Spatial re-organisation of cortical microtubules in vivo during polarisation and asymmetric division of *Fucus* zygotes

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Summary

***Fucus* zygotes polarise and germinate a rhizoid before their first asymmetrical division. The role of microtubules (MTs) in orienting the first division plane has been extensively studied by immunofluorescence approaches. In the present study, the re-organisation of MT arrays during the development of *Fucus* zygotes and embryos was followed in vivo after microinjection of fluorescent tubulin. A dynamic cortical MT array that shows dramatic re-organization during zygote polarization was detected for the first time. Randomly distributed cortical MTs were redistributed to the presumptive rhizoid site by the time of polarisation and well before rhizoid germination. The cortical MT re-organisation occurs independently of centrosome separation and nucleation. By the time of mitosis the cortical array depolymerised to cortical foci in regions from which it also reformed following mitosis, suggesting that it is nucleated from cortical sites. We confirm previous indications from immunodetection studies that centrosomal alignment and nuclear rotation occur via MT connexions to stabilised cortical sites and that definitive alignment is post-metaphasic. Finally, we show that cortical MTs align parallel to the growth axis during rhizoid tip growth and our results suggest that they may be involved in regulating rhizoid growth by shaping the rhizoid and containing turgor pressure**

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Key words: Microtubules, Polarity asymmetric division, *Fucus*

Introduction

Microtubules perform a variety of essential functions within the cell. The mitotic spindle supports chromosome segregation and determines the position of cleavage in animals (Ahringer, 2003). This array is focused at centrosomes in animals although other non-centrosomal arrays exist that are mostly involved in polarised morphologies of specialised animal cells (Keating and Borisy, 1999). In animals, cortical microtubule (MT) arrays have been described only in very specific cases, such as starfish or *Xenopus* oocytes and zebrafish zygotes, where they are involved in the establishment of the dorsoventral axis (Jesuthasan and Stahle, 1997; Schroeder and Gard, 1992; Schroeder and Otto, 1984).

By contrast, cortical MTs are a common feature of vegetative plant cells, in which they play a key role in shaping the cell by directing deposition of cellulose microfibrils (Mfs) in the cell wall and thereby cell growth (Lloyd and Chan, 2004). In plants the interphase MT array in G1 consists of cortical MTs linked to the plasmalemma and is replaced in G2 by the pre-prophase band cortical array that marks the position of the division plane. This array disappears in late prophase and is followed in succession by the mitotic apparatus and the phragmoplast. The perinuclear MT array is in continuity with the cortical array although it is still not clear whether cortical MTs arise from the translocation of perinuclear MTs or are nucleated de novo at cortical sites or both since there is evidence for both mechanisms (Burk and Ye, 2002; Cyr and Palevitz, 1995; Shaw et al., 2003).

Rather less is known about MT distributions in zygotes and embryos of higher plants, owing to their general inaccessibility for direct study. Zygotes of fucoid algae (*Fucus*, *Silvetia*) have long served as a cellular model to study fertilisation, polarisation and cell division. Oospheres are initially spherical with no detectable polarity (Jaffe, 1958). After fertilisation (AF) *Fucus* zygotes deposit a cell wall within minutes and establish an axis of polarity in response to external cues (such as unidirectional light) that becomes fixed in space (14-18 hours AF) and leads to the germination of a rhizoid at the side facing away from the incident light (16-20 hours AF) (Henry et al., 1996; Love et al., 1997; Novotny and Forman, 1974). Concomitantly zygotes progress through the first cell cycle and divide perpendicularly to the growth axis (Corellou et al., 2001). The first asymmetrical division gives rise to the rhizoid and thallus cells with distinct developmental fates (Bouget et al., 1998). The rhizoid remains the only site of growth of the embryos during several rounds of division. The primary division planes in the rhizoid are oriented transverse to the polar axis whereas the first thallus cell

division occurs parallel to the growth axis (Bouget et al., 1998).

The role of the cytoskeleton in the polarisation of fucoid zygotes and in nuclear rotation has been extensively studied (Allen and Kropf, 1992; Bisgrove and Kropf, 1998; Bisgrove and Kropf, 2001a; Hable and Kropf, 1998; Kropf et al., 1990; Quatrano, 1973). Whereas F-actin is thought to be essential for polarisation and germination MTs were found to be important for centrosomal alignment with the growth axis but dispensable for polarisation (Quatrano, 1973). Immunocytological studies of MT arrays of *Silvetia* zygotes during polarisation revealed a two-step alignment of the centrosomal axis with the growth axis (Bisgrove and Kropf, 2001a). Initially, prior to metaphase the centrosomal axis is roughly aligned with the growth axis. This alignment appears highly dependent on the connection of centrosomal MTs to the cortex and is consistent with a search-capture process in which actin plays a key role. Cortical MTs were faintly detected at the thallus in premetaphase. The exact alignment of the centrosomal axis is post-metaphasic and does not appear to require cortical MT connections. The study by Bisgrove and Kropf (Bisgrove and Kropf, 2001a) is the only report of cortical MTs in fucoid algae and showed short and rare MTs at the thallus cortex. However, such studies have employed fixation techniques that may interfere with the normal MT distributions and moreover do not allow re-organization of MTs to be followed in the same cell. The development of in vivo techniques such as microinjection of fluorescent tubulin and more recently, expression of GFP-tubulin has considerably improved our understanding of MT behaviour in other systems (Khodjakov et al., 1997; Yu et al., 2001).

In the present work we microinjected fluorescent rhodamin-labelled tubulin (RT) into *Fucus* zygotes to gain insights into the reorganisation of MTs in vivo during early development and the first cell cycles, We were able to detect for the first time in polarizing zygotes a dense cortical MT network that lies in continuity with the perinuclear array. Cortical MTs appear to originate from cortical nucleation sites. During polarisation of the zygote, they progressively re-organise from random to a polarised array localised at the rhizoid pole. MTs parallel to the growth axis appear to support germination. MT connections between the nucleus and the cortex that facilitate nuclear rotation (Bisgrove and Kropf, 2001a) were also confirmed in vivo. The re-organisation of the cortical array appears to occur before or at the same time as the perinuclear MTs re-organize from the nuclear envelope to the centrosomes. Cortical MTs transiently disappear during mitosis but cortical patches remain until early metaphase and may be involved in late alignment of the spindle. Our data also suggest that this cortical array is required for osmoregulation.

Materials and Methods

Zygote culture

Receptacles from sexually mature *Fucus serratus* adults were cut and dry stored at 4°C until use. Gametes were released by placing male or female receptacles in filtered seawater (FSW) in natural daylight after a short rinse with tap water. Fertilisation was performed by mixing sperm with oospheres. After 1 hour, zygotes were washed with FSW and filtered through a 90 μm nylon mesh. Zygotes were gently held in glass wedges (Speksnijder et al., 1989) and cultured in FSW under unidirectional blue light at 14°C. Nocodazole (Sigma, Poole, UK) was dissolved in DMSO at 10 mg ml^{-1} and further diluted in seawater before use to give a final concentration of 1 μg ml^{-1}.

Microinjection of fluorescent brain tubulin

Rhodamine conjugated tubulin (RT) was purchased from Molecular Probes (Eugene, OR). RT stock solution was centrifuged at 10,000 ***g*** at 4°C, aliquoted and frozen at –80°C. RT was diluted just before use in 50 mM KCl, 300 mM Mannitol, 5 mM Hepes pH 6.9 to achieve a final concentration of 2 mg ml^{-1}. Micropipettes fabricated from 1.2 mm filamented borosilicate glass (Harvard Apparatus, Edenbridge, UK), and dry bevelled (Roberts et al., 1993), were back-filled with the RT solution, which was maintained on ice. Zygotes were pressure microinjected using a Medical Systems (Greenval, NY) injection system. During microinjection, embryos were maintained at 10°C on a cooled microscope stage to minimize RT polymerisation during injection. Turgor pressure was reduced by adding 0.2-0.4 M sorbitol (depending on the age of the zygote) in seawater to allow injection and to minimize damage following pipette withdrawal. Based on co-injection with Oregon Green dextran 10,000 kDa (Molecular Probes), we estimate that the injected volume was less than 3% of cell volume corresponding to a maximum intracellular RT concentration of 0.06 mg ml^{-1}. After microinjection zygotes were returned to culture and allowed to recover and polarise under unidirectional blue light, for at least 1 hour during which the turgor pressure was readjusted progressively by regular media changes with a decreasing amount of sorbitol. The direction of the light was applied so that the germination site was at 90° or 180° to the injection site.

