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Terminal Dilation and Transformation of the Protein-filled ER to Form Protein Bodies in Pea (*Pisum sativum* L. var. *exzellenz*) Cotyledons

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완두 자엽에서 소포체 말단의 팽창에 의한 단백과립 발달

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ABSTRACT

Accumulations of the storage proteins in protein storage vacuole and the differentiation of protein bodies from protein-filled ER in developing pea cotyledons have been investigated using conventional and immunoelectron microscopy. To improve the fixation quality, single cells separated enzymatically from sliced cotyledons were used. At early stages of seed development osmiophilic protein accumulates in rER lumen were observed quite often. This protein-filled ER cisternae were differentiated into cytoplasmic protein bodies at late stage by the process called terminal dilations which have been considered a principal route of the formation of cytoplasmic protein bodies somewhat later in seed maturation. Immunocytochemical labellings of the vicilin and legumin show that presence of vicilin on both of the cytoplasmic PB and PD, but limited presence of legumin only on the cytoplasmic PB at intermediate stage of seed development. Immunogold labellings of Bip, ER retention protein, were observed on the inner periphery of protein deposits in protein storage vacuole. This result was regarded that Bip can recognize and retrieve misfolded protein during active accumulation of storage protein to the PD in PSV.

Key words : Pea, Storage protein, Protein-filled ER, Dilation, Immunogold labellings

INTRODUCTION

Leguminous seeds have been received great deal of attention on the considerable agricultural importance, because they provide storage materials together with cereals.

The major proportion of the accumulated reserves in

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legume seed is storage protein in the membrane-bounded protein bodies or aggregates derived from them (Murray, 1984).

These storage proteins can be used as nutrients during seed germination or at early stages of the growth of seedlings.

The accumulating process of storage proteins in developing legume seeds has been investigated by many authors with electron microscopy (Bewley & Black, 1978; Craig et al., 1979; Craig, 1988; Hinz et al., 1995; Jeong, 1997). Principal components of the storage proteins in legume seeds are globulins and albumins. These two proteins, which have been focused a lot by biochemical and cytological studies, comprise 99% of total storage proteins in pea seeds (Münz, 1989).

During seed maturation these proteins are formed in membrane-bound structures termed either protein bodies (PB, Pernollet, 1978) or protein storage vacuoles (PSV, Johnson et al., 1989). The differentiation of PSV in the preexisting vegetative vacuoles (VV) was investigated in numerous publications (Hinz et al., 1995; Robinson et al., 1995; Wink, 1993). The PSV are then believed to be transformed into PB by fragmentations or subdivisions (Robinson et al., 1995). Another development of PB in cereals was identified through the direct differentiation from the ER by the process of terminal dilation and vesiculation (Oparka & Harris, 1982; Kim et al., 1988).

Subsequent development on the PB during active accumulation of storage protein was known to be dual origin (Neumann & Weber, 1978; Adler & Münz, 1983). Additional development of PB by subdivision from PB and de novo development were suggested by Robinson et al. (1995) and Hoh et al. (1995). Diverse concepts of PB development in pea cotyledons have not yet been satisfactorily reviewed. Robinson and Hinz (1996) discussed that PB formation could not be explained by a few specific concepts but by multiple mechanisms.

This paper suggests that whether protein-filled ER at early stage of seed development can be differentiated into cytoplasmic PB by terminal dilation at late stage or not. And the accumulations of proteins to the small vesicles derived from ER at early stage would be another type of PB which are developed independently with PB from PSV would be suggested. For this purpose single cells were enzymatically separated from sliced pea cotyledons at diverse stages of growing pea and immunogold labellings were applied.

MATERIALS AND METHODS

1. Plant Materials

Because of the great amounts of starch grains in the parenchyma of pea cotyledons, sectioning of tissue specimens for electron microscopy has much troubles. To minimize this inappropriate preparations, specified variety of pea which contains reduced amounts of starch grains was recommended for ultrastructural investigations. *Pisum sativum* L. var. *exzellenz*, which has limited amount of starch grains, was used for this experiment.

Hydroponically growing pea in a greenhouse (22 ± 1°C, RH = 80%, 12,000 lux) was exclusively used for conventional electron microscopy and immunocytochemical gold labellings

2. Fixation of Cotyledon Tissue

Seeds at early (nominally 14-18 DAF : Days After Flowering), inetrmediate (19-23 DAF) and late (24-31 DAF) developmental stages were picked from greenhouse-grown pea. Fixation procedure was adopted as previous publication (Jeong, 1997) without changes.

