GENETIC COMBINING ANALYSIS OF FOOD-GRADE MAIZE: COLORED AND QUALITY PROTEIN

A Thesis

by

ADAM LYLE MAHAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Plant Breeding

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Approved by:

Chair of Committee,	Seth C. Murray
Committee Members,	Lloyd W. Rooney
	Kevin M. Crosby
Head of Department,	David D. Baltensperger

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ABSTRACT

Genetic Combining Analysis of Food-Grade Maize: Colored and Quality Protein.

(August 2012)

Adam Lyle Mahan, B.S., Iowa State University

Chair of Advisory Committee: Dr. Seth C. Murray

Maize genetic diversity includes an array of kernel colors (red, blue, purple) with blue concentrated in the aleurone and red primarily in the pericarp. Quality protein maize (QPM) is improved over normal maize in regards to grain concentration of the essential amino acids lysine and tryptophan but has not been widely adapted in part due to lower than conventional yields. These are minimally-utilized specialty corns when compared to the yellows and whites commonly grown. Red, blue, and purple pigments are antioxidant phytochemicals produced by the plant as secondary metabolites. Antioxidants have been linked to anti-cancer and other anti-inflammatory health benefits. QPM hybrids are desirable in developing countries where subsistent agriculture is commonly practiced and quality protein cereals are non-existent. These two diverse maize categories have been the subject of little breeding research compared to normal maize and the potential for high phenolic content as well as the characterization of these QPM hybrids has not been previously investigated. We evaluated 153 maize hybrids (84 colored, 69 QPM) across three locations. High heritability estimates were found for phenolic content (0.80), tryptophan (0.46), and endosperm opacity (0.82). It was

encouraging that all three traits observed little genotype by environment (GxE) interaction across diverse environments. This proved the trait analysis procedure to be robust in detecting and separating genotypes for both total phenolic content in colored maize, and amino acids in QPM. Top combiners for phenolics were the purple maize 'maize morado' and red lines, with blue, yellow and white maize performing in descending order. Within the tested hybrids, high per kernel antioxidants (measured by total phenolics) may be the answer for producing the most total phenolics, with the top hybrid yielding greater than twice the total phenolics as the top yielding yellow hybrid. The top QPM hybrid out yielded the top normal hybrid by 35 and 30% for lysine and tryptophan. Additionally, QPM endosperm opacity primarily followed an additive, mid-parent trend, with some hybrids (20%) from diverse germplasm backgrounds deviating from that trend displaying the complexity and recessive nature of multiple modifier loci. Additional agronomic and composition traits were minimally correlated with phenolics.

DEDICATION

To my wife Jennifer for showing me love, support and understanding while I follow my dreams. Also to my parents for providing the childhood necessary to mold me into the man I have become.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Seth C. Murray, for providing me the opportunity to obtain my graduate education and for the many times he pushed me to learn new things and grow as a person both professionally and personally. Thanks also to my committee members, Dr. Lloyd W. Rooney and Dr. Kevin M. Crosby for advising me throughout my research and providing answers to the many questions that arose.

Thanks to Mr. Gerald De La Fuente for increasing my knowledge of row crop agriculture, and for assisting me throughout my research. Thanks also to Mrs. Meghyn Meeks, Mr. Jim Wilborn, Mr. Ivan Barrero-Farfan, Mr. Victor Taleon, Mr. Frederico Barros, Beto Garza and all the other graduate and undergraduate students that helped make my research possible.

Thanks to the Texas Corn Producers Board, Texas AgriLife Research, and Monsanto Fellowships in Plant Breeding for funding my education and research.

Lastly, thanks to my parents and extended family that I am blessed to be a part of for their thoughts and prayers that has given me the strength and courage to pursue graduate education.

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CHAPTER I

INTRODUCTION

Maize (*Zea mays* L.) is important as a food, feed, and energy crop and the largest cereal crop by production worldwide ahead of both rice and wheat (FAO, 2011). As of 1998, the global cultivated area of maize was approx. 130 million hectares, with an estimated production of 574 million metric tons. By 2008 this had grown to 826 million tons (Vasal, 2002; FAO, 2011). The United States is the largest producer of maize (*Zea mays* L.) in the world, producing an estimated 310 million tons annually (FAO, 2011).

Yields are increasing as new hybrids are developed, and this data portrays the economic importance that maize has on the world market. Economically, maize is vital to developed nations such as the United States, and remains a staple food crop in developing countries. Maize throughout the world is most commonly yellow or white. Diverse colors are naturally occurring and encompass several pigments and phytochemical compounds which include carotenoids, anthocyanins, and phenolic compounds. These compounds are seen as purple, blue, and red. Colored maize is gaining popularity around the world due to the potential nutritive value not only from antioxidant activity in food products such as tortilla chips, but also as a source of natural food colorants for use in soft drinks and confectionaries (Betran et al., 2000).
Maize is primarily a starch source, representing 71% of the kernel (Prasanna et al., 2001), while protein accounts for 7-13% of the kernel (Moro et al., 1996). The highest

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This thesis follows the style of Crop Science.

quantity and quality protein is found within the germ (Prasanna et al., 2001). Cereal based diets are found in the developing world because high yields result in inexpensive calories but are deficient in protein quantity and quality. Continued improvement for nutrition of maize with an emphasis on protein will provide the opportunity for people living in developing countries to experience an enhanced, healthier way of life.

Limited work has been done to create pigmented maize germplasm other than the traditional white and yellow that is widely adapted to the United States (Betran et al., 2000; Widrlechner and Dragula, 1992 and 1998). Texas adapted blue maize by Texas A&M University is an example of a breeding program that has conducted research to take a soft floury maize and turn it into a maize with a harder, more vitreous endosperm that has improved resistance to insect and disease damage (Betran et al., 2000). Today blue maize lines have a harder kernel and are better suited for the hot humid conditions of the Southern United States where ear rot and kernel pathogens such as *Aspergillus flavus* are commonly found. Of the main phytochemicals found in maize, more research has been on carotenoids than anthocyanins or phenols.

Carotenoids are a class of yellow/orange pigments found in the endosperm of maize. Carotenes and xanthophylls are the two major carotenoid compounds and are responsible for the typical yellow and orange color of maize endosperm (Buckner et al., 1990). Carotenoids also make up several of the yellow and orange pigments found in nature which include fruit, vegetables, flowers, butterflies, and crayfish. These compounds also aid in pollinator attraction by contributing to the production of scents and desirable flavors (Cazzonelli and Pogson, 2010).

Anthocyanins are present in a wide range of colors perceived as orange to blue in the color spectrum. In maize they are found in blue, purple, and red types in high concentrations. The main component of anthocyanin known in maize is the compound cyanidin-3-glucoside (C3G) (Tsuda et al., 2003). The same holds true for purple wheat. In blue wheat, C3G was found to be the 2nd major anthocyanin (Abdel-Aal, 2003). Purple maize also has other antioxidant compounds, known as phenolic acids (Pedreschi and Cisneros-Zevallos, 2006). In general, the term "phenolic compounds" is used in reference to antioxidant compounds.

While anthocyanins are found in the free form, phenolic compounds are usually present in the bound form (approx. 80%) (Lopez-Martinez et al., 2009). The primary phenolic maize compound is ferulic acid, constituting 85% of total phenol content (Cabrera-Soto, et al., 2009). Total phenol content has been reported (Sosulski et al., 1982; Del Pozo-Insfran et al., 2006; De la Parra et al., 2007) in several studies but with a limited genotype, non-breeding emphasis. Like all antioxidant compounds phenolic compounds are secondary metabolites that serve several location dependent functions within the kernel. Phenols in the germ correspond to *Fusarium* spp. tolerance (Bakan et al., 2003); endosperm phenols cause graying of dough and tortilla color during nixtamalization (Salinas et al., 2007); While pericarp phenols assist in storage pest tolerance (Arnason et al., 1992).

Quality protein maize (QPM) is estimated to be a small portion of total maize planted at one million hectares, mostly in the developing world (Prasanna et al., 2001). The diversity of protein quantity and quality provided by maize (Dudley, 2007) leaves

room for further development and may lead to future QPM acreage increases. Normal (non-QPM) maize endosperm protein is naturally low in the two most essential amino acids in human nutrition, lysine (Kies et al., 1965) and tryptophan (Nelson, 1969) due to a high concentration of zein (60%) (Salamini and Soave, 1982). Albumins (3%), globulins (3%) and glutelins (34%) represent the remaining protein constituents of normal maize (Salamini and Soave, 1982) and contain higher quantities of lysine and tryptophan, among others. In QPM, there is a decrease of zeins, with a corresponding increase in the other three types of protein. (Prasanna et al., 2001). This supports the hypothesis that lysine is increased in the maize kernel through the ability to prevent or suppress the synthesis of the lower-quality zein protein (Vasal, 2002).

Lysine and tryptophan levels in QPM lines are typically observed as a 3:1 ratio, respectively (Nurit et al., 2009). Lysine is produced through the aspartic acid pathway in plants and has many enzymes involved in its production (Gaziola et al., 1999). QPM hybrids have averaged a 60% advantage in tryptophan levels in the protein and a 48% advantage in the overall grain when compared to normal checks across several diallel studies (Pixley and Bjarnason, 1993). Consumer demand for maize is expected to increase from a 1995 baseline by 50% worldwide, and sub-Saharan Africa specifically by 93% by the year 2020 (Pixley & Bjarnason, 2002), further increasing the importance of finding ways to better enhance the cereal grains that people depend on daily.

CHAPTER II

REVIEW OF LITERATURE

COLORED MAIZE

Natural colorants high in antioxidants have become important in certain parts of the world due to perceived health benefits. Japan is currently consuming more than 50,000 Kg per year of purple maize colorant in products such as soft drinks and specialty foods (Tsuda et al., 2003). One main obstacle keeping anthocyanin usage from becoming popular is that they are stable under acidic conditions but degrade under neutral/basic pH, which is a problem with many food production systems such as the alkaline cooking of tortillas/chips requiring a basic preparatory environment. Anthocyanins are water-soluble, making these compounds a great candidate for food production requiring aqueous systems as in drink and dessert production. The ability of antioxidants to provide healthful benefits has been delved into quite thoroughly in both animal and human studies.

In an obesity study, 1/3 less total lipids accumulated in the livers (Tsuda et al., 2003) of mice fed a diet high in fat along with purple maize colorant. Along with reducing lipid accumulation, antioxidants have also shown promising anti-cancer potential.

Antioxidants have been shown negative effects on leukemia and colon cancer cells. Two anthocyanins, cyanidin and malvidin, were isolated from the aleurone layer of dark-purple rice grain. These compounds were shown to cause human leukemia cells to cease, or slow replication by inhibiting the G2/M phase of the cell cycle, therefore

inducing the damaged, incomplete cells into programmed cell death, ridding the body of their deleterious behavior (Hyun and Chung, 2004).

Anthocyanin-rich extracts of grape, bilberry, and especially chokeberry, were shown to be potent against colon cancer cells, inhibiting cell growth by 50% within 48 hours of exposure (Zhao et al., 2004). The anthocyanins were also shown to have an affinity for only cancerous cells, as non-cancerous cells were not harmed during the experiment.

Adom and Liu (2002) hypothesized that anthocyanins aid in promoting colon health because the bound phytochemicals are able to resist stomach digestion and are unscathed once they reach the colon. Adom and Liu (2002) also discovered that 87% of total antioxidants in the maize they studied were present as bound phytochemicals. Phenolic acids are specifically bound within the cell-walls of plants (Bily et al., 2004). 18 strains of Mexican maize of varying pigmentation found bound phenolic levels from 136-2720 mg/100 g while the free phenolic levels were much less at 33-680 mg/100 g (Lopez-Martinez et al., 2009). Andreasen et al. (2001) discovered that both human and rat colons are able to release various phytochemicals from cereal bran and thus pave the way for these bound phytochemicals to have healthful benefits to the human diet by resisting digestion until entry into the colon has been achieved. One of the main bound phytochemicals is diferulic acid, which is ester-linked to cell wall polysaccharides. A study found that these bound phenolic antioxidants can in fact be broken down and absorbed in the gastrointestinal tract (Andreasen et al., 2001). This discovery helps

improve understanding as to why diets high in cereal bran (fiber) may help to prevent colonic and other related cancers.

The positive effects of antioxidants further support the observations made in earlier work that oxidative damage which occurs naturally in the body increases the risk of cancer and cardiovascular disease (Temple, 2000; Wagner et al., 1992; Ames, 1979). Thus antioxidants have a role to play in everyday health and more sources of these pigments must be developed.

Another place where pigmentation can be difficult and expensive to procure is in livestock rations. Supplements such as alfalfa meal and/or marigold petals to provide vitamins and other nutrients have been used (Egesel, 2003). This expense has led to the use of synthetic sources of nutrition. Synthetic pigments lacking the same nutritional profile are beginning to fall out of favor in the opinion of the consumer, and would likely be unsustainable in the developing world.

Although sorghum (Salas Fernandez et al. 2008) and genetically modified rice (Wood, 2010) contain some beta-carotene, yellow maize has been the only major food grain found to contain a significant amount of beta-carotene; a primary source of vitamin A (Buckner et al. , 1990). This is critically important in the developing world where diets deficient in vitamin A lead to eye disease in 40 million children every year and also place 140 to 250 million people at risk for related health disorders (Underwood, 2004). By locating natural polymorphisms in the lycopene epsilon cyclase portion of the carotenoid pathway, selection on the basis of favorable alleles can now be done with relative ease with the use of molecular markers (Harjes et al., 2008). This was

accomplished by an association mapping study where common diversity in maize was surveyed. Upon close observation, limiting steps in the carotenoid pathway were focused on and breeding/selection is occurring for these. Molecular markers linked to these limiting steps allow for selection that may otherwise not be possible by phenotype alone.

Natural variation in carotenoids, as well as other pigments should be used to provide maize which is better suited for individuals living in developing nations who lack access to other pigment-rich food such as fruit and vegetables. This natural variation will allow maize to be modified without the insertion of transgenes. Using maize of unique colors and gaining a better understanding of other pigment pathways will encourage maize to become a sufficient alternative source of other healthful vitamins and phytochemicals.

Antioxidants are important due to their reducing power to neutralize radical oxidative species (ROS) within the body. Nitric oxide (NO) is an example of one ROS which is produced by inflammation of body tissues. The NO production pathway was suppressed in mice with high levels of NO present when they were administered with anthocyanin C3G, supporting the antioxidant activity of anthocyanins (Tsuda, 2002). Rice is often used in food processing today and with the nutritional sector moving more towards whole grains, rice bran has gained popularity. Pigmented rice cultivars outperformed white rice cultivars (Nam et al., 2006) when several rice brans were tested for antioxidant potential.

In addition to antioxidant activity, carotenoids also act as precursors for the plant hormones abscisic acid and strigolactones (Cazzonelli and Pogson, 2010). Abscisic acid

is very important for plant responses to stress while strigolactones inhibit shoot branching and work to stimulate symbiotic relationships with fungi in some plant species (Cazzonelli and Pogson, 2010). Additionally, one of the last genes in the anthocyanin biochemical pathway, *bz2,* codes for a polypeptide similar to various other stress-related proteins within the plant (Holton & Cornish, 1995).

Anthocyanins are present within plant vegetative tissue. They are produced by certain stresses such as wounding (Ferreres et al., 1997), UV light (Mendez et al., 1999), pathogens (Dixon et al., 1994) etc. These stresses cause the production of ROS within the plant and therefore anthocyanin production is thought to be a secondary plant defense (Philpott et al., 2004).

Anthocyanins and polyphenols do not hold up well when exposed to extreme heat and/or pH change. Because maize is rarely consumed without some type of food processing it is important to identify and gain further understanding of the interactions between the pigments of interest, food processing methods, and digestion. Purple maize, originally from Peru, where the dark purple beverage, Chicha Morada is common (Nicholson, 1960) has been highly studied. Morado is deep purple maize primarily used for the production of this traditional drink, as well as dye. Chicha Morada is made by boiling both kernels and cobs along with fruit which often include quince (apple and pear relative), pineapple skin, cloves, and cinnamon (Nicholson, 1960). Although a sweet drink, Chicha Morada is also used for its medicinal benefits (Lopez-Martinez et al., 2009).

