ABSTRACT

MYERS, JENNIFER ANNE. Understanding Genetic Resistance to Bacteria Wilt Caused by *Ralstonia solanacearum* in Tomato. (Under the direction of Dr. Dilip R. Panthee).

Bacterial wilt caused by *Ralstonia solanacearum* (*Rs*) is a devastating plant disease that can cause dramatic losses in production and restrict the planting of tomatoes in large affected areas. *Rs* has a wide range of distribution over tropical and sub-tropical regions of the globe and comprises high genetic diversity within the species that enables strain differentiation. These different strains do not always affect the same plant species and are different enough that not all phenotypic responses and genetic resistance are the same across strains. The bacteria is prevalent in warm temperate to tropical climates and is thus very prevalent in the southern portions of the U.S. The pathogen persists in the soil and is a major constrain in tomato production in the southern U.S. No management practices have been found to be effective and consistent in controlling *Rs*. Due to this, growers must rely on genetic resistance in tomato varieties. However, the quantitative nature of bacterial wilt resistance makes understanding and utilizing the host resistance a challenge. Several quantitative trait loci (QTL) associated with bacterial wilt resistance have been identified throughout the genome, particularly chromosomes 6 and 12. Gene expression studies have been used to try and identify the specific gene or genes related to bacterial wilt resistance in tomato. However, many of these studies have looked at *Rs* strains endemic to other regions outside of the U.S.

The objective of this study was to use RNA sequence analysis to identify key genes that are playing a role in genetic resistance to a race I, phylotype II strain of *Rs* in tomato. Tomato lines ‘Hawaii 7998’ (HI 7998) and ‘North Carolina 359-2B(2010)’ (NC 359-2B(2010) were used as the resistant and susceptible lines for gene expression analysis. Plants were inoculated with *Rs* and a water control at five weeks old and hypocotyl tissue was harvested at 48 and 72 hours to be

used for RNA extraction and sequencing. Three (3) biological replicates were used in total. Disease ratings were also taken over time to visualize differences in resistance between the two tomato lines. Water and *Rs* treatments for each genotype were then compared to each other to identify genes being differentially expressed in response to infection.

HI 7998 plants were shown to be more resistant than NC 359-2B(2010) and had 51 genes differentially expressed in *Rs* inoculated plants compared to NC 359-2B(2010). Of those 51 genes, 18 were up-regulated, and 33 were down-regulated. Differential expression based on gene ontology (GO) terms also found 144 genes to be differentially expressed. All of these genes were considered to function in the cell wall and membrane/plasma membrane. Together these genes look to be working in structural changes in the cell in response to colonization in order to prevent the spread of *Rs* within the plant. With further validation of these genes, they could be utilized in the future to understand better how resistance to the U.S. strain of *Rs* is conveyed and how it may compare to other strains. This understanding can then potentially be used to develop new high performing tomato lines that utilize stable bacterial wilt resistance.

Understanding Genetic Resistance to Bacterial Wilt Caused by *Ralstonia solanacearum* in Tomatoes.

by
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DEDICATION

To my mom and dad for always encouraging me to pursue my goals and pushing me to be the best person I can be.

To my friends for you time, ears, and friendship that made my Master's experience the best it could be.

BIOGRAPHY

Jennifer Myers was born in Los Angeles, California and spent much of her time growing up in larger towns and cities without much exposure to agriculture. She found a love of science from a young age participating in science fairs and community events promoting science to young kids. She had always been drawn to plant science, but it wasn't until high school that she realized it was an area she wanted to pursue. In high school she learned more about plant science from an environmental standpoint and eventually decided to attend the University of Nebraska-Lincoln after graduation to get a Bachelor of Science in plant biology. It was there that she discovered a love for agriculture and plant breeding. The community was so much different at UNL than communities she had grown up in. It allowed her to fall in love with plant science not just for the discovery of something new, but for the improvement of crop species for the benefit of others. While at UNL, she spent much of her time outside of class working with several agricultural groups both on and off campus, as well as working as an undergraduate researcher with Dr. Rebecca Roston on freezing tolerance in Arabidopsis and other key plants. She also started to develop an interest in horticultural crops through classes and research and decided that was where she wanted to continue her focus. UNL was a very heavy agronomy school and so she decided to look for a good horticultural school for her Master's. North Carolina State University became one of the best things that ever happened to her after that. Coming to a very horticulturally diverse state and school and working on a project in plant breeding, plant pathology, and bioinformatics has been a perfect culmination of growing interests throughout her life.

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Chapter 1: Literature Review

1.1 Introduction

Tomatoes are considered one of the most utilized vegetable crops in the world with over 170 countries around the world currently producing tomatoes. In general, tomatoes are grown for one of two major markets, processing and fresh market. Processing tomatoes are used for products such as pasta sauce and ketchup, while fresh market tomatoes are sold whole in grocery stores, farmers markets, and restaurant trades. In 2017, the total production around the world of tomatoes yielded roughly 182 million metric tons for all markets (FAOSTAT, 2018). While the U.S. is not a top tomato producing country, tomato production remains one of the highest-grossing vegetables produced in the U.S. In 2018, the U.S. produced 14.1 million metric tons of tomatoes with a market value of roughly $1.8 billion (USDA, 2019).

In the U.S., North Carolina is a good producer of tomatoes as a southern state with a longer growing season and a warm humid climate. Overall, North Carolina ranks seventh in total tomato production in the U.S. (USDA, 2019). However, North Carolina is different from many tomato producing states in that all tomatoes produced in the state are grown for the fresh market. When comparing fresh-market tomato production in the U.S., North Carolina ranks number three behind California and Florida (USDA, 2019).

With any form of crop production comes a multitude of diseases that can affect the plant, and tomatoes are no exception. The warm, humid climate found in the Piedmont and coastal areas of North Carolina also provide an ideal environment for many pathogens to thrive and increases the disease pressure for growers. With tomatoes also being grown for the fresh market, there is extra pressure to produce healthy, good looking fruit. All in all, this puts a huge emphasis

on managing diseases in the state of North Carolina. One such disease that can devastate North Carolina tomato plants is bacterial wilt, caused by the bacterial pathogen *Ralstonia solanacearum*.

1.2: The Disease

Bacterial wilt, or southern wilt, is a vascular disease characterized by wilting of some or all of the plant as well as brown or necrotic stem tissue. Symptoms can appear on plants from the seeding stage to full maturity and occur when the pathogen colonizes the xylem tissue of the roots and stem and start to form large quantities of polysaccharides and other cell wall-degrading enzymes (Denny, 2006). Together, these virulence factors can block and degrade the xylem and other tissues causing necrosis in the stem tissue, which thereby restrict water movement in certain parts of the plant. This lack of water is what causes the characteristic wilting of this disease. As the water is blocked in the stem near the bacteria, lower parts of the stem can become waterlogged and add to the decay of the stem tissue (Planas-Marquès et al., 2020). The blockage of water in the plant also restricts nutrient movement and can result in the yellowing of the leaves. The compounds formed are also evident in the stem even before necrosis sets in. When an infected stem is cut and placed in water, the polysaccharides and bacterial cells start to stream out of the stem which can also be collected for identification purposes.

As temperatures increase, plants tend to face higher water pressure with increase transpiration and water loss. With bacterial wilt being characterized as a foliar wilting disease, increases in temperature and subsequent water loss can exacerbate the disease progression and make symptoms more prominent and fast-acting. On the other hand, in colder periods, symptoms

of bacterial wilt may be less pronounced and in certain species and cultivars, it may help in recovery from symptoms (Vaughan, 1944).

Not all plant species that are susceptible to bacterial wilt show exactly the same symptoms, and bacterial wilt can sometimes be mistaken for other plant diseases (EPPO, 2004). In tomatoes, other symptoms of bacterial wilt include increased adventitious roots on the stems, particularly below major infection sites. In bananas, bacterial wilt is sometimes confused with Panama disease and is usually distinguished by brown dry rot in the fruit. Potatoes can show symptoms in the tubers that may be confused with ring rot but do exhibit bacterial streaming, which can help distinguish it as bacterial wilt.

1.3: The Pathogen

The bacteria responsible for bacterial wilt, *Ralstonia solanacearum*, is a soil borne bacterial pathogen in the Burkholderiaceae family that prefers warm, humid environments and can persist for long periods within the soil. The bacteria generally exist in fields in patches that can change in size over time depending on several factors (Denny, 2006). Although prevalent in North Carolina, *R. solanacearum* can persist all over the world in tropical to warm temperate climates and is not limited to tomatoes. This bacterial pathogen can infect a wide range of host species all across the Solanaceae family, including crops such as peppers, potatoes, tobacco, and eggplant, as well as several other plant families, with plants such as banana, peanut, geranium, and castor bean (Kelman, 1953).

In the U.S., *R. solanacearum* has been identified mostly in the southern states as far north as Maryland and as far west as Texas with the pathogen being identified in Hawaii as well (Kelman, 1953). In North Carolina, the incidence of *R. solanacearum* has grown drastically in

the last hundred years from 39% of counties in 1917 to 71% in 1953 to over 80% reported in 2018 with reports from the mountains to the plains (Kelman, 1953; Kressin, 2018; Stanforn and Wolf, 1917).

1.3.1: The Pathogen- Detection Techniques

Since foliar wilting and other characteristic symptoms of bacterial wilt are not exclusive to this disease, different testing methods have been developed to identify *R. solanacearum* in the field. The pathogen can be isolated from soil dilutions and grown on certain selective media, such as 2,3,5-triphenyl tetrazolium chloride (TTC) media, to try and identify *R. solanacearum*, but this technique does not always isolate the bacteria from the soil for testing (Nesmith, 1979). Ouchterlony double diffusion (ODD) tests developed by Ouchterlony in 1948 have also been used to distinguish *R. solanacearum* from other bacterial pathogens in plants (Morton et al., 1965; Ouchterlony, 1948). Other serological techniques such as the use of known antigens for *Ralstonia solanacearum* have also been used to distinguish this pathogen from other bacterial plant pathogens (Schaad, 1979). Some additional testing methods involve chemical analysis of the lipopolysaccharides made by the bacteria to look for compounds such as glucose, rhamnose, xylose, heptose, 2-amino-2-deoxyglucose, and 2-keto-3-deoxyoctonate (Baker et al., 1984). However, most of these techniques require in lab testing that can take a fair amount of time to get a conclusive result. More in-field tests, such as enzyme-linked immunosorbent assays (ELISA) test, have been developed for faster turnaround in the identification of *R. solanacearum* in the field (Rajeshwari et al., 1998). Unfortunately, a drawback of ELISA tests is that they cannot distinguish between living or dead and virulent or nonvirulent cells. Of course, new detection methods that utilize techniques such as sequencing have continued to be developed for

not only specifying the presence of *R. solanacearum* in the field but also detecting the specific type and strain of *R. solanacearum* that is present (Cook and Sequeira, 1994; Guidot et al., 2007; Prior et al., 2016; Ramírez et al., 2020).

