FUNCTIONAL GENOMIC ANALYSIS OF WALNUT-NEMATODE INTERACTIONS

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ABSTRACT

Plant parasitic nematodes can devastate walnut orchards and are able to remain dormant deep in the soil for several years, beyond where traditional pesticides can penetrate. The only effective pesticides are often very toxic to beneficial organisms and are detrimental to the environment; several of the most effective are in the process of being phased-out. To develop novel methods of diagnosis and control, it is necessary to characterize pathogens and their interactions with host and non-host plants by performing genomic and functional genetic analyses. This project focuses on the interaction between walnut roots and *Pratylenchus vulnus*, an important nematode parasite. The primary goal is to develop a knowledge base of the genes involved in the interaction between the nematode and the walnut root on which it feeds. Genomics tools, including DNA sequencing and microarrays, are being utilized to create a database available to the research community. The information is being analyzed to identify walnut genes whose expression is changed by nematode infection. This study will establish relationships between nematode infection of walnut roots and the expression of specific genes in the nematode and the walnut host and will allow diagnostic tests to be developed to identify the presence and population of specific parasites within the plant's rhizosphere. In addition, functional genomic techniques are being used to suppress the expression of specific nematode genes to identify those that may be able to be targeted by novel pesticides.

OBJECTIVES

1. To survey the pattern of genes expressed in walnut roots and in the feeding nematodes that determine the outcome of an infection.
   a) Generation of expressed sequence tags (ESTs) by sequencing cDNA libraries prepared from mRNA isolated from *P. vulnus*, and from infected and uninfected walnut plants.
   b) Construction of a database of nematode and walnut genomic information compiled from the ESTs and made available to all researchers.
   c) Genomic analysis of the nematode and walnut databases to assign putative functions to particular genes and identify candidates for functional analysis.

2. To identify and validate nematode and walnut genes associated with the infection process in healthy and sick plants.
   a) Construction and analysis of walnut gene chips to compare expression levels between uninfected and infected plants.
   b) Confirmation of differentially expressed walnut genes by Taqman® real-time quantitative RT-PCR.
   c) Functional analysis of nematode genes using RNA interference in vitro.
   d) Functional analysis of nematode genes using carrot disc assays.

2006 was the first year of this four-year project. During the first ten months of the project, objectives 1a and 2c were primarily addressed.

PROCEDURES

Objective 1a. Generation of ESTs by sequencing cDNA libraries prepared from mRNA isolated from *P. vulnus*, and from infected and uninfected walnut plants.

Construction of *P. vulnus* cDNA library (PV-02): Carrot discs were inoculated with nematodes from walnut root cultures maintained in the McGranahan lab. Seven weeks later, 11 of these discs were individually soaked in 10 mL of ¼ strength M9 minimal media for three hours to remove nematodes. Worms were packed by centrifugation (300 g, for 2 minutes). RNA was isolated from 50 µL samples of packed worms using TRIzol reagent (Invitrogen, Carlsbad, CA) and was further purified with an RNA/DNA Mini Kit (Qiagen, Valencia, CA). Using the Creator SMART™ cDNA Library Construction Kit (Takara Clontech, Palo Alto, CA), mRNA was reverse-transcribed to cDNA and ligated into vector pDNR-LIB, which was subsequently electroporated into *E. coli* strain DH10ß (Invitrogen). Library titer was calculated to be 2.3 x 10^7 cfu/mL. 36 colonies were checked by PCR, resulting in 94% with inserts and an average insert size of 820 bp. The library was frozen as glycerol stocks which were subsequently delivered to the UCDavis CAES Genome Facility where 3840 colonies are being sequenced from this library.

Inoculation of Paradox clones with *P. vulnus* and harvesting of plants for RNA isolation: Ten vegetatively propagated clones of Paradox line Px1 (*J. regia x J. hindsii*) were grown in liners in a greenhouse that does not receive pesticide treatment. Half of the clones were inoculated with approximately 6400 nematodes per plant. The other half were left uninfected. Six weeks after inoculation, the leaves and roots of each plant were separated and individually frozen in liquid nitrogen and stored at -80°C. RNA was individually isolated from root and leaf samples of each plant using the hot borate method (essentially as described by Wilkins and Smart, 1996). After isolation, RNA was further purified with an RNA/DNA Mini Kit (Qiagen).

