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Quality Protein Maize Germplasm Characterized for Amino Acid Profiles and Endosperm Opacity

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RESEARCH

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ABSTRACT

Quality protein maize (QPM) is improved over normal (non-QPM) maize in grain concentrations of the essential amino acids lysine and tryptophan. Quality protein maize has a long history as tropical adapted germplasm, but little effort has been made to incorporate temperate or subtropical germplasm for temperate adaptation and interactions between different modifier loci in these backgrounds are poorly understood. A design-II mating scheme including new temperate and subtropical lines produced 69 hybrids. Large hybrid genetic variation components resulted in substantial broad-sense heritability H^2 estimates, specifically tryptophan (0.46) and endosperm opacity (0.82). A microbial assay for amino acid estimation proved robust across diverse environments with minimal genotype × environment (G×E) effects. Endosperm opacity had no G×E effects across both Texas and Iowa locations demonstrating stability for this trait. Endosperm opacity primarily followed an additive, midparent trend, with a few hybrids deviating from the trend (36%) suggesting a complex nature of multiple modifier loci across diverse germplasm. The top QPM hybrid outperformed the top commercial hybrid by 35 and 30% for lysine and tryptophan as a proportion of grain, respectively. QPM line Tx832 was a parent of top hybrids for lysine and tryptophan, and the highest noncommercial hybrids for methionine. Minimal correlations with yield and other traits suggest that future breeding should result in QPM hybrids with increasingly competitive yields.

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Abbreviations: AM, Ames, IA; CS, College Station, TX; FT-NIRS, Fourier transformed near-infrared reflectance spectroscopy; G×E, genotype × environment; GCA, general combining ability; H^2, broad sense heritability; non-QPM, normal maize; *o2o2*, Opaque-2 mutant; QPM, quality protein maize; SCA, specific combining ability; WE, Weslaco, TX.

Maize is important as a food, feed, and energy crop and the largest cereal crop by production worldwide, ahead of rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.; 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). Consumer demand for maize has steadily increased by 2.1% per year from 2000 to 2007 (Mitchell, 2008) while protein quantity of maize hybrids has decreased 0.3% per decade over the past century (Duvick et al., 2004). Improved nutritional quality can be achieved by exploiting the native genetic diversity of maize (Dudley, 2007).

Maize grain is primarily starch representing 71% of the kernel (Prasanna et al., 2001), while protein typically accounts for 7 to 13% (Moro et al., 1996). The highest quantity and quality protein of the kernel is found within the germ (Prasanna et al., 2001). Normal maize endosperm protein is naturally high in the

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zein fraction (prolamins; 60%; Salamini and Soave, 1982), which is superior in amino acids glutamine, leucine, and proline, but contains very little lysine (Kies et al., 1965) and tryptophan (Nelson, 1969). Zeins are the major storage proteins located in the protein bodies of maize endosperm (Wilson, 1991). Albumins (3%), globulins (3%), and glutelins (34%) represent the remaining protein constituents of normal maize (Salamini and Soave, 1982) and contain higher lysine and tryptophan levels relative to prolamins. Quality protein maize (QPM) results in a decrease in the lysine-deficient zeins (Prasanna et al., 2001), supporting that lysine is increased in the maize kernel through an increase of non-zein protein (Vasal, 2002).

Improvement of maize protein quality began with the discovery of the Opaque-2 mutant (*o2o2*) from an ear with soft, chalky kernels in the 1920s in Connecticut (Vietmeyer, 2000). QPM was developed from *o2o2* to improve kernel endosperm hardness and yield while maintaining elevated essential amino acid levels. QPM endosperm modification research began in India around 1964 (Prasanna et al., 2001) and CIMMYT in 1969. Compared to *o2o2* mutant maize, QPM has harder kernels that are less susceptible to mechanical damage, has a yield increase of 8 to 15%, and the plants exhibit greater resistance to disease and insect damage (Lambert et al., 1969; Salamini et al., 1970).

