博士論文

Structure and Function of the Phragmoplast

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STRUCTURE AND FUNCTION OF THE PHRAGMOPLAST

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Contents

Abbreviations

APMSF	(*p*-amidinophenyl)methanesulfonyl fluoride
BSA	bovine serum albumin
2,4-D	2,4-dichlorophenoxyacetic acid
DAPI	4',6'-diamidino-2-phenylindole
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonyl fluoride.
Tris	Tris(hydroxymethyl)aminomethane
UDP-sugar	uridinediphosphate-sugar

Summary

Two step procedure to induce synchronized cell division in higher plant cells was developed. The cell cycle of suspension cultured tobacco cells were arrested at G1/S phase by the treatment with aphidicolin, an inhibitor of DNA polymerase, then the cells were cultured in drug-free medium for 3 hours, and propyzamide, an inhibitor of plant tubulin polymerization, was added to the culture medium, and the cells were further cultured for about 5 hours. One hundred minutes after the termination of propyzamide treatment, the percentage of cells which had phragmoplast reached 60%. This synchronization protocol will greatly facilitate the study of plant cytokinesis.

Phragmoplasts were isolated from protoplasts prepared from cells whose cell cycle were synchronized in anaphase to telophase. In part 1 of this thesis, the structure of the isolated phragmoplast was observed. Actin filaments in the isolated phragmoplast were identified by decorating them with heavy-meromyosin. Most of the filaments were oriented perpendicularly or nearly perpendicularly to the equatorial plate and in about 80% of the filaments thus oriented heavy-meromyosin arrowheads pointed away from the plate. Abundant vesicles were present in the isolated phragmoplast-nuclei complex and they were associated with phragmoplast microtubules.

In part 2 of this thesis, localizations of polysaccharide synthases in the isolated phragmoplast were investigated. Isolated phragmoplasts were incubated with radiolabeled UDP-sugars and the radiolabeled products were localized by radioautography. The results indicated that UDP-glucose: glucosyltransferase (glucan synthase) was localized at the cell plate, UDP-glucose activated UDP-xylose: xylosyl transferase (possible xyloglucan synthase) was localized at the Golgi apparatus and UDP-galactose: galactosyl transferase was localized at the cell plate. Some properties of glucan synthase in the phragmoplast was also examined.

General introduction

The mechanism of cytokinesis in higher plant cells is different from that in animal cells. In animal cells, cytokinesis is achieved by cleaving the cell after chromosomes are separated. In higher plant cells, cytokinesis is achieved by the construction of a new cell wall (cell plate) between two daughter nuclei(Gunning 1982). Just after the chromosome separation, a cytoskeletal structure called phragmoplast, which contains microtubules and actin filaments, forms between two daughter nuclei. Although phragmoplast microtubules certainly play an important role in the construction of the cell plate, little is known about the mechanism whereby phragmoplast microtubules contribute to the construction of the cell plate. One of the roles of phragmoplast microtubules is probably transport of the Golgi vesicles. The role of actin filament is not known. At later phase of cytokinesis, the phragmoplast becomes annular shape and expands centrifugally, as the cell plate expands. It is commonly supposed that (Gunning 1982) before mitosis, the future division plane is decided and the position where new cell wall meets with their parental wall is marked by some specialization of its site. The expanding phragmoplast is guided to the specialized site of the parental cell wall by unknown mechanism.

Polysaccharides are major components of the cell plate. However, knowledges about distribution or control of its synthesis are limited.

Although there are many morphological and pharmacological studies of the phragmoplast, biochemical studies are hampered by the lack of suitable plant materials in which most of the cells have phragmoplast. I thought the development of the method which enables us to induce highly synchronized cell division would bring a breakthrough in the study of cytokinesis in plant cells.

In part 1 of this thesis, development of the procedure for the induction of cell-cycle synchrony, isolation of the phragmoplast, and the ultrastructure of the isolated phragmoplast are reported. In part 2 of this thesis localization of several

polysaccharide synthases are reported.

The term phragmoplast generally means the cytoskeletal component of the cytokinetic apparatus. However the phragmoplast is structurally and functionally related to membrane system. Isolated phragmoplasts were associated with daughter nuclei (part 1 and 2), and also the cell plates and the Golgi apparatus (part 2). So, in this report, I temporarily call this complex as a phragmoplast.

Part 1 INDUCTION OF SYNCHRONIZED CELL DIVISION OF TOBACCO BY-2 CELLS, AND ISOLATION OF PHRAGMOPLASTS.

1.Introduction

Cytokinesis in the plant cell is achieved by cell plate formation by an apparatus called phragmoplast. As the phragmoplast appears only in anaphase and telophase, I thought the development of the method which enables us to induce highly synchronized cell division should greatly facilitate the study of cytokinesis in plant cells.

Various methods for inducing the cell cycle synchrony in plant cells have been reported (Erikson 1966, Jouanneau 1971, Okamura et al. 1973, Nishinari and Yamaki 1976, Constabel 1977, King 1980, Nagata et al. 1982, Amino et al. 1983, Nishinari and Syono, 1986). Among them, the method using aphidicolin, an inhibitor of DNA polymerase gives the highest degree of synchrony (Nagata et al. 1982). Although it is reported that the method enables us to obtain a cell population in which about 70% of the cells are in mitotic phase, the method does not allow us to obtain a cell population in which a high proportion of cells are in some specific phase of mitosis. I attempted to develop a new method in which sequential treatment of aphidicolin and propyzamide was employed. Propyzamide was used for an plant microtubule inhibitor because the effect of this drug has been reported to be readily reversible (Izumi et al. 1983, Akashi et al. 1988).

Isolated spindles have contributed much to studies of structure, function and biochemistry of this apparatus(Sakai 1978, Hirokawa 1985, Masuda and Cande 1987). As I thought isolated phragmoplasts may offer good experimental system for the study of this apparatus, I developed the method to isolate phragmoplasts.

The presence in the phragmoplast of actin filaments were observed by fluorescent labeled phallotoxins (Clayton and Lloyd 1985, Gunning and Wick 1985, Kakimoto and Shibaoka 1987a, Palevitz 1987, Schmit and Lambert 1985, Seagull et al. 1987,

Traas et al. 1987, Schmit and Lambert 1990) as specific probes for F-actin, and immunocytochemically (Schmit and Lambert, 1987). But the role of actin filament is not known. Some workers reported that cytochalasins inhibited cytokinesis (Schmit and Lambert 1988, Traas et al. 1989, Mineyuki and Gunning 1990), whereas others reported that they does not (Mole-Bajer and Bajer 1988, Palevitz 1987, Palevitz and Cresti 1989). As the direction of force generation by actomyosin system is dependent on the polarity of the actin filament, it is important to determine the polarity of the filaments to understand the role of actin filaments in the phragmoplast. Isolation of phragmoplasts made it easy to decorate actin filaments in the phragmoplasts with heavy-meromyosin for the determination of the polarity of the filaments.

It has been well established that, the freeze substitution method is superior to the conventional method in preserving the ultrastructures of the samples for the electron microscopy. But application of this technique for the phragmoplast *in vivo* is difficult because the phragmoplast locates at the center of the vacuolated cell, and this technique can be applied for only the surface of the cells. Isolated phragmoplasts were observed using this technique.

2.Materials and methods

2. 1. Plant material

Tobacco BY-2 cells (*Nicotiana Tabacum* "Bright Yellow 2") were cultured in a modified Linsmaier and Skoog's medium(LS medium) which contained 3% sucrose, 370 mg/l KH_2PO_4, 1.0 mg/l thiamine hydrochloride and 0.2 mg/l 2,4-dichlorophenoxyacetic acid (Nagata et al. 1981). The pH of the medium was adjusted to 5.8 before autoclaving. Cells were subcultured every 7 days by transferring 2 ml of cell suspension into 95 ml of fresh medium in 300 ml Erlenmeyer flasks, and they were shaken at 104 strokes/min in the dark at 27 °C.

2. 2. Drug treatment

To test effects of microtubule polymerase inhibitors on mitosis, 3-day-old cells were cultured in the presence of propyzamide (N-(1,1-dimethylpropynyl)-3,5-dichlorobenzamide), or cremart(O-ethyl O-(3-methyl-6-nitrophenyl)N-sec-butylphosphorothioamidate), or Colchicine. It is reported that propyzamide (Akashi *et al.*, 1988) and cremart (Mita and Shibaoka, 1984) destroy plant microtubules. Propyzamide and cremart were dissolved in DMSO and added to the cell suspension. The concentration of DMSO was 0.1%, which had no effect on the cell cycle. Colchicine was dissolved in distilled water and added to the cell suspension. After 9 hour culture in the presence of the drug, nuclei were stained with 1% orcein dissolved in 50% lactic acid / 50% propionic acid and examined with light microscope.

2. 3. Synchronization of the cell cycle

To induce synchronization of the cell cycle, cells were treated with aphidicolin essentially by the method of Nagata et al.(1982) and then treated with propyzamide. About 20 ml of 7-day old subcultured cells were suspended in 95 ml of modified LS medium. Aphidicolin, dissolved at 5 mg / ml in DMSO, was added to the cell suspension to make the final concentration of 2.5 μg/ml and cells were cultured for 24 hours. DMSO at 0.1% or lower showed no effect either on microtubules or on the cell division. Cells were washed with 3% sucrose and cultured in modified LS medium. Three hours after removal of aphidicolin, propyzamide was added to the cell suspension to make the concentration of 5 μM and cells were cultured further. Propyzamide was dissolved at 6 mM in DMSO. In the presence of propyzamide, cell cycles were arrested at methaphase-like state. About five hours after addition of propyzamide when mitotic index reached plateau, cells were washed with 3 % sucrose and cultured in modified LS medium. Cells were sampled at appropriate time intervals for the determination of the percentages of cells whose chromosomes were not aligned at the equatorial plane (cells arrested at metaphase + cells in prophase), of cells whose chromosomes were aligned at

the equatorial plane (cells in normal metaphase), and of cells at anaphase and telophase.

2. 4. Isolation of phragmoplasts

The cells treated with aphidicolin and then with propyzamide were harvested 70 minutes after the termination of treatment with propyzamide and incubated for 1 hour at room temperature in a solution of wall-digesting enzymes which contained 1% Cellulase Onozuka RS (Yakult Honsha Co., Tokyo), 0.1% Pectolyase Y23 (Seishin Parmaceutical Co., Tokyo) and 0.38 M sorbitol (pH 5.6). Protoplasts released from the cells were collected and washed with a solution of 0.35 M mannitol. For electron microscopy, phragmoplasts were isolated using buffer A as described below. Protoplasts were transferred to buffer A (100 mM PIPES, pH 7.0, 1 mM $MgCl_2$, 20 mM KCl, 0.3 M mannitol, 0.3 mM PMSF, 1 μM taxol, 50 μg/ml leupeptin, 0.1% Triton X-100, 0.1 μM rhodamine-phalloidin, 0.2 mg/ml tropomyosin, 0.5 mg/ml heavy meromyosin, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol). In this buffer, the plasma membranes were dissolved and protoplasts released phragmoplasts. Released phragmoplasts were collected by low speed centrifugation (170xg for 2 min). Taxol or EGTA was added to the buffer A to preserve microtubules, and tropomyosin was added to preserve actin filaments (Kakimoto and Shibaoka 1987b). Heavy meromyosin was used to identify actin filaments (Ishikawa et al. 1969), and spermine and spermidine to stabilize nuclei (Willmitzer and Wagner 1981). Rhodamine-phalloidin was added to buffer A to stabilize actin filaments and allowed us to check whether or not actin filaments in isolated phragmoplasts can withstand the fixatives used for preparing specimens for electron microscopy. For other experiments, phragmoplasts were isolated using bufferB. Protoplasts were transferred to buffer B (100 mM PIPES, pH 7.0, 1 mM $MgCl_2$, 20 mM KCl, 0.3 M mannitol, 0.3 mM PMSF, 5 mM EGTA, 50 μg/ml leupeptin, 0.1% Triton X-100, 1 mM dithiothreitol) to dissolve the plasma membranes of the protoplasts. Released phragmoplasts were collected by centrifugation (170g for 2 minutes) and washed with buffer B from

which triton X-100 was omitted.

2. 5. Fluorescence microscopy

Cells or isolated phragmoplasts were fixed with 3.1% formaldehyde in potassium phosphate buffer (50 mM, pH 7.0) that contained 1 mM $MgCl_2$, 5 mM EGTA and 0.3 mM PMSF, for 40 minutes. Fixed cells were washed with PBS (8 mM phosphate-KOH, 0.9% NaCl) and treated with cell wall digesting enzyme solution (0.5% Cellulase Onozuka RS, 0.05% Pectolyase Y-23, 5 mM EGTA, 50 μg/ml leupeptin, 0.3 M mannitol, pH=5.5) for 5 min at room temperature, then washed with PBS and extracted for 10 min at room temperature with PBS that contained 0.1% Triton X-100. Isolated phragmoplasts were not treated with enzyme solution or Triton X-100. Cells or isolated phragmoplasts were incubated with mouse monoclonal antibody against chick brain α-tubulin (Amersham, Buckinghamshire, U. K.), diluted 1: 500 in PBS which contained 1% BSA at 27 ℃ for 2 hours, washed with PBS and stained with FITC-conjugated antibody raised against mouse IgG in rabbit (ICN ImmunoBiologicals, Lisle, IL.), diluted 1:20 in PBS which contained 1% BSA, at 27 ℃ for 2 hours. They were washed with PBS and stained with 0.5 mg/ml of DAPI, and observed with an BHS-RFK fluoreacense microscopy with standard filter sets. They were photographed with Fujichrome slide film (Fuji Film) or TriX negative film (Kodak).

