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DEVELOPMENTAL CHANGES OF ANTIPIT CELL ULTRASTRUCTURE IN THE SOYBEAN SEED COAT DURING SEED MATURATION

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Abstract

The fine structure of the antipit cells of soybean {*Glycine max* (L.) Merr.} endosperm were analyzed by transmission electron microscopy (TEM) during the rapid seed fill period. The antipit, which apposes the pit on the abaxial surface of the cotyledon, was composed of filamentous cells whose wall porosity appeared to increase during seed development. The filamentous cells were interconnected to each other and the subtending aleurone cells by plasmodesmata. During seed fill, the filamentous cells contained numerous Golgi cisternae derived vesicles that appeared to accumulate in the cytoplasm at the end of seed fill. The Golgi cisternae were well developed; rough endoplasmic reticulum (RER), which was abundant, consisted of large stacked cisternae. The Golgi cisternae were completely fenestrated and had fewer associated secretory vesicles at the end of seed fill. The stacked lamellae of RER, were progressively replaced by short tubular cisternae and small vesicles. At the end of seed fill, the filamentous cells contained numerous lipid bodies, protein bodies and amyloplasts with large starch grains. These observations suggest that the antipit consists of physiologically active tissue that may be involved in symplastic transport of nutrients to the embryo.

Key Words: Antipit, seed coat, soybean, seed, electron microscopy, Golgi system.

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Introduction

In most plant species, the endosperm, which occupies the bulk of the seed, is the most actively growing seed tissue and forms a storage compartment for starch, proteins and lipids (Galili *et al*., 1993). In some legume species, such as soybeans {*Glycine (G.) max* (L.) Merr.}, the cotyledons acquire this storage function. The endosperm in soybeans is thought to be used for nourishment of the developing embryo. However, recent results indicate that some of the endosperm remains unabsorbed during seed development (Thorne, 1981) and occurs as a structure (antipit) adhering to the inside surface of the seed coat at seed maturation (Yaklich *et al*., 1986).

The antipit is a unique convex structure that is located medially on the inside surface of the seed coat and projects inward toward the cotyledon surface. The structure was named the antipit because it is opposed to the convex pit on the abaxial (outside) surface of the cotyledon (Yaklich *et al*., 1986). The antipit is localized in the area formed by the concave pit. The central portion of the pit contains larger cells than those on the surrounding cotyledon surface. The surface of the antipit conforms to the surface of the pit, resulting in two surfaces that fit together in a tongue and groove manner. The pit and antipit appear to be ubiquitous in soybean and other *Glycine* species, but are not found in other legumes (Yaklich *et al*., 1989).

In a previous publication, we briefly described the anatomy and morphology of the antipit (Yaklich *et al*., 1986). In that study, the body of the antipit appeared to be composed of filamentous cells that were surrounded by porous cell walls. The filamentous cells were also interconnected symplastically by plasmodesmata. Some striking features of the filamentous cells were that the cytoplasm contained prodigious quantities of vesicles and Golgi cisternae. Storage products in the form of amyloplasts, protein storage vacuoles, and oil bodies were also present. These results have led us to study the antipit in greater detail to gain a more complete understanding of the anatomy and morphology of this structure during soybean seed development. The results, which are based on freeze-fracture and conventional transmission electron microscopy (TEM) observations, provide a better understanding of the possible function of the antipit during the period of rapid seed fill.

Materials and Methods

Soybean {*Glycine max* (L.) Merr.} plants, cultivars Sooty and Williams, were grown in field plots in 1987, 1989 and 1991, as described previously (Yaklich *et al*., 1979). Flowers at anthesis at nodes 3 and 4 were tagged following inception. Developing pods were sampled weekly when the period of rapid seed fill began (defined as "the period when soybeans are beginning to develop at one of the four uppermost nodes with a completely unrolled leaf"), and continued to physiological maturity (when 50% of the leaves yellow, referred to as "pods yellowing") (Fehr *et al*., 1971). Observations were restricted to the fine structure of the antipit cells during the period of rapid seed fill when protein and oil reserves were being synthesized and stored in the developing soybean embryo (Wilson, 1987).

For thin sections, seed coat tissue was excised and fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 5.0 mM $CaCl_2$ for 2 hours. After four washes with buffer, the sample was postfixed overnight at 4°C with 2.0% osmium tetroxide in 0.05 M cacodylate buffer (pH 7.2) containing 5.0 mM $CaCl_2$ and 0.8% potassium ferricyanide. Following postfixation, the tissue was washed with buffer, dehydrated in a graded series of acetone solutions and embedded in Spurr's (1969) low viscosity resin mixture. Thick sections were viewed with an Olympus BH-2 light microscope (Olympus Optical Co., Tokyo, Japan). Thin sections were stained in alcoholic 7.5% uranyl magnesium acetate and Reynold's (1963) lead citrate before viewing in a Hitachi H-500 H (NSA Hitachi Instruments, Mt. View, CA) transmission electron microscope operated at an accelerating voltage of 75 kV.

