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Legumin Accumulation in Endoplasmic Reticulum Cisternae at Early Stage of Seed Development and Protein Body Transformation in Pea Cotyledon Cells

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완두의 종자 발달과정에서 소포체 내강에 대한 저장 단백질 legumin의 축적과 단백과립 변환

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ABSTRACT

Immunoelectron microscopy of storage protein at early stage of seed development showed legumin was firstly accumulated protein in between endoplasmic reticulum (ER) cisternae, and these accumulates were differentiated into protein body (PB) by transformation at later stage. Thin sections of pea cotyledons during the later stages of seed maturation showed three morphologically different types of protein bodies. One of these, presented as rough-surfaced cisternae with terminal dilations, which contained protein deposits and were often found interdigitated between stacks of rough endoplasmic reticulum. Conventional electron microscopy at earlier stages of cotyledon development showed this protein body type initially developed from the rough ER. This transformation of endoplasmic reticulum into a protein body is believed to represent a new pathway of protein body development.

Key words : ER cisternae, Immunoelectron microscopy, Legumin, Protein bodies

INTRODUCTION

Seed storage protein synthesis, intracellular transport and deposition have been of great interest for last two decades, and the subject for numerous biochemical, cytological and immunocytochemical studies (Higgins, 1984; Shotwell & Larkins, 1988; Chrispeels, 1991; Galili et al., 1993; Robinson et al., 1995; Jeong & Park, 2001). All storage proteins at seed maturity are found in protein bodies (PB), and enter into the endomembrane system through cotranslation at the endoplasmic reti-

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culum (Müntz, 1989).

In cereals three different topographies of PB development are known, direct development out of the ER (Larkins & Hurkman, 1978), via a budding process from the protein storage vacuole (PSV, Hinz et al., 1995) and by de novo development during maturation (Hoh et al., 1995; Robinson et al., 1995).

The presence of storage proteins in the ER of maturing cotyledons has been frequently demonstrated by isolating ER fractions and by performing Western blots with appropriate antibodies (Bollini et al., 1982; Chrispeels et al., 1982; Higgins et al., 1983) This was also reinvestigated using the postembedding immunocytochemical labeling technique, which has enabled the direct localization of storage protein in the ER lumen at intermediate to late stage of seed development (Nieden et al., 1984; Craig & Goodchild, 1984; Jeong, 1999). It would seem that the condensation and aggregation of these proteins are late events in the intracellular transport associated with the storage protein maturation.

However, during seed maturation, it has been believed that the development of protein bodies couldn't only be attributed to the direct development from ER and the budding process, because the number of protein bodies were increased during the brief span of development, some 22~25 days after flowering (DAF).

Therefore, it is believed that another unidentified *in situ* pathway to the biogenesis of protein bodies may exist, and the present research was undertaken to investigate this possibility in terms of PB development in the pea system.

MATERIALS AND METHODS

Cotyledons at 21~26 DAF from 7~12 mm in length were removed from a greenhouse-grown pea (*Pisum sativum* var. *excellence*) and processed immediately for conventional electron microscopy. This variety has advantages in terms of microscopy, because it contains fewer starch grains in its cotyledons throughout seed development.

Segments of cotyledon tissue were fixed for 2 hours at room temperature in 2% glutaraldehyde in 50 mM potassium phosphate buffer at pH 6.8, for conventional electron microscopy. After three times of rinse for 20 min with the above potassium phosphate buffer, cotyledons were postfixed for 2 hours at room temperature in 2% osmium tetroxide or postfixed in 2% osmium tetroxide containing 0.8% potassium hexacyanoferrate. Post-fixed tissues were washed with the same buffer and then bloc-stained in 2% aqueous uranyl acetate for 16 hours at 4°C.

Sliced cotyledons were partially digested into single cells in mixed enzyme solutions for proper fixation, which increased the penetration of fixative without changing subcellular structure. After dehydration in an ethanol series, the slices were embedded in London Resin White (Hard Grade, Polyscience) and allowed to polymerize at 56°C for 24 hours.

Thin sections were prepared using a Leichert ultramicrotome with a diamond knife and then double-stained with uranyl acetate followed by lead citrate. Stained sections on 100-mesh nickel grids were observed under a Philips CM 10 transmission electron microscope at 80 KV.

