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Rachel Medina graduated in January 2013 with a Bachelor of Science in Biology. She began this research in 2012 with a semester grant and a summer grant funded by the Adrian Tinsley Program under the direction of Dr. Heather Marella. This project was presented at the Undergraduate Research Symposium in the summer of 2012 and in La Crosse, Wisconsin in April 2013 for the National Conference on Undergraduate Research. Rachel currently works for a nematology laboratory at Ohio State University and will be pursuing a PhD in the fall of 2013 in plant pathology.

Meloidogyne incognita *is an obligate parasitic roundworm that infects the root tips of a wide variety of host plants. This infection directly affects the crop production globally with the loss of crops such as tomatoes, carrots, and potatoes. Nematode infection manipulates the plant root by altering gene expression for nematode benefit. Genes such as At2g42005, a putative amino acid transporter, have been found to be up-regulated by nematode infection. Characterizing the role of the At2g42005 gene in* Arabidopsis thaliana *is the first step to understanding its function in nematode infection. The role of At2g42005 were explored by creation of a promoter:GUS vector and gene expression investigation by RT-PCR. The GUS vector is a molecular tool that allows for visualization of gene expression within the tissues of the plant. After plasmid cloning and bacterial transformation, a promoter:GUS vector was created for insertion into flowering plants for visual staining of gene expression. RNA was extracted from various plant tissues and RT-PCR performed to determine At2g42005 expression patterns. The gene At2g42005 was found to be expressed in all tissues examined. Further investigation of RNA expression will be done using qPCR to quantify At2g42005 levels.*

The ability to grow crop plants without the harmful use of pesticides is an important area of research due to its environmental significance. Pesticides have contaminated the applied areas, and supply water from runoff, and have harmed wildlife habitat. These factors push research to identify mechanisms for a safe alternative that can eliminate nematocide use completely. There is an important correlation between the supply and demand balance in agricultural research and world hunger. It has been estimated that one-eighth of the global population does not have enough food on a daily basis and this desperate percent only increases each day (1). The recent climate-based challenges, such as drought, have weighed heavily on industrial output for food resources (1). With food crops being harvested and used for biofuels, the limited output going towards global hunger is decreasing (1). All of these factors need to be taken into account when focusing research efforts on finding a way to increase global crop production without the use of ecologically harmful pesticides. Roughly half of the damage of these limited crops is due to nematode infection which currently can only be controlled by nematicide spraying unless plants with inhibitory defenses can be created (2). Therefore, the research proposed in this study focuses on bringing clarity to just one of the many problems facing agriculture, nematode infestation.

Arabidopsis thaliana is a small weed in the mustard family that has become an important model organism of plant biology research (3). Arabidopsis was the first plant to have its genome fully sequenced which makes it a helpful tool when working in a laboratory setting (3). Scientists now have the ability to examine the over 20,000 genes and their roles in processes throughout the plant (3). This plant is also capable of growing in potted soil or petri dishes with an agar-based media, making it ideal for laboratory research.

The species of root-knot nematode used for this research is *Meloidogyne incognita*. This species is native to the tropics and was found to grow exceptionally well on Arabidopsis (4). *Meloidogyne incognita* is an obligate endoparasite, meaning it is completely dependent on the plant host for its nutrition and spends most of its life cycle within the plant. The larvae of these parasites hatch from eggs in the soil but will return to the root when they are in the juvenile stage (4). This parasite is also responsible for roughly half the total crop damage due to infected roots which makes it a critical point of study (5). The damage to the root is caused by the formation of knots within the root systems which subsequently block the uptake and transportation of nutrients and water through the vascular system in the plant, leaving it malnourished.

Plant roots weave through the soil in search of water and nutrients. The nematode begins its infection by secreting proteins and molecules that counteract the plant's defensive mechanisms upon entry of the root. This step allows the nematode into the root cap and further into the vascular tissue of the plant. The nematodes then secrete proteins that have properties to break down or loosen the cell walls in the plant (2). Additionally, these nematode secretions reprogram the xylem parenchyma cells turning them into a "giant cell" or "feeding cell" (2). The giant cells are enlarged cells with multiple nuclei and form a knot along with increased vascular tissues (6). These feeding cells are fundamental to the nematode infection because they hold the nutrients being pulled from different parts of the plant (6). The nematode pulls nutrients towards the feeding sites through the duration of its life cycle and creates plant damage (6).

