Lotus japonicus: A New Model to Study Root-Parasitic Nematodes

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Sedentary plant-parasitic nematodes engage in complex interactions, and induce specialized feeding structures by redirecting plant developmental pathways, and parallels have been observed with rhizobial nodule development on legumes. A model legume would greatly facilitate a better understanding of the differences between parasitic (nematode) and mutualistic (rhizobia and mycorrhizae) symbioses, and we have developed *Lotus japonicus* as such a model. Conditions for efficient parasitism by root-knot nematode (*Meloidogyne* spp.) of the widely used *Lotus* "Gifu" ecotype were established. Features of *Lotus* biology, such as thin and translucent roots, proved ideal for monitoring the progress of nematode infection both on live specimens and post-staining. We examined *L. japonicus* mutants with nodulation phenotypes. One, *har1*, which is a hyper-nodulated mutant defective in a *CLAVATA1*-like receptor kinase gene, was found to be hyperinfected by *M. incognita*. However, another hypernodulated *Lotus* mutant exhibited the same level of *M. incognita* infection as wild-type plants. We also established conditions for infection of *Lotus* by soybean cyst nematode (*Heterodera glycines*). In contrast to the response to root-knot nematode, the Gifu ecotype is resistant to *H. glycines*, and elicits a hypersensitive response. This pattern of resistance recapitulates that seen on nematode-resistant soybean plants. We conclude that *L. japonicus* is a powerful model legume for studying compatible and incompatible plant–nematode interactions.

Keywords: *CLAVATA1*-like receptor kinase — *har1* — *Heterodera glycines* — Hypersensitive response — *Lotus japonicus* — *Meloidogyne* spp.

Abbreviations: HR, hypersensitive response; L2, second stage nematode larvae; RKN, root-knot nematode; SCN, soybean cyst nematode.

Introduction

Plant-parasitic nematodes are devastating pests, causing average yield loss in the world's major food and fiber crops estimated to be 12.3% annually; losses reach as high as 20% in crops such as banana (Sasser and Freckman 1987, Koenning et al. 1999). The control of plant-parasitic nematodes also comes at a considerable environmental and monetary cost. The extensive use of nematocides has led to ground water contamination, mammalian and avian toxicity, and residues in food (Thomason 1987), and consequently, many of the most effective chemicals have seen deregistration. Host resistance is the most environmentally and economically sound approach for nematode management, and in those crops where resistance is available, it has proved to be extremely valuable (Bradley and Duffy 1982). Regrettably, nematode resistance is yet to be identified for many crop plants, and even for crops for which it is available, the genetic basis is typically restricted. Ideally, durable nematode control in crop plants should be based on deployment of resistance from diverse sources. A more thorough understanding of the interactions between plant-parasitic nematodes and their host plants at the molecular level has the potential to lead to enhancement of existing resistance, and the development of novel resistance strategies (Bird 1996, Bird and Koltai 2000).

A central feature of plant-parasitism by root-knot and cyst nematodes is the induction of specialized root structures, termed giant cells and syncytia respectively, and which serve as feeding sites for the nematodes. Giant cells typically are surrounded by a gall or knot, which give root-knot nematode (RKN) its name. These unique plant structures clearly reflect a unique pattern of host gene expression, and various approaches have been used to isolate candidate genes (reviewed by Gheysen and Fenoll 2002). One subtractive cDNA cloning approach yielded hundreds of giant cell-expressed genes (Bird and Wilson 1994), yet much remains unknown about their roles in feeding-cell induction. Understanding feeding-site formation in the context of overall plant transcription has the potential to reveal the regulatory and effector networks responsible for the resistant and compatible interactions, and the development of plant models makes such global approaches possible.

It has been proposed that *Arabidopsis thaliana* be used as a model to study plant–nematode interactions (Sijmons et al. 1991) and not surprisingly, studies in *Arabidopsis* have lead to important findings, such as the role of the cell cycle in feeding-site induction (de Almeida Engler et al. 1999). Recently, *Arabidopsis* microarrays were used to compare host responses to closely related cyst nematodes (Puthoff et al. 2003). However, *Arabidopsis* does have significant limitations as a model to understand nematode–plant interactions. Although a robust
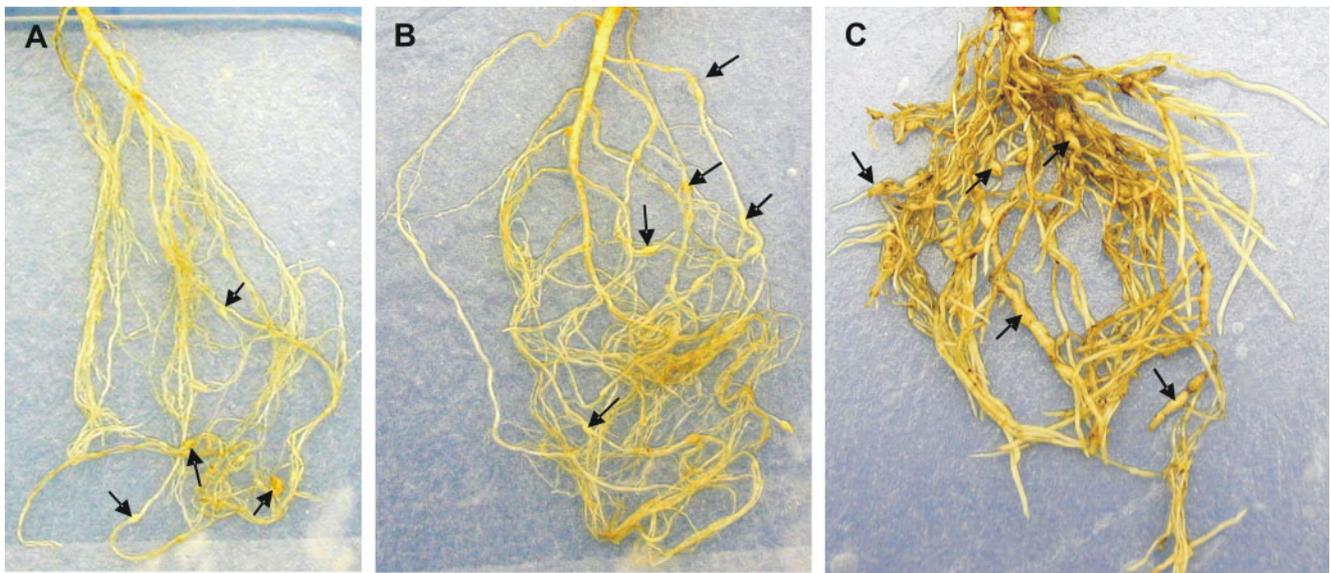
Fig. 1 Gall induction on *L. japonicus* root grown in different media by RKN, *M. incognita*. One-week-old plants were inoculated with 800 eggs, and the roots were observed 3 weeks after inoculation. Arrows indicate some of the root galls. (A) Vermiculite. (B) Clay litter. (C) 50% sand/50% peat mix.