For freeze-fracture replicas, seed coat tissue was fixed in 3.0% glutaraldehyde in 0.1 M $KHPO_4$ buffer, pH 7.2, and passed through a linear gradient of glycerol and sucrose (0 to 25%, v/v and w/v, respectively). Samples were then frozen in pre-cooled freon (155°C), freeze-fractured, coated first with platinum-carbon (Steere, 1981) and then with polycarbonate "Lexan." This procedure helped prevent breakage of the replica during subsequent thawing and aqueous acid digestion of any adhering tissue (Steere and Erbe, 1983). Cleaned replicas were recovered from water and mounted on formvar coated grids for viewing with transmission electron microscopy. Selected areas were photographed as stereo pairs at 0 and 10 degrees of tilt.

Tissue for scanning electron microscopy was prepared as described previously (Yaklich *et al*., 1989). Seeds were mounted on stubs, sputter coated with gold/ palladium and observed in a Hitachi S-530 scanning electron microscope. Images were recorded with Polaroid (Cambridge, MA) 55 P/N film.

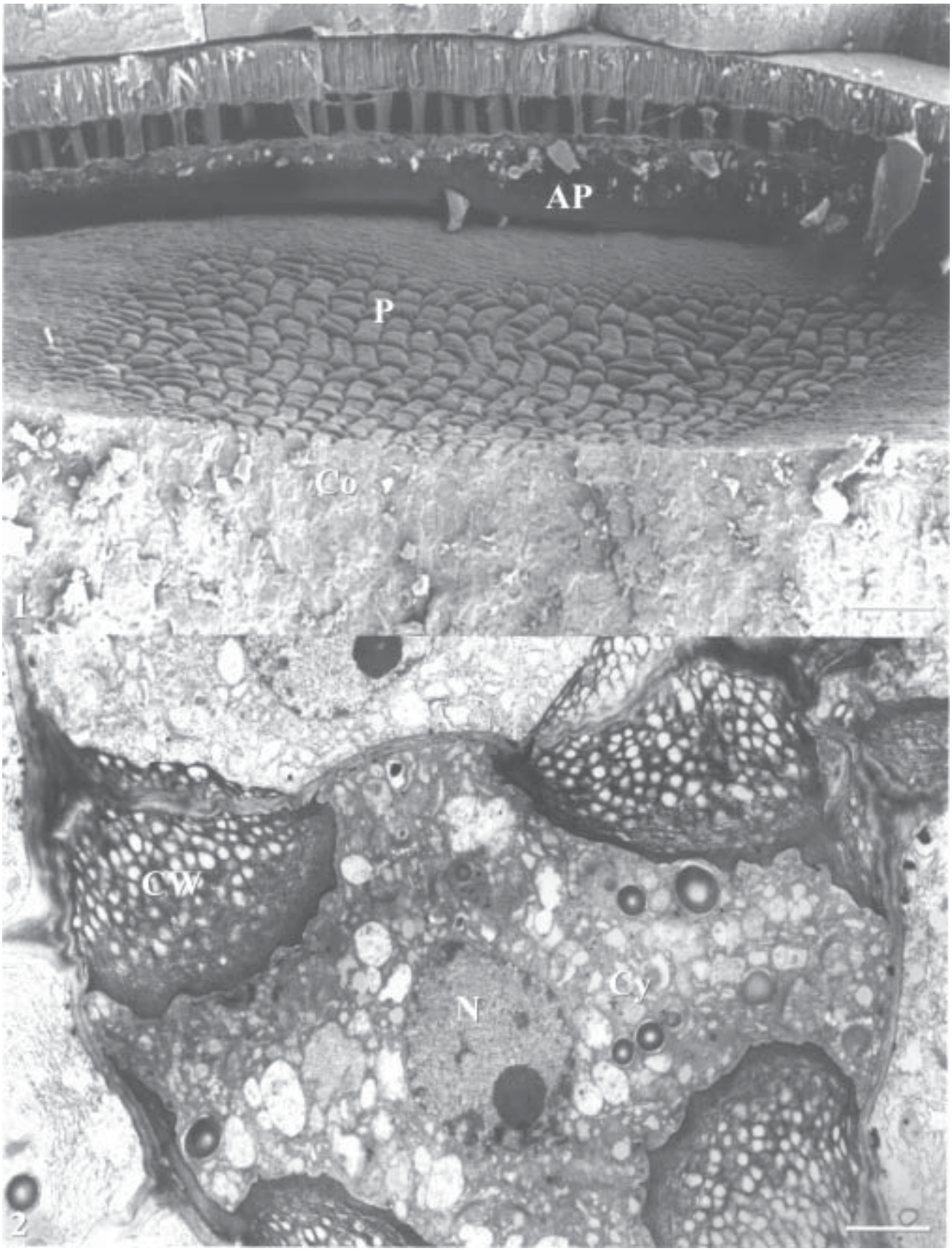
Figure 1. Scanning electron micrograph of a cross-section of a soybean seed. The pit (P) is a concave depression located medially on the abaxial surface of the cotyledon (Co) containing large epidermal cells. The antipit (Ap) is a convex structure attached to the inner surface of the seed coat and is convex in shape. Bar = 100 μm.

