Title: Co-localization of membrane and actin in growing infected cells of the *Glycine max* root nodule

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Short title: Membrane and actin in soybean root nodule

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Abbreviations:

ER, Endoplasmic reticulum; IC, infected cell; PBM, peribacteroid membrane; TEM, transmission electron microscope; UIC, uninfected cell.

Abstracts:

The root nodule of *Glycine max* (L.) Merr. is almost spherical in shape at maturity and its central tissue consists of infected cells filled with numerous symbiosomes containing bacteroids, and interspersed uninfected cells. During the growth of the nodule, the volume of each infected cell and the number of bacteroids per cell both increase, and thus abundant membranes are required for the proliferating symbiosomes. We found that in expanding infected cells there are areas adjacent to the nucleus that are devoid of bacteroids, but filled with numerous membranes and actin filaments, and that these areas are surrounded by ER membranes, indicating a perinuclear reservoir of newly formed membranes and a role for actin in delivering membranes to proliferating symbiosomes.

Introduction:

The bacterial infection of the roots of leguminous plants induces the formation of outgrowths called nodules in which symbiotic nitrogen fixation takes place. Infection by bacteria occurs through root hairs producing infection threads and the infection is discernable by root hair deformation (Higashi & Abe 1980; Napoli & Hubbell 1975; Turgeon & Bauer 1985). Simultaneous with infection, cortical cells are induced to divide, producing the nodule primordium, towards which infection threads grow; the bacteria are then released into the cytoplasm of the host cells (Goodchild & Bergersen 1966). The released bacteria are enclosed in a plant derived peribacteroid membrane (PBM) and the whole membrane bound structure is called a symbiosome (Roth *et al.* 1988). Within symbiosomes the bacteria differentiate into nitrogen fixing bacteroids (Gage 2004). The function of the PBM is to compartmentalize the bacteria and to mediate exchange of nutrients between the symbiotic partners (Whitehead & Day 1997). In addition to the cells infected by bacteria, the central tissue of the nodule contains many uninfected cells, which are known to play roles in metabolizing fixed nitrogen (Newcomb *et al*. 1985, Kaneko & Newcomb 1987, Webb & Newcomb 1987).

In legumes such as *Pisum sativum* L. and *Medicago sativum* L., the nodule meristem remains active for several weeks, giving rise to elongated indeterminate nodules that consist of cells of different developmental stages. In contrast, root nodules of *G. max* and *Phaseolus vulgaris* L. have only transient meristematic activity producing a spherical determinate nodule. The central tissue of determinate nodules consists of cells at more or less the same stage of development.

It is estimated that infected cells in a mature *G. max* nodule may contain more than 6000 symbiosomes per cell occupying up to 75% of the cell volume (Bergersen 1997). It has been observed that in the infected cells of determinate root nodules, the number of bacteroids per symbiosome increases with maturation (Whitehead & Day 1997) because at the later stage of nodule development only bacteria continue division within each symbiosome while PBM division abates (Goodchild & Bergersen 1966). On the other hand, in indeterminate nodules, bacterial division is always accompanied by PBM division, resulting in symbiosomes that contain a single bacterium each (Whitehead & Day, 1997).

It is therefore clear that during *G. max* root nodule development, many membranes must be synthesized and transported to the growing symbiosomes in order to maintain an optimal symbiotic relationship. The origin of the PBM has been studied previously, and involvement of ER, Golgi bodies, and infection thread membranes in its formation has been suggested (Cheon *et al*. 1994, Robertson *et al*. 1978, Roth & Stacey 1989). During growth of infected cells, an extraordinary amount of membranes is required to accommodate all of the symbiosomes present in one mature infected cell in a soybean nodule (Roth & Stacey 1989). However, the mechanism which provides these membranes within growing infected cells is

poorly understood. Presence of an actin cytoskeleton network was observed in infected cells of *G. max* (Whitehead *et al*. 1998) and *P. sativum* (Davidson & Newcomb 2001) root nodules and it has been suggested that it may play a role in positioning symbiosomes and supplying membranes to proliferating symbiosomes, but nothing definitive is known.