host for certain cyst nematodes, *Arabidopsis* is generally a poor host for RKN, and extensive surveys of *Arabidopsis* ecotypes have failed to reveal discernible genetic diversity in the host response (Niebel et al. 1994). Further, although *Arabidopsis* is a good model for many aspects of basic plant biology, it is not representative of all plant families, nor all plant processes. In particular, *Arabidopsis* fails to engage in symbiotic associations either with rhizobia or mycorrhizae.

Through rhizobial symbioses, legume crops directly provide one-third of human protein intake. Nematodes are major legume pests, and soybean cyst nematode (SCN) for example, is recognized as the major pathogen of soybean (Wrather et al. 2001). Genetic analyses have revealed an interplay between SCN infection and nodulation (Ko et al. 1984), but the complexity of soybean genetics has made understanding these relationships a daunting task (Opperman and Bird 1998). However, the development of the legume models *Lotus japonicus* and *Medicago truncatula* (VandenBosch and Stacey 2003) provide an alternative route to dissecting complex biological symbioses, including the mutualistic, rhizobial–plant interaction and the parasitic, nematode–plant interaction. Indeed, recent studies using *Medicago* indicated that nodulation and giant-cell formation involve shared pathways of transcriptional regulation (Koltai et al. 2001). Genetic analyses of *Medicago* accessions has revealed a rich palette of diverse host responses to root-knot and cyst nematode infection (Dhandaydham and Opperman, personal communication), further reinforcing the utility of this legume model to study plant–nematode interactions. However, *Lotus* and *Medicago* do exhibit some fundamental differences in their associations with rhizobia, with the former forming determinant, and the latter, indeterminant nod-

ules, and this is one reason why both species have been developed in parallel into models (VandenBosch and Stacey 2003). We therefore wish to use *Lotus* in parallel with *Medicago* to study both nematode–plant interactions per se, and also the interplay between nematode–parasitism and nodulation.

L. japonicus is a weed legume with a small (about 400 Mb) diploid genome, proven to be an effective model to study legume–rhizobium symbiosis (Schauer et al. 1998, Schauer et al. 1999, Nishimura et al. 2002). Advanced resources for genomic analysis (Asamizu et al. 2000), and forward (Handberg and Stougaard 1992) and reverse (Perry et al. 2003) genetic studies have been developed, as well as plant transformation techniques (Lohar et al. 2001, Stiller et al. 1997). Here we report the development of *L. japonicus* as a model legume to study plant–nematode interactions, and validate this system using two species of RKN (*M. incognita* and *M. hapla*) and SCN (*Heterodera glycines*).

