Actin cytoskeleton in the extra-ovular embryo sac of *Utricularia nelumbifolia* (Lentibulariaceae)

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**Abstract** The actin cytoskeleton in the mature female gametophyte of angiosperms has been examined in only a few dicot and monocot species. The main purposes of this study were to identify how the actin cytoskeleton is arranged in the mature extra-ovular embryo sac in *Utricularia nelumbifolia* (Lentibulariaceae). We found that the extra-ovular part of the central cell has a well-developed actin cytoskeleton: actin microfilaments formed of long strands which run longitudinally or transversally to the long axis of the embryo sac. The exerted part of the central cell, which is exposed to the environment of the ovary chamber, is highly vacuolated and in the thin peripheral cytoplasm possesses a complicated network of actin microfilaments. The epidermal cells of the placenta that are in contact with the extra-ovular part of the embryo sac are crushed. The ultrastructure data of these cells are presented. We detected the accumulation of the actin cytoskeleton between the micropylar parts of the synergids and the extra-ovular part of central cell. This actin accumulation is unusual because in typical angiosperms the micropylar parts of the synergids form the apex of the female gametophyte.

**Keywords** Actin microfilaments · Female gametophyte · Embryo sac · Egg apparatus · Central cell · Lentibulariaceae · Carnivorous plants

**Abbreviations**

- ASB Actin-stabilizing buffer
- DAPI 4′,6-Diamidino-2-phenylindole dihydrochloride
- ES Embryo sac
- AF Actin microfilaments
- PBS Phosphate-buffered saline
- SEM Scanning electron microscope

**Introduction**

In seed plants, there is a cross dialogue between two generations: the female gametophyte (embryo sac, ES) and maternal sporophyte (Ingram 2010; Bencivenga et al. 2011). Ovular tissues provide nutrients to the female gametophyte and also participate in the guidance of the pollen tube (Shimizu and Okada 2000; Chudzik et al. 2005; Palanivelu et al. 2003; Chae and Lord 2011). In most angiosperms, the female gametophyte remains in the ovule surrounded by sporophyte tissue; however, in a few genera, the female gametophyte grows outside the ovule, where it is partially exposed and may establish contact with other sporophyte tissue, e.g., in *Philadelphus, Thesium, Galium, Utricularia*, and some members of the Scrophulariaceae family (Maheshwari 1950). In *Philadelphus, Thesium*,
Galium, Torenia, and some species of Lindernia not only part of the central cell is extra-ovular but also the egg apparatus itself (Krishna Iyengar 1941; Maheshwari 1950; Higashiyama et al. 2006). In these genera, the egg apparatus occupies the micropylar-apical position in the embryo sac. In the above-mentioned genera, the most famous is the genus Torenia because studies of the extra-ovular egg apparatus in this genus (especially Torenia fournieri) confirmed the fact that the synergids are responsible for the attraction of pollen tubes in angiosperms (Higashiyama et al. 1998, 2001, 2003). These studies also provided new data about the process of fertilization (Higashiyama et al. 1997; Yuan et al. 2002) and about the symplastic communication between the central cell and the egg apparatus (Han et al. 2000). It is worth noting the genus Utricularia in any discussion of the extra-ovular egg sac. With some exceptions, a common characteristic in Utricularia species is that only the central cell grows outside, while the egg apparatus occurs in the part of the central cell which remains in the ovule (Plachno 2011). Therefore, this Utricularia feature is different from the model species T. fournieri in the egg apparatus position. In T. fournieri, the egg apparatus is exposed. The occurrence of the partially exposed embryo sac in Torenia also allows the actin cytoskeleton in living female gametophytes of this species to be studied (Huang 2002). The actin cytoskeleton in Torenia is different from the model species ovule (P. occidentalis) (P. occidentalis, 1954; Farooq and Siddiqui 1964; P. occidentalis, 1954; Higashiyama et al. 2006). In these genera, the egg apparatus occupies the micropylar-apical position in the central cell and the primary endosperm. However, in Utricularia, which possesses another type of exposed embryo sac, there is a lack of data about its actin cytoskeleton. In Utricularia, the central cell forms the haustorium which interacts with placental tissue (Khan 1954; Farooq and Siddiqui 1964; Plachno and Świątek 2008). This haustorium in some Utricularia species (subgenus Utricularia, section Utricularia) is the “prelude” for producing, after fertilization, a special placenta-endosperm syncytium (which possesses both maternal and paternal genetic material) (Khan 1954; Plachno and Świątek 2011). This syncytium has a well-developed actin cytoskeleton (Plachno et al. 2011). Moreover, it seems that Utricularia quelchii synergids have a simpler structure compared to related Genlisea and some other angiosperms (Plachno 2011). Therefore, identifying how the actin cytoskeleton is arranged in the egg apparatus of Utricularia appeared to be worth investigating.

