ABSTRACT

STOWE, KATHERINE DRAKE. Investigating the Genetic Control of Soil-Borne Pathogen Resistance and the Yellow Burley Phenotype in *Nicotiana tabacum* L. (Under the direction of Dr. Ramsey S. Lewis).

Tobacco (*Nicotiana tabacum* L.) is an economically significant crop grown throughout the world. Greater than 10% of the North Carolina tobacco crop is lost annually due to plant pathogens, with black shank and Granville Wilt (caused by *Phytophthora nicotianae* and *Ralstonia solanacearum*, respectively) causing the greatest economic losses. Development of varieties with genetic resistance provides the most economic method of reducing disease losses. Three projects were conducted to investigate and characterize the genetics of resistance to soilborne pathogens in modern tobacco cultivars. In addition, a fourth project was conducted to use map-based gene discovery to identify the genes at the *Yellow Burley 1* (Yb_1) and *Yellow Burley 2* (Yb_2) loci, the determinants of the major phenotypic difference between burley tobacco and cultivars of alternative market classes.

First, we investigated the effect of an introgressed *N. rustica* genomic region (designated as *Wz*) on black shank resistance and correlated the effects on yield and quality. DNA markers were used to transfer *Wz* to the elite flue-cured variety K 326 and nearly isogenic lines and hybrids were developed. *Wz* was found to exhibit large favorable effects on black shank resistance in seven field diverse disease environments. No evidence of a negative relationship between *Wz* and yield and/or quality was observed. Data suggest commercial value for *Wz* in flue-cured tobacco-breeding programs with the goal of developing high-yielding tobacco cultivars with resistance to multiple races of the black shank pathogen.

To better characterize *Wz*-mediated resistance, further experiments were carried out in project two. Genotypes with the *Wz* gene region were found to exhibit high levels of root resistance to both race 0 and race 1 isolates of *P. nicotianae*, but little stem resistance to either race. Little evidence of *Wz* resistance being overcome by high inoculum levels was observed, a finding that is inconsistent with *Wz* resistance being of the partial type. Finally, after several cycles of pathogen inoculation and re-isolation on K 326 *Wz*/*Wz*, isolates of *P. nicotianae* with increased potential to cause disease on *Wz*-containing genotypes were identified. The allelic variation provided by the *Wz* region will likely be most valuable when used in combination with *Php* in genetic backgrounds that also possess a medium to high level of polygenic resistance.

In project three, the genetics of the resistance to black shank and Granville wilt in tobacco cultivar K 346 was investigated. Four significant QTL were found to be associated with black shank resistance, and three QTL were found to be associated with Granville wilt resistance. The two QTL with the largest effect on black shank resistance were also found to have the greatest effects on Granville wilt resistance. This may partially explain previously observed positive correlations between resistance to black shank and bacterial wilt among current cultivars. Further investigation is needed to determine whether this observed relationship is due to pleiotropy or due to favorable coupling-phase linkages that were established during prior breeding efforts.

Finally, map-based gene discovery was used identify the genes at the Yb_1 and Yb_2 loci, the genetic determinants for the chlorophyll-deficient phenotype of the burley tobacco market class that is conferred by a double homozygous recessive genotype. The study showed that Yb_1 and Yb_2 encode for proteins with a very high degree of similarity to EGY1 from *Arabidopsis thaliana*, a

predicted chloroplast membrane-bound, ATP independent, metalloprotease. The identification of these genes represents the first reported example of map-based gene discovery in *N. tabacum*.

Investigating the Genetic Control of Soil-Borne Pathogen Resistance and the Yellow Burley Phenotype in *Nicotiana tabacum* L.

by
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DEDICATION

This dissertation is dedicated to my parents, Paul and Melissa Drake, for their unwavering support and for their constant reminders that a life full of hard work, dedication, persistence, humility, and gratitude is a life worth living.

BIOGRAPHY

Katherine Drake Stowe, daughter of Paul and Melissa Drake, was born and raised on a small family farm in Pinetops, North Carolina. Upon graduation from SouthWest Edgecombe High School in 2005, she attended North Carolina State University. She graduated with a Bachelor's degree in Polymer and Color Chemistry and a minor in Crop Science in 2009. While still an undergraduate, she met Dr. Ramsey Lewis and began working in the tobacco breeding and genetics lab where an interest in agriculture research was discovered. In 2009, she began pursuit of a Master of Science degree program in Crop Science under the direction of Dr. Ramsey Lewis. After completion of this degree in 2012, she continued in the tobacco breeding and genetics lab, pursuing a Ph.D. degree. Once completed, Katherine will serve as Research Coordinator for the North Carolina Soybean Producers Association.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Black Shank Resistance and Agronomic Performance of Flue-Cured Tobacco Lines and Hybrids Carrying the Introgressed *Nicotiana rustica* region, *Wz*

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ABSTRACT

Black shank, caused by *Phytophthora nicotianae*, is one of the most important diseases affecting tobacco (*Nicotiana tabacum* L.) production in the U.S.. Genetic mechanisms are needed that provide resistance to current races and that can be combined into cultivars that provide high yields of cured leaf with acceptable quality. Previous research identified DNA markers associated with an introgressed *N. rustica* genomic region (designated as *Wz*) found to contribute to resistance to race 0 and race 1 isolates. Objectives of the current research were to use DNA markers to transfer *Wz* into the elite genetic background of flue-cured tobacco cultivar K 326 and to develop nearly isogenic lines and hybrids with and without the race 0 immunity gene *Php*. These materials were evaluated in multiple environments for black shank resistance, yield, and quality characteristics. *Wz* was observed to positively affect resistance in the seven diverse disease environments tested. Genotypes in which *Wz* was combined with *Php* exhibited the greatest levels of resistance. No evidence of a negative relationship between *Wz* and yield and/or quality was observed. Data suggest commercial value for *Wz* in flue-cured tobacco-breeding programs with the goal of developing high-yielding tobacco cultivars with resistance to race 0 and race 1. Further studies are necessary to determine the durability of *Wz*-mediated resistance, however.

INTRODUCTION

Black shank, caused by the soil-borne pathogen *Phytophthora nicotianae*, has historically been one of the most important diseases affecting tobacco (*Nicotiana tabacum* L.) production in the U.S.. This disease is also of significant economic importance in several other tobacco-producing countries. Overall strategies used to mitigate losses from the disease include the use of genetic resistance, crop rotation, and chemical inputs. Use of genetic resistance is attractive only if it can be incorporated into cultivars without reducing yield and/or quality below commercially acceptable levels, however.

Multiple sources of resistance have been used or investigated to improve black shank resistance in cultivated tobacco. Polygenic resistance currently present in commercial flue-cured and burley tobacco cultivars is likely mostly derived from the cigar tobacco cultivar Florida 301 (Tisdale, 1931; Xiao et al., 2012). This type of resistance is believed to be effective against all known races of the pathogen. Monogenic resistance to race 0 of *P. nicotianae* is controlled by the genes *Php* and *Phl* introgressed into *N. tabacum* from *N. plumbaginifolia* and *N. longiflora*, respectively (Valleau et al., 1960; Apple, 1962a; Chaplin, 1962). Cigar tobacco cultivar Beinhart 1000 has also been studied as a potential source of a very high level of quantitative resistance (Heggestad and Lautz, 1957; Silber and Heggestad, 1963; Chaplin, 1966; Wills, 1971; Nielsen, 1992; Vontimitta and Lewis, 2012a,b), but at the present time few commercial flue-cured or burley tobacco cultivars have Beinhart 1000 in their pedigree.

Described races of *P. nicotianae* include race 0, race 1 (Apple, 1962b, 1967), race 2 (van Jaarsveld et al., 2002), and race 3 (McIntyre and Taylor, 1978; Gallup and Shew, 2010), with race 0 and race 1 currently being of greatest economic importance. To date, no known source of

genetic resistance provides complete resistance to all races of the black shank pathogen, however. In addition, these known resistance genes are often found to be associated with reduced yields and/or quality of cured leaf (Chaplin and Ford, 1958;Nielsen, 1992; Lewis, 2011), making it difficult to develop highly resistant cultivars that also produce high yields of cured leaf with acceptable quality characteristics. Additional genetic mechanisms might be of value for improving the level and expanding the range of resistance to increase the likelihood of developing high-yielding tobacco cultivars with high levels of black shank resistance.

A large number of studied accessions of *N. rustica* were previously found to exhibit extremely high levels of resistance to race 0 of *P. nicotianae* and very high levels of resistance to race 1 isolates (Nifong et al., 2011), but the nature of this resistance has not been investigated. Woodend and Mudzengerere (1992) described the introgression of a wildfire and angular leaf spot (caused by *Pseudomonas syringae* pv. *tabaci*) resistance gene on a chromosome segment designated as *Wz* from this species to *N. tabacum*. Several U.S. flue-cured tobacco cultivars known to possess the *Wz* region were observed to exhibit high levels of field resistance to black shank (Antonopoulos et al., 2010). The *Wz* region was subsequently characterized and reported to affect resistance to both race 0 and race 1 isolates of *P. nicotianae*, and DNA markers of *N. rustica* origin were identified that are associated with the introgressed region (Drake and Lewis, 2013).

Wz might be of use for extending the level and range of black shank resistance in flue-cured tobacco. It is not currently known, however, whether *Wz* affects resistance to *P. nicotianae* in diverse tobacco-producing areas. It is possible that pathogen populations exist that are not affected by *Wz*-mediated resistance. The effect of *Wz* on yield and quality of flue-cured

tobacco is also unknown. Introgressed alien chromosome segments are often deployed in heterozygous condition in tobacco to mitigate adverse effects on these important traits (Lewis, 2011). The relative level of black shank resistance in *Wz*/*Wz* homozygotes versus *Wz*/– heterozygotes has yet to be investigated. Finally it should be determined if there is a favorable phenotypic effect when *Wz* is combined into single genotypes with the race 0 immunity gene, *Php*. The objectives of this research were to use marker assisted selection to transfer *Wz* into the elite genetic background of flue-cured tobacco cultivar K 326 and to develop nearly isogenic lines (NILs) and hybrids (NIHs) with and without the race 0 immunity gene *Php*. These genetic materials were evaluated for black shank resistance in diverse disease environments in three tobacco-producing states in comparison with a set of existing cultivars exhibiting a range of black shank resistance. The materials were also evaluated for yield and quality characteristics in six different environments to determine the effect of *Wz*, if any, on these traits.

MATERIALS AND METHODS

Development of Genetic Materials

The breeding line 'Wz' was chosen as a source of the *Wz* region previously described by Woodend and Mudzengerere (1992) and Drake and Lewis (2013). Elite flue-cured tobacco cultivar K 326 was selected to be the recipient of *Wz* using the backcross breeding method. This cultivar expresses only an intermediate level of polygenic black shank resistance but has been of major commercial importance worldwide for greater than 20 yr, and most modern U.S. cultivars have high proportions of K 326 in their parentage. After initial hybridization between Wz and K

326, five cycles of backcrossing were performed to produce BC_5F_1 families. Selection for black shank resistance in the BC_1F_1 and BC_2F_1 generations was conducted via field disease nursery selection and artificial inoculation, respectively. Selection for *Wz* in the BC_3F_1 through BC_5F_1 generations was conducted via selection for associated molecular markers (Drake and Lewis, 2013). BC_5F_1 individuals carrying *Wz*-associated markers were self-pollinated to produce segregating BC_5F_2 populations that were genotyped with coupling and repulsion phase markers to predict individuals homozygous for the *Wz* region. Homozygosity for *Wz* was verified via genotyping of testcross progeny produced by hybridizing BC_5F_2 *Wz*/*Wz* individuals with K 326. Single homozygous (*WzWz*) BC_5F_2 plants were self-pollinated to produce BC_5F_3 NILs and also hybridized with K 326, cytoplasmic male sterile (*Cms*) K 326, and K 326 *Php*/*Php* (K 326 into which *Php* was backcrossed five times) to produce seed of heterozygous (*Wz*/–) F_1 NIHs. In addition, two BC_5F_2 segregants lacking the *Wz*-associated markers were selected and self-pollinated to produce seed of BC_5F_3 null segregant (–/–) families.

Field Evaluation for Black Shank Resistance

The complete set of 24 entries evaluated in field black shank disease nurseries is outlined in Table 1.1. Materials included three K 326 *Wz*/*Wz* BC_5F_3 lines, three K 326 *Wz*/– F_1 hybrids, three *Cms* K 326 *Wz*/– F_1 hybrids, three K 326 *Wz*/– *Php*/– F_1 hybrids, two K 326 BC_5F_3 null segregant families, K 326, and a series of check varieties representing a range of black shank resistance in tobacco. Field black shank resistance was evaluated in soil-borne disease nurseries in a total of seven environments in three tobacco-producing states. Experiments were conducted at the Lower Coastal Plain Tobacco Research Station (Kinston, NC), the Upper Coastal Plain Research Station (Rocky Mount, NC), and the Oxford Tobacco Research Station (Oxford, NC)

during 2012. Experiments were conducted at the Upper Coastal Plain Research Station and private farms in Sumter County, SC; Irwin County, GA; and Pierce County, GA, during 2013. For the North Carolina and Irwin County, GA, environments, randomized complete block designs were used with four replications. Experimental units consisted of single-row plots containing 20 plants. The experimental design for the Pierce County, GA, environment was a randomized complete block design with six replications and experimental units consisting of single 20-plant rows. The experimental design for the Sumter County, SC, environment was a completely randomized design with each entry being replicated four times. Experimental units consisted of single 20-plant plots. Plants were transplanted and maintained according to common agricultural practices for flue-cured tobacco production for the respective states. The number of plants killed by black shank in each plot was recorded approximately every 14 d beginning at approximately 21 d post-transplanting. Four to five separate disease evaluations were recorded for each environment.

Field Evaluation for Yield and Quality

A total of 21 genotypes were evaluated in six field environments during the 2012 and 2013 growing seasons (Table 1.2). Materials included the same three K 326 *Wz*/*Wz* BC_5F_3 families, three K 326 *Wz*/– F_1 hybrids, three *Cms* K 326 *Wz*/– F_1 hybrids, three K 326 *Wz*/–*Php*/– F_1hybrids, two K 326 BC_5F_3 null segregant families, K 326, and a series of current commercially important check cultivars. Experiments were conducted at four locations during 2012: the Cunningham Research Station (Kinston, NC), the Upper Coastal Plain Research Station (Rocky Mount, NC), the Border Belt Tobacco Research Station (Whiteville, NC), and the Oxford Tobacco Research Station (Oxford, NC). The experiments were

performed at the Rocky Mount and Oxford locations during 2013. The experimental design used in each environment was a randomized complete block design with four replications. Plots consisted of single 20-plant rows and were managed according to standard flue-cured production practices for North Carolina (North Carolina Cooperative Extension, 2013). Intra-row spacing was 56 cm at all four locations, while inter-row spacing was 122 cm at the Oxford, Rocky Mount, and Whiteville locations and 112 cm at the Kinston location.

Tobacco plots were harvested at each of the six environments in four separate leaf harvests according to the rate of ripening and maturity. Leaf harvests were flue-cured, weighed, and assigned USDA quality grades by a former USDA tobacco grader. Value 100 kg^{-1} (USD) and value ha^{-1} (USD) were calculated for each plot on the basis of the 2012 flue-cured price index (North Carolina Cooperative Extension, 2013). Fifty-gram composite samples of cured leaf were collected for each plot using a weighted mean basis. Oven-dried samples were ground and analyzed for percentage total alkaloids and percentage reducing sugars according to the method of Davis (1976).

Statistical Analysis

Area under the disease progress curve (AUDPC) was calculated for each plot at the conclusion of the black shank field experiments according to Madden et al. (2007). Analyses of variance were first performed for disease-resistance data for each environment separately using PROC MIXED of SAS 9.3 (SAS Institute, Cary, NC). For the combined analysis over environments, an analysis of variance was performed on entry means from each environment. The pooled error served as the entry × environment term in the analysis of variance, and the entry × environment mean square was used as the error term for calculation of LSDs for

comparing means averaged over environments. Statistical comparisons of genotypic classes and checks for AUDPC were conducted using appropriate LSD tests (Gomez and Gomez, 1984).

For the yield and quality experiments, an analysis of variance for measured characters was performed over environments using PROC MIXED. Genotype was treated as a fixed effect, while environment and replication were treated as random effects. Statistical comparisons of genotypic classes for measured traits of interest were conducted using Fisher's protected LSD tests and single degree of freedom contrast statements (Steel et al., 1997).

RESULTS

The set of materials outlined in Table 1.1 was evaluated for field black shank resistance in a total of seven environments. Analyses of variance for AUDPC values for each of the seven environments independently revealed significant differences between entries in each environment (Table 1.3). Race 1 was present in all environments as evidenced by high mortality in the race 1 indicator strain, NC1071 (*Php*-containing breeding line of a genetic background that would otherwise provide a low level of black shank resistance). In environments with low disease pressure (Pierce County, GA, and Sumter County, SC), it was difficult to identify significant differences between most entries for AUDPC. In all environments except one (Sumter County, SC), *Wz*-possessing genotypes exhibited significantly greater ($P < 0.05$) levels of black shank resistance than K 326 (Fig. 1.1). In no environment were *Wz*/*Wz* genotypes significantly more resistant than *Wz*/– genotypes. In addition, the presence of cytoplasmic male sterility did not affect resistance in any environment. K 326 *Wz*/–*Php*/– genotypes had significantly lower ($P < 0.05$) AUDPC values relative to *Wz*-carrying entries in only a single

environment (Rocky Mount, 2012). In environments with high disease pressure (Kinston, 2012; Rocky Mount, 2012; Irwin County, GA), *Wz*-possessing genotypes had significantly lower ($P < 0.05$) AUDPC values as compared with that for NC 71, a *Php*-containing flue-cured cultivar primarily of K 326 parentage.

Averaged over all seven environments, K 326 *Wz*/–*Php*/– genotypes exhibited the second highest level of resistance, second only to that of highly resistant cigar tobacco cultivar Beinhart 1000 (Fig. 1.1). *Wz*-containing genotypes were very comparable and not significantly different from K 346, a flue-cured tobacco cultivar with a very high level of polygenic black shank resistance. *Wz* genotypes exhibited significantly greater ($P < 0.05$) levels of resistance than NC 196 and NC 71, *Php*-containing flue-cured tobacco cultivars largely of K 326 descent. The materials of interest were also evaluated in six replicated field experiments for yield, physical cured leaf quality, and cured leaf chemistry. An analysis of variance over environments indicated there to be significant ($P < 0.05$) differences between entries for all measured characteristics (Table 1.4). Significant entry × environment interactions were observed for many of the measured traits, but these were considered of low interest because environment was considered as a random effect, and means are presented as averages over environments (Fig. 1.2). K 326 *Wz*/– and K 326 *Wz*/*Wz* genotypes produced the highest yields (kg ha^{-1}) in the experiment, and *Wz*-containing genotypes were not significantly different from K 326 for this trait. Significant differences were not observed between the *Wz*/*Wz* and *Wz*/– genotypic classes for yield.

Cured leaf of popular flue-cured tobacco cultivar NC 196 produced the highest grade index, a measure of physical cured leaf quality (Fig. 1.2). There were no significant differences

between K 326 and any of the *Wz*-containing genotypes for grade index. The same relationships were observed for value 100 kg^{-1}, also a measure of physical cured leaf quality. The three entries producing the greatest value ha^{-1} were *Cms* K 326 *Wz*/–, K 326*Wz*/–, and K 326 *Wz*/–*Php*/–, respectively. No significant differences were observed between K 326, NC 196, and any *Wz*-containing genotypes for value ha^{-1}. Some *Wz*-containing genotypes exhibited significantly lower ($P < 0.05$) levels of percentage total alkaloids as compared with K 326. No significant differences were observed between K 326 and any *Wz*-containing genotype for percentage reducing sugars.

DISCUSSION

The primary objective of flue-cured tobacco breeding is to combine necessary levels of disease resistance into cultivars that produce high yields of cured leaf with acceptable or improved cured leaf quality. Heritability of black shank resistance is generally high if good field disease nurseries are available (Vontimitta and Lewis, 2012a,b; Xiao et al., 2013), and it is not difficult to develop cultivars with high levels of black shank resistance. Indeed, cigar tobacco cultivar Beinhart 1000 exhibits extremely high levels of resistance due to the accumulation of multiple favorable alleles affecting a high level of partial resistance (Vontimitta and Lewis, 2012a,b). It has been difficult to achieve this level of resistance to multiple races of *P. nicotianae* in flue-cured tobacco without sacrificing yield and/or quality, however. Data from the current research indicate that the introgressed *N. rustica* region, *Wz*, can positively affect black shank resistance in multiple environments in three tobacco-producing states with little to no adverse effect on yield or quality. K 326 *Wz*/*Wz* and K 326*Wz*/– genotypes were significantly

more resistant than K 326 in six of seven tested environments. Race 1 was present in all of these tested environments as evidenced by a high level of mortality in NC1071 (race 1 indicator strain) in each environment. There was no strong evidence of an additive effect associated with *Wz*, as *Wz*/*Wz* homozygotes were not significantly superior to *Wz*/– heterozygotes for resistance. Likewise, the presence of cytoplasmic male sterility did not affect the impact of *Wz* in the K 326 genetic background. Averaged over all environments, the K 326 *Wz*/–*Php*/– genotype provided the second-highest level of resistance, second only to that exhibited by Beinhart 1000. In one environment, the K 326 *Wz*/–*Php*/– genotype exhibited significantly greater resistance than K 326 *Wz*/*Wz* and *Wz*/– genotypes. This indicates probable value in combining the two resistance mechanisms into single cultivars. In environments with high disease pressure, *Wz*/*Wz*, *Wz*/– and *Wz*/–*Php*/– genotypes exhibited levels of black shank resistance that were significantly greater than those exhibited by commercially successful flue-cured tobacco cultivars NC 196 and NC 71. These F_1 hybrids are largely of K 326 descent and are heterozygous for *Php*.

The data presented here do not indicate a yield penalty associated with *Wz* and therefore do not suggest a strong agronomic advantage for deploying *Wz* in heterozygous versus homozygous condition. This contrasts with several other alien disease resistance gene introgressions in tobacco, where heterozygous breeding materials typically exhibit superior agronomic performance relative to homozygous materials (reviewed by Lewis, 2011). Likewise, the results do not suggest a negative influence of *Wz* on physical cured leaf quality as indicated by grade index, value 100 kg^{-1}, and value ha^{-1} values for *Wz*-containing genotypes that were very comparable to those exhibited by K 326, per se. Chemical quality as measured by percentage total alkaloids and percentage reducing sugars was within the boundary of commercial

acceptability as defined by average determinations for check cultivars NC95, NC2326, and K 326 (Smith et al., 2003).

The data presented here suggest commercial value of *Wz* in cultivar development programs, especially when combined with *Php* in F_1 hybrids. A major unanswered question, however, is whether or not the *Wz* region contains a gene that confers a very high level of resistance (or possibly immunity) to certain isolates via a race-specific, gene-for-gene type of interaction, or whether *Wz* carries a gene or genes that contribute to a large partial resistance effect. The latter scenario might be preferable if long-term durability is desired. Gene-for-gene recognition systems can quickly be overcome via pathogen evolution. For example, continued deployment of *Php* (a race 0 immunity gene) places high selection pressure on *P. nicotianae* populations to result in rapid increased prevalence of race 1 genotypes (Sullivan et al., 2005b). Although components of partial resistance have been reported to display race specificity (Johnson, 1984; Young, 1996; Poland et al., 2008), and partial resistance does not necessarily infer long-term durability (Johnson, 1984; Keane, 2012; Montarry et al., 2012), *P. nicotianae* may be less likely to rapidly overcome resistance genes with partial effects (Parlevliet, 2002; Poland et al., 2008). Even genes with known large effects on pathogen resistance are not necessarily easily overcome (Johnson, 1984;Michelmore et al., 2013).

One piece of evidence that could be used to argue against the partial effect model is that little evidence of additivity was observed for *Wz* for resistance in the current study. In other words, when averaged over environments, *Wz*/*Wz* homozygotes exhibited only slightly lower AUDPC values than *Wz*/– heterozygotes. Additional experiments are underway to further characterize the mechanism of resistance for the gene(s) present on the *Wz* chromosome

segment. Race specificity cannot be proven until isolates are identified that are able to overcome the type of resistance under consideration (Johnson, 1984). If *P. nicotianae* populations capable of overcoming *Wz* are ultimately identified, breeding strategies where *Wz* is deployed in combination with *Php* in genetic backgrounds contributing intermediate to high levels of polygenic resistance might be optimal. Such strategies might slow development of strains with alternative virulence profiles, and resistance-breaking isolates might have lower fitness and reduced aggressiveness (Vanderplank, 1982). For example, Sullivan et al. (2005a) found race 1 isolates of *P. nicotianae* to be less aggressive and to exhibit reduced fitness as compared with race 0 isolates. Forcing a pathogen to overcome multiple monogenic resistance mechanisms could incrementally reduce aggressiveness and fitness, outcomes that could be advantageous to long-term management of the black shank pathogen (Michelmore et al., 2013). For reducing loss over time, Sullivan et al. (2005b) suggested rotating varieties between those with partial resistance and those with a combination of partial resistance and race-specific immunity genes. DNA markers for black shank resistance (Johnson et al., 2002; Drake and Lewis, 2013) are useful for pyramiding multiple resistance genes when pathogen isolates are not available to permit discriminatory selection for individual resistance factors.

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TABLES AND FIGURES

Table 1.1. Tobacco entries evaluated in field soil-borne disease environments.

Entry	No. of entries	Reason for inclusion
K 326	1	recurrent parent with intermediate level of polygenic resistance
K 326 BC_5F_3 *Wz*/*Wz* NILs†	3	*Wz*/*Wz* NIL
K 326 BC_5F_3 *Wz*/– NIHs‡	3	*Wz*/– NIH
Cms K 326 BC_5F_3 *Wz*/– NIHs	3	*Wz*/– NIH
K 326 BC_5F_3 *Wz*/–*Php*/– NIHs	3	*Wz*/–*Php*/– NIH
K 326 BC_5F_3 –/– null segregant families	2	–/– null segregant
Beinhart 1000	1	extremely high level of polygenic resistance
K 346	1	very high level of polygenic resistance
KT 209LC	1	very high level of polygenic resistance combined with *Php* gene
KT 206LC	1	high level of polygenic resistance combined with *Php* gene
NC 196	1	intermediate level of polygenic resistance combined with *Php* gene; currently most popular flue-cured tobacco cultivar in U.S.
NC 71	1	intermediate level of polygenic resistance combined with *Php* gene
NC 2326	1	low level of resistance
NC 95	1	low level of polygenic resistance
NC 1071	1	low level of polygenic resistance combined with *Php* gene; race 1 indicator line

†NIL, nearly isogenic line.
‡NIH, nearly isogenic hybrid.

Table 1.2. Tobacco entries evaluated in field yield and quality experiments.

Entry	No. of entries	Reason for inclusion
K 326	1	recurrent parent; check cultivar for U.S. Regional Minimum Standards Program
K 326 BC_5F_3 *Wz*/*Wz* NILs†	3	*Wz*/*Wz* NIL
K 326 BC_5F_3 *Wz*/– NIHs‡	3	*Wz*/– NIH
Cms K 326 BC_5F_3 *Wz*/– NIHs	3	*Wz*/– NIH
K 326 BC_5F_3 *Wz*/–*Php*/– NIHs	3	*Wz*/–*Php*/– NIH
K 326 BC_5F_3 –/– null segregant families	2	–/– null segregant
K 394	1	flue-cured tobacco of intermediate yield ability; very high level of polygenic resistance
K 346	1	flue-cured tobacco of intermediate yield ability; very high level of polygenic resistance
NC 196	1	high-yielding flue-cured tobacco cultivar carrying *Php*; currently most popular U.S. flue-cured tobacco cultivar
NC 71	1	high-yielding flue-cured tobacco cultivar carrying *Php*
NC 2326	1	check cultivar for United States Regional Minimum Standards Program
NC 95	1	check cultivar for United States Regional Minimum Standards Program

†NIL, nearly isogenic line.
‡NIH, nearly isogenic hybrid.

Table 1.3. Analyses of variance for area under the disease progress curve for tobacco genetic materials evaluated in seven field disease environments.

	Mean squares						
	Kinston, NC	**Oxford, NC**	**Rocky Mount, NC**	**Rocky Mount, NC**	**Irwin County, GA**	**Pierce County, GA**	**Sumter County, SC**
Source	2012	2012	2012	2013	2013	2013	2013
Entry	8899,431***	2416,289***	13,501,655***	5714,927***	14,628,194***	355,062***	81,186***
Replication	1297,921***	87,457	3380,103*	314,472	348,417*	118,320	–

*Significant at $P < 0.05$.
***Significant at $P < 0.001$.

Table 1.4. Analysis of variance for tobacco genetic materials evaluated for yield and quality in six North Carolina environments.