Immunocytochemistry was performed using the method described by Hoh et al. (1995) with slight modification. Primary fixation was carried out in 1.5% paraformaldehyde plus 1% glutaraldehyde in 25 mM sodium cacodylate buffer (pH 7.2) for 2 hours at room temperature. Tissues were then washed in 25 mM cacodylate buffer and placed in a secondary fixative (1% osmium tetroxide, 0.8% potassium hexacyanoferrate in 25 mM of sodium cacodylate buffer) for 2 hours at room temperature. Fixatives were removed by washing with cacodylate buffer and distilled water for 10 minutes each. The tissues were then treated with 0.5% borohydrate solution, and dehydrated in an ethanol series and infiltrated with London Resin White (Hard grade, Poly-

science), which was polymerized at 56°C for 24 hours in an oxygen free atmosphere.

Thin sections were collected on formvar-coated nickel grids and floated on TBS for 30 minutes. Blocking of the nonspecific binding of gold particles was carried out with fresh low-fatted milk (for α-TIP) and with 3% BSA+0.2% BSA-C in TBS. After blocking, the sections were incubated with anti-legumin antibody solution for 1 hour at room temperature and washed with washing solution containing 1% BSA+0.07% BSA-C in TBS. Sections were then exposed to 10 nm gold-conjugated secondary antibody at a dilution of 1 : 30 in washing solution. Grids were then rinsed in distilled water and post-stained in uranyl acetate and lead citrate before being examined under a Philips CM 10 transmission electron microscope.

RESULTS

Thin sections during early development (7~8 mm cotyledons) revealed non-uniformly distributed osmiophilic contents in the rER cisternae (Figs. 1, 2. arrowheads). The peripheral accumulation of the storage protein in PSV occurred actively at this stage (Fig. 1.). The ultrastructural features of luminal widening were identified in some portions of rER without protein accumulations (Fig. 3). It is not clear how these osmiophilic proteinaceous components could have developed into these different types of protein bodies.

In sections of pea cotyledon cells fixed at the active protein accumulation stage in the protein storage vacuole (shortly before the cessation of storage protein synthesis, 23~25 DAF; 11 mm in diameter), aggregates of osmiophilic storage proteins of three different types (designated PB_1, PB_2 and PB_3) were clearly discriminated by their topographies (Fig. 4), but these three types could not be observed concurrently at certain stage of development.

Firstly, there are large spherical vacuoles (at least 2 μm in diameter) containing storage proteins in varying degrees of aggregation or condensation. These structures have been previously described as fragmented PSV, and were designated type PB_1.

Secondly, numerous, smaller protein bodies with denser contents were classified as type PB_2. Judging by their sizes, many of them could have arisen through budding of the PSV, which begins at an early stage of seed development. Some of these protein bodies may have represented cross sections of the cisternal swellings of ER, which are found between the stacks of rough ER cisternae (Fig. 4. arrowheads). This type of protein body, derived from cisternal swellings, was named PB_2.

The cisternal portion of PB_3 stretched linearly or concentrically to the cytoplasm, this concentric spreading of PB_3 encircled PB_2 (Fig. 1. arrowheads, Fig. 4. large arrows). The width of individual areas of PB_3 was three folds than that of the intact rER (Fig. 4).

Post-embedding immunogold labeling showed that the proteinaceous osmiophilic accumulations in the lumen of the ER react positively with anti-legumin antibodies (Fig. 5. arrowheads). Nonspecific labelings were not observed on the protein accumulations in ER cisternae. Actively developing Golgi vesicles at early stage has electron dense inclusions and also specifically gold labeled against anti-legumin antibodies. Only mature vesicles with inclusions show very specific immunolabelings comparing no labelings of the imma-ture vesicles (Fig. 6. arrowheads). A lot of actively developing vesicles from ER show peripheral accumulations of storage proteins at early stage, and these accumulations leads to PB development at intermediate or late stage of seed maturation (Fig. 7).

DISCUSSION

The cotyledons of leguminous seeds have many protein bodies, which are the final sites of protein accumulations. The development of protein bodies (PB) from the protein storage vacuole (PSV) has been attributed to

fragmentation (Craig et al., 1980), division (Oparaka & Harris, 1982), and budding process (Hinz et al., 1995). In particular, these three processes have been considered to be routes to the development of protein bodies in peas.

An alternative way of describing ontogeny of the protein bodies was proposed by Hoh et al. (1995). This suggestion has been supported because of the abrupt increase of protein bodies and active accumulations of the storage proteins to the PSV between 22 and 25 DAF (Robinson et al., 1995; Jeong, 1999). The vacuoles of plant cells are usually regarded as multifunctional organelles, in particular, preexisting vegetative vacuoles are used as sites for storage proteins in pea cotyledons. These vacuoles are believed to be transformed into protein body through a process of fragmentation or subdivision (Robinson et al., 1995), which is considered to be the principal process involved in the formation of protein bodies.

