ABSTRACT

KRESSIN, JONATHAN PAUL. Bacterial Wilt (*Ralstonia solanacearum*) of Tomato (*Solanum lycopersicum*): Analyses of the Interactions of Host Resistance under Field and Greenhouse Conditions with Two Bacterial Strains, Vascular Browning of Stem, Low Temperature Shock Stress, Microbe-Associated Molecular Pattern-Triggered Immunity, and Relative Expression of Potential Resistance Loci. (Under the direction of Dr. Dilip R. Panthee and Dr. Frank J. Louws.)

Bacterial wilt (BW) (*Ralstonia solanacearum* Smith) (Rs) is a devastating soil-borne disease of the economically important tomato (*Solanum lycopersicum* L.), and is endemic in many parts of the Southeastern USA fresh-market tomato growing regions. Rs infects host vascular tissues through wounds and sites of secondary root emergence while, dramatically altering its phenotype to include production of large quantities of extracellular polysaccharides, leading to permanent wilting of susceptible hosts and degradation of vascular bundles. Management of BW is very challenging, although host resistance can be mobalized for good control by grafting susceptible commercial varieties onto resistant rootstocks. Resistance to BW is quantitative, polygenic in nature, and is heavily modulated by environmental influences and variations between regional strains. Tomato resistance remains enigmatic, but it is known that Rs multiplication and spread upon vascular colonization is suppressed. Stimulation of defense hormone signaling pathways occurs, as well as host production of reactive oxygen species (ROS). Pattern recognition receptors (PRRs) detect microbial pathogen components and stimulate defense responses through Microbe/Pathogen-associated molecular pattern (MAMP/PAMP)-triggered immunity (MTI/PTI) and effector-triggered immunity (ETI). The receptors of MTI and ETI are core components of plant immune responses, often having common motifs. Many gene loci

predictions within the tomato genome database contain these motifs and reside within the most important chromosome 6 BW resistance QTL.

The objectives of this research were to investigate tomato rootstock resistance to BW as modulated by graft wound, diverse NC localized Rs isolates, vascular browning variation, and low temperature shock stress. Additionally, I investigated the potential of using a laboratory-based assessment of MTI to predict BW resistance with general, and an Rs-specific, peptides. Lastly, I tested a selection of loci with defense-related motifs in the BW resistance QTL on chromosome 6 in order to determine if genome database mining could facilitate discovery of resistance genes when combined with relative expression analysis. Thus, summer field and winter greenhouse studies were conducted in 2013-14 using multiple tomato rootstock varieties from diverse sources in a conventional field production system with natural or artificial inoculum.

I found that neither grafting nor the NC Rs isolates significantly modulated genotype resistance levels, and the rootstocks formed a spectrum of resistance. 'RST-04-105-T' (DP Seed) has previously been reported as being moderately to highly resistant, but unexpectedly clustered with the susceptible control. 'Hawaii 7997', 'Hawaii 7998', 'Cheong Gang' (Seminis), and 'RST-04-106-T' (DP Seed) had similarly high resistance and are expected to perform well in NC grafted tomato production systems. The natural application of mid-epidemic low temperature shock stress in the greenhouse study increased the incidence of BW, leading to more effective separation of the genotypic mean wilt resistance levels. End-of-study vascular browning scores of stem cross-sections was a significant predictor of foliar wilt, improving variance assessments, and is the first report to the author's knowledge

comparing vascular browning variation with tomato resistance to BW. Stimulation of ROS production in tomato was successful using the MAMPs FlgII-28, Csp22, and a mutant version of Flg22 (Pa Flg22), but not Rs-specific Flg22. Although significant variation was observed between the genotypes, non- and self-graft treatments, and pre/post Rs inoculation, ROS production was not predictive of BW resistance. The physical location of the chromosome 6 BW resistance QTL was documented, and dozens of loci potentially related to defense responses were identified, but no clear changes in gene expression for any of the 25 target loci were observed at 3 days post inoculation in 'Florida 47' or 'Cheong Gang'. This research has important applications to NC grafted tomato production for management of BW, BW screening methodologies, and provides foundational information for MTI mobilization in the tomato-Rs pathosystem.

Bacterial Wilt (*Ralstonia solanacearum*) of Tomato (*Solanum lycopersicum*): Analyses of the Interactions of Host Resistance under Field and Greenhouse Conditions with Two Bacterial Strains, Vascular Browning of Stem, Low Temperature Shock Stress, Microbe-Associated Molecular Pattern-Triggered Immunity, and Relative Expression of Potential Resistance Loci

by
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DEDICATION

Soli Dei Gloria Christos Jesus. Your creation sings of you. Слава Богу!

To my wife Mary.

Thanks for your support, prayers, patience, and help. I love you!

To Krishna Bhattarai.

Thanks for your friendship, help, and encouragement throughout the whole Masters program process!

BIOGRAPHY

Raised in the desert Southwest, Jonathan Kressin has always had a heart for wide open spaces, the great outdoors, and the beauty of nature. His love for science budded as a child when the thrill of discovery and the inquisitive mind shook hands. Homeschooled all his life, Jonathan counts that some of his most defining and helpful life lessons were learned not in the classroom, but in the real world putting education into practice. Whether that was leadership development, music, competitive public speaking, or being around friends, life was always brought back to the questions, "What can I learn from this?" and "How can I make this be better?" He has always been a bit of a natural teacher and counselor, and seeks the wisdom to do that with compassion, humility, and grace. Though generally reserved on the outside, Jonathan is full of passions for truth, right living, and being involved in the building of that which endures. Jonathan gives all credit and thanks to Jesus Christ, his savior and Lord, for all that is good and right in him. *Soli dei Gloria!*

Jonathan's passion for plant sciences came later in highschool. He is someone who loves plants! And science… which is why he has pursued plant genetics. As a very visual thinker, the physical changes and interactions of life processes thrill him! Coming to North Carolina State for graduate school was a bit by accident. A casual after class conversation with a professor led to applying to NCSU, being accepted, and joining in a research team aimed at mobilizing basic host-pathogen research into practical solutions for those who need them. While the journey has not been a breeze, it has been good. After all, some of the hardest experiences in life are the most meaningful and most worth doing.

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LIST OF ABBREVIATIONS

Abbreviation	Description of term
• 3-OH-PAME:	3-OH-Palmitic Acid Methyl Ester
• ABA:	Plant hormone; abscisic acid
• AUDPC:	Area under disease progress curve
• Avr gene:	Avirulence gene
• bp:	Base pairs of nucleotides
• bv_:	Biovar of pathogen; Biovar 1 (bv1), Biovar 2 (bv2), etc.
• BW:	Bacterial Wilt
• chr.:	Chromosome
• cM:	Centimorgans; mapping unit used to describe relative genetic distances
• dpi:	Days post inoculation
• dpp, dap:	Days post planting, days after planting; terms are equivalent
• EPS I:	Extracellular polysaccharide I
• ETI:	Effector-triggered immunity
• HR:	Hypersensitive response
• IPM:	Integrated pest management
• JA, SA, ET:	Plant hormones: Jasmonic acid, salicylic acid, and ethylene
• LPS:	Lipopolysaccharide
• LSMean:	Least squares mean

- MAMP: Microbe-associated molecular pattern
- Mb: Megabases; 1 Mb is equal to 1,000,000 nucleotide bases
- MTI: MAMP-triggered immunity
- PAMP: Pathogen-associated molecular pattern; a subset of MAMP
- Phc: Phenotype conversion system in Rs
- phy_: Phylotype of pathogen; Phylotype I (phyI), phylotype II (phyII), etc.
- PRR: Pattern recognition receptor
- PTI: Pathogen-triggered immunity; a subset of MTI
- qRT-PCR: Quantitative real-time reverse-transcription polymerase chain reaction
- R gene: Resistance gene; classically associated with vertical resistance
- R_: Race of pathogen; Race 1 (R1), Race 2 (R2), etc.
- RCBD: Randomized complete block design
- RFLP: Restriction fragment-length polymorphisms
- ROS: Reactive oxygen species
- Rs: *Ralstonia solanacearum* (Smith)
- SAR: Systemic-acquired resistance
- SNP: Single-nucleotide polymorphisms
- SSR: Simple sequence repeats
- T2SS: Type-II secretion system found in bacteria
- T3SS: Type-III secretion system found in bacteria
- Vir gene: Virulence gene

CHAPTER 1: LITERATURE REVIEW

1.1: Introduction

Plant diseases impact food supply quality and availability, local and global markets, and human and animal health and wellbeing. Many examples of severe hardship and loss due to plant diseases have been well documented. Despite many useful advances in the ability to manage plant disease issues, it is estimated that as much as 35% of global annual food production is lost due to plant pests, with the subcategory of plant pathogens and viruses responsible for 10-15% of global production losses (Popp and Hantos, 2011; Strange and Scott, 2005).

As a species globally, tomato (*Solanum lycopersicum* L.) is known to be susceptible to at least 200 different disease-causing organisms, causing scores of devastating diseases that reduce food quality, economic value, and crop production sustainability. This review focuses on one of the many important tomato diseases, Bacterial Wilt (BW) of tomato caused by *Ralstonia solanacearum*, examining what is known about the world importance of the disease, host and pathogen biology, genetics, and specific endeavors to increase the ability to control the disease in the Southeastern United States of America (USA), especially the state of North Carolina (NC). Although there are many plants susceptible to BW, Solanaceous plants are very susceptible, including tomato, potato, pepper, tobacco, and eggplant. Most of the research on this disease has been performed with the specific interactions of *R. solanacearum* and either tomato, potato, tobacco, or *Arabidopsis thaliana*. This review, however, will focus primarily on the work done in tomato.

1.2: Bacterial Wilt Disease

Bacterial Wilt disease (BW), also known as Southern Bacterial Wilt or Southern Wilt, is a vascular wilt disease of many horticulturally important fruit and vegetable crops in the Southeastern USA and around the world. A landmark exhaustive review of the first five decades of research on BW was written by (Kelman, 1953), which helped cement the foundational works and methodologies for BW research.

Just as the name implies, BW is characterized by foliar wilting symptoms in infected hosts. Plants in the early stages of wilting are known to 'recover' when temperatures cool down during the night (Clayton and Smith, 1942; Kelman, 1953; Moorman, 2014). In Solanaceous species, the interval from first wilting to permanent wilting point can be as short as a few days or as long as several weeks, depending on environmental conditions, host age and vigor, strain aggressiveness, and the resistance level of the hosts (Gallegly and Walker, 1949; Kelman, 1953; Mew and Ho, 1976; Mew and Ho, 1977; Zehr, 1970). Internal symptoms are most apparent in fully wilted plants, which are characterized by browning of the vascular bundles leading to tissue degradation and pith necrosis (Kelman, 1953). Stems exhibiting vascular browning are usually filled with very high concentrations of bacteria (Araud-Razou et al., 1998; Vasse et al., 1995), which can be observed as a thick, milky stream oozing out of cut stems immersed in water (Moorman, 2014; Olson, 2005). Stunting is another common symptom, and occasionally foliar chlorosis is apparent in some species (Kelman, 1953; Meng, 2013). There is also an increase in adventitious root formation up the stems of tomato that is related to bacterial invasion of the large primary vascular bundles (Kelman, 1953).

Caused by the soil bacterium *Ralstonia solanacearum* Smith (Smith, 1896) (Rs), BW has a worldwide distribution between the N45 and S45 parallels in tropical, subtropical, and temperate regions with high rainfall (c.100 cm/year or more), a growing season of at least 6 months where the average winter and summer temperatures do not drop below 10 ^{o}C and 21 ^{o}C, respectively, and where the yearly average temperature is less than 23 ^{o}C (Lucas, 1975).

Of the more than 200 plant species in 50 different plant families infected by Rs (Buddenhagen and Kelman, 1964; Hayward, 1964; Hayward, 1991; Hayward, 1994; Hayward, 1995; Moorman, 2014; Olson, 2005), members of family Solanaceae are among the most economically important crops. Potato (*Solanum tuberosum* L.) and tomato (*Solanum lycopersicum* L.) were the first and second most important vegetable crops worldwide in 2001, respectively (FAO-Land and Water Division, 2013), and most commercial varieties are highly susceptible to BW. Tobacco (*Nicotiana tabacum* L.) is also an economically important crop susceptible to Rs, especially in the Southeastern USA. Other noteworthy plant species susceptible to Rs infection are: Eggplant (*Solanum melongena*), Pepper (*Capsicum* spp.) Geraniums (*Pelargonium* spp.), Banana (*Musa* spp.), and Arabidopsis (*Arabidopsis thaliana*) (Kelman, 1953; Moorman, 2014)

1.3: The Pathogen—*Ralstonia solanacearum*

Ralstonia solanacearum is an aerobic, gram-negative, rod-shaped, soil β-proteobacterium (Hayward, 1991; Palleroni and Doudoroff, 1971; Palleroni, 1984; Schell, 2000). Originally described by E.F. Smith in 1896 as the causal agent of BW (Smith, 1896), Rs has been taxonomically reclassified many times over the last century. Historical

classifications include *Bacillus solanacearum* Smith, *Phytomonas solanacearum* Smith, *Pseudomonas solanacearum* Smith, and *Burkholderia solanacearum* (Yabuuchi et al., 1992). After additional phylogenetic work, the genus *Ralstonia* was formed for a subset of bacteria from *Burkholderia* (Schell, 2000). The taxonomic identity of Rs is not entirely settled, due to recent comparative genomic studies supporting a growing opinion that Rs is actually a species complex perhaps worthy of reclassification into several new species (Genin and Denny, 2012; Remenant et al., 2010).

Strain characterization of Rs has been accomplished either by molecular biology techniques and host affinity assays (races), comparative metabolic assays (biovars), or by genomics (phylotypes) (Hayward, 1991). Rs has been classified into five races and five biovars, with a review given by (Agrios, 2005). Race 1 is found endemically in regions of North and South America (including the Southeastern USA), and South Asia, causing the majority of economic loss worldwide. Race 2 is tropical, and does not cause much economic loss in Solanaceous crops. Race 3 is a cooler climate strain highly virulent to potato, but is not present in the USA. Race 3 biovar 2 (R3 bv2) was inadvertently introduced into the USA on geraniums several decades ago, but has been eradicated or at least effectively quarantined so far. R3 bv2 is, however, still considered to be highly dangerous to American agriculture because it is highly virulent to potato and able to cause disease in more Northern temperate regions, which led to it being listed as a USDA-APHIS Select Agent (Representative Tauzin, 2002; USDA-APHIS, 2012) out of concern for its effects on the North American potato industry if it were ever to become established in the continent. Races 4 and 5 cause disease in plants of little importance in world agriculture (Agrios, 2005). Phylogenetic studies have

found that Rs strains can be grouped into four major phylotypes grouped geographically: I (Asia), IIa and IIb (Americas), III (Africa), and IV (Indonesia) (Remenant et al., 2010)

On a genetic level, Rs has an uncommon genome arrangement with two independently replicating replicons (circular genomes). The larger replicon (3.8 megabases (Mb)) codes for most of the basic proteins involved in cellular function, whereas the smaller one (1.9 Mb), previously called the mega plasmid, contains many of the pathogenicity and virulence genes (Boucher et al., 1986; Schell, 2000). In addition, the genome of some strains examined is thought to contain many possible transposable elements, which may contribute to the pathogen's high genetic variability. Genome-wide studies of representative strains of Rs from around the world suggest that the Rs species complex contains about 2,850 conserved genes and a variable genome with about 3,100 genes (Remenant et al., 2010; Remenant et al., 2011). Genetic analyses of several key housekeeping and virulence genes reveal, however, that Rs can be classified into two geographic divisions, one centered in Asia and the other in the Americas (Schell, 2000). It is also important to note that the genomes of multiple phylotypes (phy), biovars (bv), and races (R) of Rs have been sequenced and are publicly available (Salanoubat et al., 2002):
https://iant.toulouse.inra.fr/bacteria/annotation/cgi/ralso.cgi

1.4: The host—*Solanum lycopersicum*

Tomato (*Solanum lycopersicum* L.), as mentioned previously, is a very important vegetable crop around the world, being sought for its adaptable culinary qualities, as well as being developed as a model plant system for fruit development (The Tomato Genome

Consortium, 2012). Synonymous with *Lycopersicon esculentum* Mill., tomato is an herbaceous perennial dicot plant in Solanaceae with yellow flowers, pinnately compound green leaves, and either a determinate or indeterminate growth habit (Jones, 2008). Production, however, usually treats them as herbaceous annuals. The economic importance of tomato, however, is a relatively recent development of the last 120 years or so, probably restricted by a common European belief at that time that tomato fruit were poisonous (Jones, 2008). Tomato fruits are botanically a berry and have a diverse range of size, shape, color, total soluable solids, and sugar and acid content.

Tropical in nature, tomato has been bred and adapted for many environments around the world, being grown primarily in open field environments, as well as greenhouse and hydroponic production systems. *S. lycopersicum* specifically is thought to have its center of origin along the coast of Western South America (especially Peru and Ecuador), and was only introduced to Europe after being brought back with Spanish explorers in the A.D. 1500s, though widespread introduction took another 200 years (Jones, 2008). It is believed to have first been domesticated in Mexico.

Genetically, tomato has a diploid genome with 12 chromosomes ($2n = 2x = 24$) with an approximate total size of 900 Mb (The Tomato Genome Consortium, 2012). Tomato is self-fertile and easily self-pollinates, normally being bred for commercial markets as F_1 hybrids or inbreds. Open-pollinated varieties do exist as well. Within the realm of tomato breeding, especially breeding for disease resistance traits, genetically important wild relatives of tomato include: Potato, eggplant, *Solanum arcanum* Peralta, *S. chilense* (Dunal) Reiche, *S. habrochaites* S. Knapp and D.M. Spooner, *S. peruvianum* L., and especially *S.*

pimpinellifolium L. (closest wild relative (Peralta et al., 2008)). Dr. Charles Rick (1915-2002; http://tgrc.ucdavis.edu/charlie.aspx) was a pioneer in collection and preservation of tomato germplasm from around the world, and was especially well known for his work in introgressing many wild species into our domesticated varieties (Rick, 1960; The Tomato Genome Consortium, 2012). Disease resistance breeding has benefited greatly from his foundational work.

The genome sequence for *S. lycopersicum* 'Heinz 1706' was recently released (2012), showing only a 0.6% genetic divergence from *S. pimpinellifolium* 'LA1589', whereas it has an 8% divergence from potato (The Tomato Genome Consortium, 2012). All three species have sequenced genomes, which are available: http://solgenomics.net/ (The Tomato Genome Consortium, 2012). The tomato genome annotation pipeline predicted that the publically available sequence contained 34,727 protein-coding genes, though RNA sequence data only predicted 30,855. A comparison with the *Arabidopsis* genome (http://www.arabidopsis.org/) found that 31,741 predicted genes were highly similar. *S. pimpinellifolium* was also quite similar to tomato (The Tomato Genome Consortium, 2012).

On a production scale, the USA ranks second in the world for tomato production, producing 12.9 million metric tons in 2009 valued at more than $2 billion in annual farm cash receipts (Novagrim, 2010; Thornsbury, 2012). Globally, about 100 million tons of tomatoes were produced in 2010 (Novagrim, 2010; USDA-Economic Research Service, 2009). In 2010, the top 10 tomato producing countries in the world ranked by total weight of production were China, USA, India, Turkey, Egypt, Italy, Iran, Spain, Brazil, and Mexico (Novagrim, 2010). Of these nations, BW of Solanacous crops has been reported at least in

China, USA, India, Turkey (Ustun et al., 2009), Egypt (Balabel et al., 2005), Iran (Bagheri and Taghavi, 2000), and Mexico (Hernández-Romano et al., 2012), though not exclusively these.

Within the USA, tomato production is classically divided into processing and fresh market, where varieties and production systems are developed specifically to serve each production type. Varieties effective for processing type are not well adaptable to fresh market type, and vice versa. This production pattern is not replicated in many other tomato producing nations (Thornsbury, 2012). The greatest overlap of USA tomato production and BW is in the Southeastern states. Major tomato growing states with BW problems are Florida, Virginia, and NC, where tomato production is nearly entirely fresh market type. Florida was the second largest fresh market tomato producing state in 2012, growing about 35% of the USA total production. Virginia grew about 7%, and NC grew about 4% of national fresh market production (USDA-National Agricultural Statistics Service, 2013).

Apart from tomato and the previously mentioned other Solanaceous crops pathogenized by Rs, other noteworthy plant species are also infected, including alternate weed and ornamental hosts (Hayward, 1994; Kelman, 1953). Common weed hosts include: Pigweed (*Amaranthus* spp.), ragweed (*Ambrosia* spp.), horseweed (*Conyza* spp., formerly the genus *Erigeron*), cocklebur (*Xanthium* spp., especially *X. pennsylvanicum*), and jimson weed (*Datura* spp., especially *D. stramonium*) (Kelman, 1953). Common ornamental hosts include: Chrysanthemum (*Chrysanthemum* spp.), dahlia (*Dahlia* spp.), African marigolds (*Tagetes erecta*), zinnia (*Zinnia elegans*), garden petunia (*Petunia hybrid*), nasturtium (*Tropaeolum* spp.), and verbena (*Verbena* spp.) (Kelman, 1953). Also, some other

agricultural crops/herbs include: Sunflower (*Helianthus annuus* L.), cassava (*Manihot esculenta*), peanut (*Arachis hypogaea*), bean (*Phaseolus vulgaris*), sesame (*Sesamum indicum*), and members of Zingiberaceae including ginger (*Zingiber officinale*), sand ginger (*Kaempferia galanga*), turmeric (*Curcuma* spp.), and cardamom (*Elattaria* and *Ammomum* spp.) (Kelman, 1953; Kumar et al., 2014).

1.5: Host-Pathogen Interactions

Bacterial Wilt is considered by many to be one of the most economically destructive plant bacterial pathogens, but also one of the most scientifically valuable. This is due to the highly aggressive nature of Rs, as well as, the wide geographic distribution and very large host range it has (Prior et al., 1998). A survey of the community of the journal *Molecular Plant Pathology* received 458 votes for the 'Top 10' important plant pathogenic bacteria in 2012, and Rs ranked as the second most scientifically and economically important bacteria, right behind *Pseudomonas syringae* pathovars (Mansfield et al., 2012). The key to Rs pathogenicity appears to lie in its ability to produce dozens of potent and often redundant virulence factors into the host systems that suppress host defense responses, while enhancing the ability of the bacteria to spread through the surrounding tissues (Schell, 2000).

Tomato resistance to BW is known to be polygenic and quantitative in nature, with even the most resistant varieties still being colonized by Rs (Hayward, 1991). In tomato, Rs resistance does not appear to be related to the common hypersensitive response (HR) that causes localized cell death to halt pathogen invasion, but rather is related to mechanisms that traditionally have been termed tolerance (Agrios, 2005).

1.5.1: Host-Pathogen Interactions—Biological and Genetic

A valuable review of the plethora of virulence factors, their theorized relationships, and known functions was published by (Schell, 2000), and many authors have added onto the basic functional information in that review. On a molecular level, there are more than 20 different interacting gene products that allow the bacteria to be pathogenic (Schell, 2000). These host of virulence factors include many diverse proteins such as transcription initiators and repressors, membrane-bound and cytosolic chemoreceptors, mobility proteins, exogenous lytic enzymes, and the well known type-III secretion system (T3SS) (JhyGong and HsuLiang, 2012; Li et al., 2010; Nakaho and Takaya, 1993; Nakaho et al., 2000; Nakaho and Allen, 2009; Schell, 2000). More recently, (Meng, 2013) published a more succinct review of what are currently considered the most important virulence factors: Extracellular polysaccharide I (EPS I), T3SS, motility proteins, cell-wall-degrading enzymes, and a type-II secretion system (T2SS). The *hrp* operon is a finely regulated set of genes encoding production of the T3SS, which is the primary delivery system for an estimated 70-80 effectors into the host tissues (Schell, 2000). More recently, several studies have reported that Rs expresses type-IV and -VI secretion systems, as well as temperature-dependent virulence factors in some cold-adapted strains that are induced under cool temperatures (Bocsanczy et al., 2012; Bocsanczy et al., 2014; Remenant et al., 2010).

Apart from protein-based virulence factors, Rs is also well known for producing large amounts of the important virulence factor EPS I upon colonization of the host vascular bundles in the stems (Denny and Baek, 1991; McGarvey et al., 1999; Meng, 2013; Milling et al., 2011). EPS I expression is also cell density-dependent (Kang et al., 1999). The function

of EPS in the BW pathosystem is still unclear, but common hypotheses are that it either contributes to water blockage in vascular bundles or shields the bacteria cells from either plant detection or plant-produced defensive chemicals (Denny and Baek, 1991; McGarvey et al., 1999; Milling et al., 2011). Interestingly, it appears that EPS I interacts differently between resistant and susceptible tomato plants (Milling et al., 2011). Rs mutants unable to produce EPS I behaved similarly to wild type Rs in susceptible tomato varieties, whereas resistant tomato varieties had significantly higher defense responses in the presence of EPS I-producing Rs compared to the non-producing mutant strain (Milling et al., 2011).

Another important biological aspect of Rs life and pathogenicity is the ability of the bacterium to use quorum sensing signals to induce phenotypic changes that affect aspects of motility, extracellular coatings, and re-regulation of the virulence genes (Schell, 2000). A very interesting aspect of Rs quorum sensing is the effect the concentration of the sensing molecules have on expressed phenotypes of Rs, including EPS I production, motility, and expression of protein virulence factors, which will be looked at in more detail later. This effect has been termed the phenotype conversion system (Phc), which is regulated by the interactions of a core group of five genes, centered around the global regulator PhcA, effectively controlling virulence expression like a biological switch (Brumbley and Denny, 1990; Brumbley et al., 1993; Clough et al., 1994; Clough et al., 1997a; Clough et al., 1997b; Denny et al., 1998; Huang et al., 1993; Schell et al., 1993; Schell, 1996; Schell, 2000). The Phc does not appear to be unique to only Rs (Garg et al., 2000), but may be unique to *Ralstonia* spp. Some evidence exists, however, that a similar mechanism may exist in *Agrobacterium vitis* (Schell, 2000).

1.5.2: Host-Pathogen Interactions—Spatial and Temporal

The spatial and temporal disease progression of BW is complex in both biology and chemistry. Much of what is known about Rs behavior *in planta* has been revealed by a variety of microscopy and staining studies from the 1990s using wild-type and mutant Rs strains (Araud-Razou et al., 1998; McGarvey, 1999; McGarvey et al., 1999; Saile et al., 1997; Schmit, 1978; Vasse et al., 1995; Wallis and Truter, 1978). Schell's review of the components and interactions of the elaborate sensory network controlling virulence gene expression also contains an excellent section reviewing work examining the spatial and temporal interactions of Rs *in planta* (Schell, 2000). Control of virulence factors in Rs is accomplished via a complex system of regulatory proteins and quorum sensing signals, with cell density thresholds that convert Rs cells that are motile, produce siderophores, pili, etc. into relatively non-motile, EPS I-producing, cell wall-degrading pathogenic cells expressing a devastating array of virulence factors and effectors (McGarvey, 1999; Schell, 2000). Such a diverse system allows for the careful control of pathogenicity factors and resource management strategies. This intricate regulation system is consistent with the life cycle of the pathogen, allowing for precise adaptation to a variety of micro-ecosystems such as the nutrient-poor soil environment to the nutrient rich vascular cambium (Schell, 2000).

Ralstonia solanacearum lives naturally in the soil as a mobile saprophyte. Upon encountering potential hosts, the pathogen attaches to the root surfaces and forms micro colonies around lateral root emergence sites (Kelman and Sequeira, 1965; Schmit, 1978), the root elongation zone (Araud-Razou et al., 1998; Vasse et al., 1995), and entry through wounds of plant roots. Evidence suggests pili and perhaps lipopolysaccharides are involved

in virulence in tomato (Duvick and Sequeira, 1984; Romantschuk, 1992; Saile et al., 1997; Sequeira, 1985). It is known that motility, both swimming (flagallin) and twitching (pili) are important for full virulence in tomato (Tans-Kersten et al., 2001; Tans-Kersten et al., 2004). Type-IV pili have been observed as critical for facilitating this attachment via twitching motility, which is required for full virulence (Kang et al., 2002; Kang et al., 2005). The role of pili components for virulence in the early stages of BW disease in potato have recently been found to be very important (Siri et al., 2014; Wairuri et al., 2012).

At some point around the time of vascular penetration, the bacteria build up colony density significantly (Schell, 2000). Upon reaching a critical mass of 10^7 cells/mL, the pathogen changes major aspects of its phenotype via a quorum sensing signal called 3-OH-Palmitic Acid Methyl Ester (3-OH-PAME) (Flavier et al., 1997; Schell, 2000). Rs has a high sensitivity and affinity to 3-OH-PAME, as concentrations as low as 5 nM cause observable changes in the bacterium (Clough et al., 1994; Flavier et al., 1997). Upon reaching a threshold concentration, 3-OH-PAME stimulates a 50-fold increase in expression of the Phc regulon, which codes, in part, for the global regulatory protein PhcA (Schell, 2000). PhcA has been identified as the key "switch" involved in the phenotype conversion mechanism. It directly or indirectly induces or represses several important operons. PhcA has been shown to be involved in the mass production of EPS I and cellular lytic enzymes (Huang et al., 1998; Schell, 2000). PhcA also has roles in repressing swimming motility, possibly expression of the T3SS, and expression of *pglA, B, and C* (also known as *pehA, B, and C*). The *pgl* genes code for several endogenous and exogenous polygalacturonase proteins, which are involved in movement through pectin-rich areas of roots (Schell, 2000). 3-OH-PAME is produced by

PhcB and then transported outside the bacterium, where it is detected by the transmembrane receptor PhcS (Clough et al., 1997b; Flavier et al., 1997; Schell, 2000). Loss of any portion of the 3-OH-PAME recognition system, or *phcA*, leads to severe reduction of virulence.

Once the root surface colonies are established, invasion of the root occurs rapidly (<4 hours), with Rs movement being primarily through the intercellular spaces in the root cortex (Schell, 2000). Pathogen success at this stage is highly dependent on the expression of the T3SS, which is positively regulated by HrpB (Genin et al., 1992; Kanda et al., 2003). Initiation of Phc was thought to suppress T3SS expression, although recent reports indicate that the T3SS appears to remain as a very important virulence factor throughout the disease progression rather than only at the early stages of infection (Jacobs et al., 2012; Meng, 2013; Monteiro et al., 2012). Loss or inactivation of the T3SS dramatically reduces Rs pathogenicity, and prevents the ability of Rs to stimulate a hypersensitive response (HR) in some resistant species (Arlat et al., 1992; Boucher et al., 1992). Additionally, it was recently reported that sucrose is surprisingly present in tomato xylem tissues, and expression of *hrpB* is highly stimulated by sucrose (Jacobs et al., 2012).

After about 2 or 3 days post inoculation (dpi), bacteria colonize around the inner cortex and parenchyma tissues of the vasculature, likely penetrating the endodermal barrier in compromised regions (Esau, 1960; Schell, 2000). Up to this point, the bacteria exist in low, heterogeneous concentrations, causing minimal damage to the plant tissues as they travel through the intercellular spaces. Relatively little tissue damage is thought to have occurred at this point, and host defenses appear to be either suppressed or avoided at this stage based upon bacterial spread and penetration (Araud-Razou et al., 1998; McGarvey,

1999; Schell, 2000). Contrastingly, it was later determined that tomato defensive signaling pathways are stimulated as early as one day after Rs inoculation (Ishihara et al., 2012).

After about 4 or 5 dpi, the bacteria penetrate the stele (the vascular cylinder of stems and roots) and xylem vessels, and can be observed filling the stem by day 6 (Schmit, 1978; Vasse et al., 1995; Wallis and Truter, 1978), about which time most controlled inoculation experiments observe some degree of wilting symptoms in susceptible hosts. Direct stem inoculation experiments generally observe wilting symptoms several days earlier, however. Many researchers have noted that some relationship may exist between an increase in tyloses proliferation in the vascular tissues and Rs infection, though the relationship remains unclear (Kelman, 1953). It has been suggested that penetration of the vascular cylinder is accomplished either through several of the Rs-produced cellulolytic enzymes or by bursting of colonized host tyloses cells (Schell, 2000). Some evidence suggests that the T2SS is a critical component allowing colonization of the xylem vessels and general systemic infectivity (Liu et al., 2005; Tsujimoto et al., 2008). The T2SS is also involved with delivery of a concoction of cell-wall degrading enzymes (CWDEs) (Denny et al., 1990; González and Allen, 2003; Huang and Allen, 2000; Huang and Allen, 1997; Tans-Kersten et al., 1998), which may be linked to stele penetration and the observed vascular browning of the stem as BW progresses in the host.

Upon colonization of the vasculature, Rs spread through the stem is more rapid. By day 8 of infection, bacterial populations can reach $>10^{10}$ cells/cm (McGarvey et al., 1999; Saile et al., 1997), which would trigger activation of Phc, rendering the cells non-motile (Clough et al., 1997a). By this point, host plants exhibit severe wilting symptoms, and highly

susceptible plants soon reach the permanent wilting point and collapse. Interestingly, it is still a little unclear as to what is the exact cause of wilting. The prevailing views are that it occurs either from blockage of xylem vessels by masses of bacteria and/or from the large amounts of Rs-produced EPS I freely floating in the xylem vessels at this stage turning the water into sludge (McGarvey et al., 1999; Schell, 2000).

Bacteria begin to die in the stem as the host dies (Swanson et al., 2005). Rs is believed to survive in the soil on decaying plant material, associated with plant roots, or asymptomatically in weed hosts (Elphinstone, 1996; Graham et al., 1979; Granada and Sequeira, 1983). Spread of the pathogen is primarily through soil water, contaminated tools/equipment, and with contaminated seeds or seedling transplants (Agrios, 2005). Interestingly, colonized hosts are known to shed large quantities of Rs from the roots (10^5 to 10^6 CFU/mL run-off water), even prior to plant collapse (Swanson et al., 2005). Unfortunately, little is known about the life cycle of Rs within the soil environment (Schell, 2000). In contrast, it is known that the pathogen must survive the harsh environment of the soil matrix, as well as invade, colonize, and defend itself in the nutrient rich plant tissues. These are two very different and dynamic living conditions with unique resources and dangers to manage, which is likely the reason Rs pathogenicity requires such an elaborate set of virulence mechanisms and the ability to radically alter its expression profile via the Phc system (Schell, 2000).

1.6: Management of Bacterial Wilt

Management of BW is not a simple task. Like most plant pests and diseases, efforts have been made to develop an integrated pest management (IPM) strategy that combines awareness and education with balanced control methods that are economically viable, environmentally safe, and practically implementable. Strategy development has focused in sanitation methods, chemical soil fumigation control, crop management, and host resistance. While these measures have in some respects helped mitigate the spread of Rs throughout growing regions (Agrios, 2005), they provide very little help to tomato production soils once Rs becomes established. Crop rotation is ineffective due to the ability of Rs to persist seemingly indefinitely in infested fields (Chellemi et al., 1994) Rs can also be found in virgin soils recently cleared of native forest (Kelman, 1953). Additionally, use of soil fumigant chemicals does not provide adequate season-long control of BW (Enfinger et al., 1979; Jyothi et al., 2012). Sanitation of farm equipment and worker movement control can help reduce spread of Rs. Spread of inoculum via transport of symptomless infected seedlings (latent infections) continues to be a concern (Agrios, 2005; Swanson et al., 2005; Vaughan, 1944).

1.6.1: Management of Bacterial Wilt—Breeding for Resistance

Host resistance is an integral part of any effective BW IPM strategy (Hayward, 1991). The development of superior varieties with multiple disease resistance gene packages is of primary importance, including for BW resistant varieties. Breeding varieties with BW resistance and economic value has been very difficult, as is evidenced by the complete lack

of available varieties on the markets. The use of molecular markers as an aid for disease resistance breeding efforts has many success stories, including in other important tomato diseases (Ashrafi et al., 2009; Foolad and Sharma, 2005; Foolad and Panthee, 2012; Foolad, 2007). Development of molecular markers tightly associated with BW resistance is highly desirable, and may be key for development of economically viable fresh-market varieties.

There are several key factors that have plagued breeding and research efforts for BW resistance in tomato (Scott et al., 2005; Yang and Francis, 2007). The first is a very tight genetic association between BW resistance traits with traits for small fruit size, indeterminate growth habit, and bitter taste caused by fruit alkoloids (Acosta et al., 1964; Borchers and Nevin, 1954; Opena et al., 1990; Scott et al., 2005; Walter, 1967; Wang et al., 1998). In fact, in more than five decades of breeding tomato for BW resistance in large fruited varieties (>200 g fruit), there has only been one report this author is aware of where that tight association may have been broken, with a mean fruit size of 203 g and disease resistance levels statistically comparable to Hawaii 7997 (Scott et al., 2004; Scott et al., 2009). Yet, it seems some breeders have had relatively little trouble with developing processing and processing/fresh-market types with small to medium fruit size (70 g) (Monma and Sakata, 1993), though these sizes are still quite small. Generally, it seems that the more a line is selected for larger fruit size, the less resistance to BW is carried along (Scott et al., 2005). It is worth noting that making selections in the absence of disease pressure can generate lines with low resistance when high resistance was predicted (Scott et al., 2004).

The second major breeding constraint is a byproduct of the nature of host resistance, namely, the propensity of Rs to form latent infections in even the most resistant tomato

genotypes (in other susceptible species as well) making accurate detection and resistance estimates difficult (Milling et al., 2009; Nakaho and Takaya, 1993; Prior et al., 1996; Swanson et al., 2005). The difficulty with latent infections is not confined to tomato, and at least in part seems related to temperature (Milling et al., 2009; Prior et al., 1996; Zehr, 1970).

A third factor is the high degree of environmental influence modulating severity of BW, with soil temperature, soil moisture, air temperature, and soil pH effecting even gene segregation/inheritance studies (Acosta, 1978; Gallegly and Walker, 1949; Grieve, 1943; Hayward, 1991; Mew and Ho, 1977; Scott, 1996; Scott et al., 2005; Vaughan, 1944). This pattern is regularly noted where cool spring/fall planting seasons exhibit much less BW severity than warm summer months. The confounding effects of environment were noted by (Acosta, 1978) when multiple studies screening approximately 13,000 tomato plants from various crosses were not even able to conclusively determine gene effects or inheritance patterns, concluding them to be entirely additive gene effects. Several researchers have noted that resistance seems to breakdown under soil temperatures of 32 ^{o}C and higher (Barnes and Vawdrey, 1993; Mew and Ho, 1977; Scott et al., 2005). This has generally been linked to the optimum temperature range that favors maximum pathogen growth *in vitro*, which most studies report to be in the range of 30-33 ^{o}C (Kelman, 1953). It should be noted that this link has not been definitively established, with some evidence demonstrating that Rs strain performance under environmental stresses *in vitro* is not always an accurate predictor of performance *in planta* (Milling et al., 2009). Generally, BW incidence is greater under higher temperatures (especially soil), higher inoculums densities, and in younger plants (Kelman, 1953; Mew and Ho, 1976; Nakaho and Takaya, 1993). There also appears to be a floor of

about 21 ^{o}C in tomato and potato for host expression of wilting symptoms, though hosts can be become latently infected as low as 18 ^{o}C for most Rs strains (Hayward, 1991; Kelman, 1953; Meier and Link, 1923; Singh et al., 2014a). Additionally, there is some evidence that nitrogen fertilizer-based suppression of BW symptoms is mediated by temperature (Kelman, 1953). At this stage it is worth considering if some of the difficulties experienced by breeding and genetic screens may be due to susceptibility of the common leaf wilting metric to environmental variation, especially soil temperature. (Thoquet et al., 1996b) suggested that as much as 40% of BW resistance variation was due to environmental influences in their resistance QTL mapping studies. It should be noted that no other reliable non-destructive screening method is available at this time, especially for large scale field studies, other than the leaf wilt metric. An alternate assessment method was developed by (Prior et al., 1996) where they used end-of-study assessments of bacterial concentration in the stem to largely correct for the variation between cool and warm growing seasons and taking into account any latent infections. While the method is helpful for improving the accuracy of assessments, it does decrease the high-throughput potential. Additionally, they found that spread of the bacteria in the stem was a function of temperature (Prior et al., 1996). Thus, it would be very helpful for researchers to develop a monitoring system for environmental influence, and use that information to correct foliar wilt-based assessments of host resistance in tomato.

1.6.2: Management of Bacterial Wilt—Resistance Genetics and Mechanisms

A key strategy for using resistant varieties to manage BW is selection of lines that perform well in local growing regions, since resistance is often influenced by regional strains

of the pathogen (Hayward, 1991). Several multi-national studies have reinforced this need (Hanson et al., 1996; Scott et al., 2005). A comparison of BW resistance among a diverse set of species and accessions found that pepper and eggplant generally have high resistance levels, but not tomato (Lebeau et al., 2011; Wang et al., 2013). The researchers assembled a collection of 30 BW resistant accessions, including genotypes commonly used in resistance mapping studies, from these species and challenged them with 12 Rs strains representing the strain diversity of the pathogen (Lebeau et al., 2011). Noteworthy tomato genotypes tested were 'CRA66' and 'Hawaii 7996', along with diagrams describing their genetic origins. They found that the interactions grouped into six groups from highly resistant to highly susceptible, and with no clear connections to phyllotype specificity. Generally, the most aggressive strains were found in phylotypes I, IIB, and III. They also found that the resistant tomato accessions from around the world were predominantly derived from *Solanum pimpinellifolium, S. lycopersicum*, and *S. lycopersicum* var. *cerasiforme*. Curiously, another researcher reported the identification of a novel BW resistance gene derived from *S. peruvianum*, but remains unconfirmed.

A multi-national, multi-year screening study for BW in tomato was performed in the early 2000s, using 31 genotypes from 14 resistance sources (see below). Of those genotypes, only 7 were identified as having >90% survival rate across all locations; 3 were selections from Hawaii (Hawaii 7996, 7997, 7998; resistance source PI 127805A), 3 were from the Philippines (TML46, TML114, R-3034; resistance source unknown, Venus and CA67(1169), and unknown, respectively), and 1 was from North Carolina (BF Okitsu; resistance source NC 19/53-64N), which resembled the Hawaiian phenotype. Genetically, the project

suggested that resistance derived from Hawaii 7998 was probably (but not perfectly consistent) controlled by a single major gene with several smaller effect genes (Scott et al., 2005). This is generally in agreement with other inheritance studies (Hartman and Elphinstone, 1994; Mahir et al., 1993), as well as BW resistance QTL mapping studies using the closely related Hawaii 7996 (Carmeille et al., 2006; Thoquet et al., 1996a; Thoquet et al., 1996b; Wang et al., 2000; Wang et al., 2013). It should be noted that some reports suggest recessive gene action (Mahir et al., 1993; Monma and Sakata, 1993) (Singh, 1961) or partial dominance giving way to recessive (Acosta et al., 1964) in some breeding material (Sharma et al., 2006). Curiously, (Mew and Ho, 1976) found that resistance levels of tomato seedlings compared to plants at flowering stage had a low correlation ($r = +0.58$).

Generally, QTL mapping studies have found that chromosome (chr.) 6 of 'Hawaii 7996' contains a strong QTL for BW resistance over several different resistance sources and Rs strains (Race 1, phyllotypes I & II; race 3, phyllotype II) (Carmeille et al., 2006; Mangin et al., 1999; Thoquet et al., 1996a; Thoquet et al., 1996b; Wang et al., 2000). Among these studies, several other smaller effect QTLs have been reported (chr. 4, 12, 3, 11, 8, and 10), being race and sometimes phyllotype-specific (Wang et al., 2013). All but the most recent study have used restriction fragment-length polymorphism (RFLP) markers for mapping the resistance QTLs. Generally, the chr. 6 QTL is quite large, spanning around 30 cM. One report suggested that the chr. 6 QTL contained two linked resistance loci in that region, with the strength being affected by days post inoculation (dpi), where the distal (upper; away from the centromere) end is stronger during early stages of wilting (as early as 6 dpi), and broadens toward the proximal end of the chromosome (lower; towards the centromere)

(Mangin et al., 1999). The chr. 12 QTL, using a population of recombinant inbred lines (RILs) derived from 'Hawaii 7996' (resistant) x 'West Virginia 700' (susceptible; *S. pimpinellifolium*), has been reported as being related to suppression of bacterial multiplication within the tomato stem in the only study using simple sequence repeat (SSR) markers (Wang et al., 2013), as well as being a strain-specific locus (Wang et al., 2000). The first QTL mapping report, which was the only study to use an F_2 population derived from a cross of L285 (resistant) x C286 (susceptible) (L285 is *L. esculentum* var. *cerasiforme* from AVRDC), compared maps based on data from injured root drench and stem inoculation methods, and found that resistance mapped differently, with the chromosome (Chr.) 6 QTL being related more to resistance under root drench, while Chr. 10 and 7 QTLs are more important for resistance in shoot injected inoculation (Danesh et al., 1994). The QTLs on chr. 4 and 8 were weaker, and (Carmeille et al., 2006), using inoculation with R3, reported that they were only detected during the hot season. The chr. 3 QTL has been reported as a weak loci (Carmeille et al., 2006; Thoquet et al., 1996b; Wang et al., 2013).

On a finer genetic level, relatively little is known about the specific genes involved in resistance. One generally observed pattern seems to be that gene expression changes are either non-existent or much more reduced in strength and rate in susceptible varieties compared to resistant (Ishihara et al., 2012; Jyothi et al., 2012; Milling et al., 2011), implying that resistance variation may be closely associated with expression regulation, rather than simply presence/absence of resistance genes. Marker genes related to the Salicylic Acid (SA) and Ethylene (ET) defense pathways are up regulated, while the Jasmonic Acid (JA)-dependent signaling pathway is suppressed in some instances (5 dpi) (Jyothi et al., 2012;

Takahashi et al., 2014) and stimulated along with Auxin accumulation in others (1 dpi) (Ishihara et al., 2012). As early as 1 dpi, changes in gene expression were observed in as many as 140 gene loci, including pathogenesis-related (PR), hormone signaling, and lignin biosynthesis pathways in the resistant 'LS-89' (Ishihara et al., 2012). Recently, phytoalexin synthesis was also related to host responses (Lin et al., 2014). An additional aspect of host resistance mechanisms is the rapid production of reactive oxygen species (ROS). ROS is may be acting as a defense pathway singal and/or a direct defensive mechanism against Rs cells, since Rs is known to produce ROS mitigating enzymes *in planta* (Brown and Allen, 2004; Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011). In *Arabidopsis thaliana*, broad-range resistance to BW is due to the single recessive gene RRS1-R, and involves Abscisic Acid (ABA) signaling (Deslandes et al., 2003; Deslandes et al., 2002; Feng et al., 2012; Lahaye, 2004). This is not the case in tomato, as previously noted. One gene product, a Caffeoyl CoA 3-O-methyltransferase (CCoAOMT), has been reported to be associated with resistance to BW in tomato, but remains unconfirmed (Miao et al., 2008).