Figure 2. Electron micrograph of a transverse section of a filamentous cell during early seed fill. The cell wall (CW) next to the plasma membrane is rippled and appears to develop channels between adjacent cells. The filamentous cell contains dense cytoplasm (Cy). N = nucleus. Bar = 5 μm.

Results

The antipit is located on the inside surface of the seed coat and fills the cavity formed by the pit on the abaxial surface of each of the cotyledons (Fig. 1). The body of the antipit contains filamentous cells that are attached to the subtending aleurone layer. The porous cell walls are a prominent feature of the filamentous cells of the antipit (Fig. 2). The surrounding cell wall, which is net or web-like, is not the typical compact cell wall that encloses the cytoplasm of the subtending aleurone cells. Instead, the porous cell walls of each cell are expansive and have fine cell wall endings between adjacent rows of filamentous cells that do not delineate the boundary of adjacent rows of filamentous cells. This creates a web-like appearance of cell wall between adjacent rows of filamentous antipit cells. The cell wall opposing the plasma membrane is rippled. The cell wall immediately surrounding the cytoplasm changes during seed fill. In tangential sections, the cell wall appears to develop channels which increase in size and result in abundant spaces in the web-like cell wall between adjacent rows of filamentous cells. The cell wall appeared solid in freeze fracture replicas, indicating that liquid can exist within the wall proper. These cell walls form the body of the antipit that fills the cavity formed by the apposing concave pit.

During early through mid seed fill, the plasma membrane exhibits round attached vesicles that are evident in the protoplasmic (PF) and ectoplasmic (EF) faces (Fig. 3). These vesicles create an undulating or rippled membrane that delineates the cytoplasm. These attached vesicles fractured in both the split-membrane and cross-fracture configuration. Cross fractures of the plasma membrane revealed a change of pattern of intramembranous particles (IMP's), namely areas where the vesicles are attached do not contain IMPs. During mid-seed-fill, the plasma membrane undulates and becomes serrate in appearance. At physiological maturity, cross-fractures of lipid bodies can be observed in the plasma membrane.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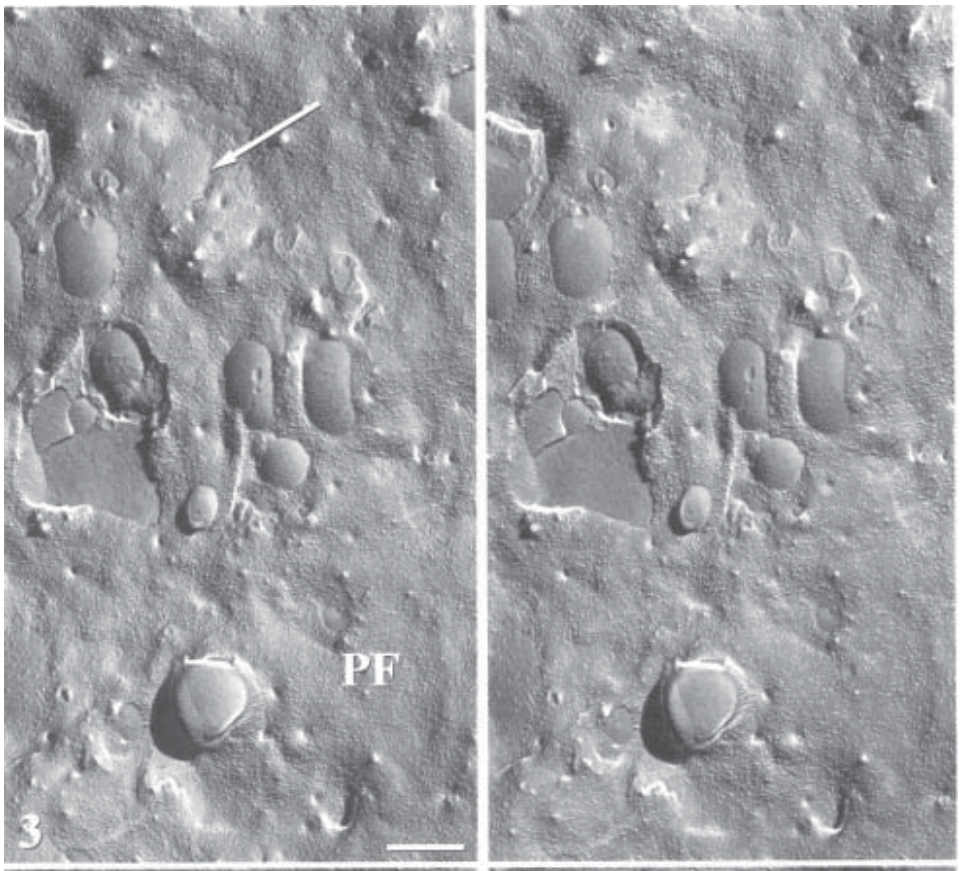


