Localization of Ca^{2+} and Ca-ATPase on wet (*Ruscus aculeatus*) and dry (*Primula officinalis*) stigma surface

ELŻBIETA BEDNARSKA

Department of Plant Cytology and Embryology, Institute of Biology,
Copernicus University, Gagarina 9, 87-100 Toruń, Poland

(Received: October 23, 1989. Revision accepted: January 19, 1990)

Abstract

Calcium is present in the surface cells of both types of stigma. The chlorotetracycline method was used to show that the papillae of the dry stigma of *Primula officinalis* are the source of fluorescence of the CTC- Ca^{2+} complex. The precipitation method (NHA) localized calcium ions mainly in the pellicula surrounding these papillae. The NHA- Ca^{2+} precipitates were localized on the plasma membrane in the papillae of the wet stigma of *Ruscus aculeatus*. Ca-ATPase activity was found in both types of stigma in sites where the presence of Ca^{2+} had been determined.

Key words: stigma, *Primula officinalis*, *Ruscus aculeatus*, calcium, Ca-ATPase localization, pollen-pistil interaction

INTRODUCTION

The stigma surface plays a crucial role in pollen-pistil interaction. The surface of a wet stigma is covered with an exudate, of a dry stigma — by a lipoprotein pellicula (rev. K n o x 1984). The exudate and the pellicula are therefore the sites of the first contact between a pollen grain and the stigma, and this is where the mechanisms inducing the germination of the pollen tube are localized. The stigma also is the site of recognition and rejection (mainly in sporophytic self-incompatibility) of incompatible pollen grains (K n o x 1984). Under *in vitro* conditions, Ca^{2+} plays an important role in the germination of pollen grains (Brewbaker and Wack 1963). The normal growth of the pollen tube is also dependent on the optimal concentration of these ions in the medium (Piction and Steer 1983). Studies conducted using $^{45}\text{Ca}^{2+}$ have shown that there is an influx of calcium ions into the pollen grain and tube during these

processes (Jaffe et al. 1975, Bednarska 1989a) and that a characteristic gradient of these ions is formed. Calcium ions accumulate near the aperture of a germinating pollen grain; in the growing pollen tube — mainly near its tip (Reiss and Herth 1978, Polito 1983, Reiss et al. 1985, Bednarska et al. 1988). The involvement of Ca^{2+} ions in pollen-pistil interactions has also been suggested. It has been found in *Brassica oleracea* that after self-pollination the pollen has a higher calcium content than after cross-pollination (Singh et al. 1989). It cannot be excluded that calcium is a messenger between the pollen grain and pistil. Ca^{2+} -dependent protein kinases occur in *Nicotiana glauca* pollen tubes germinating *in vitro* and could be involved in the calcium-mediated transduction of signals between the pistil and pollen tube (Polya et al. 1986). The response to recognition of incompatible pollen grain (self-pollen) is the accumulation of callose at the tip of the pollen tube, the so-called callose response (rev. Gaudin and Dumais 1987). The synthesis of callose in *Oenothera biennis* pollen tubes growing *in vitro* is dependent on the intracellular concentration of calcium, which is regulated through the uptake of these ions from the medium (Bednarska 1989b).

Processes dependent on Ca^{2+} probably occur on the stigma. It is supposed that, similarly as *in vitro*, calcium ions are an important link in the chain of events regulating the germination of pollen grains and growth of pollen tubes *in vivo*. In accepting this hypothesis, it must be assumed that calcium ions are present on the surface of the stigma along with mechanisms controlling their concentration. A high level of calcium in the surface cells of the *Ruscus aculeatus* stigma was shown previously using the CTC method and X-ray microanalysis (Bednarska 1989c). This paper presents the results of cytochemical studies on the localization of calcium and Ca-ATPase in mature, unpollinated stigma of *Primula officinalis* and *Ruscus aculeatus*.

MATERIAL AND METHODS

Mature, unpollinated stigmas of *Ruscus aculeatus* L. and pin morph style stigmas of *Primula officinalis* L. were fixed for electron microscopy for 1 hour in 2.5% glutaraldehyde in cacodylate buffer, pH 7.2, then rinsed 3 times in the same buffer (1 hour), post-fixed in 1% OsO_4 , dehydrated in an ethyl alcohol series and embedded in Epon 812. Ultrathin sections were stained according to Reynolds (1963) and examined in a Tesla BS 500 electron microscope.

In order to non-specifically stain the surface of *Primula officinalis* stigma, the material was incubated in a 3% aqueous solution of lanthanum chloride and fixed for electron microscopy.

