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CALCIUM ION PRESENCE AS A TRAIT OF RECEPTIVITY IN TENUINUCELLAR OVULES OF *GALANTHUS NIVALIS* L.

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In *Galanthus nivalis* during the progamic phase, both the embryo sac and somatic cells of the ovule change their ultrastructure and physiology, as observed by light, fluorescence, and electron microscopy. Fresh ovules from buds, opening flowers, and from cross-pollinated flowers were stained in toto to detect pectins, acidic polysaccharides, proteins, lipids, callose, free calcium ions and membrane-bound calcium. These substances were found only in the micropylar part of fertile ovules. All stainings were negative in sterile ovules. In EM, the somatic cells in the micropylar part of the ovule were observed to develop secretion activity. Their exudates passed to the intercellular spaces, mainly to the micropylar canal. The amount of the exudate increased after pollination. Free or loosely bound calcium ions were present in extracellular regions of the micropylar part of fertile ovules. The substances detected in the micropylar exudate of fertile ovules are suggested to support and direct pollen tube growth to the embryo sac.

Key words: *Galanthus nivalis*, ovule, micropyle, exudates, pectins, calcium.

INTRODUCTION

Ovule receptivity is crucial to fertilization and fertility in flowering plants. At the last step of pollen-pistil interaction a number of supporting mechanisms have been suggested, but still relatively little is known about the biochemical and molecular background of this process. It is generally agreed that pollen tube penetration into the micropyle of an ovule is genetically controlled, as demonstrated by research on female sterile plant mutants (Hülskamp et al., 1995; Wilhelmi and Preuss, 1996).

The behavior of the pollen tube inside the ovary differs significantly among plant species, but many authors have suggested that an attracting signal is sent from the micropyle of a fertile, receptive ovule (Mulcahy and Mulcahy, 1985; Śnieżko, 1996, 1997; Chudzik and Śnieżko, 1999a,b; Herrero, 2000). The question is whether the differences in pollen tube behavior are related to ovule receptivity and whether this receptivity is actively demonstrated or signalled. The presence of exudates in the micropylar canal has been described as a sign of ovule maturity in a number of unrelated species (Chao, 1971; Tilton, 1979; Arbeloa and Herrero, 1991; Franssen-Verheijen and Willemse, 1993; Chudzik and Śnieżko, 1999a,b; Herrero, 2000). The origin, composition and amount of micropylar substances differ among the investigated plants, and their identities and functional roles remain to be determined. Some adhesion molecules are supposed to play a key role in pollen tube guidance (Sanders and Lord, 1989; Wilhelmi and Preuss, 1996; Jauh et al., 1997; Mollet et al., 2000). The presence of free calcium ions on the pathway of pollen tube growth is another significant factor for pollen tube-pistil interactions (Bednarska and Butowt, 1995; Reger et al., 1992; Lenartowska et al., 1997).

We studied the development of *Galanthus nivalis* ovules, especially during the progamic phase, to determine the phenomena that can influence interaction with the pollen tube. *G. nivalis* seemed a good model for such work, in view of the biology of its flowering, described in our previous paper (Chudzik et al., 2002).

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MATERIALS AND METHODS

PLANT MATERIAL

Plants of *Galanthus nivalis* were grown in the Botanical Garden of Maria Curie-Skłodowska University in Lublin. Ovules were isolated at four developmental stages: (1) at the stage of the fully formed embryo sac in September; (2) at the stage of the fully formed embryo sac from flower buds in February and March; (3) freshly opened flowers in March and April; and (4) 24 and 48 h after cross-pollination.

IN TOTO STAININGS

Pollen tube growth and penetration into the ovules was observed with a fluorescence microscope (Nikon, UV 430 nm) after aniline blue staining (Śnieżko, 1996, 2000). These observations were intended to find the time of the ovules' highest receptivity (Chudzik et al., 2002).