The main purpose of this study was to identify how the actin cytoskeleton is arranged in the mature extra-ovular embryo sac in Utricularia nelumbifolia (before pollination and fertilization). The results of fluorescent light microscopy were correlated with those of transmission and scanning electron microscopy.

Material and methods

Material

The flowers of a Brazilian species of U. nelumbifolia Gardner (section Iperua, Taylor 1989) were obtained from the greenhouse collection of the Botanic Garden of the Jagiellonian University in Kraków, Poland.

Immunofluorescence of cytoskeleton

For the visualization of actin microfilaments (AF), placentas with ovules were fixed in a mixture of 4% formaldehyde (freshly prepared from paraformaldehyde) and 0.25% glutaraldehyde in an actin-stabilizing buffer (ASB; 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid), 10 mM ethylene glycol-bis(2-aminooethyl ether) N,N′N′N′tetraacetic acid, and 1 mM MgCl₂, pH 6.8), overnight at 4°C. All chemicals were obtained from Sigma (Germany) unless otherwise indicated. After fixation and three washes in an ASB, they were dehydrated in a graded ethanol series. The material was then infiltrated with Steedmans’ Wax. Then 5–10-μm sections were taken from the embedded ovaries and adhered to poly-L-lysine-coated microscope slides. The sections were dried overnight, dewaxed in ethanol, rehydrated in an ethanol–phosphate-buffered saline (PBS) series, and rinsed in PBS. After 10 min in methanol at −20°C, they were washed in PBS. Tissue sections were preincubated in 5% bovine serum albumin in PBS for 45 min, washed in PBS, and then incubated in a monoclonal antibody against actin (clone C4, ICN) overnight at 4°C. The sections were then washed in PBS and incubated for 4 h in a secondary Alexa 488-conjugated anti-mouse antibody (Molecular Probes). The slides were rinsed in PBS and the nuclei were stained by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; 5 μg/1 ml, Sigma). The slides were then mounted in an anti-fading solution Citifluor AF1 (Agar). In the control experiments, conducted in a similar manner but omitting the first antibody, no actin staining was detected (control is presented as Supplementary material). Fluorescence was examined using Nikon Eclipse E800 and Olympus BX60 epifluorescence microscopes. More than 100 ovules were examined. The quality of fixation was checked using the differential interference contrast microscopy technique. The quality of preservation of the AF varied between the embryo sacs. The embryo sacs with collapsed cells in which serious apparent osmotic damage was observed were not included in the description of the actin cytoskeleton arrangements. However, the embryo sacs in which slight plasmolysis occurred, but the actin cytoskeleton appeared intact, were included in the study.
Light and electron microscopy

For electron microscopy, placentas with ovules were isolated from ovaries and fixed in a mixture of 2.5% formaldehyde and 2.5% glutaraldehyde in a 0.05-M cacodylate buffer (pH 7.0) for 2 days then washed in a cacodylate buffer, postfixed in 1% OsO₄ in a cacodylate buffer for 24 h at 4°C, rinsed in the same buffer, treated with 1% uranyl acetate in distilled water for 1 h, dehydrated with acetone, and then embedded in Epoxy Embedding Medium Kit (Fluka). Semithin sections (0.8–1.5 μm thick) were cut using a Leica ultracut UCT ultramicrotome, stained with methylene blue and examined under an Olympus BX60 microscope equipped with a DP12 digital camera and AnalysiS 3.2 (Soft Imaging System) software. Ultrathin sections (70 nm) were cut using a Leica ultracut UCT ultramicrotome. After contrasting with uranyl acetate and lead citrate, the sections were examined using a Hitachi H500 electron microscope. The procedure for preparing samples for Scanning electron microscope (SEM) was as described earlier (Plachno and Świątek 2009, 2010). The dried tissues were sputter-coated with gold and viewed using a HITACHI S-4700 microscope (Scanning Microscopy Laboratory of Biological and Geological Sciences, Jagiellonian University).