2. 6. Electron microscopy

For electron microscopy, phragmoplasts isolated with buffer A were used. The isolated phragmoplasts were washed with PIPES-Mg buffer (100 mM PIPES, pH 7.0, 1 mM $MgCl_2$, 20 mM KCl, 0.3 M mannitol, 0.3 mM PMSF) that contained 0.1% lysine, and then with PIPES-Mg buffer. Washing with the lysine solution was designed to stabilize actin filaments (Kakimoto and Shibaoka 1987a). The washed phragmoplasts were fixed with 2.5% glutaraldehyde in 50 mM potassium phosphate buffer (pH=7.0), dehydrated with acetone series, stained *en bloc* with 0.1% hafnium chloride in acetone, and embedded in Spurr's resin(Kakimoto and Shibaoka 1987b).

The ultrastructure of the phragmoplasts was also examined by freeze-substitution electron microscopy (Van Harreveld and Crowell 1964). The isolated phragmoplasts were rapidly frozen by bringing them into contact with a copper block that had been pre-cooled with liquid nitrogen. The frozen phragmoplasts were transferred into a solution of 1% osmium tetraoxide in acetone cooled in dry ice-acetone and left for 72 hours at -80°C. The temperature of the fixed phragmoplasts was raised stepwise until it reached that of the room. After settling at room temperature for 30 minutes, the fixed phragmoplasts were stained en bloc in 0.1% hafnium chloride for 1 hour (Hatae et al. 1984) and embedded in Spurr's resin. Sections were cut and stained with uranyl acetate and lead citrate, and were examined with an electron microscope (JEM-100C, Jeol, Tokyo).

3. Results

3. 1. Effects of propyzamide on the cell cycle

Three-day-old subcultured cells were cultured for 9 hours in modified LS medium that contained various concentrations of propyzamide, cremart or colchicine. In the presence of propyzamide or cremart, chromosomes in BY-2 cells condensed to assume a metaphase-like appearance, but they were not aligned in an equatorial plate. In cells treated with 6 uM propyzamide or 1 μM cremart for 6 hours, only fragmented microtubules or none at all were present from the observation by immunofluorescence microscopy(data not shown). The mitotic index of 3-day-old subcultured cells was about 7%. In the presence of propyzamide (4 μM or higher) or cremart (0.3 μM or higher), the index increased with time and reached about 25% after 9 hours, but colchicine had no effect on the mitotic index(Fig. 1). After about 6 hours, however, adjacent chromosomes in the cells treated with propyzamide oe cremart began to fuse to produce multi-nucleate cells. Extending the treatment with propyzamide for more than 6 hours seems to be ineffective in increasing the number of cells that resume the progression through the cell-

cycle in synchrony. Effect of propyzamide on microtubules and mitosis has already reported by Akashi *et al*(1988).

3. 2. Effect of aphidicolin on the cell cycle

Using the procedure of Nagata et al. (1982), we cultured 7-day-old subcultured cells in modified LS medium that contained 2.5 μg/ml aphidicolin for 24 hours, and then transferred them to fresh 2,4-D medium and continued their incubation. Treatment with aphidicolin decreased the mitotic index of tobacco culture cells to zero, but after the cells were transferred to the medium that contained no aphidicolin the mitotic index increased with time and reached a peak of about 40% (Fig. 2). The percentage of cells having phragmoplasts, however, did not exceed 15% (data not shown).

3. 3. Synchronization of the cell cycle

The results obtained demonstrated that both propyzamide and aphidicolin were useful for inducing synchronization of the cell cycle, but the degree of synchronization induced by propyzamide or aphidicolin alone was far from satisfactory for our purposes. Thus, we attempted to induce a higher degree of synchronization by treating cells with both aphidicolin and propyzamide according to the procedure described in "Materials and Methods."

Propyzamide increased the mitotic index of aphidicolin-pre-treated cells to about 80% (Fig. 2). The increase in mitotic index was due to the increase in the number of cells whose chromosomes were not aligned in an equatorial plate (Fig. 2, 3a). After removal of propyzamide, percentage of the cells whose chromosomes were aligned in equatorial plane increased to reach a peak of 60% after 40 minutes(Fig. 2. 3c). After 40 minutes, anaphase cells began to appear, and after about 90 minutes the percentage of cells in anaphase + telophase reached a peak of 60% (Fig. 2, 3c). At this point the proportion of cells having the phragmoplast was also about 60%. I also examined the feasibility of cremart. Cremart(0.3 μM) also caused so called metaphase arrest on tobacco BY-2 cells pretreated with aphidicolin, but the

effect was not reversible.

Soon after the termination of treatment with propyzamide, microtubules began to polymerize. As shown in Fig. 4e, microtubules were observed after as little as 7 minutes, showing that the effects of propyzamide can be reversed rapidly. After 14 minutes, the mitotic spindles became evident (Fig. 4f). After 90 minutes, phragmoplasts were observed at a high frequency (Fig. 4h). Similar experiments using subcultured cells of various ages gave almost identical results, but when young subcultured cells, such as 3-day-old cells, were used, treatment with aphidicolin for 20 - 22 hours gave better results than treatment for 24 hours.

3. 4. Isolation of phragmoplasts

It takes about 60 minutes to digest cell walls and release protoplasts, and the cell cycle continues to proceed in cells in the solution of wall-digesting enzymes. However, since the progression through the cell cycle slows down as digestion of the cell walls progresses, treatment with the enzyme solution was started 30 minutes before the percentage of cells with phragmoplasts reached a peak (i.e. about 70 minutes after the termination of treatment with propyzamide). This procedure gave the maximum yield of isolated phragmoplasts.

Released protoplasts were lysed and phragmoplasts were collected from the lysate by centrifugation. The phragmoplast-rich pellet was washed with buffer B from which Triton X-100 was omitted. The purity of the isolated phragmoplasts were about 60% (Fig. 5). Since cell walls are thinner in 3-day-old subcultured cells than in 7-day-old cells, preparation of protoplasts is much easier with the former than with the latter cells.

3. 5. Association of daughter nuclei with phragmoplasts

The isolated phragmoplasts were associated with daughter nuclei (Fig. 5, 6). The phragmoplasts isolated with lysis buffer B showed no rhodamine-phalloidin staining, but were accompanied by daughter nuclei, indicating that actin filaments are not

involved in the association between phragmoplast and nuclei. The phragmoplast-nuclei complexes isolated with buffer B were incubated with Ca-PIPES buffer (100 mM PIPES, pH 7.0, 100 μM $CaCl_2$, 1 mM dithiothreitol) at 0°C for 20 minutes. Microtubules in the phragmoplast disappeared as a result of this treatment (Fig. 7b), but daughter nuclei were not separated by this treatment (Fig. 7a). Microtubules do not seem to be involved in maintaining the association between phragmoplast and nuclei.

3. 6. Ultrastructure of isolated phragmoplasts

Actin filaments as well as microtubules were present in the phragmoplast-nuclei complexes isolated with buffer A and fixed by conventional procedures (Fig. 8a,d). The vesicles that are usually observed between phragmoplast microtubules and those at the equatorial plane (Bajer 1968, Hepler and Jacson 1968) were not well preserved (Fig. 8d). Microtubules extended from the equatorial plate to the reforming daughter nuclei (Fig. 8a,b), but actin filaments were not present near the daughter nuclei (Fig. 8b). Actin filaments which bound heavy meromyosin and formed arrowheads complexes were abundantly distributed near the equatorial plate (Fig. 8d). Most of them were arranged in parallel or nearly in parallel to the spindle axis, although they were not as neatly arranged as microtubules (Fig. 8d). The direction of myosin arrowheads was examined with 200 actin filaments which were arranged in parallel or nearly in parallel to the spindle axis. As the shapes of arrowheads were not distinct in 50 filaments out of the 200, we determined the direction of the arrowheads with 150 filaments, with the result that 77% of them (115 filaments) had arrowheads pointing away from the cell plate. Cross-bridges were often observed between adjacent microtubules (Fig. 8c). Thin filaments which were not decorated with heavy meromyosin were also present in association with microtubules (Fig. 8b).

Although vesicles were only poorly preserved by the conventional method, they were well preserved by the freeze-substitution method. Abundant vesicles, which appeared to be

attached to microtubules were observed (Fig. 9a, b).

4. Discussion

Treatment with aphidicolin increased the mitotic index of tobacco culture cells, but it did not increase the percentage of cells which synchronously formed phragmoplasts to a level high enough for our purpose. This failure is due, at least partly, to the distance in the cell cycle between the phase at which aphidicolin arrests the cycle and the phase at which formation of phragmoplasts occurs. Many events intervene between these two phases and as the cells complete these various events the synchronization of the cell cycle, induced by aphidicolin, may be disturbed. The phase at which inhibitors of microtubules arrest the cycle is close to the phase at which formation of phragmoplasts occurs. Thus, in the anticipation that the use of a microtubule inhibitor would improve the method for inducing the synchronized formation of phragmoplasts, we examined the effects of microtubule inhibitors. Propyzamide (Akashi et al. 1988) and Cremart (Mita and Shibaoka, 1984) are known to be inhibitors of plant microtubule. Cremart did not give a satisfactory result because of the lack of the reversibility of its effect. The effect of propyzamide was readily reversible as shown by Akashi et al.(1988), and when applied to aphidicolin pretreated cells, it gave a fairly good synchrony. It gave the percentage of cells having phragmoplasts of about 60% and allowed us to isolate a preparation of phragmoplasts with a purity of about 60%. The two step procedure to synchronize cell cycle will be a great utility in the study of metaphase-anaphase/telophase-G1 phase transition. And isolated phragmoplasts can be used for the biochemical study of phragmoplast microtubule system.

Each isolated phragmoplast was associated with the daughter nuclei. A firm association of the daughter nucleus with the phragmoplast was demonstrated by Dawson et al. (1985) who observed that a phragmoplast isolated from an onion root meristematic cell during cell-squashing was still attached to one

of the daughter nuclei. The association between phragmoplast and nuclei was observed even in complexes which showed neither the staining of microtubules nor of actin filaments. This result suggests the involvement of cytoskeletal components other than microtubules and actin filaments in the association of the daughter nuclei with the phragmoplast. In this connection, it is noteworthy that phragmoplasts bind antibody raised against intermediate filament antigen (Dawson et al. 1985) and that thin filaments which are not decorated by heavy meromyosin are present in the isolated phragmoplast (Fig. 8b). We should examine whether such thin filaments have some connection with intermediate filaments or not.

Using isolated phragmoplast-nuclei complexes, we confirmed the presence in the phragmoplast of actin filaments by electron microscopy. Most of the actin filaments in the phragmoplast were arranged in parallel or nearly in parallel to the spindle axis and in about 80% of the filaments thus arranged heavy meromyosin arrowheads pointed away from the equatorial plate, indicating that the polarity of the filaments was opposite on two side of the plate. This result and the well-known fact that arrowheads on microfilaments in Characean plant cells point in the direction opposite to that of cytoplasmic streaming (Keraey et al. 1976) suggest the possible involvement of phragmoplast actin filaments in the transport of vesicles toward the site of formation of the cell plate.

To analyze the mechanism of transport of vesicles toward the site of formation of the cell plate, it is also necessary to examine the relationship between vesicles and phragmoplast microtubules. Although the vesicles in the isolated phragmoplast were not well preserved by the conventional method of fixation, they were preserved in satisfactory condition by the freeze-substitution method. Thus, it appears that the freeze-substitution method is superior to the conventional method for studies of the relationship between vesicles and phragmoplast microtubules. However, the freeze-substitution method does not seem to give a satisfactory result in the case of the

phragmoplast in situ, perhaps because this method is useful only for fixing specimens of 10 um thickness or less (Van Harreveld and Crowell 1964) and the phragmoplast is usually located in the center of vacuolated cells. The isolated phragmoplasts should be of great utility for studies of the relationship between vesicles and phragmoplast microtubules by freeze-substitution electron microscopy. Application of rapid freeze and deep etching procedure for this sample will be possible and it will reveal many aspects of the phragmoplast. As the isolated phragmoplast microtubules associates many vesicles, it is possible to isolate the translocater of these vesicles.

PART 2. POLYSACCHARIDE SYNTHESIS BY ISOLSTED PHRAGMOPLASTS

1.Introduction

In higher plant cells, cytokinesis is achieved by forming the cell plate between two daughter nuclei(Gunning 1982). The cell plate is formed by the coalescence of small vesicles which accumulate in the plane of cell division. Based on the position of the Golgi apparatus and the staining characteristics of the Golgi vesicles and the cell plate, it has been proposed that these vesicles are derived from the Golgi apparatus (Gunning,1982). The Golgi vesicles are supposed to contain new cell wall materials including polysaccharides. Although polysaccharides are major component of the cell plate, little is known about synthesis and localization of its constituent.

Aniline-blue, relatively specific dye for callose(1,3-β-glucan), stains the cell plate (Fulcher et al.1975). Recently, immunocytochemistry was applied to investigate the localization of polysaccharides. Northcote (1989) showed that callose is present in the cell plate but not in the Golgi apparatus using immunoelectron microscopy. Xyloglucan was localized in the Golgi apparatus and in the cell plate(Moore and Staehelin 1988).

Biochemical studies showed that a UDP-glucose: 1,3-β-glucan (callose) synthase is localized at the plasma membrane, and it is in most cases latent and only activated by perturbed conditions. Callose synthesis in the cell plate is one of the relatively rare cases where callose is synthesized *in vivo* in a non perturbed state. So, I examined several properties of glucan synthase in the isolated phragmoplasts. Though 1,4-β-glucan (cellulose) synthesis *in vitro* by plasma membrane has not been succeeded, it is believed that cellulose is synthesized at the plasma membrane *in vivo*. Enzymes capable of producing the polysaccharide-containing cell-wall components such as arabinosyl transferase (Gardiner and Chrispeels 1975), glucosyl transferase (Ray et al. 1969), xylosyl transferase (Brummell et al. 1990, Ray 1980), and fucosyl transferases (Brummell et al., Green and Northcote 1978, James and Jones 1979) have been shown to reside largely in the

Golgi apparatus from membrane fractionation studies. From these studies, it is supposed that cellulose and callose are synthesized at the plasma membrane whereas other polysaccharides are synthesized at the Golgi apparatus.

