Distribution Changes of Calcium and Programmed Cell Death in the Pistil of Litchi (*Litchi chinensis* Sonn.) Flower during Its Development

WANG Xiang-Ping[1], SU Li-Xun[3], SU Jin-Wei[2*]

[1]*Test Centre,* [2]*College of Life Sciences, Fujian Agriculture and Forestry University, Fuzhou 350002, China;* [3]*School of Life Sciences, Xiamen University, Xiamen 361005, China*

Abstract: Potassium pyroantimonate precipitation method was used for investigating calcium distribution and cell ultrastructure change during development of pistils of litchi male and female flower. The results showed that at the megasporocyte stage of female flowers, calcium precipitates was located mainly at cell wall and intercellular space of inner integument near the micropyle and style cells, and to a lesser extent in vacuoles. Vascular tissues also contained much calcium precipitates. In inner integument cells near the micropyle of male flowers, the vacuole contained most of the calcium precipitates. Calcium precipitates in style cell and vascular tissues of male flowers was sparse and seldom seen. After meiosis of megasporocyte, pistils of female flowers continued to grow and those of male flowers aborted. In female flowers, calcium precipitates concentration became lower and calcium precipitates was probably transported to the places for future pollen bourgeoning and fertilization. Cell wall calcium precipitates concentration increased in the inner integument cells near the micropyle. Calcium precipitates concentration increased from topper style cells to lower ones. In male flowers, inner integument cells near the micropyle underwent the programmed cell death (PCD): flow of calcium from vacuoles into nucleus might had triggered the PCD process. A continuous channel was formed between perinuclear space and cytoplasm membrane lumen, and calcium flowed freely between nuclear membrane and plasma membrane. At certain time and locations, calcium precipitates was newly appeared at some organelles like endoplasimic reticulum, mitochondria and peroxisomes. This calcium redistribution in cells might trigger and regulate the process of PCD. In male flowers, style cells containing no calcium precipitation soon began to degenerate.

Key words: calcium ion; litchi; pistil development; programmed cell death; ultracytochemical localization

Calcium plays a key role in cell signal transduction as a second messenger (Poovaiah and Reddy 1993). Functions of calcium in plant sexual reproduction are essential in the plant reproductive biology research. Its functions in pollen tube growth and distribution in pistil tissues have been investigated (Tian and Russell 1997; Zhang *et al*. 1997; Xie *et al*. 2005), but researches about its possible function in flower sex differentiation are few. Results of earlier researches indicated that as other separate flowers, litchi flowers have bisexual premordium at an early stage (Li 1987; Lin and Wu 1999). Before meiosis of megasporocyte, reproductive organs of male and female flowers develop synchronously. After it, stamen and pistil differentiate and flowers become unisexual through a process of programmed cell death (PCD) and selective abortion (Lin and Wu 1999). Xiao *et al*. (2003) investigated the relation between hormone and litchi sex differentiation and Su and Wang (2005) have studied the ultrastructural change of litchi male flowers during sex separation. But the above researches about litchi sex did not deal with its relationship with calcium messenger. Many other researches show that calcium participates in the regulation of PCD (He *et al*. 1996; Zhang *et al*. 2001). Calcium in the cell may stimulate the calcium-dependent endonuclease and induce DNA

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*Corresponding author (E-mail: sujinwei8@yahoo.com; Tel: 86-591-83789383).

decomposition (Heath 1998; Xu and Hanson 2000). So it is expected that calcium may also play a key role in flower sex differentiation and selective flower abortion, but there have been no related reports. The meaning of this paper was using the potassium pyroantimonate precipitation method to study the distribution of calcium through the development process of male and female flowers of litchi and investigate the cytological mechanism of function of calcium in sex determination.

1 Materials and Methods

1.1 Plant materials

Litchi (*Litchi chinensis* Sonn.) has three kinds of flowers, the most are male ones, with fewer female ones, and very few bisexual ones. At early stage of flower bud formation, both male and female flowers have bisexual primordium. And differentiation of male and female flowers begins after megasporocyte meiosis. Because it is difficult to differentiate the gender of flowers at early stage, we chose 'Yuhong' which has special characteristics of blossoming. In its first group of flowers in each year, male flowers are at the center of spike, with female ones around them.