Results

The Gifu B129 ecotype of *L. japonicus* is susceptible to RKN

As a genus, RKN has a very broad host range (Sasser and Freckman 1987) and larvae apparently enter the roots of susceptible and resistant plants indiscriminately. However, because environmental factors can influence penetration, we examined the influence of several growth parameters on 1-week-old *L. japonicus* plants (ecotype Gifu B129) for infection by either freshly isolated *M. incognita* eggs or second stage larvae (L2), which are the only two nematode stages present in soil (apart from adult males, which do not penetrate plants). Because 28.0°C is optimum for *M. incognita* hatch, we initially chose
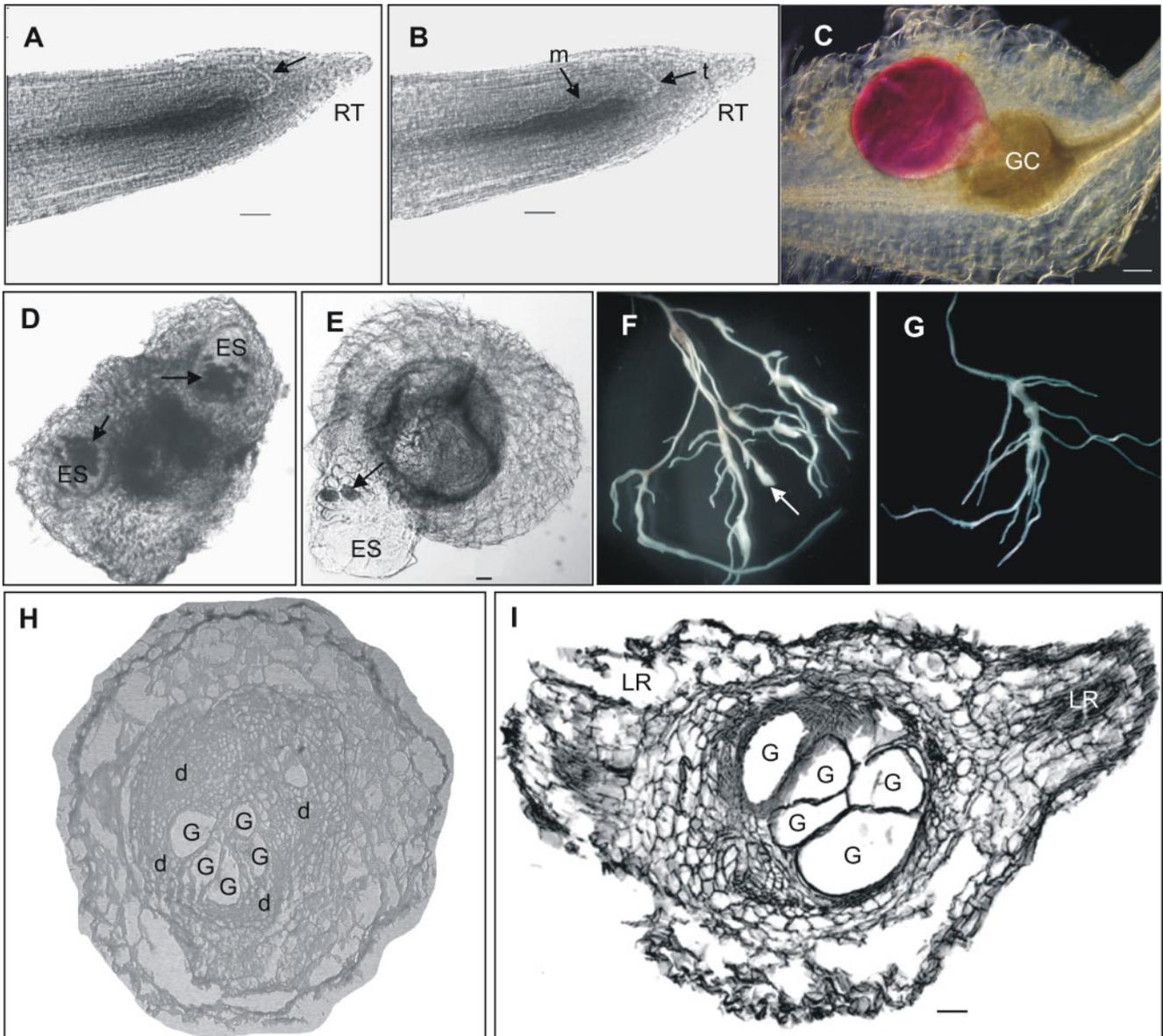
Fig. 2 *L. japonicus* root infection by RKNs. (A) An L2 (arrow) entered the root just above the root tip, and after reaching the meristem turned around in the developing vascular cylinder. (B) An L2 (t) turning around after reaching the root meristem, and another migrating in the vascular cylinder (m). (C) Acid fuchsin stained root with a female *M. incognita* (stained red) feeding on a complex of giant cells (GC). (D) A hand cross-section through a *M. incognita* induced gall showing 2 egg sacs (ES) and egg masses (arrows). (E) A hand cross-section through a *M. hapla* induced gall showing egg sac (ES) and egg mass (arrow). (F) A part of the root with galls induced by *M. incognita*. Galls induced on root tips (terminal gall, TG) stop further root growth. (G) A portion of a root with galls induced by *M. hapla*. Due to numerous lateral root originating from the galls, it appears like a 'witch's broom'. (H) Cross-section through *M. incognita* induced gall. G, giant cells; D, layer of small dividing cells around the giant cells. (I) Cross-section through *M. hapla*-induced gall. G, giant cells; LR, lateral roots. Scale bars = 100 μm.

this temperature for infection, but observed that *Lotus* plants did not grow well at a constant temperature of 28.0°C. Lowering this to 26.0°C improved plant health, and using an infection rate either of 800 eggs or 200 L2 per plant consistently yielded indistinguishable and robust infection at both temperatures. All subsequent experiments were performed at a constant temperature of 26.0°C.

