Continual Green-Fluorescent Protein Monitoring of Cauliflower Mosaic Virus 35S Promoter Activity in Nematode-Induced Feeding Cells in *Arabidopsis thaliana*

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The responsiveness of the cauliflower mosaic virus 35S promoter in feeding sites developed by both sexes of *Heterodera schachtii* and female *Meloidogyne incognita* has been studied. The objective was to establish the value of green-fluorescent protein (GFP) as a nondestructive reporter gene system for characterizing promoter activity at nematode feeding sites *in vivo*. Growth units were devised that allowed individual feeding sites in roots of *Arabidopsis thaliana* to be observed by both bright-field and epifluorescent illumination. Changes in GFP expression were visually observed under experimental conditions that resulted in chloroplast formation in syncytia but not other root cells. Changes in GFP levels altered the extent of quenching, by this protein, of red light emitted by chlorophyll within the chloroplasts under violet excitation. Image analysis provided a semiquantitative basis for simultaneous measurement of changes in GFP fluorescence and the unquenched emission by chlorophyll. GFP levels were constant in cells surrounding the syncytium induced by *H. schachtii*, but they fell progressively from 10 to 35 days postinfection within this structure. Significant reduction in GFP levels was not limited to the early part of the time course but also occurred between 27 and 35 days postinfection. GFP was detected by immunoblotting in females of *M. incognita* but not in *H. schachtii* parasitizing similar GFP-expressing roots.

Additional keywords: regulation, transgenic.

Nematodes represent a key threat to modern agricultural practice (Atkinson 1995). The obligate sedentary endoparasitic nematodes, which comprise the root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (including *Globodera* and *Heterodera* spp.), are the most agronomically important groups. Root-knot nematodes invade close to root meristems and migrate through the root in an intercellular manner before establishing feeding sites adjacent to differentiating vascular tissue. These feeding sites comprise a system of giant cells surrounded by characteristically galled cortical root tissue. Each giant cell is formed by repeated nuclear division of a single cell without concomitant cytokinesis (Sijmons et al.

1994). By contrast, cyst nematodes normally invade in the zone of root elongation and migrate in an intracellular manner, with concomitant destruction of plant cells, before initiating a single feeding cell, a syncytium, usually from a parenchymal cell within the stele. The syncytium is formed by the dissolution of adjacent parenchymal cell walls and the fusion of cell contents (Sijmons et al. 1994). Both giant cells and syncytia are highly specialized structures adapted to provide a nutrient sink from which the nematode is able to feed in an intermittent manner. Such adaptation of a plant cell to provide a nematode feeding cell involves significant alteration of normal plant gene expression (Hammond-Kosack et al. 1989).

Control of nematodes currently relies on three principal approaches: chemicals, cultural practices, and resistant varieties, often used in an integrated manner (Hague and Gowen 1987). However, the level of control provided by such conventional procedures is often inadequate to deal with the problem. This offers an important opportunity for plant biotechnology to produce effective and durable forms of nematode control with consequent reduction in environmental damage.

The identification of a promoter used to express a novel product that disrupts some aspect of nematode parasitism is a basic requirement of a molecular approach to nematode control. The wound-responsive promoter, *wun-1*, from potato responds to intracellular root migration by *Globodera pallida* but not to the less damaging intercellular migration of *Meloidogyne* spp. (Hansen et al. 1996). Several genes which show some degree of feeding-cell-specific expression, after the parasite has become established, have been reported (Gurr et al. 1991; Van der Eycken et al. 1996; Lambert and Williamson 1993; Wilson et al. 1994; Bird and Wilson 1994; Opperman et al. 1994).

