Cellular Elucidation of Mode of Defense Responses in Cotton Roots

in response to *Rotylenchulus reniformis* Infections

by

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Abstract

The hypersensitive response is an important mechanism of plant innate immunity which has been studied for decades. It is the immune response whereby a host plant R genes will recognize a specific effector gene from a pathogen and respond by programmed cell death within a small as possible perimeter of the infection site, thus preventing spread of biotrophic pathogens. In this study, we investigate if this reaction occurs as a result of nematode infection. *Rotylenchulus reniformis*, the Reniform nematode, is a semi-endoparasitic nematode posing a great threat to many breeds of cotton. In this study, a new method of examining cellular reactions to underground threats is developed. Utilizing the autofluorescence of the plant parasitic nematode, the confocal microscope can be used to investigate the behavior of these nematodes. In this study, we look at the stability of the autofluorescence expressed by the Reniform nematode and how carious cotton breeds react to the infection of said Reniform nematodes. Using root staining and confocal co-imaging, the cotton root cellular responses to infection are visualized.

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Chapter I: Literature review

The plant innate immunity model as perceived by plant parasitic nematodes: Review

Introduction

Plant parasitic nematodes (PPNs) are underground plant herbivores that attack the majority of economically important crops and cause devastating diseases and damages; responsible for over $170 billion of annual crop losses (Elling, 2013). These small and soil-dwelling pests parasitize plant roots, initially attacking the root tissue of the host plants by use of a stylet. When they puncture the cells, the stylet mechanically disrupts the host cells, and then releases a mixture of enzymes used to soften the cell walls such as cell wall degrading enzymes (CWDEs; Haegeman *et al*, 2011; Quentin *et al*, 2013). Now, important question is if and how plants and their root systems are capable of conferring disease resistance towards PPN infections? Our understanding to these questions will underpin generic repertories and modes in host defense machineries, which serve vital means in genetic engineering or molecular breeding approaches to upgrade a plant's own defense and growth capacities and improve yield and survival for food or biofuel crops. This mini-review discusses how PPN proceed their way into host plants, and the current update of plant innate immunity.

The Plant Immune Systems

In nature, plants as sessile organisms are encountering consistent and numerous attacks by a wide range of biotic competitors and consumers including bacteria, viruses, fungi, oomycetes and PPNs. To resolve the stresses, plants have evolved the ability to induce a two-layered defense system, consisting basal and R gene-mediated resistances, known also as PAMP- and effector triggered immunity, respectively (PTI and ETI). The basal, or initial, form of defense responses (PTI) is induced by detection of pathogen- or microbial-associated molecular patterns (PAMPs or MAMPs); molecules associated with the outer surface of the pathogens.

PAMPs are recognized by membrane-bound, pattern recognition receptors (PRRs) of the host cells, that rapidly accumulate signalling molecules such as calcium ion, reactive oxygen species (ROS) and salicylic acid (SA), and activate protein phosphorylation and dephosphorylation cascades which in turn initiate transcriptional and physiological changes in rendering disease resistance. PTI then manifests increased callose deposition to fortify cell wall matrices, while including systemic acquired resistance that provides long-lasting protection against secondary infection by a broad range of pathogens. (Boller and Felix, 2009; Bent and Mackey, 2007; Garcia-Brugger *et al*, 2006; Blume *et al*, 2000, Torres, 2010; Nühse *et al*, 2007; Zhang *et al*, 2007).

To counteract PTI, pathogens encode so-called effector proteins that impede the activity of PRRs and kinase cascades, inducing effector-triggered susceptibility (ETS) (Kamoun, 2007; Hann and Rathjen, 2010). Effectors are small molecules that selectively bind to a specific protein and regulate the host biological activity (Hogenhout *et al*, 2009). Effectors target host enzymes and utilize their functions. They are delivered to the host cells via various translocation apparatuses, such as the type III secretion system of some gram-negative bacteria and biotrophic interfacial complex of filamentous fungi, and target the cell surface components. Intracellular effectors are secreted by pathogens as well and these will translocate across the cellular membrane and travel to specific organelles within the host cell (Kamoun, 2006; Block *et al,* 2008). These two classes of effectors are able to influence host responses and interrupt the cellular processes which are required for PTI, thus resulting in ETS.

When host plants fall victim to ETS, a second level of defence is activated. This is achieved by the plant expressing R (resistance) proteins which recognize specific effectors. This induces Effector-Triggered immunity (ETI; Jones and Dangl, 2006). In the event of ETI, there is a theory of a gene-for-gene interaction (Flor, 1942). The host must have a dominant R-gene which corresponds with the pathogen's dominant *avr* (*avirulence*) gene (also termed as

effectors; Flor, 1942; Flor, 1955; Greenberg and Yao, 2004). The R-gene, mainly consisting of nucleotide binding domain and leucine rich repeat domain, recognizes *avr* genes by direct interaction or by help of decoy proteins, and induce ETI (Cui *et al*, 2015). ETI eventually culminates to rapid programmed cell death (PCD) at the localized sites of pathogen infections (hypersensitive response, HR). The induction of PCD hampers the development of the pathogen, provided the pathogen is biotrophic in its lifestyle, needing living host cells for survival. HR is the threshold for plant defence responses (Jones and Dangl, 2006; Cui *et al*, 2015).

A known indication of HR taking place is the preceding production of ROS, or ROS burst. The ROS functioning remains a mystery, however, the link between HR and ROS production was established by Doke (1983). The mitochondria play an important role in generating ROS and recent studies by Maxwell *et al* (2002) suggest that this is a similar case in plants. Salicylic acid, produced as a defence hormone, is able to act as an uncoupler of oxidative phosphorylation, inhibiting the respiratory chain and generation of ROS in the Mitochondria (Love *et al*, 2008).

Nematode induced PTI in plants

The most damaging PPN are sedentary in their manner of infection; once they establish a feeding and growing sites, PPN remain at the positions for an extended period of time. Therefore, they are often vulnerable to the radars of the plant immune systems (Wondafrash *et al*, 2013). It is however possible that PPN camouflage themselves with host-derived carbohydrates, since their surface outer coat surface contains lectin-like proteins, capable of binding plant carbohydrates (Spiegel *et al*, 1995). This phenomenon has not been demonstrated in plants, but observed in animals and their respective infectious nematodes (Blaxter *et al*, 1992; Maizels *et al*, 2001). Once host cells recognize the attack by PPN, plants elaborate PTI-like immune responses resulting in oxidative burst and callose deposition, as well as producing

nematode targeting cell wall degrading or modifying enzymes (Gheyson and Fenoll, 2002; Waetzig *et al*, 1992). Recently, functional profiling of nematode-derived metabolites has uncovered that ascarosides, small glycolipid compounds conserved in nematodes (Golden and Riddle, 1982; Noguez *et al*, 2012), play a role as an immune elicitor (Manosalva *et al*, 2015). The most abundant ascaroside found amongst well-known nematodes, *Caenorhabditis elegans*, *Meloidogyne incognita*, *Pratylenchus brachyurus* and *Heterodera glycines*, is ascr#18 (C11). In Arabidopsis, treatment of ascr#18 render hallmark PTI responses, accumulating transcripts of a) PTI-marker genes [Flg22-INDUCED RECEPTOR KINASE1 (FRK1) and PHOSPHATE-INDUCED1 (PHI1)], b) SA-responsive genes [PATHOGENESIS RELATED-4 (PR4) and GLUTATHIONE S-TRANSFERASE (GST)], as well as c) JA-biosynthetic genes [LIPOXYGENASE2 (LOX2) and ALLENE OXIDE SYNTHASE (AOS)]. As results, ascr#18 induce increased disease resistance towards a broad range of pathogens; virus, bacteria, oomycete, fungi and nematodes (Manosalva *et al*, 2015), suggesting that ascr#18 is a first kind of PAMP signature in PPN, and plant roots potentially share a conserved machinery, capable of perceiving ascr#18 and activating PTI responses.

Nematode effectors

For decades, there have been a large number of efforts being made to identify nematode effectors and enlighten us on their roles in communication between the nematodes and their plant hosts. Proteomic approaches have been used on *M. incognita*, including high-throughput proteomics based liquid chromatography, nano-electrospray ionization and tandem mass spectrometry (Bellafiore *et al*, 2008), revealing 486 secreted proteins potentially required for immune suppression and host cell reprogramming. With the benefits of recent advances in next-generation sequencing technologies, expressed sequence tags (ESTs) can be generated from various juvenile developmental stages (Roze *et al*, 2008; Jones *et al*, 2009; Jaouannet *et al*, 2012; Haegeman *et al*, 2013). infected plant tissues (Haegeman *et al*, 2013), micro-

aspiration of the esophageal gland cytoplasmic contents (Wang *et al*, 2001; Gao *et al*, 2003; Huang *et al*, 2004) and isolated whole glands (Maier *et al*, 2013). Ectopic expression studies have also been used for functional identification of important proteins secreted by nematodes such as Gr-VAP1 and GrCEP12 produced by *G. rostochiensis* (Lozano-Torres *et al*, 2014; Chen *et al*, 2013). Despite recent efforts, however, little has been revealed about ETI associated immune responses and regulatory modes in plant parasitic nematodes.

