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Egg Apparatus in Sexual and Apomictic Species of *Taraxacum*: Structural and Immunocytochemical Aspects of Synergid Cells

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The paper reports a comparative study of the female gametophyte and especially synergid structure in sexual and apomictic dandelions. We analyzed diploid sexually reproducing *Taraxacum linearisquameum* ($2n = 2x = 16$) and two triploids, *T. alatum* and *T. udum* ($2n = 3x = 24$), with autonomous embryo and endosperm development. There were no observed differences in the organization of the mature megagametophyte between the examined species. Both meiotically reduced and diplosporous embryo sacs showed typical polarity of the egg apparatus cells, together with development of a filiform apparatus in the synergids, but immunocytochemical analyses indicated that microtubules form longitudinal brush-like bundles adjacent to the filiform apparatus in the synergids of the sexual *T. linearisquameum*. This arrangement of cytoskeletal elements is similar to the configuration described in other amphimictic plants. The synergids of the apomictic *T. alatum* and *T. udum* show a uncharacteristic and relatively weak cytoskeleton with no brush-like bundles. We discuss the role of synergids in autonomous apomicts.

Key words: Apomixis, female gametophyte, synergid cell, filiform apparatus, microtubular cytoskeleton, *Taraxacum*.

INTRODUCTION

Most angiosperms produce seeds sexually, but agamospermy (asexual seed formation by apomixis) occurs in more than 400 genera belonging to over 40 flowering plant families (Asker and Jerling, 1992; Bicknell and Koltunow, 2004; Noyes, 2007). In both sexual and apomictic plants, development of the female gametophyte (also called megagametophyte or embryo sac) is indispensable for seed formation. Inside the ovules of sexually reproducing plants, the megasporocyte divides meiotically and gives rise to four haploid megaspores, only one of which survives and undergoes three mitotic division to form a reduced megagametophyte in which fertilization of the egg and central cells is required for embryo and endosperm formation. By contrast, in the ovules of apomictic plants, meiotic reduction may be either avoided (apospory) or altered (diplospory); that is why the sporophyte and gametophyte have the same ploidy level (Nogler, 1984; Asker and Jerling, 1992). The female gametophyte develops from a nucellus cell called an aposporous initial, or from a chromo-

somally unreduced megaspore mother cell. In the meiotically unreduced megagametophyte the egg cell develops into an embryo without fertilization (parthenogenesis). In most apomictic plants, fertilization of the central cell is required for initiation of endosperm growth (pseudogamy) but in others the endosperm develops autonomously (Nogler, 1984; Asker and Jerling, 1992; Koltunow, 1993).

In amphimictic plants, synergids are structurally specialized and highly active cells which play an essential role in many steps of the sexual reproductive process. They are of particular importance in controlling the final stages of pollen tube attraction and reception. At the micropylar pole of the synergid the cell wall is thickened and forms finger-like projections into the synergid cell cytoplasm which are known as the filiform apparatus (FA) (Willemse and van Went, 1984; Huang and Russell, 1992; Higashiyama, 2002). Many genetic, molecular and physiological studies done in the last decade, examining *Arabidopsis thaliana*, *Zea mays* and *Torenia fournieri*, have confirmed the crucial reproductive functions of synergids and have elucidated the role

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of the FA in pollen tube attraction and reception (for review see Punwani et al., 2007, 2008; Punwani and Drews, 2008; Li et al., 2009; Dresselhaus and Márton, 2009; Okuda et al., 2009; Kessler and Grossniklaus, 2011). Since embryo and endosperm development is independent of pollination in autonomous apomicts, it seems desirable to determine whether this is reflected in the structure of synergid cells. So far the structure of synergid cells has not been analyzed in detail in apomictic species, but research on the female gametophyte organization of the obligatory apomict *Chondrilla juncea* (Asteraceae) revealed that the synergid cells of this species have no FA (Kościńska-Pająk and Bednara, 2006).

