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The Feeding Behavior of Adult Root-knot Nematodes (*Meloidogyne incognita*) in Rose Balsam and Tomato

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Abstract: Meloidogyne incognita is a parasitic root-knot nematode that causes considerable yield loss in a wide range of plants. In this study we documented the movement of adult female nematodes for more than 2 hr in micro-slices of infected tomato (*Solanum lycopersicum*) and rose balsam (*Impatiens balsamina*) plants using light and video microscopy. Stylet thrusting was followed by short pumping actions of the esophagus, dorsal esophageal gland ampulla, and metacorpal bulb. Regular thrusting was normally accompanied by head turning and always preceded continuous stylet thrusting aimed at a single point (for 20 to 90 sec). Females often held the stylet in a protruded position, while pulsating the metacorpus bulb, for about 30 sec. Subsequently, the stylet was paused in a retracted position for 5 to 40 sec. This sequence of behavior took 290 to 380 sec to complete. The procedure developed in this study provides a useful cytological technique to investigate the interaction between root-knot nematodes and the giant cells formed by infected plants. Scanning electron microscopy revealed that the head of the adult nematode was located in the narrow intercellular spaces among several giant cells. The anterior part of the head of the adult was folded like a concertina, whereas that of the second-stage juvenile was not. The labial disc and medial lips of second-stage juveniles seemed expanded and sturdy, whereas those of the adult were star-shaped, appeared to be contracted, and softer. These morphological differences in the heads of adult and second-stage juveniles are discussed with respect to their movement.

Key words: cytological technique, feeding site, giant cell, *Impatiens balsamina*, micro-slices, morphology, SEM, *Solanum lycopersicum*, video light microscopy.

The root-knot nematode [*Meloidogyne incognita* (Kofoid and White, 1991) Chitwood, 1949] invades roots and induces the formation of giant cells (GCs), which are the dominant feeding sites of the nematode. Nematodes maintain GCs throughout their growth, development, and reproduction (Berg et al., 2008). The interaction between host plants and their parasitic nematodes is complex, and only limited information is available about the behavior of endoparasitic nematodes inside their host roots (Wyss and Zunke, 1986; Hussey 1989; Sijmons et al., 1991; Wyss et al., 1992). Wyss et al. (1992) used a video recorder to monitor second-stage juveniles (J2) of *M. incognita* as they invaded plant roots and moved toward the central cylinder to induce GC formation. However, it is technically challenging to record adult female root-knot nematodes in living roots, because visibility is gradually obscured as the gall tissues develop.

In this study, we sought to document the behavior of adult female nematodes within root galls of infected plants, and to compare the location of adult female and J2s in infected roots using scanning electron microscopy (SEM).

Materials and Methods

Nematode preparation: The *Meloidogyne incognita* (Kofoid & White, 1991) Chitwood, 1949 used in this study was originally isolated from Japanese yam (*Dioscorea japonica* Thunb.) in Ishikawa Prefecture, Japan. The single egg mass populations were multiplied and maintained in the greenhouse on tomato (*Solanum lycopersicum* L.) cv. Kyoryoku-Beiju (Takii Seed, Kyoto, Japan). After 40 to 50 d of cultivation, the root systems were carefully removed from the pots and washed. The egg masses that formed on the galls were collected and the J2s that hatched from these eggs were used for inoculations.

Nematode inoculation: Tomato cv. 'Kyoryoku-Beiju' and rose balsam (*Impatiens balsamina* L.) 'Tsubakizaki-kongo' (Takii Seed) were used as host plants in this study. Seeds were sown in trays (200 cells/flat) filled with seedling soil (Yosaku N150; JCAM AGRI., Tokyo, Japan). After 3 wk the seedlings were transplanted to polyethylene pots (10.5-cm diam.) filled with a mixture of seedling soil (Yosaku N150; JCAM AGRI.) and beach sand at 1:1 (v/v). A week after transplanting, these plants were inoculated by pouring 3 ml of water containing 200 J2s into each pot. The pots were maintained in a greenhouse ventilated at 25°C.

