Small RNA and Transcriptome Analysis of Cotton (*Gossypium hirsutum* L.) Susceptibility, Resistance, and Hypersensitivity to Reniform Nematodes (*Rotylenchulus reniformis*)

by

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Abstract

Reniform nematodes (RN) are semi-endoparasitic nematode species causing significant yield loss in cotton, particularly in the eastern US cotton belt. Successful RN parasitism is contingent on establishment of a syncytium, which serves as the sole nutrient source on which RN live. The syncytia are hypertrophied, multinucleate root cells with enlarged nuclei and dense cytoplasm, which resulted from the breakdown of cell walls between initial feeding cells and neighboring cells. It is believed that nematode secretions injected through their stylet, a specialized needle-like structure mouthpart, are essential in syncytium initiation and maintenance.

In response to nematode infestation, plant resistance relies on the coordination of different resistance mechanisms including specific resistance genes or proteins, several plant hormone pathways, and reactive oxygen species (ROS) that are generated in response to nematode attack. These resistance-related elements crosstalk to each other and can be seen as an integrated signaling network regulated by transcription factors and small RNAs (sRNAs) at the transcriptional (epigenetic), posttranscriptional, and/or translational levels. However, little is known about the mechanisms involved in host responses to RN infestation.

The overall objective of this project is to identify and characterize such regulatory networks in cotton root responses to RN infestation with the following specific objectives: 1) To determine transcriptom and sRNA expression in cotton roots with

different levels of RN resistance with and without RN infestation, 2) to correlate the identified sRNAs and transcriptome expression profiles and build potential regulatory networks that are important in mediating different levels of resistance to RN in cotton roots.

To accomplish these objectives, both transcriptome and sRNA libraries were constructed from RN susceptible, resistant, and hypersensitive genotypes of cotton seedlings, with and without RN infestation. The expression of transcriptome was analyzed to detect RN responsive genes and important gene differences between genotypes with varied levels of RN resistance. A number of known genes involved in generic plant-nematode interactions, as well as genes that are newly identified to be involved in cotton-RN interactions, were detected in this study. For the second objective, conserved miRNAs and lineage-specific miRNAs were identified, and the target sequences of the identified differentially expressed miRNAs were determined from the custom assembled transcriptome data. SRNA regulatory networks involving miRNAs and their negatively regulated target genes were suggested to play important roles in RN pathogenesis in cotton roots.

Taken together, the work in this study identified genes and sRNAs that are important in plant responses to RN infestation and in the genotypic variations for RN resistance, and these results will set a foundation for future research towards understanding the resistance mechanisms to RN in cotton.

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Chapter I. Introduction and Literature Review

Plants are often exposed to biotic stresses derived from viruses, bacteria, fungi, nematodes, and insects. Interactions between host plants and their pathogens determine the degree of pathogenesis observed. Successful pathogens attach to a host plant, penetrate through the physical barriers of the cell wall, and override host plant defenses. Once inside the plant, pathogens can either kill plant cells (necrotrophic pathogens), or live within host tissues without causing plant cell death (biotrophic pathogens). In response, resistant plants have evolved the ability to recognize pathogens and make timely defensive responses.

The interactions between plants and pathogens are summarized as a 'zigzag' model where plants are able to recognize pathogen-associated molecular patterns (PAMPs) derived from the pathogen utilizing pattern recognition receptors (PRRs) leading to pattern-triggered immunity (PTI) (Jones and Dangl 2006). PAMPs are invariant epitopes derived from pathogens that are: fundamental to the fitness of pathogens, absent in the host, and recognized by a wide array of potential hosts (He, et al. 2007; Schwessinger and Zipfel 2008). PRRs are cell-surface-localized receptors, usually harboring an extracellular leucine-rich repeat (LRR) domain, that recognize conserved pathogen elements (Schwessinger and Zipfel 2008).

Pathogens that successfully suppress PTI responses can release pathogenic effectors into host plants, altering host-cell structures and suppressing defense responses leading to effector-triggered susceptibility (Jones and Dangl 2006). Specific resistance

proteins (R-proteins) have also evolved in response to such effectors, to sense pathogen effectors yielding effector-triggered immunity (ETI) (Jones and Dangl 2006).

Canonical R-proteins are intracellular and often referred to as NBS-LRR proteins because they typically contain nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Hogenhout, et al. 2009; Shirasu 2009). While the LRR domain is believed to be responsible for interaction between plant receptors and pathogen effectors, the NBS domain is characterized by NTPase activity and functions as a molecular switch activating subsequent downstream signal transduction during contact with pathogen-derived effectors (Glowacki et al. 2011). R-proteins can directly sense pathogen effectors, or they can detect pathogens though other cofactors, which are direct host targets of pathogens (Glowacki, et al. 2011).

Sedentary plant endoparasitic nematodes (SPENs) are biotrophic pathogens that can cause significant yield loss in crop plants. The most well studied and crop-impactful SPENs are root-knot nematodes (RKN, *Meloidogyne* spp.) and cyst nematodes (CN, *Globodera* and *Heterodera* spp.), while reniform nematodes (RN, *Rotylenchulus reniformis* Linford & Oliveira.), a kind of plant semi-endoparasite, are also known to affect important crops such as cotton and soybean (Robinson 2007).

The life cycle of the different SPENs, i.e. RKN, CN, and RN, typically requires around three weeks under favorable environmental conditions of optimal soil moisture and temperatures. Eggs generated by female nematodes are deposited into a gelatinous matrix on the host root surface, which protects eggs from dehydration (Williamson and Gleason 2003). There are four juvenile stages, separated by molts, needed for eggs to mature into adults. While the second-stage juvenile of RKN and CN penetrates into the

host plant root, for RN it is the female young adult that is the infective stage.

Once inside the host plant, successful nematode parasitism is contingent upon establishment of a nematode feeding site (NFS), which serves as the sole nutrient source on which the nematode lives. The NFS are hypertrophied, multinucleate root cells with enlarged nuclei and dense cytoplasm, which resulted from nuclear division without cytokinesis of infected host cells (giant cells, in the case of RKN) (Williamson 1999), or breakdown of cell walls between initial feeding cells and neighboring cells (syncytia, in the case of CN and RN) (Williamson and Gleason 2003; Robinson 2007). These sedentary endoparasites ingest nutrient from the NFS through their stylet, a specialized hollow needle-like structure mouthpart. Female nematodes feeding on the host root enlarge and start to produce eggs.

Nematode effectors secreted through the stylet are essential in NFS initiation and maintenance. These effector proteins act as PAMPs or pathogenic effectors aiding nematode parasitism, conditioning host defense responses, and/or modifying host plant physiology (Davis, et al. 2004; Davis, et al. 2008; Haegeman, et al. 2012; Hewezi and Baum 2013). These nematode effectors are directly secreted into the cytoplasm of host cells to interact with components of the cell cycle, cytoskeleton, and cellular metabolism, or alternatively, they accumulate in host extracellular spaces to degrade plant cell walls and change cell wall architecture (Mitchum, et al. 2013).

In this review, signaling and signal transduction involved in plant general biotic defense mechanisms will be reviewed for plant susceptibility/resistance (S/R) to SPENs, and the nature of the integrated signaling network that determines plant responses to nematodes will be defined. Specifically, different *R*-genes mediating nematode resistance

and their upstream signaling will be reviewed. The roles of different classes of phytohormones, their synthesis and signaling, and the role(s) of reactive oxygen and nitrogen species (ROS and RNS) generation and signaling in plant responses to SPENs will be reviewed. The emerging and integrative regulatory role of small RNAs in plant S/R to SPENs will be considered then in summary.

R-proteins and their upstream signaling in plant resistance to SPENs

Initial sensing of nematode infestation can occur either extra- or intracellularly and typically involves interaction with an R-protein receptor (typically identified as an NBS-LRR proteins) (**Fig. 1.1**). Many distinct loci involved in initiating resistance responses to SPENs have been mapped to plant genomes (**Table S1.1**). The first cloned locus was *Hs1*$^{pro-1}$, predicted to encode an extracellular leucine-rich region containing protein. This was followed by the cloning of a series of other *R*-genes including: *Mi-1*, *Gpa2*, *Gro1-4*, "*Hero A*", *CaMi*, and *Ma*, that have all been shown to encode canonical intracellular NBS-LRR type R-protein receptors (**Table 1.1**).

These cloned NBS-LRR proteins can also be further classified as TIR-NBS-LRR or CC-NBS-LRR based on their amino-terminus motif (**Table 1.1**). Both TIR (toll-interleukin receptor) and CC (coiled-coil) domains are thought to be crucial in the signal transduction of innate immunity (Glowacki et al. 2011). Further investigating how different domains contribute to pathogen sensing and signal transduction will be helpful to understand *R*-gene-mediated signaling in nematode resistance. Specifically, a WRKY-like domain on the carboxyl terminus of the *Ma* gene encoded protein suggests a direct role of the Ma protein in downstream regulation of defense gene expression and plant immunity (**Table 1.1**). More discussion and comparison of these R-protein structures can

be found in detailed reviews (Fuller et al. 2008; Goverse and Smant 2013; William and Kumar 2006).

Rhg1 and *Rhg4* are two unlinked quantitative trait loci (QTLs) in soybean that appear to condition S/R to SCN and are unique when compared to the canonical *R*-gene loci mentioned above (Hauge et al. 2001). Since the discovery of these QTLs it has been hypothesized that extracellular LRR kinase-type R-proteins found in the coding regions of these QTLs conditioned resistance to SCN (Hauge et al. 2001).

However, transgenic soybean plants with over-expressed or silenced LRR-kinase genes from the *Rhg1* locus showed little change in S/R to SCN (Melito, et al. 2010), contradicting the original disclosure. Subsequently, it was shown that *Rhg1*-conditioned SCN resistance was determined by three genes encoding an amino acid transporter, an α-SNAP protein, and a wound-inducible domain protein (Cook, et al. 2012). The copy number of a 31kb repeat sequence containing these 3 genes appears to determine SCN resistance: multiple copies of the repeat produced resistance, while a single copy produced a susceptible phenotype (Cook, et al. 2012). While, the precise role of the 31 kb gene repeat remains unclear at this time, it appears that the *Rhg1* locus plays a regulatory role that may involve the expression of some type of as yet undefined *R*-gene. In addition, differentially methylated regions within *Rhg1* correlated with SCN resistance. This fact along with the observation of copy number of the 31 kb repeat-region mentioned above suggest the possibility that some type of epigenetically mediated phenomenon may play a role in *Rhg1*-mediated host plant resistance (Cook, et al. 2014) the details of which remain to be elucidated.

Rhg4-mediated SCN resistance or susceptibility is linked to two nucleotide

polymorphisms in a single copy gene encoding a serine hydroxymethyltransferase (SHMT) (Liu, et al. 2012) in contradiction to the earlier report (Hauge et al. 2001) that the *LRR*-gene near the *Rhg4* QTL locus conditioned SCN resistance. SHMT may affect plant S/R to nematodes through regulation of folate one-carbon metabolism, since folate deficiency may cause parasitizing nematode death and degradation of nematode induced syncytia.

Thus, none of the gene products from *Rhg1* and *Rhg4* resemble either extracellular or intracellular canonical R-proteins, and further work is required to elucidate the detailed roles of *Rhg1* and *Rhg4* in SCN resistance.

The best-studied example of an R-protein involvement in nematode resistance is tomato *Mi-1*. RKN resistance mediated by the *Mi-1* gene is dependent on pathogen recognition and resistance signal transmission mediated by an LRR domain internal to the Mi-1 protein (Hwang et al. 2000; Hwang and Williamson 2003) and by an ATPase activity associated with its NBS domain (Tameling et al. 2002). Subsequently, it has been shown that the extended N-terminus of the Mi-1 protein has both negative and positive regulatory roles in the activation of Mi-1 protein (Lukasik-Shreepaathy et al. 2012).

Upstream of *Mi-1*, a gene product of the *Rme1* locus is required for RKN resistance, although the *Rme1* sequence has not been cloned as yet, and thus its exact biological function is unknown (Martinez de Ilarduya, et al. 2004). In tomato, orthologs of Arabidopsis *HSP90-1* and *SGT1* were also required for *Mi-1*-mediated RKN resistance, as demonstrated by virus-induced gene silencing (Bhattarai, et al. 2007). Based on a proposed model of R-protein-mediated signaling (Glowacki, et al. 2011), the *Mi-1* encoded NBS-LRR protein, an HSP90-1 protein, and an SGT1 protein form an R-protein

signaling complex that can activate downstream signaling pathways by detection of nematode effector-induced conformational changes in the protein coded by the *Rme1* gene that may be an interacting partner of the Mi-1 protein as well as the direct target of nematode effectors (Bhattarai, et al. 2007). Similarly, PCN resistance mediated by *Gpa2* also requires the RAN GTP activating protein 2 that functions as a cofactor for *Gpa2* (Sacco et al. 2009).

As pathogen receptors, NBS-LRR proteins detect pathogen effectors and trigger ETI type resistance characterized by hypersensitive responses (HR). In accordance, several nematode effectors that may interact with plant NBS-LRR proteins that are coupled with HR have been identified. These include a PCN SPRY domain-containing protein, RBP-1, that interacts with the Gpa2 protein (Sacco et al. 2009), an RKN protein coded by the *MAP-1* gene that may interact with the tomato Mi-1 protein (Semblat et al. 2000), and another RKN encoded protein product of the *Cg-1* gene that may interact with Mi-1 protein (Gleason et al. 2008). It also could be concluded that there are other unknown nematode effectors interacting with host plant R-proteins, because HR type cell death is frequently identified in plant *NBS-LRR* genes mediating nematode resistance (Chen et al. 2007; Khallouk et al. 2011; Sacco et al. 2009; Sobczak et al. 2005; William 1999).

Moreover, recent findings involving small regulatory RNAs (sRNAs) add a new layer of complexity to the regulation of *NBS-LRR* genes during plant defense responses (Li et al. 2012; Shivaprasad et al. 2012; Zhai et al. 2011), which is discussed in greater detail below in consideration of the role of sRNAs in nematode pathogenesis.

Hormone signaling in plant S/R to SPENs

Downstream of PTI and ETI activation, plant hormone signaling induces or suppresses defense responses to nematodes through regulation of different transcription factors (**Fig. 1.1**). Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) function in plant stress responses (**Table 1.2**). Hormones are also involved in plant growth and development related to nematode infection, such as Auxin (AX) and Cytokinin (CK). AX plays an essential role in modulating plant cell morphology regulating NFS development (**Table 1.2**). CK also function as modulators in NFS development (**Table 1.2**).

Salicylic Acid (SA)

SA signaling is required in *R*-gene mediated defense responses to SPENs (Branch, et al. 2004; Kandoth, et al. 2011; Uehara, et al. 2010). Specifically, SA signaling and response genes were strongly induced in tomato plants harboring the *Mi-1 R*-gene (Molinari, et al. 2013), the "*Hero A*" gene (Uehara, et al. 2010), and the resistance QTL, *Rhg1* (Kandoth, et al. 2011). Transgenic tomato plants containing dominant *Mi-1* or "*Hero A*" alleles expressing *NahG* (encoding SA hydroxylase) exhibited reduced resistance to RKN or CN, respectively (Branch, et al. 2004; Uehara, et al. 2010). Furthermore, the SA analog benzothiadiazole (BTH), completely restored RKN resistance in *NahG* transformed tomato roots harboring an active *Mi-1* gene, but resistance was not established in susceptible plants lacking a functional *Mi-1* gene (Branch, et al. 2004).

Downstream of SA signaling, Arabidopsis *WRKY70* is required for both basal defense and *R*-gene mediated resistance (Eulgem and Somssich 2007). In tomato plants

containing *Mi-1* alleles, orthologs of Arabidopsis *WRKY70* were induced after exogenous application of SA. Similarly, attenuated *Mi-1*-mediated resistance against RKN was observed when tomato *WRKY70* was silenced (Bhattarai, et al. 2010). However, another WRKY transcription factor, *WRKY72*, was found to control *Mi-1* mediated defense responses and basal resistance to RKN, independent of SA signaling (Atamian, et al. 2012). While different *WRKYs* are differentially regulated in roots after nematode infestation (Barcala, et al. 2010; Klink, et al. 2007; Portillo, et al. 2013; Uehara, et al. 2010), their specific roles remain elusive. Yet, it is known that WRKY transcription factors regulate the expression of defense related genes in both PTI and ETI (Eulgem and Smossich 2007). Specifically, the down regulation of *WRKY6, WRKY11, WRKY17, and WRKY33* in Arabidopsis roots in response to BCN infestation has been demonstrated to favor nematode and NFS development (Ali et al. 2014).

In addition to *R*-gene-mediated resistance, genes involved in SA biosynthesis, SA signaling, and SA responses also contribute to nematode basal resistance (**Table 1.2**). The requirement of endogenous SA accumulation for resistance to SCN was demonstrated in Arabidopsis iso-chorismate synthase gene (*ICS*) mutants and *NahG* transgenic lines where each demonstrates increased SCN susceptibility (Wubben, et al. 2008). ICS is a key enzyme in the SA biosynthesis pathway (Vlot, et al. 2009). Higher levels of *ICS* expression were detected in *SAMT*-overexpressing (SA methyltransferase) soybean roots, where these plants exhibited resistance to SCN (Lin, et al. 2013). SAMT modulates SA levels by converting SA to methyl salicylic acid (MeSA) (Vlot, et al. 2009), and MeSA can function as a mobile signal, mediating systemic acquired resistance (SAR) in some plants (Park, et al. 2007).

The At*pad4* mutant at the PHYTOALEXIN DEFICIENT locus which is involved in SA signaling showed increased SCN susceptibility; overexpressing wild type At*PAD4* in soybean showed increased resistance to RKN (Wubben, et al. 2008; Youssef, et al. 2013). PAD4 acts upstream of SA in pathogen responses via interaction with ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) that has similar sequence to *PAD4* (Vlot, et al. 2009). In soybean roots, *EDS1* transcript levels were induced after infestation by both compatible and incompatible populations of SCN (Klink, et al. 2007). These findings together demonstrated that SA-upstream signaling is required in nematode resistance.

Signaling downstream of SA is largely regulated via the NON-EXPRESSOR of PATHOGENESIS RELATED 1 gene (*NPR1*) (Vlot, et al. 2009) recently identified as an SA receptor (Wu, et al. 2012). In the nucleus, NPR1 interacts with TGA transcription factors which can bind to a cis-element required for SA responsiveness (Vlot et al. 2009). Arabidopsis *NPR1*-deficient mutants showed increased susceptibility to SCN, and SUPPRESSOR OF *npr1-1* INDUCIBLE (*SNI1*) deficient mutants exhibited increased resistance to SCN (Wubben, et al. 2008). Similarly, transgenic expression of At*NPR1* conferred resistance to RN, RKN, and SCN in cotton, tobacco, and soybean, respectively (Parkhi, et al. 2010; Priya, et al. 2011). The same effect of SCN parasitism suppression was also observed in soybean root over expressing At*TGA2* (Matthews et al. 2014).

The best-studied SA responsive defense gene is *PR-1* (pathogenesis related 1) although induction of *PR-2* and *PR-5* are also used as indicators of SA signaling activation in resistance responses (Vlot, et al. 2009). Suppression of *PR-1, PR-2*, and *PR-5* gene expression in *10A06* SCN effector gene transformed Arabidopsis increased

susceptibility to SCN (Hewezi, et al. 2010). The disruption of SA-responsive defenses may be critical in at least SCN parasitism. Consistent with these findings, the suppression of *PR-1* and *PR-5* in roots where an NFS was successfully established and decrease of SCN parasitism in At*PR-5* over expressed soybean roots, support the SA-responsive defenses in nematode resistance (Barcala, et al. 2010; Hewezi, et al. 2010; Klink, et al. 2010; Matthews, et al. 2014; Portillo, et al. 2013).

In contrast, *PR-1* was highly induced in the RKN more susceptible *lox3* mutant of maize (Gao, et al. 2008). *LOX3* encodes a 9-lipoxygenase that oxidizes fatty acids to oxylipins, including JA (Mosblech, et al. 2009) and *lox3 mutant* also shows increased levels of JA and ET responsive and biosynthetic genes (Gao, et al. 2008). Exogenous foliar application of the SA analog, BTH in rice only slightly induced RKN resistance compared to the MeJA or the ET-generating compound ethephon (Nahar, et al. 2011). Thus, while SA plays a critical role in nematode S/R there are other hormones and interacting signaling pathways involved in plant responses to nematode infestation.

Jasmonic Acid (JA)

Blocking JA perception via the *COI* receptor in *Mi-1* resistant tomato plants did not compromise resistance to RKN (Bhattarai, et al. 2008; Mantelin, et al. 2013). However, negative crosstalk between the JA- and SA-signaling pathways in *Mi-1*-mediated resistance was consistent with the fact that SA-induced *WRKY70* was suppressed after treatment with the JA derivative MeJA (Atamian, et al. 2012).

JA signaling, unlike SA production and signaling appears to be required for susceptibility to SPENs, since it was shown that a mutant JA receptor (*coi-1*) led to

significantly lower numbers of RKN egg masses on RKN-susceptible tomato roots (Bhattarai, et al. 2008). In addition, JA biosynthesis increased in nematode susceptible tomato genotypes (Bhattarai, et al. 2008; Gao, et al. 2008; Ozalvo, et al. 2013).

Induction of JA biosynthesis genes (**Table 1.2**) in the Arabidopsis *lox4* mutant makes plants more susceptible to RKN infestation implicating a link between JA accumulation and nematode susceptibility (Ozalvo, et al. 2013). A similar result was found in the RKN susceptible *lox3* maize mutant where JA biosynthesis genes (**Table 1.2**) were also induced (Gao, et al. 2008). Both *LOX4* and Zm*LOX3* are induced in response to RKN infestation (Gao, et al. 2008; Ozalvo, et al. 2013). Taken together, it appears that JA biosynthesis may play a positive role in plant susceptibility to nematode (Gao, et al. 2008; Ozalvo, et al. 2013).

Direct application of JA induces RKN resistance responses in tomato (Cooper, et al. 2005) in a dose dependent manner (Fujimoto, et al. 2011), and some studies have shown that protease inhibitors may be down-stream regulators of JA-induced nematode resistance. High expression of a multicystatin-type gene and protease inhibitor encoding genes in tomato roots was observed when RKN infection was repressed (Fujimoto, et al. 2011). Similarly, JA-responsive protease inhibitor (*Pin2*) and γ–thionin-coding genes were repressed in tomato plants overexpressing the *Mj-FAR-1* gene (Iberkleid, et al. 2013). Mj-FAR-1 is a member of RKN-specific fatty acid and retinol binding family protein, and Mj-FAR-1 may play a positive role in plant susceptibility to RKN (Iberkleid, et al. 2013).

JA and ET appear to play a greater role in rice resistance to RKN than does SA. Both MeJA and ET treatment induce strong resistance to RKN correlated with strong

induction of resistance genes (Nahar, et al. 2011). Foliar treatment with JA or ET biosynthesis inhibitors increase rice susceptibility to RKN (Nahar, et al. 2011), and genes involved in JA and ET biosynthesis and signaling were mainly suppressed in rice roots and shoots after RKN infestation (Kyndt et al. 2012). Furthermore, JA was found to be an indispensable signal in rice, mediating resistance to RKN, and ET-mediated RKN resistance is dependent on JA biosynthesis (Nahar, et al. 2011). ET foliar treatment had no effect on the response to RKN infestation in rice mutants with impaired JA biosynthesis, but JA-induced defense was still functional when ET-signaling was impaired (Nahar, et al. 2011).

