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Calcium Distribution during Anther Development of Tobacco

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ABSTRACT

Calcium distribution during anther development of tobacco was investigated using potassium antimonite technique. Before the appearance of microspore mother cells, no calcium-induced precipitates ware found in sporogenous cells and somatic cells of the anther wall. Prior to the onset of microsporogenesis, abundant minute calcium precipitates appeared in the callose wall. At this stage, there were also many precipitates that accumulated in the vacuoles of the tapetum. However, fewer calcium precipitates appeared in young microspores. With microspore development many calcium precipitates accumulated in the germ pore, and then appeared in the small vacuoles of the cytoplasm. At a later microspore stage, a large vacuole formed and some precipitates were observed in the vacuole. The number of precipitates in tapetal cells decreased as microsporogenesis proceeded. After microgametogenesis began, calcium precipitates in the cytoplasm of pollen grains decreased. As pollen grains accumulated starch, a fewer were evident in the cytoplasm. These results displayed the spatial and temporal features of calcium distribution during anther development of *Nicotiana tabacum* L., suggesting that it may be related with microspore development.

Keywords: antimonite localization, microspore, bicellular pollen, *Nicoctiana tabacum* L., tapetum

INTRODUCTION

Calcium is a significant element in organisms and participates in numerous physiological functions and its role has been revealed more and more in plant development (Bush 1993, 1995; Geitmann and Palanivelu 2007). Pollen tubes cultured *in vitro* have long been used to study the relationship between Ca^{2+} and tube growth, and it has been shown that a gradient of calcium distributed within the tube tip regulates tube growth (Feijó *et al*. 1995; Malhó and Trewavas 1996; Pierson *et al*. 1996). In addition, some researchers investigated Ca^{2+} distribution in ovaries and ovules of wheat (Chaubal and Reger 1990), pearl millet (Chaubal and Reger 1992), *Nicotiana tabacum* L. (Tian and Russell 1997), *Brassica napus* (Yu *et al*. 1998), *Plumbago zeylanica* L. (Tian *et al*. 2000) and rice (Zhao *et al*. 2002), and found numerous antimonite-precipitated calcium in the micropyle, and synergids, which may attract pollen tubes growing into the embryo sac. In conclusion, calcium provides essential signaling, physiological and regulatory roles during sexual reproduction in flowering plants and the abundance of calcium is an accurate predicator of plant fertility (Ge *et al*. 2007).

Tirlapur and Willemse (1992) first observed the distribution of membrane calcium in the anther of *Gasteria verrucuosa*. Tian *et al*. (1998) found that numerous calcium precipitates accumulated within the tapetum and locule of the anther of rice during its development, and that sterile anthers displayed abnormal calcium distribution when compared to fertile anthers of a photoperiod-sensitive genetically male-sterile rice. Meng *et al*. (2000) also found more calcium precipitates accumulated in the cells of the vascular bundle of sterile anthers than in those of the fertility maintenance line of wheat. These results indicated that the abnormality of calcium distribution is related with anther development. However, the features of calcium distribution in anther were investigated only in rice and wheat so far. It is necessary to investigate if the features of calcium distribution in rice and wheat anther are special phenomena only occurred in monocotyledons or universal ones in flowering plants. The current study used potassium antimonite to localize and detect pools of loosely bound calcium in anthers of *Nicotiana tabacum* L. during their development and to investigate the relation between Ca^{2+} distribution and anther development.