1.3.2: The Pathogen- Classification

Due to the wide range of locations and hosts *R. solanacearum* can infect, there is a fair amount of diversity within the species that can be divided into several subgroups. *R. solanacearum* can be first divided into four phylotypes classified by their phylogeny and subsequently their native region (Prior and Fegan, 2005). Phylotype I originated in Asia and Oceania, phylotype II originated in the Americas, phylotype III originated in Africa, and phylotype IV originated in Indonesia and Asia. The pathogen can also be broken down into five races by host species and six biovars by ability to acidify different disaccharides and sugar alcohol containing media (Buddenhagen and Kelman, 1964; Hayward, 1964). For example, *R. solanacearum* that can infect banana in phylotype II would be considered to be race 2, and *R. solanacearum* that can acidify media with mannitol, sorbitol, dulcitol, *myo*-inositol, D-ribose, and trehalose but not lactose, maltose, or D-(+)- cellobiose would be biovar 4. Another method was also developed later for separating biovars based on the bacteria's ability to produce nitrite from nitrate and nitrogen gas from nitrate under anaerobic conditions (Hayward et al., 1990).

Although *R. solanacearum* has been classified by phylotype, race, and biovar historically, newer sequencing and genetic methods have been used to more accurately separate out subspecies and improve the classification system (Cook and Sequeira, 1994). Phylogenic trees based on highly conserved genes such as the TTSS effector gene hrpB grouped strains into groups similar to the phylotype classification; however, other genes such as phage genes and

insert sequences did not group by phylotype (Guidot et al., 2007). Other genetic sequencing and proteomics studies have also been able to confirm the prior classification system based on the Hayward et al. 1990 nitrate assays (Prior et al., 2016).

Based on historical classifications, race 1 in phylotype II is the endemic *R. solanacearum* infecting tomatoes in the U.S and is the single type that exists in fields in the U.S. (Kelman, 1953). However, race 3 biovar 2 has also been identified in the U.S. after it was brought over from offshore sites on geranium cuttings, but it has since been eradicated (Kim et al., 2003). Of course with new molecular techniques, researchers have been able to distinguish between different race 1, phylotype II strains found in the U.S. based on host species, geographical locations, and other key factors to describe the exact strain type found in different U.S. fields (Hong et al., 2012).

1.3.3: The Pathogen- Management Practices

To combat bacterial wilt in tomatoes, there are very few consistently effective methods for treating a field to reduce or eliminate *R. solanacearum*. The use of biological control agents (BCA) has shown to provide some biological control efficacy of bacterial wilt in tomato; however, these methods can be difficult to apply and inconsistent (Yuliar et al., 2015). In tomato, applying a nonpathogenic *Ralstonia pickettii* as a stem injection at 10^7 CFU/mL conferred biological control efficacy of 73% by outcompeting *R. solanacearum* (Wei et al., 2013). Using However, this is a difficult method to apply for an entire field of plants. *Ralstonia pickettii* was also tested as a soil drench, but the CFU/mL had to be increased to 10^9 to be effective and conferred biological control efficacy of 53% (Wei et al., 2013). A slightly easier method is mixing *Bacillus amyloliquefaciens* QL-5 with the soil at a density of $1x10^7$ CFU/g of soil, but the

biological control efficacy ranged from 17 to 87% in tomatoes by decreasing root colonization (Wei et al., 2011). Similar studies in pepper and eggplant had higher biological control efficacy of 61-80% and 81% respectively and may provide a different method of control (Hu et al., 2010; Ramesh and Phadke, 2012).

Other studies have found that certain soil amendments can have some effect on *R. solanacearum* populations. One such study showed that the application of urea and CaO could reduce *R. solanacearum* concentration in certain soil types but it was not consistent across all soil types tested and would always drastically increase the pH (Michel and Mew, 1998). Another study found that a foliar spray of 0.3% $CaCl_2$ on a sea-shell grit (42% CaO) amended soil could control the symptoms of bacterial wilt to an extent and still have vigorous plants, but it did not control symptoms as well as just the foliar spray which did not yield vigorous plants (Power, 1983). Although some of these methods have had some success in controlling bacterial wilt, the inconsistency remains as a problem of using certain management practices for controlling *R. solanacearum* and subsequent symptoms in the field. However, genetic resistance to bacterial wilt is still a viable possibility in tomatoes.

1.4: Genetic Resistance

Currently, certain tomato lines are considered to have varying levels of resistance to bacterial wilt. A study evaluating different tomato varieties' resistant to bacterial wilt found a *Solanum lycopersicum* line Hawaii 7996 (HI 7996) was one of the most stable sources of bacterial wilt resistance with an average survival rate of 97% (Wang et al., 1998). HI 7996 is considered to be highly resistant to bacterial wilt and is a common tomato rootstock used in grafting for bacterial wilt resistance. Although this line is considered highly resistant, it is a poor

producer of fruit, which is why it is mostly used as a rootstock. Even with the success of grafting a resistant rootstock to a good performing scion, growing and producing grafted plants can be an expensive and time-consuming process. This concern continues to drive the need for a resistant tomato line with good performance.

Another issue in current bacterial wilt resistance is how it reacts to environmental stress, particularly heat stress. The added stress of heat, both in the air and in the soil, only exacerbates bacterial wilt symptoms, even in resistant varieties (Krausz and Thurston, 1975; Mew and Ho, 1975). For a disease prevalent almost exclusively in warm temperate to tropical climates, this is a very pertinent concern for growers. Contrastingly, both resistant and susceptible lines have shown to be able to recover in cooler periods with resistant lines showing slightly better recovery (Vaughan, 1944).

The current tomato lines that have been tested for bacterial wilt resistance have not all been discovered using the same race and biovar of *R. solanacearum*, and due to the differences in subpopulations of *R. solanacearum*, this resistance is not always stable when challenged with different types of *R. solanacearum* (Jacobs et al., 2012; Kunwar et al., 2019). Unfortunately, even with different types of resistance, how tomatoes convey this resistance is still relatively unknown. In some plant species, such as Arabidopsis, it has been found that only one recessive gene controls the bacterial wilt resistance trait (Deslandes et al., 2002). This makes understanding the mode of resistance fairly easy. However, in tomatoes it has been found that there is no one gene controlling bacterial wilt resistance. Bacterial wilt resistance in tomatoes is a quantitatively controlled trait with multiple genes working together to get the varying levels of resistance (Thoquet et al., 1996a, 1996b).

Due to the quantitative nature of this trait, there are no definitive resistant and susceptible categories. Resistance and susceptibility in tomatoes exists on a spectrum of very susceptible to highly resistant and due to the variability of *R. solanacearum* in different conditions and environments, resistance is not always the same in every environment. This inconsistency makes it more challenging to identify key genes that are major contributors to resistance. However, in comparative studies of susceptible and resistant tomato lines, it was found that the resistant plants are not avoiding colonization by the bacteria (Ciampi et al., 1980; Winstead and Kelman, 1952). Resistant tomato lines seem to have a similar bacterial density as susceptible lines in the stem tissue, but only the susceptible lines show significant damage (Grimault and Prior, 1993). Even though resistant tomatoes are still being colonized by *R. solanacearum*, they are still able to function relatively normally. Therefore, whatever mechanism is causing plants to be more resistant to the bacteria is not necessarily preventative but more likely one that contains and manages the infections and symptoms.

1.4.1: Genetic Resistance- QTL

With the knowledge that multiple genes control bacterial wilt resistance, several studies have sought quantitative trait loci (QTLs) that are associated with bacterial wilt resistance. These association studies have found QTLs across a large portion of the tomato genome with significant QTLs on chromosomes 3, 4, 6, 8, 10, and 12 (Carmeille et al., 2006; Danesh et al., 1994; Thoquet et al., 1996a, 1996b; Wang et al., 2000). A study looking at recombinant inbred lines (RILs) derived from resistant H7996 and susceptible WVa700 against a race 3 in phylotype II strain of *R. solanacearum* found a major QTL on chromosome 6 and a minor QTL on chromosome 3 (Carmeille et al., 2006). When they looked at resistance in high heat, they also

found two QTLs on chromosome 4 and 8 that only had intermediate effects in the hot season. Further studies have continued to narrow down the location in the genome. Chromosomes that continued to show relevant QTLs were chromosomes 6 and 12 with some association with chromosome 4. One particular study looked closely at chromosomes 6 and 12 to try and identify key QTLs that convey bacterial wilt resistance and found two major QTLs which they called *Bwr-6* and *Bwr-12* on chromosomes 6 and 12 respectively (Wang et al., 2013). These two QTLs were correlated with low bacterial wilt incidence in tomatoes, with progeny that had both QTLs showing better resistance to those with only one QTL. The QTL on chromosome 12 was found to not be associated with resistance against race 3 in phylotype II, but it was found to contain genes related to the suppression of internal multiplication of the pathogen (Wang et al., 2013).

1.4.2: Genetic Resistance- Markers

With an idea of general locations responsible for bacterial wilt resistance, further studies can be done to narrow down the location of important candidate genes for not only conveying resistance in plants but predicting resistance as well. Since the sequencing of the tomato genome, there have been thousands of different types of genetic markers developed for a tomato that has been linked to many different traits in tomato. One such type of marker that has been used as for tomato is a simple sequence repeat (SSR) marker. Generally, SSR markers are developed by constructing a genomic library and looking for repeat motifs in the sequences (Zane et al., 2002). These types of markers, among others, can be used to try and identify markers associated with bacterial wilt resistance in tomato. When a marker or markers are found to be associated with bacterial wilt, that marker can be looked into further to try and identify nearby genes responsible for resistance, or it can be used for predicting resistance.

