

ABSTRACT

MCCACHREN, PATRICK STEPHEN. Investigating the Genetics of Flowering Time and Black Shank Resistance, and Testing a Novel Method of Paternal Haploid Isolation in Tobacco (*Nicotiana tabacum* L.). (Under the direction of Dr. Ramsey S. Lewis.)

Genetic studies have the potential to provide insight into the nature of important traits in tobacco. Recently, a large increase in the number of simple sequence repeat (SSR) markers available for tobacco has expanded the potential for mapping genes controlling quantitative traits in tobacco. We used these recently published SSRs to expand the map coverage for a 'Beinhart-1000' x 'Hicks' mapping population in order to detect new QTL affecting flowering time and resistance to the causal agent of the black shank disease, *Phytophthora nicotianae* Breda de Haan. An enhanced linkage map consisting of 276 markers was generated, and as a result, a total of nine black shank resistance QTL were detected in the improved model. In addition, four different disease severity assessments were compared for their ability to detect QTL and to explain the largest amount of the genotypic variation for black shank resistance. Area under the disease progress curve (AUDPC) was found to be the most informative of the four models, yielding the largest number of significant QTL, providing the smallest QTL confidence intervals, and explaining nearly 100% of the genetic variation. Secondly, a model consisting of 10 QTL was created to explain genetic variation for flowering time. Each of the QTL within the model had moderate to small effects on flowering time.

When a new tobacco hybrid with superior genetics has been identified and selected for commercial release, the female parent must first be converted to cytoplasmic male sterility in order to simplify the seed production process and protect the intellectual property of the parents. Haploidy in tobacco is a naturally occurring phenomenon that can be induced

or selected for. In some circumstances, haploidy could be used to expedite the conversion of male fertile breeding lines to cytoplasmic male sterility. A study was undertaken to determine the feasibility of identifying paternal haploids from seed using the 'Xanthi 35S:PAPI' method of haploid selection. Large amounts of seed were produced by crossing the purple Xanthi 35S:PAPI line as a female with three different green paternal parents: 'TN 86,' 'K 326,' and 'Narrow Leaf Madole.' Fourteen green plants were identified among the progeny from these crosses and were saved for further testing so that their ploidy level could be determined. Using flow cytometry, it was determined that of the 14 green plants, eight were true haploids. Overall, the average rate of paternal haploidy observed for the three crosses was very low, one haploid per 199,243 seed. Although the frequency is low, the high reproductive capacity of tobacco could make this a viable system for isolating paternal haploids from seed. Possibilities for increasing the frequency of paternal haploids need to be explored, however.

In an additional study, investigations were made into previous reports suggesting a link between *Tobacco mosaic virus* (TMV) infection and increased resistance to black shank among the progeny of infected tobacco plants. This study was conducted to determine the feasibility of utilizing TMV inoculation as a means of increasing the heritable black shank resistance of tobacco breeding materials. Seed was harvested from individual plants of three different genetic backgrounds that had either been inoculated with TMV or mock inoculated as controls. Progeny from these plants were grown in soil-borne disease nurseries infested with *P. nicotianae*. Highly significant differences in disease severity were observed between differing genetic backgrounds. Within a given genetic background, however, no significant increases in resistance were observed between progeny derived from TMV infected plants

and progeny from their uninfected counterparts. Overall, this technique was determined to be an ineffective means of generating *de novo* black shank resistance in tobacco.

Investigating the Genetics of Flowering Time and Black Shank Resistance, and Testing a Novel Method of Paternal Haploid Induction in Tobacco (*Nicotiana tabacum* L.)

by
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DEDICATION

This work is dedicated to my parents, William and Pam McCachren. They have given me an incredible amount of support in my academic endeavors, and most importantly, they have shown me every day what it means to be a wholehearted follower of Christ.

BIOGRAPHY

Patrick McCachren was born in Harrisburg, North Carolina, where he lived until he began college. Patrick first discovered his interest in plants when he was about four years old, and has continued studying them at some level ever since that time. In the tenth grade, he was intrigued by Mendel's work, and given access to the proper germplasm may have attempted to replicate the experiments himself. This interest followed him to North Carolina State University, where he started college in the fall of 2006.

At NCSU, Patrick majored in Turfgrass Science and began to study the work of a few turfgrass breeders including Dr. Glenn Burton, Dr. Wayne Hanna, and David Doguet. It was at this point that he decided to become involved in the breeding process. He graduated Summa Cum Laude in the fall of 2009, obtaining a B.S. in Turfgrass Science. Following the suggestion of his undergraduate plant genetics professor, Dr. Susana Milla-Lewis, Patrick began his graduate work in the spring of 2010 under the direction of Dr. Ramsey Lewis. Being a part of Dr. Lewis's breeding program, Patrick has learned the art and science of field experiment management, nursery management, and QTL mapping and marker analysis. Patrick would like to enter the private sector, and will be seeking employment upon the completion of his M.S degree.

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CHAPTER 1: LITERATURE REVIEW

Tobacco's Role in Agriculture: An Introduction

Tobacco production in the United States dates back to the colonial era, and in fact, many of the first settlers in North Carolina were farmers that moved southward from Virginia around 1650, driven by a need for fresh, fertile tobacco land (Lilly, 2012). In the many years between then and now, tobacco has largely remained an important commodity in the economy of the nation, but more particularly, the economy of North Carolina.

Tobacco production in North Carolina peaked in 1939, when 843,000 acres of tobacco were grown statewide (Lilly, 2012). Since that time, the tobacco acreage grown on both a statewide and a nationwide basis has declined steadily (Capehart, 2004). By 2011, the national flue cured tobacco acreage had retreated to approximately 206,700 acres, yielding 347 million pounds of tobacco (USDA, NASS, 2012). Nevertheless, in 2010, North Carolina flue-cured growers produced a total of 348.6 million pounds of tobacco, 77% of the national crop (Brown, 2011). According to the USDA National Agricultural Statistics Service (2012), the value of the national flue-cured tobacco crop in 2010 was \$763 million. Of that amount, the value of the flue-cured tobacco grown in North Carolina during the same time was estimated to be \$588 million (Brown, 2011).

As one might imagine, tobacco plays an important role in the North Carolina state economy and the national economy, even in the present day. For this reason, it continues to be studied as an agricultural crop, and new issues pertaining to the management of the crop continue to surface. This review will be dedicated to a discussion of some of the historical innovation and background leading up to the present day research in tobacco.

Black Shank as an Obstacle to Tobacco Production

Black shank, caused by the pathogen *Phytophthora nicotianae* (Breda de Haan) was first identified in the Dutch East Indies by Van Breda de Haan in the year 1896 (Breda de Haan, 1896). At that time, he described the pathogen as *Phytophthora nicotianae*. Although other names have been proposed (the most current revision being *Phytophthora nicotianae* var. *nicotianae*), the original name is still the most widely accepted designation for this organism.

Black shank is a destructive disease of tobacco, affecting mainly the roots and stem of the tobacco plant. Often, the earliest symptoms resemble those associated with drought stress, where plants will wilt or flop over when evapotranspiration is at its peak, but do not fully recover during the night when evapotranspiration is lower. As the disease progresses, a blackened lesion forms on the stem, starting in the crown and spreading up through the stalk, with the capability of traveling through the stem to a height of 30 centimeters or more above ground level. When such a diseased stem is found, splitting it open will often reveal darkened, dried-out pith that has separated into plate like discs (Lucas, 1975). Because this pathogen often enters through the roots before colonizing and killing the stem and root tissue, it is capable of causing total plant death. Cultivars are available, however, that carry varying levels of resistance to the disease (Shew, 1987; Antonopoulos et al., 2010). It has been shown that older root tissue is more resistant to infection by the pathogen, and that younger root tissue is more susceptible (English and Mitchell, 1989). Interestingly, cultivars that are resistant to black shank as a result of the 'Florida 301' type of quantitative resistance have been observed to produce smaller root systems, and as a result, are more prone to lodging

(Jones and Shew, 1995; Antonopoulos et al., 2010). Compared to susceptible varieties, lines with this type of resistance are less likely to develop lesions when roots are directly exposed to the pathogen (Jones and Shew, 1995). Additionally, the reduced root system in cultivars with Florida-301 resistance is a result of reduced root branching, not reduced root length. Fewer root branches necessitate fewer young growing points, thereby reducing the total number of infection courts (Jones and Shew, 1995). It is also known that the pathogen is most capable of infecting plants when temperatures are favorably warm, between about 24 and 30 degrees C (McCarter, 1967; Lucas, 1975; Jacobi et al., 1983). Fresh transplants, however, are much more susceptible than mature plants if environmental conditions for pathogenization are optimal. Plants that have been recently set in the field can be colonized by black shank if the temperature averages 20 degrees C or above during the first 5-7 days after transplanting. Temperatures above this threshold drastically increase the chances of successful infection (Lucas, 1975). In addition to temperature, precipitation can affect disease pressure. Black shank, being an oomycete, is able to spread through soil water and surface water, so it is often more prevalent in areas and years where there is average or excess rainfall or runoff (Jacobi et al., 1983; Shew, 1987).

The prevalence of black shank has increased over time. While it caused an estimated crop loss of 1% in 1973 (Lucas, 1975), losses have been greater than 2% in recent years. In 2011, 'moderate' losses due to the disease were experienced, and 2.53% of the North Carolina tobacco crop was lost that year due to black shank. This was less than the economic damage incurred in 2010 when 3.87% of the crop (approximately 6.12 million kilograms of tobacco, worth \$22.9 million) was lost due to the disease (Brown and Snell, 2011; Mila and

Radcliff, 2011; USDA, NASS, 2012). With economic losses running into the tens of millions of dollars, the pathogen has required a significant amount of attention from pathologists and breeders alike (Carlson et al., 1997; Johnson et al., 2002a, 2002b; Sullivan et al., 2005). One of the earliest responses to the disease, aside from crop rotation, was the 1943 release of ‘Oxford 1,’ the first flue-cured tobacco cultivar containing black shank resistance. Incorporation of genetic resistance into tobacco varieties has been an objective of breeders since that time (Bowman and Sisson, 2000).

Historical Approaches for Controlling Black Shank

In the earliest days, abandoning a field was the most reliable way to reduce losses due to black shank, since the disease persisted in infested fields even when 5 year rotations were used (Tisdale and Kelley, 1926). Later, resistance derived from cigar cultivar ‘Florida 301’ was introduced into flue-cured tobacco. Cultivars carrying Florida 301-derived quantitative resistance generally exhibit lower levels of root colonization than cultivars lacking this kind of resistance (Nusbaum, 1952). This quantitative resistance, though not complete, allowed growers to rotate tobacco with other crops on fields that were infested. Varieties with increased resistance could be rotated as often as every two years, whereas cultivars that had less resistance could be rotated once every four years or more (Kincaid, 1960). Since then, more complete resistance to race 0 in the roots and stem has become available through the introgression of the *Php* and *Phl* genes. Of particular interest, these *Ph* genes are also much more effective in providing race 0 resistance in the stem of the plant than Florida 301, which is only slightly resistant to race 0 infection of the stem (Csinos, 1999). In 1953, the *Php* locus was transferred from *Nicotiana plumbaginifolia* Viv. to *Nicotiana tabacum* via an

interspecific cross. After additional crossing, the line 'PD 468' was produced (Chaplin, 1962). This line exhibited inferior agronomic characteristics, however, reducing its attractiveness as a source of germplasm (Chaplin, 1962). Apple (1962b) reported a more successful independent introgression of the *Php* allele from *Nicotiana plumbaginifolia* during the development of NC1071 and similar lines. This material was resistant to race 0 and exhibited acceptable yield and quality. It is believed that this line served as the donor for the *Php* gene used widely in flue-cured tobacco breeding (R.S. Lewis, personal communication, 2012). The *Ph* gene present in 'Coker 371-Gold,' the ancestor of many of the *Ph*-bearing varieties, was found to be of the *Php* type (Johnson, 2002a), and may have been the introgression resulting from the work of Apple (1962b) (R.S. Lewis, personal communication, 2012). Unfortunately, neither the *Phl* nor *Php* genes provide resistance to race 1 of *P. nicotianae*. This new race was first documented in 1962, and has increased in prevalence since that time (Apple, 1962a). Race 1 was first discovered in Kentucky, and it can be distinguished by its ability to overcome the *Ph* genes (Sullivan et al., 2005). Race 1 arose shortly thereafter in other locations, and within five years of the initial discovery, it was present in other tobacco growing regions, including North Carolina (Apple, 1967). Much of the current tobacco land now has some amount of race 1 present (Csinos, 1994; Antonopoulos et al., 2010). This means that in many fields, the *Ph* gene has more limited value in contributing to the genetic resistance of a given variety (Antonopoulos et al., 2010).

Another means of control is provided through the use of agrichemicals. Mefenoxam and metalaxyl are the only two fungicides currently labeled for use in tobacco to control black shank (Csinos, 2005). Early application is important for proper control. Later

applications risk the plant having been infected beforehand, which greatly reduces the effectiveness of the application. Furthermore, while effective, these chemicals can be expensive and do not provide complete control of the disease. Fumigation is also an option for severe instances of disease, especially those that exist in conjunction with nematode infestations. Chloropicrin products can be used for that purpose (Mila and Radcliff, 2011).

Current Status of Quantitative Resistance in Tobacco

Because of the prevalence of race 1 of *P. nicotianae* in growers' fields and the lack of completely effective chemical control, much attention at present remains focused on quantitative resistance derived from Florida 301 (Vontimitta, 2010) and the resistance loci present in another cultivar, 'Beinhart-1000' (Vontimitta and Lewis, 2012). This latter cultivar shows a greater degree of resistance to black shank than Florida 301, although one of the important quantitative trait loci (QTL) involved in disease resistance is either linked to or possibly pleiotropic with genes conferring flavor and aroma characteristics that are not acceptable for flue-cured or burley tobacco. Of the six QTL identified by Vontimitta and Lewis (2012), two are major QTL and four are minor QTL. These six QTL were able to explain 73% of the genetic variation. Due to incomplete map coverage it is likely that a portion of the important loci in this mapping population remained unidentified during the original study (Vontimitta and Lewis, 2012).

All modern cultivars carrying the *Php* gene, including Coker 371-Gold, also carry some level of quantitative resistance derived from Florida 301 (Johnson et al., 2008; Antonopoulos et al., 2010). For this reason, these materials can be utilized directly as elite breeding lines in future breeding efforts focused on increasing the level of quantitative

resistance present in commercial cultivars. Starting with these resistant lines as elite parents, additional resistance-associated QTL could be incorporated into them, ultimately resulting in higher overall resistance to race 1.

Another source of resistance, the *Wz* gene, is also being investigated at present. The *Wz* gene, ultimately derived from *Nicotiana rustica* and currently present in Zimbabwean breeding lines, has provided promising results in laboratory tests early on and may confer high levels of partial resistance against race 1 (Antonopoulos et al., 2010). More recently, field testing of materials carrying this gene has produced results suggesting effectiveness against both race 0 and race 1. Markers have been identified for the introgressed *N. rustica* region, so it is now possible to use marker assisted selection to identify and move this major gene within breeding populations (Drake and Lewis, 2012).

Measurements Used to Quantify Disease Severity

Many measurements exist for the purpose of quantifying disease severity, including the area under the disease progress curve (AUDPC), end percent survival, logit transformation, and the disease index (DI) (Bartlett, 1936; Van der Plank, 1963; Berger, 1981; Csinos et al., 1984). AUDPC is a very accurate tool for assessing quantitative disease resistance, and works especially well for polycyclic pathogens like black shank (Kannwischer and Mitchell, 1978; Jeger and Viljanen-Rollinson, 2001). This assessment requires multiple rating intervals throughout the growing season in order to quantify the rate of disease progress. The equation for this disease assessment is best described by Shaner and Finney (1977). The disease index is similar to the AUDPC, but does not take into account the specific length of the rating interval, instead assuming a fixed interval between all rating

dates (Csinos et al., 1984). In contrast, end percent survival is a measurement normally used to provide a rapid, but less accurate, analysis based on only one final rating date. It can provide a reasonable estimate of genetic resistance and disease severity, but is best reserved for situations where resources are limited and only one rating can be taken (Das et al., 1993). Arcsine square root transformation can stabilize the variance of data expressed in this manner (Bartlett, 1936). The logit transformation as described by Berger (1981) can also be used to give a better estimate of the variance of the data. Logit transformation expresses phenotypic traits with binary outcomes such that the outcomes of the total number of samples are summarized as a ratio of the log odds of samples resulting in one outcome compared to the other.

Flowering Time and its Importance in Tobacco

Flowering time is a highly heritable trait in many crops. In soybeans, for example, high heritabilities for flowering time have been reported (Weber and Moorthy, 1952). Likewise, it is also known to be a highly heritable trait in tobacco, and most of the genetic variation is additive in nature (Robinson et al., 1954). Flowering time in tobacco is positively correlated with yield (King, 1986), and also highly correlated with leaf number (Legg et al., 1965). Tobacco is typically considered a day-neutral species, although, short day flowering tobacco types have been identified and studied (Waterkeyn et al., 1965). The short day, or mammoth, types normally do not flower until more than 40 leaves are produced (Allard, 1919; Mann and Chaplin, 1957). From a different approach, the *Many Leaves* gene has also been studied as a possible means for influencing the number of days to flowering (Clausen and Cameron, 1944; Lewis et al., 2007). Considering the focused interest on major

genes that control flowering time, it may also be interesting to investigate QTL that are already present in past and current cultivars to determine their effect on reproductive maturity and yield.

History of Tobacco Breeding

Tobacco varieties were traditionally released as inbred lines, this being the simplest means of obtaining a true breeding cultivar. Historically, hybrids have lacked substantial heterotic yield advantage to justify the extra expense of producing hybrid seed (Aycock, 1980). In contrast, most modern cultivars are F₁ hybrids, resulting in part from the use of the *Ph* and *N* genes which can significantly reduce yield and quality when present in the homozygous condition (Legg, 1982; Wernsman and Rufty, 1987; Bowman and Sisson, 2000; Parkunan et al., 2010). Additionally, tobacco seed is now available for sale in foreign markets where hybrids are preferred because their cytoplasmic male sterility makes property rights management more straightforward (Bowman and Sisson, 2000). Tobacco breeding has been narrowly limited to the use of a few parent cultivars. In the flue-cured market class, for example, 'Hicks' and 'Coker 139' are in the pedigrees of more than 75% of the varieties released between 1964-1984 (Bowman et al., 1984). Pedigree selection is the most common style of breeding used for tobacco, though single seed descent, doubled haploidy, and recurrent selection have also been used under appropriate circumstances (Wernsman and Rufty, 1987). Cross pollination of tobacco flowers must be done by hand, as natural outcrossing is very low (1-2%). Hand pollinations will generally produce 2500-3500 seed, however, making hybrid seed production possible. Plants can simply be sprayed with insecticide, bagged, and left alone if self-pollination is necessary. For cross pollinations and

self-pollinations performed by hand, hanging tags are used to identify the paternal parent of the cross as well as any other important information (Wernsman and Rufty, 1987).