Taraxacum Wigg (dandelion) makes a good model genus for comparative analysis of embryological structures and processes in amphimictic and apomictic species because it forms a polyploid complex in which ploidy and the mode of reproduction are strongly correlated: sexuality is linked to diploid dandelions, whereas polyploid species usually reproduce asexually *via* apomixis (Richards, 1973, 1989). In *Taraxacum*, apomixis involves meiotic diplospory, parthenogenesis and autonomous endosperm development (Gustafsson, 1946; Richards, 1973; Nogler, 1984; Asker and Jerling, 1992).

This paper focuses on three *Taraxacum* species with known chromosome numbers: diploid *T. linearisquameum* Soest (2n = 2x =16) and two triploids, *T. alatum* Lindb. and *T. udum* Jordan (2n = 3x = 24) (Góralski et al., 2009; http://www.binoz.uj.edu.pl). These species were also the subject of embryological research in which it was shown that *T. alatum* and *T. udum* are obligatory apomicts (Kościńska-Pająk, 2006; Musiał et al., unpubl.), and that meiotic division of the megaspore mother cell as well as Polygonum-type embryo sac formation occur in *T. linearisquameum* ovules (Musiał, unpubl. data).

In this study we used tissue clearing technique and immunofluorescence labeling to examine micromorphological aspects of synergid cells in the egg apparatus of sexual and apomictic dandelions. We put special emphasis on observations of the filiform apparatus and the organization of the microtubular cytoskeleton in synergids of sexual and apomictic plants.

MATERIALS AND METHODS

PLANT MATERIAL

The studies used dandelion capitula sampled just before and during anthesis. Inflorescences of *T. linearisquameum* and *T. alatum* were sampled from specimens growing in the private collection of Dr. Jolanta Marciniuk in Siedlce (52°10'49"N, 22°18'26"E). Capitula of *T. udum* were collected from plants randomly taken from a natural population in Pomiechowo near the embranchment of the Wkra and Narew rivers (52°27'18"N, 20°43'47"E).

TISSUE CLEARING TECHNIQUE

Inflorescences of each *Taraxacum* species were fixed in FAA and stored in 70% ethanol. Then the ovules were isolated from the flowers and cleared in methyl salicylate according to a procedure described earlier (Musiał et al., 2012). Cleared ovules were examined with a Nikon Eclipse 80i microscope fitted with Nomarski interference contrast optics.

IMMUNOCYTOCHEMICAL STUDIES

For localization of microtubules, ovaries were excised from the sampled inflorescences and immediately fixed in freshly prepared solution of 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate-buffered saline (PBS) for 4 h at room temperature. After fixation, the ovaries were rinsed three times in PBS, dehydrated in a graded ethanol series, embedded in Steedman's wax, cut 5–7 μm thick and mounted on microscope slides coated with Mayers's egg albumen by the method Bohdanowicz et al. (2005) described. The sections were dried overnight, dewaxed in absolute ethanol, rehydrated in an ethanol-PBS series, washed in PBS and preincubated in PBS with 0.1% bovine serum albumin (BSA) for 30 min at room temperature. Then the tissue sections were incubated in a humid chamber for 90 min at 37°C with monoclonal anti-mouse-β tubulin (Sigma-Aldrich Co.) diluted 1:200 with PBS containing 0.1% BSA. After 3 rinses with 0.1% BSA in PBS the sections were incubated for 12 h at 4°C with secondary antibody conjugated with FITC (Sigma-Aldrich Co.), diluted 1:100 with blocking buffer. After labeling, the slides were rinsed in PBS and the samples were treated with DAPI (1 μg/ml) in PBS for 5 min to stain the nuclei. Finally the sections were rinsed in PBS and mounted in anti-fade Citifluor.

Fluorescence was observed with a Nikon Eclipse 80i epifluorescence microscope fitted with a monochrome CCD camera.

