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Ultrastructure of Mature Embryos in the Parasitic Flowering Plant *Cuscuta japonica*

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The ultrastructural features of embryos were studied from mature dry and soaked seeds of the parasitic angiosperm *Cuscuta japonica*. Outer tangential walls in the protoderm cells were thickened and covered by a thin cuticle layer. These walls could play important roles in preventing water loss from the *Cuscuta* seedling surfaces after germination and in strengthening the surfaces against various environmental stresses. In the protoderm cells of soaked embryos, lipid materials were released into the thick outer walls through the fusion of lipid bodies with the plasma membrane. In the dry embryos were stored a large number of protein bodies with globoid crystals and lipid bodies. Numerous lipid bodies also were aligned under the plasma membrane. In both dry and soaked embryos, protein bodies were digested and transformed into small vacuoles. The degraded reserves of the lipid and protein bodies could then be mobilized to nourish subsequent germination and seedling growth. Proplastids in the soaked embryo cells contained a few thylakoids and electron-dense plastoglobuli, and crystallized phytoferritin. The phytoferritin, an iron-protein complex, would also be utilized in chloroplast development for autotrophic seedling growth.

Keywords: Cuscuta japonica, embryos, parasitic angiosperm, plastids, reserves, ultrastructure

The holoparasitic flowering genus *Cuscuta* depends on host plants to supply organic food materials, water, and minerals; its seedlings have chlorophyll especially at their tips (Dawson et al., 1994). Although the photosynthetic pigments and plastid genes within *Cuscuta* seedlings and stems, as well as the developmental structures of their haustoria, has been extensively studied, little has been reported concerning ultrastructure of *Cuscuta* embryos (Lyshed, 1992; Lee et al., 2000). Lyshede (1992) has described briefly that the ground meristem cells of *Cuscuta pedicellata* embryos are packed with lipid and protein bodies. Likewise, the shoot tips of mature embryo axes in *Cuscuta japonica* have only been briefly described at the light-microscopic level (Lee et al., 2000). Embryo cells in the seeds of many autotrophic plants are characterized by the presence of proplastids and a large number of storage materials, such as lipid and protein bodies (Rost, 1972; DeMason, 1986; Leprince et al., 1990; Perdomo and Burris, 1998; Cordova-Tellez and Burris, 2002; Walton and Wallace, 2005). These reserves are utilized during germination and seedling growth (Matsui et al., 1999; Rylott et al., 2001; Cornah et al., 2004). Therefore, the cytological study of embryos from *C. japonica* is essential to our understanding of seedling growth during the pre-parasitic or autotrophic stage. However, the ultrastructure of mature *Cuscuta* embryos has not been examined in detail. The aim of the present study is to analyze the fine structure of *C. japonica* embryos, and to relate those cytological features to future investigations of their autotrophic seedling growth. This report is the first part of a comprehensive study on the subsequent developmental structures of embryos, seedlings, and haustoria in *C. japonica*.

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MATERIALS AND METHODS

Plant Materials

Mature, dry seeds of dodder (*C. japonica* Choisy) were scarified with concentrated sulfuric acid for 45 min and rinsed in tap water, followed by distilled water for 30 min. For the soaking treatment, some were placed for 16 h on moist filter paper in Petri dishes under darkness in an incubator at 30°C. Embryos from the dry and the soaked seeds were then examined by electron microscopy.

Transmission Electron Microscopy

Embryo axes from dry and soaked seeds were sliced into approximately 1 mm^3 segments and pre-fixed in a mixture of 2.5% glutaraldehyde - 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 6.8), for 2 to 4 h at room temperature. Afterward, the materials were post-fixed in 1% osmium tetroxide, and buffered with 0.1 M sodium cacodylate buffer (pH 6.8) for 8 to 10 h at 4°C. The materials were washed in the buffer, and dehydrated in a graded acetone series before being embedded in Epon (Spurr, 1969). Thick sections were cut with an LKB-V ultramicrotome, stained with 0.05% toluidine blue, and examined under an Olympus BH2 light microscope (Japan). Thin sections, cut with an RMC MT-7000 ultramicrotome, were mounted on grids, and stained with uranyl acetate and lead citrate, then examined and photographed with a JEM 100 CXII transmission electron microscope (JEOL, Japan) at 80 kV.