I directly localized glucosyl transferase (callose synthase), UDP-glucose activated xylosyl transferase, and galactosyl transferase in the isolated phragmoplasts. Glucosyl transferase was localized at the cell plate, and UDP-glucose activated xylosyl transferase was localized at the Golgi apparatus, as has been expected. Surprisingly, galactosyl transferase was localized at the cell plate.

2. Materials and methods

2. 1. Isolation of protoplasts in anaphase to telophase

The cell cycle of BY-2 cells were synchronized by 2 step synchronization method using aphidicolin and propyzamide as described in part 1. Soon after the removal of propyzamide, cells were suspended in an enzyme solution [1% Sumizyme bulk grade (Shinnihonkagakukougyou Co., Showacho, Anjo-shi, Japan), 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo) and 0.3 M mannitol dissolved in culture medium then adjusted the pH to 5.5] in an Erlenmeyer flasks which were gently shaken at 27℃. Vigorous shaking made the protoplasts burst and gentle shaking made its cell cycle arrested at metaphase because of anoxia. So, during the enzyme treatment, the Erlenmeyer flasks were filled with 100% oxygen and shaken gently. Under this condition, chromosome segregation and cell plate formation proceeded with normal time schedule.

2. 2. Isolation of phragmoplasts

To study polysaccharide synthesis in the phragmoplasts, phragmoplasts were isolated without detergent. Protoplasts, whose cell cycles were synchronized in anaphase to telophase, were washed with a solution of 0.5 M mannitol and then suspended in ice cold buffer (buffer C: 50 mM PIPES, 0.25 mM $MgCl_2$, 300 mM

sucrose, 0.25% BSA, 1 mM DTT, 50 μg/ml leupeptin, 10 μg/ml APMSF, 10 μg/ml pepstatin A, pH 7.0). This suspension was gently passed through 16μm nylon mesh. During the passage, protoplasts were broken and released phragmoplasts. Released phragmoplasts were washed with the same buffer by centrifugation(170xg, 2 min) and resuspended in the same buffer.

2. 3. Callose synthesis by isolated phragmoplasts

Suspension of isolated phragmoplasts were mixed with the same volume of callose synthesis buffer (buffer C that contained appropriate concentrations of UDP-glucose, 10 mM cellobiose and 200 μM $CaCl_2$) and incubated for 4 min (for quantitative estimation of the product) or 15 min (for radioautographycal study and characterization of the product) at 27 ℃. The synthesis buffer contained 11 MBq/ml UDP-[6-^{3}H]glucose (American Radiolabeled Chemicals Inc.; St. Louis Mo., U.S.A.) for radioautography, 148 KBq/ml UDP-[U-^{14}C]glucose (American Radiolabeled Chemicals Inc.) for characterization of the product by enzyme digestion and 22 kBq/ml UDP-[U-^{14}C]glucose for quantitative estimation of the product. To examine the effect of pH, isolated phragmoplasts were washed with a solution which contained 0.5 M sucrose, 0.25 mM $MgCl_2$, 0.25 % BSA, 1 mM DTT, 50 μg/ml leupeptin, 10 μg/ml APMSF, 10 μg/ml pepstatin A) and resuspended in the same solution. Reactions were started by adding the same volume of solution which contained 50 mM PIPES-KOH (pH 6.0, 6.5, 7.0) or 50 mM Tris-HCl (pH 7.0, 7.5, 8.0, 8.5), 200 uM UDP-[^{14}C]glucose, 0.3 M sucrose, 0.25 mM $MgCl_2$, 200 μM $CaCl_2$, 1 mM DTT, 10 mM cellobiose and protease inhibitors as above.

2. 4. Characterization of the product by enzyme digestion

After glucan synthesis reactions, 1 ml of solution which contained 1 M NaOH, 0.5 M $NaHBO_4$ and 0.5 mg/ml of acid swollen cellulose were added to the 200 μl of the reaction mixture, boiled for 20 min, and washed with water by centrifugation. The pellet was incubated with 0.5 mg/ml of 1,3-β-glucanase

(laminarinase from mollusk; Sigma) dissolved in 20 mM sodium acetate solution. The digest was chromatographed on Whatman 3 MM paper with 1-propanol/ethyl acetate/water(7:1:2, v/v/v) at 60 °C. The chromatogram (23 cm in length) was cut into 0.5 cm fragments, and the radioactivity of each fragment immersed in 5 ml of scintillator (4 g/l of PPO and 0.1 g/l of bis-MSB in toluene) was counted with a liquid scintillation counter.

2. 5. Transfer of xylose from UDP-[^{3}H]xylose by isolated phragmoplasts

Suspension of isolated phragmoplasts was mixed with the same volume of xyloglucan synthesis buffer(buffer C which contained 56 μM UDP-[6-^{3}H]xylose(18.5 MBq/ml for autoradiography and 1.85 MBq/ml for quantitative study; NEN Research Products) 10 mM $MnCl_2$ and 4 mM unlabeled UDP-glucose) and incubated for 30 min at 27 °C.

2. 6. Transfer of galactose from UDP-[^{3}H]galactose by isolated phragmoplasts

Suspension of isolated phragmoplasts was mixed with the same volume of the buffer A, which contained 200 μM UDP-[^{3}H]galactose (148MBq/ml; American Radiolabeled Chemicals Inc.), 10 mM cellobiose and 200 μM $CaCl_2$, and incubated for 30 min at 27 °C.

2. 7. Fixation and Embedding

Isolated phragmoplasts which had been incubated with radiolabeled substrates were fixed with excess volume of 2.5 % glutaraldehyde in 50 mM cacodilate buffer (pH 7.0) which contained 250 μM $CaCl_2$ and 250 μM $MgCl_2$ for 1 hour at room temperature. They were washed with the same buffer for 3 times by centrifugation and post fixed with 1% OsO_4 for 1 hour at 0 °C. During fixation and washing, soluble materials including substrates were lost and insoluble products remained in the sample. Samples were washed with cold water, dehydrated by ethanol series and propylene oxide, and embedded in Spurr's

resin.

2. 8. Light microscope radioautography

Sections of 3.5 μm thickness were cut and mounted on slide glasses which had been coated with a mixture of the white of the egg and glycerol (1:1, v/v). Slides with sevtions were covered with radioautographic emulsion NR-M2 (Konica), exposed for 2-14 days and developed in Render (Fuji film, Tokyo, Japan). They were dipped in 2.5% glutaraldehyde in 50 mM cacodilate buffer (pH 7.0) to fix gelatin and stained with Paragon mixture, a mixture of 1 part of Paragon Multiple Stain (0.19% toluidine blue, 0.07% basic fuchsin and 30% ethanol; Paragon C. & C. Co., Inc.; Willow Ave., Bronx, N. Y. 10454, U.S.A.) and 3 part of 2% sodium tetraborate. They were mounted in Malinol (Muto Pure Chemicals Ltd. Tokyo, Japan) and photographed on a microscope (BH-2; Olympus)using DPlan Apo 10 UV PL (10 x), DPlan Apo 20 UV PL (20 x), Splan Apo 60 (60 x), DPlan Apo 100 UV (100 x) and Fujichrome reversal film (ISO 100; Fuji Film).

2. 9. Electron microscope radioautography

Sections of golden color thickness were cut, mounted on copper grids, covered with radioautographic emulsion NR-H2 (Konica) and exposed for about 1 month at 4 ℃. They were gold latencificated with a solution which contained 0.004% $AuCl_3 \cdot HCl \cdot 3H_2O$, 0.5 mg/ml KSCN, 0.6 mg/ml KBr for 10 seconds and developed in Rendor. To remove gelatin, grids were soaked in distilled water for 30 min at 37 ℃ and consequently in 0.5 M acetic acid for 30 min at 37 ℃ (Williams, 1977). After rinse, they were stained with uranyl acetate and lead citrate. Samples were observed with an electron microscope (JEM-100S, Joel, Tokyo).

2. 10. Measurements of the quantity of polysaccharides synthesized *in vitro*

Glucan synthesis reaction, which was started as described above, was stopped by dipping the test tube into ice. After adding 5 volume of 70% ethanol which contained 2 mM EGTA (pH 7)

and 5 mg/ml of carrier cellulose to the reaction mixture, the mixture was kept at 0 °C overnight, and then filtered through Whatman GF/C glass fiber filters and the residue was washed successively with 75 ml of distilled water, 20 ml of acetone, again with 75 ml of distilled water and 10 ml of acetone.

For xyloglucan synthase assays, reaction was terminated by adding 4 volume of ethanol which contained 5 mg/ml of carrier cellulose. The mixture was kept at 4 °C overnight. The mixture was filtered through Whatman glass fiber filter and the residue was washed with 100 ml of 70% ethanol and 10 ml of acetone.

The dried filter with the residue was placed in a scintillation vial with 5 ml of ACS 2 (Amersham) and the radioactivity was counted with Beckman LS9000 liquid scintillation counter.

2. 11. Fluorescence Microscopy

A suspension of isolated phragmoplasts was mixed with 1/10 volume of 37% formarin and kept for one hour at room temperature. After being washed with PBS, phragmoplasts were incubated with mouse monoclonal antibody against chick brain α-tubulin (Amersham, Buckinghamshire, U. K.), diluted 1: 500 in PBS which contained 1% BSA, at 27 °C for 2 hours. Phragmoplasts were washed with PBS and then stained with FITC-conjugated antibody raised against mouse IgG in rabbit (ICN ImmunoBiologicals, Lisle, IL.), diluted 1:20 in PBS which contained 1% BSA, at 27 °C for 2 hours. Callose in the phragmoplasts was stained with 0.1% aniline blue (Schmit, Kongen/N) in 50 mM Na_2HPO_4 and visualized by UV excitation.

3. Results

3. 1. Localization of callose in dividing BY-2 cells.

Tobacco BY-2 cells, whose cell cycle was synchronized in anaphase to telophase, were stained with 0.1% aniline blue(Fig.10). Cell plates in BY-2 cells were stained with aniline blue, which is relatively specific dye for callose

(Currier, 1957; Eschrich and Curierr, 1964; Smith and McCully, 1978).

3. 2. Isolation of phragmoplasts

In part 1 of this thesis, I described the method for isolation of phragmoplasts, in which I used Triton X-100. Although the method was proved to be suitable for the study of cytoskeleton, it won't be suitable for the study of cell-wall polysaccharide synthases, because the synthases have been considered to be present in the membrane and therefore, extractable with or labile against detergent. So, I developed a method for the isolation of phragmoplasts which does not employ detergent. Protoplasts at anaphase / telophase were passed through 16 μm nylon mesh. As the diameter of the protoplasts is about 40 μm, protoplasts were broken and released phragmoplasts by this procedure. Isolated phragmoplast retained microtubules (Fig. 11), two daughter nuclei (Fig. 11), the cell plate and the Golgi apparatus (Fig. 13).

3. 3. Incorporation of radioactivity into isolated phragmoplasts formed from UDP-[^{3}H]glucose

Isolated phragmoplasts were incubated with UDP-[^{3}H]glucose and the localization of radioactivity incorporated into insoluble materials was investigated by radioautography. Silver grains represent the sites of incorporation of radioactivity. The majority of the radioactivity incorporated from UDP-[^{3}H]glucose at the concentrations of 5 μM, 100 μM or 1 mM UDP-glucose were shown to be present at the cell plate (Fig. 12). Electron microscope radioautograph shows no significant label at the Golgi apparatus (Fig. 13).

The cell plate of isolated phragmoplasts was not stained well with aniline blue (Fig. 14). Aniline-blue positive products were formed at the cell plate when isolated phragmoplasts were incubated with 1 mM UDP-glucose. Aniline-blue positive products were formed at the whole cell plate in phragmoplasts of early developmenal stage when microtubules of which are present between two daughter nuclei, whereas mainly at the expanding site in

phragmoplasts of the late developmental stage when microtubules of which showed annular shape distribution. Aniline blue staining of the isolated phragmoplasts was faint when they were incubated with 100 μM UDP-glucose, probably because the amount of the product was small.

3. 4. Properties of callose synthase in the phragmoplasts

The rate of incorporation of radioactivity from 100 μM and 1 mM UDP-[^{14}C]glucose into phragmoplasts were nearly linear with respect to time up to 5 min (Fig. 15) and were linear with respect to phragmoplast density in the assay mixture up to 0.35 mg/ml of protein (Fig. 16). Four minutes of incubation at the protein concentrations of around 0.2 mg/ml was used for all quantitative assays. Optimum pH for the reaction was 7.0 (Fig. 17). The reaction was inhibited by 2 mM EGTA and slightly activated by 5 mM cellobiose, 5 mM laminaribiose (table 1), and activated by substrate itself (Fig. 18). Double reciprocal plot of the rate of the reaction (1/v) against the concentration of substrate(1/s) also indicates substrate activation(Fig. 19). Eighty six percent of radioactive product formed from 100 μM UDP-[^{14}C]glucose were degraded by 1,3-β-glucanase (0.5 mg/ml, 27°C, 4 hours) to release radioactive glucose (Fig. 20).

3. 5. Incorporation of radioactivity into isolated phragmoplasts from UDP-[^{3}H]xylose in the presence of high concentration of unlabeled UDP-glucose

In the absence of UDP-glucose, the amount of incorporation from 28 μM UDP-[^{3}H]xylose into isolated phragmoplasts was 4.6% of total radioactivity in assay mixture whereas addition of 2 mM UDP-glucose increased the incorporation to 11.6% of the total. In the presence of UDP-glucose, radioactivity was probably incorporated into xyloglucan, because this condition has been reported to be preferable to xyloglucan synthesis (Hayashi and Matsuda, 1981). But the possibility that the radioactivity was incorporated into other polysaccharides can not be excluded.

