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温敏雄性不育水稻培矮64S花药发育过程中钙的变化

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摘要:采用焦锑酸钾沉淀法研究了温敏雄性不育水稻(*Oryza sativa* L.)培矮64S在高温引起雄性不育与正常可育花药发育过种中Ca^{2+}的分布变化。结果表明,当培矮64S生长在较高温度条件下引起雄性不育,与可育花药相比,不育花粉母细胞中有较多的液泡、较多的Ca^{2+}沉积和较少的线粒体,并且有较多的Ca^{2+}沉积在不育花药的中间层、表皮层和绒毡层中。到四分体与单细胞花粉时期,不育花药的木质部细胞的次生加厚壁上有较多的Ca^{2+}沉淀,连接组织中的Ca^{2+}沉淀也大大增加,所有不育花粉外壁较厚而发育都不正常。在单核细胞早期,不育花粉的四分体细胞中有较明显的大液泡出现。不育花药中的Ca^{2+}在花药发育的各时期均比可育花药要多。这些结果说明在高温生长条件下,花粉母细胞发育的异常、花药中Ca^{2+}沉积的增加、绒毡层与花粉外壁发育的异常可能与培矮64S花粉败育相关。

关键词:水稻; 雄性不育; 花药; 花粉; Ca^{2+}
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Calcium Precipitate in the Anthers of Thermo-sensitive Genic Male-sterile Rice Peiai 64S

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Abstract: Peiai 64S (*Oryza sativa* L. subsp. *indica*), is a thermo-sensitive genic male-sterile (TGMR) rice. High temperature causes sterile pollens at the microsporogenesis stage. The anther morphology and the calcium precipitate were investigated in the fertile and sterile anthers of Peiai 64S under low or high temperature. There were more small vacuoles, more calcium precipitates and less mitochondria in the sterile pollen mother cells than those in the fertile pollen mother cells. More calcium precipitates were located in the epidermis, endothecium and tapetum of the sterile anther than in those of the fertile anther. Longitudinal inner cell wall of the sterile anther became thicker than that of the fertile anther at the dyad stage and uninucleate pollen stage. Some large vacuoles appeared in the tapetal cells of the sterile anthers at the early uninucleate pollen stage. All of the sterile pollens possessed malfunctioned exine. The results proposed that the abnormalities of pollen mother cell, the tapetum and the pollen exine, and more calcium precipitates in TGMR rice Peiai 64S under high temperature were related with the pollen abortion.

Key words: Rice; Male-sterile; Anther; Pollen; Calcium

The male sterility is the base of the hybrid rice (*Oryza sativa* L.), which has led to a great improvement in rice productivity. Peiai 64S is a thermo-sensitive genic male-sterile (TGMS) rice and

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is widely used in two-line hybrid rice breeding in China. Its male fertility is controlled by temperature during its sensitive stage. According to prior studies[1], the pollens are sterile when the plants are grown above 23℃, and fertile below 21℃. The maximum, minimum and optimum temperature for sterile alteration of Peiai 64S are 22.8, 21.7, and 22.5℃[2].

Calcium is known to have key cellular regulatory roles as the ubiquitous second messenger, which takes part in the regulation of physiological processes and development. The calcium concentration and its gradient are essential for microsporogenesis, pollen maturation and pollen tube growth[3], and the ovule's development[4]. The high calcium concentration and calcium precipitate have been found in leaf lamina, abnormal anthers and sterile pollens of the cytoplasmic male sterile (CMS) rice, and the photo-sensitive genic male sterile (PGMS) rice[5–6]. Here we investigated the morphology and the calcium location in the developmental sterile and fertile anthers of the TGMS rice Peiai 64S when they were grown under high or low temperature.

1 Materials and methods

1.1 Plant materials

Seeds of Peiai 64S were planted group by group in the experiment field of Guangzhou, China. Before the spike differentiation, the plants were transferred into a 30 cm × 30 cm pots and put into the growth chamber to control their male fertility. One group was grown under the male fertile condition (average 20.75℃, 22℃/19℃ day/night with 14 h daylight), and another group was grown under the male sterile condition (average 27.08℃, 30℃/23℃ day/night with 14 h daylight).