Calcium was localized by two methods: in a fluorescence microscope using chlorotetracycline (CTC, Serva) according to Reiss and Herth (1978) and in an electron microscope using N, N-naphthaloylhydroxylamine sodium salt (NHA, Fluka) according to Sobota et al. (1987). Ca^{2+} was localized on

unfixed *P. officinalis* pistils using the CTC method as described earlier (Tretyn et al. 1987). Stigma which had previously been rinsed for 15 min. with a 1 mM aqueous solution of EGTA (ethylene glycol bis-(β -aminoethyl ether)-N,N, N,N-tetraacetic acid, Serva) before being placed in CTC were used as controls. Mature stigma of *P. officinalis in situ* were also washed with a solution of EGTA. After 15 min. the EGTA solution was rinsed off with distilled water and the fluorescence of CTC was studied after 2, 4 and 6 hours.

NHA at 5 mM was added to 2.5% glutaraldehyde in cacodylate buffer, pH 7.2 and used for the incubation of stigma for 1 hour, after which they were rinsed using cacodylate buffer, pH 7.2 and postfixed in 1% OsO_4 (1 hour). Stigma that had been rinsed for 15 min. in 1 mM EGTA before being placed in the incubation solution were used as controls. The precipitates on sections of *R. aculeatus* were rinsed out for 10 min. with 1 mM EGTA pH 8.0, temp. 60°) according to Vaughan et al. (1987).

Ca-ATPase activity was located using the method of Ando et al. (1981). The stigma were incubated for 1 hour in a medium containing: 250 mM glycine-NaOH buffer (pH 9.0), 3 mM ATP, 10 mM CaCl_2 , 4 mM lead citrate, 8 mM levamisole (an inhibitor of alkaline phosphatases). Stigma incubated without ATP or Ca^{2+} were used as controls.

RESULTS

Papillae approximately $45\text{ }\mu\text{m}$ long and $11\text{ }\mu\text{m}$ wide were found on the surface of the pin morph style stigmas of *P. officinalis*. The small amount of cytoplasm in them was gathered mainly in the base, whereas in the apical part, a thin layer of cytoplasm stretched between a vacuole and the cell wall. The papillae were surrounded by a wall (about $2\text{ }\mu\text{m}$ thick), on the outer side of which no exudate was observed — dry stigma. Its surface was covered by a pellicula which was visualized by staining with lanthanum chloride (Fig. 4). The pellicula was often torn away from the cell wall because unfixed stigma were incubated in La^{2+} .

The surface of the *R. aculeatus* stigma was formed by monocellular, small papillae ($20\text{ }\mu\text{m}$ long and $10\text{ }\mu\text{m}$ wide) which were covered with copious amounts of a lipid exudate — wet stigma.

In *P. officinalis* pistils, the fluorescence of the CTC- Ca^{2+} complex was limited only to the stigma (Fig. 1), where mainly the papillae fluorized (Fig. 2). Strong fluorescence indicating the presence of membrane-bound calcium was localized in the cytoplasm layer near the wall (Fig. 2). The fluorescence of CTC- Ca^{2+} was also observed in the form of a thin layer, probably the pellicula, surrounding the cell wall, which was especially well visible when, because of plasmolysis, the strongly fluorescent cytoplasm was separated from the cell wall. Fluorescence of CTC- Ca^{2+} in the pellicula indicates that calcium ions are bound similarly there as in membranes. Fifteen-minute washing of stigma in 1 mM EGTA inhibited the fluorescence of chlorotetracycline both outside of the cell wall and in

PLATE I

Fig. 1. Fluorescence of the CTC-Ca²⁺ complex in a *Primula officinalis* stigma. s — stigma, p — style, x 100. Fig. 2. Fluorescence of the CTC-Ca²⁺ complex in the external layers of cytoplasm and in the pellicula of the papillae (arrow). x 680. Fig. 3. Lack of CTC-Ca²⁺ fluorescence in a stigma preincubated for 15 min. in 1 mM EGTA. x 680. Fig. 4. Papilla of *P. officinalis* stigma following lanthanum chloride. La³⁺ deposits visualize the pellicula. p — pellicula, cw — cell wall, cy — cytoplasm. x 25000. Fig. 5. Papilla of *P. officinalis* stigma after incubation with NHA. Electron-dense precipitates on the pellicula (arrows). High electron density of the endoplasmic reticulum in the cytoplasm (cy). p — pellicula, cw — cell wall. x 47000. Fig. 6. A *P. officinalis* stigma papilla washed in 1 mM EGTA before incubation with NHA. Complete lack of precipitates on the pellicula. cw — cell wall, cy — cytoplasm. x — 47000

