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Immunocytochemical evidence of calreticulin-like protein in pollen tubes and styles of *Petunia hybrida* Hort.

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Summary. With a polyclonal antibody raised against calreticulin (CRT) the locations where the protein occurs in unpollinated and pollinated styles of *Petunia hybrida* were localized. The epitopes binding the CRT antibody were immunolocalized preferentially in pollen tubes. In transmitting tract cells, both before and after pollination, the level of CRT was low. The protein was mainly localized in the cytosol and around dictyosomes of transmitting-tract cells. In pollen tubes, a high level of CRT was found at their tips rich in endoplasmatic reticulum, cisternae piles of reticular and/or dictyosomal origin, and vesicles. Binding sites of the CRT antibody were also found in the internal callosic cell wall of the pollen tube. These results indicate a role of CRT in cells directly participating in pollen–pistil interaction.

Keywords: Calreticulin; Calcium homeostasis; *Petunia hybrida*; Pollen–pistil interaction; Pollen tube; Transmitting tissue.

Abbreviations: CRT PAb anti-calreticulin polyclonal antibody; CRT calreticulin; IP_3 inositol 1,4,5-trisphosphate.

Introduction

In plants, as in animals, calcium ions fulfil the role of a signaling molecule inside the cell in many vital processes (Pozzan et al. 1994, Bush 1995, Clapham 1995, Dedman and Keatzel 1995, Malhó 1999). This requires rapid quantitative and qualitative changes in the content and distribution of Ca^{2+} in the cell. A fundamental role in the storage and mobilization of Ca^{2+} is played by vacuoles and endoplasmic reticulum (ER) (Kauss 1987, Johannes et al. 1991, Bush 1993). In the membranes of these organelles different Ca^{2+} transporters were found such as the Ca^{2+}/H^+ antiporter, the inositol 1,4,5-trisphosphate(IP_3)- and voltage-gated Ca^{2+} channels (Schumaker and Sze 1986, Johannes et al. 1991, Thomson et al. 1993). A specific class of proteins, called reticuloplasmins, occurs in the ER lumen, having a high capacity and low affinity for binding calcium ions (Koch 1987, Macer and Koch 1988). Due to its abundance, the ER is able to accumulate large quantities of Ca^{2+} mobilized during the stimulation of cells.

Calreticulin (CRT) is the major calcium-binding reticuloplasmin in the ER lumen of eukaryotic cells (Michalak et al. 1992). CRT has a hydrophobic retention sequence – KDEL in animals and HDEL in plants – responsible for the presence of the protein in ER (Pelham 1989, 1992; Smith and Koch 1989; Napier et al. 1992). The basic and proven function of CRT is the sequestration and mobilization of Ca^{2+} in the ER lumen and role in calcium signaling (P. Camacho and Lechleiter 1995, Hassan et al. 1995, Mery et al. 1996, Opas et al. 1996, Krause and Michalak 1997). Furthermore, other less-well-understood roles of CRT have also been suggested. This protein has recently been accepted to act as chaperone (Helenius et al. 1997, Zhang et al. 1998) and binds directly to and regulates integrins (Rojiani et al. 1991; Dedhar 1994; Jeffs 1994; Coppolino et al. 1995, 1997). There is also proof that CRT plays an active part in the regulation of the

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expression of genes (Burns et al. 1994, Dedhar 1994, Jeffs 1994, Michalak et al. 1996). Thus, knowledge obtained to date has shown that CRT is a ubiquitous and multifunctional protein (Nash et al. 1994, Crofts and Denecke 1998). In the last years, there has been an increasing interest in the characterization of CRT in plant cells. It has been characterized in spinach (Menegazzi et al. 1993, Navazio et al. 1995), barley (Chen et al. 1994), *Arabidopsis thaliana* (Benedetti and Turner 1995, Nelson et al. 1997), tobacco (Denecke et al. 1995, Borisjuk et al. 1998), pea (Hassan et al. 1995), maize (Kwiatkowski et al. 1995, Napier 1995, Dresselhaus et al. 1996), sugar beet (Opas et al. 1996), *Ginko biloba* (Nardi et al. 1998), and *Liriodendron tulipifera* (Navazio et al. 1998). These reports suggest that, also in plant cells, this protein plays fundamental roles in the calcium homeostasis.