Blue maize is currently being used to produce blue maize chips and tortillas (Betran et al., 2000; Lopez-Martinez et al., 2009). Nixtamalization, also known as lime cooking of maize for the production of tortilla chips, requires corn to be cooked in a 90-100 degree C° water bath along with calcium hydroxide. The calcium hydroxide allows for the separation of the endosperm (starch) from the pericarp of the kernel. To determine the degree of antioxidant loss from the blue maize due to nixtamalization, cooked maize was divided in half with one treatment being acidified and the other a normal treatment. The treatment of acid resulted in reduction of total antioxidant for tortillas and chips to be 11 and 17%, respectively (Del Pozo-Insfran et al., 2006). Anthocyanins were identified through spectroscopy of the maize samples in the 200-600 nm range with a unique band for glucoside derivatives occurring at 520 nm (Del Pozo-Insfran et al., 2006). The instability of anthocyanins under basic conditions were found elsewhere (Tsuda et al., 2003). The total amount of anthocyanins remaining in acidified tortillas was approximately 50%, with an 8-23% advantage over non-acidified tortillas (Del Pozo-Insfran et al., 2007). Acidification of maize products used in the nixtamalization process should be considered for special situations as in the use of colored maize. Acylated anthocyanins are less prone to color fade with increasing pH (Bakowska-Barczak, 2005). Also when nixtamalizing blue maize for food production, white endosperm is preferred as yellow tends to give the maize product a green hue, instead of the intended blue color (Betran et al., 2000). From personal experience, yellow endosperm also produces undesirable coloring for red maize as well.

Purple maize from China was examined by Yang and Zhai (2010) for anthocyanin content. This purple maize contained higher amounts of anthocyanins than found in previous studies, (Del Pozo-Insfran et al., 2006) however this could be the result of many different factors – including genotypes, environments, and laboratory methods of measurement. Five Chinese hybrids also showed great variability with the lightest colored hybrid yielding 1.27 mg/g while the darkest hybrid had 30.4 mg/g (Zhao et al., 2008). The purple maize grown in China had different growing conditions than other anthocyanin studies. These Chinese studies help to show that differences can be seen in data depending on where the study takes place.

Moreno et al. (2005) found the total anthocyanin content of four Mexican blue maize lines ranged from .54 to 1.15 mg/g, showing variation across genotypes for anthocyanins. Abdel-Aal (2006) found black rice had the greatest total anthocyanin content with 3.28 mg/g, far above maize where the purple (1.28 mg/g) and the red sweet (.61 mg/g) were the highest. So it can be said that anthocyanin content not only varies with environment but also with the genotype and intensity of the kernel color, based on this limited number of maize types and experiments.

In contrast to maize, sorghum anthocyanins tend to exhibit better stability at higher pH. Sorghum contains anthocyanins that do not have a hydroxyl group in the 3-position of the C-ring, and therefore are known as 3-deoxyanthocyanins (Dykes and Rooney, 2006). Because of this, sorghum holds promise as a commercial natural food colorant. To my knowledge no extensive characterization of maize pigments has been

undertaken, so it seems possible that these same types of anthocyanins could be found in sorghum's close relative, maize.

Other research has also been conducted to test the degradation of anthocyanins under stress. The optimum temperature for anthocyanin stability of various colored maize samples is 38 degrees C° and therefore yielded the best absorbance for total anthocyanin content (Abdel-Aal et al., 2006). The same study also found that wheat anthocyanin degrades when exposed to increasing temperatures from 65-90 degrees C°. In another stability test, purple maize extracts at pH 3 revealed a color which most closely matched that of the synthetic dye Red 40, while increasing pH above 3 resulted in a loss of color (Cevallos-Casals & Cisneros-Zevallos, 2004). This is an important find because Red 40, like other synthetic dyes may have negative health effects. (Jacobson, 2010). Promotion and availability of natural colorants is desirable to offset dependency on synthetic pigmentation.

Two main chemical configurations of anthocyanins; acylated or nonacylated have been discovered (Moreno et al., 2005; Dougall et al., 1997). The most common type found in maize leaf tissue and flowers is the nonacylated type while acylated anthocyanins were more common in grain of four samples of land race maize from Mexico (Moreno et al., 2005). These observations made from a limited number of studies and sample sizes require further research to validate these findings.

Nonacylated anthocyanins may be less desirable for food production because they exhibit less stability under variable pH (Dougall et al., 1997). On the other hand, vegetable crops show decreased bioavailability of acylated anthocyanins compared to

their nonacylated counterparts (Charron et al., 2009; Charron et al., 2007). Purple carrot juice was administered to human subjects and their plasma was measured for eight hours following consumption for the presence of anthocyanins. The nonacylated anthocyanins were present at a concentration level 4 times higher than that of acylated anthocyanins even though 76% of the total anthocyanins in the carrot juice were acylated (Charron et al., 2009). A 4-fold advantage for bioavailable nonacylated anthocyanins were also discovered in a red cabbage study (Charron et al., 2007).

No study in maize has investigated acylated vs. nonacylated bioavailability. Thus we assume that maize anthocyanins would behave in a similar fashion. C3G, the most common anthocyanin in maize, is nonacylated (Styles and Oldriska, 1972). Moreno et al. (2005) found the percentage of acylated anthocyanins among four maize genotypes ranged from 42-63.3%. Common compounds among acylated anthocyanins include caffeic, p-coumaric, ferulic, and sinapic acid (Bakowska-Barczak, 2005). Although the health implications remain unknown, other anthocyanins have also been isolated. For instance, cyanidin and malvidin were the main compounds found in blue maize (Hyun & Chung 2004), while pelargonidin was identified as a third major compound (Moreno et al., 2005).

Blue and some red maize contain pigments in both the pericarp and aleurone layer. The pigmentation found in the pericarp allows easy mechanical extraction (Moreno et al., 2005), making maize pigments accessible as food additives and other related products. Moreno found anthocyanins in the pericarp of red kernels (115.05 mg/g) were higher than that found in grape skins (Ryan and Revilla, 2003; Munoz-

Espada et al., 2004). Total anthocyanin content was recorded from flour samples of degermed kernels, pericarp, and endosperm. After an extraction with a methanol/acetic acid water mixture, spectrophotometry was used to quantify anthocyanins at 520 nm.

Colorimeters are used to give a standardized set of values to describe outward appearance of the maize kernels. Three values are given; L, a, and b. The L value represents how well light passes through the kernel. The a value measures the red:green ratio, while the b value measures the yellow:blue ratio (Del Pozo-Insfran et al., 2007; Moreno et al., 2005)

Total anthocyanin or phenol content can be measured by grinding whole kernels and adding extractive solvents (methanol, HCl). The pigments leach out into solution and their absorbance can be read using a UV-vis spectrophotometer (Lopez-Martinez et al., 2009). High-performance liquid chromatography (HPLC) is often used to analyze purified (free of kernel debris) anthocyanin extracts. It typically includes use of a column with varying gradients of solvent used to separate pigments for detection at 250 to 600 nm. The majority of bands are detected at 520 nm ((Del Pozo-Insfran et al., 2007; Moreno et al., 2005; Brenna & Berardo, 2004). The samples are compared to commercially available standards to properly identify the chemical compounds (Moreno et al., 2005).

Wet chemistry analysis of kernel pigments is undesirable to rapidly and efficiently analyze hundreds to thousands of samples in a single growing season. Because of this, wet chemistry is used to develop calibration curves for near infrared spectroscopy (Brenna & Berardo, 2004) instruments which scan a sample and based on

interactions between the light and the sample, feedback in the form of wavelength banding patterns are produced. These patterns, plugged into the proper calibration equation, can be used to give similar information achieved through wet chemistry but in a fraction of the time and cost (after initial investment of NIR equipment). Traditionally this has been done with a ground sample but at Texas A&M we are currently experimenting with whole kernel analysis. Derivations of these methods exist and individuals should use those which best fit their needs and abilities.

Anthocyanins (red and purple), carotenoids (yellow), and white pigment have been genetically characterized as controlled by dominant alleles for red aleurone (*pr1*) , colored aleurone (*c1*), colored (*r1*), and white (*y1*). The genetics behind blue and dark-purple maize is understudied (Ford, 2000). Additionally, many of the studies were conducted using only visual qualitative measurements, where more sophisticated and quantitative analysis methods may detect the presence of many modifiers. Most of this discussion covers the anthocyanin pathway. Additional factors control color expression in the aleurone layer and include anthocyaninless-1 (*a1*), anthocyaninless-2 (*a2*), bronze-1 (*bz1*), bronze-2 (*bz2*), colorless-2 (*c2*), defective kernel-1 (*dek1*), and viviparous-1 (*vp1*) (Selinger & Chandler, 1999; Styles and Ceska, 1972). Purple color is complex and requires a dominant allele at every factor for the deepest shades of purple (Styles and Ceska, 1972).

Of these factors, eight are enzyme-related genes (*a1, a2, bz1, bz2, c2, chi, pr, and* whp) which are used to catalyze the production and transport of anthocyanin as well as five genes responsible for regulation (*b, c1, pl, r, and vp1*) which control specific plant

organs from which anthocyanins are expressed throughout the plant (Hanson et al., 1996). The anthocyanin pathway is as follows: *c1-c2-r-In-a1-a2-bz1-bz2*, where the c genes regulate anthocyanin formation in the kernel but not in plant tissues, a1, a2, and r are complimentary genes. *In* causes an intensification of pigment when in the recessive form (Styles and Ceska, 1972).

As discussed, two of the main anthocyanins are derivatives of cyanidin and pelargonidin. Plants with the *pr* alleles in dominant form primarily produce cyanidin derivatives, while plants with the recessive alleles produce pelargonidin derivatives (Styles and Ceska, 1972). Cyanidin is thought to hinder the growth and division of leukemia cells and thus could be used as a selection marker to produce vegetables with increased cyanidin levels. Maize pigments are commonly found in the aleurone requiring functional copies of the *c2, a1, a2, bz1, and bz2* alleles (Selinger & Chandler, 1999).

QUALITY PROTEIN MAIZE

In the 1920's a field laborer harvesting corn by hand observed an ear with chalky, soft kernels and turned it into a Connecticut experiment station. More than 40 years later, Purdue University professor Edwin Mertz began study of amino acid profiles from a diverse group of maize. The soft kernels from the discovered ear revealed lysine content twice those of any previous sample. Mertz and Bates (1964) were the first to report on the actual nutritional advantage of the *opaque*-2 maize and identify the mutant genes. From that point on, the development of high protein, now known as quality protein maize (QPM) has been the subject of vast amounts of research (Vietmeyer, 2000).

Although *opaque-2* maize holds an advantage over normal maize in protein quality (a higher percentage of lysine, and other essential amino acids), it has drawbacks in major agronomic traits. Negative pleiotropic effects of the opaque-2 allele result in late maturity as well as lodging issues to taller plants and thinner stalks (Salamini et al., 1970). When compared to QPM, *opaque-2* maize is soft, susceptible to mechanical damage, and has reduced yields of 8-15%. The plants are less resistance to disease and insect damage (Lambert et al., 1969; Salamini et al., 1970). *Opaque-2* mutants have increased lysine, histidine, arginine, aspartic acid, glycine, and cysteine when compared to normal maize (Mertz and Bates, 1964). In the time since opaque-2 mutants were used, as many as 100 different opaque mutants have been identified. While some of these mutants are allelic many are not (M.P. Scott personal communication)

Cereals grains are the primary source of calories in the developing world but lack adequate protein quantity and quality. Furthermore, most conventional cereal grains contain ~2% lysine, less than half the amount recommended (FAO, 1985) leaving a void in human nutrition. Wheat, like normal maize is severely lysine deficient, while rice has a low, albeit more balanced amino acid profile (Graham et al., 1990). This problem can be rectified by adding legumes and other protein sources to diets. Protein supplementation has its limitations in developing countries where such protein sources are often cost prohibitive. QPM may become adopted as a staple in daily diets due to comparable yields to normal maize where subsistence agriculture is most commonly practiced. With normal maize protein having a nutritional value 40% (bioavailability) of that compared to milk (Zaidi et al., 2009).

Increased protein quality goes beyond improved amino acid composition to include bioavailability of the nitrogen contained within the protein, allowing humans and livestock alike to utilize the nutrients with increased efficiency. The amount of N retained in the body from QPM is approx. 80%, compared to 40 to 57% in normal maize (Pixley & Bjarnason, 2002). The greater concentrations of essential amino acids arginine, histidine, lysine, tryptophan, and valine in QPM when compared with normal maize (Burgoon et al., 1992), showing the results of the improvement of the quantity as well as quality of protein.

Based on a child diet study, no difference was seen between those who consumed QPM as 90% of their daily diet with those consuming a whey protein/milk diet (Graham et al., 1990). This and other studies like it in both humans and livestock have shown QPM diets can be valuable to reduce malnutrition problems where other sources of protein rich foods are seldom available (Bressani, 1992; Clark, 1978; Mertz, 1992).

Throughout the world, livestock rations increase in cost due to protein supplementation necessary for a balanced maize based diet. Comparison of QPM rations to conventional rations produced starter pigs with higher rates of gain and efficiency of gain (Burgoon et al., 1992; Lopez-Pereira, 1992; Sullivan et al., 1989). The use of QPM in livestock rations could inexpensively minimize/eliminate the reliance on soybean meal or other supplementation.

India became one of the first countries to research endosperm modification shortly after the opaque-2 mutant discovery in 1964. This is likely due in part to the importance that cereal grains have in the daily diets of the citizens living there. In 2001,

85% of maize produced in India was still utilized for human consumption. The development of QPM was an important research objective towards the improvement of dietary health (Prasanna et al., 2001). Analysis of maize with varying opaqueness began later at CIMMYT in 1969 by John Lonnquist and V. L. Asnani. Using a modified back-crossing method, maize from a variety of genetic backgrounds including tropical and subtropical along with the congruent introduction of modifier genes and selection for a harder (flintier) endosperm (Vasal, 2001) were used to develop QPM. Efforts for high protein maize development focused on incorporating the *opaque-2* gene into elite normal germplasm creating the unmistakable chalky endosperm typically resulting in a 10-15% yield reduction once converted to QPM (Vasal, 2002).

Segregating ears in crosses for the *opaque*-2 modifier genes showed kernels with a ½ opaque to ½ vitreous phenotype, while the remaining kernels on each ear were completely opaque. Lysine content however remained the same within either kernel type across same ear (Paez et al., 1969). This led to selecting vitreous kernels with some opaque markings to identify the presence of the mutant gene. Several methods have been used to screen the *o2* germplasm for harder, vitreous kernels.

The first was the light box method (Paez et al., 1969) which used the transmission of light through the kernel to determine the level of opacity. Since the amino acid profiles of the translucent and opaque portions of the same kernel were not different (Paez et al., 1969), the light box became a useful tool in converting the *o2o2* germplasm to the improved QPM. Kernels that allowed varying degrees of light to pass were kept for their hard endosperm qualities. A certain level of opacity was selected to

insure retention of the *o2* gene during screening. In 1977 Loesch et al. proposed a method of kernel shearing strength analysis which involved pressing kernels with specified amounts of pressure and determining their strength by the resulting fragments. Due to high cost and practicality to breeding programs, this method never became popular and research relied predominately upon the light box.

Once QPM sources were established, the ability to continually increase the protein quality while simultaneously improving yield and quality of elite material became the primary breeding goal. The highest levels of lysine, tryptophan, and methionine from a cross of B73o2o2 x CML 161 (both of which are included in this thesis) were found in the opaque kernels, while levels higher than normal were still found in the flinty kernels (Gutierrez-Rojas et al., 2008). Through proper kernel phenotyping, *opaque*-2 material can be recombined with existing QPM material to increase the amount of genetic variation available for developing new genotypes.

Commercial maize is grown as a hybrid; it is never grown as an inbred and rarely grown as a landrace except in subsistence production because of lower yields. Because of this, heterosis in QPM material is important. When compared to three-way or double-cross hybrids, single-cross hybrids have yielded less, leading researchers to believe that QPM inbred lines lacked the potential for heterosis (Pixley and Bjarnason, 2002). Grain protein and especially lysine and tryptophan have been shown to increase under drought stress and results suggest that grain yield and grain protein of QPM hybrids have the least stability while tryptophan and lysine levels are the most stable when analyzed across stressed and normal growing conditions (Zaidi et al., 2009). Generally, higher

grain yielding QPM hybrids exhibit lower protein yield/quality which is intuitive since most of the increases in grain yield are the result of increasing starch. This tradeoff (lower yields) makes the continual improvement of QPM a challenge. Despite this, a positive correlation between endosperm hardness improvement and other desirable agronomic traits has been found, suggesting the ability to improve multiple traits simultaneously (Pixley and Bjarnason, 1993 & 2002).

