Gene expression in giant cells induced in tomato roots by *Meloidogyne javanica*

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Abstract

Plant parasitic nematodes are major pathogens of a wide range of crops. Giant cells induced by root-knot nematodes are highly specialised cells which function as transfer cells and provide nutrients to support the growth and reproduction of the nematode. Using a differential display approach, 81 differentially displayed bands were detected between the cytoplasm of giant cells induced in tomato roots by *Meloidogyne javanica* and control tissues. Of these, 73 were up-regulated and 8 were down-regulated. Sixteen were further analysed by real-time quantitative RT-PCR. The most highly up-regulated transcript increased 56 fold in giant cells, and the greatest down-regulation was 11 fold. A time course of expression of selected transcripts using RT-PCR from giant cell enriched tissue showed similar changes. Sequenced transcripts showed significant similarity to mitogen-activated protein kinase, S-adenosylmethionine decarboxylase, cysteine synthase, cytochrome *c* reductase subunit, and ribosomal proteins. The observed gene expression patterns reflect the high metabolic rate in mature giant cells rather than processes of giant cell induction. This work has been extended to analysis using Affymetrix GeneChip microarrays. A comparison of transcripts between giant cell enriched and control tissues revealed a total of 2,448 genes with more than 2-fold changes in expression (about 10% of the 24,000 genes on the chips). Of these genes, 744 were up-regulated in nematode feeding cells and 1,704 were down-regulated. These genes have been classified into functional groups, and the results show substantial changes in gene expression in giant cells that is consistent with the function of giant cells in supporting the development of the nematode parasites.

Media summary

Root-knot nematodes are major crop pathogens which induce ‘giant’ feeding cells by re-programming gene expression in host cells: understanding these changes will lead to development of novel synthetic resistance to these pathogens.

Key Words

Root-knot nematode; *Meloidogyne*; giant cells; gene expression; differential display; microarray

Introduction

Root-knot nematodes (*Meloidogyne* spp.) infect more than 2,000 plant species and significantly reduce agricultural production (Sasser and Freckman, 1987). After hatching from eggs, second-stage juveniles invade roots of host plants, and migrate intercellularly to differentiating vascular regions. The nematodes then become sedentary and induce the formation of giant cells which act as the nutrient source for their development and reproduction (Jones, 1981; Williamson and Hussey, 1996). Between 2-12 giant cells are induced from pro-vascular cells in the differentiating vascular cylinder (Jones, 1981). They become multinucleate by repeated mitosis without cytokinesis and fill with metabolically active cytoplasm (Jones and Payne, 1978). There is an increase in the activities of many enzymes in giant cells, as well as in the rate of synthesis of DNA, ribosomal RNA and protein, compared to that in the surrounding cells, which reflects the increased metabolic activity inside giant cells (Jones, 1981; Favery *et al.*, 1998). Formation and maintenance of giant cells requires continuous stimulus from nematode esophageal gland secretions (Williamson and Hussey, 1996). The modification of normal root cells into feeding structures includes complex morphological and physiological changes, and a new pattern of gene expression. The

application of two approaches to study changes in gene expression in giant cells is presented here: differential display (DD) with real-time quantitative RT-PCR, and microarray technology.

Methods

Plant Material and Root-knot Nematode Infection

Tomato (*Lycopersicon esculentum*) cv. Grosse Lisse (Yates, Smithfield, Australia) was cultured *in vitro* as host for root-knot nematode infections as described by Hutangura *et al.* (1999). Plants were grown at a constant temperature of 25?C with a light regime of 16 h light and 8 h dark.

Extraction of cytoplasmic contents from individual giant cells

Extraction of giant cell cytoplasmic contents was carried out with a modified pressure probe system described by Wang *et al.* (2001). The extracted cytoplasmic sample was expelled into 1µl of mRNA isolation buffer (100mM Tris-HCl pH 8.0, 500mM LiCl, 10mM EDTA pH 8.0, 1% LiDS, 5mM dithiothreitol.

mRNA isolation and normalisation of amounts

mRNA from *in vitro* cultured healthy tomato roots or giant cells cytoplasm was isolated with Oligo$(dT)_{25}$ Dynabeads (Dynal, Carlton South, Australia) following the method of Wang *et al*. (2003). For reliable DD analysis of giant cell cytoplasm, semi-quantitative RT-PCR was carried out to normalise the template amount for DD (Wang et al 2003). Differences in template amounts were estimated and different dilutions of mRNA from healthy root tissue were made. Expression levels of the actin gene in the dilutions and the giant cell extracts were also compared using semi-quantitative RT-PCR. The concentrations of mRNAs from healthy root tissue and giant cell extracts were then normalised for DD.

mRNA differential display RT-PCR

Differential display analysis was carried out using modified degenerate two-base anchor primers ($ET_{12}VN$, where E is an *Eco*RI site as 5'-CGGAATTCGG-3') and 18-21 mer elongated arbitrary primers that have over 50% GC content and dG or dC at the 3' base (Zhao *et al.*, 1995) as described by Wang et al (2003).

