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JAZ Levels in Arabidopsis After Infection by Parasitic Root-Knot Nematode

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Submitted in Partial Completion of the
Requirements for Commonwealth Honors in Biology

Bridgewater State University

May 9, 2017

Dr. Heather Marella, Thesis Director
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JAZ Levels in Arabidopsis after Infection by Parasitic Root-Knot Nematode

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May 9th, 2017

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Abstract

Root-knot nematodes (*Meloidogyne spp.*) are a major problem in the United States; damaging millions of dollars' worth of crops every year. It is believed that root-knot nematodes (RKN) alter the expression of JAZ gene family members in the plant, *Arabidopsis thaliana*, but there is not much documentation on which genes change expression and why. The JAZ genes are transcriptional regulators in the signaling pathway for the plant hormone, Jasmonic acid, which controls certain aspects of plant defense. There are twelve JAZ genes in Arabidopsis but it is unclear if they each have distinct roles in Jasmonic acid defense signaling. First, the levels of JAZ gene expression in Arabidopsis roots without nematodes were examined. Then the differences in expression after the addition of root-knot nematodes to the plants were examined. Arabidopsis seedlings were grown on sterile petri dishes for two weeks then juvenile nematodes were added. After two, six, 13 and 20 days, the RNA was extracted from the roots. The RNA was turned into complimentary DNA (cDNA) by reverse transcription to use it in quantitative Polymerase Chain Reaction (qPCR). qPCR can quantitate the amount of expression for each of the twelve JAZ genes after nematode infection, compared to the uninfected plants. The JAZ genes that changed significantly in presence of the nematodes were JAZ 9 and 10. JAZ 1 and 5 showed trends of altered expression with RKN but more replicates are required. GUS staining was performed on transgenic Arabidopsis lines carrying JAZ1 and JAZ12 promoter::GUS fusions and both showed that expression was localized to the roots, specifically the RKN knots for JAZ12.

Introduction

The root-knot nematode (RKN), *Meloidogyne incognita*, is an endoparasite that infects host plants near the root tip. RKN are prevalent in agricultural settings and specifically monoxenic cultures (Sijmons et al. 1991) and have spread worldwide to a wide variety of vascular plants (Koenning et al. 1999, Jones et al. 2013). RKN need to live inside a host in order to survive because they no longer have the genes that will protect them from pathogens and without the host they would not be able to complete their life cycle (Abad et al. 2008, Jones et al. 2013). *Meloidogyne incognita*, reproduce asexually; the females create large egg sac on the outside of the knot, the structure the nematodes live in, that can contain thousands of eggs (Jones et al. 2013). Eggs contain clonal offspring and are produced through mitotic parthenogenesis (Triantaphyllou 1981 and 1985). Males are only produced under times of stress, such as too many parasites in one plant, low nutrients, or physical damage; thereby allowing the remaining females to reproduce successfully (Davide and Triantaphyllou 1967, 1968, Snyder et al. 2006, Jones et al. 2013). The eggs will hatch and release J2s, the infective stage of development. Once they have settled into the root of the plant host they will molt two more times. After the J4 molt, is the adult reproductive stage, where the female will use parthenogenesis to create eggs (Castagnone-Sereno et al. 2013).

RKN are problematic because they insert themselves in between the cells deep within the roots causing damage and extracting nutrients for feeding. The J2s damage root cells by using their needle-like stylets to puncture the cells and release enzymes that will break down the cell wall components (Jones et al. 2013). Enzymes that are produced from the subventral gland help the parasite to enter into the roots and move around with greater ease (Davis et al. 2008, Jones et al. 2003); these enzymes are potentially used to degrade the extracellular matrix to make space

for the nematode. More enzymes are produced from the esophageal glands that degrade the cell wall components like cellulose, hemicellulose and pectin to make the cell more pliable to the nematode (Abad et al. 2008, Jones et al. 2013). The nematode also produces proteins, like calreticulin, which suppress the plant immune response (Jaouannet et al. 2013). They also make effector molecules that mimic plant proteins and cause a signaling cascade that will turn on pathways to aid in invasion (Gheysen et al. 2011). These enzymes and effector molecules are not typically found in animals, but rather in bacterium and fungi, so it hypothesized that these were obtained by horizontal gene transfer (Danchin et al. 2010, Jones et al. 2013, Haegeman et al. 2011, Rybarczk-Mydlowski et al. 2012, Paganini et al. 2012).

The RKN move from the vascular tissue into the surrounding xylem parenchyma cells in the root and change their cellular identity and function. These cells become the feeding cells that allow the nematode to obtain nutrients from the host. The RKN-created feeding cells are called giant cells, due to their large size. They become large because during cellular division cytokinesis is prevented and karyogenesis continues (Williamson and Gleason, 2003). This causes the number of nuclei to increase and the amount of the other cellular components to increase as well (Gheysen et al. 2011). The organelles that are impacted are the central vacuole, which is broken up into smaller compartments, and the numbers of plastids, mitochondria, and ribosomes increase (Castagnone et al. 2013). These large cells cause distinctive swelling in the root, giving the appearance of knots on the root. Once the parasite is established at the giant cells, the glands on the dorsal end produce effector molecules that dictate the plant's response (Gheysen et al. 2011).

Not only are the giant cells physically disruptive to the root but they are also a problem because they become nutritional sinks in the plant. Photosynthetic products and other nutrients

are sent to the giant cells due to their increased metabolism (Hammes et al. 2005). Amino acid transporter gene expression is altered in order to get more amino acids, especially the essential amino acids they cannot synthesize on their own, into the giant cells (Marella et al. 2013). The essential amino acids that plants make are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Some other transporters that nematodes need in their giant cells are aquaporins, sucrose transporters and ATPases, which are recruited to the membranes in order to supply nutrients for the nematode to survive (Hammes et al. 2005). The knots prevent mineral uptake from the surrounding soil (Hammes et al. 2005), so the plant becomes more nutritionally deprived. This is not an issue for the nematode since their life cycle take about a month to complete (Gheysen et al. 2011). Altogether, this depletes the plant of its nutrients which negatively impacts growth.

Another negative effect of RKN is that they change the level of auxin, a hormone that is involved in the elongation of roots, organ development and localization of transporters (Benkova et al. 2003) so the knot will become larger. Plasmodesmata disappear in the giant cells causing cell to cell communication to decrease (Hammes et al. 2005). There is also an increase in cell wall remodeling proteins, which are induced by auxin (Gheysen et al. 2009). This occurs because the nematode wants to isolate the giant cell to reduce the immune response from the plant host (Yan et al. 2015). The plant attempts to counteract this with its hormone defense pathways.

Plants are sedentary organisms that cannot defend themselves in the typical sense, like animals, against pathogens. They do not have an adaptive immune system response but rather they use hormones to trigger an innate defense against pathogens. Plants have receptors on their cell membranes that recognize foreign antigens but sometimes the initial innate defense protein cascade is not enough so another type of defense is triggered called the inducible defense. Plants

use specialized hormones to defend themselves from the living organisms that invade or injure their body. The major defense hormones that plants have are jasmonic acid, ethylene and salicylic acid. Together jasmonic acid (JA) and ethylene defend the organism from pathogens like insects, parasites, and fungus. The other hormone, salicylic acid (SA) turns off signaling pathways that are initiated by JA and ethylene and defends the plant against pathogens like bacteria (Nahar et al. 2011). Adding methyl jasmonate (one of the compounds falling into the JA hormone class) to a variety of hormone deficient mutants aided in defense against nematodes (Nahar et al. 2011).

