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Mobilization of Food Reserves and Ultrastructural Changes in Cotyledons of Germinating Soybean Seeds and Seedlings

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Abstract

The mobilization of food reserves and ultrastructural changes in the cotyledons of germinating soybean seeds (*Glycine max* L. Merr. Cultivar Amsoy) and seedlings were studied by using light and transmission electron microscopy. When germination began, the cotyledon tissues were packed with protein and lipid bodies. Mobilization of the reserves started in epidermis and vascular bundles. After three days of seedling growth, significant reductions of protein and lipid bodies were observed; concurrently, the numbers of starch grains, glyoxysomes, and mitochondria were increased. These ultrastructural changes are discussed with reference to the metabolism of the germinating soybean seeds and seedlings.

Key words: *soybean, germination, ultrastructure, cotyledon*

INTRODUCTION

Since the advent of electron microscopy, considerable information has been available on the ultrastructural changes that occur during seed germination and seedling growth in yucca (1,2), french bean (3,4), pea (5,6), and soybean (7-10). However, none of these previous studies have provided a comprehensive sequence of changes observed in germinating seeds and seedlings, and, so far, little attempt has been made to compare the metabolic differences between the component cells within the cotyledons.

The cotyledons are the site of reserve nutrient storage and account for almost 90% of the seed mass. Cotyledons of soybean comprise an epidermis, a distinct hypodermis, storage parenchyma, and vascular bundles. Vascular bundles, surrounded by a parenchymatous bundle sheath, are situated along the boundary between adaxial and abaxial parenchyma. A cotyledon has an extensive system of intercellular spaces, which can be observed as triangles at every cell wall junction of the parenchyma, when it is viewed as a transverse section.

The purpose of this work is to follow the mobilization of food reserves and ultrastructural changes of several organelles in cotyledons of soybean seeds that play important roles in the mobilization of food reserves during seed germination and seedling growth. The observations will be discussed with reference to the metabolism of the cotyledons of germinating soybean seeds and seedlings.

MATERIALS AND METHODS

Plant material

Soybean (*Glycine max* L. Merr. cv Amsoy) seeds were surface-sterilized in 70% ethanol for 10 s, rinsed with running tap water for 10 min, and soaked for 50 min in oxygen-flushed, double-distilled water. Seeds were then layered on wet filter paper on cotton wool in a plastic box with a lid. The box was placed in the dark at 28°C.

Tissue processing

Cotyledons of germinated soybean seeds were placed in 4% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M Na phosphate buffer (pH 7.4) at room temperature. The tissues were cut with a razor blade into approximately 1 mm^3 blocks and placed in fresh buffered fixative overnight at 4°C. Fixation was followed by three buffer rinses, 10 min each, and the tissues were postfixed in 1% (w/v) OsO_4 for 4 h at room temperature by using 0.1 M Na phosphate buffer (pH 7.4). The tissues were washed three times in buffer for 30 min followed by dehydration through a series of graded acetone solutions (30, 60, 90 to 100%). Specimens were infiltrated with Spurr resin (Polysciences, Inc., Warrington, PA) according to manufacturer's instruction for a firm block, cast in aluminum weighing pans, and placed in a 60°C oven overnight.

Light microscopy

Thick sections were cut at 0.5 ~ 1.0 mm with glass knives on a Reichert Ultracut E ultramicrotome. Sections were collected with a stick applicator and placed on a water droplet on a glass microscope slide. The slide was placed on a hot plate (50 to 55°C) to remove section wrinkles and to adhere the sections to the slide. Sections were stained with 1% (w/v) toluidine blue, washed, dried in warming tray, mounted in permount, and dried on a warming tray for several days. Bright-field observations were made on a Leitz Wetzlar Orthoplan

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microscope.

Transmission electron microscopy

Ultrathin sections (silver and gray) of approximately 60 to 85 nm were cut, spread with chloroform fumes, and collected on 200- and 300-mesh copper grids. Sections were stained with 20% (w/v) aqueous or 5% (w/v) methanolic uranyl acetate, followed by 2% (w/v) lead citrate. Observations were made by Hitachi HU-11C-1 transmission electron microscope at 50KV accelerating voltage by using Dupont Cronar Ortho S Litho sheet films.

RESULTS

Mobilization of food reserves during germination and seedling growth

The earliest stage at which the ultrastructure of reserve tissues of soybean seeds examined by electron microscopy was after 1.5 h of germination. Attempts to examine the cotyledons in dry, ungerminated seeds were not very successful because of poor penetration of the fixative.

Proteins

Storage protein bodies were present in all cells of the soybean cotyledons, including vascular tissues at the beginning of germination. In storage parenchyma cells, the protein bodies were large (diameter: 2 to 15 mm) (Figs. 1a and 1b), and many small protein bodies, mostly up to 0.1 to 5 mm in diameter, occurred in epidermal and vascular tissues (Figs. 1a and 1b). The shape of the protein body was limited by a distinct single-unit membrane and had an internal structure that was usually homogeneous and without any inclusions (Fig. 1c). Sometimes, however, small globoids were found, mainly in protein bodies of storage parenchyma cells (Fig. 1d) and radicles (Fig. 1e). Protein bodies in epidermis stained less intensely than those in storage parenchyma (Figs. 1a, 1c and 1d), and protein bodies in the same cell varied in their densities of staining (Fig. 1a).