Results

Like the majority of angiosperm taxa, *U. nelumbifolia* has a so-called Polygonum-type female gametophyte. In *U. nelumbifolia*, the mature central cell can be divided into three parts: extra-ovular, central (where the secondary nucleus occurs) and the chalazal. The micropylar part of the central cell of the mature embryo sac projects out beyond the edges of the integument and grows outside the ovule (Fig. 1a, b). The embryo sac is exposed to the environment of the ovary chamber (Fig. 1a, b); however, the extra-ovular part of the central cell also penetrates the placenta tissue (Fig. 1c). This haustorial part of the central cell which penetrates the placenta has contact with a special spherical group of cells that have a thicker cell wall (“nutritive tissue”) which differentiates in close proximity to the ovule base at an early stage of the development of the female gametophyte (Fig. 1c). In the micropylar part and the extra-ovular part of the central cell, AF form long strands which run longitudinally and transversally to the long axis of the embryo sac (Fig. 2a). The exposed part of the central cell, which is in contact with the environment of the ovary chamber, is highly vacuolated (Fig. 2b). A complicated network of AF is clearly visible in the thin peripheral cytoplasm of the exposed part of the central cell.

Fig. 1 The morphology and anatomy of the ovule and embryo sac of *U. nelumbifolia*; a SEM micrograph of the ovule (Ov) and extra-ovular part of the central cell (arrow). Bar=11 μm. b SEM micrograph of the extra-ovular part of the central cell (arrows), the ovule was removed for better visualization of the central cell. Bar=200 μm. c A longitudinal section through the ovule, embryo sac, and part of the placenta showing the arrangement of actin in different types of cells (actin by antibody immunolabeling—green). Note the haustorial part of the embryo sac which penetrates the placenta tissue (HES) and long AF strands which run longitudinally to the long ES axis (small arrows); raphe (Ra), placenta (P), integument (In), placenta epidermis (Ep), central cell (Cc), hypostase (H), nutritive tissue (Nt), the extra-ovular part of the central cell (arrow). Bar=10 μm
The epidermal cells of the placenta that are in contact with the extra-ovular part of the embryo sac are crushed (Fig. 2c). In contrast to the other epidermal cells, positive signals were not observed after DAPI staining (not shown), nor were they observed in these cells after actin staining (Fig. 3a). The cell walls of these cells are partially dissolved (Fig. 2c). Thus, the extra-ovular part of the embryo sac should be considered as haustorial. In the central part of the central cell, near the secondary nucleus, bundles of microfilaments run in cytoplasmic strands (Fig. 3a) forming radial or random arrays. The chalazal part of the central cell has a cortical actin cytoskeleton in the shape of long AF arranged according to the long axis of the ES (not shown). In the synergids, AF are present throughout the cytoplasm but are concentrated at the micropylar pole (Fig. 3a) in a transverse orientation. It should be stressed that near the micropylar part of the synergids in the central cell, there are thick bundles of AF oriented longitudinally. These bundles form an actin cylinder between the synergids and the extra-ovular part of the central cell (Fig. 3b). Actin microfilaments occur around the nuclei of the synergids and also along cytoplasmic transvacuolar strands (not shown). Our observations of the actin cytoskeleton are consistent with ultrastructural data (Fig. 4a, b), e.g., a concentration of AF was detected at the micropylar synergid pole where most of the cytoplasm, nucleus, and filiform apparatuses are located (Fig. 4a). Plasmodesmata between the synergids and the central cell occur here (not shown). In addition, the part of central cell which borders the micropylar part of the synergids has cytoplasm filled with mitochondria, profiles of endoplasmic reticulum, dictyosomes, and numerous small vesicles (Fig. 4a). An egg cell is situated below the synergids (Fig. 4b). The egg nucleus and the majority of the
The actin cytoskeleton in the female gametophyte of angiosperms has been examined in only a few dicot and monocot species (e.g., Webb and Gunning 1994; Huang et al. 1993, 1999; Huang and Russell 1994). None of these species develops such a well-developed haustorial central cell which penetrates placenta tissues, as the *Utricularia* species do. Thus, the enormous haustorial, extra-ovular part of the central cell, which has a complicated network of actin microfilaments, is unique to *Utricularia*. The question is why does *Utricularia* develop this extra-ovular part of the central cell. In the *Utricularia* ovule, the funiculus lacks vascular tissue, so some *Utricularia* species have another pathway for the transport of metabolites (mainly species from the section *Utricularia*, Khan 1954; Plachno and Świątek 2008, 2009) directly from the placental nutritive tissue to the embryo sac. In addition, the influence of the extra-ovular part of the central cell on the sporophytic cells of the placenta has been observed in the case of *U. nelumbifolia* (section Iperua). It appears that this highly vacuolated extra-ovular part of the central cell of *U. nelumbifolia* squashes the epidermal cells of the placenta by physical pressure. This study indicated that, in the above-mentioned epidermal cells, there was protoplast disintegration and that there was also a partial dissolution of cell walls. Synergids are essential for attracting pollen tubes and for the fertilization process of angiosperms (Huang and Russell 1992; Higashiyama et al. 2001; Okuda et al. 2009), but it has only recently been found that the central cell also has its own role in attracting pollen tubes (Marton et al. 2005; Chen et al. 2007). It was proposed that signals from the micropylar part of the central cell could diffuse readily to the micropyle or that the signals produced by the central cell may be transported via the egg apparatus cells (Dresselhaus 2006; Chen et al. 2007; Liu et al. 2010). In *U. nelumbifolia*, the enlargement of the central cell increases the absorptive surface of the embryo sac; in addition, it may also help in signal transduction for the attraction of pollen tubes. However, further studies are necessary to determine this.