	Mean squares					
	Yield	**Value ha^{-1}**	**Value 100 kg^{-1}**	**Grade Index**	**Total alkaloids**	**Reducing sugars**
Source	kg ha^{-1}	\$ ha^{-1}	\$ 100 kg^{-1}		%	%
Environment	10,995,823***	659,092,823***	283,037***	10,698***	30.21***	1080.9***
Replication (Environment)	1191,376***	30,107,421***	9672***	311***	0.65***	33.1***
Entry	2053,302***	43,295,811***	6374***	203***	0.84***	15.8***
Entry × Environment	196,371***	4658,664**	1887*	65*	0.12*	4.7

*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$

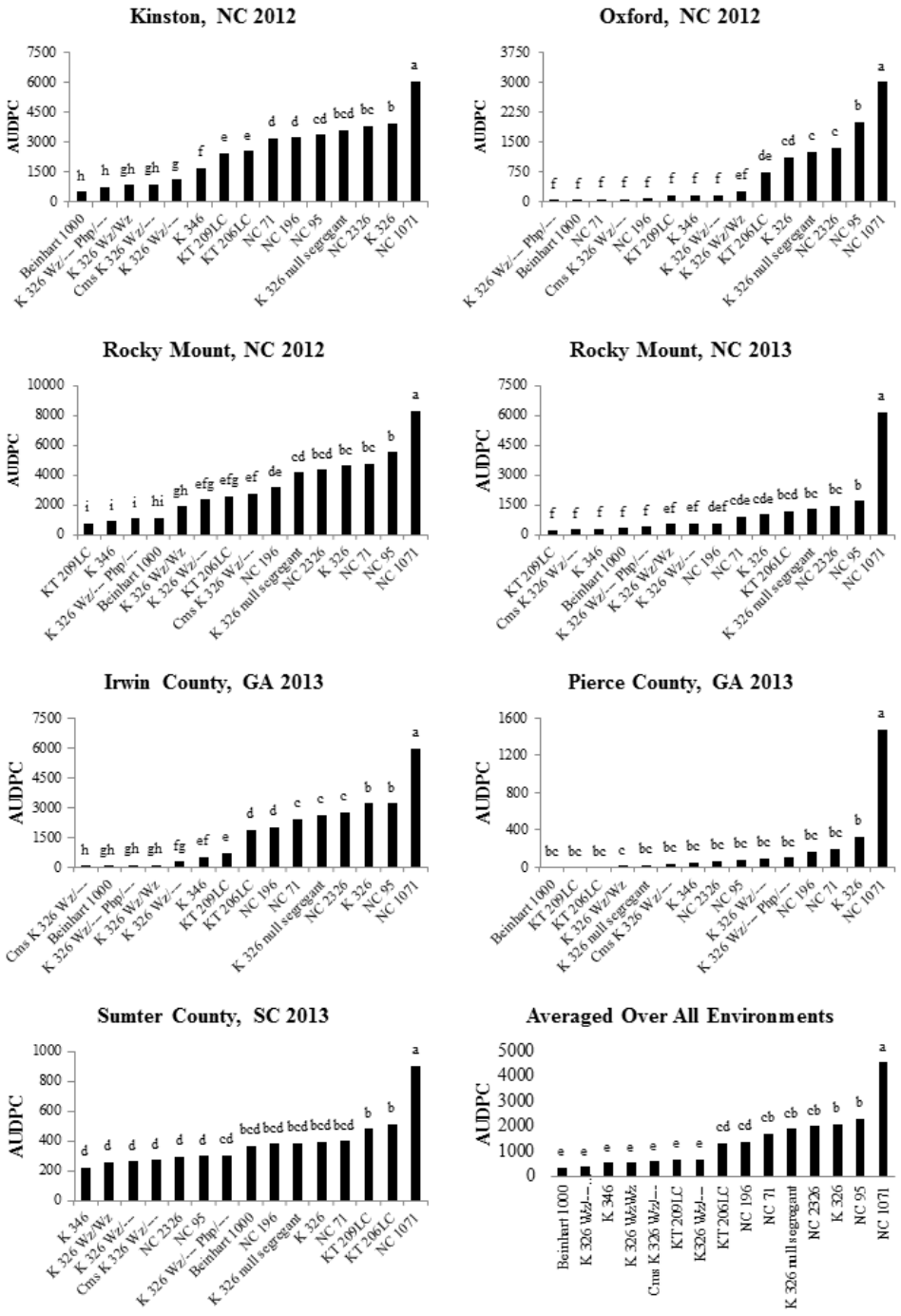

Figure 1.1. Means for area under disease progress curve (AUDPC) for various tobacco genotypic classes and tested control lines. Means are presented for each of seven disease environments individually, and entry means averaged over environments are also provided. Means with the same letter are not significantly different at the $P < 0.05$ level.

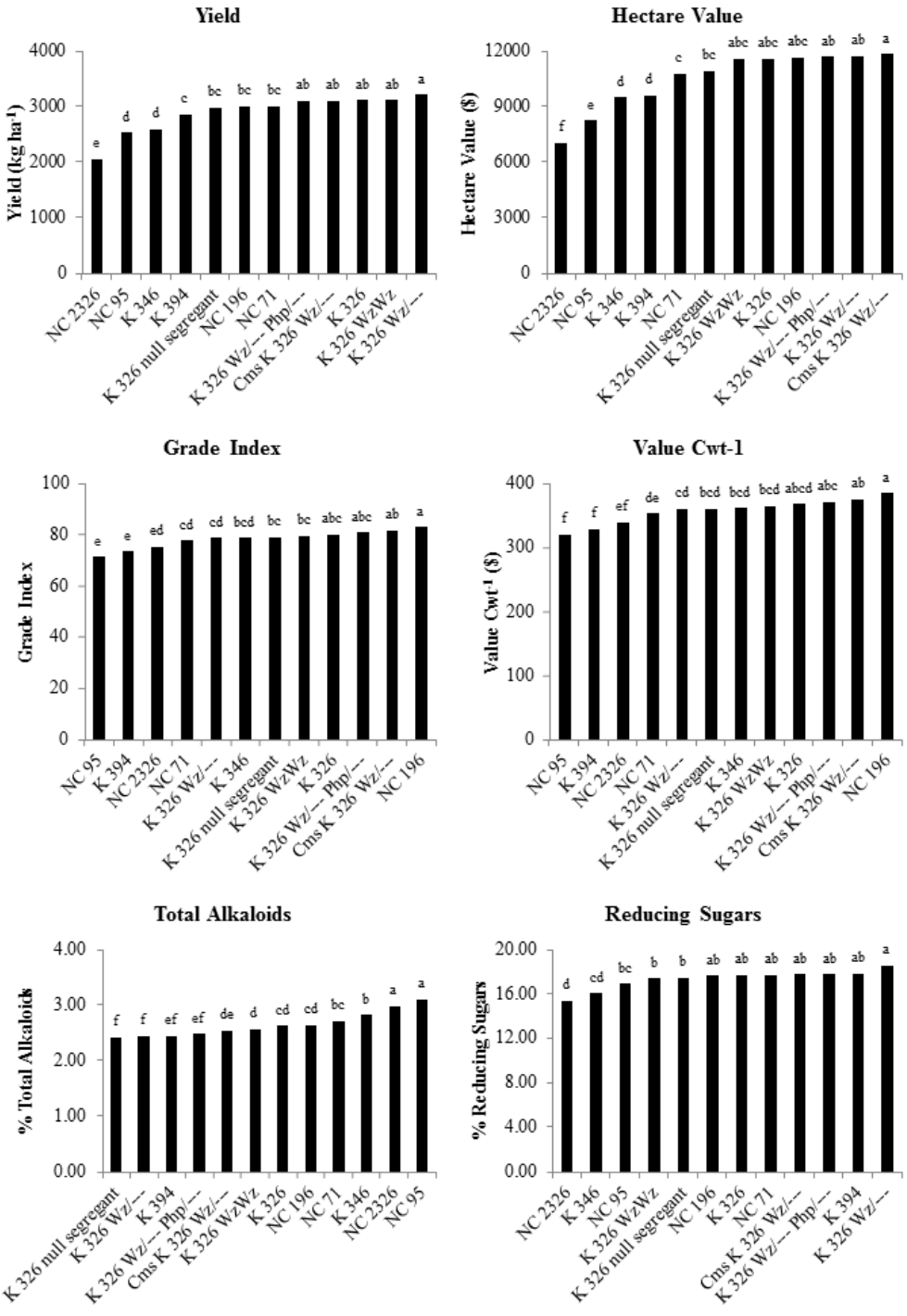

Figure 1.2. Means for yield and quality characteristics of tobacco for various genotypic classes and tested control lines. Means are averages over seven North Carolina environments. Means with the same letter are not significantly different at the $P < 0.05$ level.

Chapter 2: Characterization of *Phytophthora nicotianae* Resistance Conferred by the Introgressed *Nicotiana rustica* region, *Wz,* in Flue-Cured Tobacco

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ABSTRACT

Black shank, caused by *Phytophthora nicotianae,* is one of the most important diseases affecting tobacco worldwide. It is primarily managed through the use of host resistance that prevents or delays the onset of disease. Sources of complete resistance and partial resistance are available but no variety provides complete resistance to all known isolates. An additional source of resistance to *P. nicotianae,* designated as *Wz,* has been introgressed into *N. tabacum* from *N. rustica.* The *Wz* gene region has been found to confer high levels of resistance to *P. nicotianae*, but has not been characterized to determine if the resistance is expressed equally in root and stem tissues, if the resistance can be overcome by high inoculum densities of the pathogen, or the potential for the rapid selection of new isolates of the pathogen with increased ability to cause disease on varieties with this resistance. This study found *Wz*-mediated resistance to be most highly expressed in the roots, with only a slight observed reduction in stem-lesion size compared to susceptible controls in stem inoculation. No substantial relationships were observed between initial inoculum levels and disease development on *Wz* genotypes, a finding that is generally inconsistent with resistance being of the partial type. In root inoculations with isolates that had been adapted for one or five host generations on *Wz* plants, higher disease severities and percent root rot were observed on *Wz* genotypes inoculated with an isolate exposed to *Wz* for five host generations as compared to plants inoculated with an isolate exposed to *Wz* for only one host generation. These *Wz*-adapted isolates did not exhibit increased levels of aggressiveness on genotypes with alternative sources of partial resistance, suggesting that pathogen adaptation to *Wz* lines was specific. To reduce the potential for shifts in pathogen populations with specific virulence on *Wz,* genotypes, *Wz* resistance should be combined with other sources that confer

complete and partial resistance. Rotation of tobacco cultivars with varying mechanisms of black shank resistance is also recommended.

INTRODUCTION

Black shank, caused by the soilborne oomycete *Phytophthora nicotianae*, is one of the most important diseases affecting tobacco production in the U.S. and other tobacco producing areas of the world (Lucas 1975). Symptoms include wilting, chlorosis, necrosis of the roots and basal part of the stem, and eventually plant death. The disease is initiated in soils with above 20°C temperature, following periods of rainfall that allow soil saturation needed for the release of asexual, motile zoospores (Shew 1983). Zoospores are the primary infectious propagules of *P. nicotianae*, and can be produced every 72 h on infected roots under favorable environmental conditions (Lucas 1975; Shew and Lucas 1991; Gallup et al. 2006). The pathogen is therefore capable of producing high levels of secondary inoculum within a single growing season, a feature that increases its potential to adapt quickly to selection pressures such as host resistance. Pathogen populations and disease are managed through chemical applications, crop rotation, cultural practices, and deployment of resistant genotypes. Planting of resistant varieties is the most effective way to decrease black shank disease severity, however (Lucas 1975).

Historically, two types of host resistance have been used in the development of modern flue-cured tobacco cultivars. Quantitative resistance, derived from the cigar tobacco cultivar Florida 301 (Fla 301) and often referred to as 'Fla 301 resistance', is controlled by multiple genes and is partial and non-race specific (Smith and Clayton 1948; Chaplin 1966; Xiao et al. 2013). Florida 301 was derived from a cross between varieties Big Cuba and Little Cuba by Tisdale in the 1930s (Tisdale 1931). Varieties with Fla 301 resistance exhibit low to high levels of root resistance, but little or no stem and leaf resistance to *P. nicotianae* (Wernsman et al. 1974; Shew and Lucas 1991; Jones and Shew 1995). Some level of disease loss occurs with all varieties with

this type of resistance, and there is a strong tendency for it to be associated with lower yields and reduced leaf quality. Additional sources of partial resistance are therefore being investigated for incorporation into breeding programs.

Similar to Fla 301, cigar tobacco variety Beinhart 1000 (BH 1000) also exhibits a high level of resistance to all races of *P. nicotianae*. To the best of our knowledge, however, this cultivar has not been used in the parentage of any current flue-cured varieties, partly due to unfavorable leaf chemistry characteristics associated with resistance (Vontimitta and Lewis 2012a, b). BH 1000 originated from a selection of variety 'Quin Diaz,' and possesses a high level of root resistance with little to no expressed resistance in leaf or stem tissues (Heggestad and Lautz 1957; Chaplin 1966; Wills and Moore 1971; Tedford and Nielsen 1990; Nielsen 1992). Two major-effect quantitative trait loci (QTL), *Phn* 7.1 and *Phn* 15.1, were previously identified from BH 1000 that explained 43% of the observed variation in resistance (measured as end percent survival) in a BH 1000 × Hicks doubled haploid mapping population (Vontimitta and Lewis 2012a, b). Both *Phn* 7.1 and *Phn* 15.1 contribute to a reduced rate of lesion expansion and a subsequent delay in above-ground symptom development (McCorkle et al. 2012). The *Phn* 7.1 QTL is present in both Fla 301 and BH 1000, but *Phn* 15.1 only contributes to black shank resistance in BH 1000 (Xiao et al. 2013). This newly identified *Phn* 15.1 QTL found in BH 1000 provides another source of partial resistance that might be used to increase black shank resistance if it can be incorporated into tobacco varieties with acceptable yield levels and cured leaf quality (Vontimitta and Lewis 2012a, b).

Genes conferring complete resistance also have been used to manage black shank. Complete resistance to wild-type (race 0) *P. nicotianae* is conferred by single genes, *Php* and *Phl.*

The *Php* gene was transferred to *N. tabacum* from *N. plumbaginifolia* (Chaplin 1962), while the *Phl* gene was transferred to *N. tabacum* from *N. longiflora* (Valleau et al. 1960). Host resistance conferred by the *Php* gene is expressed in tobacco roots, stems, and leaves (Csinos 1999). In response to widespread deployment of the *Php* gene in flue-cured varieties desired by growers, there was a rapid race shift from race 0 to race 1 in production fields (Csinos 1994; Sullivan et al. 2005b; Gallup and Shew 2010). Race 1 is able to overcome both *Php* and *Phl* (Lucas 1975), and has become the dominant race where varietiess containing the *Php* gene have been planted.

A new source of black shank disease resistance introgressed from *N. rustica* and designated as *Wz* has been studied (Woodend and Mudzengerere 1992; Drake and Lewis 2013). *Wz* has been found to confer very high levels of resistance to both race 0 and race 1 isolates while having little negative impact on tobacco yield and/or quality (Drake et al. 2015). These characteristics make this source of resistance potentially very desirable for extending the level of black shank resistance in flue-cured tobacco. It is not currently known, however, whether *Wz* confers complete resistance or a high level of partial resistance. If *Wz* confers complete resistance, pathogen populations might be expected to rapidly adapt to its resistance mechanism. *Wz* resistance might be more durable if it confers a high level of partial resistance.

Multiple races of *P. nicotianae* have been reported and include races 0, 1, 2, and 3. Race 2 was reported in South Africa using KY 14 × L8, Burley 21 × L8, and Delcrest 202 as a set of differentials, but has not been widely accepted because no known resistance gene is present in Delcrest 202 (van Jaarsveld et al. 2002; Gallup and Shew 2010). Race 3, which overcomes *Phl* but not the *Php* gene, was first described in Connecticut by McIntyre and Taylor and later reported in North Carolina (McIntyre and Taylor 1976, 1978; Gallup and Shew 2010). Similar to disease

resistance controlled by single genes in other pathosystems, the black shank pathogen is able to quickly overcome complete resistance in field environments (Apple 1962; Apple 1967; Csinos 1994; Sullivan et al. 2005b; Gallup and Shew 2010).

While the use of host resistance can provide an efficient way to reduce economic losses due to plant pathogens, pathogen populations can quickly adapt to selection pressure applied by changes in management strategies. When complete resistance is continuously used in the same location, selection pressure is imposed to select for individuals in the pathogen population that are able to infect the host. This selection can results in pathogen race shifts, giving single gene resistance greatly reduced value in disease management (McDonald and Linde 2002). Complete resistance is rarely durable, and although partial resistance is generally considered durable, it can be difficult to utilize in breeding programs. Pathogen adaptation to partial resistance does occur, but usually occurs gradually, resulting in an erosion of resistance over time as opposed to the rapid breakdown of complete resistance characterized by boom and bust cycles (McDonald and Linde 2002).

Research on pathogen adaptation to partial resistance by root pathogens is generally lacking, with most studies having focused on foliar fungal pathogens. Previous research in pathosystems outside of tobacco black shank have reported an overall increase in pathogen aggressiveness characterized by increases in secondary inoculum production (Phillips and Blok 2007; Delmotte et al. 2014), lesion expansion, and infection efficiency (Leonard 1969; Villareal and Lannou 2000), decreases in latent period (Lehman and Shanner 1997), and erosion of partial resistance over time (Montarry et al. 2012). Studies on fungi, viruses, and nematodes support observations of increased pathogen aggressiveness in populations exposed to host varieties with

high levels of partial resistance when compared to populations exposed to susceptible hosts (Apple 1960; Leonard 1969; Cowger and Mundt 2002; Sullivan et al. 2005; Phillips and Blok 2007; Montarry et al. 2012). In the tobacco black shank pathosystem, pathogen adaptation to partial resistance derived from Fla 301 has been observed (Dukes and Apple 1961; Sullivan et al. 2005ab; McCorkle et al. 2015).

Because *Wz* resistance has not been characterized, and also because *P. nicotianae* has the genetic ability to quickly overcome complete resistance, several research objectives were pursued. The objectives of this study were to determine 1) if *Wz* resistance is expressed both in tobacco stem and root tissues, 2) if black shank resistance conferred by the *Wz* region can be overcome by increased inoculum densities, and 3) if *P. nicotianae* can rapidly adapt to *Wz*-mediated resistance. The overall goal was to gain insight on whether or not *Wz* provides an immunity effect versus a high level of partial resistance.

MATERIALS AND METHODS

Genetic Materials

The genetic materials used in the experiments described below were as follows. First, K 326 was selected as an elite flue-cured tobacco cultivar with a low-intermediate level of polygenic black shank resistance. This variety was used as the recurrent parent in the development of the backcross-derived *Wz* homozygous line, K 326 *Wz*/*Wz* (BC_5F_3 stage of backcrossing) and the K 326 *Wz*/-- F_1 hybrid (heterozygous for *Wz*-mediated resistance). Experimental materials also included Hicks (a variety highly susceptible to black shank), NC 1071 (a breeding line carrying the *Php* gene coupled with extremely low levels of any additional resistance), K 346 (a flue-cured

cultivar with a moderate to high level of partial resistance likely derived from Fla 301), BH 1000 (a cigar line with exceptionally high levels of partial resistance), and K 326 *Php*/*Php* (K 326 into which *Php* was backcrossed five times).

Stem Inoculations

Stems of six tobacco genotypes (K 326 *Wz*/*Wz*, K 326 *Wz*/--, K 326 *Php*/*Php*, K 326, Hicks, and NC 1071) were inoculated with either a race 0 or race 1 isolate of *P. nicotianae* in a controlled greenhouse environment to determine if *Wz* resistance is expressed in above-ground tissue. *Phytophthora nicotianae* isolates were grown in pure culture on a 5% V8 medium for 7-12 days at 28ºC. V8 medium was made by adding 20 g Bacto Agar (Fisher Scientific, Fair Lawn, NJ) to 800 ml of deionized water and 200 ml of clarified V8 juice base. Medium was autoclaved at 121ºC for 30 min. After cooling to 56 ºC, 18 ml of medium was poured into each 10 cm Petri dish after it cooled to 56ºC. Clarified V8 juice base was made by suspending 250 ml V8 juice (V8 Vegetable Juice, Campbell's Soup Company, Camden, NJ) with 2 g of $CaCO_3$ and 750 ml of deionized water. The suspension was autoclaved at 121 ºC for 10 min, filtered by suction through Celite 545 (Fisher Scientific, Fair Lawn, NJ) and then brought back to the original volume with deionized water. Sterilized toothpicks were placed on the surface of the *P. nicotianae* cultures and incubated in the dark for an additional 7 days. Sterilized toothpicks were prepared by cutting rounded wooden toothpicks (Home360 distributed by DZA Brands, LLC) in half and autoclaving them in 5% V8 broth for 30 min at 121°C three consecutive times prior to placing on the agar culture.

Plants were inoculated with infested toothpicks approximately six weeks after transplanting into 15 cm pots and when a minimum height of 30 cm was reached. Toothpicks

were inserted into the plant stems between the fourth and fifth node. Treatments were arranged in a split-plot design with race as the main-plot factor and host genotype as the sub-plot factor. Each treatment was replicated five times and the experiment was conducted three times. Lesion lengths were measured with a digital caliper each day for five days post-inoculation and area under lesion expansion curve (AULEC) was calculated for each plant. Analyses of variance for AULEC values were conducted using PROC MIXED (SAS 9.3). Race, genotype, and race by genotype interaction were considered as fixed effects, while run and replication within run were considered random effects. Comparisons of lesion expansion between genotypes were calculated with LSMEANS using a Tukey-Kramer adjustment (Kramer 1956).

Dosage Study

To determine if the high level of resistance observed in *Wz* genotypes can be overcome by high initial inoculum levels, the six genotypes used in the stem inoculation trial were inoculated in an inoculum dosage study. Cultures of race 0 and race 1 of *P. nicotianae* were grown under the same parameters as previously described. Once hyphae reached the edge of the Petri dish, sterilized oat grains were placed directly on top of cultures. Dishes were then sealed with Parafilm® and incubated at room temperature in the dark for approximately 15 days. Oat grains were first sterilized by combining 500 ml of crimped oats with 400 ml of deionized water and autoclaving at 121ºC for one hour daily for three consecutive days.

Each of the six tobacco genotypes were grown in 10 cm pots and inoculated with one of five dosage levels (1, 5, 10, 15, or 20 oat grains) of either a race 0 or race 1 isolate. Seeds were germinated on autoclaved Metro Mix 200 growing medium (Sun Gro Horticulture,Vancouver, Canada) under plastic domes at 25°C with 24 h light. Approximately two weeks after emergence,

seedlings were transplanted to individual pots containing a 2:1 peat:sand mixture. One week after transplanting, each plant was inoculated by placing the prescribed number of infested oat grains into the soil. Treatments were arranged in a split-plot design with the race/dose combination as the main-plot factor and genotype as the sub-plot factor. The experiment was conducted three times in a growth chamber in the NCSU Applied Tobacco Genetics and Chemistry Laboratory.

The growth chamber was maintained at a temperature of approximately 30°C with 24 h light, and uniform soil moisture was maintained via sub-irrigation. Sub-plots consisted of six plants, each in an individual 8 cm pot (LA 1801, Landmark Plastics, Inc.). Disease incidence was recorded each day for 45 d post-inoculation, and area under disease progress curve (AUDPC) was calculated for each sub-plot according to Madden et al. (2007). Analyses of variance for AUDPC values were performed using PROC MIXED of SAS 9.3 (SAS Institute, Cary, NC). Genotype, race, dose, and all interactions between the three classes were treated as fixed effects, and replication was treated as a random effect. Comparisons between treatments were made using LSMEANS with Tukey-Kramer adjustments for the p-values (Kramer 1956).

Isolate Adaptation

A race 0 and race 1isolate of *P. nicotianae* were selected for the adaptation study. The initial isolates were obtained from soil in a field with active black shank disease and race was confirmed based on inoculations of host differentials. Isolates were adapted on plants of K326 *Wz*/-- for five consecutive cycles of selection by inoculating K 326 *Wz*/-- plants and then and then isolating from the plant with the shortest incubation period to serve as inoculum for the next generation of selection. The most aggressive isolate from generation one (G1) and five (G5)

of selection were then used to inoculate five tobacco genotypes to characterize the nature of isolate adaptation to *Wz* and other sources of black shank resistance. The four isolates were grown in pure culture on a 5% V8 medium as previously described. Once hyphae had grown to the edge of a 10 cm Petri dish, sterile oat grains were poured on top of pure cultures of each isolate, sealed with Parafilm®, and incubated at 28ºC for 10-15 days in the dark. Oats were prepared and sterilized as previously described.

Five tobacco genotypes were sown on a 1:1 mixture of peat-lite and sand in 10 cm × 15 cm plastic cell packs in Phytotron growth chambers (North Carolina State University, Raleigh, NC). Genotypes included Hicks, K 346, BH 1000, K 326 *Wz*/-- and K 326 *Wz*/*Wz*. Seedlings were watered daily and fertilized once per week with 10-10-10 Miracle-GRO® (Scotts Miracle-Gro Company, Marysville, OH). Plants were grown at 30ºC/26ºC day/night temperature with fluorescent lighting at an intensity of 750 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 14 hrs per day. Six to seven week-old seedlings were transplanted to 10 cm plastic pots and grown for an additional four weeks at the same environmental conditions as above. Colonized oat grains were used to inoculate eight seedlings of each genotype four weeks after transplanting by placing one colonized oat grain into each of two separate 5-cm-deep holes 3 cm away from the stem. Plants were watered after inoculation to seal inoculum holes.

Treatments were organized in a randomized complete block design with each isolate × host genotype combination replicated eight times in each of two runs of the experiment. For race 0 and race 1 inoculations, each treatment block corresponded to a single growth chamber cart. Carts were moved daily to access all plants for watering and rating. A non-inoculated

susceptible Hicks plant was placed on each cart to ensure inoculum was not being transferred from one treatment to adjacent treatments.

Plants were rated at approximately the same time daily for the development of stem necrosis or significant wilting. The incubation period (time in days from inoculation to the development of above ground symptoms) was recorded for 4 weeks post inoculation. Not all plants developed symptoms within the 4 week test period, so incubation period in days was converted to disease severity values for comparison across varieties. The assigned severity values for each class were as follows: 1-6 days=10, 7-10 days=8, 11-16 days=6, 17-22=4, 23-28=2, and no symptoms at day 28=0. On day 28 of the experiment, plant roots were rinsed in water and percent root rot was visually rated by the same evaluator for all runs of the experiment. Disease severity and root rot means were calculated in SAS (Version 9.4, SAS Institute, Cary, NC), and an analysis of variance (ANOVA) table was generated using the PROC GLIMMIX command. Race 0 and race 1 experiments were analyzed separately.

RESULTS

Stem Inoculation Experiment

A significant run effect was observed for the stem inoculation experiment ($P < 0.0001$) due to variations in overall disease development across all treatments for each run. Significant differences were observed between host genotypes for AULEC with race 0 inoculations ($P < 0.0001$), with the susceptible variety Hicks having the highest AULEC, and lines with the *Php* source of single gene resistance (NC 1071 and K 326 *Php/Php)* exhibiting the lowest AULEC. In contrast, the K 326 *Wz/Wz* and K 326 *Wz/*-- genotypes developed substantial stem lesions

when inoculated, with either race. No significant differences in AULEC were observed between the K 326 *Wz*/*Wz* homozygote and the K 326 *Wz*/-- heterozygote, but both had AULEC values lower than Hicks with the race 0 isolate (Fig. 2.1). No significant differences were observed among genotypes for AULEC values after inoculations with race 1 (Figs. 2.1 and 2.2).

Dosage Study

The effects of genotype and genotype × pathogen race interaction were significant ($P < 0.0001$), but no genotype × race × dose interaction was observed ($P = 0.7046$). Hicks exhibited the highest AUDPC value with race 0 inoculations, with similar AUDPC values observed among all inoculum doses. AUDPC values were much lower on *Wz*-containing genotypes than on susceptible Hicks, but were similar AUDPC for both *Wz*-containing genotypes across all dosage levels (Fig. 2.3). In contrast, lines with *Php* single gene resistance (K 326 *Php*/*Php* and NC 1071) exhibited the lowest AUDPC values, with no disease development in these treatments. With race 1, Hicks and NC 1071 exhibited the greatest AUDPC values, while K 326 *Wz*/*Wz* and K 326 *Wz*/-- had the lowest. Similar to race 0 treatments, low amounts of disease were observed on *Wz* genotypes, although no differences were observed with the range of inoculum level tested (Fig. 2.3).

Isolate Adaptation

For the *P. nicotianae* adaptation experiment, no significant run × isolate-generation interaction was detected for disease severity for either race, and data were pooled over runs for analysis. No differences in disease severity trends were observed between race 0 and race 1 inoculations (Figs. 2.4 and 2.6). High levels of disease developed after race 0 and race 1 inoculation of the susceptible variety Hicks. Most above-ground black shank symptoms in Hicks

plants occurred within six days after inoculation. This contrasted with low observed disease severity values after inoculation with either isolate for K 346 and BH 1000, genotypes with high levels of partial resistance to *P. nicotianae* (Figs. 2.4 and 2.6). For both race treatments, no differences were observed in disease severity between G1 and G5 isolates for the genotypes Hicks, K 346, or BH 1000 ($P > 0.05$) (Figs. 2.4 and 2.6).

For the K 326 *Wz*/-- and K 326 *Wz*/*Wz* genotypes, disease severities were low after inoculations with the G1 isolates, but significantly higher ($P < 0.0001$) after inoculations with either the G5 isolates of race 0 and race 1 (Figs. 2.4 and 2.6). K 326 *Wz*/-- exhibited an increase in mean disease severity from 0.4 when inoculated with the G1 race 0 isolate, to a mean severity of 3.8 when inoculated with the G5 race 0 isolate. Similarly, for race 0 inoculations of K 326 *Wz*/*Wz*, mean disease severity increased from 0.5 for G1 inoculations to 3.6 for G5 inoculations (Fig. 2.4). This large increase in disease severity over generations of selection was also observed for K 326 *Wz*/-- and K 326 *Wz*/*Wz* genotypes for race 1 inoculations ($P < 0.0001$). Disease severity for K 326 *Wz*/-- inoculated with race 1 increased from 0.0 to 4.1 with isolates from G1 and G5, respectively. Similarly, disease severity for K 326 *Wz*/*Wz*, increased from 1.1 to 4.5 with isolates from G1 and G5, respectively.

No significant run × isolate-generation interaction was observed for percent root rot, so data from both runs was pooled for analysis. No differences in percent root rot were observed between race 0 and race 1 inoculations (Figs. 2.5 and 2.7). A high percentage of root rot was observed for susceptible variety Hicks, with all plants exhibiting 100% root necrosis, regardless of race or isolate generation. Overall, varieties K 346 and BH 1000 exhibited lower amounts of root rot as compared to Hicks for race 0 and race 1 inoculations (Figs. 2.5 and 2.7). For both

races, simple effect comparisons of host genotype × isolate-generation combinations revealed no differences in percent root rot between isolate generations one and five for Hicks, K 346, or BH 1000 ($P > 0.05$) (Figs. 2.5 and 2.7). In contrast, for K 326 *Wz*/-- and K 326 *Wz*/*Wz,* significantly greater ($P < 0.05$) amounts of root rot were observed on plants inoculated with the G5 isolates as compared to the G1 isolates for both races. For genotype K 326 *Wz*/-- inoculated with race 0, percent root rot increased from 21% with the G1 isolate to 49% with the G5 isolate. For the same genotype inoculated with race 1, percent root rot increased from 11% with the G1 isolate to 63% with the G5 isolate.

DISCUSSION

Both partial and complete sources of host resistance have been important tools in the management of black shank in tobacco since introduction of the disease in the U.S. around 1915. Cigar tobacco variety Fla 301 is the primary source of partial resistance for commercial tobacco varieties and has been heavily relied upon in disease management systems, particularly since widespread pathogen race shifts beginning in the 1990s (Tisdale 1931; Csinos 1994; Sullivan 2005ab Gallup and Shew 2010).