The pistil is a suitable model for studying the role of calcium and proteins binding Ca^{2+} in the flow of intercellular information in plants. After pollination, this organ is the site of interaction of somatic cells of the transmitting tract with the male gametophyte. The proper development and appropriate orientation of the pollen tubes require, on the one hand, a so-called tip-focussed Ca^{2+} gradient (Miller et al. 1992, Holdaway-Clarke et al. 1997). And, on the other hand, local changes in the concentration of Ca^{2+} in the cytosol of the pollen tube tip (Malhó et al. 1994, 1995; Franklin-Tong et al. 1996; Malhó and Trewavas 1996; L. Camacho et al. 2000). Until now, the sites and means of storing Ca^{2+} in the cytosol of the pollen tubes have not been identified. Both vacuoles and ER are taken into account. Current research carried out in our laboratories on *Petunia hybrida* aims to characterize the role of CRT in the development of pollen tubes. Research carried out in situ by a hybridization method revealed that the CRT gene is expressed both in the transmitting-tract cells and in the pollen tubes growing in vivo (Lenartowska et al. 2001). The aim of the current research was the immunolocalization of the CRT protein in transmitting-tract cells of the style and in the pollen tubes growing in vivo.

Material and methods

Plant material

From pistils of *Petunia hybrida* Hort. var. multiflora (commercial cultivar grown at room temperature in the Institute of General and Molecular Biology, Nicolaus Copernicus University, Toruń, Poland) we used the substigmal parts of unpollinated and pollinated styles at 6, 16, 48, and 70 h after pollination.

Sample processing

The tissue samples were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in phosphate-buffered saline (PBS), pH 7.3, for 24 h at 4 °C. Then the material was washed in the same buffer, dehydrated in ethanol concentrations, 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 two weeks at –20 °C. Ultrathin sections were cut with a glass knife on an Ultracut microtome LKB 8800 and collected on nickel grids coated with 3% Formvar (Sigma).

Western blot analysis

Unpollinated and pollinated pistils were homogenized in 0.8 ml of sample buffer twice, the two extracts were combined and centrifuged at 13 000 **g** for 15 min. The supernatant was heated at 100 °C for 2 min and then cooled and 20 µl was loaded into the well of a 12% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose gel paper at 30 V for 1 h. Immunoblot detection was done as described previously (Napier et al. 1995), using antibody dilutions of 1 : 4000 for the anticalreticulin serum (1 : 2000 for the preimmune serum) and 1 : 4000 for the secondary peroxidase anti-rabbit immunoglobulin (Sigma). The signal was developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, U.K.) and recorded on photographic film.

Immunolabelling

The sections were treated with 5% bovine serum albumin (BSA) in PBS, pH 7.0, at room temperature and the preincubation was 20 min for electron microscopy. Next, the sections were incubated with primary antibody (CRT PAb) at 1 : 500 concentration in PBS, pH 7.0, with 2% BSA and 0.1% Tween 20 for 1.5 h at 37 °C. The sections were washed in three changes of the same buffer and incubated with secondary antibody (anti-rabbit immunoglobulin G-gold conjugate, 10 mm diameter; BioCell, Cardiff, U.K.) at 1 : 100 concentration in PBS, pH 7.0, with 1% BSA for the same times and temperature conditions as with the primary antibody. The sections were washed in the buffer and finally in distilled water and stained in 2.5% uranyl acetate for 30 min.

Controls

The specificity of the immunocytochemical reaction was controlled in two ways. To verify if the CRT PAb used in the reaction bound to protein epitopes, the immunolocalization was preceded by incubation of the sections in a proteinase K solution. The incubation was to digest the protein epitopes. In the other control method the incubation with the primary antibody was omitted.

Microscopy

Stained samples were observed in Tesla BS 500 transmission electron microscope at an accelerating voltage of 60 kV. Micrographs were taken on Foton TN-12 films.