3. Fixation of Single Cells for Conventional and Immuno Electron Microscopy

After removal of testa and radicles from pea seeds, 1.5 mm segments of sliced cotyledons were palced into macerating medium containing 0.1% (w/v) macerozyme R-200 (Yakult Honshu, Japan), 0.5% (w/v), pectolyase Y-23 (Seishin, Japan), 1 mM $CaCl_2$, 10 mM sodium ascorbate, 2% (w/v) BSA, 0.7 M mannitol to adjust 800

mOsm of final osmotic pressure. These sliced segments were incubated for 1~1.5 hr at 28~30°C without shaking. After enzymatic maceration, individual cells were carefully collected and washed with washing medium containing 1 mM $CaCl_2$, 10 mM sodium ascorbate, 2% (w/v) BSA, 0.9 M mannitol for 800 mOsm of osmotic pressure.

Washed single cells were fixed in primary fixative containing 2% (w/v) glutaraldehyde, 0.5 M mannitol, 25 mM sodium cacodylate buffer (osmotic pressure of 800 mOsm, pH 7.2) for 2 hrs at 4°C. After primary fixation, 0.5 M mannitol-containing cacodylate buffer was used for washing. Secondary fixation was carried out in several changes (5 min, 10 min, 20 min) of fixative containing 2% (w/v) osmium tetroxide, 0.8% (w/v) potassium hexacyanoferrate ($K_4Fe(CN)_6$) in 50 mM cacodylate buffer at pH 7.0 before exposing to final fixative for 1 hr at room temperature.

After washing in distilled water for 10 minutes of three times, single cells were treated with an 0.5% aqueous borohydride solutions for 30 minutes to get rid of osmication, and washed again in distilled water for 10 minutes (3 times) before dehydration. After dehydration in ethanol series at room temperature, embeddment in LR White (polyscience, Hard Grade) and polymerization were carried out at 52°C for 18 hrs in the absence of air. Ultrathin sections were made using Leichert ultramicrotome (Ultracut E) and collected on formvar coated nickel grids (100 mesh). Sections were observed in a Philips CM 10 electron Microscope operating at 80 kV.

4. Immunogold Labelling

Ultrathin sections on the grid were floated on Tris-buffered saline (TBS) for 30 minutes at room temperature before blocking. Fresh low-fat milk (for α-Tip) and blocking solution containing 3% (w/v) BSA, 2% (w/v) BSA-C in TBS (for legumine, viciline and Bip) were used as blocking solution to block non-specific binding of antibodies. And then the sections were incubated on primary antibody solutions (diluted 1:200 to 1 : 500 with BSA+BSA-C for legumine and Bip, with leaf extreact+BSA+BSA-C for vicilin, leaf extreact+fresh low-fat milk for α-Tip) for 1 hour at room temperature in a moist chamber. After incubation, the sections were washed in washing solutions containing 1% (w/v) BSA, 0.07% (w/v) BSA-C in TBS for 5 minutes (3 times), and immunogold labelled with 10 nm gold-conjugated secondary antibody (Biocell, Cardiff, England). The sections were then washed in washing solutions for 5 minutes (3 times) and also washed in distilled water for 5 minutes (2 times). Sections were stained in aqueous uranyl acetate and lead citrate. Observations and photography were undertaken with Phillips CM 10 transmission electron microscope at 80 kV.

RESULTS AND DISCUSSION

Ultrathin sections of sliced cotyledon tissue at early stage of development have osmiophilic storage protein aggregates at the periphery of protein storage vacuole (PSV) compairing compressed vegetative vacuole (VV) (Fig. 1). These protein deposits shows different types of repository, i.e. some of them are separated each other and the others are attatched together to show several peaks. The presence of two vacuolar system (VV and PSV) in developing pea cotyledon at early stage was discussed as "replacement of VV into PSV" (Robinson et al., 1995). The observations of both vacuoles lying adjacent are clear in the same cell of pea cotyledon. This result might be the evidence in favor of two vacuolar hypothesis (Hoh et al., 1995). Immunocytochemical labellings on the γ-Tip are recognized only the VV, whereas on the α-Tip are restricted to the protein storage vacuoles. At intermediate stage of seed development, one can see spreadings of PD at the periphery of PSV (Fig. 2).