In this study, features of *Wz*-mediated resistance, a type of resistance that may be of value for extending the level of black shank resistance in future flue-cured tobacco cultivars, were investigated. First, we were interested in determining whether or not *Wz* was similar to the dominant gene, *Php,* gene in terms of expression of resistance in stem tissues. Complete resistance controlled by the *Php* gene is expressed in all host tissues, including the roots, stems, and leaves (Wills and Moore 1971; McIntyre 1978; van Jaarsveld et al. 2002; Gallup and Shew

2010), while complete resistance conferred by the *Phl* gene is only expressed fully in the host roots (Gallup and Shew 2010). For race 0 stem inoculations, we confirmed the high level of stem resistance conferred by *Php*, as evidenced by very low lesion expansion on *Php*-containing gentypes NC 1071 and K 326 *Php*/*Php*. Highly significant lesion expansion was observed on *Php* genotypes when inoculated with race 1, however. In contrast, *Wz* genotypes (K 326 *Wz*/-- and K 326 *Wz*/*Wz*) developed substantial stem lesions after inoculation with either race 0 or race 1. The lack of stem resistance in *Wz* genotypes makes *Wz* at least somewhat different than the gene-for-gene resistance type of resistance conferred by *Php*.

In partially resistant varieties of tobacco, black shank disease development is highly dependent upon initial inoculum levels (Ferrin and Mitchell 1986). If initial inoculum levels are high, *P. nicotianae* is capable of causing severe disease on varieties with high levels of partial resistance. High inoculum levels create more potential infections on plant roots, especially under favorable environmental conditions. A higher number of lesions can compensate for the reduced lesion size typical of resistant varieties compared to susceptible varieties, and result in severe root and crown rot. In contrast, high levels of disease can occur on susceptible varieties at any inoculum level due to higher pathogen infection efficiencies and greater rates of lesion expansion compared to more resistant plants (Ferrin and Mitchell 1986; Gallup et al. 2006). Varieties with complete resistance from the *Php* or *Phl* genes are immune to infections from race 0 of *P. nicotianae* at all inoculum levels, but race 1 can cause high levels of damage with low initial inoculum concentrations unless high levels of partial resistance are also incorporated into the variety. Partial resistance is typically controlled by multiple genes with additive effects, so

increasing levels of inoculum are typically associated with increased disease severity (Ferrin and Mitchell 1986).

To better understand the nature of resistance conferred by the *Wz* gene region, we compared tobacco genotypic responses when exposed to 1, 5, 10, 15, and 20 oat grains infested with either a race 0 or race 1 isolate of *P. nicotianae*. For race 0 inoculations, no plants of *Php*-containing genotypes exhibited disease symptoms. In contrast, for both race 0 and race 1 inoculations, small amounts of disease were observed on *Wz*-containing genotypes. These observations are inconsistent with *Wz* providing resistance via an immunity mechanism. On the other hand, no relationships were observed between initial inoculum levels and the amount of disease development on any of the genotypes tested. Also, no evidence of an additive effect associated with increasing zygosity of *Wz* was observed. Based solely upon this growth chamber inoculation data, it is therefore difficult to draw conclusions on the question of whether or not *Wz* provides a high level of partial resistance.

Black shank disease resistance conferred by the *Wz* region is likely controlled by a single gene, although the presence of multiple favorable genes on the alien introgressed *N. rustica* segment cannot be ruled out. Pathogen adaptation to *Wz* genotypes was investigated after five generations of pathogen selection for aggressiveness/virulence on *Wz* genotypes. In controlled inoculation experiments involving G1 and G5 isolates adapted on *Wz* genotypes, no differences in the level of disease or root rot were observed on Hicks, K 346, or BH 1000, which have resistance to black shank from other genetic sources. In contrast, large differences were observed in how *Wz* genotypes responded to the adapted isolates of both races. These results clearly support specific pathogen adaptation to *Wz*-containing genotypes after exposure to this

source of resistance for as few as five host generations. On *Wz* genotypes, G5 isolates were able to cause significantly greater amounts of disease and in a shorter period of time compared to G1 isolates. While it is often assumed that R-genes confer race specific, complete resistance, there are many exceptions (Niks et al. 2015). In many host pathosystems, it is common for R-genes to result in incomplete or partial resistance in the host (Lawrence et al. 1995; Franckowiak et al. 1997; Parniske et al. 1997; Andaya and Ronald 2003; Stewart et al. 2003; Smith and Hulbert 2005). For example, barley variety Trumpf shows incomplete expression of resistance to avirulent isolate Israel 202 of *P. hordei* conferred by one major R-gene, *Rph9.z* (Franckowiak et al. 1997).

Increased aggressiveness after exposure to host varieties with high levels of partial resistance has been reported in many pathosystems. For *P. nicotianae* on tobacco, increases in secondary inoculum production and aggressiveness have been reported after exposure to varieties with high levels of partial resistance (Apple 1960; Sullivan et al. 2005a). Observed increases in pathogen aggressiveness were gradual, however, resulting in a slow erosion of partial resistance derived from Fla 301 over time.

In this study, we have demonstrated that *Wz* is unlike the immunity gene, *Php*, in that its black shank resistance is not manifested in the stem. It is also unlike *Php* in the sense that it clearly does not provide an immunity effect as some disease developed on plants following inoculation with non-adapted isolates and has also been observed in field studies (Drake et al. 2015). At the current time, we do not have evidence to indicate that *Wz* confers a high level of resistance via a gene-for-gene mechanism. Nevertheless, the work presented here suggests the *Wz* region may select for specific variants in the pathogen population that render *Wz* resistance

less effective after relatively few generations of selection. Deploying *Wz* in combination with *Php* in genetic backgrounds with a medium to high level of polygenic resistance may prove to be the most effective means of developing black shank resistant tobacco cultivars. DNA markers for qualitative black shank resistance genes (Johnson et al. 2002; Drake and Lewis 2013), in addition to markers linked to QTL associated with black shank resistance (Stowe Chapter 3 2016; Vontimitta and Lewis 2012a; Xiao et al. 2013), make it possible to pyramid multiple resistance genes into a single cultivar. In a *Brassica napus-Leptosphaeria maculans* pathosystem, stacking a qualitative R-gene with quantitative polygenic resistance extended resistance provided by the *Rlm6* gene three years longer than when the gene was deployed in a susceptible background (Brun et al. 2010). Combining multiple sources of resistance could potentially slow the development of *P. nicotianae* isolates with alternative virulence profiles, as mutations in a number of different avirulence genes would need to accumulate before a population could cause high levels of disease in these plants (Michelmore et al. 2013; Vanderplank 1982).

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TABLES AND FIGURES

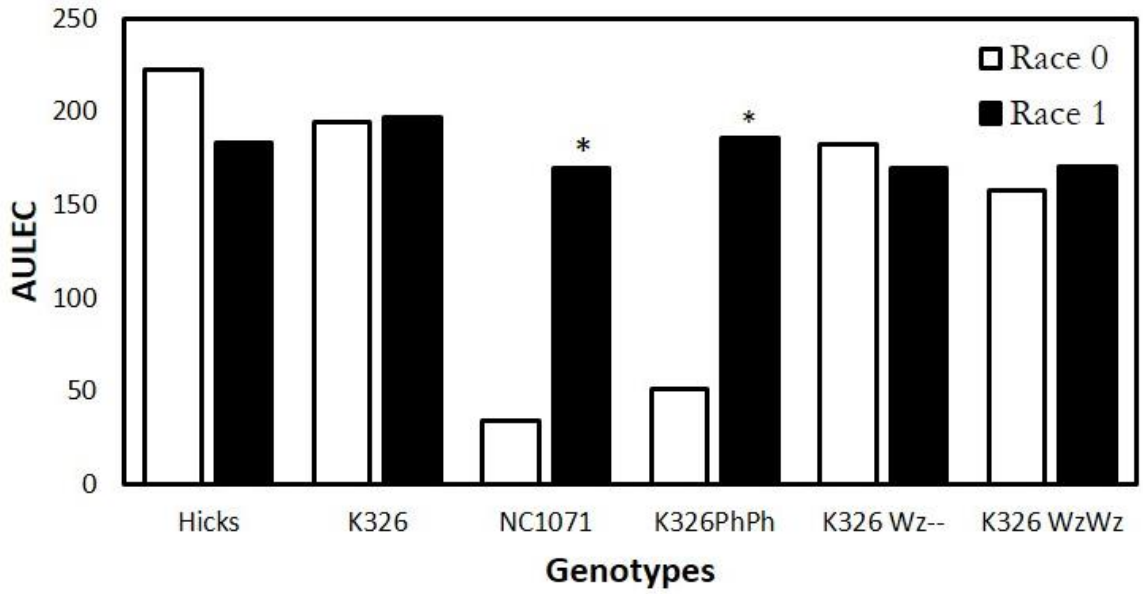

Figure 2.1. Black shank area under lesion expansion curve (AULEC) for race 0 and race 1 *Phytophthora nicotianae* stem inoculations.

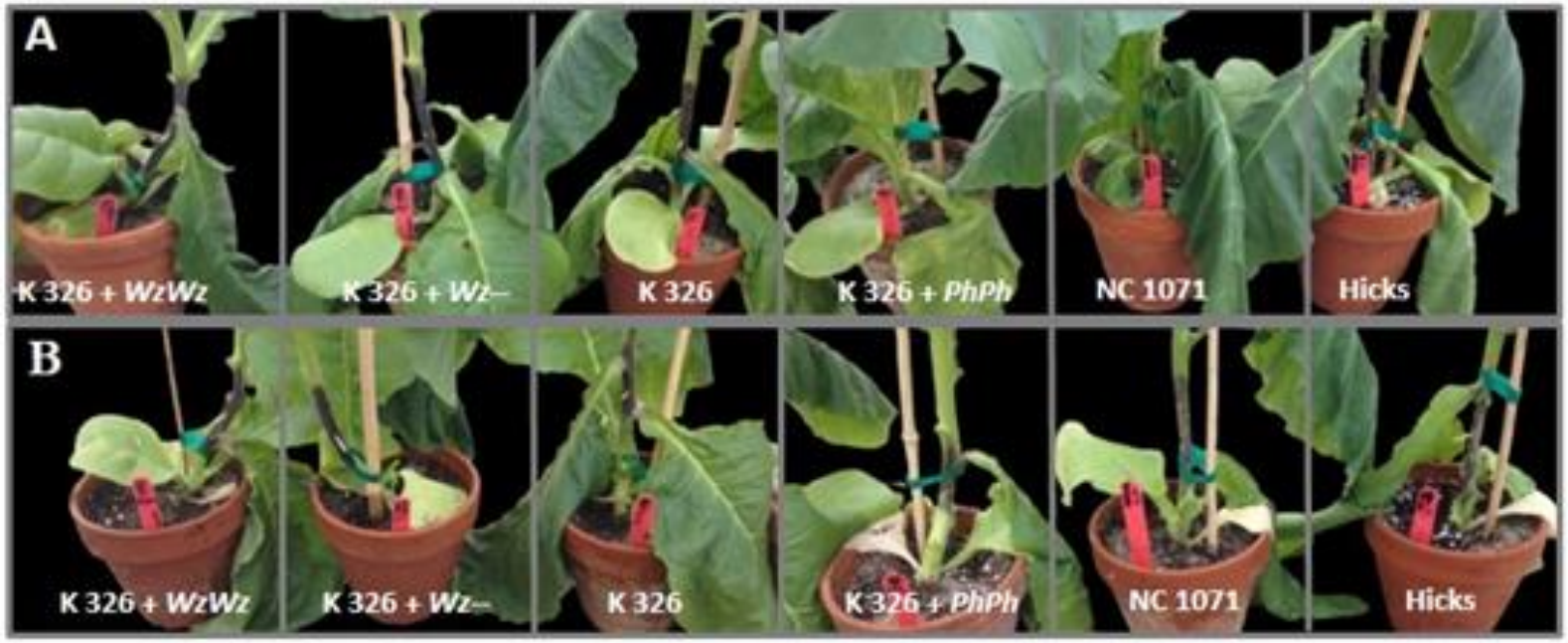

Figure 2.2. Stem inoculations using an infested toothpick inoculation method on tobacco varieties with race 0 (A) and race 1 (B) isolates of *Phytophthora nicotianae*.

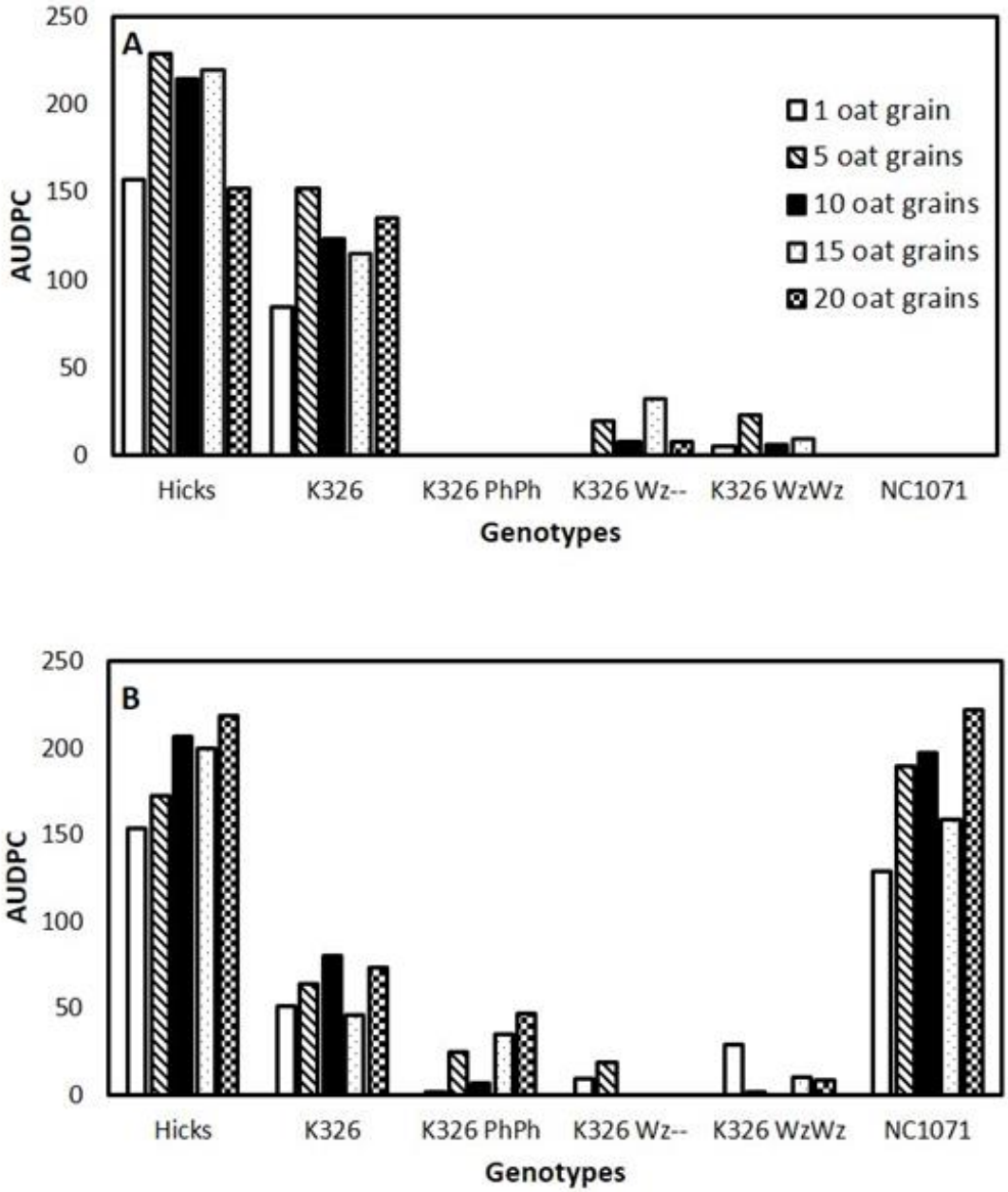

Figure 2.3. Black shank area under disease progress curve (AUDPC) for race 0 (A) and race 1 (B) inoculations with 5 levels of *Phytophthora nicotianae* inoculum.

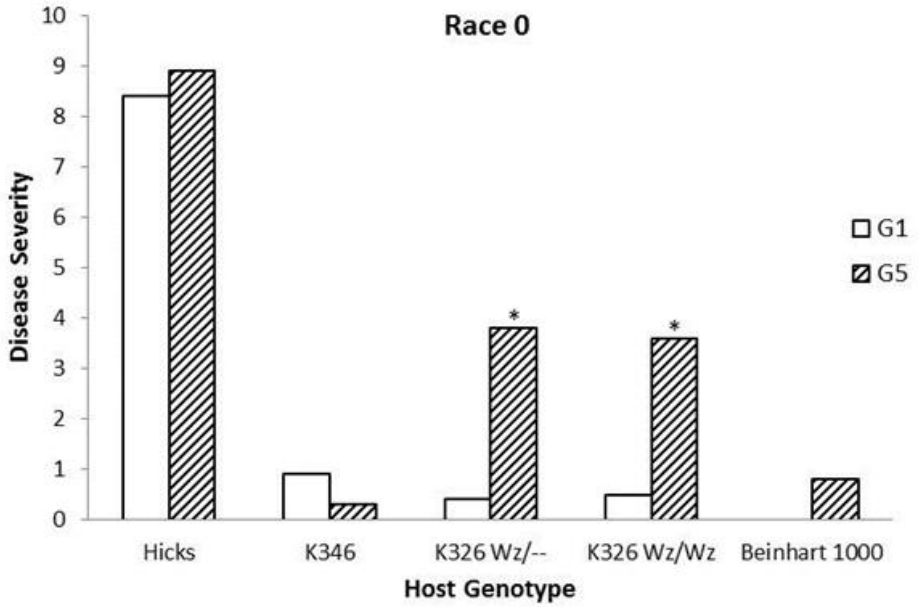

Figure 2.4. Mean disease severity (N=16) of tobacco varieties following inoculation with a race 0 *Phytophthora nicotianae* isolate exposed to host genotype K 326 Wz/-- for one generation (G1) and for five generations (G5). Disease severity values assigned by incubation period in days. Severity value of 10 for 0-6 days, 8 for 7-10, 6 for 11-16, 4 for 17-22, 2 for 23-28, and 0 for no above ground symptoms 28 days post inoculation. Significant differences between G1 and G5 inoculations for each variety indicated by "*".

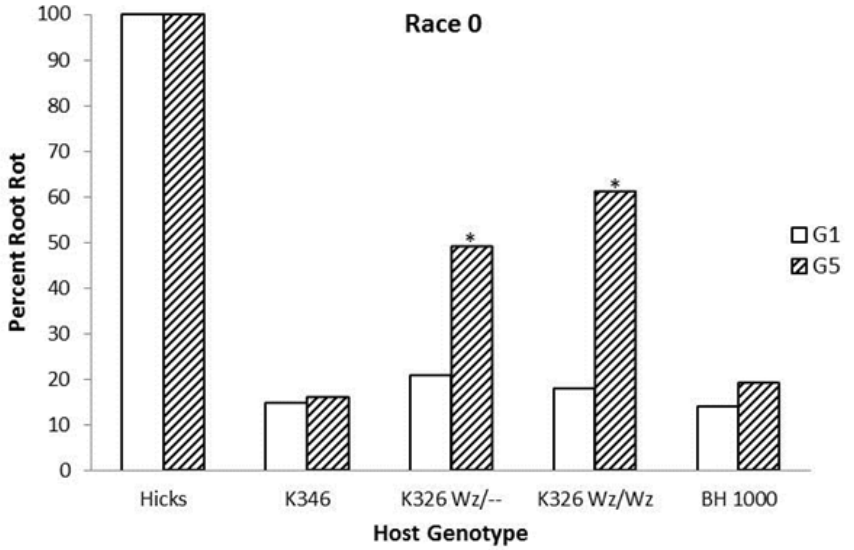

Figure 2.5. Mean percent root rot (N=16) of tobacco varieties following inoculation with a race 0 *Phytophthora nicotianae* isolate exposed to host genotype K 326 Wz/-- for one generation (G1) and for five generations (G5). Percent root rot was rated manually 28 days post inoculation on a scale of 0 to 100. Significant differences between G1 and G5 inoculations for each variety indicated by "*".

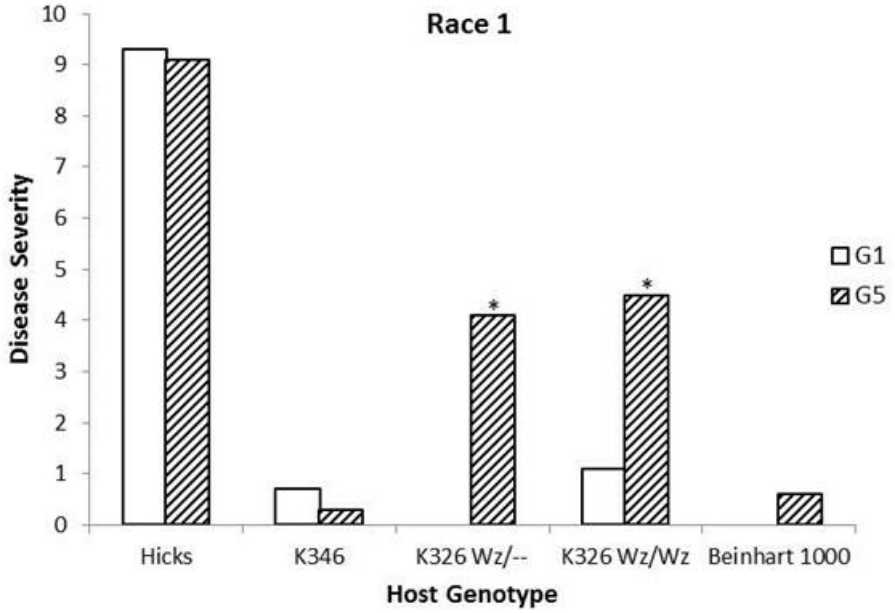

Figure 2.6. Mean disease severity (N=16) of tobacco varieties following inoculation with a race 1 *Phytophthora nicotianae* isolate exposed to host genotype K 326 Wz/-- for one generation (G1) and for five generations (G5). Disease severity values assigned by incubation period in days. Severity value of 10 for 0-6 days, 8 for 7-10, 6 for 11-16, 4 for 17-22, 2 for 23-28, and 0 for no above ground symptoms 28 days post inoculation. Significant differences between G1 and G5 inoculations for each variety indicated by "*".

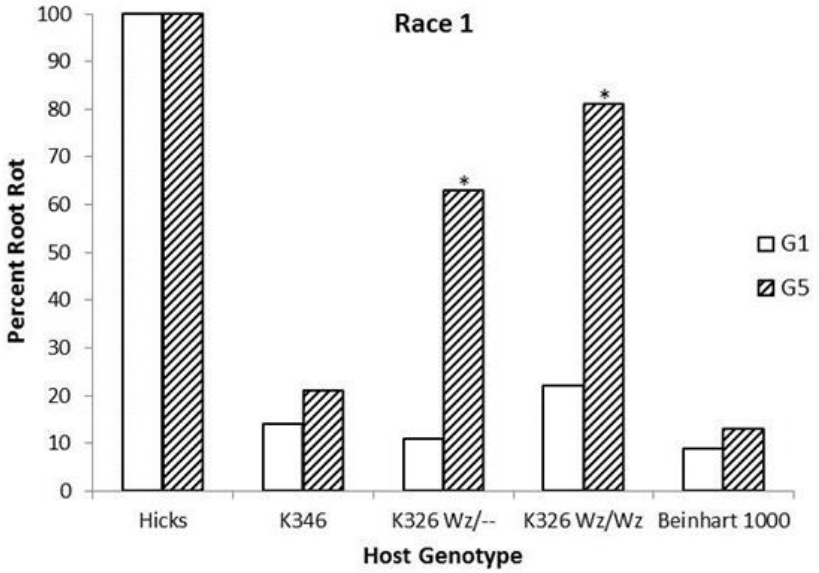

Figure 2.7. Mean percent root rot (N=16) of tobacco varieties following inoculation with a race 0 *Phytophthora nicotianae* isolate exposed to host genotype K 326 Wz/-- for one generation (G1) and for five generations (G5). Percent root rot was rated manually 28 days post inoculation on a scale of 0 to 100. Significant differences between G1 and G5 inoculations for each variety indicated by "*".

Chapter 3: Investigating the Genetics of Polygenic Resistance to Multiple Soilborne Pathogens in Tobacco

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ABSTRACT

Black shank and Granville wilt, caused by the soilborne pathogens *Phytophthora nicotianae* and *Ralstonia solanacearum*, respectively, are the most important diseases affecting tobacco production in the U.S.. A greater understanding of the genetic systems controlling resistance to these two diseases, as well as identification of DNA markers associated with genomic regions contributing to this resistance, could aid in the development of improved varieties. An evaluation of 50 historical tobacco lines for resistance to soilborne pathogens revealed a high positive correlation between resistance to black shank and resistance to Granville wilt, suggesting the possibility that some *N. tabacum* genomic regions confer resistance to both diseases. To gain further insight on this question, a QTL mapping study was initiated to investigate the genetics of resistance to black shank and Granville wilt in flue-cured tobacco cultivar, 'K 346,' which exhibits a very high level of resistance to both diseases. A recombinant inbred line (RIL) population consisting of 186 lines derived from a cross between K 346 and disease-susceptible tobacco accession 'TI1068' was generated and evaluated for resistance to both diseases in multiple field environments. The lines were also genotyped at 258 microsatellite marker loci. Four significant QTL were found to be associated with resistance to black shank (explaining 60.0% of the observed phenotypic variation), and three QTL were found to be associated with Granville wilt resistance (explaining 50.3% of the observed variation). The two QTL with the largest effect on black shank resistance (located on LG 6 and LG 7) were also found to be the QTL with the greatest effects on Granville wilt resistance. This finding may at least partially explain previously observed associations between resistance to black shank and bacterial wilt among current cultivars and within breeding populations. The LG7 QTL (*Phn7.1*) with the

largest effect on resistance to both diseases has also been found to be associated with black shank resistance in two previous mapping studies, thus further validating the effect of this genomic region on resistance to soilborne pathogens. Further investigation is needed to determine whether or not the observed relationship between favorable QTL affecting resistance to both diseases is due to the same genes (i.e. pleiotropy) or due to favorable coupling-phase linkages that were established due to prior breeding efforts.

INTRODUCTION

Black shank and Granville wilt (also known as bacterial wilt), caused by the soilborne pathogens *Phytophthora nicotianae* and *Ralstonia solanacearum*, respectively, are the two most economically important diseases affecting flue-cured tobacco production in the U.S.. These two diseases account for approximately 50% of total disease losses each year (North Carolina Cooperative Extension 2015). Strategies used to mitigate loss from both diseases include crop rotation, soil fumigation, and the use of genetic resistance. Genetic resistance is usually the most cost effective component of integrated disease management systems, but is only utilized by growers if it can be incorporated into cultivars that maintain commercially acceptable levels of yield and quality.

The black shank pathogen was first reported in the U.S. in 1915 in southern Georgia and became an economically important problem for tobacco production across the U.S. in the 1930's (Shew and Lucas 1991). Four races of *P. nicotianae* have been described, including race 0, race 1 (Apple 1962a, 1967), race 2 (van Jaarsveld, Wingfield, and Dernth 2002), and race 3 (McIntyre 1978; Gallup and Shew 2010). Only race 0 and race 1 are believed to be of major economic importance, however. A number of sources of genetic resistance to black shank have been investigated and used (Johnson et al. 2002; Vontimitta and Lewis 2012ab; Drake and Lewis 2013; Xiao et al. 2013; Drake et al. 2015), but no known source of genetic resistance provides complete resistance to all races of the black shank pathogen.

Tisdale (1931) developed the first U.S. black shank resistant cultivar, a cigar type designated as 'Florida 301.' The black shank resistance in this cultivar is of the classic polygenic type, where resistance is partial, controlled by multiple genes, and non-race specific (Smith and

Clayton 1948; Chaplin 1966; Xiao et al. 2013). Based on pedigree information, Florida 301 is believed to be the likely source of polygenic black shank resistance in modern flue-cured and burley tobacco cultivars (Xiao et al. 2013). While varying levels of polygenic black shank resistance are present in numerous flue-cured tobacco cultivars, this resistance type has been associated with lower yielding ability and reduced cured leaf quality (Chaplin and Ford 1958).

Monogenic resistance genes designated as *Php* and *Phl* were introgressed from *Nicotiana plumbaginifolia* (Apple 1962b; Chaplin 1962) and *N. longiflora* (Valleau, et al. 1960), respectively, and provide immunity to race 0 of *P. nicotianae*. Wide-scale planting of varieties carrying these genes has led to pathogen race shifts in U.S. soils and alternative races now predominate (Sullivan et al. 2005). A genomic region designated as *Wz* has also been introgressed from *N. rustica* and reported to provide very high levels of resistance to both race 0 and race 1 isolates, while having little measurable negative impact on yield and/or quality (Drake and Lewis 2013; Drake et al. 2015). Beinhart 1000, a cigar tobacco cultivar, also exhibits exceptionally high levels of polygenic black shank resistance (Vontimitta and Lewis 2012ab). Despite continued efforts to utilize this resistance in flue-cured cultivar development, however, we are currently unaware of any flue-cured variety with Beinhart 1000 in its pedigree.

The use of molecular markers over the last 10 years has provided an increased understanding of the genetic control of polygenic black shank resistance in tobacco. Previous work to identify quantitative trait loci (QTL) associated with black shank resistance in tobacco include a mapping study using a Florida 301 × Hicks recombinant inbred line (RIL) population, which led to the identification of three major QTL on linkage groups (LG) 7, 14, and 24, in addition to several QTL with smaller effects (Xiao et al. 2013). A second study described the

genetics of black shank resistance in a Beinhart 1000 × Hicks doubled haploid mapping population and identified two major QTL on LGs 7 and 15 (Vontimitta and Lewis 2012b). The QTL on LG 7, which exhibited the largest effect in the Beinhart 1000 population, was also found to have the largest effect in the Florida 301 population. The very significant QTL on LG15 found in the Beinhart 1000 population was not identified in the Florida 301 population.

Ralstonia solanacearum is a complex species that affects hundreds of plant species worldwide, including the economically-important *Solanaceous* crop plants potato, tomato, pepper, eggplant, and tobacco. The pathogen is extremely diverse and classification schemes divide the pathogen into races and biovars (Hayward 1991), or more recently, phylotypes and sequevars (Kim et al. 2016). Strains isolated from tobacco and tomato across the southeastern U.S. have been classified as race 1, biovar 1, and belong to phylotype II (Hong et al. 2012; Katawczik et al. 2016).