Results

Western blot analysis

Immunoblotting an antibody raised against maize CRT showed high specificity of this antibody in *P.*

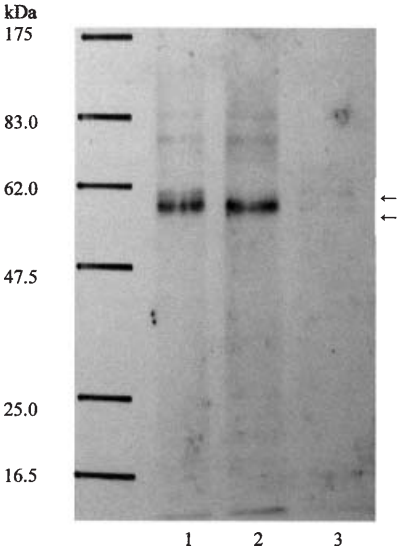

Fig. 1. Immunoblots of soluble protein fractions of *P. hybrida* pistils and *A. thaliana* root membranes with antibody to maize CRT. *1* and *2* Fractions from *P. hybrida* pistils, unpollinated and 48 h after pollination, show two bands at around 55 kDa. *3 A. thaliana* positive control shows a very faint band

hybrida pistils (Fig. 1). The CRT PAb recognized a double band around 55 kDa (Fig. 1, lanes 1 and 2) in soluble protein fractions. This result was repeated for unpollinated and pollinated pistils, thus the antibody recognized the same epitopes in the transmitting-tract cells and other tissues of the pistil and in the pollen tubes. *Arabidopsis thaliana* root membranes as positive control showed the same double band, a very faint one in this case (Fig. 1, lane 3). The control blot with exactly the same protein gave absolutely no signal at all (not shown).

Immunogold CRT localization

The immunogold method revealed the subcellular localization of epitopes binding CRT PAb in transmitting-tract cells and pollen tubes. In the transmitting-tract cells of the unpollinated style, gold particles were localized mainly in the cytosol (Fig. 2 A, B), sometimes in the dictyosomes (Fig. 2 B) and in the nucleus (Fig. 2 A). The labelling was not observed in the area of the extracellular matrix of the transmitting tissue. After pollination, no significant changes were observed in the localization of CRT in the transmitting-tract cells (data not shown).

Epitopes binding CRT PAb were also found in pollen tubes. In the tip zones of these cells, many gold particles were localized at the border between the cell membrane and the cell wall (Fig. 3 A) and in smaller numbers in the electron-transparent cytoplasmic vesicles (Fig. 3 A). The highest level of CRT PAb binding was observed in the tip cytosol zone, in which long ER channels were present (Fig. 3 B) and membranes were piled up creating a network of distensions and vesicles

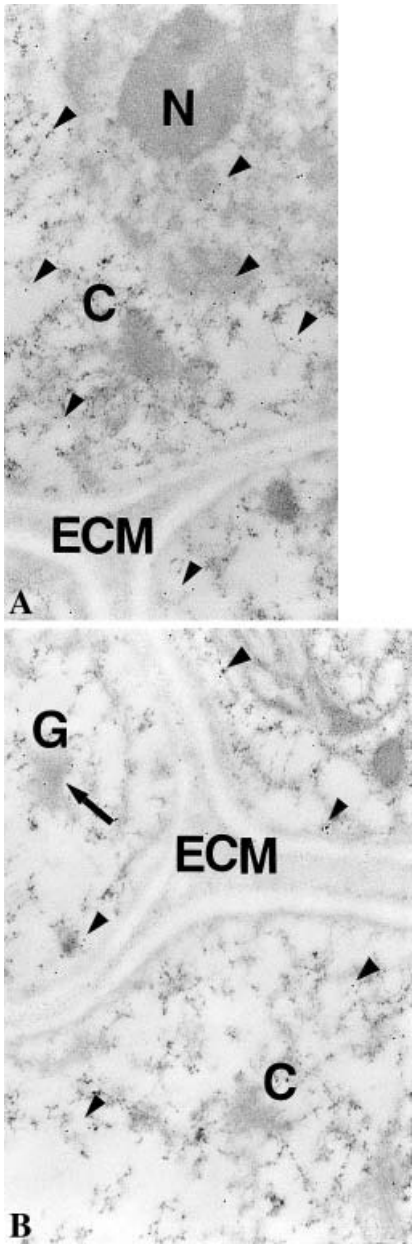

Fig. 2 A, B. Immunogold CRT PAb localization in transmitting-tract cells of unpollinated style of *P. hybrida*. Gold particles are localized in the cytoplasm (arrowheads), around dictyosomes (**B**, arrow), and in the nucleus (**A**, arrowheads), gold traces are not observed in the extracellular matrix of the transmitting tissue. A, ×62000; B, ×60000. *C* Cytoplasm; *ECM* extracellular matrix; *G* Golgi apparatus; *N* nucleus

