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RESEARCH ARTICLE

Zengfang YIN, Ruwen FAN

Selective autolysis of protoplasmic components during development of secondary-phloem sieve-tube elements in *Populus deltoides*

Abstract Observing changes in plasma components during the development of sieve-tube elements (SE) in secondary-phloem is very important in the study of their physiological function. We investigated the development of SE in the secondary-phloem of *Populus deltoides* with the aid of an electron microscope. The developmental process of SE in secondary-phloem can be divided into three stages: immature, mature and degenerated, based on the changes of plasma components and cell structures. The immature stage is the development period before the vacuole membrane was ruptured. The radial extension of cells, cell wall incrassation and generation of plastid sieve elements and P-protein occurred during this period. The mature stage started when the vacuole membrane was ruptured. Selective autolysis of protoplasmic components formed mature SEs, with the characteristics that the organelles, such as dissociative ribosome, Golgi body, endoplasmic reticulum and nuclei were disassembled quickly. Two distinctive ways of nuclear degeneration occur. One is an early degenerated nuclear membrane, with dispersed karyoplasms, joined by P-protein. The other is early agglomerated chromatin and subsequently degenerate karyotin with two clear layer structures in the nuclear membrane. One sign in the degenerated stage is the disassembled plasma membrane. During this stage, plastid membranes become disorganized, the starch grains are dispersed in the chamber of SE and the mitochondria dissembled. The callose appears synchronously in sieve plates and P-protein disaggregates. Opened sieve plates are then formed because of callose autolysis, after the protoplasm disappears completely. Finally, the physiological function of SE is lost.

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Zengfang YIN (✉), Ruwen FAN
College of Forest Resources and Environment, Nanjing Forestry University, Nanjing 210037, China
E-mail: zfyin@njfu.com.cn

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1 Introduction

Although research on the relationship between the sieve-tube element (SE) structure and its function, using transmission electron microscopy techniques, has made great strides during the last 40 years (Li and Zhang, 1983; Fahn, 1990). Much of the earlier research was conducted on the developmental process of sieve-tube elements in primary and meta-phloem, with experimental material of herbaceous plants such as *Triticum aestivum* L. (Zee and O'Brien, 1971), *Zea mays* L. (Ouyang et al., 1998) and *Arabidopsis thaliana* (Wu and Zheng, 2003). The SE of secondary phloem also plays a crucial role in the transport and cycling of organic nutrient substances in tree ontogeny. This SE of secondary phloem is formed from the differentiation of vascular cambium cells. However, little is known about the changes in protoplasm during the developmental process of SE in secondary phloem. In particular and to our knowledge, no recent literature exists about the changes of cell structures and composition of functional loss of SE.

Therefore, we have attempted to explore the selective autolysis of protoplasm components in the development of the SE of secondary phloem, using a model poplar tree, in order to provide a fundamental understanding of plant developmental biology and of forest production.

2 Material and methods

2.1 Plant material

All experimental material was derived from 1 to 2 year old healthy branches of 9-year *Populus deltoides*, grown in the nursery of the Nanjing Forestry University. The samples

were collected in December of the first year, once in January and in February of the second year, respectively. More samples were collected every five days from February 21 and every 15 days from the end of July to December. Sample collection and fixation were repeated twice.

2.2 Methods

The 5th and 10th internodes of the 1 and 2 year old branches were cut into pieces, to size 0.7 mm × 0.5 mm. The samples were prefixed with 6% glutaraldehyde in a 0.5 mmol/L dimethylarsonic acid sodium salt buffer (pH 7.4) at 4°C for two hours. The margins of the prefixed material were then cut off and this material was divided into 0.2 mm × 0.35 mm pieces. This was fixed with the same fixation solution at 4°C (or at room temperature) for 4–6 h and then washed three times using a 0.5 mmol/L dimethylarsonic acid sodium salt buffer (overnight at the second washing) and then post-fixed with 1% OsO_4 in the same buffer for 4–5 h. After washing in the buffer, the samples were dehydrated through a graded ethanol series, substituted with propylene oxide and embedded in Epon812. The sections were cut with a LKB-V ultramicrotome and stained by conventional methods with uranyl acetate and lead citrate. Finally, we observed our experimental material through a H-600 transmission electron microscope.