RESULTS

Specimens of the embryo axes were transversely sectioned for an examination that mainly focused on the structural and quantitative aspects of reserves, e.g., lipid and protein bodies,

and inclusions within the plastids. It was believed that these reserves would be utilized for subsequent germination and seedling growth. Endosperm tissues surrounding the embryo were removed during preparations for the electron microscopy. The embryo axis comprised three types of primary meristem: one layer of protoderm cells, 4 to 6 layers of ground

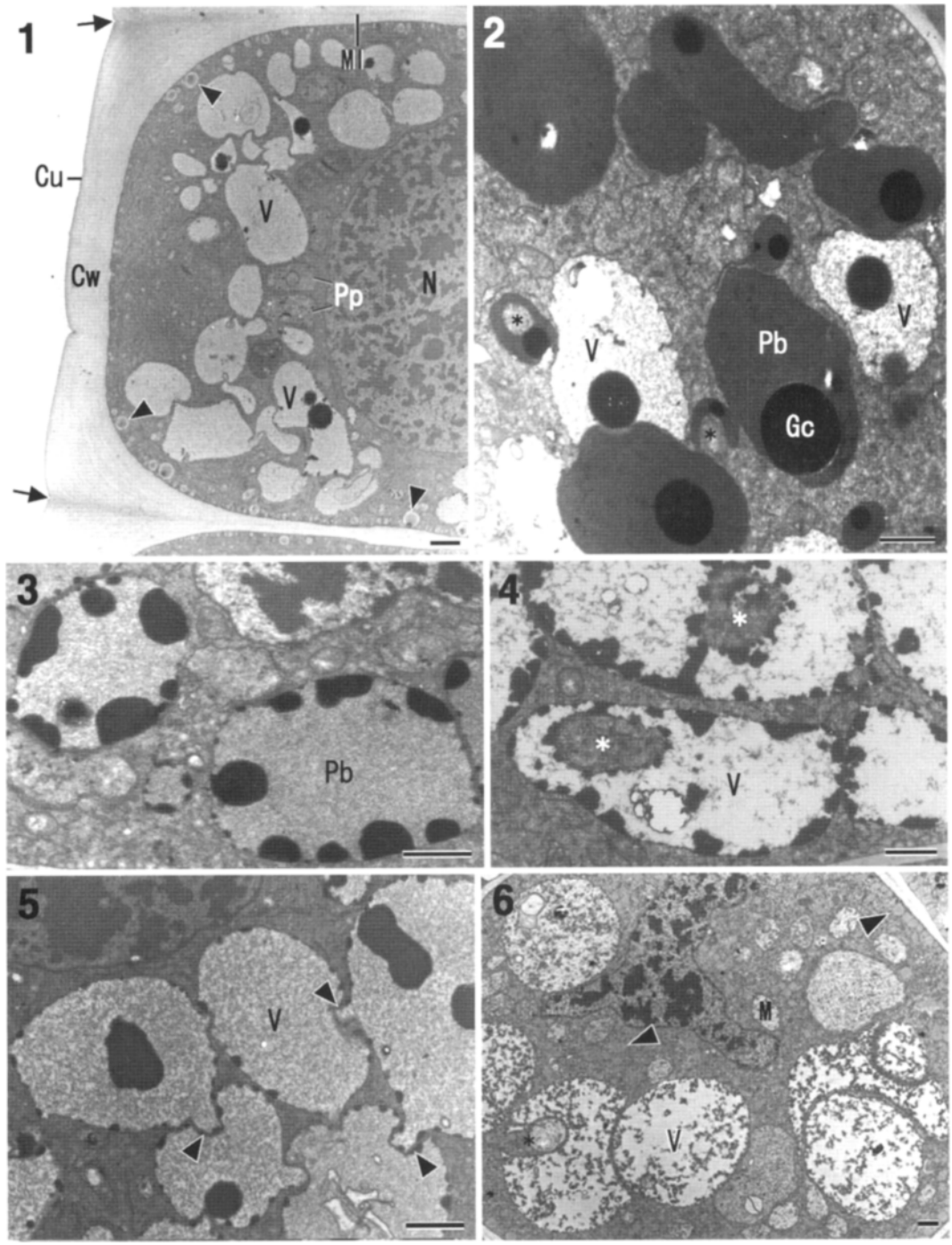

Figure 1-6. Electron micrographs of embryo cells from mature dry seed of *C. japonica*. 1-2, Protoderm cells. 1, Thick outer tangential wall (Cw) is covered by thin cuticle (Cu). Electron-dense wedges (arrows) in region where thick outer wall fuses with radial walls. Many small vacuoles (V) contain fine granules and electron-dense particles. Arrowheads indicate lipid bodies. Ml, middle lamella; N, nucleus; Pp, proplastid. Bar = 1.0 μm. 2-6, Ground meristem cells. 2, Outermost cells contain irregularly shaped protein bodies (Pb) with electron-dense globoid crystals (Gc) and small vacuoles (V) with those crystals. Cytoplasmic portions (asterisk) are sequestered in lobed region of Pbs. Bar = 1.0 μm. 3-5, Cells immediately within inner layer from outermost cells. 3, Digesting protein bodies (Pb) with fine granules and electron-dense materials along periphery. Bar = 1 μm; 4, Vacuoles (V) have electron-dense materials at periphery of tonoplasts. Cytoplasmic portions (asterisks) are sequestered by lobed vacuoles. Bar = 1.0 μm; 5, Small vacuoles (V) are fusing (arrowheads) with each other. Bar = 1.0 μm; 6, Cells in middle region of ground meristem have many small vacuoles (V) with fine fibrillar structures. Numerous lipid bodies (arrowheads) and mitochondria (M) are seen in cytoplasm, a portion (asterisk) of which is segregated in lobed region of vacuole. Bar = 1.0 μm.

meristem cells, and the procambium cells. The nuclei were generally prominent in these cells.

Cells from Dry Embryos

Protoderm cells had lipid bodies near the plasma membrane, plus proplastids and many small vacuoles with fine granular material and electron-dense particles (Fig. 1). These dense particles were similar to the globoid crystals observed in the outermost ground meristem cells (Fig. 2). The outer tangential walls of the protoderm cells were much thicker

