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Microinjected Fluorescent Phalloidin in Vivo Reveals the F-Actin Dynamics and Assembly in Higher Plant Mitotic Cells

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Endosperm mitotic cells microinjected with fluorescent phalloidin enabled us to follow the in vivo dynamics of the F-actin cytoskeleton. The fluorescent probe immediately bound to plant microfilaments. First, we investigated the active rearrangement of F-actin during chromosome migration, which appeared to be slowed down in the presence of phalloidin. These findings were compared with the actin patterns observed in mitotic cells fixed at different stages. Our second aim was to determine the origin of the actin filaments that appear at the equator during anaphase-telophase transition. It is not clear whether this F-actin is newly assembled at the end of mitosis and could control plant cytokinesis or whether it corresponds to a passive redistribution of broken polymers in response to microtubule dynamics. We microinjected the same cells twice, first in metaphase with rhodamine-phalloidin and then in late anaphase with fluorescein isothiocyanate-phalloidin. This technique enabled us to visualize two F-actin populations that are not co-localized, suggesting that actin is newly assembled during cell plate development. These in vivo data shed new light on the role of actin in plant mitosis and cytokinesis.

INTRODUCTION

A large body of evidence obtained during the last few years that reveals entirely new features of the plant actin cytoskeleton suggests that plant microfilaments may play an important role in specific properties of the higher plant cells (for review, see Lloyd, 1989). To date, these data have been obtained after labeling of fixed and permeabilized cells, either by indirect immunofluorescence or with fluorescent phalloidin (Seagull, Falconer, and Weerdenburg, 1987; Schmit and Lambert, 1987; Lloyd and Traas, 1988), as a specific probe for F-actin (Wieland, 1977; Wulf et al., 1979). The identification of an actin network in the cytoplasm of endosperm cells (Schmit and Lambert, 1985, 1987; Molè-Bajer, Bajer, and Inoué, 1988), as in various cell types (for reviews, see Staiger and Schliwa, 1987; Lloyd, 1989), showed that F-actin is, like tubulin, one of the major components of the plant cytoskeleton. Its role, however, is hardly understood. A much-debated question is to define the potential activity of actin during plant mitosis and cytokinesis (Forer, 1985, 1988; Schmit and Lambert, 1987, 1988; Molè-Bajer et al., 1988). In most cultured animal cells, mitotic induction is characterized by the disassembly of actin stress fibers (for review, see Aubin, 1981). On the contrary, in higher plant cells it has been found that the interphase cytoplasmic actin network is rearranged within the cell cortex at the onset of mitosis and remains as a permanent cage around the microtubular mitotic spindle, as seen after permeabilization, in endosperm (Schmit and Lambert, 1987) and in other higher plant cells (Traas et al., 1987; Lloyd and Traas, 1988). Microfilaments were also detected inside the spindle (Forer, 1985), but their potential activity is still unknown. Indeed, cytochalasins (Schmit and Lambert, 1988) and phalloidin may affect chromosome velocity but do not stop chromosome migration. Besides these characteristics and the unexpected behavior of the cortical actin during the process of mitosis, a particular accumulation of actin filaments is detected during strategic events that control cytokinesis, suggesting that actin may regulate plant cytokinesis.

Two steps are of particular interest. (1) At the onset of mitosis, actin filaments were detected within the preprophase band that is believed to predetermine the future cell division plane (Palevitz, 1987) and that was thought to be formed only by microtubules. (2) During cytokinesis itself, which is related to the preprophase events, actin filaments are accumulated in a wreath in the phragmoplast (Palevitz, 1987; Lloyd and Traas, 1988; Traas, Burgain, and Dumas de Vaulx, 1989).

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As actin is known to be highly sensitive to fixation and permeabilization (Lloyd and Traas, 1988), presumptive artefactual rearrangements of actin microfilaments during this procedure are still possible, particularly as far as the mitotic plant F-actin is concerned. In the hope of avoiding such current objections, our aim was to study directly the dynamics of the microfilaments in vivo. For these investigations, living mitotic endosperm cells were microinjected with low concentrations of fluorescent phalloidin at precise stages of mitosis. As chromosome movement proceeded, we could analyze the dynamics of labeled microfilaments at subsequent stages, using an image-processing system that allows fast recording without light exposure damage.

The main questions we posed were: (1) Can actin filaments be stretched by mitotic forces related to spindle microtubule/microfilament interactions, as suggested by our earlier studies? (2) What is the origin of the equatorial telophasic actin accumulation? Is phragmoplast actin a remnant of mitotic actin or is it newly polymerized at cytokinesis? Our current data give new insight into current hypotheses on the activity of actin during plant mitosis and cytokinesis.