3 Results

The acute change of cell structure and protoplasm composition occurred during the mature development and functional decline of SE of the secondary phloem. In order to aid in the description, the developmental process was divided into three stages, i.e., an immature, a mature and a degenerate stage.

3.1 Immature stage

The immature stage is defined as the developmental phase before the SE cell tonoplast was broken. In this stage, certain phenomena appeared. For example, the cells were enlarged, the cell walls thickened, and the SE plastids and P-protein were produced. In the initial stage, the cell became enlarged because of an enlarged vacuole (Fig. 1a). As cells became large, the cell walls clearly increased their thickness. The cytoplasm and nucleus were crowded into the periphery of the cell wall due to the enlarged central vacuole. Therefore, many organelles, such as bodies of the Golgi complex, the endoplasmic reticulum, the plastids and polyribosomes became deposited near the cell wall (Figs. 1b and c). The nucleus of the SE looked like the shape of an amoeba, surrounded by mitochondria (Fig. 1d), a small amount of mitochondria remained in the cytoplasm. Golgi bodies consisted of three to six layers of sacs surrounded by many Golgi vesicles. A few vesicles were connected with inflexed tonoplasts and cytoplasm membranes (Fig. 1e). The rough endoplasmic reticulum was distributed in parallel or became mesh shaped. As the SE cell wall substance accumulated, the sieve-tube plastid began to deposit amylum, which was closely connected with the inflexed tonoplasts and cytoplasm membranes (Fig. 1f). At this time, the P-protein appeared filiform, which was produced in local cytoplasm of the SE. Golgi bodies and mitochondria were often observed near the P-protein and some filiform P-proteins were distributed around the nucleus (Figs. 1c and 1g).

In the initial development stage of the SE, there were some plasmodesmata between the cell walls of neighboring SEs. A large amount of rough endoplasmic reticulum, mitochondria and a few Golgi bodies were observed near cell walls. Double zygomorphic callose platelets appeared in the cell walls, which were detached by thin layers consisting of middle lamella and primary cell walls (Fig. 2a). Large amounts of callose and cellulose then accumulated so fast that the callose and cellulose layers became wide and connecting strands were formed between two pairs of callose platelets. The callose was found in the portions of the walls between the callose cylinders surrounding the connecting strands (Fig. 2b). At the same time, the protoplast of the SE started to enter the mature selective autolysis phase.

3.2 Mature stage

The mature stage is defined as the period where the SE is developing and selective autolysis of protoplasm component SE occurs. During this stage, the obvious symbol of SE development is broken tonoplast. The protoplast became rapidly disorganized. Polyribosomes became dissociative ribosomes and then gradually disappear (Fig. 2b). Other organelles such as Golgi bodies had no clear sac membrane structure and neither did the endoplasmic reticulum (ER). The cisternae of the ER were finally broken into fragments (Fig. 1g). The tonoplast was completely disassembled, but the cytoplasmic membrane was still intact. Plastid was a kind of special organelle in SE, which could accumulate a large amount of starch and in turn became S-type SE plastids during SE development (Fig. 2c). A series of changes in the P-protein of SE, where the filiform P-protein aggregated to become an erose electronic compact substance, were also observed. The sieve plate between neighboring SEs was then developed (Fig. 2c).

The nucleus degenerated in two ways during SE development: 1) the nuclear membrane first degenerated and the karyoplasm that is closely joined to P-proteins dispersed (Fig. 2d); 2) the chromatin first agglomerated

