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Localization of pectins and Ca^{2+} ions in unpollinated and pollinated wet (*Petunia hybrida* Hort.) and dry (*Haemanthus albiflos* L.) stigma

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Abstract: The subcellular localization of Ca^{2+} ions as well as esterified and deesterified pectins in unpollinated and pollinated wet (*Petunia hybrida*) and dry (*Haemanthus albiflos*) stigma was analyzed. Stigmas with different surfaces were found to differ in Ca^{2+} and pectin localization. In a wet *Petunia hybrida* stigma, Ca^{2+} ions were present in the exudate occurring in the intercellular spaces of secretory tissue before pollination. The exudate of an unpollinated stigma was the site of the localization of large amounts of deesterified pectins. Stigma penetration by pollen tubes induced the lysis of this category of pectins. The epidermal cells walls of the dry *Haemanthus albiflos* stigma before pollination lacked free and loosely bound Ca^{2+} ions. Pollination induced an accumulation of these ions in the apoplast of the stigma epidermal cells. In cells walls of an unpollinated stigma, mainly esterified pectins were present. Their deesterification took place after pollination at the site of pollen grain adhesion and then at the site of pollen tube growth. These results have shown that wet and dry stigmas differ in pectin metabolism and in the mechanism of forming a calcium environment at the site of pollen grain germination.

Key words: Calcium - Pectins - Stigma - *Petunia hybrida* Hort.- *Haemanthus albiflos* L.

Introduction

Stigma is the natural environment of pollen grain hydration and germination. The surface of the stigma is divided into wet and dry [9]. Mature stigma of the wet type is covered by an exudate in which various proportions of carbohydrates, lipids and proteins are present. The dry stigma has little or no surface secretion at maturity. The anatomical differences in the stigma are accompanied by physiological differences, particularly apparent in the phenomenon of self-incompatibility. In general, most of the plants which have a gametophytic type of self-incompatibility have wet stigmas, whereas in plants which have dry stigmas sporophyte incompatibility occurs [16]. Wet stigmas are considered to be less selective as both non-self pollen and self-pollen germinate on their surface. Rejection of self-pollen takes place in the style. Dry stigmas are only penetrated by pollen tubes germinating from pollen grains recognized as compatible.

The site of pollen germination is the extracellular matrix (ECM) of stigma cells. In wet stigmas it is an exudate present on its surface and in intercellular spaces of secretory tissue, in dry stigmas - the surface of epidermal cell wall. Thus, the epidermal cell wall and exudate components formed during stigma maturation will play a key role in pollen grain germination. This environment should provide the nutrients required by pollen grains and an optimal level of ions which play a key role during pollen germination. Investigations performed since the 1960s have shown that Ca^{2+} ions play a fundamental role in the regulation of pollen grain germination and in the growth of pollen tubes [10]. *In vitro* investigations have demonstrated that growing pollen tubes take up Ca^{2+} ions from the medium [14] and store them in the pollen tube tip, in which a characteristic tip-to-base gradient is formed [26]. The changes in Ca^{2+} concentration in the apical area of the pollen tip are correlated with its pulsatory growth and reorientation [21, 22].

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Under *in vivo* conditions, the optimal Ca^{2+} ion concentration required for pollen grain germination should be provided by the stigma. Investigations using $^{45}Ca^{2+}$ have shown that the ions present in the pistil are taken up by the growing pollen tubes [3]. Thus it should be expected that the stigma is provided with mechanisms ensuring the formation of an optimal calcium environment at the site where pollen grain germination will take place. In the extracellular compartment, deesterified pectins are capable of binding Ca^{2+} ions [5]. Deesterified pectins formed in the apoplast as a result of the pectin methylesterase (PME) activity [29], due to the presence of free carboxyl groups bind cations and in particular Ca^{2+} ions. Bound Ca^{2+} ions may be liberated during enzymatic lysis of this pectin category. The use of deesterified pectins in Ca^{2+} storage and liberation was observed in transmission tissue of the style in *Petunia hybrida*. The increase in the level of free Ca^{2+} after pollination [17] is correlated there with the lysis of deesterified pectins strongly binding Ca^{2+} [18]. It is not known how common this mechanism is and whether it also occurs in the stigma.

The condition of the initiation of phenomena leading to the germination of pollen on the stigma is the adhesion between reproduction partners. In plants, pectins play an important role in cell adhesion [20]. Investigations in *Lilium longiflorum* have shown that in the open style acid pectins and a peptide named SCA (stigma/stylar cysteine rich adhesin) are responsible for the adhesion between the pollen tube and transmitting tract epidermis (TTE) [23, 24]. The occurrence of acid pectins in the pollen tube wall has been observed in all plant species investigated so far [15, 19, 28]. This pectin category is also present in the ECM of the transmitting tract both in closed [18] and in open styles [15]. Pectin metabolism in the stigma has not been investigated so far. Thus, the participation of pectins in adhesion between germinating pollen grains and the stigma surface remains an open question.

The aim of the present investigations was to analyze Ca^{2+} and pectin localization in unpollinated and pollinated stigmas with a wet (*Petunia hybrida*) and dry (*Haemanthus albiflos*) surface.

Materials and methods

Plant material. Commercial cultivars of *Petunia hybrida* Hort. var. multiflora and *Haemanthus albiflos* L. were grown at room temperature in the Institute of General and Molecular Biology, Nicolaus Copernicus University, Toruń, Poland. Flowers were emasculated one day before anthesis and pollinated at anthesis. Stigmas were dissected on the day of anthesis (unpollinated) as well as 6 h and 16 h after pollination, and fixed for subcellular Ca^{2+} localization or immunolabelling.

Sample processing. The material was fixed and dehydrated in ethanol and embedded in LR Gold resin (Sigma Chemical Co., St. Louis, Mo., U.S.A.). Polymerization with 1% benzoyl peroxide as the accelerator occurred for 3 days at room temperature. The sections were cut with diamond knife on a Leica Ultracut UCT microtome (Reichert-Jung, Vienna, Austria). Semithin sections for pectin localization were placed on Biobond (British BioCell, Cardiff, UK) treated microscope slides, ultrathin sections for Ca^{2+} localization were collected on the nickel grids coated with 3% Formvar (Sigma).

