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Laser Capture Microdissection and Real-Time PCR for Measuring mRNA in Giant Cells Induced by *Meloidogyne javanica*

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Abstract: The techniques of laser capture microdissection and quantitative RT-PCR were investigated as methods for measuring mRNA in giant cells induced by *Meloidogyne javanica.* Laser capture microdissection allowed precise sampling of giant cells at 1 to 3 weeks after inoculation. The expression of three genes (a water channel protein gene *Rb7,* a plasma membrane H^{+}-ATPase (*LHA4*), and a hexose kinase (*HXK1*) was measured based on mRNA extracted from tissue samples and quantitated using reverse-transcription real-time PCR. These genes were chosen arbitrarily to represent different aspects of primary metabolism. The amount of *HXK1* mRNA in giant cells was not different from that in root meristem or cortical cells when compared on the basis of number of molecules per unit tissue volume, and was similar at all sample times. Amount of mRNA for *LHA4* and *Rb7* was much greater in giant cells than in cortical cells, but only *Rb7* was also greater in giant cells than in root meristem cells. Numbers of mRNA molecules of *LHA4* increased linearly in giant cells from 1 to 3 weeks after inoculation, whereas the amount of *Rb7* mRNA was similar at 1 and 2 weeks after inoculation but increased at 3 weeks after inoculation. The amount of mRNA for these two genes was similar at all sample times in cortical and root-tip cells. Apparent up regulation of some genes in giant cells may be due primarily to the increased number of copies of the gene in giant cells, whereas for other genes up regulation may also involve increased transcription of the increased number of copies of the gene.

Key words: Gene expression, giant cells, *HXK1, LAH4,* laser capture microdissection, *Meloidogyne javanica,* mRNA, *Rb7,* root-knot nematodes, real-time PCR, reverse transcription.

When plant-parasitic nematodes of the genus *Meloidogyne* infect a host, they induce the formation of enlarged cells, termed giant cells, in the host roots that become the primary feeding site of the nematodes. Giant cells are densely cytoplasmic and multinucleate. The multiple giant cells per feeding site act as transfer cells, increasing the flow of nutrients from the host to the parasite (Bird and Loveys, 1975; Jones and Northcote, 1972). Giant cells are known to have elevated levels of many metabolites (e.g., ATP, glucose, and free amino acids) (Gommers and Dropkin, 1977) and some enzymes (Veech and Endo, 1969). Mature giant cells from pea have ca 60 nuclei/giant cell, whereas lettuce has ca 26 nuclei/giant cell (Starr, 1993). Further, nuclei from mature giant cells are polyploid, with up to 100 chromosomes/nucleus (Wiggers et al., 1990). In pea and tomato, nuclei from giant cells averaged 15.8 and 14.2 times more DNA, respectively, than the nuclei from non-infected root tip nuclei (Wiggers et al., 1990). Wiggers et al. (1991) reported that, based on four gene sequences, nuclear DNA increase in giant cell nuclei appeared to be a systematic increase of all the genomic material.

One aspect of giant cell activity and nematode development that has not been investigated in detail is the relationship between number of nuclei per giant cell and mRNA accumulation. Several studies have reported alterations of normal patterns of gene expression in giant cells (Bird and Wilson, 1994; Favery et al., 2004; Fenoll et al., 1997; Gheysen and Fenoll, 2002), but no study has related changes in gene expression specifically to giant cell or parasite development. Engler et al. (1999) reported that cell cycle inhibitors applied during the first 3 days after inoculation inhibited further development of giant cells and the nematode. No inhibition was observed when the cell cycle inhibitors were applied at 9 days after inoculation. Similarly, Wiggers et al. (2002) reported that when the multinucleate condition of the giant cells was altered by treatment with colchicine such that there were 7 nuclei/giant cell (treatment at 3 days after inoculation), then nematode development was greatly inhibited. If the colchicine treatment was delayed until 7 days after inoculation such that there were 16 nuclei/giant cell (about one third of the normal number of nuclei per giant cell), then development of nematode parasite was not affected. These observations indicate that whereas the giant cells must develop a multinucleate condition to support normal development of the nematode parasite, the normal multinucleate state of giant cells may be well above the required minimum.

