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Immunocytochemical Investigation on the Intracisternal Accumulations of Storage Protein in Pea Cotyledon Cells

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완두 자엽세포의 소포체 내강에 축적된 저장 단백질에 대한 면역세포화학적 연구

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ABSTRACT

In 1980s, the fragmentation or subdivision of protein deposits at the periphery of protein storage vacuole was suggested as the only route of PB development in pea cotyledon cells. Since then, other independant processes such as terminal dilation, transformation and de novo development have been discussed as alternative routes for PB development, and today, these multiple mechanisms of PB development are accepted as a result of active investigations. For analysis of the protein accumulations in the ER cisternae during seed development, immunocytochemical gold labellings were applyed on the single cells separated by enzymatic digestion from cotyledon tissue. Anti-legumin labellings at the early stage, and anti-vicilin labellings at the intermediate stage were observed on the protein-filled ER. The α-Tip, which is the ER retention protein, was labelled somewhat at late stage, and PPase, a sort of tonoplast membrane protein, was labelled at early stage.

Key words : α-Tip, Immunocytochemistry, Legumin, Pea, PPase, Protein-filled ER, Vicilin

INTRODUCTION

The synthesis and accumulations of storage proteins during seed maturation of peas have been of great interest for last two decades because of the cytological and immunocytochemical importances of the intracellular transport (Higgins, 1984; Shotwell and Larkins, 1988; Chrispeels, 1991; Galili et al., 1993; Jeong, 1998).

The developmental topographies of protein bodies (PB) are known to be dynamic. It has been considered

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that the protein bodies are developed from protein deposits in protein storage vacuole (PSV) by subdivision and fragmentation. The other pathways of PB development, terminal dialation of protein-filled ER cisternae and peripheral deposition of protein to the ER-derived vacuoles, have been suggested by several publications (Larkins and Hurkman, 1978; Johnson et al., 1989; Hinz et al., 1995; Hoh et al., 1995; Jeong, 1999).

Biochemical and ultrastructural studies of the developing legume seeds demonstrate that the synthesis of storage protein could be detected before protein body formation. And most of the surrounding membranes of the protein bodies are formed from the ER membrane during the early stage of seed development (Marty et al., 1995; Robinson et al., 1995; Robinson and Hinz, 1996).

Principal components of the storage proteins are legumin, vicilin and albumin, comprising 99% of the total storage proteins in peas (Münz, 1989). But the minor components such as Bip, α-Tip and Ppase play a more important function than the storage proteins themselves because they contain targetting sequences in their polypeptides (Chrispeels and Raikhel, 1992; Altschule et al., 1993; Jeong, 1998).

However, sequences of the deposition or the accumulation of proteins to the protein bodies need more investigation in spite of the existing of many publications. Therefore, sequential accumulations of storage proteins to the protein storage organelles such as PSV, ER cisternae and protein bodies will be immunocytochemically investigated. For this purpose we applied immunogold electron microscopy to the developing pea cotyledon tissues using the post embedding techniques.

MATERIALS AND METHODS

Developing pea (*Pisum sativum* var. excellence) seeds of early (16-18 DAF, Days Afetr Flowering), intermediate stage (19-23 DAF), and late stage (24-27 DAF) were removed from greenhouse grown plants. After the removal of the seed coat and radicle, tissue slices were made by using a razor blade. Single cells were immunofixed as it has been previously published (Jeong, 1999).