MATERIALS AND METHODS

Nicotiana tabacum L. was grown in a controlled environment at 27°C with a 15 h daylength illuminated at 54 μmol $m^{-2}s^{-1}$, and at 20°C with 9 h of darkness. Anthers at different developmental stages were squeezed to determine the developmental stage using a microscope and fixed 3 h at room temperature in 2% glutaraldehyde (v/v) in 0.1 mol/L phosphatic buffer (pH 7.8) containing 1% potassium antimonite ($K_2H_2Sb_2O_7.4H_2O$). The principle of potassium antimonite to precipitate calcium was introduced in detail in our recent review paper (Ge *et al*. 2007). After fixation by glutaraldehyde the samples were washed (three 30 min changes in buffered 1% antimonite) and postfixed in 1% (w/v) buffered OsO_4 containing 1% antimonite for 16 h at 4°C. Then samples were washed in buffer (three 30 min changes), dehydrated in a graded acetone series and embedded in Spurr's resin. In each stage, at least 10 anthers were embedded and 5 sectioned at 80 nm thickness and stained with 2% uranyl acetate (w/v) in 50% methanol (v/v), and then after being washed and air dried, sections were observed and photographed using a JEM-100 transmission electron microscope.

RESULTS

Calcium distribution in anther at the stage of microspore mother cell

There were no calcium-induced precipitates evident in the early microspore mother cell in young anthers (**Fig. 1**). Abundant starch grains accumulated in the cells of anther wall and connective tissue, but no calcium precipitates appeared in the cells at this stage (**Fig. 2**). Then within cells of the anther wall, calcium precipitates increased conspicuously, especially in the tapetum. Before meiosis of the microspore mother cell (MMC), a thick layer of callose

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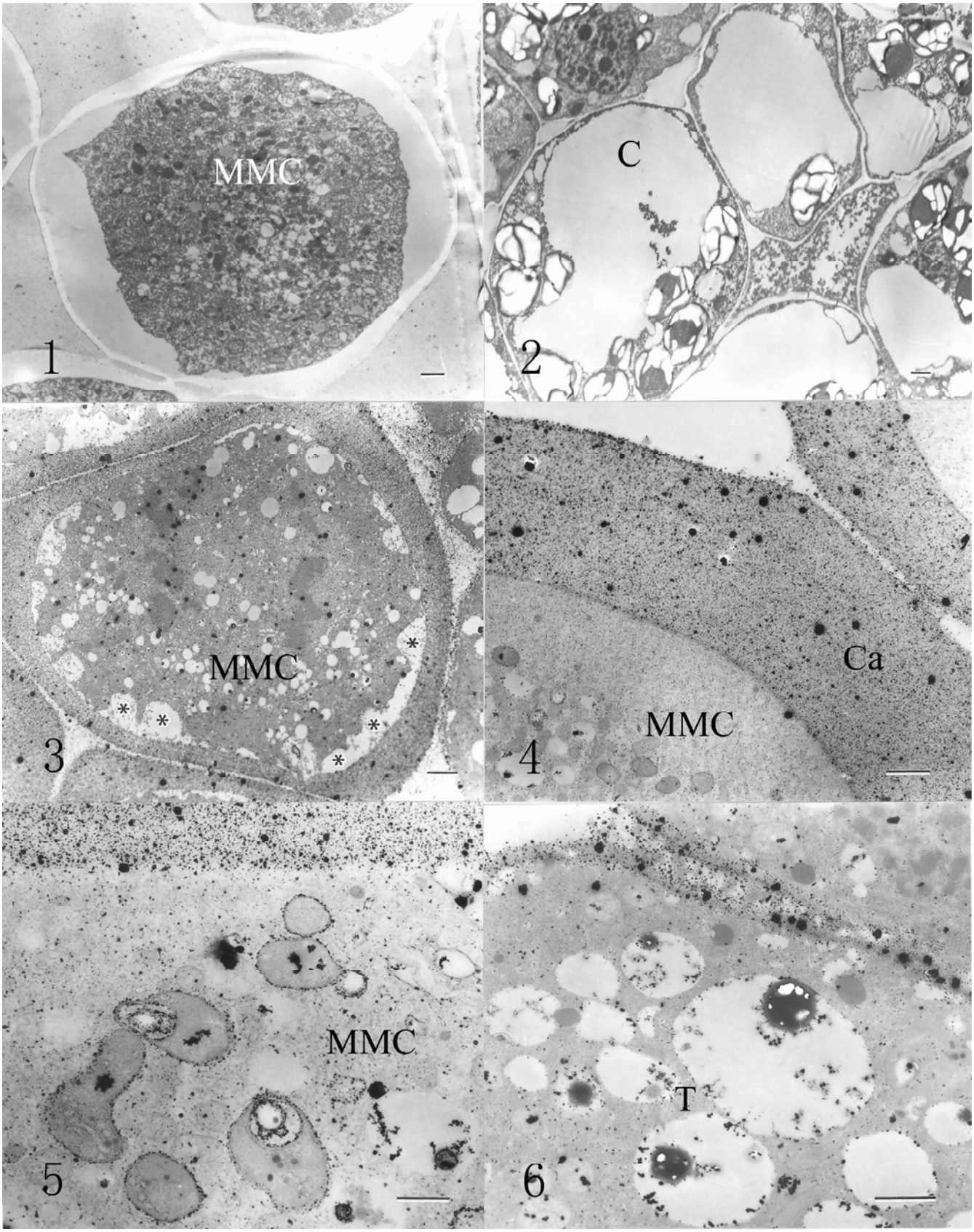