In this study, we found that in the growing infected cells of *G. max* root nodules, especially in young root nodules of 3 to 4 week old plants, there are specialized areas next to nuclei and rough ER, filled with numerous membranes and bundles of actin filaments associated with the membranes, which suggests localized production of the provision of PBM and a delivery system utilizing actin filaments.

Materials and methods

Plant materials

Seeds of soybean *Glycine max* (L.) Merr. cv. Mikawashima were sown in vermiculite supplemented with some soil from the field for inoculation and kept under 16 h light (15 Wm-2, FL 40s fluorescent lamps, Toshiba, Tokyo, Japan) per day at 25±2°C. Nodules from 3 to 5 week old plant roots were used for experiments.

Processing for light and transmission electron microscopy

Nodules were cut with a razor blade and fixed in 2% gluteraldehyde in 0.05 M potassium phosphate buffer (pH 7.0) and kept at room temperature for 2 h and at 4°C overnight. They were rinsed with the same buffer and postfixed in 2% OsO_4 in the buffer (pH 7.0) at room temperature for 1.5 h, then dehydrated in an acetone series and embedded in Spurr's resin.

For light microscopy, sections of about 0.5 µm thickness were stained with 0.05% toluidine blue in 1% Borax. For transmission electron microscopy, ultra thin sections (silver-gold) were stained with uranyl acetate and lead citrate and observed by Hitachi H-7500 TEM at an accelerating voltage of 100kV.

Processing for fluorescent microscopy

Free-hand sections were cut from soybean root nodules with a razor blade. For fluorescent labeling of actin, membrane and nucleus, we followed the method of Olyslaegers *et al*. (1998), but with some modifications. Sections were incubated in actin buffer (100 mM PIPES, 10 mM EGTA, 5 mM $MgSO_4$ and 0.3 M mannitol, pH 6.9) containing 1% (w/v) glycerol (Wako), 0.2 µM rhodamine phalloidin (Invitrogen, stock dissolved in methanol) to

label actin and $DiOC_6$ (3, 3'-dihexyloxacarbocyanine iodide, Kodak, stock dissolved in ethanol) at a final concentration of 10 μg/ml to label membrane. Both actin and membrane were stained simultaneously for 20 minutes and then Hoechst 33342 (Dojindo, stock dissolved in water) was added to the final concentration of 0.005 μg/ml in the same incubation media to label nuclei and incubated for another 15 minutes. The sections were rinsed three times in distilled water and observed with a Nikon ECLIPSE 50i microscope using the G-2A filter set to visualize labeled actin and the B-2A filter set to visualize labeled membranes and the V-2A filter to visualize labeled nuclei.

Statistical analysis

A Kruskal-Wallis one way analysis of variance (ANOVA) on ranks using the SigmaStat program was performed to evaluate the differences in infected cell size of nodules of different ages. For multiple comparisons, the data were corrected by Dunn's Method.

Results

LM observation of *G. max* root nodules

Semi-thin sections (ca. 0.5 μm thickness) of chemically fixed and resin embedded root nodules were stained with toluidine blue (Fig 1). Under the light microscope, infected cells and uninfected cells at the central portion of root nodules (Fig 1, UIC) could be clearly recognized. The infected cells are enlarged and filled with numerous symbiosomes, while uninfected cells are smaller and contain large central vacuoles. The infected cells tend to have a centrally located nucleus (Fig 1, N). Besides the nucleus, darkly stained areas (Fig 1 a, arrows) devoid of symbiosomes are often observed in young infected cells of root nodules from 3 to 4 week old plants. The intense staining of the area besides the nucleus was reduced considerably in infected cells in root nodules from 5 week old plants.