Images were acquired using a Bio-Rad 1024 confocal laser scanning microscope (CLSM) (Hemel Hempstead, UK) equipped with an argon/krypton laser. Fluorescent tubulin was excited at 568 nm and fluorescence was observed at 605 nm using a 10 nm band pass filter. Optical sections were acquired at 1.5 μm intervals unless otherwise stated. Sections were projected using the maximum brightness projection with LASERSHARP software (Bio-Rad).

Results

Fluorescent tubulin microinjection allows in vivo observation of MT reorganisation during zygotic development

Fucus zygotes were microinjected with rhodamine-labelled tubulin (RT) to examine microtubule distribution and movement during the polarization process, germination and mitosis that leads to asymmetrical division. Of zygotes that were microinjected early in development (between 2-5 hours AF, n=50) 74% polarized and germinated and of those observed after germination, 66% (n=50) entered mitosis indicating that microinjection had little deleterious effect on zygote development. Germination never occurred at the injection site but according to the light direction i.e. perpendicularly or opposite to the injection site showing that the injection was not a relevant stimulus for polarisation compared with light. In a small proportion of injected zygotes (16%, n=81) the nucleus was seen to be slightly displaced from the centre to a more peripheral position and MT bundles were very occasionally observed extending between the nucleus and the injection site (see e.g. Fig. 5D) or transiently at the site of injection (not shown). However, zygotes with those features grew as well as other injected zygotes with no apparent disruption of the timing of developmental events. To check for artefacts (such as microtubule bundling or the appearance cortical MT foci) potentially arising from injection of excessive

RT levels, varying amounts of RT varying from very low (i.e. barely detectable above cellular autofluorescence) to high (i.e. 2-fold or more than cell autofluorescence) (see Figs S1-7 in supplementary material) were injected. No differences in RT distribution were observed between zygotes or embryos injected with low or high levels of RT. These observations together with the observed MT re-organisation during polarisation and mitosis strongly indicate that the observed MT structures are not artefacts arising from tubulin microinjection.

Repeated confocal scanning of the embryos was shown to be deleterious since most embryos stopped their development after five rounds of serial scanning (i.e. 100-150 scans). To avoid this problem and to ensure a sufficient number of time points for each developmental sequence, zygotes were scanned from either the early stage of polarisation until first mitosis entry or from germination until the end of the first mitosis. Two cell embryos were also injected to observe further development.

A homogenous and dense cortical MT network is detected in unpolarised *Fucus* zygotes

27 zygotes or oospheres (three different batches) were microinjected with RT between 0 and 4 hours (AF). MT distribution was examined in zygotes using CLSM at 5-7 hours AF (Fig. 1). Tubulin was not incorporated into MTs in oospheres and the diffusion of free fluorescent tubulin from the injection site resulted in a diffuse fluorescence throughout the cytoplasmic network (Fig. 1A). By contrast, a cortical MT array was detected within minutes after injection of zygotes. In unpolarised zygotes (5-7 hours AF) randomly arranged MTs were distributed homogeneously around the cell cortex (Fig. 1B). MT arrays from the surface extended deeper into the cytoplasm. Cytoplasmic MTs (deeper than 10 μm) formed a meshwork around numerous organelles and vesicles that appeared as dark ovoid areas (Fig. 1C). Perinuclear MTs were uniformly nucleated from the nucleus through the cytoplasm towards the cortex but appeared to incorporate tubulin more slowly since some zygotes had a labelled cortex before any perinuclear MTs were detected (data not shown). One or two bright spots of fluorescence were frequently (19% n=26) associated with the nuclear surface (Fig. 1D). These most probably correspond to the centrosomes (Bisgrove and Kropf, 1998). MTs radiated from these bright spots as well as from the nuclear envelope suggesting that they may be nucleation centres.

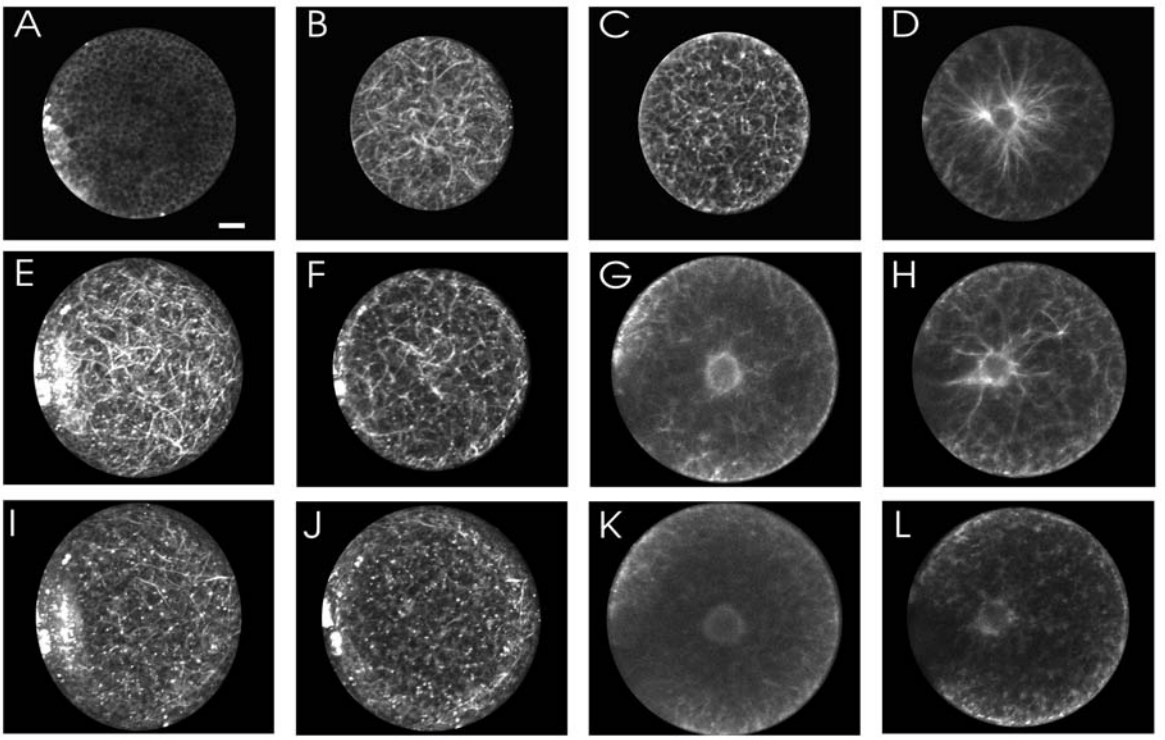

Fig. 1. Specificity of incorporation of fluorescent tubulin in different MT arrays of unpolarised zygotes. Zygotes are positioned with the light coming from the top of the page and were microinjected on the left side. (A) Oospheres 4 hours after injection (1 optical section). (B) Cortical area of a 5 hours zygote (projection of the 2 upper optical sections): MTs are homogeneously distributed and randomly oriented. (C) One 15 μm deep section shows the cytoplasmic MT array in a 6-hour-old zygote. (D) Projection of section from 30 to 38 μm deep nuclear MT array in a 6-hour-old zygote. (E-L). Effect of nocodazole on MT arrays. (E-H) 7-hour-old zygote before and (I-L) after 2 hours incubation with nocodazole. (E,I) Cortical MTs showing only a few MTs and fluorescent foci (free non incorporated tubulin) remaining after nocodazole treatment. (F) Cytoplasmic region (projection from 7.5 to 27 μm deep inside the zygote) showing a dense cortical MT array at the periphery of the zygote. After incubation with nocodazole (J) very few and short MTs remain. (G,K) Nuclear region (projection from 28.5 to 33 μm deep inside the zygote). (G) The tubulin has not been well incorporated in the nuclear area, which is only faintly labelled. (H,L) Nuclear area from another zygote before (H) and after (L) nocodazole incubation. The bright patches on the left of zygotes in A, E, I, correspond to unincorporated tubulin on the zygote surface near the injection site. Bar, 15 μm.