Cereal-based diets are common in the developing world because high yields result in inexpensive calories. Unfortunately, cereal-based diets are deficient in protein, especially essential amino acids, which QPM can improve. QPM now represents a small portion of global maize production at approximately one million hectares (Prasanna et al., 2001). In Africa, QPM is planted on approximately 200,000 ha (Krivanek et al., 2007). Some African countries (Lesotho, Malawi, and Zambia) consume maize for more than 50% of their daily calories and protein (Nuss and Tanumihardjo, 2011). Continued genetic improvement of maize protein is important to cereal-based diets because of the lack of other protein sources due to cost and availability. Children substituting QPM for normal maize experienced height and weight increases of 12 and 9%, respectively, as reported in a meta-analysis of nine studies across Ethiopia, Ghana, India, Mexico, and Nicaragua (Gunaratna et al., 2010). Additionally, animal feeding operations benefit from improved protein quality in grain to decrease their reliance on soybean meal and other protein supplements in both the developing and developed world (Burgoon et al., 1992; Lopez-Pereira, 1992).

Development and characterization of QPM lines is especially important in the United States, where only QPM lines Tx802 (Betran et al., 2003a), Tx807 (Betran et al., 2003b), and Tx811 (Betran et al., 2003c) have been released and few breeding studies have sought to improve amino acid content without using the *o2o2* allele (Olsen et al., 2003; Scott et al., 2008). Several small companies have been developing food-grade hybrids of conventional and organic maize for domestic use and concentrate heavily on protein quality and quantity (Sustainable Seed Company, Covelo, CA; Michael Fields Agricultural Institute, East Troy, WI; American Organic, Warren, IL). As of now, no QPM hybrids are commercially available for interested growers in the United States.

It is unknown if studies focused on tropical QPM germplasm and conducted in tropical locations are relevant to temperate QPM germplasm. In developing temperate QPM it is believed that there are many modifiers, which might differ across genetic backgrounds and have a complex inheritance (Bjarnason and Vasal, 1992; Lopes and Larkins, 1996; Lopes et al., 1995). There has been little work on how these modifiers affect grain opacity and hardness or if these modifiers, especially from different sources, will work synergistically or antagonistically (Wu et al., 2010).

The advantages of QPM cannot be fully realized unless hybrids are developed that people want to grow, showing composition stability over varying environments which include drought and nutrient deficient conditions. The objectives of this research were: i) to quantify the ability of newly-developed temperate QPM lines to increase protein quality (measured as amino acid concentration) in grain relative to non-QPM hybrids; ii) to identify components of variation across diverse QPM material; and iii) to further understanding of interaction between endosperm modifiers across diverse QPM material.

MATERIALS AND METHODS

Germplasm

The germplasm under investigation consisted of 14 inbred lines (Table 1) which were used to produce 69 hybrids (Supplemental Table S1). Six of the inbred lines were developed by Texas AgriLife Research of College Station, three were developed by Dr. Arnel Hallauer's program at Iowa State University, two were developed by CIMMYT, two were commercial lines (Monsanto Company 1991, 2001), and one was an improved B73 *o2o2* line. Two of the three lines from Dr. Hallauer were the result of splitting a segregating line. The design-2 mating scheme consisted of five unreleased Texas A&M University lines designated as "group A," and nine other lines designated "group B." Hybrids were made between the two groups, including some reciprocals, but none were made within groups. Missing hybrid combinations can be attributed to flowering-time asynchrony or failure to set seed.

Experimental Design

A design-II mating scheme was chosen to determine general combining ability (GCA) and interactions on a single-cross basis (Fehr, 1987; Hohls, 1996). 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 of 2010, respectively. Hybrid yield trials were conducted in CS, WE, and Ames (AM), Iowa during the summer of 2011. An additional pilot study was conducted in 2010 in both CS and WE and

