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Calcium Distribution during Anther Development in Oil Tea (*Camellia oleifera* Abel.)

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Abstract. The mechanism by which calcium regulates anther development remains unclear. This study investigated the relationship between calcium distribution and anther development in oil tea (*Camellia oleifera* Abel.) by using the potassium antimonite technique. Before the onset of microsporogenesis, abundant minute calcium precipitates appeared on the plasma membranes of microspore mother cells. Meanwhile, numerous precipitates accumulated in the tapetal cells. After meiosis, calcium precipitates appeared in young microspores. During microspore development, calcium precipitates mainly appeared in the small vacuoles of the cytoplasm. At the late microspore stage, a large vacuole formed, and the number of precipitates in the microspore decreased. The number of precipitates in the tapetal cells decreased as microsporogenesis proceeded. Then, calcium precipitates in the bicellular pollen cytoplasm again increased in number. During bicellular pollen development, the number of calcium precipitates decreased. As the pollen grains matured, only a few calcium precipitates were evident in the pollen cytoplasm. The results of this study, which show the spatial and temporal features of calcium distribution during the anther development of *C. oleifera*, suggest that calcium distribution is related to anther development.

Calcium is an essential element and an important ubiquitous messenger that participates or modulates many intracellular metabolic processes of plant growth and development (Bush, 1995; Ge et al., 2007a; Hepler and Wayne, 2005; Jones and Lunt, 1967). In flowering plants, calcium plays crucial roles in regulating sexual reproduction processes such as pollen germination, pollen tube growth (Cardenas et al., 2008; Franklin-Tong, 1999; Hepler, 1997; Heslop-Harrison, 1987; Kwack, 1967; Steinhorst and Kudla, 2013), and fertilization (Antonie et al., 1999; Digonnet et al., 1997; Santella et al., 2004; Tian and Russell, 1997; Whitaker, 2006). However, insufficient information is available on calcium distribution during microsporogenesis and anther development. The development of anthers is complicated. Cells in different anther tissues undergo different processes that lead to conspicuous changes in morphology and structure; these processes include meiosis in microspore mother cells, degeneration of tapetal cells, polarization in microspores during the formation of large vacuoles, and pollen wall formation after releasing from tetrads. Anther cell differentiation during anther development is strictly regulated in accordance with specific spatial and temporal processes, and calcium may play a role in some of these processes. Tian et al. (1998) found that numerous calcium precipitates accumulate within the tapetum and locules of *Oryza sativa* anthers during anther development. They also discovered that sterile anthers display abnormal calcium distribution compared with fertile anthers in a photoperiod-sensitive genetically male-sterile *O. sativa* line. Meanwhile, Meng et al. (2000) observed that more calcium precipitates accumulate in the cells of the vascular bundles of anthers in a sterile *Triticum aestivum* line than in those of a fertile maintainer line. These results indicate that the abnormality of calcium distribution is related to anther abortion. However, to date, the features of calcium distribution in anthers have been investigated in only a few species (Ge et al., 2007b; Kong and Jia, 2004; Qiu et al., 2009), and the mechanism by which calcium regulates anther development is unclear. In the present study, we used potassium antimonite to localize and detect pools of loosely bound calcium in anther cells of *C. oleifera*, and we investigated the relationship between calcium distribution and anther development.

Camellia oleifera, a species of the Theaceae family, is native only to China. Camellia oil can be extracted from *C. oleifera* seeds, which have been used in China for more than 1000 years (Ruter, 2002). The polyunsaturated and good phenolic content of camellia oil is up to 80% (Yu et al., 1999). Camellia oil is a high-quality cooking oil that is similar to olive oil and it stores well at room temperature (Ruter, 2002). However, the yield of *C. oleifera* is very low, which is closely related to sexual reproduction. Anther plays an important role in the sexual reproduction of *C. oleifera*. The present study aims to explore the role of calcium in anther development. This study may serve as a theoretical basis for the sexual reproduction of *C. oleifera*.

