Nondestructive Imaging of Plant-Parasitic Nematode Development and Host Response to Nematode Pathogenesis

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ABSTRACT

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The secluded lifestyle of endoparasitic plant nematodes hampers progress toward a comprehensive understanding of plant–nematode interactions. A novel technique that enables nondestructive, long-term observations of a wide range of live nematodes in planta is presented here. As proof of principle, *Pratylenchus penetrans*, *Heterodera schachtii*, and *Meloidogyne chitwoodi* were labeled fluorescently with PKH26 and used to infect *Arabidopsis thaliana* grown in microscopy rhizosphere chambers. Nematode behavior, development, and morphology were observed for the full duration of each parasite's life cycle by confocal microscopy

for up to 27 days after inoculation. PKH26 accumulated in intestinal lipid droplets and had no negative effect on nematode infectivity. This technique enabled visualization of *Meloidogyne* gall formation, nematode oogenesis, and nematode morphological features, such as the metacarpus, vulva, spicules, and cuticle. Additionally, microscopy rhizosphere chambers were used to characterize plant organelle dynamics during *M. chitwoodi* infection. Peroxisome abundance strongly increased in early giant cells but showed a marked decrease at later stages of feeding site development, which suggests a modulation of plant peroxisomes by root-knot nematodes during the infection process. Taken together, this technique facilitates studies aimed at deciphering plant–nematode interactions at the cellular and subcellular level and enables unprecedented insights into nematode behavior in planta.

Plant-parasitic nematodes are a major threat to sustainable crop production in temperate and tropical regions alike. Some of the most damaging species belong to the root-lesion (*Pratylenchus* spp.), cyst (*Globodera* and *Heterodera* spp.), and root-knot (*Meloidogyne* spp.) nematodes (11,22). All plant-parasitic nematodes are obligate biotrophs that depend on their hosts for survival. However, infection strategies can show marked differences between genera. Root-lesion nematodes are migratory endoparasites that invade roots and feed on a large number of host cells that are destroyed in the process, thereby resulting in lesions on the roots (54). In contrast, sedentary endoparasites such as cyst and root-knot nematodes induce the formation of living feeding sites in infected plant tissue and minimize cellular damage to their hosts. Syncytia and giant cells, the feeding sites of cyst and root-knot nematodes, respectively, are the sole source of nutrition for these nematodes and, therefore, essential to their survival (26). The basic principles of the life cycles of root-lesion, cyst, and root-knot nematodes are relatively similar. All four juvenile life stages (J1, J2, J3, and J4) and the development into either adult males or females are separated by molts. The first-stage juvenile (J1) remains in the egg and molts into a second-stage juvenile (J2), which hatches. In cyst and root-knot nematodes, the J2 is the infective stage that invades plant tissue and initiates the formation of a feeding site, whereas the remaining life stages (except the adult male) are sedentary (26). In root-lesion nematodes, all life stages from the J2 onward are motile and infective (54). An important difference in postinfection biology is that, in *Meloidogyne*

spp., J3 and J4 stages do not feed because they remain ensheathed in the cuticles of the previous life stage and lose their stylets. In contrast, in *Heterodera* spp., the cuticle of the previous life stage is completely shed and the J3 and J4 nematodes feed. In *Pratylenchus* spp., all vermiform life stages feed (37).