Granville wilt was first observed in 1880 in Granville County, North Carolina, and spread throughout North Carolina and other tobacco producing states over the following 30 years (Kelman 1953). Sources of genetic resistance to Granville wilt have not been as thoroughly investigated as sources of resistance to black shank, likely due to greater sensitivity of the causal agent of this disease to environmental conditions and diversity in pathogenic potential of different strains (Katawczik and Mila 2012). In the 1930's, a screen of over 1,000 *N. tabacum* accessions lead to the identification of two highly resistant genotypes: 79-X (a line derived from a cross between TI 79A and the Oriental variety, Xanthi) and TI 448A (a tobacco accession collected in Columbia) (Smith and Clayton 1948). Resistance from TI 448A has been described as polygenic in nature and is believed to be the only source of Granville wilt resistance

incorporated into U.S. flue-cured tobacco cultivars (Smith and Clayton 1948; Valleau 1952). The *Rps* and *Rxa* genes have been described and reportedly utilized by Japanese tobacco breeders (Matsuda and Ohashi 1973; Matsuda 1977), although the system used to identify and declare the presence of these genes is not clear. These genes provide only moderate resistance and are not believed to have been deployed in U.S. cultivars.

Limited studies have been conducted to identify regions associated with Granville wilt resistance in tobacco. Nishi et al. (2003) identified one major QTL originating from the Japanese burley breeding line 'W6' with a large effect on bacterial wilt resistance using a limited number of AFLP markers. Qian et al. (2013) identified two QTL with medium effects on LGs 6 and 16 in both a TI 448A × Enshu and a TI 448A × Yanyan 97 population. For both QTL in both populations, the resistant allele was contributed by the non-TI 448A parent. Other sources report Yanyan 97 to have TI 448A in its pedigree, however (Lan et al. 2014). Most recently, Lan et al. (2014) identified two major QTL on LGs 17 and 24, along with two minor QTL on LGs 6 and 12 to be associated with bacterial wilt resistance in a Yanyan 97 × Honghua Dajinyuan mapping population.

There is anecdotal evidence of a correlation between black shank and Granville wilt resistance in U.S. flue-cured tobacco. Many cultivars that exhibit high levels of resistance to black shank also tend to exhibit medium to high levels of resistance to Granville wilt, and vice versa (Fisher 2015). One possible explanation for this observation is that the same genomic regions that affect resistance to black shank also contribute to resistance to Granville wilt.

The flue-cured tobacco variety 'K 346' exhibits high levels of field resistance to both black shank and Granville wilt, and is an excellent representative of U.S. flue-cured tobacco

cultivars with high levels of disease resistance to soilborne pathogens. The high level of black shank resistance in K 346 is likely derived from the cigar cultivar Florida 301, while the high level of Granville wilt resistance is likely to have been incorporated from TI 448A, although this has never been verified for either disease. Despite the high level of resistance exhibited by K 346, this variety is grown on limited acreage due to lower yields and quality as compared to other popular cultivars. The first objective of this research was to use QTL mapping to determine the extent to which QTL significantly associated with black shank resistance in Florida 301 overlap with those identified to control resistance in modern tobacco cultivar, K 346. A second objective was to investigate the genetic correlation between resistance to black shank and bacterial wilt using a set of historical tobacco cultivars, as well as a recombinant inbred line (RIL) mapping population derived from a cross between K 346 and disease-susceptible tobacco accession 'TI 1068.' A third objective was to determine the degree of overlap between QTL controlling resistance to black shank and bacterial wilt in this mapping population.

MATERIALS AND METHODS

Genetic Materials and Disease Evaluations

A set of 50 tobacco genotypes was selected to study the phenotypic ranges and correlations for resistance to black shank and Granville wilt (Table 3.1). The set of lines included Florida 301 and TI448A, the reported sources of soil-borne disease in flue-cured tobacco. Eleven old-line cultivars were selected to represent flue-cured tobacco germplasm prior to the initiation of science-based plant breeding to improve soil-borne disease resistance. Also included were 36 flue-cured tobacco varieties of historical importance and that represented decades of tobacco improvement from the 1940's to the 1990's. No hybrids were included, and no varieties

carrying the *Php* gene were included, as the primary interest was to study polygenic resistance. Finally, Beinhart 1000 was also included in the set of 50 lines as a genotype that exhibits exceptionally high levels of polygenic resistance to black shank. These 50 lines were evaluated for black shank resistance at two North Carolina locations naturally infested with the black shank pathogen (Lower Coastal Plain Research Station, Kinston, NC; and Upper Coastal Plain Research Station, Rocky Mount, NC) in 2010 and 2011 (for a total of four environments). The set of 50 lines was also evaluated for Granville wilt incidence in a bacterial wilt nursery at the Upper Coastal Research Plain Research Station during 2014 and 2015; a bacterial wilt field at the Pee Dee Research and Education Center at Florence, SC, during 2015; and in a naturally infested bacterial wilt field on a private farm in Selma, NC, during 2015. The experimental design for each environment was a randomized complete block design with three replications. Experimental units consisted of single 14 m, 22-plant plots. Starting at approximately 40 d after transplanting, the number of plants killed by black shank or Granville wilt was recorded. This continued throughout the growing season at intervals of approximately 14 d. At the end of the growing season, area under the disease progress curve (AUDPC) was calculated for each plot using the trapezoidal method of Madden et al. (2007).

A population consisting of 180 $F_{4:5}$ recombinant inbred lines (RILs) was generated from a cross between K 346 and the black shank- and bacterial wilt-susceptible germplasm accession TI 1068. The RIL population and parental lines were evaluated for black shank incidence at the Lower Coastal Plain Research Station and Upper Coastal Plain Research Station during 2014 and 2015 (for a total of four environments). The set of materials was also evaluated for Granville wilt incidence in a bacterial wilt nursery at the Upper Coastal Plain Research Station in 2014 and

2015, and in one naturally infested field on a private farm near Selma, North Carolina, in 2015. The experimental design for each environment was a 10 × 18 alpha-lattice with three replications. The two parents were also randomized within each block for a total of 20 entries per block. Experimental units consisted of single 7 m long, 12-plant plots. Starting at approximately 40 d after transplanting, the number of plants killed by black shank or Granville wilt was recorded. This continued throughout the growing season at intervals of approximately 14 d. At the end of the growing season, area under the disease progress curve (AUDPC) was calculated for each plot.

Statistical Analysis

An analysis of variance (ANOVA) for AUDPC was performed on the 50 historical lines using PROC MIXED of SAS 9.4 (SAS Institute, Inc. 2013) for each disease separately. Genotype was treated as a fixed effect, while environment and replication were treated as random effects. Best linear unbiased estimates (BLUEs) were obtained for each line for each disease. Correlation coefficients between BLUEs for black shank and Granville wilt incidence were calculated using PROC CORR of SAS 9.4.

AUDPC data for the RIL population were subjected to a square root transformation to reduce heterogeneity of error variances. Transformed data were used to obtain a best linear unbiased prediction (BLUP) for each RIL across all locations/years for each disease using ASReml version 3 (Gilmour et al. 2009). A mixed linear model was fit with random effects for RIL, environment, RIL × environment, replication within environment, and block within replication/environment. Variance components estimated from this model were used to

calculate heritabilities on an entry mean basis per a univariate mixed model analysis described by Holland et al. (2003). Subsequent QTL analyses were carried out using the BLUPs.

Genotyping

Leaf tissue samples were collected from 10 plants per each $F_{4:5}$ family and combined into single tubes. Leaf tissues samples from single plants were collected for parental lines, K 346 and TI1068. Genomic DNA was isolated using a modified cetyltrimethylammonium bromide procedure (Afandor et al. 1993; Johnson et al. 1995) and quantified using a Hoefer fluorometer (Hoefer Scientific Instruments, Holliston, MA).

The two parents of the RIL population were initially genotyped using a total of 825 previously described primer pairs selected to amplify well-spaced microsatellite markers across each *N. tabacum* linkage group (Bindler et al., 2011). PCR reactions were performed in 10 μL volumes containing approximately 1—15 ng template DNA, 6 μL of True Allele PCR Premix (Life Technologies, Carlsbad, CA), and 10 μM of each primer. Reaction conditions consisted of 45 PCR cycles at an annealing temperature of 55°C according to Bindler et al. (2007). Forward primers were labeled with FAM, HEX, or AT550 for fragment analysis on an Applied Biosystems 310xL Genetic Analyzer (Life Technologies, Carlsbad, CA). Samples containing 1 μL of PCR product for each marker, 1 μL of Gene Scan 400 HD ROX dye standard (Life Technologies, Carlsbad, CA) and 9 μL Hi-Di formamide (Life Technologies, Carlsbad, CA) were denatured for 5 min at 95°C. Fragment analysis was performed as a multi-loading assay analyzing simultaneously up to three markers labeled with different dyes. Alleles were scored using the GeneMapper 5.0 software package (Life Technologies, Carlsbad, CA). Markers identified to be polymorphic between the parental lines were used to genotype the entire RIL population.

Map Construction and Quantitative Trait Loci Identification

A linkage map was created with R/qtl (Broman et al. 2003). Heterozygous markers were treated as missing data. Markers with genotypes on less than 130 individuals were excluded from the analysis, as well as lines with genotypes for less than 50 markers. Markers were organized into roughly 24 linkage groups based on a minimum logarithm of odds (LOD) score of 4.5. Marker orders and genetic distances were calculated with the Kosambi mapping function (Kosambi 1943) using ripple and a genotyping error probability of 0.0001. A final ripple was preformed to ensure the most likely marker order had been selected.

Following linkage map construction, QTL mapping was performed using phenotypic BLUPs for both black shank and Granville wilt resistance using Haley-Knott regression (Haley and Knott 1992) in R/qtl (Broman et al. 2003). QTL were selected using a forward/backward step-wise model selection approach with the function stepwiseqtl (Broman and Wu 2015). All pairwise interactions were considered, but no higher-order interactions were allowed. QTL were identified using a penalized LOD score criterion with three penalties (main effects penalty, heavy penalty on interaction, and light penalty on interaction) (Manichaikul et al. 2009). Values for penalties were calculated from 1000 permutation results with scantwo. After the model was finalized, the effect of each QTL was estimated using fitqtl.

RESULTS

Analyses of Historical Lines

The set of materials outlined in Table 3.1 was evaluated for black shank resistance in a total of four environments and for Granville wilt resistance in a total of three environments. Analyses of variance for black shank across all environments revealed highly significant

differences ($P < 0.0001$) among the 50 entries. AUDPC BLUEs for black shank ranged from 2.5 to 54.9, with a mean of 24.6 (Fig. 3.1). Analyses of variance for Granville wilt AUDPC values across all environments also revealed highly significant differences ($P < 0.0001$) among the 50 entries. AUDPC BLUEs for Granville wilt ranged from 5.9 to 44.7, with a mean of 22.6 (Fig. 3.1). Black shank and Granville wilt resistance (as measured by AUDPC) for the 50 historical lines was highly correlated, with a Pearson correlation coefficient ($r_{Pearson}$) = 0.85 ($P < 0.0001$).

Phenotypic Analyses of RIL Population

The set of 180 RILs and their parents (K 346 and TI1068) were evaluated for black shank resistance in a total of four environments and for Granville wilt resistance in a total of three environments. Analyses of variance with a mixed model that corrected for environmental effects on transformed AUDPC values revealed substantial variability in both black shank and Granville wilt resistance among the RILs. BLUPs ranged from 1.6 to 7.1, with a mean of 5.1, for black shank square root transformed AUDPC (Fig. 3.2a); and from 1.7 to 5.7, with a mean of 4.3 for Granville wilt square root transformed AUDPC (Fig. 3.2b). Entry mean heritability was calculated to be H = 0.95 (Standard Error = 0.01) and H = 0.65 (Standard Error = 0.05) for black shank and Granville wilt, respectively. A high genetic correlation between the two traits was observed, with $r_g = 0.89$ ($P < 0.0001$).

Genetic Linkage Map

Of the 825 microsatellite primer pairs tested, 258 (31.2%) were found to produce distinguishable, polymorphic alleles between K 346 and TI1068. A total of 252 markers were used for linkage map construction. The 252 markers were assigned to 24 linkage groups spanning a total length of 2020 cM (Fig. 3.3). The number of markers on each linkage group

ranged from 9 to 13 with an average of 10.5 markers per linkage group and an average spacing of 8.8 cM per marker. Marker order in this population was largely congruent with the high density map published by Bindler et al. (2011) (Supplemental Fig. 3.S1). Exceptions included the reordering of some markers within a linkage group, smaller pairwise differences, and a reduced overall map length.

QTL Identification in RIL Population

Using Haley-Knott regression, the stepwise model selection approach selected a final model for black shank AUDPC that included four QTL located on LGs 6, 7, 14, and 23, with no significant QTL × QTL interaction effects (Table 3.2 and Fig. 3.4). The favorable allele at the QTL on LGs 6, 7, and 23 was contributed by K 346, while the favorable allele at the QTL on LG 14 was contributed by TI 1068. The final model for Granville wilt AUDPC included three QTL located on LGs 6, 7, and 19, also with no significant QTL × QTL interaction effects (Table 3.2 and Fig. 3.4). The favorable allele was contributed by K 346 for all three QTL. A QTL on LG 14, with the favorable allele coming from TI 1068, was just below the threshold for addition into the Granville wilt model. QTL for both black shank and Granville wilt co-localized on regions on LGs 6 and 7. The QTL on LG 7 explained 38.0% and 32.1% of the phenotypic variation for black shank and Granville wilt AUDPC, respectively. The QTL on LG 6 explained 6.1% and 8.1% of the phenotypic variation for black shank and Granville wilt AUDPC, respectively. Additional QTL affecting resistance to both diseases explained less than 5% of the total phenotypic variation. The final QTL model for black shank AUDPC explained 60.0% of the observed phenotypic variation, while the final QTL model for Granville wilt AUDPC explained 50.3% of the observed phenotypic variation.

DISCUSSION

A high observed correlation ($r_{Pearson}$ = 0.85) between black shank and Granville wilt resistance in the set of 50 lines of historical importance provided initial support for the hypothesis that at least some tobacco genomic regions controlling resistance to black shank also contribute to Granville wilt resistance. Because genome-wide genotypes were not available for the set of 50 lines, however, some of the observed correlation could possibly be due to population structure or kinship. Nevertheless, the results provided initial credibility to the anecdotal evidence of genetic associations between black shank and Granville wilt resistance.

If pleiotropic effects were large contributing factors to the observed strong genetic correlations described above, one would expect the two reported sources of resistance in K 346 (Florida 301 for black shank resistance; and TI 448A for Granville wilt resistance) to exhibit some level of resistance to both diseases. As expected, Florida 301 exhibited very high field resistance to black shank and a moderate level of resistance to Granville wilt. In our experiments, however, TI 448 exhibited only low to moderate levels of resistance to Granville wilt, along with very low levels of resistance to black shank. Reasons for these observations may include (1) the *Ralstonia* populations are different today than they were in the 1940's when TI 448A was classified as resistant, (2) TI 448A isn't the source of Granville wilt resistance in K 346, (3) TI 448A accession in the current *Nicotiana* germplasm collection is not correct, or (4) the high level of Granville wilt resistance in varieties such as K 346 is due to one or a few TI 448A-derived QTL coupled with a series of additional QTL derived from alternative source material.

To more thoroughly investigate the high level of genetic resistance to both black shank and Granville wilt in flue-cured tobacco cultivar K 346, a mapping population derived from hybridization between K 346 and susceptible germplasm accession TI 1068 was studied. The mapping study identified four QTL on LGs 6, 7, 14, and 23 that affected black shank resistance and three QTL on LGs 6, 7, and 19 affecting resistance to Granville wilt. The QTL on LGs 6 and 7 affecting resistance to both diseases co-localized to the same genomic regions. The QTL identified on LG 7 had the greatest effect and controlled the largest percentage of phenotypic variation for both black shank (38.0%) and Granville wilt resistance (32.1%). This QTL was also found to have the largest effect and to control the largest percentage of variation for black shank resistance in a Florida 301 × Hicks RIL population (16.9%) (Xiao et al. 2013) and a Beinhart 1000 × Hicks doubled haploid population (25.4%) (Vontimitta and Lewis 2012b). The number of shared markers between the three studies was limited, making comparison of the location of the associated region challenging. In both the K 346- and Beinhart 1000-derived populations, however, the interval for the QTL was positioned over marker locus PT61472. In the Beinhart 1000- and Florida 301-derived populations, the interval was positioned over marker locus PT30174, suggesting a role for the same region in each population. The identification of this QTL in a third population provides further validation for the position and importance of allelic variation in this region in black shank resistance in *N. tabacum*. Given the confirmed importance of the QTL on LG 7 for black shank resistance in combination with newfound importance it has on Granville wilt resistance, fine mapping this interval for resistance to both soil-borne diseases would be valuable for more precise identification of regions associated with resistance. This would aid in the transfer of soil-borne disease resistance to other cultivars and help gain

insight on the question of linkage versus pleiotropy as contributing reasons to the correlation between the two traits.

The QTL on LG 6 had an intermediate effect for both diseases, explaining 6.1% and 8.1% of the phenotypic variation in black shank and Granville wilt, respectively. A QTL on LG 6 was also found to have an intermediate effect on Granville wilt resistance (5.4%) in a Yanyan 97 (which has TI 448A in its pedigree) × Honghua Dajinyuan $F_{2:3}$ mapping population (Lan et al. 2014), and to have an intermediate effect on black shank resistance (7.1%) in a Florida 301 × Hicks RIL mapping population (Xiao et al. 2013). The fact that few markers were shared between these studies again makes comparison difficult, but the confidence interval for this QTL was positioned over marker TM10089 in both the Yanyan 97 and Florida 301 populations, strengthening the evidence for the importance of this region for both black shank and Granville wilt resistance.

Surprisingly, a QTL on LG 24 was identified in both the Florida 301 and Beinhart 1000 black shank mapping studies, as well as in the Yanyan 97 Granville wilt mapping study, but not in the current K 346 mapping study. Limited overlapping markers make it difficult to determine if the same region on LG 24 was identified in all three studies, but this seems likely. K 346 is representative of modern flue-cured tobacco cultivars possessing high levels of polygenic resistance to multiple soilborne pathogens. Because this QTL was not identified in the K 346 mapping population, it suggests that this region may not have been incorporated in modern cultivars. High density genotyping of the 50 historical lines in the genomic regions of interest would help confirm this speculation. If modern varieties do not carry an incorporated Florida 301 region at this genomic location, it could be utilized as a novel source of resistance to both

black shank and Granville wilt. Markers associated with this region could be useful in transferring this resistance to desired genetic backgrounds. Given the low to moderate effect associated with this QTL in the other populations, it would be most useful in backgrounds that already possess the QTL on LG 6 and 7.

The finding that the two largest QTL affecting resistance to black shank in the K 346 × TI1068 population overlapped almost perfectly with the two QTL with greatest effects against Granville wilt in the same population is interesting. Further study is necessary to determine whether or not this relationship is due to the same genes affecting resistance to both diseases (i.e. pleiotropy), or due the result of favorable coupling phase linkages that were produced via selective breeding. The concept of multiple disease resistance (MDR) was first presented by Orton (1902) when a cowpea cultivar was described to be resistant to both *Neocosrnospora vasinfecta* (fungal wilt) and *Heterodera radicicola* (root-knot nematode). Since that time, many breeding programs have successfully developed varieties with MDR including those of economically-important species such as wheat, tomato, cucumbers, cabbages, and legumes (Ausemus 1943; USDA 1953; Barnes 1961; Williams et al. 1968; Nene 1988). Lines exhibiting MDR could contain many genes acting independently to confer resistance, or single genes conferring resistance to multiple pathogens. Single genomic regions and single genes conferring resistance to multiple pathogens have recently been identified. A number of qualitative MDR genes have been reported, including the *Mi-1* gene of tomato which confers resistance to both the root-knot nematode and potato aphid (Rossi et al. 1998), and the *RPS1* and *RPS4 Arabidopsis* genes which work together to confer resistance to both fungal and bacterial pathogens (*Colletotrichum higginsanum*, *Pseudomonas syringae*, and *R. solanacearum*) (Narusaka et al. 2009).

Quantitative resistance loci associated with MDR have also been reported in crops such as rice, where overlapping QTL affecting resistance to rice blast, sheath blight, and bacterial blight were detected on the same chromosome segment (Wisser et al. 2005). In addition, multiple genomic regions were found to be associated with gray leaf spot, northern leaf blight, and southern leaf blight in *Zea mays* (Zwonitzer et al. 2010; Wisser et al. 2011). The marker-trait associations identified here, coupled with an expected high-quality *N. tabacum* physical sequence that is expected to be published soon, should be useful for further studies to determine whether or not the overlap of Granville wilt and black shank resistance QTL on *N. tabacum* linkage groups 6 and 7 is due to genes with pleiotropic effects or due to favorable coupling phase genetic linkages that were generated via selective breeding.

The data reported here describes a polygenic system contributing to the high levels of field resistance to Granville wilt in flue-cured tobacco cultivar K 346. Bacterial wilt resistance in the *Solanaceae* has been most thoroughly investigated in tomato, where resistance appears to be controlled in a manner similar to tobacco. In tomato, bacterial wilt resistance is largely quantitative in nature, with many QTL mapped, and no major resistance genes being identified (Young and Danesh 1994; Thoquet et al. 1996ab; Wang et al. 2000; Wang et al. 2013). Both strain-specific and broad-spectrum QTL have been identified. A major broad spectrum QTL on tomato chromosome 6 was identified that is effective against both phylotype I and II strains, and the local genomic region is a hot spot for disease resistance genes (Carmeille et al 2006; Wang et al. 2013). Resistance genes in this region include *Mi* for root-knot nematode and potato aphid resistance, *Cf2* and *Cf5* for *Caldosporium fulvum* (tomato leaf mold) resistance, *Ol-1* for *Oidium lycopersicon* (powdery mildew) resistance, *Ty-1* for *Tomato yellow leaf curl virus* resistance, and *Am* for

Alfalfa mosaic virus resistance (reviewed by Kim et al. 2016). Studies involving grafting susceptible scions onto resistant rootstocks have demonstrated the key role of roots in host plant resistance, and grafting can be an effective strategy for managing disease in tomato production in North Carolina (Rivard et al. 2012).

Development of tobacco cultivars with high levels of resistance to root diseases and soilborne pathogens, while still maintaining yield and quality, is a primary objective of tobacco breeding programs. Results from the current study provide a greater understanding of the genetic control of black shank and Granville wilt resistance in the tobacco cultivar K 346, as well as a greater understanding of resistance to major soilborne pathogens available in tobacco germplasm, as a whole. The identification of regions associated with resistance, and molecular markers associated with those regions, will hopefully aid in the development of new tobacco varieties which have high levels of soil-borne disease resistance coupled with acceptable yield and quality. This information should contribute to future efforts to identify genes contributing to high levels of partial resistance to soilborne pathogens in *N. tabacum*.

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TABLES AND FIGURES

Table 3.1. Set of 50 historical tobacco cultivars evaluated for soil-borne disease resistance and their black shank and Granville wilt area under disease progress curve (AUDPC) values when evaluated in disease nurseries

Entry	Genotype	Pedigree	Black Shank AUDPC	Granville Wilt AUDPC
1	Florida 301 (PI 552629)	Source	6.70	16.44
2	Beinhart 1000	Source	2.45	29.89
3	TI 448A (TC 594)	Source	37.96	25.19
4	Warne (Farmer Variety) PI 552335	Farmer Variety	47.34	37.24
5	Virginia Bright Leaf (Farmer Variety) PI 552385	Farmer Variety	46.15	40.44
6	Gold Dollar (Farmer Variety) PI 552310	Farmer Variety	46.90	40.74
7	Hicks Broadleaf (Farmer Variety) PI 552397	Farmer Variety	42.65	34.97
8	Jamaica (Farmer Variety) PI 552315	Farmer Variety	46.56	34.02
9	White Stem Orinoco (Farmer Variety) PI 552337	Farmer Variety	44.28	37.92
10	Yellow Special (1943) PI 552340	Harrison Special/Lizzard Tail	54.88	44.70
11	400 (1942) PI 552341	A farmer selection likely from Harrison Special/Silk Leaf	51.70	38.22
12	Vesta 30 (1940's) PI 552769	Parentage is a BS resistant strain, Warne, and Yellow Special	21.90	19.20
13	Vesta 64 (1940's) PI 552772	[FLA 301/White Stem//White Stem/3/White Stem]XYellow Special	25.20	23.16
14	Oxford 1 (1942) PI 552320	FLA 301/Virginia Bright Leaf//Virginia Bright Leaf/3/Virginia Bright Leaf	19.52	27.20
15	Oxford 2 (1942) PI 552321	FLA 301/Virginia Bright Leaf//Virginia Bright Leaf/3/Virginia Bright Leaf	10.69	15.44

16	Oxford 3 (1942) PI 552322	FLA 301/White Stem//White Stem/3/White Stem	20.01	25.95
17	Yellow Special A (1943) PI 552378	Yellow Special/Yellow Mammoth	52.47	37.96

Table 3.1. Continued

18	Virginia Gold (1947) PI 552334	Yellow Special/Cash	43.81	34.79
19	Ox. 1-181 (1948) PI 552401	FLA 301/Virginia Bright Leaf//Virginia Bright Leaf/3/Virginia Bright Leaf	20.15	20.95
20	Golden Wilt (1949) PI 552393	Virginia Bright Leaf/TI 448A	36.96	23.26
21	White Gold (1949) PI 552400	Huggins Golden Yellow/402	42.30	33.67
22	Dixie Bright 101 (1949) PI 552383	[TI 448A/400//Oxford 1] X [FLA 301/400//400]	21.57	16.23
23	Dixie Bright 102 (1949) PI 552384	[TI 448A/400//Oxford 1] X [FLA 301/400//400]	13.16	14.75
24	Golden Cure (1950) PI 552390	Ducane/TI 706//Ducane/3/Ducane	45.88	32.14
25	Vesta 5 (1952) PI 552396	??	28.74	30.23
26	Coker 139 (1954) PI 552389	Coker Golden Cure/Oxford 1-181//Coker Golden Wilt/Dixie Bright 101	18.00	14.01
27	Dixie Bright 244 (1955) PI 551300	Dixie Bright 101//Dixie Bright 102/Bottom Special	14.51	12.33
28	Coker 187 (1956) PI 552391	Golden Wilt/Oxford 1-181	14.98	12.47
29	Coker 187-Hicks (1957) PI 552392	Coker 187/Hicks	24.82	18.76
30	SC 58 (1959) PI 552344	Yellow Special X [FLA 301/Warne] X [400/TI 448A]	19.85	25.97
31	McNair 10 (1960) PI 552422	McNair 12/Ox 1-181	21.09	34.31
32	Coker 319 (1962) PI 552426	Coker 139/Hicks	19.78	17.03
33	McNair 20 (1962) PI 552429	White Gold/224G//4*Hicks (224G = Burley line with *N. longiflora* BS resistance)	13.93	18.16
34	McNair 30 (1962) PI 552430	White Gold/224G//4*Hicks (224G = Burley line with *N. longiflora* BS resistance)	34.71	29.36

35	Coker 298 (1964) PI 552445	Coker 139/Coker 156	10.20	10.23
36	NC 2326 (1964) PI 552453	Hicks/9102//Hicks/3/Hicks/4/Hicks	20.65	25.87

Table 3.1. Continued

37	Virginia 115 (1964) PI 552458	Hicks/Coker 139	15.25	17.48
38	Coker 258 (1966) PI 552461	Coker 319/NC 95	14.52	14.72
39	Speight G-28 (1969) PI 551318	Coker 139/Oxford 1-181//NC 95	12.37	12.28
40	McNair 135 (1970) PI 551304	McNair 30/Speight G-10	16.10	7.67
41	McNair 944 (1973) PI 552494	Speight G-10/McNair 30	11.84	12.95
42	NC 82 (1979) PI 551311	6129/Coker 319	7.42	9.19
43	K 326 (1982) Gold Leaf Seed Co.	McNair 225//McNair 30/NC95	8.98	16.95
44	K 394 (1984) PI 552525	Speight G-28/McNair 944	14.12	20.57
45	K149 (1990) PI 549109	[Speight G-28/Coker 354]X[CB-139XF-105]X[Speight G-28/Coker 354]XMcNair 399	7.46	5.89
46	K 346 (1990) Gold Leaf Seed Co.	McNair 926/80241	27.22	13.37
47	NC55 (1996) Gold Leaf Seed Co.	K 326/DH 1220//K 326/Coker 371-Gold	22.92	18.67
48	Oxford 207 (1996) Gold Leaf Seed Co.	Coker 319/K 399	11.02	7.85
49	NC606 (1999) Raynor Seeds	NC 729/NC 82	7.68	8.00
50	NC810 (2001) Cross Creek Seeds	Oxford 2101/NC 729	12.42	9.25

Table 3.2. Summary of QTL selected in a K 346 x TI 1068 recombinant inbred line mapping population using Haley-Knott regression with a forward/backward stepwise selection approach. QTL associated with *Phytophthora nicotianae* (black shank) and *Ralstonia solanacearum* (Granville wilt) were identified.

