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Ultrastructure of Crystalline Inclusions in the Thylakoids of Dodder (*Cuscuta japonica*) Plastids

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The plastids from seedlings of the parasitic angiosperm *Cuscuta japonica* were ultrastructurally investigated. In shoot subapical cells from 3-d-old seedlings grown in the dark, the etioplasts contained prolamellar bodies and amorphous and dense inclusions. In the shoot subapical cells obtained from 6-d-old seedlings grown under light conditions for the last 3 d, the underdeveloped chloroplasts contained phytoferritin within the stroma as well as amorphous and dense inclusions that were limited by the thylakoid membranes. In the developing chloroplasts, electron-dense materials were detected within the transversely sectioned thylakoid lumens. This dense material presented two different images, depending upon the sectional plane. When transversely prepared, the materials appeared as somewhat thick, linear structures, whereas longitudinally sectioned thylakoids revealed very large crystalline inclusions. In the developed chloroplasts, the amounts of electron-dense material or crystalline inclusions were remarkably reduced in the thylakoid lumens, which were electron-translucent. Far fewer crystalline inclusions were observed in the developed chloroplasts of seedlings than in the developing chloroplasts. These results suggest that the crystalline inclusions may be temporarily reserved within the thylakoid lumens of chloroplasts in the *C. japonica* seedlings.

Keywords: chloroplast, crystalline inclusion, *Cuscuta japonica*, parasitic angiosperm, thylakoid, ultrastructure

Membrane-enclosed inclusions within plastids were initially reported by Hohl (1961), who observed them in the hyperplastic tissues of *Datura* and tumor tissues of *Nicotiana* and *Solanum*. Crystalline inclusions enclosed within membranes have also been found in the epidermal plastids of *Beta* leaves and stamens (Hoefert and Esau, 1975). They have postulated that these inclusions are composed of proteins that are stored under certain conditions in the epidermal cells. Chloroplasts obtained from healthy and curly-top infected *Spinacia* leaves harbor crystalline inclusions enclosed by dilated thylakoid membranes (Esau, 1975). Such inclusions completely enclosed by thylakoid membranes have been visualized using a tilting (goniometer) stage under an electron microscope (Miller et al., 1976). They have also demonstrated that the periodicity of crystals also can be demonstrated via the tilting method. Although large crystalline inclusions have been detected within the chloroplast stroma of well-watered *Salix* leaves, their numbers decline with increasing water stress, and they are completely absent in severely stressed plants (Vapaavuori et al., 1984).

Crystalline inclusions that are not enclosed by membranes have been found in the stroma of plastids from *Echinochloa* leaves (Vanderzee and Kennedy, 1982) as well as within the cytoplasm of chlorenchyma cells in *Origanum* leaves (Bosabalidis and Papadopoulos, 1983). Intrathylakoidal crystalline inclusions have been detected within the developing plastids of *Coleus*, whereas such inclusions are either absent or rare in mature or developed chloroplasts from that genus (Varkey and Nadakavukaren, 1982) as well as from *Saintpaulia* (Finer and Smith, 1983) and the leaves of *Spinacia* (Rascio et al., 1985). However, these intrathylakoidal inclusions have also been identified in fully developed chloroplasts from *Spinacia* (Miller et al., 1976; Shojima et al., 1987) and *Salix* (Vapaavuori et al., 1984).

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In contrast, Lyshede (1989) has detected plastids with a few electron-dense thylakoids and small vesicles within the young epidermal cells of green apices from 7-d-old seedlings of *Cuscuta pedicellata*. Sherman et al. (1999) have briefly described the membrane-enclosed crystalline inclusions in chloroplasts of dodder (*Cuscuta pentagona*) seedlings grown for up to 10 d.