Localization of the radioactivity incorporated into insoluble

products from UDP-[^{3}H]xylose in the presence of high concentration of unlabeled UDP-glucose were investigated by radioautography(Fig. 21, 22). Photographs taken under the light microscope showed that silver grains was present as scattered aggregates around nuclei(Fig. 21). There were a few grains at the cell plate, but the proportion of thus grains to the total was low. These observations were confirmed by electron-microscopy radioautogram (fig. 22). Silver grains were found at the Golgi apparatus like structures and at unidentified structures around nuclei, most of which were supposed to be Golgi apparatus.

3. 6. Incorporation of radioactivity into isolated phragmoplasts from UDP-[^{3}H]galactose

Isolated phragmoplasts were incubated with UDP-[^{3}H]galactose (100 μM) and the localization of the radioactivity incorporated into insoluble materials was investigated by radioautography(Fig. 23, 24). Radioactivity at the cell plate was higher at the cell plate than at other site of the phragmoplast. This suggests the presence of galactosyl transferase at the cell plate. But we should be careful because the absolute quantity of incorporation was low: though the total applied radioactivity of UDP-[3H]galactose (148 MBq/ml) was higher than that of UDP-[^{3}H]glucose (11 MBq/ml) or UDP-[^{3}H]xylose(18.5 MBq/ml), density of the silver grain was low.

4. Discussion

I reported the method to isolate phragmoplasts from tobacco BY-2 cells (part 1). Although the method which employed detergent was useful for the study of cytoskeleton, it seemed unsuitable for the study of membrane protein. Actually, I couldn't observe intact cell plate or the Golgi apparatus by electron microscopy (part 1). So, I developed a method which does not employ any detergent. The cell plate and the Golgi apparatus were observed in phragmoplasts isolated by newly

developed method which includes mechanical rupture of the protoplast.

It has been considered that callose was present in the cell plate from observation of tissues of coon roots (Fulcher 1975) and balsam roots (Jones 1977) stained with aniline blue, and from the immunocytochemical observation of bean roots (Northcote *et al.* 1989). Aniline-blue staining revealed the presence of callose at the cell plate of cultured tobacco cells. The presence of callose in the cell plates of wide variety of plant species indicates its importance.

It has been supposed that glucan are synthesized at the plasma membrane and the cell plate whereas non glucan wall polysaccharides are synthesized in the Golgi apparatus and transported to the cell plate (Moore and Staehelin 1988).

Radioautographical study showed that callose was synthesized at the cell plate as expected. The cell plates of isolated phragmoplasts were not stained well with aniline blue. Aniline blue staining in the cell plates of protoplasts were also weak (Wu, X. personal communication). Aniline blue positive materials were formed when the isolated phragmoplasts were incubated with 1 mM UDP-glucose mainly at the expanding region of the cell plate in late stage of cytokinesis. I rarely observed localized incorporation of radioactivity at the expanding region of the cell plate by radioautography of sectioned phragmoplasts, indicating that the central region of isolated phragmoplasts also form insoluble materials. Thus, the localized staining of the phragmoplasts with aniline blue seems to indicate that the radioactivity from UDP-[^{3}H]glucose is incorporated into some materials other than callose or that aniline blue can not make access to callose in the central part of the phragmoplast.

The cell plate is the rare cases where callose is synthesized in non perturbed condition. So, I investigated several properties of callose synthase in the phragmoplasts to compare with that in the plasma membrane. The reaction required trace of Ca^{2+} which was present in the phragmoplast preparation, and the reaction was inhibited by EGTA. Optimum pH was 7.0, and the

reaction was slightly activated by cellobiose and laminaribiose. Because of substrate activation of the reaction, 1/V was not linear with respect to 1/S. These properties are consistent with glucan synthesis by the plasma membrane (Hayashi *et al.* 1981, Amino *et al.* 1985, Delmer *et al.* 1977). In the presence of cellobiose as an activator, Km for UDP-glucose was roughly estimated to be 1 to 2 mM, which is higher than that reported in plasma membrane (Hayashi *et al.* 1987).

Insoluble materials which incorporated the radioactivity from UDP-[^{3}H]xylose in the presence of high concentration of UDP-glucose were localized at the Golgi apparatus. This condition is preferable to xyloglucan synthesis (Hayashi and Matsuda 1981). However radiolabeled xylose might be also incorporated into other polysaccharides species such as xylan. Biochemical studies have shown that the Golgi apparatus has enzymes which synthesize xyloglucan from UDP-glucose and UDP-xylose (Hayashi and Matsuda 1981, Brummell *et al.* 1990). Xyloglucan was shown to be present at the cell plate and the Golgi apparatus by immunoelectron microscopy (Patricia and Staehelin, 1988). The present study confirms the concept that xyloglucan in the cell plate is synthesized in the Golgi apparatus.

Not many studies have been done about galactosyl transferase in plants. Though Ali *et al.* (1986) reported the presence of galactosyl transferase in a Golgi-enriched fraction, he didn't take account of the contribution of the plasma membrane to the reaction. The present data suggests the presence of galactosyl transferase in the cell plate. It was a surprise because it has been supposed that non glucan polysaccharides are synthesized in the Golgi apparatus. It seems possible that galactose was incorporated into glycoprotein or glycolipids as well as into polysaccharides. polysaccharides, glycoprotein, or glycolipid. However as the main site of the transfer of galactose to proteins and lipids in animal cells is the Golgi apparatus (Burgerson *et al.* 1982, Jacques *et al.* 1982, Jurgen *et al.* 1982), the most plausible products in this case were polysaccharides such as galactan. But since the signal of incorporation was weak, no definite conclusion can be reached for the time being.

General discussion

Plant cytokinesis involves many processes. The division plane is believed to be determined before mitosis. And the memory of the division site is supposed to remain during mitosis at the site where the new wall and the parental wall will meet. After the chromosome separation, the phragmoplast forms and develops. The mechanism of phragmoplast formation is an important subject. Recently, Asada *et al.*(1991) showed the presence of activity of microtubule translocation at the equatorial region of the phragmoplast. This activity may contribute to the formation and/or development of the phragmoplast. The function of the phragmoplast microtubules are supposed to be the transport of new wall materials, but precise function of the phragmoplast is not known. There are many studies about plant cytokinesis, but most of these studies were performed using endosperm cells or meristematic tissues such as root tips. I developed the highly synchronized cell division system of plant cells and the method to isolate phragmoplasts. Sample of isolated phragmoplasts will be a good material for the biochemical study of the phragmoplast. Isolated phragmoplasts contain abundant microtubules and associated vesicles. These vesicles are supposed to fuse to form the cell plate in vivo. Isolation of these vesicles and its translocater will be possible.

Little is known about the synthesis of new cell wall materials including polysaccharides. Isolated phragmoplasts will contribute to the study of the control mechanism of polysaccharide synthesis.

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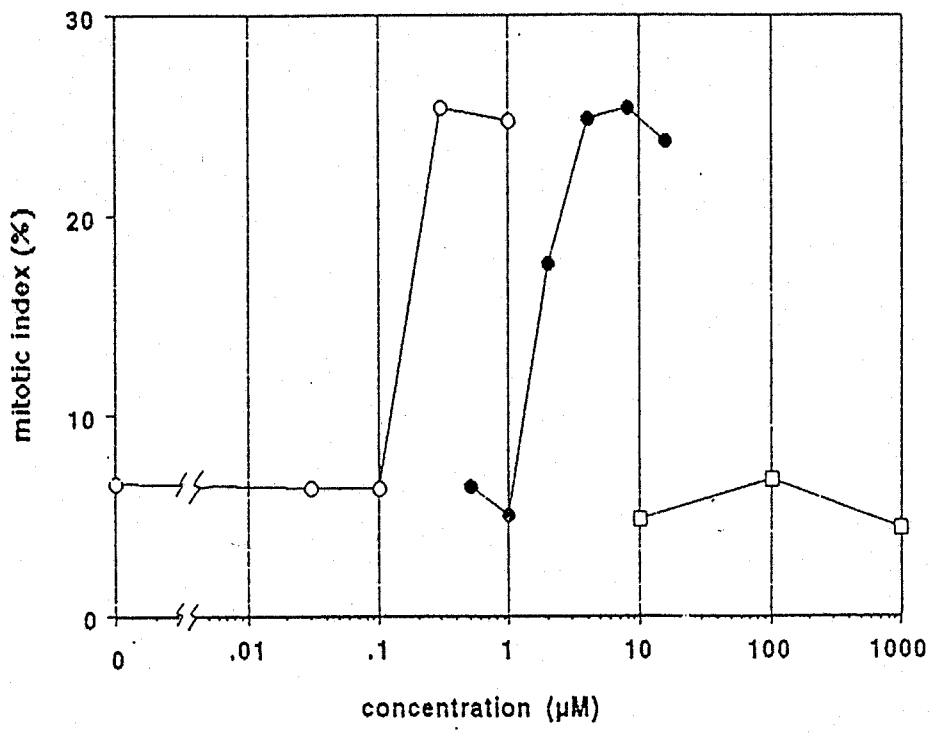


Fig. 1. Effects of microtubule polymerization inhibitors on mitotic index. Actively growing BY-2 cells were incubated for 9 hours with culture medium which contained cremart(open circle), propyzamide(closed circle) or colchicine(closed square).

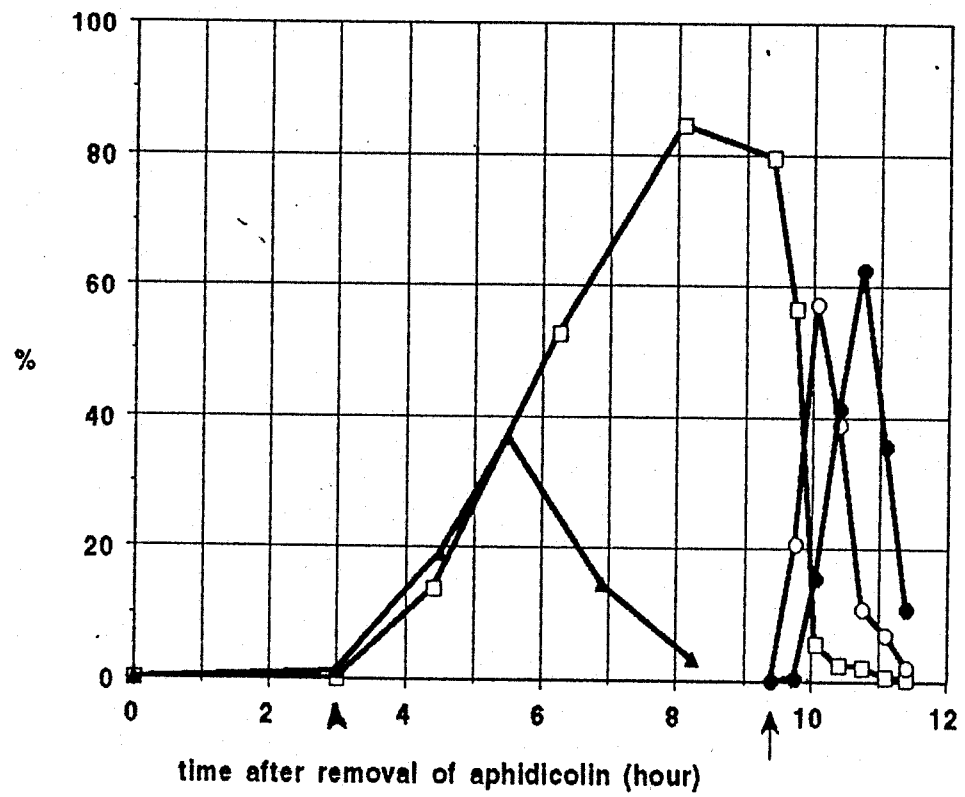


Fig. 2. Changes in the mitotic index of tobacco BY-2 cells after treatment with aphidicolin and the effect of propyzamide on it. Tobacco BY-2 cells were cultured in modified LS medium that contained aphidicolin for 24 hours and then washed and cultured in modified LS medium. The abscissa indicates time after removal of aphidicolin. Propyzamide was added at the time indicated by an arrowhead and removed at the time indicated by the arrow. Triangle: Mitotic index of cells cultured in modified LS medium after the removal of aphidicolin. Square: Mitotic index of cells cultured in modified LS medium to which propyzamide was added to make the final concentration of 5 μM. Propyzamide was added at the time indicated by an arrowhead and removed at the time indicated by an arrow. Open circle: Percentage of cells whose chromosomes are arranged at the equatorial plane. Closed circle: Percentage of cells in anaphase + telophase.