Resistance mechanisms to BW in tomato are generally accepted to be analogous with tolerance, because even in the most resistant genotypes, resistance responses are stimulated by Rs presence in the roots and stems, but they do not lead to root-based Rs inhibition, but rather to suppression of bacterial growth and spread through the stem (Grimault and Prior, 1993; Grimault and Prior, 1994; Grimault et al., 1995; Hikichi et al., 1999; Ishihara et al., 2012; Prior et al., 1996). Specifically, limitations in pathogen growth seem to be related to the restriction of pathogen spread in the stem, particularly in the spread from the protoxylem tissues to primary and other xylem tissues. This pattern was most conspicuous in the highly

resistant ‘Hawaii 7996’ (Nakaho et al., 2004). There is a correlation between host resistance and latent bacterial colonization at the midstem (6^{th} internode) (Grimault et al., 1994a), as well as evidence that Rs suppression seems to be linked primarily to host-pathogen interactions in the lower part of the stem (Grimault and Prior, 1994). Additionally, the formation of tyloses is suggested to be involved in limiting Rs movement in the stem but has not been definitively demonstrated (Grimault et al., 1994b; Kelman, 1953).

The affects of host resistance on symptomology and epidemiology of BW is slowly being quantified. Resistant tomato plants are known to decrease the rate of Rs ingress into the plant and subsequent colonization rates, multiplication and density of bacterial cells *in planta*, and affect levels and spread of ESP I through the plant tissues (McGarvey, 1999). It has been observed that a pectinase-deficient Rs mutant is related to reduced host structural defense responses, but only in resistant tomato varieties (Nakaho and Allen, 2009), suggesting that tomato defense responses are at least partially dependent upon detection of pathogen-produced virulence factors. Interestingly, the rate of pathogen spread up and down the stem from the infection site is not different, and is dependent on soil moisture and temperature, with 32 ^{o}C reported as optimum in tobacco (Kelman, 1953; Van Der Meer and Jikke, 1929). Overall rate of bacterial spread does differ by species, with differences in vessel diameters being suggested as the cause (Grieve, 1943). Yet, overall transpiration rates of healthy and infected plants only gradually deviated when wilting symptoms became advanced (greater than 1/3 of leaf surface area), suggesting that asymptomatic leaves compensated for the loss of the symptomatic leaves, which exhibited drastic reductions in transpiration rates, during initial symptom development (Grieve, 1941). Water uptake

patterns were similar to transpiration rates, with relatively high absorption rates even when bacterial blockage of xylem vessels was substantial. Even more astonishing, potato plants inoculated at the stem apex did not exhibit significant reductions in water uptake even when Rs spread had reach to the base of the stem (Grieve, 1941; Kelman, 1953). Combined, these results would suggest that hosts seem to be able to easily compensate for expected severe reductions in water movement in the plant due to bacterial occlusion of vascular bundles, implying that wilting symptoms may be linked to some other effects of Rs colonization of vasculature apart from simply constriction of water flow. It also may indicate that the ability of the pathogen to move through the stem is not determined by rate of water flow.

Although it goes beyond the scope of this review, it should also be noted that much work has been done in elucidating the effects of other non-pathogenic soil microbes, as well as simple applications of silicon or chitosan. These treatments enhance host resistance levels, but not through direct suppression of Rs growth. Rather, it appears that they indirectly stimulate the host defense pathway mechanisms in a manner that appears to be very similar to natural host resistance in tomato (Algam et al., 2013; Diogo and Wydra, 2007; Ghareeb et al., 2011a; Ghareeb et al., 2011b; Hyakumachi et al., 2013; Jogaiah et al., 2013; Kiirika and Wydra, 2012; Kiirika et al., 2013; Kloepper and Choong-Min, 2006; Li and Dong, 2013; Takahashi et al., 2014; Yi et al., 2008).

1.6.3: Management of Bacterial Wilt—Vegetable Grafting

In light of the tight genetic linkage of resistance with small fruit size and other undesirable traits, the decades of breeding efforts for development of BW resistant tomato

varieties for fresh-market production has practically been entirely unfruitful (Scott et al., 2005). Combined with growing restrictions on use of soil fumigant chemicals, and host resistance being the only effective management strategy for BW infested fields, vegetable grafting for soil-borne disease management has received growing focus over the last decade in the NC and Southeastern USA tomato growing states. Although the concept is not novel, vegetable grafting of any kind has not been incorporated into USA production systems to any significant degree, and only more recently become widely used in Asia (Sakata et al., 2007). Modern vegetable grafting originated in Japan and Korea almost a century ago, where watermelons were grafted onto gourds for increases in quality, production, and disease resistance (Ashita, 1927; Lee, 1994; Yamakawa, 1983). In Japan, the use of grafted vegetable plants for management of BW is a regular practice (Lee et al., 1998), as well as for other diseases in many crops. European vegetable growers have also widely adopted the use of grafted material in their production systems, primarily in an effort to reduce or eliminate the need to soil fumigate with the environmentally damaging methyl bromide chemistry (King et al., 2008).

Vegetable grafting is the surgical removal of a seedling top (scion) and reattachment to another seedling lower stem and root system (rootstock), where it is then placed in a low light, high humidity environment for healing before being transplanted into the field or greenhouse (Lee, 1994; Rivard and Louws, 2006; Rivard and Louws, 2008). A common, age-old practice in tree and vine crops, vegetable grafting has demonstrated potential for improving production systems as a part of an IPM strategy for management of soil-borne diseases, including BW of tomato (Lee et al., 1998; Lee et al., 2010; Louws et al., 2010;

Rivard et al., 2012). While the exact mechanisms of resistant rootstock protection on susceptible scions is not clear, it is generally expected that transduction of resistance compounds across the graft union is not the explanation, such as in BW of tomato, though some specific host-pathogen interaction examples are reported (King et al., 2008; Lee, 1994). The presence of varietal cross-talk for disease resistance expression, then, is reasonable, but must be assessed on individual rootstock pathosystems.

Grafting susceptible tomato varieties, with good commercial quality fruit production, onto soil-borne disease resistant rootstocks with high vigor substantially reduces BW incidence compared to the susceptible varieties grown alone, even in fields known to have very high natural disease pressure. Fruit yields with grafted tomato production have been found to be equivalent or even a little better when using vigorous disease resistant rootstocks, especially under disease pressure from soil-borne pathogens (Freeman et al., 2011; McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008). There are also reports suggesting that some abiotic stress tolerances can be conferred to a production system by using vigorous rootstocks, including thermal, water, and pollutant stresses (Rivard and Louws, 2006; Schwarz et al., 2010).

Vegetable grafting allows tomato growers to combine beneficial (and often counter selective) traits of rootstock and scion, especially in organic vegetable production systems where chemical control methods for disease are not available (Rivard and Louws, 2008). The main challenge for USA-based vegetable grafting is cost reduction in order to facilitate economical tomato production. Due to the necessity of using two varieties growers must purchase twice as much seed each season, and hybrid rootstock seed can be quite expensive.

Additionally, while it is possible for growers to perform their own grafting production, large-scale production in the USA is limited, with most growers in NC purchasing plants from foreign nurseries equipped for large-scale production of grafted vegetable plants. This adds the additional cost and risk of transport over long distances. Finally, if use of disease resistant rootstocks increases, it will be important to establish monitoring systems for detection of new resistance-selected races of the soil pathogens able to overcome host defenses, as is already the case with chemical and foliar host resistance systems (King et al., 2008; Louws et al., 2010). There are many benefits available for use of grafted vegetable production. Many resources are available to aid in personal education and skill development for grafting. Many of those resources are publically available: http://graftvegetables.org/ (USDA-National Institute of Food and Agriculture, 2014)

1.7: Host Resistance Genetics

Thus far, this review has incorporated many disease related terms, such as resistance genes, pathogenicity and virulence factors/genes, host defenses, single gene vs. polygenic resistance, tolerance vs. true resistance, and interactions of host and pathogen-produced components. More recent literature introduces the concepts of Pathogen-Associated Molecular Patterns (PAMPs), Microbe-Associated Molecular Patterns (MAMPs), PAMP-triggered immunity (PTI), MAMP-triggered immunity (MTI), effector-triggered immunity (ETI), Pattern Recognition Receptors (PRRs), Hypersensitive response (HR), systemic-acquired resistance (SAR), reactive oxygen species (ROS), R genes (resistance genes), and avirulence (Avr) genes. Many review articles have been written on the subjects in the last

two decades, addressing each facet in much more detail than is applicable here. For more detailed information on the complex interactions of these important plant immunity-related terms, as well as details on the historical development of our understanding of plant defense systems, and host-pathogen interactions, see these excellent reviews (Bailey-Serres and Mittler, 2006; Beckman, 2000; Bent and Mackey, 2007; Boller and Felix, 2009; Collier and Moffett, 2009; Dangl et al., 2013; Dangl and Jones, 2001; Denancé et al., 2013; Dodds and Rathjen, 2010; Flor, 1971; Jones and Dangl, 2006; Medzhitov and Janeway, 1997; Monaghan and Zipfel, 2012; Naumenko, 2013; Nicaise et al., 2009; Nürnberger and Brunner, 2002; Postel and Kemmerling, 2009; Sharma et al., 2012; Thomma et al., 2011; Vance et al., 2009; Vanderplank, 1963; Vanderplank, 1991; Yadeta and Thomma, 2013; Zipfel and Robatzek, 2010). Also, (Beckman, 2000; Hann et al., 2010; Zipfel, 2009). It should also be noted that generalized models for these systems, both in effectiveness and function, are still being debated in the published literature, with several alternative views. Additionally, several good articles have been published that focus on some of the techniques used to study MTI/PTI (Lloyd et al., 2014; Nguyen et al., 2010).

Over the last two decades, our understanding of how plants detect, respond to, and manage pathogen interactions has greatly increased. For many decades, the prevailing view was that plants had specific genes (R genes) that provided resistance to a pathogen by detecting a pathogen-expressed gene (Avr gene/Vir gene/pathogenicity factor), which was essential for successful host colonization and completion of disease cycle. Thus, an observed resistance response only occurred when both the host R gene and pathogen Avr gene (producing an elicitor) were present (and believed to directly interact, similar to vertebrate

recognition of antigens), which would stimulate a burst of reactive oxygen species (ROS) in the programmed cell death hypersensitive response (HR). When an interaction lacked one or more of these components, the plant would succumb to infection and disease. This narrow-range relationship has become known as vertical resistance or the gene-for-gene theory of plant resistance and is generally viewed as qualitative resistance, where each gene can only be identified by its counterpart in the host-pathogen relationship (Agrios, 2005; Flor, 1942; Flor, 1971; Keen, 1990; Thomma et al., 2011).

For decades there was a raging debate over whether the pathogen factor should be considered a Virulence factor or an Avirulence factor, which deeply divided the field of Plant Pathology because it was unclear if and why a pathogen would counterproductively produce a component that would stimulate host resistance and immunity (Zipfel and Robatzek, 2010). The virulence model of gene-for-gene resistance was demonstrated to be the more parsimonious model with a clear test (Vanderplank, 1991), stating:

> "For each gene that conditions resistance in the host there is a corresponding gene that conditions pathogenicity in the parasite" (Flor, 1971).

In contrast to R genes, another kind of resistance was identified where plants would remain either symptomless but colonized by the pathogen, or exhibit a distinct reduction in disease symptoms, but not presence/absence of colonization. This became known as tolerance, quantitative resistance, or horizontal resistance, and was thought to be caused by

many small effect genes with a weaker, broader range of pathogen control (Agrios, 2005; Vanderplank, 1963).

1.7.1: Host Resistance Genetics —PTI vs. ETI

The advent of the molecular genetics and genomics era allowed researchers to investigate the horizontal and vertical resistance mechanisms, perhaps synonymous with plant immunity. This led to the discovery of more complex layers of interactions between host and pathogen, along with a whole host of new terminology, which requires revising the now overly simplistic view of gene-for-gene plant immunity. It is now known that plants have a multi-layered recognition system that differentiates self and non-self molecular components (Monaghan and Zipfel, 2012). When a microbe (pathogen or other) encounters a plant, there is an interaction of cellular receptors that defines the first level of host/non-host and threat/non-threat recognition (Vance et al., 2009). This interaction is partly facilitated by plant-based, surface-localized, membrane spanning PRRs that detect conserved microbial molecular patterns of microbe-produced structures necessary for fitness (MAMPs/PAMPs) (Monaghan and Zipfel, 2012), as well as by simple physical barriers that prevent a potential pathogen from being able to establish infection (Naumenko, 2013). Many MAMPs have been characterized for plant species, including fungal chitin, bacterial lipopolysaccharides (LPS), peptidoglycans, quorum sensing factors, conserved portions of flagellin, and in some cases even plant compounds associated with tissue damage (DAMPs).

Each MAMP is detected by specific PRRs (Boller and Felix, 2009). If the plant PRRs detect these compounds, they signal for defense response called PAMP(MAMP)-triggered

immunity (PTI/MTI), which includes calcium bursts, ion fluxes, ROS production, ethylene production, stomatal closure, SA accumulation, callose deposition, and kinase stimulation causing substantial expression changes *in planta.* These PTI responses temporally range from seconds to days after detection, and PTI stimulation by specific MAMPs is sometimes species or family specific, and may even be tissue specific (Monaghan and Zipfel, 2012; Nicaise et al., 2009; Tena et al., 2011; Zipfel and Robatzek, 2010). Additionally, not all microbes present all MAMPs (Zipfel and Robatzek, 2010). A recent compilation of known MAMPs, their known PRRs, and responsive species is available (Postel and Kemmerling, 2009). PTI is sufficient to prevent colonization of most microbes, suggesting that it is the reason only a tiny fraction of microbes can be pathogenic at all (Boller and Felix, 2009), which is why some authors use the more general term MTI, since it is not simply pathogens that are detected and responded against. PTI is the standard terminology adopted from animal immune system models, but MTI is probably the more biologically accurate term. PTI is also referred to as the innate immune system or the basal defense system in plants.

If PTI is not sufficient to prevent host colonization at the extracellular level, then another level of host resistance mechanisms are activated, where intercellular receptors detect the activity of pathogen effectors injected into the host (Dangl and Jones, 2001; Dodds and Rathjen, 2010; Jones and Dangl, 2006). These seem to be most analogous to the historical concept of R and Vir genes, since they are generally characterized by having nucleotide-binding (NB) and leucine-rich repeat (LRR) domains inducing an immunity response called ETI (Dodds and Rathjen, 2010). ETI often (though not exclusively) leads to HR and SAR (Thomma et al., 2011). The end result of all these host-receptor and pathogen-component

interactions is what determines if disease will develop or not. Pathogens must suppress PTI responses in order to cause disease, with some pathogen effectors specifically targeting PRRs, and loss of PRRs often leads to hyper-susceptibility of the host (Rosli et al., 2013; Zipfel and Robatzek, 2010).

There is some debate over whether MAMPs and effectors, and their associated reception systems, are different levels of responses, with ETI being considered superior to PTI (Dangl and Jones, 2001; Jones and Dangl, 2006; Thomma et al., 2011). There is a general distinction between the two, where MAMPs are general microbial features required for fitness and survival, and effectors are very specific attacks against host machinery and contribute to pathogen virulence. Not all effectors, however, are narrowly conserved, and not all MAMPs are general microbial components. Some MAMPs (flagellin) even contribute to virulence (Thomma et al., 2011). Additionally, PRRs, which are generally thought to be constrained to the cell membrane, may be involved intracellularly, which has generally been described as the function of R genes stimulating ETI. Some of the characterized R genes have forms and functions that resemble PRRs, as well, with some PRRs able to stimulate HR and SAR, and some R genes that cannot (Thomma et al., 2011).

It is clear that PTI and ETI have many overlapping responses and that our understanding of them is not even close to complete. What should be apparent is that resistance genetics research and molecular biology research in the plant immune system is now beginning to coalesce, which is exciting. It also demonstrates that plant-pathogen interactions are more complex then researchers and breeders imagined, even as recently as two decades ago. A clear understanding of the molecular and genetic revelations of late will

be instrumental in further elucidating many of the host-pathogen interactions that have eluded researchers for many decades, none-the-least for BW of tomato.

1.7.2: Host Resistance Genetics —Relation to Bacterial Wilt

It is known that tomato detects multiple MAMPs, stimulating some degree of resistance response, including flagellin and cold shock protein (gram negative bacteria); xylanase, ergosterol, and chitin (fungi); invertase (yeast); and cutin monomers (plant cuticle) (Postel and Kemmerling, 2009). In *Arabidopsis* and tomato, a boiled extract of Rs was found to be able to stimulate host defenses (Pfund et al., 2004; Takabatake and Mukaihara, 2011), and (Pfund et al., 2004) reported that an unknown proteinaceous MAMP of about 5 to 10 kDa was responsible. (Takabatake and Mukaihara, 2011) reported that the proteinaceous compound was only effective as a pre-inoculation treatment, being rendered ineffective by the T3SS when applied to host at the same time as inoculation. Elongation factor Tu was also found to be partly responsible (Takabatake and Mukaihara, 2011). A comparative proteome analysis of resistant and susceptible tomato stems found 6 Rs-related proteins and 6 tomato-related proteins that were differentially regulated in susceptible tomato stems, but not in resistant stems. These proteins were extracted from plant midstems at 5 dpi, the time when the susceptible check genotype started wilting, and were identified as pathogenesis-related (PR), stress related, and metabolic proteins. Detailed information is available for them (Dahal et al., 2009). In another study that used a proteomics approach to compare stem cell wall proteins in resistant (Hawaii 7996) and susceptible (WVa700) tomatoes found 14 differentially regulated proteins between the two varieties. Inoculation with Rs was found to

differentially regulate expression of 15 proteins in the resistant line (7 up-regulated, 8 down-regulated) and 13 proteins in the susceptible line (5 up-regulated; 8 down-regulated) at 5 dpi. Detailed information is also available for these proteins (Dahal et al., 2010).

Tomato has the receptor LeFLS2 (FLAGELLING SENSING 2), a homolog of FLS2 receptor in *Arabidopsis,* which is responsible for detection of conserved sequences of flagellin (Flg22, FlgII-28) (Bauer et al., 2001; Chinchilla et al., 2006; Gomez-Gomez and Boller, 2000; Robatzek et al., 2007). Flg22 was the first MAMP described, and was found to be conserved among many bacterial species (Felix et al., 1999). FLS2 has tissue-specific expression localized to known bacterial entry sites, such as stomata, hydathodes, and lateral roots (Rs enters roots through lateral root openings) (Beck et al., 2014). Although motility was known to be critical for the early stages of Rs infection, (Schell, 2000) noted that the role of bacterial flagella in BW disease development was unknown. While *Arabidopsis* has many similarities to tomato, it exhibits substantial within-species variation of susceptibility to bacterial pathogens, as well as marked differences of resistance mechanisms compared to tomato (Atwell et al., 2010; Takabatake and Mukaihara, 2011). Interestingly, Rs has several amino acid differences at key positions in the conserved section of bacterial flagellin, suggesting it that those changes likely make it undetectable by FLS2 in *Arabidopsis* (Pfund et al., 2004). Additionally, the major proteiniaceous compound related to stimulation of host defense is not detected via *Arabidopcsis* FLS2, nor was it related to the Flg22 of either Rs (K60) or the common Flg22 sequence described by (Felix et al., 1999; Pfund et al., 2004). Unless the information was simply not published, no tests of the Rs (K60)-specific Flg22 sequence (Rs Flg22) have been performed in tomato. The pattern of response in *Arabidopsis*

suggests that tomato should also not detect Rs-specific Flg22. If that is the case, knowing the clear strong detection response of the conserved sequence, the unique amino acid changes of that flagellin sequence may contribute to the ability of Rs to evade recognition by host PRRs, thus allowing it to be an effective pathogen on such a wide host range. It also suggests that there remains an unknown proteineaceous component produced by Rs that elicits the strongest defense response.

The highly resistant Hawaii 7996 was found to have activated resistance expression faster and to a stronger degree than susceptible 'Bonny Best' when inoculated with Rs (Milling et al., 2011). Additionally, extracts of EPS I, besides being the arguably most important virulence factor, were found to stimulate resistance expression only in the resistant tomato line, while lack of EPS I was related to reduced ROS production *in planta*.

Production of reactive oxygen species is linked with host defense responses to Rs, and other tomato bacterial pathogens. Rs mitigation of plant-produced ROS is linked with the ability to virulently colonize tomato (Colburn-Clifford et al., 2010; Colburn-Clifford and Allen, 2010; Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011). The conserved sequences of bacterial flagellin (Flg22, FlgII-28) and cold shock protein (Csp22) are known to differentially elicite host ROS production in a wide array of tomato varieties (and *Brassica* spp.) in a rapid, transient, dose-dependent oxidative burst (Felix et al., 1999; Felix and Boller, 2003; Lloyd et al., 2014; Veluchamy et al., 2014). A direct linkage of this type of oxidative burst to host resistance is enigmatic, although pre-treatments with MAMPs prior to inoculation are known to provide a protective effect in the host in some host-pathogen interactions, and losses of MAMP detection in the host leads to hypersusceptability (Rosli et

al., 2013; Zipfel, 2009; Zipfel and Robatzek, 2010). A dose-dependent, transient ROS production was related to stimulation with live *Pseudomonas syringae* pv. *tomato* (Pto), with Flg22 being the primary component of ROS elicitation in a T3SS-independent manner (Smith and Heese, 2014). This transient ROS production event generally peaks about 30-40 minutes after stimulation with live Pto (Smith and Heese, 2014), but is generally sooner (10-20 minutes after stimulation) with the flagellin peptides (Felix et al., 1999; Lloyd et al., 2014; Veluchamy et al., 2014).

Interestingly, applications of exogenous chemistries have been noted as being able to stimulate resistance responses in tomato to BW. A list of sources for studies using these compounds has been noted previously in this paper. A few examples are highlighted here. The *Phytophthora infestans* 10-kDa extracellular elicitin protein is able to produce an HR and SAR response in *Nicotiana* spp., but not in tomato. Yet, treatments of the purified protein are able to induce substantial quantitative resistance to BW in tomato ('Micro-Tom', susceptible) via stimulation of the JA- and ET- mediated signaling pathway (Kawamura et al., 2009). Silicon is known to induce the basal defense system, with stimulation related to modifications of the cell wall structure, which leads to enhanced BW resistance (Diogo and Wydra, 2007). Bacterial LPS from a wide range of species is recognized by *Arabidopsis*, leading to a rapid burst of nitric oxide via nitric oxide synthase (NOS) and stimulation of local and systemic defense-related genes, which were previously known to be associated with hormonal signaling. Loss of NOS was linked with hypersusceptibility of *Arabidopsis* to Pto (Zeidler et al., 2004).

1.8: Final Thoughts

Tomato is a very important world crop, and BW devastates it whenever it is present. Effective management strategies are needed in order to slow the spread the pathogen and to maintain the ability to grow susceptible crops in infested fields. Tomato is being pushed into the spotlight as the model system for the Solanaceae family, which includes many economically important crops worldwide. Additionally, Rs is growing as a model system for genetic and molecular biology studies of pathogenicity. It is a scientifically rich field of study, with direct application to crops and disease problems of substantial economic importance.

The extreme host and geographic range of R. solanacearum has been a strong source of scientific curiosity. Much work has already been accomplished on unraveling the molecular mysteries surrounding the life cycle and infection mode-of-action of Rs. The important work now is to translate that knowledge to plant-microbe interactions and practical disease management strategies (Dangl et al., 2013). The BW pathosystem offers a plethora of opportunities for increasing our understanding of plant defense systems on genetic, cellular, and biochemical levels. There is much potential to be explored.

The relationship between PTI, ETI, and Rs is not very well understood. Due to the nature of tomato resistance to Rs, it would seem reasonable that PTI may be playing an important role, with multiple receptors each detecting Rs-components and amplifying the defense responses to a measureable degree, as has already been documented with the numerous QTL studies of tomato resistance to Rs. The chr. 6 QTL, then, may contain one or more larger effect-triggering receptors for one or more Rs components. The other QTLs often

associated with strain-specific responses may then be detecting other strain-specific MAMPs, adding another level of quantitative resistance. Susceptible lines, which are regularly reported as having no response or very little response, may be missing some of these important receptors, either by the complete or partial lack of the genetic information or by effective suppression by Rs effectors. The overlap of responses (ROS and hormone production, quantitative defense responses, cell wall strengthening, etc.) between PTI and Rs infection lends to the notion that resistant lines are detecting MAMPs, since there does not appear to be any examples of ETI-like responses.

Several key questions remain: Do endemic Rs strains in NC and the Southeastern U.S.A. exhibit differential virulence on known resistant rootstocks for grafted tomato production? Can the environmental variables creating substantial within genotype variation be corrected by incorporating other metrics to estimate resistance of tomato genotypes? Can a synthesis of the genetic and molecular resistance fields be effective at developing gene-linked molecular markers for marker-assisted selection of BW resistance? And, can our knowledge of innate immunity and MAMPs be translated into time saving, high throughput screening assays for non-pathogenic selection of disease resistant tomato lines?

The research that follows represents my efforts to begin to answer these important questions. Due to the influence of environmental variables on resistance, I performed experiments both in a natural field setting where tomato resistance to BW is of very practical importance, as well as in greenhouse conditions with controlled inoculum. Additionally, I adapted a laboratory-based assay for measuring MTI-related ROS production and assessed its potential for predicting resistance to BW in a moderately high-throughput manner. Finally, I

endeavored to develop a strategy for identification of putative resistance loci for fine mapping of resistance QTL and marker development that merged the solid foundation of the previous QTL studies with the wealth of information about the tomato genome now available. I investigated the modulating effects that use of localized Rs strains, a grafted tomato production system, and BW resistance variation in eleven tomato genotypes from various backgrounds had on these main objectives.

CHAPTER 1: REFERENCES

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CHAPTER 2: ROOTSTOCK SCREENINGS FOR BACTERIAL WILT DISEASE RESISTANCE IN TOMATO

Abstract:

Tomato (*Solanum lycopersium* L.) is the second most important vegetable crop in the world. Bacterial Wilt (BW), caused by *Ralstonia solanacearum* Smith (Rs), is a devastating disease of tomato in tropical and subtropical environments around the world with losses of 70-100% in the Southeast U.S. in heavily infested fields. Vegetable grafting of commercial scions onto disease resistant rootstocks has been developed as a viable management strategy for, particularly for fresh-market tomato production. BW resistance in tomato rootstock germplasm, however, is quantitative and polygenic, being substantially affected by localized Rs strains and by environmental factors, especially temperature and moisture. My objectives in this study were to investigate tomato rootstock resistance to BW as modulated by grafting, North Carolina (NC) Rs strains, vascular browning variation, and low temperature shock stress. Summer field and winter greenhouse studies using 10 variably resistant tomato rootstock lines (mixture of open-pollinated and commercial hybrids) and 'Florida 47' as susceptible commercial control was performed in 2013-14 to assess these modulating effects. The field study was performed under standard tomato production practices using natural inoculum pressure from the local endemic Rs population, assessing the effect of grafting on resistance. The potted greenhouse study was carried out in a heated glass house, assessing the effects of two contrasting NC Rs tomato isolates and low temperature shock stress on resistance. Rootstock lines including 'HI7997' and 'HI7998' and hybrid lines 'Cheong Gang'

(Seminis) and ‘RST-04-106-T’ (DP Seeds) were found to be highly resistant in the greenhouse study to both NC strains, quantitatively, but were moderately resistant in field study. Effect of grafting in field study did not affect resistance level of genotypes, though the vigorous ‘Maxifort’ was found to be significantly more susceptible in the first 5 weeks of the study than even susceptible check. Other rootstock lines including ‘CRA66’, ‘RST-04-105-T’ (DP Seeds), and several BHN selections had intermediate resistance. Rootstock hybrid ‘Maxifort’ (DeRuiter) and scion hybrid ‘Florida 47’ were highly susceptible, as anticipated. A severe, albeit fortuitous, cold weather event caused greenhouse study plants to be exposed to 9 ^{o}C for two consecutive nights, causing recoverable cold damage after disease had reached a stable plateau. Following cold stress, a second substantial phase of wilting was observed, mostly in resistant rootstocks, which again reached a stable plateau. Analyses of cold shock effects on the study suggest that cold shock stress was related to second phase of new wilt incidence, and was useful in separation of mean genotypic BW resistance levels. Greenhouse diseases scores were complimented with stem imprints on semi-selective media and ELISA to confirm Rs identity and assess frequency of latent infection. End-of-study vascular browning was assessed at 1.27 cm above soil line and included in a predictive model for wilt resistance as a second layer of analysis. Vascular browning scores were predictive of foliar wilt, fitting ANOVA model best as a covariate nested within genotype rather than simply a second response variable. Inclusion of the scores improved model predictions of foliar witling variation substantially. This research suggests that: 1) Tomato rootstock lines are resistant across localized strains of Rs in NC. 2) The presence of healed graft wounds does not modulate resistance of rootstocks. 3) End-of-study vascular browning can be useful

in improving reliability of statistical assessments of foliar wilt variation. 4) Cold shock stress may be useful in improving separation of mean BW resistance levels of tomato rootstocks once the disease progression has plateaued. These data suggest future research questions for vascular browning and cold shock interactions with the BW pathosystem, and would be the first report assessing variation in vascular browning as it relates to tomato resistance to BW to the author's knowledge.

2.1: Introduction

Bacterial Wilt (BW) is a devastating disease of the tomato (*Solanum lycopersicum* L.) in major tropical and temperate tomato growing regions around the world, including Southeastern USA and the state of North Carolina (NC) (Kelman, 1953). Caused by the soil bacterium *Ralstonia solanacearum* Smith (Smith, 1896), BW infects hosts through wounds and sites of secondary root emergence, colonizes vascular parenchyma, and leads to permanent wilt of foliage (Hayward, 1991; Kelman, 1953). The pathogen is aided in this by production of a vast array of virulence factors that either camouflage Rs cells from host detection and/or inhibit host defense responses, as well as the ability to drastically change its phenotypic profile upon colonization of hosts (Brown and Allen, 2004; Jacobs, 2013; Meng, 2013; Remenant et al., 2010; Schell, 2000). Rs populations in North America have been identified as Race 1, biovar I, phylotype II (Agrios, 2005; Hayward, 1991; Remenant et al., 2010), which are virulent on tomato and tobacco (Kelman, 1953).

Management of BW has proven to be quite difficult for tomato growers, as the pathogen persists in the soil for many years, making crop rotation ineffective (Chellemi et al.,

1994). Soil fumigation is known to effectively reduce Rs populations in the soil, but does not correlate well with season-long management (Enfinger et al., 1979; Jyothi et al., 2012). The use of host resistance to BW is the only economically viable and effective management strategy for fields already infested with the pathogen (Hayward, 1991), particularly in NC. The threat of Race 3 biovar 2 to the North American potato industry has further reinforced the importance of host resistance, especially to multiple strains of Rs (Representative Tauzin, 2002).

Unfortunately, breeding large-fruited tomato varieties with strong BW resistance has proven to be nearly impossible due to tight genetic associations of small fruit size, indeterminate growth habit, and bitter fruit taste with BW resistance (Scott et al., 2005). Resistance in tomato is quantitative in nature, and analogous to a tolerance mechanism (Scott et al., 2005). Both resistance breeding and management efforts for BW are further hampered by the propensity of Rs to form latent infections in even highly resistant hosts, as well as a large environmental influence on wilting symptoms, especially from soil temperature (Acosta, 1978; Hayward, 1991; Scott et al., 2005). Generally, pathogen growth and ability to cause wilting symptoms is maximized when soil temperature is between 30-36 ^{o}C, but inhibited below 21 ^{o}C for most strains, even though viable, virulent colonies can be extracted from symptomless plants as low as 18 ^{o}C (Barnes and Vawdrey, 1993; Gallegly and Walker, 1949; Grieve, 1943; Kelman, 1953; Meier and Link, 1923; Mew and Ho, 1977; Singh et al., 2014b; Vaughan, 1944). In contrast, there are reports that some strains of Rs produce unique virulence factors at lower temperatures (Bocsanczy et al., 2012; Bocsanczy et al., 2014).

Since strong quantitative resistance does exist, an alternative management strategy using grafted tomato production has been proposed (Louws et al., 2010; Rivard et al., 2012; Rivard and Louws, 2008). Like in other woody crops, the use of soil-borne disease resistant varieties as rootstocks with commercial hybrid scions has been demonstrated to be a viable management strategy for BW in tomato in multiple locations in the USA, including NC (Freeman et al., 2011; McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008). Previous work with a selection of BW resistant rootstocks was performed in NC fields in 2009. The study demonstrated some geographic variation in the effective control of BW when 'RST-04-105-T' was used as a rootstock (Rivard et al., 2012).

In order to better assess rootstock performance for grafted management of soil-borne diseases such as BW in NC, and to identify rootstock germplasm for reliable resistance across NC tomato growing regions, field and greenhouse screens were performed in 2013-14 on 10 different rootstock genotypes from a variety of public and private sources in field and greenhouse conditions. The main goals of the study were: 1) To replicate field rootstock resistance results from previous 2009 study, testing resistance variation with presence or absence of healed graft union wound, as well as incorporating additional tomato rootstock genotypes. 2) To verify field resistance under natural disease pressure in a controlled greenhouse setting using artificial inoculation. 3) To assess variation in rootstock resistance response to two NC Rs tomato isolates collected from contrasting environmental and geographical backgrounds (Mountain and Coastal Plain regions). 4) The greenhouse study was affected mid-experiment by a severe cold weather event, causing recoverable low

temperature stress. Thus, I assessed the effect that a fortuitous low temperature stress event played on BW disease progression in the greenhouse study.

2.2: Materials and Methods

Eleven (11) tomato lines, which included a mixture of public and private genotypes ranging from highly susceptible to highly resistant (Table 2.1), were screened for resistance to BW in field (summer 2013; Jackson Co.) and greenhouse (winter 2013-14; Wake Co.) conditions in NC. The large-fruited commercial cultivar 'Florida 47' (FL47) is a popular variety in NC, and was used as susceptible check in all experiments. Rootstock varieties were chosen based upon their popularity as a tomato rootstock for vigor ('Maxifort'), or for their variation in resistance to BW, either by company labeling ('BHN 998', 'BHN 1053', 'BHN 1054', 'Cheong Gang', 'RST-04-105-T', and 'RST-04-106-T') or previous scientific evaluations ('CRA66', 'Hawaii 7997', and 'Hawaii 7998') (McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008; Scott et al., 2005).

2.2.1: Plant Growth Conditions

Both non- and self-grafted treatment plants for the field 2013 study were sown in parallel into 96-cell plug trays 6-7 weeks before being planted on June 28th, 2013 in a field with a known history of high annual natural BW disease pressure. Seedlings were grown in the greenhouse under daily overhead watering and bi-weekly synthetic fertilization (20-20-20) at ½ label rate before being transplanted into the field. The grafting methods are

described in a subsequent section. Care was taken during transplanting to ensure graft union remained above the soil line. The field planting beds were fumigated with chloropicrin. After transplanting into the field, plants were grown under the NC standard raised bed stake-and-string with drip irrigation and white plastic mulch method as previously described in similar trials in this location (Rivard et al., 2012).

Concern of a possible viral outbreak in the Jackson Co. plants led to quarantine measures that included disconnecting the study plots from the drip irrigation system midseason (35 dap). The plots were confirmed to not be infected with suspected viral diseases about a month later (data not shown) and the quarantine was lifted, but the irrigation was not reconnected for the duration of the season. The summer of 2013, however, had been an extremely wet season with abnormally high amounts of rainfall (more than three times the average rainfall during May-August), reduced light intensity, and a 2 ^{o}C reduction in average temperature. Thus, the plants in this study remained well watered and produced an acceptable harvest despite the disruption in controlled irrigation (Silverman et al., unpublished).

The greenhouse study plants were sown into 24-cell Pro-Tray Cell Flats in October, 2013. Prior to inoculation the plants were overhead watered 1-2 times daily and periodically fertilized at ½ label rate (20-20-20). Ten days prior to inoculation, 24-cell trays were placed in hole-less trays and bottom-watered as needed with 1-2 L for the duration of the experiment to maintain equal, constant soil moisture, as well as being placed on a heating mat timed to heat during the night from 6:00 PM to 6:00 AM. Plants were fertilized once after inoculation at 9 days post inoculation (dpi) with 1 L of ½ label rate poured into bottom tray. The heating pad was removed at 26 dpi.

A severe cold weather front in the first week of January caused abnormally low temperatures for the area, with the air temperature inside the greenhouse dropping to a low of 8.9 ^{o}C two nights in a row in the middle of the BW scoring period (dpi 21-22). Experimental plants did exhibit symptoms of low temperature stress (moist loss of turgor, epinasty, some growth deformation of newest foliage at that time), although root heat from the heating pad provided some protection since experimental plants showed the least severe symptoms compared to other tomato plants in the same greenhouse.

2.2.2: Grafting Methodology

Jackson Co. plants were grafted in Fox Greenhouse facilities (Raleigh, NC) at North Carolina State University using the Japanese top-grafting (also known as tube-grafting) technique and modified healing protocol, as described previously (Rivard and Louws, 2006; Rivard et al., 2012). Briefly, 2-3-leaf stage seedlings were brought into low light indoor conditions for at least two hours prior to grafting. Sterile razor blades were used to cut rootstock plant below the cotyledons at a 45^{o} angle. For self-grafted plants, the cut top was placed back on self bottom and secured with 2 mm silicon grafting clip.

After grafting, plants were gently placed in a greenhouse propagation block under a frame covered in 3-4 layers of fine shade cloth on top and sides for about 7 days. Fine mist from hanging emitters (CoolNet Pro 4-way fogger, Netafirm) was used to regulate humidity in healing chamber. Mist interval (Heavy: 4 seconds every 4 minutes; light: 4 seconds every 8 minutes) was used to gradually reduce humidity during healing process, while removal of shade cloth layers was used to gradually increase light intensity. Care was taken to ensure

mist did not build up heavy droplets on leaves, which would cause dislocation of graft union. After the healing period, the plants were placed back in full sun greenhouses for hardening-off until being transported to field.

2.2.3: Isolate Collection and Inoculation

Tomato strains of Rs were collected and isolated from infected commercial tomato varieties in Jackson Co. (Jc) and Pender Co. (P), fields in NC (Silverman et al., unpublished). Infected stems were surface sterilized with 10% bleach solution, rinsed in sterile distilled water (dH_2O), and the cut sections were streaked on TZC agar media (Dextrose, peptone, casamino acids, agar, dH_2O; 2, 3, 5—triphenyl tetrazolium chloride (TZC)) (French et al., 1995; Kelman, 1954). Identification of species was confirmed via colony morphology on TZC and Rs-specific ELISA (Agdia, Inc.; Elkhart, Indiana). Pure culture isolates were stored in 20% glycerol in sterile dH_2O and stored in -80 ^oC freezer.

Jackson Co., NC is in the heart of the Mountain region at a field elevation of about 564 m (1850 ft.) and 35^o N latitude. It has an average yearly temperature of 12.6 ^oC (54.7 ^oF), average temperature peak in July at 22.3 +/- 7.7 ^oC (72.2 +/- 12.0 ^oF), average annual humidity of 76.07%, and an average annual precipitation of 133.8 cm (52.67 in.) peaking from May to August. Pender Co., NC borders the Atlantic Ocean in the Coastal Plains region at an elevation of about 15 m (50 ft.) and 34^o N latitude. It has an average yearly temperature of 17.1 ^oC (62.7 ^oF), average temperature peak in July at 26.8 +/- 5.4 ^oC (80.3 +/- 10.0 ^oF), average annual humidity of 79.56%, and an average annual precipitation of 134.1 cm (52.81 in.) peaking from July to September.

Inoculum for greenhouse study was made by streaking CPD plates (TZC media without the TZC) (French et al., 1995; Kelman, 1954) with either Rs isolate Jc or P. Cultures were incubated at 30 ^{o}C for 72-hours and suspended in sterile dH_2O. The inoculum concentration was determined by spreading 100 uL of a 10-fold dilution series on plates of semi-selective media (SMSA-E (French et al., 1995)), which were then incubated for 48-hours at 30 ^{o}C. The number of colony forming units (CFUs) were determined by counting the number of single colonies of the lowest countable dilution plate that had greater than 10 colonies. Isolate Jc suspension was 2.1-4.0 x 10^9 CFU/mL and isolate P suspension was 1.0-2.8 x 10^9 CFU/mL.

Plants were inoculated using the soil drench method (Kelman, 1953). Briefly, well-watered and drained plant root balls were injured with a razor blade by making two parallel cuts through length of the root ball halfway between the stem and the plastic cell wall. The root ball was then drenched with 10 mL of bacterial suspension. The plants were then covered with clean, black, plastic bags for a 48-hour incubation period without watering, after which the bags were removed and plants were bottom watered.The Jackson Co. field study has a history of high annual disease pressure, with research and breeding trials regularly performed there. Thus, natural inoclum from the endemic population of Rs in the field was used for inoculation of the Jackson Co. field study. Plants were considered inoculated upon transplant.

2.2.4: Disease Data Collection Over Time

The Jackson Co. study incorporated non- and self-grafted combinations of plants, which were arranged in in a randomized split-plot design with four replications. Genotype blocks were devided by graft-type (Table 2.2). Disease progression was measured for each plant by observing wilt incidence (± wilting) in 1-2 week intervals, with 13 total observations occurring during a period of 91 days after planting (dap). Periodically, Rs presence in wilted plants was confirmed by internal examinations of select stems for vascular browning and bacterial streaming when the cut stem was immersed in water. Additionally, isolations of Rs from the stems were performed on select wilted plants to confirm the wilting agent, as described previously.

The greenhouse study plants were arranged in a randomized complete block design (RCBD) with three replications, blocked by treatments with Rs isolates (Table 2.2). Measurements were taken of each plant, but genotype was considered the experimental unit randomized within each replication. Thus, each plant-within-genotype was a subsample, allowing us to account for additional variation during analyses. Two plants per genotype were also used as negative inoculation controls. Disease severity was assessed for each plant using a 0 to 5 severity scale, where score of 0 = healthy appearance, 1 = one leaf wilting, 2 = 2 or more leaves wilting, but not whole plant, 3 = whole plant wilting, 4 = whole plant wilting and drying out, score 5 = whole plant wilting, drying, and decaying. Thus, a score of 4 or greater meant that the plant was irrecoverably wilted, having reached permanent wilting point, and was considered dead. Plants were rated every 2-3 days over a period of 42 days post inoculation (dpi).

Greenhouse plants recovered well from the cold stress and new growth was healthy. The canopy layer that had been newly expanding at time of cold event over the next few weeks of observations often appeared to be wilting during the heat of the day, but normal BW disease progression was not observed. Thus, notations were made for plants whose scores may have been artifacts of the lingering effects of low temperature stress, and a duplicate dataset with the corrected values was created and analyzed in parallel with the original.

2.2.5: End of Greenhouse Study Assays

At experiment end (43 dpi), plant stem was crossesctioned at 1.27 cm and 3.81 cm above the soil line and blotted onto SMSA-E media (French et al., 1995) to assess frequency of latent infections and spread of culturable Rs up the stem. Cuts were made with razor blades sanitized first in 10% bleach for at least 1 minute, rinsed in sterile dH_2O, then in 70% ethanol and let air dry. The same razor blade was used on the upper cut first, then the lower, after which it was sanitized. Each cut stem height was gently pressed multiple times (13-23) against the surface of the culture media for technical replications of blots. The inoculated plates were incubated at 30 ^{o}C, and assessed after 48-hours for presence/absence of cultured Rs and degree that stem cross-sectioned area was filled with bacteria. Rs identity as causal agent of wilt in study was confirmed by testing a sample of plants and cultures representative of the experimental levels with an Rs-specific ELISA, prescence of vascular browning, and periodic bacterial streaming tests from immersed stems.

Additionally, plants were assessed for the health of stem at 1.27 cm (0.5 inch) above soil line. To do this, the extent of vascular browning in the stem cross-section was rated using a severity scale of 0 to 5, where 0 = healthy, no vascular browning spots; 1 = one or two small spots; 2 = multiple spots, browning is < 50% of vascular ring; 3 = heavy browning, browning is > 50% of vascular ring; 4 = severe browning, inner pith beginning to decay; 5 = stem dead and hollow. These values were either used raw or compared using disease index.

2.2.6: Statistical Analyses

For the Jackson Co. study, disease incidence was compared directly as %wilt incidence of each plot over time, and by calculating the area under disease progress curve (AUDPC) per day for each plot,

$$AUDPC_{incidence} = \frac{\sum_{i=1}^{n}\left\{\left(\frac{\%wilt_n + \%wilt_{n+1}}{2}\right) * (time_{n+1}time_n)\right\}}{time_{n_{total}}}$$

where: n = the individual observation point, %wilt = the percent of wilted plants per plot, and time = the days post planting for n observation period. Due to the irrigation disconnect, three AUDPC values were calculated to assess if the disconnect effected disease severity: Total period (AUDPC_total; 0-91 dap), planting to disconnect (AUDPC_aug2; 0-35 dap), and disconnect to end of observation period (AUDPC_aug2end; 35-91 dap).

Disease severity scores were compared over time by calculating a disease index, where, for each time point for plants of the same class level, the number of plants with each score level were counted and multiplied by that score, the totals were added, and the sum was

divided by the total number of plants in the class. A similar index was created for vascular browning scores.

