Cell bodies and neuronal processes of externally exposed amphid sensilla in

Bursaphelenchus xylophilus

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Externally exposed sensilla, amphids and phasmids, in the pine wood nematode Bursaphelenchus xylophilus were examined with respect to their number, wiring, and position in comparison with those reported in the free-living soil nematode Caenorhabditis elegans. When B. xylophilus was soaked in FITC solution at 5°C for a few hours, two pairs of amphid neurons were stained. The staining pattern showed their bipolar processes emanating from the cell body located dorso-laterally: one process extended toward the tip of the head, and the other was projected into the nerve ring. Overnight soaking of the nematode with FITC revealed a teardrop-shaped apparatus with triradiate symmetry at the anterior of the metacorpus and long-shaped structures running toward stylet knobs. By this method, we could not detect phasmids in B. xylophilus, the result being different from those in C. elegans. Jpn. J. Nematol. 32 (2), 45-52 (2002).

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Like other animals, most nematodes need to sense environmental cues so as to take immediate and appropriate actions to them for their survival. They sense various outside stimuli by using sensory neurons, which are abundant in the head region and also in the tail, though less so (Cooman and De Grisse, 1985; White et al., 1986). Neurons are generally classified into one of three types, sensory neurons, interneurons, or motor neurons (motoneurons) (White et al., 1986). Among sensilla, amphids and phasmids, which are present at openings in the head and tail regions, respectively, are very conspicuous for many nematodes and comprise bilaterally symmetric pairs of externally exposed chemosensory and mechanosensory organs. In the free-living soil nematode Caenorhabditis elegans, for example, bundles of 12 sensory neurons form the amphid sensilla, and bundles of 2 sensory neurons make up the phasmid sensilla (White et al., 1986). Eight of the amphid neurons and 2 phasmid neurons bundled together pass through channels, formed by sheath cells and support cells, to narrow openings where their dendrites are exposed externally to catch environmental stimuli (White et al., 1986; Mori, 1999). If these neurons carry any developmental or functional abnormality, their important functions, such as chemotaxis, thermotaxis, and mechanosensory actions as well as dauer entry or recovery, cannot be executed properly (Hedgecock et al., 1985; Perkins et al., 1986; Bargmann and Horvitz, 1991; Shakir et al., 1993; Mori, 1999).

The pine wood nematode Bursaphelenchus xylophilus also has similar sensilla, which must be essential for various taxis and mating behaviors, entrance into beetle vectors, transmission to and invasion

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of host trees, and entry into and recovery from the dispersal form (equivalent to dauer form in *C. elegans*). In *C. elegans*, mutations and cellular laser ablation causing sensory abnormalities in these neurons have provided powerful tools to analyze the properties of “sensory behaviors.” To do similar analyses in *B. xylophilus*, therefore, we first examined the normal wiring of sensory neurons, by a simple and convenient FITC-soaking or-filling method (Hedgecock et al., 1985), and compared the observation with that made of *C. elegans*.

**MATERIALS AND METHODS**

**Handling of nematodes**

*B. xylophilus*, isolate S-10, was cultured on a fungal mat of *Botrytis cinerea* grown on potato dextrose agar (PDA) according to Futai (1980).

**Fluorescin isothiocyanate (FITC) staining**

Amphid and phasmid neurons were stained by the modified method used for *C. elegans* by Hedgecock et al. (1985). A stock dye solution containing 20 µg/ml 5-fluorescin isothiocyanate (FITC) in dimethylformamide was stored at -20°C and diluted before use by adding M9 buffer (stock dye solution: M9 buffer = 1:4 v/v) as a working dye solution. Two modified methods were used as follows: First, to stain *B. xylophilus*, about 300 µl working dye solution was dropped directly onto nematodes, which had been growing on PDA plates for three or four days, and the nematodes were further incubated at 20°C overnight. After incubation, nematodes were transferred to a fresh agar plate without dye and allowed to crawl around for at least 10 minutes to remove excess FITC, anaesthetized with 1% 1-phenyl-2-propanol (unpublished result), and mounted on an agar pad prepared on a microscope slide (Sulston and Horvitz, 1977) for viewing by a confocal laser scanning microscope (ZEISS Axioplan 550) and a Nomarski differential interference contrast and fluorescence microscope (Nikon E 600). Second, to stain *B. xylophilus*, about 10 nematodes were picked up and transferred into a few drops of the working dye solution on a slide glass, and the slide was then kept at about 5°C in a refrigerator for a few hours. After the staining, the nematodes were transferred into a few drops of distilled water on a slide glass and incubated at room temperature (25°C) for about 10 minutes to remove excess FITC. Then, the nematodes were anaesthetized, mounted, and viewed as described above.