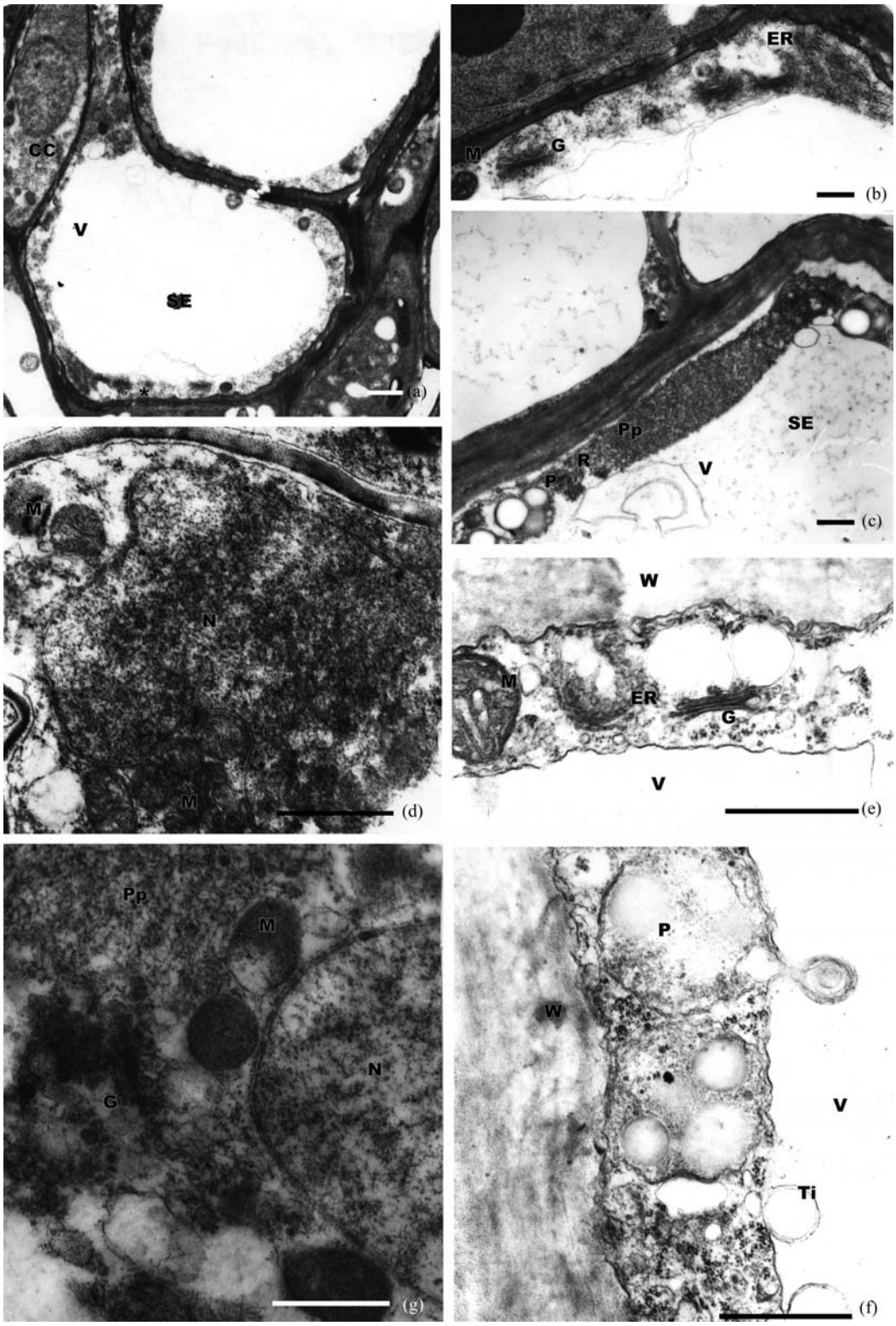

Fig. 1 Ultrastructure of sieve elements at an immature stage (bar: 1 μm)
(a) The cross section of a SE showing the sieve element (SE) and its companion cell (CC) in their immature stage; (b) Enlarged picture showing mitochondria (M), Golgi body (G), and an endoplasmic reticulum (ER) near the sieve element cell wall; (c) Another cross section of a sieve element showing sieve plastid (P) and P-protein (Pp); (d) The cross section of a sieve element showing an irregular shaped nucleus (N) surrounded by mitochondria (M); (e) The longitudinal section of sieve elements showing Golgi body (G), mitochondria (M) and an endoplasm reticulum (ER) near the sieve element cell wall; (f) The longitudinal section of a sieve element showing plastids (P) and tonoplast invagination (Ti); (g) The cross section of a sieve element showing P-protein (Pp) in a threadlike shape, Golgi body (G), mitochondria (M) and a nucleus (N).