1.2 Methods

Procedures for cytochemical localization of calcium were those of Wang *et al.* (1994) with some modifications: ovules and styles from young flowers at different developmental stages were picked under anatomical lens and submerged in a fixative of 3% glutaraldehyde / 2% potassium pyroantimonate buffered in potassium phosphate at 4°C for 3 h. Then the materials were washed with 2% potassium pyroantimonate buffered in potassium phosphate 4 times, followed by fixation with 1% osmium tetraoxide / 2% potassium pyroantimonate buffered with potassium phosphate at 4°C for 10 h. The fixed materials were washed by normal redistilled water (pH 7.0) 3 times and pH 10.0 redistilled water twice, 20 minutes each time. Then the materials were dehydrated with a graded ethanol series and embedded in Spurr resin (ERL-4206). Using RMC ultramicrotome (made in USA) to get the ultrasections, and these unltrasections were post-stained with uranyl acetate, viewed and photographed under the JEM1010 transmission electron microscope (made in Japan). For the control, sections were immersed in EGTA 1 mmol/L (pH 8.0) at 60°C for 0.5 h to remove the calcium pyroantimonate precipitates from the sections.

2 Results

2.1 Calcium distribution at megasporocyte stage

At megasporocyte stage, the inner and outer integuments of ovule were well developed. Micropyle was formed from the inner integument and the style was formed by projected out of the top ovary. The view under optical microscope was consistent with reports by Li (1987) and Lin and Wu (1999) (Photographs omitted).

In inner integument cells near the micropyle of female litchi flower, calcium precipitates concentration was relatively low and calcium precipitates were located mainly at the cell wall and intercellular space (Fig.1a).

Style cells of female flowers were compact. Calcium precipitates were found mostly at cell wall and in the vacuoles (Fig.1b). Meanwhile, vascular tissue was well developed and also contained lots of calcium precipitates, located at vascular tube wall and in the lumen, indicating intense calcium transportation (Fig.1c).

The situation in male flowers was different. Calcium precipitates in inner integument cells near the micropyle of male flower were found mostly in vacuoles, which meant that vacuoles were the main calcium reservoir (Fig. 1d).

Calcium precipitate in the style cell of male flowers was sparse and was seldom seen (Fig.1e). Vascular tissue was poor developed, containing no calcium precipitates (Fig.1f).

2.2 Calcium distribution after megasporocyte meiosis

In the inner integument cells near the micropyle of female litchi flowers 2 d after megasporocyte meiosis, calcium pyroantimonate precipitates showed no marked increase (Fig.2a). But the calcium precipitates content increases much one-day prior to blossom (Fig.2b). Some smaller precipitates were also found at the cytoplasm membrane (Fig.2b). The integument cells had features of