Trait	Linkage Group	Left Marker	Right Marker	Position (cM)	Effect (srAUDPC)	LOD	Percent phenotypic variance explained
Black Shank Resistance	7	PT61472	PT52970	6.6	-0.83	25.4	38.0
Black Shank Resistance	6	PT61581	PT50885	71.0	-0.42	5.4	6.1
Black Shank Resistance	23	PT54270b	PT50062	0.0	-0.28	4.0	4.0
Black Shank Resistance	14	PT53339	PT53687	60.8	0.29	4.4	4.4
Granville wilt Resistance	7	PT61472	PT52970	6.6	-0.45	19.1	32.1
Granville wilt Resistance	6	PT61581	PT50885	69.0	-0.28	6.0	8.4
Granville wilt Resistance	19	PT60099	PT53353	87.7	-0.18	4.0	5.5

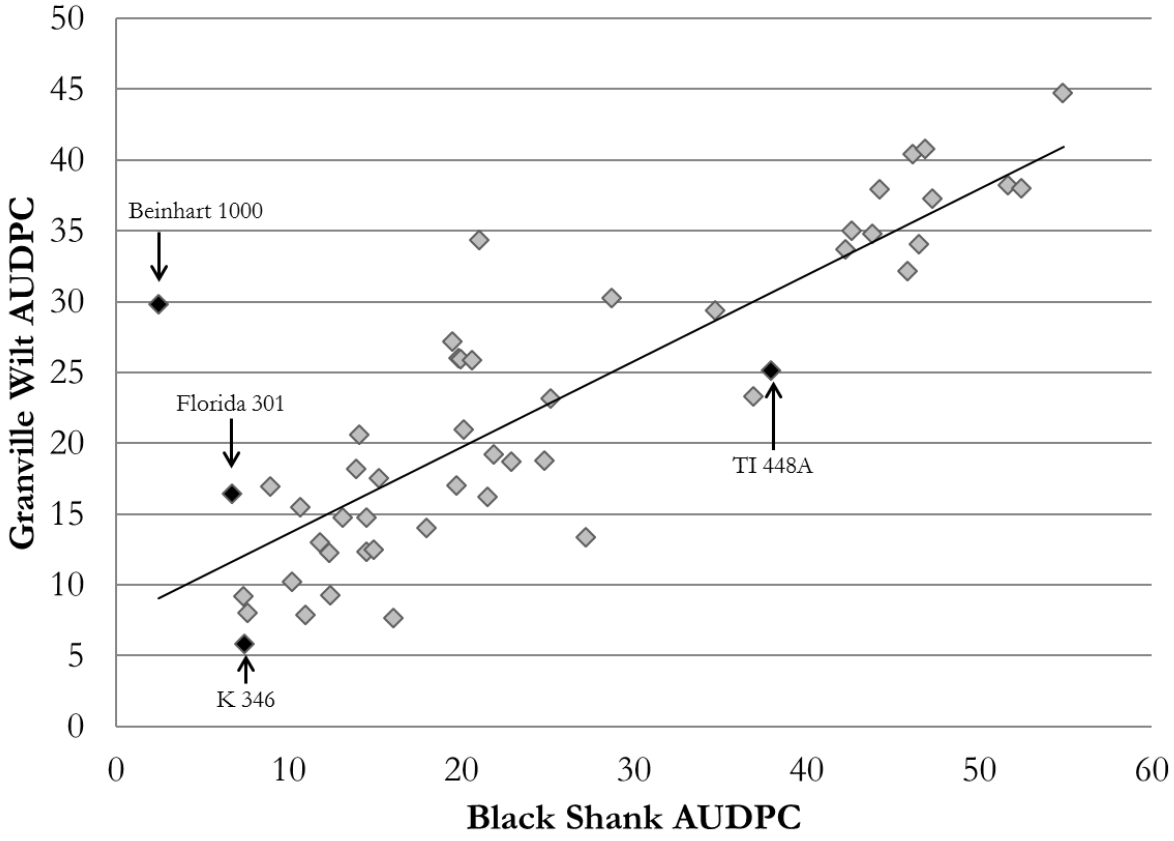


Figure 3.1. Black shank area under disease progress curve (AUDPC) versus Granville wilt AUDPC for 50 historically important tobacco lines.

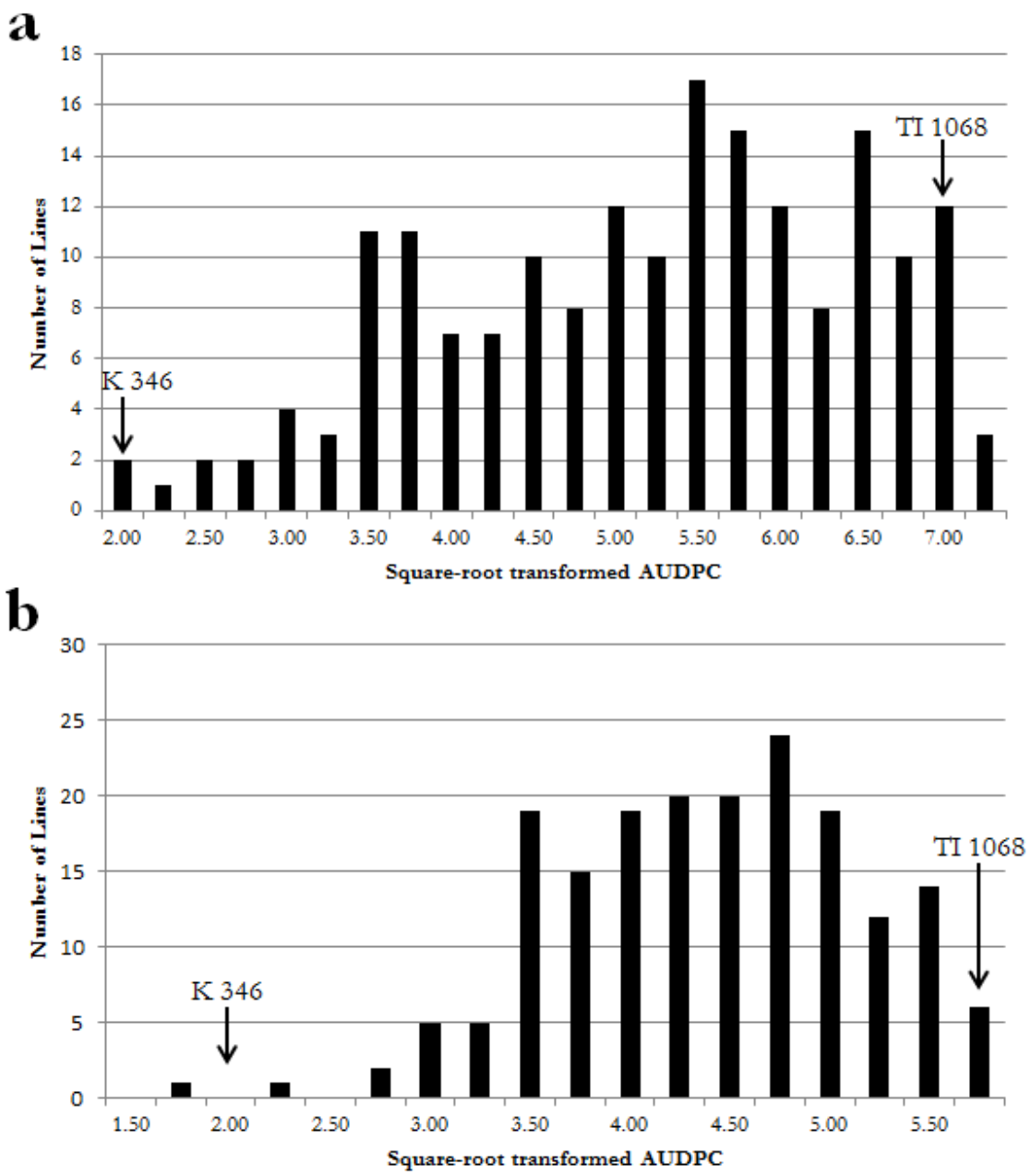
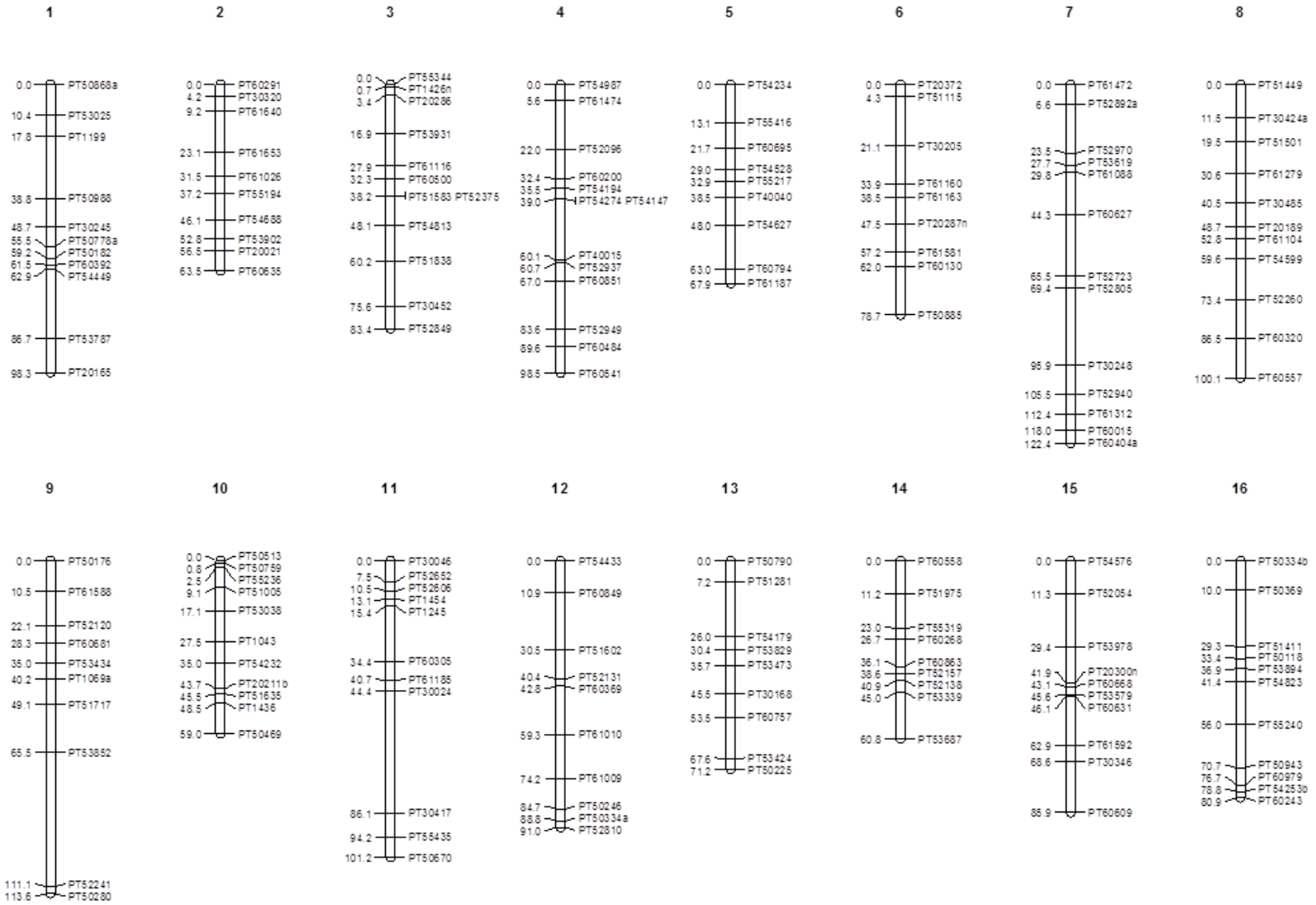
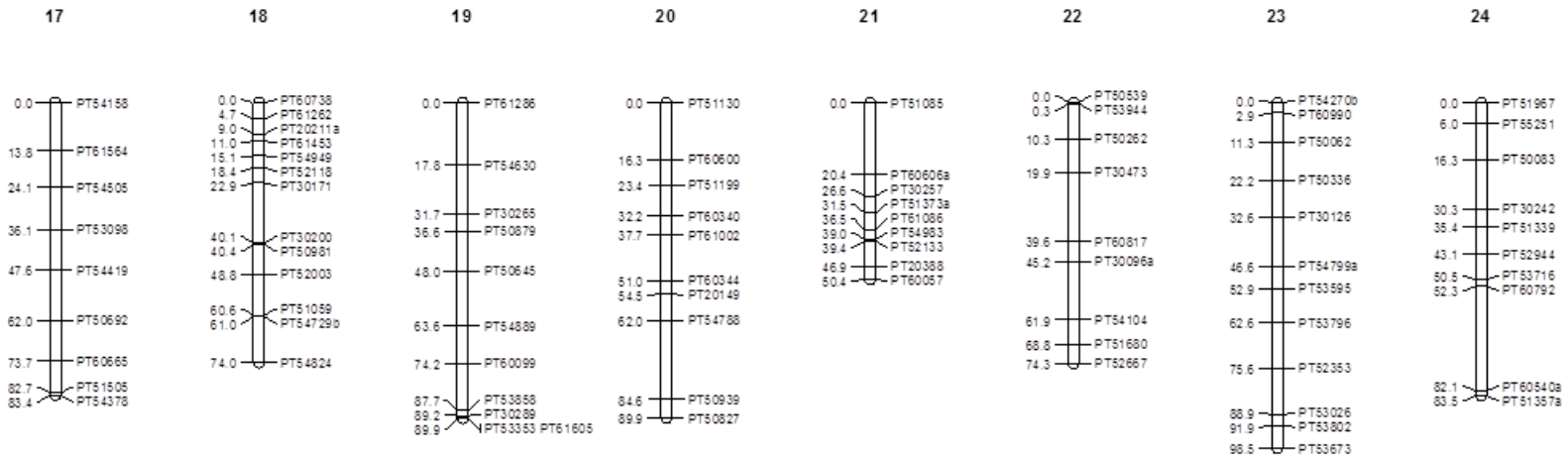


Figure 3.2. Distribution of square-root transformed area under disease progress curve (AUDPC) values for K 346 mapping population for (a) black shank and (b) Granville wilt.

Figure 3.3. Linkage map for 252 SSR markers generated using 177 recombinant inbred lines derived from the cross K 346 x TI 1068. Numbers on the left side are centiMorgans counted from the top of the chromosome.


1
0.0 PT50868a
10.4 PT53025
17.8 PT1199
38.8 PT50988
48.7 PT30245
55.5 PT50778a
59.2 PT50182
61.5 PT60392
62.9 PT54449
86.7 PT53787
98.3 PT20165
2
0.0 PT60291
4.2 PT30320
9.2 PT61640
23.1 PT61653
31.5 PT61026
37.2 PT55194
46.1 PT54688
52.8 PT53902
56.5 PT20021
63.5 PT60635
3
0.0 PT55344
0.7 PT1426n
3.4 PT20286
16.9 PT53931
27.9 PT61116
32.3 PT60500
38.2 PT51583 PT52375
48.1 PT54813
60.2 PT51838
75.6 PT30452
83.4 PT52849
4
0.0 PT54987
5.6 PT61474
22.0 PT52096
32.4 PT60200
35.5 PT54194
39.0 PT54274 PT54147
60.1 PT40015
60.7 PT52937
67.0 PT60851
83.6 PT52949
89.6 PT60484
98.5 PT60541
5
0.0 PT54234
13.1 PT55416
21.7 PT60695
29.0 PT54528
32.9 PT55217
38.5 PT40040
48.0 PT54627
63.0 PT60794
67.9 PT61187
6
0.0 PT20372
4.3 PT51115
21.1 PT30205
33.9 PT61160
38.5 PT61163
47.5 PT20287n
57.2 PT61581
62.0 PT60130
78.7 PT50885
7
0.0 PT61472
6.6 PT52892a
23.5 PT52970
27.7 PT53619
29.8 PT61088
44.3 PT60627
65.5 PT52723
69.4 PT52805
95.9 PT30248
105.5 PT52940
112.4 PT61312
118.0 PT60015
122.4 PT60404a
8
0.0 PT51449
11.5 PT30424a
19.5 PT51501
30.6 PT61279
40.5 PT30485
48.7 PT20189
52.8 PT61104
59.6 PT54599
73.4 PT52260
86.5 PT60320
100.1 PT60557
9
0.0 PT50176
10.5 PT61588
22.1 PT52120
28.3 PT60681
35.0 PT53434
40.2 PT1069a
49.1 PT51717
65.5 PT53852
111.1 PT52241
113.6 PT50280
10
0.0 PT50513
0.8 PT50759
2.5 PT55236
9.1 PT51005
17.1 PT53038
27.5 PT1043
35.0 PT54232
43.7 PT20211o
45.5 PT51635
48.5 PT1436
59.0 PT50469
11
0.0 PT30046
7.5 PT52652
10.5 PT52606
13.1 PT1454
15.4 PT1245
34.4 PT60305
40.7 PT61185
44.4 PT30024
86.1 PT30417
94.2 PT55435
101.2 PT50670
12
0.0 PT54433
10.9 PT60849
30.5 PT51602
40.4 PT52131
42.8 PT60369
59.3 PT61010
74.2 PT61009
84.7 PT50246
88.8 PT50334a
91.0 PT52810
13
0.0 PT50790
7.2 PT51281
26.0 PT54179
30.4 PT53829
35.7 PT53473
45.5 PT30168
53.5 PT60757
67.6 PT53424
71.2 PT50225
14
0.0 PT60558
11.2 PT51975
23.0 PT55319
26.7 PT60268
36.1 PT60863
38.6 PT52157
40.9 PT52138
45.0 PT53339
60.8 PT53687
15
0.0 PT54576
11.3 PT52054
29.4 PT53978
41.9 PT20300n
43.1 PT60668
45.6 PT53579
46.1 PT60631
62.9 PT61592
68.6 PT30346
85.9 PT60609
16
0.0 PT50334b
10.0 PT50369
29.3 PT51411
33.4 PT50118
36.9 PT53894
41.4 PT54823
56.0 PT55240
70.7 PT50943
76.7 PT60979
78.8 PT54253o
80.9 PT60243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4.7 PT61262
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11.0 PT61453
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18.4 PT52118
22.9 PT30171
40.1 PT30200
40.4 PT50981
48.8 PT52003
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61.0 PT547291b
74.0 PT54824
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17.8 PT54630
31.7 PT30265
36.6 PT50879
48.0 PT50645
63.6 PT54889
74.2 PT60099
87.7 PT53858
89.2 PT30289
89.9 PT53353 PT61605
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16.3 PT60600
23.4 PT51199
32.2 PT60340
37.7 PT61002
51.0 PT60344
54.5 PT20149
62.0 PT54788
84.6 PT50939
89.9 PT50827
21
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20.4 PT60606a
26.6 PT30257
31.5 PT51373a
36.5 PT61086
39.0 PT54983
39.4 PT52133
46.9 PT20388
50.4 PT60057
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10.3 PT50262
19.9 PT30473
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45.2 PT30096a
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74.3 PT52667
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88.9 PT53026
91.9 PT53802
98.5 PT53673
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6.0 PT55251
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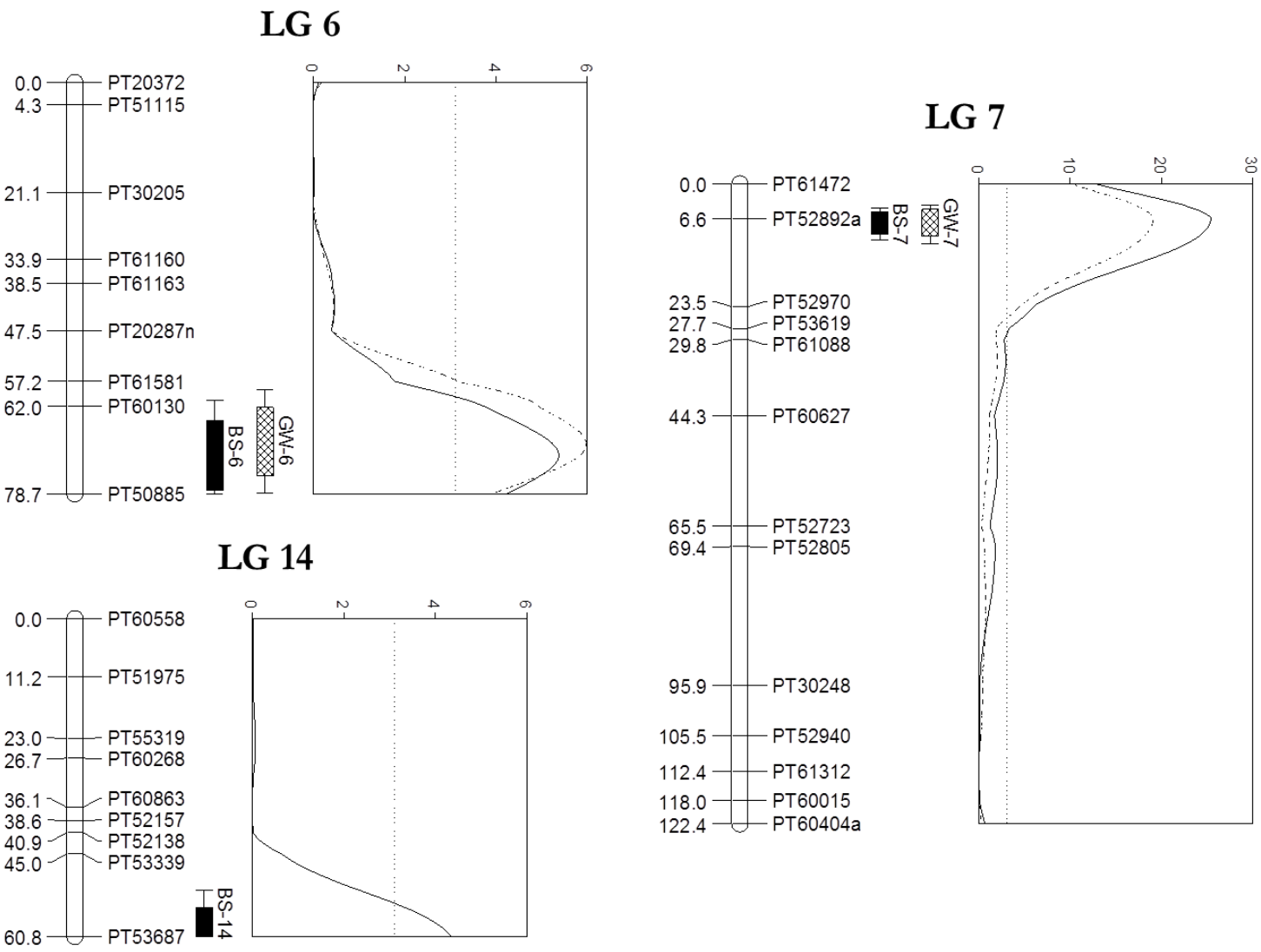
30.3 PT30242
35.4 PT51339
43.1 PT52944
50.5 PT53716
52.3 PT60792
82.1 PT60540a
83.5 PT51357a

Figure 3.4. Logarithm of odds (LOD) score peaks for linkage groups (LGs) found to be significantly associated with *P. nicotianae* or *R. solanacearum* resistance. Solid and dashed lines relate to data for *P. nicotianae* and *R. solanacearum*, respectively. Vertical bars represent 1 LOD and 2 LOD confidence intervals.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33.9 PT61160
38.5 PT61163
47.5 PT20287n
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62.0 PT60130
78.7 PT50885
BS-6
GW-6
0
2
4
6
LG 14
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11.2 PT51975
23.0 PT55319
26.7 PT60268
36.1 PT60863
38.6 PT52157
40.9 PT52138
45.0 PT53339
60.8 PT53687
BS-14
0
2
4
6
LG 7
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6.6 PT52892a
23.5 PT52970
27.7 PT53619
29.8 PT61088
44.3 PT60627
65.5 PT52723
69.4 PT52805
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BS-7
GW-7
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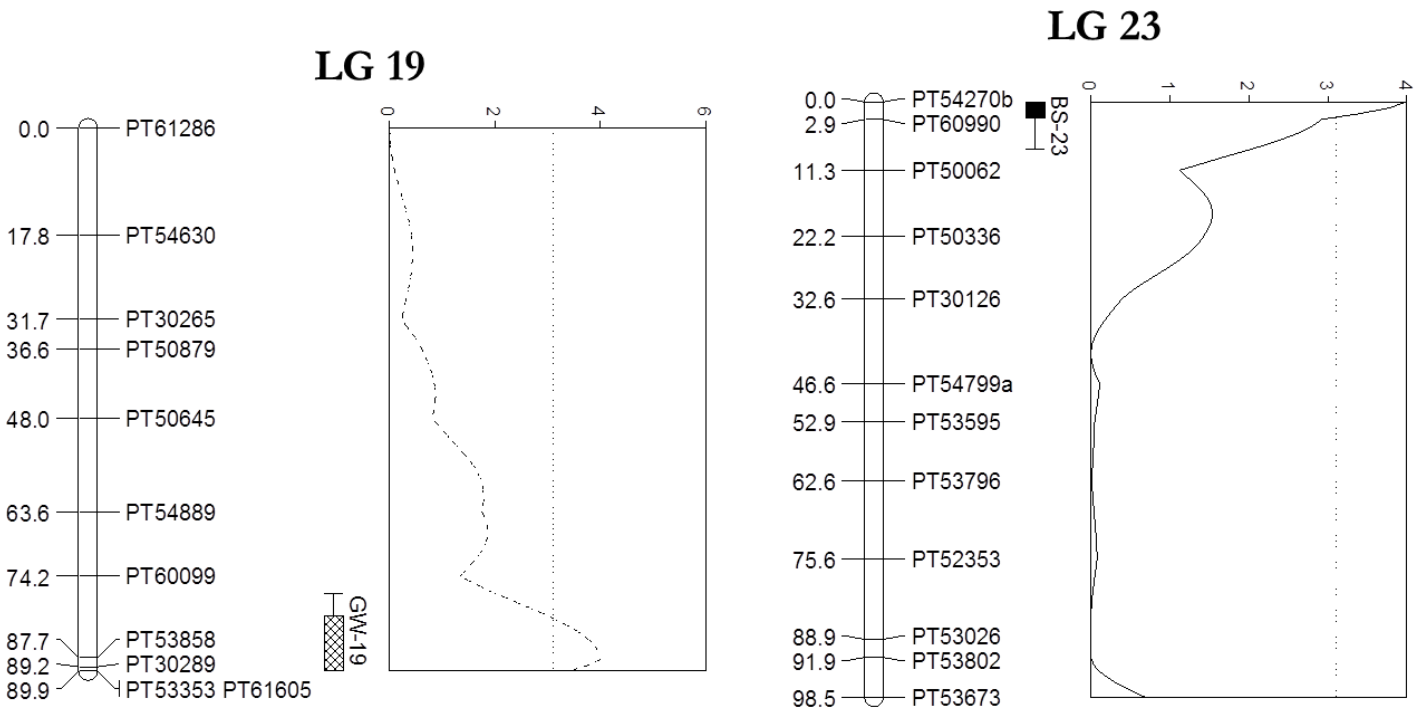
10
20
30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87.7 PT53858
89.2 PT30289
89.9 PT53353 PT61605
GW-19
0
2
4
6
LG 23
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2.9 PT60990
11.3 PT50062
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32.6 PT30126
46.6 PT54799a
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62.6 PT53796
75.6 PT52353
88.9 PT53026
91.9 PT53802
98.5 PT53673
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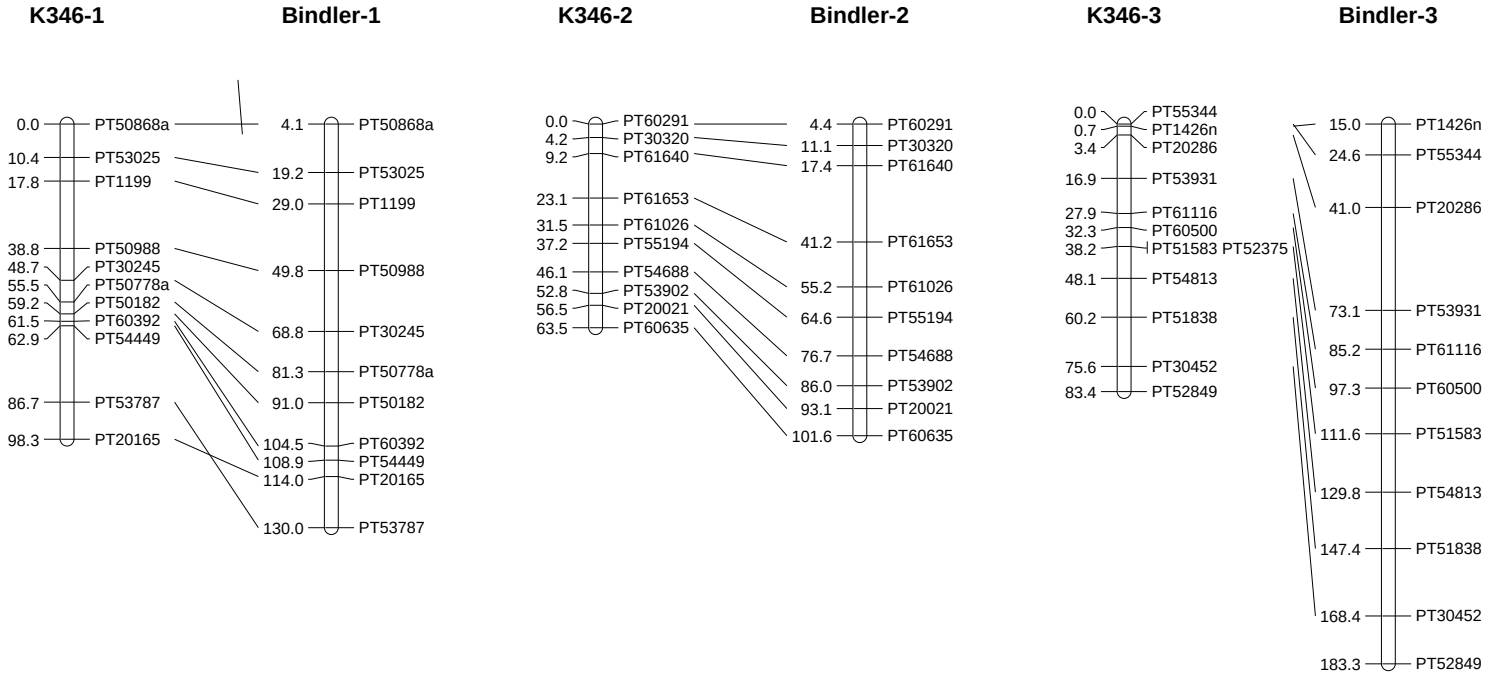
2
3
4

