生体蛍光標識による感染初期段階の内部寄生性植物線虫の植物組織内観察

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[SHORT COMMUNICATION]

In planta observation of live fluorescent plant endoparasitic nematodes during early stages of infection

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Plant endoparasitic nematodes spend most of their life cycle hidden within plant roots and worldwide cause major losses to agriculture every year. Root-knot nematodes ('RKNs', *Meloidogyne* spp.) are sedentary endoparasitic nematodes that establish permanent feeding sites within plant roots as 2nd-stage juveniles (J2), and then spend the rest of their life-cycle at this site. In contrast, root-lesion nematodes (*Pratylenchus* spp.) are migratory endoparasites that move in roots and feed on different root cells, causing typical symptoms of brown lesions.

There is great interest in studying the initial infection stages of both sedentary and migratory endoparasitic nematodes. In the case of RKNs, it is known that host cells undergo key initial changes of cell-cycle activation, DNA synthesis and nuclear division during the first 48 hr of infection and host cell selection (de Almeida Engler *et al.*, 1999; Jones, 1981; Jones and Payne, 1978; Niebel *et al.*, 1996). In the case of root-lesion nematodes, tracking invasive movement and behavior in root tissues also has potential to reveal the nature of resistant responses limiting the completion of the life-cycle. A major challenge is that current methods to identify nematodes inside plant roots during early stages of infection are either destructive or do not provide sufficient temporal or spatial resolution.

Fluorescent compounds have been used to track solute movement in host plant tissues during parasitic nematode infection (Böckenhoff *et al.*, 1996; Hofmann *et al.*, 2007; Hutangura, 1999). Expression of fluorescent proteins has also been used to study feeding cell biology (Hofmann and Grundler, 2006; Hoth *et al.*, 2005; Hoth *et al.*, 2008) and is a common approach to identify specific cell types in plant roots (Birnbaum *et al.*, 2003; Brady *et al.*, 2007; Lee *et al.*, 2006). Research on initial stages of nematode infection would thus greatly benefit from a fluorescence-based approach that would also enable invading nematodes to be identified in plant roots with high spatial resolution.

Live cyst nematodes have recently been marked fluorescently using fluorescein isothiocyanate (FITC) (Schroeder and MacGuidwin, 2007). FITC is a fluorescent fluorescein conjugate that has also been used to show uptake from solution by J2 RKNs (Rosso *et al.*, 2005). An earlier study made use of a different fluorescein conjugate, fluorescein diacetate (FDA), to label RKN juveniles and to distinguish between living and dead nematodes (Bird, 1979). FDA is a non-fluorescent conjugate that becomes fluorescent after entering a cell and being hydrolyzed by endogenous esterases. In addition to facilitating identification of live nematodes, background fluorescent signals and requirement for extensive washing are kept to a minimum because FDA does not fluoresce, and free fluorescein remains in live cells. In this study, we tested whether FDA can be applied to label individual nematodes fluorescently for direct non-destructive observation of their movement inside plant roots.

MATERIALS AND METHODS

Nematode stocks and FDA treatment:

Meloidogyne hapla Chitwood were collected from an infected field used for tomato cultivation (Niseko, Hokkaido, Japan) and stocks were maintained on tomato plants (Kyoryoku Beiju variety; Takii Seed, Tokyo, Japan) in a glasshouse. *Meloidogyne javanica* (Treub) were collected from plants of *Solanum nigrum* on Murdoch University campus (Perth, Australia) and stocks maintained on tomato plants (Tomato Grosse Lisse Improved; Terranova Seeds, Smithfield, Australia) grown in a glasshouse. For infections with RKNs, egg masses were collected from infected tomato roots grown in the glasshouse, washed once with sterile water containing 0.1% streptomycin sulfate, followed by at least two washes with sterile water. Egg masses were collected by centrifugation (1 min at 1,000 $\times g$) between each wash. The egg masses were then placed in sterile water and incubated at room temperature to hatch. Freshly hatched juveniles (J2s) were allowed to migrate through five sheets of sterile 'Kimwipe' tissues for approximately 6 hr, and then

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active nematodes were collected by centrifugation.

Root-lesion nematodes (*Pratylenchus penetrans* (Cobb)) were isolated from infected strawberry plants and stocks maintained on alfalfa callus cultures as previously described (Yamada *et al.*, 2005). For experimental treatment and infections, root-lesion nematodes of mixed stages (J2, J3, J4 and adult) were isolated using the Baermann funnel method (25°C, 3 days) after physical disruption of the callus.