Disease severity was compared statistically by calculating AUDPC scores for each plant. A modified formula was used,

$$AUDPC_{severity} = \frac{\sum_{i=1}^{n}\left\{\left(\frac{score_n + score_{n+1}}{2}\right) * (time_{n+1} time_n)\right\}}{time_{n_{total}}}$$

where: n = the individual observation point, score = the severity scale score, time = the days post inoculation for n observation period. Due to the low temperature stress event, two AUDPC values were calculated to assess if the event affected disease severity: Total period (AUDPC_total; 1-42 dpi) and the period of inoculation to observed cold stress symptoms (AUDPC_21; 1-21 dpi).

Disease incidence values were calculated from BW severity scale values for each time point in the greenhouse study. Simply, any score of 0 = -wilting; any score greater than 0 = +wilting. Incidence of plant death (score 4 or 5) was similarly calculated, where any score < 4 = -death; any score ≥ 4 = +death. Additionally, to further assess influence of cold stress, rate of new wilting (score > 0 given previous period score = 0) and plant recovery (score = 0 given previous period score > 0) were calculated at each time point and plotted over time.

Statistical analyses were performed in SAS 9.4 (SAS; Cary, NC) using analysis of variance (ANOVA, MANOVA), generalized linear mixed models (MIXED, GLIMMIX), linear regression (REG), correlation (r, ρ), and Wilcoxon signed rank tests.

2.3: Results

Unless stated otherwise, all statistical tests and reported p-values are at $\alpha = 0.05$.

2.3.1: Jackson Co., 2013 Field Study

The 2013 Jackson Co. study was performed during the summer months in the Mountain region of NC under natural inoculum pressure, assessing field resistance as modulated by grafting. Disease pressure in the field was moderate to high for the Jackson Co., 2013 study based upon disease incidence of susceptible check 'Florida 47', which reached 72.5% and 62.5% incidence for non-grafted and self-grafted, respectively (Figure 2.1). First wilting was observed at 28 dap in non-grafted treatment, and 22 dap in self-grafted treatment. Study plots were disconnected from drip irrigation system on 35 dap, but this location received excessively high amounts of rainfall during the study period, yielding an acceptable harvest (Silverman, 2015, unpublished). It is unlikely that observed wilting was due to lack of water in the plots.

Overall, regular wilting incidence of the susceptible check showed that BW progressed regularly from 28 to 91 dap, reaching the highest mean incidence of all the genotypes tested in both the non-grafted and self-grafted treatments (Figure 2.1). In contrast, the rootstock genotypes showed comparable increases in BW incidence with the susceptible check until around 38-42 dap, after which no new BW was observed. For this reason, three AUDPC values were calculated and normalized to per day for comparison. Correlation analysis of the three statistics found a highly significant correlation (0-91 vs. 0-35 = 0.648, 0-

91 vs. 35-91 = 0.998, 0-35 vs. 35-91 = 0.598 ; $p < 0.0001$ all) between all three intervals (Figure 2.2).

Overall, significant differences in AUDPC were only found in the early season (0-35 dap; adjusted $R^2 = 0.6874$), with significant effects of genotype ($p < 0.0001$) and the interaction of genotype and replication ($p = 0.0004$), but not for the other two AUDPC observation periods (adjusted $R^2 = 0.1003$ and 0.0714 for 0-91 and 35-91, respectively). In both grafting treatments, 'Maxifort' had high BW incidence early in the early season (0-35 dap), actually being the first line to show wilting symptoms regardless of graft-type, with the least squares mean (LSMean) AUDPC being significantly different from the susceptible check. 'Maxifort' did not, however, reach similar incidence levels with susceptible check by the end of the season. Unexpectedly, the mean incidence of 'RST-04-105-T' was much higher than expected for both non- and self-grafted plots in light of previous field studies in this region (Rivard et al., 2012). 'Hawaii 7998' had the lowest mean incidence in the non-grafted plots, whereas 'BHN1054' and 'Cheong Gang' were lowest in the self-grafted plots.

Comparisons of LSMean AUDPC scores surprisingly showed no significantly different variation between genotypes for either the total observation period or 35-91 dap. An investigation of wilting incidence for each genotype revealed high wilt incidence variation between replications, as evidenced by the large confidence intervals (Figure 2.1: B and D). Analyses of the plots using a nested mixed model, with graft-type nested within genotype, yielded similar results (Figure 2.3). Interestingly, 'Maxifort' again had statistically greater mean daily AUDPC when compared to all other genotypes in the early season, indicating that

it exhibited wilting symptoms significantly earlier than any other genotype tested, including the susceptible check.

2.3.2: Greenhouse Study 2013-14

The greenhouse study was done during the winter of 2013-14 in NC. Two-month-old seedlings were inoculated with two geographically distinct Rs tomato isolates in order to determine if any tomato rootstock genotypes expressed differential resistance to NC Rs strains. The first wilting was observed on 7 dpi (Figure 2.5). Wilting incidence of susceptible check 'Florida 47' was very high, reaching greater than 90% incidence within five days after first wilting was observed. 'RST-04-105-T' and 'Maxifort' exhibited a similar overall wilting incidence pattern as 'Florida 47' reaching 100% incidence by the end of the study. The three BHN lines and 'CRA66' progressed similarly, but exhibited reduced incidence (50-75%) by the end of the study compared to the susceptible check. The Hawaii lines, 'Cheong Gang', and 'RST-04-106-T' had the least incidence overall (25-41%) by the end of the study, showing none or less than 10% wilting incidence until a sharp increase in BW incidence was observed at 29 dpi.

A severe cold weather front afflicted the area in the first week of January, 2014, such that the greenhouse air temperature reached 8.9 ^{o}C for two consecutive nights (dpi 21-22). Experimental plants were affected, but recovered quickly. Concerns of error from overlap of cold shock symptoms and true BW symptoms lead to creation of a second dataset where possible wilt error caused by lingering effects of cold shock was systematically corrected. Both datasets were analyzed in parallel, and patterns from analyses were found to be present

in both sets, though disease progression overtime was inflated (Data not shown). The cold corrected dataset was deemed to be more biologically accurate for BW assessment. Therefore, only analyses from cold corrected dataset are presented.

To assess the effect of the low temperature stress event on BW disease progression, total disease incidence of the experiment was plotted over time and compared to the diagnostic measures of incidence of permanent wilt (plant death), rate of new wilting, and rate of wilt recovery (Figure 2.6). First, overall wilt incidence consistently increased from 7 to 19 dpi, followed by a plateau where no new wilting occurred for about 7-9 days, which is corroborated by the decline to 0% for rate of new wilt. This period is described as epidemic phase 1 (A). This was followed by a brief but sharp increase in disease incidence at 29 dpi, followed by another plateau where rate of new wilt again declined to about 0%. This period is described as epidemic phase 2 (B). Additionally, incidence of permanent wilt (considered to be any severity score of 4 or greater) exhibited a positive slope over the entire course of the experiment, surpassing the overall BW incidence of the first phase plateau only a few days following observed second epidemic phase, demonstrating that a portion of plants newly wilting in the second phase reached permanent wilting point. Rate of wilt recovery (reversion back to severity score of 0) was never observed to be greater than 3% at any time over the entire course of experiment. It is also interesting to note that the similarity between the intervals between inoculation to first epidemic wilt and low temperature stress to observed second epidemic wilt, which was 5-7 days in both cases.

Since it appeared that the low temperature stress was related to the second phase of wilting, it was desirable to investigate if it affected the greenhouse study differentially by Rs

isolate treatment. Overall, it could not be confirmed that the plants wilted differentially following the cold shock event. All of the genotypes exhibited an increase in BW incidence following the cold shock, and the resistant lines expressed the largest magnitude increase of newly wilting plants (Figure 2.5 and 2.7). Assessments of wilting in relation to the cold shock event for each genotype lead to the identification of three distinct patterns—Genotypes where: 1) Most of the wilting incidence occurred during the first epidemic phase ('Florida 47', 'Maxifort', 'CRA66', 'RST-04-105-T', 'BHN1053'); 2) most of the wilting incidence occurred during the second epidemic phase ('Cheong Gang', 'RST-04-106-T', 'Hawaii 7997', 'Hawaii 7998'); and 3) substantial wilting incidence occurred in both first and second epidemic phases ('BHN1054'). 'BHN998' did not display any of these patterns, instead was observed to have fairly consistent increases in new wilt occurring regularly throughout the observation period. The average progression of wilt severity scores for lines representative of the three temperature-related patterns are presented in Figure 2.7.

In order to determine the overall resistance level of each genotype, and how that may have been modified by low temperature stress, two AUDPC values were calculated for each plant and compared—Total (1-42 dpi) and first epidemic only (1-21 dpi). The values significantly correlated ($\rho = 0.915$; $p < 0.0001$), but the matrix depicted two distinct modes of linearity (data not shown), so only the Spearman correlation is reported. A paired t-test of daily AUDPCs for each plant found that the first epidemic values were significantly different from the total period values ($df = 131$, $p < 0.0001$), with a distribution of the differences being skewed toward 0.0 (data not shown). Thus, influence of cold shock appears to have increased the overall daily AUDPC values in the study. Interestingly, a Signed Rank Test

found that the overall mean genotypic ranks were unchanged between first epidemic and total period ($p = 0.0625$).

Mixed model analysis of variance for total period AUDPC was significant ($p = 0.0002$; $R^2 = 0.703$), with genotype being highly significant ($p < 0.0001$) and borderline significance for the interaction of Rs isolate and replication ($p = 0.0451$). The interaction of genotype and Rs isolate was not significant ($p = 0.6846$). Overall all, 'Florida 47' had the highest mean daily AUDPC, consistent with it being the susceptible check, for both total and first epidemic periods. 'RST-04-105-T', 'Maxifort', and 'BHN1053' were not statistically different from the susceptible check in the overall period, but 'BHN1053' was significantly different in the first epidemic (Figure 2.8). 'RST-04-105-T' was found to have the second largest daily mean AUDPC, irrespective of period. It was not significantly different from the susceptible check or susceptible 'Maxifort', but was significantly different from 'BHN998, 'Hawaii 7997', 'Hawaii 7998', 'Cheong Gang', and 'RST-04-106-T'. The Hawaii genotypes, 'Cheong 'Gang', and 'RST-04-106-T' were the most resistant genotypes, but were not statistically different from the intermediately resistant BHN lines or 'CRA66'.

Since one of the main objectives of this study was to determine if any of the genotypes expressed a differential resistance response to the Rs isolates, I present the mean daily AUDPC comparisons for the genotype by Rs isolate interaction, even though it was not statistically significant (Figure 2.9). Although no significant differential resistance responses were observed for any genotype based upon mean daily AUDPC, 'RST-04-105-T' and 'Hawaii 7997'appear to be good candidates for further assessments. It is interesting that 'Hawaii 7997' only had wilting under treatment Jc but none under treatment P.

I found significant statistical overlap in the greenhouse study despite the fact that the plants were artificially inoculated with a high concentration of Rs. Upon investigation, I found that there was unequal variation between the genotypes in a parabolic pattern when genotypes were ordered according to their mean resistance levels (mean daily AUDPC) (Figure 2.10; C). The most resistant and most susceptible lines exhibited the least model residual within genotype variation, whereas the intermediately resistant lines showed high levels of variation. The nature of this variation remains unclear.

Upon completion of the study, each plant stem was assessed for signs and symptoms of BW to confirm that wilting was due to Rs. Stem cross-sections were assessed for culturable Rs and scored for extent of vascular browning. Of the 132 plants inoculated, only four were found without any signs or symptoms of BW, suggesting Rs failed to colonize them (one each: 'CRA66', 'BHN998', 'Hawaii 7997', and 'Hawaii 7998'). In contrast, 45 inoculated plants (34.1%) never expressed wilting symptoms during the study, but Rs was detected in all except the previously mentioned four (31.1% latent infection rate), including the most resistant lines. Interestingly, even most dead stems contained culturable Rs near the soil line.

Examination of the plant stems showed that there was variation in vascular browning of stem cross-sections, thus a severity scale of 0-5 was developed in order to assess the stems at 1.27 cm above the soil line. The mean and distribution of vascular browning scores was found to fluctuate by genotype (Figure 2.10; A and B). Additionally, vascular browning scores were highly correlated ($p < 0.0001$) with daily AUDPC scores for total period ($r = 0.894$) and first epidemic ($r = 0.765$). Thus, a second level of mixed model analysis was

performed in order to determine if the addition of vascular browning information improved model predictions of foliar wilting. Initial tests using vascular browning as a second response variable along with total period daily AUDPC was not the best fit, since vascular browning scores were still significantly predictive of model residuals (data not shown). It was determined that vascular browning data fit best as a covariate nested within genotype ($F = 14.26$, $p < 0.0001$, $R^2 = 0.952$). The effects of genotype remained highly significant ($p = 0.0080$) and vascular browning nested within genotype was found to by a highly significant effect ($p < 0.0001$). Additionally, the strange pattern of large, parabolic residual variation in the model residuals was substantially reduced (Figure 2.10; compare C and D), except for 'BHN998' for some unknown reason.

Further assessments of the vascular browning scores (Figure 2.11) found that they were an effective explanation for both the raw daily AUDPC values (A), as well as the model linear predictions of daily AUDPC values (B). In a separate analysis methodology, a disease index (C) was created in the same fashion as previously mentioned for wilt severity scores, and genotypic means were used, along with model slope and intercept coefficients, to predict the mean daily AUDPC values. The predictions had the same relative genotypic rankings (data not shown) compared to the mixed model assessments prior to inclusion of vascular browning scores. This further indicated that vascular browning mean and slope information was effective at predicting relative foliar wilt resistance. Inspections of the slopes (D) found that the genotypes may cluster into six or seven slope groups, with the coefficients differing by a maximum of 0.1, generally grouping according to resistance to foliar wilt. The suggested genotypes in each cluster are: 1) 'Florida 47'; 2) 'RST-04-105-T' and 'Maxifort';

3) ‘BHN1053’; 4) ‘CRA66’ and ‘BHN1054’; 5) ‘Hawaii 7997’ and ‘BHN998’; 6) ‘Hawaii 7998’, ‘Cheong Gang’, and perhaps ‘RST-04-106-T’; and 7) ‘RST-04-106-T’.

2.4: Discussion and Conclusions

I assessed BW resistance in tomato rootstock genotypes in field and greenhouse settings, as well as investigated the modulating effects of healed graft union, NC region Rs isolates, and cold shock stress on tomato resistance levels. In addition, I provide the first known study comparing vascular browning of the stem to foliar wilting in tomato.

2.4.1: Rootstock Resistance when Grafted or Inoculated with NC Rs Isolates

A previous report (Rivard et al., 2012) suggested that ‘RST-04-105-T’ may express a differential resistance response to BW based upon geography, having moderate resistance in the Mountain region counties (Henderson Co. and Jackson Co.) and high resistance in the Coastal Plains region counties (Sampson Co., which borders Pender Co.). Variation of resistance based upon regional strains of Rs is well documented (Hayward, 1991; Hong et al., 2012; Lebeau et al., 2011; Scott et al., 2005; Wang et al., 2013; Zehr, 1970). To assess this hypothesis in all the genotypes, the greenhouse study compared BW resistance levels to two Rs tomato isolates from these regions. Susceptible control ‘Florida 47’ had the highest disease incidence and severity in both field and greenhouse studies, as expected, reaching incidence levels consistent with moderately high disease pressure (about 60-70%) in the field and very high disease pressure in the greenhouse (100%). Surprisingly, ‘RST-04-105-T’ was

found to have the second highest incidence and severity in both studies, which is generally in conflict with other reports and company labeling that 'RST-04-105-T' (DP Seeds) is moderately to highly resistant to BW (McAvoy et al., 2012; Rivard et al., 2012). My results indicate that this genotype is highly susceptible to BW strains from both regions of NC, and should not be recommended for use in NC fields with a known history of the disease.

No differential resistance response could be statistically verified in the greenhouse for any genotype. This would indicate that local endemic populations of Rs in NC are not expected to wilt these genotypes differently between regions. Previous work observed a potential differential resistance response from 'RST-04-105-T' in these regions, but it should be noted that in those studies, this rootstock was only assessed with a commercial scion grafted onto it—never the genotype non- or self-grafted, as in this study (Rivard et al., 2012). It is not expected that the presence of a susceptible commercial scion would modulate resistance expression of a rootstock towards greater resistance to foliar wilt. On the other hand, it is possible that other factors of the Coastal Plains region contributed to the differences they observed. It is true that my results observed a large difference in the mean wilting of 'RST-04-105-T', as well as in 'Hawaii 7997', but those differences were not significant. Greater statistical power in future assessments may demonstrate significant differences in resistance by Rs isolate.

Additionally, it was consistently noticed that 'RST-04-105-T', when allowed to bear leaves, exhibited a strange disorder that caused hyper leaf senescence of bottom and middle canopies working toward the apex, beginning at the tip of the terminal leaflet and progressing towards the bud. I suspect that this is a genetic disorder since it was present in all plants

under all conditions observed, including from multiple batches of seeds. In the greenhouse study, it was rare to observe more than two or three fully expanded leaves on the plants at any one time. Field study plants, with a greater access to water, nutrition, and sunlight, showed more leaf retention, but the plants still looked substantially poorer than the other genotypes (Data not shown).

In the Jackson Co. study, I was testing the hypothesis that the presence of a healed graft wound would increase susceptibility of the plant to infection by Rs. Despite the high degree of statistical overlap, some biologically meaningful information was apparent. An interaction between genotype and replication was observed, indicating that Rs presence in the field was captured by experimental placement, but remained aggregated in relation to symptom expression during the study. For the effect of grafting, I was not able to identify a significant difference in wilting between non- and self-grafted plots, suggesting that risk of infection through arial graft union is low. Other studies also observed that grafting does not significantly increase susceptibility to BW (Freeman et al., 2011; McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008), although this is the first study that compares non- and self-grafted treatments for all rootstocks. These previous studies have only made that comparison with the susceptible control, which normally reaches between 70-100% losses anyways. In those situations, a lack of resistance genetics are likely to have a much greater effect on wilting rather than a healed graft wound. It is unfortunate that the summer of 2013 was such an abnormal year for a study of this kind. Additional verification is needed before it can be reliably concluded if graft wounds do increase BW susceptibility or not. The observation that ‘Maxifort’ had significantly higher mean daily AUDPC in the early season

matches with my observations that it was the first line to wilt in the season, even if it was eventually outpaced by the susceptible control. Additionally, the effect of grafting may have amplified that pattern (Figure 2.4). It should be noted that the interaction of genotype and grafting treatment was not found to be significant in the model, thus the interaction of grafting with 'Maxifort' is not conclusive. 'Maxifort' is not known to have any resistance to BW, which the greenhouse study seems to verify, but it is known to be a highly vigorous, multi-stemmed, indeterminate rootstock. If infection occurred later in the season after 'Maxifort' plants had become well established, the sheer vigor of the line may give it the appearance of reduced susceptibility to BW by simply being able to cope longer with the presence of Rs in the vasculature.

It was found that 'BHN1053' and 'BHN1054' (BHN Seed) had moderately high disease incidence in both studies. In the greenhouse study, 'BHN1054' grouped as only significantly different from the susceptible control, and 'BHN1053' grouped with the susceptible control but had significantly greater disease than 'Hawaii 7998' and 'RST-04-106-T'. This level of BW susceptibility is greater than previously reported from work in Virginia and Florida (Freeman et al., 2011; McAvoy et al., 2012). I also observed a higher level of wilting in 'CRA66' than was previously observed in work in NC (Chellemi et al., 1994; Rivard and Louws, 2008).

Based upon these studies, rootstocks that should be recommended for BW disease resistance in NC would be 'RST-04-106-T', 'Hawaii 7997', 'Hawaii 7998', and 'Cheong Gang', which is in agreement with other regional and world evaluations of some of these lines (McAvoy et al., 2012; Rivard et al., 2012; Scott et al., 2005). Additionally, these

genotypes are expected to perform well in contact with both Western and Eastern NC strains of Rs and with a grafted tomato management system.

2.4.2: Influences of Environmental Conditions on Bacterial Wilt

I was interested to assess the effect of environmental influences on BW. In the Jackson Co. study, the high degree of within genotype disease resistance variation made meaningful statistical comparison very challenging, with only 'Maxifort' found with significantly greater mean daily AUDPC in the field study, and only in the early season (0-35 dap) interval (Only in the self-grafted treatment, depending on if graft-type was nested within genotype or each graft-type was analyzed separately.). Although more meaningful contrasts were possible in the greenhouse study, large within genotype BW resistance variation was still observed. As noted previously, it is well known that even small changes in environmental conditions can play significant roles in expression of foliar wilting, especially soil temperature, soil moisture, and air temperature (Acosta, 1978; Gallegly and Walker, 1949; Hayward, 1991; Kelman, 1953; Mew and Ho, 1977; Scott et al., 2005). The reduced average temperature and waterlogged soil conditions likely suppressed the expression of overall wilt symptoms in the Jackson Co. study. It was previously noted that due to the record rainfall during the summer season, the average temperature was reduced by 2 ^{o}C. A comparison of spring and summer studies in Guadaloupe also observed a 2 ^{o}C reduction in mean daytime temperature, which was linked to suppression of BW symptoms (Prior et al., 1996). Additionally, the reliance on natural inoculum likely contributed to the sporadic distribution of observable BW in the field, which may not have been as troublesome in a more normal year when incidence of susceptible controls have historically been reported to

be higher (Rivard et al., 2012), and by other unpublished BW screening experiences and production history of those regions of the tomato field used in the study.

Another curious pattern from the Jackson Co. study was that most of the wilt incidence occurred during observations in the week following the study irrigation disconnect. It was observed that during that week there was some lull in the rain intensity and breaks in the cloud layer allowing much more light into the field, along with increasing the air temperature noticeably. After that, the rest of the season was more heavy rain nearly on a daily basis, particularly in the afternoons when BW symptoms were mostly likely to be observed (Dialog with field manager). Thus, the environmental conditions were less conducive to observing disease progression most of the season, likely leading to a reduction in observed wilt that may have been observed in a more average season following that period of increase. The fact that the susceptible control continued to increase in incidence over the entire season, whereas even moderately resistant lines did not, reinforces this view and discourages the possibility of observed wilting around the water disconnect to simply be an indication of some drought stress yielding false positive wilt incidence.

The unexpected presence of the second phase of newly wilted plants observed at 29 dpi may indicate that cold shock stress, and perhaps other environmental stress factors, may be leveraged in BW screening studies to improve genotype mean separation, and improve reliability of greenhouse results to field environments. The low temperatures reached are not conducive to tomato or pathogen growth, and are well below the known soil temperature threshold for observing wilting symptoms (Hayward, 1991; Scherf et al., 2010; Swanson et al., 2005; Vaughan, 1944; Zehr, 1970). Additionally, cold shock stress effects were most

pronounced in the most resistant lines, but that may simply be due to the fact that all of the plants were still alive at that point, compared to the intermediately and highly susceptible lines. It is interesting to note the patterns of variation of cold shock stress observed in each genotype, with some lines seeming to be rather unaffected by cold shock ('BHN1053' and 'CRA66') versus the similarly resistant 'BHN1054'. (Mew and Ho, 1977) noted at least one tomato genotype that appeared to be fairly unaffected by increases in soil temperature. Cold shock effects on BW are not expected in field production systems, but may be important in winter greenhouse production systems. It would be very interesting to see if other shock stress factors such as heat or drought might increase BW incidence when applied in a controlled manner. Of course, since these are results of only one study so far, caution in generalization of results is wise. Followup studies are needed to compare controlled low temperature exposure in susceptible, moderately resistant, and highly resistant genotypes.

Within the greenhouse study, the pairwise comparisons between the first epidemic AUDPCs and the total period revealed a greater degree of similarity between the genotypes in the early epidemic phase than after the cold shock event (Figure 2.8; A and B). This suggests that the cold shock event and subsequent additional wilting actually improved the ability to separate the mean daily AUDPC values of the genotypes between the highly susceptible and the highly resistant lines, despite an overall increase in wilt severity following the cold shock event, particularly in the highly resistant genotypes. In retrospect, the timing of the low temperature stress could not have been more ideal, since the progression of bacterial wilt incidence had plateaued two observation periods previously, suggesting that low temperature shock stress had a causative effect on the second wilting

epidemic. (Carmeille et al., 2006) reported that wilting symptoms progressed rapidly among greenhouse seedlings with 6-8 fully expanded leaves until 15 dpi, after which it stabilized by 22-23 dpi. Unfortunately, they did not present disease progression over time data, so it is unclear if the authors were indicating that the population reached a linear rate of wilt incidence or a exhibited a horizontal plateau of disease progression, such as was observed in my greenhouse study. Many other greenhouse and controlled experiments that reported the progression of BW overtime have used either much younger plants or used a direct stem inoculation method rather than a soil soak. That being the case, while the specific day intervals may not be relatable to my work, a plateau effect in BW development in the populations has been previously observed (Dalsing and Allen, 2014; Wang et al., 2000). Resistant lines in field assessments have shown a plateauing effect in some cases when disease progression over time has been reported (Mew and Ho, 1977; Rivard et al., 2012). (Mew and Ho, 1976) observed a low positive correlation between BW resistsance assessments between young tomato plants and plants at flowering stage. In the greenhouse study, plants were older and first flowering was observed about 26 dpi in plants that were nearly four months old.

While it is impossible to demonstrate the specific effect the cold shock stress had on the host-pathogen interaction in this study, several hypotheses could be generated. First, the most likely hypothesis is that the cold shock, which was primarily a foliar effect due to overnight heating of the root zone, weakened the host such that the disease equilibrium between host defenses and pathogen attacks was altered in favor of Rs. (Walker, 1965) wrote a nice review of how changes in temperature, age, and soil moisture can affect host resistance

levels in a variety of plant-pathogen interactions. A less likely alternative hypothesis is that the wilting was due to expression profile changes within the bacteria which led to either decreased detection by the host or increased virulence. While race 1 Rs is not considered to be virulent at temperatures as low as 18 ^{o}C, other strains like race 3 biovar 2 are virulent at such temperatures, with the increase in virulence seeming to be linked to specific host-pathogen interactions *in planta*, which include low temperature expression of unique effectors (Bocsanczy et al., 2011; Bocsanczy et al., 2012; Bocsanczy et al., 2014; Jacobs et al., 2012; Milling et al., 2009; Swanson et al., 2005), though it is also reported that low temperature fluctuations typical of temperate climates negatively affected survival of Rs in tomato and other species (Scherf et al., 2010). Within both hypotheses, this change in disease equilibrium might occur in previously colonized stems or at the point of Rs penetration of the roots. The literature would generally suggest the former (reversion of latent infections) to be most likely (McAvoy et al., 2012; Milling et al., 2009; Prior et al., 1996; Scott et al., 2005; Swanson et al., 2005), while the curiously similar intervals between inoculation and cold shock stress with their respective surges in new wilting may suggest the later, that the second epidemic was related to a surge in secondary infection. A third hypothesis that might explain my observations is that the low temperature stress was inconsequential, and the observed second epidemic would have occurred anyways, which would carry some startling implications about host resistance mechanisms, host-pathogen interactions, and/or secondary infection patterns, but such a pattern has not been reported to my knowledge. Due to the difficulty of replicating such a cold shock as was experienced, it is difficult to make definitive conclusions about the modulating effect such a stress might have on the BW

pathosystem. Regardless, it should be noted that controlled seedling BW assays are often concluded around 20-25 dpi, which I was planning to do also, especially after the initial observation of cold stress effects in the experiment. Had I proceeded with experiment cleanup at that time, the second surge of new wilting would never have been observed.

2.4.3: Vascular Browning as a Significant Predictor of Foliar Wilt

The presence of vascular browning of the stem is a common symptom associated with BW, and has classically been treated as one of several diagnostic assessments of wilting pathogens (Hayward, 1991; Kelman, 1953; Winstead and Walker, 1954). During the end-of-study latent infection tests, I observed a high level of variation of vascular browning in the tomato stem cross-sections, as well as low to moderate levels of it in many plants that had never exhibited wilting symptoms during the entire study. So, I developed a severity scale to assess this variation. To the knowledge of the author, this work is the first case where genotypic variation of vascular browning has been assessed or used to help predict variation of foliar wilting.

Vascular browning scale scores of stem cross-sections were calculated at the end of the study, since they are destructive measurements. The severity score values of each plant were included into the general mixed model as a secondary level of analysis. Vascular browning scores fit best as a covariate rather than as a second response variable along with daily AUDPC. The addition of the greenhouse study vascular browning scores to the mixed model significantly increased the explanatory power of the model (R^2 = 0.703 before; = 0.952 after) and reduced the spread of residual plots. The base model residuals for

intermediate genotypes were spread just over ± 2 mean daily AUDPC, but inclusion of vascular browning scores reduced that spread by half to ± 1 mean daily AUDPC, except for 'BHN998'. Additionally, the base model residuals showed a distinct parabolic spread when genotypes were sorted by LSMean daily AUDPC, with intermediately resistant lines showing the greatest within genotype variation. Inclusion of the vascular browning scores markedly corrected this pattern of parabolic variation (Figure 2.10; compare C with D).

End-of-study vascular browning is a significant predictor of total period daily AUDPC, and my analyses suggest that this is via the resistance level of the genotype. In some sense, vascular browning may be a somewhat redundant measure of disease, yet the association between vascular browning and leaf wilt AUDPC appears to be driven by the resistance level of the genotype, since the raw scores remained significantly predictive of the model residuals where AUDPC and vascular browning were both response variables (Data not shown).

Wilting is generally thought to be simply related to high densities of bacteria blocking water flow in vessel elements (Hayward, 1991; Kelman, 1953). The importance of bacterial concentrations and spread in the stem in relation to host resistance has been documented (Grimault and Prior, 1993; Grimault et al., 1994a). Other reports have noted that foliar wilting is related to spread of Rs up the stem, seemingly irrespective of host resistance, and that the spread is a function of temperature (Prior et al., 1996). It would be very interesting to assess foliar wilting and vascular browning levels with inoculum load in order to determine the interactions. (Winstead and Walker, 1954) reported that vascular browning is generally a symptom of multiple fungal and bacterial pathogens irrespective of host, and suggested it

was related to high levels of pectin methylesterase activity, though the enzymes were not expected to be a critical factor involved in pathogen establishment. (Kelman, 1953) noted that foliar symptom development is more rapid the younger a plant is, and that vascular browning can be observed as early as 48 hours after inoculation, although that time-frame is sure to change between stem puncture or soil drench inoculation methods.

It remains unclear if foliar wilting and vascular degredation are simply two independent symptoms of Rs colonization, or if they are related in a causative manner. The nature of this relationship will dictact whether or not vascular browning should only be assessed as a dependent variable, or if analysis as a covariate nested within genotype is biologically accurate. The general pattern is that an increase in AUDPC is related to an increase in severity of vascular browning, with the greatest variation occurring in lines that are moderately resistant. I did observe, however, that the mean and spread of vascular browning was not the same for all genotypes, with even the most resistant genotypes tolerating light to moderate vascular browning, while showing very little foliar wilting. In fact, no foliar wilt was observed in plants with none to light vascular browning (severity score of 0-1) (Data not shown). Curiously, the resistant 'Hawaii 7997' had the lowest mean vascular browning, but was the fourth most resistant, while most of the plants of the top three most resistant genotypes ('RST-04-106-T', 'Cheong Gang', and 'Hawaii 7998') had moderate to heavy vascular browning (severity score of 2-3).

Is it possible that foliar wilting is at least partly the result of vascular breakdown rather than simply xylem vessels becoming clogged by high numbers of Rs cells? Lack of water is the clear source of the turgor loss, but it is not clear if disruption in water movement

is due to vessel occlusion or degredation of the vasculature, since the foundational studies have only been able to demonstrate that water movement is disrupted only in tissues containing Rs (Kelman, 1953). If wilting was only due to vascular occlusion, it may be expected that wilt would be observed prior to vascular degredation, since the pathogen replicates rapidly and mass produces exopolysaccharide I upon penetration and colonization of the vascular cambium (Denny et al., 1998; McGarvey et al., 1999; Schell, 2000). This may be, unless spread of the pathogen up the stem—shown to be important for foliar wilt symptom expression (Prior et al., 1996)—is linked to vascular degredation, or the pathogen biology predicates both growth and tissue disruption simultaneously. The answer may be that vascular degredation is linked to pathogen spread in the stem, since that has been reported to be fairly independent of transpiration rate and water uptake in the roots (Grieve, 1941; Kelman, 1953).

Another potentially important question is, is vascular browning caused by the direct activity of Rs, or is it a byproduct of either a successful or failed host resistance mechanisms (i.e. a vascular hypersensitive reaction of sorts, ability or inability to repair vascular tissues fast enough, etc.)? It is known that resistance is related to production and self-protection of reactive oxygen species (ROS) (Mandal et al., 2011), lignin biosynthesis (Ishihara et al., 2012), possibly callose deposition (Beckman, 2000), and constriction of bacteria to primary xylem tissues (Ishihara et al., 2012). If the plant ROS scavaging or cellular reinforcement systems are disrupted by bacterial effectors or not stimulated due to failure to detect Rs, then cellular damage of the host will result (Sharma et al., 2012), such as vascular browning, in which case resistance would be related to functioning cellular repair systems, which has been

reported (Mandal et al., 2011). The ability to pre-condition (prime) tomato immune responses with pre-inoculation treatments of silicon, chitosan, or *Bacillus* spp. suggests that the rate and strength of immune stimulation is key to successful defense against Rs (Diogo and Wydra, 2007; Ghareeb et al., 2011a; Hyakumachi et al., 2013; Takahashi et al., 2014). In this hypothetical case, resistance would equal reduced vascular browning and reduced pathogen spread, but may or may not prevent foliar wilting unless pathogen populations are also restricted. Alternatively, if resistance were related to a hypersensitive reaction-like oxidative burst in the vascular tissues, pathogen movement would be restricted and small areas of localized cell death would be observable as vascular browning. Thus, resistance would equal some vascular browning and constricted Rs spread in the stem. This is likely what the host is trying to do, albeit unsuccessfully, since Rs goes to great lengths to protect itself from a highly oxidative environment *in planta* (Colburn-Clifford et al., 2010; Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011; Mandal et al., 2011). These hypotheses require further testing in order to determine the true relationships between foliar wilt, pathogen concentration, pathogen spread in the stem, and vascular degredation in tomato. Further elucidation of these biological relationships will provide clearer direction for how to incorporate vascular browning assessments into predictive models for tomato resistance to BW. Assessing these factors at multiple time points at multiple stem heights in several genotypes will help elucidate these relationships.

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CHAPTER 2: TABLES AND FIGURES

Table 2.1. ***Genotypic and phenotypic information on tomato lines*.**
Genotypic and phenotypic information on the tomato lines used in the field and/or greenhouse studies for resistance to the bacterial wilt causal agent, *Ralstonia solanacearum* (Rs).

Name	Synonym	Usage	Breeding Type; Habit	NC-Mountain Rs Strain	NC-Coastal Plains Rs Strain	Rs Resistance Source	Other Resistances	Other Information	Developer or Seed Source
Hawaii 7997	HI7997; Ha7997	Rootstock	OP; SD	MR-R	R	PI 127805A	N/A		Univ. FL
Hawaii 7998	HI7998; Ha7998	Rootstock	OP; D	R	R	PI 127805A	N/A	Bacterial Spot resistance (*X. campestris* pv. *vesicatoria*)	Univ. FL
Cheong Gang	ChGng	Rootstock	N/A	R	R	N/A	Fol: 1, 2, For, RKN, ToMV		Seminis
RST-04-106-T	DP106	Rootstock	N/A	R	R	N/A	Pt, Fol: 1, 2, RKN, ToMV		DP Seeds
BHN 998	N/A	Rootstock	N/A	MR	MR	N/A	N/A		BHN Seeds
BHN 1053	N/A	Rootstock	N/A	MR-MS	MR	N/A	N/A		BHN Seeds
BHN 1054	N/A	Rootstock	N/A	MR-MS	MR	N/A	N/A		BHN Seeds
CRA66	N/A	Rootstock	OP; ID	MR-HR	MR-HR	Unknown	N/A		Univ. FL; INRA, Guadaloupe
Maxifort	Maxi	Rootstock	F1H; I	S	S	None	Pt, Fol: 1, 2, For, V, RKN, ToMV	Highly vigorous growth	Paramount Seeds; Johnny's Select Seed
RST-04-105-T	DP105	Rootstock	N/A; I	S	MS-S	N/A	Pt, Fol: 1, 2, For, V, RKN	Defoliating disorder	DP Seeds
Florida 47	FL47	Scion	F1H; D	S	S	None	Aal, Fol: 1,2, Ss, Vd	75 days to maturity	Seedway

Notes: *I- Indeterminate* *D- Determinate* *R- Resistant* *S- Susceptible* *MR- Moderately resistant* *MS- Moderately susceptible*
SD- Semi determinate *OP- Open pollinated* *F1H- F1 hybrid* *N/A- Information is NOT available* *Rs- Ralstonia solanacearum*

Table 2.2. ***Experimental design for the field and greenhouse.***
Plant numbers for the Jackson Co., NC 2013 field and Wake Co., NC 2013-14 greenhouse studies.

Field Study	Genotype	Non-graft plants/plot *	Self-graft plants/plot *	Total Non-graft	Total Self-graft
Jackson Co., 2013	FL47	10	10	40	40
	CRA66	3	3	12	12
	Cheong Gang	6	6	24	24
	Maxifort	6	6	24	24
	BHN1053	6	6	24	24
	BHN1054	6	6	24	24
	DP105	6	6	24	24
	DP106	6	6	24	24
	HI7997	6	0**	24	0**
	HI7998	6	0**	24	0**
			Total:	**244**	**196**

Greenhouse Study	Genotype	Isolate Jc plants/rep *	Isolate P plants/rep *	Total Jc	Total P
Wake Co., 2013-14	FL47	2	2	6	6
	CRA66	2	2	6	6
	Cheong Gang	2	2	6	6
	Maxifort	2	2	6	6
	BHN998	2	2	6	6
	BHN1053	2	2	6	6
	BHN1054	2	2	6	6
	DP105	2	2	6	6
	DP106	2	2	6	6
	HI7997	2	2	6	6
	HI7998	2	2	6	6
			Total:	**66**	**66**

Notes: ** 4 replications for Jackson Co., 3 replications for greenhouse, of plot*
***Due to unexpected losses during grafting procedure*

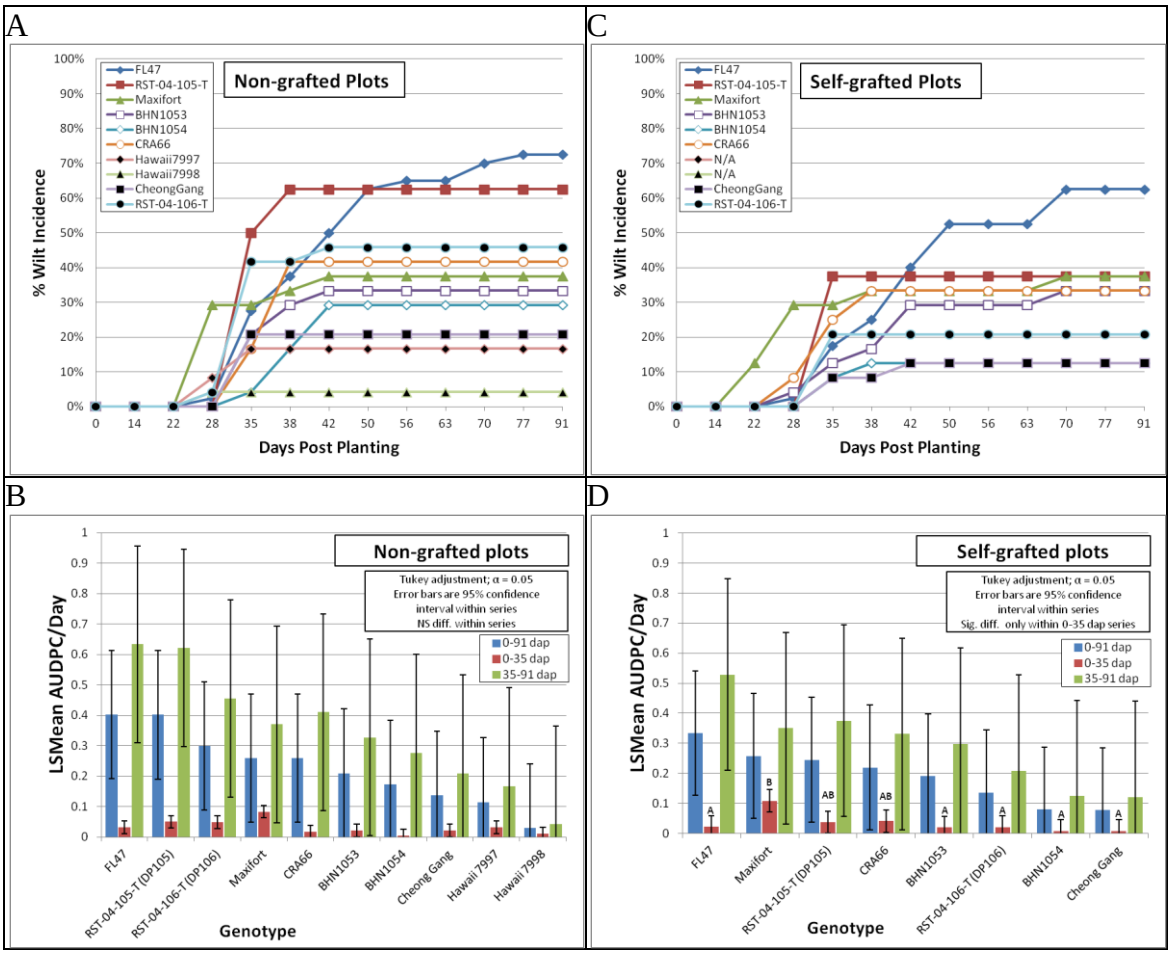
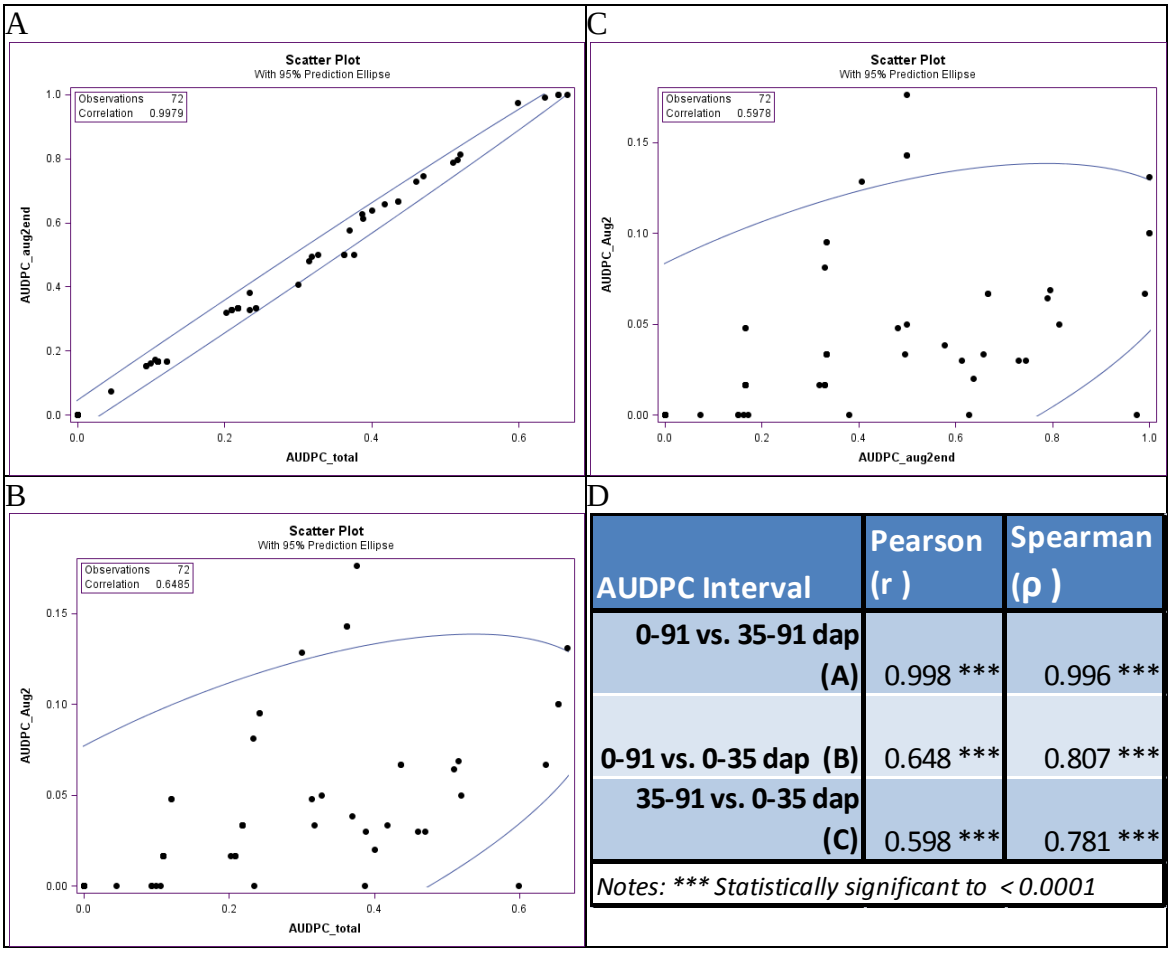


Figure 2.1. ***Bacterial wilt disease in the Jackson Co. 2013 study.***
The Jackson Co. 2013 study of bacterial wilt (*Ralstonia solanacearum*) disease incidence and least squares mean (LSMean) daily area under disease pressure curves (AUDPCs) for four replications of both non-grafted and self-grafted plots over multiple time intervals. Mean bacterial wilt incidence of ten tomato genotypes for A) non-grafted treatments and C) self-grafted treatments (Note: Self-grafted N/A series had no plots due to grafting failure for 'Hawaii 7997' and 'Hawaii 7998'). Daily LSMean AUDPC values comparing three calculation intervals: 0 to 91, 0 to 35, and 35 to 91 days after planting (dap) of B) non-grafted plots and D) self-grafted plots. Error bars within LSMean AUDPC (B and D) are 95% confidence intervals for comparison within each series, and genotypes with same letters (D) are not significantly different within each series, based upon Tukey's mean comparison test (α = 0.05).