Figure 3.S1. Comparison of the linkage map created for the K 346 x TI1060 population with the high-density linker map of Bindler et al. (2011).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Bindler-1
K346-2
Bindler-2
K346-3
Bindler-3
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86.7 PT53787
98.3 PT20165
4.1 PT50868a
19.2 PT53025
29.0 PT1199
49.8 PT50988
68.8 PT30245
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91.0 PT50182
104.5 PT60392
108.9 PT54449
114.0 PT20165
130.0 PT53787
0.0 PT60291
4.2 PT30320
9.2 PT61640
23.1 PT61653
31.5 PT61026
37.2 PT55194
46.1 PT54688
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4.4 PT60291
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17.4 PT61640
41.2 PT61653
55.2 PT61026
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76.7 PT54688
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101.6 PT60635
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0.7 PT1426n
3.4 PT20286
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27.9 PT61116
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38.2 PT51583 PT52375
48.1 PT54813
60.2 PT51838
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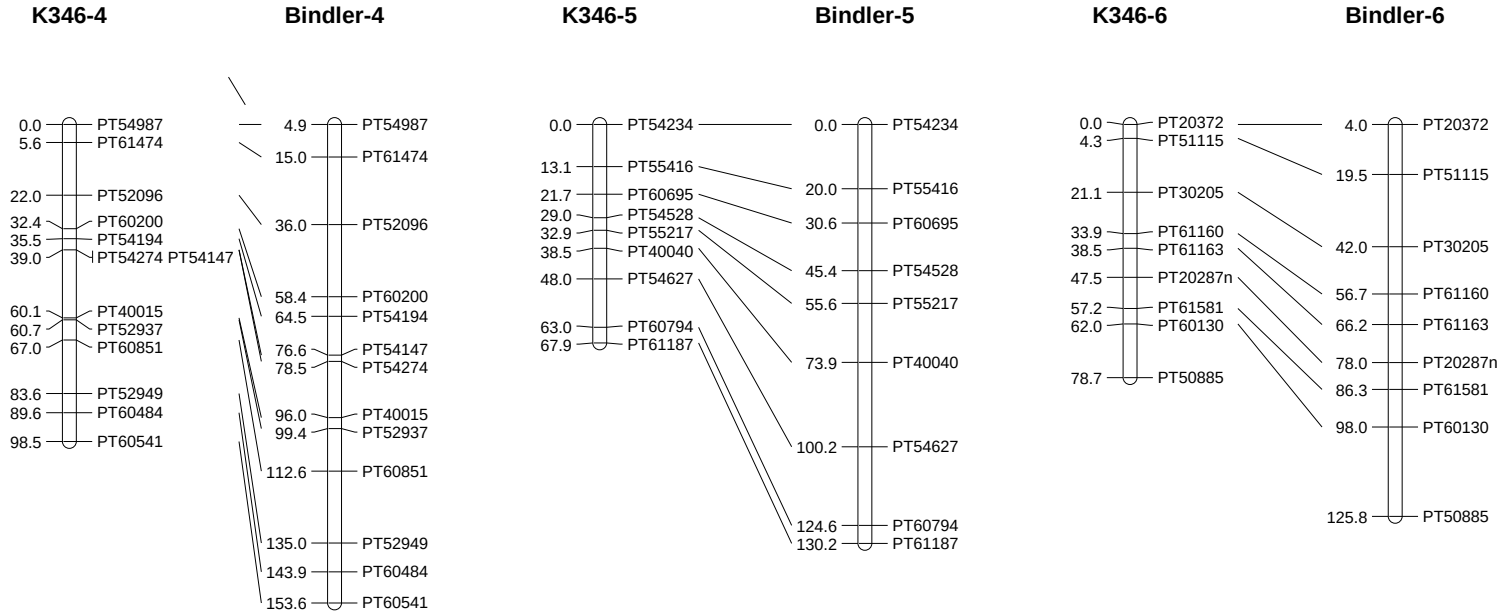
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K346-5
Bindler-5
K346-6
Bindler-6
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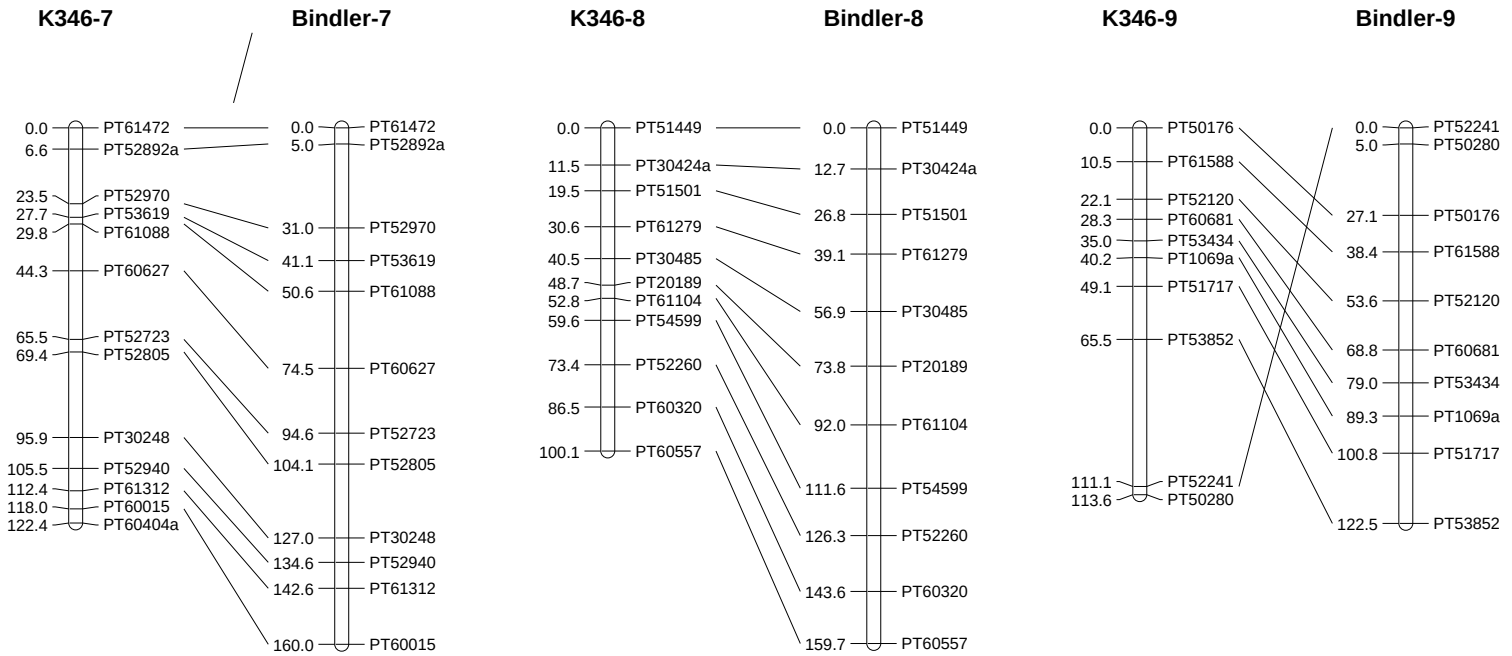
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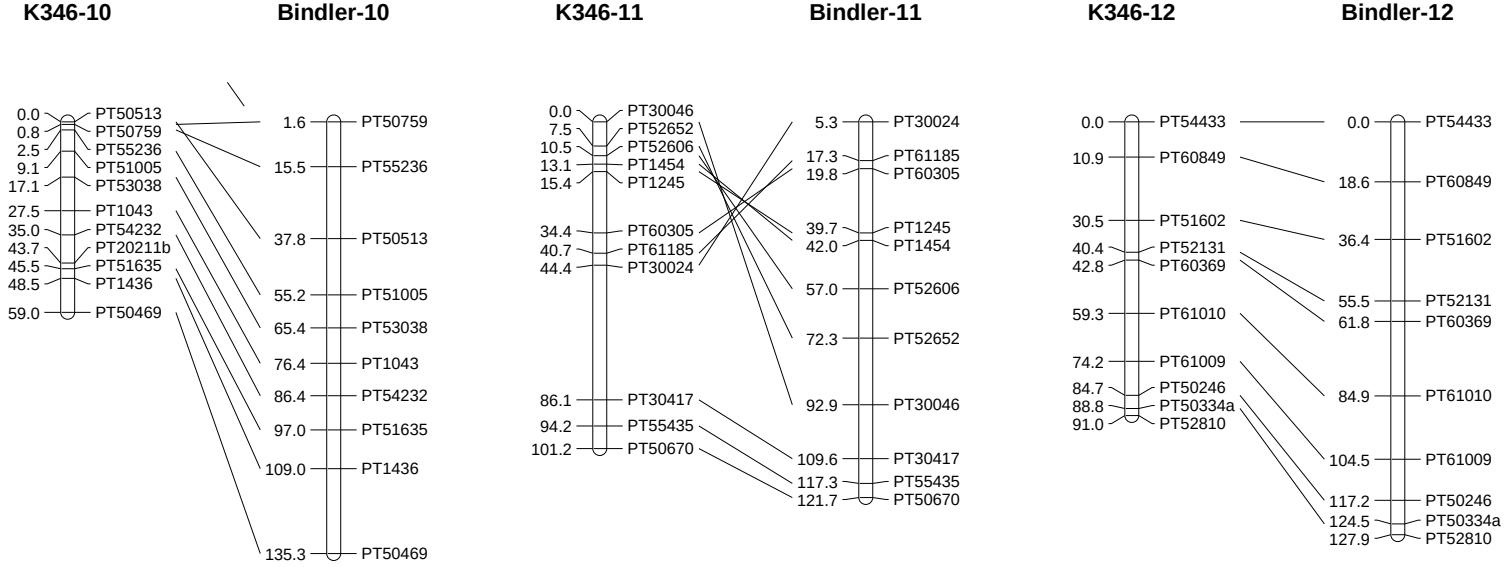
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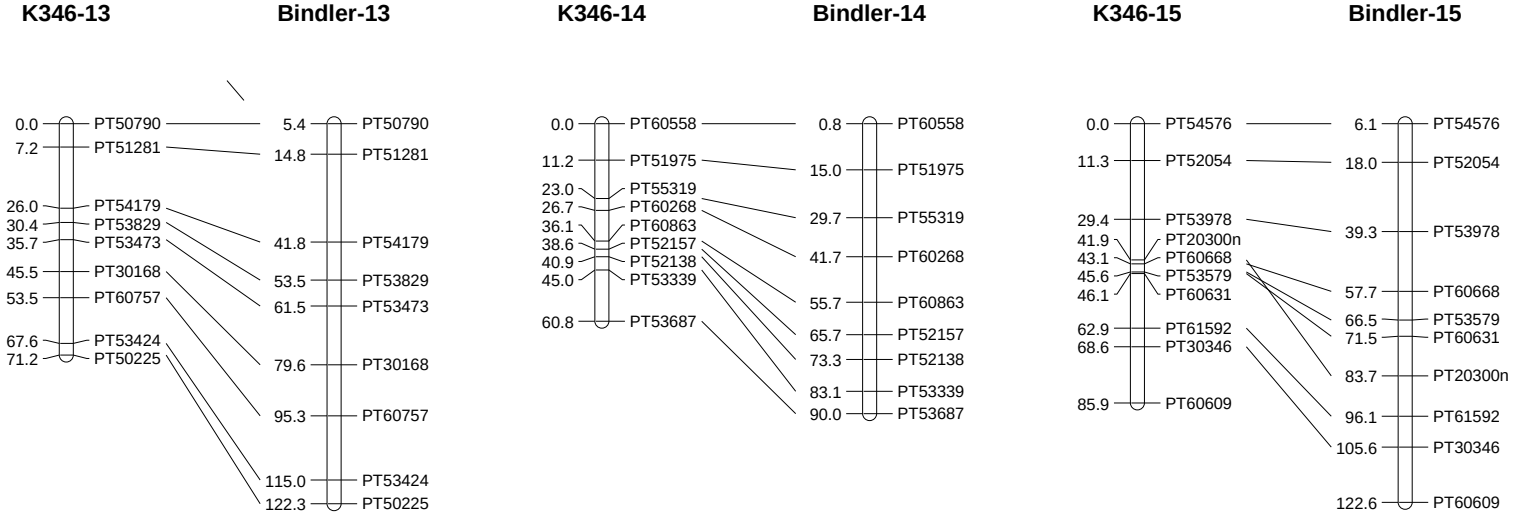
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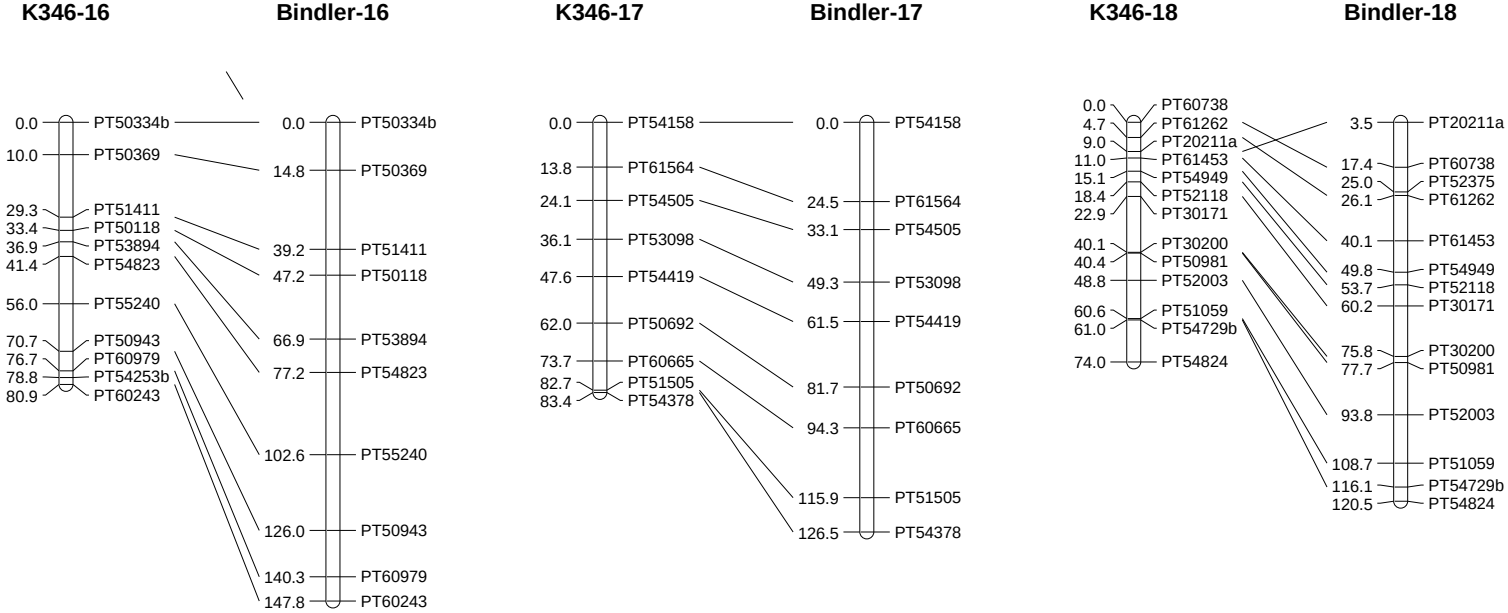
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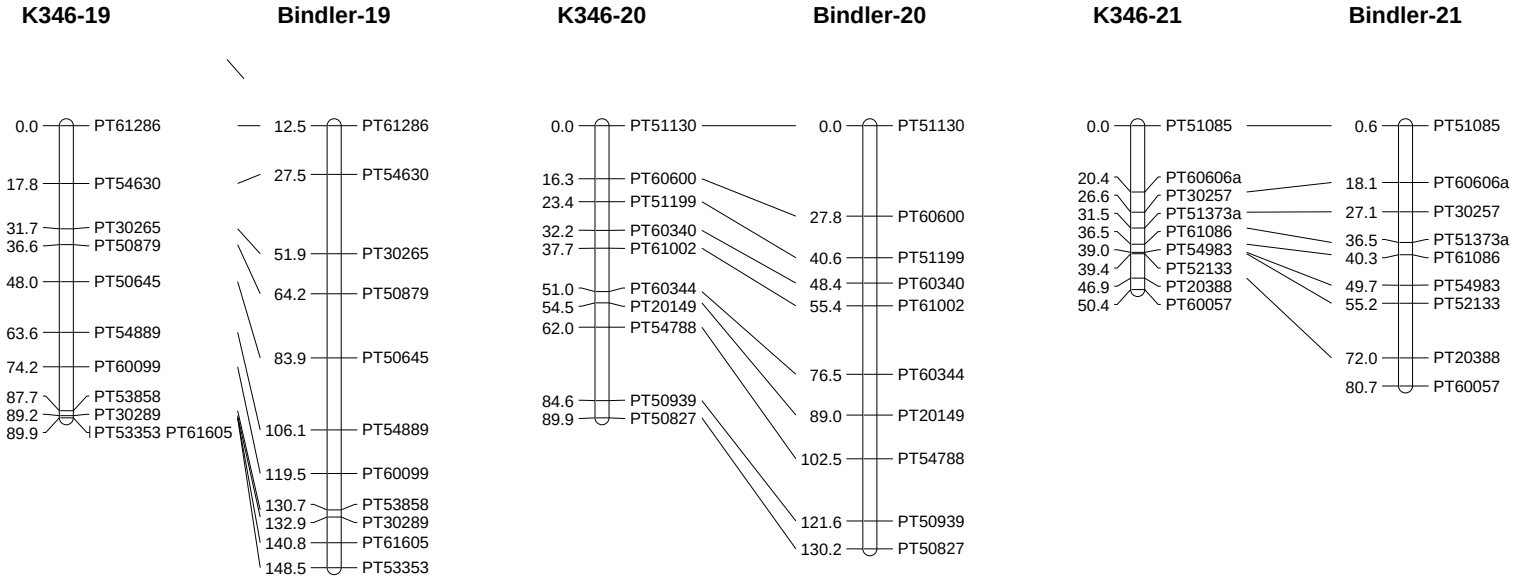
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27.5 PT54630
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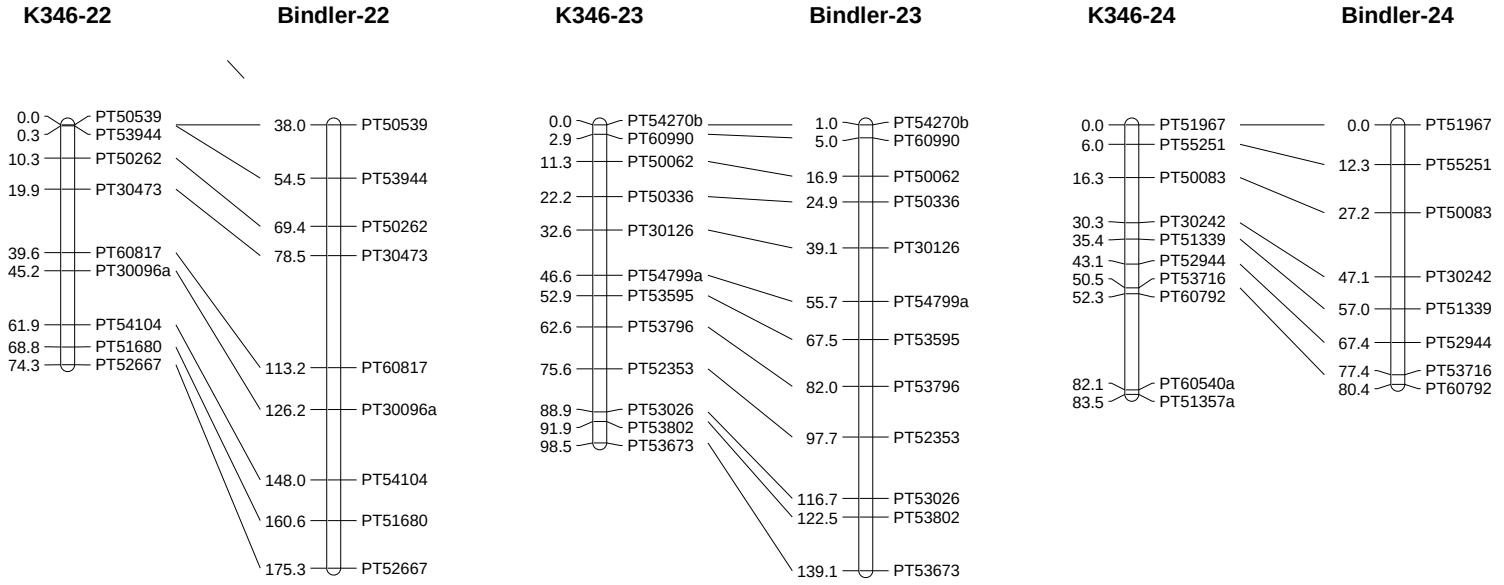
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Chapter 4: The *Yellow Burley* Loci Encode for Membrane-Bound Metalloproteases that Function in Chloroplast Maintenance in Tobacco (*Nicotiana tabacum* L.)

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Prepared for Submission to Plant Physiology

ABSTRACT

The primary distinguishing characteristic of cultivars of the burley tobacco market class is their high degree of chlorophyll deficiency, particularly on stems, stalks, and midveins, that becomes accentuated during plant maturation. This 'yellow burley' phenotype is conferred by a double homozygous recessive genotype ($yb_1yb_1\ yb_2yb_2$) at the *Yellow Burley 1* (Yb_1) and *Yellow Burley 2* (Yb_2) loci. This genetic deficiency affects the physiology of burley tobacco cultivars and translates into, among other things, reduced N utilization efficiency and increased potential for accumulation of tobacco-specific nitrosamines, a potent class of carcinogens that exists in greater amounts in tobacco products containing cured burley tobacco leaves. Through map-based gene discovery, we have shown that Yb_1 and Yb_2 encode for proteins with a very high degree of similarity to EGY1 from *Arabidopsis thaliana* which is predicted to function as a chloroplast membrane-bound, ATP independent, metalloprotease. Genetic analysis of a historical set of tobacco cultivars indicates that burley tobacco evolved via a two-step process: an 8 bp deletion in Yb_2, followed by human selection of a spontaneous homozygous 1 bp insertion in Yb_1, whereby both mutations lead to premature protein truncation. The identification of the genes at the Yb_1 and Yb_2 loci represents the first reported example of map-based gene discovery in *N. tabacum,* and their identification sheds light on the physiological differences between burley tobacco and other tobacco market classes.

INTRODUCTION

Similar to other crop species (such as wheat, cotton, maize, and rice), tobacco (*Nicotiana tabacum* L.) can be categorized into multiple market classes. These market classes, or tobacco types, can be differentiated by area of cultivation, harvesting and curing methodologies, and plant genetics. All of these variables can contribute to physical and chemical characteristics that are expected for cured leaf derived from varieties of the various market classes. By far, the most widely cultivated type of tobacco is flue-cured tobacco, with burley tobacco being the second-most widely grown. Both are used primarily in the manufacture of blended cigarettes, although burley tobacco is also used for pipe and certain chewing tobacco products.

Tobacco cultivars of the burley tobacco market class are differentiated from cultivars of other market classes by genome-wide differences that affect plant architecture, leaf type, and leaf chemistry. Perhaps the greatest distinguishing characteristic of burley tobacco varieties, however, is the high degree of chlorophyll deficiency that is most evident on the stems, stalks, and leaf midveins of burley tobacco plants and that becomes more pronounced with plant age (Figure 4.1). The chlorophyll-deficient phenotype of burley tobacco is conferred by a double homozygous recessive genotype ($yb_1yb_1\_yb_2yb_2$) at the *Yellow Burley* 1 (Yb_1) and *Yellow Burley* 2 (Yb_2) loci, which have been reported to reside on chromosome B and chromosome O, respectively (Henika, 1932; Clausen and Cameron, 1944; Stines and Mann, 1960). Only a single dominant allele at either of the two loci is required to establish the normal, more-green phenotype.

Besides affecting chlorophyll levels, the recessive alleles at Yb_1 and Yb_2 also have highly significant effects on the physiology of the tobacco plant and the chemistry of the derived cured leaf. For example, Legg et al. (1977) found the differences in chemical composition between

burley and flue-cured tobaccos to be largely attributable to alleles at the *Yb*$_1$ and *Yb*$_2$ loci. In this study, cured leaf of nearly isogenic *yb*$_1$*yb*$_1$ *yb*$_2$*yb*$_2$ 'burley' genotypes was found to be higher for NO_3-N, alpha amino N, total N, total alkaloids, ash, pH, and holocellulose; and lower for starch, reducing sugars, polyphenols, chlorides, and alkalinity of H_2O ash. More recently, Lewis et al. (2012) found the double recessive *yb*$_1$*yb*$_1$ *yb*$_2$*yb*$_2$ genotype to be associated with increased alkaloid levels, reduced nitrogen use efficiency, reduced nitrogen utilization efficiency, and increased leaf nitrate nitrogen (NO_3-N) which, in turn, likely operated to contribute to increased levels of the carcinogenic class of compounds known as tobacco specific nitrosamines (TSNAs) that are derived via nitrosation reactions with naturally occurring tobacco alkaloids. It was speculated that higher TSNA levels in *yb*$_1$*yb*$_1$ *yb*$_2$*yb*$_2$ genotypes may be at least partially due to a relatively poor capacity of such tobacco plants to assimilate accumulated nitrogen into N-containing organic molecules involved in plant growth and development (Lewis et al., 2012). The increased pool of free NO_3-N likely becomes available for reduction to nitrite (the primary nitrosating agent for TSNA formation in air-cured tobacco) by plant nitrate reductase enzymes or via microbial activity. The precise function of the gene(s) at the *Yb*$_1$ and *Yb*$_2$ loci has yet to be reported, but grafting studies have indicated the genetic differences to be expressed at the leaf level rather than the root (Crafts-Brandner et al., 1987ab).

Liu et al. (2015) speculated that *Yb*$_1$ and *Yb*$_2$ may encode for genes involved in chlorophyll synthesis as they found the precursors of δ-aminolevulinic acid, porphobilinogen, uroporphyrinogen III, coproporphyrinogen III, protoporphyrin IX, Mg-protoporphyrin IX, and protochlorophyllide in chlorophyll biosynthesis to be lower in a burley tobacco cultivar as compared to a Maryland tobacco cultivar (*Yb*$_1$*Yb*$_1$ *Yb*$_2$*Yb*$_2$, *Yb*$_1$*Yb*$_1$ *yb*$_2$*yb*$_2$, or *yb*$_1$*yb*$_1$ *Yb*$_2$*Yb*$_2$

genotype). The activity of chlorophyllase in the burley tobacco cultivar was 2.04 times as high as in that in the Maryland cultivar. These authors concluded that the yellowish color of burley tobacco was caused by low chlorophyll content, possibly due to a combination of inhibited biosynthesis and accelerated degradation of chlorophyll regulated by the enzymes δ-aminolevulinate dehydratase and chlorophyllase, respectively. Because of the observed effects of yb_1 and yb_2 on nitrogen use physiology, one could also reasonably speculate that genes involved in nitrogen assimilation or transport might also underlie the observed chlorophyll deficiency of burley tobacco plants, although Sierro et al. (2014) found few differences in gene expression in major nitrogen assimilation genes between burley and flue-cured tobacco.

Chloroplast homeostasis requires coordination of gene expression from both nuclear and plastid-encoded genes, as well as appropriate transport, processing, and degradation of their products. Insight on genes controlling chloroplast biogenesis and processes has been gathered primarily from research on mutations producing visible chlorophyll deficiencies in model species such as *Arabidopsis*. Along with genes at the *Yellow Burley* loci, reported *N. tabacum* genetic factors affecting chlorophyll deficiency include *ivory* (Gwynn and Mann, 1965), *white seedling* (Clausen and Cameron, 1950), *yellow green* (Nolla, 1934), and *sulfur* (Burk and Menser, 1964). Of these, only *sulfur* has been characterized and found to encode for a protein involved in magnesium chelation into protoporphyrin IX, the last common intermediate precursor in chlorophyll and heme biosynthesis, to form Mg-protoporphyrin IX (Papenbrock et al, 1997; Grafe et al., 1999). Identification of the genes at the *Yb* loci would provide insight on the genetic changes that led to the evolution of the burley tobacco market class. In addition, the identification of these genes would shed light on the physiological differences between burley tobacco and other tobacco

market classes, and also provide information to help understand the complex biochemical and physiological processes that occur in plastids of species other than *Arabidopsis*.

Wu et al. (2014) recently identified EMS-induced mutations in tobacco causing a white stem characteristic and found these mutations to be allelic to yb_1 and yb_2. These authors subsequently identified microsatellite markers on linkage groups 5 (3.96 cM away) and 24 (8.56 cM away) to be linked to these loci. The objectives of this research were to fine map the genomic positions of Yb_1 and Yb_2, and ultimately identify and characterize the genes underlying the yellow burley phenotype in tobacco.

RESULTS

Fine Mapping of Yb_1 and Yb_2

Three pairs of lines isogenic for alleles at the Yb_1 and Yb_2 loci were genotyped using a 30 K Infinium SNP chip. Two genomic regions were found to exhibit polymorphisms for all three pairs of nearly isogenic lines. Through co-segregation analyses with microsatellite markers on the high density microsatellite marker linkage map of Bindler et al. (2011), these SNP markers were found to reside on *N. tabacum* linkage groups (LG) 5 and 24, which were donated by progenitor species *N. sylvestris* and *N. tomentosiformis*, respectively (data not shown). Two SNP markers estimated to span a region of 1.33 cM on LG 5, and six SNP markers estimated to cover a region of 17.16 cM on LG 24, were converted to KASP markers (Figure 4.2, Supplemental Table 4.S1) for fine mapping the Yb_1 and Yb_2 loci using the BWDH8/NC1426-17//NC1426-17 and BWDH16/NC1426-17//NC1426-17 BC_1F_1 populations which were expected to segregate for the wild-type allele at one *Yb* locus and to be fixed for the recessive allele at the alternative

Yb locus. Genotyping of the parental lines of the two mapping populations (BWDH8, BWDH16, and NC1427-7) with the eight KASP markers was used to infer that the BWDH8-derived population was segregating for the markers on LG 5 and the BWDH16-derived population was segregating for the markers on LG 24.

Screening of 384 BC_1F_1 individuals in the BWDH8/NC1426-17//NC1426-17 BC_1F_1 population with the two selected LG 5 KASP markers refined the position of one *Yb* locus to 0.26 cM from SNP marker Yb5-1 (Figure 4.2). Screening of 384 BC_1F_1 individuals in the BWDH16/NC1426-17//NC1426-17 BC_1F_1 population with the six selected LG 24 KASP markers refined the position of the other *Yb* marker to a 4.37 cM interval between SNP markers Yb24-4 and Yb24-5, and 1.07 cM away from marker Yb24-4 (Figure 4.2). To identify scaffolds with potential candidate genes, SNP marker sequences most closely associated to either *Yb* locus were aligned to scaffolds from a draft *N. tabacum* genome sequence (Edwards, 2015; Edwards et. al., 2015). Three scaffolds (3,876,640 bp) with a total of 79 predicted genes were identified for LG 5 and three scaffolds (8,458,802 bp) with a total of 223 predicted genes were identified for LG 24.

Sequence similarity searches to the *Arabidopsis* genome assembly (The *Arabidopsis* Information Resource, TAIR) (www.arabidopsis.org) were carried out to determine the potential function of predicted genes. Genes predicted to be associated with nitrogen assimilation, nitrogen use physiology, or chloroplast activities were considered as potential candidate genes underlying the yellow burley phenotype. Two such candidates were identified for the genomic region of interest on LG 5 and four for the region of interest on LG 24 (Table 4.1). Full length *Arabidopsis* cDNA sequences corresponding to the six genes of interest were obtained from

TAIR and BLAST searches against genome sequence data for K 326 (Yb_1Yb_1 Yb_2Yb_2) and TN90 (yb_1yb_1 yb_2yb_2) (GCA_00715075.1 and GCA_00715135.1, respectively; www.ncbi.nlm.nih.gov). No differences were found between the K 326 and TN90 draft sequences for *N. tabacum* homologs of the *Arabidopsis* genes *CJD1*, *FtsZ2-1*, *ChlAKR*, or *RCA*. In the *CRTISO* homolog, an 80 bp deletion was identified in intron 7 of TN90, but this did not result in predicted changes in the amino acid sequence. In the *EGY1* homolog, however, 8 bp and 111 bp deletions were identified in TN90 relative to K 326, which lead to predicted alternative splicing and an extra exon.

