LOCALIZATION OF MEMBRANE-ASSOCIATED CALCIUM IN UNPOLLINATED AND POLLINATED PISTIL OF *PETUNIA HYBRIDA* HORT.

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(Received: October 10, 1994. Accepted: February 10, 1995)

ABSTRACT

In the pistil of *Petunia hybrida*, the transmitting tract and the ovules are the sites which give Ca^{2+} -CTC fluorescence. In unpollinated pistil the level of membrane-associated Ca^{2+} decreases from the stigma to the base of the style. The renewed strong rise of Ca^{2+} -CTC fluorescence appears on the placenta surface and in the ovule integuments. Following pollination, when the pollen tubes have grown through the pistil, the pattern of membrane-associated Ca^{2+} on the path stigma – ovary is reversed. The highest fluorescence is found in the base of the style. In pollinated ovules the Ca^{2+} -CTC fluorescence increases markedly. In the transmitting cells membrane-associated Ca^{2+} occurs mainly in the polar regions of the cell. During cell degeneration following pollination the cytoplasmic clusters show Ca^{2+} -CTC fluorescence.

The used *P. hybrida* cultivar is self-fertile. The selection of pollen tubes occurs mainly in the upper part of the style. The rejected pollen tubes show a steady high level of membrane-associated calcium.

KEY WORDS: *Petunia hybrida*, calcium-chlorotetracycline, pollen-pistil interaction.

INTRODUCTION

Sexual reproduction in angiosperms is accomplished through interaction between the cells of the male gametophyte and those of the pistil transmitting tract and the embryo sac. Studies in the last decade have revealed that in plants, like in animals, the regulation of many metabolic processes, including information exchange, is mediated by Ca^{2+} system (Poovaiah and Reddy 1987, Boss 1989). This has lead many researchers to be interested in the role of Ca^{2+} in sexual reproduction of angiosperms.

The role of Ca^{2+} in pollen germination *in vitro* has been known for a long time (Brewbaker and Kwack 1963). Optimum concentration of these ions in the medium (Picton and Steer 1983) and a characteristic tip-to-base calcium gradient in the pollen tube (Steer and Steer 1989, Rathore and al. 1991, Miller et al. 1992,) are essential for normal growth of the pollen tubes. It is suggested that in the growing pollen tubes, Ca^{2+} regulate the functioning of the cytoskeleton and tip directed exocytosis (Steer and Steer 1989).

Recent studies on Ca^{2+} distribution in the pistil have related mainly to the stigma surface and the embryo sac. The elevated Ca^{2+} level in the papillae of the dry stigma (Tirlapur and Shiggaon 1988, Bednarska and Karbowska 1990) as well as in the exudate and surface cells of the wet stigma (Bednarska 1989) probably points to the involvement of these ions in the reception and early events of germination of pollen *in vivo*. The rise of Ca^{2+} level in the synergid found in several of the plants studied (Chaubal and Reger 1990, 1992a, Huang and Russell 1992,) has made it possible to put forward the hypothesis that these ions participate in directing pollen tube growth towards the egg apparatus, and also in the opening

mechanism of the tube. The increase in Ca^{2+} concentration in the micropylar region after the release of the sperm cells, may – according to Chaubal and Reger (1992b) – prevent further pollen tubes from growing into the ovule.

Ca^{2+} in the pistil is taken up by germinating pollen (Bednarska 1991) and growing pollen tubes (Bednarska and Butowt 1995). This indicates that calcium ions are also involved in growth regulation of the pollen tubes *in vivo*. The present work brings the results on the localization of calcium in unpollinated and pollinated pistil of *Petunia hybrida* using chlorotetracycline.

MATERIAL AND METHODS

The material for study were unpollinated and self-, and cross-pollinated pistils of *Petunia hybrida* Hort. The pollinated pistils were taken 18-24 h (unwilted flower) and 72-78 h (perianth starting to wilt) after pollination. The studies were carried out on hand-cut longitudinal sections of stigma, transverse sections of style, isolated transmitting tissue and on sections of placenta with ovules. Transmitting tissue was isolated from three parts of the style: part I – upper 1/3 of the style, part II – middle 1/3 of the style, part III – lower 1/3 of the style.