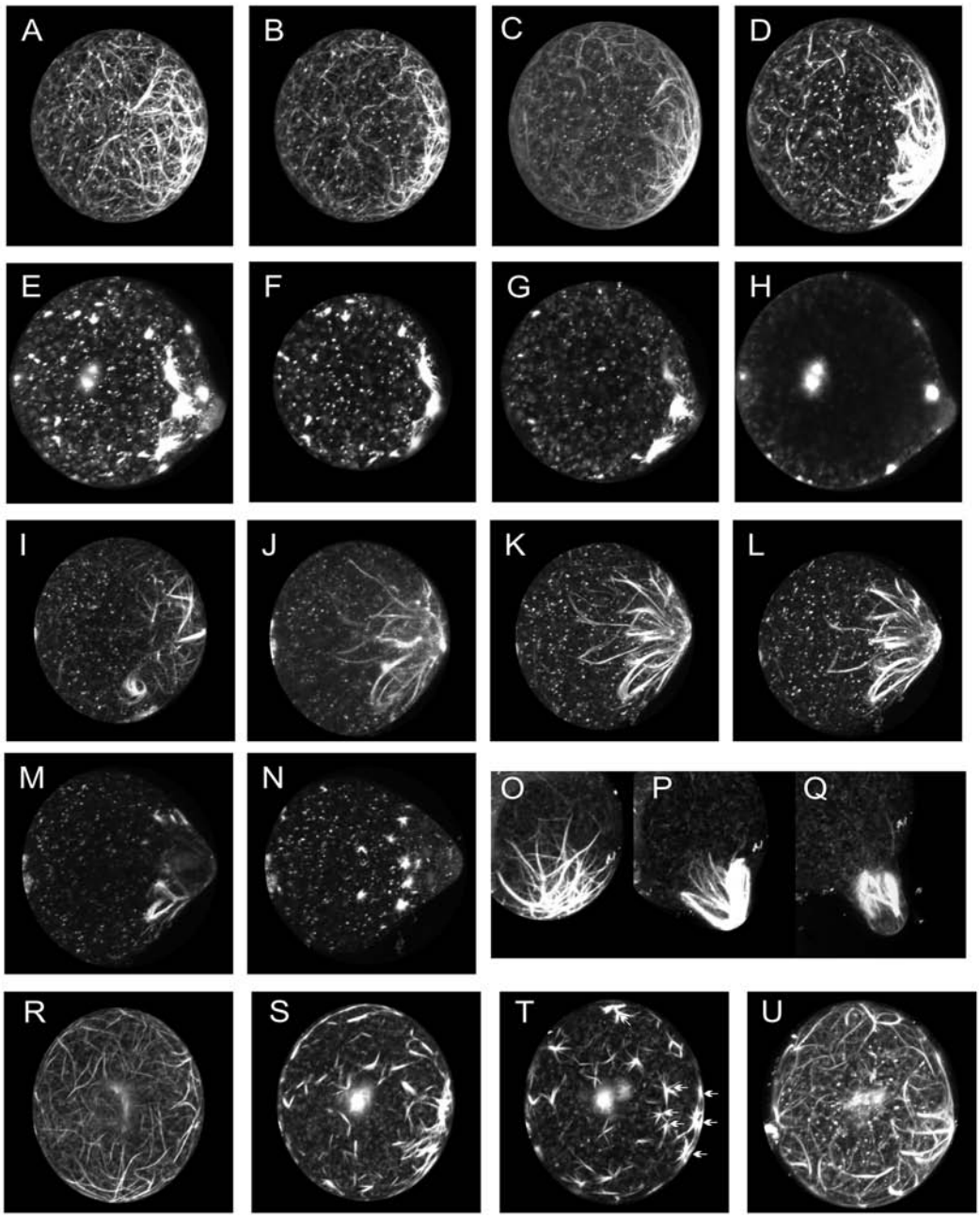

Fig. 2. Time course of cortical MT array reorganisation in polarised zygotes. (A-H) Polarisation, germination and mitosis entry in the same zygote. Light direction is from the left. (A-E) All sections are projected to reflect the shape of the zygote. (A) 17 hours (B) 19 hours, (C) 21 hours and (D) 23 hours AF showing progressive localisation of cortical MTs towards the rhizoid pole (projection of all sections). (E) 26 hours AF (projection of all sections). The rhizoid has germinated and long cortical MTs are absent in the zygotes with a prophase spindle but bright tubulin structures remain at the rhizoid collar. (F-H) Different areas of the same 26-hour-old zygote showing that tubulin structures at the collar start at the cortex and go deep inside the cytoplasm: (F) Cortical area through the upper 10 μm of the same zygote. Bright tubulin structures are localised as a collar around the rhizoid sub-apex. (G) Cytoplasmic area (11-22 μm) and (H) nuclear area (32-37 μm) with the prophase spindle. (I-N) MT re-organisation during rhizoid elongation (projection of all sections). (I) 17 hours AF with cortical MTs localised to the rhizoid hemisphere. (J,K,L) 19, 22 and 24 hours AF showing rhizoid emergence and the parallel arrangement of MTs to the rhizoid axis. (M,N) 25 and 27 hours AF: MTs depolymerise from the rhizoid tip to bright cortical star-shaped structures located at the collar. (O-Q) Projection of all sections during rhizoid germination and growth. (O) 16 hours AF showing convergence of MTs towards the site of rhizoid emergence. (P) 20 hours AF: MT bundles form a crescent at the periphery of the growing rhizoid. (Q) 27 hours AF showing disappearance of MTs from the rhizoid apex and MT bundles parallel to the growth axis extending from the rhizoid collar to a region below the apex. (R-U) Cortical network disappearance during mitosis and reformation after mitosis (projection of all sections). In this zygote rhizoid germination occurred downwards out of the horizontal scanning plane but on the right side of the zygote. (R) 16-hour-old zygote. Cortical MTs are more localised to the rhizoid hemisphere and the nuclear envelope is labelled. (S) 20 hours AF. Cortical MTs are more focused to the rhizoid and all cortical MTs are shortening. (T) Aster nucleation begins 26 hours AF showing mitosis entry. Both asters are detected in the cell centre. Only cortical star-shaped structures remain at the cortex (arrows). (U) 38-hour-old zygote showing reformation of the cortical network, particularly at the rhizoid pole. Bar, 15 μm.

We tested the sensitivity of different MT arrays in the unpolarized zygote to nocodazole which is known to be efficient in inhibiting MT polymerisation in *Fucus* zygotes (Brawley and Quatrano, 1979). All types of MT arrays of unpolarised zygotes (6 hours AF) were shown to be sensitive to this drug after 2 hours treatment and although a few MTs were barely detectable at the cortex of 2 zygotes, no MTs could be detected in the majority of zygotes (60%, n=5) (Fig. 1E-L). MT depolymerisation was not due to a general toxic effect of nocodazole since on return to seawater all zygotes germinated and the majority underwent mitosis (60%, n=5). Together these results indicate that the microinjected fluorescent tubulin was specifically incorporated into the different MT arrays of *Fucus* zygotes.