Seedling tips in *C. japonica* also have been anatomically 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 last 3 d) have lots of starch-containing amyloplasts. The mature embryo cells from the *C. japonica* contain a reserve of numerous lipid and protein bodies, as well as proplastids with a few thylakoids and phytoferritin (Lee, 2006). Furthermore, the shoot subapical cells of 3-d-old dark-grown seedlings have etioplasts with prolamellar bodies, which develop into chloroplats with thylakoids that were well organized into grana from 6-d-old seedlings grown in the light for the last 3 d (Lee, 2007). He has also found that thylakoid lumens of the chloroplasts are filled with electron-dense materials. Herein, the ultrastructural features of membrane-enclosed crystalline inclusions are described from the chloroplasts of *Cuscuta japonica* seedlings.

MATERIALS AND METHODS

Plant Materials

Mature, dormant seeds of *C. japonica* Choisy were scarified for 45 min with concentrated sulfuric acid, then 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 of some of these 3-d-old seedlings were then wrapped in wet cotton, placed in 500-mL covered beakers, and exposed to sunlight from nearby windows for an additional 3 d.

Transmission Electron Microscopy

For examination via electron microscopy, the shoot subapical portions from these 3- and 6-d-old seedlings were sliced into approximately 1 mm^3 segments and pre-fixed for 2 to 3 h at room temperature in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in a 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 Watts in a Pelco Model 3450 Laboratory Microwave Processor (Ted Pella, USA) equipped with a thermistor copper temperature probe and an auxiliary Pelco 3420 Microwave Load Cooler (Ted Pella). Afterward, the tissues were post-fixed in 1% osmium tetroxide, buffered at pH 6.8, and microwaved three times for 40 s each period. The pre- and post-fixation temperature of the oven was 45°C. These fixed segments were washed in buffer and dehydrated in a graded acetone series, for 40 s per step, in a