The ability to study the behavior of plant-parasitic nematodes during pathogenesis and to characterize the cellular changes in infected plant tissue is of great interest, not only to elucidate fundamental principles of host–parasite interactions but also to explore novel control strategies. Plant-parasitic nematodes secrete proteinaceous effectors from the esophageal gland cells into plant tissue to induce cellular and physiological changes in the host (33) but the basic mechanisms that define molecular and cellular plant–nematode interactions remain largely unknown. One of the reasons that significant advances in this area are slow is that endoparasitic nematodes are inherently difficult to study. As obligate biotrophs, they cannot be propagated without their hosts and, once they are deeply embedded in plant tissue, key events become obscured. One of the most commonly used methods to analyze plant–nematode relationships relies on fixing infected roots and cross-sectioning areas of interest. Depending on the research question of interest, cross-sectioning is often combined with histological or immunological staining and subsequent observations by light or fluorescence microscopy (2,38,47,48) or followed up with electron microscopy (14,19,23,24,44). Even though this approach has yielded valuable insights, it has shortcomings that hamper conceptual breakthroughs. Cross-sectioning plant and nematode tissue is technically demanding, destructive, and prone to fixation artifacts. Furthermore, the results give static snapshots, and do not provide a dynamic picture of cellular changes or nematode behavior over time. Whole-mounts are an alternative to cross-sections and facilitate sample preparation but are subject to the same limitations (16,49). To partially overcome these problems, previous investigators have used video light microscopy (43) or fluorescent dyes to track nematode infection (15,42). However, in these cases,

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either the dye was lost by the nematode after only a few days or the experimental setup did not allow for in-depth in planta studies. Furthermore, most approaches are based on artificial growing conditions (i.e., plants maintained in agar or as root explants, methods that can substantially alter plant–nematode interactions).

Here, we report on an optimized technique that overcomes these challenges by fluorescently labeling root-lesion nematodes (*Pratylenchus penetrans*), sugar beet cyst nematodes (*Heterodera schachtii*), and Columbia root-knot nematodes (*Meloidogyne chitwoodi*) with the lipid analog PKH26 and, subsequently, following the behavior of labeled nematodes in *Arabidopsis thaliana* grown in microscopy rhizosphere chambers (micro-ROC) (12) using confocal microscopy throughout each life cycle. PKH26 is a lipophilic fluorescent dye with long aliphatic tails that integrates into lipids and is widely used for long-term cell tracking assays in mammalian systems (41). PKH26 has been used to study the surface coat of nematodes (32,34,35) but its potential to observe live nematodes over longer periods in planta has not been examined to date.

MATERIALS AND METHODS

Nematode inoculum. Nematode stock cultures were maintained under greenhouse conditions. As host plants, *Mentha × piperita* ‘Black Mitcham’ was used for *P. penetrans*, *Beta vulgaris* ‘4430R’ (Betaseed, Shakopee, MN) for *H. schachtii*, and *Solanum lycopersicum* ‘Rutgers’ for *Meloidogyne chitwoodi*. All host plants were grown in autoclaved sand. To obtain inoculum for experiments, mixed life stages of *P. penetrans* were extracted by cutting infected mint roots into 1- to 2-cm pieces and shaking them in a dilute NaOCl solution (0.01% commercial bleach) on a rotary shaker at 200 rpm for 2 days at room temperature (23°C). The suspension was poured through a set of nested sieves (850-, 75-, and 25- μ m pore size from top to bottom). Each sieve was consecutively rinsed with water for 2 min. Mixed life stages of *P. penetrans* collected on the 25- μ m pore sieve were backwashed into a 50-ml tube and purified on a sucrose gradient following established procedures (17) before being used for subsequent experiments. *H. schachtii* cysts were collected by mixing heavily infested sand from sugar beet stock cultures with water and decanting the supernatant over a 250- μ m pore sieve. Cysts were

rinsed with water and crushed by manually rubbing them against the sieve surface, and the released eggs were collected on a 25- μ m pore sieve. *H. schachtii* eggs were purified on a sucrose gradient (17) and incubated in a modified Baermann pan at room temperature (23°C) to allow hatching of J2s. The hatch solution contained 3.14 mM ZnSO₄ at pH 7.0. Then, 4 to 7 days later, J2s were collected by centrifuging the hatch suspension for 3 min at 375 $\times$ g in a HN-S clinical centrifuge (International Equipment Co., Needham Heights, MA). The resulting nematode pellet was washed with sterile water and centrifuged once more before being used for PKH26 labeling. To obtain *M. chitwoodi* inoculum, eggs were extracted from infested tomato roots using 0.5% NaOCl (18), then purified on a sucrose gradient (17). Eggs were rinsed with water and transferred to a modified Baermann pan to hatch J2s in sterile water at room temperature (23°C). After 4 days, J2s were collected by centrifuging the hatch suspension, as described above. Nematodes were washed with sterile water and centrifuged once more before being used for PKH26 labeling.

