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ORIGINAL ARTICLE

Distribution of calcium in the stigma and style of tobacco during pollen germination and tube elongation

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Abstract Potassium antimonate was used to locate loosely bound calcium in the stigma and style of tobacco. The tobacco stigma is wet and covered by a thick layer of glycoprotein exudate at anthesis. The exudate contains abundant vesicles, which are densely labeled with calcium precipitates. When pollen grains arrive at the stigma, become hydrated, and as the pollen swells, Ca^{2+} precipitates accumulate at the aperture. Calcium precipitates that accumulate in pollen cytoplasm are initially concentrated within small vacuoles, but as germination proceeds these appear to fuse, forming prominent, densely labeled vesicles that preferentially accumulate near the proximal region of the growing tube. Although the stigma has abundant particles, few calcium precipitates are observed in the transmitting tissue from anthesis to 11 h after pollination. However, at 22 h after pollination, accumulation of calcium increases distally from the stigmatic interface with the transmitting tissue through the length of the style to the ovary. An examination of flowering plants with differing floral biology will be needed to understand the role of loosely bound calcium accumulation and its relationship to tissue-level changes in calcium uptake, maintenance of other calcium pools, including $[Ca^{2+}]_{cyt}$, and in pollen and style maturation during the progamic phase.

Keywords Calcium · *Nicotiana tabacum* · Pollen tube · Stigma · Style

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Introduction

Brewbaker and Kwack (1963) first described the requirement of calcium for in vitro pollen germination, which they found could be met by culturing pollen en masse, resulting in a "population effect," or by adding calcium to the medium. In either case, calcium proved to have an obligatory role in all pollen tube elongation and the importance of calcium in reproductive physiology has been the subject of many studies (Ge et al. 2007). In addition to myriad other metabolic roles of calcium, as a coenzyme, secondary messenger and wall-stabilizing cation, pollen tubes cultured in vitro could be attracted by the presence of a calcium gradient (Mascarenhas and Machlis 1962). These workers also found that the Ca^{2+} increased gradually from the stigma to the ovary in *Antirrhinum majus*, and this correlated well with the attraction of pollen tubes in vitro. Although promising, the role of a calcium gradient in directing pollen tubes has not proved to be universal in other plants (Glenk et al. 1971; Mascarenhas 1975). Afterwards, few reports appeared in the literature and more emphasis focused on the function of Ca^{2+} during pollen tube elongation in vitro, in a fundamentally different role, as a largely intracellular signal.

Pollen tubes cultured in vitro clearly require the absorption of external calcium and a continuous source is necessary to maintain an intracellular Ca^{2+} gradient at the tip of the pollen tube for their polar elongation (Holdaway-Clarke

et al. 1997). However, the distribution and dynamics of the external Ca^{2+} source, in vivo, on the stigma during pollination and in the style during tube elongation, has not been fully examined. The principal pool of calcium involved in this uptake is the "loosely bound" calcium pool, maintained by ionic bonds at concentrations that are far greater than in their role in intracellular signaling; this pool ultimately serves as a source for calcium, which serves in myriad roles. Such phenomena as calcium uptake during pollen germination, post-pollination redistribution of calcium in the response of stigma cells and polarization during pollen germination depend on a ready source of chemically available calcium. Pollination in lily, for example, induces an apparent increase of calcium within the intracellular matrix of the transmitting tissue (Zhao et al. 2004). In contrast to in vitro studies, Iwano et al. (2004) found that oscillations of intracellular Ca^{2+} concentration using in vivo pollinated *Arabidopsis* plants occur at greater frequency in the tip region of in vivo pollen tubes, suggesting differences between in vivo and in vitro results; likely these internal differences relate to the availability of this external calcium pool. In this study, we examine the distribution of such available calcium in the stigma and style of tobacco in vivo using potassium antimonate precipitation to better understand the role of extracellular calcium concentrations in relationship to tobacco pollen tube elongation and stigma interactions.

Materials and methods

Nicotiana tabacum L. cv. Daqingye was grown in controlled growth chambers with a 15 h day length at 27°C with plants illuminated at 54 μmol m^{-2} s^{-1} and 9 h darkness at 20°C. The tobacco style is approximately 4 cm long, and it takes pollen tubes 45 h to reach the ovary after pollination (Tian and Russell 1997). In this study, the style was divided into four segments, each 1 cm long, with the first segment including the stigma and style and the last segment ending in the ovary. We sampled the pollinated stigma after 3 h and the four different segments of the style at 11, 22, 33 and 44 h after pollination. Emasculated styles of unpollinated flowers were also observed in a similar manner.

Sections of 1 mm length were removed from the proximal (basal) end of each segment and fixed for 3 h at room temperature in 2% glutaraldehyde (v/v) in 0.1 M KH_2PO_4 buffer (pH 7.8) containing 1% potassium antimonate ($K_2H_2Sb_2O_7{\cdot}4H_2O$). These were washed in three 30 min changes of buffered 1% potassium antimonate and post-fixed in 1% (w/v) buffered OsO_4 containing 1% potassium antimonate 16 h at 4°C. After three 30 min changes of buffer, the samples were dehydrated in a graded acetone series, infiltrated and embedded in Spurr's resin. At least five samples were sectioned at each stage and stained with 2% uranyl acetate (w/v) in 50% methanol (v/v). Specimens were observed and photographed using a JEOL JEM-2100 transmission electron microscope. Calcium precipitates were examined by EDXA (energy-dispersive X-ray analysis) using an Inca 100 detector on a JEOL JEM-1230 transmission electron microscope at 100 kV. Control experiments involving chelation of calcium using EGTA and EDTA, removal of precipitates and categorization of non-antimonate based precipitates in similar tobacco reproductive tissues are reported in detail elsewhere (Tian and Russell 1997).