Although the *opaque-2* allele was the first mutant to be found which causes an increase in the quality of essential amino acids in the maize kernel, other mutant alleles were later discovered. A second allele with similar effects was identified as *floury-2,* (Nelson et al., 1965) while additional mutants *opaque-7* (McWhirter, 1971), *opaque-6*, (Ma & Nelson, 1975), *floury-3* (Ma & Nelson, 1975), and others were later discovered. None of the other mutants have resulted in an advantage over *opaque-2* alone, so the majority of research and advancement has occurred with the original mutant allele discovery with limited research investigating *floury-2* (Vasal, 2002). *Opaque-2* and *floury*-2 mutants are located on chromosomes seven and four, respectively (Vasal, 2002). Mutant opaque-7, opaque-6 and floury-3 are smaller effect mutants with similar endosperm interactions but independent of the specific *opaque-2* or *floury-2* genes (Di Fonzo et al., 1980). Although the two primary mutants share a similar amino acid profile, *floury-2* holds an advantage in methionine content (Nelson et al., 1965). As opaque-2 mutant maize was converted into today's QPM, a correlation was found between increased endosperm hardness and two loci both linked to the expression of gamma-zein storage proteins located on chromosome 7 and 7L, respectively (Lopes et

al., 1995). Close linkage was found between one of these "modifier" loci and the gamma-zein gene. The effect of the modifier loci could not be easily observed unless individuals being modified were homozygous for the opaque-2 locus, resulting in the need for opaque-2 and modifier pools of germplasm to use in crossing schemes (Lopes et al., 1995). In an earlier study of the inheritance of modified endosperm in a set of diverse *opaque-2* backgrounds Belousov (1987) found gene dosage effects on the texture of the kernel as well as incomplete penetrance (expression) of modifier genes, lending support to the Lopes et al. assertion that many genes are involved in the modification of endosperm. In one particular case the addition of modifier loci to produce vitreous QPM lines also increased production of enzymes crucial to the production of lysine while reducing lysine catabolism, resulting in a higher level of total lysine available in the kernel with improved grain hardness (Gaziola et al., 1999).

The traditional kjeldahl method estimates protein content by determining total nitrogen in the grain and multiplying that number by a factor of 6.25 in the case of maize, and 5.7 in the case of wheat (Williams, 1973). In the case of QPM, methods targeting specific amino acid content were also desirable.

Since a trademark of QPM is elevated tryptophan levels, a procedure was developed where tryptophan and glyoxylic acid react to produce a colored compound when combined with sulfuric acid and ferric chloride (Nurit et al., 2009). Analysis of the colored compound at 560 nm gave results comparable to a similar acetic acid-based colorimetric procedure (r^2=.80) as well as high pressure liquid chromatography (r^2=.71) (Nurit et al., 2009). Colorimetric analysis via another method which reacts with the

lysine in the sample has also been used and relies on detection of absorbance patterns at 400 nm (Tsai et al., 1972).

An HPLC study concluded that analysis of the alcohol-soluble prolamins made it possible to differentiate between QPM, normal and original opaque-2 maize due to the lower concentration of the prolamine fraction associated with QPM (Paulis et al., 1992). This discovery assisted in QPM breeding selection in the early stages.

A long used method for measuring lysine and other amino acid content in maize is ion-exchange chromatography (Moore and Stein, 1963). A grain sample is subjected to a negative or positively charged column and the appropriate molecules are drawn to their respective column. Lysine is an example of a positively charged amino acid and would be thus collected by the negatively-charged column. Mass spectrometry is usually performed in conjunction with liquid chromatography procedures to determine size and chemical structure of specific proteins in simple mixtures (Link, et al., 1999). Specifically, tryptophan and methionine content can be determined by a microbial-digestion method involving hydrolysis and extraction of protein and digestion by microbes (Scott et al., 2004). After an incubation period of 16-20 hours, absorbancy readings at 595 nm are able to estimate tryptophan and methionine concentrations from grain by the amount of amino acids in the microbe cells.

The use of near infrared spectroscopy to estimate protein content through analysis of banding patterns at specific wavelengths has been reported (Baye et al., 2006; Berardo et al., 2009). Of particular interest has been the ability to predict specific amino acid concentrations while avoiding the expensive lab procedures otherwise necessary. In

barley, lysine calibrations have been successfully developed (Osbourne, 2006). In terms of ground corn samples, accurate calibrations (R^2=.78-.98) were found for many amino acids including lysine, methionine, and tryptophan (Fontaine et al., 2002).

CHAPTER III

COMBINING ABILITY FOR IMPORTANT ECONOMIC, AGRONOMIC, AND COMPOSITIONAL TRAITS IN A DIVERSE SET OF COLORED (RED, BLUE, PURPLE) MAIZE [Z. *mays*]

INTRODUCTION

Throughout the world, including the US, maize is primarily grown as yellow or white grain with only a few countries such as Mexico, have food preferences for blue (Betran et al., 2000) and one country, Peru, prefers the dark-purple maize used for the popular drink Chicha Morado (Nicholson, 1960). Grain colors in maize naturally occur involving several pigments and compounds including carotenoids, anthocyanins, and phenols which create coloration in the pericarp, aleurone layer, and/or endosperm. Blue, red, and purple color is found primarily in the aleurone and/or seed coat. Anthocyanins are the primary components of these pigments while carotenoids are yellow/orange pigments found in the endosperm.

Anthocyanins, flavonoids, phenolic acids, etc. are all polyphenols that belong to a larger group of phytochemicals (Del Pozo-Insfran et al., 2007). Plant phytochemicals are secondary metabolites that may serve several location-dependent functions within the kernel and have been implicated in a number of different phenotypes (Lopez-Martinez et al., 2009). Phenolics in the germ correspond to *Fusarium* spp. tolerance (Bakan et al., 2003); endosperm phenolics cause graying of dough and tortilla color during nixtamalization (Salinas-Moreno et al., 2007); while pericarp phenolics assist in storage pest tolerance (Arnason et al., 1992). However many of these compounds are

only found in low yielding landraces and locked in germplasm repositories (Widrlechner and Dragula, 1992 and 1998).

Blue maize developed by Texas AgriLife (Betran et al., 2000) is a rare example of inbred breeding efforts in place for the improvement of colored maize. In the US only a few suppliers have one or a few hybrids and many end users rely on open-pollinated landrace cultivars such as 'Hopi Blue'. Thus, throughout the world, colored corns are often plagued by low yields and inconsistent pigments that increase the cost of consumption and may affect pigment quality.

Total phenolics have been reported (Sosulski et al., 1982; Del Pozo-Insfran et al., 2006; De la Parra et al., 2007) in several studies but with a limited number of genotypes as well as a non-breeding emphasis. Anthocyanins are present in the free form, while many phenolic compounds are bound (approx. 80%) (Lopez-Martinez et al., 2009). Ferulic acid is the primary phenolic compound in maize, constituting 85% of total phenol content (Cabrera-Soto, et al., 2009). Qualitative genetic control of red, purple, yellow, and white color in maize has been well established in classical genetic studies while quantitative variation and modifiers largely remain unexplored.

Qualitative genes conditioning anthocyanins (red, and purple), carotenoids (yellow), and white pigment are primarily controlled by dominant alleles for red aleurone (*pr1*), colored aleurone (*c1*), colored (*r1*), and white (*y1*) (Ford, 2000). Additional factors controlling color expression include anthocyaninless-1 (*a1*), anthocyaninless-2 (*a2*), bronze-1 (*bz1*), bronze-2 (*bz2*), colorless-2 (*c2*), defective kernel-1 (*dek1*), and viviparous-1 (*vp1*) (Selinger & Chandler, 1999; Styles and Ceska,

1972). Purple color is complex and requires a dominant allele for every factor for the deepest shades of purple (Styles and Ceska, 1972). This purple should not be confused with the dark-purple seen from 'Maiz Morado' a Peruvian landrace (Nicholson, 1960). The genetics behind blue is poorly understood and no genetic research into the inheritance and genetics behind 'Maiz Morado' has been reported. Still, natural colorants high in antioxidants are becoming increasingly desirable in certain parts of the world due to perceived health benefits.

The ability of antioxidants to provide healthful benefits has been thoroughly investigated in both animal and human studies. In mice fed a diet high in fat along with purple (non-morado) maize colorant, 1/3 less total lipid accumulation occurred in their livers (Tsuda et al., 2003). Antioxidants from rice have been shown to condition negative effects on leukemia (Hyun and Chung, 2004) and grape extracts have been shown to decrease colon cancer (Zhao et al., 2004). Further positive health effects of antioxidants are supported by observations that oxidative damage occurring naturally in the body increases the risk of cancer and cardiovascular disease (Temple, 2000; Wagner et al., 1992; Ames, 1979) and can be prevented through the consumption of antioxidant-rich foods. The role of antioxidants in everyday health will continue to expand and more sources of these pigments as well as easier access to those sources must be explored.

The objectives of this research was: 1) to determine the ability of colored maize lines to increase antioxidant potential (as measured by total phenolics) in grain; 2) to estimate antioxidant potential; 3) to increase understanding of inheritance of diverse kernel pigmentation; 4) to assign heterotic and combining ability groups to new colored

Texas AgriLife lines; and 5) to identify secondary traits correlated with grain color and yield.

MATERIALS AND METHODS

Germplasm

Germplasm included 11 inbred lines (Table 3-1) and the 84 produced hybrids from the diallel mating design (Table 3-2). Six of the inbred lines were developed by Texas AgriLife in College Station, two from Texas AgriLife in Lubbock, one purchased from Redwood City Seed Company (Redwood City, CA), and two commercial lines (Monsanto Company 1991, 2001). Source seed of AgriLife inbred lines originated from 2009 nurseries, with the remaining three parents ordered in 2010. The eleven lines consisted of three blue, five red, one purple, and two yellow, encompassing a wide array of kernel pigmentation (Table 3-1). In 2010, the eleven parents were mated in a full diallel (reciprocal crosses) mating design performed in CS and WE produced 84 hybrids. Missing hybrid combinations resulted from flowering time asynchrony and incompatibility of select parents.

Hybrid yield and composition trials were conducted in CS, WE, and Ames (AM), Iowa during the summer of 2011. Seed was produced in WE fall 2009 for a pilot study in summer of 2010 in both CS and WE involving a subset of 33 hybrids in unreplicated trials.

Experimental Design

The diallel mating design allowed us to estimate general combining ability (GCA), and specific combining ability (SCA) of lines (Fehr, 1987; Griffing, 1956).

Table 3-1. Germplasm by parent number including pedigree and parent name.

Parent	Pedigree	Name
1	((Lfy2361-B/Tx114 (B73w)-B Dark blue-B)Tx114/Lfy2304-B-B-B-1-2-B-B-B-2-B-B-B)	lfy Blue
2	Ethiopia15-B-5-1-B-B2-B-1-B-B-B	Ethiopia Blue
3	Red Hybrid Ear-B-1-2-2-1-B-B	Red Hybrid Ear
4	Red Ear 2-2-2-1-1-2-B-B	Red Ear
5	WX-LBRED1	Wenwei1
6	WX-LBRED2	Wenwei2
7	LH195	LH195
8	LH287	LH287
9	Ethiopia12-B-3-3-B-B2-B1-2-B-B-B	Ethiopia Blue2
10	(LAMA2002-23-1-B-B/LAMA2002-11-1-B-B)-B-B-B-B-B-1	LAMA Red
11	Maiz Morado	Maize Morado

Yield trials were planted in a randomized complete block design with 88 (CS and WE) and 84 entries (AM). Yield plots were 3.96 m in length with row widths of .76 (CS, AM) and 1.01 (WE) meters and were thinned to a population of approximately 64,000 plants ha^{-1}. Blocks were assigned as two replications per location. Due to the contamination of foreign pollen upon the ear (Xenia effect) that was first observed during a 2010 pilot study, five (WE) and three (CS, AM) ears were chosen at random to be self-pollinated and hand-harvested separately from the rest of the plot to mimic the effects of each hybrid planted in an isolated grower's field. WE and CS locations harvested all ears in the plot to obtain yield in addition to composition data. In AM only self-pollinated ears were harvested allowing for composition data only.

Table 3-2. Hybrids produced from diallel mating design. Commercial checks included BH9014VT3, BH9440W, DKC67-23, and DKC68-06.

	Parent 1	Parent 2	Parent 3	Parent 4	Parent 5	Parent 6	Parent 7	Parent 8	Parent 9	Parent 10	Parent 11
Parent 1	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Parent 2	No	-	Yes	Yes	No	No	No	No	No	No	No
Parent 3	No	Yes	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Parent 4	No	Yes	Yes	-	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Parent 5	Yes	Yes	Yes	Yes	-	Yes	Yes	Yes	Yes	Yes	Yes
Parent 6	Yes	Yes	Yes	Yes	Yes	-	Yes	Yes	Yes	Yes	Yes
Parent 7	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes	Yes	Yes	Yes
Parent 8	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes	No	No
Parent 9	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	No	No
Parent 10	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	Yes

Phenotypic Measurements

Plant height was measured in the field from the base of the plant to the tip of the tassel and ear height was measured from the base of the plant to the ear node. Flowering time was measured by the number of days from planting to when 50% of the plants were either shedding pollen (days to anthesis) or silk (days to silk). Ears were shaded and dried in a greenhouse (CS, WE) or in a forced air dryer (AM). Self Pollinated ears were shelled using a hand sheller while open pollinated ears were shelled using a mechanical ear sheller (Agriculex, Guelph, Ont., Canada) and weights were taken. Grain moisture was measured at time of harvest with a mini GAC plus (Dickey-john, Minneapolis, MN). Kernel weight was measured using a model U seed counter (International Marketing and Design Co., San Antonio, TX) to count 500 kernels per genotype which were then weighed.

Sample sizes of 50 grams were initially ground to a 2 mm fineness with a Polymix® PX-MFC 90 D mill (Kinematica Ag, Bohemia, NY) and then further ground by the Cyclone™ sample mill (UDY Corporation, Fort Collins, CO) to 1 mm fineness. Starch, crude protein, fat, and phosphorous were determined by Fourier transformed near-infrared reflectance spectroscopy (FT-NIRS). A calibration curve was developed in the Texas AgriLife corn breeding program in College Station on a Thermo Antaris II (Thermo-Fisher) FT-NIRS updated to include 20 outlier samples from this study. Wet chemistry on these samples was collected by Ward Laboratories (Kearney, NE). Samples were scanned in reflectance 32 times using a rotating cup over wavelengths from 4,000

to 10,000 wave numbers (cm^{-1}) at increments of 2 cm^{-1}, and then converted to absorbance.

Total Phenol Extraction, Quantification, and Analysis

Total phenolics were measured via the Folin-Ciocalteu method (Lopez-Martinez et al., 2009). A 0.3 gram ground subsample (see above) was weighed for each field plot and analysis was performed in triplicate to account for lab errors. Each day of analysis was noted and included in statistical analysis to account for changes in room temperature/humidity, standards, etc.

Subsamples and 25 mL of 1% HCL in methanol were placed into plastic round-bottom extraction tubes. Up to 40 field samples in triplicate were extracted and analyzed for a total of 120 observations per day. All tubes were shaken for two hours to allow for the extraction. Tubes were centrifuged to remove plant material from the supernatant.

Gallic acid was used to develop a standard curve to determine the gallic acid equivalent (GAE) estimate for total phenolics. Five standards were made with different levels of both gallic acid (0, .025, .05, .075, and .1 ml) and methanol added for a 0.1 ml total volume. The five standards were added to 1.1 mL methanol, 0.4 mL Folin reagent, and 0.9 mL ethanolamine. Ethanolamine reacts with the Folin reagent to produce a blue pigment with variation in darkness in direct correlation with the amount of gallic acid present. Each standard was also made in triplicate, and analyzed with a UV-VIS spectrometer (Shimadzu Scientific, Columbia, MD) calibrated to detect gallic acid absorbency curves at 600 nm. The standard curve consists of the five absorbance readings which were used to correlate with readings from extracted samples.

For analysis, 0.1 ml of the extracted sample was used in place of the standard in the above procedure. The extraction solution of 1% HCL in methanol is the mixture of 10 mL HCL diluted to 1000 mL with methanol. A 1:5 ratio of Folin to distilled water is used for the Folin reagent. The 0.5 M ethanolamine reagent is produced by diluting 30.9 mL ethanolamine to 1000 mL with distilled water. The 200 ppm gallic acid standard included 20 mg gallic acid dissolved in 100 mL methanol. The reaction takes place for 20 minutes before spectrometer analysis. Eight field samples (24 tubes) are analyzed per run due to time sensitivity of the reaction.