Jasmonic acid is the main defense hormone against nematodes (Nahar et al. 2011, Wasternack et al. 2016, Yan et al. 2015). JA is initially synthesized from alpha-linolenic acid, which is found in the membranes of plastids, through the lipoxygenase pathway (Liechti and Farmer, 2006, Wasternack et al. 2016). Before turning on the JA pathway, plants try to defend themselves by changing ion concentrations (specifically calcium), changing membrane potentials and by phosphorylating proteins, with mitogen-activated protein kinases (MAPK), to stop the entry of the nematode (Yan et al. 2015, Meng et al. 2013). MAPK is activated by effector molecules produced by pathogens and turns on a series of processes to try to stop a pathogen (Meng et al. 2013). However, that may not be enough for an aggressive invader, so the plant produces jasmonic acid to aid in defense. As a plant hormone, jasmonic acid is also involved in UV protection (Yan et al. 2015), senescence (Chini et al. 2016), development and metabolic pathways (Liechti et al. 2006), water deficiency and exposure to ozone (Chini et al. 2007), stamen development (Wasternack et al. 2016), root growth (Demianski et al. 2012), and to signal to neighboring plants that there is a pathogen attack (Yan et al. 2015). Some other JA responses are trichome formation, chlorophyll degradation, and anthocyanin biosynthesis (Wasternack et

al. 2016). Like animal hormones, this plant hormone creates a signaling cascade that causes a downstream response.

The active form of jasmonic acid is the conjugate, jasmonic acid – isoleucine (JA-Ile). This is produced by the enzyme JAR1. The addition of the amino acid is essential for JA to initiate signaling (Staswick et al. 2004). JA-Ile enters into the nucleus to cause the signaling cascade that turns on the genes needed for defense or other jasmonic acid functions (Li et al. 2017). The transmembrane protein, JAT1, aids in the movement of jasmonic acid-isoleucine through phospholipid bilayers. JAT1 is localized in the nuclear envelope to bring JA-Ile into the nucleus, while JA alone cannot enter into the nucleus (Li et al. 2017).

In Arabidopsis, there is a family of genes that regulate Jasmonic acid signaling, called the JAZ (Jasmonate Zim-Domain) gene family, and each of the twelve members potentially has their own role that aid in the protection of the plant (Chini et al., 2007). JAZ proteins work by repressing the genes that are induced by JA-Ile. JAZ binds to MYC2 (a transcription factor) (Wasternack et al. 2016, Pauwels et al. 2011) and recruits other proteins that aid in the repression of the JA genes. NOVEL INTERACTOR OF JAZ (NINJA) is an adaptor protein that binds directly to JAZ (specifically its N-terminus) and recruits another protein called Topless (TPL). TPL binds to HDA, which is a histone deacetylase that will condense the DNA so it cannot be transcribed. In the presence of JA-Ile, JAZ will detach from MYC2 and the JA-Ile/JAZ formation binds to a SCF complex that contains a Coronatine Insensitive 1 (COI1) subunit (Wasternack et al. 2016, Demianski et al. 2012). This COI1 protein will bind directly to the JA-Ile/JAZ formation and the SCF will ubiquitinate JAZ (Wasternack et al. 2016). This leads to its degradation by a 26S-proteasome (Wasternack et al. 2016, Chini et al. 2007). RNA polymerase will be recruited to the promoter region by MYC2 and transcribe the gene into RNA, which is

then translated into the needed proteins to protect the plant from the invader (Figure 1). MYC2 positively regulates genes that aid in the wounding response, and inhibit root growth, while it also negatively regulates necrotic pathogen resistance (Wasternack et al. 2016). JAZ and MYC2 are regulated through a negative feedback loop (Chini et al. 2007). This means that when end products are high in concentration, the protein cascade will be stopped. MYC2 and JAZ are also regulated by the circadian rhythm of the plant (Chini et al. 2016) which means that levels of each of these proteins vary during the day. This occurs because the other organisms that the plant interacts with have optimal conditions in which they are most abundant or when the plant is more susceptible to pathogen infection (Ingle et al. 2015). This allows the plant to predict infection and build defenses to lower the infection rate.

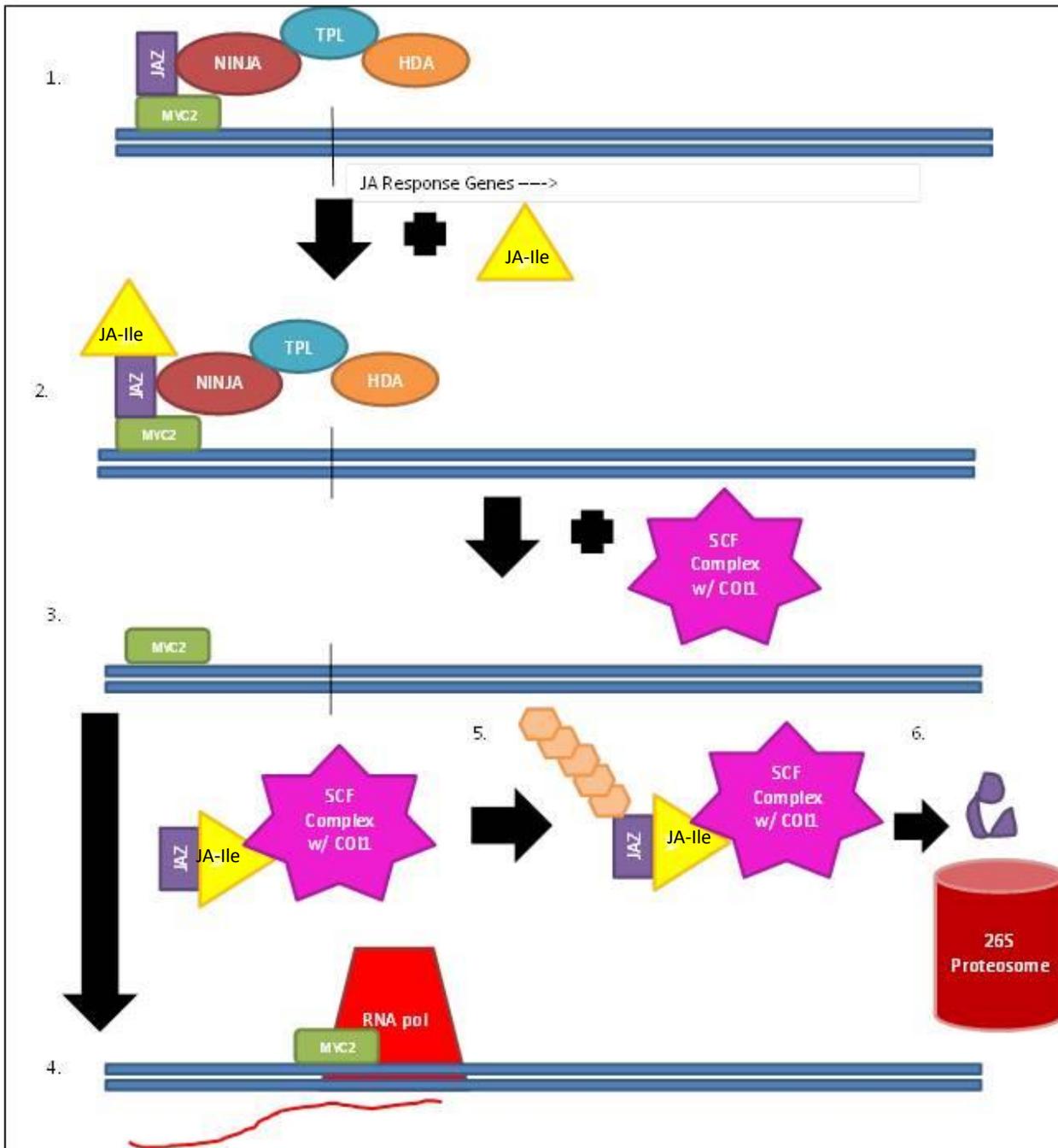
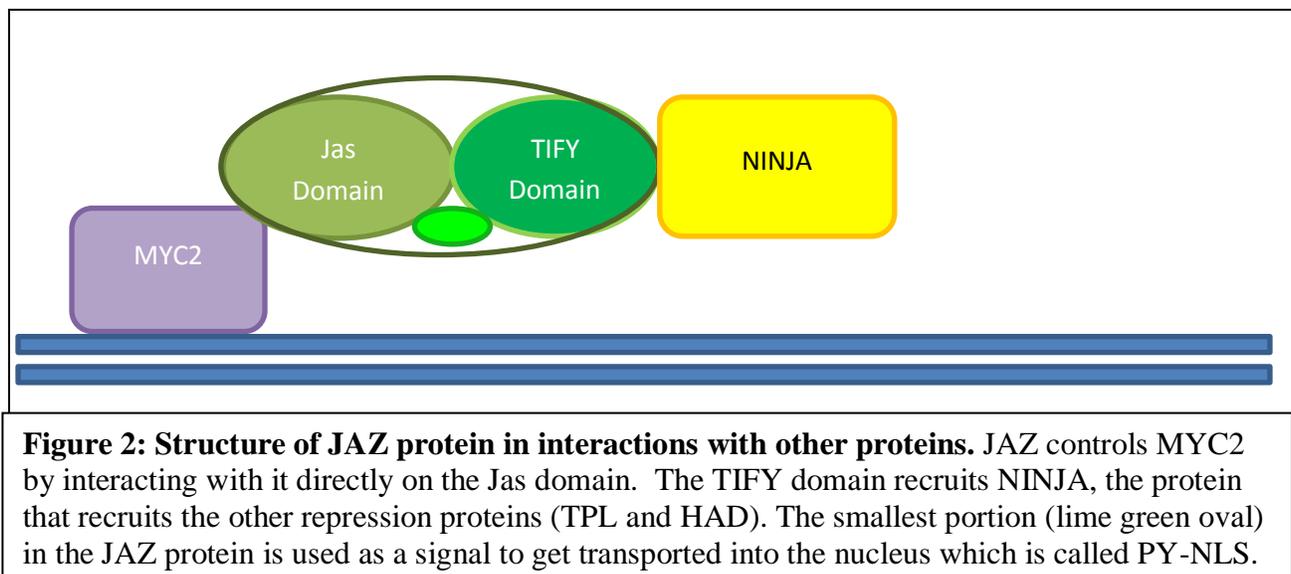