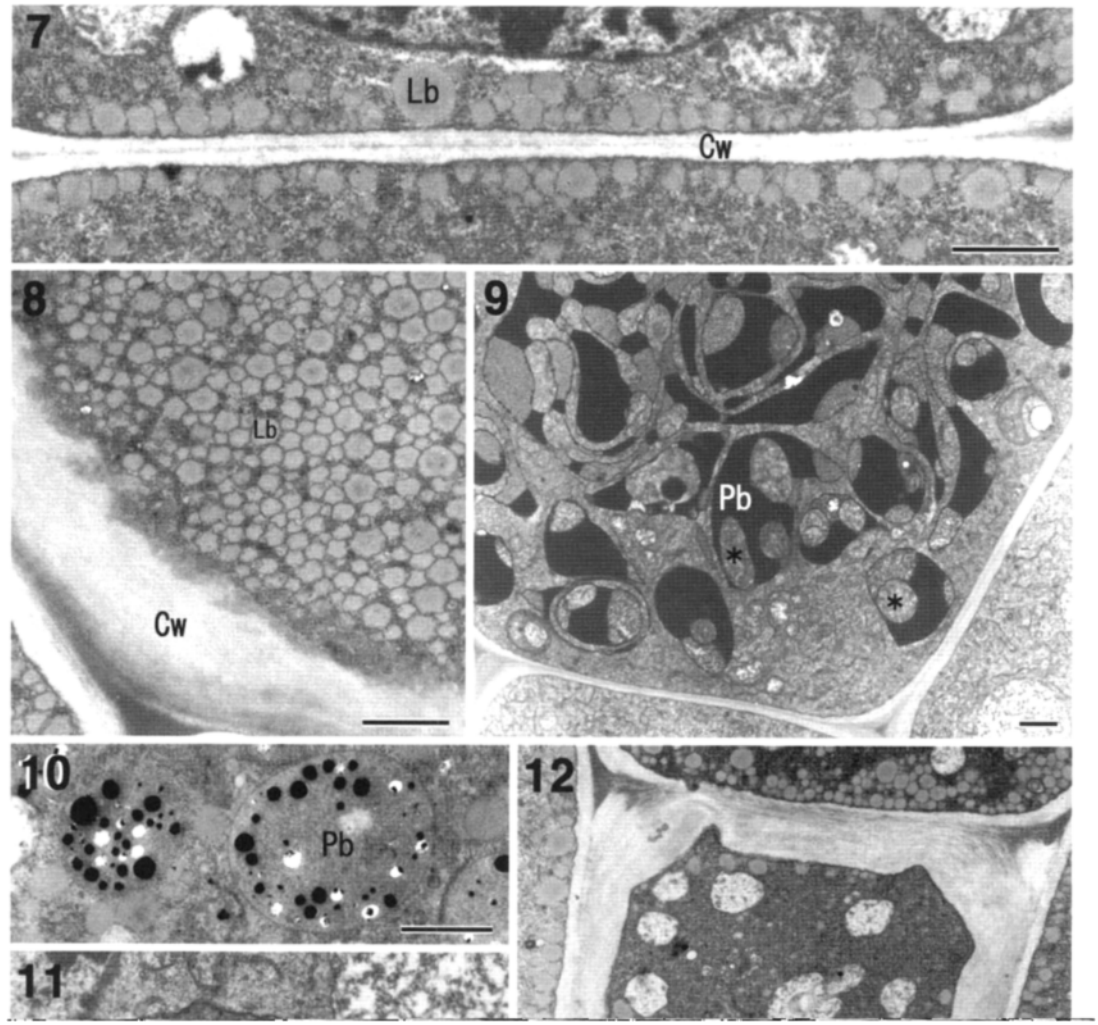

Figure 7-12. Electron micrographs of ground meristem cells in dry embryo of *C. japonica*. 7, In vacuolated cells, lipid bodies (Lb) are easily visible beneath plasma membrane. Cell walls (Cw) are transversely sectioned. Bar = 1 μm; 8, Section oblique to cell wall (Cw) shows large number of lipid bodies (Lb) arranged near plasma membrane. Bar = 1 μm; 9, Cells at innermost region of ground meristem adjacent to procambium have numerous protein bodies (Pb). Cytoplasmic portions (asterisks) are sequestered in lobed regions of Pb. Bar = 1.0 μm; 10, Cells adjacent to procambium have another type of protein body (Pb), with several small globoid crystals. Bar = 1.0 μm; 11, Most cells of dry embryo have polymorphic proplastids (Pp) with few thylakoids and electron-dense plastoglobuli. Bar = 1.0 μm; 12, Procambial cell is differentiating into xylary element (Xe) by thickening of secondary wall (Sw). Lipid bodies (Lb) in element are many fewer in surrounding ground meristem cells. Bar = 1.0 μm.

than those of the radial walls, and the outer surface was covered by a thin, electron-dense cuticle. Heavily stained wedges were present at the junction where the thick tangential wall fused with the radial walls, being continuous with the middle lamella in the latter (Fig. 1).

Ground meristem cells had a number of lipid bodies and small vacuoles with electron-dense structures. Here, the outermost cells or those immediately adjacent to the protoderm contained various-sized lipid bodies under the plasma membrane, as well as irregularly shaped protein bodies with electron-dense globoid crystals, and small vacuoles also with those crystals. Some protein bodies were lobed, and the cytoplasmic components were sequestered in that lobed region (Fig. 2). The cells just within the inner layer from the outermost ground meristem cells had numerous degrading protein bodies filled with fine granular materials (Fig. 3). These protein bodies were transformed into vacuoles (Fig. 4), with both the bodies and the electron-dense materials of the vacuoles being deposited at the periphery. The small vacuoles appeared to be fusing with each other (Fig. 5). Most cells in the ground meristem had several small vacuoles with fibrillar structures (Fig. 6). Cell organelles, including a large number of lipid bodies, occurred in most of the embryo cells. In particular, lipid bodies were arranged beneath the plasma membrane (Fig. 7). Such an alignment was confirmed via oblique sectioning of the cell wall (Fig. 8). A few cells, either at the innermost region of the ground meristem or adjacent to the procambium in cross sections of the embryo axis, were almost entirely filled with numerous protein bodies (Fig. 9).