Statistical Analysis

SAS 9.2 (SAS Institute, INC., Cary, NC, USA) PROC MIXED procedure was used to determine percent variation for all traits across all hybrids produced. PROC CORR was used for correlations across traits and proc GLM was used for mean separation. Separately, DIALLEL-SAS05 (Zhang et al., 2005) was used to produce general (GCA) and specific combining ability (SCA) estimates. Analysis within the program was based on model 1, method 4 (Griffing, 1956) which accounts for a half diallel, (no reciprocals) and no parents. The DIALLEL-SAS05 program relies on all crosses being successful in order to estimate GCA and SCA. Since the full set of reciprocals was not completed for all lines, a half diallel was analyzed instead, with three hybrids (out of 45) being substituted for their reciprocal in the program.

RESULTS AND DISCUSSION

Agronomic traits; days to anthesis, days to silk, and height were significantly and substantially different in AM than in CS or WE. The 50% silk and anthesis dates ranged

over 6 days in WE, 20 in AM, and CS in the middle with 14 (silk) and 12 (anthesis). Maturity is usually shortened in WE due to warm early season temperatures and the rapid accumulation of growing degree days while the greater maturity range in Ames suggested probable photo-period sensitivity in some hybrids. The length of plant vegetative growth is related to maturity (Sacks and Kuckarik, 2011) and the genotypes as a whole continued to grow for a longer period of time before initiating reproductive growth at the AM location. This was observed with the difference in means for height traits in AM compared to WE and CS. Because maize production in the United States is primarily in the Midwest it was important to have a test in AM to evaluate Texas material and the various traits were affected by an extreme environment to which they were not adapted.

2011 grain yields (Table 3-3) were higher in CS than WE as expected due to fewer environmental stresses in CS (moisture, temperature). Grain yield was not measured at AM because the plants were still at very high moisture at harvest; a clear effect of this Texas material being unadapted to a Midwest environment. Because of this lack of adaptation, only the three selfed ears were harvested from each plot for seed analysis. Total phenolics measured as μg/g gallic acid equivalency (GAE) demonstrated little change in mean across all three environments, unlike many other traits that differed greatly between AM and the Texas locations.

In our 2010 study, selfing the hybrid ears eliminated segregation for pigmentation on the ear. In 2011, selfing ears from each plot delivered repeated results with the exception of one parent. Parent 3 (‘red hybrid ear’) segregated in hybrid

Table 3-3. Test locations for the eleven parent diallel mating design. † Yield and moisture recorded in College Station (CS) and Weslaco (WE) only.

	College Station, TX		Weslaco, TX		Ames, IA	
Traits	Mean ± S.D.	Min, Max	Mean ± S.D.	Min, Max	Mean ± S.D.	Min, Max
Grain yield (kg ha^{-1})	7783 ± 1695	2448, 11611	6653 ± 1883	1193, 10607	N/A †	N/A†
Grain moisture g kg^{-1} (at harvest)	142 ± 17	98, 189	150 ± 19	105, 205	N/A †	N/A†
Weight (g/500k)	155 ± 20	92, 211	163 ± 20	88, 220	142 ± 26	72, 214
Days to silk (50%)	72.8 ± 2.5	67, 81	74.0± 1.1	72, 77	73.6 ± 4.55	65, 84
Days to anthesis (50%)	71.6 ± 2.4	67, 79	74.0 ± 1.1	72, 77	72.6 ± 3.98	65, 84
Plant Height (cm)	202 ± 13	165, 231	194 ± 14	137, 229	233 ± 32	100, 320
Ear Height (cm)	67 ± 11	36, 97	61 ± 9	36, 86	111 ± 21	60, 180
Composition traits						
Total phenols (µg g^{-1})	269 ± 78	180, 682	260 ±78	171, 619	264 ± 72	193, 690
Moisture g kg^{-1}	111 ± 0.3	105, 118	114 ± 2	108, 121	95.8 ± 2.3	88.9, 103.6
Fat g kg^{-1}	30.6 ± 6.9	16.9, 51.1	31 ± 7	14, 56	33.3 ± 4.2	20, 45.6
Starch g kg^{-1}	659.5 ± 17.6	614, 702.8	656 ± 17	611, 699	676.1 ± 16.1	637.1, 718.6
Crude Protein g kg^{-1}	123.1 ± 11.9	93.7, 158.7	129 ± 12	102, 165	106.7 ± 11.7	72.9, 138.1
Phosphorous g kg^{-1}	3.6 ± .1	3.2, 4.0	4 ± .1	3.2, 3.9	3.7 ± .1	3.4, 4.1

combinations with blue parents as red ears, or a mixture of blue and white kernels on the same ear. Parent 3 is fully inbred with alleles fixed after 10+ generations of selfing. The observed segregation did not occur in all crosses and seems most likely to result from possible transposon effects.

The largest differences in the five seed composition traits (moisture, fat, starch, crude protein, and phosphorous) were seen at AM and involved moisture and crude protein. AM had the highest mean starch across the test since some of these hybrids were

harvested before physiological maturity (black-layer) with starch being produced in the later reproductive stages and continuing accumulation through maturation (Hoseney, 1998). Phosphorous showed little variability as a whole (both by FT-NIRS and wet-chemistry) and further research is necessary to more accurately detect this trait with increased statistical power.

Sources of Observed Variation

The data was analyzed across all three environments (where feasible) to determine sources of variation in the study. Environment was highly significant ($P \leq 0.01$) for all measured traits except grain yield, plant and ear height, and phosphorus (Table 3-4). A replicate effect was observed for all traits excluding grain moisture, kernel weight, total phenolics, and crude protein. A small, yet significant source of variation for day of total phenolic analysis was observed, explaining a source of error that was effectively partitioned from the residual. The hybrid genetic component was highly significant and the diallel mating design allowed the genetic component to be divided into males and females (GCA) as well as the interaction between them (SCA). GCA and SCA represent additive and dominant gene action, respectively. All traits exhibited significant GCA effects, except grain moisture. With the exception of phosphorous, all traits had highly significant SCA effects.

The small (4.3%) hybrid genetic component for phosphorous does not provide conclusive evidence to support predominant GCA or SCA effects since the residual for this trait was high and therefore a lack of sufficient power to interpret existence of genotypic effects. This lack of genotypic effect does point to the possible lack of genetic

Table 3-4. The percentage of observed variation and significance each source explains. Environment (Env), Replicate (Environment) (Rep), Day of analysis (Day), specific combining ability (SCA), general combining ability (GCA), general combining ability x environment (GCA x Env), specific combining ability x environment (SCA x Env), Residual error (Res). + Traits only analyzed in CS. ++ Traits analyzed in CS and WE. *P < 0.05, **P < 0.01. Heritability estimates in () were a result of IA removal from the analysis.

				Gen			Gen*Env					
	Env	Rep	Day	SCA	GCA		SCA*Env	GCA*Env		Res	H^2	h^2
Traits					male	female		male	female			
Grain yield kg ha^{-1}	14.5	4.0**		11.3**	13.7**	14.8**	13.4**			28.3	.49	.35
Grain moisture g kg^{-1} (harvested)	9.7*			13.6**				9.3**		67.5	.15	
Weight (g/500k)	18.6**			9.5**	9.1**	7.3*	14.0**	5.8**	6.0**	29.8	.32	.20
Days to silk^{+} (50%)		3.0**		23.0**	27.1**	21.0**				25.9	.73	.50
Days to anthesis^{+} (50%)		1.8**		10.5**	39.9**	25.8**				22.0	.78	.67
Plant height^{++}	8.2	3.1**		37.7**	8.3*	9.2*		2.0*	1.9*	30.0	.62	.20
Ear height^{++}	6.0	8.1**		10.5**	11*	11.7**		4.9**	3.2*	44.7	.39	.26
Total phenol µg g^{-1}	46**		2.0**	3.0**	40.0**	3.0**	4.0**			4.0	.85	.80
Crude protein g kg^{-1}	45.1**			2.4*	13.4**	11.2**	5.0**			22.9	.49 (.60)	.45 (.52)
Starch g kg^{-1}	22.9*	5.6**		5.7**	14.6**	14.2**		1.9*	2.1**	32.9	.48 (.60)	.45 (.53)
Fat g kg^{-1}	4.2*	1.0**		3.1**	30.6**	29.2**		4.4**	4.2**	23.4	.66 (.74)	.63
Phosphorous g kg^{-1}	6.9	10.9**			2.5**	1.8*				78.0	.06	

variation for the trait, and limitations in accuracy of phenotyping, suggesting more research is necessary for both detection and interpretation of this trait.

Across all traits other than grain moisture and plant height, the additive variance (GCA) explained a higher percentage of variance than SCA as is expected in a mating design even though all parents were mated without regard to heterotic grouping. It was observed that plant height had twice as much variation attributable to SCA than GCA, especially compared with days to anthesis which was six times more influenced by GCA effects. Plant height exhibiting a heterotic effect (Beavis et al., 1991) has been previously reported. These observations point to a greater heterotic influence on plant height than flowering time. It was also encouraging that in the 2010 pilot study the percent variation from the genetic component for yield was similar (42.8%) although more variation (9.1%) came from SCA effects. Grain moisture had greater genotypic and environmental variation in the pilot study. Plant and ear height traits were similar across both years.

GCA x environment was significant for grain moisture, kernel weight, plant height, ear height, starch, and fat. SCA x environment effects were significant for grain yield, kernel weight, total phenolics, and crude protein. Only a substantial portion of genetic by environmental interaction was observed with grain yield and height resulting in important changes in genotypic rank for these traits. Small GxE interactions are important as they allow for separation among genotypes based on genetic differences, without environment as a substantial confounding factor. Agronomic traits (days to silk, days to anthesis, plant and ear height) were greatly altered by the lack of adaptation and

therefore a large GxE effect (< 50% in most cases) resulted in the AM test being dropped from those analyses.

Although significant, SCA x environment, but not GCA x environment, effects (Table 3-4) were generally a small portion of total trait variation for total phenolics. This was especially important because the AM location was included in analyzing this trait, unlike some of the agronomic traits which were too substantially affected by climatic differences. Low GxE and a low residual error term for total phenolics, suggests that genetic evaluation could be done with minimal environments, few replications, and using our same lab phenotyping protocol.

Heritability

Heritability takes into account the genetic variation in a genotype that is inherited and predictable and not affected by GxE interactions. Traits with higher heritabilities are easier to breed/select for since a large portion of the variation for the trait is transferred to the following generation. Broad sense heritability (H^2) is a heritability estimate based on all genetic variance; dominant and additive (Sleper and Poehlman, 2006). In descending order; total phenolics, days to anthesis, days to silk, and plant height had the highest H^2. Narrow sense heritability (h^2) is a heritability estimate involving only the additive portion of the genetic variance (Sleper and Poehlman, 2006). In descending order; total phenolics, days to anthesis, and fat had the highest h^2 estimates.

By excluding the AM location from the analysis for plant and ear height, variation shifted from the environment term to the hybrid genetic component. This led to a reasonable H^2 for plant height (.62) but remained low for ear height (.39). Average

expected heritabilities based on other reported studies for plant and ear height are 56.9 and 66.2% respectively (Hallauer et al., 1988). While our plant height heritability was slightly better than average, our ear height was nearly half of the average. Because the range of ear heights as a proportion of the average was higher than the range of plant heights, and because the residual error was much higher for ear height, our measurement phenotype for ear height may be less accurate.

Silk and anthesis analysis was reduced to only include CS because of missing data in WE and the extreme variation from AM. By removing AM, H^2 for silk (.73) and anthesis (.78) outperformed the reported range of 50-70% (Hallauer et al., 1988). Yield also had a higher H^2 (.49) than the reported range (< .30). The high estimations of H^2 for these traits were the product of calculating with a low number of environments and having very diverse material in the study.

Of the seed quality traits analyzed, crude protein, starch, and fat exhibited large, significant genotypic differences and thus had high H^2 and h^2 estimates. This increased slightly when analysis was done on WE and CS only. An effect from early harvest on the trial in AM, possibly inhibited the completion of grain development in some samples. Narrow-sense heritability estimates (h^2) were also high for seed composition traits due to a large portion of the genotypic variation coming from additive gene action (Table 3-4). The heritability estimate for fat corresponded well with the reported range of >.70 (Hallauer et al., 1988), supporting the efficacy of the calibration curves used for detection.

In 2010, grain yield, plant height, and ear height had similar broad-sense heritabilities while it was not possible to determine heritability for other traits due to the smaller number of genotypes tested. The similarly low ear height estimate was due to a large percentage (87.9) of variation partitioned by environment, which was not observed in 2011.

GCA Estimates of Diallel Parents

GCA estimates of inbred lines were calculated because this source of variation was significant for nearly all traits. GCA estimation of inbred lines is important because it represents the additive variation and average breeding value of the lines' contribution to all hybrids tested.

For days to silk and days to anthesis, short season (early) plants are desired to avoid the high temperatures (Khanal et al., 2011) that the southern U.S. can produce in the late summer season, and mature before freezing temperatures occur in northern locations. The most negative combiners for days to anthesis and days to pollination represent the earliest material. Not surprisingly, parent combining ability for days to anthesis was similar for days to silk with 'lfy Blue' and 'LH287', representing the low general combiners (early) and 'LAMA Red' representing the highest general combiner (late) across all environments. LAMA Red, lfy Blue, and LH287 were also substantially different in flowering time GCA between Texas and Iowa environments. This was likely because of photoperiod sensitivity effects and led to them being the respective high and low combiners. Plant height and ear height were also highly related and 'Red Hybrid Ear' and 'Red Ear' had the highest GCA for both height traits.

Across CS and WE environments, inbred LAMA Red and Red Hybrid Ear had the highest GCA values (data not shown) for grain yield. For total phenolic content, parents Red Ear and 3 Red Hybrid Ear were the top GCA performers. These two were the top performers because Maiz Morado was only in six hybrid combinations and could not be estimated with the SAS Diallel program. Looking at the raw values Maiz Morado hybrids all had approximately 2.5 times the levels of the best non-maize morado hybrid (Red Ear). The top two total phenolics parents Red Ear and Red Hybrid Ear suggests a dark red phenotype is important for producing more total phenolics/bioactive phenolics. Red maize producing higher total phenolics than blue, white, and yellow maize has been previously reported (Lopez-Martinez et al., 2009). However, Maiz Morado has been shown to have the highest antioxidant properties in maize as measured by high performance liquid chromatography (Cevallos-Casals & Cisneros-Zevallos, 2004).

Measurement of grain moisture not only sheds light on dry-down ability of the grain but also maturity of the genotype as well. 'Wenwei Red1' had the highest GCA, while 'Ethiopia blue' and lfy Blue had the lowest GCA. Expressed another way, inbred 5 was among the latest maturing parents, while 9 and 1 were among the earliest. LH287 and LAMA Red had the highest GCA for kernel weight and thus crosses with these parents yielded larger, or more dense kernels than other crosses.

SCA Estimates and Heterotic Grouping

Specific combining ability of inbred lines is important because it represents the dominance variation and allows for the interpretation of heterotic patterns that allow

grouping. This is especially important in maize where two very prominent heterotic groups in US yellow and white maize exist; stiff-stalk (SS), and non-stiff-stalk (NSS).

Hybrids LAMA Red x Wenwei Red1 (1695 kg ha^{-1}) and Ethiopia Blue x Wenwei Red1 (1381 kg ha^{-1}) were the top specific combiners (data not shown) for grain yield. This was surprising given that the combination of 'LH195' x LH287 represents a commercially important SS x NSS cross that would have been expected to be the best combiner. The worst SCA for grain yield was exhibited by hybrid LH287 x Ethiopia Blue2 (2260 kg ha^{-1}) suggesting that these parents belong to the same heterotic group.

Based on SCA values with the two commercial lines the eight other diallel parents were placed into respective heterotic groups. LH195 and 8 LH287 are known to belong to the stiff stalk, and non-stiff stalk synthetic groups, respectively. Ethiopia Blue, Red Hybrid Ear, Red Ear, and Ethiopia Blue2 combined best with LH195 and fell into the non-stiff stalk synthetic group, while lfy Blue, Wenwei Red1, and Wenwei Red2 combined best with LH287 and fell into the stiff stalk synthetic group. LAMA Red had interactions across both heterotic groups unsurprisingly since it has 100% tropical parentage. Maiz Morado also had an extremely tropical phenotype (tall, late flowering, bushy with drooping leaves) but without reciprocal crosses it was not possible to confidently place into a heterotic group.