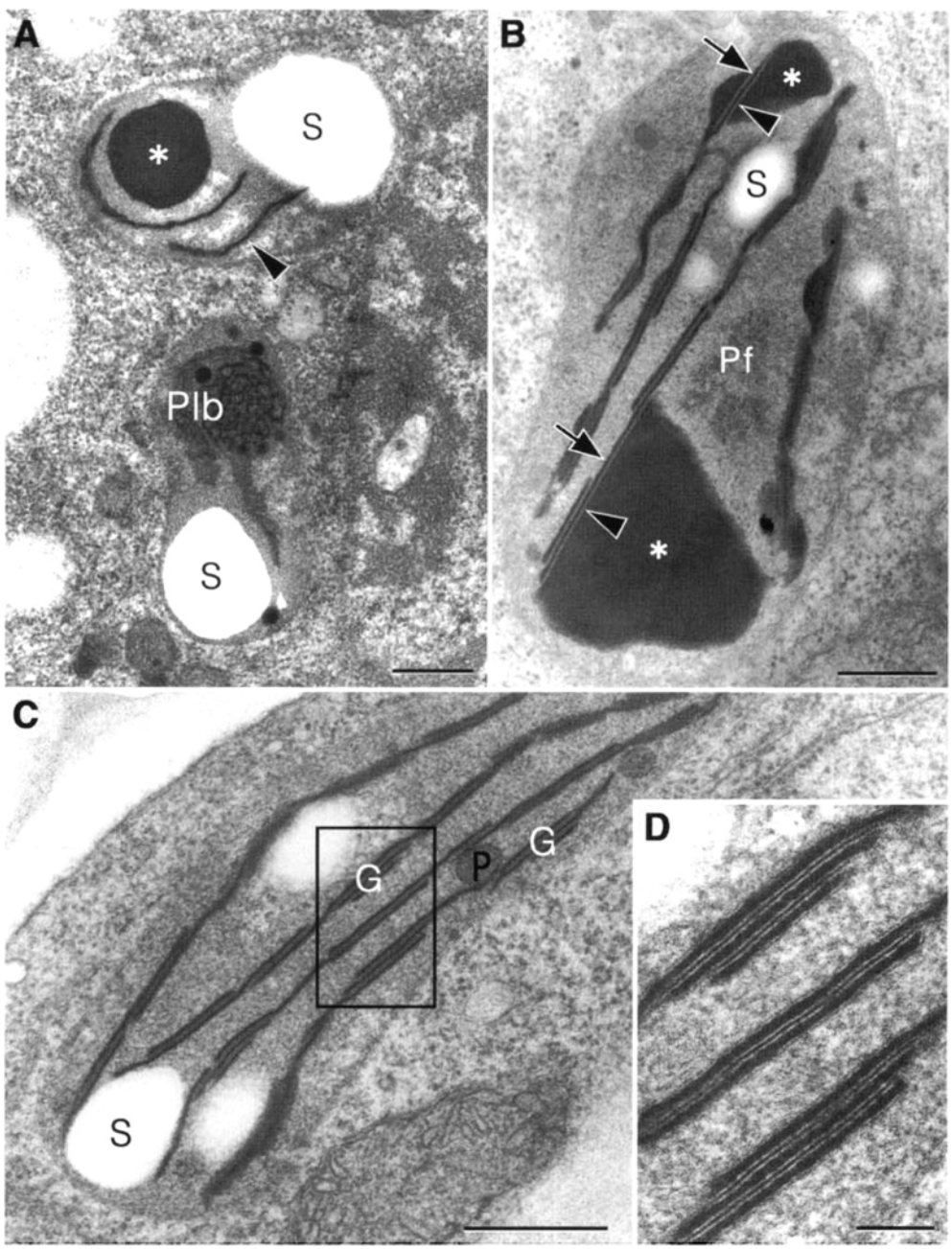

Figure 1. Electron micrographs of plastids in subapical cells from 3- (**A**) and 6-d-old (**B-D**) seedling shoots of *C. japonica*. (**A**) Protodermal cells have etioplasts with prolamellar bodies (Plb), a few darkly stained thylakoids (arrowhead), starch grains (S), and amorphous and dense inclusions (asterisks). Bar = 0.25 μm. (**B**) In underdeveloped plastids, phytoferritin (Pf) particles are aggregated within stroma, and electron-dense and amorphous inclusions (asterisks) are partially limited by single thylakoid membrane (black arrowheads) that is closely associated with transversely sectioned thylakoid (arrows) with densely stained material that appears identical to amorphous inclusions. S, starch grain. Bar = 0.25 μm. (**C**) Developing chloroplast with thylakoids forming grana (G), starch grains (S), and plastoglobuli (P). Thylakoid lumens harbor darkly stained materials. Bar = 0.5 μm. (**D**) Enlarged region marked by rectangular box in Figure 1C showing densely stained materials enclosed by thylakoid membranes. Bar = 0.1 μm.

microwave oven at 37°C. The tissues 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, then examined with a light microscope (BH2; Olympus, USA). Thin sections cut with an RMC MT-7000 ultramicrotome were mounted on grids, stained with uranyl acetate and lead citrate, then examined and photographed at 80 kV using either a JEM 100 CXII or a transmission electron microscope (H-7600; Hitachi, Japan).

RESULTS

The shoot tips from 3-d-old, dark-grown seedlings were hooked and yellowish. In their shoot subapical regions, the ground meristem cells showed proplastids with few thylakoids and starch grains. However, the protodermal cells contained etioplasts with prolamellar bodies, a few darkly stained thylakoids, starch grains, and amorphous and dense inclusions (Fig. 1A). In contrast, the hooked shoot tips of 6-d-old seedlings that had been exposed to light for the final 3 d of their treatment period were pale green. In their ground meristem cells, chloroplasts at early developmental stages had large aggregates of phytoferritin, starch grains, and amorphous and dense inclusions that were limited by the thylakoid membranes (Fig. 1B). However, both the protodermal and ground meristem cells had developing chloroplasts with thylakoids that were well organized into grana, starch grains, and plastoglobuli (Fig. 1C). The chloroplasts found in the ground meristem cells contained more starch grains than did those observed in the protodermal cells. Grana from both tissue types consisted primarily of two to three thylakoids (Fig. 1C, 2A, 3A, 4A), but stacks of four were occasionally visible. Although electron-dense material was evident in the lumens of the transversely sectioned thylakoids (Fig. 1C, D), large crystalline inclusions were observed in thylakoids that were paradermally sectioned (Fig. 3A, 4A).

