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Scanning Electron Microscopy of *Rhizobium trifolii* Infection Sites on Root Hairs of White Clover

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White clover root hairs which were inoculated with *Rhizobium trifolii* 4S (infectious strain) contained infection threads which were observed by light microscopy and scanning electron microscopy. Three morphological types of root hairs retaining infection threads were recognized. The bacteria were strongly attached between the surfaces of two plant cell walls as follows: between surfaces of a root hair tip curled back on itself, between a protuberance from a root hair and its cell surface, or between two root hair tips clinging together. An anatomical analysis documented the attachment site of the infection thread sheath from the inside of the root hair cell.

An indicator of rhizobial infection of leguminous plants is the formation of an infection thread in the root hair of the host legume. Several events precede the formation of the infection thread (19). Recently, studies which examined the adsorption of *Rhizobium* spp. cells to root hair surfaces of the host plant during the first few hours after inoculation have shown that this is an early determinant of host specificity at the species level (3, 5, 6). However, many of the primary infection steps remain unclear, for example, the mechanism of degradation of the cell wall of the root hair, the mechanism of restoration of the invaded entrance of bacteria at the root hair tip, and the process of making the infection thread. The morphology of the infection process has been described from many observations using light and transmission electron microscopy (7-9, 17, 18) and from a few observations using scanning electron microscopy (1, 4). A marked deformation by curling or branching of root hairs has been reported to be restricted to legumes inoculated with host-specific *Rhizobium* spp. cells (22). The infection process as summarized by Dart (2) indicates that bacterial invasion is always related to deformation of the root hairs. No investigations employing scanning electron microscopy have provided an anatomical analysis of the attachment site of the infection thread and the morphology of the infection thread within the root hair.

This report employs scanning electron microscopy and light microscopy to reveal the rhizobial invasion site and the infection thread attachment point in root hair cells of white clover.

MATERIALS AND METHODS

Rhizobial strain and culture media. The infectious strain of *Rhizobium trifolii* 4S was used. It was cultured in a liquid medium of mannitol-yeast (12) by using a shake culture (40 times per min on a balance arm shaker) at 28°C for 18 h. The exponential-phase bacteria were harvested at room temperature by centrifugation at 10,000 × *g* for 20 min. The bacterial pellet was washed three times with sterile water and then suspended in Fåhraeus inorganic medium (7) at a concentration of 10^6 cells per ml.

Plant culture. The plant material was white clover (*Trifolium repens* cv. Ladino). The seeds were sterilized with a solution of 0.2% formaldehyde-0.1% $HgCl_2$-99% ethanol (1:1:1, vol/vol). After the seeds were rinsed with sterile water three times, they were planted in a thin layer with sterile water in petri dishes and cultured for 3 days at 25°C under shift illumination of light (2.6 W/m^2, 400- to 700-nm wavelength; fluorescent lamps) for 16 h and dark for 8 h. After germination, the seedlings were rinsed three times with sterile water, and 10 plants were transferred to a petri dish (6-cm diameter) containing 3 ml of Fåhraeus inorganic medium and to which bacteria (10^6/ml) had been added. The inoculated plants were incubated for 7 days under the same environmental conditions used for germination.

Scanning electron microscopy. Root hairs used for material were prepared after excising the hypocotyl of the young plant. Root hairs used for anatomical analysis were freehand sectioned by using a razor under a binocular microscope. Materials for scanning electron observation were fixed for 2 h at 4°C in 0.1 M phosphate buffer containing 2.5% glutaraldehyde, pH 6.8. After dehydration through an ethanol series, they were transferred to isoamyl acetate for 30 min at room temperature and dried by the critical point method (HCP-2; Hitachi Co. Ltd., Tokyo, Japan). The specimens were coated with gold about 20 nm in thickness by an ion coater (IB-3; Eiko Engineering Co., Tokyo, Japan). A scanning electron microscope (S-450; Hitachi), was used at 15 kV.