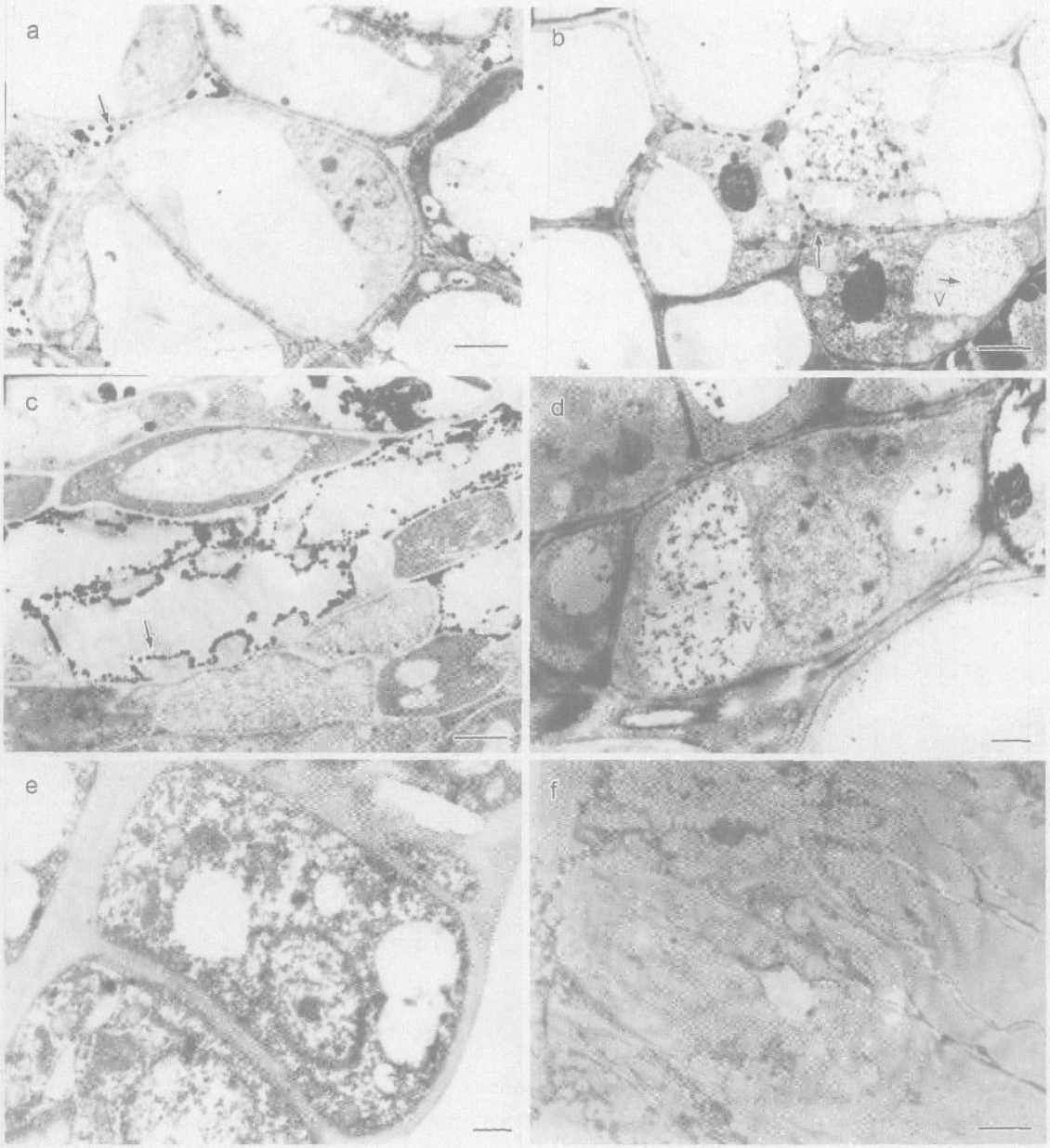

Fig.1 Calcium distribution at megasporocyte stage

a: In inner integument cells near the micropyle of female flower, calcium precipitates were mainly located at cell wall and intercellular spaces (↑) (Bar=2 μm). b: In the style cells of female flower, calcium precipitates were found mostly at cell wall and in vacuoles (v) (↑) (Bar=2 μm). c: In the vascular tissue of female style flower, calcium precipitates were found at tube wall and tube lumen (↑) (Bar=2 μm). d: Calcium precipitates in male flower inner integument cells near the micropyle were found mostly in vacuoles (v) (↑) (Bar=1 μm). e: Style cells of male flowers did not contain calcium precipitate (Bar=1 μm). f: Vascular tissues of male flowers were poor developed, containing few calcium precipitates (Bar=2 μm).

transmitting tissues: the number of organelles in the cells increased and exhibited active physiological functions (Sun *et al.* 1996). Such phenomena indicated that these cells might function in providing nutrients and energy to the future pollen tube.

Style of female flower continued to grow after megasporocyte meiosis. Style taken from the flower one day prior to blossom was about 3 mm. It was trisected and observed. Space between cells was greater than at megasporacyte stage. Calcium precipitates were located

