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Research Article

Transcriptome analysis of resistant soybean roots infected by *Meloidogyne javanica*

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Abstract

Soybean is an important crop for Brazilian agribusiness. However, many factors can limit its production, especially root-knot nematode infection. Studies on the mechanisms employed by the resistant soybean genotypes to prevent infection by these nematodes are of great interest for breeders. For these reasons, the aim of this work is to characterize the transcriptome of soybean line PI 595099-*Meloidogyne javanica* interaction through expression analysis. Two cDNA libraries were obtained using a pool of RNA from PI 595099 uninfected and *M. javanica* (J_2) infected roots, collected at 6, 12, 24, 48, 96, 144 and 192 h after inoculation. Around 800 ESTs (Expressed Sequence Tags) were sequenced and clustered into 195 clusters. *In silico* subtraction analysis identified eleven differentially expressed genes encoding putative proteins sharing amino acid sequence similarities by using BlastX: metallothionein, SLAH4 (*SLAC1 Homologue 4*), SLAH1 (*SLAC1 Homologue 1*), zinc-finger proteins, AN1-type proteins, auxin-repressed proteins, thioredoxin and nuclear transport factor 2 (NTF-2). Other genes were also found exclusively in nematode stressed soybean roots, such as NAC domain-containing proteins, MADS-box proteins, SOC1 (*suppressor of overexpression of constans 1*) proteins, thioredoxin-like protein 4-Coumarate-CoA ligase and the transcription factor (TF) MYBZ2. Among the genes identified in non-stressed roots only were Ser/Thr protein kinases, wound-induced basic protein, ethylene-responsive family protein, metallothionein-like protein cysteine proteinase inhibitor (cystatin) and Putative Kunitz trypsin protease inhibitor. An understanding of the roles of these differentially expressed genes will provide insights into the resistance mechanisms and candidate genes involved in soybean-*M. javanica* interaction and contribute to more effective control of this pathogen.

Key words: gene expression, root knot nematode, transcriptome.

Introduction

Soybean is the most important agricultural commodity in the world, both in terms of value and quantity. Besides, it is an attractive crop for the production of renewable fuels such as biodiesel. Root-knot nematode (RKN-*Meloidogyne* spp.) is a serious constraint for many crops, and can significantly affect crop productivity worldwide. In Brazil, this pathogen was responsible for economic losses of over US$52.2 million during the 1999/2000 harvest (Yorinori, 2000).

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The use of nematode-resistant cultivars is the most economical and environmentally friendly management strategy for the control of the pathogen (Boerma and Hussey, 1992). The Plant Introduction (PI) 595099, a soybean genotype that is highly resistant to RKN species and to the soybean cyst nematode (SCN) *Heterodera glycines* (Davis *et al.*, 1998), has been successfully used as a new source of nematode resistance in Brazilian breeding programs (Silva, 2001).

Many physiological changes associated with stress response in plants are controlled at the transcriptional level. Several studies of gene expression have contributed to elucidate the physiological response to infection of soybean roots with *Heterodera glycines* (Alkharouf *et al.*, 2004, 2005; Khan *et al.*, 2004; Klink *et al.*, 2005; Ithal *et al.*,

2007a,b). Through microarray analysis, it was possible to identify 429 differentially expressed genes during susceptible soybean-*H. glycines* interaction (Ithal *et al.*, 2007a). These included genes encoding enzymes involved in plant secondary metabolism, such as the biosynthesis of phenolic compounds, lignin, and flavonoids that were up-regulated early during nematode infection and remained overexpressed throughout nematode development. Similarly, genes related to stress and defense responses like pathogenesis-related proteins (PR), cell wall modification enzymes, cellular signaling proteins, and transcriptional regulation factors were consistently up-regulated. Transcript profiling analysis of developing syncytia induced in susceptible soybean by SCN showed interplay among phytohormones that likely regulate synchronized changes in the expression of genes encoding cell wall-modifying proteins. This process appears to be tightly controlled and coordinated with cell wall rigidification processes that may involve lignification of feeding cell walls (Ithal *et al.*, 2007b).

Expressed sequence tags analyzed in other plants inoculated with *Meloidogyne* spp. identified several genes similar to the ones found in compatible soybean-*Heterodera* interaction. For instance, in susceptible *Arachis* spp. inoculated with *M. arenaria*, *arp* (auxin-repressed protein) genes were up-regulated whereas cytokynine oxygenase, metallothionein and resveratrol synthase were down-regulated (Proite K, 2007, Doctoral thesis, Universidade de Brasília). The characterization of the transcriptional profile of a compatible tomato response to Javanese nematode demonstrated significant changes in the steady-state transcript levels of several functional categories, including pathogenesis-related genes, hormone-associated genes and development-associated transcription factors (Bar-Or *et al.*, 2005). Responses to *M. incognita* infection in roots of a resistant cowpea (*Vigna unguiculata* L. Walp.) genotype and a susceptible near-isogenic line showed that a greater number and proportion of genes were down-regulated in the resistant than in the susceptible genotype, whereas more genes were up-regulated in the susceptible than in the resistant genotype (Das *et al.*, 2010).

