RESISTANCE TO GRAY LEAF SPOT OF MAIZE:

UNDERLYING GENETIC ARCHITECTURE AND ASSOCIATED MECHANISMS

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Jacqueline Marie Benson

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RESISTANCE TO GRAY LEAF SPOT OF MAIZE: UNDERLYING GENETIC ARCHITECTURE AND ASSOCIATED MECHANISMS

Jacqueline Marie Benson, Ph.D.

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Gray leaf spot (GLS) is a foliar disease of maize caused by *Cercospora zeae-maydis* and *Cercospora zeina* and quantitative resistance to GLS is important for maize production. A nested association mapping (NAM) maize population, consisting of 25 populations of 150 recombinant inbred lines, was used to identify quantitative trait loci (QTL) for GLS resistance. Trials were conducted in Blacksburg, VA, in a field with high natural incidence of GLS. A multivariate mixed model was used in ASReml3 to give the best linear unbiased predictions of disease severity ratings. QTL were selected using a general linear model selection procedure in SAS 9.2. Sixteen QTL, distributed across the maize genome, were identified using a likelihood of odds (LOD) selection threshold >4. Seven of these 16 QTL displayed allelic series with significantly higher and lower effects than the common parent allele. Near-isogenic lines (NILs) extracted from heterogeneous inbred families were developed to confirm and further fine-map select QTL, targeting the loci with the greatest LOD scores from the model selection QTL analysis. Phenotypic characterization of the NILs confirmed that the loci in bins 1.04, 2.09 and 4.05 likely contribute significantly to disease resistance, with bins 1.04 and 2.09 conferring reductions in disease of 12% and 23%, respectively. In contrast, the susceptible allele in bin 4.05, which was associated with the distance between major veins, conferred an increase of 8.4%. This disease-related venation trait was confirmed using the 4.05 NILs. Genome-wide

association studies revealed candidate genes related to the production of carotenoids, anthocyanins and antioxidant compounds that may play a role in cercosporin detoxification. Expression analysis of 1.05 NILs treated with cercosporin implicated a flavin-monooxygenase gene in cercosporin detoxification. Furthermore, significant associations between NAM parental allelic effects and parental phenotypes at the microscopic level for the 1.02 and 1.06 loci implicated callose plug and phenolic accumulation, respectively, in host defense. Elucidating the genetics of quantitative disease resistance loci provides breeders with valuable information that may enhance their ability to use molecular markers as a means to rapidly introgress loci that provide quantitative disease resistance.

BIOGRAPHICAL SKETCH

Jacqueline Benson was born in Okinawa, Japan to parents Edward and Diana Benson. She is proud of the African-American and Puerto Rican heritage and being raised in a military household, where she developed a strong sense of service. Her first 18 years of life consisted of moving to various locations including Hawaii, Germany and Maryland. Those early experiences in life heightened her appreciation for travel and different cultures.

Her undergraduate studies were in biology and ecology at St. Mary's College of Maryland (SMCM) where she completed an honors thesis in Dr. Christopher Tanner's seagrass ecology lab. She worked to improve understanding of seagrass ecology as it relates to pathogen resistance and stresses in a changing environment. In her free time, she played on the SMCM women's rugby team and interned during the summer. Her internship at the National Arboretum and desire to pursue a career in service motivated her to pursue applied agricultural genetics research at Cornell University.

In Dr. Rebecca Nelson's quantitative disease resistance lab, Jacqueline employed natural allelic variation in genetic mapping populations derived from diverse maize germplasm to further dissect the genetics of gray leaf spot resistance. At Cornell she also pursued her interest in pedagogy though teaching and training opportunities. Jacqueline's long-term goals are to work on the applied side of science for the purpose of improving farmer livelihoods.

DEDICATION

To Mom and Dad

For the drive, encouragement and support

Your loving daughter, Jaci

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CHAPTER 1: INTRODUCTION

Gray leaf spot (GLS), caused by *Cercospora zeae-maydis* and *C. zeina* is one of the most common and destructive maize (*Zea mays*) diseases in the United States (US) and in other maize growing regions of the world, notably Africa (Bhatia et al., 2002). This fungal disease is characterized by rectangular, tan lesions that can increase in density to cause severe blighting of the leaves, ultimately reducing grain yield (Ward et al. 1999). The fungus flourishes in warm humid weather that facilitates spore production on the maize residue, dispersal, and development on the growing crop (Jenco and Nutter 1992).

C. zeae-maydis and *C. zeina* are both members of the phylum Ascomycota. The sexual members of this group produce distinct asci that form when the zygote divides through the process of meiosis (Campbell and Reece 2008). A mitotic division of the tetrad results in the formation of eight ordered ascospores within the ascus. When mature, the parathecium releases the asci and the spores within germinate when the environmental conditions are favorable. While the sibling species were both believed to reproduce primarily asexually, two mating types present in similar proportions were identified for both pathogens that cause GLS, which suggests that cryptic sex may occur in natural populations (Groenewald et al. 2006).

One of the most frequently cited papers on *C. zeae-maydis* pathogenesis reported external and internal growth of the pathogen on maize leaves (Beckman and Payne 1982b). To date, few studies have extended this understanding of pathogen development on and within the host (Kim et al. 2011), although Beckman and Payne (1982b) showed that after a period of high humidity,

conidium germinate on the leaf surface. Once the stomate has been detected, the fungus will form an appressorium, which requires high humidity (between 90-95%) (Thorson and Martinson 1993). *C. zeae-maydis* was observed to maintain hyphal growth on the leaf surface for up to one week before appressorium formation and penetration, which occurs within a week (Beckman and Payne 1982b). Once a spore lands on the leaf surface, a germ tube forms an appressorium over the stomate and enters the leaf, thereby exhibiting positive stomatal tropism.

Colonization of the plant results in the formation of a mycelial network within the leaf and based on the shape of the lesion, it appears that lateral hyphal growth is delimited by the sclerenchyma. After colonization of the maize leaf and formation of lesions, the fungus begins the production of stroma. Stroma, which form in the substomatal cavities of the maize leaf, can overwinter and survive climatic factors that are not ideal for within-season reproduction.

After *C. zeae-maydis* produces the stroma, specialized asexual structures called conidiophores are produced, which act as inoculum for secondary cycles. The conidiophores protrude through the stomata of the leaf and begin to produce conidia. The wind and/or water droplets then disperse the conidia, which make contact with another leaf surface and germinate, form appressoria, and colonize the plant cells. This can occur more than once in a season, making it a polycyclic organism (Stromberg and Donahue 1986b). The conidia produced from conidiophores can also survive unfavorable conditions. They can rehydrate after dry hot periods and produce secondary conidia after germinating on the conidiophore (Lapaire and Dunkle 2003). *C. zeae-maydis* can continue this microcyle conidiation for up to four cycles before expending endogenous reserves. The mycelium produced by the fungus can overwinter within

plant debris in the form of hard masses of intertwined hyphae (Payne and Waldron 1983). Sporulation of the fungal mass in the spring will result in the production of conidia as primary inoculum.

Successful colonization of the plant cells results in the formation of the distinct rectangular-shaped lesions, which appear to be delineated by the major veins of susceptible varieties (Beckman and Payne 1982b; Beckman and Payne 1983). Chlorotic lesions are the first macroscopic symptom of infection, which is followed by the development of necrotic lesions a few weeks after infection. During this period between the chlorotic fleck and lesion stages, the pathogen's actions are not well understood. More resistant varieties display fleck symptoms that, when exposed to sunlight, appear translucent and even greasy. Depending on the level of susceptibility, lesions may grow in size by further killing the cells in the region or may increase in number by dispersion of secondary inoculum.

The life-style of *C. zeae-maydis* is not well understood. Broadly speaking, biotrophs derive energy from living cells while necrotrophs derive energy from killed cells (Lewis 1973). Hemibiotrophy has been defined as an initial period of biotrophy followed by the transition into necrotrophic development (Perfect and Green 2001). However, there are many ways to describe either biotrophy or necrotrophy (Oliver and Ipcho 2004). Biotrophs are obligates, have narrow host range, induce the hypersensitive response in incompatible reactions, and are controlled by single resistance genes in a pathway dependent on salicylic acid. While necrotrophs are non-obligate, have a broad host range (often attributed to the production of toxins and cell wall-degrading enzymes), and are controlled by quantitative resistance in a pathway dependent on

jasmonic acid and ethylene. Older literature identifies the GLS pathogens as necrotrophic (Chupp 1953; Stromberg and Donahue 1986a), while recent literature suggests that GLS starts with a biotrophic phase and then transitions to a necrotrophic phase, making GLS a hemibiotroph disease (Balint-Kurti et al. 2010). Understanding the mechanisms underlying resistance to GLS may further elucidate the pathogen biology since there are contrasting mechanisms of defense against necrotrophic and biotrophic pathogens (Glazebrook 2005).

It is understood that light plays an important role in the infection cycle. The photoreceptor PHL1 was identified as being necessary for pathogenesis by *C. zeae-maydis*. Light is required for stomatal perception and infection as well (Kim et al. 2011). However, continuous light will inhibit germination, germ tube growth, and sporulation (Beckman and Payne 1982a, 1983). Additionally, blue light induces cercosporin biosynthesis but represses conidiation (Bluhm and Dunkle 2008). Cercosporin, a light-activated, nonhost-specific toxin produced by the *Cercospora* genus, is credited with the success of the genus, as it acts in plant cell death and the formation of the necrotic tissue. Gwinn et al. (1987) first reported isolation of cercosporin from *C. zeae-maydis*. Duvick (1987) showed that cercosporin was not detected in apparently uninfected tissue but was significantly detected in water-soaked lesions and mature lesions. When this source of cercosporin was extracted from the infected tissue, it caused necrosis when applied to healthy tissue and formed lesions like those used to identify symptoms of GLS.

History

While *Cercospora zeae-maydis* was first noted in Illinois to be the causal agent of GLS by Tehon and Daniels (1925), its prevalence and impact on yield were not considered significant until the

late 1970s to the early 1980s. The effects of GLS disease development on maize crops were widely underestimated until the late 1980's because of the late onset of symptoms that characterize the disease. Early reports of the effect of GLS disease development on yields of maize hybrids and inbred line performance came from studies of maize grown in Virginia, West Virginia, Tennessee, Kentucky, and North Carolina (Hilty et al. 1979; Payne and Waldron 1983; Roane et al. 1974; Rupe et al. 1982). GLS was noted to be most prevalent in regions with long periods of high relative humidity and leaf wetness (Rupe et al. 1982).

By the mid-1980s, GLS was widespread and considered to cause yield losses of up to 70% due to the associated severe blighting, stalk deterioration, and lodging throughout the mid-Atlantic maize growing regions (Latterell and Rossi 1983). A major contributing factor to the yield losses is believed to be the reduction in the photosynthetic capacity of the maize leaf due to the presence of the GLS lesions. The leaves of the upper half of the maize plant contribute to over 75% of the photosynthate moved to the ear during the grain-fill period and if production and movement of these nutrients are severely compromised by lesion development, both the size and number of kernels per ear are reduced (Allison and Watson 1966).

During this time, GLS disease symptoms and the presence of *Cercospora* spp. isolates in typical GLS lesions were also reported in South and Central America, states within the US maize belt, and maize-growing regions in South Africa. It then became apparent that the increase in disease severity and presence coincided with increasing use of no-till or conservation agriculture practices and increased planting densities (Denazareno et al. 1991; Payne and Adkins 1987). Disease severity increased with the amount of plant debris left on the field from the previous

season, so farms using tillage methods that left such residues, especially greater than 35% of debris, were found to be prone to high disease pressure the following season (Denazareno et al. 1993). In 1996, Garst Seeds Company estimated that the disease was spreading by 80 to 160 km^2 per year in the US (Ward et al. 1999). In 2002, the first report of GLS in Ontario, Canada was published, in which the authors suggested a potential role for increasing temperatures in the northward spread of the disease (Zhu et al. 2002).

By the early 1990s, reports of GLS became more frequent and work to elucidate the nature of GLS inheritance, sources of resistance via germplasm registration, and early genetic mapping studies were performed in recognition of its increased importance (Fig. 1.2). These studies were used to determine whether resistance is under the control of single, few, or multiple host genes. The data indicated that the populations have either qualitative variation or quantitative variation as a result of major gene and multiple gene control, respectively.

Cercospora zeina and the presence of GLS in Africa

Original reports of GLS classified the causal agent as a single species, *C. zeae-maydis.* While this is still the pathogen most commonly referred to in the literature as the causal agent of GLS, there are increasing indications that there are two, or possibly three, pathogens responsible for GLS lesions. Two genetically distinct groups were identified among single conidial isolates of the fungal pathogen that cause GLS (Wang et al. 1998), which were about 80% genetically distinct between groups and about 90% similar within the groups. An even greater genetic difference was reported between the two fungal pathogens of maize than between either one of them and the sorghum pathogen, *C. sorghi*. Both isolates were present among maize-growing

regions of the US but more frequently isolates from the Group I, now identified as *C. zeae-maydis,* were found in the maize belt while Group II, *C. zeina,* was localized to the east coast. Later both groups were identified in Brazil (Brunelli et al. 2008). Moreover, individual lesions on maize plants harvested on the east coast contained isolates from both groups, suggesting that the pathogens may sporulate out the same lesion (Carson et al. 2002).

Morphological differences in conidia and conidiophore structures have not been significant enough to distinguish between the two groups. Isolates from Africa were found to be more genetically related to Group II isolates, with which they also shared the properties that they grew more slowly and did not produce detectable amounts of cercosporin in culture (Dunkle and Levy 2000; Meisel et al. 2009). These Group II isolates from Africa were also more genetically diverse than the Group II isolates from the eastern US, suggesting that maize-growing regions in Africa may be the center of origin for Group II and that a bottleneck occurred before its migration to the US. Isolates collected from Nigeria were identified as being genetically distinct from either Group I or II (Sharma et al. 2010) and were more closely related to *C. apii*, *C. beticola*, and *C. sorghi* than to the known causal agent of GLS.

Group I and II of *C. zeae-maydis* were classified as genetically distinct species following phylogenetic analysis of isolates from the two groups using internal transcribed spacers as well as 5.8S rRNA, elongation factor, histone, actin, and calmodulin gene regions (Crous et al. 2006; Goodwin et al. 2001). Additionally, Group I has a faster growth rate in controlled environments, elongated conidiophores, and cercosporin production. Crous et al. (2006) determined that Group I is *C. zeae-maydis* and named Group II as *C. zeina*.

C. zeina has traditionally been considered the causal species for GLS in Africa. Since there are dramatically different cropping systems of maize that range from industrial large-scale systems to small-scale subsistence farms, the management strategies and disease impacts also vary. In addition to South Africa, GLS has been reported in Zimbabwe, Zambia, Cameroon, Kenya, Uganda, Zaire, Ethiopia, Malawi, Mozambique, Nigeria, Swaziland, and Tanzania, where maize is a major food crop produced by subsistence farmers (Lyimo et al. 2011; Ward et al. 1999).

Epidemiology

As for many polycyclic diseases, GLS development is conditioned by the amount of initial inoculum present, the rate of pathogen reproduction, and the proportion of healthy tissue remaining for the pathogen to infect. The amount of initial inoculum present is heavily influenced by conservation tillage practices and the presence of mature, infected tissue in nearby fields, since the primary inoculum develops on the debris from the previous season if it remained on or near the soil surface when optimal environmental conditions were reached (Denazareno et al. 1992; Payne and Waldron 1983). The rate of pathogen reproduction is determined by climatic factors such as humidity, rainfall, and temperature. GLS disease development is heavily influenced by these climatic variables so that even in the presence of high initial inocula, moderate to severe epidemics may fail to develop. Bhatia and Munkvold (2002) found that varietal resistance, cumulative temperature hours, and planting dates were also significantly correlated with disease development. Longitude of the field site has also been noted to significantly associate with disease and, although not tested, this may relate to cumulative temperature hours (Paul and Munkvold 2004).

Favorable environmental conditions combined with the polycyclic nature of GLS and ease of dispersion may result in epidemics that results in yield loss. Aerial spore counts are highest during the daily drop in relative humidity and increase in temperatures (Paul and Munkvold 2005). Aerial spores were sampled as early as the second half of June but were in greatest concentration at the end of the season in late September. Maximum spore production in controlled settings has been noted to occur between 25 and 30°C (Paul and Munkvold 2005). Inoculum can be wind and rain dispersed, so the conidia produced on one plant may transfer to the leaf of another maize plant. The distribution pattern of lesions on the maize plant changes throughout the season. Lesions first appear on the lower leaves and will eventually reach the higher tiers (Maroof et al. 1993) and within individual leaves, the middle and basal regions develop lesions later than the tapered ends. It was found that maize lines exhibiting long latent periods (from 14 to 28 days) as well as lower sporulation capacity fall within the moderately to highly resistant categories (Beckman and Payne 1982a; Ringer and Grybauskas 1995). In general a long latent period is found with GLS compared to other foliar diseases of maize and brings into question what the pathogen is doing between infection and necrosis, since the spores are present so early in the season. Additionally, early infection cycles, nourished by steady rainfall and sporulation, are believed to have a larger impact on overall disease development than later infections cycles.

Subsistence farmers usually plant maize on soils with low fertility and have limited resources. Additionally, growing seasons often overlap, which may exacerbate the spread of disease from the mature crop to the developing plants. Under tropical conditions, the amount of inoculum

present is highly correlated to disease development. Distance from the source of inoculum in turn affects the amount of inoculum present. No relationship was detected between disease development and the direction from the source of inoculum (Asea et al. 2002).

In the Western Highlands of Camaroon, GLS was identified as the potential cause of a 79% yield loss (Ngoko et al. 2002). Other maize diseases were identified but none was as devastating as GLS. The increased use of nitrogen, phosphorus, and potassium increases disease development (Caldwell et al. 2002; Ward et al. 1997a). Since small-scale farmers are encouraged to use chemical fertilizers for increased production but may not be able to afford the fungicides necessary to protect the crop from GLS, this strategy may also increase losses to disease. Similarly, in Nigeria, nitrogen-augmented fields were associated with increased disease development, although fertilizer application was reported to have no effect on sporulation (Okori et al. 2004). It was found that maize fertilized by composted cattle and poultry manure exhibited lower levels of GLS disease development compared to maize fertilized with manufactured mineral fertilizers (Lyimo et al. 2012).

Management

Integrated pest management practices can be used to control losses attributed to diseases or pests. These practices can include use of resistant cultivars, chemicals, and different farming practices. Ward et al. (1999) identified disease management strategies that can be used to reduce the development of GLS. These include the identification and utilization of resistant and tolerant sources in breeding for disease resistance as well as the use of fungicides, soil fertility, plant density, and irrigation to diminish disease development. Additionally, Latterell and Rossi (1983) suggested rotation, sanitation, and resistant or tolerant varieties as means for disease

management. It is often not feasible to alter agronomic practices involving plant density, irrigation, and nutrient application for disease management because these practices have such direct effects on crop yield. *C. zeae-maydis* specifically attacks the maize crop and has not been noted to survive longer than one year on maize debris so crop rotation may be a promising means of reducing disease. Additionally, removing the debris from the current season and planting a winter cover crop will facilitate management of the disease, as primary inoculum survives on the debris of the previous season and no-tillage plots exhibit the greatest aerial spore count, disease onset, and development among plots with variable tillage treatments (Payne and Duncan 1985).

Fungicides reduce disease development and yield losses amongst susceptible maize hybrids. As might be expected, the most susceptible maize hybrids had greatest response in reduced disease development to fungicide application (Ward et al. 1996). These toxic chemicals are expensive and their use in developing countries can be particularly hazardous to both growers and consumers because of limited regulation. Because smallholder farmers do not have economic or practical access to fungicides, they suffer yield losses under disease-conducive conditions unless they have access to resistant varieties.

Because susceptible hybrids respond well to the fungicides, Ward et al. (1997a) suggested that the benefits of no-till or conservation tillage practices outweigh the expense of fungicides in South Africa and therefore fungicide mixtures should be used in conjunction with conservation tillage practices to reduce GLS disease development and the risk of fungicide resistance (Ward et al. 1997b). Optimum fungicide treatment was achieved by spraying before the disease developed beyond the basal five leaves and continuing to spray until reproductive maturity

(Ward et al. 1997c). This may control disease but not give return on investment, however. A probability study on the likelihood of receiving a net return from maize yields with the application of fungicides revealed that in every scenario, the probability of returns was less for more than one application of fungicides, but that with one spray, six out of the nine scenarios provided a greater than 50% chance of net returns (Munkvold et al. 2001).

Components of epidemics and their use to plant breeders

In the mid 1980's when the late onset of GLS disease development was accepted as a threat to yield, resistance had not been incorporated into commercial germplasm; available lines with high levels of resistance had poor agronomic characteristics. Because of this, it was important to develop inoculation and screening methods and evaluate the plants developed for crop production and improvement. There are many methods that can be used to evaluate disease resistance, such as constructing a disease progress scale used for scoring germplasm, such as hybrids, inbreds, and wild ancestors, collected over time in an epidemic environment. The data collected from these studies can be used to determine the accessions that contain the best source of resistance and whether or not these sources are ideal based on the way in which the population was developed. It is important that the disease resistance breeder has a good understanding of disease epidemiology.

Plant breeders interested in developing resistant varieties use epidemic development as a means to assess susceptibility among different plant genotypes. Visible components of epidemics such as lesion development or incubation period are most often used by breeders to identify variability

in disease resistance. The methods used by breeders to assess resistance are used by plant pathologists to develop disease progress curves for models used in disease forecasting.

The disease progress curve is a plot of disease severity over time. This is attained by rating disease severity at different intervals in a select time period, often from the appearance of symptoms to the death of plants. The resulting curve reflects disease development as a result of complex interactions between components of the disease triangle. The factors included in the disease triangle are environmental variation, pathogen biology, and host biology. Epidemiologists have developed models to describe the curves of graphs for both monocyclic and polycyclic pathogens. These models started out simply with parameters that include basic infection rate, initial inoculum, and the proportion of healthy plants. They grew more complex in an attempt by epidemiologists to capture other variables of disease progress within their models. The parameters that are often not evaluated for such models include host growth, latent period length, and lesion development (Berger and Jones, 1985).

Host growth is an important parameter because it influences the observed rate of disease increase and the shape of the disease progress curve. Imagine that the proportion of the disease on a plant may be the same for two consecutive measurements one week apart if there is twice the increase in disease as well as twice the increase in leaf area during the interim of measurements. Models began to include a logistic equation to explain the increase in leaf area over time (Berger and Jones, 1985).

Another component of disease epidemics is the latent period. The latent period length is defined as the time lapse between infection and reproduction. While the latent period is difficult to assess, in certain pathosystems it is often strongly correlated with incubation period (IP), which can be estimated by the time between inoculation and visible symptom development (Xu and Robinson, 2001). Latency is variable among pathogens and between latent periods that occur in the duration of one season (Berger, 1989). The environment and other aspects of pathogen biology affect this variability among latent periods. GLS is a disease with a very long latent period so this is an important component to consider.

Lesion development is an important epidemic component because growth of lesions in either size or number contributes to total severity of disease in an epidemic (Berger et al, 1997). Lesions can either expand or increase in number depending on the pathogen. Even if the same pathogen is present, the lesion characteristics can be different depending on the host. Consequences of lesion development on the plant leaf include a decrease in photosynthetic capacity. The blighted leaf surface provides space for greater inoculum production. Lesion development is particularly important for screening GLS because IP has a very low heritability (0.18), suggesting that it should not be used as a means to screen for resistance across environments (Gordon et al. 2006).

Plant breeders are interested in slowing the progress of epidemics by breeding for host resistance to reduce losses and dependency on fungicides, which are expensive and may not be environmentally friendly. Resistance is also important because pathogen populations are capable of developing resistance to sprays and major resistance genes. There are advantages and disadvantages to breeding for horizontal resistance. Some advantages of major gene resistance

are that the mechanism is understood and that it is easier to breed for single gene resistance or pyramid a few single genes into a susceptible background. The big disadvantage is that they are often ephemeral, and in the case of resistance against GLS and many other necrotrophs, nonexistent. Single gene resistance imposes strong selection pressure on the pathogen population, often resulting in boom and bust cycles through the development of pathogen genotypes capable of evading detection by resistance genes. The other option is breeding for quantitative resistance. The mechanisms underlying quantitative resistance are not well understood and it is difficult to breed into varieties because of linkage, allelic series, and linkage drag. In addition, there could be pleiotropic loci associated with other traits and therefore trade-offs to introgression. Resistance genes in the same linkage block may be introgressed together but if there are different alleles of the same gene, all of which confer resistance, the breeder can only introgress two alleles into a susceptible background. Linkage drag, on the other hand, is the accompaniment of deleterious genes with the genes of interest during recombination.

Studies have begun to identify mechanisms of quantitative resistance and have found that they affect different stages of the disease cycle (Chung et al. 2010). These mechanisms include reduction in total number of infections, reduction in lesion expansion, reduction of sporulation, lengthening of the latent period, and increasing the number of propagules necessary to establish infection (Berger, 1977). Breeders are able to assess lesion expansion, sporulation as a function of lesion size, total number of infections based on lesion number, and latent period if it is directly related to incubation period.

Plant breeders use intentionally-induced disease epidemics in order to identify sources of resistance, screen progenies, or analyze the genetics of resistance. It is useful to know whether resistance is qualitatively or quantitatively controlled and the regions in the genome that confer this resistance. This information will aid in the introgression of these regions into the genome of a susceptible variety. They are also interested in assessing the heritability of resistance, as an indicator of the stability of resistance in different environments. In order to accomplish these genetic studies, it is useful to create an epidemic amongst the plant population with heavy and even disease pressure that permits discrimination of different levels of resistance.

In some cases, it may be possible to use natural infection, but the conditions must be consistently favorable for pathogen development. If natural heavy disease pressure exists in the field, it is unlikely that the pressure is evenly distributed throughout the population so the environmental effects should be controlled by using environmental checks or spatial analysis. This is important because breeders are evaluating the ability of the particular genotype to resist disease progression relative to the other genotypes in the population. Additionally, a consistent method for screening diseases in the greenhouse is valuable. While it is challenging to develop an epidemic in the greenhouse for GLS, progress has been made with individual leaf infections or by applying conidial suspension in early development by puncturing the whorl of plants at the V3 stage and placing a bag over the plant (Asea et al. 2005; Fleer and Partridge 2004). These greenhouse methods provide a way to identify levels of resistance and determine host pathogen interactions outside of the field.

Variability among pathogen populations may be one explanation of genotype by environment interactions (Carson et al. 2002). The variability in parasitic fitness, disease efficiency, and lesion length of *C. zeae-maydis* isolates suggests that breeders should screen for GLS disease development using a multi-isolate inoculum (Bair and Ayers 1986). After noting hybrid-location interactions, the same hybrids were planted at sites with no historical presence of GLS. The hybrids were treated with inoculum developed from isolates harvested at the previous field locations. The authors found that the hybrid-isolate interaction at the locations with no historic disease mirrored that of the previous hybrid-location interaction. Since there are two causal agents of GLS, it brings into question whether QTL identified in genetic mapping experiments performed in presence of both are indeed effective for resistance against both pathogens.

Once an epidemic has been established, it is important to have a method of measuring resistance and a known susceptible variety with which to compare to the rest of the population. It would be ideal to measure the growth and development of the pathogen, but this poses a problem because it is likely that the pathogen population size is difficult to quantify for large breeding or mapping populations. The next best method is to have a means with which to evaluate the pathogen effect on the host. Often this is done through symptom evaluation and is useful if the severity of symptoms is directly correlated to the pathogen presence. Similar to the way pathologists of certain plant diseases develop disease curves, plant breeders use lesion development as a way to measure host resistance. If the plant does not exhibit obvious disease symptoms such as lesion development (eg. some virus diseases), quantitative PCR can be used to quantify the pathogen presence (Edwards et al. 2002).

The components of disease epidemics that are of most interest to breeders are incubation period and the extent of lesion development. Incubation period is measured by recording the days between pathogen exposure and symptom appearance. The extent of lesion development can be determined by measuring lesion size and percent diseased leaf area and calculating area under the disease progress curve (AUDPC). Three to five rating sessions can be performed with nearly equal time between each session in order to develop an AUDPC for each genotype. Taking multiple ratings throughout the season is a better evaluation of resistance than just a single rating. These are variables of disease development that can be visually evaluated during the planting season. Visible disease development is especially useful because it enables the breeder to efficiently assess resistance in larger breeding or mapping populations.

Elucidating the genetics of diseases

There are different types of resistance including those under single (monogenic), few (oligogenic), and multiple (multigenic) gene control. The different controls result in either discontinuous (qualitative) variation or continuous (quantitative) variation in resistance. This variation in resistance allows breeders to determine the complexity of genetic control based on the distribution of phenotypes of F_2, F_3, or backcross populations as well as genetic or association mapping populations. The germplasm pool should be evaluated to determine the best genetic sources for constructing these populations. The F_2, F_3, and backcross generations can also be developed to assess heritability. Qualitative traits are typically easier to inherit and transfer than quantitative traits. These populations can also be evaluated for heterosis or transgressive variance in which the progeny exhibits more extreme levels of resistance or susceptibility than that of the parents due to heterozygous state or the effect of different allele combinations for

genes controlling resistance. Resistance can be scored among hybrid, backcross and mapping populations consisting of fixed recombinant inbred lines (RIL) to perform QTL analysis. RILs are desirable for genetic mapping studies because they have a fixed genotype that can be reproduced for multiple locations and multiple years. This elimination of genotypic variation for a single genotype allows one to determine the impact of planting in different environments and field seasons.

Statistical analysis can be performed to find the co-segregation of markers with the phenotype if the resistance trait is qualitatively inherited. If the trait is found to be qualitatively inherited, a bulked segregant analysis can be used instead. The efficacy of markers for MAS of the resistance phenotype can also be tested. These markers may eventually be used for the pyramiding of qualitative or quantitative genes. For these genetic mapping studies, it is useful for the markers to be distributed evenly across the genome for genetic mapping, though this is often challenging within centromeric regions of the chromosome. These markers may be used to genotype diverse germplasm as well as the resistant germplasm that has been evaluated in the field trials for crossing potential, and should be polymorphic, co-dominant markers. The markers used to assess genotypes should be polymorphic between the susceptible and the resistant germplasm.

Mechanisms underlying QTL

Genetic mapping studies have been performed to elucidate the loci and genes underlying quantitative disease resistance (QDR). The mechanisms underlying resistance, however, are not well understood. Poland et al. (2009) provided six hypotheses regarding mechanisms that

underlie QDR loci: (1) genes that underlie plant development and architecture, (2) genes with mutations or allelic changes in genes involved in basal defense, (3) genes involved in secondary metabolite production known to fend off pathogen attacks, (4) genes involved in signal transduction, (5) weak forms of *R*-genes, and (6) genes previously unassociated with pathogen defense. I hypothesize that genes underlying secondary metabolite production, plant architecture, and development are playing a specific role in GLS resistance.

Secondary metabolites. Genes involved in secondary metabolite production are hypothesized to be involved in GLS resistance through detoxification of cercosporin. Anthocyanins and phenolic acids are metabolites with antioxidant properties; these may be particularly useful when the plant is exposed to a toxin that produces active oxygen species such as cercosporin. Cercosporin is a photo-activated perylenequinone that converts molecular oxygen to active oxygen species (Daub 1982). These species include hydrogen peroxide, hydroxyl radical, superoxide, and singlet oxygen (Spikes 1989). The plant has evolved with exposure to the first three oxygen species and so has a response to these species. Superoxide dismutase, catalase, and peroxidase enzymes, produced by the plant, catalyze reactions that reduce the radical species and prevent additional damage to the cell. However, the only place that the plant is exposed to singlet oxygen species is within the chloroplasts (Young 1991). Chlorophyll is also a photo-activated molecule and is capable of producing singlet oxygen species when some of the absorbed energy is not passed to the electron transport chain. Carotenoid pigments present within the chloroplasts are capable of quenching the singlet oxygen species.

Plant architecture. Host resistance may be involved in reducing the total number of infections, reduction in lesion expansion, reduction of sporulation, and increasing the number of propagules necessary to establish infection (Berger, 1977). Quantitative resistance has been noted to affect lesion size, latent period, and sporulation (Coates and White 1994; Ringer and Grybauskas 1995; Ward et al. 1999). GLS lesion characteristics vary depending on the maize genotype, which may be indicators of host resistance mechanisms. Lesion type can also be indicative of overall disease development. Chlorotic lesions are associated with reduced lesion size, sporulation, and overall disease development (Freppon et al. 1994). When plants with chlorotic lesion types were crossed to plants with nonchlorotic lesion types, all of the progeny exhibited chlorotic lesions, which indicated that dominant interactions may be at play; however later generations exhibited segregating phenotypes, suggesting a more complex mode of inheritance (Freppon et al. 1996).

Plant maturation has a profound influence on GLS pathogenesis. The genetic control of plant development thus influences susceptibility to the disease. When testing leaf disks from plants of differing age and differential field disease development, Gwinn et al. (1987) found that it was not the varietal susceptibility in the field but the plant age that correlated to *C. zeae-maydis* infection. They found that the fungus was able to form appressoria and penetrate significantly more stomates in old tissue than in young tissue across the cultivars, even though all stomates were closed in the high humidity conditions. Additionally, there was significantly less ion leakage in older tissue from treatment with cercosporin, which was interpreted as a decrease in sensitivity to cercosporin in older tissue suggesting that the toxin may play a crucial role in young tissue and perhaps earlier stages of necrotophy. There was no significant difference found across cultivars in either fungal penetration frequency or cercosporin sensitivity. Increased evidence for plant

age playing a role in defense is supported by an experimental finding wherein identical hybrids planted in succession at three week intervals exhibited a nearly three week delay in symptom appearance, suggesting that disease development is influenced by physiological maturity. Other hypotheses include delay in inoculum levels and the establishment of high humidity microclimate by the leaf canopy, but neither explain the experimental results (Rupe et al. 1982). Additionally, after inoculating maize hybrids at different stages of crop development, Nutter and Jenco (1992) found that 90% of the yield variation could be explained by the disease severity on the middle third of the plant at the late dough stage of kernel development.

Summary

In the following chapters, the genetics of GLS are explored through use of linkage and association mapping and as well as near-isogenic lines. The NAM population was used for genetic and association mapping purposes, while the HIF populations were used for fine-mapping and confirmation purposes. Additionally, I worked to gain a greater understanding of underlying disease mechanisms through macroscopic and microscopic analysis of pathogenesis.

Figure 1.1. Maize leaves from 10 different inbred lines that found the nested association mapping population that were infected with gray leaf spot in Blacksburg, Virginia. Images were collected after dehiscence and there is an apparent continuous distribution.

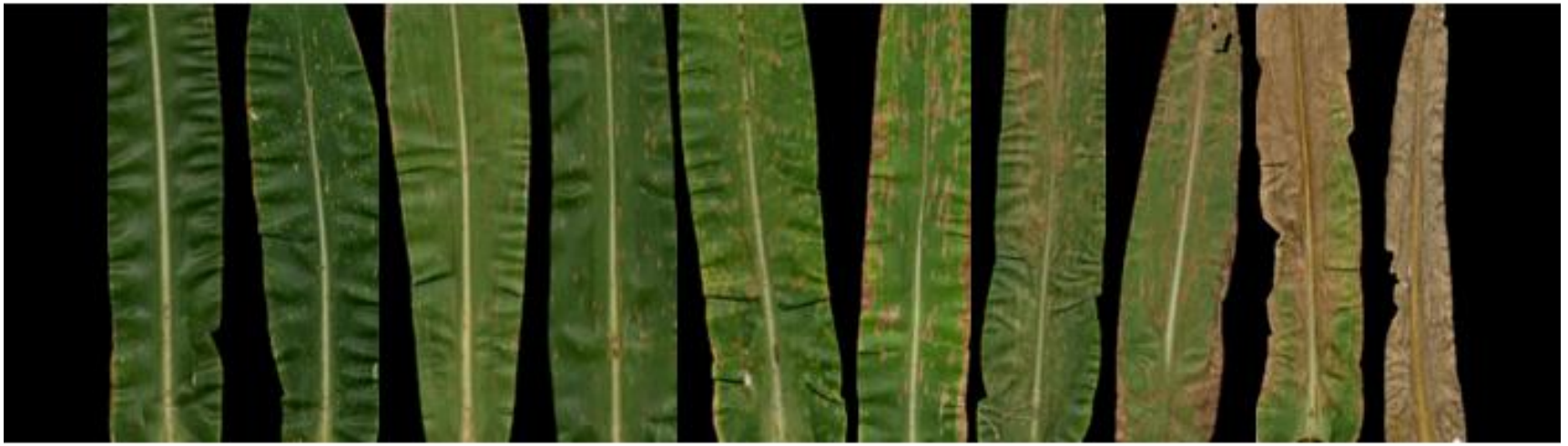
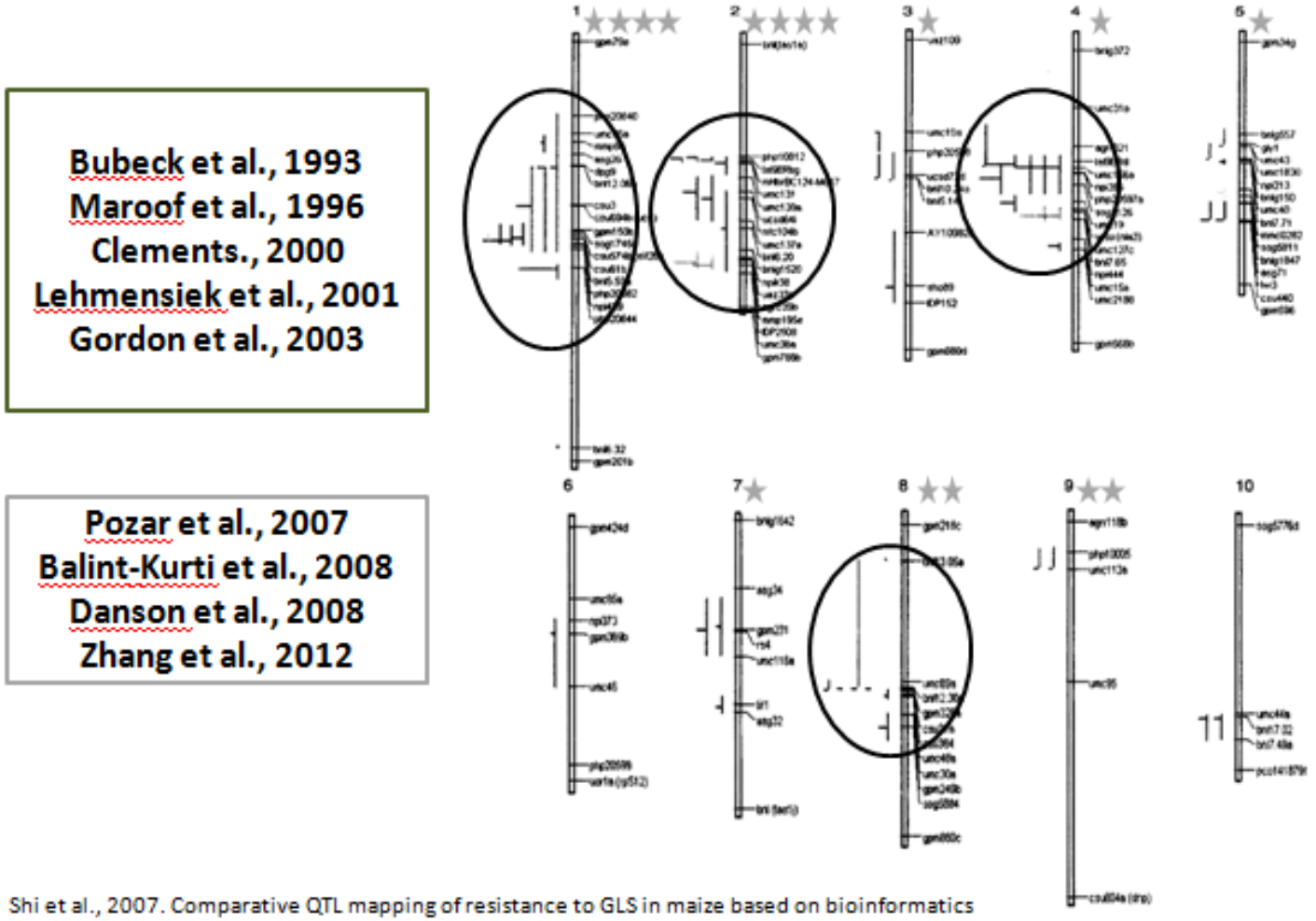


Figure 1.2. Quantitative trait loci from five studies performed from 1993 to 2003 (listed in the black box). Shi et al., 2007 compiled the QTL across the genome and identified hotspots circled in black. The gray stars indicate individual QTL on the neighboring chromosome identified among the four studies performed from 2007-2012 (listed in the gray box).



Shi et al., 2007. Comparative QTL mapping of resistance to GLS in maize based on bioinformatics

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CHAPTER 2: PLEIOTROPIC LOCI IMPLICATE STRUCTURAL AND DEVELOPMENTAL MECHANISMS IN QUANTITATIVE DISEASE RESISTANCE ASSOCIATED WITH GRAY LEAF SPOT OF MAIZE

ABSTRACT

Gray leaf spot (GLS) is a foliar disease of maize caused by *Cercospora zeae-maydis* and *Cercospora zeina*. Quantitative resistance to GLS is important for crop production. The nested association mapping (NAM) maize population, consisting of 25 populations of 150 recombinant inbred lines, was used to identify quantitative trait loci (QTL) for GLS resistance. Trials were conducted in Blacksburg, VA, on a field with high natural incidence of GLS. Lines were evaluated for disease severity three times at seven day intervals for each of three years. A multivariate mixed model was used in ASReml3 to give best linear unbiased predictions of disease severity ratings. QTL were selected using a general linear model selection procedure in SAS 9.2. QTL analysis identified 16 QTL distributed across the maize genome using a likelihood of odds (LOD) selection threshold >5. Seven of the 16 significant QTL displayed allelic series with significant effects above and below that of the B73 allele. Heterogeneous inbred families (HIF) were developed to confirm and further fine-map selected QTL. HIF analysis confirmed loci in bins 1.04, 2.09 and 4.05as contributing significantly to disease resistance. The markers associated with these loci had the greatest LOD scores from the model selection QTL analysis. The resistant alleles in bins 1.04 and 2.09 conferred reductions in disease of 12% and 23%, respectively. The susceptible allele in bin 4.05 conferred an increase of 8.4%. The 4.05 locus is also associated with distance between major veins. This disease-related venation trait was confirmed using the 4.05 NIL lines. Genome-wide association studies

revealed candidate genes related to the production of carotenoids, anthocyanins and strong antioxidant compounds that may play a role in cercosporin detoxification. It is important to elucidate the genetics underlying GLS of maize and associated mechanisms in order to increase the breeder's decision making capacities.

INTRODUCTION

Despite the vast literature on plant defense mechanisms, the genetic basis of quantitative disease resistance (QDR) is not well understood. Poland et al. (2009) described possible mechanisms underlying QDR. One hypothesis is that some genes conferring QDR are related to morphological and developmental processes such as leaf structure and flowering time (Poland et al. 2011; Wisser et al. 2006). Previous QTL studies have identified regions of the genome that confer resistance to GLS (Balint-Kurti et al. 2008; Bubeck et al. 1993; Clements et al. 2000; Danson et al. 2008; Juliatti et al. 2009; Lehmensiek et al. 2001; Maroof et al. 1996; Pozar et al. 2009). The nested association mapping (NAM) population has been used to identify the genetic architecture underlying other fungal foliar diseases (Kump et al. 2011; Poland et al. 2011). By comparing NAM mapping results for multiple traits, co-localization of multiple traits can be elucidated.

GLS is a foliar disease of maize caused by the polycyclic pathogens *Cercospora zeae-maydis* and *Cercospora zeina* (Crous et al. 2006; Tehon and Daniels 1925). GLS is considered one of the most significant maize diseases in the United States and in maize-growing regions world-wide (Ward et al. 1999). The symptoms of GLS are characterized by tan, rectangular-shaped lesions that lengthen parallel to the leaf venation. When GLS is prevalent, it can cause yield losses over 70% due to associated severe blighting, stalk deterioration and lodging (Latterell and Rossi 1983; Ngoko et al. 2002). Current sustainable agriculture practices (no-till) are conducive to disease development because primary inoculum survives on the debris of the previous season

and no-tillage plots exhibit the greatest aerial spore count, disease onset and development among plots with variable tillage treatments (Payne and Duncan 1985).

Foliar fungicides are not an ideal option because they are expensive and may have a negative impact on the environment. Additionally, fungicides may not be accessible to subsistence farmers due to price or actual proximity to the fungicide sources so therefore, it is important to develop resistant varieties.

There is a well-established relationship between flowering time and fungal disease development in maize, including for GLS (Kump et al. 2011; Poland et al. 2011; Wisser et al. 2011). Flowering time has therefore been used as a covariate in genetic and association mapping studies but has not been used to identify pleiotropic loci for disease and maturity or to infer host resistance mechanisms from the relationship. The genetic architecture of maize flowering time has also been studied using the NAM population (Buckler et al. 2009). Additionally, there is an absence of studies on the relationship between leaf venation structure and lesion development even though there is evidence that lesion expansion can contribute significantly to disease epidemics caused by members of the *Cercospora* spp. and lesions appear to be bounded by major veins (Berger et al. 1997). In the present study, we utilized the NAM population to genetically map loci conferring resistance to GLS of maize and those influencing inter-vein distance.

MATERIALS AND METHODS

Plant materials and field site

The nested association mapping (NAM) population was developed by the Maize Diversity Project as a public resource (www.panzea.org). The NAM population consists of 25 recombinant inbred line (RIL) families derived from crossing each of 25 diverse maize lines to a single reference parent, B73 (McMullen et al. 2009). Of the 5,000 lines that comprise the NAM population, 3,678 were planted on the GLS screening site of Virginia Polytechnic Institute's Whitethorne Research Farm located in Blacksburg, VA. In addition, 150 lines of the Intermated B73 x Mo17 population (IBM) population were included. Line selection was based on seed availability and predicted experimental power (Yu et al. 2008). Three replications of the population were planted in 2008, 2009 and 2010 (one replication/year). Sixteen kernels of each line were planted in 2.4 m rows with 0.3 m spacing between rows. The population was arranged in an incomplete block design augmented by blocks that contained two parental checks. The blocks were arranged by families within the NAM.

The Whitethorne Research Farm's GLS screening site was chosen for the high and even disease pressure that is routinely observed with natural inoculum. Maize has been continuously planted in the field under no-till conditions since 1985. The field was manually inoculated for three seasons prior to dependence on the natural inoculum present. The isolates VA-1, VA-2 and VA-3 originally used to inoculate the field were collected from maize fields located in Montgomery County and Wythe County of Virginia, and in 1985 and were identified as *Cercospora zeae-maydis*, which was later re-classified as *C. zeae-maydis* II, now known as *Cercospora zeina*

(Wang et al. 1998). Sporulation of *C. zeae maydis* and *C. zeina* on the residues from the previous seasons likely provided the primary inoculum for disease development on the maize plants. At the conclusion of the 2009 and 2010 seasons, diseased samples were collected at random from a subset of the parental lines. For each year, 25-50 isolations were made. Isolates were identified at the genus level based on conidial morphology and identified to the species level using colony traits when grown on potato dextrose agar (PDA). Those isolates producing the characteristic purple halo (cercosporin) and exhibiting faster colony growth were inferred to be *C. zeae-maydis*. These tests have been extensively compared with molecular typing in our laboratory and found to be reliable for distinguishing the two species (Hsieh, 2010). The majority of samples were identified as *Cercospora zeae-maydis*, and a minority (5-10%) were *C. zeina*.

Phenotypic assessment

A disease rating methodology was modified to include increments of 0.25 on a 0-5 scale (Maroof et al. 1996). Using this 20-point scale, each line was scored three times at seven-day intervals. For most lines, this rating was collected after flowering time determined by dehiscence. From the resulting disease scores, an area under the disease progress curve (AUDPC) was calculated for each line. Data on days from planting to 50% anthesis of the row (DTA) were also collected.

Images of GLS infection on maize leaves were collected in 2009 and 2010. Ear leaves were sampled from each of the RILs three times at 10 day intervals. These leaves were sampled after flowering time, when the GLS symptoms began to develop. The leaves were transferred to the lab within the day and scanned with the corresponding identification. Images were analyzed

using a program developed by Maurice Benson (University of North Carolina-Wilmington). The distance between three major and three minor veins were measured on each leaf sample and the values were averaged per NAM line. Venation structure was measured at the widest part of the leaf since leaves within the population varied in size and shape of the leaf. The program also measured the dimensions of each lesion and the number of lesions in that given area.

In order to assess sporulation in relation to lesion size, lesion samples were collected in 2011 from the parents of the NAM population. These samples were boiled in 1 M KOH and then rinsed with fresh sterile autoclaved water. The rinse was repeated several times over a two week period, resulting in cleared leaf samples that were devoid of chlorophyll and other pigments. Each sample was then placed on a slide and examined under a light microscope. The conidiophores within the lesions were counted using a manual counter and the computer-projected image. The distance between the major veins were measured using a standard metric ruler.

Analysis

The statistical software ASReml3 was used to acquire best linear unbiased predictions (BLUPs) of the GLS disease scores as described by Poland et al. (2011). The BLUPs extracted from this model were used to calculate AUDPC, which was used as the response variable in the GLMselect stepwise selection procedure with family and days to anthesis (DTA) as covariates and 1,106 common-parent-specific markers as predictor variables at a selection threshold of p-value = 1×10^{-4}. SAS v9.1.3 was used to perform QTL mapping using a general linear modeling approach.

Those markers selected as significantly contributing to the response in the stepwise selection model were identified as QTL, while the associated model estimates were considered allelic effects. The sum of allelic effects in the parental lines was compared to the parental phenotypic BLUP in order to determine the percent phenotypic variation explained by the QTL. A general linear model (GLM) was constructed using the selected markers associated with the QTL as predictors and family and flowering time as covariates. Variations of this model were used to construct confidence intervals, identify interactions, and determine least squared means (LSM) of AUDPC for a given allelic effect of a given QTL. The variance components of family and RIL were used to describe genetic variance for the NAM population. Heritabilities on an individual plot basis and on a line mean basis were estimated for the entire NAM population as described in Hung et al. (2011). A mid-parent offspring regression was used to predict narrow sense heritability. Confidence intervals were identified by removing one marker at a time from the full linear model and inserting the associated flanking markers individually until the flanking markers failed to significantly describe the response variable at $p<0.0001$ (Poland et al. 2011). QTL-QTL interactions and interactions between QTL-associated markers and non-significant markers were included in the GLM to identify significant interactions ($p<0.0001$). Pleiotropic loci and alleles affecting both flowering time and disease development were identified by substituting DTA for AUDPC as the response variable in the GLM described above.

Residuals for each chromosome were produced from the GLM by removing physically linked markers, one linkage group at a time, from the model. These residuals were submitted to NAM-GWAS at BioHPC for genome-wide SNP association using the bootstrap regression analysis

option (Tian et al. 2011). Significant GWAS hits were functionally annotated using basic local alignment search tool (BLAST). These annotations were categorized into biological groups, which include: structural components, secondary metabolites, primary metabolites, genes previously implicated in pathogen-defense, membrane transporter, development, cellular energy, cell signaling, and cell cycle. The genes on the inter-pro hit list (www.maizesequence.org) were also categorized into these same categories. A proportion z-test was used to test the abundance of genes in a given category relative to the overall abundance predicted in the maize genome.

Heterogenous inbred family development and QTL confirmation

Specific RILs that compose the NAM were selected for heterogenous inbred family (HIF) development (Tuinstra et al. 1997). Forty-three lines met the criteria of segregating at one of three QTL and being fixed at all other significant QTL. The three QTL of interest were those that corresponded to the three markers that most significantly described GLS AUDPC. These lines were selected from six subpopulations of the NAM based on the predicted effect of the allele at the specific locus on disease development.

The selected lines were selfed at Aurora, NY 2009 and then genotyped. The plants that were heterozygous at the loci of interest were selfed in 2010 winter nursery and later genotyped. Fixed lines were selected for random placement in one of six pedigree-based Latin square designs on Whitethorne Farm in Blacksburg,VA. These lines were genotyped and scored using the disease rating methodology described above. Lines within the same HIF were analyzed for significant association of phenotype and genotype at loci of interest.

RESULTS

Heritability of resistance

The NAM population exhibited a wide range of disease susceptibility among parental lines as well as within and between populations (Table 2.1). At the point of maximum variance identified among the BLUPs, the parental lines varied from 2.05 to 4.87 in their disease rating score. The mean rating for the NAM population was 3.73 with a variance of 0.24. The average variance within populations was 0.11. Broad sense heritability was calculated at 0.72 on an individual plot basis and 0.83 on a line mean basis to correct for the unbalanced design of the experiment (Hung et al. 2011). Additive genetic variance was responsible for 52% of the phenotypic variance.

Disease resistance loci, GWAS and interactions

A model selection approach to QTL mapping was used to identify loci that significantly described GLS disease progress. GLS QTL were designated as qGLSBIN#$_x$, where "q" indicates a QTL, BIN# is replaced with the genomic bin location within which the selected markers are located, and "x" denotes the genotype source of the specified allele. Sixteen markers, found on nine of the 10 chromosomes, were selected by the model at $p<4.3x10^{-5}$ (Table 2.2). The selection threshold was based on a calculation for false discovery rate (FDR). Effect sizes across parental lines varied at each locus. Each locus had allelic effects that were significantly less than, greater than, and equal to the effect of the B73 allele (Table 2.3). Conversely, each parental line had susceptible, resistant and neutral allelic factors relative to the B73 allele. There

was a significant positive correlation between the sum of the allelic effects and AUDPC for each parental line (R^2=0.33; p=0.0023).

There were a total of 146 significant GWAS hits (Table 2.4). There were genes within a 20 kb window of 63 hits, which were functionally annotated (Table 2.4). Only 41 of the genes had characterized functional annotations and these were used in the proportions test. The categorization and test summary can be found in Table 2.5. Cellular energy, development, and secondary metabolites were statistically overrepresented categories with p-values of $2.22x10^{-10}$, $2.24x10^{-15}$, and $1.23x10^{-3}$, respectively.

Inclusion of significant QTL selected by the model thus explained 78.5% of the phenotypic variation among the parental lines; inclusion of all QTL ($p<0.05$) explained 82.2% of the phenotypic variation among the parental lines. Parental LSM of AUDPC for each locus were produced by the model and used to detect allelic potential for disease reduction. Alleles at three loci were predicted to confer disease reduction of greater than 10% (Fig. 2.1).

Three two-way allelic interactions were detected. One pair of interacting loci involved two markers within confidence intervals significant for disease, while the other two involved interactions between significant and non-significant markers at $p<4.3x10^{-4}$. Interactions were entered into the GLM model as predictors of AUDPC. There was a significant interaction between qGLS_4.05 and qGLS_7.03, both QTL significant for disease.

Disease QTL confirmation and estimated effects

Three QTL were confirmed using HIF analysis (Tuinstra et al. 1997). The families were composed of RIL selfed for seven generations. Lines of the original S_5 generation that were heterozygous at one of the three loci of interest (qGLS1.04, qGLS2.09, qGLS4.05) were identified. Genotyping was performed on S_7 lines within the same HIF that were segregating for the parental alleles at the loci of interest. There were at least six lines resulting from independent recombination events, three with the B73 allele and three with the alternate allele, for each HIF described below.

The observed levels of disease were significantly different among the HIF lines for the B73 and other parent alleles (Fig. 2.2; $p<0.05$). Lines with the CML228 allele at qGLS1.04 exhibited an average of 12.1% less disease compared to the B73 allele at the same locus, while the model predicted a 2.48% disease reduction. Lines with the CML333 allele at qGLS2.09 exhibited an average of 22.3% less disease while the model predicted a 5.15% reduction. Finally, lines with the Ki11 allele at qGLS4.05 exhibited an average of 8.4% more in disease relative to lines with the B73 allele at the same locus, while the model predicted a 5.1% increase in disease.

Buckler et al. (2009) identified flowering time QTL using the NAM population, When those DTA data were associated with disease progress in Blacksburg, a quadratic relationship was identified. Since a linear relationship is preferred for general linear modeling, QTL for site-specific DTA were identified because the relationship between DTA and disease progress in Blacksburg was linear. When DTA data on the NAM population was entered as a response variable, ten of the model selected QTL co-localized with disease progress QTL (Table 2.2).

The specific allelic effects of DTA were predicted to be significant at $qGLS1.05_{CML228}$ ($p=0.0402$) and $qGLS4.05_{Ki11}$ ($p=0.0244$). When HIF DTA was entered into the GLM models, it was only found significant in the $qGLS4.05_{Ki11}$ population ($p=0.0263$) and this did not affect the LSM estimate of difference in disease between the two alleles. Additionally, the proportion tests of the annotated GWAS hits indicate that development related genes are in greater proportion than expected based on the number of development related genes in the genome.

Loci affecting inter-vein distance and conidiophore development

As with the disease QTL, loci affecting inter-vein distance (IVD) were identified using a model selection approach. Nine markers associated with IVD, found on six of the ten chromosomes, were selected by the model. Similar to the disease QTL, effect sizes for IVD QTL across parental lines varied at each locus and there were allelic effects that were significantly less than and greater than the effect of the B73 allele. A significant positive relationship between disease development (AUDPC) and the distance between major veins was detected ($p<0.0001$). No significant relationship was observed for the correlation between minor vein distance and disease development. QTL were compared across IVD and disease development phenotypes, resulting in the identification of four co-localizing intervals (Fig. 2.3).

To assess the epidemiological relevance of narrow IVD, lesion parameters and conidiophore counts were collected from 2011 lesion samples. The conidiophore counts and IVDs for the NAM parental lines were graphed and compared (Fig. 2.4). IVD accounted for 46% of conidiophore count variation. A significant positive correlation was identified between IVD and the number of conidiophores within the lesions ($p=2.34x10^{-14}$). This correlation suggested that

the smaller the distance between the major veins, the lower the conidiophore count. When entered into a model along with length, length*IVD and pedigree, IVD remained significant (Table 2.6). As expected, there was a strong relationship between pedigree and IVD, so when the two were entered into a model together, width became less significant ($p<0.0001$). If only length and width were entered into the model to determine the effect of width when conidiophore variation attributed to length was taken into account, width accounted for 41% of the variation.

Co-localizing QTL for maturity and multiple diseases

A significant negative relationship was observed between days to anthesis (DTA) and AUDPC (Fig. 2.5; $p<0.0001$). DTA QTL co-localized with disease development QTL at 10 genomic loci. There is a significant relationship between parental allelic effects for co-localizing QTL among the two traits (Figure 2.6). This relationship was weak, so the parental allelic effects of each QTL were investigated independently. Of these 12 loci, there was a significant relationship between the parental allelic effects for AUDPC and DTA only at the 3.06 locus ($p=0.0007$; $r=-0.39$).

GLS QTL also co-localized with SLB and NLB foliar disease QTL (Kump et al. 2011; Poland et al. 2011). GLS and SLB QTL co-localized at 12 loci, while GLS and NLB QTL co-localized at 11 loci. However, the relationship between parental allelic effects between the two diseases and GLS were only positively significant among NLB QTL (Fig. 2.7; $p<0.05$). There was one negative significant relationship between SLB and GLS QTL at the 3.06 locus, where DTA and NLB were also significantly associated (Fig. 2.7; $p<0.022$).

DISCUSSION

In this study, we report six novel GLS disease resistance loci. The other 10 loci co-localized with GLS QTL previously identified from other studies (Table 2.2). Of the 10 loci, seven were confirmed with two or more studies while qGLS1.06 and qGLS5.03 were identified in four or more studies (Table 2.2). Of the 10 co-localizing QTL, seven were more precisely mapped relative to previous reports (Table 2.2). Other QTL, such as $qGLS4.05_{Ki11,}$ were not finely resolved due to the low recombination rate in the interval. McMullen et al. (2009) found heavy segregation distortion on chromosome 4 within the B73 x Ki11 family. This region had a significantly greater proportion of B73 alleles than Ki11 alleles. All of the QTL mapped by Maroof et al. (1996) were also identified using the NAM strategy. The NAM study was performed on the same field site over a decade later, which is significant because the inoculum source is present on the debris from seasons past. This suggests that, while the pathogen population may have shifted, it still drives similar plant resistance mechanisms.

High heritability was identified for GLS across the NAM population (0.83). Similar heritabilities have been reported for other foliar diseases scored across the NAM population (Kump et al. 2011; Poland et al. 2011) despite differences in disease and rating methodology. Our NAM-based predictions of allelic performance at a given locus underestimated actual allelic differences measured in the field based on near-isogenic lines for three loci. However NAM estimates of the allele effect at a given locus may differ from the effect of the allele observed in field tests of near-isogenic lines. This may be attributed to the fact that the NAM estimate is based on the whole population, in which there is a wide range of disease resistance, not just on

the single bi-parental cross in which there was a highly resistant and susceptible line. The best linear unbiased prediction of the disease index ratings may be reducing the variance within a given line causing the allelic effect estimates to be deflated.

Detection of interactions among disease loci and loci not contributing significantly to disease is unusual. Such interactions were not identified in the NAM analyses conducted to date for other foliar diseases (Kump et al. 2011; Poland et al. 2013). In other systems, interactions have been reported to exist among functionally similar genes, often those that act in the same pathway (Tong et al. 2004). Byrne et al (2007) elucidated interactions through use of RNA interference methodologies.

This study revealed the relationship between IVD (distance between major veins) and GLS disease development and identified co-localizing loci that contributed to both traits. Pleiotropy was identified using the HIF family developed for the 4.05 locus, which segregated significantly for both disease and IVD. qGLS_4.05$_{Ki11}$ was associated with both wide IVD distance and susceptibility to GLS, as expected based on the positive correlation between IVD width and disease development.

Generally, a maize plant with narrower IVD can better resist gray leaf spot disease development than a plant with IVD structure. This suggests that one host resistance mechanism is related to lesion restriction. When pedigree was entered into the model, it removed much of the variation associated with width since the parameters are highly collinear as expected given that inter-vein distance should be similar for like inbreds and variable among diverse inbreds. Omitting it from

the model permitted us to determine the proportion of variance associated with IVD. Length of the lesion explained about 59% of the variation for conidiophore count. Another 41% was attributed to the width or IVD. These findings suggest that a lesion on an inbred with narrow veins will produce fewer conidiophores than that of a lesion on an inbred with wide veins. The reduced presence of conidiophores should result in reduced production of inoculum. Because *Cercospora zeae-maydis* is a polycyclic pathogen, the effect of reduced conidiophores development is compounded across multiple reproductive cycles within one season.

This study confirmed the relationship between flowering time and disease development for GLS. This relationship has been observed for other foliar diseases, namely NLB and SLB. Loci contributing to both flowering time and GLS disease development were resolved to varying degrees. A strong correlation (R^2=0.88) was observed between BLUPs of days to anthesis and disease development at the experimental location. This correlation resulted in the identification of numerous co-localizing loci between the two traits. Given that the entire population was scored for disease development post-anthesis, we would not expect a correlation between the two traits to be observed unless there are physiological processes that occur between the vegetative and reproductive stages of a maize leaf that influence pathogenesis. In a host that reaches reproductive maturity early in the growing season, disease will be greater than in a host that reaches reproductive maturity a month later (Figure 2.5).

Maroof et al. (1993) found that assessment of lesion development is best post-anthesis. However, it is apparent from the appearance of flecks and microscopic confirmation that the pathogen penetrates and begins to colonize the plant before flowering time (Beckman and Payne

1982a; Beckman and Payne 1982b). Physiological processes that occur in a maize leaf after reproductive maturity apparently enable the pathogen to enter a more destructive phase of its life cycle, killing the surrounding cells and producing conidiophores for secondary inoculum production.

When testing leaf disks from plants of differing age and differential field disease development, Gwinn et al. (1987) found that it was not the varietal susceptibility in the field but the plant age that correlated to *C. zeae-maydis* infection. They found that the fungus was able to form appressoria and penetrate significantly more stomates in old tissue than in young across the cultivars, even though all stomates were closed under the prevailing conditions of high humidity. Additional evidence for plant age playing a role in defense is provided by experimental findings wherein identical hybrids planted in succession at three week intervals exhibited a nearly three-week delay in symptom appearance, suggesting that disease development is influenced by physiological maturity. Other hypotheses, delay in inoculum accumulation and the establishment of high humidity microclimate by the leaf canopy, did not explain the experimental results (Rupe et al. 1982).

The results of this study demonstrate that structural and developmental mechanisms underlie quantitative resistance to GLS of maize and provide greater resolution to the genetic loci contributing to sources of resistance. As we begin to elucidate the mechanisms underlying quantitative disease resistance, the plant breeder's decisions regarding development and deployment of resistance will be improved, especially as the mechanisms relate to important agronomic traits such as days to reproductive maturity.

Table 2.1. Descriptive statistics for area under the disease progress curve within the nested association mapping sub-populations (subpopulation is indicated by the non-B73 parent) and among the parental lines. SE/D/V=Standard Error/Deviation/ Variance; min/max=minimum/maximum.

Parents	Mean	SE	SD	SV	Range	Min	Max	Count
B97	44.72	0.28	3.38	11.39	16.03	34.75	50.78	150
CML103	40.45	0.28	3.37	11.35	18.27	32.14	50.41	149
CML228	39.29	0.29	3.46	12.00	16.18	31.76	47.95	147
CML247	38.79	0.24	2.94	8.67	17.42	29.61	47.03	150
CML277	36.78	0.33	3.87	15.00	18.86	26.94	45.80	138
CML322	43.25	0.24	2.91	8.49	13.59	37.46	51.05	148
CML333	38.68	0.37	4.45	19.82	27.92	28.61	56.53	148
CML52	35.18	0.26	3.17	10.03	15.39	28.81	44.20	148
CML69	40.68	0.28	3.40	11.55	21.59	27.75	49.34	149
Hp301	46.81	0.25	3.01	9.05	15.28	39.11	54.39	150
IL14H	51.49	0.34	4.15	17.19	24.50	36.75	61.25	150
Ki11	45.91	0.26	3.20	10.25	16.31	38.45	54.76	150
Ki3	39.75	0.38	4.27	18.22	24.22	26.84	51.06	125
Ky21	44.34	0.31	3.82	14.58	20.58	34.27	54.85	147
M162W	42.80	0.23	2.83	8.03	13.04	36.27	49.31	150
M37W	43.87	0.32	3.85	14.82	27.47	33.20	60.67	149
Mo17	46.29	0.24	2.91	8.47	15.01	37.99	52.99	150
Mo18W	37.11	0.30	3.63	13.20	19.69	29.31	49.00	150
MS71	48.33	0.35	4.30	18.47	21.58	36.48	58.06	148
NC350	36.66	0.30	3.69	13.61	19.41	27.73	47.14	150
NC358	39.14	0.33	4.04	16.32	20.24	29.87	50.10	150
Oh43	47.04	0.22	2.74	7.51	14.83	38.27	53.11	150
Oh7B	48.41	0.35	4.25	18.04	21.20	36.82	58.02	150
P39	48.40	0.28	3.37	11.38	14.10	41.27	55.37	150
Tx303	39.51	0.25	3.10	9.60	16.49	32.36	48.85	150
Tzi8	43.70	0.44	5.06	25.57	24.41	30.33	54.73	132
All	39.97	1.26	6.56	43.01	22.27	28.26	50.53	27

Table 2.2. Quantitative trait loci (QTL) identified by nested association mapping of gray leaf spot resistance. Model selection results are given in relation to the results of previously published QTL studies. p<0.0001, Chr=Chromosome, CI=Confidence Interval; DTA=Blacksburg-specific days to anthesis; Southern Leaf Blight (SLB) QTL Source: Kump et al., 2011; Northern Leaf Blight (NLB) QTL Source: Poland et al., 2011.; Astericks indicate better resolved QTL.

GLS QTL Designation	Marker Name	p-value	Chr	Position (bp)	CI (cM)	Co-localizing GLS QTL	Other NAM QTL
qGLS1.02	PZB01957.1	2.94E-10	1	22,892,866-28,421,841	7.6		NLB
qGLS1.04	PHM5098.25	1.06E-20	1	56,747,253-83,780,725	12.1		DTA, NLB, SLB
qGLS1.06	PHM1968.22	3.15E-11	1	161,027,952-208,733,347	28.9	Clements et al., 2000; Lehmensiek et al., 2001; Maroof et al., 1996; Shi et al., 2007	NLB, SLB
qGLS2.03*	PHM6111.5	6.37E-07	2	14,836,855-22,999,224	14.3	Bubeck et al., 1993; Maroof et al., 1996	DTA
qGLS2.09*	PZA02727.1	1.94E-34	2	212,537,417-235,852,920	35.2	Gordon et al., 2003; Zhang et al., 2012	DTA, SLB
qGLS3.06	PZA00186.4	8.31E-19	3	143,898,953-180,504,690	26.3		DTA, NLB, SLB
qGLS4.05	fea2.3	4.70E-44	4	9,759,854-178,889,832	64.8	Balint-Kurti et al., 2008; Maroof et al., 1996	DTA, NLB, SLB
qGLS5.01	PZA02753.1	1.27E-07	5	5,928,250-7,985,979	8.1		DTA
qGLS5.03*	PZA02792.26	1.88E-06	5	15,138,119-30,994,484	10.8	Bubeck et al.; 1993; Clements et al., 2000; Lehmensiek et al., 2001; Shi et al., 2007; Zhang et al., 2012	SLB
qGLS5.06*	PZA02667.1	2.70E-08	5	192,167,921-207,708,797	21.9	Bubeck et al., 1993	DTA, NLB, SLB
qGLS6.02	PZA00214.1	3.98E-06	6	86,257,528-113,885,960	28.4		NLB
qGLS6.05	PZA02673.1	2.00E-08	6	118,087,791-147,224,252	15.8		NLB, SLB
qGLS7.03	PZA00986.1	8.42E-15	7	13,174,365-142,783,202	35.4	Bubeck et al., 1993; Clements et al., 2000	DTA, NLB, SLB
qGLS8.03*	PZA01470.1	2.08E-12	8	23,769,876-101,178,933	7.6	Zhang et al., 2012	DTA, SLB
qGLS8.06*	PZA03651.1	2.96E-06	8	135,091,499-156,907,035	12.6	Maroof et al., 1996; Shi et al., 2007	NLB, SLB
qGLS10.06*	PZA02663.1	2.62E-05	10	136,941,040-142,193,827	12.4	Bubeck et al., 1993	DTA, NLB, SLB

Table 2.3. Estimated allelic effects of parental lines at qGLSBIN for relative change in disease where the BIN number is listed between the Parent and AUDPC columns. The values are the coefficients of the general linear model paramter except the calculated area under the disease progress curve (AUDPC). R=resistant; S=susceptible

Parent	1.02	1.04	1.06	2.03	2.09	3.06	4.05	5.01	5.04	5.06	6.02	6.05	7.03	8.03	8.05	10.06	AUDPC
CML52	0.13	-0.69	-0.10	-0.14	-0.50	0.01	-0.67	0.31	-0.38	0.25	0.55	-0.48	-0.69	-0.87	-0.21	-0.72	28
Mo18W	0.39	-0.16	-0.50	-0.25	-0.02	0.16	-2.29	0.28	-0.19	0.20	0.35	-0.20	-0.14	-0.84	0.37	-0.96	30
CML277	-0.41	0.59	-2.25	-0.88	0.02	0.58	-0.95	0.29	-0.07	0.16	-0.45	-1.33	-0.57	-0.41	0.37	0.17	31
CML247	-0.76	-0.31	-0.11	0.21	-0.21	-0.09	-0.52	-0.08	-0.16	-0.35	0.67	-0.32	-0.57	-0.08	-0.16	-0.47	32
CML69	0.01	-0.71	-0.05	0.18	-0.88	-0.62	-0.51	-0.16	-0.39	-0.28	0.44	-0.57	-0.87	-0.04	0.31	-0.22	34
Tzi8	-0.09	-0.77	-0.62	0.46	-2.89	0.23	0.29	-0.43	-0.08	0.77	0.26	-0.81	0.20	-0.94	0.11	0.16	35
CML228	0.15	-0.56	0.03	-0.46	-0.61	-0.23	0.18	-1.07	-0.28	0.21	-0.02	-0.36	-0.71	-0.48	-0.18	-0.90	35
NC358	-0.39	-0.91	-1.12	-0.89	-0.68	-0.41	-0.04	0.20	-0.81	0.05	0.37	-0.10	-1.49	0.44	1.57	0.22	35
NC350	-0.49	-0.58	-0.56	-0.25	-1.78	0.02	-0.90	-0.14	-0.44	0.56	0.45	-0.04	-0.64	0.02	0.17	-0.57	35
CML333	0.29	-0.09	-0.11	-0.38	-1.50	0.74	-0.40	-0.13	-0.29	0.35	-0.35	0.30	-0.46	0.11	0.98	-0.13	36
Tx303	-0.35	-0.77	0.32	-0.55	-0.49	0.71	-0.14	0.47	-0.30	0.69	-0.20	-0.26	-0.37	0.31	-0.11	-0.35	36
CML103	-0.11	0.21	-0.24	-0.67	-0.97	1.26	-0.76	0.25	0.05	0.13	0.50	-0.66	-0.67	-0.39	0.54	-0.21	38
Ky21	-0.32	-0.46	-0.33	-0.49	0.04	0.41	0.47	0.38	0.74	0.49	0.45	-0.68	-1.03	0.03	-0.80	-0.54	39
CML322	-0.31	-0.21	-0.26	0.33	-0.17	1.07	-0.70	-0.27	0.30	0.17	0.14	-0.61	-0.11	-0.10	0.68	0.06	39
M162W	0.27	-0.35	-0.07	0.25	-0.09	-0.32	0.68	-0.28	0.11	0.41	0.69	-0.38	-0.63	0.45	-0.16	0.22	40
Ki3	-0.56	0.46	-0.60	-0.15	0.46	0.43	-1.11	-1.43	-0.77	0.42	0.00	-0.42	-0.76	-1.20	-0.40	-0.34	41
M37W	-0.03	-0.61	-0.48	-0.23	-0.06	0.26	0.02	-0.71	0.79	-0.10	-0.97	-0.09	0.26	0.64	0.26	-0.25	42
Ki11	0.50	-0.28	0.22	-0.23	0.37	0.19	1.24	0.47	-0.14	0.36	0.49	-0.29	-0.62	-0.51	-0.27	0.03	45
Mo17	0.13	-0.82	-0.30	0.09	0.10	0.14	0.04	0.41	-0.31	0.01	0.21	-0.20	0.30	0.01	-0.02	-0.75	45
B97	-0.51	-0.72	0.24	-0.17	-0.01	0.01	-1.36	0.06	0.59	1.23	0.63	0.25	-0.75	-0.06	0.42	-0.29	46
Hp301	-0.58	-0.81	0.38	0.27	0.14	-0.06	-0.03	0.03	-0.38	0.61	0.59	-0.46	-0.41	-0.29	-0.10	0.64	47
Oh43	0.15	-1.06	0.12	0.10	0.18	0.07	0.25	-0.14	0.23	0.29	-0.11	-0.44	-0.31	-0.51	0.18	-0.14	47
Oh7B	1.33	-0.69	-0.45	-0.07	-0.50	1.13	-1.37	-0.19	0.89	0.32	0.70	-0.19	-0.50	0.20	0.37	-0.77	48
M571	-0.33	0.07	-0.10	0.25	-0.18	0.96	-1.94	0.34	0.02	0.51	-0.92	-0.40	-0.44	0.52	0.34	-0.07	49
P39	-0.01	-0.61	0.04	0.01	0.23	0.83	-0.47	0.08	-0.41	0.52	0.16	-0.84	-0.09	-0.19	0.40	-0.26	49
Il14H	-0.54	-0.11	0.62	-1.32	0.14	1.40	-0.68	-1.04	1.16	-0.47	-0.31	-0.38	-0.70	-0.61	0.59	-0.07	51

R

S

Table 2.4. Functionally annotated genome wide association (GWA)hits. A 10 Kilobase window on either side of the GWA hit was screened for genes. Chr=Chromosome; BPP=Bootstrap posterior probability

Chr	Marker Position	allele	effect	BPP	Gene	Functional Annotation
1	2,030,770	T/C	0.62	7	GRMZM2G164696	Maize for beta 1 tubulin
1	9,931,873	C/G	0.71	10	GRMZM2G178571	*Zea mays* 1-deoxy-D-xylulose 5-phosphate synthase 1
1	21,532,972	G/A	-0.52	8	GRMZM2G086604	*Zea mays* retrotransposon Cinful-1
1	21,557,348	G/T	0.93	7	Intergenic	
1	26,273,179	C/T	1.47	8	Intergenic	
1	64,986,440	C/G	-0.51	10	GRMZM2G127181	Uncharacterized
1	74,843,220	T/C	-0.36	6	Intergenic	
1	75,268,439	G/A	-0.60	14	Intergenic	
1	173,110,701	G/A	-2.00	8	Intergenic	
1	180,262,819	C/T	-1.39	16	Intergenic	
1	183,969,702	G/T	-0.70	12	GRMZM2G080746	Uncharacterized
1	186,614,192	T/C	-1.01	35	AC205695.3_FG008	Uncharacterized
1	187,927,685	G/A	-1.87	26	intergenic	
1	198,970,183	T/C	0.74	7	Intergenic	
1	199,579,946	A/G	-0.85	7	Intergenic	
1	201,295,476	G/A	-0.63	12	Intergenic	
1	207,120,463	--/CC	-0.77	7	GRMZM2G303157	*Zea mays* T cytoplasm male sterility restorer factor 2
1	284,043,464	G/A	0.43	6	Intergenic	
1	284,048,249	C/A	0.49	6	GRMZM2G069772	*Zea mays* rust resistance protein rp3-1
1	284,837,740	A/G	0.30	9	Intergenic	
1	284,841,425	G/T	0.46	8	Intergenic	
1	284,892,880	A/C	0.34	13	Intergenic	
1	286,994,723	G/A	0.63	8	GRMZM2G371210	phytoene synthase (PSY1)
1	293,630,563	-------/GCCACAT	-2.93	7	GRMZM2G068117	*Zea mays* putative pol protein
2	13,580,508	C/G	-0.59	11	cDNA	
2	17,397,612	G/T	-0.66	7	Intergenic	
2	17,422,032	A/T	-0.75	8	GRMZM2G057131	rust resistance protein rp3-1 (rp3-1)
2	20,029,416	C/G	-0.66	10	Intergenic	
2	20,639,672	CTAGC/-----	-0.70	7	Intergenic	
2	20,971,563	C/A	-0.80	7	Intergenic	
2	21,542,600	G/C	-0.95	9	GRMZM2G425211	Uncharacterized
2	22,215,570	C/A	-0.58	7	GRMZM2G545802	putative growth-regulating factor 1
2	172,704,846	A/T	0.46	7	GRMZM2G000601	ubiquitin-conjugating enzyme E2-17 kDa
2	225,963,000	C/A	-1.49	26	Intergenic	
2	228,976,174	C/A	-1.72	7	Intergenic	
2	229,145,253	G/A	-1.35	39	GRMZM2G041642	rust resistance protein rp3-1 (rp3-1)
2	229,637,503	C/G	-2.41	7	Intergenic	
2	229,862,946	T/G	-0.93	16	GRMZM2G391164	Uncharacterized
2	230,414,321	C/T	-1.18	6	GRMZM2G152258	tropinone reductase 2

2	230,584,153	A/G	-1.89	7	GRMZM2G513206	ramosa 2 (ra2)
2	230,615,501	T/G	-0.88	10	GRMZM2G052403	Tha8 (tha8)
2	230,898,848	T/A	-0.53	7	GRMZM2G082302	Uncharacterized
3	163,420,613	G/A	0.84	12	Intergenic	
3	163,875,247	T/G	1.21	16	Intergenic	
3	163,888,333	G/A	0.81	35	Intergenic	
3	165,031,510	T/C	1.08	9	Intergenic	
3	165,509,907	G/T	0.82	7	GRMZM2G444541	*Zea mays* cultivar B73 chloroplast
3	165,792,180	T/A	0.84	16	Intergenic	
3	167,576,843	C/-	0.57	7	Intergenic	
3	202,841,878	C/T	-0.70	43	AC207628.4_FG011	*Zea mays* see2a for putative legumain
3	206,959,629	C/G	-0.53	9	GRMZM2G028568	phytoene synthase (Y1)
3	217,329,015	G/A	0.61	8	Intergenic	
4	11,272,990	C/T	-1.46	13	GRMZM2G068330	*Zea mays* protein dimerisation region containing protein
4	12,987,147	G/A	-1.19	6	GRMZM2G124593	Uncharacterized
4	39,437,101	C/T	-0.49	22	Intergenic	
4	93,594,993	T/G	-1.28	28	Intergenic	
4	137,271,558	T/G	-0.84	11	Intergenic	
4	140,210,546	C/T	-1.66	81	Intergenic	
4	143,945,947	C/T	-0.57	33	Intergenic	
4	147,941,545	---/GTC	1.05	10	Intergenic	
4	149,532,029	A/T	-0.61	9	Intergenic	
4	160,911,650	C/A	-0.70	7	Intergenic	
4	161,434,924	G/A	-0.70	6	GRMZM2G137696	*Zea mays* discolored-1 (mutant allele dsc1-Ref::Mu1)
4	245,875,432	C/A	-0.87	11	Intergenic	
5	4,244,452	C/T	-0.33	12	GRMZM2G007063	*Zea mays* opaque-2 heterodimerizing protein 1b (ohp1b)
5	4,294,169	A/C	-0.52	12	GRMZM5G801939	Uncharacterized
5	4,669,956	G/C	-0.52	11	GRMZM2G027495	*Zea mays* B73 acc oxidase (ACO35)
5	6,369,752	C/T	-1.26	7	GRMZM2G176042	Uncharacterized
5	6,418,770	A/G	-1.01	10	GRMZM2G077404	*Zea mays* arginine N-methyltransferase 2
5	6,536,883	G/C	-1.20	15	GRMZM2G095185	Uncharacterized
5	6,540,639	A/T	-1.47	15	GRMZM2G090432	Uncharacterized
5	6,588,968	A/T	-1.22	22	Intergenic	
5	7,088,865	A/G	-0.82	12	Intergenic	
5	20,486,425	--/TA	0.77	6	GRMZM2G080231	Uncharacterized
5	21,974,701	C/G	0.81	9	Intergenic	
5	25,824,240	C/A	-1.00	13	Intergenic	
5	40,275,106	T/C	0.93	16	Intergenic	
5	56,244,216	A/T	-1.03	6	Intergenic	
5	181,353,895	--/CT	0.50	6	Intergenic	
5	182,336,996	C/T	0.64	6	Intergenic	
5	194,708,029	C/G	0.40	11	GRMZM2G144097	*Zea mays* folylpolyglutamate synthase (LOC100285702)

5	197,017,506	-/G	0.57	7	Intergenic	
5	204,100,563	T/G	0.59	26	GRMZM2G029186	Uncharacterized
5	205,473,574	G/C	0.82	22	AC195458.3_FG001	Uncharacterized
6	96,317,088	T/C	0.37	9	Intergenic	
6	96,520,306	TGT/---	0.58	9	GRMZM2G472187	Uncharacterized
6	97,120,550	G/C	0.97	11	GRMZM2G131020	*Zea mays* CUE domain containing protein (LOC100282879)
6	104,876,576	G/T	-1.08	6	Intergenic	
6	108,329,244	C/G	-1.11	9	GRMZM2G701063	*Zea mays* transcriptional activator
6	109,996,701	T/C	-0.55	11	Intergenic	
6	110,193,339	C/A	-0.76	9	Intergenic	
6	110,249,748	TC/--	-0.66	11	Intergenic	
6	129,925,381	C/T	-0.42	12	Intergenic	
6	138,425,664	T/C	-0.42	6	GRMZM2G117439	Uncharacterized
6	143,322,264	-----/GCTCG	-0.36	6	Intergenic	
6	149,244,757	T/C	-0.48	6	Intergenic	
7	2,931,863	G/C	-0.74	6	Intergenic	
7	3,212,361	-------/CTCGATT	-0.56	6	Intergenic	
7	121,127,424	------/CTCCCA	-0.54	6	Intergenic	
7	123,595,279	G/A	-0.54	67	GRMZM2G154752	Uncharacterized
7	158,495,841	C/A	0.66	6	Intergenic	
7	159,611,041	-/A	-0.53	11	GRMZM2G158452	Uncharacterized
7	162,122,347	T/A	-1.37	11	Intergenic	
8	8,431,762	T/A	0.86	6	Intergenic	
8	9,495,845	C/T	0.34	7	GRMZM2G473485	*Zea mays* rust resistance protein rp3-1 (rp3-1)
8	13,658,348	G/T	1.02	6	AC187393.3_FG001	*Zea mays* heat shock factor-binding protein 1 (hsbp1)
8	20,706,941	G/A	-0.64	7	GRMZM2G080588	Z.mays GapC2
8	26,104,142	-/G	-0.64	9	GRMZM2G154221	*Zea mays* T cytoplasm male sterility restorer factor 2 (rf2a)
8	35,882,697	G/C	-0.86	10	Intergenic	
8	49,566,758	G/A	-0.90	24	Intergenic	
8	57,863,663	G/A	-1.07	30	Intergenic	
8	72,046,701	---/AAC	-0.77	7	Intergenic	
8	105,554,753	C/T	0.80	6	Intergenic	
8	141,605,883	G/A	1.08	8	GRMZM2G015735	*Zea mays* chloroplast phytoene synthase (Y1)
8	146,531,554	-/G	0.77	6	Intergenic	
8	149,501,067	A/T	0.71	6	Intergenic	
8	154,091,270	A/G	1.29	8	Intergenic	
8	158,597,202	C/T	0.42	23	Intergenic	
8	170,550,068	T/C	0.31	8	Intergenic	
8	173,107,104	C/A	-1.04	8	GRMZM2G124365	*Zea mays* B transcriptional activator (b1)
9	16,154,952	G/C	-1.15	6	Intergenic	
9	16,379,663	CAG/---	-0.87	13	Intergenic	
9	16,620,539	A/G	-0.82	14	Intergenic	

9	18,225,380	C/T	-0.93	6	GRMZM5G899123	*Zea mays* rust resistance protein rp3-1 (rp3-1)
9	99,711,027	T/C	-1.17	10	Intergenic	
9	128,943,719	G/A	0.85	11	GRMZM2G045178	Uncharacterized
9	133,801,705	C/A	1.38	10	GRMZM2G134279	*Zea mays* starch branching enzyme IIb (ae)
9	142,738,763	A/G	-0.53	9	GRMZM2G150598	*Zea mays* starch synthase I (sS1)
9	142,906,275	C/A	-0.54	40	Intergenic	
9	143,305,297	C/T	-0.51	7	Intergenic	
9	150,278,166	C/T	0.75	31	Intergenic	
10	1,429,820	C/T	0.39	20	GRMZM2G048067	Uncharacterized
10	1,529,739	C/A	0.47	20	GRMZM2G098603	*Zea mays* B73 pathosis-related protein 2
10	1,716,176	C/T	0.53	15	GRMZM2G430780	*Zea mays* NADPH-dependent reductase (a1)
10	1,888,649	C/T	0.80	14	GRMZM2G022606	Uncharacterized
10	1,918,857	A/T	0.51	14	GRMZM2G104638	Uncharacterized
10	1,922,550	G/C	0.36	14	GRMZM2G104655	Ribosome inactivating protein 1 (rip1)
10	3,691,250	C/G	0.53	6	GRMZM2G143769	*Zea mays* rust resistance protein (Rp1-D)
10	46,171,212	G/A	-0.57	7	Intergenic	
10	67,107,876	C/T	-0.42	11	Intergenic	
10	108,685,826	A/G	-0.55	23	Intergenic	
10	137,015,827	G/T	-0.56	16	GRMZM5G818664	*Zea mays* latency associated nuclear anti
10	138,301,336	C/G	-0.42	12	GRMZM2G105801	Uncharacterized
10	144,243,105	GT/--	-0.77	6	Intergenic	
10	144,489,188	G/T	-0.62	15	Intergenic	

Table 2.5. Summary statistics of functionally annotated and categorized genome wide association hits. Significant GWAS hits were functionally annotated using basic local alignment search tool (BLAST). These annotations were categorized into biological groups, which include: structural components, secondary metabolites, primary metabolites, genes previously implicated in pathogen-defense, membrane transporter, development, cellular energy, cell signaling, and cell cycle. The genes on the inter-pro hit list (www.maizesequence.org) were also categorized into these same categories. A proportion z-test was used to test the abundance of genes in a given category relative to the overall abundance predicted in the maize genome. SE: standard error.

Category	Genome (count)	GLS (count)	SE	z-value	p-value
Cell Cycle	25777	13	0.077	1.155	8.76E-01
Cell Signal	21016	2	0.073	3.837	1.00E+00
Cellular Energy	805	5	0.018	-6.237	2.22E-10
Development	205	3	0.009	-7.841	2.24E-15
Membrane Transporter	2896	0	0.033	1.399	9.19E-01
Pathogen Defense Related	4590	4	0.040	-0.626	2.66E-01
Primary Metabolite	204	1	0.009	-2.391	8.39E-03
Secondary Metabolite	4604	8	0.041	-3.027	1.23E-03
Structural Components	3442	5	0.035	-1.916	2.77E-02

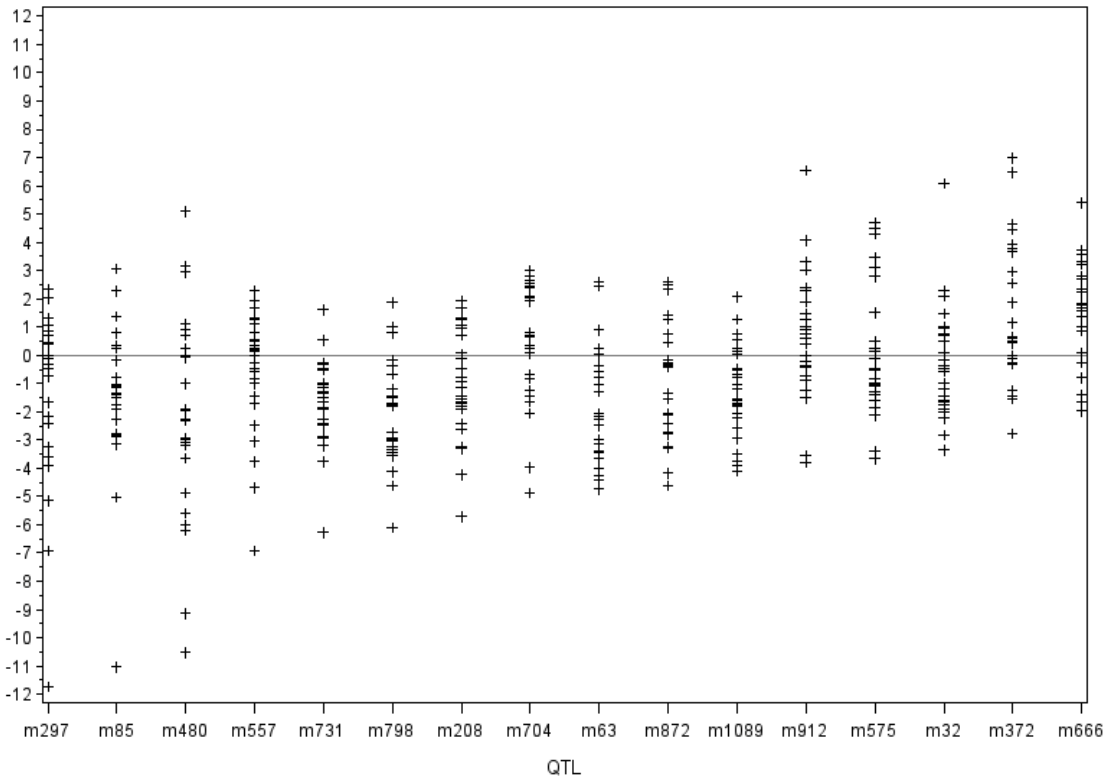


Figure 2.1. General linear model predicted percent change in disease across significant disease QTL. Each cross indicates the predicted change in disease of single allele. There are twenty-six crosses for each QTL, each representation the allele from one of the NAM parental sources.

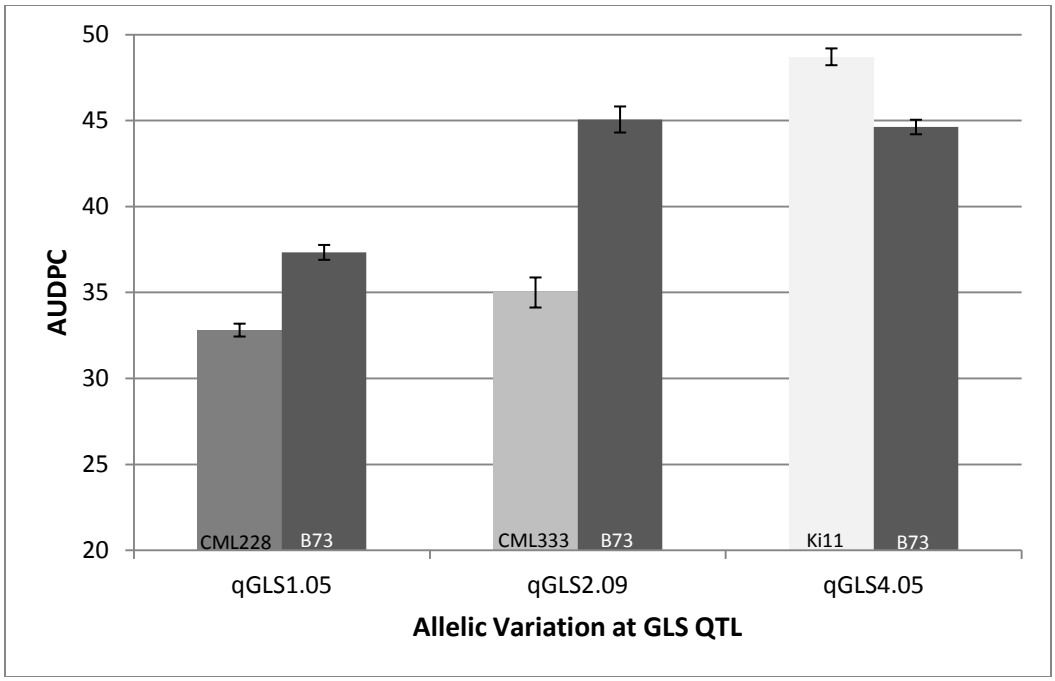


Figure 2.2. Disease development (area under the disease progress curve, or AUDPC) among heterogeneous inbred family lines across three GLS QTL.

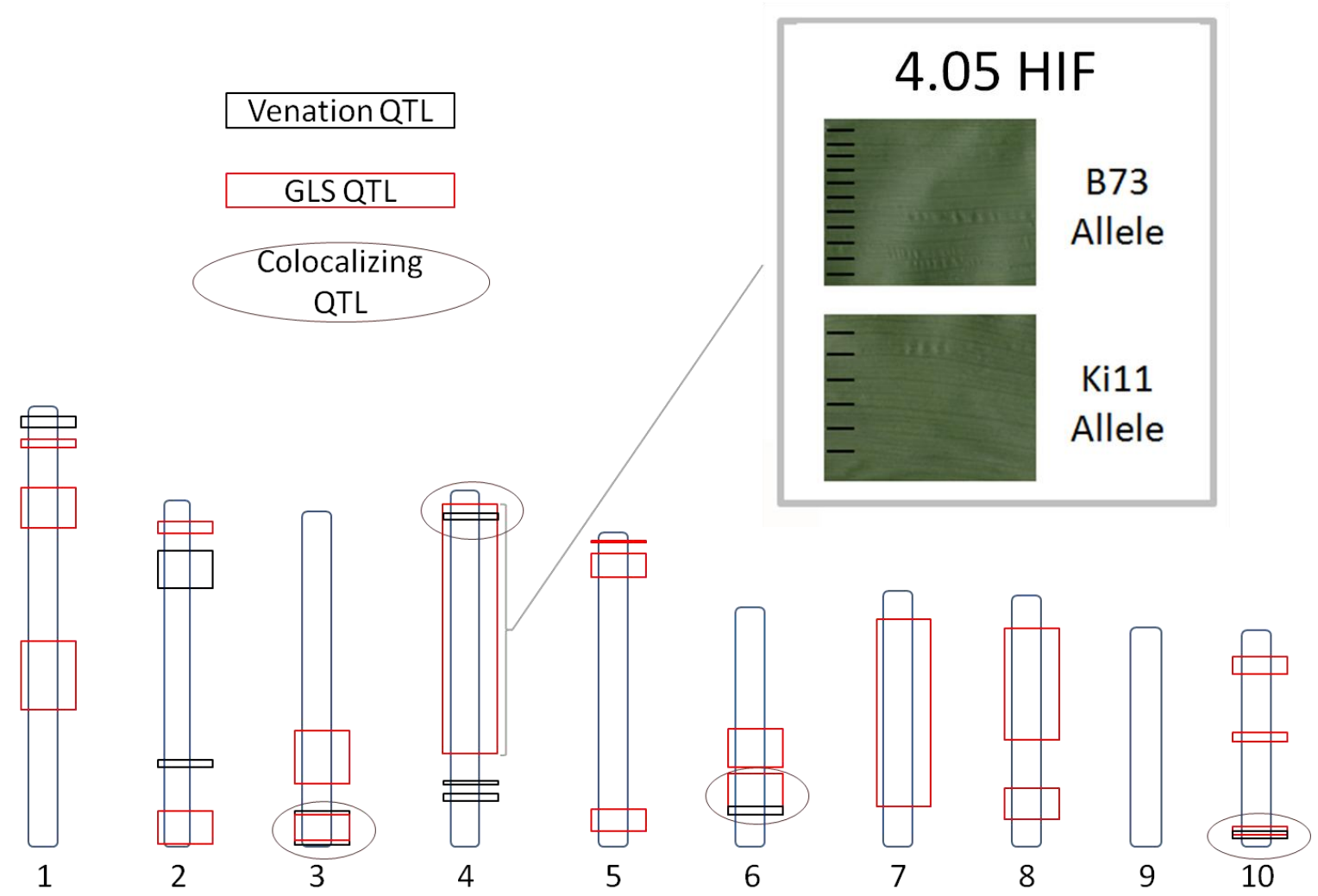


Figure 2.3. Co-localizing quantitative trait loci for gray leaf spot and inter-vein distance (IVD) and an image highlighting the difference between the IVDs from maize leaves with the B73 and Ki11 alleles at the 4.05 locus.

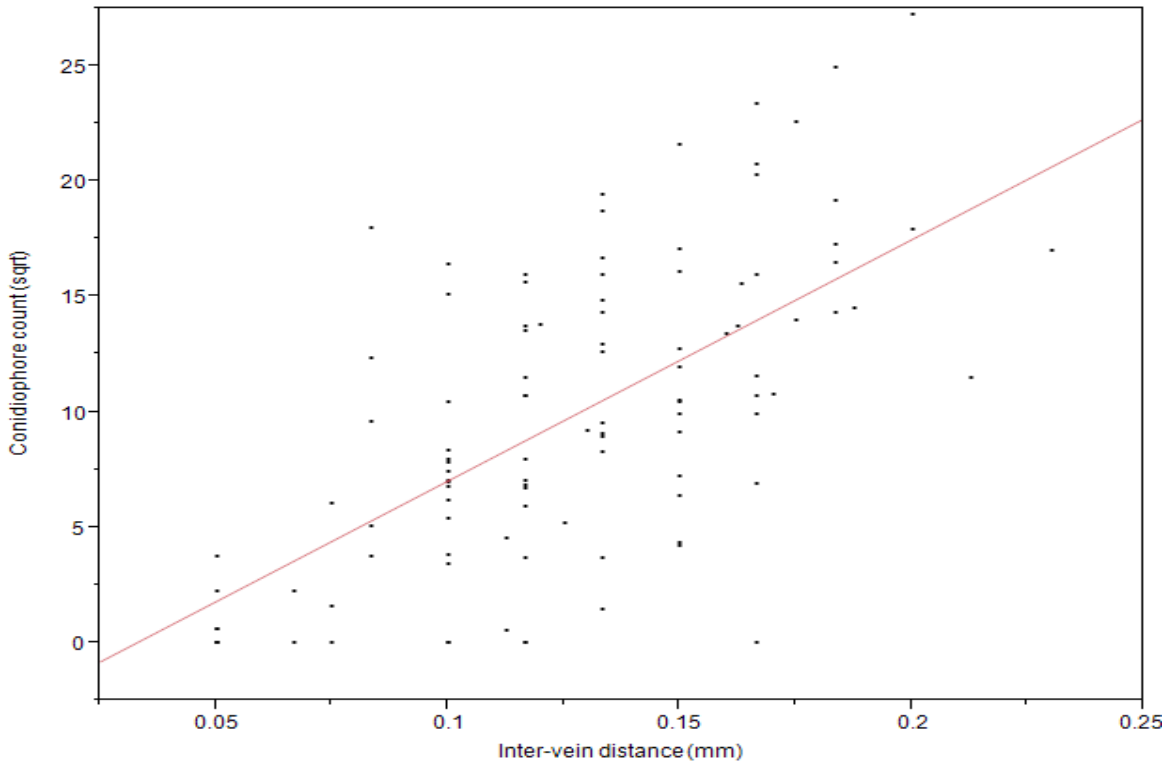


Figure 2.4. Significant relationship between inter-vein distance and square root (sqrt) of the conidiophore counts (R^2=0.43; p<0.0001)

Table 2.6. Model results with conidiophore count (square root) as the response variable. IVD: inter-vein distance; DF: degrees of freedom; Prob: probability; SS: sum of squares; TSS: total sum of squares.

Model	Source	DF	Sum of Squares	F Ratio	Prob > F	SS/TSS
1	Pedigree	21	964	6	2.03×10^{-9}	0.5906
1	Length	1	566	73	5.83×10^{-13}	0.3467
1	IVD	1	47	6	0.0159	0.0287
1	Length*IVD	1	55	7	0.0089	0.0340
2	Length	1	929	57	1.70×10^{-11}	0.5898
2	IVD	1	646	40	7.16×10^{-9}	0.4102

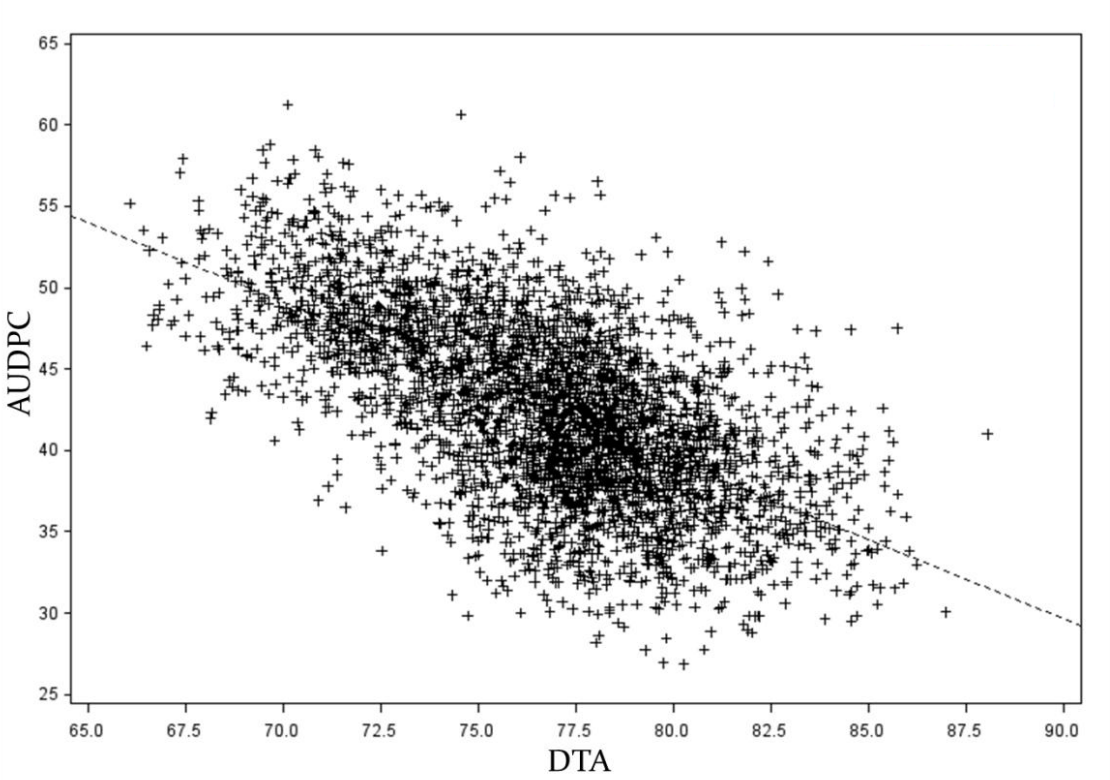


Figure 2.5. Relationship between days to anthesis (DTA) and area under the disease progress curve (AUDPC) among nested association mapping recombinant inbred lines. $p<0.0001$; $r^2=0.3812$.

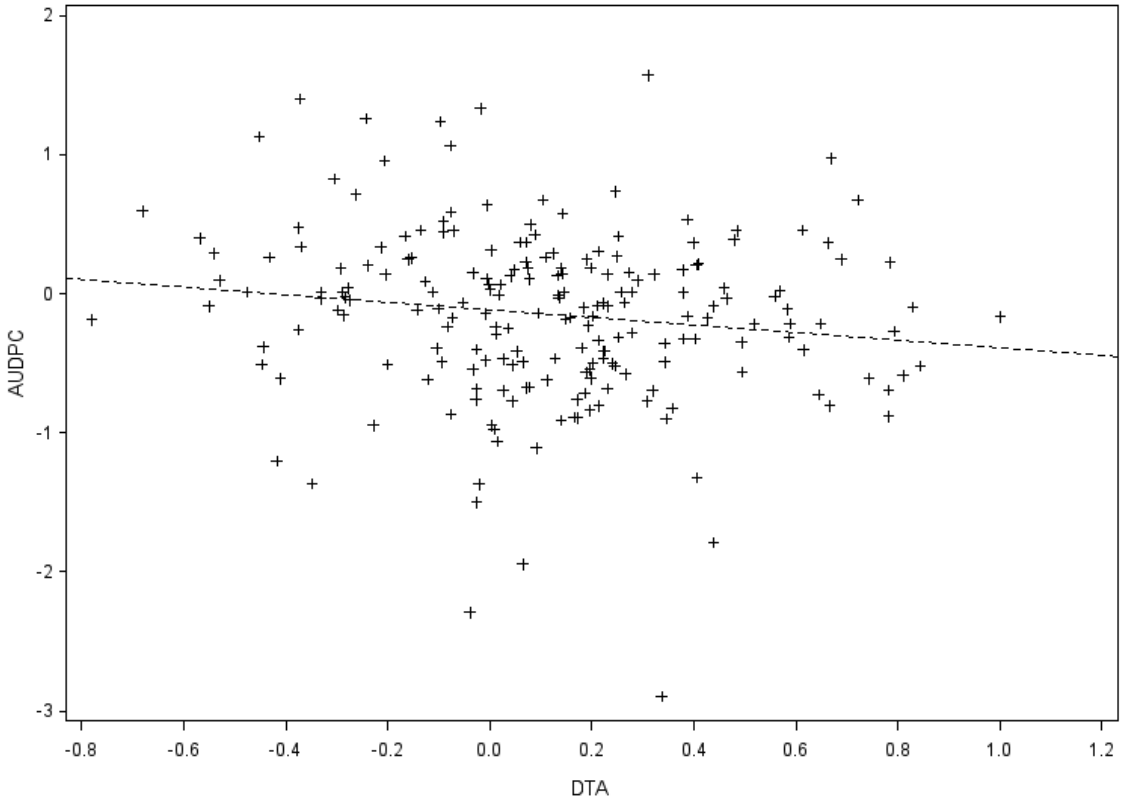
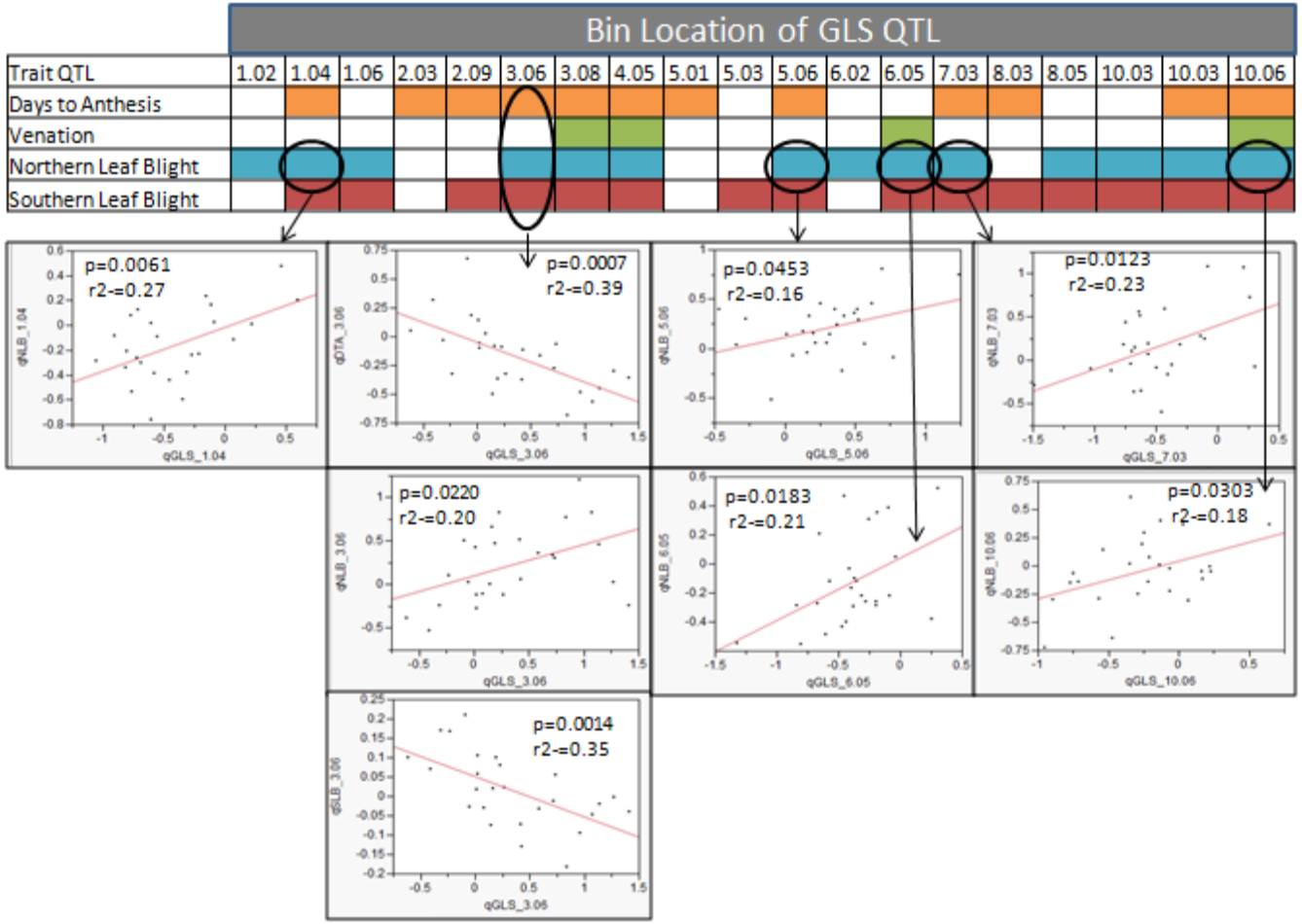


Figure 2.6. Parental allelic effects for days to anthesis (DTA) and area under the disease progress curve (AUDPC) among loci that co-localize between the two traits. p=0.0372; r^2=0.0209.

Figure 2.7. Co-localizing quantitative trait loci (QTL) across four different traits and GLS. The bin locations are located beneath the gray bar. Each colored box indicates a co-localizing QTL. Parental allelic effects for each trait and QTL were tested across those for GLS. Significant associations were circled and graphed. The r^2 and p-value are indicated.



APPENDIX 2.1: SAS CODE FOR JOINT-LINKAGE ANALYSIS, RESIDUALS FOR GENOME-WIDE ASSOCIATION, AND CONFIDENCE INTERVALS

*Joint-linkage developed by Jesse A. Poland, modified by Jacqueline M. Benson (JMB); Script for residuals and confidence intervals developed by JMB.

*Sorting

```
proc sort data=bbdtagenopheno;
  by pop;
run;
```

*Macro for inserting genetic data into selection model

```
data _null_;
length factor $14000;
factor = 'Pop ';
do m = 1 to 1106;
   factor=trim(factor) || ' (pop)m' || left(m);
end;
call symput('factor',factor);
run;
```

*Selection model

```
ods csvall file = 'C:\Documents and Settings\Jacqueline\My Documents\Jaci\NAM\BB Flowering Time\GLMSelect Results1.csv';

proc GLMSelect data=bbdtagenopheno;
class pop;
model AUDPC10= pop &factor / INCLUDE=1 SELECT=SL SLS=9.81E-4 SLE=9.81E-4 MAXSTEPS=50;
output out=outglms;
run;

%put &factor;
```

*General linear model used to develop residuals for nested association mapping-genome wide association study and compute least squared means

```
ods _all_ close;

ods csvall file = 'C:\Documents and Settings\Jacqueline\My Documents\Jaci\NAM\GLM Results1.csv';

proc GLM data=bbdtagenopheno outstat = glsQTL;
class pop;
model AUDPC10 = POP m480(POP) m297(POP) m63(POP) m372(POP) m798(POP)  m872(POP)
m575(POP) m667(POP) m709(POP) m407(POP) m1056(POP) m1048(POP) m86(POP) m206(POP)
m32(POP) m270(POP) m820(POP) m912(POP) m558(POP) m801(POP)  m585(POP) m761(POP)
m732(POP) m687(POP) m565(POP) m376(POP) m381(POP) m871(POP) m67(POP) m756(POP)
m740(POP) m1052(POP) m710(POP);
lsmeans / out = GLS10_LSmeans;
output out=chr10 r=residual;
run;
QUIT;
```

*Macro for confidence intervals

```
%Macro CI;
proc GLM data=genotypesandblups11 outstat = gls32CI&m;
class pop;
model AUDPC10 = POP days2anthesis &m(POP) m63(POP) m85(POP) m208(POP) m297(POP)
m372(POP) m480(POP)
m557(POP) m575(POP) m666(POP) m704(POP) m731(POP) m798(POP) m872(POP) m912(POP)
m1089(POP);
*lsmeans / out = NLB08_LSmeans;
output out=chr4 r=residual;
run;
quit;
%Mend CI;

%let m = m1;
%CI
%let m = m2;
%CI
%let m = m3;
%CI
…
%CI
%let m = m47;
%CI
%let m = m48;
%CI
%let m = m49;
%CI
```

```
*Continue with other selected QTL and surrounding markers
*Combine output data into one file and export

ods csvall file = 'C:\Documents and Settings\Jacqueline\My Documents\Jaci\NAM\SAS\GLS
CI\GLS32CI.csv';

DATA GLS32CI;
Set Gls32cim1 Gls32cim2 Gls32cim3 … Gls32cim47 Gls32cim48 Gls32cim49;
RUN;

ods _all_ close;

proc export data = GLS32CI
outfile = 'C:\Documents and Settings\Jacqueline\My Documents\Jaci\NAM\SAS\GLS CI\GLS32CI.csv'
dbms=csv
replace;
RUN;
```

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CHAPTER 3: FLAVIN-MONOOXYGENASE UNDERLIES A GRAY LEAF SPOT QTL

ABSTRACT

Gray leaf spot (GLS) is a foliar disease of maize caused by *Cercospora zeae-maydis* and *C. zeina*. Quantitative resistance to GLS is important for crop production. The genetics of complex diseases are increasingly elucidated; however, the mechanisms underlying quantitative disease resistance are not well understood. The quantitative trait loci (QTL) *qGLS1.04* was fine-mapped in Blacksburg, VA, on a field with heavy incidence of GLS. Plants were evaluated for disease severity three times at seven day intervals. The 1.04 QTL interval was fine-mapped from an interval of 27.0 Mb to two intervals of 6.5 Mb and 5.2 Mb. Based on this finding it is possible that multiple genes underlie highly significant QTL identified by NAM. Detoxification-related genes, specifically glutathione-S-transferases, were detected in the qGLS1.04 interval, at an abundance that was greater than expected for the overall genome based on a proportion test. Heterogeneous inbred family (HIF) lines were treated with cercosporin, the toxin produced by *C. zeae-maydis*, to test for increased expression of candidate detoxification-related genes. Treatment of the HIF lines with cercosporin resulted in increased expression of a putative flavin-monooxygenase (FMO) gene. FMOs are a family of oxidoreductases that have been previously implicated in cercosporin detoxification. Genes involved in carotenoid production were also predicted to be involved in resistance; however, no significant difference in carotenoid level was detected among the HIF lines. Increased understanding of resistance mechanisms and the specific genes underlying QTL will improve the breeder's capacity to decide which loci, source and/or genes should be utilized in the resistance breeding program.

INTRODUCTION

Plant disease resistance can be categorized as being qualitative or quantitative in nature. Complete plant resistance controlled by a single gene is considered qualitative due to the strong disease phenotype conditioned by the gene. Genes responsible for this type of resistance have been well studied and utilized in breeding programs (Hulbert et al. 2001). This form of resistance may be overcome by pathogen adaptation or may be unavailable in a given host-pathogen interaction (Vera Cruz et al. 2000). For diseases caused by necrotrophic pathogens, complete, single-gene resistance is typically unavailable. This is the case for gray leaf spot of maize.

The inheritance of quantitative disease resistance can be analyzed using genetic mapping strategies. The genetic architecture can be resolved further to understand the mechanisms involved in quantitative disease resistance, which would improve plant breeders' decision-making capacity. In addition to signal transduction and basal defense, genes underlying quantitative disease resistance are believed to play a role in mitigating the effects of microbial compounds on the host plant. Poland et al. (2009) hypothesized that genes underlying quantitative resistance loci may include those that are involved in chemical warfare.

Plant pathogens produce an array of toxic molecules and enzymes that aid in successful infection of their hosts (Kimura et al. 2001). These products can range from host non-specific to specific and come in diverse chemical forms. *Cercospora zeae-maydis*, the predominant causal agent of gray leaf spot (GLS) in the United States, produces the non-host selective toxin, cercosporin.

Much of the success of the *Cercospora* species is attributed to their production of the cercosporin toxin (Daub and Ehrenshaft 2000). Cercosporin is a photo-activated perylenequinone that converts molecular oxygen to active oxygen species (Daub 1982). These species include hydrogen peroxide, hydroxyl radical, superoxide and singlet oxygen (Spikes 1989). When the fungal pathogen releases the toxin, active oxygen species are produced within the colonized cells, which may lead to cell death and nutrient leakage without the onset of host defenses.

Plants have evolved with exposure to the first three oxygen species and so have a response to these species. Superoxide dismutase, catalase and peroxidase enzymes, produced by the plant, catalyze reactions that reduce the radical species and prevent additional damage to the cell. However, the only place that the plant is exposed to singlet oxygen species is within the chloroplasts (Young 1991). Chlorophyll is also a photo-activated molecule and is capable of producing singlet oxygen species when some of the absorbed energy is not passed to the electron transport chain. Carotenoids play a key role in quenching singlet oxygen before it damages the chloroplast or other machinery within the plant cell (Ramel et al. 2012). Other enzymes or metabolites, such as oxidoreductases and secondary metabolites with antioxidant properties, may be involved in cercosporin detoxification or reducing the damage caused by cercosporin (Daub 1987; Daub et al. 1992; Ververidis et al. 2001).

The current study was undertaken to identify genes that control GLS resistance at the quantitative resistance locus denominated *qGLS1.04*$_{\text{CML228}}$ and to determine the mechanism underlying disease resistance at this locus. This locus was identified as a GLS resistance QTL using the nested association mapping (NAM) population (Chapter 2). The confidence interval

identified was 25 Mb in size and an abundance of detoxification-related genes were noted in the interval. While 12 of the 16- other QTL identified were associated with inter-vein distance and/or flowering time, $qGLS1.04_{CML228}$ was not significantly associated with either.

We therefore hypothesized that $qGLS1.04_{CML228}$ is involved in *per-se* resistance to GLS. We further hypothesized that, if detoxification genes conditioned GLS resistance, that their expression would be up-regulated in the resistant nearly-isogenic lines (NILs) compared to the susceptible after cercosporin treatment. Additionally, we hypothesized that one or more of the genes within the region may be involved in carotenoid production because a portion of the detox-related genes were predicted to be involved in carotenoid production. In this case, we would expect increased production in the resistant NIL after treatment with cercosporin.

METHODS & MATERIALS

Confirmation & fine-mapping

The heterogeneous inbred family (HIF) strategy was used to develop nearly-isogenic lines (NILs) for fine-map the 1.04 region (Tuinstra et al. 1997). The CML228 allele was targeted, as it was predicted to provide resistance significantly different from the B73 allele (Chapter 2). A recombinant inbred line (RIL), Z003E0094, was selected from the B73 x CML228 family within the NAM. This line was selected because it segregated at the 1.04 locus and was fixed at other quantitative trait loci (QTL) predicted to be significant for disease (Chapter 2). Z003E0094 was selfed during the 2009 field season and genotyped across significant disease loci.

The seed was advanced in the winter field season and genotyped in the greenhouse. Both heterozygous and fixed lines were planted. The heterozygotes were selfed while the fixed lines were arranged in a Latin Square experimental design for disease screening in the 2010 field season. A total of 36 lines were tested for GLS response. Those lines that were heterozygous in the region implicated in disease resistance were again advanced in a winter field season, genotyped and planted this time in an incomplete block design that included both heterozygous and fixed lines for both the 2011 and 2012 field season. A total of 1,750 and 6,175 plants were screened and genotyped in the 2011 and 2012 field seasons, respectively, in order to increase power and the likelihood of identifying an advantageous recombination breakpoint.

Experimental units (rows in 2010 and individual plants in 2011 and 2012) were scored three times for disease at seven day intervals. These disease scores were used to calculate area under the disease progress curve (AUDPC), a measure of disease development. In 2010 and 2011, flowering time data was also collected for each experimental unit. The markers used for genotyping are given in Table 3.1 along with the corresponding LOD scores from the 2012 analysis.

Experimental units (lines or plants, depending on the year) with like haplotypes were analyzed together. Like haplotypes were identified as having the same genotypes flanking the segregating regions. The experimental units were analyzed using PROC GLM in SAS 9.3, where the response variable was disease development (AUDPC) and the predictors were the genotypes within the QTL confidence or fine-mapping interval. The false discovery rate (FDR) was

calculated independently for each year based on the number of markers analyzed and used as a threshold for significant associations between the disease phenotypes and marker genotypes.

Functional annotation of NAM GWAS and genes within the fine-mapping interval

A list of genes within the fine-mapping intervals, 77,242,690 to 83,780,725 Mb and 88,849,284 to 94,085,195 Mb, was exported from the maize genome browser (www.maizesequence.org). These genes were functionally annotated using BLAST. An initial inspection suggested that the region was rich in detoxification-related genes. A proportion test was used to test for the abundance of detoxification-related (DR) genes with respect to abundance in the genome. Specifically, the genes tested for abundance were oxidoreductases, glutathione-S-transferases and genes in the carotenoid synthesis pathway, phytoene synthase and 1deoxy-D-xylulose 5-phosphate synthase.

Cercosporin treatment of 1.04 isolines

Twenty-four nearly-isogenic lines (F6:8NILs) developed using the HIF strategy were grown in the greenhouse under standard maize growing conditions. Four blocks of these lines were set up in a complete block design. Half of the six plants within each block contained the susceptible B73 allele while the other half contained the resistant CML228 allele. Different lines within the same HIF family were used to account for any residual background effect resulting from regions that may still have been segregating (estimated at less than 0.5%).

At flowering time, two ear leaves of each plant were treated with 0.1 ml of 100 uM cercosporin in acetone and an acetone control using a procedure modified from Batchvarova et al. (1992).

The treatment and control were infiltrated using a needleless syringe. Both the control and cercosporin treatments were applied to the same leaf on either side of the midrib. The plants were left in constant light for 24 hours in order to activate the cercosporin. After this time period, 10 leaf punches of 6 mm in diameter were collected around each treated site (two controls and two treatments per plant). Samples collected from the lower ear leaf were used for carotenoid detection while samples from the upper ear leaf were used for expression analysis.

Carotenoids were extracted and measured from maize leaf tissue using modified procedures based on those of Alba et al. (2005) and Bushway (1986). Under low light conditions, pre-weighed tissue of about 100 mg was homogenized with 50 µl of a 0.3% $MgCO_3$ solution (w:v) and 300 µl of tetrahydrofuran (THF). Homogenization was repeated after addition of 300 µl of 0.5% butylated hydroxyl-toluene/methanol (w:v). An additional 600 µl of THF was added to the extract, which was then filtered. To the filtered extract, 50 µl of 25% NaCl and 600 µl of petroleum ether were added and the sample was vortexed well. The upper phase was dried down and 500 µL HPLC grade ethyl acetate was added in preparation for the column; this was mixed well and filtered. A sample of the extract was added to a YMC C_{30} column for reverse-phase high performance liquid chromatography (RP-HPLC).

Real-time quantitative PCR (RT-qPCR) was performed on the cercosporin- and control-treated HIFs to test for expression differences across genes hypothesized to play a role in cercosporin detoxification. Total RNA was purified from maize leaf tissue using the RNEasy Mini Kit (Qiagen). The same kit was used for DNase I digestion. The cDNA was prepared using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Primer pairs were

designed for candidate detoxification-related genes listed in Table 3.2 and used for RT-qPCR using Power SYBR Green Master Mix (Invitrogen). The data was analyzed using the Comparative CT method (Schmittgen and Livak 2008).

Flavin-monooxygenase single nucleotide polymorphisms (SNP)

SNPs between B73 and CML228 within the putative flavin-monooxygenase gene were identified using maize Haplotype Map Version 2 (HMPv2; www.panzea.org). The B73 and CML228 gene sequences were developed by aligning the SNP calls with the sequence provided by the Maize Genome Sequence Consortium. The B73 SNP calls from HMPv2 matched the reference (B73) genome. The promoter region sequence for the B73 and CML228 allele were analyzed using plant promoter prediction software (Prediction of PLANT Promoters using RegSite Plant DB, Softberry Inc.) to detect functional differences in the promoter region resulting from sequence polymorphisms between the parental lines.

RESULTS

NIL pairs were developed using the HIF strategy to confirm and fine-map the *qGLS1.04*$_{CML228}$. This locus was subsequently fine-mapped using derivatives of the same HIF population. The QTL interval spanned from 56,747,253 Mb to 83,780,725 Mb. The estimated recombination rate at this locus was 0.236 cM/Mb. The QTL was fine-mapped to two intervals of 77,242,690-83,780,725 Mb and 88,849,284-94,085,195 Mb, referred to as *qGLS1.04_1* and *qGLS1.04_2* (Fig. 3.1). The *qGLS1.04_1* interval contained 99 genes based on version 2 of the maize

genome (www.maizesequence.org), while in *qGLS1.04_2* interval there were 51. The genes within these regions were functionally annotated using BLAST. It was found that at the *qGLS1.04_1* locus, 13.1% of the genes had been implicated in the role of detoxification while at the *qGLS1.04_2*, 9.8% of the genes had been implicated in the same role.

Both intervals in the 1.04 region appeared to have a high density of defense response (DR) genes. Proportion tests revealed that putative glutathione-S-transferase genes were significantly more numerous than expected (Table 3.3). To test if any of the DR genes may be playing a role in disease resistance, the HIF lines were treated with cercosporin. Expression was up-regulated in GRMZM2G425719 by 3.44 fold (Fig. 3.2). Up-regulation of other DR genes in the regions was not detected. GRMZM2G425719 is a putative flavin-monooxygenase gene.

Forty-four polymorphisms within this gene were identified using HMPv2. Twenty of those polymorphisms were identified in the promoter region of the gene. When a plant promoter detection algorithm was used to detect functional changes between the B73 and CML228 promoter regions, a G to A substitution was identified. This change led to the detection of a functional TATA box within the CML228 allele. No significant difference was found in carotenoid levels among the treated HIF lines.

DISCUSSION

The GLS QTL at the 1.04 locus identified through nested association mapping was validated and fine-mapped using the HIF strategy. Fine-mapping genetic of this locus provides breeders with markers that are closely linked to gene(s) conditioning the disease resistance phenotype. The 1.04 QTL interval was fine-mapped from an interval of 27.0 Mb to two intervals of 6.5 Mb and 5.2 Mb. This suggests that multiple genes underlie original QTL identified by NAM. Increased marker density may help resolve one QTL into two (or more) if there is high LD in the region among the NAM founders.

Treatment of the HIF lines with cercosporin resulted in increased expression of a putative flavin-monooxygenase (FMO) gene. FMOs are a family of oxidoreductases that have been previously implicated in disease resistance. FMOs have been implicated in glucosinolate production (Hansen et al. 2007; Li et al. 2008). Glucosinolates are a class of secondary metabolites noted for their role in fungal disease resistance among the brassicaceae (Mithen 1992). Mishina and Zeier (2006) found that FMOs play a role in biologically-induced SAR. The increased expression response is likely local because the control and the cercosporin treatments were infiltrated in the same leaf. Lastly, oxidoreductases have been implicated in the degradation of the cercosporin toxin into non-toxic xanosporic acid (Taylor et al. 2006). These authors found that mutant strains of *Xanthomonas campestris* could no longer degrade cercosporin while its wild-type progenitor has this ability. All of the mutants in the study could be complemented with a genomic clone with homologous sequence to a transcriptional regulator and an oxidoreductase. The mutants had point mutations in the oxidoreductase and not the regulator but

expression of both was necessary for complementation. Since the putative FMO was upregulated by cercosporin, its role may also be in cercosporin degradation. This can be tested by transforming *Xanthomonas campestris* wild-type strains with sequences coding for the putatative flavin-monooxygenase to test if the gene is involved in cercosporin degradation. Our findings support the hypothesis that genes underlying quantitative disease resistance are involved in mitigating the effects of microbial compounds that are deployed during the pathogenesis process. Since there are multiple fine-mapping intervals underlying the 1.04 QTL, additional genes are likely to be involved in conferring resistance at this locus.

Table 3.1.Markers and associated p-values from the qGLS1.04 fine-mapping analysis.

Marker	Chromosome	Location	p-value
PZA03168.5	1	51514741	0.808
PZA01267.3	1	77242690	0.5963
PZA00752.1	1	82019775	8.687E-08
PZA01135.1	1	83780725	0.1703
PZE0188095678	1	88095678	0.2202
PZB01235.4	1	93909140	0.0007
PZE0194085195	1	94085195	0.3395
PZA02750.3	1	101421637	0.549

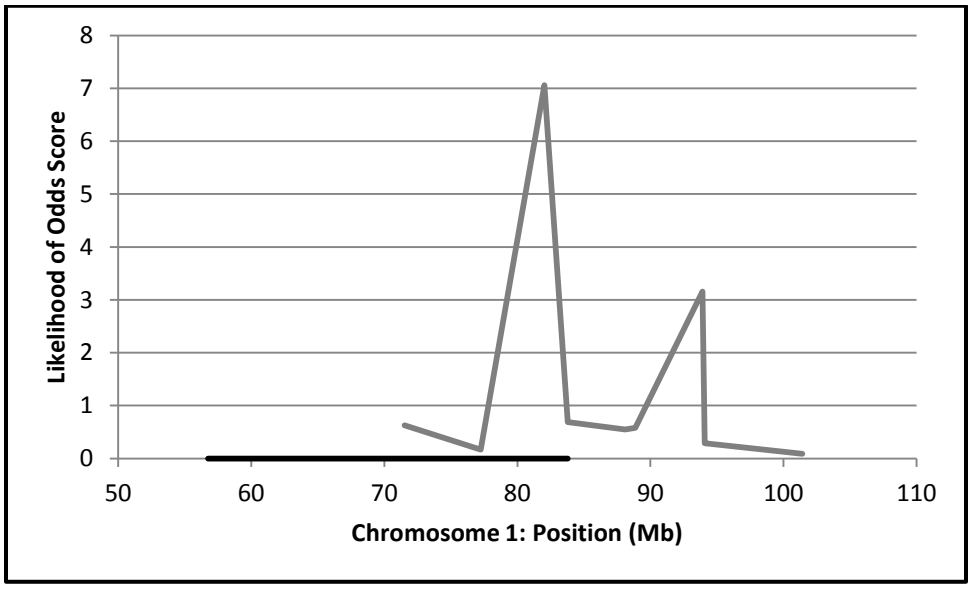

Figure 3.1. The qGLS1.04 fine-mapping region. The nested association mapping quantitative trait loci confidence interval is indicated as a black bar on the X-axis. The final fine-mapping interval using the heterogeneous inbred family method is in gray.

Table 3.2. List of gene locations on chromosome one and primer sequences used for expression tests on heterogeneous inbred family lines treated with cercosporin.

Gene	Start	End	Primer Name	Primer Sequence
GRMZM2G044383	81777188	81778493	GRMZM2G044383_F	CCTCTGCGCGTGTATCTCGTCG
GRMZM2G044383	81777188	81778493	GRMZM2G044383_R	CCTGCACCTCAGGTCCCTCCA
GRMZM2G039312	82187887	82190028	GRMZM2G039312_F	TGCACCTGCCAGATCCTGTCCA
GRMZM2G039312	82187887	82190028	GRMZM2G039312_R	GACGGCCGGGGGATGGGATT
GRMZM5G889520	82213773	82215225	GRMZM5G889520_F	CACACAGCCGCCTCTCACCG
GRMZM5G889520	82213773	82215225	GRMZM5G889520_R	GCTGGATGCTGGAAGGGTGCC
GRMZM2G162251	82578863	82582627	GRMZM2G162251_F	ACGTTCCACCACACCCACACG
GRMZM2G162251	82578863	82582627	GRMZM2G162251_R	CCAGATGGCTCAGGTAACCTCGATT
GRMZM2G099467	83400264	83404419	GRMZM2G099467_F	AGAGCACGTGGACGTGGATCTGATT
GRMZM2G099467	83400264	83404419	GRMZM2G099467_R	AACTTGGCAAAAAGACGGTTGCCCA
GRMZM2G028302	83431960	83433879	GRMZM2G028302_F	GCAAACGGGGCCCGGCATC
GRMZM2G028302	83431960	83433879	GRMZM2G028302_R	TGGCCAGAATCGGACTCGAGCG
GRMZM2G057768	84671535	84674990	GRMZM2G057768_F	CGTTCTCGCCCAGTCGCACC
GRMZM2G057768	84671535	84674990	GRMZM2G057768_R	TGCAGACCAATCAGCTCCCAACA
GRMZM2G028033	87011117	87011985	GRMZM2G028033_F	AGCACAGGAGGATTCAGAGGCT
GRMZM2G028033	87011117	87011985	GRMZM2G028033_R	TGAGTGAATCAGCGAGGGATCCAA
GRMZM2G425719	92342987	92345452	GRMZM2G425719_F	CCGTCACGCCACCAATCCCC
GRMZM2G425719	92342987	92345452	GRMZM2G425719_R	GATGCCCACTGGAGCCACCG
GRMZM2G086750	96688490	96691067	GRMZM2G086750_F	AGCCCATGAAGCGAGCAACCC
GRMZM2G086750	96688490	96691067	GRMZM2G086750_R	TGCAATTGGCGTCGTATGAAGTGA

Table 3.3. Summary statistics for functionally annotated and categorized genes within the qGLS1.04 quantitative trait loci hypothesized as putative detoxification related genes. SE: standard error.

Category	Genome (count)	qGLS1.04 (count)	SE	z-value	pvalue
1-deoxy-D-xylulose 5-phosphate synthase	4	2	0.058	-0.229	0.409
Glutathione-s-transferase	573	11	0.031	-2.100	0.018
Oxidoreductase	214	4	0.031	-0.751	0.226
Phytoene synthase	4	4	0.081	-0.328	0.372

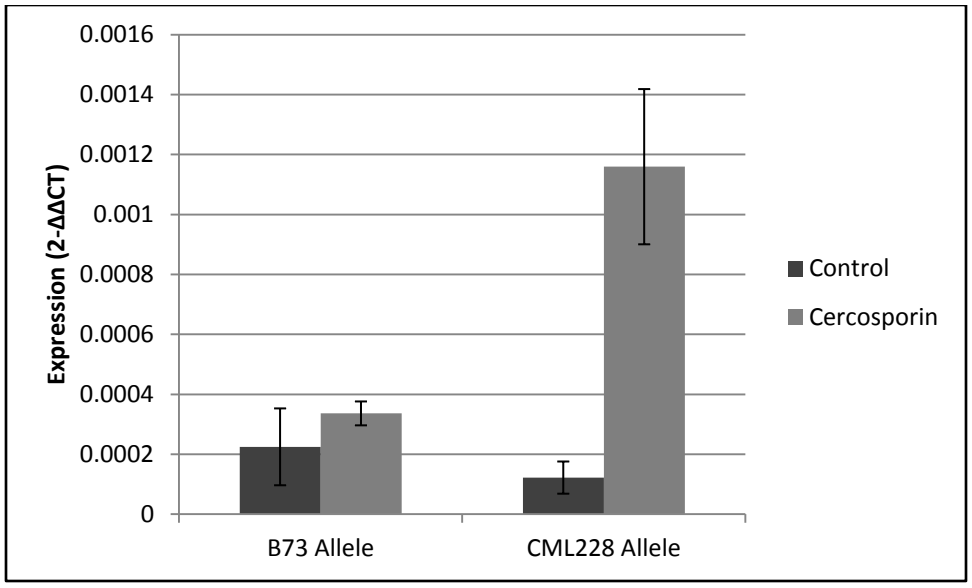

Figure 3.2. Expression differences of the putative flavin-monooxygenase among heterogeneous inbred family lines segregating at qGLS1.04 for the B73 or CML228 maize alleles. The HIF lines were treated with cercosporin or the acetone control on either side of the maize leaf midrib. There was a significant difference between the CML228 samples treated with cercosporin and the other samples in the experiment (p=0.0012).

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CHAPTER 4: HOST-PATHOGEN INTERACTIONS DURING GRAY LEAF SPOT PATHOGENESIS: INFERENCES FROM HISTOPATHOLOGY AND PLANT RESISTANCE MECHANISMS UNDERLYING DISEASE RESISTANCE IN MAIZE

ABSTRACT

Cercospora zeae-maydis and *C. zeina* are the causal pathogens of maize gray leaf spot (GLS), arguably the most devastating maize disease world-wide. In spite of the importance of the disease, the pathogens' life-style(s) is not well understood. In this study, diverse maize inbred lines were scored for microscopic indications of host response (fluorescence accumulation [FA] and callose plug formation), fungal development (extent of mycelial development and sporulation) and cell death (staining with propidium iodide). Overall disease development and host maturity were assessed in the field for the same panel of maize germplasm. The diverse maize inbred lines were selected because they had been used as the parents of the nested association mapping population (NAM), which has been used for high-resolution genetic and association mapping of GLS resistance in maize. This design made it possible to associate host responses with specific disease loci identified using the NAM. Histopathological analysis was focused on stages of pathogenesis between the formation of chlorotic and necrotic lesions by recording a spectrum of disease-related phenotypes. After entering the maize leaf, the pathogen developed along the minor veins and the adjoining cross-veins. After growth along the maize leaf venation, the pathogen developed between the mesophyll cells as reported in the literature. During the time between appearance of chlorotic flecks and necrotic lesions, growth of *C. zeae-maydis* was associated with cell death as detected by propidium iodide staining, suggesting a necrotrophic lifestyle even before the appearance of necrotic lesions. FA was significantly

associated with *qGLS1.02*, a QTL locus at which one out of four genes implicated by genome-wide association study (GWAS) analysis could be related to the fluorescence response. One gene underlying a significant association hit was a putative N(1)-acetylpolyamine oxidase (APO), which is involved in cell death and the HR response. Callose plug deposition was significantly associated with *qGLS1.06*. Three of the nine association hits at this locus appeared to be associated with components of cell wall synthesis. *C. zeae-maydis* appears to have a stealth necrotrophic life-style between the chlorotic fleck and necrotrophic lesion phase, while callose deposition likely plays a role in host defense.

INTRODUCTION

Cercospora zeae-maydis and *C. zeina* are the causal pathogens of maize gray leaf spot (GLS), arguably the most devastating maize disease world-wide. Chlorotic flecks are the first macroscopic symptom of infection, which are followed by the development necrotic lesions a few weeks after infection. During the lengthy period between the chlorotic fleck and lesion stage, the process of pathogenesis is not well understood. The present study aimed to further elucidate the pathogenesis process between the flecking and lesion stage by use of microscopic investigation linked to association genetics and QTL mapping.

Since GLS has a 14-28 day latent period (Ringer and Grybauskas 1995), one hypothesis is that the pathogen spends the period between infection and necrosis developing through the maize leaf, slowly killing the surrounding tissue and absorbing nutrients until it reaches an optimum capacity at which point necrosis occurs. This could be termed the "stealth necrotroph" hypothesis. An alternative hypothesis includes the hemibiotrophic life-style, in which the pathogen absorbs nutrients undetected on living host until a switch, at which point it begins to live on the dead cells (Divon and Fluhr 2006).

Since a description of *C. zeae-maydis* pathogenesis was published over 30 years ago (Beckman and Payne 1982), few studies have extended the understanding of pathogen development on and within the host (Kim et al. 2011). Beckman and Payne (1982) showed that after a period of high humidity, the conidia germinates on the leaf surface. *C. zeae-maydis* was observed to maintain hyphal growth on the leaf surface for up to one week before appressorium formation and

penetration, which occurred within a one-week period (Beckman and Payne 1982). Once the spores landed on the surface of the leaf, the germ tube formed an appressorium over the stomata and penetrated into the maize leaf.

The germ tube exhibited positive stomatal tropism. Once the stomata had been detected, the fungus formed appressoria, which required humidity between 90-95% to form (Thorson and Martinson 1993). Colonization of the plant resulted in the formation of a mycelial network within the plant leaves. During the time before necrosis, it was observed that colonization was intercellular and remained within the mesophyll. It also appeared as if the lateral hyphal growth was delimited by the sclerenchyma based on the shape of the lesion (Beckman and Payne 1982; Beckman and Payne 1983).

GLS exhibits a continuous spectrum of susceptibility across diverse maize germplasm and shows quantitative inheritance (Hilty et al. 1979). Quantitative trait locus (QTL) mapping studies utilizing biparental crosses have identified at least 24 genetic loci contributing to disease resistance (Balint-Kurti et al. 2008; Bubeck et al. 1993; Clements et al. 2000; Maroof et al. 1996; Zhang et al. 2012). Using the nested association mapping maize population (NAM), discussed in Chapter 2, six novel QTL were identified and previously-identified QTL were better resolved. Further, specific single-nucleotide polymorphisms were associated with resistance.

The NAM was derived from a cross between a common maize inbred parent and 25 diverse maize inbreds and is composed of 5,000 recombinant inbred lines. The size of the population provided greater power and resolution than the traditional biparental mapping populations and

the design permitted both linkage mapping and genome-wide association studies (GWAS) (Yu et al. 2008). The genetic mapping experiments provided disease effect estimates for a given parental line at a specific genetic locus. In the case of NAM, there were 25 different estimates for each of the 25 diverse lines that comprise the population across 16 loci identified in Chapter 2. These effect estimates can be associated with other traits. By associating allele effects at QTL mapped based on the phenotype of macroscopic disease development (area under the disease progress curve) with microscopic disease response phenotypes, this study also aimed to elucidate the mechanisms underlying quantitative disease resistance.

While a great deal of genetic mapping has been done on quantitative disease resistance (QDR), the underlying mechanisms of resistance are not well understood. GWAS are getting closer to addressing this issue, as significant associations can be functionally annotated to allow tentative inference regarding the locus mechanism (Freedman et al. 2011). Such results are subject to both type I and type II errors, however, and it is generally necessary to triangulate GWAS results with complementary findings to validate them. For example, associating putative disease loci identified by GWAS with loci co-segregating with disease-related host phenotypes can strengthen the case that genes confer the disease resistance or susceptibility phenotype. The following study is novel in that it combines visual analysis of pathogenesis with genetic mapping and GWAS.

Mechanisms underlying QDR are believed to be diverse. There are numerous microbial pathogenic strategies; for any given offensive strategy, there may be multiple corresponding defensive strategies. Poland et al. (2009) hypothesized that some quantitative resistance loci are

components of chemical warfare or are conditioned by genes that regulate morphology. Examples of those involved in chemical warfare include those genes involved with the production of callose and secondary metabolites. The formation of secondary metabolites and callose accumulation can be used as biochemical markers for evidence of a resistance response around infected loci (Dietrich et al. 1994). These markers can be visualized using fluorescent microscopy techniques such as aniline blue staining for detection of callose accumulation and autofluorescence of accumulated secondary metabolites such as phenolics associated with the hypersensitive response (Chung et al. 2010; Holliday et al. 1981; Koga et al. 1988).

The main objectives were to i) investigate fungal development and its relationship to temporal and spatial patterns of cell death in the maize leaf for 25 diverse maize inbreds from which the NAM population was derived, ii) analyze plant responses in the form of callose accumulation and fluorescence accumulation (FA) at the site of infection across the NAM founders, iii) relate the plant response phenotype to the corresponding genetic effects at disease resistance loci, and iv) annotate genome-wide association hits that reside within loci that had significant relationships to the plant response phenotype. To achieve these aims, the NAM parental lines were scored for microscopic indications of host response between the chlorotic and necrotic lesion stages of macroscopic pathogenesis. Microscopic phenotypes included fungal development and cell death as well as FA and the presence of callose plugs. In addition, macroscopic disease development and maturity were scored for each of the maize lines. The host responses were associated with specific disease loci.

METHODS AND MATERIALS

Plant material and field site

Twenty-five diverse maize inbred lines were planted in a complete lattice square design containing six replications of 5 x 5 randomized plots. Sixteen kernels of each line were planted in 2.4 m rows with 0.3 m spacing between rows. These maize inbreds were selected to represent maize diversity independent of phenotype, and were chosen because they had been used as the parents of the NAM population (McMullen et al. 2009). The material was planted on the gray leaf spot (GLS) screening site of Virginia Polytechnic Institute's Whitethorne Research Farm located in Blacksburg, VA. The Whitethorne Research Farm's GLS screening site was chosen for the high and even disease pressure that is routinely observed with natural inoculum.

Maize had been continuously planted in the field under no-till conditions since 1985. The field had been manually inoculated for three seasons (1985-1988) and subsequently used for GLS screening without inoculation. The isolates VA-1, VA-2 and VA-3 originally used to inoculate the field were collected from maize fields located in Montgomery County and Wythe County of Virginia. In 1985, these isolates were initially identified as *Cercospora zeae-maydis*, later re-classified as *C. zeae-maydis* II, and currently known as *C. zeina* (Wang et al. 1998). Sporulation of *C. zeae maydis* and *C. zeina* on the residues from the previous season likely provided the primary inoculum for disease development on the maize plants. At the conclusion of the 2009-2011 seasons, diseased samples were collected at random from a subset of the parental lines. For each year, 25-50 isolations were made. Isolates were identified at the genus level based on conidial morphology and identified to the species level using colony traits when grown on potato

dextrose agar (PDA). Those isolates producing a characteristic purple halo resulting from secretion of cercosporin and exhibiting faster colony growth were inferred to be *C. zeae-maydis*. These tests have been extensively compared with molecular typing in our laboratory and found to be reliable for distinguishing the two species (Crous et al. 2006). The majority of samples were identified as *C. zeae-maydis*, and a minority (~5%), were *C. zeina*.

Tissue collection

Each of the maize inbreds selected for the trial exhibited some degree of susceptibility to GLS. Symptoms ranged from chlorotic flecks to chlorotic lesions in the early stages. These early symptoms corresponded to distinct lesion types by maturity. By the end of July, all of the inbreds exhibited the early symptoms of GLS. On 28 July 2010, a minimum of six 3 x 3 cm^2 segments were cut from different maize plants within a given plot. Each segment contained an isolated chlorotic fleck or chlorotic lesion that was indicative of infection. The tissue segments were placed in 50 ml eppendorf tubes for tissue treatment. By 18 August 2010, all of the lines exhibited GLS lesion types that were characteristic for each genotype. A minimum of six leaf segments containing non-coalesced lesions were excised from different maize plants within the same plot. For both harvests, all of the tissue segments were collected between node three and node six of the maize plant. This was done in order to select tissue from the same relative position on the plant despite differences in ear and plant height across the diverse lines.

Fluorescent microscopy preparation

Each sample was immediately immersed in a 1M KOH solution in preparation for the analine blue stain. Samples were heated to boiling in an autoclave and then cooled. The samples

remained in the KOH solution for 24 hours before the solution was replaced with autoclaved water. The water was replaced with fresh water every 48 hours for two weeks and stored for later image processing. This cleared the tissue samples. Using sterile technique, the samples were removed from the eppendorf tubes and placed on a microscope slide. Three drops of a 0.1% aniline blue in K_2HPO_4 solution was placed on the samples using a Pasteur pipette. In preparation for the propidium iodide stain, the tissue samples were immediately fixed in 1.25% (v/v) glutaraldehyde for 12 hours at 4°C to prevent cell lysis. This was followed by fixation with 1% osmium tetraoxide to stabilize the cell membrane. The samples were then rinsed and stored in acetone. In preparation for microscopy, the samples soaked in a 10X PI stain solution (Sigma PA0100) for a minimum of 10 minutes before capturing images.

While contamination was minimal across the samples, when occasionally detected, the sample was not treated for fluorescent microscopy. The remaining samples were again randomized and screened blind under the microscope so that the identity of the sample remained unknown so as to not bias photographing or analyzing the pictures. These samples were observed under a Zeiss fluorescence microscope with a G365 excitation filter, a FT395 dichromatic beam splitter and an LP420 barrier filter.

Image analysis

Images of each sample were scored qualitatively for apparent plant responses and the presence of conidiophores. The images were quantitatively scored for fungal development and the level of propidium iodide (PI) stain accumulation. In the case of PI stain, four tissue samples within each of the sample types were scored to improve accuracy of the PI accumulation estimate. The

samples were assessed for the presence/absence of fluorescent accumulation and the presence/absence of callose formation. The following 0-3 scale was developed based on observations of fungal development in the maize tissue, and used to score all samples: 0 = no visible growth; 1 = inconsistent growth along the veins; 2 = continuous growth along the veins; and 3 = growth between the veins (not including cross-vein development). Examples of images for each rating are shown in Fig. 4.1.

Four samples for each genotype were scored for PI accumulation using a 0-4 scale in which 0 = no visible PI stain or minimal stain around the infection site; 1 = PI stain around infection site and minimally along the minor veins; 2 = inconsistent PI stain along minor veins; and 3 = consistent PI stain along minor veins and the yellow stain of tissue between the veins however with minimal appearance of green; and 4 = complete stain of the tissue sample and apparent necrosis void of color.

Statistical analysis

General linear models were performed in SAS 9.3 to examine the association among fungal development, conidiophore development, propidium iodide accumulation, plant reactions, AUDPC, flowering time, and tissue sample type. Replication number, row and column data were also entered into the model as covariates. Correlations were recorded in the case of quantitative responses and predictors, while significant differences between least squared means were tested using Tukey's HSD in the case of quantitative responses and qualitative predictors.

RESULTS

Understanding pathogenesis in the GLS system has been impaired by the difficulty of producing the disease under controlled conditions. The experimental design for the following experiment was not feasible in the greenhouse due to its size and the challenges with creating a GLS epidemic in the greenhouse using mature maize plants (Asea et al. 2005). We therefore collected samples from 22 diverse NAM founder lines grown in an experimental field maintained for the purpose of GLS disease screening. Plants were scored repeatedly for disease levels and for days to anthesis (DTA). Area under the disease progress curve (AUDPC) was calculated from three weekly disease ratings. As expected, a wide range of disease levels and maturity periods were observed (Table 4.1). GLS lesions were the only disease symptoms observed on the leaves from which the samples were taken. Chlorotic and necrotic lesion samples (n=300) were collected from the ear leaves before flowering and shortly after all the lines had presented lesions. Fungal growth was frequently observed in the trichome segment of the maize leaf. In leaf samples with immature necrotic lesions, it was observed that conidiophore development occurred near the trichome segment.

To relate macroscopic phenotypes (chlorotic vs. necrotic lesion type; AUDPC) to microscopic phenotypes, images of each sample were scored qualitatively for apparent plant responses and the presence of conidiophores. The images were quantitatively scored for fungal development and the level of propidium iodide (PI) stain accumulation. PI stain is used as an indicator of plant cell death (Darzynkiewicz et al. 1992). In the case of PI stain, four subsamples were scored to improve accuracy of the PI accumulation estimate. The samples were assessed for the

presence/absence of FA, which has been attributed to autofluorescence of antimicrobial molecules, and the presence/absence of callose formation (Chung et al. 2010). The samples were scored for fungal development using a scale of 0-3. This scale was based on observations of fungal development in the maize tissue. Examples of images scored for inconsistent, continuous and inter-veinal growth are shown in Fig. 4.1. Images that displayed growth between the minor veins, not including cross-vein development, were categorized as exhibiting inter-vein growth. The samples were scored for PI accumulation using a scale of 0-4.

Plant resistance response

The presence of callose plugs and FA was associated with reduced fungal development, suggesting that these plant response phenotypes may be disease resistance responses. Specifically, mean fungal development was significantly different across plant response categories (Fig. 4.2.A; $p<0.0001$). Samples with callose plug formation exhibited significantly less fungal development than samples exhibiting no apparent plant response (NAPR) or FA. Samples showing FA had intermediate levels of fungal development, and those showing NAPR had the highest level of fungal growth. Additionally, samples exhibiting callose plugs and FA developed less disease over the field season. Within the necrotic lesion sample types, the mean AUDPC was not different between the samples exhibiting callose formation and FA. However, samples exhibiting those plant responses were more resistant than those showing NAPR (Fig. 4.2.B; $p=0.0007$). There was also a significant difference for the combined data (Fig. 4.2.B; $p=0.0052$).

The FA phenotype was associated with cell death in that the average PI level, a quantitative indicator of cell death, was significantly greater for those samples exhibiting FA than for those samples containing callose formation or with NAPR ($p=0.002$). At low levels of cell death, fewer samples showed FA than exhibited callose formation or NAPR (Fig. 4.2.C; $p=0.0289$). At high levels of cell death, more samples exhibited FA than showed NAPR or callose formation (Figure 4.2.C; $p=0.0434$).

The presence of callose plugs was less often associated with lesion tissue and the presence of conidiophores than FA or NAPR. Callose formation was associated with chlorotic samples 75.8% of the time, while callose was associated with lesions 24.1% of the time. FA was associated with both chlorotic lesion samples at 58.2% of the observations and necrotic lesions about 42.7% of the time. There appeared to be NAPR in necrotic lesion samples for 57.3% of the observations and in chlorotic fleck samples 42.7% of the observations. Additionally, callose formation was exclusively associated with the absence of conidiophores (Fig. 4.2.D). FA was associated with the absence of conidiophores in 73% of the samples. Samples with NAPR were associated with the absence and presence of conidiophores at approximately the same rate of 48% and 52%, respectively.

Fungal development

On average, cell death was significantly lower in those samples exhibiting low levels of fungal development than in those samples exhibiting highly developed mycelium ($p=0.0043$). Samples of moderate fungal development were not significant from either low or high levels of development. Specifically, those tissue samples exhibiting low levels of fungal development

were observed significantly more often than those samples with greater fungal development to have no cell death, while the intermediate level of fungal development exhibited no difference between the other two levels (Fig. 4.3.A; p=0.0411). Conversely, those samples exhibiting high levels of fungal development were observed significantly more often to have high levels of cell death than those at the earlier stages of development (Fig. 4.3.A; p=0.0012).

Resistant lines were observed to have lower levels of fungal development than those that were more susceptible. There was a significant difference in mean AUDPC within the necrotic lesion samples but not in the chlorotic fleck or combined samples (Fig. 4.3.B; p=0.0002). Not surprisingly, samples with little fungal development were significantly more resistant than those samples with well-developed fungal growth. The samples exhibiting a medium level of development did not have an associated mean AUDPC that was significantly different from any of the other fungal development stages.

Resistant lines (as defined by AUDCP) were less likely to exhibit conidiophores development than susceptible lines in necrotic lesion samples. Mean AUDPC was not significantly different for chlorotic samples in the presence or absence of conidiophores (Fig. 4.4.A). This is because 0.81% of the chlorotic samples exhibited conidiophore development, while they were associated with the lesion samples 76.5% of the time. Within the necrotic lesion sample set, samples lacking conidiophore development were significantly more resistant than in the samples with conidiophores present (Fig. 4.4.A; $p<0.0001$). The difference was less significant in the combined sample set (Fig. 4.4.A; p=0.0064). Those lines lacking conidiophore development matured significantly later that those with conidiophores present (p=0.0009).

Conidiophores were always present when inter-vein fungal development was observed, but the presence of conidiophores was not exclusive to the inter-vein development category. Mean fungal development within the maize leaf tissue was significantly less in the absence than in the presence of conidiophores ($p<0.0001$). This was expected because conidiophores were most often associated with the presence of inter-vein growth. As expected, there was significantly less fungal growth in the chlorotic samples than in the lesion samples ($p<0.0001$). This is due to lesion development occurring in conjunction with the presence of conidiophores, which were almost always associated with inter-vein growth, the stage of fungal growth preceding conidiophore development.

The average cell death was significantly higher for tissue samples containing conidiophores than for those lacking conidiophore development ($p=0.0036$). At low levels of cell death, there were more observations for tissue samples lacking conidiophores than for those with conidiophore development (Fig. 4.5.A; $p=0.0071$ and 0.0138, respectively). However, there were more observations at moderate levels of cell death in the presence of conidiophores than in their absence (Fig. 4.5.A; $p=0.0014$). The average cell death was significantly less for the chlorotic fleck sample type than for the necrotic lesion samples ($p=0.012$). There were significantly more observations at low levels of cell death for chlorotic fleck tissue than for necrotic lesion tissue (Figure 4.5.B; $p=0.0217$ and 0.0322). Conversely, there were significantly more observations at moderate levels of cell death for necrotic lesion tissue than for chlorotic fleck tissue (Fig. 4.5.B; $p=0.0054$). The only PI level at which there was a distinction between mean AUDPC across frequency of observations was at PI level 2, a medium-to-low level of cell death (Fig. 4.4.B).

Those with no observations at PI level 2 were significantly more susceptible than those samples with 75% of the observations at PI level 2 (Figure 4.4.B; p=0.0354).

QTL relationship to host disease response and associated GWAS hits

Since there were 16 possible GLS QTLs to associate with host disease response, 16 pairwise tests were performed between the parental disease effects (PDE) at the given loci and callose accumulation or hyper fluorescence response, independently. This required a multiple testing threshold of $p<0.0277$. There was a significant positive relationship between the PDE of *qGLS1.02* and the hyper fluorescence response (p=0.0132) suggesting that FA is present in more susceptible lines. Additionally, there was a significant negative relationship between the PDE of *qGLS1.06* and the parental callose accumulation response (p=0.0086) suggesting that lines exhibiting greater presence of callose plugs tend to be more susceptible.

There were four significant GWAS hits within the *qGLS1.02* confidence intervals, while there were nine significant hits within the *qGLS1.06* confidence interval that had BPP over 3 (Chapter 2). Table 4.2 provides the maizesequence.org gene name and location as well as the functional annotation. The gene annotation was performed using the gene ontology website (http://www.geneontology.org). Four annotated genes at the 1.02 locus may be related to cell wall formation. There is one annotated gene at the 1.06 locus that may be related to the hyper fluorescence response.

DISCUSSION

More histology and cytology studies have been performed with *Cercospora zeae-maydis* than with *C. zeina*, but it is generally accepted that the latter goes through much of the same infection processes (Meisel et al. 2009). Because *C.zeina* was in low abundance at the experimental site (0.05% of isolates tested), it is assumed that the observations made in this study reflect infection by *C. zeae-maydis*. The mode by which the pathogens enter the leaf is well understood based on prior studies. After a period of high humidity, the conidia germinate on the leaf surface and the germ tubes grow in the direction of the stomata (Beckman and Payne 1982). *C. zeae-maydis* was observed to maintain hyphal growth on the leaf surface for up to one week before appressorium formation and penetration, which occurred within one week. The pathogen's development during the lengthy time between infection and necrotic symptom development are not well understood. During the time before necrosis, colonization was intercellular, restricted to the mesophyll, and lateral hyphal growth was delimited by the sclerenchyma (Beckman and Payne 1982). It was not clear whether the pathogen actively caused cell death or if it was nourished through a biotrophic interaction. Chlorotic spots were the first macroscopic symptom of infection; this was followed by the development necrotic lesions a few weeks after infection.

Histopathology

After entering the maize leaf, the pathogen was observed in this study to develop along the minor veins and the adjoining cross-veins. Only after growth along the maize leaf venation did the pathogen develop intercellularly between the mesophyll cells as reported in the literature (Beckman and Payne 1982). One hypothesis is that the fungus gains access to nutrients via the

maize leaf vascular structure. During the time between infection and symptom development, the fungus may reach some threshold level of growth before ramifying hyphal growth around the mesophyll cells. The branched hyphal structure was clearly associated with conidiophore development; such branched structures were not observed without the presence of conidiophores. Additionally, it was observed that the fungus developed optimally in the trichome segment of the maize leaf after entering the maize leaf at a non-selective location. The pathogen exhibited expansive hyphal development in this region, which appeared anatomically more spacious than the rest of the maize leaf. Additionally, in leaf samples with immature necrotic lesions, it was observed that conidiophore development occurred near the trichome segment, consistent with the observation of Lapaire and Dunkle (2003) that conidia developed more aggressively on trichome substrata. This region may be optimal for fungal growth resulting in the more aggressive development of conidia.

Fungal development

Propidium iodide staining was used to test whether *C. zeae-maydis* is a stealth necrotroph, causing cell death before necrotic lesions were observed. Cell death was indeed observed across samples exhibiting low levels of fungal development, though more cell death was observed in samples exhibiting fungal growth at a later developmental stage. There were lower levels of cell death in the absence of conidiophores than in their presence. At the stage of conidiophore development, the fungus had killed the surrounding cells and locally colonized the interior of the maize leaf, resulting in the formation of necrotic tissue and production of the conidiophores. Conidiophore development was generally absent in chlorotic tissue and was more often associated in tissue samples with visible lesions. Chlorotic tissue tended to show lower levels of

cell death, while lesions showed higher levels of cell death. This was expected, as lesions form as a result of cell death.

Lines that were more resistant were more likely to have had no or little fungal growth than those samples that were more susceptible, which were more likely to exhibit inter-vein growth. Those lines that were more resistant were more likely to have callose formation than those samples that were more susceptible, which were more likely to have been scored as having FA and NAPR. This is consistent with the inference that callose formation, often at the plasmodesmata, resulted in a lower mean fungal growth; it appears that the formation of callose aids in plant resistance. This is widely supported in the literature (Hématy et al. 2009).

Plant reaction

Diverse NAM parental lines were used to evaluate plant responses in order to associate defense mechanisms to specific regions of the genome. The parental disease effects at each of the 16 NAM QTL were tested against the parental plant response phenotypes measured at the microscopic level. The purpose was to identify significant associations between loci and plant resistance responses. The responses measured included callose plug deposition and FA, with the latter understood to reflect the accumulation of phenolics leaking from dead cells around the site of infection (Koga, 1988).

Callose plug deposition was negatively associated with *qGLS1.06* meaning that more resistant lines more frequently exhibited callose plugs. One of the nine GWAS hits at this locus is related to callose synthesis, which was DIMBOA-UDP glucosyl transferase, which catalyzes the

forward and reverse reaction that produces or uses UDP-D-glucose as a substrate. UDP-glucose is a substrate that can be transported to specific cellular components for the synthesis of callose and other products (Avigad and Dey 1997).

FA was positively associated with *qGLS1.02* meaning that the presence of FA was overall associated with greater levels of susceptibility. There were four GWAS hits at this locus, one which could be related with the fluorescence response. A putative N(1)-acetylpolyamine oxidase (APO) was found to underlie one of the significant GWAS hits. This gene is involved in cell death and the HR response (Marina et al. 2008). This results in autofluorescence of the region and is often measured by the fluorescence response (Yu et al. 1998). Marina et al. (2008) tested the role of polyamine oxidation in tobacco defense against biotrophic and necrotrophic pathogens. An increase in polyamine oxidase was detected after infection in both pathogens, however infection was increased by the necrotroph while it was decreased in the biotroph. While the presence of FA was significantly associated with reduced fungal growth in the micrographs, overall lines exhibiting FA were more susceptible. This supports the hemibiotrophic hypothesis and indicates that the HR response may be effective during the biotrophic phase at early stages of development but when there is a shift to the necrotrophic phase, it is no longer effective and costly compared to taking the callose plug strategy.

Samples exhibiting FA were significantly fewer at low levels of cell death and greater at high levels of cell death than the observations for the other plant reaction classification. On average, there was greater evidence of cell death for the samples exhibiting FA than for the other plant reaction categories. These results suggest that the appearance of FA is strongly associated with

cell death and that it may actually be a result of a hypersensitive response in which the plant kills the cells surrounding an infection site. The HR is a means of plant defense to restrict biotrophic pathogen growth. While the HR response is used as a means to prevent further spread of the infection, it actually facilitates the infection of necrotrophic fungal pathogens (Govrin, 2000), possibly explaining why it is not more effective at reducing overall disease development as indicated by the allelic effect association and compared to callose plug production.

CONCLUSIONS

During the time between chlorotic flecks and necrotic lesions, *C. zeae-maydis* appears to behave as a necrotroph, killing host tissue as it grows. It is stealthy in the sense it kills surrounding cells without causing full-blown necrotic lesions. This, however, does not eliminate the hypothesis of a hemibiotrophic lifestyle, in which the pathogen derives nutrients from living host cells prior to entering a necrotrophic phase. The association of the APO gene with *qGLS1.02*, together with the finding that FA is associated with lower levels of fungal development suggests that the pathogen may have a phase at which it does not depend on cell death. However, since FA is associated with susceptibility overall, this supports that the presence of FA is detrimental to the host during the necrotrophic phase.

This study contributes insights on the life-style of *C. zeae-maydis* between the chlorotic and necrotic lesion phases. At the chlorotic fleck stage, *C. zeae-maydis* slowly kills the surrounding cells as it develops along the minor venation of the maize leaf. At the necrotic lesion stage, the

pathogen has colonized the inter-vein space and developed conidiophores for secondary inoculum production. The trichome space appears to be the principal location for initial fungal development after infection.

In this study, plant resistance responses at the microscopic level were associated with specific QTL. Functional annotation of GWAS hits within these regions enabled the identification of candidate genes based on the plant resistance response. In order to confirm that the plant resistance response is associated with a specific locus, nearly-isogenic lines (NILs) should be evaluated. This experiment should observe NILs for microscopic plant resistance responses after infection with the pathogen. Mutant lines can be evaluated to test whether the identified candidate genes indeed confer resistance.

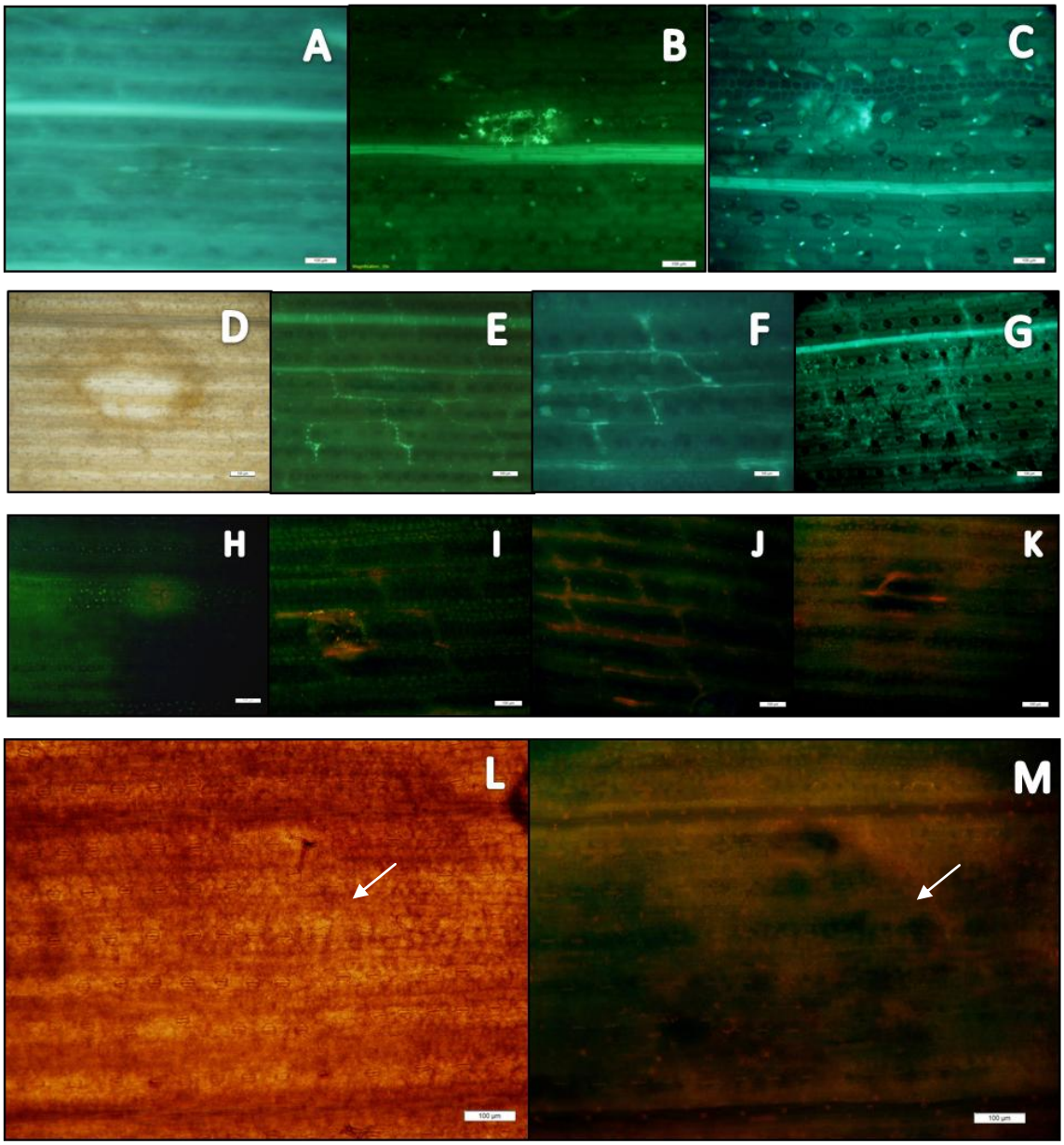

Figure 4.1. Micrographs of fungal development and cell death. Images A-G: samples treated with aniline blue (AB). Images H-M: samples treated with propidium iodide (PI). (A) No apparent plant reaction. B: Fluorescence accumulation around chlorotic spot. C: Callose plug accumulation often found at the plasmodesmata. D: Chlorotic spot under white light. E: Same sample as (D) under fluorescent light, showing inconsistent fungal growth. F: Sample exhibiting continuous fungal growth. G: Sample exhibiting conidiophores and inter-veinal growth. H-K; M: Examples of sample that would receive a score of 0-4, respectively. L: The white light image beginning to exhibit conidiophore development and the corresponding void in image M.

Table 4.1. Flowering time and disease traits of diverse maize inbred lines. DR: Disease rating AUDPC: Area Under the Disease Progress Curve; N: number.

Pedigree	Days to Anthesis	Mean DR1	Mean DR2	Mean DR3	Mean AUDPC	N
B73	68	2.86	3.11	4.79	48.5	6
B97	70	2.63	2.79	3.75	41.9	6
CML103	76	2.15	2.55	3.95	39.2	5
CML277	75	1.95	2.40	3.05	34.3	5
CML322	76	1.79	2.38	3.58	35.4	6
CML333	80	2.15	2.35	3.95	37.8	5
CML52	78	1.75	2.17	3.42	33.3	4
CML69	81	1.95	2.45	3.60	36.6	5
Hp301	68	2.55	3.10	4.25	45.5	5
Ki11	69	2.40	2.50	4.00	39.9	5
Ky21	73	2.33	2.83	3.71	41.0	6
M162W	79	2.46	2.63	3.88	40.5	6
M37W	72	1.70	2.35	3.70	35.4	5
Mo17	66	2.05	2.35	3.85	37.1	5
Mo18W	72	1.83	2.21	3.00	32.4	6
MS71	63	2.79	3.13	4.50	47.4	6
NC350	77	1.58	2.04	3.46	31.9	6
NC358	68	2.00	2.54	3.33	36.5	6
Oh43	64	2.38	2.80	4.20	42.4	6
Oh7b	75	2.67	2.92	4.42	45.2	6
P39	59	3.45	4.15	5.00	58.6	5
Tzi8	83	1.88	2.29	3.21	33.8	6

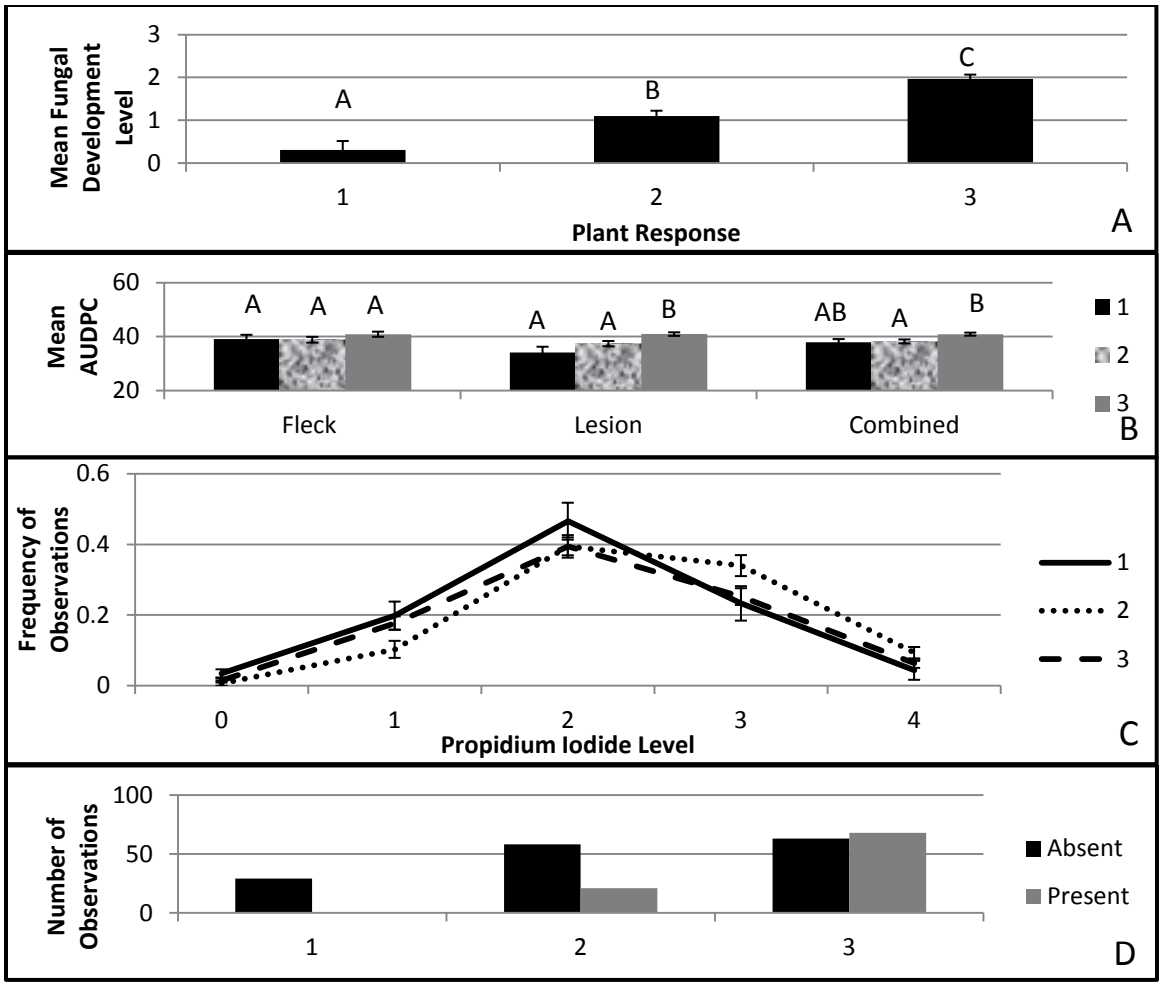

Figure 4.2. Four traits' relationship across three plant responses: (1) Presence of callose plugs, (2) fluorescence accumulation and (3) no apparent plant reaction. Sub-figures A-D: A: Mean fungal development level for each plant response. Fungal development was determined based on an increasing growth scale of 0-3 where zero is an indication of no growth, one is inconsistent growth, two is continuous growth and three is inter-vein growth. There was a significant difference between each of the plant response types with respect to the mean fungal development level ($p<0.0001$). B: Mean susceptibility level or area under the disease progress curve (AUDPC) across plant reaction phenotypes. AUDPC is calculated based on three disease rating collected post-anthesis and the time interval between data collection. These levels were determined within chlorotic fleck (Fleck), necrotic lesion (Lesion) and combined sample types. Among the necrotic lesion samples, the samples with no apparent plant response were more susceptible than those samples exhibiting callose plugs or fluorescence accumulation ($p=0.0007$). When the sample sets are combined there remains a significant difference between samples exhibiting fluorescence accumulation and those exhibiting no apparent plant reaction ($p=0.0052$). C: The frequency of observations for propidium iodide accumulation across plant response. PI accumulation was scored on a 0-4 scale where 0 indicates no accumulation to 4 which indicates complete coverage of stain. There was a significant difference between callose plug accumulation or no plant reaction and fluorescence accumulation at PI level 1 ($p=0.029$). There was also a significant difference at level 3 between callose plug accumulation or no plant reaction and fluorescence accumulation ($p=0.043$). D: The number of observations in the presence (Present) or absence (Absent) of conidiophores across plant response. The presence of callose plugs was exclusively associated with the absence of conidiophores. The fluorescence accumulation observation was associated with the absence of conidiophores in 73.4% of the samples. Samples with no apparent plant reaction were associated with the absences and presence of conidiophores at approximately the same rate of 48.1% and 52.9%, respectively.

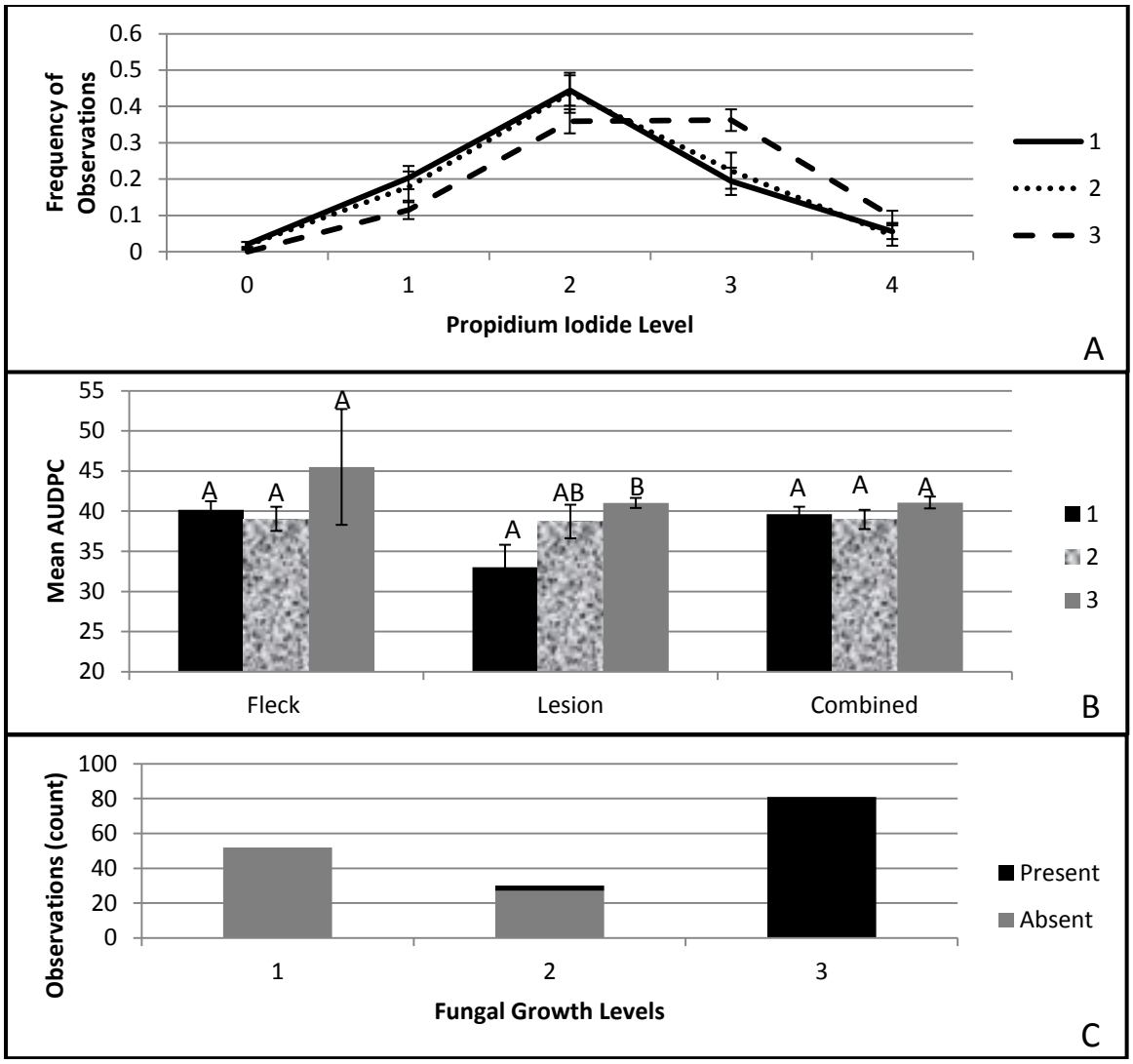

Figure 4.3. Three traits' relationship across three levels of fungal growth on an increasing scale of fungal development: (1) inconsistent growth, (2) continuous growth and (3) inter-vein growth. Sub-figures A-C: A:The frequency of observations for propidium iodide accumulation across three levels of fungal growth. PI accumulation was scored on a 0-4 scale where 0 indicates no accumulation to 4 which indicates complete coverage of stain. There was a significant difference between inconsistent growth and inter-vein growth at PI level 0 ($p=0.0411$). There was also a significant difference at level 3 between inconsistent or continuous growth and inter-vein growth ($p=0.0012$). B: Mean susceptibility level or area under the disease progress curve (AUDPC) across fungal growth. AUDPC is calculated based on three disease rating collected post-anthesis and the time interval between data collection. These levels were determined within chlorotic fleck (Fleck), necrotic lesion (Lesion) and combined sample types. The black bars indicate inconsistent growth while the speckled and gray bars indicate the continuous growth and inter-vein growth, respectively. There was no significant difference in the fleck or combined groups. However there was a significant difference among the lesion samples where the inconsistent growth development level tended to be less susceptible than the inter-vein growth development level ($p=0.0002$). C: Fungal growth levels, in the presence (Present) or absence (Absent) of conidiophores. The gray bar indicates absence of conidiophores while the black bar indicates the presence of conidiophores. Conidiophores are never present when a sample is scored as exhibiting inconsistent growth. Conidiophores are always observed when a samples is scored as exhibiting inter-vein growth.

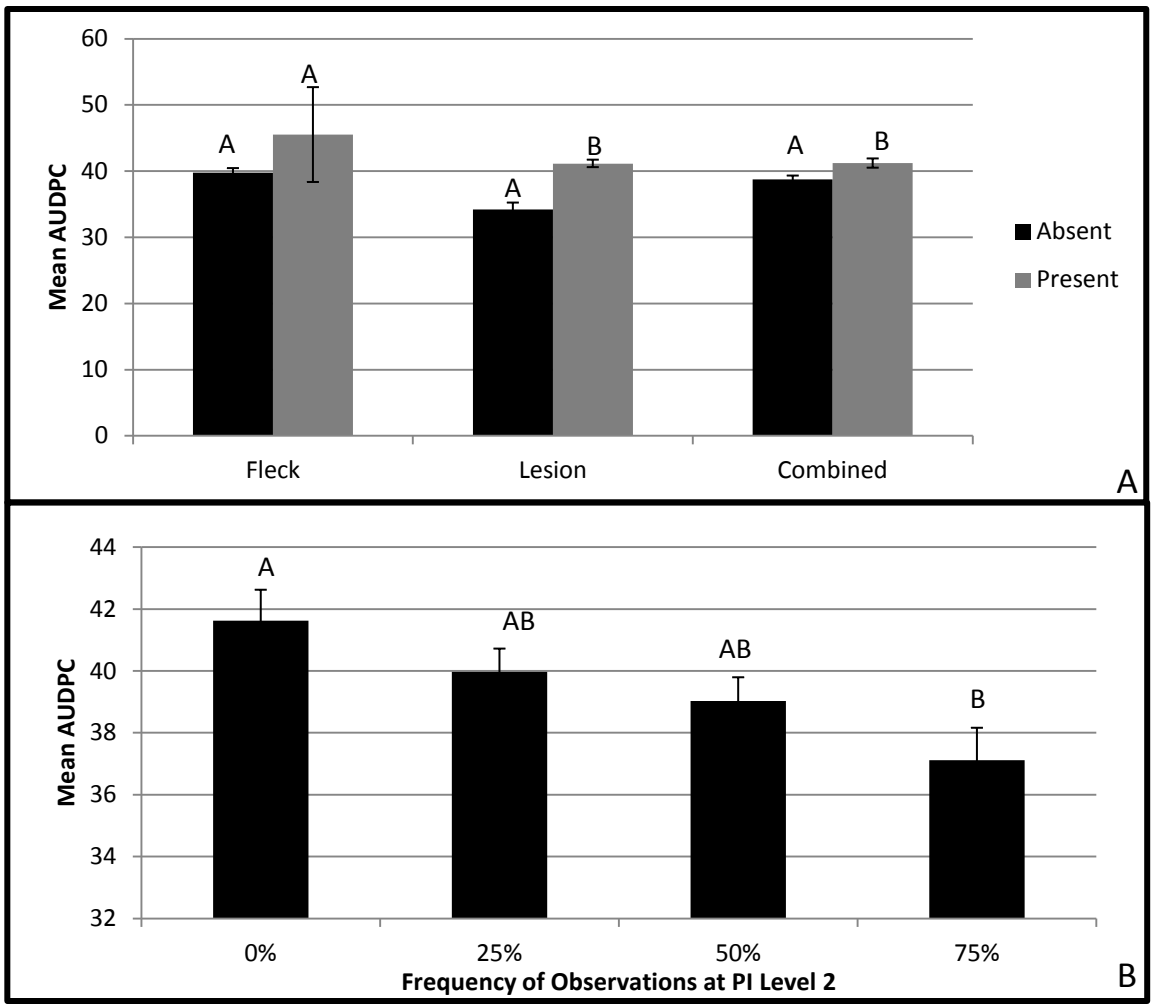

Figure 4.4. Sub-figure A: Mean susceptibility level or area under the disease progress curve (AUDPC) across the presence (Present) or absence (Absent) of conidiophores. AUDPC is calculated based on three disease rating collected post-anthesis and the time interval between data collection. These levels were determined within chlorotic fleck (Fleck), necrotic lesion (Lesion) and combined sample types. The black bar indicates the absence or conidiophores while gray bar indicate presence of conidiophores. Within the chlorotic fleck samples there was no significant difference in mean AUDPC among the presence or absence of conidiophores. Among the necrotic lesion samples, the samples with conidiophores present were more susceptible than those samples that had not yet developed conidiophores ($p<0.0001$). When the sample sets are combined there remains a significant difference between the presence or absence of conidiophores ($p=0.0064$). B: Mean susceptibility level or area under the disease progress curve (AUDPC) for samples with 0, 25, 50 & 75 percent of observations at level 2 propidium iodide (PI). For each diverse inbred line, there were a maximum of 24 samples assessed for the level of PI stain. Those lines with zero observations at PI level 2 were significantly more susceptible than those lines with 75% of their observations at PI level2 ($p=0.035$). There were no significant differences among other PI levels.

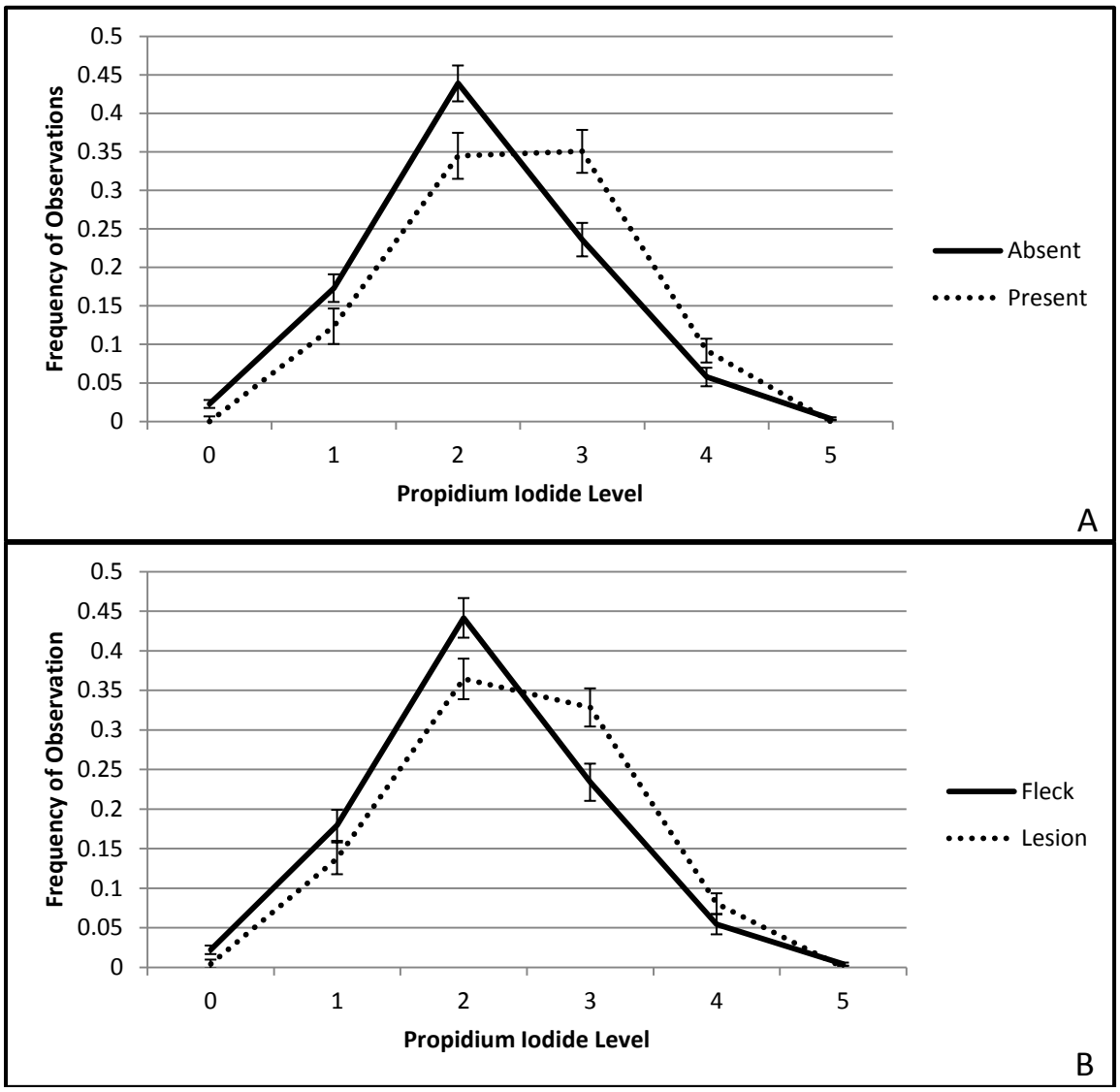

Figure 4.5. Sub-figure A: The frequency of observations for propidium iodide accumulation in the presence (Present) or absence (Absent) of conidiophores. The solid and dotted lines indicate the absence and presence of conidiophores, respectively. PI accumulation was scored on a 0-4 scale where 0 indicates no accumulation to 4 which indicates complete coverage of stain. There was a significant difference between the absence and presence of conidiophores at PI level 0, 2, and 3 with p-values of 0.007, 0.014, 0.001, respectively. Sub-figure B: The frequency of observations across five levels of propidium iodide accumulation for chlorotic fleck (Fleck) and lesion (Lesion) tissue samples. The solid and dotted lines indicate that the sample is either in the chlorotic or lesion category, respectively. PI accumulation was scored on a 0-4 scale where 0 indicates no accumulation to 4 which indicates complete coverage of stain. There was a significant difference between the chlorotic fleck and lesion sample types at PI level 0, 2, and 3 with p-values of 0.022, 0.032, 0.0054, respectively.

Table 4.2. Annotation of genome-wide association hits in *qGLS1.02* and *qGLS1.06*. Chr-Chromosome; cM-centimorgan; BPP-Bootstrap Post Posterior; GO-Gene Ontology.

qGLS	Chr	Position	cM	Allele	BPP	Effect	Maize Sequence Gene	Gene Interval	GO Annotation
1.02	1	24,770,931	41.63	C/A	4	1.02	GRMZM2G071343	24,781,228-24,786,321	N(1)-acetylpolyamine oxidase
1.02	1	26,275,081	43.19	G/A	6	1.63	GRMZM2G098714	26,326,640-26,330,061	Replication factor A-like protein
1.02	1	26,447,854	43.5	A/C	3	1.02	GRMZM2G472693	26,427,723-26,432,914	Histone-lysine N-methyltransferase
1.02	1	26,805,452	44.15	G/A	4	-0.49	GRMZM2G165622	26,818,272-26,821,649	Cysteine synthase
1.06	1	173,110,701	94.12	G/A	8	-1.16	GRMZM2G303010	173,142,735-173,144,264	XA1 Rice blast disease resistance
1.06	1	183,657,474	98.44	T/A	3	-1.27	GRMZM2G050177	183,670,348-183,677,238	Galactinol--sucrose galactosyltransferase 1
1.06	1	183,969,702	99.25	G/T	10	-0.67	GRMZM2G080775	183,993,561-184,006,197	Peroxisomal targeting signal type 1 receptor
1.06	1	180,262,819	96.69	C/T	16	-1.4	GRMZM2G161335	180,306,606-180,308,510	DIMBOA UDP-glucosyltransferase
1.06	1	186,614,192	101.33	T/C	35	-0.8	GRMZM2G362303	186,633,675-186,639,255	Cell wall-associated receptor kinase 2
1.06	1	187,927,685	102.38	G/A	26	-1.49	GRMZM5G836190	187,978,007-187,980,515	Leucine-rich repeat
1.06	1	199,579,946	113	A/G	5	-0.78	GRMZM2G386463	199,612,825-199,625,783	Fucosyltransferase 11
1.06	1	201,295,476	115	G/A	12	-0.57	GRMZM2G419024	201,403,482-201,409,528	*Uncharacterized*
1.06	1	207,120,463	119.31	--/CC	8	-0.84	GRMZM2G303157	207,126,913-207,132,165	*Uncharacterized*

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CHAPTER 5: CONCLUSIONS

This work is done in the context of a forward genetics approach to understanding the genetic architecture of foliar and ear rot fungal maize diseases (Fig. 5.1). This approach allows us to identify the regions of the genome conferring resistance and further narrow the region through fine-mapping and hypothesis testing of the candidate genes. From genetic mapping experiments, geneticists are able to identify QTL. GWAS and fine-mapping with nearly isogenic lines (NIL) allow us to narrow the QTL interval and identify candidate genes through significant SNP associations. With this information, geneticists can test hypotheses regarding candidate genes for functional variation using NILs, mutants and expression analyses. Lastly biochemical and mechanical assays can be performed to better understand the function of the genes and mechanisms underlying disease resistance. It should be noted that this approach is complementary but opposite of the reverse genetics approach in which specific genes are mutated, silenced or transformed in previously wild-type lines. Once the gene function is changed, the phenotype is screened under specific conditions to elucidate gene function. Both approaches are valid, but the forward approach is most relevant given the goal of identifying natural variation in disease resistance regions. Understanding natural variation for resistance is intended to support trait introgression into susceptible varieties by enabling the identification of resistance sources, loci and potential breeding trade-offs.

The availability of a public platform for maize genetic diversity analysis has allowed the efficient analysis of the genetic architecture of maize GLS. More specifically, the nested association mapping and 282 Diversity Panel (Flint-Garcia et al. 2005; McMullen et al. 2009) mapping

maize populations were used to identify QTL and putative SNPs associated with resistance in a broadly representative sample of maize germplasm. It was advantageous that Ed Buckler, lead developer of both populations, is located at Cornell University and is a generous collaborator. The large size and diverse nature of the NAM maize population provided us with the ability to identify numerous QTL, their effect sizes and sources for a given locus.

Over half of the QTL identified in this study co-localized with previously-identified QTL for GLS resistance. The confidence intervals were narrower in most cases, which improved the utility of marker assisted selection in developing resistant lines. GWAS was performed using NAM data because the population is derived from 25 diverse maize inbreds crossed to the common parent B73. Most of the significant GWAS hits were associated with the disease QTL, but the many GWAS hits outside of QTL regions suggest that the NAM genetic mapping strategy does not identify all QTL but may perhaps do so with a lower statistical modeling threshold.

Another great advantage to using the NAM population is that it had been screened for the foliar diseases northern leaf blight (NLB) and southern leaf blight (SLB) in addition to various other agronomic traits since it is a maize community resource used by numerous colleagues. Knowing the QTL implicated in the genetic architectures of multiple traits allows the loci that overlap between traits to be identified, suggesting loci for which pleiotropy or linkage occurs. Pleiotropy was tentatively inferred when the NAM parental allelic effects were significantly associated for more than one trait. Specific traits of interest in this dissertation were days to anthesis (flowering time), a measurement of maturity, NLB and SLB disease development and inter-vein distance.

Flowering time is an important agronomic trait because most maize-growing regions in the US require lines that mature more rapidly than tropical lines. There is a wide range of diversity in flowering time so it is important that a breeder knows the implications of introducing a disease resistance locus that is also associated with maturity. In addition to the flowering time implications of disease resistance loci, it is advantageous if a breeder is also provided with information regarding the relationship between parental allelic effects among NLB and SLB. It was found that loci resistant for two diseases may be a susceptibility factor for the third. Specifically, the GLS parental allelic effects at *qGLS3.06* were negatively associated the SLB effects while positively associated with NLB. Overall, there was a large trend of positive significant relationships between GLS and NLB. Elucidating these trade-offs improves the breeders' decision- making capacities with regard to developing resistant varieties, as well as suggesting common mechanisms underlying the resistance to multiple diseases.

Inter-vein distance (IVD) was another trait of interest because it appears that GLS lesions are delimited by the major veins of the maize leaf. It was confirmed that distance between the major veins was significantly associated with disease resistance in the NAM population. IVD was analyzed in the NAM population to identify QTL that co-localize with disease QTL. Those with narrow IVD tended to be more resistant. GLS disease development and inter-vein distance QTL co-localized in four locations across the genome, one of which was confirmed using the 4.05 HIF lines. To further understand the mechanism, the lesion dimensions were measured and conidiophores counted within each measured lesion. When length and width of the lesion were entered into a GLM with conidiophore count as a response, the width parameter was highly significant, indicating that the lesions are restricted by the major veins and more importantly the

secondary inoculum production is increased in more susceptible lines with greater inter-vein distance. Tests such as these have given us a better understanding of the mechanisms underlying quantitative resistance and support the hypothesis that plant architecture plays a major role in quantitative resistance.

The 282-line Diversity Panel was also used to identify significant marker-GLS associations. Having multiple populations also allowed us to triangulate the common candidate loci, thus giving us more evidence with regard to the robustness of the QTL and to the genes implicated in disease resistance within a given QTL. This population had previously been screened for GLS by our colleagues and collaborators in Peter Balint-Kurti's laboratory at North Carolina State University. Dr. Balint-Kurti suggested that the population be screened in Blacksburg, VA as well. This site was developed over 20 years of continous corn, no-till land management after initial treatments with *C. zeae-maydis* and *C. zeina* inoculum from maize-growing regions in VA. The field site was selected because of its reputation of reliable, evenly distributed disease pressure and the fact that the disease would progress as it does under natural field conditions. Field inconsistencies were managed by calculating BLUPs from the disease ratings, which effectively removed the field effect. GLS does not develop consistently in NY State; nor did the lab want to be implicated as the cause of subsequent GLS epidemics in NY State. Since the location is distant from Cornell, there were limited facilities, person power, and time to inoculate each plant, so the availability of a site with uniform natural inoculum was critical to success.

In addition to the NAM and 282-line Diversity Panel, fine-mapping populations were developed using the HIF strategy to confirm QTL identified through NAM and narrow the confidence

intervals. The QTL in bins 1.04, 2.09 and 4.05 were confirmed by testing for significant disease development differences among lines with the resistant and susceptible alleles. The observed allele effects were greater in the NILs than the statistical model estimated. In the future, caution should be taken with covariate selection and developing BLUPs from the disease ratings because it may be removing variance detected in the field observations. It would be worthwhile to build models that more closely approximate the change in disease development detected in the field.

In two out of the three fine-mapping cases, I was able to narrow the confidence intervals of the QTL. The 4.05 region was difficult to fine-map given the very low recombination rate in a large chromosomal segment. To identify the causal polymorphisms underlying resistance in this region, several alternative approaches can be considered: (1) Develop a new population that lacks the challenging linkage structure by use of any other parental cross that exhibits less segregation distortion thank Ki11 (McMullen et al. 2009). (2) Screen for variation in transcript levels among GWAS candidates with and without exposure to GLS. (3) Further elucidate the genetics of leaf venation, a mechanism believed to underlie resistance in the 4.05 region. The 1.05 and 2.09 fine-mapping populations can be further exploited to narrow the QTL confidence intervals. In the 1.05 population, variation in the amount of pollen shed during anthesis was consistently observed, while there was variation in drought tolerance in the 2.09 fine-mapping population. Not only are these traits interesting and agronomically important, but data can be collected to see if they co-segregate with the disease trait and map to the same location. These implications can be used to narrow candidate genes for the region and disease resistance mechanisms at the given locus.

In addition to fine-mapping, the 1.05 HIF population was treated with cercosporin in the greenhouse to test for differences in gene expression among detoxification-related candidate genes. A flavin-monooxygenase gene was found to differ in expression between the resistant and susceptible lines. HapMapV2 SNPs between the parental lines were identified for the candidate gene with significant differences in expression. One polymorphism was located in the TATA box in the promoter region. The flavin-monooxygenase gene should be sequenced among the 1.05 HIF lines used in the greenhouse experiment. Within-gene GWAS should also be performed using the HapMapV2 SNPs to implicate other causal polymorphisms. Further hypothesis testing of this sort can be done to elucidate the mechanisms underlying disease resistance.

As further means to better understand the defensive strategies of relevance to maize resistance to GLS, the host-pathogen interactions were analyzed at the microscopic level. Specifically, the details of fungal pathogenesis between the fleck and necrotic lesion stages of disease development were poorly understood, but potentially relevant to understanding pathogen offensive strategies and host resistance mechanisms. What exactly is *Cercospora zeae-maydis* doing during its extended latency period? Given the challenges of developing a GLS epidemic in the greenhouse, the field was an ideal space where large experimental designs could be implemented to explore pathogenesis. It was found that the pathogen appears to enter the maize leaf and grow along the minor venation, killing little by little along the way. The default understanding had been that the pathogen immediately begins to ramify between the minor veins without tracking the veins first. It would be ideal to use a greenhouse assay to analyze for differences between *C. zeae-maydis* and *C. zeina* pathogenesis styles, but this was not achieved.

Nonetheless, due to the size and design of the field experiment, significant associations between plant response phenotypes and genetic loci were identified. Good candidate genes were identified from significant GWAS hits within these loci that appear to be related to the plant response phenotype. The associations between these loci and host-pathogen interactions observed at the microscopic level can be confirmed by screening NILs for the plant response after exposure to the pathogens. In addition, a greenhouse setup can be used to establish which QTL, identified in a field with a mixed pathogen population, is effective in controlling either *C. zeae-maydis*, *C. zeina*, or both.

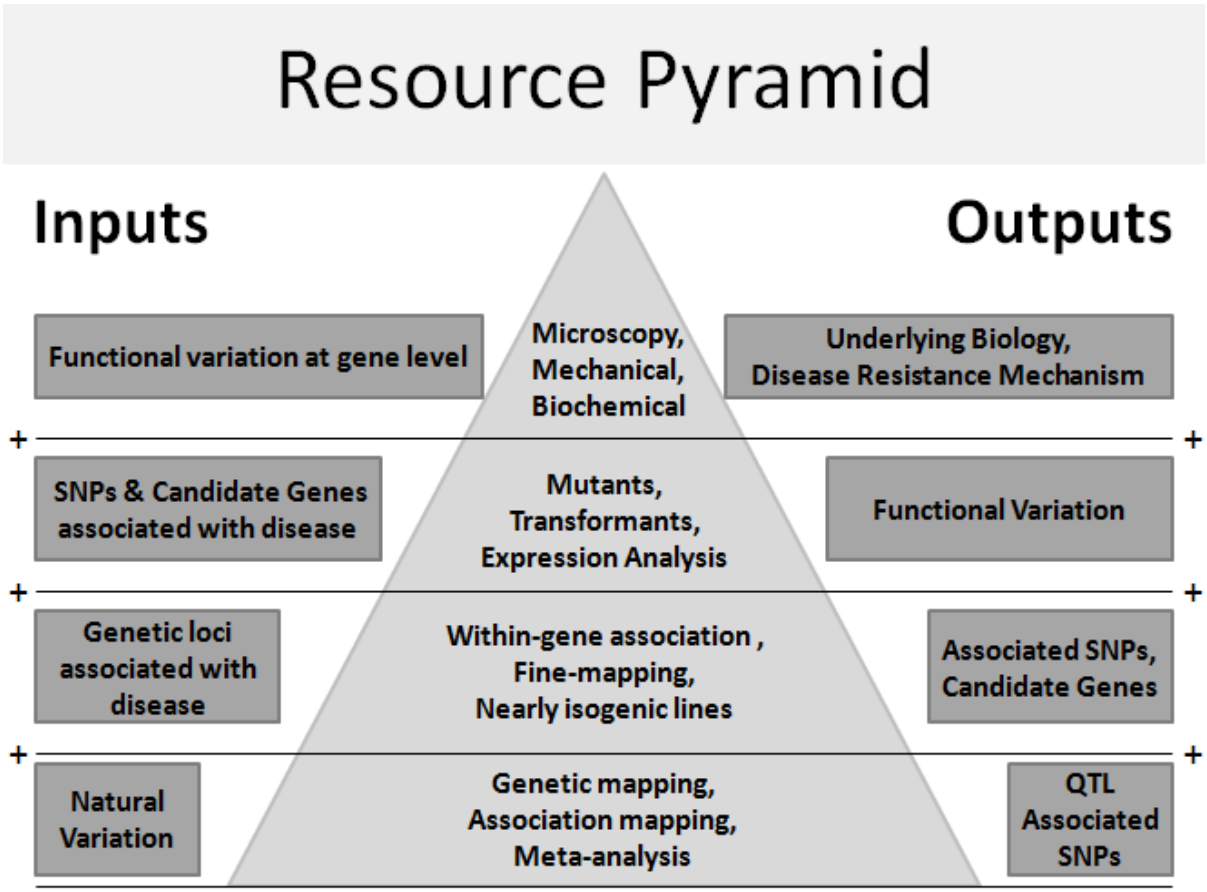

Figure 5.1. Resource pyramid utilized by the Rebecca Nelson Lab.

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