RESULTS

Plant F-Actin Detection in Living Mitotic Cells

We optimized the labeling conditions to determine the lowest doses of microinjected fluorescent phalloidin that would provide detectable fluorescence of F-actin in live cells, while avoiding as much as possible the assembly, stabilizing, and/or toxic effects of the probe. The whole F-actin pattern, as compared with fixed cells, was visualized using phalloidin concentration in the micromolar range, as described in Methods. It corresponds per cell to 10^{-9} to 10^{-8} μg of fluorescent probe; that is, 50 to 750 times lower concentrations than for earlier published data.

During microinjection that was constantly recorded in real time, the morphology of the cells was not altered and the mitotic chromosome movement progressed. In fluorescence, the diffuse staining that was observed during and immediately after microinjection was specifically and very rapidly concentrated on the microfilaments, whereas the fluorescent background disappeared. Nevertheless, some stabilizing effect resulting in thickening of the actin bundles was observed only about 40 min to 60 min after microinjection.

Recording of the in vivo labeled microfilaments at different stages of the mitotic cell had to be performed with a low light-sensitive (SIT) camera set up with real time image processing, as described in Methods; this was to limit exposure time to a few seconds only, and, thus, prevent quick fading. In fact, no antifading agents could be used because they are toxic in living cells and because they might induce in vivo alterations of the actin dynamics.

Chromosome Movement in Fluorescent Phalloidin-Injected Cells

After fluorescent phalloidin microinjection, cell preparations were maintained as described in Methods, and chromosome movement was monitored for up to 3 hr. Controls were made on nonmicroinjected and/or PBS-injected mitotic endosperm cells. In PBS-injected cells, chromosome movement was entirely comparable with noninjected cells, indicating that microinjection by itself did not disturb the process of mitosis. In all cases, chromosome condensation and nuclear envelope breakdown took place normally. Our experimentation focused mainly on metaphase to telophase transition. After microinjection of rhodamine-phalloidin, poleward chromosome migration occurred. After reaching the poles, the chromosomes decondensed in daughter nuclei in the same way as in control cells. Although the chromosome route was similar to controls, velocity of chromosome movement was slowed down in most fluorescent phalloidin-injected cells. Table 1 shows a comparison among different control cells where the rate of chromosome movement was an average of 1 μm/min, whereas it was 0.5 μm/min in the presence of the probe. The spindle final elongation in anaphase B was 15% to 30% lower than in controls, but cytokinesis occurred.

In Vivo Microfilament Dynamics during Mitosis after Phalloidin Microinjection

Recording was made using an image-processing system, as described in Methods. This system allowed us to reduce artifacts due to light damage on in vivo fluorescently labeled actin filaments.

Figure 1a illustrates a metaphase cell just after microin-

Table 1. Chromosome Velocity and Spindle Elongation in Fluorescent Phalloidin Microinjected Cells

	Kinetochore velocity, μm/min	Final spindle elongation, μm
Fluorescent phalloidin-injected cells (10)	0.5 ± 0.2	55 ± 5
Controls		
Noninjected cells (20)	1.2 ± 0.2	70 ± 5
PBS-injected cells (5)	1.0 ± 0.1	70 ± 5
FITC/BSA-injected cells (5)	1.0 ± 0.1	70 ± 5

Average cell size is 90 ± 5 μm. For each set, the number of experimented cells is indicated in parentheses.

jection of rhodamine-phalloidin. When observed in fluorescence microscopy, it reveals a supple, dense network (Figure 1c) that has developed in the cell cortex. The two opposite polar domains are composed of small regular meshes of thin criss-crossing actin bundles. In the cytoplasmic domain surrounding the mitotic spindle, such thin bundles of F-actin become parallel to the spindle axis, where they appear to be stretched when compared with previous stages. These microfilaments are located in contact or in close proximity to spindle microtubules. Optical sections through the mitotic spindle also reveal the presence of some actin filaments within the spindle microtubules, particularly kinetochore fibers. During anaphase progression, the F-actin bundles aligned more and more parallel to the spindle axis. Simultaneously, as illustrated in Figures 1d and 1e, many actin filaments could also be visualized inside the spindle, parallel to microtubule fibers. No more cytoplasmic diffuse pattern could then be seen. When those early microinjected cells were observed in telophase, i.e., about 1 hr later, the labeled F-actin network had reduced to a few thick bundles, located mostly around the daughter nuclei. No fluorescent filaments were seen in the phragmoplast of such cells (data not shown). In comparison, late-anaphase and telophase microinjected cells revealed a strong filamentous pattern at the equatorial region.

This absence of fluorescence observed in telophase living cells, previously microinjected in metaphase, indicates that the actin accumulation observed in the interzone of late anaphase injected cells seems not to be due to broken cortical microfilaments pushed by growing polar microtubules.