Ethylene (ET)

ET signaling is not involved in *Mi-1*-mediated RKN resistance (Fujimoto, et al. 2011), but ET and ET-signaling affect basal resistance to both RKN and CN (**Table 1.2**). ET-treated soybean roots exhibited increased SCN susceptibility (Tucker, et al. 2010) while the inhibitors of ET action, 1-methycyclopropene (MCP) and 2,5-norbornadiene (NBD), reduced SCN colonization in soybean roots consistent with ET playing a positive role in nematode susceptibility. Arabidopsis ET-overproducing mutants (*eto1*, *eto2*, and *eto3,* **Table 2**) demonstrate hyper-susceptibility to CN (Wubben, et al. 2001), but the same ET overproducing mutants showed reduced susceptibility (increased resistance) to RKN (Fudali, et al. 2013). Such opposing results have also been reported with ET signaling mutants in CN and RKN resistance (**Table 1.2**). For example, Arabidopsis ET receptor mutant (*etr1*) and ET signaling mutants (*ein2* and *ein3*) showed decreased susceptibility to CN (Wubben, et al. 2001); and a gene encoding UDP-glucose-4-

epimerase that contributes to CN resistance was negatively regulated by the intermediate ET-signaling genes, *EIN2* and *EIN3* (Wubben, et al. 2004). ET-insensitive mutants (*etr1, ers2, ein4*) and tomato (*Nr*) and mutant genes positively regulating ET signaling (*ein2*, *ein3*, *ein5*, and *ein7*) resulted in higher levels of RKN infestation, while the negatively regulating ET signaling mutant (*ctr1*) attracted fewer RKN (**Table 1.2**). Thus, it can be concluded that the ET biosynthesis and signaling pathways positively regulate susceptibility to CN, whereas they contribute to RKN resistance (**Table 1.2**) although the mechanistic basis of such opposing effects is unclear at this time.

Ethylene Response Factors (ERFs or EREBPs) that specifically bind to a GCC box *cis*-element sequences have been found in many PR-protein coding gene promoters (Wang, et al. 2002). *EREBP* transcription factor was induced in soybean resistant reactions but suppressed in susceptible reactions to SCN (Mazarei et al. 2011). One soybean *EREBP* (Gm*EREBP1*) appears to be involved in the induction of different classes of *PR*-genes in roots of both *GmEREBP1*-overexpressing soybean and Arabidopsis plants (Mazarei, et al. 2002), although this transgenic overexpression did not confer increased resistance to SCN in Arabidopsis (Mazarei, et al. 2007). In addition to ET-induced PR-protein coding genes *GmPR2*, *GmPR3,* and *AtPDF1.2*, SA-responsive PR-protein coding genes (*AtPR1, GmPR1*, and *AtPR2*) and JA-responsive PR-genes (*GmPR3* and *AtPDF1.2*) were also induced in *GmEREBP1* overexpressing plants (Mazarei, et al. 2007).

These studies demonstrate widely varying roles of ET biosynthesis and signaling in S/R to SPENs that vary according to the specific nematode investigated. ET may have pleiotropic effects in plant resistance to nematodes because: 1) unique mechanisms are

required for different nematode species in host attraction, as see where CN and RKN responded differently in ET biosynthesis and signaling mutants; 2) complex unknown crosstalk between ET and JA or SA signaling pathways occur during nematode infestation, as suggested by induction of different classes of *PR* proteins in *GmEREBP1* transgenic plants. Thus, it is currently difficult to determine a precise role for ET in nematode infestation, but it is clear that ET does play important roles in specific nematode pathogenesis and possibly indirectly in resistance.

Auxin (AX)

AX insensitive mutants appear to be resistant to CN compared to their wild type counterparts (Goverse, et al. 2000), and AX levels increase transiently in the expanding NFS and cells surrounding the NFS (Goverse et al. 2000; Karczmarek et al. 2004; Absmanner et al. 2013). In particular, AX responsive elements were found in the cis-element of *NtCel7* gene (Wang et al. 2007). *NtCel7*, a tobacco endo-β-1,4-glucanase gene, functions in cell-wall degradation and is strongly induced in both RKN and CN feeding cells (Wang et al. 2007). Taken together, these studies suggest that a local and transient accumulation of AX in feeding cells upon nematode infection may support NFS establishment and nematode parasitism.

Polar AX transport manipulates AX distribution in feeding cells during nematode infestation (Goverse, et al. 2000; Grunewald, et al. 2009; Lee, et al. 2011). At the beginning stages of CN infection, AX accumulates in the infection site by induced *LAX3*/*AUX1*-mediated AX import and reduced *PIN1*-mediated AX export, whereas when a syncytium is expanding, *PIN3* and *PIN4* facilitate the lateral transport of AX to the cells

surrounding the initial syncytium (Grunewald, et al. 2009; Lee, et al. 2011). Specifically, *LAX3* was demonstrated to be a direct target of the nematode secreted protein Hs19C07 (Lee, et al. 2011). Binding to Hs19C07 can activate *LAX3*, leading to subsequent syncytia development (Lee, et al. 2011).

AX effects on nematode S/R are also mediated through AX response factors (ARF) by activating or repressing AX-responsive genes (Woodward and Bartel 2005). Members of the ARF gene family are distinctly and dynamically regulated in host Arabidopsis plants in response to BCN infestation compared to uninfected plants (Hewezi et al. 2014). AX accumulation and *ARF*s appear to play a transient role in NFS initation and early development (Goverse et al. 2000; Karczmarek et al. 2004; Absmanner et al. 2013). However, continued high expression of *ARF*s in fully developed syncytia (Hewezi et al. 2014) and AX responsive mature root galls supports a functional role of AX and *ARF*s in mature NFS as well (Cabrera et al. 2014). It should be noted that *ARF*s are targets of several microRNAs and small interfering RNAs (see sRNA regulation section below, and **Table 1.3**). The detailed role of such sRNA regulation of *ARF*s in plant responses to SPENs has not been extensively investigated to date, but given the role of *ARF*s in NFS growth and development this area is likely to yield significant information on plant/nematode interactions in the future.

Downstream of *ARF*s, the AX responsive gene *LATERAL ORGAN BOUNDARIES-DOMAIN 16* (*LBD16*), which showed activation upon RKN infestation, was implicated in the induction of both root galls and lateral roots (Cabrera, et al. 2014). This study established the first molecular link between root gall induction and lateral root formation (Cabrera, et al. 2014).

In contrast to the above studies that suggested a dependence of NFS initiation and morphogenesis on AX, *AtWRKY23,* which acts downstream of *ARF*s was induced in early syncytium development during BCN infection independent of AX (Grunewald, et al. 2008). This result suggests the involvement of other pathways in the regulation of early plant responses to nematode infestation. AX is known to interact synergistically with ET in general, but ET-mediated nematode susceptibility was independent of AX (Fudali, et al. 2013; Wubben, et al. 2004). This inconsistency of AX dependence in uninfected and infected plant signaling pathways suggests the possibility of nematode secreted effectors bypassing plant AX signaling to modulate plant responses (Lee et al. 2011). It is also possible that AX-like compounds found in nematode secretions can manipulate plant S/R to nematodes (Hewezi and Baum 2013; Mitchum et al. 2013).

Cytokinin (CK)

CK signaling components are also differentially regulated during SCN infestation (Barcala, et al. 2010; Ithal, et al. 2007). It was found that in RKN infested *L. japonicus* roots, the CK-inducible gene *ARR5* was strongly induced in rapidly dividing small cells around the giant cell but absent in mature galls (Lohar, et al. 2004). Similarly, significantly fewer and smaller galls were formed on transgenic *L. japonicus* roots, when CK was degraded by overexpressed CK oxidase (Lohar, et al. 2004).

In addition to functioning downstream of *R*-gene mediated nematode resistance, different hormones are also important players in plant basal defense responses. Stress hormones SA, JA, and ET regulate plant S/R to SPENs mainly through *PR* genes or other

resistance related factors; growth hormones AX and CK primarily affect nematode parasitism though manipulation of NFS initiation and development. The specific role of each hormone in plant responses to nematodes is a bit more complex showing both plant and nematode species specificity as well as differences that depend on infestation timing. In addition, using different gene/proteins for studying hormone effects on plant-nematode interactions could lead to different conclusions because there are complex interactions between various hormone-signaling pathways. Moreover, nematode secretions should also be considered when interpreting these plant responses, since nematode secreted effectors are known to manipulate plant development and defense responses by interacting with or mimicking plant genes/proteins (Hewezi and Baum 2013; Mitchum, et al. 2013).

ROS generation and signaling in plant S/R to SPENs

ROS are chemically reactive molecules containing oxygen, including singlet oxygen, superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radical (Ray, et al. 2012). Plants constantly produce ROS as a byproduct of metabolic processes such as photosynthesis and respiration (Tripathy and Oelmuller 2012). Steady-state levels of ROS are tightly regulated by competing ROS generation and scavenging mechanisms. ROS over-accumulation usually causes oxidation of lipids, proteins, and DNA as well as other components often leading to cell death (Tripathy and Oelmuller 2012). When challenged by pathogens, ROS production increases rapidly in what is often called the oxidative burst, which can lead to local cell death (Tripathy and Oelmuller 2012). The local burst of ROS in response to pathogen infection could also be transferred

systemically in a cell-to-cell auto-propagating manner, integrating with other signaling pathways generating a SAR response (Baxter, et al. 2013; Mittler, et al. 2011; Tripathy and Oelmuller 2012).

In response to RKN infection, both RKN susceptible and resistant tomato plants showed nematode penetration into their roots (Melillo, et al. 2006; Melillo, et al. 2011). However, 48 hours post infection (hpi), significantly fewer RKN were observed in *Mi-1*-mediated incompatible infested roots than in compatible infested roots (Melillo, et al. 2006). In accordance, an oxidative burst was observed at the root infection site as soon as 12 hpi for both compatible and incompatible responses, but this was only prolonged in incompatible responses (*Mi-1* tomato responses to avirulent RKN) until 48 hpi when cell death became evident (Melillo, et al. 2006; Melillo, et al. 2011).

NADPH oxidases were the main source of ROS production in plant incompatible responses and subcellular localization of H_2O_2 production for incompatible responses followed a pattern consistently observed in HR (Melillo, et al. 2006). Collectively, these observations show that, as one of the most sensitive signals monitoring cellular metabolic changes, ROS accumulation occurs rapidly in response to RKN infection, and temporal and spatial differences in ROS (particularly H_2O_2) accumulation are crucial in determining the extent of RKN pathogenesis in host plants.

Rapid apoplastic generation of ROS has been mainly associated with pathogen resistance (Baxter, et al. 2013; Mittler, et al. 2011; Tripathy and Oelmuller 2012). Consistent with this, RKN genes encoding ROS scavenging enzymes (clade B peroxiredoxins) were more actively transcribed in parasitic stages to protect RKN from the oxidative responses of the host, and knockdown of these genes resulted in reduced

RKN parasitism (Dubreuil, et al. 2011).

Conversely, a recent study demonstrated a negative role for ROS in cell death and BCN resistance in Arabidopsis (Siddique, et al. 2014). Loss of two specific NADPH oxidase genes, *RbohD* and/or *RbohF*, required for ROS production at 24 hpi after BCN infestation, resulted in reduced BCN parasitism, smaller syncytium size, and enhanced cell death (Siddique, et al. 2014). In addition, the suppression of cell death in *RbohD* and/or *RbohF* mutants was demonstrated to be independent of SA accumulation, and an antagonistic relationship between SA and ROS was suggested because SA responsive genes were induced in *RbohD*/*F* double mutants but were suppressed in *RbohD* overexpressing Arabidopsis (Siddique, et al. 2014).

ROS also work in concert with nitric oxide (NO), the main reactive nitrogen species (RNS) found in biological systems, to control plant responses to SPENs (Melillo, et al. 2011; Yu, et al. 2012). NO is a gaseous nitrogen-containing free radical, endogenously produced by plants serving as an important mediator of defense responses (Bellin, et al. 2013). In tomato plants demonstrating an incompatible reaction to RKN, the peak generation of ROS was preceded by production of NO (Melillo, et al. 2011). Similarly, in *P. thunbergii* responding to pine wood nematode, endogenous NO levels increased coincident with a rapid increase in H_2O_2 levels, whereas H_2O_2 levels decreased when pretreated with an NO scavenger (Yu, et al. 2012).

ROS generation is always associated with plant defense responses, and rapid apoplastic generation of ROS often leads to an HR-type cell death, thus restricting the spread of the infection leading to pathogen resistance (Baxter et al. 2013). However, newly identified ROS suppression of cell death and support of BCN parasitism suggests

an activation of HR-type cell death can occur through other unknown signaling mechanisms (Feng and Shan 2014). This new finding is also consistent with ROS having distinct roles in plant S/R to SPENs.

Small RNAs may be important regulators in plant S/R to SPENs

Small regulatory RNAs (20-24 nucleotides in length) are emerging as important aspects of plant defense responses resulting from epigenetic, transcriptional, posttranscriptional, and/or translational gene regulation (Katiyar-Agarwal and Jin 2010; Ruiz-Ferrer and Voinnet 2009; Shukla, et al. 2008; Sunkar, et al. 2007). The primary classes of plant sRNAs are the short interfering RNAs (siRNAs) and the micro RNAs (miRNAs) although there are other emerging classes of sRNA that have not yet been extensively investigated in the context of pathogenesis (Axtell 2013).

miRNAs are the best studied class of sRNAs. miRNAs are derived from single stranded RNA transcripts, which form hairpin loop structures (Axtell 2013). Both miRNAs and siRNAs are processed from their double-stranded RNA precursors by DICER-like proteins (DCLs) and the resulting miRNAs and siRNAs are loaded into Argonaute (AGO) proteins to form an RNA-induced silencing complex (RISC) that can bind to target RNAs or DNAs (Axtell 2013).

Plant miRNAs and siRNAs play important roles in plant biotic stress responses by regulating genes involved in plant PTI and ETI (Katiyar-Agarwal and Jin 2010), hormone signaling (Liu and Chen 2009), ROS generation and signaling (Shukla, et al. 2008), and various other types of signaling (Ruiz-Ferrer and Voinnet 2009). Thus, sRNAs are positioned to integrate various aspects of the pathogenesis responses into regulatory

networks. Current studies further suggest that miRNAs and siRNAs are playing such regulatory roles during nematode pathogenesis in host plants (**Fig. 1.1, Table 1.3, Table S1.2**).

Genes encoding proteins associated with miRNA or siRNA biogenesis and/or function including *DCL*s, *AGO*s, *RDR*s, and genes encoding DNA methylase proteins, as well as histone methylation and deacetylation-related genes, are regulated in RKN-induced tomato root galls and RKN-infected rice roots (Ji, et al. 2013; Portillo, et al. 2013). DNA and histone methylation and histone acetylation are important mechanisms mediating epigenetic gene regulation in plants (Sahu, et al. 2013). Taken together, these results are consistent with miRNA and siRNA biogenesis and function playing an important role in plant responses to SPENs.

The biogenesis and functioning of miRNAs and siRNAs were also demonstrated to be required in plant S/R to SCN (Hewezi, et al. 2008). Responses to SCN were examined in several single, double, and triple mutants of Arabidopsis. The genes examined included genes coding for the *DCL*s and *RDR*s, various isoforms of which are involved in the production of specific miRNAs and siRNAs. Mutation in these sRNA-producing genes all displayed decreased SCN susceptibility compared to wild type (Hewezi, et al. 2008).

Predicted targets of differentially expressed miRNAs and siRNAs indicated specific roles of sRNAs in nematode pathogenesis in host plants (**Table 1.3**, **Table S1.2**). Genes encoding R-proteins, ARFs, Heat Shock Proteins, ROS scavenger Cu/Zn superoxide dismutases, and various transcription factors are all predicted to be the targets of one or more differentially expressed miRNAs or siRNAs (**Table 1.3, Table S1.2**).

Among the differentially expressed sRNAs in response to nematode infestation, Arabidopsis miR396 was down-regulated four days post SCN infestation and up-regulated seven days post infestation (Hewezi, et al. 2008). Targets of miR396, including Arabidopsis *GRF* (Growth Regulating Factors) exhibited the opposite expression trends to miR396 post SCN infestation (Hewezi, et al. 2008).

To investigate the role of miR396/*GRF* in plant responses to SCN, Arabidopsis mutants deficient in *GRF* genes or overexpressing miR396 were examined, and overexpression of miR396 and/or reduced *GRF* gene expression resulted in reduced SCN susceptibility (Hewezi, et al. 2012). Furthermore, miR396-overexpressing Arabidopsis roots produced smaller syncytia with fewer SCN infections. Similar characteristics were also observed in miR396 binding site-deficient mutants (Hewezi, et al. 2012).

Since the *GRF* gene family positively controls cell proliferation and size, the coordinated expression of miR396 and its target *GRF* genes are critical in syncytia development during SCN infection. Moreover, almost half of the genes differentially expressed in syncytia overlapped with genes differentially regulated in *GRF* deficient and miR396 resistant Arabidopsis mutants, indicating that miR396/*GRF* is an essential regulatory system in reprogramming of gene expression in SCN-induced syncytia (Hewezi, et al. 2012).

sRNA expression changes in response to RN infestation have been investigated in cotton (Li and Locy, unpublished result). It was found that specific miRNAs and siRNA sequences (including cotton miR396 and miR482 among others) exhibit distinct expression patterns in response to RN infestation in cotton genotypes differing in RN resistance and susceptibility. The spectrum of sRNA target genes derived from

differentially expressed sRNAs include genes previously implicated in plant innate immunity, hormone signaling, ROS generation and signaling, as well as sRNA biogenesis and function, and in epigenetic regulation. This analysis supports the idea that sRNAs serve to integrate a signaling network that regulates most, if not all, of the various signaling pathways discussed above.

Viral (Shivaprasad et al. 2012), bacterial (Shivaprasad et al. 2012), or fungal (Zhu et al. 2013) infection of tomato or diploid cotton (*G. raimondii*) all resulted in suppression of specific miRNAs and induction of their target *R*-genes (*NBS-LRR* genes). Co-expression of miRNAs and their *NBS-LRR* targets in tobacco caused decreased resistance to TMV (Li et al. 2012). Based on bioinformatics and experimental data generated from different plant species, some *NBS-LRR* genes can produce clustered secondary siRNAs from their mRNA transcripts in a phased manner, and miRNA targeting is required for the production of secondary siRNAs (Shivaprasad et al. 2012; Zhu et al. 2013; Zhai et al. 2011; Li et al. 2012). Furthermore, some secondary siRNAs (i.e. trans-acting siRNAs) can also target other defense related genes (Shivaprasad et al. 2012) while others have shown involvement in AX signaling regulation of root development (Fahlgren et al. 2006; Marin et al. 2010; Yoon et al. 2010). Since NBS-LRR proteins and AX signaling clearly have a role in endoparasitic nematode S/R, it is reasonable to presume that miRNA/siRNA signaling plays important roles in integrating nematode signaling systems.

Support for such a hypothesis comes from a bioinformatic analysis of sRNA regulatory networks involved in RN signaling in cotton. A RN-responsive miRNA (miR482 family) was predicted to target an NBS-LRR protein-coding mRNA that could

be cleaved into a cluster of secondary phased siRNAs (Li and Locy, unpublished results). These siRNAs also target a series of transcription factors and other proteins many of which are known pathogenesis-related genes involved in signaling pathways. This provides preliminary support for the involvement of sRNA regulatory network in cotton-RN interactions through miRNAs and NBS-LRR protein coding genes producing secondary siRNAs that have been implicated in plant innate immunity as described for other pathogens (Fei, et al. 2013; Li, et al. 2012; Shivaprasad, et al. 2012; Zhai, et al. 2011).

Overall, these studies suggested that the miRNA regulation of *NBS-LRR* gene expression via the production of secondary siRNAs is playing an important role in nematode pathogenesis in host plants, although the exact nature of such regulatory network remains to be defined. It is possible that host plants can defend themselves by down regulating specific miRNAs leading to the expression critical *R*-genes involved in nematode resistance. The suppression of *NBS-LRR* gene expression via the sRNA pathway serves as a protective mechanism for plants since large increases in NBS-LRR transcripts and protein levels could trigger cell death and/or plant HR (Qiao et al. 2013). It is also possible that infecting nematodes secrete specific effectors that induce sRNA regulatory network suppressing the expression of *NBS-LRR* genes and thus promoting nematode parasitism. Since the delivery of sRNAs between host plants and infecting nematodes can suppress the expression of genes essential for nematode pathogenesis and development (Charlton et al. 2010; Dalzell et al. 2010; Fairbairn et al. 2007; Li et al. 2010; Klink et al. 2009), it is possible that nematode effector RNAs can act as the initiators of the plant sRNA regulatory networks, although this remains to be established.

Conclusions

In conclusion, SPENs resistance in host plants appears to be initiated when plant R-proteins sense nematode secreted effectors. Detection of nematode effectors leads to massive downstream reprogramming of gene expression through various hormones, ROS, and NO signaling pathways. However, each hormone's role in particular plant-nematode interaction is unique. Detection of nematode by R-proteins also leads to a localized cell necrosis at the nematode infection site, and a local burst of ROS and NO are causally correlated with HR observed in *R*-gene-mediated nematode resistance, although ROS play a supportive role in parasitism when incompatible *R*-gene interactions are involved.

It is clear that, instead of functioning independently, different signaling factors work in concert with each other in a highly controlled regulatory network. The specific nature of nematode produced factors interacting with plant factors also plays an important role in mediating the behavior of the regulatory network developing plant responses. sRNAs are emerging as important regulators of pathogen resistance that are implicated in the regulation of crucial regulatory nodes in plant defense responses, such as *NBS-LRR* genes. Based on these findings, it could be concluded that sRNA might be the hub of plant S/R to nematodes, but additional, continuing studies are required to reveal and demonstrate the specific roles of sRNAs in various plant-nematode interactions.

The application of emergent next generation sequencing technologies and network analysis strategies will not only support the implication of canonical signaling pathways in plant S/R to nematodes, but will also play a key role in implicating pathway

cross talk and integration as we move forward.

