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Molecular Markers and Cell Cycle Inhibitors Show the Importance of Cell Cycle Progression in Nematode-Induced Galls and Syncytia

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Root knot and cyst nematodes induce large multinucleated cells, designated giant cells and syncytia, respectively, in plant roots. We have used molecular markers to study cell cycle progression in these specialized feeding cells. In situ hybridization with two cyclin-dependent kinases and two cyclins showed that these genes were induced very early in galls and syncytia and that the feeding cells progressed through the G_2 phase. By using cell cycle blockers, DNA synthesis and progression through the G_2 phase, or mitosis, were shown to be essential for gall and syncytium establishment. When mitosis was blocked, further gall development was arrested. This result demonstrates that cycles of endoreduplication or other methods of DNA amplification are insufficient to drive giant cell expansion. On the other hand, syncytium development was much less affected by a mitotic block; however, syncytium expansion was inhibited.

INTRODUCTION

Sedentary plant parasitic nematodes are pathogens that infect a wide range of economically important plant crops, causing severe losses to agriculture that can amount to more than $100 billion per year worldwide (Opperman and Bird, 1998). Because Arabidopsis is a suitable host for several plant parasitic nematodes (Sijmons et al., 1991), it can be used as a model host. The simplicity of its anatomy (Dolan et al., 1993) combined with the translucent character of the thin roots allowed us to conduct whole-mount observations and to perform a detailed study of cell cycle progression in the highly specialized feeding cells induced by sedentary nematodes.

The feeding sites caused by root knot nematodes (*Meloidogyne incognita*) and by cyst nematodes (*Heterodera schachtii*) were compared for their progression through the cell cycle. In these compatible plant–nematode interactions, second-stage infective juveniles (J_2) penetrate the roots and migrate toward the vascular cylinder. Probably as a result of salivary secretions, root cells close to the xylem are induced to become giant cells (root knot nematodes) or syncytia (cyst nematodes) that are used as a food source until completion of the nematode's life cycle (4 to 6 weeks later).

Although both feeding systems share common structural and functional features, their ontogeny differs significantly. Giant cells are generated through sequential mitoses without cytokinesis (Huang and Maggenti, 1969) and cycles of endoreduplication (Jones and Payne, 1978; Huang, 1985; Wiggers et al., 1990). Nuclear and cellular hypertrophy is apparent together with secondary cell wall depositions that form ingrowths along the xylem to facilitate solute transport. A dense cytoplasm with an increased number of organelles and proteins and numerous small vacuoles have been observed. Root cells that surround the giant cells divide, giving rise to a root knot or gall.

Cyst nematodes initiate a syncytium through elongation of the initial feeding cell, accompanied by some divisions of adjacent cells (Golinowski et al., 1996, 1997). The multinucleated state is most probably attained by cell wall dissolution of neighboring cells (Endo, 1987) rather than by mitotic activity. Increases in cytoplasmic density and nuclear volume and cell wall ingrowths along the xylem vessels have been observed for both syncytia and giant cells.

To understand better the events that take place during the

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early steps of feeding site development, we used molecular markers to monitor cell cycle progression (for a diagram, see Gheysen et al., 1997). The S (DNA synthesis) phase of the cell cycle was analyzed by measuring tritiated thymidine incorporation into DNA. To monitor S, G_2, and M phases, we examined the expression patterns of two genes encoding cyclin-dependent kinases (CDKs) (*cdc2aAt* and *cdc2bAt*) and two genes encoding mitotic cyclins (Arath;*CycB1;1* and Arath;*CycA2;1*) of Arabidopsis.

Progression through the eukaryotic cell cycle is mediated by CDKs (Morgan, 1995). The catalytic activity of the CDKs is to some extent regulated by their association with regulatory proteins, named cyclins. The *cdc2aAt* and *cdc2bAt* genes encode two structurally and functionally distinct CDKs in Arabidopsis (Ferreira et al., 1991; Imajuku et al., 1992; Segers et al., 1996, 1997). The *cdc2aAt* gene is transcribed throughout the cell cycle at a constant level, whereas *cdc2bAt* is preferentially expressed from the S phase to the G_2 phase. The expression of both CDKs is associated with actively dividing cells and cells that are competent to divide (Martinez et al., 1992; Hemerly et al., 1993; Segers et al., 1996). The steady state mRNA level of *CycA2;1* rises during S phase and reaches a maximum at the end of the G_2, whereas the level of the *CycB1;1* transcript rises during the G_2 phase, reaching a maximum at the G_2-to-M transition (Shaul et al., 1996). *CycA2;1* expression is correlated with active cell division and competence to divide (S. Burssens, unpublished results). In contrast, *CycB1;1* is expressed mainly in actively dividing cells (Ferreira et al., 1994). Here, we used *CycB1;1* expression as a marker for the G_2 and M phases, whereas *CycA2;1* expression covers additional stages of the cell cycle, including the S phase.

We have already reported the promoter activity of two of the above-mentioned cell cycle genes from Arabidopsis, *cdc2aAt* and *CycB1;1*. In these analyses, we used the β-glucuronidase (*gus*) reporter system to study root knot and cyst nematode feeding sites (Niebel et al., 1996). Here, we extend our previous study on promoter activity by analyzing *cdc2bAt–gus* and *CycA2;1–gus* gene fusions in transgenic roots infected with root knot or cyst nematodes. To confirm our GUS analyses, we used in situ hybridization experiments to monitor steady state transcript levels of the *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1* genes in galls and syncytia.

To determine whether DNA synthesis and mitosis are essential for a proper ontogeny of galls and syncytia, we blocked cell cycle progression specifically with the cell cycle inhibitors hydroxyurea (HU) and oryzalin. HU inhibits the activity of the ribonucleotide diphosphate reductase enzyme, thus depriving the cells of newly synthesized deoxynucleotide triphosphates and preventing DNA replication. The result is a block at the G_1-to-S transition (Young and Hodas, 1964). Oryzalin, a dinitroaniline herbicide, has strong binding affinity for plant tubulins (Morejohn et al., 1987) and inhibits microtubule polymerization in plant cells, resulting in cell blockage during the G_2 phase of mitosis.

By combining the results from DNA synthesis in nuclei of feeding cells with the expression analysis of cell cycle markers and the use of cell cycle inhibitors, we developed a model for gall and syncytium development involving cell cycle activity, which we present here.

RESULTS

DNA Synthesis at Nematode Feeding Sites of Arabidopsis Roots

Roots infected by root knot or cyst nematodes were taken at different time points after inoculation and incubated for 12 hr with ^{3}H-thymidine to detect DNA synthesis. Figure 1 shows DNA synthesis in infected and control Arabidopsis roots. In uninfected roots incubated with ^{3}H-thymidine, little or no label was observed in the nuclei of the vascular parenchyma, at the position where the galls or syncytia normally develop (Figure 1A). The intensity of DNA synthesis at feeding sites induced by root knot or cyst nematodes at different time points (1 to 15 days after inoculation) is summarized in Table 1. One to 2 days after inoculation with root knot nematodes, the expanding giant cells contained several enlarged nuclei that were often mitotic (Bird, 1973; Starr, 1993). Very intense labeling was seen on the nuclei of these giant cells (Figure 1B) as well as on nuclei from adjacent cells. In contrast, 9 days after inoculation, little or no labeling was observed in nuclei of gall cells (Figure 1C). In syncytia 1 to 2 days after inoculation, labeling was observed not only on syncytial nuclei but also on all nuclei of surrounding proliferating cells from the vascular cylinder (Figure 1D). At that stage, syncytium expansion was radial and mainly longitudinal, the cytoplasm became denser, and the nuclei enlarged. Nuclei of giant cells or syncytia 5 days after inoculation (Figure 1E) were more strongly labeled when compared with neighboring cells. Nine days after inoculation, nuclei from syncytia and mainly of neighboring cells showed that DNA synthesis continued to occur (Figure 1F).