Osmiophilic storage protein aggregates in ribosome-free cisternae have been described in legume cotyledons during the late stages of cotyledon maturation (Craig et al., 1979; Adler & Müntz, 1983; Craig, 1986). The cisternae are frequently found within stacks of rER, and have luminal diameter of several times wider than that of the rER. These cisterna dilate, usually terminally, into protein-filled vacuoles. Vicilin has been shown to be present in such cisternae (Craig & Goodchild, 1984; Craig, 1986; Jeong & Park, 2001).

The present study shows that individual ER cisternae, with ribosomes on their surfaces are filled with osmiophilic substances. Moreover, this osmiophilic material was uniformly distributed in the ER lumen, and it is believed that this protein-filled ER transforms into the protein bodies, which could be considered to be an alternative explanation for protein body development during the short period required for seed maturity in pea cotyledons.

When pea cotyledons were subjected to a cold treatment (4°C) before fixation, these cisternae were shown to have connections to the PSV, and were therefore, interpreted as being part of a highly extensive and ramifying vacuolar system. However, cold treatment is known to produce drastic changes in the plant endomembrane system, and often leads to abnormal membrane fusions and proliferation (Mollenhour et al., 1975). The present investigation resolved this issue by demonstrating that the protein-filled cisternae are indeed of rER origin. Through the continued accumulation of storage proteins in the rER, and possibly their lateral transport into terminal dilations, a hitherto undescribed type of PB was found to be formed in developing pea cotyledons during the later stages of maturation.

Although PB_1 and PB_2 develop directly out of the rER in cereals, PB_3 of the pea differs from the cereal type of PB in at least two respects. Firstly, the surface of the ER-derived PB in cereals is typically covered with ribosomes, even when the storage proteins, prolamins, are transgenically expressed in tobacco leaves (Bagga et al., 1995). As is the case for other PB in pea cotyledons, the membrane of PB_3 does not bear ribosomes after completion of PB development. However, it is interesting to note that when storage proteins begin to aggregate in the lumen of rER cisternae, the surface of ER where protein aggregates were found without ribosomes.

Secondly several investigations have suggested an important role for the binding protein, Bip, in the biogenesis of PB in cereals (Boston et al., 1991; Zhang & Boston, 1992; Li et al., 1993). Although certain amino acid sequences are required for their supra-molecular assembly (Geli et al., 1994), it is believed that Bip facilitates the folding of the prolamin storage polypeptides, thereby enabling them to aggregate in the lumen of the ER. However, Bip was found to be immunocytochemically localized on the luminal surface of storage protein deposits in pea cotyledons at early to intermediate stage of development (Robinson et al., 1995), but Bip was not localized on the transformed PB at late stage in this investigation.

Otherwise, in this study, the specific labelings of anti

-legumin antibodies to the protein accumulates in ER cisternae and to the secretory vesicles of Golgi complex at early stage could be interpreted that the legumin accumulation occur prior to the other protein accumulation in pea cotyledons.

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＜국문초록＞

완두 종자 발달의 이른 시기에 특징적으로 조면 소포체 내강에 단백질이 축적되는데, 이 단백질에 대한 전자현미경적 면역세포 화학적 반응을 실시한 결과 legumin으로 확인 되었다. 이 단백질은 소포체 내강에 점점 축적되고 소포체 끝이 부풀어서 단백과립으로 발달하였다. 완두의 단백과립 발달 과정은 3가지 유형이 확인 되었는데, 단백질 저장 액포의 분절에 의해서 형성된 제 1 형 단백과립, 가장자리에 단백질이 축적된 단백질 저장 액포의 budding에 의해서 형성된 제 2 형 단백과립, 그리고 단백질 저장 소포체의 끝이 부풀어서 형성된 제 3 형 단백과립으로 구분되었다. 제 3 형 단백과립은 수정 후 23～25일 사이의 짧은 기간에 급격하게 발달되어 자엽세포를 가득차게 만드는 것으로 확인 되었으며, 이러한 유형은 지금까지 알려지지 않은 새로운 단백과립 발달 과정으로 생각된다.