Light microscopy and video recording: Thirty-day-old galled root tissues infected with *M. incognita* were collected from greenhouse-grown rose balsam and tomato. The galls were sectioned into180-μm-thick slices using a micro-slicer (DTK-3000; Dosaka EM, Kyoto, Japan). Living nematodes that had not been injured during sectioning were selected for observation with light microscopy. The behavior of adult females was monitored using light microscopy and recorded with a digital camera (HC-300Z; Fujix, Tokyo, Japan) and HDD & DVD video recorder (DVR-HG865; Mitsubishi Electric, Tokyo, Japan). Images of the stylet and the relevant scenes acquired every 0.1 sec were printed using computer software (Digital Creation Gear Digizo; Princeton Technology, Tokyo, Japan) for video analysis. To evaluate the movement of the stylet, distances between the stylet knobs and the lips were measured in consecutive images (Fig. 1A). At least 50 root galls from each plant species were observed.

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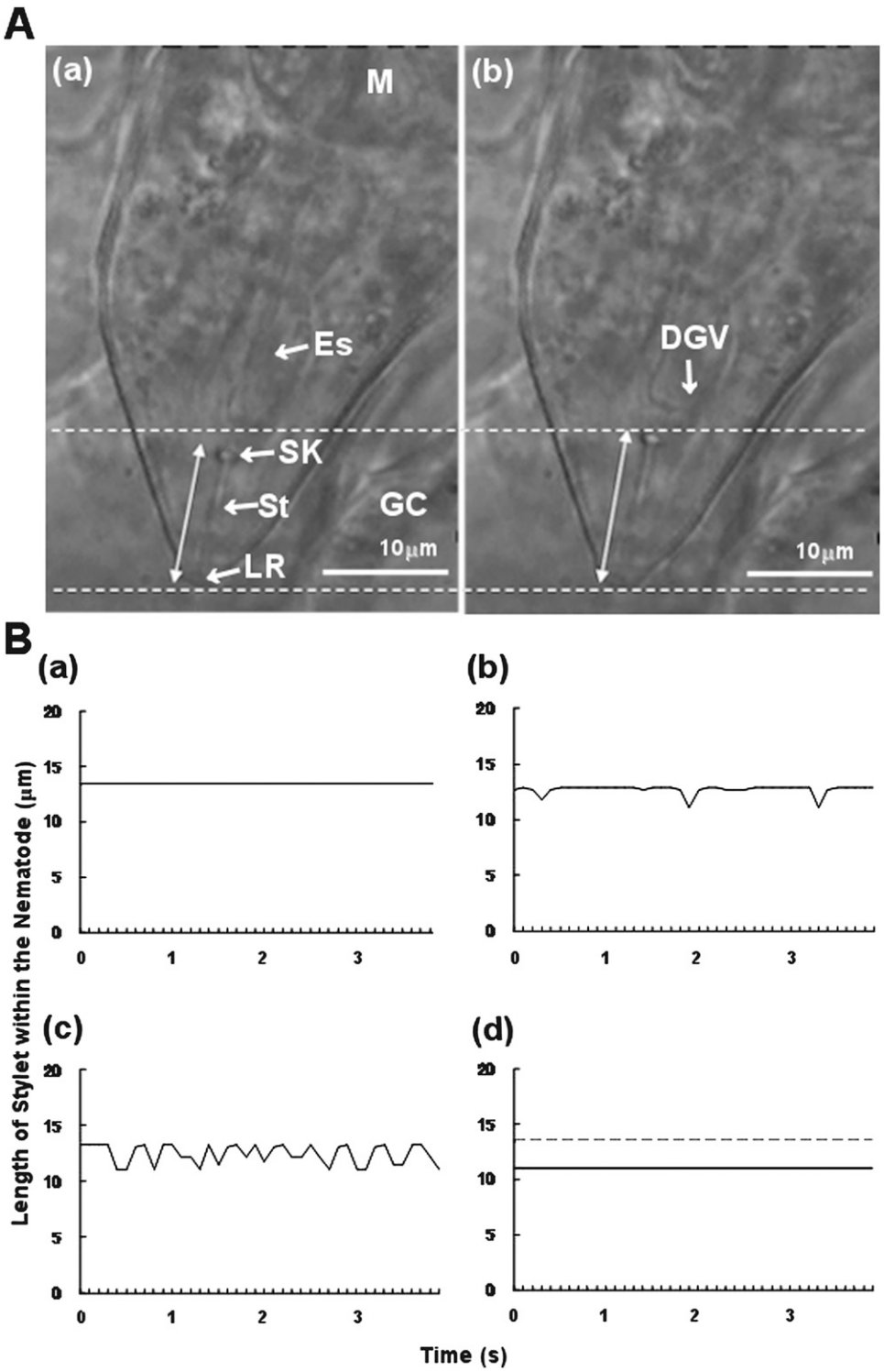