Electron dense proteins are actively accumulating from the periphery of small sized PB in the cytoplasm at

this stage. Protein-filled ER strands (arrow in Fig. 2) encircled the cytoplasmic PB. These findings could not be observed at early and late stage of seed development.

Positive labellings of anti-Bip polyclonal antibody on the PSV by immunocytochemistry revealed that the PSV was originated from ER (Boston et al., 1991; Fontes et al., 1991; D'Amico et al., 1992; Pedrazzini et al., 1994). Since antibiotics treatment on plant cells can prevent core glycosylation of glycoprotein (Denecke et al., 1991), retention of storage protein in the ER after antibiotics treatment was considered a inducible incident.

Fixation of seed for electron microscopy has some troubles because of the storage materials such as starch, lipid and proteins as well as phytin globoids and crystalls (Mollenhauer and Totten, 1971; Kim, 1984; Craig, 1986; Jeong, 1997). To minimize this trouble and to improve fixation quality single cells from cotyledon tissue were released by enzymatic maceration. This ptotocol overcome nearly all of the troubles during fixation and preparation of samples (Jeong, 1997).

Putative storage protein (Figs. 3-4, arrows) in the rough ER lumen was characteristically observed at early stage of seed development. Specifically ribosomes are not attatched on this sector of the ER where osmiophilic storage proteins are retained. As the seed maturation proceeds, the accumulations of the protein in the ER lumen are observed quite often between rER stacks (Fig. 5). The width of protein-filled ER is twice or three times larger than rER cisternae without osmiophilic accumulations in the lumen.

Dramatic swellings (Fig. 5. arrowheads, Fig. 6. arrows) at the terminus of protein-filled ER were considered the next step of PB differentiation.

These swellings contain electron dense materials and the electron density of these materials increased at late stage of seed maturation (Fig. 7). It has been investigated that the outgrowth of protein-filled ER was to be differentiated into cytoplasmic protein bodies (Hoh et al., 1995).

The osmiophilic aggregates in the rER lumen was confirmed as storage proteins, but it is not clear that these proteins will be remained in ER lumen until PSV differentiation or transported out of the ER to the other protein bodies. The retention time of legumin and vicilin in the ER under normal growth condition was known to be several hours in plants (Chrispeels, 1982; Bollini et al., 1982) and less than 30 minutes in animals (Braakmann et al., 1991) except some examples of longer periods (Lodish et al., 1987). These protein-filled ER in maturing pea cotyledons was already observed by Craig (1986), but these findings were obtained under cold stress. The terminal-and-active dialtions of protein-filled ER at late stage might be the principal development of protein bodies in pea cotyledons. Especially this transformation of PB could not be observed at the other stage of seed maturation.

ER vesicles are known to be the recipients of storage proteins in cereals (Hilling & Ameluxen, 1985; Coraggio et al., 1988; Rosenberg et al., 1993). At early stage (17 DAF) a lot of small vesicles, derived from ER, retaining peripheral small osmiophilic aggregates compairing large protein deposits in PSV (Figs. 8, 9). These vesicular depositions were resulted independently with protein deposits in PSV (Fig. 9). These vesicles with osmiophilic accumulations would be transformed into PB, but this transformation will be descussed in later publication.

Immunolocalization of the legumin antibody show very specific labellings only on the cytoplasmic PB but no labellings on the protein deposits in PSV (Fig. 10) compairing peripheral labellings on the protein deposits and strong labellings on the cytoplasmic protein bodies (Figs. 11, 13). And α-Tip antibody localization on the PB are specific at the margin of the cytoplasmic protein bodies (Fig. 12). The localization of Bip antibody on the protein deposits in PSV show inner-peripheral labellings. Postembedding immunogold labellings against α-Tip would be used to identify VV and PSV (Robinson et al., 1995). The positive labellings of the α-Tip in Fig. 12 could be interpreted that the origin of the mem-

brane of this cytoplasmic PB was PSV. This descussion had also been supported by prior publications (Johnson et al., 1989; Marty-Mazars et al., 1995; Hoh et al., 1995).

The differential distributions of the vicilin and legumin on the protein deposits and cytoplasmic protein bodies indicate that accumulations of specific proteins could be taken place at intervals during seed development. At seed maturity, it is necessary to try double labellings of different antibodies on the same sections. Craig and Millerd (1981) already tried immunocytochemical labellings of the vicilin and legumin, and they concluded that one protein body contain both of the proteins. On the contrary of their observations, uneven distribution of vicilin and legumin on the protein body before seed maturity are considerd as delay of the specific protein depositions.