Results

Calcium distribution in stigma

The stigma of tobacco is wet, due to secretion. The secretion drop could be observed by naked eyes after the flowers were emasculated, but not pollinated. Some papillar cells elongate near the margin of the stigma (Fig. 1; Tables 1, 2). At anthesis, the papillar cells and epidermal cells are highly vacuolated, with many flocculent calcium precipitates in their large vacuoles, but few precipitates outside the cell (Figs. 2, 3). A thin layer of material is present on the surface of stigma. At 2 h after anthesis, the surface of the stigma is covered by a layer of material about 100 μm in thickness, which appears electron dense. There are many vesicles on the stigma surface that are low in electron density, exhibiting evident differences in electron density compared with the surrounding material (Fig. 4). When pollen grains are loaded on the stigma, they will germinate on the stigma surface and some pollen grains move into the stigma exudate. At this time, calcium precipitates increase in the cell wall of the stigma and in the vesicles on the stigma surface (Fig. 5). To identify the precipitates in the vesicles (Fig. 6), they were examined using EDXA (Fig. 7) and were confirmed to include two overlapping peaks composed of antimony and calcium, which are characteristic of calcium antimonate precipitates. Tian and Russell (1997) also report other control experiments in tobacco gynoecial tissues that confirm the identification of these precipitates as calcium antimonate.

Calcium changes in the germinating pollen grain

After pollen lands on the stigma, the grains contact the stigma surface and begin the hydration process prior to tube emergence (Fig. 8). Upon hydration of the pollen grains, some calcium precipitates appear on the surface of

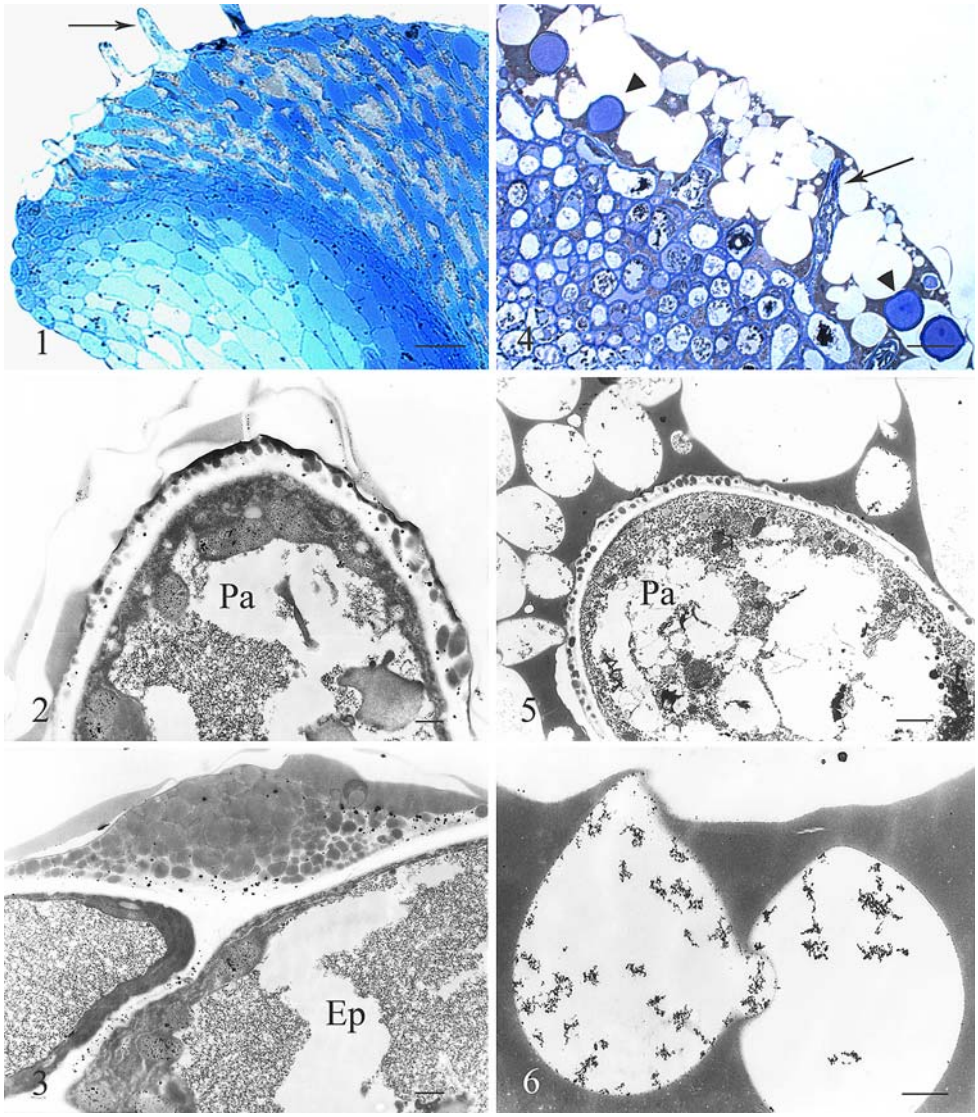


Fig. 1–6 **Fig. 1** Tobacco stigma before anthesis showing papilla (*arrow*). *Bar* = 100 μm. **Fig. 2** Flocculent calcium precipitates accumulate in the large vacuole of a papillar cell (*Pa*) and some in cell walls. *Bar* = 0.5 μm. **Fig. 3** Calcium precipitates occur in cell walls of the stigma surface and near secreted exudate material. Newly synthesized exudate protein (*Ep*) is visible in the epidermal cell. *Bar* = 0.5 μm. **Fig. 4** Tobacco stigmatic surface 2 h after anthesis, showing immersed pollen grains (*arrowheads*) in a thick layer of vesicle-containing exudate. *Unlabeled arrow* indicates papillar cell. *Bar* = 50 μm. **Fig. 5** Papillar cell (*Pa*) and stigma surface with calcium precipitates accumulating in vesicles. *Bar* = 2 μm. **Fig. 6** Flocculent calcium precipitates accumulate within exudate vesicles. *Bar* = 0.5 μm