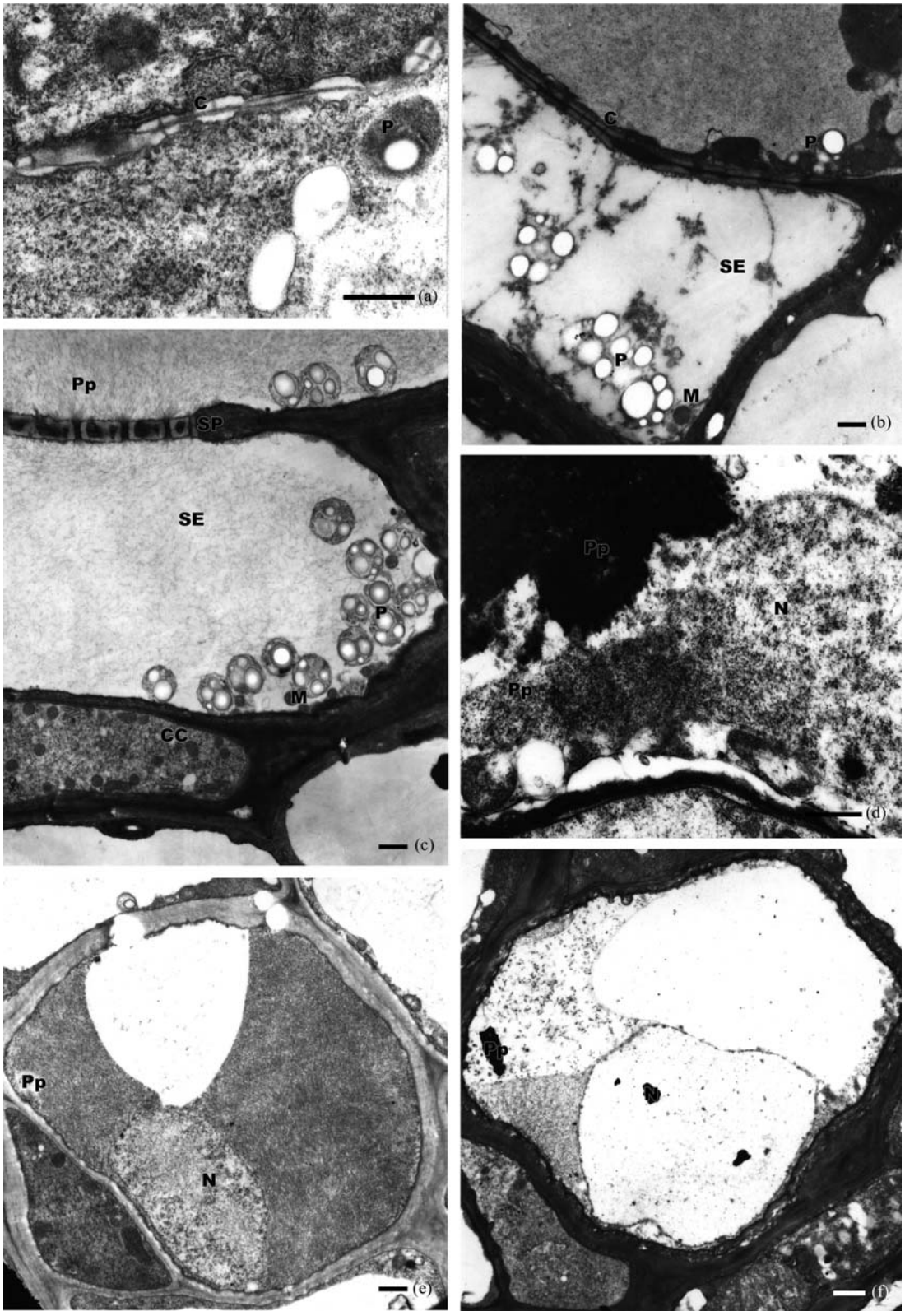

Fig. 2 Ultrastructure of sieve elements at a mature stage (bar: 1 μm)
(a) The cross section of a sieve element showing the forming of callose walls; (b) The cross section of a sieve element showing the sieve element in its initial developmental stage; (c) The cross section of a sieve element showing the structure of a sieve element at a mature stage; (d) The cross section of a sieve element showing dispersion of karyotin and the rupture of a nuclear membrane; (e) The cross section of a sieve element showing the nucleus (N) at an initial, degenerate stage; (f) The cross section of a sieve element showing a nucleus (N) at a later degenerate stage, the karyotin gradually disappearing and an intact nuclear membrane.