The Role of Molecular Studies in Tobacco Breeding

QTL mapping and other molecular marker studies have been carried out in tobacco since the 1990's to meet a variety of research goals. One of these early attempts successfully identified the location of the *Rk* gene that confers resistance to the root knot nematode (*Meloidogyne incognita*) using RAPD markers (Yi et al., 1998). Another such experiment used RAPDs to identify a genomic deletion associated with PVY resistance in the line 'Virgin A Mutante.' This research led to the suggestion that Okinawa 1 and Kerti No. 1 be used as donor lines for the resistance trait, because they appeared to have either a very small deletion, or simply a loss of function of the allele. These two cultivars were thought to be missing the smallest portion of the genome, while other lines were thought to have larger chromosomal deletions that conferred the resistance, along with agronomic inferiority (Noguchi et al., 1999). AFLPs became popular following RAPDs, and their use continues to the present day. A reasonable number of studies have been conducted using this type of marker. Lewis et al. (2005) used AFLPs to map the location of the *N* gene in tobacco. The *N* gene is responsible for the hypersensitive response that results from exposure to the *Tobacco mosaic virus* (TMV) pathogen, and markers linked to this trait could be used in marker assisted backcrossing. While AFLPs were popular during their time and are still used in tobacco, advancing technology and simpler protocols for microsatellite markers led the tobacco community to transition to microsatellites in the late 2000's.

Currently, the most advanced marker used in tobacco is the simple sequence repeat (SSR), or microsatellite marker. The first SSR map for tobacco was published by Bindler et al. (2007), and comprised a total of 293 loci covering 1930 centimorgans. These new SSRs are advantageous because they only amplify one or a few bands of DNA and they produce reliable, codominant results that typically require little effort to score compared to older marker protocols. The initial SSR map was expanded when Bindler et al. (2011) published an updated version including 2363 loci covering a map distance of 3270 centimorgans. This second map displays significant improvements over other existing marker resources in tobacco, and it provides a marker density great enough to support in-depth marker based breeding efforts. SSRs have been used in a limited number of studies to date, as a result of their relatively young age. One such SSR-based mapping study was performed by Vontimitta and Lewis (2012), and resulted in a number of black shank resistance QTL being detected from a relatively wide intraspecies cross. Moon et al. (2009) utilized the SSR map to genotype 702 diverse tobacco accessions from the U.S. *Nicotiana* germplasm collection in order to analyze their diversity. As a result of this study, accessions were clustered to form seven distinct groups, with each group corresponding roughly to a specific geographical region of origin. SSRs, despite their recent advent in tobacco and their functionality and ease of use, may soon be superseded by a newer technology in the form of a SNP chip (R.S. Lewis, personal communication, 2011).

QTL Mapping in Tobacco

Historically, QTL mapping has been performed only sparingly in tobacco. This is largely due to the low levels of marker polymorphism that were observed in tobacco using

the early marker protocols. In the past, genotyping projects were largely limited to identification of markers that were associated with traits under the control of a single gene. For example, Lewis et al. (2007) was able to locate a major QTL affecting flowering time and yield that had been introgressed into a 'Red Russian' background using AFLP markers. There were also some early attempts aimed at mapping the genomes of diploid tobacco relatives using RAPDs and RFLPs, including work done by Lin et al. (2001) and Suen et al. (1997). This kind of work was the norm until Bindler et al. published the first SSR map of tobacco in 2007. Upon the release of the first SSR map, it became possible to use the more straightforward SSR protocol to genotype larger numbers of known loci, place them on a map in a more meaningful way, and analyze them to determine where QTL might exist. Vontimitta (2010) was able to compare the resistance loci identified within Beinhart-1000 to the resistance loci derived from Florida 301 and found that some of the QTL were likely present in both lines, some were present in only one line, and some showed differential effects between the two lines. Vontimitta and Lewis (2012) were also able to detect six black shank resistance QTL in a Beinhart-1000 x Hicks mapping population. These were the first two instances where genome-wide mapping was employed with the intent of mapping multiple QTL within a given population or parent line, and more such studies may follow.

The ultimate goal of mapping studies is to link markers with the trait(s) of interest in such a way that the trait(s) can be tracked and selected for during multiple generations of crossing. This allows breeders to perform simple, non-destructive molecular tests in place of laborious phenotypic data collection. One such instance where marker technology has been used in tobacco is in backcrossing the *Many Leaves* QTL into favorable genetic backgrounds,

including K326, Speight 168, and NCTG-61 (D.P. Eickholt, personal communication, 2012). In this experiment, the introgression of the *Many Leaves* QTL increased acre value significantly in both the Speight 168 and NCTG-61 backgrounds.

Mechanisms of Haploidy in Tobacco

Haploidy is a naturally occurring phenomenon in tobacco (de Nettancourt and Stokes, 1960). Early on, haploid technology was applied through the identification of haploid-diploid twins at the cotyledonary stage. This method exploited rare seeds that produce two seedlings, one being a haploid and the other a diploid. Another early method took advantage of the hypersensitive response (HR) to TMV conferred by the *N* gene present in one of the parents. This protocol called for the crossing of a female *n* gene plant with a male *N* gene plant. All of the progeny plants were inoculated at the first true leaf stage and hypersensitive plants, those containing the parental genome carrying the *N* gene, were discarded as being F₁ hybrids. The remaining plants that did not exhibit the HR were considered to be putative gynogenic haploids (de Nettancourt and Stokes, 1960).

Since both of the early protocols were laborious, more innovation was needed to make the process viable on a large scale. Two years later, it was discovered that haploids could be identified through the use of seedling genetic markers, where the haploids take on a different appearance or characteristic than diploids. Burk (1962) crossed a plant carrying the *yg/yg* (*yellow-green*) recessive genotype onto various other cultivars that bore the dominant phenotype *Yg/Yg*. Whenever a seedling with the yellow-green phenotype was identified, it was considered a probable haploid (*yg/--*), while the vast majority of other plants were assumed to be diploid F₁ hybrids (*Yg/yg*). This method was much simpler because visual

identification of haploids was rapid, yet, the technique had one major disadvantage. The parent from which haploids are derived must contain at least one copy of the *yg* gene, if not two, and all haploids identified by this method are of the *yg* genotype and phenotype. Because no tobacco cultivar is of the *yg/yg* genotype, this method was not practical for use in tobacco breeding (Lewis and Rose, 2011).

Later, the mechanisms of paternal haploidy were explored via the induction of androgenesis from microspores (Bourgin and Nitsch, 1967). In this instance, excised stamens were cultured on sterile media in an incubator until haploids were regenerated. These plantlets could then be moved to the greenhouse after growing to a sufficient size (Nitsch and Nitsch, 1969). This method was simple, did not require the presence of a specific gene or phenotype during the haploid selection process, and produced large numbers of haploids. However, Arcia et al (1978) and Burk and Matzinger (1976) both reported that the resulting dihaploids were lower yielding, averaging 15% lower than the parent lines in one study, and inferior for the purposes of cultivar development. Reed and Wernsman (1989) reported that the yield loss associated with anther culture was associated with increases in the nuclear DNA content of the doubled haploid lines.

A later attempt at producing haploids utilized wide crossing between *N. africana* and *N. tabacum* (Burk et al., 1979). This system was simple and practical from a breeding standpoint because no extra genes were required for haploid selection, and because gynogenic haploids produced using this method gave way to doubled haploids with superior agronomic performance relative to the anther culture derived doubled haploids (Wernsman et al., 1989). In this breeding scheme, a *Nicotiana tabacum* line acting as the female is crossed

with *N. africana*. Greater than 99% of the resulting seedlings fail to survive past the cotyledonary stage because of a hybrid lethality mechanism. The few surviving seedlings are a mixture of interspecific hybrids and haploids, the latter of which can be easily identified by eye with experience. This method is thought to give rise to haploids at a rate approximating the frequency of normal, spontaneous gynogenic haploidy (Burk et al., 1979). This system has proven to be effective, even preferable in many cases, for the purposes of gynogenic haploid production. It does not work well for the production of paternal haploids, however, because the reciprocal cross is not easily made (Gerstel et al., 1979).

Goodsell (1961) was able to recover cytoplasmic male sterile androgenic haploids in maize (*Zea mays* L.) at a very low rate of three in approximately 250,000 seeds. These plants were all restored to diploidy by pollination of the haploid ear, proving that cytoplasm transfer can be made via paternal haploidy. Later, Chase (1963) agreed, reporting the rate of paternal haploidy to be 80 fold lower than maternal haploidy, or approximately 1 in 80,000 seed. This type of work was performed in tobacco more recently with a dominant lethal allele, *Rac-*, which confers a rootless phenotype. The deficiency induced by the *Rac-* allele can be used to identify paternal haploids. This method calls for the crossing of normal rooted (*rac+/rac+*) plants onto a *Rac-* parent. Seedlings resulting from hybridization with the *Rac-* parent are rootless and die, while the haploid plants survive and grow normally (Horlow et al., 1993). It is very difficult to maintain the *Rac-* genotype, however, because the rootless plant must be successfully grafted onto a suitable rootstock in order to survive (Pelletier et al., 1987).

A suitable dominant genetic marker that is easy to manipulate and maintain did not exist until recently, when the *PAP1* gene from *Arabidopsis thaliana* was transformed into *Nicotiana tabacum* 'Xanthi' under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Xie et al., 2006). As a result of *PAP1* overexpression, this line becomes intensely purple within approximately 1 week of germination, depending on environmental factors. Haploids resulting from the cross of Xanthi 35S:*PAP1* x non-35S:*PAP1* will be green in color, and can be rapidly distinguished from purple F₁ hybrids (Lewis and Rose, 2011). This system may be less efficient for haploid production, because available data indicate the rate of haploidy to be sevenfold less than the rate observed in crosses with *N. africana*. It could be useful for the production of androgenic haploids, however (Lewis and Rose, 2011).

Following any of these methods of haploid production, it becomes necessary to reconstitute a diploid from the haploid in order to continue the line from seed. Traditionally, chromosome doubling has been done by the leaf midvein culture method described by Kasperbauer and Collins (1972). *In vitro* midvein culture may induce somaclonal variation, however, whereas reproduction by seed does not (Larkin and Scowcroft, 1981). Colchicine has also been used for this purpose, but often produces erratic results (Burk et al., 1972). In maize, Goodsell (1961) was able to restore diploidy by pollinating the ears of haploid plants with diploid pollen. Some of the seeds produced from this cross turned out to be fertile diploids. Correspondingly, Burk (1962) reported that, while tobacco haploids have no detectable pollen fertility, haploid plants can set a few viable seed when crossed with a diploid plant acting as the pollen donor. The cross pollination technique is not used routinely to restore diploidy, possibly as a consequence of the heteroploid plants often resulting from

this type of cross (Burk, 1962). More importantly, in many instances restoring diploidy by outcrossing would introduce significant heterozygosity into the restored diploid, thus defeating the purpose of haploidy for most breeding applications. As the sole exception, this method may become important as a means of introducing cytoplasmic male sterility into elite lines (Chase, 1952; Goodsell, 1961; Lewis and Rose, 2011). In this case, an inbred diploid version of the haploid line would already exist, and could be used as the paternal parent in the cross without risk of introducing unwanted heterozygosity.

Tobacco Mosaic Virus as a Pathogen and a Mutagen

Tobacco mosaic virus (TMV) is a viral pathogen of tobacco and many other plants. Advanced cases of TMV are the most obvious, producing a mottling or mosaic pattern on the leaves, while early symptoms typically include areas of the leaf near the vein becoming a much lighter shade of green than the surrounding tissue, a phenomenon referred to as vein clearing (Lucas, 1975). TMV is easily spread through contact, and the infection it produces can sometimes affect entire fields, which can be devastating to crop yields (Hull, 2009). Currently, resistance to this disease is provided by the *N* gene, a dominant gene that causes a hypersensitive response distinguished by small local lesions of brown, necrotic tissue surrounding the infection site. This resistance gene is derived from the wild relative *Nicotiana glutinosa*, and was introduced to *Nicotiana tabacum* via crosses with the synthetic amphidiploid *Nicotiana digluta* (Clausen and Lammerts, 1929; Holmes, 1938). Another form of resistance that has been experimented with, but never deployed, is conferred by the transformation of tobacco with the TMV coat protein. This type of resistance reduces, and in some cases eliminates, the symptomology of TMV infection (Abel et al., 1986).

Occasionally, viruses have been reported to act as mutagenic agents. The first reports of this kind were in maize, where aberrant segregation ratios for major genes were observed as a result of infection with *Barley stripe mosaic virus* (Sprague et al., 1963; Sprague and McKinney, 1966, 1971). Since then, similar observations have been made in tobacco, where *Tobacco mosaic virus* inoculation resulted in various epigenetic changes (Boyko et al., 2007). In tobacco, TMV was reported to induce a systemic signal in the plant that increased the homologous recombination rate. Specifically, large amounts of recombination were observed in a genomic region homologous to the *N* gene (Boyko et al., 2007). Additionally, DNA methylation patterns were abnormal. While overall methylation of the genome increased, certain sites surrounding regions homologous to the *N* gene were found to be substantially hypomethylated (Boyko et al., 2007). TMV infection has also been reported to cause broader epigenetic changes resulting in greater tolerance to a number of stresses, possibly as a result of increased *PR1* expression, a response often associated with systemic acquired resistance (Kathiria et al., 2010). The outcome of TMV inoculation is thought to be dependent on the time and intensity of the infection, as well as other environmental factors. One of the most interesting phenomena associated with TMV inoculation is the potential for an increase in black shank resistance among some of the progeny lines (Kathiria et al., 2010). In this study, the progeny of TMV-infected individuals experienced, on average, less than 33% disease incidence when inoculated with black shank, while 87% disease incidence was observed for the control group.

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CHAPTER 2:

**MAPPING ADDITIONAL QTL AFFECTING BLACK SHANK RESISTANCE AND
FLOWERING TIME IN TOBACCO**

Abstract

Recently, the number of simple sequence repeat (SSR) markers available for tobacco (*Nicotiana tabacum* L.) has increased dramatically. This extra information has improved genomic coverage and enhanced opportunities for accurately mapping quantitative trait loci (QTL). We used these recently published SSRs to extend the map coverage for a 'Beinhart-1000' x 'Hicks' mapping population in order to detect new QTL affecting flowering time and resistance to the causal agent of the black shank disease, *Phytophthora nicotianae* Breda de Haan. An enhanced linkage map consisting of 276 markers was generated, and as a result, a total of nine black shank resistance QTL were identified in the new model. Three of these were previously undetected. Four different disease severity assessments were compared for their ability to detect QTL and to explain genotypic variation for black shank resistance. Of these assessments, the area under the disease progress curve (AUDPC) was found to be the most informative of the four, yielding the largest number of significant QTL while explaining nearly 100% of the genetic variation. Secondarily, a model consisting of 10 QTL was created to explain genetic variation for flowering time. In contrast to the situation for black shank resistance, the model explaining flowering time consisted of QTL having only moderate to small effects.

Introduction

Relatively few QTL mapping studies have been conducted in tobacco to this point. Early marker-trait association studies were generally focused on traits under the control of single genes (Yi et al., 1998; Noguchi et al., 1999; Lewis et al., 2005). Although some of the early work was aimed at mapping the genomes of wild relatives of tobacco (Suen et al.,

1997; Lin et al., 2001), the first linkage map for cultivated tobacco was not reported until 2007, with the publication of a 293 locus SSR-based map covering 1930 centimorgans (cM) (Bindler et al., 2007). Because these SSR markers are anchored on a linkage map, it is relatively simple to choose primers that will amplify DNA within a given region of interest to the researcher. Additionally, SSRs are more reproducible and exhibit greater polymorphism compared to older marker techniques (Moon et al., 2009). As a result of these benefits, SSR markers have permitted researchers to more efficiently conduct genetic investigations within this species. In one study, a preliminary effort was directed at the identification of SSR markers associated with QTL affecting black shank resistance derived from the cigar tobacco cultivar 'Beinhart 1000' (Vontimitta and Lewis, 2012b). Since that time, an updated edition of the SSR map has been published (Bindler et al., 2011) that contains a total of 2362 loci covering a map distance of 3270 cM. This information might be used to improve genomic coverage in the mapping population of Vontimitta and Lewis (2012b), permit identification of previously undetected QTL, and increase the resolution of interesting QTL.

Significant interest is currently focused on mitigating crop loss caused by black shank, a destructive pathogen of tobacco (Antonopoulos et al., 2010). This disease affects all of the major tobacco producing areas except Brazil (Gallup et al., 2006), and is historically the most important disease affecting tobacco production in the southeastern US. While total crop losses in North Carolina due to black shank were approximately 1% in 1973 (Lucas, 1975), losses have increased in recent years. In 2010 and 2011, crop loss was estimated at 3.87% and 2.53%, respectively (Mila and Radcliff, 2011). The economic impact of this disease is significant because of the high value of tobacco on a per-hectare basis. In 2010,

losses totaled approximately 6.12 million kilograms of tobacco, worth an estimated \$22.9 million (Brown and Snell, 2011; UDSA, NASS, 2012).

