International Journal of Plant Developmental Biology ©2009 Global Science Books

ATPase Changes in Rice Anthers

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ABSTRACT

This study investigated ATPase distribution using a lead precipitation technique during rice anther development. The ATPase reaction precipitates were localized in the nuclei of microspore mother cells (MMC), and a few precipitates were detected in the cytoplasm. Low amounts of precipitate were also located in the anther wall, with the exception of tapetal cell nuclei. Following meiosis in the MMC, the precipitates in epidermal cells, endothelium and middle layer cells increased noticeably on the plasma membrane and in the nearby cytoplasm. Numerous precipitates were observed in the pollen wall during pollen development. The pollen wall exine was constructed during microspore development, and the exine precipitates were derived from tapetal cells. The intine was constructed during the bicellular pollen stage, and the intine precipitates originated from the pollen vegetative cell. The vegetative cell contained more precipitates than the generative cell. The amount of precipitate between the two pollen grain sperm cells also differed. The physiological functions of ATPase located in different cells and cellular components during rice anther development were analyzed.

Keywords: development, microspore, *Oryza sativa*
Abbreviations: ATPase distribution in rice anthers. Lin *et al*.

INTRODUCTION

In eukayotic cells, ATPase is the metabolic enzyme integral to ATP hydrolysis, and ATP serves to transport chemical energy in cells to drive metabolism (Pederson and Carafoli 1987). Therefore, exploring cellular ATPase distribution can elucidate cellular material transportation and energy metabolism processes in cells (Serrano 1989; Fedorova 1999). Studies of ATPase distribution in the gynoecia of several plants have been reported (Mogensen 1981, 1985; He and Yang 1991a, 1991b; Tian and Shen 1993), but few studies on anther ATPase distribution are available. Deng *et al*. (1990) employed physiological methods to analyze ATP levels in fertile and sterile anthers in a photoperiod sensitive rice genic male sterile line. Results indicated differences in ATP activity between both anthers. Anther structure in higher plants is complex. Four layers of cells constitute the anther wall, and each layer is unique in structure, function and developmental fate. Pollen development in the anther locula is also a rapid process, both in morphological and structural changes. For example, the microspore undergoes the following events: cytoplasmic recombination, nucleus relocation, unequal cytoplasmic division, a large vacuolar formation and its decomposition in bicellular pollen. All of these biological processes are closely related to energy metabolism, and the amount of ATPase can indirectly reflect the state of cellular energy metabolism. The present study used a lead precipitation technique to identify ATPase distribution during rice anther development. In addition, the developmental features of anther cells following ATPase distribution were analyzed.

MATERIALS AND METHODS

The rice cultivar Nongken 58 (derived from *Oryza sativa* L. *japonica*) was chosen for this study. Plants were grown under field conditions at Xiamen University in south China. When the plantlets produced five to six leaves, a short 10 h daylight treatment was applied for 20 d to induce stem tip differentiation for reproductive growth.

ATPase is located in the cell, and hydrolysis of ATP releases a phosphate that reacts with Pb^{2+} in the incubated solution to form a precipitate, indicating the position of ATPase (Robinson *et al*. 1983). This protocol determines the location and level of ATPase precipitate in the cell, and therefore suggests the amount of energy metabolism occurring in different cellular components. ATPase distribution was analyzed from the microspore mother cell (MMC) to the mature pollen stages. The anthers were collected at five stages of anther development: (1) microspore mother cells; (2) meiosis; (3) microspores; (4) bicellular pollen; and (5) tricellular pollen (mature pollen), based on pollen development. The anthers from different flowers located at the middle part of the inflorescence were fixed in 0.5% glutaraldehyde and 4% polyformaldehyde in 50 mmol/L sodium cacodylate buffer (pH 7.2) for 4 h at room temperature. Following fixation, the anthers were washed in three changes of 50 mmol/L sodium cacodylate buffer with a 30 min period between washes. Anthers were subsequently washed in three changes of 50 mmol/L Tris-maleate buffer (pH 7.2) and incubated in ATPase reactive solution containing 2 mmol/L adenosine 5′-Triphosphate (Sigma-Aldrich), 3 mmol/L $Pb(NO_3)_2$, and 5 mmol/L $MgSO_4$ in 50 mmol/L Tris-maleate buffer (pH 7.2) for 3 h at room temperature. Following enzyme reaction, anthers were washed in three changes of 50 mmol/L Tris-maleate buffer and then three changes of 50 mmol/L sodium cacodylate buffer at 4°C, and postfixed in 1% OsO_4 in 50 mmol/L sodium cacodylate buffer for 16 h at 4°C. The fixed anthers were washed in three changes of double distilled water, dehydrated in a graded acetone series, and embedded in Epon 812 resin. At least 10 anthers from each stage were examined. Sections were observed with a JEM-100CX Ⅱ transmission electron microscope without staining. Two additional controls were conducted: (1) Adenosine 5′-Triphosphate was omitted from the reaction solution in which no precipitates were produced; and (2) NaF was added to the reaction solution to inhibit ATPase hydrolysis in the cells.