PKH26 labeling. In total, $\approx$ 50,000 mixed life stages of *P. penetrans* and J2s of *H. schachtii* and *M. chitwoodi* were transferred to separate 1.5-ml microcentrifuge tubes and resuspended in 1 ml of sterile water before 1 μ l of PKH26 (stock solution 1×10^{-3} M) from the MINI26 PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, St. Louis, MO) was added. Each tube was inverted several times to mix the nematode suspensions, then incubated in the dark at room temperature for 15 min. To remove excess dye, nematodes were washed five times by repeated transfers to 50 ml of sterile water followed by centrifuging at 375 $\times$ g for 3 min in a clinical centrifuge as described above. Stained nematodes were immediately used to inoculate *Arabidopsis*. One aliquot of each batch of PKH26-labeled inoculum was stored in water at 4°C to observe fluorescence of stained nematodes outside the host plant.

Micro-ROC. *A. thaliana* ecotype Col-0 seed were incubated in sterile water at 4°C for 2 days, after which seed were planted individually in micro-ROC (Advanced Science Tools, Pullman, WA) containing moistened Sunshine number 1 potting mix (Sun Gro Horticulture, Agawam, MA) (Fig. 1). Plants were grown in a growth room with a 14-h photoperiod (300 to 400 mEm⁻² s⁻¹) and a day-and-night temperature cycle of 20 and 15°C, respectively. Ten days after planting, when the four-leaf stage was reached, each seedling was inoculated with $\approx$ 2,000 stained nematodes by pipetting 100 μ l of nematode suspension between the glass slide and the nylon mesh of each micro-ROC. Beginning at 3 days after inoculation (DAI), plants were carefully watered and fertilized with modified Knop’s media (43) without sucrose. For each nematode species, 15 micro-ROC containing one *A. thaliana* seedling each were inoculated. As controls, uninfected plants and unlabeled nematodes were used. All experiments were conducted twice.

***Arabidopsis* px-YFP line.** Binary plasmid px-YFP (TAIR accession CD3-982) (36) was introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform *Arabidopsis thaliana* ecotype Col-0 using the floral dip method (7). This created an *Arabidopsis* line in which all peroxisomes were labeled fluorescently by translationally fusing the peroxisomal targeting signal 1 (PTS1, Ser-Lys-Leu) to the C terminus of the yellow fluorescent protein (YFP). Transformants were selected by screening germinating seed on one-half strength Murashige & Skoog basal salt media (Caisson Labs, North Logan, UT), supplemented with 3% D-sucrose, 0.6% Daishin agar, timentin at 0.5 g/liter, and glufosinate ammonium (Plant Media, Dublin, OH) at 10 μ g/ml. Second-generation (T₂) seed were used in micro-ROC experiments as described above, except that plants were inoculated with unlabeled *M. chitwoodi*. Fifteen micro-ROC with one plant each were analyzed and the experiment was conducted twice. Wild-type *Arabidopsis* and uninfected px-YFP plants were used as controls. For each time point (3, 8, 12, 19, and 27 DAI), the num-

Fig. 1. *Arabidopsis* grown in microscopy rhizosphere chambers (micro-ROC). For all experiments, a single *Arabidopsis* seedling was grown in each micro-ROC. The developing root system grows between a nylon mesh and glass slide but has access to the potting mix. The glass slide is covered with a black plastic film (removed here) to block out light. Nematode inoculum was pipetted between the nylon mesh and glass slide to maximize infection.

