Ann. Bot. Fennici 46: 101–106 ISSN 0003-3847 (print) ISSN 1797-2442 (online)
Helsinki 30 April 2009

Calcium distribution in developing anthers of lettuce (*Lactuca sativa*)

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Received 10 Sep. 2007, revised version received 14 Feb. 2008, accepted 4 Mar. 2008

Qiu, Y. L., Liu, R. S., Wei, D. M. & Tian, H. Q. 2009: Calcium distribution in developing anthers of lettuce (*Lactuca sativa*). — *Ann. Bot. Fennici* 46: 101–106.

Potassium antimonite was used to locate calcium in the anthers of lettuce (*Lactuca sativa*). There are no calcium precipitates in young anthers. After meiosis of microspore mother cells, calcium precipitates first appear in the tapetal cells, from which some small secretive vesicles containing many calcium precipitates are secreted into the locule. At a late stage of the microspore, tapetal cells completely degenerate and their protoplasts move into the locule with many calcium precipitates. The calcium precipitates increase in the early microspores, and in the exine. When the microspores form some small vacuoles containing calcium precipitates, and those vacuoles then fuse to form a large one, the calcium precipitates evidently decrease. The large vacuole of bicellular pollen grain discomposes and calcium precipitates again appear in the cytoplasm and then decrease. When the pollen matures, most calcium precipitates are located in its exine with only a few in the cytoplasm.

Key words: anther, calcium, development, lettuce (*Lactuca sativa*), microspore, pollen

Introduction

Anther structure in higher plants is very complex and the development is rapid. The four layers of the anther wall display various morphologies and structures after differentiation. Pollen development through the two mitotic cycles is also a rapid process. Research on calcium distribution in anther development in higher plants has rarely been reported, and calcium function is not well understood (Ge *et al*. 2007). Tirlapur and Willemse (1992) observed a polar distribution of membrane calcium in young *Gasteria verrucosa* microspores, where the calcium is mainly associated with the area opposite to what later becomes the colporal region. Subsequently, there is a shift in the polarity, and most of the membrane calcium in older microspores is regionalized towards the colporal region. In *Chlorophytum elatum,* vegetative cells have a higher calcium precipitate content than do generative cells (Gorska-Brylass *et al*. 1997/1998), suggesting that calcium may regulate differentiation of both cell types. Tian *et al*. (1998) used potassium antimonite to detect Ca^{2+} in fertile and sterile anthers of a photoperiod-sensitive genic male sterile

rice. Abundant calcium precipitates accumulated in the loculi and on the surface of the pollen grains, but not in the cytoplasm of fertile microspores. In contrast, calcium precipitates were abundant in the middle layer and endothecium of sterile anthers, with only a few precipitates in the tapetum (Tian *et al*. 1998).

The calcium precipitated by potassium antimonite is exchangeable cellular Ca^{2+} that is sufficiently loosely bound to combine with antimonite (Wick & Hepler 1982). The concentration of calcium precipitated by antimonite in cells or tissue is higher than that of free calcium. The physiological function of precipitated calcium may be different from the function of free calcium in pollen tube growth *in vitro*. In the current study, we used antimonite to precipitate loosely-bound calcium and examined its distribution in the developing anthers of lettuce.

Material and methods

Lettuce (*Lactuca sativa*) was grown in a controlled growth chamber at 27 °C with a 15-h day length and illumination at 54 μmol m^{-2} s^{-1}, and at 20 °C for 9 h in the dark. Anthers, sampled at different developmental stages, were fixed for 3 h at room temperature in 2% glutaraldehyde (v/v) in 0.1 mol l^{-1} phosphatic buffer (pH 7.8), containing 1% potassium antimonite ($K_2H_2Sb_2O_7 \times 4H_2O$). Antimonite can bind Ca^{2+} more easily than can potassium, to form calcium antimonite precipitate (Wick & Hepler 1982), which can be identified with electron microscopy because the precipitates have a high electron density (Tian & Russell 1997). After pre-fixation, the samples were washed (three 30 min washes in buffered 1% antimonite) and post-fixed in 1% (w/v) buffered OsO_4, containing 1% antimonite for 16 h at 4 °C. The samples were then washed in buffer (three 30 min washes), dehydrated in a graded acetone series and embedded in Spurr's resin. For each experiment, at least 5 anthers were sectioned (80 nm thickness) and stained with 2% uranyl acetate (w/v) in 50% methanol (v/v). Then after being washed and aired, the samples were observed and photographed using a JEM-100 transmission electron microscope.