‘Florida 301’ was the first functional source of genetic resistance used in the development of black shank resistant cultivars. The quantitative type of resistance derived from Florida 301 has been successfully deployed in a large number of cultivars, although, it is only partially effective for reducing crop loss (Johnson et al., 2008). The first flue-cured tobacco cultivar to carry resistance to black shank was ‘Oxford 1,’ released in 1943 (Bowman and Sisson, 2000). Later, the *Php* gene was introgressed into *Nicotiana tabacum* from *Nicotiana plumbaginifolia*, and became a popular source of complete resistance to race 0 (Chaplin, 1962; Johnson et al., 2008). This resistance mechanism offers no resistance against the other races, however. Due to the wide-scale planting of *Php*-containing cultivars, a rapid rise in the prevalence of race 1 has been observed in growers’ fields, and the *Php* gene currently has reduced utility (Sullivan et al., 2005). For this reason, it would be beneficial if high levels of partial resistance could be selected for and subsequently deployed in commercial cultivars to combat this disease. It is thought that planting varieties expressing partial resistance will cause a shift towards strains of *P. nicotianae* that are more aggressive (Gallup et al., 2006; Dukes and Apple, 1961), although a cultivar rotation program involving multiple modes of black shank resistance may help to reduce the selection pressure for increased pathogen aggressiveness. Rotations intended to reduce selection pressure placed on race 0 have been described by Sullivan et al. (2005). Rotating between various resistance genes would benefit from the identification of new allelic variability affecting resistance, and the subsequent movement of those genes into elite germplasm. As

mentioned above, some preliminary work has focused on identifying QTL associated with black shank resistance in Beinhart 1000, a cigar tobacco cultivar exhibiting the highest known level of quantitative black shank resistance in *N. tabacum* (Vontimitta and Lewis, 2012b). Additional QTL mapping using the population of Vontimitta and Lewis (2012b) could be valuable for identifying favorable alleles affecting black shank resistance. Subsequently, marker assisted selection could be used as a means of integrating those alleles into breeding lines and commercial cultivars (Vontimitta and Lewis, 2012b). Utilizing the dense SSR map published by Bindler et al. (2011), it should be possible to increase marker density in areas of the genome that are currently unmapped for the population of Vontimitta and Lewis (2012b), which may lead to the discovery of new black shank resistance QTL.

In addition to the identification of new QTL, it would be interesting to compare the effectiveness of different measures of disease severity to determine which type of measurement provides the most accurate resolution of QTL affecting resistance. Few studies of this type exist. One experiment conducted in apple (*Malus domestica* Borkh.) compared the significance of QTL detected using both AUDPC and percent lesion length (lesion length ÷ shoot length) at multiple rating dates post-inoculation (Khan et al., 2006). A second study in winter rapeseed compared the robustness of QTL detection for both end percent death and disease index (Pilet et al., 1998). It would be beneficial if some of the common phenotypic measures used in QTL mapping, including end percent survival (Vontimitta and Lewis 2012a, 2012b) and disease index (DI) (Chaerani et al., 2007), could be compared to the AUDPC in order to gain insight on how each of these different techniques relate to each

other, and ultimately to find out which techniques may be the most useful for QTL identification.

Flowering time is another very important trait in tobacco because days to flowering is positively correlated with leaf number, and consequently yield (Lewis et al., 2007). Very late flowering can be undesirable, however. Two major genetic systems (photoperiod sensitivity controlled by recessive alleles at two loci, and the *Many Leaves* QTL) have been studied for the purpose of delaying flowering time in tobacco (Clausen and Cameron, 1944; Waterkeyn et al., 1965; Lewis et al., 2007). Breeders have experimented with the effects of these genes (Wernsman and Matzinger, 1980), but neither source of variability has yet been deployed widely in commercial cultivars. If moderate delays in flowering time were desirable, it may be possible to utilize the variation present within current germplasm, as most variation for flowering time is controlled by genes with small additive effects (Matzinger et al., 1962). Specifically, increased understanding of the genetic control of flowering time might allow breeders to make strategic, marker-based decisions that favorably influence tobacco yields.

The primary objective of this study was to conduct additional SSR genotyping in the Beinhart 1000 x Hicks doubled haploid mapping population of Vontimitta and Lewis (2012b) in order to increase the genomic coverage beyond what has been previously reported. Following this, the increased data set was re-analyzed to determine if additional QTL affecting black shank resistance could be detected. As an added measure, four different types of disease assessments were compared to determine which was the most valuable for identification of QTL affecting resistance. The final objective of this experiment was to

determine a QTL model explaining flowering time within the Beinhart 1000 x Hicks mapping population.

Materials and Methods

Origin of the Beinhart-1000 x Hicks Mapping Population

For this experiment, a mapping population consisting of 116 doubled haploid (DH) lines was used. To produce these lines, Beinhart-1000 was crossed with Hicks, and the F₁ from this cross was hybridized with *N. africana* according to the method described by Burk et al. (1979). Haploids identified within the progeny of this cross were subsequently chromosome doubled using the tissue culture technique described by Kasperbauer and Collins (1972). This population is the same as the DH population described by Vontimitta and Lewis (2012b).

Phenotypic Data Analysis

Phenotypic data for this experiment was identical to the data used in Vontimitta and Lewis (2012b). Field data was collected from evaluation of the mapping population and parents in three soil-borne disease nursery environments during 2007 and 2008. In 2007, the experiment was evaluated at the Upper Coastal Plain Research Station (UCPRS) and the Lower Coastal Plain Research Station (LCPRS), but in 2008, it was evaluated only at the Lower Coastal Plain Research Station. The experimental design in each environment was a randomized complete block design with three replications. Experimental units were single plots 7 m in length, with a between-row spacing of 120 cm, and a within-row spacing of 56 cm. Each plot contained approximately 12 plants. In the 2007 UCPRS environment, plots were rated at 17, 29, 40, 50, 60, 70, 80, 92, 102, 113, and 123 days after the initial stand

count. In the 2007 LCPRS environment, plots were rated for disease at 20, 34, 44, 54, 65, 76, and 86 days after the initial stand count. In the 2008 LCPRS environment, the experiment was rated 27, 41, 55, 72, 84, and 97 days after the initial stand count. During each rating, plants were scored individually as being either dead or alive. Four different measures of disease were calculated (the arcsin square root transformed end percent survival, the AUDPC, the disease index, and the logit transformed end percent survival) so that a comparison could be made between the different rating systems. Before performing QTL analysis, the results from the first three of the four measurement types were combined into entry means by averaging each line across all reps and locations for each trait. Arcsin square root transformation was calculated as:

$$\text{Arcsin square root transformation} = \sin^{-1} \sqrt{P},$$

where P is the percent of plants that survived until the last rating date. AUDPC was calculated from the equation:

$$AUDPC = \sum_{i=1}^{n-1} \left[\frac{y_i + y_{i+1}}{2} \right] [t_{i+1} - t_i],$$

where n equals the total number of rating dates, i indexes each individual rating date, y equals the percentage of plants that died from black shank at any time before the i^{th} rating date, and t equals the number of days elapsed between the initial rating and the i^{th} rating (Madden et al., 2007). Even though the AUDPC was skewed toward susceptibility, previous research has shown that log transformation and square root transformation of the data does not significantly change the outcome of QTL mapping (Cobos et al., 2006). Data was therefore

left untransformed for ease of interpretation. Disease index, a similar measure employed by Csinos et al. (1984), was calculated from the formula:

$$Disease\ Index = \frac{\sum_{i=1}^n X_i \left[100 - \left((i - 1) \frac{100}{n} \right) \right]}{I},$$

where n is the total number of rating dates after the initial stand count, i indexes each individual rating date, X is the number of plants that have died between the i^{th} date and the $i-1^{\text{th}}$ date, and I is equal to the number of plants in each plot at the time of the initial stand count.

For the fourth measurement, the logit transformed end percent survival, entry means were formed by first summing the total numbers of plants at the initial stand count and the final stand count across reps, environments, and years. These entry means were then transformed using SAS software's PROC LOGISTIC function (SAS Institute, Inc., Cary, NC, USA), although this transformation was applied with caution, as Berger (1981) stated that skewness in the data may lead to erroneous conclusions when the logistic transformation is applied.

Flowering time data were collected during 2007 and 2008 at the Central Crops Research Station in Clayton, NC. The experimental design during each year was a randomized complete block design with two replications per year. Each plot consisted of a single five plant row. Individual plots were considered to be flowering when one-half of the plants in a plot exhibited pink flowers. For the purposes of QTL mapping, data were

averaged across reps and years to produce entry means. DH Entry 13 was excluded from this set because no data were available for 2008 due to a lack of seed for that line.

SSR Marker Genotyping

DNA was isolated using the CTAB procedure described by Vontimitta and Lewis (2012b). SSRs were amplified in 15 μL volumes containing: 5 μL of template DNA (5 ng/ μL), 1.5 μL of 10x PCR buffer, 1.5 μL of 20 mM MgSO_4 , 1 μL of 5M betaine, 1.2 μL of 2.5mM dNTP's, 0.15 μL of 1 μM forward primer stock, 0.75 μL of 1 μM reverse primer stock, 0.75 μL of 1 μM M13 primer stock, 0.2 μL of Taq (5 u/ μL), and 2.95 μL of dd H_2O . The M13 primer used in these reactions was labeled as described by Schuelke (2000), except that IRDye 700 and IRDye 800 (LI-COR Biosciences, Lincoln, NE) fluorescent tags were used instead of FAM.

Thermocycling consisted of a 5 minute denaturation step at 94° C, followed by 30 cycles of denaturation, annealing, and extension at 94°C for 30 sec, the T_m of the primer for 45 sec, then 72°C for 45 sec, respectively. Following this, another eight cycles of denaturation, annealing, and extension were executed at 94°C for 30 sec, the T_m of the primer minus 3°C for 45 sec, and 72°C for 45 sec, respectively. The program ended with a five minute extension step at 72°C.

Gels were run on a LI-COR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE) using 8% polyacrylamide gels. The run conditions were set to 1500 volts, 40mA, 40 watts, and a temperature of 45°C. Each run was 2.5 hours in duration. LI-COR IRDye 700 and IRDye 800 ladders, each 50-350 base pairs in length, were loaded along with the DNA

samples, and gels were used for up to three consecutive runs each. AFLP Quantar Pro v.1.0 (Keygene Products, B.V., The Netherlands) was used to score SSR gel images.

Using this technique, 242 of the SSR primer pairs published in Bindler et al. (2011) were screened for polymorphism between the two parents, Beinhart-1000 and Hicks. Primer pairs were selected based upon their map position according to Bindler et al. (2011), so that previously unmapped regions could be accounted for in the new map. Seventy-two polymorphisms were detected using these new primers. Polymorphic markers were subsequently used to genotype the entire population of DH lines. One SSR primer pair, PT60410, amplified two polymorphic bands, while all others amplified only one polymorphic band. Data collected from this experiment was merged with the data published in Vontimitta and Lewis (2012b) in order to assemble a more complete linkage map for the DH population.

QTL Analysis

Genotypic data were imported into JoinMap 4 (Van Ooijen, 2006) to construct a linkage map using Kosambi's maximum likelihood parameter (Kosambi, 1943). Only 116 of the DH lines were used during the mapping process, as lines 13 and 37 were excluded because of a large amount of missing marker data for each. Additionally, markers that remained unlinked after mapping were excluded from the final map.

Linkage map data were exported to Windows QTL Cartographer V2.5_010 (Wang et al., 2011) for QTL mapping. Composite interval mapping (CIM) (Zeng, 1993, 1994) was used for initial QTL identification. Before performing CIM, the LOD significance threshold for each disease measure, as well as for days to flower, was determined by testing with 1000

permutations, at a significance level of 0.05 according to the method of Churchill and Doerge (1994). The CIM standard model was used for mapping all traits, and walk speed was set to 1 cM intervals. Following this, the multiple interval mapping (MIM) function of QTL Cartographer (Kao et al., 1999) was used to generate a final model for each measured trait. QTL were accepted into the initial MIM model when the LOD score for a given peak in the composite interval map was greater than 2.0, with a minimum distance of 25 cM between QTL peaks. The initial model was modified by performing multiple iterations of searching for, repositioning, and testing the significance of QTL in the model. When the model was saturated, epistasis was added and all QTL and epistatic interactions were tested again in an iterative fashion as above. Models were only modified when doing so led to a decrease in the Bayesian Information Criterion (BIC) (Piepho and Gauch, 2001). Upon completion of the MIM procedure, the total amount of genetic variation for each trait was compared to the entry mean heritability for that trait. Entry mean heritability was calculated using a program based on PROC MIXED and PROC IML, written for the SAS system (Holland et al, 2003; Holland, 2012). For traits where the total amount of genetic variation explained by the QTL model exceeded the entry mean heritability, the QTL with the smallest effect was removed, and the remaining model was re-estimated, according to Robertson-Hoyt, et al. (2006) and Zwonitzer et al. (2009). This procedure was repeated until the variation explained by the model was equal to or less than the entry mean heritability. MapChart (Voorrips, 2002) was used to graphically represent the linkage map, as well as the results from QTL analysis.

Results

Phenotypic Data

Entry mean values for the 116 DH lines and the parents are shown in Appendix A for each of the four disease severity measurements and days to flowering. Arcsin square root transformed and logit transformed end percent survival data ranged from 0 to 1.38 and -14.66 to 5.48, respectively, where large values correspond to higher end percent survival. AUDPC values and DI values ranged from 1.44 to 53.48 and 2.07 to 63.64, respectively, where large values indicate increased susceptibility. Entry mean days to flowering ranged from 63 to 89.25 days after transplanting. Figures 1-5 show histograms of the distributions of the doubled haploid lines for each of the five measurements.

Linkage Mapping

SSR markers were initially compiled into 24 groups, and only 14 markers remained unassigned to those groups. Twelve of the remaining unassigned markers were manually assignable to the initial groupings via strongest cross linkages, corroborated with the high density reference map published by Bindler et al. (2011). Two of the markers, PT20165 and PT61019, shared no appreciable linkage with any other loci, and without corroborating evidence, their location could not be established using the high density reference map. Four of the six unlinked markers (PT20286, PT61153, PT55416, and PT20291-2) described by Vontimitta and Lewis (2012b) were successfully placed on the linkage map, which is shown in Figure 6. Addition of the new genotypic data increased the total number of mapped markers to 276 and extended the total map length to 2798.3 cM.

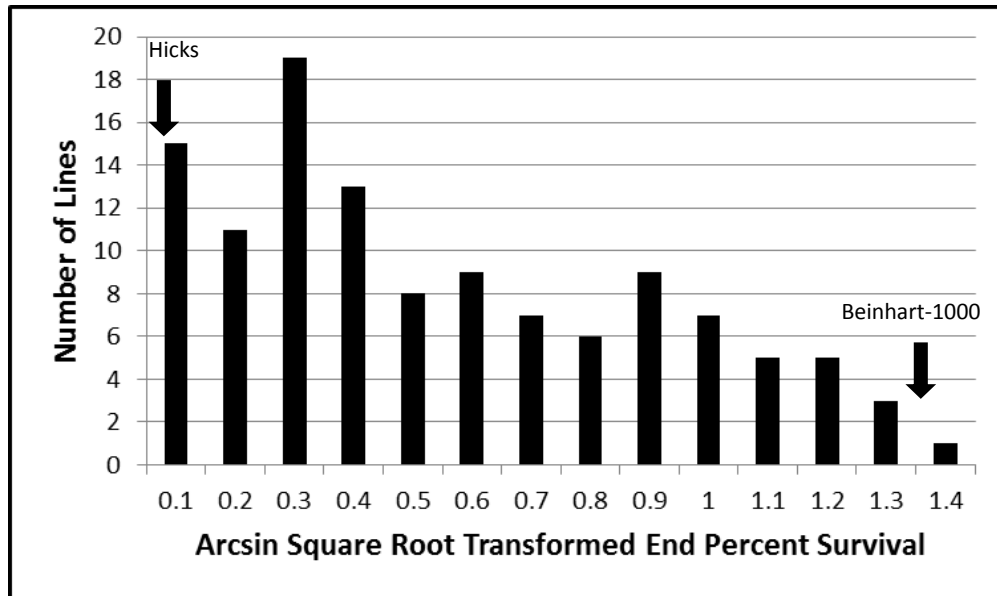


Figure 1: Histogram showing the number of lines for each level of arcsin square root transformed end percent survival

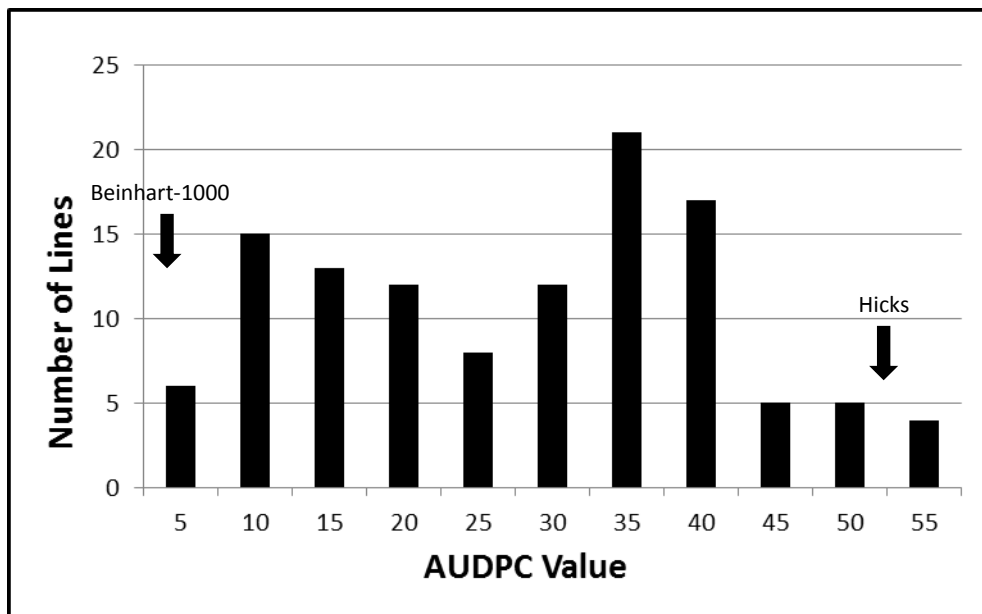


Figure 2: Histogram showing the number of lines for each level of the AUDPC.