Ca^{2+} localization was studied cytochemically using the pyroantimonate method. Stigmas were fixed for 24 h in cold (4˚C) fixative solution consisting 2% potassium antimonate ($K_2H_2Sb_2 \cdot 4H_2O$), 0.5% glutaraldehyde, 2% paraformaldehyde and 0.1 M phosphate buffer, pH 7.4. Material was then washed in five 20-min changes of the same buffer and postfixed in 1% OsO_4 in phosphate buffer, pH 7.4, for 30 min. Stigmas fixed without pyroantimonate were used as controls. The presence of Ca/Sb precipitates was investigated using energy-dispersive X-ray microanalysis [3].

For immunolabelling of pectins, the stigmas were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.4, for 24 h at 4˚C. Two categories of primary monoclonal antibody (MAb) were used: JIM5 against homopolygalacturonic acid epitopes and JIM7 against the epitopes of highly esterifed (mainly by methyl groups) forms of that acid (the antibodies were provided by Dr. J.P. Knox, Centre for Plant Biochemistry and Biotechnology, University of Leeds, UK). The sections were first treated with 2% bovine serum albumin (BSA) in PBS buffer (pH 7.4) at room temperature for 1 h and then incubated with primary antibodies JIM5 or JIM7, at 1:50 in PBS buffer (pH 7.4) with 0.2% BSA, overnight at 4˚C. Next, the sections were washed in three changes of the same buffer and incubated with goat anti-rat IgG Cy3 secondary antibodies (Sigma), 1:100 in PBS buffer (pH 7.4), with 0.2% BSA with the same time and temperature conditions as with the primary antibodies. The control was performed with the omission of primary antibodies. The sections were washed in buffer and finally in distilled water, dried at room temperature and covered with 0.5% diaminobenzene.

Microscopy. Ultrathin sections were examined without staining in JEOL 1010 transmission electron microscope at an accelerating voltage of 80 kV. Semithin sections after immunolabelling were examined in an Olympus U-RFL-T photomicroscope equipped with epifluorescence and filter system appropriate for Cy3 fluorescence. Micrographs were taken with Olympus Camedia C-2000Z Digital camera.

Results

Immunofluorescence localization of pectins

The wet stigma of *Petunia hybrida*. In the unpollinated stigma, differences were seen in the binding level of JIM7 and JIM5 antibodies in the glandular zone and the stigma cortex. The JIM7 MAb was localized mainly in the cortex, whereas the signal present in the glandular zone was markedly lower (Fig. 1A). In glandular tissue, the fluorescence of the JIM7 MAb-Cy3 complex occurred in the form of clusters (Fig. 1C). In the secretory cells, the signal was localized in the cytoplasmic granulations (Fig. 1E). The JIM5 MAb recognizing unesterified pectins was localized mainly in the glandular zone, in the stigma cortex fluorescence was much lower (Fig. 1B). A stronger signal was observed on the surface of the stigma (Fig. 1D). In the glandular tissue the fluorescence of JIM5 MAb-Cy3 complex was mainly localized in the exudate present in intercellular spaces of the secretory tissue (Fig. 1F).

Six hours after pollination, the general pattern of the JIM7 and JIM5 MAbs labelling in the stigma tissues was similar to that in the unpollinated stigma. A strong signal of JIM7 MAb-Cy3 complex was observed in the stigma cortex and of JIM5 MAb-Cy3 in the glandular zone (Fig. 2B). Almost no signal of JIM7 MAb-Cy3 (Fig. 2A) was found in the glandular zone, whereas the fluorescence of the JIM5 MAb-Cy3 complex was significantly decreased on the pollinated surface of the stigma (Fig. 2B).

Sixteen hours after pollination a large number of pollen tubes were found in the stigma. In the glandular zone, the JIM7 MAb coupled to Cy-3 was observed mainly in the pollen tube tips penetrating it (Fig. 2C). At the same time a clear decrease was seen in the level of JIM5 antibody binding to the exudate of the glandular zone. Large regions of the glandular zone with pollen tubes growing through them were nearly completely devoid of fluorescence signal (Fig. 2D).

Pollen grains were seen on the stigma surface after pollination. In the pollen sporoderm, a strong fluorescence signal was seen after incubation with both JIM7 and JIM5 antibody (Fig. 2A-C).

The control, where primary antibodies were omitted, showed no signal in the cortical tissues and weak autofluorescence in the glandular zone and strong in the pollen sporoderm (Fig. 5A, B).

The dry stigma of *Haemanthus albiflos*. The *H. albiflos* stigma is built of 3 thin lobes. The receptive surface is formed by the cuticle-covered walls of the epidermal cells on the internal side of the lobes which are in contact with the canal of the open style.

In cells of the unpollinated stigma, mainly the JIM7 Mab was demonstrated (Fig. 3A). The strongest signal of JIM7-Cy3 complex was shown by epidermal cell walls on the internal receptive surfaces of the stigma lobes. The level of fluorescence in epidermal cell walls covering the external surface of the stigma lobes was lower (Fig. 3A). Binding of the JIM7 MAb was also found in cytoplasmic granulations of epidermal and sub-epidermal stigma cells.

The level of the JIM5 Mab-Cy3 signal in the cells of the *H. albiflos* stigma was very low (Fig. 3B). A weak fluorescence of the JIM5 MAb-Cy3 complex was seen only in the epidermal cell walls on a internal surface of the stigma lobes.

Six hours after pollination an increase in the fluorescence of JIM7 MAb-Cy3 complex in the walls of all stigma cells was observed (Fig. 4A). The number and intensity of the fluorescent granulosities occurring in the cytoplasm of epidermal and subepidermal cells clearly increased.

The level of JIM5 antibody binding to the walls of the pollinated stigma cells was still low (Fig. 4B). A strong signal was only observed in the walls of these epidermal cells to which the pollen grain adhered. The presence of an increased JIM5 MAb signal was also observed in the intercellular spaces. The sporoderm of germinating pollen grain showed a differentiated fluorescence level after incubation with the JIM7 and JIM5 antibodies. A strong fluorescence of the JIM7 Mab-Cy3 complex was found in the whole sporoderm (Fig. 4A). Localization of JIM 5 MAb in the sporoderm formed a specific gradient: a high level of fluorescence was observed in the region in which the pollen grain was in contact with the epidermal cells, and a lower one outside it (Fig. 4B).