Figures 3 and 4. Stereo micrographs of freeze-fracture replica of the plasma membrane. Bars = 1 μm.

Figure 3. Membrane fractures and cross fractures of vesicles of the protoplasmic face (PF). Some of the membrane fractured vesicles contain intramembrane particles (arrow).

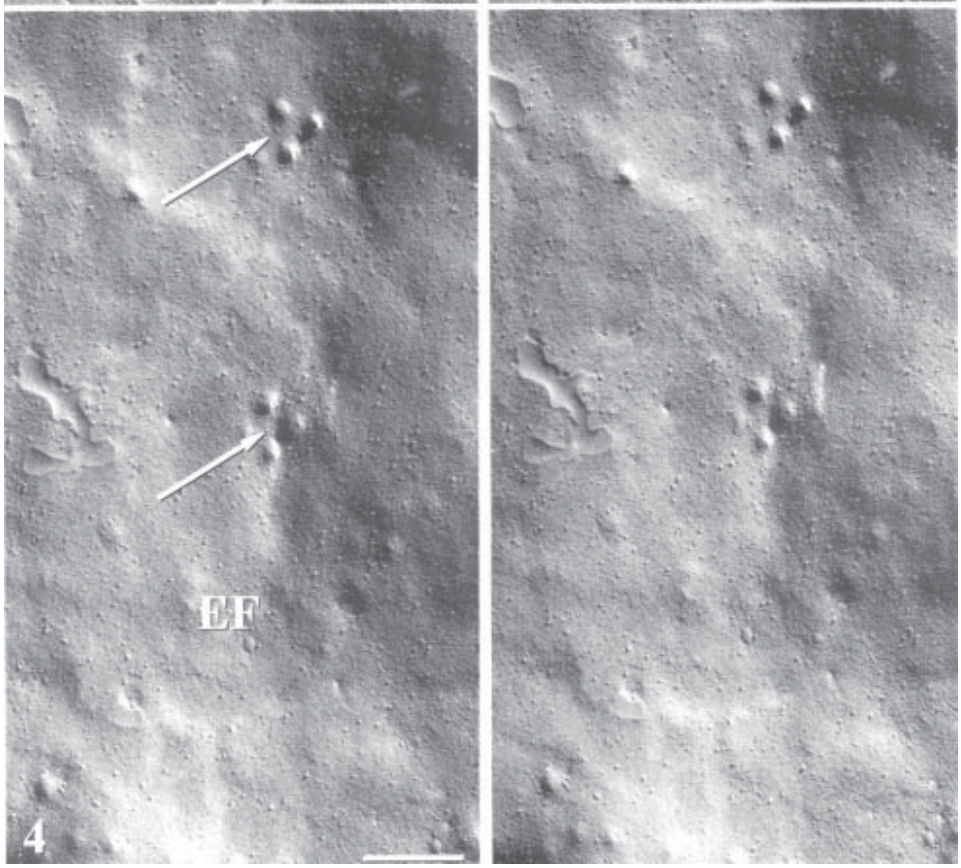
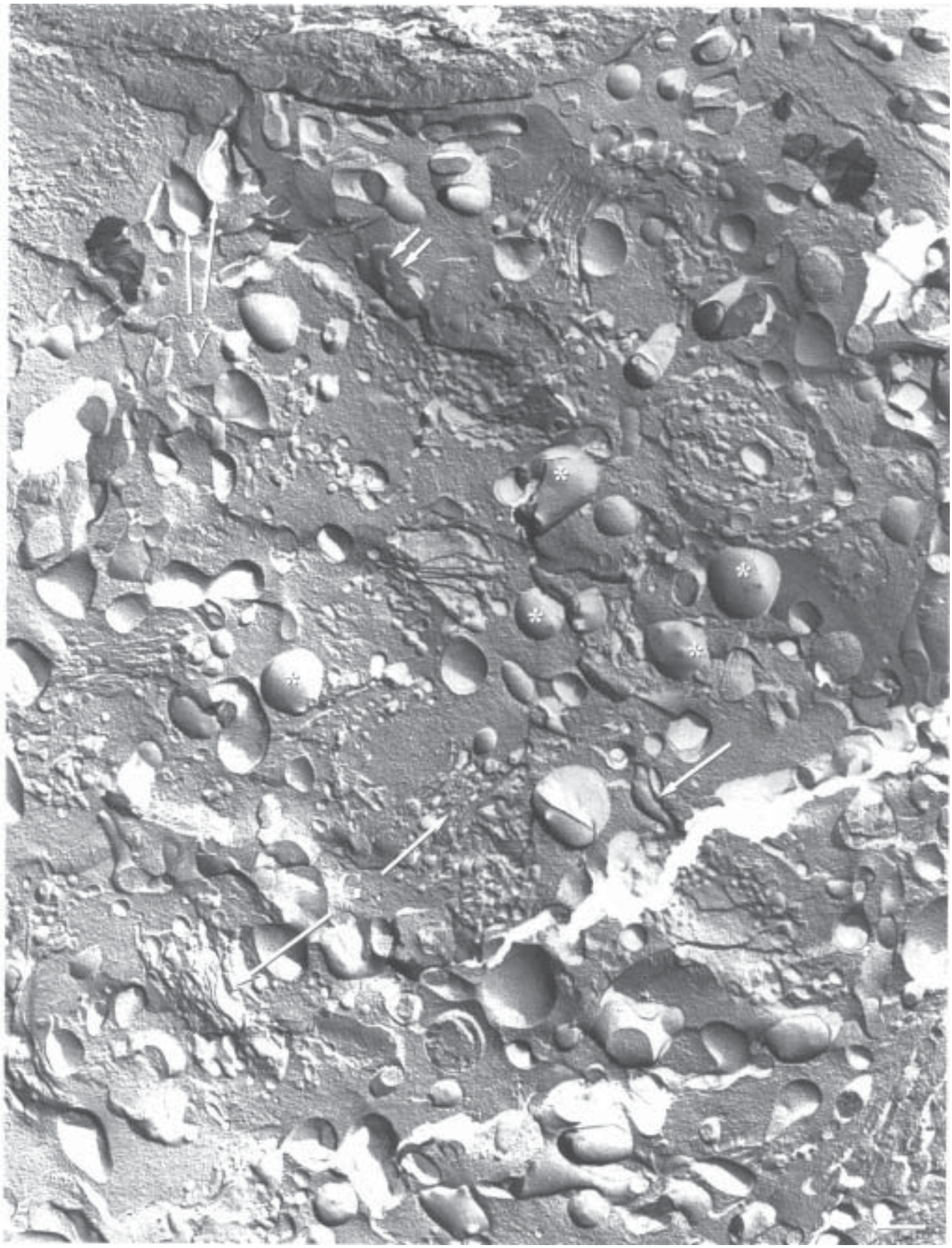
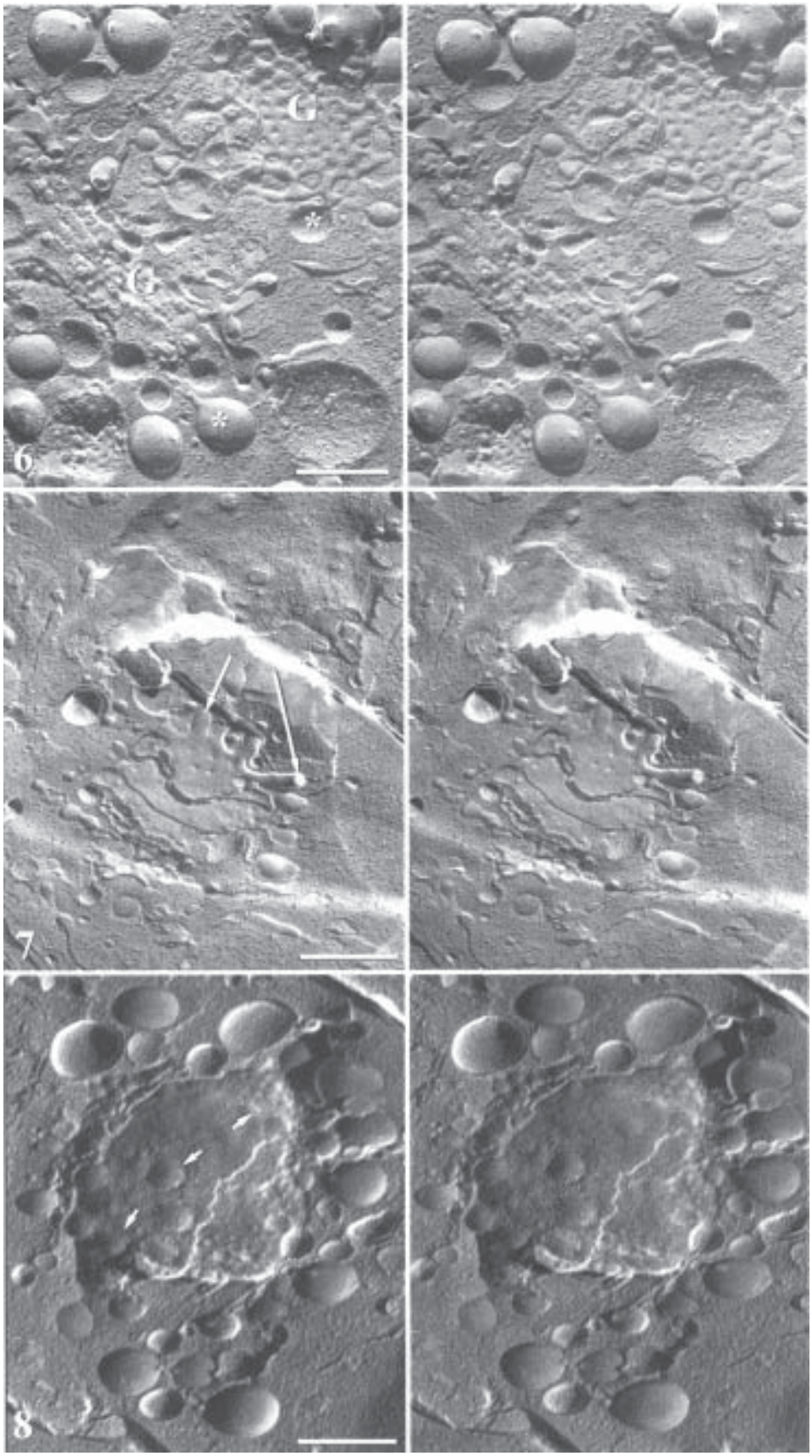