When a short pulse (3 hr) of ^{3}H-thymidine was applied to infected roots, all nuclei of giant cells were strongly labeled 1 to 5 days after inoculation, whereas on nuclei of surrounding cells, only few or no silver grains could be observed (data not shown). Similarly, syncytia 5 days after inoculation were more strongly labeled when compared with neighboring cells (data not shown). Nuclei of giant cells at similar stages (5 days after inoculation) stained with 4′,6-diamidino-2-phenylindole (DAPI) showed $>$10 chromocenters that were larger than those of nuclei in the surrounding cells. In nuclei of syncytia, the number and the size of chromocenters were variable (data not shown). Chromocenters were distinct and easy to count in interphase nuclei.

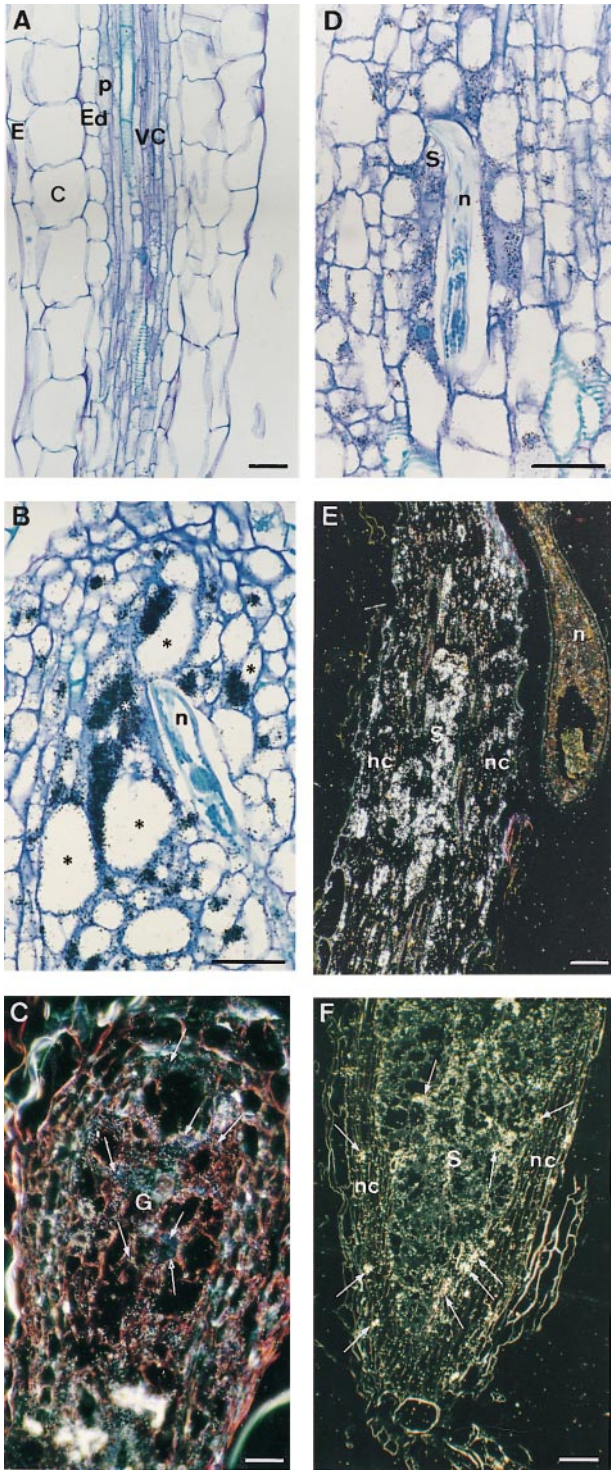

Figure 1. DNA Synthesis in Arabidopsis Roots Infected with Root Knot or Cyst Nematodes.

(A) to **(F)** show microscopy of median longitudinal toluidine blue–stained sections of Arabidopsis roots uninfected and infected with root knot or cyst nematodes and labeled for 12 hr with ^{3}H-thymidine.

With bright-field optics, **(A)**, **(B)**, and **(D)** show silver grains as black dots representing the labeled nuclei. In **(C)**, **(E)**, and **(F)**, dark-field optics were applied for sections at lower magnification to better visualize silver grains (white dots).

(A) Section through an uninfected root.

(B) Section through a gall 1 day after inoculation.

(C) Section through a gall 9 days after inoculation. Arrows indicate the nuclei.

(D) Section through a syncytium 1 day after inoculation.

(E) Section through a syncytium 5 days after inoculation.

(F) Section through a syncytium 9 days after inoculation. Arrows indicate labeled nuclei.

Asterisk, giant cell; C, cortex; E, epidermis; Ed, endodermis; G, gall; n, nematode; nc, neighboring cell; p, pericycle; S, syncytium; VC, vascular cylinder. Bars = 25 μm.

Table 1. DNA Synthesis in Galls and Syncytia Monitored at Different Time Points after Inoculation

	Time[a]			
Cells	1 DAI[b]	5 DAI	9 DAI	15 DAI
Giant cells	++++	+++	±	—
Galls	+++	+++	±	±
Syncytia	++	+++	+	±
Neighboring cells	++	++	++	+

[a] ^{3}H-thymidine incorporation was measured after 12 hr of incubation of galls and syncytia induced by root knot and cyst nematodes, respectively. Intensity (++++, very strong; +++, strong; ++, medium; +, weak; ±, very weak; −, below detection) of DNA synthesis (observed as silver grains) is the average of 20 sectioned galls or syncytia. For these data, the definition of galls excludes giant cells.
[b] DAI, days after inoculation.

Promoter Activities, Transcript Levels, and Effect of Cell Cycle Inhibitors on Transcription of Cell Cycle Genes at Nematode Feeding Sites

Promoter activity and transcript levels of *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1* genes in nematode-infected cells were analyzed by performing GUS assays and whole-mount in situ hybridization (WISH). Here, we report the expression pattern of *cdc2bAt* and *CycA2;1* in galls and syncytia by analyzing promoter–*gus* fusions at different time points after nematode infection. Details from *cdc2aAt–gus* and *CycB1; 1–gus* studies have been published previously (Niebel et al., 1996).

Figure 2 shows the promoter activity of *cdc2bAt* and the effect of inhibitors. In uninfected Arabidopsis plants, strong and slightly patchy expression of *cdc2bAt* (Figure 2F) and *CycA2;1* (S. Burssens, unpublished results) was observed in the root meristem, most probably because of cell cycle phase–specific expression. Promoter activity from the two genes was also observed in the vascular cylinder above the root meristems. When juveniles of root knot or cyst nema-

todes were migrating within the roots, a decrease in promoter activity of *cdc2bAt* (Figures 2A and 2G) and *CycA2;1* (data not shown) was detected. Induced levels of *gus* expression in feeding sites caused by root knot or cyst nematodes at different time points are summarized in Table 2. These results are illustrated in Figures 2A to 2C and 2G to 2I for *cdc2bAt*. The expression pattern of *CycA2;1* was similar to that of *cdc2bAt*; therefore, no figures are shown. Both feeding cell types show promoter induction of the two genes in the early stages of the infection process. This activation also occurred in the neighboring cells, being especially pronounced in the case of syncytia. In addition, lateral roots close to the infection sites (of both types of nematodes) often showed a zone in the apical meristem where *cdc2aAt* and *CycB1;1* transcripts were less abundant (Figure 2J). WISH analysis confirmed this expression pattern.