Sixteen hours after pollination, the pollen tubes growing in the direction of the open style were visible on the receptive stigma surface. At that time after pollination, an increase was observed in the level of the signal of MAbs JIM7 as well as JIM5 conjugated with Cy-3 (Fig. 4C, D). The intensity of JIM5 Mab-Cy3 complex fluorescence was particularly striking. The signal was localized in thickened layer of the extracellular matrix at the site of pollen tube growth (Fig. 4D).

Both antibodies used in the investigations bound to germinating pollen tubes. JIM7 MAb was localized in the cytoplasm and the cell wall of pollen tube tips (Fig. 4C), whereas the JIM5 MAb-Cy3 complex only bound to the wall of the pollen tube (Fig. 4D).

In the control material incubated without primary antibodies, no fluorescence was detected (Fig. 5C).

Subcellular localization of Ca^{2+} ions

The wet stigma of *Petunia hybrida*. In the unpollinated wet stigma of *P. hybrida*, Ca/Sb precipitates were localized within the exudate, which was present in the intercellular spaces of the glandular tissue (Fig. 6A).

Six hours after pollination in the exudate surrounding germinating pollen grains, almost no Ca/Sb precipitates were localized (Fig. 6B,C). Sixteen hours after pollination pollen tubes penetrated the intercellular space of the stigma secretory tissue. At this stage of development, numerous small precipitates were localized on the surface of the pollen tube and single ones in its cytoplasm (Fig. 6C).

The dry stigma of *Haemanthus albiflos*. The epidermal cells of the unpollinated stigma of *H. albiflos* was covered by a continuous cuticle layer (Fig. 7A).

In the cells of unpollinated stigma, Ca/Sb precipitates were localized only in the cytoplasm. In the epidermal cells, the precipitates were present in the cytosol and inside vesicles (Fig. 7A). The number of precipitates in subepidermal cells was higher than in epidermal cells. They were mostly localized within large vacuoles, on the internal side of the tonoplast (Fig. 7B). Numerous Ca/Sb precipitates were observed in the vascular bundle. The site of their localiz-

ation was an internal thickened surface of the xylem cell walls (Fig. 8C).

After pollination at the site of adhesion of the pollen grain to the stigma epidermal cell a foot was visible (Fig. 7C, D). The pollination induced significant changes in the localization of Ca/Sb precipitates. Six hours after pollination accumulation of precipitates was visible in the epidermal cell wall which was in direct physical contact with the pollen (Fig. 7C). The presence of germinating pollen grains caused a disruption of the continuity of the cuticle covering the epidermal cells of stigma (Fig. 7D,E). In these regions, a specific gradient of Ca/Sb precipitates was observed. At the site where the pollen grain adheres to the epidermal cell, only single small precipitates were localized in the cell wall (Fig. 8D). The number of precipitates and their size in the epidermal wall increased with the distance from the adhesion zone (Fig. 8E). The highest number of Ca/Sb precipitates occurred in the region of the wall which was still covered by the cuticle.

Sixteen hours after pollination pollen tubes were visible on the surface of the epidermal cells. In the apoplast of epidermal cells to which pollen tubes adhered, a change in the distribution of Ca/Sb precipitates was observed. In the cell wall only single precipitates were localized, whereas a large number of them occurred on its surface (Fig. 7F). Numerous small precipitates were present on the pollen tube wall surface. The effect of such a distribution of Ca/Sb precipitates was their accumulation at the site of adhesion of the pollen tube to the surface of the epidermal cell wall. In the pollen tube, Ca/Sb precipitates were localized mainly in electron-transparent thickenings of the internal wall, probably at the site of callose plugs formation (Fig. 8F). At the same time a thickening of the epidermal cells ECM was observed in lower part of the stigma, in the vicinity of the entry into the style canal. If this area was not yet penetrated by pollen tubes, ECM of the epidermal cells contained many large Ca/Sb precipitates (Fig. 8A). Precipitates were also visible in vesicles which underwent fusion with the plasmalemma. The presence of pollen tubes caused significant changes in the localization of Ca/Sb precipitates in this region of the stigma. They were no longer observed in the ECM but were present on the surface of the epidermal cell wall and around pollen tubes (Fig. 8B). Accumulations of Ca/Sb precipitates also occurred in the zones of adhesion of pollen tubes growing next to each other (Fig. 8B).

