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Ultrastructure and Development of Seedlings of the Parasitic Weed *Cuscuta japonica*

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Shoot subapical cells in the parasitic angiosperm *Cuscuta japonica* seedlings were ultrastructurally studied. Seedlings were grown for 3 d in the dark and then for an additional 3 d in sunlight. Under either type of illumination, most cells in the primary meristem contained several vacuoles with or without electron-dense particles. These vacuoles were believed to be derived from degraded protein bodies with globoid crystals that were stored in the embryos. As growth progressed, the reserves were gradually depleted, while various cell organelles increased. This indicated that those storage reserves were utilized for seedling development and that, concurrently, cellular metabolism in the seedling cells converted from a quiescent to an active state. When seedlings were exposed to sunlight, etioplasts with prolamellar bodies developed into chloroplasts possessing thylakoids that were well-organized into grana. These observations suggest that *C. japonica* seedlings might exist autotrophically and photosynthesize during a free-living stage prior to parasitizing their hosts.

Keywords: chloroplast, *Cuscuta japonica*, parasitic angiosperm, seedling, ultrastructure

Members of the parasitic angiosperm *Cuscuta* have no leaves and roots, so they must obtain organic nutrients and water from their host plants (Parker and Riches, 1993; Dawson et al., 1994). Neither chlorophyll and thylakoids nor the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) has been detected in post-parasitic *Cuscuta europaea* (Machado and Zetsche, 1990), *Cuscuta grandiflora*, and *Cuscuta odorata* (van der Kooij et al., 2000). Although the deletion of photosynthetically related genes has been found in the post-parasitic *C. europaea* (Freyer et al., 1995), several *Cuscuta* species do contain chlorophyll, especially in the tips of the seedlings (Panda and Choudhury, 1992; Dawson et al., 1994) and various species are capable of photosynthesis. For example, photosynthetic pigments have been detected in the pre-parasitic seedlings and parasitic stems of *Cuscuta campestris* (Dinelli et al., 1993). Several photosynthetic proteins, including Rubisco, have been found in the stems of parasitic *Cuscuta reflexa* (Hibberd et al., 1998) and in the pre-parasitic seedlings and parasitic stems of *Cuscuta pentagona* (Sherman et al., 1999). Finally, photosynthetic plastid genes have been reported in parasitic *Cuscuta* stems (Machado and Zetsche, 1990; van der Kooij et al., 2000; Revill et al., 2005). Thus, this genus cannot strictly be considered only a holoparasite.

Lyshede (1985) has made anatomical observations of chloroplasts with starch grains in the epidermis and cortex of *Cuscuta pedicellata* seedling tips. In the cells of 7-d-old *C. pedicellata* seedling tips, the plastids in young epidermal cells contain a few electron-dense thylakoids and small vesicles, and young cortical cells have plastids filled with large starch grains (Lyshede, 1989). Most developed chloroplasts have well-organized granal thylakoids in *C. pentagona* seedling tips grown for up to 10 d during their pre-parasitic stage (Sherman et al., 1999). Seedling tips in *Cuscuta japonica* also have been examined at various growth stages (Lee et al., 2000). For example, the shoot subapical cells of 3-d-old dark-grown seedlings and 6-d-old seedlings (grown under sunlight for an additional 3 d after that dark period) contain abundant starch-containing amyloplasts. Furthermore, the mature embryo cells from that species have a reserve of numerous lipid and protein bodies, as well as proplastids with a few thylakoids and phytoferritin (Lee, 2006).

The aim of the present study is to describe the ultrastructural changes that occur at various developmental stages when seedlings of *C. japonica* are grown in either darkness or sunlight. This investigation into the relationship of those structural features to subsequent autotrophic growth is part of a comprehensive study on the development of embryos, seedlings, and haustoria from *C. japonica*.

MATERIALS AND METHODS

Plant Materials

Mature, dry seeds of *C. japonica* Choisy were scarified with concentrated sulfuric acid for 45 min, and rinsed in tap water followed by distilled water. They were placed on moist filter paper in Petri dishes and germinated in the dark in an incubator at 30°C. The roots on some 3-day-old dark-grown seedlings were then wrapped with wet cotton and placed in 500-mL covered beakers, and those plants were exposed to sunlight from nearby windows for an additional 3 d. These 3- and 6-d-old seedlings were then sampled for examination by transmission electron microscopy.