In this work we used EST sequence analysis from cDNA libraries of soybean PI 595099 roots at 6, 12, 24, 48, 96, 144 and 192 h after infection (h.a.i.) with *M. javanica* to assess the gene expression changes during this interaction. This study could lead to new target genes for nematode control and identify candidates for broadening plant resistance to this pathogen through over-expression or gene silencing.

Material and Methods

Nematode inoculum

M. javanica eggs cultured on susceptible tomato host plants were extracted from roots using 0.5% bleach solution (Boneti and Ferraz, 1981) and cleaned with caulim (Coolen and D'Herde, 1972). Eggs were hatched at room temperature and J_2 were collected in fresh deionized water.

Root infections for microarray analysis

Soybean PI 595099 seeds were surface sterilized using 10% (v/v) bleach solution, sown in sterilized sand and maintained under controlled environmental conditions at 26.7 ± 2.0 °C temperature and a 16-h photoperiod. After three days, the seedlings were transplanted to seedling growth pouches with sterilized substrate. Eight days after transplant each plantlet was inoculated with 500 J_2 *M. javanica* larvae in 5 mL of deionized water. Five repetitions of inoculated and non-inoculated roots (mock control) were collected at 0, 6, 12, 24, 48, 96, 144 and 192 h after inoculation (h.a.i.). Infected and non-infected plants were arranged in a completely randomized design under greenhouse conditions.

RNA isolation

Total RNA from nematode infected and non-infected roots was isolated using Trizol (Invitrogen) reagent and cleaned with RNeasy Mini Kit for RNA cleanup (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. RNA was treated with RNase-Free DNase (Qiagen) to digest any remaining genomic DNA. RNA was quantified using a UV-spectrophotometer and its quality and integrity was examined in 1.2% agarose gel containing ethidium bromide.

cDNA cloning

Two cDNA libraries were constructed comprising the control and the pooled infected root tissues from all intervals. The libraries were prepared using the SMART cDNA Library Construction Kit (Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. Briefly, the double-stranded cDNAs were fractioned and cloned in the pTriplEx 2 vector of the same kit according to the manufacturer's protocol. The library was amplified in *Escherichia coli* DH-5 cells (Invitrogen), placed on LB agar and grown overnight at 37 °C. Plasmid preparations of the individual transformants were performed in 96-well plates.

EST sequencing

cDNA inserts were sequenced using specific primers PTR2 (5'CCGCATGCATAAGCTTGCTC3' - Reverse) and PTF2 (5'GCGCCATTGTGTTGGTACCC3' - Forward) at Embrapa Genetic Resources and Biotechnology, Brazil. Nucleotide sequences and predicted amino acid sequences were analyzed using the SisGen software (Pappas *et al.*, 2008) and Fisher (1932) statistical tests to reveal differential gene expression (Table 1). The criteria applied were a minimum of 30-base similarity between sequences and at least 90% identity. Semiautomatic annotation was performed by BlastX 2.2.3 (Altschul *et al.*, 1990),

Table 1 - Differentially expressed genes in soybean (PI 595999) resistant roots uninfected and infected with *M. javanica*.

Cluster	[1]PIN (115 reads)	[2]PII (246 reads)	Statistical tests (p-values) Stekel	Audic-Claverie	Fisher	Blast best hit
Contig7	4	2	1.52	0.037	0.089	Hypothetical protein
Contig3	1	11	1.92	0.048	0.114	Metallothionein-like protein
Contig6	0	6		0.068	0.182	SLAH4 (SLAC1 Homologue 4)
Contig8	0	5		0.1	0.33	SLAH1 (SLAC1 Homologue 1)
Contig9	0	5		0.1	0.33	Zinc finger, AN1-type; A20-type
Contig1	1	7	0.81	0.164	0.44	Auxin-repressed protein
Contig4	4	6	0.14	0.252	0.732	Thioredoxin
Contig10	2	3	0.07	0.29	0.656	ACL098Cp
Contig16	1	5	0.35	0.291	0.669	Auxin-repressed protein
Contig2	3	10	0.24	0.3	0.762	Nuclear transport factor2 (NTF-2)
Contig5	3	5	0.05	0.313	0.714	60S ribosomal protein
11	19	65	-	-	-	-

[1]PIN = PI 595099 uninoculated; [2]PII = PI 595099 inoculated with *M. javanica.*

SwissProt (Bairoch and Apweiler, 1997) and sequences were clustered according to their putative functions by using COG (*Clusters of Orthologous Groups of Proteins*) (Tatusov *et al.*, 2003). The sequences were grouped into contigs. The EST database is housed at the Soybean Genome Project Database (SGPD).