The plant uses transport proteins to move substances around the cell. The importance of transporters within the parasitic interaction between plants and root-knot nematodes (RKN) during the infection and giant cell growth has been established (5). Analysis of gene expression in the roots of an infected plant has revealed that there are a total of 634 transporters involved, 50 of which changed immediately upon initiation of the infection (5). Of those 50 transporters, 26 were upregulated during the infection which potentially implicates the nematode directly using these during the infection (5). The nematode may be using these transporters not only in the forming of the giant cells but also in nutrient storage (5).

By studying upregulated transporters, it may be possible to understand how they are important for the nematodes within the physiology of the plant. The transporter gene of interest for this research is the gene At2g42005. This gene was chosen as a focus due to previous research showing upregulation of this transporter by fourteen percent in giant cells (H. Marella, unpublished). This Arabidopsis gene is 1200 nucleotide base pairs long and transcribed into a putative amino acid transporter. A mutant Arabidopsis lacking At2g42005 expression showed significantly fewer nematode egg masses than the wild-type control plants (H. Marella, unpublished results), indicating that the At2g42005 gene is required for full nematode infectivity. When At2g42005 mutant plants were grown on agar plates and compared to wild-type plants, they showed increased growth in lateral roots as compared with wild-type. This data indicates that the At2g42005 gene is necessary for proper root growth and development and as such provides the first clue to its natural function in the plant (H. Marella, unpublished results). Since this particular Arabidopsis gene has not been previously studied, the goal of this study was to determine the function of At2g42005 in the plant and investigate its role in nematode infection. It was expected that At2g42005 would be found to show expression within the roots and that that it will be directly seen to increase expression with the infection of root-knot nematodes. In order to test this hypothesis, a promoter: GUS vector was constructed and qualitative RNA analysis of the At2g42005 gene was performed.

MATERIALS AND METHODS

TOPO Cloning

The initial step was amplifying the promoter region of the gene At2g42005 from wild-type Arabidopsis DNA. The primer sequencing were as follows: At2g42005PRO – FWD 5' TGT TTG TTT GTG TTC CTC AAG 3', Atg42005PRO – REV 5' CTC TAT ACC TGA TTA GAG ATG GGC TC 3', At2g42010UTR – FWD 5' TAA TAA GGA AGG ATC CAG TGG CAC 3'. The UTR region primer was used due to a small promoter region for the gene At2g42005 and possible regulatory elements found within the gene At2g42010 UTR region. The At2g42005 primers created a 248 bp fragment whereas the UTR primers from At2g42010 created a 465 bp fragment. The PCR cycling conditions were 95°C for 30 sec, 51°C for 30 sec and 72°C for 1 min for 30 cycles with Platinum® Taq enzyme. The PCR reactions were examined by gel electrophoresis. Samples were selected for gel extraction performed using a Qiagen Quick Gel Extraction protocol. PCR reactions resulted

in two specific samples of At2g42005BIG3 and At2g42005Small2. The DNA products were then cloned in a pCR8/GW/TOPO TA Cloning Kit to create the promoter vector

The At2g42005 promoter/TOPO vector was transformed into competent One Shot® *E. coli* cells which were grown in SOB (Super Optimal Broth) medium and plated onto LB (Luria-Bertani) spectinomycin (spec) plates for plasmid selection after 24 hours of incubation. The DNA was extracted from these cells using a Wizard Plus SV Minipreps DNA Purification System and protocol. An enzyme digestion was performed on the DNA products from both samples, At2g42005BIG3 and At2g42005Small2 for gel electrophoresis analysis.