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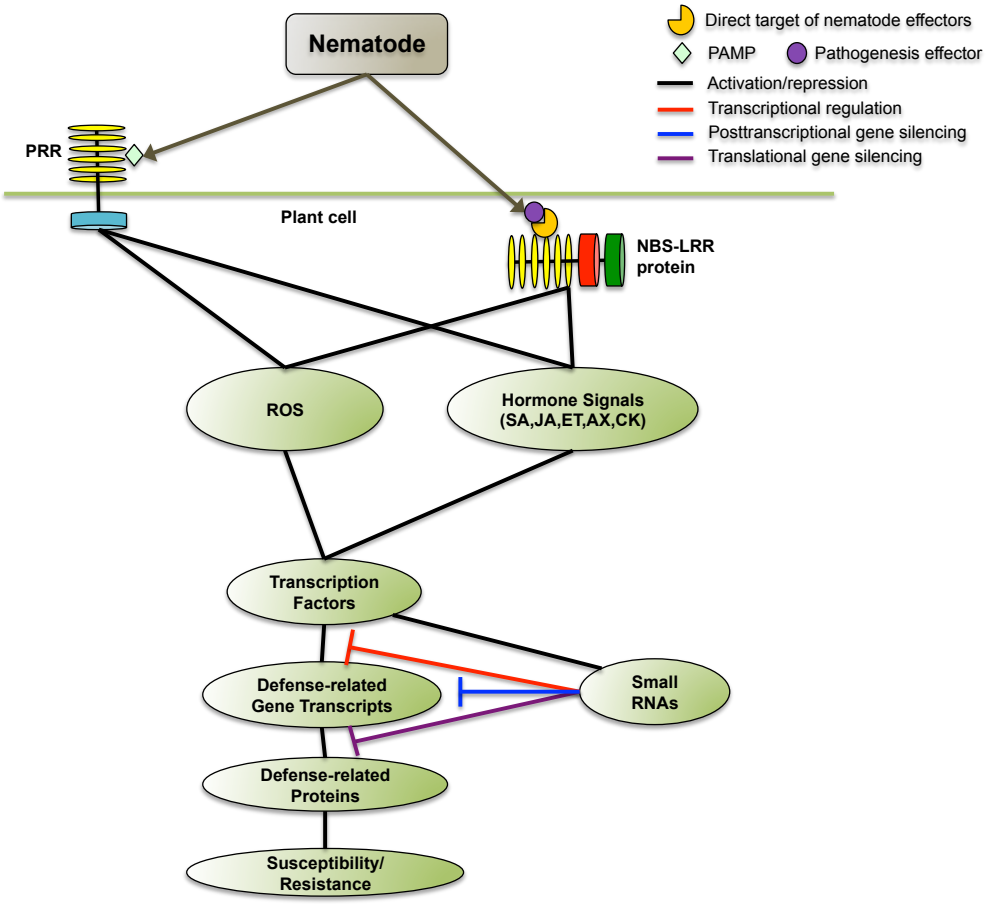

Figure 1.1 The integrated signaling network in plant responses to nematodes

Sedentary endoparasitic nematodes attack host plants and secrete various effectors functioning as PAMPs or pathogenic effectors. Upon recognition of invading nematodes with plant transmembrane extracellular R proteins or intracellular NBS-LRR proteins, ROS and various hormone-signaling pathways are activated. Different transcription factors and small RNAs regulate plant defense related factors at transcriptional, post-transcriptional, and/or translational levels leading to plant S/R to nematodes.

Table 1.1 Cloned plant genes for nematode resistance

Gene/Loci	Plant	Nematode	Encoded Protein(s)	Reference(s)
CaMi	*C. annuum L.*	RKN: *M. incognita*	CC-NBS-LRR	Chen et al. 2007
Gpa2	*S. tuberosum*	PCN: *G. pallida*	CC-NBS-LRR	Van der Vossen et al. 2000
Gro1-4	*S. tuberosum*	PCN: *G. rostochiensis*, type Ro1	TIR-NBS-LRR	Paal et al. 2004
Hero A	*S. pimpinellifolium*	PCN: *G. rostochiensis* types Ro1, Ro3 and Ro5; *G. pallida* types Pa2 and Pa3, and Luffness	CC-NBS-LRR	Ernst et al. 2002; Sobczak et al. 2005
Hs1^{pro-1}	*B. procumbens*	BCN: *Heterodera schachtii*	Amino-terminus leucine-rich region	Cai et al.1997
Ma	*P. cerasifera*	RKN: all species tested	TIR-NBS-LRR-WRKY	Claverie et al. 2011
Mi-1	*S. peruvianum*	RKN: *M. incognita*, *M. javanica*, *M. arenaria*	CC-NBS-LRR	Milligan et al. 1998; Vos et al. 1998
Rhg1	*G. max*	SCN: *H. glycines* type 0	An amino acid transporter, an α-SNAP protein, and a wound-inducible domain protein	Cook et al. 2012
Rhg4	*G. max*	SCN: *H. glycines* type 0	SHMT	Liu et al. 2012

RKN: root knot nematode; PCN: potato cyst nematode; BCN: sugar beet cyst nematode; SCN: soybean cyst nematode; SHMT: serine hydroxymethyltransferase

Table 1.2 Hormone biosynthesis and signaling genes in plant responses to nematodes

Gene /protein	Function	Regulation	Nematode	Tissue	Plant	Reference
Salicylic acid						
At*NPR1*	Receptor	Required for resistance	SCN; RKN; RN	WR	Arabidopsis; tobacco; cotton	Wubben et al. 2008; Priya et al. 2011; Parkhi et al. 2010
		Contribute resistance	SCN	WR	Soybean	Matthews et al. 2014
At*PAD4*	Signaling	Required for resistance	RKN; SCN	WR	Soybean; Arabidopsis	Youssef et al. 2013; Wubben et al. 2008
EDS1	Signaling	Upregulated	SCN	WR	Soybean	Klink et al. 2007
At*TGA*	Signaling	Contribute resistance	SCN	WR	Soybean	Matthews et al. 2014
ICS	Synthesis	Upregulated	SCN	WR	*SAMT* transgenic soybean	Lin et al. 2013
		Required for resistance	SCN	WR	Arabidopsis	Wubben et al. 2008
NahG	Hydrolysis	Increase susceptibility	SCN	WR	Arabidopsis	Wubben et al. 2008
PR1	Response	Down regulated	RKN	Galls; GC	Tomato; Arabidopsis	Portillo et al. 2013; Barcala et al. 2010
		Induced	RKN	WR	Maize *lox3* mutant	Gao et al. 2008
PR5	Response	Suppressed	SCN; RKN	GC; SN	Arabidopsis; soybean	Barcala et al. 2010; Klink et al. 2010
SAMT	Metabolism	Required for resistance	SCN	WR	Soybean	Lin et al. 2013
SNI1	NPR1 suppressor	Contribute susceptibility	SCN	WR	Arabidopsis	Wubben et al. 2008
Jasmonic acid						
AOC	Synthesis	Induced	RKN	WR	Arabidopsis *lox4* mutant; Maize *lox3* mutant	Ozalvo et al. 2013; Gao et al. 2008
AOS	Synthesis	Induced	RKN	WR	Arabidopsis *lox4* mutant; Maize *lox3* mutant	Ozalvo et al. 2013; Gao et al. 2008
γ - thionin	Response	Suppressed	RKN	WR	Tomato transgenic Mj-FAR-1	Iberkleid et al. 2013
COI-1	Receptor	Required for susceptibility	RKN	WR	Tomato	Bhattarai et al. 2008
Multicystatin	Response	Contribute resistance	RKN	WR	Tomato	Fujimoto et al. 2011
OPR	Synthesis	Induced	RKN	WR	Arabidopsis *lox4* mutant; Maize *lox3* mutant	Ozalvo et al. 2013; Gao et al. 2008
PIs	Response	Contribute	RKN	WR	Tomato	Fujimoto et al. 2011

		resistance				
		Suppressed	RKN	WR	Tomato transgenic Mj-FAR-1	Iberkleid et al. 2013
LOX8	Synthesis	Induced	RKN	WR	Maize *lox3* mutant	Gao et al. 2008
Ethylene						
CTR1	Signaling	Contribute susceptibility	RKN	WR	Arabidopsis	Fudali et al. 2013
EIN2	Signaling	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
		Contribute susceptibility	CN	WR	Arabidopsis	Wubben et al. 2001
EIN3	Signaling	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
		Contribute susceptibility	CN	WR	Arabidopsis	Wubben et al. 2001
EIN4	Receptor	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
EIN5	Signaling	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
EIN7	Signaling	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
ERS2	Receptor	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
ETO1	Synthesis	Contribute susceptibility	RKN	WR	Arabidopsis	Fudali et al. 2013
		Contribute resistance	CN	WR	Arabidopsis	Wubben et al. 2001
ETO2	Synthesis	Contribute susceptibility	RKN	WR	Arabidopsis	Fudali et al. 2013
		Contribute resistance	CN	WR	Arabidopsis	Wubben et al. 2001
ETO3	Synthesis	Contribute susceptibility	RKN	WR	Arabidopsis	Fudali et al. 2013
		Contribute resistance	CN	WR	Arabidopsis	Wubben et al. 2001
ETR1	Receptor	Contribute resistance	RKN	WR	Arabidopsis	Fudali et al. 2013
		Contribute susceptibility	CN	WR	Arabidopsis	Wubben et al. 2001
ETR3	Receptor	Contribute resistance	RKN	WR	Arabidopsis; Tomato	Fudali et al. 2013; Mantelin et al. 2013
Auxin						
AUX1	Importer	Induced	RKN & BCN	NFS	Arabidopsis	Mazarei et al. 2003
LAX1	Importer	Induced	BCN	SN	Arabidopsis	Lee et al. 2011
LAX3	Importer	Induced	BCN	SN	Arabidopsis	Lee et al. 2011
LBD16	Response	Support galls and GCs	RKN	WR	Arabidopsis	Cabrera et al. 2014
PIN1	Exporter	Support SN development	BCN	WR	Arabidopsis	Goverse et al. 2000; Grunewald et al. 2009
		Decreased over time	BCN	SN	Arabidopsis	Grunewald et al. 2009

PIN2	Exporter	Support SN development	BCN	WR	Arabidopsis	Goverse et al. 2000
PIN3	Exporter	Support SN development	BCN	WR	Arabidopsis	Grunewald et al. 2009
		Increased over time	BCN	SN	Arabidopsis	Grunewald et al. 2009
PIN4	Exporter	Support SN development	BCN	WR	Arabidopsis	Grunewald et al. 2009x
		Increased over time	BCN	SN	Arabidopsis	Grunewald et al. 2009
PIN7	Exporter	Support SN development	BCN	WR	Arabidopsis	Grunewald et al. 2009
		Decreased over time	BCN	SN	Arabidopsis	Grunewarld et al. 2009
Cytokinin						
AHK3	Receptor	Down regulated	SCN	WR	Soybean	Ithal et al., 2007
AHK4	Receptor	Down regulated	SCN	WR	Soybean	Ithal et al., 2007
ARR4	Signaling	Down regulated	RKN	GC	Arabidopsis	Barcala et al., 2010
At-*ARR5*	Signaling	Down regulated	RKN	GC	Arabidopsis	Barcala et al., 2010
		Only expressed in dividing cells around GC	RKN	GC	*Lotus japonicus*	Lohar et al., 2004
ARR9	Signaling	Upregulated	SCN	WR	Soybean	Ithal et al., 2007
At*CKX*	Oxidation	Reduced galls	RKN	GC	*Lotus japonicus*	Lohar et al., 2004
Zm*CKX*	Oxidation	Reduced galls	RKN	GC	*Lotus japonicus*	Lohar et al., 2004

WR: Whole root; GC: Giant cells; SN: syncytium

Table 1.3 Examples of soybean cyst nematode responsive small RNAs

Small RNA	Regulation	Target function
miRNA		
gma-miR1510ab-3p[1]	Down in S & R	HEX TF
gma-miR1515[1]	Down in S & R	Autophagy protein
gma-miR156[1]	Down in S & R	
ath-miR156[2]	Down 4dpi; up 7dpi	SBP domain protein
gma-miR159bcf[3]	Down in S & R	MYB TF
gma-miR160[3]	Down in S & R	
ath-miR160[2]	Down 4dpi	ARF
gma-miR162[1]	Down in S & R	Embryo-related protein
ath-miR164[2]	Down 4dpi & 7dpi	
gma-miR164[1]	Down in S & R	NAC
gma-miR166a-5p[1]	Down in S & R	HD-ZIP TF
gma-miR167[1]	Down in S & R	
ath-miR167[2]	Down 4dpi & 7dpi	ARF
ath-miR168[2]	Down 4dpi; up 7dpi	AGO protein
gma-miR169[1]	Down in S; up in R	
ath-miR169[2]	Up 7dpi	Nuclear factory
gma-miR171b[1]	Down in S & R	
ath-miR171b[2]	Down 4dpi; up 7dpi	Polyubiqutin protein; TCP family TF
gma-miR172[1]	Down in S & R	Heat shock cognate protein; AP2 TF
ath-miR172a[2]	Up 7dpi	HSP, AP2 TF; TCP family TF
gma-miR319[3]	Up in S & R	TCP family TF, plasma membrane intrisinc protein
gma-miR390b[1]	Up in S; down in R	Unknown protein
gma-miR394a[1]	Down in S; up in R	NADP+
ath-miR396a[2]	Down 4dpi; up 7dpi	GRF
gma-miR397ab[3]	Down in S & R	60s ribosomal protein; multicopper oxidase
ath-miR398a[2]	Down 4dpi & 7dpi	CSD
gma-miR408[3]	Down in S & R	Oxidoreductase
gma-miR482a-5p[1]	Down in S & R	NA
gma-miR5374[1]	Down in S; up in R	Disease resistance protein-like protein MsR1
gma-miR5674[1]	Down in S & R	PPR-containing protein
siRNA		
ath-siRNA41[2]	Up 4dpi & 7dpi	Similar to TOR1
ath-siRNA46[2]	Up 4dpi & 7dpi	Disease resistance protein
ath-siRNA52[2]	Down 4dpi; up 7dpi	MAPKKK13
ath-siRNA29[2]	Up 4dpi; down 7dpi	LTR/Gypsy
ath-siRNA32[2]	Down 4dpi & 7dpi	RC/Helitron
ath-siRNA50[2]	Up 4dpi; down 7dpi	Glycosyl hydrolase family 17 protein
ath-siRNA9[2]	Up 4dpi & 7dpi	Oxidoreductase

Down: downregulated; Up: upregulated; S: susceptible genotype; R: resistant genotype
[1]: was tested using Solexa sequencing
[2]: was tested using RT-PCR
[3]: was tested using Solexa sequencing and RT-PCR
(Hewezi et al. 2008; Li et al. 2012; Hewezi et al. 2012)

Chapter II. Transcriptome Analysis of Cotton (*Gossypium hirsutum* L.) Susceptibility, Resistance, and Hypersensitivity to Reniform Nematodes (*Rotylenchulus reniformis*)

Abstract

Reniform nematode is a semi-endoparasitic nematode species causing significant yield loss in numerous crops, including cotton. In response to reniform nematode infestation, plants demonstrate susceptible, resistant, and/or hypersensitive responses. In this study, we report a transcriptome RNA-seq analysis measuring transcript abundance in reniform nematode susceptible upland cotton (*Gossypium hirsutum*) genotypes (DP90 and SG747 combined as one sample), resistant genotype (BARBREN-713), and hypersensitive genotype (LONREN-1) with and without RN infestation. Over 90 million trimmed high quality reads were assembled into 84,711 and 80, 353 contigs with the *G. arboreum* and the *G. raimondii* genomes as references respectively. A total of 20,202 contigs were differentially expressed in response to reniform nematode infestation in at least one genotype, and they were annotated and classified into different gene ontology (GO) categories. Many genes involved in cell wall, hormone metabolism, redox reactions, secondary metabolism, transcriptional regulation, and stress responses were distinctly regulated in different genotypes. Gene expression analysis among different genotypes without reniform nematode infestation was also studied. By comparing locations of differentially expressed genes to known reniform nematode resistant quantitative trait loci, a list of resistance genes (*R*-genes) were identified, which may play important roles in regulating cotton susceptible, hypersensitive, and resistance responses to reniform nematodes. Overall, this study presents the first global gene expression analysis using different genotypes of *G. hirsutum* roots with and without reniform

nematode infestation. The differentially expressed genes identified in this study can serve as the basis for further functional analysis.

Introduction

Reniform nematodes (RN, *Rotylenchlus reniformis*) are semi-endoparasitic nematode species causing significant yield loss in cotton, particularly in the eastern US cotton belt (Robinson 2007). Successful RN parasitism is contingent on establishment of a syncytium, which serves as the sole nutrient source on which RN live. Syncytia are hypertrophied, multinucleate root cells with enlarged nuclei and dense cytoplasm that result from the breakdown of cell walls between initial feeding cells (usually an endodermal cell) and neighboring cells (Agudelo et al. 2005). It is believed that nematode secretions injected through their stylet, a specialized needle-like structure mouthpart, are essential in syncytium initiation and maintenance (Hewezi and Baum 2013; Mitchum et al. 2013). To date, a number of sequences homologous to other sedentary plant parasitic nematode effectors have been identified from RN EST assemblies (Wubben et al. 2010), however, none of them have been experimentally studied.

During the last decade, many studies using microarray or RNA-seq technology were conducted to characterize plant responses to sedentary plant endo-parasitic nematodes (SPENs) including root knot nematodes (RKN, *Meloidogyne* spp.) and cyst nematodes (CN, *Globodera* and *Heterodera* spp.) (Barcala et al. 2010; Ithal et al. 2007; Jammes et al. 2005; Klink et al. 2010; Kydnt et al. 2012; Mazarei et al. 2011; Portillo et al. 2012; Puthoff et al. 2007; Uehara et al. 2010). Based on the results from gene expression, molecular, and physiological studies, it was proposed that the host plant

responses to nematodes relies on the coordination of different resistance mechanisms including specific resistance genes or proteins, several plant hormone pathways, and reactive oxygen species (ROS) that are generated in response to nematode attack (Li et al. 2014b). These resistance-related elements can crosstalk to each other and be viewed as an integrated signaling network regulated by transcription factors and small RNAs (sRNAs) at the transcriptional, posttranscriptional, and/or translational levels (Li et al. 2014b).

The cloning of a number of resistance genes (*R* genes) that confer nematode resistance underlies nearly all progress in the field of plant-nematode interaction studies to date. Most of these cloned *R* genes were predicted to encode canonical intracellular R-protein receptors, which contain nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domains (Li et al. 2014b). Intracellular NBS-LRR-type R-protein receptors and extracellular LRR domain-containing proteins are known to recognize invading pathogen elements and trigger plant innate immunity responses (Jones and Dangl 2006).

Hypersensitive responses (HR) that can involve a localized programmed cell death (PCD) response and/or generation of reactive oxygen species (ROS), typically H_2O_2, at the pathogen infection site are typically observed in plant immunity responses (Jones and Dangl 2006). While the main purpose of PCD is to prevent the spread of the pathogen, rapid generation of ROS at the pathogen infection sites can not only trigger PCD locally, but can also be transferred to systemic tissues in a cell-to-cell auto-propagating manner and participate in the systemic acquired resistance (SAR) (Baxter et al. 2013; Mittler et al. 2011).

Upland cotton, *G. hirsutum*, is a natural allotetraploid species that likely arose from interspecific hybridization between ancestral diploid species having an A-like

genome (present day *G. arboreum*) and a D-like genome (present day *G. raimondii*) (Chen et al. 2007). In 2012, two groups separately published their assembled *G. raimondii* whole genome sequences (Paterson et al. 2012; Wang et al. 2012). Subsequently, the draft genome of *G. arboreum* became available in April 2014 (Li et al. 2014a). Without the complete genome sequences of *G. hirsutum*, the genome sequences of *G. arboreum* and *G. raimondii* are important resources that can facilitate the genome-wide transcriptome analysis of *G. hirsutum*.

At the present time, RN management in upland cotton is heavily dependent on nematicide application, which is costly and environmentally unsustainable. Hence, breeding for increased resistance to RN in cotton is an important objective (Robinson 2007). In 2007, two cotton-breeding lines with resistance to RN, LONREN-1 and LONREN-2 were released by United States Department of Agriculture (USDA) (Bell et al. 2014). The RN resistance was transferred to *G. hirsutum* from wild diploid species, *G. longicalyx*, which is immune to RN (Bell et al. 2014). Codominant simple sequence repeat (SSR) BNL3279_114 marker was used to follow introgression of the RN resistance quantitative trait loci (QTL) Ren^{lon} (Bell et al. 2014).

In a test field containing high levels of RN infestation, mild to severe stunting was reported for the LONREN-1 and LONREN-2 genotypes (Sikkens et al. 2011). Subsequently, root necrosis and a progressive decrease in root mass, typical of an HR, were also observed on the two LONREN lines with increased RN inoculum levels (Sikkens et al. 2011). BARBREN-713 was later released by USDA as another RN resistance genotype, based on its good performance in RN resistance and promising agronomic potential without HR (USDA release note). BARBREN-713 was developed

by crossing and backcrossing *G. barbadense* (tetraploid) accession GB713, a RN resistant line (Guitierrez et al. 2011), with the RKN resistant cultivar Acala Nem-X (USDA release note).

The RN resistance of BARBREN-713 is primarily due to a homozygous QTL locus Ren^{barb2} flanked by SSR markers BNL3279_105 and BNL4011_155, whereas QTL Ren^{barb3} also contributes RN resistance to BARBREN-713 (USDA release note). In addition, BARBREN-713 is also homozygous for SSR markers CIR316_202 and BNL1231_197, which flank the *rkn-1* locus for RKN resistance (Wang et al. 2006). BNL3279_105, BNL569_131, and CIR316_202 SSR markers were used to follow the resistance QTL in BARBREN-713 (USDA release note). It should be noted that the marker BNL3279 with different fragment lengths was associated with QTLs from both RN resistance resources: *G. longicalyx* and *G. barbadense*. Besides, BNL3279 with the amplicon size of 132bp was also in the flanking regions of Ren^{ari}, an RN resistance locus from *G. aridum* (Romano et al. 2009).

To understand the importance of BNL3279 in the cotton response to RN, identifying the important regulatory genes that are located in the BNL3279-linked QTL region, and comparing the sequences of each allele at this gene locus among the different resources and/or their derived genotypes is the best available first step in defining a potentially important *R*-gene for RN in cotton.

In this study, a global gene expression analysis was conducted using root RNA-seq data obtained from different genotypes of *G. hirsutum* with and without RN infestation. BARBREN-713 was selected as the resistant (R) genotype, LONREN-1 was selected as the hypersensitive (HR) genotype, and two genotypes, DP90 and SG747, were

pooled together and used as RN susceptible (S) genotypes. The aim of this study was to identify comparative gene expression responses from the RN S/R/HR genotypes, and to identify the important regulatory gene candidates located close to the RN resistance QTLs.

Results and discussion

Sequencing and transcriptome assembly results

To obtain a global view of gene expression changes in response to RN infestation in cotton, six paired-end (100bp) cDNA libraries were generated from cotton roots including two libraries from susceptible genotypes (DSU: DP90 & SG747 with no RN infestation; DSI: DP90 & SG747 with RN infestation), two libraries from hypersensitive genotype, LONREN-1 (L1U: LONREN-1 with no RN infestation; L1I: LONREN-1 with RN infestation), and two libraries from resistant genotype BARBREN-713 (B713U: BARBREN-713 with no RN infestation; B713I: BARBREN-713 with RN infestation). This generated over 150 million raw reads from all libraries (**Table 2.1**). After adaptor trimming and removal of low quality reads and reads shorter than 30bp, over 93 million reads (61% of the total raw reads) were obtained (**Table 2.1**).

These paired-end sequences from all samples were pooled together to construct two sets of reference transcriptome assemblies using *G. arboreum* and *G. raimondii* genome sequences as references (**Table 2.2**) (see methods for details). The final assemblies contained 84,711 contigs that could have been derived from the A2 genome (assembly using *G. arboreum* genome sequences as references) and 80,353 contigs that could have been derived from the D2 genome (assembly using *G. raimondii* genome

sequences as references) and were used as cotton root reference transcriptome for subsequent gene expression analysis (**Table 2.2**). Notably, A2 and D5 contigs exhibited similar assembly statistics (**Table 2.2**) and length distributions, with ~45% contigs were 100-500bp and ~55% contigs were greater than 500bp (**Fig. 2.1**).