Materials and Methods

Camellia oleifera anthers selected at different developmental stages were squeezed and examined under a microscope to determine their developmental stages. The anthers were then fixed at room temperature for 3 h in 2% glutaraldehyde (v/v) in 0.1 mol·L^{-1} phosphate buffer (pH 7.8) that contains 1%

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potassium antimonite ($K_2H_2Sb_2O_7 \cdot 4H_2O$). The use of potassium antimonite to precipitate calcium was detailed by Ge et al. (2007b). The samples were fixed in glutaraldehyde, washed three times for 30 min each in buffered 1% antimonite (w/v), and then postfixed in 1% OsO_4 (w/v) containing

development, some small vacuoles appeared in the cytoplasm and these vacuoles contained calcium precipitates (Fig. 2A). The number and size of vacuoles in the microspore increased and these vacuoles were distributed in the peripheral cytoplasm, which increased the number of calcium precipitates around the vacuoles (Fig. 2B).

Late microspore stage. The small vacuoles fused to form large vacuoles, and the number of calcium precipitates decreased in the cytoplasmic matrix. The calcium precipitates were mainly located on the vacuolar membrane, but many also appeared on the microspore plasma membrane. The pollen intine and exine were found outside the membrane, and some precipitates were specifically located in the baculum of the exine (Fig. 2C). As the anthers underwent further development, small vacuoles in the microspores fused to form a large vacuole, which marked the late stage of microspore development. When the large vacuole formed, the microspore nucleus moved to the periphery and displayed unequal distribution. The number of calcium precipitates in the late microspores decreased, whereas the precipitates on the plasma membranes and in the exines completely disappeared (Fig. 2D).

At the late microspore stage, the cytoplasm matrix of anther epidermal cells still contained many calcium precipitates, but only a few were present in the endothecium and the middle cell layer (Fig. 2E). Meanwhile, the structure of the tapetal cells became indistinct and their cytoplasmic electron density increased, suggesting that the cells began to degenerate. The number of precipitates decreased in the degenerating tapetal cells (Fig. 2F).

To confirm that the calcium in late microspores dissolved into the large vacuole, we performed energy-dispersive X-ray spectral analysis of semithin sections of microspores (Fig. 3). Although the calcium precipitates at the late microspore stage disappeared, X-ray spectral analysis revealed that some calcium remained in the large vacuole (Fig. 4). This result suggested that calcium dissolved in the large vacuole.