For histochemical reactions, intact ovules with the placenta were isolated from the ovary and immediately stained with one of the following:

1) 0.02% ruthenium red water solution, coloring pectins deep red (Luft, 1971);
2) 1% alcian blue 8GX in 3% acetic acid and 0.05% $MgCl_2$, pH 2.5, detecting acidic polyanions such as acidic polysaccharides (Scott et al., 1964);
3) 1% amido black (naftol blue black) solution in 7% acetic acid, for proteins, according to Fischer (1968);
4) 2% alizarin red S solution adjusted with diluted ammonia to pH 4.2 for free calcium ion deposits, as described by McGee-Russell (1957);
5) Sudan black, for lipid localization (Pearse, 1972). Lipid exudate was also detected as a side effect on specimens observed by fluorescence microscopy, because it absorbed chlorophyll extracted from other tissues and then emitted red light;
6) 10^{-4} chlortetracycline (CTC) solution in distilled water supplemented with 2% saccharose (Reiss and Herth, 1978), to localize membrane-bound calcium. Ovules were stained in toto for 10 min and observed with a fluorescence microscope (Labophot-2, Nikon) equipped with a V-2A excitation filter (EX 380–420 nm, BA 450 nm). Microphotographs were taken on Fuji color ASA 400 film. Two types of control reactions were made, according to Bednarska (1995): (1) samples untreated with CTC; (2) ovules washed for 10 min in 1 mM EGTA and then placed in CTC solution.

The ovules stained in toto were gently squashed, mounted in glycerol and observed with a light microscope (Carl Zeiss, Jenaval). Microphotographs were taken on Kodak color ASA 200 film.

ANATOMICAL STUDIES

Some pistils were prepared according to the routine paraffin method and stained by PAS reaction for anatomical study and starch grain localization (Pears, 1972).