Promoter: GUS Gateway Cloning

From samples that showed correct size bands, the corresponding DNA was sent out for sequencing to verify the plasmid contained the At2g42005 promoter/TOPOvector from previous steps. *E. coli* cells containing the pBGWFS7 GUS binary vector were grown on LB-spec plates, cultured, and DNA extracted similarly to the TOPO vector. The two DNA vectors were then incubated in a LR Clonase® (Life Technologies, Carlsbad, CA, USA) reaction with the enzyme XhoI, which is a restriction enzyme used to cut the TOPO vector backbone. This consisted of the vector containing the At2g42005 promoter/TOPO vector, the destination GUS vector, and TE buffer. This process incorporates the two vectors into the final product of the promoter:GUS vector. The mix was vortexed with LR Clonase® II enzyme then incubated before deactivating with Proteinase K solution. Competent cells were grown in SOB medium with the LR Clonase product and put onto LB-spec plates for plasmid selection overnight. Following the bacterial culture, DNA was purified, digested with BamHI, and finally gel electrophoresis was performed for confirmation of vector presence.

RT-PCR

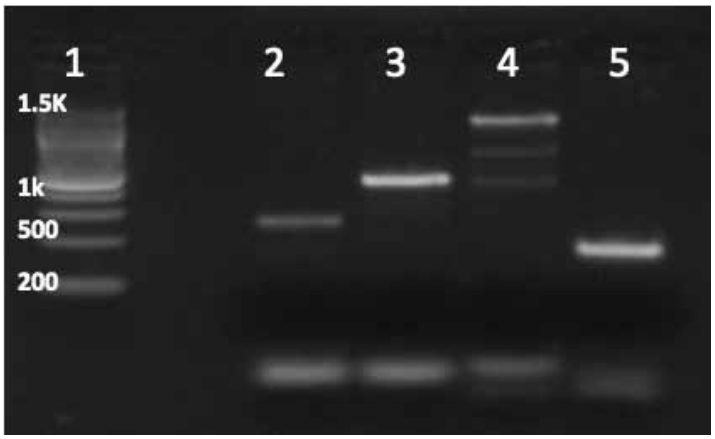
RNA extraction was performed on wild-type plants grown on agar plates in a controlled incubator and within soil. The soil-grown plants were collected at the age of 6 weeks and the plate-grown plants at 2 weeks for RNA extraction. The RNA was extracted using liquid nitrogen to freeze the selected tissues while grinding with a mortar and pestle. This was done for the tissues of stem, shoot, flower, root, whole seedling, rosette leaves, and cauline leaves. The RNA was used for RT-PCR using the Invitrogen SuperScriptIII kit, creating copies of cDNA. Reverse-transcription uses primers created specifically for the gene At2g42005 and target its presence in any of the tissues. The primer sequences were as follows: At2g42005 FWD 5' ATG GGT TTG GAG GAA CAA GG 3', At2g42005 REV 5'CAC AAG ACT GCT CAC CAC TC 3' for the At2g42005 gene target. This created a 882 bp product. The primer sequence for the control UBP22 gene was as follows: UBP22 FWD 5' TGT TTA GGC GGA ACG GAT AC 3', UBP22REV 5' GCC AAA GCT GTG GAG AAA AG 3'. This created a 158 bp product. The cDNA products were incubated with primers and LifeTechnologies PCR Supermix in a PCR reaction to give amplified regions where the gene is being expressed. This product was examined by gel electrophoresis beside a UBP22 gene as a control for each sample to show the expression level present.

Results

The original PCR product, which was the promoter region for At2g42005, was amplified and extracted from a successful gel electrophoresis. There were two samples taken one from a larger and smaller band found and both from the gene At2g42005 from wild-type Arabidopsis since two different forward primers were used (Figure 1). Transformation of the bacteria *E. coli* yielded colonies that had the At2g42005 promoter/TOPO vector within them. After an enzyme digest with EcoRI, the gel generated had appropriate bands found in each sample tested (Figure 2). These samples were sent for DNA sequencing to confirm the proper DNA was cloned. Next, the At2g42005 promoter/TOPO DNA was used to create the Promoter:GUS vector using Gateway technology. The expected resulting vector was found in only two of the 12 samples taken from colonies that grew on the LB spectinomycin agar plates (Figure 3). To confirm the results of this vector prior to sequencing, a gel was run following a digest with the restriction enzyme BamHI to verify the results (Figure 4). Both of these clones have the At2g42005 promoter driving the expression of GUS in a binary vector capable of incorporating into the Arabidopsis genome.