FIG. 1. Stylet thrusting of an adult female *Meloidogyne incognita* nematode. A. Light micrographs showing stylet thrusting in a micro-slice of a root gall of *Impatiens balsamina*. The stylet (a) protruded and (b) retracted 0.1 sec later. The upper dotted line indicates the level to which the stylet knobs retracted, and the lower line shows the level of the lip region. The arrows show the distance between the lip region (LR) and stylet knobs (SK). Es: esophagus; DGV: dorsal gland valve; GC: giant cell; M: metacorpus; St: stylet. B. Four typical patterns of stylet thrusting behavior in the giant cells of *Solanum lycopersicum* root galls micro-slices were distinguished by measuring the length between the lip region and stylet knob every 0.1 sec: (a) paused stylet thrusting, (b) regular stylet thrusting (one thrust per second), (c) rapid stylet thrusting (three to five thrusts per second), and (d) paused insertion (several tens of seconds). The dotted line represents the maximal distance between the lip region and retracted stylet knobs.

Scanning electron microscopy: The interfaces between adult nematodes and GCs in root galls were observed using SEM. Micro-sliced root-knot specimens containing an adult nematode were immersed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 2 hr at 24°C. Postfixation with 1% osmium tetroxide was

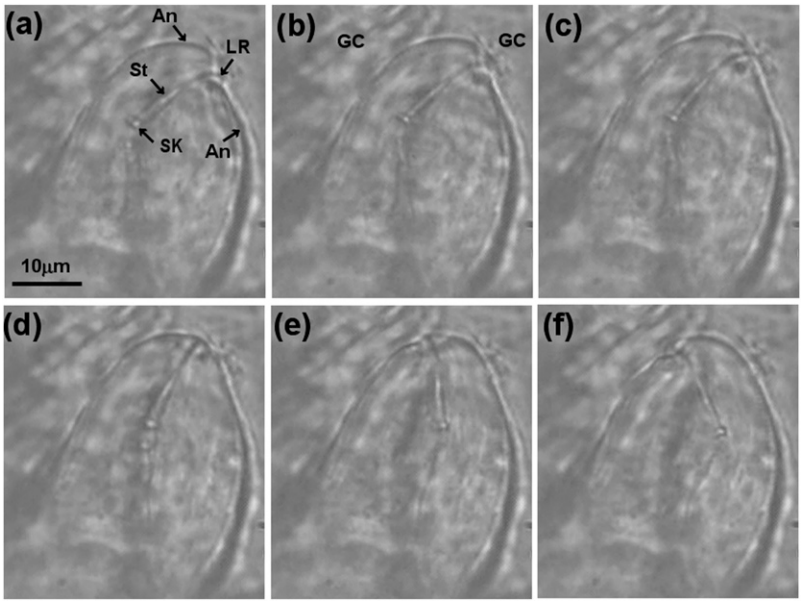

FIG. 2. Stylet and head mobility of adult female *Meloidogyne incognita* in *Impatiens balsamina* root galls. (a) 0 sec (start of movement), (b) 3 sec later, (c) 6 sec later, (d) 9 sec later, (e) 12 sec later, and (f) 15 sec later. Note that only the lip region (LR), stylet knobs (SK), and nearby annulations (An) were moving, whereas head parts that were posterior to the stylet knobs were stationary. GC: giant cell; St: stylet.