1.2 Methods for TEM

The spikes at different developmental stages were harvested and fixed for experiment. The pollen fertility was checked with I_2-KI staining protocol and the seed setting. Preparation of TEM observation was described by Tian *et al*[5]. Anthers of the Peiai 64S that was grown under low temperature or high temperature were collected at different developmental stages based on stages of pollen development, respectively. At least ten anthers from different spikelet located at middle spike-stalk of the inflorescence were fixed and at least six anthers from each treatment were examined. Anthers were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L KH_2PO_4 buffer (pH 7.8) containing 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ for 4 h at room temperature, washed in five changes of 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$ in 0.1 mol/L KH_2PO_4 buffer (20 min each) and post-fixed in 1% OsO_4 for 16 h at 4℃ in 0.1 mol/L KH_2PO_4 buffer containing 1% $K_2H_2Sb_2O_7 \cdot 4H_2O$. Anthers were then washed in four changes of 0.1 mol/L KH_2PO_4 buffer without antimonate, dehydrated in a graded ethanol series and embedded in Epon 812 resin. Sections were stained with uranyl acetate and observed with a JEM1010 transmission electron microscope. Two additional controls were conducted: (1) $K_2H_2Sb_2O_7 \cdot 4H_2O$ was omitted from solutions during processing; (2) selected grids with specimens containing calcium precipitates were incubated in a solution of 0.1 mol/L EGTA (pH 8.0) for 1 h to remove precipitates.

2 Results

2.1 Fertility alternation of Peiai 64S by temperature

Except for the fertility of pollens, no obvious phenotypic difference of Peiai 64S was observed when the plants were grown under the high or under low temperature as described by Liao and Yuan[1]. In this experiment, the pollens were completely abnormal when the plants were grown under the high temperature (>27℃), and both of their pollen staining rate by I_2-KI and the seed setting rate were 0%; but the average staining rate of pollens and the seed setting rate were 78.2% and 69.8%, respectively, when the plants were grown under low temperature (<21℃) (data not shown).

2.2 Calcium distribution in microsporogenesis stage

At the pollen mother cell stage, few Ca^{2+} precipitates were found in fertile anthers under low temperature. There was nearly no Ca^{2+} precipitate in

the anther wall and pollen mother cell (Plate I: 1, 2). There were a lot of mitochondria in the pollen mother cells, but no Ca^{2+} precipitate (Plate I: 3). Few small Ca^{2+} precipitates were in the parenchyma cell wall of the vascular bundle (Plate I: 7); but no Ca^{2+} was found in the connective tissue (Plate I: 8).

However, under the high temperature, Ca^{2+} distribution in the sterile anthers was different from that in the fertile anthers at the pollen mother cell stage. A few Ca^{2+} precipitates were in the endothecium cells of the sterile anthers (Plate I: 4). The sterile pollens had less mitochondria than that of fertile anthers, and many Ca^{2+} precipitates deposited in the cytoplasm and on the plasma membrane of sterile anthers (Plate I: 5, 6). A few of Ca^{2+} precipitates were found on the plasma membrane and in the mitochondria of phloem cells (Plate I: 10), and the same phenomena happened on the second cell wall of xylem and in the connective tissue (Plate I: 11).

Under the low temperature, vacuolated epidermis and endothecium cells had no Ca^{2+} precipitate; few Ca^{2+} precipitates were found in the interstitial space of epidermis and endothecium at the dyad stage (Plate Ⅰ: 9). Many Ca^{2+} precipitates were found on the plasma membrane and vacuole membrane of tapetal cells. There were many small mitochondria in the dyad. A few of small Ca^{2+} precipitates deposited in the pollen sac and no Ca^{2+} in the dyad, but a few Ca^{2+} precipitates deposited around the callose of the dyad (Plate Ⅱ: 13, 15). Comparing with the pollen mother cell stage, Ca^{2+} quantity increased in the connective tissues too, and the same change happened to the xylem. A few of large Ca^{2+} precipitates were found in phloem cells (Plate Ⅱ: 14).

However, Ca^{2+} precipitates in the sterile anthers at the dyad stage under the high temperature increased greatly. Many Ca^{2+} precipitates deposited on plasma membrane and in the cytoplasm of the epidermis and tapetum (Plate Ⅰ: 12). A few of Ca^{2+} precipitates deposited on the callose around the dyad, and no Ca^{2+} precipitate was found in the dyad, there were more small vacuoles in the dyad under the high temperature than that under the low temperature (Plate Ⅱ: 16, 20). Few Ca^{2+} precipitates deposited on the xylem cell wall and phloem cells. Many Ca^{2+} precipitates were found on plasma membrane and in the cytoplasm of the connective tissue, especially on its interstitial space (Plate Ⅱ: 17). Obviously, there were much more Ca^{2+} precipitates in sterile anthers than that in fertile anthers.