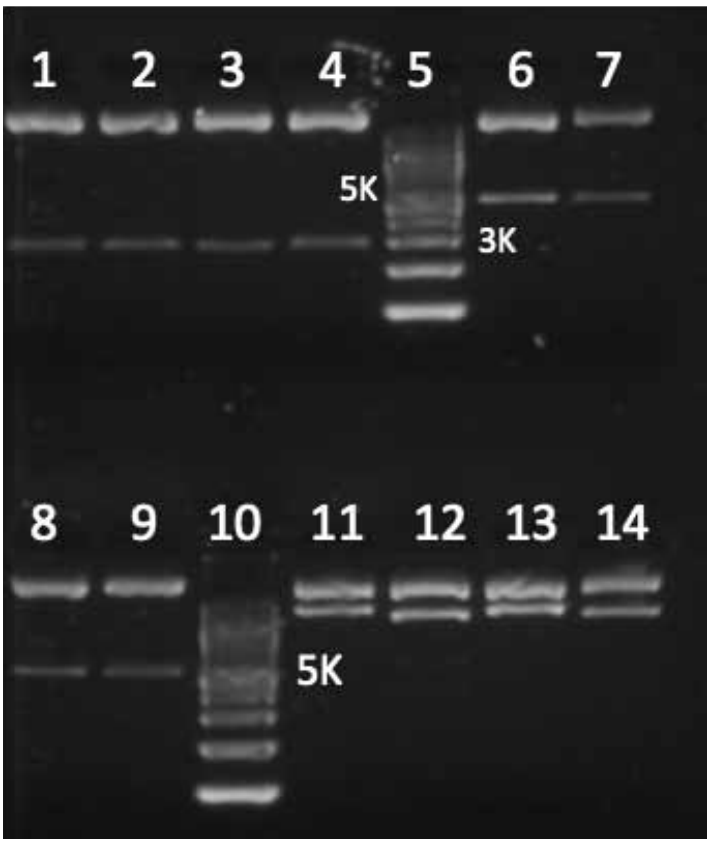
The RNA extraction and RT-PCR yielded evidence for At2g42005 expression in each tissue examined (Figure 5). At2g42005 expression was also detected in samples collected at stages 2 weeks and 6 weeks. The root, shoot, and whole seedling samples were collected from the 2-week-old plate-grown Arabidopsis. The stem, rosette leaf, cauline leaf, and flower samples were collected from the 6-week-old soil-grown Arabidopsis plants.

Figure 1. PCR Amplification of Promoter region of At2g42005.



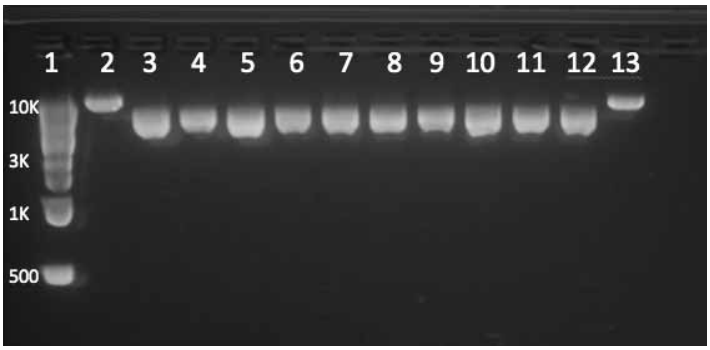
This gel has the following lanes: DNA (100bp) Ladder (1), At2g42005 Pro FWD/REV2 (2), At2g42005 UTR FWD/REV (3), At1g47670 FWD/REV (4), and UPB22 FWD/REV (5). The last two samples were used as positive controls and only the first two bands were cut out for DNA extraction. The UBP22 is an Arabidopsis housekeeping gene and is used as a positive control.