Figure 4. Region between adjacent filamentous cells illustrating plasmodesmata (arrows) on the ectoplasmic face (EF).

Figure 5 (on the facing page). Micrograph of freeze-fracture replica of a filamentous cell with numerous Golgi cisternae (G) and attached vesicles (V) at the plasma membrane. Tubules (single arrow) are attached to a Golgi cistern and endoplasmic reticulum (double arrow) is found apposed next to the Golgi cistern. Vesicles are present with fractured connections and protuberances (asterisks). Bar = 1 μm.





Figures 6-8. Freeze-fracture replicas illustrating Golgi of filamentous cells during seed fill.

Figure 6. Stereo micrograph of Golgi (G) *en face* (left) and cross section of a Golgi stack showing attached vesicles (asterisks). Bar = 0.5 μm.

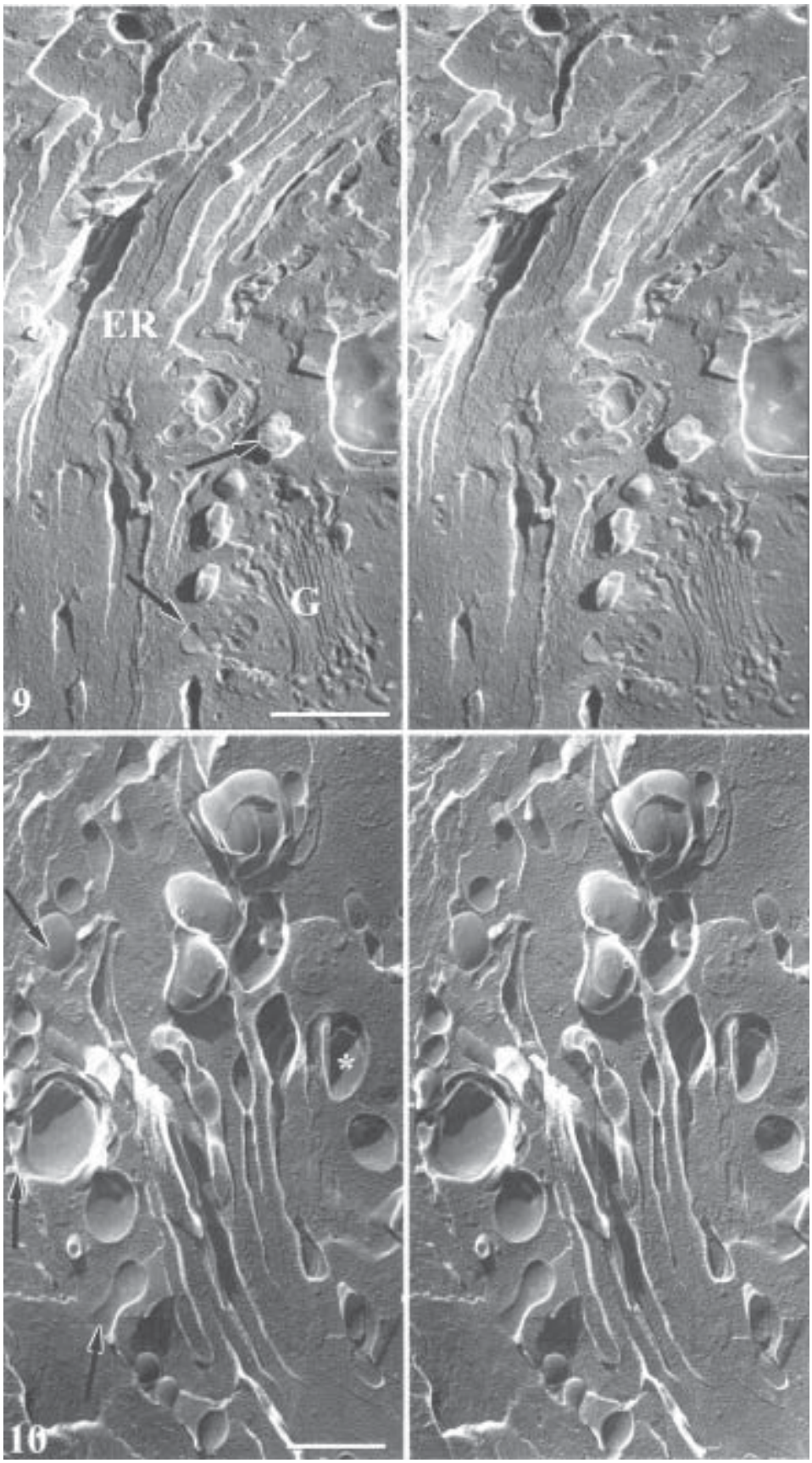
Figure 7. Stereo micrographs fractured normal to the cisternal stack. Tubules are attached to the cisternae (arrows). Bar = 1 μm.