Real-time quantitative RT-PCR

For selected cDNA fragments identified by direct sequencing, forward and reverse primers were specific to the nucleic acid sequences of the fragments, and real-time quantitative PCR analysis of the expression of various genes was then carried out using SYBR Green with an ABI PRISM 7700 Sequence Detector (Applied BioSystems) (see Wang *et al*. 2003). Quantification of the transcript level of the 16 cDNA fragments was normalised to the expression of the actin gene in giant cell cytoplasm and healthy root extracts.

Affymetrix microarrays

Giant cell enriched and equivalent control tissues were harvested from infected *Arabidopsis thaliana* roots. Total RNA was isolated from 2,000 dissected galls and 500 mg control tissue using Trizol: the quality and quantity of RNA was checked using an Agilent Bioanalyser. About 5 µg intact RNA from each sample was used to synthesise first-strand cDNA via reverse transcription with a T7-$(T)_{24}$ primer. The cDNA was used to generate biotin-labelled cRNA by *in vitro* transcription using the Enzo BioArray RNA transcript labelling method. Yield and the quality of the labelled cRNA was checked by spectrometer and electrophoresis. The cRNA was fragmented to short sequences: 15 µg of cRNA was used for each sample to hybridise to the Arabidopsis ATH1 genome array chip. Detection of the labelled cRNA with streptavidin-phycoerythrin and was achieved by scanning the chip. Duplicated samples for both infected and control tissues were set up to increase the accuracy of the result. Data generated was analysed using Affy Microarray Suite 5.0 software.

Results

Differential display RT-PCR

DDRT-PCR was done using 44 different primer combinations, and 81 differentially displayed bands (180bp - 800bp) with intensity differences between healthy root tissue and giant cell cytoplasmic extracts were identified. These 81 DD bands were: 73 up-regulated and 8 down-regulated. The reproducibility of the DD analysis was confirmed by repeating experiments with a number of primers and different mRNA samples as templates. Direct sequencing was undertaken to screen differentially displayed bands and 27 unique sequences were obtained. Details of these bands are presented in Table 1. For these bands the deduced amino acid sequences were compared to known protein sequences in GenBank using Blastp to identify possible homologues: 9 fragments showed significant identity to known sequences. For the other cDNA fragments, comparison of nucleotide sequences with the EST database in GenBank was carried out. The results indicate that all the DD bands, except two had at least one significant match with known EST sequences (Table 1). The deduced amino acid sequences of the matched longer ESTs were then compared with known protein sequences in the database

Table 1. Characteristics of sequenced DD bands.

a: Numbers in brackets for each gene show the levels of expression in giant cells cf control; tissue. b: homology at amino acid level. * no significant identity to known protein: homology determined by the longer EST sequence.

cDNA fragments	Accession no.	Up-regulation [a]	Homology [b]	Expect value
ZW0103001	BU666907	GC (3.3)	Cysteine Synthase (AF044172)	4e-16
ZW0103002	BU666908	GC	None	
ZW0103003	BU666909	GC (22.2)	SAMDC (S74514)	1e-22
ZW0103004	BU666910	GC (12.7)	GAPDH (AJ133422)*	76-31
ZW0103005	BU666911	GC (9.9)	None	
ZW0307001	BU666912	GC (13.9)	None	
ZW0805001	BU666913	GC (4)	60S ribosomal protein L23A (AC004218)	1e-22
ZW0805002	BU666914	GC (7.2)	None	
ZW0903001	BU666915	GC (15.6)	60S ribosomal protein L31 (AF237624)	5e-38
ZW0903002	BU666916	GC (11.2)	60S acidic ribosomal protein P1 (AF361624)	4e-18
ZW0903003	BU666917	GC	putetive Peroxin (AC022457)	2e-26
ZW0903004	BU666918	GC	60S ribosomal protein L31 (AF237624)	5e-38
ZW1008005	BU666919	GC (10.4)	MAP kinase (AAG51978)	7e-04
ZW1008006	BU666920	HT	None	
ZW1307002	BU666921	HT (10.7)	None	
ZW1901001	BU666922	GC	Immunophilin (U96925)*	3e-47
ZW2703003	BU666923	GC (56.5)	None	
ZW2703004	BU666924	GC	GTP-binding protein (T03717)*	9e-60
ZW2703007	BU666925	GC	SBP-like 12 (AJ132096)	2e-13
ZW2703008	BU666926	HT	None	
ZW30050020	BU666927	GC (6.3)	60S r-ptotein L37A (AJ131732)*	3e-34
ZW30050025	BU666928	GC	None	
ZW3107003	BU666929	GC (6.5)	None	
ZW3107004	BU666930	GC	*Arabidopsis* unknown protein (AC007658)*	6e-53
ZW3107005	BU666931	GC (14.7)	Cyt *c* reductase subunit (CAA55860)*	3e-28
ZW3107009	BU666932	GC (18.8)	None	
ZW31070012	BU666933	GC	None	

Quantitative RT-PCR assay

Sixteen fragments were analysed by real-time quantitative RT-PCR to confirm their differential expression. The differences in expression for the 16 candidate fragments are summarised in Table 2.