the intine in association with the germinal pore, with few precipitates occurring in the pollen cytoplasm (Fig. 9). As the pollen intine swells, the germinal pore protrudes and more precipitates appear in the pollen cytoplasm. Some small vacuoles contain calcium precipitates in the cytoplasm (Fig. 10). A thick layer of precipitates accumulates on the inner surface of the small vacuoles in association with the endoplasmic reticulum cisternae surrounding the vacuoles, suggesting that calcium precipitates were dissolved in the cisternae and were transported into small vacuoles (Fig. 11). With pollen hydration, the pollen tube began to protrude through the exine of the pollen wall (Fig. 12). The surface of the tube tip has flocculent material on it and is characterized by small calcium precipitates. Many small calcium precipitates appear in the cytoplasm of the pollen tube (Fig. 13), suggesting that calcium moves into the pollen cytoplasm through the germinal pore during hydration. Accompanying tube elongation, the electron density of the pollen tube cytoplasm decreases. Numerous small vacuoles occur in the tube, with a large vacuole forming in the pollen grain as the pollen cytoplasm vacates the grain (Fig. 14). The large vacuole contains fusion products of smaller ones in the tube, and many calcium precipitates accumulate in the large vacuole (Fig. 15). Following tube elongation and entry of most of the cytoplasm into the pollen tube, the pollen grain becomes empty and flocculent calcium precipitates accumulate in the large vacuole (Fig. 16). At this time, calcium precipitates are mainly concentrated in small vacuoles, which ultimately accumulate within the large pollen grain vacuole. A layer of calcium precipitates still occurs on the surface of the tube tip (Fig. 17), suggesting that calcium in the tube tip is potentially taken up from stigma surface vesicles and appears to accumulate in

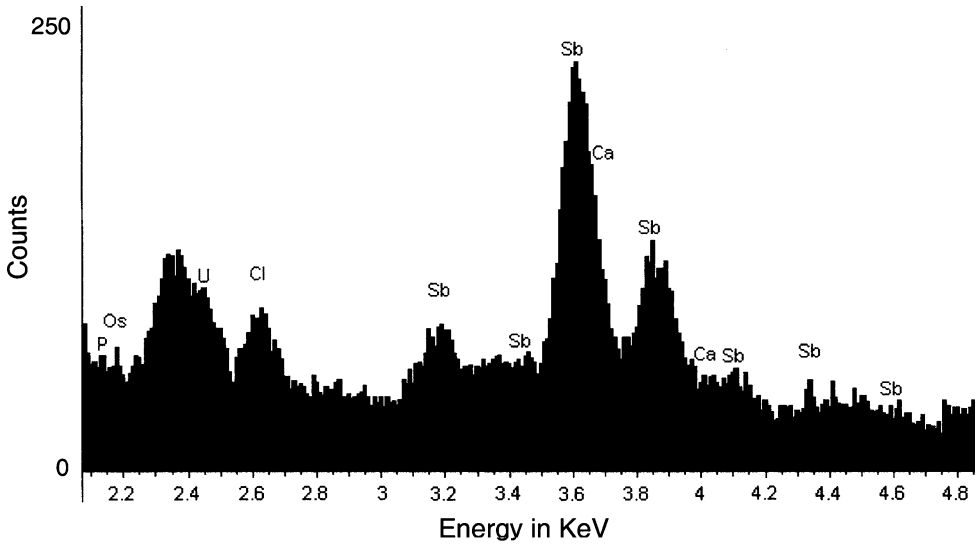


Fig. 7 Energy-dispersive X-ray analysis spectrum of tobacco stigma thin section. Overlapping peaks of Ca and Sb confirm the identity of calcium antimonate precipitates