Further studies have identified a unique cell wall modifying effector MAP-1, produced from root-knot nematodes. MAP-1 was originally identified as an avirulence factor produced during the J2 pre-parasitic stage and suggested to be involved in early recognition stages between the host and the nematode (Semblat *et al*, 2001). Vieira *et al* (2011) later hypothesized that MAP-1 may be involved in the physical interactions between plant ligands along the nematode path or the wall of enlarged cells, or perhaps both. These functions are characteristic of effectors which supress the host defence responses and allow easier establishment of a feeding site for the nematode. MAP-1 may play a role in the specificity of the interaction between the plant and nematode (Vieira *et al*, 2011). It has also been revealed that *M. incognita* map-1 homologues into a cluster of expansin-like protein encoding genes (Danchin *et al*, 2010). Expansins are thought to function in loosening of cell walls, establishing that they are involved in early stage parasitism of *M. incognita* (Qin *et al*, 2004). GrEXPB2, isolated from *G. rostochiensis*, is a unique expansin protein lacking the carbohydrate-binding domain and induces chlorosis in tobacco and cell death in tomato and potato plants (Ali *et al*, 2015). A similar expansin protein, HaEXPB2, was identified in *H. avanae* and caused cell death in *Nictonia benthamiana* (Liu *et al*, 2016). It was evident that these effectors were important in activation of plant defense responses when the signal peptides were deleted from the hosts and the symptoms did not occur. This emphasizes the importance of these cell-wall modifying elicitors in their contribution to pathogenicity and recognition by the host plant.

HsCBP (*H. schachtii* cellulose-binding protein) interacts with pectin methylesterase of Arabidopsis to promote pectin-modifying enzymes and allowing cell wall-degrading enzymes easier access to cell wall polymers (Hewezi *et al*, 2008). When the receptor *PEM3* was knocked in the Arabidopsis alternative results were observed, indicating the importance of a compatible receptor for this effector. Effectors are able to mimic plant compounds which bind to the host proteins, causing changes in the host hormone signalling and balance. Wang *et al* (2011) found cyst nematodes to secrete CLE-like (CLAVATA3/ESR) peptides which play an important role in the meristematic differentiation process. Hs19C07 interacts with the auxin influx transporter of Arabidopsis plasma membrane, LAX3. Here Hs1907 modulates influx in syncytia, facilitating development, providing evidence of nematode effectors driving processes through direct targeting of hormone transport proteins (Lee *et al*, 2011). MiDO5 was recently characterized as an important pathogenicity factor, as this effector from *M. incognita* was overexpressed in plants and caused a spike in production of the effector, resulting in stimulation of strong shoot growth. This effector was shown to interact with TIP2 (tonoplast intrinsic protein) suggesting involvement in formation of giant cells used for feeding by the root-knot nematode, thus an important factor for the pathogen survival and reproduction (Xue *et al*, 2013). Recently, hypervariable effectors (HYP) were identified in *Globodera pallida*. Gr-HYP effectors are a highly complex gene family based on repeat domain amino acid sequences. The show unparalleled diversity between others of the same population, with no two nematodes possessing the same genetic complement of Gp-HYP1 or Gp-HYP3. While their function is not entirely clear, it is proposed that Gr-HYP effectors are essential for successful infection (Eves-van den Akker *et al*, 2014).

In conclusion, there is a wide variety of effectors that have been identified and are still under investigation for origin and functionality. There are many different methods of effector

identification in nematodes, and further research is needed to extend knowledge on nematode ETS and ETI.

Programmed Cell Death: Suppression and Manipulation

Questions have arisen as to the possibility of nematodes causing HR in the roots of a host plant (Li *et al*, 2015). As HR is considered to be the ultimate stage of ETI, this is an interesting and very important question. Although nematodes causing HR is such an important area of study, infection of root-knot nematodes is usually associated with reprogramming of the plant cell development instead of the host cell death (Caillaud *et al*, 2008). There are protease inhibitors, CPI-2 (cysteine) and API-2 (aspartyl), found in root-knot nematodes, with the function of protecting the root-knot nematode intestines from dietary proteases (De Maere *et al*, 2005). However, there are examples of these protease inhibitors shown to regulate programmed cell death in plant hosts (del Pozo and Lam, 1998). This shows that there are components of the nematodes with multiple functionality dependent on the host interaction and that they could be related in the HR process. In contrast, there are a few studies proposing HR-dependent R-genes in plants, such as *Mi-1*-mediated resistance in tomato, the speculation of HR occurring when resistant coffee cultivars were found to have cell death at the sites of nematode infection proposed to be from *Mex-1*-mediated resistance in coffee and *Me*$_3$-mediated resistance in pepper (Williamson, 1999; Anthony *et al*, 2005; Pegard *et al*, 2005). A more recent study determined that Ca^{2+} channels play an important role in mediating $R_{Mc1(blb)}$, from *M. chitwoodi*, triggered HR in potato plants (Davies *et al*, 2015). This was confirmed with a biphasic ROS burst in the $R_{Mc1(blb)}$ potatoes. In a study on the root-knot nematode's ability of cell growth manipulation (Bellafiore *et al*, 2008), two kinds of cysteine proteinases inhibitors were found. The cysteine proteinases are involved in initiation and progression of programmed cell death (Beers *et al*, 2000). In addition, after successful recognition of the pathogen by the host, an early reaction is an oxidative burst of ROS production. It was analysed that during cyst

nematode (*Heterodera schachtii*) infection of Arabidopsis, the expression of RbohD and Rboh F are the main source of ROS production (Siddique *et al*, 2014). This study proposed that the invasion of the roots and migration causes cellular damage and triggers cell death. The infection increases NADPH oxidase-produced ROS which disrupts the signalling of programmed cell death responses between the neighbouring cells within the site of infection, thus supporting growth and development of the nematodes (Siddique *et al*, 2014).

A recent study investigated the effectors GrEXPB2 and HaEXPB2 expansin effector and their contribution to cell death in the leaves of *Nicotiana benthamiana* (Liu *et al*, 2016). GrEXPB2 and HaEXPB2 no longer induced cell death symptoms induced when the signal peptide was deleted, indicating the importance of the signal peptide. This is also an indication that nematode effectors are indeed able to cause cell death patterns in leaves, and if similar responses to HR occur in roots from other pathogens, there can be no argument that it is indeed possible for nematodes to cause HR in the root system of plants.

Perspective

It is evident that there are many more areas of research to be investigated when it comes to plant defence responses against parasitic nematode infections. Although HR has been studied for many years when it comes to foliar infection by bacteria and viruses, there is little known, in comparison, when it comes to nematodes causing a similar response in the root system. Although HR is the final stage of plant ETI, its effectiveness as a defence reaction can be questioned. It can be considered beneficial to the plant if it is caused by a migratory nematode that needs living cells to continue travelling throughout the plant to feed and develop, however, sedentary nematodes will not travel to these neighbouring cells that are killed off as a result of HR. It could then be considered as a liability if the cells are killing themselves unnecessarily. However, if HR is preventing the spread of the disease symptoms, such as the wilting and

stunting of the above ground growth, HR can be considered as highly beneficial to the host plant.

Chapter II: Nematode Fluorescence Study

Rotylenchulus reniformis Exhibits Green Autofluorescence

Introduction

Autofluorescent material is a compound that will fluoresce when excited by specific wavelength, thereby emitting a specific colour. It can be used as a marker of age and health within tissues of various organisms (Terman and Brunk, 2006). However, this differs depending on the quality of the autofluorescence and at what excitation and colour they fluoresce. For instance, in *Caenorhabditis elegans* that red autofluorescence correlates with health, blue autofluorescence indicates recent death of the organism or tissue and green autofluorescence is a combination of the previous two and cannot be considered as a marker for health and death of the tissues (Pincus *et al*. 2016). Forge and MacGuidwin (1989) found that different nematodes, plant parasitic and free-living, would lose their fluorescence when killed by excessive heat, freezing and formaldehyde application, indicating that the autofluorescence is certainly an indication of viability.

The green fluorescence of interest in this study is relatable to studies with fluorescin isothiocyanate (FITC) which is a hydrophobic compound used for protein labelling and neural structure mapping of *C. elegans* (Hedgecock *et al*, 1985; Shakir *et al*, 1993). The uptake of FITC by *Heterodera glycines*, a plant parasitic nematode, was investigated for the strengthening of fluorescence as a marker in the nematodes and if this would affect the mortality and life cycle of the nematodes (Schroeder and MacGuidwin, 2007). It was found

that the nematodes expressed increased fluorescence over their autofluorescence when exposed to FITC. However, there was a decrease in hatching rate of the nematode, but no effect to the infection rate of the exposed nematodes in comparison with the control.

In this study, we investigate the green autofluorescence found in the plant parasitic nematode, *Rotylenchulus reniformis*, commonly known as the Reniform nematode. The fluorescent compound within the nematodes are extracted to determine the stability of the fluorescent material under acidic, basic, reducing and oxidizing conditions. The use of this nematode component in confocal microscopy and imaging of the nematode interaction with the host is also investigated.