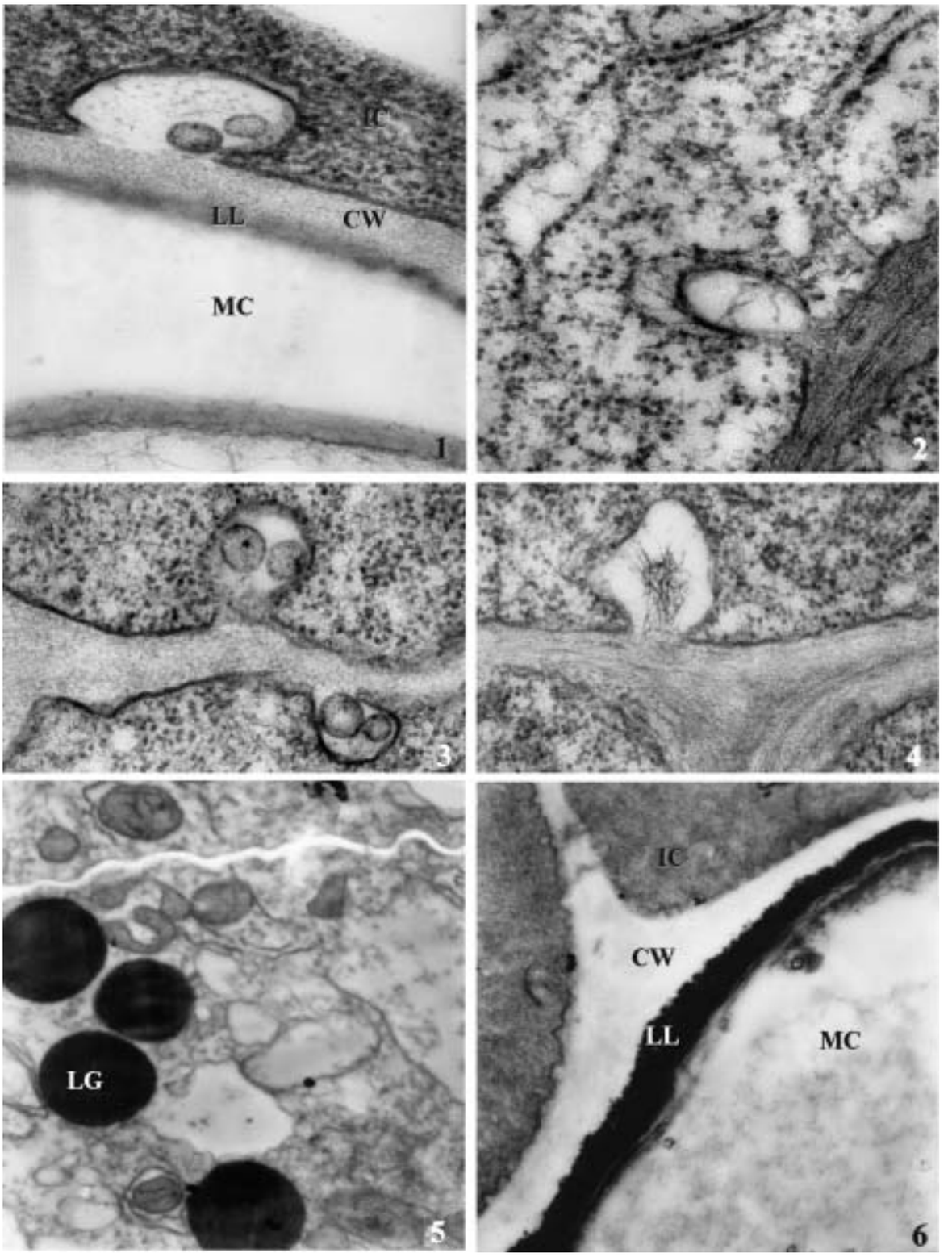
ULTRASTRUCTURAL STUDIES

For transmission electron microscopy, ovules were fixed in 3% glutaraldehyde in cacodylate buffer (pH 7.2) for 4 h at room temperature under vacuum, and then overnight at 4˚C. Then the material was postfixed with buffered 2% osmium tetroxide at 4˚C for 24 h. After rinsing in cacodylate buffer and dehydration, the samples were embedded in Epon 812 resin (Sigma). Ultrathin sections, poststained with uranyl acetate and lead citrate, were observed with a JEM 1200 EX electron microscope. Microphotographs were taken on Foton TN-12 film.

SELECTIVE LOCALIZATION OF CA^{2+} USING POTASSIUM PYROANTIMONATE TREATMENT

Selective localization of Ca^{2+} was performed according to the Wick and Hepler (1982) method, modified by Bednarska and Butowt (1994). Ovules were fixed in 4% glutaraldehyde containing 1% potassium hexahydroxyantimonate (V) [$KSb(OH)_6$] in 0.1 M phosphate buffer, pH 7.4 for 24 h at 4˚C. Samples were rinsed and postfixed in 1% OsO_4. After rinsing in phosphate buffer, the ovules were dehydrated and embedded in LR White acrylic resin and polymerized for 24 h at 60˚C. Ultrathin sections (90 nm) were collected on meshed copper grids. Sections

Fig. 1. Fragment of micropylar canal, not filled with exudate, in ovule from flower bud; a fine layer of lipid substances is on the cell wall surfaces. TEM, × 16 000. **Figs. 2–4**. Exocytotic activity in cells of micropylar part of inner integument in fertile ovules isolated from flower buds, just before anthesis. TEM, × 30 000. **Fig. 5**. Lipid globules in cytoplasm of micropylar part of inner integument in ovules isolated in autumn. TEM, × 14 000. **Fig. 6**. Fragment of micropylar canal, filled with exudate, in fertile ovule isolated from open flower; a thick layer of lipid substances is on the cell wall surfaces. TEM, × 16 000. MC – micropylar canal; LL – lipid layer; CW – cell wall; IC – inner integument cell; LG – lipid globule.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without poststaining were observed with a JEM 1200 EX transmission electron microscope with attached ASID 10 scanning TEM (STEM) at 80 KV. Microphotographs were taken on Foton TN-12 film. The presence of Ca/Sb in the precipitates was investigated by energy-dispersive X-ray microanalysis of 120 nm sections stabilized with evaporated carbon, mounted on a graphite specimen holder. Induced X-rays were collected in 200 s spectra and analyzed with an EDS LINK AN 10,000 dispersive spectrometer interfaced to the TEM. About 30 precipitates randomly selected from each fixation were analyzed.