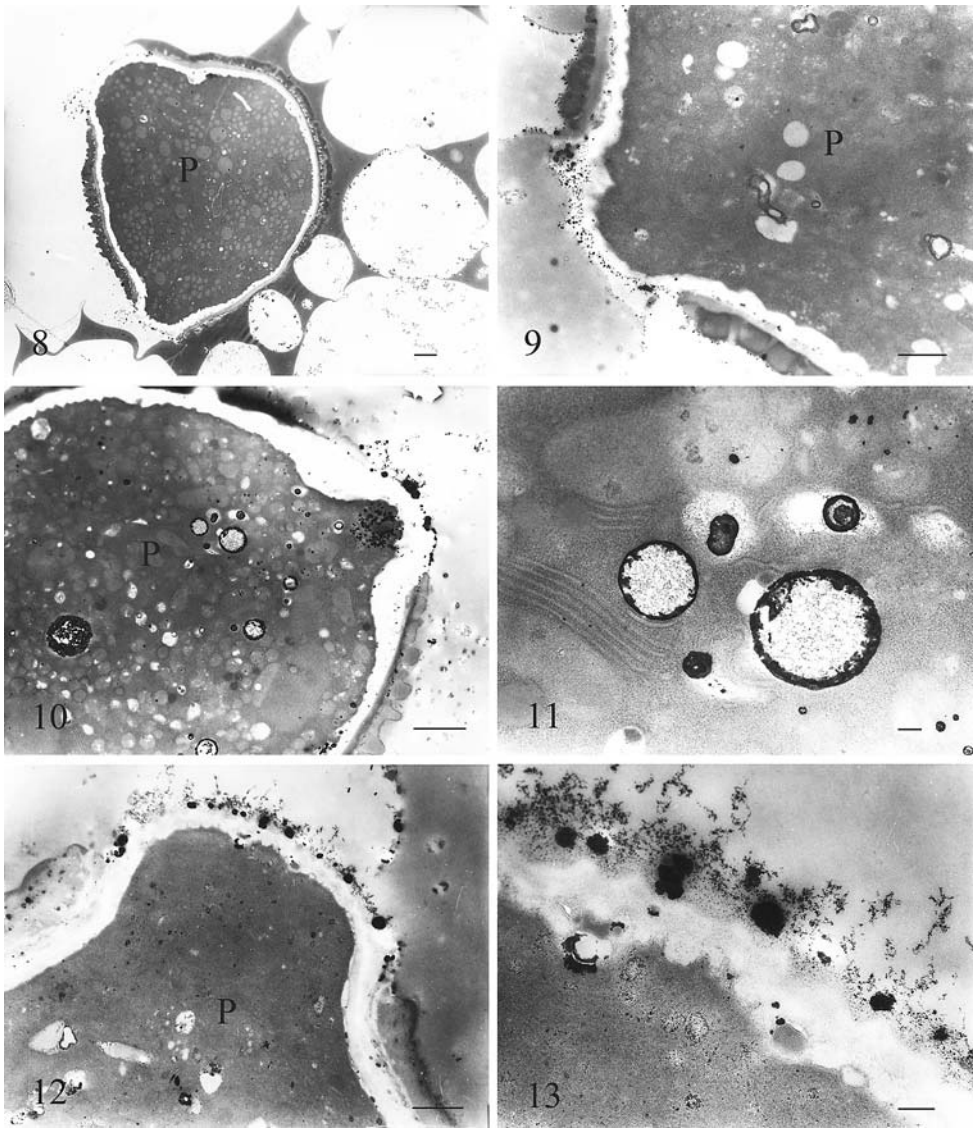


Fig. 8–13 **Fig. 8** Upon pollination, pollen grains do not contain conspicuous small vacuoles in the cytoplasm. *Bar* = 2 μm. **Fig. 9** During pollen hydration, calcium precipitates are observed on and near the germinal pore. *Bar* = 1 μm. **Fig. 10** Small vacuoles form and numerous calcium precipitates accumulate in the pollen cytoplasm as germination proceeds. *Bar* = 2 μm. **Fig. 11** A layer of calcium precipitates accumulate on the inner surface of small vacuoles. *Bar* = 0.2 μm. **Fig. 12** Flocculent precipitates accumulate on and near the developing tube tip as initial protrusion occurs. *Bar* = 1 μm. **Fig. 13** Tiny calcium precipitates form in pollen cytoplasm (magnified from Fig. 12). *Bar* = 0.2 μm