PLATE II

Figs. 7-8. *A. aculeatus* stigma papilla after incubation in NHA. Fig. 7 — precipitates on the plasma membrane and in mitochondria (arrows). Fig. 8 — the section after washing with 1 mM EGTA — partial whitening of the precipitates. m — mitochondrium, cw — cell wall, pl — plasma membrane, cy — cytoplasm. x 47000

Fig. 9. The activity of Ca-ATPase in *P. officinalis* stigma papillae. Enzymatic reaction products (arrows) in the pellicula (p). cw — cell wall, cy — cytoplasm, pl — plasma membrane. x 30000. Fig. 10. Ca-ATPase activity control — no precipitates on the pellicula. cw — cell wall, cy — cytoplasm. x 25000. Fig. 11. Ca-ATPase activity in *R. aculeatus* stigma papillae. The product of the enzymatic reaction on the plasma membrane (pl). cw — cell wall, cy — cytoplasm. x 45000. Fig. 12. Control of Ca-ATPase activity — without substrate (ATP). No precipitates on the plasma membrane. x 45000

the cytoplasm (Fig. 3). The inhibition of fluorescence of the CTC- Ca^{2+} complex in stigma *in situ* was a reversible process. After about 6 hours following chelation of calcium with EGTA the fluorescence of CTC- Ca^{2+} in papillae resumed. This points to the renewed appearance of calcium ions in the papillae of the receptive stigma of *P. officinalis*.

In the electron microscope, the precipitation method revealed calcium ions on the pellicula of the *P. officinalis* stigma in the form of tiny and somewhat larger electron-dense NHA- Ca^{2+} precipitates (Fig. 5). The electron density of the cytoplasm increased around the endoplasmic reticulum, which may be indicative of the presence of Ca^{2+} in these membranes. After washing in 1 mM EGTA no NHA- Ca^{2+} precipitates were formed on the pellicula (Fig. 6) with the endoplasmic reticulum also losing its distinct, electron-dense nature.

Calcium ions were localized on the plasma membrane and in the mitochondria of the papillae of the *R. aculeatus* stigma. Electron-dense precipitates appeared on the plasma membrane and in the mitochondria after the cytochemical reactions. The methods used in this study did not give unequivocal results showing Ca^{2+} in the exudate on the surface of the stigma, although earlier studies using CTC and X-ray analysis did show the presence of calcium there (Bednarska 1989c). The precipitates were partially removed when the sections were washed in 1 mM EGTA, which indicated that calcium was involved in their formation (Fig. 8).

The Ca-ATPase of the papillae of *P. officinalis* stigma occurred mainly in the pellicula. Irregularly shaped electron-dense precipitates were formed all over the surface of the pellicula as the result of cytochemical reactions (Fig. 9). Tiny precipitates were also found on the plasma membrane of the papillae. After incubation of the stigma in a medium lacking Ca^{2+} or ATP the precipitates formed only sporadically on the surface of the pellicula (Fig. 10).

Ca-ATPase occurred mainly in the plasma membrane of the *Ruscus aculeatus* stigma processes. Numerous irregularly shaped electron-dense precipitates were present there (Fig. 11). No precipitates were formed in reactions carried out without the substrate (Fig. 12).

DISCUSSION

It has been shown in this study that the surface cells of both types of stigma are places where calcium is present. The NHA technique was used to demonstrate that calcium ions are found mainly on the plasma membrane and in mitochondria of *R. aculeatus* papillae. It can thus be assumed that they participate in the regulation of the inter- and extracellular (exudate) level of calcium. The high level of calcium in the surface cells and in the exudate of *R. aculeatus* stigma had previously been determined using the CTC method and X-ray analysis (Bednarska 1989c).

Calcium also accumulates in the papillae of the dry stigma of *P. officinalis*. It

is found in their pellicula and cytoplasm. The studies presented here are the first direct confirmation of the presence of Ca^{2+} on the surface of the dry stigma. The presence of calcium in the pellicula was determined using two methods — fluorescence of the CTC- Ca^{2+} complex and the formation of NHA- Ca^{2+} precipitates. Chelation of calcium ions with EGTA prevented both the fluorescence of CTC and the formation of NHA- Ca^{2+} precipitates. Demonstrating the presence of calcium in the pellicula by these two methods shows that both free and bound calcium ions occur there. The fluorescence of CTC- Ca^{2+} is commonly considered to be a method of identifying membrane-bound calcium (Casswell 1979). In spite of the fact that the pellicula does not have a structure typical for biological membranes, it still has many of their properties, e.g. adenyl cyclase and ATPase activities (Gaudé and Dumas 1986). The fluorescence of CTC in the pellicula is probably the result of similar binding of Ca^{2+} as in membranes, which would be more evidence that this protein film has properties of a biological membrane. The possibility that calcium occurs in the pellicula is supported, howbeit indirectly, by the binding of La^{3+} ions, which react with calcium-binding sites (rev. Shal'ai and Tavassoli 1982). The pellicula was visualised with the use of La^{3+} ions in *Silene vulgaris* (Heslop-Harrison et al. 1975) and in *Brassica oleracea* (Gaudé and Dumas 1986).