The frequency of the appearance of darkly stained areas devoid of bacteroids besides the nucleus and the size of the infected cells are shown in Fig 2. Average length of the infected cells containing nuclei in semi-thin sections increased significantly from 34 μm in nodules of 3 week old plants to 60 μm in nodules of 5 week old plants ($P = < 0.001$, ANOVA on ranks). Cells of 5 week old plant root nodules were significantly bigger in size compared to 3 or 4 week old plant root nodules; however, there was no significant difference in the infected cell size of 3 and 4 week old plant root nodules (Multiple comparisons, Dunn's Method). A darkly stained area adjacent to the nucleus was observed in 50 % of the cells containing nuclei visible in the sections of nodules of 3 week old plants; this decreased to 44 % for nodules of 4 week

old plants. In the nodules of 5 week old plants, 27 % of the cells containing visible nuclei possessed an area devoid of bacteroids besides the nucleus.

TEM observation of *G. max* root nodules

Ultrathin sections of root nodules from 3 to 4 week old nodules cut subsequent to the semi-thin sections for LM were observed with TEM. An area equivalent to the darkly stained portion besides the nucleus is shown in Fig 3 a. The area is filled with numerous membrane structures of various sizes, outlined by rough ER membranes. Bundles of microfilaments were frequently observed and appeared to be associated with the membranes. Numerous vesicles or cross sections of tubular structures containing fibrous material (Fig 3 a, arrows) also occur in this area. Similar structures were observed close to the PBM of developing symbiosomes and appeared to be incorporated into PBM (Fig 3 b, arrowheads). The structure with fibrous inclusions (Fig 3 c, arrows) and a possibly related tubular structure (Fig 3 c, double arrows) were observed in the same section occasionally.

Fluorescent microscope observation of *G. max* root nodules

A *G. max* root nodule section fluorescently stained for nucleus, membrane and actin microfilament simultaneously is shown in Figs 4 a-c, which illustrate the same portion of a nodule showing Hoechst 33342 stained nuclei (arrows in Fig 4 a, arrows in Figs 4 b and c also indicate the localization of nuclei), localization of membrane stained with DiOC6 (Fig 4 b), and localization of actin stained with rhodamine-phalloidine (Fig 4 c). Intense fluorescence indicating localization of actin filaments and membranes tended to co-localize besides the nuclei, although intensity of fluorescence for actin and membranes was not always equivalent. In some cases, intense fluorescence for actin with relatively weak fluorescence for membrane, in other cases the opposite was observed. Furthermore, the most intense areas of fluorescence indicating localization of actin filaments and membranes, respectively, did not completely overlap, but they often were found adjacent to each other.

Discussion:

The spherical shape of determinate root nodules in *G. max* is formed after a short bout of meristemic activity, yet nodules continue to get larger for several weeks. The observation that the average size of infected cells in nodules of plants increased significantly from week 3 to 5 would seem to explain a considerable part of the overall nodule size increase in the absence of meristemic activity as caused by the growth of each infected cell.

Infected cells of 3 week old plant nodules are more or less spherical, have centrally located nuclei, and are packed with symbiosomes containing bacteroids. The infected cells of 5 week old plant nodules are often elongated and packed with symbiosomes to fill the cytoplasm. This implies that enormous amounts of peribacteroid membranes (PBMs) surrounding the symbiosomes have to be provided within a growing infected cell, and that the membranes must continue to be delivered during maturation. Roth & Stacey (1989) have estimated that 21,500 μm^2 of new membrane was required to accommodate all symbiosomes present in one mature infected cell in a *G. max* nodules, whereas the plasma membrane area of the infected cell was estimated at only 2,800 μm^2.