Received: 24 January, 2009. Accepted: 9 March, 2009.

Original Research Paper

RESULTS

Before the formation of microspore mother cells (MMC), ATPase precipitates were low in the anthers. However, following the MMC stage, the ATPase precipitates increased and displayed an anther specific distribution.

Anthers at microspore mother cell stage

Microspore mother cells exhibited a large nucleus in the central part of the cell. Many small vacuoles were observed in the cytoplasm, but other organelles were inconspicuous. Few ATPase reaction precipitates were located in MMCs (**Plate I-1**). At this developmental stage, the anther wall was differentiated and consisted of four cell layers: the epidermis, endothecium, middle layer, and tapetum. The epidermis began vacuolization at this stage, and a large vacuole occupied the central region, which pushed the cytoplasm to the edge of the cell. ATPase reaction precipitates were primarily located in the vacuoles and plasma membranes of epidermal cells. Endothecium cells were also vacuolized. The precipitates were mainly located on the plasma membrane of the endothecium cells. Large vacuoles were observed in the middle layer cells, which displayed low cytoplasm electron density. Few precipitates were observed in the middle layer cells, with negligible amounts in nuclei, and some in the plasma membrane. The tapetum cells are the innermost layer of the anther wall, and were structurally divergent from the other three cell wall layers. The tapetal cells exhibited a large nucleus, high cytoplasm density, and few vacuoles. Increased precipitate was noted in the tapetum cell nuclei and plasma membrane; however, more precipitate was located in the membrane's outer tangential plane than the inner tangential face, with a polarity distribution of the ATPase reaction precipitates evident along the membrane (**Plate I-2**). The vascular bundle in the anther connective had differentiated and vessel elements appeared thick-walled with high amounts of precipitate on the inner surface of the vessel. Precipitates were also distributed on the plasma membrane of parenchyma cells in the vascular bundle, but fewer in the cytoplasm (**Plate I-3**).

Anthers at meiosis stage

During meiosis of MMC, precipitate observed in the cytoplasm showed a small increase, and precipitate was also detected in some vacuoles (**Plate I-4**). In the four layers of the anther wall, the precipitate distribution displayed the following changes: the nuclei and cytoplasm density of tapetal cells decreased, and consequently the precipitates decreased; many small vacuoles appeared in the cytoplasm; the precipitates in the middle layer cells showed a marked increase, particularly in the nuclei and plasma membrane; and vacuolization of the epidermis and endothecium was more evident, with a layer of precipitates located in the plasma membrane of the two tissues (**Plate I-5**). Following development, many precipitates accumulated on the inner and outer tangential wall of tapetal cells, although some precipitates were observed in the cytoplasm (**Plate I-6**). The vascular bundle associated with the anthers exhibited many precipitates on the inner surface of the vessel elements, and on the plasma membrane of parenchyma cells.

Anthers at microspore stage

When the microspores were released from the tetrad, many precipitates were evident in the nucleoli, some in the karyoplasm but few in the cytoplasm. A transparent zone was observed on the microspore surface at the time of release (**Plate II-1**). At the onset of exine synthesis, some ATPase reaction precipitates appeared on this transparent zone (**Plate II-2**). Next, sporopollenin material was deposited on the transparent zone. The density between sporopollenin and ATPase reaction precipitate was different and easily determined. The microspore cytoplasm contained many small vacuoles, mitochondria, and plastids, but these organelles were not identified because no structural differentiation could be discerned at this time. Following microspore development, ATPase reaction precipitates increased in its cytoplasm (**Plate II-3**). When sporopollenin material accumulated to form pollen primexine, a region of the transparent zone thickened in an orbicular pattern to form a germ pore rudiment, a thickening not observed in the central region (**Plate II-4**). In the cells of the anther wall, the epidermis, endothecium, and middle layer cell precipitates increased, however the precipitates did not increase in tapetal cells (**Plate II-5**). The radial wall of the tapetal cells dissolved, resulting in a large gap between the cells. Concurrently, Ubisch bodies appeared in the inner tangential wall of tapetal cells. As microspores developed, the exine thickened to form three structural layers, including the tectum, baculum, and foot-layer. ATP reaction precipitates were high in these layers and accumulated on the exine surface. In the germ pore region, the outer loop of the pore developed into a multilayer structure bearing large areas of precipitate. Under the pore, a region of low electronic density appeared in the microspore cytoplasm, and a few ATPase reaction precipitates appeared in the cytoplasm and on the pore surface (**Plate II-6**). In the anther wall, the precipitates increased notably in tapetal cells, particularly along the inner tangential plane. On the surface of Ubisch bodies, many precipitates were also detected (**Plate II-7**). During formation of the large microspore vacuole, the tapetal cells degenerated; the heterochromatin displayed high electronic density in the nucleus and many precipitates appeared in the karyoplasm. Furthermore, organellar profiles were not identified and many precipitates accumulated in the cytoplasm (**Plate II-8**). The ATPase reaction precipitates in the anther vascular bundle were unchanged, with numerous precipitates still located on the inner surface of the vessel elements and the plasma membrane of parenchyma cells.