Plate I Calcium distribution in anther at the stage of microspore mother cell. (**1**) A few precipitates of antimonite-label are in an early microspore mother cell (MMC). ×4,050. Bar = 1 μm. (**2**) At the same time, few precipitates are in the cells of the connective tissue (C) of the anther. ×3,000. Bar = 1 μm. (**3**) A microspore mother cell (MMC) during meiosis reveals numerous calcium precipitates. The cellulose wall (asterisks) is being digested. ×4,050. Bar = 1 μm. (**4**) Enlargement of **Fig. 3**; A greater number and larger size of the calcium precipitates are evident in the MMC callose wall than in the cytoplasm. ×10,050. Bar = 1 μm. (**5**) Numerous precipitates also appear to be freely dispersed in the rest of the cytoplasm as well. ×15000. Bar = 0.5 μm. (**6**) In tapetal cells (T), precipitates are located within vacuoles and on the inner tangential face. ×15,000. Bar = 0.5 μm.

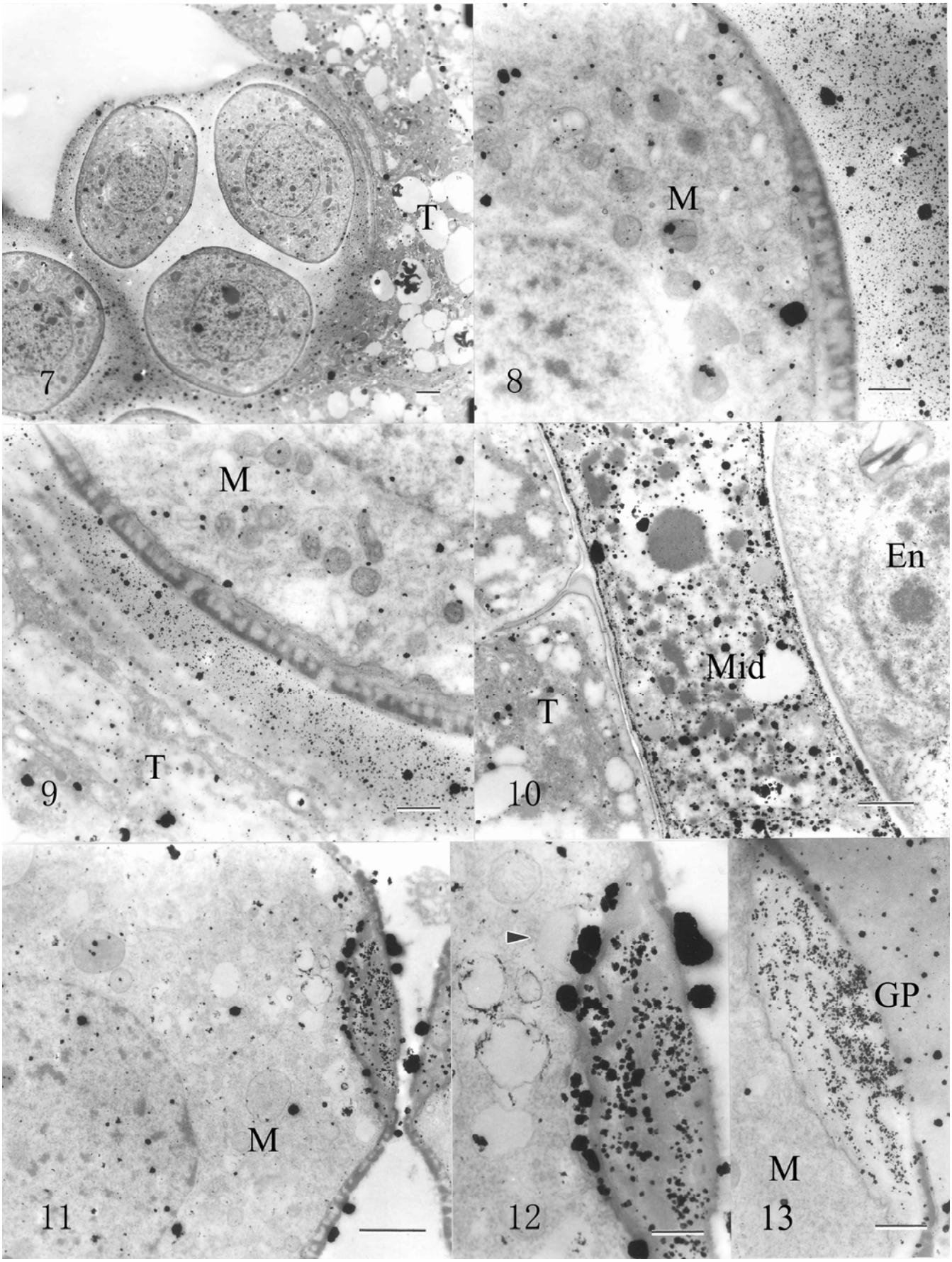

Plate II Calcium distribution in anthers at the early microspore stage. (**7**) Tetrads adjacent to the tapetum (T) with more precipitates evident in surrounding callose walls than in the walls between microspore cells. ×4,000. Bar = 1 μm. (**8**) Enlargement of **Fig. 7**; fewer precipitates in the cytoplasm of microspore (M) although many in the adjacent callose wall. ×8,000. Bar = 1 μm. (**9**) Fewer precipitates are evident in tapetal cells at the same time. ×8,000. Bar = 1 μm. (**10**) however, numerous calcium precipitates have accumulated in the cells of the middle wall layer (Mid), but very few are evident in the endothecium (En). T represents tapetum. ×10,050. Bar = 0.5 μm. (**11**) A young microspore just released from a tetrad with numerous calcium precipitates localized in the germ pore. ×10,050. Bar = 1 μm. (**12**) Enlargement of **Fig. 11**. Note some small vacuoles (arrowhead) forming within the cytoplasm adjacent to the pore. Calcium precipitates appear to be deposited on the membrane of these vacuoles. ×15,000. Bar = 0.5 μm. (**13**) Numerous calcium precipitates still ac-cumulate in germ pore during microspore development. ×10,050. Bar = 0.5 μm.