To test the conservation and divergence between the A2-derived and the D5-derived contigs, reciprocal BLASTN searches using an e-value score below 1e-6 (Guan et al. 2014) was used to determine that 85.1% of the A2-derived contigs and 89.9% of D5-derived contigs were shared (**Fig. 2.2 A**), suggesting that there was substantial conservation between the A- and D-subgenomes of *G. hirsutum*. Sequences of A2-derived contigs, D5-derived contigs, and EST collections in Cotton Gene Index 11 (CGI11, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=cotton) were also compared to examine the transcriptome converge and novelty in the newly assembled contigs using the method described by Guan et al. (2014). As a result, around 85% of CGI11 EST sequences were homologous of A2- and D5- subgenome derived contigs, suggesting the good coverage of known cotton ESTs in the self-assembled contigs (**Fig. 2.2 B**). Using the reverse query, 22.6% of both A2 and D5 contigs were unique and didn't match any sequence in CGI11 (**Fig. 2.2 B**). Thus, the newly assembled root contigs may have both good depth of coverage and unique assembled ESTs not found in existing EST collections making them useful as references for downstream analysis.

Global transcriptome changes involved in *R. reniformis* responses in *G. hirsutum* roots

To determine how many genes were differentially expressed in the 3 genotype classes and whether there was variation before and after RN infestation, the number of expressed transcripts at varying expression levels as reflected by the number of reads per kilobase of transcript per million mapped reads (RPKM) for each library is shown in **Table 2.3**. Out of 165,064 A2-derived and D5-derived contigs, approximately 50% were expressed in each library based on the criteria of RPKM $\geq$ 2, while ~30% of the contigs had expression values of RPKM $\geq$ 5 (**Table 2.3**). In general, similar numbers of genes were expressed in different genotypes and RN infestation did not seem to have effects on the number of differentially expressed genes.

In order to determine the regulation of cotton root contigs in response to RN infestation, differential expression analysis (see methods for details) was performed between RN uninfested and infested libraries for each genotype. As a result, 9,407 contigs were RN responsive in DS, 8,531 in L1, and 5,842 in B713 (**Fig 2.3 A; Fig S2.1**). Among them, 6,596, 6,210, and 4,137 RN responsive contigs were identified in DS, L1, and B713 respectively (**Fig. 2.3 A**). There were 1,554 RN responsive contigs common between DS and L1, 448 between L1 and B713, and 938 between DS and B713 (**Fig. 2.3 A**). In addition, 319 contigs were differentially expressed in all three genotypes after RN infestation (**Fig. 2.3 A**). Of these RN responsive contigs in different genotypes, more contigs were up regulated in DS and L1 after RN infestation, while more contigs were down regulated in B713 after RN infestation (**Fig. 2.3 B**).

Functional annotation of RN-responsive contigs

Following differential expression analysis, all RN responsive contigs were annotated through blastx against *G. raimondii* and *G. arboreum* gene models. Based on the gene ontology terms assigned for each RN responsive contig, several GO categories contained a high number of genes (**Fig. 2.4**). The expression of contigs involved in "transcriptional regulation", "stress response", "hormone metabolism and signaling", "secondary metabolism", "cell wall biosynthesis and degradation", and "redox reactions" were among the categories of genes known to have significant involvement in plant nematode interactions and will be discussed in more details in the following sections.

Cell wall-related genes

During plant-RN interactions, successful RN parasitism is contingent upon the establishment of syncytia, which resulted from breakdown of cell walls between the initial feeding cells and their neighboring cells (Robinson 2007). To date, many genes functioning in cell wall loosening have been identified in susceptible plant responses to RKN and/or CN infestation (Jammes et al. 2005; Wieczorek et al. 2006; Ithal et al. 2007a; Ithal et al. 2007b).

A total of 137 cell wall-related contigs were RN responsive in at least one genotype, including 90 contigs involved in cell wall loosening and/or degradation and 47 contigs involved in cell wall synthesis (**Table 2.4; Table S2.1**). Among these RN responsive contigs, most contigs involved in cell wall loosening and degradation were up regulated in susceptible genotype DS and down regulated in hypersensitive genotype L1, after RN infestation. While fewer contigs were statistically differentially expressed in

B713I compared to B713U, relatively more were down regulated (**Table 2.4; Table S2.1**).

As the initial physical barrier to protect plants from pathogen attack, partial dissolution of the plant cell wall matrix is required for the successful progression of RN parasitism (Agudelo et al. 2005). Thus, the more up regulated RN responsive cell wall loosening and degradation contigs in DSI and more down regulated ones in L1I and B713I, appeared to be in line with the their respective level of RN resistance. Moreover, the stimulation of more expansin and cellulose synthase contigs in susceptible genotype DS (**Table 2.4; Table S2.1**), are necessary for cell wall relaxation during the formation of RN induced root syncytia (Hematy et al. 2014).

In addition to being a passive physical barrier, plant cell walls can also serve as a "sensor" of pathogens to activate various cellular signaling pathways (Hematy et al. 2014). Other studies have found that resistance to specific pathogens including bacteria, fungi, and aphids can be enhanced in plants with less cellulose produced but more activated stress hormones (i.e. salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and/or abscisic acid (ABA)) signaling (Hematy et al. 2014). These findings are in part consistent with the inhibition of cell wall synthesis-related contigs in L1I and B713I (**Table 2.4; Table S2.1**).

Hormone metabolism and signaling related genes

Hormones are important players in both *R*-gene mediated resistance and plant basal defense responses to invading nematodes (Li et al. 2014b). They either regulate plant S/R to SPENs through pathogenesis related (*PR*) genes or other resistance related

factors (in the case of SA, JA, and ET), or affect nematode parasitism through manipulation of NFS initiation and development (in the case of auxin (AX) and cytokinin (CK)) (Li et al. 2014b).

In this study, a total of 111 contigs involved in hormone synthesis and signaling pathways were found to be RN responsive in at least one genotype, including 57 contigs involved in AX pathway, 5 involved in CK pathway, 16 involved in JA pathway, 9 involved in ET pathway, and 3, 4, 17 involved in SA, ABA, and GA pathway, respectively (**Table 2.5 A; Table S2.2**).

It should be noted that all 9 AX export related contigs were up regulated in DSI compared to DSU, 1 was repressed in L1I compared to L1U, and none of them was statistically differentially expressed in B713I compared to B713U (**Table 2.5A; Table S2.2**). Local and transient accumulation of AX in nematode feeding cells has a supportive role in NFS establishment and development (Li et al. 2014b), suggesting the positive role of the up-regulated AX export-related contigs in RN induced syncytium development.

In addition to the AX export-related contigs, contigs involved in AX responses were also differentially expressed (**Table 2.5 A; Table S2.2**). Specifically, *ARF9* and *ARF19* orthologs were strongly repressed in L1I and B713I compared to their RN uninfested counterparts, and *ARF8* ortholog was greatly down regulated in DSI (**Table 2.5 B**). The ARF family contains transcription factors that activate or repress AX-responsive genes, including the *AUX/IAA*, the *SAUR*, and the *GH3* families of genes. The specific role of each AX responsive factor and gene in plant nematode interaction is unclear, however, in Arabidopsis-BCN compatible interactions, *ARF9* and *ARF19* were strongly induced in syncytium and the neighboring cells at the early stage (2-3DPI) of

nematode infestation, whereas *ARF8* had a limited expression in BCN infected root throughout the time observed (1-10DPI) (Hewezi et al. 2014).

Similar to AX, CK is known to affect nematode parasitism through manipulation of NFS initiation and development (Li et al. 2014b). In this study, 2 orthologs of CK biogenesis gene *IPT1* were both greatly down regulated in L1I (**Table 2.5**). *CKX*, which degrades CK, was also statistically down regulated in L1I (**Table 2.5**). The *LOG* gene regulates CK levels by the conversion of inactive CK nucleotides to active free bases, in this study, 2 RN responsive *LOG* gene orthologs were identified (**Table 2.5**).

JA biogenesis appeared to play a positive role in susceptibility to RKN (Li et al. 2014b). Most JA biogenesis-related contigs in this study were down regulated in L1I compared to L1U, though no obvious expression trend can be concluded for DSI vs. DSU or B713I vs. DSU (**Table 2.5 A; Table S2.2**). JA-responsive genes (*JAZ*) are transcriptionally up regulated by JA, and are repressors of JA signaling by inhibiting transcription factors that regulate early JA-responsive genes (Chico et al. 2008). Both JAZ repressor proteins and their downstream transcription factors playing a role in plant pathogen interactions have been reported (Chung et al. 2008; Chico et al. 2014). In this study, 4 *JAZ* orthologs were RN responsive, with all of them induced in susceptible genotype DS after RN infestation, whereas no statistical differential expression was detected in L1I or B713I (**Table 2.5**). Besides *JAZ*, 1 orthologs of *JMT*, a stress responsive gene functioning in generation of MeJA by JA methylation (Seo et al. 2000), also exhibited significant up regulation in response to RN infestation in the DS genotype (**Table 2.5**). Although these data do not indicate the direct role of JA accumulation in

plant S/R to RN, they do suggest the importance of *JAZ* and *JMT* in plant susceptibility to RN.

Current research suggested that ET has pleiotropic effects in plant responses to nematodes, which might be mediated through regulation of different classes of stress-responsive genes via ERFs (Li et al. 2014b). It was found that ET biogenesis-related genes were mostly up regulated in DSI compared to DSU and down regulated in L1I compared to L1U (**Table 2.5; Table S2.2**).

As for SA pathway, 3 orthologs of *SAMT* were identified to be RN responsive in this study (**Table 2.5**). *SAMT* modulates SA levels by converting SA to methyl SA, and MeSA can function as a mobile signal, mediating SAR in some plants (Park et al. 2007). In plant-nematode interactions specifically, overexpressing *SAMT* conferred resistance to SCN in soybean (Lin et al. 2013). Moreover, SA was proposed to form a self-amplifying feedback loop with ROS (i.e. H_2O_2) in potentiating plant HR (Vlot et al. 2009). Thus, the specific differential expression of *SAMT* orthologs in L1I is consistent with *SAMT* playing a role in RN resistance and hypersensitive cell death responses.

ABA functions as a widespread growth inhibitor; it inhibits cell division and cell expansion, but promotes cell differentiation (Culter et al. 2010). ABA responsive GRAM domain-containing protein ABA responsive 1 (ABA1) and HVA22 protein have been associated with hypersensitive type cell death (Choi and Hwang 2011; Guo and Ho 2008). Both GRAM domain-containing protein coding gene and *HAV22* orthologs were differentially regulated in response to RN infestation (**Table 2.5 B**). In addition, Aldehyde oxidase 2 (AO2) and ABA responsive element binding factor 2 (ABF2) orthologs were also RN responsive (**Table 2.5 B**). AO2 protein catalyzes the last step of

ABA synthesis and *ABF2* gene encodes a BZIP-type transcription factor that regulates downstream ABA induced gene expression (Culter et al. 2010).

Redox, secondary metabolism, pathogenesis, and other stress-related genes

Global gene expression analysis identified over-represented RN responsive contigs involved in plant redox reactions, secondary metabolism, and pathogenesis-related responses (**Table 2.6 A; Table S2.3**). Over 70 contigs annotated as *Heat Shock Proteins* (*HSP*) and more than 50 nodulin annoated contigs were RN responsive in at least one genotype (**Table 2.6 A; Table S2.3**). Besides, several contigs categorized as HR-related, GRF (growth regulating factors), and sRNA biogenesis also exhibited statistical differential expression in response to RN infestation (**Table 2.6 B)**.

143 contigs involved in plant redox reactions were RN responsive (**Table 2.6 A; Table S2.3**). Among them, most contigs annotated as peroxidase were up regulated in DSI vs. DSU, down regulated in L1I vs. L1U, but not statistically differentially regulated in B713I vs. B713U (**Table 2.6 A; Table S2.3**). There are three main classes of plant peroxidases, of which apoplastic localized class III peroxidases can either act as H_2O_2 scavengers or generate H_2O_2, depending on the specific physiological conditions (O'Brien et al. 2012). Although the specific classes of these 79 peroxidases are unclear, we cannot rule out the possibility that they were involved in cell death in DS and L1.

Ascorbate peroxidase, dismutase and catalase belong to plant class I peroxidase family, and they serve as ROS scavengers, along with thioredoxin and glutaredoxin (Gonzalez-Rabade et al. 2012). Notably, 35 thioredoxin annotated contigs showed differential expression, with more contigs down regulated in DSI vs. DSU but up

regulated in L1I vs. L1U, in a reverse direction as compared to the expression patterns of contigs annotated as peroxidase (**Table 2.5 A**). While HR type cell death has been frequently observed in L1 under high level of RN infestation (Sikkens et al. 2011), the induction of contigs annotated as ROS scavenger might be in part responsible for the HR, playing a positive role in L1 resistance against RN (Spoel and Loake 2011).

In addition to contigs involved in redox reactions, 220 contigs involved in secondary metabolism that were RN responsive in at least one genotype (**Table 2.6 A; Table S2.3**). The phenylpropanoid pathway, specifically, has a known role in plant defense against pathogens resulting from cell wall strengthening effects, making more stress hormone (i.e. SA), and serving as an antioxidant in ROS scavenging (Camera et al. 2004; Pourcel et al. 2006). Accordingly, in all three genotypes after RN infestation, the numbers of up-regulated contigs involved in phenylpropanoid pathway were more than those that were down regulated (**Table 2.6 A; Table S2.3**). 28 contigs annotated as dirigent-like proteins were RN responsive in our data (**Table 2.6 A**). Dirigent proteins play important roles in plant secondary metabolism especially the biosynthesis of lignin, which strengthens plant cell walls and helps plants defend against pathogens (Pickl and Schaller 2013). In cotton responses to RN, lignin-type deposits in thickened cell walls were observed in RN infected root cells from both compatible and incompatible reactions at 6 DPI (Agudelo et al. 2005). Lignin deposition was greater in plant HR responses resulting inreinforcment of the cell walls surrounding pathogen infection sites; thus creating a barrier to inhibit the spread of the infection. As such, all of the RN responsive dirigent-like protein contigs were up regulated in the L1 genotype after RN infestation

(**Table 2.6 A; Table S2.3**), which showed a hypertensive-type cell death response after RN infestation (Sikkens et al. 2011).

Chitinase and thaumatin-like proteins are both PR proteins that have been shown to be induced in response to pathogen infection and are often associated with the production of antimicrobial secondary metabolites (Glazebrook 2001). Others have found that genes coding for both proteins were differentially expressed upon RKN or CN infection (Ithal et al. 2007; Kyndt et al. 2012; Uehara et al. 2010), and thaumatin like *PR-5* gene has been well correlated with nematode resistance (Barcala et al. 2010; Hewezi et al. 2010; Matthew et al. 2014; Portillo et al. 2013). In this study, contigs annotated as chitinase were significantly up regulated in DS, while thaumatin-like protein annotated contigs were up regulated in B713 (**Table 2.6 A; Table S2.3**). PR-3-type chitinase is JA- and ET-inducible, whereas the thaumatin-like class is classified as PR-5 and is SA-inducible (van Loon et al. 2006). These data, together with others' results suggested the positive role of *PR-5* gene in RN resistance.

Heat shock proteins (HSPs) are a group of proteins involved in the folding and unfolding of other proteins, and their expression is increased in response to hyperthermia and other environmental stress (Vierling 1991). HSP90 protein is required in R protein *Mi-1*-mediated RKN resistance (Bhattarai et al. 2007). Contigs annotated as different families of *HSPs* including *HSP20*, *HSP40*, *HSP70*, and *HSP90* were RN responsive as determined from expression analysis (**Table 2.6 A; Table S2.3**).

Transcription factors coding genes

Transcription factors are important regulators in plant responses to nematode infestation by inducing or suppressing defense-related genes (Li et al. 2014b). 294 contigs annotated as transcription factors were RN responsive (**Table S2.4**). **Table 2.7** listed the most abundant RN responsive transcription factor families in different genotypes.

MYB protein families are involved in the regulation of flavonoid biosynthesis, root hair patterning, and lateral root formation (Dubos et al. 2010). ERF transcription factor expression is regulated by plant hormones, including SA, JA, ET, CK, and ABA (Gutterson and Reuber 2004). WRKYs are well known as key regulators in plant innate immunity (Rushton et al. 2010), and different *WRKY* genes have been shown to regulate plant responses to nematode infestation (Ali et al. 2014; Atamian et al. 2012; Bhattarai et al. 2010). HSF transcription factors regulate the expression of heat shock molecular chaperones (HSPs) (Scharf et al. 2011). The SCARECROW transcription factor in the GRAS protein family is known to regulate root radial patterning (Bolle 2004). The TGA transcription factor in the bZIP family was demonstrated to contribute SCN resistance when overexpressed in soybean (Matthews et al. 2014), and NACs are central components of plant innate immunity hormone signaling and ROS signaling (Puranik et al. 2012). The MYC2 transcription factors in the bHLH family are known to be master regulators in JA signaling (Kazan and Manners 2013).

In addition, post-transcriptional regulation appears to be an important aspect of the expression regulation of the above transcription factors. Specifically, transcripts of MYBs are targets of both miRNAs and trans-acting small interfering RNAs (Dubos et al.

2010), which were proposed to be another important layer in the regulation of plant responses to nematode infestation (Li et al. 2014b).

Differentially expressed contigs between three genotypes without *R. reniformis* infestation

Expression analysis was also conducted among the susceptible, hypersensitive, and resistant genotypes without RN infestation to determine the genotypic differences between the genotypes. 4,171 and 5,984 contigs demonstrated increased abundance in L1U and B713U, respectively, compared to DSU. Whereas 8,503 and 4,709 contigs had decreased abundance in L1U and B713U, respectively (**Fig. S2.2 A**). Similar sets of significantly regulated biological pathways were identified for L1U vs. DSU and B713U vs. DSU (**Fig. S2.2 B**).

Association of differentially expressed contigs to *R. reniformis* resistance QTLs

The sequences of six SSR markers (BNL3279, BNL4011, BNL1721, BNL569, CIR316, and BNL1231), genetically linked to RN resistance QTLs, were retrieved from the cotton EST database (Xie et al. 2011). Five of these SSR marker sequences (all except BNL1721 that only mapped to *G. raimondii* genome) mapped to both the *G. arboreum* (Li et al. 2014a) and the *G. raimondii* (Paterson et al. 2012) genomes (**Table 2.8**).

To identify the contigs that were differentially expressed between genotypes that were located near one of the RN-resistance QTL SSR markers, the differentially expressed contigs were mapped against the *G. aboreum* and*G. raimondii* genomes. The chromosome location of each of these contig was noted and compared with those of SSR

markers, to find the contigs that mapped within 1Mb of the different SSR markers (Radwan et al. 2011).

Notably, transcripts annotated as *R*-genes accounted for 40% of the total contigs mapping within 1Mb of the different SSR markers (**Table S2.3**), all but one of these *R*-genes had a higher expression in L1U or B713U compared to DSU (**Fig. 2.5 A**). Chromosome locations 1Mb distal to BNL3279 (highlighted in yellow) are the hot spots where most of these differentially expressed R-gene annotated contigs clustered, including those annotated as LRR receptor kinase, TIR-NBS-LRR class resistance protein, and NB-ARC domain-containing resistance proteins (**Table 2.8; Fig. 2.5 A**).

Both extra-cellular LRR domain containing R-receptor and intra-cellular R-receptor proteins, which was typically identified as NBS-LRR type R proteins, can sense invading pathogen-associated molecular patterns (PAMPs) and trigger plant innate immunitiy as well as various downstream defense responses (Jones and Dangl 2006).

In plant-nematode interactions, a series of *R*-genes have been cloned, that condition resistance to either RKN or SCN, and most of them encode canonical intracellular NBS-LRR type R-receptor proteins (Li et al. 2014b). While *R*-genes of this type have not to date been correlated with RN resistance, the fact that a large number of *R*-gene-annotated contigs are located in the vicinity of the RN resistance QTLs and they were either up regulated in L1U or B713U compared to DSU, makes possible the hypothesis that one or more of these mapped sequences could be an *R*-gene type receptor protein involved in cotton S/R to RN infestation. Particularly, 290 contigs annotated as *R*-gene were statistically differentially expressed in response to RN infestation in at least one genotype (**Table S2.6**). In addition, over 50% of these RN responsive R-gene

annotated contigs were located on chromosome 4 of *G. arboreum* and chromosome 7 of *G. raimondii*, where BNL3279, BNL4011, CIR316, and BNL1231 were mapped (**Fig. 2.5 B**).

Overall conclusions

Gene expression and metabolic studies have identified genes involved in various signaling pathways that regulate plant responses to RKN and/or CN. In the present study, RNA-seq was used to investigate global gene expression patterns in plant susceptibility, hypersensitivity, and resistance to reniform nematodes. The data presented indicate that cell and cell wall architectures, hormone signaling, ROS levels, cell death pathways, pathogenesis related genes and genes involved in phytoalexin pathways were distinctly modulated between the RN susceptible, hypersensitive, and resistant genotypes. By correlating the list of differentially expressed genes between genotypes and QTL maps, several *R*-genes that could be considered in future functional analysis were highlighted. Further examination of the putative roles of these R-genes in RN susceptibility, hypersensitivity, and resistance is required.

Materials and methods

Plant material and stress treatment

Four genotypes of cotton were selected: DP90, SG747, LONREN-1, and BARBREN-713. Cotton seedlings were infested with *R. reniformis* two weeks after planting and root samples were collected at 0, 1, 3, 5, and 10 days post infestation.

Samples at 0 day were taken as uninfested controls, while samples from 1, 3, 5, and 10 days post-infestation were pooled and considered the *R. reniformis* infested samples.

cDNA library construction

Total RNA was extracted from uninfested and infested root samples using the hot borate method (Wan and Wilkins, 1994). Root samples from DP90 and SG747 were combined evenly for RNA extraction. After mRNA purification using GenElute mRNA miniprep kit (Sigma), six cDNA libraries were constructed using Mint cDNA synthesis kit (Evrogen). They are DP90 and SG747 uninfested cDNA library (DSU), DP90 and SG747 infested cDNA library (DSI), LONREN-1 uninfested cDNA library (L1U), LONREN-1 infested cDNA library (L1I), BARBREN-713 uninfested cDNA library (B713U), and BARBREN-713 infested cDNA library (B713I). The constructed libraries were sequenced on illumina 2000 HiSeq sequencer at the Genomics Core Facility at Emory University.

Processing of cDNA sequencing reads and contigs assembly

Before assembly, raw sequencing reads were trimmed by removing adaptor sequences, ambiguous nucleotides, low quality sequences, and short read length sequences (length below 30bp) with CLC Genomic workbench (version 5.5.1). The quality of raw reads and trimmed reads was checked by fastQC software (version 0.10.1). Given the large size of the data, a Trinity in silico normalization of the full data set was conducted before assembly, to reduce memory requirements and improve assembly runtime. Subsequently, the in silico normalized reads were aligned to the bowtie2 (Langmead et al. 2012) built *G. raimondii* genome (version 2, Paterson et al. 2012) reference and *G. arboreum* genome (Li et al. 2014a) reference with tophat2 (Kim et al.