Since the legumin is unglycosylated protein at early stage of seed development, this protein can be observed in cis-Golgi networks (Robinson et al., 1995). That's because why vicilin is first accumulated on the protein deposits in PSV by vesicular transport (Fig. 11, see also Higgins, 1984; Hinz et al., 1995). Since vicilin is known the first detectable protein in PSV (Higgins, 1984), unglycosylated legumins should be retained in the ER for a long period of time.

It is well known that Bip is present in the ER and direct development of PB from ER because of the presence of Bip in the PB (Zhang and Boston, 1992). Such PB have ribosomes at the cytoplasmic face of the membranes (Larkins & Hurkmann, 1978). In cereals the retention of prolamins, which are dissolved in ethanol but not in water, salt solution or alkaline solutions (Beevers, 1976), in the ER lumen and then transfered to PB (Taylor et al., 1985; Rubin et al., 1992).

Even though the KDEL sequences are not contained in the prolamin of cereals, how they can be retained in the ER? According to Vitale et al. (1993) nonexportable aggregates are associated in the ER retention proteins. Bip has been known that it has KDEL signals and functioning protein folding in the lumen of rER (Hurtely & Helenius, 1989). The inner-peripheral labellings of Bip on the PD in this experiment have been regarded that Bip can recognize and retrieve the mis-folded proteins or retain them to block deposition to the PD during active accumulation of storage protein in PSV.

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<국문초록>

완두 종자에 축적되는 저장물질은 주로 전분과 단백질로서 이러한 저장물질 때문에 고정이나 전자현미경 관찰 시료를 제작하기가 쉽지 않다. 따라서 자엽을 얇게 절편을 만들고 효소를 사용하여 단일세포로 분리한 다음 고정하여 관찰하였다. 완두의 저장단백질이 축적되는 단백질 저장 액포는 종자발달의 이른 시기에 기존의 액포를 둘러싸고 발달하게 되므로서 액포는 수축되고 단백질 저장 액포는 점점 발달하여 그 가장자리에 단백질 덩어리가 축적되게 된다. 이와는 별도로 종자발달의 이른 시기에 조면소포체의 내강에 전자밀도가 높은 단백질이 축적되기 시작하여 늦은 시기에 이 소포체의 끝이 부풀어서 구형의 단백과립으로 발달하였다. 완두종자의 저장단백질은 주로 vicilin과 legumin으로서 단백과립에대한 면역세포화학적 방법으로 확인한 결과 vicilin은 세포질에 발달된 작은 단백과립과 단백질 저장액포의 가장자리에 축적된 단백질 덩어리에 모두 반응하였으나 legumin은 세포질의 단백과립에만 반응하였다. 또한 소포체에 존재하는 단백질인 Bip은 단백질 저장액포에 축적된 단백질 덩어리의 안쪽 가장자리에만 반응하였다. 이는 단백질이 활발하게 축적되고있는 시기에 특징적으로 작용하는 Bip의 기능과 관련되는 것으로 사료된다.

FIGURE LEGENDS

Fig. 1. Thin section of cotyledon tissue at early stage of development (18 DAF) shows compressed vegetative vacuoles (VV) surrounded by developing protein storage vacuoles (PSV). This PSV can be recognized by the presence of protein deposits (PD) at the periphery of PSV. CW: cell wall, bar = 2 μm

Fig. 2. Active deposition of storage prtotein in the protein body(PB) and protein storage vacuole (PSV) at intermediate stage of development (22 DAF). Arrow indicates protein-accumulated ER. bar = 2 μm

Fig. 3. Retention of putative storage proteins (arrows) in the lumen of rER cisternae was observed at early stage of seed development. bar = 0.3 μm

Fig. 4. Terminal dilation from both ends of protein-retaining ER (arrows) at same stage with Fig. 3. Ribosomes are still attatched on the dilated ER. G: Golgi complex, bar = 0.4 μm

Fig. 5. Protein-filled ER (arrows) are terminally dilated (arrowheads) to form protein body (PB) at late stage of development. Note the intercisternal presence of protein-filled ER between rER cisternae. bar = 1 μm

Figs. 6-7. Dramatic dilations (arrows) of ER were observed to form protein bodies at late stage of seed maturation. The irregular shape of PB at this stage would be transformed into globular PB. bar = 2 μm (Figs. 6, 7)