AUDPC Interval	Pearson (r)	Spearman (ρ)
0-91 vs. 35-91 dap (A)	0.998 ***	0.996 ***
0-91 vs. 0-35 dap (B)	0.648 ***	0.807 ***
35-91 vs. 0-35 dap (C)	0.598 ***	0.781 ***

*Notes: *** Statistically significant to < 0.0001*

Figure 2.2. ***Correlation analysis for bacterial wilt in Jackson Co. 2013 Study*.**
Jackson Co. 2013 field study correlation analyses for bacterial wilt (*Ralstonia solanacearum*) area under disease progress curves (AUDPCs) calculated from disease incidence per plot. Pearson correlations for comparison of three different daily AUDPC scores: 0-91, 0-35, and 35-91 days after planting (A, B, and C). Lines depict range of 95% prediction interval. D) Summary table of each AUDPC interval correlation for Pearson and Spearman calculation methods and statistical significance level. A, B, and C related to the respective charts in this panel. For each comparison, n = 72 plots.

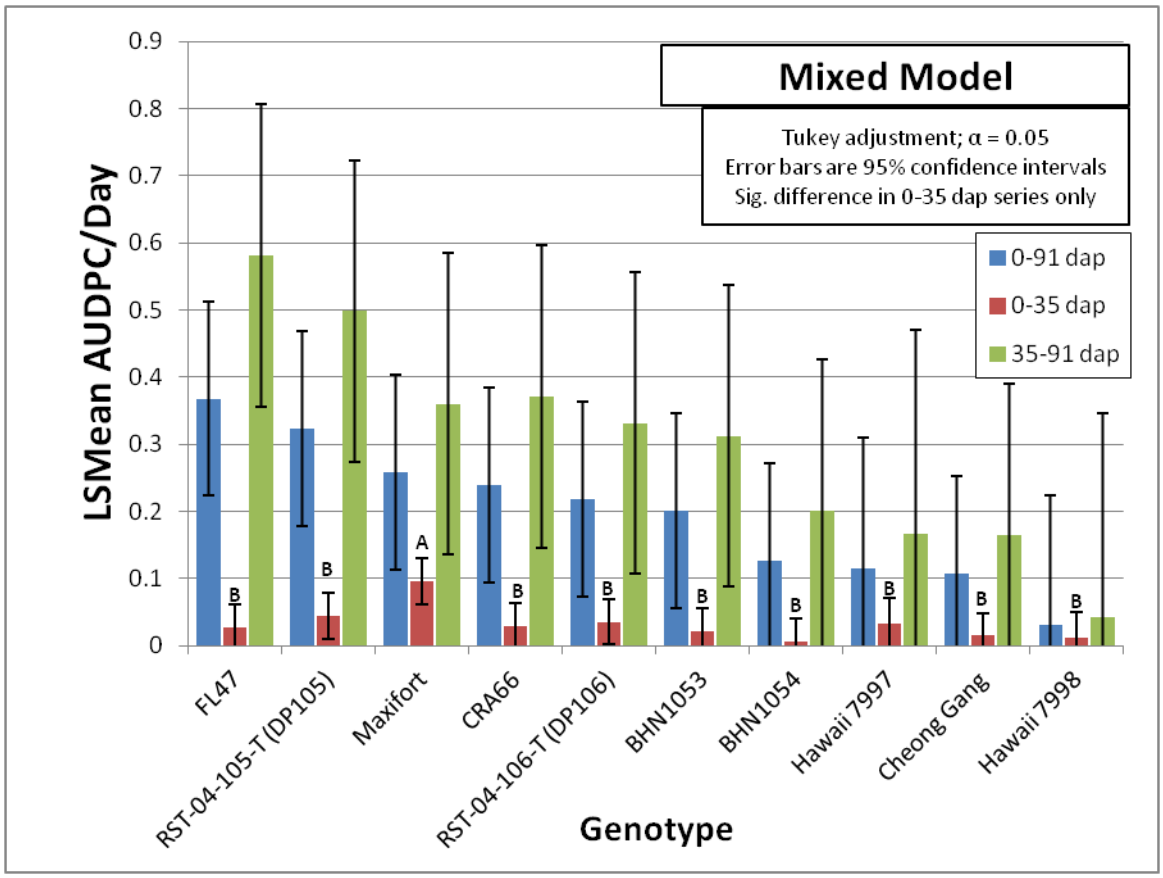


Figure 2.3. ***Bacterial wilt in Jackson Co. 2013 study assessed over three time intervals.***
The Jackson Co. 2013 study of bacterial wilt (*Ralstonia solanacearum*) least squares mean (LSMean) daily area under disease pressure curves (AUDPCs) for four replications of ten tomato genotypes. Daily LSMean AUDPC values comparing three calculation interval series: 0 to 91, 0 to 35, and 35 to 91 days after planting (dap). Error bars are 95% confidence intervals for comparison within series. Both 0-91 dap and 35-91 dap series showed no significant differences between genotypes within the respective series, and genotypes with same letters for 0-35 dap are not significantly different, based upon Tukey's mean comparison test (α = 0.05).

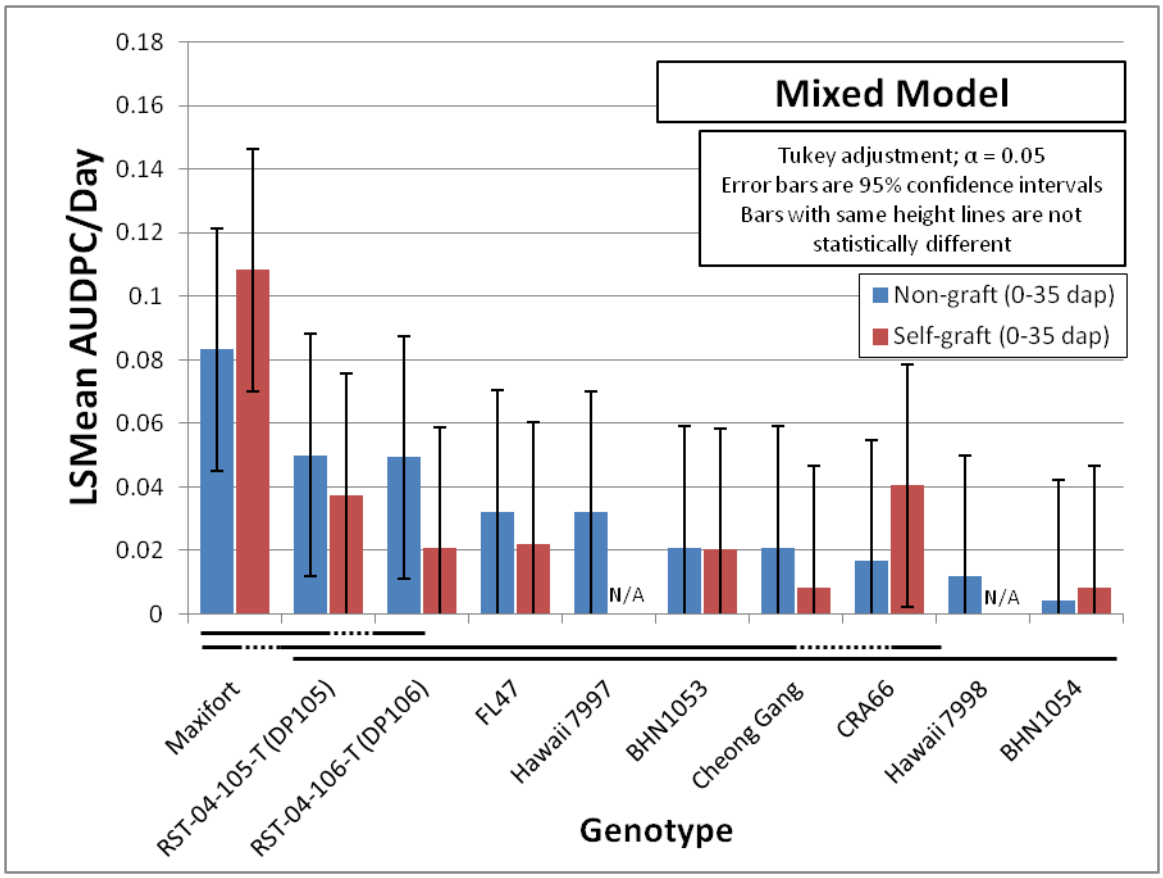


Figure 2.4. ***Comparison of the effect of grafting on genotypes in Jackson Co. 2013 study.***
The Jackson Co. 2013 study of bacterial wilt (*Ralstonia solanacearum*) least squares mean (LSMean) daily area under disease pressure curves (AUDPCs) for four replications of ten tomato genotypes for observation period of 0-35 days after planting (dap). Daily LSMean AUDPC values for each non-grafted and self-grafted genotype are compared. Error bars are 95% confidence intervals for comparison within series. Columns with the same height solid line under the horizontal scale are not significantly different (dashed line represents excluded columns), based upon Tukey's mean comparison test (α = 0.05). 'Hawaii 7997' and 'Hawaii 7998' columns are absent due to failure of grafted plants in healing process, and is marked with N/A.

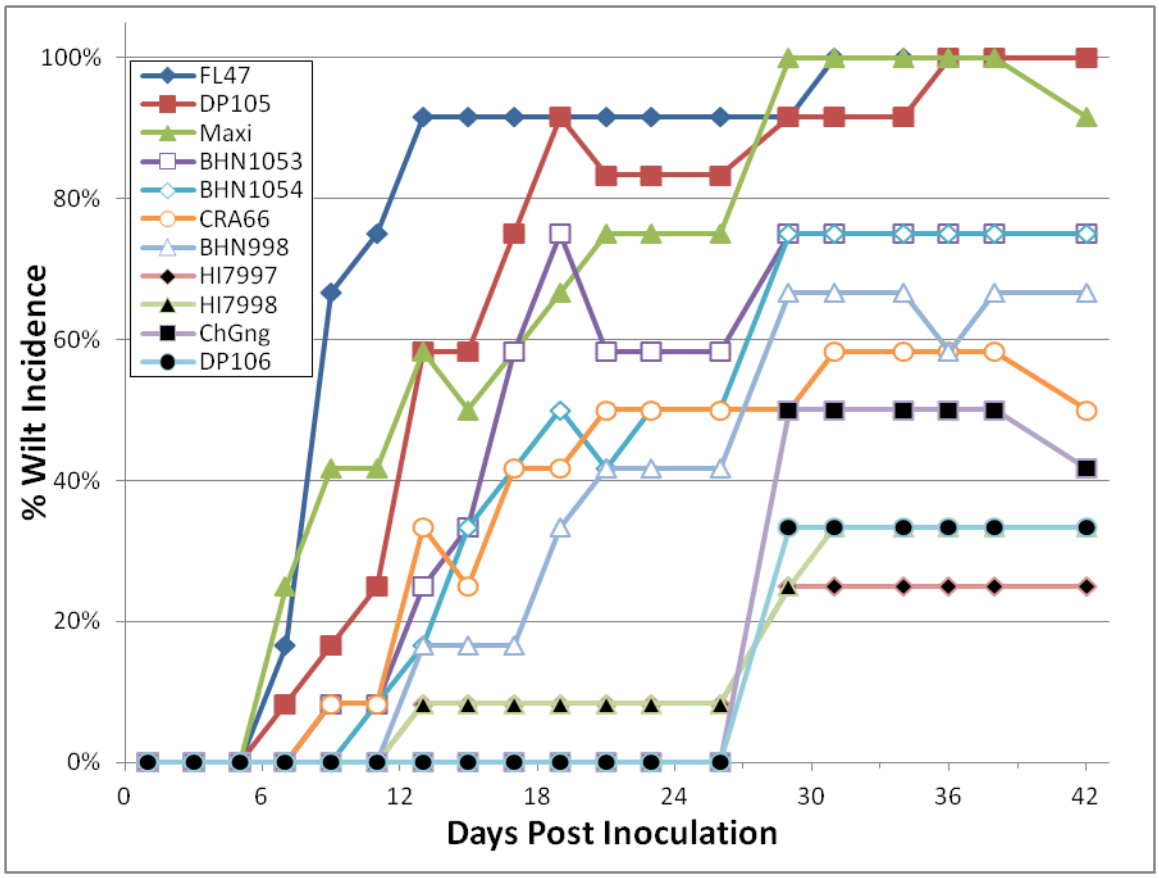


Figure 2.5. ***Bacterial wilt incidence in the winter 2013-2014 greenhouse study.*** **Greenhouse 2013-14 study of bacterial wilt incidence (*Ralstonia solanacearum*) (Rs) in eleven tomato genotypes and two geographically distinct tomato Rs isolates with three replications per isolate. Each series represents the total incidence of foliar wilting for both Rs isolates and all replications.**

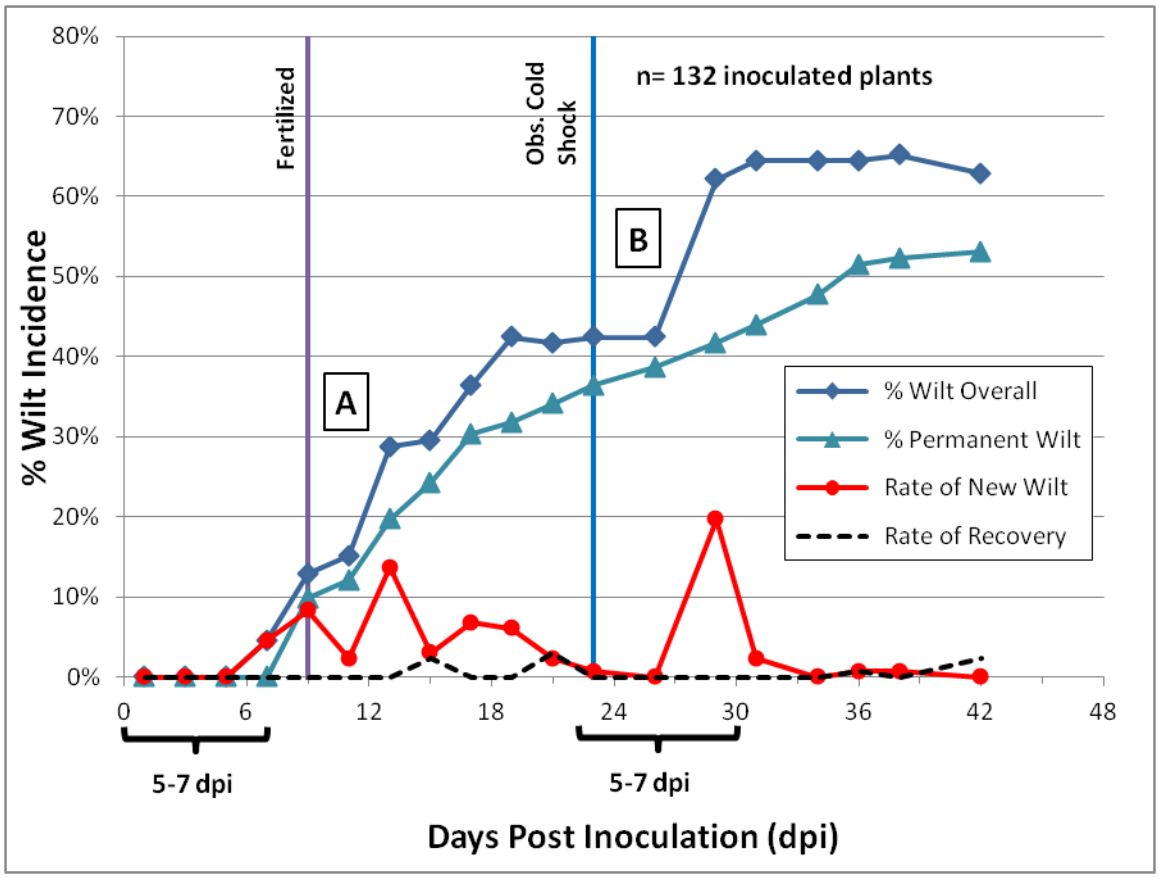


Figure 2.6. ***Overall bacterial wilt incidence in the greenhouse study highlighting the effect of cold shock stress.*** **Disease incidence of bacterial wilt (*Ralstonia solanacearum*) as modulated by mid-experiment low temperature shock stress to greenhouse tomatoes. The experiment was performed in a heated glasshouse from Dec., 2013 to Feb. 2014. 8-week-old tomato plants (mixed genotypes) were inoculated with 10 mL of 3.05 x 109 cfu/mL of isolate Jc or 1.90 x 109 cfu/mL of isolate P. Due to severe cold weather event, temperatures inside greenhouse dropped to low of 8.9 oC two nights in a row (21-22 dpi). Visible low temperature stress symptoms observed on 23 dpi, but the plants recovered. Plants were scored for wilt severity using a 0-5 scale, with 0 being healthy and 5 being permanently wilted and decaying. Vertical bars denote fertilization event (1/2 label rate; 20-20-20) and date of observed low temperature stress symptoms. Diamonds represent the percent wilt of all inoculated plants (n = 132) at each observation time point where severity score > 0. Triangles represent the percent of inoculated plants that have reached severity score of 4 or 5, which is defined as having reached permanent wilting point. Circles represent the percent of plants with scores > 0 at each time point, given the previous period score = 0. Dashed line represents the percent of plants with scores = 0, given the previous period score > 0. Brackets denote the similarity of range between inoculation and low temperature stress events with beginning of new phase of wilt incidence. A) First consistent period of increase in wilting incidence followed by a plateau of no new wilting; the first epidemic phase. B) Second consistent period of increase in wilting incidence followed by a plateau of no new wilting; the second epidemic phase. Second phase is comprised of moderately to highly resistant lines.**

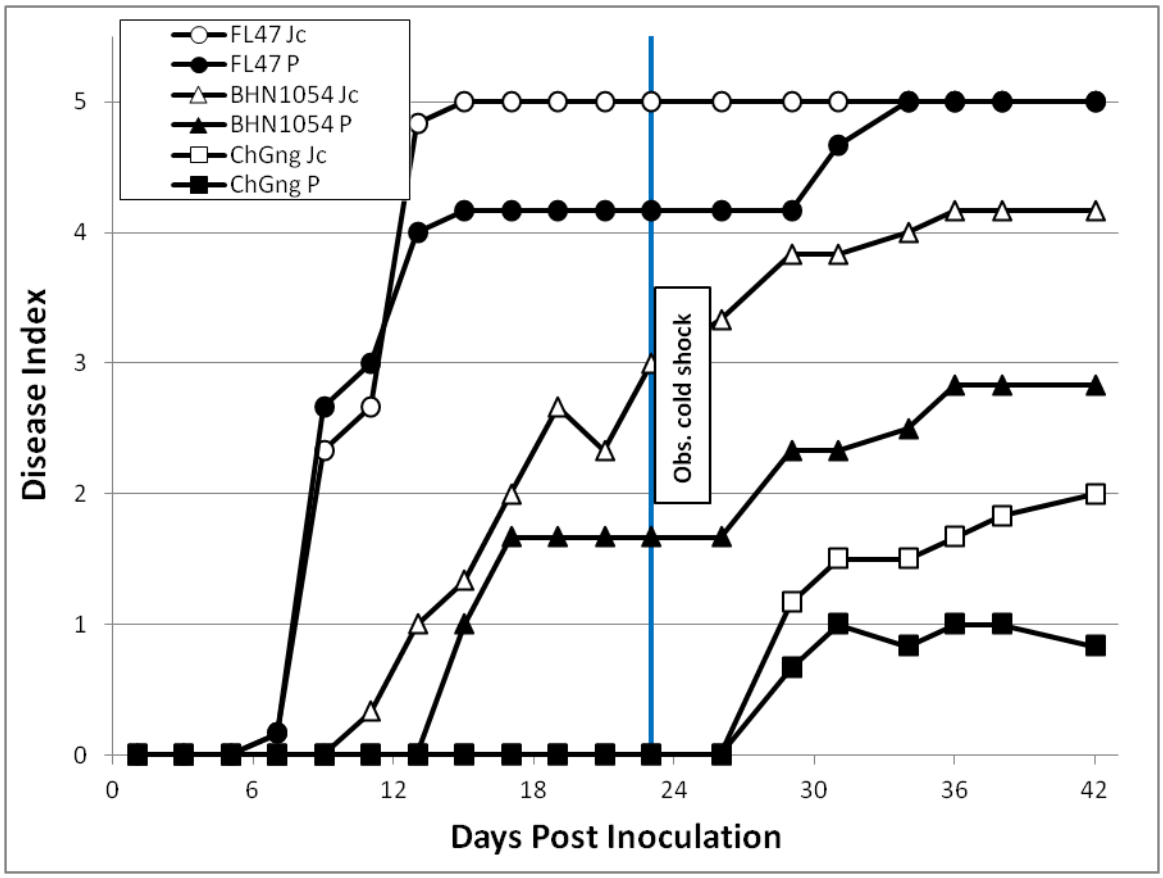


Figure 2.7. ***Representative bacterial wilt progression affected by cold shock stress.***
Progression of bacterial wilt (*Ralstonia solanacearum*) (Rs) mean disease index representative of three patterns modulated by temperature. Plants were scored for wilt severity using a 0-5 scale, with 0 being healthy and 5 being permanently wilted and decaying, and disease index was calculated for each. Mean disease index lines represent the mean of three replications. Each line represents the mean disease index of each genotype within Rs isolate treatment over time. For sake of clarity, standard error bars not included but have significant overlap between Jc and P isolates of each genotype. Vertical bar represents date of observed low temperature stress symptoms.

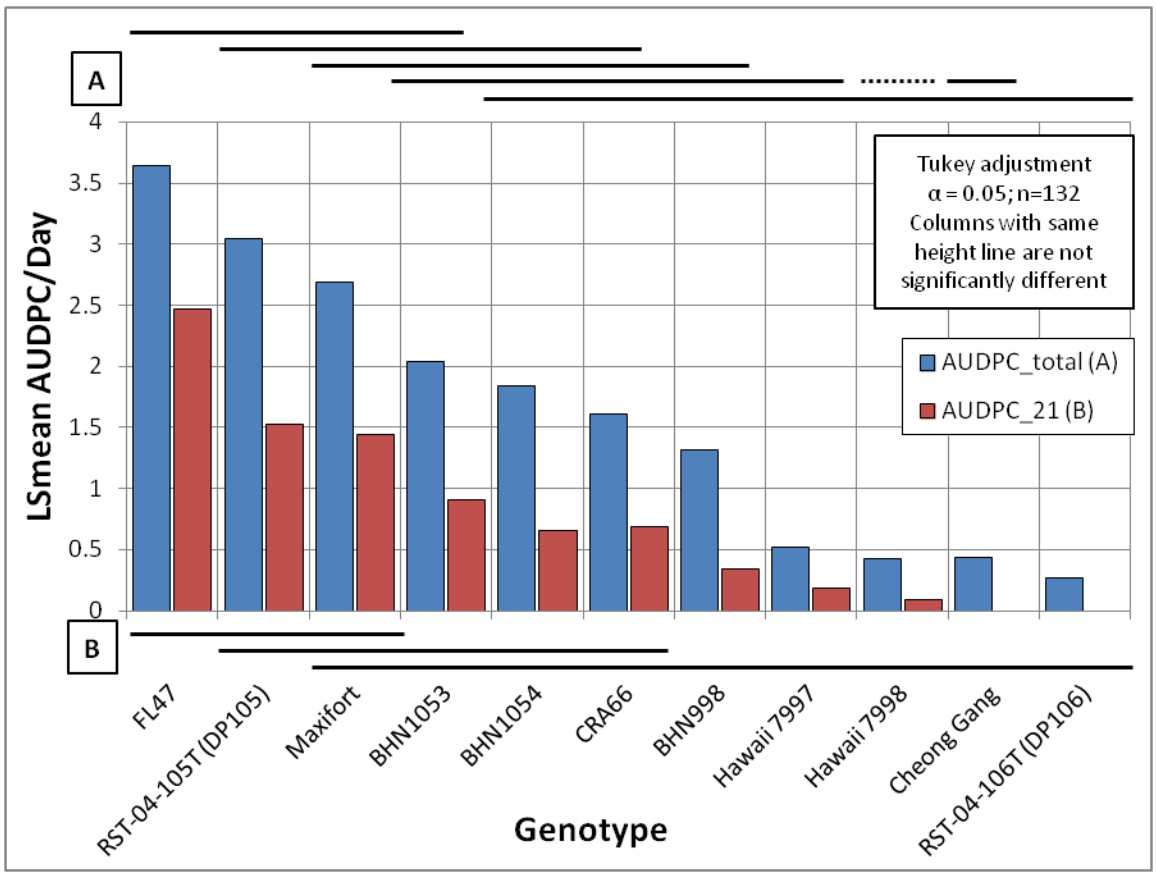


Figure 2.8. ***Genotype resistance to bacterial wilt in the greenhouse study assessed over two time intervals.***
The greenhouse 2013-14 study of bacterial wilt (*Ralstonia solanacearum*) least squares mean (LSMean) daily area under disease pressure curves (AUDPCs) for three replications comparing eleven tomato genotypes. Daily LSMean AUDPC values for total observation period of 0 to 42 and period of 1 to 21 days post inoculation (dpi). LSMean separation of mean daily AUDPCs for A) total period and B) 0 to 21 dpi by genotype. Columns with the same height solid line are not significantly different (dashed line represents excluded columns), based upon Tukey's mean comparison test (α = 0.05).

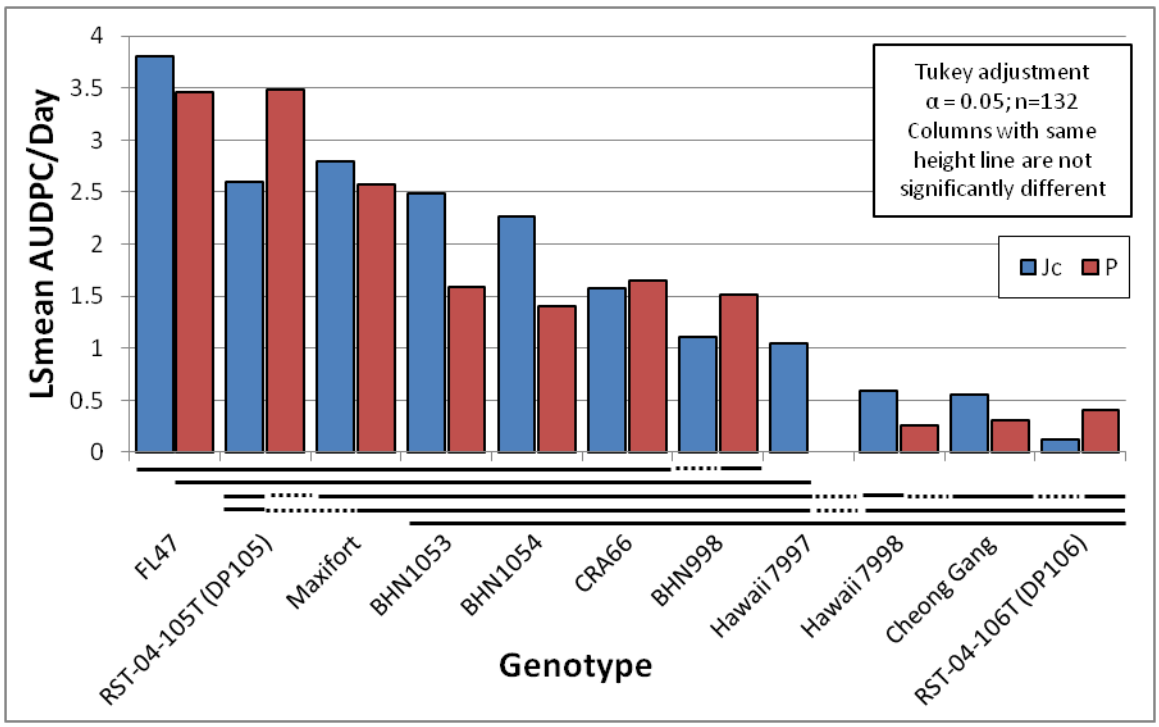


Figure 2.9. ***Genotype resistance to two contrasting isolates of Ralstonia solanacearum in the greenhouse study.*** **The greenhouse 2013-14 study of bacterial wilt (*Ralstonia solanacearum*) (Rs) least squares mean (LSMean) daily area under disease pressure curves (AUDPCs) for three replications comparing eleven tomato genotypes within two Rs isolates from tomato; Jc and P were collected from stems of wilting plants from Jackson County and Pender County, NC, respectively. Species was determined by colony morphology on semi-selective media and Rs-specific ELISA. Daily LSMean AUDPC values for total observation period of 0 to 42 days post inoculation (dpi). Columns with the same height solid line are not significantly different (dashed line represents excluded columns), based upon Tukey's mean comparison test (α = 0.05).**

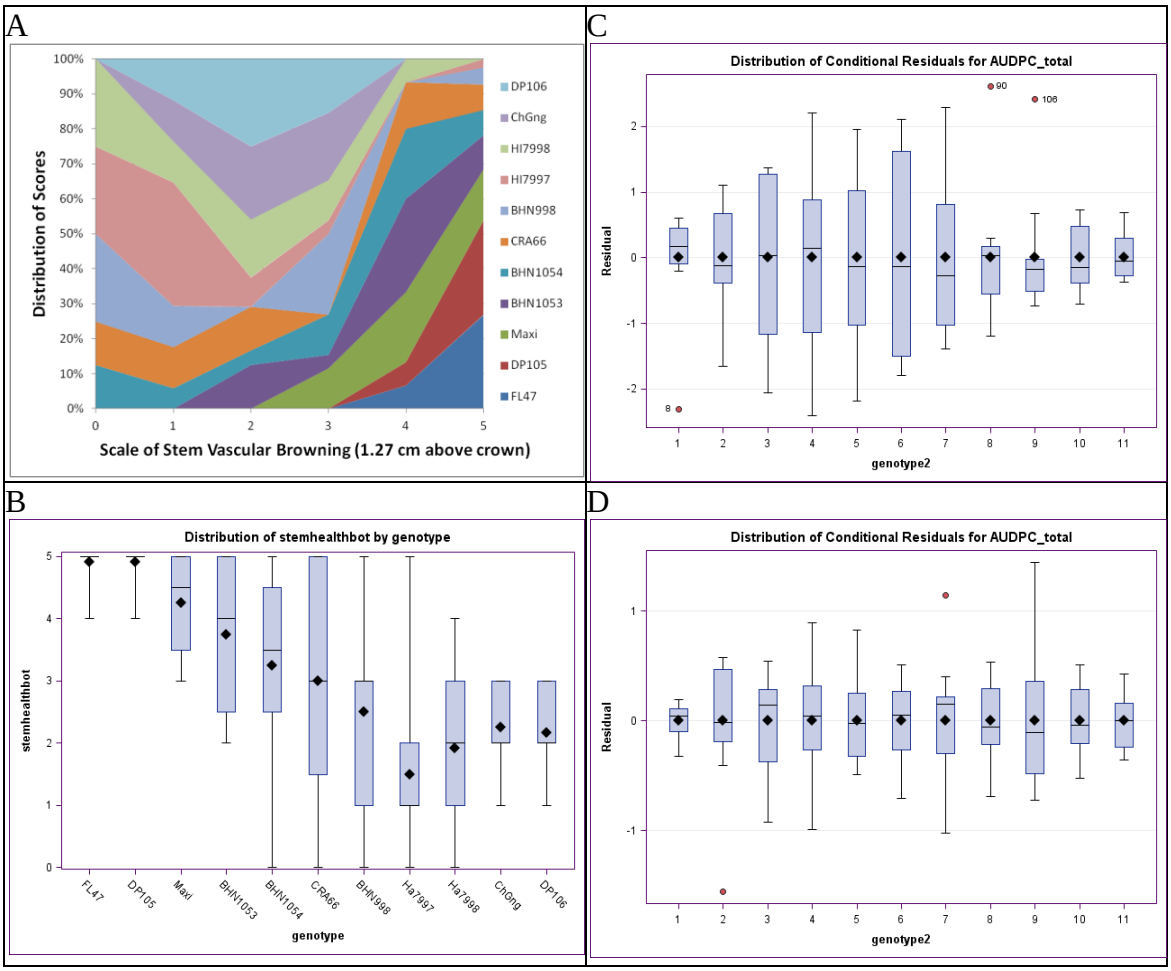


Figure 2.10. ***Assessment of vascular browning of tomato stems in the greenhouse study.***
The greenhouse 2013-14 study analyses of end-of-study vascular browning scores in tomato genotypes inoculated with bacterial wilt (*Ralstonia solanacearum*) (Rs). Plant stem cross-sections were rated for amount of vascular browning at 1.27 cm above soil line using a 0 to 5 severity scale, where 0 = no visible vascular browning and 5 = stem dead and hollow. Rs presence in stems were confirmed by blotting cross-sectioned stems on semi-selective media, assessing for growth of Rs on media, and periodic Rs-specific ELISA. "Genotype2" axes are BW resistance rankings of the study genotypes based upon previous LSMean AUDPC, where 1 is most susceptible and 11 is least susceptible. A) Distribution area of vascular browning scores within each genotype as a function of percentage of total scores (n = 132). B) Box-and-whisker plots showing distribution of vascular browning (stemhealthbot) by genotype. Diamonds within boxes represent mean of scores by genotype. C) Box-and-whisker plots depicting distributions of mixed model analysis of variance predicting total period daily area under disease pressure curves (AUDPCs) grouped by genotype and sorted by largest least squares mean (LSMean) (1) to smallest (11). Scale of residual is standard deviations from the mean (diamonds within boxes). D) Same model residual outputs as C, but after inclusion of vascular browning scores nested within genotype. Red dots (C and D) are considered by model as outlier values, which are identified.

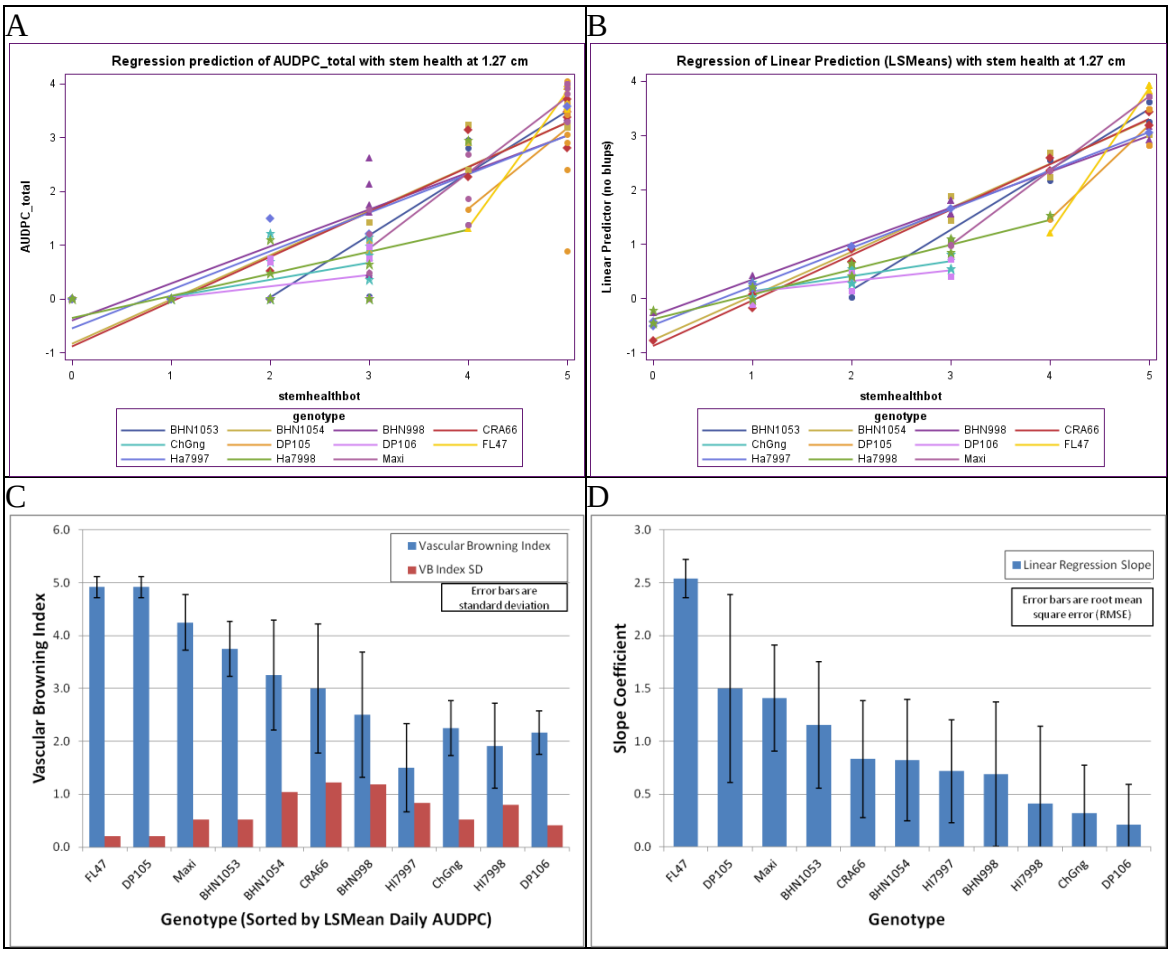


Figure 2.11. ***Assessment of stem vascular browning for prediction of foliar wilting by genotype in the greenhouse study.*** **The greenhouse 2013-14 study regression analysis of end-of-study vascular browning scores in tomato genotypes inoculated with bacterial wilt (*Ralstonia solanacearum*) (Rs) to predict daily area under disease pressure curve (AUDPC) values from observed leaf wilting over period of 1 to 42 days post inoculation (dpi), grouped by genotype. Plant stem cross-sections were rated for amount of vascular browning at 1.27 cm above soil line using a 0 to 5 severity scale, where 0 = no visible vascular browning and 5 = stem dead and hollow. Rs presence in stems were confirmed by blotting cross-sectioned stems on semi-selective media, assessing for growth of Rs on media, and periodic Rs-specific ELISA. AUDPC values were calculated from foliar wilting severity scale of 0 to 5, where 0 = healthy; no wilt and 5 = whole planting wilting, drying, and decaying. Vascular browning scores (stemhealthbot) regression predictions grouped by genotype of A) daily AUDPC values and B) mixed model analysis of variance predictions. C) Distribution of mean vascular browning indexes and index standard deviations when genotypes are sorted largest to smallest by their respective LSMean daily AUDPC values. Error bars are standard deviations between three replications. D) Distribution of mixed model slope coefficients for each genotype regression equation using of vascular browning to predict daily AUDPC. Error bars are model root mean square errors for each slope.**

CHAPTER 3: MICROBE-ASSOCIATED MOLECULAR PATTERN TRIGGERED IMMUNITY AND ITS RELATIONSHIP TO BACTERIAL WILT OF TOMATO

Abstract

The plant immune system is a complex series of dynamic interactions analogous of an arms race between host pattern recognition receptors (PRRs) detecting conserved microbe/pathogen-associated molecular patterns (MAMPs/PAMPs), pathogen avoidance of PRR detection, pathogen-produced effectors for disruption of host machinery and signaling mechanisms, and host MAMP- and effector-triggered immunity responses (MTI and ETI, respectively). The economically important tomato (*Solanum lycopersicum* L.) is attacked by the soil-borne bacterial pathogen *Ralstonia solanacearum* (Rs), causing bacterial wilt (BW) once host roots and vasculature are penetrated and colonized. During BW pathogenesis, increases in reactive oxygen species (ROS) production is observed, which may be related to the similarly observed rise of ROS production upon MAMP detection in plants. Here, MAMPs were used to artificially stimulate ROS production in tomato leaf disks, which was measured by a luminol and peroxidase-based assay, in order to investigate the relationships between MTI-related ROS production and resistance to BW of tomato. The ROS responses in eleven tomato genotypes in field and greenhouse conditions were characterized, investigating the specific effects of genotype, graft-type, and inoculation with two North Carolina tomato isolates of Rs on production curve magnitude and spread over time when elicited by four MAMP peptides, including the Rs-specific conserved sequence of the bacterial flagellin (Rs Flg22). Additionally, these results were compared with the BW

resistance levels of the genotypes in each study. It was observed that the ROS curve characteristics varied significantly between tomato genotypes, that the presence of a healed graft wound significantly increased peak magnitude, and that inoculation with Rs increased ROS uniformity between genotypes and drastically modulated curve peak time, duration, and total ROS production. Additionally, elicitation with a transpositional mutant form of the general flagellin sequence (Pa Flg22) was still able to elicit ROS production comparatively to another conserved flagellin region (FlgII-28) and cold shock protein (Csp22). Finally, it was observed that tomato genotype resistance to BW is not related to ROS stimulation with any of the four MAMPs tested, and that Rs Flg22 is essentially not detected by eleven genotypes of tomato. This work is the first report to the author's knowledge investigating MTI-related ROS production curve characterization and modulations by genotype, grafting, and inoculation with Rs, providing foundational information and research questions for future investigations.

3.1: Introduction

Plants have a complex system of extra- and intercellular receptor channels for the detection of microbial, damage-related, and self/non-self components (Boller and Felix, 2009). Plant detection of microbes occurs through the interaction of specific host pattern recognition receptors (PRRs) with microbial components known as microbe/pathogen-associated molecular patterns (MAMPs/PAMPs), which are generally conserved among microbial species and critical for fitness (Boller and Felix, 2009; Postel and Kemmerling, 2009). MAMPs were first discovered in investigations of host-pathogen interactions, but are

now considered to be a first-line detection of any microbe (Vance et al., 2009). MAMPs have been identified in fungi, yeast, oomycetes, brown algae, and both gram positive and negative bacteria, some of which are recognized by many plant families and others only by a few (Postel and Kemmerling, 2009). Host detection of MAMPs leads to signaling cascades that transitorily adapt plant phenotypes to cope with the presence of the microbe, causing immunity to colonization, which has become known as MAMP-triggered immunity (MTI; also PTI is an older term) (Monaghan and Zipfel, 2012). This system of perception forms the broad-spectrum foundation of the plant immune system, which has shown many similarities to innate immunity in animals, however the plant immune system is not adaptive and is not based upon specialized immune cells, as in animals (Jones and Dangl, 2006; Monaghan and Zipfel, 2012; Postel and Kemmerling, 2009; Vance et al., 2009). Coupled with MTI is the presence of another layer of the plant immune system that leads to host immunity. This layer involves specific detectors for the presence, or effects, of specific microbe-produced components intended to aid in host colonization (effectors), becoming known as effector-triggered immunity (ETI) (Dangl and Jones, 2001; Jones and Dangl, 2006; Monaghan and Zipfel, 2012). The degree of receptor and signaling cascade overlap between MTI and ETI is not well understood, leading some to suggest they may or may not be separate systems (Jones and Dangl, 2006; Thomma et al., 2011). Combined, MTI and ETI provide a multi-layed system of defense responses against invading microbes. The ability of an organism to evade, survive, or suppress these systems is what determines if it can be a successful plant pathogen or not (Jones and Dangl, 2006; Zipfel and Robatzek, 2010). Perhaps the best evidence for the importance of MTI to plant immune responses is the fact that pathogenic microbes must

target and disrupt the host PRRs or the downstream signaling components of MTI in order to be fully virulent (Göhre and Robatzek, 2008; Hann et al., 2010).

Although many of the components and interactions of these signaling cascades remain unclear, many PRRs involved in the initial detection have been characterized (Boller and Felix, 2009; Zipfel and Robatzek, 2010). Stimulation of PRRs is associated with many biochemical and transcriptional changes, such as a transitory burst of reactive oxygen species (ROS) that includes hydrogen peroxide (H_2O_2) (Nicaise et al., 2009; Sharma et al., 2012; Tena et al., 2011). ROS are produced by normal plant metabolic processes, but are excessively produced under various environmental stress conditions, including pathogen colonization (Bailey-Serres and Mittler, 2006; Sharma et al., 2012). In fact, MAMP-induced ROS production is a characteristic response aspect of MTI (Boller and Felix, 2009). ROS can be quite damaging to cellular components, thus plants and microbes produce ROS scavenging antioxidant compounds and enzymes, including peroxidase in plants, to mitigate cellular damage. ROS production in plants is used for direct defense against invading microbes and as signals for plant processes such as response to environmental stimuli, growth, and programmed cell death. A key aspect of this system is the balance between production of these toxic compounds and mitigation ability (Bailey-Serres and Mittler, 2006; Sharma et al., 2012).

The first bacterial MAMP identified as a trigger of plant MTI was bacterial flagellin, a key component of swimming motility. A small, 22-amino acid N-terminal region of the protein was identified as a major elicitor of what is now known as MTI, and that it is conserved across many diverse species of bacteria (Felix et al., 1999). The sequence from

Pseudomonas aeruginosa has become known as Flg22, and was shown to elicit broad spectrum resistance to both *Pseudomonas syringae* pv. *Tomato* DC3000 and *Botrytis cinerea* (Zipfel, 2009). Since then, other bacterial MAMPs have been discovered, including a 22-amino acid RNA-binding region of bacterial cold shock protein (Csp22) (Felix and Boller, 2003) and another portion of the bacterial flagellin for *Pseudomonas syringae* pv. *tomato* not far from Flg22 that is 28-amino acids long (FlgII-28) (Cai et al., 2011).

Further investigation of Flg22 demonstrated that it was detected in *Arabidopsis* by the membrane-spanning FLAGELLIN SENSING II (FLS2) protein, which was characterized by having a leaucine-rich repeat domain and receptor kinase activity (Chinchilla et al., 2006). FLS2 is expressed *in planta* in places of common bacterial entrance in shoots and roots, such as stomatal openings, hydathodes, and lateral roots (Beck et al., 2014). Homologs of FLS2 have been discovered in tomato (*Solanum lycopersicum*) (Robatzek et al., 2007) and other species (Boller and Felix, 2009) and the interaction of Flg22 and FLS2 show some distinct differences between tomato and *Arabidopsis*. Additionally, mutations in key positions of Flg22 are known to effect plant perception drastically (Felix et al., 1999). Mutations in key positions of Csp22 are also known to have this effect (Felix and Boller, 2003). FlgII-28 is detected by some Solanaceous species, but not by *Arabidopsis*, unless mutations alter the sequence (Clarke et al., 2013). Additionally, FLS2, which detects Flg22, is not the receptor for FlgII-28, since tomato plants with that gene silenced have an unaltered response, and *Nicotiana benthamiana* transformed with FLS2 does not gain detection ability of FlgII-28 (Clarke et al., 2013). Csp22 detection is only known to occur in some Solanaceous species thus far, including tomato (Felix and Boller, 2003; Postel and Kemmerling, 2009).