Results

EST validation

Throughout the analysis of the two (RKN-infected and mock control) sequenced cDNA libraries a total of 2,112 sequence reads were obtained and 877 accepted (41%). The valid ESTs were distributed in 195 clusters, these being 79 contigs, and 116 singletons. From these, 55 contigs originated from inoculated (Table 2) and 24 from non-inoculated (Table 3) roots. *In silico* comparison of the two libraries using the statistical tests of Stekel *et al.* (2000), Audic andClaverie (1997) and Fisher (1932) ($p < 0.005$) revealed 11 contigs with significant variation in their transcript levels (Table 1). These transcriptional changes might result from the up-regulation of transcription level or from reduced mRNA expression due to nematode infection.

Functional classification of ESTs homologous to genes of known function

Overall, the most abundant transcripts observed in PI 595099 roots included ESTs encoding genes involved in cell communication/signal transduction [(zinc finger, AN1-type; A20-type); (Nuclear transport factor 2 (NTF-2)], followed by hormonal regulation (Auxin-repressed protein), cellular metabolism [(SLAH4 (SLAC1 Homologue 4); SLAH1 (SLAC1 Homologue 1)], regulation of cell-environment interaction, cellular defense (metallothionein-like proteins), protein synthesis (60S ribosomal proteins) and resistance metabolism (thioredoxin) (Figure 1 and Table 1).

Discussion

In general, the onset of responses governing pest resistance in plants depends on the genotype/species, magnitude and rapidity in which the genes are expressed during the infection. Recently modulated transcript abundance was demonstrated in resistant and susceptible soybean roots during SCN interactions (Mazarei *et al.*, 2011) and also during susceptible soybean- RKN interaction (Ibrahim *et al.*, 2011). The goal of this work was to describe transcriptional changes in PI 595099 resistant soybean line roots during the early stages (6, 12, 24, 48, 96, 144 and 192 h) of infection with *M. javanica*. The *in silico* functional characterization of the transcribed reads from both libraries revealed a number of genes related either to biotic or abiotic stresses. Here, among the defense responses of PI 595099 towards *M. javanica* are included up-regulated genes encoding for zinc finger, AN1-type; A20-type; Nuclear transport factor 2 (NTF-2); Auxin-repressed protein; SLAH4 (SLAC1 Homologue 4); SLAH1 (SLAC1 Homologue 1); metallothionein-like proteins, 60S ribosomal proteins and thioredoxin.

Metallothioneins belong to a family of cysteine-rich low molecular weight metal-binding proteins in which the presence of thiol groups promotes its high affinity to heavy metal ions, such as zinc and copper (Inácio AF, 2006, Master's thesis, Escola Nacional de Saúde Pública – FIOCRUZ). Plants expressing metallothioneins better tolerate soils and substrates that are rich in heavy metal ions and are capable of mitigating the damage caused by reactive oxygen species (ROS) (Chiang *et al.*, 2006), which is associated with hypersensitive response (Wong *et al.*,

Table 2 - Expressed genes during soybean (PI 595099) root and *M. javanica*- interaction.

