TRANSCRIPTOME AND PROTEOME ALTERATIONS DURING THE NEMATODE-SOYBEAN INTERACTION

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ABSTRACT

Prince, A. M., F. H. Khan., B. F. Matthews, N. Islam, and S. S. Natarajan. 2014. Transcriptome and proteome alterations during the nematode-soybean interaction. Nematropica 44:137-145.

Plant-parasitic nematodes are important pests that cause an estimated worldwide loss of $100 billion annually. The root-knot and cyst nematodes are of major economic importance to soybean. Consequently, the need for comprehensive understanding of these nematodes directs researchers to combine the transcriptome and proteome analyses to determine the interplay between alterations in soybean gene expression and nematode parasitism. Microarray studies have found several thousand alterations in transcript abundance during nematode parasitism; proteomics has allowed analysis of thousands of root and nematode proteins. The current knowledge of root-nematode interactions is mostly based on transcriptomic data with few proteomic studies. The root-knot and soybean cyst nematode proteins are the topic of this review because they play an important role in manipulation of soybean root cell biology. A better understanding of these proteins and the altered soybean proteins in response to nematode parasitism would help in the discovery of new nematode resistance targets.

Key words: Heterodera glycines, *Meloidogyne incognita*, nematodes, protein, soybean.

RESUMEN

Prince, A. M., F. H. Khan, B. F. Matthews, N. Islam, and S. S. Natarajan. 2014. Alteraciones transcriptómicas y proteómicas durante la interacción soja-nematodo. Nematropica 44:137-145.

Los nematodos parásitos de plantas son importantes plagas de los cultivos que causan unas pérdidas anuales estimadas en $100 billones a nivel mundial. Los nematodos formadores de agallas en las raíces y los quísticos tienen además, una gran importancia económica en el cultivo de la soja. Consecuentemente, existe la necesidad de un conocimiento profundo sobre estos nematodos que conduce a los investigadores a combinar análisis trascriptómicos y proteómicos que permitan determinar la relación entre alteraciones en la expresión génica de la soja y el parasitismo del nematodo. Estudios por micromatrices han encontrado miles de alteraciones en la abundancia de transcritos durante el parasitismo por nematodos; la proteómica ha permitido el análisis de miles de proteínas de las raíces y de los nematodos. El conocimiento actual sobre las interacciones raíz-nematodo se basa principalmente en datos transcriptómicos con unos pocos estudios proteómicos. Las proteínas de nematodos agalladores de raíces y del nematodo quístico de la soja constituyen el tema de esta revisión, debido a que juegan un papel importante en la manipulación de la biología celular de la raíz de soja. Un mejor conocimiento de estas proteínas y de las proteínas de soja alteradas en respuesta al parasitismo por los nematodos podría ayudar en el descubrimiento de nuevos objetivos para la resistencia a nematodos.

Palabras clave: Heterodera glycines, *Meloidogyne incognita*, nematodos, proteína, soja.