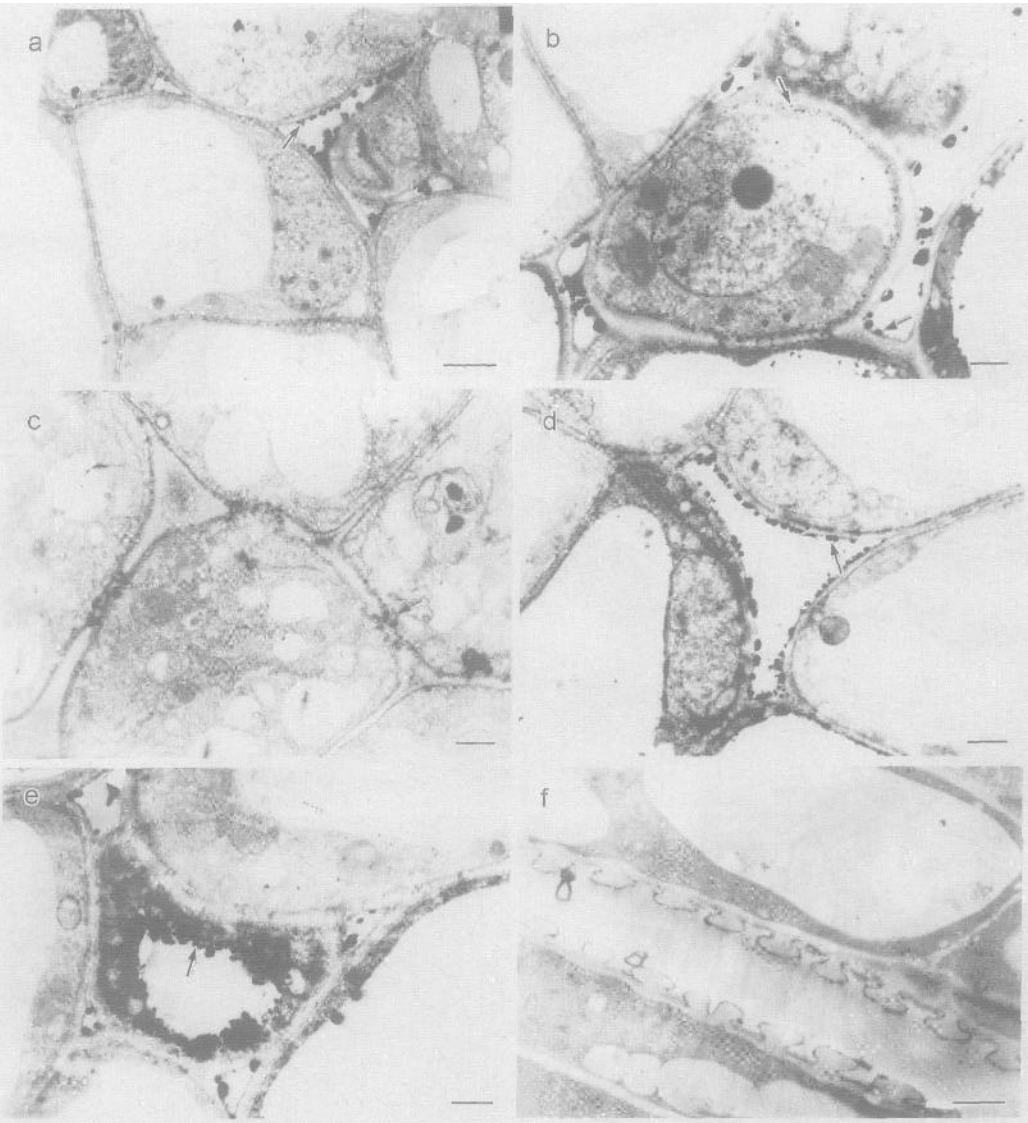

Fig.2 Calcium distribution in female flowers after megasporocyte meiosis

a: In the inner integument cells near the micropyle two days after megasporocyte meiosis, calcium precipitates at cell wall (↑) (Bar=2 μm). b: In the inner integument cells near the micropyle one day before blossom, calcium precipitates at cell wall increased and such precipitates were found in the cytoplasm membrane (↑) (Bar=1 μm). c: One day before blossom, the upper section of the style contained few (↑) calcium precipitates and the space between cells is greater than at the megasporacyte stage (Bar=1 μm). d: One day before blossom, calcium precipitates at cell wall indicating that calcium concentration of middle section was higher than that of the upper section (↑) (Bar=1 μm). e: One day before blossom, calcium precipitates at cell wall indicating that calcium concentration (↑) of lower section was higher than middle section (↑) (Bar=1 μm). f: One day before blossom, calcium precipitates concentration in vascular tissue has reduced greatly (Bar=2 μm).

mostly at the outer surface of cell wall and in the intercellular space (Fig.2c,d,e). The upper section (close to the stigma) of the style contained fewer calcium precipitates which mainly at the cell wall (Fig.2c). And calcium precipitates concentration of middle section was higher (Fig.2d), and the lowest section was the highest (Fig.2e). This up-to-low calcium concentration grade in the style might be propitious for the entrance of pollen tube through the style into the ovule (Xie *et al*. 2005).

Also, through comparison of the calcium precipitates content in the vascular tissues just before blossoming with those at megasporacyte stage, it had

been found that the concentration had reduced greatly (Fig.2f). It is expected that calcium transportation in vascular tissues was almost finished. As a whole, calcium precipitates was mostly located in the apoplast system like cell wall and cell cavity and calcium precipitates distribution through the pollen tube track tissues such as micropyle and style was denser than in other adjacent tissues. This calcium precipitates distribution shows a close relationship with future pollen tube growth.

From megasporacyte meiosis to blossom, pistil of male flowers gradually shrinked and flowers were taken and observed every 2 d until one day before blossom. As the photographs show, 2 d after megasporocyte meiosis, calcium precipitates of inner integument cells near the micropyle were located mainly in the vacuoles, less in the cytoplasm and cell wall (Fig.3a). Then these cells began to undergo programmed cell death as below:

In the nucleus, at early period, calcium in vacuoles

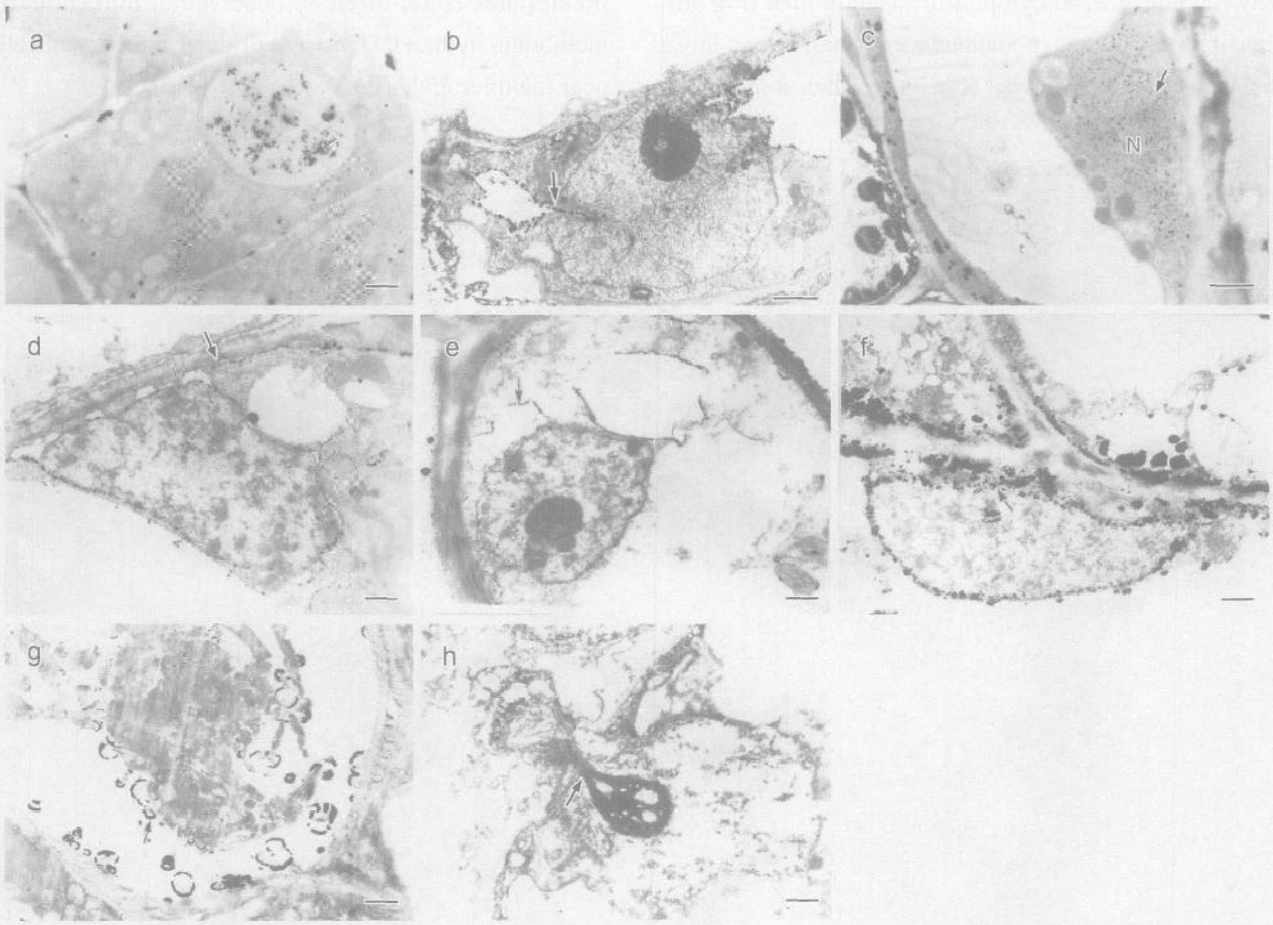

Fig.3 PCD process of male flower inner integument cells near the micropyle after megasporocyte meiosis (Part one)
a: Two days after megasporocyte meiosis (before PCD), calcium precipitates were mainly located in vacuoles (v) (↑) and cytoplasm, cell wall containing only a little (Bar=1 μm). b: Calcium precipitates distribution indicating that calcium precipitates in vacuoles were invading into the nucleus (↑) (Bar=1 μm). c: Calcium precipitates distribution indicating that calcium has suffused into the nucleus (N) and the precipitates are relatively smaller (↑) (Bar=1 μm). d: Calcium precipitates gradually congregate at nuclear membrane. Note that the continuous channel between nucleus membrane and plasma membrane, which contained calcium precipitates (↑) (Bar=500 nm). e: Nucleus begins to contract and nuclear membrane is outspreading (↑) (Bar=500 nm). f: Nuclear membrane and cytoplasm membrane are partly cracking and calcium is released out of the cell (↑) (Bar=500 nm). g: Nuclear membrane cracks and vesicles full of calcium are released (↑) (Bar=500 nm). h: Nucleus (N) shrinks and is migrating through cell wall (↑) (Bar=500 nm).

invaded into the nucleus (Fig.3b) and then calcium precipitates could be observed suffused in the nucleus under electron microscope (Fig.3c). Then calcium precipitates gradually concentrated at the nuclear membrane (Fig.3d). This calcium redistribution process in the cell resembles the situation with nucellus of rice intergument cells during PCD (Wei *et al*. 2002). Because perinuclear space and plasma membrane are continuous and nuclear pores can serve as free channels for irons and large molecule transportation, calcium translocation between nucleus and cytoplasm was unlimited (Fig.3d). Calcium precipitates in nucleus were smaller than those in vacuoles and at cell wall (Fig.3a,c). Then some nuclei began to contract and nuclear membrane outspreaded (Fig.3e); some nuclei moved close to the cell wall, nuclear membrane and plasma membrane partly cracked. Calcium had been released out of the cell (Fig.3f); in other cases, nuclear membrane cracked and vesicles full of calcium precipitates were released (Fig.3g). At last nucleus shrank and a nucleus migrated through the cell wall into a neighboring cell (Fig.3h).