It is likely that giant cell development requires a unique pattern of gene expression. Additionally, the high levels of metabolic activity characteristic of giant cells and their multinucleate condition suggest that high levels of gene expression, which may be distinct from up regulation, are needed for fully functional giant cells. The amount of mRNA may appear to be elevated (up-regulated) in giant cells because of the increased number of copies of the gene in the multinucleate giant cells. This would be distinct from cases where an increased amount of mRNA is present because there is both an increased number of copies of a gene and expression of that gene is actually up-regulated. Wang et al. (2003) used microaspiration to collect mRNA from mature giant cells and found that transcript levels of several genes of unknown function

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were 3 to 56 times greater in giant cells than in healthy roots. Microaspiration, however, could not be used to collect samples from giant cells less than 2 weeks old. When mRNA was extracted from samples enriched for giant cells, but which also contained other host cells, a maximum increase of 2.2-fold was observed for a gene that was increased 56-fold based on microaspiration; thus, simple enrichment of samples for giant cells was not adequate for studies of gene expression in giant cells. Wang et al. (2003) did not compare amount of RNA in giant cells to other specific root cells, but to whole roots.

One objective of this research was to test laser capture microdissection (LCM) as a means of sampling giant cell cytoplasm with increased precision. Laser capture microdissection is a technique by which individual cells can be harvested from tissue sections by moving selected cells to an adhesive film with a laser beam (Kerk et al., 2003). When the laser beam is focused microscopically on a specific cell, an adhesive film between the laser source and tissue sample is stretched by the laser beam until it contacts the sample. The sample then sticks to the film and the film can be transferred to a microfuge tube for further processing. The second objective of this research was to compare the levels of mRNA of selected genes important in primary metabolism in giant cells to levels in selected non-infected root tissues. The genes chosen for this study included a hexose kinase (*HXK1*) as a key enzyme in glycolysis, a membrane bound H^+-ATPase (*LHA4*), and the water channel protein *Rb7,* which is reported to be up regulated in giant cells (Opperman et al., 1994)

Materials and Methods

Sample collection and fixing: Tomato (*Lycopersicon esculentum* cv. Rutgers) seeds were germinated in seed-germination paper (Anchor Paper Company, Saint Paul, MN) at 25 °C for 2 days. The germinating seeds were then transplanted into sand in 50-cm^3 tubes and grown at 27 °C with a 12-hour light/dark cycle. Eggs of *M. javanica* were extracted from infected tomato plants using NaOCl (Hussey and Barker, 1973) and second-stage juveniles (J2) hatched as described by Vrain (1977). Tomato seedlings were inoculated at 2 weeks of age with 100 to 150 *M. javanica* J2/seedling.

Galled tomato roots were collected at 1, 2, and 3 weeks after inoculation (WAI). Samples were fixed for 4 to 24 hours at 4 °C in 10 volumes of freshly prepared 3:1 (v/v) ethanol:acetic acid immediately after being collected from roots (Zhu et al., 2003). During fixation samples were subjected to 15 min of vacuum (400 mm Hg) on ice to assist infiltration of the fixative. Control non-infected root tips and cortical tissue were collected at the same times and fixed using the same method.

Fixed tissue was dehydrated at room temperature in a graded series of ethanol (70% to 100% ethanol). Following ethanol dehydration there were three changes in XS-3, a xylene substitute (Statslab Medical Products, Lewisville, TX), for 30 min to 1 hr each. Finally, tissue samples were embedded in paraplast (Surgipath, Richmond, IL) at 60 °C.