Grain moisture, kernel weight, days to silk, and days to anthesis all had a number of hybrids with high SCA. Plant height and ear height traits had no significant SCA effects. Crude protein and starch were the only kernel composition traits with significant SCA effects, Red Ear x LH195 and Red Hybrid Ear x Red Ear respectively.

The full diallel was attempted to determine if heterotic patterns existed as these colored lines were unclassified, and to also determine if there were heterotic patterns/reciprocal effects for total phenolics. There were too few hybrids with significant SCA effects for total phenolics to effectively determine heterotic groups for the trait. Although only the half diallel was used to estimate genetic effects, analysis showed no significant effects from successful reciprocals.

The specific combination of Red Ear x Lama Red (29 ug g^{-1}) and lfy Blue x Red Ear (27 µg g^{-1}) had the highest SCA for total phenolics. However, in the context of the variation of total phenolic variation in this experiment this high SCA represented a small portion. Seven Maiz Morado hybrids were the top overall performers, representing much of the variation which could not be used in SCA analysis because of a lack of hybrid combination with all parents. The lowest SCA for total phenolics was -40 (µg g^{-1}) and involved the top general combiners Red Ear and Red Hybrid Ear.

Using Proc Mixed analysis, total phenolics had a very high percent variation coming from male SCA. This large imbalance was the result of Maiz Morado being used as a pollen donor since this line failed to develop useable ears at our CS and WE nursery locations. This parent also produced hybrids with total phenolic content 2.5 times that of the top yellow hybrid combinations and 1.8 times that of the top non-morado hybrid (lfy Blue x Red Ear) so the effects on the male portion of GCA were very large. Removing crosses with this parent balanced out the SCA effects between male and females as expected but did not substantially change the genetic effects or heritability.

Means Separation for Yield and Total Phenols

Commercial check DKC67-23, LH195 x LAMA Red, LAMA Red x LH287, Red Hybrid Ear x Wenwei Red1, and commercial check BH9440W represented the top grain yielding hybrids across WE and CS. None of the top five across locations were repeated in both WE and CS when analyzed separately, demonstrating the difference between these environments and the GxE interaction for yield. Commercial inbred lines LH195 and LH287 were expected to be high yielding but phenolics were unsurprisingly low. Genotype Red Hybrid Ear x Wenwei Red1 holds promise for commercial production of colored maize as it represents a red x red cross. Comparing CS and WE, commercial hybrid DKC67-23, along with the commercial inbreds were in the top five for CS but not in WE. Although similar locations, tropical germplasm typically performs better in the more extreme weather in WE, while temperate germplasm is typically higher yielding in CS. Commercial hybrid DKC67-23, parents 7, and 8 were developed primarily for Midwest climates which CS more closely mirrors than WE, helping to explain why none of the top five hybrids across locations were shared in the individual analyses of WE and CS.

Economics govern the crops that farmers grow. Interestingly, the top hybrid for total phenols, lfy Blue x Maiz Morado yielded approximately 0.40 kg more total phenols than the top hybrid for grain yield while yielding nearly half as much in grain. With these results it would seem reasonable for farmers to successfully profit from colored maize production as long as a premium was placed on antioxidant yield. For total phenolics, the top hybrids averaged across six observations were as follows; 1 x 11, 10 x

11, 3 x 11, 4 x 11, and 7 x 11. As noted earlier, Maiz Morado (parent 11) combined particularly well and produced the highest total phenolics. The six hybrids made with Maiz Morado were the six highest for phenolics across all locations. For WE and CS, the top five hybrids were the same as those across all three locations with ranking changed slightly. For the test in AM, LAMA red x Maiz Morado and Red Ear x Maiz Morado were replaced in the top five with Wenwei Red2 x Maiz Morado and Wenwei Red1 x Maiz Morado. Wenwei Red1 and Wenwei Red2 were developed for the high plains of Texas where the climate is more closely matching that of Iowa, which could explain their increased performance. Although based on our results a climate effect would be expected more for grain yield than total phenolics, genotypes such as LAMA Red containing tropical parentage may show a slight decrease in total phenolic development because of a shortened growing season in cooler environments. Depending on available premiums for maize antioxidant production, it may be more viable to grow the lower grain yielding colored types, especially if it is possible to raise the current yield of hybrid 1 x 11, through breeding efforts.

Parent lfy Blue, Red Ear, and Maiz Morado all have alleles that increase total phenolics beyond our GCA predictions. This may shed light on the hypothesis that the "stacking" of color alleles is possible. We visually observed that red x blue hybrids produced grain that was segregating for red and purple on the ear, consistent with red alleles being dominant and blue alleles being recessive. With 'maize morado' the dark color does not visually allow us to detect any other pigments. The phenolic analysis suggests, however, that both red and blue pigments and/or pathways are operating with

the maize morado pathway, as they had higher phenolic levels. The most intriguing example of this was Hybrid 1 (lfy blue) x 11 (‘maize morado’) and will be the target of future breeding endeavors. Hybrid 1x11 was the top hybrid in each location individually and significantly higher than all other hybrids across locations. It was higher than the additive sum of the blue + morado (best hybrid combinations) pigments suggesting there may be epistasis in this pathway. This was not due to smaller kernel size (compared with the other maize morado hybrids) which was thought to be a partial explanation.

There have been few studies of the Peruvian landrace Maiz Morado, but its color and its inheritance are quite unique. Its color has been biochemically characterized (Cevallos-Casals and Cisneros-Zevallos, 2004) but its genetics have not. An interesting genetic phenomenon of Maiz Morado is that when crossed with another line as a male the F_1 seed color is that of the female parent, while F_2 seed always displays the dark purple to nearly black phenotype. However, if Maiz Morado is used as a female, the F_1 seed color is Maiz Morado. This genetic behavior is a maternal phenotypic effect (Roach and Wulff, 1987). This behavior differs from the other dominant genes; the tissues surrounding the embryo and endosperm all derive from maternal cytoplasm. These tissues eventually develop into other kernel structures such as the pericarp. Red parents exhibited dominance in crosses as males or females with the exception of maize morado combinations (which cannot be observed), and yellow parents exhibit dominance in the endosperm both as males and females. Both blues acted in a recessive manner in F_2 seed, and the F_1 seed color was indicative of the maternal parent color. When selfed, ‘red x

blue' hybrid ears were red with a few kernels segregating for a darker pigment, suggesting an interaction with the blue gene(s) that act in a recessive manner.

While the pigments appear to have the largest effect on increasing phenolics, these compounds are present in multiple structures within the maize kernel, including the endosperm (Cabrera-Soto et al., 2009). This was observed as the yellow corns in this study (LH195 x LH287 and checks) still had significant levels of total phenolics. Yellow corn has previously demonstrated three times the total phenolics as white (Lopez-Martinez et al., 2009), and Del Pozo-Insfran et al. (2007) found anthocyanins were undetectable in white corn. However, yellow pigments are not required for total phenolics as the white corn in this study had a significant amount of total phenolics but was valued in the bottom 15% of the genotypes tested. There probably are compounds that have more genetic effects in total phenolics in addition to other, less visible, antioxidants in the grain.

Therefore, while the color compounds have large qualitative effects, and they could be stacked to maximize antioxidants, likely additional quantitative genes and modifiers could be selected to increase antioxidants. Because of small-effect modifiers, likely the large genetic variation composed of qualitative genetic effects has been selected for and future antioxidant gains in colored maize will come from the careful identification and selection for the small-effect quantitative modifiers.

Correlations Among Traits

Correlations between traits are important to identify broad generalizations that might confound our primary trait estimates as well as determining secondary traits that

would be good to simultaneously select for. Overall there were many significant, albeit small correlations between traits (Table 3-5). Days to silk and days to anthesis were most highly correlated. This is expected because synchrony between the male (tassel) and female (silk) must be maintained for reproductive growth and can be affected by climatic stresses and a lack of adaptation (Buckler, et al., 2009). Plant and ear height were highly correlated as previously reported (Meghji et al., 1984).

Grain yield correlated positively with plant and ear height and negatively with days to silk and days to anthesis. Taller plants typically have more leaves and therefore are able to produce more energy from increased photosynthesis (Lee and Tollenaar, 2007). While we did not observe any issues with lodging in our screening environments, tall plants are generally more susceptible to lodging due to the ear sitting higher on the plant (Duvick and Cassman, 1999). Maiz Morado, the highest performing hybrid for total phenolics, was the tallest of the diallel parents, which explains the significant positive correlation between total phenolics and plant (.17) and ear height (.30). Several significant correlations were observed for composition (Table 3-5). Fat had a large negative correlation with starch and a positive correlation with protein. Starch had a large negative relationship with crude protein. Starch was also negatively correlated with phosphorous while crude protein was positively correlated with phosphorous. The large negative correlation between starch and crude protein is not surprising because protein is produced in the early reproductive stages whereas starch is produced later (Hoseney, 1998). When environmental stresses become deleterious to plant growth close to physiological maturity, they cause reduced starch. As starch, fat, protein, phosphorus,

Table 3-5. Pearson's correlations for primary and secondary traits. * P < 0.05, **P < 0.01.

Traits	Grain yield kg ha^{-1}	Total phenol µg/g	Weight (g/500k)	Days to silk	Days to anthesis	Plant height	Ear height	Moisture g kg^{-1}	Fat g kg^{-1}	Starch g kg^{-1}	Crude protein g kg^{-1}	Phosphorous g kg^{-1}
Grain moisture (harvested)	-.03	.12*	.16**	.18**	.23**	.10	.10	.12*	.01	-.04	.04	-.03
Grain yield kg ha^{-1}	1	.01	.14**	-.15**	-.15**	.20**	.18**	-.07	.01	.06	-.11*	.04
Total phenol µg/g		1	-.06	-.21**	-.28**	.17**	.30**	-.16**	.29**	-.30**	.07	.09
Weight (g/500k)			1	-.10	.09	.11*	-.01	.07	-.09	.10	-.05	-.13*
Days to silk				1	.85**	-.02	-.15**	.05	-.08	-.11*	.18**	.06
Days to anthesis					1	-.14**	-.29**	.11*	-.13*	-.05	.18**	-.02
Plant height						1	.54**	-.11*	.09	-.02	-.04	.03
Ear height							1	-.05	.34**	-.18**	.07	.12*
moisture g kg^{-1}								1	-.07	.18**	-.03	-.15**
fat g kg^{-1}									1	-.45**	.10	.22**
starch g kg^{-1}										1	-.77**	-.29**
crude protein g kg^{-1}											1	.15**

and other grain components are reported on a percent basis, they are zero sum, in that as one increases, one or more of the others must decrease.

Conclusion

The Folin-Ciocalteu method for total phenolics extraction (Cabrera-Soto et al., 2009; Del Pozo-Insfran et al., 2007; Lopez-Martinez et al., 2009) was highly effective in separating genotypes and variance parameter estimation. Total phenolic content was related to kernel phenotype with dark purple Maiz Morado and deep-red hybrids containing higher phenolic composition than blue and yellow maize.

The Ames location provided an extreme environment which allowed us to screen for suitability of these lines in hybrid combination and they were unadapted. Traits such as starch, crude protein, etc. were affected by harvesting prior to complete dry-down, GxE was not a major contributor to variation for total phenolics; showing stability for kernel color and antioxidants, even at elevated harvest moisture.

The additive variance observed in this diallel was lower than typically seen due to the diverse array of parents and disregard for heterotic grouping. Lfy blue, Red hybrid ear, Wenwei Red1, and Maiz morado had potential for research into colored maize grain yield and/or high yielding total phenolic types. Maize morado holds promise as a maternally inherited dark purple pigment line which also can be modified with proper hybrid combinations and continued development.

CHAPTER IV

CHARACTERIZATION OF QUALITY PROTEIN MAIZE GERMPLASM FOR ENHANCED AMINO ACID PROFILES AND RELATED TRAITS

INTRODUCTION

Maize is important as a food, feed, and energy crop and the largest cereal crop by production worldwide, ahead of both rice and wheat (FAO, 2011). The United States is the largest producer of maize (*Zea mays* L.) in the world, producing an estimated 316 million metric tons annually (FAO, 2011). In 2010 Texas ranked 12th in the United States with 7.7 million metric tons, or 2.4% of the national total (USDA-NASS, 2011). Maize is primarily a starch source, representing 71% of the kernel (Prasanna et al., 2001), while protein accounts for 7-13% of the kernel (Moro et al., 1996). The highest quantity and quality protein of the kernel is found within the germ (Prasanna et al., 2001). Cereal based diets are common in the developing world because high yields result in inexpensive calories but are deficient in protein quantity and quality (especially essential amino acids) necessary for the human diet. Due to this deficiency, continued improvement of overall nutrition in maize with an emphasis on improved protein will allow developing countries the opportunity to experience an enhanced, healthier diet (Gunaratna et al., 2010). Additionally, animal feeding operations in both the developed and developing world could benefit from improved quantity and quality protein in grain to decrease the expense of soybean meal and other protein supplements (Burgoon et al., 1992; Lopez-Pereira, 1992).

Improvement of maize protein quality received interest in the 1920's when a chalky ear with soft kernels was discovered by a farmer in Connecticut (Vietmeyer, 2000). More than 40 years later, kernels from that ear were analyzed and found to have elevated amino acids, and further research discovered the *opaque-2* (*o2o2*) mutant primarily responsible for the elevated amino acid profile (Mertz et al., 1964). Although *opaque-2* maize holds an advantage over normal maize in protein quality (a higher percentage of lysine, and other essential amino acids), the mutant also has drawbacks in many agronomic traits. Negative pleiotropic effects caused by the *o2o2* allele result in later maturity, as well as lodging issues due to taller plants and thinner stalks (Salamini et al., 1970).

Quality protein maize (QPM) was developed to improve kernel endosperm hardness and improve yield of the *opaque-2* mutant germplasm while maintaining an enhanced amino acid profile. Research into endosperm modification began in India (1964) (Prasanna et al., 2001) and later at CIMMYT (1969). QPM, compared to *o2o2* maize without modifiers, is harder and less susceptible to mechanical damage, has a yield increase of 8-15%, and the plants exhibit greater resistance to disease and insect damage (Lambert et al., 1969; Salamini et al., 1970).

Compared to conventional yellow maize, and to a lesser extent white maize, QPM takes up a small portion of the total maize land area at 2.5 million acres and has had much less extensive emphasis on research and development (Prasanna et al., 2001). Because of the high genetic diversity of maize protein quantity and quality, QPM has room for further development (Dudley, 2007). Normal (non-QPM) maize endosperm

protein is naturally high in the zein fraction (60%) (Salamini and Soave, 1982) resulting in a short supply of the two most essential amino acids in human nutrition, lysine (Kies et al., 1965) and tryptophan (Nelson, 1969). Albumins (3%), globulins (3%) and glutelins (34%) represent the remaining protein constituents of normal maize (Salamini and Soave, 1982) and contain higher concentrations of both lysine and tryptophan, among others. Most conventional cereal grains (rice, wheat, in addition to maize) contain ~2% lysine, less than half the recommended amount (FAO, 1985). In QPM there is a decrease in the lysine deficient prolamin (zein) fraction, with a corresponding increase in the other three fractions; albumins, globulins, and glutelins (Prasanna et al., 2001). This supports the hypothesis that lysine is increased in the maize kernel through the synthesis of non-prolamine protein (Vasal, 2002).

Lysine and tryptophan levels in QPM lines are positively correlated (Nurit et al., 2009). Some QPM hybrids have averaged a 60% advantage in tryptophan levels in the protein fraction and a 48% advantage in tryptophan grain concentration when compared to normal checks across several diallel studies (Pixley and Bjarnason, 1993).

Only three temperate QPM lines; Tx802, Tx807, Tx811 (Betran et al., 2003; Choe et al., 1976) have been released in the US and few breeding studies have sought to improve amino acid content without using the o2o2 allele (Scott et al., 2008; Olsen et al., 2003). It is not clear if the many studies on tropical QPM material in tropical locations would be relevant to temperate QPM material. It is believed that there are many modifiers for QPM, and that these modifiers might be different across genetic backgrounds with complex inheritance (Lopes and Larkins, 1996; Lopes et al., 1995;

Bjarnason and Vasal, 1992). There has been little work to identify how these modifiers affect grain opacity and hardness (Wu et al., 2010). Additionally it is not known if these modifiers, especially from different sources will work synergistically or antagonistically.