Construction of walnut root and leaf cDNA libraries (WRO-02, WRN-02, WLO-02, WLN-02): A portion of the RNA from the plants in each set of clones was pooled and used to construct cDNA libraries WRO-02, WLO-02, WRN-02 and WLN-02. cDNA libraries were constructed as described above for library PV-02. Table 1 details all libraries which have been constructed for Paradox clone Px1 as well as for *P. vulnus*. Titer and insert size of each library was determined as described above. These data are shown in Table 2. The libraries were frozen as glycerol stocks which were subsequently delivered to the UCDavis CAES Genome Facility where 3840 colonies are being sequenced from each of the four walnut libraries.

Objective 2c. Functional analysis of nematode genes using RNA interference in vitro.

Selection of *P. vulnus* genes for functional analysis: In 2004, 2485 EST sequences for *P. vulnus* were deposited into the NCBI GenBank database. These ESTs were subsequently aligned to form 242 contigs using Vector NTI software (Invitrogen). A BLAST analysis was performed to compare the *P. vulnus* contigs with *Caenorhabditis elegans* sequences to identify homologs of *C. elegans* genes that should produce observable phenotypes when suppressed. Because the initial experiments will establish the effectiveness of the protocols, only genes meeting the following criteria were included:

- Only *P. vulnus* contigs were used, to increase the accuracy of the sequence and to improve the chance that these genes are expressed at a detectable level. Singletons were not considered because they may be expressed at a low level.
- Each contig member must be homologous (tblastx E-value $\leq 10^{-10}$) to the same gene or members of a gene family in *C. elegans*.
- All reported RNA interference (RNAi) experiments for the homologous *C. elegans* gene must consistently show phenotypes of reduced fecundity (e.g., sterile, embryo lethal, larval lethal, etc.) for candidate genes or wild-type phenotypes for negative control genes, with a minimum of two RNAi experiments for each *C. elegans* gene.
- The length of each contig must be sufficient to allow for a 400 bp segment to be used for RNAi and a 100+ bp segment to be used for real-time PCR. (The real-time PCR amplicon is outside of the RNAi sequence to ensure that no artifacts of in vitro transcription will be detected.)

Based on the *C. elegans* phenotypes, five genes were chosen whose suppression should result in reduced fecundity when compared with untreated nematodes. An additional three genes with wild-type RNAi phenotypes were chosen to act as negative controls. These genes are detailed in Table 3. Primers and probes for Taqman® quantitative real-time RT-PCR (ABI Biosystems, Foster City, CA) were designed and validated for each gene to ensure that transcript levels can be measured. This design and validation was performed at the UCDavis Lucy Whittier Molecular and Diagnostic Core Facility.

Construction of in vitro transcription vectors for *P. vulnus* genes: A 400 bp segment of each of the eight *P. vulnus* genes and a GFP non-nematode negative control were cloned into the plasmid vector pDU19 (Ambion, Austin, TX) between T3 and T7 promoters on opposite strands. (An example is shown in Figure 1.) This produced a series of vectors, pDP19-PV010 through pDP19-PV194, corresponding to each gene listed in Table 3.

Generation of dsRNA by in vitro transcription: Two templates are prepared by PCR for each nematode gene, one with the T7 primer and the other with the T3 primer. Gene-specific reverse primers are used to amplify the 400 bp section of the specific pDP19-PV vector. Multiple PCR reactions are run to generate high concentrations of each template.