The studies on bacterial MAMPs have been performed in relation to several important foliar bacterial pathogens of tomato and other crop species, namely the Pseudomonades and the Xathomonades (Cai et al., 2011; Clarke et al., 2013; Felix et al., 1999). *Ralstonia solanacearum* Smith (Rs) is a soil-borne bacterial pathogen of tomato, and was classified as a Pseudomonad for many decades before being reclassified into a new genus (Genin and Denny, 2012; Remenant et al., 2010; Schell, 2000; Smith, 1896). Rs is the causal agent of bacterial wilt (BW) of tomato, a devastating disease not only of tomato, but over 200 plant species in over 50 different plant families in tropical to warm temperate regions with high rainfall (Buddenhagen and Kelman, 1964; Hartman and Elphinstone, 1994; Hayward, 1964; Hayward, 1991; Moorman, 2014; Olson, 2005). Bacterial wilt invades host through wounds and openings in the roots around sites of secondary root emergence, colonizes the host vasculature, and produces large amounts of extracellular polysaccharides, leading to permanent wilting of the host (Schell, 2000). Tomato resistance to BW is quantitative and polygenic, with general and strain-specific quantitative trait loci for resistance known (Acosta et al., 1964; Carmeille et al., 2006; Jyothi et al., 2012; Lebeau et al., 2011; Mangin et al., 1999; Sharma et al., 2006; Thoquet et al., 1996a; Thoquet et al., 1996b; Wang et al., 1996; Wang et al., 2000; Wang et al., 2013). Breeding for large-fruited tomato varieties with resistance to BW has proved essentially unfruitful over the last several decades due to tight genetic associations between small fruit size and resistance (Scott et al., 2005). Grafting of BW susceptible large-fruited tomato varieties onto BW resistant rootstocks has emerged as a viable management strategy for BW infested fields, especially in the Southeastern USA where fresh-market production of large-fruited tomato varieties predominates (Freeman et

al., 2011; Louws et al., 2010; McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008).

Twitching and swimming motility has been demonstrated to be critical for Rs virulence via natural root penetration, but not for direct inoculation of tomato stems (Schell, 2000; Tans-Kersten et al., 2001), suggesting that it plays an important role prior to stem penetration when the bacteria are moving through root tissues in low concentrations. Stem penetration and vascular colonization generally occurs about 4 to 5 days post inoculation (Schell, 2000). Additionally, it is known that Rs encounters a ROS-rich environment during invasion of the plant (Colburn-Clifford and Allen, 2010; Colburn-Clifford et al., 2010; Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011), and that tomato produces ROS upon inoculation with Rs (Ishihara et al., 2012; Mandal et al., 2011). Additionally, boiled extracts of Rs have been shown to stimulate host defenses in a protective manner, and that the primary eliciting factor in *Arabidopsis* is a proteinacous compound, reported as between 5 to 10 kDa (Pfund et al., 2004) or > 10 kDa (Takabatake and Mukaihara, 2011).

The Rs-specific epitope of Flg22 was characterized from strain K60 and compared to the effects that boiled extracts of Rs and Rs aflagellate mutants had on *Arabidopsis* seedling growth (Pfund et al., 2004). It was concluded that the Rs-specific flagellin was not the major defense eliciting factor detected by *Arabidopsis* or *Nicotiana benthamiana*, since treatments with it did not lead to restricted seedling growth. This was surprising since flagellin from a wide range of bacterial species had been implicated as the major elicitor of the oxidative burst (Felix et al., 1999), to which the researchers noted that the differences in recognition systems between *Arabidopsis* and tomato, as well as the medium of detection assessment

(seedling growth vs. cell culture ROS production) may be responsible for this discrepancy of results, since the authors did not test tomato in their work (Pfund et al., 2004). As previously noted, differences in flagellin perception are now known between Arabidopsis and tomato (Clarke et al., 2013), among other characterized MAMPS (Postel and Kemmerling, 2009).

Therefore, I set out to test a variety of tomato genotypes with varying levels of resistance to BW for the ability to detect the Rs-specific Flg22 (Rs Flg22) peptide sequence by measuring the associated oxidative burst. As previously demonstrated (Felix et al., 1999), stimulating tomato leaf disks with the conserved region of bacterial flagellin triggers a burst of ROS that can be quantified using a luminescence assay with luminol and peroxidase from Horseradish (HRP). Thus, I set out to characterize this oxidative burst in tomato elicited by Rs Flg22, FlgII-28, Csp22, and a mutant form of Flg22 (Pa Flg22). Along with that, I wanted to assess the diversity of response in multiple tomato varieties in multiple growing conditions in the presence of BW pressure to assess the potential of using MTI-related ROS assessment to facilitate advanced screening and selection of bacterial wilt resistant varieties in the breeding pipeline. Within this objective, I wanted to compare ROS production variation before and after inoculation with two geographically distinct NC isolates of Rs. Additionally, I assessed the effect that a healed graft wound would have on the production of ROS in tomato.

3.2: Materials and Methods

Tomato plants from the same field and greenhouse studies described in Chapter 2 were used in this work. Briefly, one commonly used large-fruited commercial variety with

known susceptibility to BW ('Florida 47') and ten tomato rootstock varieties from diverse sources were assessed for resistance to BW and for variation in ROS production when stimulated by four different MAMP peptides. BW resistance variation is reported in Chapter 2.

The field study was performed during the summer of 2013 in a Western NC field under natural inoculum pressure from the endemic population of Rs (source of Rs tomato isolate Jc). Study plants were transplanted to the field on June 28th. Due to seed source problems at the time, 'BHN998' was not included in the field study. Non- and self-grafted treatments were assessed in a nested split-plot block design with four replications where graft-type was nested as blocks within genotype plots. Due to unexpected losses during the grafting process, no self-grafted 'Hawaii 7997' or 'Hawaii 7998' plots were planted, only non-grafted plots. The grafting process and BW assessment methodologies are previously described in Chapter 2.

The greenhouse study was performed during the winter of 2013-14 in a heated glasshouse environment with plants potted in 24-cell plastic trays in soil-less media. All eleven genotypes were assessed in a randomized complete block design with three replications by two NC Rs isolates from tomato (Jc and P) as the blocking factors. Artificial inoculation using the root cut and soil drench method was performed. Rs isolate information, as well as BW inoculation and assessment methodologies are previously described in Chapter 2.

3.2.1: MAMP Assay Design

For the Jackson Co. field study, two plants from the middle of each plot were sampled during the day, and two leaf disks were taken from each plant per plate. All nested plots were sampled. One peptide was applied per plate, and identical plates for each peptide were sampled concurrently. Four wells were left blank as a no tissue control. First sampling of study plants occurred 21-23 days after planting (dap) and second sampling occurred 29-31 dap. Due to disease concerns stated in Chapter 2, the second period of sampling was disrupted, leaving an incomplete dataset. Thus, the first period of sampling only is reported.

Similarly for the greenhouse study, two leaf disks were cut from each plant in the study, with one peptide treatment per plate. All replications were sampled, including the negative control. Four wells were left blank, as before, as no tissue controls. The first sampling of plants occurred 3-6 days before inoculation, and second sampling occurred 1-3 days post inoculation (dpi). Both periods are reported here.

Four short peptide sequences previously identified as MAMPs were synthesized (EZBiolabs, Carmel, IN) and their ability to elitic ROS production was tested: Cold shock protein (Csp22), 22- and 28-amino acid lengths of bacterial flagellin from *Pseudomonas aeruginosa* (Pa Flg22 and FlgII-28), and bacterial flagellin from *Ralstonia solanacearum* (Table 3.1). The Pa Flg22 sequence used was a mutant epitope from what is commonly used (Felix et al., 1999), where the first 6 amino acids on the N-terminus were translocated to the C-terminus, retaining their internal order and orientation.

3.2.2: MAMP Assay Protocol

In order to quantify MTI in each tomato genotype, I artificially stimulated and measured the burst of H_2O_2 from tomato leaf disks using a luminol and HRP luminescence reaction adapted from previous studies (Felix et al., 1999; Keppler et al., 1989; Lloyd et al., 2014; Veluchamy et al., 2014). 2 mm round leaf disks were cut from the second or third leaf from the apical meristem and placed right-side-up in white, 96-well plates (Lumitrac 200; 96-well plates with 392 uL total well volume; Greiner-style with flat bottoms and chimney well; Phenix Research Products, Candler, NC) filled with 200 uL dH_2O; one leaf disk per well. Leaflet mid-veins were purposefully avoided, and sampling pattern preferred apical leaflet. Once plates were filled, they were covered with aluminum wrap and incubated for 18-24 hours at room temperature.

After the incubation period, the water was removed by pipette and 100 uL of reaction mix was added. Each reaction mix was made from frozen aliquots of each component stored in -20 ^{o}C immediately prior to application. 24 uL of 17 ug/uL luminol (Sigma-Aldrich, no. A8511; dissolved in DMSO), 24 ul of 10 ug/uL HRP (Type VI-A, Sigma-Aldrich, no. P6782; dissolved in dH2O), and 12 uL of MAMP peptide (Synthesized by EZBiolab; dissolved in dH_2O) were pipetted into 12 mL of dH_2O in a light-proof tube and mixed by rapid inversion. MAMP peptide aliquot concentrations were either 100 nM (FlgII-28, Pa Flg22, and Rs Flg22) or 500 nM (Csp22) for final reaction concentrations of 0.1 and 0.5 nM, respectively. The reaction mix was prepared under low, indirect lighting since luminol is light sensitive, and applied to each well either by multi-channel pipette (Jackson Co. study) or by automatic injection (greenhouse study). The plate was then placed into the luminometer. Luminescence

was immediately measured by either a GloMax 96 Microplate Luminometer (Promega, Madison, WI; used for Jackson Co. 2013 field samples) or a Synergy2 Multi-mode Microplate Reader (Biotek, Winooski, VT, with automatic injection system; used for greenhouse 2013-14 samples). Measurements of relative light output (RLUs) were taken every two minutes for 31 cycles (Synergy2) or every four minutes for 15 cycles (GloMax).

3.2.3: Data Analyses

Data was processed using Excel 2007 (Microsoft, Redmond, WA) and SAS version 9.4 (2013) (SAS, Cary, NC). Background luminescence was calculated from blank wells and removed from final values. Any well that exhibited no reaction (No distinct pattern compared to background luminescence) was considered a null reaction and not included in plant averages. Mean RLU progression over time was used to assess modulations of curve patterns (peak height, peak time, curve shape) by genotype, treatments (graft-type or Rs isolate), and sampling times. Total, average, and maximum RLU values were calculated for statistical comparisons.

For the Jackson Co. study, nested plot averages were used for comparison, and were calculated as the mean of the two plants, generating 72 average plot data series per peptide for statistical comparisons. Two plot values were excluded from analyses due to being extreme outliers. For the greenhouse study, genotype averages per plant were used, calculated as the mean of the two plants, generating 154 plant data series per peptide. Two plant values were removed from analyses due to being extreme outliers.

Statistical comparisons were made using Pearson Correlation (r) and general linear models. The same general linear models used in the field study in Chapter 2 to assess variation of area under disease pressure curves were used to assess the variation of total and maximum RLUs. Similarly, for the greenhouse study total and maximum RLUs, the Chapter 2 linear model was modified into a repeated measures ANOVA in order to compare the variation of the two complete sampling events (Pre- and post-inoculation).

3.3: Results

I set out to assess variation in MAMP-elicited ROS production from tomato leaf disks. Overall, I found that elicitation with FlgII-28 gave the strongest overall ROS production in both studies, followed by Pa Flg22, then Csp22 (Figure 3.3). Rs Flg22 peptide elicited little-to-no response in comparison. Interestingly, FlgII-28 exhibited not only the largest overall maximum in both studies, but also elicited the slowest decline in ROS production. The time from elicitation to the average curve maximum for each peptide in the Jackson Co. field study was 16, 8, 8, and 0 minutes post elicitation for FlgII-28, Pa Flg22, Csp22, and Rs Flg22, respectively. Peak time in the greenhouse study is discussed below. Curve raw values are not comparable between studies because luminometers used for measurements were not the same.

I assessed light output using three separate metrics—total, mean, and maximum—but found that, within each peptide, total and mean RLU had a highly significant complete correlation (r = 0.98-1.0 in every case; $p < 0.0001$ for all) with each other, whereas each to maximum had a somewhat lower, though still highly significant, correlation (r = 0.7-0.9; $p <$

0.0001) (Jackson Co. data shown as representative example) (Table 3.4). I concluded that total and average are redundant measures and total RLU was a more biologically meaningful term. Thus, only total and maximum RLUs are reported here. I also often found highly significant ($p < 0.001$) correlations, albeit lower, within each metric between Csp22, FlgII-28, and Pa Flg22 in the greenhouse study (Table 3.5), and between Csp22 and FlgII-28, Csp22 and Pa Flg22, and FlgII-28 and Rs Flg22 in the Jackson Co. study (Table 3.4). Significant correlations with Rs Flg22 were sporadic.

In each study, I observed two plots (or plants) that were extreme outliers. The greenhouse study outliers were substantial, being more than 80 standard deviations from the mean of the rest of the plants. These were removed from the analyses. In the Jackson Co. study, the outliers were still large (> 5 standard deviations away from the mean of the other plots), but the effect of those outliers on the ANOVA models was presented for comparison (Table 3.2, "All" vs. "-Out"). It was observed that removal of those two data series increased the predictability of the model and the ability to detect significant variation. Specific results are reported for only the "-Out" model.

3.3.1: Effects of Genotype on ROS Production

The effect of genotype was found to be a significant predictor of ROS for Csp22, FlgII-28, and Pa Flg22 in both studies, and a significant predictor of ROS for Rs Flg22 in the greenhouse study only (Tables 3.2 and 3.3). Overall, I did observe a large amount of variation within genotypes for MTI-related ROS production. That being the case, I was still able to observe significant differences between genotypes and treatments in both studies. I

observed that the susceptible control (‘Florida 47’) generally had low elicited ROS over all peptides. In the Jackson Co. study (Figure 3.1, left) I observed that the genotype comparisons between total and maximum RLUs were similar within each peptide, but were not identical. More reordering was present between peptides. For Csp22, ‘CRA66’, Hawaii 7997’, and ‘RST-04-105-T’ were high ROS producers, whereas ‘Florida 47’, ‘Maxifort’ and ‘Hawaii 7998’ were low ROS producers. For FlgII-28, ‘Hawaii 7997’ was a high ROS producer and had a significantly higher maximum than the other genotypes. The rest of the genotypes were not significantly different from each other. For Pa Flg22, ‘RST-04-105-T’ and ‘Maxifort’ were high ROS producers, and the others were similar to each other. No differences were observed for Rs Flg22.

The greenhouse study (Figure 3.2) exhibited similar trends as the Jackson Co. study, and differences between genotypes were observed. For Csp22, no differences between genotypes were observed for total, but maximum did show significant differences, with ‘Maxifort’, ‘RST-04-105-T’, and ‘Hawaii 7998’ being generally high ROS producers and the others were rather similar. For FlgII-28, ‘RST-04-106-T’ and ‘Maxifort’ were generally higher ROS producers and the rest were similar. For Pa Flg22, ‘RST-04-105-T’, ‘BHN1053’, and ‘RST-04-106-T’ were higher ROS producers, but were only significantly different from ‘Cheong Gang’ for total. ‘RST-04-105-T’ had the highest maximum and was significantly different from all the other genotypes. For Rs Flg22, ‘Hawaii 7997’ and ‘Maxifort’ were high ROS producers and were significantly different from ‘Cheong Gang’.

3.3.2: Effects of Healed Graft Wound on ROS Production

In the Jackson Co. study, non- and self-grafted blocks were nested within genotype plots. I observed that ROS production was significantly modulated upwards by the effect of grafting for all peptides except FlgII-28 (Table 3.2). The effect was noticeably clearer after the removal of two extreme outliers. I observed that grafting only increased the height of the curve, but did not modulate either duration, peak time, or time of initiation (Figure 3.1, right side; Figures 3.4 and 3.5). Significant increases in ROS due to grafting in both total and maximum ROS production were also observed. For Csp22, 'CRA66', 'RST-04-105-T', 'RST-04-106-T' had the greatest increases in total and maximum ROS production. For FlgII-28, the effect of grafting was not found to be statistically significant. For Pa Flg22, 'CRA66' and 'RST-04-105-T' exhibit the most increase due to grafting. For Rs Flg22, 'BHN1054' and 'RST04-105-T' exhibited the greatest increase of maximum ROS production.

3.3.3: Effects of Inoculation on ROS Production

In order to assess the effects of Rs inoculation on the curve characteristics of MAMP-triggered ROS production, the greenhouse study plants were measured before and after the inoculation event and ROS production values were compared using repeated measures ANOVA. I found that the effect of inoculation was a significant modulating factor of total ROS production with all MAMPs except Pa Flg22, and of maximum with all MAMPs except Csp22 (Table 3.3). The ANOVA prediction models exhibited an overall decrease in significance and explanatory power post inoculation for maximum ROS production with all peptides, as well as for total ROS production when treated with FlgII-28 and Rs Flg22.

Curiously, Csp22 and Pa Flg22 total ROS production models increased in explanatory power after inoculation. Genotype was found to be a significant between factor effect for both total and maximum ROS in all four peptides, although genotype only modulated total ROS production for FlgII-28, Pa Flg22, and Rs Flg22 between pre- and post-inoculation. The effect of inoculation was quite pronounced in the effect of genotype, where differences in ROS were less apparent after inoculation, particularly for total production when elicited with FlgII-28, Pa Flg22, and Rs Flg22 (Figure 3.2). Total ROS production exhibited an overall reduction after inoculation, but maximum ROS was unchanged or even enhanced.

I also observed that several characteristics of the ROS production curves were modulated by the inoculation event (Figure 3.6, A-D). Mean RLU over time curves for each peptide showed that the post-inoculation decay in ROS production post-peak was faster, curve peak became more pointed, and curve reached maximum sooner in time by about 2, 16, and 8 minutes after elicitation for Csp22, FlgII-28, and Pa Flg22, respectively. Rs Flg22 exhibited an overall flattening decrease of what little ROS production was observed prior to inoculation. Overall, no affect by Rs isolate was observed, except in the interaction of plants inoculated with isolate P, where plants stimulated with FlgII-28 saw a significant increase in maximum ROS production (Figure 3.6, E-F). These patterns remained consistent across every genotype measured (Figure 3.7).

3.3.4: Relationship between Bacterial Wilt Resistance and ROS

In order to determine the relationship between MTI-related ROS production and BW resistance, total and maximum RLUs for each peptide were compared with the area under

disease pressure curve values (Chapter 2) for each plot (plant) from the respective studies. No significant correlation was detected between any peptide at either sampling time with foliar wilting from the greenhouse study (Table 3.6). The same lack of correlation was found for every peptide in the Jackson Co. study. Additionally, comparisons of mean ROS by genotype within each peptide frequently found no significant differences between susceptible control and genotypes that expressed high BW resistance, although susceptible control was generally always in the lower half of the means for ROS production (Figures 3.1 and 3.2). In fact, the highly susceptible lines 'RST-04-105-T' and 'Maxifort' were often among the highest means in the Jackson Co. study (Figure 3.1) and the greenhouse study (Figure 3.2), respectively, whereas highly resistant lines were often found with low mean ROS.

3.4: Discussion and Conclusions

I have investigated characteristics of the tomato production of ROS when leaf cores are elicited by four MAMP peptides in eleven tomato genotypes with varying resistance levels to BW in two contrasting environments. I provide foundational information on the comparative behavior of MTI-related ROS production over time using several visual and statistical metrics, demonstrating that tomato genotypes show significant variation in total and maximum ROS production. This result is in agreement with other studies comparing levels MTI-related ROS production in heirloom tomatoes and *Brassica rapa* (Lloyd et al., 2014; Veluchamy et al., 2014). I observed significant correlations between the same measures of different peptides, along with some specific variation in perception of MAMP peptides by genotype, suggesting that elicitation of a strong response to one peptide is likely

to relate to a strong response in another, but should not be presumed. This is also the first report to my knowledge investigating factors modulating MTI-related ROS production curve characteristics in tomato plants. Other studies in humans and other animals have shown that immunity-related ROS production is modulated by type and concentration of herbal plant lysates (Mahomoodally et al., 2012) and *Salmonella* serovars (He et al., 2012). An increase in thiamine was found to stimulate host ROS production systems and help overcome suppression of *Arabidopsis* ROS response by *Sclerotinia sclerotiorum* (Jun et al., 2013). In citrus, elevated levels of oxidative stress were observed during soil flooding, and an oxidative burst was observed upon drainage (Hossain et al., 2009). The timeline of ROS production following leaf wounding in *Medicago truncatula* (Soares et al., 2011) is not consistent with what I observed in the assay following MAMP stimulation, nor between several days after inoculation. It should be noted that the study did not use MAMP stimulation of ROS production nor was ROS assessed using a peroxidase reaction method. I found that presence of a graft wound is associated with increased levels of ROS production, and that the inoculation process for BW (root cut and soil drench with high concentration of bacterial suspension) appears to be responsible for dramatic changes in ROS production peak and longevity characteristics. I also found that treatments with Rs isolates only affected maximum ROS production when stimulated with FlgII-28 following inoculation. It would be interesting to see if this pattern could be duplicated using a wider range of Rs races and localized strains, since such variation is known to affect host resistance levels (Chandrashekara et al., 2012; Grimault and Prior, 1994b; Hayward, 1991; Kelman, 1953; Naidoo et al., 2011; Scott et al., 2005; Zehr, 1970).

The fact that Pa Flg22 had the second largest elicitation overall in both studies is surprising, especially considering the substantial sequence changes compared to the commonly used Flg22. Single amino acid changes in key positions were shown to dramatically reduce or eliminate tomato perception of Flg22 (Felix et al., 1999). Yet, my results would indicate that transposition of the first six amino acids, while retaining sequence identity and order, did not eliminate host perception. This may indicate that amino acid identity is more important to flagellin perception than specific positions within the conserved region.

A confusing aspect of particularly the greenhouse experiment is the presence of significant replication effects in many of the peptide responses. Part of this pattern is likely influenced by the sampling procedure, especially in the greenhouse. In order to facilitate comparisons between each peptide, reaction plates for all four peptides were sampled in parallel, and due to the physical constraints of the assay, only one replication (Jc and P treatments combined) could be sampled on the same day. Since it is not known what factors may influence ROS production in this assay, it is possible that day-to-day variations in temperature, light intensity, humidity, etc. may play a modulating role in preconditioning tomato ROS production systems. Predisposing environmental effects on plant host responses are not unreasonable, and are known to occur in even some host-pathogen interactions (Foster and Walker, 1947; Gallegly and Walker, 1949; Kendrick and Walker, 1948). Here, I have provided evidence that simply the presence of a healed graft wound appears to precondition or "prime" plant systems to produce elevated levels of ROS. In a broader perspective, the ability of other chemistries and even non-pathogenic microorganisms to

precondition host resistance responses to Rs is becoming well documented (Algam et al., 2013; Diogo and Wydra, 2007; Ghareeb et al., 2011a; Ghareeb et al., 2011b; Hyakumachi et al., 2013; Kiirika and Wydra, 2012; Kiirika et al., 2013; Takahashi et al., 2014; Yi et al., 2008). It would be very interesting to compare the effects of those systems and chemistries on this MTI-related oxidative burst with these MAMP peptides to assess the effects on curve magnitude, peak time, endurance, etc., as I have done here with grafting and inoculation.

Previous studies have demonstrated that a common feature related to stimulation with MAMPs is a rapid burst of ROS (Boller and Felix, 2009; Cai et al., 2011; Felix et al., 1999; Felix and Boller, 2003; Lloyd et al., 2014; Veluchamy et al., 2014). In field and greenhouse studies, I confirmed this phenomenon for Csp22, FlgII-28, and Pa Flg22 in eleven tomato genotypes with a range of characteristics and genetic backgrounds, but not for Rs Flg22. Although a small amount of ROS production was observed in some tomato genotypes elicited by Rs Flg22 in the greenhouse, the patterns were not confirmed in the field study. Additionally, the patterns and magnitude of the response were not comparable to either Pa Flg22 or FlgII-28. These results, along with the highly irregular pattern of elicitation between studies and genotypes, are highly suggestive that the Rs-specific conserved region of the bacterial flagellin is not an effective elicitor of the rapid, transient ROS production in tomato. This may be due to an inability of tomato to detect the Rs epitope of the conserved region of bacterial flagellin, or that any such detection is not with PRRs that effectively stimulate a rapid burst of ROS production. In view of other work done with the Rs-specific flagellin in *Arabidopsis* and *Nicotiana benthamiana* (Pfund et al., 2004; Takabatake and Mukaihara, 2011), and in light of the knowledge that flagellin perception occurs in other species of

Solanaceae and Brassicaceae (Lloyd et al., 2014; Postel and Kemmerling, 2009), my results reinforce the hypothesis that the amino acid sequence differences found in the Rs-specific flagellin molecule may be a key factor allowing Rs to infect such a wide range of hosts. It is well supported that hosts detect Rs, produce an oxidative burst with ROS, and that Rs has several mechanisms to defend itself from ROS damage (Brown and Allen, 2004; Colburn-Clifford et al., 2010; Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011; Jacobs, 2013; Kiba et al., 2003; Milling et al., 2011; Nazeem et al., 2011; Yim et al., 2013), but it does not appear to be related to detection of bacterial flagellin in either tomato or *Arabidopsis* (Pfund et al., 2004), suggesting that part of the reason Rs may be such an effective pathogen is not simply due to the ability to suppress host defenses with effectors, but also avoidance of flagellin detection by host until it penetrates the xylem vessels, where it then sheds the flagellin and becomes non-motile (Schell, 2000; Tans-Kersten et al., 2001). The importance of this conclusion is amplified by the recent determination that plants specifically position the flagellin-detecting PRR FLS2 in regions where bacterial pathogens generally enter the host (Beck et al., 2014).

In assessing the relationship between MAMP-induced ROS production and resistance to BW, I was unable to demonstrate a link between the two plant responses. The lack of correlation between MTI-related ROS production and foliar wilting would seem to indicate that the early oxidative burst is not directly related to host resistance mechanisms. Thus, ROS production, as stimulated by the MAMPs Csp22, FlgII-28, Pa Flg22, and Rs Flg22, is not a predictor of resistance to this pathogen. The relationship between stimulation of ROS by MTI and BW disease resistance remains unclear. This lack of relationship is curious, considering

how well documented ROS production is for BW resistance, as previously noted. It may be that the mechanisms involved in host resistance are equally triggered by this initial burst of ROS production in resistant and susceptible genotypes, or that the defense signaling uses other receptors detecting other elicitors and/or alternative signaling pathways. The still unknown elicitor in boiled Rs extracts may provide the link of this relationship (Pfund et al., 2004; Takabatake and Mukaihara, 2011). It would be interesting to now assess this MTI-related ROS production system using the boiled extract of Rs and see if any significant correlations are discovered. It seems clear from these studies, however, that neither flagellin nor cold shock protein elicitation of this rapid burst of ROS production in tomato is related to BW resistasnce.

CHAPTER 3: REFERENCES

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CHAPTER 3: TABLES AND FIGURES

Table 3.1. ***MAMP peptide information.***
Microbe-associated molecular pattern peptide sequences used for artificial stimulation of reactive oxygen species production, along with relevant source information and sequence comparisons. The Flg22 sequence is included to highlight differences in the mutant epitope Pa Flg22. The previously reported Flagellin 22 consensus sequence is included for reference to denote highly conserved regions where R. solanacearum-specific sequence diverges.

MAMP	Amino Acid Sequence	Length	Protein Source	Organism Source	Source
Csp22 *	AVGTVKWFNAEKGFGFITPDDG	22	Cold Shock Protein (CSP)	*Micrococcus lysodeikticus*	Felix and Boller, 2003
FlgII-28 *	ESTNILQRMRELAVQSRNDSNSSTDRDA	28	Flagellin (FilC)	*Pseudomonas syringae* T1	Cai et al., 2011
Pa Flg22 ~	SRINSAKDDAAGLQIA***QRLSTG***	22	Flagellin (FilC)	*Pseudomonas aeruginosa*	This study
Rs Flg22	QRLSTGMRVNSAQDDAAAYASA	22	Flagellin (FilC)	*Ralstonia solanacearum* K60	Pfund et al., 2004
Flg22	QRLSTGSRINSAKDDAAGLQIA	22	Flagellin (FilC)	*Pseudomonas aeruginosa*	Felix et al., 1999
Flagellin 22	-RLSSGLRINSA-DDAAG--I-	Consensus	Flagellin (FilC)	Eubacteria	Felix et al., 1999
Notes:	**At time of manuscript composition, sequence discrepancies at position 21 and 23 for Csp22 and FlgII-28 were discovered between source sequence and one used in this study, respectively. Bolded position used is a D and S; reported is G and A, for Csp22 and FlgII-28, respectively.*				
	~Sequence used is a transpositon mutant epitope based on Felix et al., 1999. First six amino acids (Bold Italics) were transferred from the N-terminus to the C-terminus, retaining internal order and orientation.				

Table 3.2. ***Analysis of variance of MAMP-ellicited ROS production from the Jackson Co. study.***
Combined results of ANOVAs predicting total and maximum relative light units (RLUs) stimulated by four separate MAMP peptides for the Jackson Co. 2013 field study. RLU values are based upon photon measurements from 96-well plates filled with 2 mm leaf disks in the presence of luminol, horse radish peroxidase, and specific MAMP peptide (Csp22, FlgII-28, Pa Flg22, or Rs Flg22). Table values are p-values of each respective F-test, except for adjusted R-square rows. Significance levels are noted based upon number of degrees of freedom (DF). Genotype is comprised of 10 genotypes with varying levels of known resistance to bacterial wilt (BW), and graft-type compares non- and self-grafted plants. Two plot means were identified as extreme outliers over multiple peptides, and were removed from the analysis (BHN1054, self-graft, rep 3; CRA66, self-graft, rep 4). Total all and Total –Out allow comparison of models with or without these two plot outliers, respectively.

ANOVA P-values ~		Csp22				FlgII-28			
Model Effects	DF	Total All	Total -Out	Max All	Max -Out	Total All	Total -Out	Max All	Max -Out
Adjusted R-square	--	*0.4779*	*0.6270*	*0.3318*	*0.6067*	*0.3508*	*0.4689*	*0.5252*	*0.5826*
Model	47	0.0121 *	0.0012 **	0.0703 NS	0.0019 **	0.0582 NS	0.0186 *	0.0056 **	0.0030 **
Genotype	9	0.0005 ***	<.0001 ***	0.0162 *	<.0001 ***	0.0121 *	0.0025 **	0.0006 ***	0.0002 ***
Replication	3	0.5278 NS	0.3404 NS	0.2862 NS	0.0666 NS	0.4449 NS	0.0847 NS	0.1827 NS	0.0414 *
Graft-Type(Genotype)	8	0.0217 *	0.0134 *	0.0700 NS	0.0139 *	0.2775 NS	0.3647 NS	0.2140 NS	0.3111 NS
Genotype*Replication	27	0.1765 NS	0.0586 NS	0.2848 NS	0.0185 *	0.1002 NS	0.0557 NS	0.0164 *	0.0175 *
		Pa Flg22				Rs Flg22			
Model Effects	DF	Total All	Total -Out	Max All	Max -Out	Total All	Total -Out	Max All	Max -Out
Adjusted R-square	--	*0.6703*	*0.7038*	*0.6449*	*0.7317*	*0.1698*	*0.2289*	*0.3045*	*0.3173*
Model	47	0.0002 ***	0.0002 ***	0.0004 ***	<.0001 ***	0.2410 NS	0.1801 NS	0.0904 NS	0.0935 NS
Genotype	9	<.0001 ***	<.0001 ***	<.0001 ***	<.0001 ***	0.0805 NS	0.2518 NS	0.0125 *	0.0604 NS
Replication	3	0.0316 *	0.0064 **	0.0061 **	0.0003 ***	0.4340 NS	0.2833 NS	0.5734 NS	0.5068 NS
Graft-Type(Genotype)	8	0.0070 **	0.0174 *	0.0038 **	0.0065 **	0.0772 NS	0.0359 *	0.0582 NS	0.0423 *
Genotype*Replication	27	0.0048 **	0.0052 **	0.0222 *	0.0074 **	0.6803 NS	0.5324 NS	0.4285 NS	0.4161 NS

Notes: *~ P-values calculated from model F-values. Adjusted R-square is actual value, not P-value.*
, **, * Significant at α = 0.05, 0.01, and 0.001, respectively. NS is not significant.*

Table 3.3. ***Analysis of variance of MAMP-ellicited ROS production from the greenhouse study.***
Combined results of repeated measures ANOVAs predicting total and maximum relative light units (RLUs) stimulated by four separate MAMP peptides for the greenhouse study. RLU values are based upon photon measurements from 96-well plates filled with 2 mm leaf disks in the presence of luminol, horse radish peroxidase, and specific MAMP peptide (Csp22, FlgII-28, Pa Flg22, or Rs Flg22). Table values are p-values of each respective F-test, except for adjusted R-square rows. Significance levels are noted based upon number of degrees of freedom (DF). Genotype is comprised of 11 genotypes with varying levels of known resistance to bacterial wilt (BW), and treatment compares blocking factor of two NC Rs isolates used for comparative inoculation. Two plant means were identified as extreme outliers over multiple peptides, and were removed from the analysis (BHN1054, isolate Jc, rep 1, plant 2; Hawaii 7998, isolate P, rep 1, plant 2). Total Pre and Total Post allow comparison of repeated measures outputs for each peptide pre- and post-inoculation; respectively.

ANOVA P-values ~		Csp22				FlgII-28			
Model Effects	**DF**	**Total Pre**	**Total Post**	**Max Pre**	**Max Post**	**Total Pre**	**Total Post**	**Max Pre**	**Max Post**
Adjusted R-square	--	*0.1994*	*0.3054*	*0.3621*	*0.2706*	*0.3686*	*0.4561*	*0.4095*	*0.2200*
Model	65	0.0548 NS	0.0064 **	0.0014 **	0.0142 *	0.0012 **	<.0001 ***	0.0003 ***	0.0384 *
Rs Isolate (Trt)	1	0.8288 NS	0.6621 NS	0.6182 NS	0.2332 NS	0.9495 NS	0.1534 NS	0.5813 NS	0.0021 **
Replication (Rep)	2	0.0419 ***	0.0002 ***	0.052 NS	0.062 NS	<.0001 ***	0.0002 ***	<.0001 ***	0.018 *
Trt*Rep	2	0.0861 NS	0.876 NS	0.0552 NS	0.819 NS	0.3407 NS	<.0001 ***	0.2579 NS	0.1859 NS
Genotype	10	0.0746 NS	0.0981 NS	<.0001 ***	0.0157 *	<.0001 ***	0.319 NS	<.0001 ***	0.1111 NS
Trt*Genotype	10	0.2181 NS	0.1381 NS	0.0534 NS	0.5709 NS	0.9998 NS	0.0012 **	0.998 NS	0.183 NS
Genotype*Rep(Trt)	40	0.1476 NS	0.0277 *	0.1124 NS	0.0174 *	0.1219 NS	0.0068 **	0.1096 NS	0.2201 NS
		Pa Flg22				**Rs Flg22**			
Model Effects	**DF**	**Total Pre**	**Total Post**	**Max Pre**	**Max Post**	**Total Pre**	**Total Post**	**Max Pre**	**Max Post**
Adjusted R-square	--	*0.4538*	*0.3467*	*0.4826*	*0.1841*	*0.2494*	*0.1209*	*0.3041*	*0.0345*
Model	65	<.0001 ***	0.0022 **	<.0001 ***	0.0702 NS	0.022 *	0.1677 NS	0.0066 **	0.3923 NS
Rs Isolate (Trt)	1	0.6891 NS	0.198 NS	0.9388 NS	0.8359 NS	0.381 NS	0.5277 NS	0.4157 NS	0.4765 NS
Replication (Rep)	2	<.0001 ***	0.0122 *	<.0001 ***	0.0413 *	0.4101 NS	0.8821 NS	0.7397 NS	0.1169 NS
Trt*Rep	2	0.5695 NS	0.0338 *	0.1424 NS	0.0319 *	0.024 *	0.6256 NS	0.0105 *	0.0464 *
Genotype	10	0.0054 **	0.1525 NS	<.0001 ***	0.0992 NS	0.0015 **	0.0334 *	0.0031 **	0.3846 NS
Trt*Genotype	10	0.9358 NS	0.0148 *	0.9957 NS	0.5483 NS	0.2736 NS	0.0027 **	0.0923 NS	0.1788 NS
Genotype*Rep(Trt)	40	0.6856 NS	0.0152 *	0.7711 NS	0.1173 NS	0.1852 NS	0.864 NS	0.0465 *	0.7529 NS
Repeated Measures Effects		**Csp22**	**Csp22**	**FlgII-28**	**FlgII-28**	**Pa Flg22**	**Pa Flg22**	**Rs Flg22**	**Rs Flg22**
Between:	**DF**	**Total**	**Max**	**Total**	**Max**	**Total**	**Max**	**Total**	**Max**
Rs Isolate (Trt)	1	0.6983 NS	0.2295 NS	0.3671 NS	0.0029 **	0.3124 NS	0.8408 NS	0.3281 NS	0.262 NS
Replication (Rep)	2	0.0116 *	0.212 NS	0.0074 **	0.0002 ***	<.0001 ***	0.0101 *	0.4589 NS	0.5176 NS
Trt*Rep	2	0.1139 NS	0.177 NS	0.0247 *	0.4113 NS	0.8233 NS	0.2639 NS	0.0351 *	0.2866 NS
Genotype	10	0.0294 *	<.0001 ***	0.0043 **	<.0001 ***	0.0073 **	0.0021 **	0.0023 **	0.0055 **
Trt*Genotype	10	0.3728 NS	0.134 NS	0.1968 NS	0.3368 NS	0.3792 NS	0.8043 NS	0.181 NS	0.08 NS
Genotype*Rep(Trt)	40	0.0639 NS	0.0177 *	0.0441 *	0.2665 NS	0.3604 NS	0.2273 NS	0.1754 NS	0.393 NS
Within:									
Sampling Time	1	<.0001 ***	0.3686 NS	<.0001 ***	0.0006 ***	0.1025 NS	<.0001 ***	<.0001 ***	<.0001 ***
Time*Trt	1	0.9862 NS	0.6533 NS	0.4191 NS	0.016 *	0.671 NS	0.8681 NS	0.481 NS	0.8914 NS
Time*Rep	2	0.0114 *	0.0174 *	<.0001 ***	0.0066 **	<.0001 ***	<.0001 ***	0.4032 NS	0.2208 NS
Time*Trt*Rep	2	0.1842 NS	0.2039 NS	0.0036 **	0.104 NS	0.0491 *	0.0036 **	0.0247 *	0.0024 **
Time*Genotype	10	0.222 NS	0.1667 NS	0.0002 ***	0.4833 NS	0.042 *	0.1108 NS	0.0014 **	0.114 NS
Time*Trt*Genotype	10	0.0898 NS	0.2396 NS	0.3327 NS	0.3829 NS	0.4241 NS	0.4927 NS	0.2415 NS	0.1776 NS
Time*Genotype*Rep(Trt)	40	0.1719 NS	0.1151 NS	0.0481 *	0.1295 NS	0.1991 NS	0.154 NS	0.2695 NS	0.107 NS

Notes: *~ P-values calculated from model F-values. Adjusted R-square is actual value, not P-value.*
, **, * Significant at α = 0.05, 0.01, and 0.001, respectively. NS is not significant.*

Table 3.4. ***Correlation analysis of ROS production comparing peptide in the Jackson Co. study.***
Pearson correlations for the Jackson Co. study relating tomato leaf disk production of reactive oxygen species (ROS) curve characteristics (total, average, and maximum production) in all combinations. ROS was measured using a luminol and peroxidase-based assay where tomato leaf disks produced ROS over time when elicited with one of four peptides (Csp22, FlgII-28, Pa Flg22, and Rs Flg22) and relative light output was measured every 4 minutes for 56 minutes. Significance levels are indicated.

Response	Csp22_ Total1	Csp22_ Avg1	Csp22_ max1	FlgII_28_ Total1	FlgII_28_ Avg1	FlgII_28_ max1	Pa_Flg22_ Total1	Pa_Flg22_ Avg1	Pa_Flg22_ max1	Rs_Flg22_ Total1	Rs_Flg22_ Avg1	Rs_Flg22_ max1
Csp22_Total1	1	0.99999 ***	0.82504 ***	0.30642 **	0.32568 **	0.32106 **	0.46047 ***	0.50147 ***	0.37736 **	0.21371 NS	0.21935 NS	0.31863 **
Csp22_Avg1	0.99999 NS	1	0.82415 NS	0.30516 *	0.32437 **	0.31942 **	0.46098 ***	0.50191 ***	0.37765 **	0.21419 NS	0.21982 NS	0.31909 **
Csp22_max1	0.82504 ***	0.82415 ***	1	0.40353 ***	0.42855 ***	0.51133 ***	0.25839 *	0.29853 *	0.22434 NS	0.07618 NS	0.08569 NS	0.15507 NS
FlgII_28_Total1	0.30642 **	0.30516 *	0.40353 ***	1	0.99488 ***	0.93752 ***	0.12494 NS	0.16784 NS	0.08321 NS	0.24017 *	0.2429 *	0.28036 *
FlgII_28_Avg1	0.32568 **	0.32437 **	0.42855 ***	0.99488 ***	1	0.93718 ***	0.12831 NS	0.178 NS	0.08298 NS	0.24317 *	0.24995 *	0.28633 *
FlgII_28_max1	0.32106 **	0.31942 **	0.51133 ***	0.93752 ***	0.93718 ***	1	0.08197 NS	0.12065 NS	0.07484 NS	0.11748 NS	0.12289 NS	0.17367 NS
Pa_Flg22_Total1	0.46047 ***	0.46098 ***	0.25839 *	0.12494 NS	0.12831 NS	0.08197 NS	1	0.98724 ***	0.96819 ***	0.16107 NS	0.16109 NS	0.15674 NS
Pa_Flg22_Avg1	0.50147 ***	0.50191 ***	0.29853 *	0.16784 NS	0.178 NS	0.12065 NS	0.98724 ***	1	0.93425 ***	0.18689 NS	0.19102 NS	0.18966 NS
Pa_Flg22_max1	0.37736 **	0.37765 **	0.22434 NS	0.08321 NS	0.08298 NS	0.07484 NS	0.96819 ***	0.93425 ***	1	0.07438 NS	0.07115 NS	0.06494 NS
Rs_Flg22_Total1	0.21371 NS	0.21419 NS	0.07618 NS	0.24017 *	0.24317 *	0.11748 NS	0.16107 NS	0.18689 NS	0.07438 NS	1	0.99791 ***	0.95983 ***
Rs_Flg22_Avg1	0.21935 NS	0.21982 NS	0.08569 NS	0.2429 *	0.24995 *	0.12289 NS	0.16109 NS	0.19102 NS	0.07115 NS	0.99791 ***	1	0.95687 ***
Rs_Flg22_max1	0.31863 **	0.31909 **	0.15507 NS	0.28036 *	0.28633 *	0.17367 NS	0.15674 NS	0.18966 NS	0.06494 NS	0.95983 ***	0.95687 ***	1

Notes: *Pearson correlation (r)* **, **, *** Significant at α = 0.05, 0.01, and 0.001, respectively. NS is not significant.*

Table 3.5. ***Correlation analysis of ROS production comparing peptide in the greenhouse study.***
Pearson correlations for the greenhouse study relating tomato leaf disk production of reactive oxygen species (ROS) curve characteristics (total and maximum production) in all combinations. ROS was measured using a luminol and peroxidase-based assay where tomato leaf disks produced ROS over time when elicited with one of four peptides (Csp22, FlgII-28, Pa Flg22, and Rs Flg22) and relative light output was measured every 2 minutes for 62 minutes. Significance levels are indicated.

