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PROJECT PLAN/RESEARCH GRANT PROPOSAL

Project Year Year 3

Anticipated Duration of Project 4 years

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Location UCD

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Project Title: Functional genomic analysis of walnut-nematode interactions.

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Commodity(s) Walnut

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Abstract

Plant parasitic nematodes can devastate mature perennial crops, such as walnut, 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 utilized to create a database available to the research community. The information will be analyzed to identify walnut genes whose expression is changed by nematode infection. This will allow diagnostic tests to be developed to identify the presence and population of specific parasites within the plant's rhizosphere. Using functional genomic techniques, the expression of specific nematode genes will be suppressed to identify those that may be able to be targeted by novel pesticides.

In the first two years we have focused on developing a database for identifying genes expressed during infection. Our study will establish relationships between nematode infection of walnut roots and the expression of specific genes in the nematode and the walnut host. In the last two years we will emphasize expression and functional analysis to identify specific nematode genes that may be targeted by novel pesticides or walnut genes that can be used to screen for tolerant progeny in a conventional breeding program. This information will increase our understanding not only of the underlying causes of susceptibility and/or resistance but will facilitate the development of DNA-based diagnostic and therapeutic strategies to detect and treat these infections.

Problem and its Significance

The California walnut industry suffers significant annual losses to soil related pests and diseases in the form of unsaleable nursery stock, lowered productivity from infected trees, and increased susceptibility of infected plants to other pathogens/pests and to adverse environmental conditions (Agrios, 1997). In particular, plant parasitic nematodes are very difficult to eradicate. Existing chemical nematicides are extremely toxic, not only to pathogenic nematodes, but also to beneficial organisms, birds, fish and mammals. Many are also phytotoxic, so can only be used before planting (Chitwood, 2002). Due to these concerns, several nematicides have been banned, including methyl bromide, which is being phased out. The remaining alternatives, such as chloropicrin, are less effective against nematodes (Martin, 2003). The development of new broad-spectrum nematicides is unlikely, given the high costs involved in licensing and bringing such products to market. Orchards and vineyards cannot easily be replanted even after rotation with non-host crops because some plant parasitic nematodes, including *P. vulnus*, can remain viable for several years in decaying roots deep in the soil profile, beyond where fumigants can penetrate. Subsequent plantings of clean nursery stock become infected once their roots grow into the deep soil layers (McKenry, 2003).

P. vulnus can be a serious pest in walnut orchards. All commonly used walnut rootstocks (English, black and Paradox) demonstrate susceptibility to nematodes (McKenry, 2004). The primarily mechanical injury interferes with the passage of water and nutrients between the roots and leaves of the plant. Symptoms of *P. vulnus* infection include stunted growth, dieback, chlorotic leaves, as well as cracking and blackening of the roots, leading to death of feeder roots (Ayoub, 1980). When the population of nematodes is high, feeding can cause significant stress to the host plant, resulting in reduced yield and greater susceptibility to bacterial and fungal pathogens entering through the wound sites (Westerdahl and McKenry, 1995).

New methods must be developed to prevent, diagnose and effectively combat nematode infestations. Such approaches require an understanding of the molecular biology of both the nematode and the host plant, as well as the interactions between the plant and the parasite (Chitwood, 2002). However, many pathogens and plants have not been characterized on a molecular basis. There is a need to conduct basic genomic research and to generate databases for gene discovery. This can be accomplished by building libraries of genetic sequences and comparing them to those of better-characterized organisms so that hypothetical functions can be assigned. These data then become a community resource, available to researchers worldwide.

This research is utilizing the most recent technological advances to produce such a database and to identify genes that are differentially expressed in infected walnut roots. This will allow diagnostic tests to be developed to identify the presence and population of specific parasites within the plant's rhizosphere as well as to diagnose asymptomatic plants, an early warning much before plants demonstrate the symptoms of a severe infection. Analysis of the differential gene expression between tolerant and susceptible selections should provide genetic markers to screen for nematode tolerance in germinated seedlings, and may identify pathways that could be exploited in the design of novel nematicides.

Research on plant parasites has been primarily conducted on root-knot and cyst nematodes of the genera *Meloidogyne*, *Globodera* and *Heterodera*. Other plant parasitic species have received little attention in molecular studies, mainly due to their microscopic size and the difficulty of studying organisms that can complete their entire life cycles within a root. In particular, there is no information that might shed light on the reasons why some plants are susceptible hosts, while others are unaffected. The interaction between root lesion nematodes and their host plants has not been studied on a molecular scale. Characterization of nematode genes may provide novel targets for future nematicides to provide therapeutic treatment of infested orchards with low

to no toxicity to other organisms. Accurate field testing, combined with specifically targeted pesticides, will allow growers to better manage their pesticide use, saving money currently spent on application of broad-spectrum agents.