Figure 2. EcoRI Restriction Digest of At2g42005 promoter/TOPO vectors.



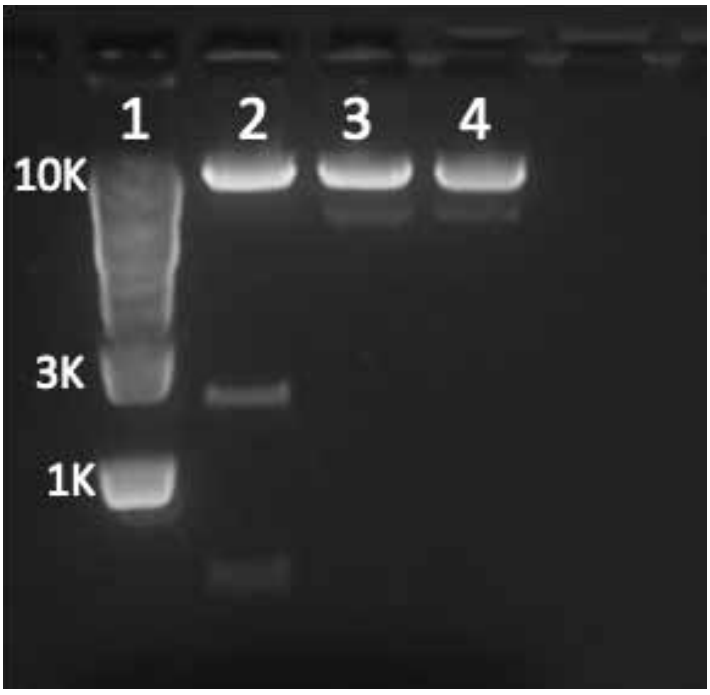
The top bands found on each sample show the TOPO vector present in the DNA samples. The first four lanes show results from cells transfected with At2g42005Small, with a DNA (100kp) ladder in the next lane (1-5). Lanes 6-9 show At2g-42005Big cells with a DNA ladder in lane 10. Lanes 11-14 are from LHT8 transfected cells, another gene that is being studied for later uses (11-14).

Figure 3. Promoter:GUS BamHI Digestion Products.



This depicts a DNA (100kp) ladder (1) and multiple samples of At2g42005Big3 samples 1-6 (2-7) followed by At2g42005Big2 samples 1-6 (8-13). Based on the restricted mobility of the plasmid in the gel, the result shows that the only samples with correct Promoter: GUS insert are At2g42005Big3-1 (lane 2) and At2g42005Big2-6 (lane 13).

Figure 4. Restriction Enyzme (BamHI) digest to confirm the promoter: GUS vector.



