Expression of the Arabidopsis *MCM* Gene *PROLIFERA* During Root-Knot and Cyst Nematode Infection

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Accepted for publication 21 August 2002.

ABSTRACT

Huang, X., Springer, P. S., and Kaloshian, I. 2003. Expression of the Arabidopsis *MCM* gene *PROLIFERA* during root-knot and cyst nematode infection. *Phytopathology* 93:35-41.

Expression of the Arabidopsis *thaliana* gene *PROLIFERA* (*PRL*) was examined during development of root-knot and cyst nematode feeding sites. These obligate plant parasites establish specialized feeding structures in roots that allow them to withdraw nutrients from the host. In the process of establishing feeding sites, nematodes alter cell cycle regulation. *PRL* is normally expressed specifically in dividing cells at all stages

of plant development and was used here as a marker for cell division. *PRL* expression, reported from a *PRL::GUS* fusion protein, was detected in nematode feeding sites of both root-knot and cyst nematodes from the earliest stages of infection in both giant cells and syncytia. However, unlike other cell cycle genes, expression of *PRL* was detected only occasionally in cells surrounding the feeding sites. *PRL::GUS* activity persisted until late in the infection cycle, past the time when other cell cycle genes are expressed. These data indicate that some aspects of the *PRL* expression pattern during nematode infection differ from that of other cell cycle genes.

Nematode infections pose a significant agronomic problem, causing yield losses in many agronomically important plants. Root-knot nematodes, *Meloidogyne* spp., and cyst nematodes, *Heterodera* spp., are the two most important and devastating nematode species that infect agronomic plants. These nematodes are both sedentary endoparasites that form an intimate relationship with their host by modifying plant cells to withdraw nutrients (6,8). Upon infection, nematodes induce the formation of feeding sites, which are termed giant cells in the case of root-knot nematodes and syncytia in the case of cyst nematodes. Nematode development and reproduction fully depends on the establishment and maintenance of these highly specialized structures. Feeding sites are established by the infective-stage juvenile (J2) nematodes, which then become sedentary and undergo subsequent molts to become mature adults (37). Most *Heterodera* spp. have a sexual mode of reproduction while the most prevalent species of root-knot nematodes (*M. incognita*, *M. javanica*, and *M. arenaria*) typically undergo a parthenogenetic mode of reproduction, although males do exist.

The mechanism controlling the formation of the nematode feeding site is not understood, however it is clear that nematodes in these two genera affect some fundamental aspect of cell division. Cytological observations have revealed that a J2 cyst nematode penetrates the root tissue and migrates intracellularly. In the cortex or the vascular cylinder, the J2 is able to establish a feeding site, which is presumably initiated by the injection of glandular secretions from a specialized mouth part, the stylet. A single syncytium forms by cell wall dissolution between the initial cell and a number of neighboring cells (7). The transfer cell-like syncytium can include up to 200 cells and serves as nutrient reservoir for development of the cyst nematode (20). A J2 root-knot nematode enters the root behind the tip and migrates

intercellularly toward the root apex, where it turns around and migrates upward toward the zone of elongation. Feeding sites are normally established in the zone of root differentiation, where secretions from the esophageal glands of the infective J2 are thought to induce several parenchyma cells in the vascular cylinder to become multinucleate giant cells. Several giant cells are induced around the head of the feeding nematode, each containing up to 100 nuclei as a result of repeated mitosis without cytokinesis (17,36,38). At the same time, the cells surrounding the giant cells undergo hypertrophy to form a gall-like structure on the plant root.

Although the mechanisms of the formation of syncytia and giant cells are likely to be different, they share common features: both are multinucleate, metabolically highly active, and adapted to serve as a nutrient source for the nematode. Both types of nematode feeding sites have cell wall ingrowths adjacent to the vascular cylinder to facilitate solute transport, dense granular cytoplasm with many organelles, and numerous enlarged amoeboid-shaped nuclei (1,20–22).

A number of studies to identify genes that are expressed in nematode feeding sites have been performed and genes that are induced during giant cell or syncytium development have been identified. Among these are genes involved in a wide variety of biological processes, including defense responses, cell wall structure, water relations, and transcriptional regulation (2,24,27–29). In particular, the activation of cell cycle genes has been extensively studied (14). Transcription of Arabidopsis genes *cdc2aAt* and *cdc2bAt*, which encode cyclin-dependent kinases (CDKs), and *CycA2;1* and *CycB1;1*, which encode mitotic cyclins, is activated upon induction of both root-knot and cyst nematode feeding sites. Additionally, the cell cycle inhibitors hydroxyurea and oryzalin have been shown to block the development of both galls and syncytia, demonstrating that cell cycle progression is necessary for establishment of the feeding sites (5).