After 9 h of germination, internal degradation of protein bodies was observed in epidermis and hypodermis (Fig. 1f). However, protein bodies in storage parenchyma still showed intense staining (Figs. 2a and 2b). As germination proceeded, changes in the internal structure of protein bodies were observed, as evidenced by an increase in electron-transparent areas (Figs. 3a and 3b) and a decrease in staining intensity (Figs. 3e and 3f), both of which suggest partial hydrolysis of proteins.

Between days 2 and 6, the protein bodies underwent several changes, which differed within each cell and within different regions of the cotyledons. In storage parenchyma cells, fusion of the protein bodies were often observed (Fig. 2d). Subsequently, larger and more irregularly shaped protein bodies appeared by day 3 (Fig. 2f). By day 6, these larger bodies showed a significant decrease in staining (Fig. 3f). The cells of storage tissue were mostly depleted of stainable storage protein, but the flocculent material remained visible by day 10 (Fig. 5d) In vascular bundles, each protein body matrix broke down in an irregular pattern, usually beginning in the center of the matrix at day 2 (Figs. 3a and 3b). The entire matrix of each protein body eventually becomes flocculent at day 5 (Fig. 3e). The flocculent matrix was almost gone by day 8, leaving protein vacuoles (Fig. 5a). In epidermis, vacuolating protein bodies were observed at day 5 (Fig. 3d).

Lipids

Lipid bodies, along with protein bodies, constituted the most abundant organelles within soybean cotyledons. They were tightly packed in the cytoplasm of cotyledon cells at the early stages of germination (Figs. 1c, 1d, 1e, and 1f). Lipid bodies, which stained uniformly gray, appeared to be bound by a thin electron-dense lamella, which is called a half-unit membrane. Usually, lipid bodies were round and oval, and the size of lipid bodies was smaller on the average than the protein bodies (Figs. 1c and 1d).

Lipid bodies closely associated with cisternal endoplasmic reticulum (ER) were commonly seen in storage parenchyma cells after 9 h of germination (Figs. 2a and 2b). At day 2, lipid bodies were scattered in cytoplasm (Fig. 2d). Significant reduction of lipid bodies was observed at day 3 in epidermis, where the degradation of protein bodies was mostly completed (Fig. 3c), which was the case in vascular bundles at day 5 (Fig. 3e). The number of lipid bodies in storage parenchyma cells was significantly decreased at day 6, when protein body matrix became very flocculent. Lipid bodies showed an affinity for the inner side of plasma membrane (Fig. 3f).

In contrast to the starch grains and protein bodies, the lipid bodies were the last of the soybean seed reserves to be utilized completely. Frequently, a few undegraded lipid bodies were present in all cotyledon cells at the late stages of seedling growth, although no starch grains and protein bodies were present then, and some cytoplasmic organelles had been broken down (Figs. 4b and 5c).

Starch

No starch grains were observed in any of the cotyledons cells at the early stages of germination (Figs. 1c and 1d). Starch grains were abundant in all cotyledon cells between days 3 and 6 (Figs. 2f, 3c, and 3f). At late stages of seedling growth, starch grains disappeared completely in the epidermis (Fig. 4b), whereas few degraded starch grains were still found in vascular bundle and storage parenchyma cells (Figs. 4a and 25a).

Ultrastructural changes during germination and seedling growth

Mitochondria

At the early stages of germination, up to 9 h, mitochondria were only recognizable as membrane-bound organelles with no internal structure. Part of the outer membrane was distended so that it appeared tenuous (Fig. 1c). At 30 h, there were no recognizable cristae in the mitochondria (Fig. 2c). Mitochondria became more numerous, and the number of cristae increased after day 3 (Figs. 3c and 3d). At day 5, in