Embedded tissues were cut with a microtome into 5-µm-thick ribbons. The ribbons were floated on water on glass slides at 42 °C to stretch. The slices were air-dried and stored in the dark at 4 °C in a dessicator. Before LCM, paraffin on the slides was removed by three rinses in xylene for 3 min each, followed by rinsing in ethanol (70%, 95%, 95%, 100%, and 100% ethanol). The slices were soaked in diethyl pyrocarbonate (DEPC)-treated water for 10 min and then allowed to air-dry.

Laser capture microdissection: The Pix-Cell II LCM (Arcurus Engineering, Inc., Mountain View, CA) system was used to microdissect cells from prepared samples. The laser beam was adjusted to stretch a 7.5-µm-diam. portion of the thermoplastic film on the surface of capture caps over the target cells. Power settings were 50 to 60 mW, with a laser pulse duration of 700 µseconds according to manufacturer's instructions. Control samples consisted of root meristem and cortical cells collected from similar-sized areas as the giant cells but from non-infected roots. Giant cell samples at different times after inoculation were standardized based on the stage of nematode development. Samples collected at 1 WAI were associated with slightly swollen J2, at 2 WAI all samples were associated with greatly swollen J2, and at 3 WAI all samples were associated with females that had just begun egg production.

Because of the unique nuclear condition of giant cells and because activity of traditionally used house-keeping genes are unknown, mRNA levels were measured on the basis of sample volume rather than by comparison to expression of a house-keeping gene, as is common for most experiments on gene expression. Sample volume was calculated based on 5-µm-thick samples multiplied by the surface area. Surface area of tissue transferred by the laser beam to each capture cap was calculated digitally by a computer program written in MatLab.

RNA extraction: CapSure macro LCM caps (Arcturus Bioscence, Mountain View, CA) with captured cells were fitted to 0.5-ml microfuge tubes containing 200 µl of Tri Reagent (Molecular Research Center, Cincinnati, OH), and the tubes were inverted and incubated on ice until extracted. For extraction of total RNA, the caps with Tri Reagent were incubated at room temperature for 5 min. The caps were then removed from the tubes, 40 µl chloroform was added to each tube, and the tubes were vortexed for 15 sec. After incubating for 5 min at room temperature, the tubes were centrifuged at 12,000*g* at 4 °C for 15 min. The aqueous phase was transferred to a fresh tube and 100 µl isopropanol/tube was added to precipitate RNA. The tubes were mixed

well, incubated at room temperature for 5 min and then centrifuged at 12,000*g* for 8 min at room temperature. The pellets were washed with 75% ethanol, and tubes were centrifuged at 7,500*g* at room temperature for 5 min. The pellets were air-dried and the RNA was resuspended in 20 µl of DPEC-treated water. RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 (NanoDrop Technologies, Inc., Wilmington, DE).

Real-time reverse transcription PCR: Primers for quantitative PCR were designed using DNA Star (DNASTAR Inc., Madison, WI) to produce a product of 100 to 150 bp that flanked a region containing at least one intron. The genes tested and primer sequences are listed in Table 1.

The Qiagen Onestep RT-PCR kit (Qiagen, Inc., Valencia, CA) was used to perform RT-PCR. The RT-PCR mixture contained 1 × reaction mix (400 µM each of dATP, dTTP, dCTP, and dGTP, 2.5 mM $MgSO_4$), 0.5 µM of the forward and reverse primers, and 0.1 µl enzyme mix. Thermal cycling conditions were 50 °C for 30 min for reverse transcription, 95 °C for 15 min for HotStarTaq DNA polymerase activation, and 10 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min for PCR. Four to 5 µl RNA (300 pg to 200 ng) of total RNA template was used for each assay. The Qiagen QuantiTect SYBR Green PCR kit was used throughout as 25-µl reactions in a Smart Cycler (Cepheid, Sunnyvale, CA). Thermal cycling conditions were 95 °C for 15 min for HotStarTaq DNA polymerase activation, and 45 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min for PCR. Fluorescence from SYBR Green I was detected at 494 and 521-nm wavelength by a fluorescent detection system. The cycle threshold (C_t) values for each reaction were calculated automatically by the Smart Cycler detection software by determining the point in time (PCR cycle number) at which the fluorescence exceeded 10 times the computer-determined standard deviation for background. The size of the PCR product was confirmed electrophoretically.