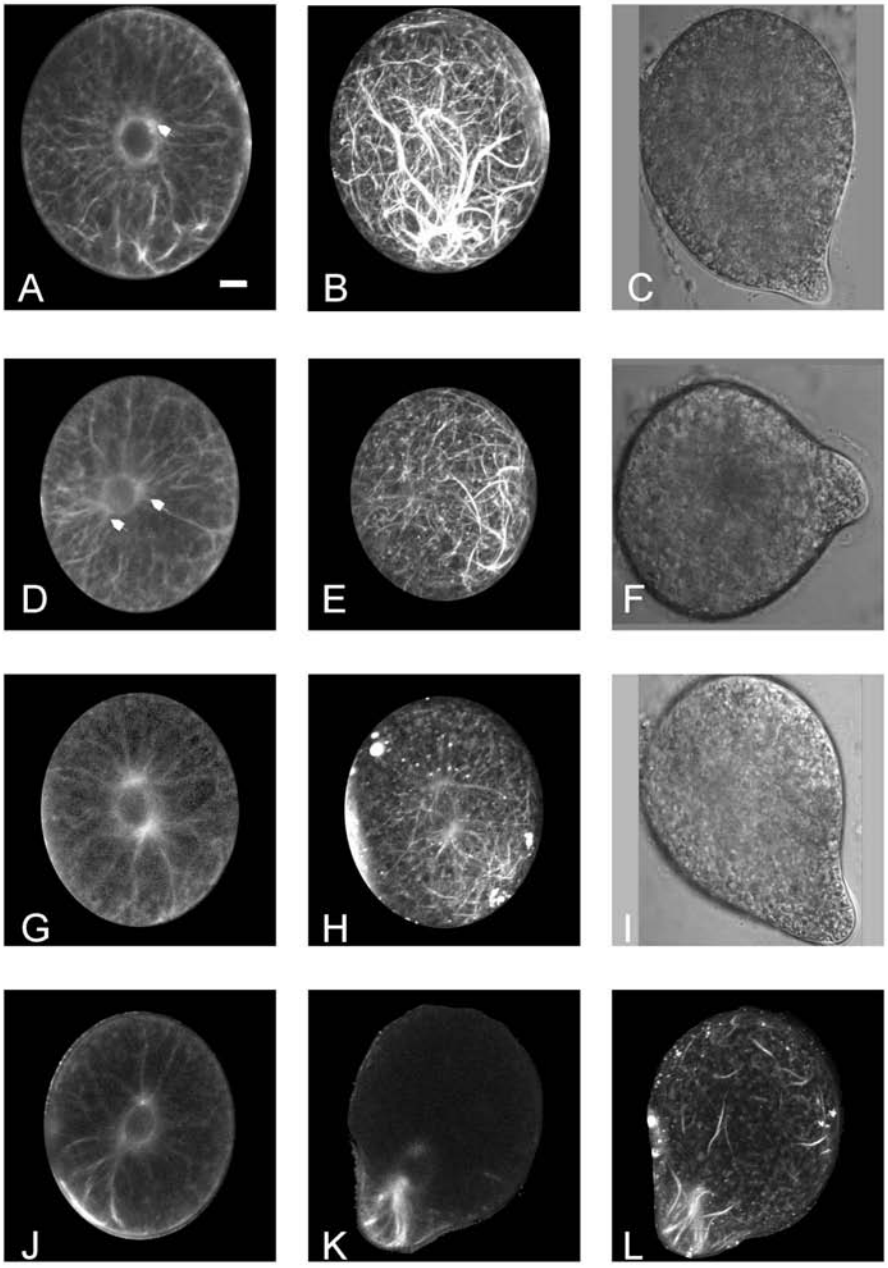

Fig. 3. Perinuclear MT re-organisation in zygotes having localised cortical MTs. Four representative zygotes are shown (A-C), (D-F), (G-I), (J-L). (A,D,G,J) Nuclear region (projection of 5-6 sections) and (B,E,H) cortical region (projection of all sections) of 10-hour-old zygotes. In all zygotes cortical MTs are located at the hemisphere where germination will occur. (C,F,I) Transmitted images of germinated zygotes at 28 hours AF. (A) Perinuclear MTs are homogeneously nucleated from the nuclear envelope and thicker MTs extend toward the cortex of the future rhizoid. Two non-separated centrosomes are detected (arrow). (D) Centrosomes (arrows), almost aligned with the growth axis start to nucleate MTs. (G) MT nucleation occurs from centrosomes that are aligned with the growth axis. (J) MTs radiating in opposite directions from both centrosomes (aligned with the growth axis) towards the cortex. (K) Nuclear area of the same zygote 25 hours AF. Only MTs connected to the rhizoid site remain and the nucleus is pulled toward that site in the germinated zygote. (L) Projection of all sections of the same zygote. The bright patches in H and L correspond to unincorprated tubulin aggregate on the zygote surface at the site of injection. Bar, 15 μm.

The cortical network is localised at the future germination site by the time of polarisation

Clear changes in the MT networks were observed as zygotes developed (Fig. 2). Interestingly the cortical array became preferentially localized at one hemisphere in spherical zygotes as early as 10 hours AF (80%, *n*=15) (see Figs 3 and 6). In the 6 batches examined (*n*=42) cortical MTs localised at the rhizoid pole in ungerminated zygotes (Fig. 2A-D). Cortical MTs were first broadly localised to one hemisphere of the cell and became focused at a more precise site (Fig. 2A-C). As germination occurred cortical MTs formed an even denser and concentrated array (Fig. 2D), and eventually formed a crescent at the nascent rhizoid tip (see Fig. 2P). During rhizoid emergence the cortical MTs reoriented parallel to the growth axis and progressively disappeared from the thallus pole (Fig. 2I-L,O). As zygotes entered mitosis the cortical MT array was dramatically reduced to star-shaped patches from which the cortical MTs had originally elongated. These remained brightly labelled at the cell cortex (Fig. 2E,N,S). These cortical structures were more concentrated at the sub-apical region of the rhizoid where they were often aligned as a collar that extended over 10 μm into the cytoplasm (Fig. 2E,N) and were also seen at the rhizoid apex. The time sequence acquisition shown in the lower row of Fig. 2 shows the disappearance of the cortical network at mitosis entry and its subsequent reformation following mitosis (Fig. 2R-U). Although the reformation of MT was not followed from the beginning the re-formed MTs appeared to originate from sites close to the star-shaped foci to which they retract at mitosis entry (arrows in T).

Cortical MT reorganisation occurs independently of perinuclear MT re-organisation

We next investigated more precisely the concordance between cortical and perinuclear MT reorganisation (Fig. 3). Cortical MT localisation to the future germination site was detected as early as 10 hours AF (90%, *n*=21). Among the 10 hours zygotes, 75% (*n*=20) were detected with one or two nucleated centrosomes. In the remaining 35% cortical MTs were localised before the nucleation switch from the nuclear envelope to the centrosomes had occurred (Fig. 3A,B).

Perinuclear MTs progressively focused from around the nuclear envelope to the centrosomes, which separated around

the nucleus (68.6%, *n*=67). (Fig. 3A,D,G). Centrosomal MTs diverged in opposite directions towards the cortex (Fig. 3G,J). Once the centrosomes were nucleated, one of them was always connected via MTs to the site where cortical MTs were localized even before the centrosomal alignment with the growth axis was achieved (Fig. 3D). These observations suggest that the re-organisation of cortical MTs takes place before or concomitant with perinuclear re-organisation but that it does not depend upon the reorganisation of the perinuclear array.