Table 2. Differences in expression level of genes between giant cell cytoplasmic contents and healthy root tissue, determined by the quantification of template amounts by real-time quantitative RT-PCR.

cDNA fragments	Actin normalisation (CtH/CtG)	ΔCt (CtH-CtG)	Actual fold difference ($2^{\Delta Ct}$)
ZW0103001	1.003	1.74	3.33
ZW0103003	1.017	4.47	22.16
ZW0103004	1.017	3.67	12.73
ZW0103005	1.017	3.30	9.85
ZW0307001	1.000	3.80	13.93
ZW0805001	0.992	2.01	4.03
ZW0805002	0.992	2.85	7.23
ZW0903001	1.000	3.96	15.56
ZW0903002	1.000	3.48	11.16
ZW1008005	1.000	3.38	10.41
ZW1307002*	1.000	**-3.42**	10.70
ZW2703003	1.000	5.82	56.49
ZW30050020	1.017	2.66	6.33
ZW3107003	1.000	2.7	6.50
ZW3107005	1.000	3.88	14.72
ZW3107009	1.000	4.23	18.77

CtH and CtG are the mean Ct value from triplicate sample from healthy root tissue and giant cell cytoplasmic contents.

Microarray results

This work revealed 744 genes up-regulated more than 2-fold in feeding sites, and 1,704 down-regulated genes. The 744 up-regulated genes have been classified into different functional groups using Affymetrix annotation and other literature resources (Table 3). Further data analysis is being carried out to search for common motifs in promoter regions of the up-regulated genes to identify nematode responsive elements.

Table 3 Summary of functional classes of up-regulated genes and 3 representative genes from each class

Functional class	Three representative genes [a]
General metabolism	Polygalacturonase (27.9), L-asparaginase gene (21.1), isocitrate dehydrogenase (21.1)
Transcription factors	homeobox protein (64), CONSTANS (22.6), AP2 domain transcription factor (9.8)
Plant defence / pathogen related	beta-1,3-glucanase (22.6), two disease resistance protein (18.4 and 8.6)
Signal transduction	serine/threonine kinase (32), GTP-binding protein (4), calmodulin (2.8)
Cell structure / maintenance	integral membrane protein (29.9), proline-rich protein (24.3), pectate lyase (5.7)
Transport proteins	nitrate transporter (39.4), lipid transporter (10.6), hexose transporter (3.7)
DNA and RNA processing	splicing factor (7.5), RNA helicase (6.1), histone H2B (4)
Phytohormone related	ABA-responsive protein (42.2), EREBP (9.8), auxin induced protein (4.6)
Nodulin related	nodule inception protein (14.9), nodulin-like protein (4.9), major intrinsic protein (3.2)
Others	CLE3 (5.7), APG precursor (5.3), light regulated protein (4.9) Hypothetical, unknown, putative proteins (52, 32, 24.3)

[a] *The number in parentheses following each gene represent degree of up-regulation compared to control root tissue.*

Of the 744 up-regulated genes, 41% encode hypothetical, putative, or unknown proteins. The functional classification is: general cellular metabolism (17.7%), transcription regulation (9.8%), plant defence (7.4%), signal transduction (4.8%), cell structure and maintenance (4.4%), transport activity (4.2%). However, since these data were generated in one experiment, expression of selected transcripts is being checked by quantitative RT-PCR and *in situ* RT-PCR.

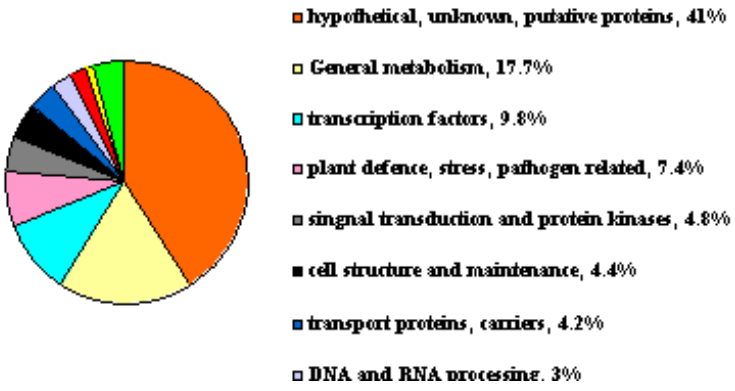

Figure 1. Functional categories of up-regulated genes identified by microarrays.

Conclusion

These results reflect the fact that giant cells have very active cytoplasm related to their function. Many genes identified in previous work are found in this data. In addition, several hundred genes have been identified here that have not been identified before in plant-nematode interactions.

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