Figure 8. Stereo micrograph fractured normal to the cisternal stack. Vesicles appear to be forming in the cisternae (arrowheads) and not in the fenestrated region. Bar = 1 μm.

Figures 9 and 10. Freeze-fracture replicas showing endoplasmic reticulum during seed fill.

Figure 9. Stereo micrograph of a filamentous cell showing a stack of endoplasmic reticulum (ER) and fractures of tubules (arrows). G = Golgi.

Figure 10. Stereo micrograph of a filamentous cell illustrating budding of vesicle (asterisk) from endoplasmic reticulum and vesicles attached at the plasma membrane (arrows). Bars = 1 μm.



Plasmodesmata are present in the plasma membrane and cell walls between adjacent filamentous cells and the subtending aleurone layer (Fig. 4). These structures provide symplastic continuity between adjacent filamentous cells and the subtending aleurone cells. The plasmodesmata are found singly and associated in groups of up to seven. No particular number or arrangement of plasmodesmata occurs within a group. Endoplasmic reticulum (ER) is found tightly apposed to the plasmodesmata and the plasma membrane.

Numerous Golgi cisternae occur in the antipit cells and are associated with aggregations of vesicles. Golgi cisternae occur in different shapes including crescent, C, or circular and are frequently located together near the endoplasmic reticulum (Fig. 5). The number of cisternae per stack varies from five to eight and displays different degrees of fenestration (Fig. 6). Cisternae are observed with tubelike projections as well as with the netted fenestrated appearance (Fig. 7). Fenestration becomes more prominent during seed fill when some cisternae contain swollen areas on or at the border of the fenestrae (Fig. 8). Fractures toward the end of seed fill exhibit mainly the fenestrated cisternae.

The endoplasmic reticulum is observed as lamellar and tubular forms (Fig. 9). The cisternae are frequently stacked and in close apposition to the Golgi cisternae. Large vesicles appear to be budding off stacked cisternae of ER (Fig. 10). ER is observed opposed to the plasma membrane throughout seed fill.

Vesicles are very prominent in the cytoplasm and are found attached to the plasma membrane. They appear to accumulate during the end of seed fill. Vesicle formation by the Golgi cisternae occurs either as finger-like projections or through fenestration and vesicle formation. Many vesicles have several residual out-growths, indicating multiple points of attachment to the Golgi cisternae during formation (Fig. 5). Vesicles are also observed attached to each other. Free vesicles around the Golgi cisternae range in diameter from 0.4 to 1.1 microns. Vesicles also appear to form at the periphery of the ER. The vesicles associated with ER are close to the plasma membrane. The ER is surrounded by large vesicles that are free or attached to the plasma membrane.

The antipit cells develop an extremely dense cytoplasm during active seed fill. In addition to the organelles described above, the cytoplasm contains mitochondria and stored reserves in amyloplasts, protein storage vacuoles, and lipid bodies. As in the case of aleurone cells, the lipid bodies tend to migrate to the plasma membrane and surround the protein storage vacuoles at the end of seed fill.