Transmission Electron Microscopy

Shoot subapical portions of the seedlings were sliced into approx. 1-mm^3 segments and pre-fixed for 2 to 3 h at room temperature in a mixture of 2.5% glutaraldehyde - 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 6.8). They were then exposed twice to microwave radiation for 10 and 20 s at 70% of the maximum 800-watt voltage in a Laboratory Microwave Processor (Pelco Model 3450; Ted

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Pella, USA) that was equipped with a thermistor copper temperature probe and an auxiliary Microwave Load Cooler (Pelco 3420; Ted Pella). Tissues were post-fixed in 1% osmium tetroxide, buffered at pH 6.8, and microwaved three times for 40 s each. Segments were then washed in buffer and dehydrated in a graded series of acetone (30, 50, 70, 90, and 100%), at 40 s for each step, in a microwave oven at 37°C. The tissue pieces were then infiltrated and embedded with Spurr's resin (Spurr, 1969). Thick sections cut with an LKB-V ultramicrotome were stained with 0.05% toluidine blue, and examined under an Olympus (USA) BH2 light microscope. Thin sections cut with a RMC MT-7000

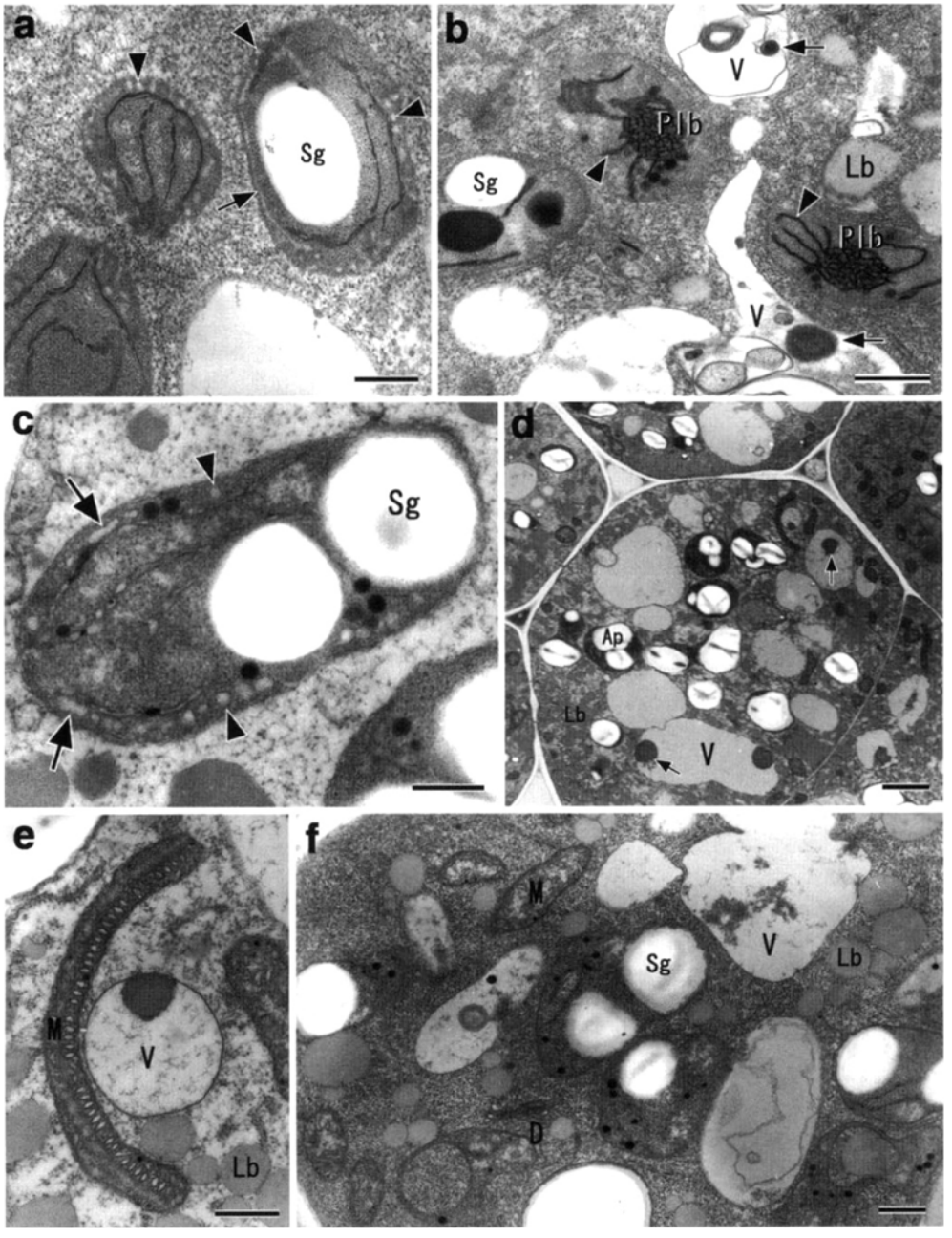

Figure 1. Electron micrographs of shoot subapical cells from 3-d-old *C. japonica* seedlings grown under darkness. (**a-b**) Protoderm cells. (**a**) Plastids have a few electron-dense thylakoids and starch grains (Sg). Several small vesicles (arrowheads) and electron-translucent thylakoid (arrow) are shown at periphery. Bar = 0.5 μm. (**b**) Etioplasts have prolamellar bodies (Plb) to which electron-dense thylakoids (arrowheads) are continued. Vacuoles (V) possess electron-dense