To further extend the characterization of cell cycle gene expression during nematode feeding site establishment, we have examined the expression of the Arabidopsis *PROLIFERA* (*PRL*) gene during root-knot and cyst nematode infections. *PRL* encodes

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Publication no. P-2002-1101-01R

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a minichromosome maintenance (MCM) protein that is normally expressed in dividing cells. MCM proteins act as part of a complex that functions to regulate the initiation of DNA replication during S phase of the cell cycle (23). The MCM complex binds to replication origins during the G₁ phase of the cell cycle, and replication is triggered during S phase by displacement of the complex from chromatin. The PRL protein is localized to the nucleus in a cell cycle-dependent manner, entering the nucleus during G₁, and disappearing from the nucleus during mitosis (33). During the course of typical plant development, *PRL* is expressed only in dividing cells and not in cells undergoing endoreduplication (16, 33). Consistent with a predicted role in DNA replication, disruption of *PRL* causes megagametophyte and embryo lethality (33, 34). Additionally, *prl* mutant embryos show defects in cytokinesis (16). These observations suggested either that *PRL* plays an additional role in some aspect of cytokinesis or that a novel cytokinesis checkpoint, coupled to DNA replication, exists in Arabidopsis (16). We show here that *PRL* is expressed during both root-knot and cyst nematode infections.

MATERIALS AND METHODS

Nematode cultures. Root-knot nematode, *M. javanica* strain VW4, was obtained from V. M. Williamson, University of California, Davis, and maintained on susceptible tomato, *Lycopersicon esculentum* cv. UC82b. The beet cyst nematode, *H. schachtii*, was obtained from Ole Becker, University of California, Riverside, and maintained on sugarbeet, *Beta vulgaris*. Root-knot nematode eggs were extracted from infected roots by processing in 0.52% sodium hypochlorite in a Waring blender for 2 min at high speed (19). Egg and root debris were collected on 25-μm pore sieve, and J2s hatched as previously described (25).

Cysts of beet cyst nematodes were extracted from roots and soil according to Caswell et al. (4). Cyst nematode J2s were collected in a similar way to root-knot J2s, except that a 4 mM ZnSO₄ solution was used to stimulate hatching. Two days after incubation at room temperature, hatched J2s of both species were collected and suspended in water. J2s were pelleted by centrifugation, rinsed twice in sterile water, and surface-sterilized in 0.01% HgCl₂ for 5 min followed by four additional rinses with sterile water.

Plant material and growth conditions. *Arabidopsis thaliana* (L.) Heynh. transgenic plants containing a gene trap *Dissociation* (*DsG*) transposable element (35) inserted into the *PRL* gene were used in these experiments (34). The *DsG* element contains a β-glucuronidase (*GUS*) reporter gene, therefore *prl::DsG* plants express a *PRL::GUS* fusion protein, regulated by the *PRL* promoter, via a transcriptional/translational fusion (34). Seeds collected from selfed *prl::DsG* plants were surface-sterilized and cold-treated at 4°C for 48 h. Sterilized seeds were plated on nutrient media supplemented with 1% purified agar (Sigma Chemical, St. Louis) in 9-cm petri dishes (35). For root-knot nematode infections, seeds were sown on Knop medium (32) supplemented with 1% sucrose, whereas for beet cyst nematode

infections, Gamborg's B5 medium with 2% sucrose was used (18). Plates were placed on an incline in a growth chamber at 21 to 22°C with a light regime of 16-h light and 8-h darkness.

In vitro inoculation of cyst and root-knot nematodes. Four days after seeding, seedling roots were inoculated with the surface-sterilized J2s of either root-knot or beet cyst nematodes. An inoculum of 20 to 30 nematodes per seedling was used. *M. javanica*-infected seedlings were incubated at 24°C and *H. schachtii*-infected seedlings were incubated at 22°C. To ensure precise timing of nematode infections and initiation of feeding sites, 1 day after inoculation (DAI), seedlings were transferred to fresh media plates to avoid further nematode attack.

GUS histochemical assay and nematode staining. Infected whole plants or roots were used for histochemical localization of GUS activity as previously described (33). The *DsG* insertion in *PRL* was segregating in the population, and plants containing the *PRL::GUS* fusion were identified by the presence of stained root tips.

To visualize the nematodes inside the roots, tissue was first cleared in 0.5% sodium hypochlorite for 1 min and washed extensively in water to eliminate traces of the bleach. Nematodes were then stained by boiling in acid fuchsin in a microwave. After the staining solution was cooled to room temperature, plant tissues were destained in an acetic acid, glycerol, and water (1:1:1) solution. The destaining solution was changed several times until plant tissues had destained. When the nematodes developed such that their bodies protruded outside the root, the acid fuchsin step was omitted in order to avoid detaching them from the root.

