

THE DEVELOPMENT OF THE FEMALE GAMETOPHYTE IN *FRAGARIA* × *ANANASSA* DUCH. CV. SELVA

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Megasporogenesis and female gametophyte development were investigated in ovules of the everbearing strawberry *Fragaria* × *ananassa* Duch. cv. Selva. Observations of thin sections revealed that ovule development starts from the formation of a nucellus and coincides in time with the beginning of receptacle overgrowth. The most characteristic feature during nucellus differentiation is the formation of a multicellular archesporium, beginning from at least two cells. Analysis of female gametophyte development indicated that in addition to the meiotic mode, female gametophytes develop by an apomeiotic mode of Antennaria type. Asynchronous development of female gametophytes of different origin occurs. The mature, eight-nucleate, seven-celled female gametophyte of meiotic origin is cylindrical and slightly curved. It occupies the central part of the nucellus. The egg apparatus, consisting of an egg cell and two synergids, is formed in the micropylar part of the female gametophyte; the opposite chalazal pole is occupied by antipodal cells. Besides the ovule in which only one seven-celled female gametophyte finally develops, ovules with a different number of cells were observed to initiate female gametophyte development. Some ovules contain a nucellus with a tetrad of linearly arranged megaspores surrounded by enlarged cells, each of which has the potential to develop into an apomictic female gametophyte. After degeneration of some post-meiotic cells or developmentally advanced female gametophytes, some of the chalazal cells initiated female gametophyte development.

Key words: *Fragaria* × *ananassa*, megasporogenesis, multicellular archesporium, female gametophyte, ovule, apomixis.

INTRODUCTION

Strawberry (*Fragaria* × *ananassa* Duch.) (family Rosaceae, subfamily Rosoideae) is an important cultivated species. In Poland it is also of great economic importance and is second only to apples in volume of fruit production. Poland ranks fourth among the world's strawberry producers, hence the demand for improved varieties.

Strawberry is a hybrid between two wild American species, *Fragaria virginiana* and *F. chiloensis* (Darrow, 1966). Both species are natural auto-octoploids with 56 chromosomes. *Fragaria* × *ananassa* Duchesne is believed to be the product of spontaneous crossing between the two species in Europe in the middle of the 18th century. As a result, *F.* × *ananassa* has the very complex genetic structure of an autoallooctoploid species, with a genome consisting of 56 chromosomes ($2n=8x=56$)

coming from both parents in equal numbers (Darrow, 1966). Almost all contemporary varieties probably are direct descendants of one hybrid plant obtained in Europe about 250 years ago (Staudt, 1961). The genetic variation among modern varieties comes from segregation and crossover events within a small gene pool, with a relatively small component resulting from mutations accumulated over the last two and a half centuries. Some cultivars are obtained by backcrossing with one of the wild ancestors (Marta et al., 2004; Luby et al., 2008).

Strawberry flowers have a structure characteristic for Rosaceae. The typical flower has five parts, although mutants with more elements have been reported. The majority of plants have perfect flowers, but imperfect (pistillate) forms exist and are important in breeding. The pistils are arranged in a regular spiral on the apical portion of the receptacle. The ovary of each monocarpelate pistil develops into

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a true fruit – a small achene located on the surface of the overgrown receptacle (Darrow, 1966). Each achene contains one seed (Winton, 1902; Davis et al., 2007). The strawberry exhibits open pollination. Self-incompatibility mechanisms have not been reported in the octoploid species of *Fragaria* (Davis et al., 2007), but seedlings obtained from self-pollination are fragile as a consequence of inbreeding. Strawberry varieties are propagated in horticultural production vegetatively by rooting young plants developing in the nodes of runners. Strawberry breeding is still based on intra-specific crossing and selecting the best genotypes of the offspring. For this reason, studies of its sexual reproduction and embryology are important as basic research and for breeders. Proper formation of fruits of commercial strawberry cultivars increases the economic benefits of crop production. Among many factors, undisturbed development of functional achenes definitely plays a role in the growth of a false fleshy fruit consisting mainly of an enlarged floral receptacle (Mudge et al., 1981; Strik and Proctor, 1988). Arriza et al. (2011) suggested that nonfunctional achenes might result from disruption of post-fertilization processes, such as embryo abortion. Processes occurring during ovule development, especially megasporogenesis and megagametogenesis, presumably are critical periods for the development of functional achenes.