In *U. nelumbifolia*, the actin cytoskeleton of the central and chalazal parts of the central cell have an arrangement very similar to other plants where F-actin is distributed in the cortical and perinuclear region and also along the transvacuolar strands (*Plumbago* Huang et al. 1993; *Zea*, *Torenia* Huang et al. 1999; *Arabidopsis* Webb and Gunning 1994) or along the amyloplasts when the central cell is rich in starch (*Arabidopsis* Webb and Gunning 1994). Yuan et al. (2002), using living *Torenia* embryo sacs and microinjection experiments, showed that in the central cell, AF form a very dynamic system that is dependent on the phases of the development of the flower (time of anthesis) and pollination. These authors found thick bundles of AF which connected the secondary nucleus to the micropylar, cortical cytoplasm near the egg apparatus. A similar actin cylinder was found in the central cell of *Galanthus nivalis* (Bednara 2003). According to Yuan et al. (2002), actin in the central cell not only participates in cytoplasm streaming and
organelle movements but also in the migration of the secondary nucleus to the region of the egg apparatus. As was mentioned above, in Torenia and Galanthus, the actin cylinder which connects the central cell and the egg apparatus was observed (Yuan et al. 2002; Bednara 2003). An actin cylinder-like structure was also observed in this study, however in a different location: between the micropylar parts of the synergids and the extra-ovular part of the central cell. This accumulation of actin cytoskeleton may participate in the transport of signals between the cytoplasm of the central cell and the synergids, especially since there are plasmodesmata which connect the micropylar parts of the synergids with the central cell. However, this should be experimentally proven.

Generally, in angiosperm synergids, actin microfilaments are mostly aligned longitudinally with the long cell axis (Arabidopsis Webb and Gunning 1994; Torenia Fu et al. 2000). In contrast, the actin microfilaments in U. nelumbifolia were aligned transversely with the long cell axis; this is probably connected with their simpler synergid structure as compared to other angiosperms (Plachno 2011) and with the orientation and structure of the filiform apparatus in this genus. In Torenia, dense actin filaments are located near the region of the filiform apparatus. Later, after the degeneration, actin forms a cap near the filiform apparatus (Fu et al. 2000). Similarly to Torenia, AF have also been detected in this region in Utricularia synergids.

The four following actin arrangements have been described for plant egg cells: (1) AF in the chalazal pole, (2) a cortical arrangement, (3) AF in cytoplasmic strands, and (4) a random arrangement (Webb and Gunning 1994; Huang et al. 1993, 1999; Huang and Russell 1994; Bednara 2003). Similar to other angiosperms, the AF concentrate at the chalazal pole of the egg cell in U. nelumbifolia. The poor AF cytoskeleton in the Utricularia egg may be connected to the inactive physiological stage (resting phase) of the egg cell of angiosperms or actin signals may simply label globular actin.

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Fig. 4 Ultrastructure of the egg apparatus of U. nelumbifolia and an immunostaining of the actin microfilaments in the egg cell; a contact between the micropylar part of the synergid and the central cell; synergid (S), synergid nucleus (sn), central cell (Cc), filiform apparatus (Fa). Bar=1 μm. b Ultrastructure of the egg apparatus, egg nucleus (Egn), egg cell (eg), central cell (Cc), synergid (S). Bar=1.3 μm. c Poor actin cytoskeleton in egg cell: actin microfilaments (arrows), egg nucleus (Egn), vacuole of the egg cell (V). Bar=10 μm.
Conclusions

This study has shown that the *U. nelumbifolia* embryo sac has some unusual characteristics in AF arrangement comparison with typical angiosperms: The extra-ovular part of the central cell has a well-developed actin cytoskeleton—actin microfilaments formed of long strands run longitudinally and transversally to the long axis of the embryo sac. The exerted part of the central cell, which is exposed to the environment of the ovary chamber, is highly vacuolated, and in the thin peripheral cytoplasm, a complicated network of actin microfilaments was present.

The accumulation of the actin cytoskeleton between the micropylar parts of the synergids and the extra-ovular part of the central cell is unusual because in typical angiosperms the micropylar parts of the synergids form the apex of the female gametophyte.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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