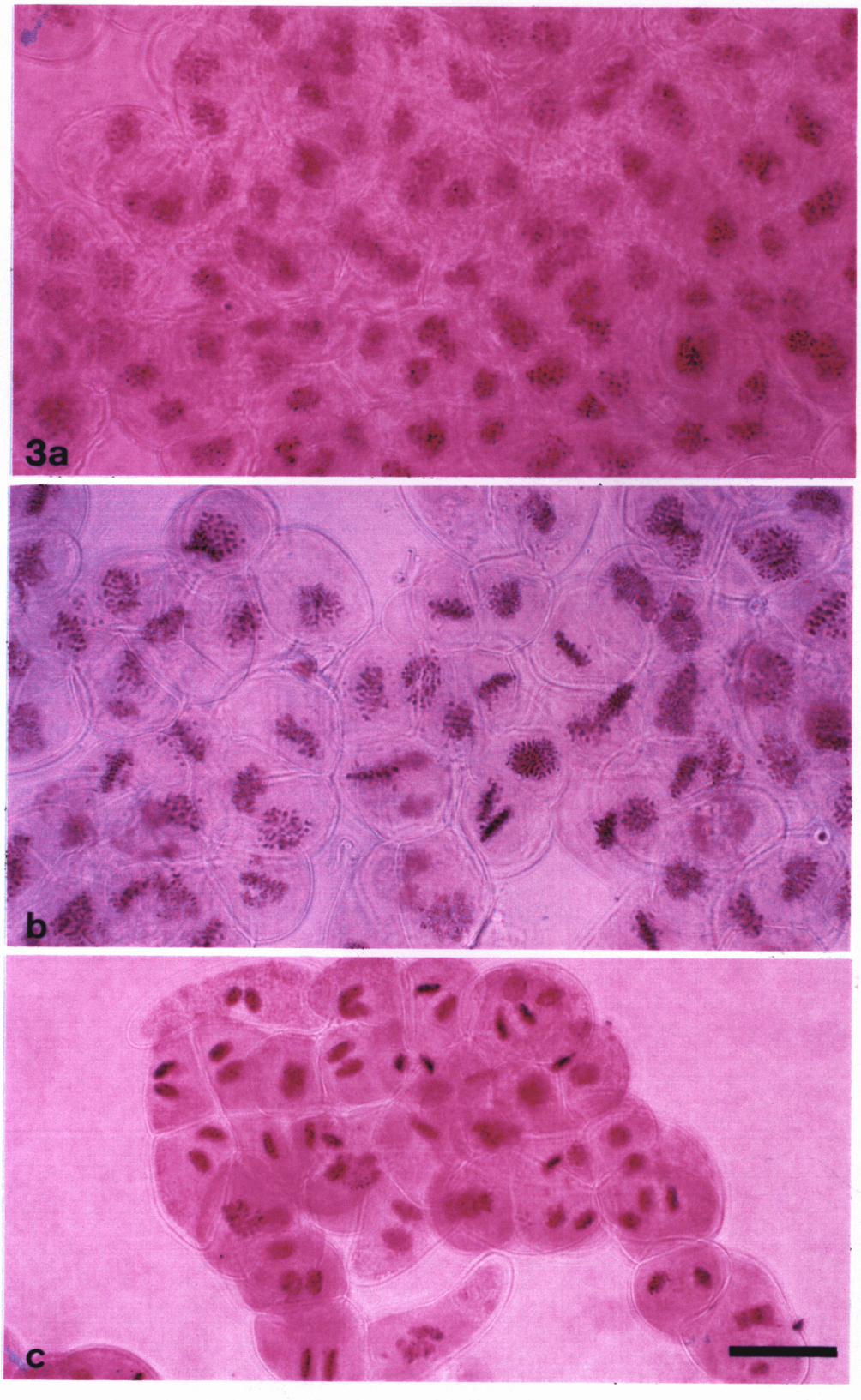


Fig. 3. Synchronized cell division of tobaccco BY-2 cells. Cells were stained with orcein just before removal of propyzamide (a), 40 min (b), 90 min (c) after removal of propyzamide. Bar=50 μm

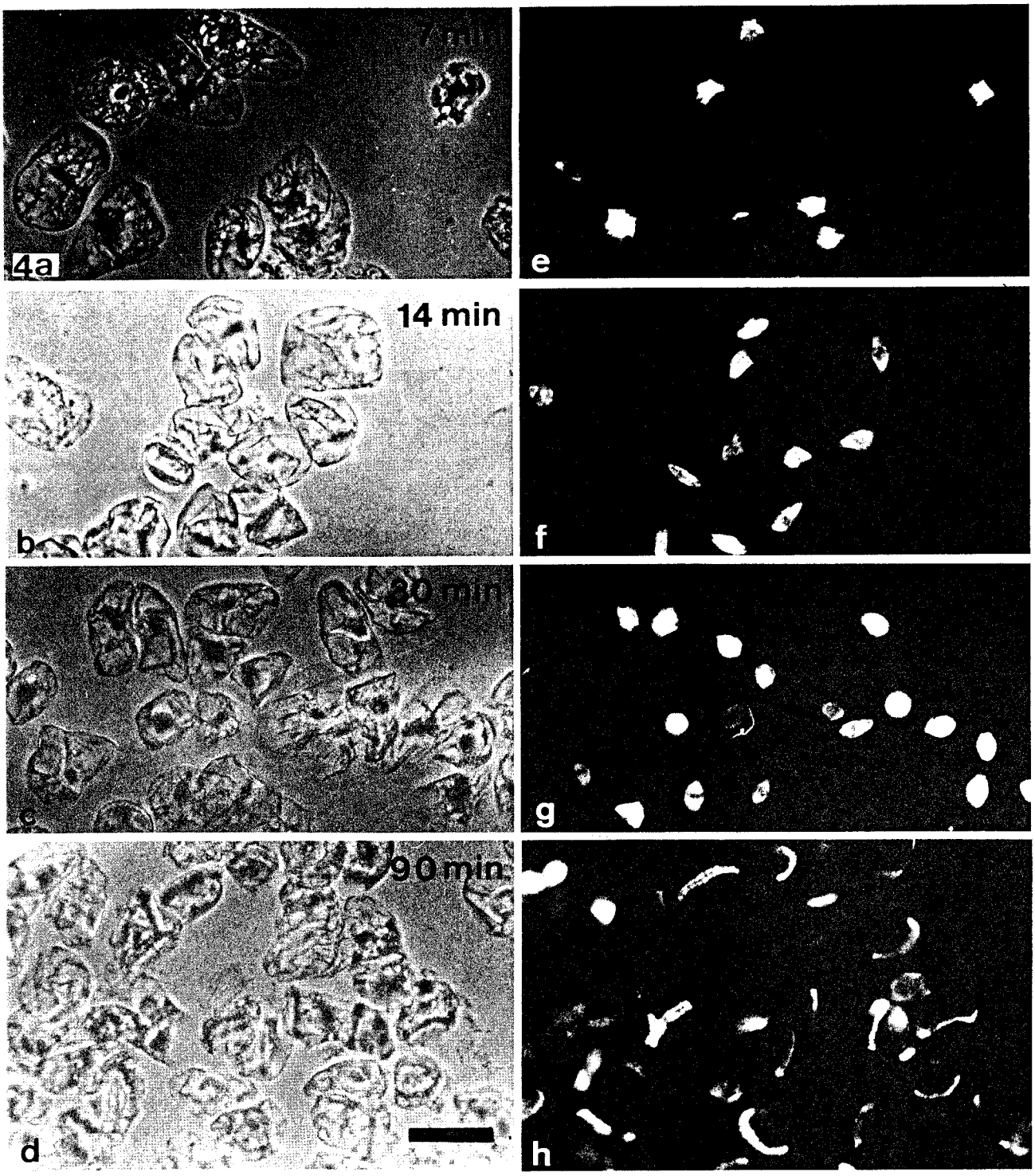


Fig. 4. Synchronized formation of phragmoplasts after the termination of the treatment with propyzamide described in the legend to Fig. 2. After the termination of treatment with propyzamide, cells were sampled at appropriate time intervals and stained for microtubules. Time after the termination of treatment with propyzamide is given in minutes in the photographs. (a)-(d)Phase contrast micrographs. (e)-(h)Immuno-fluorescence micrographs. Bar = 50 μm

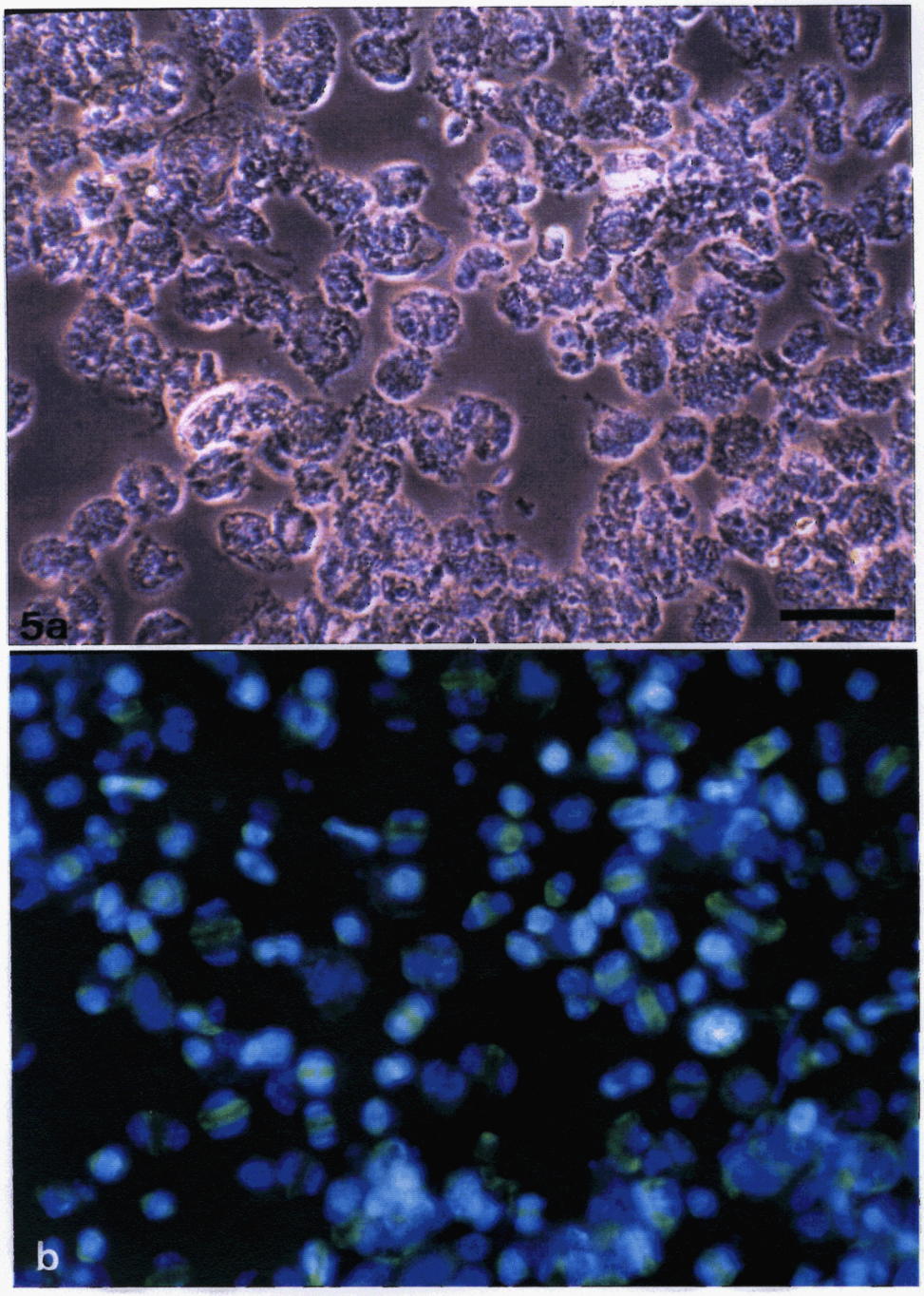


Fig. 5. Phragmoplast-nuclei complexes isolated from cell-cycle synchronized tobacco BY-2 cells. (a) Phase contrast micrographs. (b)Phragmoplasts were double stained for microtubules (green) and nuclei (blue). bar=50 μm.

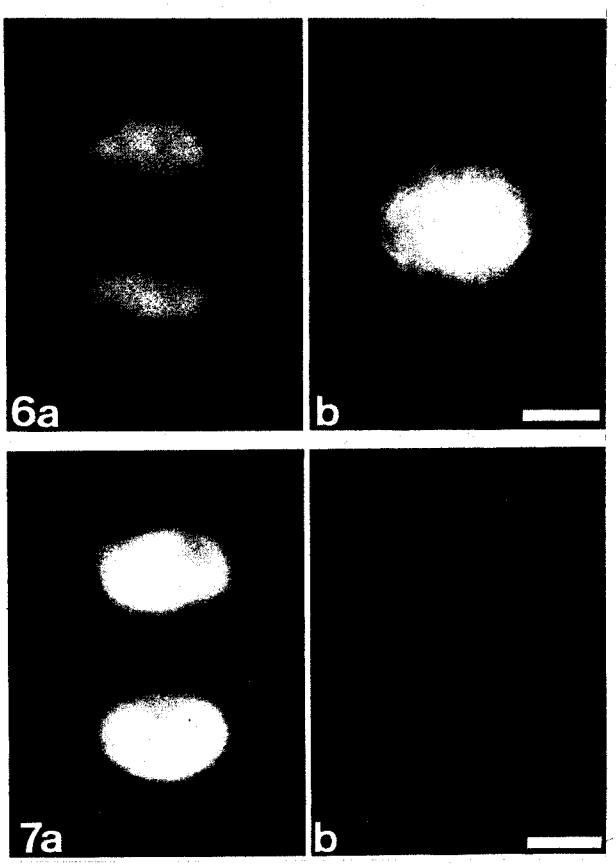


Fig. 6. A phragmoplast-nuclei complex isolated from a tobacco BY-2 cell by treatment with buffer B and double stained with DAPI (a) and anti-α-tubulin antibody (b). Note that the phragmoplast (b) is associated with the daughter nuclei (a).
Bar= 10 um

Fig. 7. Phragmoplast-nuclei complexes isolated from tobacco BY-2 cells by treatment with buffer B, incubated in 100 μM $CaCl_2$ for 20 minutes at $0^{o}C$, and double stained with DAPI (a) and anti-α-tubulin antibody (b). Note that phragmoplast microtubules are destroyed by treatment with $CaCl_2$ (b), but a pair of daughter nuclei is not separated by this treatment (a). Bar = 10 μm

Fig. 8. Electron micrographs of a phragmoplast-nuclei complex isolated from tobacco BY-2 cell by treatment with buffer A. (a) A whole complex. Boxed areas (b), (c), and (d) are shown at higher magnification in Figs. 10(b), (c), and (d), respectively. (b) Near the reforming daughter nucleus, abundant microtubules, but no actin filaments, are seen. Some microtubules are associated with thin filaments that are not decorated by heavy meromyosin. (c) Cross bridges between adjacent microtubules. (d)Near the equatorial plate, actin filaments decorated by heavy meromyosir are seen. They are oriented perpendicularly or nearly perpendicularly to the plate. Note that arrowheads point away from the plate in most of the filaments. (a) Bar = 5 μm (b)-(d) Bar = 0.5 μm