Calcium was localized using chlorotetracycline (CTC; Serva Heidelberg, FRG) after Reiss and Herth (1978). The unfixed material was placed for 10 min in a water solution of 10^{-4}M CTC and 2% saccharose. Samples were observed with a fluorescence microscope (Fluoval, Zeiss, Jena, FRG) equipped with a BG 12 excitation filter. For monitoring Ca^{2+} – CTC emission a narrow-pass interference barrier filter with a peak at 532 nm was used. Photographs were taken on a

Figs 1-4. Ca^{2+} -CTC fluorescence in unpolinated pistil.

Fig. 1. Stigma – fluorescence of exudate (e) and of stigmatic tissue (S). x 160.

Fig. 2. Transverse section of style at the base of stigma – fluorescence of transmitting tract (T) and of vascular bundles (b). x 100.

Figs 3-4. Transmitting cells. x 400.

Fig. 3. In cells from style part I strong fluorescence of polar regions, i.e. under transverse walls.

Fig. 4. In a cell from style part II the fluorescence is localized in polar regions and in cytoplasm among vacuoles and starch grains; the cell next to it is nearly completely devoid of fluorescence.

Fig. 5. Placenta with ovules – fluorescence of placenta surface and of the site of funiculi emergence (arrow); in ovules Ca^{2+} -CTC fluorescence is found in the external integument cells. x 100.

black and white HL 400 ASA film (Foton, Poland). Control samples were: (1) untreated with CTC, (2) washed for 10 min in 1 mM ethylene glycol-bis-(β -aminoethyl ether)-N,N,N-tetraacetic acid (EGTA; Serva, Heidelberg, FRG) and then placed in CTC solution.

The level of the Ca^{2+} -CTC fluorescence of the transmitting tissue was based on percentage of cells showing fluorescence, compared with the total number of cells (5 flowers, 80-100 cells from each style part).

RESULTS

Unpollinated pistil

In the unpolinated pistil of *P. hybrida* the Ca^{2+} -CTC fluorescence occurs throughout the whole transmitting tract. The fluorescence decreased on the path from the stigma to the base of the style (Figs 6A, B, Tab. 1A). A strong rise of fluorescence was observed in the placenta and the ovule (Fig. 5).

In the stigma, the fluorescence was localized in the surface exudate and in the stigmatic cells (Fig. 1). In the style Ca^{2+} -CTC fluorescence was limited to the centrally located transmitting tissue and the two vascular bundles (Fig. 2). The highest number of fluorescent transmitting cells was below the stigma (ca. 56%) and diminished towards the ovary. In the middle part of the style there were ca. 31% of fluorescent cells and only ca. 10% before the ovary (Tab. 1A). This indicates a stigma-to-ovary gradient of Ca^{2+} -CTC in the transmitting tissue of the *P. hybrida*.

The transmitting cells show polarity in Ca^{2+} -CTC distribution. In the cells beneath the stigma a high fluorescence was noted in the polar regions, that is under the transverse walls (Fig. 3). In the lower parts of the style, in the more elongated cells, Ca^{2+} -CTC fluorescence was localized also in the cytoplasm among the vacuoles and starch grains (Fig. 4).

In the placenta the fluorescence accumulated on its surface and in the sites of emergence of funiculi (Fig. 5). In unpolli-

Fig. 6. Ca^{2+} -CTC fluorescence in transmitting tissue of unpollinated pistil.
Fig. 6A. Just beneath stigma most cells shows fluorescence of plasmalemma and polar cytoplasm. x 200.
Fig. 6B. In transmitting tissue from style part III fluorescence is visible in scattered cells. x 160.
Fig. 7. Ca^{2+} -CTC fluorescence in transmitting tissue 72 h after pollination. x 160.
Fig. 7A. Nearly complete decline of fluorescence in transmitting cells of style part I.
Fig. 7B. Comparatively strong fluorescence of cytoplasm in transmitting cells of style part III (arrow – pollen tube).

TABLE 1. Percentage of cells showing Ca^{2+} -CTC fluorescence in successive style parts of unpollinated pistils (A) and pistils 72 h after pollination (B).

A	%	PISTIL					% OF FIVE PISTILS
		1	2	3	4	5	
	P						
	I	70	49	64	45	52	56.0
	II	39	25	41	22	30	31.4
	III	15	28	11	7	10	10.2
B	I	39	28	36	31	42	35.2
	II	58	69	85	49	75	67.2
	III	93	87	99	98	100	95.4

% – percentage of fluorescence cells, P – part of style

nated ovules increased fluorescence was observed in the epidermal cells of the external integument.