Additionally, two types of control were made on the sections: some were observed without potassium pyroantimonate treatment, and others were incubated before fixation in 100 mM EGTA (pH 7.8) for 1 h to remove free calcium ions.

RESULTS

Galanthus nivalis L. is a monocot plant with tenuinucellar ovules. Flower development proceeds in two phases, before and after winter, as described by Chudzik et al. (2002). The ovules develop to the stage of embryo sac formation in autumn, but they reach the fully mature stage in the spring after flower opening and pollination. Fertile and sterile ovules are present inside the ovaries. They differ in size and shape, so it was possible to distinguish and compare them in the same conditions.

ANATOMY AND MORPHOLOGY OF OVULES

Ovules of *Galanthus nivalis* are anatropic, bitegmic and tenuinucellar. Meiotic cells, and later the embryo sac, lie under one layer of nucellar epidermis. At the tetrad stage, both integuments completely cover the nucellus and form a micropylar canal. The space inside the micropylar canal is narrow and irregular. At the micropylar pole, the embryo sac has the egg cell and two synergids with the filiform apparatus distinctly stained by PAS reaction. Below the egg apparatus is a central cell with a large vacuole, and on the chalazal pole are three antipodals clinging together closely.

In young ovules, the cells surrounding the micropylar canal have dense cytoplasm with sparse dictyosomes and vesicles. The cell walls are thin, with a fine layer of cuticle on the surface of the micropylar canal. At that stage there is no exudate in the micropylar canal, and the cells surrounding it are similar to the adjacent cells in the integument.

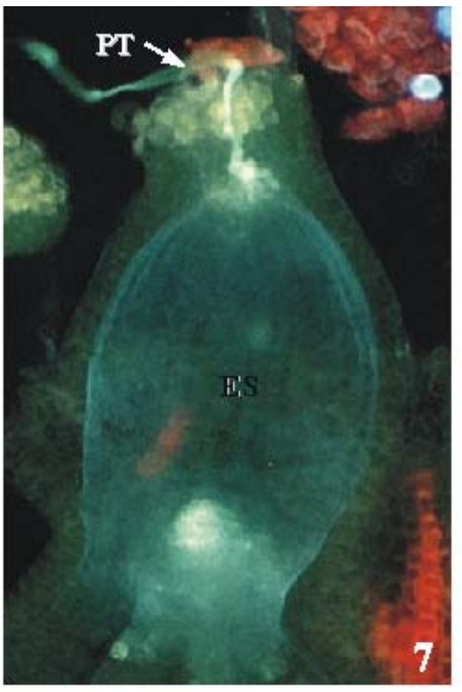
In ovules taken from flower buds just before anthesis, the cytoplasm of the cells surrounding the micropylar canal was very rich in RER, dictyosomes, vesicles and lipid globules. Exocytosis of fibrillar material or small globules was often observed (Figs. 1–4). In ovules 48 h after anthesis, the ER lost ribosomes but was still forming cisternae. Numerous lipid globules, accumulating as storage materials in the cytoplasm of the nucellar and integumental cells, were clearly visible in TEM. In the ovules observed in autumn, lipid globules were large and abundant, especially in the micropylar part of the ovule (Fig. 5). In ovules at the anthesis stage, lipid substances were not so abundant inside the cells, but a relatively thick layer of cuticle and electron-dense exudate in the micropylar canal was seen (Fig. 6). No starch grains were detected in the somatic cells of the ovules or in the embryo sacs after staining with PAS reaction.

After aniline blue staining, fluorescence microscopy made it easy to distinguish fertile ovules – containing the embryo sac – from sterile ones (Figs. 7, 8). The anatomy of the micropylar tissues of the two types of ovules is similar, but there are no generative cells inside the nucellus of the sterile ovule. In these ovules, at the anthesis stage the micropylar canal observed in TEM was empty and never penetrated by the pollen tube.