Thin sections, on the formvar coated nickel grids (hexagonal 100 mesh), were floated on tris buffered saline for 30 minutes for equal blocking of the binding of antibodies. Blocking solutions were applied to minimize the non-specific binding of primary antibody for 30 minutes at room temperature. Fresh low-fat milk for the α-Tip, 3% (w/v) BSA plus 2% (w/v) BSA-C in TBS for Bip, Legumin, Vicilin and PPase were used. After blocking, the sections were incubated with diluted primary antibody for 1 hour in a humid chamber. Primary antibodies were diluted 1 : 200 to 1 : 500 with BSA and BSA-C or milk containing leaf extract. Unbinding antibodies on the surface of the sections were washed out with washing solution that contained 1.5% BSA plus 0.07% BSA-C in TBS (4 × 5 minutes) and were exposed to 10 nanometer gold-conjugated secondary antibody. Then the grids were washed again in the same washing solution as just mentioned and were subsequently put in triple distilled water (2 × 5 minutes each). After washing, antigene-antibody binding was stabilized and fixed in 1% glutaraldehyde for 10 min. Post staining in uranyl acetate and lead citrate after washing in DW were carried out for precise observation with CM 10 transmission electron microscope at 80 kV. Uranyl acetate and lead citrate staining were not carried out in some sections in order to enhance the observation of the gold particles.

RESULTS

Because of a burst during agitation and centrifugation, final recovery of the protoplasts were reduced. For this reason, we used the enzymatic maceration method of the sliced tissue to obtain single cells (Jeong, 1999). This method was appropriate for immunofixation, because the concentration of fixative is minimized for the preservation of the ultrastructure and antigenecity of the cotyledon cells, which improves the quantity of immu-

nolabellings.

Thin sections of the single cells embedded in LR White were immunolabelled with gold-conjugated secondary antibody. But without incubation to the primary antibody, they were found to be almost free of gold particles (data not shown).

Dense immunogold labellings with gold-conjugated antibodies were observed for protein accumulations and protein bodies in all sections of this experiment. The strand of protein accumulation in the ER was labelled by anti-legumin antibody-gold conjugates at the early stage of seed development (Fig. 1 arrows), whereas protein deposits (PD) at the periphery of protein storage vacuole (PSV) were free of immunolabelled gold particles (Fig. 1).

Specific labellings of anti-legumin antibody on the cytoplasmic protein body and ER-derived protein accumulations were observed at the early stage of seed development (Figs. 2-5). However, ER cisternae without protein accumulations showed no gold particles (Fig. 5. arrow).

Immunogold detection of vicilin in the intracisternal compartment of pea cotyledon cells at the intermediate stage was very specific (Fig. 3). Protein bodies, which were formed by terminal dilation of the protein-filled ER, showed gold conjugated anti-vicilin antibody labellings (Fig. 3. arrowheads). The gold-conjugated secondary antibody with legumin and vicilin were labelled on the protein accumulations in the intracisternal compartments of the ER (Figs. 1-3, 5).

Immunogold localization of the α-Tip antibody at intermediate to somewhat late stage of seed development was very clearly visible in the cisternal compartment (Fig. 4 arrowheads), and in the cytoplasmic protein body (Fig. 6). These labellings were not observed at the early stage of seed development.

Gold particles which were conjugated with secondary antibody of PPase, on the membrane of the protein body derived from the protein-filled ER (Fig. 7), were very closely observed. However, the protein deposit at the periphery of PSV showed no labellings of PPase antibody (Fig. 7).

DISCUSSION

Numerous fine structural investigations on the development of the protein storage vacuole from the vegetative vacuole (VV) at early stage of seed maturation have been well carried out in pea cotyledon cells. After the ontogeny of PSV, synthesis and accumulation of the storage protein have been started at the early stage of seed maturation (Hoh et al., 1995).

Surrounding membrane of the protein body is supposed to be derived from the vegetative vacuole in some plant species (Rubin et al., 1992). However there has been no strong evidence for this observation because these vacuoles are filled with storage proteins by fusion of transport vesicles from the ER or Golgi complex (Altschule et al., 1993). But, by fusion of the ER/Golgi vesicles with VV, the VV transforms into PSV in which the storage proteins accumulate. Therefore this discussion needs further investigation because of the chemical differences between VV and PSV (Oberbeck et al., 1994).