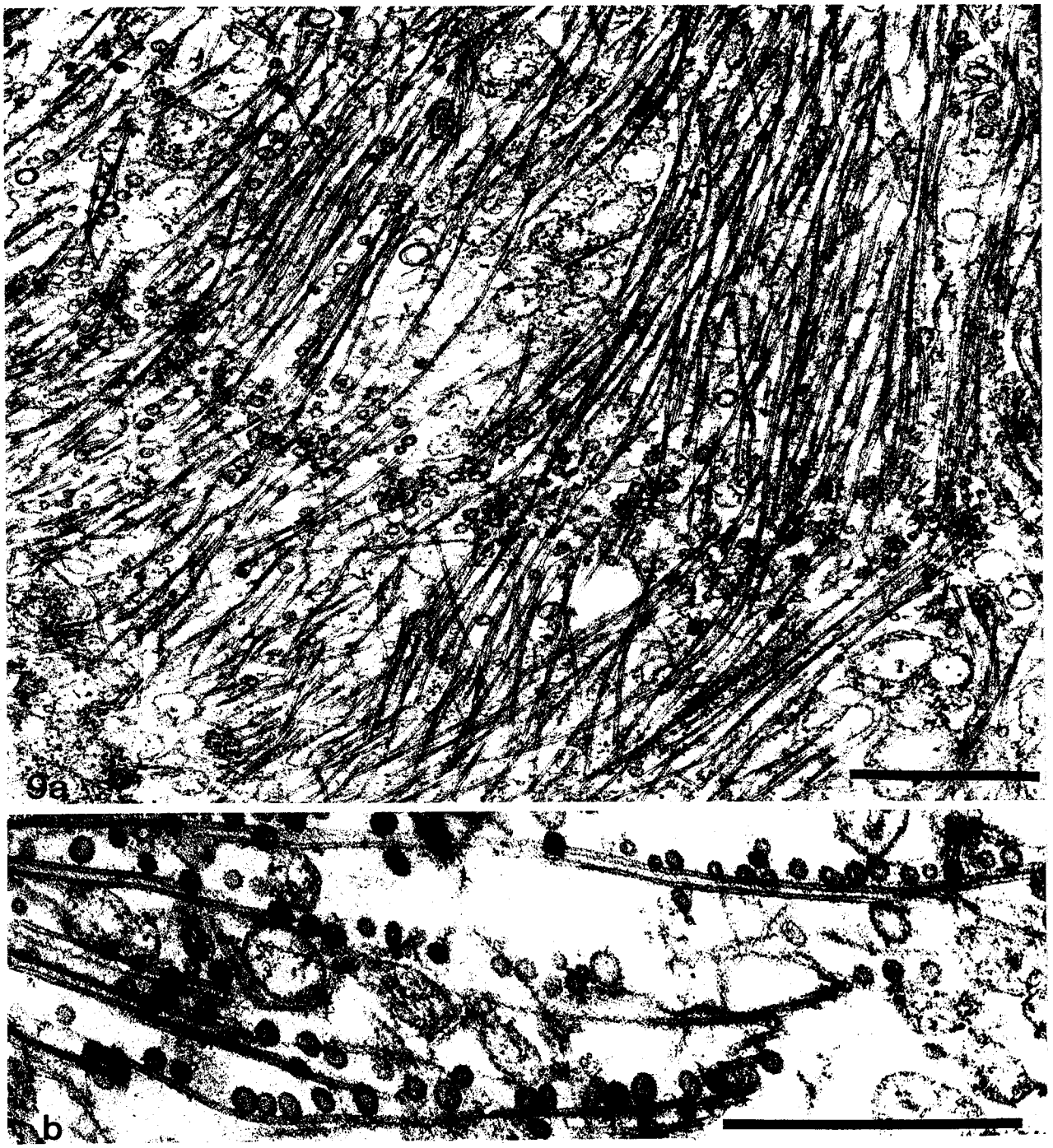


Fig. 9. (a), (b)Electron micrographs of a part of a phragmoplast isolated from a tobacco BY-2 cell by treatment with buffer A and . fixed by freeze-substitution method. Abundant, electron-dense small vesicles are seen attached to the phragmoplast microtubule. Bar = 1 μm

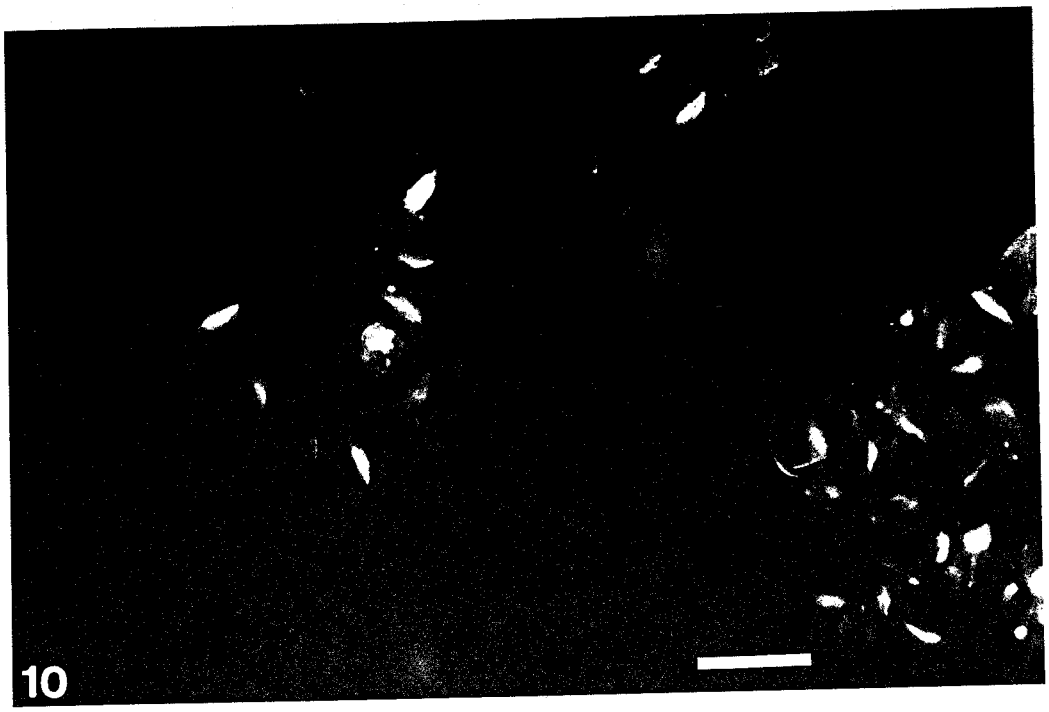


Fig. 10. Distribution of callose in BY-2 cells. Tobacco BY-2 cells, whose cell cycle was synchronized in anaphase to telophase, were stained with aniline blue. Aniline blue stainable material was localized at cell plates. Bar=50 μm

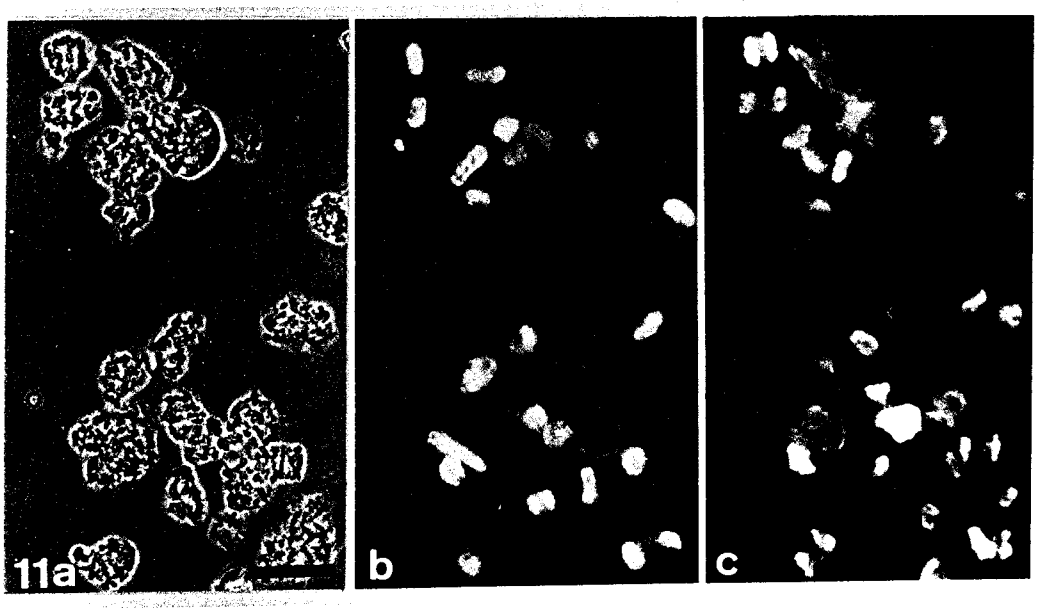


Fig. 11. Phragmoplasts which were isolated by passing through nylon mesh. a) Phase-contrast micrograph. b)Immunofluorescence micrograph which visualizes microtubules. c)Stained with DAPI for nuclei. Arrowheads indicates isolated phragmoplasts. Bar=50 μm

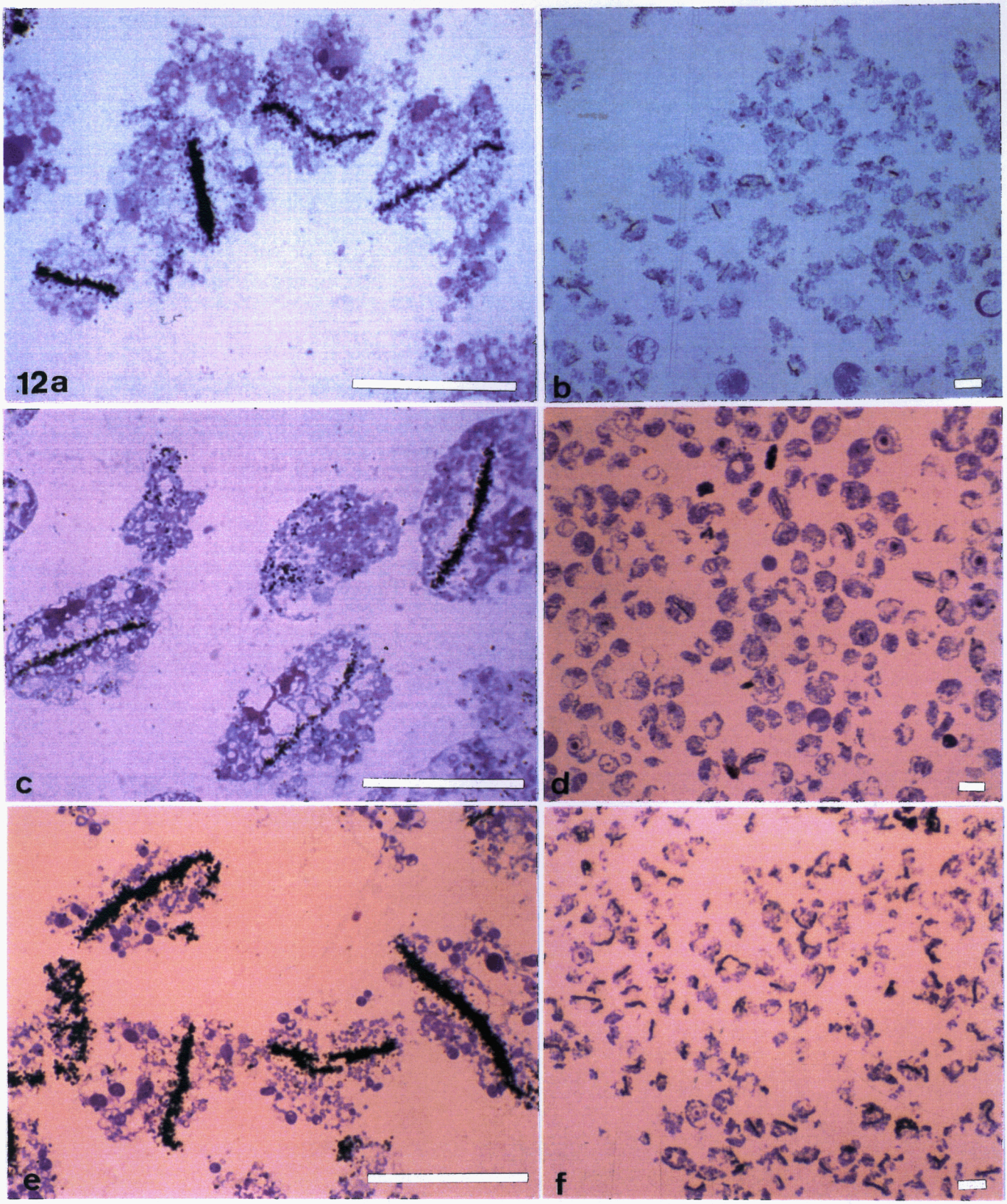


Fig. 12. Radioautographs of sectioned phragmoplasts which reveal the incorporation of the radioactivity from UDP-[^{3}H]glucose into isolated phragmoplasts. UDP-[^{3}H]glucose was used at 5 µM (a, b), 100 µM (c, d) and 1 mM (e, f). Silver grains are present at the cell plate. Bar=30 µm

Fig. 13. Electron microscope radioautograph which reveals the incorpolation of radioactivity into isolated phragmoplasts from 5 μM UDP-[^{3}H]glucose. Note that silver grains are localized at the cell plate. No grains are seen on the Golgi apparatus (G). N: nucleus. Bar=5 μm

