

Calcium distribution and function during anther development of *Torenia fournieri* (Linderniaceae)

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Potassium antimonite was used to locate calcium in the anthers of *Torenia fournieri* (Linderniaceae). Abundant calcium precipitates accumulate in the microsporocyte cytoplasm. After meiosis, calcium precipitates are abundant on the microspore wall, as well as the callosic wall of each tetraspore. A large number of calcium precipitates also occur on the outer membranes of the tapetal cells, and in the intercellular spaces of the endothecium and middle layer. The quantity of calcium precipitates in the cytoplasm and nucleus increases at the early microspore stage, then gradually decreases until pollen maturation. Calcium precipitates on the pollen wall gradually increase from the early microspore stage until pollen maturation. Numerous calcium precipitates are observed around the Ubisch bodies. The relation between the distribution of calcium and mitosis, nuclear displacement, the formation of the pollen cell wall, as well as the possible functions of anther walls and Ubisch bodies in the transportation of calcium to the mature pollen are discussed.

Key words: anther wall, calcium, pollen, *Torenia fournieri*

Introduction

Calcium, an important and ubiquitous messenger in plant cells, has been shown to play a crucial role in plant growth and development (Tian & Yuan 2000). The important roles of calcium in regulating pollen germination and pollen tube growth (Franklin-Tong 1999), as well as in fertilization (Digonnet *et al.* 1997, Antonie *et al.* 1999, Yu *et al.* 1999) have attracted attention of plant development biologists.

Anther structure is complex. The four cell layers of the anther wall are clearly different in structure and function. Studies on calcium distribution in male reproduction have mainly focused on pollen germination and pollen tube growth (Tirlapur & Cresti 1992, Tirlapur & Willemsee 1992), while far less is known about calcium's role in anther development. Calcium distribution in the anther has been studied in only few plants. Calcium accumulation is correlated with the failure of pollen development and with pollen

abortion in photoperiod-sensitive cytoplasmic male-sterile rice (Tian *et al.* 1998), Honglian-Yuetai cytoplasmic male sterile rice (Li *et al.* 2001), wheat (Meng *et al.* 2000) and *Brassica campestris* ssp. *chinensis* var. *communis* (Xie *et al.* 2005). In gymnosperms, Kong and Jia (2004) studied calcium distribution in the anther of *Larix principis-rupprechtii* and discussed the relation between the distribution of calcium precipitates on the tetrad walls, the formation of the pollen wall, and the possible function of epidermis, middle cells, tapetum and Ubisch bodies in transportation of Ca^{2+} to the pollen. However, in the model plant *Torenia fournieri*, only a few studies have been conducted on calcium distribution in male reproductive structures and only one on the distribution of calcium during the process of pollen germination and pollen tube growth (Yao & Zhao 2004).

In the current study, potassium antimonite precipitation is used to visualize calcium localization in the anthers of *T. fournieri*, in order to explore the role of calcium in anther development.

Material and methods

Plants of *T. fournieri* were grown in the South China Botanical Garden, the Chinese Academy of Sciences. Anthers were collected at different developmental stages based on the stages of

pollen development. At least ten anthers from different flowers were fixed, and at least five anthers from each treatment were examined. Anthers were fixed in 2% glutaraldehyde in 0.1 M KH_2PO_4 buffer (pH 7.8) containing 1% $K_2H_2Sb_2O_7 \times 4H_2O$ for 12–24 h at 4 °C, washed in three changes of 1% $K_2H_2Sb_2O_7 \times 4H_2O$ in 0.1 M KH_2PO_4 buffer (0.5 h each) and postfixed in 1% OsO_4 for 16 h at 4 °C in 0.1 M KH_2PO_4 buffer containing 1% $K_2H_2Sb_2O_7 \times 4H_2O$. The postfixed tissues were washed in three changes of 0.1 M KH_2PO_4 buffer without antimonite, dehydrated in a graded ethanol series and embedded in Epon 812 resin. Ultra-thin sections (80 nm) were cut using a Leica-Ultracut S ultramicrotome, stained with uranyl acetate and observed with a JEM-1010 transmission electron microscope at 90 kV (Sajo *et al.* 2005). Two additional controls were used: (i) $K_2H_2Sb_2O_7 \times 4H_2O$ was sometimes omitted from solutions during processing; (ii) selected grids with specimens containing calcium precipitates were incubated in a solution of 0.1 M EGTA for 1 h at 37 °C to remove precipitates (Tian & Russell 1997).

