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MICROFILAMENT CYTOSKELETON OF ENDOSPERM CHALAZAL HAUSTORIUM OF *RHINANTHUS SEROTINUS* (SCROPHULARIACEAE)

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The actin cytoskeleton of endosperm and of the mature endosperm chalazal haustorium cell of *Rhinanthus serotinus* was examined by immunohistochemistry and epifluorescence microscopy. A prominent actin cytoskeleton composed of numerous cross-linked filaments is present at the distal pole of the chalazal haustorium cell. Thick, longitudinally oriented bundles of microfilaments localize in transvacuolar cytoplasmic strands. A meshwork of delicate actin filaments surrounds the large polytene nuclei; some of the filaments radiate from the nuclear envelopes. Abundant and clearly visible actin filaments also occur at the proximal pole of the haustorium cell. A network of microfilaments in cortical cytoplasm and F-actin arrays associated with nuclei are found in endosperm proper cells.

Key words: *Rhinanthus serotinus*, cytoskeleton, F-actin, immunolabelling, rhodamine-phalloidin, endosperm, chalazal haustorium.

INTRODUCTION

Numerous studies suggest that the main function of the endosperm, which develops as a result of double fertilization, is transfer of nutritive substances to the embryo during development (Raghavan, 1976; 1986; Vijayaraghavan and Prabhakar, 1984). In many angiosperms the endosperm forms micropylar and/or chalazal haustoria penetrating the ovular tissue (Johri and Ambegaokar, 1984). Schmid (1906) reported that Scrophulariaceae develop both micropylar and chalazal endosperm haustoria. The karyological differentiation of the haustoria in the genus *Rhinanthus* is accompanied by a polyploidization process (Tschermak-Woess, 1957). Polytenic nuclei with huge DNA content are characteristic of rapidly growing, highly specialized and synthetically active plant cells. However, little is known about the ultrastructure and cytoskeleton in these highly polyploid plant cells. So far, ultrastructural studies on the chalazal haustorium have been done only in *R. minor* (Nagl, 1992) and *R. serotinus* (Bohdanowicz et al., 1993). The results of the second study suggest that extensive microfilament arrays are present in the haustorium cell. F-actin is present in nearly all plant cells as part of the cytoskeleton, which plays an important role in the organization of their cytoplasm. This is generally accepted and has been visualized (Parthasarathy et al., 1985; Traas et al., 1987). In the last few years, immunofluorescence techniques have revealed the importance of the actin cytoskeleton during endosperm development (Muench et al., 2000; Nguyen et al., 2001, 2002) and organelle movements (Boevink et al., 1998; Olyslaegers and Verbelen, 1998; Kandasamy and Meagher, 1999). On the other hand, no immunostaining work has been done on the relationship between microfilaments and cell morphogenesis in highly polyploid plant cells.

This paper reports observations of the actin cytoskeleton visualized within the mature endosperm chalazal haustorium and endosperm proper cells of *Rhinanthus serotinus* (Scrophulariace) using immunolabelling and rhodamine-phalloidin staining.

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MATERIALS AND METHODS

PLANT MATERIAL

Plants of *Rhinanthus serotinus* (Schönheit) Oborny growing in natural habitats in Rumia near Gdynia were used in the study.

FLUORESCENCE LABELLING OF F-ACTIN

Ovules were excised from ovaries and immediately pretreated for 15–60 min in 400 µM m-maleimidobenzoic acid N-hydroxysuccinimide ester (MSB, Sigma) (Sonobe and Shibaoka, 1989; Huang and Russell, 1994) in actin-stabilizing buffer (ASB) to which 5% DMSO (dimethyl sulfoxide) was added to permeate the cells (Traas et al., 1987). ASB consisted of 50 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]), 10 mM EGTA (ethylene glycol-bis[β-aminoethyl ether] N,N,N',N'-tetraacetic acid), and 1mM $MgCl_2$, pH 6.8. After preincubation the ovules were rinsed three times (10 min each) in the ASB and fixed immediately in 4% formaldehyde freshly prepared from paraformaldehyde in ASB containing 5% DMSO for 4 h at room temperature. Then the ovules were rinsed in ASB (3×) and incubated with 0.33 µM rhodamine-phalloidin (Rh-phalloidin, Sigma) in ASB containing 5% DMSO for 1.5 h. The samples were treated with wall-degrading enzyme [1% cellulase (Sigma) in ASB] for 1 h after three rinses in ASB. Following several rinses in ASB, nuclei were stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). The endosperm proper and endosperm chalazal haustorium were isolated from ovules under a stereomicroscope and placed directly on a microscope slide. Controls processed in a similar manner but without Rh-phalloidin staining showed no actin fluorescence.