RNA Expression Analysis

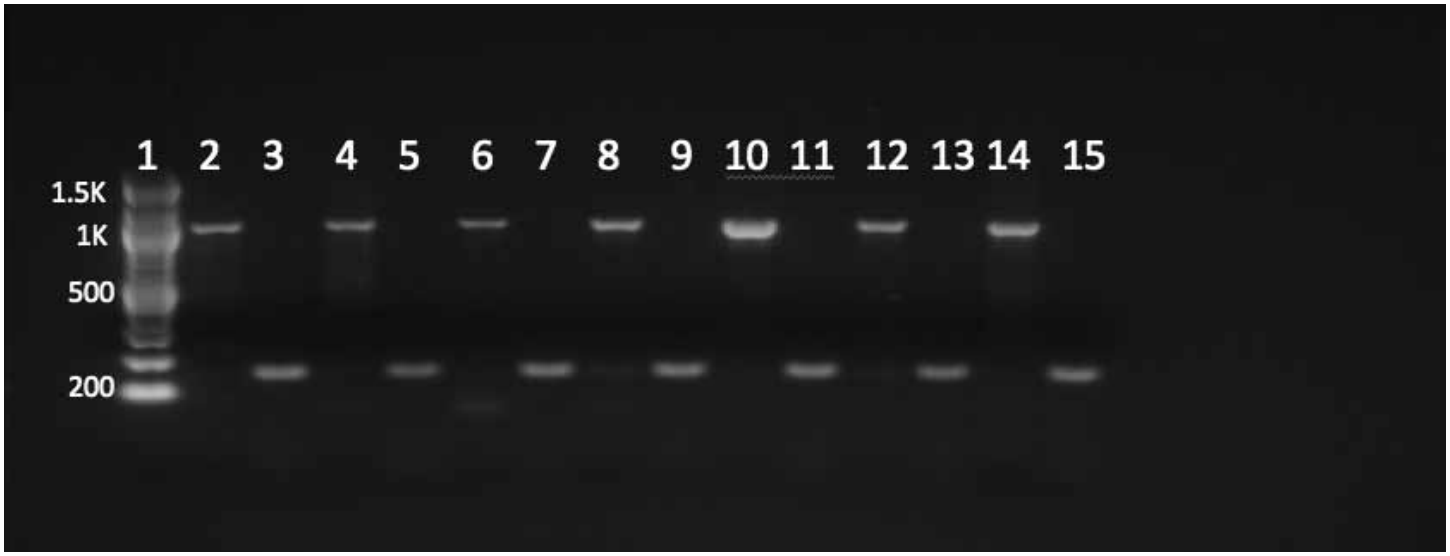


Figure 5. The various tissues collected and tested for expression levels of At2g42005 within them from wild-type Arabidopsis. Each sample has a corresponding UBP22 control. The first lane is a ladder (1) followed by samples in even-numbered lanes and controls in the odd-numbered lanes. The samples were as follows: seedling (2-3), shoot (4-5), root (6-7), stem (8-9), flowers (10-11), rosette leaves (12-13), and cauline leaves (14-15). All tissues tested demonstrate expression of the At2g42005 gene.

This gel was conducted to verify the data found in Figure 3. The first lane is the DNA ladder (1), followed by: pBGWSF7 GUS binary vector as a positive control (2), At2g42005Big3-1 (3), At2g42005Big2-6 samples (4). The control is found to have multiple bands due to three cuts being made by the restriction enzyme; yet when the promoter is cloned into the vector properly one of those restriction sites is lost resulting in two cuts from the enzyme (lanes 3 and 4).

Discussion

The upregulation of the gene At2g42005 that initiated this study has much larger implications with nematode infection. Amino acids are a requirement for nematode nutrition (7). Since the nematode is unable to synthesize all the amino acids that they need, amino acid transporters are essential for the nematode infection process and a much needed area of study. This is due to the nematode's inability to synthesize all the amino acids that it needs (7). It has been shown with various other amino acid transporters which are similar to At2g42005, such as AAP6, that upregulation is found directly within the feeding cells that the nematodes induce (7). The damage created by nematodes redirecting amino acids for their own benefit is seen in crop plants globally. This is due to the sink effect the nematodes create with their genomic hijacking of the host plant which is defenseless to such infection.

The At2g42005promoter:GUS vector was created to be utilized later in research. This molecular tool will be introduced into *Agrobacterium* for the transformation of flowering wild-type plants. By performing this next step, the plant can be GUS stained to visualize At2g42005 expression. Difficulties were encountered on the protocol at both the initial promoter amplification as well as the transformation of the final promoter: GUS product vector. The protocols were adapted to the specific gene being worked with and were successful afterwards.

The results for the RNA extraction and RT-PCR indicate that At2g42005 is being expressed throughout the entire plant and at multiple stages within its lifecycle. This was unexpected and causes a need for further investigation for the importance of At2g42005 within the plant. It was hypothesized that At2g42005 would be found expressed in the roots due to this being the site of nematode infection, but its expression elsewhere was unknown. The next stage of research will be to translate the qualitative data reported here and apply a quantitative approach. This will be done using qPCR to measure the various expression levels of At2g42005 within plant tissues. Overall, this study holds promise in the identification of the genes necessary to avert nematode infection without the use of nematicides.

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