ABSTRACT

KRESSIN, JONATHAN PAUL. Resistance Dynamics of Tomato and the *Ralstonia solanacearum* Species Complex: Assessing Resistance Mechanisms and Methods for Practical Evaluation in Breeding and Pathology Programs Within a Diverse Set of Rootstock and Founder Germplasm. (Under the direction of Drs. Dilip R. Panthee and Frank J. Louws).

The breeding history of tomato (*Solanum lycopersicum* L.) resistance to bacterial wilt (the *Ralstonia solanacearum* species complex) has been riddled with enigmas, conflicting reports, and decades of low genetic gain. The most efficacious resistance has been available for more than 40 years, yet it has only been effectively deployed in rootstock varieties because of the extreme difficulty of introgressing high levels of resistance into large-fruited, pleasant tasting genetic backgrounds. These resistant rootstocks have been a primary driver of the adoption of grafted tomato production in North Carolina and elsewhere, yet the quantitative resistance in tomato does not prevent the bacteria from invading roots and hypocotyl tissue where the graft union resides; rather it predominantly restricts the ability of the pathogen to colonize adjacent xylem cells. This has led to major questions about why a grafted management strategy is actually effective.

We explored several enigmatic aspects of the bacterial invasion patterns over time and space in grafted tomato combinations (susceptible to highly resistant) with luciferase- and green fluorescent protein-labeled strains to better understand why vegetable grafting endures as a viable management strategy. These results were then harmonized into a wilt resistance model that provides phenotypic context for resistance assessments and breeding. We then focused on methods for more effectively quantifying resistance in a larger panel of diverse rootstock and resistance founder germplasm. We specifically explored the relationship between colonization and wilt resistance with the vascular browning phenomenon at the tissue-system level, quantifying variation across germplasm, establishing assessment metrics, and exploring its
viability as a non-transient indicator of bacterial wilt resistance that could be applied in a more high-throughput manner to breeding. Furthermore, we performed field and greenhouse resistance screens over multiple years and locations to further identify useful field-level resistance within the diversity panel germplasm. A selection of these lines (mostly hybrid rootstocks and a few founders) were then tested as rootstocks for wilt resistance and commercial productivity in a multi-location variety trial across North Carolina, contrasting disease and no-disease conditions.

Through destructive sampling and novel imaging techniques, our results suggest that tomato resistance induces a series of tug-of-war arenas that are sequentially overcome for the bacteria to effectively colonize the plant—root invasion, root proliferation, radial vascular colonization, vertical xylary translocation (unclear), and pith/cortical tissue invasion. The work also clearly highlights key aspects of this quantitative tug-of-war dynamic that results in a mostly binary disease response—wilt. Wilt development is predominantly related to the tug-of-war dynamic in the basal hypocotyl, where effective resistance suppresses total vascular bundle invasion, not just invasion within bundles. Wilt is related to a spatial and density-dependent threshold of hypocotyl colonization, which appears to be entirely determined by the rootstock. Ultimately, resistant rootstocks protect susceptible scions through a combination of limiting the extent of xylem volume blockage in the hypocotyl, but not necessarily by preventing the pathogen from crossing the graft union. Our work also suggests that resistance failure is not necessarily related to selection of resistance-breaking pathogen strains, but rather a simple loss of the colonization*quarantine tug-of-war. The characterization of vascular browning variation revealed a tight relationship between spatial colonization and vascular browning severity, which was strongly positively correlated with wilt development, suggesting that disease screens can
employ an end-of-study assessment of resistance to further discern superior resistance among otherwise similarly wilt resistant varieties.

Finally, our field and greenhouse tests of the wide diversity germplasm panel provide evidence-based germplasm recommendations for disease resistance breeding and applied management decisions for North Carolina, with applications for resistance rotation and diversity. Furthermore, the lines tested as rootstocks indicate that the same varieties should work well across the soil types and environmental conditions of the state, which means that tomato growers have a strong set of resistant rootstocks from diverse breeding backgrounds available for high performance management of bacterial wilt that they can use in rotation without compromising resistance or yield performance. Additionally, we have identified novel candidates for additional introgression breeding efforts to further bolster the diversity of resistant material and help safeguard these materials against resistance breakdown for many decades, which can be supported with these new tools for resistance quantification within the framework of the host-pathogen dynamics at each important anatomical battlefield.
Resistance Dynamics of Tomato and the *Ralstonia solanacearum* Species Complex: Assessing Resistance Mechanisms and Methods for Practical Evaluation in Breeding and Pathology Programs Within a Diverse Set of Rootstock and Founder Germplasm.

by
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DEDICATION

This work is dedicated to the creativity and ingenuity of the Lord Jesus Christ, who has seen fit to let me search them out and discover what He has done. So much of this work, from the very beginning idea to investigate the vascular browning phenomenon, was through His gentle nudge and setting up so many little things along the way that helped formulate and crystalize the ideas contained in this work. Truly:

*It is the glory of God to conceal a matter; to search out a matter is the glory of kings.* ~Solomon, King of Israel (Proverbs 25:2, NIV)

This work is also dedicated to the many tomato breeders, pathologists, and farmers who have endured generations of frustration working to manage bacterial wilt. I hope this work will help bring an end to that otherwise bleak future. We are in this struggle together; let us see it through to completion.

Finally, this work is dedicated to my wife Mary, who willingly gave up a job offer in chemical engineering to support me in pursuit of a PhD at NC State. This work would quite literally not have occurred without that sacrifice and her continued support throughout the journey.
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 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 people. Jonathan gives all credit and thanks to Jesus Christ, his savior and Lord, for all that is good and right in him. Soli de Gloria!

Jonathan’s passion for plant sciences came later in high school. 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.
ACKNOWLEDGMENTS

Soli deo gloria.

My wife Mary Kressin.

My co-advisors Dr. Dilip Panthee and Dr. Frank Louws.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description of term</th>
</tr>
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<tbody>
<tr>
<td>BH</td>
<td>Basal hypocotyl</td>
</tr>
<tr>
<td>BW</td>
<td>Bacterial wilt</td>
</tr>
<tr>
<td>CG</td>
<td>A graft combination that would be used commercially</td>
</tr>
<tr>
<td>chr.</td>
<td>Chromosome</td>
</tr>
<tr>
<td>DH</td>
<td>Distal hypocotyl</td>
</tr>
<tr>
<td>dpi</td>
<td>Days post inoculation</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>H</td>
<td>‘Hawaii 7996’</td>
</tr>
<tr>
<td>Ix</td>
<td>Internode 1, 2, 3, or 6</td>
</tr>
<tr>
<td>LUX</td>
<td>Luciferase operon; <em>LuxCDABE</em></td>
</tr>
<tr>
<td>M</td>
<td>‘Marmande’</td>
</tr>
<tr>
<td>NG</td>
<td>Non-graft type</td>
</tr>
<tr>
<td>QTL</td>
<td>Quantitative trait loci</td>
</tr>
<tr>
<td>rX-sX</td>
<td>RootstockX-scionX graft combination, where X=cultivar</td>
</tr>
<tr>
<td>RG</td>
<td>Reciprocal graft—the reverse of the commercial graft type</td>
</tr>
<tr>
<td>Rs</td>
<td><em>Ralstonia solanacearum</em> species complex</td>
</tr>
<tr>
<td>S</td>
<td>‘Shield’</td>
</tr>
<tr>
<td>SG</td>
<td>Self-graft; plants of the same variety as rootstock and scion</td>
</tr>
<tr>
<td></td>
<td>Standard abbreviations for the metric system were used</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
<tr>
<td>VB</td>
<td>Vascular browning</td>
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</table>
1.1: Introduction.

The host-pathogen interactions between tomato (*Solanum* spp. L.) and the *Ralstonia solanacearum* (Rs) species complex are as economically devastating as they are complexly enigmatic. Since Erwin F. Smith penned the original description of bacterial wilt (BW) and the pathogen that caused it 122 years ago (Smith, 1896), an enormous amount of research and breeding has occurred to manage this disease spanning hundreds of plant species. Yet, BW is still a major, economically devastating disease of global significance in the most important vegetable species of the Earth—Potato, tomato, eggplant, and pepper (Elphinstone, 2005; Mansfield et al., 2012).

While most vegetables are not typically thought of as subsistence crops in the same way as wheat, rice, or dry beans (with the exception of potato), they do form integral components of a vitamin-rich, nutrient balanced, and culinarily diverse food system. Furthermore, the agricultural systems most threatened by the presence of Rs are the same regions that host the largest human population densities, the most vulnerable of subsistence farmers, and the environments most suitable for pathogens like Rs to thrive—i.e. the tropical and subtropical belt of the Earth running through much of South and Central America, most of Africa, the Middle East, Southeastern Asia, and the South pacific zones of Oceana.

The most recent population growth projections suggest that the population of the Earth will increase from the current 7.6 billion to 8.6 billion people in another 12 years (UN Department of Economic and Social Affairs, 2017). Further extrapolations suggest an additional 1.2 billion people will be present 32 years from now. While issues of food access, distribution
capacity, and social and political stability cannot be ignored, there remains the necessity to increase production capacity to feed the growing human population globally.

Not long ago, it was estimated that 35% of global food production are lost due to pathogens and pests, with plant pathogens and viruses accounting for about 10 to 15% of production losses (Popp and Hantos, 2011; Strange and Scott, 2005). It is also projected that half of the future population growth by 2050 will occur in 9 countries: India, Nigeria, the Democratic Republic of the Congo (DRC), Pakistan, Ethiopia, the United Republic of Tanzania, the United States of America, Uganda, and Indonesia. There is a clear connection between poorer nations with a much larger reliance on subsistence farming and major population growth projections. *R. solanacearum* has been reported as causing regular economic losses in Solanaceous and other crops in 8 of those 9 countries (Elphinstone, 2005). It is present in the DRC as well. The global importance of bacterial wilt management is clear for providing vegetable nourishment for those rapidly growing nations.

This review builds upon a previous review (Kressin, 2014), which provides a detailed overview of tomato, *Rs*, BW, and many aspects of the genetic, spatial, and temporal dynamics of the host-pathogen interactions. It also addresses practical management through host genetics deployed as rootstocks. This review will focus on literature that was either not addressed in that review, is more recent, or provide more details of certain portions of the research field that are most relevant to this work.

**1.2: Distributions and economic impacts of bacterial wilt.**

The distribution of BW has been studied much more thoroughly than the economic impacts of the disease. CAB International and the EPPO collaborated to assess the global
distribution of Rs by race (CABI/EPPO, 1999). While race 1 and 3 are damaging for Solanaceous crops, North America only has race 1 (Agrios, 2005). The race designations are not related to host genetics but rather host preference and are no longer being consistently used in the literature. Maps 138 and 783 from 1977 and 1999 (the most recent one available) show the distribution of the Rs species complex and race 1, respectively (CABI/EPPO, 1977; CABI/EPPO, 1999).

*R. solanacearum* is also categorized by four phylotypes, and the connection between phylotypes, biovars, and races has been basically established (Prior and Fegan, 2005). The global population structure of Rs is quite complex, leading to the recent reclassification of the Rs-species complex into three species, based upon genetic, biochemical, and host patterns. Focusing on Solanaceae, the structure currently stands as: Phylotype I (*R. pseudosolanacearum*; all Solanaceae; Africa, Europe, Eastern Asia), phylotype IIA (*R. solanacearum*; minus *Capsicum* spp.; North and South America mostly with some African spots), phylotype IIB (*R. solanacearum*; especially aggressive on potato; Caribbean to South America, Africa, and spots in Europe the South Pacific), phylotype III (*R. pseudosolanacearum*; all Solanaceae; Africa), and phylotype IV (*R. syzygii*; South Pacific and Eastern Asia) (C. H. Lin et al., 2014; Prior et al., 2016; Ramsubhag et al., 2012; Remenant et al., 2010; Safni et al., 2014; Wicker et al., 2011; Yahiaoui et al., 2017).

Within the Southeastern USA, historically endemic native populations of Rs are present, although they appear to contain a much lower genetic diversity compared to the global situation (Hong et al., 2012; Jimenez-Madrid et al., 2016). Florida has a small pocket of phylotype I (Hong et al., 2012; Wicker et al., 2011). Populations of Rs have been reported in over 80% of the counties in North Carolina, with strains in the Mountain region seeming to be a more recent,
growing problem (Kelman, 1953; Rivard et al., 2012, and internal strain libraries). Figure 1.1 shows where Rs has historically been identified and where our modern strain libraries have come from, which gives a sense of where Rs is a current problem.

There are not many reports on the actual economic impact of Rs in crops either globally or nationally. Some crops in some regions have thorough reports and/or loss estimates, which has been summarized fairly recently (Elphinstone, 2005). Economic losses fluctuate, partial due to changes in the types of crops grown where Rs is present.

For example, Rs is still considered a major pathogen of tobacco, and the significance was very large historically in NC, leading to substantial abandonment of tobacco fields in NC (Kelman, 1953). More recently, there has been a significant increase in the importance of Rs across the Southeastern USA for tobacco, and losses in NC and SC alone in 1998 were estimated at $40 million USD (Elphinstone, 2005). *R. solanacearum* has also historically caused losses in tomato, potato, peanut (groundnut), and eggplant (Kelman, 1953). There is some effort to convert abandoned tobacco farms into vegetable farms in recent years, which is likely to lead to a resurgence of BW issues.

Within tomato specifically, overall losses are not well reported. At the most recent Tomato Breeders Roundtable meeting in Ohio, USA (April, 2018), a roundtable summary report of breeding needs/challenges was given from representatives of the global tomato breeding industry. They frequently mentioned BW as the second most important bacterial disease to their respective sectors, following bacterial spot (*Xanthomonasi* spp.) (personal observation).

The last global review of production losses for tomato are summarized (Elphinstone, 2005). In Taiwan, losses ranged from 15 to 55%; in India, 10 to 100%; Australian estimates are 5 to 15% of the national crop, and hotspot regions spike to 70%. A large survey of Ugandan
tomato production districts found Rs in 88% of them. Within the USA, estimates are still spotty. In SC, it has been estimated that 15% of the tomato fields have pockets of Rs, while yield losses overall range from 1 to 5%.

*R. solanacearum* is present in every major tomato production region in NC, as well as 80% of the counties, although it is not clear what percentage of acreage is affected (Figure 1.2). Where hot spots occur, losses are easily between 70 and 100% (Kressin, 2014; Rivard et al., 2012; Rivard and Louws, 2008; Silverman, 2015). Florida production losses are generally less severe with a greater diversity of Rs strains, but hot spots are similar and can cause substantial losses under ideal conditions (Ji et al., 2007; McAvoy et al., 2012). In Virginia, the Eastern Shore region is where most of the tomato production is centered, and Rs is a major pathogen in that area in a similar manner to NC and FL (McAvoy et al., 2012; Wimer, 2009).

1.3: Impacts of temperature on bacterial wilt symptomology and host-pathogen physiology.

There is a rather strange influence of soil temperature and air temperature on BW expression that has frustrated pathologists and breeders for decades (Acosta, 1978; Nakaho et al., 1996; Prior et al., 1996; Scott et al., 2005). Soil temperature has a magnitude greater impact on disease development than air temperature. This phenomenon has never been adequately explained apart from the general aspect of Rs living in the soil and infecting the roots.

The impact of air temperature is clearly related to the vapor pressure deficit (VPD). As temperature rises, the capacity of the air to hold moisture increases, thereby increasing the evaporation potential of water in the leaves. When the stomates are open, higher temperatures increase the water loss of the leaves. When the supply of water to the leaves is restricted or cutoff, the plants will wilt. As soon as the flow rate into the leaves becomes greater than the loss,
either because of reduced restriction, greater flow capacity, or reduced loss potential, wilt will recover. Thus, wilt is affected by highly quantitative physiological processes, yet is a mostly binary phenomenon determined by an ebb and flow of host*environment interactions.

Furthermore, the optimal temperature ranges for growth and nutrient uptake by Rs and tomato are offset by about 5 °C (30 °C and 25 °C, respectively), while both seem to reach their maximum heat tolerance and rapidly decline after another 5 to 10 °C increase (Criddle et al., 1997; Gallegly and Walker, 1949b; J. B. Jones Jr., 2008; Kelman, 1953; Mew and Ho, 1977; Singh et al., 2014; Tindall et al., 1990; Vaughan, 1944). This means that when the environment is becoming more stressful for tomato, it is becoming more optimum for Rs. Tomato vitality declines a few degrees cooler than Rs. Appendix A discusses this more fully.

1.4: Diversity of bacterial wilt resistant germplasm.

The lack of effective chemical or cultural control methods for BW are constantly noted (Chellemi et al., 1994; Driver and Louws, 2002; Enfinger et al., 1979; Lemaga et al., 2001; Satou et al., 2006). This leaves host resistance of crop abandonment. Efficacious resistance is available and has been around for many decades.

A worldwide test of 35 tomato lines known to carry some level of resistance was conducted across 11 countries in the presence of local strains. The fields were arranged in a randomized complete block design with two to four replications of at least 20 plants each. Resistance was evaluated by plant survival (wilt incidence at the end of the trial) (J. Wang et al., 1998). There was also a greenhouse test using a highly virulent and aggressive strain from Southeast Asia, Pss4.
The germplasm in the global test represented 15 sources or combinations of BW resistance, while 7 entries had an unknown resistance source. The top 7 most overall resistant entries were noted as tracing back to only three resistance sources, or they possibly all came from selections from the Philippines (Scott et al., 2005; J. Wang et al., 1998). Excluding duplicates, three lines developed by the University of Hawaii breeding program were the 1st, 3rd, and 5th most resistant (Hawaii 7996, 7997, and 7998, respectively), while ‘BF-Okitsu’ (Syn. ‘BF-Okitsu 101’) from Japan was 2nd, ‘TML46’, ‘TML114’, and ‘R3034’ from the Philippines were 4th, 6th, and 7th, respectively, which all exhibited an average of 90% survival over the 11 locations. The frequently examined ‘CRA66’ from INRA was 8th at an average of 87.3% survival.

Apart from sheer disease resistance, noteworthy lines from this work were: ‘L285’, a local Taiwan landrace that had been the resistant donor in the first BW mapping study (Danesh et al., 1994). ‘CRA66’, which features prominently in many breeding programs and management tests, has been used to help characterize the latent infection and invasion pattern phenomena, identifying the hypocotyl as a key location for resistance (Grimault and Prior, 1993; Grimault and Prior, 1994a; Prior et al., 1996). ‘Fla7421’ (later released as ‘Neptune’) is the germplasm source that has yielded the only known breeding line to potentially have successfully combined large fruit size (>200 g) and high levels of BW resistance (Scott et al., 2004; Scott et al., 2009; Scott et al., 1995).

And the Hawaii 7000 series: ‘Hawaii 7996’ has become the globally recognized most broadly resistant line, and has been the resistance donor parent in all other bacterial wilt QTL mapping studies (Carmeille, Luisetti, Besse, Prior et al., 2006; Mangin et al., 1999; Thoquet, Olivier, Sperisen, Rogowsky, Prior et al., 1996; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot
et al., 1996; J. Wang et al., 2000; J. Wang et al., 2013). ‘Hawaii 7998’ was the resistance source for ‘LS-89’, a popular rootstock in Japan that became the subject used to greatly improve our understanding of mechanisms of host resistance on both anatomical and gene expression levels (Ishihara et al., 2012; Nakaho et al., 1996; Nakaho et al., 2000; Nakaho et al., 2016; Nakaho et al., 2004). This line has also had a significant impact on bacterial spot resistance (*Xanthomonas* spp.) (J. B. Jones and Scott, 1986; Scott et al., 2011). ‘Hawaii 7997’, apart from being a frequently utilized source of resistance for breeding, was also the resistance source for the previously mentioned ‘Neptune’ lineage (Scott et al., 1995).

Later, several breeders put together a report where they very thoroughly reviewed the literature and notes about the history of tomato BW breeding efforts from the 9 major public breeding programs in the last century (The Universities of North Carolina, Florida, Hawaii, Puerto Rico, and the Philippines, the Horticultural Research Station in Japan, the Asian Vegetable Research and Development Center in Taiwan [AVRDC], and the French research institutes INRA\(^1\) and IRAT\(^2\)) (Daunay et al., 2010). They reconstructed the flow of resistant germplasm between these programs, notable breeding releases, and traced the available pedigree notes as far back as possible to identify the founder lines.

It was reported that most of the resistance sources still used today were developed between the 1950s to 1970s, which remain the best in the world, especially the Hawaii 7000 series from the University of Hawaii. There was substantial germplasm flow in and out of these

\(^1\) INRA: Institut National de la Recherche Agronomique.
\(^2\) IRAT: Institut de Recherche en Agronomique Tropicale, which is now part of CIRAD as the Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement.
programs, and the breeding notes are often incomplete or sometimes conflict with later published journal papers.

The most glaring of these discrepancies is in the resistance source for the lines Hawaii 7996, 7997, and 7998 (and tangentially 7981) (Daunay et al., 2010). The Hawaii breeding program started using resistance from the North Carolina program, where ‘PI 129080’ and a mysterious “Beltsville No. 3814” were prominently featured.

Back in Hawaii, Acosta (1964) noted that additional resistance being bred into some of the Hawaii germplasm was obtained (either through breeding or from someone else) from “PI 127805A” and was field selected for 9 generations in Hawaii. The notes suggest the source was a S. pimpinellifolium line received from D.C. McGuire, although the line names referenced were not the same (Daunay et al., 2010). Several lines were released originating from this material—‘Kewalo’ and ‘5808-2’.

The first mention of the Hawaii 7000 series lines was in the 1970s and subsequent literature in the 1990s reported that the resistance came from “PI 127805A” as well (Daunay et al., 2010). Personal correspondence in the late 1970s and early 1980s, which surfaced later, however, suggested those lines were sister selections from some initial highly variable selection called “HSBW” (presumably “Heat Set Bacterial Wilt”), having a resistance source originating from the Philippines.

No publications were made of this line or the development of the Hawaii 7000 series, unfortunately. Gene banks have ‘PI 127805’, which is labeled as a S. pimpinellifolium accession collected in Peru (USDA GRIN system), but the “A” designated line appears to have vanished into annals of history. Thus, the origin of the most resistant, most studied, and likely most bred
resistance sources remain unclear. They could be the combination of five separate resistance sources.

A similar but less complex situation was noted for ‘CRA66’, where it may be a unique source unto itself selected from lines out of the Caribbean or be either the progeny or parent of ‘OTB2’ from Japan, which traces its origin back to the North Carolina germplasm of the 1950s (Daunay et al., 2010). Further reports highlight numerous additional sources of potentially independent resistance, which have been bred to various extents in various programs around the world, such as those that fed into the Florida and Georgia breeding programs in the 1970s and 1980s (Jaworski et al., 1987; Sonoda et al., 1979).

Several reports from the most recent BW resistance mapping project published SSR markers flanking five important resistance loci. The very large (~30 cM), broad-spectrum, major effect QTL on chromosome 6 was finally divided into four distinct but adjacent loci (Bwr-6a through 6d) (J. Wang et al., 2013). A major strain-specific locus (against phylotype I) was located on chromosome 12 (Bwr-12) and was linked with suppression of internal multiplication of the Rs in the stem. Near isogenic lines derived from a non-‘Hawaii 7996’ source (‘CLN2585D’; otherwise unknown) with or without Bwr-12 demonstrated that it is crucial for resistance to the highly aggressive strain Pss4 (phyloptype I from Taiwan) (P. Hanson et al., 2013).

Furthermore, a collection of resistance germplasm was screened using the chromosome 6 and 12 QTL markers (Ho et al., 2013). The results indicated that ‘L285’, ‘Venus’, ‘CRA66’, and ‘IRAT-L3’ were homozygous for all four of the Hawaii-type (H) chromosome 6 QTL markers but were homozygous for the ‘WVa700’-type (W) chromosome 12 QTL markers. This may explain the absence of any QTL detection on chromosome 12 when ‘L285’ resistance was
mapped using a phylotype I, biovar 4 strain (Danesh et al., 1994). Furthermore, ‘Saturn’, the sister line of ‘Venus’ had the opposite pattern, being homozygous for the H markers on chromosome 12, but having the W or other marker alleles for all four of the chromosome 6 QTL.

While these markers are based upon a moderately fine QTL map, they suggest that some of the differences between resistance levels has to do with the number of these QTL that a line possesses. These markers have been used for pyramiding the Bwr loci with other disease packages (P. Hanson et al., 2016).

Another recent report of markers for BW resistance in Vietnam used a bulked-segregant analysis of progeny derived from the same crossing population ‘Hawaii 7996’ x ‘WVA700’ (Truong et al., 2015). They identified 6 RAPD primers that exhibited polymorphisms linked to the resistant pool of F9 RILs used. One was converted to a codominant sequence characterized amplified region (SCAR) marker and all were tested in a wider germplasm panel of 92 lines. The markers only tested positive in the ‘Hawaii 7996’ source. It remains unclear how useful these markers may be. No other Hawaii 7000 series lines were tested.

1.5: Tomato plant colonization patterns and the graft union variable.

There are a lot of unknown aspects and curious phenomena about the host-pathogen dynamics of Rs colonization activities, especially with how resistance impacts those dynamics. One of those features is bacterial density within the stem. All resistant sources are more or less readily colonized by Rs at least into the hypocotyl. ‘LS-89’ has been the subject of many experiments about this subject, which was the most planted resistant rootstock in Japan in the 1990s to early 2000s. The previously described ‘CRA66’ was also frequently tested.
A series of experiments were conducted using the resistant ‘LS-89’ and the susceptible ‘Ponderosa’ (Nakaho, 1997a; Nakaho, 1997b; Nakaho et al., 2004; Nakaho and Allen, 2009). Using a soil inoculation method on trimmed roots, the resistant ‘LS-89’ remained symptomless for 14 days and the bacterial densities in the taproot (TR) through first internode (I$_1$) ranged from $10^6$ to $10^7$ CFU/g FM from 4 to 14 days post infection (dpi). Meanwhile, the susceptible ‘Ponderosa’ reached nearly 100% wilting and had bacterial densities from TR to I3 between 8.5 to 9.3 log(CFU/g FM) at 14 dpi (Nakaho, 1997a).

Another set of experiments using stem inoculation just above the cotyledons demonstrated that when the inoculum density was $10^6$ CFU/mL, no wilt was observed in ‘LS-89’ and the mean bacterial density in the upper hypocotyl was steadily between 7.2 to 7.7 log(CFU/g FM) from 4 to 14 dpi (Nakaho, 1997b). When the inoculum density was increased to $10^9$ CFU/mL, the density curve over time was shifted higher by about 1.0 to 1.5 log(CFU/g FM) and 100% of the plants developed wilt. The disease severity index was only about 50%, however. Additionally, higher densities of bacteria were observed at each height location in the plant, especially at the highest point measured (internode 5).

In other studies with mutant strains of Rs on ‘LS-89’, the line began to show wilt symptoms about the time that the bacterial density levels in the upper hypocotyls reached about 7 to 8 log(CFU/g FM). This apparent threshold was the same when plants were inoculated with either the Wt strain K60 or strain K60-509, a triple mutant that lacked three of the four pectinases Rs produces (PehA, PehB, and Pme) (Nakaho and Allen, 2009).

Yet, in a different series of experiments using stem inoculation on 5 to 6 leaf stage plants, no wilt was reported in ‘LS-89’ even though bacterial densities plateaued at ~8.3 log(CFU/g FM) by 6 dpi, and the plants still did not wilt by 30 dpi. Yet, the susceptible ‘Ponderosa’ reached 10
log(CFU/g FM) by 8 dpi and were severely wilted (Ishihara et al., 2012). Even the authors seemed surprised by the density results but did not speculate on why the plants did not wilt even though they were grown in the same conditions as their previous work.

Finally, experiments with a broader range of resistant lines demonstrated that plants of varying resistance levels (0 to 30 % wilt) maintained mean bacterial density levels below 8.0 log(CFU/g FM), while lines that never exhibited wilting maintained densities below about 7.2 log(CFU/g FM) (Nakaho et al., 2004).

These reports consistently dance around a density threshold pattern. Lines that have no wilting, or perhaps only low levels, seem to be able to cap bacterial densities in the stem at less than 10^8 CFU/g FM at the extreme upper limit. Lines that readily wilt exhibit bacterial densities 10 to 100 times greater. Resistance caps bacterial growth, although the mechanisms remain poorly understood.

Another aspect of colonization is the ability of Rs to invade vertically up the plant xylem. Nearly all studies examining Rs invasion up the height of the plant have not considered the effect of grafting. For example, the highly resistant ‘LS-89’ exhibited a rapid increase in infected plants without any development of wilting, plateauing at ~90% by 8 days post inoculation (dpi) (Nakaho, 1997a). The colonization frequency of these plants was assessed at 14 dpi from the taproot to the 5th internode and compared with the susceptible ‘Ponderosa’. It was found that ‘Ponderosa’, with 100% wilt, had 100% colonization at all locations measured. The same investigation of the ‘LS-89’ plants indicated that 90% were infected in the upper hypocotyl and ~25% were infected to the 5th internode. This suggested a vertical restriction of bacterial colonization rates in the resistant line.
Most commercial grafting practices attach BW susceptible cultivars to highly resistant rootstocks in the upper hypocotyl or 1st internode. Plants grafted in this manner obtain season-long protection from BW that is equivalent to the non-grafted rootstock, as well as produce a harvest often 1 to 2 months after the non-grafted susceptible scion varieties have begun to wilt (Cardoso et al., 2012; Grimault and Prior, 1994a; Kressin, 2014; McAvoy et al., 2012; Nakaho et al., 1996; Onduso, 2014; Rivard et al., 2012; Rivard and Louws, 2008). The previous non-grafted study indicated that Rs readily colonized the upper hypocotyl of ‘LS-89’, suggesting that the scions should also become infected in a grafted combination.

Of the three grafted studies that looked at aspects of colonization (Nakaho et al., 1996; Nakaho et al., 2004), two of them show that Rs can reach the basal hypocotyl in as little as 14 to 21 dpi. They suggest that either Rs does not pass the graft union into the susceptible or that, when the bacterium crosses over it, the plant wilts. But the connection is not solid. Self-grafted ‘LS-89’ plants exhibited about a 69% colonization rate above the graft union without any wilt development (Nakaho et al., 1996). 35% of the susceptible ‘Ponderosa’ scions were latently infected even though they were grafted onto ‘LS-89’.

Later in a second round of grafting experiments, ‘Hawaii 7996’ was included and the plants were inoculated at the flowering stage with 10^7 CFU/mL (Nakaho et al., 2004). Self-grafted ‘Ponderosa’ and ‘Ponderosa’ grafted onto the ‘LS-89’ rootstock reached 100 and 97% wilt by 14 dpi, indicating that greater disease development in the ‘LS-89’ treatments compared to the previous tests. When ‘Hawaii 7996’ was the rootstock only 32% of the susceptible scions wilted. Curiously, the ‘Hawaii 7996’ rootstock treatment exhibited about 39% latently infected scions at 14 dpi, while ‘LS-89’ only had 3% due to the high levels of wilting. Curiously, the latently infected ‘Ponderosa’ scions grafted onto ‘Hawaii 7996’ had bacterial densities of 6.8
log(CFU/g FM) at 1 cm above the graft union while the rootstock had 6.1 log(CFU/g FM) below the graft union.

The third grafted study tested colonization rates at several heights, including below and above the graft union, but did not specifically report the extent of latently infected scions. In two experiments, when the root was either ‘CRA66’ or ‘Caraibo’, no wilt was observed (compared to 100% and 50% wilt in ‘Floradel’, respectively). Essentially no hypocotyl colonization was observed in ‘CRA66’, but about 64-68% of ‘Caraibo’ hypocotyls were colonized below the graft union, while 40% and 20% of the susceptible ‘Floradel’ scions on ‘Caraibo’ were colonized above the graft union or 10 cm above the graft union, respectively (Grimault and Prior, 1994a). Thus, when the roots were ‘Caraibo’, 40% of the susceptible scions were latently infected.

It is difficult to suggest how resistant rootstocks could maintain a relatively wilt-free state for an entire season when these controlled environment/glass house experiments indicate Rs is capable of reaching and/or crossing the graft union within several weeks of inoculation. Furthermore, substantial rates of latently infected susceptible scions have been reported. This may imply a rootstock-scion resistance cross-talk where resistance is somehow conferred to the susceptible scion. Comparative mechanism studies, however, have only been performed on non-grafted material.

1.6: Vascular browning as a component of bacterial wilt symptomology.

Vascular browning (VB) is a brown discoloration of the vascular tissues, especially the xylem, that is associated with fungal and bacterial pathogens that migrate through the vascular system (Agrios, 2005). Historically, VB has regularly been reported for BW all the way back to the Erwin Smith in 1896. It was used as a visual marker for colonization that could be observed
sometimes prior to, but usually with, wilting in tomato plants (Kelman, 1953; Smith, 1896). While VB development is not unique to Rs, it is part of a typical bacterial wilt diagnosis when it is combined with bacterial streaming and the foliar wilt profile (Agrios, 2005).

Only a handful of reports have investigated VB specifically. Early studies from the 1950s and before reported aspects of VB during investigations of wilting mechanics, where it was usually noted as absent or mildly or strongly present (Gothoskar et al., 1953; Husain and Kelman, 1958b; Winstead and Walker, 1954). These studies were primarily aimed at settling debates over the cause of wilting—toxins, cellular components, or bacterial density and exopolysaccharide (EPS) slime.

Vascular browning is connected to bacterial pectinase activity. Solutions of purified pectinases from *Fusarium* spp. and Rs were shown to induce VB in cut stems within 2 days, which correlated with more advanced wilting symptoms (Winstead and Walker, 1954). The authors concluded that pectinases were insufficient for gaining entry into the host but were important for the development of VB and vessel plugging.

A few years later, another series of experiments further examined the impact of Rs pectinases in BW of tomato (Husain and Kelman, 1958b). They found that liquid culture filtrates containing these pectinases were capable of rapidly macerating the stem tissues of tomato cuttings placed in the purified solution. There seemed to be a connection between the breakdown of host tissue and some wilt development, although the connection was weak. Concurrently, the same authors presented a major report implicating the Rs EPS slime as the major factor determining wilt development, which is now the consensus view (Husain and Kelman, 1958a).

In the 1960s, the subject was reviewed, which discounted the model that pectinases caused wilting through the release of cellular materials that subsequently plug the vessels
(Buddenhagen and Kelman, 1964). It was noted that VB development in the fungal wilts was attributed to the oxidation and polymerization of phenols by the plant polyphenol oxidase. There were also old reports of some Rs strains from potato in Portugal and Kenya that cause wilt on potato but little or no VB in the vascular ring of the tubers, although they seemed to be rare exceptions (Kelman, 1953).

*R. solanacearum* produces one pectin methylesterase (Pme), three polygalacturonase (PG; PehA, PehB, and PehC) enzymes, and two cellulolytic enzymes (Egl and CbhA), but no pectate lyases that can directly hydrolyze pectin (Schell, 2000). More recently, the importance of pectinases and cellulases in colonization and wilt development have been demonstrated and reviewed (Denny et al., 1990; Huang and Allen, 2000; Huang and Allen, 1997; Liu et al., 2005; Meng, 2013; Nakaho and Allen, 2009; Schell, 2000). These reports are summarized below.

Lack of the PG PehB exhibited slightly reduced virulence. Loss of the PG PehA can delay wilt development and death two-fold, especially when inoculating via soil drenching compared to direct stem inoculation. Lack of both PGs reduces the rate of stem invasion and colonization, but the results were not validated in later pectinase gene deletion studies. Losses of the one or both cellulase enzymes (Egl and CbhA), however, did consistently delay colonization and wilt development. Lack of Pme does not have an obvious effect on colonization or wilt development. Furthermore, pectinase activity in tomato has been implicated with contributing to the resistance-specific induction of structural defenses, likely through host detection of released oligogalacturonides (Nakaho and Allen, 2009).

In other work, the putative modes of action of the pectinases have been explored in relation to host resistance and basal defense priming (Diogo and Wydra, 2007; Kurabachew and Wydra, 2014; Wydra et al., 2005; Wydra and Beri, 2006; Wydra and Beri, 2007). Generally,
Pme activity improves the ability of the PGs to attack the complex pectin chains, which is less severe in silicon-primed tomato plants. Furthermore, Rs inoculation leads to changes in the degree of rhamnogalacturonana I branching between resistant and susceptible genotypes. These changes localize to the xylem parenchyma and the vessel cell walls.

Following the previously mentioned VB studies in the 1950s, basically no research has been performed. In the early 1990s a report emerged testing gene dosage effects for BW resistance in leaf-axil-inoculated or naturally infested field tomatoes using a diallele crossing scheme (Anand et al., 1993). What made this report unique was that it used VB severity and the degree of bacterial oozeing as a way of tracking colonization and as a selection criterion.

A 0 to 3 VB scale typical of Fusarium wilt research was adapted for the BW work. The plants were destructively harvested 100 days after transplanting, the stems were split in half lengthwise, and VB was scored as: 0 = no browning; 1 = light brown color, spread restricted to 2 cm from the point of inoculation; 2 = light brown color spread more than 2 cm; 3 = dark brown color, widespread browning of vascular tissue.

The researchers observed substantial differences in wilt development, and a reevaluation of that data indicates that the VB score was positively correlated with wilt incidence in a threshold-like manner and also with the bacterial ooze score (Chapter 3, Supplemental 3.6A-B). Unfortunately, the report does not indicate the bacterial density used for inoculation. They also used a direct stem artificial inoculation method rather than soil drenching. Furthermore, the VB scale measured vertical severity but not radial.

A more recent report from Kenya used the same assessment method in field and potted glass house experiments with grafted tomatoes for management of BW (Onduso, 2014). In this
case, they examined a local wild tomato line alongside ‘Cheong Gang’ and ‘Shin Cheong Gang’ as rootstocks grafted with the susceptible ‘Anna F1’ scion.

The field sites relied upon natural disease pressure, while the glass house experiments were artificially inoculated. The potted plants were inoculated by: lightly injuring the roots on one side of the plant with a scalpel, pouring 4 mL of $10^9$ CFU/mL bacterial suspension over the injured roots, and then adding 100 mL of sterile water to disperse the inoculum through the 5 L pot. The process was repeated the next day on the other side of the plant. There were 5 plants per pot and three replicate pots. The field plants were destructively sampled after harvesting (100 dpi), whereas the potted experiments were sampled after 60 dpi. VB and bacterial ooze severities were measured, representing a total of 2 years over 5 locations.

The results parallel the previous study described, where VB severity was positively correlated with wilt percent and bacterial oozing scores; the resistant rootstock treatments exhibited significantly less wilting, VB severity, and bacterial ooze than the non-grafted susceptible treatment. The work also suggests that resistant materials showing the same levels of wilting have detectable differences in VB severity.

1.7: Host-pathogen interactions and their modulation by genetic resistance.

The natural invasion pathway of Rs in tomato is as follows: from the soil to the root epidermis, through the apoplastic spaces of the root cortex, penetration of the stele primarily through natural breaks in the casparian strip at sites of lateral root emergence, into the xylem vessel lumen, and vertically up the xylem tissues into the hypocotyl and stem of the plant (Schell, 2000; Vasse et al., 2005). The whole process can take as long as 7 days or as little as 3 days depending upon the age of the host, the soil temperature, and the inoculum density (Ishihara
et al., 2012; Nakaho et al., 1996; Nakaho, 1997b; Prior et al., 1996; Schell, 2000). The consensus is that wilt development occurs based upon the invasion condition of the stem, not the invasion of the roots (Rs is not considered a root rot pathogen, although it does cause some damage) (Grimault and Prior, 1993; A. C. Hayward, 1991; Vasse et al., 2005).

It is well established that resistance is both quantitative and polygenic, but there is very little consensus for how many genes are involved and how the alleles are expressed (dominant, recessive, additive, etc.) (Acosta et al., 1964; Acosta, 1978; Anand et al., 1993; Grimault et al., 1995; Scott et al., 2005). Furthermore, no documented resistance keeps the plants clean—I.e. prevents invasion and colonization of the roots and hypocotyl (Nakaho et al., 1996; Nakaho et al., 2004; Prior et al., 1996).

It is also well established that resistance is induced as early as 1 day after infection by the presence of pathogen factors (exopolysaccharides) and likely plant cell wall components released by the pathogen (Ishihara et al., 2012; Milling et al., 2011; Nakaho and Allen, 2009). Our previous work further demonstrated that tomato does not detect the typically conserved region of the Rs-specific bacterial flagellin like it can with other bacterial pathogens like *Xanthomonas* spp. or *Psuedomonas syringae* (Beck et al., 2014; Bhattarai et al., 2016; Felix et al., 1999; Kressin, 2014; Veluchamy et al., 2014).

Resistance to Rs involves multiple facets of structural reinforcement that effectively slow and/or quarantine the bacteria to small regions of vascular tissue (Nakaho et al., 2004), such as reinforcement of pit membranes, callose and lignin deposition, development of an electron-dense coating along the xylem vessel lumen, enhanced expression of β-1,3-glucanase genes related to apoplastic accumulation in cells surrounding infected xylem vessels, changes in the composition of the pectin layer and cell wall protein profiles, and possibly a more coordinated deployment of
tyloses and/or gums (Dahal et al., 2009; Dahal et al., 2010; Grimault et al., 1994; Ishihara et al., 2012; Nakaho et al., 2000; Nakaho and Allen, 2009; Wydra and Beri, 2006).

Furthermore, a recent report comparing hypersensitive response-marker genes in the stem concluded that vascular-based reactions contribute to the quantitative resistance (Nakaho et al., 2016). Curiously, this upregulation was not obviously associated with localized cell death (most xylem cells are already “dead”) but rather aggregated materials in a manner typical of a hypersensitive response reaction. Furthermore, leaf infiltrations with Rs resulted in localized cell death and reduced pathogen proliferation in the resistant ‘LS-89’ (resistance derived from ‘Hawaii 7998’) but not in the susceptible ‘Ponderosa’, which is another key feature of that resistance response.