particles (arrows) and membranous structures. Lb, lipid body; Sg, starch grain. Bar = 0.5 μm. (**c-f**) Ground meristem cells. (**c**) In cells at outer region of ground meristem, plastids have small vesicles (arrowheads) along periphery and several electron-dense plastoglobuli. Vesicles appear to fuse with each other to form thylakoids (arrows). Sg, starch grain. Bar = 0.5 μm. (**d**) Cells in middle region of ground meristem have several small vacuoles (V) with electron-dense particles (arrows), starch-containing amyloplasts, and lipid bodies (Lb). Bar = 2 μm. (**e**) Elongated mitochondrion (M) with well-developed cristae. Small vacuole (V) includes electron-dense particles. Lb, lipid body. Bar = 0.25 μm. (**f**) Several cells in middle region of ground meristem have such organelles as ribosomes, dictyosomes (D), mitochondria (M), and vacuoles (V) with granular and fibrillar materials. Plastids have starch grains (Sg), a few thylakoid membranes, and electron-dense plastoglobuli. Lb, lipid body. Bar = 1 μm.

ultramicrotome were mounted on grids, stained with uranyl acetate and lead citrate, and examined and photographed with JEM l00 CXII or Hitachi (Japan) H-7600 transmission electron microscopes, at 80 kV.

RESULTS

The transversely sectioned portions of shoot subapices from *C. japonica* seedlings consisted of three tissue types: protoderm, ground meristem, and procambium. Most of the primary meristem cells contained conspicuous nuclei.

Cells from 3-D-Old Seedlings

The shoot tips of 3-d-old seedlings were hooked and yellowish. In their subapical regions, the protoderm cells had thick outer tangential walls that were identical to those pre-