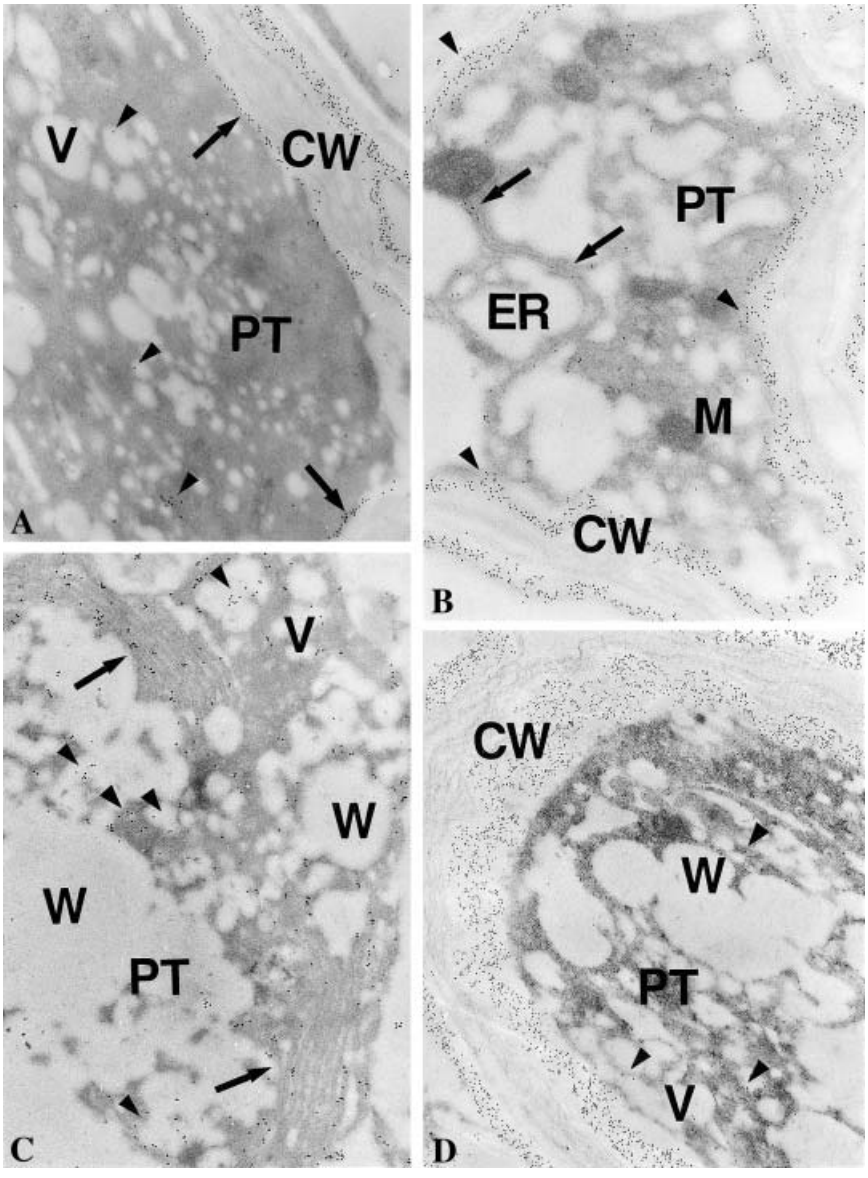

Fig. 3 A–D. Immunogold CRT PAb localization in *P. hybrida* pollen tubes growing in vivo. **A** 6 h after pollination, in the tip zone of pollen tube many gold particles are localized between the cell membrane and the cell wall (arrows) and only sparse gold particles are present in the electron-transparent vesicles (arrowheads). ×60500. **B** 16 h after pollination, the labelling is observed in the subtip cytosol zone of the pollen tube, in which long ER elements are present (arrows), a high level of CRT PAb binding is also observed in the inner, callosic cell wall of that cell (arrowheads). ×85000. **C** 16 h after pollination, gold particles are localized in membranes piled up creating a network of distensions (arrows) and in vesicles (arrowheads), vacuoles are devoid of labelling. ×51000. **D** Selective CRT PAb binding in the cell wall of the pollen tube 48 h after pollination; the antibody is only bound to epitopes in the inner, callosic cell wall, the fibrillar outer cell wall is devoid of labelling, a small amount of labelling is observed in the cytoplasmic vesicles (arrowheads). ×50000. *CW* Cell wall; *ER* endoplasmic reticulum; *M* mitochondrion, *PT* pollen tube, *V* vesicles, *W* vacuole