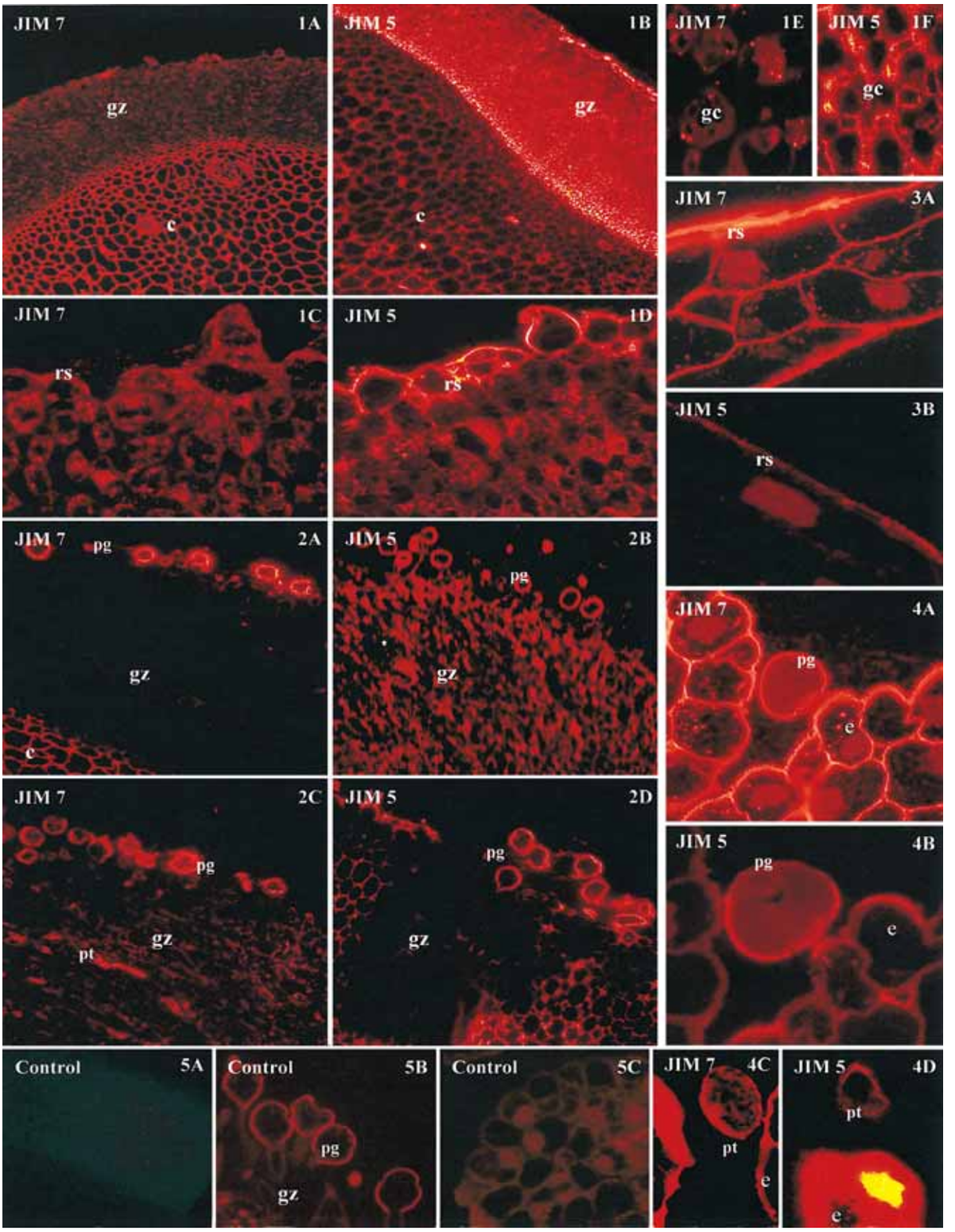
In the control, *i.e.* in material which was fixed without the addition of pyroantimonate, electron dense precipitates did not occur (Fig. 8D, E, F).

The presence of Sb/Ca in the precipitates observed in the investigated material was checked using X-ray analysis (Fig. 6D, E). In energy-dispersive X-ray spectra acquired from the precipitates present in the pyroantimonate-fixed stigmas, prominent Sb peaks (Lα-3.60 keV, Lβ - 3.84 keV) overlapping the Ca peaks (Kα - 3.69 keV, Kβ - 4.01 keV) were observed.

Discussion

The performed investigations have shown that the two investigated anatomically different types of stigmas differ in pectin metabolism and in the mechanism of forming a calcium environment indispensable for correct germination of pollen grains.