The essential amino acids provided by QPM could prove valuable to countries where consistent, alternative protein sources are hard to obtain. Consumer demand for maize increased by 2.1 percent per year from 2000-2007 (Mitchell, 2008), further increasing the importance of enhancing the cereal grains that people depend on daily. Because growth reductions in grain based diets can be virtually eliminated in humans and animals when replaced with QPM diets, QPM is viable option to bridge the protein gap in areas where cereals are heavily relied on (Bressani, 1992; Clark, 1978; Mertz, 1992; Graham et al., 1990).

The protein advantages of QPM cannot be realized unless genotypes are developed that people want to grow, those that show composition stability over varying environments, including drought and nitrogen deficient conditions. The objectives of this research were: 1) to determine the ability of newly developed temperate QPM lines to increase protein quality (measured as amino acid concentration) in grain relative to non-QPM hybrids; 2) to identify components of variation in QPM material; 3) to increase understanding of interaction between endosperm modifiers; 4) to assign heterotic and combining ability groups to Texas AgriLife QPM lines; and 5) to identify secondary traits correlated with protein quality and yield.

MATERIALS AND METHODS

Table 4-1. Germplasm by parent number and their respective pedigree, names, kernel color, and type. Colors included yellow (Y), white (W), and orange (O).

Male	Female	Pedigree	Name	Color	Type
1		([CLQ06901 x B97] - F2)-2-3-3-1-1-B2	Hallauer1	Y	QPM
2-1		([B99 x CLQ06901]-F2)-1-5-1-1-1-1-B1	Hallauer2-1	Y	QPM
2-2		([B99 x CLQ06901]-F2)-1-5-1-1-1-1-B2	Hallauer2-2	Y	QPM
3		CML 161	CML 161	O	QPM
4		B73 o2o2	B73 o2o2	Y	*o2o2*
5		CML 176	CML 176	W	QPM
6		LH195	LH195	Y	Normal
7		LH287	LH287	Y	Normal
8		(Tx811-B x CML 176-B)-B-B-B-B-1-B-B-B-B-B	Tx829	W	QPM
	9	(Tx802 x Ko326y)-18-1-1-1-B-B/CML161-B-4-B-B-B-B-1	Tx830	Y	QPM
	10	((Ko326y x Tx806)-6-1-1-1-B-B/CML161)x(Tx802/CML161))-2-B-B-B-B-1	Tx831	Y	QPM
	11	(P69Qc3HC107-1-1#-4-2#-4-B-B-1-4-B-B-B-B-B X CML 193)-B-B-2-B-B-B-B-1	Tx832	Y	QPM
	12	Pop. 69 Templado Amarillo QPM-B-B-B2-12-B-B-B-B-B	Tx833	Y	QPM
	13	((B104/(Tx802 x Ko326y)-18-1-1-1-B-B)x(Tx714/(Ko326y x Tx806)-6-1-1-1-B-B))-B-B-2-B-B-B-1	Tx834	Y	QPM

Germplasm

14 inbred lines (Table 4-1) were used to produce 69 hybrids, and four commercial hybrids were included as checks (Table 4-2). Six of the inbred lines were developed by Texas AgriLife of College Station, three from Arnel Hallauer's program at Iowa State

University, two from the International Maize and Wheat Improvement Center (CYMMIT), two commercial lines (Monsanto Company 1991, 2001), and an improved B73 *o2o2* line. Two of the three lines from Hallauer was a result of splitting a line that was segregating for an endosperm modifier. Source seed of inbred lines originated from 2009 and 2010 nurseries, with the commercial lines ordered in 2010. The fourteen lines consisted of nine yellow QPM, two white QPM, two yellow normal maize types, and one opaque-2 line. In 2010, the parents were mated in a design-2 where five unreleased TAMU lines were designated as "female", and the other nine were designated "male". Hybrids were made between the two groupings, including some reciprocals, but not within the male and female groups. Missing hybrid combinations can be attributed to flowering time asynchrony and incompatibility of select parents.

Hybrid F_1 seed was produced at the Texas AgriLife Experiment Stations in College Station (CS) and Weslaco (WE), Texas during the summer and fall months of 2010, respectively. Hybrid yield trials were conducted in CS, WE, and Ames (AM), Iowa during the summer of 2011. Additionally a pilot study was conducted in 2010 in both CS and WE which included a subset of hybrids (32) in unreplicated trials from seed made in WE 2009 winter nursery.

Experimental Design

A design-II mating design was chosen to determine general combining ability (GCA) and interactions on a single-cross basis (Fehr, 1987; Hohls, 1996). Yield trials were planted in a randomized complete block design with 88 entries (CS and WE) and 84 entries (AM). Yield plots were 3.96 m in length with row widths of 0.76 (CS, AM)

Table 4-2. Hybrids produced from design-II mating design. Commercial checks included BH9014VT3, BH9440W, DKC67-23, and DKC68-06.

	Male 1	Male 2-1	Male 2-2	Male 3	Male 4	Male 5	Male 6	Male 7	Male 8	Female 9	Female 10	Female 11	Female 12	Female 13
Male 1	-	-	-	-	-	-	-	-	-	No	Yes	Yes	Yes	Yes
Male 2-1	-	-	-	-	-	-	-	-	-	Yes	Yes	Yes	Yes	Yes
Male 2-2	-	-	-	-	-	-	-	-	-	Yes	Yes	Yes	Yes	No
Male 3	-	-	-	-	-	-	-	-	-	No	Yes	Yes	No	No
Male 4	-	-	-	-	-	-	-	-	-	Yes	Yes	Yes	Yes	Yes
Male 5	-	-	-	-	-	-	-	-	-	No	No	No	No	No
Male 6	-	-	-	-	-	-	-	-	-	No	Yes	Yes	Yes	Yes
Male 7	-	-	-	-	-	-	-	-	-	No	Yes	Yes	Yes	Yes
Male 8	-	-	-	-	-	-	-	-	-	No	No	No	No	No
Female 9	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	-	-	-	-	-
Female 10	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	-	-	-	-
Female 11	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	-	-	-	-	-
Female 12	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	-	-	-	-	-
Female 13	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes	-	-	-	-	-

and 1.01 (WE) meters and were thinned to a population of approximately 64,000 plants ha^{-1}. Blocks were assigned as two replications per location. Due to the contamination of foreign pollen upon the ear (Xenia effect) that was observed during our 2010 pilot study and previously reported in QPM studies (Hossain et al., 2008; Pixley and Bjarnason 1994); five (WE) and three (CS, AM) ears were chosen at random to be self-pollinated and harvested separately to mimic the effects of each hybrid planted in an isolated grower's field. WE and CS locations harvested all plot ears to obtain yield and composition data. In AM, only self-pollinated ears were harvested allowing composition data only.

Phenotypic Measurements

Plant height was measured in the field from the base of the plant to the tip of the tassel and ear height was measured from the base of the plant to the ear node. Flowering time was measured by the number of days from planting to when 50% of the plants were either shedding pollen (days to anthesis) or silk (days to silk). Ears were shaded and dried in a greenhouse (CS, WE) or in a forced-air dryer (AM). Self pollinated ears were shelled using a hand-sheller while open pollinated ears were shelled using a mechanical ear sheller (Agriculex, Guelph, Ont., Canada) and weights were taken. Grain moisture was measured at time of harvest with a mini GAC plus (Dickey-john, Minneapolis, MN). Kernel weight was measured using a model U seed counter (International Marketing and Design Co., San Antonio, TX) to count 500 kernels per genotype which were then weighed. Endosperm opacity ratings were determined visually from 1 (completely opaque) to 5 (completely translucent) separately on 50 kernels and average

for each sample similar to the method outlined in Scott et al. (2004).

Sample sizes of 50 grams were initially ground to a 2 mm fineness with a Polymix® PX-MFC 90 D mill (Kinematica Ag, Bohemia, NY) and then further ground by a Cyclone™ sample mill (UDY Corporation, Fort Collins, CO) to 1 mm fineness. Starch, crude protein, fat, and phosphorous were determined by Fourier transformed near-infrared reflectance spectroscopy (FT-NIRS). A calibration curve was developed in the Texas AgriLife corn breeding program in College Station on a Thermo Antaris II (Thermo-Fisher) FT-NIRS updated to included 18 outlier samples from this study. Wet chemistry on these samples was conducted by Ward Laboratories (Kearney, NE). Ground samples were scanned in reflectance 32 times using a rotating cup over wavelengths from 4,000 to 10,000 wave numbers (cm^{-1}) at increments of 2 cm^{-1}, and then converted to absorbance; whole kernel samples were also scanned but were found to be less predictive.

Amino Acid Estimation and Analysis

Tryptophan, Methionine, and Lysine were estimated using a microbiological method based on E. *coli* strains auxotrophic for tryptophan, methionine, or lysine as performed by Scott et al. (2004). Samples prepared as above were used in the assay and the concentration of each amino acid was calculated using linear regression onto a standard curve. These values are estimates and serve to rank genotypes and are suited to the high-throughput needs of modern breeding programs. This method has been proven highly effective to estimate amino acid profiles in maize grain (Scott et al., 2004; Gutierrez-Rojas et al., 2008).

These estimations of amino acid contents were used directly and also divided by total protein content. This is because when breeding/selecting for QPM, it is important to improve amino acid content while simultaneously maintaining adequate protein levels.

Statistical Analysis

SAS 9.2 (SAS Institute, INC., Cary, NC, USA) PROC MIXED procedure was used to determine percent variation for all traits across all hybrids produced. Variance components were determined using an all random model and heritability estimates were also obtained from the calculations in Figure 4-1.

σ^2_{GCA} =	σ_A
$\sigma^2_{GCA \times E}$ =	σ^2_{AE}
σ^2_{SCA} =	σ^2_D
$\sigma^2_{SCA \times E}$ =	σ^2_{DE}

$$H^2 = (\sigma^2_A + \sigma^2_D) / (\sigma^2_A + \sigma^2_D + \sigma^2_{AE} + \sigma^2_{DE} + \sigma^2)$$

$$h^2 = \sigma^2_A / (\sigma^2_A + \sigma^2_D + \sigma^2_{AE} + \sigma^2_{DE} + \sigma^2)$$

Figure 4-1. Genetic variance estimates and heritability calculations. Additive (σA), additive by environment (σ2AE), dominance variance (σ2D), and dominance by environment genetic variance (σ2DE) estimated as general combining ability (σ^2_{GCA}), general combining ability by environment ($\sigma^2_{GCA \times E}$), specific combining ability (σ^2_{SCA}), and specific combining ability by environment ($\sigma^2_{SCA \times E}$). Broad (H^2) and narrow (h^2) sense heritabilities.

General combining ability (GCA) was estimated for lines and specific combining ability (SCA) was estimated for specific male x female line interactions; GCA and SCA estimates were obtained using fixed effects. Observed percent variation was determined from covariance parameter estimates as calculated by PROC MIXED. Covariance parameter estimates for each source of variation was summed for a grand total and each individual estimate was then divided by this sum. PROC CORR was used for Pearson correlations across traits and PROC GLM was used for means separation analysis. For the analysis of amino acids, in order to account for variation among assays carried out in different micrometer plates a two-step model was used where the first model ran 'plate' as a random variable and produced new residuals which were run on the main model to determine significant effects (Table 4-4); these results were similar but improved (less residual error) compared to a model without the 'plate' effect.

RESULTS AND DISCUSSION

Agronomic traits; days to anthesis, days to silk, and height were significantly and substantially different in AM than in CS or WE (Table 4-3). The 50% silk and anthesis dates ranged over 6 days in WE, 20 (silk) and 21 (anthesis) in AM, and CS was in the middle with 16 (silk) and 13 (anthesis). This shortened maturity in WE and lengthened maturity in AM was likely due to rapid growing degree day accumulation and photo-period sensitivity and was also seen in a completely different set of germplasm (Mahan et al., submitted). The difference in means for height traits in AM (approx. 0.67 meter) compared to WE and CS demonstrated a lack of adaptation. Because maize production in the United States is primarily in the Midwest it was important to have a test in AM to

evaluate Texas material adaptation and to determine how various traits were affected by an extreme environment to which they were not adapted.

2011 grain yields (Table 4-3) were limited to CS due to lost WE data and yield was not measured in AM because the plants were still at very high moisture at harvest. Because of elevated moisture in AM, only the three selfed ears were harvested from each plot for seed analysis. This was a clear result of this Texas material being unadapted to the Midwest AM environment.

Endosperm opacity had little variation across all three environments, an important aspect for the trait, as visual recognition of opaqueness verifies the presence of *o2o2* genes (Mertz, 1992; Vasal, 2002). In our 2010 study, selfing the hybrid ears eliminated segregation on the ear from foreign pollen that was seen in the open-pollinated material. In 2011, selfing ears from each plot delivered the same result with the exception of segregation for endosperm color for white/yellow crosses. Among the four seed composition traits (fat, starch, crude protein, and phosphorous) the largest differences were seen at AM for crude protein (Table 4-3). It was interesting to observe that AM had the highest mean starch across the test since some of these hybrids were harvested shortly before physiological maturity (black-layer) and starch is produced in the later reproductive stages, continuing accumulation through maturation (Hoseney, 1998). Phosphorous showed little variability in these samples (both by FT-NIRS and wet-chemistry) and further research is necessary to more accurately detect this trait with increased statistical power. Based on their respective means, lysine and tryptophan displayed stability across environments while methionine was considerably lower in

Table 4-3. Test locations for the fourteen parent design-II mating design. † Yield and moisture were recorded in College Station (CS) only. Methionine, lysine, and tryptophan assay results are estimates only.

	College Station, TX		Weslaco, TX		Ames, IA	
Traits	Mean ± S.D.	Min, Max	Mean ± S.D.	Min, Max	Mean ± S.D.	Min, Max
Grain yield† (kg ha^{-1})	7595 ± 1444	3766, 11047				
Grain moisture g kg^{-1} (at harvest)	136 ± 23	82, 210				
Weight (g/500k)	146 ± 16	115, 205	152 ± 20	75, 206	126 ± 28	56, 190
Days to silk (50%)	74 ± 3	67, 82	74 ± 1.5	72, 77	75 ± 5	66, 84
Days to anthesis (50%)	72 ± 2.5	67, 79	74 ± 1.5	72, 77	75 ± 4	65, 85
Plant Height (cm)	202 ± 11	168, 229	192 ± 13	140, 221	269 ± 22	205, 315
Ear Height (cm)	76 ± 11	48, 102	67 ± 11	36, 102	136 ± 16	95, 190
Endosperm opacity	3.09 ± 0.87	1.10, 4.40	3.10 ± 0.79	1.16, 4.28	2.95 ± 0.82	1.08, 4.38
Composition traits						
Moisture g kg^{-1}	107.5 ± 3.4	97.7, 114.7	108.5 ± 4.2	99.1, 118.3	96.3 ± 4.0	85.3, 107.4
Fat g kg^{-1}	37.9 ± 6.4	26.1, 58.8	39.9 ± 7.5	19.7, 64.3	39.1 ± 6.0	29.9, 59.3
Starch g kg^{-1}	667.0 ± 15.1	616.7, 704.3	655.7 ± 15.2	614.9, 694.9	668.2 ± 18.8	604.8, 706.5
Crude Protein g kg^{-1}	115.3 ± 10.5	90.4, 150.2	121.0 ± 10.3	99.3, 148.6	101.6 ± 12.6	75.0, 145.4
Phosphorous g kg^{-1}	3.5 ± 0.10	3.2, 3.9	3.7 ± 0.10	3.4, 4.0	3.7 ± 0.10	3.4, 3.9
Methionine (assay)	0.134 ± .050	0.060, .267	0.142 ± .055	0.053, .253	0.087 ± .013	.040, .130
Lysine (assay)	0.081 ± .013	0.055, .111	0.088 ± .016	0.056, .127	0.081 ± .012	.044, .133
Tryptophan (assay)	0.097 ± .012	0.070, .134	0.100 ± .013	0.066, .133	0.102 ± .013	.045, .133
Methionine (protein adj.)	1.17 ± 0.44	0.52, 2.40	1.16 ± 0.43	0.41, 2.23	0.87 ± 0.11	0.66, 1.23
Lysine (protein adj.)	0.71 ± 0.13	0.47, 1.12	0.73 ± 0.15	0.44, 1.13	0.81 ± 0.14	0.57, 1.41
Tryptophan (protein adj.)	0.85 ± 0.13	0.59, 1.14	0.83 ± 0.13	0.51, 1.15	1.03 ± 0.15	0.70, 1.40

AM. AM also had the largest range for lysine, tryptophan, and methionine, corresponding with the late maturing hybrids having the least mature seed at time of

harvest. Methionine and lysine content as a proportion of kernel protein was higher in AM, an effect of lower overall protein content in AM. Observations of lower protein in AM may be due to lower starch in Texas locations, a result of drought stress.