Stock solutions of FDA (Sigma-Aldrich Japan K.K., Tokyo, Japan) were prepared as described (Bird, 1979). Working solutions of 0.01%, 0.001% and 0.0001% FDA were then prepared by dilution in distilled water. Stock solutions of 10% w/v resorcinol (Sigma-Aldrich) were prepared freshly by dissolving in distilled water. Working solutions of 1% w/v resorcinol were then prepared by dilution in distilled water. Freshly hatched *M. hapla* J2s or isolated *P. penetrans* were incubated in FDA solutions either with or without 1% resorcinol in the dark for 2 or 4 hr as described in the results. Following incubation, nematodes were washed once with sterile water by centrifugation (1 min at 1,000 $\times g$) and resuspended in a sterile solution of 0.2% agar. Treated nematodes were then either observed directly, or used for plant inoculations.

Plant materials and nematode infection:

Tomato plants used for infection were purchased from Takii Seed (Tokyo, Japan; Kyoryoku Beiju variety). Infections of *Arabidopsis thaliana* were carried out using either Col-0 wild-type, or the S17 transgenic line (Lee *et al.*, 2006) containing a green fluorescent protein (GFP) gene downstream of the At2g22850 gene promoter that drives expression in pericycle cells at the phloem pole in roots.

Seeds of tomato and *Arabidopsis* were sterilized by incubation in 5% NaOCl, 0.0025% Triton X-100 for 5 min, followed by 5 washes in sterile water. Seedlings were cultivated on sterile media as previously described (Hutangura *et al.*, 1998), except only 2 seeds were added to each dish and plates were kept at 23°C. Treated and untreated *M. hapla* J2s were applied to tomato plants at 9 days after sowing (das). Treated and untreated *M. hapla* J2s, *M. javanica* J2s and *P. penetrans* were applied to *Arabidopsis* plants at 10 das. Each experiment was carried out with at least four separate plates (1 to 2 plants per plate) and independently repeated at least in duplicate. For infections, approximately 20 nematodes were applied per plant. Following inoculation, plants were kept in the dark and roots examined by confocal microscopy at either 1 day post inoculation (dpi) or 3 dpi as described in the results. Acid fuchsin staining of plants roots was also carried out as previously described by Byrd *et al.* (1983).

Microscopic observation of nematodes and roots:

Bright-field and epifluorescent wide-field microscopy was performed with a Nikon AZ100 Multizoom microscope (Nikon Instech, Tokyo, Japan) fitted with a mercury fiber illuminator (Intensilight; Nikon Instech) and a GFP-B filter unit (Excitation 460-500 nm, Dichroic 505 nm, Emission 510-560 nm; Nikon Instech). Images were captured with either a DS-2MBWC (for fluorescence and monochrome images) or a DS-Fi1 (for color images) camera head connected to DS-U2 control unit USB interface (Nikon Instech), and saved using NIS-Elements Imaging Software (Nikon Instech). Laser confocal microscopy of *M. javanica* in *Arabidopsis* roots was performed using a Nikon Diaphot-TMD inverted microscope attached to a BioRad MRC-1024 Laser Scanning Confocal Imaging System with a 488 nm excitation wavelength and 500-540 nm emission wavelength. Laser scanning confocal microscopy of *P. penetrans* in *Arabidopsis* roots was performed using a Leica SP2 confocal microscope with a 488 nm excitation wavelength and 500-540 nm emission wavelength. All raw images were saved using the relevant microscope software and then processed using ImageJ software.

RESULTS AND DISCUSSION

Low concentrations of FDA marked live RKNs:

A 0.01% solution of FDA was previously shown to produce discrete patterns of fluorescence specifically in live RKNs (Bird, 1979). Resorcinol has also recently been used to stimulate uptake of solution by J2 RKNs (Huang *et al.*, 2006; Rosso *et al.*, 2005), and thus freshly hatched *M. hapla* J2 were first treated with a solution of 0.01% FDA and 1% resorcinol. Similar to that described by Bird (1979), discrete fluorescent particles were observed within *M. hapla* J2 nematodes, however, fluorescent precipitates also appeared in the solution during treatment and the treated nematodes appeared to lose active movement. Ten-fold dilutions of FDA were then tested to reduce the background fluorescence. A 0.001% FDA, 1% resorcinol solution was found to provide comparable fluorescence and greatly reduced background fluorescent precipitate, although nematodes still appeared to lose activity (data not shown). These results were consistent with resorcinol causing reduced nematode viability, and thus a solution of 0.001% FDA alone was next tested. Following 2 hr exposure to 0.001% FDA alone, *M. hapla* juveniles retained their active movement and showed a similar pattern of fluorescence to that observed in previous treatments (Fig. 1A, movie available upon request). The same successful fluorescent marking with