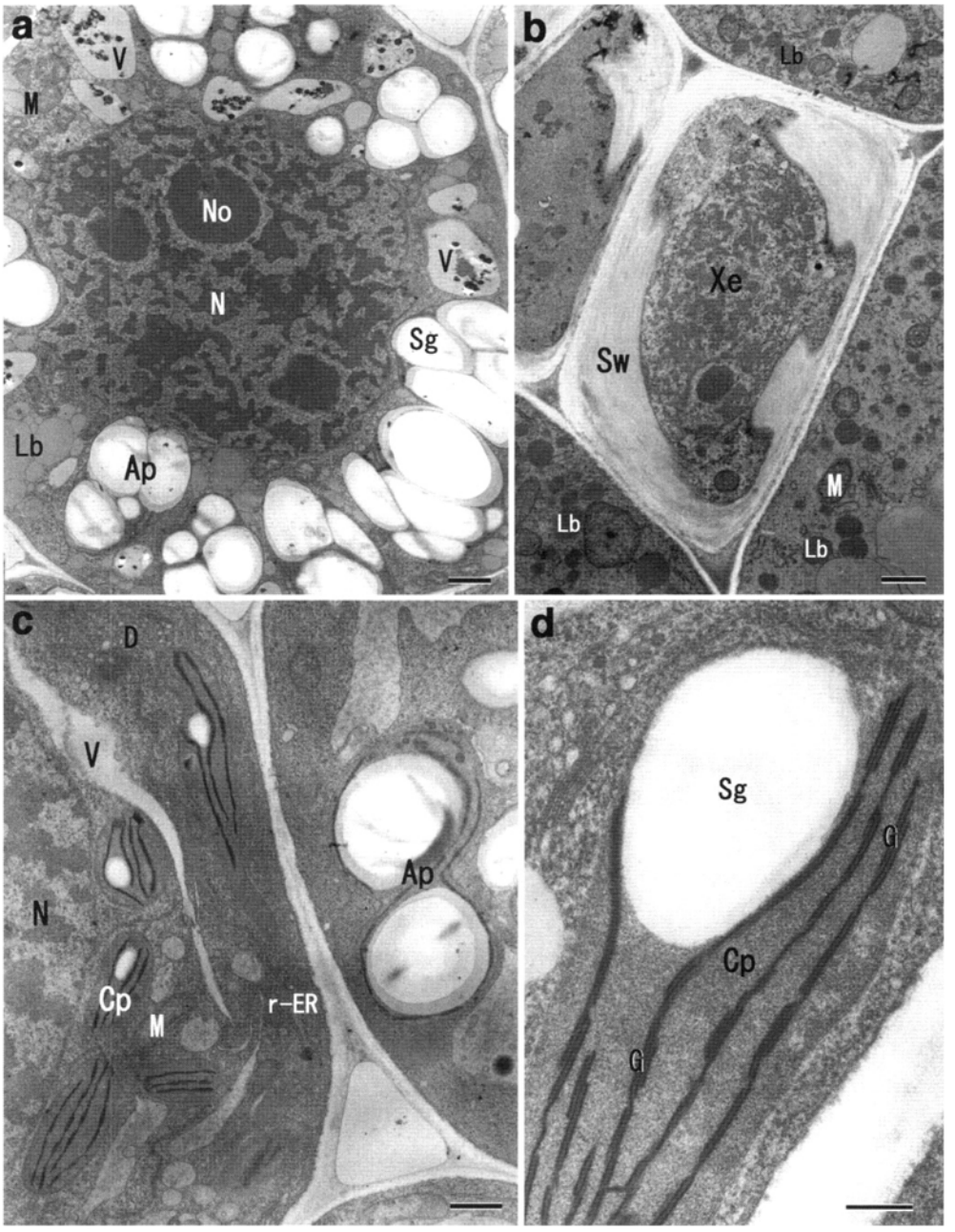

Figure 2. Electron micrographs of shoot subapical cells from 3-d-old dark-grown seedlings (**a-b**) and 6-d-old plants (**c-d**) grown for additional 3 d under sunlight. (**a**) Cells at innermost region of ground meristem have many amyloplasts (Ap) filled with starch grains (Sg), several small vacuoles (V) with electron-dense granules, lipid bodies (Lb), and mitochondria (M). N, nucleus; No, nucleolus. Bar = 1 μm. (**b**) Xylary elements (Xe) differentiated from procambium cells in central region of shoot subapex have thickened secondary walls (Se). Ground meristem cells surrounding Xe have many lipid bodies (Lb). M, mitochondria. (**c**) Protodermal cells (at left) have dense cytoplasm containing dictyosomes (D), mitochondria (M), rough endoplasmic reticulum (r-ER), and vacuoles (V). Chloroplasts (Cp) possess starch grains (Sg) and electron-dense thylakoids. Outermost ground meristem cell (at right) has constricted amyloplast (Ap) with starch grains. N, nucleus; V, vacuole. Bar = 1 μm. (**d**) Grana (G) stacks of chloroplast (Cp) consist of two to three thylakoids with lumens darkly stained. Sg, starch grain. Bar = 0.25 μm