Identification of VV and PSV in this investigation was done by immunogold labellings of the α-Tip antibody to the protein bodies. Specific labellings of the α-Tip antibody-conjugated gold particles on the protein accumulations and protein bodies indicate that these strands and the PB were derived from the ER. This discussion was also suggested by Hoh et al. (1995), Marty-Mazars et al. (1995) and Jeong (1999).

The development of PB from PSV by the process of fragmentation or subdivision of the protein lumps after peripheral budding has been considered as the only route of PB formation since it was first suggested (Craig et al., 1980). However, as a result of active investigations on the PB development, several other ontogenies of protein bodies have been presented in legume seeds (Hinz et al., 1995; Hoh et al., 1995; Robinson et al., 1995). Especi-

ally, the multiple mechanism of protein body development was suggested by Robinson and Hinz (1996). They concluded that more than three modes of protein body formation can be observed during seed development; each of these topologies have been periodically operated *in vivo*.

Globulins (legumin and vicilin) and albumins are two principal storage proteins in pea seeds and these two proteins have also been focused by biochemical and cytological studies because they constitute 99% of the total storage proteins (Hinz et al., 1995). Especially legumin and vicilin synthesis and accumulation in the developing legume seeds have been studied with precision.

The rate of vicilin synthesis is higher than that of legumin during the early stage of seed development. And It was known that the legumin synthesis increases rapidly while the vicilin accumulation seems to decrease at its intermediate stage (Higgins, 1984; Hinz et al., 1995).

We observed immunocytochemical gold labellings of the legumin in the protein filled ER at the early stage, whereas the vicilin labellings were specifically observed at the intermediate stage. Because of the specificity of the immunogold labellings of legumin and vicilin on the accumulation in the protein-filled ER, legumin is thought to have accumulated earlier than vicilin. But further investigation was needed because this result was different from that of the prior investigations (Higgins, 1984; Hinz et al., 1995). The priorities of vicilin biosynthesis in their studies were not investigated immunocytochemically in the protein-filled ER but investigated biochemically in the whole seed.

In this investigation, legumin accumulation in the protein-filled ER was thought to have priority over the vicilin accumulation at the early stage in pea seed development. And it was confirmed by the specific labellings of the vicilin antibody in the protein-filled ER at the intermediate stage (Jeong, 1998).

Melory and Herman (1991) have shown that the expression of the α-Tip in the PSV of soybean cotyledon is not corelated with the presence or concentration of storage protein. The α-Tip was known to be expressed in seed tissue during whole stage of seed development (Höfte et al., 1992).

We have been able to confirm the presence of the α-Tip and PPase in this study. Antibodies prepared against the α-Tip labelled protein-filled ER (Fig. 4) as it did against the cytoplasmic protein body (Fig. 6). High density gold particles conjugating with the secondary antibody of PPase was very closely observed on the membrane of the ER-derived PB (Fig. 7).

PPase polypeptides were known to be present in the membrane of protein bodies in pea cotyledons, which was shown by Western blotting by immunogold labellings (Robinson et al., 1996). It was suggested that PPase is present in the PSV at the early stage, and that the α-Tip is present somewhat at a later stage of cotyledon development.

Although, Maeshima et al. (1994) did not find any PPase labellings in the protein bodies of pumpkin seed, we confirmed that PPase, one of the typical enzymes in tonoplast, was present on the PSV in developing pea cotyledons at the early stage. Especially, protein bodies derived from the protein-filled ER showed strong labellings of this enzyme. This result indicated that the PPase and V-ATPase are synthesized de novo and become inserted into the membrane of the PSV at the early stage of seed development. However it is thought that a precise investigation is necessary to reclarify the differences between pea and pumpkin seeds.