Following the formation of a large vacuole in the microspore, which pushed the cytoplasm and nucleus to the periplasm, the microspore displayed a polarity that resulted in unequal cell division. The precipitates were unevenly distributed in the microspore nucleus, and possibly resided in chromatin condensing regions (**Plate III-1**). A region of low electronic density appeared in the microspore cytoplasm, and some precipitates were located just under the exine where the pollen intine was forming (**Plate III-2**). During late microspore developmental stages, the cells of the anther wall changed as follows: the epidermis, endothecium and middle layer became highly vacuolized; one large vacuole was located in each cell; and some precipitates appeared in the cytoplasm. Tapetal cells had degenerated, the cytoplasm showed high electronic density, and organellar profiles were indistinguishable (**Plate III-3**).

Anthers at the bicellular pollen stage

After microspore cell division, bicellular pollen consisted of one large vegetative cell and one small generative cell. The generative cell remained close to the pollen wall, and a transparent zone differentiated it from the vegetative cell. No evident structural differences existed between the cells, and a few precipitates were observed. Before decomposition of the large vacuole comprising most of the vegetative cell, the precipitates increased, but precipitate increases were not seen in the generative cell (**Plate III-4**). As the large vacuole of the vegetative cell decomposed into many small vacuoles, the precipitates in its cytoplasmic matrix increased (**Plate III-5**). During bicellular pollen development, numerous precipitates accumulated on the tectum surface, in the exine baculum, and the pollen wall intine (**Plate III-6**), suggesting the construction of exine and intine required increased energy.

The generative cell separated from the intine and moved into the vegetative cell cytoplasm after the large vacuole in the vegetative cell decomposed. The vegetative cell cytoplasm increased in density and initiated starch accumulation.