performed in the same buffer for 2 hr at 24°C. Specimens were then dehydrated in a graded series of ethanol, immersed in t-butyl alcohol (2-methyl-2-propanol), and subjected to freeze drying (ES-2030; Hitachi, Tokyo, Japan). The specimens were coated with 8 nm of platinum by ion spatter (E-1010; Hitachi) and observed with a field emission SEM (S-4700; Hitachi) at 15 kV. At least 30 root galls from rose balsam were observed.

RESULTS AND DISCUSSION

The movement of adult nematodes in root microslices was monitored using light microscopy, and recorded video images for more than 2 hr. Stylet thrusting was followed by short pumping actions of the esophagus, dorsal esophageal gland ampulla, and metacorpal bulb (Fig. 1A). By measuring the differences in length between the lip region and stylet knob at 0.1-sec intervals, we determined the time interval between protrusion and retraction of the stylet (Fig. 1B). Whereas retracted stylets were 13.5-μm long inside the nematode, protruded stylets were between 10.5- and 12.0-μm long inside the nematode. Therefore, we speculate that stylets protrude by up to 3 μm. Based on our observations we grouped the thrusting behavior into four categories: (i) paused stylet thrusting (i.e., the stylet is fully

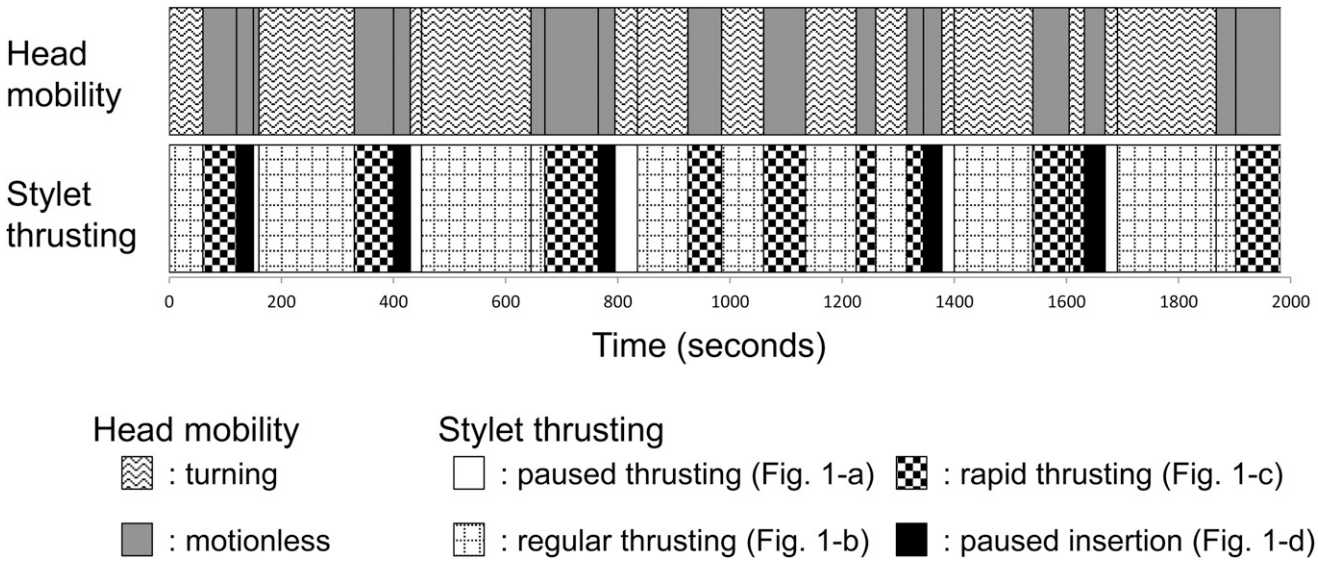

FIG. 3. Time-dependent changes in head mobility and stylet thrusting behavior of *Meloidogyne incognita* adult female in the giant cells of micro-slices of *Solanum lycopersicum* root galls. The head was turning or motionless, and patterns of stylet thrusting behavior are shown as (a) paused thrusting, (b) regular thrusting, (c) rapid thrusting, and (d) paused insertion, as in Fig. 1B.