Cell cycle blockers (HU and oryzalin) were applied to different transgenic plants containing promoter–*gus* gene fusions

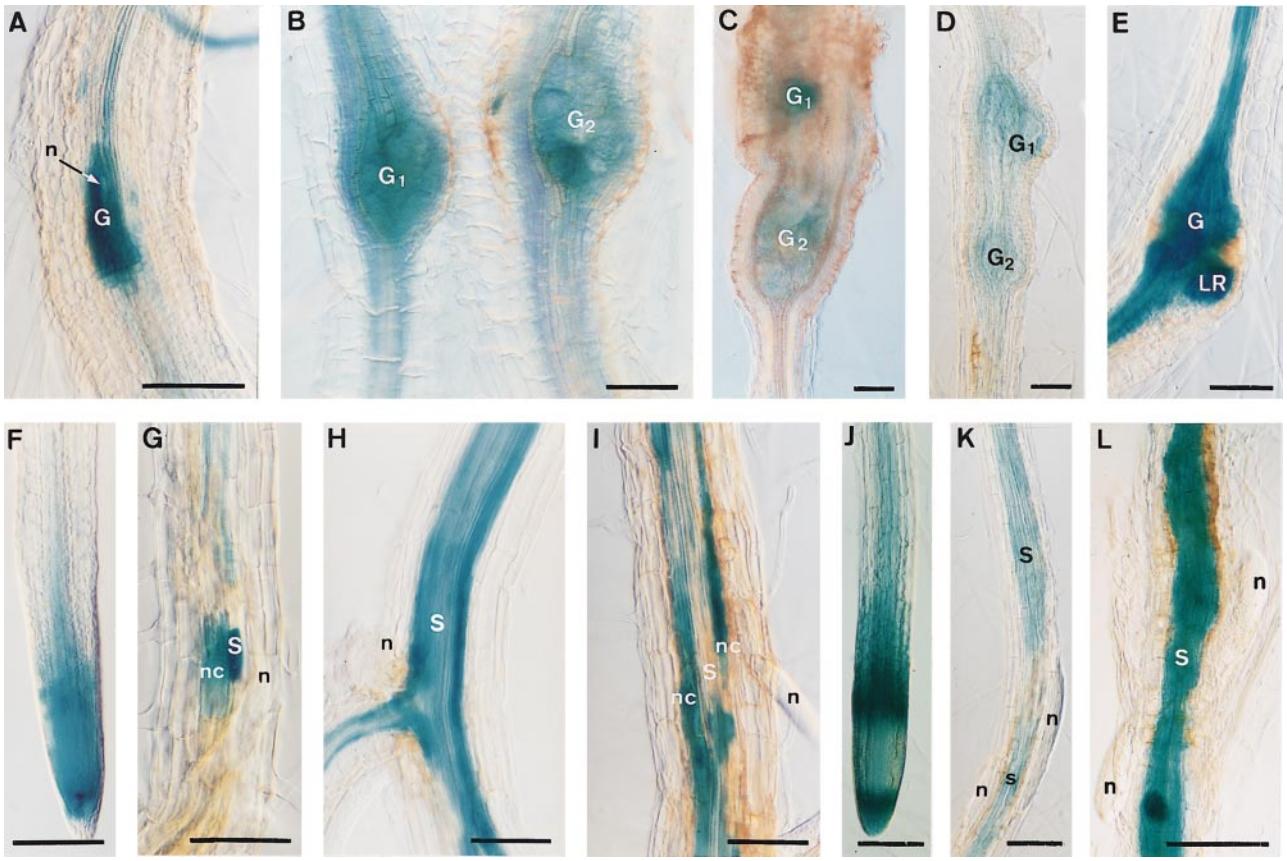

Figure 2. Promoter Activity of *cdc2bAt–gus* and the Effect of Cell Cycle Inhibitors on the Expression of *cdc2bAt–gus* in Arabidopsis Plants Infected with Root Knot or Cyst Nematodes.

(A) *cdc2bAt–gus* expression in a gall 1 day after inoculation.
(B) *cdc2bAt–gus* expression in galls 5 days after inoculation. Gall 1 is younger than is gall 2.
(C) *cdc2bAt–gus* expression in galls 9 days after inoculation. Gall 1 is younger than is gall 2.
(D) *cdc2bAt–gus* expression in galls 5 days after inoculation, the last 48 hr of which were with HU treatment.
(E) *cdc2bAt–gus* expression in a gall 5 days after inoculation, the last 48 hr of which were with oryzalin treatment.
(F) *cdc2bAt–gus* expression in an uninfected root.
(G) *cdc2bAt–gus* expression in a syncytium 1 day after inoculation.
(H) *cdc2bAt–gus* expression in a syncytium 5 days after inoculation.
(I) *cdc2bAt–gus* expression in a syncytium 9 days after inoculation.
(J) *cdc2bAt–gus* expression in a lateral root neighboring a syncytium.
(K) *cdc2bAt–gus* expression in a syncytium 5 days after inoculation, the last 48 hr of which were with HU treatment.
(L) *cdc2bAt–gus* expression in a syncytium 5 days after inoculation, the last 48 hr of which were with oryzalin treatment.
G, gall; G_1, gall 1; G_2, gall 2; LR, lateral root; n, nematode; nc, neighboring cell; S, syncytium. Bars = 100 μm.

Table 2. Promoter Activity of *cdc2bAt* and *CycA2;1* in Galls Induced by Root Knot Nematodes and in Syncytia Induced by Cyst Nematodes Monitored at Different Time Points after Inoculation

		Time[a]			
Gene	Cells	1 DAI[b]	5 DAI	9 DAI	15 DAI
cdc2bAt	Giant cells	++++	+++	+	—
	Gall	+++	++	±	—
	Syncytia	++++	+++	±	±
	Neighboring cells	++	++++	+++	+
CycA2;1	Giant cells	++++	++++	+	—
	Gall	+++	+++	±	—
	Syncytia	++++	+++	+	±
	Neighboring cells	+++	++++	++	±

[a] Intensity of GUS staining (++++, very strong; +++, strong; ++, medium; +, weak; ±, very weak; −, below detection) is the average of 30 galls or syncytia. Definition of galls for these data excludes giant cells.
[b] DAI, days after inoculation.

of *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1*. Promoter activities of the four cell cycle genes in infected seedlings untreated and treated with the two cell cycle blockers are summarized in Table 3. HU treatments of infected transgenic plants showed much less GUS staining in galls and syncytia (shown for *cdc2bAt* in Figures 2D and 2K, respectively) compared with untreated infection sites (Figures 2B and 2H). Oryzalin-treated galls or syncytia often showed higher GUS activity (shown for *cdc2bAt* in Figures 2E and 2L, respectively). HU and oryzalin treatments not only affected *gus* expression in the infected transgenic plants analyzed but also caused a delay in gall and syncytium development.

To confirm that the observed GUS patterns reflect the endogenous transcript levels of *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1*, we performed WISH experiments with infected and uninfected roots at different developmental stages. Transcripts of the four cell cycle genes were localized in feeding sites induced by root knot and cyst nematodes in different stages of development. In situ results are summarized in Table 4 and illustrated in Figures 3A to 3F. No hybridization signal was detected in WISH experiments with sense probes in roots infected with either root knot or cyst nematodes at all stages analyzed (Figures 3G and 3H for a gall and a syncytium hybridized with a *cdc2aAt* sense probe 1 day after inoculation, respectively).

Cytological Analysis of Nematode Feeding Site Development in Roots Treated with Cell Cycle Inhibitors

Infected roots were treated with the cell cycle inhibitors HU or oryzalin to investigate how DNA synthesis and mitosis affected the formation and development of a gall or a syncytium. To control whether these inhibitors interfered with the viability of nematodes, we incubated second-stage juveniles at various concentrations of HU (up to 100 mM) or oryzalin (up to 30 μM) for 3 days, and potassium permanganate staining, used as an indicator for dead nematodes (Jatala, 1975), was found to be negative. After these treatments, nematodes could still penetrate into the roots and complete their cycle. Because nematodes seemed not to be affected by several days of incubation at high concentrations of HU or oryzalin (100 mM and 30 μM, respectively), these high inhibitor concentrations were applied in 48-hr treatments. For longer incubations (up to 40 days), lower concentrations of the inhibitors were used (100 μM HU and 2 μM oryzalin) to avoid possible long-term effects on nematode development and reproduction.