The accumulation of Ca^{2+} on the surface of both types of stigma permits the conclusion that processes along with their specialized surface in the form of the pellicula or exudate are adapted to biological protection of the calcium environment at the site where the first contact between the stigma and the pollen grain takes place and the germination of the pollen grain begins. It is well known that calcium ions play a key role in the germination and growth of the pollen tube *in vitro* (Brewbaker and Kwack 1963, Picton and Steer 1983). The calcium gradient that is formed during this process (Reiss and Herth 1978, Polito 1983, Reiss et al. 1985, Bednarska et al. 1988) is done so with the use of exogenous Ca^{2+} ions (Jaffe et al. 1975, Bednarska 1989a). The uptake of these ions from the medium is regulated by calcium channels. The blocking of the channels reduces pollen germination (Bednarska 1989a) and disrupts the polarization of pollen tubes (Reiss and Herth 1985). In addition, *in vitro* the amount of Ca^{2+} taken up from the medium regulates the synthesis of tube callose, whose deposition at the pollen tube tip *in vivo* is an important element of rejection of self-incompatible pollen grains (self-pollen), the so-called callose response (see rev. Gaudé and Dumas 1987). Lowering the uptake of Ca^{2+} inhibits the synthesis of callose, while increasing it induces the synthesis of this polysaccharide at the tip of the tube (Bednarska 1989b). Studies on *Brassica oleracea* have shown that after self-pollination, pollen grains are characterized by a higher calcium level than after cross-pollination (Singh et al. 1989). The authors suggest that this is the result of different amounts of calcium ions taken up during self- and cross pollination from the intra- and extracellular pool of calcium, e.g. from the cell wall. The presence of calcium on

the surface of the stigma, as demonstrated in this study and previously (Bednarska 1989c), probably serves the germinating pollen grains. The amount of Ca^{2+} taken up from the surface of the stigma may regulate the germination of the pollen and the synthesis of callose at the tip of the pollen tube. It may thus be an important factor in pollen-pistil interaction. This would require mechanisms controlling the accumulation of Ca^{2+} on the surface of the stigma and the uptake of these ions by the germinating pollen grains. The results obtained in this study point to the possibility that this pool is replenished on the surface of the *P. officinalis* stigma after the action of EGTA and point to the existence of a mechanism regulating the Ca^{2+} level on the surface of the stigma. It should be assumed that this mechanism is a calcium pump related to the activity of Ca-ATPase. This enzyme has been localized in the plasma membrane of both types of stigma and, in addition, in *P. officinalis* it is also present in the pellicula. ATPase is also present in the pellicula and plasma membrane of *Brassica oleracea* papillae (Gauze and Dumas 1986). Maybe the complex of pollen and pistil S proteins, which is formed after pollination, affects the activity of Ca-ATPase, thus the freeing of Ca^{2+} and regulates the calcium channels, thus affecting the uptake of these ions by the germinating pollen grains.

Acknowledgments

I am grateful to Doc. Alicja Górska-Brylasek for helpful discussions and to Prof. Bohdan Rodkiewicz for the critical reading of the manuscript.

REFERENCES

- Ando T., Fujimoto K., Mayhara H., Ogawa K. 1981. A new one-step method for the histochemistry and cytochemistry of Ca-ATPase activity. *Acta Histochem. Cytochem.* 14: 705-707.
- Bednarska E., 1989a. The effect of exogenous Ca^{2+} ions on pollen grain germination and pollen tube growth — investigations with the use of $^{45}\text{Ca}^{2+}$, and Verapamil, La^{3+} and ruthenium red. *Sex. Plant Reprod.* 2: 53-58.
- Bednarska E., 1989b. The effect of intracellular calcium level regulators on the synthesis of pollen tube callose in *Oenothera biennis* L. *Acta Soc. Bot. Pol.* 58: 39-50.
- Bednarska E., 1989c. Localization of calcium on the surface of stigma in *Ruscus aculeatus* L. — studies by the chlorotetracycline and X-ray microanalysis. *Planta* 179: 11-16.
- Bednarska E., Tretyn A., Górska-Brylasek A., 1988. Localization of Ca^{2+} and peroxidase activity in zone of callose synthesis in *Haemanthus albiflos* L. pollen tube. *Acta Physiol. Plant.* 10: 237-241.
- Brewbaker J., Kwack B.H., 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *Amer. J. Bot.* 50: 859-865.
- Caswell A., 1979. Methods for measuring intracellular calcium. *Int. Rev. Cytol.* 56: 145-181.
- Gauze T., Dumas C., 1986. Organization of stigma surface components in *Brassica oleracea*: a cytochemical study. *J. Cell Sci.* 82: 203-216.
- Gauze T., Dumas C., 1987. Molecular and cellular events of self-incompatibility. *Int. Rev. Cytol.* 107: 333-366.