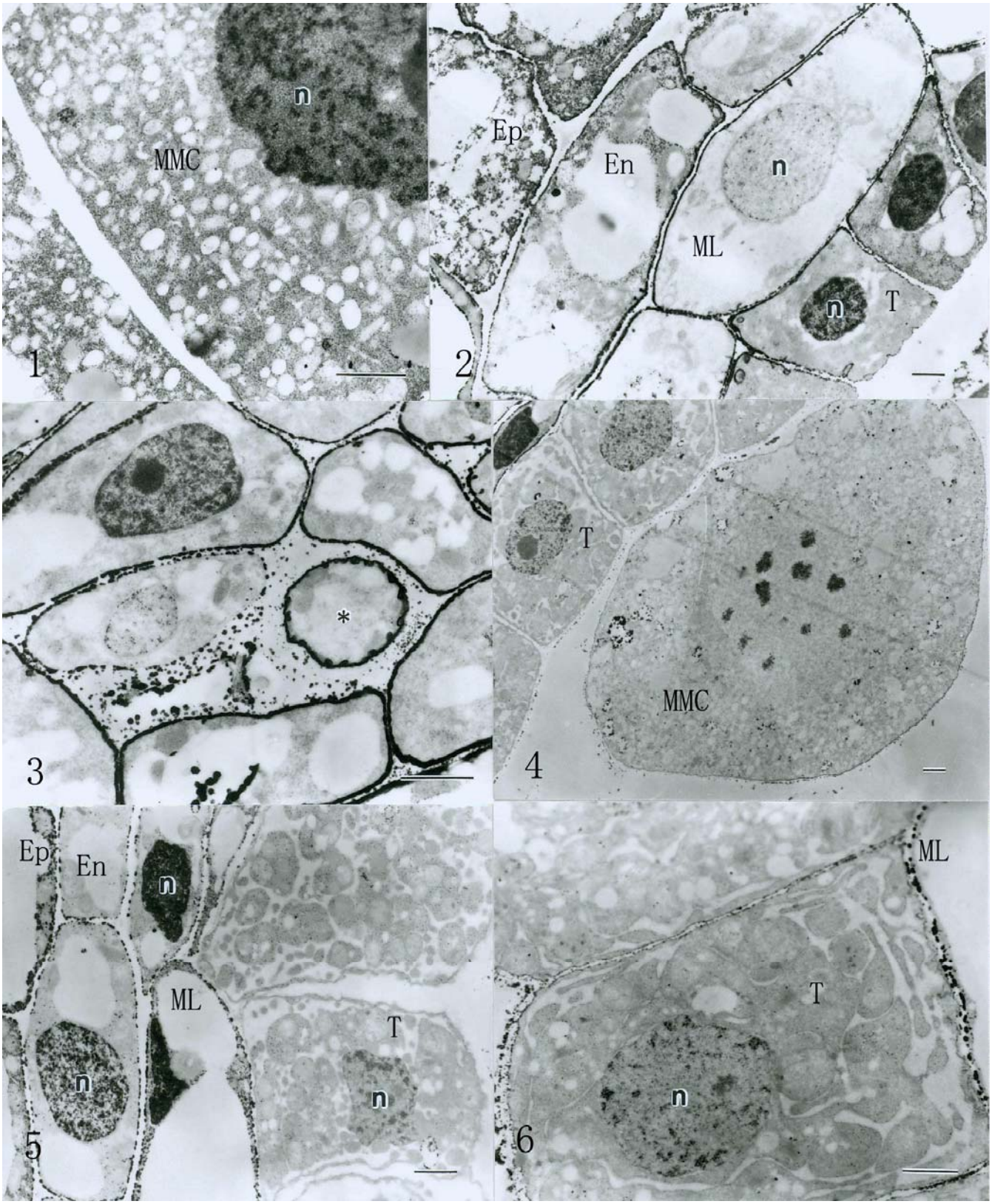

Plate I (**1**) There were many ATPase precipitates (ppts) in the nucleus but only a few in the cytoplasm of microspore mother cells (MMC). ×10 666; Bar = 1 μm; (**2**) The anther wall consisted of four layers of cells: epidermis (Ep), endothecium (En), middle layer (ML) and tapetum (T). Some ppts appeared on the plasma membrane of the cells and also in the nuclei of tapetal cells. n: nucleus. ×6 666; Bar = 1 μm; (**3**) Vascular bundle of anther had been differentiated and many ppts were formed on the inter surface of vessel (asterisk) and on the plasma membrane of parenchymal cells. ×10 666; Bar = 1 μm; (**4**) A small number of ppts were observed in the microspore mother cell (MMC) during meiosis. A decrease in the ppts of tapetal cell nuclei (T). ×3 600; Bar = 1 μm. (**5**) There were more ppts in the nuclei of middle layer (ML) and endothecium (En) but a few in the cytoplasm of tapetal cells (T) after the meiosis of MMC. Ep: epidermis; n: nucleus. ×6 666; Bar = 1 μm; (**6**) Tapetal cells (T) exhibited numerous endoplasmic reticula at this time. No ppts were formed in the cytoplasm of tapetal cells, but many in the inner and exterior tangential walls of the cells. ML: middle layer; n: nucleus.×8 933; Bar = 1 μm.

The intine thickness increased and exceeded that of the exine. Numerous large ATPase reaction precipitates accumulated in the intine. In the cytoplasm, adjacent to the plasma membrane, many small vacuoles contained precipitates, which fused to the plasma membrane and secreted the contents into the intine (**Plate III-7**). At this point, the tapetum was completely degenerated, and only a tapetal membrane remained with some Ubisch bodies attached to the