Comparison with the F-Actin Distribution in Fixed and Permeabilized Cells

The in vivo dynamics of the labeled network observed after phalloidin microinjection was compared with the one determined on a series of fixed cells at different stages of mitosis. Cell preparations were immunolabeled using monoclonal anti-actin antibodies raised again chicken-gizzard actin or stained with fluorescent phalloidin. This was described previously (Schmit and Lambert, 1987) and is illustrated in Figure 2 for comparison purposes. In mid-anaphase, Figures 2a and 2b, the actin pattern corresponds to that observed in vivo, just after phalloidin microinjection in metaphase or early anaphase. In late anaphase, as seen Figures 2c and 2d, the F-actin organization has clearly changed at the equatorial region. It corresponds to an increase of actin bundles that have invaded the interzone, parallel to the spindle axis. This accumulation occurs before the two microtubular half-spindles are connected with each other, i.e., during polar microtubule growth. Later on, the actin filaments are localized as a wreath intermingling with the phragmoplast microtubular ring.

In Vivo Microfilament Distribution after Double Phalloidin Microinjection

In the hope of understanding the origin of the actin wreath observed during cytokinesis, we performed a double microinjection in the same cell: a first injection in metaphase, using rhodamine-phalloidin, followed by a second one in late anaphase, using fluorescein isothiocyanate (FITC)-phalloidin. This experiment is illustrated in Figure 3. The reorganization of rhodamine-labeled microfilaments was followed from metaphase to late anaphase over a period of 1 hr. The F-actin pattern corresponded to that described in Figure 1. When chromosomes reached the poles, as illustrated in Figures 3d to 3f, only a few rhodamine-labeled bundles were observed at the equator, whereas the cortical network appeared composed of thick actin bundles. Such bundles are only observed after long exposure to the probe and are not seen in fixed or freshly microinjected cells. Therefore, even at low concentration, phalloidin may induce some bundling effects on labeled actin filaments. The same cell was then re-microinjected with FITC-phalloidin (Figures 3g to 3i) to determine whether new polymerized, unlabeled, and, therefore, undetectable bundles were formed during anaphase development. The FITC-labeled pattern of F-actin differed completely from the rhodamine-labeled network. Many thin, criss-crossing bundles appeared in the cortex and in the interzone, showing that actin filaments were now labeled with the second probe. This staining appeared to be as supple and thin as the one observed just after the first injection. Only a very few microfilament bundles were co-stained with the two fluorochromes, suggesting that the remnant rhodamine-labeled filaments were saturated with the fluorochrome after the first injection.

In Vivo Actin Distribution during Cytokinesis in Phalloidin-Injected Telophase Cells

In addition to these experiments, we studied actin distribution during later stages of cytokinesis in freshly single microinjected telophase when the cell plate is already organized, as seen in Figure 4. The in vivo actin pattern is comparable with that in Figure 1d, indicating an accumulation of microfilaments at the equator, while criss-crossing actin bundles remain clearly detected in the cortex.

DISCUSSION

Fluorescent labeled phalloidin, introduced as an actin-specific drug (Wieland, 1977), appeared to be a powerful