Results

Calcium distribution

Microspore mother cell stage

Early microspore mother cells are densely cytoplasmic with a centrally located nucleus. Some organelles, such as plastids and mitochondria, appear similar to those in undifferentiated tissue. In these cells, some vacuoles contain sporadic calcium precipitates. Calcium precipitates also appear on the surface of microspore mother cells (Fig. 1A). At this early stage, the tapetum, middle layer, endothecium and epidermis of the anther walls are still differentiating. The anther epidermal cells contain large vacuoles, which displace the cytoplasm to the periphery. The cells of endothecium and middle layer display large centrally located nuclei. The tapetal cells are larger than cells in the other three layers and have centrally located nuclei. Some calcium precipitates were observed in the cells of anther walls and in the cell walls between tapetal cells (Fig. 1B).

Tetrad stage

After meiosis of a microspore mother cell, the four young microspores are compartmentalized by a common callose wall and the tetrahedral tetrad is wrapped in a callose wall (Fig. 1C). Early tetrad microspores are densely cytoplasmic and have large centrally located nuclei. They contain few calcium precipitates. At this stage, the endothecium cells are highly vacuolated. The tapetal cells become anomalous. Their inner tangential planes become unevenly, and the gaps between them increase. Their nuclei appear anomalous, and many endoplasmic reticulum cisterns appear in the cytoplasm. The number of calcium precipitates increases in the tapetal cytoplasm after the formation of vacuoles which contain abundant precipitates. This suggests that calcium is transported by a transmembrane pathway (Fig. 1D).