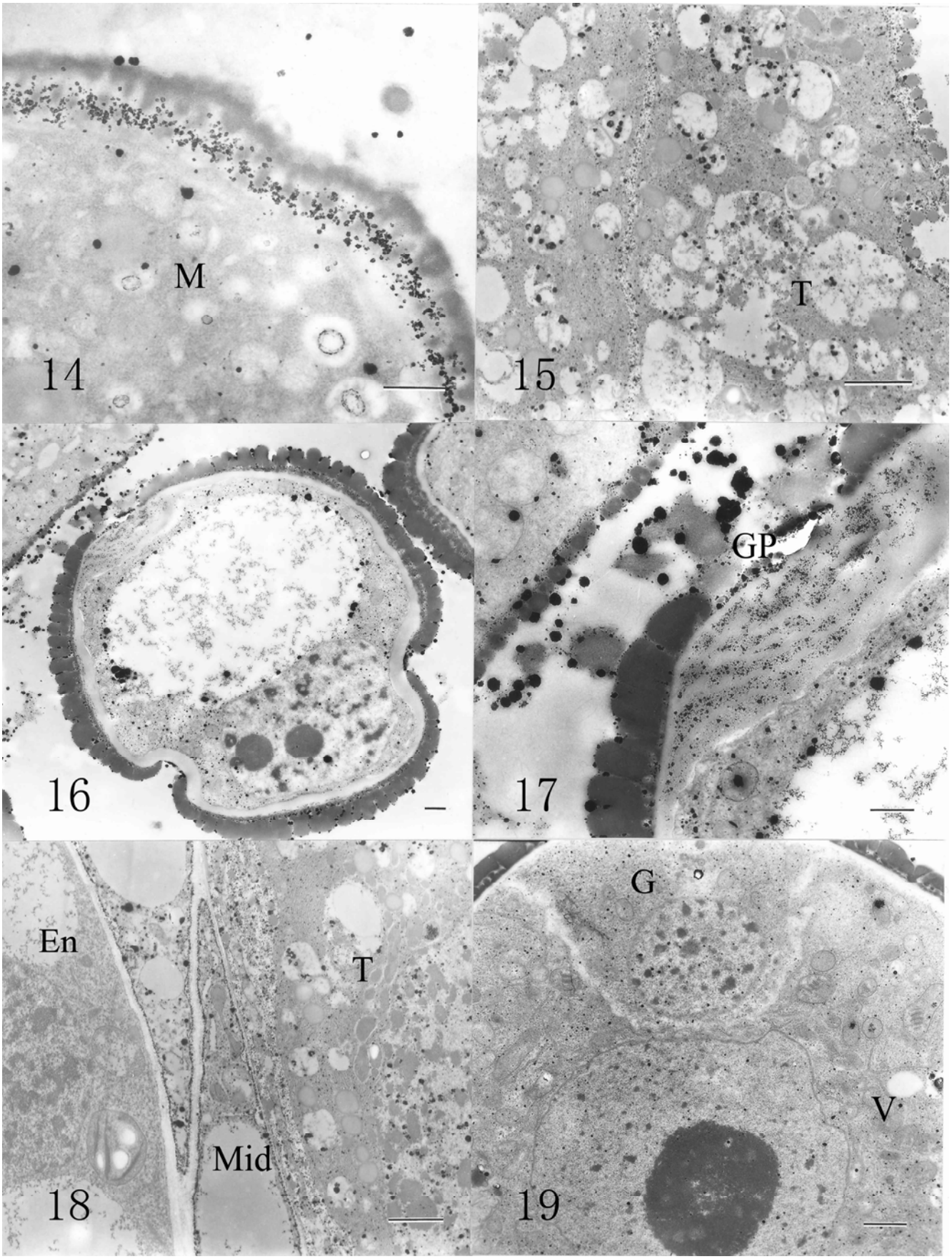

Plate III Calcium distribution in anthers at the late microspore stage. (**14**) Numerous calcium precipitates accumulate in the site of forming intine of a developing microspore. ×10,050. Bar = 1 μm. (**15**) At this stage, tapetal cells (T) display more calcium precipitates during intine formation than before (compare to **Fig. 9**). ×12,000. Bar = 1 μm. (**16**) At late stage microspore, a large vacuole has formed and some large precipitates have accumulated on the vacuole membrane. Note the precipitates in the intine disappear. ×4,000. Bar = 1 μm. (**17**) Enlargement of **Fig. 16**, there are still numerous small precipitates within the germ pore (GP), and some large precipitates located on the surface of Ubisch bodies being transported to the surface of the microspore. ×15,000. Bar = 0.5 μm. (**18**) The precipitates are evidently decreased in the cells of the middle layer (Mid) and tapetum (T) at late microspore stage. ×10,050. Bar = 1 μm. (**19**) After microspore division, no quantitative differences in calcium precipitates were observed between the generative cell (G) and vegetative cell (V). ×8,000. Bar = 1 μm.