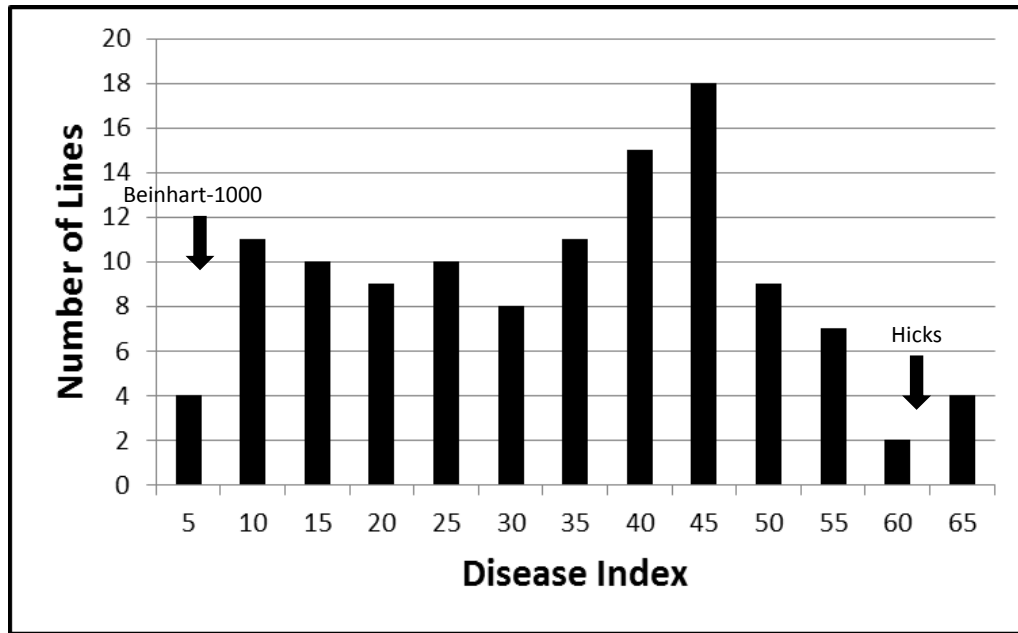


Figure 3: Histogram showing the number of lines for each level of the disease index.

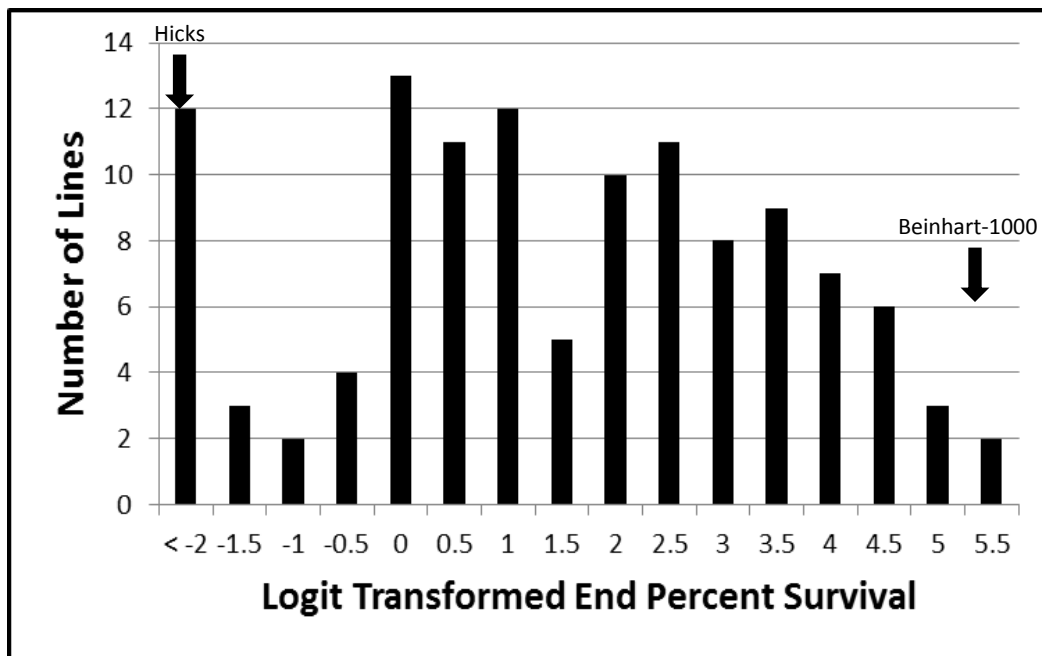


Figure 4: Histogram showing the number of lines for each level of logit transformed end percent survival.

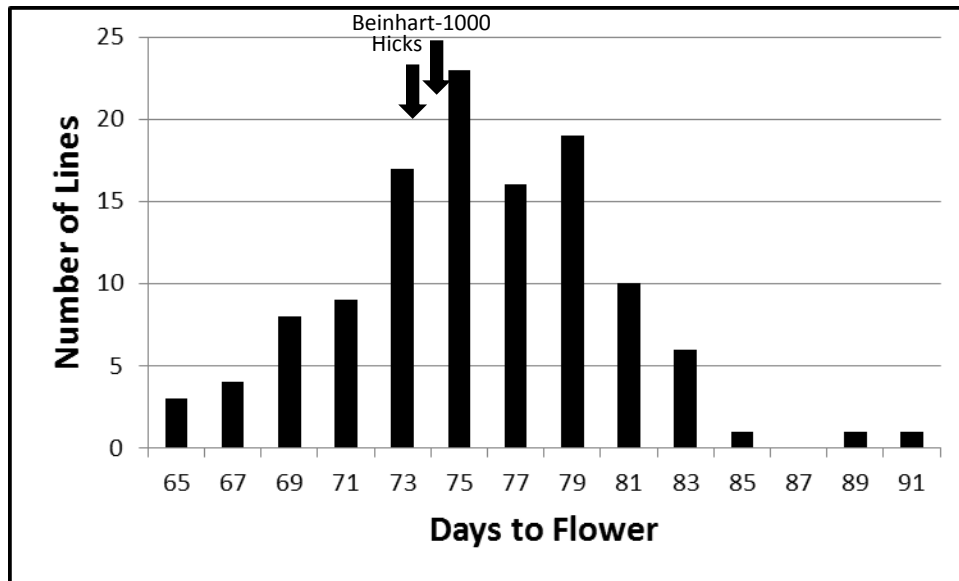
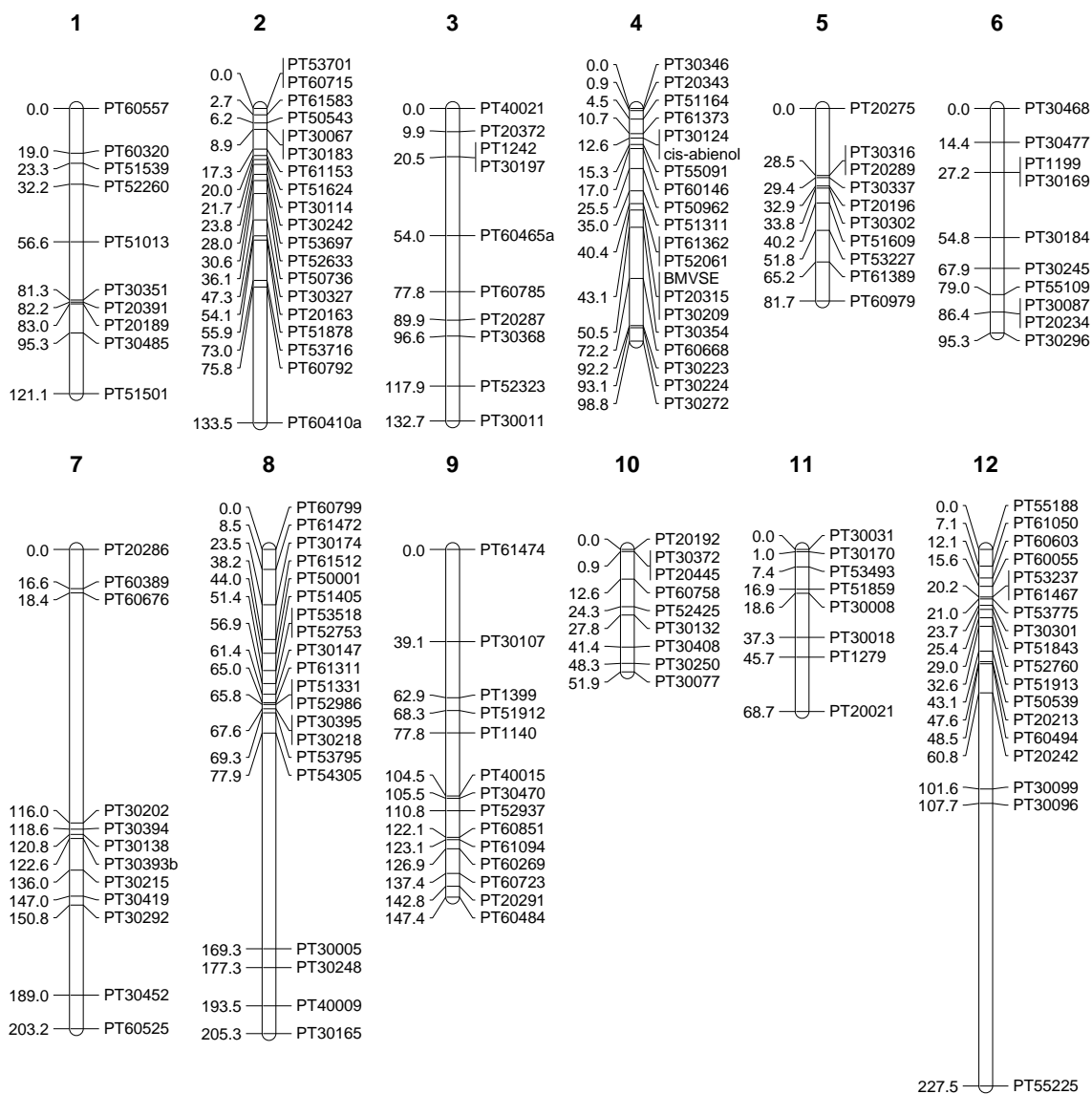
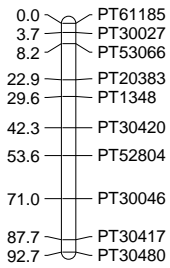


Figure 5: Histogram showing the number lines flowering within each time interval.

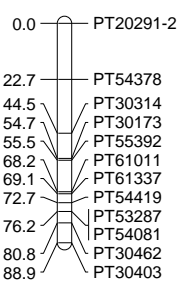
Figure 6: Revised SSR map of the Beinhart-1000 x Hicks mapping population.



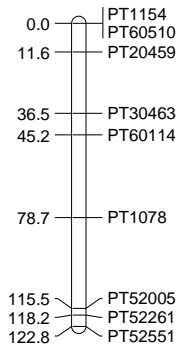
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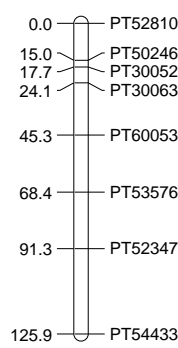
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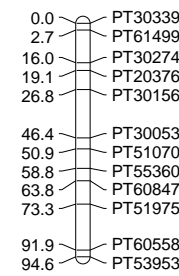
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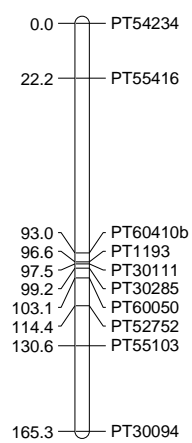
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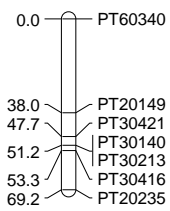
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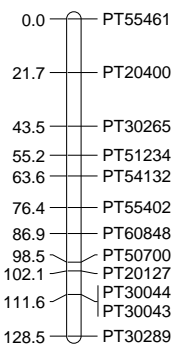
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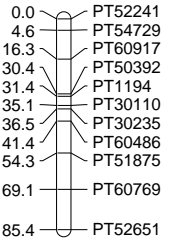
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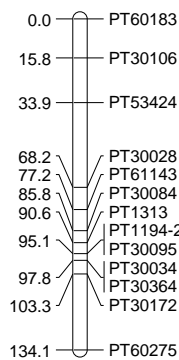
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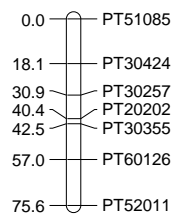
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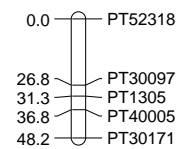
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23



24



QTL Analysis

After extending the linkage map, MIM analysis was used to produce new models for each of the four black shank resistance measurements and flowering time. Table 1 provides a summary for each of these models, allowing quick comparison of their entry mean heritabilities, the number of QTL in each model, and the amount of variation explained by each of the five measurements.

It is interesting to note that 10 QTL were initially detected for the AUDPC, more than for any other disease assessment. Additionally, the QTL detected for the AUDPC exhibited greater LOD scores than the QTL associated with alternative disease assessments. The model for AUDPC was initially over fit, as the amount of phenotypic variation explained by this QTL model was greater than the entry mean heritability. Therefore, the three least significant components of the model, one QTL and two epistatic interactions, were dropped in order to reduce the amount of variation explained to a level just below the entry mean heritability. Following removal of these components, the final model for the AUDPC was still able to explain the largest portion of the genetic variation (nearly 100%) relative to models for the other disease resistance assessments. For comparison, the final model for logit transformed end percent survival explained just over half of the genetic variability, while the other two models explained an intermediate amount. It is important to note that most of the QTL remained in the same positions under each of the different disease models, though some minor changes in position were observed for smaller or less densely mapped QTL. Ten QTL were identified that affected flowering time, in addition to one epistatic interaction. The

Table 1: Entry mean heritabilities, percent of the variation explained, and the number of QTL detected using MIM for each of the five traits.

Trait	Heritability (Entry Mean)	Phenotypic Variation Explained	Genotypic Variation Explained	Number of QTL Detected
Arcsin Square Root Transformed End Percent Survival	0.9379	0.7620	0.8113	8
Area Under the Disease Progress Curve (AUDPC)	0.8445	0.8409	0.9934	9
Disease Index	0.9216	0.7992	0.8655	8
Logit Transformed End Percent Survival	0.9622	0.4952	0.5146	5
Flowering Time	0.8446	0.7829	0.9269	10

individual effects of flowering time QTL were generally small to medium, as two copies of the QTL conferring the greatest effect only altered the days to flowering by 1.91 days.

Tables 2-6 detail the linkage group and position (in cM) for each QTL, in addition to their individual effects, expressed in terms of the portion of genotypic and phenotypic variation explained. If epistasis was detected in a given model, it is defined in the table as such. For epistatic interactions, the two QTL involved in the interaction are provided.

A more detailed graphical representation of the QTL identified for each of the four disease severity measures is presented in Figure 7. For each QTL, graphs are shown for all of the models that were able to detect that QTL. Figure 8 depicts all 10 of the flowering time-related QTL using the same 1- and 2-LOD error bars. In the flowering time model, two of the 10 QTL were found on a single linkage group, meaning that only nine distinct linkage groups were reported to affect flowering time in this population.

Table 2: QTL detected for multiple interval mapping of arcsin square root transformed end percent survival, along with their positions, effects on the phenotype, and the percent of the phenotypic and genotypic variation explained by each.

QTL	Linkage Group	Position (cM)	Additive Effect ^a	% σ^2_p	% σ^2_g
1	2	8.9	0.0974	2.9	3.09
2	4	11.7	0.386	22.2	23.67
3	8	20.5	0.4269	32.1	34.22
4	9	140.4	0.1017	2.4	2.56
5	11	16.4	0.1618	4.5	4.80
6	13	92.7	-0.1732	2.3	2.45
7	14	80.8	0.1783	3.8	4.05
8	15	66.3	0.1759	5.9	6.29

^aEffect on the arcsin square root transformed end percent survival, where positive numbers indicate that the favorable allele is contributed by Beinhart-1000, and negative numbers indicate the favorable allele is contributed by Hicks.

Table 3: QTL detected for multiple interval mapping of AUDPC, along with their positions, effects on the phenotype, and the percent of the phenotypic and genotypic variation explained by each.

QTL	Linkage Group	Position (cM)	Additive Effect ^a	% σ^2_p	% σ^2_g
1	1	27.3	-2.4038	1.8	2.13
2	2	21.8	3.4135	2.4	2.84
3	4	11.7	12.5244	18.1	21.42
4	8	20.5	16.0653	35.5	42.01
5	9	105.5	5.3049	5.3	6.27
6	11	16.9	5.5501	3.5	4.14
7	13	92.7	-7.2415	3.8	4.50
8	14	80.8	6.6048	4.6	5.44
9	15	63.2	7.0585	6.9	8.17
Epistasis	1 x 15	-----	-2.16	2.04	2.41

^aEffect on the AUDPC, where positive numbers indicate that the favorable allele is contributed by Beinhart-1000, and negative numbers indicate the favorable allele is contributed by Hicks.

Table 4: QTL detected for multiple interval mapping of the disease index (DI), along with their positions, effects on the phenotype, and the percent of the phenotypic and genotypic variation explained by each.

QTL	Linkage Group	Position (cM)	Additive Effect ^a	% σ^2_p	% σ^2_g
1	2	8.9	3.8943	2.6	2.82
2	4	11.7	15.6326	20.9	22.67
3	8	20.5	18.8687	34.6	37.53
4	9	105.5	5.8273	4.7	5.10
5	11	16.9	6.1081	3.4	3.69
6	13	92.7	-9.0959	4.1	4.45
7	14	80.8	7.1361	3.8	4.12
8	15	64.3	7.5108	5.7	6.18

^aEffect on the DI, where positive numbers indicate that the favorable allele is contributed by Beinhart-1000 and negative numbers define favorable alleles that are contributed by Hicks.

Table 5: QTL detected for multiple interval mapping of logit transformed end percent survival, along with their positions, effects on the phenotype, and the percent of the phenotypic and genotypic variation explained by each.

QTL	Linkage Group	Position (cM)	Additive Effect ^a	% σ^2_p	% σ^2_g
1	4	12.6	3.2696	8.9	9.25
2	8	22.5	5.1183	25.1	26.09
3	9	126.9	2.3917	7.3	7.59
4	13	92.7	-2.2834	3.8	3.95
5	14	80.8	2.1203	4.4	4.57

^aEffect on the logit transformed end percent survival, where positive numbers indicate that the favorable allele is contributed by Beinhart-1000, and negative numbers indicate the favorable allele is contributed by Hicks.