ber of peroxisomes was counted in three areas of three images each in infected and uninfected roots following previously established procedures (9). Briefly, three 0.01-mm² areas (100 by 100 μ m) were cropped from each image using Adobe Photoshop. Using ImageJ, cropped RGB color images were changed to 8-bit black-and-white images. The “threshold” function was used to designate black pixels as peroxisome area. The “analyze particles” function was used to count pixel groups (number of peroxisomes) and area fraction (area percentage). This technique was unable to differentiate between overlapping peroxisomes, which were counted as one organelle and possibly reduced the true number of peroxisomes in some instances. To circumvent this problem, the total area of peroxisomes was estimated ([area fraction/100] $\times$ 0.01 mm²). All data were analyzed for statistical significance using a *t* test at α 0.05 using SAS 9.2 software.

Confocal microscopy. Infected *Arabidopsis* roots and uninfected controls grown in micro-ROC were observed using an LSM 510 META laser scanning microscope (Zeiss, Jena, Germany) beginning at 3 DAI and extending for up to 27 DAI. Excitation wavelengths were set at 514 nm (argon) and 543 nm (HeNe) with emission wavelengths of 503 to 530 nm (green) and

560 to 615 nm (red). In addition, nematodes stained with PKH26 were observed on standard microscopy glass slides 3 days after being labeled using the same parameters. For px-YFP roots and respective controls, the excitation wavelengths were set at 514 nm (argon) with the emission wavelength at 560 nm. Subsequent image processing was performed with ImageJ and Adobe Photoshop.

RESULTS

***P. penetrans* behavior and life cycle in *Arabidopsis* roots.** Immediately after being labeled with PKH26, all vermiform life stages of *P. penetrans* began to show fluorescence in the lip region, esophagus, and intestine. At 3 days after labeling, pronounced staining was visible, especially in lipid droplets in the intestine of *P. penetrans* that had been stored at 4°C (Fig. 2A). Increased mortality of labeled *P. penetrans* or overtly toxic effects of PKH26 compared with untreated nematodes were not observed. To examine whether micro-ROC facilitates observation of root-lesion nematode behavior in planta and throughout the life cycle of the parasite, *Arabidopsis* grown in micro-ROC were inoculated with mixed life stages of *P. penetrans* that were labeled

Fig. 2. *Pratylenchus penetrans* stained with PKH26. **A**, *P. penetrans* 3 days after labeling with PKH26 outside of plant, stored at 4°C until imaging. Intense fluorescence of intestinal lipids. All other pictures show nematodes in planta. **B**, *P. penetrans* at 3 days after inoculation (DAI) in *Arabidopsis* roots and **C**, at 19 DAI. **D**, Molting female at 19 DAI; arrow indicates metacarpus. **E**, Resting male at 26 DAI, arrow indicates spicules. **F**, Anterior portion of female during migration through epidermal tissue at 27 DAI. **G**, Migrating female at 27 DAI with deposited egg (arrow). **H**, Deposited eggs at 27 DAI (arrows). Scale bars = 50 μ m.

with PKH26. The nematodes migrated toward and invaded the roots as early as 1 DAI. At 3 DAI, the fluorescence of lipid droplets temporarily decreased in PKH26-stained *P. penetrans* inside roots but rapidly recovered in feeding nematodes (Fig. 2B and C). Nematodes were observed for up to 27 DAI, at which point *P. penetrans* completed its life cycle. The intensity of PKH26 fluorescence was strong throughout the duration of the experiments and did not weaken at 27 DAI. Importantly, micro-ROC enabled observation of characteristic *P. penetrans* behavior throughout the nematode's life cycle, including intracellular migration (Fig. 2B and C), feeding (Fig. 2C and D), coiling and resting inside host cells (Fig. 2E and F), and deposition of eggs inside host tissue (Fig. 2G and H). In addition, distinct morphological features such as the intestine, metacarpus, vulva (Fig. 2D), and spicules (Fig. 2E) were visible in labeled nematodes inside roots.