Within the stem, there are a number of reports that have tried to quantify variation in the protein populations of the xylem walls and xylem sap (Dahal et al., 2009; Dahal et al., 2010; Ishihara et al., 2012; Planas-Marques et al., 2018; Wydra and Beri, 2006). Dozens of proteins are differentially expressed between resistant and susceptible lines with or without infection by Rs. The earlier studies were able to narrow some down to various pathogenesis, stress-related, and metabolic proteins that were differentially regulated upon Rs infection, but no strong candidates were settled on as definitively conferring resistance. The arabinogalatan protein, a cell-surface protein that plays a role in plant growth, development, and is especially abundant in xylem cell walls, exhibited increase abundance in plant cell wells of susceptible lines, but do not seem to change in resistant (Wydra and Beri, 2006).

Furthermore, transcriptome analysis of the resistant ‘LS-89’ found that out of the 140 genes that were differentially expressed upon infection, pathogenesis-related, hormone signaling, and lignin biosynthesis genes were upregulated compared to ‘Ponderosa’ (Ishihara et al., 2012).
A β-1,3-glucanase gene, which degrades callose, was the top upregulated gene, and was uniquely expressed in resistant cultivars. The validation efforts indicated that glucanases localized to xylem and pith tissues around vessels that were colonized by Rs, primarily in the apoplastic spaces. This led the authors to the hypothesis that the apoplast may be an important site for tomato defense responses to Rs infection (Ishihara et al., 2012). Additionally, UV autofloresence of pith tissues near primary bundles increased, along with lignin biosynthesis genes, suggesting Rs-induced lignification of the pith.

Very recently, a report was made comparing the changes in the active proteome profiles of the leaf apoplast in the resistant ‘Hawaii 7996’ and the susceptible ‘Marmande’ before and after inoculation with Rs (Planas-Marques et al., 2018). They used activity-based protein profiling, a novel technique that allows the pull down of active protein families via small molecular probes that bind to active sites. They found a variety-specific induction of two protein classes upon infection—papain-like cysteine proteases and serine hydrolases in ‘Hawaii 7996’, which have been linked to plant immunity in other pathosystems and have been reported in the apoplastic space of tomato. The P69 class of proteases were found to be post-translationally activated. Furthermore, they suggest that the resistant line is more innately prepared for Rs invasion because the susceptible line exhibited much greater changes in the protein network structure.

There are also resistance mechanisms at work when Rs is migrating from the rhizosphere into the roots that serve to delay and generally reduce the extent of invasion, although it does not prevent hypocotyl colonization (Caldwell et al., 2017; Vasse et al., 1995; Vasse et al., 2005). The resistant ‘Hawaii 7996’ was reported to have two subclasses of roots (long and thinner with very few root hairs; short and thicker with numerous long root hairs) that are differentially colonized
by Rs (normally colonized vs. restricted with no xylem penetration, respectively), while the
susceptible ‘Supermarmande’ only has one type (numerous root hairs) (Vasse et al., 2005).

Based on the published images, both types of roots are clearly laterals, although it is not
clear what is the main root class they branch from, either the same or different (Zobel and
Waisel, 2010). At the seedling stage, the main tomato roots are the taproot with some laterals and
basal roots with many laterals (personal observations). Additionally, the root based defensive
mechanisms employed by the highly resistant line to an aggressive wilt-type strain were similar
to those employed by the susceptible line to defend against a non-pathogenic strain, noting
physical signs of a possible root-based HR in both lines (Vasse et al., 2005).

Analysis of the root epidermal, cortical, and vascular cell invasion patterns revealed
interesting patterns as well (Schenk et al., 2018; Vasse et al., 2005). The single root type of
‘Supermarmande’ was predominantly colonized in the intercellular grooves of the root zone of
elongation and the intercellular cracks around sites of lateral root emergence. The cortical
intercellular spaces were filled with bacteria, forming longitudinal lines of infection along the
endodermis. Stele penetration seemed correlated with natural modifications to the casparian strip
in the previously mentioned locations, and xylem vessels were entered by degradation of the
cellulose and pectin primary cell wall components between the lignin reinforcements of the
secondary cell wall. Further cell-to-cell movement was facilitated by degradation of the primary
cell wall-comprised pit membranes.

The two root types of ‘Hawaii 7996’ exhibited contrasting colonization patterns (Vasse et
al., 2005). The thick short roots were more heavily colonized on the root surface, but invasion
around lateral root cracks and root tips remained localized without the intercellular lines of
bacteria in the cortex and vascular colonization was not observed. In contrast, the thin long roots
had much less epidermal colonization, but exhibited similar cortical invasion as the susceptible. Vascular colonization was less frequent at both the lateral root cracks and the elongation zone than in the susceptible. A dense vascular coating and amorphous polyphenolic compounds were also frequently observed in resistant roots to the Wt strain, perhaps similar to what has been observed in stems (Nakahō et al., 2000; Nakahō et al., 2016; Nakahō and Allen, 2009; Vasse et al., 2005).

A recent report adds to the root story by demonstrating that resistance in both ‘Hawaii 7996’ and ‘CRA66’ delays the root invasion and stele colonization process by about 24 hours (under their conditions), with a reduced extent of the root vascular core becoming infected (Caldwell et al., 2017). This correlated with reduced colonization within the vascular bundles of the hypocotyl. The root phenotype is not necessarily responsible for the hypocotyl colonization phenotype, however. Further work is needed clarify that relationship.

The xylem vessel size at the time of inoculation was examined but no clear distinctions were found between the two resistant and two susceptible lines tested (Caldwell et al., 2017). This further supports the view that resistance is not related to innate differences in vessel diameter.

1.8: Developments in assessment technology for bacterial wilt.

There have been a number of important, novel developments in BW assessment technology that will play an important role in our research. A Spanish-French research effort was developed to advance the capacity for easily transforming Rs with genetics of interest. They developed a chromosomal insertion tool that employs homologous recombination to insert plasmid DNA into a known region of the Rs genome, which can easily be switched out with
other constructs or transferred into another strain (Monteiro, Solé et al., 2012). The insertion tool—the pRC system (*Ralstonia* chromosome)—is advantageous for several reasons:

1) The researchers used a GATEWAY cassette, which is an Invitrogen product developed in the late 1990s and used extensively in molecular biology for efficient DNA fragment-transfer between plasmids. This system allows cloning of any promoter::gene combination in and out of the insertion site.

2) The pRC uses a homology site in the Rs genome for construct insertion via a double recombination event. This fixes the construct in the genome, making it stable over generations of cells.

3) Because of the specificity of the system, the insertion site is consistent rather than random, which ensures that only one copy of a fragment is inserted in a known region, thereby greatly reducing any concerns about variability in expression from multiple copies, chromosomal folding, or off-target effects on pathogen virulence.

And, 4) they developed three systems that vary in their antibiotic selection markers—gentamycin, tetracycline, and kanamycin, making them compatible with most of the current Rs mutants in the literature, and is compatible for multiple restriction enzymes.

The pRC tool was originally validated in the phylotype I strain, GMI1000, by expressing the *LuxCDABE* operon (LUX) driven by several promoters (Monteiro, Genin et al., 2012). The system provided a non-invasive, real-time assessment method for measuring promoter activity that can be used *in vivo* either with extracted bacteria or *in situ* within the plant. They tested expression of the type III secretion system (T3SS) and the promoter driving production of exopolysaccharide (EPS) (Monteiro, Genin et al., 2012). EPS is the main virulence factor that clogs the plant xylem vessels and induces wilting, while the T3SS is a crucial pathogenicity
factor that delivers a cocktail of host-disrupting effector molecules into the plant cells (Meng, 2013).

The validation work further demonstrated that the T3SS remains actively transcribed during later stages of infection when the bacteria reach high cell densities in the plant, which was opposite of the prevailing view (Monteiro, Genin et al., 2012). The result then led to the discovery of a second, novel regulatory system that affects the expression of the T3SS in a manner independent of the plant cell-contact detector prhA (Paola Zuluaga et al., 2013). This pathway triggers the signal cascade classically associated with T3SS expression (Coll and Valls, 2013).

Furthermore, they noted that the expression of this alternate T3SS-induction pathway was linked to an unknown small molecule or environmental cue in planta that was not one of the amino acids or sugars normally present in the apoplastic or xylary fluids of tomato (Paola Zuluaga et al., 2013). Of the hydroxycinnamic acids involved in host-pathogen interactions as precursors of lignin, ferulic acid, as well as oleanolic acid, was later found to stimulate expression of the T3SS, but it does so via the classic prhA signaling pathway (Wu et al., 2015).

Since then, the pRC system has been mobilized for in planta live screening for resistance responses in potato breeding germplasm (Cruz et al., 2014; Ferreira et al., 2017). Using the LUX reporter system previously mentioned in a Uruguayan strain highly virulent on potato (phytotype IIB), they found a correlation between whole-plant light production and the onset of wilting. Furthermore, the light output was quantitative, and was useful for detecting latently infected plants in a high-throughput evaluation program. The method allowed for non-destructive tracking of colonization, dissemination, and multiplication patterns of Rs in the potato plants.
They also added another strain that switched out the LUX operon for green fluorescent protein (GFP), which was used for precise tissue localization studies using confocal microscopy (Ferreira et al., 2017). The light-labeled strains were used to validate the mechanisms of resistance—reduction of pathogen spread from the roots and the base of the stem up into the aerial parts of the stem, limited invasion of the xylem vessels, and reduced multiplication within the resistant plants.

A similar kind of LUX imaging of whole plants was recently performed in resistant and susceptible accession of pepper (Du et al., 2017). Using a more traditional transformation technique of cell electroporation, they made seven transformants of a phylotype I strain virulent on pepper and chose the best one that most resembled the wilt type virulence and morphology.

A strong, positive relationship was observed between luminescence of whole plants and the bacterial population densities within the stems. After testing 100 pepper accession in a disease screen, they took the most resistant and assessed the luminescence dynamics. The resistant line prevented large numbers of bacteria from developing in the stem (capped around $10^7$ and $10^5$ CFU/g of tissue in the roots and collar, respectively), while further vertical invasion was restricted. The downside of the assessment technique was that luminescence was basically never observed in the resistant plant, thus detection of latently infected resistant plants was not possible from the whole plant luminescence (Du et al., 2017).
1.9: The need for a fresh perspective on evaluation and breeding for BW resistance in tomato.

The above reports in potato and pepper are the first notable adaptations of more novel resistance assessments for active BW resistance breeding applications in Solanaceous crops. More recent work in eggplant BW resistance also shows advancement, where modern genotyping-by-sequencing approaches were mobilized for identifying multiple resistance QTL and the cloning of a major resistance gene, providing at least one gene-linked marker and multiple QTLs that can be selected (Lebeau et al., 2013; Salgon et al., 2017; Salgon et al., 2018; Xi’ou et al., 2015).

Such applications in tomato breeding are either absent or completely impractical. Little-to-no progress has been made in recent years in public breeding for BW resistance. The recent moderate resolution genetic map from ‘Hawaii 7996’ has been the most helpful, and the only information to be actively deployed by some for breeding (P. Hanson et al., 2016; J. Wang et al., 2013). The private sector breeding efforts have yielded some progress by developing tomato rootstocks with high levels of BW resistance combined with other important soil-borne disease packages. These efforts have only adapted tandem repeat-based marker technology, so far as has been disclosed.

The improvement in our understanding of the mechanisms and genetics conferring BW resistance in tomato has not been translated in a manner that can be deployed in a breeding program. It is obvious that microscopic examinations, bacterial counts, and histological staining are not high-throughput enough, and thus too costly, to process hundreds of crosses yielding thousands of breeding lines and progenies every year.
Our previous work explored two novel assessment methods for BW resistance (Kressin, 2014). We tested the viability of using microbe-associated molecular pattern-triggered immunity (MAMP; MTI), as measured by the burst of reactive oxygen species, to predict BW resistance. This would have been a high-throughput assessment method that was non-destructive. Unfortunately, no correlations were observed with resistance. This was predominantly because of the discovery that tomato does not recognize the Rs-specific flagellin protein sequence (Kressin, 2014).

We also reported preliminary data suggesting that the vascular browning phenomenon in the stems of infected plants exhibited measurable variation that was positively correlated with host resistance, so much so that it was possible to better separate the panel of 14 lines with the browning symptomology than with wilt severity. In contrast, no differences were observed in hypocotyl colonization among the resistant and susceptible material (Kressin, 2014).

If tomato breeding for BW resistance is to overcome many of the hurdles that have stalled applied progress for several decades, then novel assessment methods and technologies need to be evaluated. Furthermore, the great revelations about tomato BW resistance mechanisms needs to be adapted for large-scale applied breeding and pathology. Tomato BW research and development is badly in need of translational phenomics.

1.10: Key questions and phenomena to resolve.

In summary, there are some key questions and phenomena to be explored in order to advance the basic understanding of BW phenomena and resistance dynamics. Resolving these issues will improve tomato breeding for BW resistance by improving the ability to mobilize the
pathological information about the host-pathogen interactions into a well deployed assessment system. They questions are:

1) Why do tomato plants actually wilt? Put another way, how does a polygenic resistance lead to a basically binary wilt response?

2) Following the first, why do even highly resistant germplasm exhibit some wilt?

3) What is the physiological and structural meaning of the apparent growth cap that differentiates resistance and susceptible germplasm?

4) Since highly resistant rootstocks still get colonized to the hypocotyl with moderate to high frequency, how do susceptible scions survive season-long and produce competitive yields when they are grafted to the hypocotyl? Is there rootstock-scion resistance cross-talk?

5) Mechanistically and structurally, what are breeders selecting for, and how can those features be tracked in a breeding program?

6) What is the nature of the relationship between vascular browning variation, wilt development, and host resistance? Is it useful for breeding and pathological evaluation?

7) Can some of these new imaging techniques provide data sufficiently useful and sufficiently high-throughput for application to BW breeding programs?

8) How much diversity of resistance is available in the global germplasm, and how much has been incorporated into commercial hybrid rootstocks?

9) Can growers rotate resistance genetics? Do all resistance sources perform well across the environments, soils, and pathogen strains of North Carolina and the greater Southeastern USA? What lines exhibit unique variation that is pathologically and agronomically suitable for rotation?
1.11: Miscellaneous novelties in bacterial wilt research.

In an effort to report on recent developments in the BW literature, there are several interesting reports that do not necessarily influence this research but seemed worth making a note of. On the molecular side, a collaborative effort recently mobilized the Rs genomes to investigate the phylogeographic variations in the quarantined race 3 biovar 2 (phylotype IIB) strains in order to understand possible routes of geographic spread (Clarke et al., 2015).

It was found that most of the disseminated isolates tested in Europe, Africa, and Asia belong to one clonal lineage, whereas the diversity of the South American strains support the view that the quarantine group of strains originated from that continent. Core effector screens were then performed in tomato, eggplant, pepper, tobacco, and lettuce germplasm using an Agrobacterium-mediated transient expression method to search for resistance sources. Several potential sources of resistance were found, suggesting that segregating populations from these lines could be mapped using the transient expression assay.

The work did not, however, validate the results with any actual inoculations. It also did not report the specific results of the tomato lines tested, only that they were drawn from a panel developed for diversity to Rs and Psuedomonas spp. pathogens that included “cv. Hawaii” (Clarke et al., 2015; Wroblewski et al., 2009).

On the management side, a report from Slovenia investigated the antibacterial activity of wild mushroom extracts on Rs growth in vitro and their value for plant protection applications (Erjavec et al., 2016). A number of mushroom species were observed whose extracts inhibited Rs growth. Several provided some protective activity when the stems were co-inoculated with Rs + extract. The active substances within the two best species extracts were proteins around 180 kDa in size.
1.12: Figures

Figure 1.1: Distribution of Ralstonia solanacearum in the USA.
Distribution of Rs in the USA in states where occurrence was found to be common, with losses from slight to severe. Redrawn from (Kelman, 1953).
Figure 1.2: Distribution of *R. solanacearum* in North Carolina.

Historical occurrence of Rs in NC counties as of 1952 (Kelman, 1953) and locations that our modern strain libraries were isolated from (red: tomato; blue: tobacco; purple: historic locations isolated from mostly tomato, and tobacco, but also eggplant, peanut, and potato.) Note: five additional counties have reported Rs since the 1953 survey, all in tomato.
CHAPTER 2: SPATIAL-TEMPORAL DYNAMICS OF THE TOMATO-BACTERIAL WILT PATHOSYSTEM-A HARMONIOUS MODEL FOR WILT DEVELOPMENT AND HOST RESISTANCE

2.1: Abstract

Bacterial wilt of tomato is caused by the globally important soil-borne pathogen *Ralstonia solanacearum*, a species complex. During host invasion, the bacteria infect the roots by migrating through the apoplastic spaces. They then penetrate the vascular cylinder, colonize the xylem tissue, and rapidly invade vertically into the hypocotyl and stem tissues, resulting in rapid wilt development leading to plant collapse. Efficacious host resistance is available, which has been characterized as limiting (but not preventing) spatial invasion of the stem xylem vessels and capping off bacterial proliferation within the stem. This quantitative resistance is currently deployed in rootstocks for grafted management of bacterial wilt. Curiously, the most resistant germplasm does not prevent colonization of the hypocotyl, and can exhibit low incidences of wilting, leading to questions about the host-pathogen dynamics within the context of grafted plants. Grafting experiments suggest the possibility of a rootstock-scion resistance crosstalk. We explored aspects of the spatial and temporal dynamics of colonization and bacterial proliferation in a 3-dimensional anatomical context with a mixture of grafting combinations of resistant and susceptible tomato germplasm. Through the application of novel imaging methods using light-reporter strains of GMI1000 (phylotype I), wilt development was found to be connected with a specific bacterial density threshold that is subsequently related to spatial invasion at the vascular tissue-system level in the hypocotyl. Furthermore, the work demonstrates/validates novel aspects of resistance in the roots and stems. A partial leaf wilt symptom is documented that highlights the importance of assessing wilt severity rather than simple wilt incidence. By tracking bacterial
invasion from root to shoot apex, the work verifies that *R. solanacearum* can regularly cross the graft union from resistant to susceptible tissues, but doing so does not lead to an obvious bloom in either bacterial density or spatial invasion. The results do not validate the hypothesis that resistant lines reduce the rate of vertical invasion. Finally, we propose an integrated tug-of-war model of invasion, wilt development, and how resistance modulates those parameters at each anatomically and physiologically important stage. The model relates to the anatomical changes occurring in the root-shoot junction, as well as other known resistance mechanisms and wilt-related phenomena. This work provides a phenotypic context for further investigations and breeding assessments in the bacterial wilt pathosystem.

2.2: Introduction

Bacterial wilt (BW) of tomato (*Solanum* spp.) is a major soil-borne disease of global economic importance, historical significance, and scientific interest (A. C. Hayward, 1991; Kelman, 1953; Mansfield et al., 2012). Bacterial wilt is caused by the *Ralstonia solanacearum* species complex (Rs), a vascular wilt pathogen with a very large host range. Many of the most globally important food, oil, fiber, and ornamental crops are impacted by Rs, as well as numerous weed species (A. C. Hayward, 1994; Kelman, 1953; Kumar et al., 2014). Additional hosts continue to be reported, most recently in *Rosa* spp. (Tjou-Tam-Sin et al., 2017) and *Cucurbita maxima* (She et al., 2017).

In Solanaceous hosts, Rs enters the roots through wounds and sites of lateral root emergence, colonizes the xylem tissue, moves up into the stem, and causes a rapid, permanent wilt through a combination of high bacterial densities and mass-production of extracellular polysaccharides (Grimault and Prior, 1993; A. C. Hayward, 1991; McGarvey et al., 1999; Meng,
2013; Schell, 2000). Within affected tomato fields, losses of susceptible varieties range dramatically (regularly 70 to 100% in heavily infested fields in North Carolina and under controlled inoculation) (P. Hanson et al., 2016; McAvoy et al., 2012; Rivard et al., 2012; Scott et al., 2005).

Strong quantitative resistance in tomato has been available for many decades. (Acosta, 1978; Daunay et al., 2010; Henderson and Jenkins Jr., 1972; Scott et al., 2005; J. Wang et al., 1998). Resistance, however, has only been successfully deployed in rootstocks due to a seemingly unbreakable linkage between small fruit size and high levels of resistance (Panthee et al., 2017; Scott et al., 2005). Vegetable grafting for soil-borne disease management is practiced around the world on a commercial scale where resistant rootstocks are surgically combined with susceptible scions (King et al., 2008; Louws et al., 2010). Resistance to BW is regularly deployed in a number of commercial hybrid and public inbred rootstocks, which can provide effective, season-long, field-level BW management.

There are several extremely enigmatic aspects of BW of tomato that are especially meaningful for BW management through vegetable grafting. These stem from core aspects of our fundamental understanding of the host-pathogen interactions. First, the most highly resistant varieties available do not prevent root, hypocotyl, or stem colonization by Rs. For example, studies of the resistant rootstock ‘LS-89’ indicated that the upper hypocotyl could reach infection rates of 90% within 14 days following a root inoculation with \(10^7\) CFU/mL of Rs suspension (Nakaho, 1997a). This leads to high latent infection rates in the stem, although fluctuations by strain, temperature, and inoculum density variation are reported (Ciampi and Sequeria, 1980; Grimault and Prior, 1993; Kressin, 2014; Nakaho et al., 1996; Nakaho et al., 2004).
Commercial vegetable grafting practices attach BW susceptible cultivars to highly resistant rootstocks in the upper hypocotyl or 1st internode. Plants grafted in this manner obtain season-long protection from BW and produce a harvest often 1 to 2 months after the non-grafted susceptible scion varieties have begun to wilt (Cardoso et al., 2012; Grimault and Prior, 1994a; Kressin, 2014; McAvoy et al., 2012; Nakaho et al., 1996; Onduso, 2014; Rivard et al., 2012; Rivard and Louws, 2008). Yet, nearly all studies examining Rs invasion up the height of the plant have not considered the effect of grafting.

Only a handful of studies have examined stem colonization dynamics as it impacts plants with susceptible scions grafted onto resistant rootstocks for BW management. Two studies showed that Rs can reach the basal hypocotyl in as little as 14 to 21 dpi and can cross the graft union (Nakaho et al., 1996; Nakaho et al., 2004). When the susceptible ‘Ponderosa’ was grafted onto the resistant rootstock ‘LS-89’ (the most resistant Japanese rootstock in use at the time) was examined, about 35% of the susceptible ‘Ponderosa’ scions were latently infected (Nakaho et al., 1996). In another set of experiments, the highly resistant ‘Hawaii 7996’ rootstock was added. Disease development was much greater in the experiment, yet ‘Hawaii 7996’ allowed about 39% of the susceptible scions to become latently infected 14 days after inoculation (Nakaho et al., 2004).

Another study examining a different resistance source reported that when the rootstock was either ‘CRA66’ or ‘Caraibo’ (a progeny of ‘CRA66’), no wilt was observed (Grimault and Prior, 1994a). Essentially no hypocotyl colonization was observed in ‘CRA66’ rootstock treatments, but susceptible scions on the ‘Caraibo’ rootstock treatment exhibited latent infection rates of 40% above the graft union and 20% as high as 10 cm above the graft union. It remains a mystery how graft combinations like these can maintain a relatively wilt-free state for an entire
season. The relatively high latent infection rates in the susceptible scions suggests either a rootstock-scion resistance cross-talk or that invasion above the hypocotyl is not meaningful for wilt development. Unfortunately, comparative resistance mechanism studies have only ever been performed using non-grafted material.

Another enigma has to do with the relationship between wilt development (a generally binary response) and the quantitative nature of the resistance mechanisms. Tomato is a diploid with 12 chromosomes (chr.) (2n=2x=24). BW resistance has been mapped in the most resistant genotype world-wide—‘Hawaii 7996’ (Carmeille, Luisetti, Besse, Chiroleu et al., 2006; Mangin et al., 1999; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996; J. Wang et al., 2000; J. Wang et al., 2013), as well as to a limited extent in ‘L285’(Danesh et al., 1994). These quantitative trait loci (QTL) mapping studies have reported up to 5 strong resistance loci (1 on chr. 12 and 4 clustered on chr. 6), as well as small-effect loci on 5 other chr. (3, 4, 8, 10, and 11). Various loci have been associated with variation in overall resistance strength (chr. 6 and 12), race and phylotype-specific resistance (all but chr. 6), days post infection (dpi) (chr. 6), suppression of bacterial multiplication in the stem (chr. 12), and even root drench verses stem inoculation methods in ‘L285’ (chr. 6 had a bigger impact on root drench, while chr. 7, and 10 on stem puncture).

Bacterial wilt resistance is complex and quantitative, affecting many aspects of the host-pathogen interactions over time and space. Wilt resistance is induced (Ishihara et al., 2012; Milling et al., 2011), and has been correlated with chemical and structural changes. Wilt resistance has been linked with a number of phenotypes: 1) restriction of radial cell-to-cell invasion in the vascular tissues of the hypocotyl (Grimault et al., 1994; Nakaho, 1997a; Nakaho, 1997b; Nakaho et al., 2004). 2) A vascular tolerance for high densities of bacteria in the roots.
and stem (Grimault and Prior, 1993). 3) Reinforcement and thickening of xylem cell walls and pit membranes (Nakaho et al., 2000). 4) Innate variation in root morphology and reduced cortical invasion (Vasse et al., 1995; Vasse et al., 2005). 5) Delayed colonization of the root stele and reduced overall proliferation in resistant roots (Caldwell et al., 2017). 6) A reduction in the frequency of vertical invasion in the stem tissues (Prior et al., 1996). 7) Production of tyloses and gums in invaded xylem vessels, albeit with an uncertain degree of importance (Grimault et al., 1994). 8) Lignin biosynthesis and deposition (Ishihara et al., 2012), and possibly callose deposition (Beckman, 2000). And, 9) a suggested vascular hypersensitive response (Nakaho et al., 2016). It is important to understand what resistance mechanisms are most related to the development of the disease, and how the mechanisms all work in harmony to maintain a turgid, productive state in resistant germplasm.

A final enigma relates to the invasion pathway of Rs in tomato. Plants are composed of three vegetative tissue-systems—Ground (pith, cortex, mesophyll, etc.), vascular (xylem and phloem), and epidermal. These tissue-systems build the various organs. During invasion and colonization of tomato tissues, Rs must successfully traverse through all three of these systems and their associated unique cell types. It must also navigate through apoplastic (middle lamella) and pseudo-symplastic (xylem vessel lumens and xylary pits) pathways. It must migrate through the anatomically and functionally diverse root, hypocotyl, and stem organs. Finally, in order to produce economically meaningful disease, it must do so very rapidly, as quickly as 4 to 6 days (Ishihara et al., 2012; Schell, 2000).

Each tissue-system, organ, and cell-type can provide unique barriers to colonization and antagonistic defenses against bacterial proliferation that Rs must penetrate, bypass, suppress, or evade. Grafting and structural investigations regularly identify the hypocotyl region as the key
region determining the fate of the plant (Grimault and Prior, 1994a; Nakaho et al., 1996; Nakaho et al., 2004). Furthermore, the effect of resistance on reducing colonization of single vascular bundles is well documented (Caldwell et al., 2017; Nakaho et al., 2000; Nakaho et al., 2004). But a systematic investigation into the invasion patterns at a whole-plant and tissue-system level are lacking. What is so unique about the hypocotyl region that allows it to largely determine the fate of the plant?

It is logical that connecting key resistance mechanisms to disease development must consider Rs invasion at the tissue system and organ level. But, in order to mobilize this understanding of resistance mechanisms into applied plant breeding and pathology, they must be measurable in a higher throughput manner than what light and electron microscopy and histological staining can offer. Towards this goal, new tools and pipelines are being developed and tested for potato breeding programs that employ Rs strains transformed to constitutively express the luciferase operon (LUX) or the green fluorescent protein (GFP) (Cruz et al., 2014; Ferreira et al., 2017; Monteiro, Solé et al., 2012; Monteiro, Genin et al., 2012).

This work explores the adaptation of these technologies towards tomato breeding and basic host-pathogen discovery applications. In the process, this work endeavors to resolve the three previously highlighted enigmatic phenomena—colonization dynamics in grafted tomatoes, quantitative host-pathogen interactions leading to basically binary wilting, and the impact of whole-plant system zones on disease development. We tested the hypotheses that: 1) Rs crossing the graft union into susceptible tissue happens and does not cause wilt initiation, 2) the wilting and bacterial density patterns are both related to a critical spatial colonization threshold within the vascular volume of the stem, and 3) the hypocotyl is a bottleneck zone that drives the disease system because it is the location of the root-to-shoot vascular transition. Finally, we propose a
harmonious model for BW development in tomato at the whole-plant level that integrates the known resistance phenomena.

2.2: Materials and methods

2.2.1: Plant materials and growth

We performed controlled environment disease experiments on potted grafted seedlings. We chose to use three tomato lines of *S. lycopersicum*—one highly susceptible commercial scion and two resistant rootstocks with unique breeding backgrounds. Tomato seeds of ‘Marmande’ (M; highly susceptible scion (Monteiro, Genin et al., 2012), Leroy Merlin Seed), ‘Shield’ (S; moderately resistant commercial hybrid rootstock, Rijk Zwaan), and ‘Hawaii 7996’ (H; highly resistant, public open-pollinated breeding rootstock) were sown on the surface of potting medium in 5 cm bottom-watered pots covered with plastic wrap for germination in a growth chamber (Either a FITOCLIMA 1200, Aralab, Portugal, or a SCLAB S.L., Barcelona, set at 27 °C or 25 °C, respectively) with 60% humidity under 12h day/night LED or fluorescence lighting (light intensity of 120-150 μmol·m⁻²·s⁻¹). After about 8 to 11 days the seedlings were transferred singly to fresh pots of the same type and grown under the previous conditions. The plants were bottom watered with water to about 2 cm deep after the soil began to dry and the plants began to wilt a little (about every 2 to 3 days) to encourage root growth and hardening. No fertilizers were ever applied, and no signs of nutrient stress, stunting, or pot bound roots were observed; the plants continued to grow in a healthy manner.
2.2.2: Standard “soil” grafting

Once the stems became 1.5 to 2.0 mm in diameter (between 9 to 18 days), they were top-grafted using a growth chamber—adapted version of the method of Rivard and Louws (2006) with the following adaptations: We grafted all the plants about 2 cm below the cotyledons and used a steeper angle cut (about 70°). We were producing multiple batches of plants in overlap, so we placed each tray of 24 pots with grafted plants into a plastic tub (Altuna 2594005, Stewart Garden, United Kingdom) with a clear vented lid filled with 3 to 4 cm of water. The lids were misted with water and the vents were closed. The tubs were placed in the growth chambers and the above lights were turned off. Over the next 8 days, we acclimated the grafted plants back to light, and then to ambient humidity, misting the lids as needed. The timeframe for healing was: 36 to 48 hours in the dark; 24 hours at 10% light; 24 hours at 50% light; 24 hours at 100% light and vents opened; 48 hours with lid partly opened; 24 hours with lid removed; removed from tub. After the healing process, plants were grown under the previous conditions until inoculation.

2.2.3: In vitro grafting

Seeds of each genotype were surface sterilized in 35% bleach and 0.02% Triton-X 100 for 5 minutes and then rinsed thoroughly with sterile distilled water (at least 5 times). They were then sown onto sterile filter paper placed on Murashige and Skoob medium in square culture plates (Sudelab S.L., Barcelona). The plates were sealed with surgical tape (to allow some airflow) and grown in a walk-in tissue culture growth chamber (Inkoa, Vizcaya, Spain) set at 22 °C, 16/8h day/night with fluorescent lighting. In order to match up hypocotyl diameters, seeds of ‘Hawaii 7996’ were placed in the chamber 24 hours before ‘Shield’ and ‘Marmande’, which were kept at 4 °C in the meantime.
About 8 to 13 days after sowing, the seeds had sprouted and were just barely beginning to develop the first true leaf. Using sterile tools and a laminar flow hood, the cotyledons were removed and the plants were cut at a perpendicular angle about 1 to 2 cm below the cotyledons. The parts were then transferred to fresh plates without filter paper—the scion on one plate and the rootstock to the other. Care was taken to keep lids closed as much as possible to reduce desiccation. After 8 plants were transferred, the same process was performed on the other variety for the graft combination. The two pieces were then gently maneuvered so that the stems laid flat and the cut surfaces were touching. No clips or other stabilizing devices was used other than the surface tension of the moisture on the plate. Self-grafted combinations were made by reattaching the two parts of the same variety. The plates were gently covered, sealed as before, and placed standing upright in racks back in the tissue culture growth chamber with the same light and temperature regime.

After about 12-20 days, successfully healed plants were transferred to the same pots and soil conditions as the previously mentioned soil-grafted plants. In order to acclimate the plants to the growth chambers, they were placed in small acclimation boxes with transparent, vented lids (HORTOSOL, Germany) and placed in the same growth chamber conditions as the soil-grafted plants under full light. The vents were opened after 24 hours, and the trays were removed from the acclimation boxes. The plants grew under the same conditions as the soil-grafted plants until inoculation.

2.2.4: Bacterial strains and inoculum preparation

We used two modified strains of the well-known GMI1000 isolate of the Rs species complex (phyloptype 1, now considered to be the distinct species *Ralstonia pseudosolanacearum*)
(Safni et al., 2014) although the disease symptomology is indistinct from the North American phylotype IIA strains). Strain 372 constitutively expressed the luciferase reporter operon (\textit{LuxCDABE}; GMI1000-LUX or simply LUX), while strain 528 constitutively expressed the green fluorescent protein (GMI1000-GFP or simply GFP).

Bacterial inoculum was prepared by streaking each strain on solid Phi medium amended with 10 µg/ml gentamicin, 0.005% 2,3,5-triphenyl tetrazolium chloride (TZC), and 0.5% glucose and incubated for at least 24 hours at 28 °C. Virulent-type colonies were picked and transferred to sterile liquid Phi medium with the same amount of gentamicin and incubated on a shaker at 28 °C. After incubating overnight, the ~10^9 CFU/mL liquid culture was spectrophotometrically adjusted with distilled water to 10^7 CFU/mL (OD_{600}=0.01) for the desired volume of inoculum. An aliquot of the strain 372 suspension was tested for luminescence to validate the expected appropriate expression.

At least 1 day before inoculation, the plants were examined for graft quality and any plants with physically unstable unions were removed. The grafting clips were removed and the plants were transferred to the infection chamber (the same FITOCLIMA 1200 conditions as previously described set at 27 °C air temperature). The soil was allowed to dry somewhat overnight to encourage inoculum retention.

Each experiment was inoculated when the plants had reached the 7 to 9 true leaf stage, which ranged from 17 to 24 and 40 to 44 days after grafting (45 to 51 and 50 to 57 days after sowing) for the soil-grafted and \textit{in vitro}-grafted experiments, respectively. The roots were lightly injured by piercing the soil at the 4 corners of the pots all the way through with a 1 mL pipetted tip and then poured 40 mL of the above bacterial suspension or distilled water over each root
ball. Care was taken to prevent the bacterial suspension from being poured onto the stem or leaves.

The plants were immediately placed into the infection growth chamber and not watered for 24 hours, afterwards the soil was kept evenly moist for the duration of the experiment. The inoculation parameters were the same for both the LUX and GFP reporter strains.

2.2.5: Experimental design

We performed several initial tests to optimize our experimental design, conditions, and assessment procedures. We principally used the LUX-labeled strain for the replicated experiments, while the GFP-labeled strain was used with extra plants of each treatment. Our final experimental design involved 2 grafting methods (soil and in vitro), 3 destructive harvests (3, 6 or 7, and 10 dpi), 7 graft combinations. The grafting treatments are designated as rootstockX-scionX (rX-sX)—self-graft (SG; M, S, and H), the commercially used graft combination (CG; rS-sM and rH-sM), and the reciprocal-graft (RG; rM-sS and rM-sH). Each unique combination of factors usually had 3 plants as subsamples but were occasionally less or more depending on grafting success. Each experiment was repeated twice. A 3rd experimental batch was observed non-destructively up to about 20 dpi in order to generate disease progress curves.

When the plants were harvested, they were assessed at the whole plant level for wilting, and then were destructively assessed at 7 locations within each plant—taproot (TR), basal (BH; just above the first root emergence) and distal hypocotyl (DH; within ~2 cm below and including the cotyledons) regions, the graft-union region (G; 1 to 2 cm around the union) and internodes 1, 2, 3, and 6 (I1 through I6, respectively). The plants were arranged in trays for each harvest such that all the plants for a particular strain*harvest time were in the same tray exposed
to the same water source. Additionally, the subsamples of each graft combination were arranged in rows within each harvest tray. Treatments having ‘Marmande’ roots were interspersed between rows having ‘Shield’ or ‘Hawaii 7996’ roots to minimize secondary inoculum pressure variation from bacterial leakage from infected roots. Due to space limitations, experiments using each grafting method had to be performed separately, thus each experiment was the unique combination of graft-method*time replication, and was a full 3*7*7 factorial within each experiment. Destructive harvests of strain 528-inoculated plants were harvested at variable dpi and focused on the TR, BH, DH, and I1 locations. They were otherwise treated the same. Water mock-inoculated control plants for each graft combination and method were also included.

### 2.2.6: Non-destructive assessments

At each harvest and for the disease curves for all experiments, the plants were scored for wilt severity using either a percentage of wilting leaves or a wilt severity scale, as well as simple incidence (presence/absence). The percentage leaf wilt was determined by counting the number of wilting leaves and dividing by the total number of leaves on the plant (partially wilting leaves were counted as ½ a leaf). We used a 0 to 5 wilt scale that we use in field and greenhouse wilt assessments (see chapter 3, figure 3.1; after (Kressin, 2014) with some modification), where 0 = healthy, no wilt; 1 = 1 to 9% of canopy is wilting; 2 = 10 to 49% canopy wilt; 3 = 50 to 90% canopy wilt; 4 = 91 to 100% canopy wilt; 5 = permanent wilting point, plant collapse, tissues beginning to dry/decay.
2.2.7: Destructive assessments

After scoring wilt severity, the plant roots were washed and lightly surface-sterilized. To
do this, the soil-less potting media was removed with water and finger rubs, the roots were
soaked for at least 1 minute in clean water amended with bleach (~5% bleach), and then soaked
for at least 1 minute in another beaker of clean water without bleach. Then the leaves were
removed with a razor blade and the clean plants were placed under moist paper towels and
misted periodically.

In order to help assess bacterial invasion at the whole-plant level, an adapted version of
the LUX reporter system previously developed for the bacterial wilt of potato pathosystem was
used (Cruz et al., 2014). Whole-plant luminescence images were collected from plants inoculated
with strain 372 or water using a ChemiDoc Touch imaging system (Bio-Rad, Spain). The bare
plant stems were cut into 2 to 3 pieces no greater than 13 cm long and long roots were trimmed
so that they would fit on the imaging system tray. In all images, the pieces were arranged from
left-to-right from root to apex. Grayscale pictures were taken using a 0.5 second exposure.
Luminescence was measured using a 5-minute exposure time with the 3x3 sensitivity setting
(moderately high resolution). Positive luminescence points were determined by eye as an
obvious darker color than the tissue background. Overexposed pixels were indicated with red,
but none of the images ever reached that point.

Following the whole-plant imaging, each plant was dissected at the previously described
7 locations (Supplemental 2.1C) with a razor blade, which was sterilized in 95% ethanol + flame
between each plant. Beginning with I6, transverse cuts were made at each location moving
towards the TR. A series of measurements were performed at each location:
1) Each cut surface was imprinted at least twice on solid Phi medium (described previously) and incubated at 28 °C for 24 hours. Variation in the amount of Rs growth in each imprint allowed for scoring the amount of bacteria growth given the area of the imprint: 0 = no growth; 1 = 1 to 25% of the area is filled; 2 = 26 to 50% of the area is filled; and 3 = 50 to 100% of the area is filled (Kressin, 2014). The plates were observed for another 24 hours to ensure that no Rs growth appeared in locations scored as 0. If there was visible growth, the location was scored as 1 (this occurred often enough in tissues where Rs was not detected by any other means that it was worthwhile perform).

2) The severity of vascular browning (VB) was scored (presented in chapter 3, figure 3.2).

3) A 0.5 cm section was excised and placed into a sterile 2 mL tube filled with 200 uL of sterile distilled water and incubated at room temperature for at least 20 minutes. After anywhere from 10 minutes to 1 hour, the tubes were placed in a luminometer (FB 12, Berthold Detection Systems, Germany). After a 10 second incubation in the machine, relative light units per second (RLU/s) were measured for 10 seconds. The final mean level was recorded.

And, 4) two transverse slices about 1 mm thick were made and arranged according to location on an empty sterile culture plate humid box (after the pilot tests, we settled on only taking measurements on the BH through I1). In addition, the two halves of a radial slice through a 1 to 2 cm section around the graft union, as well as the two transverse sections at the bottom of the segment were also arranged in the box. The lid was kept closed as much as possible and frequently misted to keep the slices turgid. Generally, the plants of 3 to 4 graft treatments could fit in a single plate. It was not necessarily feasible to maintain the same rotation of slices coming from the same plant, thus the locations of signal in adjacent slices should not be directly
compared spatially. The plates were then placed into the previously mentioned ChemiDoc Touch imager, the lid was removed, and grayscale, ultraviolet (UV), and luminescence images were taken. The light and luminescence settings were the same as the whole-plant images, but were taken at a closer zoom. The UV image exposure rates were automatically determined by the software (generally about 0.2 to 0.4 seconds). Post-capture image adjustments for the whole-plant and sliced samples were made to standardize the images using the Bio-Rad Image Lab software (version 4.0 or better). For the tissue location slices, high and low signal intensity images were needed in order to give tissue localization clarity between low and high bacterial densities. For the high intensity spots, high = 1000, low = 400, gamma = 1.15; for low intensity spots, high = 3000, low = 400, and gamma = 1.15.