HISTOCHEMICAL FEATURES OF MICROPYLAR PART OF FERTILE OVULES

Histochemical staining of unfixed ovules showed that the micropylar region of the integuments and

Fig. 7. Ovule with well-formed embryo sac, 48 h after cross-pollination. Note the presence of the pollen tube in the micropyle. Fluorescence micrograph, aniline blue. × 400. **Fig. 8**. Sterile ovule isolated from open flower; nucellus has no embryo sac. Fluorescence micrograph, aniline blue. × 400. **Fig. 9**. Fertile ovule at anthesis stained for pectins with ruthenium red. × 150. **Fig. 10**. Ovule isolated from unpollinated flower stained for free calcium ions with alizarin red. × 200. **Fig. 11**. Ovule with fragment of placenta from unpollinated flower, stained for lipid exudate with Sudan black. × 150. **Fig. 12**. Lipid exudate in micropylar canal and on the top of the inner integument (arrow) in ovule taken from open flower. Fluorescence micrograph. × 250. **Fig. 13**. Ovules taken 24 h after pollination, stained for free calcium ions with alizarin red. × 120. **Fig. 14**. Ovule taken 24 h after pollination, stained with CTC. × 120. PT – pollen tube; II – inner integument; F – funiculus; ES – embryo sac; M – micropyle.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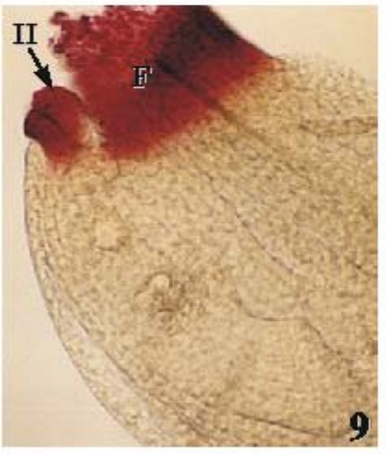
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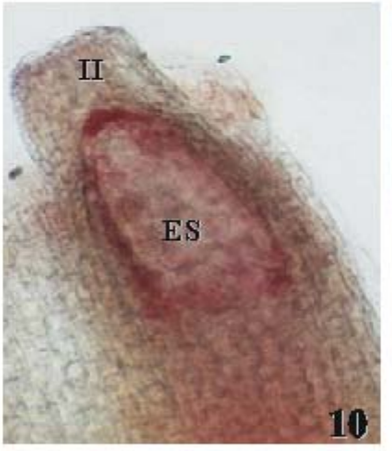
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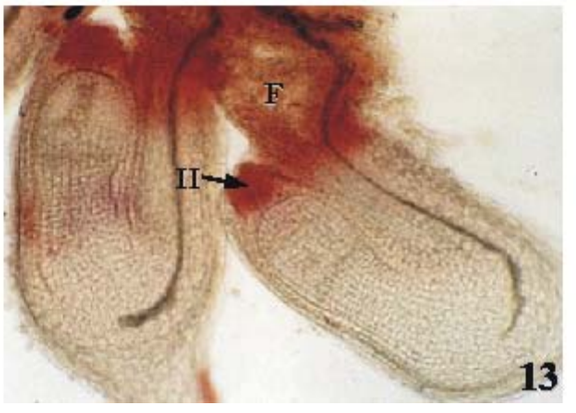
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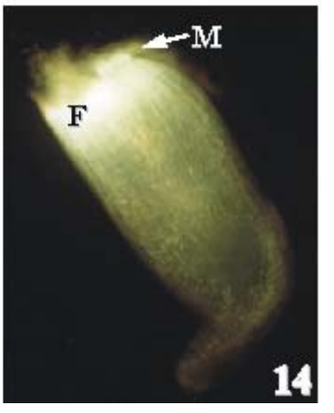
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the apex of the nucellus differ from other tissues of this organ. In the ovules isolated in September, positive reactions for the presence of pectins and acidic polysaccharides were observed only in the micropylar part of the inner integument. Other histochemical stainings were negative at this stage of development. Similar results were obtained in the ovules isolated from flower buds in February.

In older ovules isolated from open but unpollinated flowers, pectins (Fig. 9), acidic polysaccharides and proteins were detected. Positive reactions for these substances were located mainly in the micropylar part of the inner integument. Free calcium ions were detectable only around the embryo sac (Fig. 10). The presence of a lipid exudate was seen by light (Fig. 11) and fluorescence microscopy (Fig. 12).