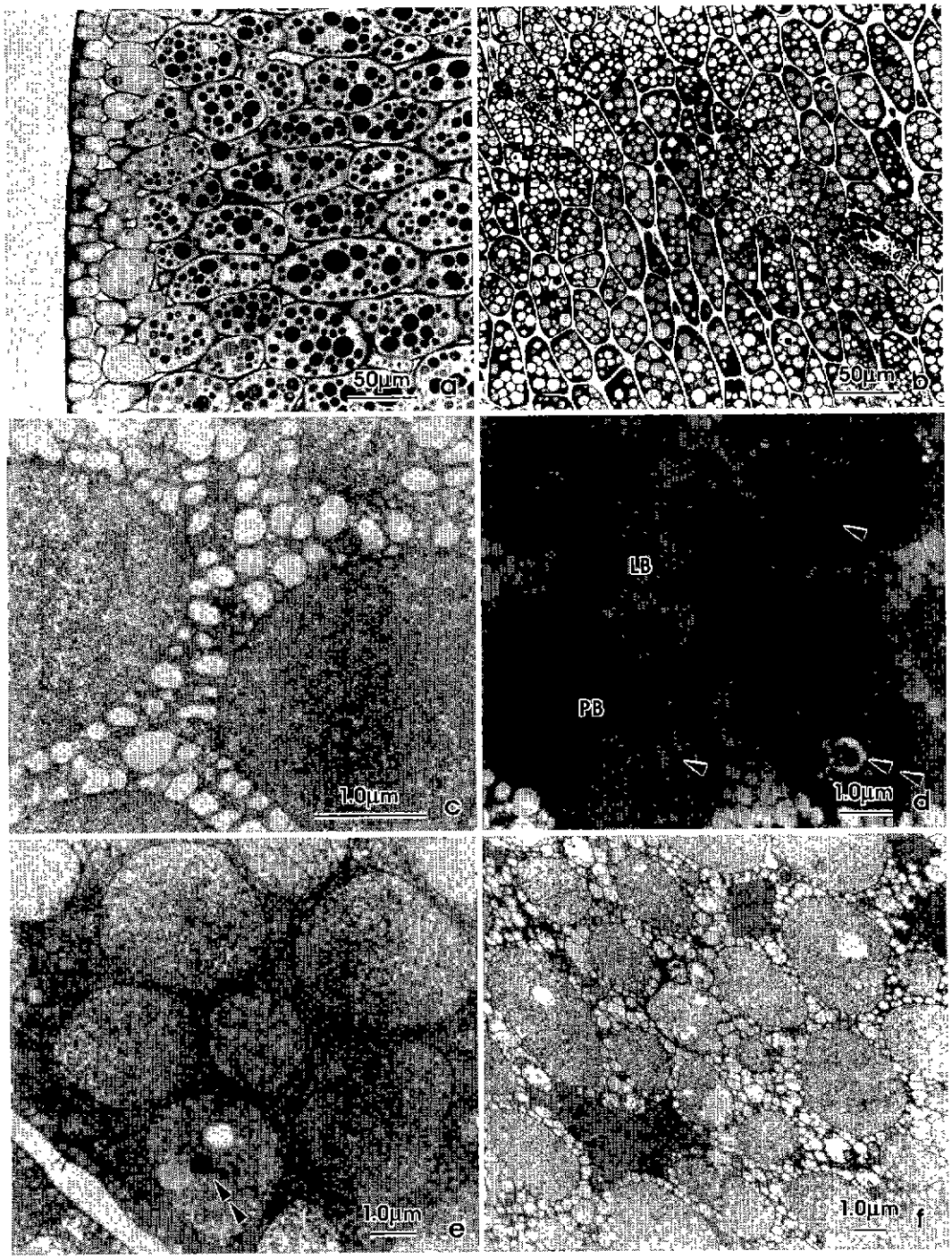

Fig. 1. a, Cotyledon after 1.5-h germination. Protein bodies of epidermal cells stain less intensely than those of storage parenchyma cells; 247×. b, Cotyledon: 1.5-h germination showing procambium and storage parenchyma; 239×. c, 1.5-h-old epidermal cell: protein bodies (PB) with homogenous matrices; 20,400×. d, 1.5-h germination; storage parenchyma cell: cotyledon is packed with protein bodies and lipid bodies (LB). Note globoids in protein bodies (arrow); 9,500×. e, 9-h-old radicle:displaced globoid (arrow); 8,700×. f, 9-h-old epidermal cell with internal degradation of protein bodies; 5,700×.

some cells of epidermis, mitochondria became dark, and their cristae were swollen (Fig. 3d). The mitochondrial darkening and swelling of cristae were increased in many cells of cotyledons between days 6 and 10 (Fig. 5c). Furthermore, pleomorphic mitochondria, ranging in shape from spherical to very elongated, were often observed at the late stages of seedling growth (Fig. 5c).

Plastids

At the beginning of germination, no plastids were observed. After 9 h of germination, few plastids were seen in all cotyle