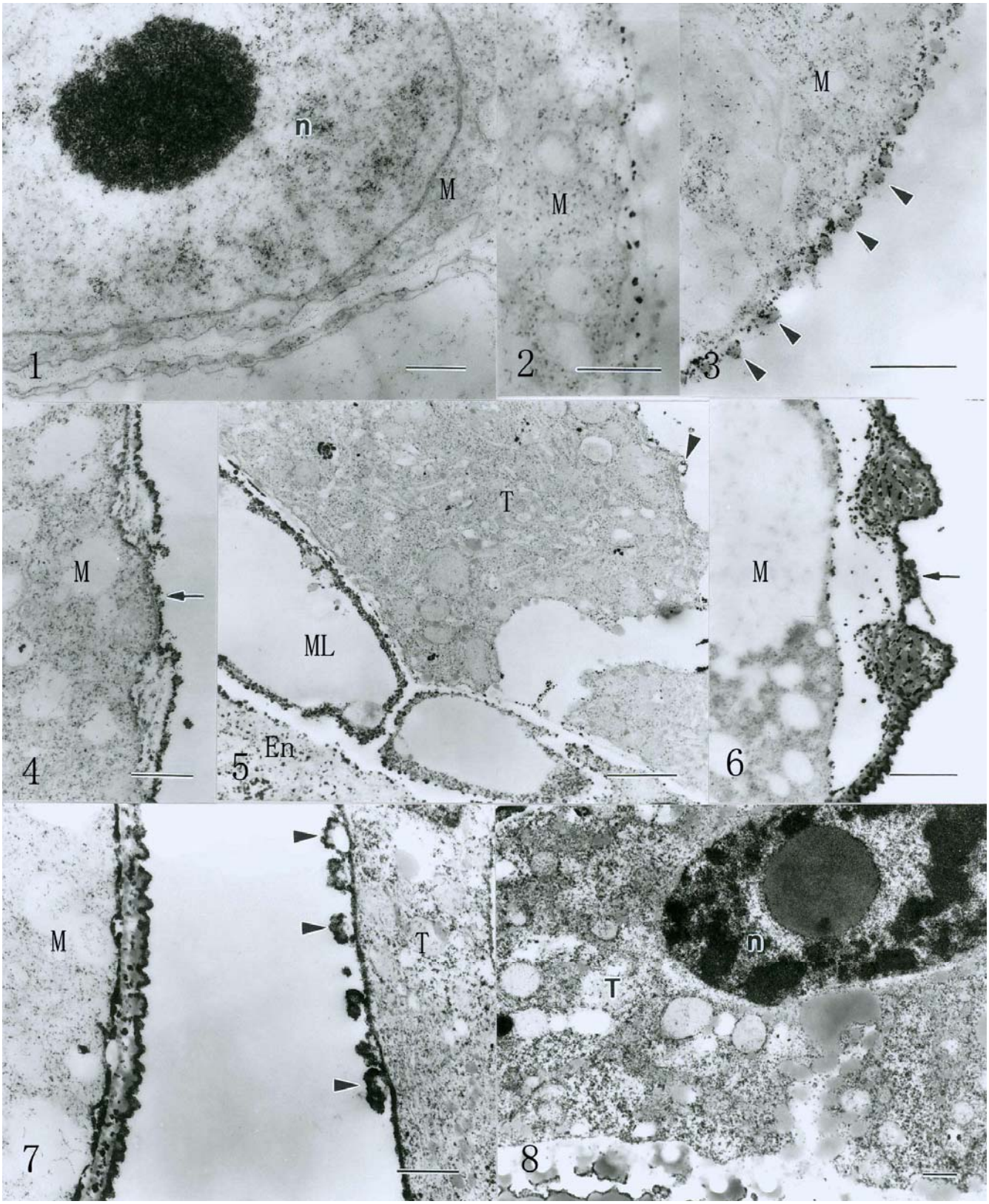

Plate II (**1**) Numerous precipitates (ppts) were located in the nucleolus, a few in the nucleus matrix (n), and ppts were absent from the cytoplasm of a microspore (M) just released from a tetrad. A transparent strap appeared on the surface of the microspore and no ppts were formed. ×18 666; Bar = 0.5 μm; (**2**) Some ppts began to form in the transparent strap on the plasma membrane of the microspore (M) during anther development. ×26 666; Bar = 0.5 μm; (**3**) Some sporopollenin granules (arrowheads) began to accumulate on the transparent strap formed on the plasma membrane of a microspore, which formed the microspore (M) exine. ×26 666; Bar = 0.5 μm; (**4**) Some regions of the transparent strap enlarged and formed a microspore (M) germination pore (arrowhead). ×18 666; Bar = 0.5 μm; (**5**) A few ppts were observed in tapetal cells (T), and many in the cells of the middle layer (ML) and endothecium (En). Some Ubish bodies (arrowhead) also appeared on its inner tangential face. ×10 666; Bar = 1 μm; (**6**) Numerous ppts were formed in the microspore (M) germination pore (arrowhead). ×10 666; Bar = 1 μm; (**7**) Numerous ppts were formed in the cytoplasm of tapetal cells (T), and some Ubish bodies (arrowheads) appeared on its inner tangential face. The exine of the microspore (M) was completely formed by this time. ×18 666; Bar = 0.5 μm; (**8**) The number of ppts markedly increased in the tapetal cells (T), especially in the nuclei (n) and nucleoli at the late microspore stage. ×13 333; Bar = 0.5 μm.