Figure 1: Model of JAZ action in JA signaling. The transcription factor MYC2 sits on the G-box motif in the promoter region of the JA response genes. JAZ binds to the MYC2 to prevent other transcription factors and RNA polymerase from binding and transcribing the DNA into RNA. JAZ recruits other proteins that aids in repression; HDA is a histone deacetylase that condenses the DNA (1). After JA is produced by oxylipases in the plastids, the JA-Ile molecule enters the nucleus where it will interact with the JAZ protein (2). This causes JAZ and the proteins attached to it to detach from MYC2. A SCF Complex with COII subunit will attach to JAZ (3). Since there are no proteins repressing the expression of the JA genes, RNA polymerase will be recruited to the promoter region and the gene will be transcribed (4). The SCF complex will ubiquitinate the JAZ protein (5). This will lead to the degradation of JAZ by a 26S-proteasome (6).

JAZ proteins have two major domains, the Jas domain and the TIFY domain. They do not have a DNA binding domain because they bind to the transcription factor, like MYC2. The Jas domain on the N-terminus interacts with the transcription factors (Bai et al. 2011). The TIFY domain has a ZML [ZIM (Zinc-finger like protein inflorescence meristem) like proteins] portion or has another Jas domain (Vanholme et al. 2007, Bai et al. 2011). The TIFY domain in JAZ interacts with NINJA protein to suppress transcription (Bai et al. 2011). Since JAZ must be sent into the nucleus in order for it to perform its function, there is a nuclear localization signal (NLS) within the protein structure. Lee et al. (2006) found that there is a portion of the JAZ protein that is called the PY-NLS, which is hydrophobic or basic in nature (Figure 2). When JAZ genes are highly expressed there is a repression of the JA response genes. On the other hand when there are low levels of a JAZ gene that means that those genes that are normally repressed will be expressed.



Each of the twelve JAZ genes in *Arabidopsis* may have a slightly different function; based on differences in the coding sequence and expression pattern (figure 3). The known functions of the JAZ family members are listed in Table 1. JAZ 4 and JAZ 8 are mainly involved in senescence (Chini et al. 2016), therefore we hypothesize that they will have low levels in the roots. Several JAZ members appear important for bacterial defense; Chini et al. (2016) did an experiment with a JAZ 10 mutant which was very sensitive to the bacterial pathogen, *Pseudomonas syringae*. Another experiment by de Torres Zabala et al. (2016) also showed that JAZ 5 works alongside JAZ 10 in defense against *P. syringae* (Chini et al. 2016). Demianski et al. (2012) found that JAZ 6, JAZ 9 and JAZ 10 are all induced by bacterial infection. They also found that JAZ10 knockout plants were more susceptible to bacterial infection, so there must be a defense pathway that JAZ10 supports.

Table 1: Description of how the 12 JAZ genes control functions inside the plant.

The known functions of the 12 JAZ genes are listed in order to make hypotheses about which genes may change in presence of nematode infection.

<u>GENE</u>	<u>Function</u>
JAZ1	JAZ 1 controls embryogenesis, formation of somatic embryos, induces the expression of IAA biosynthesis (Mira et al. 2016, Grunewald et al. 2009), male fertility (Figuroa et al. 2015), resistance to fungal pathogens (Jiang et al. 2016) and interactions with GA (Li et al. 2014).
JAZ2	JAZ 2 controls stomatic dynamics by being at high levels in the guard cells (Gimenez-Ibanez et al. 2017), and regulation of defense metabolites (Yan et al. 2014).
JAZ3	JAZ 3 controls the genes responsible for insect resistance (Moa et al. 2017), root growth inhibition under environmental stresses (Valenzuela et al. 2016), the functions of YAB1/FIL, such as axial patterning, growth of lateral organs, and activity in the SAM (stem apical meristem) (Boter et al. 2015).
JAZ4	JAZ 4 controls senescence (Jiang et al. 2014) and cold stress responses (Hu et al. 2013).
JAZ5	JAZ 5 controls pathogen growth alongside JAZ10 (de Torres Zabala et al. 2016).
JAZ6	JAZ 6 controls rhythmic susceptibility in plant defense (Ingle et al. 2015).
JAZ7	JAZ 7 controls senescence, flowering, and plant defense against pathogens like fungi (Thatcher et al. 2016) and root length (Yan et al. 2014).
JAZ8	JAZ 8 is unique because it is expressed only in the leaves and is not degraded like the other JAZ proteins (Shyu et al. 2012).
JAZ9	JAZ 9 has been used for biochemical experiments that involve the JAZ genes but there is no function found to date.
JAZ10	JAZ 10 controls partial inactivation of photoreceptor phytochrome B (phy B) (Cerrudo et al. 2016), works with JAZ 5 to repress pathogens (de Torres Zabala 2016), and Cargnel et al. (2014) found that there is a link between these two functions.
JAZ11	JAZ 11 has no research on how it functions to this date.
JAZ12	JAZ 12 has research on how protein interactions in the cell but not its overall function in the plant.