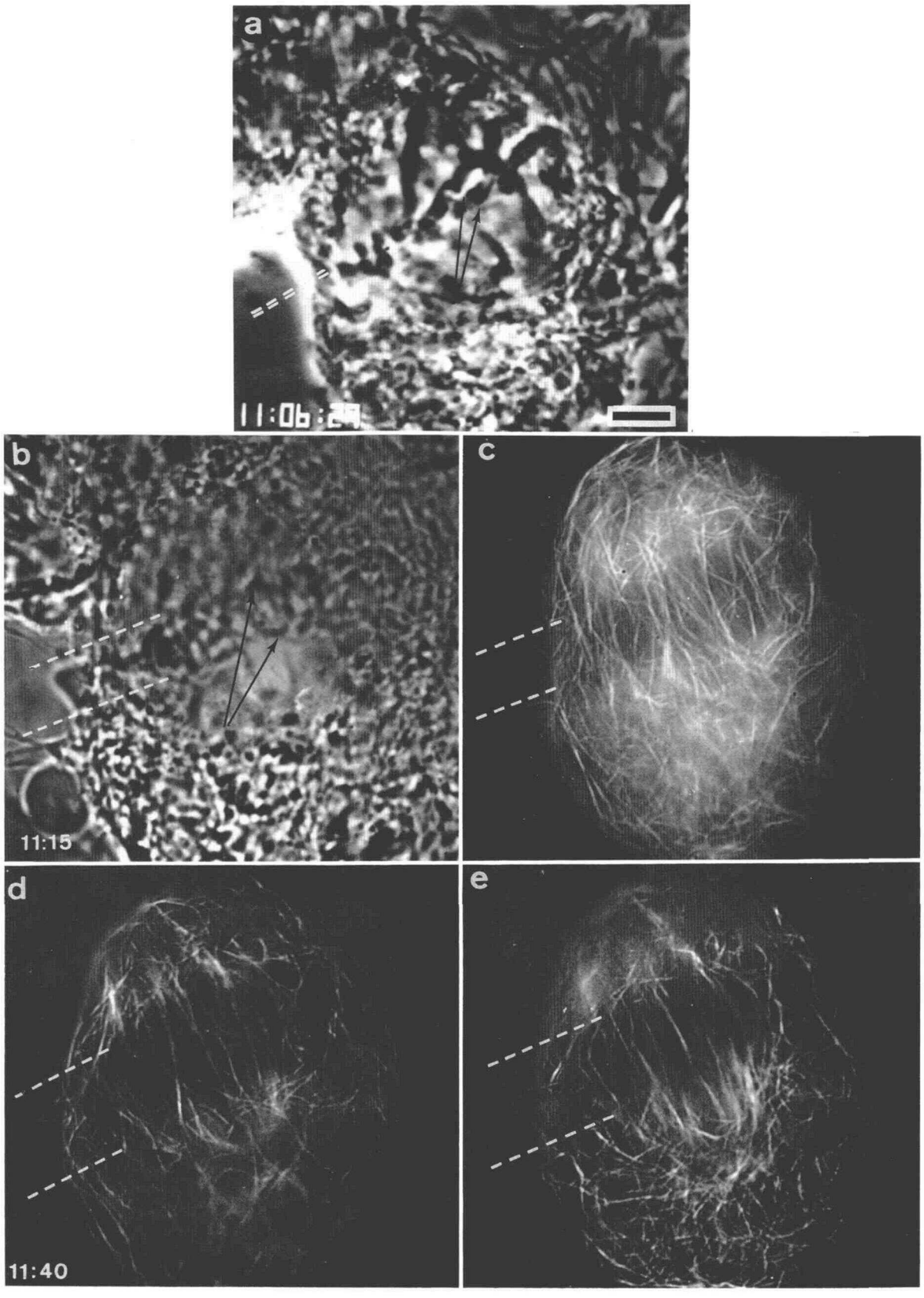

Figure 1. In Vivo F-Actin Dynamics in a Rhodamine-Phalloidin Microinjected Cell during Metaphase-Anaphase Transition.