Since chromosomes 6 and 12 have been shown to have some major QTLs associated with bacterial wilt, markers specifically targeted to those chromosomes would be beneficial in understanding resistance. In 2009, a research group used 13 bacterial artificial chromosomes (BACs) to develop 21 SSR markers on chromosome 6, with some being associated with the *Bwr-6* QTL associated with bacterial wilt resistance (Geethanjali et al., 2010). The QTL region was associated with marker SLM6-17 at 47.9 cM and SLM6-53 at 75.1 cM. In 2010, the same group used the same techniques for developing SSR markers for chromosome 12 and found 16 SSR markers across 11 BAC clones (Geethanjali et al., 2011). No direct association with known bacterial wilt QTLs were identified.

Another genetic marker useful for identifying genes responsible for bacterial wilt resistance are single nucleotide repeat (SNP) markers. In a genome-wide association study (GWAS) comparing entire genome sequences between a susceptible and a resistant tomato variety, differing SNPs between the two varieties can help identify key markers for resistance. In total, 5259 SNPs were found to differ between resistant and susceptible varieties; however, only 265 were found to be in coding regions of the genomes, with many of the markers being on chromosomes 6 and 12 (Kim et al., 2018). SNP *Solyc12g009690.1* was one of the markers tightly associated with the *Bwr-12* QTL and could be a good marker to use for selecting bacterial wilt resistant varieties in the future.

1.4.3: Genetic Resistance- RNA-seq

With more specific QTLs and markers identified, sections of the genome can be used to experiment further and find specific genes that are responsible for bacterial wilt resistance. One such method for finding genes affecting bacterial wilt resistance is through RNA sequencing

(RNA-seq). By converting all extracted RNA into cDNA and sequencing it, researchers can get a wide range of data that can be analyzed for a variety of information (Haas and Zody, 2010). This can include identifying changes in the expression levels of specific genes. When comparing the expression levels of an infected plant and a healthy, control plant, the change in expression levels could indicate an important reaction the plant is having that may convey resistance or susceptibility to a pathogen.

In *R. solanacearum* infected tomatoes, this technique could be used to ascertain potential genes that are changing in expression levels upon infection. A study involving bacterial wilt in eggplants was able to find several potential genes whose expression level had changed upon infection and whose biological process ontology fit with the nature of the disease, such as plant hormone signaling pathways genes (Chen et al., 2018). These genes could be ultimately responsible for the plant's resistance or susceptibility. This method could also help better understand what types of genes are responsible for resistance and the mechanisms by which it occurs. Changes in gene expression levels can be seen by comparing infected susceptible and resistant tomato lines while using their non-infected counterparts as a baseline. Using a range of susceptible and resistant lines also helps to get a better understanding of the quantitative nature of resistance. Comparing these expression changes across multiple time points can also be used to see how the plant responds to *R. solanacearum* infections over time.

In a study looking at the gene expression levels of resistant tomato varieties under a water mock inoculation versus a race 1 in phylotype I *R. solanacearum* inoculation, 156 genes were found to have a change in expression levels (Ishihara et al., 2012). A susceptible comparison, however, did not find any significant changes in expression levels under the same two conditions. The resistant tomato line found 146 genes to be upregulated and 10 genes were found

to be down regulated. Of these genes, most were found to be a type of catalytic gene in both up and down regulated genes. Gene types included plant hormone signaling, carbohydrate metabolism, and lignin biosynthesis. Some or all of these genes could play a major role in encoding bacterial wilt resistance in these tomatoes.

1.5: Final Thoughts and Research Objectives

Even though researchers are continuing to uncover more and more about the genetic capability of tomatoes and its ability to confer resistance to bacterial wilt, there is still more to be learned. With the use of QTLs and genetic markers, researchers are able to identify specific regions of the genome that are important in encoding specific functions in bacterial wilt resistance. Using gene expression levels and RNA sequencing data can help to understand better how these regions of the genome truly function and play a role in allowing the plant to survive.

Here, two studies will be done regarding tomato resistance to a strain of race 1 *R. solanacearum* in phylotype II found in eastern North Carolina. The objective of the first study was to look at significant QTLs for several economically important traits and how they relate to bacterial wilt resistance in the progeny of a cross between a highly resistant parent and a highly susceptible parent. The objective of the second study was to look at changes in gene expression in two *R. solanacearum* inoculated tomato lines, one susceptible and one resistant, over time to identify key genes in bacterial wilt resistance.

Genetic and sequencing tools have truly had a significant impact on understanding how genetic resistance to bacterial wilt functions and will continue to make an impact going forward. The more researchers and breeders understand how mechanisms of genetic resistance to bacterial

wilt functions in tomatoes, the easier it becomes to breed and grow new tomato lines that have efficacious and consistent resistance to bacterial wilt and are high performing varieties.

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Chapter 2: RNA Sequencing Study for Bacterial Wilt Resistance in Tomato

Abstract

Bacterial wilt caused by the soil borne pathogen *Ralstonia solanacearum* (Smith) is one of the most devastating diseases in tomato (*Solanum lycopersicum*). The bacteria thrive in warm temperate to subtropical climate zones, making the southern U.S. an ideal environment for this devastating disease that can heavily impact regional production. Devastating tomato production, *Ralstonia solanacearum* can causes foliar wilting to total plant death, among other symptoms, in tomatoes. With limited options for controlling the disease in the field, the objective of this study is to identify and understand genetic resistance to bacterial wilt in tomatoes. Previous studies have identified potential sources of genetic resistance to several different strains of *Ralstonia solanacearum*, as well as several quantitative trait loci (QTL) linked to bacterial wilt resistance in tomatoes. To determine differential gene expression involved in bacterial wilt caused by a U.S. strain of *Ralstonia solanacearum* in tomatoes, we selected 'Hawaii 7998', a highly resistant line, and 'NC 359-2B(2010)' as a highly susceptible line, with treatments of *Ralstonia solanacearum* inoculum and a water control. Infected and noninfected stem tissue from both tomato lines were harvested 48 and 72 hours after inoculation and RNA was extracted for RNA sequencing analysis. Gene expressions were compared across samples to identify candidate genes that are being differentially expressed between samples. A comparison between *Ralstonia solanacearum* and water inoculated HI 7998 showed 51 genes being differentially expressed, whereas the same comparison in NC 359-2B(2010) showed no differentially expressed genes. Differentially expressed genes found in HI 7998 were classified into 30 different gene ontology categories. Biological process type genes had 1 to 6 genes being differentially expressed and are

likely functioning in small changes across the cell, potentially conferring pathogenic defense. Differentially expressed genes functioning in cell and plasma membrane and cell periphery suggest cell structural changes to protect the cell and prevent the pathogen from spreading in the plant. Using identified candidate genes, future studies can target verification of major genes that are responsible for bacterial wilt resistance, the elucidation of resistance mechanisms, and the utilization of bacterial wilt resistance in future tomato lines.

2.1: Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is a devastating foliar wilt disease that affects several crop species across many important plant families, such as the Solanaceae and Musaceae families. The bacteria *Ralstonia solanacearum* has been found in regions all over the world and can exist in warm temperate to tropical climates (Kelman, 1953). In the U.S., *Ralstonia solanacearum* has been identified from as far north as Maryland and as far west as Texas, as well as in Hawaii (Kelman, 1953). The pathogen is highly diverse genetically, in its distribution and host range and has been divided into four phylotypes that largely correspond to geographical regions, five races discerned by differential responses on host species, and six biovars discerned by substrate utilization (Buddenhagen and Kelman, 1964; Hayward, 1964; Prior and Fegan, 2005). This classification also affects how the disease is controlled as management practices and genetic resistance are not always efficacious against all *Ralstonia solanacearum* strains.

In most plant species, bacterial wilt is characterized by localized wilting and necrotic tissue, which can progress to total plant death. This can also cause yield loss and a decrease in fruit quality in the plants infected. In North Carolina, bacterial wilt has a major effect on tomato

growers, particularly with the extra pressure of growing tomatoes for the fresh market. The bacteria, *Ralstonia solanacearum,* is a soil borne pathogen that enters the plant through damage in the roots and grows in the xylem and adjacent cells of the stem where it forms polysaccharides and other cell degrading compounds to produce key symptoms of bacterial wilt (Denny, 2006). *Ralstonia solanacearum* tends to exist in the field in patches that are more localized, but once the bacteria exist in the field, it tends to remain in the soil for very long periods of time. The size and distribution of *Ralstonia solanacearum* in the field can change over time but very little can be done to control the bacteria. Techniques such as supplementing soils and foliar sprays have been tested for controlling bacterial wilt and *Ralstonia solanacearum* populations, but none have been found to be consistently effective (Michel and Mew, 1998; Wei et al., 2011, 2013; Yuliar et al., 2015). With little to no management practices available for controlling bacterial wilt in the field, the use of genetically resistant plant material remains the best method for growing tomatoes in infested fields.

Currently, there are tomato lines that have some level of genetic resistance to *Ralstonia solanacearum*, but how exactly this resistance is conveyed is still unknown. Previous studies looking at the inheritance and phenotypic range of bacterial wilt resistance have found that bacterial wilt resistance is not controlled by one single gene in tomato but is very quantitative in nature (Thoquet et al., 1996a, 1996b). The complicated nature of how resistance to *Ralstonia solanacearum* is controlled makes understanding the nature of genetic resistance tougher. Quantitative trait loci (QTLs) associated with bacterial wilt resistance in tomato have been found in six (6) of the twelve (12) chromosomes with some only being significant in the hot season (Carmeille et al., 2006; Danesh et al., 1994; Thoquet et al., 1996a, 1996b; Wang et al., 2000).

By knowing that resistance to bacterial wilt in tomatoes is a quantitative trait and with sources of bacterial wilt resistance available, gene expression studies can be used to get a better picture of what specific genes are playing an important role in resistance. A previous gene expression study in bacterial wilt of eggplant found potential key genes in resistance based on the expression levels of the genes and their gene ontology (Chen et al., 2018). In similar studies in tomato bacterial wilt resistance, 156 genes had been found to have been differentially expressed upon infection by a race 1 *Ralstonia solanacearum* in phylotype I endemic to Asia and Oceania (Ishihara et al., 2012). Similar to the study in eggplant, many of the genes identified fit into a very similar category of gene type, including plant hormone signaling pathways.
Due to the complicated nature of *Ralstonia solanacearum* classification and distinction, it has been found that resistance to bacterial wilt is not always efficacious across different races and strains (Jacobs et al., 2012; Kunwar et al., 2019). This prompts a look into resistance mechanisms in tomato to endemic U.S. *Ralstonia solanacearum* strains in phylotype II. In the U.S., a *Solanum lycopersicum* line and some of its progeny from a Hawaii breeding program have been utilized as the main source of genetic resistance to a race 1, phylotype II strain of *Ralstonia solanacearum* as it has been found to be the most stable source of resistance (Wang et al., 1998). The tomato line ‘Hawaii 7996’ (HI 7996), as well as tomato lines ‘Hawaii 7998’ (HI 7998) and ‘Hawaii 7997’ (HI 7997), have been utilized in several U.S. tomato breeding programs for establishing commercial bacterial wilt resistance. However, the incorporation of bacterial wilt resistance into well performing tomato lines has been challenging and leaves room for more studies to understand how this resistance is conveyed.