In vitro transcription to generate dsRNA: T3 and T7 MEGAscript® kits (Ambion) are used to transcribe gene specific RNA. For each nematode gene, one reaction with T3 polymerase enzyme and one reaction with T7 polymerase enzyme are run. A minimum of 500 ng of appropriate template is used for each in vitro transcription reaction. Reaction components and conditions are per the manufacturer's instructions, with each in vitro transcription incubated at 37°C for 4-5 hours. Subsequently, the two complementary RNA strands are mixed together and annealed by first incubating at high temperature (5 minutes at 95°C, followed by 15 minutes at 70°C) followed by gradual cooling (2°C per minute to 4°C). The dsRNA is subsequently purified by lithium chloride precipitation. Single stranded RNA transcripts and annealed dsRNA concentrations are measured by spectrophotometer and checked for integrity by running on a 2% agarose gel.

Induction of RNAi in *P. vulnus* by soaking in dsRNA: RNAi is induced in *P. vulnus* by soaking the nematodes in an optimized solution containing dsRNA and 10-25 mM resorcinol. In the lab, populations of *P. vulnus* are maintained in vitro on roots of germinated Paradox (line Px1) somatic embryos. Nematodes are then subsequently transferred to carrot discs in vitro. Carrots provide two advantages over walnut roots for use in these experiments: First, carrot discs can be maintained for several months in vitro without the need for media; second, carrots are available in much greater quantity than walnut roots, allowing for many experimental replications. *P. vulnus* are subsequently isolated from carrot discs by soaking each disc in ¼ strength M9 minimal media for several hours. To induce RNAi, 1000 individuals per replicate are soaked for 24 hours in the solution containing dsRNA and resorcinol. After soaking, the nematodes are washed 2-3 times in ¼ strength M9 minimal media and left to recover for 48 hours. The media is then drawn off and the nematodes are dissolved in 1X ABI buffer and frozen. The samples are then delivered to the UC Davis Lucy Whittier Molecular and Diagnostic Core Facility where the Taqman analysis is performed.

RESULTS AND DISCUSSION

Objective 1a. Generation of ESTs by sequencing cDNA libraries prepared from mRNA isolated from *P. vulnus*, and from infected and uninfected walnut plants.

Current status of EST sequencing from *P. vulnus* and Paradox cDNA libraries

EST library sequencing is currently in progress and is expected to be concluded in January 2007. When finished, 3840 sequences will have been generated from each of the five libraries. Table 4 shows the number of sequences generated as of December 6, 2006. Table 1 shows the projected number of high quality ESTs that are expected to be generated from each library by the completion of sequencing.

Objective 2c. Functional analysis of nematode genes using RNA interference in vitro.

The cellular process by which double-stranded RNA (dsRNA) activates sequence-specific degradation of mRNA transcripts is believed to have evolved as a viral defense mechanism. RNA interference (RNAi) is triggered by dsRNA and certain ssRNA (for which the complementary strand is synthesized by an RNA-dependent RNA polymerase). The dsRNA is cleaved by a specific RNase III-type endonuclease (Dicer) into fragments (approximately 21 nt long with 2 nt 3′ overhangs) termed small interfering RNA (siRNA). The siRNA duplexes are subsequently incorporated into ribonuclotide-protein complexes (siRNPs), where the strands are separated. In an ATP-dependent process an siRNP is activated to form the RNA-induced silencing complex (RISC). There, the single-stranded siRNA guides the RISC to cleave complementary mRNAs, resulting in silencing of gene expression (Meister and Tuschl, 2004; Dykxhoorn *et al*., 2003).

RNAi in *C. elegans* was first documented when Fire *et al*. (1998) demonstrated that dsRNA was far more effective than either sense or antisense ssRNA in suppressing specific gene expression. Maeda *et al*. (2001) optimized RNAi by soaking as an efficient high-throughput method. Briefly, cDNA is amplified by PCR using primers that add promoters for bacteriophage T3 and T7 RNA polymerase. These amplicons are then transcribed in vitro to produce dsRNA. Worms are soaked in a solution containing the dsRNA and then transferred to feeding plates. Phenotypes

in *C.elegans* are observed in the P0 generation (soaked nematodes) and the F1 generation (their progeny). This procedure has been used successfully to induce RNAi in several species of plant parasitic nematodes (Fanelli et al., 2005; Bakhetia et al., 2005; Rosso et al., 2005; Urwin et al. 2002).