Contig	Blast best hit	Organism	Accession code	E-value	Number of ESTs PIN[1]	Number of ESTs PII[2]	Total number of ESTs
Contig1	Auxin-repressed protein-like protein, Positives = 48/72 (66%)	*Nicotiana tabacum*	AY183722.1	1e-11	2	12	14
Contig2	Nuclear transport factor 2 (NTF-2), Positives = 113/124 (91%)	*Arabidopsis thaliana*	sp|Q9C7F5.1|NTF2_ARATH	7e-57	3	10	13
Contig3	Metallothionein-like protein 2 (MT-2)	*Cicer arietinum*	sp|Q39459.2|MT2_CICAR	5e-15	1	11	12
Contig4	Thioredoxin-like 4, Positives = 77/104 (74%)	*Arabidopsis thaliana*	sp|Q8LDI5.2|TRXL4_ARATH	4e-27	5	6	11
Contig5	60S ribosomal protein L23,Positives = 92/93 (98%)	*Zea mays*	gb|ACG48540.1|	5e-69	3	5	8
Contig6	SLAH4 (SLAC1 HOMOLOGUE 4) Positives = 134/176 (76%)	*Arabidopsis thaliana*	ref|NP_001077757.1|	5e-49	0	7	7
Contig8	SLAH1 (SLAC1 HOMOLOGUE 1); transporter Positives = 79/113 (69%),	*Arabidopsis thaliana*	ref|NP_176418.2|	2e-29	0	6	6
Contig9	Zinc finger A20 and AN1 domain-containing stress-associated protein 8 (OsSAP8) Positives = 127/169 (75%)	*Oryza sativa*	sp|A2YEZ6.2|SAP8_ORYSI	8e-56	0	5	5
Contig10	ACL098Cp, Positives = 28/59 (47%)	*Ashbya gossypii*	ref|NP_983306.1|	2.7	2	3	5
Contig11	N-methyltransferase Positives = 133/160 (83%)	*Arabidopsis thaliana*	ref|NP_565246.1|	8e-70	0	4	4
Contig13	Ubiquitin-conjugating enzyme E2-17 kDa 8 Positives = 148/148 (100%)	*Arabidopsis thaliana*	834173 UBC8	2e-82	0	4	4
Contig14	Probable glutathione S-transferase (Heat shock protein 26A) (G2-4), Positives = 56/57 (98%)	*Glycine max*	gb|AAG34798.1|AF243363_1	2e-25	0	4	4
Contig16 CL1Contig2	Auxin-repressed protein Positives = 69/131 (52%)	*Zea mays*	gb|ACG37064.1|	5e-13	1	5	6
Contig17	Probableaquaporin PIP-type 7a (Turgor-responsiveprotein 7ª, 31), Positives = 107/108 (99%)	*Medicago truncatula*	gb|AAK66766.1|AF386739_1	1e-54	0	3	3
Contig18	Histone-lysine N-methyltransferase ASHR1, Positives = 30/54 (55%)	*Arabidopsis thaliana*	sp|Q7XJS0.2|ASHR1_ARATH	6.1	1	2	3
Contig19	Auxin response factor 2 (ARF1-binding protein) (ARF1-BP) Positives = 49/57 (85%).	*Lycopersiconesculentum*	gb|ABC69711.1|	9e-16	0	3	3
Contig20	Adenine phosphoribosyl transferase 1 (APRT 1), Positives = 164/174 (94%)	*Solanum tuberosum*	gb|ABB86271.1|	2e-81	0	3	3
Contig21	Acetyl-CoAcarboxylase, alpha subunit Positives = 31/56 (55%)	*Flavobacterium sp. MED217*	ref|ZP_01059904.1|	34.7	0	3	3
Contig25	Chloroplast 50S ribosomalprotein L14, Positives = 94/94 (100%).	*Glycine max*	ref|YP_538801.1|	5e-45	0	3	3
Contig33	Acyl carrier protein, mitochondrial precursor (ACP) (NADH-ubiquinoneoxidoreductase 9.6 kDasubunit) (MtACP-1), Positives = 79/120 (65%)	*Arabidopsis thaliana*	sp|P53665.1|ACPM_ARATH				
9e-25	0	2	2				
Contig34	Kinesin light chain 3 (Kinesin light chain KLCt) Positives = 122/137 (89%)	*Arabidopsis thaliana*	gb|AAM63491.1|	1e-54	0	2	2
Contig35	Predicted protein Positives = 30/57 (52%)	*Pichia guilliermondii*	gb|EDK41815.2|	0.90	0	2	2
Contig36	Putative non-LTR retroelement reverse transcriptase, related Positives = 49/115 (42%)	*Medicago truncatula*	gb|ABN08132.1|	1e-06	0	2	2

Table 2 (cont.)