This study looks a gene expression levels of two tomato lines, one highly resistant and one highly susceptible, to better understand how genetic resistance to an endemic U.S. race 1,

phylotype II strain of *Ralstonia solanacearum* is conveyed and how resistance differs from other strains. This could provide the opportunity to incorporate known mechanisms of bacterial wilt resistance into breeding lines to produce tomatoes that are both resistant and well performing. This could also allow for a better knowledge of differences in resistance mechanisms between different strains of *Ralstonia solanacearum*.

2.2: Materials and Methods

Three (3) tomato lines were grown in the greenhouse (fall 2018; Wake Co.) and inoculated with *Ralstonia solanacearum* to assess bacterial wilt symptoms. Infected plants were compared to an identical water inoculated control. Publicly available line 'Hawaii 7996' (HI 7996) is bacterial wilt resistance. A related line 'Hawaii 7998' (HI 7998), shares most of the same resistance as HI 7996 and was used as the main bacterial wilt resistant genotype. A North Carolina advanced tomato breeding line 'North Carolina 359' (NC 359-2B(2010)) is a breeding line developed by the North Carolina State University tomato breeding program and was used as the bacterial wilt susceptible genotype.

2.2.1: Plant Growth Conditions

All tomato lines were sown in 48-cell Pro-Trays Cell Flats using Fafard 2P promix for seedling with a single seed per cell. Separate trays were sown for *Ralstonia solanacearum* and water inoculations and randomized on the greenhouse bench. Seeds were planted at three different times for three biological replicates on July 30, 2018, August 20, 2018, and October 16, 2018. Sown trays were placed on a clean heating mat set at 83°F and were later moved to the

greenhouse bench after 7 to 10 days. Plants were overhead watered 1-2 times per day and fertilized at ¼ to ½ the label rate (20-20-20).

2.2.2: *Ralstonia solanacearum* Inoculation

A frozen stock of a race 1, phylotype II strain of *Ralstonia solanacearum* (Rs) collected from Jackson Co., North Carolina was streaked on a CPD plate and was allowed to grow in an incubation chamber for two days. The plate was then suspended with DI water until all surface contents of the plate were dissolved. This solution was poured into a container and filled with more DI water to total 4.5L and mixed thoroughly. An inoculation loop of Rs from a second plate was added to the mixture to obtain the target colony forming unit (CFU) per milliliter of bacteria at 10^7 CFU/mL. Two 10-fold serial dilution series from 10^{-1} to 10^{-8} were done and dilutions 10^{-4} to 10^{-8} were streaked on new CPD plates and placed in the incubator to obtain an exact CFU/mL for the inoculum (Table 2.1). Clean DI water was also prepared and streaked on another CPD plate for a control.

Plants were inoculated 5 weeks after planting. The plants' roots were injured by pushing a sterile razor blade down in the plant media roughly a centimeter away from the stem on either side of the plant root ball. Half of the trays were inoculated using 10mL of DI water at each plant and placed on a heating pad set at 83°F. The other half of the trays were inoculated using 10mL of the Rs inoculum at each plant and placed on a separate heating pad set at 83°F to avoid cross contamination.

2.2.3: Plant Sampling and Observations

All observations were recorded on a scale of 0 to 5, where 0 = no wilting, 1 = 1 to 9% of the plant is wilting, 2 = 10 to 49% of the plant is wilting, 3 = 50 to 90% of the plant is wilting, 4 = 91 to 100% of the plant is wilting, and 5 = plant collapse, plant drying and decaying. 24 hours post inoculation, plants were observed for bacterial wilt symptoms. Disease rating for all plants across all three replications were used to find disease incidence (DI) by using the following equation: $DI = \frac{\Sigma(disease\ rating\ x\ number\ of\ plants\ at\ that\ rating)}{total\ number\ of\ plants\ x\ highest\ disease\ rating} x\ 100$. Six (6) HI 7998 tomato plants from the water inoculation tray were taken out, and 1cm of the hypocotyl stem tissue of each of the six (6) plants was removed and pooled in a 2 mL tube and place immediately in liquid nitrogen (Figure 2.1). Tubes were later stored in a freezer set at -80°C. This process was repeated for the NC 359-2B(2010) water treatment, HI 7998 Rs treatment, and NC 359-2B(2010) treatment. Stem imprints of all plants were taken in a SMSA-E antibiotic media plate, which was placed in an incubator. This sampling technique was repeated at 48 hours, 72 hours, and 96 hours post inoculation. Temperature inside the greenhouse, weather and plant number varied across time points and replication and could have an effect on the disease severity and gene expression (Table 2.2). Remaining plants were continually observed after sampling every two to three days until disease progression reached a plateau. Stem imprints were also observed two days after being placed in the incubator to see if plants were infected or not. This method was repeated twice for three biological replicates.

2.2.4: RNA Extraction

Tissue samples from both tomato lines across all three replications at the 48-hour and 72-hour time points (24 samples in total) were removed from the freezer and kept in liquid nitrogen

until they were ready to be used in individual RNA extraction. Frozen hypocotyl tissue was placed in a mortar and pestle with liquid nitrogen to be ground into a fine powder. Ground up tissue was moved into a 10mL tube and placed in liquid nitrogen for extraction. This step was repeated for all 24 samples. From there, 100mg of the plant tissue was added to a 1.5mL tube with 1mL of Trizol and vortexed to mix before sitting at room temperature for 5 minutes. Chloroform (200µL) was then added to each tube and vortexed again before sitting at room temperature for 15 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C. The aqueous layer of each sample was transferred to a new 1.5mL tube with 500µL of isopropanol and allowed to sit at room temperature for 10 minutes. Tubes were then centrifuged at 12,000g for 8 minutes at 4°C. The supernatant was removed without disturbing the pellet and 1mL of 75% ethanol was added to each tube and vortexed. Tubes were centrifuged again at 7,500g for 10 minutes at 4°C. Ethanol was removed without disturbing the pellet and tubes were left to aid dry for 15 minutes in the flow hood. Remaining RNA was suspended in 25µL of milliQ water and 1µl was used to measure RNA concentration.

A Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA) was used to clean up the RNA. A solution with 240µL DNase reaction buffer, 24µL DNase I, and 1536µL milliQ water was made and 75µL of the solution was added to each clean 1.5mL tube on ice. 25µL of RNA from each sample were each added to one of the new tubes. Tubes were then incubated at 37°C for 10 minutes. After, 1µL of 0.5M EDTA was added to each tube and spun down. A new lysis solution was made with 12mL of lysis solution and 120µL of 2-ME. 500µL of the new lysis solution was added to each tube and mixed. Binding solution (500µL) was then added to each tube and mixed immediately. The contents of each tube (700µL) was added to a red binding column and placed in a collection tube to be centrifuged at max speed for 1 min. Flow through

was discarded and the remaining contents of the tubes were used to repeat the previous step. Flow through was discarded again and 500µL of wash solution 1 was added to each column and centrifuged for 30 seconds at max speed. Flow through was discarded and the previous step was repeated with wash solution 2. Flow through was discarded and the column was placed in a new 1.5mL tube. 50µL of elution solution was added to each column and let sit for about 1 minute before centrifuging for 1 min at max speed. 1µL of the flow through was placed in the Nanodrop for RNA concentration and the remaining RNA was stored at -80°C.

2.2.5: RNA Sequencing and Analysis

RNA samples were submitted to NovoGene (CA, USA) for RNA sequencing and RNA sequencing analysis. Samples were quality controlled using nanodrop, 1% agarose gel electrophoresis, and Agilent Bioanalyzer 2100 (Agilent Technologies, CA, USA) before library construction. One microgram of RNA per sample were used in sequencing libraries using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacture's recommendations. mRNA was enriched using poly-T oligo-attached magnetic beads, fragmented using divalent cations under elevated temperatures in NEBNext First Strand Synthesis Reaction Buffer (5X), and then used in first strand cDNA synthesis using random hexamer primers and M-MuLV Reverse Transcriptase (RNase H). Second-strand synthesis was then performed using DNA Polymerase I and RNase H with remaining overhangs converted to blunt ends by exonuclease/polymerase activities. The final cDNA library then underwent purification by AMPure SP system (Beckman Coulter, Beverly, USA), size selected with 3 µL USER Enzyme (NEB, USA), and adaptor-ligated at 37°C for 15 minutes followed by 5 minutes at 95°C. Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primers were

used in PCR, with PCR product later purified using AMPure XP system. Library quality was then assessed on the Agilent Bioanalyzer 2100 system. cBOT Cluster Generation System was used to cluster index-coded samples with PE Cluster Kit cBot-HS (Illumina) according to the manufacturer's instructions. Library preparations were then sequenced on an Illumina platform and 125 bp/150 bp paired-end reads were generated.

Raw data in FASTQ format from Illumina were then transformed to sequence reads by base calling. Reads were filtered for adaptor contamination, uncertain nucleotides in more than 10% of either read, and low-quality nucleotides in more than 50% of the read. Q20, Q30 and GC content was also calculated from cleaned up data. Reads were then mapped to the reference genome using HISAT2 with the index of the reference genome built using hisat2 2.1.0 (Kim et al., 2014). Gene expression levels were then measured by transcript abundance by counting reads that map to the genes or exons using HTseq v0.6.1 (Anders et al., 2015). Reads were normalized across samples using fragments per kilobase of transcript sequence per millions base pairs sequenced (FPKM) (Trapnell et al., 2010). Differential expression analysis of water inoculated samples versus *Ralstonia solanacearum* inoculated samples were performed using the DESeq R package (1.18.0) (Anders and Huber, 2010, 2012). P-values were then adjusted using the Benjamini and Hochberg's approach for controlling false discovery rate (Benjamini and Hochberg, 1995). Differentially expressed genes were then used in Gene Ontology (GO) enrichment analysis using GOseq R package (1.18.0) (Young et al., 2010).