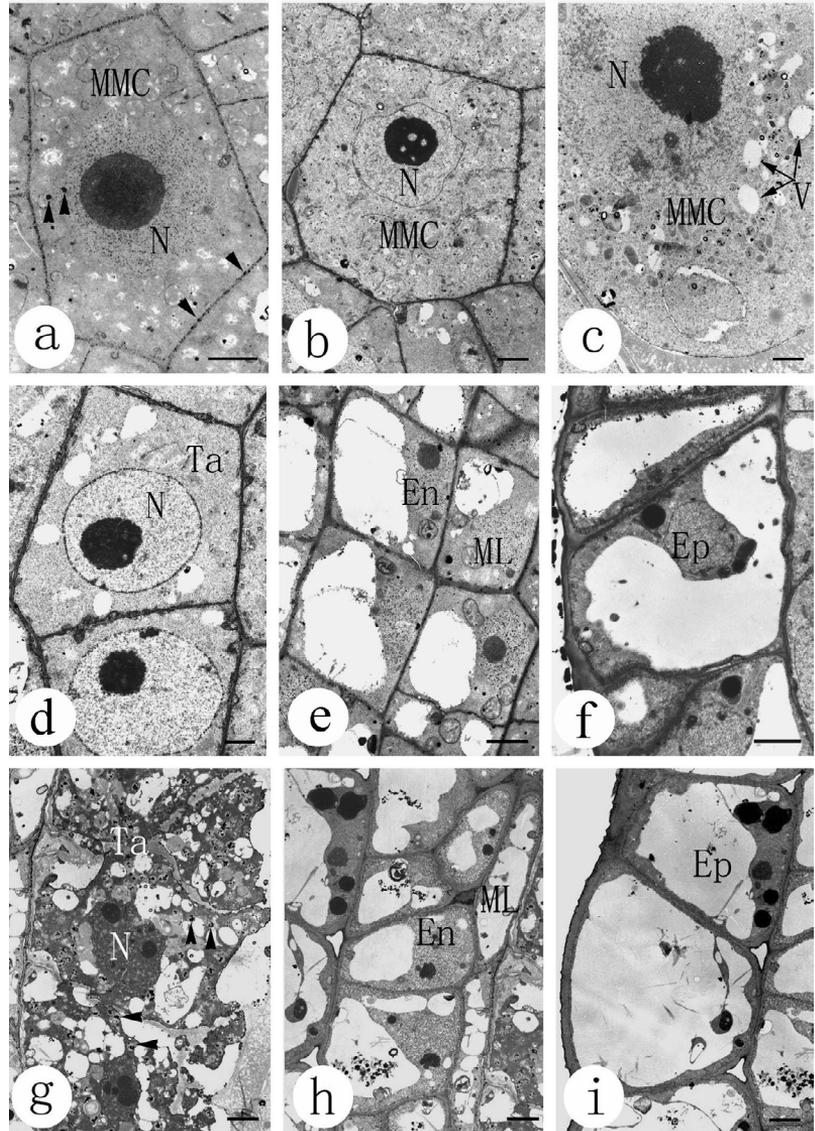
Results

Calcium distribution was analyzed from the microspore mother cell to the mature pollen stage (Table 1).

Table 1. Comparison of relative abundance of calcium-induced precipitates in anthers of *Torenia fournieri*. Relative abundance: – = no precipitates; + = few precipitates; ++ = common precipitates; +++ = abundant precipitates; ++++ = very abundant precipitates; × = degenerated cells.

| | Microspore mother cell | Tetrad | Early microspore | Late microspore | Microgametophyte | Mature pollen |
|--------------|------------------------|--------|------------------|-----------------|------------------|---------------|
| Anther wall | | | | | | |
| Epidermis | + | ++ | + | ++ | + | ++ |
| Endothecium | + | +++ | + | +++ | ++++ | +++ |
| Middle layer | + | ++ | ++ | × | × | × |
| Tapetum | + | ++ | +++ | × | × | × |
| Ubisch body | | | + | +++ | +++ | ++ |
| Pollen | | | | | | |
| Exine | | + | ++ | +++ | ++++ | ++++ |
| Intine | | | + | +++ | ++++ | – |
| Cytoplasm | +++ | + | ++ | + | ++ | + |
| Nucleus | – | – | +++ | + | + | + |
| Callose | | +++ | | | | |

Fig. 1. *Torenia fournieri*. Microspore mother cell stage. — **a**: Numerous calcium precipitates (arrowheads) in polyhedral microspore mother cell (MMC). — **b**: A microspore mother cell and nucleus (N) showing little change in the number of calcium precipitates. — **c**: Small vacuoles (V) in a round microspore mother cell. Calcium precipitates have decreased on the cell surface. — **d–f**: At the early microsporocyte stage only a few calcium precipitates appear in cells of the anther wall: tapetum (Ta), middle layer (ML), endothecium (En) epidermis (Ep). — **g–i**: At the later microsporocyte stage, there is a decreased number of calcium precipitates (arrowheads) in the cells of the anther wall: tapetum (Ta), middle layer (ML), endothecium (En) epidermis (Ep). Nucleus (N). Scale bars: **a, c, d** = 1 μ m; **b, e, f–i** = 2 μ m.