Figs. 1-4. Immunofluorescence localization of esterifed (JIM7 MAb) and unesterifed (JIM5 MAb) pectins in wet stigma of *P. hybrida* and dry stigma of *H. albiflos.* **Fig. 1.** Unpollinated stigma of *P. hybrida*. **A, C, E** - Localization of esterifed pectins. **A.** Strong fluorescence in the apoplast of the cortex tissue, in the glandular zone only a weak signal is observed; × 10. **C.** On the stigma surface and in the glandular zone the signal occurs in small clusters; × 40. **E.** In the glandular cells, granular localization in the cytoplasm is observed; × 100. **B, D, F** - Localization of unesterifed pectins. **B.** Strong fluorescence in the glandular zone, while weak one in the cortex tissue; × 10. **D.** Stronger signal can be seen in the stigma receptive surface; × 40. **F.** In the glandular zone, the fluorescence is mainly localized in the exudate between the secretory cells; × 100. **Fig. 2.** Pollinated stigma of *P. hybrida*. **A, B** - 6 h after pollination. **A.** Localization of esterifed pectins. Strong signal in the apoplast of cortical region, the glandular zone is nearly completely devoid of the fluorescence; × 40. **B.** Localization of unesterifed pectins. Decrease in fluorescence in the stigma surface, a small area devoid of the signal is visible in the glandular zone (asterisk); × 40. **C, D** - 16 h after pollination. **C.** Localization of the esterifed pectins. In the glandular zone fluorescence is observed mainly in the growing pollen tube tips ; × 40. **D.** Localization of unesterifed pectins. Large regions of the glandular zone penetrated with pollen tubes are nearly completely devoid of fluorescence signal; × 40. A red fluorescence can be observed in the pollen grain sporoderm after incubation with JIM7 and JIM5 MAb. **Fig. 3.** Unpollinated stigma of *H. albiflos*. **A.** Localization of esterifed pectins. Fluorescence in the walls and in the form of clusters in the cytoplasm of all stigma cells. The strongest signal occurs in the internal receptive surface of the stigma lobe; × 40. **B.** Localization of unesterifed pectins. Weak fluorescence in walls of epidermal cells on the receptive surface of the stigma lobe; × 40. **Fig. 4.** Pollinated stigma of *H. albiflos*. **A, B** - 6 h after pollination. **A.** Localization of esterifed pectins. Strong fluorescence in the apoplast of all stigma cells and in the form of clusters in their cytoplasm. The signal is also present in the pollen grain sporoderm; × 40. **B.** Localization of unesterifed pectins. Increased fluorescence in the walls of epidermal cells in the vicinity of the pollen grain. In the pollen grain, the signal can be seen in the sporoderm regions which are in contact with the stigma surface; × 100. **C-D** - 16 h after pollination. **C.** Localization of esterifed pectins. The signal in the apoplast of stigma epidermal cells and in the pollen tube - in the cell wall and in the form of clusters in the cytoplasm; × 100. **D.** Localization of unesterifed pectins. A distinctly increased fluorescence in the thickened layer of the epidermal apoplast of the stigma. In the pollen tube the signal is limited to the cell wall; × 100. **Fig. 5.** Controls. In *P. hybrida* no signal is observed in the unpollinated stigma **(A)**; after pollination a autofluorescence occurs in the pollen sporoderm and glandular zone **(B)**. In *H. albiflos* stigma, a weak autofluorescence of the cell nuclei can be seen **(C)**. gz - glandular zone, gc - glandular cell, c - cortex, rs - receptive surface, pg - pollen grain, pt - pollen tube, e - epidermal cell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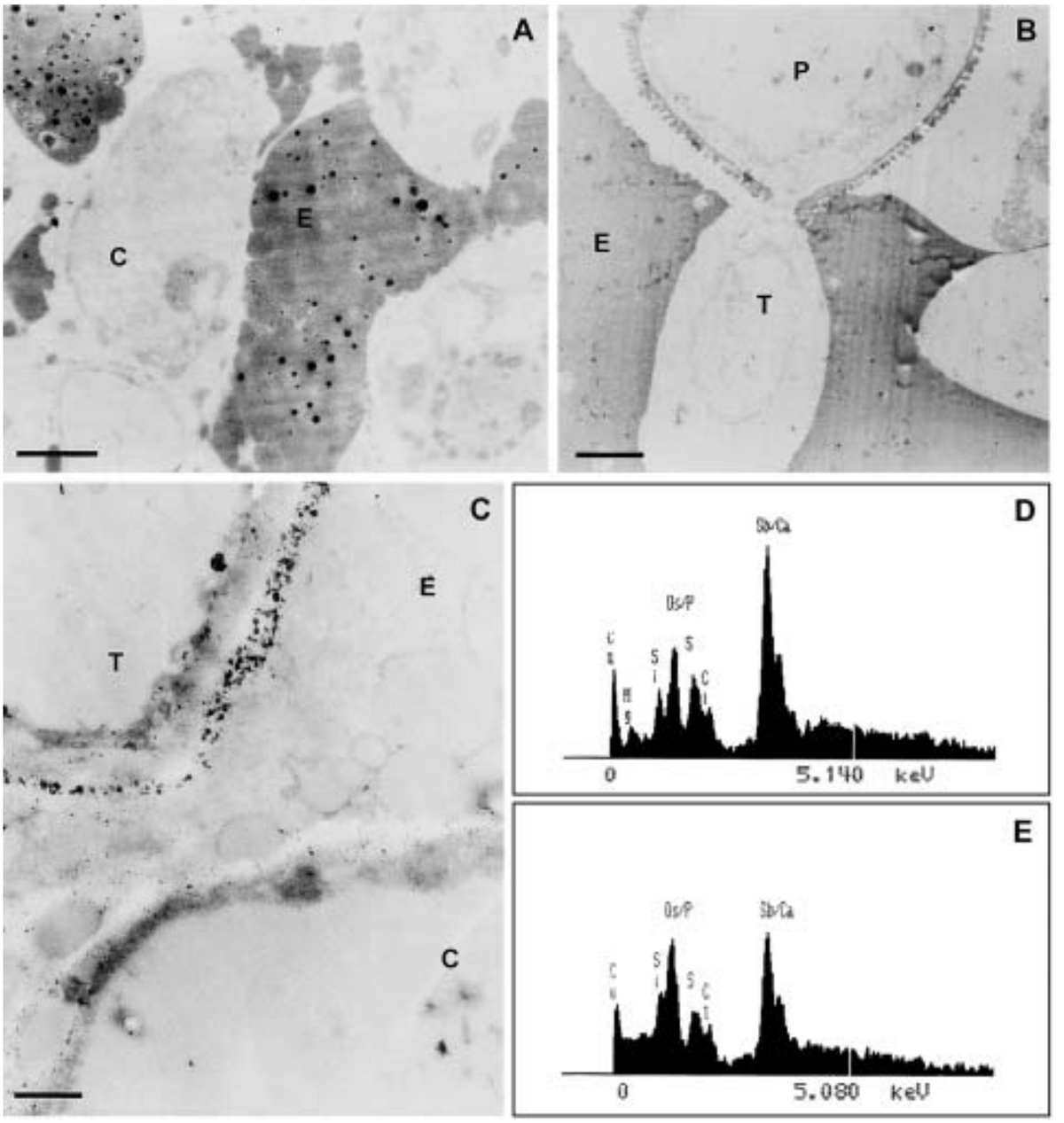
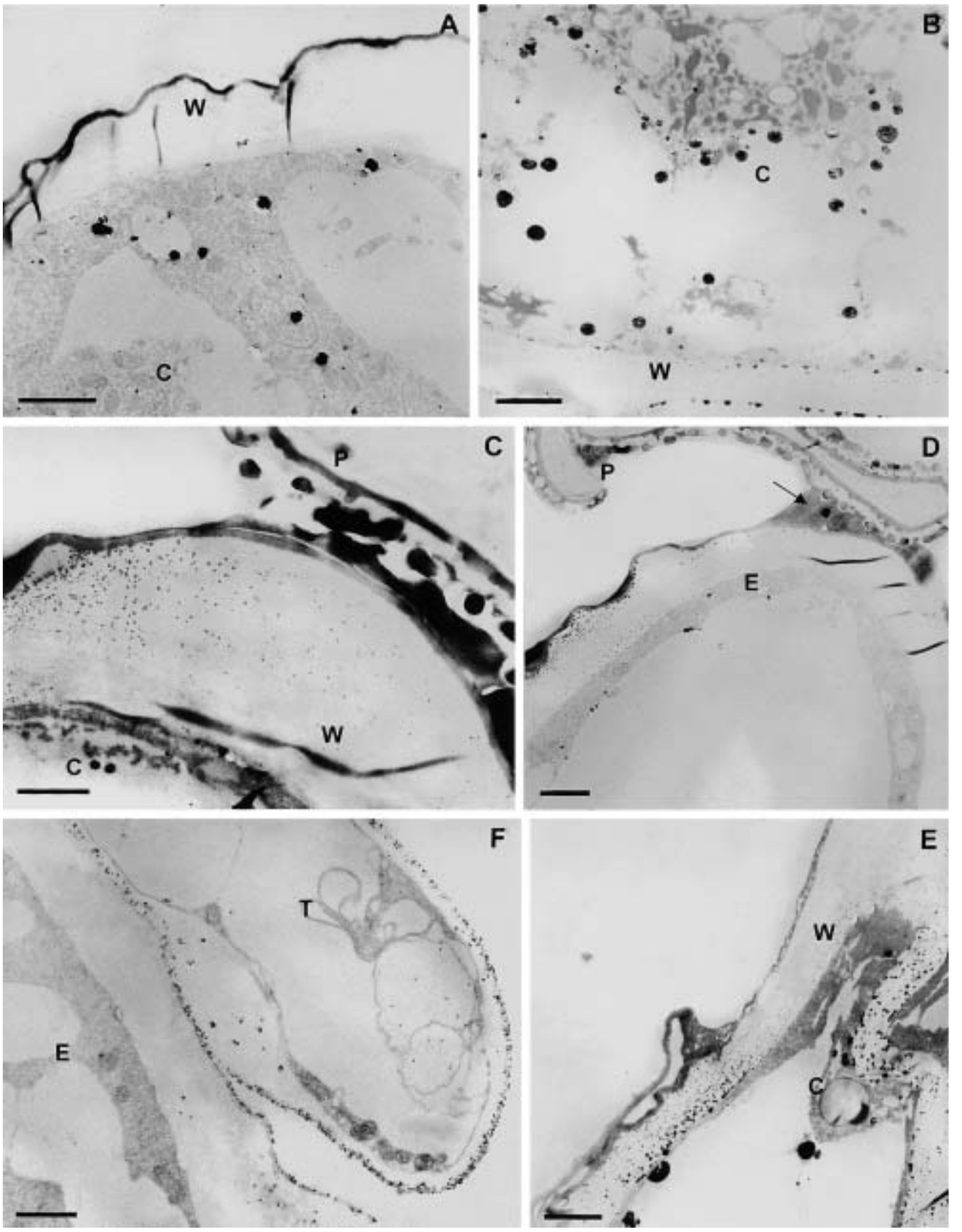