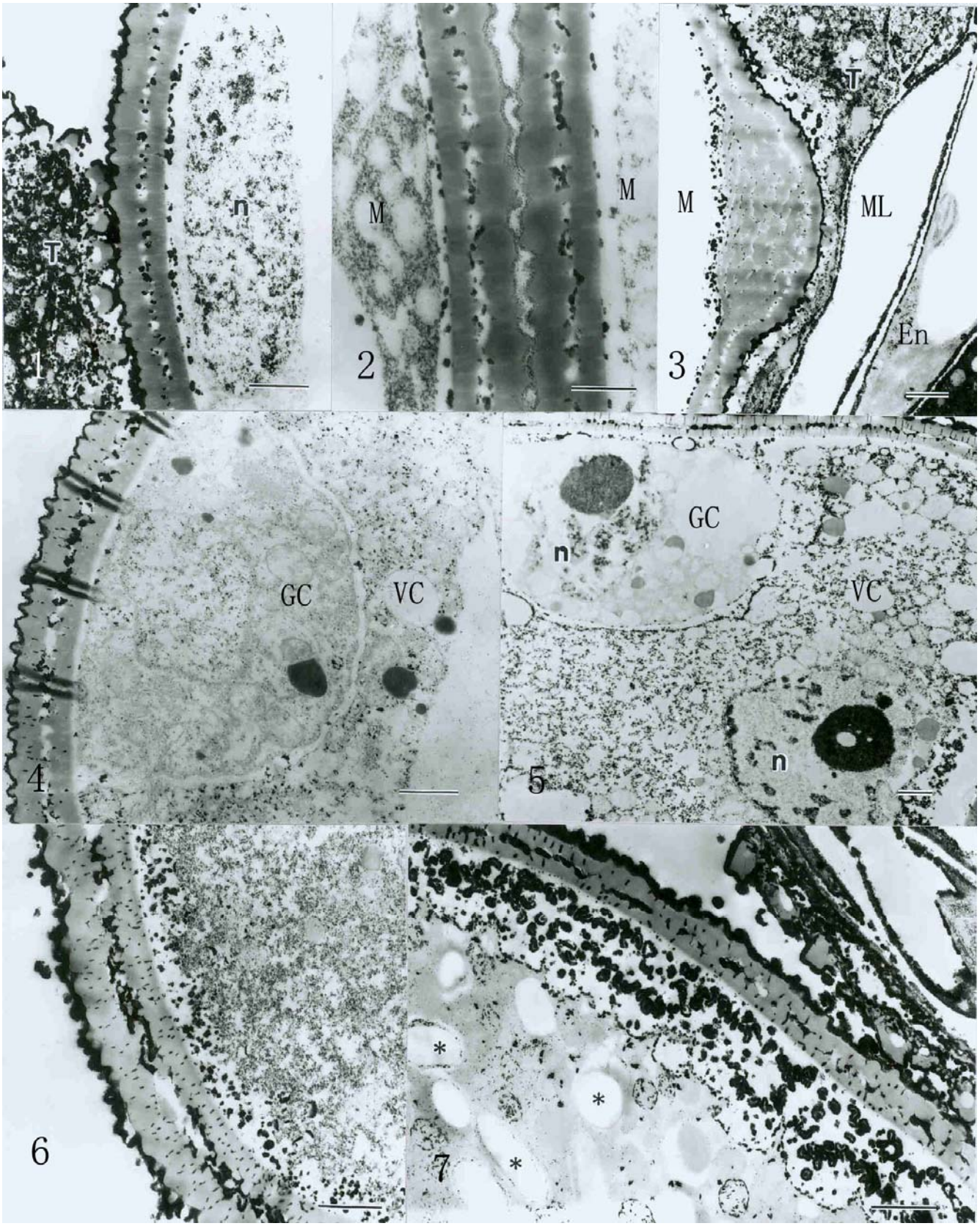

Plate III (**1**) The number of precipitates (ppts) increased in the nucleus (n) of the microspore before division. Tapetal cells (T) began to degenerate. ×8 933; Bar = 1 μm; (**2**) A transparent strap was formed on the inner surface of the microspore (M) exine (Ex) and a few ppts were observed. ×9 833; Bar = 1 μm; (**3**) A microspore (M) germination pore facing tapetal cells (T), where it noticeably decreased in size. ML: middle layer; En: endothecium. ×6 666; Bar = 1 μm; (**4**) Early stage of 2-cellular pollen; more ppts were observed in the vegetative cell (VC) than in the generative cell (GC). Ex: exine of microspore. ×8 933; Bar = 1 μm; (**5**) The amount of ppts in the nucleus (n) of a vegetative cell (VC) and that of generative cell (GC) appeared different. ×5 094; Bar = 1 μm; (**6**) The number of ppts increased in the transparent strap and appeared on the inner surface of the exine (Ex), suggesting that the pollen intine (In) was formed at this time. ×10 666; Bar = 1 μm; (**7**) As the pollen grains initiated starch synthesis (asterisks), many small vesicles containing ppts were transported into the intine (In). Ex: exine of pollen. ×10 666; Bar = 1 μm.

membrane. Precipitates still appeared in the cells of anther vascular bundles, with most located on the plasma membrane of the bundle cells, and some in the nuclei (**Plate IV-1**). These observations suggested active transport of material into the anthers.