Materials and Methods

Nematodes

Nematode eggs were collected with the sucrose centrifugation technique (Jenkins, 1964). Using a modified baermann funnel method (Viglierchio and Schmitt, 1983), the Reniform eggs were hatched in warm water and filtered through kimwipe paper. Small unwanted particles were then removed. Nematodes were then observed under a light microscope to determine maturity of the nematodes.

Nematode inoculation

Cotton seeds of the cotton line Barbren-713, a known tolerant host of Reniform nematodes (Li *et al*, 2015) were planted in sandy soil and inoculated using a micropipette with 2000 Reniform nematodes on the day of planting. Soil was kept moist with water daily for 14 days in a growth chamber at 30 °C. Carefully, the roots were collected and rinsed of remaining soil particles with clean water for confocal imaging.

Confocal imaging

A small fraction of the nematodes was placed on a long cover slip with an additional cover slip placed above it to spread the water and nematodes apart. The light microscope was used to identify maturity and sex of the nematodes and their eggs. The confocal microscope was used with the FITC filter-set with an excitation of 488 nm.

Before confocal imaging of the cotton roots, a staining solution was needed as the cotton roots do not fluoresce on their own as the nematodes do. The roots were submerged in one milligram per litre of propidium iodide (PI) for five minutes. When collecting images of the root-nematode interaction, a co-imaging of two filter-sets were used. FITC was used with 488 nm excitation for the nematodes as above and the PI solution staining the root cells was excited at 561 nm with the TRITC filter-set.

Compound extraction

The collected Reniform nematodes were concentrated to the highest possible density. They were then ultra-sonicated twice to break down the cell membranes of the nematodes. The sample was then centrifuged again for one minute at 1000 rpm and the supernatant was collected as the nematode fluorescence.

Fluorescence stability

Stability was determined by a time course spectrum using increased levels of potential pH, hydrogen peroxide (H_2O_2), and dithiothreitol (DTT) were measured with the Cytation 3 fluorometer to determine stability in various conditions. An acidic to basic range of pH levels were used including 3.3, 4.8, 5.9, 7.0, 8.0, and 9.2. This was applied with these the use of TRIS-HCL (tris(hydroxymethyl)aminomethane hydrochloric acid) at the various pH levels added to the samples. For both H_2O_2 and DTT 0 µM, 5 µM, 10 µM, 25 µM, 50 µM, and 100 µM were

combined with a measured amount of H_2O to produce a final volume of 100 µL for each sample. Each of the eighteen solutions were placed into their own well in a 96-well plate. Two microliters of nematode fluorescence were pipetted into each of the eighteen wells. Using the Cytation 3 Imagining Reader, the emission and excitation of the nematode fluorescence was measured and recorded. The emission range used was 300-700 nm and the excitation point used was 425 nm. To determine stability of the fluorescence, the emission and excitation was measured at starting time, thirty minutes, one hour, 2 hours, three hours and 4 hours. The control was determined by averaging the data at 0 hours. Control groups for the experiments were not treated with any chemicals and were measured after extraction.

Results

Reniform nematodes produce autofluorescence in intestinal tract

Under confocal microscopy, the entirety of the nematodes could be viewed with an emission filter and the fluorescent compound within the nematodes was visible under an excitation of 488 nm confocal laser (Figure 1). Various stages of the Reniform development were recorded under confocal microscopy, including the egg stage, juvenile stage and the mature stage, both male and female. The autofluorescence emits from the gut of the nematode at all growth stages with exception to the eggs, which appear to completely fluoresce.

PI solution and nematode autofluorescence can be combined to create interaction imaging

Reniform autofluorescence in combination with the PI solution staining of the plant roots makes it possible to view a high detailed real-time interaction between the Reniform and the cotton roots (Figure 2). The Reniform protrudes out of the cotton root as the posterior remains on the outside of tits host. The PI solution creates a clean red bordering shape of each cell of the roots to distinguish between each of them.

Reniform autofluorescent material is a stable compound

The Reniform fluorescence decreased steadily from 1 to 4 hours at different concentrations of pH, H_2O_2 and DTT. For different pH levels (Figure 3A), pH 3.3 and pH 8.0 had a decreased intensity of approximately 30 watts per square meter (W/m2). The 9.2 pH intensity decreased a small amount between 10 to 20 W/m2. The pH 4.8, 5.9 and 7.0 changed intensity drastically over the course of 4 hours, decreasing between 100 and 175 W/m2. All of the DTT concentrations diminished (Figure 3B). The 5.0 μM and 50 μM concentrations decreased the most significantly, dropping from 395 to approximately 200 W/m2. The data showed similar results for H_2O_2 (Figure 3C), all reducing. The 10 μM concentration decreased significantly of approximately 130 W/m2 from 1 to 4 hours, whereas the 50 μM concentration decreased only 20 W/m2.

Discussion

The autofluorescence in the nematode proves to be a useful characteristic for identification of parasitic nematodes on the roots of plants. The green autofluorescence against the red PI solution creates a high-quality image for detection of nematode presence as well as identification of the feeding site of the nematode. The autofluorescence as an indicator of viability (Forge and MacGuidwin, 1989) may also indicate of the nematode is still alive at the site of infection (Figure 2). As the Reniform nematode eggs are autofluorescent, it is also possible to see if the female is bearing eggs that have the ability to hatch and produce juvenile nematodes.

The Reniform autofluorescent material is a stable compound as there is not much degradation over time. Treatment with DTT, a reducing agent, is frequently used to reduce the disulfide bonds of proteins by decreasing the number of binding sites or to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins (Vauquelin

1979). The different concentrations of DTT decreased the fluorescence intensities more disjointedly than those of the H_2O_2 levels, which decreased contiguously, however, the compound still had fluorescent qualities under these conditions, showing its stability.

The autofluorescent compound of the Reniform is also able to retain its quality when the nematode is exposed to PI solution (Figure 2). This shows evidence that the autofluorescent material within the gut of the nematode is well protected even when the nematode is feeding on the cotton root. It is also interesting to note that the fluorescent material had a higher intensity at acidic levels of pH (Figure 3A). This makes sense as the fluorescent material is found in the gut, or digestive tract, of the nematodes which is acidic for breakdown of food.

In conclusion, the fluorescent material is degraded after extensive exposure to both reducing and oxidizing conditions (Figure 3B and C). The degradation under these conditions can be supported by the studies that show nematodes losing their fluorescence when they are killed, as the body of the nematode will likely oxidize as they can no longer control the diffusion of oxygen into the bodies (Pincus, *et al*, 2016; Forge and MacGuidwin, 1989). However, when protected by the Reniform nematode structure, the fluorescence will remain stable within the nematode during all stages of its life cycle, even during infection.