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

Group B	Group A	Pedigree	Name	Color	Type	Endosperm opacity
1		([CLQ06901 × B97]-F2)-2–3-3–1-1-B2	Hallauer1	Y	QPM	2.1
2-1		([B99 × CLQ06901]-F2)-1–5-1–1-1–1-B1	Hallauer2-1	Y	QPM	1.5
2-2		([B99 × CLQ06901]-F2)-1–5-1–1-1–1-B2	Hallauer2-2	Y	QPM	4.2
3		CML 161	CML 161	O	QPM	3.7
4		B73 *o2o2*	B73 *o2o2*	Y	*o2o2*	1.0
5		CML 176	CML 176	W	QPM	3.8
6		LH195	LH195	Y	Normal	4.2
7		LH287	LH287	Y	Normal	3.9
8		(Tx811-B × CML 176-B)-B-B-B-B-1-B-B-B-B-B	Tx829	W	QPM	2.2
	9	(Tx802 × Ko326y)-18–1-1–1-B-B/CML161-B-4-B-B-B-B-1	Tx830	Y	QPM	2.0
	10	((Ko326y × Tx806)-6–1-1–1-B-B/CML161)x(Tx802/CML161))-2-B-B-B-B-1	Tx831	Y	QPM	1.7
	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	3.5
	12	Pop. 69 Templado Amarillo QPM-B-B-B2–12-B-B-B-B-B	Tx833	Y	QPM	4.4
	13	((B104/(Tx802 × Ko326y)-18–1-1–1-B-B)x(Tx714/(Ko326y × Tx806)-6–1-1–1-B-B))-B-B-2-B-B-B-1	Tx834	Y	QPM	1.6

included a subset of hybrids (32) in unreplicated trials from seed produced in WE 2009 winter nursery. Four commercial hybrids (BH9014VT3, BH9440W, DKC67–23, and DKC68–06) were planted as checks. Yield trials were planted in a randomized complete block design with 73 entries in CS (30°32′ N; 96°25′ W) and WE (26°10′ N; 97°56′ W) and 69 entries in AM (42°1′ N; 93°46′ W). Yield plots were single row, 4.0-m in length, with row widths of 0.8 (CS, AM) and 1.0 (WE) m and thinned to 64,000 plants ha^{-1}. To facilitate plant health CS had 103, 84, and 9 kg ha^{-1} of N, P, and Zn applied, respectively, and AM and WE had 120 and 73 kg ha^{-1} N applied, respectively. Blocks were assigned as two replications per location. Due to the contamination of foreign pollen on the ear (xenia effect) that was observed during our 2010 pilot study and previously reported in other QPM studies (Hossain et al., 2008; Pixley and Bjarnason 1994), five (WE) or three (CS, AM) ears were randomly chosen for self pollination and harvested separately to mimic the effects of each hybrid planted in an isolated grower's field. At the WE and CS locations, all plots were hand harvested to obtain yield and composition data. In AM, only self-pollinated ears were harvested allowing for kernel composition data.

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 top ear node. Flowering time was measured by the number of days from planting to when 50% of the plants were shedding pollen (days to anthesis) or silk (days to silk). Ears were shaded from sunlight and dried in a greenhouse (CS, WE) or in a forced-air dryer (AM). Self-pollinated ears were processed using a hand sheller while open-pollinated ears were shelled using a mechanical ear sheller (Agriculex, Guelph, ON, Canada) and weights were taken. Grain moisture was measured at time of shelling with a mini GAC plus (DICKEY-john, Minneapolis, MN). Kernel weight (500 k) was measured using a model U seed counter (International Marketing and Design Co., San Antonio, TX). Endosperm opacity light-box ratings were determined visually, using a scale of 1 (completely opaque) to 5 (completely translucent) separately on 50 kernels and averaged for each yield trial sample similar to the method outlined in Scott et al. (2004). Endosperm opacity light-box ratings were also determined for inbred seed produced in 2010 at the same time as the yield trial samples and later from 2012 samples grown during a seed increase.

Sample sizes of 50 g were ground initially to a 2-mm fineness with a Polymix PX-MFC 90 D mill (Kinematica Ag, Bohemia, NY) and then to a 1-mm fineness with a Cyclone sample mill (UDY Corporation, Fort Collins, CO). Starch, crude protein, fat, and phosphorous were determined by Fourier transformed near-infrared reflectance spectroscopy (FT-NIRS) using a calibration curve developed by the Texas AgriLife corn breeding program in College Station on a Thermo Antaris II (Thermo-Fisher) FT-NIRS updated to include 18 outlier samples from this study. Wet chemistry for the additional samples was conducted by Ward Laboratories (Kearney, NE). Ground samples were scanned in reflectance 32 times using a rotating cup over wavelengths from 4000 to 10,000 wave numbers (cm^{-1}) at increments of 2 cm^{-1}, and then converted to absorbance as recommended by the manufacturer. Whole kernel samples were also scanned but were found to be less predictive.