For many hosts (such as tomato and tobacco), the highest rates of experimental RKN infection are achieved by growing the plants in river bottom sand, and sand has the added advantage of being easy to remove for post-infection studies. However, we found that sand did not support typical *Lotus* root development, but rather lead to short, thick, and wavy roots. We therefore tested several "soil types" for their ability to both

support normal root development and permit efficient nematode invasion. Plants were fertilized weekly with Hornum's nutrient solution, and otherwise watered with tap water as needed. Vermiculite and clay litter were easy to wash off, but failed to provide robust infection; in vermiculite (Fig. 1A) and clay litter (Fig. 1B), the infection rate was low and those root galls which did develop were small. There was no apparent difference between vermiculite and clay litter in terms of root infection and gall size. In contrast an equal sand/peat mixture supported both normal root development and robust nematode infection (Fig. 1C), typically yielding approximately 30 root galls per plant at 3 weeks after inoculation. The kinetics of RKN infection in vermiculite and clay litter also appeared to be slower than in the sand/peat mixture. Visible root galls were apparent on roots grown in the sand/peat mixture a week after inoculation with RKN eggs whereas it took about 2–3 weeks on vermiculite and clay litter raised plants for galls to be detected. Inoculation of *Lotus* roots with *M. hapla* eggs (800 eggs per plant) under the conditions optimized for *M. incognita* gave an equivalent and reproducible number of infections.

Characteristics of RKN infection in L. japonicus

L. japonicus roots are translucent, and permit observation of invading nematodes. RKN L2 enter the root just above the root tip, and orient themselves along the length of the root (Fig. 2A). They migrate to the root meristem, and then larvae turn around and migrate upward in the vascular cylinder (Fig. 2A, B) where they initiate feeding sites, characterized by the induction of stereotypic giant cells. Characteristic nematode development ensues, leading to the sedentary adult female (Fig. 2C) which lays eggs into a proteinaceous egg sac on the surface of the root (Fig. 2D, E). The pattern of nematode development was identical for *M. incognita* and *M. hapla* (Fig. 2D, E). Gall formation was evident as swelling of the root around the feeding sites (Fig. 2F, G). Root galls induced by *M. incognita* were consistently larger (Fig. 2F) than those induced by *M. hapla* (Fig. 2G), which, compared to galls induced by *M. incognita*, had a greater propensity to spawn lateral roots, leading to the typical 'witches broom' appearance (Fig. 2G). Heavy infection of root tips by *M. incognita* frequently lead to the development
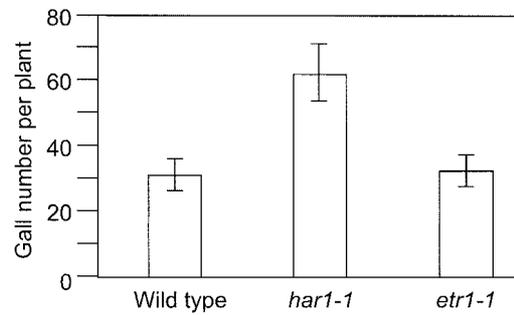

Fig. 3 Gall number per plant induced by *M. incognita* 3 weeks after inoculation of *L. japonicus* ecotype Gifu B129 (wild type), *hypernodulation and aberrant root formation* mutant (*har1-1*), and the ethylene resistant line (*etr1-1*), which is transgenic for the dominant, *Arabidopsis etr1* gene. Each plant was inoculated with 800 eggs, and galls were counted 3 weeks after inoculation. The same experiment was carried out twice with 8–12 plants in each experiment for each genotype. Error bars represent 95% confidence intervals of respective means.
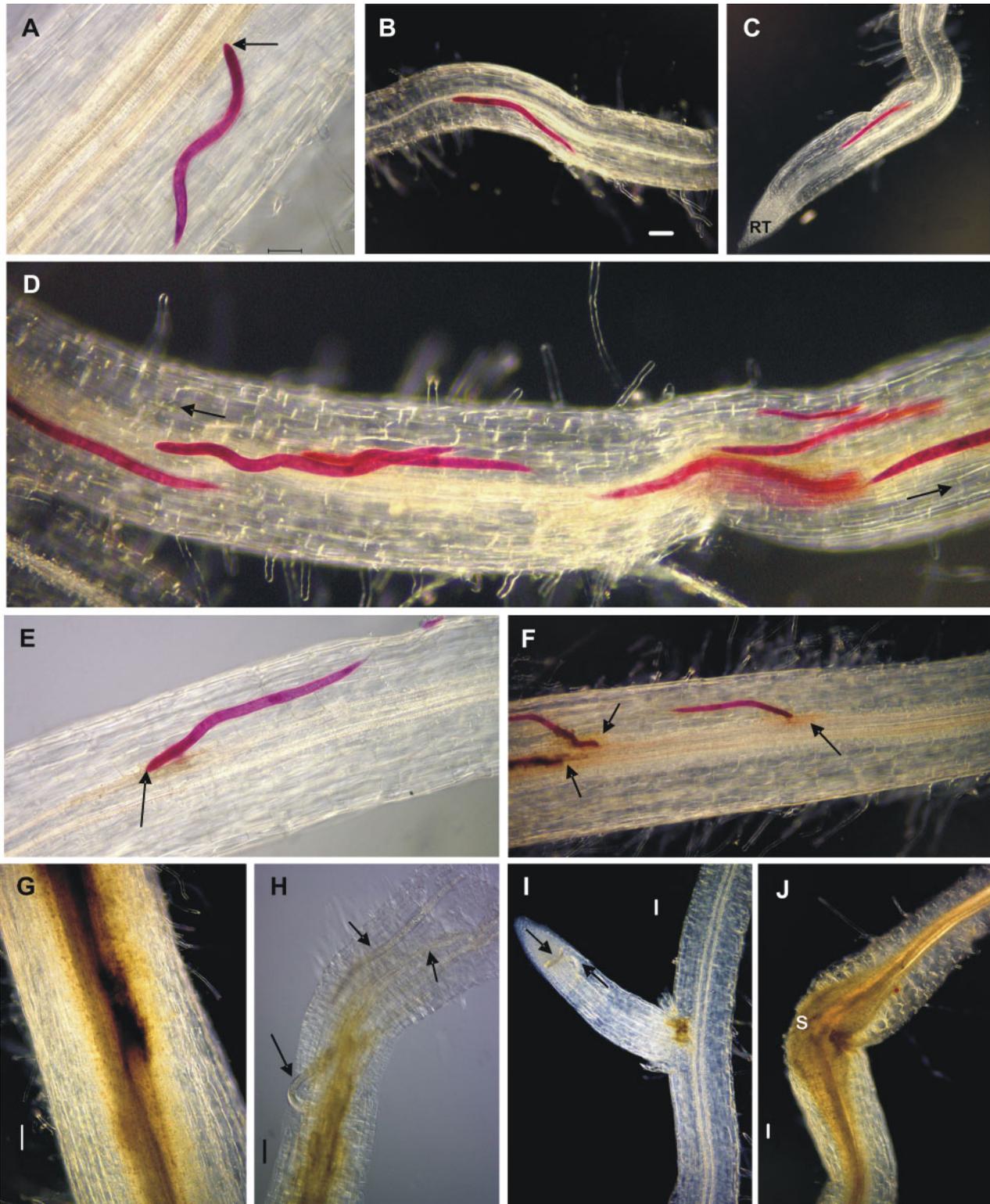
of terminal root galls, and the concomitant cessation of root growth from the tip meristem (Fig. 2F).

