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Organization of the actin cytoskeleton during megasporogenesis in *Gasteria verrucosa* visualized with fluorescent-labelled phalloidin

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SUMMARY

The organization of the actin cytoskeleton was studied during megasporogenesis in *Gasteria verrucosa.* Ovules were gently squashed to set free meiocytes, dyads and tetrads. The unfixed cells were permeabilized with Nonidet P-40 and DMSO, and actin filaments were visualized with fluorescent-labelled phalloidin. During premeiosis, actin filaments elongated and were distributed throughout the cytoplasm. During prometaphase actin filaments encaged the future area of chromosome transport. At metaphase, bundles of fluorescent actin filaments were observed in the spindle zone. In the dyad and tetrad, F-actin was redistributed. A large quantity of actin filaments marked the functional megaspore in particular. Before pachytene, at diakinesis and after the selection of the megaspore, actin filaments elongated, which coincided with an increase in the cell volume. Comparison of these data with the findings on the microtubular arrays during megasporogenesis in *Gasteria* leads to the conclusion that actin filaments and microtubules may co-distribute at several stages during megasporogenesis, i.e. in phragmoplasts and in spindles, as has recently been reported for the process of microsporogenesis in *Gasteria.*

Key-words: actin, cytoskeleton, megasporogenesis, phalloidin.

INTRODUCTION

Actin plays a role during cytoplasmic streaming (review, Jackson 1982; root hairs, Emons 1987), during the growth of pollen tubes at the tip (Pierson *et al.* 1986) and during nuclear migration (Palevitz 1980) and karyokinesis (Tiwari *et al.* 1984; Staiger & Schliwa 1987; Van Lammeren *et al.* 1989). Associations between actin filaments and microtubules in dividing plant cells have been reported in the preprophase band (see Palevitz 1987) the phragmoplast (Sheldon & Hawes 1988) and in the mitotic (Forer & Jackson 1979; Schmit & Lambert 1987) and meiotic (Traas *et al.* 1989; Van Lammeren *et al.* 1989) spindles. The function and localization of actin have recently been reviewed by Staiger & Schliwa (1987).

In the present paper, actin filaments are visualized during megasporogenesis in *Gasteria verrucosa* to determine the distribution of F-actin. The amount of co-distribution between

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actin filaments and microtubules is evaluated in a comparison of the results with data on the microtubular configurations during megasporogenesis (Bednara *et al.* 1988; Willemse & Van Lammeren 1988). The results are also compared with data on the microtubular (Van Lammeren *et al.* 1985) and F-actin (Van Lammeren *et al.* 1989) distribution during microsporogenesis in *G. verrucosa.*

MATERIALS AND METHODS

Gasteria verrucosa (Mill.) H. Duval plants were grown in a greenhouse at approximately 21°C and with 16-h illumination per day. Ovaries were dissected from flower buds at various stages of development. Up to 50 ovules were sampled for each stage. The experiment was repeated twice. The ovules were gently pressed between two slides to set free the meiocytes, dyads and tetrads. This procedure was successful until the formation of vacuoles caused the enlargement of the functional megaspore (Willemse & Bednara 1979). The ovules were squashed in an extraction buffer (0·1 M Pipes containing 0·1 M mannitol, 10 mM EGTA, 0·05% (v/v) Nonidet P-40, 5% (v/v) DMSO) and incubated for 1–1·5 h at 20°C (Traas *et al.* 1987) between a poly-L-lysin coated object slide and a cover slide. A microchamber was prepared by fastening the sides of the covering slide onto the object slide with paraplast. In the microchamber, which prevented the dislocation of the immobilized cells, two openings were left at opposite sides to enable the addition and removal of fluids.