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<국문초록>

완두 자엽세포에 대한 저장단백질 과립의 발달은 단백질 저장 액포의 가장자리에 축적된 단백질의 fragmentation에 의해서 이루어지는 것으로 알려져 왔다. 그러나 최근 이 외에도 terminal dilation, transformation, de novo development 등의 독립적인 과정이 각각 다른 시기에 관찰되므로서 단백과립의 발달은 이러한 여러 과정이 모두 나타나는 복합과정임이 알려졌다. 이러한 과정과는 별도로 종자발달의 이른시기에 소포체 내강에 축적되는 저장단백질과 여기서 발달하는 단백과립에 대하여 규명하고자 발달중인 종자의 자엽으로부터 단일 세포를 얻어서 면역세포 화학적 반응을 실시하였다. 그 결과 종자발달의 이른 시기에는 legumin이, 중간시기 이후에는 vicilin이 축적되므로서 단백질이 축적된 소포체가 단백과립으로 발달하는 것으로 나타났다. 소포체 내강에 존재하는 단백질인 α-Tip은 비교적 늦은시기에, tonoplast membrane protein인 PPase는 이른시기에 각각 면역세포화학적 반응이 관찰되었다.

FIGURE LEGENDS

Fig. 1. Immunoelectron micrograph of anti-legumin antibody labellings on the protein accumulations (arrows) in the ER cisternae show gold particles in comparison with no labellings on the protein deposits (PD) at the periphery of protein storage vacuole (PSV). M: Mitochondria, VV: Vegetative Vacuole

Fig. 2. Immunogold labellings with anti-legumin antibody on the cytoplasmic protein body and protein accumulation in the ER (arrows) show very strong labellings.

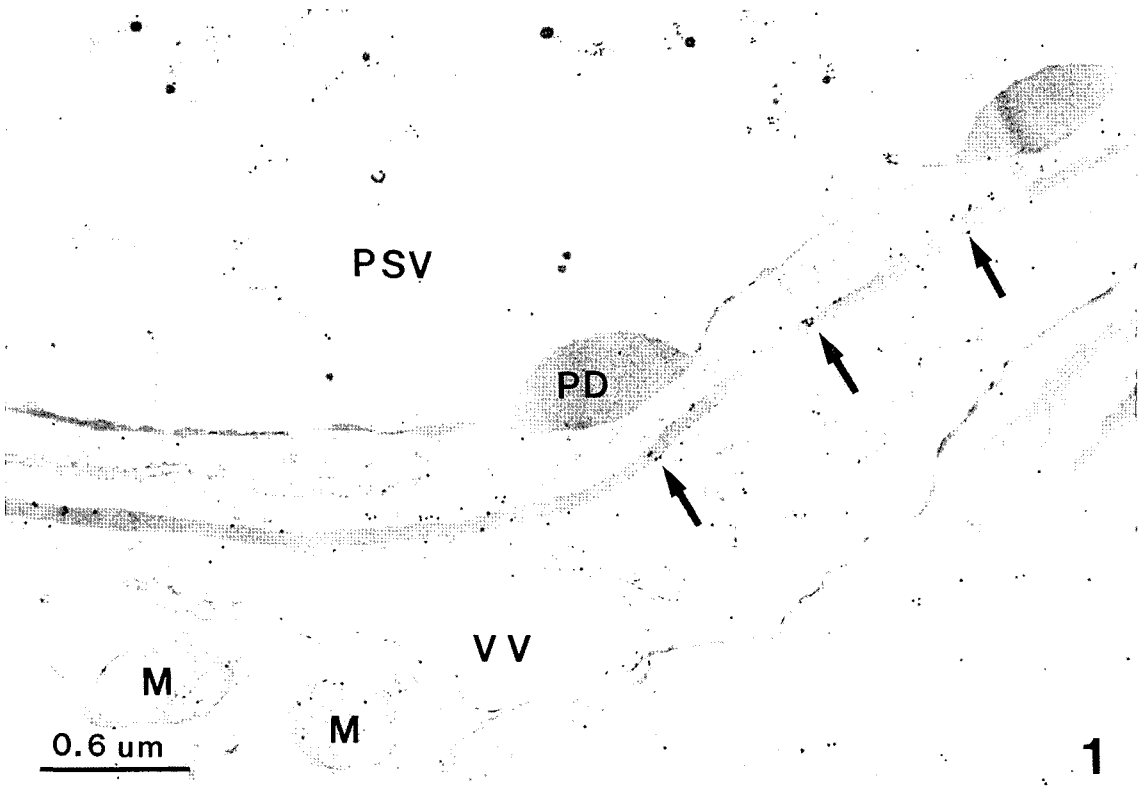
Fig. 3. Protein-filled ER and terminal dialtions (arrowheads) are labelled with anti-vicilin antibody at intermediate stage of seed development.