Fig. 6. Subcellular localization of Ca^{2+} ions in wet stigma of *P. hybrida*. **A.** In unpollinated stigma numerous Ca/Sb precipitates are localized in the exudate present in the intercellular spaces of secretory tissue; bar = 2 μm. **B.** In the exudate among germinating pollen tubes the number of Ca/Sb precipitates is very low; bar = 5 μm. **C.** 16 h after pollination. In exudates penetrated by pollen tubes, no Ca/Sb precipitates can be seen. Numerous small precipitates are localized on the surface of the pollen tube wall and single ones in its cytoplasm; bar = 1 μm. E - exudate, C - secretory cell, T - pollen tube, P - pollen grain. **D, E.** X-ray spectra of the precipitates occurring on the surface of the pollen tube wall of *P. hybrida* **(D)** and in the epidermal cell apoplast of pollinated stigma of *H. albiflos* **(E)**. Prominent Sb peaks (Lα-3.60 ke V, Lβ - 3.84 ke V) overlapping the Ca peaks (Kα - 3.69 ke V, Kβ - 4.01 ke V) are visible.

Fig. 7. Subcellular localization of Ca^{2+} ions in dry stigma of *H. albiflos*. **A, B** - Unpollinated stigma. **A.** In the cuticle-covered epidermal cell, Ca/Sb precipitates are localized in the cytosol and in cytoplasmic vesicles; bar = 2 μm. **B.** Accumulation of precipitates in cortex cells; bar = 2 μm. **C- F**. 6 h after pollination. **C.** Numerous small Ca/Sb precipitates in the epidermal cell wall at the site of pollen grain adhesion; bar = 1 μm. **D, E** - Fragments of the stigma surface, to which the germinating pollen grain is attached by a foot (arrow); bar = 2 μm. **D**. In the epidermal cell walls in the vicinity of adhering pollen only single Ca/Sb precipitates are visible. **E.** In the region of the cell wall distant from the site of pollen adhesion and still covered with the cuticle the number of precipitates is distinctly higher. Large single precipitates are present in the epidermal cell cytoplasm. **F.** 16 h after pollination. Ca/Sb precipitates are localized mainly on the surface of the epidermal cell wall and around the pollen tube; bar = 1 μm. W - cell wall, C - cytoplasm, E - epidermal cell, T - pollen tube.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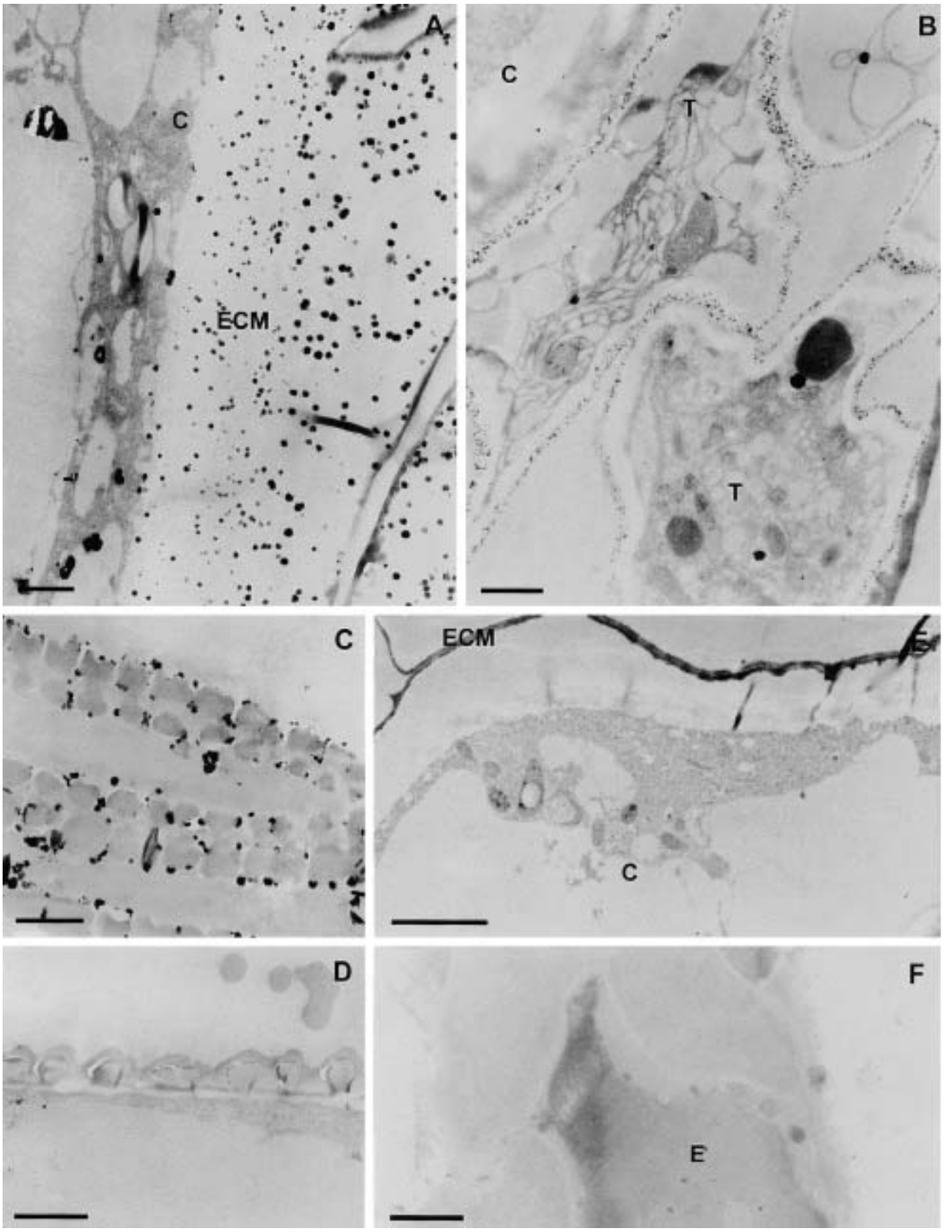
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Wet Petunia hybrida stigma