Cells that were released from the ovules were washed in Pipes buffer that contained 4 mM EGTA for 10 min and stained in the dark, partly with 10 μl 10^{-6} M rhodamine–phalloidin, in a Pipes buffer containing 0·1 M mannitol and 10 mM EGTA for 40 min at 20°C, and partly with 10^{-6} M phalloidin–FITC (Sigma) in the same buffer, where the treatment with extraction buffer was reduced to 10 min at 20°C. Some cells were rinsed in buffer for 15–20 min and embedded in Citifluor (Citifluor Ltd, London, U.K.) containing 20% Mowiol 4-88 (Hoechst, Frankfurt am Main, FRG), others were observed directly after the administration of phalloidin–FITC. Several stages of megasporogenesis were investigated simultaneously to exclude variations in the staining procedure. Rhodamine and FITC were compared with respect to their intensity of staining and fading.

Sections were observed with epifluorescence microscopy using a Leitz Ortholux microscope with Xenon 150 W illumination (excitation filter BP 530–560 nm, barrier filter LP 580 nm) and a Nikon Labophot microscope with HBO illumination (EX 470–490, BA 515). The objective used was a Nikon Plan Achromat 50 × oil (N.A. 0·50–0·85). Micrographs were made from representative specimen on Ilford XP1 400 and Kodak Ektachrome P800-1600.

RESULTS

The majority of the ovules excised from an ovary exhibited synchrony with respect to the development of the megaspore mother cells. During meiosis I and II, various division stages were found within one ovary. Actin filaments were stained with rhodamine–phalloidin and phalloidin–FITC in two successive experiments. The actin filaments proved to be well stained in both experiments and the distributions of actin filaments were similar and provided sufficient release of meiocytes, dyads and tetrads.

During zygotene and pachytene, the megameiocytes exhibited randomly oriented arrays of small fluorescent rods or short bundles of actin distributed throughout the cytoplasm (Fig. 1a). This distribution persisted until diplotene. At late diplotene, the