2.3: Results

All statistical tests and P-values are at a = 0.05.

2.3.1: Disease Incidence

All water inoculated tomato plants showed no sign of bacterial wilt across all observation points. Stem imprints of water inoculated controls also showed no signs of *Ralstonia solanacearum* colonization. In all three replications, NC 359-2B(2010) inoculated with *Ralstonia solanacearum* showed higher disease incidence and faster disease progression than *Ralstonia solanacearum* inoculated HI 7998 (Figure 2.2a). The level of bacterial wilt disease incidence for HI 7998 at 17 dpi was 42.22, whereas the level of bacterial wilt disease incidence for NC 359-2B(2010) at 17 dpi was 88.89 (Figure 2.2b). HI 7998 plants inoculated with *Ralstonia solanacearum* also tended to have less of the plant show wilting symptoms than NC 359-2B(2010) inoculated with *Ralstonia solanacearum* (Figure 2.2c,d). Some HI 7998 plants showed no bacterial wilt symptoms but were colonized by *Ralstonia solanacearum* based on stem imprint assays. A comparison of HI 7998 and HI 7996 plants inoculated with *Ralstonia solanacearum* showed similar bacterial wilt disease progression in both lines with the bacterial wilt disease incidence of HI 7996 at 37.78 at 17 dpi (Figure 2.3a,b). *Ralstonia solanacearum* inoculated HI 7996 plants also showed similar phenotypic responses to HI 7998 (Figure 2.2c,d). Overall, bacterial wilt symptoms were not observed until around 96 hours (4 days) post inoculation. All three replications showed similar disease progression in both tomato lines.

2.3.2: Gene Expression Analysis

RNA sequencing for each sample gave between 37,000,000 and 48,000,000 clean reads in which 91-96% mapped to the reference genome. In total, 18,457 genes were looked at between HI 7998 and NC 359-2B(2010) in both treatments with 357 genes uniquely expressed in *Ralstonia solanacearum* inoculated HI 7998, 92 genes in water inoculated HI 7998, 103 genes in

Ralstonia solanacearum inoculated NC 359-2B(2010), and 96 genes in water inoculated NC 359-2B(2010) (Figure 2.4). When looking at differentially expressed genes, 51 genes were found to be differentially expressed between *Ralstonia solanacearum* and water inoculated HI 7998 at a significance threshold of an adjusted p-value <0.05 (Table 2.3). Of those 51 differentially expressed genes, 18 were found to be up-regulated and 33 were found to be down-regulate (Figure 2.5). The comparison between *Ralstonia solanacearum* and water inoculated NC 359-2B(2010) did not find any significant differentially expressed genes.

A cluster analysis of the differentially expressed genes showed a high level of similarity between gene expression levels in *Ralstonia solanacearum* and water inoculated NC 359-2B(2010) (Figure 2.6). Gene expression levels were also very similar in *Ralstonia solanacearum* and water inoculated HI 7998 with the exception of the third grouping of genes on the distal end of the cluster that appeared to be up-regulated in *Ralstonia solanacearum* inoculated HI 7998 and not differentially expressed or down-regulated in water inoculated HI 7998 (Figure 2.6). The comparison of NC 359-2B(2010) and HI 7998 genes show the first grouping of genes were mostly up-regulated in NC 359-2B(2010) and mostly down-regulated in HI 7998. The second grouping showed the opposite pattern with genes in NC 359-2B(2010) being mostly down-regulated and genes in HI 7998 being mostly upregulated. The third grouping of genes showed a similar pattern between both NC 359-2B(2010) treatments and water inoculated HI 7998 with mostly down-regulated genes. Genes in the third grouping of *Ralstonia solanacearum* inoculated HI 7998 was more highly up-regulated in comparison.

Genes were also found to be significantly enriched in *Ralstonia solanacearum* versus water inoculated HI 7998 based on their gene ontology (GO) term. GO terms were fit in either biological processes or cellular components category with a total of 144 differentially expressed

genes overall (Table 2.4). The genes in the cellular component category consisted of cell wall periphery, plasma membrane, and membrane GO terms with 25, 23, and 56 genes being differentially expressed respectively (Figure 2.7a). Overall, 47 genes were up-regulated, and 57 genes were down-regulated. In the biological processes category, 11 genes were involved in the cell wall organization or biogenesis, 8 genes were involved with plant-type cell wall organization or biogenesis, 7 genes were involved in plant-type cell wall biogenesis, 8 genes were involved with cell wall biogenesis, and 6 genes were involved with plant-type secondary cell wall biogenesis (Figure 2.7b).

2.4: Discussion

Tomato line HI 7998 is a related line of HI 7996, which is considered to be one of the most stable source for bacterial wilt resistance in tomato (Wang et al., 1998). However, despite its elite heritage, HI 7998 has never been considered as resistance to bacterial wilt as HI 7996. In this study, HI 7998 plants showed similar levels of phenotypic resistance to bacterial wilt as HI 7996 plants, and thus HI 7998 provided a good source of genetic resistance for the expression study. Due to the shared lineage between HI 7998 and HI 7996 and their observed similar level of bacterial wilt resistance, it is likely that gene expression responses in HI 7996 would be similar to those seen in HI 7998. The age of the plants in this study could have had a factor in this similarity in resistance, but the use of HI 7998 instead of HI 7996 in the gene expression study could also yield new and novel genes not seen in HI 7996.

The disease progression of the three tomato lines also followed a similar pattern despite having different levels of resistance (Figure 2.2 and 2.3). All tomato lines did not show any symptoms of bacterial wilt until at least three- or four-days post inoculation in all plants tested.

When comparing resistant and susceptible lines in the seedling stage, HI 7998 was observed to be more resistant to bacterial wilt than NC 359-2B(2010) with several HI 7998 plants not seeing any bacterial wilt symptoms despite evidence of colonization. On the other hand, there were a hand full of HI 7998 plants that did express bacterial wilt symptoms during observation, some to even total plant death. Infection by *Ralstonia solanacearum* was confirmed in all stem imprints. This lack of resistance in all HI 7998 plants could be due to the plant being fairly young upon infection, or that the quantitative nature of bacterial wilt resistance means resistance phenotypes are not always consistent within a single line. No plants from any line showed recovery from bacterial wilt symptoms and could potentially show that once a plant in this study started to show symptoms, it would eventually die. Historically, the stability of bacterial wilt resistance has been known to be weak in tomatoes and could be playing a role in this discrepancy (Grimsley and Wang, 1997). This variation could also have to do with any number of environmental or external factors that have been shown to affect bacterial wilt resistance, including temperature, soil content, and moisture (Krausz and Thurston, 1975; Messiaen, 1989; Mew and Ho, 1975).

In gene expression, the number of genes being uniquely expressed in HI 7998 inoculated with *Ralstonia solanacearum* was roughly three times the number of genes uniquely expressing in water inoculated HI 7998 and both NC 359-2B(2010) treatments. This helps solidify the idea that there is a genetic change in HI 7998 that is responding to infection to convey the resistance phenotype seen in the plants. The 357 genes uniquely expressed in HI 7998 inoculated with *Ralstonia solanacearum* show great potential for novel gene expression that conveys bacterial wilt resistance in HI 7998. With over three (3) times the number of uniquely expressing genes in *Ralstonia solanacearum* inoculated HI 7998, there is a possibility of new genes being expressed during infection in the resistant plant that may play a major role in that resistance. When looking

at how gene expression is changing between *Ralstonia solanacearum* inoculated and water inoculated plants, the 51 differentially expressed genes in HI 7998 versus zero found in NC 359-2B(2010) further illustrate that the difference in bacterial wilt resistance between the two lines is genetic, and the change is occurring in the resistant line. In this study, more than half of the genes differentially expressed in HI 7998 were down-regulated, which differed from previous studies that found 146 of the 156 differentially expressed genes to be up-regulated in the resistant LS-89 tomato line (Ishihara et al., 2012). This could show that there is a difference in genetic resistance mechanisms being utilized in the two tomato lines, or it could be due to a difference in the *Ralstonia solanacearum* strains being used.

The genes that were found to be differentially expressed spanned the entire genome with at least one gene from each chromosome present. Each chromosome had at least two (2) genes differentially expressed with chromosome 9 having the most differentially expressed genes with 7 of the 51 genes. However, it is likely that some of these genes are false positives and are not key genes in bacterial wilt resistance.

The differences in gene expression levels seen in *Ralstonia solanacearum* inoculated HI 7998 also helps to identify gene groups that may have expression level changes in response to *Ralstonia solanacearum*. The heat map of differential gene expression shows many similarities between both treatments of HI 7998 and both treatments of NC 359-2B(2010) with a distinct difference between the two line. The third grouping of genes deviate the most dramatically in HI 7998 plants inoculated with *Ralstonia solanacearum* compared to the other three (3) samples. The group of highly expressed genes in *Ralstonia solanacearum* inoculated HI 7998 show a distinct change from the same gene grouping which are generally lowly expressed in the other three samples. The group 1 (G1) cluster also shows a small grouping negatively expressed in

Ralstonia solanacearum inoculated HI 7998 that is generally more highly expressed in the other three (3) samples.

The genes that were found to be different in *Ralstonia solanacearum* inoculated HI 7998 versus water inoculated HI 7998 based on GO terms were found in two (2) major categories (biological process and cellular component), each with a further gene break down within each category. There were more GO terms within biological processes categories, each with between six (6) and eleven (11) genes being differentially expressed. This could be the result of several small changes that are occurring within the cell in response to the pathogen and acting as pathogenic defense. The gene classifications within the cellular component category had the greatest number of differentially expressed genes in three (3) different GO terms and is likely the result of a more complicated change in the cell. With over 50 of the genes identified functioning in membrane make up, gene expression could be changing how the cell is built up and reinforced through the cell and plasma membranes to prevent the spread of bacteria in the plant. When looking at the GO terms in each category, all seem very similar to each other and are likely functioning in cell wall, membrane, and plasma membrane for pathogenetic defense. However, most of the genes are being down-regulated which implies the biosynthesis and fortification of the cell wall and membranes is decreasing in response to infection. This could mean that the plant is preventing the growth and development of cells to contain the bacteria in already infected cells or prevent natural weak spots in cells as a result of cell growth.