2013) respectively. Both *G. raimondii* and *G. arboreum* genome guided assemblies of the normalized reads into contigs and genes were carried out using Trinity with default parameters (Grabher et al. 2011). Contigs assembled with *G. raimondii* and *G. arboreum* genome sequences as references were considered D5 and A2 subgenome sequences respectively.

Expression analysis

To compute expression values of assembled contigs in each library, the trimmed reads from each library were aligned to the combined set of contigs using bowtie2, and RSEM (Li and Dewey 2011) was executed to estimate expression values of every contig based on the resulting alignments. The expression values of assembled contigs in each library were presented in RPKM (reads per kilobase of transcript per million mapped reads).

Expression analysis and Kal's statistical analysis were conducted in CLC genomic workbench (version 5.5.1). A contig was considered to be differentially expressed if the FDR corrected P-value given by the above analysis was smaller than 0.01, and the fold change in RPKM normalized counts was more than 2. The online tool BioVenn (Hulsen et al. 2008) was used for the construction of Venn diagrams. The R statistical package was used for the construction of heat maps.

Functional categorization of differentially expressed contigs

Functional categorization of contigs was performed using MapMan ontology (Thimm et al. 2004), the MapMan mapping for *G. raimondii* is available at http://mapman.gabipd.org/. The GO terms for the *G. arboreum* genes were assigned based on their sequence homology to *G. raimondii* genes.

Determining location of contigs on A2 and D5 subgenomes

The sequences of six SSR markers (BNL3279, BNL4011, BNL1721, BNL569, CIR316, and BNL1231), genetically linked to QTLs that are significant associated with *R. reniformis* resistance (Dighe et al. 2009; Gutierrez et al. 2011), were retrieved from the Cotton EST database (Xie et al. 2011). To identify their position on A2 and D5 subgenomes, SSR marker sequences were blasted against the *G. raimondii* and *G. arboreum* genome sequences respectively. Similarly, the assembled A2 and D5 subgenome sequences were blasted against the *G. raimondii* and *G. arboreum* genome sequences with only the topblast result kept to determine their location. The locations of differentially expressed contigs that were annotated as R-genes were then compared with the locations of SSR markers, to identify the differentially expressed R-genes resided 1Mb within the SSR markers mapped loci.

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Table 2.1 Summary statistics of sequenced reads from each library

Libraries	DSU	DSI	L1U	L1I	B713U	B713I	Total
Raw reads	19,778,108	18,687,950	14,897,934	14,136,410	42,536,320	42,906,218	152,942,940
High quality reads (%)	11,118,360 (56.22%)	11,928,236 (63.83)	10,329,006 (69.33%)	8,258,406 (58.42%)	26,240,970 (61.69%)	25,504,478 (59.44%)	93,379,456 (61.01%)

Table 2.1 Summary of assembly results

	A2 contig[1]	D5 contig[2]
Total transcripts	84,711	80,353
N50 (bp)	1,405	1,347
Average length (bp)	876	849
Min length (bp)	201	201
Max length (bp)	6,118	5,776

[1] Contigs assembled with *G. arborem* genome sequences as references
[2] Contigs assembled with *G. raimondii* genome sequences as references

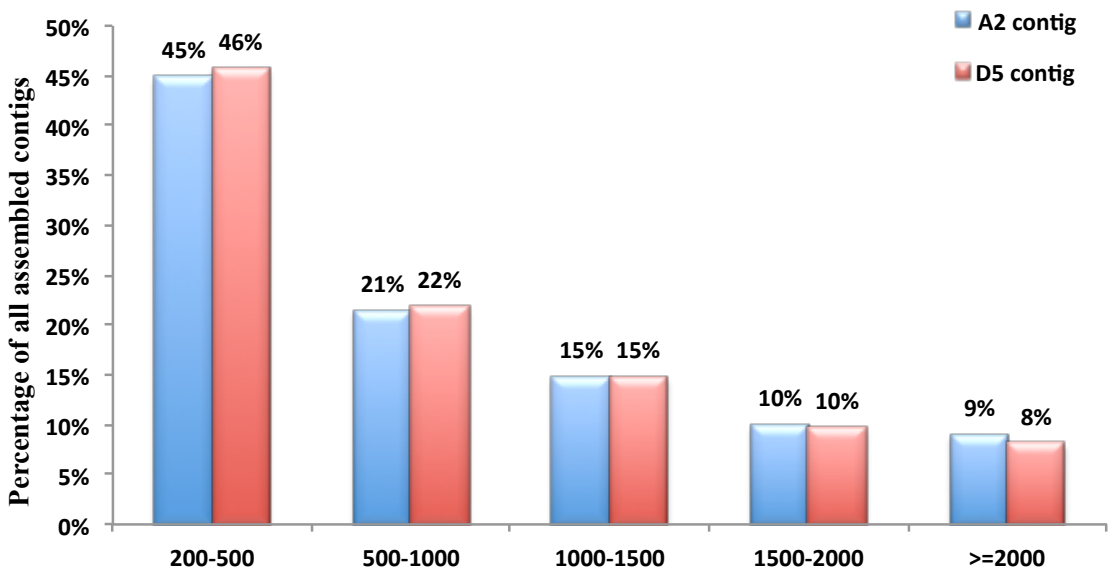

Figure 2.1 Length distribution of assembled contigs

A2 contigs: contigs assembled with *G. arborem* genome sequences as references
D5 contigs: contigs assembled with *G. raimondii* genome sequences as references

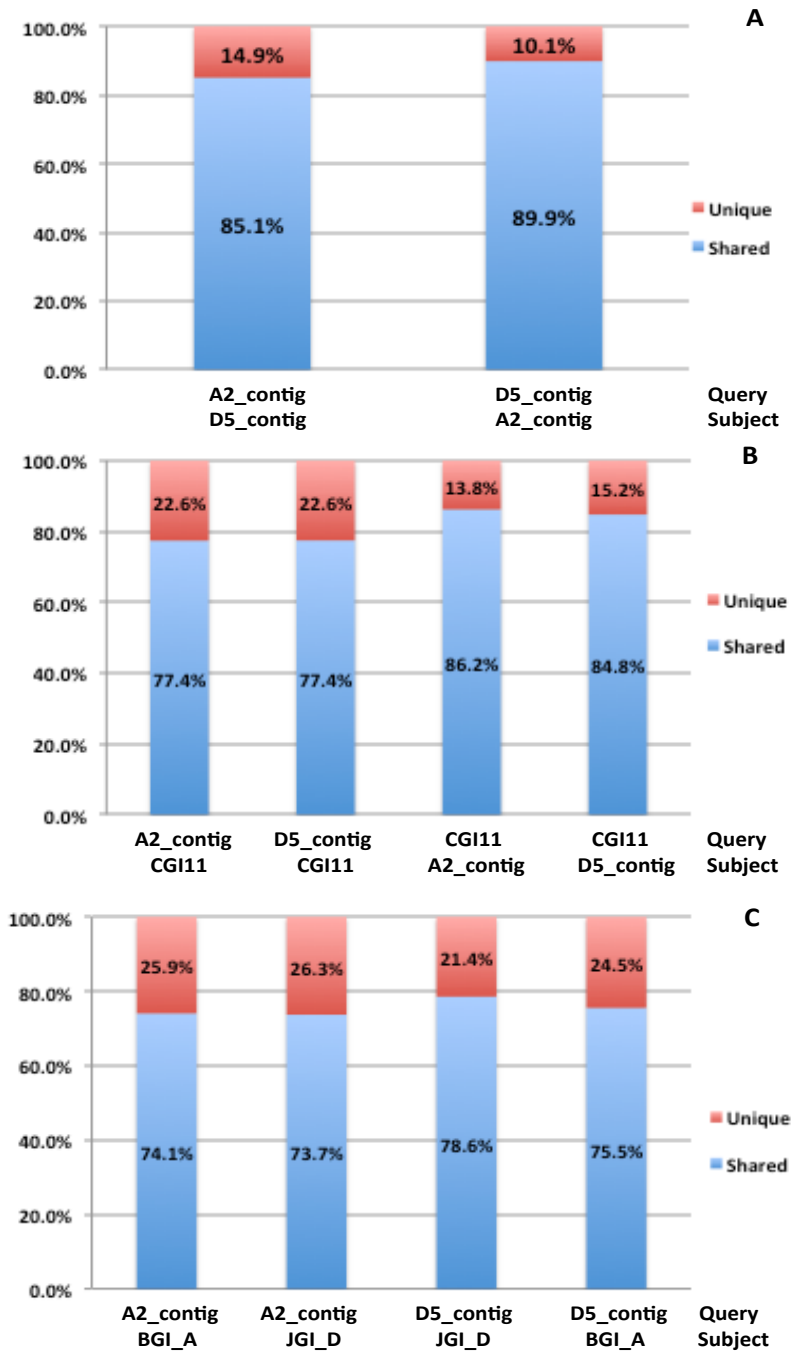

Figure 2.2 Sequence comparisons between assembled contigs with public cotton transcript datasets

A: Sequence comparison between A2 contigs and D5 contigs using reciprocal blastn; **B:** Sequence comparison between A2 contigs and D5 contigs with CGI11 ESTs using reciprocal blastn; **C:** Sequence comparisons between A2 contigs and D5 contigs with BGI_A (gene models predicted from published G. aobreum genome sequences) and JGI_D genes (gene models predicted from published G. raimondii genome sequences).

Table 2.3 The number of genes/transcripts expressed in each library

Library	RPKM ≥ 1 (%)	RPKM ≥ 2 (%)	RPKM ≥ 5 (%)	RPKM ≥ 10 (%)
DSU	97,446 (61%)	80,499 (51%)	47,746 (30%)	27,450 (17%)
DSI	101,253 (64%)	83,174 (52%)	48,146 (30%)	27,113 (17%)
L1U	90,340 (57%)	76,286 (48%)	46,265 (29%)	27,043 (17%)
L1I	92,217 (58%)	77,888 (49%)	47,422 (30%)	27,613 (17%)
B713U	109,526 (69%)	92,148 (58%)	53,049 (33%)	28,867 (18%)
B713I	107,922 (68%)	88,507 (56%)	50,380 (32%)	27,709 (17%)

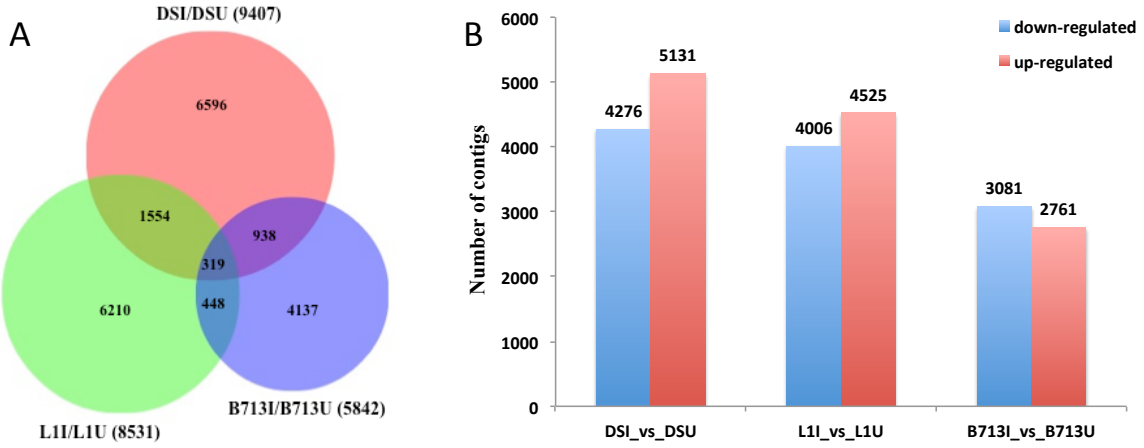

Figure 2.3 Number of RN-responsive contigs

A: Venn diagram shows the number of RN-responsive contigs in each genotype; **B:** The number of up- and down- regulated contigs in each genotype in response to RN infestation.

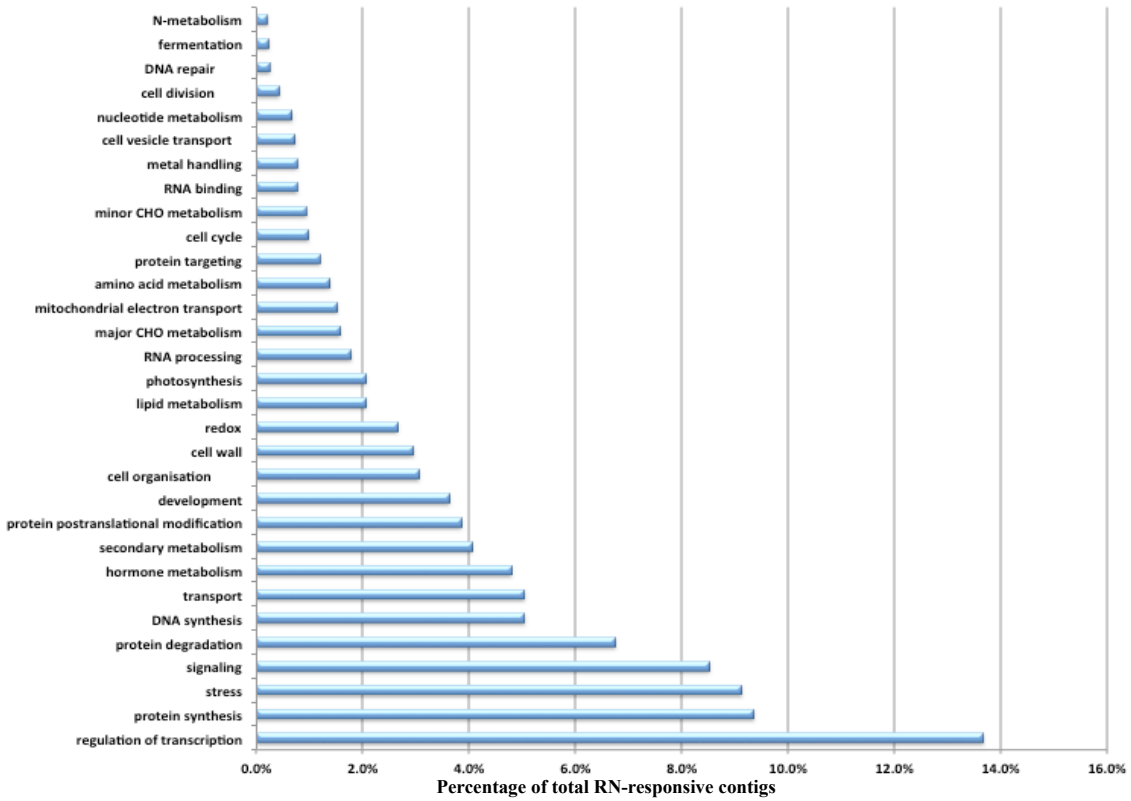

Figure 2.4 Gene Ontology categories of RN-responsive contigs

Table 2.4 Categories and numbers of RN-responsive contigs involved in cell wall biosynthesis and degradation

	DSI vs. DSU		L1I vs. L1U		B713I vs. B713U		
Cell Wall related pathways	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Total**
Cell wall loosening & degradation							**90**
Xyloglucan endotransglucosylase	28	0	0	22	1	0	35
Pectinase	13	6	6	5	1	5	31
Expansin	7	4	1	11	1	2	18
Mannan-xylose-arabinose-fucose	0	0	0	3	0	1	4
Cellulases and beta -1,4-glucanases	0	0	0	0	2	0	2
Cell wall synthesis							**47**
Cellulose synthesis	13	3	5	8	3	7	30
Cell wall precursor synthesis	6	0	1	8	0	3	15
Hemicellulose synthesis	0	2	0	0	0	0	2

Table 2.5 RN-responsive contigs involved in hormone metabolism and signaling

A. Categorires and numbers of RN-responsive contigs involved in hormone metabolism and signaling

Hormone related	DSI vs. DSU		L1I vs. L1U		B713I vs. B713U		
pathwasy	Up	Down	Up	Down	Up	Down	Total
Auxin							**57**
AX_export	9	0	0	1	0	0	9
AX_response_ARF	0	1	0	2	0	1	3
AX_response_SAUR	0	7	9	9	2	1	25
AX_response_AUX/IAA	2	3	1	5	1	1	12
AX_response_GH3	4	0	2	3	0	1	8
Cytokinin							**5**
CK_biogenesis	0	0	0	2	0	0	2
CK_degradation	0	0	0	1	0	0	1
CK_activation	0	1	1	0	0	0	2
Jasmonic acid							**16**
JA_biogenesis	2	2	2	6	2	1	11
JA_responsive	4	0	0	0	0	0	4
JA_metabolic	1	0	0	0	0	0	1
Ethylene							**9**
ET_biogenesis	4	0	0	3	1	1	9
Salicylic acid							**3**
SA_metabolic	0	0	2	1	0	0	3
ABA							**4**
ABA_response	1	1	0	2	0	1	3
ABA_biogenesis	0	0	0	1	0	0	1
Gibberellic acid							**17**
GA_response	1	6	2	10	1	1	17

B. List of RN responsive contigs involved in hormone metabolism and signaling

ID	DSU	DSI	L1U	L1I	B713U	B713I	Gene model	Annotation
D5 GG23405|c2 g1 i1	0.1	0.1	25.2	0.1	32.4	0.1	Gorai.007G026900.1	ARF 9
D5 GG26293|c1 g1 i1	0.1	8.2	27.4	0.1	8.7	0.1	Gorai.001G017000.2	ARF 19
D5 GG365|c1 g1 i1	32.3	0.1	0.1	0.1	0.1	6.9	Gorai.006G008700.1	ARF 8
A2 GG30971|c0 g1 i1	0.1	0.1	65.4	0.1	0.1	8.9	Gorai.012G122700.1	IPT 1
D5 GG3779|c0 g1 i1	0.1	0.1	65.4	0.1	0.1	8.9	Gorai.012G122700.1	IPT 1
D5 GG8102|c7 g1 i2	5.3	3.3	20.8	3.6	2.3	1.7	Gorai.013G014800.1	CKX 6
A2 GG25175|c1 g1 i2	0.1	5.9	0.1	34.4	0.1	0.1	Cotton A 29655	LOG
D5 GG14279|c0 g1 i2	21.0	0.1	0.1	0.1	0.1	8.5	Cotton A 00255	LOG
D5 GG21297|c1 g1 i1	0.1	45.7	0.1	0.1	0.1	0.1	Gorai.009G330500.3	JAZ 3
D5 GG10735|c1 g1 i4	15.5	38.8	15.5	14.2	4.9	5.2	Gorai.011G062000.1	JAZ 6
D5 GG12672|c0 g1 i3	11.1	48.7	11.2	17.5	0.7	0.1	Gorai.002G021800.1	JAZ 10
D5 GG12672|c0 g1 i2	23.5	47.5	20.6	5.7	6.3	4.1	Gorai.002G021800.1	JAZ 10
D5 GG4054|c0 g1 i1	0.1	10.1	0.1	0.1	0.1	0.1	Gorai.005G245200.1	JMT
D5 GG11052|c0 g1 i2	23.1	35.2	52.6	21.9	7.7	10.8	Gorai.011G100800.1	SAMT
D5 GG8133|c0 g1 i1	7.0	1.4	5.3	26.8	9.1	6.6	Gorai.013G029200.1	SAMT
D5 GG8133|c0 g1 i2	6.6	3.8	2.0	22.4	7.0	6.6	Gorai.013G029200.1	SAMT
D5 GG1339|c0 g1 i1	0.1	0.1	12.9	0.1	0.1	0.1	Gorai.006G118300.2	AO 2
D5 GG20031|c3 g1 i1	14.3	3.9	21.3	3.9	27.5	6.2	Gorai.009G275700.1	ABF 2
D5 GG5776|c2 g2 i1	0.1	14.8	0.1	0.1	0.1	0.1	Gorai.005G212400.1	GRAM
D5 GG26414|c1 g1 i1	57.8	12.5	139.0	46.9	28.9	12.8	Gorai.001G057000.1	HVA22

Table 2.6 RN-responsive contigs involved in stress response-related pathways

A. Category and number of RN responsive-contigs involved in stress response-related pathways

	DSI vs. DSU		L1I vs. L1U		B713I vs. B713U		
Pathways	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Total**
Redox							**143**
peroxidase	34	7	16	35	1	2	79
ascorbate peroxidase	2	0	0	2	0	0	4
dismutases and catalases	1	2	1	2	2	0	8
thioredoxin	3	17	14	6	2	3	35
glutaredoxins	0	6	5	6	2	0	17
Secondary metabolism							**220**
isoprenoids metabolism	14	34	30	13	3	8	81
phenylpropanoids metabolism	41	23	33	22	11	8	111
dirigent like	6	11	15	0	1	1	28
Pathogenesis related							**33**
chitinase	6	0	6	6	3	1	20
thaumatin	2	3	1	2	6	1	13
Other							**135**
HSP20	4	23	1	41	2	1	52
HSP40	2	1	2	5	3	0	11
HSP70	0	2	0	3	1	2	4
HSP90	3	0	0	0	0	2	3
nodulin	22	6	9	15	2	12	57
HR related	0	0	2	0	0	0	2
GRF	1	1	0	0	0	0	2
sRNA biogenesis	1	0	0	3	0	0	4

B. List of RN responsive contigs involved in stress response-related pathways

ID	DSU	DSI	L1U	L1I	B713U	B713I	Gene model	Annotation
A2 GG28654|c0 g1 i1	93.5	79.7	41.9	91.4	38.8	48.4	Gorai.006G070600.1	HR inducing
D5 GG10821|c1 g1 i1	73.3	65.2	26.6	69.6	56.5	89.8	Gorai.011G172100.1	HR inducing
A2 GG16065|c3 g1 i1	1.8	13.2	6.7	3.7	20.1	14.0	Cotton A 07040	AGO/DCL
A2 GG4990|c0 g3 i1	35.0	42.7	63.6	19.4	14.5	24.0	Cotton A 34017	AGO/DCL
D5 GG21417|c2 g1 i1	0.1	0.1	92.2	0.1	0.1	0.1	Cotton A 30349	AGO/DCL
A2 GG15921|c0 g1 i1	0.1	0.1	92.2	0.1	0.1	0.1	Cotton A 30349	AGO/DCL
D5 GG940|c4 g1 i1	0.1	21.7	0.1	0.1	0.1	0.1	Gorai.006G157300.1	GRF4
D5 GG24354|c2 g1 i1	54.1	0.1	0.1	0.1	0.1	0.1	Gorai.007G092400.3	GRF9

Table 2.7 Family and number of RN-responsive contigs annotated as transcription factors

	DSI vs. DSU		L1I vs. L1U		B713I vs. B713U		
Families	**Up**	**Down**	**Up**	**Down**	**Up**	**Down**	**Total**
MYB	19	5	7	13	3	6	48
ERF	13	6	6	10	6	5	44
WRKY	10	3	10	9	2	1	29
HSF	5	2	2	3	2	3	14
GRAS	6	2	4	1	0	2	11
BZIP	4	2	3	0	2	0	10
NAC	0	1	1	2	1	2	7

Table 2.8 Mapping positions of RN resistance QTLs-associated SSR markers

SSR	Sequence length (bp)	Chromosome	Start	End
BNL3279	619	A2 Chr10	74030524	74029915
BNL4011	337	A2 Chr4	133838455	133838116
BNL569	228	A2 Chr13	57120961	57121190
CIR316	282	A2 Chr4	130667024	130666734
BNL1231	289	A2 Chr4	104793412	104793685
BNL3279	619	D5 Chr7	56142481	56143089
BNL4011	337	D5 Chr7	54550456	54551582
BNL1721	241	D5 Chr13	30666694	30666468
BNL569	228	D5 Chr13	47353483	47353704
CIR316	282	D5 Chr7	59563000	59563272
BNL1231	289	D5 Chr7	57124822	57125006

A2: genome of *G. arboreum*; D5: genome of *G. raimondii*

Figure 2.5 R-receptor genes expression between genotypes and in response to RN infestation

A: Expression of RN resistance associated QTLs/SSRs adjacent R-receptor contigs between genotypes; **B**: Chromosome distribution of RN-responsive R receptor contigs RLK: leucine-rich repeat receptor kinase; TNL: TIR-NBS-LRR class disease resistance protein; N: NB-ARC domain-containing resistance protein; A2: genome of *G. aroboreum*; D5: genome of *G. raimondii*

Chapter III. Small RNA Analysis of Cotton (*Gossypium hirsutum* L.) Susceptibility, Resistance, and Hypersensitivity to Reniform Nematodes (*Rotylenchulus reniformis*)

Abstract

Reniform nematodes are semi-endoparasitic nematode species causing significant yield loss in cotton. In response to reniform nematode infestation, plants demonstrate susceptible (S), resistant (R), and/or hypersensitive responses (HR). In this study, we combined high-throughput sequencing with computational analysis to identify small RNAs involved in cotton S/R/HR. The sequence analysis confirmed the expression of 88 conserved miRNAs in cotton roots and identified 214 lineage-specific miRNAs, most of which mapped to specific loci in the *G. arboreum* and *G. raimondii* genomes. Furthermore, a comparison of miRNAomes revealed that 49 miRNAs were potentially associated with cotton S/R/HR reactions to reniform nematodes. By correlating the expression profile of miRNAs and the transcriptomes constructed from the same tissue under the same treatments, the regulatory roles of differentially expressed miRNAs in S/R/HR were primarily associated with "stress responses" and "transcriptional regulation". Moreover, isomiRNAs were identified from this study, and the expression of isomiRNAs for the first time was shown to be involved in cotton S/R/HR to reniform nematodes. Overall, this study presents the first small RNA expression analysis using different genotypes of *G. hirsutum* roots with and without reniform nematode infestation. The sRNA-target gene pairs identified in this study can serve as the basis for further functional analysis.