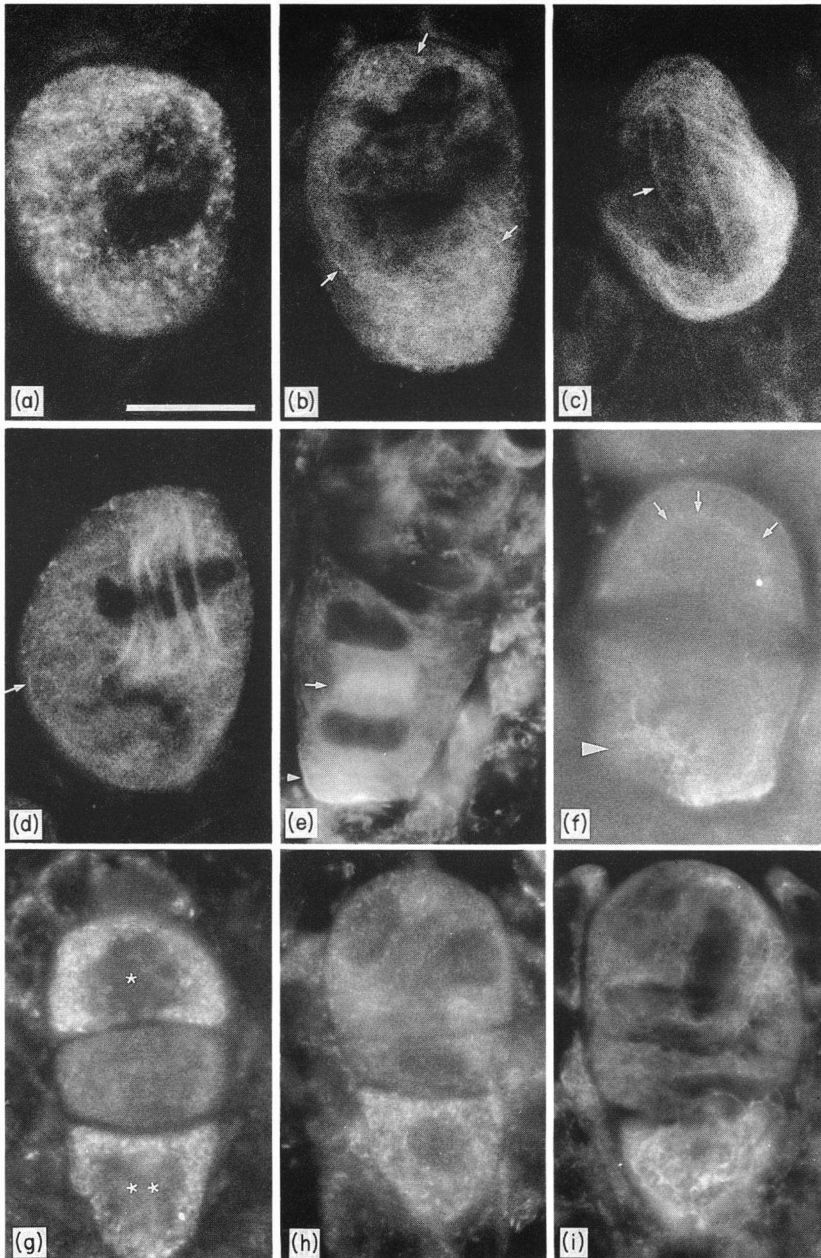

Fig. 1. Rhodamine–phalloidin labelled F-actin in meiotic cells, isolated from ovules by gentle squashing, during megasporogenesis of *Gasteria* visualized by epi-fluorescence microscopy. The micropylar side of the ovules is always at the top of the micrographs. The bar represents 20 μm. (a) Meiocyte at zygotene stage with short actin filaments or rods distributed throughout the cytoplasm. (b) Late diplotene stage. Note the higher number and the increased length of long bundles of actin that are distributed throughout the cytoplasm (arrows). (c) Diakinesis. Long bundles of actin run in a polar direction and encage the nuclear region (arrow). (d) Metaphase I. F-actin is present in the spindle. Note the presence of actin filaments in the surrounding cytoplasm (arrow). (e) Young dyad. Actin filaments are present in the phragmoplast (arrow) and in the cytoplasma especially at the chalazal pole (arrow head). (f) Dyad with few actin filaments around the nuclear membrane of the micropylar cell (arrows) and in the basal region of the chalazal cell (arrow head). (g) Triad. Actin filaments are predominantly found in the undivided dyad cell (*) and the future megaspore (**). (h) Young tetrad. The distribution of actin filaments throughout the cytoplasm is similar as found in the triad stage. (i) Old tetrad. Actin filaments have elongated. The functional megaspore shows the highest amount of actin filaments.

intensity of actin fluorescence had increased and longer actin filaments were observed throughout the cytoplasm (Fig. 1b). Long bundles of actin filaments surrounded the previous nuclear region (Fig. 1c) at diakinasis. Prominent actin bundles were observed in the spindle zone at metaphase I (Fig. 1d). In addition, a regular and loose network of actin filaments was present throughout the cytoplasm. After telophase I, an even fluorescence was observed in the phragmoplast between the two daughter nuclei (Fig. 1e), which indicates the presence of thin actin filaments that were aligned parallel and very close to each other. Actin filaments were also present in other parts of the cytoplasm, particularly at the chalazal pole (Fig. 1e).

The cellular dyad contained a limited number of actin filaments (Fig. 1f). In the micropylar cell the greater part of the actin filaments surrounded the nuclear membrane. Actin filaments were also present at the basal side of the chalazal cell. In the triad, predominantly short filaments were found in the future megaspore and the non-functional, undivided micropylar dyad cell. The middle cell contained few actin filaments (Fig. 1g). In young tetrads a comparable situation existed (Fig. 1h), however, it was found that the number of actin filaments had decreased in the non-functional micropylar megaspores. In older tetrads, long bundles of actin ran throughout the cytoplasm of all megaspores and formed a dense network in the functional megaspore (Fig. 1i).