PAS REACTION

Polysaccharides were visualized on paraffin sections of ovaries by the PAS reaction using acriflavine-Schiff's reagent (Wędzony, 1996 and references therein). The sections were deparaffinized, rehydrated to 70% ethanol, oxidized for 5 min in periodic acid solution (70 ml 100% ethanol, 0.8 g H_5IO_6,
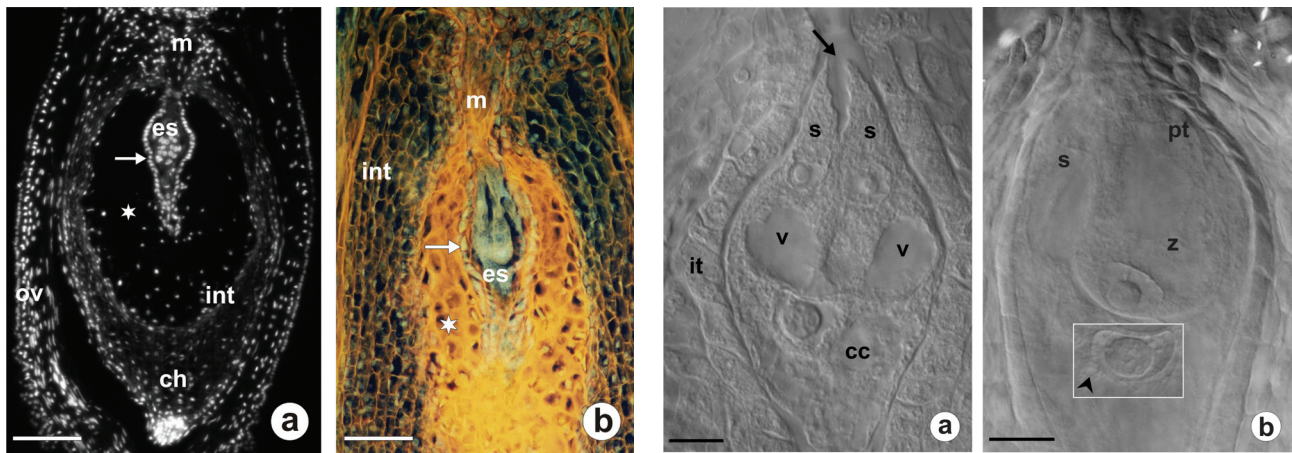
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Fig. 1. Ovary and ovule structure of *Taraxacum* species. (**a**) *T. udum*. Longitudinal section of unilocular ovary with anatropous unitegmic ovule after DAPI staining; asterisk indicates zone of thick-walled integumentary cells, (**b**) *T. alatum*. Ovule after PAS reaction, note orange staining in PAS-positive thickened walls (asterisk) of integumentary cells surrounding endothelium (arrow). ch – chalazal pole; es – embryo sac; int – integument; m – micropyle; ov – ovary wall. Bar in (a) = 100 μm; in (b) = 50 μm.

10 ml 0.2 M sodium acetate, and supplemented to 100 ml with distilled water). Than the samples were stained in acriflavine-Schiff's reagent for 15 min at room temperature. After washing in running tap water, the slides were dehydrated in a graded ethanol series, embedded in Entellan and stored in the dark at 4°C. The acriflavine-Schiff's reagent was prepared by dissolving 0.1 g acriflavine (Sigma-Aldrich Co.) in solution containing 1 g $K_2S_2O_5$, 10 ml 1N HCl and 85 ml distilled water.