Because the susceptibility to RNAi by soaking differs between species of nematodes, it is necessary to optimize the soaking conditions for each species. In 2006, three soaking experiments have been performed to determine optimal concentrations of resorcinol, a compound previously shown to induce the uptake of dsRNA in root knot nematodes (Rosso et al., 2005), and the relative reduction in transcript levels of three different genes. As shown in Figure 2, it appears that concentrations of 10 mM and 25 mM resorcinol reduce transcript levels significantly when compared to controls containing no resorcinol or treatments with higher concentrations of resorcinol. Additional optimization experiments should be completed by early 2007. At that time, a statistically significant number of replicates will be performed on each of the eight genes to verify that transcription can be suppressed by RNA interference.

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Table 1. cDNA Libraries from *P. vulnus* and Paradox Walnut Rootstock

Library	Year	Organism/ Tissue	Source/Treatment	ESTs
PV-01	2004	*P. vulnus*	In vitro: On germinated Px1 somatic embryos	2485
PV-02	2006	*P. vulnus*	In vitro: On germinated Px1 somatic embryos	~3400*
WRO-01	2004	Paradox (clone Px1) roots	In vitro: germinated somatic embryos	~500
WRN-01	2004	Paradox (clone Px1) roots	In vitro: germinated somatic embryos infected with *P. vulnus*	~600
WRO-02	2006	Paradox (clone Px1) roots	Plants in liners in greenhouse	~3500*
WLO-02	2006	Paradox (clone Px1) leaves	Plants in liners in greenhouse	~3000*
WRN-02	2006	Paradox (clone Px1) roots	Plants in liners in greenhouse infected with *P. vulnus*	~3400*
WLN-02	2006	Paradox (clone Px1) leaves	Plants in liners in greenhouse infected with *P. vulnus*	~3400*

*Based on 3840 total sequences multiplied by percentage high quality reads as of 12/6/06 (see Table 4)

Table 2. Titers for cDNA Libraries from *P. vulnus* and Paradox Walnut Rootstock

Library Name	Species/ Cultivar	Tissue	Titer cfu/mL	Clones checked	Clones with insert	Insert size range, bp	Average insert size, bp
PV-02	*Pratylenchus vulnus*	Whole, mixed stage	2.3×10^7	34	94%	300-1700	820
WLN-02	Paradox Px1	Leaves from infected plants	3.3×10^6	28	100%	300-1700	800
WRN-02	Paradox Px1	Roots from infected plants	1.7×10^7	28	100%	300-1500	650
WLO-02	Paradox Px1	Leaves from control plants	2.3×10^6	28	100%	300-1700	840
WRO-02	Paradox Px1	Roots from control plants	3.0×10^6	28	100%	300-1750	690

Table 3. *Pratylenchus vulnus* Genes Chosen for Initial RNA Interference Experiments

P. vulnus contig	*C. elegans* homolog	*C. elegans* gene product	*C. elegans* RNAi Phenotype
Pv-010	C50C3.6 (prp-8)	U5 snRNP spliceosome subunit	Sterile, Larval Lethal
Pv-035	F54C9.5 (rpl-5)	60S ribosomal protein L5	Embryo Lethal, Sterile, Larval Arrest
Pv-057	F54C1.7 (pat-10)	Troponin C	Sterile, Embryo/ Larval Lethal
Pv-058	F17E9.9 (his-34) T10C6.11 (his-4)	H2B histone	Sterile, Embryo Lethal, Larval Arrest
Pv-066	Y54E2A.11a (eif-3.B)	Translation initiation factor	Sterile, Embryo/ Larval Lethal, Larval Arrest, Sterile Progeny
Pv-015	M60.4a	N/A	Wild-type
Pv-130	F21F3.1	Similar to certain monooxygenases	Wild-type
Pv-194	JC8.8	Uncharacterized protein with conserved cysteine	Wild-type

Table 4. Status of EST Sequencing from *Pratylenchus vulnus* and Paradox cDNA libraries

Library Name	Species/ Cultivar	Tissue	Sequences completed*	High Quality	Average Length**
PV-02	*P. vulnus*	Whole, mixed stage	2304	2055 (89%)	507 bp
WLN-02	Paradox Px1	Leaves (infected plants)	1536	1344 (88%)	507 bp
WRN-02	Paradox Px1	Roots (infected plants)	2304	2047 (89%)	463 bp
WLO-02	Paradox Px1	Leaves (control plants)	3072	2378 (77%)	434 bp
WRO-02	Paradox Px1	Roots (control plants)	1536	1398 (91%)	504 bp

* As of December 6, 2006.

