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Ultrastructure of Initial Cytological Changes of Cowpea in Root Nodule Formation

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Cytological changes of cowpea root at the early stage of root nodule formation (within 5 days after inoculation) were viewed by light and electron microscopy. The root region affected by the rhizobial infection, which was composed of a radial array of cortical cells, had prominent cell divisions, mostly anticlinal in the inner cortical cells and in addition oblique and periclinal in the outer cells. An infected root hair cell (or root hair-producing epidermal cell) had numerous infection threads and degenerated cytoplasm. Nodule meristem was formed adjacent to the infected root hair cell, and characterized by dense cytoplasm, prominent nucleus, numerous small vacuoles, and increased plastids, containing infection threads as well. Bacterial cells were dividing inside the infection thread, the wall materials of which appeared to be dissolved and accumulated in small vacuoles. Inner cortical cells contiguous to the nodule meristem appeared to be actively dividing and dedifferentiating; however, they were not infected by the rhizobia. These structural characteristics are similar to those in the *Bradyrhizobium*-soybean association previously reported, and may reflect the similar cytological process in cowpea in the early nodule formation.

Keywords : cell division, cowpea, infection thread, nodule meristem.

Rhizobia induce the formation of root nodules in leguminous plants, converting atmospheric nitrogen to ammonia. The nodule formation comprises a series of events; early signal exchange between the plant and bacteria, rhizobial infection on root hair and cortical cells, release of bacteria into cell cytoplasm, nitrogen fixation and assimilation, and senescence of the nodule (Fisher and Long, 1992; Long, 1996). It is well known that host specificity is determined by early signal exchange between the symbiotic partners on the basis of low molecules such as flavonoids from plants and lipooligosaccharides from bacteria (Brewin, 1993; Lerouge et al., 1990; Peters and Verma, 1990).

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Early events in the infection of soybean by *Bradyrhizobium japonicum* (formerly *Rhizobium japonicum*) were described light and electron microscopically (Turgeon and Bauer, 1982; 1985). Structures of fully developed nodules have also been well visualized, such as bacterioids compartmented in peribacteroid membrane in infected cells, and inducing specialized cytological changes for ureide production in uninfected cells (Newcomb et al., 1985). On the other hand, relatively little is known about ultrastructural aspects of the infection process from infection thread formation to nodule development. As Calvert et al. (1984) noted that relatively few root infections developed into nodules, the process from bacterial infection to nodule development may be an important period for a successful association between prokaryotic and eukaryotic symbionts.

During the ultrastructural work on cowpea (*Vigna ungiculata* cv. Georgia 21) root, we found a *Bradyrhizobium*-infected root specimen 5 days after transplanting into the naturally contaminated soil with the bacteria. The root specimen had been prepared for electron microscopy using a procedure described in a previous work (Kim et al., 1999); the cowpea root tissue was fixed in Karnovsky's fixative for 4 hr, post-fixed in 1% osmium tetroxide for 2 hr, dehydrated in an ethanol series, and embedded in Spurr's epoxy resin (Spurr, 1969).

Cross sections (1 μm in thickness) of the infected tissue were made by thick sectioning with a glass knife on a Sorval ultramicrotome, and stained with 1% toluidine blue. The cross section of the infected root tissue revealed that an array of cells from the epidermis to inner cortex appeared different from surrounding cells, involving parenchymatous cells with newly formed cell walls, distinctive nuclei, and sometimes increased cytoplasmic contents (Fig. 1). Cell division was more prominent in outer cortex, which might be in the process of nodule meristem formation, than in inner cortex. The newly formed cell walls (daughter cell walls) were mostly anticlinal in the inner cortical cells, while oblique or periclinal cell divisions were common in the presumed nodule meristematic cells. Some daughter cell walls appeared to be continuous between adjacent layers of contiguous cortical cells. Turgeon and Bauer (1982) reported that cortical