RESULTS

R. trifolii 4S, infectious strain, invaded root hairs and formed infection threads (Fig. 1-5). The attachment site of the infection thread in-

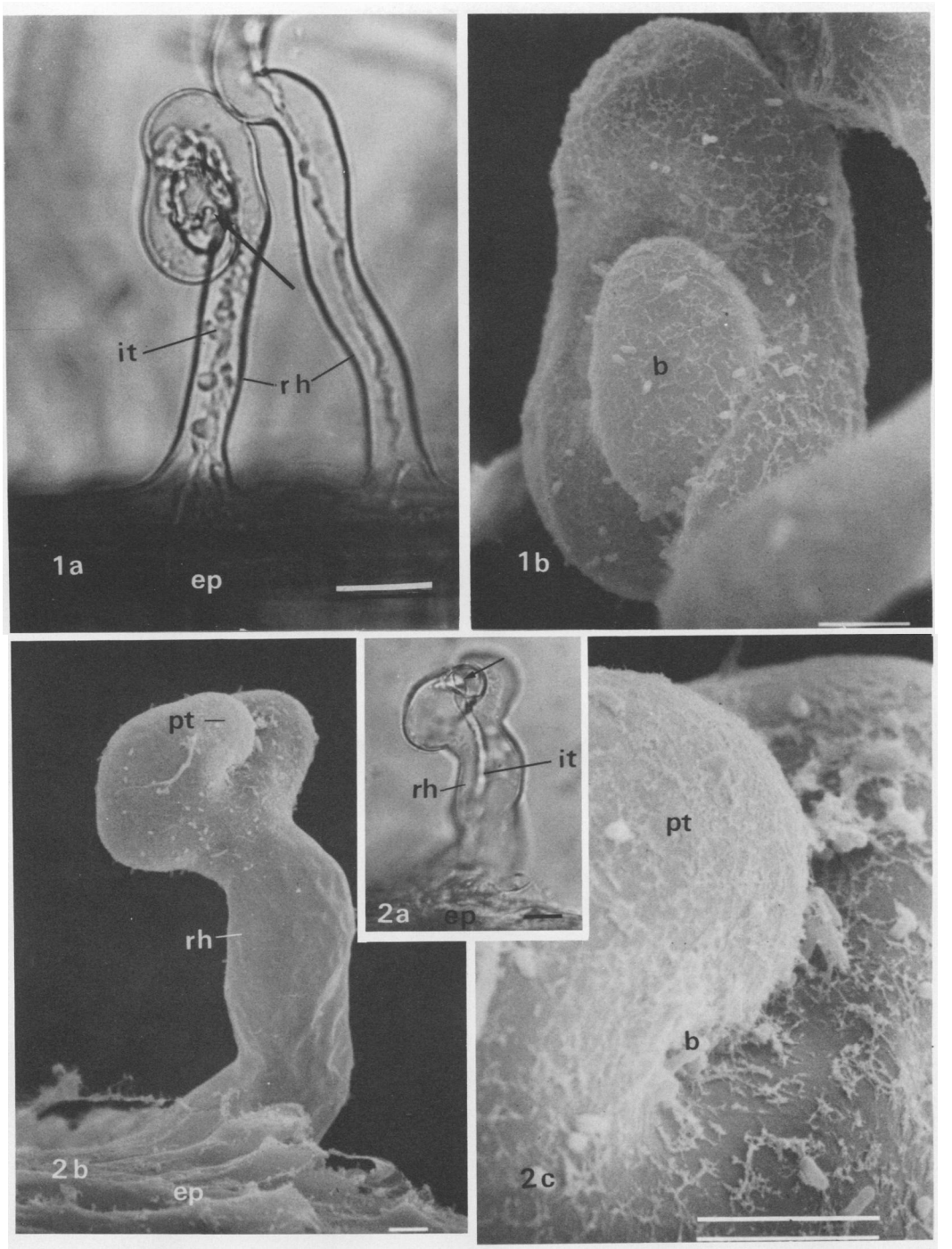

FIG. 1. *(a) Light photomicrograph of a root hair cell containing an infection thread. The arrow indicates the starting point of an infection thread. This is the typical form of Rhizobium-infected root hair cell. Bar, 2 μm. (b) Scanning electron micrograph of the curling root hair tip shown in (a). The bacteria are attached tightly to the root hair cell wall. Bar, 5 μm. Abbreviations: rh, root hair cell; it, infection thread; b, Rhizobium cell; ep, epidermis.*

FIG. 2. *(a) Light photomicrograph of a protuberant tip of root hair cell. The infection thread starts from the inside tip of the protuberance (arrow). Bar, 10 μm. (b) Scanning electron micrograph of a protuberance from the root hair tip tightly attached to the root hair cell wall. Bar, 5 μm. (c) Enlargement of the invasion entrance. Bar, 5 μm. Abbreviations: rh, root hair cell; it, infection thread; ep, epidermis; pt, protuberant tip; b, Rhizobium cell.*

side the root hair cell wall and its developing course were confirmed by light microscopy (Fig. 1a, 2a, and 3a). The profiles from scanning electron microscopy of the same infected root hairs are shown in Fig. 1b, 2b, and 3b, and highly magnified observations of the invading area of bacteria are exhibited in Fig. 2C. Three morphological types of infected root hairs can be recognized. In the first type (Fig. 1), bacteria were tightly enfolded by a core of curling root hair. In the second type (Fig. 2), the infection thread was initiated between a small protuberant branch from the root hair and the outside cell wall. In the third type (Fig. 3), two linear hair tips were entangled with each other, and infection threads were initiated at the point of contact and extended to the base of each of the hair cells.