Despite the numerous investigations of the biochemical or physical properties of strawberry fruits and tissue culture methods for obtaining improved cultivars, many issues are still unresolved, especially those related to processes involved in sexual reproduction of this plant. Investigations concerning the formation of the female gametophytes in strawberry species are limited to a few cultivars. In this work we studied the steps of female gametophyte formation during consecutive stages of ovule development in *F. × ananassa* cv. Selva. The presented findings focus mainly on megasporogenesis and female gametophyte development, the critical transitional periods between sporophytic and gametophytic generations and probably the most crucial stage of seed development.

MATERIALS AND METHODS

We used strawberry (*F. × ananassa* Duch.) cv. Selva (everbearing cultivar) plants grown in the Botanical Garden of Maria Curie-Skłodowska University in Lublin (Poland). Flower buds were collected at different developmental stages from plants grown in an experimental field and immediately fixed in Navashin fixative (formalin, acetic acid, chromic acid; 3:3:0.5) at room temperature for 48 h. Fixed flower buds were dehydrated in a graded ethanol

series (10% to 96%) for 15 min at each concentration and immersed in absolute ethanol 3 times for 15 min each. The plant material was then infiltrated in solutions of absolute ethanol and benzene (3:1, 1:1, 1:3; 20 min each) and embedded in paraffin at 59°C. For light microscopy, paraffin samples were sectioned 8 µm thick with a rotary microtome and stained with aqueous solutions of 1% safranin O (Sigma-Aldrich Cat. No. S8884) and ethanol solutions of 0.5% light green.

For localization of insoluble polysaccharides, sections were stained by the PAS reaction (Schiff's reagent, periodic acid) according to Pearse (1972). For callose detection, samples were examined by fluorescence microscopy (UV 330–360 nm) after staining with 0.05% aqueous solution of aniline blue (Clark, 1981).

Stages of megasporogenesis and female gametophyte were observed with a Nikon Optiphot II optical microscope. Micrographs were taken with a Nikon Coolpix 4500 digital camera. Only the most characteristic photographs were chosen for documentation.

RESULTS

Initial development of *Fragaria* × *ananassa* ovules on the placenta coincides with the beginning of receptacle overgrowth. Ovule development starts from formation of the nucellus, which derives from both the epidermal and subepidermal layers. Each layer forms segments consisting of cells lying in rows and forming a multilayered nucellus typical for the crassinucellate ovule. Formation of the archesporium begins very often from at least two cells, which are much larger than the other nucellus cells (Figs. 1, 2). After division along the long axis in each of the initial archesporial cells, the number of archesporial cells increases and a multicellular archesporium is thus formed. Archesporial cells participate in the formation of the nucellus via periclinal, asymmetric divisions in the micropylar region. Two or three layers of parietal tissue are formed during the early stages of nucellus development. The archesporial cells are surrounded by the elongated, lens-shaped cells of the nucellus.

The first meiotic division results in the formation of a dyad, the two cells of which may be similar or considerably differ in size (Fig. 3). Both dyad cells may be accompanied by strongly elongated cells (markedly larger than other nucellus cells) adhering tightly to the dyad. The prophase I megasporocyte wall contains a very small amount of callose. At the dyad stage, the callose in the wall separating the two cells shows strong fluorescence when the callose-containing cell walls thicken. In the other walls of the dyad the occurrence of callose is limited to a few,