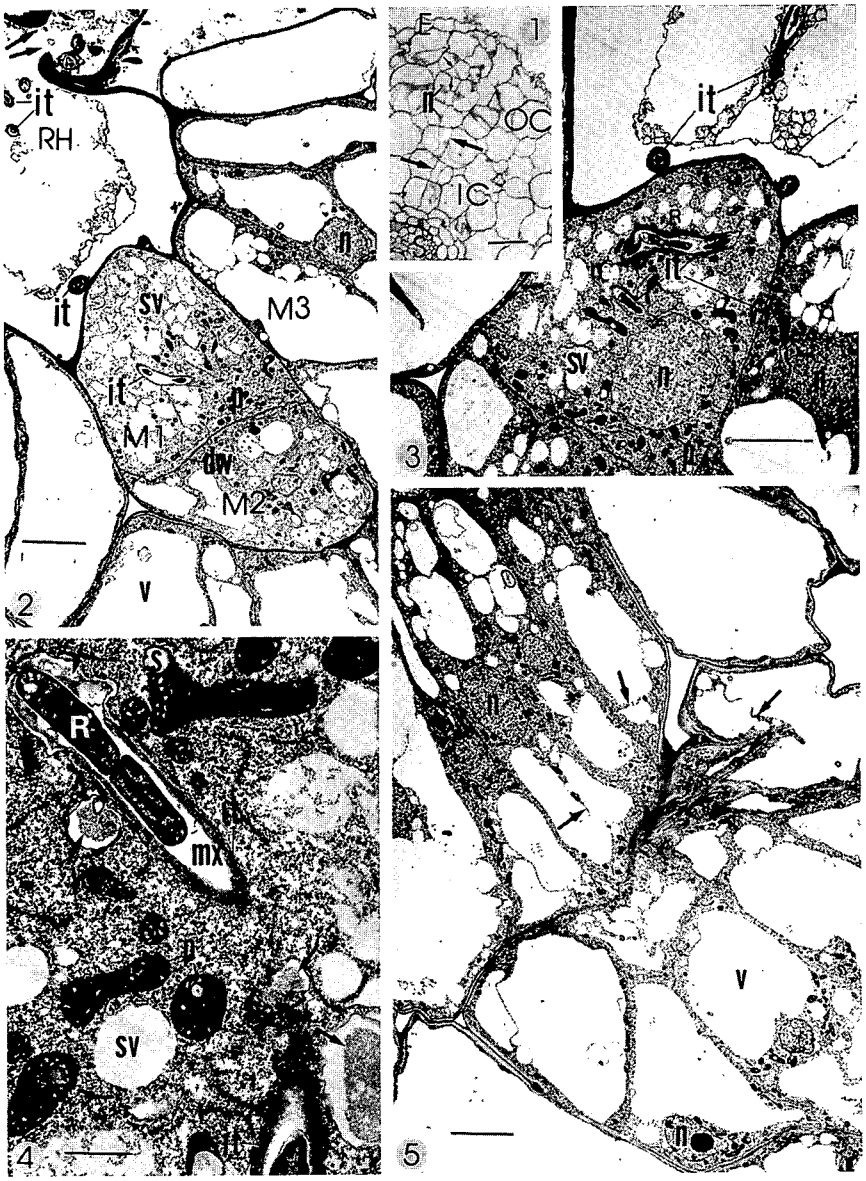

Fig. 1. Cross section of infected root tissue with stimulated cellular division even in inner cortex (IC), indicated by anticlinal daughter cell walls (large arrows). The meristem-looking cells in outer cortex (OC), characterized by prominent nucleus (n) and oblique and periclinal daughter cell walls (small arrows). It is not clear whether nodule morphogenesis occurred in the stele (S). E: epidermis. Bar = 20 μm.