2.3 Calcium distribution in pollen developmental stage

Quantity of Ca^{2+} in anthers increased with the development of anthers. After meiosis, the tetrads develop to the young microspores. The uninucleate pollen just released from the tetrad possesses abundant uniform cytoplasm and no Ca^{2+} was found around the sexine. The tapetal cells hold dense cytoplasm, and some propollenin formed around the inner longitudinal plasma membrane, where we found many Ca^{2+} precipitates (Plate Ⅱ: 18). At the late uninucleate pollen stage, the uninucleate pollen possessed a large vacuole. Many Ca^{2+} precipitates deposited on the sexine and cytoplasm. There were a few Ca^{2+} precipitates deposited in the epidermis and endothecium, Ca^{2+} quantity increased in the interstitial space. The Ubish bodies formed completely and a few Ca^{2+} precipitates located on its surface (Plate Ⅱ: 19).

Under the high temperature, the uninucleate pollen released from the tetrad had some vacuoles with some dark matrix. The inner longitudinal cell wall of tapetal cells thickened and had same large vacuoles, which had a lot of small Ca^{2+} precipitates and few propollenin deposited on plasma membrane (Plate Ⅱ: 21). The tapetum of fertile anthers had no such thick wall and the Ca^{2+} precipitates, the propollenin were found facing the pollen sac. The thick cell walls here might block the transportation of the Ca^{2+} and the nutrient from anther wall to pollen sac. Most of pollens disintegrated and only a few could develop at late uninucleate pollen stage. There was no obviouse exine all the time; the demarcation between sexine and nexine was not distinct. Some pollen caved and nearly no Ca^{2+} precipitate was found in disintegrated pollens (Plate Ⅱ: 22). The Ca^{2+} distribution was obviously different from that in fertile anthers. A layer of large

Ca^{2+} precipitates appeared on the second wall of conduits. Some irregular Ca^{2+} precipitates deposited on plasma membrane and mitochondria of cells in the connective tissue. Much more such precipitates were found in sterile anthers than that in fertile anthers.

After pollen abortion completely, Ca^{2+} precipitates on sexine were more than that at late uninucleate pollen stage. Ca^{2+} quantity in anther wall decreased a little. Part of tapetal cells degenerated and a layer of Ca^{2+} precipitates deposited on the inner longitudinal wall of tapetal cells, and no Ca^{2+} precipitate was found in epidermis and endothecium, which was similar to that in fertile anthers. Few Ca^{2+} precipitates were found in vascular tissues and few small Ca^{2+} precipitates deposited on plasma membrane and in cytoplasm. The annular conduits degenerated and a few small Ca^{2+} precipitates were found in the degenerated cytoplasm. But there were only a few of Ca^{2+} precipitates in the spiral conduits. Ca^{2+} quantity on plasma membrane of connective tissue increased compared with prophase. Many Ca^{2+} precipitates located on the interstitial space were less compared with that in fertile anthers (data not shown).

3 Discussion

Regulation of the cytosolic calcium concentration is required for normal cell growth. Calcium distribution during the anther's development had been discussed in *Gasteria verrucosa*[3] and wheat (*Triticum aestivum*)[7] *etc*. The reason of Ca^{2+} used as the messenger might rely on its strict temporal, spatial distribution and the low concentration in cells. When the plants were stimulated or regulated physiologically, the combinative calcium in "calcium bank" (vacuole, mitochondria, chloroplast) was released to fulfill the messenger process[8-9]. The potassium antimonate technique was a useful method to check the free Ca^{2+} in cells for its convenience, delicacy, and nicety. The sterile anthers of Peiai 64S under high temperature accumulated much more calcium in the mother pollen cells, the anther cell walls and the connective tissue compared to the fertile anthers under low temperature.