Microspore mother cell stage

The microspore mother cells are initially diamond shaped and in close contact with each other, but gradually become oval and separate before meiosis. They enlarge greatly during the microsporocyte stage while retaining a large nucleus, abundant organelles, and acquiring some small vacuoles. Abundant calcium is found in the cytoplasm, and around the vacuoles (Fig. 1a–c).

The microsporangium wall consists of an epidermis, endothecium, middle layer and tapetum. During the microsporocyte stage the cells of the

epidermis and endothecium become vacuolated, while the cells of the middle layer flatten. At this stage, calcium is seldom found in the cells of the epidermis, endothecium or middle layer, except for a small amount that occurs along the cell walls (Fig. 1e, f, h, i).

During the early microsporocyte stage the tapetal cells are regular in shape, and contain large nuclei. As the microspore mother cells become oval (Fig. 1c), the inner periclinal and radial walls of the tapetal cells dissolve, and some irregular invaginations appear (Fig. 1g). At this stage, calcium precipitates are localized on

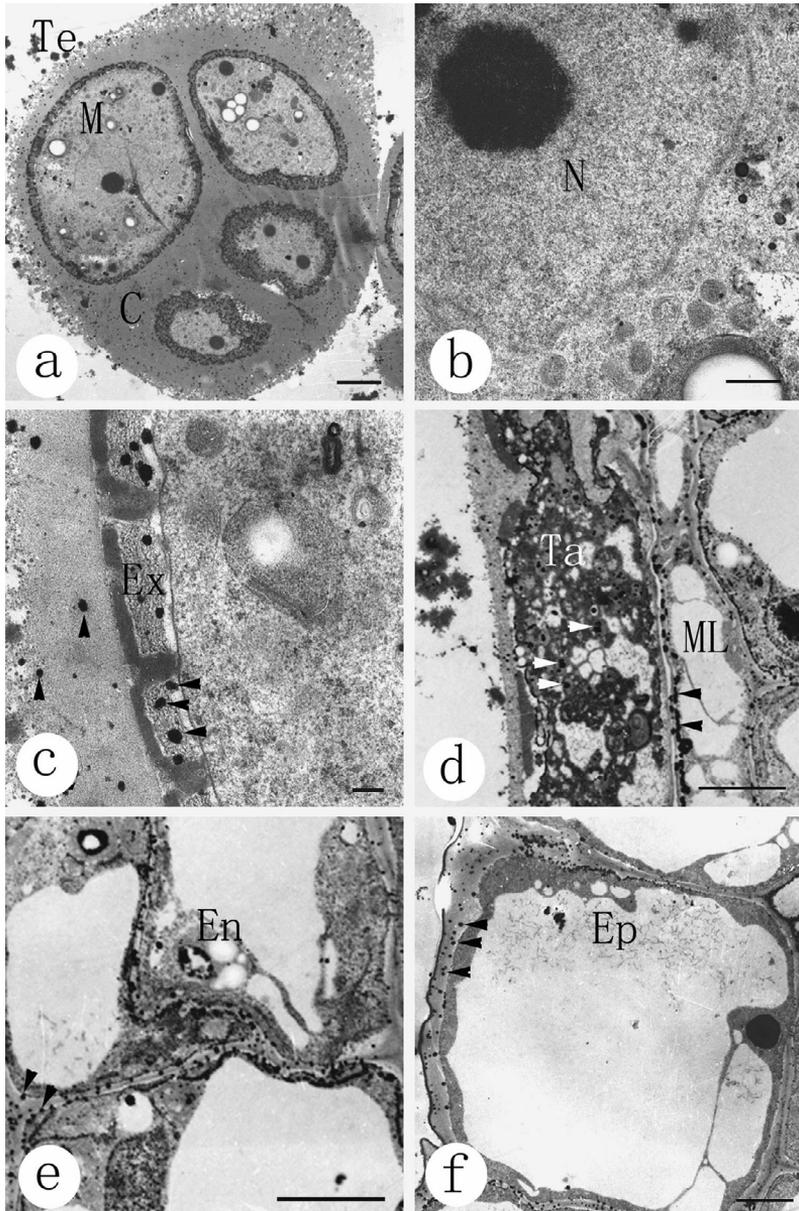


Fig. 2. *Torenia fourneri*. Tetrad (Te) stage. — **a**: Only a few calcium precipitates occur in the cytoplasm of the microspore (M), but there are abundant calcium precipitates in the callose sheath (C). — **b**: A few calcium precipitates occur in the nucleus of the tetrad microspores. — **c**: Calcium precipitates (arrowheads) are present in the developing exine (Ex) and callose sheath. — **d**: Calcium precipitates (arrowheads) in degenerating tapetal (TA) and middle layer (ML) cells. — **e**: Endothecium (En) with large numbers of calcium precipitates. — **f**: Vacuolating epidermis cell (Ep) with many calcium precipitates (arrowheads). Scale bars: **a**, **d–f** = 2 μ m; **b** = 500 nm; **c** = 200 nm.

the cell membrane, tonoplasts, and in the cytoplasm of the tapetal cells (Fig. 1g).