Other studies have also found genes acting in defense mechanisms to be differentially expressed, but they tend to be signaling and other catalytic genes that function in defense (Chen et al., 2009; Ishihara et al., 2012). However, this could be attributed again to the differences in genetic resistance mechanisms in different tomato lines or the differences in the strain of

Ralstonia solanacearum infecting the plants. Given that resistance has been shown to not always be conveyed across *Ralstonia solanacearum* strains, this could be the reasoning for these differences (Jacobs et al., 2012; Kunwar et al., 2019). Some studies have also found structural changes that are happening within the plant in response to *Ralstonia solanacearum* infection (Diogo and Wydra, 2007; Wydra and Beri, 2006, 2007). Many of these structural changes are occurring in the cell walls of the plant and specifically in xylem cells in response to how the bacteria move and affect the plant (Wydra and Beri, 2006). Specific changes occurring in the xylem cells occurring as a result of *Ralstonia solanacearum* also include the build-up of lignin to block off specific cells and prevent the spread of the bacteria into other parts of the plant (Ishihara et al., 2012).

Several genes were found throughout this study to be differentially expressed based on different criteria and many of them may play a major role in conveying resistance to a race 1, phylotype II *Ralstonia solanacearum* strain in tomato line HI 7998. However, more studies would have to be conducted to verify that these genes are truly associated with bacterial wilt resistance. Verified genes could compared across other tomato varieties with different levels of resistance to bacterial wilt to see how those genes are changing in expression in different tomato resistance levels. These genes could also be looked at across time to see how these genes and gene expression are changing over time to get the best understanding how these genes are working in giving the resistant phenotype. The addition of GO terms and identifying specific function of genes responsible from this study and future studies can also help gain an understanding of how the plant responds on a physical level to *Ralstonia solanacearum* and how that response can change over time. Verified genes could also be used to look at how those genes associate with other genes nearby and potential identify resistance mechanisms that are not

linked with small fruit size and lower yields generally associated with bacterial wilt resistant tomato plants. With a more specific idea of what genes are responsible for bacterial wilt resistance, breeders and geneticists can utilize these genes for creating tomato lines with better and more stable resistance in tomatoes as well as better performing tomato lines that are also resistant to bacterial wilt.

Chapter 2: References

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Chapter 2: Tables

Table 2.1. Concentration of *Ralstonia solanacearum* inoculum in CFU/L for each replication. The target concentration was 10^7 CFU/L. Concentrations were taken from two rounds of serial dilutions and averaged to get a rough estimate of the concentration of the inoculum. Inoculum concentrations were within 1 to 2 E+07 CFU/mL of each other.

	Inoculation 1	***Inoculation 2***	***Inoculation 3***
Conc A	1.10E+07	2.40E+07	4.40E+07
Conc B	1.20E+07	1.80E+07	2.6E+07
mean	1.15E+07	2.10E+07	3.50E+07
sd	5.00E+05	3.00E+06	9.00E+06

Table 2.2. Environmental conditions affecting sampling and subsequent analysis. Weather conditions and plant numbers for each sample from both tomato lines, water and *Ralstonia solanacearum* (Rs) treatments, and both time points across all three replications during tissue harvest. Replications spanned from the end of summer to the beginning of fall and thus temperatures and humidity in the greenhouse decreased.

	Treatment	***Timepoint***	***Replication***	***# of plants***	***Temp. °F***	***Weather***
HI 7998	Water	48H	1	6	106	sunny
			2	6	98	sunny
			3	6	78	rainy
		72H	1	6	110	sunny
			2	6	88	cloudy
			3	6	76	rainy
	Rs	48H	1	6	106	sunny
			2	6	98	sunny
			3	6	78	rainy
		72H	1	6	110	sunny
			2	6	88	cloudy
			3	6	76	rainy
NC 359	Water	48H	1	6	106	sunny
			2	6	98	sunny
			3	6	78	rainy
		72H	1	6	110	sunny
			2	6	88	cloudy
			3	6	76	rainy
	Rs	48H	1	6	106	sunny
			2	6	98	sunny
			3	6	78	rainy
		72H	1	6	110	sunny
			2	4	88	cloudy
			3	4	76	rainy

Table 2.3. Differentially expressed genes between water inoculated HI 7998 and *Ralstonia solanacearum*. Fifty-one genes had a log_2 fold change in gene expression between *Ralstonia solanacearum* and water inoculated HI 7998 that resulted in an adjusted p-value <0.05. One gene had zero expression in the water treatment and some expression in the *Ralstonia solanacearum* treatment which gave a log_2 fold change of infinity and is indicated by a *.

Gene ID	Up/Down -regulated	Log_2 Fold Change	Adjusted P-value	Gene description
Solyc08g082130.2	Down	-1.6203	0.0066539	
Solyc01g005590.2	Down	-1.4186	0.0090987	
Solyc01g103360.2	Down	-1.4047	0.0090987	Glucuronoxylan 4-O-methyltransferase 1
Solyc11g012590.1	Down	-1.4436	0.0090987	CASP-like protein
Solyc02g032840.1	Down	-1.3925	0.010791	
Solyc11g069540.1	Down	-1.321	0.010791	
Solyc02g094050.2	Down	-1.3645	0.011985	
Solyc04g079480.2	Down	-1.3344	0.024629	
Solyc04g040210.2	Down	-1.3206	0.024855	
Solyc01g088780.2	Down	-1.399	0.026625	
Solyc02g077720.2	Down	-1.4039	0.026625	
Solyc03g005480.2	Down	-1.5177	0.026625	WAT1-related protein
Solyc04g058150.2	Down	-1.448	0.026625	Metallothionein-like protein type 2 A
Solyc08g075290.1	Down	-1.432	0.026625	
Solyc08g082120.2	Down	-1.3394	0.026625	
Solyc09g097890.2	Down	-1.3304	0.026625	Cytochrome b561 and DOMON domain-containing protein
Solyc12g013650.1	Down	-1.4577	0.026625	
Solyc12g014200.1	Down	-1.2423	0.026625	Protein trichome birefringence-like 31
Solyc03g120120.2	Down	-1.2852	0.02822	
Solyc04g007590.1	Down	-1.3939	0.02822	WAT1-related protein
Solyc07g065940.1	Down	-1.3035	0.02822	
Solyc09g009620.1	Down	-1.4338	0.02822	
Solyc09g089890.1	Down	-1.2999	0.02822	
Solyc11g008880.1	Down	-1.2325	0.028908	CRIB domain-containing protein RIC4
Solyc07g062210.2	Down	-1.2178	0.029684	
Solyc00g313930.1	Down	-1.6793	0.037643	-//-
Solyc09g008660.2	Down	-1.4153	0.037643	
Solyc10g049580.1	Down	-1.245	0.037643	
Solyc11g069250.1	Down	-1.4194	0.037643	
Solyc06g082860.2	Down	-1.2733	0.038076	
Solyc02g083480.2	Down	-1.1828	0.042145	Peroxidase
Solyc06g051350.2	Down	-1.1463	0.045825	
Solyc04g014550.2	Down	-1.2661	0.045975	

Table 2.3 (continued)

Gene ID	Up/Down -regulated	Log_2 fold change	Adjusted p-value	Gene description
Solyc10g007960.1	Up	3.8182	0.0090987	Allene oxide synthase 3
Solyc12g009560.1	Up	1.3383	0.011985	EIN3-binding F-box protein 1
Solyc09g074270.2	Up	1.4892	0.024855	
Solyc01g109320.2	Up	3.0052	0.026625	Protein DETOXIFICATION
Solyc02g082630.2	Up	2.4266	0.026625	
Solyc05g021580.2	Up	3.4948	0.026625	
Solyc07g006560.2	Up	2.5789	0.026625	
Solyc08g021890.2	Up	1.5961	0.026625	
Solyc02g038740.2	Up	1.6049	0.02822	3-hydroxy-3-methylglutaryl coenzyme A reductase
Solyc04g076980.2	Up	1.724	0.02822	
Solyc05g054860.1	Up	1.4296	0.02822	
Solyc10g076240.1	Up	3.3435	0.02822	Peroxidase
Solyc11g062220.1	Up	1.2778	0.034317	
Solyc06g053570.2	Up	1.4168	0.042546	
Solyc00g233480.1	Up	2.3938	0.042604	-//-
Solyc10g008230.1	Up	2.1579	0.042604	
Solyc09g097960.2	Up	2.194	0.045825	
Solyc09g090210.2	Up	Inf*	0.048172	

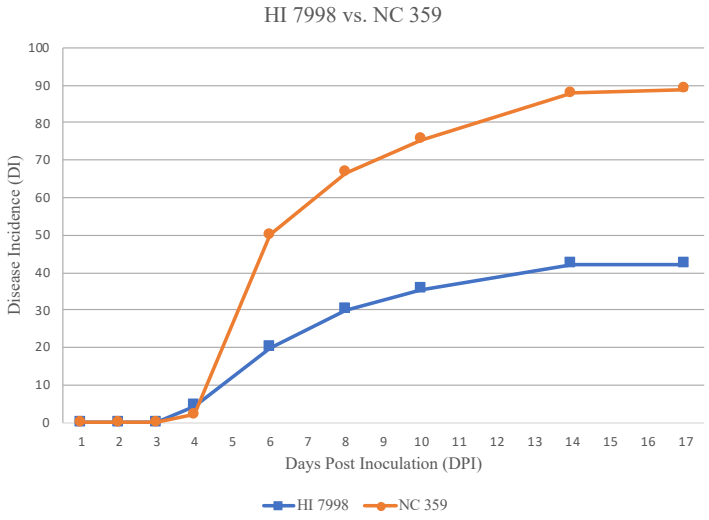
Table 2.4. Significantly expressed genes categorized by gene ontology (GO) terms. 144 genes were differentially expressed based on GO terms falling into two major categories: biological processes and cellular components. GO terms were found significant with an adjusted p-value of <0.05.