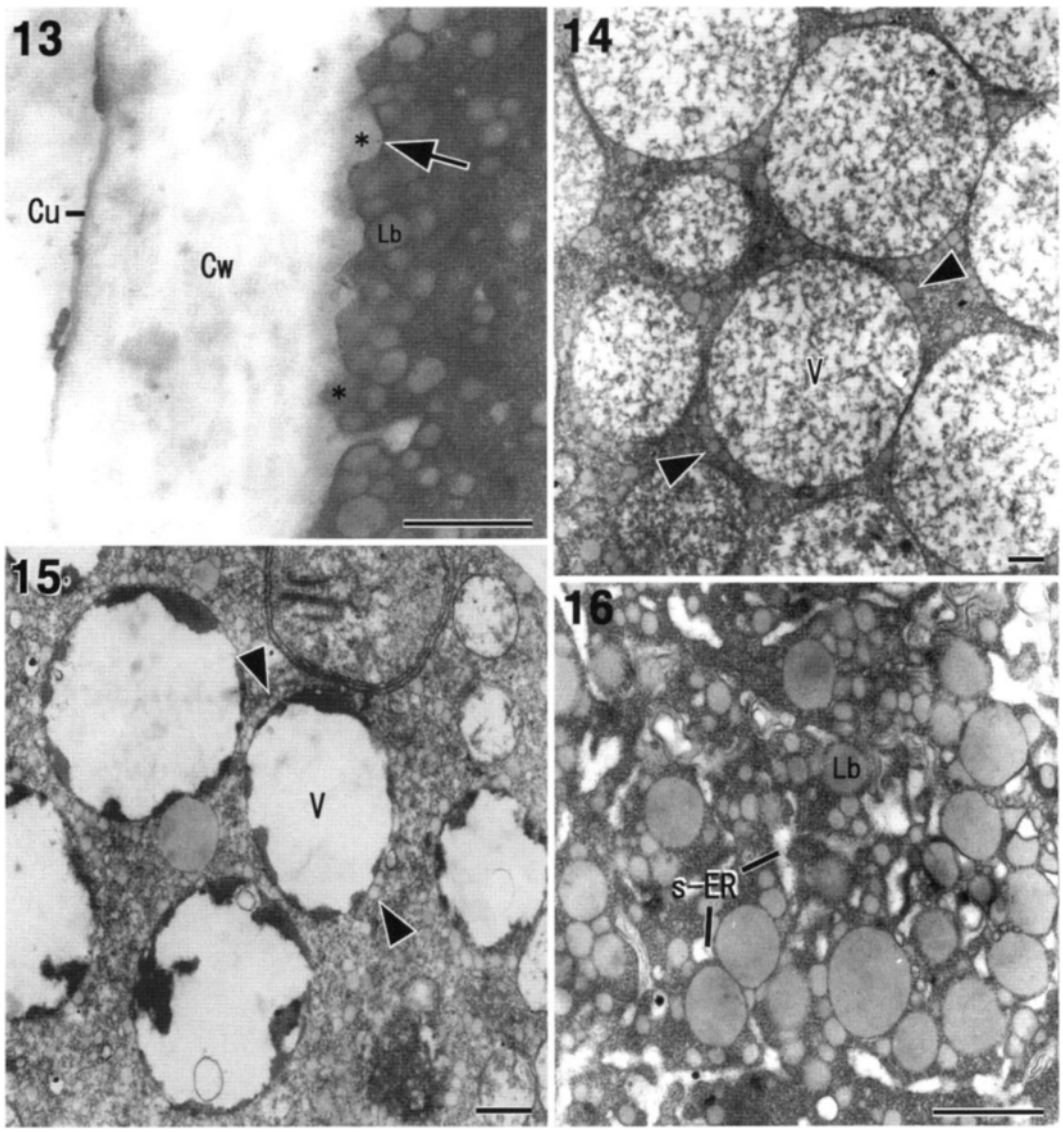

Figure 13-16. Electron micrographs of embryo cells from seeds of *C. japonica* soaked for 16 h. 13, Protoderm cells have numerous lipid bodies (Lb) near plasma membrane, which are fused with membrane (arrows); the latter show wavy or protruded regions (asterisks) with same density as lipid bodies. Contents of bodies are released into thickened outer tangential wall (Cw) covered by thin cuticle (Cu). Bar = 0.5 μm. 14-16, Ground meristem cells. 14-15, Most cells have several small vacuoles (V) filled with either fibrillar structures (Fig. 14) or electron-dense materials along periphery of tonoplast (Fig. 15). Small lipid bodies (arrowheads) are seen around vacuoles. Bars = 1.0 μm in Figures 14 and 15; 16, Some cells are filled with innumerable lipid bodies (Lb) on smooth endoplasmic reticulum (s-ER). Bar = 1.0 μm.