To better visualize morphology of galls induced in *Lotus* by *M. incognita* and *M. hapla* we examined transverse sections (Fig. 2H, I). Not surprisingly, each gall contained one or more giant cells, surrounded by several layers of densely packed, small dividing cells. Consistent with the smaller overall size of galls induced by *M. hapla*, these galls consistently had fewer layers of dividing cells around giant cells, compared to *M. incognita* induced galls. Interestingly, lateral roots originate in the dividing cells around the giant cells (Fig. 2I).

RKN hyperinfects a L. japonicus har1 mutant

The ability to perform efficient genetic analyses was a major impetus in establishing *Lotus* as a model legume, and mutants with altered patterns of rhizobial nodulation have been recovered. We wished to examine various classes of mutant for a phenotypic response to nematodes. One mutant, *har1-1* (*hypernodulation and aberrant root formation1-1*), is a hypernodulating mutant of *L. japonicus* (Wopereis et al. 2000). In the absence of rhizobial inoculation, the *har1-1* phenotype is a bushy root system with enhanced lateral roots. Rhizobial inocu-

Fig. 4 Nomarski photographs showing response of *L. japonicus* to SCN (*Heterodera glycines*) infection. Two-week-old plants were inoculated with 1,000 eggs per plant. Roots were stained with acid fuchsin 6 d (A–F) and 10 d (G–J) after inoculation. Live nematodes obtained bright red color after staining. Dead nematodes stain faintly red and fade quickly. (A) A second stage larva (L2) penetrated a mature portion of the root, and oriented its anterior (arrow) towards the vascular cylinder through the cortex. (B) An L2 migrating along the vascular cylinder. (C) An L2 penetrated the root above the root tip (RT), and is shown migrating upwards along the vascular cylinder. (D) Two groups of L2 migrating in opposite directions (arrows). (E) An L2 with its anterior in the vascular cylinder (arrow). Browning of root cells around the nematode head indicating a hypersensitive response (HR). (F) Necrotic tissues due to HR around anterior ends of three different L2 (arrows). (G) Severe tissue browning and necrosis in the root vascular bundle due to infection by L2 10 d after inoculation. (H) Dead L2s in the root (arrows) that did not acquire the acid fuchsin stain. One of the L2 was trying to migrate out of the root. Note the HR in the vascular bundle. (I) Shrunken and dead L2s in the root tip (arrows) and an HR (brown spot) in the junction of lateral and main roots. Some L2 penetrated the root through the junction of main and lateral roots. (J) A scyncytium-like structure seen rarely on *Lotus* root. Note the extensive browning of tissues in the structure and also in the vascular cylinder. Stained nematodes are not visible, indicating that either they were dead (and hence did not stain), or were absent, having migrated from the root.

lation induces a drastic inhibition of root and shoot growth in these plants concomitant with the loss of auto-regulation of nodulation (Wopereis et al. 2000). Intriguingly, independently isolated alleles of this gene (Kawaguchi et al. 2002) also showed increased development of arbuscules following infection by arbuscular mycorrhizal fungi (Solaiman et al. 2000). We inoculated wild-type and mutant *Lotus* strains with *M. incognita* and found that on average, *har1-1* plants had about

twice the number of galls (62 galls per plant) than found on wild type (Fig. 3). However, unlike the response to rhizobium inoculation, the *har1-1* strain did not exhibit a drastic inhibition of root and shoot growth in following RKN inoculation, nor was and expanded zone of the root system galled, as is the case after inoculation of *har-1* alleles with rhizobia (Wopereis et al. 2000) or mycorrhizae (Solaiman et al. 2000).