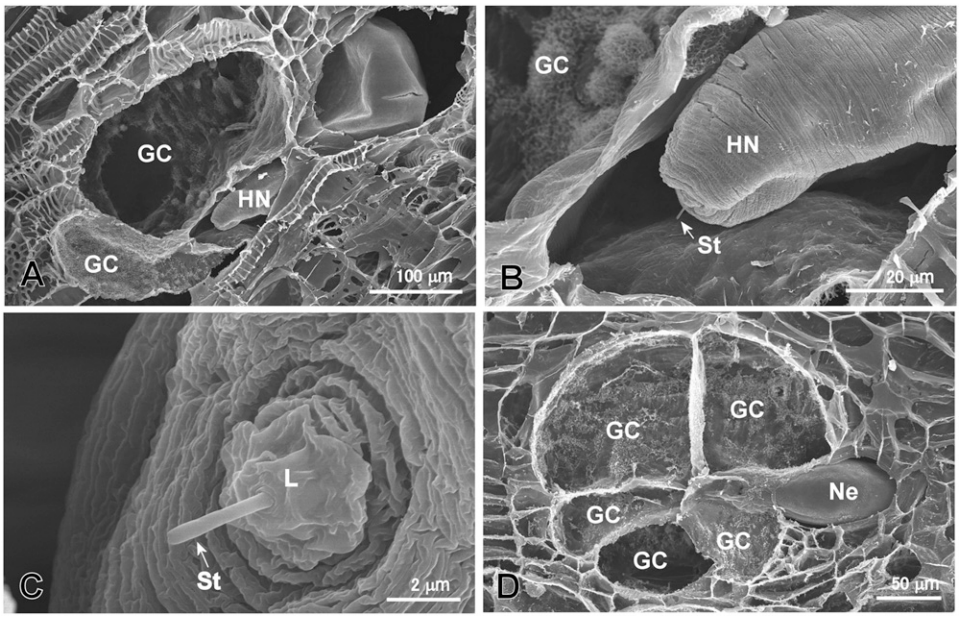

Fig. 4. Scanning electron micrographs of an adult female *Meloidogyne incognita* nematode in an *Impatiens balsamina* root gall micro-slice. A. Adult nematode close to giant cells (GCs). Note that the nematode's head (HN) is positioned for feeding from the GCs. B. Head of nematode close to GCs. Enlarged area of panel A showing the anterior end of the female with protruded stylet. Note the protruding stylet at the apex of the head. C. Lip of the same female shown in panel B. Stylets protrude to a length of about 3.1 μm. D. Head of an adult female nematode close to GCs. Note that the upper region of the nematode's head is positioned for convenient feeding from five GCs. L: lip; Ne: nematode; St: stylet.

retracted), (ii) regular stylet thrusting (i.e., the stylet thrusts at GCs at a rate of one thrust per sec), (iii) rapid stylet thrusting (i.e., the stylet thrusts at a rate of three to five thrusts per sec), and (iv) paused stylet insertion (i.e., the stylet is protruded, but stationary) (Fig.1B).

The lip region of the female adult nematode's head moved all direction, and also anterior region of the head expanded forward and contracted backward in the narrow gap among giant cells (Fig. 2). This behavior was accompanied by simultaneous thrusting of the stylet. When the annulations on one side of the head expanded, the folds on the other side contracted, as reported previously for a mature root-knot nematode (Linford, 1937). The outline and annulations of the anterior region of the head changed when the lip region moved, whereas that of posterior to the stylet knob hardly moved and did not appear to undergo morphological changes (Fig. 2).