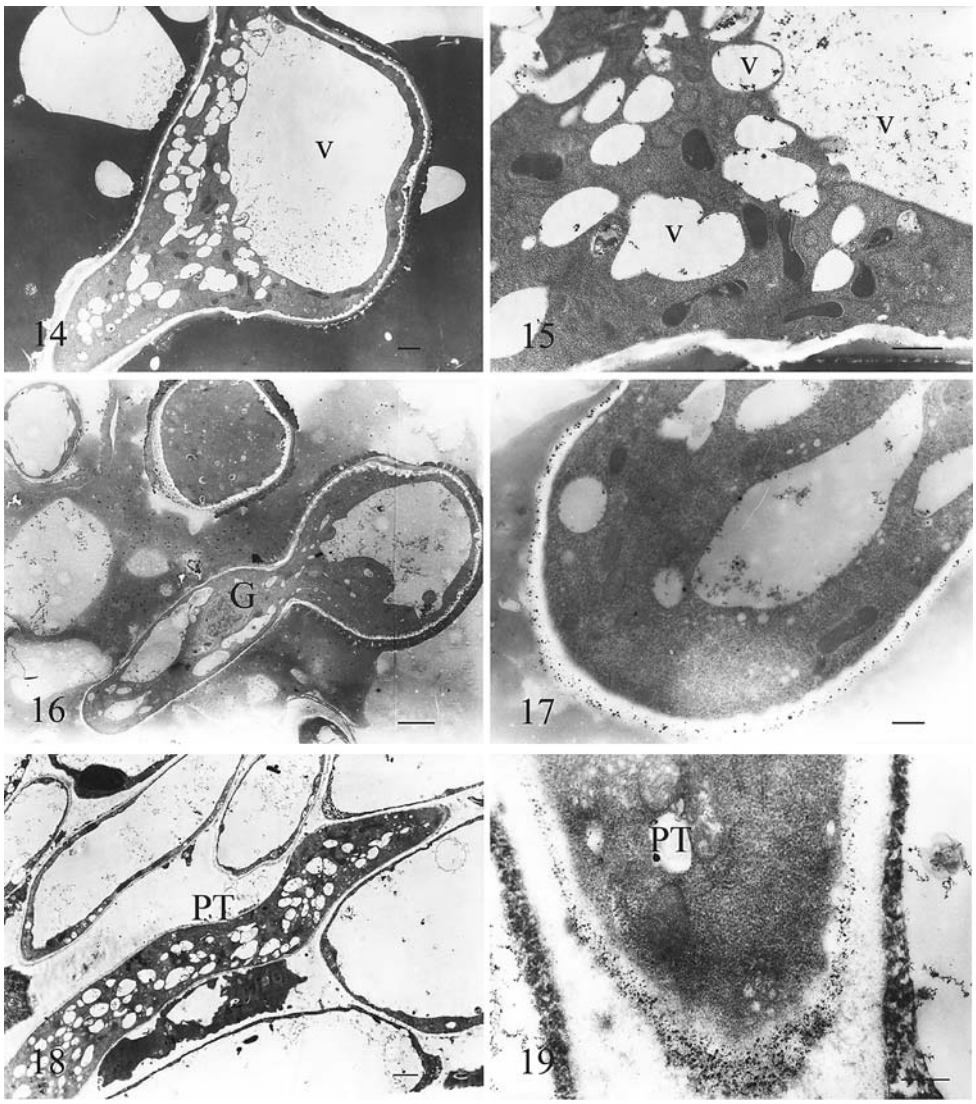


Fig. 14–19 **Fig. 14** Upon emergence of the tube, numerous small vacuoles appear inside the tube and a single large displacement vacuole in the pollen grain. *Bar* = 2 μm. **Fig 15** Ca^{2+} precipitates in the vesicles and are released into large vacuoles (magnified from Fig. 14). *Bar* = 1 μm. **Fig. 16** As the tubes elongate, cytoplasm migrates into the tube, and flocculent calcium precipitates accumulate in the large vacuole. *Bar* = 5 μm. **Fig. 17** Calcium precipitates accumulate on the surface of growing pollen tube and in vacuoles. *Bar* = 0.5 μm. **Fig. 18** Pollen tube (*PT*) penetrates between transmitting tissue cells. *Bar* = 2 μm. **Fig. 19** Obliquely sectioned pollen tube (PT) reveals numerous calcium precipitates line the wall of the tip and surround the extracellular matrix of the transmitting tissue. *Bar* = 0.5 μm

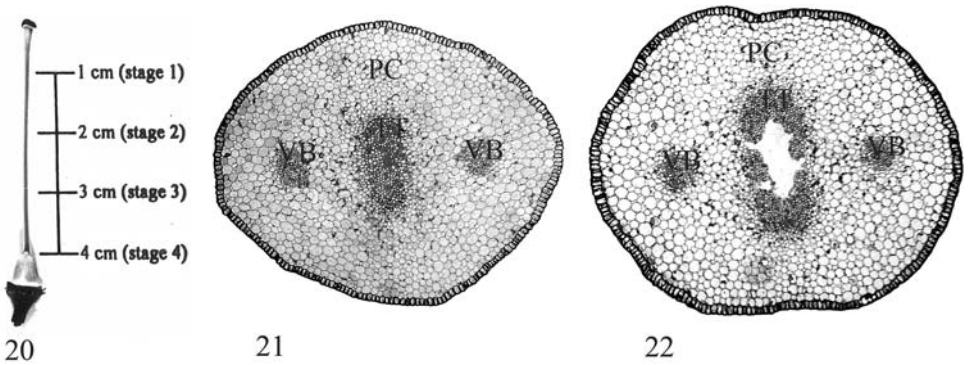


Fig. 20–22 **Fig. 20** Tobacco gynoecium at anthesis. Cut positions of the style at 1 cm intervals represent penetration thresholds for tubes at 11, 22, 33 and 44 h after pollination. **Fig. 21** Transverse section of a closed style at 1–3 cm from the stigma shows solid transmitting tissue (*TT*), parenchyma cells (*PC*) and vascular bundle (*VB*). Magnification ×60. **Fig. 22** Transverse plane of tobacco style at 4 cm shows an open transmitting tissue. *TT* transmitting tissue, *PC* parenchyma cells, *VB* vascular bundle. Magnification ×60