Peiai 64S is one of the widely used typical thermo-sensitive genic male-sterile rice lines and the expression of male-sterile genes is controlled mainly by the temperature. It is fertile under the low temperature (<21℃) and it behaves male sterile under the high temperature (>23℃)[1]. There are many forms and operations of abnormalities at different time in different male sterile types[10]. Feng *et al.*[11] found the sterile microsporocyte of Peiai 64S under high temperature began to appear some abnormalities from the meiotic prophase, and all of its microspores became finally abnormalities. The observations reported here demonstrated the three items of important abnormalities under the male-sterile temperature.

First, the Ca^{2+} subcellular distribution was different between the sterile and fertile anthers at the pollen mother cell stage, a lot of Ca^{2+} precipitates were found in sterile anthers. Some microsporocyte deposited many Ca^{2+} precipitates. We also found that almost half of the microsporocyte of Peiai 64S exhibited abnormality under the high temperature. Some abnormal cells had a lot of shout-swollen endoplasmic reticula and abnormal nuclei. The start time to appear the Ca^{2+} difference between male-sterile rice and the fertile rice of Peiai 64S was much earlier than that of CMS or PGMS rice. Tian *et al*[5] and Li *et al*[12] found that the different distribution of Ca^{2+} between the male-sterile and fertile material of a photo-sensitive genic male-sterile rice, and Honglian-Yuetai rice (a CMS type) happened at the uninucleate and binucleate stage, respectively. The photo-sensitive genic male-sterile rice was a kind of two-line male sterile rice, which the pollen abortion mainly happened at the late uninucleate stage. Honglian-Yuetai rice was a kind of three-line male sterile rice that pollen abortion mainly happened at the binucleate stage. The Peiai 64S used in our study was a kind of thermo-sensitive male-sterile rice. All of these indicated the different male sterile rices had the different time appearing abnormal Ca^{2+} distribution in anthers.

The second, the tapetum developed abnormally under the high temperature. At the meiotic stage and

early uninucleate pollen stage, the inner longitudinal cell wall of tapetal cells in male-sterile anthers appeared thicker than that in fertile anthers and there were many vacuoles at the early uninucleate pollen stage. Many Ca^{2+} precipitates deposited around the plasma membrane of the tapetal cells, where the thick cell wall hinders the direct contact with the uninucleate pollen. During anther development, the tapetum play an important role in the development of the pollens[13]. Therefore, we propose here the abnormality of the tapetum is related with the development of pollens. Tirlapur and Willemse[3] regarded that Ca^{2+} was transported from anther wall to the clinandrium, and the tapetum supplied Ca^{2+} to the pollen after tetrad stage. Therefore, the thicker wall in tapetal cells might hinder the Ca^{2+} transportation to the clinandrium.

The third, pollen wall developed abnormally. In our investigation, the exine developed abnormally, the microspore formed disorganized pollen wall. The deeper inner longitudinal tapetal cell wall, the abnormal tapetum, and the abnormal exine probably caused a block in the synthesis or the transport of cell material, which were related with pollen breakdown.

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Explanation of plates

1～3, 7～9, 13～15, 18～19: The fertile anthers; 4～6, 10～12, 16～17, 20～22: The sterile anthers.

Plate I

1～8, 10～11: Anthers at the pollen mother cell stage; 1, 4: The anther wall at pollen mother cell stage, no calcium precipitate appears in the epidermis (Ep), endothecium (En) and the middle layer (Ml) of the fertile anther; a few of calcium precipitates (arrow) appear in the endothecium cells of the sterile anther; 2, 5: The pollen mother cell (P); 3, 6: Part magnification of the pollen mother cell. Less mitochondrion (M) and abundant calcium precipitates accumulate on plasma and in the cytoplasm of the sterile pollen mother cell comparing to the fertile pollen mother cell; 7, 10: The parenchyma cell of the vascular bundle. Few calcium precipitates appear on parenchyma cell (Pa) wall of the fertile anther, but more calcium in parenchyma cell and on the parenchyma cell wall of the sterile anther; 8, 11: The connective tissue of anther. No calcium precipitates in the connective tissue (C) of the fertile anther, but some in that of the sterile anther; 9, 12: The anther wall, a lot of calcium precipitates deposit on plasma membrane and in the cytoplasm of the epidermis (Ep), endothecium (En) and tapetum (Ta) of the sterile anther, only a few calcium precipitates in tapetum of the fertile anther at the dyad stage;