**Silver nitrate staining**

Nervous system staining by using silver nitrate (Hooper, 1986) was used for *B. xylophilus*.

**RESULTS**

**FITC staining by overnight soaking at 20°C**

Overnight soaking of *B. xylophilus* in FITC at 20°C revealed a teardrop-shaped apparatus (Fig. 1.a), which was located with triradiate symmetry at the anterior of the metacorpus (Fig. 1.b), and detected long-shaped structures running toward stylet knobs (Fig. 1.a, c). Phasmids could not be detected in *B. xylophilus*. 
Fig. 1. An FITC-filled live *B. xylophilus* female, soak-stained overnight at 20°C. (a) Projection image of cephalic region. Arrow shows a teardrop-shaped apparatus. Bar, 20 µm. (b) Transverse section image at the posterior region of the metacorpus (position is indicated by the arrowheads in (a)) reconstructed by ZEISS LSM 5 Image Browser. Pharyngeal apparatus is stained in triradiate symmetry. Dorsal is top. (c) Nomarski differential interference contrast image and (d) fluorescent image of cephalic region. Bar, 10 µm.
Fig. 2. FITC-filled amphid neurons in a live *B. xylophilus* female, soak-stained in FITC dye for a few hours. APAR/L represents AmPhid Anterior Right/Left, and APPR/L indicates AmPhid Posterior Right/Left. (a) Left view of amphid neurons. Processes (arrowheads) of the sensory neurons emanate from the two cell bodies (arrows) to the tip of the head. The cell bodies are situated posterior to the dorso-laterally positioned nerve ring (NR). Presynaptic processes (PP) from the cell body to the NR can be seen between the metacorpus and the cell body. Anterior is left; dorsal is top. Bar, 20 μm. (b) Projection image of (a) superimposed with Nomarski optics.
**FITC staining by soaking at 5°C**

When *B. xylophilus* was soaked with FITC at 5°C, the right pair (APAR and APPR) and the left pair (APAL and APPL) of the sensory neurons were positioned 15-20 μm posterior and dorso-lateral to the nerve ring, and the processes of their cell bodies were oriented toward the tip of the head (Fig. 2.a, b). These neurons were stained in larvae, adults, males, and females.

**Silver nitrate staining**

We also tried silver nitrate staining (Hooper, 1986), but none of the *B. xylophilus* sensory neurons were visibly stained.

**DISCUSSION**

Amphidial apertures in the head of many nematodes extend laterally or dorsoventrally (Cooman and De Grisse, 1981), and pairs of amphidial openings in 18 species of Aphelenchoidea are situated laterally (Hooper and Clark, 1980). In the genus *Bursaphelenchus*, *B. abruptus* has pore-like amphidial openings dorso-medially located in lateral cephalic sectors (Giblin-Davis et al., 1993). *B. xylophilus* also has these openings laterally (Steiner and Buhrer, 1934; Yik and Birchfield, 1981). In the present FITC-staining, some individuals were observed to have the cell bodies and processes of the amphidial neurons situated dorso-ventrally, although we are aware that the long and slender body of this nematode could be easily distorted while it was anaesthetized or mounted on the agar pad. In *C. elegans*, six of the 8 externally exposed amphid neurons were stained by FITC soaking (Hedgecock et al., 1985; Perkins et al., 1986; Shakir et al., 1993). These amphid neurons have been identified as chemosensory, mechanosensory, and osmosensory receptors (Hedgecock et al., 1985; Perkins et al., 1986; Shakir et al., 1993; Mori, 1999). Two pairs of amphid neurons, APAR/L and APPR/L, were detected in *B. xylophilus* (Fig. 2). In *C. elegans*, two of the sensory neurons pass through an amphidial pore but could not be filled with FITC (Hedgecock et al., 1985). The amphids are also described as comprising 3 to 15 receptors in Secernentea (Cooman and De Grisse, 1981). Thus, it is possible that other neurons exist in *B. xylophilus* amphids, which function as mechanosensory or thermosensory receptors, although our FITC soaking method did not detect them.