Objectives

Two primary objectives are being addressed in this research:

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 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 to 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 *Medicago truncatula* root assay.

Objectives 1a and 1b have been completed. Work on objectives 1c and 2 is ongoing. Research plans specific to year three of the project are described in the following section.

Work Plans and Procedures

Objective 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 ESTs by sequencing cDNA libraries prepared from mRNA isolated from *P. vulnus*, and from infected and uninfected walnut roots. Nearly 13,000 new walnut expressed sequence tags (ESTs) were generated, along with 3327 new ESTs for *P. vulnus*. Details of these can be found in the walnut research report.
- b) Construction of a database of nematode and walnut root genomic information compiled from the ESTs and made available to all researchers. The ESTs derived in Objective 1a were processed and submitted to the NCBI database by the UC Davis CAES Genome Facility. These sequences were combined with all previous *Juglans* sequences in the NCBI database (primarily *Juglans regia* ESTs) and to generate a set of 8622 consensus sequences representing unique genes.

- c) Genomic analysis of the nematode and walnut databases to assign putative functions to particular genes and identify candidates for functional analysis. The compiled *P. vulnus* contigs and singleton EST sequences will be compared by BLAST analysis with EST and genomic sequences from *C. elegans*, which will be used as a reference nematode due to the high level of organized sequence information available. Databases used will include the NCBI NR and Wormbase (www.wormbase.org). BLAST comparisons will also be made against all other available nematode sequences. Sequence similarity between *P. vulnus* and *C. elegans* may indicate conserved function, while unique genes may represent proteins that have evolved in *Pratylenchus* and which may be necessary for plant parasitic functions. Further analysis using InterProScan (The InterPro Consortium, 2002) will be performed to categorize proteins by metabolic function using Gene Ontology categories (Gene Ontology Consortium, 2000). This analysis will identify candidate genes for subsequent RNAi experiments, after suppression of the initial set of genes has been validated.

The compiled walnut contigs and singletons will also be analyzed by comparison with the NCBI NR database using TBLASTX to assign putative functions. Further analysis using InterProScan will be performed to categorize proteins by metabolic function using Gene Ontology categories.

Objective 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. Microarray analysis is being performed using the NimbleGen NimbleChip 4-plex gene expression array (Roche NimbleGen Inc., Madison, WI). These chips can be fabricated quickly, contain probes specified by the user, and are relatively inexpensive, making them ideal for studying gene expression in non-standard organisms that do not have extensive sequence data available. Our laboratory has successfully used similar microarrays to analyze fruit development in citrus and apple.

A probeset for a NimbleChip 4-plex walnut array was developed from 8457 unique walnut genetic sequences. The arrays include sequences obtained in this project as well as those from *J. regia* and *J. hindsii* (parental species of Paradox) already available in the database. Twelve samples were initially processed. Labeled antisense-RNA (cRNA) synthesized from the mRNA isolated in 2006 was hybridized to the microarrays to identify candidate genes differentially regulated by nematode feeding. Three biological replicates were processed from each of the two tissues (leaves and roots) of infected and uninfected Px1 plants, with one plant representing a single biological replicate to allow robust statistical analysis.

In 2007, a longitudinal study was conducted to determine the time course of a nematode infection on walnut: 37 Px1 clones were grown in the greenhouse; 17 of these were inoculated with *P. vulnus*. Infected and uninfected plants were harvested and frozen at five different timepoints: prior to inoculation (uninfected only), and at one, two, four and eight weeks post-inoculation. RNA was isolated from two tissue types (leaves and roots) from each plant. 56 of these RNA samples (27 leaf and 29 root) were processed and hybridized to the validated microarrays.

Expert assistance on the design of these experiments and statistical analysis of microarray data is being obtained from the Bioinformatics and Data Analysis research group at the UC Davis Institute for Data Analysis and Visualization. The analysis will determine the amount of variability in the system (between biological replicates, treatments, and tissue types) and will identify genes that are differentially expressed between infected and uninfected walnuts.