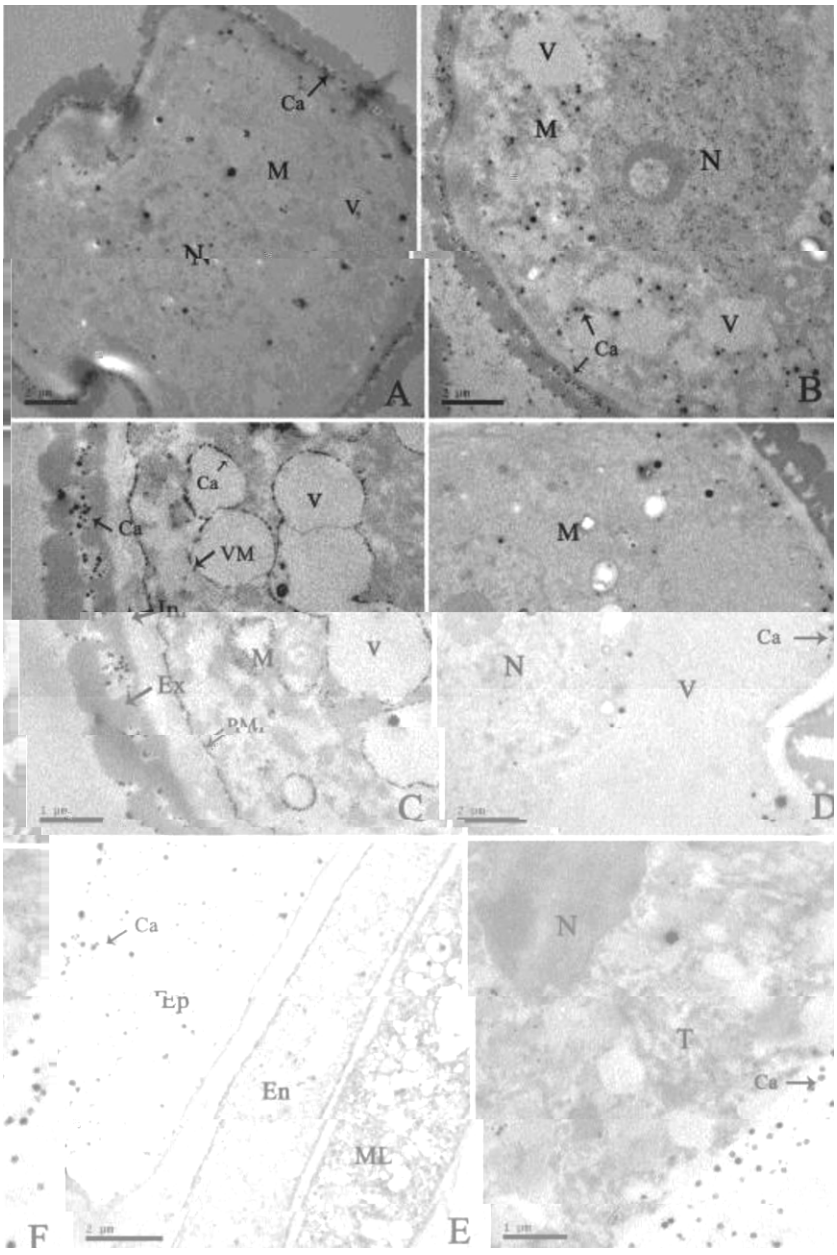

Fig. 2. Calcium precipitate distribution in *Camellia oleifera* anthers at the microspore stage. (**A**) At the early microspore stage, some scattered precipitates are located in small vacuoles. (**B**) The sizes of small vacuoles of early microspores increase, and calcium precipitates are specifically located near these vacuoles. (**C**) The small vacuoles fuse to form large vacuoles, and many precipitates are located on the vacuole membrane. (**D**) In late microspores, a large vacuole forms, and the number of precipitates in the microspore cytoplasm clearly decreases. (**E**) Many precipitates remain in the epidermal cells. (**F**) Meanwhile, many precipitates observed outside the tapetal cells. Ca = calcium; En = endothecium; Ep = epidermis; Ex = exine; In = intine; M = microspore; ML = middle layer; N = nucleus; PM = plasma membrane; T = tapetum; V = vacuole; VM = vacuolar membrane. Bars: (**A**, **B**, **D**, **E**) 2 μm, (**C**, **F**) 1 μm.

Bicellular pollen grain stage. After asymmetrical mitotic division of the microspore, a bicellular pollen grain was formed. This pollen grain consisted of a large vegetative cell and a small generative cell. At the early bicellular pollen stage, the large vacuoles in the vegetative cell disaggregated, and the density of the pollen cytoplasm increased. In the early bicellular pollen grains, the number of calcium precipitates increased again (Fig. 5A). This result suggested that the calcium that dissolved in the large vacuole of the vegetative cell returned to the cytoplasm after large vacuole disaggregation. Then, the generative cell separated from the pollen intine, became spherical, and moved into the vegetative cell. The number of calcium precipitates observed between the generative and vegetative cells was the same (Fig. 5B).

At the early bicellular pollen stage, changes occurred in the anther wall cells; the calcium precipitates in the large vacuoles of the epidermal and endothecium cells became