viously sampled from soaked and dry embryos (Lee, 2006). Plastids contained a few electron-dense thylakoids, starch grains, and many small vesicles and electron-translucent thylakoids at their peripheries (Fig. 1a). The protoderm cells possessed etioplasts with a few starch grains and prolamellar bodies from which extended electron-dense thylakoids. These cells had partially developed vacuoles with electron-dense particles and membranous structures (Fig. 1b). In cells from the outer region of the ground meristem, some plastids contained thylakoids that appeared to have been formed by the fusion of small vesicles in the peripheral region (Fig. 1c). Cells in the middle region of the ground meristem had several small vacuoles with electron-dense particles, amyloplasts with starch grains, and numerous lipid bodies (Fig. 1d). Elongated mitochondria with well-developed cristae also were sporadically found (Fig. 1e). Many cells in the ground meristem possessed various cell organelles in the dense cytoplasm, including dictyosomes, mitochondria, polyribosomes, vacuoles with granular and fibrillar materials, and plastids with a few starch grains, thylakoids, and elec-

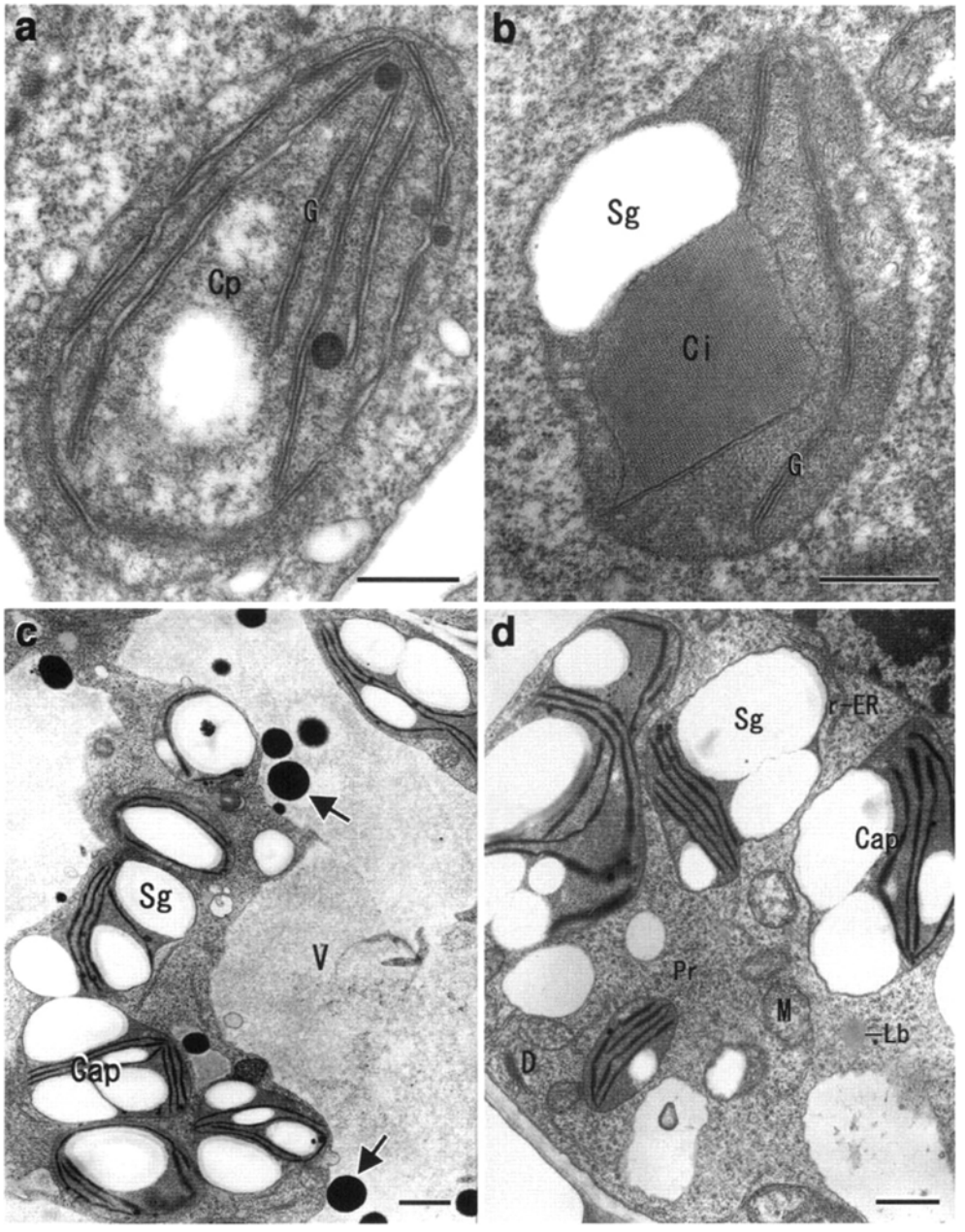

Figure 3. Electron micrographs of shoot subapical cells from 6-d-old seedlings. (**a-b**) Plastids in protoderm cells. (**a**) Chloroplast (Cp) has thylakoids well-organized into grana (G). Note that thylakoid lumens are electron-translucent. Bar = 0.5 μm. (**b**) Plastid contain thylakoids organized into grana (G), starch grains (Sg), and crystalline inclusion (Ci). Bar = 0.5 μm. (**c**) Cells in outermost ground meristem contain numerous chloroamyloplasts (Cap) with starch grains (Sg) and electron-dense thylakoids, and have large vacuoles (V) with several electron-dense particles (arrows). Bar = 1 μm. (**d**) Cells in middle region of ground meristem have various cell organelles, including dictyosomes (D), lipid bodies (Lb), mitochondria (M), polyribosomes (Pr), and rough endoplasmic reticulum (r-ER). Chloroamyloplasts (Cap) possess starch grains (Sg) and electron-dense thylakoids. Bar = 1 μm.