We recently reported an anti-feedant approach to nematode control (Atkinson 1993; Urwin et al. 1995). This may provide a generic defense against nematodes through the use of promoters which express in a constitutive or root-specific manner (Atkinson and Lilley 1997). We demonstrated that transgenic delivery of an engineered rice cysteine proteinase inhibitor (Oc-1ΔD86) modified from oryzacystatin-I (Oc-I) had a major effect on disrupting the growth and development of *G. pallida* on tomato hairy roots (Urwin et al. 1995) and of *Heterodera schachtii* and *Meloidogyne incognita* on *Arabidopsis* (P. E. Urwin, unpublished). These studies used the cauliflower mo-

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saic virus (CaMV) 35S promoter to direct expression of the Oc-IAD86 inhibitor protein. GUS expression studies have previously suggested that the CaMV 35S promoter is strongly down-regulated by *H. schachtii* and *M. incognita* (Goddijn et al. 1993). This result contrasts with our demonstration that CaMV 35S provides a useful level of nematode control. Since correct spatial and temporal promoter activity is central to the successful delivery of a defense, we investigated the expression pattern of the pBI121-derived CaMV 35S promoter used in our constructs.

We describe a novel growth system for *Arabidopsis thaliana*, which allows the continual *in vivo* microscopic analysis of plant roots and provides a convenient system for following the fate of individual nematodes. We have used this system to monitor the growth and development of both *H. schachtii* and *M. incognita* and the consequent effects on the level of expression of CaMV 35S using the green-fluorescent protein (GFP) reporter gene. We also demonstrate uptake of GFP during feeding by *M. incognita*, but not by *H. schachtii*.

RESULTS

Plant growth and nematode development.

Arabidopsis plants developed and flowered in growth units like the one illustrated in Figure 1 over a time period similar to that of plants grown in parallel in magenta pots. The nematode infection rate for both *H. schachtii* and *M. incognita* was approximately 15% of the juveniles with which roots had been inoculated. Their parasitic development progressed at a rate similar to that reported previously (Sijmons et al. 1991), and high numbers of eggs were associated with females within 35 days of inoculation. Eggs were extruded from the vulva of fe-

Fig. 1. Growth unit used to allow the continual observation of green-fluorescent protein expression in *Arabidopsis* roots.

male *H. schachtii* at 28 days postinfection (p.i.), and body wall tanning had commenced for many individuals at 35 days p.i. Egg masses of *M. incognita* were first apparent at 25 days p.i. The development of identified individuals could be followed in the growth units, and their sex could be determined retrospectively when sexual dimorphism was evident. The shape factor of roundness describes the outline of the nematode. The roundness value is calculated from the ratio of perimeter squared to area, with a minimum value of unity for a circle. For *H. schachtii*, changes in shape gave roundness values of 4.44 and 2.4 for the female at 7 and 10 days p.i. and values less than 2 at 14 days p.i. and afterward. Roundness values less than 2 are characteristic of developing fourth-stage juvenile or adult females rather than other juvenile stages or adult males (Atkinson et al. 1996). The development of *H. schachtii* females was observed from 7 to 35 days p.i., and that of *M. incognita* females from 7 to 40 days p.i. One representative individual of each species was photographed: *H. schachtii* at 7, 10, 14, and 35 days p.i. and *M. incognita* at 28 days p.i. Female *H. schachtii* induced the formation of syncytia with the long, thin outlines that characterize parasitism of this host (Sijmons et al. 1991). The syncytium was evident as a yellow green region under bright-field illumination from 10 days p.i., and this facilitated the measurement of its outline at each time point. The extent of the syncytium at 14 days p.i. is indicated by asterisks in Figure 2L and M. Once the syncytium had become established, by 10 days p.i., no significant increase in its outline area was subsequently observed. Its yellow green coloration was due to a low density of chloroplasts, which can develop in syncytia in response to limited exposure to light but were absent elsewhere in these roots and those of uninfected GFP-expressing or wild-type plants under the same growth conditions. Syncytia with a green coloration under bright-field illumination were not usually associated with juveniles that became male, but in a few individuals a green area was evident in the vicinity of the head (Fig. 2Z). A representative male was photographed from 7 days p.i. (Fig. 2P–W). It emerged from its cuticle and left the site of study between 17 and 21 days p.i.

Expression of GFP in uninfected root tissue.