- b) Confirmation of differentially expressed walnut genes by Taqman® real-time quantitative RT-PCR. Walnut genes whose expression is significantly changed by nematode feeding will be identified through statistical analysis of the microarray data. Differential expression of these genes will be confirmed by Taqman® real-time quantitative RT-PCR. The identification of differentially expressed genes will allow molecular diagnostic tests of nematode infection to be developed in a subsequent research project. It is expected that between 10 and 15 differentially expressed walnut genes will be identified for Taqman confirmation in each of the microarray experiments described in Objective 2a. The transcript level of each gene will be measured from RNA used for each of the biological replicates in the microarray experiments. We anticipate a minimum of 68 samples will be processed over the course of this research, with each sample tested for the transcript level of each differentially expressed walnut gene. The results of the Taqman assays will be analyzed statistically and compared to that of the microarrays to confirm that specific genes are differentially expressed and could be used for the development of diagnostic tests.
- c) Functional analysis of nematode genes using RNA interference in vitro.

RNA interference (RNAi) will be used as a functional genomics tool to suppress the expression of specific *P. vulnus* genes. 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 first isolated from carrot discs by washing with sterile water. 2000 individuals per replication are used for each gene to be silenced. After 24 hours of soaking in an optimized solution containing in vitro transcribed dsRNA, the nematodes are frozen for transcript analysis.

Transcript analysis will utilize Taqman® real-time quantitative RT-PCR to determine if transcription of the targeted gene has been suppressed in comparison with that of nematodes which did not undergo dsRNA soaking and nematodes that were subjected to soaking in dsRNA of negative control genes. In *C. elegans* the RNAi phenotype is transmitted from parent to offspring (Fire et al., 1998). However, *C. elegans* has a much shorter life cycle than *P. vulnus* and reproduces hermaphroditically. It is unknown whether the induction of RNAi by soaking in *P. vulnus* will result in durable suppression of the target gene, or whether this suppression can be efficiently transmitted to offspring. Therefore, in this study, analysis of transcript level will be performed on nematodes frozen immediately after soaking and on those removed from roots after varying incubation times to determine the relative amounts of suppression over the experimental period.

- d) Functional analysis of nematode genes using *Medicago* root assay. RNAi can be induced in *C. elegans* through feeding on *E. coli* that transcribe appropriate dsRNA (Kamath and Ahringer, 2003). Therefore, it is hypothesized that gene expression in plant parasitic nematodes can be suppressed by RNAi by feeding on plant cells expressing dsRNA. Previously, Boutla et al., (2002) extracted RNA from plants demonstrating post-transcriptional gene silencing (PTGS) of Green Fluorescent Protein (GFP). Injection of the RNA extracts into *C. elegans* expressing GFP resulted in silencing of GFP at comparable levels to that seen when in vitro transcribed dsRNA was injected.

This is a novel method that will be tested in this study. Our lab has previous experience in successfully applying the technology of RNAi to combat plant disease. That research showed that expression of hairpin

dsRNA homologous to the *iaaM* and *ipt* oncogenes of *Agrobacterium tumefaciens* results in a plant that does not develop crown gall disease, even when inoculated with highly virulent strains of *A. tumefaciens* (Escobar et al., 2001, 2003). RNAi is induced by transcription of the dsRNA construct, which results in degradation of the oncogene transcripts after infection. The system was shown to be effective in Arabidopsis, tomato and walnut (Escobar et al., 2002).

The current research will attempt to generate plant cell lines resistant to *P. vulnus* by inducing RNAi in the nematodes to specific genes whose suppression results in phenotypes with little or no population growth. Hairy root cultures are a means by which constructs can be quickly and efficiently tested to evaluate this hypothesis, and have previously been used successfully to express protease inhibitors, which were ingested by and subsequently inhibited *G. pallida* (Urwin et al., 1995). In this study, genes that have been successfully silenced by soaking nematodes in dsRNA (see Objective 2c) will be screened using a hairy root system in *Medicago truncatula* (Boisson-Dernier, 2001), a model plant that is phylogenetically closely related to walnut.

The gene segment will be the same as that used for dsRNA soaking, and will exclude start and stop codons, so that the transcript cannot be translated into a functional protein in the plant. The gene segment will be cloned in both orientations (hairpin) into a binary vector, suitable for *Agrobacterium*-mediated transformation, to allow simultaneous transcription and annealing of both sense and antisense strands. The resulting binary vector plasmids will be transformed into *Agrobacterium rhizogenes* by electroporation. (See Figure 1.)