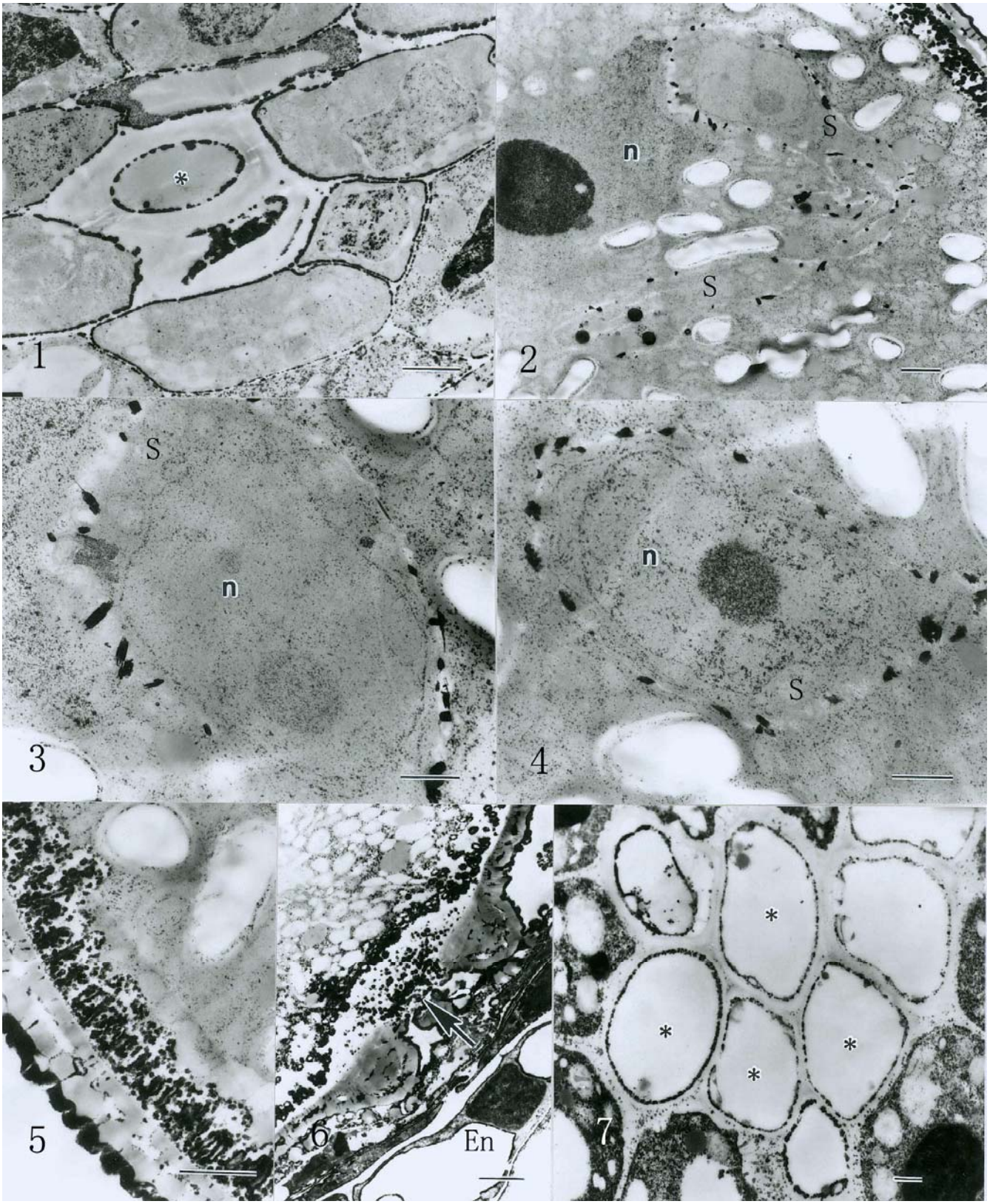

Plate IV (**1**) The inter surface of a vessel (asterisk) and the plasma membrane of parenchyma cells from an anther vascular bundle; numerous precipitates (ppts) were maintained at the 2-cellular pollen stage. ×8 933; Bar = 1 μm; (**2**) The generative cell divided to form two sperm cells (S) in a pollen grain. ×6 666; Bar = 1 μm; (**3**) Sperm cell (S) with a large nucleus (n) contained fewer ppts than a vegetative cell. ×18666; Bar = 0.5 μm; (**4**) The nucleus (n) of one sperm cell (S) contained more ppts than that of the other (**3**). S: starches. ×18 666; Bar = 0.5 μm; (**5**) A large number of ppts were still formed in the intine (In) at the 3-cellular pollen stage. The intine became increasingly thinner. Ex: exine of pollen; S: starches. ×13 333; Bar = 1 μm; (**6**) A large number of ppts in the germination pore (arrowhead) remained at the 3-cellular pollen stage. ×8 933; Bar = 1 μm; (**7**) Although some ppts on the vessel surface were retained (asterisks), cells of the anther bundle, and the parenchyma bundle cells began to degenerate two days before anthesis. ×5 333; Bar = 1 μm.