tron-dense plastoglobuli (Fig. 1f). Cells at the innermost region of the ground meristem contained many starch-filled amyloplasts and small vacuoles with several small, electron-dense granules (Fig. 2a). In the central region of the shoot subapices, the xylary elements differentiated from procambial cells had thickened secondary walls and contained fewer lipid bodies than in the surrounding ground meristem cells (Fig. 2b).

Cells from 6-D-Old Seedlings

Shoot tips from 6-d-old seedlings (i.e., 3 d dark, then 3 d light) were hooked and pale green. In their subapices, both protoderm and ground meristem cells contained dense cytoplasm that included abundant ribosomes and various organelles. The outer tangential walls of the protoderm cells also were thickened. Although protein bodies and small vacuoles containing electron-dense particles and fibrous materials were much less numerous than in the cells of the dark-grown 3-d-old seedlings, large, fused vacuoles were frequent. Lipid bodies were remarkably reduced in number compared with those in the cells of 3-d-old seedlings. Chloroplasts contained a few starch grains and well-developed thylakoids (Fig. 2c, d, 3a). Grana stacks in the chloroplasts of the protoderm and ground meristem mainly consisted of two to three thylakoids; their lumens were filled with darkly stained materials (Fig. 2d). For other chloroplasts, the thylakoid lumens were electron-translucent (Fig. 3a). In the cells of the protoderm and ground meristem, some plastids revealed crystalline inclusions as well as granal thylakoids and starch grains (Fig. 3b).

The outermost cells of the ground meristem contained numerous chloroamyloplasts that possessed starch grains and electron-dense thylakoids, and had large vacuoles with electron-dense particles (Fig. 3c). The cells in the middle region of the ground meristem showed abundant cell organelles, e.g., dictyosomes, mitochondria, polyribosomes, rough endoplasmic reticula, and several chloroamyloplasts (Fig. 3d). Cells located at the innermost region of the ground meristem had a number of amyloplasts with starch grains, a few thylakoids, and some crystalline inclusions. Plastoglobuli in the plastids were less dense and fewer in number, but were larger than those observed in the 3-d-old seedlings.