Term Type	Description	Corrected P-value	Up	Up Gene Names	Down	Down Gene Names
Biological Process	plant-type secondary cell wall biogenesis	0.000377	0	-	0	Solyc02g086430.2, Solyc01g103360.2, Solyc11g069250.1, Solyc06g075220.1, Solyc09g007420.2, Solyc09g007660.1
	cell wall biogenesis	0.001831	0	-	0	Solyc11g069250.1, Solyc09g007660.1, Solyc06g075220.1, Solyc09g007420.2, Solyc12g014200.1, Solyc02g086430.2, Solyc01g103360.2, Solyc11g008880.1
	plant-type cell wall biogenesis	0.001831	0	-	0	Solyc01g103360.2, Solyc11g069250.1, Solyc02g086430.2, Solyc11g008880.1, Solyc09g007660.1, Solyc09g007420.2, Solyc06g075220.1
	plant-type cell wall organization or biogenesis	0.01041	0	-	0	Solyc09g007660.1, Solyc09g007420.2, Solyc06g075220.1, Solyc11g069250.1, Solyc11g008880.1, Solyc01g103360.2, Solyc02g086430.2, Solyc02g083480.2
	cell wall organization or biogenesis	0.02428	0	-	0	Solyc11g008880.1, Solyc12g014200.1, Solyc01g103360.2, Solyc07g062210.2, Solyc02g086430.2, Solyc02g083480.2, Solyc09g007660.1, Solyc09g007420.2, Solyc06g075220.1, Solyc06g051350.2, Solyc11g069250.1
Cellular Component	membrane	0.001108	24	Solyc05g053600.2, Solyc06g062920.2, Solyc09g009350.2, Solyc02g090560.2, Solyc12g036140.1, Solyc05g050360.2, Solyc08g077490.1, Solyc06g076800.2, Solyc07g063730.1, Solyc09g090210.2, Solyc12g009520.1, Solyc01g109320.2, Solyc05g054860.1, Solyc02g080070.2, Solyc02g089090.2, Solyc09g075020.2, Solyc02g092860.2, Solyc11g010330.1, Solyc03g044160.1, Solyc08g021890.2, Solyc10g008230.1, Solyc12g094650.1, Solyc09g098030.2, Solyc02g038740.2	24	Solyc11g069540.1, Solyc04g081890.1, Solyc06g083440.2, Solyc12g005690.1, Solyc12g013650.1, Solyc09g089890.1, Solyc01g088780.2, Solyc02g094050.2, Solyc07g048050.1, Solyc04g007590.1, Solyc09g007420.2, Solyc10g084980.1, Solyc07g062210.2, Solyc10g049580.1, Solyc02g081890.2, Solyc03g007360.2, Solyc11g008880.1, Solyc06g075220.1, Solyc11g012590.1, Solyc08g075290.1, Solyc09g097890.2, Solyc01g005590.2, Solyc03g005480.2, Solyc06g051350.2, Solyc09g008660.2, Solyc12g014200.1, Solyc06g082860.2, Solyc11g069250.1, Solyc04g040210.2, Solyc09g007660.1, Solyc10g080380.1, Solyc06g054320.1
	plasma membrane	0.01041	11	Solyc05g054860.1, Solyc02g080070.2, Solyc02g089090.2, Solyc02g090560.2, Solyc10g008230.1, Solyc05g050360.2, Solyc03g044160.1, Solyc09g090210.2, Solyc07g063730.1, Solyc05g053600.2, Solyc06g062920.2	11	Solyc10g049580.1, Solyc03g007360.2, Solyc11g008880.1, Solyc11g069250.1, Solyc04g040210.2, Solyc11g012590.1, Solyc06g075220.1, Solyc09g007660.1, Solyc03g005480.2, Solyc01g088780.2, Solyc02g094050.2, Solyc04g007590.1
	cell periphery	0.017024	12	Solyc05g053600.2, Solyc06g062920.2, Solyc07g063730.1, Solyc09g090210.2, Solyc02g089370.2, Solyc02g089090.2, Solyc02g080070.2, Solyc05g054860.1, Solyc02g090560.2, Solyc05g050360.2, Solyc03g044160.1, Solyc10g008230.1	12	Solyc03g005480.2, Solyc01g088780.2, Solyc02g094050.2, Solyc04g007590.1, Solyc02g083480.2, Solyc11g069250.1, Solyc04g040210.2, Solyc06g075220.1, Solyc11g012590.1, Solyc09g007660.1, Solyc10g049580.1, Solyc03g007360.2, Solyc11g008880.1

Chapter 2: Figures

Figure 2.1. Tomato plants at the time of inoculation. Plants were 5 weeks old at time of sampling. Tissue samples for RNA extraction were harvested from a 1 cm section of hypocotyl tissue represented by a red rectangle.

A



B

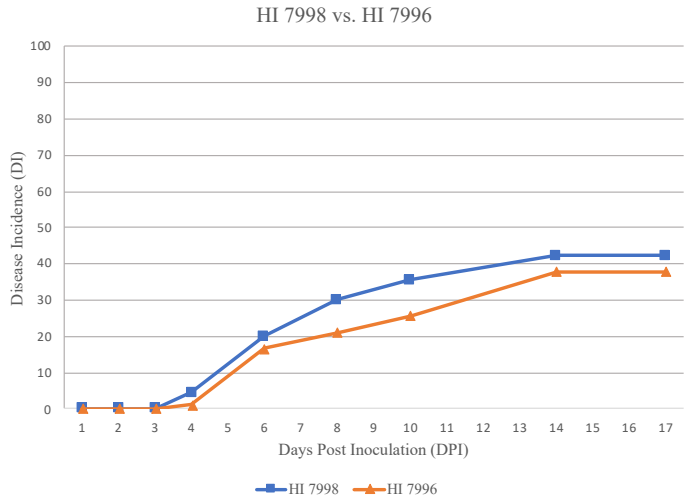
	n	Days post inoculation (dpi)						
		3	4	6	8	10	14	17
HI 7998	18	0	4.44	20	30	35.56	42.22	42.22
NC 359	18	0	2.22	50	66.67	75.56	87.78	88.89

C

D

Figure 2.2. Bacterial wilt response in resistant HI 7998 and susceptible NC 359-2B(2010). (A-B) Bacterial wilt disease incidence (DI) over time after inoculation with *Ralstonia solanacearum*. Despite being resistant, some HI 7998 plants still exhibited bacterial wilt symptoms, but NC 359-2B(2010) showed higher disease incidence overall. Phenotypic response to Ralstonia solanacearum in HI 7998 (C) and NC 359-2B(2010) (D) compared to a water inoculated control at 12 dpi.

A



B

		Days post inoculation (dpi)						
	n	3	4	6	8	10	14	17
HI 7998	18	0	4.44	20	30	35.56	42.22	42.22
HI 7996	18	0	1.11	16.67	21.11	25.56	37.78	37.78

C

D

Figure 2.3. Bacterial wilt response in resistant lines HI 7998 and HI 7996. (A-B) Bacterial wilt disease incidence (DI) over time after inoculation with *Ralstonia solanacearum*. Both lines show similar rates of disease incidence despite HI 7996 being considered more resistant. Phenotypic response to Ralstonia solanacearum in HI 7998 (C) and HI 7996 (D) compared to a water inoculated control at 12 dpi.

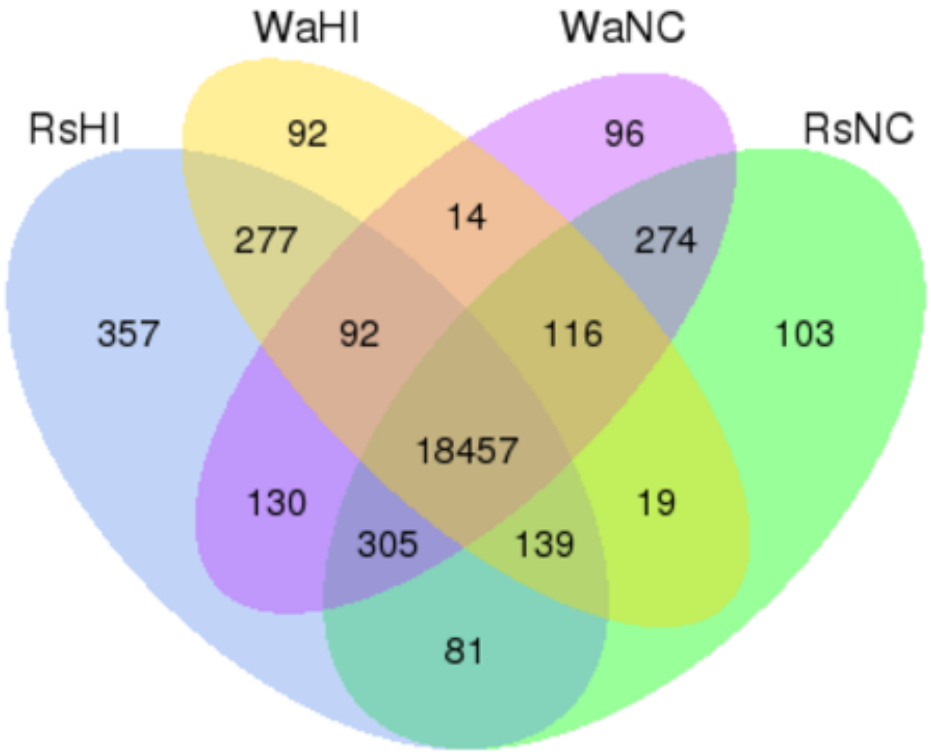


Figure 2.4. Number of uniquely expressed genes between water and *Ralstonia solanacearum* inoculated HI 7998 and NC 359-2B(2010) tomato lines. Ven diagram of number of genes being uniquely expressed in *Ralstonia solanacearum* inoculated HI 7998 (RsHI) and NC 359-2B(2010) (RsNC) and water inoculated HI 7998 (WaHI) and NC 359-2B(2010) (WaNC) as well as each combination of samples. The 357 uniquely expressing genes in Ralstonia solanacearum inoculated HI 7998 show a dramatic increase over the 92, 96, and 103 uniquely expressing genes in the remaining three samples.