Table 6: QTL detected for multiple interval mapping of flowering time, along with their positions, effects on the days to flowering, and the percent of the phenotypic and genotypic variation explained by each.

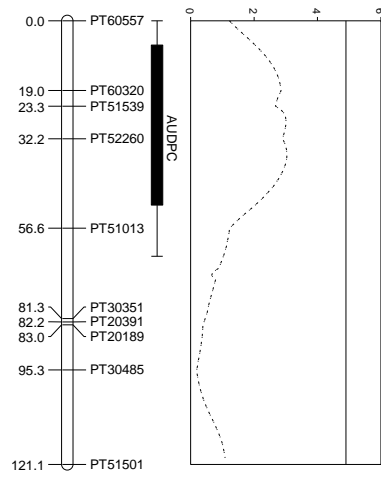
QTL	Linkage Group	Position (cM)	Additive Effect ^a	% σ^2_p	% σ^2_g
1	2	73	1.4762	8.9	10.57
2	4	95.1	1.7239	15.6	18.45
3	6	11	-1.108	7.5	8.90
4	9	144.9	-1.2854	6.9	8.16
5	10	0	-0.924	4.2	4.94
6	14	69.1	1.9143	17.5	20.77
7	15	0	1.3234	4.7	5.58
8	16	24.1	-0.8533	5.8	6.90
9	21	18.3	-0.9663	3.8	4.50
10	21	82.1	-1.0447	2.3	2.71
Epistasis	6 x 16	-----	-0.7187	1.0	1.21

^aEffect, in days to flowering, where positive numbers indicate that the Beinhart-1000 allele increases days to flowering and negative numbers indicate that the Hicks allele increases the days to flowering.

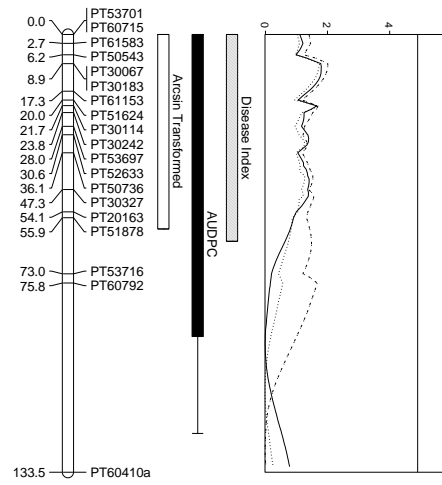
Figure 7: QTL affecting black shank resistance in the Beinhart 1000 x Hicks mapping population.

Note: in this figure, “Arcsin Transformed” represents output from the arcsin square root transformed end percent survival data, while “Logit Transformed” indicates output from the logit transformed end percent survival data. The box segment of the QTL error bars represents the 1-LOD confidence interval, and the whiskers (where applicable) define the 2-LOD confidence interval for the QTL.

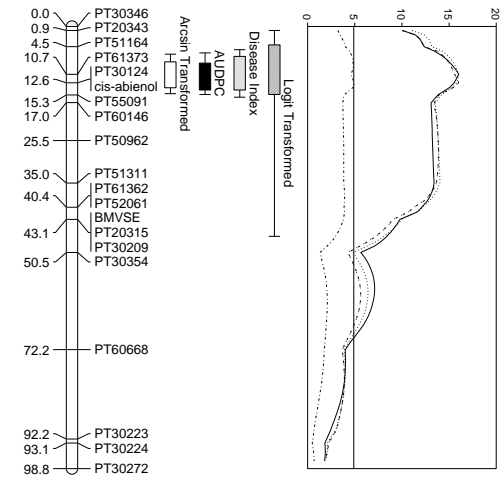
Linkage Group 1



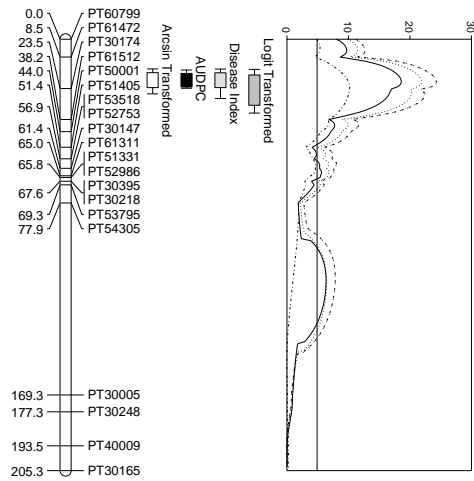
Linkage Group 2



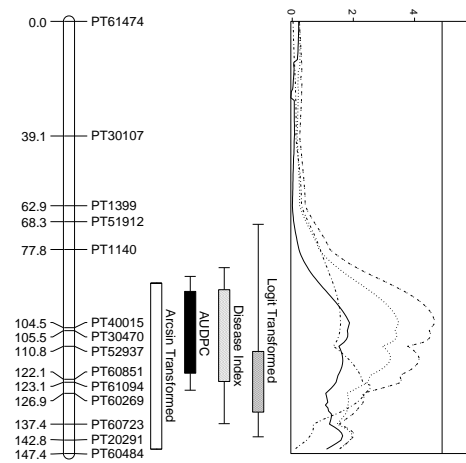
Linkage Group 4



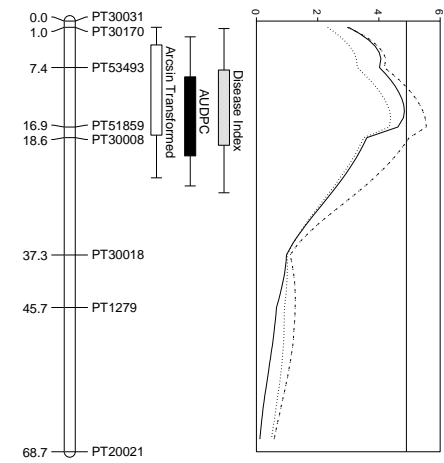
Linkage Group 8



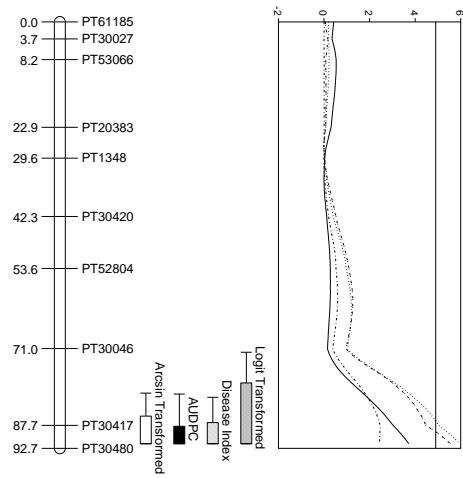
Linkage Group 9



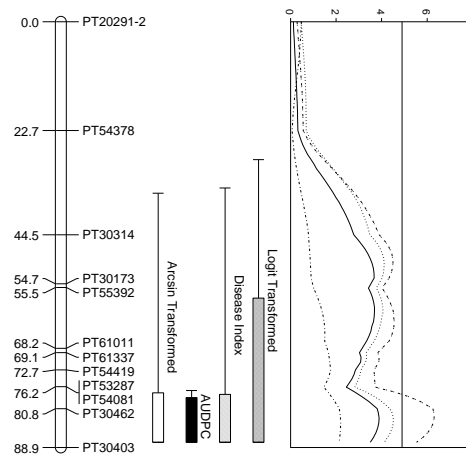
Linkage Group 11



Linkage Group 13



Linkage Group 14



Linkage Group 15

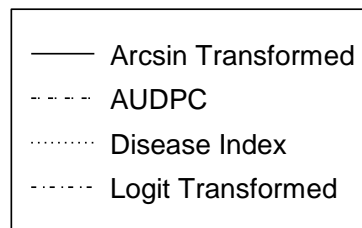
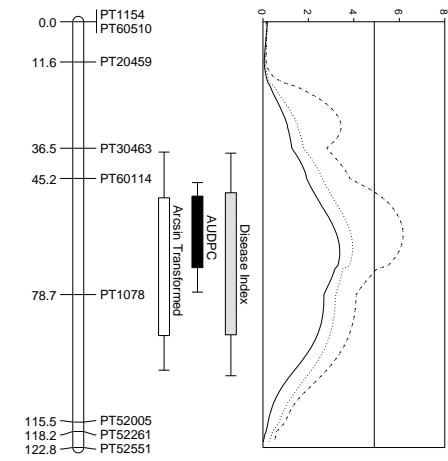
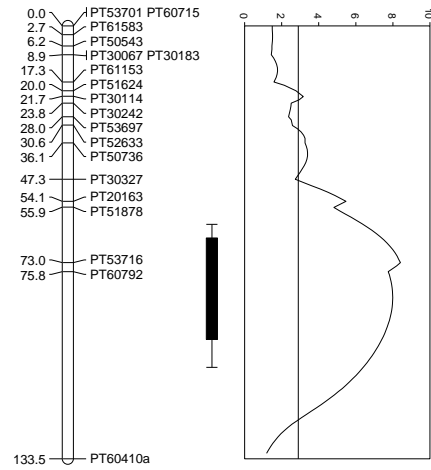


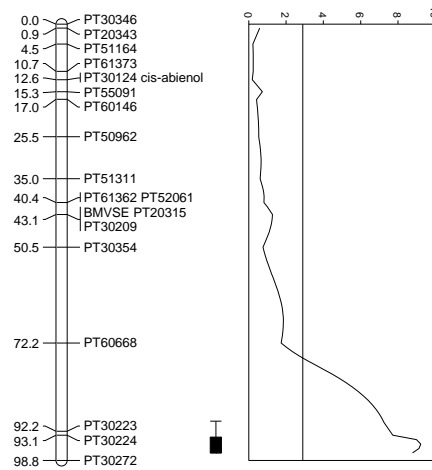
Figure 8: QTL affecting flowering time in the Beinhart-1000 x Hicks mapping population

Note: The box segment of the QTL error bars represents the 1-LOD confidence interval, and the whiskers (where applicable) define the 2-LOD confidence interval for the QTL.

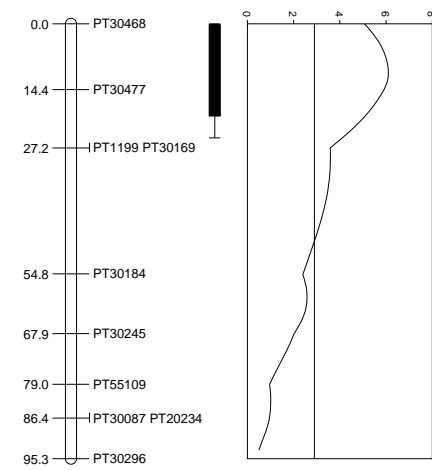
Linkage Group 2



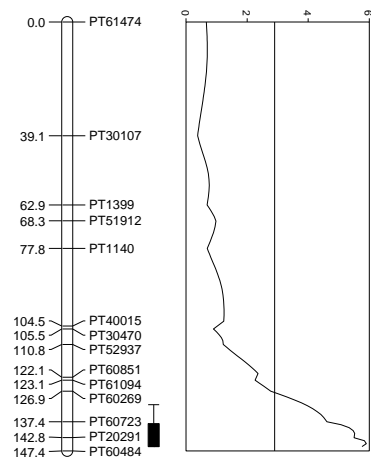
Linkage Group 4



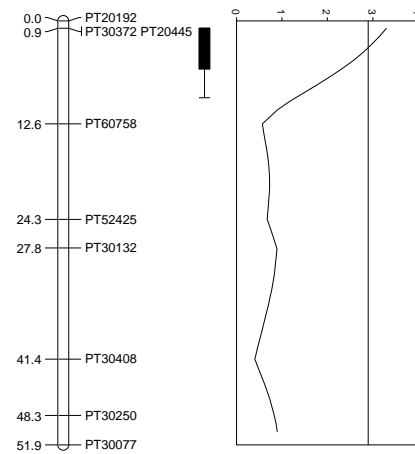
Linkage Group 6



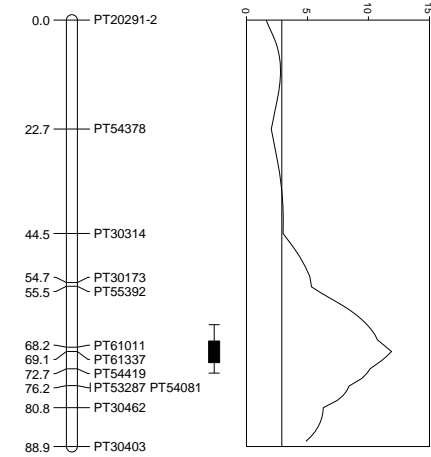
Linkage Group 9



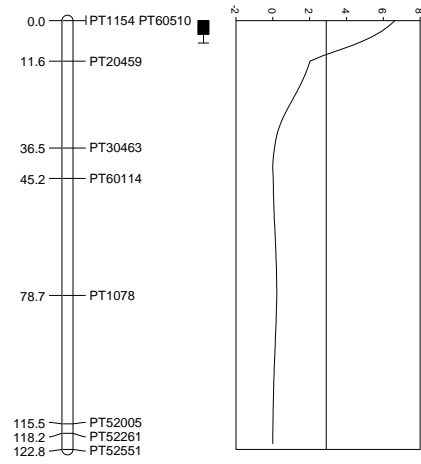
Linkage Group 10



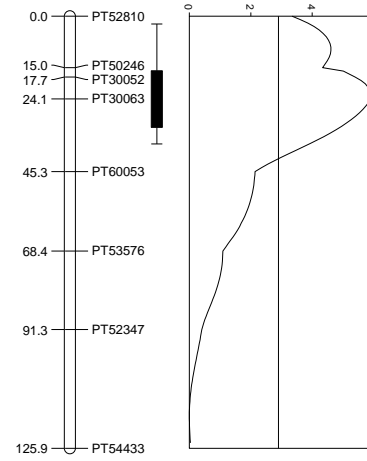
Linkage Group 14



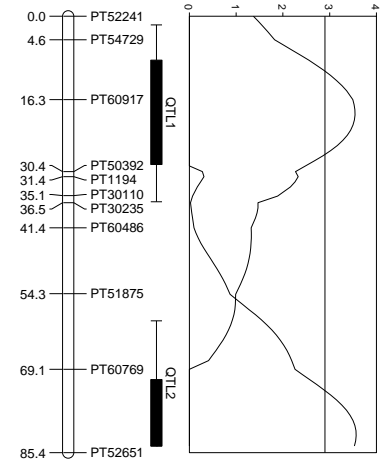
Linkage Group 15



Linkage Group 16



Linkage Group 21



Discussion

Black Shank Resistance QTL Analysis

The new black shank resistance QTL models described here provide a number of improvements over that previously reported by Vontimitta and Lewis (2012b), largely as a result of the increased map data obtained from the new SSR markers that were added. Among these improvements, three new QTL were found on linkage groups 1, 13, and 15. The identification of the QTL on linkage group 15 is especially important as it is the third largest QTL in the population, controlling approximately 8% of the genetic variation for AUDPC. Two of the original QTL identified within this population changed position appreciably after the addition of the newly genotyped markers. As a result of increased marker density on linkage group 9, the corresponding QTL on this linkage group was reported to be positioned around 105.5 cM on the new map, as determined by the two measurements that reported the greatest LOD scores for the QTL. In contrast, the initial map localized this QTL somewhere closer to PT 20291, approximately 40 cM away. The new location does, however, fall within the large original error bar outlined by Vontimitta and Lewis (2012b). Additionally, the QTL on linkage group 14 moved a significant distance from the original location under all four QTL models describing disease resistance. The new position for this QTL fell well outside of the original 1-LOD error bar published by Vontimitta and Lewis (2012b). This change in position is not well understood, because the marker order did not change and no new markers were added to this region. It is possible, however, that the change in map distances or the increase in map data on the distal end of the linkage group had some effect.

In this experiment, QTL detected using the AUDPC data were the best resolved and most explanatory as a whole, compared to those identified using the alternative disease severity measurements. As a byproduct, the AUDPC consistently gave LOD scores approximately equal to or higher than those for the other disease severity measures, effectively providing a greater degree of confidence in the QTL detected by the AUDPC as compared to the other measures. As a side effect, the higher LOD scores observed for genomic regions associated with resistance led to the declaration of an additional significant QTL on linkage group 1 that was not identifiable using the other measures of disease severity. QTL identified using the AUDPC model generally yielded smaller 1-LOD and 2-LOD confidence intervals, as compared to those produced by the other QTL models. This allows a potentially smaller segment of the donor line genome to be transferred throughout the crossing process when using marker assisted selection. This would be important for regions derived from Beinhart 1000, because this donor line has cigar characteristics that are undesirable for flue-cured or burley tobacco.

Possibly the most important distinction regarding the AUDPC was the increase in the amount of genetic variation that could be explained within this population. The AUDPC encompasses a number of parameters affecting observed disease levels, including the initial disease severity, the rate at which the epidemic progresses, and the duration and final severity of the disease. The AUDPC then summarizes the data with a single numerical value (Madden et al., 2007). This makes the AUDPC especially powerful for detecting differences in the resistance levels of cultivars expressing quantitative disease resistance against a polycyclic disease, such as black shank (Shew, 1987; Jeger and Viljanen-Rollinson, 2001;

Jiang et al., 2007). The disease index is also capable of taking into account the features described above. In contrast to the AUDPC, however, the disease index does not account for varying time intervals between ratings. It instead assumes that all intervals are equal. These attributes are a major contribution to the advantage of the AUDPC calculation over the other methods. It is therefore not surprising that the AUDPC was able to explain the largest amount of the genetic and phenotypic variation, almost 100% and 84%, respectively, whereas the other QTL models explained between 51-87% of the genotypic variation and 50-80% of the phenotypic variation.

Flowering Time QTL Analysis

Flowering time, which is a highly heritable trait, was found to be controlled by 10 QTL in this model. The two largest QTL were able to modify flowering time by just less than two days each, and accounted for 17.5% and 15.6% of the phenotypic variation, respectively. These effects are probably not of a sufficient magnitude to justify the use of marker assisted breeding in order to introduce a given QTL into a desired genetic background. In cases like this where relatively minor modifications to flowering time are desired, there is often substantial genetic variation that results from many different tobacco crosses such that introgression of alternative alleles from donor lines would normally not be necessary (Robinson et al., 1954). If lines or varieties with wider differences in their flowering times were used for mapping, it may be possible to find QTL that have moderate to larger effects, however. The *Many Leaves* genomic region, for example, is capable of delaying flowering by an average of 10 days in the heterozygous condition and 15 days in the homozygous condition (D.P. Eickholt, personal communication, 2012). For these reasons, it

may be more expedient to exhaust the natural variability for flowering time within a given cross before taking pains to introgress alleles from other, external sources such as Beinhart-1000 or Hicks.