INTRODUCTION

Soybean (*Glycine max* L.) is the most important legume worldwide, and it is cultivated for both protein and oil (Messina and Liu, 1997). Most soybean-parasitic nematodes are restricted to the roots, and the best studied are the root-knot and cyst nematodes (Perry and Moens, 2011). The root-knot nematode (RKN), *Meloidogyne incognita*, has a wide host range and causes large yield losses worldwide (Moens *et al*., 2009). It is an obligate parasite that causes significant damage to a broad range of host plants and is able to infect more than 1,700 plant species (Sasser *et al*., 1983). The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the major widely distributed biotrophic pathogen of soybean. Soybean cyst nematode infection causes about $2 billion yield losses annually in the world (Niblack *et al*., 2004). The sedentary RKN and SCN induce a change in selected root cells of the host to form feeding structures. Cyst nematodes induce a syncytium (Grundler *et al*., 1998) while the RKN induce giant cells (Jones and Payne, 1978). During parasitism, nematodes puncture the cell wall of the plant cell with their stylet, withdraw nutrients, and secrete effector proteins into the plant cells, which induce alterations in the local and systemic gene expression patterns in the plant (Gheysen and Mitchum, 2011). The basis of an incompatible nematode-plant interaction is plant recognition of specific nematode effectors, followed by successful activation of plant defenses (Jones and Dangl, 2006). Destruction of the feeding site results in nematode death. Klink *et al.* (2010a) reported that *G. max* reacts to the presence of the nematode before the nematode initiates the formation of its feeding site by differential expression of genes in soybean roots. When susceptible soybean roots fail to prevail over infection, the nematode continues its lifecycle, while in a resistant plant, roots resist infection by localized changes in the syncytium (Kim *et al.*, 1987; Endo, 1991). The RKN forms galls and feeds from a giant cell in the susceptible interaction. In the resistant interaction, the RKN does not form large galls and giant cells in soybean roots (Matsye *et al*., 2011; Hewezi and Baum, 2013).

SOYBEAN ROOT GENES AND PROTEINS RELATED TO NEMATODE INFECTION

Research on soybean nematode parasitism tackles basic questions in soybean-parasite interaction. One of the most important questions is how nematodes can modulate the soybean defense pathway. Genetic studies indicate that there are naturally occurring resistance genes (Resistance *Heterodera glycines* (Rhg) that confer partial resistance to specific populations of soybean cyst nematode. Genes that confer resistance, located at the Rhg1 and Rhg4 locus, have recently been identified (Cook *et al*., 2012; Liu *et al*., 2012). Proteome and microarray analyses of the soybean root, aimed at discovering plant defense-related proteins in roots, have been studied in soybean infected with nematode in several laboratories (Table 1). Despite the various histological studies describing the cellular phenotypic alterations associated with syncytia formation and degeneration in soybean (Riggs *et al*., 1973; Acedo *et al*., 1984), the molecular mechanisms underlying syncytium collapse are poorly understood. Soybean resistance to *Heterodera glycines* Ichinohe is classified into two different responses, the *G. max* ([Peking/PI 548402])- and *G. max* ([PI 88788])-types. Microarray analyses by Klink *et al*. (2011) comparing these different resistant responses showed gene expression alterations in the pericycle, including up-regulation of genes, encoding a protein that responds to arachidonic acid, as well as a protease inhibitor, in *G. max* ([Peking/PI 548402]) as compared to *G. max* ([PI 88788]). The up-regulation of these and other genes may explain the rapid and potent reaction in *G. max* ([Peking/PI 548402]) as compared to *G. max* ([PI 88788]). During the past several years, extensive Gene Chip data from soybean infected with *H. glycines* have been published (Ithal *et al*., 2007; Puthoff *et al.*, 2007; Tucker *et al*., 2007; Klink *et al*., 2007a, 2007b, 2009b, 2010a, 2010b, 2011; Matsye *et al.*, 2011). A whole-roots Gene Chip analysis of *G. max* infected with *H. glycines* showed altered expression of many genes, including genes encoding xyloglucan endotransglycosylase and eight α-expansin sequences (Ithal *et al*., 2007). Xyloglucan endotransglycosylase genes encode enzymes that metabolize the xyloglucan fraction of the soybean cell wall. Expansins are extracellular plant proteins involved in cell wall alterations, but they do not possess any enzymatic activity. These enzymes may contribute to syncytium formation by aiding in the dissolution of the cell wall (Yennawar *et al.*, 2006; Pant *et al*., 2014).