Autoradiography of infection sites treated for 48 hr with HU (100 mM) followed by 12 hr of ^{3}H-thymidine incubation revealed that DNA synthesis was inhibited (data not shown). The effect of oryzalin on the seedlings could be seen by the swelling of root tips caused by the excessive radial expansion, mainly of meristematic cells (Baskin et al., 1994). Sections of oryzalin-treated infection sites showed larger but no mitotic nuclei. Furthermore, cell cycle gene expression analysis in the inhibitor-treated plantlets confirmed the effectiveness of both HU and oryzalin treatments (see above).

Table 3. Effect of Cell Cycle Inhibitors on Promoter Activity of *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1* in Galls Induced by Root Knot Nematodes and in Syncytia Induced by Cyst Nematodes at an Early Stage of Infection[a]

		Treatment[b]		
Gene	Cells	Control	Hydroxyurea	Oryzalin
cdc2aAt	Giant cells	+++	±	++++
	Galls	+++	±	++++
	Syncytia	+++	±	+++
	Neighboring cells	++++	±	+++
cdc2bAt	Giant cells	+++	±	++++
	Galls	++	±	++++
	Syncytia	+++	±	+++
	Neighboring cells	++++	±	+++
CycB1;1	Giant cells	+++	±	+++
	Galls	++	+	++++
	Syncytia	+++	±	++++
	Neighboring cells	++++	+	++++
CycA2;1	Giant cells	++++	±	++++
	Galls	+++	±	++++
	Syncytia	+++	±	+++
	Neighboring cells	++++	±	+++

[a] The infection stage was 5 days after inoculation.
[b] Definition of galls for these data excludes giant cells. GUS intensity (++++, very strong; +++, strong; ++, medium; +, weak; ±, very weak) is the average of 30 galls or syncytia.

Table 4. Transcript Levels of *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1* in Galls Induced by Root Knot Nematodes and in Syncytia Induced by Cyst Nematodes Monitored at Different Time Points after Inoculation

		Time[a]			
Gene	Cells	1 DAI[b]	5 DAI	9 DAI	15 DAI
cdc2aAt	Giant cells	++++	+++	++	±
	Galls	+++	+++	±	±
	Syncytia	++++	+++	++	+
	Neighboring cells	++	++++	++	±
cdc2bAt	Giant cells	++++	+++	+	±
	Galls	+++	++	±	±
	Syncytia	++++	+++	±	±
	Neighboring cells	+++	++++	+++	±
CycB1;1	Giant cells	++++	+++	±	—
	Galls	±	++	—	—
	Syncytia	++++	+++	±	—
	Neighboring cells	±	++++	+++	±
CycA2;1	Giant cells	++++	++++	+	±
	Galls	+++	+++	±	±
	Syncytia	++++	+++	±	—
	Neighboring cells	+++	++++	+++	±

[a] Control hybridization with sense probes was negative. WISH intensity (++++, very strong; +++, strong; ++, medium; +, weak; ±, very weak; −, below detection) was determined. Definition of galls for these data excludes giant cells.
[b] DAI, days after inoculation.

Forty-eight hours after the transfer of Arabidopsis seedlings to medium with high concentrations of HU or oryzalin, attempts were made to infect these plantlets, but penetration of root knot or cyst nematodes did not occur. Thickening of epidermal cell walls caused by the applied chemicals was apparent and might have prevented penetration of the larvae by producing a mechanical barrier (Griffin and Anderson, 1979). Decreasing concentrations were tested until juveniles penetrated into the roots and migrated toward the vascular cylinder. However, in the presence of inhibitors (100 μM HU or 2 μM oryzalin), galls and syncytia failed to initiate, and no mature females were observed after long incubations (40 days).

Therefore, treatments with HU or oryzalin were applied to the Arabidopsis seedlings after inoculation with nematodes. One day after inoculation with root knot or cyst nematodes, HU or oryzalin treatment for 48 hr prevented induction of new feeding sites and inhibited the development of just-initiated giant cells or syncytia. This effect is clearly seen in oryzalin-treated giant cells (Figure 4A) when compared with untreated (Figure 4B) infection sites. Due to this treatment, the induced giant cells were small and contained fewer nuclei than expected at that stage, and the cytoplasm was denser than in surrounding cells, demonstrating that an initial stimulus had occurred at that stage (1 day after inoculation and treated for 48 hr), but further development was blocked (Figure 4A). In contrast, untreated infection sites at the same time point contained larger giant cells with several nuclei and very dense cytoplasm (Figure 4B). Lengthy treatments of seedlings inoculated with root knot or cyst nematodes with HU or oryzalin (applied 1 day after inoculation) resulted in retarded gall or syncytium development and nematodes that started to feed but did not mature (Figure 4C for root knot nematode and HU treatment). Nematodes did not stain with potassium permanganate, indicating that they were still alive, but they could not complete their life cycle.

HU or oryzalin treatments of 48 hr on root knot nematode–infected seedlings 3 days after inoculation resulted in giant cells with fewer nuclei compared with untreated seedlings 5 days after inoculation (cf. Figures 4D and 4E for HU treatment). Furthermore, the giant cell cytoplasm was less dense, vacuoles were larger, and galls were smaller than in infected roots of the control. Nuclei from oryzalin-treated giant cells were larger than those of HU-treated cells (data not shown). To check whether juveniles of the root knot nematodes could mature into adult females with these treatments, we kept infected seedlings at the same stage (3 days after inoculation) for 37 days in medium containing a low HU or oryzalin concentration. At 40 days after inoculation, nematodes in HU- or oryzalin-treated infection sites were alive (no staining with potassium permanganate), but they were not able to mature completely (Figure 4F).

At early stages of infection (1 day after inoculation), we sometimes observed a toluidine blue–stained structure resembling a cell wall between two nuclei in HU-treated or untreated giant cells (Figure 4G). Calcofluor white staining for cellulose was negative. Moreover, at early developmental stages, nuclei of giant cells often formed a contiguous semicircle-like structure, as seen in the binucleate cell shown in Figure 4G.

After HU or oryzalin treatment of seedlings infected with cyst nematodes (3 days after inoculation and a 48-hr inhibitor treatment), syncytia contained larger vacuoles and less dense cytoplasm than in control-infected roots. Furthermore, the cells neighboring the syncytia were elongated with large vacuoles (Figures 4H and 4I for HU and oryzalin, respectively). Interestingly, when treated with inhibitors, syncytia were able to expand, incorporating neighboring cells (Figure 4H for HU). In untreated roots at the same time of infection, cells neighboring the syncytium contained enlarged nuclei and a dense cytoplasm resembling meristematic cells (Figure 4J). When plantlets infected with cyst nematodes (3 days after inoculation) were kept for a 37-day period at a low HU concentration, syncytia were smaller than in untreated roots; consequently, most nematodes did not mature (Figure 4K), and only a small fraction of the cyst nematodes completed their life cycle. In infected seedlings that were kept for a long period at low concentrations of oryzalin, syncytia were less expanded; however, several females ma-