IMMUNOLOCALIZATION ON SECTIONED MATERIAL

The ovules were fixed as described above and then used according to the procedure of Vitha et al. (1997, 2000). After fixation and three rinses in ASB, they were dehydrated in a graded ethanol series containing 10 mM dithiothreitol (DTT, Sigma) (Brown et al., 1989) to minimize the background of cytoplasm. Then the plant material was infiltrated with Steedman's Wax: polyethylene glycol 400 distearate (Aldrich) and cetyl alcohol (Sigma) in a 9:1 (w/w) proportion. After polymerization of the wax, 10 µm sections were made from the embedded ovules and stretched on a small drop of distilled water on microscope slides coated with Mayer's egg albumen. The sections were dried overnight, dewaxed in ethanol, rehydrated in an ethanol-PBS series and rinsed in ASB. After 10 min in methanol at -20˚C, they were rinsed in ASB and then incubated with mouse anti-actin monoclonal antibody (Mab) (clone C4, ICN; diluted 1:1000) overnight at 4˚C. Then the sections were rinsed (3×) in ASB and incubated for 4 h in secondary Alexa 488-conjugated anti-mouse (Molecular Probes; diluted 1:800) antibody. The slides were rinsed in PBS, and the nuclei stained by DAPI. Then the sections were treated with 0.01% toluidine blue to diminish the autofluorescence of the cell walls and mounted in an antifading solution (Citifluor, Agar). In the control experiments, conducted in a similar manner and omitting the first antibody, no actin staining was detected.