GFP expression in roots was observed using epifluorescence with a violet excitation filter and a long-band-pass emission filter for frequencies greater than 495 nm. Under these conditions an untransformed root of *Arabidopsis* with an established female *H. schachtii* grown in the dark was barely visible under the photographic conditions used later (Fig. 2E). The emerald green fluorescence emission present in Figure 2D is due to excitation of GFP in the roots of an uninfected transformed plant. Chloroplasts emit red fluorescence when excited by violet light, as observed by Sheen et al. (1995). This effect is shown by a root of a wild-type *Arabidopsis* plant grown in the light specifically to stimulate chloroplast formation (Fig. 2B). The high chloroplast density colored the roots green under bright-field illumination (Fig. 2A). A root of a transformed plant expressing GFP and grown in the light also appears green from chloroplast formation when viewed under the same conditions (Fig. 2C), but the emission of red fluorescence from the chloroplasts under violet excitation was completely quenched by the strong emerald green emission of GFP (Fig. 2D).

Expression of GFP in roots infected with *H. schachtii*.

Figure 2H–O shows a developmental time course of a typical female *H. schachtii* on a root expressing GFP under the control of the CaMV 35S promoter at 7, 10, 14, and 35 days

p.i. The photographs are paired images under bright-field and fluorescence illumination. Each shows a length of root containing a nematode and its feeding site (ir) plus an adjacent noninfected root (nr) of the same plant as a positive control

(Fig. 2L and M). In the fluorescent images, the expression of GFP appears as an emerald green coloration. There is very limited autofluorescence in the plant roots, as shown earlier (Fig. 2E).

At 7 days p.i., the head was already positioned as later. This indicates that the syncytium had been initiated, but changes in its GFP level had not resulted from initial parasite establishment. The area of root exhibiting a faint yellow fluorescence around the body of the nematode in Figure 2I is consistent with a limited response of plant cell walls following invasion. The presence of chloroplasts was used as a basis for detecting the outline of the syncytium. It also allowed changes in GFP expression within the syncytium to be readily observed. A syncytium containing chloroplasts at low density is intense green when viewed under violet light if GFP is present at a high level. However, it has a red or orange yellow coloration if GFP is not present at sufficiently high levels to quench the chloroplast emissions completely. Therefore a change in the coloration of a syncytium is expected if CaMV 35S is down-regulated and appreciable expression of GFP is lost. This effect allowed the syncytium formed by a female *H. schachtii* to be detected by a faint orange red fluorescence at 10 days p.i. The intensity of this coloration was more fully apparent from 14 to 35 days p.i. The loss of GFP expression was restricted to the syncytium and did not spread to adjacent root tissue during the course of the experiment. Figure 2X and Y shows a different female nematode, which was first examined at 21 days p.i. In this case the syncytium had not developed chloroplasts, since the roots had not previously been exposed to light, and in the absence of the associated red autofluorescence a reduction in green GFP fluorescence can be directly observed.

Male *H. schachtii* did not normally influence GFP expression to the extent of a visible color change during their development. A time course of a representative male is shown in Figure 2P–W. The limited yellow coloration, particularly evident in Figure 2U, is again associated with cell walls. Occasionally, down-regulation by a male did influence coloration around the head of the animal (the orange area in Fig. 2 α), but this was extremely limited in comparison to the effect induced by females.

Analysis of GFP levels within a syncytium.

Image analysis using a color camera allowed individual mean gray levels to be determined for both the green and the red components of each fluorescent image. This was used to confirm that the interpretation of color changes observed by eye was reliable. Measurements were taken in an area in the light path either adjacent to or within the syncytium in 10 dif-