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CHAPTER 3:**A NOVEL METHOD FOR IDENTIFYING PATERNAL HAPLOIDS IN TOBACCO
BASED ON *PAP1* OVEREXPRESSION**

Abstract

Haploidy in tobacco (*Nicotiana tabacum* L.) is a naturally occurring phenomenon that can be induced or selected for, in some circumstances, for the purposes of breeding. Some of its potential uses include the production of doubled haploids and the conversion of male fertile breeding lines to cytoplasmic male sterility. This study was undertaken in order to determine the feasibility of identifying paternal haploids using the ‘Xanthi 35S:PAPI’ method of haploid selection. The purple Xanthi 35S:PAPI line was crossed as a female with three different green paternal parents: ‘TN 86,’ ‘K 326,’ and ‘Narrow Leaf Madole.’ Seed resulting from these crosses were planted out in large quantities, and fourteen green seedlings were identified from amongst the progeny and retained for further analysis. Flow cytometry was used for the purpose of separating haploids from other genetic anomalies, and it was determined that eight of the fourteen green plants were true haploids. Overall, the rate of paternal haploidy observed for the three crosses was one haploid per 199,243 seed. Related research suggests that improvements could be made to increase the frequency of paternal haploids identifiable through the Xanthi 35S:PAPI system. For example, generation of a line of Xanthi 35S:PAPI which expresses an altered centromere-specific histone may increase the rate of paternal haploidy to a level well above that of any other method attempted to date.

Introduction

Haploidy is a naturally occurring phenomenon in tobacco (de Nettancourt and Stokes, 1960) that can be exploited for a number of purposes including rapid inbreeding and the efficient creation of transgenic lines of tobacco that do not segregate for an inserted gene of interest (Lewis et al., 2007). Another potentially desirable application for haploid plants is

the rapid conversion of male fertile lines to cytoplasmic male sterility, a process that is necessary for female parents of new tobacco hybrids (Goodsell, 1961; Chase, 1963; Lewis and Rose, 2011).

The earliest haploid isolation system in tobacco relied on the identification of haploid-diploid twins arising from a single seed. Twins were detectable at the cotyledonary stage when seed was germinated in clear dishes. Of the twin seedlings, one would normally be haploid and the other would be diploid (de Nettancourt and Stokes, 1960). Another early technique took advantage of the hypersensitive response (HR) to *tobacco mosaic virus* (TMV) conferred by the *N* gene. This protocol involved the crossing of female *n/n* plants with male *N/N* plants. All of the resulting progeny were inoculated with TMV at the first true leaf stage, and plants exhibiting the necrotic hypersensitive response (HR) conferred by the *N* gene were discarded as being true F₁ hybrids. Plants remaining after this selection procedure were assumed to be *n/-* plants, and were considered putative maternal haploids (de Nettancourt and Stokes, 1960).

Since both of these early protocols required large amounts of labor to obtain only a few haploids, more innovation was needed to make the use of haploids viable on a large scale. To this end, it was later discovered that haploids could be identified through the use of seedling genetic markers, where the haploid seedlings take on a different phenotype than the diploid seedlings. Burk (1962) crossed a plant carrying the double recessive *yg/yg* (yellow-green) genotype onto various other cultivars possessing the homozygous dominant genotype *Yg/Yg*. Seedlings resulting from this cross could be separated by color into two groups. The vast majority of seedlings were of the normal green phenotype (*Yg/yg*) and were considered

to be true F₁ hybrids. Those having a lighter yellow-green phenotype (*yg/--*) were considered putative haploids. This method of haploid identification was less laborious because visual selection of haploids was rapid. Unfortunately, the yellow green genetic marker technique was never widely applicable because the use of this recessive-type marker necessitated that the material from which haploids were to be generated carry the recessive *yg* allele. Normal breeding materials are not of this genotype. Additionally, all of the haploids isolated using this method would be of the yellow green phenotype, something that is not acceptable in tobacco cultivars.

Mechanisms of paternal haploidy have also been explored via androgenesis from microspores (Bourgin and Nitsch, 1967). In this method, stamens are placed on culture media and grown until plantlets appear. Then, individual plantlets can be separated and cultured until they are ready for transplanting into pots (Nitsch and Nitsch, 1969). This method is simple, does not require the presence of a specific gene or phenotype during the haploid selection process, and is capable of yielding large numbers of haploids. Arcia et al. (1978) and Burk and Matzinger (1976) reported reduced yield potential among the resulting dihaploids, however. Doubled haploid lines averaged 15% lower yields than the parent lines in one study, and were inferior for the purposes of cultivar development. Reed and Wernsman (1989) reported that the yield loss due to anther culture was associated with an increase in the nuclear DNA content of the doubled haploid lines. Additionally, this method of paternal haploid isolation is not capable of transferring the nuclear genome of the paternal parent into the cytoplasm of the maternal parent, a desirable characteristic for the purpose of converting lines to cytoplasmic male sterility.

A later attempt at haploid selection utilized wide crossing between *N. africana* and *N. tabacum* (Burk et al., 1979). This system was practical from a breeding standpoint because no extra genes were required for haploid selection, and because gynogenic haploids did not suffer the same reduction in agronomic performance that was associated with anther culture-derived haploids (Wernsman et al., 1989). In this breeding scheme, an *N. tabacum* line acting as the female parent is crossed with *N. africana*. The overwhelming majority of resulting seedlings are true F₁ hybrids and do not survive past the cotyledonary stage because of an interspecific lethality mechanism. Surviving seedlings are a mixture of aneuploid interspecific hybrids and haploids, the latter of which can be recognized visually with experience. The rate of haploidy in this system was reported to correspond to the frequency of normal, spontaneous gynogenic haploidy (Burk et al., 1979). This system has proven to be effective for gynogenic haploid production, but does not work well for the production of paternal haploids from seed because the reciprocal cross suffers low viability when *N. africana* is used as the maternal parent (Gerstel et al., 1979).

Goodsell (1961) was able to recover cytoplasmic male sterile androgenic haploids in maize (*Zea mays* L.) at a very low rate of three haploids among the 250,000 seeds that were screened. These three plants were all successfully restored to diploidy by pollinating silks of the haploid individuals with pollen from diploid plants. The resulting plants retained the cytoplasmic male sterile condition of the maternal parent, demonstrating that cytoplasm transfer can be made via the production of paternal haploids from seed followed by chromosome doubling. Chase (1963) also reported a low rate of paternal haploidy from seed, approximately 80-fold lower than the rate of maternal haploidy, or roughly 1 in every 80,000

seed. The frequency of paternal haploids from seed was also investigated in *N. tabacum* by using a dominant lethal allele, *Rac-*, which confers the rootless character (Pelletier et al., 1987; Horlow et al., 1993). The phenotype induced by the *Rac-* plants can be used to identify paternal haploids. This method involves the crossing of normal rooted (*rac+/rac+*) plants with a rootless *Rac-* maternal parent. F₁ hybrids resulting from this cross are rootless and die, while haploid plants survive and grow normally (Horlow et al., 1993). It is very difficult to maintain the *Rac-* parent, however, because the rootless genotype must be successfully grafted onto a suitable rootstock in order to survive (Pelletier et al., 1987).

A suitable dominant seedling marker that is easy to manipulate and maintain did not exist until recently, when the *PAP1* gene from *Arabidopsis thaliana* was transformed into *N. tabacum* 'Xanthi,' under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter to induce overexpression of the transgene (Xie et al., 2006). This line will be referred to as 'Xanthi 35S:*PAP1*' in the following discussion. As a result of *PAP1* overexpression, Xanthi 35S:*PAP1* seedlings develop an intense purple coloration within approximately one week to 10 days after germination, depending on environmental factors. Haploids resulting from a cross between any non-35S:*PAP1* individual and Xanthi 35S:*PAP1* will be green in color, and can be rapidly distinguished from F₁ hybrids (Lewis and Rose, 2011). This system may be less efficient for haploid production, because the rate of maternal haploidy was found to be sevenfold lower than the rate obtained through crosses with *N. africana*. Even so, it could be used for the production of androgenic haploids from seed, as crosses can also be conducted using Xanthi 35S:*PAP1* as the female parent. Low numbers of paternal haploids

might be found among the progeny from such a cross, although little data exist on the frequency at which paternal haploids might arise from seed (Lewis and Rose, 2011).

Materials and Methods

Seed Production

Seed for this experiment was produced through hand pollination. Xanthi *35S:PAP1* was used as the maternal parent in crosses with three tobacco varieties: ‘TN 86,’ ‘K 326,’ and ‘Narrow Leaf Madole.’ These varieties are representative of the burley, flue-cured and dark tobacco market classes, respectively, and each of the three is susceptible to TMV. Fifteen pollinations were completed for each of the three crosses every day, except for the first day when five pollinations were made for every cross due to a lack of sufficient quantities of mature flowers. Tags were attached to the pedicels of pollinated flowers to indicate the date of pollination. Pollinating an equal amount of flowers per cross per day was important as a means of controlling for the day to day variability in the rate of haploidy that might have resulted from the variation of environmental conditions over time. Crosses were made on 34 different days between November 13, 2010 and December 19, 2010.

Haploid Identification

Due to the large number of seed involved in this experiment, it was necessary to estimate seed count by weight. To this end, three sets of 1,000 seed were counted and weighed for each of the three crosses. These three repetitions were averaged to determine the mean weight per 1,000 seed for each cross. To germinate the first 16 seed lots, 16 cm x 22 cm plastic trays were filled with Metro Mix 200 (Sun Gro Horticulture, Bellevue, WA) and seeded to a density of 284-426 seed per dm². Beginning with the 17th seed lot, Fafard 2 Mix

(Conrad Fafard, Inc., Agawam, MA) was used as the germination media, and seed were germinated in 26 cm x 51 cm trays seeded to a density between 339 and 452 seed per dm². Growth room temperature was kept at approximately 26° C during this time. Where damping off occurred, the quantity of seedlings lost to *Pythium* spp. were estimated and subtracted from the total amount of seed. Following germination, those seedlings that remained green approximately 7-10 days after germination were ultimately transferred to 25 cm wide pots in the greenhouse, and considered to be putative haploids.

The ploidy level of putative haploids was tested via flow cytometry. The nuclear DNA content of each putative haploid was compared to the DNA content of diploid control variety, K 326. The genomic content of this line was considered to be approximately 10.04 pg of DNA per nucleus (Johnston et al., 1999). Nucleus extraction and staining was performed using a CyStain PI Absolute P kit (Partec North America, Inc., Swedesboro, NJ). For each sample, one leaf section from the diploid control and a second from a putative haploid were co-chopped with a razor for 60 seconds in a petri dish containing 600 µL of nuclei extraction buffer. Each leaf section was approximately 1 cm² in size. Following chopping, the solution was filtered through a 30 µm filter, and 1.8 mL of Cystain PI Absolute P staining buffer was added. The solution was incubated in the dark for 60-120 minutes before analysis was performed. A FACSCalibur flow cytometer (Becton-Dickinson Biosciences, San Jose, California) fitted with a 15 mW argon laser emitting a 488 nm excitation wavelength was used to detect propidium iodide fluorescence (FLA-2). The flow cytometer was programmed to run 5,000 events for each of the samples, and debris was gated out to produce the final histograms.

Three green plants (GH11-73-2, GH11-73-4, and GH12-005-4) were suspected to be diploid based on their appearance and fertility. These individuals were tested a second time, using diploid maize as the control instead of diploid tobacco, to prevent overlap of the individual peaks. Nuclear DNA content was calculated as the (putative haploid peak / diploid standard peak) x 10.04 pg, where 10.04 pg is the nuclear DNA content of *N. tabacum* cv. Xanthi. DNA contents for GH11-73-2, GH11-73-4, and GH12-05-4 were calculated as (5.72 / maize peak) x putative haploid peak, where 5.72 pg is the nuclear DNA content of maize (Johnston et al., 1999; Dolezel and Bartos, 2005). A plant was considered to be a true haploid if its genomic content was approximately one half the size of the genomic content for the K 326 standard.

Detached leaves from each putative haploid were inoculated with TMV according to Rufty et al. (1987), as an additional measure of assurance of the presumed haploid or F₁ hybrid status of each plant. Xanthi 35S:*PAPI* is homozygous for the *N* gene, which confers resistance to TMV via a hypersensitive response (HR). Because the three paternal parents in this study were all TMV susceptible, true paternal haploids were expected to be susceptible to the disease, whereas plants containing the maternal genome were expected to exhibit resistance via the easily observed *N*-mediated HR.

Results

Haploid Isolation

During this experiment, approximately 2.4 million seed were produced. Space and time limitations permitted only 1.6 million of these seed to be germinated, however. From the seed that were germinated, a total of 14 green seedlings were identified and transplanted into

Table 7: Attributes of green plants resulting from all three crosses with Xanthi 35S:PAP1.

Seed Lot Number	Paternal Parent	Unique Identifier	TMV Response	Percent of the Diploid <i>N. tabacum</i> Genomic Content	Nuclear DNA Content (pg)	Pollen Fertility
2	K326	GH11-70-1	Susceptible	No Data	No Data	Fertile
4	TN86	GH11-73-1	Susceptible	49.8%	5.00	Sterile
18	Narrow Leaf Madole	GH11-75-1	Susceptible	49.5%	4.97	Sterile
22	TN86	GH11-73-2	Hypersensitive Response	106% [‡]	10.64 [‡]	Fertile
22	TN86	GH11-73-3	Susceptible	50.2%	5.04	Sterile
26	K326	GH11-74-2	Susceptible	49.1%	4.93	Sterile
26	K326	GH11-74-3	Susceptible	49.7%	4.99	Sterile
27	Narrow Leaf Madole	GH11-75-2	Susceptible	49.7%	4.99	Sterile
28	TN86	GH11-73-5	Susceptible	50.8%	5.10	†
31	TN86	GH11-73-4	Susceptible	105% [‡]	10.54 [‡]	Fertile
41	K326	GH11-74-4	Susceptible	50.3%	5.05	†
47	K326	GH12-05-4	Susceptible	105.9% [‡]	10.63 [‡]	†
59	K326	GH12-05-5	No Data	No Data	No Data	†
63	Narrow Leaf Madole	GH12-06-1	Susceptible	74.1%	7.44	†

†Plant was not flowering by the conclusion of the experiment

‡Data are estimates of tobacco genomic content based on the use of diploid *Zea mays* DNA standards

larger pots. A full table of the results, broken down by seed lot, can be found in Appendix B. Table 7 describes the characteristics of each green plant in detail, except for GH12-05-5, a green plant originating from seed lot 59, which died at the cotyledonary stage before any data could be collected. Additionally, flow cytometry results are not available for GH11-70-1, as this plant was determined to be a fertile diploid and discarded before flow cytometry data was obtained.

GH11-70-1 was determined to be a diploid, as it was fertile, produced seed through self-pollination, and exhibited the physical characteristics of a diploid. This plant was found among a seed lot derived from a cross involving K 326 as the paternal parent, but the identified plant expressed the burley tobacco phenotype. Therefore, this individual was likely the result of seed contamination rather than haploidy. GH11-73-2 was determined to be diploid due to the HR expressed when it was inoculated with TMV, and its petiolate leaves displayed slight purple coloration when immature despite its otherwise green phenotype. Two other green plants, GH11-73-4 and GH12-05-4, were also found to be fertile diploids. It is possible these plants were seed contaminants, as there were no phenotypic characteristics indicating that these plants were the result of hybridization with the Xanthi parent. In contrast to these exceptions, haploids were sterile, green, and similar in phenotype to their paternal parent (Figure 9).

Haploids produced a propidium iodide-A value of 154-168, while the diploid standards registered a value approximately twice as large. Figure 10 shows the histogram for a typical haploid plus a diploid control. Figure 11 diagrams the nuclear DNA content of the



Figure 9: Left: GH11-74-3 (a K 326 haploid) has a green phenotype and complete self-sterility, as indicated by the premature death of the ovaries and pedicels of mature flowers. Right: GH-11-73-2 (a Xanthi 35S:PAPI x TN 86 diploid) exhibits a green phenotype, though it expresses the petiolate trait of Xanthi 35S:PAPI and sets fertile seed capsules.

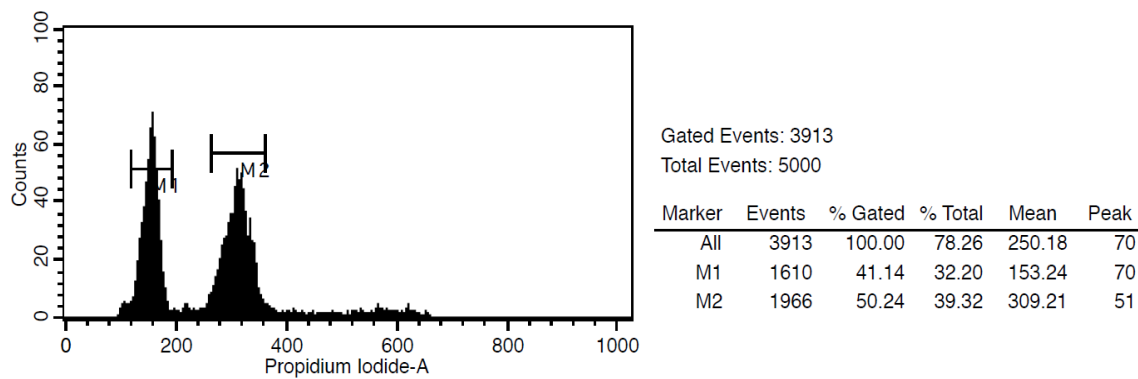


Figure 10: Histogram of all gated events for haploid individual GH11-75-1 (M1), and the K 326 diploid tobacco control (M2).

