Evidence Against Amplification of Four Genes in Giant Cells Induced by *Meloidogyne incognita*

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Abstract: Giant-cell DNA was isolated from pea (*Pisum sativum*) inoculated with *Meloidogyne incognita* and used in slot blots to test for selective sequence amplification. Four sequences representing low (ribulose 1,5-bisphosphate carboxylase and actin), mid-level (histone 3), and highly repetitive (large ribosomal repeat) sequence DNA were used as probes. Known amounts of root-tip DNA and giant-cell DNA were blotted onto hybridization membranes and probed. The signal strength on autoradiographs containing equal amounts of root-tip DNA and giant-cell DNA were compared with a scanning densitometer. No difference in signal strength between equal amounts of root-tip DNA and giant-cell DNA was found. Thus, for the probes tested, there is no difference in copy number and, hence, no selective DNA sequence amplification has occurred.

Key words: DNA, gene amplification, host–parasite relationship, *Meloidogyne incognita*, nematode, nucleus, pea, *Pisum sativum*, root-knot nematode, slot blot.

Specific gene amplification in response to developmental or environmental triggers is known in some eukaryotic organisms. Examples are the amplified ribosomal DNA genes in the oocyte of *Xenopus* (4) and genes responsible for egg shell production in *Drosophila* (12). In plants, environmental stresses can induce amplification of repeated sequence DNA; for example, amplification of ribosomal genes in flax (9). Resistance to N-(phosphonomethyl)-glycine (glyphosate) in *Daucus carota* tissue culture cells was due to amplification of the 5-enolpyruvylshikimic acid-3-phosphate synthase gene (14). Other cases of sequence amplification in plants are known, but in most cases the nature of the sequences is not understood beyond the fact they represent heterochromatic or highly repetitive sequences (20).

Meloidogyne-induced giant-cell nuclei are aneuploid and polyploid (2,15,23) with greatly elevated DNA levels. The DNA content per nucleus, however, does not correlate with ploidy level (1,23). Additionally, DNA per giant-cell nucleus continues to increase after mitotic activity ceases (23). These observations have led some researchers (17,23) to propose that endo-reduplication of DNA may occur in giant-cell nuclei. The objective of this study was to examine giant-cell DNA for specific sequence amplification utilizing the slot-blot technique for quantifying sequence number (8). This procedure involves blotting both target DNA and known amounts of a standard DNA in narrow bands on a hybridization filter. After hybridization and autoradiography, signal strength from autoradiographs can be related to sequence number from the standard series. The relative or absolute copy number of sequences complementary to the probe in the target DNA can be calculated from these data. The slot-blot technique is able to discriminate as little as a 15% increase in the concentration of a given DNA sequence (10).

MATERIALS AND METHODS

DNA isolation: All DNA was isolated by direct phenol extraction of plant material (22). DNA from uninfected roots of pea (*Pisum sativum* L. cv. Little Marvel) was obtained by growing seedlings in ragdolls (5) for 6 days and then isolating DNA from root-tip tissue. To isolate DNA from giant cells, peas were grown and inoculated with 50 juveniles of *M. incognita* (Kofoid & White) Chitwood per seedling in ragdolls and allowed to develop for 3 weeks. Infected root sections were excised from the
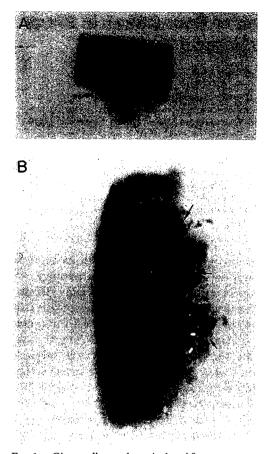
FIG. 1.   Giant-cell complexes isolated from pea roots at 3 weeks after inoculation with *Meloidogyne incognita* and typical of those used as the source of giant-cell DNA. Note numerous, enlarged feulgen stained nuclei of giant cells (arrows). A) Giant-cell complex initiated by a single nematode with associated vascular tissue. B) Giant-cell complex initiated by three nematodes and associated vascular tissue.

roots and, with the aid of a stereomicroscope, the cortical tissue and adult nematodes were removed, leaving the vascular bundle and the giant-cell complexes. The resulting specimens, predominantly giant cells and associated vascular elements (Fig. 1), were stored at −70 C in buffer (50 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20% glycerol) until sufficient tissue (approximately 0.5 g) had been collected to allow DNA isolation (20). DNA from root tips and giant cells was resuspended in sterile distilled water and stored at −20 C.

DNA probes: Four DNA probe sequences, representing segments of the genes for actin, histone 3, ribulose 1,5-bisphosphate carboxylase (rubisco), and ribosomal RNA, were used to probe the giant-cell DNA (Table 1). *Escherichia coli* JM101 was transformed separately with each probe sequence, according to a previously described procedure (13), and stored in glycerol at −70 C. Plasmids containing the cloned probe were isolated from transformed cells by a rapid alkaline extraction procedure (3). Probe sequences were isolated from plasmid vectors by treatment of the plasmids with the appropriate restriction endonucleases (Table 1) and then radiolabeled with ^{32}P with a random primed labeling kit (United States Biochemical Corp., Cleveland, OH). Unincorporated nucelotides were removed with a Bio-Rad D50 column (Bio-Rad, Hercules, CA).