The thylakoid membrane in which these crystalline inclusions were enclosed was partially visible when the granum disc was obliquely sectioned (Fig. 2A, 3A). In this case, the membrane enclosing those inclusions was apparent at higher magnifications (Fig. 2B, 3B). Likewise, the membrane was evident when the median plane of the granum disc was sectioned longitudinally (Fig. 4A). These inclusions showed a lattice substructure of alternating electron-dense (dark) and electron-translucent (light) regions that occurred at regular intervals.

For some chloroplasts, the transversely sectioned thylakoid lumens at the edges of the thylakoid membranes in which the inclusions were enclosed revealed no darkly stained material (Fig. 4A). In the developed chloroplasts (Fig. 4B), the thylakoids were well organized into grana, and the thylakoid lumens were far less electron-dense than those featured in Figure 1C-D, and 3A.

DISCUSSION

Etioplasts with prolamellar bodies and electron-dense inclusions were found in the shoot subapical cells of 3-d-old

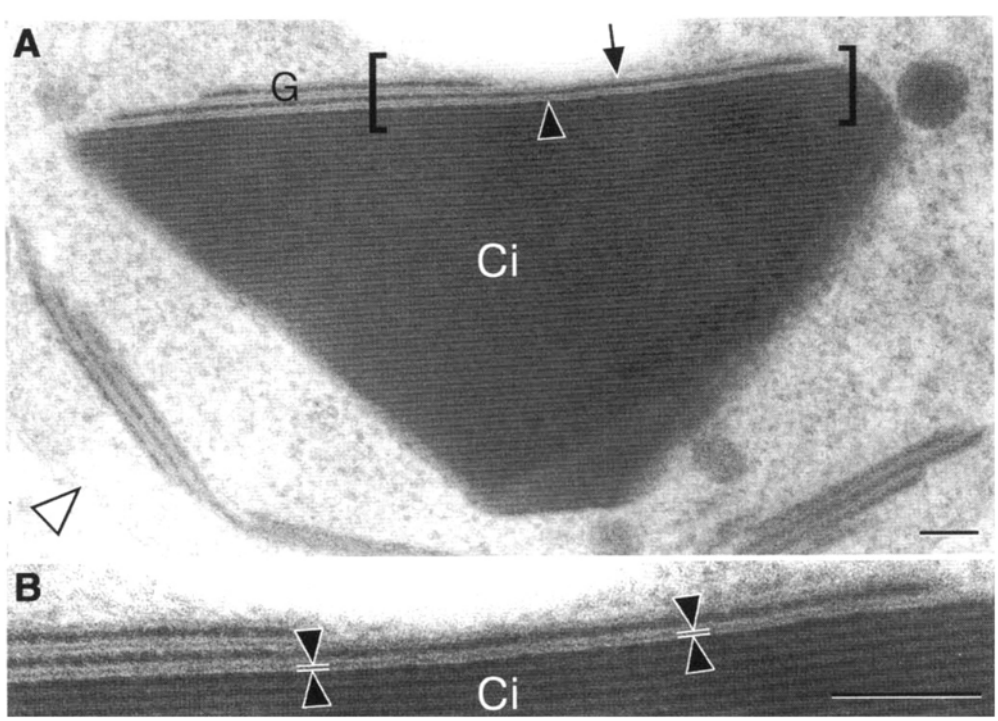

Figure 2. Electron micrographs of developing chloroplasts in subapical cells of 6-d-old seedling shoots from *C. japonica*. (**A**) Large triangular crystalline inclusion (Ci) is enclosed by thylakoid membrane (black arrowhead) that is in close contact with transversely sectioned thylakoid (arrow). G, granum. Ci shows parallel substructure. White arrowhead indicates chloroplast envelope. Bar = 0.1 μm. (**B**) Enlarged image of region marked by brackets in Figure 2A shows thylakoid membrane enclosing crystalline inclusion (Ci). Membrane appears as thin light line (two pairs of black arrowheads). Bar = 0.1 μm.