Figures

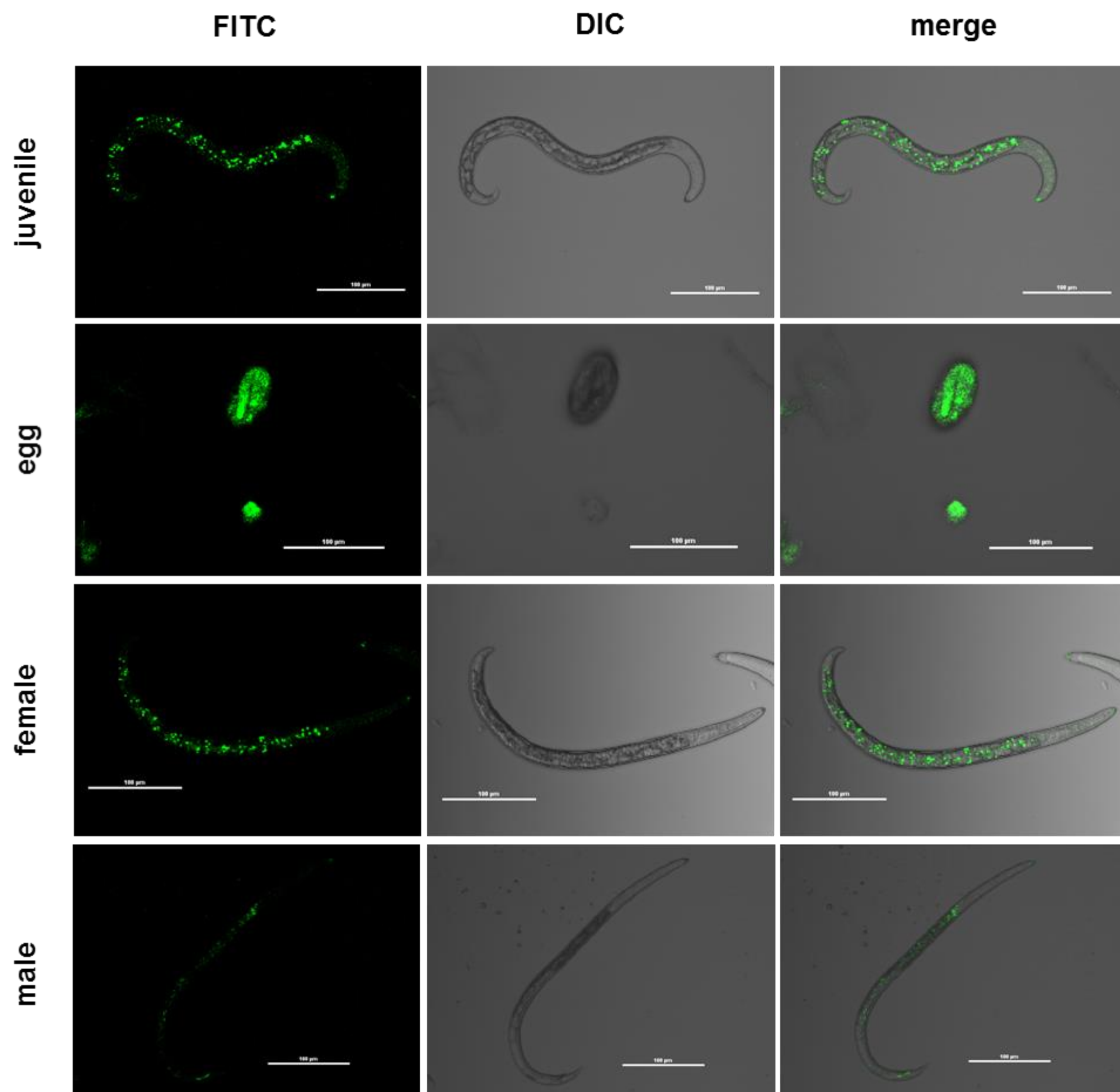

Figure 1. Reniform nematodes express autofluorescence throughout various life stages. The Reniform nematodes express green fluorescence within the intestine at an excitation of 488 nm under confocal microscopy at all stages including juvenile, mature male or female as well as when still inside the egg. This fluorescence is easily visible within the gut of the Reniform nematode and can be utilized for view of the nematode under confocal microscopy.

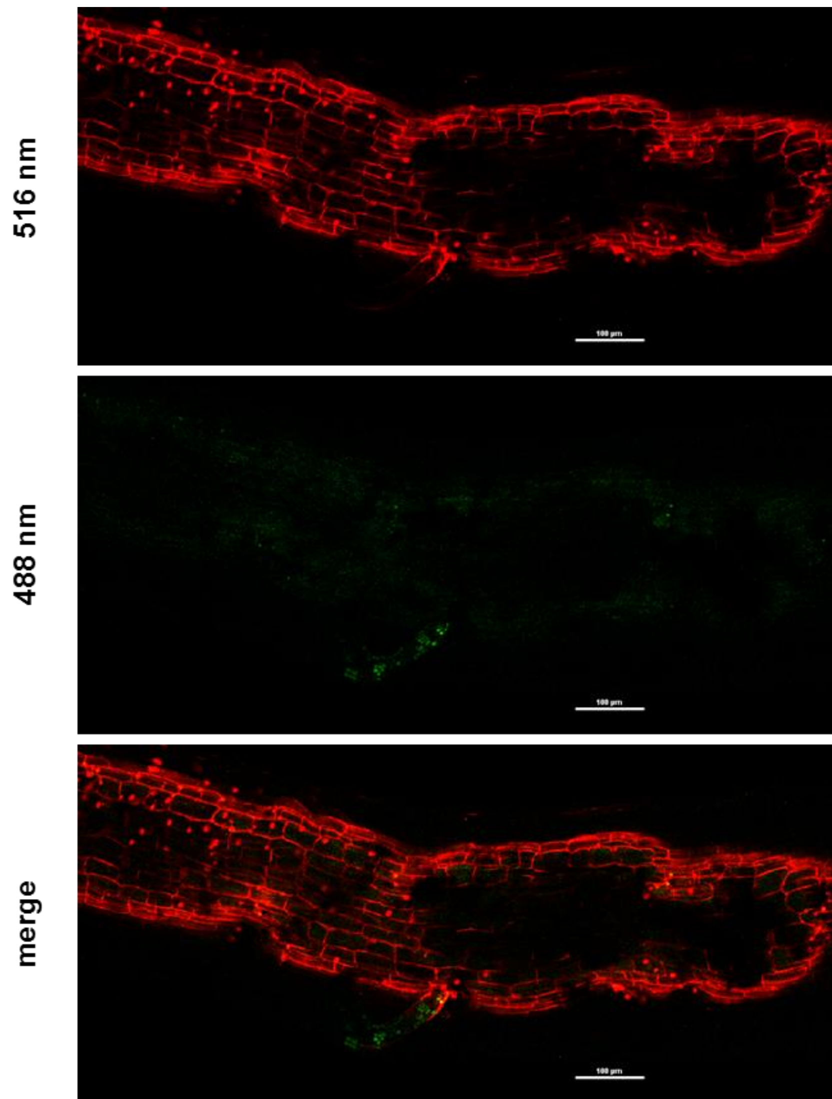

Figure 2. Real-time co-imaging of Reniform nematode establishing a feeding site on cotton root. The confocal microscope is used to capture images at two separate emission filters, FITC and TRITC, with excitations at 488 nm (green) and 561 nm (red), respectively. The green autofluorescence is easily distinguished from the red PI fluorescence, creating a real-time interaction image of the Reniform nematode on the cotton root.

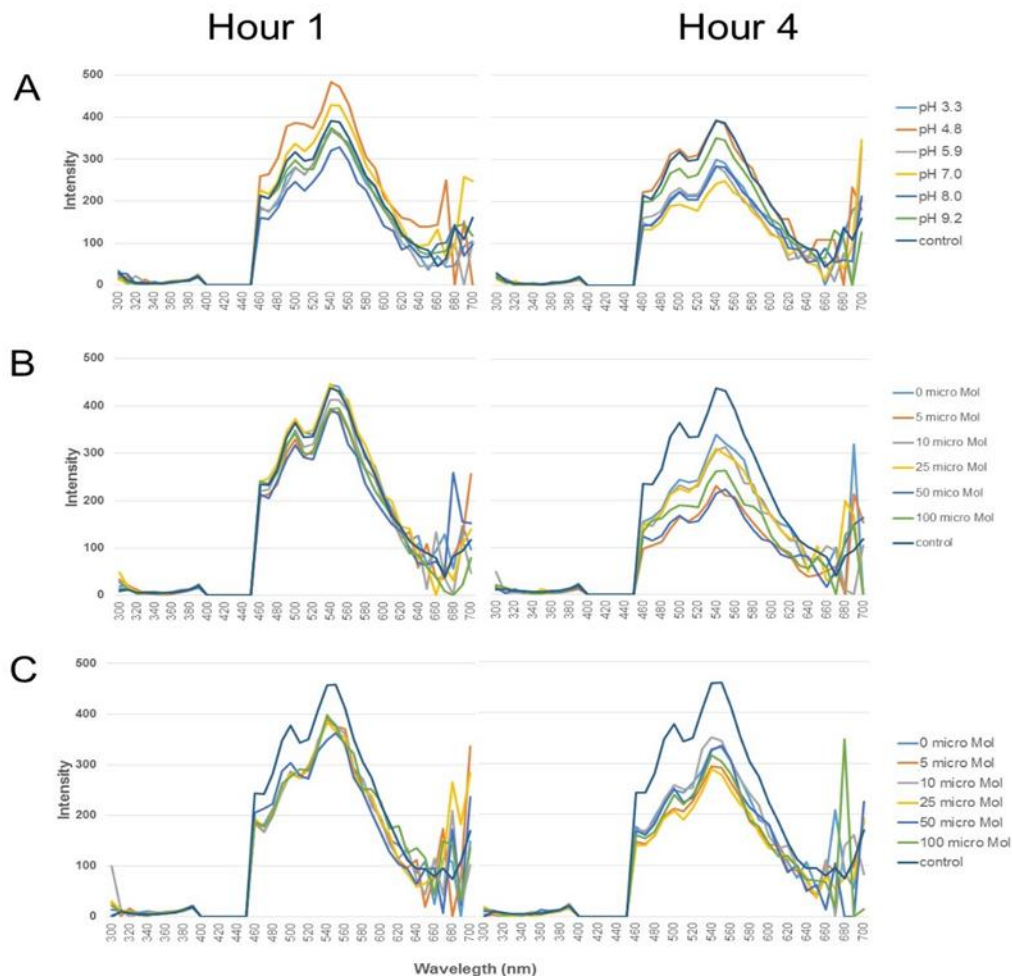

Figure 3. Average fluorescence intensity versus various wavelengths under different conditions. A) The intensity readings when treated at various pH levels. B) The intensity readings at various DTT levels. C) The intensity readings at various levels of H_2O_2.