The microarray analysis of susceptible and resistant whole roots showed differential gene expression during their response to *H. glycines* (Klink *et al*., 2007a; Matsye *et al*., 2011). Furthermore, gene expression in syncytial cells has been studied by microarray analysis of syncytial cells collected by laser capture microdissection (LCM). Roots inoculated with SCN were fixed and embedded in paraffin. Cross-sections were placed on special LCM slides with a thin membrane. Syncytial sections were identified and cut using a laser, which also cut the membrane, so the syncytial cells were released and dropped into a sterile microfuge cap. RNA was extracted from the syncytial cells for gene expression analysis (Klink *et al*., 2010b, 2011; Ithal and Mitchum, 2011). Many genes associated with cell wall modifications were found to be up-regulated in root pieces colonized by SCN. However, two xyloglucan endotransglucosylase/hydrolase transcripts were distinctively down-regulated in the SCN-colonized soybean roots (Tucker

Table1. Summary of transcriptome and proteome analyses of soybean cyst and root-knot nematodes.

Nematode	Population	Plant	Infection time points	Type of analysis	Reference
Heterodera glycines	SCN race 3	*Glycine max* Huipizhi Heidou and Liaodou 15	30 dpi	Gel-based proteomics	Liu *et al*., 2011
Heterodera glycines	NL1-RHg	*Glycine max*		Gel-based proteomics (2DE LC-MS/MS)	Chen *et al*., 2011
Heterodera glycines	SCN race 2	*Glycine max* TN02-226 and TN02-275	3,6,9 dpi	Affymetrix GeneChip and Quantitative real-time RT-PCR	Mazarei *et al*., 2011
Heterodera glycines	HG type 0	*Glycine max* NIL 34-23 and NIL 34-3	10 dpi	Gel-based proteomics (2DE ESI-MS/MS)	Afzal *et al*., 2009
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* PI88788	9 dpi	Affymetrix GeneChip	Klink *et al*., 2010a
Meloidogyne incognita			In vitro secretome	Gel-free proteomics (nanoLC ESI MS/MS)	Bellafiore *et al*., 2008
Heterodera glycines	NL1-RHg, HG-type 7 and TN8, HG-type 1.3.6.7	*Glycine max* Peking	12 hpi, 3 and 8 dpi	Affymetrix GeneChip	Klink *et al*., 2007a
Heterodera glycines	PA3, HG-type 0	*Glycine max* Williams 82	2,5,10 dpi	Affymetrix GeneChip	Ithal *et al.*, 2007
Heterodera glycines	NL1-RHg, HG-type 7, race 3	*Glycine max* Williams 82	8, 12, 16 dpi	Affymetrix GeneChip	Puthoff *et al*., 2007
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* Kent	6, 12, 24 hpi and 2,4,6,8 dpi	cDNA microarray	Alkharouf *et al*., 2006
Heterodera glycines	NL1-RHg, HG-type 7	*Glycine max* Kent	2 dpi	cDNA microarray	Khan *et al*., 2004
Meloidogyne incognita			In vitro secretome	Gel-based proteomics	Jaubert *et al*., 2002
Heterodera glycines	NL1-RHg	*Glycine max* Kent	3 dpi, 8 dpi	LCM analysis	Klink *et al*., 2010b

et al., 2007). Much of the current gene expression analysis of the interaction of SCN with soybean has been conducted using microarrays. However, next generation sequencing promises to provide even more information by revealing undiscovered genes, alternative gene transcripts, and other information. For example, Matsye *et al*. (2011) examined the interaction of SCN with two different soybean genotypes with different defense responses to SCN. One genotype, Peking, responds quickly to SCN, while the second genotype, PI 88788, responds more slowly. This study overlaid transcript abundance on biochemical pathways to visualize a portion of the data. Distinct transcript profiles were noted for the defense responses of these two different soybean genotypes. Studies such as these provide more information about gene expression than is possible with microarrays.