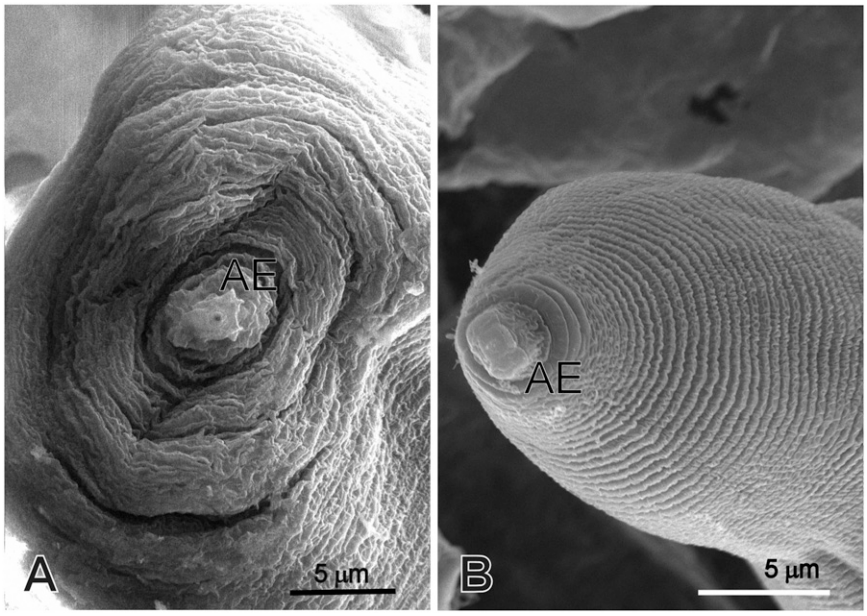

Fig. 5. Comparison of adult female and swollen J2 of *Meloidogyne incognita* in *Impatiens balsamina* root galls. Scanning electron micrographs of (A) adult female and (B) J2 anterior ends. Note that the adult's female head is folded like a concertina, such that the anterior end (AE) is able to move freely in various directions, whereas the head of the J2 is not. The lips of the J2 appear to be sturdier than those of the adult female.

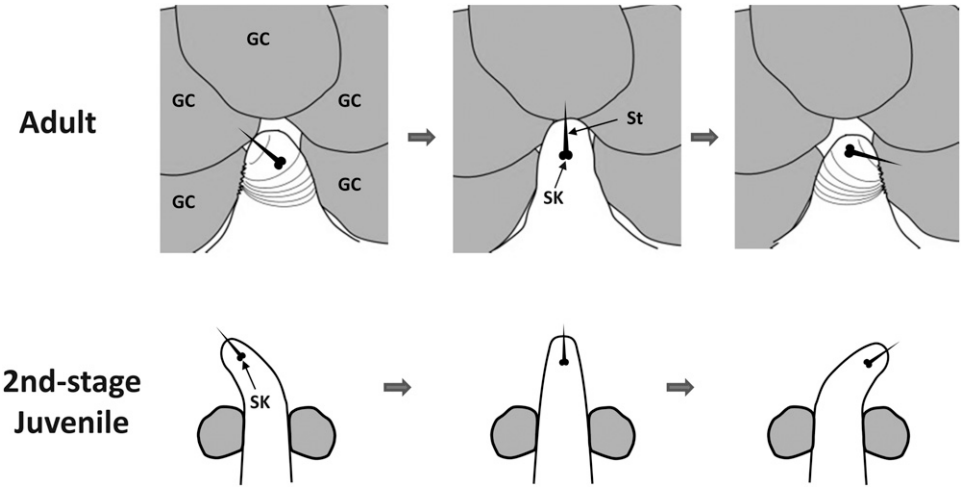

FIG. 6. Schematic representation of stylet and head mobility of adult female and swollen J2 *Meloidogyne incognita* in *Impatiens balsamina* root galls. The anterior region of the adult's female head is folded like a concertina, whereas, that of the J2 is not. GC: giant cell; St: stylet; SK: stylet knobs.