There have been no reports on the presence of phasmids either in *B. xylophilus* or in some other nematodes, such as *Aphasmatylenchus*, *Neotylenchus*, and species in Anguinidae (Cooman and De Grisse, 1981). In the caudal region of *B. xylophilus*, 7 copulatory papillae are located around the male cloaca (Steiner and Buhrer, 1934; Nickle et al., 1981; Yik and Birchfield, 1981). In Anguinidae, phasmid-like structures, whose presence or absence is one of taxonomic indices (Sturhan and Rahi, 1996), were observed in dorso-lateral positions outside lateral fields in the post-medial region of the body. By staining acetylcholinesterase of *B. xylophilus*, Matsuura (1986) identified amphidial neurons, a nerve ring, dorsal and ventral cords. He also observed intense staining in the anterior region of male cloaca, but did not refer to phasmid sensilla. We also failed to detect phasmid neurons in the present experiment by FITC soaking of *B. xylophilus*. This is in contrast to several observations made of *C. elegans* wherein 2 phasmid neurons were stained by FITC soaking (Hedgecock et al., 1985; Perkins et al., 1986; Shakir et al., 1993).

Nematode sensilla typically consist of a neuronal part and two non-neuronal parts. That is, the non-neuronal parts of a socket cell and a sheath cell surround the neuronal part of a bipolar neuron. Long thin
extensions and belt-like desmosomes connect a neuronal cilium to the sheath cell, the sheath to the socket cell, and the socket cell to the hypodermis (Cooman and De Grisse, 1981). When B. xylophilus was stained with FITC overnight, teardrop-shaped apparatuses were observable at the anterior region of the metacorpus (Fig. 1.a, c). A transverse section image of the posterior region of the metacorpus, reconstructed by the ZEISS LSM 5 Image Browser, showed that these apparatuses had a triradiate symmetry (Fig. 1.b). In other nematodes, such as C. elegans and species in Tylenchida and Dorylaimida, the lumen of the pharynx has a cuticle lining and displays triradiate symmetry (Robertson, 1979; Chen and Wen, 1982; White, 1988). In contrast, the teardrop-shaped apparatuses were positioned anterior to the metacorpus, and the long-shaped structures elongated from these apparatuses toward the stylet knob (Fig. 1.a, c), suggesting that these were cells of the procorpus or muscles to move the stylet.

The present study has provided a basis for rational functional analysis of each amphidial and phasmidial neuron both by physical ablation, such as laser killing of a target neuron, and by genetic ablation, such as mutational disturbances of a target neuron. For instance, laser ablation experiments revealed, among 12 amphidial sensory neurons of C. elegans, neurons regulating chemotactic, thermotactic, and osmotic behaviors as well as dauer formation (Bargmann and Horvitz, 1991; Mori, 1999). Because there is a plethora of mutations causing neuronal abnormalities in sensory organs of C. elegans, we herein refer only to the review by Riddle et al. (1997). To find the relations between the neurons (APAR/L, APPR/L in Fig. 2) in B. xylophilus and their behaviors (mating behavior, entrance into beetle vectors, transmission to and invasion of host trees, entry into and recovery from the dispersal form, and other behaviors that require neuronal processings) should open a new door to the understanding of genetic and biological controls of the pine wilt disease.

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和文摘要

マツノザイセンチュウの外部に開口したアンフィド感覚子と神経細胞、神経突起の配置

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マツ材線虫病の病原体であるマツノザイセンチュウ Bursaphelenchus xylophilus および自活性
土壌線虫 *Caenorhabditis elegans* の外部に開口した感覚子であるアンフィドとファスミドの神経を FITC で染色し、細胞の数、神経突起の配置を比較した。*C. elegans* では、アンフィドを構成する12の神経細胞のうち6つの神経細胞と、ファスミドを構成する2つの神経細胞が染まり、神経突起が虫体側方を走っていた。一方、マツノザイセンチュウでは神経細胞が中部食道球の後方に背側左右2つずつ確認され、そこから神経環および頭部の感覚子に向かって虫体側方を走る神経突起が観察された。また、マツノザイセンチュウを20℃で一晩染色すると、中部食道球前方に3放射相称の涙滴形のものと、そこから口針のノブまで伸びる細長いものが観察された。マツノザイセンチュウのファスミドは確認できなかった。