Chapter III: Hypersensitivity in Cotton Roots

Characterization of Root-Associated Hypersensitive Response in Cotton-Nematode Interactions: A novel method for analysis of nematode-root interactions

Introduction

For many decades, the plant immune system has been a complex territory of study. Plants need to defend themselves against many threats in the form of fungi, viruses, bacteria, insects, oomycetes and nematodes. The plant defence system against pathogens consists of two main levels. The initial, or basal, form of defence is induced by detection of Pathogen- or Microbial-Associated Molecular patterns (PAMPS or MAMPs). This basal defence system is known as PAMP-triggered immunity (PTI) (Bent and Mackey, 2007). When this layer of defence is overcome by the pathogen, a second level of defence is initiated. This is achieved by the plant expressing R (resistance) proteins which recognize specific effectors. This induces Effector-Triggered immunity (ETI) (Jones and Dangl, 2006), related to the hypersensitive response (HR) in a gene-for-gene interaction. The host must have a dominant R-gene which corresponds with the pathogen's dominant *avr* (avirulence) gene (Greenberg and Yao, 2004). Evidence of this interaction is in studies of the tomato Pto resistance gene corresponding to the avrPto gene product produced by *Pseudomonas syringae* pv. *tomato* (Tang *et al*., 1997). Once this detection has taken place, an oxidative burst occurs and Reactive Oxygen Species (ROS) are produced (Morel and Dangl, 1997). The production of ROS is mediated by NADPH activities. These activities are encoded by Rbohs (Respiratory burst oxidase homologs) to which plants contain several (Zurbrigen *et al*., 2009; Siddique *et al*., 2014). The role of ROS in plant defence will differ depending on the plant-pathogen interaction, though the oxidative burst often occurs before HR as NADPH oxidase activity is required to induce ROS production in the apoplast (Zurbrigen *et al*., 2009; Davies *et al*., 2015).

Rotylenchulus reniformis, common name Reniform, is a semi-endoparasitic species of nematode. Cotton (*Gossypium hirsutum* L.) yield losses caused by Reniform are as high as $170 billion per annum (Elling, 2013). The female Reniform nematodes penetrate the root cortex and establish a permanent feeding site in the stele, the single nematode becomes sedentary and will not move from this feeing site. The head of the nematode body will remain embedded in the root tissue and the tail, or posterior, while remain outside of the root tissue and will swell into a kidney shape, as the Reniform matures and continues its life cycle when producing eggs (Robinson *et al*., 1997; Linford and Oliveira, 1940).

Questions have arisen about the plausibility of the HR response occurring in the root system of a host plant (Li *et al*. 2015). Understanding the processes and mechanisms of HR is important to breeders who can utilize these qualities of the plants in fields of crop production. By strengthening the aspects of the plants that provide resistance to plants will result in lower crop losses caused by pests. Plant parasitic nematodes such as *Meloidogyne incognita* (root-knot) have cell reprogramming capabilities, creating giant cells or syncytia to allow for feeding on the root the root system in their life cycle (Caillaud *et al*., 2008). These cell reprogramming mechanisms may be beneficial to the nematodes instead of the host, however it is an important aspect to consider when studying the mechanism of reprogramming cell suicide in the roots as a reaction to the threat. A more recent study showed Ca^{2+} channels playing a role in mediating $R_{Mc1(blb)}$, from *M. chitwoodi*, which triggers HR in potato plants (Davies *et al*. 2015).

In this study, we investigate the possibility of an HR response occurring in a specific cotton line, Lonren-1, when under threat of the Reniform nematode. Proposed to be a hypersensitive cotton line (Li *et al*, 2015), this specimen was used with the semi-endoparasitic Reniform nematodes to investigate the cell death patterns without the need to physically destroy the roots for analysis, as the Reniform posterior remains on the outside of the roots. By developing a method of analysis through confocal microscopy, we were able to develop a novel system for

real-time imaging of the cellular reaction between a plant root and a nematode. For confirmation of the HR response, methods of ROS staining on the roots for physical evidence and enrichment analysis for genomic evidence were performed in this experiment.

Methods and Materials

Phenotypic reaction

To establish the different phenotypic reactions to the infection of Reniform nematodes, three specific cotton lines were used to compare the reaction of a proposed hypersensitive line (Lonren-1), a susceptible line (SG-747) and a tolerant line (Barbren-713). The tolerant line is not a resistant line as the nematodes will still establish a feeding site, however, the cotton will continue to grow at a normal rate. A clean soil medium was prepared in 50 mL conical tubes for all treatments. Seeds were sterilized in 10% chlorox for five minutes before being rinsed and planted in the soil. Treated seeds were inoculated at the time of planting with 2000 juvenile Reniform nematodes. The tubes were treated with water daily for two weeks before data collection. Control plants were treated in the same manner without inoculation of nematodes. All cotton plants were placed in a clean growth chamber at 30 °C.

Rotylenchulus reniformis infection curve

The samples were treated in the same manner as above for planting of the cotton seeds and inoculation of the Reniform nematodes. Cotton roots were collected and excess soil was rinsed off the roots. The cotton roots were then examined under a light microscope and the number of Reniform nematodes attached to the roots, or established a feeding site, were counted. The data collection of the roots was taken at two day intervals, starting with eight days post-inoculation and ending with 18 days post-inoculation.

Confocal microscopy imaging

Using a simple light microscope was not an appropriate way to investigate the cell death patterns on the roots when a Reniform nematode established a feeding site on the root. In this study, a new method of real-time analysis was developed to attain high quality imaging of both the nematode and the cotton root cells which were affected by the interaction. To establish imaging of the cotton roots under confocal microscopy, control cotton plants were grown for two weeks in soil. When cotton roots were collected, they were rinsed of soil and placed in propidium iodide (PI) at a concentration of one milligram per liter for five minutes. The cotton roots were analyzed under the confocal microscope at an excitation wavelength of 561.5 nm with the TRITC filter-set to excite the PI solution in the cell walls of the root cells, emitting a red colour, to produce a high-detailed image of the cotton roots. This procedure was repeated for cotton that was planted in soil at the same time as inoculation with 2000 juvenile Reniform nematodes. The reniform nematodes are autofluorescent and can be seen under confocal microscopy at an excitation wavelength of 488 nm with the FITC filter-set which emits a green colour. Using co-imaging of the 561.5 nm and 488 nm, a real-time image of the infection of the Reniform in the cotton root was attained for each of the cotton lines.

The above protocol was repeated for reactive oxygen species (ROS) burst analysis of the infected cotton samples. However, before PI staining, the roots were placed in one 50 µM of 2,7-dichlorofluorescin acetate for 30 minutes for staining of ROS products in the roots cells. The ROS production is visible with the same parameters and filter-set as the nematode fluorescence.

Results

Phenotypic reaction

After 14 days of growth in a clean chamber at 30 °C, the cotton plants were removed from the soil and each line of cotton was compared for their different reactions to the infection of

nematodes (Figure 1). When compared to the control group (Figure 1A), Barbren-713 were noticeably the least effected by the infection of the Reniform nematodes as both their root and shoot growth remained strong in comparison to the other cotton lines. SG-747 roots took the largest amount of damage to both the tap root and the lateral roots, whereas the Lonren roots only showed a loss of lateral root growth and an extended tap root growth. The control group, however, all grew relatively the same as each other (Figure 1 B), providing evidence that each cotton line has a different phenotypic reaction to the infection of Reniform nematodes.

Infection curve

To determine the ideal time for analysis of the cotton root reaction to the infection of Reniform nematodes, the infection rate of the Reniform nematodes to the cotton roots had to be established. The average number of nematodes per 10 samples were calculated and compared with variables of days post inoculation and the cotton line used (Figure 2). It was determined that the number of nematodes establishing an infection would increase for all cotton lines between 12 and 16 days, placing the 14 days mark as an ideal time for analysis under confocal microscopy, as most Reniform nematodes will have recently infected the cotton roots.

Confocal imaging

The importance of developing a protocol for clear imaging of the cellular reaction which takes place upon infection of the Reniform nematodes was indeed crucial. It was established that the PI solution was effective in creating a clear image of the root cells emitting a red colour of the cell walls and the stacked images could create a three-dimensional image of the root and its cells (Figure 3). It is also important to note that the autofluorescence of the Reniform nematode is utilized in this experiment as it emits a green colour when excited at 488 nm under confocal microscopy (Figure 4). Using the different excitation wavelengths, separate images could be superimposed upon each other, a method of co-imaging, to allow a more detailed look at the

reaction of the root cells to the plant cells, in contrast to light microscopy methods such as staining the nematodes with acid fuchsin (Figure 5A; Thies *et al*, 2002), which requires bleaching of the roots which ultimately destroys the cells under observation and does not provide a clear image. The co-imaging of nematodes on the stained root cells was achieved (Figure 5B).

Once the protocol had been established, the cell death patterns at the Reniform infection sites were analyzed to determine if a HR response is plausible (Figure 6). Upon infection of the susceptible host, SG-747, the roots show poor quality of growth and darker areas where cells have died, however these patterns are not localized. When looking at the Lonren-1 roots, the sunken, dark area surrounding the nematodes is within a small perimeter, suggesting a localized cell death pattern. The Barbren-713 on the other hand shows a well-established feeding site on the cotton root, however no clear cell death patterns, appearing unaffected by the presence of the Reniform nematode.

ROS production

Using a staining solution of 2,7-dichlorofluorescin acetate, the production of ROS can be seen under confocal microscopy excitation of 488 nm (green). To illustrate the ROS production on the cotton roots, the co-images were made as was for the cell death pattern analysis (Figure 7). After ROS staining, it is clear that there was no ROS production in the Barbren-713 and the SG-747 roots after Renifrom infection (Figure 5A and B). However, there is apparent evidence of ROS production in the Lonren-1 cotton roots (Figure 5C), indicating a large spike in production of ROS.