The origin of PBMs has been studied ultrastructurally and biochemically (for review, Whitehead & Day, 1997). Ultrastructural observations suggested the involvement of infection thread membranes, ER membranes, and Golgi body derived vesicles in the formation of PBM. It has been shown that a phospholipid component (Mellor *et al.* 1985) and a PBM protein (Cheon *et al* 1994) are produced in ER membranes. Roth & Stacey (1989a) have reported that in *G. max* root nodules, bacteria are released from infection threads near the nucleus, in the presence of abundant ER membranes. Furthermore, from studies with ineffective nodules produced by mutant bacterial strains, the authors concluded that a signal from bacteria may be required to induce synthesis of sufficient ER by plant cells to form symbiosome (Roth & Stacey 1989b).

We found that in growing infected cells of young root nodules, there are specific areas adjacent to the nucleus and ER membranes filled with large amounts of membranes. Since these areas were found mostly in young growing infected cells which require abundant PBM, and since the ultrastructure suggests that the membranes may be newly synthesized from ER, it seems reasonable to assume that those membranes are produced locally to be transported and utilized as PBM. The location of the membrane production besides the nucleus also indicates that it may be associated with the original bacterial release from the infection thread (Roth and Stacey, 1989a) although no infection thread or its remnant could be discerned in the 3 week old plant nodules.

An extensive network of actin filaments in infected cells of developing *G. max* root nodules was observed by confocal microscopy, and an association of actin filaments with symbiosomes has been suggested (Whitehead *et al*, 1998); however no co-localization of actin and membranes has so far been reported. We observed with TEM that abundant bundles of filaments were associated with newly synthesized membranes, possibly from ER, besides the nucleus. Fluorescent labeling with rhodamine-phalloidine proved that the filaments were actin. Further simultaneous staining of membrane, actin and nuclei clearly indicated that the perinucleic area that stained darkly with toluidine blue in resin embedded semi-thin sections consisted of membranes and actin.

From these observations, it can be concluded that there is a specialized area adjacent to the nucleus in which abundant membranes are produced, possibly by the ER, and which serves

as a temporary reservoir for membranes to provide PBMs required within the developing infected cell. Numerous bundles of actin filaments localized at the same time would seem to be involved in membrane delivery to the developing symbiosomes within the cell. There is very likely a mechanism to integrate actin into the membranes for delivery, although it is not known how this happens in *in vivo* root nodules. In an *in vitro* experimental system, the production of tubular structures by incorporating actin into artificial membranes has been reported (Miyata & Hotani, 1992, Honda *et al*. 1999). As our micrographs show many membranous vesicles or cross sections of tubules at the site of developing symbiosomes it would seem possible that these structures are in fact created by the integration of membranes and actin.

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Figure Legends

Fig 1. Light micrographs of cross sections of a soybean root nodule from (a) a 3 week old plant; (b) a 5 week old plant. Infected cells and uninfected cells (UIC) are clearly recognizable. Infected cell size in the 5 week old plant nodule is considerably larger than in the 3 week old plant nodule. Many infected cells of the 3 week old plant nodule possess specialized areas (arrows in a) adjacent to nuclei (N) which stain intensely with toluidine blue. The specialized area (arrow in b) is reduced in the 5 week old plant nodule. Bars = 10 μm.

Fig 2. Infected cell size and occurrence of specialized areas adjacent to nuclei in nodules of 3 to 5 week old plants. The size of infected cells whose nuclei appear in the semi-thin sections was measured. Infected cell sizes of nodules from different plant ages are represented by grey bars. Data are means ± SE. Values with the same letter are not significantly different. The frequency of occurrence of a specialized area adjacent to nuclei in infected cells is shown as a line graph.

Fig 3. Transmission electron micrographs of infected cells of soybean root nodules from (a, b) a 3 week old plant; (c) a 5 week old plant. (a) Numerous membranes in vesicular form and as bulky masses (asterisks) are delineated by ER membranes (ER) besides the nucleus (N). Abundant bundles of microfilaments (MF) appear to be interacting with the membranes. Arrows indicate vesicles with inclusions. B: Bacteroid. (b, c) Vesicles with inclusions (arrows) are frequently observed in the vicinity of developing symbiosomes containing bacteroids (B). They often appear to be incorporated into peribacteroid membranes (arrowheads). Membranes with tubular structure can be recognized occasionally (double arrows). Bars = 1µm.