RESULTS

The ovary of *Taraxacum* species is inferior and unilocular, with one basal, anatropous, unitegmic and tenuinucellate ovule. The ovules of the studied dandelion species showed no essential differences in structure. They have a massive integument exhibiting conspicuous zonal differentiation (Fig. 1a,b). The inner epidermal cells of the integument transform into an endothelial layer surrounding the megagametophyte (Fig. 2a), and around the endothelium is a characteristic zone of thick-walled cells. In this part of the integument, due to strong thickening of the cell walls, protoplast size is reduced considerably and the cells progressively degenerate, as evidenced by the weak signal after DAPI staining (Fig. 1a). Positive results of the sensitive PAS reaction showed that the material deposited in prominent thick cell walls is rich in water-insoluble polysaccharides (Fig. 1b).
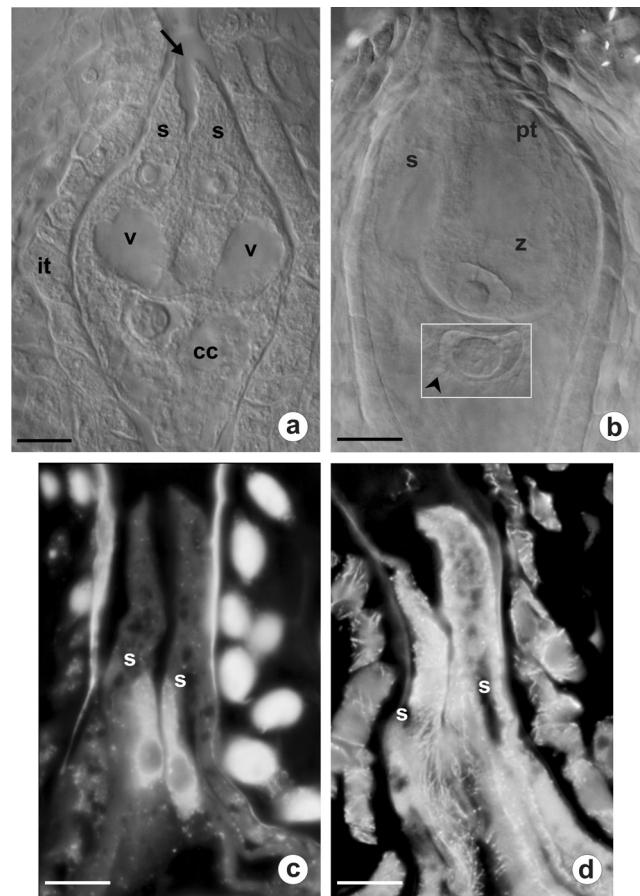

Fig. 2. Mature female gametophyte of *T. linearisquameum*, (a) and (b) images obtained from cleared material using Nomarski DIC optics. (**a**) Synergids in embryo sac just before anthesis, arrow points to filiform apparatus, (**b**) Embryo sac after pollen tube entry, zygote and remnants of pollen tube visible, arrowhead indicates sperm nucleus adjacent to secondary nucleus (insert), (**c**) Micropylar region of synergids with well visible nuclei visualized by DAPI staining, (**d**) Cytoskeleton of synergids with bundles of longitudinally oriented microtubules. cc – central cell; it – integumentary tapetum (endothelium); pt – remnants of pollen tube; s – synergid cell; v – vacuole; z – zygote. Bars = 10 μm.