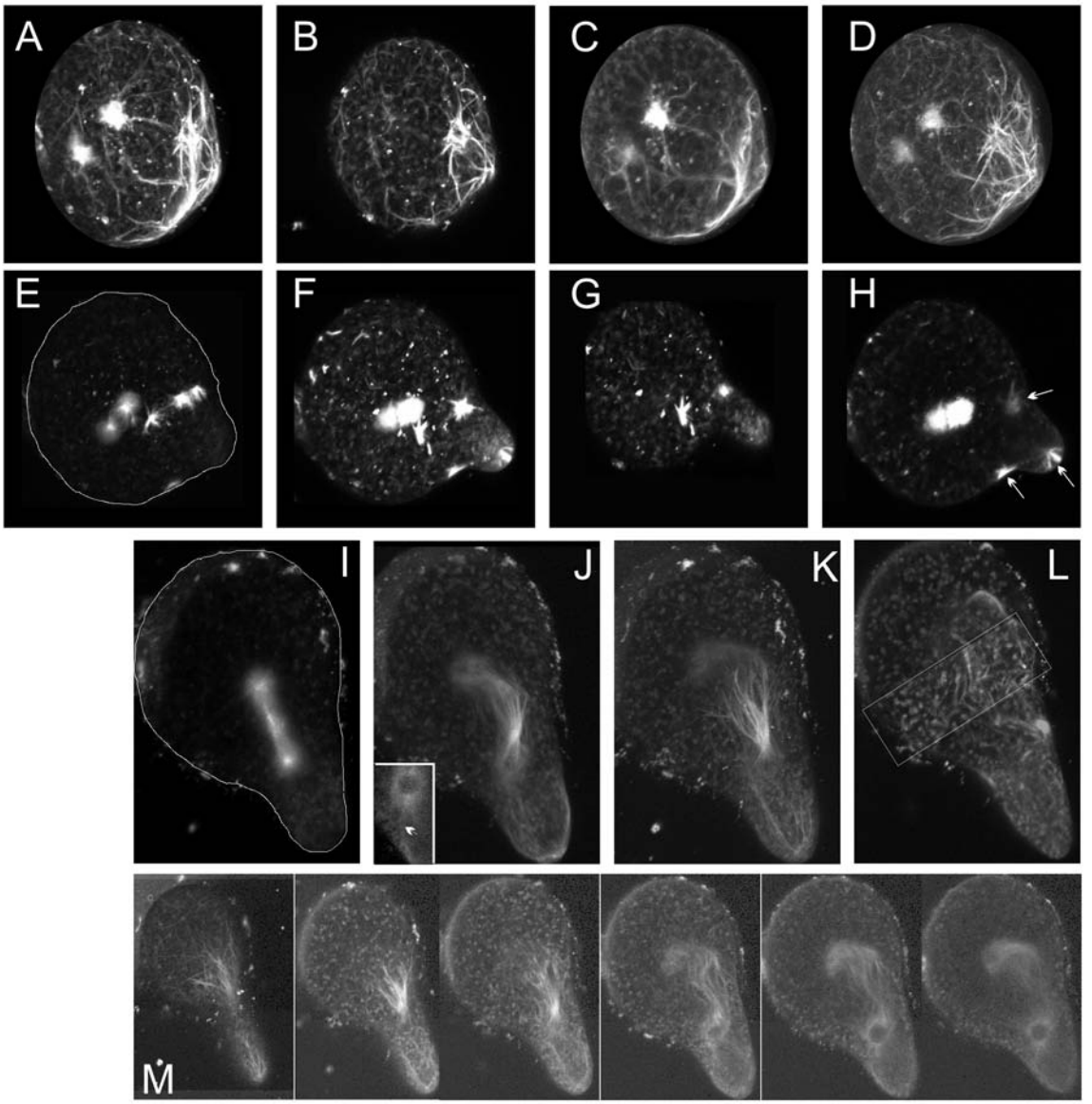

Fig. 4. MT behaviour during mitosis entry. Upper panel (A-H): Mitosis entry and spindle rotation. (A-C) 22 hours AF. (A) Projection of all sections. Nuclear MTs are nucleated at centrosomes and are connected to the cortex of the rhizoid collar and to the rhizoid tip. (B) Projection of 6 upper cortical sections. Cortical MTs are nucleated at the rhizoid collar and are oriented parallel to the growth axis. (C) Nuclear area (projection of 6 sections). One of the centrosomes is connected via two long MT bundles to peripheral MTs. (D) 23 hours AF, the centrosomal axis has rotated slightly. The rhizoid has elongated and the rhizoid MT array is nucleated from a cortical site at the rhizoid collar. (E) 26 hours AF: Prophase spindle and cortical MT structures at the cortex of the collar (projection of all sections). All other cortical MTs have disappeared. Outline of the zygote is shown white. (F,G,H) 30 hours AF, respectively, all sections, seven upper cortical sections and nuclear area (projection of 12 sections). The metaphase spindle has rotated to become almost aligned with the growth axis. Cortical MT structures are present at the rhizoid collar and the rhizoid apex (arrows). (I-L) Projection of all sections during mitosis exit: (I) telophase spindle at 22 hours AF. Spindle asters are separated by a thick MT bundle. (J) At 23 hours AF nuclear envelopes are reformed and MTs appear at the rhizoid apex. In the inset, the focus on one deep section shows faint MT connections between the new rhizoid nucleus and the cortex. (K) At 24 hours AF MTs extend from both nuclei toward the future division plane at the centre of the embryo while cortical MTs reappear in the rhizoid. (M) Different sections of the zygote shown in (K) sampled every 6 μm. In the upper section cortical MTs are detected in both the thallus and the rhizoid. Numerous fine MTs connect both nuclei and interdigitate at the centre of the embryo. (L) 26 hours: cytokinesis is achieved. In the rectangle contrast has been enhanced to show MTs organised in the spindle interzone at the division plane. MTs extend horizontally to the edge of the embryo and perpendicularly other nuclear MTs reach the same plane. Bar, 15 μm.

By the time of germination, centrosomes were seen to be separated around the nucleus in most of the zygotes (74%, *n*=27). The nucleus rotated so that the centrosomal axis became roughly aligned with the growth axis before mitosis. However this process failed in some of the zygotes (33% *n*=27) and the nucleus did not rotate so that some of the zygotes entered mitosis with a spindle perpendicular to the growth axis and were arrested at metaphase (see Fig. 2H).

MT arrays during mitosis

Zygotes entered mitosis between 20-25 hours AF (*n*=36). To follow mitosis in the same zygote we had to scan at close time intervals. However, this often resulted in a metaphasic arrest of nuclear division (61% *n*=22). Telophase figures were observed in neighbouring injected zygotes that were only scanned once (30% *n*=11). The time of mitosis progression (from prophase to cytokinesis) was estimated to be 2-4 hours (*n*=3), consistent with data reported for *Fucus spiralis* (Corellou et al., 2001).

At mitosis entry, the mitotic spindle formed and was roughly aligned with the growth axis in most of the microinjected zygotes (58%). However, in the remaining cases the spindle was not aligned until just prior to mitosis (Fig. 4A-H). When this late alignment was observed cortical MT foci remained until the spindle was aligned (Fig. 4F-H). In about 30% of the zygotes astral MTs extending from the basal aster of the mitotic spindle towards the rhizoid region were detectable (not shown). The connection of the nucleus to the rhizoid region disappeared as zygotes progressed into metaphase (not shown). Anaphase spindles were rarely seen probably due to the rapidity of this process. In contrast telophase figures were detected in 39% of the zygotes suggesting that this event is much slower (Fig. 4I,L). Fig. 4 shows early telophase: the spindle asters were separated by a large bundle of MTs (Fig. 4K). Nuclear envelope reformed and exhibited nucleating activity since it was clearly labelled by the fluorescent tubulin (Fig. 4J,M). At telophase a faint connection of the basal nuclei to the rhizoid tip was detected in 81% of the zygotes (Fig. 4J inset and M). Concomitantly cortical MTs reformed at the rhizoid (Fig. 4K,M) and thallus pole. Newly formed nuclei remained connected via MT bundles emanating from the nuclear envelope until the subsequent cytokinesis occurred (Fig. 4L). Multiple fine perinuclear MTs were still interdigitated whereas new transverse MTs appeared at the centre of the embryos. Nuclear MTs remained connected to the cell plate that was extending transversely towards the cell cortex.