Fig 4. Fluorescent micrographs of infected cells of soybean root nodules of 4 week old plant. (a) Nuclei (arrows) are fluorescently stained with Hoechst 33342; (b) Membranes stained with $DiOC_6$; (c) Actin filaments stained with rhodamine phalloidin. Images in (a) to (c) represent the same portion and arrows indicate the position of nuclei. Bar = 10 µm.

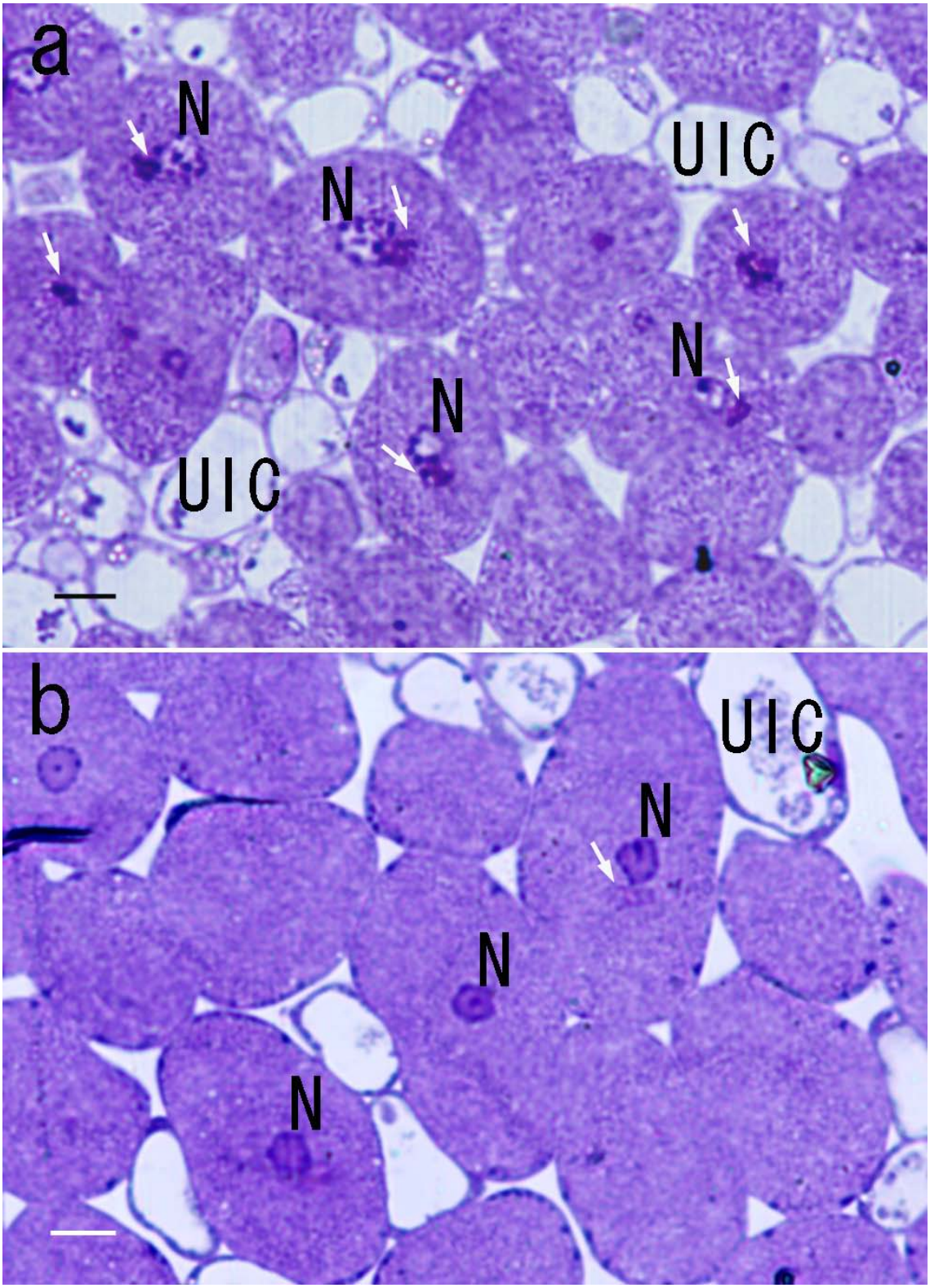

Fig.1

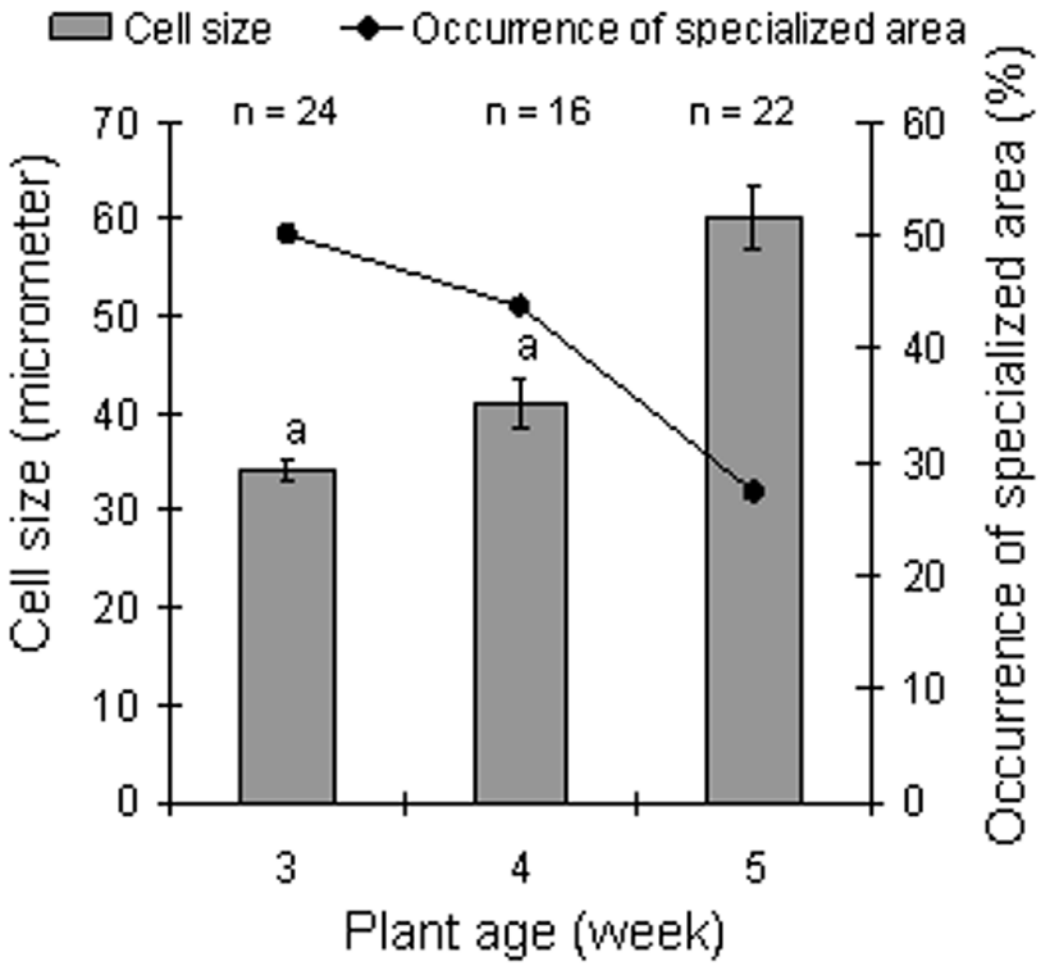
Cell size
Occurrence of specialized area
n = 24
n = 16
n = 22
a
a
Cell size (micrometer)
Occurrence of specialized area (%)
Plant age (week)
0
10
20
30
40
50
60
70
3
4
5