For plants inoculated with the GFP-labeled strain 528, none of the luminescence-based measurements were performed. Imprints and VB severity were measured as described above. Binocular microscopy was performed with a UV fluorescent lamp and DP71 (12.5 megapixels) color camera system equipped to a SZX16 stereo microscope (Olympus). Incident lighting was used for bright field observation, as well as with the UV and GFP fluorescence filters (BP330-385 BA420 and BP460-495 BA510IF, respectively). Transverse sections of stem were excised from various locations in the plants (typically TR to I1) and placed in sterile humid box plates with or without a layer of 1% agar gel underneath. The lids were misted frequently. The microscope camera software was white-balanced, and then the sample plates were placed over a black background. Entire transverse sections were imaged with the three filters with the 1x objective combined with the 2x to 2.5x zoom. Taproot slices were imaged with a size-appropriate higher level of magnification (typically 6 to 8x). The same software parameters were used per filter to capture the images.
The root-to-shoot vascular transition was examined in tomato seedlings of ‘Cherokee Purple’ at the 4 to 5 leaf stage. Seeds were sown in a spring glass house in Faferd 2P soilless potting media and germinated on a heating pad at 30 °C. The seedlings were lightly fertilized using a 20-20-20 nitrate-based water-soluble commercial fertilizer. Upon reaching the desired size, plants were harvested, the growing medium was gently washed off, and the plants were hand-sectioned from TR to I1 using a razor blade. Transverse sections of TR, hypocotyl, and stem were histologically stained with toluidine blue for 10 to 20 seconds, and then were quickly rinsed several times with clean water. The slices were then fresh-mounted in water on glass microscope slides with coverslips and imaged at 40x and 100x magnification with compound microscopy—Leica DM E microscopes (Meyer Instruments, Houston) equipped with Infinity1-2 2.0-megapixel CMOS digital cameras (Lumenera Corp., Ontario). Toluidine blue stains xylem tissues green to green-blue, fibers pale blue-green, and parenchyma tissues red-purple (Parker et al., 1982).

2.2.8: Data and statistical analyses

Wilt severity, VB severity, and colonization indexes were calculated for each plant as a percentage of the maximum possible score. These values were then used for statistical comparisons. Additionally, the wilt severity index and the percent leaf wilt (PLW) variables were basically identical with a nearly 1:1 relationship (n = 259; PLW = 0.202(wilt index) – 0.002; \( \rho = 0.99791; p < 0.0001 \)) and both were also highly correlated with wilt incidence (\( \rho_{\text{wilt index}} = 0.93241, \rho_{\text{PLW}} = 0.92304, p_{\text{both}} < 0.0001 \)). Thus, our analyses focused on the wilt index rather than PLW since it incorporates the aspect of plant death.
The relationship between RLU/s and CFU/mL was used to calculate bacterial density within the 0.5 cm tissue. The plant tissue was left in the tube for the imaging process in order to prevent the artificial loss of Rs signal. The correlation between luminescence and either Rs gene expression or presence \textit{in planta} has been documented (Cruz et al., 2014; Ferreira et al., 2017), but it needed to be validated for our specific strain, crop, and experimental conditions. To do this, dilution plating was performed on a subset of the 0.5 cm stem/root pieces from both pilot and replicated experiments that represented the range of RLU/s we were observing (0 to \(~2.4\) million). We compared water blanks, mock-inoculated, and strain 372-inoculated plants with or without symptoms, as well as stem and TR tissue types. After at least a 1-hour incubation at room temperature to allow bacteria to ooze from the tissue, water from each tube was serially diluted. Two technical replications of 10 or 20 uL samples from each dilution were used (more volume was plated if the RLU/s were less than 100 for TR and 300 for stem tissues). These samples were dropped onto solid Phi media and the drops were allowed to air dry in a clean air hood. They were then sealed with parafilm and incubated overnight at 28 °C. Once microcolonies became visible (~16 to 20 hours later), colonies per drop were counted, and the average of the two technical reps was recorded. Dilutions containing 10 to 150 colonies were used for the calculations.

To avoid reporting bacterial densities that were inflated by the tissue background (false positives), the bacterial density formulas were complemented with the other methods of detecting or inferring the presence of Rs that were performed. For example, the stem imprint data was highly sensitive, able to detect even the lowest bacterial densities observed by the plate counts. Occasionally, there were samples where the observed RLU/s/mL were clearly above the background signal or that exhibited a visible luminescence signal, but the imprint failed to grow
Rs. In those cases, it was assumed to be a real infection. In addition, we never observed VB at any time in any mock-inoculated plants, but regularly observed it in inoculated plants. These additional measurements allowed for the creation of variables indicating a highly probable positive colonization at each location. A tissue location was considered colonized if the imprint was positive for Rs growth, the RLU/s were clearly greater than the tissue background (TR threshold set to 200; stem thresholds set to 450), or there was VB. Based upon these indicator variables, we calculated the bacterial density within each 0.5 cm piece of tissue using the regression equation where the indicator was positive, otherwise bacterial density was considered to be 1 CFU/mL.

Due to some unexpected and unexplainable grafting losses, some experimental units had 1 or 2 plants for a given harvest (mean = 2.87; mode = 3). More extremely, in the case of 3 experimental units (out of 84), we chose to maintain more plants in earlier harvests to validate phenomena of interest, which meant that three units were missing entirely (in vitro rep 1 harvest 3 rS-sS LUX; soil rep 2 rH-sH LUX harvests 2 and 3). To compensate, we nested the main effect of harvest under grafting method and grafting combination during analysis.

We used SAS 9.4 for all statistical analyses, particularly the procedures UNIVARIATE, MEANS, CORR, REG, MIXED, and GLIMMIX for generating the LSMEANS and HISTOGRAM outputs. All pairwise comparisons of means were computed with the Tukey-Kramer p-value adjustment for multiple comparisons for an overall $\alpha = 0.05$. No significant differences in wilt parameters were observed between in soil and in vitro grafting methods (Supplemental 2.2A), nor for any other variable measured, so grafting method contrasts are not presented for the other variables.
2.3: Results

How does Rs practically cause wilting symptoms, and why do even highly resistant plants still experience some wilt? Does Rs reach the graft union in grafted plants? If so, what is the result? Is there any evidence of rootstock-scion interactions between resistant and susceptible germplasm? What is the relationship between the multiple, quantitative resistance mechanisms/phenomena and binary wilt induction, and what features are most useful for measuring these resistance mechanisms in a higher-throughput manner?

2.3.1: Wilt development.

Wilt development under our pilot test conditions occurred at just after 72 hours and plateaued by 10 days (Figure 2.1A and Supplemental 2.2C). Based on the disease progression for each grafted treatment in the preliminary tests, we chose to destructively harvest plants at three timepoints: 1) before wilt development, when there was the greatest probability of the plants being infected (3 dpi); 2) once the susceptible controls were nearly fully wilted and resistant plants might start showing wilt symptoms (7 dpi); and, 3) when the resistant plants exhibited some wilt incidence, susceptible plants were all permanently wilted and beginning to decay, but before meaningful data was lost due to desiccation (10 dpi).

Two conditions leading to wilt were observed—whole-plant and partial leaflet wilt (Supplemental 3B). While dissecting plants of the second wilt condition, it was obvious from our imaging and the VB that Rs had colonized a primary xylem bundle that fed the wilting leaves and invaded the leaflets, but not other primary xylem bundles at the same height (data not shown). This is in contrast to the whole-plant wilt in which Rs was not detected in the leaves themselves, suggesting wilt was related to the interactions in the stem.
Strong differences in wilting were observed between the grafted treatments. The performance of the plant was entirely dependent upon what was the rootstock (Figure 2.1A and 2.1B white diamonds). In contrast, the differences in plant colonization between grafted treatments was greatly reduced compared to wilt severity, and the pattern was the same (Figure 2.1B, black bars). No significant differences in wilt parameters were observed between in soil and in vitro grafting methods (Supplemental 2.2A, nor for any other variable measured). There were also significant differences over sampling times (p<0.0001) for wilt index and plant colonization, which increased significantly after sampling 1 and then plateaued (data not shown). In our conditions, resistant rootstocks strongly reduced wilt development of susceptible ‘Marmande’ scions in a quantitative manner, such that the grafting treatments sharing the same root genotype were nearly identical, regardless of the scion genotype.

2.3.2: Wilt-related density threshold by height and host resistance level.

We were interested in the factors that affected wilt development, and how those factors were modulated by the host resistance level. We left the excised tissue in the tubes during the height-based luminescence measurements in order to prevent the loss of signal from any bacteria remaining in the tissue. That tissue exhibited a low background fluorescence in the water controls (250 to 400 RLU/s for stem and 50 to 150 for TR; sterile distilled water was 39 ± 5.5 RLU/s). Fresh tissue weights indicated a positive, linear relationship with the RLU/s background signal that was related to tissue type (data not shown). Tissue subsamples from inoculated plants that exhibiting RLU/s values clearly above the background luminescence (n=35) were used to establish the relationship between CFU/mL and RLU/s (Supplemental 2.1A). Because RLU/s is
dependent upon the sample volume, the values were adjusted for the volume of the liquid in the tube (200μL) to be RLU/s/mL.

The relationship between bacterial density and RLU/s/mL in the dilution plating subsamples was found to be significantly correlated, regardless of tissue type and wilt status (log$_{10}$ transformed, $ρ = 0.95972$, $p<0.0001$). The data for both the taproot and stem tissues best fit a power function, where: $\text{CFU/mL} = 573.52 \times (\text{RLU}/\text{s/mL})^{0.9878}$, $R^2 = 0.9211$. Thus, bacterial densities $\geq 8 \times 10^4$ (TR) or $8 \times 10^5$ (stem) had low accuracy and should be considered more qualitative, while densities above that could be measured quantitatively with much greater accuracy (Supplemental 2.1A).

The log(CFU/mL) values of each height-based location within the plant, as well as the maximum CFU/mL observed over all locations, were correlated with the wilt severity scores. A significant positive correlation was detected ($ρ = 0.61846$ to $0.73437$; $p<0.0001$). To gain more information about the association, the frequency of wilt severity was plotted against the maximum bacterial density observed in each plant. Inspection of the trends at 1.0 log increments indicated a rapid shift from no wilt to complete wilt over a 1 log increment ($10^7$ CFU/mL) (Figure 2.2A). Zooming in on the log(CFU/mL) range of 7.0 to the maximum observed (9.1) indicated that wilt induction began in a narrow range of 7.1 to 7.7 log(CFU/mL), after which wilt progressed rapidly to the permanent wilting point above 8.0 log(CFU/mL) in a somewhat binary manner (Figure 2.2B).

We then considered if the density-related wilt threshold was related to a specific tissue location. Plotting the density of each plant over the height revealed that all wiling plants crossed the density threshold range at some point along the stem, while healthy plants never crossed it (Figure 2.2C, Marmande SG given as an example). Close inspection of the plots indicated that
the overwhelming pattern was that the threshold was crossed in the hypocotyl region first. In rare instances, the bacterial density only reached the wilting threshold in the upper canopy (I3 and I6), but wilt was still observed. The wilt threshold appeared to be a feature of the stem rather than the TR, because whenever the TR had a bacterial density above $7.0 \log(\text{CFU/mL})$ and the stem density was less than that, no wilt was observed (data not shown). Furthermore, the patterns and density threshold values were the same in all three tomato cultivars, regardless of the level of resistance (data not shown). More resistant genotypes simply reduced the number of plants that reached the wilt threshold. Thus, the tomato plants wilted when the bacterial density reached a specific threshold, regardless of the host resistance level and location in the stem.

2.3.3: Effects of resistance on invasion and bacterial density in the roots.

In order to assess colonization at the whole-plant level, whole-plant luminescence was measured. The whole-plant images provided the lowest detection sensitivity of all the methods employed in these experiments, but was useful for tracking densities in the range of $5 \times 10^6$ CFU/mL and greater (data not shown). A side-by-side comparison of grafting treatments exhibited a striking phenomenon. Whenever the root tissue was ‘Marmande’, substantial luminescence intensity was visible in the TR and basal roots (Figure 2.3A, red circles). In contrast, whenever the roots were ‘Shield’ or ‘Hawaii 7996’, essentially no luminescence was observed coming from the roots, even in plants where the scion intensities and wilt severities were equivalent with plants having ‘Marmande’ roots (Figure 2.3A, blue circles). The pattern was extremely consistent. A re-examination of previous whole-plant images from the pilot experiments, as well as unrelated in vitro screens of very young seedlings, exhibited the same pattern (data not shown).
The whole-plant images suggested that the root systems of resistant genotypes suppressed bacterial proliferation in a manner distinct from the stem tissues. To test this, the bacterial densities in the 0.5 cm pieces of excised taproots were compared across grafting treatments. The results indicated that the resistant rootstocks had a significantly reduced mean bacterial density in the TR (about 2- to 3-fold less CFU/mL) compared to treatments where the rootstock was ‘Marmande’ (Figure 2.3B). It might be that the density reduction was skewed by a reduction in TR colonization (some early infections appeared to enter the hypocotyl before the TR, presumably through the basal roots). Thus, the invasion frequencies and percent of locations invaded were analyzed more broadly to include the BH and DH. Significant reductions in root/hypocotyl colonization incidence and index were observed when the roots were resistant, which ranged from a 20 to 41% decrease (Figure 2.3C). The basic pattern of differences between grafting treatments were the same for all three variables—density, invasion incidence, and invasion index. Collectively, these results indicate that both the ‘Hawaii 7996’ and ‘Shield’ rootstocks suppress bacterial density in the roots and reduce the frequency of plant invasion.

2.3.4: Colonization patterns below and above the graft union.

To improve our understanding of the dynamics of vertical movement of Rs, we assessed multiple variables at 7 locations from TR to I6. The hypothesis was that Rs would cross the graft union regularly without compromising the health of the plants. Overall, of all the inoculated plants in all the experiments (n=241), colonization was not detected in 22% (mostly resistant rootstock treatments at 3 dpi), while 15% were colonized only below the graft union and 63% were colonized to some level above the graft union. 47% of all plants were colonized all the way to I6. 71% were colonized in the BH and 61% were colonized in the DH (just above the graft
union). In contrast, 39% exhibited some level of wilting. Thus, Rs regularly crossed the graft union, leading to a 22% scion latent infection rate.

In order to breakdown the colonization patterns, several trends were examined over height (internode)—bacterial density (Figure 2.4A, C, and E) and mean colonization incidence (Figure 2.4B, D, and F). It was observed that both variables exhibited nearly identical patterns. For both, the main effect levels for grafting treatment and height exhibited significant differences (p < 0.0001) while the interaction did not (p = 0.8704 and 0.9973 for density and colonization incidence, respectively). This indicated that the slope trends did not vary between unique combinations of grafting treatment and height. For both bacterial density and colonization incidence (of the 7 heights), the main effect means of the grafting treatments were relatively similar to what was observed for the wilt index—it was entirely dependent upon what was the rootstock (Compare figures 2.4A-B with Figure 2.1B). Additionally, increasing height was inversely related to bacterial density and colonization incidence. The TR and BH locations had the highest mean bacterial density and colonization incidence and it steadily decreased moving towards the stem apex (Figure 2.4C-D). No significant interactions were observed between any other variable and height.

Within each grafting treatment, the steepest decline in either bacterial density or colonization incidence was found in the hypocotyl region, diminishing somewhat or flat-lining from I1 to I6 (Figures 2.4E-F). The only other noteworthy feature of the change in bacterial density and colonization incidence was that in rH-sM treatments, the resistant TR and BH locations had significantly higher levels (p= 0.0451 to p<0.0001) than the susceptible tissue locations above the graft union. This trend was not as strong in the rH-sH treatment, where only the TR was significantly greater than I6.
A comparison of density and colonization trends between grafting treatments at the same heights revealed that rH-sH exhibited significantly lower levels than rM-sM at all 7 locations (p=0.0250 to p<0.0001 and p=0.0452 to p=0.0011, respectively), while rS-sS was only consistently less at the TR (p=0.0450 for both). Furthermore, rH-sM was not different from rH-sH at any location for either density or colonization incidence (p=1.0000 to p=0.8857 and p=1.000 to p=0.8980).

On the other hand, rH-sM was significantly lower than rM-sM at all locations for density (p=0.0072 to p=0.0002) and from the DH upwards for colonization incidence (p=0.0017 to p=0.0002). Similarly, bacterial density and colonization levels observed in the reciprocal treatment rM-sH were never different than rM-sM (p=1.0000 to p=0.7872 and p=0.9974 to p=0.9972). The reciprocal was also significantly greater than rH-sH (p=0.0002 to p<0.0001 and p=0.0076 to p<0.0001) at every height. The respective patterns in both variables were the same for the relationships between grafting treatments with ‘Shield’ rather than ‘Hawaii 7996’, but with less distinction. In summary, it appears that there was a main effect of “tomato stem” on the bacterial density and colonization trends over height, while the rootstock genetics simple adjusted the base intercept.

It was difficult from the density and colonization means to clearly inspect for an association of vertical invasion of the stem and wilt development. All plants of rM-sM colonized to I6 (n=35) were wilting. A closer inspection of the grafted treatments revealed that 16.7% (n=30) of rH-sH and 18.5% (n=27) of rS-sS plants were colonized all the way up to I6 without wilting, while 5.4% (n=37) of rH-sM and 2.7% (n=37) of rS-sM plants were colonized to I6 without wilting. For the reciprocal treatments, 2.7% (n=37) of rM-sS and 13.5% (n=37) of rM-
sH were latently infected up to the I6. Thus, there was not an obvious genotype-specific effect on Rs reaching the highest measured location in the plants.

*Ralstonia solanacearum* was detected above the graft union in 98.92% (n=93) of all wilted plants regardless of the severity of wilting. In contrast, a plant-by-plant inspection of all non-wilted plants at 10 dpi revealed that 25% (n=4) of rS-sM and 22% (n=9) of rH-sM were latently infected above the graft union, compared to 50% (n=4) of rS-sS and 50% (n=8) of rH-sH plants. Thus, we observed that the presence of Rs above the graft union was associated with wilting, but latently infected scions were detected frequently. There appeared to be a numerical trend where crossing the graft union into a susceptible scion increased the risk of wilt development, but sizable percentages of latently infected exceptions were observed.

### 2.3.5: Xylem colonization at the vascular bundle and tissue-system levels.

We thought that the wilt and bacterial density patterns might be explained by the transverse colonization patterns at the vascular tissue-system level. Towards this finer level of inspection, we assessed luminescence localization patterns of transverse stem sections across the grafted treatments. The xylem tissues in all three tomato genotypes were arranged into four primary bundles in the hypocotyl connected by the interfascicular cambium. As secondary growth progresses, secondary bundles develop within the interfascicular cambium (typically 2 to 4 in lower stem in our experiments).

As the height increases, the vascular bundles fragmented into additional discrete bundles. While the lignification of the entire cambial ring was present, there was little-to-no development of vessel elements in the interfascicular cambium other than the secondary vascular bundles (the blue rings in the UV images of figures 2.6 and 2.7). The interfascicular cambium was comprised
nearly entirely of xylem parenchyma and possibly some xylary fibers. Additionally, the hypocotyl region exhibited more secondary xylem and interfascicular cambial development than did the internodes, as well as overall volume of xylem tissue. Thus, most of the bulk water flow through the stem would have been conducted predominantly through the primary and secondary bundles.

Overlays of the luminescence images with the corresponding light and UV images clearly localized the GMI1000-LUX signal to the xylem ring, and single spots matched the location of primary and secondary xylem bundles (data not shown). A highly heterogenous bacterial invasion pattern was observed within the xylem tissues of infected plants of all levels of resistance (Figure 2.5). The first signs of vascular invasion were almost always localized with the primary bundles. Some secondary bundles were usually invaded next, along with additional primary bundles. Invasion of the interfascicular cambium and ground tissue system (pith and cortex) were more related with higher levels of vascular colonization.

As height increased, both the number of infection spots and the overall intensity of the spots diminished somewhat; the most spots and most intense signal was observed in the hypocotyl region typically (Figure 2.5). We often observed that one or two bundles could be colonized from the BH up to I1 and beyond while the bundles on the other side of the stem exhibited no detectable luminescence signal. This pattern was typical of the early infection stage of treatments with ‘Marmande’ roots, as well as for treatments with resistant rootstocks that were latently infected but otherwise healthy. Occasionally, the invasion margin was detected for single bundles (e.g., Figure 2.5, rS-sS with density of 7.30 and wilt of 0. Compare BH with higher slices.).
Additionally, we observed that the graft union did not appear to bottleneck the vertical movement of Rs since the intensity of the luminescence signal was generally steady along the length of the radial section containing the graft union. This was the same for both grafting methods. Poor graft union reconnection would lead to a bottleneck where vascular bundle continuity was not re-established (e.g. gaps from air pockets or mismatched angles; data not shown).

Most interestingly, when the maximum observed bacterial density and wilt severity levels were overlaid with the colonization localization images, a clear pattern was highlighted—increases in bacterial density and the induction and development of wilt were both related to additional primary and secondary bundles being colonized (Figure 2.5). There appeared to be a critical tipping point of about 3 primary bundles and some secondary bundles colonized or 4 primary bundles colonized for wilt to be observed. In the case of the previously mentioned partial leaf wilting, typically one or both of the primary bundles feeding into that leaf would be colonized all the way up and into the leaf, but bundles on the opposite side of the stem remained clear (Figure 2.5, footnoted plant).

This spatial colonization pattern similarly matched with the passing of the wilt-related density threshold. This pattern was the same regardless of resistance level. The hypocotyl region very consistently exhibited the highest number of colonized bundles compared to the higher portions of the plant, so much so that it was obvious that most plants of all grafting treatments developed wilting because of the hypocotyl colonization levels (the exception being the partial leaf wilting). Thus, it appears that both bacterial density and wilt development are linked to a proportion of total vascular volume being colonized. Resistance kept the number of colonized bundles below that critical volume (i.e. maintains heterogenous colonization patterns).
2.3.6: Pith and cortical tissue invasion in the stem.

In order to examine the tissue-system distribution of GMI1000 in more detail, a series of inoculations were performed using the GFP-transformed strain, focusing on the several ‘Marmande’ and ‘Hawaii 7996’ grafting combinations from the TR to I1. The light, UV, and GFP filters confirmed the same tissue localization patterns and relationship with wilting that had been observed with the LUX strain (Figures 2.6 and 2.7). The excitation wavelengths of GFP and lignin autofluorescence overlap partially, thus we found that the best contrast between the two signals was obtained using the UV filter, displaying the xylem tissues as a light blue color and the GFP as bright green. This method provided a more detailed picture of the colonization patterns of the grafting treatments at the vascular tissue-system.

During the imaging process, a striking contrast was observed in the level of invasion of pith and cortical tissues between ‘Marmande’ and ‘Hawaii 7996’ germplasm. In plants of grafting treatment rM-sH, we observed that the susceptible rootstock tissue can be heavily colonized in the vascular tissue such that the plant wilts and there is substantial invasion of the parenchyma tissues of the pith and cortex (Figure 2.6). In these ground tissues, most of the signal appears to be concentrated in the apoplastic spaces. Examining plants of various levels of severity indicated that this invasion originates from the infected vascular bundles. Upon passing the graft union into the resistant scion tissue, however, the xylem to ground tissue invasion was substantially reduced back to predominantly just the vascular tissues. This reduction was consistently maintained at higher locations in the plants as well.

A comparison of these RG plants with the SG ‘Marmande’ and ‘Hawaii 7996’ indicated that invasion of the ground tissue-system was related to a more late-stage of disease development and more severe wilting (Figure 2.7). Additionally, comparisons of the BH and I1 heights within
these self-grafted treatments revealed that rM-sM plants consistently exhibited similarly strong
ground tissue invasion below and above the graft union (Figure 2.7, red box). rH-sH plants
consistently exhibited similarly reduced levels of ground tissue invasion below and above the
graft union (figure 2.7, blue box). This trend was maintained in plants sampled at higher dpi (up
to 23 dpi; data not shown). Yet in the RG treatments, the ground tissue-system invasion patterns
were distinct to the genotype being observed in the section. Thus, even when rM-sH plants
experienced severe wilting symptoms and very high bacterial loads in the pith and cortex, the
ability of Rs to colonize the those tissues was drastically reduced upon entry of the resistant
tissue.

2.3.7: The root-to-shoot vascular transition in tomato.

The previously described findings further reinforced the importance of the hypocotyl
region in determining the fate of tomato plants to BW, regardless of the genotype combination. It
may be that the hypocotyl was key because it contained the root-to-shoot vascular transition.
Literature searches revealed very little information about how the transition occurs in tomato, nor
how it might impact dynamics of colonization and wilting. This junction was briefly explored.
Dissected seedlings of tomato ‘Cherokee Purple’ histologically stained revealed that the vascular
transition from the root anatomical arrangement to the stem occurred rapidly (Figure 2.8).
Beginning where the taproot begins to swell into the hypocotyl, most of the rearrangement
occurred within a few centimeters, being nearly finished just above the soil line. It transitions
much more gradually up the rest of the hypocotyl until it is fully transitioned by the first
internode.
It was observed that the polyarchic xylem core of the taproot became displaced by islands of pith (typically four or five) that rapidly expand, coalescing to form the pith of the hypocotyl (Figure 2.8). The vascular tissue becomes isolated into the primary bundles with a thin connection of interfascicular cambium. During the destructive harvests of the grafting treatments, this same basic pattern was regularly observed in all three of the genotypes (data not shown). Thus, at the very beginning of the stem vascular transition, only a few centimeters below the soil line, Rs confronts both xylem and pith tissues. Basal roots (Zobel and Waisel, 2010) were frequently observed emerging from the transition zone.

2.4: Discussion

This work tests several hypotheses about important BW phenomena related to the host-pathogen colonization dynamics and mechanisms of resistance. The work focuses on whole-plant and tissue-system-level patterns over space and time, and how those patterns are modulated by host resistance. Several new and emerging assessments tools are described, with the aim of capturing disease-critical information with higher throughput assessment techniques. In the process, several novel aspects of host resistance were identified and/or clarified. Finally, a strong connection was made between various colonization phenomena and wilt development, contextualized to the anatomy of the plant stem.

2.4.1: Wilt resistance reactions between each genotype and grafting combination.

We assessed BW resistance dynamics in the popular but highly susceptible ‘Marmande’, the commercially successful rootstock ‘Shield’, which has been the most planted rootstock for BW in North Carolina (NC) in the past several years (data not shown), and the open pollinated
‘Hawaii 7996’. We considered ‘Shield’ to be moderately resistant overall. It has behaved as highly resistant in a NC field with moderate disease pressure (Suchoff et al., 2015). In more systematic assessments it has shown an intermediate resistance level in NC fields with strong disease pressure (growers reports and chapters 3 and 4 of this work). ‘Hawaii 7996’ was found to exhibit very strong levels of resistance over 12 locations around the world and controlled greenhouse inoculations (Wang et al., 1998). It has also been the resistant donor parent in nearly every QTL mapping study for BW resistance (Carmeille, Luisetti, Besse, Prior et al., 2006; Mangin et al., 1999; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996; J. Wang et al., 2000). Resistance in ‘Hawaii 7996’ is governed by a major locus on chr. 12 (Bwr-12) and four clustered loci on chr. 6 (Bwr-6a to 6d), along with multiple other small effect loci on other chromosomes.

We hypothesized that the spatial-temporal phenotypes of ‘Shield’ would be more intermediate between ‘Marmande’ and ‘Hawaii 7996’. In field and greenhouse experiments with strong disease pressure, ‘Shield’ begins to separate from other lines more mid-to-late season after substantial fruit set has occurred and plants focus more on fruit enlargement (chapter 3 and 4 of this work). This is long after the susceptible plants have collapsed and dried. Based on this, it was included in an effort to identify phenotypic features related to resistance breakdown compared to ‘Hawaii 7996’. There was concern, however, that such a divergence would occur too late in the disease progression for these experiments to detect, which seemed to be the case in the destructive experiments (Figure 2.1).

In these grafted experiments, ‘Hawaii 7996’ and ‘Marmande’ were highly contrasting. ‘Shield’ had numerically higher numbers of wilted plants and wilt index in the destructive experiments but was not significantly different from ‘Hawaii 7996’ (Figures 2.1B). The
intermediate nature of ‘Shield’ became somewhat more apparent in some combinations by 21 dpi (Figure 2.1A). ‘Shield’ also exhibited numerically higher bacterial densities in the stem and locations colonized than did ‘Hawaii 7996’ (Figure 2.4). Thus, it behaved somewhat intermediately, but we were not able to conclusively identify what aspects of resistance were weaker. These experiments were not able to conclusively reproduce the moderate resistance level of ‘Shield’ that we have observed previously, at least not within the 10 day window. It is likely that whatever small differences we observed may be amplified in a field setting by the longer growth and development timeframe.

This work used exclusively GMI1000, a phylotype I strain from South America, which was recently reclassified into the new species *Ralstonia pseudosolanacearum*. The strain populations in the Southeastern USA are all phylotype IIA, which remain *R. solanacearum* (Hong et al., 2012; Safni et al., 2014). This difference is not likely to limit the application of these outcomes to applied applications. Both are tomato strains from the Americas and appear to have similar aggressiveness on susceptible and partially resistant materials. Substantial gene flow into phylotype I appears to have come from phylotype IIA and IIB populations, and tomato resistance to phylotype IIA is generally similar or greater than resistance to GMI1000 (Prior et al., 1990; Rivard and Louws, 2008; J. Wang et al., 1998; Wicker et al., 2011). Thus, this work may be applied quite broadly across Rs strains.

2.4.2: The wilt*bacterial density relationship.

At a physiological level, wilt develops because the rate of water flow into the leaves drops below the rate of flow out of the leaves (transpiration). The rate of out flow is determined by the vapor-pressure deficit, a combination of air temperature and relative humidity. Wilt-
inducing reductions in water flow are related to bacterial occlusion of xylem tissues through shear numbers and production of the exopolysaccharide slime EPS I (Buddenhagen and Kelman, 1964; Husain and Kelman, 1958a).

This work demonstrates that wilt development is related to a bacterial density threshold in the stem tissue, which was observed in our conditions to be a narrow range between 7.1 and 7.7 log(CFU/mL) in a 0.5cm piece of tissue (Figure 2.2). The work also demonstrates that the stem bacterial density uniformly decreases with increasing height (Figure 2.4). The concept of a density threshold in the BW pathosystem is not original to our work. This work merely articulates and characterizes it in much greater detail than previous studies.

Other tomato researchers have reported bacterial density trends that are comparable to ours. A comparison of several resistant lines with the susceptible ‘Floradel’ measured colonization and density results from taproots, collar (hypocotyl), and middle stem after anywhere from 10 to 25 days after inoculation (Grimault and Prior, 1993). The resistant lines exhibited a mean density plateau of about $10^6$ CFU/g fresh material (FM; 2 to 3 cm pieces) while the susceptible continued to increase up to $10^8$ CFU/g FM. In another set of experiments, the same authors demonstrated that ‘Floradel’ rootstocks exhibited high mortality rates and the bacterial densities in the hypocotyl were in the range of 7.5 log(CFU/g FM) (Grimault and Prior, 1994a). On the other hand, resistant rootstocks exhibited no wilting, had lower colonization frequencies, and contained bacterial densities in the hypocotyl ranging from 5.0 to 6.6 log(CFU/g FM). Interestingly, in all grafted combinations, they observed a decline in density and colonization frequencies at higher locations in the plant (Grimault and Prior, 1994a).

A series of experiments were conducted using the resistant ‘LS-89’ and the susceptible ‘Ponderosa’ (Nakaho, 1997a; Nakaho, 1997b; Nakaho et al., 2004; Nakaho and Allen, 2009).
Bacterial densities in the resistant TR through I1 ranged from $10^6$ to $10^7$ CFU/g FM up to 14 dpi. ‘Ponderosa’ reached nearly 100% wilting and had bacterial densities from TR to I3 between 8.5 to 9.3 log(CFU/g FM) at 14 dpi (Nakaho, 1997a). Another set of experiments demonstrated that when the inoculum density was increased from $10^6$ to $10^9$ CFU/mL, ‘LS-89’ shifted from no wilt to 100% wilting and bacterial densities in the upper hypocotyl shifted from between 7.2 to 7.7 log(CFU/g FM) to about 1.0 to 1.5 log higher (Nakaho, 1997b). Higher densities of bacteria were observed at each height location in the plant, especially at the highest point measured (internode 5). One report suggests that ‘LS-89’ can tolerate latent bacterial densities up to ~8.3 log(CFU/g FM) for at least 30 dpi (Ishihara et al., 2012). Even the authors seemed surprised by the density results but did not speculate on why the plants did not wilt even though they were grown in the same conditions as their previous work.

Experiments with a broader range of resistant lines demonstrated that plants of varying resistance levels (0 to 30 % wilt) maintained mean bacterial density levels below 8.0 log(CFU/g FM), while lines that never exhibited wilting maintained densities below about 7.2 log(CFU/g FM) (Nakaho et al., 2004). Mutant Rs studies also suggest a wilt threshold. ‘LS-89’ began to show wilt symptoms about the time that the bacterial density levels in the upper hypocotyls reached about 7 to 8 log(CFU/g FM), which was the same for both the wild-type K60 or the K60-509 strain, which is a triple mutant that lacks three of the four pectinases (PehA, PehB, and Pme) (Nakaho and Allen, 2009).

Even more broadly, a series of experiments compared tomato, pepper, and eggplant colonization (Grimault and Prior, 1994b). The analysis was divided between wilted and non-wilted plants. Across multiple cultivars of each species, plants that were wilting exhibited bacterial densities in the hypocotyl of 9.28 to 10.00 log(CFU/g FM), which remained the same or
slightly decreased as height increased. For non-wilting tomato plants, bacterial densities in the susceptible ‘Floradel’, resistant ‘Caraibo’, and resistant ‘Hawaii 7996’ were 6.15, 4.15, and 5.44 log(CFU/g FM). The non-wilting eggplant was generally lower between 2.92 and 5.38 log(CFU/g FM) and latently infected pepper was 7.37 to 8.37 log(CFU/g FM).

In potato, the difference between tolerant and susceptible cultivars appears to be the suppression of stem bacterial densities (Ferreira et al., 2017). Susceptible lines exhibited between ~6.0 to ~9.0 log(CFU/g FM), while tolerant lines maintained mean bacterial densities below 6.0 log(CFU/g FM). No differences in bacterial density were detected in the roots at either 2 or 7 dpi. These results are quite similar with our results and suggest that the wilt threshold exists in eggplant, pepper, and potato. The specific threshold levels are probably unique to each species. Also, the threshold would be expected to fluctuate somewhat across environmental conditions and plant hardiness.

2.4.3: Bacterial density as a component of Rs invasion of xylem volume.

An unresolved enigma in the literature relates to how resistant germplasm so effectively cap the bacterial density in the stem. This work further defines the wilt*density relationship, linking it with the spatial distribution of Rs at the tissue system level. Increases in bacterial density are related to a higher proportion of the stem that is colonized. This work also shows that a specific proportion of the vascular tissue needs to be colonized before sufficient water flow to the leaves is cut off, leading to wilting.

At the tissue-system level, the literature has generally suggested that invasion and proliferation of the xylem bundles are relatively uniform—each bundle is colonized similarly such that radial invasion is similar for all the primary and secondary vascular bundles. Whether
this view has been purposefully promulgated or not, evidence of uniform bundle colonization in the vascular tissue system is lacking. The literature is quite clear that various cultivars can exhibit major differences in colonization within infected bundles, as well as differences in the frequency of infection for populations of those lines (Caldwell et al., 2017; Nakaho et al., 1996; Nakaho, 1997a; Nakaho et al., 2016; Nakaho et al., 2004).

The literature is silent, however, concerning information about the colonization differences between bundles and how that might impact wilt development or relate to host resistance. This connection is critical. Anatomically, water flow is primarily vertical through the perforation plate, but also laterally through the paired pits (Esau, 1960; van der Schoot and van Bel, 1989). Thus, a pocket of occluded xylem conduits should not affect water movement substantially because the bulk flow can bypass blocked vessels via lateral transfer to adjacent open vessels. Dye-based water flow studies in infected plants have shown that the pathway up the stem was erratic. When stems were partially cut below a leaf, effectively disrupting the xylem tissues directly supporting it, the waterflow on the opposite, undamaged side of the stem would move up to the apex, over to the damaged side, and back down to support the leaves on the cut side (Grieve, 1936; Grieve, 1941; Kelman, 1953).

It has also been reported that the transpiration rates of infected plants did not significantly decrease until advanced wilt had developed (Grieve, 1936; Grieve, 1941; Kelman, 1953). Yet, leaf-level transpiration rates dropped sharply for wilted leaves compared to turgid. They concluded that the transpiration rates of the healthy leaves compensated for the wilting leaves by increasing their own respective transpiration rates. Clearly, the colonization patterns of single xylem bundles are only meaningful if the other bundles are similarly colonized.
Our work clearly shows that bundle colonization is highly heterogenous, even in the susceptible ‘Marmande’ (Figure 2.5). The relationship between spatial colonization and wilt development was quite similar between the susceptible and the resistant lines. In our conditions, colonization of at least 3 primary bundles and possibly a portion of the secondary bundles is needed before a plant exhibited wilting (excluding the partial leaflet wilt due to petiolule invasion). A rough visual estimation amounts this to be between ½ to ¾ of the water conducting cells, since very little development of vessel elements and trachieds were observed in the interfascicular cambium. It is clear that further development and cell differentiation in the vascular cambium would increase the proportion of the vascular ring required to cut off the same volume of water.

2.5.4: Bacterial proliferation in resistant roots.

We were not intending to investigate resistance phenotypes in the roots. But, differences in colonization frequency and bacterial density were observed in the roots of the resistant lines compared to the susceptible (Figure 2.3). This was a fortuitous addition from the stem observations. The pattern of a lack of a strong root-based luminescence signal resistant roots was especially curious when resistant and susceptible rootstock plants with similar signals in the stem were compared. They exhibited strongly contrasting signals in the roots. Thus, the root-based bacterial suppression seemed to behave independent of the status of the aerial organs. It even persisted when the plants had reached the permanent wilting point and had begun to decay.

Bacterial densities in resistant roots were reduced compared to susceptible (Figure 2.3B). Smaller differences in colonization incidence in the roots and hypocotyl were also observed (Figure 2.3C). ‘Hawaii 7996’ and ‘Shield’ had significantly reduced invasion incidence
compared to treatments where the root was ‘Marmande’. The distinctions were stronger between grafting treatments when we compared the colonization index (percent of total) for the three lowest locations (TR, BH, and DH), indicating that fewer locations were colonized based on resistant roots. Differences in TR colonization incidence and density have been reported where the resistant roots contain as low as 2 log(CFU/g FM) less bacteria than the susceptible roots and have similar or reduced colonization frequencies (Grimault and Prior, 1993; Grimault and Prior, 1994a; Grimault and Prior, 1994b).

During root invasion, Rs encounters several environments where resistance may be at work. Root invasion is facilitated by lateral root emergence. Everything inside the endodermis (casparian strip in most mature roots) is termed the stele. The pericycle cell layer (root cambium) is just internal of the endodermis and serves as the source of lateral root emergence. The process breaks through the casparian strip. Lateral roots are considered to be the primary site of entrance for Rs, as well as invasion of root tips where the casparian strip has not been established (Esau, 1960; Fahn, 1982; Schell, 2000; Vasse et al., 1995; Vasse et al., 2005). It is not known if host resistance affects the viability of lateral root sites for bacterial entry. If there were, it might help explain the reduced frequency of invaded roots and hypocotyl.

Alternatively, Rs also migrates through the root cortex via apoplastic pathways (Schell, 2000). During this invasion pathway, it encounters and must mitigate an oxidatively stressful environment (Flores-Cruz and Allen, 2009; Flores-Cruz and Allen, 2011). Penetration through the apoplastic spaces is greatly facilitated by the four extracellular pectinases the pathogen produces—three polygalacturonases and one pectin methyl esterase (Huang and Allen, 2000; Huang and Allen, 1997; Nakaho et al., 2000; Schell, 2000).
A dense vascular coating and amorphous polyphenolic compounds were also frequently observed in infected resistant roots, similar with what has been observed in stems (Nakaho et al., 2000; Nakaho et al., 2016; Nakaho and Allen, 2009; Vasse et al., 2005). A root-based hypersensitive response has also been proposed (Vasse et al., 2005). Thus, resistant roots may be producing chemistries with either bactericidal or movement inhibitors that make the root apoplast a stressful environment for bacterial cell growth. Alternatively, the same effect might be produced by a reduction of root/apoplast exudates that reduce Rs food supplies.

It was recently reported that host resistance delays Rs migration through the cortex and stele penetration by about 24 hours (Caldwell et al., 2017). Furthermore, resistance appears to suppress bacterial invasion within the roots (although the roots of the resistant lines and the susceptible ‘WVa700’ are challenging to compare because they were so different in size.). In that report, the transverse sections of the roots seemed to indicate that by 144 hours (6 days), Rs is colonizing the root cortex in the susceptible lines, but only the stele in the resistant lines.

Other microscopic studies of the root colonization reported that ‘Hawaii 7996’ develops two types of secondary roots (class identity is not clear), which are differentially colonized (Vasse et al., 1995; Vasse et al., 2005). This reduced several aspects of epidermal, cortical, and vascular invasion, which would affect the overall mean bacterial density of the roots. Root microscopy studies noted that cell-to-cell movement was facilitated by degradation of the primary cell wall-comprised pit membranes (Schenk et al., 2018; Vasse et al., 2005).

Collectively, those patterns are consistent with our findings that within resistant roots, Rs does not invade/proliferate well in the cortical tissues. Resistance reduces root invasion frequencies (somewhat) and bacterial density in the roots (strongly). In contrast, Rs seems to readily proliferate in stem tissues it can invade, regardless of the host resistance level.
2.5.5: Pith and cortical tissue invasion.

We observed that resistant lines strongly reduce invasion of cortical and pith tissues, helping confine Rs to the vascular tissues (Figures 2.6 and 2.7). This pattern was most apparent in the RG treatments, where ‘Marmande’ was the rootstock and the resistant line was the scion. A strong distinction was observed in the extent to which ground tissue was invaded between resistant and susceptible genotypes infected with the GFP strain. The effect was clearly related to the genetics of the tissue, because it was even apparent at the graft union (Figure 2.6A). The extent of ground tissue invasion coincided with the development of wilting, where it was more substantial at high severities of wilting.

A reexamination of the transversely sectioned slices exhibiting luminescence indicated a similar trend (Figure 2.5, rM-sM). Substantially more signal was observed from the entire section in the rM-sM tissue compared to the self-grafted resistant lines at the same level of wilting; independent spots were masked by signal from the pith and cortex. These observations are consistent with immunofluorescence microscopy of EPS I in hypocotyl tissue that showed strong apoplastic signal in the pith of susceptible ‘Marion’ while no signal was observed in the pith of ‘Hawaii 7996’ (McGarvey et al., 1999). In another report, Rs was observed colonizing at least three of the four primary vascular bundles as well as regions of pith tissue in ‘WVa700’ within 144 hours after inoculation, whereas in ‘Hawaii 7996’ it was limited to small clusters of cells in the primary xylem bundles (Caldwell et al., 2017).