Two root hair cells containing infection threads are illustrated in Fig. 4a, a low-magnification scanning electron micrograph of the cross section of a root. This figure shows the relative size of the infected root hair and the root. A longitudinal section of a small protuberant branch viewed from inside of a root hair in Fig. 4a is shown in Fig. 4b. A starting point of an infection thread is seen at the inside of the branch (indicated by arrow). It coincides with the morphological type shown in Fig. 2 in that the starting point of the infection thread is inside the protuberance. Figure 5a and b are longitudinal sections of an infected root hair which has curled like the first type. At the most interior part of the fold of the hair tip, infection threads are clearly recognized dividing into three branches from one invasion entrance 5 μm in diameter, and a simple infection thread (about 1.5 μm in diameter) is also observed. These figures suggest the possibility that more than one bacterium can invade one root hair. These infection threads must possess a strong resistance against physical shock since they withstood the various treatments of specimen preparation.

DISCUSSION

In the root hairs of leguminous plants inoculated with host-specific *Rhizobium* spp., deformations such as curling or contorted growth and branching are found (2, 21). However, there is no clear understanding of the relationship between the deformation of root hairs by *Rhizobium* spp. and bacterial invasion. The starting sites of infection threads, which are formed between cell walls, are classified into three kinds. Observation by scanning electron microscopy

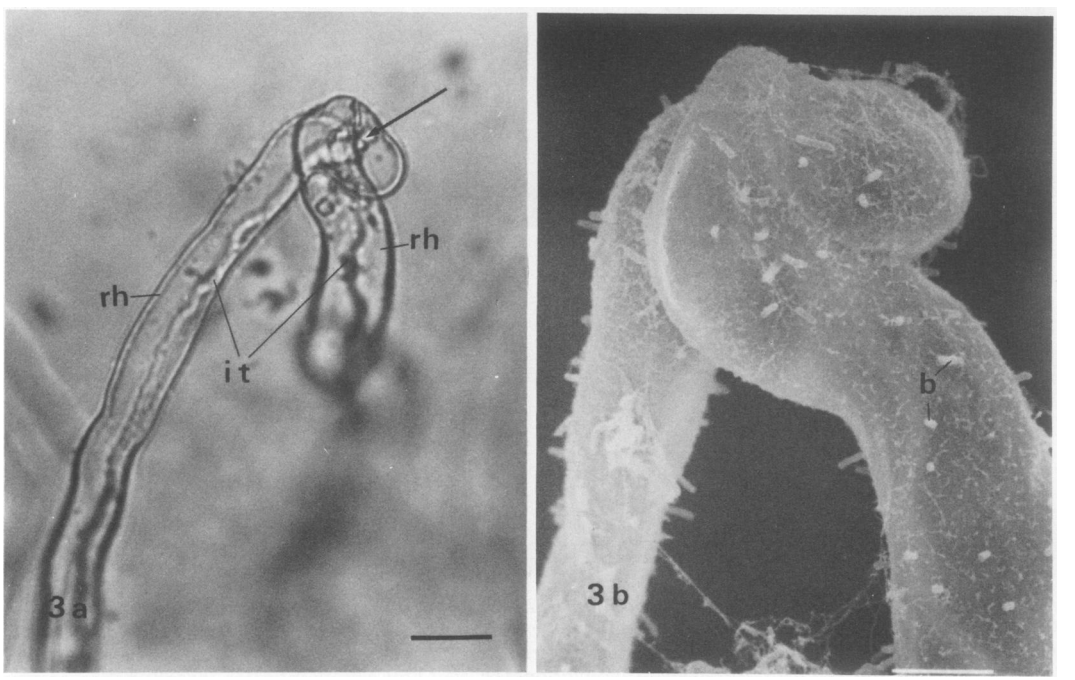

FIG. 3. *(a) Light photomicrograph of two entangled linear root hair tips. The infection thread extends to the base of each root hair cell. The arrow indicates the starting point of two infection threads. Bar, 10 μm. (b) Scanning electron micrograph of the entangled tips in contact with the cell walls. Bar, 5 μm. Abbreviations: rh, root hair cell; it, infection thread; b, Rhizobium cell.*