Standard curves for quantitative real-time PCR: PCR products were purified using a Qiagen Gel Purification Kit. The fragments were cloned into pCR 2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen, Inc., Carlsbad, CA). The inserts were sequenced to confirm identity with expected PCR products. The plasmids were extracted using Qiagen QIAprep Spin Miniprep Kit. The plasmid concentrations were quantified spectrophotometrically, and 10-fold serial dilutions were made. The C_t values were calculated by the Smart Cycler software. Calculations for each dilution set included standard deviation and average C_t. Regression analysis of the relationship between C_t values and known concentration (ranging from 10 to 10^8 molecules for each amplification product) gave r^2 values of 0.97 to 0.99 for each gene fragment (Table 2).

All data were subjected to analysis of variance using the SAS (SAS Institute, Cary, NC) general linear model to determine treatment effects. Mean separations, when appropriate, were by least significant differences. Because of the variability in the data, $\log_{10}$ transformation of the numbers of RNA molecules per sample also was used during data analysis.

Table 1. Primer sequences for tomato genes *Rb7, LHA4,* and *HXK1.*

Gene	Putative gene function	Primer sequences
Rb7	Water channel	5′-CACTGTTGCTTGCCTCCTC
		5′-CAATGGGTGCAATGGTTCC
LHA4	Plasma membrane H^+-ATPase	5′-AGTGGAAAGGCTTGGAATAAC
		5′-AGGTTTGATGCTTCTGGTGG
HXK1	Hexose kinase	5′-TGGGGTAATTTTAGGTCATCC
		5′-TCTGCGTAAAATTTCTCCCAAGTA

Table 2. Relationships C_t values and number of template molecules for *LHA4, Rb7,* and *HXK1.*

Gene	Standard curve formula
LHA4	log2 (number of template molecules) = 43.04 − 1.40 Ct (r^2 = 0.98)
Rb7	log2 (number of template molecules) = 43.35 − 1.24 Ct (r^2 = 0.97)
HXK1	log2 (number of template molecules) = 44.21 − 1.255 Ct (r^2 = 0.99)

Results

All of the giant cells of a feeding site induced by a single nematode were collected and combined for each replicated sample. Laser capture microdissection was preformed on four to six serial sections from each individual feeding site with all of the samples captured on a single cap to provide sufficient tissue for analysis. Four to six sections of tissue samples from the root meristem or the cortex with a similar area as a feeding site were collected also. Each sample from giant cells yielded about 1µg total RNA. At least four replicated samples were collected for each tissue sample type at each harvest time.

Based on real-time RT-PCR, the amount of *LHA4* mRNA in giant cells was high at 3 weeks after inoculation, with ca 1890 ± 391 molecules/μm^3 of tissue (Fig. 1). Root meristem cells had similar levels with a mean of 2037 ± 1320 molecules/μm^3 of tissue. Cortical cells had only 354 ± 154 molecules/μm^3 of tissue. Analysis of variance revealed that the amount of *LHA4* mRNA in cortical cells was different ($P < 0.05$) from that in giant cells and root meristem cells, but that the latter two cell types had similar numbers of molecules of *LHA4* mRNA. The number of *LHA4* mRNA molecules increased linearly ($r^2 = 0.528$, $P < 0.05$) in giant cells from 1 to 3 WAI but was similar ($P > 0.10$) at all sample times in cortical and root-tip cells.