ferent roots parasitized by female nematodes (including the root shown in Fig. 2H–O) over a time course from 7 to 35 days p.i. At 7 days p.i. the outline of the syncytium was not well defined, and syncytial measurements were taken from an area of the root adjacent to the head of the nematode. Values on a linear scale of 0 to 255 were derived, representing increasing intensity of fluorescence in the green and red components of the light detected by the color camera. The mean values of these measurements are given in Figure 3. Linear regression established that the green component of the fluorescence from regions adjacent to the syncytium did not significantly change from 7 to 25 days p.i. (Fig. 3A). In contrast, the negative slope of the regression line for green emissions from the light path that included the syncytium established a significant fall in intensity over the period of measurement (*t* test on slope, $P < 0.001$). Furthermore, the intensity of green light was lower at 35 days p.i. than at 27 days p.i. and all earlier time points (one-way ANOVA, *a priori* contrasts, $P < 0.001$). Therefore, a decline in emissions from GFP was detected between 27 and 35 days p.i., and the change was not restricted to the early period of the time course. The intensity of red emissions from cells around the syncytium was consistently low throughout the time course (Fig. 3B). This is consistent with the lack of chloroplasts in these cells. In contrast, the changes in red emissions establish that chlorophyll concentrations increased from 7 to 14 days p.i. and then remained at this higher level for the remainder of the time course. A curve was fitted to this changing pattern using polynomial regression.

Expression of GFP in roots infected with *M. incognita*.

No down-regulation of the CaMV 35S promoter was observed in response to infection by the female root-knot nematode *M. incognita* (Fig. 2F and G). Every gall appeared to express GFP at a high level, and the fluorescence was intense. GFP emissions from the gall tissue tended to mask emissions from the centrally lying giant cells. However, the giant cells shown in Figure 2F do seem to be associated with the intense emission evident in Figure 2G.

Uptake of GFP by *H. schachtii* and *M. incognita*.

We have recently shown by Western blot analysis that both *H. schachtii* and *M. incognita* are capable of ingesting the cystatin Oc-1AD86, a protein of 11.2 kDa (P. E. Urwin et al., unpublished). Definition of the upper size limit of proteins that can be ingested by nematodes is important both for understanding nematode nutrition and digestive processes and for design of molecules for nematode control. The availability of GFP-expressing plants has allowed us to determine if nema-

Fig. 2. Activity of the cauliflower mosaic virus (CaMV) 35S promoter analyzed by fluorescence of green-fluorescent protein (GFP) in *Arabidopsis* infected with *Heterodera schachtii* and *Meloidogyne incognita*. **A** and **B**, Bright-field and fluorescent images, respectively, of a typical wild-type C24 *Arabidopsis* root grown in the light. **C** and **D**, Bright-field and fluorescent images, respectively, of a typical GFP-transformed *Arabidopsis* root grown in the light. **E**, Fluorescent image of a wild-type C24 *Arabidopsis* root infected with female *H. schachtii* and grown in the dark. **F** and **G**, Bright-field and fluorescent images, respectively, of a typical *M. incognita* female at 28 days postinfection (p.i.) on a plant expressing GFP. **H–O**, Paired bright-field and fluorescent images, respectively, of a typical *H. schachtii* on GFP expression at 7 days p.i. (**H** and **I**), 10 days p.i. (**J** and **K**), 14 days p.i. (**L** and **M**), and 35 days p.i. (**N** and **O**). Asterisks in **L** and **M** indicate the extent of the syncytium. **P–W**, Paired bright-field and fluorescent images showing the effect of a typical male *H. schachtii* on GFP expression at 7 days p.i. (**P** and **Q**), 10 days p.i. (**R** and **S**), 14 days p.i. (**T** and **U**), and 21 days p.i. (**V** and **W**). **X** and **Y**, Effect of a female *H. schachtii* on GFP expression in a plant grown in the dark. The syncytium has not developed chloroplasts, resulting in the absence of red autofluorescence when it is viewed under violet light. The extent of the syncytium, in which lowered GFP expression is evident, is marked with asterisks. **Z** and α , Effect of a male *H. schachtii* in which localized down-regulation of the CaMV 35S promoter can be observed in α as an orange area adjacent to the head of the nematode. The bars represent 100 μ m in **A–D** and 300 μ m in **E– α** . n = Nematode; h = head of nematode; ir = infected area of root; nr = noninfected area of a second root.