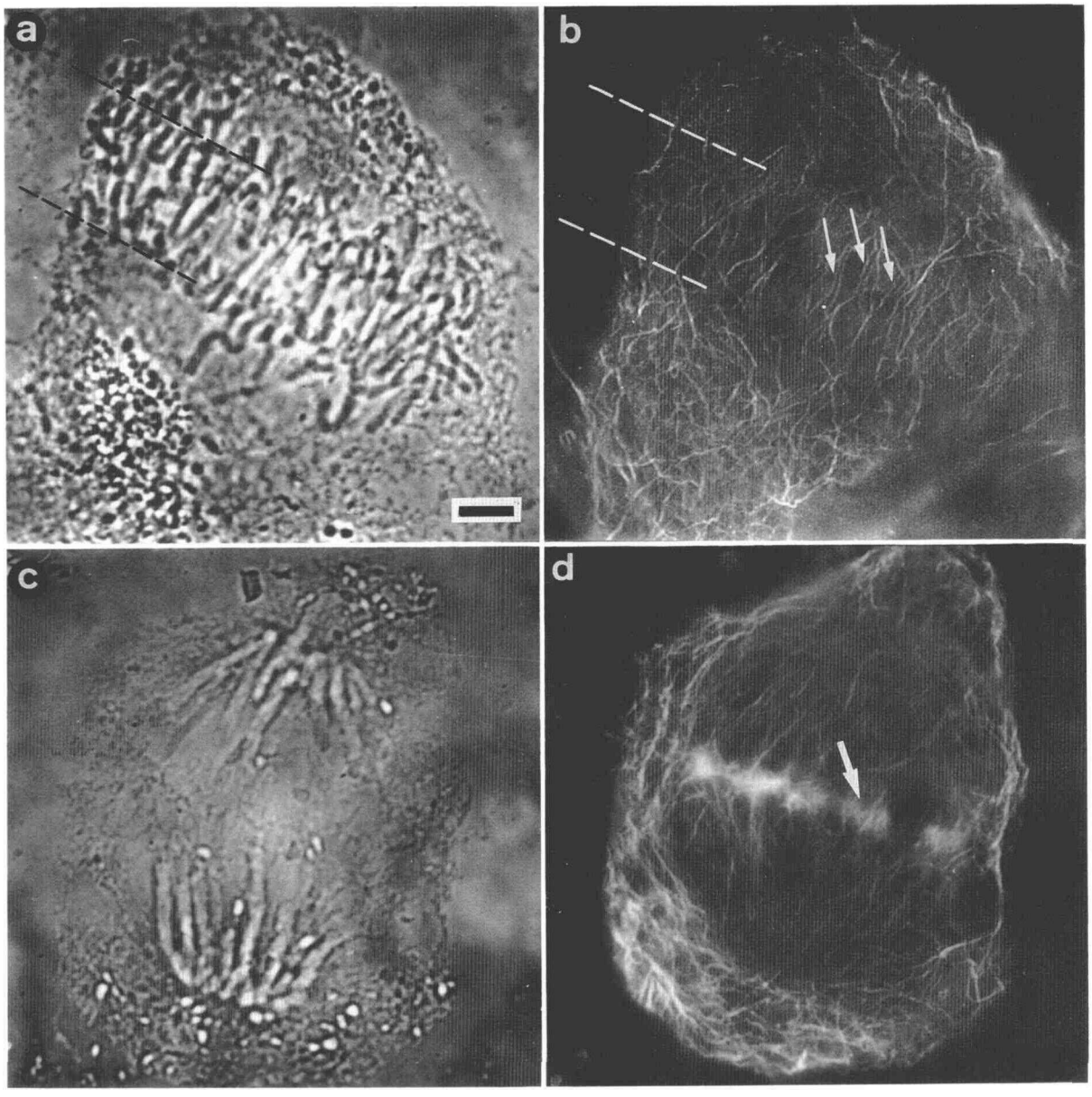

Figure 2. F-Actin Distribution in Fixed Cells after Rhodamine-Phalloidin Staining.

(a) and **(b)** A mid-anaphase cell, observed in phase contrast and fluorescence microscopy, respectively. The dotted lines underline a chromatid separation of 24 μm. In the interzone, the cortical actin meshes are observed parallel to the spindle axis (arrows) while they intermingle as a network at the polar regions, as described for living cells.
(c) and **(d)** A late-anaphase cell in phase contrast and its corresponding F-actin organization. The actin cage is still present around the mitotic apparatus. An equatorial actin accumulation (arrow) takes place before polar and phragmoplast microtubule invasion.
Bar = 10 μm.

Figure 1. (continued).

(a) Phase contrast microscopy of the cell in metaphase, just after injection of rhodamine-phalloidin (1 μM).
(b) and **(c)** The same cell in anaphase, 9 min later. The sister chromatids (arrows) just begin their polar migration. (Dotted lines show 10-μm separation.) Around the spindle, the cortical dense actin network forms a basket that is stretched at the equatorial region. At the polar regions, microfilaments form a regular network with criss-crossing bundles, without particular orientation.
(d) and **(e)** Thirty four minutes after injection, the cortical microfilaments remain as thin, straight cables. Numerous actin filaments co-localize with spindle microtubules as thicker bundles.
Bar = 10 μm.