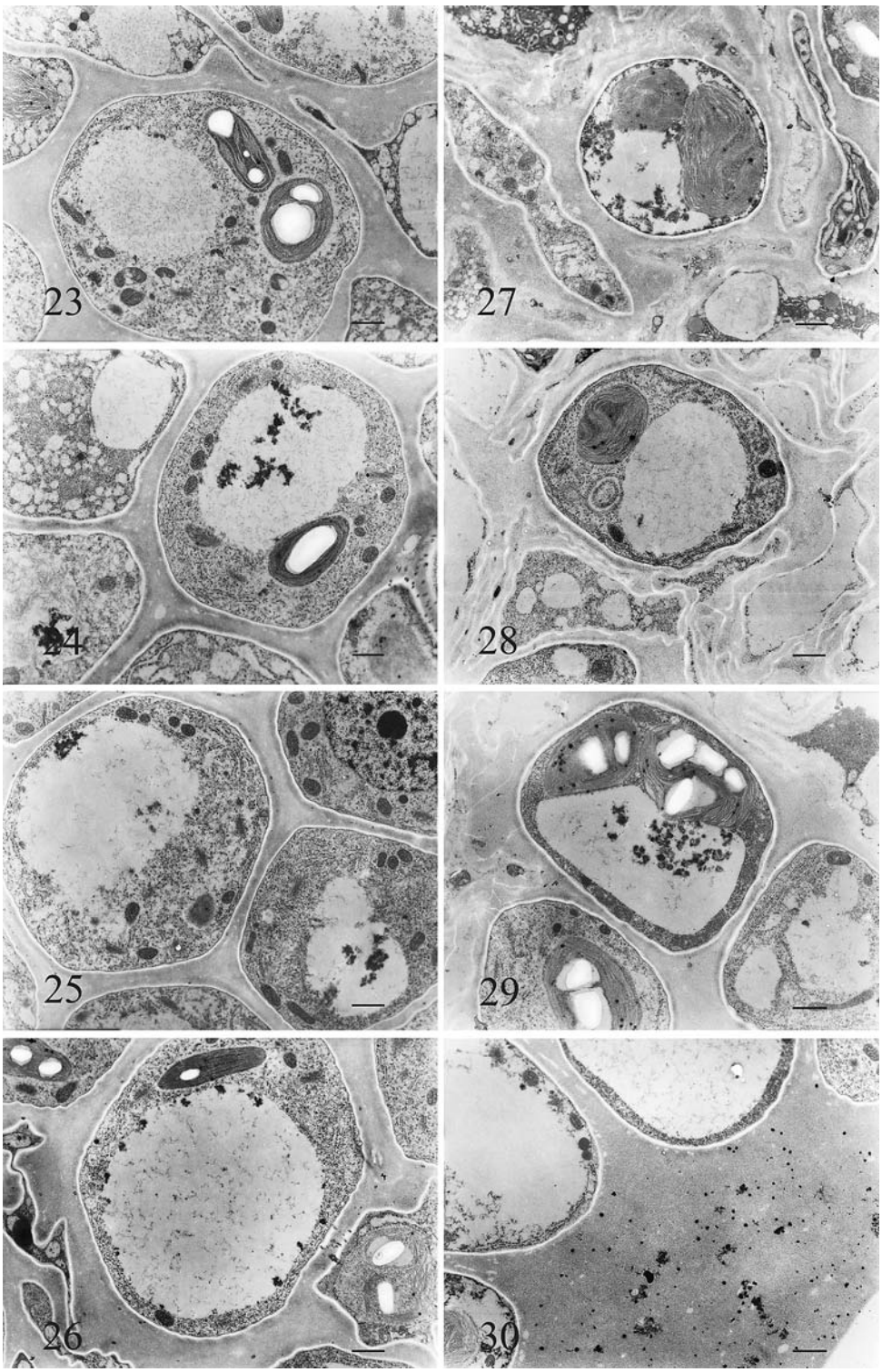
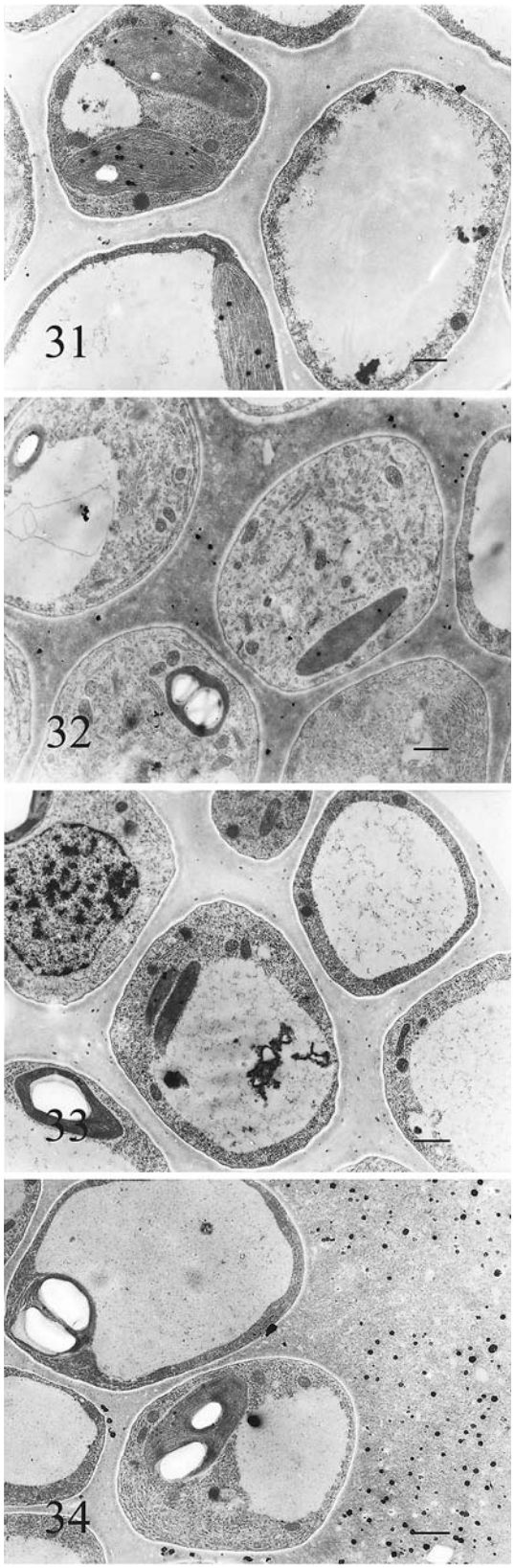


Fig. 23–30 Fig. 23–26 Anthesis in transmitting tissue within the four different segments; there are no significant differences in the number of calcium precipitates. **Fig. 27–30** One day after anthesis, the transmitting tissue at 4 cm accumulates more calcium precipitates in the extracellular matrix (Figs. 29, 30), displaying accumulation of calcium in the transmitting tissue. *Bar* = 1 μm

small vacuoles in the tube cytoplasm. After penetrating the stigma, the pollen tube elongates between the cells of transmitting tissue of style (Fig. 18). There are still many tiny calcium precipitates that appear to accumulate at the tube tip (Fig. 19).

Calcium distribution in stylar tissue

Pollen tubes take approximately 44 h to penetrate the 4 cm long tobacco style and reach the ovary (Fig. 20). Near the stigma, the style is solid (Fig. 21), but distally becomes



◀ **Fig. 31–34** Emasculated and unpollinated flowers at 1 day after anthesis. Transmitting tissue at 4 cm also displays significantly greater accumulation of calcium precipitates in the extracellular matrix, indicative of a calcium gradient in the stylar transmitting tissue. *Bar* = 1 μm

hollow near its connection with the ovary (Fig. 22). The interior of the style is organized into two regions in transverse section: the central transmitting tissue (TT) and the surrounding parenchyma tissue (PC), with two vascular bundles (VB) between regions of transmitting tissue. The central TT has slender, long cells, with far thicker cell walls and extracellular matrix material that is characterized by higher electron density between the cells. Regions in the style are easily distinguished according to their morphology. Both vascular bundles consist of characteristic vascular cells and associated parenchyma cells. Some calcium precipitates are located on the inner surface of xylem elements, suggesting a potential supplementary track for the movement of calcium. Tobacco pollen tubes grown in the extracellular matrix between the cells of the transmitting tissue contain numerous small calcium precipitates that accumulate at the tip (Fig. 19). Small calcium precipitates occur in the cells of transmitting tissue, suggesting that Ca^{2+} may be available for uptake from these stylar tissues.