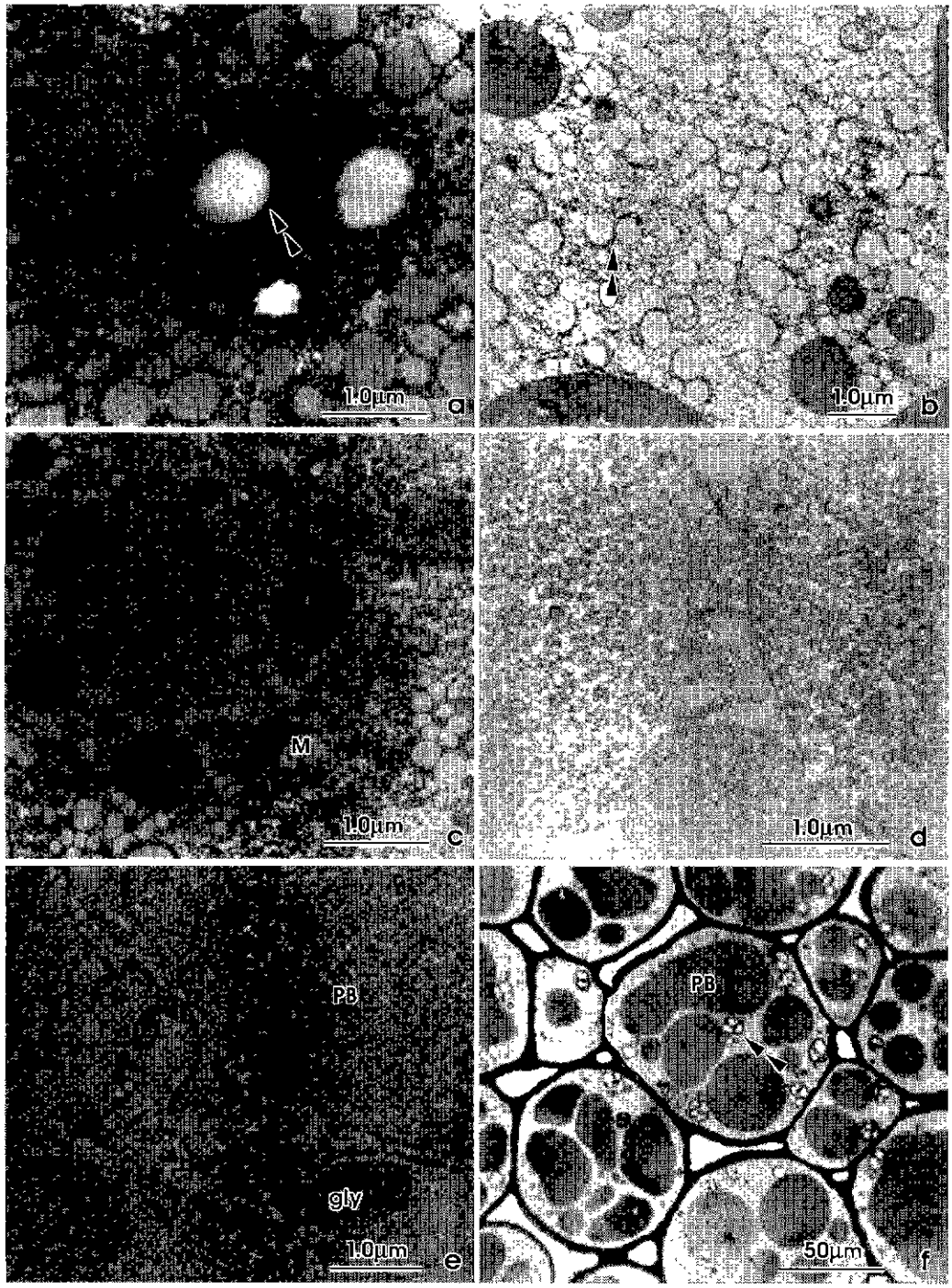

Fig. 2. a, 9-h-old storage parenchyma cell: electron-transparent regions (which may be starch) (arrow); 18,500×. b, 9-h-old storage parenchyma cell; 12,000×. c, 30-h-old adaxial epidermal cell: showing mitochondria (M) with few cristae. Endoplasmic reticulum is wrapped around lipid body (arrow); 18,600×. d, 2-d-old storage parenchyma cell with appressed enlarged protein bodies; 22,500×. e, 2-d-old storage parenchyma cell with enlarged protein bodies (PB), mitochondrion in division and a glyoxysome (gly); 16,300×. f, 3-d-old storage parenchyma cell with enlarged protein bodies (PB) and plastids containing starch grains; 375×.

don cells. The membranes surrounding plastids were well defined, but internal lamellae were absent or only partly represented. Each plastid had a granular matrix with ribosomes and small electron-transparent regions. No starch grains were observed. Plastids were quite often irregularly shaped so that, in sections, they often appeared to contain invaginations of cytoplasm with ribosomes and lipid bodies (Fig. 2a). Plastids containing large, conspicuous starch grains in a dense stroma were prominent in all cells of cotyledon between day 3 and 6 (Figs. 2f, 3c, and 3f). At day 7, plastids in epidermal cells