Pollinated pistil

The used cultivar of *P. hybrida* proved to be self-fertile. After both self- and cross-pollination seeds were set up. However, irrespective of the pollination, the pollen tubes growing in the pistil were selected and the development of some of them was inhibited. The inhibition of the pollen tubes took place mainly in part I of the style. The rejected male gametophytes were characterized by strong fluorescence of the grain and the pollen tube over its entire length (Figs. 8A, B). The pollen tubes which grew through the style inhibition zone generally showed no fluorescence. Strong fluorescence was visible in the transmitting cells (Fig. 8C, D).

3-4 days after pollination the pollen tubes reached the ovary and penetrated into the ovules. In the pistil grown through by the pollen tubes the pattern of the Ca^{2+} -CTC fluorescence on the path stigma-ovary became reversed (Figs 7A, B, Tab. 1B). The number of transmitting cells showing fluorescence was highest, ca. 95%, in the part of the style adjoining to the

Fig. 8. Ca^{2+} -CTC fluorescence in the same pistil 24 h after pollination. x 160. In the stigma (Fig. 8A) and in transmitting tissue of style part I (Fig. 8B) strong fluorescence of rejected pollen grains and tubes. Fig. 8C-D. The lower part of style; (8C – fluorescence, 8D – bright field). Pollen tubes (PT) growing in this region show no fluorescence; it can be seen in transmitting cells (TC). x 200.

Fig. 9. Intense fluorescence of internal tissues of pollinated ovule. Pronounced increase of fluorescence in micropyle (arrow). x 160.

Fig. 10. Transmitting cells 72 h. after pollination. x 400.

Fig. 10A. In the cells from style part I fluorescence occurs only in scattered cytoplasmic clusters.

Fig. 10B. In the cell from style part III fluorescence of polar region (arrow) and cytoplasmic clusters can be seen.

ovary (part III). Their number gradually decreased towards the stigma (about 67% cells in part II and about 35% cells in part I). At that time after pollination, the stigma showed practically no Ca^{2+} -CTC fluorescence. The fluorescence pattern of the style transmitting cells was also changed. In the cells from part I Ca^{2+} -CTC fluorescence occurred only in scattered cytoplasmic clusters, while the fluorescence of polar regions was disappearing (Fig. 10A). The transmitting cells from lower parts of the style showed Ca^{2+} -CTC fluorescence of the polar regions and the large cytoplasmic clusters (Fig. 10B).

After the pollen tubes penetrated into the ovules, considerable increase of fluorescence was observed in it (cf. Fig. 5 with 10). The placenta remains the site of a high Ca^{2+} -CTC fluorescence.

Control tests have shown that the transmitting tract of the pistil and the ovules of *P. hybrida* do not exhibit autofluorescence and washing in EGTA prevents the formation of Ca^{2+} -CTC complex.

DISCUSSION

The method used in the study makes it possible to reveal membrane-associated calcium (Timmers et al. 1989) and high free calcium concentration in the vicinity of hydrophobic sites (Tsien 1989). In the pistil of *P. hybrida* this pool of calcium is localized preferentially in the transmitting tract and the ovules. The parenchymatic cells surrounding the transmitting tissue showed no fluorescence. The distribution of calcium revealed by CTC in unpollinated and pollinated pistil is different. In unpollinated pistil the level of membrane-associated Ca^{2+} decreases from the stigma to the base of the style. The highest number of cells with Ca^{2+} -CTC fluorescence occurs just beneath the stigma and diminishes towards the ovary. Renewed rise in Ca^{2+} -CTC fluorescence is observed on the placenta surface and in the ovule's integument. The previous study has revealed that the total and ionic calcium is steady throughout the length of the unpollinated style (Mascarenhas 1966, Glenk et al. 1971) or increases towards the ovary (Tirlapur 1988). Because of different methods used it is difficult to compare these contradictory results. A large increase of the total and ionic calcium on the path from the lower part of the style to the placenta was noted in *Antirrhinum majus* and *Oenothera longiflora* (Glenk et al. 1971).