Possible mechanisms explaining this phenomenon may be contained in the validation efforts of the tomato transcriptome analysis comparing infected ‘Ponderosa’ and ‘LS-89’ (Ishihara et al., 2012). Of the 140 ‘LS-89’ genes that were differentially expressed upon infection, pathogenesis-related, hormone signaling, and lignin biosynthesis genes were
upregulated. A class III acidic β-1,3-glucanase was the top upregulated gene, and was uniquely expressed in resistant cultivars. Two other classes of glucanase were also upregulated in ‘LS-89’. Plant β-1,3-glucanases degrade β-glucan (callose), are part of the PR-2 family of pathogenesis-response genes, and perform important functions in cell division, plasmodesmata trafficking (by regulating callose turnover), abiotic stress tolerance, flowering and seed development, and fungal defenses (directly hydrolyze fungal cell walls) (Balasubramanian et al., 2012). Tissue localization experiments indicated that the glucanases localized to xylem and pith tissues around vessels that were colonized by Rs, primarily in the vessel lumens and apoplastic spaces near infected xylem bundles.

In the same report, UV autoflorescence of pith tissues near primary bundles increased, along with lignin biosynthesis genes, suggesting Rs-induced lignification of the pith (Ishihara et al., 2012). We did not, however, observe an obvious enhancement of autoflorescence between mock and Rs inoculated susceptible or resistant lines in our experiments. Collectively, these two features led the authors to the hypothesis that the apoplast may be an important site for tomato defense responses to Rs infection (Ishihara et al., 2012). Our results clearly validate that hypothesis.

The GFP signal strength was most intense around the pith and cortical cells, suggesting that colonization of the apoplast was predominant. Pectin is a major component of the apoplast. Stem-based assessments of the impacts of Rs and resistance on the pectin structures found innate differences in the degree of methyl-esterification of homogalacturonan (HG) (Wydra and Beri, 2006). Rs also induced changes in the degree of methyl-esterified HG, the amount of arabinogalactan protein in the xylem cell walls, and the degree of rhamnogalacturonana I branching between resistant and susceptible genotypes. Tissue localization experiments
identified these compounds to the xylem parenchyma and the vessel cell walls (Wydra and Beri, 2007).

2.5.6: Vertical invasion dynamics.

Differences in vertical invasion by resistance level have been suggested (Grimault et al., 1994; Nakaho et al., 1996; Nakaho et al., 2004; Prior et al., 1996). Specifically, in ‘LS-89’, obvious reductions in colonization frequency and bacterial density were apparent as height increased, while ‘Ponderosa’ had 100% colonization at all heights. These reports note that inoculum density and average air temperature impacted the rate of vertical movement. The development of tyloses was proposed as a possible mechanism affecting vertical movement. The issue is not settled in the literature; it remains unclear to what extent resistance reduces vertical colonization in the hypocotyl or stem tissues.

In our experiments, there were overall difference between grafting treatments for bacterial density and proportion of locations invaded (p<0.0001). Also, height levels exhibited significant differences (p<0.0001). Differences were not detected in the rate of change over height between grafting treatments overall or over sampling times (p= 0.8561 to 0.9973). The trend in our experiments was that the TR and/or BH were the most frequently colonized locations and exhibited the highest densities of bacteria. These declined gradually as height increased, which is partly due to the pooled samplings (Figure 2.3E-F). Thus, we were not able to detect resistance-based restriction of vertical invasion from TR to plant apex.
2.5.7: Colonization around the graft union and implications for wilt development.

If Rs were able to cross the graft union from a resistant rootstock into a susceptible scion, several possible phenomena might be observed: 1) An increase in bacterial density above the graft union, 2) increased rates of vertical and radial invasion, and 3) higher levels of wilt development. This would be expected under the hypothesis that the resistant rootstock does not affect the host-pathogen interactions in the susceptible scion.

Alternatively, if resistance was conferred across the graft union (rootstock-scion resistance cross-talk), the colonization and wilt factors of the susceptible scion should resemble the resistant rootstock. The reciprocal arrangement should also resemble the resistance phenotype (assuming no root-based signal). Furthermore, mechanistic evaluations (such as cell wall thickening and pit membrane reinforcement) should be apparent in the susceptible tissue similar to the resistant.

The present data partially support both hypotheses, suggesting that a third hypothesis is necessary. Bacterial density did not drastically vary on either side of the graft union, but simply declined as a function of height in a resistance-independent manner (Figure 2.4E). This is particularly clear when the CG and RG treatments are compared to each respective SG treatment. For example, the grafted treatment rH-sM exhibited nearly identical density levels as the rH-sH at all heights measured rather than increasing in the ‘Marmande’ tissue. In the same way, the reciprocal rM-sH was nearly identical with the rMsM treatment at all heights. These relationships were the same for the percent of locations colonized (Figure 2.4F), suggesting that none of the scion treatments affected vertical rates of invasion differently from the associated rootstock. Furthermore, there was no apparent difference in the number of colonized bundles on
either side of the graft union, other than the general pattern of occasional reduction by height (Figure 2.5). These data support a resistance cross-talk hypothesis.

In contrast, the presence of resistance in the scion had no apparent affect on the host-pathogen interactions of the rootstock treatment (Figures 2.4E-F and 2.5). For example, bacterial density and invasion patterns in ‘Marmande’ roots were unchanged by the presence of either ‘Hawaii 7996’ or ‘Shield’ scions. Not even the pith and cortical invasion patterns appeared affected (data not shown).

The GFP strain was used to examine the CG treatments to try and resolve the issue. This was nearly impossible to do when the rootstock was ‘Hawaii 7996’ because of the higher frequency of clean plants and the strong reduction of bacterial growth in the hypocotyl. There were simply not enough plants where Rs crossed into the susceptible scion to draw any conclusions. When it did, no changes in colonization distribution were observed. We collected samples below and above the graft unions for each treatment for electron microscopic examination of cell wall and pith membrane reinforcement. The results are not yet available. The results partially support a no cross-talk model, unless the resistance phenotypes are only expressed when resistant roots and/or BH tissues are present.

Curiously, wilt thresholds were nearly always crossed in the BH tissue first, regardless of resistance type (Figure 2.2C, representative of all graft treatments). In contrast, the density patterns in the TR had no apparent impact on stem colonization patterns and wilt development—plants did not wilt because of bacterial density levels in the TR. Although we did observe root-specific resistance activity, it did not appear to affect the scion (Figure 2.3A).

Further plant-by-plant examinations of colonization, density, and wilting show several aspects of the dynamic clearly (data not shown): 1) Wilting plants always had Rs above the graft
union. 2) A proportion of susceptible scions were latently infected, including at 10 dpi when wilt
development had plateaued. And, 3) increases in invasion or bacterial density after crossing into
the susceptible scion were very rare. If they occurred, it tended to occur further away from the
graft union (I3 and I6). Therefore, we can only conclude that penetration of the susceptible scion
somewhat increases the risk of wilt development. Perhaps our experimental conditions did not
provide enough time for that dynamic to become obvious.

Thus, the data suggest that if resistance cross-talk is occurring, it is either a root- or only
BH-based signal (not DH) that only affects shoot resistance phenotypes. This is a rather tenuous,
complicated model. An alternate, simpler hypothesis is that no meaningful cross-talk is
occurring, thy hypocotyl drives the colonization (and thus density) patterns of the entire stem,
and vertical invasion into susceptible scions has a low impact on wilt development. In simpler
terms, plants wilt because the hypocotyl resistance fails or is absent. Therefore, grafting with
resistant rootstocks seems to work because of a combination of reduced invasion frequency and
reduced extent of vascular bundle colonization in the hypocotyl, which maintains the bacterial
densities below the wilt threshold. Any rootstock-scion resistance cross-talk is either curiously
expressed and/or may decay in efficacy in higher heights of the plant as if it were being diluted.

2.4.8: The hypocotyl as the key location determining wilt.

This work validates that the hypocotyl region, particularly the basal end, is the key
battleground location leading to wilt development and plant collapse. It is the first line of defense
for the aerial portions of the plant, and the last location for basal and hypocotyl roots that
contribute to the overall water supply for the canopy. It is the bottleneck between water uptake
and water loss. The findings indicate that wilt, bacterial density, and colonization frequency
patterns are all entirely dependent upon what is the rootstock (Figures 2.1, 2.3, and 2.4). This is consistent with other experiments using ‘CRA66’ and ‘Caraibo’ rootstocks (Grimault and Prior, 1994a) and field grafted experiments with a number of resistant rootstocks (Kressin, 2014; McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008; Silverman, 2015).

The results are not, however, entirely in agreement with the grafting experiments using ‘LS-89’ grafted to ‘Ponderosa’ (Nakaho et al., 1996; Nakaho et al., 2004). In these reports, the graft combination of resistant rootstock and susceptible scion developed an intermediate level of wilt between the resistant and susceptible lines alone. No explanation is provided for the results other than the suggestion that the susceptible scion was penetrated and bacterial activity developed in an unrestricted manner.

The discrepancies with the rest of the grafting literature and the current findings may be related to differences in the experimental parameters, as well as the specific host genetics. In the present experiments, slightly older or younger plants were used (7 to 9 true leaves and no flowers here vs. 6 to 7 leaves or flowering stage then). Different temperature conditions (28 °C day/night vs. 25/20 or 30/25 °C day/night) were also used as well. The experiments used the same inoculum density (10^7 CFU/mL) and general strain type (phytotype I tomato strains; GMI1000 vs. 8107 and 8231). It is possible that the other strains are more aggressive than GMI1000.

The differences may also have been related to cultural practices. Grafted transplants going into production fields are carefully hardened prior to planting by withholding a controlled amount of water. The stress of the field keeps the plants in a hardened state for the duration of the season as long as the grower does not apply too much nitrogen fertilizer.

In our first pilot test we observed 80% wilt (46% wilt index; n=10) in non-grafted ‘Hawaii 7996’ over 6 and 10 dpi compared to 100% wilt and index (n=10) in the non-grafted
‘Marmande’. The high disease levels of ‘Hawaii 7996’ seemed to be clearly related to the softness of the plants (data not shown). After that pilot test, we were careful to harden our plants by controlling the watering frequency, allowing them to experience light water stress between watering events. This kept ‘Marmande’ rootstock treatments at 100% wilting, but reduced the wilting levels of the resistant treatments dramatically. In the ‘LS-89’ grafted reports, no comments were made about these parameters other than growing the grafted plants in greenhouse conditions at 25/20 °C day/night prior to inoculation.

Furthermore, because this work strongly validates the importance of the hypocotyl region in the resistance interaction, it is possible that disease screens using rooted tomato cuttings (Carmeille, Luisetti, Besse, Prior et al., 2006) or direct stem infections (Ishihara et al., 2012; Nakaho et al., 2016) are likely missing important components of resistance.

2.4.9: The vascular anatomy of the hypocotyl and implications for colonization and wilting.

The transition of the vascular anatomy from roots-to-shoots is the primary structural variation that makes the hypocotyl unique from all other plant organs. This feature may hold the key to how the hypocotyl governs the fate of the plant-Rs interaction. This work adds specific details about how the transition occurs in four rather diverse tomato genotypes—two unique large-fruited heirlooms, a small fruited hybrid rootstock, and an old inbred rootstock with a wilder genetic makeup.

The structural patterns of the vascular transition vary across plant families and even species, but general patterns are quite consistent within groups (Basconsuelo et al., 2002; Esau, 1960; Fahn, 1982). Generally, dicot roots arrange the vascular tissues in a central core of xylem and phloem rays surrounded by the root cortex and epidermis—no pith. The transition occurs
around the basal plate, which is established when the embryo is formed. This arrangement transitions to several major groups of stem anatomical arrangements depending on the plant species. The vascular transition occurs by a displacement of the vascular tissue with pith and the fragmentation of the vascular core into discrete bundles. This process is usually completed by at least the first true leaves (Esau, 1960; Fahn, 1982).

Tomato stems exhibit an amphiphloic siphonostele arrangement type—a continuous ring of vascular cambium with mostly external phloem (H. E. Hayward, 1938). Discrete bundles of phloem also develop in the pith, typically near the oldest xylem. The vascular cambium contains islands (medullary rays) of 3 or 4 primary bundles and several secondary bundles, surrounding a substantial pith core (Coaker et al., 2002). The cortex is relatively thinner and is composed of the same basic cell types as the pith.

We briefly examined the vascular rearrangement at the tissue system level. It was observed that the anatomical arrangement of the tomato vascular tissues changed rapidly near the basal plate as the TR diameter begins to swell into the hypocotyl diameter (Figure 2.8). In most cases, Rs was first detected in the excised TR segments alone or also in the BH. It was occasionally observed that the BH was colonized before the TR, suggesting that the basal roots arising from the transition zone are alternative points of entry to the stem. This was also frequently observed in the whole-plant luminescence images where the first detectable Rs signal localized to basal roots (Figure 2.3A).

During the GFP imaging, it was also observed that the basal roots would nearly always arise from the primary vascular bundles (data not shown). Thus, Rs invasion of a basal root would provide easy access to one vascular bundle but would then have to navigate around the cambium or across the pith in order to colonize the other bundles. This may help explain the
heterologous bundle colonization patterns where some bundles become colonized to the upper parts of the plant while adjacent bundles apparently remained bacteria-free.

2.5.10: A harmonious model for wilt development and resistance—the tomato-Ralstonia tug-of-war.

This work has endeavored to connect the various phenomena associated with Rs invasion, proliferation, and wilt development in tomato plants with the spatial-temporal dynamics of host colonization. It also endeavors to explain how the quantitative resistance of tomato impacts this dynamic. In order to connect all the phenomena, we propose a model that integrates these phenomena, termed the tomato-Ralstonia tug-of-war (Figure 2.9). The game of tug-of-war involves multiple people on each end of a rope trying to pull the other team over the center line. It is a quantitative back-and-forth interaction that leads to a binary response.

Host invasion and subsequent resistance activity can be thought of in five physio-anatomical arenas: Root invasion, root colonization, stem vascular bundle invasion, vertical invasion up the stem, and pith/cortex invasion. The outcome of death-by-permanent-wilting seems to require Rs to navigate and/or triumph in each of these contests, each of which are quantitatively defended by host resistance. Some mechanisms appear to be active in several of the arenas and others may be present only in one.

Root invasion is suppressed through at least structural, chemical, and possibly a root-based hypersensitive response. Root colonization is likely suppressed similarly and may employ the same mechanisms as pith/cortex invasion. Resistance to bundle invasion employs radial cell-to-cell restriction through at least structural means and may overlap with pith/cortical invasion resistance. Vertical invasion, if it is a true resistance feature, would be suppressed through
deployment of tyloses, gums, and possibly a stem hypersensitive response. Pith/cortex invasion is likely restricted by structural modifications and possibly the stem hypersensitive response.

The purpose of the tug-of-war model is to facilitate a deeper understanding of the phenotypic aspects of a highly quantitative resistance. It can also guide further investigations that seek to link resistance phenotypes with genetics. This would flow well into more applied applications such as breeding. Furthermore, the links between spatial anatomy and known phenomena help contextualize and clarify many confusing aspects of BW: 1) low levels of resistance failure even in the most resistant germplasm. 2) The impacts of soil temperature and air temperature on the host-pathogen dynamics. 3) Phenotypic links with resistance genetics. 4) Impacts of the host genetic background (i.e. abiotic stress tolerances). And, 5) Strain variation for virulence and aggressiveness. These are discussed further in chapter 5 and would be useful areas of further research.
2.7: Figures

Figure 2.1: Typical disease development in our conditions for seven grafting combinations of ‘Marmande’ (S), ‘Shield’ (MR), and ‘Hawaii 7996 (HR).

Plants at the 8 to 10 leaf stage were inoculated with Rs strain 372 (GMI1000-LUX) by pouring 40 mL of a 10^7 CFU/mL bacterial suspension over lightly injured roots. The plants were placed in a growth chamber at 12 h day/night at a constant temperature of 27°C. Batches of plants was assessed either non-destructively (1) or destructively (4) for wilt at 11 or 3 time timepoints, respectively, using a 0 to 5 wilt severity score (0: no wilt; 1: 1 to 10% wilt; 2: 11 to 49% wilt; 3: 50 to 89% wilt; 4: 90 to 100% wilt; 5: permanent wilting point). The scores were converted into an index and mean wilt levels are shown, along with the standard errors. Colonization was also determined in the destructive batches using stem imprints, luminescence, wilting, and vascular browning. A) Non-destructive mean wilt index curves or B) destructive mean wilt indices and colonization frequencies for seven grafting combinations were calculated. Significant differences between grafting treatments were calculated at 12 and 21 days post inoculation or averaged over time. Case-specific values with the same letter are not significantly different (Tukey-Kramer adjustment for pairwise comparisons for an overall α=0.05). ****: means are significantly different at the p<0.0001 level.
Figure 2.2: Relationship between bacterial density in the plant and wilt severity (0 to 5 scale). Distribution of wilt severity over the maximum log colony forming units (CFU)/mL A) or zoomed in on the range of 7.0 to 9.1 B). 259 tomato plants inoculated by pouring 40 mL of GMI1000-LUX suspension over lightly injured roots and destructively assessed at 3, 6 to 7, or 10 days post infection (dpi) at 7 locations within the plant (tap root, basal and distal hypocotyls, and internodes 1 to 3 and 6). Relative light units were measured from 0.5 cm sections of stem or root tissue at each location and converted to CFU/mL. Stem imprints on Phi + gentamycin medium were used to correct false positive CFU/mL values caused by the tissue background luminescence. C) CFU/mL were plotted over location for each plant of with red lines indicating plants with a wilt score > 0 (n=24) and blue indicating non-wilting plants (n=12). Data are of the self-grafted Marmande treatment, but all the grafted treatments exhibited the same pattern regardless of cultivar but are not shown for the sake of clarity. Erratic red lines are the result of tissue decay and desiccation common at 10 dpi. The dashed black line indicates the approximate density threshold for wilt initiation. T: tap root; B and D: basal and distal hypocotyl, respectively.
Figure 2.3: Root-level resistance phenotypes.
Grafted plants were inoculated with GMI1000-LUX and destructively harvested at 3, 6 or 7, and 10 dpi. Whole plant luminescence (LUX) and light images were taken and select images from the second sampling point are shown as examples. The plants had to be cut into several pieces to fit them in the image area and were laid out from left to right, root to apex. The plant outline is background light from photosynthetic tissues, while the darker the spot, the more intense the luminescence signal. Saturation level was never reached. Wilt severity levels are indicated, while + or - indicates if GMI1000-LUX was detected or not, respectively, in the plant through the other assessment methods described here. Circles highlight the roots of each plant, while red indicates roots that are of the susceptible ‘Marmande’ and blue indicates roots of the resistant ‘Shield’ or ‘Hawaii 7996’. B) Mean bacterial densities (CFU/mL) in 0.5 cm of taproot for each grafting treatment averaged over the sampling times. Graft combinations are indicated with r: rootstock, s: scion, and treatments with the same letter are not statistically significant (α = 0.05, Tukey-Kramer adjusted). C) Mean invasion of the taproot and hypocotyl positions (3 loci total) averaged over the sampling times expressed as: Incidence—a simple presence/absence of invasion at any locus (black uppercase letters); or index—the percentage of invaded loci (white lowercase letters).
Figure 2.4: Invasion dynamics over vertical height.
Aspects of GMI1000-LUX proliferation (A, C, and E) and invasion (B, D, and F) in grafted tomato plants at 7 locations (taproot, basal and distal hypocotyl, and internodes 1, 2, 3, and 6). Plants were destructively harvested over time (3, 6 to 7, and 10 dpi) and bacterial density was calculated from luminescence measurements on 0.5 cm pieces of excised tissue at each location. Additionally, colonization incidence was determined by the ability to detect GMI1000-LUX at each location by stem imprints, luminescence, or vascular browning. Overall means of Log(CFU/mL) and percent invasion of the 7 locations are shown for grafting treatment (A-B, p < 0.0001), location (C-D, p < 0.0001), and grafting treatment*location (E-F; ns, p = 0.8704 and 0.9973, respectively). Error bars are the standard error and columns with the same letter are not statistically significant (Tukey-Kramer adjusted; α=0.05)
**Figure 2.5: Xylem colonization is heterogeneous and spatially related to wilt development.**

A) High and low intensity luminescence images for tissue localization of GMI1000-LUX invasion in sections of self-grafted tomato stems (r: rootstock; s: scion; M: Marmande; S: Shield; H: Hawaii 7996). Images are representative of different wilt severity stages (0 to 5) and the maximum observed log(CFU/mL) in each plant is noted. Gray outlines are the background luminescence of the plant tissue, while black spots indicate the intensity of luminescence signals. Saturation levels were never reached. Arrangement and cut-type from top to bottom is: I1: internode 1, transverse; DH: distal hypocotyl, transverse; GR: graft union, radial; GT: grafter union bottom, transverse; BH: basal hypocotyl, transverse. Transverse sections are about 1.5 mm thick with two technical replicates, while both halves of the radial section are shown. Circles indicate small, faint spots of luminescence. BioRad Image Lab software settings are: gamma = 1.15, high = 1000 or 3000 (high vs. low intensity, respectively), and low = 400. M = location where maximum density was observed.

<table>
<thead>
<tr>
<th>Wilt: Max^w log(CFU/mL)</th>
<th>rM-sM</th>
<th>rS-sS</th>
<th>rH-sH</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.67</td>
<td>6.10</td>
<td>6.25</td>
</tr>
<tr>
<td>1</td>
<td>6.55</td>
<td>7.30</td>
<td>6.95</td>
</tr>
<tr>
<td>5</td>
<td>8.06</td>
<td>7.70</td>
<td>7.30</td>
</tr>
<tr>
<td>2*</td>
<td>8.38</td>
<td>7.02</td>
<td>7.74</td>
</tr>
</tbody>
</table>

* 2.5/8 leaves wilting at 15 with highest CFU/mL
Figure 2.6: Resistance reduces pith and cortex invasion in the stem.
Reciprocally grafted plants (rootstock Marmande; scion Hawaii 7996) were inoculated with 40 mL of $10^7$ GMI1000-GFP via the soil drench method on lightly injured roots. At various dpi, plants were scored for wilt severity (0-5) and destructively harvested. Approximately 1.5 mm slices were removed from multiple locations in the plants (T: taproot; BH: basal hypocotyl; G: graft union; DH: distal hypocotyl; I1: internode 1) and imaged under a dissecting scope equipped with ultraviolet (UV) fluorescent lighting. Pictures were taken with bright field (BF) and UV filters. A) An example plant sectioned from T through I1 showing a dramatic reduction in GFP signal in non-vascular tissues of the resistant scion compared to the susceptible rootstock. The dotted line indicates the graft union (transverse section through the angled union). Single-line scale bar = 1 mm; double-line bar = 0.2 mm. B) Additional paired UV images of reciprocally grafted plants at various wilt severities showing contrasts between ground-tissue invasion of BH in susceptible rootstock and I1 of resistant scion tissues.
Figure 2.7: Radial invasion of the pith and cortex in self-grafted susceptible and control. Radial restriction of GMI1000-GFP between internode 1 (I1) and basal hypocotyl (BH) of self-grafted ‘Marmande’ (rM-sM; red box) and self-grafted ‘Hawaii 7996’ (rH-sH; blue box). Images were collected in the same manner as figure 5. Scale bar = 1 mm.
Figure 2.8: The anatomical profile of the tomato root-to-shoot vascular transition. The general anatomical pattern of xylem arrangement from roots to shoots in ‘Cherokee Purple’ seedlings, which resemble the broad patterns in ‘Marmande’, ‘Shield’, and ‘Hawaii 7996’. Seedlings at the 4 to 5 true leaf stage were washed of soil and dissected from TR to I1. Fresh transverse sections were histologically stained in toluidine blue solution for about 10 to 20 seconds, then rinsed several times with clean water. The sections were fresh-mounted on glass slides with coverslips and imaged with light microscopy at 40x to 100x magnification. Scale bars are 100 um (double line) or 200 um (single line). Toluidine blue stains lignified tissues blue-green and primary cell walls purple-red (Parker et al., 1982).
### Figure 2.9: Tug-of-war model of the tomato-R. solanacearum pathosystem in susceptible and resistant germplasm.

A generalized model of the stages of tomato invasion and colonization (Col) by *R. solanacearum* (Rs; red arrows) and the interactions that resistance quantitatively suppresses (orange T arrows) that determine the disease fate of the plant. +: the degree of colonization detected.
Supplemental Figure 2.1: 
Assessment methods for tracking bacterial density by height.

A) Relationship between relative light units per second (RLU/s) per mL of water and CFU/mL of LUX-labeled strain GMI1000. 0.5 cm sections of stem or washed tap root of inoculated or mock-inoculated tomato plants were excised and placed in 2.0 mL Eppendorf tubes with 200 uL of sterile dH2O. After at least 1-hour incubation at room temperature, luminescence was measured with an FB 12 Luminometer (Berthold Detection Systems, Germany). RLU/s output was normalized to the volume of liquid. 10 uL of water from each was 10-fold serial diluted and 10 to 20 uL were plated twice as drops on Phi medium containing gentamycin. Bacteria colonies were counted after drying the drops and incubating the plates at 28 °C for about 24 hours. Plates were observed for another 24 hours to validate negative spots. CFU/mL were then calculated.

B) Schematic of assessment locations for each plant in our experimental design. After scoring wilt, plant roots were washed, surface sterilized in bleach water, rinsed, and the leaves were trimmed off. Whole plants were dissected at 7 locations from the top down. Razor blades were alcohol and flame sterilized between each plant.
Supplemental Figure 2.2: Wilt development in our experimental conditions.

A) Mean wilt index between grafting methods. Non-significant differences ($\alpha = 0.05$, Tukey-Kramer adjusted) are denoted with the same letters within bars (black, capitalized) or diamonds (white, lowercase).

B) Two wilt assessment methods were contrasted—percent of leaves wilting and canopy wilt severity score (0 to 5 scale; 0: no wilt; 1: 1 to 10% wilt; 2: 11 to 49% wilt; 3: 50 to 89% wilt; 4: 90 to 100% wilt; 5: permanent wilting point.

C) Mean wilt index of two experimental replications for grafting methods over grafting treatment and sampling time. Error bars = standard error.
Supplemental Figure 2.3: Full plant and partial leaf wilt symptoms.
A) Examples of the two types of wilt observed in our experiments. Typical whole-plant wilt where Rs is not present in wilting leaves (rM-sS; most wilted plants) and partial leaf wilt resulting from GMI1000-LUX invasion (rS-sS; black LUX signal in wilting leaf alongside healthy leaf; low frequency of resistant plants). B) Field grafted tomatoes (resistant rootstock with susceptible scion) showing early wilt very similar to partial leaf wilt in A).
CHAPTER 3: SCREENING FOR RESISTANCE MECHANISMS THROUGH
ASSESSMENTS OF WILT SEVERITY, VASCULAR BROWNING, AND
COLONIZATION—A PROOF OF CONCEPT

3.1: Abstract

Bacterial wilt of tomato is a global disease caused by the soil-borne *Ralstonia solanacearum* species complex. The most resistant tomato germplasm available was developed in the 1970s, however high levels of resistance have not been successfully introgressed into large fruited varieties (>200 g). This is because of a very strong linkage drag with small fruit size, undesirable horticultural qualities, and a strong influence of soil and air temperature that have confounded meaningful breeding gains for resistance introgression. Introgression into rootstock germplasm has been successful, generating highly efficacious lines for bacterial wilt management via vegetable grafting. Important gains have been made in understanding the host-resistance mechanisms, and newer, more biologically-relevant tools are available for resistance assessments. But, such phenotypic and genetic discoveries have not been well translated or adopted into applied breeding and pathology programs, which generally still rely only on a binary wilt incidence metric. Wilt is related to a quantitative spatial colonization of a volumetric threshold portion of the vascular ring, which in turn largely determines bacterial density, regardless of resistance level.

A series of greenhouse and growth chamber experiments with grafted and non-grafted germplasm were performed on a panel of 15 tomato lines expressing a spectrum of highly susceptible to highly resistant phenotypes. Using the phenotypic context for the known resistance mechanisms provided by the *Ralstonia*-tomato tug-of-war model, they test if the vascular
browning (VB) symptomology can be used to visually assess spatial colonization, bacterial density, and, thus, resistance strength. A novel VB scale was developed to facilitate quantification. Vascular browning was closely associated with the tissue localization of \textit{R. solanacearum}. Both phylotype I and IIA strains exhibited similar patterns, where VB development required at least 6 days after inoculation to develop, after which it was closely associated with bacterial localization in the vascular tissues of transverse stem sections. Browning severity was positively correlated with both bacterial density and wilt development, with the strongest correlations occurring at the basal and distal hypocotyl. The lowest correlations were found with the taproot and internode 6. The resistance levels of the germplasm were precisely distinguished by VB severity, somewhat better than by wilt severity. Dynamics of the VB phenomena over time and space are discussed. The importance wilt severity over wilt incidence for resistance assessment was reinforced through the observation of multiple wilt phenotypic variants.

This work is the first of its kind assessing VB severity and its association with wilt induction and resistance mechanisms. It is a useful, intransient, visual indicator of bacterial localization, thus making it less environmentally sensitive than foliar wilt. The method is substantially more high-throughput and much less costly than microscopy, but effectively measures resistance at the vascular tissue-system level. It is useful for germplasm seedling screens for resistance discovery, controlled environment basic research projects, progeny tests, and QTL mapping during an end-of-study destructive assessment, in combination with a rapid stem imprinting technique and novel bacterial density assessments.
3.2: Introduction

Tomato (*Solanum lycopersicum*), other members of Solanaceae, and over 200 plant species across 50 plant families are hosts to the soil-borne pathogen *Ralstonia solanacearum* species complex (Rs), which causes bacterial wilt (BW) (A. C. Hayward, 1991; A. C. Hayward, 1994; Kelman, 1953). Bacterial wilt is a vascular disease characterized by rapid wilting of foliage, vascular browning (VB), milky ooze in infected plant stems, and foliar chlorosis depending upon the host (Agrios, 2005; A. C. Hayward, 1991).

Plant xylem tissues become highly colonized by the pathogen, leading to wilt development that is caused by a combination of bacterial occlusion and pathogen-excreted exopolysaccharide slime that clogs water flow (Buddenhagen and Kelman, 1964; Husain and Kelman, 1958a). Plants collapse after reaching the permanent wilting point. *R. solanacearum* infects tomato through sites of lateral root emergence and the zone of elongation. It then colonizes the stele, moves rapidly through the xylem tissues into the hypocotyl, radially colonizes the vascular bundles of the stem, and induces wilt (Araud-Razou et al., 1998; McGarvey et al., 1998; Nakaho et al., 2004; Schell, 2000; Vasse et al., 1995; Vasse et al., 2005).

Efficacious quantitative resistance is available in tomato, which has been commercially deployed via rootstocks for grafted management of BW (Louws et al., 2010; McAvoy et al., 2012; Rivard et al., 2012; J. Wang et al., 1998; J. Wang et al., 2013). Standalone fresh-market varieties with BW resistance are very rare to non-existent because of a very strong genetic linkage drag with small fruit size, as well as linkages with bitter taste and undesirable horticultural traits (Acosta et al., 1964; Acosta, 1978; Scott et al., 2009; Scott et al., 2005). Furthermore, there is a strong environmental influence on wilt development, particularly soil and air temperature (Kelman, 1953; Mangin et al., 1999; Nakaho et al., 1996; Scott et al., 2005).
Regional strain differences and a tendency for Rs to latently infect plants further complicate management and breeding efforts (Chellemi et al., 1994; Kelman, 1953; Lebeau et al., 2011; Nakaho et al., 1996).

Field and greenhouse assessments of wilt incidence assessments have revealed as many as 11 resistance loci mapped over 8 chromosomes (3, 4, 6, 7, 8, 10, 11, 12). Only the chromosome 6 loci is consistently detected in all the mapping studies, but it spans nearly 1/3 of the chromosome arm (Carmeille, Luisetti, Besse, Prior et al., 2006; Danesh et al., 1994; Mangin et al., 1999; Thoquet, Olivier, Sperisen, Rogowsky, Prior et al., 1996; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996; J. Wang et al., 2000; J. Wang et al., 2013). Many of the loci are suggested to be strain specific, which may explain how 6 of the 7 mapping studies do not share much overlap even though they are all based on the same interspecific mapping population—‘Hawaii 7996’ x ‘West Virginia 700’.

Meanwhile, segregation studies suggest single major resistance loci. Genetic inheritance studies suggest expression patterns from major dominant all the way to entirely additive effects. The most resistant sources appear to inherit resistance in a seemingly simple manner, but as has been noted, “…with bacterial wilt resistance in tomato nothing is simple…” (Scott et al., 2005). It is at least clear that some of the breeding difficulties are related to the strain differences and environmental variation.

Physiologically, wilt is largely a binary variable that is transient until the permanent wilting point is reached. It is well established that partially wilting plants recover between day and night conditions, when they are moved to a lower temperature, and have increased frequencies of wilt as temperatures increase (Kelman, 1953). The apparent wilt resistance level
of any given line can shift quite dramatically given a change of a few degrees centigrade (Acosta, 1978; Nakaho et al., 1996; Prior et al., 1996; Scott et al., 2005).

Field-level assessment methods (and sometimes greenhouse/controlled environments) have historically used a simple incidence of wilting methodology to interrogate the genetic components of resistance. Yet, the genetic basis for resistance remains largely unresolved to-date with many uncertain or sometimes conflicting reports about gene number, expression patterns, and genomic localization (Scott et al., 2005). Despite nearly 60 years of effort, applied management of BW through host genetics has remained marginal at best, apart from more recent successes in rootstock germplasm.

In contrast, controlled environment experiments investigating cellular and molecular aspects of the host-pathogen dynamic use wilt severity scales to assess wilting (typically a 0 to 4 scale based on percent canopy wilt) (Liu et al., 2005; Milling et al., 2011; Nakaho et al., 1996; Nakaho et al., 2004). Furthermore, they also generally include some assessment of colonization (latent infection rates) and bacterial density measurements. These metrics yield quantitative outputs that are more appropriate to the nature of resistance. Such assessment methods have not been incorporated into field breeding and pathology so far.

Wilt resistance has been connected predominantly with the radial restriction of cell-to-cell movement within the vascular bundles of the hypocotyl. This is made possible via structural reinforcement of the pit membranes, cell wall thickening, and the development of an electron dense vascular coating (Caldwell et al., 2017; Grimault et al., 1994; Nakaho et al., 2000; Nakaho et al., 2004; Nakaho and Allen, 2009). A stem-based hypersensitive response has also been suggested (Nakaho et al., 2016). Grafting experiments clearly show that the hypocotyl region is the critical site of interaction determining wilt resistance (Grimault and Prior, 1994a; Nakaho et
al., 1996; Nakaho et al., 2004). Resistance also affects root colonization, but is not substantial enough to prevent stem colonization (Caldwell et al., 2017; Vasse et al., 1995; Vasse et al., 2005).

Assessment of these resistance mechanisms is challenging, especially for applied applications like breeding programs because of the labor, specialized skill requirement, cost, and low-throughput of microscopic examination. These factors are a major reason breeders and field pathologists still rely upon the wilt metric. Furthermore, measuring wilt is non-destructive, which allows for saving seed from survivors. Simple survivor selection, however, makes it difficult to recollect the parental levels of wilt resistance (Scott et al., 1993).

In chapter 2, the connection between spatial colonization of the vascular tissue-system and bacterial density in the stem with the induction of wilt symptoms was clearly demonstrated. The data indicated that wilt development was determined by a critical density threshold, which was in turn determined by a spatial colonization threshold of the vascular bundles. The disease-suppressing effect of resistance on these parameters was localized to the hypocotyl. The various colonization stages and resistance mechanisms were then linked together in the Ralstonia-tomato tug-of-war model.

More direct measurements of resistance mechanisms would provide much greater accuracy for assessing resistance, especially if they were not transitively determined by the environmental conditions, like wilting is. The luminescence-based system used in chapter 2 greatly improved the ability to more rapidly assess colonization and bacterial density in a larger pool of samples, bacterial density was dictated by the spatial restriction. Therefore, if the spatial colonization could be measured, it would explain bacterial density and wilting. Visual (macroscopic) indicators of colonization would greatly improve the assessment throughput.
Vascular browning (VB) is a common phenomenon associated with vascular colonization and BW disease diagnosis. It is generally simple to observe either as peeled stems or in transverse sections. It has been assessed before in tomato, although the literature lacks a thorough characterization of the variation and relationship with colonization and resistance. The browning has been associated with oxidation of phenolic compounds released during host colonization, particularly the activity of pathogen-secreted pectinases (Agrios, 2005; Buddenhagen and Kelman, 1964; A. C. Hayward, 1991; Husain and Kelman, 1958b; Kelman, 1953; Pegg, 1989; Smith, 1896; Winstead and Walker, 1954).

Vascular browning is a common feature associated with other wilt-related fungal and bacterial diseases. The few researchers that have employed a VB assessment have adapted methods developed previously for BW or Fusarium Wilt or do not cite a method source. The published methods assess either vertical VB in split stems or a percentage of stem browning (without specifying assessment locations or types of cuts) (Anand et al., 1993; Mandal et al., 2013; Onduso, 2014). What each report has in common, however, is that lines exhibiting wilt resistance also exhibit a reduced level of VB, and there are significant differences between resistance levels based on VB assessments.

Previously, our initial investigation into VB examined two transversely sectioned locations in the hypocotyl at the end of a rootstock resistance screen using Rs isolates from NC (phylotype IIA). Using a panel of germplasm representing the range of highly susceptible to highly resistant, we observed and quantified substantial differences in the severity of browning in the vascular cambium, which were related to the observed resistance levels (Kressin, 2014). Further analysis indicated that VB variation was positively correlated with both wilt index and the area under the disease progress curve (AUDPC) for wilt severity (Silverman et al.,
unpublished). Pairwise comparisons of wilt AUDPC or the VB index indicated that VB severity allowed for additional separation of the genotypes compared to wilt AUDPC. Therefore, this work examines the VB phenomena in much greater detail, linking it with colonization patterns, bacterial density, and wilt development. Furthermore, we test the usefulness of VB as a visual indicator of resistance as it relates to spatial colonization of the vascular tissues.

3.3: Materials and Methods

For this work, four growth chamber experiments with grafted plants inoculated with GMI1000 (phytotype I) and two greenhouse experiments with non-grafted plants inoculated with an aggressive field tomato isolate from the Mountains of North Carolina (Jc; putatively phylotype IIA, sequevar 7 (Hong et al., 2012)) were performed.

3.3.1: Plant growth, design, and inoculation conditions—Greenhouse and growth chamber

The growth chamber experiments were performed in the Fall of 2017 and have been previously described in chapter 2. In those experiment, VB variation was assessed in each plant at each height-based location for each sampling time. The susceptible ‘Marmande’, moderately resistant ‘Shield’, and highly resistant ‘Hawaii 7996’ were used in a total of 7 grafted combinations.

The greenhouse experiments were performed in 2016 with non-grafted plants and are detailed below. A total of 14 tomato cultivars ranging from highly resistant to highly susceptible were assessed. For each experiment, seeds were bulk sown in rows in trays filled with the previously mentioned soilless propagation mix and germinated on heating pads set at 27 °C. After 25 days the plants were transplanted into 24-cell propagation inserts in plastic STF-1020-
OPEN trays with holes (11-0605-1 and 11-3000-1, respectively; T.O. Plastics Inc., Hummert International). The plants were fertilized biweekly with the previously described fertilizer solution to maintain healthy, active growth.

Each greenhouse experiment incorporated 12 genotypes representing the full range of resistance levels, five destructive batch-harvests, and four replications grown over two side-by-side heating pads (Table 3.1). We used a randomized complete block design where replication was the blocking factor. Two replications were placed side-by-side on each heating pad. The heating pads were identical and used a common temperature monitoring probe. In order to control for any heating pad edge effects or inconsistencies over the length of the pad, the tray positions within the rep blocks were randomized for each rep and the position of each genotype within each tray was uniquely randomized, thus making the experiments a split-split-plot design with subsampling and a repeated measures component at the split-split-plot level (Oehlert, 2010).

Two plants of each genotype were grown in a single tray as subsamples and each tray was an entire rep*sampling unit. Thus, for each sampling event, one tray from each rep was destroyed and every plant was dissected at 6 height-based locations—basal and distal hypocotyl and internodes 1, 2, 3, and 6 (BH, DH, I1 through I6, respectively). In the growth chamber experiments with grafted plants, the taproot (TR) was also assessed. The trays were assigned to the sampling events from the beginning of the experiments. In the Spring, destructive harvests were conducted at 6, 13, 20, 41, and 48 dpi, whereas the Fall harvests were at 7, 33, 38, 58, and 65 dpi. Furthermore, the Fall experiment included a full factorial water-inoculated treatment to control for any non-Rs related VB development and compare growth rates.

The plants were grown until the 7 to 9 leaf stage before inoculating. The Spring experiment plants were sown in late January, inoculated 57 days later, and observed for 48 days,
while the Fall/Winter experiment plants were sown in mid-October, inoculated 54 days later, and observed for 65 days. Several days before inoculation, the plants were placed back on the heating pads for the duration of the experiment, which were increased to 29.4 °C in the Spring experiment and 28.3 °C in the Fall.

The reporter strain system was not available for the greenhouse experiments at the time, and we wanted to use a North Carolina strain rather than GMI1000. Inoculum was prepared as follows: 20% glycerol stock cultures of strain Jc from the mountains of North Carolina were streaked on solid CPD or TZC medium (French et al., 1995) and incubated at 30 °C for 48- to 72-hours. The TZC plates were used to verify virulent colony morphology. Plates were then flooded with distilled water, the colonies were suspended, and mixed at the ratio of 15 plates per liter, which is 10⁹ CFU/mL. After mixing thoroughly, aliquots were 10-fold serially diluted, spread on CPD or TZC plates, and incubated as before. Dilutions containing 20 to 100 colonies were counted and the bacterial density of the inoculum culture was determined. In the Spring the inoculum density was 2.17 x10⁹ CFU/mL and in the Fall/Winter it was 1.67 x10⁹ CFU/mL.