** Average length of sequence after trimming for quality and vector sequences.

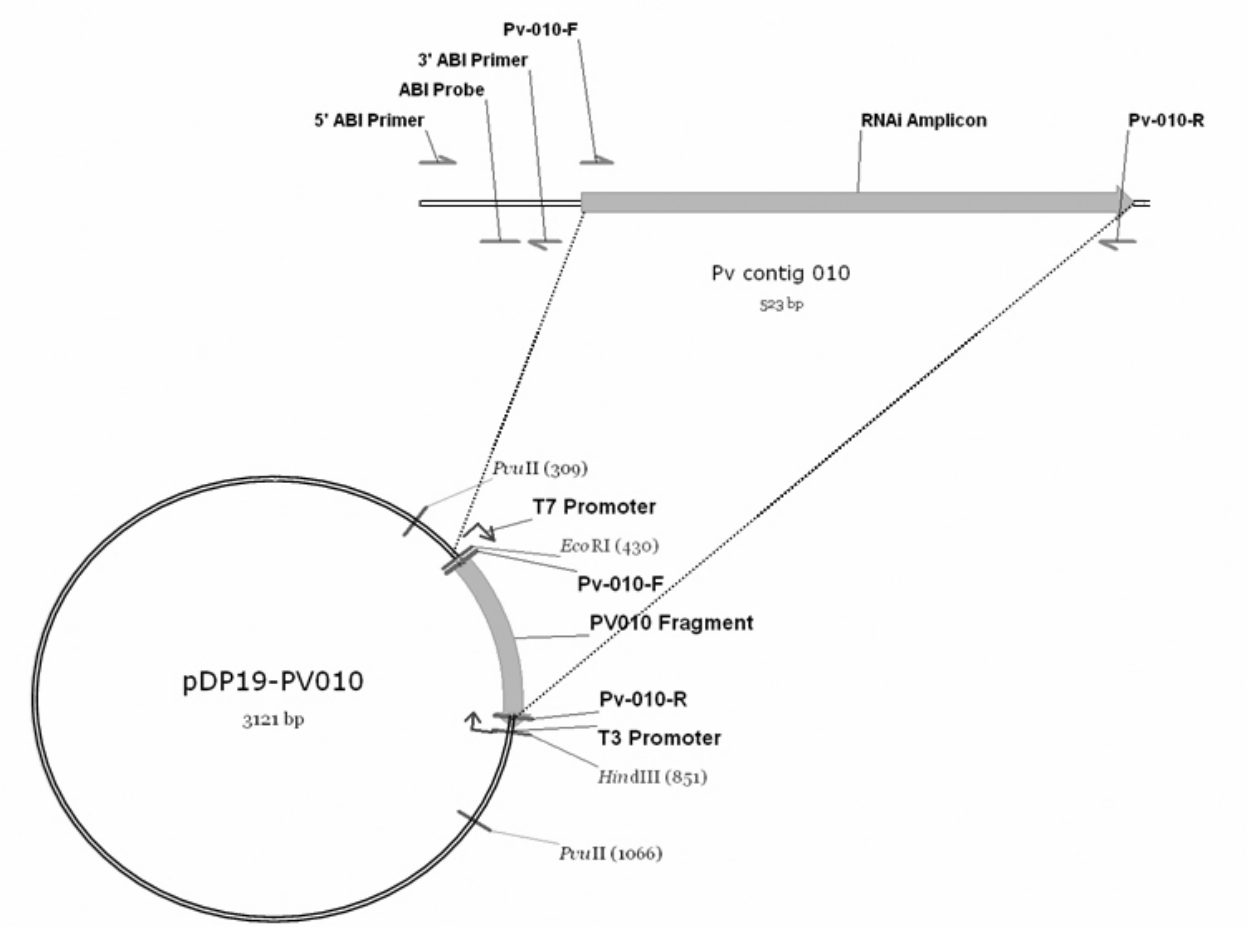

Figure 1. Construct for in vitro transcription of RNAi amplicon. The contig Pv-010 corresponds to a gene in *P. vulnus* to be targeted for silencing by RNAi. Primers and probes for Taqman® quantitative RT-PCR analysis were designed at the 5′ end of the sequence. A non-overlapping 400 bp RNAi amplicon is cloned into the vector pDP19, where it is flanked by the T7 and T3 promoters on opposing strands. Each strand is separately transcribed and the RNA annealed to form dsRNA.

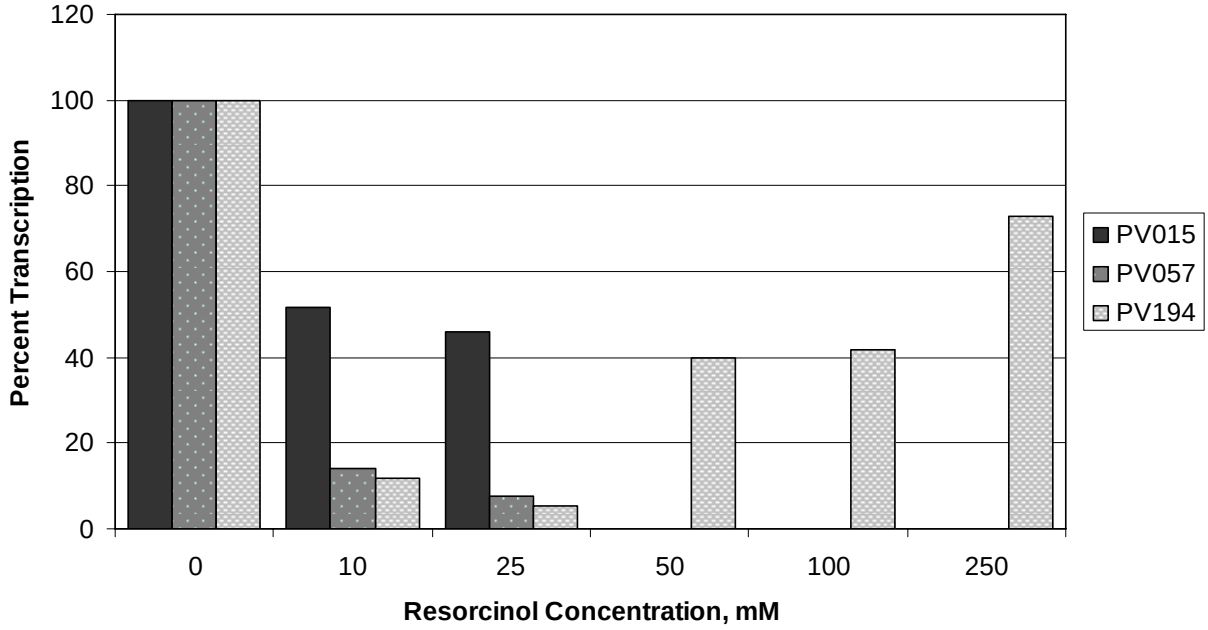

Figure 2. Suppression of *P. vulnus* Gene Expression by RNAi. Each bar represents an average of 2 or 4 samples (of approximately 1000 nematodes each) that had been soaked in a solution of dsRNA corresponding to a specific nematode gene (i.e.., PV015, PV057 or PV194) and resorcinol at varying concentrations. Percent transcription for each treatment is compared to negative controls with no resorcinol, which are shown at 100% transcription. (Please note that the goal of these experiments was to determine the concentration of resorcinol that gave the best suppression, and the individual samples do not represent statistical replicates. Therefore, no error bars are shown.)