Fig.2

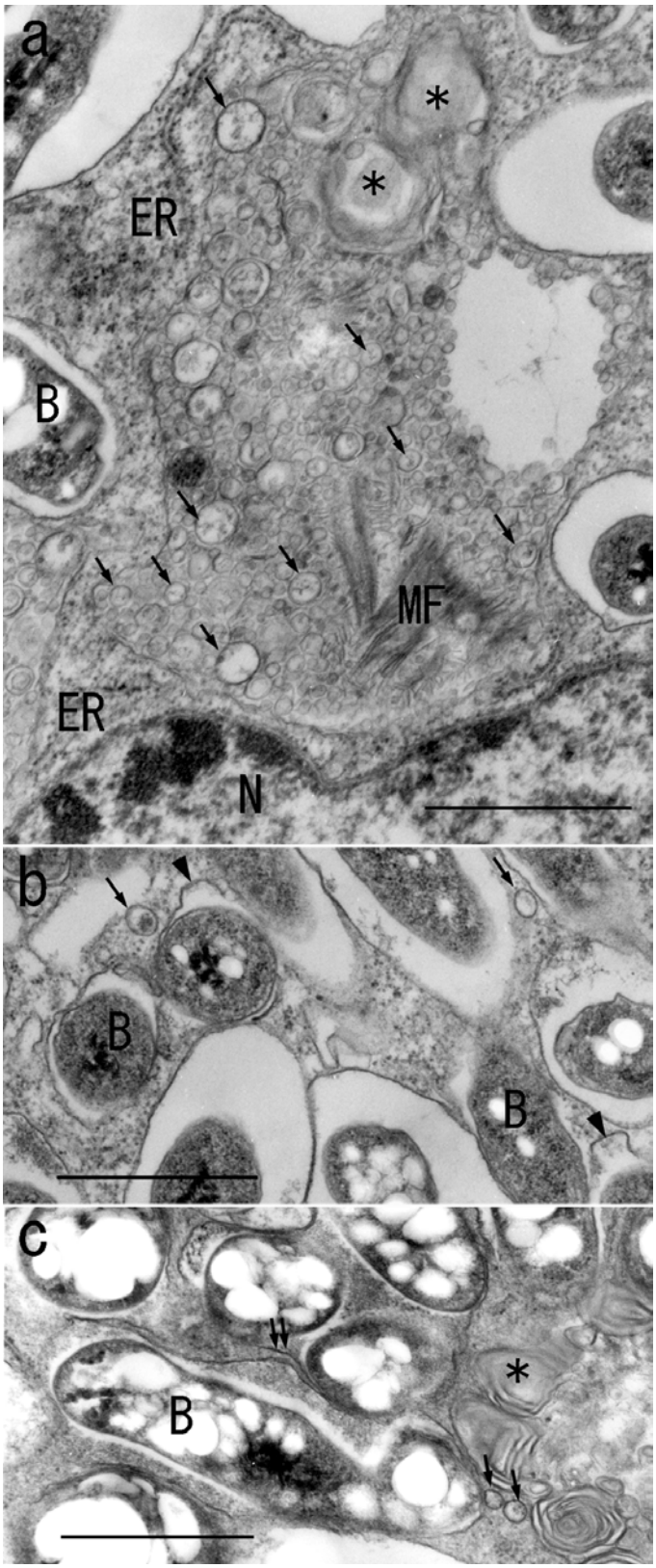