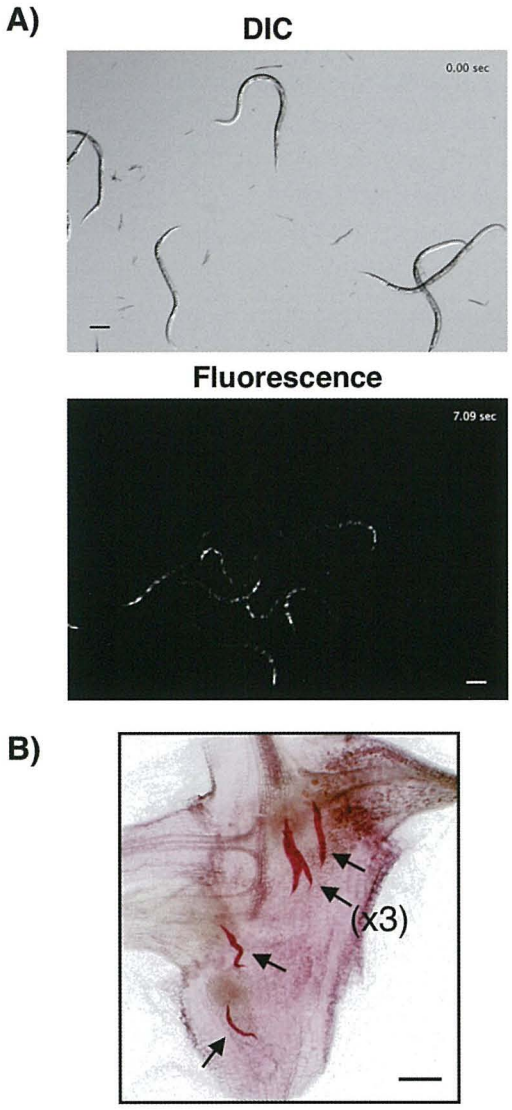

Fig. 1. Fig. 1. Low concentrations of FDA treatment resulted in marked, active root-knot nematodes.
A: Freshly hatched *Meloidogyne hapla* were treated with 0.001% FDA for 2 hr in the dark and examined by wide-field bright-field (upper panel, 35 msec exposure) or epifluorescence (lower panel, 265 msec exposure) microscopy. Both images are part of a movie series (available upon request). Scale bar = 50 μm. B: *M. hapla* J2s treated with 0.001% FDA were inoculated onto tomato plants growing *in vitro*. Nematodes were detected by acid fuchsin staining 7 days post inoculation. Scale bar = 250 μm.

active movement and no background precipitate was observed in over four independent repeats with *M. hapla*, and was also observed in three independent repeats with *M. javanica*. Based on this data, 0.001% FDA alone was selected as the preferred treatment for fluorescent labeling of live RKNs. To confirm that treated RKNs remained viable, *M. hapla* treated with 0.001% FDA were next applied to tomato plants growing on sterile media. Galls consistent with nematode infection were clearly observed on tomato roots within the first four days of inoculation.