(Fig. 3 C). Gold particles occurred, above all, on the surface of membranes creating cisternae as well as on and around the area of some vesicles. The level of labelling of pollen tube cytosol was distinctly lower on those cross sections on which only single ER cisternae, vesicles, and mitochondria were visible (compare Fig. 3 B and C). In these zones of the pollen tubes, numerous gold particles were observed in the outer cell membrane compartment on the area of the inner cell wall (Fig. 3 B). A dramatic increase in the level of labelling of pollen tube walls was observed on cross sections of the older parts of the pollen tubes (Fig. 3 D). The antibody was only bound to epitopes present in the inner, callosic cell wall of the pollen tubes. The fibrillar outer cell wall was devoid of labelling. The labelling was also still observed in the cytoplasmic vesicles (Fig. 3 D). Vacuoles of various sizes occurring in the pollen tubes were devoid of labelling (Fig. 3 C, D). In the cytosol of old pollen tubes, which had been growing for tens of hours, CRT PAb was localized mainly in the area of irregular vesicles, in the oval "fibrillar cores" present there (Fig. 4 A). These vesicles underwent exocytosis, their oval fibrillar cores were visible in the inner cell wall of the pollen tubes (Fig. 4 A).

In order to determine the degree of specificity of the immunogold reaction, a control reaction was carried out. In the material in which incubation with the