Contig	Blast best hit	Organism	Accession code	E-value	Number of ESTs PIN[1]	Number of ESTs PII[2]	Total number of ESTs
Contig37	ATP synthase 6 kDa subunit, mitochondrial Positives = 20/23 (86%)	*Solanum tuberosum*	sp|P80497.1|ATPY_SOLTU	4e-05	0	2	2
Contig38	Transcription factor MYBZ2 Positives = 131/131 (100%)	*Glycine max*	gb|ABI73970.1|	6e-119	0	2	2
Contig39	Thioredoxin-like protein 1 Positives = 149/169 (88%)	*Zea mays*	gb|ACG24478.1|	1e-66	0	2	2
Contig41	No hit blast				0	2	2
Contig42	Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit DAD1 (Defender against cell death 1) (DAD-1) (AtDAD1), Positives = 111/113 (98%),	*Arabidopsis thaliana*	ref|NP_174500.1|	3e-52	0	2	2
Contig43	Anaphase-promoting complex subunit 11 (APC11) (Cyclosome subunit 11), Positives = 67/83 (80%)	*Mus musculus*	sp|Q9CPX9.1|APC11_MOUSE	2e-36	0	2	2
Contig44	Unnamed protein product Positives = 39/54 (72%)	*Vitis vinifera*	emb|CAO40176.1|	5e-05	1	1	2
Contig46	Inner membrane magnesium transporter mrs2, mitochondrial precursor (RNA-splicing protein mrs2), Positives = 58/120 (48%),	*Schizosaccharomyces pombe*	sp|P87149.1|MRS2_SCHPO	8e-05	0	2	2
Contig48	F-box/LRR-repeat protein 16 (F-box and leucine-rich repeat protein 16) Positives = 131/144 (90%)	*Malus x domestica*	gb|ACB87911.1|	6e-57	0	2	2
Contig50	Glucose-6-phosphate 1-dehydrogenase, cytoplasmicisoform (G6PD) Positives = 89/93 (95%)	*Solanum tuberosum*	gb|ABB55386.1|	1e-44	0	2	2
Contig51	Hypothetical protein MtrDRAFT_AC136139g5v2, Positives = 35/38 (92%).	*Medicago truncatula*	gb|ABE93033.1|	8e-12	0	2	2
Contig52	USP family protein Positives = 27/30 (90%)	*Zea mays*	gb|ACG42306.1|	6e-06	0	2	2
Contig53	Unnamed protein product Positives = 111/119 (93%)	*Vitis vinifera*	emb|CAO42347.1|	6e-53	0	2	2
Contig54	39S ribosomal protein L41-A, mitochondrial precursor Positives = 79/89 (88%)	*Arabidopsis thaliana*	ref|NP_568574.1|	8e-35	0	2	2
Contig57	Chalconereductase Positives = 89/106 (83%)	*Sesbania rostrata*	emb|CAA11226.1|	3e-33	0	2	2
Contig58	Probable rhamnose biosynthetic enzyme 1 Positives = 94/103 (91%)	*Arabidopsis thaliana*	sp|Q9SYM5.1|RHM1_ARATH	7e-46	0	2	2
Contig60	Ubiquinol—cytochrome-c reductase-like protein Positives = 83/85 (97%)	*Arabidopsis thaliana*	dbj|BAD95225.1|	5e-43	0	2	2
Contig61	Translation initiation factor IF-2 Positives = 39/80 (48%)	*Plasmodium yoelii*	ref|XP_730210.1|	3.0	0	2	2
Contig62	UPF0497 membrane protein At2g28370, Positives = 49/55 (89%)	*Arabidopsis thaliana*	sp|Q9SKN3.1|U4977_ARATH	1e-20	0	2	2
Contig63	MADS-box protein SOC1 (protein suppressor of constans overexpression 1) Positives = 77/82 (93%)	*Glycine max*	gb|ABC75835.1|	3e-33	0	2	2
Contig64	SAP domain-containing protein Positives = 84/117 (71%)	*Arabidopsis thaliana*	ref|NP_201151.2|	3e-22	0	2	2
Contig65	No blast hit	-	-	-	0	2	2
Contig66	Histone H2A.F/Z Positives = 115/116 (99%)	*Arabidopsis thaliana*	emb|CAA73155.1|	4e-54	0	2	2
Contig68	Hypothetical protein MGG_13574 Positives = 28/54 (51%)	*Magnaporthe grisea*	ref|XP_001408018.1|	5.9	0	2	2
Contig69	No significant	-	-	-	0	2	2

Table 2 (cont.)

Contig	Blast best hit	Organism	Accession code	E-value	Number of ESTs PIN[1]	Number of ESTs PII[2]	Total number of ESTs
Contig70	Ferredoxin Positives = 102/118 (86%)	*Zea mays*	gb|ACG39554.1|	4e-43	0	2	2
Contig71	HMG1/2-like protein (Protein SB11) Positives = 119/121 (98%)	*Glycine max*	sp|P26585.1|HMGL_SOYBN	3e-33	0	2	2
Contig72	ABI5 binding protein A1 Positives = 47/54 (87%)	*Triticum aestivum*	dbj|BAG12827.1|	3e-25	0	2	2
Contig73	NAC domain-containing protein 29 (ANAC029) (NAC2) Positives = 64/102 (62%)	*Arabidopsis thaliana*	sp|O49255.1|NAC29_ARATH	2e-17	0	2	2
Contig74	At3g08610 Positives = 58/62 (93%)	*Arabidopsis thaliana*	gb|AAP21180.1|	4e-23	0	2	2
Contig77	Metallo-beta-lactamase superfamily protein Positives = 48/105 (45%)	*Alcanivorax* sp.	gb|EDX88588.1|	1.4	0	2	2
Contig79	4-Coumarate—CoA ligase-like 5 (Peroxisomal OPC-8:0-CoA ligase 1), Positives = 175/199 (87%).	*Oryza sativa*	sp|Q10S72.1|4CLL4_ORYSJ	2e-87	0	2	2
55	-	-	-	-	19	170	189

[1]PIN = PI 595099 uninoculated; [2]PII = PI 595099 inoculated with *M. javanica.*

2004). In *Arachis* spp., metallothionein-3 expression was observed only in roots inoculated with *M. arenaria* race 1 (Proite K, 2007, Doctoral thesis, Universidade de Brasilia). Infected roots of PI 595099 over-expressing the metallothionein gene might use its protein product as a defense mechanism, acting directly in the cell, affecting ROS concentration, in order to avoid damage to the cell wall and even nucleic acids.