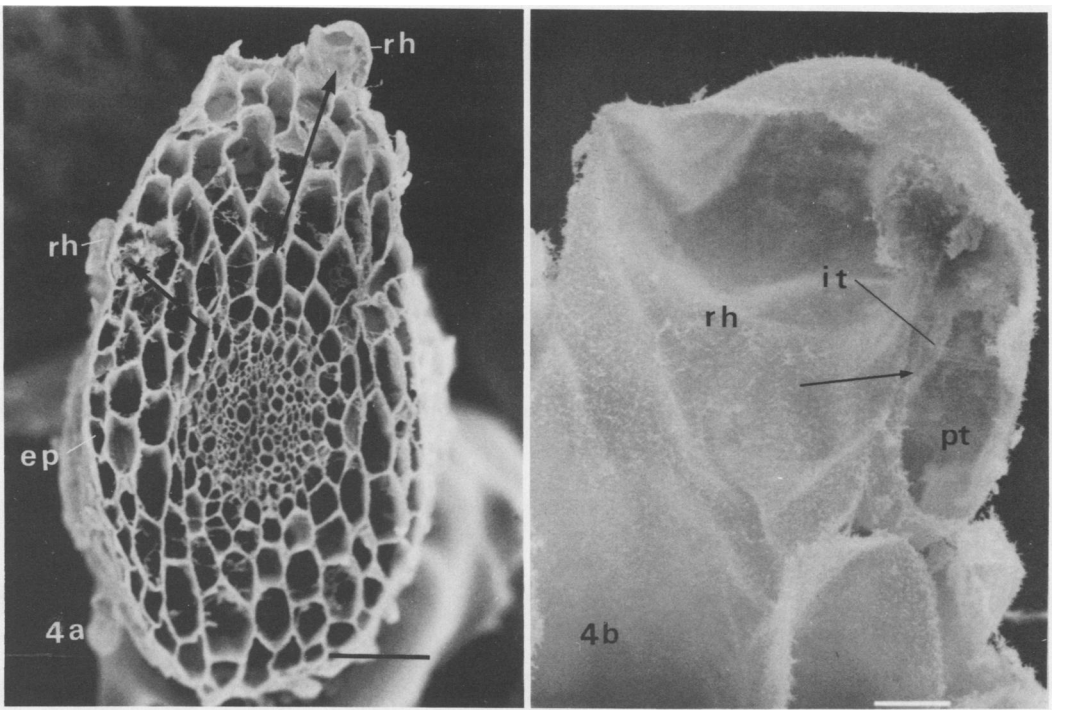

FIG. 4. *(a) Low-magnification scanning electron micrograph of the cross section of a clover root. The arrows indicate root hair cells with infection threads. Bar, 50 μm. (b) Scanning electron micrograph of the longitudinal section of a root hair cell with a protuberance. The arrow indicates the starting point of the infection thread. Bar, 5 μm. Abbreviations: rh, root hair cell; it, infection thread; ep, epidermal cell; pt, protuberant tip.*

has advantages for the research of such special points, which cannot be clearly seen by light microscope. In all types, bacteria produce cell wall occlusions in which one side is certainly the tip area of the root hair or the tip of the branch. Napoli et al. (17, 18) demonstrated that the bacterial floc attaches to the surface of the root hair tip at the initial step of infection; subsequently, infection starts before the root hair deformation, as determined by using transmission electron microscopy. We could not observe the bacterial flocculation on the surface of root hair tips because of the critical point method drying treatment used for scanning electron observation. However, if the infection started without the attachment of bacteria to the root hair cell wall, pairs of root hair tips would not become entangled as in type 3 (Fig. 3), in which two infection threads extended toward the bases of two root hairs from the same starting point. Napoli and Hubbell (18) described a very rare infection type in which an undeformed root hair has an infection thread in it; however, we could not observe it by scanning electron microscopy.