Fig. 3. Image analysis of the mean red and green components of fluorescent images of an *Arabidopsis* root transformed with green-fluorescent protein, viewed under fluorescent conditions. **A**, ■ = Mean level of green emissions from inside the syncytium; □ = mean level of green emissions from outside the syncytium. **B**, ■ = Mean level of red emissions from inside the syncytium; □ = mean level of red emissions from outside the syncytium. Values are means $\pm$ SE.

todes are able to take up the larger GFP (28 kDa). Western blot analysis demonstrated the presence of GFP in *M. incognita* but failed to detect the protein in *H. schachtii* (Fig. 4).

DISCUSSION

The time course for the development of *H. schachtii* and *M. incognita* in growth units used for this work is similar to that on *A. thaliana* grown in conventional containers by Sijmons et al. (1991). The syncytium induced in roots of *A. thaliana* by *H. schachtii* reached a maximum size in outline at 10 days p.i. and had the elongated outline described previously (Sijmons et al. 1991). The results establish that the observation growth units allow time-course studies of the parasite and its feeding site by both bright-field and epifluorescence microscopy. This has particular value for studying changes in promoter activity *in planta* with GFP as a reporter. Both up- and down-regulation of promoters occurs in nematode feeding cells and has been reported in previous work (Goddijn et al. 1993). The GFP reporter system has the advantage of not requiring the de-

Fig. 4. Western blot detection of green-fluorescent protein (GFP) in *Heterodera schachtii* and *Meloidogyne incognita*. Lane 1, *M. incognita* from GFP-transformed *Arabidopsis*. Lane 2, *M. incognita* from wild-type C24 *Arabidopsis*. Lane 3, *H. schachtii* from wild-type C24 *Arabidopsis*. Lane 4, *H. schachtii* from GFP-transformed *Arabidopsis*. Lane 8, plant material expressing GFP. Lanes 5, 6, and 7 are blank.

structive sampling for visualization which characterizes most studies based on GUS.

Chloroplasts occur in root cells grown in the light, including the syncytium induced by *H. schachtii* in infected roots of *Arabidopsis* (Sijmons et al. 1991). Preliminary observation established that chloroplasts occurred in the syncytia but not in the surrounding root cells when plants were grown with only brief exposure to light for observation as described in this work. In uninfected roots grown under high levels of light to induce chloroplasts in a range of cells, the red light emitted by chlorophyll under violet excitation is quenched when GFP is also expressed. Consequently, cells emitting both chlorophyll and GFP-derived fluorescence appear any color in the spectrum from red to green, according to the extent of the effect. Such quenching resulting in a yellow appearance of the emission was previously reported for maize protoplasts (Sheen et al. 1995). The color shift establishes that down-regulation for GFP occurs within 10 days p.i. for syncytia associated with females of *H. schachtii*. The effect is rarely detected for males.

The visual color shift is of value for those lacking image analysis equipment. Our equipment had the advantage of allowing quantification of both increases and decreases in green and red emissions. The green fluorescence emitted from GFP in the light path of the syncytium fell progressively from 10 to 35 days p.i., while the emissions from surrounding cells remained constant. Only a partial reduction in green emissions could be anticipated, even if GFP was absent from the syncytium, because the protein was expressed in the sleeve of cells surrounding this structure. However, the reading at 35 days p.i. was significantly lower than at all other occasions except 31 days p.i. Therefore, GFP was present in the syncytium until at least 27 days p.i. A similar analysis for the red emissions from chlorophyll establishes an increase in this pigment within the syncytium from 7 to 14 days p.i. and approximately constant levels subsequently. The cells surrounding the syncytium maintained a constant low level of red emissions. This presumably represents a background level for red, since these cells lacked obvious chloroplasts.