Plate II

13～17, 20: Anthers at the dyad stage; 13, 16: The dyad. A few of calcium precipitates deposit on the callose (arrows) around the dyad and no calcium precipitate in the dyad, but more small vacuoles (triangles) in the dyad of the sterile anther than the fertile anther; 14, 17: The connective tissue of anther; 15, 20: The vascular bundle (Vb). More calcium precipitates appear in the xylem (X) and phloem (Ph) cells of

the sterile anther than the fertile anther; 18 ~ 19, 21 ~ 22: Anthers at the uninucleate stage; 18, 21: The early uninucleate pollen and the tapetum. In the male-sterile anthers (21), the inner longitudinal cell wall of tapetal cells thickens and has same large vacuoles in tapetal cells; 19, 22: The uninucleate pollen and the anther wall. In the male-sterile anthers (22), no obviously formed exine all the time; some pollen caved and nearly no Ca^{2+} precipitate is found in these disintegrated pollens.

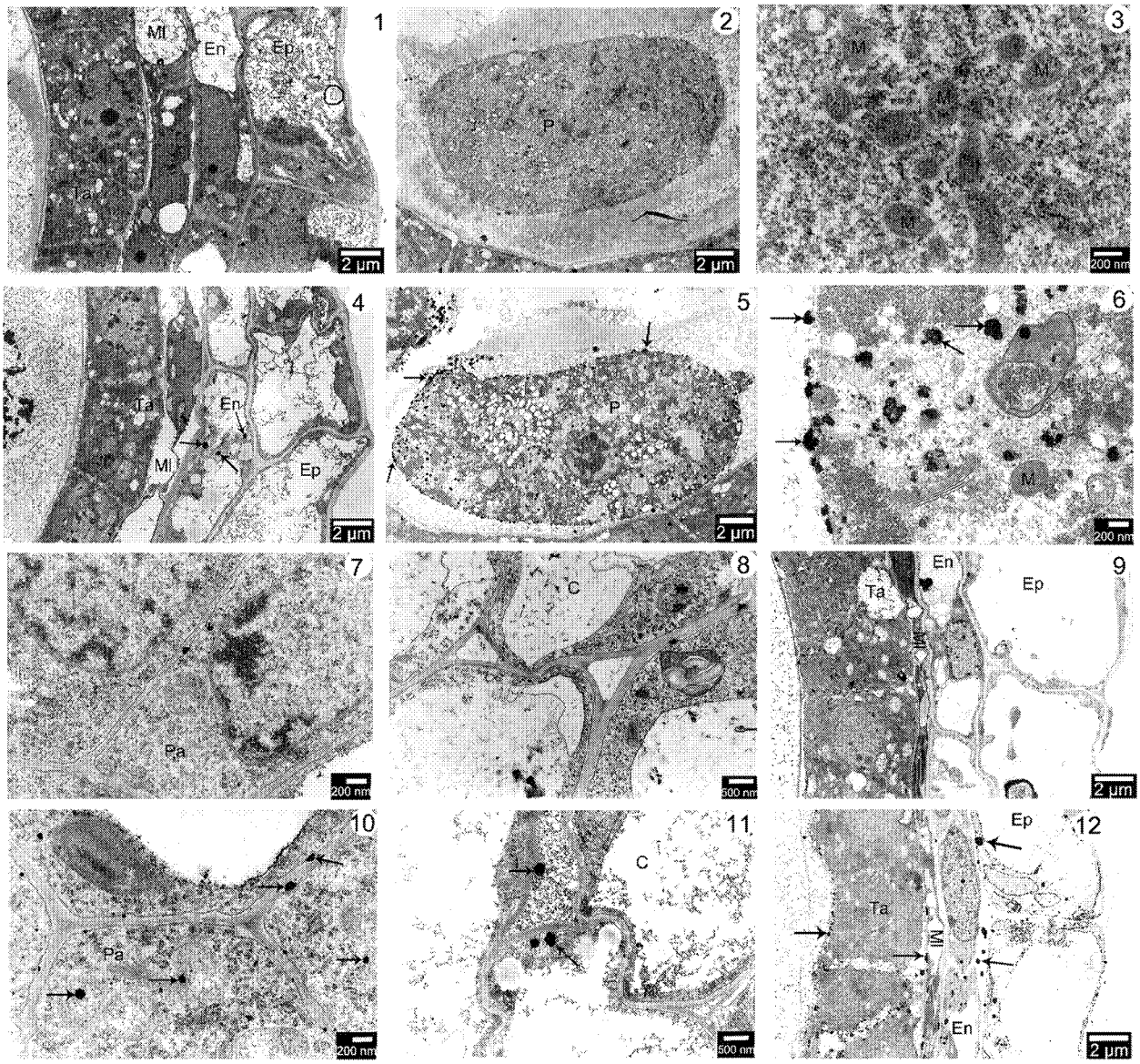

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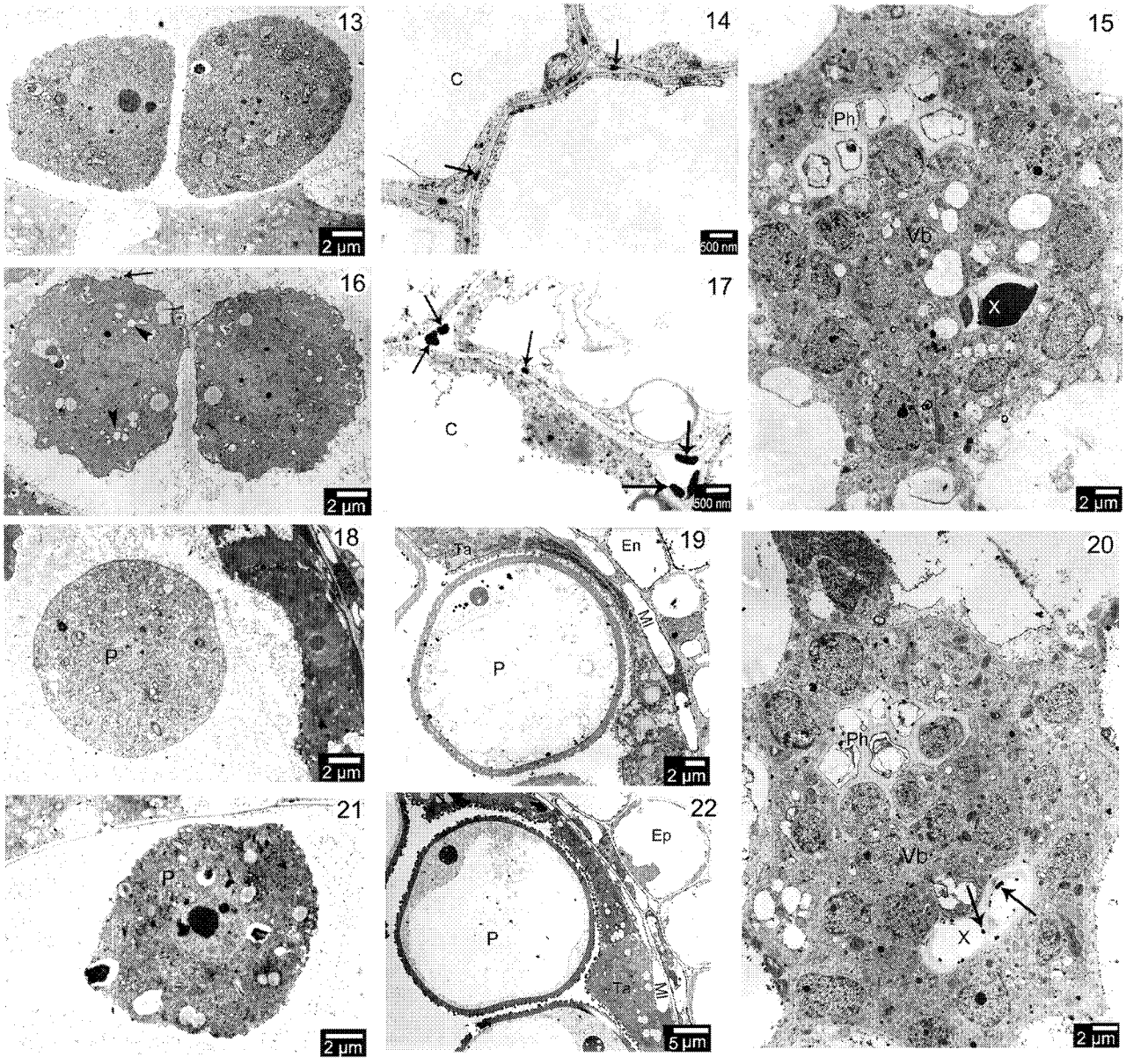
Plate Ⅰ



XIA Kuai-fei, et al　　Plate Ⅱ