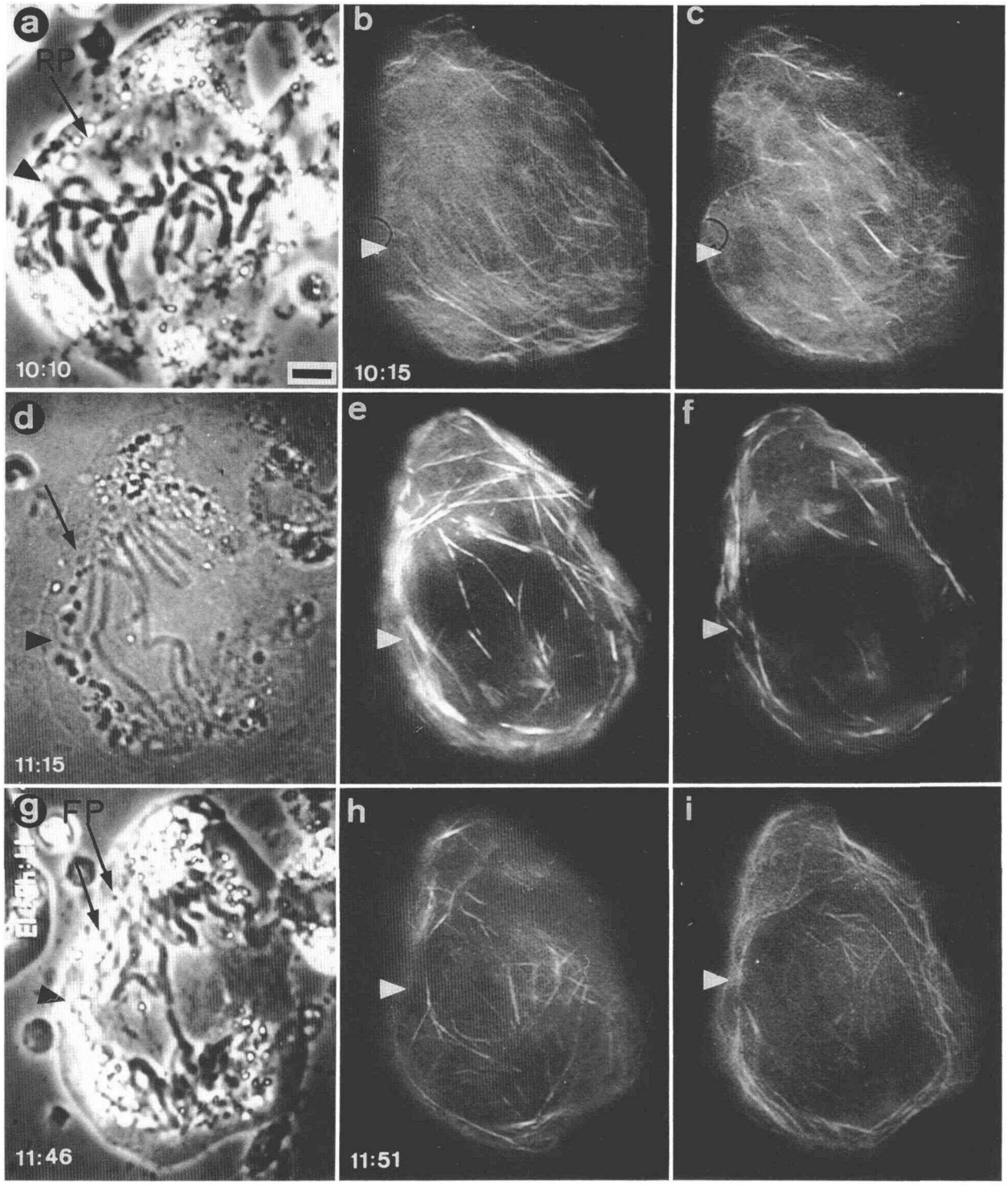

Figure 3. F-Actin Dynamics during Anaphase Development Revealed by Double Phalloidin Microinjection.

(a) Metaphase cell, observed in phase contrast microscopy, just after rhodamine-phalloidin microinjection (RP→).
(b) and **(c)** The same cell, 5 min later, observed in fluorescence microscopy. It reveals the organization of all microfilaments present at this metaphasic stage of mitosis, forming a dense network surrounding the spindle.

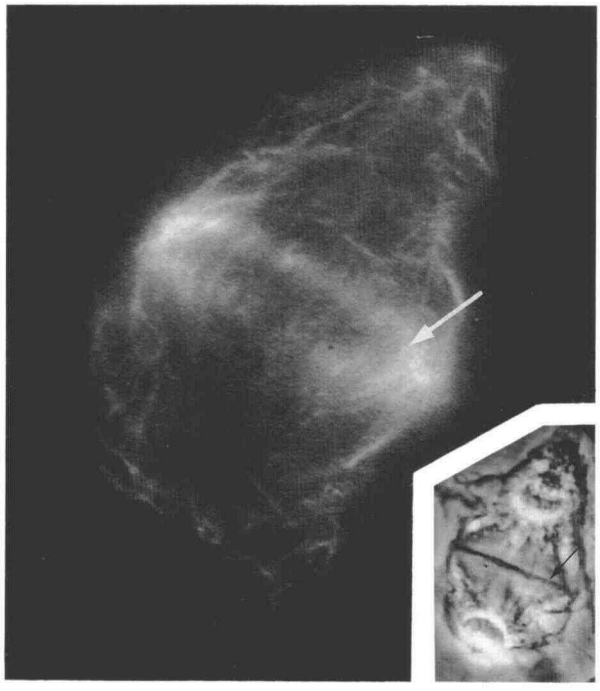

Figure 4. F-Actin Distribution in a Living Telophase Microinjected Cell.

Microinjection of rhodamine-phalloidin in telophase reveals short actin bundles forming a wreath at the equator where the cell plate is developed (arrows). These microfilaments, observed 5 min after injection, are distributed parallel to the spindle axis and are connected with the cortical F-actin network, organized in the cytoplasm surrounding the daughter nuclei. Inset: the cell in phase contrast, at the moment of injection.