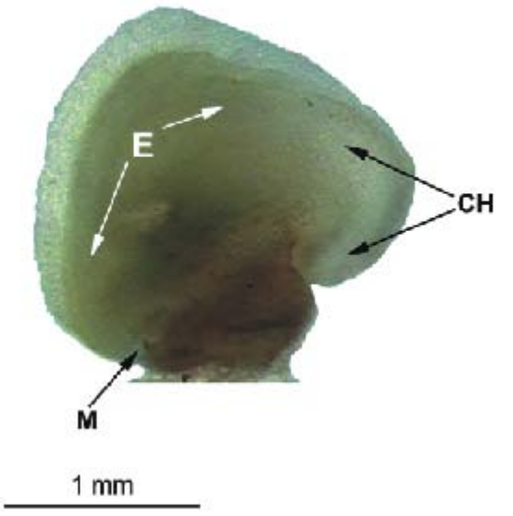
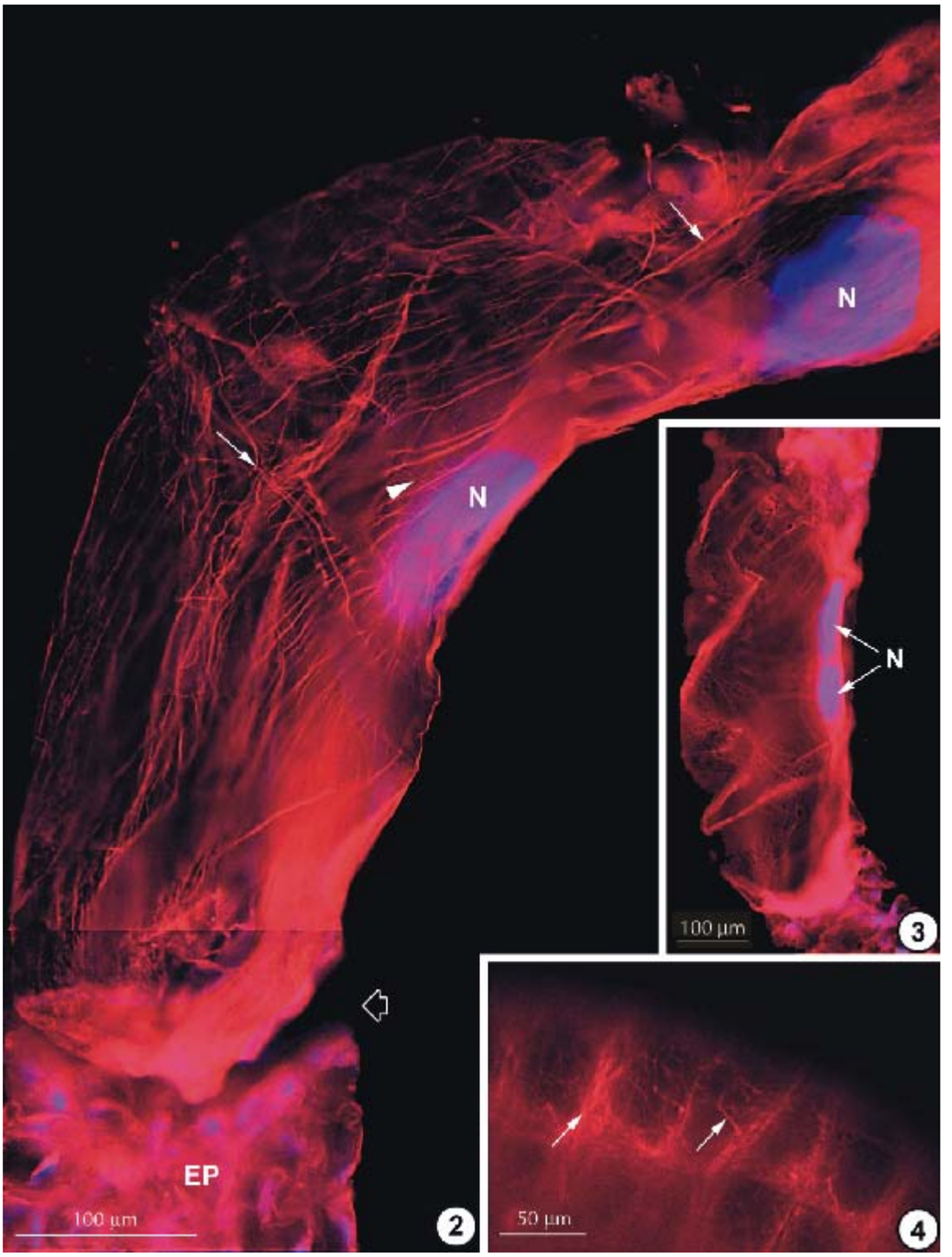


Fig. 1. Young seed of *Rhinanthus serotinus*. CH – chalazal haustorium; E – endosperm proper; M – micropylar haustorium.

MICROSCOPY

Fluorescence was observed with a Nikon Eclipse E 800 epifluorescence microscope, using the following filter blocks: G-2A (EX 510-560 nm, DM 575, BA 590) to visualize actin stained with Rh-phalloidin; B-1E (EX 470-490 nm, DM 505, BA 520-560) to observe actin labeled with Alexa 488; and UV-2A (EX 330-380, DM 400, BA 420) to examine DAPI-stained nuclei. Photographs were taken on Kodak T-max film, ASA 400. Colored image processing was performed using Adobe Photoshop.



Figs. 2–4. Images of F-actin by rhodamine-phalloidin labelling (red); nuclei by DAPI staining (blue). **Fig. 2.** F-actin filaments (arrows) run parallel to long axis of chalazal haustorium cell. Network of actin filaments (arrowhead) surrounding the nuclei (N) and occurring at the proximal pole (open arrow) attaches to the endosperm proper (EP). **Fig. 3.** F-actin bundles in chalazal haustorium cell, two large nuclei (N). **Fig. 4.** Network of microfilaments (arrows) in endosperm proper cells.

RESULTS

During the development of endosperm in *Rhinanthus serotinus* two types of haustoria are formed: micropylar and chalazal (Fig. 1). The growing chalazal haustorium crushes the surrounding tissue and remains attached to the endosperm proper only through the proximal pole, while the distal pole penetrates the ovular tissue. We focused our studies on detection of microfilaments (MF) in different parts of the haustorium cell (Figs. 2–3, 8–9) and in endosperm proper cells (Figs. 4–7), including the cytoplasm surrounding cellular organelles. The fully developed chalazal haustorium is a large kidney-shaped cell 800 μm long and 250 μm in diameter. Two conspicuous nuclei with polytene chromosomes are situated halfway along the length of the cell (Figs. 2, 3).