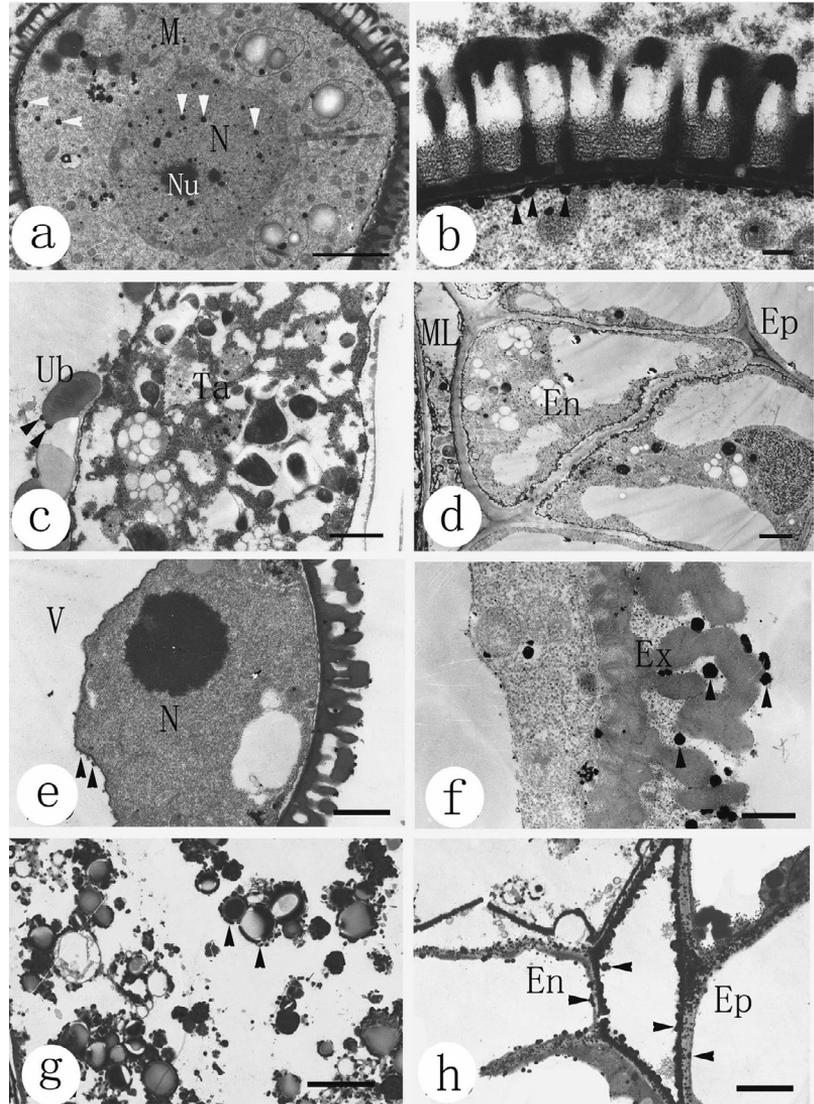
Tetrad stage

Post-meiotic tetrads can be either tetrahedral or decussate, and are surrounded by thick callosic walls (Fig. 2a). Each microspore is spherical, contains a large number of plastids and mitochondria, and has a prominent and centrally

located nucleus. The exine begins to form at this stage.

At the tetrad stage, a few calcium precipitates occur in the cytoplasm and the nuclei (Fig. 2b), and abundant calcium precipitates are found in the callose sheath and the pollen exine (Fig. 2c). During tetrad formation, the epidermis and endothecium cells contain large vacuoles, which displace the cytoplasm to the periphery of the cell (Fig. 2e, f). At the stage when the microspores are released into the locule, the cells of