Following pollination, when the pollen tubes have grown through the pistil, the distribution of membrane-associated Ca^{2+} on the path stigma – ovary is reversed. Ca^{2+} -CTC fluorescence increases towards the base of the style. In the upper parts of the pistil fluorescence gradually declines. This phenomenon reflects undoubtedly the progressive degenerative processes in the cells of transmitting tissue. In the pollinated ovules, developing into seeds, Ca^{2+} -CTC fluorescence increases markedly.

The reversal membrane-associated calcium gradient in the style is associated with change in the fluorescence pattern in the transmitting cells. In the cells of the unpollinated pistil the highest level of Ca^{2+} -CTC fluorescence is found in the polar regions, that is in the place of interconnections between the cells. Occurring of Ca ions, associated with the transversal plasmalemma and the membranes of endoplasmic reticulum in the polar cytoplasm, in the part of transmitting cells of *P. hybrida* was recently found by pyroantimonate method (Bednarska and Butowt 1995). Such distribution of Ca^{2+} may suggest that these ions take part in transmitting signals along the transmitting tissue of the style. Changes in electric potential in the transmitting tissue as an effect of pollination have been

recorded in *Petunia* by Linskens and Spanjers (1973). In transmitting cells from the degenerating tissue, grown through by pollen tubes, the polar distribution of membrane-associated calcium declines. Inside the cell, the Ca^{2+} -CTC fluorescence shows irregular bands and clusters.

The *P. hybrida* cultivar used in the present study is self-fertile. However, we deal here with the phenomenon of pollen tubes' selection. The main zone of inhibition of the pollen tubes is localized at a short distance from the stigma. Rejection of part of pollen tubes in the self-fertile *P. hybrida* has been described recently by Crusan (1993). The mechanisms of selection of pollen tubes in self-fertile plants are not well understood. They are different from those in gene S action (see discussion in Crusan 1993).

The rejected pollen tubes show a high Ca^{2+} -CTC fluorescence level over all their lengths. The disappearance of Ca^{2+} gradient in non-growing pollen tubes has been reported from *in vitro* studies (Rathore et al. 1991, Miller et al. 1992). A rise in calcium level, including Ca^{2+} detected by CTC, has been found in the course of rejection of self-incompatible pollen grains on the stigma of the sporophytic *Brassica oleracea* (Singh et al. 1989). The results obtained in the present study demonstrate that the lack of calcium gradient and rise of the membrane-associated Ca^{2+} are also associated with the inhibition of the development of pollen tubes *in vivo*. These phenomena need not be related with the self-incompatibility system.

ACKNOWLEDGEMENTS

This work was supported by the Polish Committee for Scientific Research (KBN), No. 6P203 04204.

LITERATURE CITED

- BEDNARSKA E., 1989: 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., 1991: Calcium uptake from the stigma by germinating pollen in *Primula officinalis* L. and *Ruscus aculeatus* L. *Sex. Plant. Reprod.* 4: 36-38.
- BEDNARSKA E., KARBOWSKA J., 1990: Localization of Ca^{2+} and Ca-ATPase in papillae of maturing stigma in *Pharbitis nil* L. *Phytomorphology* 40: 323-329.
- BEDNARSKA E., BUTOWT R., 1995: Calcium in pollen-pistil interaction in *Petunia hybrida* Hort. II. Localization of Ca^{2+} ions and Ca^{2+} -ATPase in unpollinated pistil. *Folia Histochem. Cytobiol.* 33: 43-52.
- BOSS W.F., 1989: Phosphoinositide metabolism: Its relation to signal transduction in plants. In: *Second Messenger in Plant Growth and Development*, Liss AR ed. Inc. 29-56.
- BREWBAKER J.L., KWACK B.H., 1963: The essential role of calcium ion in pollen germination and pollen tube growth. *Am. J. Bot.* 50: 859-865.
- CHAUBAL R., REGER B.J., 1990: Relatively high calcium is localized in synergid cells of wheat ovaries. *Sex. Plant Reprod.* 3: 98-102.
- CHAUBAL R., REGER B.J., 1992a: Calcium in the synergid cells and other regions of pearl millet ovaries. *Sex. Plant Reprod.* 5: 34-46.
- CHAUBAL R., REGER B.J., 1992b: The dynamics of calcium distribution in the synergid cells of wheat following pollination. *Sex. Plant Reprod.* 5: 206-213.
- CRUZAN M.B., 1993: Analysis of pollen-style interaction in *Petunia hybrida*; the determination of variance in male reproductive success. *Sex. Plant Reprod.* 6: 275-281.