All the histochemical stainings mentioned above gave positive results in the micropylar part of the inner integument 24 h after cross-pollination. Also, a strong positive reaction for free calcium ions and membrane-bound calcium appeared (Figs. 13, 14). In unfertile ovules, the histochemical reactions did not stain any part of them in spite of distinct positive results on the placenta, where the exudate is secreted already in autumn.

LOCALIZATION OF FREE AND LOOSELY BOUND CALCIUM IONS AT SUBCELLULAR LEVEL

Localization of free and loosely bound calcium ions at the subcellular level was done in ovules after potassium pyroantimonate treatment and observed in TEM. In the ovules obtained from flower buds, sparse Ca/Sb precipitates were located in the somatic cells of the micropyle, mainly inside the cytoplasm, vesicles, plastids, nucleus, and the middle lamellae of cell walls. Calcium ions were absent from the micropylar canal and the space between the integuments and placenta. Numerous Ca/Sb precipitates were located in the inner layer of the embryo sac cell wall, formed as a loose network of fibrils, and near the plasmalemma of the embryo sac (Fig. 15). The outer layer of this cell wall had a compact structure with parallel fibrils, and no Ca/Sb precipitates were present (Figs. 15, 16).

In the micropylar region of the ovules taken from open unpollinated flowers, the distribution of Ca/Sb precipitates was similar to that in the flower buds. There was a difference in the presence of precipitates in the intercellular spaces of the micropylar nucellus (Fig. 17), and numerous precipitates appeared in the substance filling the space between the placenta and the micropyle of the ovule. In these regions, Ca/Sb precipitates formed round structures and stripes.

A noticeable increase of Ca^{2+} content in the extracellular regions of the micropylar part of the inner integument was found 24 h after pollination. Ca/Sb precipitates were clearly visible in this tissue, mainly inside the small vesicles near the plasmalemma and in the cell walls (Figs. 18–21). Numerous Ca/Sb precipitates appeared inside the micropylar canal, especially in the space above the micropylar pole of the embryo sac, where the nucellar cells undergo lysis. Accumulation of calcium was still visible in the inner layer, but not in the outer layer of the embryo sac cell wall.

In sterile ovules, accumulation of Ca^{2+} in extracellular regions was not seen even after pollination.