***H. schachtii* behavior and life cycle in *Arabidopsis* roots.** After PKH26 and micro-ROC were used to observe the migratory endoparasite *P. penetrans* in planta, the technique was tested in sedentary endoparasitic nematodes. *H. schachtii* infective J2s were labeled and used to inoculate *Arabidopsis* grown in micro-ROC, as detailed above. Similar to *P. penetrans*, *H. schachtii* infective J2s began to show PKH26-induced fluorescence immediately after being exposed to the dye. At 3 days after labeling, intestinal lipid droplets and the lip region displayed strong fluorescence in labeled J2s that were stored at 4°C (Fig. 3A). PKH26 did not have any obvious effects on the viability of J2s, which oriented themselves toward host roots, explored, and finally invaded roots at the tips and in the region of cell differentiation. The fluorescence of lipid droplets temporarily decreased in nonfeeding infective J2s at 10 DAI (Fig. 3B) but quickly increased in feeding J3s at 14 DAI and even surpassed the staining intensity of J2s in later life stages (late J3 and J4) at 22 DAI and beyond (Fig. 3C to G). In developing females, fluorescent lipid droplets decreased and almost disappeared in adult females and cysts (Fig. 3H and I). Although lipid staining decreased in adult females and cysts, residual PKH26 staining of the remaining lipid droplets, together with autofluorescence in the outer layers of the cyst, enabled a clear visualization of these important life stages. Combined with PKH26, micro-ROC enabled observation of all phases of the *H. schachtii* life cycle in great detail in planta, including migration through host tissue (Fig. 3B), feeding (Fig. 3C and D), molting (Fig. 3E), development of vermiform males and female cysts (Fig. 3G to I), oogenesis (Fig. 3H and I), and formation of the syncytium (Fig. 3J). Furthermore, morphological details of live *H. schachtii* were clearly visible in planta, and included the esophagus, metacarpus (Fig. 3D), intestine, (Fig. 3D to G), shed cuticle during molt (Fig. 3E), ovaries (Fig. 3H), and vulval cone (Fig. 3I).

***M. chitwoodi* behavior and life cycle in *Arabidopsis* roots.** Similar to the other nematode species studied, *M. chitwoodi* infective J2s began to display fluorescence immediately upon exposure to PKH26. At 3 days after labeling, the lip region and lipid droplets in the intestine of J2s that were stored at 4°C showed a strong fluorescent signal (Fig. 4A). As in *P. penetrans* and *H. schachtii*, PKH26 did not have any obvious effects on the ability of *M. chitwoodi* infective J2s to locate and penetrate host roots. The labeled J2s migrated toward the tips of *Arabidopsis* roots and invaded roots at the zone of cell elongation. At 3 DAI, stained J2s could be clearly observed during their intercellular migration in root tissue. The fluorescence intensity of their intestinal lipid droplets was high at 3 DAI; decreased by the time they had become parasitic J2s, when they began to induce the formation of feeding sites (Fig. 4B to F); and returned to strong intensity in late feeding life stages at 15 DAI (Fig. 4G). In contrast to developing *Heterodera* nematodes, which break through the root cortex, especially in roots with a small diameter such as *Arabidopsis* and thereby facilitate imaging, *Meloidogyne* nematodes remain deeply em-

bedded in root tissue and are surrounded by a gall. As expected, gall formation made it challenging to clearly visualize morphological details in adult *M. chitwoodi* females at 27 DAI, when the life cycle was complete with commencement of egg laying and observations ended (Fig. 4H and I). However, PKH26 stained the cell membranes of giant cells, which greatly facilitated nondestructive observation of feeding site morphology and development and showed the increase in giant cell size compared with infected plant cells (Fig. 4D and F).