DISCUSSION

Reserves

A large number of lipid bodies and protein bodies with globoid crystals are stored in the embryo cells of *C. japonica* (Lee, 2006). In the study presented here, such reserves also were found in the primary meristem cells of seedlings. Here, the only fine structural evidence of lipid utilization may have been the gradual decrease in quantity for subsequent germination and growth (Table 1). Most cells in the shoot tips contained several small vacuoles, many of them with electron-dense particles. These vacuoles were transformed from the numerous protein bodies stored in the embryo cells, in which the vacuoles have electron-dense globoid crystals (Lee, 2006). In this current investigation, those particles also occurred in the vacuoles of the protoderm cells (Fig. 1b) and ground meristem cells (Fig. 1d, e, 2a) in 3-d-old dark-grown seedlings, as well as in the outermost ground meristem cells of 6-d-old dark/light-grown seedlings (Fig. 3d). The dense particles are believed to be the globoid crystals that remain after protein bodies are degraded during germination and early seedling growth. The globoid crystals possess phytate as an iron storage compound in the seed protein bodies (Pergo et al., 1998; Maroder et al., 2003). Crystals that are made from minerals may persist in an insoluble state within the vacuoles of seedling cells, as they do in the embryos of *C. japonica* (Lee, 2006).

Reserves were gradually depleted during two stages of seedling growth, while the cell organelles increased. The tendency here to reduce their quantities was more evident

Table 1. Comparison of major characteristics from cells of embryos and seedlings of *C. japonica*.

Characteristic	Dry embryos*	Soaked embryos*	Under darkness: S-sa[a] in 3-d-old seedlings	Under sunlight for additional 3 d: S-sa[a] in 6-d-old seedlings
Cell organelles	(+)	(+)	++	+++
Protein bodies	+++	+++	++	(+)
Lipid bodies	+++	+++	++	+
Plastid inclusions				
phytoferritin	None	++	None	None
plastoglobuli	(+)	++	+++	(+)
crystals	None	None	None	+
thylakoids	(+)	(+)	++	+++
starch grains	None	None	+[1)] or +++[2)]	+)[3)] or +++[4)]
Plastid types	Pp[b]	Pp	Ep[c] (Plb[d]) in pg[e] Ap[g] in Gm[h]	Cp[f] in Pd Cap[i] in Gm

*Lee (2006)

+++, abundant; ++, many; +, few; (+), fewer

[a]Subapices of shoots transversely sectioned; [b]Proplastid; [c]Etioplast; [d]Prolamellar body; [e]Protoderm; [f]Chloroplast; [g]Amyloplast; [h]Ground meristem; [i]Chloroamyloplast

[1)]In etioplasts from protoderm; [2)]In amyloplasts from ground meristem; [3)]In chloroplasts from protoderm; [4)]In chloroamyloplasts from ground meristem

compared with what occurred in the embryo cells (Lee, 2006; Table 1). This indicates that the embryo storage reserves are utilized and mobilized for germination and seedling growth and that, coincidentally, the cellular metabolism of seedlings is converted from a quiescent to a highly active state (Bewley, 1997; Herman and Larkins, 1999).

Plastids

Soaked embryo cells in *C. japonica* contain proplastids with a few thylakoids, plastoglobuli, and phytoferritin in their stroma (Lee, 2006). In contrast, no phytoferritin was found in the plastids of seedling cells here (Table 1). Phytoferritin is an iron-protein complex that is stored 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). Phytoferritin plays a role in iron reserves (Harrison and Arosio, 1996), and an Fe deficiency may cause chlorosis when the thylakoid components for photosynthesis are diminished (Winder and Nishio, 1995). Because plastids in these seedling cells were shown to have developed thylakoids, this suggests that phytoferritin is used in the formation of chloroplasts during germination and early seedling growth (Reid et al., 1998).

Although no starch-containing plastids exist in *C. japonica* embryonic cells (Lee, 2006), anatomical study has revealed that numerous starch grains are present in the subapical ground meristem cells of 3-d-old dark-grown seedlings (Lee et al., 2000). This feature was ultrastructurally confirmed here (Fig. 1a-d, f, 2a; Table 1). Nevertheless, their growing conditions inhibited photosynthesis. Two explanations for this difference in components are possible. First, the grains that accumulate in young seedlings may arise from starch-bearing amyloplasts in the endosperm cells surrounding the embryo, their existence having been previously reported in the seeds of *Cuscuta* plants (Kuijt, 1969; Lyshede, 1984). Because the shoot apices of 2-d-old seedlings are covered with the remaining endosperm tissue (Fig. 4 in Lee et al., 2000), it may be assumed that those stored starch grains are digested and supplied to the embryo during the germination and early-growth stages. Second, starch grains in the subapical cells of the seedling shoot may result from certain biochemical pathway(s), e.g., gluconeogenesis, by which sugars are converted from lipid and protein bodies stored in the embryo cells (Li and Ross, 1990; Eastmond et al., 2000; Rylott et al., 2003; Cornah et al., 2004). Further study is needed to evaluate that second possibility.