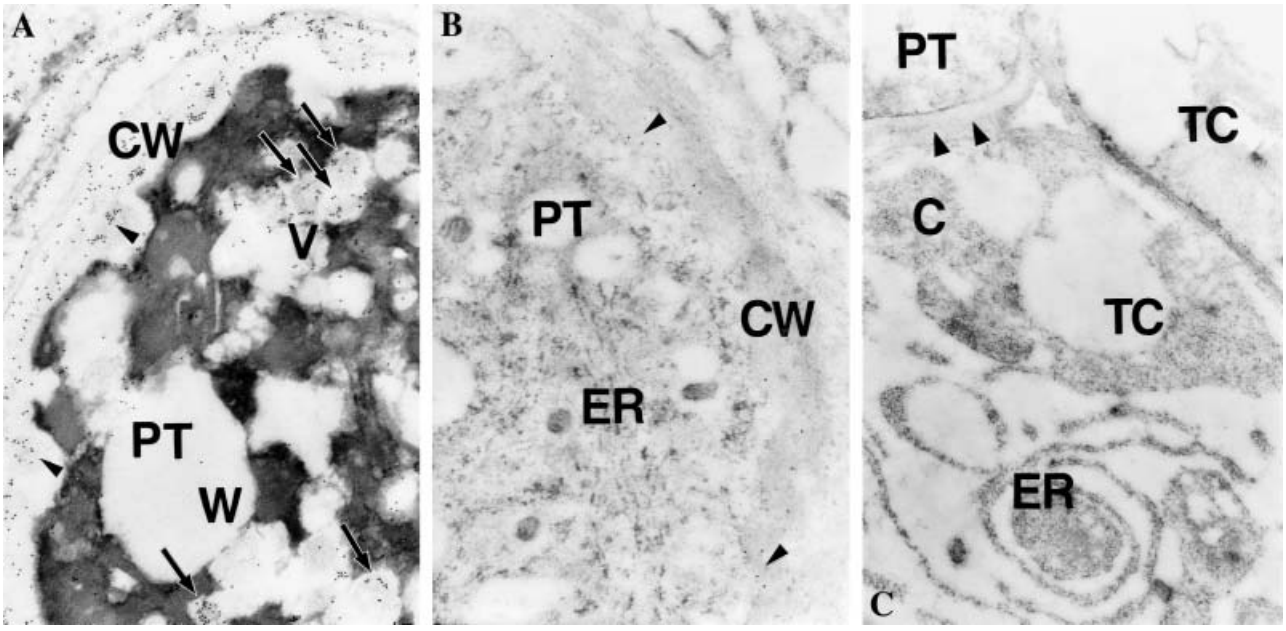

Fig. 4. **A** A substigmal part of the style 70 h after pollination. CRT PAb binding is localized mainly in the area of irregular vesicles, in the oval fibrillar cores present there (arrows), these vesicles underwent exocytosis, their oval fibrillar cores are visible in the inner cell wall (arrowheads). ×64000. **B** and **C** Controls of specificity of immunogold reaction. **B** In the material in which incubation with the primary antibody was conducted by cleavage in a solution of proteinase K only single gold particles are found in the inner, callosic cell wall of the pollen tube (arrowheads). ×65000. **C** The control reaction conducted by omitting incubation with the primary antibody showed no labelling in the transmitting-tract cells and in the callosic cell wall of the pollen tube (arrowheads). ×7000. *C* Cytosol, *CW* cell wall, *ER* endoplasmic reticulum, *PT* pollen tube, *TC* transmitting-tract cell, *V* vesicles, *W* vacuole

primary antibody was conducted by cleavage in a solution of proteinase K, the level of labelling was very low (Fig. 4 B). Only single gold particles were found in the area of the inner, callosic cell wall of the pollen tubes (Fig. 4 B). The control reaction conducted by omitting incubation with the primary antibody showed no gold traces in the ER and cytosol of the transmitting-tract cell and in the callosic cell wall of the pollen tubes (Fig. 4 C).

Discussion

Our study revealed the presence of CRT in the transmitting-tract cells and pollen tubes of *P. hybrida*. In both types of cells, the protein was localized in the area of various cell compartments. In the transmitting-tract cells, CRT was mainly localized in the cytosol, sometimes around dictyosomes, and rarely in the nucleus. Earlier research using the hybridization method in situ revealed that in the transmitting-tract cells of the *P. hybrida* pistil mature transcripts of the CRT gene occur on the ER surface (Lenartowska et al. 2001). This suggests that the CRT protein is localized primarily in the ER, which is the site of mRNA translation of the CRT gene. The presence of CRT in the dictyosomes of transmitting-tract cells may reflect the posttranslational modification of the protein in the area of the Golgi cisternae, hence, due to the presence of a retention sequence, escaped CRT can return back to the ER or can be transported to other cell compartments (Gomord et al. 1999; Pagny et al. 1999, 2000; Vitale and Denecke 1999). The occurrence of CRT around dictyosomes was reported by immunocytochemistry also in protoplasts isolated from the embryos of *Nicotiana plumbaginifolia* (Borisjuk et al. 1998). The capacity of CRT to leave the ER has also been described in animals. The protein is localized in the acrosomal vacuoles of mammalian spermatozoa, where it participates in the process of fertilization, which requires a high level of Ca^{2+} (Nakamura et al. 1993).

In the cytosol of pollen tubes, the highest level of CRT was revealed in the zone where ER elements were present. Research on the expression of the CRT gene in pollen tubes of *P. hybrida* growing in vivo showed that its mRNA localizes on the ER surface (Lenartowska et al. 2001), the ER is therefore the site of CRT translation. Past research carried out by