These bodies varied in shape and size, exhibiting lobed regions in which portions of the cytoplasm were frequently contained, as in Figure 2. However, these bodies had no globoid crystals.

The ground meristem cells around the procambium had another type of protein body, which contained several small, electron-dense globoid crystals (Fig. 10). In the dry embryo, the proplastids were varied in their morphology, having few thylakoids and electron-dense plastoglobuli (Fig. 11). In the central region of the embryo axis, a procambial cell wall was thickened by the deposition of secondary wall materials; these xylary cells had many fewer lipid bodies than in the ground meristem cells surrounding itself (Fig. 12).

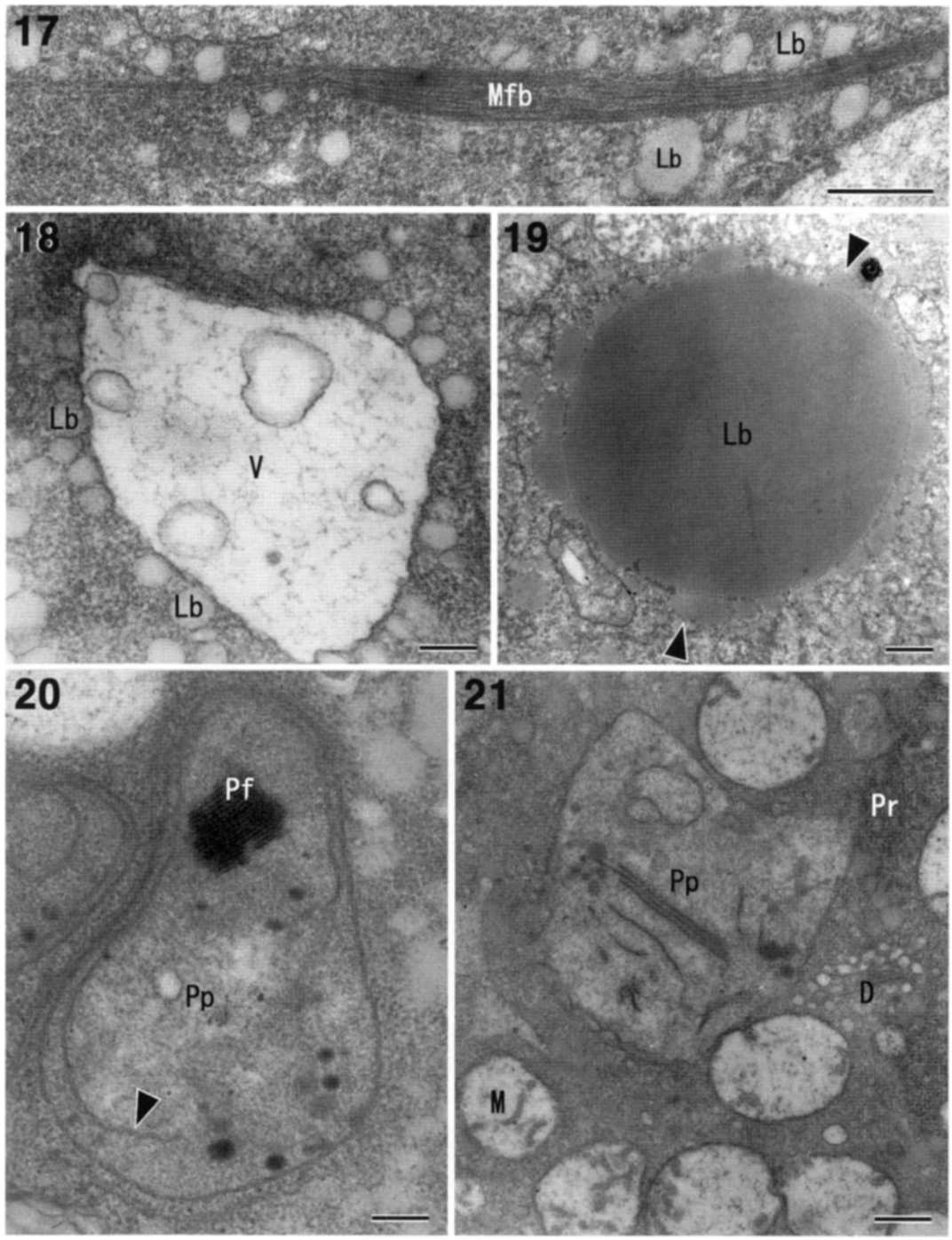

Figure 17-21. Electron micrographs of ground meristem cells from embryos of *C. japonica* seed soaked for 16 h. 17, Lipid bodies (Lb) are closely associated with microfilament bundle (MFB). Bar = 1.0 μm; 18, Lipid bodies (Lb) seem to be incorporated into vacuole (V). Bar = 0.5 μm; 19, Large lipid body (Lb) is surrounded by several small bodies (arrowheads). Bar = 1.0 μm; 20, Proplastids have crystallized phytoferritin (Pf), a few thylakoid membranes (arrowheads), and electron-dense plastoglobuli. Bar = 0.25 μm; 21, Most cells contain cell organelles including dictyosomes (D), mitochondria (M), polyribosomes (Pr), and proplastids (Pp). Bar = 0.5 μm.

Cells of Soaked Embryos

The cytological features of embryo cells that had been pre-soaked for 16 h were generally similar to those of the dry-embryo cells, but with some obvious changes in their cellular structure. In the protoderm cells, numerous lipid bodies were arranged beneath the plasma membrane, releasing their enclosed material into the thickened outer tangential wall (Fig. 13).

In most cells of the ground meristem, intact protein bodies (Fig. 2) were not visible, but several small vacuoles were seen. These vacuoles were filled with fine fibrillar materials (Fig. 14) and contained electron-opaque substances along the tonoplast (Fig. 15). Small lipid bodies were present around these vacuoles. Many protein bodies similar to those in the dry embryo (Fig. 10) also were found in the cells at the innermost region of the ground meristem. Several of those cells were packed with innumerable lipid bodies on the smooth endoplasmic reticulum (Fig. 16), some of which were closely associated with a microfilament bundle (Fig. 17). Those lipid bodies also appeared to be incorporated into the vacuoles (Fig. 18), and were often aggregated among themselves. The larger lipid bodies were closely surrounded by several smaller bodies (Fig. 19). Proplastids contained crystallized phytoferritin, a few thylakoid membranes, and electron-dense plastoglobli, but they had no starch grains (Fig. 20). Most cells exhibited organelles, including dictyosomes, mitochondria, and polyribosomes, in greater numbers than those counted in the dry-embryo cells (Fig. 21).