In the cells of 3-d-old dark-grown seedlings here, plastids containing a few electron-dense thylakoids and small vesicles were very similar to the ones that have been observed in the young epidermal cells of green apical tips from 7-d-old *C. pedicellata* seedlings (Lyshede, 1989). The structural feature of those small vesicles and their fusion at the periphery of the plastids (Fig. 1a, c) may be evidence of thylakoid biogenesis via this vesicle formation and fusion. These current results agree with those previously described, in which vesicles are derived from the budding of the inner membrane of the chloroplast envelope and are then fused with each other to form thylakoids (Hoober et al., 1991; Morre et al., 1991; Kroll et al., 2001; Vothknecht and Westphal, 2001; Westphal et al., 2001).

Under dark conditions, proplastids in *C. japonica* embryo cells have few thylakoids and no phytoferritin (Lee, 2006). When 3-d-old dark-grown seedlings in the current study were subsequently exposed to sunlight, etioplasts with prolamellar bodies in the protoderm cells were converted into chloroplasts with thylakoids that were well-organized into grana. Moreover, in the ground meristem cells, amyloplasts were transformed into chloroamyloplasts (Table 1). This light-induced conversion of etioplasts into chloroplasts corresponds with observations of various autotrophic higher plants (Mühlethaler and Frey-Wyssling, 1959; VanderZee and Kennedy, 1982; von Wettstein et al., 1995; Staehelin, 2003).

In the plastids of 3- (Fig. 1a, b) and 6-d-old seedlings (Fig. 2d), the thylakoid lumens accumulated electron-dense materials. Previous researchers also have reported that the thylakoid lumens of underdeveloped or young chloroplasts are much more electron-dense than those analyzed from more developed or mature chloroplasts (compare Fig. 1a, b, and 2d with Fig. 3a; Pettigrew and Vaughn, 1998). Further discussion of the plastids with darkly stained thylakoids (Fig. 2b) and crystalline inclusions (Fig. 3b) will be discussed in a future publication. The grana from developed chloroplasts (Fig. 3a) mainly consisted of two to three thylakoids, and no unstacked thylakoid regions were noticed between them. The grana stacks are similar to those found in the tips of preparasitic *C. pentagona* seedlings that are grown for up to 10 d (Sherman et al., 1999) as well as those that occur in the stems of post-parasitic *C. reflexa* (van der Kooij et al., 2000). Chloroplasts from *C. japonica* have much less organized thylakoids than those of such autotrophic higher plants as *Elodea* (Mühlethaler and Frey-Wyssling, 1959), *Zea* (Rascio et al., 1980; Gunning and Steer, 1996), *Gossypium* (Pettigrew and Vaughn, 1998), and *Nicotiana* (Staehelin, 2003). Though their thylakoids are poorly organized into grana stacks, the *C. japonica* chloroplasts are capable of photosynthesis, thereby enabling seedlings to grow autotrophically. The photosynthetic efficiency of *C. indecora* is greater while its seedlings remain free-living than after they become parasitic (Pattee et al., 1965). Furthermore, during the vegetative growth phase, seedlings of *C. campestris* contain more chlorophyll than do their eventually parasitic stems (Dinelli et al., 1993). Finally, photosynthetic proteins, e.g., ribulose 1,5-bisphosphate carboxylase/oxygenase, phosphoribulokinase, and plastocyanin, are greatly reduced after *C. pentagona* seedlings parasitize their host plants (Sherman et al., 1999).

In conclusion, as the growth of *C. japonica* seedlings progressed, storage reserves were gradually depleted while the numbers of various cell organelles increased. This indicates that those reserves are utilized during seedling development and that cellular metabolism is being converted from a quiescent to an active state. The phenomena of thylakoid biogenesis in the plastids and light-induced conversion of etioplasts to chloroplasts suggests that *C. japonica* seedlings can survive autotrophically during their free-living stage, in the absence of a host plant. It is possible that this stage of autotrophic growth may increase the time available during which these seedlings can parasitize suitable hosts.

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