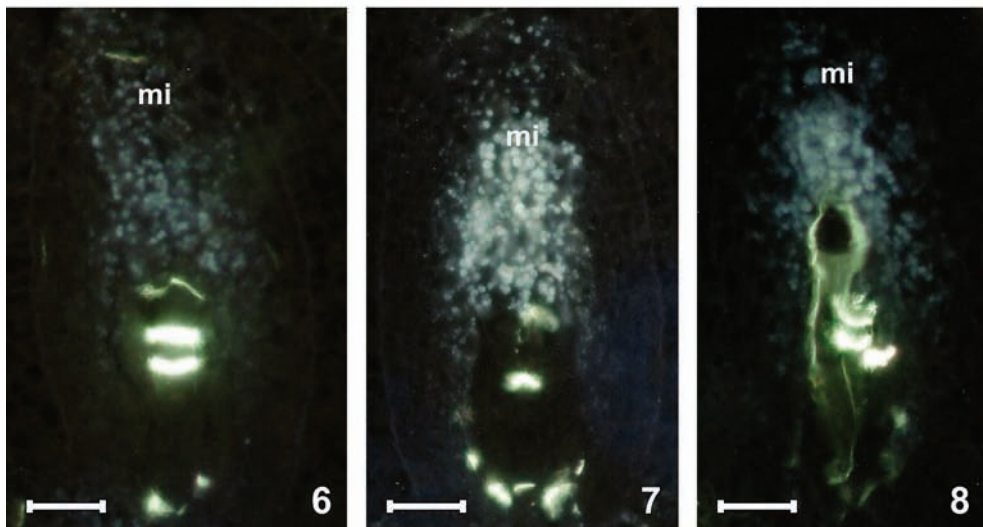
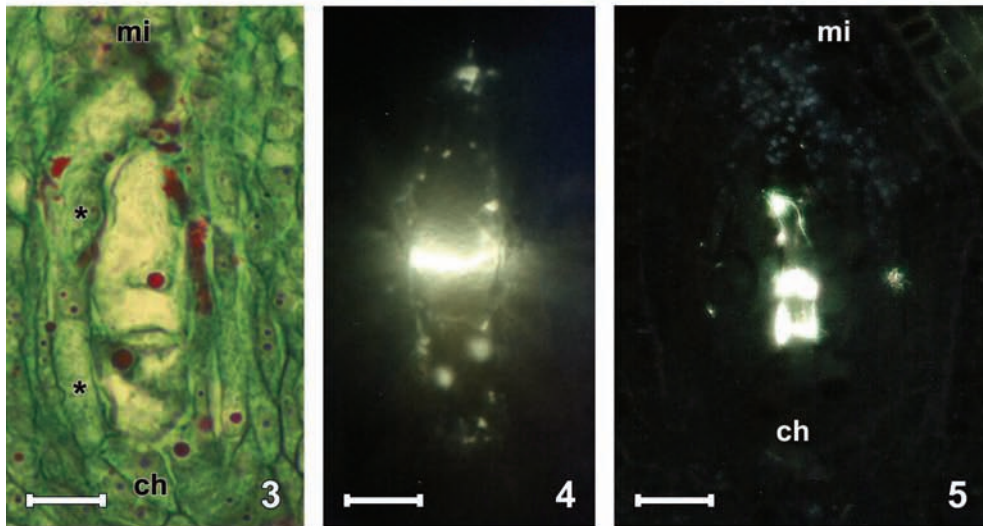
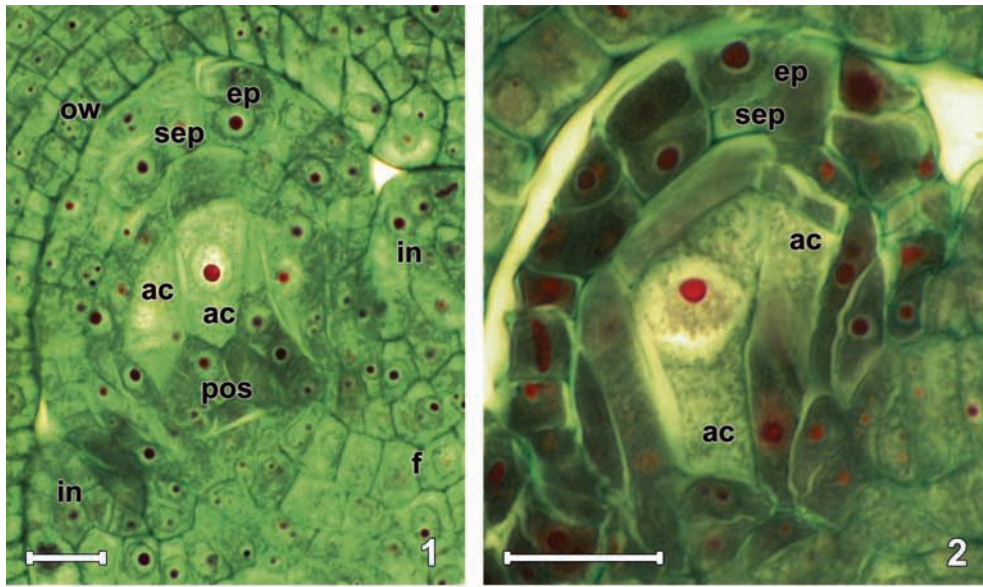
different-sized, irregularly distributed dot-like deposits (Fig. 4). Strong fluorescence of callose is visible in both thick transverse walls of the triad. Additionally, at the triad stage, callose is present at the tip of the micropylar triad cell (Fig. 5). The cells of the micropylar part of the nucellus contain amyloplasts with starch grains, which exhibit autofluorescence. At the megaspore tetrad stage, small deposits of callose occur in the chalazal megaspore (Fig. 6). The quantity of such callose deposits increases at the tetrad stage at the same time that the amount of autofluorescing starch grains increases in the region of the nucellus where the pollen tube will grow (Fig. 7). The developmental processes taking place in the ovule can run asynchronously. A dyad, a triad of megaspores and a female gametophyte were found inside one ovule (Fig. 8).