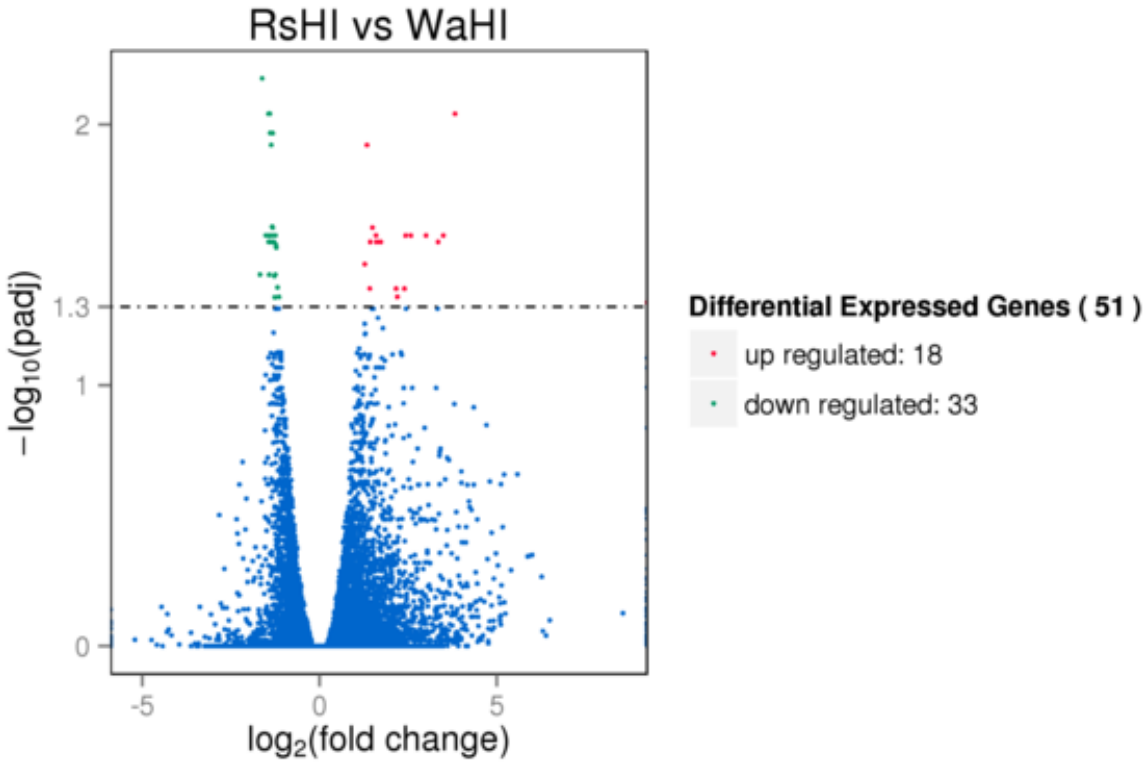


Figure 2.5. Differentially expressed genes between water and *Ralstonia solanacearum* inoculated HI 7998. Volcano plot of differential gene expression between *Ralstonia solanacearu*m inoculated HI 7998 (RsHI) and water inoculated HI 7998 (WaHI). Statistical significance of $\log_{10}$ change between samples set at 1.3 with 51 genes being differentially expressed. No genes passed the significance threshold when comparing *Ralstonia solanacearum* and water inoculated NC 359-2B(2010).

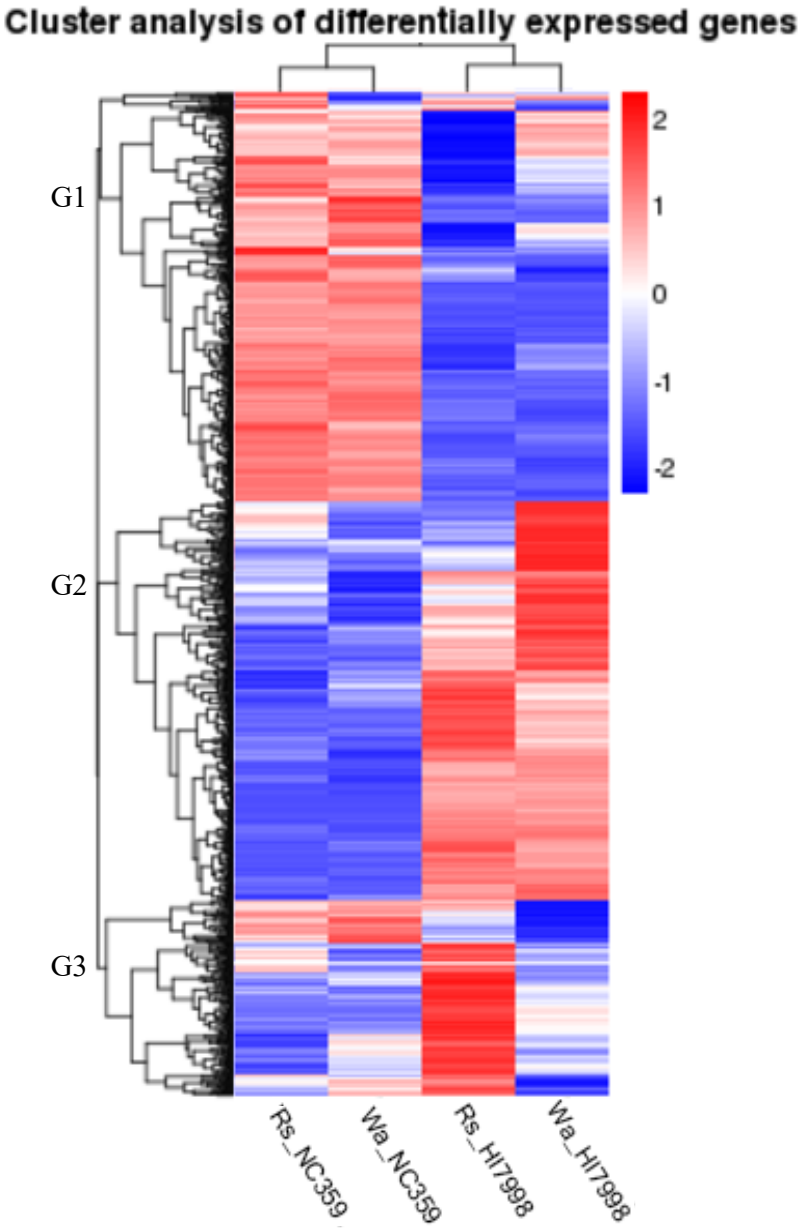


Figure 2.6. Comparison of levels of differential gene expression in water and *Ralstonia solanacearum* inoculated HI 7998 and NC 359-2B(2010) lines. Heat map of expression levels of various genes in *Ralstonia solanacearum* inoculated HI 7998 (Rs_HI7998) and NC 359-2B(2010) (Rs_NC359) and water inoculated HI 7998 (Wa_HI7998) and NC 359-2B(2010) (Wa_NC359). Red color indicates high levels of expression and blue indicates low levels of expression. Clusters were found using $\log_{10}$(FPKM+1) value. Genes clustered in three major groups, group 1 (G1), group 2 (G2), and group 3 (G3) for comparisons.

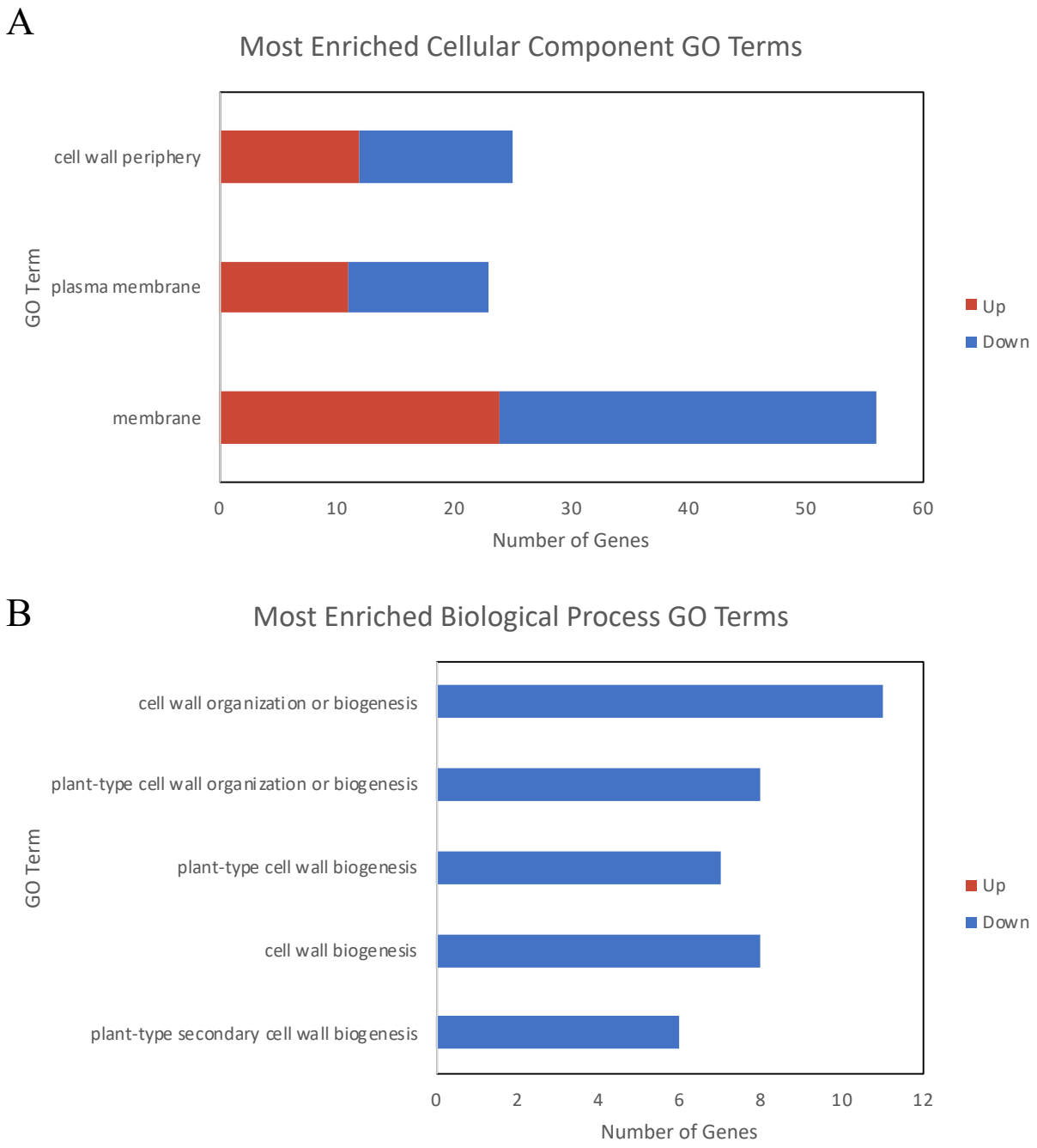


Figure 2.7. Break down of gene type in differentially expressed genes between *Ralstonia solanacearum* and water inoculated HI 7998. (A) 104 genes were found to be differentially expressed in the cellular component category consisting of three (3) GO terms. 47 of those genes were up-regulated and 57 were down-regulated (B) 40 genes were found to be differentially expressed in the biological process category with five (5) GO terms. All genes in this category were down-regulated.