Discussion

Confocal co-imaging procedure

With confocal microscopy and co-imaging of separate filter-sets allows different colour visuals, we can successfully create images of nematode and root cell interactions. With this novel experimental procedure, we can view a high detailed cellular interaction between the nematode and its host root cells. With the transparent characteristic of the nematode structure, it is difficult to view the nematodes without having to stain the nematodes, also killing them with heat and products such as acid fuchsin (Thies *et al*, 2002). With this confocal co-imaging method, there is no heat application and substances harmful to the nematode survival before analysis under the confocal microscope. This is a real-time imaging system, utilizing the autofluorescence of the nematode, emitting a green image to superimpose on a red image, being the PI solution stain of the cotton roots. The stacking of the images allows for a three0dimesional structural view of the roots and the nematode interaction with the roots (Figure 6).

Phenotypic reaction to Reniform infection

The phenotypic reaction of the cotton lines to the Reniform nematodes is expected. The Barbren-713 showed very little reaction to the nematode infection both at a macroscopic view (Figure 1) and a microscopic view (Figure 6). Barbren-713 is considered a tolerant host, as resistance implies some sort of reaction or avoidance to the pathogen. In this case, the Reniform nematodes are still able to establish a feeding site and continue their life cycle whilst the Barbren-713 continued to grow normally in comparison to the control group. The SG-747 cotton line also showed expected results of a shortened tap root and all around stunting of the plant, as this line of cotton is susceptible to nematode infection. The Lonren-1 displayed a phenotype of a curious loss of lateral roots. This could possibly be a result of nematode infection on the smaller and weaker lateral root growth of the cotton and the cell death caused weakness in the stability and structure of the lateral roots, causing them to easily fall off as they

are so young. This would not be a beneficial reaction for the Lonren-1 cotton plants as the would then struggle to establish a stable root structure.

Reniform-cotton infection curve

The results of the infection curve may seem contradictory to studies which see a higher population density in soils with susceptible cotton lines as opposed to resistant cotton lines (Starr *et al*, 2007), however this study investigated the number of nematodes established infection on the cotton. These numbers make more sense when taking into consideration the size of the plants and how many more infection sites are available on the roots. As Barbren-713 is a tolerant host, the size of the root system is far bigger than the susceptible host (Figure 1). This allows more surface area for more nematodes to feed as competition for space remains a factor in infection rates. A further approach could be taken to select a specific number of lateral roots per plant when counting the nematode infection, however this will still create the variable of which sites of the roots is more desirable to the nematode.

Hypersensitive response

The Lonren-1, which is the proposed hypersensitive cotton line (Li *et al*, 2015), had a small perimeter of cell death surrounding the area of infection, distinct to the characteristics of the Barbren-713 and Sg-747 lines (Figure 6). Barbren-713 displayed no cell death patterns or reactions to the nematode presence, nor did it show any ROS production upon ROS staining (Figure 7). SG-747 may have had cell death, but in a larger, more disorganized perimeter than Lonren-1. The SG-747 also did not produce ROS. Lonren-1 shows production of ROS, indicating a burst of ROS production, which is a precursor to the HR response (Zurbrigen *et al*, 2010). This may provide evidence that an HR response, or a similar defense mechanism, is responsible for the stronger resistance to Reniform attack than the susceptible hosts. However, the HR response is a localized reaction of the host to the infection site (Jones and Dangl, 2006).

The ROS production (Figure 7) in the Lonren-1 cotton roots is not in a small area, but scattered across the cotton root. A possible reason for this type of scattered ROS production may be in response to the Reniform nematode having punctured various sites before establishing an infection site, causing each cell to react separately to the attack.

Conclusion and future

Although the hypersensitive response is meant to be an immune response, implying it acts towards the host plant's best interest from a health and crop yield perspective, an HR response is of no use to the host in this specific scenario. The HR response prevents the spread of a biotrophic pathogen by programmed cell death of the surrounding cells at the site of infection (Morel and Dangl, 1997). The Reniform nematode is sedentary and therefore does not move once it has established an infection site. This reaction may be useful if bred in the host plant for resistance against migratory nematodes such as *Radopholus similis* or *Pratylenchus spp.* (Moens and Perry, 2009).

Although ROS production provides evidence of a possible HR response in the Lonren-1, it is important to determine if these traits match up to the HR response on a genetic level. Through further bioinformatic research, we will investigate the genes that are more highly expressed than others upon Reniform infection of the Lonren-1 cotton plants and compare these genes with data of known hypersensitive reactions to determine if this is the same response that takes place as a part of the plant immune system, or if this may only be an associated reaction.

Figures

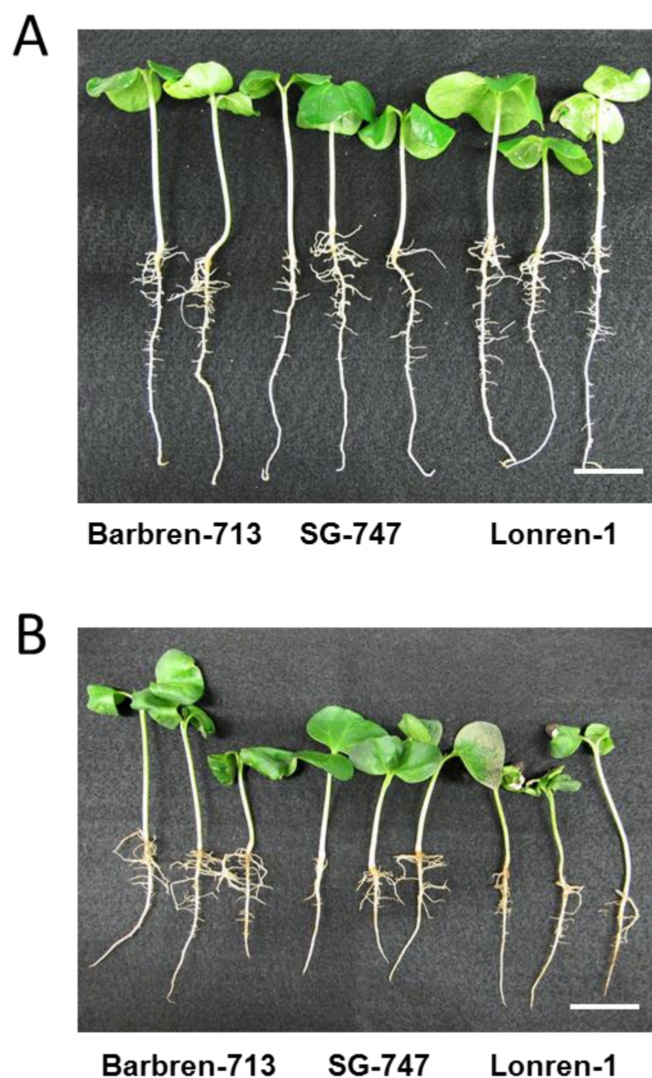

Figure 1. Phenotypic reaction of cotton lines to Reniform infection. A) Cotton plants were grown under sterile condition with no infection of Reniform to establish a control whereby the plants will grow uninfected. B) The cotton lines were infected with 2000 nematodes at time of planting and displayed each displayed a different phenotypic reaction. The white bar in the bottom right indicates one inch measurement.