and the karyotin then degenerated, but the nuclear membrane still remained clear in the two-layered structures. Consequently, the electronic transparency of karyoplasm in the SE increased and the local nuclear envelope broke. The electronic transparency of the karyoplasms was similar to that of the cytoplasm region without the presence of P-protein (Figs. 2e and 2f).

The protoplasm component of mature secondary phloem SE was distributed in the peripheral chamber of the cell, which consisted of a cytoplasm membrane, mitochondria, sieve-tube plastids and a few Golgi bodies. The P-protein appeared in a mesh shape in the SE and was connected to neighboring SEs in filiform through the sieve pores (Fig. 2c).

3.3 Degenerate stage

The functional degeneration symbol in the secondary phloem SE was the broken cytoplasm membrane. The major changes in this process included:

1) a contractile cytoplasmic membrane, an inflexed membrane in the form of extended tubules or sacs and plasmolysis phenomena (Figs. 3a and 3c);

2) the degenerated outer membrane of SE plastids resulting in starch grains dispersed in the SEs (Figs. 3b and 3c);

3) a cushion of sieve plates was laid down from large amounts of callose and disintegrated P-proteins (Figs. 3d and 3e);

4) the last degenerated mitochondrion (Fig. 3f);

5) the autolyzed callose of sieve plates after the cytoplasm had disappeared from the SE, formed openings in the sieve pore and lost completely its function in the SE (Fig. 3g).

4 Discussion

4.1 Selective autolysis of the protoplast

The protoplast selective autolysis process of SE in *Populus deltoides* started with a broken tonoplast. Some mature SE was found to possess an intact vacuole during the development of secondary phloem in *Ulmus americana* (Evert and Deshpande, 1969). Zee and O'Brien (1971) also reported that there was a sort of vacuole residue in the mature SE of *Triticum aestivum* L.. The tonoplast was present in the initial, developmental stage of SE in secondary phloem in *Populus deltoides*, but it did not remain for long before partly disintegrating. At the same time, the protoplast began to be selectively autolyzed. There was no vacuole residue in mature SEs.

The most distinctive transformation was that the polyribosome disintegrated to become free ribosome during protoplast selective autolysis, resulting in decreased electron density of the cell. Another highly changed organelle was the Golgi body. The Golgi bodies were abundant in the immature stages. The structure of the Golgi sacs became blurred at the later developmental stage and finally broke up into coated vesicles surrounding the filiform P-protein. The investigation by Hoefert (1979) in *Thlaspi arvense* L. indicated that the vesicles derived from Golgi bodies were related to the aggregation of the cell wall substance and P-protein formation. The same phenomena were observed in *Populus deltoides*. Therefore, we can infer that the coated vesicle of Golgi body is related to P-protein formation. The changes of rough endoplasmic reticulum are much more complex. For example, the enlarged lamella of endoplasmic reticulum resulted in connecting with the protoplasm components, such as plasma membrane and mitochondria and taking up the cytoplasm components. The lamella of endoplasmic reticulum was broken up into vesicles which did not become a smooth endoplasmic reticulum until the mature SE was developed. Esau and Gill (1971) thought that the smooth endoplasmic reticulum could be stacked in the mature SE and that it was a kind of important source of hydrolytic enzymes in the selective protoplast autolysis process of SE.

The mitochondrion was the last disintegrated organelle. Some divertive transformation of mitochondria distribution took place at the initial developmental stage of SE. Most of the mitochondria were surrounded by the nucleus and a few were distributed in the cytoplasm. In particular, the mitochondria surrounding the nucleus appeared as local electron transparent area, which looked like a "budding" phenomenon. The cytochrome c in mitochondria was recognized as a necessary factor introducing cell apoptosis (Kluck et al., 1997; Yang et al., 1997). During the development of SE in *Populus deltoides*, the change in the structure of mitochondria near the nucleus could have a certain relationship with that of the results from previous studies (Kluck et al., 1997; Yang et al., 1997). We inferred that local electron transparent areas of mitochondria could be present and the development of SE before the SE degeneration stage, which is closely related to the development of SEs. Another reason might be a sort of unstable state representation in the active metabolism stage of SE. Furthermore, the disintegration of mitochondria was related to the functional loss of SE during this degeneration stage.