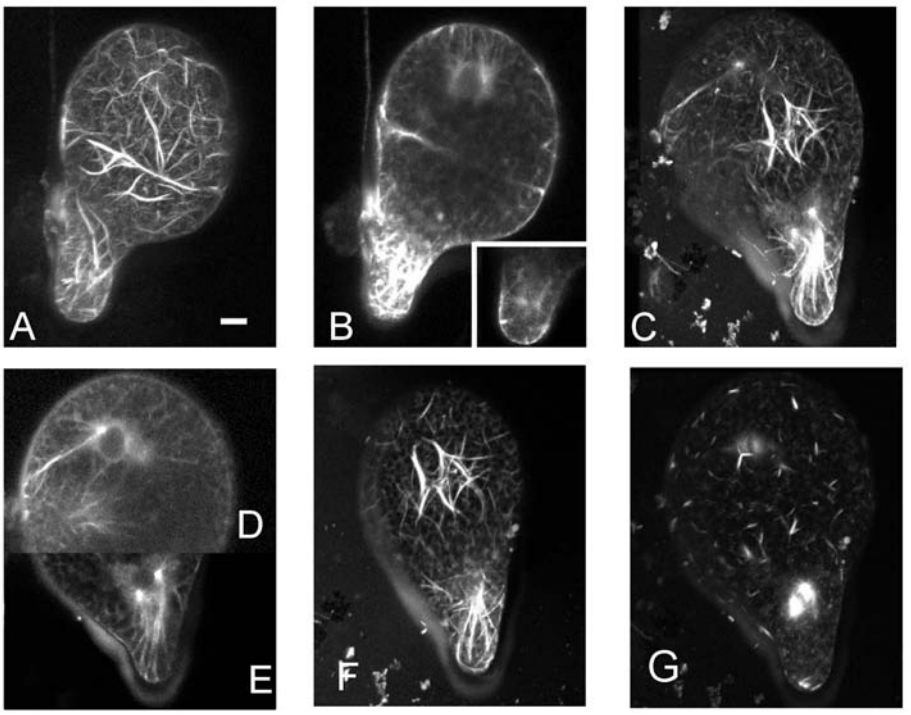

Fig. 5. Mitosis in 2 cell embryos. (A,B) Interphase in a 2 cell embryo. Cortical MT arrays are reformed in both the thallus and the rhizoid. (A) Projection of all sections, (B) single equatorial optical section. MTs connect the thallus nucleus to the upper cell cortex. MT arrays in the rhizoid are detected in the same plane. Inset shows the rhizoid nucleus with two centrosomes that separate around the nucleus. (C,D) Mitosis entry in a 2 cell embryo: (C) A few cortical MTs probably connected with nuclei are seen in both the thallus and the rhizoid cell. (D) One nuclear section in the thallus. A bundle of MTs connects one centrosome to the injection site. The other centrosome is connected through MT bundles to the cortex of the embryo (see C). (E) The rhizoid nucleus is rotating. Both centrosomes separated around the nuclei are connected to the rhizoid apex. (F) 3 hours later cortical MTs are reduced to star shaped structures and metaphase spindles are seen in both the rhizoid and the thallus cells (G). Note that the thallus spindle is perpendicular to the growth axis whereas the spindle in the rhizoid is not yet aligned with this axis (projection of all sections). Bar, 15 μm.

MT arrays in two-cell embryos behave similar to zygotes

Late injection of zygotes (*n*=3) and of the rhizoid cell of two-cell embryos (*n*=9) allowed us to follow MT behaviour during mitosis in the rhizoid and thallus cells since tubulin moved readily from one cell to the other cell at this stage and was incorporated into the MT network of both cells (Fig. 5A). The thallus cell nucleus was connected to the cell apex through centrosomal and perinuclear MTs as well as to the division plane (Fig. 5B,F). In the rhizoid both centrosomes were seen connected to the rhizoid tip and occasionally also with the division plane (Fig. 5C,E). MTs connected to the rhizoid tip may serve in nuclear rotation and alignment with the growth axis before mitosis (Fig. 5E). Cortical MTs behave in a similar way as for the first mitosis, i.e. only a few cortical foci were detectable as cells entered mitosis (Fig. 5G).

MT function in the polarizing zygote

To gain insight into MT function during polarisation and germination the sensitivity of MT arrays to nocodazole was examined in polarising zygotes, (i.e. around the time of nuclear rotation). We also tested the susceptibility of polarising and germinated embryos to osmotic changes after MTs disruption by nocodazole and

observed the development of zygotes continuously incubated in nocodazole.

MT arrays were disrupted by incubating 11-hour-old RT-injected zygotes in nocodazole for 2 hours (n=15). However, in contrast to the almost complete disruption of MTs observed in 6 hour zygotes (see Fig. 1E-L), cortical MTs were still detectable at the rhizoid pole in 60% of the nocodazole-treated polarised zygotes (Fig. 6A). Thus, cortical MTs at the rhizoid pole of polarised zygotes were less sensitive to nocodazole than cortical MTs of unpolarised zygotes (see Fig. 1). Most of cytoplasmic MTs were disrupted by nocodazole, but in contrast to the unpolarised zygotes, a few MTs connecting the nucleus to the germination site remained (53% of zygotes) (Fig. 6A). Those MTs originated either directly from the nuclear envelope or from the centrosome closer to the germination site.

Zygotes incubated with nocodazole for 2 hours AF were significantly more sensitive to hypo-osmotic shock. 22% of control 12-hour-old zygotes burst when the osmolarity of the seawater was reduced to 40% whereas 71% bursting was observed for nocodazole treated zygotes (Fig. 6B). Similarly, germinated embryos (24 hours AF) treated with nocodazole burst more than controls (58% and 20% respectively). Polarising embryos burst at the presumptive rhizoid site and germinated embryos burst close to the rhizoid apex. Zygotes grown from the time of polarisation with nocodazole could germinate albeit with a slight delay but exhibited abnormal