Sources of Observed Variation

The data was analyzed across all three environments (where feasible) to determine sources of variation in the study. Due to the large climatic differences between AM and the Texas locations, two ANOVA tables were created, one for trait analysis across all locations (Table 4-4) and the other for analysis limited to the two Texas locations (Table 4-5). All references to traits are considering the data from table 4-5, unless otherwise noted. Environment was highly significant ($P \leq 0.01$) for kernel weight, days to anthesis, ear height, and starch, and significant ($P \leq 0.05$) for days to silk, and phosphorous. A replicate effect (highly significant) was only observed for plant height, crude protein, fat, and tryptophan (protein adjusted) reflecting minimal field variation for most other traits. The hybrid genetic component was highly significant and the design-II mating scheme allowed the genetic component to be divided into males and females (GCA) as well as the interaction between them (SCA) across the 'male' and 'female' groups. GCA and SCA represent additive and dominant gene action, respectively. All traits exhibited highly significant or significant GCA effects. With the exception of kernel weight, phosphorous, and methionine, all traits had highly significant or significant SCA effects.

Across all traits, the additive variance (GCA) explained a higher percentage of variance than SCA as is expected in a mating design, even though parents were labeled

Table 4-4. The percentage of observed variation across three locations (CS, WE, AM) and significance each source explains and the broad (H^2) and narrow (h^2) sense heritabilities. Environment (Env), Replicate (Environment) (Rep), specific combining ability (SCA), general combining ability (GCA), general combining ability x environment (GCA x Env), specific combining ability x environment (SCA x Env), residual error (Res). + Traits analyzed in CS AM only. *P < 0.05, **P < 0.01.

			Gen			Gen*Env					
	Env	Rep(Env)	SCA	GCA		SCA*Env	GCA*Env		Res	H^2	h^2
				Male	Female		Male	Female			
Grain yield kg ha^{-1}			11*	11*	34**				44	0.56	0.45
Grain moisture g kg^{-1}			9*	32**	12**				47	0.53	0.44
Weight (g/500k)	30**			29**		7**	5**	6**	23	0.41	0.41
Days to silk^{+} (50%)	47**		2**	20*			11**	14**	6	0.42	0.38
Days to anthesis^{+} (50%)	43*		2*	20*		2**	16**	13**	5	0.38	0.34
Plant height	90**		2**				4**	1**	4	0.18	
Ear height	89**		0.5*	3**	1*	0.5*	2*	1*	3	0.41	0.36
Crude protein g kg^{-1}	36**	2**		8*	5**	13**	8**		28	0.21	
Starch g kg^{-1}	9	2**			22**	19**	17**		32	0.24	
Fat g kg^{-1}	0.2	0.6**		13.4**	3.6*	4.7**	1.7**	3.0**	72.8	0.17	
Phosphorous g kg^{-1}	28*	4**			1**				67	0.01	
Methionine				14*	2**		13**		71	0.16	
Lysine			13**	10*					76	0.23	0.10
Tryptophan			5**	41**			4**	2**	48	0.46	0.41
Endosperm opacity		1**	5**	68**	8**				18	0.82	0.77
Methionine (prot. adj.)					2*		17**		81	0.02	
Lysine (prot. adj.)				20**	5**	14**			61	0.25	
Tryptophan (prot. adj.)	1	1*	4**	44**	7**				43	0.56	0.52

Table 4-5. The percentage of observed variation across CS and WE and significance each source explains and the broad (H^2 and narrow (h^2) sense heritabilities. Environment (Env), Replicate (Environment) (Rep), specific combining ability (SCA), general combining ability (GCA), general combining ability x environment (GCA x Env), specific combining ability x environment (SCA x Env), residual error (Res). + Traits analyzed in CS only. *P < 0.05, **P < 0.01

			Gen			Gen*Env					
	Env	Rep(Env)	SCA	GCA		SCA*Env	GCA*Env		Res	H^2	h^2
				Male	Female		Male	Female			
Weight (g/500k)	4	1*	5**	53**			5**	5**	26	0.62	0.56
Days to silk$^+$ (50%)			8**	63**	9**				20	0.8	0.72
Days to anthesis$^+$ (50%)			10**	54**	10**				26	0.74	0.64
Plant height	28	3**	9**	21**	7**				32	0.54	0.42
Ear height	25**			30**	12**	7**			27	0.55	
Crude protein g kg^{-1}	10	3**	12**	37**					38	0.56	0.43
Starch g kg^{-1}	22**		9**	22**	14**				33	0.58	0.46
Fat g kg^{-1}	4	2**	6**	52**	23**				14	0.85	0.79
Phosphorous g kg^{-1}	37*				5*				55	0.08	
Methionine				22*	2*		8**		68	0.24	
Lysine			10**	12**					78	0.22	0.12
Tryptophan			1**	58**					41	0.59	0.58
Endosperm opacity			6**	66**	9**				20	0.8	0.74
Methionine (prot. adj.)					3*		18**		79	0.03	
Lysine (prot. adj.)			10**	23**					67	0.33	0.23
Tryptophan (prot. adj.)	1	1*	7**	50**	6**				34	0.65	0.58

as ‘male’ and ‘female’ without regard to heterotic grouping. As an example plant height had 9% variation attributable to SCA, but overall had more variation from GCA (28%). In maize, plant height usually displays more dominance variation as plant height typically exhibits a large heterotic effect (Beavis et al., 1991). A large SCA for plant height was most likely not observed in this study because not all parents were allowed to cross with each other, thus lessening the occurrence of favorable heterotic crossing. It was also encouraging that in the 2010 pilot study the percent variation from the genetic component (SCA and GCA) for yield was similar (52%) although more variation (17%) came from SCA effects.

Among all traits investigated, the smallest hybrid genetic component was for phosphorous (5%) and it did not provide conclusive evidence to support predominant GCA or SCA effects since the residual for this trait was high and we therefore lacked sufficient power to identify genotypic effects.

Overall, most traits had more variation from the male term than the female. This could have a variety of explanations including unequal distribution of parents (nine males, five females), and distribution of germplasm diversity. Although the five females included germplasm derived from CIMMYT, Texas, and Tennessee sources, they were all developed within the Texas AgriLife program. The nine males included lines from breeding programs in Iowa, CIMMYT, Texas, and US commercial programs.

GCA x environment was significant for all traits excluding kernel weight, days to anthesis, days to silk, methionine, tryptophan, and endosperm opacity. SCA x environment effects were significant for kernel weight, and ear height. A substantial

amount of genetic by environmental interaction was only observed for flowering time resulting in important changes in genotypic rank for these traits. Limited GxE interactions are useful as they allow for effective separation among genotypes based on genetic differences, without environment as a substantial confounding factor, accelerating the process of breeding/selection for traits. Agronomic traits (days to silk, days to anthesis, plant and ear height) as well as crude protein, starch, fat, and phosphorous were greatly altered by the lack of adaptation and therefore a large GxE effect (< 50% in most cases) resulting in the separate ANOVA's.

Although significant, genetic x environment effects were generally a small portion of total trait variation for amino acid estimates. This was especially important because the AM location had substantial effects on many of the other traits. Greater hybrid genetic component, due to decreased variation in GxE and error components allowed for better amino acid evaluation of genotypes. Including the AM location still allowed for substantial variation among genotypes despite undesirably high residuals, suggesting that amino acid profiles can be selected for and improved with minimal number locations and replications even in conditions to which the varieties are marginally adapted.

The lack of GxE variation for endosperm opacity as well as similar genotype variation between Table 4-4 and 4-5 suggests that endosperm modification is very stable across a wide variety of environments. This small GxE effect had been previously reported but not across such a diverse set of environments (Gutierrez-Rojas et al., 2008).

Heritability

Heritability takes into account the genetic variation in a genotype that is inherited and predictable and not affected by GxE interactions. Traits with higher heritabilities are easier to breed/select for since a large portion of the variation for the trait is transferred to the following generation. Broad sense heritability (H^2) is a heritability estimate based on all genetic variance; dominant and additive (Sleper and Poehlman, 2006). In descending order; fat, endosperm opacity, and starch had the highest H^2. Narrow sense heritability (h^2) is a heritability estimate involving only the additive portion of the genetic variance (Sleper and Poehlman, 2006). In descending order; fat, endosperm opacity, and starch also had the highest h^2 estimates.

By analyzing CS and WE separate from AM for plant and ear height, variation shifted from the environment term to the hybrid genetic component (GCA). This led to reasonable H^2 for plant height (0.54) and ear height (0.55). Average expected heritabilities based across other reported studies for plant and ear height are 56.9 and 66.2% respectively (Hallauer et al., 1988).

Similarly, flowering time analysis was substantially effected by environment. Additionally, incomplete data for WE led to only CS being analyzed for flowering time (Table 4). By analyzing CS separately, H^2 for silk (0.80) and anthesis (0.74) overestimated the reported range of 50-70% (Hallauer et al., 1988). Yield had a higher H^2 (0.56) than the reported range (< .30). Overestimations for flowering time and yield were the result of calculating with a single environment and having very diverse material in the study.

Of the seed quality traits analyzed, crude protein, starch, and fat exhibited large, significant genotypic differences and thus had high H^2 and h^2 estimates. Estimates were reported here from Table 4-5 as the early harvest on the trial in AM may have inhibited the completion of grain development in some samples, resulting in elevated residual error and GxE effects accounted for by the lack of adaptation to the AM location (Table 4-4). Endosperm opacity was the only trait which actually observed an improved H^2 when AM was included, showing its stability and lack of environmental effect.

Narrow-sense heritability estimates (h^2) were high for seed composition traits due to a large portion of the genotypic variation coming from additive gene action (Table 4-5). The heritability estimate for fat (0.85) corresponded well with the reported range of >0.70 (Hallauer et al., 1988), thus separately supporting the efficacy of the calibration curves. H^2 estimation for crude protein (0.56) also fell into the previously reported range of 0.54-0.73 (Dudley et al., 1971, Dudley et al., 1975). H^2 estimates for tryptophan, lysine, and methionine across all locations were 0.46, 0.23, and 0.16 respectively; higher than previously reported in normal maize using the same phenotyping method (Scott and Blanco, 2009). Other estimates of heritability for lysine have ranged from 0.17-0.72 (Dudley et al., 1971) and 0.62 for tryptophan (Motto, 1979).

When adjusted for protein content, lysine and tryptophan exhibited similar H^2 to their non-adjusted values displaying a possibility for researchers to simultaneously improve both amino acid and overall protein content.

In 2010, grain yield had a nearly identical H^2 as did days to silk and days to anthesis (when compared with table 4-5 values) while height traits were similar across

both years in the Texas locations. It was not possible to determine heritability for other traits due to the smaller number of genotypes formally tested for these traits.

GCA Estimates of Design-II Parents

GCA estimates of inbred lines were calculated because this source of variation was significant for all traits excluding phosphorous. GCA estimation of inbred lines is important because it represents the additive variation and average breeding value of the lines contribution to all hybrids tested. All estimates of GCA and SCA were calculated using the most appropriate model (Table 4-4 or Table 4-5) because for some traits the variation from the AM environment was too large to provide the most accurate description of the genetic material in this study.

For days to silk and days to anthesis, short season (early) plants are desired to avoid the high temperatures (Khanal et al., 2011) that the southern U.S. can produce in the late summer season, and mature before freezing temperatures occur in northern locations. Female parent 'Tx832' represented the only significant and negative general combiner (early) for either of the flowering time traits (silk) and 'CML 161' and 'Hallauer2-1' represented the highest significant general combiner (late) for both flowering time traits. It is important to note that although these parents have the significant GCA; this estimate is reflective of their hybrid combinations and may lead to them not being the earliest and latest parents in other studies. Plant height and ear height were also highly related, with 'B73 *o2o2*' having the highest GCA for ear height and the 2nd highest GCA for plant height behind only 'LH195'.

Top general combining parents (high and low) for endosperm opacity score were LH195 (0.81), CML 161(0.60), B73 *o2o2* (-1.43) and Hallauer2-1 (-0.81). LH195 is a commercial, normal maize line while B73 *o2o2* is an unmodified opaque-2 line, thus it was not surprising that they represented the high and low general combining parents for endosperm opacity.

All GCA estimations for lysine were negative and insignificant, while many GCA estimates for tryptophan were significant with B73 *o2o2* (0.014) and Hallauer2-1 (0.009) leading the way for the male parents. Tx831 (0.004), 'Tx832' (0.004), and 'Tx833' (0.003) exhibited significant GCA within the female group. QPM maize displays elevated lysine and tryptophan levels, yet normal maize continues to hold an advantage in methionine content.

For methionine estimation, LH195 (0.064), 'LH287' (0.037), and 'Hallauer2-2' (0.038) had the highest significant GCA while all female parents estimated negative GCA with only 'Tx831' (-0.15) being significant. Normal maize displays elevated methionine levels when compared to *o2o2* and QPM (Scott et al., 2004). Thus it was not surprising that commercial parents LH195 and LH287 combined most favorably to produce elevated levels of methionine when compared with non-commercial, QPM crosses.

SCA Estimates

Specific combining ability of inbred lines is important because it represents the dominance variation and allows for the selection of those parents which combine to produce the most suitable, trait specific hybrid. This is especially important in maize

where two very prominent heterotic groups in US yellow and white maize exist; stiff-stalk (SS), and non-stiff-stalk (NSS). Loose interpretation of heterotic patterns based on superior yielding hybrids with LH195 (SS) and LH287 (NSS) allowed for more formal grouping of the five previously unclassified parents of interest in this study from the female group which based on their almost solely tropical backgrounds would be hard to otherwise place. Tx830, Tx831, and Tx833 historically yield well with SS germplasm. All three 'females' also performed well with the SS heterotic group in this study and were classified as NSS. Tx832 yielded well with both LH195 and LH287, which is often expected for tropical germplasm in hybrid combinations. Tx834 yielded too poorly across all hybrids, preventing clear heterotic group placement.

Days to silk, days to anthesis, and plant height all had a number of hybrids with high SCA. Ear height had no significant hybrids. Endosperm opacity had a single significant positive and negative hybrid combination; Tx832 x Hallauer2-1 (0.75) and Tx832 x LH195 (-0.65), representing the narrow variation of Tx832 to produce a positive hybrid combination, but not a broad variation which would have translated to Tx832 having a high GCA as well. Crude protein, starch, and fat all had significant SCA effects. Of the amino acids analyzed, only methionine lacked SCA effects, not surprising since normal maize lines were restricted to the 'male' group while all five of the females were QPM lines.

Hybrids Tx830 x LH195 (0.009), Tx834 x Hallauer2-1 (0.007), 'Tx829' x Tx834 (-0.010), and Tx829 x Tx832 (-0.009) had the highest and lowest SCA for lysine estimates. Parent Tx832 was a component of both high and low-combining lysine

hybrids, an example of the importance of proper parent selection. Hybrids Tx831 x B73 *o2o2* (0.0106), Tx832 x B73 *o2o2* (0.0104), and Tx833 x Hallauer2-2 (-0.0083) were the highest and lowest for tryptophan. B73 *o2o2* was a parent for 3 of the 4 top hybrids for SCA of lysine and tryptophan. It was not surprising that B73 o2o2 ranked towards the top for SCA and GCA for amino acids as the line contains a full *o2o2* mutant.

Means Separation for Yield and Amino Acids

Commercial check DKC67-23, BH9014VT3, Tx831 x LH195, Tx832 x B73 *o2o2,* and Tx832 x LH195 represented the top grain yielding hybrids in CS. Commercial inbred lines LH195 and LH287 were expected to be high yielding but the highest LH287 hybrid was substantially lower (29.5 bu/a) than DKC67-23, the top yielding hybrid. Although limited to one location, it was promising that four QPM x QPM hybrids out-yielded two of the commercial checks in the test (data not shown). For the 2010 pilot study, Tx832 x B73 *o2o2* was the 5th highest yielding QPM hybrid (11th overall in 2010), while hybrids LH 195 x Tx811 (3rd-7th), LH195 x Tx812 (5th-4th), and LH195 x Tx813 (7th-10th) were similarly ranked from the 2010 and 2011 studies, displaying relative stability for these particular QPM hybrids.

Economics govern the crops that farmers grow. Interestingly, the top QPM hybrid for yield, Tx832 x B73 *o2o2* was also in the top 5 for lysine and tryptophan content. For QPM to gain popularity among modern day growers, the nutritional benefits of enhanced amino acid profiles must also be accompanied with a limited amount of yield drag, in order for possible grain premiums to be enough to allow growers to remain profitable.