In mitochondria of normal litchi pistil cells, no calcium precipitates could be seen (Fig.1d). But the precipitates could often be observed at mitochondria membrane in the PCD process of inner integument cells near the micropyle (Fig.4a).

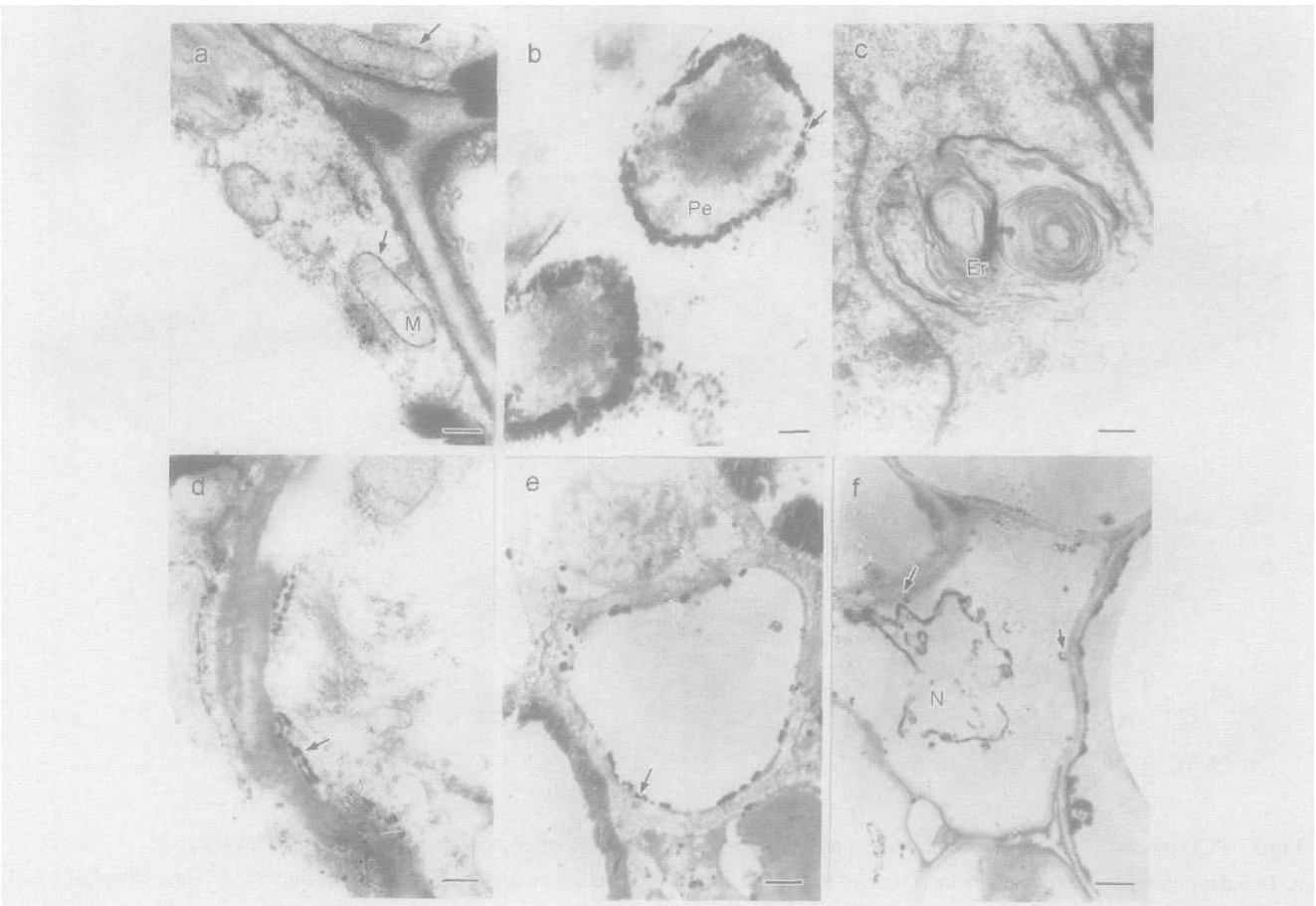

Fig.4 PCD process of male flower inner integument cells near the micropyle after megasporocyte meiosis (Part two)
a: Calcium precipitates begin to be located at the mitochondria (M) membrane (↑) (Bar=300 nm). b: Peroxisomes (Pe), note calcium precipitates at the membrane of one of them (↑) (Bar=100 nm). c: Endoplasmic reticulum (Er) twists in the cytoplasm to form concentric circles, and no calcium precipitates is observed (Bar=200 nm). d: Some short endoplasmic reticula contain calcium precipitates (↑) (Bar=200 nm). e: Calcium precipitates were located at cell wall (↑) (Bar=1 μm). f: The remnant of nucleus (N) releases vesicles full of calcium precipitates to the cell wall (↑) (Bar=1 μm).

In peroxisomes, which were not found in normal pistil cells but appeared during PCD, calcium precipitates were seen at its membrane (Fig.4b).

In endoplasmic reticulum, normal litchi pistil cells did not contain calcium precipitates in endoplasmic reticulum which was the same as reported by Wang *et al*. (1994). As our other study showed, endoplasmic reticulum and peroxisomes were also important organelles in plant programmed cell death (Su and Wang 2005). In PCD process, some long endoplasmic reticulum appeared to be twisted in the cytoplasm to form concentric circles, and no calcium precipitate was observed (Fig.4c). But some short endoplasmic reticulum contained calcium precipitates and were moved with them to the inner surface of cell wall (Fig.4d).

In cytoplasm, due to the change in the cytoplasm membrane penetrability, calcium moved from outside of the cell to the inside (Fig.3c). Calcium precipitates concentration in cytoplasm increased consequently. Change of calcium distribution would induce a series of genetic metabolism alterations (Wang *et al*. 1994). In the late period, calcium was transported out of cell with other cell materials. But even at the latest stage, when the cell was almost empty, some calcium precipitates were still located at the inner surface of cell wall (Fig.4e), or aggregated at vesicle membranes on cell wall (Fig.4f). Cell wall of the cells undergoing PCD was always complete and this was contrast with cell wall disintegration of style cells, which did not contain calcium precipitates (Fig.5). This might result from calcium precipitates at cell wall.

PCD processes of inner integument cells near the micropyle of male flower were not synchronous.

Style cells of male flowers degraded soon after megasporacyte meiosis and observation of the style one day before blossom showed that the cells had degraded and the cell became structureless (Fig.5). These tissues lacking calcium precipitates showed a broken cytoplasm membrane structure, confusion of endomembrane system, and disappearance of intercellular compartmentation. And these phenomena such as endomembrane confusion and cell wall disintegration are identical with other plants lacking calcium (Miao *et al*. 1997).

Fig.5 One day before blossom, style cells of male flowers were structureless (Bar=5 μm)

In grids of control sections treated with EGTA, the calcium precipitates became transparent, which proves that they were really calcium pyroantimonate precipitates (Fig.6).

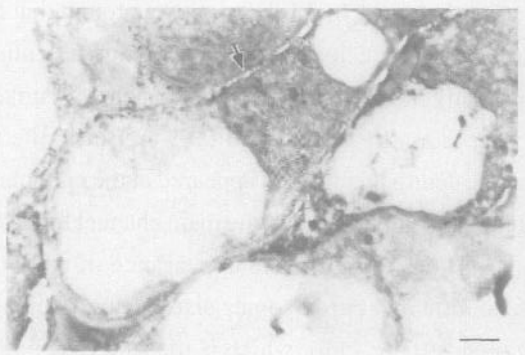

Fig.6 Control section
In grids of control sections, style cells of female flowers, the precipitates become transparent (↑) (Bar=1 μm).

3 Discussion

Increase in calcium concentration is characteristic of PCD (Nicotera and Orrenius 1998; Qiu *et al*. 2005). In the developmental process of inner integument cells near micropyle of litich male flowers, it was discovered that change in calcium concentration of the cell do not have an obvious relation with cell death and rather, the alteration of ultrastructural distribution was the main reason. As the present paper shows, when calcium precipitates was

located in vacuoles, the inner integument of male flowers developed normally (Fig.3a). But when vacuole calcium invades into nucleus, PCD could be seen soon (Fig.3b, d). This result supports Heath's conjecture that calcium accumulation is the main reason for PCD (Heath 1998).

About the signal of calcium in the nucleus, there are two kinds of views: one is that perinuclear space and plasma membrane lumen are continuous and calcium translocation between nucleus and cytoplasm is unlimited; the other is that calcium in the nucleus is not the result of simple diffusion of calcium between nucleus and cytoplasm and there is an independent calcium regulation system in the nucleus (Liu *et al.* 2001). As is reported in this paper, perinuclear space and plasma membrane are continuous and serve as free channels for calcium in the PCD process (Fig.3d). This result supports the first view, and it is not reported by now.