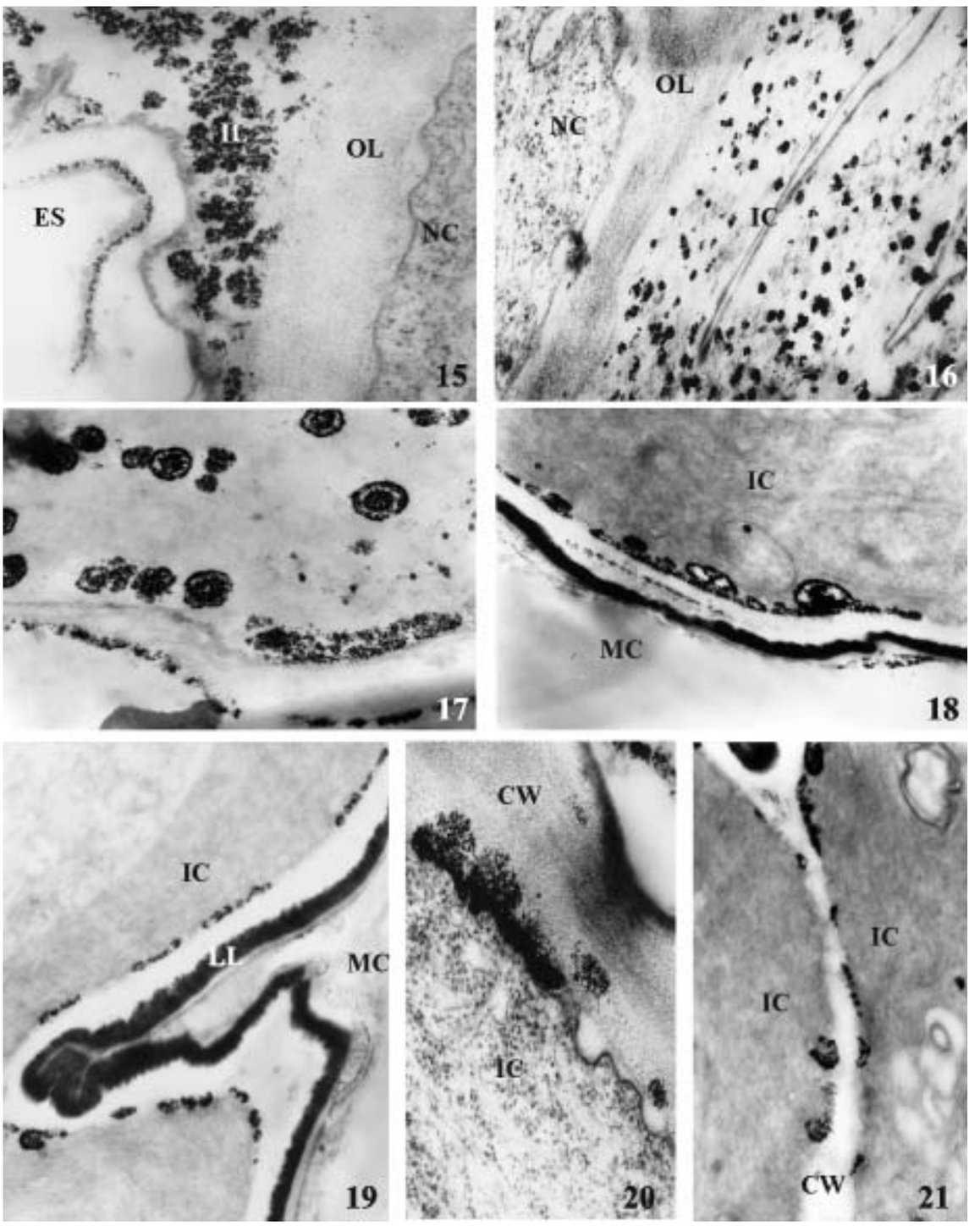
The Ca/Sb precipitates were examined by X-ray microanalysis (Fig. 22). In energy dispersive X-ray spectra acquired from the selected precipitates, prominent (Lα – 3.60 kV, Lβ1 – 3.84 kV) and minor (Lβ2 – 4.1 kV) Sb peaks were detected. The Sb Lα and Lβ1 peaks overlapped the Ca peaks (Kα – 3.69 kV, Kβ – 4.01 kV). The K (Kβ1 – 3.31 kV) and P (Kα – 2.013 kV) peaks were detected. In the analyzed precipitates no significant Na and Mg peaks were found.

This method of calcium localization was verified by control reactions. In sections of ovules untreated with potassium pyroantimonate, no precipitates were localized in the tissue. In the material treated with EGTA, only rare precipitates were visible before fixation, mainly inside the cells.

DISCUSSION

During the final stage of the progamic phase, the receptivity of the ovule is a crucial factor in positive interaction with the approaching pollen tube. It has to be attracted and directed to the micropyle and next to the fertile embryo sac.

In *G. nivalis*, development of the female gametophyte and of the somatic parts of the tenuinucellar ovule are mutually dependent processes. The micropylar part of the ovule is formed by one layer of nucellar cells covering the embryo sac and by the top part of the inner integument, surrounding the micropylar canal. It is the site of pollen tube penetration, as in the ovules of many other plants (Chao, 1971; Tilton, 1979; Arbeloa and Herrero, 1991; Franssen-Verheijen and Willemse, 1993). The presence of the mature embryo sac inside the ovule is necessary for the differentiation of somatic cells.



Figs. 15, 16. Ca/Sb precipitates in cell wall of embryo sac in young ovules. TEM, × 30 000. **Fig. 17**. Ca/Sb precipitates in exudate filling the space between the placenta and integuments in ovule from unpollinated flower. TEM, × 60 000. **Figs. 18–21**. Free calcium ions located near plasmalemma in cells surrounding micropylar canal of ovule taken from pollinated flower. TEM, Figs. 18, 19, 21 × 16 000, Fig. 20 × 40 000. ES – embryo sac; IL – inner layer of embryo sac cell wall; OL – outer layer of embryo sac cell wall; NC – nucellar cell; IC – integumental cell; MC – micropylar canal; LL – lipid layer; CW – cell wall.