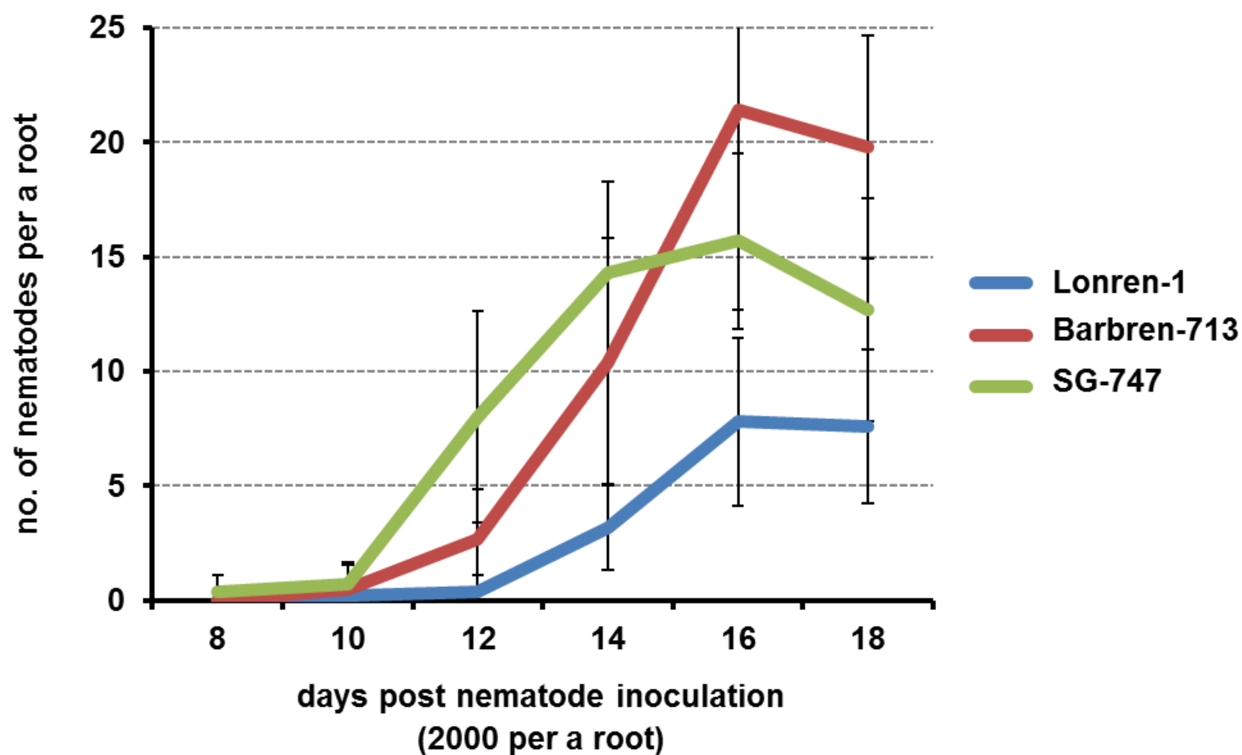

Figure 2. Reniform nematode infection curve. Lonren-1, Barbren-713 (tolerant) and SG-747 (susceptible) cotton lines (n = 10) were inoculated with 2000 Reniform nematodes and the infection rates compare each cotton line and determine the ideal time for confocal analysis.

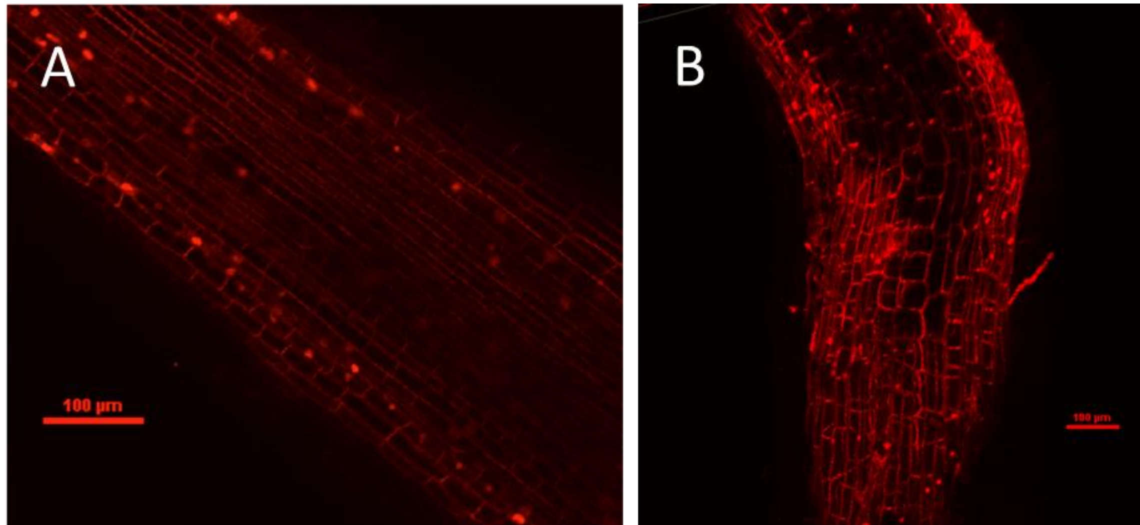

Figure 3. Confocal images of cotton roots. Untreated cotton roots of Lonren-1 were stained with PI (propidium iodide) solution and examined under confocal microscopy on A) one plane for two-dimensional imaging and B) stacked planes to provide a three-dimensional structure

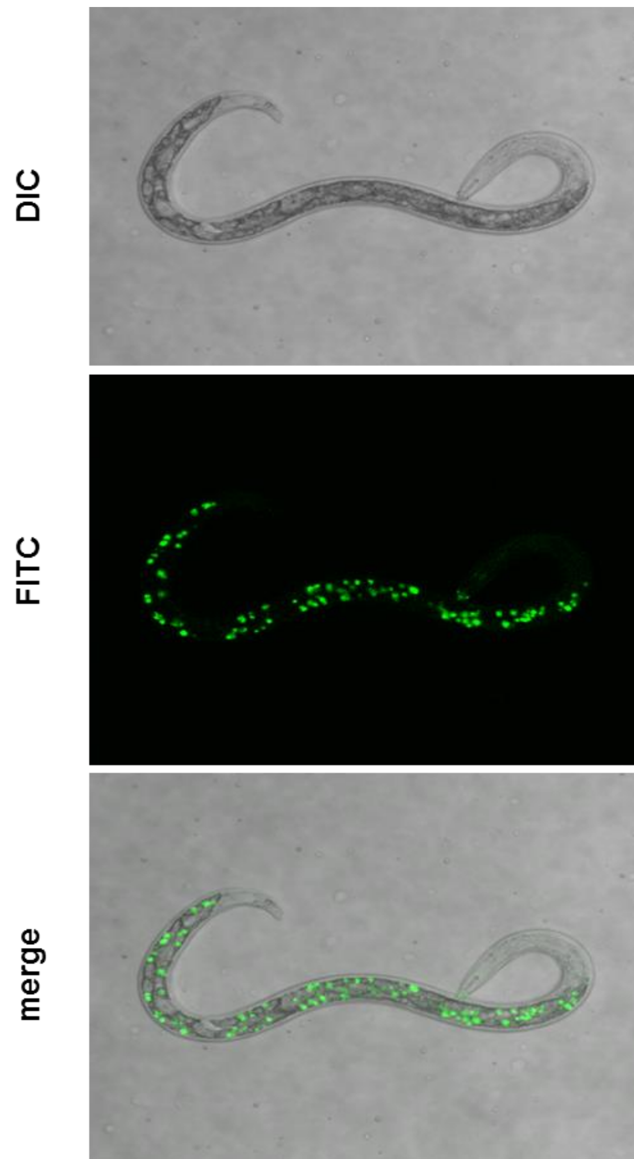

Figure 4. Juvenile Reniform nematodes expressing autofluorescence at 488 nm excitation.

The Reniform nematodes are autofluorescent at 488 nm excitation with an FITC filter-set.

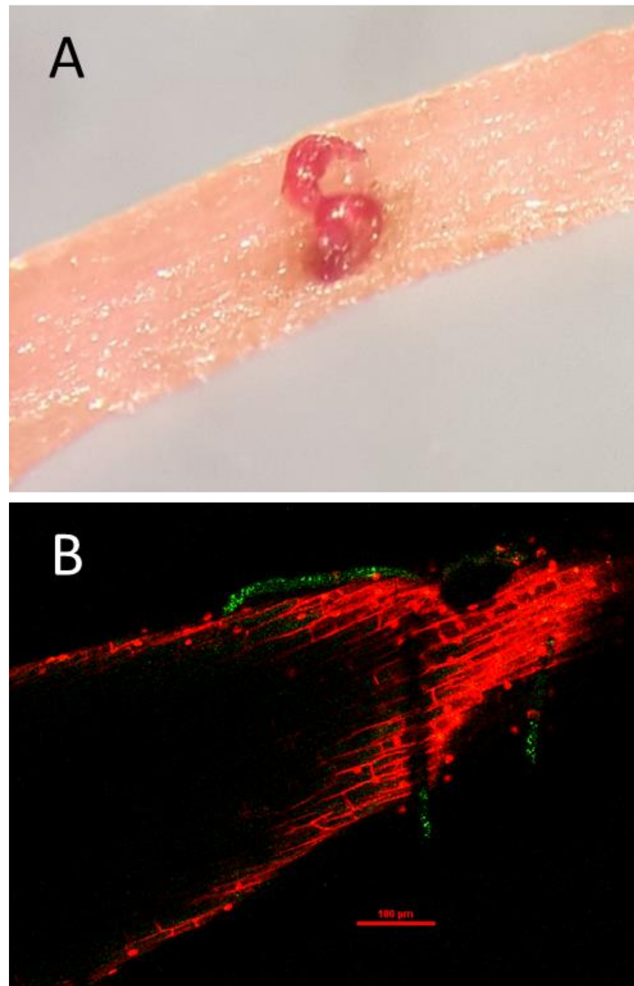

Figure 5. Acid fuschin staining method versus confocal microscopy. The comparison of the cellular imaging of the A) acid fuschin method and B) the novel co-imaging confocal method, allowing a more detailed cellular view of the root-nematode interaction.