SLAC1 (Slow Anion Channel-Associated 1) has been shown to be essential for stomata closure in response to CO_2, abscisic acid, ozone, light/dark transitions, humidity, calcium ions, hydrogen peroxide and nitric oxide (Negi *et al.*, 2008). The two *SLAC1* genes (*SLAH4* and *SLAH1*), expressed only in inoculated root libraries, are possibly involved in ionic regulation, suggested as a defense mechanism of this genotype.

The gene that encodes a 60S ribosomal protein was also significantly regulated in stressed PI 595099 roots. This gene plays an important role in the elongation step of protein synthesis and in this study it might be related to an increase in protein synthesis from genes involved in the defense response to *M. javanica*. In *Poncirus trifoliata* its expression was up-regulated when infected by *Citrus tristeza virus* (Cristofani-Yaly *et al.*, 2007).

There are other transcripts that might be induced in resistant soybean roots during nematode infection, such as the zinc finger protein (Zinc finger, AN1-type, A20-type), which belongs to the gene superfamily *SAP* (*Stress Associated Proteins*). Members of this family have been classified according to the number of Cys-His residues that bind the zinc ion (Ciftci-Yilmaz and Mittler, 2008) and are involved in DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding (Laity *et al.*, 2001). In cDNA libraries of *Poncirus trifoliata* infected with *Citrus tristeza virus* (CTV), zinc finger genes were up-regulated, suggesting the importance of this gene in the plant response to viral infection (Cristofani-Yaly *et al.*, 2007). The exclusive expression of this gene in PI 595099 inoculated roots may indicate the activation of cellular metabolism related to stress in an attempt to control larvae development.

Two *arp* (*Auxin Repressed Protein)* genes were differentially expressed in response to nematode infection (Table 1). These genes were previously described in strawberry (Reddy and Poovaiah, 1990), tobacco (Steiner *et al.*, 2003) and pepper (Hwang *et al.*, 2005). They are very similar to genes involved in a dormancy mechanism, with dormancy gene expression being repressed by auxin (Reddy and Poovaiah, 1990; Brinkler *et al.*, 2004; Shimizu *et al.*, 2006). The expression of *arp* genes is associated with several stresses, such as water stress (Kohler *et al.*, 2003), salt and low temperature (Hwang *et al.*, 2005), fungus (Coram and Pang, 2006), as well as nematode infection (Alkharouf *et al.*, 2004; Proite K, 2007, Doctoral thesis, Universidade de Brasília), among others. Nevertheless, little is known of

Table 3 - Expressed genes in uninoculated soybean (PI 595099) roots.

Contig	Name	Organism	Accession code	E-value	Number of ESTs PIN[1]	Number of ESTs PII[2]	Total number of ESTs
Contig7	Hypothetical protein Positives = 66/70 (94%)	*Vitis vinifera*	emb|CAN65763.1	3e-31	4	2	6
Contig12	ADP-ribosylation factor Positives = 181/181 (100%)	*Hyacinthus orientalis*	gb|AAT08648.1|	2e-99	3	1	4
Contig15	Polyprotein Positives = 29/58 (50%)	*Potato virus Y*	gb|ABC70481.1	0.6	3	0	3
Contig22	No hit blast	-	-	-	3	0	3
Contig23	Methionine-R-sulfoxidereductase B1 protein Positives = 130/148 (87%)	*Capsicum annuum*	gb|ABO64854.1|	1e-72	3	0	3
Contig24	Ser/Thr protein kinase Positives = 31/31 (100%).	*Lotus japonicus*	dbj|BAD95894.1|	7e-10	2	1	3
Contig26	Wound-induced basic protein, Positives = 46/47 (97%),	*Vitis vinifera*	emb|CAO15234.1|	5e-17	2	1	3
Contig27	No hit blast	-	-	-	3	0	3
Contig28	Auxin-repressed 12.5 kDa protein, Positives = 52/55 (94%)	*Robinia pseudoacacia*	gb|AAG33924.1|	1e-23	3	0	3
Contig29	60S ribosomal protein L27a-2 Positives = 124/134 (92%).	*Arabidopsis thaliana*	sp|Q9LR33.1|R27A2_ARATH	5e-54	3	0	3
Contig30	No hit blast	-	-	-	3	0	3
Contig31	Putative Kunitz trypsin protease inhibitor Positives = 109/109 (100%.)	*Glycine max*	gb|ACA23205.1|	2e-59	3	0	3
Contig32	Metallothionein-like protein 1 Positives = 45/57 (78%)	*Trifolium repens*	sp|P43399.1|MT1_TRIRP	8e-13	3	0	3
Contig40	Cysteine proteinase inhibitor (Cystatin) Positives = 88/94 (93%),	*Vigna unguiculata*	sp|Q06445.1|CYTI_VIGUN	5e-41	2	0	2
Contig45	Grx_I1 - glutaredoxin subgroup III, Positives = 94/170 (55%)	*Zea mays*	gb|ACG27551.1|	2e-27	2	0	2
Contig47	Ethylene-responsive family protein, Positives = 97/121 (80%)	*Arabidopsis thaliana*	ref|NP_194639.1|	7e-42	2	0	2
Contig49	Histidine-containing phosphotransfer protein 1 Positives = 130/154 (84%)	*Arabidopsis thaliana*	sp|Q9ZNV9.1|AHP1_ARATH	7e-46	2	0	2
Contig55	Putative mitochondrial ABC transporter ATM1b Positives = 39/79 (49%)	*Antonospora locustae*	gb|AAY27418.1|	5.1	2	0	2
Contig56	Hypothetical protein Positives = 73/77 (94%)	*Vitis vinifera*	emb|CAN70604.1|	5e-32	2	0	2
Contig59	Ribosomal protein L19 Positives = 78/82 (95%)	*Hyacinthus orientalis*	gb|AAT08672.1|	5e-26	2	0	2
Contig67	Small nuclear ribonucleoprotein, putative Positives = 52/53 (98%)	*Arabidopsis thaliana*	gb|AAM63846.1|	1e-22	2	0	2
Contig75	Hypothetical protein OsI_20016 Positives = 32/63 (50%)	*Oryza sativa*	gb|EEC79253.1|	9.0	2	0	2
Contig76	Putative lysine decarboxylase, Positives = 31/32 (96%)	*Musa balbisiana*	dbj|BAG70979.1|	5e-08	2	0	2
Contig78	Far-red impaired responsive family protein / FAR1 family protein, Positives = 178/235 (75%)	*Arabidopsis thaliana*	ref|NP_567085.1|	2e-65	2	0	2
24					61	6	67