- GLENK H.O., WAGNER W., SCHOMMER O., 1971: Can Ca^{++} ions act as a chemotropic factor in *Oenothera* fertilization? In: Pollen: Development and Physiology, ed J. Heslop-Harrison, London, Butterworths, pp 255-260.
- HUANG B-Q., RUSSELL S.D., 1992: Synergid degeneration in *Nicotiana*: a quantitative, fluorochromatic, and chlorotetracycline study. Sex. Plant Reprod. 5:151-155.
- LINSKENS H.F., SPANJERS W., 1973: Changes of the electric potential in the transmitting tissue of *Petunia* styles after cross- and self-pollination. Incompat. Newslett. 3: 81-85.
- MASCARENHAS J.P., 1966: The distribution of ionic calcium of the gynoecium *Antirrhinum majus*. Protoplasma 62: 53-58.
- MILLER D.D., CALLAHAM D.A., GROSS D.J., HEPLER P.K., 1992: Free Ca^{2+} gradient in growing pollen tubes of *Lilium*. J. Cell. Sci. 101: 7-12.
- 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.
- POOVAIAH B.W., REDDY A.S.N., 1987: Calcium messenger system in plants. RCR Crit. Rev. Plant. Sci. 6: 47-103.
- RATHORE K.S., CORK R.J., ROBINSON K.R., 1991: A cytoplasmic gradient of Ca^{2+} is correlated with the growth of lily pollen tubes. Develop. Biol. 148: 612-619.
- 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.
- SINGH A., PERDUE T.D., PAOLILLO D.J. Jr., 1989: Pollen-pistil interaction in *Brassica oleracea*: cell calcium in self and cross pollen grains. Protoplasma 151: 57-61.
- STEER M.W., STEER J.M., 1989: Pollen tube tip growth. New Phytol. 111: 323-358.
- TIMMERS A.C.J., de VRIES S.C., SCHEL J.H.N., 1989: Distribution of membrane-bound calcium and activated calmodulin during somatic embryogenesis of carrot (*Daucus carota* L.). Protoplasma 153: 24-29.
- TIRLAPUR U.K., 1988: Atomic absorption spectrometric estimation of gynoecial and non-gynoecial tissue calcium in *Ipomoea palmata* Forss. J. Plant Sci. Res. 4: 77-80.
- TIRLAPUR U.K., SHIGGAON S.V., 1988: Distribution of calcium and calmodulin in the papillate cells of stigma surface, visualized using chlorotetracycline and fluorescing calmodulin binding phenothiazines. Ann. Biol. 4: 49-53.
- TSIEN R.Y., 1989: Fluorescent indicators of ion concentration. Methods in Cell Biology 30: 127-156.

LOKALIZACJA WAPNIA ZWIĄZANEGO Z BŁONAMI W NIEZAPYLONYM I ZAPYLONYM SŁUPKU *PETUNIA HYBRIDA* HORT.

STRESZCZENIE

W szlaku transmisyjnym słupka oraz zalążkach *P. hybrida* występuje fluorescencja Ca^{2+} -CTC. W niezapylonym słupku poziom Ca^{2+} -związanego z błonami obniża się w kierunku podstawy szyjki słupka. Ponowny silny wzrost fluorescencji występuje na powierzchni placenty i w osłonkach zalążka. Po przerośnięciu słupka przez łagiewki pyłkowe następuje odwrócenie gradientu Ca^{2+} -związanego z błonami na drodze znamię-zalążnia. Najwyższa fluorescencja tkanki transmisyjnej występuje u podstawy szyjki słupka. W zapylonych zalążkach fluorescencja Ca^{2+} -CTC wyraźnie wzrasta.

W komórkach transmisyjnych Ca^{2+} -związany z błonami zlokalizowany jest głównie w ich regionach biegunowych. Podczas pozapyleniowej degeneracji tych komórek fluorescencja Ca^{2+} -CTC występuje w cytoplazmatycznych agregatach.

Użyta odmiana *P. hybrida* jest samozgodna. Selekcja łagiewek pyłkowych odbywa się głównie w górnej części szyjki. Odrzucone łagiewki pyłkowe wykazują równomierny, wysoki poziom Ca^{2+} -związanego z błonami.

SŁOWA KLUCZOWE: *Petunia hybrida*, wapń-chlorotetracyklina, interakcja pyłek-słupek.