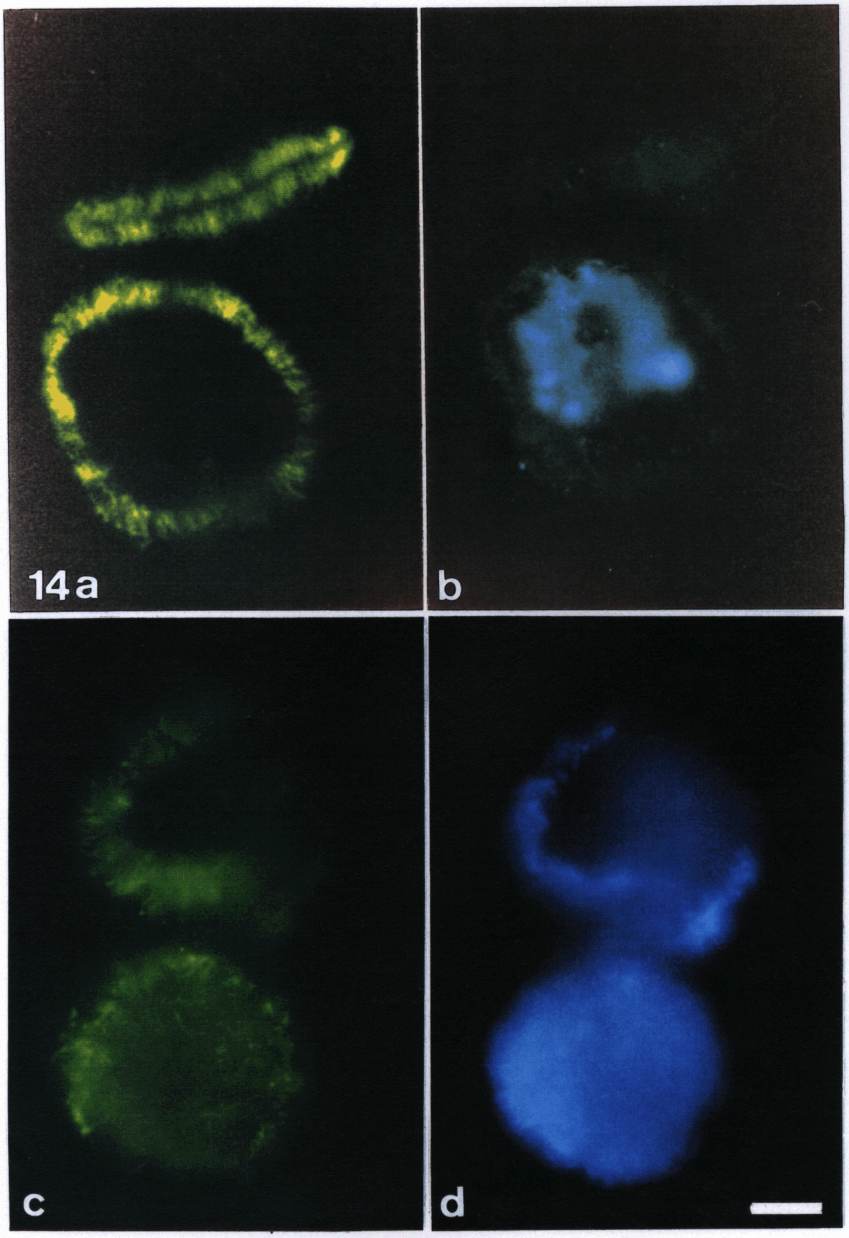


Fig. 14. a, b) Isolated phragmoplasts were stained with anti-tubulin antibody(a) and with aniline blue(b). Aniline-blue stainings were faint. c, d) Isolated phragmoplasts were incubated with 1 mM UDP-glucose and stained with anti-tubulin antibody (c) and with aniline-blue (d). Aniline-blue positive material was formed at the cell plate. Phragmoplasts of early phase (c, d; lower one) formed anilin-blue positive material in whole region of cell plates and phragmoplasts of late phase (annular shaped; c, d: upper one) mainly at the expanding region of the cell plate. Bar=10 μm

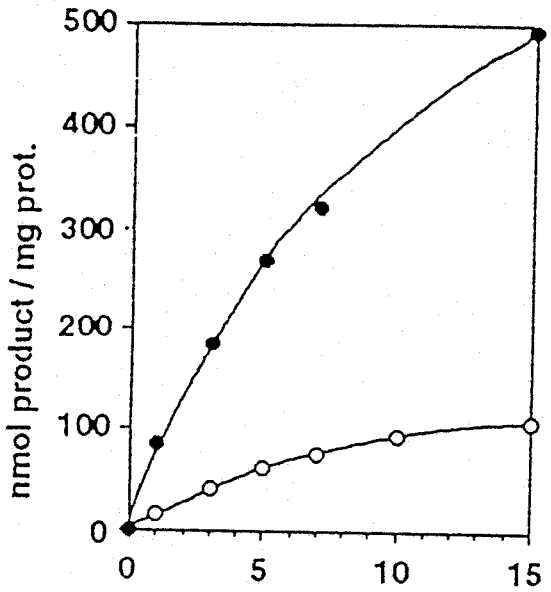


Fig. 15.

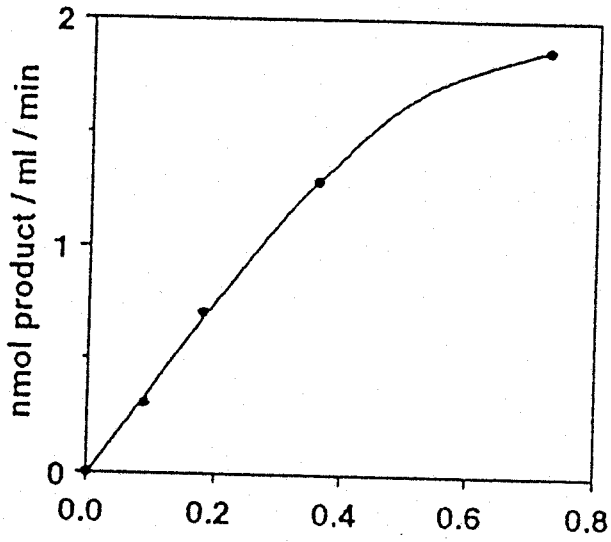


Fig. 16.

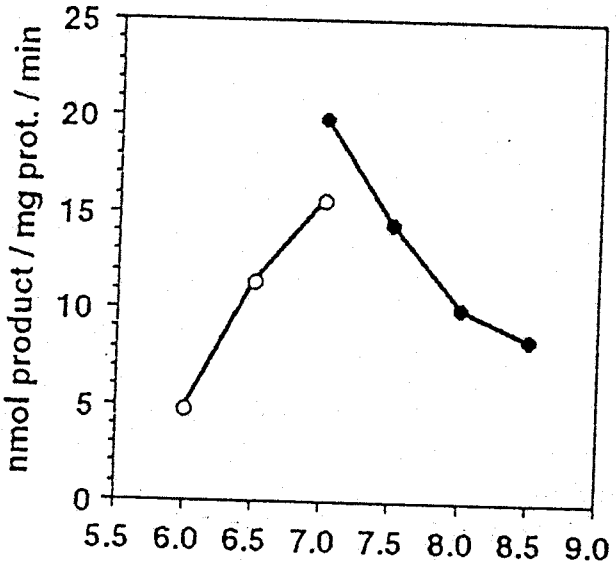


Fig. 17.

Fig. 15. Time course of glucan synthesis from UDP-[^{14}C]glucose. UDP-[^{14}C]glucose was used at 100 μM (open circle) and 1 mM (closed circle).

Fig. 16. Effect of the density of phragmoplasts (measured as protein concentration) on glucan synthesis from UDP-[^{14}C]glucose. UDP-[^{14}C]glucose was used at 100 μM.

Fig. 17. Effect of pH on glucan synthesis from UDP-[^{14}C]glucose. UDP-[^{14}C]glucose was used at 100 μM. PIPES buffer (open circle) and Tris buffer (closed circle) were used.

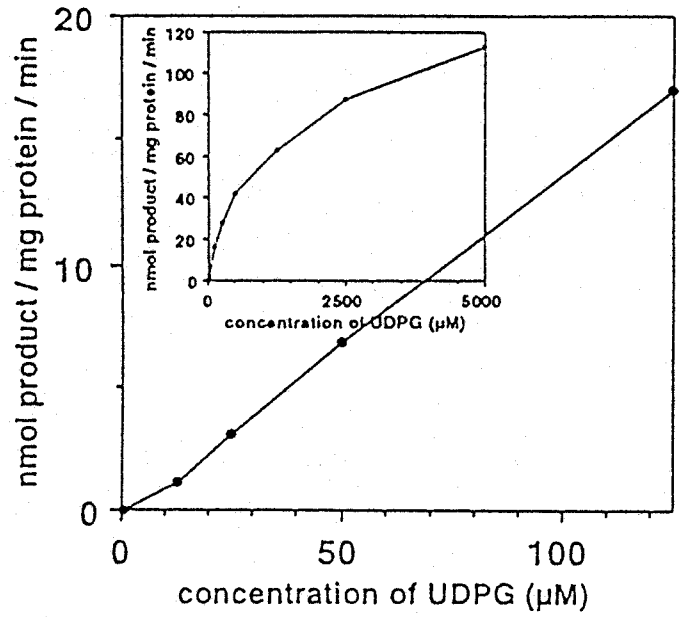


Fig. 18. Glucan synthase activity at varying concentrations of UDP-[^{14}C]glucose.

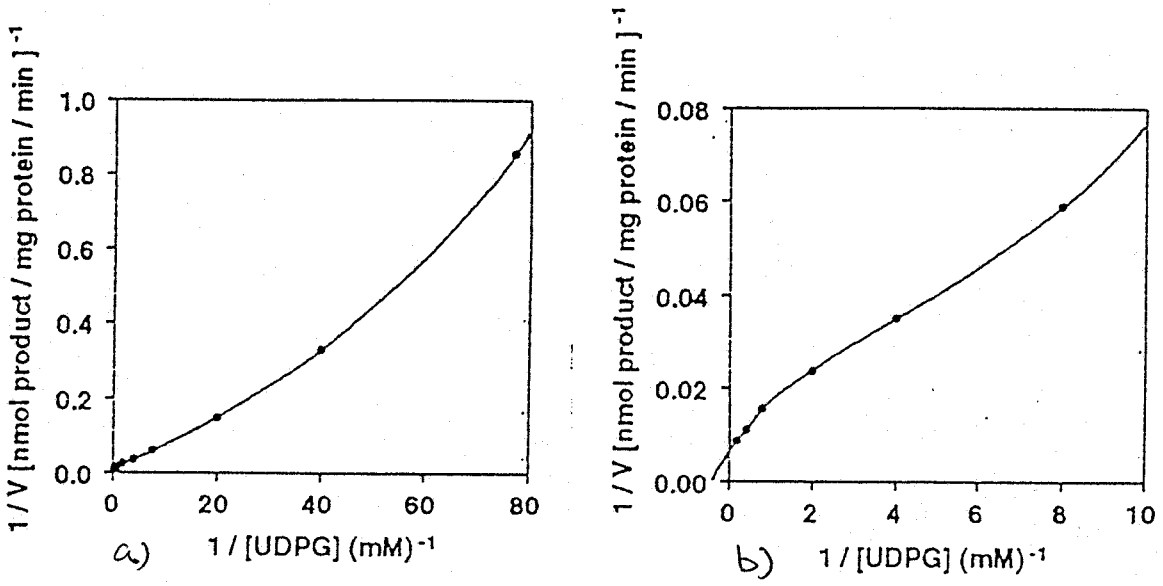


Fig. 19. a, b) Double reciprocal plot of glucan synthase activity versus UDP-[^{14}C]glucose concentration.