Comparing the top hybrid combinations across methionine, lysine, and tryptophan, several of the top hybrids for methionine were crosses with the LH195 or LH287 commercial parents, or they were commercial checks. There was no commercial material in the top 10 for lysine or tryptophan, reinforcing the gap in readily available commercial hybrids for improved amino acid quality and the opportunity to elevate levels of essential amino acids with QPM hybrids. The top QPM hybrid out yielded the top normal hybrid by 35 and 30% for lysine and tryptophan, respectively; less than previously reported by Pixley and Bjarnason, 1993 (48%). Top hybrid rankings for amino acids did not change greatly when amino acid estimates were adjusted for protein content. This follows since each amino acid should have greater representation when protein content is increased.

An important question we sought to address in this study was whether different endosperm modifiers of *o2o2* exist in these lines of diverse backgrounds and what type of gene action they exhibit. Expected mid-parent endosperm opacity was determined for each hybrid combination using the endosperm opacity ratings for each individual parent. We found in general the mid parent values of the inbreds fit the hybrid values with a few exceptions suggesting mostly additive effects. Observed endosperm opacity followed an additive, mid-parent trend with an R^2 of 0.76 supporting a substantial linear relationship between expected and observed endosperm opacity (Figure 4-2). Parents Tx832 and B73 *o2o2* are on opposite sides of the spectrum in terms of endosperm opacity with B73 *o2o2* (score of 1.0) having completely opaque kernels, while Tx832 (3.52) is in between the semi vitreous to mostly vitreous phenotype. The 12 hybrids created with these

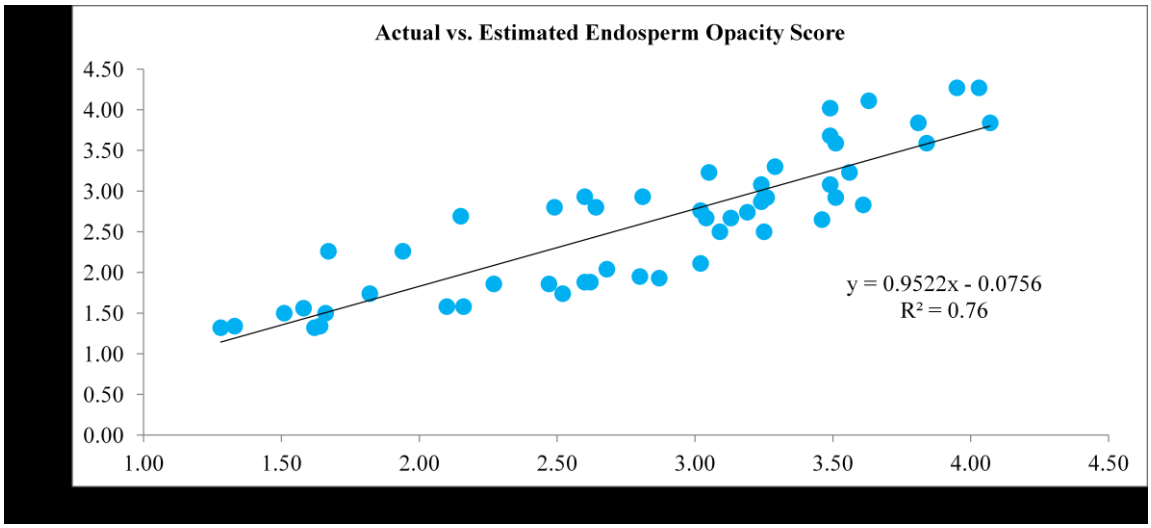

Figure 4-2. Plot of actual endosperm opacity score and expected mid-parent.

parents (reciprocals included) have average endosperm opacity of 1.65, substantially lower than the expected score (2.26). Across all 69 hybrids, 15 were substantially below (≥ -0.50) their expected endosperm opacity ratings and 3 were above their expected rating (≥ +0.50). More than 20% of the hybrids failed to reach their mid-parent rating; reflecting the complexity and recessive nature of the modifier loci.

Source germplasm of endosperm modifiers also appeared to play a role as Iowa State parents were in 10 hybrids and CYMMIT parents were in three hybrids which deviated substantially from the expected endosperm opacity. This suggests that there are different modifiers for opacity in some of these lines and follows our hybrid only analysis suggesting that opacity is partially dominant but primarily additive.

To take advantage of the grouping structure of the design-II mating and to more effectively compare our ‘female’ QPM lines of interest, means separations were also

performed based on seven groups (Table 4-6). Five groups represented each of the five QPM lines, and the remaining two represented commercial inbred parent and commercial checks, respectively.

As expected for yield, the commercial checks were significantly the highest yielding (9415 kg ha^{-1}) while the remaining groups were statistically similar with the exception of Tx834 being significantly lower than all groups (5774 kg ha^{-1}). It was surprising that the commercial inbred group did not have significantly higher yields than the QPM groups; showing that it takes two 'good' yielding inbred parents to obtain very high yields.

For lysine and tryptophan, there was no significant variation between female groups but they were all significantly higher than the commercial groups. This observation shows that while QPM holds an advantage over normal maize, breeding and testing efforts are necessary to find the specific crosses which perform better than the test mean. Adjusting amino acid estimates for protein content allowed for statistical separation of female groups, revealing the variation for protein among hybrid combinations (Table 4-6). Increased variation and subsequent rank changes suggests that a greater emphasis on multiple-trait breeding (amino acids and total protein) should be of priority in future QPM development.

Correlations Among Traits

Correlations between traits are important to identify broad generalizations that might confound our primary estimates as well as determining secondary traits that would

Table 4-6. Hybrid grouping by 'female', commercial inbred cross, and commercial check. Lysine (Lys), Tryptophan (Trp), Methionine (Met) content and protein adjusted content. Different letters represent significant differences.

Hybrid group	# of obs	Grain yield	# of obs.	Lys	# of obs.	Lys (prot. adj.)	# of obs.	Trp	# of obs.	Trp (prot. adj.)	# of obs.	Met	# of obs.	Met (prot. adj.)
Commercial checks	8	150A	22	0.076B	22	0.654C	23	0.084C	23	0.742D	24	0.137B	24	1.181AB
Commercial crosses	34	125B	91	0.077B	90	0.666C	97	0.091B	95	0.792D	100	0.143A	99	1.213A
Tx830 crosses	20	115B	53	0.087 A	52	0.741B	56	0.103A	55	0.889C	56	0.119D	54	0.996BC
Tx831 crosses	24	124B	66	0.083 A	64	0.751B	63	0.105A	61	0.951AB	66	0.103F	64	0.931C
Tx832 crosses	24	128B	60	0.09A	60	0.835A	64	0.103A	64	0.952AB	66	0.127C	66	1.151AB
Tx833 crosses	20	121B	55	0.085 A	55	0.799AB	57	0.104A	57	0.984A	58	0.118D	58	1.085ABC
Tx834 crosses	16	92C	44	0.087 A	44	0.758B	45	0.105A	45	0.918BC	46	0.106E	46	0.92C

Table 4-7. Pearson's correlations for primary and secondary traits measured in 'male' x 'female' combinations. *P < 0.05, **P < 0.01

Traits	Grain yield	Endosperm opacity	Methionine	Lysine	Tryptophan	Weight (g/500k)	Days to silk	Days to anthesis	Plant Height
Grain moisture	0.09	0.21*	0.13	-0.04	-0.24**	0.22**	0.05	0.01	0.24**
Grain yield		0.23**	0.01	0.12	-0.17*	-0.01	0.39**	0.44**	0.18*
Endosperm opacity			0.18**	-0.18**	-0.53**	0.29**	-0.03	-0.02	-0.09
Methionine				-0.05	-0.19**	0.38**	-0.21**	-0.23**	-0.40**
Lysine					0.41**	-0.13*	0.06	0.07	-0.06
Tryptophan						-0.37**	0.13*	0.16**	0.20**
Weight (g/500k)							-0.33**	-0.40**	-0.53**
Days to silk								0.88**	0.36**
Days to anthesis									0.46**
Plant height									
Ear height									
Crude protein									
Fat									
Starch									
Phosphorous									
Methionine (prot. adj.)									
Lysine (prot. adj.)									

Table 4-7. Continued.

Traits	Ear height	Crude protein	Fat	Starch	Phosphorous	Methionine (protein adj.)	Lysine (protein adj.)	Tryptophan (protein adj.)
Grain moisture	0.28**	0.02	0.00	-0.03	0.03	0.13	-0.01	-0.18*
Grain yield	0.25**	-0.27**	0.13	0.06	-0.07	0.1	0.27**	0.05
Endosperm opacity	-0.12*	-0.03	-0.31**	0.13*	-0.11*	0.21**	-0.14**	-0.37**
Methionine	-0.43**	0.44**	-0.19**	-0.21**	-0.04	0.95**	-0.32**	-0.46**
Lysine	-0.07	0.1	0.22**	-0.16**	0.11*	-0.10	0.76**	0.20**
Tryptophan	0.22**	0.01	0.31**	-0.21**	0.14**	-0.23**	0.32**	0.69**
Weight (g/500k)	-0.55**	0.35**	-0.27**	0.02	-0.11*	0.31**	-0.34**	-0.52**
Days to silk	0.34**	-0.19**	0.27**	-0.04	-0.01	-0.17**	0.22**	0.27**
Days to anthesis	0.43**	-0.26**	0.27**	-0.05	0.15**	-0.18**	0.26**	0.33**
Plant height	0.95**	-0.46**	0.04	0.11*	0.09	-0.34**	0.27**	0.53**
Ear height		-0.49**	0.06	0.13**	0.09	-0.33**	0.28**	0.57**
Crude protein			-0.10*	-0.70**	0.15**	0.17**	-0.55**	-0.71**
Fat				-0.32**	0.22**	-0.18**	0.23**	0.27**
Starch					-0.39**	0.00	0.31**	0.35**
Phosphorous						-0.10*	-0.01	0.01
Methionine (prot. adj.)							-0.17**	-0.28**
Lysine (prot. adj.)								0.63**

be good selection criteria. Overall there were many significant correlations between traits (Table 4-7). Days to silk and days to anthesis were highly correlated with one another. This is expected because synchrony between the male (tassel) and female (silk) must be maintained for reproductive growth and can be affected by climatic stresses and a lack of adaptation (Buckler, et al., 2009). Highly correlated flowering time, as well as plant/ear height has also been previously reported (Meghji et al., 1984; Mahan et al., submitted).

Grain yield correlated positively with plant height and ear height and insignificantly with flowering time as observed with hybrids grown in the same field in an unrelated study (Mahan et al., submitted). Taller plants typically have more leaves and therefore are able to produce more energy from increased photosynthesis (Lee and Tollenaar, 2007). While we did not observe any issues with lodging in our screening environments, tall plants are generally more susceptible to lodging due to the ear sitting higher on the plant (Duvick and Cassman, 1999).

Kernel weight and endosperm opacity were related; softer, starchy endosperm has an opaque phenotype while vitreous/hard endosperm is typically heavier, thus a substantial, significantly positive correlation (0.31) was observed between these two traits. Endosperm opacity was negatively correlated with tryptophan (-0.41). As endosperm becomes increasingly more vitreous (modified), concentrations of lysine and tryptophan begin to decrease as a result of moving further away from the original *o2o2* phenotype.

Methionine (0.95), lysine (0.76), and tryptophan (.69) had descending Pearson correlations between raw amino acid estimations and those after protein content adjustment. The extremely high correlation for methionine coupled with the poor heritability for the protein adjusted methionine value (Tables 4-4 and 4-5) suggests thebulk of the variation for this trait lies with the estimated value, and is independent of protein content. The correlations for lysine and tryptophan show that while protein quantity is a substantial factor, it is not the only determining factor. Lysine and tryptophan were positively correlated (0.41) but not as highly as reported by Hernandez and Bates, 1969 (0.85). Methionine (-0.21, -0.23) and tryptophan (0.13, 0.16) were significantly correlated with flowering time. The negative methionine correlation can be best explained by commercial material containing the highest amounts of methionine and flowering the earliest. Interestingly, only tryptophan had a small, negative and significant correlation with yield suggesting the ability of QPM hybrids to resist yield linkage drag with increased amino acid content.

Several significant correlations were observed for other compositional traits and these findings were consistent with Mahan et al. (submitted).

Conclusion

The microbial method for amino acid content estimation proved effective to separate genotypes and estimate genetic variance parameters. Across the three amino acids examined, normal maize contained larger quantities of methionine, while QPM contained larger quantities of essential amino acids tryptophan and lysine. Thus, it was

not surprising to find positive, significant GCA estimates for parents of the male group but none for the all-QPM female group.

Variation in protein quantity resulted in substantial rank changes for lysine and tryptophan, representing the importance of selecting for total protein content, along with protein quality in the form of amino acids. Because top hybrids for amino acid estimation were similar to their protein adjusted counterparts, a two-trait selection breeding process appears promising.

The Ames location provided an extreme environment with which we found amino acid estimation to be robust overall. It additionally allowed us to screen for suitability of these lines in hybrid combination and they were found to be unadapted. Although traits such as starch, crude protein, etc. were affected by harvesting prior to complete dry-down, GxE was not a major contributor to variation for amino acids; the early harvest may underestimate amino acid profiles, but still allow for effective genotype separation, even at high harvest moisture.

QPM hybrid Tx812 x B73 o2o2 displayed stability across test years and potential for further research into QPM hybrids which produce elevated levels of essential amino acids lysine and tryptophan and are also high yielding when compared to normal maize grown today.

Endosperm modification was not affected by diverse environments and primarily follows an additive, mid-parent trend, with some hybrids deviating from that trend displaying the complexity and recessive nature of multiple modifier loci. In addition

genetic background effects on modifier expression and/or different modifier loci may be found across this QPM panel.

CHAPTER V

CONCLUSION

Based on a review of literature coupled with the results of these studies, colored and quality protein maize both have room for further development to increase their potential for adoption as niche production sectors of a large cereal crop but these "to be released" Texas A&M inbred lines show promise. The interaction between the various color pathways is not understood but breeding for this successful interaction will allow for the continued development of colored maize lines with increased antioxidant potential. Although antioxidant potential was estimated, further analysis of top performing hybrids (HPLC, Mass Spectrometry) would allow for the identification of specific phytochemical compounds and allow for better characterization of these estimates.

The antioxidant estimates (total phenolic content) were used as a training population to develop a calibration curve for an FT-NIR which will allow for antioxidant estimation of future ground samples without the added time and expense of lab analysis. A similar calibration curve for amino acids was also an expected outcome from this thesis. The microbial amino acid assay is being further modified to determine if this is feasible. It may be determined that a more expensive amino acid analysis may be necessary to develop a calibration curve and this would probably be done with a smaller subset of the plot samples from this QPM study if this direction were taken in the future.

One unfortunate limitation to the colored study was that only one red parent had white endosperm. Because yellow endosperm is unfavorable for alkaline cooking of

tortilla chips due to undesirable color change (graying), it would have been beneficial to have more than five total parents with white endosperm (three blue, one red, and maiz morado). Similarly in terms of endosperm color, of the 14 QPM parents two had white endosperm. All crosses with these two 'males' thus segregated for yellow/white kernels, which is undesirable for hybrid selection even if said crosses resulted in high grain/amino acid yields, but may lead to new inbred lines if selection occurred for endosperm color.

A major factor of this study that added to its uniqueness was the inclusion of Ames, IA as a yield testing location in both studies. The level of adaptation or lack thereof for the germplasm in this study was not clear beforehand nor was the effects of adaptation on total phenolics and amino acids. Although most agronomic traits were largely affected, these composition/ quality traits of interest saw minimal GxE and error variation. During hand harvest in Ames, it was concerning to find that prior to drying the harvested ears, many of the genotypes were displaying kernels with partial/incomplete pigmentation. Observing the genotypes post-drying allowed the kernels to finish pigmentation throughout the entire kernel. This led to the assumption that pigmentation development progresses with grain dry down, and provides evidence of the potential for growing colored corn in climates with shorter growing seasons, without deleterious effects caused by artificial grain drying systems.

Overall, lab assays allowed for the statistical separation of genotypes in both studies herein. Each of the main traits of interest showed varying degrees of robustness across environments and allowed for better understanding of the respective germplasm.

Although the mating designs were effective in characterizing previous uncharacterized lines, continued work is required to continue to obtain data on these parental lines to allow for their eventual release so that others may benefit from the work included in this thesis.

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