Meanwhile, plants were inoculated by lightly injuring the root ball with a scalpel, placing the plants in an additional tray without holes, and pouring 10 mL of the bacterial suspension over the roots. The plants were not watered for 24-hours and lightly watered on days 2 and 3. Bottom trays were frequently monitored for dryness and were watered as needed, but the plants were not allowed to sit in constant water. In the Fall experiment, the trays were removed after the first sampling.

In both experiments, the daily minimum and maximum air temperatures were recorded by a max/min thermometer suspended in the middle of the air, shaded by a wooden hood. Daily mean air temperatures were calculated ([min+max]/2). Soil temperatures were periodically
monitored in the Spring to ensure that the temperatures were within a few degrees of the heating pad setting of 29.5 °C, while in the Fall experiment we systematically measured soil temperatures at the time of each harvest—the soil temperature at the center of each harvested root ball was measured to the same depth using a high precision digital thermometer (9878 Digital Pocket Thermometer, Taylor, -40 °C to 260 °C ± 1 °C; Hummert International).

In the Fall experiment, there was an incident of insecticidal soap burn shortly before inoculation that reduced plant growth. The plants were more heavily fertilized to encourage recovery. Although the plants appeared to recover well, it was not clear if this event and subsequent recovery period had any effect on the overall wilt development.

3.3.2: Data collection and analysis

In the greenhouse and growth chamber experiments, wilt severity was assessed once at the time the plants were destructively harvested. Based on our experience of how wilt seemed to progress in the canopy of field grown plants (Figure 3.1A), a 0 to 5 severity scale was used, which was somewhat modified from previous work: 0 = healthy, no wilt; 1 = 1 to 9% of canopy is wilting; 2 = 10 to 49% canopy wilt; 3 = 50 to 90% canopy wilt; 4 = 91 to 100% canopy wilt; 5 = permanent wilting point, plant collapse, tissues beginning to dry/decay (Figure 3.1B). This provided some additional connection between the field and seedling assays. In the growth chamber experiments, the percentage of wilting leaves on each plant was also measured. The partial leaf wilting was scored counted as 0.5 leaves.

In order to monitor if the plants were actively growing through the duration of the greenhouse experiments, plant height was measured at the time of destruction and normalized with either the first destructive harvest (Spring) or the height at the time of inoculation (Fall).
Each plant was destroyed by transversely sectioning them at the previously described locations (Supplemental 3.1). Also, the height of each cut location in each plant was measured from the soil line.

Colonization was assessed at each height via stem imprints on solid semi-selective media by pressing each sectioned location several times against either Phi medium (growth chamber experiments; see chapter 2) or SMSA-E medium with cycloheximide for fungal inhibition (greenhouse) (French et al., 1995). The Phi plates were incubated at 28 °C for about 24-hours while the SMSA-E plates were incubated for about 72-hours at 30 °C. Afterwards, they were scored for Rs growth using a semi-quantitative measurement of average growth in the imprinted area over the technical reps; 0 = no growth; 1 = 1 to 25% of the area is filled; 2 = 26 to 50% of the area is filled; and 3 = 50 to 100% of the area is filled (Supplemental 3.2). The plates were incubated for an additional 24-hours and then rechecked to ensure no positive growth was missed from very low numbers of bacteria; new growth was scored as 1.

Following the imprinting, each location was visually rated for the severity of VB. A somewhat modified scale was used based on our early work with VB variation to better assess the degree of browning in the vascular tissues (Kressin, 2014). A 0 to 5 scale was used, where: 0 = healthy, no vascular browning; 1 = 1 to 9% browning of the vascular tissues; 2 = 10 to 49% browning; 3 = 50 to 90% browning; 4 = 91 to 100% browning; 5 = 100% browning and tissue becoming dry/decayed (Figure 3.2). Because of the minimal development and colonization of the interfascicular cambium in the growth chamber experiments, the VB ratings relied mostly upon the primary and secondary vascular bundles for many of the internode locations. In comparison, browning of the interfascicular cambium was clear and frequent in the greenhouse experiments.
The binocular microscopy work was previously detailed in chapter 2. A few pertinent details are noted here. Periodically misting the samples and keeping them covered as much as possible was sufficient to make many observations at a time. We experimented with placing the tissue sections on ~1% water agar media, which greatly reduced the desiccation rate. The samples had to be spaced wider and misted much lighter, otherwise adjacent slices could be contaminated through bacterial movement in the free water drops.

Statistical analyses were performed with SAS 9.4. The most used procedures were UNIVARIATE, MEANS, CORR, REG, MIXED, and GLIMMIX for generating the LSMEANS and HISTOGRAM outputs. All pairwise comparisons of means were computed with the Tukey-Kramer p-value adjustment for multiple comparisons for an overall $\alpha = 0.05$. Height was considered a fixed main effect variable because the repeated measures model did not improve the fit statistics.

3.4: Results

3.4.1: Wilting assessments

Following inoculation, the first wilting was observed in the susceptible controls. In the growth chamber experiments wilt was observed at the end of the third day, while in the greenhouse experiment first wilt was observed on the fourth or fifth day. Differences in wilt levels were observed over batch samplings in both experiments. In the growth chamber experiments, no wilt was ever observed at the time of the first sampling (3 dpi), while in the greenhouse generally only the most susceptible lines were partially wilting at the first sampling (6 to 7 dpi) (Figure 3.3A-B). Wilt progressed rapidly over the next several days and then
plateaued between 7 to 10 dpi in the growth chamber experiments and by 13 dpi in the greenhouse experiments.

Overall, wilt severity was higher in the Fall experiment compared to the Spring, despite having the same colonization rates (Figure 3.3B and D). The general ranking of lines was not affected. Lines behaving as more moderately resistant, however, exhibited a greater increase in wilting compared to the most resistant lines.

The average soil and air temperatures in the Fall experiment they were 29.1 ± 1.9 °C and 25.7 ± 2.7 °C, respectively, while the mean daily mean air temperature in the Spring experiment was 26.4 ± 1.9 °C. The soil temperatures in the Spring were not rigorously monitored like in the Fall, ranging from 28 °C to 34 °C depending on the day and amount of sunshine hitting the soil. Soil temperatures were not monitored in the growth chamber experiments, but the air temperatures were maintained at 27 °C (data not shown).

To explore the reliability and usefulness of the wilt severity scale, it was compared to the percent leaf wilt by counting the number wilted leaves per plant out of all the leaves. It was observed that the percent leaf wilt and wilt scores were essentially identical (Supplemental 3.4). Since the wilt severity score includes the aspect of plant death (score of 5) and is much more practical for field-level evaluations, we chose to rely upon it for all further analyses.

Several aspects of wilting were observed in the experiments: 1) a rapid complete plant wilt where the leaves are not necessarily invaded by Rs, 2) a partial plant wilt that may recover (such as can be observed sometimes in the field), and 3) a partial leaf wilt that tends to lead to defoliation if the plant does not fully succumb to the disease (Figure 3.1C). Each of these cases were considered equivalent to each other using a wilt incidence metric, but severity scales allow for differentiation of these cases.
Significant differences in wilt severity were observed between genotypes and grafting treatments. In the growth chamber grafted experiments wilt, was entirely determined by what genotype was the rootstock—the scion genotype had no effect on wilt development (Figure 3.3C and chapter 2). In the greenhouse non-grafted experiments, we observed strong differences in wilting between genotypes (Figure 3.3D and Supplemental 3.5).

‘Florida 47’ and ‘Maxifort’ were susceptible controls, which exhibited consistently high wilt indices between 68 to 97% of the maximum score and were not different from ‘RST-04-105-T’, ‘BHN669’, and ‘Shield’ (Fall). Furthermore, ‘Maxifort’ (Spring) was not different from ‘BHN1087’ (Fall), ‘RST-04-106-T’ (Fall), or ‘Hawaii 7998’ (Fall). In the Spring, ‘Shin Cheong Gang’ only had less wilting than the susceptible controls, while in the Fall it was also significantly different from ‘Shield’ (Fall), ‘BHN1087’ (Fall), ‘RST-04-106-T’ (Fall), ‘RST-04-105-T’, and ‘BHN669’. ‘Hawaii 7997’, ‘CRA66’, and ‘Shin Cheong Gang’ were the most stable between seasons (16, 17, and 22 index points, respectively) while ‘Shield’, ‘RST-04-106-T’, and ‘BHN1087’ were the least stable (54, 39, and 37 index points, respectively).

3.4.2: Vascular browning severity overlap with Rs colonization.

In all the experiments, high frequencies of latent infections were observed. In the growth chamber experiments, the differences in colonization rates by grafted treatment were much smaller compared to the differences in wilt severity (Figure 3.3C). These results are discussed in more detail in chapter 2.

In the greenhouse experiments, differences in plant colonization were not detected between any genotypes in either season—all lines exhibited greater than 84% mean colonization
up to 100% (Figure 3.3D). A rapid invasion up the stem was observed even at the first sampling (data not shown).

Substantial variation in browning levels was observed in both the greenhouse and growth chamber experiments. In order to determine if VB severity could be used to visually assess colonization, the relationship of VB and Rs localization was compared microscopically in the growth chamber experiments using the GFP labeled strain. It was observed that VB overlapped with the portions of the vasculature that were colonized by Rs, while degradation of the vascular tissues was not obvious (Figure 3.2 middle and bottom). In contrast, invasion of the pith and cortical tissues was not well indicated by browning development. This trend was consistent across germplasm exhibiting a range of wilt resistance levels.

Tissue necrosis was only apparent in the greenhouse experiments several weeks after inoculation. In the growth chamber experiments, a VB score of 5 was rarely observed and the tissue typically desiccated before substantial pith necrosis was observed. In both experiments, there was a trend where more substantial pith degradation was related to a reduction in the ability to detect and recover Rs and an increase in other bacterial contaminants (data not shown).

3.4.3: Vascular browning development over time.

In order to better characterize VB over time, it was assessed at multiple time points following inoculation. In the growth chamber experiments, the first harvest occurred before the initial wilting, and colonization rates were high, but rather erratic (Figure 3.3A). By the second and third harvests, the infection rates rose dramatically in all lines. In the greenhouse experiments, the first harvest occurred after the initial wilt development, and >90% of the
sampled plants were infected to some degree in the stem (Figure 3.3B). The colonization rates remained stably higher over the other four samplings.

Development of VB exhibited a consistent pattern over time. It was significantly low in the first harvests of all the experiments (3 to 7 dpi). In both the Spring and Fall greenhouse experiments, VB was clearly delayed compared to the frequency of invaded plants and wilt development (Figure 3.5A compared to 3.3B). By the second harvest (at least 13 dpi), however, VB had developed substantially and consistently overlapped with the Rs-positive stem imprints (data not shown). In the growth chamber experiments, there were also significant differences between samplings within grafting method*treatment, as expected, where VB increased over sampling times (data not shown).

In the Spring experiment, VB development increased numerically over the destructive sampling events from harvests 2 to 5, but only sampling 5 exhibited significantly different levels of VB than samplings 2 and 3, while sampling 4 was intermediate. In the Fall experiment, there was numerical fluctuation in VB between samplings 2 to 5 but they were not significantly different. In the growth chamber grafted experiments, invasion of the hypocotyl and stem was detectable at 3 dpi, with a somewhat reduced frequency in resistant material (Figure 3.3A). Development of VB, however, was only consistently observed at 6 dpi and later in all germplasm, which then overlapped very closely with invaded vasculature (Figure 3.5B).

The vasculature was substantially less developed in the growth chamber experiments compared to the greenhouse. Thus, VB scoring was aided by taking an additional transverse section and placing it on a white background to improve the color contrast. This was not practiced in the greenhouse experiment. For the binocular microscopic examinations in the growth chamber, proper white-balancing was crucial for capturing life-like images of VB. In
summary, vascular browning development occurred 6 to 13 days after inoculation and 3 to 7 days after initial wilt development of the susceptible controls. VB was then contiguous with infection and vascular tissue localization in all germplasm examined.

3.4.4: Vascular browning severity patterns by stem height.

In order to further quantify the relationship between VB and colonization, VB over height trends were assessed. Height-based location was analyzed as a fixed effect because there was no improvement to the model fit statistics by considering it to be a repeated measures factor. The repeated measures model was also difficult to fit because of congruence issues related to the number of parameters.

In both the growth chamber and the greenhouse experiments, there were differences between the main effect levels of location within the plant (p < 0.0001; Figure 3.7A-B). The taproot exhibited significantly less VB than the hypocotyl, which had the most, while the 6th internode exhibited less VB than all other locations. It was very difficult to score browning in the taproot, mostly because of its small size, lack of a color contrast with the cortical tissues, and radial polyarchic vascular arrangement. There was a consistent decrease in VB severity as height increased from the basal hypocotyl towards the shoot apex (Figure 3.7A-B).

The interaction of location*season in the greenhouse experiments gives a sense of experiment-to-experiment consistency. There were significant differences in the mean stem VB severity by season; levels were higher in the Fall compared to the Spring (p = 0.0191) (Figure 3.7B). The overall height trend was not affected.

Differential changes in VB were also detected for the interactions of stem location*sampling within experiment. These changes were related to slope changes between the
samplings, where the first sampling was nearly flat, while VB increased more substantially closer to the hypocotyl compared to the shoot apex as time increased (data not shown).

In the growth chamber experiments, there were no differences in VB between the main effect of grafting method (in vitro vs. soil, p = 0.4024) or any of the interactions with grafting method (p = 0.0683 to 0.9998) except for small differences between grafting method*internode (p = 0.0142).

3.4.5: The vascular browning relationship with bacterial density and wilt development.

In chapter 2, it was observed that wilt development was related to a spatially-predicated bacterial density threshold. Thus, we investigated the relationship between VB and those parameters. Since there was a clear delay in VB development, the analyses were compared with and without the first sampling event.

In both experiment groups, significant positive correlations (all p < 0.0001) were observed between VB severity in all heights of the plants with both bacterial density and wilt development (Figure 3.4A-C). The trends were quite similar across variables and experiments with the strongest correlations being observed at the hypocotyl region and the weakest correlations at the taproot and 6th internode regions.

Furthermore, the highest correlations were observed in the growth chamber experiments by including all sampling events (Figure 3.4A-B). Curiously, excluding the first sampling event in the greenhouse experiments strengthened the relationships overall (Figure 3.4C). This was related to the unique relationships of those time points with the colonization and disease progression patterns.
In the growth chamber experiments, the relationship between bacterial density and VB severity by height-based location exhibited coefficients ranging from $\rho = 0.5196$ to $0.7708$ (Figure 3.4A). Within the same experiments, VB variation at each height was also strongly positively correlated with the wilt severity of the plant, ranging from $\rho = 0.5752$ to $0.8593$ (Figure 3.4B). Bacterial densities were not measured in the greenhouse experiment. But the relationship of VB and wilt was similar to the growth chamber experiments, exhibiting correlation coefficients of $\rho = 0.6917$ to $0.8470$ (Figure 3.4C).

In chapter 2, histograms of wilt severity over bacterial density helped illustrate the wilting threshold (Figure 2.2A). The strongest correlations were detected in the hypocotyl region. Thus, the same type of paired analysis was conducted for wilt severity and VB at the BH. This revealed similar threshold patterns as in chapter 2. Histograms indicated that wilt development in the growth chamber experiments largely began at a VB severity or 2 or 3 and the permanent wilting point (wilt score of 5) was largely associated with a VB score of 3 or 4 (Figure 3.4D). This pattern was also present in the greenhouse experiments, but was shifted towards higher VB scores, where initial wilt development was associated with a VB score of 3 or 4 and permanent wilting was associated with a VB score of 5 (Figure 3.4E).

### 3.4.6: Vascular browning severity for measuring wilt resistance.

In chapter 2, it was observed that the wilting threshold was the same regardless of the host resistance level. Having observed the strong relationships between VB severity, bacterial density, and wilt severity, we tested if VB severity behaved similarly and if it was useful for measuring wilt resistance. The main effects of genotype/grafting treatment were compared at the BH location based on the above findings.
In the growth chamber experiments, significant differences in VB severity (p < 0.0001) were observed between the grafting treatments (Figure 3.6A). Whenever the rootstock was the susceptible ‘Marmande’, significantly higher levels of VB were observed compared to whenever the rootstocks were the moderately resistant ‘Shield’ or ‘Hawaii 7996’. Numerically, grafted treatments with ‘Shield’ rootstocks exhibited numerically higher VB than treatments with ‘Hawaii 7996’ rootstocks. Furthermore, the VB levels were unchanged by whatever variety was the scion, although ‘Marmande’ scions were always numerically higher than resistant scions.

Similarly strong differences were observed in the greenhouse experiments. The main effects of experiment, and genotype nested in experiment, were significant (p<0.0001). There was greater VB development in the Fall experiment compared to the Spring (Figure 3.5A, 3.6D and 3.7B). Pairwise comparisons of the mean VB indices for each genotype indicated that the susceptible controls ‘Florida 47’ and ‘Maxifort’ were significantly different from all lines except for ‘RST-04-105-T’, and ‘BHN669’ (Figure 3.6D). Additionally, ‘Maxifort’ (Spring/Fall) was not different from ‘Shield’ (Fall), ‘BHN1087’ (Fall), or ‘RST-04-106-T’ (Fall).

Furthermore, many genotypes were significantly different between Spring and Fall, but the Fall levels were always greater. In the Spring, ‘Shin Cheong Gang’ exhibited significantly less VB than all other lines, but in the Fall it was not different from ‘CRA66’, ‘Cheong Gang’, ‘Hawaii 7998’, ‘RST-04-106-T’, ‘Hawaii 7997’, or ‘BHN998’. A closer inspection of the mean differences between Spring and Fall within each resistant genotype found that ‘RST-04-106-T’, ‘Shield’, and ‘Shin Cheong Gang’ exhibited the largest increases in VB severity in the Fall at 25.4, 22.4, and 20.1 index points, respectively, while ‘CRA66’, ‘Hawaii 7997’, and ‘BHN1087’ increased the least at 4.9, 7.8, and 13.0, respectively.
Plotting the mean wilt and VB indices of each genotype/grafting treatment indicated a tight, positive, linear relationship between them in both the growth chamber (Figure 3.6B, $R^2 = 0.9499$) and the greenhouse experiments (Figure 3.6C, $R^2 = 0.9183$). Interestingly, the linear trendlines in both experiments are remarkably similar, varying mostly by intercept ($y = 0.4540x + 10.267$ and $y = 0.5494x + 21.891$ for the growth chamber and greenhouse, respectively).

The pattern of VB change by height was also examined. Within the grafted growth chamber experiments, the height-based change in VB between germplasm was different ($p = 0.0004$). Treatments containing resistant rootstocks exhibited the main effect pattern of a small, non-significant, incremental decrease in VB as height increased (Figure 3.7C, solid lines). Treatments containing the susceptible ‘Marmande’ rootstock exhibited a significant ($p = 0.0353$ to $p <0.0001$) change in VB severity over height (Figure 3.7C, dashed lines). There was a more obvious reduction in VB in the TR compared to the stem tissues, and the VB decline as height increased was steeper.

Furthermore, in treatments having ‘Marmande’ roots, the reduction in browning severity in the stem decreased quickly in the resistant scion tissue. In contrast, the decline was much more gradual in the susceptible scion tissue (Figure 3.7C, comparison between dashed lines). At a given height, most of the differences in those treatments were numeric, since the only significant differences occurred between rM-sM and rM-sS at I3 and I6 ($p = 0.0310$ and $0.0440$, overall $\alpha = 0.05$). In chapter 2 (Figure 2.4E-F), however, no such treatment-specific changes were detected in either bacterial density or the frequency of invaded locations over height.

Interestingly, in the greenhouse experiment, the VB trends for each genotype*height were not different ($p = 0.2934$), nor were they for genotype*height*sampling ($p = 1.0000$). There was a common effect of “tomato stem” on VB development and patterns along the stem (Figure
3.7D). The differences in height were sufficiently explained by the main effects of height and genotype; each line exhibited a common slope but different intercepts. There were, however, differences in the interaction of genotype*sampling (p < 0.0001). Browning increased more substantially in susceptible genotypes compared to resistant over time (data not shown). Thus, we did not observe genotype-specific changes in VB severity by height like what was detected in the growth chamber grafted experiments. The resistance level of the host did not appear to modulate the VB patterns along the length of the plants. And, resistant plants accumulated VB at a slower rate than susceptible over time.

3.5: Discussion

This work explores the possibility of connecting resistance mechanisms to higher throughput assessment methodologies that can be deployed in large-scale pathology and breeding pipelines with the aim of improving accuracy, reducing environmental “noise”, and more directly selecting for the critical points of the bacterial wilt tug-of-war.

3.5.1: Use of wilt severity assessments in applied BW research and development.

Wilting caused by Rs can visually be described in a semi-quantitative manner using wilt severity scales. Chapter 2 and this work further validates this principle, while also showing that wilt progression is determined by a narrow range of spatial invasion that also determines bacterial densities within the plant. This work documents wilting patterns that can be distinguished by severity scales. Furthermore, the connection between spatial invasion, bacterial density, and wilt development suggest that time to wilt and rate of wilt severity development are
important factors for BW resistance. A simple wilt incidence metric cannot quantify these aspects effectively.

While the use of wilt severity scales for controlled environment testing is not new to BW research, they have not yet been adopted in tomato breeding and pathology screens for gene mapping and selection. This is troublesome because using a binary assessment tool to evaluate a polygenic, quantitative resistance system is causing a loss of important information and reducing the ability to discern real differences between germplasm.

The most recent QTL mapping study (J. Wang et al., 2013) has provided the most detailed dissection of resistance QTL to-date based on large greenhouse seedling wilt assays, yet only a wilt incidence assessment was employed. The mapping work was performed on F$_6$ and mostly F$_9$ recombinant inbred lines, yet loci resolution was only somewhat improved over previous maps from earlier generations of the same population (Carmeille, Luisetti, Besse, Prior et al., 2006; Mangin et al., 1999; J. Wang et al., 2000; J. Wang et al., 2013). While other factors such as recombination frequency and marker density in the target regions also impact the resolution of mapping efforts, a wilt severity assessment would likely have improved their analyses, especially if it had been combined with colonization and VB end-of-study assessments.

Recent breeding and field pathology reports still relied upon the percentage of wilting plants, employing no other mechanism-related information for selection or screening purposes (P. Hanson et al., 2016; McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008).

Substantial progress has been made recently for resistance dissection in eggplant, identifying a major resistance gene and several QTL, as well as clarifying the gene interaction patterns (Lebeau et al., 2011; Lebeau et al., 2013; Salgon et al., 2017; Salgon et al., 2018). That work employed various wilt severity scales plus end-of-study colonization assessments as part of
the phenotyping process. More recently, wilt severity scales are also being used in potato breeding and pathology seedling tests (Boschi et al., 2017; Ferreira et al., 2017).

One concern with wilt severity scales is the added labor time necessary to score the plants on a large scale. In our experience, switching to a wilt severity assessment does increase the assessment time to a degree, but it still allows processing of hundreds of plants per hour. Furthermore, wilt incidence can simply be calculated from the wilt severity scores.

3.5.2: The nature of vascular browning and disease quantification.

This report is the first of its kind that rigorously quantifies variation in VB severity in the tomato-*Ralstonia* interaction and connects it to resistance mechanisms and phenotypes. The subject has received little-to-no attention in the literature. Historically, VB has regularly been reported in disease diagnostic programs all the way back to the Erwin Smith in 1896. It was used as a visual marker for colonization that could be observed sometimes prior to, but usually with wilting in tomato plants (Kelman, 1953; Smith, 1896).

While VB development is not unique to Rs, it is part of a typical bacterial wilt diagnosis when it is combined with bacterial streaming and the foliar wilt profile (Agrios, 2005). There are old reports of some Rs strains from potato in Portugal and Kenya that cause wilt on potato but little or no VB in the tubers, although they seem to be rare exceptions (Kelman, 1953).

Only a handful of reports have investigated VB specifically. Early studies from the 1950s and before reported aspects of VB during investigations of wilting mechanics, where it was usually noted as absent or mildly or strongly present (Gothoskar et al., 1953; Husain and Kelman, 1958b; Winstead and Walker, 1954). Vascular browning was connected to bacterial pectinase activity (Winstead and Walker, 1954). The authors concluded that pectinases were
insufficient for gaining entry into the host but were important for the development of VB and vessel plugging.

*R. solanacearum* produces one pectin methylesterase (Pme), three polygalacturonase (PG; PehA, PehB, and PehC) enzymes, and two cellulolytic enzymes (Egl and CbhA), but no pectate lyases that can directly hydrolyze pectin (Schell, 2000). Liquid culture filtrates containing these pectinases were capable of rapidly macerating the stem tissues of tomato cuttings placed in the purified solution (Husain and Kelman, 1958b). Wilting, however, was linked to Rs-produced EPS slime (Husain and Kelman, 1958a). Development of VB in the fungal wilts was attributed to the oxidation and polymerization of phenols by the plant polyphenol oxidase (Buddenhagen and Kelman, 1964).

More recently, the importance of pectinases and cellulases in colonization and wilt development have been demonstrated and reviewed (Denny et al., 1990; Huang and Allen, 2000; Huang and Allen, 1997; Liu et al., 2005; Meng, 2013; Nakaho and Allen, 2009; Schell, 2000). Pectinases and cellulases affect Rs virulence via facilitating root invasion and stem colonization. Furthermore, pectinase activity in tomato has been implicated with contributing to the resistance-specific induction of structural defenses, likely through host detection of released oligogalacturonides (Nakaho and Allen, 2009).

The putative modes of action of the pectinases have been explored in relation to host resistance and basal defense priming (Diogo and Wydra, 2007; Kurabachew and Wydra, 2014; Wydra et al., 2005; Wydra and Beri, 2006; Wydra and Beri, 2007). They generally affect the branching patterns of the pectin network and some imbedded proteins. These changes localize to the xylem parenchyma tissues and the vessel cell walls.
The dynamic of VB severity in the genotype*location interaction was curious. In the greenhouse experiments, VB severity declined as the height increased, but the rate of decline was not different by genotype (Figure 3.7B and D). The differences between genotypes were based on the initial VB levels in the basal hypocotyl. The patterns were similar in the growth chamber experiments, but treatments with susceptible roots declined differently from treatments with resistant roots as height increased; resistant roots exhibited a very flat slope, while treatments with susceptible roots declined sharply after I1. Furthermore, the rM-sM treatment had enhanced VB at the I2 and I3 locations compared to the RG treatments. While the affects were not very strong statistically, it was apparent.

In contrast, in chapter 2 it was shown that both the frequency of colonization and the bacterial density at each location declined similarly with increasing height in all grafted treatments (Figure 2.4), just like was observed with the pattern of VB in the greenhouse experiments. It may be that the different patterns of VB had to do with the pattern of desiccation and bacterial containment to the vascular tissues between the grafted treatments.

Lines with susceptible roots died quickly and would often collapse by bending around the 2nd or 3rd internode and rapidly desiccating up and down the stem from that point. This often meant that internodes 2 to 6 were often the same or higher VB score than the hypocotyl and internode 1, which desiccated slower. Furthermore, multiple cases were observed where this collapse had occurred and internode 6 was free of bacteria and VB, as though the plant collapse had affected the ability of the pathogen to continue to rapidly invade vertically.

When the scion was susceptible, this collapse pattern was very common, while when the scion was resistant, the stems did not collapse as frequently. This likely led to an artificial inflation of the VB scores around the mid-stem desiccation region, which then would drop
quickly around the 3\textsuperscript{rd} and 6\textsuperscript{th} internodes. This pattern was not observed in the greenhouse experiment as the dead plants either desiccated upright or desiccated so quickly that the sampling events did not catch the intermediate stage. Furthermore, the strong restriction of ground tissue invasion in resistant stems on susceptible rootstocks that was demonstrated in chapter 2 appeared to improve the resilience of the stems against collapsing.

3.5.3: Vascular browning links to resistance

In the early 1990s a report emerged testing gene dosage effects for BW resistance in leaf-axil-inoculated or naturally infested field tomatoes using a diallele crossing scheme (Anand et al., 1993). What made this report unique was that they used VB severity and degree of bacterial oozing as part of their selection criteria as a way of tracking colonization. They adapted a 0 to 3 VB scale typical of Fusarium wilt research, where the plants were destructively harvested 100 days after transplanting, the stems were split in half lengthwise.

Vascular browning was scored as: 0 = no browning; 1 = light brown color, spread restricted to 2 cm from the point of inoculation; 2 = light brown color spread more than 2 cm; 3 = dark brown color, widespread browning of vascular tissue. A reevaluation of that data indicates that the VB score was positively correlated with wilt incidence and the bacterial ooze score (Supplemental 3.6A-B).

A more recent report from Kenya used the same assessment methods in field and potted glass house experiments with grafted tomatoes for management of BW (Onduso, 2014). In this case, they examined a local wild tomato line alongside ‘Cheong Gang’ and ‘Shin Cheong Gang’ as rootstocks grafted with the susceptible ‘Anna F1’ scion. The field sites relied upon natural disease pressure, while the potted experiments were artificially inoculated twice by pouring 4 mL
of $10^9$ CFU/mL bacterial suspension over injured roots and then dispersing it with 100 mL of sterile water. Plants were destructively harvested at 100 dpi and assessed for VB and bacterial ooze.

The Kenyan results represent a total of 2 years over 5 locations. In summary, VB severity was positively correlated with wilt percent and bacterial oozing scores. The work highlights that resistant materials showing the same levels of wilting have detectable differences in VB severity. In both studies, however, there was a pattern where lines with no wilting and small levels of VB did not yield bacterial ooze when they relied upon natural inoculation from the infested field. In our growth chamber work we occasionally encountered plants where bacterial signal was not observed in specific VB spots, especially when they had a deep brown color (data not shown). This may be due to a local extinction of bacteria.

The main drawback of the two previous studies is that the VB assessment method did not allow vertical and radial browning patterns to be distinguished. The VB assessment method used in the present work does allow for this because it focuses on transverse sections of the whole stem. Furthermore, it was designed in light of observed variation in tomato stems from a variety of resistance levels. The studies are, however, the only ones we are aware of that quantify VB severity within the last 60 years.

A somewhat tangential report investigating defense response elicitors for inducing resistance to Rs incorporated an assessment of VB (Mandal et al., 2013). The susceptible tomato ‘Arka Meghali’ was pretreated with either chitosan, salicylic acid, or jasmonic acid 7 days before inoculation, and then assessed for wilt and VB after 4 weeks. A 1 to 5 VB severity scale was adapted from Fusarium wilt research that assesses the percent of the vascular tissue that is brown (Ishikawa et al., 2005). The report was unique in that VB severity was used to measure of disease
severity in relation to wilting and it was assessed in a transverse section of the basal stem rather than in vertically split stems. All three elicitors effectively suppressed both wilt development and VB severity compared to the control, with salicylic acid having the greatest effect (Mandal et al., 2013). Furthermore, the elicitor treatments induced increases in the phenolic content, lignin deposition, and related gene expression in the roots. The report further reinforces the connection between reduced BW and reduced VB with enhanced host defense responses.

3.5.4: Vascular browning severity effectively tracks colonization.

In order to use VB severity as an indicator of Rs invasion, it was critical to demonstrate overlapping tissue localization of VB and Rs cells. This work clearly connects those features and identifies the appropriate window of time before VB is consistently detected (Figures 3.2 and 3.5). UV-fluorescence binocular microscopy was used for this purpose, which required very little sample preparation, less skilled labor, less specialized equipment, and a greater volume of samples to be processed compared to confocal microscopy. This is the first report conclusively connecting these features, especially on a whole-stem level.

The vertical invasion rates were very rapid in both experiments. Across all the experiments, Rs was often detected above I2 by 6 to 7 dpi, regardless of resistance (data not shown). The results were most dramatic in the greenhouse experiments where the inoculum density was higher. These results of vertical colonization rates up the stem are similar to other reports (Nakaho, 1997a; Nakaho, 1997b).

The UV filter was used primarily rather than the GFP or GFPA filters. It was important to clearly distinguish positive GFP signal from the background autofluorescence of the vascular tissue because the emission spectra of UV-excited GFP heavily overlaps with that of lignin. With
the GFP and GFPA filters, it was difficult to visually distinguish sparsely infected cells from bright cell walls in the absence of Rs starting to fill the vessel lumen (Supplemental 3.3). Under UV lighting, the natural colors of the vessels and Rs signal were more easily distinguished visually, although with some loss of signal intensity. The reduction in signal was considered to be less important since the work focused on the tissue systems as a whole.

The sensitivity of the stem imprints was compared with microscopic and dilution plating detection methods. With rare exception, the imprints were as sensitive as confocal microscopy and somewhat more sensitive than the binocular microscopy and dilution plating (data not shown). Furthermore, the growth pattern in the stem imprints on the media resembled the extent of vascular bundle colonization (data not shown). Thus, the stem imprinting method is a highly sensitive, very rapid, semi-quantitative method for assessing colonization, which can be very useful for applied applications. Furthermore, it would be readily adaptable for a biolog-type of culture system that can be combined with a quantitative, serological assessment to infer bacterial density in the stem.

3.5.5: Applications to applied research and breeding.

One of the aims of this research was to identify easily quantifiable phenomenon closely associated with resistance mechanisms that are neither transient (like wilting) nor heavily influenced by environmental variation (such as soil and air temperature). This work clearly established that VB severity can be used to differentiate resistant germplasm with a high degree of accuracy. This is because it effectively approximates the extent of vascular colonization and, thus, bacterial density within the stem. Furthermore, a close inspection of the mean separation in the greenhouse experiments found that VB severity allowed for additional separation of lines that
otherwise had the same levels of wilting (compare Figure 3.6D and Supplemental 3.5). Therefore, VB assessments can be very useful for more directly measuring critical BW resistance mechanisms.

Once VB had developed, it was a very stable phenotype over time. For example, wilt levels in sampling batch 4 in the fall experiment was significantly lower than sampling 2 (Figure 3.3B). In contrast, only numerical differences in VB levels were observed between samplings 2 and 4 (Figure 3.5). Similarly, the numerical increase in wilting in the Spring experiment at sampling 5 can also be observed in the VB trends. Furthermore, in the growth chamber experiments, it was occasionally observed that areas of VB were associated with greatly reduced bacterial signal, suggestive of bacterial death (data not shown). These results indicate that plants do not “recover” from VB. Thus, VB is not transient. The severity could, however, decrease over time relative to vascular volume if radial colonization rates were less than the secondary growth rates of the stem.

The environmental impact on wilt and VB severity can be seen in the relative levels within the growth chamber and greenhouse experiments (Figure 3.6A and D). The air temperatures in the growth chamber were identical between the four unique experiments, and subsequently, no differences in either wilt (p = 0.9953) or VB (p = 0.4024) indices were observed between grafting types (2 independent experiments each). This indicates the experimental consistency in the same environment.

In contrast, there were significant differences between the Spring and Fall experiments with regards to wilt and VB indices (p < 0.0001 for both). The differences cannot be attributed to differences in temperature since both experiments experienced the same mean of air temperature and general range of soil temperatures. The Spring experiment experienced the most extreme soil
and air temperatures of the two experiments with 44 more days above 30 °C air temperature (data not shown).

The differences between Spring and Fall experiments could be attributed to the previously mentioned plant damage and subsequent enhanced fertilization. It is unknown if the insecticidal soap burn affected root health in any way. The fertilizer contained a nitrate-based nitrogen source, which was shown to be beneficial to Rs virulence due to effects on root attachment, initial infection activity, ATP production, and detoxification in the low oxygen environment of the xylem fluid (Dalsing and Allen, 2014; Dalsing et al., 2015). The differences might also be related to light intensity and photoperiod, could have reduced the growth rate of the plants in the Fall compared to the Spring. Finally, the differences could be affected by higher soil water levels in the Spring compared to the Fall, which may have been suppressive of disease development.

The impact of plant culture methods and growth/stress conditions on the wilt*VB relationship is strong. A comparison of growth chamber and greenhouse experiments indicates that there is a clear shift in the VB*wilt thresholds (Figure 3.4D-E). Comparing the environments indicated that the growth chamber had the most stable temperature and least intense lighting, thus causing the lowest relative water stress on the plants. This had an overall affect on the vascular development and hardening status of the plants. In the interfascicular cambium, while contiguous in all the experiments, was more developed in the greenhouse. VB was regularly observed in the interfascicular cambium in the greenhouse experiments, but very rarely in the growth chamber.

There were obvious differences in the consistency of the colonization frequencies between the growth chamber and greenhouse experiments (Figure 3.3). This is most likely
related to the differences in bacterial densities used for inoculation. The greenhouse plants were exposed to about 60x more bacteria and soil temperatures at least 1.5 °C higher than in the growth chamber experiments. Colonization and wilt development increase with increasing inoculum densities and soil temperature, but decrease as plants age (Mew and Ho, 1976; Mew and Ho, 1977; Nakaho et al., 1996; Nakaho, 1997b). The reasoning for the difference in inoculation conditions had to do with optimizing each condition for maximum disease pressure and infection rates while retaining strong wilt resistance contrasts, given the age and development of the plants. In the greenhouse work in particular, we have consistently used $10^8$ to $10^9$ CFU/mL inoculum for testing plants about 60 days old before with good success (Kressin, 2014; Silverman, 2015).

For applied research, VB assessments fit very well as an end-of-study destructive measure once wilt development plateaus. This application was where the potential value of VB severity for resistance screening was identified (Kressin, 2014). Germplasm seedling screens for resistance discovery, controlled environment basic research projects, progeny tests, and QTL mapping are all areas to which this research is most readily adaptable. We have also experimented with applying VB assessments to field BW trials but the value of that application is unclear, mostly due to the practical deployment of such assessments (data not shown).

Assessments of VB can be easily combined with colonization and bacterial density measurements without much added cost or labor. The stem imprints were highly effective at providing a rapid, semi-quantitative assessment of colonization. There are several ways to add bacterial density assessments. The hypocotyl is clearly the best region to assess (Figure 3.4). These measurements fit very well into a rapid assessment pipeline for processing high numbers of samples, at a relatively low cost and low skill level.
Furthermore, the use of the luciferase-labeled reporter strain for a rapid assessment of bacterial density allowed for a very rapid measurement of bacterial density in planta in a very low cost, simple way. The system required some dilution plating to establish the relationship for the specific experimental conditions, but the correlation was very strong and consistent over germplasm and experiments. By leaving the tissue in the tube, an incubation step was not even necessary. Thus, the density quantification process took merely the time to cut out a 0.5cm piece of tissue, place it in a tube with sterile water, and measure it for 20 seconds.

One of the values of the reporter system is that it can be readily cloned into any Rs strain of interest, allowing broad application to research and breeding programs around the world (Monteiro, Solé et al., 2012). The system has already been deployed for basic research in Rs promoter expression in planta and for evaluating potato germplasm for resistance (Cruz et al., 2014; Ferreira et al., 2017; Monteiro, Genin et al., 2012).

One drawback to take into consideration when deploying these methodologies at the end of the study is that the ability to recover Rs sharply drops as the tissues begin to desiccate and/or rot. Thus, plants should probably be harvested once they reach the permanent wilting point (score 5). Even then, the recovery was Rs was always most successful from the hypocotyl because it desiccated last.
3.6: Figures

**Figure 3.1: Wilt severity scale and types of wilt observed.**

Wilt severity scales used in A) field evaluations and B) greenhouse and growth chamber experiments. 0 = healthy, no wilt; 1 = 1 to 9% of canopy is wilting; 2 = 10 to 49% canopy wilt; 3 = 50 to 90% canopy wilt; 4 = 91 to 100% canopy wilt; 5 = permanent wilting point, plant collapse, tissues beginning to dry/decay. C) Contrasts of whole-plant (top), partial-leaf (bottom), and partial-plant (right) wilting phenotypes observed in greenhouse, growth chamber, or field bacterial wilt experiments. Light and luminescence (LUX) images highlight the presence of absence of leaf invasion.
Figure 3.2: Vascular browning severity scale used in greenhouse and microscopic examinations.

Vascular browning (VB) severity scale levels used for isolates Jc (greenhouse) or GMI1000-GFP (strain 528; growth chamber). We used a 0 to 5 scale, where: 0 = healthy, no vascular browning; 1 = 1 to 9% browning of the vascular tissues; 2 = 10 to 49% browning; 3 = 50 to 90% browning; 4 = 91 to 100% browning; 5 = 100% browning and tissue becoming dry/decayed. The dashed red line highlights the range of tissue conditions within score level 5. Bright field (BF) images show the VB severity while the UV or GFP-filtered images (bottom row) show the colonization signal from the GFP-labeled strain (green) or tissue autofluorescence (light blue/pale yellow is lignin autofluorescence; red are chloroplasts) when the samples (middle row) were exposed to UV light. The camera settings were the same for all the UV images. Scale bars represent 1 mm. White arrows highlight positive VB or colonization locations.
Figure 3.3: Wilt development over time and germplasm.
Wilt development and plant colonization in the growth chamber (A and C) and greenhouse (B and D) experiments. About 55-day-old tomato plants were inoculated by lightly injuring the roots and then drenching the root ball with 40 mL of GMI1000-LUX at 10^7 CFU/mL (growth chamber) or 10 mL of NC strain Jc at 10^9 CFU/mL (greenhouse). Plants were batch harvested and assessed for wilt severity. Pairwise differences in the mean wilt indexes were determined between sampling events (A-B), grafting treatments (C), and genotypes (D) using Tukey-Kramer adjusted p-values (α = 0.05). The plants were also assessed for colonization using a combination of wilt development, stem imprints, luminescence, and vascular browning measurements, and were similarly analyzed as the proportion of plants colonized by grafting treatment (C) or genotype (D). Treatments with the same letter are not significantly different. Bars represent the standard error.
Figure 3.4: Relationship of wilt and vascular browning severity.
Pearson correlations between vascular browning severity and A) bacterial density or wilt severity (B-C) for multiple height-based locations within the plants, with or without including the data points from the first sampling event (3 days post inoculation for the growth chamber experiments and 6 or 7 days post inoculation in the greenhouse experiments.) The proportion of plants for each vascular browning severity level at the basal hypocotyl location were calculated for each wilt severity level in D) the growth chamber and E) greenhouse experiments.
Figure 3.5: Temporal delay of vascular browning development after inoculation.
A) Differences in the level of vascular browning development in the greenhouse experiments between sampling events. Following inoculation with strain Jc, plants were destructively harvested five times for assessment of vascular browning severity in the stem. Samplings 1 to 5 occurred 6, 13, 20, 41, and 48 days post inoculation (dpi) and 7, 33, 38, 58, and 65 dpi for the spring and fall experiments, respectively. Each column represents the main effect of sampling where means with the same letter are not significantly different (Tukey-Kramer adjusted p-values; overall α=0.05). Bars represent the standard error. B) Binocular microscopy images of transversely sectioned stems exhibiting high levels of colonization by GMI1000-GFP (right; UV) with the accompanying brightfield (left; BF) image exhibiting no vascular browning at 4 dpi compared to strong browning development at 9 dpi.
Figure 3.6: Variation vascular browning severity between germplasm and wilt development. Relationships between vascular browning (VB) indexes by A) grafting treatment, D) genotypes within experiment, and their subsequent correlation with the mean wilt index levels (B and C, respectively). Means with the same letter are not significantly different (Tukey-Kramer adjusted p-values; overall α=0.05). Bars represent the standard error.
**Figure 3.7: Vascular browning over height and height*germplasm.**

Variation in vascular browning severity between each height-based location in the plants for the main effect of location for the A) grafted growth chamber and B) non-grafted greenhouse experiments, grafting treatment by location (C, growth chamber), and example genotypes by location (D, greenhouse). Means with the same letter are not significantly different (Tukey-Kramer adjusted p-values; overall α=0.05). Bars represent the standard error. FL47 = ‘Florida 47’, Shd = ‘Shield’, HI97 = ‘Hawaii 7997’, SChG = ‘Shin Cheong Gang’; -F = Fall, -S = Spring.
### Table 3.1: Summary of germplasm tested.

Germplasm tested in the greenhouse experiments with the number of plants inoculated, the expected relative level of resistance, the season each was tested, and the seed source. HS: highly susceptible; MS: moderately susceptible; MR: moderately resistant; HR: Highly resistant.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Inoculated Plants</th>
<th>Resistance</th>
<th>Season</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florida 47</td>
<td>80</td>
<td>HS</td>
<td>Spring/Fall</td>
<td>Seedway</td>
</tr>
<tr>
<td>Maxifort</td>
<td>80</td>
<td>HS</td>
<td>Spring/Fall</td>
<td>Paramount Seed</td>
</tr>
<tr>
<td>RST-04-105-T</td>
<td>80</td>
<td>HS</td>
<td>Fall</td>
<td>DP Seeds</td>
</tr>
<tr>
<td>BHN1053</td>
<td>80</td>
<td>MS</td>
<td>Spring</td>
<td>BHN Seed</td>
</tr>
<tr>
<td>BHN669</td>
<td>80</td>
<td>MS/MR</td>
<td>Fall</td>
<td>BHN Seed</td>
</tr>
<tr>
<td>BHN1087</td>
<td>80</td>
<td>MR/HR</td>
<td>Spring/Fall</td>
<td>BHN Seed</td>
</tr>
<tr>
<td>Shield</td>
<td>80</td>
<td>MR</td>
<td>Spring/Fall</td>
<td>Rijk Zwaan</td>
</tr>
<tr>
<td>BHN998</td>
<td>80</td>
<td>HR</td>
<td>Spring</td>
<td>BHN Seed</td>
</tr>
<tr>
<td>Cheong Gang</td>
<td>80</td>
<td>HR</td>
<td>Spring/Fall</td>
<td>Seminis</td>
</tr>
<tr>
<td>CRA66</td>
<td>79</td>
<td>HR</td>
<td>Spring/Fall</td>
<td>UF/NCSU</td>
</tr>
<tr>
<td>Hawaii 7997</td>
<td>80</td>
<td>HR</td>
<td>Spring/Fall</td>
<td>UF/NCSU</td>
</tr>
<tr>
<td>Hawaii 7998</td>
<td>80</td>
<td>HR</td>
<td>Spring/Fall</td>
<td>UF/NCSU</td>
</tr>
<tr>
<td>RST-04-106-T</td>
<td>80</td>
<td>HR</td>
<td>Spring/Fall</td>
<td>DP Seeds</td>
</tr>
<tr>
<td>Shin Cheong Gang</td>
<td>80</td>
<td>HR</td>
<td>Spring/Fall</td>
<td>Seminis</td>
</tr>
</tbody>
</table>
3.8: Supplemental Figures

**A**

- Hypocotyl-
- ------------------------
- Internodes---------------------

Basal Distal 1 2 3 6

**B**

hypocotyl roots  cotyledons  node 1 / leaf 1

basal roots

graft union

Taproot

hypocotyl

Basal Distal 1 2 3 6

**Supplemental Figure 3.1: Height-based locations tested.**

Diagrams of the plant height-based locations that were dissected and assessed within each plant (thick arrows) in the A) greenhouse and B) growth chamber experiments related to other anatomical features.
Supplemental Figure 3.2: Example of stem imprint method and growth variation.
Stem imprint examples exhibiting Rs colonization at multiple heights and 1 or 4 plants per plate in the A) greenhouse and B) growth chamber experiments for stem imprints after 72- and 24-hour incubations at 30 and 28 °C on SMSA-E or Phi medium, respectively. C) Closeup of variation in bacterial growth within each stem imprint region that were scored for the degree of growth: 0 = no growth (not shown); 1 = 1 to 25% of the area is filled; 2 = 26 to 50% of the area is filled; and 3 = 50 to 100% of the area is filled. Dashed circles indicate approximate area of the stem that was imprinted.
**Supplemental Figure 3.3: Light filters used in microscopic tissue localization of GFP signal.** Comparison of the light filters (UV, GFP, and GFPA) used on the binocular microscope for differentiation of GFP and xylem tissue autofluorescence at 11.5x and 2x magnification. Scale bars are 0.2 mm (double) or 1 mm (single). Plants were inoculated with Rs strain 528 (GMI1000 constitutively expressing GFP) and dissected at 8 dpi. Stem imprints were taken from taproot to the 6th internode. The negative imprint plants did not have Rs detected at any location. All tissue presented are of ‘Marmande’. Camera settings were the same within the combination of magnification and filter.
Supplemental Figure 3.4: Relationship between wilting score and the percentage of wilted leaves from the growth chamber grafted experiments.
Supplemental Figure 3.5: *Genotypic differences in wilt severity between germplasm.*
Expansion of figure 3.3D showing pairwise similarity of means. Means with the same letter are not significantly different (Tukey-Kramer adjusted p-values; overall α=0.05). Bars represent the standard error.
Supplemental Figure 3.6: Relationship between wilt and bacterial ooze with vascular browning severity in published literature.

Comparison of the mean VB severity values with A) the mean wilt incidence or B) mean bacterial ooze score in a mixture of parents and crosses artificially inoculated at the leaf axil before transplanting or under natural soil infestation and destructively assessed 100 days after transplant. Both the VB and bacterial ooze scores were rated using a 0 to 3 scale. For VB of split stems: 0 = no browning; 1 = light brown color, spread restricted to 2 cm from the point of inoculation; 2 = light brown color spread more than 2 cm; 3 = dark brown color, widespread browning of vascular tissue. For ooze, a piece of cut stem just above the roots in a tube of water: 0 = no ooze; 1 = thin strand of ooze not continuous, flow stops after 3 minutes; 2 = continuous thin white, flow not restricted; 3 = heavy ooze, turning the water turbid within 2 minutes. The means represent 3 or 15 plants per genotype with 3 replicates for the VB/ooze and wilt incidence variables, respectively. Data from Anand et al. (1993), tables 3 to 5.
CHAPTER 4: BACTERIAL WILT RESISTANCE DIVERSITY WITHIN FOUNDER AND ROOTSTOCK GERMPLASM, AND THEIR VIABILITY FOR BREEDING AND GRAFTED MANAGEMENT APPLICATIONS

4.1: Abstract

Bacterial wilt (BW) of tomato is caused by the *Ralstonia solanacearum* species complex (Rs), which was recently divided into three species based on phylogenetic grouping of global strains. All three species cause economical losses of tomato and other Solanceous crops around the world, as well as in the Southeastern USA. Quantitative resistance has been assessed for many decades, and the most globally resistant sources have been available since the 1970s. The combination of large fruit size (>200 g) and high levels of resistance have been very difficult to obtain due to strong linkage drag, thus leading to the deployment of these lines in a grafted management system with commercially competitive susceptible scions. Not all resistance sources perform well against all strains. Rotation of resistance genetics is desired but the most widely used rootstock germplasm are commercial hybrids with undisclosed resistance sources. Thus, a panel of 41 lines plus 10 susceptible controls was obtained in order to assess their viability and diversity for management and breeding applications against North Carolina strains of Rs. The panel contains BW resistance founders, important breeding introgressions, and commercially deployed rootstocks that represent at least 15 putatively unique resistance sources. A field-first approach was used in three field*year experiments combined with two greenhouse seedling screens that incorporated colonization and vascular browning assessments. A subset of commercial hybrids and several public sources were tested as rootstocks for resistance and yield parameters in a 5-location grafted variety trial across environments. The diversity panel was also
phenotyped for multiple morphological and developmental traits as a first step towards further genetic relatedness testing. The lines clustered into 5 groups, predominantly by resistance level. A number of resistance sources performed well in NC, some of which are new candidates for resistance introgression. Multiple rootstocks provided efficacious resistance and competitive yields from multiple breeding sources, allow for a rootstock rotation program.

4.2: Introduction

Tomato production can be plagued by the presence of the soil-borne pathogen *Ralstonia solanacearum* (Rs), a species complex that causes bacterial wilt (BW) of Solanaceous crops, among many others (A. C. Hayward, 1991; A. C. Hayward, 1994; Prior and Fegan, 2005). Considered by many in the molecular plant pathology community to be economically and scientifically the second-most important plant bacterial pathogen (following the collective *Pseudomonas syringae* pathovars), it continues to cause economic loss around the world (Elphinstone, 2005; Jimenez-Madrid et al., 2016; Mansfield et al., 2012; Ramesh et al., 2014; Ramsubhag et al., 2012; Wicker et al., 2009). As a global pathogen in warm, moist temperate to tropical regions of the world, management of Rs is a current need, including in the Southeastern USA (CABI/EPPO, 1977; CABI/EPPO, 1999; Hong et al., 2012; Kelman, 1953).

The global population structure of Rs is quite complex, leading to the recent reclassification of the Rs-species complex into three species, based upon genetic, biochemical, and host patterns. Focusing on Solanaceae, they are: Phylotype I (*R. pseudosolanacearum*; all Solanaceae; Africa, Europe, Eastern Asia), phylotype IIA (*R. solanacearum*; minus *Capsicum* spp.; North and South America mostly with some African spots), phylotype IIB (*R. solanacearum*; especially aggressive on potato; Caribbean to South America, Africa, and spots in
Europe the South Pacific), phylotype III (R. pseudosolanacearum; all Solanaceae; Africa), and phylotype IV (R. syzygii; South Pacific and Eastern Asia) (C. H. Lin et al., 2014; Prior et al., 2016; Ramsubhag et al., 2012; Remenant et al., 2010; Safni et al., 2014; Wicker et al., 2011; Yahiaoui et al., 2017).

Within the Southeastern USA, historically endemic natural populations of Rs are present, although they appear to contain a much lower genetic diversity compared to the global situation (Hong et al., 2012; Jimenez-Madrid et al., 2016). Populations of Rs have been reported in over 80% of the counties in North Carolina, with strains in the Mountain region seeming to be a more recent, growing problem (Kelman, 1953; Rivard et al., 2012, and internal strain libraries). The lack of chemical or cultural practices to effectively manage BW leave genetic resistance or crop abandonment as the only options for producers (Chellemi et al., 1994; Driver and Louws, 2002; Enfinger et al., 1979; Lemaga et al., 2001; Satou et al., 2006).

Efficacious BW resistance is available, coming from mostly undomesticated or partially domesticated S. lycopersicum. Resistance can also be found in close relatives, such as S. lycopersicum var. cerasiforme, S. pimpinellifolium, and several S. peruvianum (Daunay et al., 2010; Kim et al., 2016; Miao et al., 2008; Miao et al., 2009; J. Wang et al., 1998). Breeding for BW resistance has been challenging for a number of reasons. 1) Environmental influence on wilt development is substantial, as well as 2) regional strain variation (Barnes and Vawdrey, 1993; Kelman, 1953; Nakaho et al., 1996; Opena et al., 1990; Scott et al., 2005). 3) There is substantial linkage drag with small fruit size, which only one program has recently suggested to have broken (Acosta, 1978; Scott et al., 2004; Scott et al., 2009; Scott et al., 1993; Scott et al., 2005).
4) Resistance is polygenic and quantitative, being conditioned by up to 11 resistance loci mapped over 8 chromosomes (3, 4, 6, 7, 8, 10, 11, 12), while only the chromosome 6 loci is consistently detected in all the mapping studies (Carmeille, Luisetti, Besse, Prior et al., 2006; Danesh et al., 1994; Mangin et al., 1999; Thoquet, Olivier, Sperisen, Rogowsky, Prior et al., 1996; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996; J. Wang et al., 2000; J. Wang et al., 2013). The chromosome 6 locus (Bwr-6a through 6d) seems to provide the bulk of the resistance across strains. The chromosome 12 loci (Bwr-12) is extremely important for BW resistance in Southeastern Asia and the South Pacific (P. Hanson et al., 2013; P. Hanson et al., 2016), but it may not play a major role in resistance to North American strains (Ho et al., 2013; Kressin, 2014, and chapter 3). For example, ‘CRA 66’ is not reported to contain Bwr-12, yet it exhibits strong BW resistance that is no different from the Hawaii germplasm. Overall, differences in resistance strength between various germplasm sources have been reported in parts of North America (Jaworski et al., 1987; Sonoda and Augustine, 1978; Sonoda et al., 1979).

Thus, high levels of resistance have not been successfully introgressed into commercial tomato varieties where large fruit (>200 g) are required. It has instead been deployed in rootstock material for a grafted management system, with great success globally (Arwiyanto et al., 2015; Cardoso et al., 2012; Grimault and Prior, 1994a; Kressin, 2014; McAvoy et al., 2012; Onduso, 2014; Rivard et al., 2012). The pool of rootstocks is fairly large, especially for recommendation purposes. Most are private sector hybrids and some are open-pollinated public breeding and resistance founder lines.

Many of the public rootstock germplasm were included in a global testing effort of a worldwide germplasm panel of 35 accessions for BW resistance across 12 locations, plus a greenhouse test. The top 7 most overall resistant entries were noted as tracing back to either three
resistance sources or all coming from selections from one or more unknown Philippine lines (Scott et al., 2005; J. Wang et al., 1998). Excluding duplicates, three lines developed by the University of Hawaii breeding program were the 1st, 3rd, and 5th most resistant (Hawaii 7996, 7997, and 7998, respectively). The frequently examined ‘CRA66’ from INRA was 8th.

Later, several breeders put together a report that very thoroughly reviewed the literature and notes about the history of tomato BW breeding efforts from the 9 major public breeding programs in the last century (The Universities of North Carolina, Florida, Hawaii, Puerto Rico, and Philippines, the Horticultural Research Station in Japan, the Asian Vegetable Research and Development Center in Taiwan, and the French research institutes INRA3 and IRAT4) (Daunay et al., 2010). They reconstructed the flow of resistant germplasm between these programs, along with notable breeding releases. The available pedigrees notes were traced as far back as possible to identify the founder lines. It was discovered that most of the resistance sources still used today were developed in the 1950s to 1970s, which remain the best in the world.

There was substantial germplasm flow in and out of the breeding programs, and the breeding notes are often incomplete or conflict with later published journal papers. The most glaring of these discrepancies is about the resistance source for the lines Hawaii 7996, 7997, and 7998 (and tangentially 7981), which is commonly noted in the more modern literature as being developed from “PI 127805A”. No publications were made of the development of these lines and gene banks have only have ‘PI 127805’, which is labeled as a S. pimpinellifolium accession

3 INRA: Institut National de la Recherche Agronomique.
4 IRAT: Institut de Recherche en Agronomique Tropicale, which is now part of CIRAD as the Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement.
collected in Peru (USDA GRIN system). The review concluded that the origin of the most resistant, most studied, and likely most bred resistance source (Hawaii 7996) remains unclear. It could be from ‘PI 127805’, from an unknown Philippine source, or the combination of five separate resistance sources from at least two breeding programs (Daunay et al., 2010). A similar but less complex situation was noted for ‘CRA66’, the second most studied resistance source.

Collectively, these sources indicate that a large number of resistance founder lines have been discovered and at least bred for some duration of time, yielding moderate to excellent sources of resistance. More modern rootstock development, however, has been performed predominantly by private industry. Thus, the pedigrees and/or resistance sources are not revealed. It is likely that most of the commercially available rootstocks today trace their origin to ‘Hawaii 7996’ and its sister lines.

An integral part of disease management is the use of resistance variation, either in rotation or combined together, in order to help safeguard against selection for resistance-breaking strains, such as has been documented in many crops. In order to adequately safeguard resistance to BW for the next century, breeding and management strategies need to consider resistance diversity combined with regional efficacy. A handful of different rootstocks have been tested for field efficacy in parts of NC, identifying several that perform well for both BW resistance and overall scion productivity (McAvoy et al., 2012; Rivard et al., 2012; Rivard and Louws, 2008). Our more recent efforts to expand that pool of rootstock options has been successful for the Mountain region of NC, but we lack good information for the Piedmont and Coastal Plains regions, which have very different soil types, climates, and possibly different strains (Kressin, 2014; Silverman, 2015).
Genetic relationships can be inferred based on DNA polymorphism data. Lines sharing polymorphism fingerprints are expected to be more related because of identity by descent, which can be estimated statistically (Weir et al., 2006). Most publicly available genetic data for BW resistance is based on PCR-based marker technology. The best, most recent data uses simple sequence repeats (SSR; also called microsatellites), which are a class of repetitive DNA comprised of tandem repeats of 2 to 5 nucleotides long (E.g. “AT”, “CCG”, etc.). They can vary in repeat number, are multi-allelic, and co-dominant. SSRs can be highly polymorphic within a population but are fairly stable over generations.

SSRs are commonly used as markers for genetic analyses and breeding in tomato, as well as many other organisms. The most recent BW resistance mapping efforts have employed genome-wide SSR markers for identifying resistance QTLs (Geethanjali et al., 2010; J. Wang et al., 2013). SSR markers for several of the discovered QTLs have been used for wider germplasm tests, QTL*strain effects, and selection in a breeding program (P. Hanson et al., 2013; P. Hanson et al., 2016; Ho et al., 2013). SSR markers have also been recently employed for assessing genetic diversity between Solanum and Malus species (Adeniji et al., 2012; Potts et al., 2012), genetic relatedness between cultivars within sugarcane (Chen et al., 2009), Sorghum accessions (Uptmoor et al., 2003), soybean (Ghosh et al., 2014), and rice (Kanawaipep et al., 2011), and for assessing population structure in diverse rice germplasm (Nachimuthu et al., 2015).

This work reports on efforts to collect BW resistant rootstocks and founders and assess their level of resistance across North Carolina environments and controlled inoculations. A subset were then tested for their efficacy and productivity as rootstocks for commercial grafted production systems across North Carolina, especially from Coastal Plains and Piedmont fields exhibiting strong natural disease pressure. Finally, aspects of genetic diversity among these lines
were assessed based on phenotypic similarity. This sets up the panel for future genetic testing. The work has applications for future resistance introgression and current management recommendations for North Carolina.

4.3: Materials and Methods

This work incorporates three field*year (field diversity experiments) and two greenhouse resistance screens (greenhouse diversity experiments) of non-grafted germplasm from the diversity panel. Additionally, it reports the first year of a 5-location grafted rootstock variety trial across NC during the summer of 2017 where both field resistance and yield were recorded. Because we do not have access to research fields that can be artificially inoculated, all the field tests were performed in commercial tomato fields naturally infested with Rs in cooperation with local tomato growers. The field locations were Mountain 1 (MT; heavy natural Rs infestation), the Mountain Research Station (MRS; no BW history), Piedmont (PD; low to moderate natural Rs infestation), Coastal Plains 1 (CP1; heavy natural Rs infestation), and Coastal Plains 2 (CP2; low to moderate natural Rs infestation). Finally, it includes phenotypic clustering of the diversity panel in anticipation of further genetic testing for genetic diversity and the available markers for the Bwr QTL as reported from the World Vegetable Center (AVRDC) (P. Hanson et al., 2013; P. Hanson et al., 2016; Ho et al., 2013). Genetic testing is not included in this report.

4.3.1: Diversity panel germplasm collection and growth conditions.

A collection of rootstocks and resistance founder germplasm assembled to form the resistance diversity panel. Literature searches, gene bank data mining, and company queries were performed looking for important and novel commercial rootstocks and resistance founder
germplasm. The selection criteria were based upon company/gene bank labeling, previous reports testing lines for bacterial wilt resistance, NC resistance introgression populations, or reports of lines with resistance derived from perhaps otherwise untested sources. About 110 genotypes were identified as potential founders or commercially important resistant rootstocks (data not shown). Several lines were included in the list based upon possible association with known founders. Progeny of founder lines were generally excluded, unless the founder was not available.

Seed procurement was attempted for each line. Samples were obtained for 41 lines. They represent 7 companies (Asahi, Seminis, Takii Seed, BHN Seed, DP Seeds, Sakata Seed, and Rijk Zwaan), 2 public gene banks (C.M. Rick Tomato Genetics Resource Center at UC Davis and the USDA National Plant Germplasm System), and 2 tomato breeding programs (North Carolina State University and the University of Florida). Due to a quarantine issue for shipping tomato seeds to the USA, seeds were not successfully obtained from the AVRDC genebank, where most of the lines of interest were stored. This germplasm formed the resistance diversity panel, along with a collection of 10 susceptible rootstock, commercial hybrid, heirloom, and important breeding parent controls. Not all the seeds were obtained at the same time, and many lines required a seed increase first. Thus, not all genotypes were included in all the experiments. Line information and numbers tested in each of the 5 non-grafted experiments are summarized in Table 4.1.

A subsample of 12 total lines from 10 different sources were chosen for the rootstock variety trial. These were selected primarily from private seed company availability, were developed for NC, or were known good candidates for grafting. A new rootstock line being considered by Seminis (DR6258TX) was not included in the diversity panel but was tested in the
variety trial. 6 of the rootstocks were new for NC tests. 2 other rootstocks had been examined either only in 1 year previously and/or not in either the Coastal Plains or the Mountain regions. The older rootstocks were selected based on recommendations from previous work (Kressin, 2014; Rivard et al., 2012; Rivard and Louws, 2008; Silverman, 2015). Table 4.3 summarizes basic information about the rootstocks tested in the variety trial.

All of the rootstock seeds for these experiments were donated by the listed supplier. The NC-based Tri-Hishtil grafting company in Mills River obtained the hybrid seed, grew the plants, and grafted all the commercial material, while the NC State grafting team obtained, grew, and grafted all the NCSU-supplied seed and the non-grafted scion controls. All the grafted plants were high quality, standard single-leader seedlings—i.e. not pinched.

4.3.2: Diversity field experimental design.

The non-grafted experiments tested 25, 36, and 39 lines from the diversity panel in the 2016 MT, 2017 MT, and 2017 CP1 locations alongside 2, 4, and 7 susceptible controls, respectively. A duplicate of the 2016 MT trial was planted in the PD location. Wilt development was very poor in that section of the field and is not discussed further.

For each trial, seeds were sown in 72-cell propagation inserts in plastic STF-1020-OPEN trays with holes (11-0700-1 and 11-3000-1, respectively; T.O. Plastics Inc., Hummert International) and germinated on heating pads set at 26.6 °C. The plants were fertilized biweekly with 20-20-20 soluble fertilizer (07-5400-1, Hummert International) to maintain healthy, active growth. 1 week before transplanting to the field, the seedlings were moved outdoors onto a concrete pad in full sun to allow them to harden.
All the fields were the standard plasticulture system with drip irrigation used in North Carolina (Ivors and Sanders, 2010). Table 4.2 provides details about site-specific preparation, conditions, cultural management, etc. for each grafted trial location. These conditions were either the same or with slight modification for the non-grafted germplasm field tests each year, which were carried out in adjacent plots.

The seedling plugs were transplanted to the field 4 to 5 weeks after sowing, with 4 to 6 plants per plot (occasionally less due to poor germination). In the 2017 CP1 trial the plants were spaced closer (14 inches; 35.5cm) than the adjacent grafted variety trial to conserve space. In all experiments, the genotypes were randomized within each replication block and laid out in a snake pattern across the rows, with the blocks arranged side-by-side across all the rows. Transplant survival was determined one week after planting and the minimal losses were replaced if possible.

Wilt severity was generally scored weekly to biweekly using the previously described 0 to 5 scale (chapter 3, Figure 3.1) for the duration of the experiment, which lasted until harvest time (77 to 83 days; 7 to 8 observations). The plants were generally not pruned, and were trellised, fertigated, and sprayed for pests and diseases along with the rest of the grower’s surrounding tomato crop.

4.3.3: Diversity greenhouse seedling resistance assays.

Two greenhouse resistance assays were performed with non-grafted seedlings of the diversity panel under artificial inoculum pressure. Seeds were sown into the previously described 72-cell trays, with multiple seeds per plug. The seeds were germinated and fertilized as
mentioned above. Following germination, the plugs were thinned as needed to about 2 plants per plug.

An average of 11.2 plants were inoculated 47 days after sowing with 10 mLs of $10^9$ CFU/mL of NC isolate Jc, previously isolated from a susceptible tomato line in the MT field (Kressin, 2014; Silverman, 2015). The root drench on lightly injured roots method described in chapters 2 and 3 was used. Isolate culture and inoculation procedures were previously described in chapter 3. Two additional plugs of each line were inoculated with sterile water over lightly injured roots. Table 4.1 notes the specific number of plants inoculated for each line in each experiment.

The plants were scored for wilt severity several times a week for the first 2 weeks using a 0 to 5 score (previously described in chapter 3, figure 3.1), and then less frequently until the experiments were terminated. Experiment 1 was terminated at 31 dpi, while experiment 2 was terminated at 67 dpi. Upon termination, each plant was: 1) given a final wilt score, 2) was inspected for any indication of stem rot that would indicate the presence of latent damping off pathogens, 3) dissected at the basal hypocotyl with a sterilized razor blade and the severity of vascular browning was assessed using a 0 to 5 score (previously described in chapter 3, figure 3.2), and 4) stem-imprinted on SMSA-E media (French et al., 1995). Imprint cultures were incubated at 30 °C and assessed for Rs growth 48 to 72 hours later as described in chapter 3. Because of the length of the experiments, many wilted plants completely desiccated and were therefore not imprinted because they rarely yield Rs. Those plants were considered colonized, based on prior experience.
4.3.4: Rootstock variety trial design.

The plants for the grafted variety trial were largely provided by Tri-Hishtil (Mills River, NC). For those that were provided internally, the seeds were sown, germinated, grown, fertilized, and hardened in the same manner as the field diversity experiments.

The NCSU rootstock treatments were grafted at about 18 to 21 days after sowing. Each experiment used a locally adapted scion variety of the grower’s choice. The same top grafting method described in chapter 2 and in previous work was used (Kressin, 2014; Silverman, 2015). The plants were healed under fine mist under multiple layers of shade cloth and acclimated back to normal conditions within 8 days, after which they were hardened normally. The seedlings were planted 5 to 6 weeks after sowing (at least 14 days following grafting). Prior to transplanting the grafted plants were sorted for graft quality by removing the clips, visually inspecting the union, and giving each plant a moderate shake to test for structural integrity. Plants that endured were used for planting.

Table 4.2 provides details about site preparation, conditions, cultural management, etc. for each grafted trial location. The fields were laid out in a randomized complete block design with 4 replication blocks arranged side-by-side such that each block spanned all the rows. Grafting treatment plots were randomized within each block. Each plot had between 8-12 plants depending on the location while a few plots had 1 to 6 plants due to lower germination/graft survival (Jackson, Haywood, and Brunswick co. tests). Between each plot, a non-grafted susceptible guard plant of the local scion variety was planted.

Out of curiosity, and because we had a surplus of the treatment, we decided to test if burying the graft union on a known highly resistant rootstock treatment (‘Shin Cheong Gang’) would lead to compromised resistance after the scion had rooted. So, two sets of plots of that
rootstock combination were planted—the recommended shallow depth where the root ball is just barely covered with soil, and where the graft union was purposefully buried below the soil line. All other treatments were planted at the recommended depth.

At the time of harvest, the market-type fruits of the local grower (vine ripe or mature green) were handpicked on a weekly basis and sorted according to size. The fruit quality scale had a high tolerance for fruit blemishes because we were primarily interested in fruit number and weight. Inedible fruit were culled, but the rest were considered marketable if they matched the small through jumbo sizes. Sorting for size was performed using a home-made field sizer system (Supplemental 4.3, left). The exception was the Mountain Research Station trial where a mechanical sorter was used with the gates set for the same diameters as the bucket system. The fruit diameter classes in the staked tomato production guide developed by the USDA and NC State were used (Ivors and Sanders, 2010; USDA-Agricultural Marketing Service, 1991). The USDA maximum sizing scales were used for cull through extra large, with the addition of the NC suggested “jumbo” grade (Supplemental 4.3, right). Plots were harvested 3 to 5 times and each size class was counted and weighed.

4.3.5: Phenotyping the diversity panel.

For the principle component analysis (PCA) and cluster analysis, the diversity panel was phenotyped for several morphological/developmental traits—Species, fruit color, fruit class, fruit size (partial), fruit shape, and growth type. The traits were scored as possible after the tomato descriptor, with additional levels added as needed (IPGRI, 1996).

The species scale had 6 levels from 1 to 6—Solanum habrochaites x S. lycopersicum (E.g. ‘Maxifort’), S. lycopersicum, S. lycopersicum var. cerasiforme, S. lycopersicum or S.
pimpinellifolium (gene bank listings conflict; e.g. ‘PI 129080’), S. peruvianum, S. pimpinellifolium, respectively.

Fruit color had 6 levels—green, yellow, orange, pink, red, purple, respectively. Fruit shape had 10 levels, adding “irregular” as a score of 9 before “other” as a score of 10 from the descriptor. Growth type had 5 levels, adding “semi-dwarf” (E.g. ‘LA3526’) as a score of 2 before “Determinate” at score 3 from the descriptor.

Fruit class describes the market type ordered by generally size. The scale was coded from 1 to 6 as cherry, grape, cherry/irregular, comparai, plum, and beefsteak, respectively. Fruit size was determined in the PD location in 2016 by performing a once-over harvest. The fruit were weighed and the average fruit weight determined. Because only a subset of the diversity panel was present in that experiment, however, fruit size was not included in the further analyses.

4.3.6: Data collection and analysis.

Wilt data was taken for every plant in every experiment. The susceptible guard plants were also assessed as applicable to get a sense of the the distribution and uniformity of bacterial wilt development. In the greenhouse every plant was scored for vascular browning severity and colonization also. The severity scores were converted into indices by dividing the values by 5 and multiplying by 100, which yields the percent of possible severity. The area under the disease progress curve (AUDPC) for wilt index was also calculated over the duration of the experiments:

\[ AUDPC = \sum_{i=1}^{n} \left( \frac{score_n + score_{n+1}}{2} \right) \times (T_{n+1} - T_n) \]

where, score = the severity scale value, n = time point, T = days after planting, and i = the number of individual observation points. A handful of plants showing infection with Tomato Spotted Wilt Virus (TSWV) at any time during the seasons were excluded from the wilt analysis.
Furthermore, wilt symptoms in the beginning of the epidemic were visually assessed for the presence of pith necrosis and any positive plants were excluded from the analysis.

Yields were measured in the grafted trials. The total season marketable yields per plant were calculated by dividing the plot yield by the number of plants, excluding any plants lost to diseases other than BW (E.g. TSWV, pith necrosis, etc.), which were minor. The yield per area were calculated by multiplying the mean yield per plant by the plants per acre (or hectare) estimate based on the local field setup (Table 4.1). Furthermore, the total fruit weight of all classes and harvests were summed and divided by the total number to get average fruit weight. The distributions of the mean number of fruit were also compared by grafting treatment.

SAS 9.4 (SAS, Cary NC) was used for all statistical analyses, particularly the MEANS and GLIMMIX procedures. Gaussian distributions were used for all variables with the identity link function for the LSMEAN outputs. All pairwise comparisons were performed with the Tukey-Kramer adjustment for multiple comparison testing for an overall α = 0.05.

In both years of the diversity panel trials in the MT location (especially 2016) there was a row effect where wilt development was slower and with lower incidence in an outside edge row than the rest of the rows. The analysis model was adjusted for these inconsistencies by including spatial variables. Comparisons were made of models with the fixed effects of row alone, row and position, or both factors as a spatially repeated measures adjustment [type=SP(POW) (row position)].

The row only as a fixed effect model was chosen for all the analyses since: 1) position was not a significant factor either as a fixed effect (p=0.4789) or in the spatial model (p=0.7233). 2) There were only slight improvements in the fit statistics (AICc) for the spatial model vs. the fixed effect model (6899.1 vs. 6909.0, respectively). And, 3) the models taking position into
account strongly warped the mean wilt compared to the observed wilt such that some lines exhibiting wilt became negative while highly resistant controls showing no wilt (‘Shin Cheong Gang’ and ‘Hawaii 7997’) were indicated as having wilt. Row was a significant factor in all models (p<0.0001).

The PCA and cluster analysis included the wilt AUDPC values. Because the AUDPCs were based on different observation periods, the daily mean AUDPC was calculated by dividing the final sum by the observation period. Because not all lines were present in all experiments, the daily AUDPC values of each genotype were averaged. The mean daily AUDPC was used for the analyses. Fruit size was not included in the analyses but was used to validate the fruit class scale order.

The PCA incorporated the wilt and morphological/physiological observations of each line. The SAS PRINCOMP procedure was used for the analysis. Two cluster analyses were performed—one with only the single daily AUDPC variable and the other that added the other phenotypic traits. The SAS CLUSTER procedure was used for the analysis. The main clusters from the wilt-only analysis were labeled as BW1 through BW5 from most resistant to most susceptible. These clusters of germplasm were tracked after the addition of the other phenotypic information and labeled appropriately.

4.4: Results

4.4.1: Field-level wilt resistance of the diversity panel.

This work has evaluated a total of 51 lines representing a substantial proportion of the commercial hybrid rootstock markets and resistance founder lines, plus multiple susceptible controls. A field first approach was used to evaluate the germplasm for resistance efficacy in
North Carolina. In 2016, strong disease pressure was successfully obtained in the MT location but not in the PD location (data not shown). The MT and CP1 locations were tested in 2017 and both exhibited strong disease development. The CP1 location was not available in 2016.

In the MT location in both years there was an effect of row, where one outside row of the 7-row block developed less disease and more slowly compared to the other rows (Figure 4.1A-B). This was not related to the number of susceptible controls in the rows, but had to do with a shift in the location of the block into soils that were typically drive rows or rows of sweet corn in previous years. The wilt development in the CP1 location was equivalent across the rows (Figure 4.1C).

In the MT location, the variation between replication blocks was large in 2016 (data not shown). The variation was related to the rate of wilt initiation, where one of the reps was more on the edge of the historical hotspot while the other was right in the middle of it. This made pairwise differentiation of the season-long AUDPC means difficult between the genotypes (data not shown). Greater clarity between genotypes was achieved by analyzing the end-of-season wilt indices (Figure 4.2). The numerical spread of the means was as expected in the susceptible and resistant controls.

The most resistant germplasm in the MT 2016 trial were the Hawaii lines (7997 and 7998), rootstocks from Seminis (‘Cheong Gang F1’ and ‘Shin Cheong Gang F1’), several lines from BHN Seed (1087 and 669). In close second were ‘Hawaii 7981’ from DP Seeds (RST-04-106-T), ‘Venus’, ‘Dai-honmei’ from Asahi, ‘LA3526’, ‘IRAT-L3’, and ‘CRA66’. Several others including ‘BHN1053’ and ‘Shield’ from Rijk Zwaan were more moderately resistant. ‘Maxifort’ exhibited uncharacteristically low wilt, was in the heart of the low wilt row, and was similar to
‘Mountain Magic’, ‘PI 126408’, and ‘LA2701’. Lines clearly grouping with the other susceptible controls were ‘PI 127805’, ‘PI 129080’, and ‘RST-04-105-T’ from DP seeds.

In the 2017 season, substantial differences were observed between germplasm in both the MT and CP1 locations (Figure 4.3). The susceptible controls were highly wilted and the reps were much more consistent. Across both fields, the most consistently resistant material included all of the first and second groups of most resistant lines from 2016, and new lines added to the group included: ‘Armada’ from Takii Seed, ‘Hawaii 7996’, two introgression lines from the NCSU breeding program with resistance derived from ‘Hawaii 7997’ and ‘Hawaii 7998’, ‘PI 263722’, ‘PI 251323’, ‘PI 406994’, and ‘Bowman’ from Sakata Seed.

Seed from survivors of ‘Venus’ at the end of the 2016 MT location test were bulk harvested. Both the selection ‘Venus bulk-Jc’ and the original source were planted again in 2017 in both locations. In the MT location, they performed the same, but in the CP1 location, the bulk selection was obviously numerically less wilted (Figure 4.3).

‘Shield’ was again intermediately resistant in the MT but grouped with the most resistant in the CP1 location (Figure 4.3). ‘CLN1466E’ grouped with most of the most resistant lines in the CP1 location but exhibited significantly more wilt in the MT location. In opposite manner, ‘PI 479211’ exhibited more wilt in the CP1 location than the MT location. Both ‘LA2701’ and ‘PI 126408’ exhibited significantly more wilt in the CP1 location but grouped with the highly and moderately resistant material in the MT location, which was opposite of the previous year.

As in 2016, ‘RST-04-105-T’ from DP Seeds grouped with the susceptible lines in both locations, as did ‘PI 390009’, ‘PI 129080’, ‘PI 303814’, ‘PI 127811’, ‘PI 127805’ (Figure 4.3). Several family lines from the population NC14251 grouped with the susceptible controls. NC 14251 is a double backcross of ‘Hawaii 7998’ into the large-fruited susceptible ‘NC358-
Furthermore, several of family lines exhibited greater wilting in the CP1 location compared to the large-fruited ‘Florida 47’ and ‘Cherokee Purple’ controls. They were not different from several other susceptible controls like ‘Bonny Best’ and ‘Red Morning’. As expected ‘WVa700’ was highly susceptible.

4.4.2: Greenhouse wilt screens of the diversity panel.

Two greenhouse seedling tests were performed to compliment the field studies, focusing on resistance to very strong disease pressure. A total of 816 plants across both experiments were inoculated with $10^9$ CFU/mL of the isolate Jc originally obtained from the MT location. End-of-study destructive harvests were performed to assess colonization and VB.

Some wilt and VB were observed in some water controls in each experiment. No Rs was detected in those controls using the stem imprint method, nor did the wilting pattern match that caused by Rs (it was a slow wilt where the whole plants would partially wilt but never reach the permanent wilting point and die). Close inspection of the basal hypocotyl in those plants showed external necrosis, suggesting a low degree of a fungal damping off pathogen. Furthermore, there seemed to be no distinction between resistant and susceptible lines showing symptoms within the water controls.

Several plant samples from the affected water controls were submitted to the NCSU Plant Disease and Insect Clinic for diagnosis. Pythium spp. were detected in one sample and Botrytis on the stem of another. The rest cultured Fusarium spp., which were suspected to be secondary pathogens (PDIC, personal communication). No Rhizoctonia spp. were found. To try and control for possible contamination in the Rs-inoculated plants, notes were made of any plants that were
suspected of also being contaminated with damping off pathogen. Thirty-nine plants were found. Those were excluded from the analyses.

Significant differences in wilt development and VB severity in the basal hypocotyl were found within the inoculated treatments. *R. solanacearum* was isolated from most living plants with rare exception (data not shown). It was also isolated from some plants that were fully desiccated (data not shown). The colonization data were confounded by the desiccation and possible damping off contamination. Desiccated plants were assumed to be colonized unless there were obvious signs of damping off. This made meaningful analyses of colonization rates tenuous.

In the experiment (T1), first wilting was observed in ‘Bonny Best’ at 5 dpi, while most of the wilt epidemic began between 7 and 12 dpi. A moderate separation between genotypes was observed with regards to mean AUDPC for wilt index, while the final wilt incidence closely resembled the AUDPC pattern (Figure 4.4A).

In T1, the most resistant lines were ‘BHN998’, ‘Hawaii 7996’, ‘Shin Cheong Gang F1’, ‘Bowman’, and ‘BHN1087’, which basically very little to no wilt exhibited no wilt and were significantly different from all the susceptible controls. Strangely, ‘RST-04-105-T’ developed very little wilt and was significantly lower than all the controls except ‘Fletcher’. The next numeric group of lines that exhibited numerically greater wilting were: ‘Hawaii 7997’, ‘CRA66’, ‘LA3526’, ‘BHN1053’, ‘Hawaii 7998’, and ‘IRAT-L3’, which were all significantly less wilted than ‘Bonny Best’, while some were different from all the controls except ‘Fletcher’.

The intermediately resistant lines were no different from any of the susceptible or resistant controls, which included lines such as ‘CLN1466EA’, ‘BHN669’, the two NC14251 lines, ‘Hawaii 7891’, ‘Venus’ and ‘Venus bulk-Jc’, ‘RST-04-106-T’, ‘Cheong Gang F1’ (which
had a higher percentage of apparent damping off contamination, ‘LA2701’, and ‘Shield’. ‘RST-04-106-T’ and ‘Cheong Gang F1’ exhibited the most apparent damping off contamination, at least twice that of the other lines (data not shown). Three lines were interspersed with the susceptible controls: ‘PI 126408’, ‘PI 129080’, and ‘PI 127805’.

In the second experiment (T2), the overall disease pressure appeared to be greater (Figure 4.4B). More plants reached the permanent wilting stage, however there were more substantial differences between the mean AUDPC wilt index values. First wilting occurred between 5 and 8 days, with many plants exhibiting wilt at 8 dpi. Fewer lines grouped with the least wilted lines. Interestingly, the ‘Venus’ field selection from the 2016 MT location exhibited the lowest wilting, while the original population exhibited significantly greater wilting. Curiously, ‘LA2701’ also exhibited the lowest wilting, alongside ‘Shin Cheong Gang F2’, ‘LA3526’, ‘BHN998’, and ‘Hawaii 7996’. Significant differences were very stepwise.

Other lines that were no different from ‘Hawaii 7996’ were ‘Shin Cheong Gang F1’, ‘PI 390009’, ‘CLN1466EA’, ‘RST-04-106-T’, ‘CRA66’, ‘Hawaii 7997’, and ‘PI 251323’ (Figure 4.4B). These lines exhibited less than half of the AUDPC wilt index values of the most susceptible controls. The next group of lines with higher wilting were: ‘BHN1087’, ‘PI 263722’, ‘Venus’, ‘PI 129080’, ‘Cheong Gang’ (F1 and F2), NC14251-207, and ‘Shield’. The latter two lines and the rest with higher wilt levels incrementally overlapped more and more with the susceptible controls, beginning with ‘Fletcher’. These included ‘PI 479211’, ‘PI 127805’, ‘Hawaii 7998’, ‘BHN1053’, ‘RST-04-105-T’ ‘PI 406994’, ‘Bowman’, and ‘Hawaii 7981’. Lines that were interspersed among the susceptible controls were ‘IRAT-L3’, ‘PI 127811’, ‘BHN669’, ‘PI 303814’, NC14251-187, and ‘PI 126408’.
Colonization was assessed using stem imprints onto semi-selective media. The rates of colonization were high in both experiments. Most germplasm were highly infected (75% and higher), while only a few lines exhibited frequencies between 40 and 75% (Figure 4.5A-B, diamonds). In T1, ‘CRA66’, ‘RST-04-105-T’, ‘Shin Cheong Gang F1’ exhibited colonization levels of 50, 62.5, and 72.7%, respectively. In T2, ‘Venus bulk-Jc’, ‘LA2701’, ‘CLN1466EA’, ‘PI 251323’, ‘LA3526’, ‘BHN998’, and ‘PI 390009’ were colonized between 41.6 and 72.7%. The pattern of colonization was somewhat analogous to the trend of VB development.

Vascular browning severity was assessed in the basal hypocotyl of each plant at the end of the greenhouse experiments. Significant differences in VB index were observed between the germplasm in both experiments (Figure 4.5A-B, columns). In T1, four lines exhibiting the same levels of very little to no wilting exhibited significant differences in the mean VB index levels. ‘Hawaii 7996’ had significantly less VB than ‘Shin Cheong Gang F1’ and ‘BHN998’ while ‘RST-04-105-T’ was intermediate, and they all had substantially less VB levels than ‘Bowman’. Furthermore, lines exhibiting moderately low levels of wilting exhibited substantially increasing levels of VB. ‘Hawaii 7996’, despite having the lowest VB severity, exhibited 100% colonization of the basal hypocotyl.

In T2, the mean VB levels were all around inflated compared to T1, but substantial differences were observed between genotypes. A pattern similar to experiment 1 was observed, where lines with the lowest wilting generally had the lowest VB index, although ‘Shin Cheong Gang F2’ was similarly wilted with ‘LA2701’ and ‘BHN998’ but exhibited significantly higher levels of VB. Curiously, ‘CLN1466EA’ had significantly greater wilt than ‘BHN998’, ‘LA2701’, and ‘Venus bulk-Jc’, yet it had exhibited nearly identical levels of VB.
A regression of the final wilt index with the VB index in each experiment exhibited a tight, positive, linear relationship that was significantly correlated (p<0.0001) (Figure 4.6A). In both experiments, the regression slopes were nearly identical; in T1 = 0.5481 and T2 = 0.5439. In contrast, the final wilt indices of each experiment were much more loosely related, although the trend was still clearly positive (Figure 4.6B).

4.4.3: A subset of the resistance germplasm as rootstocks for field management of BW.

A subset of rootstocks and founder germplasm was tested in five locations as rootstocks in a grafted variety trial. In these experiments, BW pressure was very strong in the CP1 and MT locations but was essentially non-existent in the CP2 and PD locations. The MRS location was a no-disease control. In the CP1 field, first wilt symptoms were observed 15 days after planting, primarily in the susceptible guard plants. In contrast, the first wilt development was observed in the MT field at 23 days after transplanting. The Mountain field was cooler and had been fumigated, whereas the CP1 field had not. The overall vigor of the plots in CP1 was much lower than the other fields and had the strongest bacterial spot and bacterial wilt pressures (data not shown). The vigor in the PD and MT locations was excellent. CP2 exhibited moderate vigor.

The AUDPC wilt index values for each plot revealed strong differences by rootstock treatment for BW resistance in the MT and CP1 locations (Figure 4.7A-B). The susceptible non-grafted local scion variety was rapidly highly wilted in both fields. In both locations, ‘Shin Cheong Gang F1’, ‘CRA66’, ‘Armada F1’, and DR6258TX (Seminis test name) exhibited the lowest wilting. Additionally, ‘BHN 1087’, ‘RST-04-106-T’, and ‘Hawaii 7998’ performed moderately well in the CP1 location (Figure 4.7A) but grouped with the highly resistant lines in the MT location (Figure 4.7B).
Furthermore, the NC rootstocks NC13192 and NC13194 (test names) were tested in the MT location only due to a low availability of seed. They exhibited almost no wilting and grouped with the most resistant rootstocks, as did ‘Bowman’, ‘RST-04-106-T’, and ‘BHN 1087’. ‘Shield’ exhibited wilt in a delayed manner compared to the susceptible controls, reaching 91.5 and 68.5% wilt incidence by the end of the season in the CP1 and MT locations, respectively. This rendered it as an intermediate AUDPC value, which grouped with ‘Bowman’ and ‘RST-04-106-T’ in the CP1 location, but singly in the MT location. Finally, the NC rootstock NC1238-16 (test name) has bacterial speck and root knot nematode resistance. It was tested for the possibility of multiple bacterial disease resistance. The line exhibited substantial wilt development in the MT location, being statistically and numerically equivalent to the susceptible control non-grafted scion.

4.4.4: The effect of burying the graft union on resistance efficacy.

It is a standard recommendation that the graft unions remain above the soil line to prevent scion rooting (std). Out of curiosity, the ‘Shin Cheong Gang F1’ treatment was duplicated, and the second set of plots were planted deep, purposefully bury the graft union below the soil line (deep). Plants were occasionally non-destructively examined for scion rooting, which was observed in both locations, which was absent in the plots planted at the standard depth. No significant differences were detected between the std and deep treatment for wilt development at either location, only a small, numerical increase in wilt development (Figure 4.7A-B). In contrast, the susceptible guard plants between each plot were heavily wilted by midseason, as well as adjacent susceptible rootstock and control plots (Supplemental 4.3).
4.4.5: Productivity of susceptible scions grafted onto diverse rootstocks.

Fruit yields were analyzed in several ways: total yield per acre (kg), mean fruit size overall (kg), and mean fruit number within each size class (Figures 4.8 through 4.13). The overall trend was that the most resistant rootstocks exhibited the highest yields, whereas yield parameters were largely the same in the absence of BW pressure. Cull weights and numbers were very low in all experiments, so only the analyses for the marketable yield parameters are reported.

In the CP1 field, ‘Shin Cheong Gang F1’ std yielded the most fruit weight and had the largest average fruit size, although it was only different from the susceptible control or ‘Shield’ and the control, respectively (Figure 4.8A-B). ‘Armada F1’, the deep buried ‘Shin Cheong Gang F1’, DR6258TX, and ‘CRA66’ yielded numerically greater fruit weight than the control, and had significantly greater average fruit weight. The deep buried ‘Shin Cheong Gang’ yields were only a little bit lower than the std treatment. ‘Shield’ produced the lowest yields of the rootstock treatments.

In the CP2 location, which developed essentially no BW, there were no differences in yield by weight or size, although the mean yields per acre did differ by about 4000 kg between the lowest (‘BHN1087) and the highest (DR6258TX) numerical performers (Figure 4.9A-B).

In the PD location, the public rootstocks, the non-grafted control, and ‘Shin Cheong Gang F1’ deep plots were planted 1 week later than the rest of the experiment because of delays in production. Some differences in yield parameters were observed, despite essentially no wilt development (Figure 4.10A-B). All the hybrid rootstocks yield greater fruit weight (up to 15000 kg per acre more) and exhibited significantly larger fruit (up to a 65% increase) than the non-grafted control and the public rootstocks (‘CRA66’ and ‘Hawaii 7998’), with a little statistical
overlap at the low end. At this location ‘Armada F1’ was the top performer numerically for the yield parameters, yielding about 8000 kg more fruit per acre than the ‘Shin Cheong Gang F1’ buried deep.

At the MRS location—the no BW control field—significant differences in the yield parameters were observed across rootstock treatments (Figure 4.11A-B). There were no significant differences between the hybrid rootstocks and the non-grafted control, although all the hybrid rootstocks produced numerically greater fruit weight (up to about a 6000 kg per acre increase) and larger fruit (about an 11% increase in the mean). Furthermore, there was a numerically obvious decrease in fruit weight coming off of the ‘CRA66’ and NC13194 treatments, while only NC13192 exhibited a significant decrease.

The NC-developed rootstocks that were grown as non-grafted plots yielded less fruit weight and smaller fruit than the susceptible control (Figure 4.11A-B), although the fruit size was greatly improved compared to the non-grafted resistance donor (‘Hawaii 7998’). The non-grafted treatment of NC13194 yielded numerically less fruit weight and significantly smaller average fruit size than the non-grafted ‘Mountain Majesty’ control, but it was the best performer of the non-grafted rootstocks.

Finally, in the MT location, strong differences in yield were observed, which paralleled the wilt development trend (Figure 4.12A-B). All the rootstock treatments except NC1238-16 yielded significantly more fruit weight per acre and had a greater mean fruit weight than the non-grafted controls and rootstocks. Despite having similar wilt development, the yield per acre value of the ‘Hawaii 7998’ grafted treatment was obviously numerically reduced compared to the other rootstocks with similar levels of wilting. ‘Armada F1’ produced the greatest yield but was
numerically imperceptible from several others. The deep buried ‘Shin Cheong Gang’ yields were only a little bit lower than the std treatment. ‘Shield’ yielded the poorest of the hybrid rootstocks.

At each harvest, the fruit were sorted by size, counted, and weighed. Some overall patterns for fruit number by size class were observed. In the CP1 and CP2 locations (‘Red Morning’ scion), the diameter distributions were fairly evenly distributed between medium to extra-large, and basically no jumbos (Figure 4.13A-B). The best yields were related to enhanced numbers of fruit, especially mediums and extra larges, while the lower yields were characterized by an overall reduction in fruit numbers across the size categories, especially of extra larges (Figure 4.13A). In the absence of BW (CP2), the numbers and distributions by size category were identical (Figure 4.13B).

In the PD location (‘Red Mountain’ scion), most of the fruit were extra-large, then large, then jumbo (Figure 4.13C). There was a clear upwards shift in size distributions from the susceptible control to the hybrid rootstocks, where nearly half of the fruit from the control were larges and no jumbos while the hybrid rootstocks were half to about ¾ extra larges and jumbos. Comparing the susceptible control with the ‘CRA66’ and ‘Hawaii 7998’ yield about 15 and 30% fewer fruit than most of the hybrid rootstocks, respectively. It is not clear how much of these trends are because of the heavy clay soil type or the 1-week delayed planting.

In the MRS and MT locations (‘Mountain Majesty’ scion), the fruit were nearly all extra-large and jumbo, with more jumbos in the no-disease field (MRS) and more extra-larges in the heavily diseased field (MT) (Figure 4.14A-B). NC 13194 produced about 1/3 more fruit than the next highest treatment, although the size and quality still need substantial improvement. At MRS, there was greater fluctuation in the distribution of jumbo fruit, especially comparing ‘CRA66’, the susceptible control, and ‘Armada F1’ (Figure 4.14A). There were also obvious fluctuations in
the number of larges and extra larges produced. Among the resistant rootstocks in the MT location, similar patterns were present, while the susceptible control produced very few fruits because of the severe wilt development (Figure 4.14B).

Furthermore, the rootstocks varied in the propensity to produce extra larges and jumbos more exclusively. Compared to the susceptible control in the MRS location, several of the rootstocks appeared to shift mediums and larges into larger size classes without necessarily changing the overall fruit numbers (Figure 4.14A-B). This pattern was also apparent among healthy rootstock treatments in the MT location.

4.4.6: Cluster and principal component analyses.

The phenotypic characteristics of each line were analyzed using cluster and principal component analyses (PCA). In order to relate the disease data across locations, the daily mean AUDPC was analyzed. The lines grouped into five major resistance clusters, noted as BW1 to BW5 for least to most wilt development, respectively (Figure 4.15). Alongside the disease data, five phenotypic characters were assessed: species, fruit color, fruit class, fruit shape, and growth type. A correlation analysis indicated a range of positive and negative trends from -0.4429 to +0.4684 (Table 4.4). Most variable combinations were not significantly correlated. Fruit color and fruit class were significantly positively correlated (p = 0.0005), while growth type, fruit shape, and species were significantly negatively correlated with fruit class (p = 0.0011, 0.0268, and 0.0452, respectively).

The addition of the phenotypic characters to the cluster analysis identified four main clusters, with one cluster seeming to exhibit two sub-clusters (Figure 4.16). Upon closer inspection, it was observed that the genotypic clustering was similar to the mean daily AUDPC-
derived clustering alone, with some modification. BW3 (containing ‘Shield’, among others) clustered between BW4 and BW5. Also, PI127805 clustered with BW5 instead of BW4 in a wild species/interspecific hybrids subgroup. BW4 also contained a similar subgroup.

Five out of the six principal components explained 95% of the variance, while the first two components explained >50% (Table 4.5). The variable effect breakdown for each principal component indicated strong positive and negative influences for each variable; no variable had little-to-no effect overall (Table 4.6). Principal component 1 primarily reflected major effects of all the variables except wilt severity. Principal component 2 heavily reflected the normalized wilt severity, while fruit class and shape have relatively low impacts. Principal component 5 and 6 were moderately affected by wilt severity. Principal component 3 heavily reflected species and fruit shape variables, while fruit class and disease are of no affect. Principal component 4 heavily reflected fruit shape and fruit color variation, while disease and growth type were very marginal. Principal component 5 heavily reflected growth type, while fruit shape was marginal. The final principal component heavily reflected variation in fruit class and fruit color, while fruit shape and growth type were low.

4.5: Discussion

4.5.1: The diversity of the diversity germplasm panel.

The resistance diversity panel contains resistant germplasm from around the world, and the phenotypic potential of those lines for BW management in NC was assessed. The primary objective was to relate genetic diversity with phenotypic success as stand-alone lines and rootstocks for grafted management. This work provides substantial phenotypic evidence that can
be linked with further genetic testing. This work readily applies to introgression breeding, resistance pyramiding, and resistance rotation for applied field management of BW.

Based on known heritage, the founder germplasm represents three species/varieties of tomato collected from at least 10 countries, while the rootstock germplasm represents 10 unique breeding programs around the world. Based on pedigree*collection region, the diversity panel is expected to potentially contain at least 15 unique resistance sources. Furthermore, the initial germplasm searches revealed about 110 lines representing these and potentially additional resistance sources, of which only 41 lines were able to be obtained for this work (data not shown). This pool of resistance remains largely undescribed genetically and phenotypically. The extent to which these sources have been introgressed into modern commercial rootstocks is either unknown because of poor records or undisclosed.

4.5.2: Stability and viability of the diversity germplasm panel.

This work focused on three important aspects of disease resistance breeding—resistance strength, stability, and performance in applied management settings. Towards this, three field studies and two greenhouse seedling tests were performed on non-grafted germplasm were performed, representing 5 unique time*location environments. Furthermore, 5 grafted field trials were performed with a subset of the best germplasm in the panel, providing additional wilt resistance information and yield data.

In each field experiment (except MRS), the aim was to provide strong, natural BW pressure, which was achieved at two locations (MT and CP1; Figures 4.2 and 4.3). The greenhouse experimental aim was to supply very strong disease pressure to help separate the highly resistant from the moderately resistant, as well as to assess the germplasm for
colonization patterns and VB severity. The results further validated the connection between VB, wilt development, and resistance quantification (Figure 4.6A).

Others have assessed some of the founder germplasm and commercial rootstocks for resistance efficacy and yield potential. The cluster analyses identified 5 clear resistance groups across the experiments (Figure 4.15 and Supplemental 4.4). Cluster BW5 contained nearly all of the susceptible controls. The overall trends of each line are discussed in light of other reports:

First, the PI (public introduction; USA) lines 129080, 127805, 303814, 127811, NC1238-16 and the commercial hybrid ‘RST-04-105’ regularly exhibited substantial wilting in both field and greenhouse tests, clustering with the susceptible controls in the BW4 and BW5 groups (Figure 4.15). This indicates that they do not appear to contain meaningful resistance against NC strains, all of which have been reported to be phylotype IIA, sequevar 7 (Hong et al., 2012). This is quite unexpected, especially considering that the Hawaii germplasm are generally thought to have been selected from ‘PI 127805’ and the NC state program was working with ‘PI 129080’ in the 1950s and 60s for the development of ‘Venus’ and ‘Saturn’ (Daunay et al., 2010; Henderson and Jenkins Jr., 1972). In this work, ‘Venus’ clearly clustered with the Hawaii germplasm as moderately to highly resistant (Figure 4.15). ‘PI 303814’ was tested based on any possible connection to the mysterious “Beltsville PI 3814” that was also noted as contributing to ‘Venus’ and ‘Saturn’ (Daunay et al., 2010; Henderson and Jenkins Jr., 1972).

‘PI 127811’ was chosen because of gene bank labeling as “199”, which may have connected it with “UPR199” from the University of Puerto Rico breeding program. Neither of these lines appeared to contain meaningful BW resistant in our experiments. As expected, ‘WVa700’ was highly susceptible (Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996; Thoquet, Olivier, Sperisen, Rogowsky, Prior et al., 1996).
Second, a number of lines exhibited partial resistance at least in some experiments, spanning the clusters BW2 to BW4. They were the PI lines 126408, 390009, and 479211, ‘LA2701’, and ‘CLN1466EA’. ‘PI 126408 exhibited some resistance in both locations of 2017 and less in 2016, but really failed in the greenhouse. The Florida breeding program identified ‘PI 126408’ as in the top three most resistant sources in the 1970s (Sonoda and Augustine, 1978; Sonoda et al., 1979). It was also reported to be highly resistant in the Western Shore of Virginia (Wimer, 2009). The line also featured as a resistant donor for the Georgia breeding program line ‘219-1-2 BWT’, which also exhibited substantial year-to-year variability (Jaworski et al., 1987).

The line also has unique fruit. The fruit size and shape are mixed, producing small 3 to 4 locule pink cherry types, somewhat square small to medium pink types, and apparently fused irregular medium to large types that are red (data not shown). The seed source may have been contaminated with another line, however.

‘PI 390009’ was noted in the AVRDC gene bank records as being a local tropical variety from Thailand or Taiwan with resistance to BW and viruses, and it did not seem to perform very well in the 2017 field locations. It did better in the MT location than the CPI location and performed surprisingly well in the second greenhouse under inoculation from the MT strain.

‘PI 479211’ was the only line listed as “CRA” in the USDA GRIN genebank. It was noted as being from Columbia with good fruit set under high heat and humidity. The line may harbor some intermediate level of BW resistance based on both the 2017 field trials and the second greenhouse experiment.

‘LA2701’ was labeled as BW resistant in the TGRC genebank and was reported as having some use for grafted management of BW in China (Zhang et al., 2010). It performed poorly in the MT 2016 experiments, moderately in the T1 experiment, and quite well in the 2017
MT and CP1 locations as well as in T2. The T2 results could have been due to a higher frequency of failed colonization by Rs in the greenhouse (Figure 4.5B).

‘CLN1466EA’ is a released variety from the AVRDC breeding program and was chosen because of the combination of moderate heat stress tolerance and BW resistance derived from ‘CRA84-26’. It was previously evaluated in NC growth chamber inoculations and exhibited a moderate level of resistance (Silverman, 2015). Other lines from that family (‘CLN 1466 J’ and ‘CLN 1466 P’) exhibited moderately high resistance in Nepal fields (Timila and Joshi, 2007). ‘CLN1466EA’ exhibited consistently moderate resistance in our experiments, with significantly greater wilt development in the MT location verses the CP1 location in 2017. It clustered with ‘PI406994’ in BW3 (Figure 4.15).

Third, another group of lines showed moderate to high resistance, clustering mostly in BW2 and BW3, and just dipping into BW1 (Figure 4.15). These were ‘IRAT-L3’, ‘Shield’, ‘BHN1053’, ‘LA3526’, ‘PI 406994’, ‘PI 251323’, ‘Hawaii 7981’, ‘BHN669’, ‘Venus’ (both), ‘Hawaii 7998’, and ‘Bowman’. ‘IRAT-L3’ was developed from the resistance source ‘UPR199’ out of the Puerto Rico breeding program and is most resistant against phylotype IIA strains (Lebeau et al., 2011). It is moderately resistant to some phylotype I and III strains, but is slaughtered by some phylotype IIB strains (alongside ‘Hawaii 7996’ ‘CRA66’). It has also been reported to contain some resistance to strains of *Verticillium dahliae* race 2 (all strains that overcome the *Ve1* gene) (Baergen et al., 1993; Gold et al., 1996; Laterrot, 1984). ‘IRAT-L3’ is currently being investigated for race 2 resistance in NC (internal research).

‘Bowman’ is a new rootstock on the market that has good to excellent BW resistant in this work. It is also labeled for Verticillium wilt race 2 resistance, having been tested in Japan and South Africa (Bryan Zingel, Sakata Seed, personal communication). It is also unique as the
only line in the panel with bright orange fruit. Thus, it is not likely to have the same sources of resistance as ‘IRAT-L3’.

‘LA3526’ (cultivar name ‘L04012’ when donated) is listed as BW resistant by the TGRC gene bank. It was found to be useful for grafted resistance in China (Zhang et al., 2010). It was part of the AVRDC breeding program back in the early 1990s. ‘PI 251323’ was the resistant source for the ‘GA 1405’ group of lines (Jaworski et al., 1987). The line was one of the important sources of resistance in the Georgia breeding program. It performed similar to several BHN lines, ‘Bowman’, and ‘Venus’. ‘PI 406994’ was collected from Panama and fed into the AVRDC breeding program, alongside ‘Venus’ from North Carolina (Daunay et al., 2010; Henderson and Jenkins Jr., 1972).

‘Venus’ (and sister line ‘Saturn’) have been used as resistance sources for several breeding programs, but the results were intermediate (P. M. Hanson et al., 1996; Jaworski et al., 1987). Their resistance was derived from ‘PI 129080’ and “Beltsville PI 3418” (Henderson and Jenkins Jr., 1972). The mysterious Beltsville line appears to have vanished into the dustbins of history (Daunay et al., 2010). ‘Venus’ was one of the only lines harvested in both the MT and PD locations in 2016, where the mean fruit weights were 175 g and 85 g (mean = 130 g), respectively. While the range was large, it exhibited the largest fruit of the BW1 and BW2 clusters. Furthermore, the one generation of bulk survivor selection of ‘Venus’ from the MT location in 2016 (“Venus-Bulk Jc”) exhibited greater resistance overall than did the stock ‘Venus’. The selection was in the BW1 cluster while the stock was in the middle of BW2.

‘Hawaii 7981’ is best known for being the source of HypR-mediated resistance to bacterial spot of tomato race T3 (Xanthomonas perforans), and, as a sister line with the other Hawaii 7000 series, it also contains BW resistance (J. B. Jones et al., 1998; Scott et al., 2001;
Similarly, ‘Hawaii 7998’ is more well studied as a BW resistance source, but also contains the Rx3-mediated resistance to bacterial spot race T1 (*Xanthomoas euvsicatoria*) (J. B. Jones and Scott, 1986; Scott et al., 2011). ‘Hawaii 7998’ progeny segregated almost as a single-gene resistance, but further breeding has not validated that hypothesis (Scott et al., 2005). It has also been tested in NC as a rootstock, providing good resistance but lacking yield vigor (Kressin, 2014).

‘Shield’ performed as moderately resistant in this work (chapters 2, 3, and this one) and was significantly more wilted than any of the other rootstocks tested here (except NC1238-16-F3). It was previously tested as a rootstock in the PD location, where it performed as highly resistant under moderate disease pressure (Suchoff et al., 2015).

‘BHN669’ was released as a standalone cultivar with intermediate resistance to BW, which has been reported in the Eastern Short of Virginia and in Florida (Hong et al., 2011; Wimer, 2009). It tied with ‘Venus’ for the largest mean fruit size in the BW1 and BW2 clusters (134 g). ‘BHN1053’ performed very similarly but with fruit about half the size (64 g). As a rootstock, ‘BHN1053’ was tested in NC, Florida, and Virginia where it exhibited moderate resistance (Kressin, 2014; McAvoy et al., 2012; Silverman, 2015) The rootstock tests are consistent with the present findings here and in chapter 3.

Fourth, the last group of lines showed high levels of resistance, spanning clusters BW1 and BW2. No line remained perfectly healthy in all experiments. These included: ‘Hawaii 7996’, ‘Hawaii 7997’, ‘Shin Cheong Gang’, ‘Cheong Gang’, ‘CRA66’, ‘Armada F1’, ‘BHN998’, ‘BHN1087’, ‘RST-04-106-T’, ‘PI 263722’, and the NC lines NC13192-F3 and NC13194-F3. The NC lines were tested internally and selected for resistance in the MT location in previous
years. Their resistance was introgressed from ‘Hawaii 7998’ into NC-adapted, large-fruited backgrounds.

‘PI 263722’ was used as a resistance source in the Georgia breeding program as the resistant donor for ‘GA 1565-2-4 BWT’, and also in the Florida program (Jaworski et al., 1987; Sonoda and Augustine, 1978; Sonoda et al., 1979). It performed comparable to ‘CRA66’ but with greater variability (Figure 4.15 and Supplemental 4.4).

‘Armada F1’ was suggested to us by Dr. Jay Scott from the University of Florida breeding program because of possible resistance to both race 1 and race 3 Rs (Phylotypes IIA and IIB generally, including the quarantined group race 3 biovar 2) (personal communication, 2015). It did extremely well in these tests.

‘CRA66’ and ‘Hawaii 7996’ are the most studied and probably bred resistant lines available. They have been used for decades in various studies about the host-pathogen interactions of tomato*Rs and resistance genetics studies (too many references to note). They were the 1st and 10th most resistant entries in a global test, yet their origins are among the most murky (Daunay et al., 2010; J. Wang et al., 1998). ‘CRA66’ came out of the INRA breeding program but has an uncertain origin. It is not clear if it is a truly unique source of resistance that fed into INRA or it was bred from either ‘OTB2’ in Japan or an unknown wild S. lycopersicum var. cerasiforme. It is rather unique in that it has pink fruit, like ‘PI 126408’.

‘Hawaii 7996’ was the most resistant line in a worldwide study in the late 1990s. It has also been the resistant parent of all but one QTL mapping study (Danesh et al., 1994; Thoquet, Olivier, Sperisen, Rogowsky, Prior et al., 1996; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996; J. Wang et al., 2013). It was supposedly derived from “PI 127805A” after about 9 generations of selection in Hawaii (see previous discussion above). It is also not clear if it is a
sister line to the other Hawaii germplasm tested here or if it was their progenitor (Daunay et al., 2010).

The rest of the rootstocks have either already been discussed, were previously tested in NC at various times, and/or are part of the current rootstock recommendations. Some have also been tested in Virginia, Florida, and Kenya (Freeman et al., 2011; Kressin, 2014; McAvoy et al., 2012; Onduso, 2014; Rivard et al., 2012; Rivard and Louws, 2008; Silverman, 2015).

4.5.3: The viability of old and new rootstocks for commercial grafted management of BW.

The rootstock evaluations both now and from the previously mentioned work indicate that there are multiple rootstocks with equally high levels of BW resistance that provide efficacious resistance in both the Coastal Plains and the Mountain regions. ‘Bowman’ may not be a good fit in the Coast Plains (Figure 4.7A). ‘Shield’ performed the worst of the commercial rootstocks in both locations and is probably not a good fit for NC (Figure 4.7). In general, the other rootstocks are a good fit for BW management across NC.

The lack of disease development in two of the four disease locations was unfortunate. We have to rely upon grower information many times to get good infested hotspots. While field resistance tests require strong, uniform disease pressure, it is in the best interest of the grower to suppress and aggregate disease hotspots as much as possible.

Desirable levels of BW natural BW pressure were obtained in the CP1 and MT locations. The same batch of plants were used to plant both CP1 and CP2, so CP2 can be considered a no-disease contrast with CP1, much like between the MT and MRS locations.

The lack of disease development at the PD location was frustrating. The grower has actually decided to return to non-grafted tomato production because the BW severity has fall off
so much. A major factor for this is likely a shift in management practices, where in the last several years the grower has been adding nearly 2 tons of high-calcium lime to the soil each season, and has successfully increased his soil pH to around 7.4 (personal communication). These amendments coincide with when we started seeing a reduction in BW severity in experiments at that location. Surprisingly, no issues of blossom end rot were present in the grafted experiment, despite the pH shift. The PD location is a heavy clay soil, which may help explain this.

The buried graft union results were unexpected. No obvious compromise in resistance was observed by allowing the scion to root. This phenomenon requires addition investigation, and the data is too preliminary (only 1 year) to recommend any changes in planting method. We plan to replicate the test in 2018.

Strong differences in yield were observed between the fields and rootstock treatments. Due to the differences in the scion variety, management, and cultural practices, it is not meaningful to compare the fields of each region. By far the strongest factor affecting yield was how much BW developed within the grafted treatment plots (compare the disease vs. no disease fields). More disease development meant more dead plants and poorer yields. In the absence of disease development, very little differences were observed between rootstocks grafted with the commercial cultivars and the non-grafted standard. Rootstocks with the same basic amount of disease also tended to produce the same yields, which is excellent news for grafted management of bacterial wilt, because it means growers can rotate rootstocks and maintain both the same levels of resistance and yields.

In the absence of disease, most rootstocks do not give a yield penalty compared to the susceptible control. This was often associated with having the largest mean fruit weight overall.
The increase in fruit weight appeared mostly linked to increases in the size partitioning of the fruit towards large size categories, with increases in fruit number being a secondary factor. Similar performance has been observed previously in NC, Virginia, Florida, and Kenya with various subsets of these rootstocks (Freeman et al., 2011; Kressin, 2014; McAvoy et al., 2012; Onduso, 2014; Rivard et al., 2012; Rivard and Louws, 2008; Silverman, 2015).

As for yield performance, ‘Shin Cheong Gang’ and ‘Armada F1’ were the most productive based on weight per unit area. While most of the other rootstocks at each location were statistically the same, those lines regularly the top performers. The story was somewhat unique in the PD location compared to the others, which was likely a factor of the heavy red clay soil type. ‘Armada F1’ was clearly the best rootstock for that region in the absence of meaningful disease pressure (Figure 4.10A-B). In the rest of the regions, most of the rootstocks are reasonable recommendations for yield performance.

This work supports the general view that grafted field tomato production is only economically viable when there is a soil-borne disease issue to manage. In the absence of disease, factors such as cultural management, soil type, fumigation, and foliar disease management have a much greater impact on grafted plant yields than the rootstocks tested here. The exception is ‘Hawaii 7998’ that consistently yielded poorer than most of the other rootstocks despite having similar resistance levels. It is not a great fit for grafted management because of the yield penalty.

On a positive note, the NC rootstocks NC13192 and NC13194 appear to be correcting some of the apparent lack of vigor/tissue incompatibility issues of ‘Hawaii 7998’, while retaining high levels of wilt resistance (Compare those lines in Figures 4.7B and 4.12A-B). Curiously, ‘Hawaii 7996’ did not appear to negatively impact yields when used as a rootstock in previous
experiments grafted with the heirloom ‘German Johnson’ (Rivard and Louws, 2008). Additional work is needed to further improve the grafted yields of the NC rootstocks compared to the current commercial hybrids.

Breeders for decades have been trying to break the small-fruit and resistance link, but so far no stand-alone, large fruited varieties have been released (Scott et al., 2009; Scott et al., 2005). As expected, the non-grafted rootstocks had much smaller fruit than the susceptible control, even though the NCSU program has been breeding resistant material with the locally adapted large-fruited material. ‘NC13194-F3’ has the most promise so far in combining larger fruit size with high levels of wilt resistance but it needs further improvement for quality fruit. Thus, the long-term outputs of the tomato breeding efforts will most likely provide diverse, well-adapted, highly resistant rootstocks that can be grafted with any commercially competitive scion variety. NC13192 and NC13194 seem to have sufficient resistance but just need more vigor. These efforts are important because most of these rootstocks were bred for production in Asia, rather than N. America, and so they will not be as adaptable the Southeastern USA region.

Additional testing is needed to verify the patterns observed for ‘Bowman’, ‘Armada F1’, DR6258TX, and the NC rootstocks before adding them to recommended rootstock list for NC. But, these results suggest they could be unique additions for rootstock rotation practices. Furthermore, this work adds substantial evidence favoring ‘Shin Cheong Gang’ for the rootstock recommendations. ‘CRA66’ remains a viable, productive option for NC growers, especially since it is open-pollinated, which allows for cheap seed production. But, it may not perform well in heavy clay soils. ‘RST-04-106-T’ and ‘BHN1087’ remain productive options as well but are a little more susceptible under very strong disease pressure. ‘Bowman’ is unclear. ‘Shield’ is not a good fit for NC BW management.
4.5.4: Diversity of bacterial wilt resistance genetics.

Bacterial wilt resistance derived from ‘Hawaii 7996’ has been moderately fine mapped (J. Wang et al., 2013). Only one study has examined the distribution of the BW resistance loci in a wider population of germplasm (Ho et al., 2013). This work shares several lines in common: ‘Hawaii 7996’, ‘WVa700’, ‘CRA66’, ‘IRAT-L3’, and ‘Venus’. The authors tested the allele patterns of each marker for the four loci in the chromosome 6 QTL and the one chromosome 12 QTL. They found that ‘Venus’, ‘CRA66’, and ‘IRAT-L3’ were homozygous for all four of the Hawaii-type (H) chromosome 6 QTL markers but were homozygous for the ‘WVa700’-type (W) chromosome 12 QTL markers. Furthermore, ‘Saturn’, the sister line of ‘Venus’ had the opposite pattern, being homozygous for the H markers on chromosome 12, but having the W or other marker alleles for all four of the chromosome 6 QTL. While these markers are not based on a truly fine map, they suggest that some of the differences between resistance levels has to do with the number of QTL that each line possesses.

Curiously, ‘CRA66’ was more resistant than both ‘IRAT-L3’ and ‘Venus’ overall in our work, yet they are reported to have the same major QTL profiles (Ho et al., 2013). ‘Hawaii 7996’ has all of them and is the most resistant. The lack of the chromosome 12 QTL does not seem to have a major effect on the resistance efficacy of ‘CRA66’ in NC. All four lines group with the other Hawaii germplasm in the cluster analyses for BW resistance alone (Figure 4.15) or with the other phenotypic characters included (Figure 4.16).

The limitations of this sort of marker testing, however, are that all of the QTL mapping activities in the last 20 years have been using the donor resistance of ‘Hawaii 7996’. ‘CRA66’, and many of the other lines tested presently, may have novel QTL compared ‘Hawaii 7996’. The very first QTL map used ‘L285’ as the resistant parent. That work identified a minor resistance
loCi on chromosome 7 related to shoot inoculation that has never been found in the Hawaii-based maps, as well as a chromosome 10 loci that has only been found again in one report based on ‘Hawaii 7996’ (Danesh et al., 1994; Thoquet, Olivier, Sperisen, Rogowsky, Laterrot et al., 1996).

The absence of any elongated fruit types (grape, plum, etc.) in any of the resistance diversity panel is noteworthy (Table 4.1). Bacterial wilt resistance introgression into these elongated genetic backgrounds appears to be lacking in rootstock germplasm, while the trait is not present in wild and undomesticated resistant germplasm. This suggests that elongated types may not provide any added value for rootstock development for BW resistance.
4.6: Figures

**Figure 4.1: Field variation in wilt development by row.**
Analysis of bacterial wilt variation by field-row in the 2016 and 2017 diversity panel resistance screens. Every plant was periodically scored for wilt severity over the course of the season using a 0 to 5 scale and the values were converted into wilt indices by dividing each score by 5 and multiplying by 100. The mean area under the disease progress curves (AUDPC) for wilt index were calculated and analyzed for 2016 (A) and 2017 (C), as well as the end-of-season wilt index (B) in 2016. Rows in the mountain location (A, B, and C gray columns) are not necessarily the same between years. The variation by row was modeled as a fixed effect variable. Bars represent the standard error. Means with the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall $\alpha = 0.05$).
Figure 4.2: Mean differences between genotypes for the final wilt index in 2016. Individual means were adjusted for based on the overall variation between the main effect of row, yield some values that are somewhat outside the real bounds of the variable (0 to 100). Gray columns highlight lines that were used as susceptible controls. Means sharing the same horizontal line are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall $\alpha = 0.05$). Dashed error bars represent the Tukey-adjusted 95% confidence intervals.
Figure 4.3: Genotypic means for the diversity panel germplasm by field in 2017.
Black columns (CP1) represent the Coastal Plains 1 location in Pender county while the gray columns (MT) represent the mountain location in Jackson county, North Carolina. CP1 means sharing the same horizontal line are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall $\alpha = 0.05$). Many differences were also present for the MT means, but they are not shown for simplicity sake. Dashed error bars represent the Tukey-adjusted 95% confidence intervals. Because the two locations were assessed for about the same period (79 days for CP1 and 77 days for MT), the between locations pairwise comparisons are also shown (* = $p<0.05$; _ = genotype pair absent; ~ = $p\geq0.05$).
Figure 4.4: Mean differences in wilt AUDPC in the greenhouse experiments.

Greenhouse resistance tests on seedlings of the diversity panel germplasm following inoculation with 10 mL of $10^9$ CFU/mL of NC isolate Jc on lightly injured roots. 47 day-old plants were used and kept on a heating pad set at 29.5 °C. All plants were periodically scored for wilt severity using a 0 to 5 scale (8 and 7 times for experiments 1 and 2, respectively) and wilt indices were calculated for each plant. The means represent 5 to 24 plants (mean is 11.18) per genotype, and the experiment was repeated twice. Some additional genotypes were added to the second experiment, so the results of each are shown, where experiment 1 (A) tested 32 genotypes while experiment 2 (B) tested 41. Columns represent the mean AUDPC wilt index while the diamonds represent the final wilt incidence for each genotype. Means sharing the same horizontal line are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall $\alpha = 0.05$). Because the experiments were assessed for different lengths of time with substantially greater wilt in the second experiment, pairwise tests across experiments are not shown.
Figure 4.5: Mean differences in vascular browning severity compared to colonization incidence in the greenhouse experiments.

End-of-experiment assessments of vascular browning severity and plant colonization for greenhouse experiments 1 (A) and 2 (B). Each plant was destructively harvested and assessed for vascular browning severity at the basal hypocotyl using a 0 to 5 severity scale. The mean indices were calculated as previously for wilt severity. Means sharing the same horizontal line are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall α = 0.05). Each stem was imprinted on SMSA-E media, which was incubated for 48 to 72 hours and then assessed for the presence of Rs. Based on the combination of plate growth and wilt development, each plant was noted as colonized or disease/pathogen free. The diamonds indicate the total proportion of plants that were colonized for each genotype. Error bars represent the standard error.
Figure 4.6: Linear relationships between wilt and vascular browning parameters in the greenhouse experiments.

A) The relationship between the final wilt index of each genotype linearly regressed with the vascular browning index for each experiment, as indicated by the dotted and dashed lines for experiment 1 (T1) and experiment 2 (T2), respectively. B) The relationship between wilt index AUDPC values in T1 regressed with T2 by genotype.
Figure 4.7: Wilt development in the grafted variety trials.
Wilt severity analysis between treatments in the grafted variety trial locations that exhibited strong disease pressure—Coastal Plains 1 (CP) and Mountain (MT). Wilt severity was assessed periodically from transplanting to mid harvest using a 0 to 5 scale. The analyses of the AUCPD values (columns) and final wilt incidence (diamonds) for each grafting treatment from two locations are shown. Columns sharing the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall α = 0.05). All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
**Figure 4.8: Yield parameters at the Pender county location.**

A) Marketable yield per acre and B) average fruit weight of the grafted variety trial at the Coastal Plains 1 location (CP). Plots were harvested for vine-ripe tomatoes for three weeks, and the plants were stripped on the final harvest. The fruit were graded for size, counted, and weighed. The average yield per plant was extrapolated to plants per acre. Columns sharing the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall α = 0.05). All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
Figure 4.9: Yield parameters at the Brunswick county location.

A) Marketable yield per acre and B) average fruit weight of the grafted variety trial at the Coastal Plains 2 location (CP2). Plots were harvested for vine-ripe tomatoes for four weeks, and the plants were stripped on the final harvest. The fruit were graded for size, counted, and weighed. The average yield per plant was extrapolated to plants per acre. Columns sharing the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall α = 0.05). All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
**Figure 4.10: Yield parameters at the Rowan county location.**

A) Marketable yield per acre and B) average fruit weight of the grafted variety trial at the Piedmont location (PD). Plots were harvested for mature green tomatoes twice, and the plants were stripped on the final harvest. The fruit were graded for size, counted, and weighed. The average yield per plant was extrapolated to plants per acre. Columns sharing the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall $\alpha = 0.05$). All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
**Figure 4.11: Yield parameters at the Haywood county no-disease control location.**

A) Marketable yield per acre and B) average fruit weight of the grafted variety trial at the Mountain Research Station location (MRS). Plots were harvested for vine-ripe tomatoes for five weeks, and the plants were stripped on the final harvest. The fruit were graded for size, counted, and weighed. The average yield per plant was extrapolated to plants per acre. Columns sharing the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall α = 0.05). All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
Figure 4.12: Yield parameters at the Jackson county location.