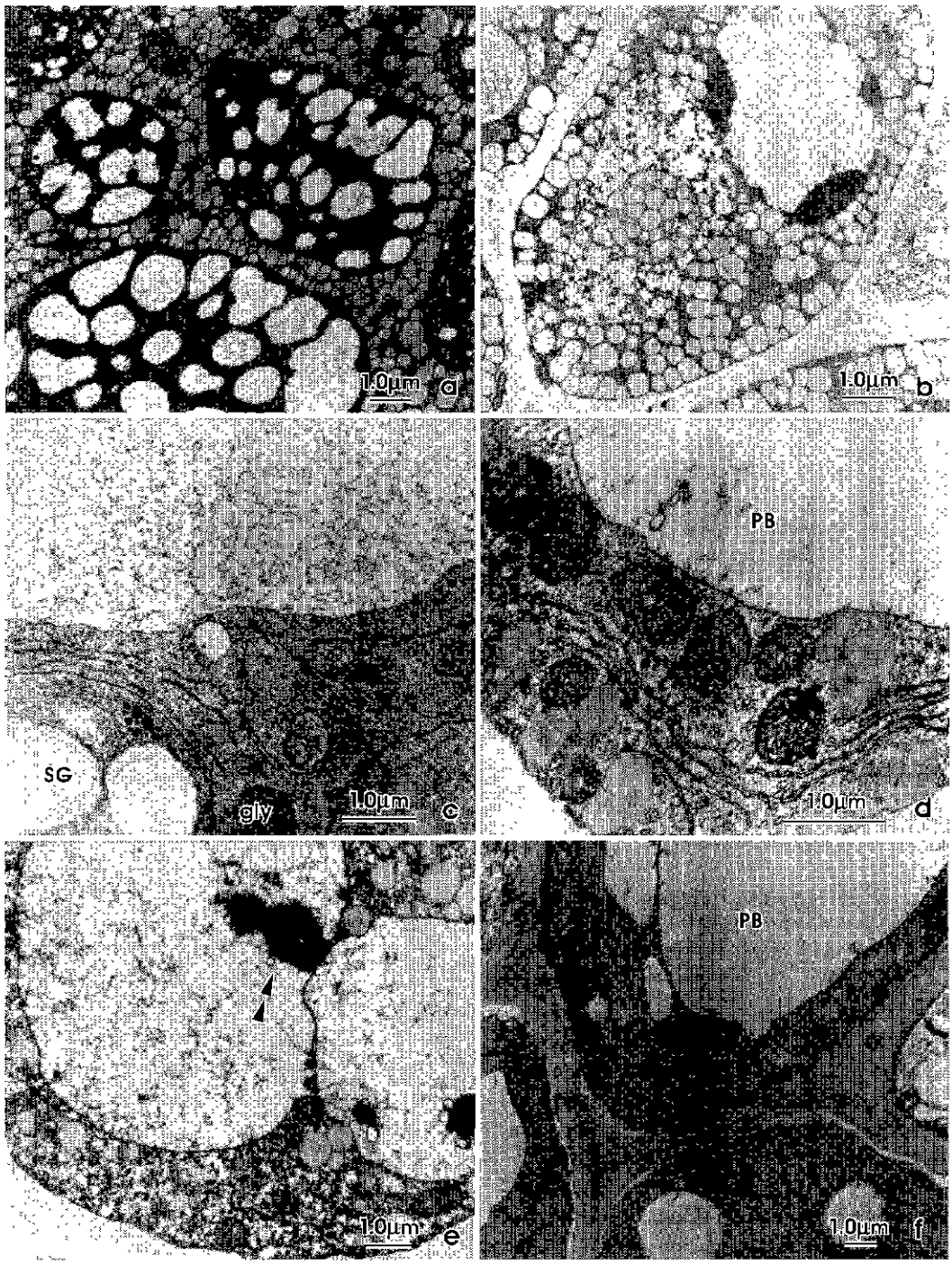

Fig. 3. a, 2-d-old vascular bundle cell: showing internal degradation of protein bodies; 7,000×. b, 2-d-old vascular bundle cell with flocculent protein bodies; 9,000×. c, 3-d-old epidermal cell with starch grains (SG) in plastid, endoplasmic reticulum, and glyoxysomes (gly) associated with endoplasmic reticulum. Number of lipid bodies is less; 13,000×. d, 5-d-old epidermal cell showing parallel array of endoplasmic reticulum and numerous mitochondria, protein body (PB) is almost vacuolated; 18,560×. e, 5-d-old vascular bundle cells: vesicles (arrow) in protein bodies appear as empty single-membrane-coated spheres; 8,200×. f, 6-d-old storage parenchyma cells: staining intensity of protein bodies (PB) is significantly decreased, and lipid bodies show an affinity to the inner side of plasma membrane; 5,400×.

began to disintegrate but contained prolamella bodies and a few osmiophilic globules (Fig. 4b). Plastids in the vascular bundles and storage parenchyma cells at this stage remained intact and occasionally contained small starch grains (Figs. 4a and 5a).

Glyoxysomes

Glyoxysomes were absent at the early stages of germination. However, the cells of the cotyledons after 2 days of ger-