immunocytochemical methods showed the reticular localization of CRT in plants (Denecke et al. 1995, Napier et al. 1995, Opas et al. 1996, Borisjuk et al. 1998) but did not allow to discriminate whether the antibody bound to the cytosol surface or the inner surface of the ER membranes. Assuming that the observed labelling reflects the intrareticular localization of CRT, it should be accepted that these organelles take part in the regulation of calcium homeostasis in the pollen tubes of *P. hybrida* growing in vivo. This basic function of CRT has been shown in all animal and plant cells investigated hitherto (Opas et al. 1991, 1996; Michalak et al. 1992; Bastianutto et al. 1995; Hassan et al. 1995; Mery et al. 1996; Golovina and Blaustein 1997; Krause and Michalak 1997; Meldolesi and Pozzan 1998; Nardi et al. 1998; Navazio et al. 1998). The precise regulation of the cytosolic level of Ca^{2+} in pollen tubes is vital to its correct growth and orientation (Miller et al. 1992; Mascarenhas 1993; Pierson et al. 1994, 1996; Li et al. 1996; Hepler 1997). Active calcium channels at the tip are the site of constant Ca^{2+} uptake from the extracellular space (Pierson et al. 1996). The maintenance of the tip-focussed gradient of the calcium requires a reduction in the concentration of these ions in the cytosol with increasing distance from the pollen tube tip. This can take place through the sequestration of Ca^{2+} in cytoplasmic organelles or their removal beyond the cell membrane. It is also known that reorientation of the direction of growth of the pollen tubes is possible due to IP_3-dependent, asymmetric changes in the level of calcium ions in the tip (Franklin-Tong et al. 1996). Which of the cytoplasmic organelles are equipped with the mechanisms for the sequestration and the IP_3-dependent release of Ca^{2+} has not yet been determined. In the light of our results it is possible to suggest that in the *P. hybrida* pollen tubes containing CRT, the ER is the site of the accumulation of a mobile pool of Ca^{2+} which can be activated on the IP_3-dependent path in response to an extracellular signal. The presence of CRT was not observed in the vacuoles, which confirms the specificity of the antibody used. It is known that CRT does not occur in these organelles (Opas et al. 1996), which are recognized as major stores of Ca^{2+} (Bush 1995). A different system of calcium sequestration operates there (Randall 1992).

In the area of the cytosol of pollen tubes, CRT is also localized in electron-transparent tip vesicles and in the cores of secretory vesicles occurring in old pollen tubes. Importantly, accumulation of CRT at tips of growing root hairs was reported by Baluška et al. (2000). It had already been proven earlier in *Nicotiana alata* (Li et al. 1995) and in sugar beet (Pennel et al. 1989) that not only polysaccharides but also proteins are transported in secretory vesicles. This indicates that in the pollen tubes CRT may undergo posttranslational modifications and become a secretory protein. The first place in which CRT appears apart from the reticular system (ER, Golgi, vesicles) is the border between the cell membrane and the primary cell wall. This might suggest that the periplasmatic localization of CRT participates in the adhesion of the extracellular matrix to the cell membrane. In vitro research has revealed that in animals CRT binds with integrins (Dedhar 1994, Jeffs 1994, Coppolino et al. 1995). The presence of integrin-like proteins has also been observed in plants, such as in the cell membrane of the cells of the root cap of *A. thaliana* and in rhizoids of *Chara* sp. (Katembe et al. 1997).

In older subtip zones of *P. hybrida* pollen tubes, a high level of CRT PAb binding is observed in the inner, callosic cell wall. The specificity of the reaction is confirmed by the preincubation of the material in a solution of proteinase K which slowed CRT PAb binding and proves that the antibody reacted with protein epitopes. It was shown earlier that in the callosic inner cell wall, besides the 1,3-β-glucan polymer, proteoglycans such as arabinogalactan proteins and extensin-like glycoprotein occur (Li et al. 1992, Rubinstein et al. 1995, Dahiya and Brewin 2000). Baluška et al. (1999) reported that CRT remains associated with callosic pit fields at the cell periphery in plasmolyzed maize root cells. The present study reveals an association of CRT with callose deposition, suggesting that CRT or a CRT-like protein, probably capable of binding Ca^{2+}, is also localized in the callosic cell wall of the pollen tube. Localization of Ca^{2+} by the pyroantimonate method showed that free calcium ions can occur in the inner cell wall of *P. hybrida* pollen tubes (Lenartowska et al. 1997). This phenomenon is not common, which suggests that these ions are released from the site of their sequestration, e.g., during the partial lysis of the callosic cell wall. The neutral polymer 1,3-β-glucan does not bind Ca^{2+}. It cannot be ruled out, then, that these ions are sequestrated with the participation of CRT or a CRT-like protein. An attractive hypothesis would suggest that the callosic cell wall in pollen tubes is the site of accumulation of calcium removed from the subtip cytosol with the aim

of maintaining the tip-focussed gradient of Ca^{2+}. More precise research is required, however, to verify this hypothesis.

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