Microscopic analysis. Stained roots were examined with a stereo microscope to identify infection sites. Root segments containing infection sites were mounted in 50% glycerol and visualized using Differential Interference Contrast optics on a Leica DMR compound microscope. Images were captured with a SpotRT digital camera (Diagnostics Instruments, Sterling Heights, MI).

RESULTS

***PRL* expression in root-knot nematode feeding sites.** The *PRL* gene is required for megagametophyte and embryo development and is expressed in dividing cells throughout plant development (16,33). We used a gene trap line that reports *PRL* expression from a *GUS* reporter gene to examine *PRL* expression during nematode infection (34). The gene trap line contains a *DsG* element inserted near the 3' end of the *PRL* gene, resulting in a transcriptional and translational fusion of *PRL* to *GUS*. Previous studies have shown that GUS activity in this line accurately reports *PRL* expression (16,33,34). We examined *PRL* expression during root-knot nematode infection over 27 days (Table 1). Plants were sampled daily until 6 DAI and every 3 to 4 days thereafter. In uninfected roots, GUS activity was confined to lateral root primordia and to the tips of primary and lateral roots as previously reported (34). GUS activity in both infected and uninfected roots frequently localized to spots corresponding to nuclei, consistent with the previously reported localization (33). During root-knot

TABLE 1. *PRL::GUS* activity in nematode feeding sites

Cell	Days after inoculation ^a											
	1	2	3	4	6	9	12	15	16	19	20	25
Giant cells	+	+++	nt	++++	++++	++++	++++	++++	nt	–	nt	nt
Gall	+/-	+/-	nt	+/-	–	–	–	–	nt	–	nt	nt
Root tip	+++	+++	nt	+++	+++	+++	+++	+++	nt	+++	nt	nt
Syncytia	+	nt	++	+++	++++	nt	++++	nt	++++	nt	+++	–
Neighboring cells	+/-	nt	+/-	+/-	–	nt	–	nt	–	nt	–	–
Root tip	+++	nt	+++	+++	+++	nt	+++	nt	+++	nt	+++	++

^a Intensity (++++, very strong; +++, strong; ++, medium; +, weak; +/-, occasionally detectable; –, not detectable; and nt, not tested) was based on average of observations on 20 to 40 samples.

nematode infection, GUS activity was first detected as early as 1 DAI (Fig. 1A). At this time, nematodes had penetrated the root at the elongation zone, and in many roots nematodes had migrated to the vascular bundle and begun to feed. Many roots were infected by more than one J2. Root swelling was observed at the site of nematode feeding. Weak GUS activity near the head of the feeding J2 was detected in the majority of roots observed. At 2 DAI, nematode feeding sites had been established and GUS activity localized to enlarged cells near the head (Fig. 1B). Staining in these cells, which were presumed to be giant cells, was more intense than at 1 DAI. We cannot rule out the possibility that in addition to giant cells some neighboring cells were also expressing *GUS*. GUS activity in developing galls at 3 to 6 DAI was of comparable intensity (Fig. 1C and D; Table 1). During this time, J2s were observed to undergo morphological changes consistent with their normal development (12). These changes include a decrease in length and increase in width (Fig. 1D). GUS staining encompassed an increasingly larger area consistent with the enlargement of giant cells, which are multinucleate. In addition, individual nuclei undergo successive rounds of endoreduplication (36). Lateral roots often initiated from the developing galls (Fig. 1D). From 6 to 15 DAI, GUS activity remained high and was detected only in giant cells and not in other cells comprising the gall (Fig. 1E and F). During this time, J2s of root-knot nematodes underwent one or two molts, developing into J3 or J4 stage. At 19 DAI, GUS activity was no longer detectable in giant cells (Fig. 1G). At this stage, the bodies of female nematodes protruded outside the gall. At 23 to 24 DAI, approximately 50% of females were laying eggs, which were visible inside a gelatinous matrix deposited near the vulva (Fig. 1H). At this time, adult males also emerged from the root.