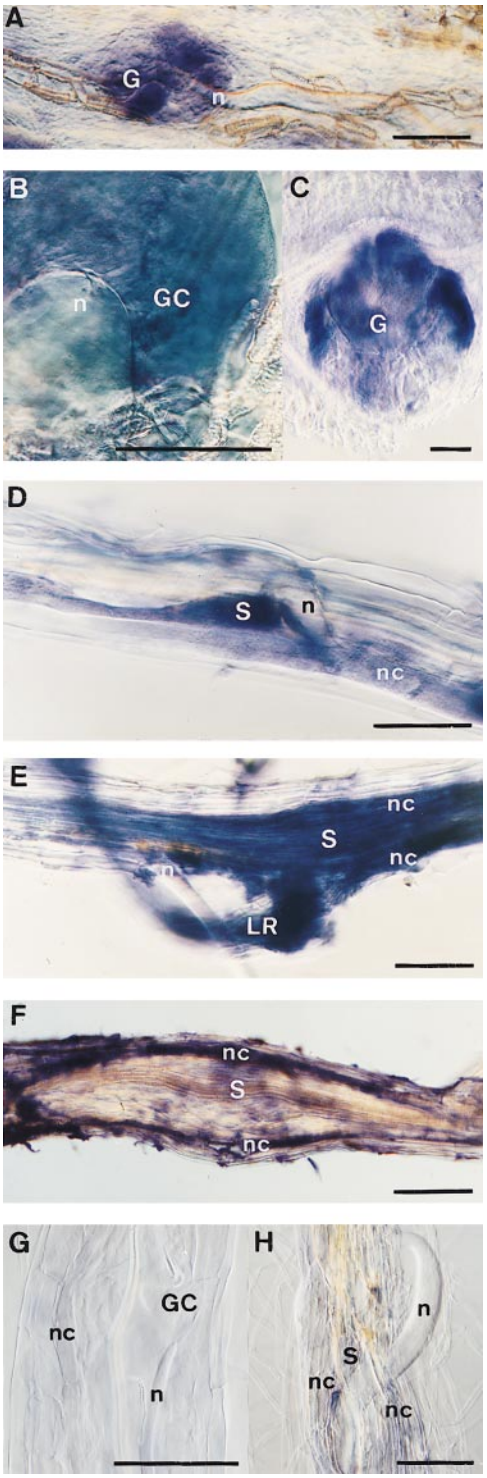

Figure 3. WISH with Galls and Syncytia.

The purple stain represents the hybridization signal.
(A) Gall 1 day after inoculation hybridized with an antisense *CycA2;1* probe.
(B) Section cut with a Vibroslicer of a gall 5 days after inoculation hybridized with an antisense *cdc2aAt* probe. A nematode is visible with the stylet inserted into a giant cell.
(C) Section cut with a Vibroslicer of a gall 5 days after inoculation hybridized with an antisense *CycB1;1* probe.
(D) Syncytium 1 day after inoculation hybridized with an antisense *cdc2bAt* probe.
(E) Syncytium 5 days after inoculation hybridized with an antisense *CycB1;1* probe.
(F) Syncytium 9 days after inoculation hybridized with an antisense *CycA2;1* probe.
(G) Gall 1 day after inoculation hybridized with a sense *cdc2aAt* probe.
(H) Syncytium 1 day after inoculation hybridized with a sense *cdc2aAt* probe.
G, gall; GC, giant cell; LR, lateral root; n, nematode; nc, neighboring cell; S, syncytium. Bars = 100 μm.

tured and could form cysts containing eggs (Figure 4L) that hatched into viable stage 2 juveniles.

When roots infected with root knot or cyst nematodes were transferred later than 9 days after inoculation to medium containing the inhibitors, both types of nematodes could efficiently complete their cycle. Table 5 summarizes the data on nematode reproduction after long incubations with HU or oryzalin.

Mitotic figures have been repeatedly observed in giant cells (Owens and Novotny Specht, 1964; Dropkin, 1965; Huang and Maggenti, 1969; Bird, 1973; Rohde and McClure, 1975; Starr, 1993) but not in syncytia. An increase in mitotic figures in onion roots appeared after release from HU treatment (Brulfert and Deysson, 1971; Clain and Brulfert, 1980). Therefore, we applied HU to determine whether mitosis occurred in syncytia after treatment with this inhibitor. After staining with DAPI, we observed mitotic figures in squashed gall cells. Condensed chromosomes were not visible in syncytia; however, they were visible in surrounding cells.

DISCUSSION

This study analyzed cell cycle progression in nematode-induced galls and syncytia in Arabidopsis. Cell cycle phases in cells involved in the formation of feeding sites were defined based on results of DNA synthesis, WISH experiments, and promoter activity of four cell cycle genes coupled with the use of cell cycle blockers. In our study, we combine cell cycle markers and inhibitors to evaluate the relevance of DNA synthesis and mitosis on feeding site formation and development.

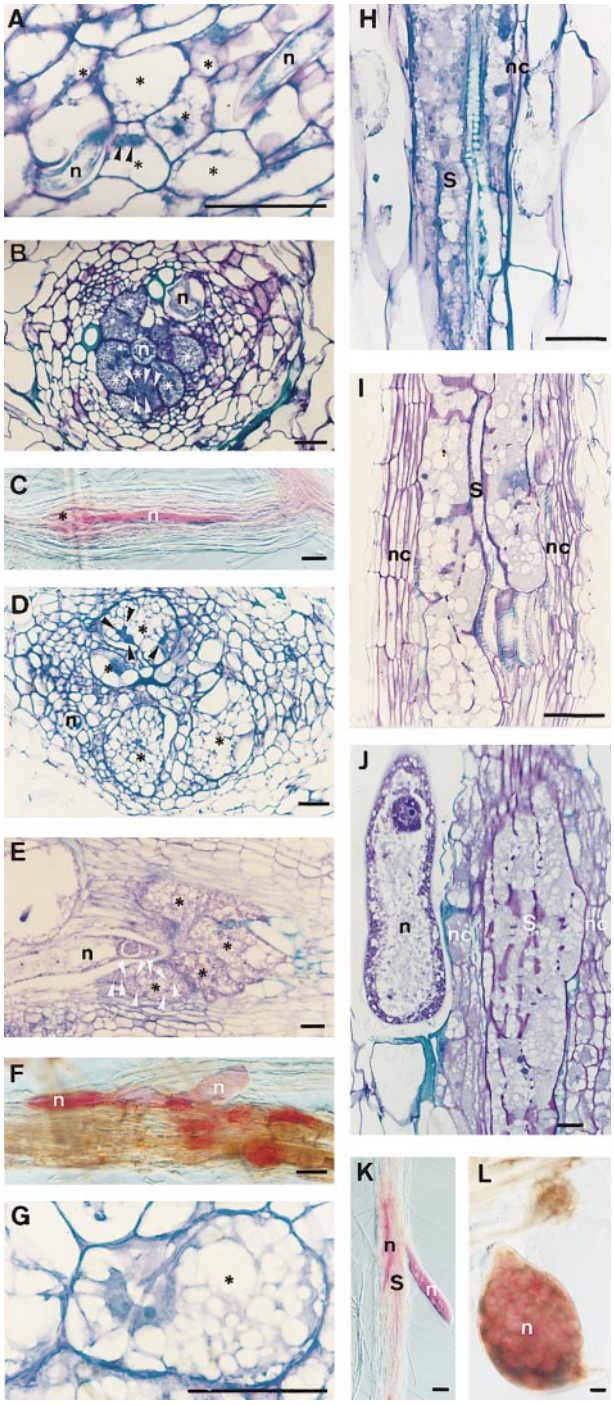

Figure 4. Microscopic Analysis of Arabidopsis Roots Infected with Root Knot or Cyst Nematodes Untreated and Treated with Cell Cycle Inhibitors.

(A) to **(L)** show bright-field microscopy of toluidine blue–stained sections of Arabidopsis roots infected with root knot or cyst nematodes, except for **(C)**, **(F)**, **(K)**, and **(L)**, which are acid fuchsin–stained whole infection sites.