Fig. 4. Protein-filled ER shows positive labellings (arrowheads) with anti-α-Tip antibody. In order to enhance the labelling density, this tissue was not post-fixed with osmium tetroxide.

Fig. 5. Immunogold labellings of protein-filled ER (arrowheads) with anti-legumine antibody are very specific at early stage of cotyledon development. Immunogold labellings were not observed in the ER (arrow) without protein accumulations.

Fig. 6. Remarkable and homogenous labellings of the α-Tip antibody on the protein bodies derived from protein-filled ER comparing peripheral labellings of PPase in fig. 7.

Fig. 7. Immunogold labellings with PPase at the periphery of the protein body derived from protein-filled ER at early stage of development. Note the absence of gold labellings on the protein deposit (PD) in the protein storage vacuole.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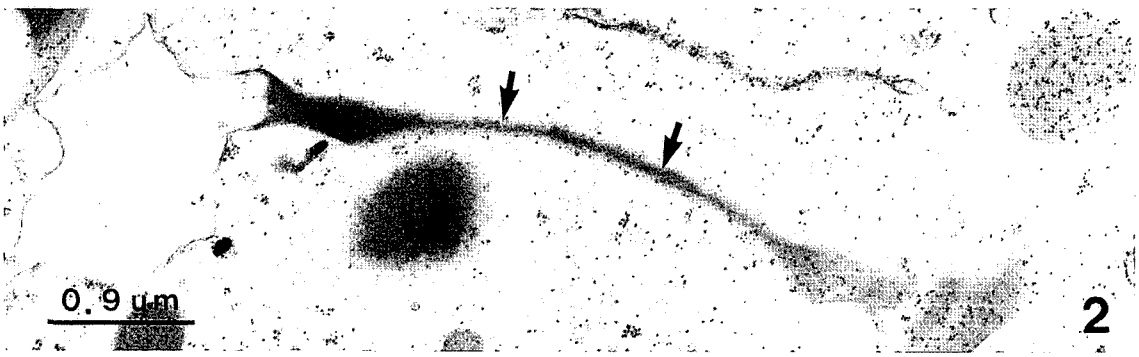
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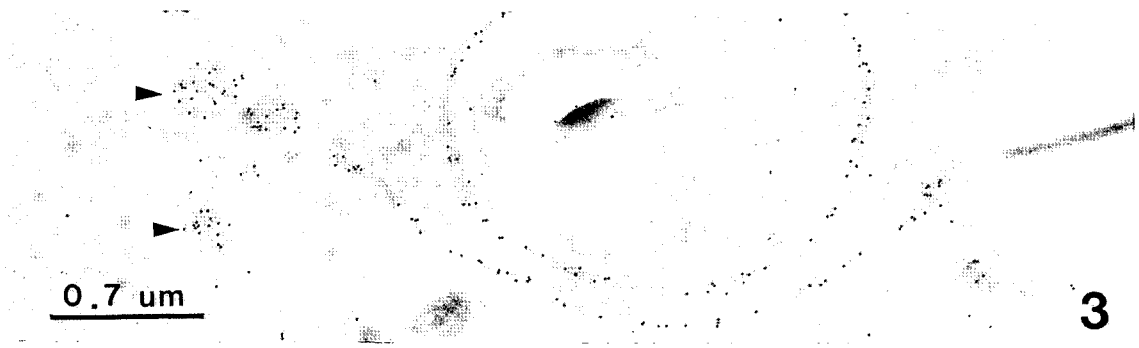
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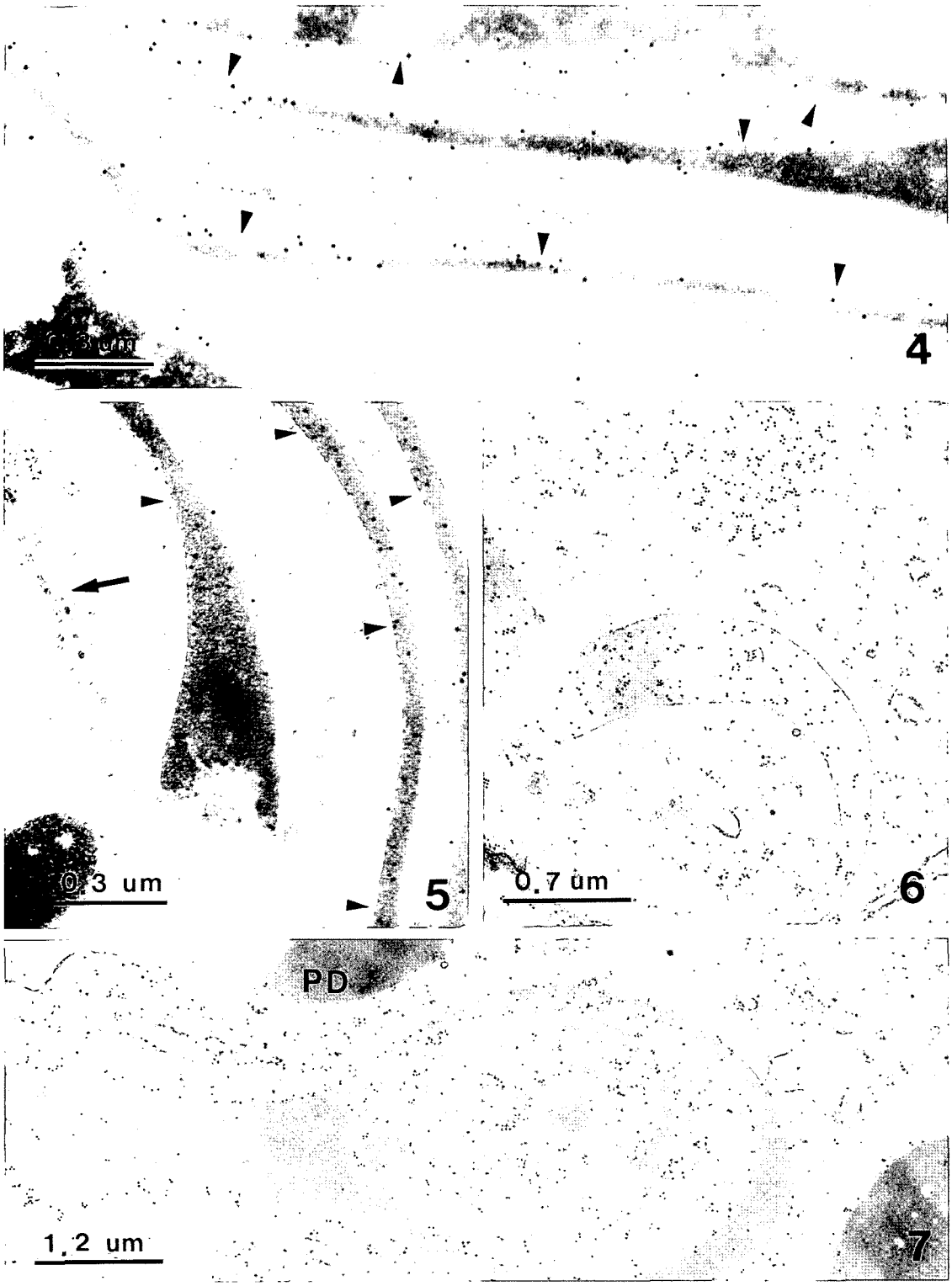
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