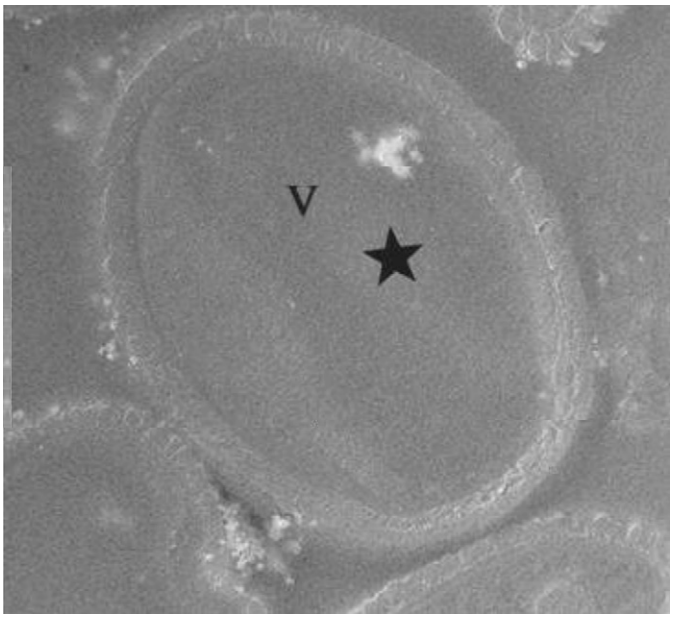

Fig. 3. The pollen grain with a large vacuole of *Camellia oleifera* at the microspore stage was sectioned (1 μm thick) and pasted on a slide. The asterisk indicates the large vacuole position in a microspore, which was analyzed by using an X-ray detector. V = vacuole.

smaller and formed abundant flocculent materials (Fig. 5C). The tapetal cells degenerated further and the cytoplasm matrix was reduced. No calcium precipitates accumulated in the degenerated tapetal cells (Fig. 5D). After pollen development, the generative cell inside the vegetative cell became elongated. Fewer calcium precipitates were present in the generative cell than in the vegetative cell (Fig. 5E). Near anthesis, only a few calcium precipitates were located in the small vacuoles in the mature pollen grains. However, many calcium precipitates were still present on the pollen surface (Fig. 5F).

Discussion

Before meiosis occurred in the MMCs of *C. oleifera*, the anthers accumulated numerous calcium precipitates, which were mainly located in the callose wall of the MMCs. During intine formation, calcium began to appear in the intine at the tetrad stage. After young microspores were released from the tetrad, calcium appeared between the endexine and the intine. At the mature pollen stage, a large number of calcium precipitates was present on the pollen exine. The pollen wall began to form at the tetrad stage. Previous studies showed that the cell wall is one of the largest calcium stores in plant cells and that pollen walls contain the most calcium in pollen grains (Kong and Jia, 2004). Furthermore, the pollen of *Gasteria verrucosa* (Tirlapur and Willemse, 1992) and *Lilium longiflorum* (Reiss et al., 1985) can release calcium during the hydration and early germination phases. Thus, we proposed that the accumulation of calcium in the callose walls of MMCs and the tetrad might be correlated with the formation of pollen walls. Moreover, the mature pollen wall might be a storage of calcium for pollen-pistil interaction and pollen germination.

In this study, there is an interesting phenomenon. The calcium precipitates were present in the cytoplasm of young microspores, particularly within small vacuoles. Then, the