Anthers in the tricellular pollen stage

Prior to pollen maturation, the generative cells divided and produced two sperm cells, forming a tricellular pollen grain. The two sperm cells were positioned near the vegetative nucleus (**Plate IV-2**). The nucleolus of the vegetative cell exhibited high electronic density, and many small precipitates were located in the nucleus and cytoplasm. The amount of precipitate differed between the two sperm cells and the nucleoli (**Plate IV-3, 4**). These results suggested that dif-

ferent activity levels and hence different energy requirements exist for each sperm cell. A transparent zone between sperm plasma membranes and inner plasma membranes of vegetative cell surrounded the two sperm cells, and large precipitates were situated in this zone. The numerous precipitates continued to accumulate in the intine, and the intine became increasingly thinner through time (**Plate IV-5**). The pollen germ pore remained oriented toward the anther wall, and numerous precipitates were observed at the intine of the pore (**Plate IV-6**). Only the epidermis and endothecium were preserved in the anther wall. In the vascular bundle of nearly mature anthers, the precipitates on the inner surface of the vessel decreased sharply. However, in the bundle parenchyma cells, the electronic density of the nucleus increased, and small vacuoles appeared in the cytoplasm, with an increase in cytoplasmic precipitates (**Plate IV-7**).

DISCUSSION

Distribution of ATPase during rice anther development

Anthers are comprised of a diversity of tissues, the result of rapid differentiation of cells that forms several different tissue types during anther development. The cells of the anther wall form four different layers, including the epidermis, endothecium, middle layer, and tapetum. Each of these cells exhibits different morphology, structure and function (Bhojwani and Bhatnagar 1979). Meiosis of MMC occurs in the anther locules, and results in four haploid microspores. Microspore division is unequal, therefore one large vegetative cell and one small generative cell are produced. The generative cell divides to form two sperm cells, which display little cytoplasm and no cell wall (Knox 1984). All of these rapid changes in anther cell morphology and structure must keep pace with physiological processes occurring in the cells. At the MMC stage of rice anther development, differences in ATPase distribution were evident among the four anther cell wall layers. The results showed that the tapetum nuclei contained more precipitate than the other three cell layers, showing the tapetum maintained higher metabolic activity. Following meiosis in the MMC, nuclei of the middle layer and endothecium cells exhibited an increase in precipitate, indicating the metabolic activity of these cells was elevated at this developmental stage. During microspore development, a few ATPase precipitates appeared in the early microspores, with the exception of the nucleus. An increase in ATPase was also observed at the late microspore development, a stage that transforms the microspore into the male gametophyte. During bicellular pollen development, the precipitates in vegetative cells were greater than in generative cells, indicating that vegetative cells maintained higher levels of ATPase than generative cells, which can be explained by the vegetative cell function. Vegetative cells serve to construct the pollen intine wall and synthesize starches (Knox 1984). After the generative cell divides and forms two sperm cells, a difference in ATPase distribution appeared between the two sperm cells, which may reflect a viability difference between the two sperm cells. ATPase distribution in rice anther development displayed both spatial and temporal features, which reflected different physiological states and functions of anther cells and tissues.

ATPase distribution and function in cells

ATPase is an abundant and widespread enzyme in living organisms that hydrolyzes ATP to provide cellular energy (except in chloroplastids) (Pederson and Carafoli 1987). ATPase distribution in a cell is related to the specialized function of each cell. Almouzni and Mechali (1988) suggested that nuclear ATPase plays an important role in nucleosome formation and stability, and also provides energy for ribosome synthesis in the nucleolus. Feijó *et al*. (1999) observed ATPase in pollen grain and tube distribution in *Agapanthus umbellatus* and found Ca^{2+}-ATPase was located largely in mitochondria and endoplasmic reticulum, Mg^{2+}-ATPase in small vacuolar membranes, and K^{+}-ATPase primarily in the plasma membrane. ATPase in the plasma membrane was thought to serve in absorption and secretion of material into or out of cells (Serrano 1989). In the present study, in the late microspore stage, ATPase precipitates markedly increased in the plasma membrane of epidermal, endothecal, and the middle layer, indicating the cells were transporting nutrient material into the locule. Plasma membrane ATPase and its role in nutrient transport were supported by the distribution of ATPase in the vascular bundle, where precipitates were distributed on the plasma membrane of parenchyma cells. The vessel does not directly enter the locule, and parenchyma cells indirectly transported nutrients to the anther locule. Therefore, many ATPase precipitates appeared on the plasma membrane of the parenchyma cells which may be related with active transportation of nutrient material.

The pollen wall was the primary location of ATPase precipitates, which indicated that construction of the exine and intine has high energy demands. However, the exine and intine ATPase was derived from different cells. During exine construction, the sporopollenin originated in Ubisch bodies of the tapetum. ATPase precipitates deposited on the Ubisch bodies were transported to the microspore surface to participate in exine construction. The pollen wall intine was formed during the bicellular pollen stage, and the vegetative cell secreted small vacuoles containing ATPase precipitates into the intine. Therefore, the ATPase used on exine and intine formation was derived from different cells at different times during development. The tapetum ATPase was sporophytic in origin, and the ATPase from vegetative cells was derived from gametophytic tissue. These results demonstrated the diversity of processes and derivation of energy sources involved in pollen wall construction.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (No. 30670126).

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