Fig. 3. *Torenia fournieri*. Stages of microspore development. — **a–d**: Early microspore stage. **a**: Abundant calcium precipitates occur in the nucleus and cytoplasm of a young microspore. **b**: Calcium precipitates in the peripheral cytoplasm. **c**: Only a few calcium precipitates occur in the degenerating tapetal cells, and early-formed Ubisch bodies (Ub). **d**: Degeneration of the middle layer. The frequency of calcium precipitates is lower in the endothecium cells than at previous stages, and only a few calcium precipitates appear in the epidermal cells. — **e–h**: Late microspore stage. **e**: Microspore with few calcium precipitates located on the tonoplast, and few in the nucleus. **f**: Many calcium precipitates occur in the exine. **g**: After the tapetum degenerates the Ubisch bodies are surrounded by a large number of calcium precipitates. **h**: Numerous calcium precipitates appear in the endothecium, epidermis, and in the intercellular spaces surrounding these cells. Scale bars: **a, d, g, h** = 2 μm ; **b** = 200 nm; **c, e** = 1 μm ; **f** = 500 nm. Arrowheads = calcium precipitates.



the middle layer and tapetum gradually degenerate, and the tapetal cells contain numerous small vacuoles and many organelles (Fig. 2d). At this stage there are more calcium precipitates on the cell membrane and tonoplasts than at previous stages. The amount of calcium in the epidermis and endothecium also increases (Fig. 2e and f).

Early microspore stage

Free microspores are released into the anther locule by the dissolution of the callose sheath.

Each microspore is circular, with a dense cytoplasm and a prominent central nucleus (Fig. 3a). Plastids and mitochondria appear similar to those in undifferentiated tetrads. Quite a few calcium precipitates are observed in the microspore nucleus and cytoplasm (Fig. 3a), and abundant calcium precipitates are deposited on the surface of the intine, but are lacking from the exine (Fig. 3b). There is little calcium in the epidermis or endothecium (Fig. 3d), with only a few precipitates in the intercellular spaces. At this stage, the cells of the middle layer begin to break down, with few calcium precipitates on the plasma

membrane and tonoplasts of these cells (Fig. 3d). As the tapetum degenerates, Ubisch bodies appear on the inner surface of the locule (Fig. 3c), and calcium precipitates increase in the tapetal cells and appear around the Ubisch bodies (Fig. 3c).

Late microspore stage

A central vacuole develops in each microspore causing the spore to enlarge in relation to the surrounding cells. The nucleus now assumes a peripheral position (Fig. 3e). At this stage, abundant calcium precipitates are found on the surface of the exine (Fig. 3f), with only a few precipitates in the cytoplasm and the nucleus (Fig. 3e). Calcium precipitates increase in the anther walls, especially in the tapetal cells and on the surface of Ubisch bodies (Fig. 3g). As the middle layer cells dissolve, calcium accumulates in the epidermis, endothecium cells and in the intercellular spaces surrounding these cells (Fig. 3h).

Microgametophyte formation and mature pollen stages

The mitotic division of the microspore nucleus results in the formation of two unequal cells, a large vegetative and a smaller generative cell. During mitosis of the microspore nucleus a large number of calcium-induced precipitates appear in the cytoplasm, but few occur in the nucleus (Fig. 4a). At the end of mitosis, the generative cell gradually separates from the pollen wall. During this time, only a few calcium precipitates are observed in the generative cell, but numerous precipitates occur on the pollen wall (Fig. 4b). As the generative cell becomes spindle shaped and is enclosed in the vegetative cell, are numerous calcium-induced precipitates appear on the pollen wall, but few in the cytoplasm (Fig. 4c, d). At this stage, the intine has fully developed, the tapetal cells have degenerated, and there are abundant calcium-induced precipitates associated with the Ubisch bodies (Fig. 4e). The number of precipitates in the endothecium remains high, but their number has noticeably decreased in the epidermis (Fig. 4f).