- Heslop-Harrison J., Heslop-Harrison Y., Barber J., 1975. The stigma surface in incompatibility responses. *Proc. R. Soc. B* 188: 287-297.
- Jaffe L.A., Weisenseel M.H., Jaffe L.F., 1975. Calcium accumulations within the growing tips of pollen tubes. *J. Cell Biol.* 67: 488-492.
- Knox R.B., 1984. Pollen-pistil interaction In: Cellular interaction. H.F. Linskens, J. Heslop-Harrison (eds.), Springer-Verlag, Berlin-Heidelberg-New York-Tokyo, pp. 508-608.
- Picton J.M., Steer M.W., 1983. Evidence for the role of Ca^{2+} ions in tip extension in pollen tubes. *Protoplasma* 115: 11-17.
- Polito V.S., 1983. Membrane-associated calcium during pollen grain germination: a microfluorometric analysis. *Protoplasma* 117: 226-232.
- Polya G.M., Miccuci V., Rae A.L., Harris P.J., Clarke A.E., 1986. Ca^{2+} -dependent protein Phosphorylation in germinated pollen of *Nicotiana glauca* an ornamental tobacco. *Physiol. Plant.* 67: 151-157.
- Reiss H.D., Herth W., 1978. Visualization of the Ca^{2+} gradient in growing pollen tubes of *Lilium longiflorum* with chlorotetracycline fluorescence. *Protoplasma* 97: 373-377.
- Reiss H.D., Herth W., 1985. Nifedipine-sensitive calcium channels are involved in polar growth of lily pollen tubes. *J. Cell Sci.* 76: 247-254.
- Reiss H.D., Herth W., Nobiling R., 1985. Development of membrane- and calcium-gradients during pollen germination of *Lilium longiflorum*. *Planta* 163: 84-90.
- Reynolds E.S., 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17: 208-212.
- Shaklai M., Tavassoli M., 1982. Lanthanum as an electron microscopic stain. *J. Histochem. Cytochem.* 30: 1325-1330.
- Singh A., Perdue T.D., Paolillo Jr., 1989. Pollen-pistil interaction in *Brassica oleracea*: cell calcium in self and cross pollen grain. *Protoplasma* 151: 57-61.
- Sobota A., Skoczylas B., Głowacka S.K., 1987. Analysis of cytochemical procedure of precipitation of calcium ions by N,N-naphthaloylhydroxylamine (NHA) in *Acanthamoeba* cells. *Acta Protozool.* 26: 145-152.
- Tretyn A., Bednarska E., Górska-Brylarska A., 1987. Localization of Ca^{2+} ions and peroxidase activity in the zone of callose wall synthesis in dyads and tetrads of *Larix decidua* Mill. *Cell Biol. Intern. Rep.* 11: 157-164.
- Vaughan M.A., Mulkey T.J., Goff Ch.W., 1987. Localization of calcium in roots and microsomal membranes of corn by direct pyroantimonate precipitation. *J. Histochem. Cytochem.* 35: 781-788.

Lokalizacja wapnia i Ca-ATPazy na suchym znamieniu Primula officinalis i mokrym znamieniu Ruscus aculeatus

Streszczenie

Komórki powierzchniowe obydwu typów znamion są miejscem występowania wapnia. Metodą chlorotetracyklinową wykazano, że wyrostki suchego znamienia *Primula officinalis* są źródłem fluorescencji kompleksu CTC- Ca^{2+} . Metodą precypitacyjną (NHA) jony wapniowe zlokalizowano głównie w pelikuli otaczającej wyrostki. W wyrostkach mokrego znamienia *Ruscus aculeatus* precypitaty NHA- Ca^{2+} zlokalizowane są na plazmalemie. W obydwu typach znamion w miejscach występowania Ca^{2+} stwierdzono aktywność Ca-ATPazy.