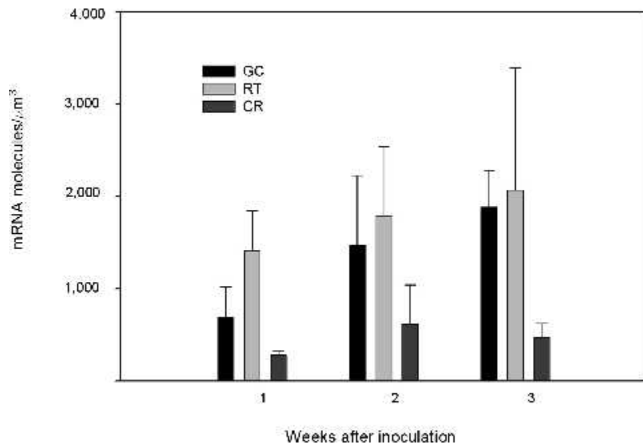

Fig. 1. Number of mRNA molecules of *LHA4* in giant cells induced by *Meloidogyne javanica,* and in root-tip and cortical cells of non-inoculated tomato at 1, 2, and 3 weeks after inoculation based on laser capture microdissection and quantiative RT-PCR. Values are means ± standard deviations of at least four samples of each tissue type at each time.

At 3 WAI, a mean of 1257 ± 1165 *Rb7* molecules/μm^3 of tissue was detected in giant cell tissue compared to 178 ± 28 molecules/μm^3 of tissue in root meristem tissue and 48 ± 29 molecules/μm^3 of tissue in cortical cells (Fig. 2). Because of the high variability in the data, values for the giant cells were different from those of the cortical cells at $P = 0.10$ but not different from numbers in root meristem cells. When data were $\log_{10}$ transformed, the numbers of *Rb7* molecules in each tissue type were different from the other tissue types ($P = 0.03$) at 3 WAI. *Rb7* mRNA was present in greater amounts in giant cells at 3 WAI than it was at 1 or 2 WAI ($P < 0.05$) but did not increase linearly with time. In contrast, amount of *Rb7* mRNA in cortical and root-tip cells was similar at all sample times ($P > 0.10$).

The amounts of *HXK1* mRNA at 3 weeks after inoculation was low in giant cells, root tip cells, and cortical cells, ranging from 4.38 ± 3.68 molecules/μm^3 tissue for root meristem cells to 11.75 ± 17.5 molecules/μm^3 tissue for giant cells. Analysis of variance indicated that there was no difference in expression of this gene among these cell types or with sample time.

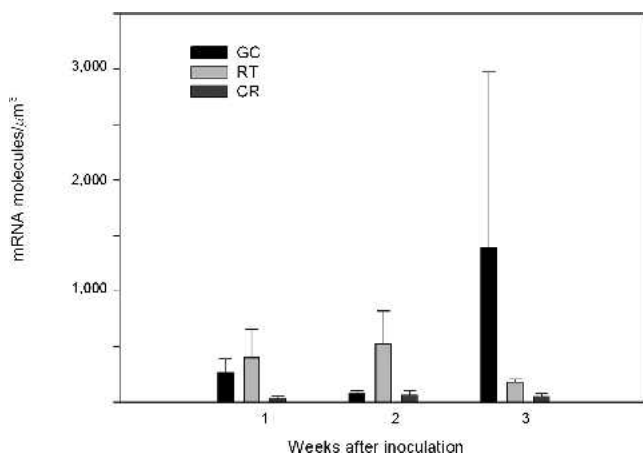

Fig. 2. Number of mRNA molecules of *Rb7* in giant cells induced by *Meloidogyne javanica,* and in root-tip and cortical cells of non-inoculated tomato at 1, 2, and 3 weeks after inoculation based on laser capture microdissection and quantiative RT-PCR. Values are means ± standard deviations of at least four samples of each tissue type at each time.