The *EGY1* (*ethylene-dependent gravitropism-deficient and yellow green protein 1*) homolog (hereafter referred to as *NtEGY1*) was investigated further because of the predicted dramatic amino acid sequence differences between TN90 and K 326. Considering the allotetraploid nature of *N. tabacum*, where the S and T genomes were contributed by *N. sylvestris* and *N. tomentosiformis*, respectively (Clarkson et al., 2005; Lim et al., 2004), the *NtEGY1* sequence was BLAST searched against the *N. tabacum* genome sequence to identify potential highly similar sequences. A predicted gene with an 89% nucleotide identity was identified as a potential homeolog, and tentatively designated as *NtEGY2*. BLAST comparisons of the *NtEGY2* sequence from K 326 and TN90 revealed a 175 bp deletion followed by many other five to ten bp deletions and a single insertion of a T nucleotide in TN90 relative to K 326.

PCR primers EGY1-F and EGY1-R designed to amplify only a segment of *NtEGY1* and to detect the 8 bp deletion in TN 90 (Table 4.S2 and Table 4.S3) were tested on K 326, TN90, and the parents of the two mapping populations (BWDH8, BWDH16, NC 1427-17). The 8 bp deletion was found to be present in TN90 and also BWDH8, but not in BWDH16. Genotyping

of 1056 BC_1F_1 plants from BWDH8/NC1426-17//NC1426-17 BC_1F_1 population (which segregates 1:1 for the normal : chlorophyll-deficient phenotype) for this deletion revealed complete cosegregation between the chlorophyll deficiency and the 8 bp deletion, thus highly suggesting *NtEGY1* to be the gene at one of the two *Yb* loci. We suggest *NtEGY1* to be $NtYb_2$ because $NtYb_2$ was identified through monosomic analysis to reside on chromosome O that was originally contributed by *N. sylvestris* (Clausen and Cameron, 1944), and because the 8 bp deletion marker co-segregates with SNP and microsatellite markers located on *N. tabacum* linkage group 5 which was determined to have been inherited from *N. sylvestris* (Bindler et al., 2011). In addition, *NtEGY1* exhibits 99% sequence identity with the most similar sequence from the genome of *N. sylvestris* (GCF_000393655.1), while it exhibits only 87% nucleotide identity with the most similar gene from the genome of *N. tomentosiformis* (GCF_000390325.1)

To determine the predicted amino acid sequence differences between the wild-type and mutant $NtYb_2$ (*NtEGY1*) alleles, the corresponding cDNA strands were isolated and sequenced from K 326 and TN90. Because of the high degree of sequence similarity between *NtEGY1* and *NtEGY2*, it was difficult to specifically amplify each cDNA separately. Therefore, it was assumed that cDNAs of both were being simultaneously amplified. PCR products were separated using gel electrophoresis and fragments were cloned and sequenced. It was possible to distinguish cDNAs corresponding to *NtEGY1* from *NtEGY2* based upon the presence of a 9 bp insertion in the former that was also predicted based upon the genomic sequence in GenBank.

Analysis of the $NtYb_2$ cDNA sequences revealed a T → C substitution at base 618, a A → T substitution at base 621, and an 8 bp deletion of bases 623 to 630 (exon 2) in TN90, which causes a frame-shift and the creation of a premature stop codon, resulting in a truncated protein

that is predicted to be missing a C-terminal 337 amino acid region compared to the native protein (Figure 4.2 and Figure 4.S1).In comparing the *NtEGY2* cDNA sequences from TN90 and K 326, the only difference found was an insertion of a T nucleotide at base 1448 of the cDNA (exon 9) in TN90. This insertion causes a frame-shift and creates a premature stop codon, resulting in a truncated protein that is predicted to be missing a C-terminal 67 aa region compared to the native protein. The wild-type *NtEGY1* and *NtEGY2* cDNAs are predicted to produce proteins with 97% amino acid similarity (Figure 4.2, Figure 4.S1, and Figure 4.S2).

Because of the identification of a 1 bp insertion leading to a predicted truncated NtEGY2 protein, it was hypothesized that *NtEGY2* might correspond to *NtYb*$_1$. KASP primers, EGY2_kasp (Table 4.S1), were designed to detect the T insertion. Because of the extremely high degree of sequence similarity between *NtEGY1* and *NtEGY2* in the vicinity of the 1 bp insertion, it was difficult to design primers specific to the *NtEGY2* homeolog. Nested PCR was therefore carried out with primers EGY2-nF and EGY2-nR (Table 4.S2 and Table 4.S3) prior to genotyping of the KASP marker. After testing the marker on K 326, TN90, and the parents of the two mapping populations (BWDH8, BWDH16, NC 1427-17), it was determined that TN90 and BWDH16 carried the mutation. A total of 1040 individuals from the BWDH16/NC1426-17//NC1426-17 BC_1F_1 population were genotyped with the marker and complete cosegregation was observed between the presence of the 1 bp insertion and the yellow burley phenotype. This provided extremely strong evidence that *NtEGY2* also encoded for a gene at one of the *Yb* loci.

We suggest *NtEGY2* to be *NtYb*$_1$ because *NtYb*$_1$ was identified through monosomic analysis to reside on chromosome B that was originally contributed by *N. tomentosiformis* (Clausen and Cameron, 1944), and because the recessive allele at this locus marker co-segregates with

SNP and microsatellite markers located on *N. tabacum* linkage group 24 which was determined to have been donated by *N. tomentosiformis* (Bindler et al., 2011). Furthermore, a BLAST search of the *N. tomentosiformis* genome sequence using *NtEGY2* reveals a sequence with 99% sequence similarity.

Comparisons of Genomic DNA Sequences

Genomic DNA sequences for $NtYb_1$ and $NtYb_2$ were isolated from both K 326 and TN90. Each of the *Yb* loci has 10 exons and 9 introns. Analysis of the $NtYb_2$ genomic sequence confirmed the substitutions and 8 bp deletion in exon 2 in burley tobacco cultivar, TN90 (Figure 4.2). The 111 bp deletion present in the draft genome sequence was not identified, and is likely an assembly error in the K 326 draft genome sequence. The deletion and these substitutions were the only differences observed between TN 90 and K 326 in $NtYb_2$. Analysis of the genomic sequence of $NtYb_1$ confirmed the insertion of a T in exon 9 of TN90 (Figure 4.2). No other differences between TN 90 and K 326 were observed for Yb_1.

Yb Loci are Members of the M50 Family of Metalloproteases

$NtYb_1$ encodes for a predicted protein consisting of 547 amino acids with a molecular mass of 58.6 kD. $NtYb_2$ encodes a predicted protein of 544 amino acids with a molecular mass of 58.4 kD. BLASTP searches using the $NtYb_1$ and $NtYb_2$ predicted wild-type protein sequences indicated that both gene products contain conserved domains consistent with the Site-2 protease (S2P), zinc metalloprotease (MEROPS) M50 family. Both proteins share 72% identity with the *Arabidopsis* protein encoded by the *EGY1* locus, a chloroplast targeted member of the MEROPS M50 family (Chen et al., 2005). In addition to similarities to *EGY1*, both proteins share high homology to proteins from several other plant species (Figure 4.3). As expected, the $NtYb_1$

encoded protein has 100% identity to a predicted chloroplastic zinc metalloprotease in *N. tomentosiformis*, and the *NtYb*$_2$ encoded protein has 100% identity to a predicted chloroplastic zinc metalloprotease in *N. sylvestris*. Both encoded proteins share 90% identity with a predicted chloroplastic metalloprotease from *Solanum tuberosum* and the same protein from *S. lycopersicum* encoded by the *lutescent2* locus (Barry et al., 2012).

EGY-like proteins are characterized by three conserved domains, GNLR, HEXXH, and NXXPXXXLDG that are all present in the *NtYb*$_1$ and *NtYb*$_2$ genes (Figure 4.S2). The GNLR motif is a novel signature unique to EGY1 and EGY-like proteins, while the HEXXH and NPDG motifs are predicted active sites, with the HEXXH pentapeptide predicted to be the metal-binding site for zinc metalloproteases (Chen et al., 2005). The 8 bp deletion leading to early termination in the *NtYb*$_2$ encoded protein in burley tobaccos results in loss of both the HEXXH and NPDG domains. All three characterizing motifs are present in the *NtYb*$_1$-encoded protein of TN90, however, suggesting the 67 C-terminal amino acids to be essential to protein function.

Chen et al. (2005) demonstrated the EGY1 protein to be localized to the chloroplasts using an EGY1-GFP fusion protein. *In silico* prediction of the *NtYb* encoded proteins using the Predotar prediction server (https://urgi.versailles.inra.fr/predotar/predotar.html) estimated a 0.95 and 0.94 probability for plastid localization for the *NtYb*$_1$ and *NtYb*$_2$ proteins, respectively.

Analysis of Historical Tobacco Varieties Provides Insight on Evolution of Burley Tobacco

To provide insight on the genetic changes that may have led to the evolution and commercial adoption of the burley tobacco market class, DNA was isolated from a total of 24 lines of tobacco that included 13 burley tobacco cultivars of historical importance, four Maryland tobacco cultivars (thought to be highly related to modern burley cultivars), and an assortment of seven additional old-line, historical cultivars that may have been involved in the evolution of modern burley tobacco (Table 4.2). Each of these varieties was genotyped for the 1 bp insertion in *NtYb*$_1$ and the 8 bp deletion in *NtYb*$_2$. As expected, all commercial burley tobacco cultivars plus Judy's Pride, Barnett Special, Kelly Brownleaf, and Kelly Burley were homozygous for both mutations (Table 4.2). All Maryland tobacco cultivars (Md 10, Md 59, Md 609, and Md 64) plus Wilson were homozygous wild-type at *NtYb*$_1$, but were homozygous for the mutant allele at *NtYb*$_2$. All other genotyped accessions were homozygous for the wild-type allele at both loci.

DISCUSSION

The first objective of this research was to fine map the genomic positions of the *Yb*$_1$ and *Yb*$_2$ loci. We were able to use SNP markers to initially fine map the locations of *Yb*$_1$ and *Yb*$_2$ to small intervals on linkage groups 5 and 24 of the map of Bindler et al. (2011). This is in agreement with the study of Wu et al. (2014), who also mapped these two loci to larger intervals on the same linkage groups. Ultimately, we were able to identify candidate genes with a high degree of similarity to a gene designated as *EGY1* from *A. thaliana* (Chen et al., 2005). Experiments involving marker cosegregation provided confirming evidence that *NtEGY2* and

NtEGY1 correspond to *NtYb*$_1$ and *NtYb*$_2$, respectively. Prior map-based gene discovery efforts in *N. tabacum* were not successful, most likely due to a general lack of polymorphism as revealed by most molecular marker systems. The identification of the genes at the *Yb*$_1$ and *Yb*$_2$ loci represents the first reported example of map-based gene discovery in *N. tabacum*, and was greatly facilitated by genome sequence information now available for this species (Sierro et al., 2013a,b; Sierro et al., 2014; Edwards 2015; Edwards et al. 2015).

Protein sequence alignments indicated the Yb$_1$ and Yb$_2$ proteins to be very closely related to EGY1 and other members of the diverse M50 family of metalloproteases (with members from bacteria, plants, and animals) that are involved in regulated intramembrane proteolysis (RIP) in response to metabolic status (Osborne and Espenshade, 2009). Specifically, *EGY1* encodes a membrane-bound, plastid-targeted, and ATP-independent metalloprotease site-2 protease that is required for development of thylakoid grana, a well-organized lamellae system, and accumulation of chlorophyll and chlorophyll a/b binding proteins in chloroplast membranes (Chen et al., 2005). The unique GNLR motif of EGY-like proteins was found only in orthologs of higher plants and cyanobacteria (Chen et al., 2005) and is lacking from other members of the M50 family, suggesting the *EGY1* locus may have originated from a cyanobacterial ancestor (Sokolenko et al., 2002). *EGY1* is one of several thousand nuclear-encoded genes involved in controlling the diverse functions of chloroplasts. Such proteins are synthesized in the cytosol and transported into chloroplasts through a process facilitated by translocon complexes on the chloroplast envelope (Schein et al., 2001). Yb1 and Yb2 proteins are strongly predicted to carry chloroplast transit peptides.

A number of chloroplast-localized proteases have been reported (Sakamoto, 2006; Kato and Sakamoto, 2010; Adam and Sakamoto, 2014). Although the role of these proteases in chloroplast biogenesis and homeososstasis is not precisely clear, they may be involved in protein quality control or removal of oxidatively damaged proteins in conjunction with adaptation to changing light intensities, nutrient stress, or senescence (Adam and Sakamoto, 2014). Chen et al. (2005) have speculated that the EGY1 thylakoid membrane protein may function, either directly or indirectly, with FtsH metalloproteases to regulate the maintenance and assembly of PSII complexes, or that EGY1 may play a role in the turnover and assembly of membrane-associated PS I. Guo et al. (2008) suggested that EGY1 may function in lipid metabolic pathways because the metalloprotease family is closely related to a sterol-regulatory element binding protein site 2 protease, which regulates SREPT-dependent lipid biosynthesis pathways.

In *Arabidopsis, EGY1* mutants exhibit smaller and reduced numbers of plastids in endodermal cells, along with reduced granal thylakoids, poorly developed lamellae networks, and significantly decreased accumulation of chlorophyll a/b binding proteins, leading to a yellow-green plant phenotype (Chen et al., 2005; Guo et al., 2008). *AtEGY1* mutants exhibit chlorotic phenotypes throughout plant development. *NtYb* mutants, in contrast, are generally not distinguishable from wild-type individuals until about 40 days after germination (under good growth-promoting conditions). At this time, the chlorophyll deficiency becomes most obvious on plant stems and leaf midveins. As the tobacco plant ages, the chlorophyll deficiency becomes more obvious in all above-ground plant parts. In this way, *NtYb* mutations are more similar to mutations in the EGY1-like gene, *lutescent2* (*L2*), from tomato, where there is an enhanced rate of chlorophyll loss in leaves and fruits as they mature (Barry et al., 2012). The developmental

aspects of mutations in *EGY1*-like genes between *Arabidopsis*, tomato, and tobacco are not understood at the current time.

Chlorophyll-deficient mutants are generally not desirable to plant breeders, with the exception of those working with ornamental species. In the case of tobacco, however, the evolution and selection of a chlorophyll-deficient mutant led to the development of a novel market class that ultimately became a primary component of the American blend cigarette. Identification of the genes underlying the yellow burley phenotype provides insight on the genetic events leading to the development of this market class of cultivated tobacco. According to Garner et al. (1936), a type of tobacco known as 'burley' was being grown in the 1800's in northern Kentucky and southern Ohio that produced cured leaf that was very light, chaffy, almost free of gum, and reddish or cinnamon in color. These characteristics made it suitable for the manufacture of a certain type of chewing tobacco known as fine cut. This type of tobacco was not of the chlorophyll-deficient type found in the burley tobacco of today and, according to Garner et al. (1936) there is little doubt that the original burley tobacco was a form of Maryland Broadleaf, which resembles modern burley tobacco in terms of plant architecture. According to Garner et al. (1936):

> 'In 1864, George Webb, a farmer in Brown County, Ohio, observed in his tobacco seedbed, which is said to have been planted with Burley seed from Bracken County, Kentucky, a number of seedlings having a peculiar chlorotic appearance – that is, they were somewhat deficient in green coloring matter. The mature plants, though otherwise normal, showed an almost pure cream color instead of the normal light green. The leaf cured to a light yellowish red color, and the light, chaffy, porous characteristics of the

> parent Burley were accentuated. Because of the peculiar creamy color of the stalk and the midrib and veins of the leaf, and the very pale greenish yellow color of the leaf web when mature, the new variety was called White Burley to distinguish it from the old Burley, which came to be known as Red Burley.'

After the discovery and use of White Burley, Red Burley was still primarily used for chewing tobacco, but the lighter grades of White Burley began to be used for smoking products, especially in cigarette blends (Gage, 1937). Gage (1937) stated, 'Had it not been for this evolutionary process, which dates back to the chance discovery of one or two plants of unusual characteristics, Burley would still be a dark, air-cured tobacco suitable for little except chewing purposes instead of the single most important single type of tobacco produced in this country.'

Identification of the genes underlying the yellow burley character permits a retrospective genetic analysis of the mutants initially reportedly discovered by George Webb. Historical and genotypic information suggest that White Burley was derived from a tobacco genotype that resembles modern-day Maryland tobacco. It is straightforward to hypothesize that the type grown in southern Ohio already carried a mutation in one of the yellow burley genes. Indeed, Stines and Mann (1960) evaluated progeny derived from crosses involving 20 non-chlorophyll-deficient tobacco varieties and identified only two varieties, both of the Maryland type (Posey and Catterton) that possessed a mutant allele at one of the two loci. Further results suggested that these two cultivars are of the $Yb_1Yb_1\_yb_2yb_2$ genotype and carry the mutant allele on chromosome O. Aycock (1976) crossed Burley 21 with seven different Maryland varieties and found six (Catterton, Md 10, Wilson, Md 59, Md 609, and Md 64) to contain one dominant gene, and 'Moore' to contain two dominant genes for normal chlorophyll content. Our

genotyping results mostly agree with this, as Wilson and the four Maryland tobacco cultivars were all homozygous for the 8 bp deletion on linkage group 5, and homozygous for the wild-type allele on linkage group 24. Of the lines that were genotyped, we found materials that carried the *NtYb*$_1$ mutation but not the *NtYb*$_2$ mutation. These results highly support the hypothesis that modern burley tobacco was selected as a spontaneous mutation in a variety resembling modern-day Maryland tobacco and that this selection was due to a spontaneous 1 bp insertion in *NtYb*$_1$. In contrast to the results of Stines and Mann (1960), who reported Catterton and Posey to possess mutant alleles at one of the two loci, we found these varieties to be homozygous for the wild-type alleles at each locus. These discrepancies could be due to genetic heterogeneity that surely exists in these old-line cultivars, or due to the possibility that the accessions now labeled with these names are not the same as those used by Stines and Mann (1960).

Besides shedding light on the evolution of the burley tobacco market class, this study also identified gene-specific markers that can be useful in marker-assisted breeding. Perhaps of greater future interest, however, is the finding that genes controlling events related to chloroplast maintenance can play a role in affecting a range of other measured characteristics in the tobacco plant and derived cured leaf (Legg et al., 1977). This should not be surprising as the chloroplasts are the site of synthesis for amino acids, fatty acids, carotenoids, and vitamins. For example, Lewis et al. (2012) found the double recessive *yb*$_1$*yb*$_1$ *yb*$_2$*yb*$_2$ genotype to be associated with increased alkaloid levels, reduced nitrogen use efficiency, reduced nitrogen utilization efficiency, and increased leaf nitrate nitrogen (NO_3-N) which, in turn, likely operated to contribute to increased TSNA levels. Use of plant genetics to reduce the potential for TSNA formation in cured tobacco leaves is an area of significant interest, and most efforts have focused on the

reduction of alkaloid precursors (Lewis et al., 2008, 2010) or modifying genes associated with the nitrogen assimilation pathway for the purpose of reducing levels of nitrosating agents (Lu et al., 2015). Data from the current research suggest altering genetic variability at loci other than those directly involved in alkaloid biosynthesis or nitrogen assimilation might also play a role in the potential for using plant genetics to reduce TSNAs levels in tobacco.

MATERIALS AND METHODS

Mapping of *Yb*$_1$ and *Yb*$_2$

We previously had available to us three pairs of lines that were created to be nearly isogenic for the dominant and recessive alleles at the *Yb*$_1$ and *Yb*$_2$ genomic regions. To create these materials, the recessive *yb*$_1$ and *yb*$_2$ alleles were transferred from burley tobacco cultivar Ky 16 to flue-cured tobacco (*Yb*$_1$*Yb*$_1$*Yb*$_2$*Yb*$_2$) cultivars SC58, NC95, and Coker 139 using eight cycles of backcrossing followed by multiple generations of self-pollination to establish homozysosity for the *yb*$_1$ and *yb*$_2$ alleles conferring the chlorophyll deficient phenotype. DNA was isolated from the six genotypes using a modified cetyltrimethylammonium bromide procedure (Afandor et al., 1993) and genotyped with a 30 K Infinium iSelect HD BeadChip SNP chip (Illumnia Inc., San Diego, CA). Genomic regions containing polymorphisms that differentiated the nearly isogenic lines were identified and corresponding SNP markers of interest were converted to Kompetitive Allele Specific PCR (KASP) markers (Semagn et al., 2014) by LGC Genomics (Beverly, MA) (Table 4.S1).

To develop mapping populations for fine mapping both *yb*$_1$ and *yb*$_2$, we first generated a set of doubled haploid (DH) lines segregating for the yellow burley phenotype by hybridizing

burley tobacco cultivar Ky 14 with flue-cured tobacco cultivar K 346, isolating haploid plants via pollination of the F_1 hybrid with *N. africana* according to Burk et al. (1979), and doubling the chromosome number of resulting haploid plants according to Kasperbauer and Collins (1972). Doubled haploid lines BWDH6 and BWDH8 were subsequently found to be homozygous for a dominant *Yb* allele at only one locus, while line BWDH16 was found to be homozygous for the dominant allele at the opposite *Yb* locus (unpublished data).

To ultimately develop progenies suitable for fine mapping Yb_1 and Yb_2, BWDH8 and BWDH16 (of either the $Yb_1Yb_1\ yb_2yb_2$ or $yb_1yb_1\ Yb_2Yb_2$ genotype) were both hybridized with burley tobacco breeding line NC1427-17 ($yb_1yb_1\ yb_2yb_2$). Corresponding F_1 hybrids were then backcrossed to NC1427-17 to develop progenies that were expected to segregate 1:1 for the yellow burley phenotype. Approximately 1000 BC_1F_1 progeny from each family were grown in a field at Clayton, NC, scored for the chlorophyll-deficient phenotype, and genotyped with KASP markers corresponding to SNPs found to be closely linked to either Yb_1 or Yb_2.

SNP markers found to be closely linked to either Yb_1 or Yb_2 were aligned to a *N. tabacum* draft sequence (Edwards, 2015; Edwards et al., 2015) to identify scaffolds that might contain Yb_1 or Yb_2 candidate genes. Genes predicted to be involved in nitrogen assimilation, nitrogen use physiology, or chlorophyll maintenance were considered as potential candidate genes underlying the yellow burley phenotype. GenBank sequences for flue cured cultivar K 326 ($Yb_1Yb_1Yb_2Yb_2$) and burley tobacco cultivar TN 90 ($yb_1yb_1\ yb_2yb_2$) were investigated for polymorphisms in these candidate genes. Primers were designed to permit genotyping for polymorphisms of interest (Table 4.S2 and Table 4.S3) and used to genotype approximately 1200 plants from the BWDH8/NC1426-17//NC1426-17 and BWDH16/NC1426-17//NC1426-17 BC_1F_1

populations to determine the degree of linkage between the polymorphism of interest and the genes controlling the yellow burley phenotype.

Isolation and Cloning of *Yb$_1$ and Yb$_2$* cDNA

RNA was extracted from leaf tissue of six-week old plants of K 326 (*Yb$_1$Yb$_1$Yb$_2$Yb$_2$*) and TN 90 (*yb$_1$yb$_1$ yb$_2$yb$_2$*) plants using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the SuperScript First-Strand Synthesis System for RT-PCR with oligo(dT) (Invitrogen, Carlsbad, CA). The coding region of *Yb* candidate genes was amplified by PCR from first-strand cDNA from K 326 and TN 90 using the primers cYb-F and cYb-R (Table 4.S2 and Table 4.S3) that were designed based on *Yb$_1$* and *Yb$_2$* predicted sequences using *N. tabacum* draft genome sequence information. Because few nucleotide differences existed between *Yb$_1$* and *Yb$_2$* candidates at either the 5' or 3' ends, it was not possible to design primers specific to either homeolog. Bands were therefore excised from agarose gels and purified with the Monarch DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA). Fragments were cloned into the pCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA) and transformed into NEB 5-alpha competent *E. coli* cells (New England Biolabs). Sequencing of individual clones derived from each cultivar was carried out using vector primers and subsequent analysis revealed that both *Yb$_1$* and *Yb$_2$* candidate fragments had been amplified.

Isolation of *Yb$_1$* and *Yb$_2$* Genomic DNA

Genomic DNA was isolated from plants of K 326 and TN90 using a modified cetyltrimethylammonium bromide procedure (Afandor et al., 1993). The genomic DNA of each candidate gene was amplified in six fragments of approximately 1250 bp each using PCR with Q5 High-Fidelity DNA polymerase (New England Biolabs) and primers described in Table 4.S1

Table 4.S2. To ensure specific amplification of the desired gene, primers were designed such that the last basepair on the 3' end was a SNP between *Yb*$_1$ and *Yb*$_2$. Fragments were excised, purified, and cloned into pCR-Blunt. Sequencing was carried out using vector primers and, for longer sequences, internal gene sequence primers (Table 4.S2 and Table 4.S3).

Genotyping of Historical Maryland Varieties

To gain insight on the evolution of the yellow burley phenotype with respect to available historical information, a series of historical cultivars believed to be related to the evolution of the burley tobacco market class (Garner et al., 1936; Gage, 1937; Stines and Mann, 1960; Aycock, 1976) were genotyped for the polymorphisms discovered in the *Yb*$_1$ and *Yb*$_2$ loci. Fragments covering the *Yb*$_1$ mutation location were amplified using Q5- High-Fidelity DNA polymerase (NEB) and primers gYb1-F6 and gYb1-R6 (Table 4.S2 and Table 4.S3). PCR products were cleaned using the Monarch PCR DNA Cleanup Kit (New England Biolabs) and sequenced with gYb1-R6. Fragments covering the *Yb*$_2$ mutation location were amplified using primers gYb2-F2 and gYb2-R2 (Table 4.S2 and Table 4.S3). PCR products were cleaned and sequenced with gYb2-F2.

Sequence Analysis and Bioinformatics Resources

DNA sequences were aligned using Vector NTI-AlignX (Thermo Fisher Scienfific, Walthmam, MA). Individual genomic DNA fragments were concatenated into full genomic sequences using Vector NTI-ContigExpress. The prediction of genes within genomic DNA sequence was performed with the FGENESH hidden Markov model-based gene structure prediction program (www.softberry.com). cDNA sequences were aligned to genomic sequences with Spidey (www.ncbi.nlm.nih.gov/spidey). Amino acid alignments were performed using

MUSCLE (Edgar, 2004). *Yb* orthologs were identified from different organisms by BLAST searching the NCBI database or the Phytozyme database using the full-length amino acid sequences of Yb_1 or Yb_2. Multiple sequence alignment of selected results was constructed using ClustalOmega. The phylogenetic tree was reconstructed using PHYLIP (Felsenstein, 2005) and visualized with R/ape (Paradis et al., 2004).

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TABLES AND FIGURES

Table 4.1. Annotated gene sequences of interest on scaffolds linked to either *Yb1* or *Yb2* loci.

N. tabacum		**Homology with *Arabidopsis***		
Scaffold	**Gene Name**	**Protein Homolog**	**Gene Symbol**	**Descriptor**
Nitab4.5_0000963	Nt5g09630050	AT1G08640	CHLOROPLAST J-LIKE DOMAIN 1 (CJD1)	Encodes a chloroplast membrane protein CJD1. Influences fatty acid composition of chloroplast lipids.
Nitab4.5_0000963	Nt5g09630080	AT2G36250	FtsZ2-1	Encodes one of two FTsZ proteins. Involved in chloroplast division.
Nitab4.5_0000613	Nt24g06130020	AT5G35220	ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN 1 (EGY1)	Membrane-associated and ATP-independent metalloprotease. Required for development of both thylakoid grana and a well-organized lamellae system in chloroplast and required for the accumulation of chlorophyll and CAB binding proteins.
Nitab4.5_0000721	Nt24g07210080	AT1G06820	CAROTENOID ISOMERASE (CRTISO)	Encodes carotenoid isomerase. Catalyzes the isomerization of poly-cis-carotenoids to all trans carotenoids.
Nitab4.5_0002170	Nt24g21700040	AT2G37770	CHLOROPLASTIC ALDO-KETO REDUCTASE (ChlAKR)	Encodes a NADPH-dependent aldo-keto reductase. Localizes to the chloroplast where it may play a role in detoxifying reactive carbonyl compounds that threaten to impair the photosynthetic process.
Nitab4.5_0002170	Nt24g21700100	AT2G39730	RUBISCO ACTIVASE (RCA)	Nuclear-encoded chloroplast protein. Required for the light activation of rubisco and involved in jasmonate-induced leaf senescence.

Table 4.2. Historic tobacco varieties genotyped for SNPs within the *Yb* loci.