[1]PIN = PI 595099 uninoculated; [2]PII = PI 595099 inoculated with *M. javanica*.

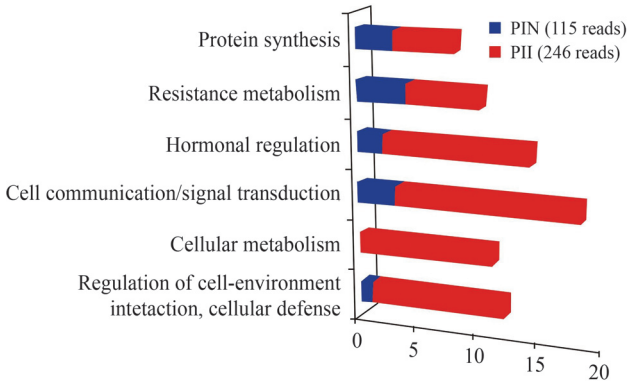

Figure 1 - Frequency distribution for differentially expressed genes in soybean (PI 595999) resistant roots uninfected (blue) and infected (red) with *M. javanica*.

the importance of *arp* genes during plant-nematode interaction (de Almeida-Engler *et al.*, 1999). This study provides insights into the *arp* gene as a possible target of future investigation during plant stress responses, especially in nematode-interactions, since root knot formation is controlled by plant hormones such as auxin (Kim *et al.*, 2007)

In addition to the genes mentioned previously, two *Thioredoxin* (*Trxs*) genes were found exclusively in the cDNA libraries from stressed soybean roots. Trxs are small proteins with a redox-active disulfide bridge and are important regulatory elements in plant metabolism (Gelhaye *et al.*, 2005). Two new *Trxs* isoforms were found specifically in legume with redox potential values similar to those of the classical Trxs, and one of them was shown to act as a substrate for the *Medicago truncatula* NADP-Trx reductase (Alkhalfioui *et al.*, 2008). In tomato, it was first demonstrated that a CITRX (Cf-9-*interacting binding thioredoxin*) plays a role in the regulation of plant disease resistance induced through Cf-9 (Rivas *et al.*, 2004). The *Trx* gene revealed herein as differentially expressed in soybean infected roots may exert negative regulation on plant metabolism and then enhance defense and hypersensitive response (HR).

Gene transcripts with homology to *Nuclear Transport Factor 2* (*NTF2*) were significantly up-regulated in infected soybean roots. A previous study has shown that the overexpression of an *NTF2* (*IAtNF2a*) blocked the nuclear import of a plant transcription factor in *Nicotiana benthamiana* leaves, indicating that the excess of *AtNTF2a* disrupted nuclear import of a small multifunctional GTPase (Ran) involved in nucleo-cytoplasmic transport, mitotic spindle assembly, and nuclear envelope formation, in a Ran-binding dependent manner (Zhao *et al.*, 2006). The *NTF2* gene was up-regulated threefold in PI595099 stressed roots, and this gene is probably contributing to an occasional abnormal cellular disorganization associated with nematode infection observed in these roots (data not shown).