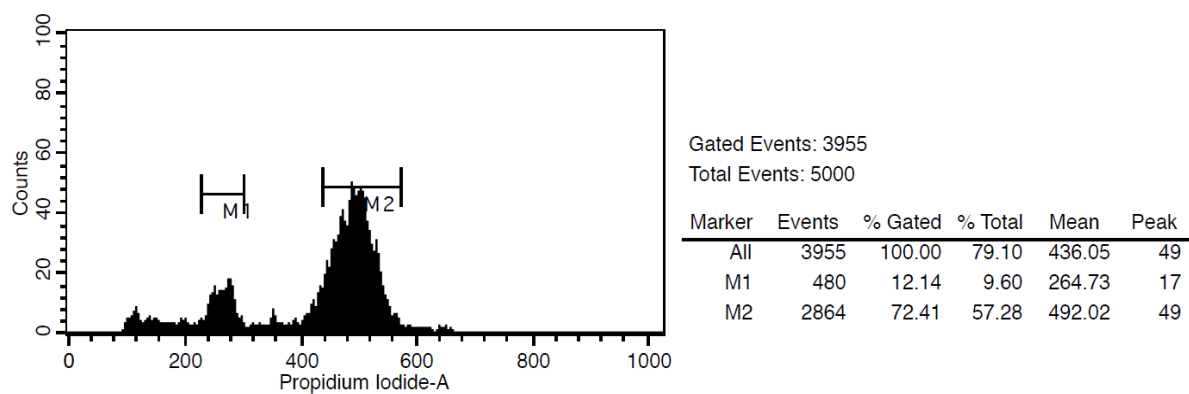


Figure 11: Histogram of all gated events for diploid maize (M1) and the diploid green plant GH11-73-2 (M2).

diploid green plant GH11-73-2 alongside a maize standard. Figure 12 and Figure 13 show the histograms for green plants containing the diploid chromosome number, alongside the diploid maize standard. Figure 14 is a histogram of the aneuploid plant designated GH12-06-1 alongside the diploid K 326 standard. Because this plant was immature at the time of leaf tissue harvest, leaf cells were still undergoing mitosis. For this reason, a G2 peak can be seen on the histogram in Figure 14, at an x-value approximating 497. Table 8 details the origin of all true paternal haploids detected during this experiment. Broken down by paternal parent, the rate of paternal haploidy was one haploid per 175,837 seed (.000569%) for TN 86, one haploid per 158,313 seed (.000632%) for K 326, and one haploid per 295,745 seed (.000338%) for Narrow Leaf Madole. Overall, this equated to a rate of .000502%, or one paternal haploid per 199,243 seed.

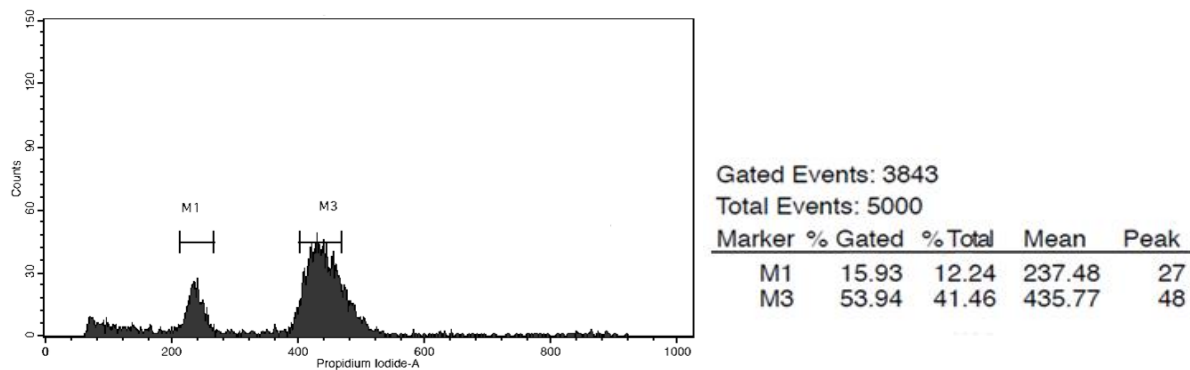


Figure 12: Histogram of all gated events for GH11-73-4 (M3) and the diploid maize control (M1).

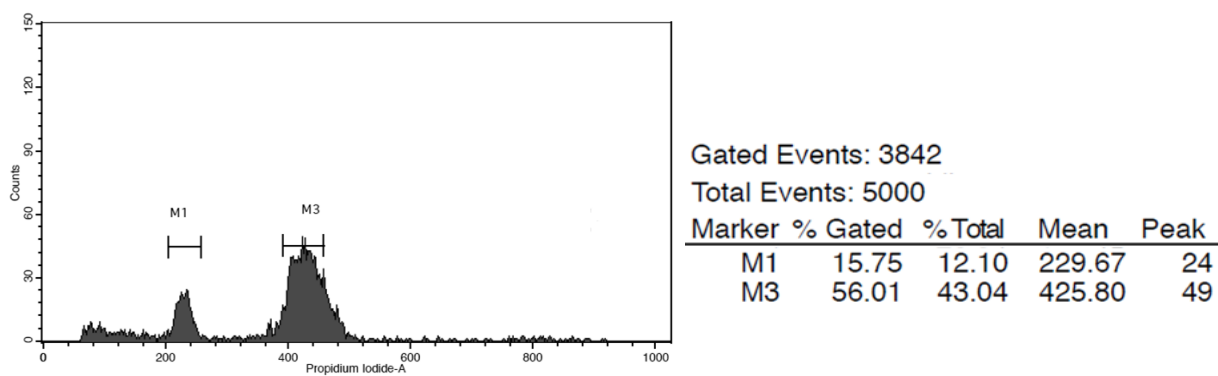


Figure 13: Histogram showing gated events for samples of GH12-05-4 (M3) and the diploid maize control (M1).

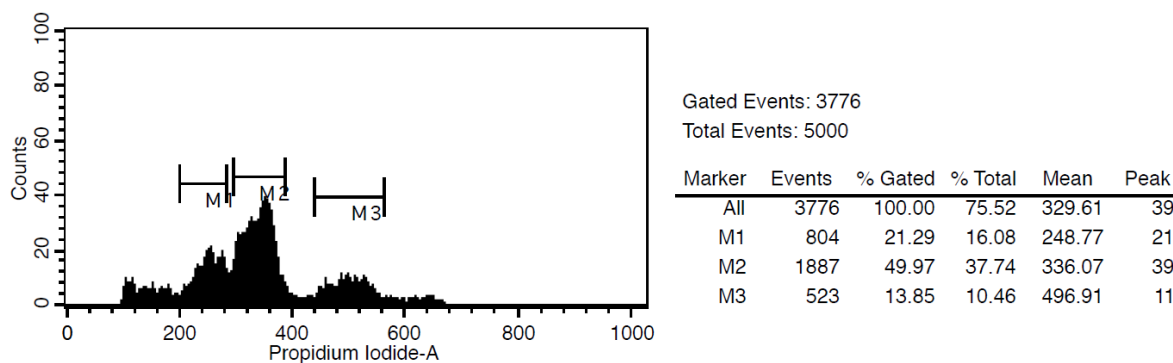


Figure 14: Histogram of gated events for samples of GH12-06-1 (M1) and the diploid tobacco standard (M2). Note: the M3 marker represents the G2 peak of GH12-06-1.

Table 8: True paternal haploids detected among the 1.6 million progeny that were tested.

Seed Lot	Paternal Parent	Pollination Date	Haploid
4	TN86	11/14/2010	GH11-73-1
18	Narrow Leaf Madole	11/18/2010	GH11-75-1
22	TN86	11/20/2010	GH11-73-3
26	K326	11/21/2010	GH11-74-2
26	K326	11/21/2010	GH11-74-3
27	Narrow Leaf Madole	11/21/2010	GH11-75-2
28	TN86	11/22/2010	GH11-73-5
41	K326	11/29/2010	GH11-74-4

Discussion

The capability of using paternal haploidy as a means for converting maize inbred lines to cytoplasmic male sterility has been recognized for years (Goodsell, 1961; Kermicle, 1969). The method described in this paper provides a similar system for the production of paternal haploids in tobacco. Unlike previous paternal haploid production methods (Burk, 1962; Pelletier et al., 1987), this approach does not require the paternal parent to contain a certain gene or be of a specific genetic background, nor does it require special cultural techniques like grafting in order to maintain the maternal parent. These advantages are strongly offset, however, by the very low rate of paternal haploidy that was observed. In this experiment, the frequency of paternal haploidy was 0.00000502. A rate of approximately tenfold greater was observed using the *Rac*- selection method, as reported by Horlow et al., (1993). Burk (1962) reported a frequency of paternal haploidy of 0.0004, a rate approximately 100 fold greater than the frequency observed in the present study.

To increase the efficiency of the Xanthi *35S:PAP1* method of paternal haploid isolation, it would be desirable to have an approach that would increase the frequency of paternal haploidy. In maize, the rate of paternal haploidy was increased through the use of genetic variability such as the *indeterminate gametophyte* mutation (Kermicle, 1969) and the RWS inducer line (Rober et al., 2005). More recently, a haploid production method was described for *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh) that should be widely applicable across different species due to its reliance on a CENH3 histone mutation (Ravi and Chan, 2010). This process uses an altered form of the centromere specific histone CENH3, produced by the *GFP-tailswap* transgene, within a line of *Arabidopsis* that lacks

expression of the wild-type CENH3 histone. The substituted GFP-TAILSWAP histone allowed mitosis to proceed normally, but when crossed with a line bearing the wild-type CENH3 histones, the mutant chromosomes have a strong tendency to segregate away, leaving haploids comprised of chromosomes from the wild-type parent. Due to pollen sterility, the *GFP-tailswap* line is best suited as a female in breeding crosses, where the paternal parent is a wild-type line. Ravi and Chan (2010) reported that 25-45% of the viable progeny from a *GFP-tailswap* x wild type cross were haploids. This represents a significant increase in the frequency of paternal haploidy compared to other methods that have been tested in tobacco to this point. If a Xanthi *35S:PAPI* mutant could be identified that lacked function at the *cenh3* site, and the *GFP-tailswap* transgene could be successfully used to breed a *cenh3* null plus *GFP-tailswap* homo- or hemizygous individual, the combination could be used to easily select for haploid progeny using the dominant *PAPI* marker.

Following the successful production of paternal haploids, it becomes necessary to double the haploid into a fertile diploid once again. The most widespread method used to double tobacco haploids is the tissue culture method described by Kasperbauer and Collins (1972). Tissue culture, however, can be detrimental to the performance of the resulting doubled lines due to somaclonal variation (Larkin and Scowcroft, 1981), some of which originates from increased retrotransposon activity during the culture process (Hirochika, 1993). Because of the potential for undesirable genetic changes that could be induced through tissue culture, it would be preferable to restore the diploid condition through an alternative means. Colchicine has also been used in the past for doubling haploids (Burk et al., 1972), but results can be erratic. Using the cross pollination method, Horlow et al. (1993)

were able to pollinate paternal haploids with diploid pollen in order to produce a small amount of diploid seed. In the context of using paternal haploidy as a means of converting lines to cytoplasmic male sterility, this would be accomplished by crossing male sterile haploid individuals with their male fertile counterpart. If this process were used to convert tobacco lines to cytoplasmic male sterility, it would require only two generations and less than one year to complete. In the first generation, the cytoplasmic male sterile haploid inducer line (CMS Xanthi *35S:PAPI* with *GFP-tailswap*) would be crossed with a male fertile line that needs to be converted to cytoplasmic male sterility. In the second generation, haploid plants derived from the above cross would be crossed as females with the male fertile diploid progenitor line. Resulting viable seed should be cytoplasmic male sterile diploids. In this way, it is hoped that the process would be optimized to a degree where it could be widely applicable to tobacco breeding programs.

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CHAPTER 4:

**EFFECTS OF TMV INFECTION ON HERITABLE BLACK SHANK RESISTANCE
IN TOBACCO**

Abstract

Previous reports have suggested a link between *Tobacco mosaic virus* (TMV) infection and increased resistance to black shank (*Phytophthora nicotianae* Breda de Haan) in the progeny of inoculated tobacco plants (*Nicotiana tabacum* L.). This study was conducted to determine the feasibility of utilizing TMV inoculation as a means of generating *de novo* black shank resistance in tobacco. Seed was harvested from 36 individual plants of three different genetic backgrounds that had either been inoculated with TMV or left uninoculated to serve as control groups. Progeny from these plants were grown in soil-borne disease nurseries infested with *P. nicotianae* to determine if TMV infection may have caused any heritable changes in black shank resistance. While highly significant differences in disease severity were observed among genetic backgrounds, no significant increases in resistance could be observed between progeny derived from TMV infected plants and progeny derived from their uninfected counterparts. One progeny line derived from a TMV infected plant exhibited significantly increased susceptibility over the control group, possibly as a result of a type I error. Overall, this technique was determined to be an ineffective means of generating *de novo* black shank resistance in tobacco.

Introduction

Black shank is a destructive disease of tobacco, and is capable of killing the entire plant. Black shank caused losses of 3.87% and 2.53% to North Carolina's flue-cured tobacco production in 2010 and 2011, respectively (Mila and Radcliff, 2011). Genetic resistance has been a part of many N.C. tobacco growers' overall management strategies to reduce economic loss from black shank ever since the 1943 release of 'Oxford 1,' the first flue-cured

tobacco variety carrying black shank resistance (Bowman and Sisson, 2000). To this point in time, the transfer of genetic resistance into superior varieties has required the crossing of resistance genes into lines with otherwise acceptable agronomic characteristics. Once these crosses have been made, it takes considerable time and effort to inbreed the resulting lines and select those that have acceptable yield, quality, and disease resistance. It would be beneficial if new genetic variability affecting black shank resistance could be generated and rapidly introduced into elite genetic backgrounds.

To this end, viruses have been reported in the past to act as mutagenic agents. In maize, *Barley stripe mosaic virus* was reported to cause aberrant segregation ratios for major genes (Sprague et al., 1963; Sprague and McKinney, 1966, 1971). More recently, TMV was found to cause heritable epigenetic changes in tobacco. Inoculation with this virus can cause hypomethylation of some genomic regions, while other regions become hypermethylated (Boyko et al., 2007). Hypomethylated areas generally correspond to regions that contain resistance genes or regions with sequences homologous to such genes (Boyko et al., 2007). As a result of TMV infection, a broader response in the overall stress tolerance of tobacco has been reported. This may be the result of increased *PR1* expression, a reaction which is often associated with systemic acquired resistance (Kathiria et al., 2010). The epigenetic outcome of TMV infection is thought to be dependent on the duration and intensity of the disease, as well as other environmental factors. From an applied standpoint, one of the most interesting phenomena associated with TMV inoculation is the potential for increased black shank resistance in the progeny of some infected individuals (Kathiria et al., 2010). In the above report, the progeny of infected individuals experienced, on average, less than 33%

disease incidence when inoculated with black shank, while 87% disease incidence was observed for the control group. If novel genetic variability affecting field resistance to black shank could be generated by inoculating breeding lines and cultivars with TMV, this could expand the tobacco breeders' toolbox for developing resistant cultivars.

Materials and Methods

Seed Production and Preparation

In this experiment, three different tobacco cultivars were used: 'Narrow Leaf Madole' (a dark tobacco cultivar), 'K326' (a flue-cured cultivar), and 'TN86' (a burley tobacco cultivar). These varieties exhibit low to intermediate levels of black shank resistance. On June 28, 2010, twelve plants of each variety were seeded, and these were subsequently transplanted to 25 cm wide pots in the greenhouse approximately one month later. On September 21, 2010, six plants of each variety were inoculated with TMV, according to the method of Rufty et al. (1987), while the other six were mock inoculated with buffer and carborundum. Inoculation consisted of rubbing two leaves from each plant with a cotton swab that had been immersed in the inoculum. Plants inoculated with TMV were moved to an adjacent greenhouse unit maintained under similar environmental conditions, in order to prevent accidental spread of the virus to the mock inoculated plants. In order to increase the duration of exposure to TMV, both the TMV inoculated group and the control group were decapitated (topped) on October 25, 2010. This action delayed flowering and seed set by four to six weeks and increased the time of exposure to the virus. Seed was harvested between December 13, 2010 and January 20, 2011, as soon as sufficient numbers of mature

capsules were formed. Seed was treated with a solution of 10% chlorine bleach solution prior to seeding in float trays as a precautionary measure against the spread of the virus.

Field Analysis of Black Shank Resistance

Transplants for this experiment were all grown using the float tray system at the Central Crops Research Station in Clayton, NC. Progeny were evaluated for black shank resistance in two soil-borne disease nurseries at the Lower Coastal Plain Tobacco Research Station (Kinston, NC) and the Upper Coastal Plain Research Station (Rocky Mount, NC) during 2011. The experimental design was a randomized complete block design (RCBD) with four replications at each location. Plots consisted of single rows 15 m long containing approximately 25 plants each. Plants were spaced approximately 56 cm apart within rows and approximately 120 cm apart between rows. The number of surviving plants for each plot were recorded at Kinston beginning on May 13, and at 13, 29, 42, 55, 71, and 84 days thereafter. At Rocky Mount, data collection began May 18. Subsequent data was collected at 12, 24, 37, 50, 66, 79, and 93 days after the initial rating. Plants were scored individually within rows as either dead or alive, and plants that died due to other causes were removed from the data on subsequent rating dates in order to prevent erroneous conclusions.

From this data, the Area under the Disease Progress Curve (AUDPC) was derived for each plot as:

$$AUDPC = \sum_{i=1}^{n_i-1} \left[\frac{y_i + y_{i+1}}{2} \right] [t_{i+1} - t_i]$$

where n equals the total number of rating dates, i indexes each individual rating date, y equals the percentage of plants in a given plot that died from black shank at any time before the i^{th} rating date, and t equals the number of days elapsed between the initial rating and the i^{th} rating (Madden et al., 2007). SAS software PROC MIXED (SAS Institute, Inc., Cary, NC, USA) was used to analyze the AUDPC data, and CONTRAST statements were used to compare the entry mean AUDPC for each family derived from a TMV infected plant to the average of the control families within its respective genetic background. CONTRAST statements were also used to compare the mean of all families derived from the TMV inoculated plants to the mean of all lines derived from the mock inoculated plants and to compare the means for each genetic background with the others.