A large vacuole traversed with several cytoplasmic strands occupies the major part of the haustorium. These cytoplasmic strands connect the cytoplasmic layer adjacent to the cell wall with the cytoplasm assembled around the large nuclei and at both poles of the haustorium cell (Figs. 2–3). Thick, longitudinally oriented MF bundles localize in these cytoplasmic strands (Figs. 2–3, 9). Organelles, especially plastids (not shown) are numerous within the strands. An actin meshwork composed of delicate filaments surrounds the polytene nuclei (Figs. 2–3); some of the filaments radiate from the nuclear surface. Abundant and clearly visible actin filaments occur at the proximal pole of the haustorium cell (Figs. 2–3). At the chalazal end of the haustorium cell are a distinct and very developed actin cytoskeleton with numerous cross-linked microfilaments (Fig. 8) as well as a high number of mitochondria (data not shown).

In endosperm proper cells F-actin aggregates are also observed. An extensive network of actin filaments running throughout the cytoplasm is located in the cortical region of the endosperm cells (Fig. 4). These F-actin arrays congregate around the nuclei; some envelop the nuclei, and others appear to radiate from the nuclear surface (Figs. 5–7).

DISCUSSION

It is well known that in Scrophulariaceae the endosperm produces chalazal haustoria (Schmid, 1906; Arekal, 1963). Generally, the haustorium cell is a rapidly developing, short-lived organ associated with the nourishment of the embryo during its development (Raghavan, 1976; 1986; Vijayaraghavan and Prabhakar, 1984). The mature chalazal haustorium of *Rhinanthus serotinus* is a huge, long cell containing two large nuclei with polytene chromosomes (Bohdanowicz et al., 1993). The enormous size of the haustorium cell and its nuclei is connected with multiplication of the basic nuclear DNA content. Polytene chromosomes are only found in highly specialized giant plant cells. Polytene structures in the endosperm chalazal haustorium have been described in three species of *Rhinanthus* (Tschermak-Woess, 1957), *R. minor* (Nagl, 1992) and *R. serotinus* (Bohdanowicz et al., 1993).

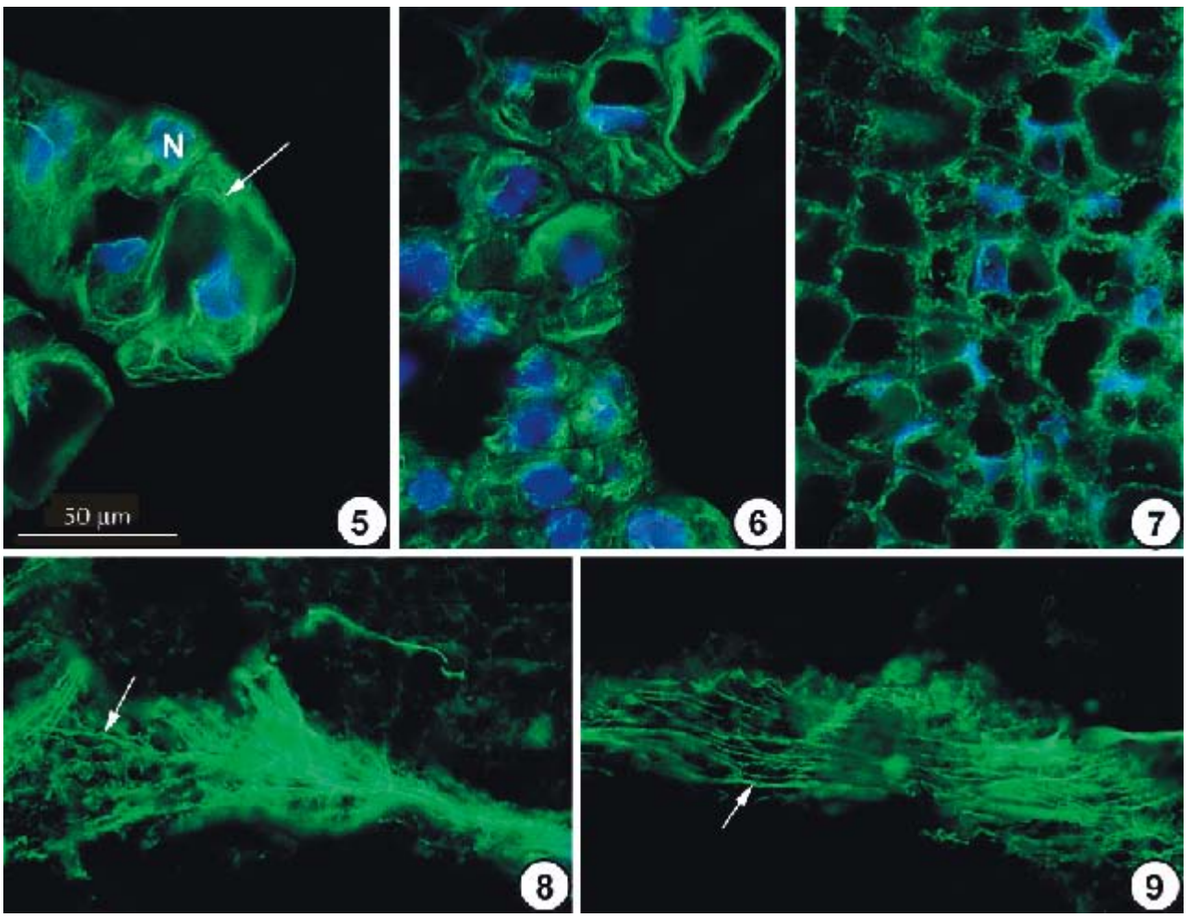
The optimized protocol used in our study allowed for better preservation and penetration of Rh-phalloidin and antibodies into the cells, hence better staining of microfilaments. The main features of the actin cytoskeleton in the *R. serotinus* haustorium cell are as follows: it contains numerous MF congregating around the nuclei and radiating from the nuclear envelopes; the actin network is extremely abundant at the chalazal end of the cell; and thick, longitudinally oriented MF bundles are present in cytoplasmic strands traversing the main vacuole.