tool in the investigation of the plant actin cytoskeleton over the last few years (for review, see Lloyd, 1989). In this report, we have shown that fluorescent phalloidin, as a small peptide of about 1 kD, diffuses instantaneously after microinjection into the living endosperm cells and binds specifically to actin filaments within less than 1 min, whereas G-actin does not combine with the probe. Because of their natural lack of a cell wall, the Haemanthus endosperm cells allow direct access to the plasma membrane for microinjection without any pre-enzymatic treatment that could induce artifactual reorganization of the cytoskeleton, particularly in the cell cortex. As phalloidin is known to have a stabilizing effect on F-actin, this may influence the natural microfilament turnover. Therefore, the microinjection of derivatized plant actin that is competent to co-assemble with endogenous actin would be an excellent marker to study the natural dynamics of the actin pool. Unfortunately, such a probe is not yet available; therefore, we focused on fluorescent phalloidin in our current investigation. Microinjected cells, followed during subsequent stages of mitosis, almost instantly revealed the unexpected importance of the F-actin cytoskeleton as a dynamic architecture that surrounds the plant mitotic spindle. Comparison with our previous observations on fixed cells stained with higher phalloidin concentrations (Schmit and Lambert, 1985, 1987) indicated that, in vivo, the whole intracellular F-actin is labeled, even when using very low phalloidin concentration. No cytoplasmic fluorescent aggregates have ever been found in plant cells, in contrast to animal cells with higher probe concentrations (Wehland and Weber, 1981).

In vivo fluorescent labeled F-actin confirmed the presence of linear actin bundles parallel to the spindle axis in anaphase, suggesting that living microfilaments are organized as an elastic cage on the surface of the microtubular spindle, whereas chromosomes progress toward the poles. In the polar cell domain, where no mitotic forces are developed, the F-actin meshes are smaller and remain as a network with criss-crossing microfilaments without particular orientation. Previous observations (Schmit and Lambert, 1988) in fixed cells suggested that this actin network surrounding the microtubular spindle is submitted to mitotic forces, inducing a "stretching" of the bundles in the equatorial zone, but artifacts due to fixation and permeabilization cannot be left out. Such actin structural changes during mitosis require dynamic cross-links that could be displaced and could assemble/disassemble. An elasticity of actin polymers was observed in vitro (Sato et al., 1985) when weak forces were applied; comparatively slow-acting mitotic forces could create a similar elasticity in vivo. Using immunogold labeling on fixed mitotic cells, our previous results (Schmit and Lambert, 1988) showed that microfilaments intermingle and interact with microtubules both at the surface and in the spindle, suggesting the presence of cross-linking proteins. Those interactions are also described for animal cells (Euteneuer et al., 1987). Until now, plant-actin-associated proteins remained mostly unchar-

Figure 3. (continued).

(d) to **(f)** The same cell in late-anaphase, 65 min after RP injection. A reorganization of the rhodamine-labeled F-actin distribution is observed. Thick bundles are detected in the cortex, but only a few of them remain at the equatorial region.
(g) to **(i)** The same cell 75 min after the first RP injection and 5 min after a second injection of FITC-labeled phalloidin (FP→). The distribution of the FITC-labeled microfilaments shown in **(h)** and **(i)** in late-anaphase/telophase is completely different from the rhodamine-labeled pattern in **(e)** and **(f)**. New labeled, thin F-actin bundles are visualized in the cortex and at the equator.
Bar = 10 μm; ▶ = reference mark.

acterized, although myosin was found to be involved in cytoplasmic streaming (Kato and Tonomura, 1977).

As we have shown, anaphase chromosome migration continues in phalloidin-injected cells, although the chromosome movement is slowed down by approximatively half in treated cells. This slowing of chromosome velocity is associated with phalloidin itself, as microinjection of PBS buffer or fluorescent BSA has not induced such changes, indicating that microinjection is not the cause. Final separation of chromosomes and spindle elongation are significantly reduced in the presence of phalloidin, as was also observed after cytochalasin treatment (Schmit and Lambert, 1988). Such mitotic alteration may reflect a modification of the dynamics of the actin cage, although actin itself cannot be considered as a mitotic motor. It has been shown that phalloidin can interfere with the F-actin/G-actin equilibrium in a concentration-dependent way by shifting it to actin polymerization and stabilization against denaturing agents (Dancker et al., 1975; Wieland, 1977; Colluccio and Tilney, 1984). Such a stabilizing effect was observed during our experiment when the living cell was in the presence of fluorescent phalloidin for more than 1 hr, as, for example, in the double microinjection experiment. Using an image-processing system allows very fast integration (see Methods): rhodamine-phalloidin fading is minimized as well as cytoskeletal alterations that may be due to illumination (Wilson, Fuller, and Forer, 1987).

It is also well established that phalloidin tightly binds to actin filaments (Wehland and Weber, 1981) and cannot be released under light exposure. Therefore, it is highly improbable that the absence of microfilaments observed at the equator in telophase cells microinjected in metaphase should be due to rhodamine-phalloidin fading and/or relocation.