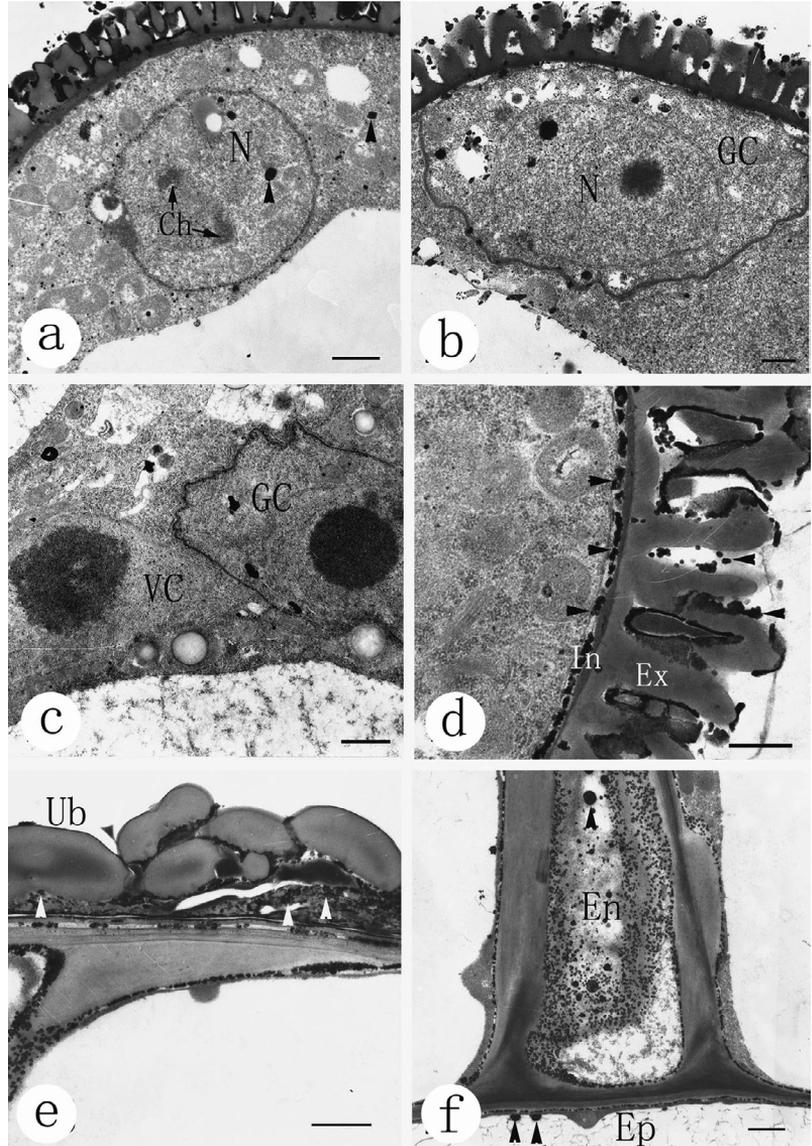
The pollen grains are two-celled at the time of anther dehiscence, and contain numerous starch grains and lipid droplets. Only a few calcium precipitates are located in the nucleus and cytoplasm of the generative cell at this stage (Fig. 5a). Although numerous precipitates are still present in the exine, few precipitates occur in the intine (Fig. 5b). In the anther wall, occasional calcium precipitates still occur in the epidermis (Fig. 5d), and abundant precipitates are found in the endothecium and surrounding the Ubisch bodies (Fig. 5c).

Discussion

Calcium is known to be involved in cellular responses to environmental stimuli (Bush 1995, Nayyar 2003). Kong and Jia (2004) found that calcium was abundant in the plastids of the microspore mother cell of *Larix principis-rupprechtii* during meiosis, and suggested that calcium might be related to starch hydrolysis and thus provide nutrition for meiosis. In our study, abundant calcium precipitates were also found in the microspore mother cells. We suggest that the accumulation of calcium in the cytoplasm of the microspore mother cells might be correlated with the meiosis of these cells. At the early microspore stage, a large number of calcium precipitates accumulate in the nucleus and cytoplasm. This accumulation might be related to the displacement of the nucleus at this stage. In later stage microspores, accumulations of calcium appear on the tonoplast of the large vacuole, and may be correlated with the formation of this vacuole.

During anther development of several plants, calcium precipitates accumulate in the anther wall (Tian *et al.* 1998, Xie *et al.* 2005, Qiu *et al.* 2005). The accumulation of calcium on the mature pollen wall might reflect calcium storage for pollen germination (Gong *et al.* 1995, Ma *et al.* 1998). In *Torenia fournieri* calcium precipitates are found (i) abundantly on the callosic wall around microspore, and sparsely on the exine, in the tetrad stage; (ii) regularly distributed on the exine, in the early microspore; (iii) not only on the exine, but also on the intine, when the intine is fully developed; (iv) on the exine, but not on the intine, in the mature pollen.

Fig. 4. *Torenia fournieri*. Formation of the microgametophyte. — **a**: A few calcium precipitates appear in the cytoplasm and nucleus (N) as the chromosomes (Ch) begin to separate during mitosis. — **b**: Generative cell (GC) partially attached to the pollen wall, and containing a few calcium precipitate in its cytoplasm and nucleus (N). — **c**: Vegetative cell (VC), and generative cell now no longer attached to the outer wall, both containing few calcium precipitates. — **d**: Pollen grain with completely formed intine (In). The borderline between the intine and exine can be clearly seen. Numerous calcium precipitates are present in both the intine and exine. — **e**: Ubisch bodies associated with numerous calcium precipitates. — **f**: Numerous calcium precipitates located in the endothecium cells (EN), while few calcium precipitates occur in the epidermal cells. Scale bars: **a, c, e, f** = 1 μ m; **b, d** = 500 nm. Arrowheads = calcium precipitates.