The second mutant we tested was a transgenic *Lotus* line carrying the *etr1-1* allele of the *A. thaliana ETR1* gene (Chang et al. 1993). This dominant allele confers both an ethylene resistant and a rhizobial hypernodulation phenotype to the transgenic *Lotus* plants (Lohar et al., submitted). In contrast to the results with *har-1-1*, the difference in gall number between wild-type and ethylene resistant plants (33 galls per plant) was not statistically significant (Fig. 3). Hence, one mutant *Lotus* line with a nodulation phenotype (*har1*) also exhibits a nematode-infection phenotype, whereas another (the *etr* transgenic line) does not. Further, the nematode-galling phenotype seen on the *har1* plants is not a direct recapitulation of the rhizobial-nodulation phenotype, reinforcing the power of *Lotus* as a tool to dissect subtle and complex biological interactions.

SCN induces an HR in L. japonicus

Two-week-old *Lotus* plants grown at a constant temperature of 26.0°C in either clay litter or an equal mix of sand and peat were inoculated with approximately 1,000 SCN eggs, and scored for infection 6 d (Fig. 4A–F) and 10 d (Fig. 4G–J) later by staining roots with acid fuchsin. We independently scored the root systems of 15 *Lotus* plants and found that infection by SCN in clay litter was rare. However, we found a large number of SCN L2 in all roots observed 6 d after inoculation in the sand/peat mixture. After penetrating the epidermis, L2 oriented their anterior through the cortex towards vascular bundle (Fig. 4A). They then turn to migrate parallel to the vascular bundle in search of cells to induce feeding sites (Fig. 4B–D). Unlike RKN L2, SCN larvae enter the root at any point on the root surface, from the root tip (Fig. 4C) to mature root zones (Fig. 4A, B, D), with some L2 apparently entering the root at the site of lateral root eruption (Fig. 4I). Also in contrast to invading RKN larvae, SCN L2 migrate both acropetally and basipetally from the penetration site (Fig. 4D).

Cell necrosis or browning was not apparent at the point of root entry, or in the epidermis or cortex through which larvae migrated. However, when observed 6 d after inoculation many vascular cells in close proximity to the anterior of the nematode displayed a hypersensitive response (HR) and turned brown and necrotic (Fig. 4E, F). These HR reactions were coincidental with larval feeding, and became increasingly conspicuous, occupying a large part of the vascular cylinder by 10 d after inoculation (Fig. 4G). At this stage nematodes were sometimes seen migrating out of the root (Fig. 4H), indicating that they had failed to establish feeding sites. At 10 d after inoculation the nematodes stained only lightly and transiently with acid fuchsin, indicating that they were either dead or dying of starvation. Consistent with this interpretation was the presence of shrunken nematodes (Fig. 4I). Occasionally an HR was observed with no nematode being clearly present (Fig. 4I), likely reflecting that the still motile larvae had migrated from the root. After careful examination of fifteen *Lotus* root systems inoculated with SCN we observed just two occurrences of the syncytial cells indicative of feeding site establishment (Fig. 4J). However, these syncytia displayed a strong HR. Collectively, our results indicate that the Gifu B129 ecotype of *L. japonicus* is resistant to the MO line of SCN, and that this resistance involves the induction of an HR at the feeding site. However, SCN L2 could enter the *Lotus* root, and migrate through it without inducing an HR.

Discussion

Root-knot and soybean cyst nematodes are economically important, obligate parasites that induce unique structures in host roots. However, little is known about the plant genes required for these specialized structures which serve as feeding sites for the nematode. The use of model plants for which extensive genomic information is available offers obvious advantages over crop plants in the study of the complex host biology associated with feeding site induction and function, and *A. thaliana* has proven tractable (Sijmons et al. 1991, de Almeida Engler et al. 1999, Puthoff et al. 2003). However, a recent study (Koltai et al. 2001) showed that the parasitic plant–nematode interaction elicits pathways in common with mutualistic legume–rhizobium symbiosis, suggesting that comparing and contrasting findings from various host–microbe associations might be particularly rewarding. Further, since both symbioses (parasitic and mutualistic) are economically important, a model legume is a practical choice to study both interactions in the same system, and our results confirm that *L. japonicus* is an excellent model for such research.

As a first step in assessing *Lotus* for its suitability as a nematode host, we examined physical parameters. The choice of growth medium for root–nematode experiments is important, as soil texture affects the severity of root infestation. Sandy loams and coarser textured soils are more conducive to infection in the field than heavy or clay soils (Sasser 1954, Raski and Johnson 1959, Thomason and Lear 1959), and researchers typically use coarse textured soils such as river bottom sand for RKN studies in the greenhouse (de Ilarduya et al. 2001, Ammiraju et al. 2003). However, unlike tomato, we found that *Lotus* roots develop poorly in sand alone, and so we tested various growth media for nematode infection. An equal mix of sand and peat was found to be optimal, supporting both good root development and efficient infection by the several nematode species tested. Several other "soil types", including clay litter and vermiculite were found to sustain robust *Lotus* root systems, but nematode infection was retarded in these media. A constant temperature of 26.0°C was found to be optimal both for *Lotus* growth and nematode infection.