Introduction

Reniform nematodes (RN, *Rotylenchlus reniformis*) are semi-endoparasitic nematode species causing significant yield loss in cotton, particularly in the eastern US cotton belt (Robinson 2007). Successful RN parasitism is contingent on establishment of a syncytium, which serves as the sole nutrient source on which RN live. The syncytia are hypertrophied, multinucleate root cells with enlarged nuclei and dense cytoplasm, which resulted from the breakdown of cell walls between initial feeding cells (usually an endodermal cell) and neighboring cells (Agudelo et al. 2005). It is believed that nematode secretions injected through their stylet, a specialized needle-like mouthpart, are essential in syncytium initiation and maintenance (Hewezi and Baum 2013; Mitchum et al. 2013). To date, a number of effectors from other sedentary plant parasitic nematodes have been identified from RN EST assemblies (Wubben et al. 2010), however, none of them have been experimentally studied.

Small regulatory RNAs (sRNAs), ranging from 20 to 24 nucleotides in length, regulate gene expression at the transcriptional, posttranscriptional, and/or translational level (Katiyar-Agarwal and Jin 2010; Ruiz-Ferrer and Voinnet 2009; Shukla, et al. 2008; Sunkar, et al. 2007). Short interfering RNAs (siRNAs) and the micro RNAs (miRNAs) are the best studied classes of sRNAs although there are other emerging classes of sRNA that have not yet been extensively investigated in plant (Axtell 2013).

miRNAs are the best studied class of sRNAs, and they are derived from single stranded RNA transcripts that form hairpin loop structures (Axtell 2013). SiRNAs are derived from double stranded RNA precursors, whose formation is dependent or independent of miRNAs or other siRNAs triggers (Axtell 2013). Both miRNAs and siRNAs are processed from their precursors by DICER-like proteins (DCLs) and the

resulting miRNAs and siRNAs are loaded into Argonaute (AGO) proteins to form an RNA-induced silencing complex (RISC) that can bind to target RNAs or DNAs (Axtell 2013).

Plant miRNAs and siRNAs play important roles in biotic stress responses by regulating genes involved in plant innate immunity (Katiyar-Agarwal and Jin 2010), hormone signaling (Liu and Chen 2009), ROS (reactive oxygen species) generation and signaling (Shukla, et al. 2008), and various other types of signaling (Ruiz-Ferrer and Voinnet 2009).

Current studies suggest that miRNAs and siRNAs play important regulatory roles during nematode pathogenesis in host plants. Genes encoding proteins associated with miRNA or siRNA biogenesis and/or function including DCLs, AGOs, RDRs, and genes encoding DNA methylase proteins, as well as histone methylation and deacetylation-related genes, are regulated in root knot nematode (RKN, *Meloiddogyne incognita*)-induced tomato root galls and RKN-infected rice roots (Ji, et al. 2013; Portillo, et al. 2013). The biogenesis and functioning of miRNAs and siRNAs were also demonstrated to be required in plant S/R to soybean cyst nematodes (SCN, *Heterodera glycines*) (Hewezi, et al. 2008). Responses to SCN were examined in several single, double, and triple mutants of Arabidopsis. The genes examined included genes coding for the DCLs and RDRs, various isoforms of which are involved in the production of specific miRNAs and siRNAs. Mutation in these sRNA-producing genes all displayed decreased SCN susceptibility compared to wild type (Hewezi et al. 2008). In addition, sRNA sequencing studies suggested that, in response to RKN/SCN infestation, miRNAs and siRNAs regulate plant S/R through genes encoding resistance receptor proteins, auxin response

factors, Heat Shock Proteins (HSP), ROS scavenger Cu/Zn superoxide dismutases, and various transcription factors (Hewezi et al. 2008; Li et al. 2012). Specifically, miR396 has been demonstrated to dynamically regulate *GRF* (Growth Regulating Factors) genes to control the initiation and development of syncytia (Hewezi et al. 2012).

The term "isomiRNA" was created by Morin et al. (2008), representing sRNA variants of known miRNAs registered in miRBase. It was proposed that miRNA precursors forming atypical secondary structures, and their inaccurate processing by DCL protein caused the formation of isomiRNAs (Zhang et al. 2013; Morin et al. 2008). While the biological significance of isomiRNAs in plant has not been investigated to date, differential expression of isomiRNAs has been implicated in a variety of human diseases including cancer (Babapoor et al. 2014; Boele et al. 2014; Li et al. 2013), type II diabetes (Baran-Gale et al. 2013), and Huntington's disease (Marti et al. 2010).

Upland cotton, *G. hirsutum* is a classic natural allotetraploid that arose from interspecific hybridization between an A genome-like and a D genome-like ancestral diploid species (Chen et al. 2007). Present day *G. arboreum* (A-genome) and *G. raimondii* (D-genome) are two of the closest relatives of the original tetraploid progenitors (Chen et al., 2007), and in 2012, two groups separately published *G. raimondii* whole genome sequence assemblies (Paterson et al. 2012; Wang et al. 2012). Subsequently, the draft genome of *G. arboreum* became available in April 2014 (Li et al. 2014). Without the complete genome sequences of *G. hirsutum*, the genome sequences of *G. arboreum* and *G. raimondii* combined serve as reasonable models for genome-wide studies of *G. hirsutum*.

At the present time, RN management in upland cotton (*G. hirsutum*) is heavily dependent on nematicide application, which is costly and environmentally unsustainable. Hence, breeding for increased resistance to RN in cotton is an important objective (Robinson 2007). In 2007, two cotton-breeding lines with resistance to RN, LONREN-1 and LONREN-2 were released by United States Department of Agriculture (USDA) (Bell et al. 2014). The RN resistance in LONREN genotypes was transferred from wild diploid species *G. longicalyx*, which is immune to RN (Bell et al. 2014). But in the field test with high levels of RN infestation, mild to severe stunting was reported for the LONREN-1 and LONREN-2 genotypes (Sikkens et al. 2011). Root necrosis and a progressive decrease in root mass, typical in plant hypersensitive responses (HR), were also observed on the two LONREN lines with increased RN inoculum levels (Sikkens et al. 2011). BARBREN-713 was later released by USDA as another RN resistance genotype, based on its good performance in RN resistance and promising agronomic potential without HR (USDA release note). BARBREN-713 was developed by crossing and backcrossing *G. barbadense* (a tetraploid species) accession GB713, a RN resistance germplasm (Guitierrez et al. 2011), with the RKN resistance cultivar Acala Nem-X (USDA release note).

In this study, conserved and lineage-specific miRNAs were identified, and miRNA expression analysis was conducted using root sRNA-seq data obtained from different genotypes of *G. hirsutum* with and without RN infestation. BARBREN-713 was selected as the resistant (R) genotype, LONREN-1 was selected as the hypersensitive (HR) genotype, and another two genotypes, DP90 and SG747, were pooled together and used as RN susceptible (S) genotypes. In addition, isomiRNAs were identified and for the

first time shown to be involved in cotton S/R/HR responses to RN. The aim of this study was to identify the specific responses of miRNA expression from the RN S/R/HR genotypes, and to identify the important regulatory miRNA/isomiRNA-target gene pair candidates.

Results and Discussion

Small RNA sequencing data overview

Six small RNA libraries were generated from cotton roots including two libraries from susceptible genotypes DP90 & SG747 either without RN infestation (DSU) or with RN infestation (DSI), two libraries from the hypersensitive genotype, LONREN-1 either without RN infestation (L1U) or with RN infestation (L1I), and two libraries from resistant genotype BARBREN-713 either without RN infestation (B713U) or with RN infestation (B713I).

Over 124 million raw reads were generated from Illumina deep sequencing (**Table 3.1**). To simplify the sequencing data, all identical sequence reads in each sRNA library were grouped and converted into unique sequence tags with associated read counts of the individual sequence reads. After removing the reads of low quality and masking adaptor sequences, over 25 million sRNA reads ranging from 19 to 25nt in length representing just over 5 million distinct sequences were obtained (**Table 3.1**). Of the clean small RNA sequences after trimming, about 99% were left after excluding sequences matched with non-coding RNAs, including rRNA, tRNA, snRNA, and snoRNA sequences (**Table 3.1**).

The fully sequenced and currently best-assembled genome sequence of *G. arboreum* (Li et al. 2014) and *G. raimondii* (Paterson et al. 2012) were used as references of the *G. hirsutum* A2 and D5 subgenomes, respectively, and the *G. hirsutum* unigenes from CGI11 were used as references for small RNA mapping (Paterson et al., 2012). Over 2.2 million unique sRNA sequences (corresponding to over 12 million reads) exactly matched the genome sequences of *G. raimondii*, *G. arboreum*, or the CGI11 unigene set (**Table 3.1**). Notably, an almost equal percentage of unique sequences (or total reads) exactly matched either the *G. raimondii* or the *G. arboretum* genome sequences (**Fig. 3.1**), suggesting an equal usage of gene homeologs from the A2 and D5 subgenomes in *G. hirsutum* root tissue. Furthermore, the sequences matching the *G. arboreum* and/or *G. raimondii* genomes accounted for about 96% of the total reference-matching sRNA sequences, leaving only 4% of the sRNA sequences uniquely matching the CGI11 unigene set (**Fig. 3.1**). This extends the observation that the majority of protein coding genes are shared between the two diploid subgenomes (Paterson et al., 2012) to the tetraploid to include at least root-expressed sRNA producing genes.

The abundances of sRNA raw sequences, clean sequences, sequences excluding other non-coding RNAs, and reference-matched sequences were also examined for each of the six sRNA libraries and were reported in **Table S3.1**. The data for each individual library in fact correspond proportionately quite well with the summarized total data discussed above and thus do not deserve further comment here.

Of the over 2.2 million sRNA sequences matching the reference genomes (total sRNA library), the most abundant length of sRNAs were the sequences 24nt in length (32.7%), followed by sequences 23nt (14.3%) and 21nt (13.1%) in length (**Fig. 3.2 A**).

This length distribution is consistent with the length distribution of cotton small RNAs found in other studies with other tissues (Liu et al. 2014; Wei et al. 2013; Xue et al. 2013). The total sRNA sequences in each library exhibited similar length distribution as well except for B713I library, which showed a relatively higher abundance of 19-21nt small RNAs and lower abundance of 23-25nt small RNAs (**Fig. 3. 2 B**).

Identification of conserved and lineage-specific miRNAs in cotton roots

The published genome sequences of *G. aoboreum* and *G. raimondii*, as well as the unigenes of *G. hirsutum* in CGI11 were used as the references for miRNA hairpin-like structured precursor identification. As a result, a total of 1,140 potential miRNA precursor loci were identified, including 614 precursor loci for 193 miRNAs from *G. arboreum* genome (**Table 3.2**), 478 precursor loci for 207 miRNAs from *G. raimondii* genome (**Table 3.2**), and 48 precursors for 37 miRNAs from *G. hirsutum* unigenes (**Table 3.2**). Of the 48 unigene precursors, only 10 were unique and not found in either of the genome references used while 7 were identical to sequences found the *G. raimondii* genome but had no apparent homolog in the *G. arboreum* genome. The remaining 31 precursors from the unigene set were found either in the *G. arboreum* genome alone, or in both the reference genomes with less than 1 mismatched base. Thus, of the 1,102 total unique precursors identified 1,092 of these precursors are associated with the best available models of the A2 and D5 genomes, and only 10 appear not to be accounted for by these assemblies at this time.

The 1,102 unique putative precursors that were identified produce 302 putative mature miRNA sequences found in the 6 root sRNA libraries used in this study (**Table 3.2**). Of these 302 miRNAs, 190 (63%) were 22nt in length or less while 37% were 23nt

or 24nt in length (data not shown). The 302 putative mature miRNAs included 88 conserved miRNAs found in miRBase21 and 214 lineage specific miRNAs (ls-miRNAs). Of the conserved miRNAs, 83 (94%) were 22nt in length or less while only 6% were over 22nt in length. Correspondingly, 96% of the mature miRNAs greater than 22nt in length were ls-miRNAs, and thus, only 50% of the ls-miRNAs were in the canonical length classes less than 23nt in length. This likely results from the fact that there are but a few sequences in miRBase greater than 22nt in length, and that many of the ls-miRNAs have non-canonical lengths making it difficult to interpret the significance of these putative miRNAs.

Conserved miRNAs in cotton roots

Many conserved miRNAs were predicted to have multiple precursor loci (**Table S3.2**). Notably, miR2949a, miR479a, and miR482e precursors were only identified from the unigenes of *G. hirsutum* in CGI11 (**Table S3.2**). MiR2949 is registered as a *G. hirsutum*-specific miRNA in miRBase 21, and miR479 is conserved across several plant species but was only registered as a tetraploid cotton miRNA currently. Thus, in addition to suggesting the miRNA sequence conservation between *G. hirsutum* and its diploid progenitors, our data indicate that new miRNA loci or different species of miRNAs evolved during the evolution of the *G. hirsutum* genome. However, the complete genome sequences of *G. hirsutum* are required to further demonstrate such observation.

88 unique sRNA sequences belong to 54 families representing 163,727 reads were identified as miRNAs that show sequence homology to Viridiplantae mature miRNAs registered in miRBase 21 (http://www.mirbase.org/). This number of mature miRNAs is

much smaller than the number of *Gossypium* mature miRNAs deposited in miRBase 21 (378). Most of those unidentified miRBase *Gossypium* miRNAs were *Gossypium*-specific, and were initially identified from *G. hirsutum* fibers (Xue et al. 2013) or ovules (Wang et al. 2012). Thus, these data reflected a root-specific expression pattern of miRNAs.

Among the identified miRNAs, 42 are conserved across at least two genera of Viridiplantae (classified as class I in **Table S3.3**). 18 have been identified in other genera of Viridiplantae but have not been reported for *Gossypium* (class II in **Table S3.3**), and the rest 29 classes III miRNAs are *Gossypium*-specific. Most of the class I and class II miRNAs had higher normalized expression values compared to class III miRNAs (**Table S3.3**), suggesting their essential roles in a variety of plant species. miRNA 159, 166, and 396 were the three most abundantly expressed families that are highly conserved across vascular plant species (Cuperus et al. 2011). Other class I highly-conserved miRNAs, including miR162, miR482, and miR167, and other class II miRNAs including miR1448, miR3954, and miR319, as well as the class III *Gossypium*-specific miR3476, miR2949, miR2947, and miR7495 also exhibited total abundance of more than 1000 RPM. In contrast, most of the low abundance miRNAs were *Gossypium*-specific (**Table S3.3**).

Lineage-specific miRNAs in cotton roots

Along with the identification of conserved miRNAs, lineage-specific miRNAs (ls-miRNAs) were also identified in this study. Ls-miRNAs are defined as those miRNAs derived from identifiable eligible miRNA precursor(s) that have more than two mismatches to mature miRNAs registered in miRBase 21 (see “material and methods”

section). In total, 214 unique small RNA sequences were identified as ls-miRNAs, including 129 ls-miRNAs from 470 loci mapped to *G. arboreum* genome, 136 from 313 loci mapped to *G. raimondii* genome, and 14 from 17 loci mapped to the CGI11 *G. hirsutum* unigene set (**Table 3.2; Table S3.2**).

The identified mature ls-miRNAs sequences ranged from 20-24nt in length, with 102 sequences being 24nt in length while 82 sequences were 21nt in length. Sequences 20, 22, and 23nt in length were significantly lower in abundance (**Table 3.3**) which is consistent with other similar observations (Xue et al. 2013). As was previously shown, most of the miRNAs 21nt in length (48/82) had 5' terminal uridine nucleotides (Xue et al. 2013), whereas a majority of the miRNAs 24nt in length (84/102) had 5' terminal adenine nucleotides (**Table 3.3**). Previous studies have shown that AGO1 usually harbors 21 and 22nt miRNAs with a 5' terminal uridine directing the cleavage of target mRNAs at post-transcriptional level (Bologna and Voinnet 2014), while AGO4 preferentially recruits 24nt small RNAs with a 5' terminal adenine directing the methylation of target DNA loci (Bologna and Voinnet 2014). Thus, the different features of the length classes of the ls-miRNAs predicted in this study might be correlated with their different biological functions in cotton roots.

Although the majority of the ls-miRNAs are of low abundance, there are 6 miRNAs (ls-miR159, ls-miR172, ls-miR43, ls-miR174, ls-miR51, and ls-miR171) whose total normalized expression values exceed 500 RPM (**Table S3.4**), suggesting their important regulatory roles in cotton root tissues. Moreover, when comparing to miRNAs identified from other tissues of *G. hirsutum*, including leaf, square, boll, and flower, all of

these ls-miRNAs are preferentially expressed in the root tissue (Hu, Ph.D Dissertaion, 2013).

In summary, miRNAs conserved in Viridiplantae registered in miRBase 21 were identified. Many *Gossypium* fiber- or ovule-specific miRNAs were not identified probably due to their low expression values in the root tissue. In addition, 214 lineage-specific miRNAs were identified from these data for the first time.

Potential important miRNAs in cotton S/R/HR to RN

Differential miRNA expression profile between genotypes

To identify miRNAs, which can affect the genotypic variance between the susceptible control genotypes DP90 and SG747 (DS), the hypersensitive genotype LONREN-1 (L1), and the resistant genotype BARBREN-713 (B713), miRNA expression was compared between the three genotypes without RN infestation. Two pairs of expression analysis were conducted: L1U vs. DSU, and B713U vs. DSU.

As a result, 23 conserved miRNAs and 15 ls-miRNAs were statistically differentially expressed in at least one of the above two genotypic comparisons (**Fig. 3.3**). Of these differentially expressed miRNAs, 21 were differentially expressed in L1U compared to DSU, including 3 miRNAs expressed in the L1U library at least 1.5 fold higher than in the DSU library, and 18 miRNAs expressed in the DSU library at least 1.5 fold higher than in the L1U library (**Fig. 3.3**). 23 miRNAs were differentially expressed in B713U compared to DSU, including 15 miRNAs expressed in the B713U library at least 1.5 fold higher than in the DSU library, and 8 miRNAs expressed in the DSU library at least 1.5 fold higher than in the B713U library (**Fig 3.3**). It is known that LONREN-1 exhibited HR-type responses under high levels of RN infestation (Sikkens et al. 2011);

BARBREN-713 showed good RN resistance without yield penalty. Thus, those miRNAs that were distinctly regulated in L1U compared to DSU could be involved in HR response, and miRNAs that showed up- or down- regulation in B713U library compared to DSU library could act as positive or negative regulators in BARBABREN-713-specific resistance. Among these differentially expressed miRNAs, miR166a, miR167a, miR403a, miR482b, and ls-miR171 were all expressed at least 1.5 fold higher in L1U and B713U libraries compared to DSU library (**Fig 3.3**), suggesting that they are negative regulators controlling RN basic resistance in cotton roots. Nevertheless, miR7495c accumulated to a greater extent in DSU than in L1U, but accumulated to a lesser extent B713U than in DSU (**Fig 3.3**), suggesting that miR7495c is correlated with the different levels of RN tolerance between LONREN-1 and BARBREN-713 (Sikkens et al. 2011).

RN-responsive miRNAs

To elucidate the potential regulatory roles of cotton miRNAs in responses to RN infestation, the expression profiles of miRNA were also investigated between the uninfested and infested sRNA libraries for each genotype. Three pairs of expression analysis were conducted: DSI vs. DSU, L1I vs. L1U, and B713I vs. B713U. 42 miRNAs were identified as RN-responsive miRNAs, which showed statistical differential expression in at least one of the above comparisons. Most miRNAs that were differentially expressed between genotypes were RN-responsive, except for miR1448a, miR166d, miR171b, miR398b, ls-miR123, ls-miR163, and ls-miR171 (**Fig. 3.3; Fig. 3.4**). In addition, 11 miRNAs that were not differentially expressed between genotypes

were detected to be RN-responsive, including miR156a, miR156b, miR164a, miR164b, miR2118a, miR3476a, miR396d, miR482a, miR482d, miR482e, and ls-miR78.

Among these 42 RN-responsive miRNAs, all 17 RN-responsive miRNAs identified from LONREN-1 were induced; equal numbers of miRNAs were induced (9) or repressed (9) in the susceptible DS genotype, and most of (24 out of 26) the RN-responsive miRNAs identified from BARBREN-713 were repressed (**Fig. 3.4**). These RN-responsive miRNAs were classified into eleven groups (group I, II, III, IV, V, VI, VII, VIII, IX, X, XI), each demonstrating different miRNA accumulation profiles in response to RN infestation (**Fig. 3.4**).

Group I consists of 15 miRNAs (miR2949a, miR8011a, miR482a, miR482e, miR403a, miR396c, ls-miR212, ls-miR174, ls-miR96, ls-miR175, ls-miR95, ls-miR194, ls-miR94, ls-miR53, and ls-miR78), whose accumulation was only statistically repressed in B713I compared to B713U (**Fig. 3.4**). This expression profile suggests that these 15 miRNAs are negative regulators in RN resistance that need to be suppressed in response to RN to facilitate RN resistance in BARBREN-713.