Results

Differences in black shank resistance were highly significant between different genetic backgrounds ($P < 0.0001$) (Figure 15). Overall, Narrow Leaf Madole exhibited an AUDPC of 50.39, making it the most susceptible of the three lines in this experiment, while K 326 was the most resistant with an AUDPC of only 21.56. TN 86, with an AUDPC of 29.02, fell in between but was only slightly more susceptible than K 326. No significant differences were found when the AUDPC of the control group, averaged across all three varieties, was compared to the AUDPC of the groups derived from TMV inoculated plants. Likewise, no interaction of ‘inoculation’ x ‘variety’ was observed. In contrast, when the progeny derived from individual TMV-inoculated plants of a given variety were compared to the average of the controls for that variety, only one of the experimental lines was found



Figure 15: Narrow Leaf Madole (left) was highly susceptible compared to K 326 (right).

to have a significantly different AUDPC. Entry 19, a progeny line derived from a TMV inoculated TN 86 plant, exhibited a significantly higher AUDPC ($p=.0193$) relative to the average AUDPC for the progeny from the six uninoculated TN 86 control plants. Entry means AUDPC's for each of the 36 progeny lines can be found in Table 9.

Table 9: Entry mean AUDPC, and Tukey grouping, for each of the 36 experimental lines.

Tukey Grouping	Mean	Entry	Variety	Inoculum
A	55.099	32	Narrow Leaf Madole	TMV
A	53.328	15	Narrow Leaf Madole	Mock
A	52.1	31	Narrow Leaf Madole	TMV
A	51.469	35	Narrow Leaf Madole	TMV
A	51.283	16	Narrow Leaf Madole	Mock
A	50.781	18	Narrow Leaf Madole	Mock
A	50.026	13	Narrow Leaf Madole	Mock
A	49.696	14	Narrow Leaf Madole	Mock
AB	48.338	17	Narrow Leaf Madole	Mock
AB	48.042	33	Narrow Leaf Madole	TMV
AB	47.803	34	Narrow Leaf Madole	TMV
ABC	46.683	36	Narrow Leaf Madole	TMV
BCD	35.868	19	TN 86	TMV
CDE	32.998	24	TN 86	TMV
DEF	32.251	20	TN 86	TMV
DEF	32.213	4	TN 86	Mock
DEF	30.796	2	TN 86	Mock
DEF	30.731	21	TN 86	TMV
DEF	30.494	22	TN 86	TMV
DEF	28.323	6	TN 86	Mock
DEF	27.406	12	K 326	Mock
DEF	26.069	1	TN 86	Mock
DEF	25.032	5	TN 86	Mock
DEF	24.889	9	K 326	Mock
DEF	24.601	29	K 326	TMV
DEF	24.14	10	K 326	Mock
EF	21.963	23	TN 86	TMV
EF	21.569	26	K 326	TMV
EF	21.486	3	TN 86	Mock
EF	20.15	8	K 326	Mock
EF	20.025	27	K 326	TMV
EF	19.643	11	K 326	Mock
EF	19.611	30	K 326	TMV
F	19.243	7	K 326	Mock
F	18.881	25	K 326	TMV
F	18.614	28	K 326	TMV

Discussion

In this experiment, TMV inoculation proved to be an ineffective means of increasing the level of black shank resistance intrinsic to an already established cultivar. Three different genetic backgrounds were studied in this experiment, in the event that one background responded differently than the others. Despite the widely different cultivars tested, there were no detectable significant differences in AUDPC for any of the lines derived from TMV-infected plants as compared to the average of their respective controls, except for the case of Entry 19. In this case, the progeny derived from a TMV-infected TN 86 plant exhibited an entry mean AUDPC of 35.87, which is significantly greater than 27.32, the average AUDPC of the controls. In no case were progeny derived from a TMV-infected plant found to be more resistant than the control group. Taking into account the large number of comparisons that were made, it is possible that Entry 19 was significantly different as the result of a type I error rather than a true difference in genetic resistance. For these reasons, the method proposed by Kathiria et al. (2010) was determined to be an ineffective means of increasing the black shank resistance of an established line of tobacco.

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APPENDICES

Appendix A

Comparison of entry-mean values for each of the four different methods used to assess black shank disease severity. Additionally, entry-mean values for days to flowering are included on the far right.

Entry	Line	AUDPC	Disease Index	Arcsin Transformed End Percent Survival	Logit Transformed End Percent Survival	Days to Flowering
1	DH05B 710-2	38.63	47.04	0.167	-0.749	68.5
2	DH05B 710-3	31.29	38.36	0.325	0.609	73.75
3	DH05B 710-6	37.77	46.20	0.194	-0.442	77.5
4	DH05B 710-12	53.48	63.64	0.000	-14.657	68.25
5	DH05B 710-14	12.34	15.61	0.890	3.220	63
6	DH05B 710-15	36.23	44.08	0.208	-0.422	73.5
7	DH05B 710-28	46.64	55.73	0.000	-14.657	75.5
8	DH05B 710-33	32.71	40.60	0.165	-0.749	88
9	DH05B 710-40	45.12	54.11	0.000	-14.657	75.25
10	DH05B 710-42	43.62	52.72	0.000	-14.657	84.5
11	DH05B 710-47	21.63	26.98	0.571	1.934	65.25
12	DH05B 710-50	36.47	45.80	0.259	0.178	72
14	DH05B 710-53	13.67	18.38	0.705	2.454	69.25
15	DH05B 710-58	9.18	11.99	0.971	3.569	74.5
16	DH05B 710-59	8.41	11.19	0.866	3.150	72.75
17	DH05B 710-60	18.95	22.94	0.775	2.747	80.75
18	DH05B 710-65	30.73	37.58	0.345	0.797	74.25
19	DH05B 710-72	31.75	39.30	0.329	0.676	77.75
20	DH06B 162-30	42.12	50.67	0.000	-14.657	77.25
21	DH05B 1252-52	13.29	17.05	0.806	2.938	73.5
22	DH05B 1252-60	31.29	38.81	0.240	-0.007	77.75
23	DH05B 1252-84	31.26	39.00	0.256	0.128	71
24	DH05B 1252-95	18.89	25.92	0.490	1.554	68.75
25	DH05B 1252-100	6.67	7.30	1.132	4.322	79.5
26	DH05B 1252-101	26.98	34.92	0.340	0.707	74.75
27	DH05B 1252-116	3.80	5.25	1.127	4.288	75.25
28	DH05B 1252-118	34.65	42.82	0.257	0.138	77

Entry	Line	AUDPC	Disease Index	Arcsin Transformed End Percent Survival	Logit Transformed End Percent Survival	Days to Flowering
29	DH05B 1252-133	37.01	45.28	0.273	0.242	73.75
30	DH05B 1252-137	17.52	22.45	0.672	2.365	78
31	DH05B 1252-148	13.80	18.10	0.838	3.026	70.25
32	DH05B 1252-175	8.15	11.84	0.882	3.190	65
33	DH05B 1252-184	20.28	27.12	0.515	1.644	73.5
34	DH05B 1252-199	11.03	14.68	0.827	2.972	81.25
35	DH05B 1252-208	17.43	21.73	0.777	2.752	72.5
36	DH05B 1252-225	34.49	42.39	0.258	0.138	79.75
38	DH05B 1252-229	25.50	32.67	0.445	1.325	75
39	DH05B 1252-233	6.75	9.16	1.058	3.931	89.25
40	DH05B 1252-240	5.17	6.42	1.170	4.523	81.75
41	DH05B 1252-251	27.03	33.23	0.369	0.994	73.5
42	DH05B 1252-252	32.47	40.30	0.213	-0.228	78.5
43	DH05B 1252-257	37.59	46.02	0.093	-1.894	80.5
44	DH05B 1252-277	6.99	9.68	0.968	3.559	76.75
45	DH05B 1252-280	34.57	42.47	0.196	-0.461	74.75
46	DH05B 1252-284	39.06	47.55	0.000	-14.657	76.5
47	DH05B 1252-285	35.51	43.88	0.194	-0.480	75.5
48	DH05B 1252-291	35.03	42.67	0.237	-0.017	74.75
49	DH05B 1252-292	35.94	44.32	0.096	-1.838	79.25
50	DH05B 1252-293	4.56	7.57	1.035	3.783	77.25
51	DH05B 1252-297	41.58	50.02	0.089	-1.894	79
52	DH05B 1252-298	13.66	16.82	0.817	2.952	79
53	DH05B 1252-300	3.53	3.91	1.246	4.994	74.75
54	DH05B 1252-302	34.19	41.23	0.283	0.281	72.25
55	DH05B 1252-303	28.65	35.45	0.355	0.861	81
56	DH05B 1252-304	30.96	38.77	0.368	0.881	75.5
57	DH06B 173-2	15.77	19.67	0.773	2.731	70
58	DH06B 173-3	27.36	32.94	0.404	1.058	76.5
59	DH06B 173-4	11.64	13.93	0.919	3.352	75.75
60	DH06B 173-5	35.89	43.38	0.331	0.676	68.5
61	DH06B 173-7	25.98	32.87	0.416	1.175	73.75
62	DH06B 173-11	31.50	39.81	0.276	0.281	68
63	DH06B 173-13	45.02	54.37	0.000	-14.657	70.75

Entry	Line	AUDPC	Disease Index	Arcsin Transformed End Percent Survival	Logit Transformed End Percent Survival	Days to Flowering
64	DH06B 173-14	32.66	40.73	0.236	-0.046	69.5
65	DH06B 173-16	24.27	30.82	0.485	1.507	81.25
66	DH06B 173-18	11.46	13.73	0.949	3.486	72.75
67	DH06B 173-20	6.67	8.65	1.050	3.945	73.25
68	DH06B 173-24	24.17	30.36	0.490	1.542	71.25
69	DH06B 173-25	17.64	21.73	0.695	2.439	77.5
70	DH06B 173-26	46.49	56.10	0.000	-14.657	71.5
71	DH06B 173-27	35.28	43.59	0.190	-0.480	70.25
72	DH06B 173-30	20.75	26.63	0.547	1.805	64.75
73	DH06B 173-31	14.88	19.17	0.741	2.592	74
74	DH06B 173-35	30.34	38.89	0.395	1.058	76.5
75	DH06B 173-39	16.93	22.44	0.612	2.114	79.25
76	DH06B 173-40	37.83	46.68	0.215	-0.228	79.75
77	DH06B 173-41	51.74	61.24	0.000	-14.657	69.75
78	DH06B 173-49	5.64	7.01	1.136	4.373	76.5
79	DH06B 173-67	40.96	49.65	0.137	-1.182	73.25
80	DH06B 173-68	24.29	30.64	0.471	1.465	66.5
81	DH06B 173-69	33.99	41.93	0.187	-0.508	74.5
82	DH06B 173-81	28.53	36.30	0.297	0.504	77.25
83	DH06B 173-83	22.96	29.56	0.545	1.826	75.5
84	DH06B 173-85	15.69	22.27	0.583	2.002	78
85	DH06B 173-86	33.41	40.41	0.325	0.630	78.25
86	DH06B 173-87	7.95	9.48	1.069	4.019	69.25
87	DH06B 173-90	25.21	28.16	0.651	2.266	76.75
88	DH06B 173-101	30.71	37.89	0.253	0.128	72.5
89	DH06B 173-104	16.72	23.27	0.632	2.153	72.75
90	DH06B 173-114	50.67	60.16	0.000	-14.657	78.75
91	DH06B 173-121	31.24	38.66	0.300	0.429	67.5
92	DH06B 173-130	12.53	15.06	0.922	3.363	68.5
93	DH06B 162-1	31.38	39.27	0.279	0.301	74.5
94	DH06B 162-2	24.43	29.52	0.540	1.780	75.25
95	DH06B 162-3	29.81	37.72	0.292	0.409	82.25
96	DH06B 162-4	12.48	14.77	0.873	3.152	72.75
97	DH06B 162-5	28.57	34.47	0.474	1.500	71

Entry	Line	AUDPC	Disease Index	Arcsin Transformed End Percent Survival	Logit Transformed End Percent Survival	Days to Flowering
98	DH06B 162-8	6.44	7.82	1.079	4.059	63.75
99	DH06B 162-9	11.24	17.88	0.622	2.186	72.5
100	DH06B 162-11	36.42	44.50	0.208	-0.257	72.25
101	DH06B 162-12	26.17	32.79	0.359	0.839	78
102	DH06B 162-13	44.59	53.74	0.000	-14.657	66.25
103	DH06B 162-14	18.44	24.04	0.628	2.169	79.5
104	DH06B 162-16	1.44	2.07	1.262	5.139	78.75
105	DH06B 162-17	14.33	21.11	0.598	2.043	72.5
106	DH06B 162-18	37.59	45.73	0.134	-1.164	74.75
107	DH06B 162-19	8.26	11.06	0.965	3.541	75
108	DH06B 162-20	45.49	53.46	0.179	-0.498	78
109	DH06B 162-21	31.32	39.29	0.240	-0.046	68.5
110	DH06B 162-26	9.01	12.55	0.897	3.265	74.25
111	DH06B 162-27	8.51	10.11	0.999	3.685	77.25
112	DH06B 162-32	26.34	31.41	0.520	1.670	77
113	DH06B 162-33	35.09	43.38	0.159	-0.831	74
114	DH06B 173-46	2.94	3.89	1.234	4.854	81
115	DH06B 173-47	7.83	8.65	1.112	4.240	72.75
116	DH06B 173-52	19.05	25.84	0.597	2.043	71.5
117	DH06B 173-55	35.67	42.76	0.346	0.807	70
118	DH06B 173-102	15.59	21.13	0.706	2.508	78.25
119	Beinhart-1000	2.28	2.51	1.378	5.476	74.5
120	Hicks	52.73	62.25	0.000	-14.657	73.75

Appendix B

This table shows the paternal parent, date of pollination, and number of seed and mature capsules for each seed lot.

Additionally, the number of green plants found in each seed lot is included along with the identifier(s) assigned to them.

Seed Lot	Paternal Parent	Date of Pollination	Number of Seed	Number of Mature Capsules	Number of Green Plants	Unique Identifier
1	TN 86	11/13/2010	8550	5	0	
2	K 326	11/13/2010	9430	5	1	GH11-70-1
3	Narrow Leaf Madole	11/13/2010	10900	5	0	
4	TN 86	11/14/2010	18300	15	1	GH11-73-1
5	K 326	11/14/2010	22,970	15	0	
6	Narrow Leaf Madole	11/14/2010	20000	13	0	
7	TN 86	11/15/2010	25700	15	0	
8	K 326	11/15/2010	28000	15	0	
9	Narrow Leaf Madole	11/15/2010	29100	16	0	
10	TN 86	11/16/2010	26800	15	0	
11	K 326	11/16/2010	21100	16	0	
12	Narrow Leaf Madole	11/16/2010	30900	15	0	
13	TN 86	11/17/2010	24300	15	0	
14	K 326	11/17/2010	25000	16	0	
15	Narrow Leaf Madole	11/17/2010	30700	15	0	
16	TN 86	11/18/2010	20420	15	0	
17	K 326	11/18/2010	24930	14	0	
18	Narrow Leaf Madole	11/18/2010	31030	15	1	GH11-75-1
19	TN 86	11/19/2010	22880	15	0	

Seed Lot	Paternal Parent	Date of Pollination	Number of Seed	Number of Mature Capsules	Number of Green Plants	Unique Identifier
20	K 326	11/19/2010	27410	15	0	
21	Narrow Leaf Madole	11/19/2010	31800	15	0	
22	TN 86	11/20/2010	20540	14	2	GH11-73-2, GH11-73-3
23	K 326	11/20/2010	22750	15	0	
24	Narrow Leaf Madole	11/20/2010	25970	15	0	
25	TN 86	11/21/2010	20900	15	0	
26	K 326	11/21/2010	20430	15	2	GH11-74-2, GH11-74-3
27	Narrow Leaf Madole	11/21/2010	28280	15	1	GH11-75-2
28	TN 86	11/22/2010	23900	15	1	GH11-73-5
29	K 326	11/22/2010	18720	13	0	
30	Narrow Leaf Madole	11/22/2010	20600	15	0	
31	TN 86	11/23/2010	22480	15	1	GH11-73-4
32	K 326	11/23/2010	11330	15	0	
33	Narrow Leaf Madole	11/23/2010	21100	14	0	
34	TN 86	11/23/2010	17070	13	0	
35	K 326	11/23/2010	14170	15	0	
36	Narrow Leaf Madole	11/23/2010	17370	15	0	
37	TN 86	11/24/2010	23720	15	0	
38	K 326	11/24/2010	0	0	0	
39	Narrow Leaf Madole	11/24/2010	19340	11	0	
40	TN 86	11/29/2010	31130	15	0	
41	K 326	11/29/2010	22570	14	1	GH11-74-4
42	Narrow Leaf Madole	11/29/2010	28750	15	0	
43	TN 86	11/30/2010	28920	15	0	
44	K 326	11/30/2010	22540	14	0	

Seed Lot	Paternal Parent	Date of Pollination	Number of Seed	Number of Mature Capsules	Number of Green Plants	Unique Identifier
45	Narrow Leaf Madole	11/30/2010	30610	15	0	
46	TN 86	12/1/2010	25000	13	0	
47	K 326	12/1/2010	23520	15	1	GH12-005-4
48	Narrow Leaf Madole	12/1/2010	28380	12	0	
49	TN 86	12/2/2010	28110	15	0	
50	K 326	12/2/2010	31130	15	0	
51	Narrow Leaf Madole	12/2/2010	38200	15	0	
52	TN 86	12/3/2010	32260	15	0	
53	K 326	12/3/2010	31010	15	0	
54	Narrow Leaf Madole	12/3/2010	34180	15	0	
55	TN 86	12/4/2010	30610	15	0	
56	K 326	12/4/2010	29360	15	0	
57	Narrow Leaf Madole	12/4/2010	30390	15	0	
58	TN 86	12/5/2010	29270	15	0	
59	K 326	12/5/2010	27320	15	1	GH12-005-5
60	Narrow Leaf Madole	12/5/2010	33060	14	0	
61	TN 86	12/6/2010	28650	15	0	
62	K 326	12/6/2010	23250	15	0	
63	Narrow Leaf Madole	12/6/2010	32830	15	1	GH12-006-1
64	TN 86	12/7/2010	18000	8	0	
65	K 326	12/7/2010	18000	8	0	
66	Narrow Leaf Madole	12/7/2010	18000	8	0	