Figs. 8-9. Peripheral accumulations of storage proteins to the vesicles (arrows) derived from ER at early stage of seed development would be transformed into another type of PB. This PB would be developed concurrently and independently with those from protein deposits (PD) in PSV. bar = 2 μm (Fig. 8), 1 μm (Fig. 9)

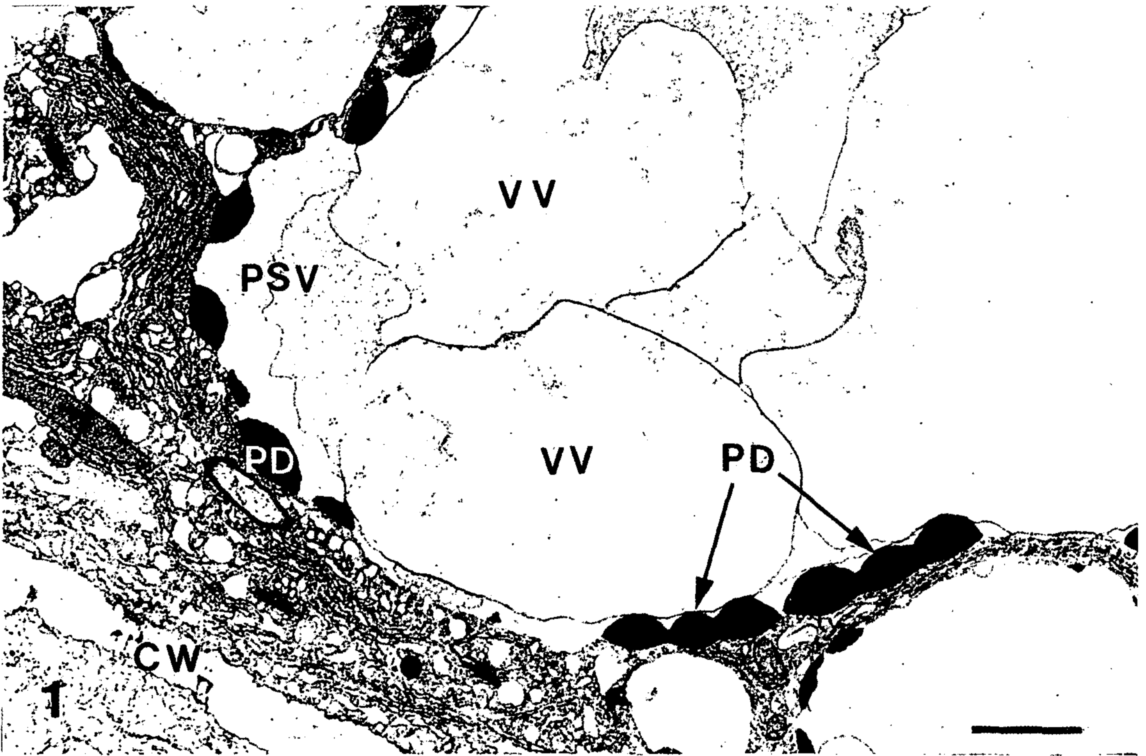
Figs. 10-11. Immunocytochemical localizations of legumin and vicilin on the protein deposits (PD) at the periphery of protein storgae vacuole (PSV). Note unlabellings of anti-legumin antibody on the PD (Fig. 10) compairing labellings of anti-vicilin antibody on the PD and cytoplasmic protein body (Fig. 11). bar = 0.5 μm (Figs. 10, 11).

Fig. 12. Immunocytochemical localizations of α-Tip in developing protein bodies show the marginal labellings on the PB. bar = 0.5 μm

Fig. 13. Immunocytochemistry of anti-vicilin antibody on the cytoplasmic protein bodies show strong labellings. bar = 4 μm

Fig. 14. Immunocytochemical localizations of the Bip homologue are specific at the inner periphery of the protein deposit (PD) in the protein storage vacuole (PSV). bar = 0.3 μm