Fig.3

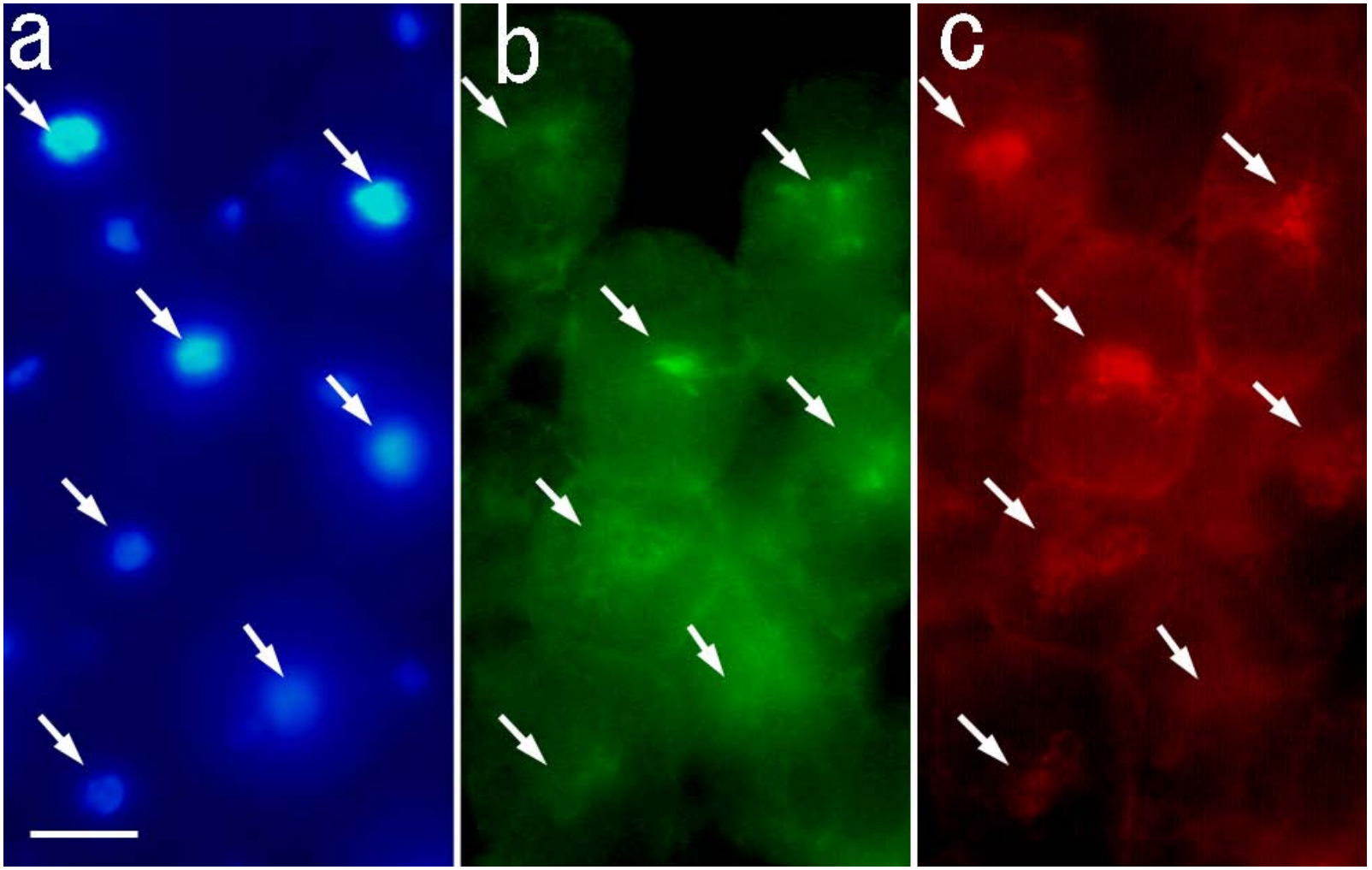

Fig.4