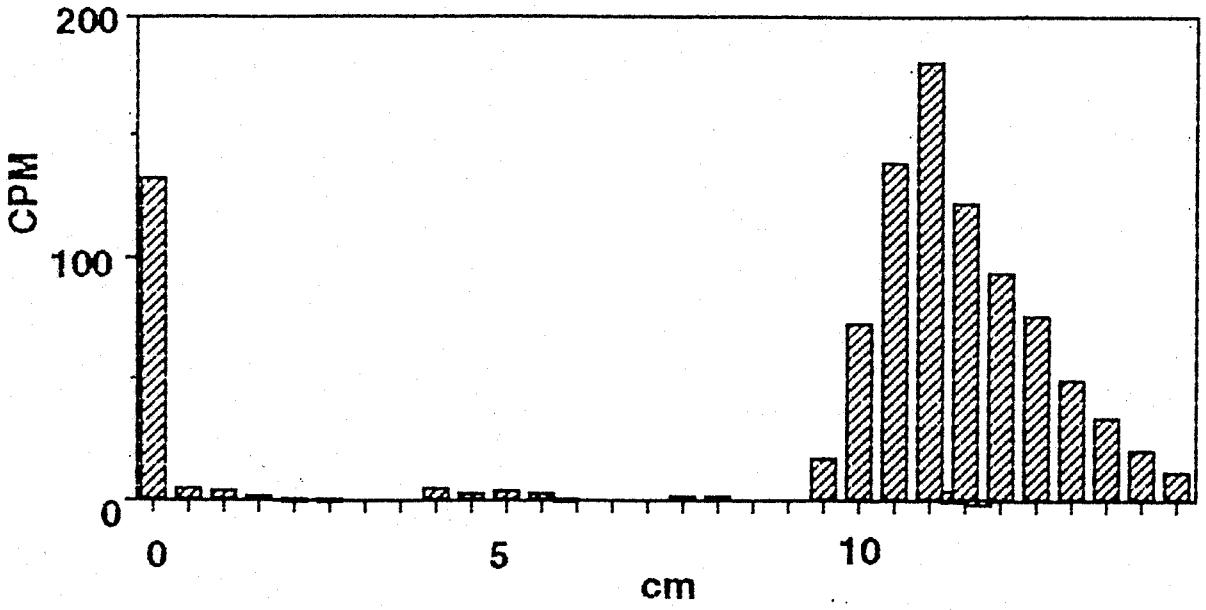


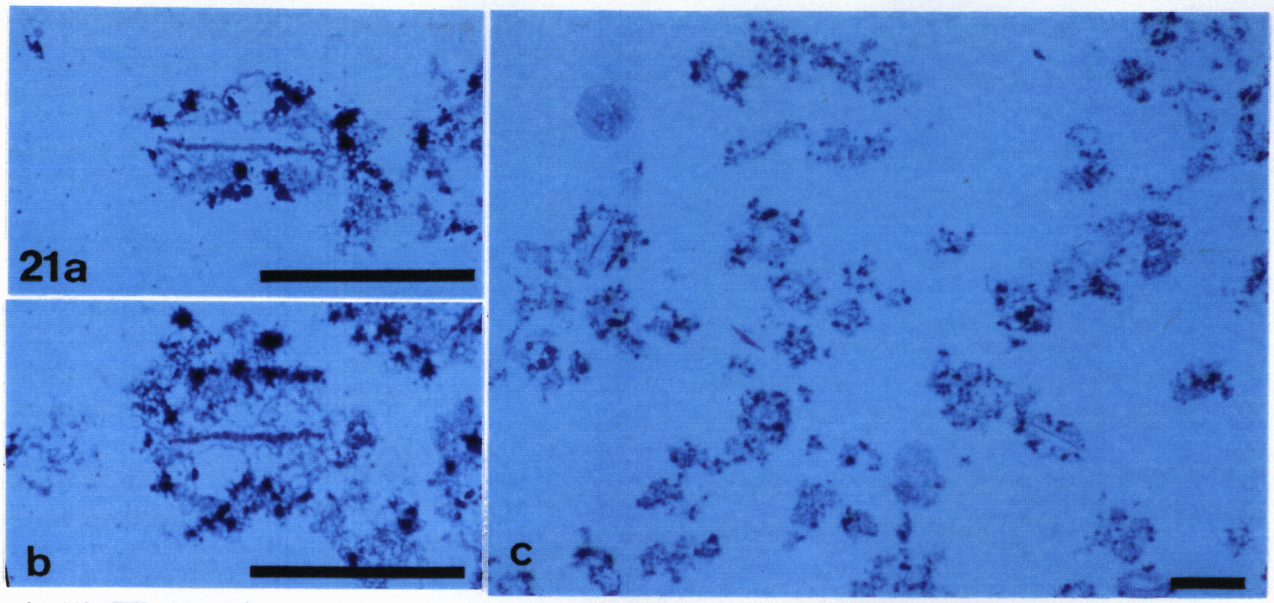
Fig. 20. Products formed from 100 μM UDP-[^{14}C]glucose were digested with 0.5 mg/ml of 1,3-β-glucanase at 27 ℃ for 4 h (materials and methods) and the digest was analysed by paper chromatography.

Table 1

Factors affecting glucan synthesis

Incubation mixture	Glucan synthase activity (nmol / mg protein / min)
Standard system	13.1
minus Ca^{2+}	11.4
plus Ca^{2+} (1mM)	11.4
plus EGTA (2mM)	2.7
plus Mg^{2+} (1mM)	10.0
plus Mg^{2+} (5mM)	6.2
plus cellobiose (5mM)	13.1
plus laminaribiose (5mM)	14.1
minus Ca^{2+}, plus cellobiose (5mM)	13.5
minus Ca^{2+}, plus laminaribiose (5mM)	14.9

The standard system contains: isolated phragmoplasts (175 μg protein/ml), 50 mM PIPES-KOH (pH=7.0), 0.3 M sucrose, 0.25 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 50 μg/ml leupeptin, 10 μg/ml APMSF and 100 μM UDP-[^{14}C]glucose. The values in the brackets are final concentration in the assay mixture.



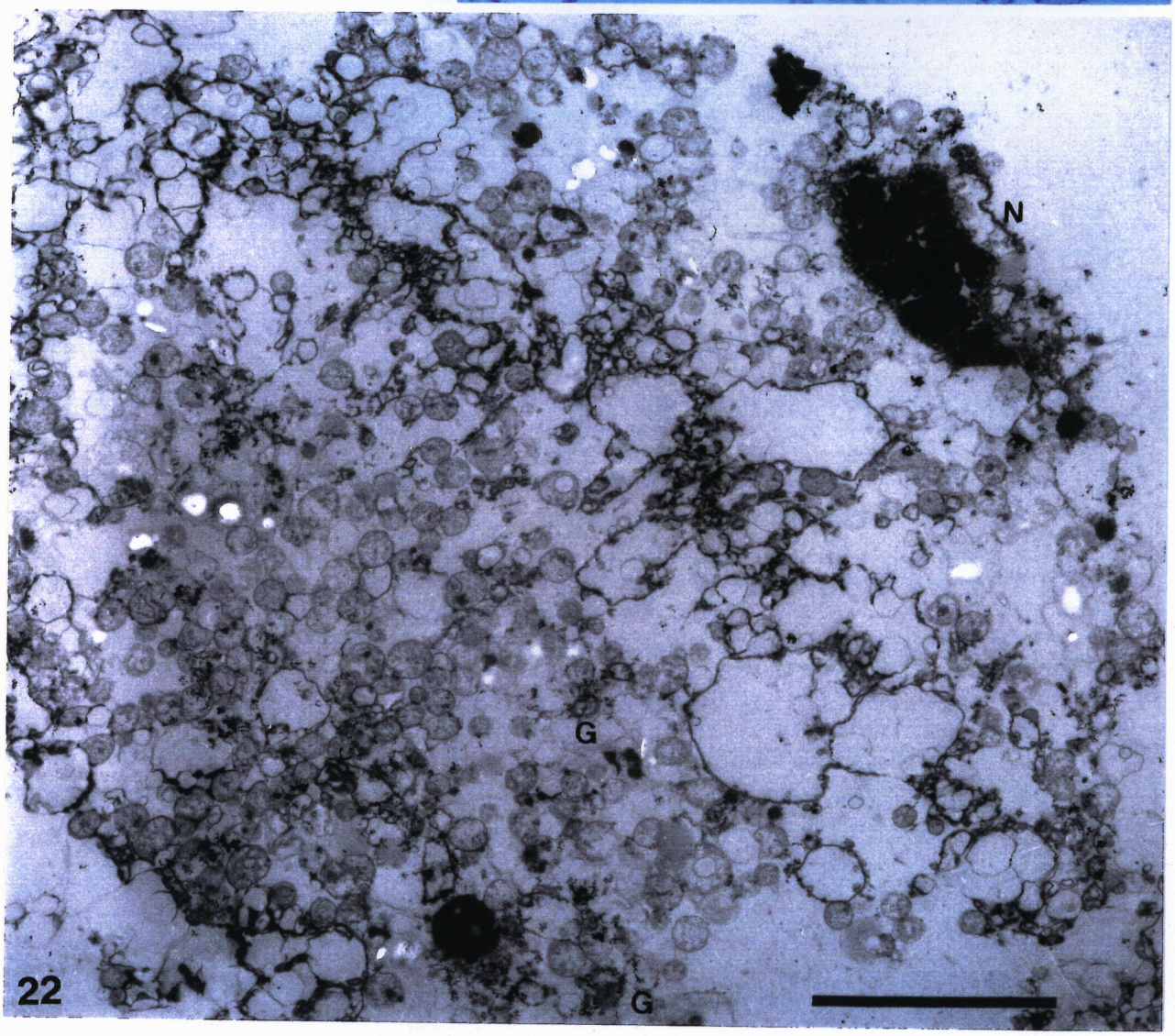
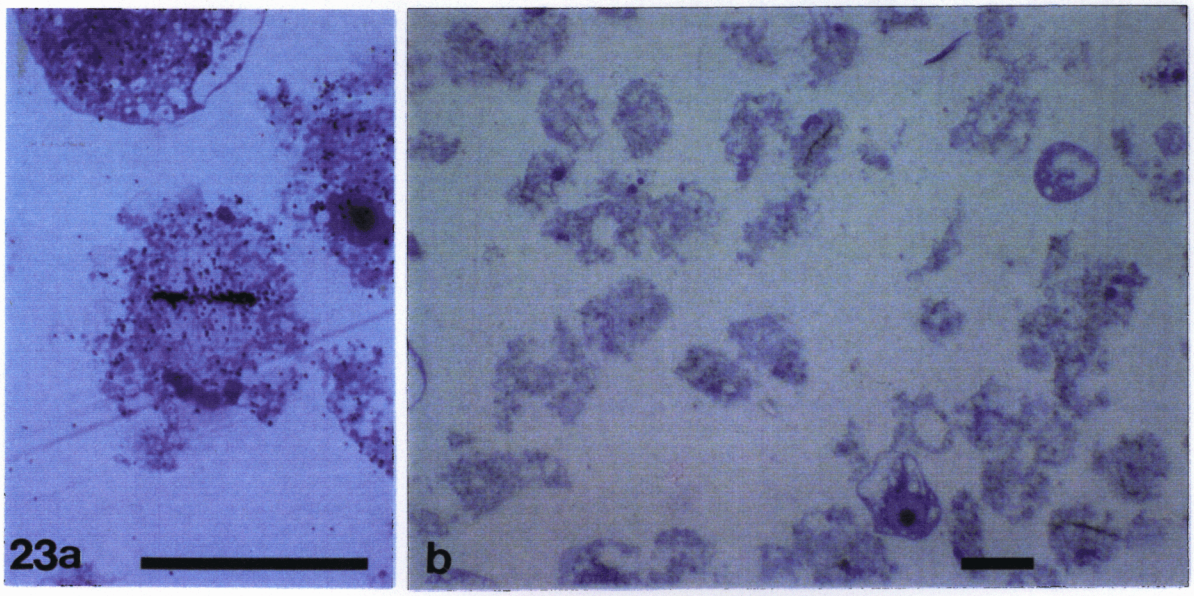


Fig. 21. a, b, c,) Radioautographs of sectioned phragmoplasts which reveal the incorpolation of the radioactivity from UDP-[^{3}H]xylose (28 μM) in the presence of unlabeled UDP-glucose (2 mM). Silver grains existed as scattered aggregate around nuclei. Bar=30 μm

Fig. 22. Electron microscope autoradiograph of sectioned phragmoplasts which reveal the incorpolation of the radioactivity from UDP-[^{3}H]xylose (28 μM) in the presence of unlabeled UDP-glucose (2 mM). Radioactivity exists at Golgi-apparatus-like structures (G) and unidentified structure around nuclei. N: nucleus. Bar=5 μm



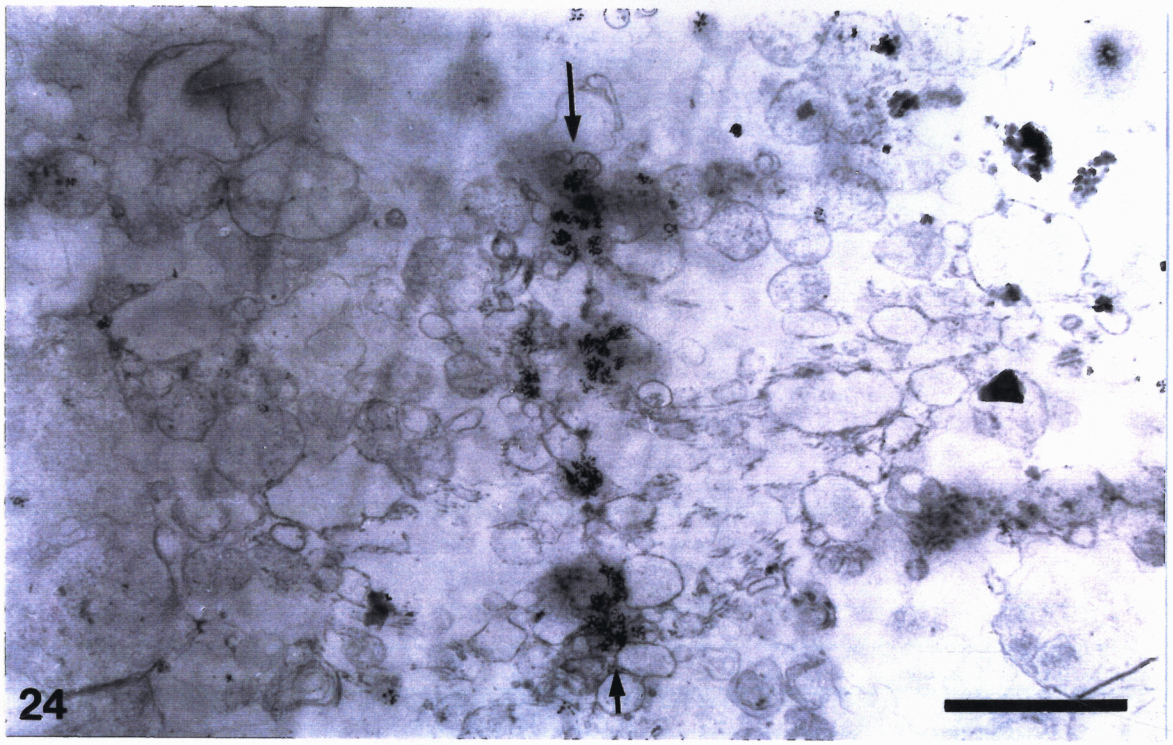


Fig. 23. a, b) Radioautographs of sectioned phragmoplasts which reveals the incorporation of radioactivity from UDP-[^{3}H]galactose (100 µM). Density of silver grains is high at the cell plate. Bar=30 µm

Fig. 24. Electron microscope radioautograph which reveals the incorporation of radioactivity from UDP-[^{3}H]galactose. Arrows indicate equatorial plane. Bar=2 µm