FIGURE LEGENDS

Fig. 1. Thin sections of 8 mm pea cotyledon cells showing a developing PSV (protein storage vacuole) with peripheral accumulation of storage protein. The arrowheads point to a PB_3 precursor and to osmiophilic deposits in the rER cisternae. bar = 2 μm

Fig. 2. The initiation of protein accumulation (arrowheads) in the rER cisternae was occurred at early stage of seed development in pea cotyledon cell. bar = 0.1 μm

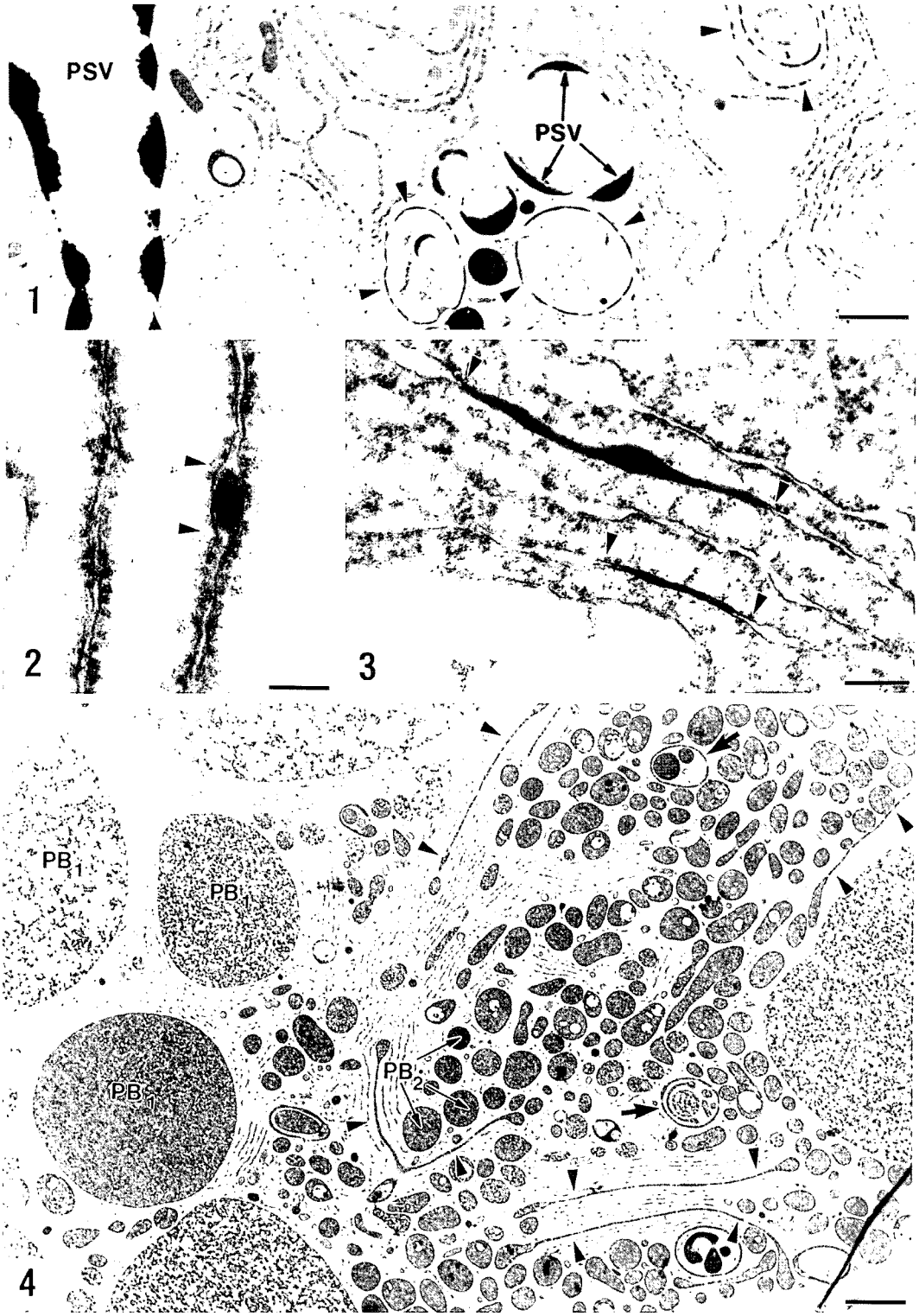
Fig. 3. Storage protein accumulations in between rER cisternae (arrowheads) at intermediate development of pea seed are clearly observed . bar = 0.2 μm

Fig. 4. Electronmicrograph of a thin section through a storage parenchyma cell in a 11 mm diameter pea cotyledon. Three, morphologically distinct, types of protein bodies (PB) are recognizable : two spherical protein bodies PB_1, and PB_2 and a third, PB_3 (indicated by arrowheads) which is basically cisternal in character but has the terminal swellings of prominent ER. bar = 4 μm