Usually the active megaspore elongates and becomes highly vacuolated: it clearly begins to differentiate into the female gametophyte. The oval nucleus of the megaspore is surrounded with cell organelles. Amyloplasts with large starch grains are scattered in the nucellar tissue adjacent to the megaspore (Fig. 9). After karyokinesis, a two-nucleate female gametophyte is formed, with nuclei located at the chalazal and micropylar poles and separated by a large vacuole (Fig. 10). In this case, the third micropylar megaspore developed a two-nucleate female gametophyte. The other megaspores in the nucellus may exhibit female gametophyte development: the terminal, chalazal megaspore beneath the two-nucleate female gametophyte often possesses a large nucleus and nucleolus. The oval and highly vacuolated two-nucleate female gametophyte is surrounded by flattened, elongated nucellus cells. The absence of remnants of degenerated megaspores near the female gametophyte points to the apomictic rather than post-meiotic origin of this structure (Fig. 11). In one nucellus, besides the two-nucleate female gametophyte developed from the

chalazal megaspore, two adjacent nucellus cells initiated female gametophyte development (Fig. 12). Karyokinesis in the two-nucleate stage results in the formation of a four-nucleate female gametophyte. Growth of the four-nucleate stage is accompanied by disintegration of adjacent nucellus tissue and defunct megaspores (Figs. 13, 14). The micropylar pole of the four-nucleate stage is surrounded by cells filled with amyloplasts. The mature, eight-nucleate, seven-celled female gametophyte is cylindrical and slightly curved. It occupies the central part of the nucellus. An egg apparatus consisting of an egg cell and two synergids (Fig. 15) is formed in the micropylar part of the female gametophyte, while the opposite chalazal pole is occupied by antipodal cells (Fig. 16). An adjacent, partially developed female gametophyte may occur in the chalazal region of the ovule.

In nucelli in each of which one female gametophyte finally developed, various aberrations were observed. Some of the nucelli contained linear triads in the central region, accompanied by atypically numerous, enlarged and vacuolated cells, which potentially could develop into apomictic female gametophytes (Fig. 17). The cells in the center of the nucellus differed significantly in structure from the surrounding small, flattened and elongated cells. The enlarged cells of the nucellus contained dense cytoplasm and large nuclei with large nucleoli as well as a varying number of vacuoles. The number of cells developing into female gametophytes varied. Sometimes only a few cells show clear features of development into female gametophytes (Fig. 18). After degeneration of post-meiotic and nucellar cells located in the micropylar part of the nucellus, some of the chalazal cells initiated female gametophyte development (Fig. 19). Some of the aposporic female gametophytes reached the two-nucleate stage in the chalazal region of the ovule (Fig. 20). These two-nucleate female gametophytes showed, atypically,

Figs. 1–8. Megasporogenesis in *Fragaria × ananassa* cv. Selva. Longitudinal sections. Figs. 1–3. Staining with light green and safranin. Figs. 4–8. Staining with aniline blue. Bars = 15 µm. **Fig. 1.** Longitudinal section of crassinucellar ovule in early stage of development. Nucellus composed of epidermal (ep) and subepidermal (sep) layers. Two archesporial cells (ac) are larger than the other nucellus cells. Note postament cells (pos) and inner integument (in) at base of forming nucellus. ow – ovary wall; f – funiculus. **Fig. 2.** Nucellus with two archesporial cells (ac) derived by longitudinal division of the original archesporial cell. Elongated, lens-shaped cells of nucellus surround archesporial cells. ep – epidermal layer; sep – subepidermal layer. **Fig. 3.** Dyad. Both cells show high vacuolization, and the micropylar cell is larger than the chalazal one. Note elongated, enlarged cells adjacent to dyad cells (asterisked). mi – micropylar pole; ch – chalaza. **Fig. 4.** Fluorescence of callose in dyad walls. Wall separating dyad cells shows strong fluorescence, while the other walls show irregularly distributed dot-like callose deposits of different sizes. **Fig. 5.** Triad with strong fluorescence of callose in two transversal walls and at tip of micropylar megaspore. Note autofluorescence of starch grains in micropylar part of nucellus. mi – micropylar pole; ch – chalaza. **Fig. 6.** Tetrad of megaspores with callose fluorescence. Chalazal megaspore with modest quantity of callose deposits at its chalazal tip. Note thinner callose wall between central and micropylar megaspores. mi – micropylar pole. **Fig. 7.** Tetrad of megaspores with enlarged chalazal megaspore with some callose deposits in chalazal part of nucellus. Intensive autofluorescence of starch grains visible along the possible route of pollen tube growth. mi – micropylar pole. **Fig. 8.** Fluorescence of callose in transverse walls of dyad and triad and in wall of developing female gametophyte. mi – micropylar pole.