DISCUSSION

Walls of Protoderm Cells

The protoderm cells in *C. japonica* embryos were characterized by thick, outer tangential walls that were covered by a thin cuticle layer. These features are similar to those reported for 7-day-old *C. pedicellata* seedlings (Lyshede, 1989). It is thought that those thick outer walls aid in preventing water loss from the *Cuscuta* seedling surfaces after germination and in strengthening the surfaces against mechanical stresses from the environment.

In the protoderm cells of soaked embryos, numerous lipid bodies near the plasma membrane appeared to release their contents into the thick outer walls by fusing with that membrane. These observations are consistent with previous reports, i.e., that the derivatives of lipids are the major structural component of a secreted plant cuticle (Tanaka et al., 2001; Suh et al., 2005). Kunst and Samuels (2003) have also demonstrated that one transport mechanism is the direct transfer of cuticular lipids to the plasma membrane. Likewise, Pighin et al. (2004) have found that the ABC (adenosine triphosphate binding cassette) transporter protein, which is localized in the plasma membrane, exports lipids from the epidermal cells to the plant surface. A thin cuticle may ensure that the seedling is not readily drowned under wet soil conditions (Lyshede, 1989). Concurrently, the cuticle layer that overlays the outer wall surfaces of the protoderm may act as a vital hydrophobic barrier over the aerial surfaces of *C. japonica* during germination and seedling growth, thereby limiting nonstomatal water loss (Riederer and Schreiber, 2001; Nawrath, 2006) and protecting the seedlings from chemicals and biotic aggressors (Müller and Riederer, 2005).

The radicle in *Cuscuta* plants is degenerated after germination and the seedling surface becomes covered by the cuticle (Lyshede, 1985), potentially causing a problem with water absorption. The wedges in the region where the thick outer wall fuses with the radial walls are considered pectinaceous because they are continuous with the middle lamella in the radial cell walls. This pectic substance is abundant in the cell wall underneath the cuticle (Jeffree, 1996) and may be synthesized to overcome mechanical stress between fused cell walls (Sieber et al., 2000). Nevertheless, although this cuticle layer is thought to be a preventive barrier to water loss, its permeability has been demonstrated (Kerstiens, 1996; Helbsing et al., 2000). Therefore, it is conceivable that water from fog or dew that is taken up across the cuticle enters the *Cuscuta* seedling through those hygroscopic pectin-containing wedges in the protoderm cell walls.