The GFP reporter system clearly established that the CaMV

35S promoter was active in the gall tissue surrounding the feeding site of *M. incognita*. The high emission intensity prevented certain detection of expression in giant cells. However, GFP was detected in *M. incognita* by immunoblotting, which provides strong circumstantial evidence for its presence in giant cells. This is consistent with the intense emissions from the region of the giant cells evident in Figure 2G. GFP was not detected in females of *H. schachtii*, although it was present in the syncytium. Microinjection into the syncytium induced by this species has established that *H. schachtii* takes up fluorescent dextrans of 20 but not 40 kDa (Bockenhoff and Grundler 1994). Recent immunoblotting experiments have shown that both *H. schachtii* and *M. incognita* ingest proteinase inhibitors of 11.2 kDa (P. E. Urwin et al., unpublished). This suggests that the exclusion limit for globular proteins in *H. schachtii* is between 11.2 and 28 kDa, whereas the upper limit in *M. incognita* exceeds 28 kDa. The site of exclusion of macromolecules by *H. schachtii* may be the feeding tube (Bockenhoff and Grundler 1994). This structure is frequently replaced during feeding (Wyss 1992). Possibly one of its functions is to act as a prefilter, preventing blockage by macromolecules and organelle fragments in the nutrient flow toward the animal. Possibly GFP is not detected in female *H. schachtii* because it is rapidly degraded by intestinal proteinases. Both cysteine and serine proteinase activities occur in the intestine of *Heterodera glycines* (Lilley et al. 1996), and a cysteine proteinase activity of *H. schachtii* is inhibited during development on transgenic plants expressing a cystatin (P. E. Urwin et al., unpublished). However, cysteine proteinases also occur in *M. incognita* (unpublished results). Therefore it is more likely that differences in uptake rather than digestive proteinases explain the presence of GFP in *M. incognita* only.

Previous work reported that the CaMV 35S promoter did not provide expression in the gall of *M. incognita* or the syncytium of *H. schachtii* on *Arabidopsis* (Goddijn et al. 1993). Even if no GFP is taken up by *H. schachtii*, it seems unlikely that GFP could persist after early down-regulation of the promoter over the time course to 35 days p.i. Therefore we conclude that the CaMV 35S promoter has some activity in the syncytium until at least 27 days p.i. Another possibility is that GFP is actively transported into the feeding cell via the symplastic pathway. If so, it is necessary for GUS not to be imported into the syncytium to fit observations to date. Our results are valid for the CaMV 35S promoter derived from the commercially available plasmid pBI121 (Clontech, Palo Alto, CA) but may not apply to other, deleted versions of this promoter that show different responsiveness to nematodes (P. Burrows, personal communication).

This work establishes the potential of GFP as a reporter system for studying changes in the responsiveness of promoters to nematodes *in vivo*. Evidence suggests that *H. schachtii* excludes a globular protein of 28 kDa. The definition of exclusion limits for different economic nematodes has important implications for the design of any transgenic resistance approach that involves the uptake of proteins by the oral route.

MATERIALS AND METHODS

Plant transformation.

The construct pBI121 CaMV35S-mGFP was kindly provided by J. Haseloff, MRC, Cambridge. The *gfp* gene in this

construct had been modified to remove the cryptic splice sites in the encoded protein, to allow expression in *Arabidopsis* (Haseloff and Amos 1995). The construct was electroporated into *Agrobacterium tumefaciens* (Shen and Forde 1989) and introduced into *Arabidopsis thaliana* ecotype C24 by *A. tumefaciens*-mediated transformation of roots as described by Clarke et al. (1992). T1 seed was collected from individual plants; Aracons (Beta-Tech, Gent, Belgium) were used to ensure self-fertilization.

Plant growth.