Inside the ovule the female gametophyte develops, and as in other angiosperms it remains deeply embedded in the sporophyte tissues of the ovule. The mature megagametophytes of the examined species do not differ in organization. Both the meiotically reduced embryo sacs of *T. linearisquameum* and the diplosporous embryo sacs of *T. alatum* and *T. udum* contain a three-celled egg apparatus comprising the egg cell and two synergids at the micropylar pole, the central cell with a secondary nucleus, and usually three antipodal cells at the chalazal pole. The egg apparatus and central cell form a female germ unit which occupies the largest
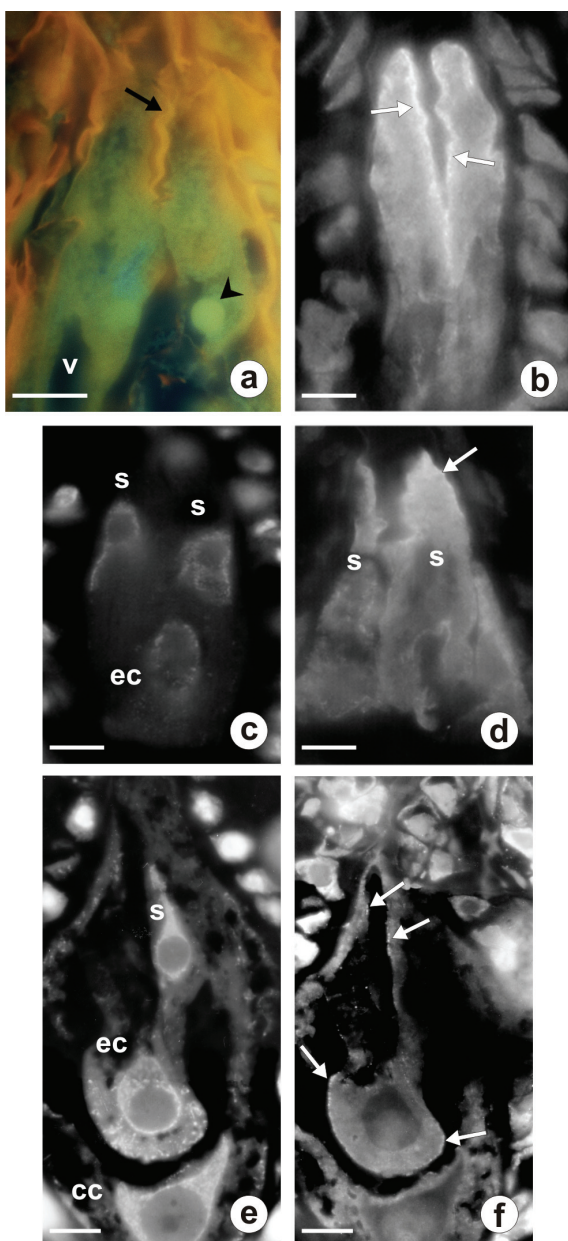
Fig. 3. Micropylar pole of mature female gametophyte of *T. alatum*. (**a**) Synergids after PAS treatment, FA visible as zigzag-shaped PAS-positive orange cell wall (arrow), arrowhead shows nucleus, (**b**) Micropylar region of synergids after tubulin immunodetection, arrows indicate spot fluorescence of cortical microtubules, (**c**) Egg apparatus after DAPI staining, showing position of nuclei in egg cell and synergids, (**d**) Cytoskeleton of synergids, cortical microtubules visible mainly close to micropylar end, no brush-like arrangement of microtubules present, (**e**) Micropylar part of mature embryo sac after DAPI staining, note numerous DNA-containing organelles in cytoplasm surrounding egg nucleus, (**f**) Micropylar part of mature embryo sac after tubulin immunodetection, scanty cytoskeleton visible both in egg cell periphery and synergid (arrows). cc – central cell; ec – egg cell; s – synergid cell; v – vacuole. Bar in (a, e, f) = 10 μm; in (b–d) = 12.5 μm.
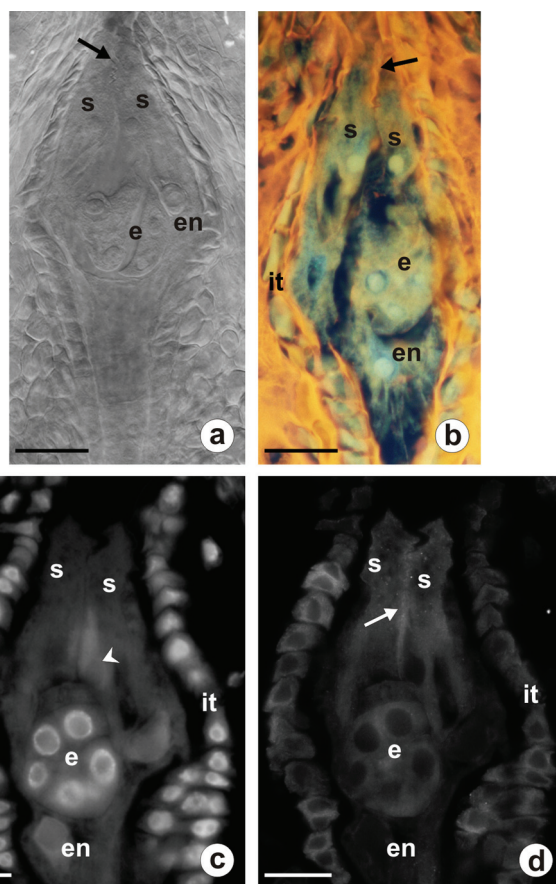