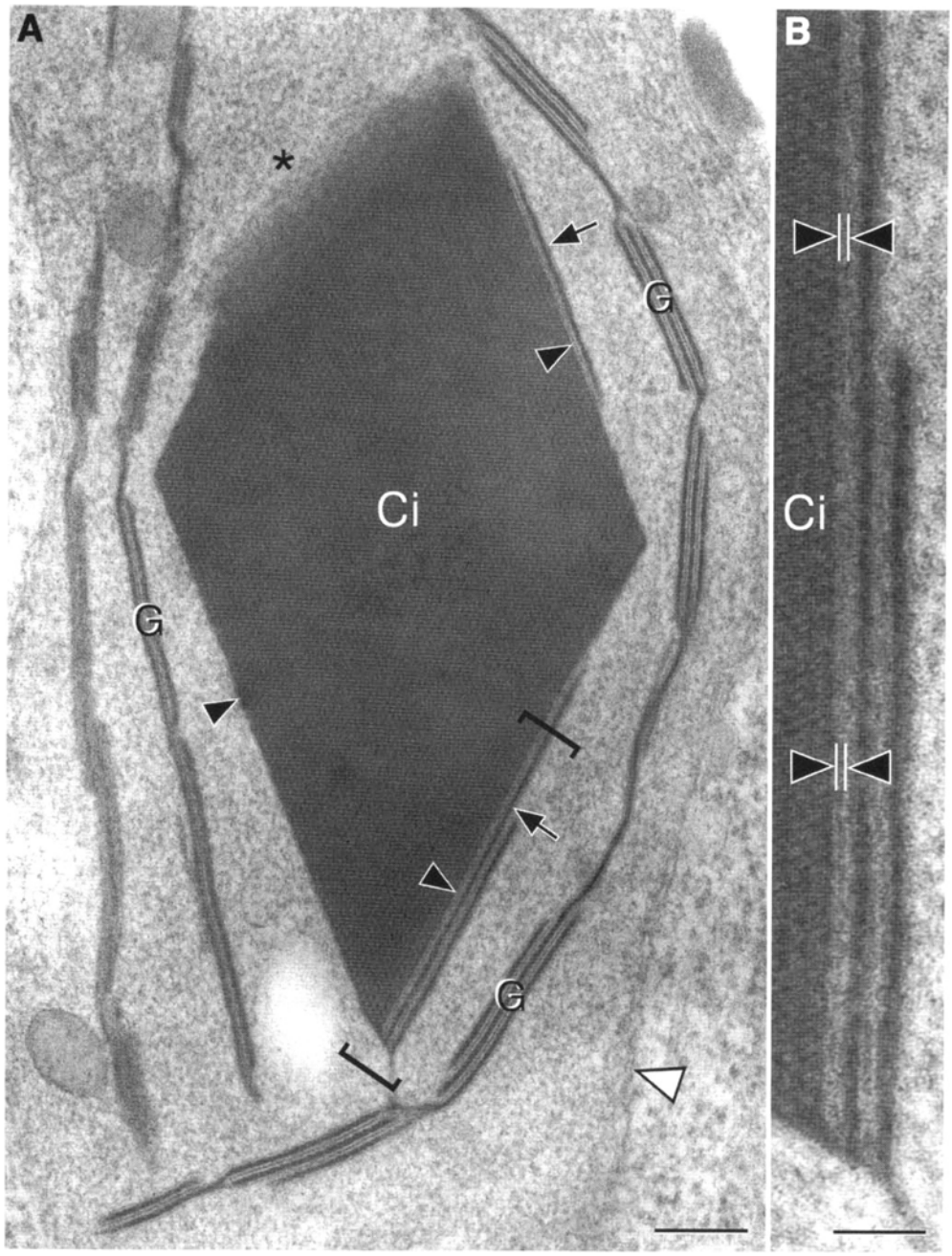

Figure 3. Electron micrographs of developing chloroplasts in subapical cells of 6-d-old seedling shoots from *C. japonica*. (**A**) Developing chloroplast evidencing large crystalline inclusion (Ci) enclosed by a single thylakoid membrane (black arrowheads), which exhibits two sets of periodicities in lattice substructure. Transversely sectioned thylakoids (arrows) are closely associated with Ci. Lumens of several granal (G) thylakoids around Ci are filled with electron-dense material. Asterisk indicates region in which limiting membranes do not appear. White arrowhead shows plastid envelope. Bar = 0.1 μm. (**B**) High-magnification micrograph of portion (black arrowheads) indicated by brackets in Figure 3A, which exhibits crystalline inclusion (Ci) enclosed by single thylakoid membrane (two pairs of black arrowheads). Bar = 0.025 μm.