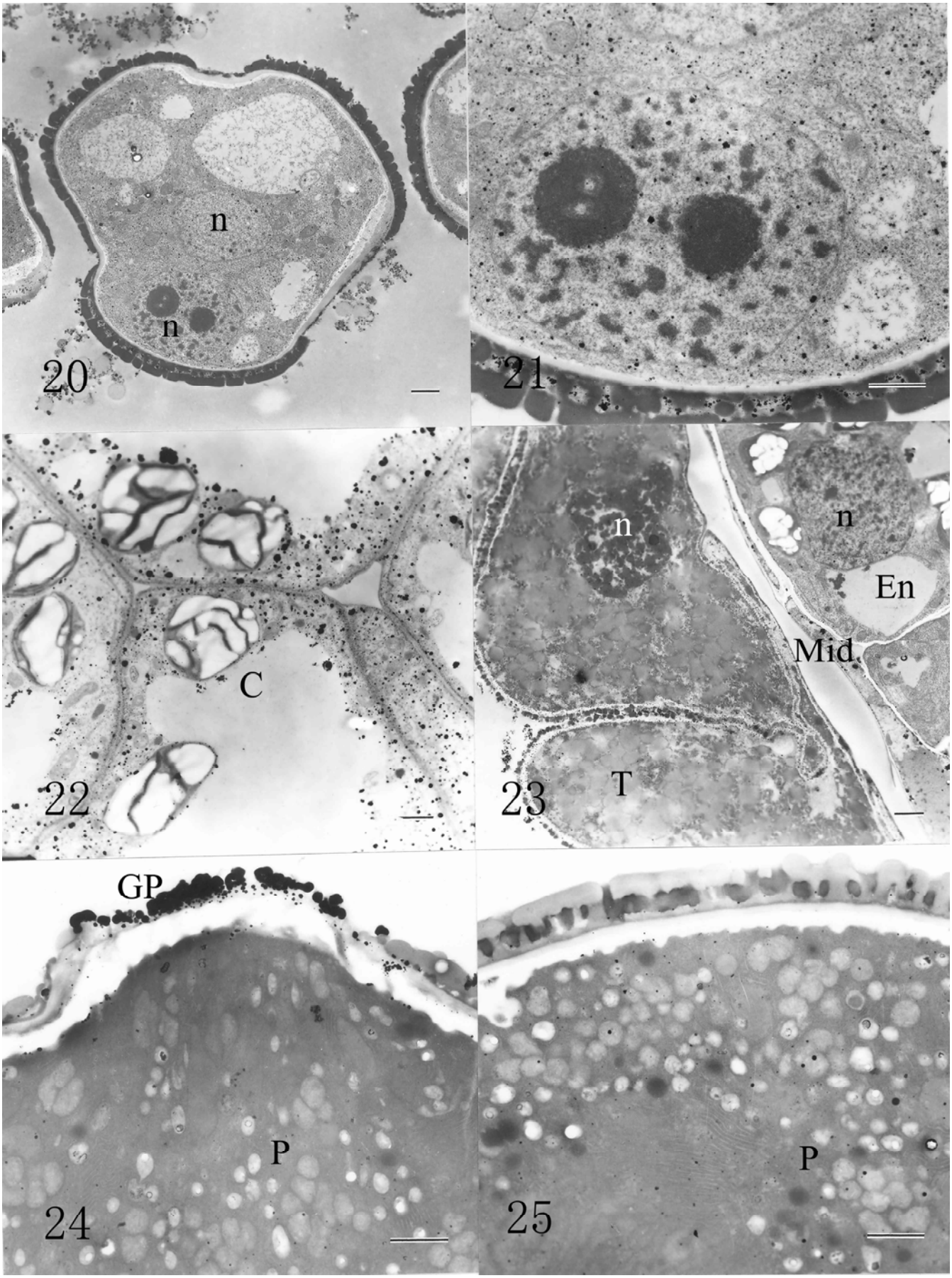

Plate IV Calcium distribution in anther at the bicellular pollen stage. (**20**) The large vacuole separated into several smaller ones in the bicellular pollen grain. Both n are nucleus of vegetative and generative cells. ×4,000. Bar = 1 μm. (**21**) Enlargement of **Fig. 20**, calcium precipitates appear in slightly greater numbers in vegetative cell comparing to **Fig. 19**. ×10,050. Bar = 1 μm. (**22**) Calcium precipitates increased markedly in the connective cells (C) at this time comparing to **Fig. 2**. ×6,000. Bar = 1 μm. (**23**) Calcium precipitates decreased sharply in the cytoplasm of degenerating tapetal cells (T). Mid: middle layer; En: endothecium. ×6,000. Bar = 1 μm. (**24**) Numerous precipitates still accumulated on the surface of the germ pore (GP) of a nearly mature pollen grain (P). ×10,050. bar = 1 μm. (**25**) A few precipitates are in the cytoplasm of a nearly mature pollen (P). ×10,050. Bar = 1 μm.

wall formed around each MMC. When MMC began to divide its cellulose wall dissolved and became some vacuoles (see asterisk). No calcium precipitates appeared in these vacuoles although numerous precipitates accumulated in the callose wall (**Fig. 3**), and the size of the precipitates was larger and the number greater than in MMC cytoplasm (**Fig. 4**). In MMC cytoplasm, calcium precipitates accumulated on the membranes of some organelles and in vacuoles (**Fig. 5**). In the tapetal cells of the anther wall at this same time, some calcium precipitates concentrated in vacuoles. When the vacuoles containing calcium precipitates were secreted into the locule, numerous small precipitates appeared on the surface of the inner tangential wall (**Fig. 6**).

Calcium distribution in anthers at the microspore stage

After meiosis of MMC, each newly formed microspore underwent a developmental process leading to changes in its morphology and internal structure. Numerous calcium precipitates remained in the callose wall of each tetrad but they were fewer in the walls between microspores than in the wall surrounding each tetrad (**Fig. 7**). Microspores in each tetrad began to produce their exine, and only a few calcium precipitates appeared in the cytoplasm of each microspore (**Fig. 8**). In the cells of the anther wall, calcium precipitates decreased in tapetal cells than before (**Fig. 9**), but they sharply increased in the middle layer of anther wall at this time (**Fig. 10**). However, the cells of the epidermis and endothecium never accumulated much calcium precipitate.