Fig. 4. Female gametophytes of apomictic *Taraxacum* species from closed flowers just before anthesis. (**a**) *T. alatum*. Embryo sac containing globular embryo and endosperm, note presence of two synergids still functioning, arrow indicates zigzag-shaped wall separating synergid cells; image obtained from unstained cleared material using Nomarski DIC optics, (**b**) Embryo sac of *T. alatum* after PAS reaction. Embryo at globular stage, endosperm and two persisting synergids visible, arrow shows PAS-positive orange-stained FA, (**c–d**) Embryo sac of *T. udum* with globular embryo surrounded by endosperm and two synergids: (**c**) DAPI staining shows flattened synergid nuclei (arrowhead), (**d**) Arrow points to weak spot fluorescence in synergids after immunocytochemical reaction with antitubulin. e – embryo; en – endosperm; it – integumentary tapetum; s – synergid cell. Bar in (a, b) = 25 μm; in (c, d) = 20 μm.

part of the embryo sac (Fig. 2a). In the studied *Taraxacum* species the egg cell and synergids are pear-shaped cells with distinct polarity. The chalazal poles of the synergids are highly vacuolated and the nuclei are in the micropylar region where most of the cytoplasm is located (Figs. 2a,c, 3a,c,e), while the egg cell has a chalazally situated nucleus and the micropylar region is filled by a large vacuole (Fig. 3e).

In *T. linearisquameum* the synergid cell wall at the micropylar end is markedly thickened and forms a filiform apparatus (Fig. 2a). In this species the synergids showed intensive fluorescence after immunolabelling. A high concentration of tubulin was disclosed mostly at the micropylar pole. At the tip of the cells a dense network of microtubules was visible; below the tip the tubulin formed bundles more or less parallel to the longitudinal synergid axis (Fig. 2d). Mature megagametophytes of *T. linearisquameum* were penetrated by the pollen tube through one of the synergids. At anthesis, at the micropylar pole of the embryo sac we most often observed only one typically polarized synergid and remnants of a pollen tube which destroyed the second synergid cell in penetrating the female germ unit (Fig. 2b). In the central cell of some embryo sacs a sperm nucleus was visible close to the secondary nucleus (Fig. 2b). These observations confirm that seed formation in diploid *T. linearisquameum* initiates after pollination and double fertilization.

In the autonomous apomicts *T. alatum* and *T. udum* a thickened wall of irregular outline was also clearly seen at the micropylar contact site of two synergid cells (Fig. 3a) but the configuration of the synergids' microtubular cytoskeleton differed from that observed in synergid cells of the diploid sexually reproducing dandelion. In apomictic species the synergid cytoskeleton was relatively poor and observed mainly at the micropylar end of the cells (Fig. 3b,d). Spot fluorescence was mostly visible in the periphery of the synergids, indicating a circular distribution of the cortical microtubules. No characteristic brush-like arrangement of microtubules was found in the synergids of any of the analyzed embryo sacs of *T. alatum* and *T. udum*. The cytoskeleton was also scanty in the egg cells of the studied diplosporous megagametophytes. Aggregations of a few microtubules were detected mainly in the chalazal region of the egg cell, especially in the part of cytoplasm close to the cell wall (Fig. 3f), whereas DAPI staining showed the presence of numerous organelles in the cytoplasm surrounding the egg nucleus (Fig. 3e). In *T. alatum* and *T. udum* we found that the embryo and the endosperm begin to develop just before anthesis in the closed capitula, and that the seeds form without the participation of pollen. At the micropylar pole of embryo sacs containing a globular embryo and cellular endosperm, the presence of two intact synergids was significant (Fig. 4 a–d). In embryo sacs analyzed at this developmental stage the synergids showed no signs of degeneration. They retained their typical shape and polarity and nuclei were distinctly visible in the cells (Fig. 4a, b). In some embryo sacs, however, the immunolabelling of tubulin was very weak in synergid cells and their nuclei were flattened and showed low fluorescence after DAPI staining (Fig. 4c, d). Most likely these are signs of initiation of synergid degeneration. Presumably the synergids maintained their physiological activity up to the globular embryo stage; possibly they play a role in nutrition of the embryo sac and the developing embryo.