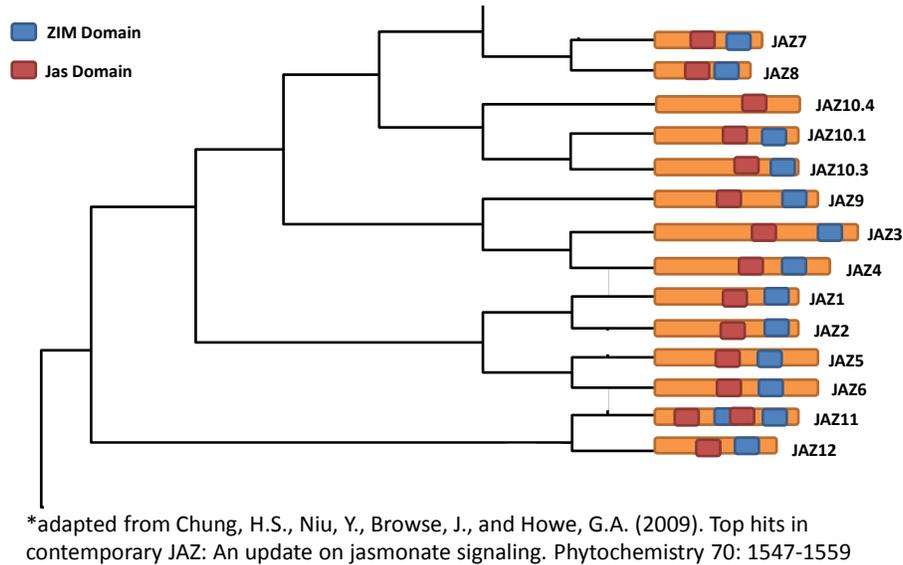


Figure 3: JAZ Family Gene Tree showing how each of the JAZs are related. JAZ is the family of proteins involved in repression of JA response genes. Each of the genes have a Jas domain, that recruits proteins to the DNA for repression, and a ZIM domain which will interact with transcription factor.

Jasmonic acid is important when RKN infect plant roots because it triggers defense mechanisms, but sometimes JA signaling can be manipulated by the RKN, either through biosynthesis or signaling pathways, to allow infection (Bhattarai et al. 2008; Nahar et al., 2011). Some of the other defense proteins may be altered by the nematode as well (Yan et al. 2015). Grunewald et al. (2009) used GUS staining to show where the JAZ1 protein was localized in the plant, and it expresses specifically in the knots where nematodes are feeding. It appears that the RKN are manipulating the plant's own defensive system to their advantage, which has been observed previously for another plant hormone, auxin (Gheysen and Mitchum, 2011). However, it is not clear which genes in the jasmonic acid pathway are being manipulated. The JAZ gene family is one possible target that the nematodes might use.

I am interested to see how the levels of these twelve JAZ genes change in the presence of the root-knot nematode to determine which, if any, are being manipulated by the nematode during parasitism. Using real-time qPCR, I measured the levels of 11 of the 12 JAZ genes of nematode-infected and uninfected roots to identify those with altered levels of expression during a time course of RKN infection of Arabidopsis. Additionally, I examined the localization of JAZ1 and JAZ12 expression in RKN-infected roots using Arabidopsis carrying JAZ promoter::GUS reporter constructs. This provides visual confirmation of the qPCR results and tells us which cell types in the root are involved. I hypothesized that the JAZ gene transcripts that protect the plant's cells from invaders will increase during the initial phase of infection but will decrease after infection is established. Additionally I hypothesized that the JAZ gene transcripts that are involved in environmental stress responses, like water depletion, will increase over the course of infection.

Materials and Methods

JAZ Gene Expression qPCR Analysis

First, Arabidopsis seeds of the Columbia wild-type were surface sterilized with bleach and ethanol in a sterile laminar flow hood and plated on Gamborg's growth media supplemented with 2% sucrose at pH 6.1. These seeds grew for 14 days in an incubator under 8 hours day/16 hours night at 23°C. Five hundred infective J2 nematodes were added to the Arabidopsis plates (Marella et al., 2013).

Since nematodes infect the roots of its host, root samples were collected at 4 different time points after RKN infection (day 2, day 6, day 13, and day 20) from root samples with and without RKN, frozen in liquid nitrogen and stored at -80°C. Only one biological replicate was run. Once all samples were collected RNA was extracted from the roots using the Qiagen RNeasy Plant Mini Kit. After quantification on a Nanodrop 1000 spectrophotometer, the smallest amount of RNA extracted, 32.9 nanograms, was converted into cDNA using the Invitrogen SuperScriptIII cDNA Synthesis Supermix, using oligo dT as the primer, following the manufacturer's protocol.

The diluted cDNA (1:10) was used as the template for qPCR with JAZ specific primers (Table 2). A comparison to an internal control gene that had similar amounts to the housekeeping gene protein phosphatase 2A subunit A3 (PP2AA3) was used to normalize the data (Demianski et al., 2012). A 10µL reaction was run containing 1 µL of diluted cDNA and BioRad Sso Advanced SYBR Green Supermix. The qPCR was performed for 40 cycles of 95°C for fifteen seconds then 60°C for one minute on the Applied Biosystems QuantStudio 6 qPCR machine

using the $\Delta\Delta C_T$ Gene Expression method on the system software. The melt curve was obtained to verify a single product. qPCR was performed with three technical replicates for each sample.

. *Promoter JAZ::GUS Gene Staining*

JAZ promoter::GUS plants for JAZ1 and JAZ12 have been generated by previous members of the Marella lab (unpublished data). The seeds of the JAZ1 promoter::GUS and JAZ12 promoter::GUS transgenic lines were sterilized and plated as described above. The J2 nematodes were added after two weeks and staining was performed at 5, 7 and 11 days after infection. The seedlings were submerged in the GUS staining buffer in multiwell plates overnight at 37°C (Jefferson, 1987). Photos of stained roots were taken on using Olympus DP25 camera mounted on the Olympus SZ61 microscope to visualize which tissues show expression of JAZ1 and JAZ12.