The translucent nature of *L. japonicus* roots permits continuous, non-destructive monitoring of the invasion process at least to the stage where feeding sites are induced using standard Nomarski optics. Similar observations have been made in *Arabidopsis* (Wyss et al. 1992), but computer enhancement of video images was required to achieve satisfactory resolution in this species. As is the case on *Arabidopsis* (Wyss et al. 1992), we observed that RKN larvae preferentially penetrate *Lotus* just above the root tip. In contrast, however, SCN L2 freely penetrate any portion of the *Lotus* root. This differs from the situation in soybean, where the larvae exhibit a strong preference for penetrating the root 1 cm or more above the root tip (Kinloch 1998). Similarly, Wyss (1997) reported that beet cyst nematode (*Heterodera schachtii*) L2 invade *Arabidopsis* roots predominantly in the zone of elongation (above the root tip).

Behavior within the *Lotus* root also was seen to be different for root-knot and cyst nematodes. Following penetration of the epidermis, we observed that *M. incognita* L2 initially migrate through the cortex towards the root tip, and because no cellular damage was evident, we presume that migration is through the apoplast, as is the case in other hosts examined (Wyss et al. 1992). At, or just prior to the root tip meristem, RKN L2 turn, and migrate basipetally into the vascular cylinder. SCN larvae also migrate within and parallel to the vascular cylinder, but because they are able to penetrate the root at any position along its length, their migration is apolar. In *Arabidopsis*, root tissue at the site of infection and along the path of migrating SCN became necrotic (Grundler et al. 1997). A similar observation has been reported for *H. schachtii* in which larval death accompanies necrosis of cortical cells in the roots of resistant sugarbeet (Kleine et al. 1997). In contrast, SCN does not elicit tissue browning/necrosis either at the site of entry or during migration in the cortex in *Lotus*.

The end of the migratory phase within the vasculature for both species corresponded with the onset of feeding site induction, but the outcome was very dependant on the nematode species. Morphologically distinct feeding sites (syncytia) were rarely apparent following infection with SCN. Rather, a necrotic, HR ensued, indicating that the tested host ecotype (Gifu) is resistant to the nematode line (MO). It appeared that the HR was induced only after attempts by the invading worms to initiate feeding sites in *Lotus*. This pattern strongly recapitulates the pattern reported for SCN-resistant soybean plants (Endo 1991, Kim et al. 1987), suggesting that the response of *Lotus* to SCN is more similar to the response of resistant soybean plants than is that of *Arabidopsis*. The formation of rare syncytia by SCN in *Lotus* root indicates that in spite of strong resistance response, *Lotus* is fully competent to respond to signals from the nematode and is able to respond by inducing feeding structures. We suspect that certain combinations of *L. japonicus* accessions and SCN races will produce fully compatible interactions. The availability of compatible and resistant lines would facilitate the mapping of *R*-loci, and as cloning genes by chromosome walking is a routine procedure in *L.*

japonicus (Krussel et al. 2002, Nishimura et al. 2002), *Lotus* should become a useful plant for cloning interesting genes in the pathway of SCN–legume interactions.

In contrast, both tested RKN species (*M. incognita* and *M. hapla*) were able to initiate giant cell formation from vascular parenchyma cells in the stele, and continue development through three molts to sexual maturity, confirming that *L. Japonicus* is bona fide host for RKN. It is important to stress that other combinations of nematode isolate/species with different *Lotus* ecotypes may give different patterns of resistance or susceptibility to those described here, and we plan to examine a range of *Lotus* accessions.

As a prelude to a thorough survey of host responses to plant-parasitic nematodes by the many *L. japonicus* mutants exhibiting phenotypes associated with symbioses with microorganisms (VandenBosch and Stacey 2003), we examined two mutants we suspected might exhibit a nematode phenotype. The first mutant was chosen because it hypernodulates following rhizobial inoculation (Wopereis et al. 2000), and carries a single base change in the endogenous *Lotus har1* gene. The second mutant we examined was a transgenic, ethylene resistant *L. japonicus* line we constructed, and which we showed also to be hypernodulated by rhizobia (Lohar et al. submitted).

The *Lotus har-1* gene has recently been cloned, and found to encode a CLAVATA1 (CLV1) like receptor kinase (Krussel et al. 2002, Nishimura et al. 2002). The *har1-1* allele has a single base change which leads to premature termination, and because it lacks the extracellular, receptor domain, it likely is a null. In *Arabidopsis*, CLV1 forms a hetero-dimer with CLV2, which perceives the apoplastic peptide ligand, CLV3 (Rojo et al. 2002) to control stem cell identity in shoot meristems. In the absence of rhizobial inoculation, *har1-1* produces an increased number of, but shortened, lateral roots, leading to a bushy root system (Wopereis et al. 2000). In the presence of compatible rhizobia, *har1-1* displays a hypernodulation response. Further, not only do the roots become hypernodulated, but there is a drastic inhibition of root and shoot growth (Wopereis et al. 2000). Remarkably, the root mutant phenotype is restored when normal *har-1* activity is present in the shoot. Thus, the *har1-1* phenotype is shoot controlled. It has been proposed that HAR-1 may interact with a root derived signal (and possibly with other proteins) in the shoot, and that the interaction induces a feed back regulation of nodulation and lateral root formation in the root (Krussel et al. 2002, Nishimura et al. 2002). It is not unreasonable to speculate that the hypothesized root-derived signal may be a CLV3-like peptide. Intriguingly, it has recently been proposed that SCN produces such molecules (Olsen and Skriver 2003), possibly to mimic an endogenous plant ligand (Bird 1996), although the role of these nematode peptides awaits a functional test. A computational screen (Olsen and Skriver 2003) failed to identify similar peptides in RKN, but the libraries from which the *Meloidogyne* sequences were obtained (McCarter et al. 2003) were not specifically enriched for sequences from secretory glands, as had been the SCN

libraries. We showed that *M. incognita* establishes approximately twice the number of galls on *har1-1* plants compared to a wild-type host. It would be interesting to compare the response of *har1-1* plants to SCN invasion, but as we discussed, the Gifu ecotype of *Lotus* is resistant to SCN.