A) Marketable yield per acre and B) average fruit weight of the grafted variety trial at the Mountain location (MT). Plots were harvested for vine-ripe tomatoes for five weeks, and the plants were stripped on the final harvest. The fruit were graded for size, counted, and weighed. The average yield per plant was extrapolated to plants per acre. Columns sharing the same letter are not significantly different based on multiple-comparison tests with Tukey-Kramer adjust p-values (overall $\alpha = 0.05$). All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
**Figure 4.13: Fruit number distributions by size class for the Coastal Plains and Piedmont locations.**

The mean distribution of fruit by size class in the grafted variety trial for the Coastal Plains 1 (A) and 2 (B) locations, along with the Piedmont (C). The plots were harvested for vine-ripe (AB) or mature green (C) tomatoes two to four times and the plants were stripped on the final harvest. The fruit were graded for size and counted. Error bars represent the standard error. All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
Figure 4.14: Fruit number distributions by size class for the Mountain region locations.
The mean distribution of fruit by size class in the grafted variety trial for the Mountain Research Station (A) and Mountain (B) locations. The plots were harvested for vine-ripe) tomatoes five times and the plants were stripped on the final harvest. The fruit were graded for size and counted. Error bars represent the standard error. All treatments were grafted as rootstocks except for those designated with “NG”, which were non-grafted. All treatments were planted at the standard depth for grafted tomatoes (soil just covering the rootball; std), while treatments designated with “deep” were planted such that the soil covered the graft union.
Figure 4.15: Cluster analysis of daily mean AUDPC.
Clustering of the least-squares mean AUDPC values for wilt severity. Each genotype*experiment mean was normalized to daily AUDPC and the grand mean over experiments was used for the cluster analysis. Left ledger is the genotype and the coded resistance source (according to the right-side legend). Sources with “?” are putative connections based on gene bank passport information and educated guess-work. Main resistance clusters are labeled.
Figure 4.16: Cluster analysis of phenotypic variables.
Clustering of the least-squares mean AUDPC values for wilt severity along with five other phenotypic characters. Each genotype*experiment mean was normalized to daily AUDPC and the grand mean over experiments was used for the cluster analysis. Left ledger is the genotype. Right legend indicates the variables used in the analysis. Main branch labels relate to disease clusters in previous figure.
### 4.7: Tables

**Table 4.1: Diversity panel and controls.**

Summary information and numbers tested in each experiment.

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<tr>
<td>PI127805</td>
<td>--</td>
<td>8</td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td>50.75 ± 12.45</td>
<td>S. pim.</td>
<td>Cherry</td>
<td>Founder</td>
<td>Self</td>
<td>GRIN</td>
<td></td>
</tr>
<tr>
<td>PI129080</td>
<td>T702</td>
<td>6</td>
<td>12</td>
<td>10</td>
<td>12</td>
<td>12</td>
<td>47.81 ± 5.53</td>
<td>S. ly. or S. pim.</td>
<td>Campari</td>
<td>Founder</td>
<td>Self</td>
<td>GRIN</td>
<td></td>
</tr>
<tr>
<td>PI251323</td>
<td>LA0418</td>
<td>--</td>
<td>12</td>
<td>10</td>
<td>--</td>
<td>11</td>
<td>19.91 ± 14.68</td>
<td>S. ly. or S. pim.</td>
<td>Campari</td>
<td>Founder</td>
<td>Self</td>
<td>GRIN</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.1: (continued).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Variety</th>
<th>Year</th>
<th>Type</th>
<th>Characteristics</th>
<th>Origin</th>
<th>Potential</th>
<th>Rootstock</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI263722</td>
<td>199</td>
<td>12</td>
<td>10</td>
<td>12 14.99 ± 2.03</td>
<td>S. ly.</td>
<td>Campari</td>
<td>UPR199?</td>
<td>GRIN</td>
</tr>
<tr>
<td>PI303814</td>
<td>4929</td>
<td>11</td>
<td>10</td>
<td>10 54.55 ± 1.863</td>
<td>S. per.</td>
<td>Cherry</td>
<td>Beltville #3814?</td>
<td>GRIN</td>
</tr>
<tr>
<td>PI390009</td>
<td>--</td>
<td>12</td>
<td>10</td>
<td>11 33.70 ± 1.62</td>
<td>S. ly.</td>
<td>Campari</td>
<td>Self</td>
<td>AVRDC</td>
</tr>
<tr>
<td>PI406994</td>
<td>W&amp;C 0176</td>
<td>12</td>
<td>10</td>
<td>12 27.40 ± 2.714</td>
<td>S. ly.</td>
<td>Campari</td>
<td>Self</td>
<td>GRIN</td>
</tr>
<tr>
<td>PI479211</td>
<td>--</td>
<td>11</td>
<td>10</td>
<td>10 35.98 ± 1.75</td>
<td>S. ly.</td>
<td>Campari</td>
<td>&quot;CRA&quot;</td>
<td>GRIN</td>
</tr>
<tr>
<td>Red Morning</td>
<td>--</td>
<td>48</td>
<td>10</td>
<td>-- 45.74 ± 1.20</td>
<td>S. ly.</td>
<td>Beefsteak</td>
<td>--</td>
<td>Harris Seeds</td>
</tr>
<tr>
<td>Red Mountain</td>
<td>--</td>
<td>--</td>
<td>10</td>
<td>-- 54.14 ± 0.0</td>
<td>S. ly.</td>
<td>Beefsteak</td>
<td>--</td>
<td>Harris Seeds</td>
</tr>
<tr>
<td>RST-04-105-T</td>
<td>DP 105</td>
<td>12</td>
<td>10</td>
<td>10 39.32 ± 1.91</td>
<td>S. hab x S. ly.</td>
<td>Cherry</td>
<td>Rootstock</td>
<td>DP Seeds</td>
</tr>
<tr>
<td>RST-04-106-T</td>
<td>DP 106</td>
<td>12</td>
<td>10</td>
<td>12 15.82 ± 1.26</td>
<td>S. ly.</td>
<td>Cherry</td>
<td>Rootstock</td>
<td>DP Seeds</td>
</tr>
<tr>
<td>Shield</td>
<td>61-802</td>
<td>12</td>
<td>10</td>
<td>5   31.69 ± 1.67</td>
<td>S. ly.</td>
<td>Campari</td>
<td>Rijk Zwaan</td>
<td>Rijk Zwaan</td>
</tr>
<tr>
<td>Shin Cheong Gang F1</td>
<td>--</td>
<td>12</td>
<td>10</td>
<td>11 6.74 ± 1.02</td>
<td>S. ly.</td>
<td>Cherry</td>
<td>Seminis</td>
<td>Seminis</td>
</tr>
<tr>
<td>Shin Cheong Gang F2</td>
<td>--</td>
<td>12</td>
<td>10</td>
<td>-- 7.42 ± 0.52</td>
<td>S. ly.</td>
<td>Cherry</td>
<td>Seminis</td>
<td>NCSU</td>
</tr>
<tr>
<td>Venus</td>
<td>PI645370</td>
<td>12</td>
<td>8</td>
<td>8   10 20.79 ± 1.71</td>
<td>S. ly.</td>
<td>Beefsteak</td>
<td>Founder</td>
<td>GRIN</td>
</tr>
<tr>
<td>Venus-Bulk Jc</td>
<td>--</td>
<td>12</td>
<td>10</td>
<td>12 12 8.92 ± 1.18</td>
<td>S. ly.</td>
<td>Beefsteak</td>
<td>Founder</td>
<td>NCSU</td>
</tr>
</tbody>
</table>

Daunay et al. (2010) TGC 60: p.6
Table 4.1: (continued).

<p>| | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>WVa700</td>
<td>PI204996</td>
<td>--</td>
<td>--</td>
<td>10</td>
<td>--</td>
<td>40.44</td>
<td>± 0</td>
<td>S. pim.</td>
<td>Cherry</td>
<td>Genetics</td>
<td>--</td>
</tr>
</tbody>
</table>

2-3See Daunay et al. (2010) Tomato Genetics Cooperative report. 60: p.6; AVRDC = World Vegetable Center.
Table 4.2: Location, field preparation, and cultural practices used for each location.

<table>
<thead>
<tr>
<th>Location:</th>
<th>Coastal Plains 1</th>
<th>Coastal Plains 2</th>
<th>Mountain Research Station</th>
<th>Mountain 1</th>
<th>Piedmont</th>
</tr>
</thead>
<tbody>
<tr>
<td>County:</td>
<td>Pender</td>
<td>Brunswick</td>
<td>Haywood</td>
<td>Jackson</td>
<td>Rowan</td>
</tr>
<tr>
<td>Elevation (ft):</td>
<td>41</td>
<td>43</td>
<td>2638</td>
<td>1848</td>
<td>783</td>
</tr>
<tr>
<td>Observation period:</td>
<td>79</td>
<td>86</td>
<td>78</td>
<td>77</td>
<td>76</td>
</tr>
<tr>
<td>Total harvests:</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Days to harvest (type):</td>
<td>65 (Red ripe)</td>
<td>64 (Red ripe)</td>
<td>71 (Red ripe)</td>
<td>71 (Red ripe)</td>
<td>70 Mature green</td>
</tr>
<tr>
<td>Local Scion:</td>
<td>Red Morning</td>
<td>Red Morning</td>
<td>Mountain Majesty</td>
<td>Mountain Majesty</td>
<td>Red Mountain</td>
</tr>
<tr>
<td>Soil type:</td>
<td>Norfolk loamy fine sand and Goldsboro fine sandy loam</td>
<td>Goldsboro fine sandy loam</td>
<td>Cullowhee-Nikwasi complex, fine sandy loam</td>
<td>Hemphill clay loam</td>
<td>Cecil sandy clay loam to clay</td>
</tr>
<tr>
<td>Soil Fumigation:</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Plastic:</td>
<td>Black</td>
<td>Silver reflective on white</td>
<td>Black</td>
<td>White</td>
<td>White on black</td>
</tr>
<tr>
<td>Number of rows:</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Row spacing:</td>
<td>5 feet</td>
<td>5 feet</td>
<td>5 feet</td>
<td>5 feet</td>
<td>5 feet</td>
</tr>
<tr>
<td>Plant spacing:</td>
<td>18 inch</td>
<td>22 inch</td>
<td>18 inch</td>
<td>18 inch</td>
<td>22 inch</td>
</tr>
<tr>
<td>Total Plots:</td>
<td>44</td>
<td>28</td>
<td>64</td>
<td>68</td>
<td>44</td>
</tr>
<tr>
<td>Pruning:</td>
<td>none</td>
<td>2 suckers</td>
<td>2 suckers</td>
<td>2 suckers</td>
<td>2 suckers</td>
</tr>
<tr>
<td>Expected wilting (S):</td>
<td>70 to 100%</td>
<td>50 to 100%</td>
<td>0%</td>
<td>70 to 100%</td>
<td>5 to 50%</td>
</tr>
<tr>
<td>Plants per acre:</td>
<td>5808</td>
<td>4752</td>
<td>5808</td>
<td>5808</td>
<td>4752</td>
</tr>
</tbody>
</table>
Table 4.3: Information about the tomato materials used for the multi-location grafted variety trial experiments.

<table>
<thead>
<tr>
<th>Rootstock</th>
<th>Interest</th>
<th>Type</th>
<th>Use</th>
<th>Testedx</th>
<th>Locations</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>1238-16-F3</td>
<td>New</td>
<td>NCSU breeding line</td>
<td>Rootstock</td>
<td>CG,NG; std</td>
<td>2</td>
<td>NCSU</td>
</tr>
<tr>
<td>NC13192-F3</td>
<td>New</td>
<td>NCSU breeding line</td>
<td>Rootstock</td>
<td>CG,NG; std</td>
<td>2</td>
<td>NCSU</td>
</tr>
<tr>
<td>NC13194-F3</td>
<td>New</td>
<td>NCSU breeding line</td>
<td>Rootstock</td>
<td>CG,NG; std</td>
<td>2</td>
<td>NCSU</td>
</tr>
<tr>
<td>CRA66</td>
<td>Old</td>
<td>Open-pollinated</td>
<td>Rootstock, Founder</td>
<td>CG; std</td>
<td>4</td>
<td>NCSU; others</td>
</tr>
<tr>
<td>Hawaii 7998</td>
<td>Old</td>
<td>Open-pollinated</td>
<td>Rootstock, Founder</td>
<td>CG; std</td>
<td>4</td>
<td>NCSU; others</td>
</tr>
<tr>
<td>Armada F1</td>
<td>New</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std</td>
<td>5</td>
<td>Takii Seed</td>
</tr>
<tr>
<td>BHN 1087 (RT1054)</td>
<td>Old</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std</td>
<td>5</td>
<td>BHN Seed</td>
</tr>
<tr>
<td>Bowman</td>
<td>New</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std</td>
<td>5</td>
<td>Sakata Seed</td>
</tr>
<tr>
<td>DR6258TX</td>
<td>New</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std</td>
<td>5</td>
<td>Seminis</td>
</tr>
<tr>
<td>RST-04-106-T</td>
<td>Old</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std</td>
<td>4</td>
<td>DP Seeds</td>
</tr>
<tr>
<td>Shield</td>
<td>Newish</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std</td>
<td>5</td>
<td>Rijk Zwaan</td>
</tr>
<tr>
<td>Shin Cheong Gang</td>
<td>Newish</td>
<td>Hybrid</td>
<td>Rootstock</td>
<td>CG; std, deep</td>
<td>5</td>
<td>Seminis</td>
</tr>
<tr>
<td>Mountain Majesty</td>
<td>Local</td>
<td>Hybrid</td>
<td>Local scion</td>
<td>NG; std</td>
<td>2</td>
<td>Harris Moran</td>
</tr>
<tr>
<td>Red Morning</td>
<td>Local</td>
<td>Hybrid</td>
<td>Local scion</td>
<td>NG; std</td>
<td>2</td>
<td>Harris Moran</td>
</tr>
<tr>
<td>Red Mountain</td>
<td>Local</td>
<td>Hybrid</td>
<td>Local scion</td>
<td>NG; std</td>
<td>1</td>
<td>Harris Moran</td>
</tr>
</tbody>
</table>

CG: Commercial graft use; NG: Non-grafted; std: recommended planting depth of just coving the root ball with soil; deep: planted deep so that graft union was buried from sight.
Table 4.4: Pearson correlation matrix for phenotypic variables used for principal component analysis.

<table>
<thead>
<tr>
<th></th>
<th>Daily mean AUDPC</th>
<th>Species</th>
<th>Fruit color</th>
<th>Fruit class</th>
<th>Fruit shape</th>
<th>Growth type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily mean AUDPC</td>
<td>--</td>
<td>0.1672</td>
<td>0.1384</td>
<td>0.2425</td>
<td>0.6136</td>
<td>0.3502</td>
</tr>
<tr>
<td>Species</td>
<td>0.1964</td>
<td>--</td>
<td>0.6047</td>
<td>0.0452</td>
<td>0.7976</td>
<td>0.3302</td>
</tr>
<tr>
<td>Fruit color</td>
<td>-0.2104</td>
<td>-0.0742</td>
<td>--</td>
<td>0.0005</td>
<td>0.1551</td>
<td>0.3380</td>
</tr>
<tr>
<td>Fruit class</td>
<td>0.1666</td>
<td>-0.2818</td>
<td>0.4684</td>
<td>--</td>
<td>0.0268</td>
<td>0.0011</td>
</tr>
<tr>
<td>Fruit shape</td>
<td>0.0724</td>
<td>0.0368</td>
<td>-0.2020</td>
<td>-0.3100</td>
<td>--</td>
<td>0.3280</td>
</tr>
<tr>
<td>Growth type</td>
<td>-0.1335</td>
<td>0.1391</td>
<td>-0.1369</td>
<td>-0.4429</td>
<td>0.1397</td>
<td>--</td>
</tr>
</tbody>
</table>

Table 4.5: Eigenvalues and proportion of variation for each principal component.

<table>
<thead>
<tr>
<th>Eigenvalue</th>
<th>Difference</th>
<th>Proportion</th>
<th>Cumulative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.9754</td>
<td>0.3292</td>
<td>0.3292</td>
</tr>
<tr>
<td>2</td>
<td>1.2571</td>
<td>0.2095</td>
<td>0.5387</td>
</tr>
<tr>
<td>3</td>
<td>0.9976</td>
<td>0.1663</td>
<td>0.7050</td>
</tr>
<tr>
<td>4</td>
<td>0.8043</td>
<td>0.1341</td>
<td>0.8391</td>
</tr>
<tr>
<td>5</td>
<td>0.6691</td>
<td>0.1115</td>
<td>0.9506</td>
</tr>
<tr>
<td>6</td>
<td>0.2965</td>
<td>0.0494</td>
<td>1.0000</td>
</tr>
</tbody>
</table>

Table 4.6: Eigenvectors for each principal component by variable.

<table>
<thead>
<tr>
<th></th>
<th>Prin1</th>
<th>Prin2</th>
<th>Prin3</th>
<th>Prin4</th>
<th>Prin5</th>
<th>Prin6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daily mean AUDPC</td>
<td>0.020378</td>
<td>0.804417</td>
<td>0.000233</td>
<td>0.021536</td>
<td>0.466147</td>
<td>-0.36707</td>
</tr>
<tr>
<td>Species</td>
<td>0.292908</td>
<td>0.313417</td>
<td>0.74974</td>
<td>0.237609</td>
<td>-0.33708</td>
<td>0.289461</td>
</tr>
<tr>
<td>Fruit color</td>
<td>-0.45934</td>
<td>-0.28802</td>
<td>0.346152</td>
<td>0.595303</td>
<td>0.122798</td>
<td>-0.46558</td>
</tr>
<tr>
<td>Fruit class</td>
<td>-0.61581</td>
<td>0.181612</td>
<td>-0.03034</td>
<td>0.128937</td>
<td>0.264539</td>
<td>0.707294</td>
</tr>
<tr>
<td>Fruit shape</td>
<td>0.365874</td>
<td>0.081144</td>
<td>-0.50745</td>
<td>0.75622</td>
<td>-0.06332</td>
<td>0.161776</td>
</tr>
<tr>
<td>Growth type</td>
<td>0.43555</td>
<td>-0.36354</td>
<td>0.244225</td>
<td>0.014078</td>
<td>0.76159</td>
<td>0.195626</td>
</tr>
</tbody>
</table>
4.8: Supplemental Figures

Supplemental Figure 4.1: *Map of NC with counties highlighting field locations.*
Stars highlight germplasm trial locations--Jackson, Haywood, Rowan, Brunswick, and Pender counties (left to right). The non-grafted germplasm field tests were conducted at Jackson and Pender county locations while the grafted variety trial was conducted at all five locations. The Haywood location was the NC Department of Agriculture’s Mountain Research Station and was a no disease contrast for the Jackson county field.

<table>
<thead>
<tr>
<th>Class</th>
<th>Maximum diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cull</td>
<td>&lt;2-4/32 &lt;5.4</td>
</tr>
<tr>
<td>Small</td>
<td>2-9/32 5.8</td>
</tr>
<tr>
<td>Medium</td>
<td>2-17/32 6.4</td>
</tr>
<tr>
<td>Large</td>
<td>2-25/32 7.1</td>
</tr>
<tr>
<td>Extra-large</td>
<td>3-15/32 8.8</td>
</tr>
<tr>
<td>Jumbo</td>
<td>&gt;3-16/32 8.9</td>
</tr>
</tbody>
</table>

Supplemental Figure 4.2: *Fruit size grading system (left) and diameters used to classify each size class (right).*
Fruit from each plot were hand harvested and sorted by size by placing through the smallest possible class. Fruit that did not pass through the extra-large hole were classed as jumbo. Additionally, inedible fruit were classed as cull, such as the result of insect feeding or cracking that was oozing. After sorting all the fruit from the plot, the fruit from each bucket were counted and weighed.
Supplemental Figure 4.3: Contrast of bacterial wilt resistance efficacy between susceptible roots and resistant rootstocks.
Supplemental Figure 4.4: Daily mean AUDPC of the wilt index for the diversity panel and controls.

The mean AUDPC wilt index means from each experiment were divided by the observation period to produce daily averages for cross experiment comparison. The overall genotypic mean across the experiments were calculated. The bars are color coded according to the major clusters in Figure 4.15. Error bars are the standard deviation of the experiments. Genotypes lacking error bars were only tested in one experiment.
CHAPTER 5: OVERALL RESEARCH IMPLICATIONS AND FUTURE DIRECTIONS

5.1: The 10,000 foot view.

Bacterial wilt of tomato is a major disease problem in NC, and the lack of large-fruited, high quality resistant scion material has been the fundamental driver of the adoption of rootstocks for management of the disease. We have put a lot of research effort into providing practical short-term and long-term management solutions for the NC tomato industry.

In the short-term we have historically focused on evaluating rootstocks already developed and we have continued to evaluate emerging rootstock germplasm for efficacy in NC production systems (chapter 4, partial). Many researchers have wrestled with this difficult disease over the years, and we are encouraged by our successes for short-term management through grafting with resistant rootstocks.

In the long-term we want to advance our basic understandings of the host-pathogen interactions of BW wilt, particularly with how they are modulated by genetic resistance (chapter 2). This requires the ability to translate our mechanistic understanding into something breeders can select for that provides a high degree of predictive accuracy and high-throughput. We need more translational phenomics in BW of tomato research (chapter 3).

Finally, breeding programs are only as good as the diversity they contain, so the identification, quantification, and introgression of diverse resistance sources/variants into regionally adapted, productive genetic backgrounds should always be the true long-term objective of BW research (chapter 4).

This work has sought to do just that. We have generated a strong set of evidence strengthening and amending our basic and applied understanding of the BW host-pathogen
interactions, how that relates to disease development, and how to more effectively interrogate the diversity of resistance so that it can be mobilized in rootstock development towards sustainable deployment.


In chapter 1, a series of 9 key questions was presented concerning several economically important and scientifically enigmatic phenomena surrounding the BW pathosystem, and how to connect the wealth of basic resistance research to applied breeding and management practices. This work attempted to address each of those points. Some were addressed more fully than others, but it does attempt to fill the knowledge gaps and bridge the basic and applied worlds of BW resistance in tomato.

In chapter 2 we presented the mobilization of novel imaging and bacterial detection techniques for application to the economically relevant field of vegetable grafting for BW management. In the process, we attempted to harmonize our results with the findings of many other researchers into a syntenous model describing the anatomical invasion stages of Rs in tomato and how resistance modulates each facet. This model provides a phenotypic context for resistance evaluation and breeding that finally helps resolve longstanding questions about how bacterial density relates to disease development, how resistance restricts that growth, and what part of the plant is the key tipping point in the binary wilt balance.

Vascular cell-to-cell movement restriction (via xylary pit reinforcement) and the pith/cortical invasion restriction seem to render the strongest resistance phenotypes. These two also appear to affect both early- and late-stage resistance activity. Due to the complex nature of cell wall and pectin structures, they are each likely to be controlled by several genes. This profile
seems to closely match what has been described for the resistance QTLs BWR-6 and possibly also BWR-12 (Danesh et al., 1994; Mangin et al., 1999; J. Wang et al., 2013). Additional phenotyping efforts in the established recombinant inbred lines that have been used for mapping may be able to link these phenotypes with specific QTL.

Although we did not specifically examine the environmental influences on colonization and disease development, the tug-of-war model readily explains how those inputs impact the balance of spatial constriction and invasion—they tip the balance out of the optimum of tomato and into the optimum of Rs.

In addition, the tug-of-war model explains the impact of inoculation techniques on wilt development, where: injured roots bypass cortical migration resistance; higher inoculum densities increase the probability of successful vascular bundle invasion because of sheer cellular bombardment with cutting enzymes; stem inoculation generally involves artificial disruption of cell walls giving unrestricted access to a greater proportion of the xylem volume and bypasses whatever preparation time the root invasion process gives to the stem; and it explains the age factor because older plants develop a greater volume of xylem that must be invaded and plugged, and the hardening process stimulates more vascular development and water loss preventative barriers like waxy cuticles.

The model also explains how VB severity can be related to resistance and disease development, because it is a phenotypic marker of spatial colonization. In chapter 3, that model was applied to a broader rootstock germplasm assessment that first defined the variation and then connected it with meaningful resistance mechanisms. In chapter 4 we returned to the very applied area of resistance diversity as it impacts sources for breeding and deployment as rootstocks. While the field application was last, in many ways our previous work began this
whole process as a field-first approach—We went into the controlled environments and the laboratory to explore what we knew worked.

We have ongoing research further searching for evidence of a rootstock-scion crosstalk mechanism using electron microscopy of our grafted plants in chapter 2. We are also trying to improve our understanding of the chemical nature of the VB itself by exploring the application of Raman spectroscopy to this phenomenon. Finally, this work has opened up many more questions and hypotheses for future research that can come out of the understanding of the host-pathogen tug-of-war model.

1) Can we use the available Bwr QTL markers to help parse out what aspects of the tug-of-war model they each impact? E.g.: If Bwr-12 is related to suppression of internal multiplication of the pathogen in the stem (J. Wang et al., 2013), what spatial dimension(s) are being compromised when it is absent? Greater cell-to-cell invasion through the pit membranes? Greater frequency of bundles colonized? Higher bacterial densities within the same volume of infected cells? Greater ability to invade the pith and cortical tissues? Ability to proliferate in resistant roots also?

2) Other Solanaceous hosts seem to share the same density capping pattern and spatial quarantining of the pathogen in the stem. Would a thorough examination of those species reveal a similar tug-of-war model? Are the genetic features conditioning resistance the same across Solanaceae?

3) Do all resistance sources share the same resistance mechanism? The VB data in chapters 3 and 4 suggest they do. Are we missing novel mechanisms or resistance QTL in the many other resistance sources available?
4) If the resistance mechanisms are the same across resistant germplasm or even species, what explains the strain specific responses? What spatial and anatomical aspects of the invasion pathway are compromised by the unique effector profiles of those diverse strains?

5) If we made a new QTL map based on bacterial density, VB severity, or number of xylem bundles invaded, would we obtain better resolution? Would it yield loci adjacent to the major QTL governing fruit size on chromosomes 2, 3, and 11? Would it improve the consistence of gene action studies?

5.3: Practical implications of the tug-of-war resistance dynamic

1) Field-level sporadic failure of highly resistant germplasm does not necessarily indicate the presence of a resistance-breaking strain of the pathogen. Rather, it is likely to be a simple case where the host-pathogen tug-of-war has tipped in favor of Rs. Indeed, the very allowance of latent infections in highly resistant material is advantageous for protecting the efficacy and durability of the resistance response. The bacteria are not qualitatively killed off like with a typical hypersensitive response. Thus, there is a very low selection pressure for resistance-breaking strains to become dominant in the field. This is important for management recommendations.

2) The tug-of-war model reconciles the strange influence of soil temperature and air temperature on BW expression that has frustrated pathologists and breeders for decades (Acosta, 1978; Nakaho et al., 1996; Prior et al., 1996; Scott et al., 2005). Soil temperature has a magnitude greater impact on disease development, which is logical because the most important tug-of-war arena is in the roots leading to the basal hypocotyl at the soil line.
Furthermore, the optimal temperature ranges for growth and nutrient uptake by Rs and tomato are offset by about 5 °C (30 °C and 25 °C, respectively), while both seem to reach their maximum heat tolerance and rapidly decline after another 5 to 10 °C increase (Criddle et al., 1997; Gallegly and Walker, 1949b; J. B. Jones Jr., 2008; Kelman, 1953; Mew and Ho, 1977; Singh et al., 2014; Tindall et al., 1990; Vaughan, 1944). This means that when the environment is becoming more stressful for tomato, it is becoming more optimum for Rs, and tomato declines a few degrees cooler than Rs. Thus temperature increases will tip the tug-of-war balance towards Rs by improving the metabolic and enzymatic capabilities of the pathogen and increase the rate of water loss from the host, which shifts the wilt threshold to a lower level of tissue colonization and bacterial density.

3) The nature of each pathogen-by-resistance interaction arena strongly suggests multiple host genetic mechanisms providing defense, yet there seem to be two overall mechanisms—chemical bombardment (phenolics, ROS) and structural containment (lignin, callose, electron dense vascular coatings). The QTL mapping studies indicate two major loci on chr. 6 and 12 that seem to provide the bulk of the resistance, and the chr. 6 loci can be broken down into 4 adjacent QTLs (P. Hanson et al., 2013; P. Hanson et al., 2016; J. Wang et al., 2013).

This may help explain the confusing and often conflicting gene segregation studies: The critical components of resistance seem to segregate as one major loci in ‘Hawaii 7996’ (Grimault et al., 1995) or two paired gene loci (Scott, J., Nov. 2015, personal communication), and ‘Hawaii 7998’, a sister line to 7996, somewhat fits a single major gene model with several minor genes (Scott et al., 2005). Yet, substantial environmental and strain effects often cloud the genetic interpretations (Acosta, 1978; Scott et al., 1993; Scott et al., 2005).
The tug-of-war model indicates that the tug-of-war dynamics are the source of this confusion—resistance is active, but when genetic analyses only consider the binary wilt result, there is too much noise caused by the five-arena resistance. Thus, genetic analyses that include information on key marker phenomena, such as restriction of radial invasion, are likely to yield better results. There may be additional resistance mechanisms or allelic variation in the known germplasm that have not yet been characterized, since nearly every mechanistic study has used resistance of, or derived from, ‘Hawaii 7996’, ‘Hawaii 7998’, and ‘CRA66’.

4) The nature of the host genetic background is likely to play a supporting role for the plant. Innate tolerances for heat and drought stress should shift the wilt threshold more in favor of the plant by improving growth and water retention under stressful conditions. The rate of secondary vascular development—growing more roots and vascular tissues—should also help the host by increasing the number of cells that must be colonized to reach the wilt threshold.

5) Differences in strain pathogenicity and aggression may not follow the classic gene-for-gene concept of many other disease resistance traits in tomato, but rather a mixture of the capability to evade species and cultivar specific detection mechanisms, differences in metabolic and enzymatic potential \textit{in planta}, and specific effector cocktails that more or less effectively suppress the hosts multiple resistance systems and metabolic machinery. This seems to be the case for the highly quarantined race 3 biovar 2 phylotype IIB-sequevar 1 strains (A. A. M. Bocsanczy et al., 2011; A. M. Bocsanczy et al., 2012; A. M. Bocsanczy et al., 2014; A. M. Bocsanczy et al., 2017; Clarke et al., 2015; Colburn-Clifford and Allen, 2010; Milling et al., 2009; Swanson et al., 2005).
5.4: What do we do with all this diversity?

One of the outcomes of this work has been the cataloging of genetic resistance sources. In this work we identified over 110 lines that are either resistance founders, important resistance introgressions, or commercial hybrids. The number of lines is becoming large enough to start thinking about performing a genome-wide association (GWAS) mapping project for BW resistance, like what was recently performed on 360 tomatoes for domestication and fruit size traits (T. Lin et al., 2014). Yet, a quick comparison of our list with theirs only found two lines in common—‘WVa700’ and ‘Hawaii 7998’.

If both pools of germplasm were combined, and additional progeny from these founders were added, there would easily be nearly 600 lines for association mapping, and less than half would need to be sequenced. The 360 lines are all likely to be susceptible, unless new resistance sources are discovered. Coupled with the advancements in the reliability of the refined tomato reference genome, and the tomato breeding industry would have a very useful genomic resource.

At the recent Tomato Breeders Roundtable meeting in Ohio (February, 2018), the tomato industry began discussions of beginning a pan-genome project by generating five or more new reference genomes to integrate with the ‘Heinz 1706’. This would complement a GWAS very nicely because it would readily improve marker discovery among tomato groups and breeding pools.

5.5: Next steps in rootstock development.

We have really lacked a reliable rootstock resistance evaluation in the Coastal Plains region of NC. In the last several years we have kept getting low disease pressure or non-BW disease pressure (Kressin, 2014; Silverman, 2015). In this work, we finally got a good field test
outside of the Mountains. Because of our work in chapter 4, our recommendations for managing BW across NC have new evidence supporting our existing recommendations, as well as further confidence about making recommendations in the Coast Plains region.

In addition, it is clear that NC growers have multiple rootstocks from several private and public breeding programs that provide good yields and high levels of BW resistance season-long. Growers should be encouraged to start rotating rootstocks in order to help protect them from selecting for more aggressive strains in the native field populations of Rs. While some of the new rootstocks available to NC growers show great promise, our recommendations about them are preliminary. We need at least an additional year of testing to make sure the patterns are stable over seasons, which is planned for the summer of 2018.

We have strong resistance. Now, further rootstock development should focus on productivity, multiple disease resistance (especially for Verticillium dahliae race 2), abiotic stress tolerance, and exploring rootstock traits conferred to the scion. Examples of the last might be: resistance to foliar pathogens and pests, dwarfing traits, etc. We have preliminarily examined if the brachytic gene (br) in a rootstock might condition shortened internodes to an heirloom and commercial hybrid variety (data not shown).
REFERENCES


APPENDIX
APPENDIX A: HEAT STRESS PHYSIOLOGY ON TOMATO AND BACTERIAL WILT

1.1: Temperature effects on tomato and *Ralstonia solanacearum* (Rs), independently

Any discussion of heat stress effects on plant/pathogen growth and development must consider the type and strength of the stress. The stress may be acute or chronic, each with varying degrees of severity based upon the degree, duration, and rate of the change (Wahid et al., 2007). Generally, a transient 10-15 °C temperature increase compared to the ambient is considered to be a heat shock/stress in experiments, although more chronic-style experiments in tomato have used a 4 °C range in average daily temperature (Peet et al., 1998).

For the sake of a context for discussion, let us say that two different summers are experienced in NC. The first is cooler, with an average state temperature of about 24 °C. The second is abnormally hot, with an average temperature of 29 °C. The hot summer is an increase of 5 °C (about a 10 °F change) continuously, making it a chronic, moderate stress factor. NC tomato production is exposed to about half of the heat-zones of the Southeastern US (American Horticultural Society, 1995), which means the scenario would cause the peak summer temperatures of Pender county at the coast would become the peak temperatures for Jackson county in the heart of the Mountain region (Table A1).

Air temperature is a primary source of heat damage on tomato growth, physiology, and productivity. The optimum air temperatures for tomato have been calculated in several ways. A respiration-based description of the plant growth rate indicates that temperatures for leaf metabolism (CO₂ production) leading to growth begins at 12 °C, peaks at 21 °C (greatest metabolic efficiency), and stops at 37 °C (Criddle et al., 1997). Growth and uptake experiments indicate the optimum day/night air temperature ranges for tomato are 18.3/18.3 to 29.5/21.0 °C.
(J. B. Jones Jr., 2008), while the critical low/high temperature thresholds for any growth are about $<10/35^\circ C$ (Hansen et al., 1994; Went, 1957). These values match closely to the metabolic efficiency thresholds.

Soil temperature also affects tomato shoot and root growth, water uptake, and the uptake rates of most of the plant nutrients, especially potassium and nitrate (not boron and molybdenum, and iron has conflicting reports) (J. B. Jones Jr., 2008; Tindall et al., 1990). The root temperature thresholds are similar to air temperature, but have less flexibility. The optimum temperature is about 25 °C, with a high temperature damage threshold of about 32 °C.

The effects of temperature stress on the plant are also modulated by the tissue type and developmental stage of the plant (Wahid et al., 2007). For plants in general, heat stress affects a plethora of physiological functions. Tomatoes specifically have an upper heat damage threshold of 30 °C and especially 35 °C, which means the scenario would push Jackson Co. to more regularly having warm summers that peak above 30 °C, and Pender Co. would regularly expose tomatoes to above threshold temperatures in the summer (Table A1). This kind of heat stress would lead to decreases in: fruit set (through pollen/ovule viability and flower/embryo abortion), seed development and germination, vegetative growth, flowering time and morphology (most conspicuously, an exerted style outside the pollen cone), fruit size, and fruit ripening (Foolad, 2005; Peet et al., 1998; Wahid et al., 2007).

Molecularly, this kind of heat stress will adversely disrupt: meiosis, photosynthesis (photosystem II and ATP generation, along with carbon assimilation through reduced levels and activity of Rubisco), and membrane stability (solute leakage) (Wahid et al., 2007; G. Wang et al., 2015). The heat stress will also increase water loss and heat shock protein concentrations in the cells. Plants, especially in the Pender Co.-type conditions, will be stunted and have poor yields,
which may threaten the economic viability of summer tomato production in the warmer parts of the Southeast, especially in hotter years, but it is not as likely to dramatically affect tomato viability in Jackson Co.-like environmental zones.

Since Rs grows primarily in a soil environment, alongside the tomato roots, air temperature variation does not have a major effect on the pathogen, apart from soil warming. Soil temperature is the main driving force. Rigorous investigation of temperature effects on pathogen growth, physiology, and nutrient uptake have not been done like they have for tomato. Studies have focused more on cold stress adaptation and survival.

A rough estimate of growth in cultures at various temperatures showed a lot of variation by strain, but a summary of multiple studies suggests that 8-18 °C is the minimum temperature for growth, 27-37 °C is optimum, 35-41 °C is the maximum, and the thermal death point is 45-55 °C (Figure A1) (Kelman, 1953). The growth optimum for Rs is about 5 °C warmer than tomato. Several authors have noted a sharp drop off in growth after 32-35 °C (Grieve, 1943; Singh et al., 2014). Thus, if the average soil temperatures increased concurrent with the air temperatures, Rs fitness would not likely be negatively affected in the Southeast, but would either remain unchanged or exhibit somewhat of an enhanced growth rate.

1.2: Likely changes in recognition, infection, and disease development at higher temperature.

The effects of temperature on the interaction of tomato and Rs are strong and have been regularly studied, mostly in susceptible lines (Acosta, 1978; Gallegly and Walker, 1949b; Grieve, 1943; Mew and Ho, 1977; Nakaho et al., 1996; Prior et al., 1996; Scott et al., 2005; Singh et al., 2014; Vaughan, 1944). An increase in soil temperature has a much stronger effect
on wilt development than does air temperature (Figure A2). Nakaho et al. (1996) demonstrated that a 5 °C temperature increase (from 25/20 °C to 30/25 °C day/night) in growth chamber seedling experiments led to increased rates of infection and wilting, and partially resistant lines exhibited a stronger contrast between the temperature regimes than did susceptible. Vaughan (1944) observed the same phenomenon, and wilt development generally plateaued about 32 °C. Several researchers have reported that increases in temperature led to faster and stronger initiation of wilt symptoms (Grieve, 1943; Mew and Ho, 1977; Singh et al., 2014; Vaughan, 1944).

There appears to be a high temperature threshold where resistance “breaks down” at about 30-32 °C (Krausz and Thurston, 1975; Mew and Ho, 1977; Nakaho et al., 1996; Singh et al., 2014). The nature of this phenomenon is unknown, and a few lines do not exhibit that pattern, at least not up to 32 °C (Mew and Ho, 1977). Colonization tests in field-grown tomatoes showed that Rs more consistently reached higher up the stem (6th internode, midstem; compared to collar) in the summer season compared to the spring, even though there was only a 2 °C change in the average temperature (Prior et al., 1996).

Collectively, a 5 °C increase in average temperature would be expected to increase the frequency of resistance “break down” events, maximize wilt initiation and frequency, and increase bacterial invasion up the stem, which implies that grafted management of bacterial wilt with resistant rootstocks may lose efficacy due to greater frequencies of scion colonization. The observation of temperature “insensitive” resistance is curious, but has not been well characterized. It is possible that there is heat stress tolerance mixed with bacterial wilt resistance in those lines.
The enhancement of the rate of infections and wilt development in both susceptible and resistant tomato plants as temperature rises is very curious. The literature is not clear about what mechanisms may be causing this shift. Little is known about how tomato and Rs recognize one another during pathogenesis, or about the biology of Rs in the rhizosphere (Schell, 2000). Root exudate-based attraction is plausible. Even less is known about how temperature variation modulates those parameters. Rs perceives contact with plant cell walls via an outer membrane receptor called PrhA, which in turn induces a protein signal cascade that initiates pathogenic behavior (Peeters et al., 2013).

Perhaps the most parsimonious answer lies in the fact that Rs invasion and colonization relies heavily on excreted pectinolytic enzymes, both in susceptible and resistant tomato lines (González and Allen, 2003; Huang and Allen, 2000; Nakaho and Allen, 2009), and that resistance to the pathogen involves structural growth/repair components (Nakaho, 1997a; Nakaho, 1997b; Nakaho et al., 2004; Nakaho and Allen, 2009). Therefore, a heat stressed tomato host, having moderate to sharp reductions in carbon assimilation, nutrient uptake, and enhanced water stress, would be required to defend against a pathogen exhibiting uncompromised or perhaps enhanced growth rates. As the strength of the tomato wanes, the strength of the pathogen optimizes, leading to Rs outpacing the tomato plant in growth.

A 5 °C increase in average temperature across the Southeast would shift the ecosystem towards the ideal for Rs and away from the ideal for tomato. This is supported by the observation that greater concentrations of Hoagland solution (2x and 3x) in sand cultured tomatoes and increased day length (6, 12, 18 hours) led to reduced wilt development (Gallegly and Walker, 1949a; Gallegly and Walker, 1949b). Co-chaperone-based stabilization of carbon assimilation improved heat stress mitigation, drought tolerance, and bacterial wilt resistance resistance (G.
Wang et al., 2014; G. Wang et al., 2015). In other words, greater ability to obtain nutrients and assimilate carbon leads to enhanced structural defense capabilities.
1.3: Tables

Table A1: Average temperature profiles for important tomato bacterial wilt trial locations. Mean temperature (C) summary of NC tomato growing counties in the Mountain (Jackson Co.) and Coastal Plains (Pender Co.) regions, and how those would change with a 10 °F (5 °C) increase in the mean yearly temperature. Values were obtained from the US weather station data collected from 1980 to 2010 (Source: U.S. Weather Station Data, 2010a,b;).

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1.4: Figures

Figure A1: Temperature effects on *R. solanacearum* growth.
A) Temperature effects on growth of *R. solanacearum* strains from various regions of the world (Redrawn from: Kelman, 1953, table 4). Means and mode were calculated from values reported for each category. Error bars for the mean are the standard deviation; error bars for the mode are the maximum/minimum ranges reported.
**Figure A2: Temperature effects on wilt development.**
A) Impact of temperature variation on the mean wilt index of infected tomato plants (Redrawn from: Gallegly and Walker, 1949b).

1.5: References

Appendix references are included in the main references section of this work.