Table 2: Primers for the 11 JAZ genes used and a housekeeping gene used during qPCR.		
Forward and reverse primers were obtained from Demianski et al. (2012) and used to amplify the amounts of the genes of interest. PP2AA3 is a housekeeping gene that should be expressed in every cell so is a good indicator of whether or not the cDNA was prepared properly.		
Gene	Forward Primer	Reverse Primer
JAZ1	5'-GTCGTGGCTCGGTTTAGCAG-3'	5'-GGCTTGAGGGTGGTTAGCATT-3'
JAZ2	5'-ACCCAGCTTGGCTCAGTTCA-3'	5'-CTTCTGGGTCGGACAGGCT-3'
JAZ3	5'-AAGTAGCACAAACGGAAGTCCG-3'	5'-GACGTCAAGCCTCTTGTCGC-3'
JAZ4	5'-AGGTTCCAGTCAGCAAGACCA-3'	5'-GGTTCGGACATGGAGACTGTG-3'
JAZ5	5'-CAAGCCAGAGATTGTAACCGG-3'	5'-TGTGACGACTGTCCTGCCTC-3'
JAZ6	5'-AACGAGTTCGGGAACAATG-3'	5'-ACCTGATGTTGCTGCCAG-3'
JAZ7	5'-TGAGAAGTTCAGACGGTTCGG-3'	5'-TCGAGTCGAATTGTTTGGGAT-3'
JAZ9	5'-TGCTGTCGAAGAACGAGGGT-3'	5'-CTTCCCCCATTCTCTAGCTGC-3'
JAZ10	5'-GGTCGCTAATGAAGCAGCATC-3'	5'-TCTGTCTCCATCGACGACTCG-3'
JAZ11	5'-GACGGCATCGATTTGTCCAC-3'	5'-TGCTTCCGAAGTCGTAGCAGA-3'
JAZ12	5'-TCTCGTTTTGCAGATCCACG-3'	5'-GCCAATGCCTCCTTGAATA-3'
PP2AA3	5'-AACGTGGCCAAAATGATGC-3'	5'-AACCGCTTGGTCGACTATCG-3'

Results

Ten of the twelve JAZ genes were analyzed by qPCR to determine gene expression of each over time. Most genes amplified properly, shown by only one melt curve peak present for each gene. JAZ 4 failed to produce a clean melt curve and JAZ 8 was not expressed in the roots. The JAZ gene expression was normalized to the housekeeping gene PP2AA3. The PP2AA3 C_T mean score ranged from 25.9 to 26.6 for all samples, so the amplification signal crossed the threshold at similar points, indicating consistency amongst the samples. I compared the day of extraction with RKN to the same day sample without RKN then compared to the other three days of infection. This shows how the levels of the different JAZ genes change over developmental time in the roots.

When determining relative changes in the gene expression the time points were compared relative to the same time point without nematodes sample. The “without RKN” samples display the normal progression of the gene’s expression over time in roots. While the “with RKN” samples display how the infection changes the gene’s expression level. The expression of JAZ 1, 5, 9, and 10 changed compared to the non-infected controls (Figures 4-7). The expression of JAZ 2, 3, 6, 7 and 11 did not show any alteration of expression over time or with RKN infection in the roots (data not shown). JAZ 12 expression (Figure 8) did not change as detected by qPCR.

Significantly Different JAZ Gene Expression

The expression of JAZ9 significantly increases 3.5 fold with RKN on day 2 compared to without RKN on the same day (Figure 4). Then JAZ9 gene expression drops to stable levels as time continues. The gene expression without RKN shows normal expression of this gene over root development time.

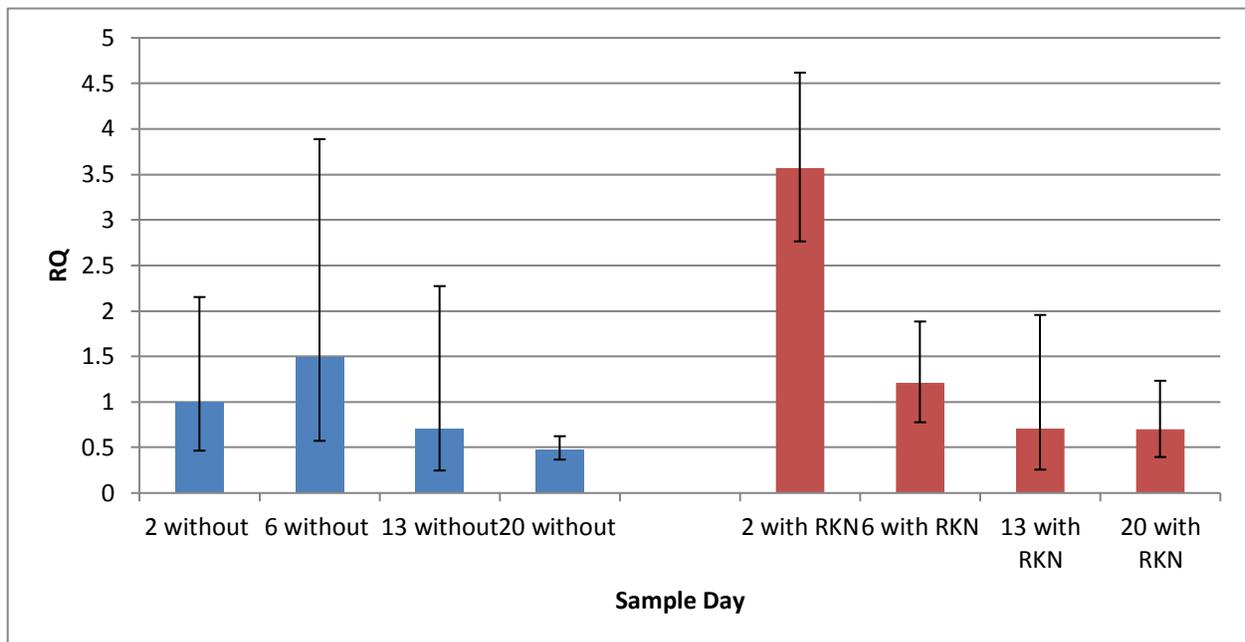


Figure 4: JAZ9 expression over 4 different time points show a significant increase early in infection. The values at each time point were compared to day 2 without RKN to determine relative increase or decrease. Day 2 with RKN is the only time point that is significantly different than without RKN. RQ stands for relative quantity based on fluorescence readings. The error bars are the range of three technical replicates.

The expression of JAZ10 in RKN infected roots increases significantly on day 2 and day 20 compared to the without RKN controls (Figure 5). On days 6 and 13 the RKN infected roots do tend to have higher JAZ10 expression but the level of error is not a significant. The 7-fold increase in expression at 2 days with RKN tapers off to a 2.3-fold increase by day 20. The days without RKN show the normal expression of JAZ10 over time in the roots.