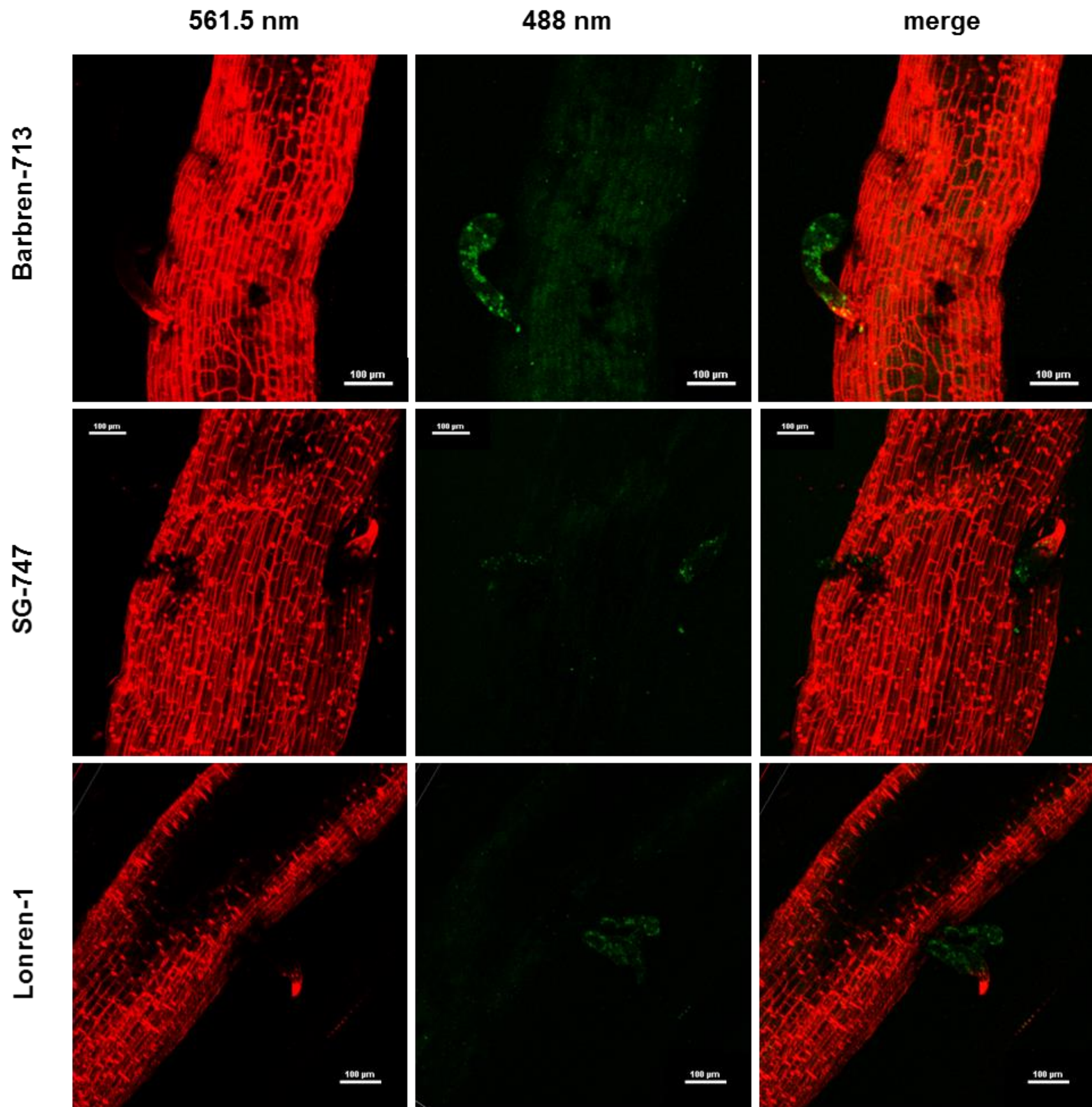

Figure 6. Cell death patterns of various cotton line sunder confocal microscopy. Using the co-imaging confocal method of root imaging, Barbren-713, SG-747 and Lonren-1 cotton roots could be viewed with nematode infection site expressed by the green autofluorescence. Darkened areas indicate cell collapse and death.

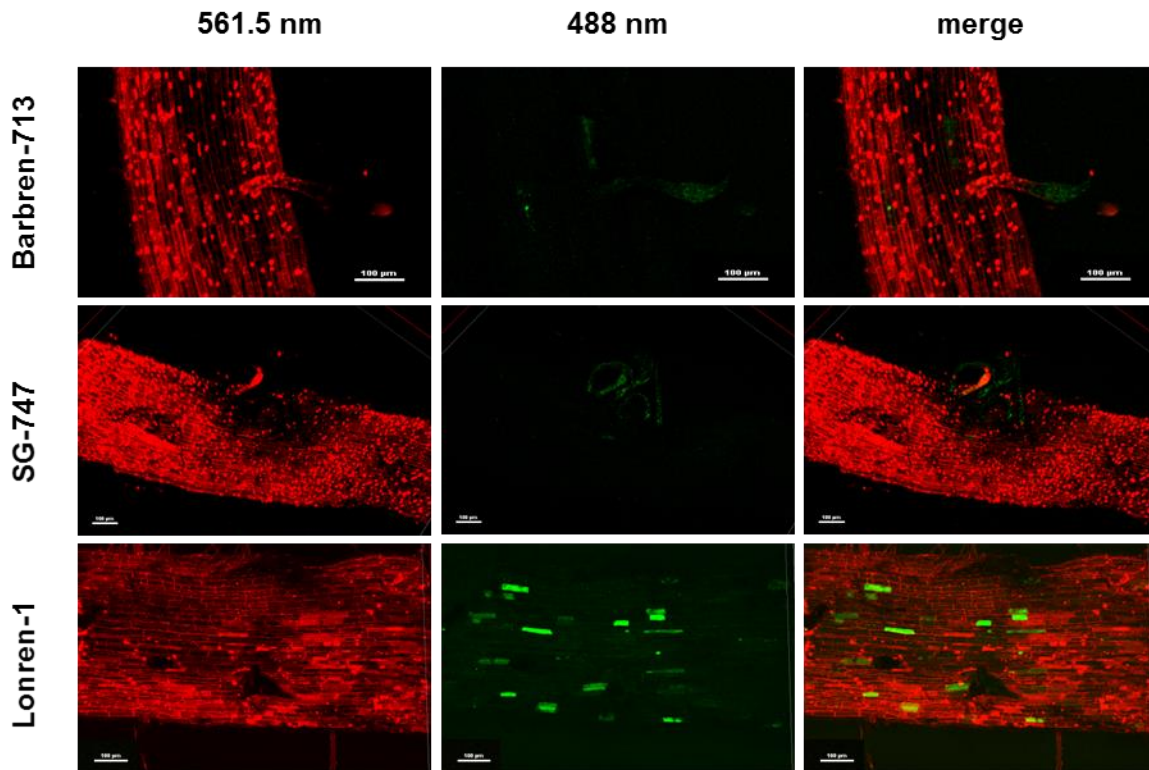

Figure 7. ROS staining and confocal micros copy of various cotton lines. The ROS stain is excited by the same wavelength as the nematode autofluorescence, therefore green sites are an indication of ROS production. Lonren-1 was the only plant displaying ROS production upon nematode infection, however the production was not uniform or localised.

Chapter IV: Overall conclusion

During this study, I investigated the possibility of cotton roots expressing a hypersensitive response when attacked by Reniform nematodes. By comparing various lines of cotton, using the proposed hypersensitive, a susceptible and a tolerant line, the phenotypic reactions of the cotton to Reniform attack could be determined for each line. I developed a real-time co-imaging method to see the cellular interaction between the Reniform nematodes and their hosts.

1) Before developing a protocol for viewing of the Reniform nematode and cotton interaction, I needed to figure out the infection rate of Reniform nematodes on young cotton plants. It was important the roots be younger and smaller, as these were going to be used on the confocal microscope. The results, however, were surprising, as each plant was inoculated with as many as 2000 nematodes and only a small fraction of the nematodes established a feeding site, indicating their low infection efficacy to young cotton roots.
2) Determining the autofluorescent material was stable in the Reniform nematodes was an important aspect of the study with regards to developing the protocol. For successful co-imaging, the nematode autofluorescent material had to remain stable and visible when stained with PI solution. The stability of the autofluorescent material is high as through many different treatments, as well as many days kept in cold condition, the nematodes retained their autofluorescence.
3) This was the first method of real-time confocal imaging on a plant with such large roots. Although studies had been done through staining of roots and confocal microscopy, these studies used roots of Arabidopsis, which have far smaller and thinner roots than cotton. This method is the first if its kind to attain real-time cellular interaction images between nematodes and cotton roots, utilizing the autofluorescence of plant parasitic

nematodes and the semi-endoparasitic nature of the Reniform nematode. I was able to create clean and clear images of all the cotton lines as well as view the cellular death patterns surrounding the sites of infection.

4) Using ROS staining with the confocal microscope, I was also able to show that the ROS burst, which is characteristic of an HR response, was only detected in the proposed hypersensitive line of cotton, Lonren-1. This takes the study a step further in confirming that this may be HR in the cotton roots.
5) Further studies will be done with bioinformatic to confirm if this reaction is HR or if it is associated to HR in anyway. By comparing the gene expression during Reniform nematode infection on the hypersensitive cotton roots and the gene expression of know HR cases in reaction to bacteria or viruses, I will be able to confirm further information about these reactions.

In conclusion, this study has answered many questions about HR as a reaction to nematode infections, as well as developed a novel system for real-time imaging of nematode and roots interactions using confocal microscopy.

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