At anthesis, there were no differences in the distribution of calcium precipitates in the cells of the whole style (Figs. 23, 24, 25, 26) and this would persist until 11 h after pollination, when pollen tubes are estimated to have elongated 1 cm into the style. A calcium gradient appeared in the transmitting tissue at 22 h after pollination (Figs. 27, 28, 29, 30), when pollen tubes are estimated to have elongated 2 cm into the style: the calcium precipitates were still exiguous in the upper part at 1–3 cm of the style (Figs. 27, 28, 29), but they increased conspicuously in the transmitting tissue at 4 cm of the style (Fig. 30). At 44 h after pollination, when the leading pollen tubes reach the ovary, the calcium gradient still remains in the transmitting tissue.

Calcium distribution in the style of unpollinated flowers

The stigma and style of the flowers emasculated but not pollinated were also examined in order to explore the relationship between calcium distribution in the pistil, germination of pollen grains and pollen tube elongation. At 1 day after anthesis (1.5 days after emasculation), calcium precipitates do not exhibit any obvious differences in the upper transmitting tissue (in the first 3 cm of the style) when pollination was withheld. However, cells of transmitting tissue in the proximal 1 cm of the style still contained more calcium precipitates than in the distal style (Figs. 31, 32, 33, 34). This result was same as in the pollinated flowers, suggesting that the occurrence of calcium accumulation in the transmitting tissue is independent of

Table 1 Calcium-induced precipitates on the tobacco stigma by relative abundance before anthesis, 2 h after pollination and 3 h after pollination

Tissue involved	Structural compartment	Before anthesis	Developmental stage 2 h after pollination	At 3 h after pollination
Stigma surface	Matrix	+	–	–
	Vesicle	–	+++	+++
Epidermal cell	Cell wall	++	++	–
	Cytoplasm	++	+++	+
	Vacuole	++++	++++	+++
	Matrix	++	++	++++
Papilla cell	Cell wall	+	+	–
	Cytoplasm	++	++	++
	Vacuole	++++	++++	++++
Pollen grain	Pollen wall	/	+	+
	Cytoplasm	/	++	–
	Vacuole	/	++	+++
	Germ pore	/	++++	++++
	Pollen tube tip	/	/	++++

At least six samples were examined at each stage to complete the data for this table. Individual samples varied by no more than one unit from those given here

Relative abundance: – no precipitates (ppt), + uncommon (1–19 ppt/μm^2), ++ common (20–39 ppt/μm^2), +++ abundant (40–59 ppt/μm^2), ++++ very abundant (60 or more ppt/μm^2)

Table 2 Calcium-induced precipitates in the extracellular matrix of the transmitting tissue of tobacco by relative abundance in each stylar segment position and at each developmental stage listed

Stylar segment	Anthesis	Developmental stage 1 day post-emasculation	At 1 day post-pollination
I, Stigma/distal style	–	+	–
II, Mid-distal style	–	–	–
III, Mid-proximal styletage	–	–	–
IV, Proximal style/ovary	–	+++	+++

At least six samples were examined at each stage to complete the data for this table. Individual samples varied by no more than one unit from those given here

Relative abundance: – no precipitates (ppt), + uncommon (1–19 ppt/μm^2), ++ common (20–39 ppt/μm^2), +++ abundant (40–59 ppt/μm^2), ++++ very abundant (60 or more ppt/μm^2)

pollination. In flowers in which pollination was withheld for 3 days (3.5 days after emasculation), some calcium precipitates accumulated in the cells of stigma, suggesting a trend of calcium accumulation in the transmitting tissue from the ovary toward the stigma in the post-mature styles of unpollinated flowers.

Discussion

During fertilization in flowering plants, a series of interactions occur between male and female cells and tissues when pollen tubes elongate through the diploid sporophytic tissue of the pistil. These interactions facilitate pollen tube entry into the embryo sac and optimize the success of the deposited sperm cells in their fusion with the egg and central cell. The role of calcium in this process has received particular attention, as it appears to provide a universal signal with pleiotropic effects involving attraction, long and short-distance communication, cellular fusion and cell signaling (Ge et al. 2007). The current study focuses on stores of calcium that are loosely bound, much more abundant than free calcium and available to meet the calcium requirements of myriad metabolic functions.

Ca^{2+} distribution in the stigma

Calcium is regarded as a requirement for vigorous germination and pollen tube growth. As noted before, when pollen grains are germinated in vitro in small numbers, rates of germination and elongation are impaired unless more pollen is added (the so-called "population effect"), but this may be overcome by adding more calcium (Brewbaker and Kwack 1963). Uptake of calcium by germinating pollen grains has been demonstrated in several reports (Reiss and Herth 1978; Polito 1983; Bednarska 1989). Feijó et al. (1995) hypothesized that pollen hydration may trigger increased turgor, allowing calcium uptake through stretch-activated calcium channels. However, all of these reports were in vitro and few reports concern calcium dynamics during pollen germination in vivo, particularly on the stigma and in the style. Recently, Iwano et al. (2004) used the sensitive yellow cameleon probe to sense signaling concentrations of $[Ca^{2+}]_{cyt}$ to report in detail on cytoplasmic free Ca^{2+} dynamics in pollen grains and papillar cells during pollination of *Arabidopsis*. Their study revealed three stages of Ca^{2+} uptake/increase: (1) at pollen grain attachment, soon after pollen hydration, (2) after pollen protrusion and tube initiation, and (3) during pollen penetration of the stigmatic surface. Ultimately, the source of such calcium must be met by metabolically available calcium, which is maintained in much more abundant stores through ionic binding, largely in association with protein and carbohydrates present within and between cells (Ge et al. 2007). This pool is readily labeled by precipitation with antimonate, which forms distinctive stable calcium precipitates in competition with other anions (Tian and Russell 1997). The loosely bound Ca^{2+} examined in this study, therefore, represents a likely source for this available calcium and is relevant to reproductive success. Thus, it should be noteworthy when a pool of available calcium,

which is far more abundant than the cytosolic fraction varies dramatically.