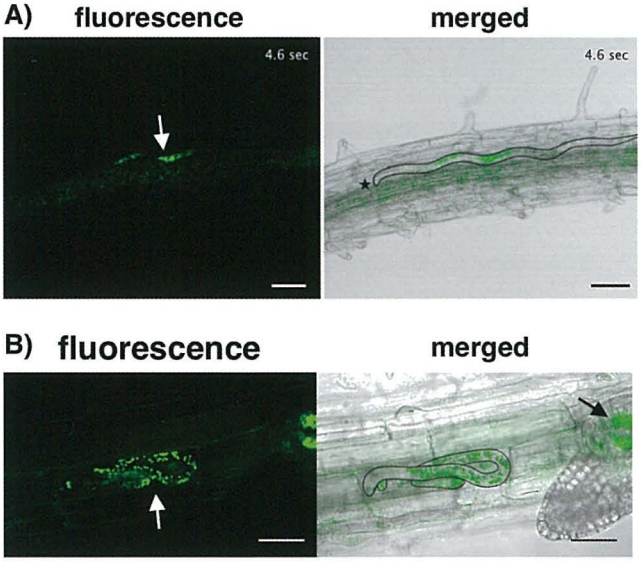

Fig. 2. Fluorescent endoparasitic nematodes can be identified in plant roots by epifluorescence microscopy.
A: FDA-treated freshly hatched *Meloidogyne javanica* were inoculated onto *in vitro* cultures of *Arabidopsis* and examined by confocal microscopy at 3 days post inoculation. B: FDA-treated *Pratylenchus penetrans* were inoculated onto *in vitro* cultures of the S17 transgenic *Arabidopsis* line and examined by confocal microscopy after 3 days.
Fluorescence images (pseudocolored green) are shown in the left panels with location of nematodes indicated by white arrow. To demonstrate the spatial location of the nematode within the root, the same fluorescence image is shown merged with the bright field image in the right panels. Nematodes are outlined with a black line and anterior end marked with star. Fluorescence due to expression of GFP in specific root cells is shown with black arrow. Scale bars = 50 μm.

These galls were further confirmed to contain multiple infections and enlarged RKNs consistent with developing juveniles at 7 days post inoculation (dpi) (Fig. 1B). This demonstrated that treated fluorescent RKNs did indeed remain viable and were able to invade plant roots and successfully initiate infection sites.

Infecting fluorescent RKNs were observed within plant roots:

During the early stages of infection prior to changes in root cell morphology, it is difficult to distinguish whether the nematode has actually begun feeding or is still moving through the root. A common method to identify nematodes inside plant roots is acid fuchsin staining, however this is a destructive assay that kills both the nematode and plant cells. This destruction of nematode and root tissue inhibits subsequent molecular or biochemical analysis of the early infection stages. Fluorescent *M. javanica* RKNs that had been treated with 0.001% FDA could be readily identified within host *Arabidopsis* roots (Fig. 2A) and were confirmed

to be active by observation of nematode movement (movie available upon request). The fluorescence from FDA treatment remained present for at least four days after treatment.

Root-lesion nematodes were auto-fluorescent and also observed within plant roots:

The above results demonstrated that FDA was a suitable live stain for RKNs during infection of host roots. We next asked whether the same approach could be used for the root-lesion migratory endoparasitic nematode, *P. penetrans*. Similar to that for RKNs, *P. penetrans* remained active following treatment with 0.001% FDA solution. However, unlike RKN J2s, *P. penetrans* also displayed readily observed green autofluorescence even in the absence of FDA treatment. Both juveniles and adults of *P. penetrans* displayed similar levels of this autofluorescence. To test whether either fluorescence remained present during root infection and enabled direct observation in plant roots, *P. penetrans* were inoculated onto transgenic plants expressing GFP specifically at phloem poles and roots were examined at 1 dpi by confocal microscopy. Both FDA-treated and untreated nematodes showed suitable green autofluorescence that could readily be detected in *Arabidopsis* roots in conjunction with detection of GFP expression in specific root cells (Fig. 2B). At present, the source of the autofluorescence in *P. penetrans* is not known. It is also unclear whether this is influenced by previous culture conditions or whether it also exists in other root-lesion nematode species. However, the fact that it was not observed in RKNs indicates further analysis of this autofluorescence may provide important insight into root-lesion nematode biology.

The advantage of the FDA fluorescence and root-lesion autofluorescence is that the green fluorescence is a similar wavelength to that of GFP commonly used as a gene reporter in plant research. Particularly in the case of *Arabidopsis*, many transgenic lines now exist that contain GFP under control of promoters driving distinct expression in specific root cell types (Birnbaum *et al.*, 2003; Brady *et al.*, 2007; Lee *et al.*, 2006). As far as we are aware, this is the first published report of a fluorescent marked plant parasitic nematode being directly observed alive and moving inside a plant root. This provides an exciting new strategy for studying early host biological events associated with successful establishment of parasitic nematode infection sites.