Line	TC Number[1]	Stem Color	*Yb*$_1$ Genotype[2]	*Yb*$_2$ Genotype[3]
Posey	512	Green	Yb_1Yb_1	Yb_2Yb_2
Catterton	494	Green	Yb_1Yb_1	Yb_2Yb_2
Green Brior	40	Green	Yb_1Yb_1	Yb_2Yb_2
Gertz	496	Green	Yb_1Yb_1	Yb_2Yb_2
Sweeney	514	Green	Yb_1Yb_1	Yb_2Yb_2
Moore	511	Green	Yb_1Yb_1	Yb_2Yb_2
Wilson	517	Green	Yb_1Yb_1	yb_2yb_2
Md 10	498	Green	Yb_1Yb_1	yb_2yb_2
Md 59	501	Green	Yb_1Yb_1	yb_2yb_2
Md 64	502	Green	Yb_1Yb_1	yb_2yb_2
Md 609	505	Green	Yb_1Yb_1	yb_2yb_2
Barnett Special	1	White	yb_1yb_1	yb_2yb_2
Judy's Pride	49	White	yb_1yb_1	yb_2yb_2
Kelly Brownleaf	50	White	yb_1yb_1	yb_2yb_2
Kelly Burley	51	White	yb_1yb_1	yb_2yb_2
Burley 1	3	White	yb_1yb_1	yb_2yb_2
Burley 2	4	White	yb_1yb_1	yb_2yb_2
Burley 11A	5	White	yb_1yb_1	yb_2yb_2
Burley 21	7	White	yb_1yb_1	yb_2yb_2
Ky 1	52	White	yb_1yb_1	yb_2yb_2
Ky 5	53	White	yb_1yb_1	yb_2yb_2
Ky 9	54	White	yb_1yb_1	yb_2yb_2
Ky 14	57	White	yb_1yb_1	yb_2yb_2
Ky 16	59	White	yb_1yb_1	yb_2yb_2

[1] 'TC' indicates 'Tobacco Cultivar in the U.S. *Nicotiana* germplasm collection
[2] *Yb*$_1$ genotype determined by testing for for 1 bp insertion
[3] *Yb*$_2$ genotype determined by testing for for 8 bp deletion

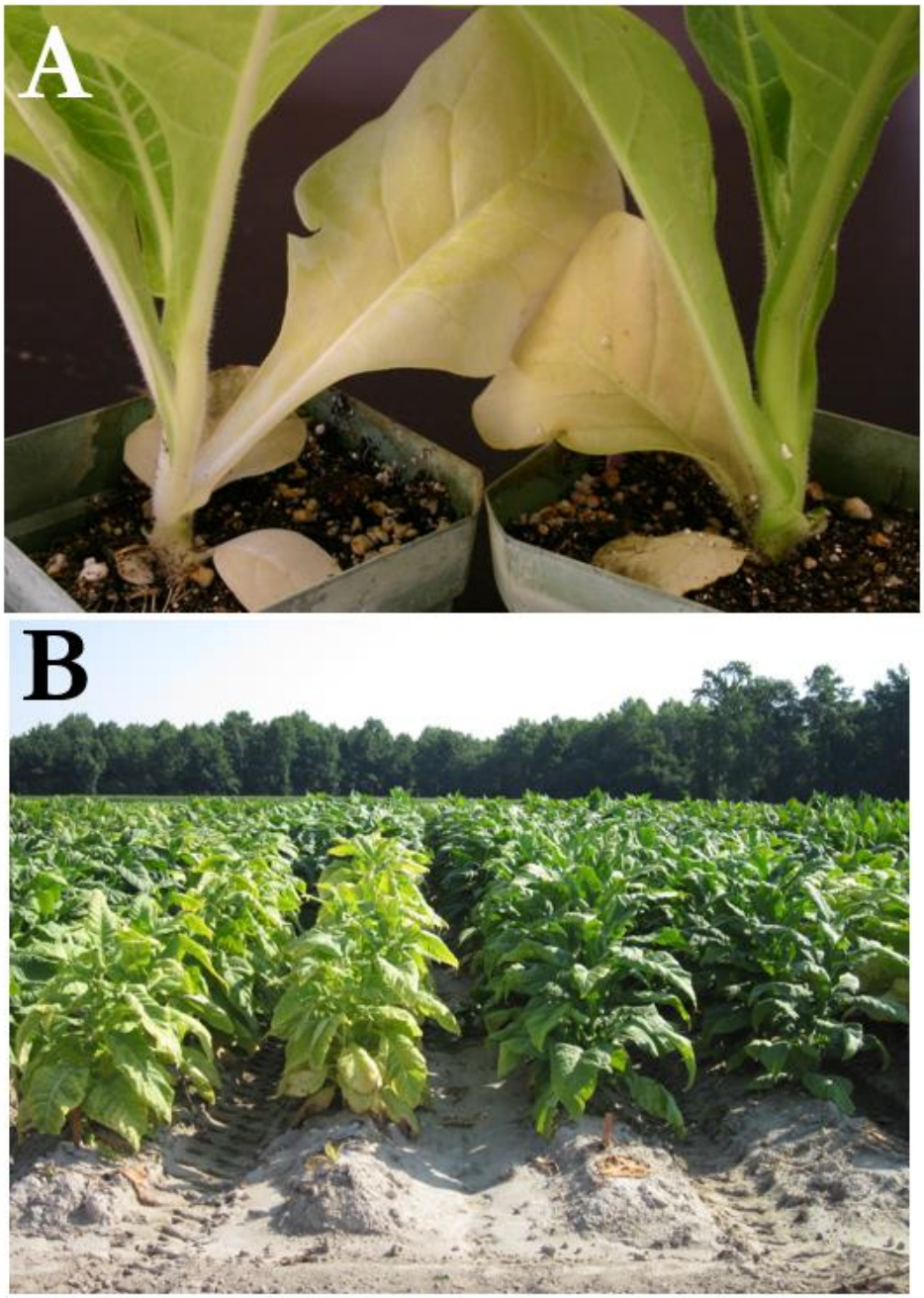

Figure 4.1. Phenotypic differences between *yb1yb1yb2yb2* and *Yb1Yb1Yb2Yb2* genotypes. A) TN90 (*yb1yb1yb2yb2*) (left) and K 326 (*Yb1Yb1Yb2Yb2*) (right). B) SC58 normal (right) and SC58 mutant (*yb1yb1yb2yb2*) (left).

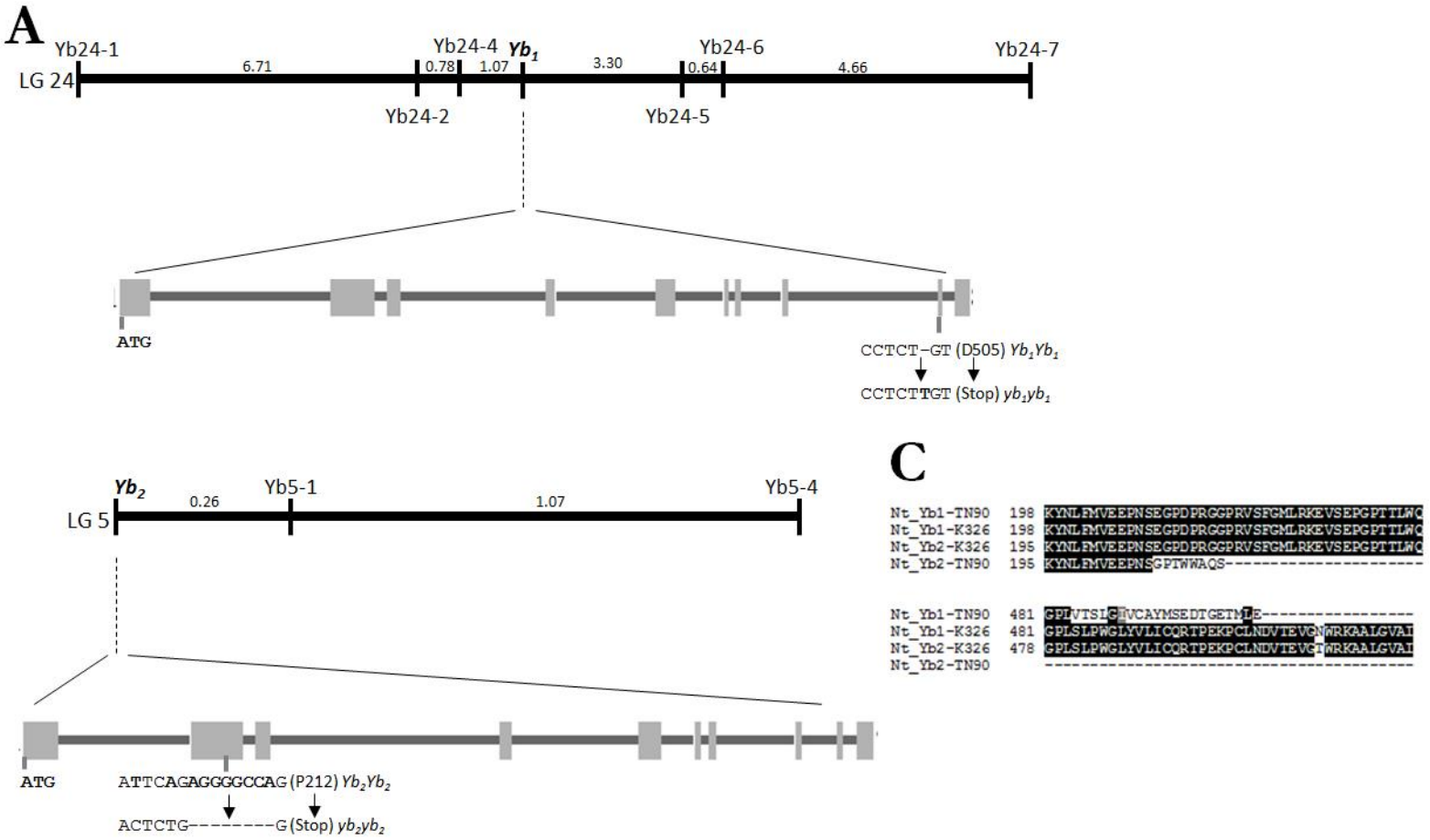

Figure 4.2. Positional cloning of the (A) *Yb1* gene and (B) *Yb2* gene. Dark lines are drawn to represent relative positions of genetic markers. The genetic distance between these markers is shown in cM. Grey rectangles indicate exons. (C) Consequences of *Yb* mutations. The 8 bp deletion in exon 2 of *Yb2* results in a frame shift, leading to the incorporation of 8 spurious amino acids before terminating in a premature stop codon. The insertion of a T in exon 9 of *Yb1* results in a frame shift that leads to the incorporation of 21 spurious amino acids before terminating in a premature stop codon.

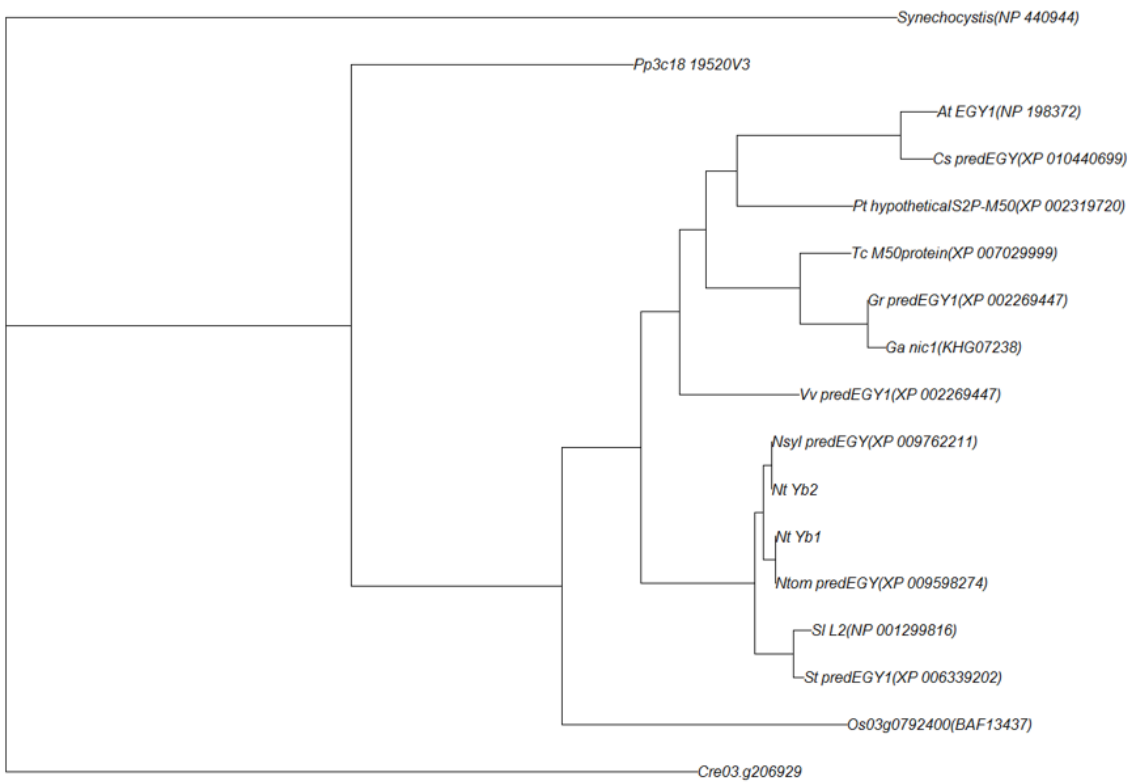

Figure 4.3. Phylogeny of Yb_1 and Yb_2 related proteins. The dendrogram was constructed using the maximum likelihood method. Branch lengths are scaled according to the expected fraction of amino acids changed. GenBank accession number are given for each branch except for the Cre03.g206929 and Pp3c18_19520V3 which are based on annotations of the *Chlamydomonas reinhardtii* and *Physcomitrella patens* genomes available through the Phytozome database (www.phytozome.net).

Table 4.S1. Sequences submitted to LGC Genomics (Beverly, MA) for conversion to KASP primers. SNPs are noted with brackets.

Primer Name	DNA Sequence
Yb5-1	TTAATAATAGTTCCTTTTTGACCGCGGGTACGAGGGCTAGCGTGAAATTGGCCACGAAGT[T/C]TGC CTAGATTTGAGACTTAATAGACGTTCGGGGTTGATACTCAATGTCGTACCTACTAAT
Yb5-4	GACTACTATCATTGCCAGTCTCAACCTGTTCTGGTTCAAGCTTCAGTTCCACGTCCTCTC[T/C]ATCAG TCTCTGCAGGCTCAACTGTTTCGATCTCCTTGTGTTCTGGAACTTCTTTAGAATC
Yb24-1	TAGATATTTGCCTCTTGTAGCTGGTTTACCATTTATAAAGCTTTTTTTCCTGTTGCCGCAT[A/G]ATGA AGTGTAATGTTGTCTCTATTATCTGTTAAAGACTTGTGATGTAGCTTGGTGTTCTG
Yb24-2	GAATCCTATGCTGGGATTAAGCCAAGCTGGTTTAGCTGCATCGGGATTTAATCCTTCGTT[T/C]GTG GGGATTGGTGCTGGTTATGATATAAATAGCATTAATCCGGGCGTTTTAGGTAGTAGT
Yb24-4	GTGGAATCTAAGGAGGGTAAAGTGTAAGCAAGTTTATCCTTGTCCTAGAAAGGTAGAGAT[A/G]TT GTTTCCGATAGATCCTTGGCTAAAAAAACGCAGAAAAGAAGCAGTGTCAACAAGTAAT
Yb24-6	GCAATATAAAAGTCAATACTAGTCTTAACGGTGTGAGGCCTTTTGGAAAAACTTGTACGG[T/G]CTT GGTCCAAAACAGACAATATCACACCATTTAAGAGTATCTTTAGGCCGTTTTAGTCCG
Yb24-7	GACGGTATGGTTAAAGATGTATATACGGAAGACTTCTCGATCACCATCGGAGAGAAATGC[T/G]TAT TTTTATGTCTCTTTTGGCCCAATGGAGAGCCCTTATTTATAAATGTAGAAGATTGAA
EGY_2kasp	GCTTAGTGTTGCAAATTGTACAGTCACTTAAAACATTCTGTTTGTGCTGCTATCAGCTTGGTGGACC TCT[T/_]GTCACTTCCTTGGGGATTGTATGTGCTTATATGTCAGGTAATAAAAATACATTGCTTTATG AAGTATTATGCTTGTGTAATAATATCGCCGTCCTATTTCATGGTATA

Table 4.S2. Oligonucleotides used for each task in map-based identification of Yb1 and Yb2 genomic DNA and cDNA.

Task	Oligonucleotides Used
Genotyping of EGY1 deletion	EGY_1-F/EGY_1-R
Genotyping of EGY1 homeolog insertion	EGY_2-nF/EGY_2-nR followed by EGY_2-kasp
Amplification of Yb_1 cDNA	cYb-F/cYb-R
Amplification of Yb_2 cDNA	cYb-F/cYb-R
Amplification of Yb_2 genomic fragment 1	gYb2-F1/gYb2-R1
Internal sequencing of Yb_2 genomic fragment 1	gYb2_Int1
Amplification of Yb_2 genomic fragment 2	gYb2-F2/gYb2-R2
Amplification of Yb_2 genomic fragment 3	gYb2-F3/gYb2-R3
Internal sequencing of Yb_2 genomic fragment 3	gYb2-Int3
Amplification of Yb_2 genomic fragment 4	gYb2-F4/gYb2-R4
Amplification of Yb_2 genomic fragment 5	gYb2-F5/gYb2-R5
Amplification of Yb_2 genomic fragment 6	gYb2-F6/gYb2-R6
Amplification of Yb_1 genomic fragment 1a	gYb1-F1a/gYb1-R1a
Amplification of Yb_1 genomic fragment 1b	gYb1-F1b/gYb1-R1b
Amplification of Yb_1 genomic fragment 1c	gYb1-F1c/gYb1-R1c
Amplification of Yb_1 genomic fragment 1d	gYb1-F1d/gYb1-R1d
Amplification of Yb_1 genomic fragment 2	gYb1-F2/gYb1-R2
Amplification of Yb_1 genomic fragment 3	gYb1-F3/gYb1-R3
Amplification of Yb_1 genomic fragment 4	gYb1-F4/gYb1-R4
Amplification of Yb_1 genomic fragment 5	gYb1-F5/gYb1-R5
Internal sequencing of Yb_1 genomic fragment 5	gYb1-Int5
Amplification of Yb_1 genomic fragment 6	gYb1-F6/gYb1-R6
Internal sequencing of Yb_1 genomic fragment 6	gYb1-Int6
Amplification of fragment with Yb_2 deletion in Maryland lines	gYb2-F2/gYb2-R2
Sequencing of Yb_2 deletion fragment in Maryland lines	gYb2-F2

Table 4.S3. Oligonucleotides used in map-based cloning of *Yb1* and *Yb2* genomic DNA and cDNA.

Primer Name	Primer Sequence (in 5' to 3' direction)
EGY1-F	AAGAGTAGCTGCATGATGTGAAGA
EGY1-R	CTGGCCCCTCTGAATTAGGTTCCTC
EGY2-nF	GCAATCGTTGTCCAGTGTCTA
EGY2-nR	TTTCCGACCTCTGTTACATCA
cYb-F	ATGGGAACGCTAACGAGCTGCAGTTTCAGCACAATGAAT
cYb-R	TCAAAAGCTGGTTACAAGACCTATACCTAGTTCTTCTGCAAG
gYb2-F1	ATGGGAACGCTAACGAGCTGCAGTTTCAGCA
gYb2-R1	CCGTGGATATTGTTGGCTGCCAAGAAATT
gYb2-Int1	CCGGTGTCAATTTCTTCCAGA
gYb2-F2	AAGGTTGATCCCAGTGATGTGAAGATA
gYb2-R2	CCAACACACCATATGCCAGTGGTAT
gYb2-F3	CCGTTTGTGGATTCTGCTATACCAC
gYb2-R3	GGAATGCTTAATTTCACATTCCTTGGAAAC
gYb2-Int3	CTGATTTAGCTATGCTAGTTTGC
gYb2-F4	GAAATTGGGCATTTTCTGGCTGCG
gYb2-R4	GCAAAAGGACCCACAAGCGAAATA
gYb2-F5	CGAAAAGCAAAGGTAGATATTTCGCTTGT
gYb2-R5	CCAATAAGTGATCCTTTCCCAAAGGC
gYb2-F6	GCCTTTGGGAAAGGATCACTTATTGG
gYb2-R6	CCACTACGTACAGTCAAAAGATCAGTATGAA
gYb1-F1a	CCTCACCATCTTCCAATTACATTTTTCACCATTG
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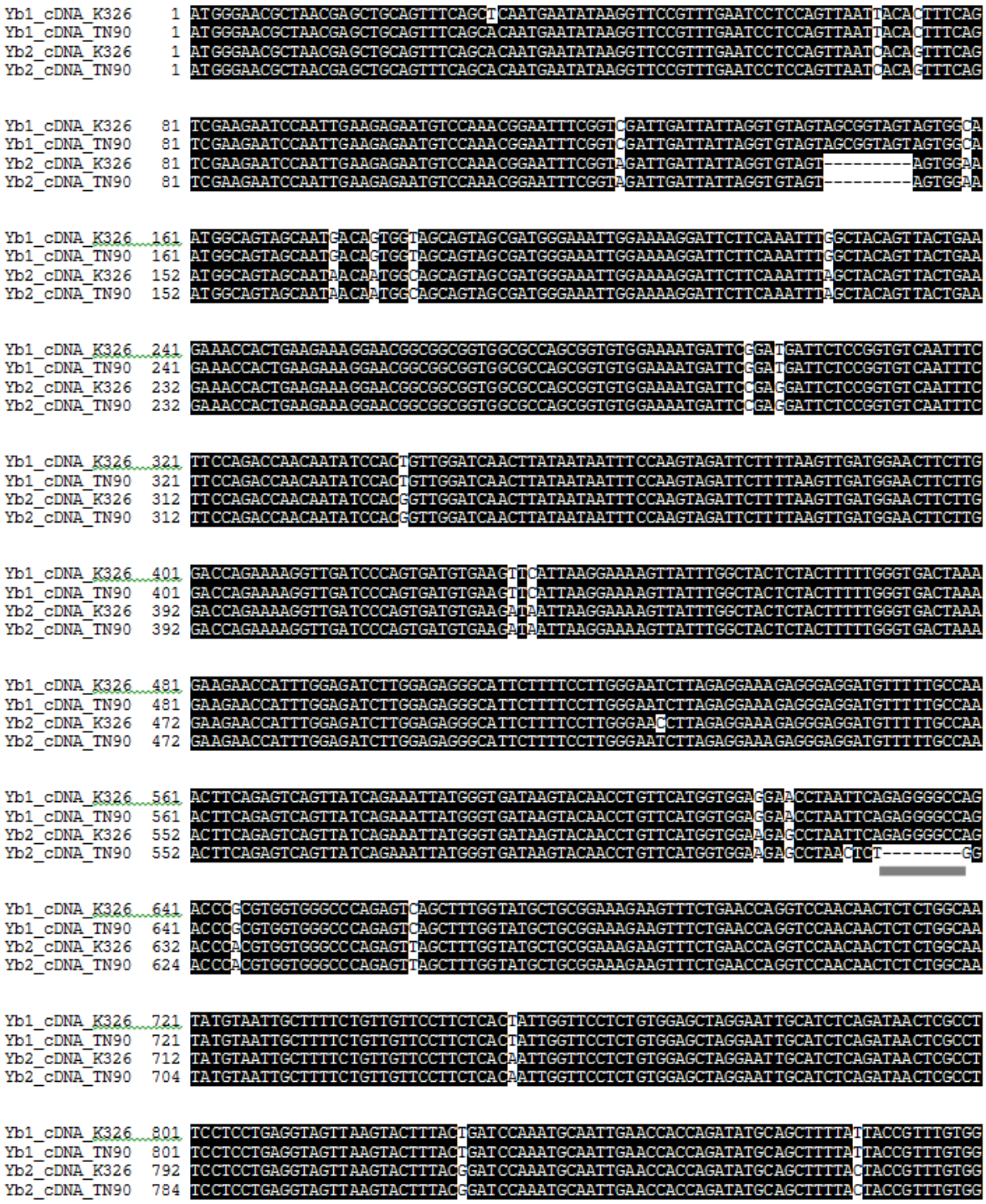
Figure 4.S1. cDNA alignments of *Yb* genes. Alignments were generated using Vector-NTI AlignX. Polymorphisms are indicated with a white background. The mutations in TN 90 which result in the yellow burley phenotype are underlined with solid gray lines.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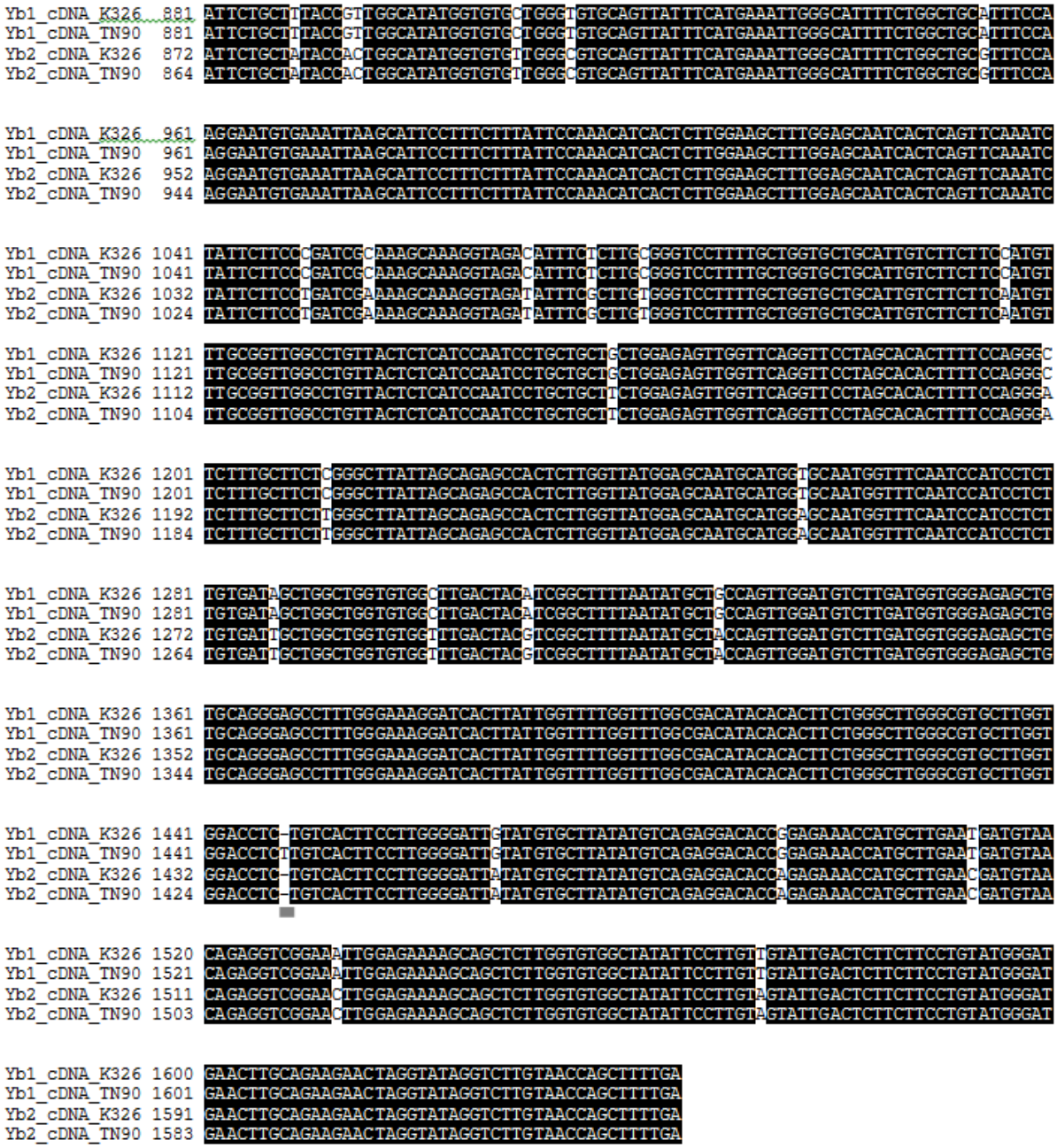
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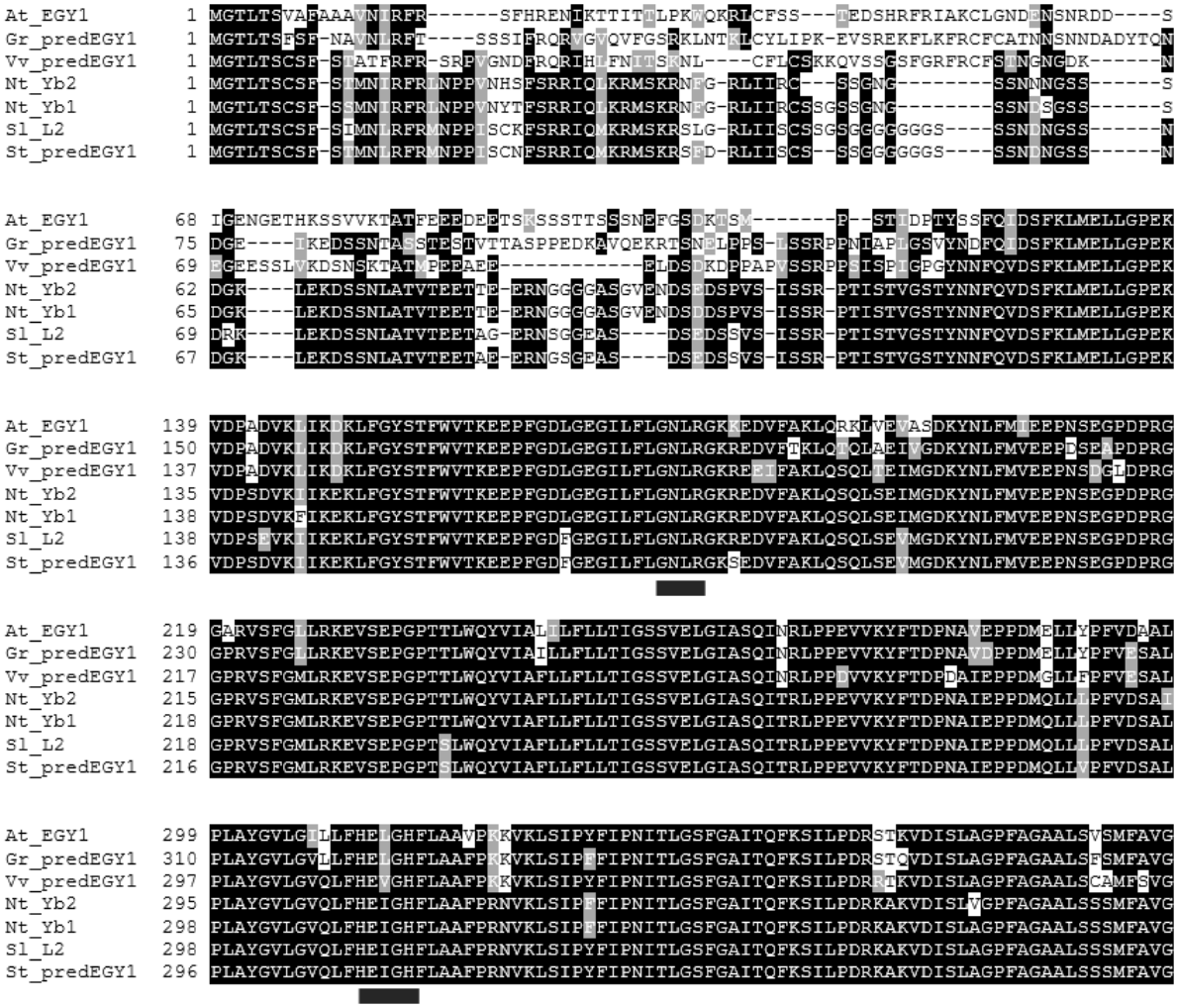
Figure 4.S2. Amino acid alignments of Yb related proteins. Alignments were generated using MUSCLE. Conserved amino acids are indicated by shaded squares. The three conserved domains, GNLR, HEXXH, and NPDG are underlined with black lines. Accession numbers are as follows: *Arabidopsis* EGY1 (NP_198372), *Gossypium raimondii* predicted EGY1 (XP_012492480), *Vitis vinifera* predicted EGY1 (XP_002269447), *S. lycopersicum* L2 (NP_001299816), *S. tuberosum* predicted EGY1 (XP_006339202).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