Singh and Srivastava (1972) observed that the outer membrane and the matrix of plastids degenerated and crystalloids appeared in a free state at the later differentiation stage of meta-phloem SE in *Zea mays* L. leaves. However, Walsh and Evert (1975) thought that the plastid was intact in better preserved SEs. Ouyang et al. (1998) found the phenomena that the partial outer membranes of plastids and mitochondria had disintegrated, based on the research in the process of SE in primary phloem in *Zea mays* L. leaves. Possible reasons could be the impact of outer membrane stability on the sharp changes in the

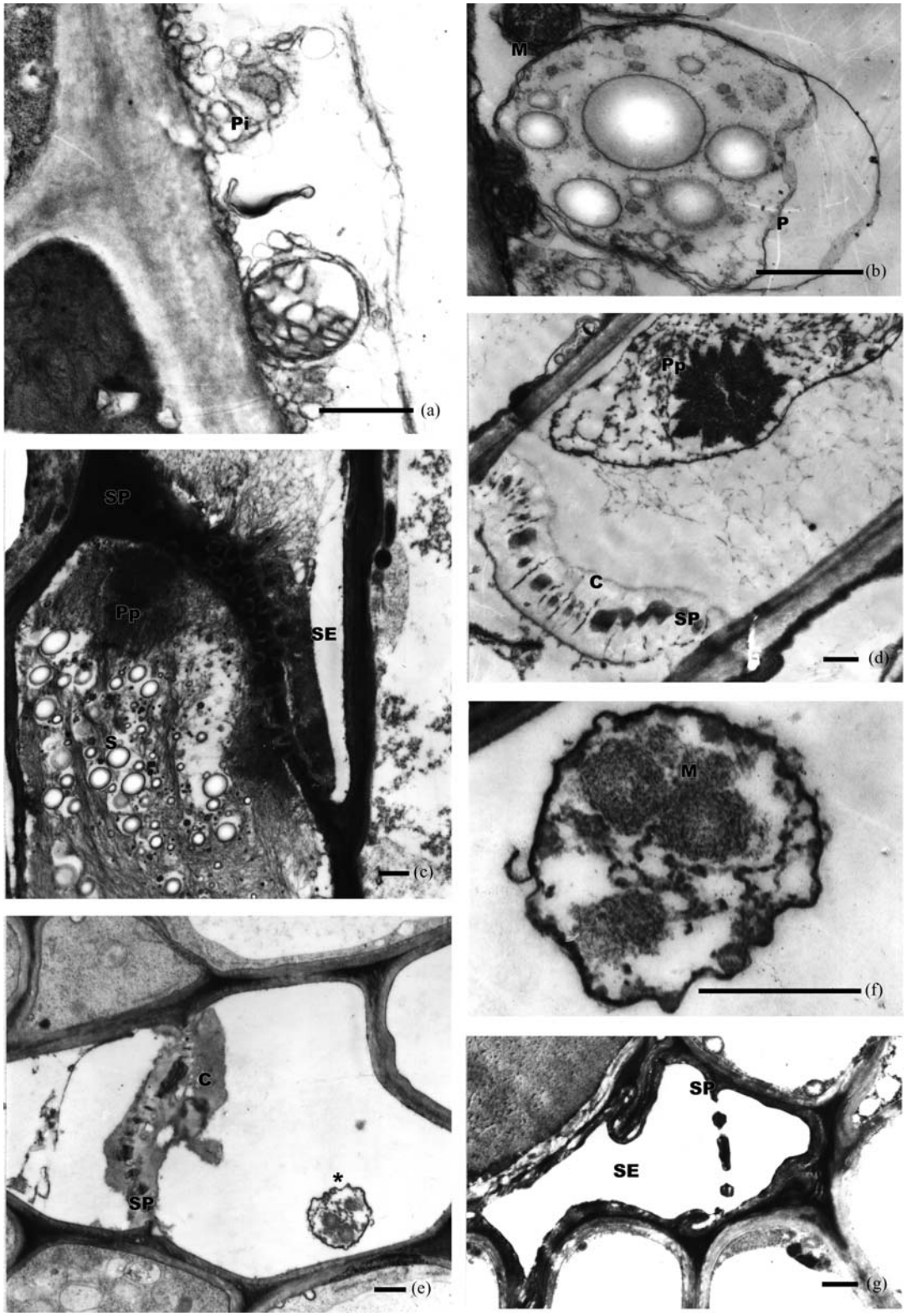

Fig. 3 Ultrastructure of sieve element at the degenerate stage (bar: 1 μm)
(a) The longitudinal section of a sieve element showing a plasma membrane of a disaggregated sieve element (Pi); (b) The cross section of a sieve element showing a disaggregated sieve plastid (P); (c) The longitudinal section of a sieve element showing starch grain from the S-plastid dispersion in the chamber of a sieve element; (d) The longitudinal section of a sieve element showing disaggregated P-protein (Pp) and a callose wall (C) in a sieve plate; (e) The cross section of a sieve element showing a sieve element in its degenerate stage; (f) A five times enlarged picture, showing disorganized mitochondria (M); (g) The cross section of a sieve element without a function and an opened sieve plate (SP).

inner-environment during the process of SE differentiation resulting in rupture in the fixation process and the disintegration of plastids and mitochondria during SE differentiation. Wang and Hu (1993) concluded that many ribosomes that had disappeared had resulted in a retrogression of plastids and mitochondria based on their investigation of the process of microsporogenesis in *Gossypium hirsutium* L.. A similar conclusion was also provided by Ouyang et al. (1998). Our research indicated that the degeneration of the outer membrane was a normal process because most of the disorganization appeared at the degeneration stage of SE. The lumen of the double-layer membrane of plastids in SEs was enlarged and disappeared due to rupture. The starch grains dispersed in the filiform P-proteins of SE. With the functional loss of SE, the starch grains gradually degenerated and in the end disappeared completely.

4.2 Degeneration of the nucleus in SE