Fig. 15. Immunolocalization of the legumin on the protein body derived from putative protein-filled ER shows very specific labellings. bar = 0.3 μm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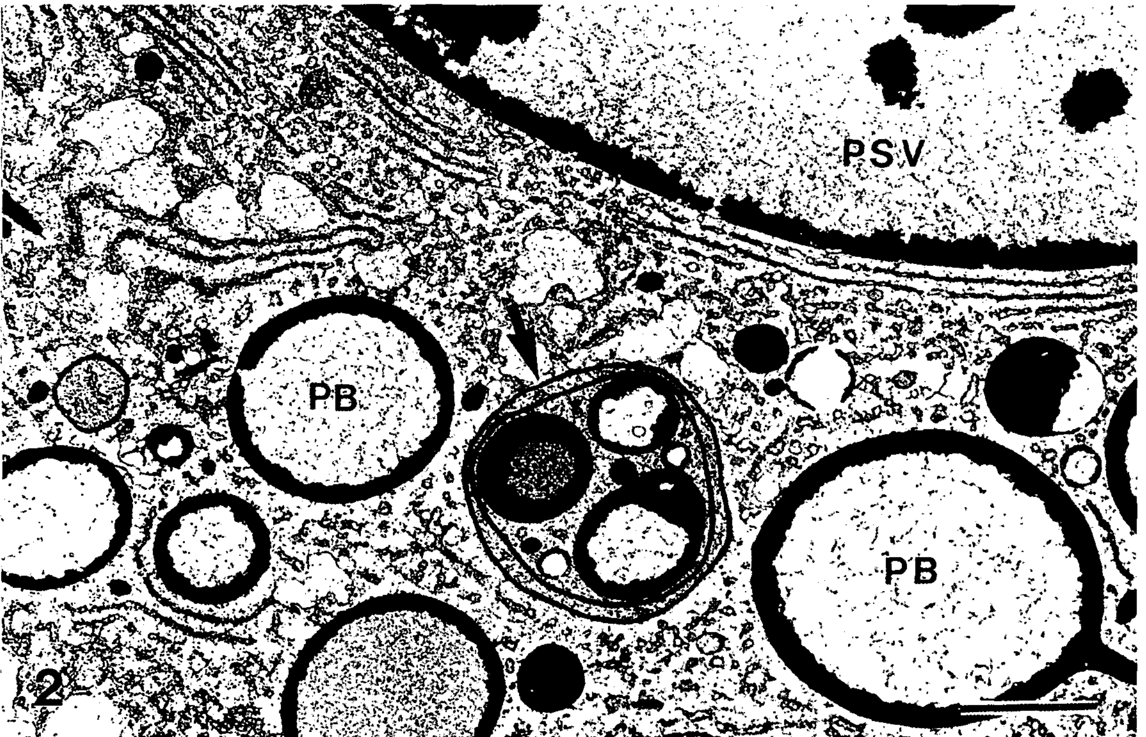
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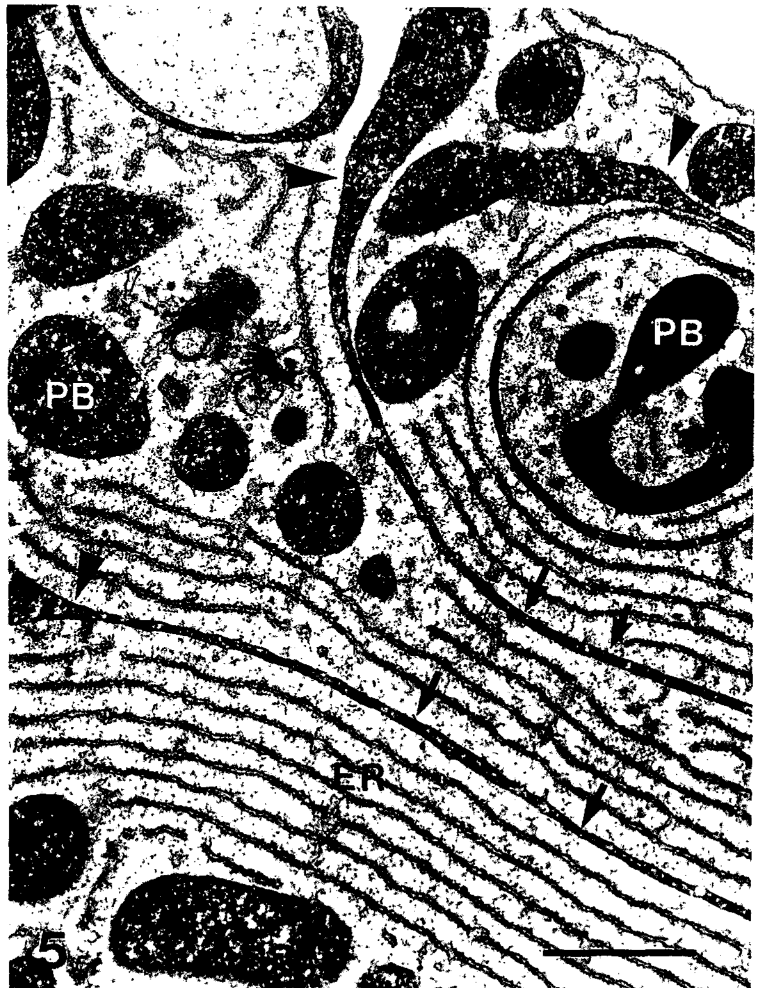
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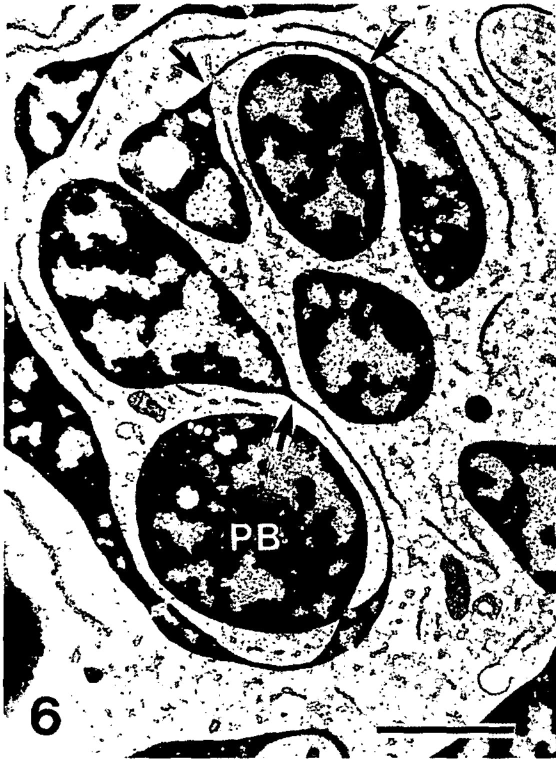
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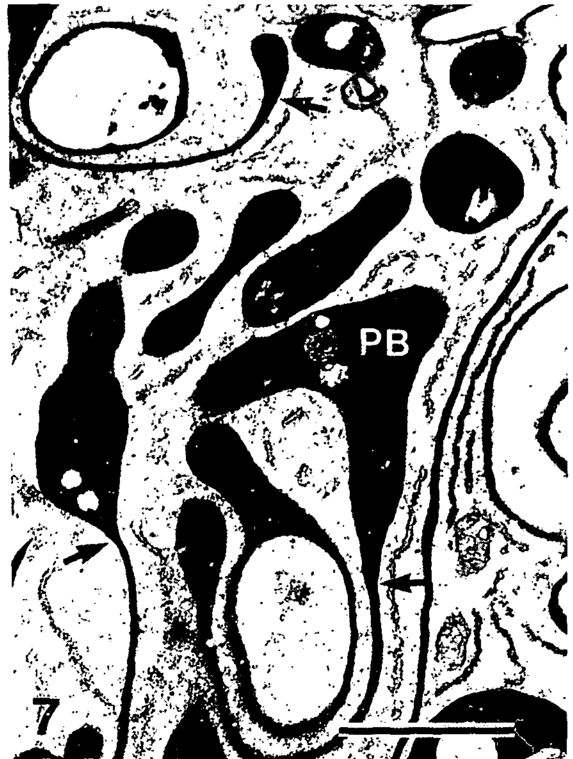
6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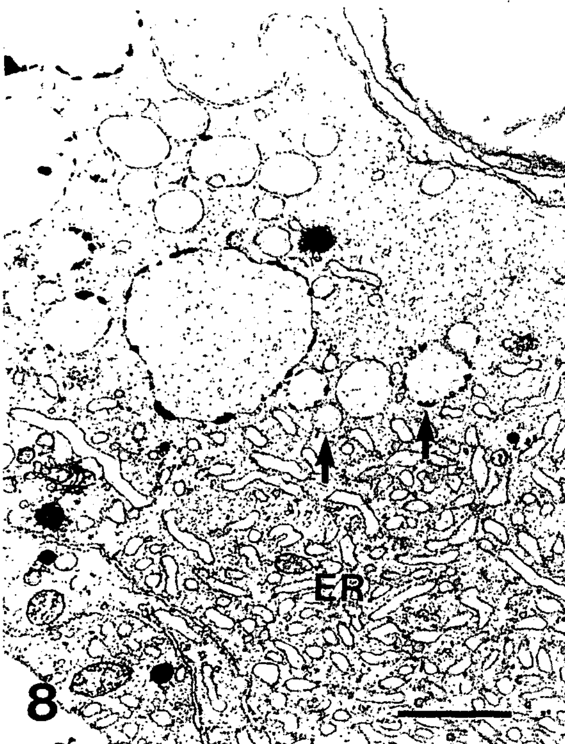
7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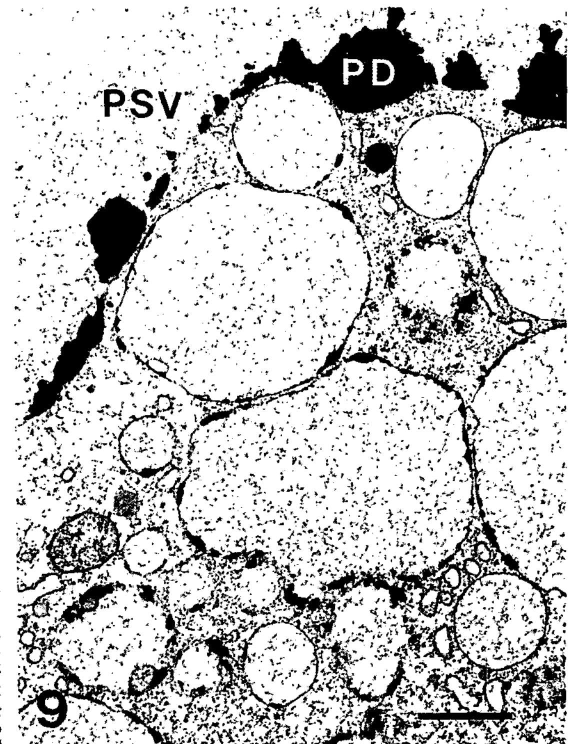
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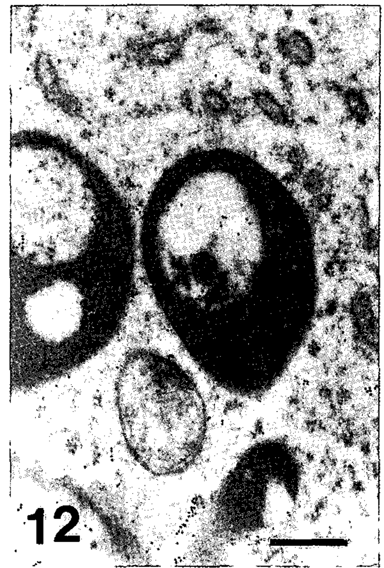
PSV
9