The central phenotype of RKN on *har1* plants (i.e. hypergalling) is equivalent to the principal rhizobial and mycorrhizal phenotypes (Solaiman et al. 2000). However, in contrast to the outcome of rhizobial inoculation, RKN infection does not cause discernible inhibition of root and shoot growth after inoculation with nematodes, indicating that nematode infection does not trigger a systemic regulation of overall plant development. In the absence of specific experimental evidence, a direct relationship between RKN proteins and the *Lotus har1* locus must remain conjectural, and indeed more trivial reasons for the hypergalling phenotype can be envisioned. For example, Wopereis et al. (2000) reported that the density of lateral root primordia and emerged lateral roots (number per unit length of root) were at least three times higher in *har1-1* plants compared to wild type. Thus, the increased gall number in *har1-1* might be due to the increased number of lateral roots providing additional targets for nematode penetration, although on wild-type plants the number of potential penetration points far exceeds the actual number of galls.

Like *har1-1* plants, ethylene resistant *L. japonicus* plants have been shown to be hypernodulated by rhizobia (Lohar et al. submitted). However, we found the level of RKN infection in ethylene-resistant *Lotus* plants to be similar to wild-type plants. Thus, ethylene may not play a direct role in interaction between *Lotus* root and RKNs.

Collectively, our results indicate that *L. japonicus* is a good model legume for studying legume–nematode interactions. Further, because *Lotus* is a good system to study legume–rhizobium and legume–mycorrhizae symbioses, comparing these beneficial symbiosis with the harmful symbiosis involving parasitic nematodes will likely be rewarding.

Materials and Methods

Plant material and growth conditions

Wild-type (ecotype Gifu B129) and mutant *L. japonicus* lines were treated identically. Construction of the *Lotus* line transgenic for the *A. thaliana etr1-1* mutant allele of *ETR1* is described elsewhere (Lohar et al. submitted). Transgenic over-expression of *etr1-1* in the Gifu ecotype of *L. japonicus* confers ethylene resistance and a hypernodulation phenotype following rhizobial infection (Lohar et al. submitted). The *har1-1* mutant was obtained by Szczyglowski et al. (1998), and has been characterized extensively for its hypernodulation and short bushy root phenotype (Wopereis et al. 2000, Krussel et al. 2002, Nishimura et al. 2002). Seeds were scarified and sterilized in concentrated sulfuric acid for 5 min, and then washed for 4–5 times with sterile water. Seeds were germinated on wet filter papers in the dark at room temperature for 2 d prior to transfer to either fine vermiculite, clay litter, or a mixture of river bottom sand and peat (1/1, v/v) in 5×5 cm plastic cups. Plants were maintained in the NCSU phytotron at a constant temperature (26.0°C or 28.0°C) with a 16-h light/

8-h dark regimen. Plants were watered with tap water daily and fertilized weekly by watering with 1× Hornum's nutrient solution (5 mM NH_4NO_3, 3 mM KNO_3, 1.2 mM $MgSO_4$, 720 μM NaH_2PO_4, 50 μM Fe-EDTA (9% Fe), 7 μM $MnSO_4$, 20 μM H_3BO_3, 2 μM $CuSO_4$, 1 μM $ZnSO_4$, 300 nM Na_2MoO_4, pH 6.8).

Plants were inoculated with nematodes 2 weeks after planting, and galls were scored 3 weeks after inoculation.

Nematode culture and inoculation

RKNs (*M. incognita* and *M. hapla*) were maintained in the greenhouse on tomato plants, and egg masses were extracted with 0.5% (v/v) NaOCl, and second stage larvae hatched as described by Hussey and Barker (1973). Plants typically were inoculated with about 800–1,000 eggs or 150–200 RKNs infective larvae (L2) at 1–2 weeks after germination. Inoculated roots were destructively observed for root galls beginning 1-week post inoculation.

SCN MO (*H. glycines*) was maintained in the greenhouse on soybean. SCN eggs were isolated after homogenizing the cysts in a glass homogenizer and rinsing the eggs onto a sieve with a 25-μm mesh (Cairns 1960). Two-week-old *Lotus* plants were inoculated with 1,000 freshly isolated eggs.

Staining and microscopy

Soil was rinsed from roots, which then were cleared in 2% (v/v) NaHOCl for 4 min, followed by rinsing and soaking in water for 15 min. Roots were boiled for 30 s in 0.01% acid fuchsin in 150 mM acetic acid, cooled, rinsed and stored in distilled water. Stained roots were mounted on microscope slides and photographed either using a compound microscope equipped with Nomarski DIC optics, or a stereomicroscope.

Unstained galls were prepared for observation and photography under the light microscope either by hand-sectioning as fresh tissue, or were fixed, dehydrated, infiltrated with paraffin, and 25 μM sections cut using a rotary microtome as described by Graham and Joshi (1995).

Acknowledgments

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