Our observations revealed some differences between sexual and apomictic species within the same genus in the configuration of the microtubular cytoskeleton in synergids. The simpler arrangement of the cytoskeleton in the synergids of *T. alatum* and *T. udum* apparently is connected with the apomictic mode of reproduction, that is, parthenogenesis and autonomous endosperm formation.

DISCUSSION

Apomixis can be considered as spatial and temporal deregulation of the sexual pathway, directed by critical epigenetic mechanisms (for review see Koltunow and Grossniklaus, 2003; Rodrigues and Koltunow, 2005; Grimanelli, 2012; Rodriguez-Leal and Vielle-Calzada, 2012). The ovule is the site of fundamental processes relevant to sexual and apomictic reproduction. Although the same embryological structures are involved in the seed production of apomicts and sexual plants, the structural details of the egg apparatus in autonomous apomicts remain open territory for researchers, especially with regard to synergids. Most angiosperms have two synergids, which are sister cells conserved in their structural and physiological features and involved in attraction and reception of the pollen tube (reviewed in Higashiyama, 2002; Punwani and Drews, 2008; Li et al., 2009). A unique structure of synergids is the presence of the filiform apparatus, whose morphology varies widely among species but whose organization appears to be similar within family (Willemse and van Went, 1984; Huang and Russel 1992). Although the FA most often takes the form of a thickened and elaborate cell wall with numerous finger-like projections, within the family Asteraceae it was frequently observed as only wall thickening, for example in *Helianthus annuus* (Newcomb 1973). In the investigated apomictic *T. alatum* and *T. udum* as well as in amphimictic *T. linearisquameum* the micropylar part of the synergid wall is thickened, visible as a zigzag-shaped structure. However, the synergids of apomictic dandelions exhibited a poorly developed microtubular cytoskeleton having quite a different arrangement than the one observed in synergids of amphimictic *T. linearisquameum*. Only a few studies of the configuration of the embryo sac cytoskeleton have been made. These investigations of species including *Gasteria verrucosa* (Willemse

and van Lammeren, 1988), *Nicotiana tabacum* (Huang and Russell, 1994), *Zea mays* (Huang and Sheridan, 1994) and *Arabidopsis thaliana* (Webb and Gunning, 1994) have revealed that the microtubules in synergid cells usually are longitudinally oriented in bundles that form brush-like structures often adjacent to the FA. In *T. linearisquameum* synergids the microtubule arrangement resembled that described in the above-mentioned amphimictic species. On the other hand, the configuration of the scanty microtubular cytoskeleton in the synergids of autonomous apomicts *T. alatum* and *T. udum* was similar to that previously described in the synergids of the obligatory apomict *Chondrilla juncea* (Kościńska-Pająk and Bednara, 2006). In these apomictic species the microtubules were cortically distributed in the synergid cytoplasm and did not form longitudinal bundles. Our present observations, revealing differences between the microtubular cytoskeleton of synergids in sexual and apomictic species within the same genus, seem to support an earlier suggestion (Kościńska-Pająk and Bednara, 2006) that the configuration of the cytoskeletal elements of synergids may be related to the mode of reproduction.

The role of the synergid cells remains an open question in species whose embryo and endosperm development is independent of any contribution from pollen, but there has been some progress recently in, for example, *Brachiaria brizantha*, where synergids were found to be involved in autonomous embryo development (Alves et al., 2007; Silveira et al., 2012). In this species, molecular analysis of the temporal and spatial expression of certain cDNA sequences by *in situ* hybridization in ovaries of apomictic and sexual plants indicated that synergids may play a crucial role not only at the moment of fertilization but also in the autonomous development of an unreduced egg cell. Our observations of long-persisting synergids in the embryo sacs of apomictic dandelions call for detailed research aimed at clarifying the possible involvement of these cells in transport of nutrients.

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