When microspores were released from tetrads, numerous calcium precipitates accumulated in the germ pore, and some small vacuoles formed in the nearby cytoplasm (**Fig. 11**). The vacuoles originated within the germ pore and some calcium precipitates attached on its membrane (**Fig. 12**), suggesting a way for calcium to be relocated within the microspore from the germ pore. The germ pore always accumulated numerous calcium precipitates during microspore development (**Fig. 13**). When microspores formed the intine of the pollen wall, numerous calcium precipitates specially accumulated in the site of intine (**Fig. 14**), suggesting that calcium may be related with intine formation. Concurrently with microspore development, the cytoplasm of tapetal cells displayed more vacuoles comparing with before and the number of calcium precipitates increased again, most of them in small vacuoles (**Fig. 15**). At the late microspore stage, a large vacuole displaced the microspore nucleus to a periplasmatic region. This polarization occurred before the first division of the developing male gametophyte (**Fig. 16**). At this time, the intine formation was complete and no calcium precipitates remained in it. However, small calcium precipitates increased in the cytoplasm and some larger precipitates were present around the edges of the vacuole and many small ones were suspended within it. Calcium precipitates combined with sporopollenin material from tapetal cells were secreted into the locule, and then much smaller calcium precipitates appeared in the wall layers of the germ pore (**Fig. 17**). The germ pore of microspore always displayed numerous calcium suggests that it is main entrance of materials into microspore and so is calcium. At the same time, the number of calcium precipitates in tapetal cells and the cells of the middle layer decreased, and the cells of the middle layer formed a large vacuole (**Fig. 18**).