MiR396b and miR3954a made up group II, whose accumulation was repressed in both DSI and B713I compared to their uninfested counterparts. The accumulation of Group III miRNAs (miR7495c, miR396a, and miR156b) was only reduced in DSI compared to DSU. While the reduction of group II miRNAs may contribute plant basic resistance to RN in both DSI and B713I, group III miRNAs are probably negative regulators in RN susceptibility.

MiR7495a and miR827a belong to group IV, and their abundance was induced in L1I compared to L1U but repressed in DSI compared to DSU (**Fig. 3.4**). The increased

accumulation of these two miRNAs may lead to the resistance and/or HR responses observed in LONREN-1, whereas their decreased abundance in DSI compared to DSU may support RN successful parasitism on cotton roots.

Group V includes four miRNAs, miR159a, miR3476a, miR482c and miR482b that were induced in L1I compared to L1U, repressed in B713I compared to B713U, and remained unchanged in DSI compared to DSU (**Fig. 3.4**). The different expression patterns of these four miRNAs in L1I vs. L1U and B713I vs. B713U suggested that they play a role in the RN HR responses. Similarly, the increased abundance of group VI miRNAs only in L1I vs. L1U suggested that miR166b, miR166a, miR164b, and ls-miR172 are involved in the HR responses observed in LONREN-1.

The accumulation of the three group-VII miRNAs (miR164a, miR482d, and miR156a) only in DSI suggested that they are positive regulators in RN susceptibility, whose increased abundance is necessary for RN susceptibility and stable expression is required for RN resistance in LONREN-1 and BARBREN-713.

Group VIII consists of four miRNAs (miR396d, ls-miR159, miR166c, and miR2118a), whose accumulation was increased in both L1I and DSI but remained unchanged in B713I compared to their uninfested counterparts (**Fig. 3.4**). Combined with the phenotypic difference between LONREN-1 and the susceptible genotypes, these data indicated that the group VIII miRNAs are positive regulators in RN susceptibility but are also involved in HR responses in LONREN-1. Ls-miR51 is the only miRNA in group IX, whose up regulation in DSI vs. DSU and down regulation in B713I vs. B713U (**Fig. 3.4**) suggested its negative role in RN resistance.

Three miRNAs were RN-responsive in all three comparisons examined, including miR162a, ls-miR43, and miR167a, which form group X (**Fig. 3.4**). Specifically, the expression pattern of ls-miR43 implied its involvement in RN HR responses, and the expression pattern of miR167a indicated its positive role in RN resistance in both LONREN-1 and BARBREN-713. Group XI miRNA (miR319a) was only statistically induced in B713I compared to B713U, whose accumulation may be required for RN resistance. These studies indicate that there are a number of possible roles for miRNAs in mediating RN resistance, hypersensitivity, and susceptibility responses in cotton roots.

Combined analysis of the miRNA expression profile between genotypes and in response to RN infestation

By combining the miRNAs' expression data between genotypes and in response to RN infestation, the potential role of all the differentially expressed miRNAs were summarized in **Table 3.4.**

In general, if the same role was concluded for a specific miRNA from the comparisons between genotypes and in response to RN infestation, that particular role was determined to be the potential function of the miRNA in cotton S/R/HR to RN. In this way, the potential functions of miR159a, miR166b, miR166c, miR3954a, miR403a, miR482c, miR7495a, miR827a, ls-miR172, ls-miR194, ls-miR43, and ls-miR51 were determined (**denoted as class I in Table 3.4**). For miRNAs that were only detected to be differentially expressed between genotypes or in response to RN infestation, the only role that was concluded was determined to the potential function of the miRNA in cotton S/R/HR to RN. Thus, the potential functions of miR1448a, miR166d, miR171b, miR398b,

ls-miR123, ls-miR163, and ls-miR171 were determined based on their expression data between genotypes, whereas the functions of ls-miR78, miR156a, miR156b, miR164a, miR164b, miR2118a, miR3476a, miR396d, miR482a, miR482d, and miR482e were determined based on their expression data in response to RN infestation (**class II in Table 3.4**).

Some differentially expressed miRNAs appear to have distinct roles based on their expression data between genotypes and in response to RN infestation, the potential functions of these miRNAs were determined based on their combined expression data. For instance, ls-miR159 was suggested to be a positive regulator in RN resistance based on its expression data between genotypes. However, based on its expression data in response to RN infestation, ls-miR159 was suggested to be a positive regulator in RN HR responses and susceptibility. Thus, the finalized potential function of ls-miR159 was determined to be a positive regulator in cotton basic/non-race specific resistance to RN defining this ls-miRNA as a class III miRNAs (**Table 3.4**). In addition, a number of miRNAs that were distinctly regulated in LONREN-1 or BARBABREN-713 may play a role plant "HR" or "R" responses to RN (**table 3.4**), but it cannot be determined whether they have a genotype-specific positive or negative role.

IsomiRNAs could play an important role in cotton S/R/HR to RN infestation

isomiRNAs identified from cotton roots

During the identification of miRNAs, it was noticed that multiple sRNA variants mapped to the same miRNA loci as the reference mature miRNA sequences. These small RNA variants were referred as isomiRNAs and the isomiRNAs for conserved miRNAs

identified in this study were listed in **Table S3.5.** Compared to their corresponding cognate miRNA sequences, these isomiRNA sequences varied at either or both ends and can be classified into three categories: 1) 3'-end variants exhibiting different nucleotide only at 3'-end compared to their corresponding reference miRNA sequences; 2) 5'-end variants exhibiting different nucleotide only at 5'-end compared to their corresponding reference miRNA sequences; 3) 3'-and 5'- variants exhibiting different nucleotide on both ends compared to their corresponding reference miRNA sequences.

In total, 454 isomiRNA sequences were identified for all conserved miRNAs, except for miR166d, miR171c, miR171d, miR398b, miR477d, miR7495a, miR7504b, and miR7505a. Of these 454 isomiRNAs, variants with different nucleotides at both 3' and 5' ends compared to their corresponding cognate miRNA sequences were the most frequent (215), followed by 3' variants (161), and isomiRNAs categorized as 5' variants were the least frequent (78) (**Fig. 3.5 A**). It was found that 3' variants had a much higher average abundance compared to the other two types of isomiRNAs (**Fig. 3.5 B**). Following the same method, 1,903 isomiRNAs were identified for ls-miRNAs classified in this study, showing the similar kind of frequency and abundance distributions.

Differential expression of isomiRNAs and their potential roles in cotton S/R/HR to RN

The expression of isomiRNAs between genotypes was also examined following the same method for miRNAs expression analysis. Based on the statistical analysis, 24 isomiRNAs were differentially expressed between the three genotypes (**Table S3.6**). 17 out of the 24 isomiRNAs were variants of differentially expressed miRNAs (**Table S3.6**). Nine had the same expression patterns as their corresponding cognate miRNA sequences,

and 8 had different expression patterns compared to their corresponding cognate miRNA sequences (**Table S3.6**). Of the 24 differentially expressed isomiRNAs, 15 were 3' & 5' variants compared to their corresponding reference miRNAs, 8 were 3' variants, and only 1 was 5' variant (**Table S3.6**).

When analyzing the expression profile of the isomiRNAs in response to RN infestation, 28 isomiRNAs were shown to be RN responsive (**Table S3.7**). Twenty-four out of the 28 RN-responsive isomiRNAs were variants of RN-responsive miRNAs, with 9 having the same expression patterns from their corresponding cognate miRNA sequences, and 15 having different expression patterns from their corresponding cognate miRNA sequences (**Table S3.7**). In addition, of these 28 RN-responsive isomiRNAs, 18 were 3' variants compared to their corresponding reference miRNAs, 10 were 3' & 5' variants, and no 5' variants were detected (**Table S3.7**).

Following the same method described for differentially expressed miRNAs, the expression results for all the differentially expressed isomiRNAs were combined, and their potential roles in cotton S/R/HR to RN were investigated (**Table 3.5**).

The only genome-wide survey of isomiRNA in plant was conducted by Zhang et al. (2012) in larch. They indicated that the nucleotide(s) trimming from and/or addition to miRNA sequences 5' and/or 3' end are associated the degradation of miRNAs, and miRNAs are more intended to be degraded from the 3'-end than from 5'-end (Zhang et al. 2012). Their proposal partially explained the higher frequency and abundance of the 3'-variants identified from this study if isomiRNAs are degradation products of their mature cognate miRNAs. However, the high accumulation level of some isomiRNAs

identified in this study is consistent with a biologically significant role for these isomiRNAs.

The sequence characterization of isomiRNAs identified from here suggested that consistent with the findings of isomiRNAs in mammalian cells (Pantano et al. 2010; McGahon et al. 2013), modification in the 3' end of the cotton mature miRNA is more frequent. According to the differential expression analysis, several highly expressed isomiRNAs exhibited genotype- and/or treatment- specific expression pattern. In addition, several isomiRNAs identified from this study accumulated more abundantly than their corresponding cognate miRNAs in a specific library. Taken together, these data further allow the inference that one form of miRNA sequence is more preferentially selected in a specific genotype or a under specific condition, in this case, with or without RN infestation.

When combining all the significantly regulated isomiRNAs identified from this study, it was found that less than half of them (17 out of 37) were 3' and 5' variants, more than half (19 out of 37) were 3' variants, and only 1 was 5' variants (**Table 3.5**). In addition, the majority of these differentially expressed isomiRNAs (27 out of 37) exhibited a 5'-terminal uridine (**Table 3.5**). These differentially expressed isomiRNAs are of high abundance and might regulate target gene expression in a similar mechanism as their reference miRNAs, because 5'-terminal uridine is crucial for a miRNA sequence to be recognized for post-transcriptional regulation (Bologna and Voinnet 2014).

Combined analysis of RN S/R/HR-associated miRNAs/isomiRNAs and their target genes

To characterize the specific function of miRNAs in cotton S/R/HR to RN, targets of differentially expressed miRNAs from self-assembled contigs were predicted. By integrating the expression profile of miRNAs identified from this study, and the expression profile of contigs identified from transcriptome study (see Chapter 2), the expression levels of 181 miRNA-target pairs were negatively correlated (**Table S3.8**). When GO categories were assigned to these targets, "stress" and "regulation of transcription" were the top categories with the most abundant target genes that were categorized. In addition, genes involved in "redox reactions", "cell wall metabolism", "secondary metabolism", and "hormone metabolism" were also identified among these targets (**Fig 3.6**).

In addition to miRNAs, the negatively regulated target genes for significantly regulated isomiRNAs were also identified from self-assembled cotton root tissue contigs. As a result, 81 isomiRNA-target genes pairs were identified (**Table S3.8**). Similar to miRNAs, "transcriptional regulation" and "stress" are the two predominant GO categories for targets of the differentially expressed isomiRNAs (**Fig. 3.6**).

When examining the predicted target genes for miRNAs, it was found that several pathways are co-regulated by miRNAs involving in HR responses and miRNAs serving as negative regulators in RN resistance (**Fig. 3.7 A**). Among these co-regulated genes, plant cytochrome P450 enzymes are required for the biosynthesis of many secondary metabolites including gibberellins, abscisic acid, and plant defense substances (Zhao et al. 2014). *ACO* gene catalyzes the last step of ethylene (ET) biosynthesis (Wang et al. 2002). ET overproducing reduced RKN resistance (Fudali et al. 2013) but demonstrated hyper-susceptibility to CN in host plants (Wubben et al. 2001). *RLK*s and *NB-ARC*s co-

targeted by multiple miRNAs are plant resistance (R) proteins that can sense invading pathogens and trigger plant innate immunities and various downstream defense responses (Jones and Dangl 2006). Moreover, HR-type responses characterized as localized programmed cell death are typically observed in these *R*-genes-mediated plant immunity responses (Jones and Dangl 2006). The last type of genes annotated as ubiquitins are key for the regulation of many processes such as the ROS, hormone signaling, and programmed cell death (Trujillo and Shirasu 2010). These data suggested that the accumulation of the above five types of genes are required for plant resistance to RN, it also suggested that the expression levels of these genes are critical in mediating plant HR responses. In concert, large increases in plant *R*-gene transcript and protein levels were proposed to trigger cell death and plant HR (Qiao et al. 2013). Here, through negatively controlling the accumulation of critical genes in root tissues, miRNAs were suggested to serve an essential regulatory node in determining cotton S/R/HR to RN.

Similarly, four types of genes were identified to be co-regulated by isomiRNAs with distinct regulatory roles in plant responses to RN (**Fig. 3.7 B**). Among them, SANT domain-containing and *bHLH* gene were predicted to be required in plant resistance to RN. The accumulation level of SANT domain-containing and thaumatin-like genes was also suggested to be critical for cotton HR responses to RN. Thaumatin-like genes may be a negative regulator in mediating RN resistance, and *bHLH* may play a positive role in plant HR to RN, and *HSP40* (Chaperon DnaJ) gene expression level was suggested to be important in determining plant resistance to RN. Notably, two isomiRNAs of miR2949 shared the same types of target genes although they were concluded to play different

roles in plant responses to RN (**Fig. 3.7 B**). This kind of regulatory network was also identified for a pair of isomiRNAs of miR159 (**Fig. 3.7 B**).

When comparing the targets of differentially expressed isomiRNAs with their corresponding reference miRNAs, it was found that, some of the isomiRNAs share target genes with their corresponding cognate miRNAs, whereas several isomiRNAs gained new targets or were predicted to negatively regulate distinct targets (**Table 3.5**).

High sequence complementarity between miRNAs and their targets is a prerequisite for strong posttranscriptional silencing. IsomiRNAs, varying in size and sequence compared to their cognate miRNAs may be loaded into different AGO proteins and regulate alternative targets, thus resulting in diverse regulatory outcomes (Li et al. 2014). Such regulatory mechanisms have been characterized in human embryonic stem cells, where isomiRNAs have distinct targets compared to their corresponding reference miRNAs (Hinton et al. 2014). Nevertheless, isomiRNAs with overlapping targets compared to their reference canonical miRNAs may improve the sensitivity of the miRNA-target networks in cotton S/R/HR to RN; isomiRNAs regulating distinct targets/pathways compared to their cognate miRNAs may fine tune critical gene expression in cotton root tissues for specific responses to RN infestation (Cloonan et al. 2011).

Conclusion

In the present study, small RNA-seq was used to identify conserved miRNAs and lineage-specific miRNAs and their global expression patterns in cotton susceptibility, hypersensitivity, and resistance to reniform nematodes were examined. A total of 1,140

potential precursors for root-specific miRNAs were identified in the model A- and D-genomes of *G. hirsutum*. Six hundred fourteen hairpin precursors were predicted that produce 88 miRNAs previously reported in some plant (miRBase 21) while 478 predicted precursors were produce 214 novel miRNA species having more than 2nt mismatches with known miRNAs (defined here as novel lineage specific miRNAs). It can be concluded that the majority of these predictions are credible miRNA candidates since they are both expressed at significant levels in at least 1 sRNA library, and many of them demonstrate differential expression levels in response to RN infestation or between genotypes.

Additionally, a total 2,357 length variants of the miRNAs (isomiRNAs) were also uncovered. While many of these have low expression values making their roles relevant difficult to conclude, there are at least some of these that demonstrate significant expression and apparent biological relevance in the context of RN responses. Such observations have recently been made in model systems, and thus, this study lends support to the notion that isomiRNAs can play important biological functions in addition to their cognate miRNAs.

The data presented indicate that miRNAs control plant S/R/HR to RN through a wide range of biological pathways, including “stress-related”, “transcription regulation”, “redox-related”, “cell wall synthesis”, “hormone metabolism”, “calcium signaling”, and “secondary metabolism” pathways. By correlating the expression profile of significantly regulated miRNAs with their negatively regulated targets, several miRNA-target gene pairs that could be considered in future functional analysis to further examine their putative roles in RN susceptibility, hypersensitivity, and resistance were uncovered. This,

in conjunction with complete transcriptome analysis, provides a basis for the development of sRNA regulatory networks involved in defining RN resistance, susceptibility, and hypersensitivity in cotton roots.

Materials and methods

Plant material and stress treatment

Four genotypes of cotton were selected: DP90, SG747, LONREN-1, and BARBAREN-713. Cotton seedlings were infested with reniform nematode two weeks after planting and root samples were collected at 0, 1, 3, 5, and 10 days post infestation. Samples at 0 day were taken as uninfested controls, while samples from 1, 3, 5, and 10 days post infestation were pooled and considered the reniform nematode infested samples.

Small RNA library construction

Total RNA was extracted from uninfested and infested root samples using hot borate method (Wan and Wilkins, 1994). Root samples from DP90 and SG747 were combined evenly for RNA extraction. Total RNA prepared as above was used to prepare sRNA libraries for sequencing on the Illumina platform according to manufacturer's protocol at the Hudson Alpha Institute, Huntsville, AL. Six sRNA libraries were constructed. They are a reniform nematode uninfested sRNA library derived from DP90 and SG747 genotypes (DSU), a reniform nematode infested sRNA library derived from infested DP90 and SG747 genotypes (DSI), a reniform nematode uninfested sRNA library derived from the LONREN-1 genotype (L1U), a reniform nematode infested sRNA library derived from the LONREN-1 genotype (L1I), a reniform nematode uninfested sRNA library derived from the BARBREN-713 genotype (B713U), and a

reniform nematode infested sRNA library derived from the BARBREN-713 genotype (B713I).

Preprocessing of small RNA sequencing data

All the sequencing data were initially processed by removing the 3'-adaptor sequence using CLC genomic Workbench 5.8 (CLC bio, Aarhus, Denmark). Any sequences without adaptor matches were excluded from further analyses. Subsequently, sequences between 19nt and 25nt with quality score greater than 0.05 were mapped to the rRNA, tRNA, snRNA, and snoRNA deposited in NCBI (http://www.ncbi.nlm.nih.gov) and Rfam database (http://www.sanger.ac.uk/software/Rfam) using the Bowtie 1.0.0 sequence aligner (Langmead et al. 2010) to remove the non-coding RNAs. After excluding the non-coding RNAs, the retained small RNA sequences were mapped to the genome of *G. raimondii* version 2 (G.raimondii_v2) (Paterson et al. 2012), the genome of *G. arboreum* (Li et al. 2014), and the EST sequences of Cotton Gene Index 11 (descried as *G. hirsutum* unigenes) using Bowtie. Only sequences with exact matches were retained for further analyses.

Identification of conserved, lineage-specific microRNAs, and isomiRNAs

Potential miRNA precursors were determined using mireap (http://sourceforge.net/projects/mireap), with the maximal distance between putative miRNA and miRNA* of 450nt, the maximal free energy of -30 kal mol-1, the maximal copy number of miRNAs on reference of 500, and default values for other parameters. After this analysis, the remaining putative miRNA were filtered by two additional features of plant miRNAs: single strand bias (≥0.9, the total reads of sRNAs from the sense strand of miRNA precursor divided by the total reads of sRNA from both strands of

miRNA precursors) and abundance bias (≥0.6, the total reads of the most abundant three sRNAs derived from putative miRNA loci divided by the total reads of sRNAs matching the miRNA precursors) (zhai et al. 2011; Jeong et al. 2011). Lastly, CentroidFold (Sato et al. 2009) was used to check the remaining putative miRNAs' secondary structures. MiRNAs passing the above criteria with two or fewer mismatches to mature miRNAs in miRBase 21 (http://www.mirbase.org) were annotated as conserved miRNAs, and the remainders were annotated as lineage specific miRNAs (ls-miRNAs). Sequences derived from miRNA loci that failed to be annotated as miRNAs were considered as miRNA variants or isomiRNAs.

Expression analysis and target prediction of miRNAs and isomiRNAs

To investigate the differentially expressed miRNAs and isomiRNAs between libraries, the abundance of "reference-matched sRNAs" (see "preprocessing of small RNA sequencing data") in a given library was used for the normalization of read abundance, which was denoted as RPM (reads per million reads). Expression analysis and Kal's test were conducted in CLC genomic Workbench (version 5.5.1). A specific miRNA or isomiRNA was considered to be differentially expressed if the FDR corrected P-value given by the above analysis was smaller than 0.05, and the fold change value in RPM normalized sequence counts was more than 1.5.

Target of differentially expressed miRNA and isomiRNA was predicted using psRNATarget (Dai et al. 2011), with the self-assembled cotton root contig sequences as reference targets.