To better understand the effects of *M. incognita* infestation on roots, Ibrahim *et al*. (2011) studied the *G. max* gene expression in galls formed in roots. Their microarray results showed that some members of the cyclin-dependent kinases family were differentially expressed, which may be related to plant nuclear division observed during giant cell formation resulting from *M. incognita* feeding. Moreover, the study showed up-regulation of many genes responsible for cell wall remodeling and extension in soybean roots including xyloglucan endotransglycosylase/ hydrolase, endoxyloglucan transferase A2, pectin esterase, and endo-1,4-glucanase with a concomitant down-regulation of cellulose synthase during the early time points of infestation. On the other hand, their experimental data suggested that *M. incognita* directs the metabolism of a soybean root cell to increase the production of nucleotides required for nuclear division, which occurs during giant cell formation. For example, the gene encoding glucose-6-phosphate isomerase is up-regulated, and this enzyme provides precursors leading to the production of nucleotides required for DNA replication.

NEMATODE GENES AND PROTEINS INVOLVED IN SOYBEAN INFECTION

Much work has been put into the characterization of nematode effector proteins in recent years. Nematode parasitism genes encode secreted effector proteins that alter the host cell to form a feeding site. Studies aimed at the identification of nematode effectors have mainly focused on nematode genes regulated during parasitism with special emphasis on genes expressed in the oesophageal glands (Ithal *et al*., 2007; Elling *et al*., 2009). A list of candidate effector proteins secreted by the nematode into the plant tissues to promote infection has been developed (Bellafiore *et al*., 2008; Lu *et al*., 2009). However, the exact role of most of these proteins during root invasion or during feeding cell induction and maintenance remains unknown. Only a few of these proteins have been studied (Bakhetia *et al*., 2007, 2008). Bellafiore *et al*. (2008) directly identified 486 proteins secreted by *M. incognita*. The main limitation to carrying out precise protein identification at that time was that the *M. incognita* genome sequence had not yet been reported. The authors partially overcame this limitation using mass spectrometry and identified proteins that contain segmental sequence identity in four databases, namely parasitic nematode proteins (http://www.nematode.net), NCBI nr nematode proteins, *M. incognita* EST database from INRA-Sophia Antipolis, and NCBI nr plant proteins, depending on the conservation of protein sequences between species that enables a protein database from one species to partially substitute for a database from the cognate species.

Some secretory proteins from the nematode oesophageal glands show similarities with plant proteins. Plant CLAVATA3/ESR-related (CLE) peptides have diverse roles in plant growth and development. Proteins from the CLAVATA3/ESR (CLE)-like family have been identified only in plants and plant nematodes (Mitchum *et al*., 2008; Lu *et al*., 2009). Surprisingly, the secretome of Brugia malayi, one of the three causative agents of lymphatic filariasis in humans, was observed to overlap that of *M. incognita*, suggesting a common parasitic mechanism between nematodes of animals and plants. Most of the identified proteins were involved in stress response, detoxification, protein folding, energy metabolism, protease inhibitors, and proteins with putative nuclear localization (Bellafiore *et al*., 2008). The *M. incognita* proteins involved in protein degradation were over-expressed in parasitic endophytic third-stage juveniles (J3) in comparison with pre-parasitic exophytic second-stage juveniles (J2), and glutathione S-transferases are secreted during parasitism. These are required for completion of the nematode life cycle by remodeling the plant responses from *M. incognita* infection (Dubreuil *et al*., 2007). Two cysteine proteases encoding cathepsin L-like enzymes have also been isolated from feeding female *M. incognita* (Shingles *et al*., 2007). In addition, Jaouannet *et al*. (2012) identified three genes expressed in the oesophageal glands of *M. incognita* J2 that encode secreted protein effectors using comparative genomics. One of these genes, Mi-EFF1, is a predicted nuclear localization signal probably involved in manipulating nuclear functions of giant cells. Many of the gene products, such as reactive oxygen species (ROS) modifying enzymes, are involved with establishment of a successful parasitic interaction. The ROS-producing and ROS-scavenging enzymes from both the pathogen and the host affect the redox state at the host-pathogen interface. Dubreuil *et al*. (2011) showed that *M. incognita* Clade B peroxiredoxin genes are more actively transcribed in parasitic stages in the hypodermis and pseudocoelum. Klink *et al*. (2009b) reported that *G. max* genotype (Peking) interacts differently with two different populations of *H. glycines*