Many compounds involved in plant defense are synthesized in the phenylpropanoid biosynthesis pathway, such as lignin and phytoalexins. Several stress-induced phenylpropanoids can lead to cell wall polymerization, which is the first physical barrier for pathogen resistance (Dixon and Paiva, 1995). MYB represents the largest transcription factor family in *Arabidopsis thaliana* (Chen *et al.*, 2005) and is reported to contribute to defense response and regulatory processes in higher plants (Yanhui *et al.*, 2006). The expression patterns herein observed for MYB TFs might be indirectly involved in soybean cell wall resistance, and we infer that they may prevent larvae from penetrating, and therefore would reduce and/or delay gall formation, as observed in PI 595099 roots (data not shown).

An important gene encoding a NAC-domain protein (such as *NAM, ATAF1* and *CUC2* genes) was detected in the stressed PI 595099 libraries only (Contig73, Table 2). Members of this superfamily of transcription factors possess an N-terminal conserved amino acid sequence named NAC domain and are widely distributed in the plant genomes. The importance of this protein family in a range of biological processes has been reviewed by Olsen *et al.* (2005). These processes include embryonic, floral and vegetative development, lateral root formation, senescence and auxin signaling, as well as defense and wounding stresses. Members of this family were extensively studied in *A. thaliana*, which contains more than 90 representatives of NAC domain proteins (*AtNAC*). It has been also reported that the *AtNAC2* gene plays a role in the ethylene and auxin signaling pathways, and is involved in the salt stress response and lateral root development (He *et al.*, 2005).

Another member of this family, the *SND1* gene (*Secondary wall-associated NAC Domain proteins*), is a key regulator compound in the secondary wall of *A. thaliana* fibers (Zhong *et al.*, 2006). In studies based on the Afimettrix soybean GeneChip, NAC transcription factor probe sets were consistently induced in the resistant TN02 line and suppressed in the susceptible soybean TN02-275 line sister during SCN race 2-interaction (Mazarei *et al.*, 2011). The role of this gene in the resistance response to *M. javanica* infection in soybean is unknown, but it might be induced by ethylene during injuries caused in the roots by larvae, or in cell-wall strengthening during J_2 penetration.

Aquaporin transcripts, such as PIP (plasma membrane intrinsic protein), one of the four groups of plant aquaporins, were also represented in the RKN-infected roots. Aquaporin is a water channel protein that shows increased expression levels in cell membranes and has an important function in cell expansion and division (Okubo-Kurihara *et al.*, 2009). Aquaporin genes have been demonstrated to be associated with *H. glycines*-inoculated soybean roots (Klink *et al.*, 2005) and rice leaves resistant to *Magnaporthe grisea* (Jantasuriyarat *et al.*, 2005), indicating that these genes might have relevant roles in plant defense responses. We infer that the presence of *aquaporin*

PIP in PI 595099 stressed roots may elicit the plant defense machinery via water deficit signaling.

Many other genes encoding proteins involved in plant defense were identified in this study, such as the DAD1 (defender against cell death 1) protein, MADS-box protein SOC 1 (suppressor of overexpression constans 1) protein, cytochrome C reductase, *SAP* (*Stress Associated Proteins-domain*), glutathione-S-transferase, as well as proteins related to secondary metabolism pathways, such as chalcone synthase and 4-coumarate-CoA. Further studies on these genes will certainly contribute considerably to the understanding of the PI 595099 resistance mechanisms to *M. javanica*.

It was expected that the gene expression pattern in non-stressed roots would reflect normal root development, and not surprisingly, several ESTs encoding proteins that are involved in plant stress response, including Ser/Thr protein kinase, putative Kunitz trypsin protease inhibitor, cysteine proteinase inhibitor, ethylene-responsive family protein and Metallothionein-like protein 1, were represented in the libraries (Table 3). Apparently, the presence of these genes at a low level might indicate an efficient basal resistance, or an injury response due to root development. Provided that these genes have been described to be involved in both injury and insect attack response (Singh *et al.*, 2008; Luo *et al.*, 2009), certain features of the PI 595099 resistance mechanism are probably present in the plant even before pathogen penetration, and the genes discussed in this study (and probably others) are up-regulated so as to to fully express the resistance phenotype.

In conclusion, this study provided a global profile of gene expression changes in soybean PI 595099 during RKN attack, elucidating some elements involved in an incompatible interaction with *M. javanica*. Validation of the most relevant genes by quantitative PCR should provide a better understanding of RKN parasitism of soybean and aid in the identification of potential targets for genetic improvement of several crops. In addition, histological characterization studies, by monitoring various time points in the penetration and development of *M. javanica* juveniles (J2) in soybean PI 595099 roots, will provide insights by associating these plant resistance responses with the RKN interaction, and this is the subject of our current studies.

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Internet Resources

Soybean Genome Project Database (SGPD, http://bioinfo03.ibi.unicamp.br/soja/ (September 21, 2011).