PSV
PD
10

PSV
11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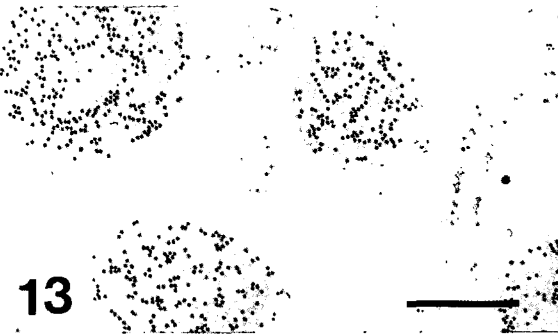
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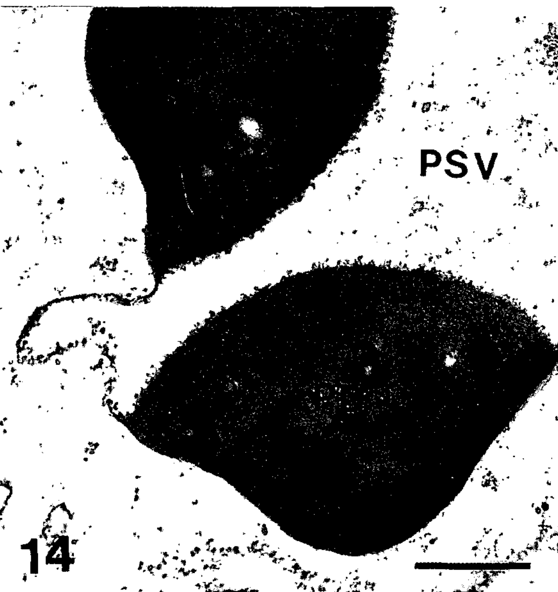
PSV
14

15