In unpollinated stigma of *P. hybrida*, free and loosely bound Ca^{2+} was localized above all extracellularly in the electron-opaque secretion present in intercellular spaces of glandular tissues. Investigations with the use of X-ray microanalysis did not show the presence of calcium in the exudate present on the *P. hybrida* stigma [13]. It cannot be excluded that X-ray microanalysis of whole stigmas in a scanning microscope made possible only the analysis of the surface of the exudate, whereas the PA method made it possible to localize free and loosely bound Ca^{2+} also within the stigma tissues. The presence of calcium in the exudates of the wet stigma of *Ruscus* has been demonstrated both by the method of X-ray microanalysis as well as by the CTC method [1]. Ca^{2+} ions on the surface of the wet stigma of *P. hybrida* necessary for germination of pollen grains are additionally supplied there by pollen grains. Earlier investigations have shown that in this species calcium occurs in the pollen coat [2] and in calcium crystals attached to the sporoderm derived from the anther [12]. This indicates that in the formation of the calcium environment on the wet *P. hybrida* stigma both partners of reproduction take part.

The performed immunocytochemical investigations have shown that in the unpollinated *P. hybrida* stigma, both esterified and deesterified pectins occur. Their localization was different in cortex cells and in secretory tissue and the exudates. In the apoplast of cortex cells, mainly esterified pectins were present, however in the secretory tissue both pectin categories occured. In the cytoplasm of secretory cells, clusters of esterified pectins were located, while in extracellular areas both on cell walls and in the exudates mainly deesterified pectins were present. Such a localization indicates that the secretory cells synthesize methylesterified pectins which undergo an intense deesterification process in extracellular areas.

After pollination, a degradation of deesterified pectins was observed. Their level decreased in these stigma regions which were penetrated by pollen tubes. The process of lysis of deesterified pectins appears to be a result of a direct contact between the growing pollen tubes and the ECM of secretory tissue. In the stigma regions which were not penetrated by pollen tubes, the level of this category of pectins was still high.

Our earlier investigations have shown that deesterified pectins which strongly bind Ca^{2+} are also present in the intercellular matrix of the transmitting tissue of the style of the unpollinated *Petunia hybrida* pistil [18]. The participation of this pectin category in Ca^{2+} storage appears, however, to be different in the stigma and style. In transmitting tissue of the pollinated style, lysis of deesterified pectins [18] is accompanied by a strong increase in Ca^{2+} levels in the extracellular matrix [17]. In this "stiff" region of the transmitting tract, this category of pectins probably is strongly bound to Ca^{2+}. In contrast, after pollination, in exudate of the stigma the level of free and loosely bound Ca^{2+} was very low, since almost no Ca/Sb precipitates were localized there. This indicates that the lysis of deesterified pectins in stigma of *P. hybrida* is not accompanied by the liberation of high levels of Ca^{2+} ions. Deesterified pectins present in the semiliquid stigma exudates are probably poorly cross linked by means of Ca^{2+}. Pectin lysis is performed by the polygalacturonase (PG), acting on deesterified pectins [4, 27]. Polygalacturonase occurs in the pollen coat and is secreted by germinating pollen tube [6, 11, 27]. The present investigations suggest that in the *P. hybrida* stigma exudate conditions enabling the functioning of pollen PG are created already before pollination.

Dry Haemanthus albiflos stigma

In the dry stigma of *H. albiflos*, the localization of both Ca^{2+} ions and pectins was different from that in the wet stigma of *P. hybrida*. Before pollination, the apoplast of epidermal cells of the dry stigma of *H. albiflos* contained no Ca^{2+} ions as shown by the PA method. In these cells, the relatively low level of Ca^{2+} ions was only localized in the cytoplasm. In cortex cells, the Ca^{2+} level was distinctly higher than in epidermal cells. These ions were also localized in the area of the xylem, indicating that they are transported to the stigma cells by the vascular bundle.