Hybridization of probes to giant-cell DNA: GeneScreen Plus hybridization membrane

TABLE 1.   DNA probes used to detect gene amplification in giant-cell nuclei induced in *Pisum sativum* by *Meloidogyne incognita*.

Original probe	Plasmid vector	Probe length (kb)	Restriction enzyme	Description of gene segment
pSac3	pBR322	3.0	Hind III	Actin gene from *Glycine max*
pGmr3	pBR325	4.5	Eco RI	Large ribosomal repeat containing 18S-28S rRNA genes from *G. max*
pSrs2.1	pBR325	2.10	Eco RI	Ribulose 1,5-bisphosphate carboxylase small subunit gene from *G. max*
pH3	pUC9	0.42	Eco RI	Histone 3 gene from *Arabidpsis thaliana*

Restriction endonucleases required to excise probe sequences from the plasmid vector.
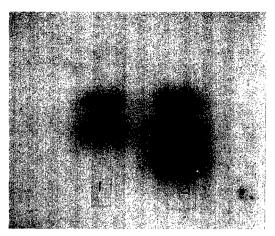
FIG. 2. Autoradiograph of ribosomal DNA probe pGmr3 hybridized to DNA from giant cells (lane 1) and uninfected root-tip cells (lane 2) using a slot-blot apparatus. Concentrations of DNA applied to the slot blot were 5, 10, and 15 μg/slot from bottom to top for root-tip DNA and 5 and 10 μg/slot for giant-cell DNA.

TABLE 2. Comparison of signal strengths of densitometer readings of autoradiographs of four DNA probes hybridized to root-tip DNA and DNA from giant cells induced by *Meloidogyne incognita* on *Pisum sativum*.

DNA conc.	Peak height (cm)			
	pSrs2.1	pSac3	pGmr3	pH3
Uninfected root-tip DNA				
15 μg	11.50	11.85	19.70	9.45
10 μg	10.40	11.50	17.95	7.15
5 μg	9.10	10.90	16.40	5.60
R^2	0.92	0.91	0.94	0.96
Giant-cell DNA				
10 μg	10.20	11.50	17.90	7.00
5 μg	9.00	10.85	16.30	5.75

R^2 values are correlation coefficients for fit of data a linear model of the form $Y = mX + b$.

RESULTS AND DISCUSSION

Densitometer scans of autoradiographs (e.g., Fig. 2) showed that each of the four probes hybridized to root-tip and giant-cell DNA. The relationship between root-tip DNA concentration and signal strength was linear with correlation coefficients greater than 0.91 (Table 2). Because the amount of hybridization of probes to giant cell DNA was not different from the amount of hybridization to root-tip DNA, we concluded that there was no selective amplification of the genes represented by the sequences of the DNA probes used in this study.

Where sequence amplification has been confirmed in plants, it is most often the highly repetitive or heterochromatic sequences that are amplified. In protocorms of the orchid *Cymbidium*, AT-rich heterochromatin appears amplified (19). Heterochromatin amplification also occurs in *Crepis capillaris* (21) and *Phaseolus coccineus* (11). In environmentally stressed flax, amplified sequences were of highly repeated sequences, particularly the 5S ribosomal gene (9). In our study, we examined sequences that represented virtually unique sequence DNA, mid-level repetitive, and high-level repetitive sequences. The rubisco gene and actin gene exist as small multigene families (7,18); histone 3 is present in ca. 100 copies (6), and the ribosomal repeat is present in ca. 3,900 copies (16). Although the results

(New England Nuclear, Boston, MA) was cut to fit the slot-blot apparatus (Steed Engineering Co., Palo Alto, CA) and pretreated according to manufacturer's instructions. The slot-blot apparatus was pretreated with salmon sperm DNA and then assembled according to established protocols (8). Root-tip DNA at 5, 10, and 15 μg/slot and giant-cell DNA at 5 and 10 μg/slot were applied to the hybridization membrane in the slot-blot apparatus according to manufacturer's instructions and incubated for 1 hour at room temperature. The apparatus was then disassembled and the membranes were allowed to dry overnight. Hybridization and post-hybridization washes were completed under high stringency conditions at 65 C according to GeneScreen Plus instructions, except that dextran sulfate was omitted from the hybridization solution, and the hybridization and wash temperature was 55 C for the actin and ribosomal RNA gene probes.

Autoradiographs of labeled probes hybridized to root-tip and giant-cell DNA were scanned on a EC Apparatus Corporation (St. Petersburg, FL) densitometer. Signal strength was determined from peak heights (8).

were negative for the probes used in this study, the possibility of specific amplification of other sequences cannot be ruled out.

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