***M. chitwoodi* infection modulates peroxisome abundance in giant cells in *Arabidopsis*.** To test whether the micro-ROC technique is useful for analyzing subcellular changes in nematode-infected plant tissue, a transgenic *Arabidopsis* line (px-YFP) in which the peroxisomal targeting signal 1 (PTS1, Ser-Lys-Leu) is fused to the C terminus of YFP and, thereby, fluorescently labeling peroxisomes (36), was inoculated with *M. chitwoodi* infective J2s. Root tissue peroxisomes were evenly distributed throughout uninfected roots (Fig. 5). At 3 DAI, *M. chitwoodi* initiated feeding site formation and the number and area of peroxisomes per cell significantly increased in infected root cells (Figs. 5 and 6). At 8 DAI, the number of peroxisomes was higher in infected plant cells when observed with the microscope (Fig. 5). However, due to technical constraints that did not allow differentiating single peroxisomes that overlapped in the microscope image, the counting technique used indicated that there was no statistically significant difference in the number of peroxisomes between infected and uninfected cells at 8 DAI (Fig. 6A). Measuring the area of peroxisomes was not affected by these technical constraints (see Materials and Methods) and showed a significant increase in the area of peroxisomes in infected cells at 8 DAI (Fig. 6B). At later stages of the infection cycle, the relative distribution of peroxisomes reversed. At 12 DAI, there were significantly fewer peroxisomes in giant cells than in the surrounding plant cells and uninfected areas. This trend continued throughout the remaining time points at 19 and 27 DAI, when the life cycle of *M. chitwoodi* was complete and observations ended (Fig. 5D to G). Taken together, we found that the number of peroxisomes increased in young giant cells compared with uninfected plant tissue whereas, in later stages of the *M. chitwoodi* life cycle, there were significantly fewer peroxisomes in nematode feeding sites relative to in neighboring uninfected plant tissue.

DISCUSSION

Attempts to study live nematodes in their hosts go back to the early 1940s with the pioneering experiments of Linford, who developed root observation boxes to observe *Meloidogyne* spp. (27,28). Even though this method allowed root development in soil, thereby having the advantage of offering relatively natural growing conditions, Linford's design did not enable prolonged in planta studies. Early in planta nematode behavior could be imaged inside the root observation boxes but nematode migration events and later behavior could only be followed in dissected roots, which limited the usefulness of this technique (28). Significant breakthroughs in plant-nematode imaging were made by growing *Arabidopsis* in agar under axenic conditions, infecting plants with surface-sterilized nematodes, and observing the resulting interactions with video-enhanced contrast light microscopy (14,43,50,51). A major advantage of this approach was that it was nondestructive and enabled long-term observations of live nematodes in planta. Subsequent studies made use of fluorescent dyes or plant reporter genes to visualize details of live plant-nematode interactions (6,45). However, even minor changes in the composition of artificial plant growth media can have a major impact on the infection rate of nematodes and, therefore, alter the relationships with the host, which creates a major challenge when using axenic growth conditions (43). Furthermore, imaging of live nematodes during the infection process would greatly benefit

from a fluorescent label that could be applied to the parasite and facilitate following its behavior in planta. Plant-parasitic nematodes are known to take up fluorescent dyes such as acridine orange, fluorescein isothiocyanate, or fluorescein diacetate (4,5,

15,42). However, these dyes proved to be of relatively little value in plant–nematode interaction studies because they were not stable throughout the entire life of the nematode, thereby obscuring late parasitic events (15,42).