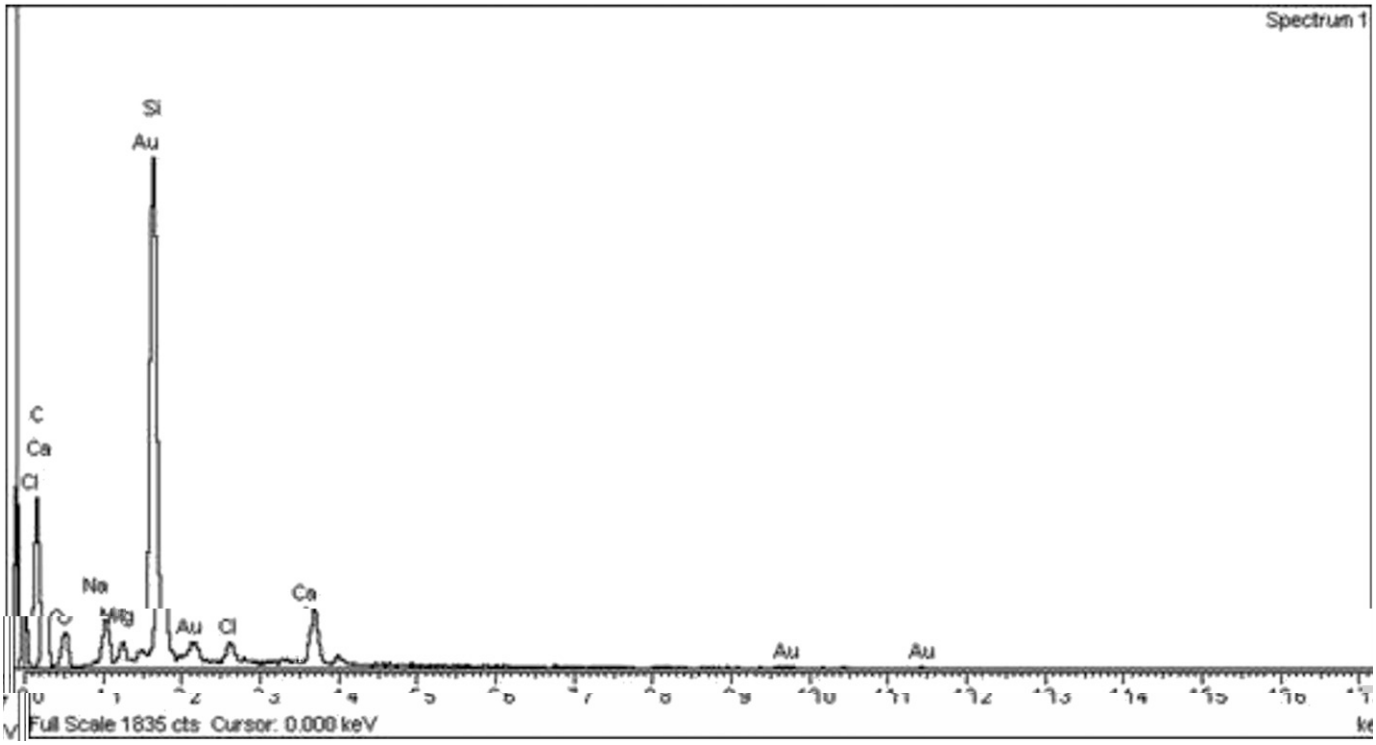

Fig. 4. Energy-dispersive X-ray spectra of semithin sections of large vacuoles in microspores of *Camellia oleifera* microspores. Calcium was present in the large vacuole.

small vacuoles coalesced to form a large vacuole and the calcium precipitates disappeared from the late microspores. Hepler and Callaham (1987), Hepler and Wayne (1985), Izant (1983), and Wick et al. (1985) demonstrated that calcium is associated with critical events of mitosis in plant cells. After the microspore divided to produce a bicellular pollen grain, the large vacuole in the vegetative cell disaggregated and calcium precipitates reappeared in the vegetative cell cytoplasm. Then, the number of calcium precipitates in the bicellular pollen grains continued to decrease. Calcium is involved in the cell division and polarized tip growth process of pollen tubes (Tirlapur and Willemse, 1992). The calcium distribution pattern observed during *C. oleifera* anther development exhibited spatial-temporal features; high levels of calcium appeared in *C. oleifera* anthers at a specific time (at the microspore stage) and in a specific region (vacuole formation of microspores). This result suggests that calcium regulates large vacuole formation in microspores during pollen development in *C. oleifera*.

The physiological function of calcium during anther development remains unknown. Calcium can regulate many cellular functions according to its cellular location, binding, and solubility (Hepler and Wayne, 2005). Calcium also appears to play a unique and important role in plant cells during wall accretion, vacuolar turgor maintenance, and stomatal movement (Bolwell, 1993; De Silva et al., 1985a, 1985b; Inoue and Katoh, 1987; Schwartz et al., 1988). However, a few studies have examined calcium distribution during anther development and the physiological function of calcium in anther development has not previously been proposed. In the present study, calcium precipitates in anthers mostly accumulated during microspore development. Thus, the presumed functions of calcium in pollen development are as follows: 1) regulating the activation of voltage-dependent ion channels (Hedrich and Neher, 1987); and/or 2) providing osmotic pressure to aid in the formation of vacuoles within a microspore, which contributes to the polarization of the cytoplasm preceding the unequal division of the microspore. In this study, we presumed that the physiological function of calcium during anther development is regulating large vacuole formation in the microspore, which distributes microspore nuclei unequally and initiates microgametogenesis. As pollen matures, calcium

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