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Table 3.1 Summary statistics of small RNAs sequenced from roots

	Total	Unique
Raw sequencing reads	124,333,052	
Sequences after adaptor, low quality, & length trimming	25,769,461	5,017,902
After excluding tRNA, rRNA, snRNA, and snoRNAs	17,825,234	4,946,108
	69%	99%
Reference matched	12,316,972	2,277,861
	100%	100%
G. arboreum	10,832,121	1,636,536
	88%	72%
G. raimondii	10,739,431	1,507,087
	87%	66%
***G. hirsutum* unigene**	8,987,336	766,556
	73%	34%

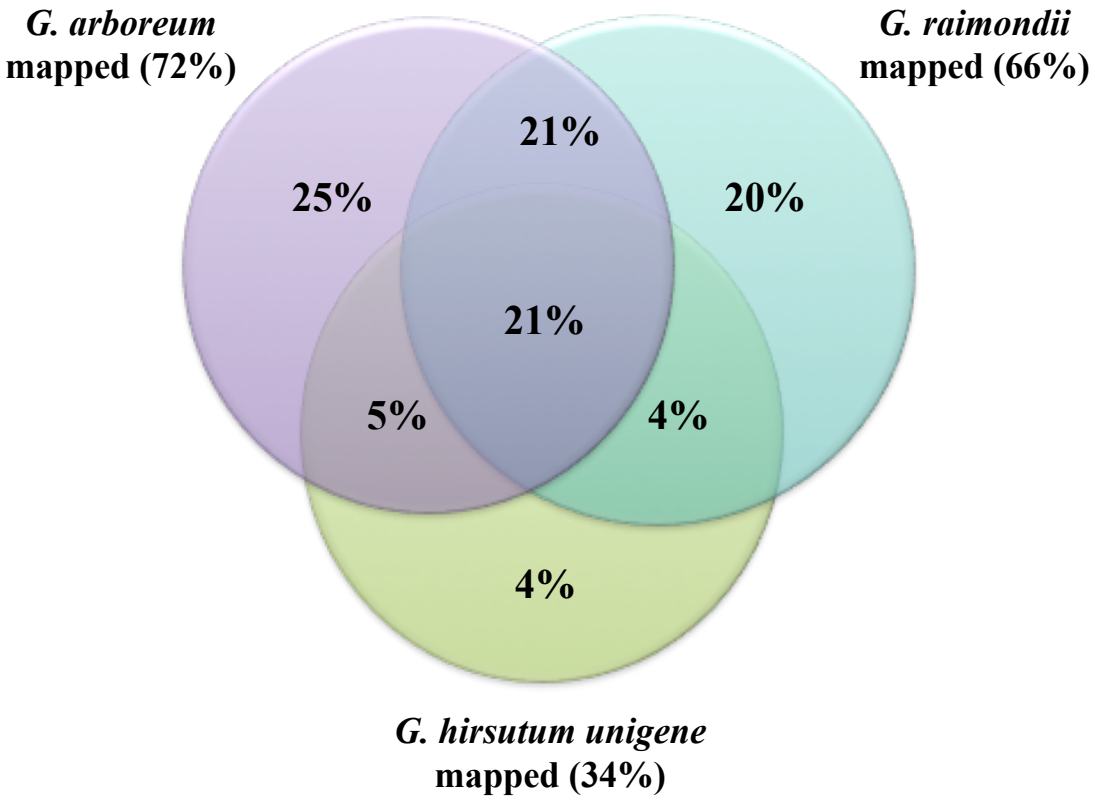

Figure 3.1 Venn diagram showing the percentage of sRNAs mapped to different references

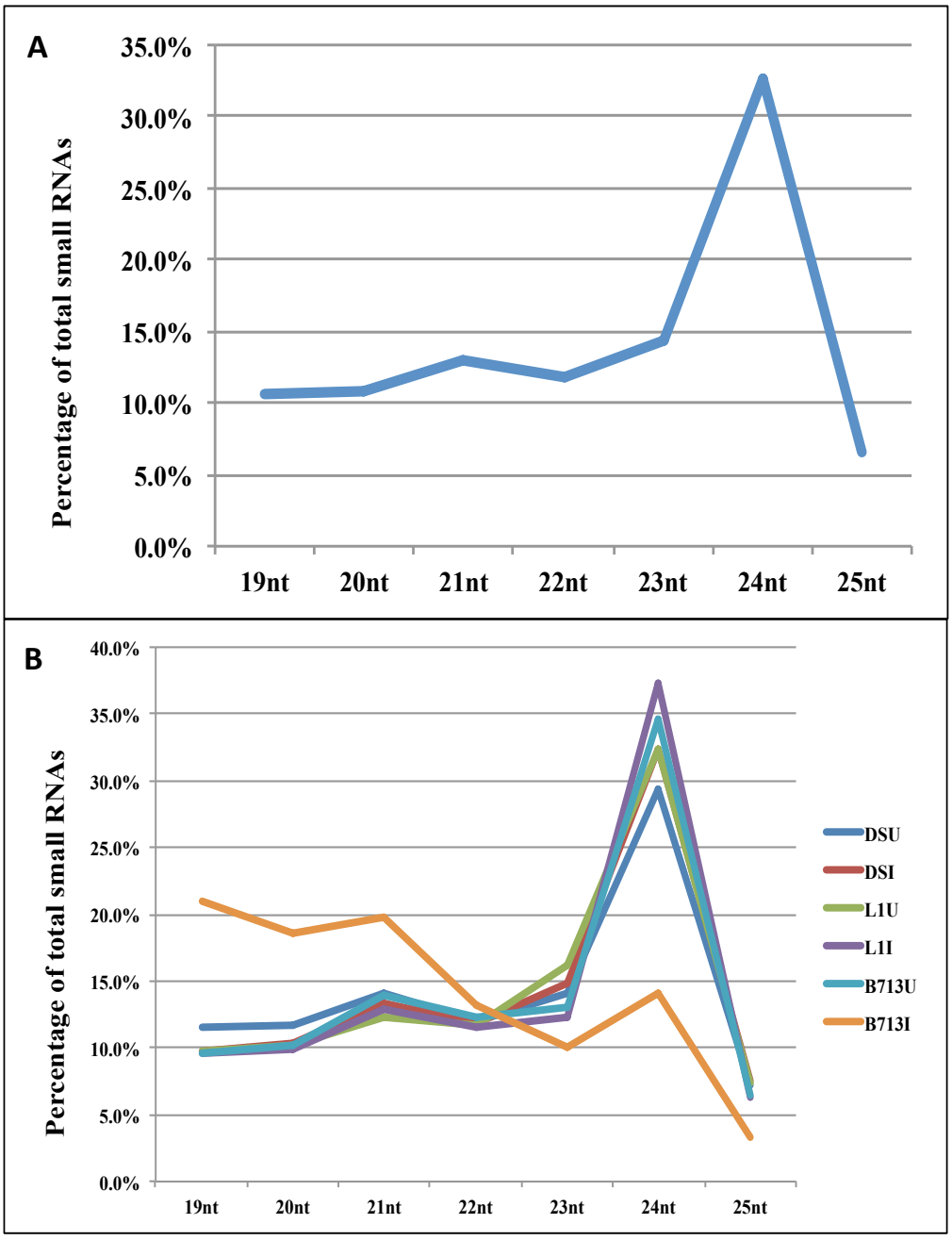

Figure 3.2 Length distributions of sRNA sequences matching the reference genomes

A: combined total sRNA libraries; **B:** total sRNAs in each of he six libraries separately.

Table 3.2 Number of miRNAs and precursors identified from different references

	Total	*G. arboreum*	*G. raimondii*	*G. hirsutum* unigene
Total miRNA	302	193	207	37
Precursor	1,140	614	478	48
Average precursors per miRNA	3.77	3.18	2.31	1.3
Conserved miRNA	88	64	71	23
Precursor	340	114	165	31
Average precursors per miRNA	3.86	2.25	2.32	1.35
Lineage-specific miRNA	214	129	136	14
Precursor	800	470	313	17
Average precursors per miRNA	3.74	3.64	2.3	1.21

Table 3.3 The 5' terminal nucleotide and lengths of the 214 lineage-specific miRNAs

5’ terminal	**miRNA length (nt)**					
	20	**21**	**22**	**23**	**24**	**Total**
A	1	19	2	3	84	109
U	4	48	8	1	8	69
C	1	15	8	0	4	28
G	1	0	0	1	6	8
Total	7	82	18	5	102	214

ID	L1U/DSU	B713U/DSU
miR319_a	1.5	-1.0
miR162_a	2.2	-1.2
ls_miR_51	1.9	-1.3
miR1448_a	-1.1	-2.1
miR396_a	-1.1	-2.1
ls_miR_194	-1.3	-2.2
miR7495_a	-6.0	1.1
miR3954_a	-5.0	-1.3
miR171_b	-4.6	-1.1
miR827_a	-4.4	-1.2
miR166_c	-2.0	-1.1
miR159_a	-2.0	1.3
miR166_b	-1.9	1.0
miR482_c	-1.8	-1.2
miR396_b	-1.8	1.1
miR2949_a	-1.7	-1.1
ls_miR_172	-4.6	-1.3
ls_miR_43	-2.8	-1.2
ls_miR_171	-6.1	-2.1
miR482_b	-2.7	-1.7
miR403_a	-2.2	-1.6
miR167_a	-1.6	-2.4
miR166_a	-1.5	-1.8
miR7495_c	-54.8	1.6
miR166_d	-2.8	12.2
miR398_b	1.3	3.6
miR8011_a	1.7	2.1
miR396_c	2.9	5.9
ls_miR_212	-11.6	14.7
ls_miR_159	1.5	7.8
ls_miR_174	-1.2	3.9
ls_miR_175	1.3	5.3
ls_miR_53	1.3	2.3
ls_miR_95	3.6	6.3
ls_miR_163	1.6	2.2
ls_miR_123	1.3	7.7
ls_miR_94	1.4	4.0
ls_miR_96	2.0	3.2

Figure 3.3 Heatmap showing the differentially expressed miRNAs between genotypes

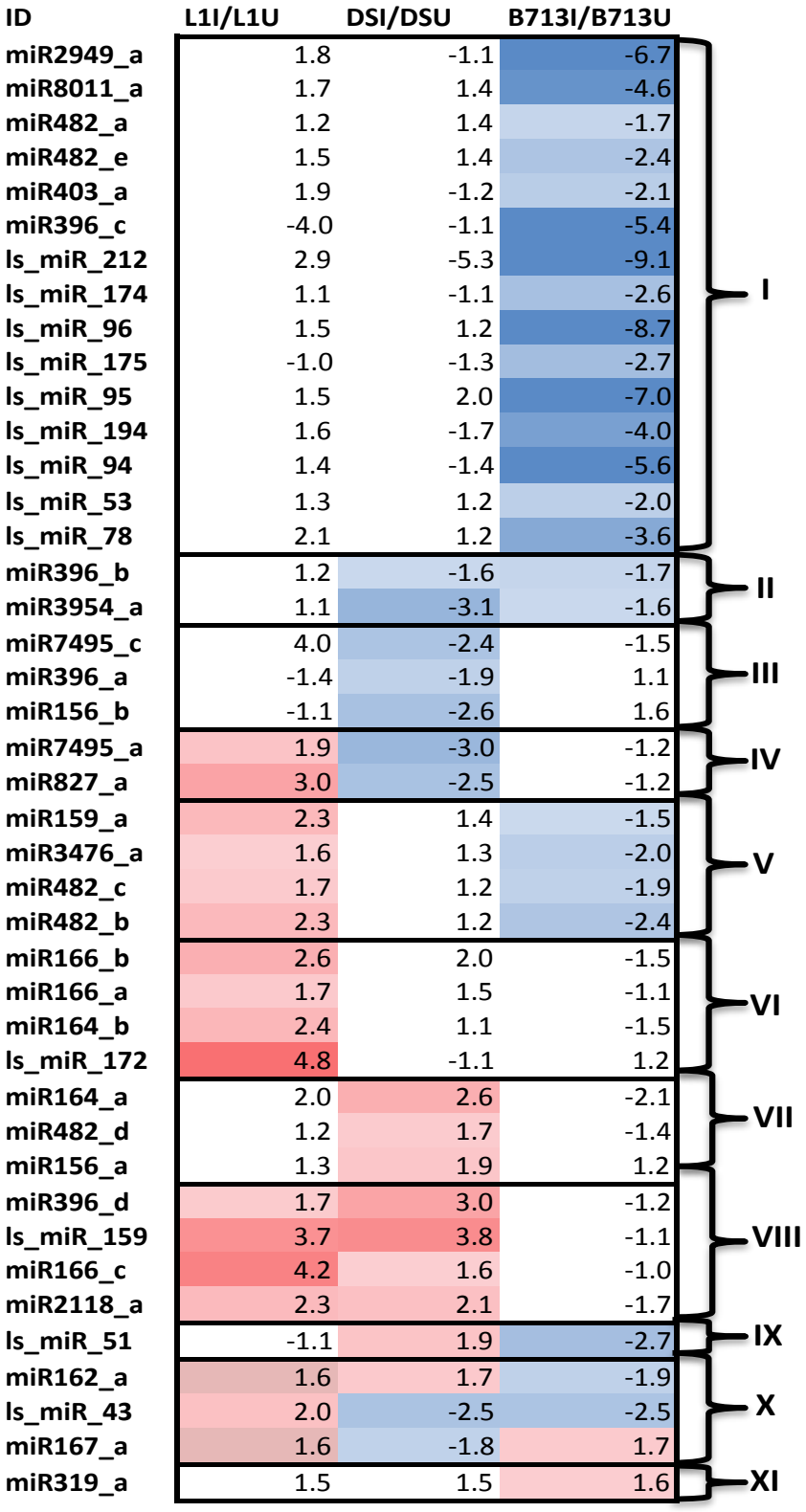

Figure 3.3 Heatmap showing the RN-responsive miRNAs

Table 3.4 Cotton S/R/HR-related miRNAs and their predicted targets

miRNA	Function	Class	Targets
miR827 a	HR	I	ACO; cytochrome P450; Pectin-lyase-like
miR159 a	HR	I	AX efflux carrier; GH3; cellulose synthase; HSP90.1
miR482 b	HR	III	B-ZIP 9; Ca-binding EF-hand; protease; HSP70; NB-ARC; Zinc finger
miR482 c	HR	I	Calcium-binding EF hand; Dof-type Zinc finger
miR166 a	HR	III	glucuronidase 3; TPR
miR7495 a	HR	I	HSP20; HSP70; protein kinase; retrotransposon gag; TPR-like
miR7495 c	HR	III	HSP70; protein kinase; retrotransposon; TPR-like;
miR3476 a	HR	II	lectin; plant peroxidase
miR164 b	HR	II	NA
miR166 b	HR	I	NA
miR166 c	HR	I	NA
miR162 a	HR	III	Peptidase A1
ls-miR 43	HR	I	RLK; nuclear transport factor
ls-miR 172	HR	I	RLK; Ubiquitin-like
miR171 b	HR	II	SCARECROW-like 13; ATPase
ls-miR 194	Negative for R	I	ACO; HLH; MYB
miR396 b	Negative for R	III	Cytochrome P450
ls-miR 171	Negative for R	II	Galectin; HLH; portein kinase; Ubiquitin-like
miR2949 a	Negative for R	III	lacccase 5; peptidase
miR3954 a	Negative for R	I	monooxygenase; NAC 014; protein phosphatase; Ran BP2/NZF Zinc finger
ls-miR 78	Negative for R	II	NA
miR403 a	Negative for R	I	NA
miR482 a	Negative for R	II	NA
miR482 e	Negative for R	II	NA
miR1448 a	Negative for R	II	NB-ARC, O-methyltransferase; Hydroxymethylglutaryl-CoA reductase
miR396 a	Negative for R	III	RLK
ls-miR 51	Negative for R	I	rotamase CYP3; splicing factor 3B subunit
miR156 b	Negative for S	II	NA
miR167 a	Positive for R	III	Armadillo-type; fatty acid hydrolase; NADP-binding; phosphate transporter; tubulin
ls-miR 123	Positive for R	II	NA
ls-miR 163	Positive for R	II	NA
miR166 d	Positive for R	II	NA
miR319 a	Positive for R	III	NA
miR398 b	Positive for R	II	Ribosomal protein L31e
ls-miR 159	Positive for R	III	Unknown function
miR156 a	Positive for S	II	NA
miR164 a	Positive for S	II	NA
miR482 d	Positive for S	II	NA
miR396 d	Positive for S & HR	II	NADH-ubiquinone oxidoreductase; WRC
miR2118 a	Positive for S & HR	II	Unknown function
miR396 c	R	III	Glutathione S-transferase
ls-miR 174	R	III	HSP 40, histone
ls-miR 175	R	III	HSP 40, histone
ls-miR 94	R	III	Ribosomal protein
ls-miR 95	R	III	Ribosomal protein
ls-miR 96	R	III	Ribosomal protein
miR8011 a	R	III	Ribosomal protein
ls-miR 53	R	III	Unknown function
ls-miR 212	R	III	Zinc finger; oxygenase; Ribosomal protein

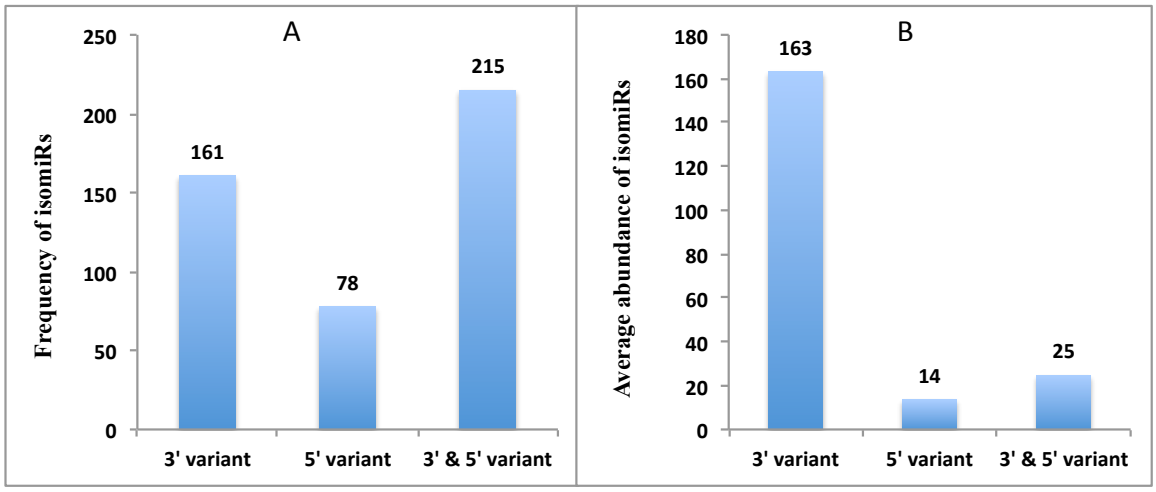

Figure 3.5. Frequency and Average abundance of different types of isomiRNAs

Table 3.5. Cotton S/R/HR-related isomiRNAs and their predicted targets

ID	Type	Function	Targets
iso-ls-miR155	5' & 3'	Negative for R	bHLH; Calectin; Myosin; protein kinase; Zinc finger
iso-ls-miR156	5' & 3'	Negative for HR	bHLH; Ubiquitin-like; Zinc finger
iso-miR166a	5' & 3'	Negative for R	Chalcone and stilbene synthase
iso-miR396a|b	3'	Negative for R	dirigent-like; WRC; metalloendopeptidases
iso-miR3476a	3'	Positive for HR	Fatty acid hydroxylase; signal transduction response regulator
iso-ls-miR172	5' & 3'	HR	Glycoside hydrolase; Thaumatin; protein kinase
iso-ls-miR172	5' & 3'	HR	Glycoside hydrolase; Thaumatin; protein kinase; ACO
iso-ls-miR159	3'	Positive for R	HSP 40
iso-ls-miR159	5' & 3'	R	HSP 40
iso-ls-miR174	5' & 3'	Positive for R	HSP 40
iso-ls-miR174	5' & 3'	R	HSP 40; DREB & EAR motif; histone
iso-miR3954a	3'	Negative for S	monooxygenase; NAC 014; Ran BP2/NZC Zinc-finger
iso-miR3954a	3'	Negative for S	monooxygenase; protein phosphatase
iso-ls-miR174	5' & 3'	R	NA
iso-ls-miR45	5' & 3'	R	NA
iso-miR1448a	5'	Negative for R	NA
iso-miR156a	3'	HR	NA
iso-miR159a	3'	Positive for R	NA
iso-miR166a|b|c	3'	Positive for HR	NA
iso-miR166a|b|c	3'	Positive for HR	NA
iso-miR166c	5' & 3'	Negative for R	NA
iso-miR166c	5' & 3'	Positive for R	NA
iso-miR2947a	3'	Negative for R	NA
iso-miR396a	3'	Negative for R	NA
iso-miR396b	3'	Negative for S	NA
iso-miR482b	5' & 3'	Negative for R	NA
iso-miR162a	3'	Positive for S	Peptidase A1
iso-miR2949a	3'	HR	Peptidase; SANT domain
iso-miR2949a	3'	Negative for R	Peptidase; SANT domain
iso-miR7508a	5' & 3'	Positive for HR	Ribosomal protein
iso-ls-miR43	3'	Positive for HR	RLK; Nuclear transporter
iso-ls-miR172	5' & 3'	Positive for R	Thaumatin

iso-miR482e	3'	Positive for S Negative for HR	Thioredoxin
iso-miR319a	5' & 3'	& positive for R	U box
iso-miR319a	5' & 3'	Positive for R	U box
iso-miR167a	3'	Positive for S & HR	Unknown function
iso-miR2118a	3'	Positive for S	Unknown function

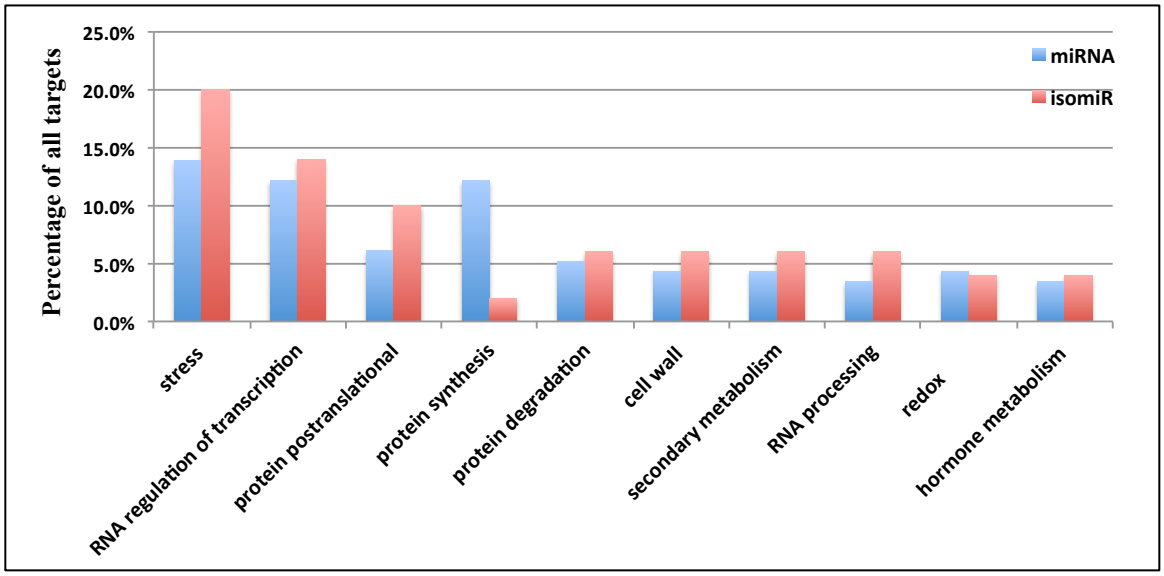

Figure 3.6. The top 10 GO categories of miRNAs and isomiRNA target

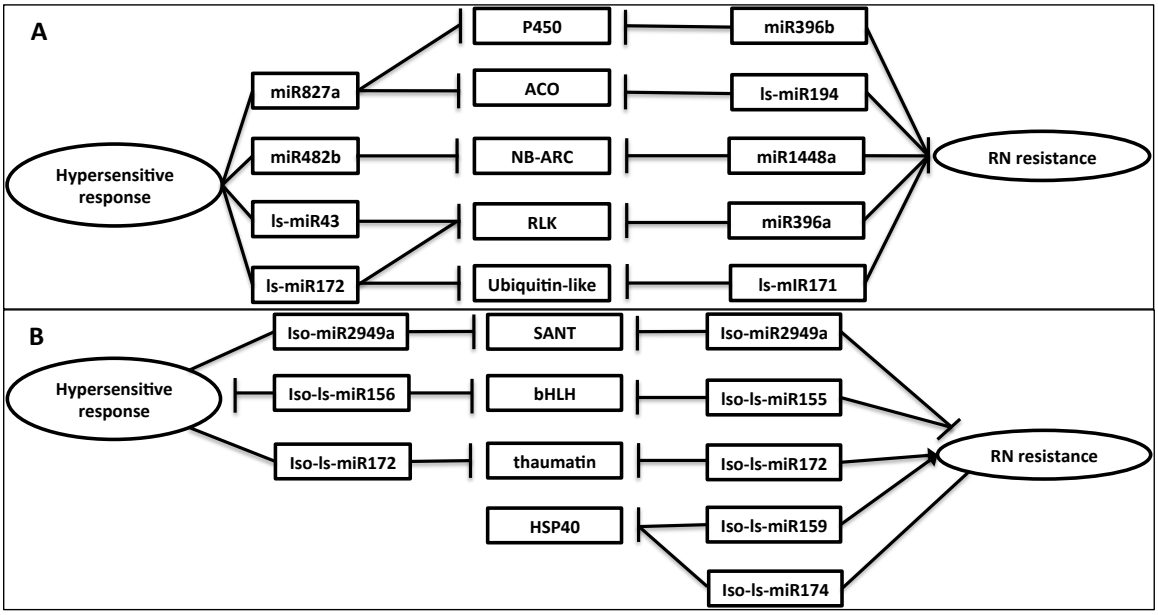

Figure 3.7. Potential functional network of RN S/R/HR-related miRNAs/isomiRNAs in cotton roots

A: Network of miRNAs involved in RN S/R/HR to RN; **B:** Network of isomiRNAs involved in RN S/R/HR to RN.