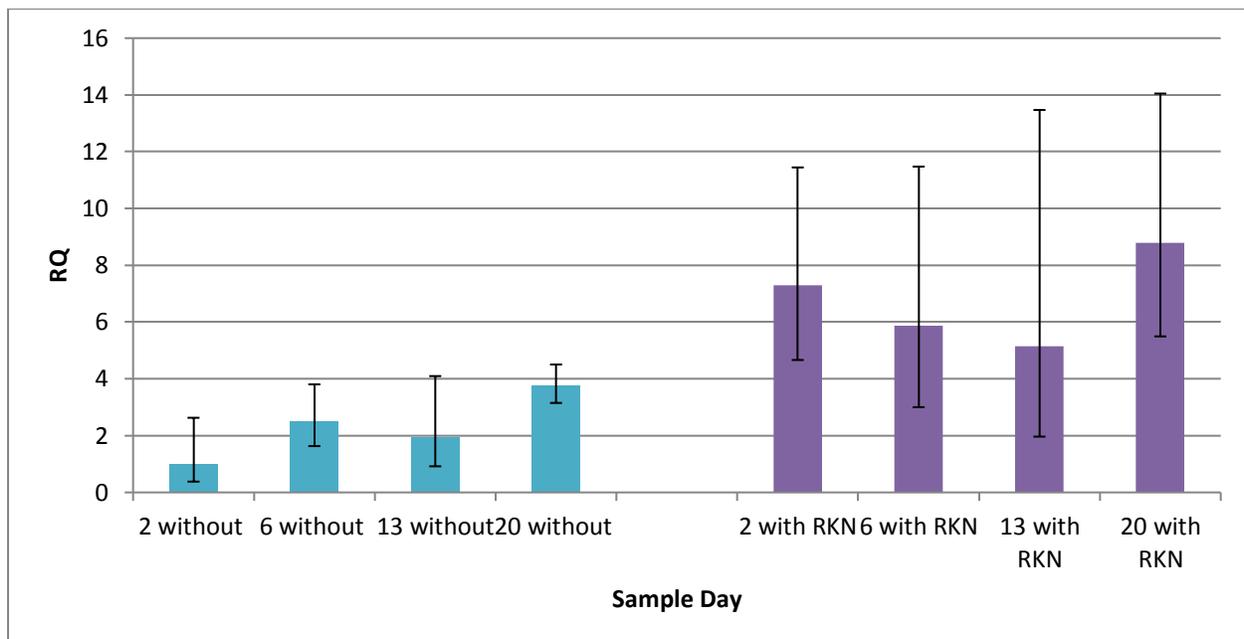


Figure 5: JAZ10 gene expression over 4 different time points with and without RKN shows a significant increase. JAZ10 expression was monitored by qPCR. Each time point has a larger amount of JAZ10 expressed with RKN than without. Day 2 and 20 were significantly higher with RKN. RQ stands for relative quantity based on fluorescence readings. The error bars represent the range of three technical replicates.

JAZ Genes with Potential Differential Expression Trends

The expression of JAZ 1 shows variable expression levels over time (Figure 6). The Day 6 with RKN increases significantly compared to the day 2 RKN. But afterwards there is a trend towards increasing expression in day 13 and day 20 time points that is not significantly different than the 2 day time point. The time points without RKN show the normal progression of JAZ1 expression during root development.

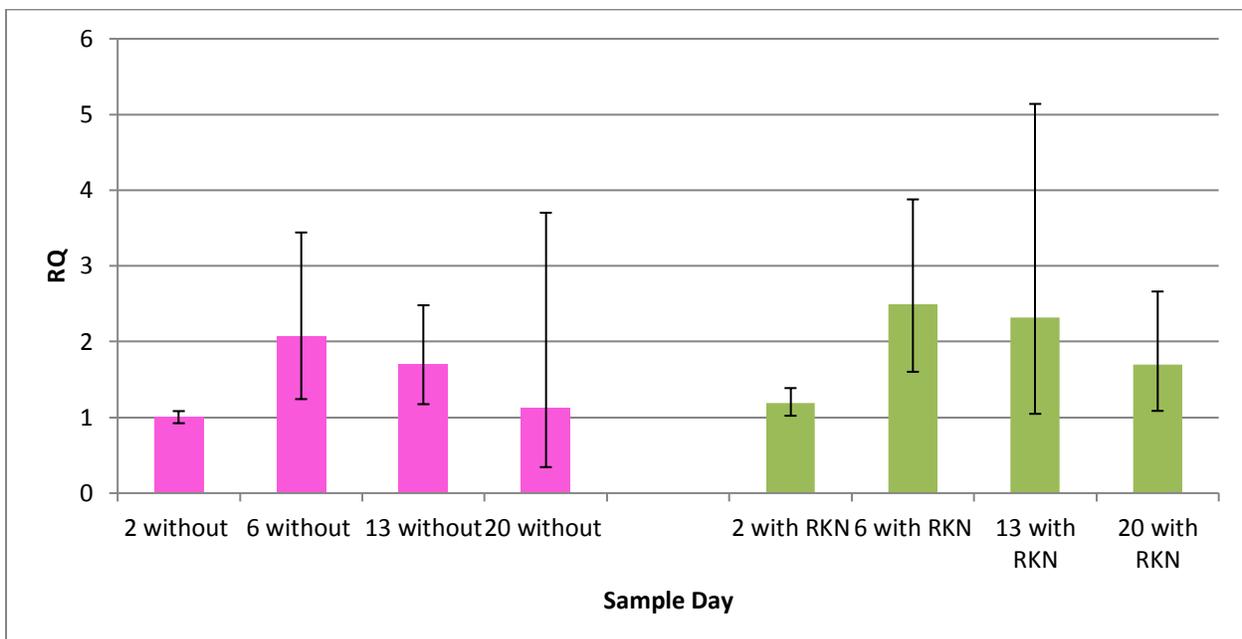


Figure 6: Gene expression of JAZ 1 during RKN infection over 20 days with 4 time points. Day 2 without RKN was the time point that was set to 1 and the other data points are relative to this sample. A housekeeping gene, PP2AA3, was used to normalize the data. JAZ1 expression peaks at the Day 6 time points with and without RKN. The time points without RKN show the normal expression of JAZ1 in plant growth. RQ stands for relative quantity. The error bars represent the range of three technical replicates.

The expression of JAZ 5 generally increased over time, however due to the amount of error, significance cannot be established (Figure 7). The pattern is that each time point with RKN has higher expression than the samples without RKN, even though there was not a significant difference between any of the time points. The days without RKN show the normal development of JAZ5 in wild type Arabidopsis, which seems to indicate an increase in expression as the root develops.

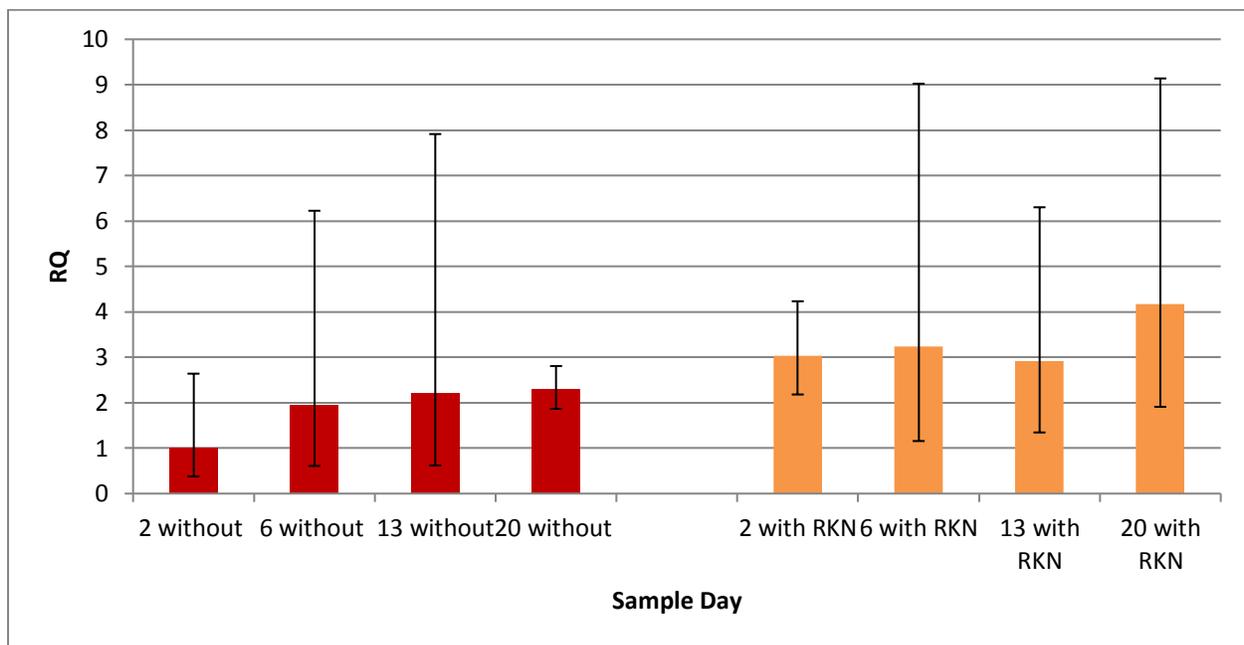


Figure 7: JAZ 5 gene expression during infection of RKN over 4 different time points compared to no infection controls. JAZ5 increased over time and increased in amount compared to no infection control at the same time points. There is no significant difference between any particular time point with RKN and without RKN. RQ stands for relative quantity. The error bars represent the range of the three technical replicates.