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英文論文（本報・短報）の和文摘要

土壌締め固め・リアルタイムPCRを介した土壌からのキタネグサレセンチュウの定量検出

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土壌締固めを介したリアルタイムPCR法を用いて、土壌中で線状の形態で存在するキタネグサレセンチュウ（以下RLN）の検出を検討した。RLNに汚染された黒ボク土および灰色低地土を50 mlの採土円筒に充てんし、土壌締固め機で土壌を締固めてからDNAを抽出した。土壌を締固めると*Ct*値が対照区（締固めなし）よりも1.0~2.5回小さくなったため、土壌締固めにより、RLNのDNAを効率的に検出できるようになることが示唆された。RLNを含まない黒ボク土と灰色低地土にRLNを既知数添加し、土壌締固めとリアルタイムPCRを行ったところ、*Ct*値とRLN頭数に有意な相関関係が認められた（黒ボク土：$r^2 = 0.993$、$P < 0.01$　灰色低地土：$r^2 = 0.965$、$P < 0.01$）。そのため、土壌締固め法を介したリアルタイムPCRによるRLNの定量検出が可能と判断された。検出限界は風乾土20 gあたり黒ボク土で25頭、灰色低地土で10頭であった。検出限界の向上のために、DNA抽出法の改良が必要であると推察された。

宿主探索活性の高い*Caenorhabditis japonica*耐久型幼虫を集める簡易方法

田中龍聖・奥村悦子・吉賀豊司

*Caenorhabditis japonica*はベニツチカメムシに随伴する細菌食性線虫である。*Caenorhabditis japonica*耐久型幼虫は主にカメムシ雌成虫体表面から一年を通じて検出されることから、線虫とベニツチカメムシとの親密な関係があると考えられるが、そのカメムシ雌特異的随伴や*C. japonica*の生活史については不明な点が多い。*Caenorhabditis japonica*の生物学や耐久型幼虫の行動を理解するため、宿主探索活性の高い*C. japonica*耐久型幼虫を選択的に集めるための簡易な方法を開発した。また、この方法で得られた耐久型幼虫はベニツチカメムシに随伴できることを確認した。

SDSを用いて線虫1頭からPCR増幅に直接供試可能なDNAを抽出する方法

酒井啓充

PCR増幅のために線虫1頭からDNAを抽出する方法はこれまでさまざまに報告されているが、通常は粗抽出液がそのままPCR増幅に供されている。SDSはDNA抽出によく用いられる界面活性剤であるが、PCR増幅を阻害するため、線虫1頭からDNAを抽出する方法には用いられてこなかった。そこで、SDSを用いて線虫1頭からPCR増幅に直接供試可能なDNAを抽出する方法を考案した。実体顕微鏡下でサツマイモネコブセンチュウ幼虫1頭を滅菌ろ紙片でつぶし、0.1% SDSを含む抽出バッファー4 µl中に虫体を潰したろ紙片を入れて、50℃・2時間の後95℃・10分間の温度処理を施し、196 µlの滅菌水を加えてDNA抽出液とした。得られた抽出液各5 µlを用いて、Powers and Harris（1993）のプライマーを用いてミトコンドリアDNAのPCR増幅を行ったところ、供試した10頭分すべてにおいて約1.6 kbの目的の産物が得られた。核リボソームDNAのITS領域についても、オオハリセンチュウおよびクルミネグサレセンチュウから本抽出法により得られた抽出DNAを用いてPCR増幅した結果、増幅産物が得られた。本DNA抽出法は、用いる抽出バッファーの構成が単純であること、特別なキット類を使用する必要がないことなどから、適用しやすい手法である一方、線虫の同定診断に十分適用可能な手法である。

生体蛍光標識による感染初期段階の内部寄生性植物線虫の植物組織内観察

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内部寄生性植物線虫の感染初期の研究は重要であるが、寄主植物根内の線虫を生きたまま観察できないことが障害となっていた。そこで、二酢酸フルオレセイン（FDA）の0.001％溶液を用い、生きたネコブセンチュウとネグサレセンチュウを蛍光標識して、非破壊的な蛍光観察を行った。その結果、FDAで処理したネコブセンチュウは生物活性を保っており、寄主のトマト根内で感染場所が検出できることが示された。実験系として用いたシロイヌナズナでは、寄主植物根の中に侵入した線虫を落射蛍光顕微鏡下で直接観察することができ、FDA由来の蛍光が処理後少なくとも4日間検出された。FDAで処理したネグサレセンチュウも容易に寄主植物に感染したが、ネグサレセンチュウは検出可能な自己蛍光を示したため、非破壊的な検出のためのFDA処理は必要ないことが分かった。この報告は、線虫感染の初期段階において、高度な時空間分析に基づく非破壊的解析が可能であることを実証した。