Calcium distribution in anther at the bicellular pollen stage

After an asymmetrical mitotic division of a microspore, a bicellular pollen grain is formed which consists of a larger vegetative cell and a smaller generative cell. Initially, there was no difference in the number of calcium precipitates observed within these two cells (**Fig. 19**). Then the large vacuole of the vegetative cell disaggregated into several small ones (**Fig. 20**), and calcium precipitates in the cytoplasm of vegetative cell increased again (**Fig. 21**). Meanwhile, many calcium precipitates accumulated in the cells of the connective tissue, as well as starch synthesis leading to amyloplasts (**Fig. 22**). Concurrently, tapetal cells began to degenerate, the structural integrity of these cells became less distinct and calcium precipitates within these cells decreased sharply (**Fig. 23**). These phenomena suggest that the anther's requirement for calcium and nutritional material decreases, leading to calcium and polysaccharide material accumulation in cells of the connective tissue. When starch began to accumulate in bicellular pollen grains, the electron density of the cytoplasm of these pollen grains increased. With bicellular pollen development, numerous calcium precipitates still accumulated on the surface of the germ pore (**Fig. 24**), and they were sharply diminished in the cytoplasm of bicellular pollen (**Fig. 25**).

DISCUSSION

The development of the anther is a very rapid and complicated process. The cells within different tissues of the anther exhibited conspicuous changes of morphology and structure, such as degeneration of tapetal cells, microspore cytoplasm became more highly polarized as a large vacuole formed, and pollen wall formation after released from tetrad. At the stage of meiosis of the microspore mother cell, calcium precipitates first appeared in tapetal cell cytoplasm and then accumulated in vacuoles which were secreted into the locule of the anther. After this movement, tapetal cells began degenerating. Calcium precipitates began to accumulate in the cytoplasm of young microspores just released from a tetrad. Within these microspores, precipitates accumulated within small vacuoles which then coalesced to form a single large vacuole. After mitosis of each microspore leading to the bicellular pollen grain, the large vacuole appeared to disaggregate into many smaller ones and calcium precipitates appeared in these small vacuoles again. Then calcium precipitates in the bicellular pollen grain decreased. The Ca^{2+} distribution in the anther of tobacco displays spatial-temporal features following its development. The results of higher level Ca^{2+} appeared in special time of tobacco anther is quite similar to that reported in rice and wheat, where high calcium concentration is located in special region of anther during its development (Tian *et al*. 1998; Meng *et al*. 2000).

What is the presumed physiological function of Ca^{2+} during anther development? Ca^{2+} can regulate many cell functions according to its cellular location, binding or solubility, such as regulating the activities of enzymes when combined with them, acting as signal ions between cells, forming an essential component of the cell structure, and possibly acting as second messengers (Bush 1995). Ca^{2+} also appears to have a uniquely important role in plant cells during wall accretion, vacuolar turgor, and in stomatal movement (de Silva *et al*. 1985a, 1985b; Inoue and Katoh 1987; Schwartz *et al*. 1988; Bolwell 1993). The presence of Ca^{2+} within plant cells also appears related to the transport and accumulation of amino acids (Rickauer and Tanner 1986) and the metabolism of carbon-hydrated compounds (Brauer *et al*. 1990). However, only a few reports that have dealt with the calcium distribution during anther development and no information related the Ca^{2+} physiological function in anther development was proposed. In the present study, calcium precipitates in anthers were mostly accumulated during microspore development. Thus, two possible functions of Ca^{2+} in pollen development are: providing osmotic pressure to aid the formation of a large vacuole within a microspore, which contributes to polarization of the cytoplasm preceding unequal division of the microspore, and secondly, Ca^{2+} contributes to the intine formation because numerous calcium precipitates located in the site of intine during its forming. For confirming above hypothesis, it is necessary to do further research to open up the mechanism of Ca^{2+} physiological roles in anther development of flowering plants.

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