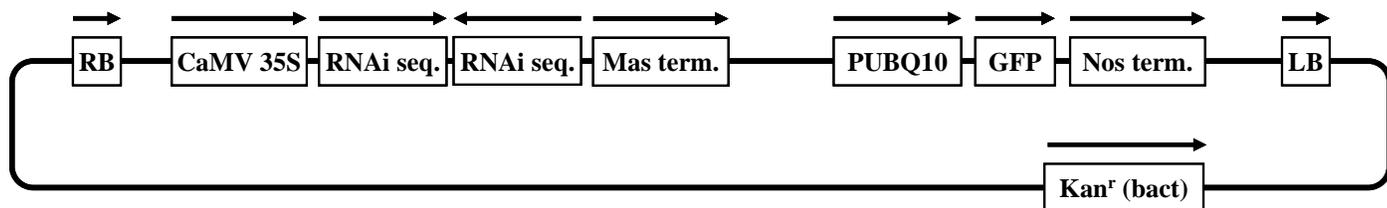


Figure 1. Binary vector used for hairy root transformation. cDNA corresponding to a portion the gene to be silenced (“RNAi seq.”) is inserted as a hairpin following the CaMV 35S promoter. After transformation by *Agrobacterium rhizogenes*, transcription in planta will produce double-stranded RNA (both sense and antisense RNA strands). (Backbone vector is pB7 from J.E. Lincoln.)

A. rhizogenes is a plant pathogen, characterized by the production of adventitious roots with many root hairs at the site of infection. Virulent strains of *A. rhizogenes* possess Ri (root-inducing) plasmids containing T-DNA (transfer DNA) that is incorporated into the infected plant cell’s nuclear genome. Expression of a subset of the genes (termed *rol* genes) on the T-DNA is responsible for the growth of adventitious roots (Meyer et al., 2000).

Medicago roots will be transformed in vitro with *A. rhizogenes* containing the binary vector. Transcript levels in the resulting hairy root cultures will be measured by Taqman® real-time quantitative RT-PCR and Northern blotting. Although it might be expected that expression of dsRNA in plant cells would result in its immediate degradation to siRNA, generally, in plant RNAi experiments, many transformants must be evaluated to find a few that effectively silence the gene. In some lines transformed with silencing constructs, significant levels of full-length RNA have been shown to be present (Viss et al, 2003). If, however, excessive degradation of the longer dsRNA to siRNA occurs, it may be necessary to inhibit the endogenous

plant RNAi machinery. This may be accomplished by the addition of a silencing suppressor gene, such as Hc-Pro, to the T-DNA in the binary vector (Roth et al., 2004).

Wild-type *P. vulnus* will be placed on *Medicago* hairy roots expressing dsRNA and allowed to feed for at least four weeks. Phenotypes and mRNA levels in the nematodes will be determined as described in Objective 2c above.

It is possible that genes that are silenced by the soaking method may not be silenced by hairy root feeding. If this occurs, it may be due to a lower concentration of RNA transcript found in the plant cells compared to that used for soaking. To investigate, the soaking experiments will be repeated at lower RNA levels to determine the lowest dsRNA concentration needed for RNAi induction. However, it is also possible that RNAses present in *P. vulnus* salivary secretions may completely degrade the dsRNA before ingestion. This, in itself, would be a significant outcome of these experiments, particularly if the specific RNase could be identified.

Timetable

Milestone	Begin Date	End Date	Objective
1	01/31/2006	09/30/2006	Generation of ESTs by sequencing cDNA libraries prepared from mRNA isolated from <i>P. vulnus</i> , and from infected and uninfected walnut roots.
2	01/31/2006	06/30/2008	Functional analysis of <i>P. vulnus</i> genes by soaking in dsRNA to induce RNAi in nematodes including Taqman analysis.
3	07/01/2006	03/31/2007	Construction of a database of nematode and walnut root genomic information.
4	10/01/2006	06/30/2007	Genomic analysis of the nematode and walnut databases to assign putative functions to particular genes and identify candidates for functional analysis.
5	01/31/2007	09/30/2007	Construction and analysis of initial walnut microarrays to compare expression levels between uninfected and infected plants.
6	10/01/2007	12/31/2007	Taqman validation of differentially expressed walnut genes from first microarray experiment
7	02/01/2008	01/30/2010	Functional analysis of <i>P. vulnus</i> genes by expression of dsRNA in <i>Medicago</i> root assays to induce RNAi in nematodes, including Taqman analysis.
8	01/01/2008	08/31/2008	Construction and analysis of validated walnut microarrays to compare expression levels between uninfected and infected plants.
9	09/01/2008	12/31/2008	Taqman validation of differentially expressed walnut genes from second microarray experiment
10	01/01/2009	09/30/2009	Microarray analysis of temporal changes in walnut gene expression as a result of nematode feeding
11	10/01/2009	01/30/2010	Taqman validation of differentially expressed walnut genes from longitudinal microarray experiment

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