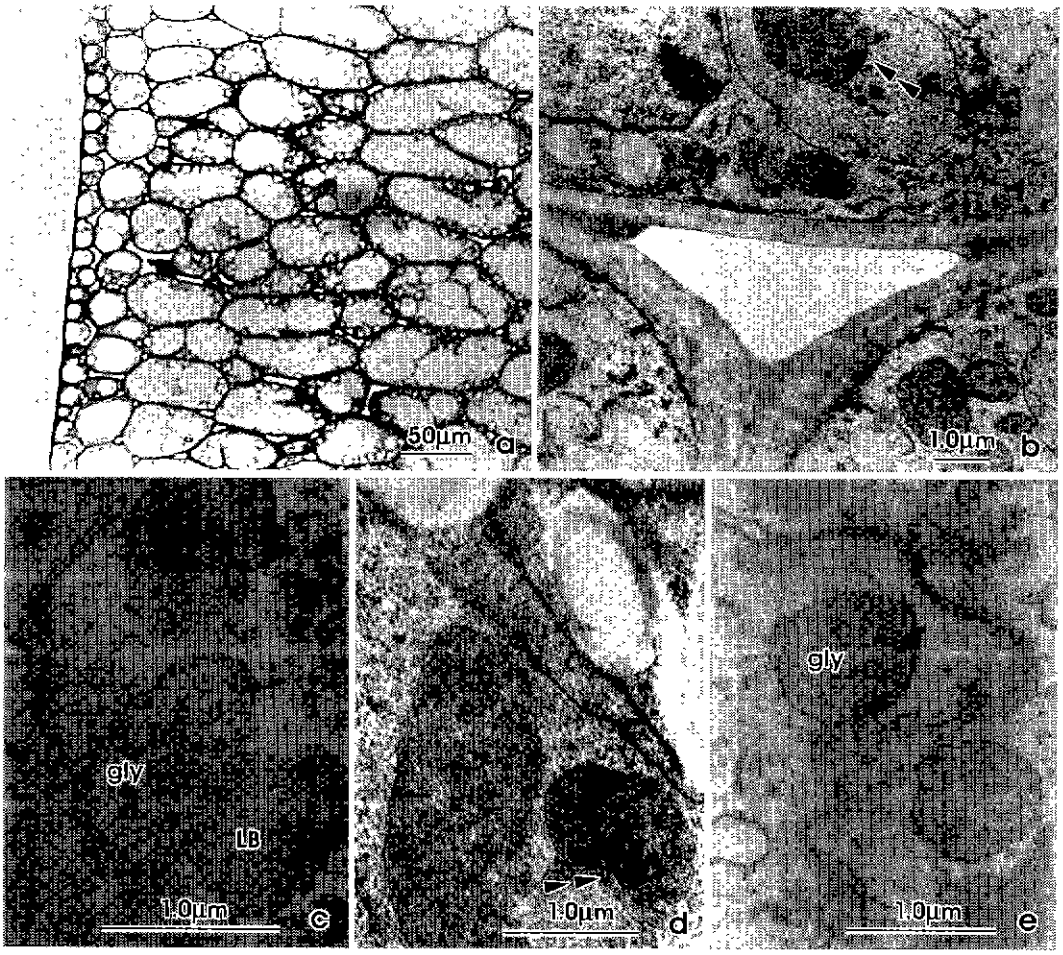

Fig. 4. a, 7-d-old storage parenchyma cell: cotyledon of seedling shows vacuolated epidermis; 235×. b, 7-d-old epidermal cells: prolamellar body related to photoinduction (arrow); 8,400×. c, 7-d-old cotyledon: glyoxysome (gly) with flocculent matrix is associated with lipid body (LB); 28,600×. d, 7-d-old cotyledon: glyoxysome associated with endoplasmic reticulum (arrow); 23,500×. e, 7-d-old cotyledon: glyoxysome (gly) associated with endoplasmic reticulum; 24,100×.

mination contained few glyoxysomes, and the matrices of the glyoxysomes were uniformly dense (Fig. 2e). Between days 3 and 7, glyoxysomes were commonly observed in all cells of the cotyledons (Figs. 3c, 4b, 4c, 4d, and 4e). Close associations of glyoxysomes with the endoplasmic reticulum (Figs. 3c, 4d, and 4e) and lipid bodies (Fig. 4c) were frequently found. At the late stages of seedling growth, flocculent matrices of glyoxysomes were frequently observed (Figs. 4c, 4e, 5c, and 5d).

Endoplasmic reticulum (ER)

After 9 h of germination, short segments of ER cisternae were often seen in close association with and wrapping around lipid bodies in storage parenchyma and epidermis (Figs. 2a, 2b, and 2c). Short cisternal ER segments appeared more numerous after 2 day of germination (Figs. 2d and 2e) and were present by day 10 (Fig. 5d). Relatively long ER cisternae and parallel arrays of ER along plasma membrane became prominent in epidermal cells between days 3 and 5 (Figs. 3c and 3d). At day 9, long cisternal ER segments formed concentric figures in epidermal cells (Fig. 5b).

DISCUSSION

The ultrastructure of dry seeds was difficult to preserve because there was little penetration of fixative because of the compactness of soybean cotyledon tissues. Cotyledons sampled after 1.5 h of germination were taken as tissue representative of the beginning of germination. It is recognized that the possibility of some changes during the 12 h fixation period cannot be excluded. However, most reserve mobilization and ultrastructural changes took place after day 3 of seedling growth. This 1.5 h period, therefore, is arbitrarily designated as the beginning of germination.

The abundance of storage reserves, such as lipid and protein bodies, in the cotyledons after 1.5 h of germination was striking. Two types of protein bodies were observed in storage parenchyma cells, one with globoids and, the other, without any inclusions. This is in agreement with the results of Lott (11) and Lott and Buttrose (12).