(A) Section through a gall 3 days after inoculation, the last 48 hr of which were with oryzalin treatment.
(B) Section through a gall 3 days after inoculation.
(C) Whole root, stained with acid fuchsin, with a reddish juvenile of the root knot nematode 40 days after inoculation, after 39 days of treatment with HU.
(D) Section through a gall 5 days after inoculation, the last 48 hr of which were with HU treatment.
(E) Section through a gall 5 days after inoculation.
(F) Whole root stained with acid fuchsin, showing reddish female root knot nematodes 40 days after inoculation, after 37 days of oryzalin treatment.
(G) Section through a giant cell 1 day after inoculation, showing a cell wall–like structure between the nuclei.
(H) Section through a syncytium 5 days after inoculation, the last 48 hr of which were with HU treatment.
(I) Section through a syncytium 5 days after inoculation, the last 48 hr of which were with oryzalin treatment.
(J) Section through a syncytium 5 days after inoculation.
(K) Whole root stained with acid fuchsin, showing reddish juveniles of the cyst nematode 40 days after inoculation, after 37 days of treatment with HU.
(L) Whole root stained with acid fuchsin, showing a reddish mature female cyst nematode 40 days after inoculation, after 37 days of treatment with oryzalin.
Asterisk, giant cell; n, nematode; nc, neighboring cell; S, syncytium. Arrowheads in **(A)**, **(B)**, **(D)**, and **(E)** indicate nuclei. Bars = 25 μm for **(A)**, **(B)**, **(D)**, **(E)**, **(G)**, and **(H)** to **(J)**; bars = 50 μm for **(C)**, **(F)**, **(K)**, and **(L)**.

DNA Synthesis in Galls and Syncytia

Analysis of DNA synthesis provided information on which cells in a feeding site went through the S phase of the cell cycle. At early stages of gall formation, ^{3}H-thymidine incorporation into nuclei of giant cells was higher than that in surrounding cells. Such a high level of DNA synthesis also has been reported in giant cells of tomato (Rubinstein and Owens, 1964) and cotton (Rohde and McClure, 1975) infected with root knot nematodes. These and our data suggest that the mitotic cycle might be shorter in giant cells than in uninfected root cells and/or that alternating endoreduplication and mitotic cycles might occur. Our data as well as previous data demonstrate that giant cells are not only multinucleated but also have enlarged nuclei when compared with normal plant cells.

At later stages of gall development, DNA synthesis or mitosis was not often detected, implying that at that time, giant cells reached the required DNA level to maintain a high metabolic activity until the nematode's life cycle was completed. Consistently, mitosis had previously been observed rarely in mature giant cells of pea, tomato, lettuce, and broad bean (Starr, 1993).

Table 5. Effect of Cell Cycle Inhibitors Applied at Different Days after Inoculation on the Reproduction (%) of Root Knot and Cyst Nematodes

	Treatment[a]					
	HU (%)			Oryzalin (%)		
Nematodes	1 DAI[b]	3 DAI	9 DAI	1 DAI	3 DAI	9 DAI
Root knot 40 DAI (egg masses)	0	0	76	0	0	85
Cyst 40 DAI (cysts)	0	5	60	0	35	63

[a] A total of 20 plants were infected each with 50 nematodes. One, 3, and 9 DAI are the time points at which the infected plantlets were transferred to medium with low concentrations of the inhibitors, followed by incubation until 40 DAI. Eggs within cysts and egg masses hatched into infective stage 2 juveniles. Percentages were estimated by counting how many nematodes completed their cycle compared with the number of juveniles that penetrated the Arabidopsis roots.
[b] DAI, days after inoculation.

During early syncytium development (5 days after inoculation), more DNA synthesis was observed in nuclei of syncytia when compared with the surrounding cells. DNA synthesis has been reported in syncytia of soybean infected with the cyst nematode *H. glycines* (Endo, 1971a, 1971b); however, in contrast to our results, lower incorporation was observed in feeding cells than in adjacent cells. Because until now mitosis has never been convincingly demonstrated in cyst nematode feeding cells, active DNA synthesis suggests that endoreduplication cycles occur in syncytia, resulting in nuclear hypertrophy. In contrast to galls, syncytia and mainly their neighboring cells still showed ^{3}H-labeled nuclei at later stages (9 days after inoculation) of development, suggesting that syncytium development continues for a longer period than does that of galls. Our cytological analysis also showed that nuclei of neighboring cells were large, indicating that endoreduplication cycles might occur in these cells before syncytium incorporation. Confirming our results, Golinowski et al. (1996) have reported that cell division and nuclear hypertrophy precede incorporation of neighboring cells into the syncytium until late stages of development.

Results on short pulses of ^{3}H-thymidine on infected roots showed that at early stages of infection, the majority of cells are synthesizing DNA, and the other phases of the cell cycle are shorter or absent in nuclei of feeding cells compared with surrounding cells.

Arabidopsis has a chromocentric nuclear organization that is often observed in plants with small chromosomes and low DNA content (Lafontaine, 1974; Nagl and Fusenig, 1979). Chromocenter number usually reflects the number of chromosomes. All interphase nuclei of giant cells and several nuclei of syncytia contained more than the expected number of 10 chromocenters. Chromocenters were also larger than those in nuclei of neighboring cells, probably because of overlapping, fusion, or polyteny (Kabir and Singh, 1989). A detailed confocal analysis of these nuclei will reveal whether somatic association of chromosomes and/or polyploidy occurs in nuclei of feeding cells.

Expression Analysis of the *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1* Genes

Promoter–*gus* fusion analysis and WISH with four cell cycle genes of Arabidopsis allowed us to follow other phases of the cell cycle. The obtained GUS patterns of the four cell cycle genes were similar to the transcript levels detected in the WISH experiments.

During migration, juveniles often caused a decrease in expression of the promoter–*gus* fusions in the root, indicating the downregulation of cell cycle activity, which is probably the result of stress on cells surrounding the nematode. Stress may lead to a cell cycle arrest and reduction of the expression of cell cycle genes (Logemann et al., 1995). In contrast, within the first hours of feeding by root knot or cyst nematodes, the four genes were strongly expressed in feeding cells before any cell divisions were visible. Thus, the initial stimulus from the nematode rapidly induced the cells to reenter or progress through the cell cycle. GUS staining and detection of mRNA of *cdc2aAt*, *cdc2bAt*, and *CycA2;1* in nondividing cells surrounding the initiating giant cells and syncytia also demonstrate the competence for division of these cells that is needed for gall and syncytium development. During initial gall and syncytium development, strong expression of the *CycB1;1* gene suggests the prevalence of the G_2 phase. In addition, DNA synthesis data and expression of *cdc2bAt* and *CycA2;1*, strong expression of a mitotic cyclin (*CycB1;1*), and the absence of mitotic figures in syncytia indicate that the cell cycle proceeds at least until late G_2.

During gall development, the different stain intensities between different giant cells for the *cdc2bAt* and the cyclin genes observed in WISH experiments as well as in GUS assays confirm that distinct giant cells within the same gall are at different stages of the cell cycle, as has been previously suggested (Bird, 1961; Rubinstein and Owens, 1964). The mRNA level of *cdc2bAt* and the two cyclin genes in galls and syncytia was more transient than for *cdc2aAt*, probably because of the broader expression profile of the latter.

In contrast to the normal expression pattern (Hemerly et al., 1993; Ferreira et al., 1994), a reduction in promoter activity (Vercauteren et al., 1995) and mRNA levels of *cdc2aAt* and *CycB1;1* was observed in a particular region of the lateral root meristem from lateral roots that neighbor galls and syncytia. A similar inhibition zone was reported when plantlets containing the *cdc2aAt–gus* or *CycB1;1–gus* constructs were treated with indole-3-acetic acid or α-naphthaleneacetic acid (Hemerly et al., 1993; Ferreira et al., 1994). In

addition, infected roots showed consecutive lateral root formation, and *CycB1;1–gus* activity was seen in pericycle cells mainly close to syncytia (data not shown). Analogous results were previously observed during auxin treatments on uninfected Arabidopsis roots (Ferreira et al., 1994). An increase in auxin levels has been reported to induce cells in G_1 to progress through the cell cycle and to endoreduplicate (Lur and Setter, 1993a, 1993b). By causing changes in auxin levels in infected roots, nematodes might induce root cells at the G_1 phase to proceed into the cell cycle and/or to initiate endoreduplication. Altered levels of phytohormones have been observed in root knot nematode–infected roots (Bird, 1962; Yu and Viglierchio, 1964; Viglierchio and Yu, 1968).