Fig. 3. *Heterodera schachtii* stained with PKH26. **A**, *H. schachtii* second-stage juveniles (J2s) 3 days after labeling with PKH26 outside of plant, stored at 4°C until imaging. Intense fluorescence of intestinal lipids. All other pictures show nematodes in planta. **B**, Intracellular migration of J2 at 10 days after inoculation (DAI). **C**, J3 at 14 DAI begins to break through root cortex. **D**, Developing J3 or J4 with median bulb (arrow) at 22 DAI. **E**, Molting J3 at 23 DAI. Arrows indicate shed cuticle. **F**, Developing female at J4 stage, 23 DAI. **G**, Developing male at J4 stage, 23 DAI. **H**, Adult female during oogenesis (arrow indicates developing eggs) at 23 DAI. **I**, Mature eggs (top arrow) and vulval cone (bottom arrow) at 28 DAI. **J**, Syncytium (arrow) at 28 DAI. Scale bars = 50 μ m.

To resolve these problems, an optimized method to observe plant–nematode interactions based on fluorescently labeling nematodes with PKH26 and following behavior inside roots using plants grown in micro-ROC was developed. The technique has

distinct advantages over previously used imaging methods. Most importantly, it enables nondestructive long-term observations of plant–nematode interactions over the entire life of the parasite. Furthermore, host plants are grown in potting soil and roots are

Fig. 4. *Meloidogyne chitwoodi* stained with PKH26. **A**, *M. chitwoodi* second-stage juveniles (J2s) 3 days after labeling with PKH26 outside of plant, stored at 4°C until imaging. Intense fluorescence of intestinal lipids. All other pictures show nematodes in planta. **B**, Migrating J2 at 3 days after inoculation (DAI). **C**, Late parasitic J2 and developing gall at 12 DAI. **D and E**, J2 (arrow) at 12 DAI during intercellular migration. **F**, Early giant cell formation at 12 DAI. **G**, J3 or J4 breaking through the root cortex at 15 DAI. **H and I**, Posterior end of female surrounded by gall tissue at 27 DAI with egg (arrow). Scale bars = 50 μ m.

not exposed to light. These growing conditions sidestep potential problems with media composition that can influence the ability of nematodes to infect (43) and avoid the need to surface-sterilize nematodes with compounds such as mercuric chloride, which is

lethal at high concentrations and can have a negative impact on host-parasite interactions, even at low concentrations, by interfering with nematode neurotransmission, olfaction, and locomotion (31). Roots grown with exposure to light show a greater total

Fig. 5. Peroxisomes tagged with yellow fluorescent protein (YFP) in transgenic *Arabidopsis* roots infected with *Meloidogyne chitwoodi*. **A**, Distribution of YFP-tagged peroxisomes in uninfected roots 22 days after germination in microscopy rhizosphere chambers. **B**, Early *M. chitwoodi* gall formation at 3 days after inoculation (DAI). **C**, Increased peroxisome concentration in developing gall region compared with surrounding uninfected root tissue at 8 DAI. **D to G**, Reduction of peroxisome concentration in developing gall region compared with surrounding uninfected root tissue at 12 DAI (**D**), 19 DAI (**E**), and 27 DAI (**F** and **G**). Scale bars = 50 μ m.

length, more branching, more root hairs, and bursts of reactive oxygen species (52,53), traits that can influence nematode infectivity. Additionally, exposure of nematode-infected root systems to light is known to cause the formation of chloroplasts in feeding sites of sedentary endoparasites (40,45). Recent studies provide evidence for molecular cross-talk between photosynthesis, plant immunity, and chloroplast-derived reactive oxygen species involved in defense responses (13,20,55). Plant–nematode interaction studies based on culture conditions that permit permanent light exposure of roots grown in agar are altering the relationships between the nematode and its host, thereby possibly inviting systematic errors and confounding variables that can easily be avoided with the technique presented here. In this study, we have limited our observations to *A. thaliana* due to its ease of culture but micro-ROC have been used to study root biology in a wide variety of plants, including tobacco, wheat, and onion (M. Knoblach, *unpublished data*). The versatility of the micro-ROC growing system and the ability of PKH26 to stain a wide range of plant-parasitic nematodes suggest that this technique can be applied in diverse plant–nematode pathosystems.