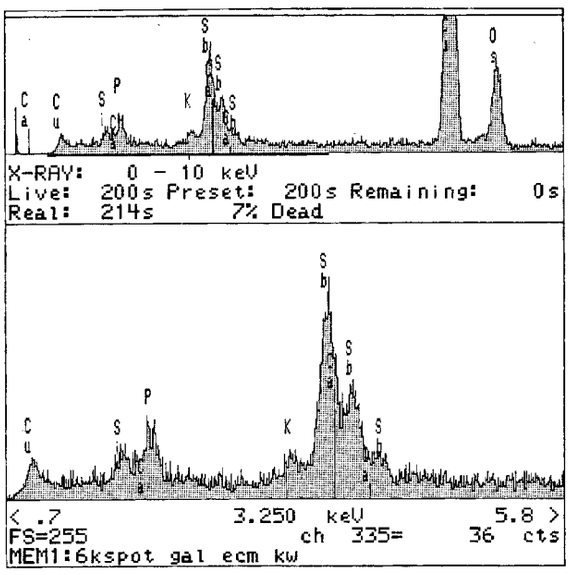


Fig. 22. Diagram from energy-dispersive X-ray analysis of ultrathin sections of *G. nivalis* ovules prepared by the Sb/Ca precipitation method.

Secretion activity at this site is important for preparing a suitable pathway for pollen tube growth. All histochemical stainings applied on fresh ovules showed a very distinct difference between fertile and sterile ones. In sterile ovules the development of somatic cells in the micropylar part is inhibited, and they remain meristematic or parenchymal. The ultrastructure of organelles, cell walls and intercellular spaces shows no traces of secretion.

In fertile ovules of *G. nivalis* the somatic part begins its differentiation before anthesis but becomes very active after flower opening and pollination. Pollination also becomes a stimulus for the full maturation of the embryo sac (Chudzik et al., 2002). Secretion from somatic cells is strictly limited to the region between the micropyle and egg apparatus. The micropylar canal becomes filled with dense exudate containing proteins, lipids, pectins and free calcium ions. All these substances can support pollen tube growth, but the presence of pectins and calcium ions is mentioned in many publications as especially important. They are secreted during the progamic phase from somatic cells; this was confirmed by EM and X-ray analysis of Ca/Sb precipitates.

Pectins colored by ruthenium red are widely distributed in the intercellular spaces between the cells of the integument and inside the micropylar canal. Free calcium ions are spotted in the same region to a great extent, but especially in the surroundings of the egg apparatus of the embryo sac. The free calcium ions are adsorbed on the network of polysaccharide chains of pectins; from this deposit they could be easily taken by the growing pollen tube. Access to free calcium ions is an important factor during pollen tube growth, as described in other plants and also shown in vitro (Brewbacker and Kwack, 1963; Malhó et al., 2000; Pierson et al., 1996). The functions of pectins and calcium ions as substances supporting and attracting the pollen tube are overlapping roles, a suggestion made in many publications (Brewbacker and Kwack, 1963; Reger et al., 1992; Vennigerholz, 1992; Malhó et al., 2000; Bednarska, 1995; Bednarska and Butowt, 1995; Jauh and Lord, 1996; Pierson et al., 1996; Lenartowska et al., 1997; Mollet et al., 2000).

Pectins can be a source of nutrients for the pollen tube. They are hydrolyzed to simple sugars by enzymes secreted from the pollen tube (Taylor and Hepler, 1997). Pectins can also play a role in forming the smear background on the pollen tube pathway. The pectin-covered surface enables attachment of the pollen tube, which is important for its growth. Such thigmotropism has been described inside the ovary in *Lilium* and demonstrated in vitro (Sanders and Lord, 1989; Jauh et al., 1997; Mollet et al., 2000).

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