Fig. 2. Initial nodule meristem adjacent to the infected root hair cell (RH) (or root hair-producing epidermal cell), showing dense cytoplasm, small vacuoles (sv), increased plastids (p), and periclinal daughter cell wall (dw), containing infection thread (it). The outermost site is probably a pocket of folded root hair, the cell wall of which has been dissolved (arrows). Numerous infection threads (it) were observed in the root hair cell in which the cytoplasm was degenerated, attached to the outside wall of the infected cortical cell. When observing serial sections, infection threads were found in the three meristematic cells of M1, M2 and M3. n: nucleus, v: vacuole. Bar = 5 μm.

Fig. 3. Another serial section of Figure 2 with higher magni- fication, showing the meristematic cells, characterized by pro- minent nuclei (n), dense cytoplasm, increased small vacuoles (sv), and increased organelles such as plastids (p). *Bradyrhizobium* cells (R) were located inside of the infection threads (it), indicating the penetration of rhizobia into cortical cells via the infection thread formation. Bar = 5 μm.

Fig. 4. Higher magnification of the cytoplasm of the meristem M1 in Fig. 2, showing the detailed structure of infection threads (it) and plastids (p). *Bradyrhizobium* cells (R) are dividing inside the infection thread. Around the tips of infection threads, infection thread wall materials appeared to be dissolved and accumulated (arrows) in small vacuoles (sv). Many plastids had irregular or vermiform shapes with no or small starch granules (s). mx: matrix. Bar = 1 μ.m

Fig. 5. Dividing and dedifferentiated cortical cells located inside and contiguous to the infected outer cortical cells in Fig. 2. The outer cells, having anticlinally divided daughter cells, seemed to be more active in cell division and more differentiated than the inner cells. However, no infection thread was observed in these dividing cells. Note formation of small vacuoles by compart-mentalizing (arrows) vacuole (v). n: nucleus. Bar = 5 μm.

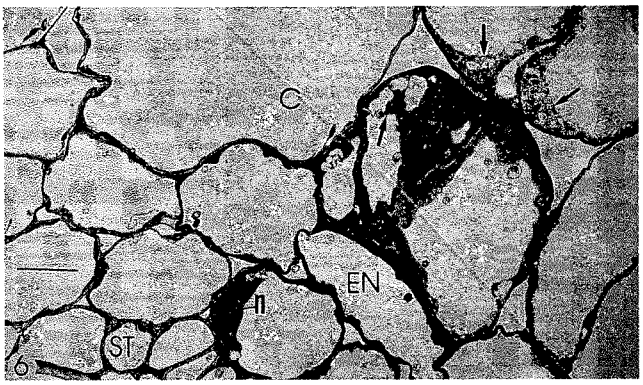

Fig. 6. Electron microscopy of the innermost portion of the root tissue affected by the *Bradyrhizobium* infection, showing innermost cortical cells (C) initiated dedifferentiation with increased cytoplasm (arrows) and probably new cell division (see the same innermost cortical cell in Fig. 1, showing cell division). It is not clear whether endodermis (EN) and cells inside the stele has been affected by the *Bradyrhizobium* infection and started morphogenesis. ST: sieve tube. n: nucleus. Bar = 5 μm.

cell divisions were mainly anticlinal at 48 hr after inoculation, but that they were oblique or periclinal as well as anticlinal when forming nodule meristem 4 days after inoculation. These aspects indicate anticlinal cell division may precede meristematic cell division for nodule development.

The area of tissue examined by light microscopy was sectioned 80-90 nm in thickness with a diamond knife to visualize the fine structure of the whole area. The sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 100 CX electron microscope. As shown in Figure 2, the outer layer consisted of root hair cells (or root hair-producing epidermal cells) and adjacent cortical cells, and the outermost site is probably a pocket of folded root hair, of which the cell wall has been dissolved (arrows). Numerous infection threads were observed in the root hair cells in which the cytoplasm was degenerated, and their remnants were attached to the outside wall of the meristematic cell. Cortical cells were either dedifferentiated or undifferentiated. In serial sections of the area, infection threads were found in the three meristematic cells of M1, M2 and M3. Figure 3 is a serial section of Figure 2, showing typical meristematic cells, containing prominent nuclei, dense cytoplasm, increased small vacuoles, and increased organelles such as plastids. Bacterial cells were found in the inside of infection threads, indicating that rhizobia penetrate into cortical cells via the infection thread formation.