The expression of JAZ12 is relatively stable through each of the time points with and without RKN (Figure 8). There is no statistically significant change observed for this gene by qPCR.

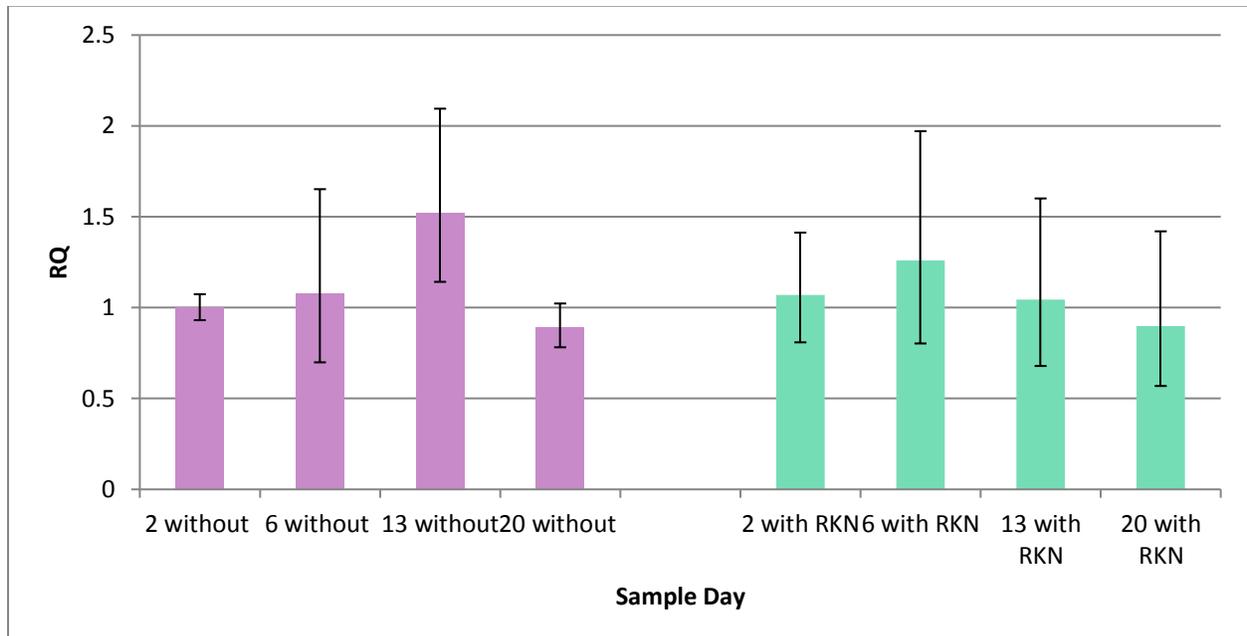


Figure 8: JAZ12 gene expression in the roots of Arabidopsis shows no change in expression. JAZ12 expression was monitored over time with and without RKN by qPCR. To determine relative variation the day 2 without RKN time point was set to an RQ of 1. There is no significant difference in JAZ12 expression between the time points or RKN treatment. RQ stands for relative quantity. The error bars represent the standard deviation of three technical replicates.

JAZ::GUS Staining

To determine which cells show expression of JAZ genes transcripts, promoter::GUS lines were tested under RKN infection. This test will be able to tell us where the JAZ genes of interest are localized in the root. Promoter::GUS staining uses an artificial construct placed into transgenic Arabidopsis plants that gives a blue color in the cell types where the gene promoter is active (Jefferson, 1987). Promoter::GUS staining is a well-established method to study specific genes in plant-nematode interactions (Barthels et al., 1997; Hammes et al., 2005; Marella et al., 2013). A JAZ1::GUS line was previously tested and showed localization in the roots' knots (Grunewald et al. 2009). To test where JAZ1 and JAZ12 gene expression is localized, GUS staining was performed on three different lines of transgenic Arabidopsis seedlings: JAZ1::GUS A1, JAZ1::GUS C and JAZ12::GUS. These plants carry a transgene of the specific JAZ promoter driving the expression of a GUS reporter. During the staining process, the β -glucuronidase (GUS) enzyme cleaves 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) to reveal the cells expressing the promoter and therefore the gene of interest. Both JAZ1::GUS lines had bacterial contamination so the plants were completely blue when stained due to over expression of JAZ1 in the defense response (Figure 9). Bacterial infection was determined by observation. JAZ12::GUS showed expression in knots at days 5 and 7 and in lateral root tips and lateral root buds in non-infected roots (Figure 9).

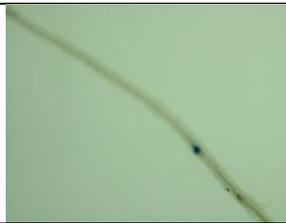
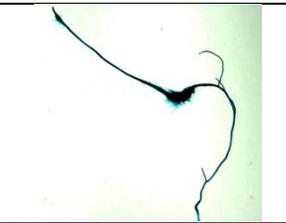
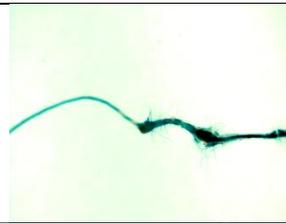
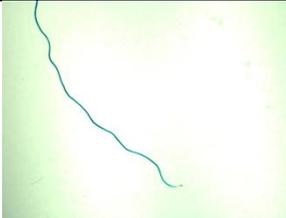
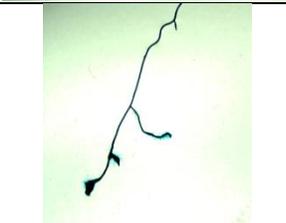
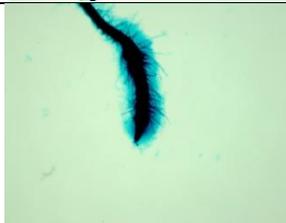
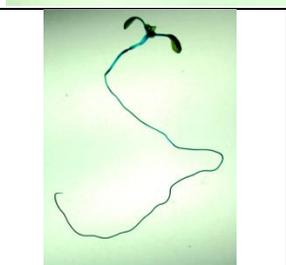
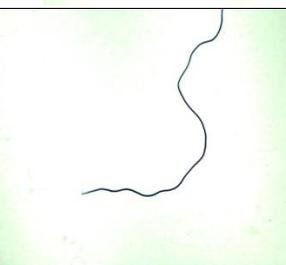
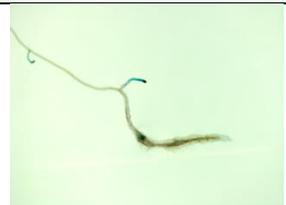
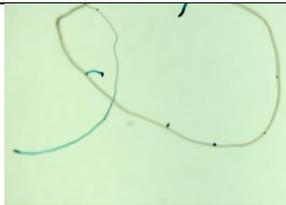
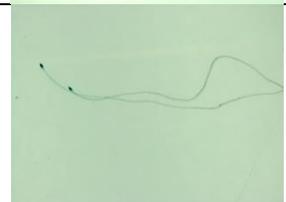
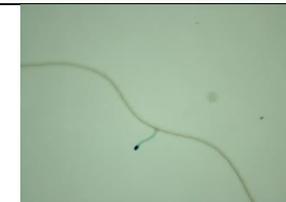
GENE		DAY 5	DAY 7	DAY 11
JAZ1:: GUS A1	With RKN			
	Without			
JAZ1:: GUS C	With RKN			
	Without			No expression
JAZ12:: GUS	With RKN			
	Without			