Discussion

The use of LCM coupled with quantitative RT-PCR proved to be a useful system for the precise sampling of giant cell tissue at different stages of development. In most quantitative PCR experiments a house-keeping gene is used as an internal standard. We did not believe this would be appropriate in our studies because of the distinct possibility that even such house-keeping genes, although not strictly up-regulated, would be expressed at higher levels in giant cells than in other cell types. Therefore, amounts of mRNA in giant cells relative to other root tissues was evaluated based on estimated sample volume. Because of the unique characteristics of giant cells, comparison of giant cells to whole root extracts was considered to be inappropriate. Root meristem and cortical cells were chosen as control tissues because they represent root cells with high and low levels, respectively, of metabolic activity. In a similar study published while the this paper was in review, Ramsay et al. (2004) also demonstrated the potential of LCM with quantitative RT-PCR for measuring specific mRNA molecules in giant cells as early as 3 days after inoculation. Our study differs from Ramsay et al. (2004) in that they used actin and mitogen-activated protein kinase as internal standards rather than sample volume for measuring mRNA and they did not compare amount of mRNA in giant cells to that of other root cells.

The genes *HXK1, LHA4,* and *Rb7* were chosen to represent different aspects of primary metabolism. *Rb7* was also selected because it is reported to be strongly up-regulated in giant cells (Opperman et al., 1994). It was surprising that *HXK1,* a key enzyme in glycolysis, did not exhibit increased levels of expression in the giant cells. Tomato has at least one other hexose kinase (*HXK2*) (Altschul et al., 1997); however, expression of *HXK2* was not measured and may have given a different response.

Amounts of *LHA4* and *Rb7* mRNA in giant cells were 5-fold and 26-fold, respectively, greater at 3 WAI than in cortical cells. Further, the number of mRNA molecules for these genes was greater at 3 WAI than at earlier sample times. The numbers of nuclei per giant cell also would be expected to increase during this period of development (Starr, 1993). However, no actual correlation analysis between amount of mRNA and numbers of nuclei per giant cell was attempted because it was not possible to make accurate counts of nuclei in tissue prepared for LCM. The amounts of mRNA for these

two genes in giant cells at 3 weeks after inoculation were similar to those reported by Wang et al. (2003) for several genes, mostly of unknown function, from mature giant cells using microaspiration. The use of LCM, however, permitted sampling of giant cells at earlier stages of development than was possible using microaspiration.

The high level of *Rb7* mRNA in giant cells relative to the root meristem cells may be due to both its up regulation (Opperman et al., 1994) and to increased numbers of copies of the gene in giant cells. Additionally, that this increase is evident only after the nematode completes its last molt and is beginning egg production suggests a greater specific need for this protein than at earlier periods of giant cell and nematode development. In contrast, whereas the amount of *LHA4* mRNA was much higher in giant cells than in cortical cells, the level of expression in giant cells was similar to that in root meristem cells. This gene does appear to not be up-regulated, but rather higher levels of mRNA appear due to the increased numbers of copies of the gene per sample volume.

Direct comparisons of mRNA in these different tissues is difficult because of the differences in numbers of nuclei per unit of tissue. In cross-sectional area, giant cells at 3 weeks after inoculation were 56 times as large as root meristem cells but only 1.9 times as large as cortical cells (data not shown). Thus, giant cells have similar numbers of nuclei on a total sample volume basis, as do root meristem cells, but a far greater number of nuclei than do cortical cells. Additionally, because giant cell nuclei are greatly polyploid (Huang and Maggenti, 1969; Wiggers et al., 1990) they have also multiple copies of each gene per nucleus. Thus, a distinction between apparent up regulation due to increased transcription per copy of a gene and that due simply to a greater number of copies of each gene must be made.

Giant cell development is a complicated process that undoubtedly involves altered patterns of gene expression relative to other root cells. Additionally, giant cell function to sustain development of the nematode parasite obviously requires a higher rate of many metabolic processes than is typical of most root cells. Thus, there are likely altered levels of gene expression necessary to sustain giant cell function that may be distinct from altered gene expression needed for differentiation. Gross comparisons of gene expression are unlikely to distinguish these different, but possibly overlapping phenomena. The LCM system, especially when coupled with a variety of tools of molecular genetics will greatly assist in unravelling this complex system.

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