Great size differences in protein bodies were observed; vascular bundle and epidermal cells have relatively small bodies,

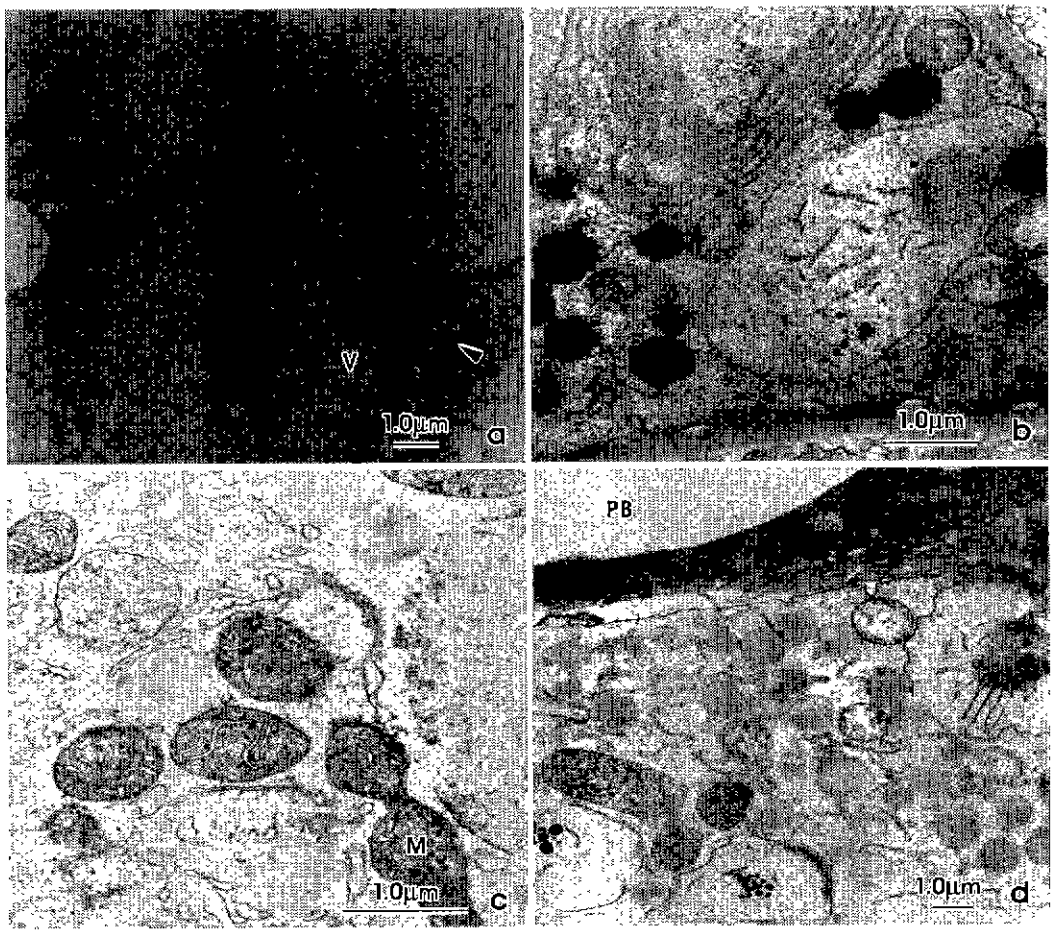

Fig. 5. a, 8-d-old vascular bundle cell: various sizes of electron-transparent vacuoles (V) and starch grains (arrow) are seen; 7,380×. b, 9-d-old epidermal cell: concentric endoplasmic reticulum; 15,180×. c, 9-d-old epidermal cell: dark, highly organized (metabolically very active) mitochondria (M) appear in; 21,160×. d, 10-d-old storage parenchyma cell: stainable material seen in protein body (PB) and lipid bodies still present; 6,800×.

whereas storage parenchyma cells have very large ones. Similar observations were made in squash cotyledons (13). Many agree that the protein bodies vary in size from one plant to another and from one tissue to another (4,14,15).

The variability in the degree of staining of protein bodies in a cell may reflect simple differences in composition or in protein concentration. This variation can be partly explained by the findings of Tombs (7) who isolated two fractions of soybean protein bodies that have different densities of 1.29 and 1.30 g/cm^3.

The presence of a limiting membrane around each protein body in dormant seeds has been convincingly shown (11,15, 16). Evidence for the existence of intact membranes around protein bodies during protein body degradation is also accumulating (5,10,16). These observations suggest that the soluble products accumulate within the protein bodies and then leak through the membranes into the cytoplasm (5) while the protein bodies are undergoing autolysis (17).

The observed pattern of protein body degradation was more complex than those described for *Glycine* (7,8,10,15), or for other legumes, including *Pisum* (5,6), *Phaseolus* (3,4), and *Vigna* (17). The pattern in the cells around the vascular bundles, where the protein is degraded with little or no coalescence of bodies, is like that described for *Pisum sativum* (5). Possible instances of vacuolar fusion were encountered in these cells. The pattern in epidermis and storage parenchyma, where the bodies swell, coalesce, and then are vacuolated, was the one most frequently described (3,7,10,15). A similar pattern was found in seeds of *Yucca schidigera*, where the protein bodies in the embryo coalesced before breaking down; however, the ones from the perisperm disappeared directly (1).