C. japonica seedlings grown in the dark. When some of those seedlings were grown for an additional 3 d in the light, the chloroplasts from their shoot subapical cells revealed crystalline inclusions with a latticed substructure within their thylakoid lumens. These inclusions in the longitudinally sectioned thylakoids corresponded to the electron-dense material previously reported from the lumens of transversely sectioned thylakoids (Lee, 2007: personal communication with Dr. Brian E. S. Gunning, Australian National University). Therefore, this current study is the first to describe two different images of thylakoid content generated from the chloroplasts of *C. japonica*. Finer and Smith (1983; Fig. 1B) have earlier noted transversely sectioned thylakoid lumens that have electron-dense materials in the mature chloroplasts of palisade cells from the leaves of *Saintpaulia*. However, no such observations had previously been published regarding the structure of such thylakoid lumens, even though other researchers had found relevant structures quite similar to

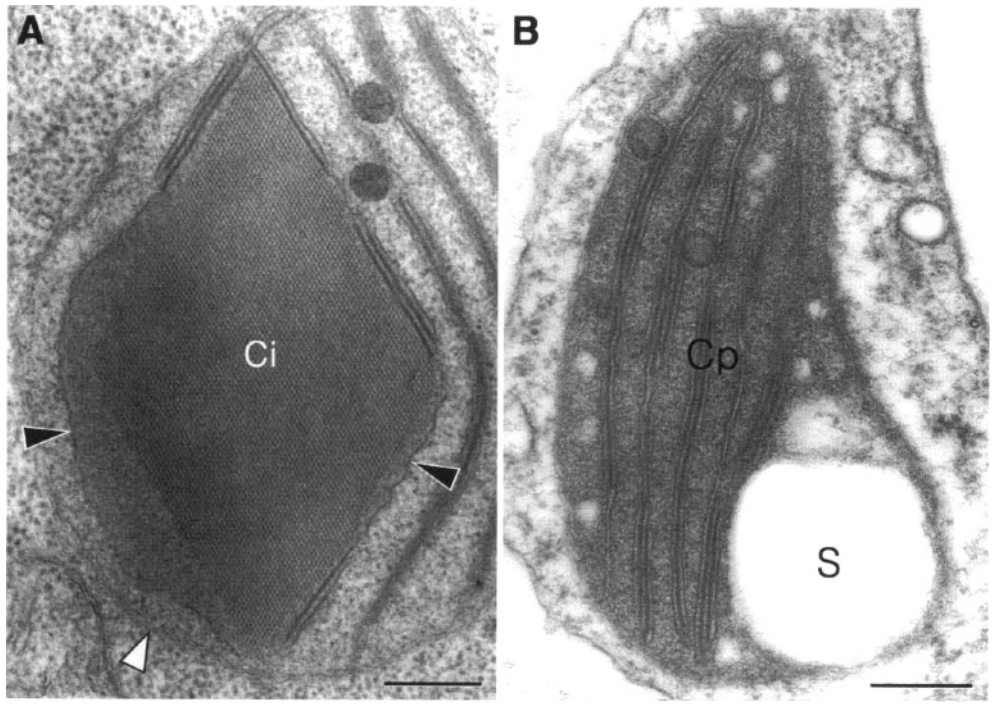

Figure 4. Electron micrographs of developing (**A**) and developed chloroplasts (**B**) in subapical cells from 6-d-old seedling shoots of *C. japonica*. (**A**) Thylakoid membrane (black arrowheads) enclosing crystalline inclusion (Ci) with clearly visible lattice substructure. White arrowhead indicates plastid envelope. Bar = 0.25 μm. (**B**) Developed chloroplast (Cp) has thylakoids organized into grana, and thylakoid lumens are electron-translucent. S, starch grain. Bar = 0.25 μm.