PKH26 labeling was extremely stable for the duration of the life cycle of all three nematode species tested here. The dye accumulated predominantly in the intestinal lipid granules and, to a lesser degree, in the surface coat of nematodes. In our experiments, we saw no obvious change in the behavior and biology of the nematodes stained with PKH26 and, thus, have no reason to believe that other aspects of the nematodes' biology are affected by the staining. In nonfeeding J2s of *H. schachtii* and *M. chitwoodi*, the intensity of PKH26 fluorescence temporarily decreased but, once feeding commenced and the intestinal lipid reservoirs were replenished, fluorescence returned to high intensity in juveniles and young adults before it decreased again in adults. This pattern is consistent with the changes in lipid concentration in developing nematodes and the abundance of lipid droplets in the intestine (1,10,25,30). Furthermore, lipid droplets are dynamic and can separate into smaller droplets or merge into larger ones (39), which suggests that the observed changes in PKH26 fluorescence throughout feeding and nonfeeding nematode life stages mirror this dynamic by incorporating stain from older, previously labeled lipid droplets into newly formed ones. Nematodes were labeled fluorescently almost immediately upon PKH26 exposure, which points at a diffusion mechanism across cell membranes rather than ingestion of the dye. However, PKH26 was unable to cross the nematode egg shell and did not label developing juveniles inside eggs (data not shown). Interestingly, we found that the giant cells of PKH26-labeled root-knot nematodes also displayed PKH26-specific fluorescence. Giant cells are known to develop massive ingrowths of the plasma membrane,

which increase their internal surface area 10 times or more and lead to a corresponding increase in membrane lipids (23). Thus, it is feasible that the giant-cell-specific fluorescence observed here results from incorporation of PKH26 that was released into the feeding site by the nematode. In control plants that were infected with nonlabeled nematodes, no comparable fluorescence could be observed.

To validate the use of micro-ROC to image nematode-induced changes in plant cells during the infection process, the response of peroxisomes to root-knot nematode infection was studied. We found that the number of plant peroxisomes strongly increased in early giant cells as early as 3 DAI compared with uninfected cells. At later stages of the infection process, peroxisomes were significantly less abundant in giant cells than in uninfected plant cells. Peroxisomes are found in all eukaryotes and are involved in both reactive oxygen species decomposition and production (8). Hydrogen peroxide, a universal cell stress signal, can induce the formation of new peroxisomes in plants and animals (29). The changes in peroxisome abundance in root-knot nematode-infected plant cells observed in this study mirror changes in the transcript level of peroxisome-specific genes in infected plants. Using differential display assays, Vercauteren and co-authors (46) showed that the *Arabidopsis* peroxidase gene ATP6a was induced in young giant cells and endodermal cells at 3 to 7 DAI but was restricted to endodermal cells at later time points between 14 and 21 DAI, which corresponds to the location of peroxisomes observed here. Similarly, *Arabidopsis* peroxidase and catalase genes were found to be downregulated in giant cells in large-scale microarray experiments (3,21). We hypothesize that root-knot nematodes induce, directly or indirectly, a degradation of peroxisomes in developing giant cells to protect their feeding sites from peroxisome-derived reactive oxygen species involved in plant defense responses.

In summary, the combined use of micro-ROC and fluorescent labels has the potential to significantly advance fundamental understanding of plant–nematode interactions. Further, it has broad appeal for studying rhizosphere biology in general when applied to other plant-associated organisms such as bacteria, oomycetes, or fungi.

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Fig. 6. Quantification of yellow fluorescent protein-tagged peroxisomes in *Meloidogyne chitwoodi*-infected and uninfected *Arabidopsis* roots. **A**, Number of peroxisomes per 0.01 mm² of root. **B**, Area of peroxisomes (1 × 10⁻³ mm²) per 0.01 mm² of root.

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