The duration of the head movement and stylet thrusting patterns as illustrated in Fig.1B is diagrammed in Fig. 3. Regular thrusting was usually accompanied by head turning and always preceded rapid stylet thrusting aimed at a single point (for 30 to 90 sec). After rapid thrusting, females often exhibited paused stylet thrusting (paused insertion), during which the metacorpus bulb pulsated for about 30 sec. Subsequently, pausing was observed for 5 to 40 sec. The sequence of behaviors described above took 290 to 380 sec to complete. Therefore, it seems that regular thrusting corresponds to searching for suitable thrusting points, rapid thrusting corresponds to inserting the stylet into GCs, and paused thrusting corresponds to feeding and/or secretion. Wyss et al. (1992) showed that the J2 had characteristic behavioral sequences, including stylet-tip protrusion, metacorpal bulb pumping, and rapid stylet and head movement. The sequences described for J2s are similar to those observed for female adults in this study. In *Heterodera schachtii* A. Schmidt, 1871 J2, the stylet remained protruded while the metacorpus pulsated for about 1 hr (Wyss and Zunke, 1986). In *M. incognita* J2, the pulsations lasted for only tens of seconds (Wyss et al., 1992). Wyss and Grundler (1992) speculated that *M. incognita* adult females exhibited similar feeding cycles as J2, based on the presence of numerous feeding tubes in GCs (Hussey and Mims, 1991). Therefore, *M. incognita* nematodes appear to have relatively short and regular feeding cycles both in the J2 and adult female stages.

After confirming with light microscopy that the microslices contained an adult female nematode, the microslices were analyzed using a SEM. The nematode's head was located in the narrow intercellular space close to the center of the gall, where the corners of several GCs converged (Fig. 4A,D). The intercellular space containing the anterior end of the nematode appeared to be too narrow to permit free movement. The stylet (0.44 μm in diam.) protruded from the apex of the head (Fig. 4B,C), to a length of 3.1 μm (Fig. 4C). The length of the protruded stylet shown in Fig. 4C corresponded with the maximum length calculated from the stylet thrusting behavior observed in light micrographs (Fig. 1). This length is sufficient to penetrate GC walls, which 0.8- to 2.3-μm thick (Hussey and Mims, 1991). Stylet puncture sites in tomato root GC walls were found to have a maximum width of 0.43 μm, based on transmission electron micrographs (Hussey and Mims, 1991). This value was coincident with the diameter of the stylet (0.44 μm) determined by SEM (Fig. 4C).

The anterior region of the adult head was folded like a concertina (Figs. 4C; 5A), whereas that of the J2 was not (Fig. 5B). Based on the shape of the anterior region of the adult head, the anterior annulations of the adult appeared to enable the head to move freely in all directions, as we observed using light microscopy (Fig. 2). On the other hand, the annules in the anterior region of the J2 head was not folded (Fig. 5B), and J2s changed position by moving their entire anterior end (Wyss et al., 1992). In the J2 stage, somatic muscles run along the entire length of the nematode, whereas they are limited to the anterior end of the female adults (Elsea, 1951) because they lose most of the somatic muscles, except for those in the anterior end (Bird 1967, 1971). These reports support the notion that adult females turn their heads by moving only the anterior portion, and that this motion is directed by both the lip region and the stylet knob. A schematic representation of stylet and head mobility of the adult female and J2 *M. incognita* in *I. balsamina* is shown in Fig. 6.

The labial disc and medial lips of the J2 are dumbbell-shaped (Eisenback and Hirschmann, 1979) and appear thick and sturdy (Fig. 5B), whereas those of the adult female are star-shaped and thin, and appear to be weak (Fig. 5A). The sturdy structure observed in the J2

may be used to invade root cells. It was previously reported that J2 rubbed their lip against the wall surface at the junction between the two cells and that point was weakened (Wyss et al., 1992). In contrast, the adult nematode, which is embedded in the root, has no need to penetrate the cell wall, and the annulations of the anterior end are therefore specialized for feeding from GC. Thus, the cytological method presented in this study provides insight into the feeding behavior of root-knot nematodes in the GCs of infected plants.

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