those now presented here in Figure 1C-D, 2A, and 3A. Plastids containing a "few electron-dense thylakoids" have been described briefly by Lyshede (1989; Fig. 3, inset), in a study of epidermal cells from the green apices of 7-d-old *C. pedicellata* seedlings. Nevertheless, the structures of those crystalline inclusions were not addressed in that earlier work. Moreover, Sherman et al. (1999) have detected crystalline inclusions surrounded by a distended thylakoid membrane in the chloroplasts of *C. pentagona*, but they are much smaller than the ones described herein, and also evidence far less clear latticework. This electron opacity of the granal thylakoids has also been noted in the youngest chloroplasts of the leaves of *Gossypium hirsutum*, whereas the developed chloroplasts in that species have electron-translucent granal thylakoids (Pettigrew and Vaughn, 1998). Such a phenomenon might be attributed to the activity of polyphenol oxidase, which causes those thylakoids to appear quite electron-dense. However, that previous research also did not investigate those crystalline inclusions within the thylakoids. In the current study, the thylakoid lumens of older, more developed chloroplasts were electron-translucent (Fig. 4A, B). This feature has also been noted in the most developed chloroplasts of pre-parasitic *C. pentagona* seedlings grown for up to 10 d (Sherman et al., 1999) as well as in those of post-parasitic stem cells of *C. reflexa* (van der Kooij et al., 2000).

Here, the dense materials or crystalline inclusions within the thylakoid lumens were frequently observed in underdeveloped chloroplasts, but these characteristics were markedly reduced in the fully developed organelles. These results are in agreement with those from earlier studies (Varkey and Nadakavukaren, 1982; Finer and Smith, 1983; Rascio et al., 1985). Consequently, one might consider the plastids with thylakoid lumens forming electron-dense materials or crystalline inclusions to be developing chloroplasts that would differentiate into mature or developed ones, as in Figure 4B. In the latter case, the thylakoid lumens possessed far less electron opacity than in the former (Fig. 1C, 2A, 3A) as a result of the light-induced conversion of etioplasts into chloroplasts. Therefore, one might suggest that the photosynthetic capability of these *Cuscuta* seedlings may ensure their survival until they can parasitize the host plant (Lee, 2007).

The cause of such crystalline inclusion formation has not yet been clearly elucidated. Vapaavuori et al. (1984) have previously reported the existence of large, crystalline inclusions within the chloroplast stroma of well-watered *Salix* leaves, with their numbers diminishing as water stress increases. In terms of their chemical natures, these membrane-enclosed inclusions in the plastids are generally considered proteinaceous. Sprey (1976) has reported the presence of ribulose 1,5-diphosphate carboxylase (RuDPCase) within the thylakoid lumens of spinach chloroplasts, and has presumed that enzyme segregation into those lumens renders them inactive as a result of crystallization. However, Shojima et al. (1987) have immunocytochemically demonstrated, in spinach chloroplasts, that RuDPCase cannot be recognized in the intrathylakoidal crystalline inclusions. Recent studies have shown that the thylakoid lumens contain approximately 200 different photosynthetic proteins (Peltier et al., 2002; Schubert et al., 2002), some of which are transported into and across the thylakoid membrane (Robinson et al., 2000). Therefore, it is feasible that

the intrathylakoidal crystalline inclusions may be transitorily stored in the developing chloroplasts.

In summary, visualization of the thylakoid lumens in developing chloroplasts from the subapical cells of 6-d-old *C. japonica* seedling tips has revealed electron-dense materials as two distinctly different images, depending upon the sectional plane of those lumens. Specifically, those materials appear as thick linear structures in the transversely sectioned thylakoids, whereas large crystalline inclusions evidencing a lattice substructure can be observed in the longitudinally sectioned ones. These inclusions are far fewer in developed chloroplasts than in the less mature organelles. In the former, the thylakoid lumens show considerably less electron opacity than is noted in the developing chloroplasts. This suggests that crystalline inclusions within the thylakoids are associated with chloroplast development in the seedlings of *C. japonica*.