Early microspore stage

Microspores are released from tetrads after the

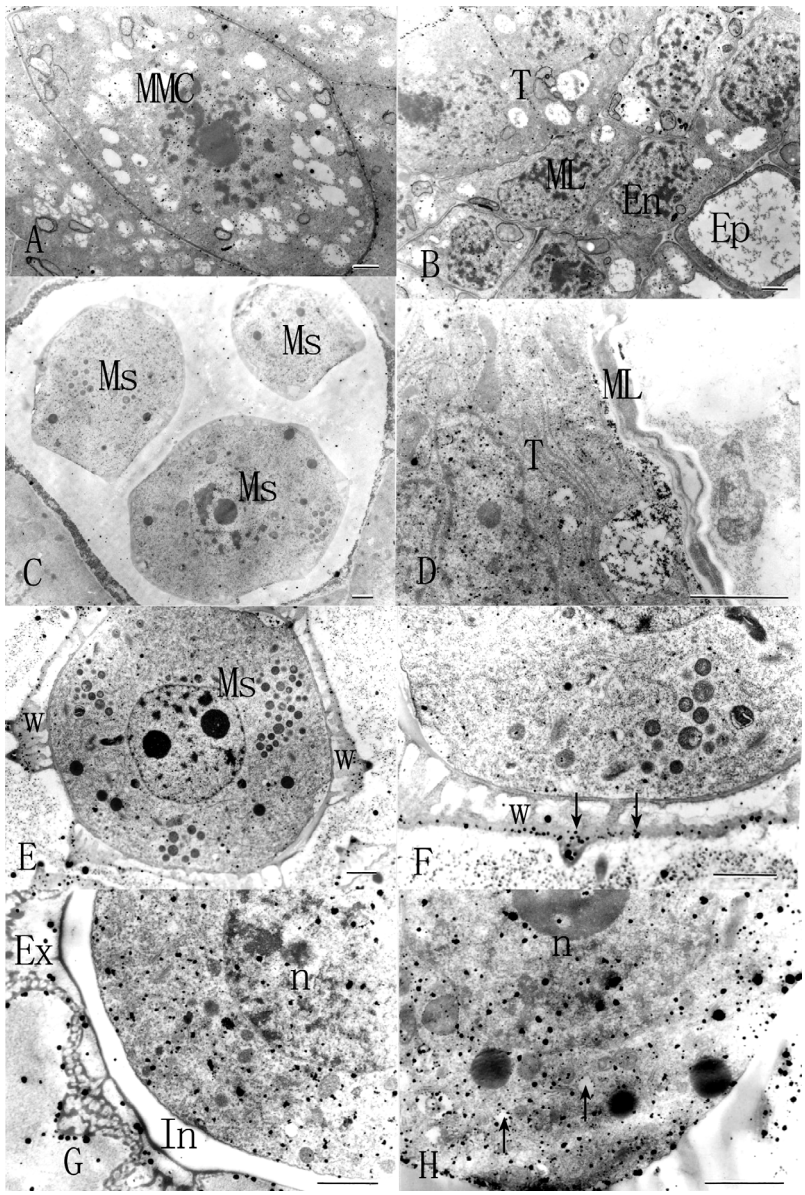

Fig. 1. Calcium distribution in developing anther cells of *Lactuca sativa*. Bar = 1 μm. — **A**: No calcium precipitates appear in microspore mother cell (MMC) but a few on the cell surface. — **B**: Anther wall just differentiated consists of four layers: epidermis (Ep), endothecium (En), middle layer (ML) and tapetum (T). All of the cells contain a few calcium granules. — **C**: No calcium granules in microspores (MS) of tetrad. — **D**: Calcium precipitates increase in tapetal cell (T). ML: middle layer. — **E**: A young microspore (MS) just released from tetrad with its exine (W). — **F**: Calcium precipitates (arrows) increase in microspore and on its exine (W). — **G**: Calcium precipitates increase in microspore cytoplasm and nucleus (n), and some in exine (Ex) but not in intine (In). — **H**: Microspores begin to form small vacuoles containing calcium precipitates (arrows).

callose wall breaks down. The four microspores display centrally located nuclei and many undifferentiated organelles in their dense cytoplasm (Fig. 1E). At this stage, the young microspores still contain a few calcium precipitates, and some well-regulated precipitates appear in its developing exine (Fig. 1F). During microspore development, calcium precipitates are significantly increased (Fig. 1G). The pollen intine forms during this stage, and while calcium precipitates appear in the exine, there are none in the intine. Small vacuoles appear in the microspore cytoplasm containing a few calcium precipitates (Fig. 1H). In the anther wall, the structure and calcium content of the epidermis and endothecium does not change. However, some changes do occur in the middle layer and tapetum. The cells of the middle layer are degenerated and cannot be clearly seen. The electron density of the tapetal cells increase significantly, and abundant cal-