Somly (1984) pointed out that endoplasmic reticulum also stores calcium. In this paper, it was found that there was no calcium precipitates at endoplasmic reticulum in the normally developed pistil cells, which was agreed to the observation of Wang *et al.* (1994). But in the PCD process, calcium precipitates appeared at the endoplasmic reticulum and might serve as the main channel for calcium transportation (Fig.4d). The appearance of calcium in mitochondria and peroxisomes also occured during the PCD process (Fig.4a,b), which is the first case reported in plant. Researches on animal programmed cell death discovered that mitochondrion is the receptor and amplifier of the death signal. Factors inducing cell death such as excessive calcium will cause a decline of $\Delta\psi m$ between the two sides of mitochondria membrane and increase in membrane permeability, thereby trigger PCD (Kroemer *et al.* 1997). And the distribution mechanism of calcium precipitates at mitochondria and peroxisomes in programmed cell death still needs further research.

Both male and female flowers have bisexual anlagen when they are budding, but after megasporacyte meiosis, female gametophyte of female flowers grows and that of male flower degrades. As Ye *et al.* (1992) reported, blastocyst cavity of litchi formed by inner integument is essential for female gametophyte and the embryo to develop normally. As the result of the present paper shows, PCD of inner integument cells of male flower cells may be the important cause for its female gametophyte abortion (Fig.4e,f). And our report means that calcium, as second messenger, its distribution has important triggering function on PCD process of flower sexdifferentiation.

Calcium concentration and distribution in style cells of female flowers change from megasporacyte stage to blossom stage. At first, at megasporacyte stage, the vascular tissues contained lots of calcium precipitates showing that active transport was undergoing (Fig.1c). But the style taken one day prior to blossom had little calcium precipitates in the vascular tissue (Fig.2f). This may be a sign that calcium transport in the style has stopped. And calcium has been transported to the places of the pollen tube growth in pistil. Calcium gradient in style tissue has been seen in some plant species (Xie *et al.* 2005), but in some other species no gradient was observed (Zhang *et al.* 1997; Yu *et al.* 1999). There is still no final conclusion currently. Also, calcium precipitates was not distributed evenly in the style of litchi female flower with lower concentration near the stigma and higher near the ovule end (Fig.2c,d,e). This gradient may induce the entrance of pollen tube into ovule through the style (Tian and Russell 1997). Calcium gradient in style of tobacco is formed after fertilization, which is latter than in litchi, and the deferment of calcium gradient formation may be closely related to that tobacco flowers are bisexual and have long style. The pistil matures earlier than the stamen (Xie *et al.* 2005). Litchi flowers are mostly unisexual and have short style. So calcium gradient is formed before blossom and is well prepared for fertilization after blossom. This phenomenon indicates that calcium distribution in style may be related to many other factors, which need further investigations.

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荔枝雌蕊发育过程中钙分布变化与细胞程序性死亡

王湘平[1]，苏力寻[3]，苏金为[2*]
福建农林大学[1]测试中心，[2]生命科学学院，福州 350002；[3]厦门大学生命科学学院，厦门 361005

摘要：应用焦锑酸钾沉淀法研究了荔枝雌花和雄花雌蕊发育过程中钙的分布变化。在大孢子母细胞阶段，雌花近珠孔内珠被细胞和花柱细胞的钙沉淀颗粒主要分布在细胞壁和细胞间隙，少部分在液泡；雌花花柱维管细胞中含有很多的钙沉淀颗粒；在雄花的近珠孔内珠被细胞钙沉淀颗粒大多在液泡中；雄花花柱细胞和维管细胞中钙沉淀颗粒很少。大孢子母细胞减数分裂后，雌花雌蕊继续发育，雄花雌蕊败育。雌花维管中的钙沉淀颗粒数量减少，可能被转运到将要发生花粉萌发和受精的部位。雌花近珠孔内珠被细胞壁的钙沉淀颗粒分布增加，花柱细胞从上(近柱头)到下(近子房)钙沉淀颗粒量递增。雄花近珠孔内珠被细胞发生程序性死亡：液泡中的钙进入细胞核启动细胞程序性死亡，核周隙与质膜腔形成连续的通道，钙在核与细胞质之间的流动不受限制；在特定的时间段，钙沉淀颗粒出现在线粒体、过氧化物体和线型内质网的外膜上。钙在细胞中重新分布可能触发和调节细胞程序性死亡的进程。缺乏钙沉淀颗粒的雄花花柱细胞迅速解体。

关键词：钙离子；荔枝；雌蕊发育；细胞程序性死亡；超微细胞化学定位
中图分类号：Q945

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*通讯作者(E-mail: sujinwei8@yahoo.com; Tel: 0591-83789383)。