Discussion

The surface of the antipit is the complement of the surface of the pit, indicating that the cellular imprint is a direct result of pressure on a porous cell wall surface. This observation would explain the complementary nature and the "tongue and groove" appearance of the cotyledonary pit and the antipit of the seed coat. The "tongue and groove" appearance probably results from compression of the antipit tissue during growth and also from maturation and desiccation of the seed. The final size and shape of the antipit results from filling the cavity of the pit with porous, pliable cell wall.

Our observations demonstrate that the Golgi system was actively secreting vesicles and that the ER may also be producing vesicles. Vesicle formation was continuous throughout seed development, and an accumulation of vesicles occurred at the end of seed fill. We have no evidence that this accumulation of vesicles represents regulated exocytosis (Burgoyne and Morgan, 1993). The observations that vesicle formation in the Golgi system may occur at the periphery of cisternae, from cisternae with finger-like projections, and from swollen areas inside the fenestrae, indicated that Golgi cisternae may have different functions controlled by temporal regulation. TEM data suggested that the vesicles may differ because they stained differently. Fewer than 1% of the vesicles cross-fractured. However, those vesicles that did cross-fracture did not appear to show differences in their matrix. Therefore, the vesicles may be similar in membrane configuration.

Our results allow us to speculate on the functional role of the antipit for the developing embryo. The retention of the antipit indicates that it is not metabolized. The two soybean lines examined here and those examined in a previous study (Yaklich *et al*., 1989) exhibited considerable variation in the structure of the pit and antipit. The combined results showed that the antipit was found as a prominent structure in some soybean lines but almost non-existent or barely discernable in other lines of *G. max*. Seven other *Glycine* species that were examined in a previous study contained a well-developed antipit (Yaklich *et al*., 1989). *Glycine max* was the only species that contained lines with minimal antipit tissue, and interestingly, this is the only *Glycine* species that has been extensively cultivated and selected for modern agriculture.

The antipit may function in seed fill as suggested by the abundant organelles that are present. The filamentous cells are contiguous with the aleurone layer, and their cytoplasm is also connected symplastically through plasmodesmata (Yaklich *et al*., 1992). The aleurone cells also have abundant organelles and secrete vesicles that migrate to the cell wall. Unlike the filamentous cells, the aleurone layer encircled the inside surface of the seed coat, had prominent cell walls at maturity, and appeared to contain more ER. The apparent secretory activity of both of these cell types leads us to suggest that the cells have a nutritive function associated with seed development.

Our data suggest that the antipit cells may function in three ways, namely secreting compounds for the development

of the porous cell wall, secreting metabolites for the development of the embryo, and providing a source of storage reserves for maturation or subsequent germination of the embryo. Secretion must be associated with the development of the porous cell wall. This process may be constant throughout seed fill because the antipit fills the pit cavity. Furthermore, previous studies reported that the endosperm tissue and cotyledon surface are in close contact with each other (Yaklich *et al*., 1984, 1986). Rainbird *et al*. (1984) demonstrated that the seed coat is involved in the active transformation of compounds that are utilized by the embryo. These transformations include the metabolism of ureide and amino acids. The role of the antipit may be more passive but could involve nutrient flow to the developing embryo. The changes in structure of the porous cell wall between adjacent rows of filamentous cells indicates nutrient passage may occur through the antipit because the space within the porous cell wall increased with seed fill. Unless specific areas of the seed coat release nutrients, we can assume that at least part of the nutrients pass through the antipit to the developing embryo. Likewise, the ultrastructure of the cytoplasm of the filamentous cells and their interconnections with the aleurone by plasmodesmata would also signify that these cells are capable of symplastic transport of nutrients with the aleurone cells. An earlier report (Hsu *et al*., 1984) indicated that the antipit contained more glutamate synthase activity. We suggest that the filamentous cells either transform glutamate to glutamine or possibly function during embryo development by transforming amino acids for secretion to the developing embryo for protein synthesis. On a dry weight basis the seed coat metabolizes ureide more actively than the soybean pod tissue (Rainbird *et al*., 1984).

Finally, a characteristic of viable seed tissue capable of renewed metabolic processes during imbibition is the storage of food reserves during development. To this regard, the role of the aleurone tissue in the degradation of storage reserves in the germination of cereal grains has been documented. Whether the soybean aleurone and antipit tissue have a metabolic function at the initiation of imbibition is not known. The presence of storage reserves in the aleurone and antipit tissue suggests that they contribute to the germination of the embryo. This indicates that these tissues could resume metabolic activity during germination and thereby contribute to the expansion of the seed coat during imbition or other seed germination physiology.

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Editor's Note: All of the reviewer's concerns were appropriately addressed by text changes, hence there is no **Discussion with Reviewers**.