***PRL* expression in beet cyst nematode feeding sites.** We examined GUS activity in the *PRL* gene trap line after infection with beet cyst nematodes. Infection was monitored over a 35-day period (Table 1). Plants were sampled daily for the first 6 DAI, and every 3 to 4 days until day 20. Samples were also taken at 25 and 35 DAI. During the first 24 h, J2 beet cyst nematodes had penetrated the roots, migrated to the vascular bundle, and initiated a feeding site. As with root-knot nematode infection, GUS activity was detected early in the infection process of cyst nematodes. At 1 DAI, faint GUS activity was localized to the area near the head of the feeding nematode (Fig. 2A). From 1 to 6 DAI, the intensity of GUS staining progressively increased (Fig. 2B to D). The syncytium elongated dramatically and multiple foci of GUS activity corresponding to individual nuclei were observed. At the early stages of syncytium formation, *GUS* expression may have also been observed in neighboring cells (Fig. 2C). During this period, cyst nematode J2s developed normally (30). GUS intensity remained high through 16 DAI (Fig. 2E and F) and individual nuclei were enlarged consistent with earlier reports of endoreduplication (14). As the syncytia developed, lateral roots were often induced from the surrounding tissue and expansion of the syncytium caused the root to swell (Fig. 2E). Nematodes underwent two additional molts and fourth-stage juveniles appeared at 12 DAI. As the nematodes enlarged in size, their bodies protruded onto the surface. Adults appeared at 16 DAI (Fig. 2F) and copulation was observed. At 20 DAI, GUS staining intensity diminished (Fig. 2G) and GUS activity was no longer visible at 25 DAI (Fig. 2H).

DISCUSSION

Cell cycle genes have previously been shown to be activated in both root-knot and cyst nematode feeding sites. Furthermore, the normal plant cell cycle is perturbed in both giant cells and syncytia. In both cases, cells are induced to re-enter the cell cycle. In giant cells, aberrant cell cycles that include both the activation of mitosis in the absence of cytokinesis (acytokinetic mitosis) and endoreduplication are observed. In syncytia, aberrant cell cycles

appear to be limited to endoreduplication. Previous studies have focused on expression of the cell cycle genes encoding mitotic cyclins and CDKs (14). In Arabidopsis, the response of the CDK genes *cdc2aAt* and *cdc2bAt* and the cyclin genes *CycA2;1* and *CycB1;1* has been extensively investigated. *cdc2aAt*, *cdc2bAt*, and *CycA2;1* are expressed both in actively dividing cells and in cells that are competent to divide (3,15,31), whereas *CycB1;1* expression appears to be limited to dividing cells (11). In this study, we have examined the expression of the MCM gene *PRL* during induction of nematode feeding sites using a gene trap line that reports *PRL* expression via a transcriptional/translational fusion to the *GUS* gene.

We observed GUS activity corresponding to *PRL* expression during the initiation and development of both giant cells and syncytia. GUS activity was detected during the very early stages of feeding site formation by both root-knot and beet cyst nematodes (Figs. 1A and 2A), indicating a rapid induction of *PRL* expression. GUS activity reached high levels in both giant cells and syncytia over the first few days of infection. In both cases, foci of GUS staining were observed consistent with the previously reported nuclear-localization of the PRL protein (33).

Syncytia are thought to increase in size by incorporation of neighboring cells via cell wall dissolution (10). Little if any mitosis has been reported during syncytium formation, but observations of DNA synthesis and increase in nuclear size indicate that nuclei undergo endoreduplication (9,26). *PRL* is normally expressed in dividing cells and not in cells that are undergoing endoreduplication (33). For example, *PRL* expression in the first pair of rosette leaves was not detected in 10-day-old seedlings (33). In contrast, a continual increase in DNA content was observed in the first pair of rosette leaves until 21 days after germination, indicating the prolonged occurrence of endoreduplication in these cells (13). Thus, *PRL* expression coincided with reported patterns of cell division in leaves and was not detected at later stages when endoreduplication occurs (33). Therefore, the detection of *PRL* expression in developing syncytia was initially unexpected. Expression of *CycB1;1*, which is normally expressed only in dividing cells, has also been observed in syncytia (5). Because *CycB1;1* is expressed beginning at the G₂ phase of the cell cycle, its expression in syncytia led to the conclusion that the cell cycle in syncytial nuclei must proceed until late G₂ (5). The expression of *PRL* and *CycB1;1* in nondividing syncytia suggests that cells have re-entered the cell cycle as if they were undergoing a normal division, and arrested prior to mitosis.