during resistant and susceptible reactions. Differential expression and false discovery rate analyses identified genetic expression patterns for these two populations of *H. glycines* (incompatible population, NL1-RHg and compatible population, TN8) both before and after they undergo resistant or susceptible reactions. These analyses identified differentially expressed parasitism genes at 12 hr, 3 d, and 8 d post infection, including genes that are suppressed in the incompatible population and are important during the parasitic stages of *H. glycines*. Recent microarray analyses, using the Affymetrix Soybean Genome Array examining two genetically related soybean lines, TN02-226 and TN02-275, inoculated with the same SCN population, provided additional insights into the specific alterations in gene expression of a susceptible and a resistant reaction, which helped to identify genes involved in host defense. The results of this study showed that the expression levels of 162 transcripts changed significantly in the resistant line, of which 84 increased while 78 decreased. However, in the susceptible line, 1,694 transcripts changed significantly, of which 674 increased while 1,020 decreased. The altered genes were associated with metabolism, transcription, cell wall modification, plant defense, and signal transduction (Mazarei *et al*., 2011).

The roles of soybean cyst nematode parasitism genes are not well-defined. However, a group of *H. glycines* genes with potential functions during soybean-nematode interactions have been identified by an extensive in silico study (Elling *et al*., 2009). These include genes for secretory proteins that change expression with the onset of parasitism, signal peptide-bearing gene products with similarity to plant histone deacetylase, and *H. glycines* genes that are conserved in soil-living microbes or soybean. Soybean cyst nematodes produce three known serine proteases, *H. glycines* serine protease-I, -II, and –III, which may play a role in protein turnover and digestion (Lilley *et al*., 1997). Moreover, cyst nematodes secrete proteins from their dorsal gland that are similar to plant proteins. In different gene expression studies, over 60 differentially expressed parasitism secreted proteins have been identified from the oesophageal gland cells of *H. glycines* (Wang *et al*., 2001; Gao *et al*., 2001; 2003). Furthermore, *H. glycines* secretes effector proteins that function as peptide imitators of plant CLAVATA3 / ESR (CLE)-like peptides and are possibly involved in root cells reprogramming to form syncytium (Wang *et al*., 2010).

RNAi gene silencing is one tool currently being used to explore the functions of nematode genes and as a novel way to inhibit nematode development. Several soybean cyst nematode genes have been silenced, including a gene-encoding, small ribosomal protein 3a (Hg-rps-3a [accession number CB379877]) and 4 (Hg-rps-4 [accession number CB278739]), synaptobrevin (Hg-snb-1 [accession number BF014436]), and a spliceosomal SR protein (Hg-spk-1 [accession number B1451523.1]) (Klink *et al*., 2009a). A second tool that shows promise is the overexpression of plant genes to confer resistance to nematodes (Matsye *et al*., 2012; Matthews *et al*., 2013; Youssef *et al*., 2013; Maldonado *et al*., 2014a, 2014b; Matthews *et al*., 2014; Pant *et al*., 2014;Youssef and Matthews, 2014).

Proteomic technologies are powerful tools for examining proteome alterations caused by mutations, genetic modifications, or responses to different stimuli including nematode infection (Görg *et al*., 2000; Dubey and Grover, 2001; Afzal *et al*., 2009). Recent progress in the availability of immobilized pH gradient (IPG) strips with different pH ranges, image analysis software, and modern mass spectrometers, together with the establishment of protein databases, have significantly increased the accuracy of protein characterization from complex protein mixtures and offer high-throughput analysis. For proteomic analysis, a number of separation steps are usually used to decrease sample complexity. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) separation involves first separating proteins based on their isoelectric point. The second step in two-dimensional electrophoresis (2DE) is to separate proteins based on molecular weight using polyacrylamide gel electrophoresis (Natarajan *et al*., 2005).