several vacuoles instead of one large central vacuole separating two nuclei. In contrast to the degenerated post-meiotic cells visible in different sections in the same nucellus, apomictic female gametophytes potentially would become mature female gametophytes. Degeneration at the four-nucleate stage also occurs (Fig. 21). In such a case, neighboring enlarged nucellus cells undergo differentiation as female gametophytes. In most cases, development is asynchronous. On the same plane, three developmentally advanced female gametophytes were observed rather sporadically (Fig. 22). Two of the three were separated by a transverse wall, indicating their origin from two megaspores.

DISCUSSION

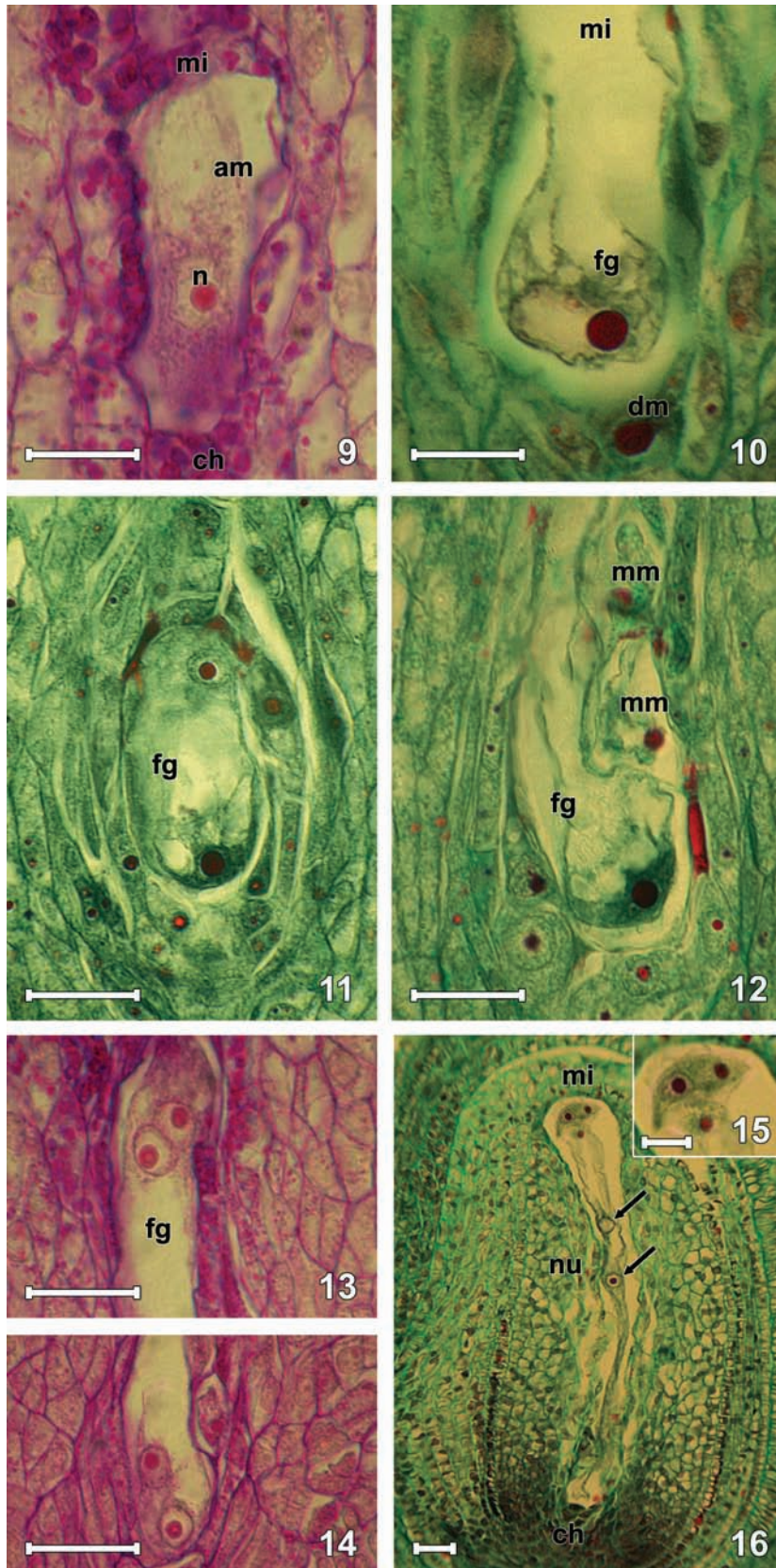
This investigation showed that the most characteristic feature of nucellar differentiation in *Fragaria* × *ananassa* cv. Selva is the formation of a multicellular archesporium. This kind of archesporium usually originates from two or more archesporial cells differentiated from centrally situated nucellus cells, and has been reported in several *Fragaria* species (Konstantinov, 1966; Niemirowicz-Szczytt, 1984; Kunz and Gröber, 1988; Baturin, 1997, 2009). Multicellular archesporia were found in *Fragaria* × *ananassa* varieties such as Roscinskaja, Rubinovaja (Ruby) and Negritenok (Pickaninny) (Konstantinov, 1966). According to those authors, the tendency to develop supplementary female gametophytes varies in different strawberry varieties, being most apparent in those showing higher yields. In *Potentilla* (Rutishauser, 1948) and in *Rubus* (Christen, 1950), two other genera of Rosaceae, the multicellular archesporium is formed by two divisions of a single primary archesporium to produce a varying number of secondary archesporial cells. Archesporium formation in *F.* × *ananassa* cv. Selva is similar to that described for *F.* × *ananassa* cv. Mieke Schindler and the wild species *F. moschata* (Kunz and Gröber, 1988).

The data we obtained support, in part, Kunz and Gröber's (1988) suggestion that the female gametophytes originate by different modes. They emphasized that female gametophytes in ovules in *F. ananassa* cv. Mieke Schindler and in the wild species *F. moschata* differed either in origin or in developmental processes. Our observations of the successive stages of development indicate that the female gametophytes in *F.* × *ananassa* cv. Selva may develop from megaspores or by Antennaria-type apomixis, referred to as mitotic diplospory. In this type, the megasporocyte divides three times without meiosis and produces an unreduced female gametophyte. One example of this development pattern is the two-nucleate stage shown in Figure 11.

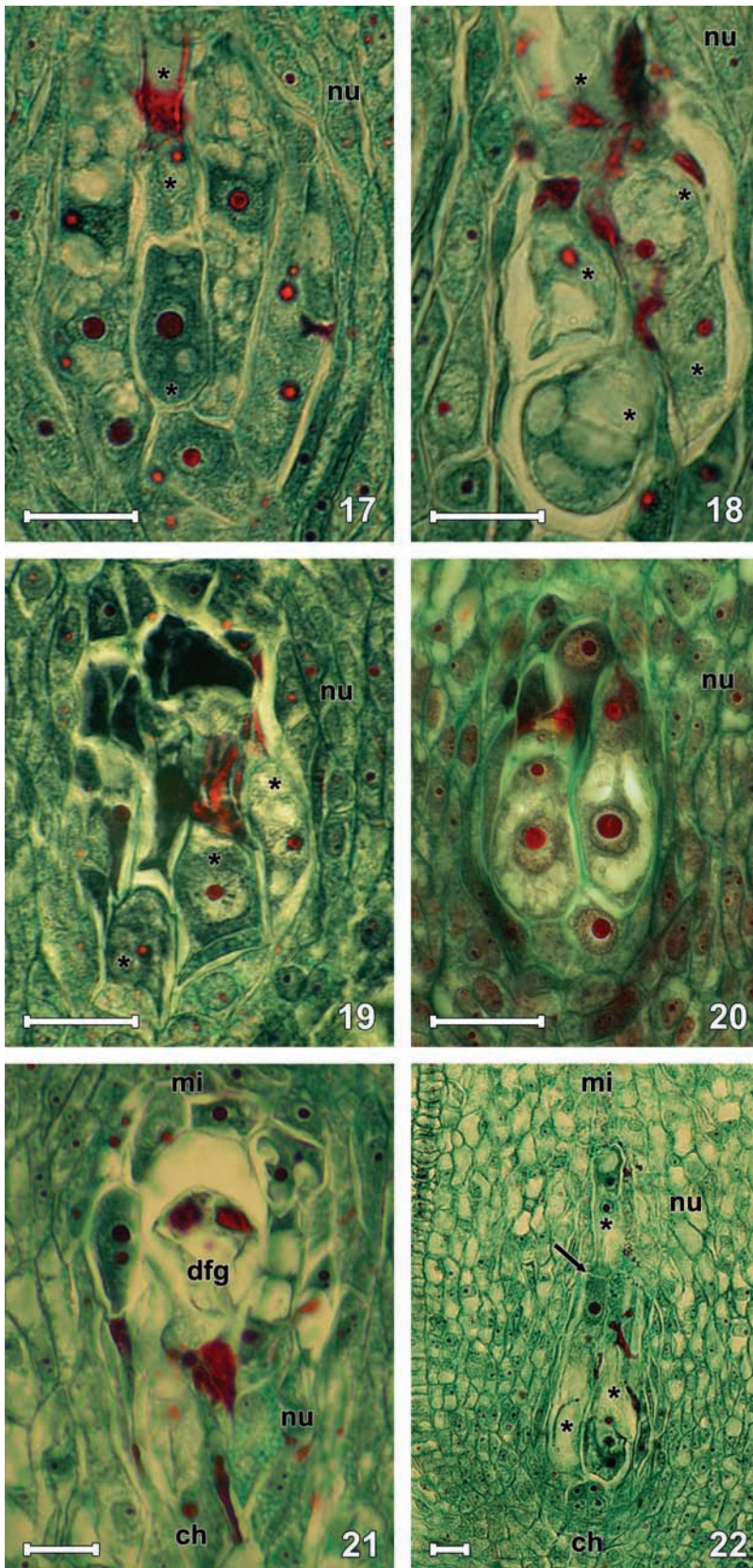
Ultrastructural changes during the process of diplospory were described in *Poa nemoralis* and *Poa palustris* (Naumova et al., 1999). Despite changes in metabolic activity, the transformation of the megasporocyte to produce a one-nucleate diplosporous female gametophyte and its division to produce a two-nucleate stage was accompanied by an increase in cell size and reorganization of the nuclei and cytoplasm. A prominent feature of such a transformation was thickening of the cell wall, with the final effect of cell isolation.

In female gametophyte development following the *Taraxacum* type as described for strawberry by Kunz and Gröber (1988), meiotic prophase is initiated but then interrupted so that two unreduced dyad cells are formed. A mitotic division in one of them gives rise to the female gametophyte. Similar events were also described for *F. moschata* and *F.* × *ananassa* cv. Mieke Schindler (Kunz and Gröber, 1988). In the ovules of both species the chalazal cell of the dyad initiates female gametophyte development. In *F.* × *ananassa* cv. Selva we did not observe this type of female gametophyte development. We found a size difference in the cells of the dyad, resulting from the first meiotic division. The larger dyad cell, most often the chalazal member, develops into a female gametophyte. In our observations, a female gametophyte was also formed from the micropylar cell of the dyad. Adjacent to the dyad, markedly enlarged cells may also initiate female gametophyte development.

This study of the strawberry cultivar Selva showed that female gametophytes may develop in either meiotic or apomeiotic modes, and that female gametophytes of different origin may develop asynchronously. Similar observations have been reported in different *Fragaria* species (Kunz and Gröber, 1988; Caranta et al., 1996). Other recent research may raise some doubt as to whether apomixis truly exists in *Fragaria*, and the matter should therefore be treated cautiously. According to Nosrati et al. (2010), a RAPD study of matroclinal progeny from experimental crosses in the genus *Fragaria* showed no evidence for apomixis. However, asexual reproduction of megagametophytes, described as gametophytic apomixis, occurs in plants belonging to Rosaceae. This type of apomixis was investigated in different flowering plants as several variant mechanisms (Talent, 2009). In Rosaceae, both diplospory and apospory have been noted in the same species. Both diplospory and apospory have been described even in the same ovule (Czapik, 1996; Jankun and Kovanda, 1988; Nybom, 1988; Nygren, 1967; Koltunow and Grosniklaus, 2003; Savidan, 2000). Polyembryony was observed in different *Fragaria* × *ananassa* cultivars. According to Caranta et al. (1996), additional apomictic embryos may arise from cells of an unreduced female gametophyte. To



Figs. 9–16. Developing female gametophytes of *Fragaria* × *ananassa* cv. Selva. Longitudinal sections stained by PAS reaction (Figs. 9, 13, 14) and with light green and safranin (Figs. 10 – 12, 15, 16). Bars = 15 μ m in Figs. 9–15, 30 μ m in Fig. 16. **Fig. 9.** Enlarged and highly vacuolated active megaspore with starch grains gathered around nucleus. Amyloplasts with large starch grains scattered around active megaspore. am – active megaspore; n – nucleus; mi – micropylar pole; ch – chalaza. **Fig. 10.** Chalazal fragment of two-nucleate female gametophyte (fg). Note squamous shape of cell with large nucleus and nucleolus adjacent to chalazal pole of female gametophyte. mi – micropylar pole; dm – degenerating megaspore. **Fig. 11.** Two-nucleate, oval female gametophyte (fg) with nuclei separated by central vacuole. Elongated, flattened cells surround female gametophyte. **Fig. 12.** Fragment of two-nucleate female gametophyte (fg) formed from chalazal megaspore, and two considerably smaller nucellar cells at early stage of female gametophyte development (mm). **Figs. 13, 14.** Four-nucleate female gametophyte with pair of nuclei at micropylar and chalazal poles. Micropylar pole of female gametophyte (fg) surrounded by cells filled with amyloplasts. Note degenerated cells at side wall of female gametophyte at left. **Figs. 15, 16.** Mature, cylindrical and slightly curved seven-celled female gametophyte with egg apparatus at micropylar pole (mi) in right top corner (Fig. 15), two polar nuclei (arrows) in central cell (antipodal cells on a different plane are not visible in this section). Oblique section shows fragment of second female gametophyte in chalazal region of ovule. nu – nucellus.



Figs. 17–22. Central fragments of *Fragaria × ananassa* cv. Selva nucelli. Longitudinal sections stained with light green and safranin. Bars = 15 µm. **Fig. 17.** Central part of nucellus with linearly arranged triad (each cell asterisked) surrounded by enlarged, vacuolated nucellus cells. nu – nucellus. **Fig. 18.** Several enlarged nucellus cells (asterisked) beginning to differentiate into female gametophytes. nu – nucellus. **Fig. 19.** Three enlarged nucellus cells (asterisked) differentiated into female gametophytes at chalazal pole of nucellus. Remnants of degenerated postmeiotic cells visible above developing nucellus cells. nu – nucellus. **Fig. 20.** Aposporic, two-nucleate female gametophytes (with small vacuoles) developing in chalazal region of ovule. Remnants of degenerated postmeiotic cells visible in different sections. nu – nucellus. **Fig. 21.** Micropylar part of degenerated, postmeiotic, four-nucleate female gametophyte. Enlarged nucellus cells adjacent to female gametophyte undergoing early stage of differentiation. nu – nucellus; ch – chalaza; mi – micropylar pole; des – degenerating female gametophyte. **Fig. 22.** Fragments of three developing female gametophytes (asterisked) on the same plane. Note cell wall between two female gametophytes (arrow). nu – nucellus; ch – chalaza; mi – micropylar pole.

verify those hypotheses, investigators should use molecular markers to determine the exact origin of strawberry embryos, whether meiotic or apomictic. In recent years advances have been made in understanding the molecular mechanisms controlling apomixis (Rodrigues and Koltunow, 2005). Asexual seed development by apomixis may be an important tool in breeding programs (Szkutnik, 2010).

During female gametophyte development we observed various deviations from the series of events described above. Some ovules contained a nucellus with a tetrad of linearly arranged megasporocytes surrounded by enlarged cells, which had the potential to develop into apomictic female gametophytes. In one ovule there were different numbers of cells which developed into female gametophytes. After degeneration of some post-meiotic cells or developmentally advanced female gametophytes, some of the chalazal cells initiated female gametophyte development.

Our results from aniline blue staining clearly showed that the megasporocytes underwent meiosis. During megasporogenesis in *F.* × *ananassa* cv. Selva, callose appears at the beginning of this process. Callose occurs in a very small quantity in the wall of the prophase I megasporocyte, and larger amounts in the wall between the dyad cells. As a rule, in most angiosperms this β -1,3-glucan is detected in the wall of the prophase I megasporocyte (Rodbkiewicz, 1970; Rodbkiewicz and Kuran, 1971; Noher de Halac and Harte, 1975; Williams et al., 1984; Śniezko and Harte, 1984; Abramova et al., 2003). However, in the andromonoecious shrub *Caesalpinia gilliesii* callose does not occur around the megasporocyte but is deposited in the walls of nucellar cells at the chalazal pole of the ovule (Calvino and Carrizo Garcia, 2009). Bicknell and Koltunow (2004) emphasized that callose deposition during gametophyte development differs between apomictic and sexually reproduced plants, a difference that can sometimes be useful as a marker for the presence or absence of apomixis. In *F.* × *ananassa* cv. Selva, the walls at the later stages of megasporocyte division and early stages of female gametophyte development contain irregularly distributed deposits of callose.

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