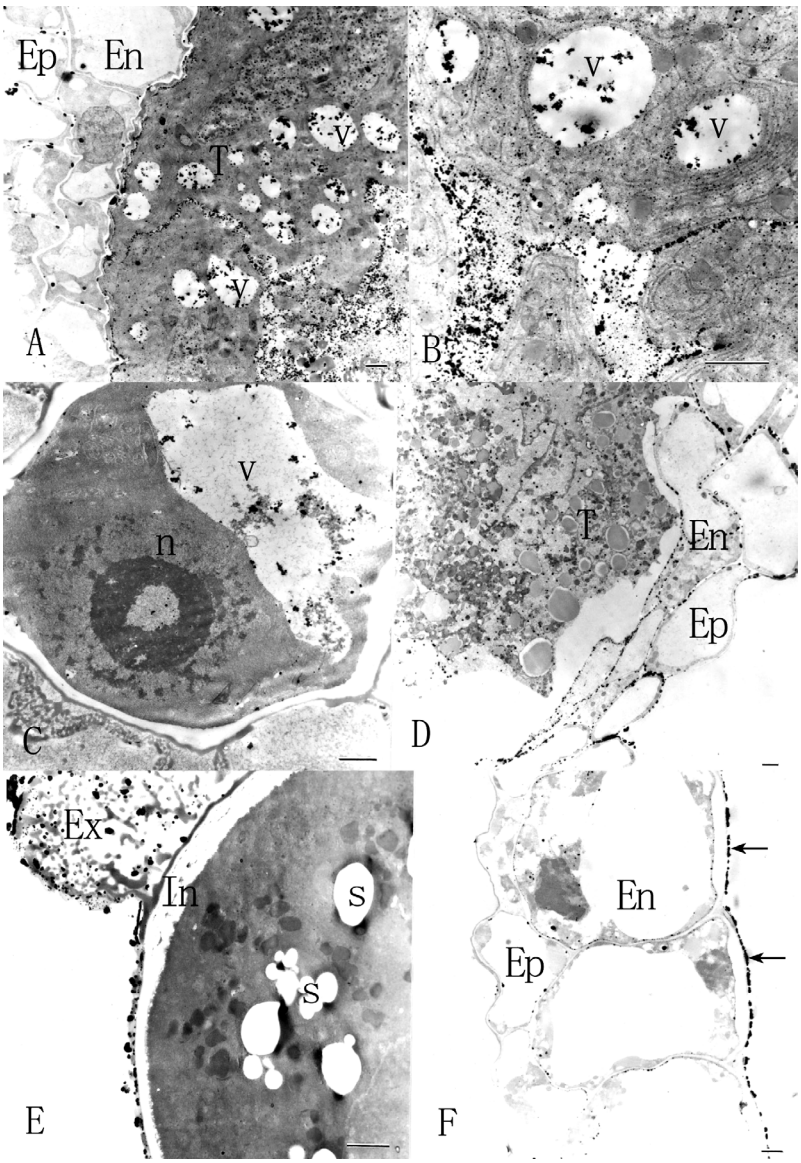

Fig. 2. Calcium distribution in developing anther cells of *Lactuca sativa*. Bar = 1 μm. — **A**: At the same stage as in Fig. 1H, tapetal cells (T) formed many small secretory vacuoles (v) containing abundant calcium precipitates. Ep: epidermis; En: endothecium. — **B**: Small calcium precipitates are distributed in cytoplasm and large ones in vacuoles (v) of tapetal cells. Abundant calcium precipitates accumulate in extracellular gaps between tapetal cells. — **C**: Microspore at late stage forms a large vacuole (v) containing some large calcium precipitates and meanwhile they sharply decrease in microspore cytoplasm and nucleus (n). — **D**: The cytoplasm mass of tapetum moves into locule during its degenerating, and many calcium precipitates appear in epidermis (Ep) and endothecium (En). — **E**: After the large vacuole decomposes in bicellular pollen, it begins to synthesize starches (s). There are still many calcium granules in exine (Ex) but a few in intine (In). — **F**: At the same stage, the anther wall consists only of two layers of epidermis (Ep) and endothecium (En). Calcium precipitates sharply decrease in both cells but some on tapetum membrane (arrows).

cium precipitates appear in their cytoplasm (Fig. 2A). Later, some of the precipitates accumulate in small vacuoles, which are then secreted into the locule. After that, calcium precipitates in the tapetal cells decrease, and accumulate in extracellular gap (Fig. 2B) and the inner tangential plane. Also at this time, many endoplasm reticulum cisterns and lipid drops appear in the tapetal cytoplasm, and the electron density of the tapetal cytoplasm decreases.

Late microspore stage

During microspore development, a morphological change occurs where a large vacuole forms, which pushes the nucleus into the peripheral area. This creates a polarity in the microspore, in preparation for an uneven division. Therefore, this large vacuole plays a significant role in pollen differentiation. In the late microspore stage, calcium precipitates decrease in the cytoplasmic

matrix, but some precipitates were detected in the large vacuole (Fig. 2C). In the anther wall, the tapetum degenerates and becomes a periplasmodium that moves into the locule (Fig. 2D). The periplasmodium contains many organelles and some calcium precipitates, but the number of precipitates decreases sharply. Meanwhile, many calcium precipitates appear on the surface of cells of the epidermis and endothecium. This suggests that the degeneration of the tapetum stops calcium movement and causes calcium to accumulate in the epidermis and endothecium.

Bicellular pollen stage

After microspore division, the bicellular pollen begins to accumulate starch. The electron density of the vegetative cell increases. Few calcium precipitates are present in the pollen cytoplasm and intine, while some are present in the exine and on its surface (Fig. 2E). With the degeneration of tapetum, only two layers of cells, the epidermis and endothecium, constitute the anther wall. Few calcium precipitates appear in both cell types, and some precipitates accumulate in the tapetal membrane (Fig. 2F).

Discussion

Most studies of calcium and its role during sexual reproduction of angiosperms have concentrated in the calcium signal function of pollen tube growth *in vitro* (Ge *et al*. 2007), but there are few studies of calcium distribution and its physiological function for anther development. In the research of calcium distribution in fertile and sterile anthers of rice (Tian *et al*. 1998) and wheat (Meng *et al*. 2000), abundant calcium moves to the loculi of fertile anthers at the microspore stage, and most calcium precipitates accumulate on the surface of the microspore, but only few in its cytoplasm. In sterile anthers, fewer calcium precipitates appear in the loculi and more are located in the microspore. The results indicate a requirement for abundant calcium during anther development, and the difference of calcium distribution in fertile and sterile anthers suggests that calcium content may influence male sterility (Tian *et al*. 1998, Meng *et al*. 2000).