At later stages of gall development (~9 days after inoculation), weak or no expression of *cdc2bAt* and the two cyclin genes, weak *cdc2aAt* expression, no H^3-thymidine incorporation, and the absence of mitotic figures suggest that no nuclear division or DNA synthesis is required when giant cells are sufficiently developed. Comparable late stages of syncytium development showed that cells neighboring the feeding cell still expressed the *cdc2aAt*, *cdc2bAt*, *CycB1;1*, and *CycA2;1* genes. Therefore, we presume that these cells are still cycling, allowing syncytia to reach a critical size necessary for the cyst nematodes to complete their life cycle properly.

Cell Cycle Inhibitors Used to Evaluate the Influence of DNA Synthesis and Mitosis on Feeding Site Formation

The development of galls and syncytia clearly involves cell cycle activity. Therefore, we questioned whether cell cycle inhibitors, such as HU and oryzalin, which are known to block DNA synthesis and mitosis, respectively, would influence feeding site development. HU (Glazer and Orion, 1984, 1985; Stender et al., 1986), oryzalin (Orum et al., 1979), and several other inhibitors, such as maleic hydrazide (Davide and Triantaphyllou, 1968), colchicine (Gershon, 1970), morphactin (Orion and Minz, 1971), BAS 083 (Romney et al., 1974), chlorpropham, *S*-ethyl dipropylthiocarbamate (Griffin and Anderson, 1979), and dimethyl tetrachloroterephthalate (Romney et al., 1974), have been applied to infected plants to control nematode development in crop species. Low concentrations of cell cycle blockers reduced or blocked feeding site development; however, in these reports, no detailed cytological analysis or evaluation of treatments on the ontogeny of the feeding sites was performed.

Ours and previous results (Orum et al., 1979; Glazer and Orion, 1984) have shown that short incubations of stage 2 juveniles with high concentrations of HU or oryzalin did not affect nematode infectivity. However, data resulting from prolonged treatments, even at low concentrations of HU or oryzalin, need to be interpreted with care. Juvenile nematodes do not seem to require DNA synthesis and cell division to grow and mature, with their reproductive systems being an exception (Pai, 1928; Hyman, 1951; Gershon, 1970). Furthermore, higher concentrations of DNA synthesis inhibitors than those used in our experiments are required to impair nematode reproduction and longevity (Kisiel et al., 1972; Ford and Shackney, 1977; Ramseier et al., 1977). When plants were transferred at later stages after inoculation to low concentrations of HU or oryzalin, nematodes were able to mature and complete their life cycles. It was also reported that the herbicide oryzalin does not bind to animal tubulins, suggesting that only plant cells are directly affected by the oryzalin treatment (Morejohn and Fosket, 1984; Morejohn et al., 1987; Hugdahl and Morejohn, 1993). Furthermore, nematodes have a strong and impermeable cuticle (Cox et al., 1981; Reddigari et al., 1986) that acts most probably as a barrier against penetration of applied chemicals. Therefore, we believe that the cell cycle inhibitors have no direct effect on nematode development and maturation.

The fact that oryzalin disturbs cytoskeleton organization in plant cells was also taken into consideration. Our results showed that under oryzalin treatment, DNA synthesis occurred in galls and syncytia and that neighboring cells were able to be fused into the syncytia, allowing proper feeding site development.

Nematodes penetrate but are unable to induce feeding sites on plantlets grown on low concentrations of either inhibitor, indicating that cell cycle arrest in roots interferes with the ability of the plant to respond to the presence of the parasite. Therefore, according to our results, DNA synthesis and mitosis would be essential for the primary establishment of both galls and syncytia. Indeed, blocking DNA synthesis before syncytium initiation stopped later events, such as cell fusion. Although nuclear divisions seem not to take place in syncytia, an initial mitotic stimulation cannot be excluded during feeding site initiation.

HU treatment of seedlings early after infection with root knot and cyst nematodes (3 days after inoculation) arrested gall development and affected less strikingly syncytium development, respectively. The fact that a few cysts and no root knot nematodes matured suggests that cell cycle progression is more important for the early developmental stages of a gall than of a syncytium. This difference might be explained by the fact that giant cells and syncytia become multinucleated because of sequential mitosis and cell fusion, respectively. With HU treatment, feeding cells contained a much less dense cytoplasm, indicating that it might be the lack of nutrient supply that inhibits the development of juveniles.

Inhibition of mitosis by oryzalin at early stages of infection resulted in arrested giant cell development and in less expanded syncytia. Root knot nematodes could not mature, and only a fraction of the cyst nematodes could complete their life cycle. Therefore, mitosis is important for the development of both galls and syncytia. Mitosis does not seem to occur inside the syncytia, but cell division of surrounding cells appears essential for the radial enlargement of the feeding site.

Incubation of feeding sites with inhibitors at late stages (9 days after inoculation) showed that feeding sites were apparently sufficiently developed to allow nematode matura-

tion. The lower maturation rates of cyst compared with root knot nematodes show that at this stage syncytium expansion seems to be more disturbed by the treatments than are giant cells.

Several cytological reports suggest the absence of mitotic activity in syncytia (Endo, 1964, 1987; Endo and Veech, 1970). Here, we used cell cycle blockers to detect mitotic figures in feeding cells. When seedlings were released from HU blocking at different time points, mitotic figures were seen in giant cells but never in syncytia. The distribution of nuclei heterogeneous in size and shape along the dense cytoplasm is characteristic of a mature syncytium. On the other hand, giant cells have their nuclei clustered and often organized in a semicircle, indicating consecutive rounds of mitosis.

Cell wall–like structures between the nuclei were more often present in HU-treated than in untreated giant cells, possibly because inhibitors slow down cell cycle progression (Navarrete et al., 1979; Clain and Brulfert, 1980), and consequently, the stage at which cell wall–like stubs are present is more easily detected. The absence of staining for cellulose excludes the possibility that these stubs would be invaginated giant cell walls. According to Jones and Payne (1978), alignment of cell plate vesicles in giant cells proceeds normally, but the vesicles subsequently become dispersed and cytokinesis is arrested. Such cell wall–like stubs between nuclei or a semicircular organization of nuclei was never observed in syncytia.

Blocking gall and syncytial cells with HU at the G_1-to-S phase of the cell cycle clearly caused a decrease in GUS staining at nematode feeding sites of *cdc2bAt–gus*, *CycB1;1–gus*, and *CycA2;1–gus* plants. In contrast, when galls or syncytia were arrested by oryzalin, the promoter activity of the four cell cycle genes was higher than in untreated infection sites, which is consistent with their high expression during the G_2 phase of the cell cycle. These data confirm the efficacy of the cell cycle blockers.

Initiation and maintenance of galls and syncytia depend on the stimuli coming from the nematode that trigger altered gene expression in the host (Bird, 1996; Williamson and Hussey, 1996). In summary, DNA synthesis and transcriptional activation of genes that are key regulators of the cell cycle demonstrate that nematodes can induce cells to reenter or progress through the cell cycle. Comparison of HU- and oryzalin-blocked cells allowed us to determine the importance of DNA replication independent from mitosis. Gall and syncytium establishment requires DNA synthesis and progression until the late G_2 phase or mitosis, because nematodes cannot initiate a feeding site when host cells are blocked at the G_1-to-S phase or late G_2-to-M phase.

On the other hand, once a gall or a syncytium is initiated, its development is differently affected by the inhibitors. We have shown that DNA synthesis is essential for both gall and syncytium development. When mitosis was blocked, gall development was completely inhibited, indicating that cycles of endoreduplication or other ways of DNA amplification are insufficient to drive giant cell expansion. In the case of syncytia, a mitotic block only affected the radial expansion of a syncytium by preventing neighboring root cells from dividing. However, the longitudinal expansion of a syncytium, which occurs via cell wall dissolution, was not affected. This phenomenon results in less developed feeding sites, leading to improper maturation of the infecting nematodes that depend on the nutrient supply from fully developed syncytia. Our results support the idea that syncytium development involves several cycles of endoreduplication bypassing mitosis. It will be of interest to determine whether syncytial cells contain inhibitors secreted by the cyst nematodes that specifically block the activity of M phase–promoting factors and/or induce unknown S phase–related protein kinases.