Response	Total1_ Csp22	Total2_ Csp22	Max1_ Csp22	Max2_ Csp22	Total1_ FlgII_28	Total2_ FlgII_28	Max1_ FlgII_28	Max2_ FlgII_28	Total1_ Pa_Flg22	Total2_ Pa_Flg22	Max1_ Pa_Flg22	Max2_ Pa_Flg22	Total1_ Rs_Flg22	Total2_ Rs_Flg22	Max1_ Rs_Flg22	Max2_ Rs_Flg22
Total1_Csp22	1	0.10025 NS	0.82119 ***	0.12535 NS	0.52867 ***	-0.03119 NS	0.49275 ***	0.05165 NS	0.30516 ***	0.10752 NS	0.29907 ***	0.20202 *	0.3347 ***	0.06941 NS	0.28474 ***	-0.02283 NS
Total2_Csp22	0.10025 NS	1	-0.0007 NS	0.88337 ***	-0.02535 NS	0.38196 ***	-0.07062 NS	0.42061 ***	-0.23883 **	0.50187 ***	-0.19969 *	0.43483 ***	0.20206 *	0.34749 ***	0.07739 NS	0.03928 NS
Max1_Csp22	0.82119 ***	-0.00074 NS	1	0.11401 NS	0.45137 ***	-0.06334 NS	0.54506 ***	0.04784 NS	0.39071 ***	0.02709 NS	0.49544 ***	0.15506 NS	0.26217 **	0.02092 NS	0.22876 **	-0.06618 NS
Max2_Csp22	0.12535 NS	0.88337 ***	0.11401 NS	1	0.04588 NS	0.24413 **	0.05047 NS	0.40946 ***	-0.12635 NS	0.40481 ***	-0.06586 NS	0.41802 ***	0.27356 **	0.25212 **	0.17524 *	0.06726 NS
Total1_FlgII_28	0.52867 ***	-0.02535 NS	0.45137 ***	0.04588 NS	1	-0.10776 NS	0.92295 ***	0.04763 NS	0.43941 ***	0.09185 NS	0.37839 ***	0.21084 *	0.37723 ***	0.0338 NS	0.32061 **	-0.0747 NS
Total2_FlgII_28	-0.03119 NS	0.38196 ***	-0.0633 NS	0.24413 **	-0.10776 NS	1	-0.1254 NS	0.69149 ***	-0.18568 *	0.44403 ***	-0.16804 NS	0.29796 ***	-0.01606 NS	0.23031 **	-0.06202 NS	-0.0254 NS
Max1_FlgII_28	0.49275 ***	-0.07062 NS	0.54506 **	0.05047 NS	0.92295 ***	-0.1254 NS	1	0.08796 NS	0.45898 ***	0.02436 NS	0.49004 ***	0.16815 NS	0.36703 ***	-0.0005 NS	0.35722 ***	-0.11936 NS
Max2_FlgII_28	0.05165 NS	0.42061 ***	0.04784 NS	0.40946 ***	0.04763 NS	0.69149 ***	0.08796 NS	1	-0.08445 NS	0.36163 ***	-0.00817 NS	0.4061 ***	0.15796 NS	0.12137 NS	0.04148 NS	0.01609 NS
Total1_Pa_Flg22	0.30516 ***	-0.23883 **	0.39071 ***	-0.1264 NS	0.43941 ***	-0.18568 *	0.45898 ***	-0.08445 NS	1	-0.08432 NS	0.90956 ***	0.02597 NS	0.13659 NS	0.00811 NS	0.13679 NS	-0.00337 NS
Total2_Pa_Flg22	0.10752 NS	0.50187 ***	0.02709 NS	0.40481 ***	0.09185 NS	0.44403 ***	0.02436 NS	0.36163 ***	-0.08432 NS	1	-0.05731 NS	0.81882 ***	0.15451 NS	0.24663 **	0.0244 NS	-0.01957 NS
Max1_Pa_Flg22	0.29907 ***	-0.19969 *	0.49544 ***	-0.0659 NS	0.37839 ***	-0.16804 NS	0.49004 ***	-0.00817 NS	0.90956 ***	-0.05731 NS	1	0.09728 NS	0.11139 NS	-0.00138 NS	0.12349 NS	-0.03532 NS
Max2_Pa_Flg22	0.20202 *	0.43483 ***	0.15506 NS	0.41802 ***	0.21084 *	0.29796 ***	0.16815 NS	0.4061 ***	0.02597 NS	0.81882 ***	0.09728 NS	1	0.12928 NS	0.11757 NS	0.00886 NS	-0.04868 NS
Total1_Rs_Flg22	0.3347 ***	0.20206 *	0.26217 **	0.27356 **	0.37723 ***	-0.01606 NS	0.36703 ***	0.15796 NS	0.13659 NS	0.15451 NS	0.11139 NS	0.12928 NS	1	0.15892 NS	0.81947 ***	-0.02822 NS
Total2_Rs_Flg22	0.06941 NS	0.34749 ***	0.02092 NS	0.25212 **	0.0338 NS	0.23031 **	-0.0005 NS	0.12137 NS	0.00811 NS	0.24663 **	-0.00138 NS	0.11757 NS	0.15892 NS	1	0.01802 NS	0.31758 ***
Max1_Rs_Flg22	0.28474 ***	0.07739 NS	0.22876 **	0.17524 *	0.32061 ***	-0.06202 NS	0.35722 ***	0.04148 NS	0.13679 NS	0.0244 NS	0.12349 NS	0.00886 NS	0.81947 ***	0.01802 NS	1	-0.10758 NS
Max2_Rs_Flg22	-0.02283 NS	0.03928 NS	-0.0662 NS	0.06726 NS	-0.0747 NS	-0.0254 NS	-0.11936 NS	0.01609 NS	-0.00337 NS	-0.01957 NS	-0.03532 NS	-0.04868 NS	-0.02822 NS	0.31758 ***	-0.10758 NS	1

Notes: *Pearson correlation (r)* **, **, *** Significant at α = 0.05, 0.01, and 0.001, respectively. NS is not significant.*

Table 3.6. ***Correlation analysis of ROS production with the Jackson Co. study.***
Pearson correlations relating tomato leaf disk production of reactive oxygen species (ROS) curve characteristics (total and maximum production) for the Jackson Co. and greenhouse studies. Both studies compare curve characteristics to plant area under disease progress curves (AUDPC) and genotype. Greenhouse study also compares with pre- and post-inoculation, Rs isolate, and replication while the Jackson Co. study also compares with graft-type (non- or self-graft). ROS was measured using a luminol and peroxidase-based assay where tomato leaf disks produced ROS over time when elicited with one of four peptides (Csp22, FlgII-28, Pa Flg22, and Rs Flg22) and relative light output was measured every 2 minutes for 62 minutes. Significance levels are indicated.

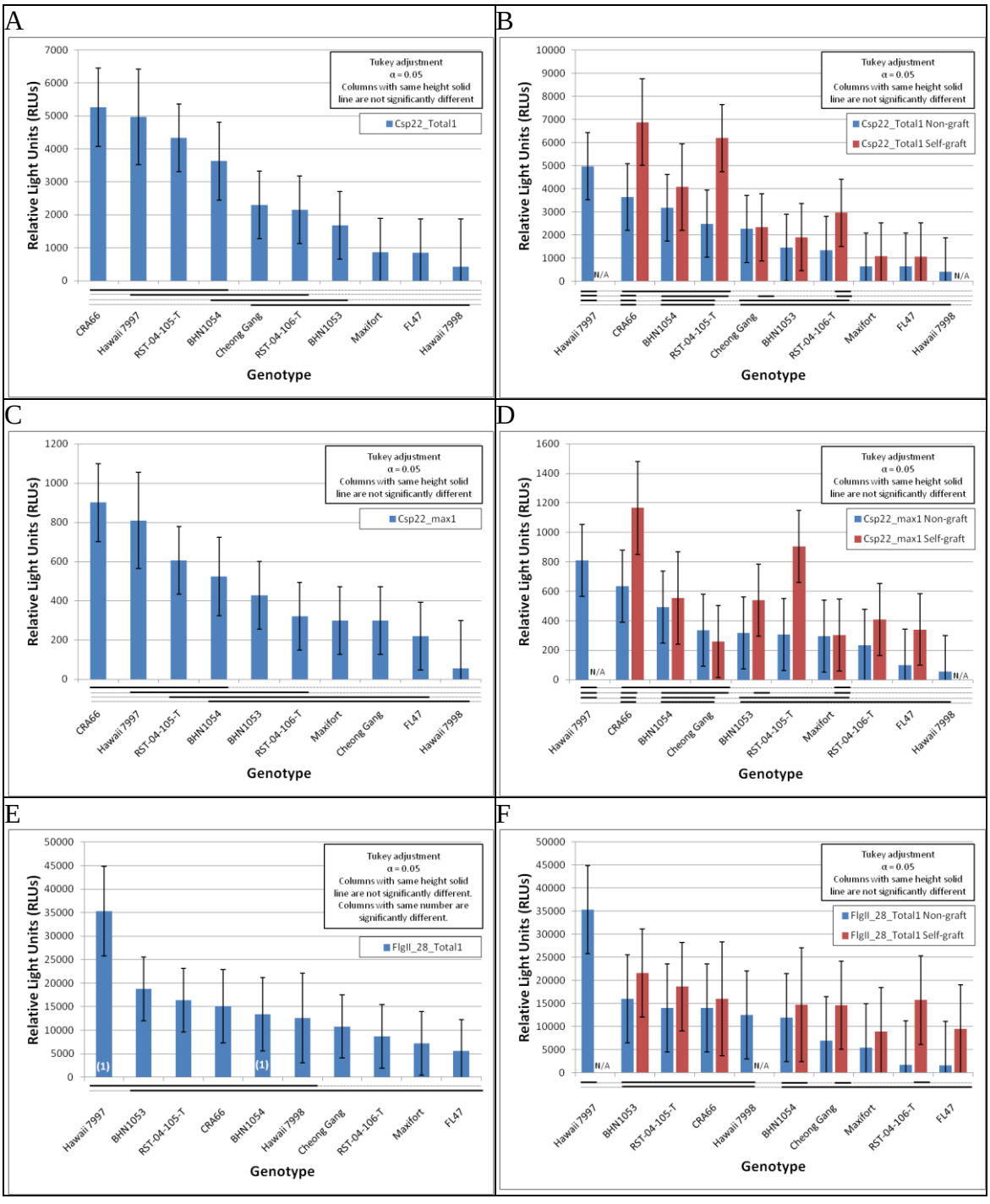
Response: Greenhouse	AUDPC		Rs Isolate	Genotype	Replication
	1-43 dpi	1-21 dpi			
Total1_Csp22	-0.03693 NS	-0.04200 NS	-0.02322 NS	0.11727 NS	-0.18847 *
Total2_Csp22	-0.16280 NS	-0.10697 NS	-0.04296 NS	0.09729 NS	0.06071 NS
Max1_Csp22	0.08760 NS	0.05304 NS	-0.04099 NS	-0.10283 NS	-0.15080 NS
Max2_Csp22	-0.00535 NS	0.02908 NS	-0.10117 NS	-0.07595 NS	-0.01351 NS
Total1_FlgII_28	0.03833 NS	0.02227 NS	0.01298 NS	0.06449 NS	-0.39908 ***
Total2_FlgII_28	-0.08806 NS	0.02018 NS	0.08935 NS	-0.00618 NS	0.22913 **
Max1_FlgII_28	0.15248 NS	0.12299 NS	0.04435 NS	-0.11927 NS	-0.40469 ***
Max2_FlgII_28	-0.09376 NS	-0.01927 NS	0.23151 **	-0.03448 NS	-0.01801 NS
Total1_Pa_Flg22	0.02948 NS	-0.02032 NS	0.02739 NS	-0.07322 NS	-0.32769 ***
Total2_Pa_Flg22	-0.05666 NS	0.00944 NS	0.08457 NS	0.01530 NS	0.13255 NS
Max1_Pa_Flg22	0.10592 NS	0.04190 NS	0.00144 NS	-0.19062 NS	-0.33734 ***
Max2_Pa_Flg22	-0.03869 NS	-0.02575 NS	-0.00619 NS	0.01402 NS	-0.05628 NS
Total1_Rs_Flg22	-0.01153 NS	-0.00754 NS	-0.06082 NS	-0.02875 NS	-0.06770 NS
Total2_Rs_Flg22	-0.08939 NS	-0.10802 NS	-0.04780 NS	0.12546 NS	0.02637 NS
Max1_Rs_Flg22	0.04070 NS	0.04082 NS	-0.04788 NS	-0.10268 NS	-0.04715 NS
Max2_Rs_Flg22	-0.07527 NS	-0.06827 NS	-0.06636 NS	0.10440 NS	0.15526 NS

Response: Jackson Co.	AUDPC			Genotype	Graft-Type
	0-91 dap	0-35 dap	35-91 dap		
Csp22_Total1	0.04688 NS	-0.01110 NS	0.05032 NS	0.05124 NS	0.21971 NS
Csp22_Avg1	0.04677 NS	-0.01220 NS	0.05030 NS	0.05105 NS	0.21954 NS
Csp22_max1	-0.06740 NS	0.05566 NS	-0.07574 NS	-0.05303 NS	0.22625 NS
FlgII_28_Total1	-0.09768 NS	0.02398 NS	-0.10493 NS	0.05954 NS	0.13845 NS
FlgII_28_Avg1	-0.09204 NS	0.03369 NS	-0.09982 NS	0.05930 NS	0.13435 NS
FlgII_28_max1	-0.07731 NS	0.08922 NS	-0.08909 NS	0.06476 NS	0.10644 NS
Pa_Flg22_Total1	0.15531 NS	0.17675 NS	0.14837 NS	-0.21756 NS	0.21844 NS
Pa_Flg22_Avg1	0.18082 NS	0.20036 NS	0.17320 NS	-0.20249 NS	0.20181 NS
Pa_Flg22_max1	0.15472 NS	0.20710 NS	0.14514 NS	-0.25536 NS	0.22577 NS
Rs_Flg22_Total1	-0.06162 NS	-0.20185 NS	-0.04753 NS	0.17791 NS	0.09281 NS
Rs_Flg22_Avg1	-0.05160 NS	-0.19220 NS	-0.03781 NS	0.16343 NS	0.08440 NS
Rs_Flg22_max1	-0.08016 NS	-0.19884 NS	-0.06731 NS	0.25106 *	0.11974 NS

Notes: *Values are Pearson correlation (r)*

, **, * Significant at α = 0.05, 0.01, and 0.001, respectively. NS is not significant.*

Figure 3.1. ***Comparisons of ROS production by genotype and graft-type in the Jackson Co.study.***
Panel comparisons of least square means from the Jackson Co. 2013 field study for total and maximum RLUs for each of four MAMP peptides by genotype. Left-hand column panels (A, C, E, G, I, K, M, and O) are between genotype comparisons, and right-hand column panels (B, D, F, H, J, L, N, and P) are graft-type within genotype comparisons. Row pairs (A and B, C and D, etc.) are the specific curve descriptor within its respective MAMP. Tukey adjustment was used for pair-wise comparisons, and error bars are the associated 95% confidence intervals for each mean. Columns with the same height solid line are not significantly different from each other. Numbers on bars designate significantly different means unable to be visualized by line comparisons. Due to unexpected losses during grafting process, no plots of self-grafted Hawaii 7997 or Hawaii 7998 were planted; absence is noted by N/A.


A
Relative Light Units (RLUs)
Tukey adjustment
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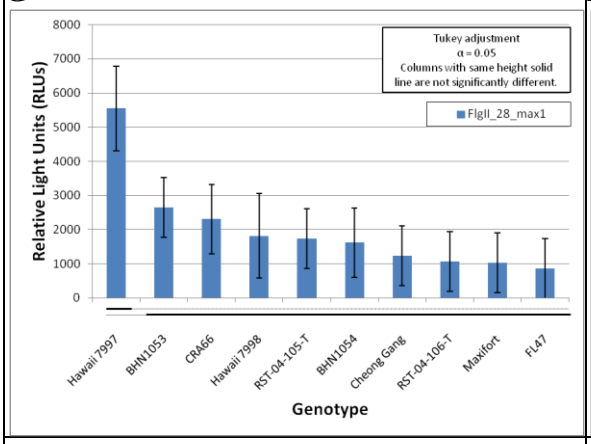
Columns with same height solid line are not significantly different
Csp22_Total1
CRA66
Hawaii 7997
RST-04-105-T
BHN1054
Cheong Gang
RST-04-106-T
BHN1053
Maxifort
FL47
Hawaii 7998
Genotype
B
Relative Light Units (RLUs)
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different
Csp22_Total1 Non-graft
Csp22_Total1 Self-graft
N/A
Hawaii 7997
CRA66
BHN1054
RST-04-105-T
Cheong Gang
BHN1053
RST-04-106-T
Maxifort
FL47
Hawaii 7998
Genotype
C
Relative Light Units (RLUs)
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different
Csp22_max1
CRA66
Hawaii 7997
RST-04-105-T
BHN1054
BHN1053
RST-04-106-T
Maxifort
Cheong Gang
FL47
Hawaii 7998
Genotype
D
Relative Light Units (RLUs)
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different
Csp22_max1 Non-graft
Csp22_max1 Self-graft
N/A
Hawaii 7997
CRA66
BHN1054
Cheong Gang
BHN1053
RST-04-105-T
Maxifort
RST-04-106-T
FL47
Hawaii 7998
Genotype
E
Relative Light Units (RLUs)
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Columns with same number are significantly different.
FlgII_28_Total1
(1)
(1)
Hawaii 7997
BHN1053
RST-04-105-T
CRA66
BHN1054
Hawaii 7998
Cheong Gang
RST-04-106-T
Maxifort
FL47
Genotype
F
Relative Light Units (RLUs)
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different
FlgII_28_Total1 Non-graft
FlgII_28_Total1 Self-graft
N/A
N/A
Hawaii 7997
BHN1053
RST-04-105-T
CRA66
Hawaii 7998
BHN1054
Cheong Gang
Maxifort
RST-04-106-T
FL47
Genotype

G


Tukey adjustment
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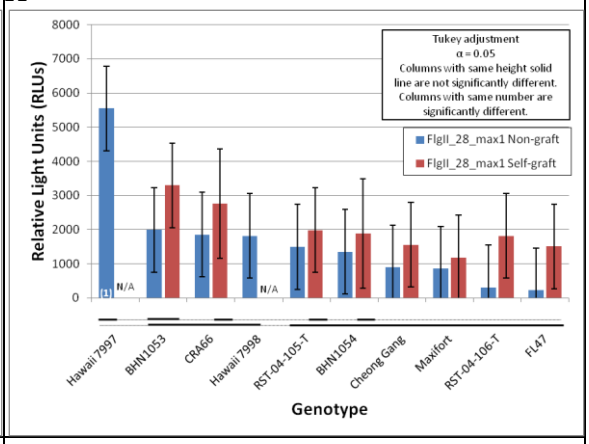
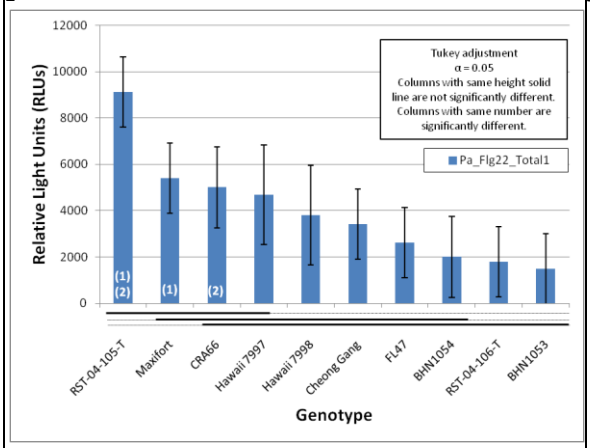
Columns with same height solid line are not significantly different.
FlgII_28_max1
Relative Light Units (RLUs)
8000
7000
6000
5000
4000
3000
2000
1000
0
Hawaii 7997
BHN1053
CRA66
Hawaii 7998
RST-04-105-T
BHN1054
Cheong Gang
RST-04-106-T
Maxifort
FL47
Genotype

H


Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Columns with same number are significantly different.
FlgII_28_max1 Non-graft
FlgII_28_max1 Self-graft
Relative Light Units (RLUs)
8000
7000
6000
5000
4000
3000
2000
1000
0
(1)
N/A
N/A
Hawaii 7997
BHN1053
CRA66
Hawaii 7998
RST-04-105-T
BHN1054
Cheong Gang
Maxifort
RST-04-106-T
FL47
Genotype

I


Tukey adjustment
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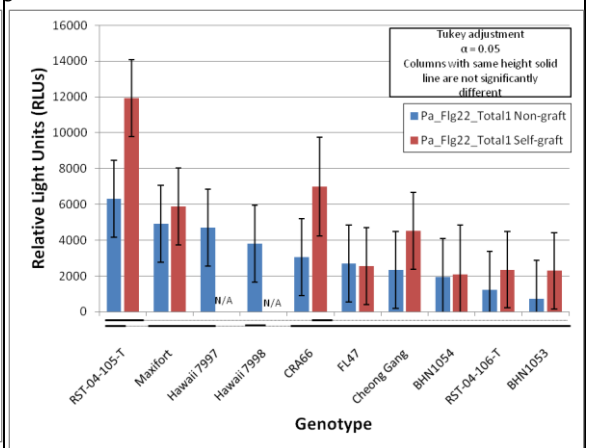
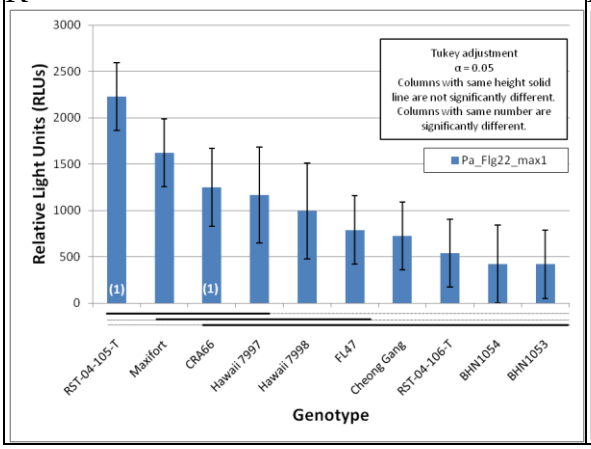
Columns with same height solid line are not significantly different.
Columns with same number are significantly different.
Pa_Flg22_Total1
Relative Light Units (RLUs)
12000
10000
8000
6000
4000
2000
0
(1)
(2)
(1)
(2)
RST-04-105-T
Maxifort
CRA66
Hawaii 7997
Hawaii 7998
Cheong Gang
FL47
BHN1054
RST-04-106-T
BHN1053
Genotype

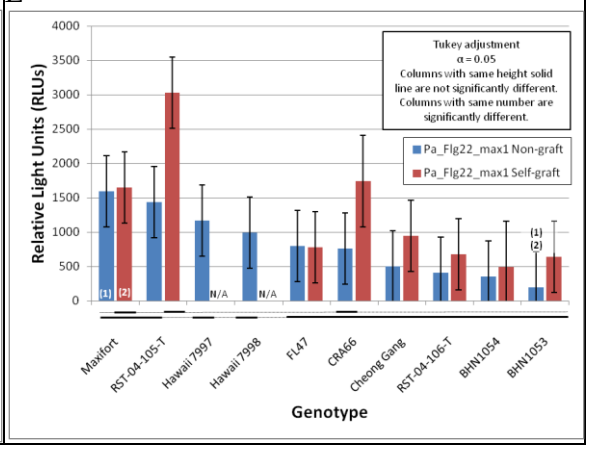
J


Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different
Pa_Flg22_Total1 Non-graft
Pa_Flg22_Total1 Self-graft
Relative Light Units (RLUs)
16000
14000
12000
10000
8000
6000
4000
2000
0
N/A
N/A
RST-04-105-T
Maxifort
Hawaii 7997
Hawaii 7998
CRA66
FL47
Cheong Gang
BHN1054
RST-04-106-T
BHN1053
Genotype

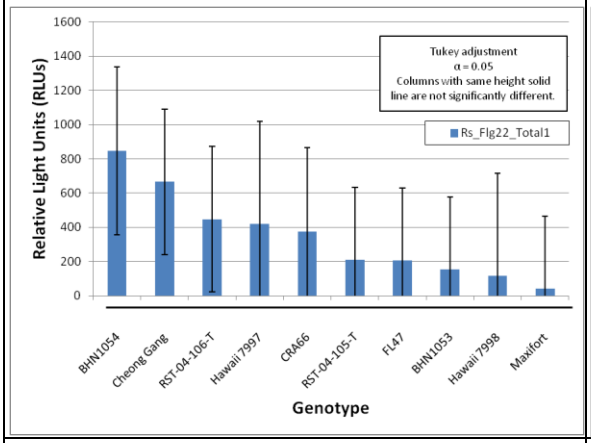
K


Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Columns with same number are significantly different.
Pa_Flg22_max1
Relative Light Units (RLUs)
3000
2500
2000
1500
1000
500
0
(1)
(1)
RST-04-105-T
Maxifort
CRA66
Hawaii 7997
Hawaii 7998
FL47
Cheong Gang
RST-04-106-T
BHN1054
BHN1053
Genotype

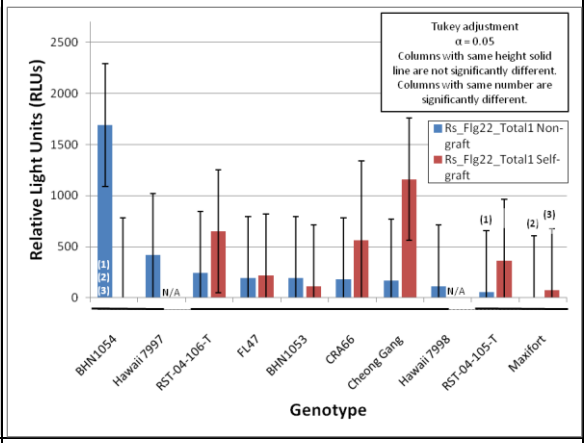
L


Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Columns with same number are significantly different.
Pa_Flg22_max1 Non-graft
Pa_Flg22_max1 Self-graft
Relative Light Units (RLUs)
4000
3500
3000
2500
2000
1500
1000
500
0
(1)
(2)
N/A
N/A
(1)
(2)
Maxifort
RST-04-105-T
Hawaii 7997
Hawaii 7998
FL47
CRA66
Cheong Gang
RST-04-106-T
BHN1054
BHN1053
Genotype

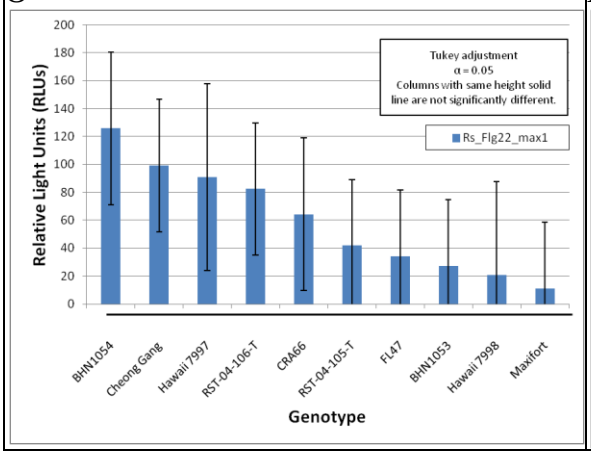
M


Relative Light Units (RLUs)
1600
1400
1200
1000
800
600
400
200
0
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Rs_Flg22_Total1
BHN1054
Cheong Gang
RST-04-106-T
Hawaii 7997
CRA66
RST-04-105-T
FL47
BHN1053
Hawaii 7998
Maxifort
Genotype

N


Relative Light Units (RLUs)
2500
2000
1500
1000
500
0
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Columns with same number are significantly different.
Rs_Flg22_Total1 Non-graft
Rs_Flg22_Total1 Self-graft
(1)
(2)
(3)
N/A
BHN1054
Hawaii 7997
RST-04-106-T
FL47
BHN1053
CRA66
Cheong Gang
Hawaii 7998
RST-04-105-T
Maxifort
Genotype

O


Relative Light Units (RLUs)
200
180
160
140
120
100
80
60
40
20
0
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Rs_Flg22_max1
BHN1054
Cheong Gang
Hawaii 7997
RST-04-106-T
CRA66
RST-04-105-T
FL47
BHN1053
Hawaii 7998
Maxifort
Genotype

P

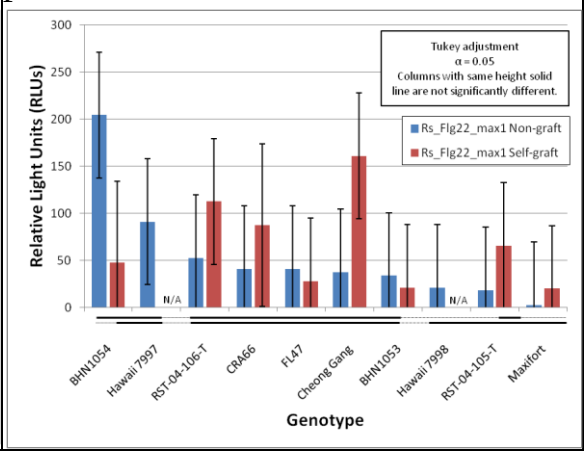
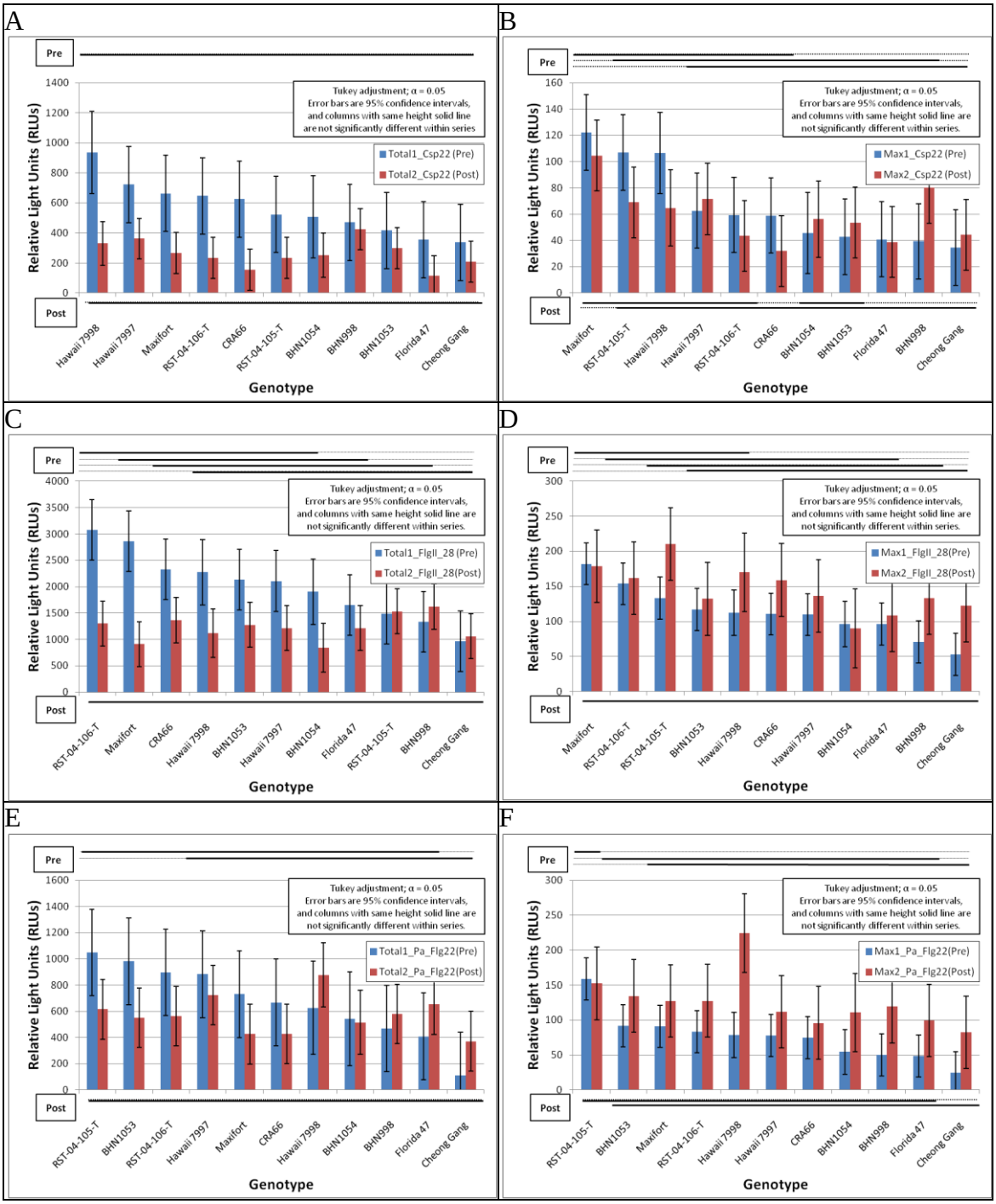
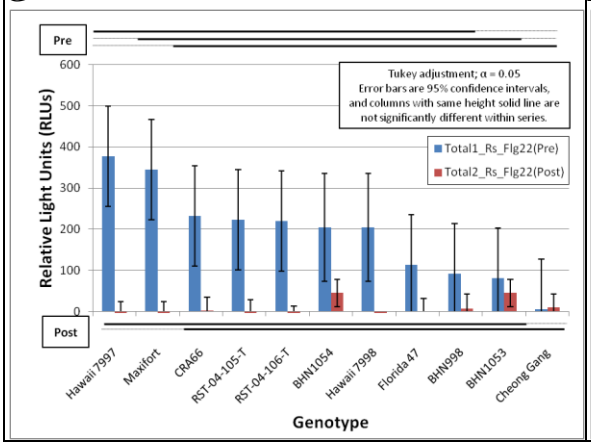

Relative Light Units (RLUs)
300
250
200
150
100
50
0
Tukey adjustment
α = 0.05
Columns with same height solid line are not significantly different.
Rs_Flg22_max1 Non-graft
Rs_Flg22_max1 Self-graft
N/A
BHN1054
Hawaii 7997
RST-04-106-T
CRA66
FL47
Cheong Gang
BHN1053
Hawaii 7998
RST-04-105-T
Maxifort
Genotype

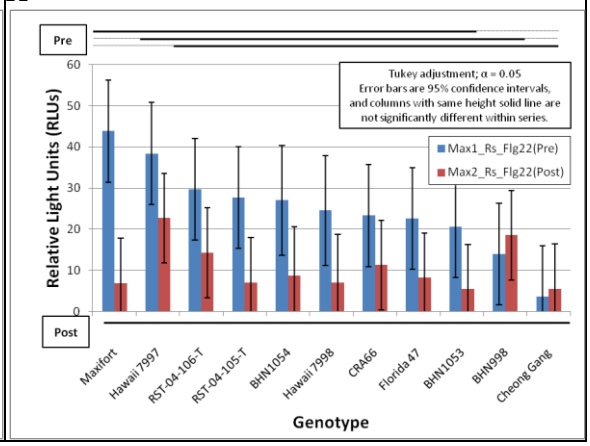
Figure 3.2. ***Comparison of ROS production modulated by inoculationwith Rs in the greenhouse study.*** **Comparisons of Pre- and Post-inoculation with *Ralstonia solanacearum* least square mean RLUs from the greenhouse 2013-14 study for four MAMP peptides and 11 genotypes. Panels compare total (A, C, E, and G) and maximum (B, D, F, and H) mean RLUs by genotype for both time points for each MAMP peptide. Repeated measures ANOVA with a Tukey adjustment was used for pair-wise comparisons, and error bars are 95% confidence intervals within each series. Columns in each series with the respective same height solid line are not significantly different from each other.**


A
Pre
Post
Tukey adjustment; α = 0.05
Error bars are 95% confidence intervals, and columns with same height solid line are not significantly different within series
Total1_Csp22 (Pre)
Total2_Csp22 (Post)
Relative Light Units (RLUs)
Hawaii 7998
Hawaii 7997
Maxifort
RST-04-106-T
CRA66
RST-04-105-T
BHN1054
BHN998
BHN1053
Florida 47
Cheong Gang
Genotype
B
Max1_Csp22 (Pre)
Max2_Csp22 (Post)
C
Total1_FlgII_28 (Pre)
Total2_FlgII_28(Post)
D
Max1_FlgII_28(Pre)
Max2_FlgII_28(Post)
E
Total1_Pa_Flg22(Pre)
Total2_Pa_Flg22(Post)
F
Max1_Pa_Flg22(Pre)
Max2_Pa_Flg22(Post)

G


Pre
Post
Tukey adjustment; α = 0.05
Error bars are 95% confidence intervals, and columns with same height solid line are not significantly different within series.
Total1_Rs_Flg22(Pre)
Total2_Rs_Flg22(Post)
Relative Light Units (RLUs)
600
500
400
300
200
100
0
Hawaii 7997
Maxifort
CRA66
RST-04-105-T
RST-04-106-T
BHN1054
Hawaii 7998
Florida 47
BHN998
BHN1053
Cheong Gang
Genotype

H


Pre
Post
Tukey adjustment; α = 0.05
Error bars are 95% confidence intervals, and columns with same height solid line are not significantly different within series.
Max1_Rs_Flg22(Pre)
Max2_Rs_Flg22(Post)
Relative Light Units (RLUs)
60
50
40
30
20
10
0
Maxifort
Hawaii 7997
RST-04-106-T
RST-04-105-T
BHN1054
Hawaii 7998
CRA66
Florida 47
BHN1053
BHN998
Cheong Gang
Genotype

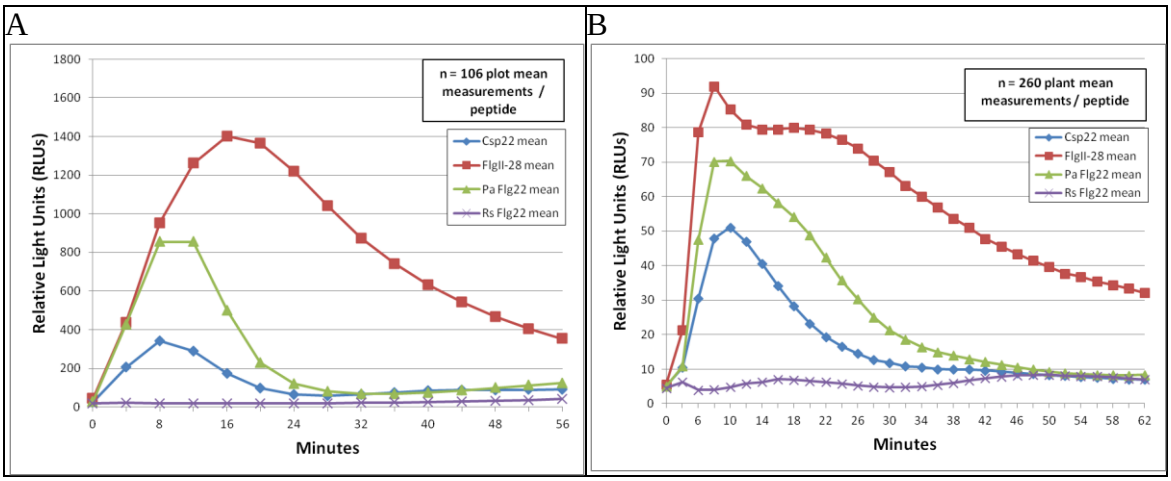


Figure 3.3. ***Comparison of average ROS production curves between the field and greenhouse studies using four peptides.*** **Overall mean relative light units (RLUs) over time measured from tomato leaf disks stimulated with four MAMP peptides (Csp22, FlgII-28, Pa Flg22, Rs Flg22) for the Jackson Co. 2013 field study (A) and the greenhouse 2013-14 study (B). Tomato leaf disks from each study were placed in 96-well plates, incubated overnight in distilled water, and then treated with a solution of luminol, HRP, and MAMP peptide. Plates were immediately placed in luminometer in order to capture photon emissions from luminescence reaction. Each well was measured every 4 minutes (Jackson Co.) or 2 minutes (Greenhouse). Scales are not comparable since experiments were performed on separate machines.**

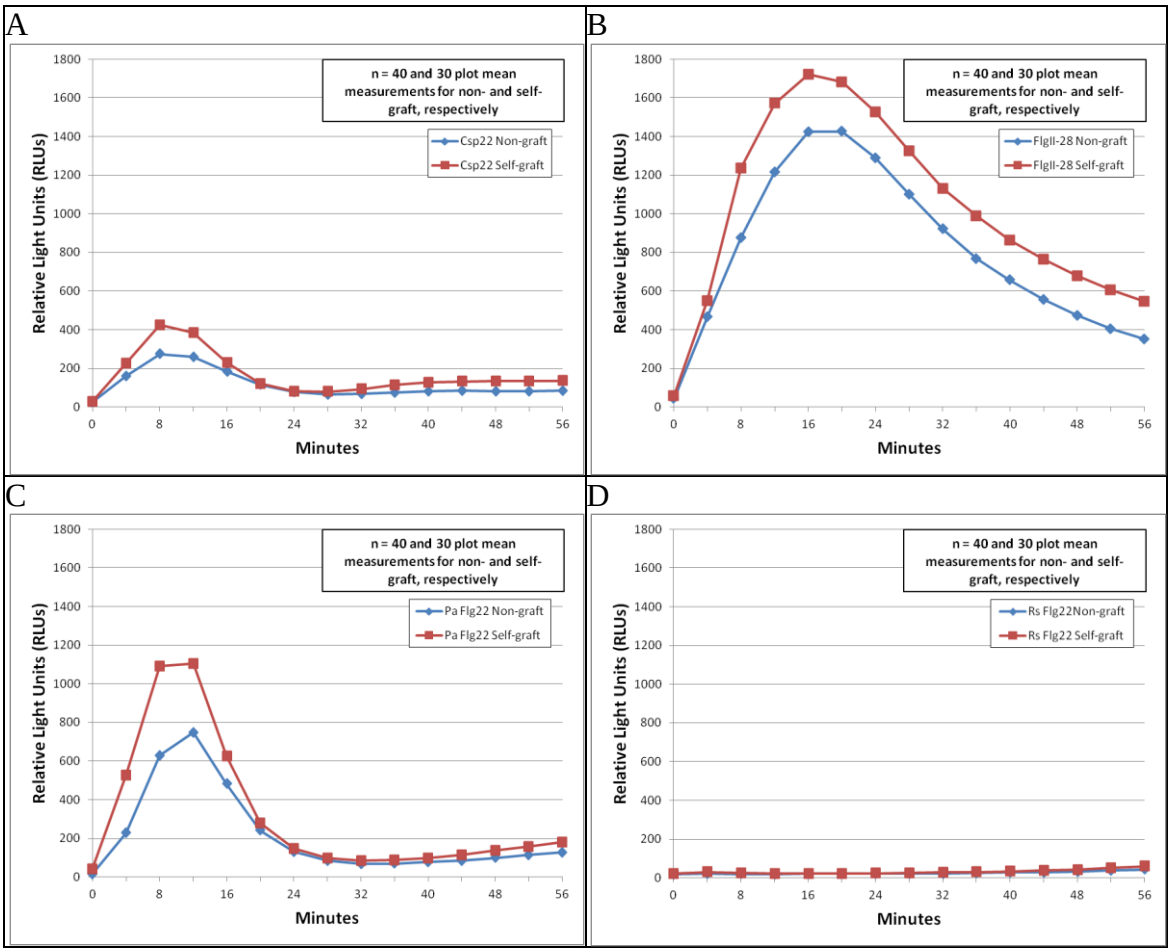
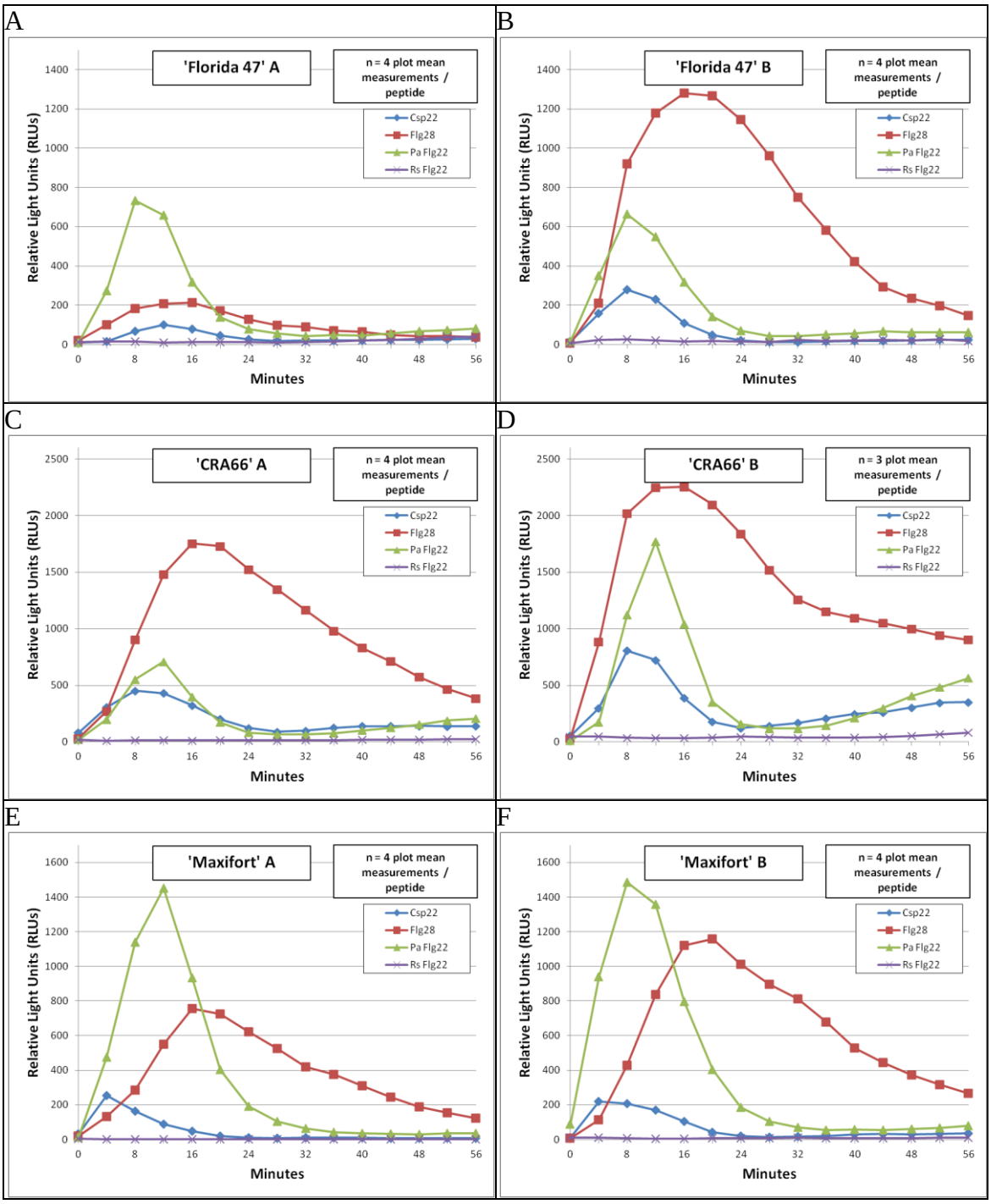
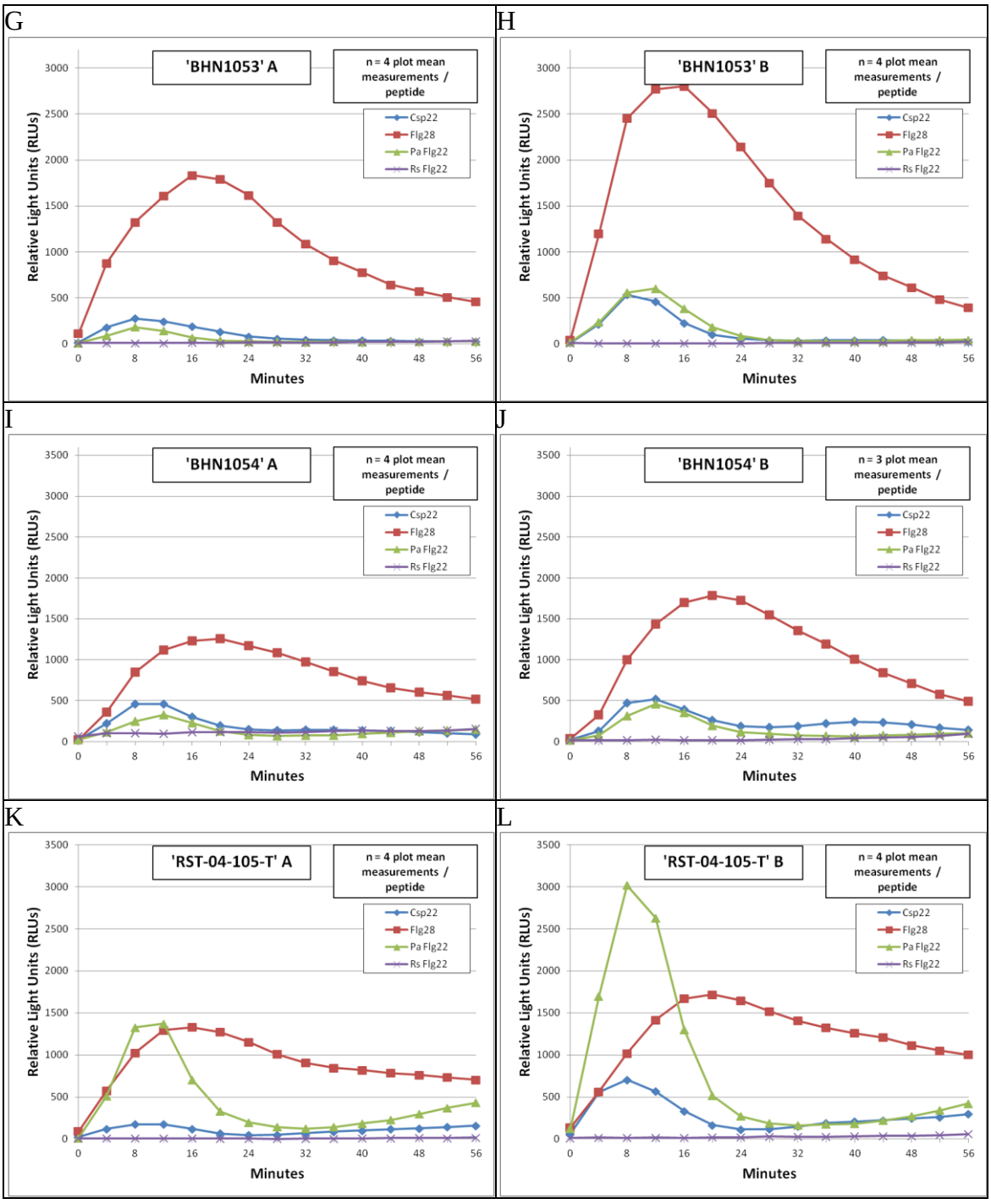
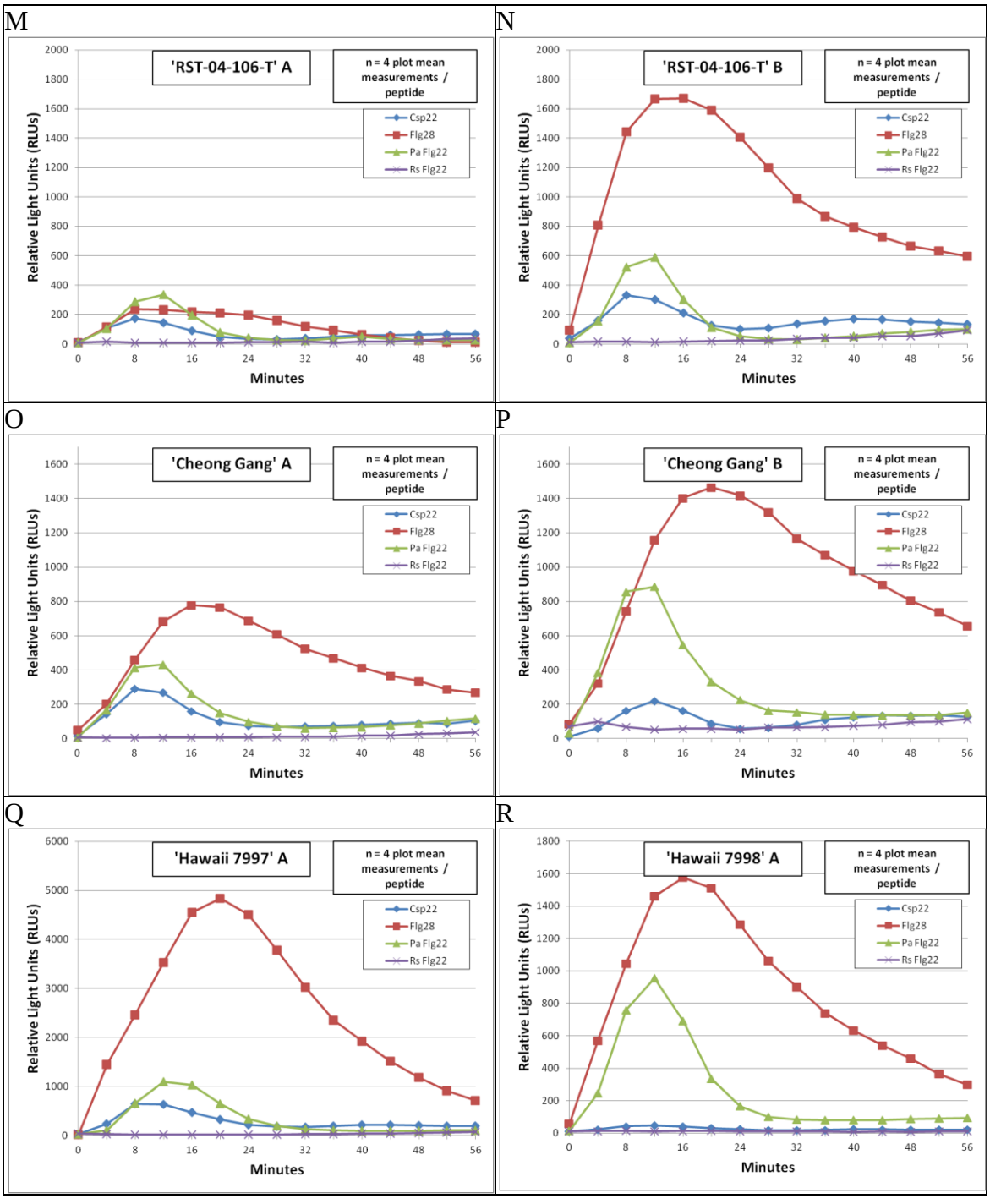


Figure 3.4. ***Effect of grafting on average ROS production curves.***
Panel of overall mean RLUs for the Jackson Co. 2013 study comparing effect of grafting treatment on ROS production over time, as measured by luminol and HRP-based assay. ROS production was stimulated by the MAMP peptides Csp22 (A), FlgII-28 (B), Pa Flg22 (C) and Rs Flg22 (D).