During development, the calcium distribution changes in the lettuce anther tissue. Few calcium precipitates appear in young anthers before meiosis of microspore mother cells. After meiosis, the precipitates significantly increase, and calcium also moves into the locule. Following the increase in calcium in the anther wall, the epidermis and endothecium differentiate and the tapetum degenerates. After calcium precipitates accumulate in the microspore cytoplasm, it forms small vacuoles, which fuse together to form a large vacuole containing calcium precipitates. The calcium precipitates in the cytoplasmic matrix decrease at this time, suggesting that the calcium is now concentrated in the large vacuole. After microspore mitosis, the large vacuole in the vegetative cell of the bicellular pollen decomposes. The calcium precipitates again increase in the cytoplasmic matrix, and the bicellular pollen begins to synthesize starch. These results all indicate that calcium levels are involved with the regulation of anther differentiation and development.

The tapetum is a layer of somatic tissue that transports nutritional materials into the locule. In rice (Tian *et al*. 1998) and wheat (Meng *et al*. 2000), the tapetum is secretory. Many calcium precipitates are located on the surface of Ubisch bodies and are likely involved with transport into the loculi. However, in lettuce, the tapetum is amoeboid and there are no Ubisch bodies, so the mechanism of calcium transfer by the tapetum must be different from that of rice and wheat. Calcium precipitates in lettuce tapetum are transferred into the locule by secretory vesicles. Therefore, the mechanism of calcium transfer by tapetal tissue depends on the type of tapetum.

The tapetum degenerates by programmed cell death (Wang *et al*. 1999). Calcium is known to play an important role in inducing programmed cell death in plant cells (Ning *et al*. 1999). Wang *et al*. (2001) found that pollination may induce apoptosis of tobacco, and apoptosis was related to the expression of calcium and calmodulin-dependent protein kinase T1. During the development of lettuce anthers, there are few calcium precipitates in the tapetum at the micro-

spore mother cell stage. However, after meiosis, calcium increases significantly in the tapetum, which may be a factor of initiating degeneration. Therefore, the dynamics of calcium distribution in the tapetum is tightly related to its degeneration and may trigger its programmed cell death.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (No. 30670126). The authors thank Prof. C. Peterson for his helpful comments and revision of the manuscript.

References

Ge, L. L., Tian, H. Q. & Russell, S. D. 2007: Calcium function and distribution during fertilization in angiosperms. — *Am. J. Bot.* 94: 1046–1060.

Gorska-Brylass, A., Butowt, R. & Rodriguez-Garcia, M. I. 1997/1998: Distribution of loosely-bound calcium in the vegetative and generative cells of the pollen grains in *Chlorophytum elatum*. — *Biol. Plantarum* 40: 169–181.

Meng, X. H., Wang, J. B. & Li, R. Q. 2000: Effect of photoperiod on Ca^{2+}-ATPase distribution in photoperiod-sensitive cytoplasmic male-sterile wheat during anther development. — *Acta Bot. Sin.* 42: 15–22.

Ning, S. B., Wang, L. & Song, Y. C. 1999: Programmed cell death in plants — a new emerging research field. — *Devel. Reprod. Biol.* 8: 71–100.

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., Kuang, A., Musgrave, M. E. & Russell, S. D. 1998: Calcium distribution in fertile and sterile anthers of a photoperiod-sensitive genic male-sterile rice. — *Planta* 204: 183–192.

Tirlapur, U. K. & Willemse, M. T. M. 1992: Changes in calcium and calmodulin levels during mirosporogenesis, pollen development and germination in *Gasteria verrucosa*. — *Sex. Plant Reprod.* 5: 214–223.

Wang, L., Wang, Y., Zhang, M. & Lu, Y. 2001: Pollination induced apoptosis in tobacco related to expression of calcium/calmodulin-dependent protein kinase T1. — *Devel. Reprod. Biol.* 10: 53–60.

Wang, M., Hoekstra, S., Van Bergen, S., Lamers, G. E. M., Oppedijk, B. J., Heijden, M. W., Priester, W. & Schilperoort, R. A. 1999: Apoptosis in developing anthers and the role of ABA in this process during androgenesis in *Hordeum vulgare* L. — *Plant Mol. Biol.* 39: 489–501.

Wick, S. M. & Hepler, P. K. 1982: Selective localization of intracellular calcium with potassium antimonite. — *J. Histochem. Cytochem.* 30: 1190–1204.