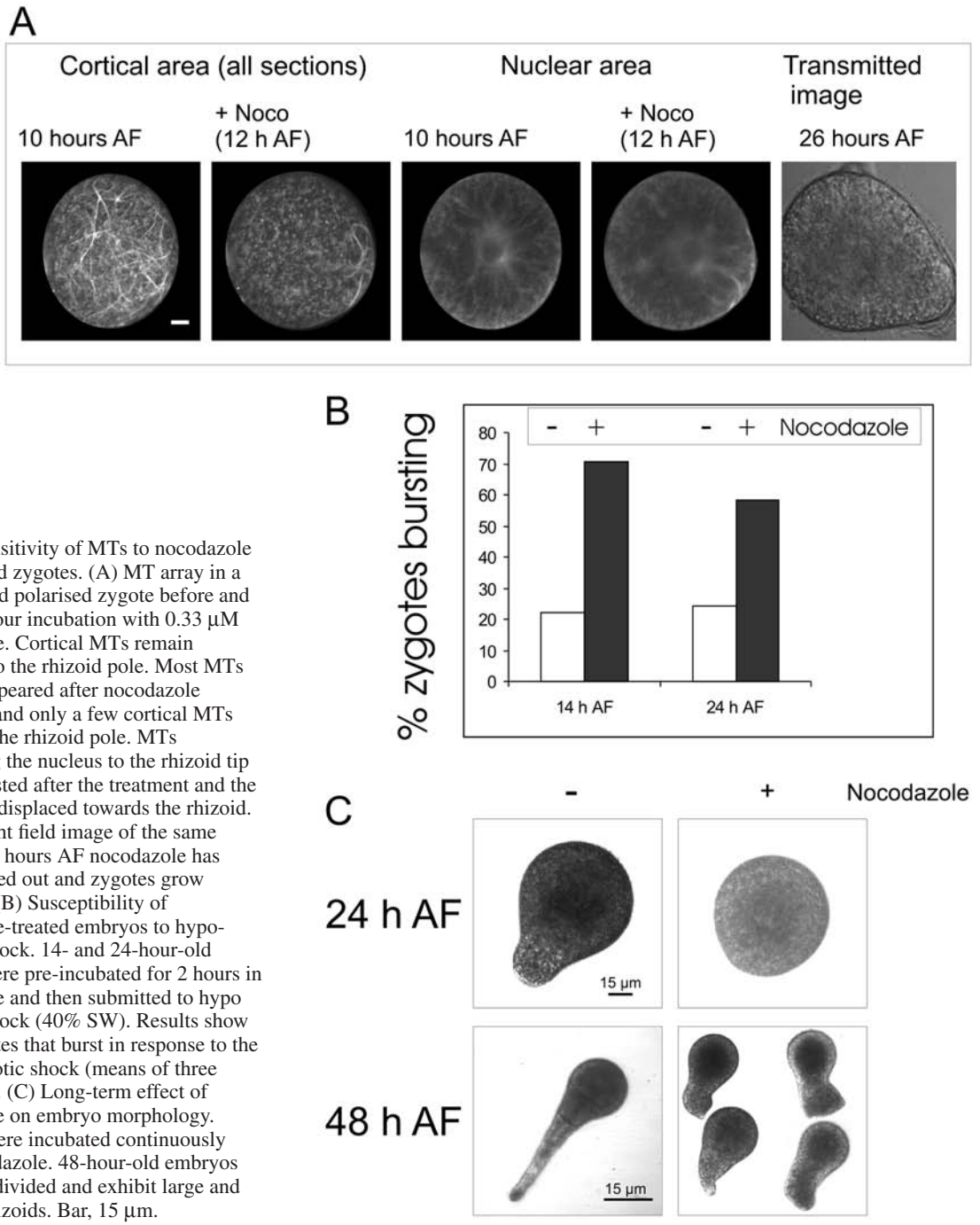

Fig. 6. Sensitivity of MTs to nocodazole in polarised zygotes. (A) MT array in a 10-hour-old polarised zygote before and after a 2 hour incubation with 0.33 µM nocodazole. Cortical MTs remain localised to the rhizoid pole. Most MTs have disappeared after nocodazole treatment and only a few cortical MTs remain at the rhizoid pole. MTs connecting the nucleus to the rhizoid tip have persisted after the treatment and the nucleus is displaced towards the rhizoid. In the bright field image of the same zygotes 26 hours AF nocodazole has been washed out and zygotes grow normally. (B) Susceptibility of nocodazole-treated embryos to hypo-osmotic shock. 14- and 24-hour-old zygotes were pre-incubated for 2 hours in nocodazole and then submitted to hypo osmotic shock (40% SW). Results show % of zygotes that burst in response to the hypo-osmotic shock (means of three replicates). (C) Long-term effect of nocodazole on embryo morphology. Zygotes were incubated continuously with nocodazole. 48-hour-old embryos remain undivided and exhibit large and broader rhizoids. Bar, 15 µm.

rhizoid morphology (Fig. 6C) typically with broader and shorter rhizoids.

Thus disrupting MTs increased the sensitivity of unpolarised and polarised zygotes to osmotic changes and constant inhibition of MT polymerisation deeply affected rhizoid morphology.

Discussion

Microinjection of fluorescent tubulin has allowed in vivo confocal imaging of MT arrays and their re-organisation during the development of *Fucus* zygotes and embryos. This approach has revealed, for the first time, the existence of a dynamic cortical MT array that shows dramatic re-organization during zygote polarization. This cortical array was not detected by immunocytochemistry of tubulin in a range of earlier studies on *Fucus* embryos (Allen and Kropf, 1992; Bisgrove and Kropf, 1998; Bisgrove and Kropf, 2001a; Brawley et al., 1976; Kropf et al., 1990; Nagasato et al., 1999), possibly as a result of damage by the cell wall digestion and plasma membrane permeabilisation procedures (Bisgrove et al., 1997; Brawley and Quatrano, 1979). Interestingly, in vivo observation of MTs shows that randomly distributed cortical MTs are redistributed to the presumptive rhizoid site by the time of polarisation and well before rhizoid germination. Moreover, our observations strongly suggest that the cortical array is nucleated from cortical sites. This occurs independently of centrosome separation and nucleation. Our results further suggest that cortical MTs may be involved in regulating rhizoid growth by shaping the rhizoid and containing turgor pressure. Finally our observations support previous indications from immunodetection studies of fucoid MTs that centrosomal alignment and nuclear rotation occur via MT connexions to stabilised cortical sites and that definitive alignment is post-metaphasic.

What is the origin of the cortical MT array?

Cortical MTs are a common feature of plant cells and the question of whether the cortical array arises from the nuclear envelope or from the cortex is still debated. In synchronized BY- 2 tobacco cells overexpressing a GFP-tubulin construct, in vivo observation from mitosis exit to G1 indicates that MTs are first nucleated at the new nuclear envelope and subsequently elongate to the cell cortex (Yoneda and Hasezawa, 2003). However γ-tubulin was seen first accumulating at the nuclear surface but then spread to the cell cortex along with MTs. Since γ-tubulin is a marker of microtubule organizing centres (MTOCs) this suggests that while the nuclear surface may act as the origin of cortical MTs further reorganisation of the cortical MT array occurs at the cell cortex where new MTOCs are put in place (Kumagai et al., 2003). Moreover, a recent detailed study of the cortical MT array of *Arabidopsis* shows that new MTs can be initiated at the cell cortex (Shaw et al., 2003), supporting the existence of cortical nucleation sites in plants. In *Fucus* zygotes, fertilisation most probably triggers the polymerisation of MT arrays since labelled MTs are not detected in oospheres. The lack of labelling of MTs in oospheres suggests that the MT network is stable and thus not incorporating tubulin. The randomly arranged cortical MT array of the zygote extends for about 10 μm beneath the plasmalemma. Optical sectioning indicates that the cortical array lies in continuity with a perinuclear array consisting of long MTs radiating from the nuclear envelope. Whether the initial cortical MTs originally arise from the growth of perinuclear MTs or from the polymerisation de novo of new MTs from cortical nucleation sites that may have translocated from the nuclear envelope could not be resolved in the present study. However, the involvement of cortical nucleation sites in the subsequent organization of cortical MTs is supported by several observations. Firstly, in numerous freshly injected young zygotes nuclear and/or perinuclear arrays were barely detectable whereas MTs were readily apparent in the cortex. This probably reflects a slower incorporation of labelled tubulin at the nuclear envelope rather than a screening effect of the cell, since increasing the power of the confocal scanning laser still failed to reveal perinuclear MTs in these zygotes. Thus, cortical nucleation sites may be more dynamic than those at the nuclear envelope in young zygotes. Second, the cortical array becomes polarised to the presumptive rhizoid site before the re-organisation of the perinuclear array, which suggests that both arrays are independent. Thirdly, while further work is needed to show definitively that that cortical MT arrays polymerise from the cortical MT foci, the location of the persisting cortical MT foci during mitosis correlates with the region in which MTs reform following mitosis. Whether MT are nucleated de novo from cortical sites or whether the nucleation centres or MTs are first located at the nuclear envelope and are then re-directed to the cortex after mitosis as is suggested to occur in BY-2 cells (Kumagai et al., 2003; Kumagai et al., 2001) could not be clearly determined. However, we observed that at telophase the nuclear envelope was actively nucleating MTs whereas cortical MTs had not yet reformed by the time of mitosis exit. Finally it has been postulated that nuleating material may disperse to the cortex in vegetative plant cells because of the lack of centrioles to focus MTs (McDonald et al., 1993; Palevitz, 1993). Since *Fucus* zygotes possess centrioles (Brawley et al., 1976; Nagasato et al., 1999), it seems that another process may have influenced the selection of cortical MTOCs in this system.

What is the role of the cortical MT array?