Figure 3.5. ***ROS production for each genotype comparing four peptides and the effect of grafting.***
Mean RLUs for the Jackson Co. 2013 study comparing effect of grafting treatment on ROS production over time, as measured by luminol and HRP-based assay. ROS production was stimulated by the MAMP peptides Csp22 (blue diamonds), FlgII-28 (red squares), Pa Flg22 (green triangles) and Rs Flg22 (purple stars). Plots were either non-grafted ("A" in name; A, C, E, G, I, K, M, O, Q, and R) or self-grafted ("B" in name; B, D, F, H, J, L, N, and P). Each row pair (A and B, C and D, etc.) is the same genotype. Due to unexpected losses during grafting process, no plots of self-grafted Hawaii 7997 or Hawaii 7998 were planted. Only curves from non-grafted treatment of those lines are presented (Q and R).


A
'Florida 47' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
B
'Florida 47' B
n = 4 plot mean measurements / peptide
C
'CRA66' A
n = 4 plot mean measurements / peptide
D
'CRA66' B
n = 3 plot mean measurements / peptide
E
'Maxifort' A
n = 4 plot mean measurements / peptide
F
'Maxifort' B
n = 4 plot mean measurements / peptide


G
'BHN1053' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
H
'BHN1053' B
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
I
'BHN1054' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
J
'BHN1054' B
n = 3 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
K
'RST-04-105-T' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
L
'RST-04-105-T' B
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes


M
'RST-04-106-T' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
N
'RST-04-106-T' B
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
O
'Cheong Gang' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
P
'Cheong Gang' B
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
Q
'Hawaii 7997' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes
R
'Hawaii 7998' A
n = 4 plot mean measurements / peptide
Csp22
Flg28
Pa Flg22
Rs Flg22
Relative Light Units (RLUs)
Minutes

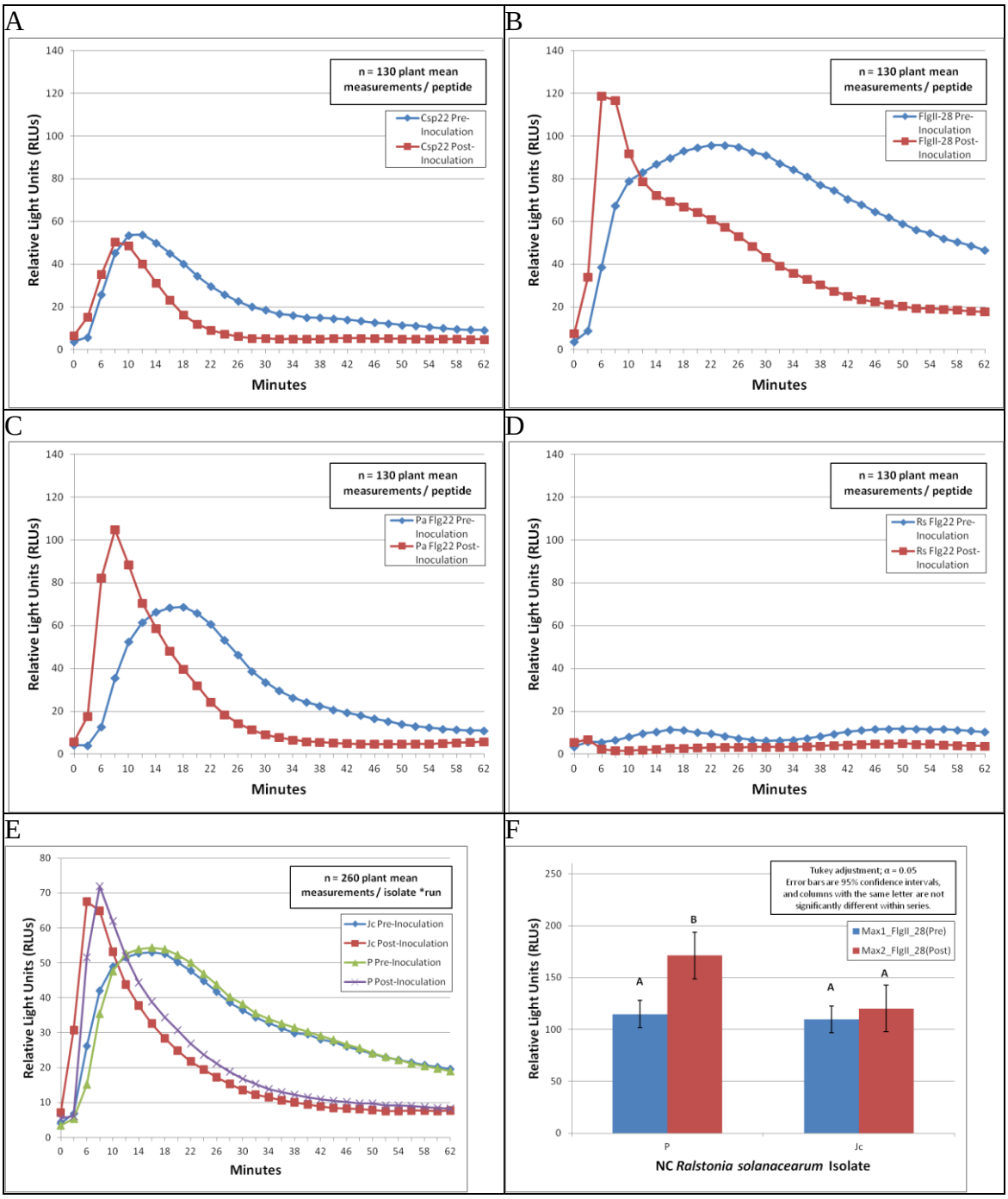
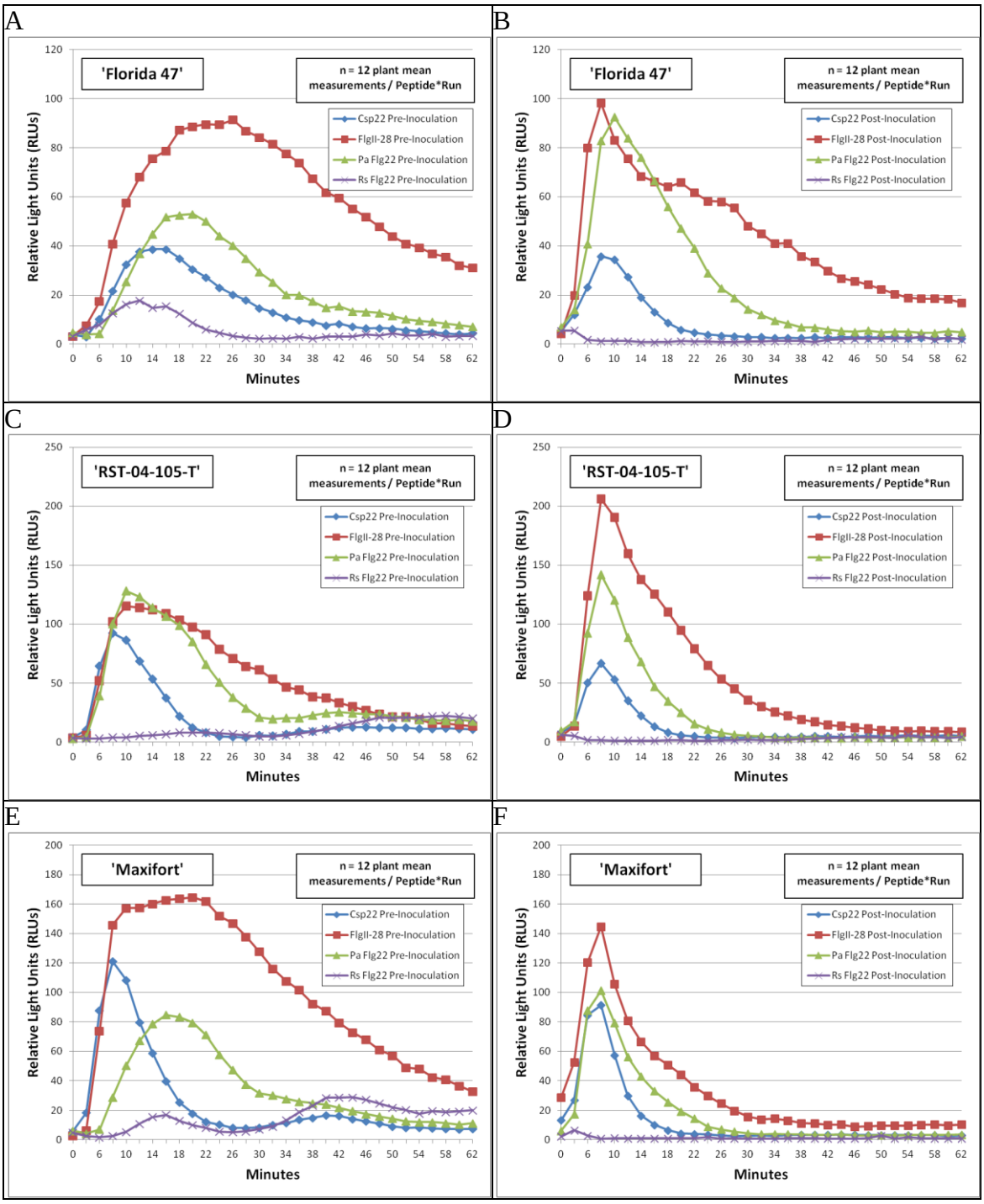
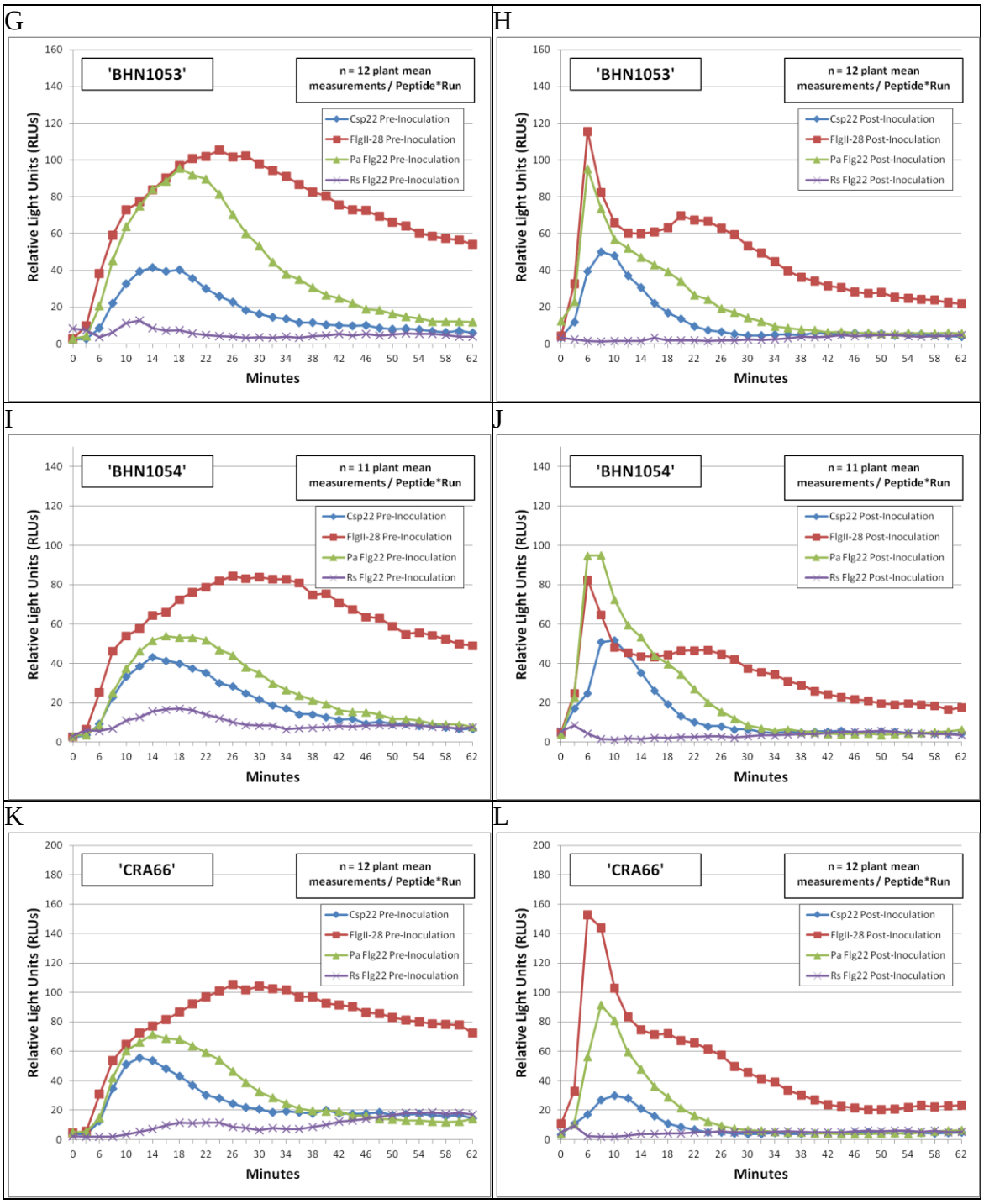


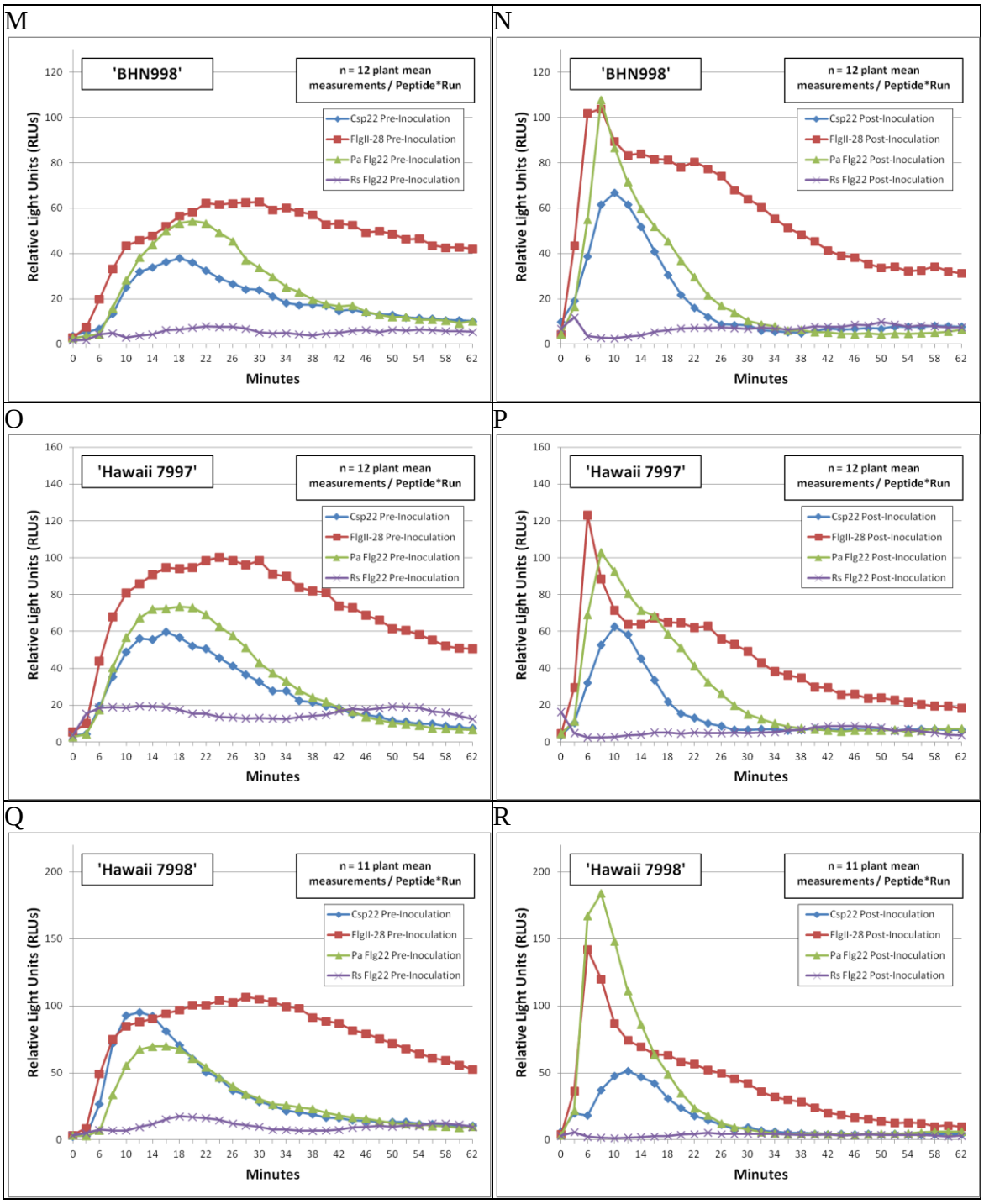
Figure 3.6. ***Effect of inoculation on ROS production curves and peak maximum.***
Panel of overall mean RLUs for the greenhouse 2013-14 study comparing ROS production over time for pre- and post-inoculation sampling points, as measured by luminol and HRP-based assay. ROS production was stimulated by the MAMP peptides Csp22 (A), FlgII-28 (B), Pa Flg22 (C), and Rs Flg22 (D). Blocking factor of treatment with Rs tomato isolates Jc and P is compared over per- and post-inoculation (E). Bar graph depicts Tukey adjusted least squares means from repeated measures ANOVA of mean maximum RLUs for FlgII-28 in Rs treatment blocks before and after inoculation (F). Error bars are 95% confidence intervals, and bars with same letter are not significantly different, within each series.

Figure 3.7. ***ROS production for each genotype comparing four peptides and the effect of inoculation with Ralstonia solanacearum.***

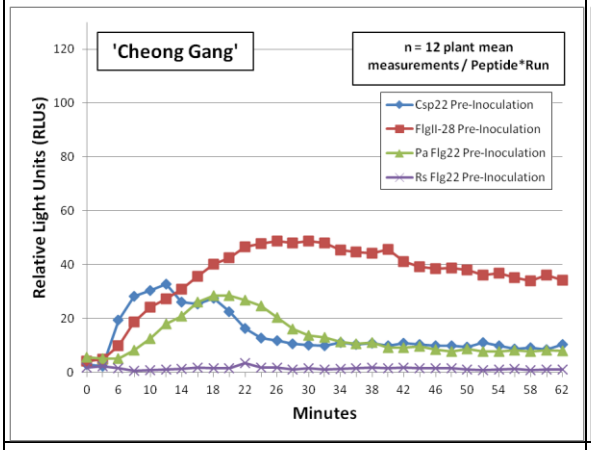
Mean RLUs for the greenhouse 2013-14 study comparing mean ROS production over time for before and after inoculation, as measured by luminol and HRP-based assay. ROS production was stimulated by the MAMP peptides Csp22 (blue diamonds), FlgII-28 (red squares), Pa Flg22 (green triangles) and Rs Flg22 (purple stars). Panels compare pre-inoculation (A, C, E, G, I, K, M, O, Q, S, and U) or post-inoculation (B, D, F, H, J, L, N, P, R, T, and V) curve profiles. Each row pair (A and B, C and D, etc.) is the same genotype.


A
'Florida 47'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
B
'Florida 47'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes
C
'RST-04-105-T'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
D
'RST-04-105-T'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes
E
'Maxifort'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
F
'Maxifort'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes

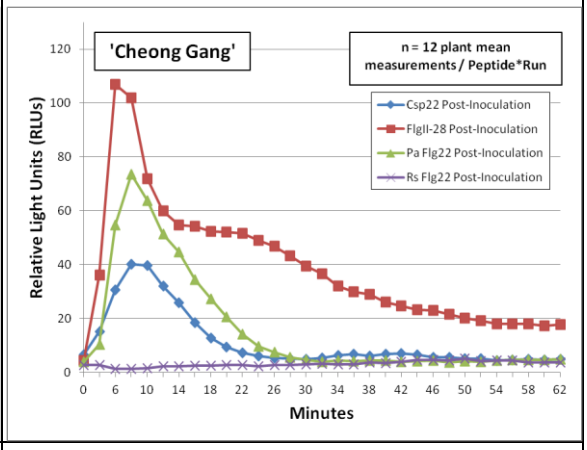

G
'BHN1053'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
H
'BHN1053'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes
I
'BHN1054'
n = 11 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
J
'BHN1054'
n = 11 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes
K
'CRA66'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
L
'CRA66'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes


M
'BHN998'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
N
'BHN998'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes
O
'Hawaii 7997'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
P
'Hawaii 7997'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes
Q
'Hawaii 7998'
n = 11 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes
R
'Hawaii 7998'
n = 11 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes

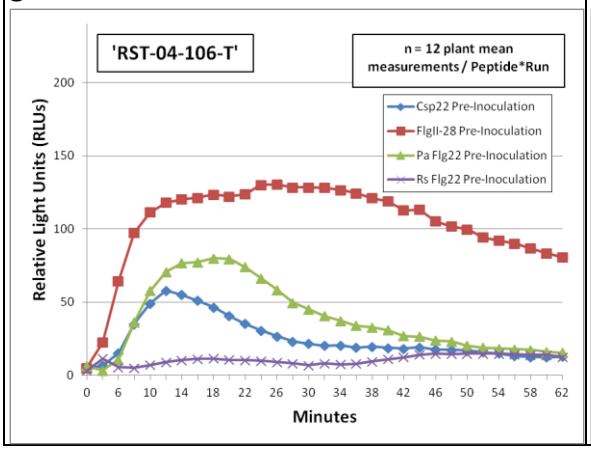
S


'Cheong Gang'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes

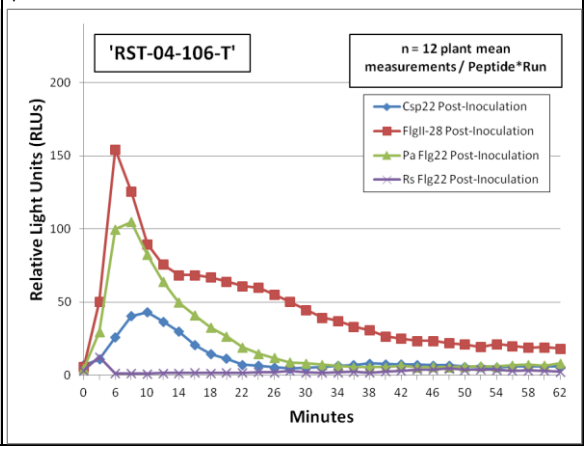
T


'Cheong Gang'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes

U


'RST-04-106-T'
n = 12 plant mean measurements / Peptide*Run
Csp22 Pre-Inoculation
FlgII-28 Pre-Inoculation
Pa Flg22 Pre-Inoculation
Rs Flg22 Pre-Inoculation
Relative Light Units (RLUs)
Minutes

V


'RST-04-106-T'
n = 12 plant mean measurements / Peptide*Run
Csp22 Post-Inoculation
FlgII-28 Post-Inoculation
Pa Flg22 Post-Inoculation
Rs Flg22 Post-Inoculation
Relative Light Units (RLUs)
Minutes

CHAPTER 4: CONCURRENT GENE DISCOVERY AND GENE-LINKED MARKER DEVELOPMENT FOR BACTERIAL WILT RESISTANCE USING A GENOME-BASED RELATIVE EXPRESSION ANALYSIS METHOD

Abstract:

Bacterial wilt (BW) of tomato (*Solanum lycopersicum* L.) is caused by the invasion of roots and colonization of host vasculature by the soil-borne pathogen *Ralstonia solanacearum* Smith (Rs). Tomato resistance to BW is controlled by multiple quantitative trait loci (QTL) that suppress pathogen growth and multiplication in the vascular tissues, but does not prevent host colonization. The strongest and most consistently observed BW resistance QTL is located on the upper portion of chromosome 6, but no specific resistance genes have been identified. Here I present an alternative method for resistance gene identification using relative expression analysis of gene targets that exhibit sequence similarity with motifs of known resistance gene receptors. I documented the physical region of the chromosome 6 QTL in the recently released tomato genome, along with many gene prediction loci in that region. Quantitative real-time RT-PCR, with an index of 6 reference loci, was used to assess 23 of these targets in two tomato genotypes with contrasting BW resistance at 3 days post inoculation (dpi) with two North Carolina tomato isolates of Rs. I also tested a putative resistance gene located on chromosome 2 using the same method. I noted that the resistance QTL on chromosome 6 spans a length of about 20 to 40 megabases (Mb), with the most significant markers clustering together in a region of about 3.9 Mb. I also identified dozens of loci with similarities to known resistance gene motifs, but overall I

detected no definitive changes in expression patterns at 3 dpi, though 11 targets were stably expressed. I did find some evidence that several loci may be down regulated in both genotypes by Rs inoculation with one of the isolates, but not the other. I also present some evidence that may suggest that the putative resistance gene on chromosome 2 may be down regulated by Rs inoculation in the susceptible genotype, but not the resistant. I discuss the value of this alternative approach utilizing genomic database information for fine mapping the resistant QTL and gene identification. This work provides foundational information uniting genomic mapping studies with the published tomato genome, as it relates to BW resistance on chromosome 6.

4.1: Introduction

Bacterial wilt (BW), caused by the soil-bacteria *Ralstonia solanacearum* Smith (Rs) (Smith, 1896) is a devastating disease of over 200 plant species in 50 different plant families around the world, including the economically important tomato (*Solanum lycopersicum* L.) (Buddenhagen and Kelman, 1964; Hayward, 1964; Hayward, 1991; Hayward, 1994; Hayward, 1995; Kelman, 1953; Moorman, 2014). Tomato resistance to BW is quantitative and polygenic in nature, and even the most resistant lines are not able to prevent colonization of the vasculature (Acosta, 1978; Grimault and Prior, 1993; Milling et al., 2009; Scott et al., 2005; Swanson et al., 2005). Sources of BW resistance in tomato have been identified from three different world collection sources—Hawaii, Philippines, and North Carolina (Scott et al., 2005; Wang et al., 1996; Wang et al., 1998). Breeding for resistance in large-fruited varieties has proved quite difficult due to genetic linkages between resistance and undesirable

traits such as indeterminate growth type and small fruit size, as well as large environmental influences upon expression of disease symptoms, especially from soil temperature and moisture (Acosta, 1978; Hayward, 1991; Mew and Ho, 1977; Scott et al., 1993; Scott et al., 2005). BW resistance is also known to be affected by race and even localized strains of Rs (Hanson et al., 1996; Hayward, 1991; Lebeau et al., 2011; Naidoo et al., 2011; Zehr, 1970). Resistance mechanisms remain unclear, although it is generally agreed upon that tomato suppress growth and multiplication of Rs in the vascular tissues (Nakaho et al., 2004; Schell, 2000). Curiously, while resistance only appears to affect growth in the stem, it has been shown that tomato detects Rs presence and initiates defensive responses while the pathogen is still penetrating through the root systems, as well as upon vascular colonization (Colburn-Clifford and Allen, 2010; Colburn-Clifford et al., 2010; Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011; Ishihara et al., 2012; Mandal et al., 2011).

Resistance genetics vary between host species. In *Arabidopsis thaliana*, resistance to BW is determined by the single recessive gene RRS1-R (Deslandes et al., 2003; Deslandes et al., 2002; Lahaye, 2004), and also helps provide dual resistance to bacterial and fungal pathogens (Narusaka et al., 2009). Resistance to BW in non-hosts or with non-pathogenic strains is related to host-induction of the hypersensitive response (HR) in some cases (Kiba et al., 2003). In other cases, resistance mechanisms appear to be differing degrees of tolerance to Rs populations (Grimault and Prior, 1994b). In tomato, despite a multitude of genetic studies around the world, the inheritance patterns of resistance remains surprisingly unclear, with some reports indicating recessive, partially dominant, single-gene partial resistance, multiple-gene additive, and various combinations of each (Acosta, 1978; Hartman and

Elphinstone, 1994; Mahir et al., 1993; Scott et al., 1993; Scott et al., 2005; Sharma et al., 2006). In the highly resistant 'Hawaii 7998', resistance may be controlled by a single major effect gene along with several smaller effect genes (Scott et al., 2005). The multitude of quantitative trait loci (QTL) mapping studies predominantly using 'Hawaii 7996' generally support this view, placing the major effect gene(s) on the upper portion of chromosome 6, and other smaller gene effects variably on chromosomes 12, 4, 10, 3, 11, 8, and 7 (Table 4.1) (Carmeille et al., 2006; Danesh et al., 1994; Mangin et al., 1999; Thoquet et al., 1996a; Thoquet et al., 1996b; Wang et al., 2000; Wang et al., 2013). There are some indications that some of the smaller effect loci appear to be related to strain-specific resistances, and the QTL on chromosome 12 was recently reported to be associated with the suppression of Rs in the stem (Carmeille et al., 2006; Wang et al., 2000; Wang et al., 2013). Additionally, another report noted that the significance of the chromosome 7 and 10 QTLs were only detectable when plants were directly inoculated in the stem, but not when inoculated via the more natural root-drench method (Danesh et al., 1994).

In the last few years one report suggested they identified a specific gene related to disease resistance, and markers for resistance (Miao et al., 2008; Miao et al., 2009), but the report has not been verified. Overall no conclusive discoveries have been made of clear resistance genes. The first tomato transcriptome study under Rs inoculation found that the resistant LS-89 substantially increased expression of 146 genes, 13 of which had significant increases in expression even 1 day post inoculation (dpi). Genes showing increases in expression included hormone signaling (jasmonic acid and ethylene) defense-related signaling pathways, and a β-1,3-glucanase, but no specific resistance genes were identified.

Also, pathogenicity-related and lignin biosynthesis genes increased in expression upon Rs inoculation (Ishihara et al., 2012). Other gene expression studies have identified increases in salicylic acid (Milling et al., 2011). An important general observation is that gene expression patterns between susceptible and resistant varieties demonstrate that Rs-induction of defense pathways is either non-existent or severely reduced in susceptible lines compared to resistant, and that resistant lines respond faster than susceptible (Ishihara et al., 2012; Jyothi et al., 2012; Milling et al., 2011).

The stimulation of reactive oxygen species (ROS) upon Rs invasion has been reported (Flores-Cruz and Allen, 2009; Mandal et al., 2011). ROS are used by plants for both signaling in the host and for direct pathogen defense (Sharma et al., 2012). The importance of ROS production for host resistance has been noted in other host-pathogen interactions (Bindschedler et al., 2006; Das et al., 2008; Davies et al., 2006; Jun et al., 2013; Mandal et al., 2008; Melillo et al., 2006; Sahebani and Hadavi, 2009). Related studies using exogenous applications of either commensal micro-organsisms or applications of silicon and other chemicals stimulates host defense systems in quite similar ways as reported for inoculation with Rs, including either jasmonic acid, ethylene, or ROS signaling pathways, providing a protective-conditioning effect against pathogen attacks, as though the plant system were being primed for defense (Algam et al., 2013; Ghareeb et al., 2011a; Hassan and Abo-Elyousr, 2013; Hyakumachi et al., 2013; Takahashi et al., 2014; Yi et al., 2008). Interestingly, a similar protective affect is observed in *Arabidopsis* and tomato when treated with boiled extracts of Rs 24 hours prior to inoculation with Rs (Takabatake and Mukaihara, 2011). The boiled extract compound has not yet been identified, but was determined to be

one or more proteinaceous compounds no smaller than 5-10 kDa that is not the Rs flagellin (for *Arabidopsis*), and hosts respond in a manner consistent with stimulation of the innate immune system (Pfund et al., 2004; Takabatake and Mukaihara, 2011).

The nature of tomato BW resistance on a mechanistic, transcriptomic, and biochemical level suggests that tomato recognizes and responds to Rs using innate immunity. The innate immune system is based upon host recognition of conserved microbe-associated molecular patterns (MAMPS) by pattern recognition receptors (PRRs) that induce MAMP-triggered immunity (MTI) (Boller and Felix, 2009; Postel and Kemmerling, 2009; Zipfel, 2009). Another layer of host defense is the highly-specific effector-triggered immunity (ETI), where plants recognize the presence or effects of pathogen-produced attack compounds. Plants generally respond to pathogen effectors with a strong oxidative burst known as the hypersensitive response (HR) (Boller and Felix, 2009). Some researchers suggest that ETI and MTI are distinct systems, with ETI generally being superior to MTI (Dangl and Jones, 2001; Jones and Dangl, 2006). Others disagree, pointing out many cases of activity similarity and overlap (Thomma et al., 2011). Several PRRs have been identified thus far, including FLAGELLIN SENSING II (FLS2), which is a membrane spanning receptor that detects a conserved region of bacterial flagellin (Boller and Felix, 2009; Chinchilla et al., 2006; Robatzek et al., 2007). FLS2 is also, consequently, a case of clear MTI activity, but is known to stimulate ETI-like responses in some cases (Thomma et al., 2011). Regardless of these differing views, it is well demonstrated that plant receptors associated with immune responses generally have similar features, including leucine-rich repeats (LRRs), nucleotide binding sequences (NBS), and receptor-like kinase activity (RLK) (Boller and Felix, 2009).

LRR domains are important for protein-protein interactions, generally providing recognition specificity, while NBS domains are generally associated with modulations in gene expression (Collier and Moffett, 2009). RLK domains are important for signal transduction in phosphorylation cascades. Most PRRs are membrane-spanning LRR-RLKs, also known as receptor protein kinases (RPKs), while many of the receptors classically associated with ETI are NBS-LRRs (Boller and Felix, 2009; Collier and Moffett, 2009; Tena et al., 2011).

In 2012, the genome sequence for tomato became publically available (The Tomato Genome Consortium, 2012), and more recently a revised and improved genome assembly has been made available (ITAG2.40). 'Heinz 1706' was the core variety used for this genome assembly, and a draft assembly of *S. pimpinellifolium* 'LA1589' is also available. Tomato is a diploid organism with a genome comprised of about 900 megabases (Mb) arranged in 12 chromosomes (2n = 2x = 24), exhibiting only about 0.6% sequence variation from *S. pimpinellifolium* (The Tomato Genome Consortium, 2012). Using a combination of bioinformatic algorithms and RNA sequence data, thousands of putative gene and gene fragment loci have been identified, and some predictions of function have been annotated based upon known sequence domains (www.Solgenomics.net).

Molecular markers are helpful for improving the efficiency and efficacy of the breeding pipeline for disease resistance traits, as well as being very important for resistance gene pyramiding in breeding lines (Foolad and Sharma, 2005; Foolad, 2007; Panthee and Chen, 2010; Robbins et al., 2010). I set out to utilize the tomato genome database to help identify resistance genes for BW in tomato, as well as concurrently develop gene-linked markers useful for marker assisted selection of BW resistance. My objectives in this study

were to: 1) Identify resistance QTL positions in the annotated sequence database; 2) Identify potential PRRs and resistance gene receptor loci for genetic screening; and 3) Assess the expression response of these loci in a highly resistant and highly susceptible tomato variety inoculated with Rs. I also wanted to try and verify the presence of the putative resistance gene on chromosome 2 (Miao et al., 2008).

4.2: Materials and Methods

Genetic material for this study was sampled from the same greenhouse 2013-14 disease resistance screening study as previously detailed in Chapter 2 of this thesis. Briefly, eleven tomato genotypes representing a range of BW resistance levels were grown in black 24-cell plastic trays in greenhouse conditions during the winter of 2013-14. 'Florida 47' was used as a susceptible control. Plants were grown in a randomized complete block design with three replications and two plants per genotype were subsamples, with Rs isolate as the blocking factor. Two plants per genotype were also grown as non-inoculation controls. Plants were inoculated with two tomato Rs isolates (Jc and P) from contrasting NC environments using the root-cut and soil drench method. Inoculated plants were incubated without watering in black plastic bags for 48-hours after inoculation. Plants were grown on heating pad providing soil heat from 6 PM to 6AM.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was used for assessment of relative expression in the highly susceptible 'Florida 47' and highly resistant 'Cheong Gang' at 3 dpi from a mixture of leaf and root tissues, and comparisons were made between genotypes and between Rs isolate treatments. The protocol used here was based

upon the manufacturer's instructions and a synthesis of the methods of similar work (Ishihara et al., 2012; Lilly et al., 2011; Rotenberg et al., 2006), as well as recommended best practices (Udvardi et al., 2008).

4.2.1: Identification of Potential Bacterial Wilt Resistance Loci

For this study, I chose to focus on the major resistance QTL on the upper portion of chromosome 6 (Carmeille et al., 2006; Danesh et al., 1994; Mangin et al., 1999; Thoquet et al., 1996a; Thoquet et al., 1996b; Wang et al., 2000; Wang et al., 2013), since it has been detected consistently over many resistance QTL studies for BW in tomato (Table 4.1). The genomic region associated with this QTL was determined by comparing the locations of the RFLP markers associated with the chromosome 6 resistance QTL from each mapping study, comparing both tightly associated markers and the range of markers associated with high LOD scores. Thankfully, six of the seven studies used the same parents to establish their mapping population. The genetic maps used by each study were based upon the first QTL mapping study from 1994 derived from 67 polymorphic RFLP markers (Danesh et al., 1994) and the previous work at chromosome and marker assemblies in comparison to the potato genome (Gebhardt et al., 1991; Tanksley et al., 1992). Subsequent studies added additional markers periodically (Thoquet et al., 1996a; Wang et al., 2000). Markers in the resistance QTL region were then identified in the physical genome using the site search engine using both the ITAG2.30 and ITAG2.40 assemblies (Table 4.2). Gene models were noted and descriptive information is detailed where marker sequences overlap.

Loci within the chromosome 6 BW resistance QTL region were identified by visual scans of the putative gene models developed by the interactive EuGene software (http://www.mia.inra.fr/en, INRA, Paris, France). Selection criteria were the presence of one or more predicted protein motifs: NBS, LRR, and/or RLK. These were identified from the annotation descriptions. Additionally, annotations such as "resistance-related protein" and similar descriptions were selected. A subset of 23 loci were chosen for relative expression analysis (Table 4.3). Since a caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) was putatively identified as a resistance gene (Miao et al., 2008), the published primer sequences were also included. For comparison, an additional primer pair for that loci was selected from the related annotation in the tomato genome, which was located on chromosome 2, since the original pair product size and optimum annealing temperature did not match closely with the rest of the target loci PCR primers. The reference genes used were Actin 41, ubiquitin 3, and elongation factor 1-alpha, and two separate primer pairs were used per gene. Primers for these reference genes have previously been quantified for qRT-PCR in tomato, and the published primer sequences were used (Rotenberg et al., 2006). qRT-PCR primers for the targets were designed using Primer 3 based upon the ITAG2.30 genome assembly, except for the previously stated putative resistance gene. Primer design criteria were: About 150 bp product size, 40-60% GC content, lengths at least 18 nucleotides, and a melting temperature of about 60 ^{o}C. The sequences of each primer pair were tested for multiple hits within the NCBI database, and only pairs that were reasonably close enough to amplify a product were chosen. Primers were synthesized using standard desalting (Invitrogen, Grand Island, NY).

4.2.2: Genetic Material and cDNA Library Creation

The greenhouse study plants were sampled for leaf and root tissue at 3 dpi. One leaflet from the 1st or 2nd leaf from the plant apex and a comparable amount of healthy root tissue from each genotype was pinched off of two plants per Rs isolate and replication and pooled in a 1.5 mL RNase-free tube, so each tube contained two biological replications. Each tube was immediately flash-frozen in liquid nitrogen and then stored at -80 ^{o}C until used in RNA extraction. Negative inoculation controls were also sampled for use as control samples in relative expression analysis.