Flocculent protein bodies were observed in most of the cotyledons between days 2 and 7. In the epidermis and vascular bundles, this type of protein body was observed earlier than in storage parenchyma cells because of earlier hydration and degradation. These occurrences might be due to the internal degradation of protein matrix by proteinase existing in protein bodies (18), not the result of fixation artifacts (19).

Some technical difficulties were met in monitoring the deg-

radation of globoids in protein bodies. Because globoids are brittle, they are not penetrated well by fixatives and resins (12) As a result, they tend to shatter when cut and fall out of sections. This occurrence left a hole or small fragments of globoid around globoid areas, as shown in Figs. 1d and 1e.

Another conspicuous change during germination and seedling growth was degradation of lipid bodies in the cytoplasm. At day 3, a significant decrease in the number of lipid bodies was observed. Concurrently, starch grains and glyoxysomes appeared, and mitochondria became numerous. Similar observations have been made by many researchers in germinating seeds (3,5,10,15).

It was difficult to follow the fate of lipid body degradation in this material. However, it seems reasonable that the storage fats become hydrolyzed by lipase and that the fatty acids degraded after transfer into glyoxysomes through the glyoxylate cycle (20). Characteristic association observed between glyoxysomes and lipid bodies (Fig. 4c) also reflects their mutual involvement in gluconeogenesis through the conversion of fatty acids to carbohydrates (21).

During germination and seedling growth of several legume species, starch is depleted (3,6,22), but soybeans seemingly accumulate starch grains in plastids as a result of gluconeogenesis (21). Biochemical data have shown that the reserve carbohydrates of soybean cotyledons consist primarily of low molecular weight oligosaccharides, particularly sucrose, stachyose, and raffinose (23), and at maturity the cotyledons contain approximately 1% starch (24). However, many electron microscopic observations confirm the accumulation of starch in soybean seedlings and other non-legume species as a transient reserve material (2,8,10,15).

The starch content rapidly declined during the late stages of seedling growth. No starch grains were observed in epidermal cells at day 7, whereas degrading and small starch grains were often found in the plastids of storage parenchyma and vascular bundle cells. A combined α-amylase and α-glucosidase system was reported to be responsible for starch utilization during the late stages of seedling growth (21). Short segments of ER in association with lipid bodies were conspicuous after 9 h of germination. This profile was also observed by Harris and Chrispeels (25). The role of this association was not elucidated. However, Bain and Mercer (5) presumed that fat material was used directly in the formation of the ER membrane system.

The existence of ER in cotyledon cells became most prominent between days 3 and 5 when the most active reserve mobilization was observed. The role of ER in germinating seeds and seedlings is not completely understood; however, many researchers suggest that it plays an important role in the transport of the breakdown products of the storage reserves (5,26), ribonuclease and proteinase, to the protein bodies (27). It has been suggested that ER is involved in the biogenesis of glyoxysomes by a process of invagination (9,28,29) A direct attachment between glyoxysome membrane and ER shown in Figs. 4d and 4e and the similarity in polypeptide composition between ER and glyoxysomal membranes (28,30) strongly support this suggestion.

A tendency for the ER to become oriented in parallel lamellae along the cell periphery and appearance of concentric ER, as observed in Figs. 3d and 5b, seems to be characteristic of aging (3).

Mitochondria at the early stages of germination (up to 9 h) showed little evidence of typical cristae and stroma, although they may be able to maintain certain oxidative functions (31). The rapid development of numerous cristae and dense stroma after 2 days of germination may be related to the rapid initiation of respiration in soybean seeds (32). At the late stages of seedling growth (days 5 to 90), mitochondria with dark matrices and swollen cristae were often observed in some cells. This change was ascribed to the increased permeability of cell and mitochondrial membrane (5) and was interpreted as a degenerative change (3).

The general pattern of storage reserve mobilization in soybean cotyledons was found to be like that described by Smith (6). The reserves disappeared first from the vascular bundles and epidermis. In the storage tissue, reserve mobilization began in adjacent cells around vascular bundles and epidermis and gradually progressed to the central storage tissue. At day 7, the epidermis completely lacked starch grains in plastids and protein bodies, but lipid bodies were still observed in all cells of cotyledons. It seems clear from this observation that lipid is the last storage reserve to be utilized completely during soybean seedling growth. This sequence has been shown also for yucca seeds (2).

A similar pattern of reserve mobilization was reported in *Pisum* (6), where degradation began at the periphery of the cotyledons. This result did not seem to show any correlation with the distance from the vascular bundles. Different zonation of reserve mobilization was reported in *Phaseolus* (3,4) and *Vigna* (17) where reserve mobilization began in the cells farthest from the epidermis and from the vascular tissue; after 4 days of seedling growth, the central regions were vacuolated, while the cells around the vascular bundles and under the epidermis were still packed with reserves. Yomo and Taylor (33) confirmed this observation by reporting that protease activity was greatest in the cells farthest from the vascular bundles in cotyledons of germinating *Phaseolus vulgaris*.

With this understanding of the basic patterns of hydrolysis of reserves, we may be better prepared to understand the complex metabolic controls that are associated with the breakdown and utilization of storage reserves.

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