Reserves

Protoderm and ground meristem cells in *C. japonica* embryos contained innumerable lipid and protein bodies. These reserves have also been observed in many other species (Rost, 1972; Coimbra and Salema, 1994; Pergo et al., 1998; Walton and Wallace, 2005). For example, Lyshede (1992) has reported that the ground meristem cells of *C. pedicellata* embryos are packed with lipid and protein bodies. In the current study, lipid bodies in the dry embryos were frequently aligned along the plasma membrane. This phenomenon also has been found in plant embryos during seed drying and maturation (DeMason, 1986; Leprince et al., 1990; Misra et al., 1993). Lipid bodies migrate toward the cell wall when maize seeds are exposed to drying conditions (Perdomo and Burris, 1998; Cordova-Tellez and Burris, 2002), such lipid alignment next to the cell walls may change cell water relations, leading to more organized dehydration during seed drying. Lipid bodies were closely associated with and incorporated into vacuoles. They may become engulfed or autophagocytized by the vacuoles in embryo cells, as in germinating *Ricinus* seeds (Schwarzenbach, 1971). Vegetative vacuoles that are transformed from protein bodies or protein storage vacuoles function as lytic compartments containing various hydrolases (Marty, 1999). Lipolytic enzyme activity increases during the mobilization of storage lipids in germinating seeds (Matsui et al., 1999; Rylott et al., 2001; Cornah et al., 2004).

In the *Cuscuta* ground meristem studied here, protein bodies with globoid crystals were frequently seen in the outermost and innermost cells, whereas those bodies were digested and vacuolated in the remaining cells. These protein bodies have proteases and become degraded in the storage cells (Bethke et al., 1998; Jiang et al., 2001), after which the amino acid reserves can be mobilized to nourish the embryos and seedlings (Müntz et al., 2001; Shutov et al., 2003). During germination, storage protein bodies are transformed into vegetative vacuoles in the seed reserve tissues (Rost, 1972; Bewley, 1997; Herman and Larkins, 1999; Marty, 1999). Here, globoid crystals were found in the pro-

tein bodies, as has been reported for many other plant embryos (Maldonado and Lott, 1991; Coimbra and Salema, 1994; Pergo et al., 1998; Walton and Wallace, 2005). Electron-dense particles were detected in several small vacuoles of the protoderm cells (Fig. 1). These were thought to be the globoid crystals that remained after the protein bodies were degraded. Previous energy-dispersive X-ray analysis has revealed that these globoid crystals contain mineral nutrients, e.g., P, K, and Mg, in the protein bodies of seeds in many species (Maldonado and Lott, 1991; Pergo et al., 1998; Maroder et al., 2003). Phytate is the most common iron storage compound, typically being located in the globoid crystals of seed protein bodies. Thus, at the embryo stage, the crystals that comprise minerals may remain in an insoluble state in the vacuoles of some cells.

Proplastids

Proplastids in the dry embryo cells had no phytoferritin and fewer thylakoids and plastoglobuli than those containing phytoferritin in the soaked embryo cells. However, phytoferritin was absent from the seedling plastids (unpublished data). Plant cells may store iron in the form of phytoferritin, an iron-protein complex, in the plastid stroma (Gunning and Steer, 1996). This protein is more commonly deposited under dark or low-light growing conditions (Reid et al., 1998), when photosynthesis is inactive (Wildman and Hunt, 1976). Phytoferritin plays a role in iron metabolism, with a dual function of detoxification and reserve (Harrison and Arosio, 1996).

To conclude, the thick outer tangential walls in the protoderm cells of *C. japonica* embryos were covered with a thin cuticle, which may have prevented water evaporation from the *Cuscuta* seedling surfaces after germination and have strengthened the surfacces against mechanical and environmental stresses. In the protoderm cells of soaked embryos, the secretion of lipid materials into those thick outer walls produced this cuticle. Here, lipid and protein bodies stored in the embryo cells were degraded, then mobilized to nourish the germinating embryo and support subsequent seedling growth.

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