The occurrence of abundant calcium in association with the receptive stigma is documented for a variety of species, as is its rapid uptake and accumulation in the pollen. External calcium has been shown to be necessary to support the free calcium needed to build intracellular gradients of in vitro-grown pollen tubes (Nobiling and Reiss 1987; Obermeyer and Weisenseel 1991; Pierson et al. 1994; Holdaway-Clarke et al. 1997). When a pollen grain lands on the stigma, it becomes hydrated and absorbs sufficient Ca^{2+} to be detectable using calcium antimonate. Such calcium is potentially derived from vesicles and these seem to reflect coordinated depletion after pollination.

In tobacco, the stigma is wet, covered by a broad layer of stigmatic secretions at the surface at anthesis and there is no immediate barrier to germination. Sunflower, which has a drier stigma, also maintains external calcium stores that are much more abundant on the receptive surface of the stigma, especially outside and inside the papillae, than on non-receptive surfaces (Zhang et al. 1995). Calcium precipitates are also reported to be abundant in the intercellular matrix of the stigmatic tissue of cotton (Zhang et al. 1997) and on the stigmatic surface of *Brassica oleracea* following pollination, particularly where the pollen grain lands and tube germinates on the stigmatic papillae (Elleman and Dickinson 1999). Universal occurrence of such stores of calcium in and on stigmatic surfaces is in conformity with the in vitro requirement for exogenous calcium during pollen germination and tube elongation.

Ca^{2+} gradient distribution in the style

After evidence that calcium gradients did not exist in the style of a number of flowering plants (Glenk et al. 1971; Mascarenhas 1975), published reports did not again appear until antimonate precipitation was used in combination with TEM to provide greater resolution. In sunflower (Zhang et al. 1995) and cotton (Zhang et al. 1997), substantial accumulations of calcium precipitates were observed in the cell wall and the cytoplasm of the transmitting tissue of style, but very few precipitates were observed in the surrounding parenchyma tissue. In petunia (Lenartowska et al. 1997), Ca^{2+} content increased in extracellular regions of the transmitting tissue preceding the penetration of the pollen tubes. Yu et al. (1999) observed many tiny calcium precipitates in the cell wall of the rice style, which has no traditional transmitting tissue. Likewise, Zhao et al. (2004) examined Ca^{2+} accumulation in the lily style and reported that pollination induces an increase in calcium precipitates in the transmitting tissue. These results indicate that an environment of abundant calcium exists along the tube elongating pathway that appears to meet the demand of pollen tubes for Ca^{2+} during their growth in vivo. Differences in Ca^{2+} abundance, however, were not mentioned in the styles in these plants. In this study, tobacco styles appear to accumulate a significant Ca^{2+} increase in the basal segment near the ovary appearing at 22 h after pollination.

Although no Ca^{2+} gradients were described in the plants mentioned above, sufficient calcium stores existed in the transmitting tissue to indicate that local abundance correlates well with fertilization and loosely bound Ca^{2+} is more than adequate to meet their nutritional needs (Tian et al. 1998). The styles of the above-mentioned species (except lily) are relatively short, and the tube can grow to the embryo sac in very short time, so relatively smaller stores of available calcium may be required to support the Ca^{2+} uptake needs of pollen tubes in the style. Correspondingly, species with longer styles having a longer duration of tube growth may require larger stores to support elongation to the embryo sac.

The length of the tobacco style is up to 4 cm; it takes pollen tubes 45 h to reach the ovary (Tian and Russell 1997). Interestingly, Ca^{2+} accumulation in the ovule is directly related to pollination, unlike that in the style, and changes in loosely bound calcium are known to be involved in cellular signaling, potentially through their role as principal source of free cytoplasmic Ca^{2+} levels. Two studies provide such evidence. First, the earliest visible evidence of functional divergence between the receptive and the persistent synergid are changes in the labeling pattern of loosely bound calcium, which precede by almost a day to the outwardly visible degenerative changes in the cytoplasm of the receptive synergid (Tian and Russell 1997). Second, in megasporogenesis, abortive megaspores show a depletion of loosely bound calcium immediately prior to visible changes in their cellular ultrastructure (Qiu et al. 2008). Changes in loosely bound calcium may therefore precede evident $[Ca^{2+}]_{cyt}$ and ultrastructural changes in programmed cell death and also in other cellular signaling as well. Pollination induction of accumulation of loosely bound calcium in the style was reported in *Petunia hybrida* (Lenartowska et al. 1997) and in lily (Zhao et al. 2004); however, Ca^{2+} accumulation in tobacco styles is delayed until 22 h after pollination, demonstrating that pistil maturity occurs after anthesis. During tube elongation in the style, embryo sacs complete their preparation for tube penetration, with accumulation of Ca^{2+} and calcium precipitates in the filiform apparatus and distinctive patterns that indicate which of the synergids would be receptive (Tian and Russell 1997).

Clearly, the biology of flowering plants, such as tobacco and *Arabidopsis*, differ significantly. The former contains thousands of ovules in its ovary, whereas the latter

typically contains less than 30. Add to this, the presence of wet versus dry stigma, open versus closed style, and bicellular versus tricellular pollen, and it is evident that we have examined a limited range of the reproductive variability present in angiosperms. Examination of a greater diversity of flowering plants with differing floral biologies will be needed to understand the role of calcium accumulation and dynamics in the context of gynoecial structure, evolutionary patterns and maturation in the pollen and style during the progamic phase.

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