Pollination induced the accumulation of Ca^{2+} ions in the apoplast of the *H. albiflos* stigma epidermal cells. At the site of contact of the pollen grain with the stigma cell a foot was formed and the continuous cuticle was torn, probably as the result of the activity of the cutinase secreted from the pollen grain [8]. Before the pollen grain germination, the presence of free and loosely bound Ca^{2+} was observed in the epidermal cell wall

Fig. 8. Subcellular localization of Ca^{2+} ions in the lower part of *H. albiflos* stigma 16 h after pollination. **A.** Accumulation of Ca/Sb precipitates in the extracellular matrix of stigma epidermal cell in the region not penetrated by pollen tubes. Precipitates also occur in cytoplasmic vesicles which undergo fusion with the plasmalemma; bar = 1 μm. **B.** Pollen tubes growing on the stigma surface - Ca/Sb precipitates are present on the pollen tube - epidermal cell wall boundary and in the zone of adhesion of pollen tubes to each other; bar = 500 nm. **C.** Ca/Sb precipitates in xylem cells of the vascular bundle of the *H. albiflos* stigma; bar = 2 μm. **D, F.** Control - stigmas fixed without addition of PA. Lack of electron-opaque precipitates in xylem cells **(D)**, epidermal cells apoplast of the pollinated *H. albiflos* stigma **(E)** and the exudates of the unpollinated *P. hybrida* stigma (Fig. 8 F); bar = 2 μm. C - epidermal cell, ECM - extracellular matrix, T - pollen tube, E - exudate.

which was in physical contact with the pollen grain. Thus, the effect of pollination of *H. albiflos* dry stigma is the formation of an environment containing free and loosely bound Ca^{2+} ions at the adhesion site of the pollen. The post-pollination increase in the calcium level in epidermal cells wall of the dry stigma was described earlier in *Brassica oleracea* using the chlorotetracyclin method and X-ray microanalysis [7, 13]. Investigations in *Zea* using indicators of cytosolic Ca^{2+} ($[Ca]_c$) have demonstrated that pollination causes within seconds an increase in $[Ca]_c$ level in stigma trichomes [25]. This is accompanied by changes in membrane potential on the pathway stigma-ovary. The results of present study and investigations in *Brassica oleracea* [7, 12] and *Zea* [25] suggest that in epidermal cells of dry stigmas pollination induces both the increase of the $[Ca]_c$ level as well as accumulation of these ions in the apoplast. In the first case, Ca^{2+} ions probably participate in pollen-stigma signalling [25]. Ca^{2+} accumulation in apoplast of stigma epidermal cells appears to be a secondary phenomenon linked to the supply of the germinating environment of the pollen with Ca^{2+} ions.

During pollen germination, the Ca^{2+} level in the stigma epidermal cell wall in the area of pollen adhesion decreased. However, an increase was observed in the area of cell walls distant from the site of adhesion of pollen grains. Calcium-rich regions of the epidermal cell wall were still covered with a cuticle. This indicates that these regions of the stigma epidermis were not penetrated by pollen tubes. An especially high post-pollination accumulation of Ca^{2+} in the apoplast of stigma epidermal cells of *H. albiflos* occurred at its base, in the region of the transition to the style canal, where no adhering pollen grains were observed. This was an area in which pollen tubes also did not grow. Thus, adhesion and germination of pollen induce the accumulation of free and loosely bound Ca^{2+} in the apoplast of stigma epidermal cells befor their penetration by pollen tubes. The Ca^{2+} level in the epidermal cells of the *H. albiflos* stigma decreased significantly after its direct contact with pollen tubes. Cell walls to which pollen tubes adhered were almost completely devoid of Ca/Sb precipitates. This suggest that pollen tubes take up Ca^{2+} accumulated in the apoplast of stigma epidermal cells. Uptake of pistil Ca^{2+} by pollen tubes was noted earlier in *P. hybrida* [3].

The accumulation of free and loosely bound Ca^{2+} in the apoplast of the receptive cells of the dry *H. albiflos* stigma raises the question of their origin. Investigations have demonstrated with the use of antibodies JIM5 and JIM7 that in the cell walls of the unpollinated stigma predominantly esterified pectins weakly binding Ca^{2+} are present. After pollination, the level of this category of pectins increases. The presence of esterified pectins in the cytoplasm of stigma cells indicates that they are still synthesized and secreted to the apoplast. An increased level of deesterified pectins strongly binding Ca^{2+} was observed only after pollination at the site of adhesion of pollen grains and subsequently in an area into which the pollen tubes grew. This indicates that in the dry *H. albiflos* stigma, pectin deesterification is induced by pollination and germination of pollen tubes. It thus appears that pectins present in the apoplast of stigma epidermal cells do not play the role of a significant Ca^{2+} store. Probably these ions are secreted after pollination from the epidermal cell protoplast. The hypothesis that the source of calcium required for pollen tube germination on the dry *Brassica oleracea* stigma are papilla cells was proposed by Iwano *et al*. [13]. Using X-ray microanalysis they observed that after compatible pollination the calcium concentration increased near the surface of the papilla. Investigations of Ca^{2+} subcellular localization performed in *H. albiflos* appear to confirm this hypothesis. The presence of Ca/Sb precipitates in vesicles undergoing fusion with the plasmalemma indicates the process of post-pollination secretion of Ca^{2+} ions from epidermal cells of the stigma.

In most of species possessing dry stigmas, pollen selection already takes place on the stigma [16]. Only pollen grains recognized as compatible germinate on its surface. Hence, the environment of pollen germination is provided with the optimal level of Ca^{2+} ions and deesterified pectins which are a substrate for PG only after pollination with compatible pollen.

In both analyzed types of stigmas, the localization of Ca^{2+} and pectins in pollen tubes was identical. Ca^{2+} was localized on the pollen tube surface both at the site of their adhesion to the ECM of the stigma and in the areas in which they adhered to each other. The pattern of pectin localization in pollen tubes did not differ from that known from earlier investigations [15, 18, 19, 28]. On the apex of pollen tubes, both in the cytoplasm and the cell wall, esterified pectins are present. Deesterified pectins were only found in the cell wall. In both investigated types of stigmas, pollen tubes grow into an environment in which deesterified pectins capable of binding Ca^{2+} are present. This indicates that this category of pectins and Ca^{2+} ions play an important role in adhesion of pollen tubes to each other and to the apoplast of the dry and to the ECM of the wet stigma. The participation of acid pectins together with the SCA peptide (stigma/stylar cysteine-rich adhesin) in the adhesion of pollen tubes to each other and to the ECM of the style canal was documented in *Lilium* [23, 24]. This mechanism may be common and occur in the whole transmitting tract of the pistil regardless of its anatomical structure. Ca^{2+} are present on the surface of pollen tubes also growing in the style, both in the transmission tissue of the closed *Petunia hybrida* pistil [18] as well as in the canal of the open *Lilium longiflorum* pistil [15]. In the ECM of both types of styles deesterified pectins also occur [15, 18].

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