We were also interested in determining the possible origin of actin involved in cell plate development (Schmit and Lambert, 1985, 1987, 1988). The origin of this cytokinetic actin is entirely unknown. Two hypotheses are suggested: (1) new actin may assemble at the equator in response to the unknown signals that control cytokinesis or (2) equatorial actin found in telophase is the result of a passive accumulation of microfilaments that have been broken by the polar microtubules that elongate toward the equator and push them toward the developing phragmoplast.

Double microinjection of a mitotic cell in metaphase and late anaphase, using differently labeled phalloidins, is a new experimental procedure that may help to explain actin origin. Our data suggest that a new set of actin filaments assembles in telophase, which, therefore, favors the first hypothesis. If it is assumed that most of the intracellular F-actin is labeled by the first phalloidin injection, and that phalloidin stabilizes F-actin, no microfilament, or very few, should be labeled after the second microinjection. This is not the case. The labeling of a new set of thin actin filaments by freshly introduced phalloidin (second injection) indicates that a pool of G-actin remained unpolymerized in the cytosol, even 1 hr after the first phalloidin injection, and was induced to polymerize during the process of anaphase, particularly at the equatorial region where cytokinesis is programmed. The signals that may control and trigger such new assembly of actin are entirely unknown. This shows that the stabilizing effect of phalloidin that is revealed by filament bundling after long exposure to the probe is restricted to the first labeled polymers because the second microinjection reveals thin filaments organized in a supple network.

Our data shed new light on the plant dynamics suggesting that F-actin may play an important role in plant mitosis, mostly in the control of spindle elongation and in cytokinesis. Whether the cytoskeletic actin has properties similar to those found in the preprophase band, or if it corresponds to a particular isoform population, is entirely unknown.

METHODS

Cell Preparations

Living mitotic endosperm cells of the Blood Lily (*Haemanthus katherinae*) were spread on phytagar (GIBCO Laboratories, Chagrin Falls, OH) -smeared coverslips and maintained alive in a wet chamber at room temperature as previously described (Molè-Bajer and Bajer, 1968; Schmit et al., 1983; Bajer and Molè-Bajer, 1986; Schmit and Lambert, 1987). Cells at precise stages of mitosis were pre-selected under phase contrast microscopy, and the preparations were then transferred to the injection set-up.

Microinjection Procedure

Curved glass micropipettes of 1 mm inner diameter were loaded with fluorescein-phalloidin or rhodamine-phalloidin (Molecular Probes Inc., Junction City, OR; R-432 and R-415) at concentrations from 0.66 to 3.3 μM in PBS (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4), pH 7.0, and mounted on a Leitz micromanipulator. The needle apex was broken just before injection and the volume of injected medium was tested so as not to exceed 10 pL. Cell preparations (coverslips) were placed in a wet Petri dish 5 cm in diameter, and the preselected mitotic cells were rapidly pressure-injected under constant recording on a JVC recorder (NCR, model CR-6600E) equipped with 3/4-inch cassettes. Immediately after injection, the preparations were replaced in the microchamber, as previously described (Molè-Bajer and Bajer, 1968), to avoid dehydration. All reagents were purchased from Merck (Strasbourg, France) unless otherwise specified.

Microscopy

Microinjection of selected cells was performed on a Zeiss inverted microscope (IM 35) equipped for phase-contrast and fluorescence microscopy. During microinjection, the cells were observed under phase contrast with a PH2 FLUAR 40/0.75 objective. After injec-

tion, fluorescent labeled living cells were immediately controlled under proper excitation filters, using an HBO 50 lamp. The preparation was then transferred to a Leitz Orthoplan microscope equipped for epifluorescence with an HBO 100/W2 lamp as a light source. The cells were observed with a fluorescence 63/1.30 Leitz objective and rhodamine or FITC excitation/emission filters.

As a living fluorescent marker for actin, rhodamine-labeled phalloidin was preferred to FITC-phalloidin because of its excitation properties and lesser toxicity and fading. FITC was only used as a second probe for double microinjection.

Image Recording and Processing

Fluorescent labeled mitotic cells were analyzed using a low-light SIT video camera (Heinman, Wiesbaden, Federal Republic of Germany) connected to an image-processing system (Crystal Sapphire, Quantel France, Montigny le Bretonneux). Image integration (25 images/second) in real time allowed us to minimize the cytoskeletal damage that we found, as did other authors (Wilson et al., 1987), when increasing concentrations of the probe and/or illumination exposures. Successive observations were carried on at different mitotic stages. The digitalized images were then improved by background subtraction and crisp mask application.

Photography

High-resolution images were visualized on a Sony monitor (PVM 122 CE), and pictures of the processed images were taken on the screen with a Canon T70 36-mm camera. Direct micrographs of fixed cells were taken with a Wild Leitz Vario Orthomat camera. Chromosome movement was determined on sequential video recording of each cell, taken every 20 min. Agfa pan 100 professional and Kodak T MAX 400 films were used.

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