Figure 9: GUS staining in the roots of transgenic Arabidopsis at different time points after infection of J2 root-knot nematodes. Transgenic plants carrying JAZ promoter::GUS fusion constructs were generated and tested in the third generation. JAZ 1::GUS had two independent lines of GUS insertions. These seeds lines were grown on agar plants in an incubator for 2 weeks then infected with J2s. After 5 days, 7 days, and 11 days Arabidopsis seedlings were placed in the GUS staining buffer and incubated overnight at 37°C. Blue GUS staining indicates expression of the JAZ gene promoter. Lines JAZ1::GUS A1 and C had contamination present so there is a higher amount of staining due to higher defenses used across the whole plant not just inside knots, as expected. JAZ12::GUS expression is in the knots formed during nematode infection and at the root tips.

Discussion

JAZ genes are not only involved in direct attack against a pathogen, they also turn genes off that are energetically taxing to the organism. This is important because the plant wants to use its energy effectively to get rid of RKN. Most JAZ gene functions have not been documented to date, which makes it challenging to predict their involvement in root-knot nematode defense (Table 1). At first I hypothesized that the genes primarily involved in defense would increase in first few days and then decrease due to the infection progressing. Then later in infection the genes involved in environmental stress would increase due to loss of nutrients and water to the nematode feeding site and the nematode wanting to keep the plant out of a stressed state. The observed changes were a significant increase on day 2 with RKN for JAZ9 (Figure 4). JAZ10 also had a significant increase (Figure 5). JAZ 1 (Figure 6) and JAZ 5 (Figure 7) showed some possible trends in expression with RKN infection over time. Lastly JAZ12 had no expression change over time by qPCR (Figure 8); but when the GUS staining was done on JAZ12 there is a change in expression (Figure 9).

JAZ 9 expression spikes on day 2 with RKN (Figure 4). The function of JAZ 9 is unknown (Table 1); it is interesting that its expression increased significantly on only the earliest day of infection and then levels out. The potential function could be the initial defense mechanisms. Experiments should be performed on JAZ 9 knockout mutants to examine the plants for growth phenotypes, as well as changes in the level of nematode infection.

JAZ 10 expression displayed an induction with RKN present. Day 2 and 20 were significantly different with RKN than without (Figure 5). According to Cerrudo et al. (2016), JAZ10 inactivates phytochrome B, which is one of the photoreceptors involved in many plant

processes, like photomorphogenesis. So the large induction of JAZ10 means that phytochrome B is repressed and not functioning at high levels in infected roots, which may change nutrient movement. JAZ10 works alongside JAZ5 in defense (de Torres Zabala et al. 2016).

JAZ 5 expression displayed an increasing trend during progression of the experiment (Figure 7). JAZ 5 is involved with defense against the pathogen *P. syringae* (de Torres Zabala et al. 2016). This bacterium infects whatever part of the plant that it comes intact with. JAZ 5 might work similarly in defending the plant from both, bacteria and nematode, since it is expressed during both bacteria and nematode infection.

JAZ 1 is involved in the repression of auxin levels (Mira et al. 2016, Grunewald et al. 2009) and gibberellin (GA) interactions (Li et al. 2014) so the trend in expression of this gene (Figure 6) might mean the nematode is manipulating these hormones through JAZ1. A few studies have been performed on the levels of auxin during RKN infection (Absmanner et al. 2013, Gheysen and Mitchum, 2011). Karczmarek et al. (2004) used an auxin-responsive element called DR5 to test for the presence of auxin near the RKN feeding sites. GUS promoter DR5:*gusA* staining showed that there was a high concentration of auxin inside the giant cells at early time points which then diminished.

JAZ 12 is interesting because the GUS staining shows expression in the knots (Figure 9), but the qPCR expression is pretty stable throughout each time point (Figure 8). JAZ 12 appears to only be turned on in the knots and not the majority of the root tissue. Repeating the qPCR using only RNA from the knots should give a more precise qPCR reading from the infected roots. There is currently no research on how JAZ12 functions so experiments should be performed on mutants to check for plant growth and nematode infection phenotypes.

JAZ 2, 3, 6, 7 and 11 had no change in expression throughout the progression of time (data not shown). This could be due to developmental changes not in the time frame of the experiment performed, induction by other forms of stress, like water depletion, or that like JAZ12, they are only expressed in the knots and not at high levels in the whole root during RKN infection. These genes should be rechecked by qPCR for expression in isolated knot tissue or by creation of promoter::GUS lines.

JAZ 8 was not examined during the time of our experiment because there is data that shows that it is not expressed in roots (Heather Marella, personal communication), which was the tissue used in these experiments. The qPCR primers for JAZ4 did not meet quality control as they had two peaks in the melt curve analysis. Demianski et al. (2012) designed these primers for leaf tissue but the JAZ genes are spliced differently in separate portions of the plant, so this may account for multiple spikes in the melt curve. JAZ4 could be tested in the future with the development of new qPCR primers. The GUS staining data revealed expected patterns with blue staining localized to areas of infection (Figure 8). There was some bacterial contamination in two out of the three lines that were plated so those seed lines had more staining across the whole plant, not just the roots like originally expected for JAZ1 (Grunewald et al. 2009). Both of the contaminated lines were expressing JAZ 1 promoter, which is a gene that is used to regulate auxin and GA levels within the plant. Therefore, it makes sense that during bacterial infection that the whole plant would turn blue. This is due to the shutdown of normal growth processes and the switch to defense mechanisms. Auxin and GA are used for plant growth and development and the plant shifts energy away from growing when there is an invader. The JAZ genes are expressed in the knots because that is where they receive the signal from the nematode. This change in JAZ levels could lead to a signaling cascade that might spread through the whole

plant. Grunewald et al. (2009) had also found similar results when they used JAZ1::GUS promoter lines.

A potential future project is to combine the JAZ mutant lines for JAZ9, 10, 1 and 5 to determine if it is more resistant. One double mutant of high interest is that of *jaz5* and *jaz10*, which show cooperative effects (de Torres Zabala et al. 2016). Because both have been shown to increase in the presence of nematodes, therefore a double mutant, which has the ability to express more defense proteins, might be able to keep the RKNs out of the root. Another double mutant would be a *jaz1* and *jaz7* combination. Both are involved in root development and would be interesting to see how the RKN infection progresses with altered root development.

Using a qPCR approach, I have shown that JAZ9 and 10 are expressed significantly more in RKN infected roots at various time points. There are also the potential trends for increased expression of JAZ1 and 5. The induction of JAZ12 in the feeding site was demonstrated by promoter GUS fusion. These combined experiments show that only certain JAZ family members are induced by nematode infection throughout the root. These are only the first steps to providing plants better control of their defense mechanisms against root-knot nematodes and similar pathogens.

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