To facilitate continual microscopic examination of roots, plants were effectively grown in "two dimensions" between glass plates (200 × 180 mm) (Fig. 1). Three plastic spacers (1.5 mm thick) were placed at the sides and bottom edge of a glass plate, and a fourth spacer was placed approximately 5 cm from the top edge. MS medium (0.5×) supplemented with 1% sucrose and 0.4% phytigel (Sigma Chemical Co., Dorset, U.K.) was poured on the plate between the spacers and allowed to set, and then the fourth spacer was removed. A 10- to 14-day-old *Arabidopsis* plant grown in a petri dish containing the medium described above was placed on the plate with only the roots on the medium and the aerial tissue in the top quarter of the plate. Where appropriate, the roots were then infected with nematodes. Another set of three spacers (1.5 mm thick) was added on top of the first set, followed by a second glass plate. The sides and bottom were sealed with waterproof tape and the top edge with micropore tape. The lower three quarters of the cassette, containing the medium and the roots, was covered with aluminum foil to prevent exposure to light, and the plates were held together with small fold-back clips (Fig. 1). On initial observation of a root system, no chloroplasts were apparent. However, on subsequent observations, chloroplasts had developed in syncytia, because the root systems had been exposed to light during examination. These cassettes were stored vertically in Sanyo MLR3500 growth cabinets with a 16-h day length and light intensity at 6 $\mu\text{mol sec}^{-1} \text{m}^{-2}$.

Nematode infection.

Eggs of *M. incognita* were recovered from stock colonies (ex Imperial College, London) and set to hatch as described by Atkinson et al. (1996). Cysts of *H. schachtii* (ex Maribo, Denmark) and second-stage juveniles (J2s) were surface-sterilized before use. Cysts were incubated in 0.1% malachite green for 30 min at room temperature and rinsed in running tap water for 1 h prior to soaking overnight at 4° C in an antibiotic cocktail containing streptomycin sulfate (8 mg ml⁻¹), penicillin G (6 mg ml⁻¹), polymyxin B (6.13 mg ml⁻¹), tetracycline (5 mg ml⁻¹), and amphotericin B (1 mg ml⁻¹). The cysts were washed and set to hatch in filter-sterilized tap water. An overnight hatch of J2s was counted and sterilized sequentially for 5-min periods with each of the following antibiotics: 0.1% streptomycin sulfate, 0.1% penicillin G, 0.1% amphotericin B, and 0.1% cetyltrimethylammonium bromide (Cetrimide, Sigma). J2s were collected by microcentrifugation for 10 s between treatments and were finally washed extensively in filter-sterilized tap water before use.

A 10- μl aliquot containing 100 J2s of *H. schachtii* or *M. incognita* was pipetted onto the roots of an individual *Arabidopsis* plant.

Image capture and analysis.

Bright-field images were viewed with a Leica microscope (model DMRB), and fluorescent images were viewed with an Olympus microscope (model BH2) at an excitation wavelength of 395 to 415 nm (violet light) and a cut-off emission filter at 495 nm. Images for analysis were captured with a color camera (Kappa CF15 MCC) mounted on the Olympus microscope. The camera output was connected to a frame grabber, and analysis was controlled through a software package (Leica Quantimet 500+C). The captured measurements were transferred to a cascaded window of a spreadsheet (Microsoft Excel 5) by the use of a macro routine. Data sets were saved as worksheets and subsequently transferred to a statistical package (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL) resident on the hard drive of the computer. This was used to carry out regression and one-way ANOVA (Snedecor and Cochran 1989).

Detection of GFP by Western blot analysis.

Adult females of *H. schachtii* and *M. incognita* were removed from either C24 wild-type or transgenic *Arabidopsis* plants expressing GFP and placed in water. Samples of *H. schachtii* comprising 40 individuals were collected from each C24 or GFP-expressing *Arabidopsis*. Two similar-sized samples of *M. incognita* were collected. The nematodes were homogenized in a microcentrifuge tube using a plastic pestle. The soluble protein fraction was collected by centrifugation ($13,000 \times g$ for 10 min) in 20 μ l of 0.5 $\times$ phosphate-buffered saline and subjected to Western blot analysis. An anti-GFP primary antibody (Clontech, U.K.) and an anti-rabbit secondary antibody conjugated with horseradish peroxidase were used, and detection of positive samples was carried out by HRPL (Horseradish Peroxidase Luminescent Visualization System) according to the manufacturer's instructions (National Diagnostics, Atlanta, GA).

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