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LITERATURE CITED

Bosabalidis AM, Papadopoulos D (1983) Ultrastructure, organization and cytochemistry of cytoplasmic crystalline inclusions in *Origanum dictamnus* L. leaf chlorenchyma cells. J Cell Sci 64: 231-244

Esau K (1975) Crystalline inclusion in thylakoids of spinach chloroplasts. J Ultrastruct Res 53: 235-243

Finer JJ, Smith RH (1983) Structure and development of plastids in epidermal cells of African violet (*Saintpaulia ionantha* Wendl.) in culture. Ann Bot 51: 691-695

Hoefert LL, Esau K (1975) Plastid inclusion in epidermal cells of *Beta*. Amer J Bot 62: 36-40

Hohl HR (1961) Plastids and tumorigenesis. Amer J Bot 48: 528

Lee KB (2006) Ultrastructure of mature embryos in the parasitic flowering plant *Cuscuta japonica*. J Plant Biol 49: 384-391

Lee KB (2007) Ultrastructure and development of seedlings of the parasitic weed *Cuscuta japonica*. J Plant Biol 50: 213-219

Lee KB, Park JB, Lee S (2000) Morphology and anatomy of mature embryos and seedlings in parasitic angiosperm *Cuscuta japonica*. J Plant Biol 43: 22-27

Lyshede OB (1989) Electron microscopy of the filiform seedling axis of *Cuscuta pedicellata*. Bot Gaz 150: 230-238

Miller KR, Bloodgood RA, Staehelin LA (1976) Crystals within thylakoids: A structural analysis. J Ultrastruct Res 54: 29-36

Peltier JB, Emanuelsson O, Kalume EE (2002) Central function of the luminal and peripheral thylakoid proteome of *Arabidopsis* determined by experimentation and genome-wide prediction. Plant Cell 14: 211-236

Pettigrew WT, Vaughn KC (1998) Physiology, structural, and immunological characterization of leaf and chloroplast development in cotton. Protoplasma 202: 23-37

Rascio N, Colombo PM, Viccia FD, Chitano P (1985) Intrathylakoidal crystal appearance during the vital cycle of spinach chloroplasts. Protoplasma 126: 153-157

Robinson C, Woolhead C, Edwards W (2000) Transport of proteins into and across the thylakoid membrane. J Exp Bot 51: 369-374

Schubert M, Peterson UA, Haas BJ, Funk C, Schroder WP, Kieselbach T (2002) Proteome map of the chloroplasts lumen of *Arabidopsis thaliana*. J Biol Chem 277: 8354-8365

Sherman TD, Pettigrew WT, Vaughn KC (1999) Structural and immunological characterization of the *Cuscuta pentagona* L. chloroplast. Plant Cell Physiol 40: 592-603

Shojima S, Nishizawa NK, Mori S (1987) Do intrathylakoidal inclusions really contain RUBPCase. Protoplasma 140: 187-189

Sprey B (1976) Intrathylakoid occurrence of ribulose 1,5-diphosphate carboxylase in spinach chloroplasts. Z Pflanzenphysiol 78: 85-89

Spurr A (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J Ultrastruct Res 26: 31-43

van der Kooij TAW, Krause K, Dorr I, Krupinska K (2000) Molecular, functional and ultrastructural characterisation of plastids from six species of the parasitic flowering plant genus *Cuscuta*. Planta 210: 701-707

Vanderzee D, Kennedy RA (1982) Plastid development in seedlings of *Echinochloa crus-galli* var. *oryzicola* under anoxic germination conditions. Planta 155: 1-7

Vapaavuori EM, Korpilahti E, Nurmi AH (1984) Photosynthetic rate in willow leaves during water stress and changes in the chloroplast ultrastructure, with special reference to crystal inclusions. J Exp Bot 35: 306-321

Varkey PJ, Nadakavukaren MJ (1982) Influence of leaf differentiation on the developmental pathway of *Coleus* chloroplasts. New Phytol 92: 273-278