Fig. 5. Osmiophilic deposits in rER have specific immunocytochemical labelings (arrowheads) with anti-legumin antibodies. These deposits were observed in cells of 8 mm sized seed. bar = 0.2 μm

Fig. 6. Secretory vesicles of Golgi complex were immunocytochemically labeled with anti-legumin antibodies. These labelings indicate that the legumin was accumulated at early stage of seed development. Arrowheads indicate immature Golgi vesicles without labelings. bar = 0.1 μm

Fig. 7. Peripheral accumulations (arrows) of storage protein in vesicles derived from ER. These accumulations may lead to ontogeny of protein bodies during brief span of seed development. PSV:Protein Storage Vauole. bar = 2 μm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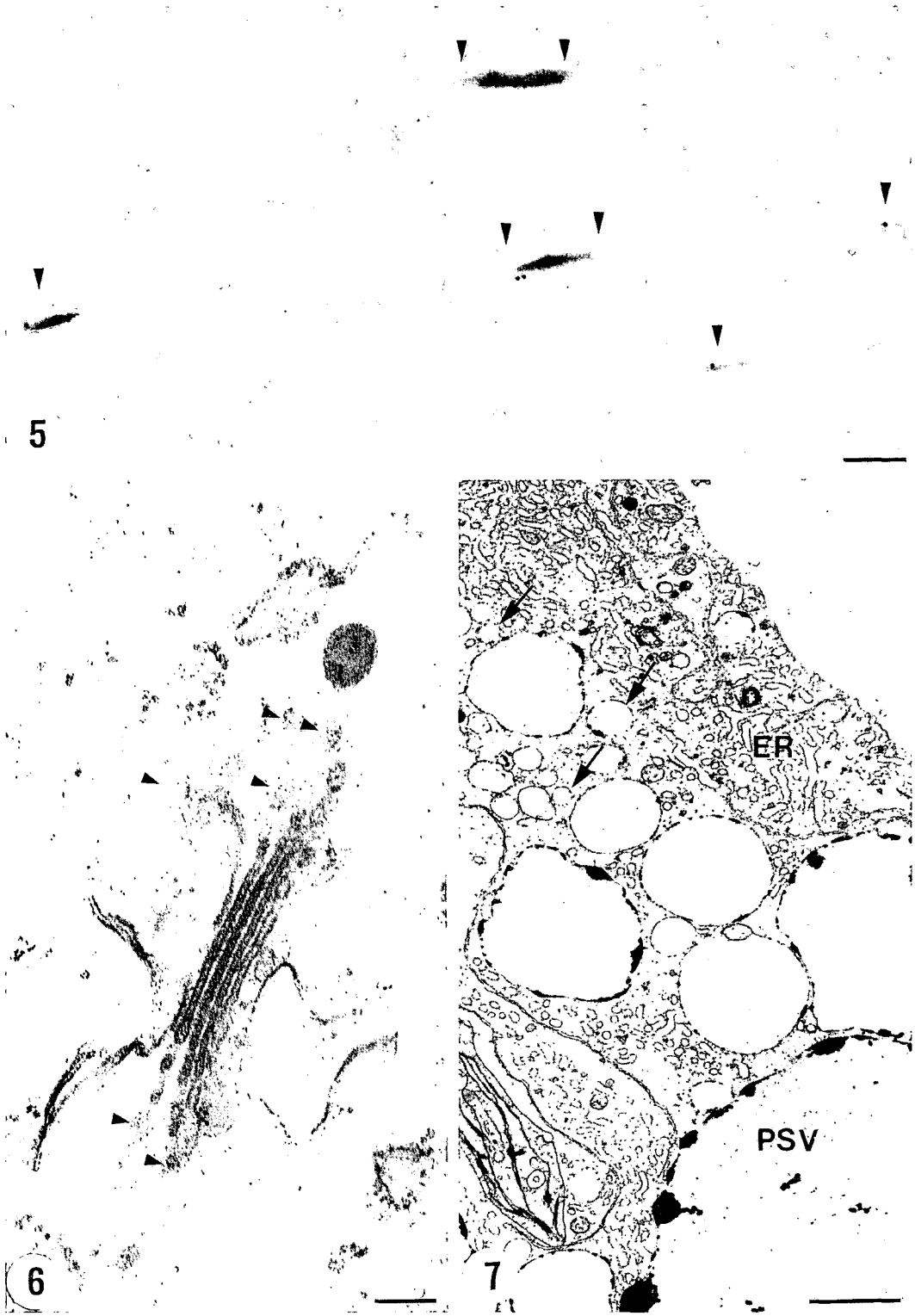
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