As with other cell cycle genes, *PRL* expression was observed in giant cells during root-knot nematode infection. However, *PRL* expression was rarely, if at all, detected in the surrounding gall tissue (Fig. 1). In contrast to our observations, high levels of DNA synthesis and expression of cell cycle genes have previously been reported in gall tissue (5). *PRL* is expressed in dividing cells and does not appear to play a role during endoreduplication (33). Although we cannot be certain that there is no *PRL* expression in gall tissue, it is clear that if expressed, *PRL* is at much lower levels than are *cdc2aAt*, *cdc2bAt*, and *CycA2;1* transcripts (5,26). The observed marginal levels of *PRL* expression indicate that very little cell division occurs in gall tissue. However, other cell cycle genes are abundantly expressed in gall tissue, and in addition, high levels of DNA synthesis have been reported. These observations are consistent with the occurrence of endoreduplication in these cells. In agreement, only low levels of *CycB1;1* transcripts were detected in gall tissue (5,26). *CycB1;1* is normally expressed during the G₂ and M phases of the cell cycle, and therefore transcripts should be present in dividing cells. Although the role of *cdc2aAt*, *cdc2bAt*, and *CycA2;1* during endoreduplication is not clear, these genes are not exclusively expressed in dividing cells, but also in cells that are competent to undergo division (3,15,31). Giant cells do not undergo traditional cell division, but instead undergo acytokinetic mitosis and individual nuclei undergo endo-

Fig. 1. *PROLIFERA* gene (*PRL*) expression reported by a *PRL::GUS* fusion protein in giant cells formed during root-knot nematode infection of Arabidopsis roots. GUS activity at **A**, 1 day after inoculation (DAI), arrowhead shows position of GUS activity; **B**, 2 DAI; **C**, 4 DAI; **D**, 6 DAI; **E**, 12 DAI; **F**, 15 DAI; **G**, 19 DAI; and **H**, 27 DAI. n = nematode, gc = giant cell, lr = lateral root, g = gall, and em = egg mass. Bars = 100 μ m (bar in **A** refers to **A** to **F**; bar in **G** refers to **G** and **H**).

Fig. 2. *PROLIFERA* gene (*PRL*) expression reported by a *PRL::GUS* fusion protein in syncytia formed during beet cyst nematode infection of *Arabidopsis* roots. GUS activity at **A**, 1 day after inoculation (DAI), arrowhead shows site of GUS activity; **B**, 3 DAI; **C**, 4 DAI; **D**, 6 DAI; **E**, 12 DAI; **F**, 16 DAI; **G**, 20 DAI; and **H**, 25 DAI. n = nematode, s = syncytium, and lr = lateral root. Bars = 100 μ m (bar in **A** refers to **A** to **D**; bar in **E** refers to **E** to **G**).

reduplication. While *cdc2aAt*, *cdc2bAt*, and *CycA2;1* expression is not strictly limited to dividing cells, the expression of *PRL* and *CycB1;1* is restricted to those cells undergoing division (11, 16,33). The expression of these genes in nondividing giant cells suggests that these cells may initially re-enter the cell cycle as a normal cell division and arrest prior to mitosis, similar to what appears to occur in syncytia.

GUS activity in both giant cells and syncytia was observed until late in the infection cycle. In giant cells, intense GUS staining persisted until 15 DAI, at which time the root-knot nematodes were in the J3 stage. In syncytia, GUS activity was detected until 20 DAI, at which time the cyst nematodes were mature. In both giant cells and syncytia, expression levels of the cell cycle genes *cdc2aAt*, *cdc2bAt*, *CycA2;1*, and *CycB1;1* were seen to diminish earlier than this, with GUS activity from promoter:*GUS* fusions decreasing in intensity between 5 and 9 DAI (5). Our experiments differ in that we have examined *PRL* expression using a *PRL::GUS* fusion protein. It is possible that GUS activity from the fusion protein is detectable after the cessation of transcription, and thus it may not be appropriate to directly compare our results with those of other studies using promoter::*GUS* fusions. However, previous studies have shown that this fusion accurately reflects *PRL* transcript accumulation (16,33). *PRL::GUS* activity mirrored *PRL* expression, as measured by in situ hybridization. Therefore, we do not believe that the persistence of *PRL::GUS* activity in our studies is simply due to an increased stability of the fusion protein. Instead, the persistence of GUS activity may be explained by an arrest of the cell cycle at a stage prior to mitosis. A *PRL::GFP* fusion protein was previously shown to enter the nucleus during G₁ and exit the nucleus during mitosis (33). The *PRL::GUS* fusion might persist in the nucleus for a long period of time in the absence of mitosis. Because *PRL* functions as part of a protein complex, it might be protected from degradation in these cells. This is consistent with conclusions that the cell cycle in syncytia is blocked in G₂ and may suggest that a similar cell cycle arrest occurs in giant cells.

ACKNOWLEDGMENTS

This research was supported by the University of California Agricultural Experiment Station grant to I. Kaloshian. We thank J. Baldwin, Department of Nematology, University of California, Riverside, for critical reading of the manuscript.

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