Figure 4 is a higher magnification of the cytoplasm of meristem cell, M1, showing the detailed structure of infection threads and plastids. Bacterial cells were observed to divide in the inside of an infection thread. Around the tips of infection threads, infection thread wall materials appeared to be dissolved (arrows) (Fig. 4). Many plastids had irregular or vermiform shapes with no or small starch granules. Bassett et al. (1977) suggested that rhizobia might be released from the infection thread by the disintegration of thread wall and compartmentalization of the disintegrated wall material in membrane-bound vesicles derived from the membrane surrounding the thread. Bal (1985) also showed that the disappearance of infection thread after the rhizobial release is attributed to degradation within large vacuoles derived from the fusion of small vacuoles. Our study indicates that infection thread degradation may start at the early stage of infection. However, electron-lucent matrix in the infection thread was still remained around the bacterial cells. Neither rhizobia released into the cytoplasm nor bacterioids with peribacteroid membrane derived from plasma membrane (Cheon et al., 1993; Verma and Hong, 1996) were observed in the meristematic cells.

All cells located in the array of affected cells under the meristematic cells were in the process of dedifferentiation and cell division (Fig. 5). No infection thread was observed in these dividing cells. The outer cells seemed to be more active in cell division than the inner cells. Even the innermost cortical cells also showed dedifferentiation (Fig. 6). On the other hand, there was no cytological evidence that endodermis, pericycle and phloem cells had been affected and started nodule morphogenesis.

There are variations in nodule formation among rhizobia-plant associations. The structural features of cowpea in early nodule formation are similar to those of *B. japonicum*-soybean system (Calvert et al., 1984; Turgeon and Bauer, 1982), but different from those of *R. meliloti*-alfalfa system (Ardourei et al., 1994; Dudley et al., 1987), in which nodule primordia form in outer and inner cortex, respectively. Though the infection stages of the root section observed in this study could not be determined exactly, it was considered to be the stages from IV to VII because inner cortical cells were in active mitotic state, and the root stele was not differentiated yet (Calvert et al., 1984). Those stages are from 60 hr to 3-4 days after inoculation. In addition the structural changes in cowpea were similar to those described in a *Bradyrhizobium*-soybean association at 4 days after inoculation, in which a nodule meristem developed in outer cortex next to the infected root hair that was degenerated (Turgeon and Bauer, 1982). In cowpea, many centers of cell division became minute protrusions on the root surface at 4 days after inoculation (Teres and Pueppke, 1987). Based on these aspects, the infection stage of cowpea we examined is believed to be 4 days after inoculation.

The earliest cytological events in root nodule formation are mitosis in hypodermal cells within 12-24 hr (Calvert et al., 1984) or cytological dedifferentiation and mitosis in inner cortex within 21-24 hr after inoculation of root nodule bacteria (Dudley et al., 1987), which occurs at the same

time or earlier than root-hair curling, the characteristic early event (Dazzo and Hubbell, 1982; Vasse and Truchet, 1984; Yao and Vincent, 1969). In this study, we observed that the infection of nodule bacteria stimulate cell dedifferentiation and mitosis in uninfected inner cortical cells as well as outer meristematic cells. The progress of dedifferentiation or cell division of the uninfected cells was proportional to distance from the meristematic cells; more progressed in the cells adjacent to the meristematic cells. This indicates that stimulating factors might be transported progressively into the inside of the infected root. The structural features in our study also suggest that cell division and dedifferentiation in nodule formation may be parallel to or independent of the rhizobial infection.

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