METHODS

Plant Material

Surface-sterilized seeds from *Arabidopsis thaliana* C24 and transgenic plants harboring the *cdc2aAt*, *cdc2bAt*, Arath;*CycB1;1*, and Arath;*CycA2;1* promoter–β-glucuronidase (*gus*) fusions were sown on K1 germination medium (Valvekens et al., 1988). Petri dishes were kept under growth chamber conditions with a light regime of 16 hr of light and 8 hr of darkness. Ten days after germination, seedlings were transferred to Knop medium (Sijmons et al., 1991) and kept slightly inclined to allow roots to grow on the surface of the medium and to facilitate nematode infection.

Nematode Inoculation

Axenically grown tomato roots transformed with *Agrobacterium rhizogenes* 15834 were used to propagate the root knot nematode *Meloidogyne incognita* from in vitro–obtained egg masses (Verdejo et al., 1988). Egg masses of the root knot nematodes were collected and allowed to hatch in sterile water at 25°C. After 5 days, infective juveniles (J_2) were collected and used to inoculate Arabidopsis seedlings in Knop medium. Roots from white mustard (*Sinapis alba*) were used to propagate the cyst nematode *Heterodera schachtii*. Arabidopsis seedlings were inoculated with second-stage infective juveniles (J_2) hatched in root exudate from rapeseed (*Brassica napus*). Except for experiments performed 1 day after inoculation, plantlets were always transferred 2 days after inoculation with root knot or cyst nematodes to fresh medium (with or without inhibitors or ^{3}H-thymidine). To avoid additional infections and to ensure more precise timing of harvested infection sites, this step was important.

^{3}H-Thymidine Incorporation

Infected seedlings were transferred to Knop medium containing 2 μCi mL^{-1} methyl-^{3}H-thymidine (47 Ci $mmol^{-1}$) (Amersham, Aylesbury, UK) 12 hr, and 3, 5, 9, and 15 days after inoculation. Labeling was done for 3 hr (short-pulse experiments) or 12 hr (overnight). Infection sites were fixed, embedded, sectioned, and stained as

described below, and ^{3}H-thymidine incorporation was detected on sections by microautoradiography.

Whole-Mount in Situ Hybridization

The whole-mount in situ hybridization (WISH) procedure was performed on entire infection sites or galls sliced with a Vibroslicer (Campden Instruments, London, UK). Fresh slices of developing galls (starting from 5 days after inoculation) were made to permit probe and antibody penetration. Clones of the *cdc2aAt* cDNA (Hemerly et al., 1993), *cdc2bAt* cDNA (Segers et al., 1996), *CycB1;1* cDNA (Ferreira et al., 1994), and *CycA2;1* cDNA (S. Burssens, unpublished data) were used to generate sense and antisense probes. Infected roots were hybridized essentially as described by de Almeida Engler et al. (1998). Comparable time points as for the ^{3}H-thymidine experiments were analyzed (3, 5, 9, and 15 days after inoculation).

Hydroxyurea and Oryzalin Treatments

To test the possible toxicity of the cell cycle inhibitors hydroxyurea (HU) and oryzalin, we incubated hatched J_2 juveniles of root knot or cyst nematodes for 3 days in Knop medium containing high (100 mM HU and 30 μM oryzalin), intermediate (50, 30, 10, and 2 mM HU and 15 μM oryzalin), or low (100 μM HU and 2 μM oryzalin) concentrations of inhibitors. A sample of treated nematodes was observed under Nomarski optics, and the remaining infective juveniles were used to inoculate Arabidopsis seedlings. A fraction of the nematodes was stained with 0.5% $KMnO_4$ to check nematode mortality (Jatala, 1975): dead nematodes stain amber to deep brown, whereas living ones do not.

To confirm whether DNA synthesis was inhibited, autoradiography was performed with squashes and sections of galls and syncytia treated for 48 hr with HU, transferred to the same medium containing ^{3}H-thymidine, and incubated for 12 hr. Typically, swollen root meristems were observed during oryzalin treatments (Baskin et al., 1994).

Arabidopsis seedlings that were kept 48 hr in Knop medium containing the inhibitors (HU or oryzalin in low to high concentrations) were inoculated with root knot or cyst nematodes to test whether nematodes were able to penetrate and infect roots under these conditions. Cell cycle arrest of infected Arabidopsis C24 and transgenic plantlets harboring promoter–*gus* fusions was induced by incubations in K1 medium containing high concentrations of HU (100 mM) or oryzalin (30 μm). Seedlings were transferred to medium with the inhibitors 1, 3, 5, 9, and 15 days after inoculation and kept for 48 hr. Longer incubations in medium containing the inhibitors HU (100 μM) and oryzalin (2 μM) (up to 40 days, which is normally more than sufficient for nematodes to complete their cycle) also were performed to investigate whether nematodes could mature and complete their life cycle. To examine whether nematodes were alive under these conditions, a fraction of the infected roots that were treated and untreated with the inhibitors was stained with potassium permanganate, as mentioned above. For microscopic observation of nematodes, the other fraction of the infected roots was stained with acid fuchsin, as described by Daykin and Hussey (1985).

To catch mitotic figures in galls and syncytia, a DNA inhibitor (HU) was used to synchronize cells. Infected seedlings were incubated with a high concentration (100 mM) of HU for 24 and 48 hr and returned to Knop medium. This point is referred to as time 0, and the following timepoints correspond to the time after recovery. Samples were collected after 8, 10, 12, 20, and 26 hr (Brulfert and Deysson, 1971, 1973), and the DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI).

DNA Staining with DAPI

For observations of nuclei and mitotic figures, whole infection sites were fixed with 4% formaldehyde in 50 mM Pipes buffer, digested with 1% cellulysine and 0.5% macerozyme for 15 min, and slightly squashed on poly-L-lysine–coated slides (10 mg mL^{-1}). Subsequently, samples were stained with 1 μg mL^{-1} DAPI (Sigma) in water, mounted in Citifluor (Agar Scientific, Stansted, UK), and examined with an Axioscop (Zeiss, Jena, Germany).

Fixation and Embedding for Morphology

Infected roots were fixed in 2.0% glutaraldehyde in 50 mM Pipes buffer, pH 6.9, and subsequently dehydrated and embedded in a butylmethacrylate and methylmethacrylate mixture (4:1), as described by Gubler (1989). Embedding medium from sectioned material was dissolved with acetone, stained with 0.05% toluidine blue in 1% borax, and mounted in Depex (Sigma). Microscopy was performed using bright-field optics.

Histochemical GUS Assays

Histochemical assays of GUS activity were performed essentially as described by Jefferson (1987), with minor modifications. For this purpose, seedlings were infected in the presence of the inhibitors or were transferred to medium with inhibitor 1, 3, 5, 9, and 15 days after inoculation. Treatments were done either for 2 days in medium with high concentrations of HU or oryzalin or for up to 40 days in medium with low concentrations of the inhibitors. Infected treated plantlets and infected untreated plantlets were incubated with 90% ice-cold acetone for 30 min and washed twice with 100 mM sodium phosphate buffer, pH 7.4. Samples were then incubated for 1 hr to overnight in an enzymatic reaction mixture (1.0 mg mL^{-1} 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, 0.5 mM potassium ferrocyanide, and 0.5 mM potassium ferricyanide) in sodium phosphate buffer at 37°C in the dark. The reactions were stopped by washing three times for 10 min in sodium phosphate buffer. Samples were subsequently fixed in 2.0% glutaraldehyde and transferred to a chlorallactophenol-clearing solution (Beeckman and Engler, 1994). Infection sites were microscopically analyzed by using Nomarski optics.

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