In the few reports on microfilament organization (Derksen et al., 1986; Staiger and Schliwa, 1987), a distinct actin network connected to a meshwork of microfilaments enveloping the cell nucleus has been described. The prominence of the nucleus-and-microfilament organization in the haustorium indicates that, besides other functions, microfilaments may play a role in nuclei positioning.

Recently the participation of the cytoskeleton and especially actin filaments in mRNA transport has been reported (Nasmyth and Jansen, 1997; Muench et al., 1998). The multiplication of nuclear genomes suggests high gene activity in the chalazal haustorium of *Rhinanthus*. Probably the highly developed actin cytoskeleton around polytene nuclei is also involved in the intracellular distribution of nuclear products in this huge cell.

The dense actin network and numerous mitochondria observed at the chalazal end of the haustorium are probably connected with the labyrinth of the transfer wall, formed and functioning there (Nagl, 1992; Bohdanowicz et al., 1993).

This study clearly showed the presence of long, thick actin bundles associated with numerous plastids in cytoplasmic strands traversing the haustorium cell. The regular formation of these actin arrays suggests their fundamental role in the movement of organelles inside such a large cell. The role of actin in the movement and localization of organelles has been confirmed by numerous fluorescence



Figs. 5–9. Images of F-actin by antibody immunofluorescence labeling (green); nuclei by DAPI staining (blue). **Fig. 5.** Bundles of F-actin (arrow) in endosperm proper cells. N – nuclei. **Fig. 6.** Rich microfilament network throughout the cytoplasm in endosperm proper cells. **Fig. 7.** Radial arrays of microfilaments emanating from endosperm nuclei. **Fig. 8.** Abundant bundles of actin filaments (arrow) at distal pole of chalazal haustorium cell. **Fig. 9.** Longitudinally oriented microfilaments (arrow) in cytoplasmic strand in haustorium cell. Bar in Fig. 5 corresponds to all figures.

studies (Boevink et al., 1998; Olyslaegers and Verbelen, 1998; Kandasamy and Meagher, 1999).

In endosperm proper cells of *R. serotinus*, the cortical actin cytoskeleton network and F-actin arrays associated with nuclear envelopes are essentially similar to those of other angiosperm species (Muench et al., 2000; Nguyen et al., 2001, 2002).

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