Gong *et al.* (1990) presented data to show that the cell wall contains one of the largest calcium stores in the cell, and that the pollen wall has the highest calcium level of any region in a pollen grain. Gong *et al.* (1995) also found that there is a significant Ca^{2+} release from pine and tobacco pollen during the hydration and early germination phase. Our results show that the accumulation of calcium in the callosic wall is correlated with the formation of the pollen wall, and are consistent with the hypothesis that changes in calcium distribution can influence the

accretion and formation of the exine and intine. The accumulated calcium may also play a role in pollen germination.

Calcium distribution in the microsporangia and on the Ubisch bodies suggests a gradual transportation of calcium from the microsporangia, wall to the pollen during pollen development (Kong & Jia 2004). A large amount of calcium is transported into the locule in normal fertile anthers, but little calcium is transported in sterile anthers (Tian *et al.* 1998, Meng *et al.* 2000, Li *et al.* 2001). These results suggest that

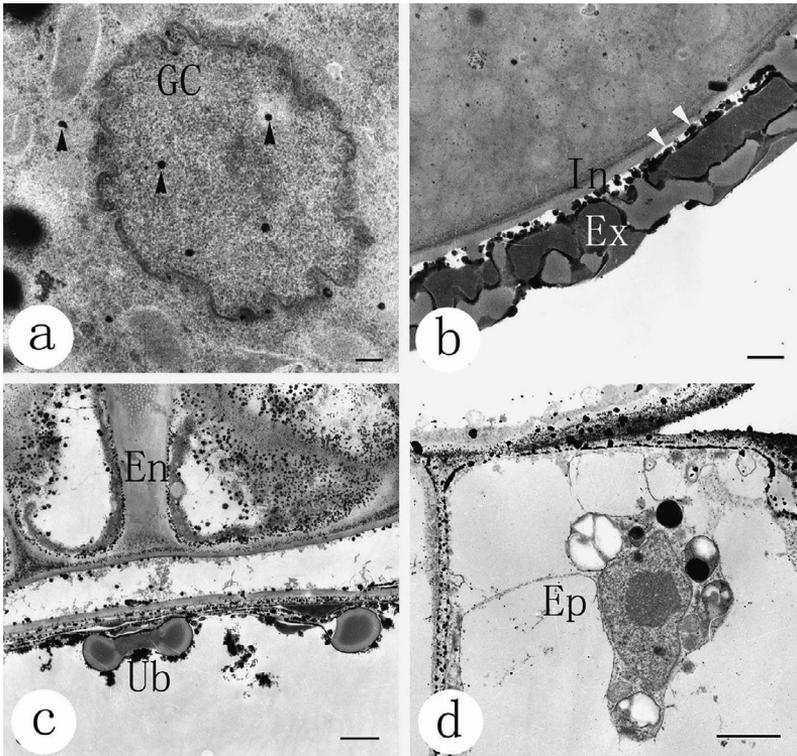


Fig. 5. *Torenia fournieri*. Mature pollen stage. — **a**: Generative cell enclosed in vegetative cell cytoplasm, both containing a few calcium precipitates. — **b**: Pollen wall with no calcium precipitates in the intine, but numerous precipitates in the exine. — **c**: Endothecium (En) and Ubisch bodies (Ub) with numerous calcium precipitates. The remnants of the tapetum contain a few precipitates. — **d**: Epidermal cell with a greater number of calcium precipitates than at the two-celled stage. Scale bar: **a** = 200 nm; **b** = 500 nm; **c** = 1 μ m; **d** = 2 μ m. Arrowheads = calcium precipitates.

the distribution of calcium is correlated with pollen abortion. In the present study, calcium in the pollen wall increases, and calcium in the anther wall decreased. These results suggest that calcium trends to be transported into the microsporangium locule.

The most important function of the tapetal cells is to provide nourishment for microspore development. During periplasmoidal tapetum development in lettuce (*Lactuca sativa*), a large number of calcium precipitates accumulate in the tapetal cells and move into the locule with the tapetal protoplasts (Qiu *et al.* 2005). Unlike lettuce, the anther wall of *T. fournieri* has a secretory tapetum. As the tapetal cells dissolve, calcium is precipitated onto the Ubisch bodies.

Tapetal cell degradation is a process of programmed cell death (Tian 2002). Wang *et al.* (2001) confirmed that the Ca^{2+} signal system is involved in programmed cell death during anther development. Our results support this conclusion as calcium-induced precipitates increase as the tapetum degenerates.