The cortical MT array is known to be essential for plant morphogenesis. A role for cortical MTs in controlling the orientation of cellulose Mf deposition is supported by numerous studies including the analysis of an *Arabidopsis* mutant for katanin (MT severing protein), in which aberrant cortical MTs and cellulose Mfs are found (Burk and Ye, 2002). Cortical MTs were also shown to be required to maintain cellulose synthase localisation (Burk and Ye, 2002; Gardiner et al., 2003). However a simple causal link between the orientation of cortical MTs and the orientation of Mfs (Baskin, 2001), has recently been challenged since the mor-1 *Arabidopsis* mutant, in which cortical MT organization is disrupted can still display ordered cellulose Mf assembly (Himmelspach et al., 2003).

It has been proposed that, in brown algae, F-actin would have a predominant role in cellulose Mf orientation in the apparent absence of extensive cortical MTs (Katsaros et al., 2002). A pharmacological study on spherical *Silvetia* zygotes showed that the disruption of F-actin, but not MTs, hampered

cellulose deposition and had an effect on cell wall strength (Bisgrove and Kropf, 2001b). Furthermore, based on the assumption that cortical MTs were absent it was concluded that F-actin rather MTs would guide cellulose synthase. The discovery, shown here, of cortical MTs that reorient parallel to the growth axis in tip growing zygotes suggests that MTs may indeed play a role in cell wall formation. This could occur in association with F-actin since our unpublished results indicate that cortical MT stability depends upon F-actin integrity (see Fig. S7 in supplementary material). It would thus be interesting to investigate the effects of MT disruption on cellulose Mf orientation. Further investigation of the interactions between actin and MTs is also warranted since Bisgrove and Kropf (Bisgrove and Kropf, 2001a) showed that latrunculin B did not disrupt MTs associated with the mitotic apparatus.

The arrangement of cortical MTs has not been described in spherical walled plant cells to our knowledge. *Fucus* zygotes are natural protoplasts that build up their cell wall during the first 4 hours of development and undergo tip growth after polarisation (Brownlee and Bouget, 1998; Novotny and Forman, 1974; Stevens and Quatrano, 1978). It may be that the randomly arranged cortical MTs underlie a random orientation pattern of cellulose Mfs. Randomly arranged MTs are reported to occur when plant protoplasts are prepared enzymatically. MTs become progressively rearranged in BY-2 cells as cell wall is regenerated and cells start to elongate (Hashimoto, 2003).

The re-orientation of cortical MTs from a random orientation to one parallel to the emerging rhizoid growth axis parallels the orientation of MTs in tip growing plant cells such as root hairs or pollen tubes (Smith, 2003). MTs are known to play an important role in regulating directionality and stability of apical growth (Bibikova et al., 1999). MT inhibitors partially block gymnosperm pollen germination and growth, producing swollen apices (Anderhag et al., 2000) similar to that which we observed when zygotes were continuously incubated with nocodazole and reported earlier in response to the microtubule stabilizing drug taxol (Kropf et al., 1990). MTs may therefore have an important role in directing and shaping rhizoid growth. Moreover, the significantly higher sensitivity of nocodazole-treated zygotes (germinated or not) to hypo-osmotic shock indicates that MTs are involved in osmoregulation. This finding is in contrast with Bisgrove and Kropf (Bisgrove and Kropf, 2001b) that found no effect of another MT depolymerising agent, oryzalin, on the response of *Silvetia* zygotes to hypo-osmotic treatments. Species differences in the sensitivity to the hypo-osmotic treatment could account, at least in part, for this discrepancy. *Silvetia* spp. inhabit the upper shore zone and experience greater fluctuations in their natural osmotic environment than the lower shore *Fucus serratus* which was used in the present study. Our own unpubished data indicate that lower shore species are less tolerant to imposed hypo-osmotic shocks than those growing on the upper shore. Finally, F-actin, but not MTs, is thought to be essential for the localisation of morphological determinants in fucoid zygotes (Hable and Kropf, 1998; Kropf et al., 1989; Shaw and Quatrano, 1996). However, as cortical MTs were seen localised at the presumptive rhizoid pole before germination and since a 2 hours treatment with nocodazole was not sufficient to completely disrupt polarised cortical MTs (a time that was used in previous experiments to test the role of MTs in polarisation) (Quatrano, 1973) it is pertinent to reconsider a role for MTs in polar axis fixation and determinant localisation. Focusing of the MT network at the animal pole has been reported in *Xenopus* oocytes at stage VI and is known to support morphogenetic determinant transport at this pole (Gard et al., 1997; Miller et al., 1999). Studies of polarization and MT organization in response to a wider range of MT disrupting agents will be required to assign a more definitive role for cortical microtubules in zygote polarization.

Mechanism of MT array reorganisation

F-actin is an early marker of the nascent growth axis in *Fucus* zygotes and cortical F-actin is redistributed to the presumptive rhizoid pole early in development (Hable and Kropf, 2000; Kropf et al., 1989). Because of its close interactions with MTs (Yarm et al., 2001) F-actin is very likely to be involved in regulation of MT reorganisation in *Fucus* zygotes. In yeast, the molecular factors involved in the search-capture mechanisms of MTs that underlie nuclear rotation are localised at the cortex via F-actin (Schuyler and Pellman, 2001). In fucoid algae cortical stabilisation complexes involving cell wall connections (Kropf et al., 1988) are presumably put in place before the switch of MT nucleation from the nuclear envelope to the centrosomes since cortical MTs are localised at the rhizoid pole and stabilised before centrosomes are nucleated. Once the nucleation switch is achieved, centrosomal MTs radiate in opposite directions from each centrosome towards the thallus and the rhizoid cortex.

Our results corroborate those of Bisgrove and Kropf (Bisgrove and Kropf, 2001) which indicate that centrosomally arising MTs in fucoid algae obey a search-capture mechanism checking the cell cortex and being captured by cortical adhesions that are more concentrated at the rhizoid cortex. This could determine the pre-metaphasic centrosomal alignment. However in contrast to the closely related *Silvetia* (Bisgrove and Kropf, 2001) MT connections between the nucleus and the rhizoid pole occur before rhizoid germination in *Fucus* and may additionally serve to direct deposition of cell wall components to the rhizoid pole as it was shown for zygotes of the fucoid alga *Hormosira* (Schoenwaelder and Clayton, 1999).

Centrosomal and spindle alignment with the growth axis

The mechanism of centrosomal alignment with the growth axis has been extensively studied in fucoid algae. Previous work combining tubulin immunodetection and pharmacological approaches (Bisgrove and Kropf, 1998; Bisgrove and Kropf, 2001a) showed that initial pre-metaphasic centrosomal alignment relies on a search-capture mechanism occuring progressively before mitosis entry (at germination time) whereas final post-metaphsic centrosomal alignment does not involve cortical adhesion but is linked by an unknown mechanism to the elongation of the mitotic apparatus in anaphase/telophase. Thus, none of the treatments known to disrupt cortical adhesions hampered post-metaphasic spindle alignment (Bisgrove and Kropf, 2001a). Likewise, in the present study most injected zygotes had their centrosomal axis roughly aligned with the growth axis at mitosis entry and thus only minor rotation of the axis occurred during mitosis. No

spindle-to-cortex connections could be detected during metaphase and anaphase, and telophase spindles were impressively elongated. Telophase spindles were always better aligned than metaphase spindles. We further show that rhizoid nuclear-cortical MT connections reappeared at telophase while the thallus nucleus was seen connected to the upper cortex of the thallus cell after cytokinesis.

In conclusion, these studies provide new insights into the role of the cytoskeleton in polarization and division plane alignment. The previously undescribed occurrence of a dense cortical MT network in fucoid zygotes and embryos and its re-distribution during polarization together with the relative insensitivity of polar-localized MTs to pharmacological disruption indicates a fundamental role in zygote polarization that needs to be investigated further. The persistence of cortical MT foci at the rhizoid pole and the re-growth of the cortical network from these foci suggest that they may form a component of a spatial memory during cell division.

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