Recently, Chen *et al*. (2011)built a full proteome 2-DE reference map of *H. glycines* J2 stage and the identified proteins were characterized by their function in diverse biological processes. The reference map represented 816 different proteins and showed 20 secreted proteins of *H. glycines* including β-1,4-endoglucanase, pectatelyase, and expansin, which are involved in plant cell wall degradation. A distinguishing feature of this work is that the author examined gene expression at the translational level using 2D-PAGE and further analyzed the identified proteins function using the Gene Ontology (GO) database.

Another 2-DE analysis compared resistant soybean root (cv. Huipizhi Heidou, Chinese black soybean) infected by *H. glycines* Race 3 with the susceptible cultivar Liaodou 15 by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS). This resulted in 16 differentially expressed and identified proteins known to be involved in defense, energy and metabolism, suggesting they may play a role in soybean resistance to *H. glycines*. The author further reported that some proteins including trypsin inhibitor p20, Xylem serine proteinase 1 precursor, triosephosphate isomerase, caffeoyl coenzyme A 3-O-methyltransferase were up-expressed while putative RNA polymerase III and ATP synthase beta chain were down-expressed in the resistant samples (Liu *et al*., 2011).

In a related study focusing on proteome and metabolome, changes between resistant and susceptible near isogenic lines (NIL) of soybean

roots were reported. Comparisons were made between control and treated NIL 10 d post SCN infestation (dpi). 2D-PAGE and quadruple time-of-flight tandem mass spectrometry were utilized for proteome analysis, and gas chromatography-mass spectrometry was used in metabolite analysis (Table 1). The altered proteins and metabolites in roots after SCN infestation in the resistant NIL included nine up-regulated proteins and metabolites in the glutathione pathway. Moreover, interactome analysis showed that chaperonin, F1-ATPase, multicatalytic endopeptidase, thaumatin-like (TL) protein, cytosolic HSP, and triose-P isomerase were interacting indirectly through two to seven intermediates. The protein functions included transcription factors, chaperonins, signal transduction factors, and metabolism involved in energy generation. The author suggested that the regulation of protein degradation and protein transport across the nuclear membranes might be important for resistance to SCN (Afzal *et al*., 2009). Despite their excellent resolving power, 2D gels are limited in several respects. One problem is the sensitivity and reproducibility of detection (Gygi *et al*., 2000). A second potential problem with 2D gel separations is due to post-translational and proteolytic modifications. A third limitation is under-representation of membrane proteins, which account for approximately 30% of total proteins, thus limiting full proteome characterization (Stevens and Arkin, 2000). In the future, data from proteomics will be integrated with microarray data so changes in transcript levels and protein abundance can be used to provide a better picture of events occurring within the plant and within particular cells during nematode infection.

CONCLUSION

Soybean-parasitic nematodes are a major problem for soybean agriculture both in developed and developing countries. A number of biotechnology-based approaches have been described to understand the soybean nematode interactions involving resistant and susceptible genotypes. However, there is still a lack of information on many aspects of soybean-nematode interaction. Moreover, the advances in transcriptome and proteome analyses of soybean root–nematode interactions have demonstrated an overlap in the signaling pathways during the early time points of nematode infection of soybean roots. The goal is to identify the full proteome of both nematode and soybean to better understand how nematode proteins interact with soybean root cells. Proteomic approaches could lead to the discovery of more nematode protein effectors as potential targets for the development of nematode resistant soybean varieties.

ACKNOWLEDGMENT

The authors gratefully acknowledge support from a grant from United Soybean Board project number Y9254.

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Received: *8/XI/2013*
Recibido:

Accepted for publication: *21/V/2014*
Aceptado para publicación: