ABSTRACT

ZILA, CHARLES THOMAS. Utilizing Exotic Maize Germplasm to Improve Fusarium Ear Rot Resistance and Validate a Photoperiod Flowering Time QTL. (Under the direction of James B. Holland).

Tropical maize germplasm is a potential source of novel alleles to improve the disease resistance and yield potential of elite temperate maize. However, several obstacles exist when incorporating exotic germplasm into U.S. breeding programs, such as photoperiod sensitivity and other undesirable agronomic characteristics of the donor sources.

A few studies have assessed the performance of exotic Fusarium ear rot resistance alleles in adapted germplasm, but the relationship between resistance in inbred parent lines and their derived topcross hybrids is poorly understood. In this study, I evaluated a set of $BC_4F_{3:4}$ and $BC_4F_{4:5}$ lines derived from backcrosses of the highly resistant but unadapted inbred line GE440 to the elite proprietary inbred line FR1064. These lines were crossed to the susceptible tester NC478 to develop topcross F_1 hybrids. Fusarium ear rot resistance was evaluated on both the topcross hybrids and the inbreds *per se*, and agronomic performance of the hybrids was also evaluated. The results of the study indicate that disease resistance is not correlated to yield potential or flowering time, but *Fusarium* resistance of inbreds *per se* is strongly correlated with resistance in their topcross hybrids. This result suggests that indirect selection for inbred resistance is an effective way to improve hybrid resistance in this population. Furthermore, I was able to recover two lines with hybrid resistance superior to FR1064 but no different for grain yield, grain moisture, flowering time, height, or lodging.

Sensitivity to daylength is a major obstacle to using novel disease resistance alleles from unadapted germplasm. Locating and validating quantitative trait loci (QTL) conferring photoperiod sensitivity may give breeders the ability to effectively select against this trait

when incorporating exotic germplasm into temperate breeding programs. Traditional QTL studies relying on recombinant inbred lines (RILs) are often limited in their utility to precisely define QTL intervals due to limited recombination within the intervals. The use of heterogeneous inbred families (HIFs) is one method to overcome this obstacle. I evaluated a set of HIFs in both artificial and field conditions to validate and refine a previously identified photoperiod flowering time QTL interval on chromosome 10. Using these HIFs, I was able to narrow the region from 25.9 cM to less than 1 cM. A homolog of the rice photoperiod sensitivity gene *Ghd7* lies within this 1 cM interval.

Utilizing Exotic Maize Germplasm to Improve Fusarium Ear Rot Resistance and Validate a Photoperiod Flowering Time QTL

by
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DEDICATION

To my mom and dad, for encouraging me and supporting my education for the better part of two decades. Whether it was problems with a school project, a broken down Jeep Wrangler, or just needing a home-cooked meal, you kept the gears running smoothly from Westville to West Lafayette to Raleigh. Also, to Katie: through all the dirt, pollen, grease, and sunburns I accumulate throughout the day, you give me something to look forward to when I come home at night.

BIOGRAPHY

Charlie Zila was born in 1986 to Chuck and Jan Zila. Raised with his younger brother Jim on a small family farm in northwest Indiana, Charlie was guided in his early years by his father, a corn and soybean farmer, and his mother, a nurse. Upon graduation from high school in 2004, he attended Purdue University to complete a degree in Plant Breeding & Plant Genetics.

During his tenure at Purdue, Charlie was fortunate to have Dr. Herb Ohm as both his advisor and employer. While working in the Ohm Lab, he had the opportunity to conduct a small research project phenotyping a wheat population for Fusarium head blight resistance. In the summer of 2007, Charlie spent time working under the mentorship of Martin Medina at a Monsanto corn breeding station in Lebanon, IN. After graduation in May 2008, he would garner further industry experience under the mentorship of Dr. Maqsood Rehman at a Monsanto soybean breeding station in Oxford, IN.

Charlie moved with his fiancée Katie Kroening to North Carolina at the end of 2008. He started work on a Master's thesis in the spring semester with Dr. Jim Holland at North Carolina State University, working on validating a photoperiod flowering time response QTL in tropical corn and also breeding for resistance to Fusarium ear rot in corn. Upon graduation, Charlie will continue on in the Holland Lab for a PhD and to further unlock the mystery that is Fusarium ear rot resistance.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Literature Review

Breeding for Improved Resistance to Fusarium Ear Rot and Fumonisin Contamination

Fusarium ear rot, caused by the fungi *Fusarium verticillioides* (Sacc) Nirenberg (formerly *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg, is likely the most widespread disease afflicting corn ears in the United States and is present in many arable regions of the world (van Egmond et al., 2007). Colonization by *F. verticillioides* is prevalent in the warm dry conditions common in the southern United States and lowland tropics (Miller and Trenholm, 1994), although the pathogen can be found overwintering in plant residue wherever corn is grown (White, 1999). *F. verticillioides* may enter the cob by airborne conidia infecting down the silk channel or through feeding damage by corn earworm (Koehler, 1942); it can also infect the stalk and spread systemically to invade the ear through the ear shank (White, 1999). Symptoms of Fusarium ear rot can include localized white or pink mycelia growth on the ear, a "starbursting" pattern on affected kernels, and complete destruction of the kernels in severe cases (Eller et al., 2008).

In addition to causing yield losses due to decreased grain yield and quality, *F. verticillioides* and *F. proliferatum* produce a mycotoxin called fumonisin; the toxin has been associated with equine leukoencephalamalacia and porcine pulmonary edema and is also a suspected carcinogen (Miller and Trenholm, 1994; Marasas, 1996; Presello et al., 2008). In 2007, the European Union set regulations limiting fumonisin contamination to 1 part per million (ppm = $\mu g\ g^{-1}$) for human foods and to less than 0.2 ppm for infant foods; likewise, the Food and Drug Administration recommends no more than 2 ppm to 4 ppm of

contamination for many milled corn products (Center for Food Safety and Applied Nutrition, 2001; Kyprianou, 2007). However, fumonisin is often found in significant concentrations in grain and food products across the United States (Sydenham et al., 1991; Shelby et al., 1994). A study in North Carolina in 2000 tested 14 popular hybrids for fumonisin contamination; the most resistant and susceptible hybrids had fumonisin concentrations of 7 ppm and 177 ppm respectively, and most hybrids averaged between 20 ppm to 40 ppm (Heiniger and O'Neal, 2002).

Although levels of Fusarium ear rot and fumonisin contamination can be reduced indirectly by controlling disease vectors via pesticide applications or the use of transgenic pest resistance (Munkvold et al., 1997; Munkvold et al., 1999), the most effective method to control the disease is through the use of hybrids possessing genetic resistance. Resistance to Fusarium ear rot is highly quantitative (King and Scott, 1981; Nankam and Pataky, 1996; Clements et al., 2004), and no fully immune genotypes have been discovered. Furthermore, it has been difficult to incorporate resistance genes into elite germplasm (Bush et al., 2004). Robertson et al. (2006) estimated heritability on an entry mean-basis of 0.47 for ear rot resistance and 0.75 for fumonisin contamination resistance in a backcross population of (GE440 × FR1064) × FR1064; in addition, entry mean-basis heritability in a NC300 × B104 recombinant inbred line population was 0.80 for ear rot resistance and 0.86 for fumonisin content. Genotypic correlations between ear rot resistance and fumonisin content were 0.96 and 0.87, respectively. These high genotypic correlations indicate that visual selection for ear rot resistance should be effective in reducing susceptibility to fumonisin contamination in inbred populations. ELISA assays can be used to directly select for resistance to fumonisin

contamination; however, these assays are quite expensive and require more time to perform than visual ear rot scores. Although directly assessing fumonisin contamination levels would allow for more precise selection of fumonisin resistant lines, indirect selection for ear rot should improve the chances of recovering lines with lower fumonisin contamination susceptibility.

Previous studies have identified the inbred line GE440 as a potential source of resistance to Fusarium ear rot and fumonisin contamination (Clements et al., 2004; Robertson-Hoyt et al., 2006; Robertson et al., 2006). Unfortunately, GE440 has many undesirable agronomic characteristics, such as white kernel color, poor root lodging resistance, and overall poor yield. However, Robertson-Hoyt et al. (2007) conducted a study to examine the relationship between the disease resistance of GE440 and agronomic performance in a $BC_1F_{1:2}$ population using the susceptible commercial line FR1064 as the recurrent parent; they reported that disease resistance traits were not strongly correlated with agronomic traits. This suggests that when using GE440 as a source of resistance, combined selection for increased disease resistance and agronomic performance should be possible.

As only a few studies have been completed assessing the heritability and performance of Fusarium ear rot resistance in inbred populations *per se*, the correlation between resistance observed in inbred lines *per se* and resistance observed in topcross hybrids is not well understood (Robertson et al., 2006; Eller et al., 2010). This may be due in part to the difficulty in finding appropriate testers to evaluate resistant inbred lines. Studies conducted by Robertson-Hoyt et al. (2007) and Eller et al. (2010) evaluated topcross hybrids derived from crosses of Fusarium-resistant backcross lines to the commercial single cross F_1 tester

FR615×FR697; the former study found no significant variation for ear rot resistance among the hybrids tested, and the latter study found no significant variation for fumonisin contamination among the hybrids tested. Eller (2009) hypothesized that the use of FR615×FR697 as a tester was a hindrance to the expression of disease resistance variation among hybrids as FR697 was found to have a high level of resistance when evaluated as an inbred *per se*.

A primary objective of this thesis is to test the hypothesis that the high level of resistance in GE440 can be introgressed into the elite proprietary line FR1064 without having a negative effect on agronomic performance of topcross hybrids of the backcross-derived inbred lines. In addition, I am interested in determining the effectiveness of direct selection for improved resistance in the topcross hybrids compared to indirect selection for resistance in the inbred lines *per se*.

Backcrossing is most effective when the donor parent is not seriously deficient in traits other than those of interest (Fehr, 1987). Although GE440 is rather exotic and possesses many poor agronomic characteristics, it was chosen as the resistance donor because similar levels of resistance have not been identified in more adapted germplasm (Clements et al., 2004). Maize as a whole is a highly genetically diverse crop, but elite germplasm in the United States is derived from a relatively narrow genetic base, which may leave maize production vulnerable to new or emerging pests and pathogens (Smith, 2007). Gouesnard et al. (1996) and Goodman (2004) both highlighted the importance of incorporating tropical maize into temperate breeding programs as potential sources of novel

disease resistance and other valuable traits. However, tropical maize can be difficult to use in the United States because of photoperiod sensitivity (Goodman, 1999).

Validation of a Photoperiod Flowering Time Response QTL in Tropical Maize

Maize (*Zea mays* L. ssp. Mays) is a short-day crop that was domesticated in Mesoamerica roughly 9,000 years ago (Troyer, 1999; Matsuoka et al., 2002). As Native Americans adapted maize to more northern latitudes over the course of history, temperate races of maize became day neutral as photoperiod sensitivity was selected against (Gouesnard et al., 2002). Similarly, selection against photoperiod sensitivity is believed to have also occurred in both sorghum and pearl millet in Africa, both close relatives to maize (Craufurd et al., 1999; Saidou et al., 2009). In the United States, most current maize hybrids are descended from only a handful of temperate open-pollinated varieties which in turn were derived from a mixture of two of 150 to 230 known races (Goodman and Brown, 1988; Troyer, 1999). The relatively narrow genetic base of temperate germplasm may leave the U.S. maize crop genetically vulnerable to new pathogens or mutations in current pathogen races and biotypes (Smith, 2007). Tropical germplasm has often been cited as a source of genetic diversity to improve the narrow base of U.S. temperate germplasm (Gouesnard et al., 1996; Goodman, 2004).

One obstacle to incorporating exotic maize germplasm into U.S. breeding programs is photoperiod sensitivity, which is quite common in tropical maize lines. Photoperiod sensitive maize lines grow taller, have higher total leaf numbers, and flower significantly later when grown in the long-day environments typical of the corn growing regions of the United States as compared to the short-day environments nearer the equator (Allison and Daynard, 1979; Warrington and Kanemasu, 1983; Goodman and Brown, 1988). For maize, long-day environments are characterized by day lengths longer than 13 hours (Ellis et al.,

1992). Late flowering can have adverse effects on a maize crop. In the Midwest, rainfall typically becomes less prevalent in July; coincidentally, delayed pollination may make a crop more drought-susceptible and consequently reduce yield (Schweitzer, 2007; Jung and Müller, 2009). Furthermore, late flowering also delays seed set, grain filling, and grain maturation; all of these factors may contribute to reduced yield as well as delayed harvest and increased risk to a late season frost.

Significant variation for flowering time due to both photoperiod sensitivity and vernalization requirements have been observed and well documented in the model species *Arabidopsis thaliana*, a long-day plant (Caicedo et al., 2004; Shindo et al., 2005; Imaizumi and Kay, 2006). Caicedo et al. (2004) reported that two genes, *FRIDGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*), were responsible for vernalization response in Arabidopsis, and both have haplotype distributions that vary significantly across latitudes. *FLC* is an autonomous floral repressor gene that is activated by *FRI* but is inhibited under vernalization. In addition, Shindo et al. (2005) found that a loss-of-function in the *FRI* gene conferred a strong selective advantage by reducing flowering time. Correspondingly, Imaizumi and Kay (2006) showed that the floral promoter *CONSTANS* (*CO*), which is responsible for activation of the florigen gene *FLOWERING LOCUS T* (*FT*), is under circadian regulation and only activates under long daylengths. Genes under the control of the circadian clock (the "daily timer") respond to specific changes in daily daylengths (Samach and Coupland, 2000).

Flowering time in *Arabidopsis* has been shown to be controlled by four different pathways – the autonomous pathway, the gibberellin pathway, the vernalization pathway, and the photoperiod sensitivity pathway (Putterill, 2001; Simpson and Dean, 2002). Genes in the

autonomous pathway affect flowering time regardless of daylength. The gibberellin pathway promotes flowering by responding to the phytohormone gibberellic acid. As mentioned above, the vernalization pathway accelerates flowering time if the plant is exposed to a prolonged period of cold temperatures, and the photoperiod sensitivity pathway responds to daylength. All four pathways have been shown to actively interact with one another. *CO*, which is modulated by the circadian clock and daylength, activates the florigen gene *FT* under long daylengths, but *FT* can also be activated if vernalization inhibits *FRI* from activating *FLC* (which represses *FT*) or if the autonomous floral promoter *FCA* is up-regulated to overcome the action of *FLC* (Quesada et al., 2003; Caicedo et al., 2004; Searle et al., 2006; Imaizumi and Kay, 2006). Application of gibberellic acid can also induce flowering under the short-day conditions in which *CO* would not be activated, but the mechanisms of how gibberellic acid precisely interacts with the other pathways is currently poorly understood (Michaels and Amasino, 1999; Simpson and Dean, 2002).

Many of the genes identified controlling flowering time in *Arabidopsis* have been shown to be highly conserved across other species, although their specific functions seem to vary across species (Kojima et al., 2002; Izawa, 2007; Slotte et al., 2007; Hecht et al., 2007; Kwak et al., 2008). Kojima et al. (2002) noted that the rice gene *Heading date1* (*Hd1*) is a homolog of *CO*, even though *Hd1* is activated in short-day conditions while *CO* is activated in long-day conditions. Later, Izawa (2007) reported that the *Heading date3a* (*Hd3a*) gene in rice, an ortholog of *FT*, is controlled by the antagonistic actions of *Hd1* and *Early heading date1* (*Ehd1*). Furthermore, the authors reported that significant variation exists for both *Hd1* and *Ehd1* across latitudes. The functions of many other conserved genes in addition to *Hd1*

have diverged among taxa over the course of evolution. Although *FLC* and *FRI* are conserved in *Capsella bursa-pastoris* (a close relative to *Arabidopsis*), Slotte et al. (2007) discovered that the genes *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *TIMING OF CAB EXPRESSION1* (*TOC1*) were much more important for flowering time regulation than either *FLC* or *FRI*. Wheat, barley, and other temperate grasses lack homologues to *FLC*; instead, Yan et al. (2004) demonstrated that the gene *VRN2* was responsible for vernalization response in wheat, and a loss of function of *VRN2* leads to vernalization insensitivity.

Xue et al. (2008) identified *Ghd7* as a gene controlling a quantitative trait locus (QTL) responsible for delaying flowering time, increasing plant height, and increasing the number of spikelets per panicle in rice under long daylengths. *Ghd7* encodes a CCT (*CO*, *CO*-like, and *TIMING OF CAB1*) protein domain that has no sequence homology with *Arabidopsis*. *Ehd1*, which is essential to the activation of *Hd3a* and is downstream of *Ghd7* in the flowering time regulatory pathway, has recently been shown to be severely repressed by *Ghd7* under long daylengths (Itoh et al., 2010). Allelic variation for *Gdh7* is strongly associated with latitude, with stronger alleles of *Ghd7* being found more often in tropical southern Asia and weaker or non-functional variants being found more frequently in northern growing areas (Tsuji et al., 2011).

Many quantitative trait loci (QTL) have been identified contributing to flowering time in maize (Chardon et al., 2004), but only a few flowering time genes have been cloned, such as *DWARF8* (*D8*), *DELAYED FLOWERING1* (*DLF1*), *VEGETATIVE TO GENERATIVE TRANSITION1* (*VGT1*), and *INDETERMINATE1* (*ID1*) (Thornsberry et al., 2001; Colasanti et al., 2006; Muszynski et al., 2006; Salvi et al., 2007; Colasanti and Coneva, 2009).

Although variation in *D8* and *VGT1* differ among latitudes, neither gene is a photoperiod flowering time response gene, but rather they may be flowering time *per se* genes (Camus-Kulandaivelu et al., 2006; Ducrocq et al., 2008; Camus-Kulandaivelu et al., 2008). Moreover, *VGT1* appears to only account for a small proportion of flowering time variation in maize (Buckler et al., 2009).

QTL mapping studies are typically the first step in the process of uncovering genes for various traits. Even though over 100 genes conferring various sensitivities to photoperiod, light intensity, light quality, and temperature are suspected to contribute to flowering time in *Arabidopsis*, the large effect loci *FRI* and *FLC* are consistently detected across studies (Koornneef et al., 2004). A few large effect photoperiod-induced flowering time genes have been identified in sorghum that have some sequence homology with maize, suggesting that similar large effect photoperiod sensitivity QTLs may exist in maize (Lin et al., 1995; Childs et al., 1997).

Numerous previous studies have identified several QTL across the maize genome controlling photoperiod flowering time response (Koester et al., 1993; Moutiq et al., 2002; Chardon et al., 2004; Briggs et al., 2007; Wang et al., 2008; Buckler et al., 2009; Ducrocq et al., 2009; Coles et al., 2010). Across numerous populations and environments, the studies have consistently highlighted the importance of a region on chromosome 10 to flowering time and photoperiod response in maize. Analysis by Koester et al. (1993) found QTL affecting flowering time and plant height on chromosomes 1, 8, and 10, with the QTL located on chromosome 8 conferring photoperiod sensitivity. Moutiq et al. (2002) reported several QTL contributing to photoperiod flowering time sensitivity with a QTL on chromosome 10

having the largest effect (associated with 46% of the phenotypic variation). Chardon et al. (2004) reported a large number of flowering time QTL in a meta-analysis of many previous maize QTL mapping studies. Of the QTL reported, six had effects observed consistently across studies; one each localized to chromosomes 1 and 9 and two each localized to chromosomes 8 and 10. Briggs et al. (2007) found a large effect day-length responsive gene on chromosome 10 in a maize-by-teosinte population, while Wang et al. (2008) found QTL for flowering time, plant height, and leaf number photoperiod sensitivity clustered in bin 10.04. Buckler et al. (2009) reported many QTL, each with relatively small effects, for flowering time under long daylengths in the maize nested association mapping (NAM) population, but the QTL on chromosome 10 had the largest observed allelic effects.

Coles et al. (2010) mapped photoperiod response QTL in multiple temperate – by – tropical maize populations and reported that the chromosome 10 QTL had by far the largest effect on phenotypic variation for flowering photoperiod responses (R^2 = 0.39). Coles et al. (2010) mapped the chromosome 10 photoperiod QTL (referred to as *ZmPR4*) using four recombinant inbred line (RIL) populations: B73 × CML254, CML254 × B97, B97 × Ki14, and Ki14 × B73. B73 and B97 respond as temperate daylength-neutral inbred lines and CML254 and Ki14 as tropical photoperiod-sensitive inbred lines. Photoperiod sensitivity was assessed based on six phenotypic traits: days to anthesis (DTA), days to silking (DTS), plant height (PH), ear height (EH), leaf number (TLN), and anthesis-silk interval (ASI). *ZmPR4* was localized to a 25.9 centiMorgan (cM) interval of the linkage map between markers PZA00337.4 and umc1827 (between 86.3 Mbp and 108.2 Mbp on the AGPv1 physical map, http://maizesequence.org) on chromosome 10. These results are consistent

with those obtained by Briggs et al. (2007), Wang et al. (2008), and Ducrocq et al. (2009). However, the study provided evidence that there may be two distinct QTL in this region, one contributing to flowering time (mapped with very high resolution to a less than 1-cM region) and the other contributing to plant height and leaf number. In fact, the flowering time QTL localized to a 1 cM region within the Coles et al. (2010) interval, and this coincides with the Ducrocq et al. (2009) fine map position.

Ducrocq et al. (2009) mapped a photoperiod flowering time response QTL to a 170 kbp interval inside of the *ZmPR4* region in a near-isogenic line population. Within their interval, Ducrocq et al. (2009) identified a gene encoding a CCT protein domain that is homologous to *Ghd7*, a heading date regulator in rice (Xue et al., 2008).

Linkage-based QTL mapping typically does not permit the pinpointing of QTL regions to less than a 10-30 cM interval, which in turn restricts the effectiveness of marker assisted selection (MAS) for the QTL (Remington et al., 2001). The most critical limiting factor contributing to the relatively low resolution on QTL linkage maps is a lack of recombination events within the QTL region. Fine-mapping is often used to follow-up initial QTL studies by specifically selecting lines that result from recombination events within the target QTL region. To increase the effectiveness of fine-mapping, the genetic backgrounds of lines within a fine-mapping population should be genetically identical outside of the QTL interval, thereby converting a quantitative trait into a Mendelian trait (Alonso-Blanco and Koornneef, 2000). A line in which only one locus is segregating is known as a near-isogenic line (NIL); NILs are typically constructed through backcrossing, which is both time and resource intensive (Fehr, 1987; Tuinstra et al., 1997). An alternative to producing NILs through

backcrossing is to create heterogeneous inbred families (HIFs). In large RIL populations after several successive generations of inbreeding, individual RILs may still segregate at a few loci; HIFs are produced by identifying RILs that are still segregating only around the target QTL region and then subsequently selfing those lines to produce additional recombination within the QTL region (Tuinstra et al., 1997). A major goal of this thesis is to use the HIF approach to validate and further delineate the *ZmPR4* QTL region defined by Coles et al. (2010).

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CHAPTER 2: Relationships Between Inbred Line *per se* Fusarium Ear Rot Resistance, Testcross Resistance, and Testcross Agronomic Performance in Maize

Abstract

Fusarium ear rot, caused by *Fusarium verticillioides* (Sacc.) Nirenberg, is a disease prevalent in maize in the southeastern United States responsible for grain contamination with the mycotoxin fumonisin. Resistance to the disease is quantitatively inherited, and immune genotypes are not known to exist in elite US germplasm. In addition, little is understood about the relationship of *Fusarium* resistance in inbred seed parents and their derived hybrid cultivars, nor of the relationship between hybrid resistance and agronomic performance. In this study we examined resistance in a highly selected set of lines *per se* derived from the backcross of the unadapted resistant line GE440 into the agronomically superior propriety line FR1064, as well as topcrosses derived from the susceptible tester NC478 to the selected lines. We recovered 53 $BC_4F_{3:5}$ and $BC_4F_{4:6}$ lines with superior resistance to the recurrent parent FR1064, and one line with a level of resistance similar to GE440. Two lines were identified that produced topcross hybrids with significantly better resistance than the FR1064 topcross check but were the same for grain yield and six other agronomic characteristics. Strong genetic correlations were detected between inbred *per se* resistance and topcross resistance (95% for ear rot resistance and 90% for fumonisin accumulation), and higher heritabilities in the inbred lines compared to topcross hybrids suggest that indirect selection in inbred parents may be more effective at improving hybrid resistance than direct selection in hybrids if the correct tester is chosen. No significant correlations were detected between

resistance and grain yield, plant height, ear height, or flowering time, suggesting that resistance to *Fusarium* can be improved without adverse consequences on agronomic performance.

Introduction

Fusarium ear rot is the most widespread disease affecting corn ears in the United States. The disease is prevalent in dry high-humidity environments, such as the coastal region of North Carolina and other southeastern states (Miller and Trenholm, 1994). In addition to decreasing grain yields and test weights, the fungus also produces a mycotoxin called fumonisin that is associated with diseases in livestock and humans (Miller and Trenholm, 1994; Marasas, 1996; Presello et al., 2008). Corn produced in North Carolina often contains levels of fumonisin that exceed recommended FDA limits for livestock and human consumption (Center for Food Safety and Applied Nutrition, 2001; Heiniger and O'Neal, 2002).

The best management strategy for Fusarium ear rot is the use of resistant hybrids; however, most popular hybrids are susceptible to the disease (Bush et al., 2004). A few studies have shown that *Fusarium* resistance in inbred lines is moderately to highly heritable but is heavily environmentally influenced. Bolduan et al. (2009) reported heritabilities of 0.88 for fumonisin content and 0.65 for ear rot resistance in a random sample of 21 inbred lines from Germany and Canada. Based on the estimated variance components, Bolduan et al. (2009) also computed theoretical heritabilities for ear rot resistance for differing levels of the number of environments (±1 environment), number of replicates per environment (±1

replicate per environment), and number of plants evaluated within the experimental plot (10 plants versus 5 plants). Increasing or decreasing the number of replicates and the number of plants evaluated changed the estimated heritability relatively little ($\Delta h^2 \leq 0.04$ and $\Delta h^2 \leq 0.02$, respectively), while altering the number of environments had a much more dramatic effect ($\Delta h^2 \leq 0.10$).

Previous studies have identified inbred line GE440 as an excellent potential source of Fusarium ear rot resistance to improve parent inbred lines (Clements et al., 2004). In addition, previous research has shown that it is possible to backcross the resistance from GE440 into the adapted background of the proprietary inbred FR1064 (Robertson et al., 2006; Robertson-Hoyt et al., 2007; Eller et al., 2010). Robertson et al. (2006) evaluated 215 $BC_1F_{1:2}$ families derived from the first backcross of GE440 into the susceptible commercial line FR1064 and reported heritabilities of 0.75 for fumonisin content and 0.47 for ear rot resistance, indicating that significant genotypic variation existed within the population for disease resistance. Eller et al. (2010) evaluated 78 $BC_4F_{1:3}$ lines in 2007 that were advanced from the Robertson et al. (2006) $BC_1F_{1:2}$ families, and 19 of the most resistant $BC_4F_{1:3}$ lines in 2008. A combined analysis revealed significant genetic variation for both ear rot resistance and fumonisin content among the $BC_4F_{1:3}$ lines. Five $BC_4F_{3:4}$ sublines were derived for each of four of the top $BC_4F_{1:3}$ lines and were also tested for disease resistance in 2008; significant variation was observed for Fusarium ear rot among sublines derived from a common $BC_4F_{1:3}$ parent. In addition, significant genotypic variation was observed among the 19 selected $BC_4F_{1:3}$ lines for several agronomic traits, including plant height, ear height, and flowering time. These results indicate that even when dealing with a highly selected subset

of lines from the original Robertson et al. (2006) study, variation among and within the selected lines is known to exist.

The primary objective of this study was to incorporate the *Fusarium* resistance of GE440 into the elite background of FR1064 while maintaining high agronomic performance by further exploiting within line variation. The specific objectives of this study were the following goals: (i) identify highly uniform and homozygous BC_4-derived lines with the greatest resistance to Fusarium ear rot and fumonisin contamination derived by Eller et al. (2010), (ii) assess if indirect selection for *Fusarium* resistance in the inbred lines *per se* is an effective strategy for improving resistance in testcross hybrids, and (iii) determine if improved resistance has a negative impact on agronomic performance of topcross hybrids.

Materials and Methods

Population Development

The GE440 × FR1064 backcross population (referred to as GEFR, for short) was developed by crossing GE440 (derived from the open-pollinated variety Hastings Prolific) to the susceptible inbred line FR1064 (an improved B73 type) and then backcrossing for four generations (Figure 2.1). Robertson et al. (2006) evaluated 215 $BC_1F_{1:2}$ families for *Fusarium* resistance, and ten families with the lowest fumonisin content were chosen for further backcrossing to FR1064 using a modified single seed backcrossing scheme. Up to 100 unique BC_2F_1 families were created, and large family sizes were used to advance the population for two more backcrosses. No intentional selection was applied at any backcrossing step. In the summer of 2007, Eller et al. (2010) evaluated a set of 79 $BC_4F_{1:3}$

families for both ear rot resistance and fumonisin content. Superior families were identified and selected primarily on the basis of ear rot resistance; all lines were also self-pollinated and individual ears harvested. Between five and ten $BC_4F_{3:4}$ ear rows from the selected $BC_4F_{1:3}$ families were evaluated for disease resistance and subsequently selfed in the summer of 2008. Best linear unbiased predictors (BLUPs) were calculated to estimate ear rot scores and fumonisin contents for both years of data (Eller et al., 2010).

In early spring 2009, new selections were made to generate lines for topcrossing and disease evaluation. Individual ears were selected using the 2007 and 2008 BLUP data; more emphasis was given to fumonisin content rather than ear rot scores. The best twelve $BC_4F_{1:3}$ families from Eller et al. (2010) were identified, and ten new lines per each family were generated from $BC_4F_{4:5}$ or $BC_4F_{3:4}$ single ear stocks. Eight families had $BC_4F_{4:5}$ seed available, and four families had only $BC_4F_{3:4}$ seed available. In total, 120 new lines were generated (80 $BC_4F_{4:5}$ lines and 40 $BC_4F_{3:4}$ lines). The $BC_4F_{1:3}$ GEFR lines evaluated by Eller et al. (2010) were expected to be fixed homozygous for FR1064 alleles at 93.75% of all loci and segregating for FR1064 and GE440 alleles at the other 6.25% of loci. By comparison, the $BC_4F_{4:5}$ and $BC_4F_{3:4}$ lines in this study are expected to be more uniform. Within a $BC_4F_{3:4}$ line, approximately 96.09% of loci are expected to be fixed for FR1064 alleles, 2.34% are expected to be fixed for GE440 alleles, and the other 1.56% loci are expected to be segregating. Within $BC_4F_{4:5}$ lines, 96.5% of loci are expected to be fixed for FR1064 alleles, 2.73% are expected to be fixed for GE440 alleles, and the other 0.78% are expected to be segregating.

Field Evaluation of Inbred GEFR Lines – Experimental Design

In 2009, the 80 $BC_4F_{4:5}$ and 40 $BC_4F_{3:4}$ experimental GEFR lines were evaluated for disease resistance at four locations in North Carolina (Clayton, Jackson Springs, Kinston, and Lewiston-Woodville). In addition to the experimental lines, the entry list included six instances of FR1064 and two instances of GE440 to bring the total number of entries to 128. The experimental plots were single rows arranged in a randomized 8 × 16 α-lattice block design with two replications in each of the four locations for a total of 1024 experimental plots. Based on results from the 2009 field season, 60 experimental GEFR lines (including the 50 most resistant and 10 most susceptible to Fusarium ear rot) were selected to be tested again in 2010. Not enough seed remained from the $BC_4F_{4:5}$ and $BC_4F_{3:4}$ stocks for testing, so $BC_4F_{4:6}$ and $BC_4F_{3:5}$ seed from the summer 2009 nursery was used instead. The same four locations used in 2009 were chosen again to evaluate the selected subset of lines. In addition to the 60 selected GEFR lines, the entry list included six instances of FR1064, two instances of GE440, and two instances of NC478 to bring the total number of entries to 70. NC478, the topcross parent for the hybrid study, was included in this year's study to confirm its susceptibility to Fusarium ear rot. The experimental plots were single rows arranged in a randomized 7 × 10 α-lattice block design (Patterson and Williams, 1976) with two replications in each of the four locations for a total of 560 experimental plots. At harvest, the Jackson Springs experimental plots were abandoned due to poor seed set due to severe drought stress.

Field Evaluation of Testcross GEFR Hybrids

In the summer nursery 2009, each of the 120 GEFR lines as well as FR1064 and GE440 were topcrossed to NC478 (using NC478 as a female parent) to produce F_1 hybrid seed. NC478 is a public inbred line with a mixture of temperate non-Stiff Stalk and tropical pedigree origin ([(PH X105A × H5)Agroceres 155] × NC262A; http://www.cropsci.ncsu.edu/maize/germplasm_450_498.html) with good combining ability with Stiff Stalk maize lines, but a high level of susceptibility to Fusarium ear rot observed in North Carolina nursery plots (Major Goodman, personal communication). Based on the inbred line selections from the 2009 inbred disease study, 60 testcross hybrids were chosen to evaluate that corresponded to the selected inbred GEFR lines. The final entry list included two instances of the NC478×FR1064 testcross, one instance of the NC478×GE440 testcross, and one instance each of the commercial check hybrids Pioneer brand 31G66 and DeKalb brand DK697 to bring the total number of entries to 64.

The testcross hybrids were evaluated for disease resistance in each of the four locations chosen to evaluate the GEFR inbred lines *per se* (Clayton, Jackson Springs, Kinston, and Lewiston-Woodville). The experimental plots were single rows arranged in a randomized 8 × 8 α-lattice block design with two replications in each of the four locations for a total of 512 experimental plots. Each plot was planted with 25 kernels and was then thinned to a uniform stand of 20 plants.

The testcross hybrids were also evaluated separately for yield and agronomic characteristics. The same locations used for the hybrid disease study were also used for the hybrid yield trial. The experimental plots were double rows arranged in a randomized 8 × 8

α-lattice block design with two replications in each of the four locations for a total of 512 experimental plots. Each row of the plot was planted with 25 kernels, and the whole plot was then thinned to a uniform stand of 40 plants.

Inoculation Technique

The inoculation method used in this study is described in detail by Robertson et al. (2006). Six isolates of *Fusarium verticillioides* (referred to as NC-i6, NC-i7, NC-i9, NC-n16, NC-n17, and NC-n22) were used for inoculation; these isolates were originally collected and identified by Eller (2009) from diseased maize fields in North Carolina. All isolates used were selected based upon their ability to cause ear rot and produce high levels of fumonisin; these isolates can be obtained through the Fusarium Research Center collection (http://frc.cas.psu.edu/). The six isolates were cultured independently on a medium of potato dextrose agar (Fisher Scientific, Pittsburgh, PA). Conidia were collected from the cultures by rinsing each Petri plate with distilled water and using a paint brush to help loosen conidia. After straining the conidia suspension of the six combined isolates through cheesecloth to remove agar debris, the solution was diluted to approximately 2×10^6 conidia mL^{-1}.

The inoculation technique was the same for both inbred and hybrid disease studies, as well as between 2009 and 2010. Each experimental plot was inoculated twice. The first inoculation occurred approximately one week after silking by injecting 5 mL of the conidia suspension into the silk channel of the primary ear of the first twelve plants in each experimental plot. The second inoculation occurred approximately two weeks after silking by injecting 5 mL of the conidia suspension directly into the base of the ear of the same

plants inoculated during the first week. Direct correspondence with station managers at distant locations allowed for proper timing of inoculations at those locations. Injections were administered using an Allflex draw-off syringe (Allflex USA, DFW Airport, TX) fitted with a 16-gauge veterinary needle. Syringes were connected to a modified Solo backpack sprayer (Solo, Newport News, VA) to hold the conidia suspension; one drop of Tween-20 was added to each liter of conidia suspension to break the surface tension of the suspension.

Phenotypic Trait Measurements

During the growing season, percent stand, anthesis date and silking date (Clayton only), plant height (cm), ear height (cm), root lodging, and stalk lodging information were collected for each plot in all experiments. Anthesis dates and silking dates were recorded as the date when 50% of plants within the plot were shedding pollen or silking, respectively. Plant and ear heights were measured on a random sample of three plants within each plot. Plant height was measured as the height (cm) from the soil to the topmost node (directly below the tassel); ear height was measured as the height from the soil to the node connected to the shank of the primary ear. Root lodging and stalk lodging were recorded as the number of plants lodged with intact stalks and the number of plants lodged with broken stalks, respectively. Root-lodged plants are defined as plants leaning greater than 30% from vertical but with intact stalks, while stalk-lodged plants are defined as plants with stalks broken or cracked below the primary ear.

At maturity, ten of the twelve inoculated ears were collected by hand from each experimental plot of the inbred and hybrid disease evaluation trials. Each of the ten ears

within each experimental plot was visually scored for Fusarium ear rot symptoms. Scores were assigned to each ear in increments of 5% from 0% to 100% diseased based on the percentage of the ear presenting disease symptoms.

At maturity, each plot in the hybrid yield trials was mechanically harvested and yield and grain moisture content (%) were recorded.

Fumonisin assay

Ears that were visually scored for ear rot were bulked together within each plot and shelled. The bulk plot sample was then ground into a fine powder using a Romer II Series Mill (Romer Labs, Union, MO). A 20-g sample of ground grain from each plot was then used to assay for fumonisin content. The amount of fumonisin B1 present in each plot sample was tested using Diagnostix fumonisin ELISA assay kits (Diagnostix, Mississauga, ON, Canada).

Statistical Methods – Inbred and Hybrid Disease Evaluations

The MIXED procedure in SAS Version 9.2 software (SAS Institute Inc, 2010) and ASReml 3.0 software (Gilmour et al., 2009) were used to analyze percent ear rot and fumonisin content data collected from the two field seasons (2009 and 2010) of the experiment. The inbred and hybrid disease evaluations were both analyzed separately in the same fashion. In total, four locations from 2009 and three locations from 2010 were analyzed for the inbred study, and four locations from 2010 were analyzed for the hybrid study. In both studies, squared residuals were correlated with predicted values for both ear

rot score and fumonisin content, violating the usual assumptions of the analysis of variance. Therefore, all analyses were performed on a natural logarithmic transformation on both responses.

Each location was analyzed separately using SAS PROC MIXED to determine the best fitting model at each location for inbred *per se* ear rot, inbred *per se* fumonisin content, topcross ear rot, and topcross fumonisin content. Random effects were chosen using Akaike's Information Criterion (Akaike, 1974) to compare four different models at each location: a simple model fitting only entry as a fixed effect, a randomized complete block model adding a random replication effect, an α-lattice model adding random replication and block within replication effects, and an anisotropic correlated errors model (Brownie et al., 1993). Once the random effects were selected, fixed first, second, third, and fourth-order polynomial trend terms in both the row and column field directions were added to the model (Kirk et al., 1980; Tamura et al., 1988; Bowman, 1990). Only trend terms significant at $P = 0.01$ were selected to remain in the model. All models were weighted by the number of ears scored within each plot, and all fixed effects were tested using a Kenward-Roger adjustment for degrees of freedom (Kenward and Roger, 1997).

After the best model was determined in each location, three combined analyses were performed on the inbred study using ASReml to nest the various spatial models within each location. 2009 and 2010 locations were combined and analyzed separately, and then the two years were combined to create a full dataset. Only one combined analysis was needed for the hybrid study due to the fact it was only evaluated in 2010. For the purpose of simplicity in the across year analysis, specific year × location combinations were considered unique

environments. The model in each case consisted of a fixed entry effect, random location and entry × location interaction effects, a heterogeneous error variance structure within each location, and the various spatial effects nested within their respective locations. The spatial effects modeled within each location for the inbred study can be found in Table 2.1, and the spatial effects modeled within each location for the hybrid study can be found in Table 2.2. Least square means were computed for entries across locations.

Heritabilities of ear rot resistance and fumonisin content were calculated on a both a plot-basis and an entry mean-basis (Holland et al., 2003) for both the inbred and hybrid studies. The same models as above were used except that entry was considered a random effect sampled from the potential population of all possible selected GEFR inbred lines in order to obtain a variance component for entry. Heritability on a plot-basis was estimated as

$$\widehat{H}(per-plot\ basis) = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \hat{\sigma}_{GL}^2 + \hat{\sigma}_\varepsilon^2}$$

Heritability on an entry mean-basis was estimated as

$$\widehat{H}(entry\ mean\ basis) = \frac{\hat{\sigma}_G^2}{\hat{\sigma}_G^2 + \frac{\hat{\sigma}_{GL}^2}{l} + \frac{\hat{\sigma}_\varepsilon^2}{lr}},$$

where $\hat{\sigma}_G^2$ is the estimated genetic variance, $\hat{\sigma}_{GL}^2$ is the estimated entry × location variance, $\hat{\sigma}_\varepsilon^2$ is the estimated residual variance, *l* is the number of locations, and *r* is the number of replications per location.

Genotypic correlations between Fusarium ear rot resistance and fumonisin content for both the inbred and hybrid studies were computed by multivariate mixed models analyses in

ASReml fitting entry and location as random effects. The genotypic correlation ($\hat{r}_{gij}$) between rot resistance and fumonisin was estimated as

$$\hat{r}_{gij} = \frac{\hat{\sigma}_{Gij}}{\hat{\sigma}_{Gi}\hat{\sigma}_{Gj}},$$

where $\hat{\sigma}_{Gij}$ is the estimated genotypic covariance between rot resistance and fumonisin content, $\hat{\sigma}_{Gi}$ is the estimated genotypic standard deviation for rot resistance, and $\hat{\sigma}_{Gj}$ is the estimated genotypic standard deviation for fumonisin content (Holland, (2006).

Lastly, a multivariate mixed model in ASReml was used to compute a genotypic correlation between inbred rot resistance and hybrid rot resistance using individual location LSMEANs for the inbreds and their corresponding testcross hybrids. The LSMEANs used to calculate the correlation are from only the locations in which both the inbreds and the hybrids were evaluated (Clayton 2010, Kinston 2010, and Lewiston-Woodville 2010). The model statement in ASReml was specified as

$$Y_{INB}, Y_{HYB} = Trait + Trait.Line + Trait.Location,$$

where Y_{INB} is the inbred *per se* rot score variate, Y_{HYB} is the hybrid rot score variate, $Trait$ fits the mean for both disease variates, $Trait.Line$ fits the random genotype effect for each disease variate, and $Trait.Location$ fits the random location effect for each disease variate. Each term in the model was associated with one variance component for each trait and a covariance component for the two traits. A correlation between inbred fumonisin content and hybrid fumonisin content was also calculated in the same way as rot resistance.

Statistical Methods – Hybrid Yield Evaluations

PROC MIXED in SAS Version 9.2 (SAS Institute, Cary, NC) and ASReml (VSN International, Hemel Hempstead, UK) were used to analyze the yield data collected from the three locations of the 2010 hybrid yield trial. Before analysis, yield measurements were adjusted for ears dropped during harvest and to a common moisture content of 15.5%. Yields were then converted to the more intuitive unit of bushels of grain per acre (bu A^{-1}). Within-location models were selected for grain yield in the same way as described in the inbred disease study. Additionally, the combined analysis and least square means were computed in the same way as the inbred study. Table 2.3 describes the spatial terms nested within each location of the hybrid yield trial.

Other agronomic traits (plant height, ear height, percentage of erect plants calculated by subtracting the root lodging and stalk lodging scores from the stand count and then dividing by stand count, days to anthesis, days to silking, and moisture at harvest) were analyzed by fitting a model including environment, replication within environment, block within replication, genotype, and genotype-by-environment interaction. Genotypes were considered fixed effects and all other effects were random. Heterogeneous error variances were modeled. Although spatial models can produce least square means with greater precision than traditional block designs, choosing the correct spatial model can take considerable time for complicated field designs and be computationally intensive (Brownie et al., 1993; Casanoves et al., 2005). Therefore, the traits deemed to be of greatest importance in this study (inbred ear rot, inbred fumonisin content, hybrid ear rot, hybrid fumonisin content, and hybrid grain yield) were analyzed using spatial models, while the simpler α-

lattice model was used to analyze other agronomic characters. Least square means for hybrid the CORR procedure in SAS was used to estimate correlations between agronomic traits and disease traits in the GEFR hybrids (checks were dropped from the data set). The data set used for PROC CORR consisted of the least square means from the combined spatial analyses across locations of ear rot resistance, fumonisin content, and yield in addition to least square means generated for the other six agronomic traits from the α-lattice analyses.

Results and Discussion

Inbred Disease Evaluations

Significant ($P < 0.001$) genotypic variation for both ear rot resistance and fumonisin content was observed among inbred lines in the summer 2009 evaluation. The mean ear rot observed in the 120 experimental GEFR entries ranged from 5.0% to 63.4% with an overall mean of 16.6%. The mean fumonisin content observed in the experimental entries ranged from 5.7 ppm to 60.0 ppm with an overall mean of 21.2 ppm. FR1064, the susceptible recurrent parent, exhibited ear rot severity and a fumonisin content of 30.7% and 33.6 ppm, respectively. GE440, the resistant donor parent, exhibited ear rot severity and a fumonisin content of 0.8% and 6.3 ppm, respectively. Heritability on an entry mean-basis was 49% for rot resistance and 34% for fumonisin content with a genetic correlation of 74% between the two traits.

After analysis of the summer 2009 data, the most resistant 50 lines were selected for further testing in 2010, as well as ten of the most susceptible lines. Significant genotypic variation was observed among entries in the summer 2010 study for both ear rot resistance

and fumonisin content ($P < 0.001$ for both). The 2010 field season proved to be more conducive to *Fusarium* infection and development than the previous year. The mean ear rot observed in the 60 experimental GEFR entries ranged from 15.8% to 71.9% with an overall mean of 37.8%. The mean fumonisin content observed in the experimental entries ranged from 20.8 ppm to 130.7 ppm with an overall mean of 55.4 ppm. FR1064 displayed ear rot severity of 68.5% and a fumonisin content of 75.9 ppm, while GE440 displayed ear rot severity of 1.2% and a fumonisin content of 14.7 ppm. In addition, NC478, the tester for the hybrid evaluations, had an ear rot severity of 84.6% and a fumonisin content of 151.9 ppm. Heritability on an entry mean basis was 63% for ear rot resistance and 49% for fumonisin content with a genetic correlation of 81% between the two traits.

Combining data across the two field seasons resulted in improved heritability estimates for the two disease characteristics. Entry mean heritability based on the combined data set was 64% for ear rot resistance and 49% for fumonisin content with a genetic correlation of 74% between the two traits. Mean ear rot resistances across years ranged from 8.4% to 89.6% among the original 120 experimental entries with an overall mean of 24.5% (Table 2.4). Likewise, the mean across years and locations for fumonisin content ranged from 9.7 ppm to 81.5 ppm with an overall mean of 34.1 ppm (Table 2.4). The two-year means for ear rot and fumonisin content in FR1064 were 43.2% and 47.9 ppm, respectively, while the means for GE440 were 0.9% and 9.1 ppm, respectively. Of the original 120 experimental entries, 53 had least square means for ear rot resistance and fumonisin content that were significantly better than FR1064. One experimental line, GEFR400-9-5-2-3-8, had a least square mean for fumonisin content (9.7 ppm) very similar to that of GE440 (9.1 ppm;

Table 2.4). Further selections within this subset of lines will be based on the combined data set due to the robust nature of the estimates compared to either field season alone.

Hybrid Disease Evaluation

Significant genotypic variation was observed among the testcross hybrids for ear rot resistance and fumonisin content in the summer 2010 study ($P < 0.001$ for both). Of the 60 GEFR inbred lines evaluated in the same field season, 59 had corresponding topcross hybrids evaluated for disease; one line did not have sufficient testcross seed for inclusion in the study. The evaluated testcross hybrids had ear rot severity scores ranging from 7.3% to 17.4% with an overall mean of 11.7% (Table 2.4). By comparison, the NC478×FR1064 topcross hybrid had an ear rot score of 15.7% while the NC478×GE440 topcross hybrid had a score of 3.7%. Pioneer hybrid 31G66 is indicated as being *Fusarium* tolerant (www.pioneer.com); it had an ear rot score of 10.8%. Fumonisin contents varied from 10.3 ppm to 34.1 ppm in the GEFR testcross hybrids with an overall mean of 20.5 ppm (Table 2.4). Fumonisin contents for NC478×FR1064, NC478×GE440, and Pioneer 31G66 were 27.1 ppm, 7.3 ppm, and 14.5 ppm, respectively. Heritability on an entry mean basis was 16% for ear rot resistance and 18% for fumonisin content with a genetic correlation of 88% between the two traits.

A genetic correlation was computed between inbred disease resistance and the disease resistance of their derived testcross hybrids. The correlation between inbred ear rot resistance and testcross ear rot resistance was 95%, while the correlation between inbred fumonisin content and testcross fumonisin content was 90%. These relatively high

correlations combined with moderate inbred entry mean heritabilities suggest that indirect selection for inbred *Fusarium* resistance is likely more effect at improving hybrid resistance rather than direct selection for hybrid resistance. The efficiency of response to indirect selection versus direct selection for ear rot resistance can be given as

$$\frac{CR_{HYB}}{R_{HYB}} = \hat{r}_{ROT}\sqrt{\frac{\widehat{H}_{INB}}{\widehat{H}_{HYB}}},$$

where CR_{HYB} is the correlated response of hybrid resistance by selection on inbred *per se* resistance, R_{HYB} is the direct response of selection on hybrid resistance, $\hat{r}_{ROT}$ is the genetic correlation for ear rot resistance between the inbred lines are their derived hybrids, $\widehat{H}_{INB}$ is the entry mean heritability of inbred rot resistance, and $\widehat{H}_{HYB}$ is the entry mean heritability of hybrid rot resistance (Falconer and Mackay, 1996). The efficiency of response to indirect selection for fumonisin content is estimated in the same fashion. Efficiencies greater than one indicate that indirect selection for inbred *per se* resistance is more effective than directly selecting for hybrid resistance. Using the calculated correlations and entry mean heritabilities from only the summer 2010 data, the efficiency of indirect selection for ear rot resistance is 1.88 and for fumonisin content is 1.49. Both of these values indicate that the end goal of improving hybrid *Fusarium* resistance could be more readily achieved by selecting for resistance in the inbred parents. The high relative efficiency of indirect selection based on inbred *per se* information arises due to the high genetic correlation between inbred and topcross hybrid values and the substantially greater variation observed among inbred lines compared to among their topcross hybrids (Figures 2.2 and 2.3).

Hybrid Yield Evaluation

Although conducive to *Fusarium* development, the 2010 field season was poor for corn grain yield. Temperature measurements collected at Raleigh-Durham International airport (RDU) by the National Weather Service marked June 2010 as the hottest June on record since measurements began in 1944, and July 2010 was the second hottest July on record (http://www.weather.gov). Many locations received little rainfall during the crucial flowering months of June and July. Lewiston-Woodville was hit particularly hard by drought conditions. Precipitation measurements collected at the Peanut Belt Research Station by the North Carolina State Climate Office indicate that this location received only 5.7 cm of rain in June (compared to a 30-year average of 8.0 cm), and July brought only 3.2 cm of rain (compared to a 30-year average of 8.8 cm) (http://www.nc-climate.ncsu.edu). Even with the difficulties of the field season, significant genotypic variation was detected for yield among the testcross hybrids ($P < 0.001$). Across-location means for the 59 testcross hybrids ranged from 105.5 bu A^{-1} to 137.6 bu A^{-1} with an overall mean of 118.9 bu A^{-1} (Table 2.4). The NC478×FR1064 and NC478×GE440 check hybrids had mean yields that differed significantly at $P = 0.05$ of 130.7 bu A^{-1} and 109.6 bu A^{-1}, respectively. Thirty-three of the 59 testcross hybrids had mean yields that did not differ significantly from NC478×FR1064 at $P = 0.05$. Commercial hybrids 31G66 and DK697 had mean yields of 138.2 bu A^{-1} and 147.0 bu A^{-1}, respectively.

Significant genotypic variation among hybrids was also observed for days to anthesis ($P = 0.0003$), days to silking ($P = 0.0231$), plant height ($P < 0.0001$), ear height ($P < 0.0001$), and grain moisture ($P = 0.0004$). No significant variation was observed for percentage of

erect plants ($P = 0.4015$). No significant correlations were observed between either ear rot or fumonisin contamination resistance and days to anthesis, days to silking, plant height, ear height, or yield (Table 2.5). The lack of significant correlations indicates that it should be possible to recover a number of lines from the experiment that have high disease resistance without the excessive plant or ear height, late maturity, high lodging, or low yield potential of the donor parent of resistance, GE440.

Conclusions

The results of the inbred disease study indicate that selection has been successful in recovering advanced backcross lines with significantly better ear rot resistance and fumonisin contamination levels than the recurrent parent FR1064. Fifty-three of the original 120 selections had ear rot scores and fumonisin contents that were both significantly better than FR1064. Additionally, we have been able to identify one inbred line (GEFR400-9-5-2-3-8) that not only has ear rot resistance significantly better than FR1064 but also consistently displays fumonisin content levels that do not differ significantly from the resistance donor parent GE440. The relatively high number of resistant lines remaining in testing allows for more flexibility in selecting on other non-disease related traits, such as agronomic characteristics like plant height and flowering time.

A few of the more resistant lines also have closely related susceptible sister lines (derived from the same $BC_4F_{1:3}$ family) that could be potentially valuable in developing a high resolution mapping population to pinpoint resistance QTL. All sister lines that were derived from the same BC_4F_1 plant are expected to be segregating at no greater than 6.25%

of loci between them. GEFR400-9-5-2-3-8 and its sister line GEFR400-9-5-2-3-5 have the largest contrast in *Fusarium* resistance phenotypes in this study with ear rot scores and fumonisin contents that differ by 31.4% and 47.3 ppm, respectively (Table 2.6). Other phenotypically distinct sister line pairs detected in this study are also presented in Table 2.6.

Previous studies of the relationship between inbred resistance per se and resistance in topcross hybrids were confounded by generally low disease levels and by partial resistance of the tester. Eller et al. (2010) found no significant relationship between inbred per se resistance and hybrid resistance, concluding that the desired gains in hybrid resistance were not achieved by indirect selection on inbred per se resistance. However, Eller (2009) believed that these findings were the result of a poor choice of tester. Moderate levels of resistance in the FR615×FR697 single cross tester that was used in that study were hypothesized to have masked resistance coming from the GEFR side of the testcross. Moreover, Clements et al. (2004) found significant variation among testcross hybrids when using FR1064 as a tester for non-Stiff Stalk experimental lines. We believe that the use of NC478 as a tester in this study allowed for effective evaluation of the experimental lines as significant variation for resistance was observed in the hybrid disease study, although observed variation in the testcrosses was less than that of the inbred lines *per se* (Figures 2.2 and 2.3). Relatively high correlations between inbred *per se* and hybrid ear rot and fumonisin content scores (0.95 and 0.90, respectively), combined with higher inbred entry mean heritability compared to hybrid entry mean heritability, indicate that in this population indirect selection on inbred *per se* resistance should be more effective at improving testcross hybrid resistance than directly selecting for resistance in the hybrids.

The results of this study indicate that selecting for resistance to *Fusarium* should not unduly affect agronomic performance in testcross hybrids. No significant correlations were revealed between resistance and flowering time, yield, plant height, or ear height. These results are consistent with both Robertson-Hoyt et al. (2007) and Eller et al. (2010). Robertson-Hoyt et al. (2007) studied the relationship between *Fusarium* resistance and agronomic traits in testcrosses using $BC_1F_{1:2}$ ancestors of the GEFR population in this study. They found no significant relationship between resistance and flowering time or ear height. Additionally, no relationship was detected between grain yield and fumonisin content, and only a small relationship (r=0.29) was detected between grain yield and ear rot resistance. Eller et al. (2010) examined relationships between resistance and agronomic characteristics in the inbred lines *per se*; no significant correlations were identified between resistance and plant height, ear height, or flowering time. All of the preceding results indicate that it is possible to introgress *Fusarium* resistance from unadapted sources without negatively impacting hybrid grain yield and agronomics. Two inbred lines, GEFR399-1-5-1-3-4-2 and GEFR399-2-10-6-2-5-1 (Table 2.4), have been recovered that have ear rot scores and fumonisin contents less than FR1064 (on both an inbred *per se* and topcross basis) and do not differ significantly than the FR1064 topcross check for either grain yield or any of the other six agronomic traits evaluated. Several other inbred lines have been identified with *Fusarium* resistance consistently superior to FR1064 but may be lacking in one or a few hybrid agronomic traits (Table 2.4). Given the nature of the 2010 field season, combined with the fact that the topcross hybrids have only been evaluated in one year, at minimum one more year of observation will be necessary to definitively determine whether or not these

other GEFR candidates also possess improved *Fusarium* resistance with key agronomic traits similar to (or even better than) FR1064.

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Table 2.1. Traditional block effects and spatial effects fit in final selected analysis model within each environment of the GEFR inbred disease study for ear rot scores and fumonisin content. R^n and C^n denote *n*-th order row and column polynomial trend terms, respectively.

Trait	Year	Location	Block or spatial effect(s)
Ear rot	2009	Clayton	rep, block(rep), C^3
Ear rot	2009	Jackson Springs	anisotropic correlated errors, R^1, C^2
Ear rot	2009	Kinston	anisotropic correlated errors
Ear rot	2009	Lewiston-Woodville	rep
Ear rot	2010	Clayton	R^2, R^4, C^2
Ear rot	2010	Kinston	rep, block(rep)
Ear rot	2010	Lewiston-Woodville	rep, block(rep)
Fumonisin	2009	Clayton	anisotropic correlated errors, R^2
Fumonisin	2009	Jackson Springs	rep, block(rep), C^1, C^2
Fumonisin	2009	Kinston	anisotropic correlated errors
Fumonisin	2009	Lewiston-Woodville	anisotropic correlated errors
Fumonisin	2010	Clayton	rep, block(rep), R^4, C^4
Fumonisin	2010	Kinston	rep, block(rep), C^1
Fumonisin	2010	Lewiston-Woodville	R^3

Table 2.2. Traditional block effects and spatial effects fit within each environment of the 2010 GEFR hybrid disease study for ear rot scores and fumonisin content. R^n and C^n denote *n*-th order row and column polynomial trend terms, respectively.

Trait	Location	Spatial term(s)
Ear rot	Clayton	C^1
Ear rot	Jackson Springs	anisotropic correlated errors, R^3
Ear rot	Kinston	none
Ear rot	Lewiston-Woodville	anisotropic correlated errors
Fumonisin	Clayton	rep, block(rep)
Fumonisin	Jackson Springs	none
Fumonisin	Kinston	none
Fumonisin	Lewiston-Woodville	rep, block(rep)

Table 2.3. Spatial effects fit within each environment of the 2010 GEFR hybrid yield trial analysis for grain yield. R^n and C^n denote *n*-th order row and column polynomial trend terms, respectively.

Location	Spatial term(s)
Clayton	anisotropic correlated errors, R^2, C^1, C^3
Kinston	anisotropic correlated errors, C^3
Lewiston-Woodville	anisotropic correlated errors

Table 2.4. Least square means for twelve superior GEFR lines and checks for Fusarium ear rot resistance and fumonisin content as inbreds *per se* (averaged across seven environments) and for Fusarium ear rot resistance, fumonisin content, and grain yield as topcrosses to the tester NC478 (averaged across four environments for disease traits and three environments for grain yield).

	Inbred *per se*		Topcross to NC478								
	Fusarium ear rot	Fumonisin content	Fusarium ear rot	Fumonisin content	Grain yield	Grain moisture	Days to anthesis	Days to silking	Plant height	Ear height	Erect plants
Experimental lines	%[†]	ppm[†]	%[†]	ppm[†]	bu A^{-1}	%	d	d	cm	cm	%
GEFR397-8-2-2-1-2-1	17.6[‡]	21.8[‡]	10.7[‡]	18.8	128.9	15.5	65.1	65.8	202.7	107.2	94.4
GEFR397-8-2-2-1-3-1	13.9[‡]	14.0[‡]	7.4[‡]	10.3[‡]	112.8	15.5	65.7	67.2	210.2	110.4	90.9
GEFR397-8-2-2-1-3-2	10.5[‡]	14.0[‡]	9.3[‡]	15.8[‡]	116.4	15.4	66.9	67.5	207.1	104.3	92.1
GEFR399-1-5-1-3-4-2	14.4[‡]	16.1[‡]	9.0[‡]	12.2[‡]	119.6	14.7	63.5	64.1	196.4	95.8	92.5
GEFR399-1-5-1-3-5-2	18.0[‡]	16.1[‡]	10.0[‡]	16.3[‡]	116.0	14.9	63.7	64.1	191.7	91.6	92.6
GEFR399-2-10-6-2-1-1	14.9[‡]	15.4[‡]	13.4	19.0	124.4	15.5	63.9	65.0	202.6	94.4	94.0
GEFR399-2-10-6-2-5-1	20.9[‡]	22.3[‡]	9.8[‡]	16.2[‡]	119.2	15.3	65.4	65.9	194.4	93.4	92.4
GEFR399-2-3-1-1-1	13.7[‡]	21.8[‡]	10.4[‡]	20.7	115.8	15.5	64.5	65.8	197.5	94.3	94.2
GEFR399-2-3-1-2-2-1	22.6[‡]	27.0[‡]	8.8[‡]	17.0	127.0	15.5	64.4	64.9	196.7	95.9	93.6
GEFR399-2-3-1-2-4-1	19.2[‡]	23.2[‡]	15.1	18.4	127.9	15.5	65.9	67.1	197.4	98.9	94.5
GEFR400-9-5-2-3-1	26.6[‡]	18.1[‡]	10.8[‡]	17.2	137.6	16.0	66.1	66.7	196.8	99.6	94.9
GEFR400-9-5-2-3-8	8.4[‡]	9.7[‡]	10.8[‡]	16.1[‡]	117.1	16.1	64.7	65.7	192.9	92.5	93.5
Checks											
FR1064	43.2	47.9	15.7	27.1	130.7	15.2	64.9	65.2	201.1	98.2	95.0
GE440	0.9[‡]	9.1[‡]	3.7[‡]	7.3[‡]	109.6	16.3	68.6	68.7	235.6	132.2	81.9
NC478	51.6	79.7[‡]	-	-	-	-	-	-	-	-	-
Pioneer 31G66	-	-	10.8[‡]	14.5[‡]	138.2	14.7	64.7	65.1	204.1	87.8	95.1
DeKalb DK697	-	-	12.4	23.4	147.0	15.2	67.8	69.3	206.4	106.2	97.0
Mean of experimental lines	24.5	34.1	11.7	20.5	118.9	15.4	65.1	65.8	199.6	98.4	93.4
Average LSD (0.05)	-	-	-	-	12.6	0.5	1.5	2.0	8.2	6.0	4.3

[†]Backtransformed from least square means from the analysis of natural log of the trait.
[‡]Significantly different from the FR1064 check at $P = 0.05$ on the transformed scale. Least significant difference is not appropriate on original scale.

Table 2.5. Pearson's correlation coefficients among of least square means for disease and agronomic traits of GEFR line topcross hybrids evaluated in three locations in 2010.

	Fusarium ear rot	Fumonisin content	Days to anthesis	Days to silking	Plant height	Ear height	Erect plants	Grain moisture	Yield
Fusarium ear rot	1								
Fumonisin content	0.63***	1							
Days to anthesis	NS	NS	1						
Days to silking	NS	NS	0.88***	1					
Plant height	NS	NS	0.32*	0.29*	1				
Ear height	NS	NS	0.41**	0.39**	0.84***	1			
Erect plants	0.28*	0.56***	NS	NS	NS	NS	1		
Grain moisture	NS	NS	NS	NS	NS	NS	NS	1	
Yield	NS	NS	NS	NS	NS	NS	0.36**	NS	1

NS is not significant at $P = 0.05$.
*Significant at $P = 0.05$.
**Significant at $P = 0.01$.
***Significant at $P = 0.001$

Table 2.6. Side-by-side comparisons of six pairs of GEFR sister lines contrasting for Fusarium ear rot and fumonisin content least square means (averaged across seven environments). Each pair is derived from the same $BC_4F_{1:3}$ family as described in Eller et al. (2010).

Experimental line	Generation	Fusarium ear rot	Fumonisin content
		%[†]	ppm[†]
GEFR397-8-10-8-4-3-2	$BC_4F_{4:6}$	22.0	27.2
GEFR397-8-10-8-4-5-1	$BC_4F_{4:6}$	44.0	69.6
GEFR399-1-5-1-3-3-2	$BC_4F_{4:6}$	27.8	55.5
GEFR399-1-5-1-3-4-2	$BC_4F_{4:6}$	14.4	16.1
GEFR399-2-10-6-2-1-1	$BC_4F_{4:6}$	14.9	15.4
GEFR399-2-10-6-2-2-1	$BC_4F_{4:6}$	36.4	43.7
GEFR399-3-7-4-2-4-1	$BC_4F_{4:6}$	17.0	22.7
GEFR399-3-7-4-2-5-1	$BC_4F_{4:6}$	38.9	50.1
GEFR400-9-5-2-3-5	$BC_4F_{3:5}$	39.8	57.0
GEFR400-9-5-2-3-8	$BC_4F_{3:5}$	8.4	9.7
GEFR412-13-6-1-4-3-2	$BC_4F_{4:6}$	41.3	61.0
GEFR412-13-6-1-4-5-2	$BC_4F_{4:6}$	24.5	29.1

[†]Backtransformed from least square means from the analysis of natural log of the trait.

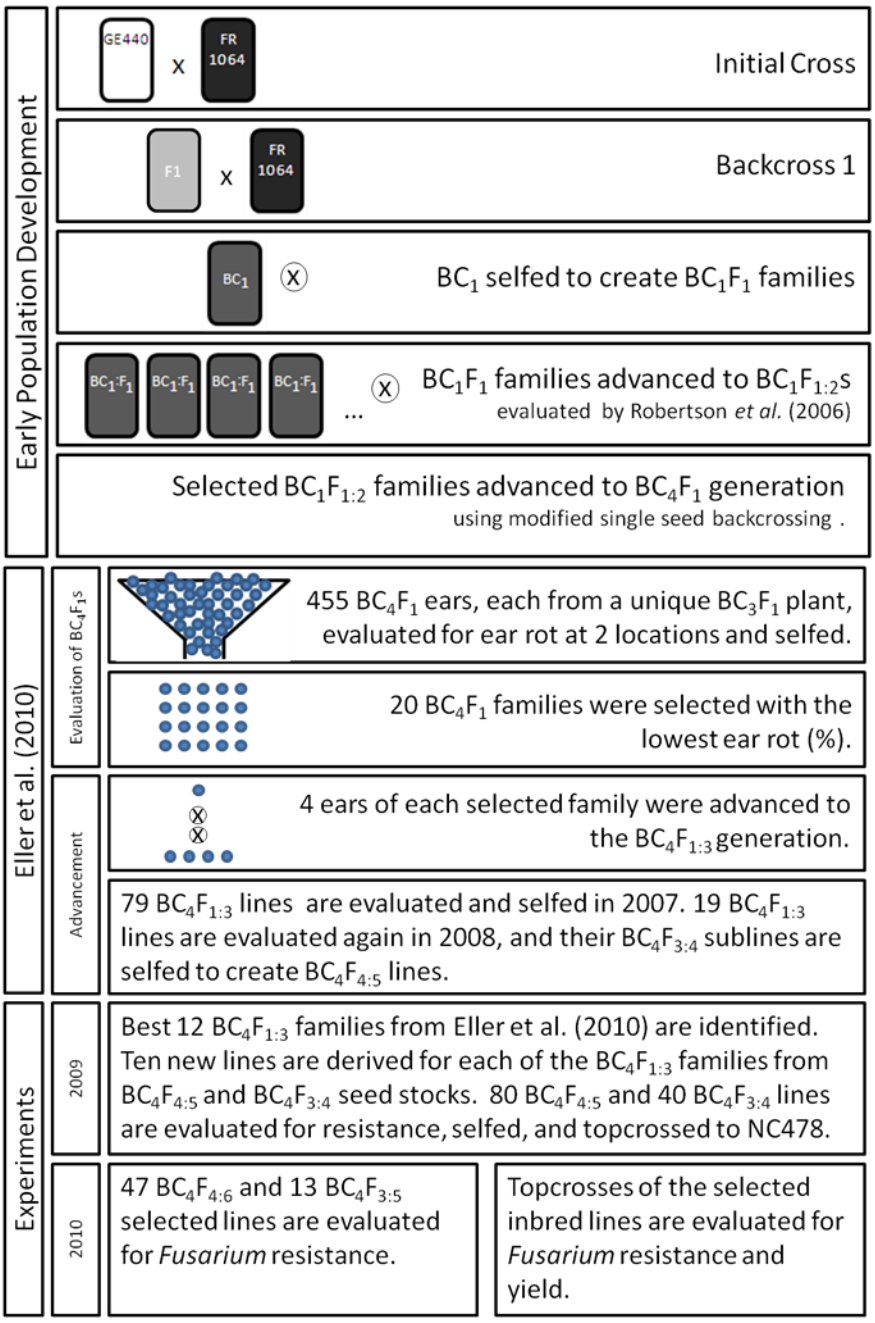

Figure 2.1. Diagram showing GEFR population development and line evaluation (adapted from Figure 1 in Eller et al. 2010).

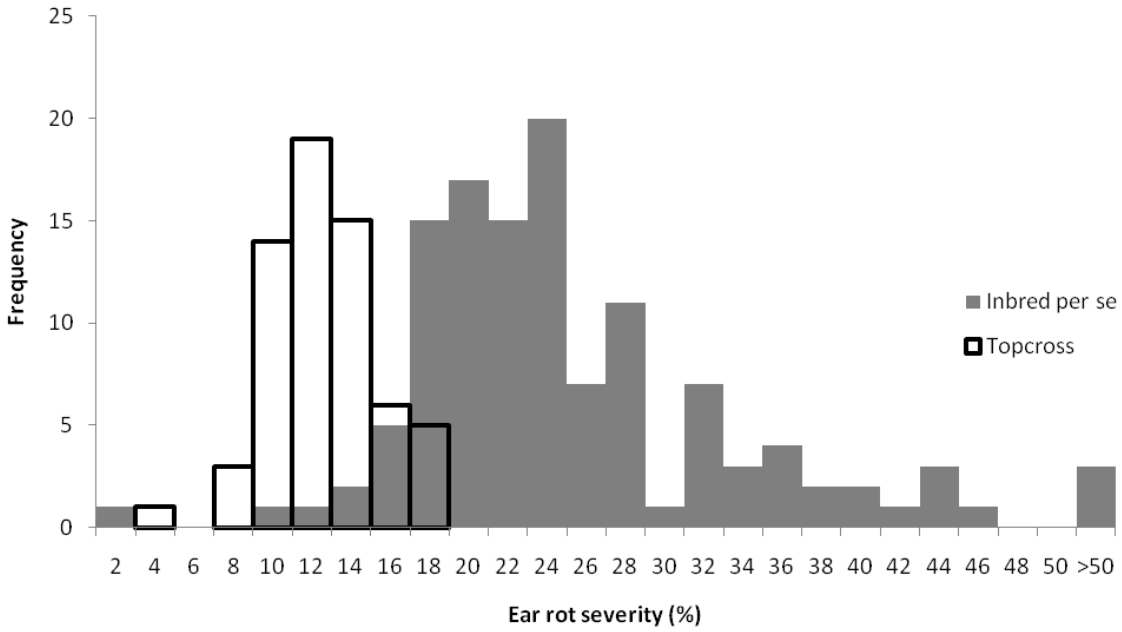

Figure 2.2. Histogram showing the distribution of least square means for Fusarium ear rot resistance from the combined GEFR inbred disease analysis (averaged across seven environments) and the topcross disease analysis (averaged across four environments).

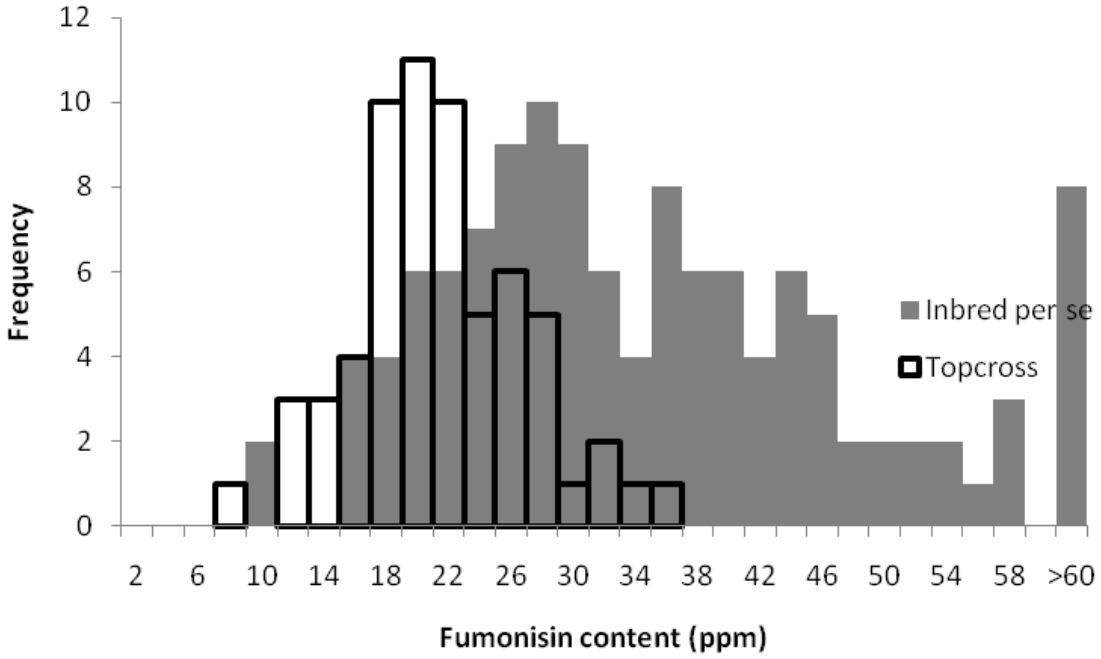

Figure 2.3. Histogram showing the distribution of least square means for fumonisin content from the combined GEFR inbred disease analysis (averaged across seven environments) and the topcross disease analysis (averaged across four environments).

CHAPTER 3: Validation of a Photoperiod Flowering Time Response QTL in Tropical Maize

Adapted from

Coles, N.D., C.T. Zila, and J.B. Holland. 2011. Allele effect variation at key photoperiod response QTL in maize. Crop Sci. 51: 1028-1035.

Abstract

Tropical maize represents a valuable genetic resource containing unique alleles not present in elite temperate maize. The strong delay in flowering in response to long daylength photoperiods exhibited by most tropical maize hinders its incorporation into temperate maize breeding programs. A previous study has identified a strong photoperiod response QTL in a 25.9 cM region on chromosome 10. Using a set of heterogeneous inbred families (HIFs), this photoperiod response QTL position was validated and narrowed to a slightly less than 1 cM region on chromosome 10.

Introduction

The genetic diversity of elite temperate maize (*Zea mays* L.) germplasm is reduced relative to global intraspecific variation, resulting from intense phenotypic selection for increased yield and crop uniformity over the past century (Tenaillon et al., 2001; Goodman, 2004). The relatively narrow genetic base of temperate maize renders it more vulnerable to evolving pathogen biotypes and may limit future gains in productivity (Smith, 2007). Genetically more diverse tropical maize represents a valuable genetic resource that could be used to enhance the diversity and productivity of temperate maize (Gouesnard et al., 1996; Goodman, 2004). Unfortunately, most tropical germplasm is poorly adapted to temperate growing environments. A major component of the poor adaptation of tropical maize to temperate environments is the response of tropical germplasm to long-day photoperiods. At daylengths greater than 13 hours, photoperiod sensitive maize exhibits delayed flowering, increased plant height, and a greater total number of leaves (Allison and Daynard, 1979; Kiniry et al., 1983; Warrington and Kanemasu, 1983).

In the tropical regions of Mexico where maize was first domesticated, precipitation rates and daylengths cycle annually. Specifically, the dry season in Central Mexico occurs from December to April when daylengths are increasing, and the wet season occurs from June to September when daylengths are decreasing (Bullock, 1986; Medina García et al., 1998; Ruiz Corral et al., 2008). Because water stress during flowering can reduce fertilization and seed set, maize and its predecessor teosinte likely evolved photoperiod sensitivity to synchronize their reproductive phases to the wetter, short-day growing season (Ribaut et al., 1996; Campos et al., 2006). A similar evolution of photoperiod sensitivity is believed to have occurred in sorghum (Craufurd et al., 1999). Latitude 20º N passes through Central Mexico, and at this latitude the daylength shifts from 13.3 hours on June 16 to 12.23 hours on September 16 (United States Naval Observatory, 2011). Thus, maize adapted to this region is quite sensitive to a one-hour shift in photoperiod. In contrast, temperate maize populations that were introduced to long-day environments during the spread of maize growing culture throughout North and South America were selected to be photoperiod insensitive. The major U.S. corn producing region is centered north of 40º N, where daylength is almost 14 hours on May 1, increasing to 15 hours on June 16, and not decreasing below 13 hours until September 3 (United States Naval Observatory, 2011). The timing of flowering under these much longer daylengths is greatly delayed in tropical maize, reducing the grain filling period (which must be completed before the first frost) and yield, and increasing grain moisture at harvest. These responses can mask the expression of favorable alleles carried by tropical germplasm, resulting in a major barrier to the introgression of tropical germplasm into temperate maize.

Photoperiod response can be eliminated in both temperate × tropical and tropical × tropical populations through phenotypic selection over several generations, resulting in

agronomically superior temperate inbred and hybrid lines (Hallauer and Sears, 1972; Hallauer, 1994; Holland and Goodman, 1995; Goodman, 1999; Lewis and Goodman, 2003; Nelson and Goodman, 2008). Methods to efficiently and accurately select against photoperiod sensitivity across a wide variety of tropical germplasm could accelerate the introgression of valuable alleles from tropical maize into temperate maize.

Marker-assisted selection can facilitate plant breeding by determining which members of a segregating population carry detrimental or unfavorable alleles, even when the effects of such alleles are masked by epistasis or incomplete penetrance (Xu and Crouch, 2008). A comprehensive study of the QTL governing maize photoperiod sensitivity would determine which photoperiodic alleles are common to certain tropical maize populations, and the results of such a study could be used to select among tropical photoperiod sensitive parental lines in order to produce completely tropical populations segregating for photoperiod insensitivity. Indeed, it is possible to produce photoperiod insensitive progeny from the cross of two photoperiod sensitive parents (Holley and Goodman, 1988; Goodman, 1999).

A small number of photoperiodic response QTL have been identified in common across distinct temperate × tropical maize populations and independent environments (Moutiq et al., 2002; Wang et al., 2008; Ducrocq et al., 2009; Coles et al., 2010) raising the possibility that a substantial proportion of the photoperiodic flowering time variation may be controlled by a few loci. Meta-analysis of numerous maize studies indicated that six flowering time QTL were detected regularly across diverse mapping populations (Chardon et al., 2004). Four of these six QTL are located in the same genomic regions as major photoperiodic QTL identified by Coles et al. (2010) in a joint QTL analysis of four temperate × tropical populations. Of the four genomic regions identified by Coles et al. (2010), a QTL identified on chromosome 10 (*ZmPR4*) had the

largest effect on photoperiod response, accounting for up to 40% of the phenotypic variation in photoperiod response. Furthermore, Coles (2009) hypothesized that the use of heterogeneous inbred families (HIFs) could prove useful in validating and refining the position of the chromosome 10 QTL.

Coles (2009) developed a set of heterogeneous inbred families derived from the B73 × CML254 recombinant inbred line (RIL) mapping population used in Coles et al. (2010). The primary objective of this study was to validate and refine the position of the major chromosome 10 photoperiod response QTL in heterogeneous inbred families segregating for alleles only in the region of its initial map position.

Materials and Methods

Heterogeneous Inbred Family (HIF) Development

HIFs were derived from a single recombinant inbred line from the B73 × CML254 RIL population described by Coles et al. (2010) (Figure 3.1). One F5:7 RIL was chosen to derive HIFs because it was segregating at markers flanking the ZmPR4 region on chromosome 10 but homozygous elsewhere in the genome. Several plants of the RIL were self-pollinated and harvested individually to form F7:8 lines. Up to 72 seedlings of each F7:8 line were genotyped at 19 SSR markers in the interval from bnlg210 to umc1115 (Figure 3.2). A sample of plants representing contrasting homozygous classes for the entire interval, homozygous recombinant classes from different recombination events, as well as partly heterozygous recombinant progenies from each line was self-pollinated to form $F_{8:9}$ lines. $F_{8:9}$ lines were increased by self-fertilization; one set of plants within each line was harvested individually to form $F_{9:10}$ lines, and a second set of plants from each line was harvested in bulk to form $F_{8:10}$ lines. Thirteen $F_{9:10}$ lines

(coded as 1304-1-1-1 to 1304-1-1-13) derived from a single $F_{8:9}$ line that was heterozygous in two regions near the QTL were genotyped individually at four SSR markers in the heterozygous regions, and genotypes at surrounding loci were interpolated based on grandparental F_8 genotype data and the four directly genotyped loci (Figure 3.3).

Phytotron Experiment

HIFs representing contrasting parental haplotypes and several recombination events within the *ZmPR4* region were chosen for photoperiod sensitivity study evaluations under controlled conditions in the North Carolina State University Phytotron (http://www.ncsu.edu/phytotron/). Experimental entries included 21 $F_{8:10}$ generation lines, 13 $F_{9:10}$ generation lines, and two instances each of B73 and CML254, resulting in a total of 38 entries per replication.

The experimental design was a randomized complete block design with six replications repeated across two planting dates, one month apart. Experimental units were 15 cm-diameter plastic pots sown with several seeds of a single line. Seedlings were thinned after emergence to a single plant per pot. The growing medium was a mixture of peat moss and vermiculite, and pots were watered twice daily with a nutrient solution. Within a planting date, pots were arranged in a randomized complete block design within a single growth chamber and subjected to a photoperiod regime of 18 h day/6 h night with temperatures set to 30° day/26° night. Illumination was provided by a mixture of 1500 ma cool-white fluorescent and 100 W incandescent lamps with red:far red light ratios of 0.838. Lamps were separated from the chamber by a plexiglass barrier. The light levels measured about one m above the chamber floor were 500 (+/- 15) μmol m^{-2} s^{-1}. Relative humidities in the chambers were typically above 70%.

More detailed environmental specifications are available at Saravitz et al. (2009); see description of "B chambers."

Plants were grown under long daylength photoperiod growth chamber conditions for 30 d, which Coles et al. (2010) demonstrated was sufficient to induce a very strong delayed flowering photoperiod response in CML254. After 30 d, the plants were moved to a greenhouse with 12 h of artificial lighting and temperatures set at 25°. In the greenhouse, plants were watered once daily and fertilized with slow-release fertilizer. Days to tassel emergence (DTE), days to anthesis (DTA), and plant height (PH) measurements were recorded for each individual. Least square means were calculated for each trait and line combination using SAS Proc Mixed (SAS Institute Inc, 2010). Genotypes were considered fixed effects, whereas planting date, replication (nested within planting date), and genotype × planting date interaction were considered random effects. Analysis of variance was conducted on the line least square means to test the null hypothesis of no marker effect for each SSR locus by testing the variation among marker class means using the pooled variation within marker class means.

Field Experiment

Five HIF lines from the phytotron study with sufficient seed quantities were chosen for a field evaluation under long daylengths in the summer of 2009. The experimental design was a randomized complete block arrangement of the five HIF lines plus B73 with four replications each at four locations: Colombia, MO; Clayton, NC; Ithaca, NY; and Madison, WI. Measurements were collected for days to anthesis (DTA), days to silking (DTS), plant height (PH), ear height (EH), and total leaf number (TLN). Anthesis-silk interval (ASI) was calculated as the difference between DTS and DTA.

Locations were first analyzed separately using SAS Proc GLM; linear combinations were used to estimate the effect of the testing region between the two subsets of HIFs. Locations were then combined and analyzed using SAS Proc Mixed. Genotypes were treated as fixed effects, and location, replication (nested within location), and genotype × location interaction were included as random effects. All pairs of genotypes were compared using the pdiff option of the lsmeans statement in Proc Mixed.

Results

We had sufficient seed of five HIF lines for replicated field evaluations in North Carolina, Missouri, New York, and Wisconsin (Figure 3.3). The lines flowered very late in New York, so data were not available from this location. In the combined analysis across the other three locations, all HIFs were significantly later flowering (days to anthesis or silking), taller (ear or plant height), and had more leaves than B73 (Table 3.1). The HIF flowering phenotypes fell into two distinct and significantly different groups. HIFs 1304-1-3C and 1304-7 flowered 4.5 to 7.4 d later than HIFs 1304-21-6B, 1304-21-12B, and 1304-16C (Table 3.1). These two groups consistently differed for allelic constitution at markers defining an interval on the IBM map from positions 225.7 to 253.5 (equivalent to about 7 cM,Balint-Kurti et al., 2007). The two lines homozygous for CML254 alleles in this region represented the later flowering group, whereas the three lines homozygous for B73 alleles in this region represented the earlier flowering group (Figure 3.3). The two later flowering lines differed from each other genotypically at markers flanking this region, providing further evidence that the flowering time QTL is delimited by this 7 cM region (Figure 3.3). Some pairs of HIFs in the two groups did not differ for plant and ear height, and no variation for leaf number was observed among these HIFs (Table 3.1).

Sufficient seed was available to conduct a replicated controlled long-daylength environment evaluation of a larger group of HIFs, including the same five used in the field evaluation. Three recombination events within the 7 cM QTL region defined in the field study were represented in this larger group of HIFs (Figure 3.3). Tassel emergence, anthesis date, and plant height varied significantly among the HIFs evaluated in the growth chamber and greenhouse study. The flowering time QTL region identified in the field study also had highly significant effects on flowering time in the controlled environment study (Figure 3.3). The additional recombinations represented by the HIFs in this study further delimited the flowering time QTL region to three consecutive markers (umc2348, umc1995, and umc1246), encompassing IBM positions 244.6 to 248.2, an interval slightly less than 1 cM (Figure 3.4). In this region, plants homozygous for the CML254 allele flowered about 6 d later than the plants homozygous for the B73 allele. Two smaller QTL peaks were observed for PH, one at the same position as the flowering time QTL and a second QTL near IBM position 283 (Figure 3.4). At both QTL, the homozygotes with CML254 alleles were taller than B73 homozygotes. These results provide further evidence for the presence of two separate QTLs in the region, affecting flowering time and plant height differently, as suggested by Coles et al. (2010).

Conclusion

These HIF results are consistent with the position of a strong flowering time or photoperiod response QTL in this region (Moutiq et al., 2002; Wang et al., 2008; Buckler et al., 2009; Coles et al., 2010), which Ducrocq et al. (2009) fine-mapped to a 170kb interval around 94.0 Mbp on the maize AGPV1 physical map (www.maizesequence.org), including sequences just upstream of a homolog of *Ghd7*, which controls flowering time in rice (Xue et al., 2008).

Ghd7 encodes a CCT protein domain that has been shown to severely suppress rice flowering time under long daylengths, and non-functional variants of this gene are associated with more temperate adapted varieties of rice in Asia (Xue et al., 2008; Tsuji et al., 2011).

Overall, the HIF approach was an effective method to further fine map the region around *ZmPR4*. Generation of the HIF families took roughly one year's worth of labor, compared to the much longer amount of time it takes to generate NIL families. However, difficulties with seed production limited the ability to generate and maintain a large number of unique HIF families, subsequently limiting the ability to detect and evaluate unique recombination events within the *ZmPR4* region. Therefore, a larger HIF collection with more novel recombination events would be needed to narrow the *ZmPR4* region even further yet.

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Table 3.1. Least square means of heterogeneous inbred family and check entry lines across three environments for days to anthesis (DTA), days to silk (DTS), anthesis-silk interval (ASI), ear and plant heights, and total leaf number.

Line	DTA	DTS	ASI	Ear height	Plant height	Leaf number
	d			m		
1304-1-3C	86.7 a[†]	87.9 a	1.0 ab	1.31 a	2.32 a	27.0 a
1304-7	86.2 a	88.1 a	1.8 b	1.27 ab	2.31 ab	27.1 a
1304-21-6B	81.5 b	81.0 b	-0.7 c	1.26 ab	2.25 ab	26.5 a
1304-21-12B	81.7 b	81.1 b	-0.6 c	1.23 ab	2.26 ab	26.6 a
1304-21-16C	81.3 b	80.7 b	-0.7 c	1.16 b	2.17 b	26.4 a
B73	69.8 c	69.8 c	-0.1 ac	0.81 c	1.66 c	20.1 b

[†]Least square means followed by the same letter within a trait are not significantly different at the 0.05 probability level.

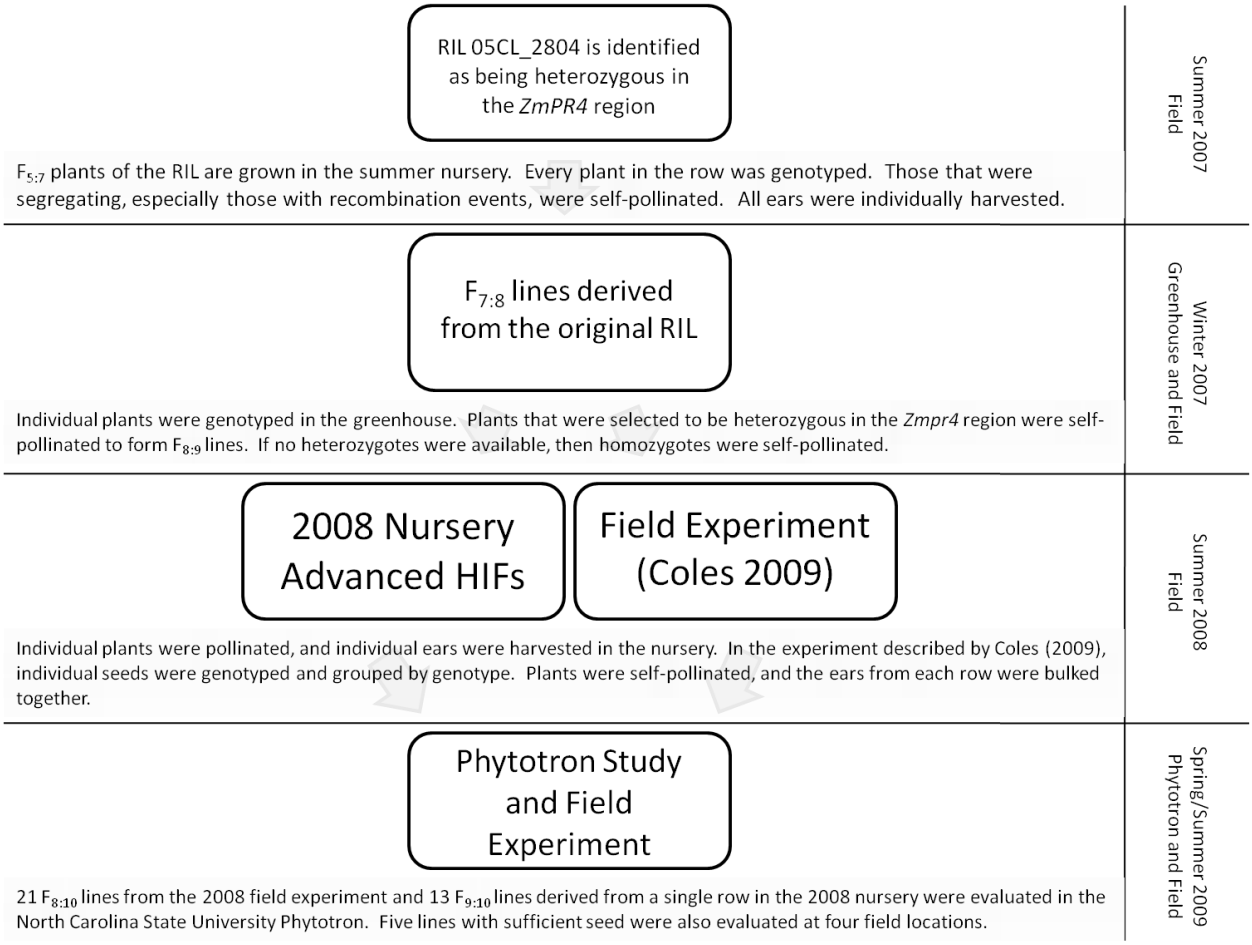
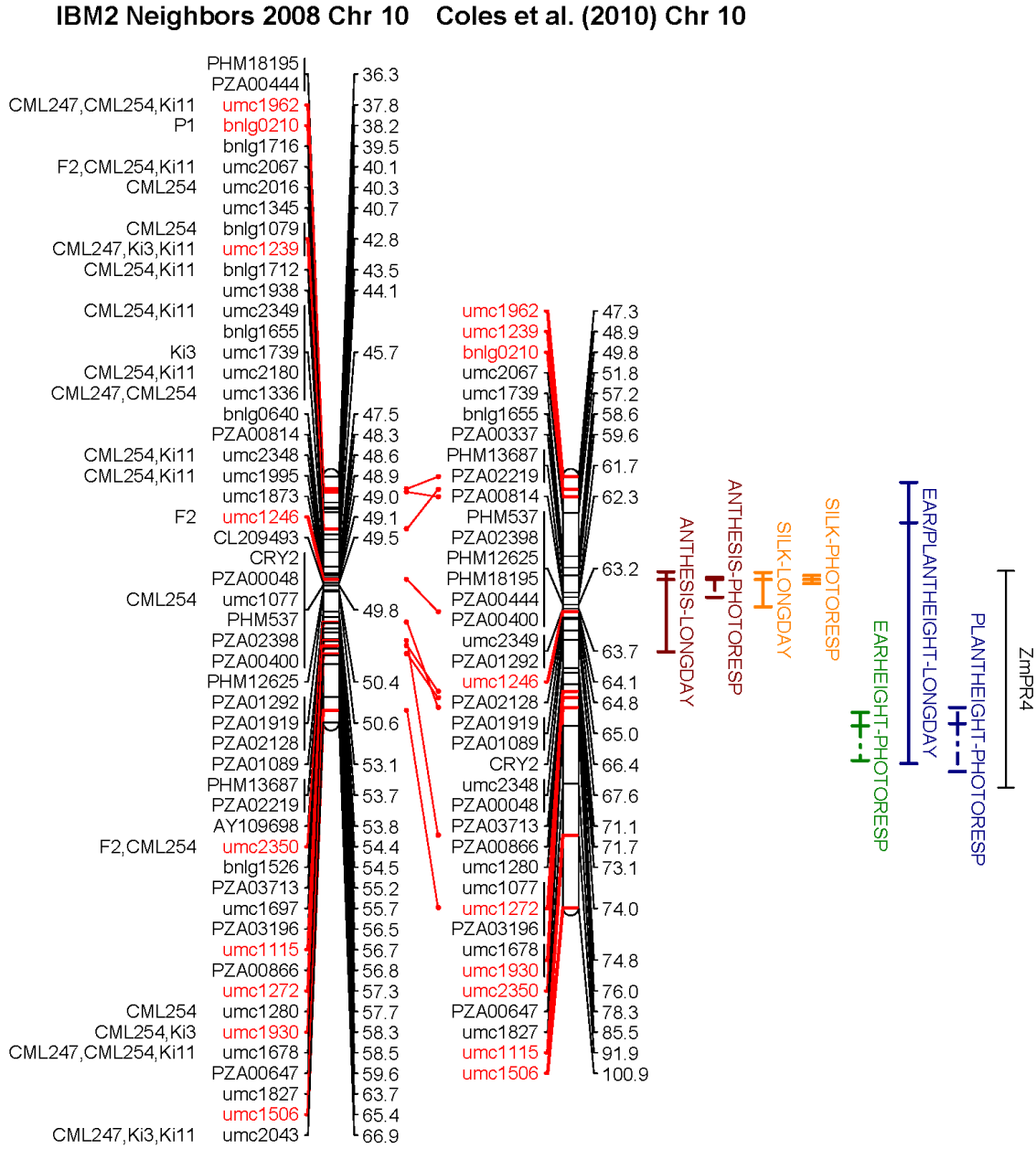


Figure 3.1. Diagram showing how the ZmPR4 HIFs were derived from a single recombinant inbred line (adapted from Figure 4.1 in Coles 2009).

Figure 3.2. Linkage map of a segment of chromosome 10 containing the key photoperiod response QTL region *ZmPR4* as described by Coles et al. (2010). The segment is shown two different ways; the segment on the right of the pair contains markers ordered according to the analysis of Coles et al. (2010), while the segment on the left contains markers ordered according to the IBM2 Neighbors 2008 map as a reference. Markers that were scored on one or more of the populations in the Coles et al. (2010) study are indicated to the left of each IBM2 segment. "F2" indicates that the marker was scored on the Ki14×CML254 F_2 population. "CML247", "CML254," "Ki3," or "Ki11" indicate that the marker was scored on the backcross population derived from that donor parent, respectively. In addition to markers scored on the populations in this study, other markers are shown to provide a framework for each region in question. Markers highlighted in red are part of the backbone of the IBM2 Neighbors 2008 map, meaning that these markers are well-ordered on that map. QTL bars represent the 2-LOD support interval of the QTL position as shown by Coles et al. (2010). The middle hash mark of each bar represents the maximum likelihood position of the QTL. "Longday" refers to QTL that were indentified under long daylength environments, while "photoresp" refers to QTL associated with the photoperiod response.


IBM2 Neighbors 2008 Chr 10
Coles et al. (2010) Chr 10
PHM18195 36.3
PZA00444
CML247,CML254,Ki11 umc1962 37.8
P1 bnlg0210 38.2
bnlg1716 39.5
F2,CML254,Ki11 umc2067 40.1
CML254 umc2016 40.3
umc1345 40.7
CML254 bnlg1079
CML247,Ki3,Ki11 umc1239 42.8
CML254,Ki11 bnlg1712 43.5
umc1938 44.1
CML254,Ki11 umc2349
bnlg1655
Ki3 umc1739 45.7
CML254,Ki11 umc2180
CML247,CML254 umc1336
bnlg0640 47.5
PZA00814 48.3
CML254,Ki11 umc2348 48.6
CML254,Ki11 umc1995 48.9
umc1873 49.0
F2 umc1246 49.1
CL209493 49.5
CRY2
PZA00048
CML254 umc1077 49.8
PHM537
PZA02398
PZA00400
PHM12625 50.4
PZA01292
PZA01919 50.6
PZA02128
PZA01089 53.1
PHM13687
PZA02219 53.7
AY109698 53.8
F2,CML254 umc2350 54.4
bnlg1526 54.5
PZA03713 55.2
umc1697 55.7
PZA03196 56.5
umc1115 56.7
PZA00866 56.8
umc1272 57.3
CML254 umc1280 57.7
CML254,Ki3 umc1930 58.3
CML247,CML254,Ki11 umc1678 58.5
PZA00647 59.6
umc1827 63.7
umc1506 65.4
CML247,Ki3,Ki11 umc2043 66.9
umc1962 47.3
umc1239 48.9
bnlg0210 49.8
umc2067 51.8
umc1739 57.2
bnlg1655 58.6
PZA00337 59.6
PHM13687 61.7
PZA02219 62.3
PZA00814
PHM537
PZA02398
PHM12625 63.2
PHM18195
PZA00444
PZA00400 63.7
umc2349
PZA01292 64.1
umc1246 64.8
PZA02128 65.0
PZA01919
PZA01089 66.4
CRY2 67.6
umc2348
PZA00048 71.1
PZA03713 71.7
PZA00866 73.1
umc1280
umc1077 74.0
umc1272
PZA03196 74.8
umc1678 76.0
umc1930 78.3
umc2350 85.5
PZA00647 91.9
umc1827 100.9
umc1115
umc1506
ANTHESIS-LONGDAY
ANTHESIS-PHOTORESP
SILK-LONGDAY
SILK-PHOTORESP
EARHEIGHT-PHOTORESP
EAR/PLANTHEIGHT-LONGDAY
PLANTHEIGHT-PHOTORESP
ZmPR4

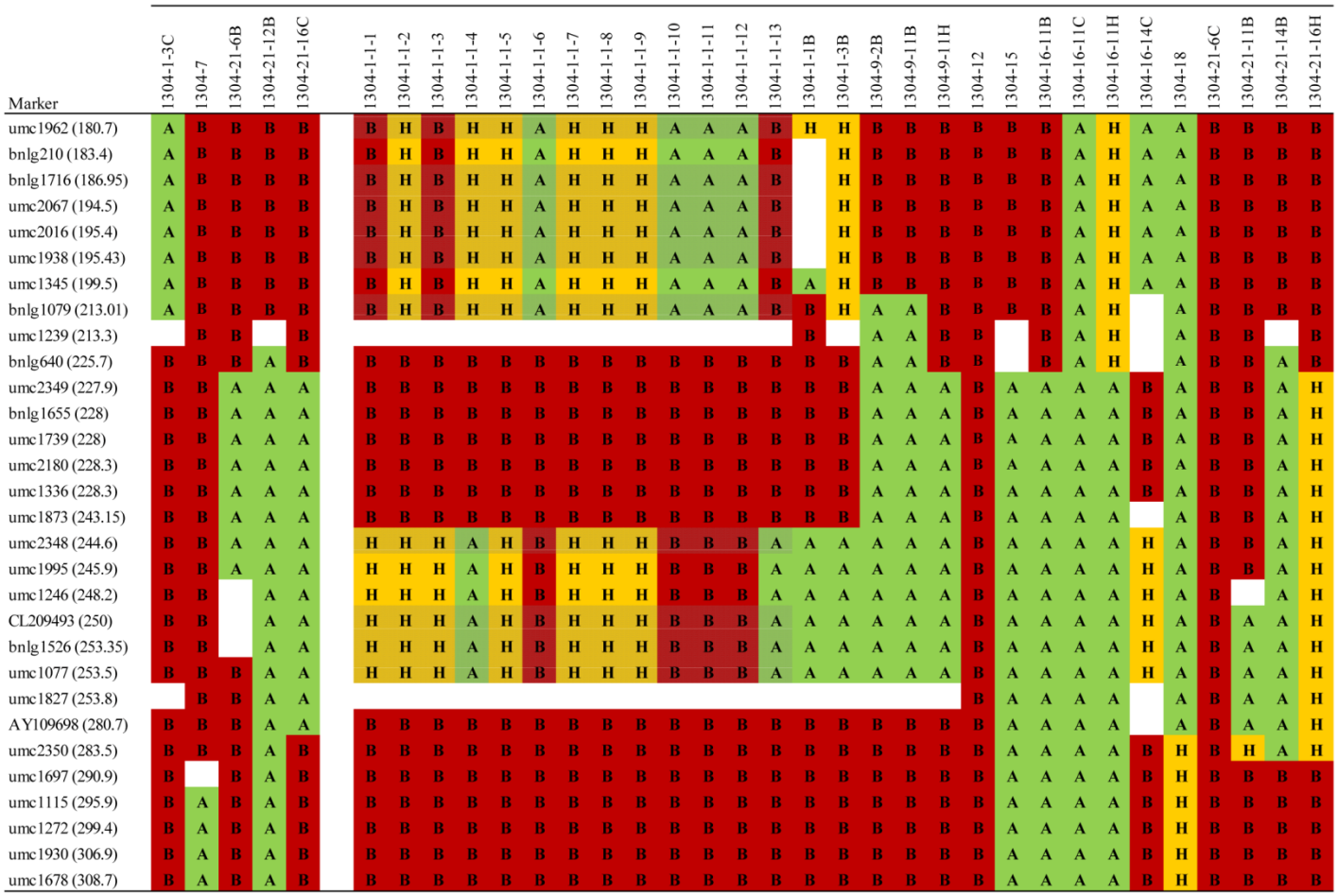


Figure 3.3. Graphical genotypes for HIFs tested in the 2009 field and phytotron experiments. The five leftmost HIFs displayed were tested in the field experiment; all HIFs shown were tested in the phytotron study. Loci that are homozygous for the B73 allele are denoted by "A", while loci that are homozygous for the CML254 allele are denoted by "B" and those heterozygous are denoted by "H". Shaded loci indicate interpolated marker scores. IBM2 2008 positions are indicated in parentheses next to each marker.

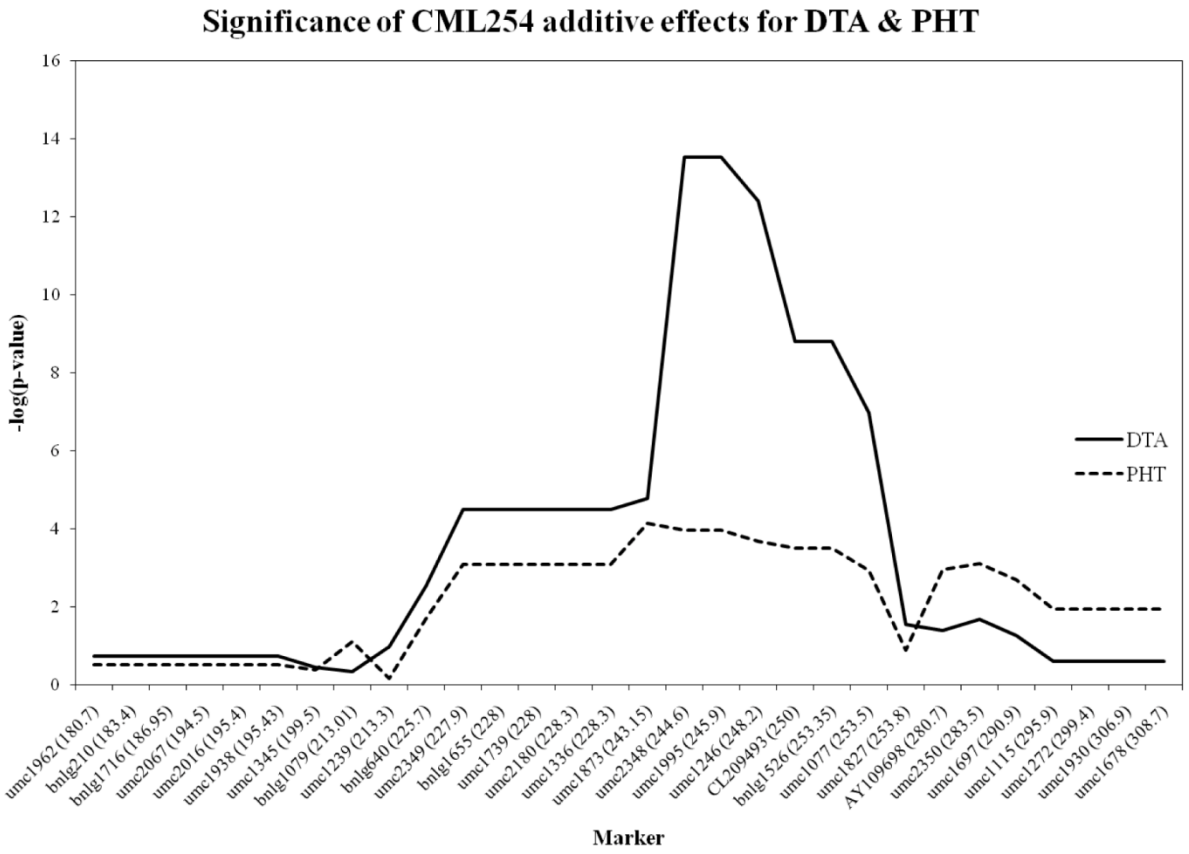


Figure 3.4. Significance (negative log of *p*-value) of additive effects at SSR loci in the ZmPR4 QTL region for days to anthesis (DTA) and plant height (PH). Markers are ordered according to the IMB2 2008 neighbors map, and positions are indicated in parentheses to the right of the marker names.