DISCUSSION AND CONCLUSION

Fluorescent-labelled phalloidin has often been used to visualize actin filaments in plant cells. It has been shown that similar patterns of actin filaments are evident after immunocytochemical staining with fluorescent-labelled monoclonal anti-actin unless cytochalasin B and D are used (Tang *et al.* 1989). Competition experiments with rhodamine and unlabelled phalloidin have been described elsewhere (Van Lammeren *et al.* 1989). Moreover it has recently been verified that rhodamine–phalloidin labelled filaments are composed of actin (Lancelle & Hepler 1989). During premeiosis, megaspore mother cells showed a random distribution of short actin bundles in the cytoplasm. This pattern clearly differed from the distributions found hereafter. As the various developmental stages were labelled simultaneously, the differences in length were not due to the staining procedures but represented a premeiotic elongation of actin filaments.

During the premeiotic phase, a comparable distribution of microtubules was found in megaspore mother cells of *Gasteria* (Bednara *et al.* 1988). Hence, a co-distribution of F-actin and microtubules is conceivable. Intermingling arrays of microtubules and actin filaments have, however, often been observed (Staiger & Schliwa 1987) and certainty about a co-distribution should be obtained by the simultaneous labelling of F-actin and microtubules, experiments which are presently being executed. It is not clear whether the actin filaments that are present at the chalazal pole play a role in the selection of the functional megaspore. During diplotene, however, the microtubules disappeared into the periferal cytoplasm (Bednara *et al.* 1988), while the actin filaments persisted. This indicates that the presence of cytoplasmic microtubules is not a prerequisite for the stability of cytoplasmic F-actin. Such a prerequisite was reported for F-actin in the meiotic spindles during microsporogenesis in *Gasteria* (Van Lammeren *et al.* 1989). It is not clear whether microfilaments present at the chalazal pole play a role in the selection of the megaspore that will become the megagametophyte.

Before diakinesis, a reallocation of the actin filaments in the megaspore mother cells resulted in the formation of polar-directed actin filaments, which encage the site of the

future spindle, as was also found in the mitotic cells of *Haemanthus* (Schmit & Lambert 1987). We observed actin filaments in the spindle region at metaphase I. As pole-to-pole filaments exhibited a similarity in distribution to that of the spindle microtubules (Bednara *et al.* 1988), a co-distribution is possible. Moreover, a co-distribution of F-actin and microtubules is likely in the phragmoplast. The even fluorescence found in that region clearly differs from the short bundles of actin filaments at the zygotene stage and points to a close alignment of thin bundles of actin filaments in the phragmoplast. Co-distributions are also suggested in spindles and phragmoplasts during the microsporogenesis of *Gasteria* (Van Lammeren *et al.* 1989). This might point to similar functions during meiosis in both microsporogenesis and megasporogenesis. Recently it has been suggested that the movement of bundles of microfilaments closely aligned with microtubules in *Physarum polycephalum* depends on the integrity of the microtubular cytoskeleton (Uyeda & Furuya 1989). Moreover it has been pointed out that the structural association of microtubules and actin filaments implies a functional relationship with respect to organelle movement (Pierson *et al.* 1989).

In the dyad some actin filaments were observed near the nuclear membrane, but, in contrast to the microtubules (Willemse & Van Lammeren 1988), no clear pattern of radiating actin filaments was found.

In the triad and tetrad the random distribution of F-actin in the cytoplasm reappeared, but at the late tetrad stage the actin filaments were elongated and predominantly found in the functional megaspore. The latter pattern differed in intensity from the microtubular configuration. Microtubules were predominantly present in the non-functional megaspores of the young tetrad (Bednara *et al.* 1988). Thereafter, in the late tetrad stage the microtubules reappeared in the functional megaspore (Willemse & Van Lammeren 1988). The meaning of the occurrence of both microtubules and cytoplasmic F-actin in the functional megaspore remains to be elucidated.

Bundles of actin elongated during three stages: (i) at zygotene, (ii) at diakinesis and (iii) at the tetrad stage, especially in the functional megaspore. These periods preceed an enlargement and elongation of the cell. The elongation of bundles of actin filaments may be related to the increase in cell volume. In cell division, bundles of actin share the zone of chromosome transport, as do the microtubules. The present paper points out the dynamic organization of the actin cytoskeleton during female meiotic cell division.

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