One important feature of a SE compared with other types of cells was that it still had some physiological function after the nucleus had disappeared. Wu and Zheng (2003) found that the degenerated nucleus was a kind of typical cell death procedure during the SE of primary phloem differentiation of *Arabidopsis thaliana* root. Walsh and Evert (1975) reported that the nuclear degeneration belonged to a pyknotic pathway. The nucleus did show itself to be a rip-panel before its degeneration and the nuclear membrane folded inward to become tubular. Similar phenomena were found during the nuclear degeneration of the SE in primary phloem in *Zea mays* L. leaves (Ouyang et al., 1998). Besides, Ouyang et al. (1998) found a close relationship among the selective autolysis of protoplast, nuclear degeneration and endoplasmic reticulum activity. Some previous research indicated that the nucleus of SE could be degenerated through chromatolysis. Hoefert (1980) observed this phenomenon during the development of SE in *Thlaspi arvense* L.. In contrast, some higher electronic density substances were formed after nuclear degeneration of SE. Most of the literature attributes this phenomenon to chromatin agglomeration. In our research, we found that the nucleus of SE in *Populus deltoides* had degenerated in two ways. One was based on chromatolysis, similar to that of *Thlaspi arvense* L. (Hoefert, 1980). Chromatin of the nucleus gradually disappeared, but the nuclear membrane remained intact, and the local nuclear membrane began to break up later on. We inferred that the ribosomes entered the nucleus from the broken nuclear membrane or the nuclear pore complex and it might be the same as the electron density between the nucleus and the lumen of cells. Obviously, the degenerated nucleus was well surrounded by P-protein. Another degenerated nucleus was totally different from the above observation. The chromatin of the nucleus did not agglomerate or dissolve but dispersed and formed into homogenous substances with high electron density. Likewise, this kind of degenerated nucleus connected closely with P-protein and shared the same electron density. Furthermore, the nuclear membrane broke so that the karyoplasm spilled over. Consequently, we thought the karyoplasm was transformed into P-protein.

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