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References

- Antonie, A. F., Faure, J. E., Cordeiro, S., Dumas, C., Rougier, M. & Feijo, J. A. 1999: A calcium influx is triggered and propagates in the zygote as a wave front during in vitro fertilization of flowering plants. — *Proceedings of the National Academy of Sciences of the United States of America* 97: 10643–10648.
- Bush, D. S. 1995: Calcium regulation in plant cells and its role in signaling. — *Annual Review of Plant Physiology and Plant Molecular Biology* 46: 95–122.
- Digonnet, C., Aldon, D., Leduc, N., Dumas, C. & Rougier, M. 1997: First evidence of a calcium transient in flowering plants at fertilization. — *Development* 124: 2867–2874.
- Franklin-Tong, V. E. 1999: Signaling and modulation of pollen tube growth. — *Plant Cell* 11: 727–738.
- Gong, M., Li, Y. & Tsao, T. H. 1990: Calcium messenger

- system in plants. — *Chinese Bulletin of Botany* 7(3): 19–29.
- Gong, M., Yang, Z. H. & Tsao, T. H. 1995: Initiating effects of calcium on pollen germination and its regulative role in pollen growth. — *Acta Scientiarum Naturalium Universitatis Pekinensis* 31(3): 238–249.
- Kong, H. Y. & Jia, G. X. 2004: Calcium distribution during pollen development of *Larix principis-rupprechtii*. — *Acta Botanica Sinica* 46(1): 69–76.
- Li, R. Q., Zhu, Y. G., Meng, X. H. & Wang, J. B. 2001: The distribution of calcium in the pollen and connective tissue of Honglian-Yuetai cytoplasmic male sterile rice. — *Acta Agronomica Sinica* 27: 230–233.
- Ma, L. G., Xu, X. D., Cui, S. J. & Sun, D. Y. 1998: The involvement of phosphoinositide signaling pathway in the initiatory effects of extracellular calmodulin on pollen germination and tube growth. — *Acta Phytophysiologica Sinica* 24(3): 196–200.
- Meng, X. H., Wang, J. B. & Li, R. Q. 2000: Effect of photoperiod on calcium distribution in photoperiod-sensitive cytoplasmic male-sterile wheat during anther development. — *Acta Botanica Sinica* 42: 15–22.
- Nayyar, H. 2003: Calcium as environmental sensor in plants. — *Current Science* 84: 893–902.
- Qiu, Y. L., Liu, R. S., Xie, C. T., Yang, Y. H., Xu, Q. & Tian, H. Q. 2005: The character of calcium distribution in developing anther of lettuce (*Lactuca sativa* L.). — *Acta Biologiae Experimentalis Sinica* 38(5): 377–386.
- Sajo, M. G., Furness, C. A., Prychid, C. J. & Rudall, P. J. 2005: Microsporogenesis and anther development in Bromeliaceae. — *Grana* 44: 65–74.
- Tirlapur, U. K. & Cresti, M. 1992: Computer-assisted video image analysis of spatial variation in membrane associated Ca^{2+} and calmodulin during pollen hydration, germination and tip growth *Nicotiana tabacum* L. — *Annals of Botany* 69: 503–508.
- Tirlapur, U. K. & Willemsse, M. T. M. 1992: Changes in calcium and calmodulin levels during microsporogenesis, pollen development and germination in *Gasteria verrucosa*. — *Sexual Plant Reproduction* 5: 214–223.
- Tian, H. Q. 2002: Programmed cell death during sexual reproduction in Angiosperms. — *Journal of Plant Physiology and Molecular Biology* 28(3): 161–168.
- Tian, H. Q., Kuang, A. X., Musgrave, M. E. & Russell, S. D. 1998: Calcium distribution in fertile and sterile anthers of photoperiod-sensitive genic male-sterile rice. — *Planta* 204: 183–192.
- Tian, H. Q. & Russell, S. D. 1997: Calcium distribution in fertilized and unfertilized ovules and embryo sacs of *Nicotiana tabacum* L. — *Planta* 202: 93–105.
- Tian, H. Q. & Yuan, T. 2000: Calcium functions in fertilization process in Angiosperms. — *Acta Phytophysiologica Sinica* 26(5): 369–380.
- Wang, L., Wang, Y., Zhang, M. & Lu, Y. T. 2001: Pollination-induced apoptosis in tobacco related to expression of calcium/calmodulin-dependent protein kinase T1. — *Developmental and Reproductive Biology* 10: 53–60.
- Xie, C. T., Yang, Y. H., Qiu, Y. L., Ge, L. L. & Tian, H. Q. 2005: Calcium distribution in fertile and sterile anthers of a genic male sterile Chinese Cabbage. — *Journal of Plant Physiology and Molecular Biology* 31(6): 615–624.
- Yu, F. L., Zhao, J., Liang, S. P. & Yang, H. Y. 1999: Ultra-cytochemical localization of calcium in the gynoecium and embryo sac of rice. — *Acta Botanica Sinica* 41: 125–129.
- Yao, C. Y. & Zhao, J. 2004: Effects of calcium and boron on pollen germination and pollen tube growth of *Torenia fournieri*. — *Journal of Wuhan Botanical Research* 22(1): 1–7.