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, #74904) according to the manufacturer's instructions. Briefly, individual frozen samples were weighed in the tube, and then ground to powder under liquid nitrogen using a clean mortar and pestle rinsed with RNase-free distilled water and sterilized in autoclave at 121 ^{o}C for 1 h. Sterile mortar and pestle were stored at -20 ^{o}C until used. Frozen powder was placed in a clean, RNase-free 1.5 mL tube and 600 uL of buffer RLT (with 5% β-mercaptoethanol) was added and vortexed. Tissue lysate was added to QIAshredder columns, centrifuged, and supernatant was transferred to a new RNase-free tube without disturbing pellet. 0.5x volume of ethanol was added and mixed by pipette. The solution was then added to a silica column and washed. On column DNA digest was performed using RNase-Free DNase Set (Qiagen, #79254) according to the manufacturer's instructions. DNase solution was added to each column and incubated at room temperature for at least 15 minutes. After final washes, the RNA was eluted with 30 uL RNase-free water into clean 1.5 mL tubes (kit-provided) and stored at -80 ^{o}C.

RNA concentration was quantified using a nanodrop spectrophotometer (Thermo Scientific) and adjusted to 100 ng/uL total RNA with RNase-free water. RNA integrity was assessed by visualizing 10 uL of 100 ng/uL stock on a 1.2% agarose formaldehyde denaturing gel with 10 uL GelRed (10,000X; Biotium, #41003, Hayward, CA) per 100 mL gel. Samples were mixed with 5x loading dye (RNase-free) in a 4:1 sample:dye ratio, incubated in 65 ^{o}C water bath for 2 minutes, cooled on ice, and then loaded into gel matrix. Gel was run at 100 v for about 2 hours. Gels were visualized using UV light, and sample integrity was determined by assessing band clarity and ratio of ribosomal bands.

cDNA libraries were created using first-strand synthesis with AffinityScript qPCR cDNA synthesis kit (Agilent Technologies, #600559, Santa Clara, CA) according to the manufacturer's instructions. For each reaction, 500 ng of total RNA was mixed in a 20 uL reaction with RNase-free water, 2x reaction mix, oligo dT mix, and RT/block enzyme. PCR reaction protocol was: 5 minutes at 25 ^{o}C, 15 minutes at 42 ^{o}C, 5 minutes at 95 ^{o}C, and held at 4 ^{o}C in thermocycler (T100, BioRad). Two independent cDNA reactions were synthesized, combined in order to mitigate variation due to cDNA synthesis, and diluted to 2.5 ng/uL. The final library mixture for each sample was divided into two aliquots and one was stored at -80 ^{o}C as a backup to minimize freezing degradation.

4.2.3: qRT-PCR Reactions

qRT-PCR was used to quantify relative expression changes for the targets using the BrilliantII SYBR Green qPCR master mix (Agilent Technologies, #600828) according to the manufacturer's instructions in 96-well format. One cDNA sample was used per reaction set,

which contained all 25 target primers and 6 reference gene primer sets with two technical replications for each primer pair. Two no-primer controls were also included. Briefly, 2x SYBR Green reaction mix, RNase-free water, reference dye (diluted 1:500), and 2.5 ng of cDNA sample were added together and mixed by pipette. Then, 48.5 uL of mix was transferred to reaction tubes and 1.5 uL of forward/reverse primer mix was added for a final primer concentration of 300 nM. The solutions were mixed by pipette and 25 uL of the final mixtures were transferred to the technical replication tubes. The reaction set was centrifuged briefly to collect any drops and all bubbles were removed. Reactions were recorded using the Mx3005P qPCR thermocycler and software (Agilent Technologies—Stratagene). The reaction protocol was: 10 minutes at 95 ^{o}C for initial denaturing; 40 cycles of 30 seconds at 95 ^{o}C, 30 seconds at 55 ^{o}C, measure reaction tubes, 90 seconds at 72 ^{o}C; 7 minutes at 72 ^{o}C for final elongation; melting curve analysis from 55 to 95 ^{o}C. Representative samples of qRT-PCR products were visualized on a 1.0% agarose gel with 10 uL GelRed per 100 mL gel in TAE buffer to determine size and number of amplification products. Products were run at 80 v for about 1 hour.

4.2.4: Expression Data Processing and Determination of Fold-Expression Changes

The data from each reaction set was exported from Mx3005P software as the raw output normalized to reference dye (Rn). As recommended by (Udvardi et al., 2008), these files were assessed using the program LinRegPCR (http://www.hartfaalcentrum.nl/index.php?main=files&sub=LinRegPCR) in order to determine the optimum window-of-linearity of the log-linear phase of the amplification curve

(Ruijter et al., 2009). The reaction efficiencies and threshold cycle (C_T) values were calculated from the software determined baseline for each reaction tube. The common baseline calculation was used for most reactions, with occasional individual baseline calculations used to improve the similarity of technical replications. The average amplification efficiencies for each primer pair over all samples and conditions was used for all future analyses.

The stability of the reference loci was tested using the Excel macro program BestKeeper-1 (http://www.gene-quantification.com/bestkeeper.html), which uses pair-wise correlations for the analysis (Pfaffl et al., 2004). All six reference gene primer pairs were validated as stable over all samples and conditions. These were then used for calculation of a reference index for use in determining fold-changes of expression of target loci using REST (Relative Expression Software Tool) 2009 (Qiagen and M.W. Pfaffl) (Pfaffl, 2001; Pfaffl et al., 2002; Vandesompele et al., 2002). The program default settings were used, except that the number of calculation iterations was increased from 1000 to 4000 since the user guide suggested it may improve the accuracy of the calculated values. Calculations of fold-change were determined for: Overall samples, Rs isolate within each genotype, and each genotype within Rs isolate. The calculation algorithms test the hypothesis that variation between sample and control group is due to random chance.

4.3: Results

In order to develop gene-linked markers, I used the publically available sequence annotations in the tomato genome database (www.Solgenomics.net) to guide identification of

potential resistance loci. I selected gene predictions that exhibited features often related to detection of pathogens and defense-related proteins from within the large BW resistance QTL on chromosome 6.

4.3.1: Potential Resistance Loci

I observed that the major resistance QTL on chromosome 6 of the tomato genome spanned a length of about 21.5 to 44.2 Mb (ITAG2.40), depending on if the upper marker border is CD67 or the adjacent *Cf-2*, respectively. The survey of gene prediction models in previous studies have indicated that the strongest resistance associations occurred with four RFLP markers CT184, TG118, TG73, and TG153, synthesizing all seven of the studies together (Table 4.1, "Best Marker") (Carmeille et al., 2006; Danesh et al., 1994; Mangin et al., 1999; Thoquet et al., 1996a; Thoquet et al., 1996b; Wang et al., 2000; Wang et al., 2013). These markers were found to cover a region of 3.9 Mb, spanning positions 36,066,281 to 39,925,555 base pairs (bp) (ITAG2.40) (Table 4.2). Several marker locations were not able to be located in the tomato genome annotations, including CP18. CP18 was previously mapped right in the middle of the group of highly significant marker associations (Danesh et al., 1994; Thoquet et al., 1996a; Wang et al., 2000). I noted that each marker physically located within the genome also overlapped to some degree with a gene model prediction, which could be important for future studies.

Within that large resistance QTL, I identified more than 50 loci that matched the selection criteria (data not shown) scattered throughout the region. Of those identified, I selected 23 for relative expression analysis (Table 4.3), and tested a chromosome 2 locus for

a putative resistance gene (Miao et al., 2008). The chromosome 6 targets selected spanned a range of 10.9 Mb, spanning positions 29,721,918 to 40,629,337 bp. This region covered a large portion of the upper end of the resistance QTL region, including full overlap of the region containing the most significant resistance associated markers. Within this region, I identified targets that shared sequence similarity with known protein motifs such as: Coiled-coils (CC)-NBS-LRR, many NBS-LRR and LRR resistance-related proteins motifs, F-box/LRR repeats, a clathrin heavy chain, an autophagy protein, and disease resistance-responsive family motifs.

4.3.2: Relative Expression Analysis

I tested two tomato genotypes with contrasting BW resistance levels under inoculation with two NC tomato isolates of Rs for changes in relative expression compared to non-inoculated control plants. BestKeeper-1 analysis revealed that all 6 reference loci were stable over all reaction conditions and genotypes (Table 4.4). Also, 11 of the 25 target loci exhibited stable amplification, 2 targets may or may not be stable, and 12 were too variable for reliable analysis. Overall, I saw no significant changes in expression between isolates or between genotypes ($p > 0.05$). Several reliable primers showed significant changes in expression under certain conditions. Primer set Q3-Q4short exhibited a low significant change in expression in 'Florida 47' only, showing a reduction in regulation by a factor of 0.394 when inoculated with Rs. Additionally, primer sets NC-BW-2607B and NC-BW-3578B exhibited a moderately significant reduction in expression by a factor of 0.775 and 0.783, respectively, over both genotypes when inoculated with isolate P. Other targets

mathematically exhibited significant differences, but they were not found to have consistent amplification.

4.4: Discussion and Conclusions

I set out to identify and quantify gene expression potentially involved in tomato resistance to BW, leaveraging the recently released tomato genome database to aid in QTL localization and target selection. The relative expression levels of each target were compared in a highly susceptible and a highly resistant tomato genotype at 3 dpi (Chapter 2). The qRT-PCR results were assessed with a statistically rigorous approach using a comparative index of 6 reference loci and amplification efficiency corrected C_T values.

The survey of the chromosome 6 resistance QTL revealed over 50 loci that met the selection criteria, but I was only able to test 23 of them. The target identification and expression analyses highlighted several important observations. First of all, a vault of valuable bioinformatic information is available for the molecular geneticist and breeder for identifying testable genetic loci for fine mapping resistance or any other QTL related trait with relatively little effort. Utilizing this resource was very helpful for "gene discovery" of testable loci. The QTL mapping studies have provided a valuable foundation of resistance-associated markers, and now those physical sequences and spatial locations are available. The publishing of the tomato genome (The Tomato Genome Consortium, 2012), as well as the more recent revised sequence assembly has made the database a valuable tool for designing novel and productive gene identification studies.

Secondly, while a lot of information is available at the researcher's fingertips, careful thought must be given to timely and cost-efficient assessment methods. This research used a qRT-PCR approach because of its high level of detection sensitivity, and because any positive findings would then have a gene-linked marker already developed. In retrospect, however, in order to effectively and efficiently assess the diversity of expression of the identified loci in time, over Rs diversity, and in multiple contrasting genotypes, a higher throughput method is desirable. This would also allow tracking of the polygenic defense signaling pathways, which was not practical in this study. Assessments like qRT-PCR would be better for secondary validation of results from a high throughput sequencing or microarray platform, as has been done previously (Ghareeb et al., 2011a; Ishihara et al., 2012; Rosli et al., 2013).

Thirdly, the difficulty of trying to separate the resistance genes from the genetics of small fruit size and indeterminate plant growth suggests that genes determining all three characteristics may reside side-by-side in the approximately 20 to 40 Mb region cataloged here. QTL mapping studies always have to balance resolution with available resources and time. Thus, a "wash, rinse, and repeat" method of fine mapping using more plants, greater numbers of markers, and additional generations of recombination events becomes more and more difficult the finer one gets to the target. Here I propose an alternate approach using the newly developed tools of the genomics revolution in tomato and a little investigative prowess. While major genes related to resistance may not be annotated in the genome, it is worth assessing the defense-related targets already discovered. The classic fine-mapping techniques will be needed if it can be determined that the major BW resistsance loci have not

already been discovered *in silico*, albeit unknowingly, by the tomato genome annotation efforts.

Lastly, during my investigations of the BW QTL region, it was noted that many single nucleotide polymorphic markers are already mapped to the genome, and may be useful in concert with the RFLP maps already established. Additionally, I compared the target loci sequences to the NCBI database using BLAST and found multiple loci that exhibited high sequence similarity with predicted late blight (LB) and tobacco mosaic virus (TMV)-related resistance genes (data not shown). I was not looking for these genetic aspects, but it would be interesting to see if there might be loci related to resistance mechanisms of these genes located in the same place as BW resistance genes. Perhaps some of the loci may be involved in resistance responses to all three diseases.

Since previous work had shown elevated expression levels of defense signaling genes within the first few days following inoculation (Ishihara et al., 2012), I assessed the material at 3 dpi. First of all, I noted variation of amplification stability among the transcript targets. Since I was careful to test each primer pair for duplicate amplification loci in known tomato sequences, this report may suggest that these genes are stably expressed in either leaf or root tissues of tomato. Overall I did not detect clear changes in gene expression for the reliable targets, which may be related to the time of sampling or the loci involved. It is also possible that the genetic components for BW resistance are expressed in a vascular tissue-specific manner. It is unclear at what level the host expresses the genes at the end of the signaling cascades that actually provide the resistance, if that is the mechanism involved. It may also be that the resistance genes are in the group of loci not yet tested, or that they yet remain

undefined by computer algorithms. It has been suggested that the importance of portions of the chromosome 6 QTL shifts over time, with the upper portion being more important initially and then shifting more towards the lower portion (Mangin et al., 1999; Wang et al., 2000). Most of the tested target loci in this work were focused in the upper portion of the QTL, with some overlap to the lower portion. It may be that some of the targets may not have had enough time to express those changes, although most of the targets were concentrated in the upper protion of the QTL. Future experiments should assess this region in a time series. I did uncover some evidence that may suggest that the previously reported CCoAOMT gene on chromosome 2 (Miao et al., 2008) may be suppressed by Rs in 'Florida 47' but not in 'Cheong Gang'. It should be noted that no BW resistance QTL has been reported on chromosome 2, but also the resistance source used in the QTL mapping studies was derived from 'Hawaii 7996' (*S. lycopersicum*) while (Miao et al., 2008) used several other lines including LA 1364 (*S. peruvianum*), which may be the reason for the discrepancy.

I also found some evidence that inoculating tomato with NC Rs isolate P (from Pender Co., NC) may cause some reduction in expression of two other loci, while isolate Jc (from Jackson Co., NC) does not. NC-BW-2607B amplifies a portion of a putative gene that is predicted to code an autophagy protein 5-like product. Autophagy is the predominant system by which proteins are degraded in the vacuole and lysosomes of plants and animals, and is related to being marked with a ubiquitination signal (Yoshimoto et al., 2004). Autophagy proteins are coded by *atg* genes, and are known to play a role in the apoptotic process (I.e. programmed cell death) in animals. Autophagy protein 5 is specifically involved in formation of double membrane vesicles, including the membranes of positive-strand RNA

viruses (Guévin et al., 2010). The NC-BW-3578 target has sequence homology with a predicted LB resistance protein R1A-10-like product in potato (*Solanum tuberosum*) (NCBI BLAST search).

BW disease is a very dynamic, complicated pathosystem (Schell, 2000), and to get a good grasp of the range of interactions and modulating factors, qRT-PCR will be helpful for specific verification of identified loci, but may not be the most ideal testing platform for discovery of a few "needles in the haystack," so to speak. The QTL mapping studies and sequenced genome have greatly reduced the range of possible resistance loci locations, and should be taken advantage of. This work provides a novel approach to BW resistance gene identification in tomato utilizing those resources in concert, along with documentation of the physical location of the chromosome 6 BW resistance QTL, gene expression data, and descriptive information for 23 loci in that region. I also provide preliminary evidence that may reinforce the validity of the recent report of a resistance gene being identified on chromosome 2 (Miao et al., 2008), as well as providing database information about that loci.

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CHAPTER 4: TABLES AND FIGURES

Table 4.1. ***Summary of the bacterial wilt resistance QTL mapping studies in tomato.***
Summary of the bacterial wilt (BW) resistance quantitative trait loci (QTL) mapping studies in the tomato. Source includes cross of contrasting parents (resistant x susceptible). The QTLs were mapped to portions of chromosomes (Chr.) and upper, lower, and strongest marker boundaries are noted. Markers were either restriction fragment length polymorphisms (RFLP) or simple sequence repeats (SSR) from bacterial artificial chromosomes.

Source	Population Type; number	Chr.	Percent of Wilt Variation	Marker Names			Marker Type	Notes
				Upper Border	Lower Border	Best Marker		
Danesh et al., 1994								Used Rs strain UW364 (R1 bv 4, China)
L285 x C286	F2; 71	6	30.2-77.3	TG118	TG365	CT184	RFLP	
		7	24.4	TG51b	TG135	--	RFLP	Only significant for shoot inoculation
		10	38.2-24.6	TG230	TG285	TG255b	RFLP	
Thoquet et al., 1996a								Used Rs strain GMI8217 (R1, French West Indies)
Hawaii 7996 x WVa700	F2; 200	4	6.6-10.9	CD49	TG464	TG268	RFLP	
		4	6.1-10.4	TG464	GP165	GP165	RFLP	
		6	17.7-21.4	TG178	CP18	TG118	RFLP	
		11	10.4	--	--	GP162	RFLP	
Thoquet et al., 1996b								Used Rs strain GMI8217 (R1, French West Indies)
Hawaii 7996 x WVa700	F2 clones, F3	3		CP6	O5	GP226	RFLP	
	families; 200, 20x200	4		CD73b	TG464b	K12?	RFLP	
		4		GP165	GP165	GP165	RFLP	
		6		<TG118	CT109	TG118, TG73	RFLP	
		8		CD40	GATA1		RFLP	
		10		PAL2	CP105		RFLP	
		11		D6b	O10	O10	RFLP	Specific to F2 clones
Mangin et al., 1999								Used Rs strain GMI8217 (R1, French West Indies)
Hawaii 7996 x WVa700	F3; ~3500	6a						Cf-2 (6a) may contain multiple strain specific
				Cf-2	CP18	TG118	RFLP	resistance traits, while 6b is more general
		6b		CP18	TG162	TG73, TG406	RFLP	Visible in younger plants; shifts to 6a over time
Wang et al., 2000								Used Rs strain Pss4 (R1 bv3, Taiwan)
Hawaii 7996 x WVa700	F3 families;	6		CF-2	TG406	TG118-TG73	RFLP	
		12		Ct120	K4a	TG564	RFLP	Strain specific locus
Carmeille et al., 2006								Used Rs strain JT516 (R3 phy II, Reunion Is.)
Hawaii 7996 x WVa700	F2:3, Recombinant	3		TG515	K4d	--	RFLP	
	inbred lines	4		CD49	TG464	--	RFLP	Only detected in hot season
		6	7.1-17.2	TG153	TG240	TG153	RFLP	
		8		CD40	CT135	--	RFLP	Only detected in hot season
Wang et al., 2013								R1 phy I and R2 phy II (Indonesia, Philippines, Taiwan, Thailand, and Reunion)
Hawaii 7996 x WVa700	RIL; 188. Same	3						Weak loci
	mapping population	6		SLM6-47	SLM6-94		SSR	Strong over multiple strains
	as Carmeille et al.,	12						Related to suppression of internal multiplicaiton
	2006			SLM12-9	SLM12-2		SSR	of the pathogen in the stem.

Notes:
Hawaii 7996: Solanum lycopersicum (Lycopersicon esculentum)
WVa700: Solanum pimpinellifolium (Lycopersicon pimpinellifolium)
L285: L. esculentum var. cerasiforme

Table 4.2. ***Detailed loci information for chromosome six bacterial wilt resistance QTL markers.***
RFLP markers associated with bacterial wilt resistance QTL on chromosome 6. Physical locations are noted in ITAG2.30 and ITAG2.40 assemblies. Locations were determined by locating each sequence in the available tomato genome assembly (www.Solgenomics.net). Any sequence-linked gene models that marker sequences overlap with are noted, along with any functional information available. The marker Cf-2 is the coding sequence of a tomato resistance gene against *Cladosporium fulvum* (Dixon et al., 1996).

Marker	Type	Chr.	Sequence-Linked Loci	Sequence Length (bp)	ITAG2.3 Start (bp)	ITAG2.4 Start (bp)	Source	Description
Cf-2	RFLP	6	Solyc06g008300.2	3,403	2,161,344	2,164,746	Tomato genome	Resistant to races of *Cladosporium fulvum* (*Passalora fulva*). Dixon et al., 1996
CD67 ~	RFLP	6	Solyc06g035760.2	1,788	21,596,705	24,907,105	Tomato genome	Cytochrome P450
TG178	RFLP	6	--	--	--	--	Tomato-EXPEN 2000	Position: 10.00
TG232	RFLP	6	--	--	--	--	Tomato-EXPEN 1992	Position: 10.90
TG325	RFLP	6	--	--	--	--	Tomato-EXPEN 2000	Position: 22.00
TG118 (Fwd)	RFLP	6	Solyc06g053320.2	10,547	32,556,081	36,066,281	Tomato genome	Polyadenylate-binding protein
TG118 (Rev)	RFLP	6	" "	" "	32,557,923	36,068,123	Tomato genome	" "
TG153	RFLP	6	--	--	--	--	Tomato-EXPEN 2000	Position: 33.00
CP18	RFLP	6	--	--	--	--	Tomato-EXPEN 1992	Postion: 35.00
CT184	RFLP	6	Solyc06g061150.2	12,670	35,545,672	39,155,772	Tomato genome	Identical to marker CT272. AP-2 complex subunit mu
TG73 (Fwd)	RFLP	6	Solyc06g063200.1	3,425	36,314,222	39,924,322	Tomato genome	Glutamate receptor-like gene associated with plant development and defense
TG73 (Rev)	RFLP	6	" "	" "	36,315,455	39,925,555	Tomato genome	" "
TG240 (Fwd)	RFLP	6	Solyc06g065390.2	3,344	37,196,678	40,806,778	Tomato genome	50S ribosomal protein L21
TG240 (Rev)	RFLP	6	" "	" "	37,198,457	40,808,557	Tomato genome	" "
TG406 (Fwd)	RFLP	6	Solyc06g066640.2	1,407	38,268,123	41,878,223	Tomato genome	Photosystem I reaction center subunit VI-1, chloroplastic
TG406 (Rev)	RFLP	6	" "	" "	38,269,235	41,879,335	Tomato genome	" "
TG365 (Fwd)	RFLP	6	Solyc06g068680.2	5,485	38,968,134	42,578,234	Tomato genome	Respiratory burst oxidase-like protein
TG365 (Rev)	RFLP	6	" "	" "	38,968,990	42,579,090	Tomato genome	" "
TG162 (Fwd)	RFLP	6	Solyc06g073330.2	7,629	41,577,022	45,187,122	Tomato genome	Lysyl-tRNA synthetase
TG162 (Fwd)	RFLP	6	" "	" "	--	45,187,171	Tomato genome	" "
CT109	RFLP	6	Solyc06g074820.2	2,351	42,749,561	46,359,661	Tomato genome	Aquaporin-like protein
Notes:	CC: Coiled coil; LRR: Leucine rich repeat; NBS: Nucleotide binding sequence							
	~ Putative marker identity in tomato genome.							

Table 4.3. ***Loci information and primers for relative expression analysis targets.***
Target loci in the bacterial wilt resistance QTL on chromosome 6 and select loci from chromosome 2 were selected for relative expression analysis. Putative gene sizes and descriptive information were compiled from both the annotated tomato genome (www.Solgenomics.net) and BLAST (NCBI) searches. The physical locations in ITAG2.30 and ITAG2.40 are presented for comparison, since the loci identification and primer design were completed prior to release of ITAG2.40. Primer sequences for qRT-PCR amplification of cDNA are shown. Reference gene information appended at bottom.

Primer	Use	Chr.	Target Loci	Target Length (bp)	ITAG2.3 Start (bp) *	ITAG2.4 Start (bp) *	Source	Description	Forward	Reverse
Q3-Q4short	Target	2	Solyc02g093270.2	1404	54,179,675	54,178,272	Tomato genome	Caffeoyl-CoA O-methyltransferase family 3	CCCTGCACTACCTGTTCTTG	CCATTCCATAGGGTGTTGTC
Q3-Q4	Target	2	NCBI: EU161983	729 ~	N/A	N/A	Miao et al., 2008	Solanum lycopersicum caffeoyl-CoA O-methyltransferase (CCoAOMT) mRNA	ATGGCAAGCAATGGAGAAA	TTAACTGATGCGTCGGCAA
NC-BW-2607B	Target	6	Solyc06g043140.2	1094	26,072,237	29,721,918	Tomato genome	Autophagy protein 5	AGGAGGAGGAACTGAAGCAC	CAGGTACCCAATTCGAGGAG
NC-BW-2605B	Target	6	Solyc06g043110.1	165	26,051,734	29,743,350	Tomato genome	Disease resistance-responsive family protein	TGGCGTTTACCTCCATGATG	GAATGAATTTCCATGAATCCGC
NC-BW-2601B	Target	6	Solyc06g043080.2	2180	26,011,928	29,781,141	Tomato genome	Disease resistance-responsive family protein	TTAGGCCCTGATGGATTGAG	CGATCACGACAAACAACACC
NC-BW-2842B	Target	6	Solyc06g048910.1	5006	28,429,688	31,939,888	Tomato genome	CC-NBS-LRR, resistance protein	GGAAGTCTCCACTCAAGCAC	GCTGACTGGCCTTCTATCAA
NC-TY-3103B	Target	6	Solyc06g051310.2	11290	34,542,438	34,542,438	Tomato genome	Clathrin heavy chain	CATTGGCTTTGCCTGCTC	TGCCTGCCCAGTTCTGTT
NC-BW-3362B	Target	6	Solyc06g054320.1	555	33,626,366	37,136,566	Tomato genome	Disease resistance response	GATGGCTTCACAGAGCGAAC	CCAGTGCCTCCAACAATAGG
NC-BW-3369B	Target	6	Solyc06g054430.1	279	33,695,819	37,206,019	Tomato genome	F-box/LRR-repeat protein 4	ACGCTTGTGCACTTGTCTG	TACAAGTGGAATCGGGAGAG
NC-BW-3370B	Target	6	Solyc06g054440.2	5696	33,700,999	37,211,199	Tomato genome	F-box/LRR-repeat protein 14	GACACATGTAGGGCTGAGGT	ATGAAGCCTGCGTCAGTTAG
NC-BW-3410B	Target	6	Solyc06g059810.1	6550	34,103,561	37,713,661	Tomato genome	F-box family protein	GAAGCACCACCTATTCCATC	CCATTTATCCTCTCCGACCA
NC-BW-3578B	Target	6	Solyc06g062440.2	3814	35,784,240	39,390,527	Tomato genome	CC-NBS-LRR, resistance protein	GTGAAGGTTGCTCTCTGCATC	GCCATATCACCCAAATCCTG
NC-TY-3670B	Target	6	Solyc06g064680.1	2286	36,704,723	40,314,823	Tomato genome	NBS-LRR, resistance protein	GAAGAACTCTGCCTCAGTGTTG	CTTGGGGGGATCTCCTTGATC
NC-BW-3671B	Target	6	Solyc06g064690.1	690	36,715,264	40,325,364	Tomato genome	NBS, resistance protein fragment	CCACGGTGATCCTTATTCTC	CTCTCAAACCTGCTTCCAGT
NC-BW-36721B	Target	6	Solyc06g064710.1	375	36,721,924	40,332,024	Tomato genome	NBS-LRR resistance protein-like protein	CGTCACAAGTGTCGAGTTCC	TAGACAGCTCCTCAAGCACA
NC-BW-36725B	Target	6	Solyc06g064720.1	2256	36,725,787	40,335,887	Tomato genome	NBS-LRR, resistance protein	TGCATTTGATGGAAGTCTCG	GGTGTAGGTGTGATATCCACTCA
NC-TY-3672B	Target	6	Solyc06g064720.1	2256	36,725,787	40,335,887	Tomato genome	NBS-LRR, resistance protein	TGCCAGATTCATGGGCTACT	GCTGCAGAGTTTGGAGATGG
NC-TY-3673B	Target	6	Solyc06g064750.1	2292	36,739,600	40,349,700	Tomato genome	NBS-LRR, resistance protein	GGTTGTCTTGATTGCTGGAATTT	ACTTCAACAGATGGTCTACTGGG
NC-BW-3675B	Target	6	Solyc06g064760.1	2289	36,753,875	40,363,975	Tomato genome	NBS-LRR, resistance protein	GGCTGTTTGTTGACGGAAG	TCTGAAGCTTCCCATTCTTTC
NC-BW-3676B	Target	6	Solyc06g064780.1	279	36,765,822	40,375,922	Tomato genome	NBS, resistance protein fragment	GTGGAGAACCTTCCACCAAAC	CGAAATAGGCCAGCGAAAC
NC-BW-36767B	Target	6	Solyc06g064790.1	1388	36,767,552	40,377,652	Tomato genome	NBS-LRR resistance protein-like protein	CAAGTTGTGGATGGCTGAAGAG	CCCTGAGATTTGAAGGGAAGAC
NC-BW-3692B	Target	6	Solyc06g065000.1	1800	36,925,986	40,534,287	Tomato genome	CC-NBS, resistance protein fragment	GATCATTGGCCAGCTCCTT	GGAAACAGTGCACTTAGCACGA
NC-BW-37013B	Target	6	Solyc06g065120.1	663	37,014,377	40,623,815	Tomato genome	LRR, resistance protein fragment	CAATTCGGGAACTGCCATC	CCAGCATAAAGCTCCTCCAA
NC-BW-37018B	Target	6	Solyc06g065140.1	929	37,018,235	40,628,335	Tomato genome	LRR, resistance protein fragment	GAAGTTGTCAAGGAACCGAAGG	GAGATGGCCAATGGAAGGA
NC-BW-37019B	Target	6	Solyc06g065150.1	942	37,019,237	40,629,337	Tomato genome	LRR, resistance protein fragment	TGTGGTCCAGTCCTCTCTCAA	CAGGATTCAACACACCTGCTC
Act41	Reference	--	NCBI: U60479	1150	--	--	Rotenberg et al., 2006	Actin	GCTCTTGACTATGAACAGGAAC	AAGGACCTCAGGACACCG
Act41B	Reference	--	NCBI: U60479	1150	--	--	Rotenberg et al., 2006	Actin	CCAGGTATTGCTGATAGAATGAG	CTGAGGGAAGCCAAGATAGAG
Ubi3R	Reference	--	NCBI: X58253	2374	--	--	Rotenberg et al., 2006	Ubiquitin 3	GCCGACTACAACATCCAGAAGG	TGCAACACAGCGAGCTTAACC
Ubi3RB	Reference	--	NCBI: X58253	2374	--	--	Rotenberg et al., 2006	Ubiquitin 3	ACTCTTGCCGACTACAACATCC	CTCCTTACGAAGCCTCTGAACC
EF-1	Reference	--	NCBI: X14449	1692	--	--	Rotenberg et al., 2006	Elongation factor 1-alpha	GATTGGTGGTATTGGAACTGTC	AGCTTCGTGGTGCATCTC
EF-1B	Reference	--	NCBI: X14449	1692	--	--	Rotenberg et al., 2006	Elongation factor 1-alpha	CTCCGTCTTCCACTTCAGG	TCAGTTGTCAAACCAGTAGGG

Notes: * Targets were designed from ITAG2.3. Target locations shifted downstream in genome 3.604 +/-0.059 Mbp in ITAG2.4.
~ Only mRNA sequence available. All other lengths are full DNA sequence lengths.

Table 4.4. ***Relative expression analysis results for the chromosome six QTL targets.***
The relative expression analysis results for 25 target loci and 6 reference genes tested by qRT-PCR in 'Florida 47' and 'Cheong Gang'. Reliability of results were determined by assessing the ranges of the standard errors of the mean CT and 95% confidence intervals of the normalized expression for each primer, along with significant fit to linear model. Reliable primers are bolded to aid reading. Also, Ct values larger than 30 were considered suspicious results unless the target exhibited good consistency of amplification. The reference loci were combined into an index used for normalization of all targets compared with the respective negative inoculation controls. Normalized expression is the fold-change factor for that loci and conditions, where a value < 1.0 is down regulated, and a value > 1.0 is upregulated. Significance levels are noted. Table continues on next page…

Primer	Use	Reliable Loci Primer?	Avg. Ct Over All Conditions	LinRegPCR	BestKeeper-1 Index			Overall Genotypes and Rs Isolate				Florida 47: Jc and P			
				Efficiency	Std. Error +/- Ct	Pearson (*r*)	Linear Model	Norm. Express.	Standard Error Range	95% Confidence Interval		Norm. Express.	Standard Error Range	95% Confidence Interval	
Q3-Q4short	**Target**	**Yes**	**23.409**	**0.975**	**0.906**	**0.956**	*******	**0.633**	**0.401 - 1.057**	**0.291 - 1.236**	**NS**	**0.394**	**0.295 - 0.507**	**0.284 - 0.550**	*****
Q3-Q4	Target	No	32.139	0.857	1.373	0.825	***	0.685	0.346 - 1.643	0.149 - 2.457	NS	0.433	0.340 - 0.642	0.294 - 0.749	*
NC-BW-2607B	**Target**	**Yes**	**26.178**	**0.940**	**0.903**	**0.940**	*******	**0.753**	**0.671 - 0.990**	**0.260 - 1.054**	**NS**	**0.638**	**0.557 - 0.829**	**0.299 - 0.971**	**NS**
NC-BW-2605B	Target	No	30.900	0.741	7.643	-0.019	NS	0.031	0.000 - 4.826	Range > 100.0	NS	5.956	Range > 10.0	Range > 100.0	NS
NC-BW-2601B	Target	No	31.039	0.758	7.539	0.035	NS	0.000	0.000 - 0.440	Range > 100.0	*	0.550	Range > 10.0	Range > 100.0	NS
NC-BW-2842B	Target	No	30.729	0.829	5.796	-0.452	NS	0.026	0.000 - 1.713	Range > 100.0	NS	41.921	Range > 10.0	Range > 100.0	NS
NC-TY-3103B	**Target**	**Yes**	**28.930**	**0.821**	**0.831**	**0.960**	*******	**0.905**	**0.748 - 1.137**	**0.522 - 1.353**	**NS**	**0.932**	**0.831 - 1.090**	**0.626 - 1.151**	**NS**
NC-BW-3362B	**Target**	**Yes**	**25.801**	**0.920**	**0.758**	**0.901**	*******	**0.961**	**0.704 - 1.429**	**0.532 - 1.723**	**NS**	**1.016**	**0.745 - 1.531**	**0.624 - 1.618**	**NS**
NC-BW-3369B	**Target**	**Yes**	**28.912**	**0.912**	**1.022**	**0.884**	*******	**0.813**	**0.630 - 1.117**	**0.185 - 1.446**	**NS**	**0.778**	**0.624 - 1.128**	**0.263 - 1.250**	**NS**
NC-BW-3370B	**Target**	**Yes**	**27.381**	**0.967**	**0.969**	**0.934**	*******	**0.768**	**0.636 - 1.030**	**0.240 - 1.341**	**NS**	**0.718**	**0.602 - 1.054**	**0.302 - 1.336**	**NS**
NC-BW-3410B	Target	No	28.701	0.734	7.580	0.113	NS	0.001	0.000 - 0.674	0.000 - 1.032	*	0.001	0.000 - 0.179	0.000 - 0.762	NS
NC-BW-3578B	**Target**	**Yes**	**26.976**	**0.942**	**0.831**	**0.955**	*******	**0.783**	**0.723 - 0.977**	**0.364 - 1.051**	**NS**	**0.717**	**0.648 - 0.875**	**0.398 - 0.966**	**NS**
NC-TY-3670B	Target	Maybe	34.324	0.578	1.060	0.644	*	0.653	0.436 - 1.161	0.258 - 1.805	NS	0.802	0.455 - 1.375	0.403 - 1.679	NS
NC-BW-3671B	Target	Mabye	33.521	0.985	0.663	-0.091	NS	0.987	0.364 - 2.182	0.243 - 10.918	NS	0.882	0.437 - 3.121	0.306 - 4.426	NS
NC-BW-36721B	Target	No	34.891	0.960	1.160	-0.324	NS	1.176	0.357 - 3.903	0.078 - 25.630	NS	1.191	0.320 - 6.556	0.296 - 15.275	NS
NC-BW-36725B	**Target**	**Yes**	**32.218**	**0.930**	**0.718**	**0.759**	******	**0.818**	**0.541 - 1.424**	**0.285 - 1.742**	**NS**	**1.249**	**0.986 - 1.665**	**0.753 - 1.750**	**NS**
NC-TY-3672B	**Target**	**Yes**	**29.459**	**1.003**	**0.752**	**0.653**	*****	**0.561**	**0.332 - 0.928**	**0.206 - 1.382**	**NS**	**0.596**	**0.343 - 1.228**	**0.294 - 1.362**	**NS**
NC-TY-3673B	**Target**	**Yes**	**30.686**	**0.961**	**1.000**	**0.658**	*****	**0.460**	**0.221 - 0.857**	**0.168 - 1.818**	**NS**	**0.420**	**0.221 - 1.116**	**0.181 - 1.620**	**NS**
NC-BW-3675B	Target	No	31.284	0.876	3.723	0.181	NS	4.778	Range >10.0	Range > 100.0	NS	9.380	Range > 10.0	Range > 100.0	NS
NC-BW-3676B	Target	No	32.781	0.916	2.355	0.197	NS	3.555	0.716 - 7.057	Range > 100.0	NS	1.450	0.765 - 5.318	0.345 - 7.353	NS
NC-BW-36767B	Target	No	30.707	0.676	9.238	-0.049	NS	72.528	Range >10.0	Range > 100.0	NS	199.281	Range > 10.0	Range > 100.0	NS
NC-BW-3692B	**Target**	**Yes**	**30.379**	**0.891**	**0.903**	**0.892**	*******	**0.752**	**0.503 - 1.037**	**0.397 - 1.210**	**NS**	**0.726**	**0.561 - 0.954**	**0.481 - 1.044**	**NS**
NC-BW-37013B	Target	No	34.524	0.923	0.984	-0.110	NS	0.985	0.317 - 2.747	0.140 - 10.078	NS	2.550	1.219 - 7.994	0.593 - 12.953	NS
NC-BW-37018B	Target	No	31.673	0.798	5.807	0.240	NS	40.267	Range >10.0	Range > 100.0	NS	16.359	Range > 10.0	Range > 100.0	NS
NC-BW-37019B	Target	No	28.938	0.739	9.129	-0.049	NS	0.172	Range >10.0	Range > 100.0	NS	93.356	Range > 10.0	Range > 100.0	NS
Act41	Reference	Yes	24.556	0.940	0.663	0.979	***	1.005	--	--	--	1.052	--	--	--
Act41B	Reference	Yes	23.644	0.986	0.584	0.982	***	1.030	--	--	--	1.075	--	--	--
EF-1	Reference	Yes	20.938	0.953	0.806	0.985	***	0.902	--	--	--	0.842	--	--	--
EF-1B	Reference	Yes	21.230	0.954	0.790	0.973	***	0.858	--	--	--	0.811	--	--	--
Ubi3R	Reference	Yes	21.000	0.950	0.655	0.982	***	1.089	--	--	--	1.071	--	--	--
Ubi3RB	Reference	Yes	23.260	0.824	0.654	0.958	***	1.147	--	--	--	1.209	--	--	--
Notes:	*, **, ***: Significance at α = 0.05, 0.01, and 0.001, respectively. NS = Not significant.														

Table 4.4. Continued from previous page.

Primer	Use	Geong Gang: Jc and P				Jc with both				P with both			
		Norm. Express.	Standard Error Range	95% Confidence Interval		Norm. Express.	Standard Error Range	95% Confidence Interval		Norm. Express.	Standard Error Range	95% Confidence Interval	
Q3-Q4short	**Target**	**1.018**	**0.857 - 1.214**	**0.794 - 1.281**	**NS**	**0.640**	**0.427 - 1.053**	**0.319 - 1.164**	**NS**	**0.627**	**0.372 - 1.020**	**0.318 - 1.261**	**NS**
Q3-Q4	Target	1.084	0.424 - 2.388	0.416 - 2.597	NS	0.644	0.339 - 1.225	0.188 - 2.353	NS	0.728	0.348 - 1.833	0.198 - 2.282	NS
NC-BW-2607B	**Target**	**0.888**	**0.765 - 1.041**	**0.714 - 1.047**	**NS**	**0.731**	**0.543 - 1.041**	**0.255 - 1.058**	**NS**	**0.775**	**0.704 - 0.845**	**0.674 - 0.915**	******
NC-BW-2605B	Target	0.000	0.000 - 0.694	0.000 - 0.966	NS	0.018	Range > 10.0	Range > 100.0	NS	0.053	0.000 - 3.487	Range > 100.0	NS
NC-BW-2601B	Target	0.000	0.000 - 0.000	0.000 - 0.000	*	0.000	0.000 - 0.184	Range > 100.0	*	0.000	0.000 - 0.504	Range > 100.0	*
NC-BW-2842B	Target	0.000	0.000 - 0.000	0.000 - 0.000	**	0.138	Range > 10.0	Range > 100.0	NS	0.005	0.000 - 0.756	0.000 - 5.573	NS
NC-TY-3103B	**Target**	**0.878**	**0.808 - 0.982**	**0.791 - 1.037**	**NS**	**0.878**	**0.642 - 1.176**	**0.476 - 1.386**	**NS**	**0.932**	**0.791 - 1.111**	**0.738 - 1.157**	**NS**
NC-BW-3362B	**Target**	**0.910**	**0.746 - 1.259**	**0.710 - 1.369**	**NS**	**0.841**	**0.599 - 1.111**	**0.491 - 1.718**	**NS**	**1.099**	**0.807 - 1.535**	**0.719 - 1.632**	**NS**
NC-BW-3369B	**Target**	**0.851**	**0.636 - 1.060**	**0.546 - 1.318**	**NS**	**0.824**	**0.714 - 1.296**	**0.178 - 1.503**	**NS**	**0.803**	**0.634 - 1.037**	**0.549 - 1.079**	**NS**
NC-BW-3370B	**Target**	**0.822**	**0.701 - 0.975**	**0.589 - 1.047**	**NS**	**0.772**	**0.628 - 1.172**	**0.235 - 1.366**	**NS**	**0.764**	**0.640 - 0.957**	**0.579 - 1.001**	**NS**
NC-BW-3410B	Target	0.002	0.000 - 0.633	0.000 - 0.915	**	0.002	0.000 - 0.895	0.000 - 1.077	NS	0.000	0.000 - 0.138	0.000 - 0.608	***
NC-BW-3578B	**Target**	**0.855**	**0.764 - 0.991**	**0.751 - 1.046**	**NS**	**0.783**	**0.634 - 1.006**	**0.357 - 1.054**	**NS**	**0.783**	**0.732 - 0.837**	**0.709 - 0.867**	*****
NC-TY-3670B	Target	0.531	0.437 - 0.687	0.294 - 0.811	NS	0.758	0.511 - 1.144	0.451 - 1.368	NS	0.563	0.362 - 0.922	0.251 - 1.853	NS
NC-BW-3671B	Target	1.106	0.843 - 1.647	0.627 - 1.861	NS	1.022	0.457 - 1.868	0.330 - 6.819	NS	0.954	0.292 - 2.595	0.216 - 11.697	NS
NC-BW-36721B	Target	1.162	0.678 - 3.690	0.192 - 3.893	NS	1.361	0.479 - 3.899	0.386 - 7.324	NS	1.017	0.244 - 6.960	0.061 - 31.790	NS
NC-BW-36725B	**Target**	**0.535**	**0.478 - 0.704**	**0.240 - 0.830**	**NS**	**0.942**	**0.582 - 1.419**	**0.551 - 1.731**	**NS**	**0.710**	**0.383 - 1.377**	**0.238 - 1.684**	**NS**
NC-TY-3672B	**Target**	**0.529**	**0.421 - 0.699**	**0.239 - 0.777**	**NS**	**0.668**	**0.517 - 0.805**	**0.507 - 1.185**	**NS**	**0.472**	**0.264 - 0.943**	**0.206 - 1.384**	**NS**
NC-TY-3673B	**Target**	**0.502**	**0.425 - 0.716**	**0.221 - 0.789**	**NS**	**0.546**	**0.270 - 1.031**	**0.180 - 1.892**	**NS**	**0.386**	**0.221 - 0.683**	**0.167 - 1.062**	**NS**
NC-BW-3675B	Target	2.434	Range > 10.0	Range > 100.0	NS	17.577	Range > 10.0	Range > 100.0	NS	1.299	0.434 - 4.059	0.211 - 10.543	NS
NC-BW-3676B	Target	8.720	Range > 10.0	Range > 100.0	NS	7.768	Range > 10.0	Range > 100.0	NS	1.627	0.872 - 3.553	0.727 - 10.131	NS
NC-BW-36767B	Target	26.397	Range > 10.0	Range > 100.0	NS	842.616	Range > 10.0	Range > 100.0	NS	6.243	Range > 10.0	Range > 100.0	NS
NC-BW-3692B	**Target**	**0.780**	**0.641 - 1.050**	**0.414 - 1.132**	**NS**	**0.806**	**0.650 - 1.034**	**0.429 - 1.126**	**NS**	**0.702**	**0.495 - 1.007**	**0.384 - 1.250**	**NS**
NC-BW-37013B	Target	0.381	0.229 - 0.777	0.113 - 1.112	NS	0.962	0.324 - 2.657	0.254 - 5.628	NS	1.009	0.388 - 3.241	0.115 - 12.061	NS
NC-BW-37018B	Target	99.113	Range > 10.0	Range > 100.0	NS	475.121	Range > 10.0	Range > 100.0	NS	3.413	1.285 - 9.863	0.733 - 40.961	NS
NC-BW-37019B	Target	0.000	Range > 10.0	Range > 100.0	NS	0.084	Range > 10.0	Range > 100.0	NS	0.351	Range > 10.0	Range > 100.0	NS
Act41	Reference	0.959	--	--	--	0.953	--	--	--	1.059	--	--	--
Act41B	Reference	0.987	--	--	--	0.970	--	--	--	1.093	--	--	--
EF-1	Reference	0.966	--	--	--	0.919	--	--	--	0.886	--	--	--
EF-1B	Reference	0.908	--	--	--	0.910	--	--	--	0.810	--	--	--
Ubi3R	Reference	1.107	--	--	--	1.108	--	--	--	1.069	--	--	--
Ubi3RB	Reference	1.088	--	--	--	1.168	--	--	--	1.126	--	--	--
Notes:	*, **, ***: Significance at α = 0.05, 0.01, and 0.001, respectively. NS = Not significant.												