Ljunggren and Fåhraeus (14) reported induction of polygalacturonase from host root hair cells by compatible rhizobial exopolysaccharide. Many investigators have not confirmed this hypothesis (13, 15, 20). Hunter and Elkan (11) also did not find hydrolytic enzymes which were induced by any rhizobial cell or cell materials. However, they suggested that highly localized increases in enzyme activity might occur during invasion. More recently, the production of hydrolytic enzymes from rhizobia was confirmed (10, 16). On the basis of these facts, a suitable hole must be opened in the cell wall of the root hair for bacterial entry. The size of the invaded entrance is about 1.5 to 5.0 μm in diameter (Fig. 5). After the bacteria penetrate into the root hair cell, the invasion entrance must be enclosed by some mechanism. It is reasonable to presume that contact and adhesion of overlapping cell walls act as a protective mechanism against leakage of cytoplasmic materials from the root hair cells during bacterial penetration. We consider that, after the initial step of *Rhizobium* infection, the infective bacteria induce the deformation of the host root hairs by some interaction between the host roots and the bacteria when the bacteria are tightly occluded in the close space between root hair cell walls during these

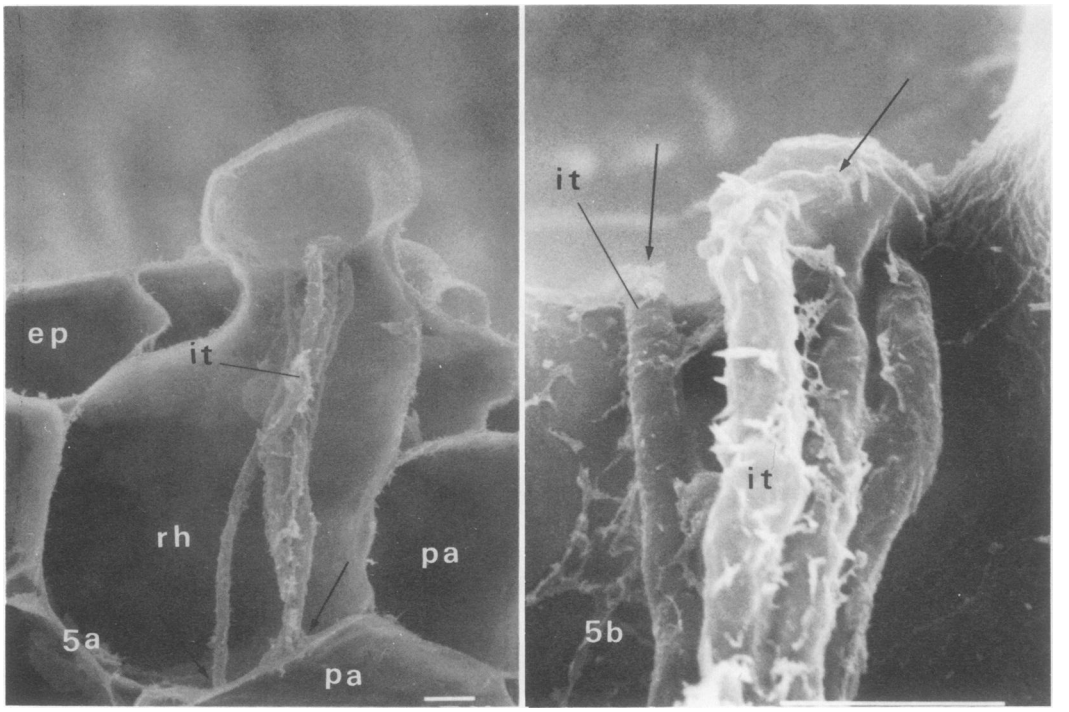

FIG. 5. *(a) Scanning electron micrograph of the longitudinal section of a root hair cell retaining infection threads. This root hair cell curls towards the opposite side. One infection thread branches into three from a part of an entrance, and the other is a single thread. The ends of a sheath of these infection threads touch on part of a connected cell wall of a root hair and a parenchyma cell (arrows). Bar, 5 μm. (b) Enlargement of the invasion entrance part (indicated by arrows) in (a). Bar, 5 μm. Abbreviations: pa, parenchyma cell; ep, epidermal cell; rh, root hair cell; it, infection thread.*

deformations; subsequently, they degrade the tips of the root hair cell walls and enter the root hair cells by constructing the infection thread.

The infection thread passes through the root hair cells to the adjoining sponge (parenchyma) cells (Fig. 5). The sheath of the infection thread tightly attaches to the connected cell walls of the root hair and parenchyma cells. An additional interesting question is whether the conditions of bacterial invasion are the same when bacteria degrade the cell walls of root hair tips and those of the parenchyma or cortex cells.

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