Amino Acid Estimation and Analysis

Amino acid concentrations were quantified via a microbiological method as performed by Scott et al. (2004). To array the samples, 10-mg ground samples of 1-mm fineness were placed randomly in 96-well plates, leaving 10 wells empty for standards. To extract and hydrolyze protein, 50 mM KCl adjusted to pH 2.0 with HCl containing 0.2 mg of pepsin were added to each well and each plate was shaken for 16 h at 37°C. The plates were centrifuged and 5 μL of supernatant for each amino acid as well as 5 μL of standard to the 10 empty wells were transferred to a second plate for analysis. Each plate was inoculated with auxotrophic bacteria in 100 μL M9 minimal media for either lysine (KL334; Birge and Low, 1974), tryptophan (CAG18455; Singer et al., 1989), or methionine (P4X; Jacob and Wollman, 1961). Incubation with shaking at 37°C followed for 16 h for methionine and tryptophan

Table 2. Trait means for College Station (CS) and Weslaco (WE), Texas and Ames, Iowa (AM).

	CS		WE		AM	
Traits	Mean ± SD	Min, Max	Mean ± SD	Min, Max	Mean ± SD	Min, Max
Grain yield (kg ha^{-1})	7595 ± 1444	3766, 11047	N/A†	N/A†	N/A†	N/A†
Grain moisture g kg^{-1} (at harvest)	136 ± 23.0	82, 210	N/A†	N/A†	N/A†	N/A†
500 kernel weight (g)	146 ± 16.0	115, 205	152 ± 20.0	75.0, 206	126 ± 28.0	56.0, 190
Days to silk (50%)	74 ± 3.0	67, 82	74 ± 1.5	72, 77	75 ± 5.0	66, 84
Days to anthesis (50%)	72 ± 2.5	67, 79	74 ± 1.5	72, 77	75 ± 4.0	65, 85
Plant Height (cm)	202 ± 11.0	168, 229	192 ± 13.0	140, 221	269 ± 22.0	205, 315
Ear Height (cm)	76.0 ± 11.0	48.0, 102	67.0 ± 9.00	36.0, 102	136 ± 16.0	95.0, 190
Endosperm opacity	3.1 ± 0.9	1.1, 4.4	3.1 ± 0.8	1.2, 4.3	3.0 ± 0.8	1.1, 4.4
Composition traits						
Methionine‡	0.134 ± 0.050	0.060, 0.267	0.142 ± 0.055	0.053, 0.253	0.087 ± 0.013	0.040, 0.130
Lysine‡	0.081 ± 0.013	0.055, 0.111	0.088 ± 0.016	0.056, 0.127	0.081 ± 0.012	0.044, 0.133
Tryptophan‡	0.097 ± 0.012	0.070, 0.134	0.100 ± 0.013	0.066, 0.133	0.102 ± 0.013	0.045, 0.133
Methionine (protein adjusted)	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 adjusted)	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 adjusted)	0.85 ± 0.13	0.59, 1.14	0.83 ± 0.13	0.51, 1.15	1.03 ± 0.15	0.70, 1.40
Moisture g kg^{-1}	108 ± 3.4	98, 115	109 ± 4.0	99.0, 118	96.0 ± 4.0	85.0, 107
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 ± 15.1	617, 704	656 ± 15.2	615, 695	668 ± 18.8	605, 707
Crude Protein g kg^{-1}	115 ± 10.5	90.4, 150	121 ± 10.3	99.3, 149	102 ± 12.6	75.0, 145
Phosphorus g kg^{-1}	3.5 ± 0.1	3.2, 3.9	3.7 ± 0.1	3.4, 4.0	3.7 ± 0.1	3.4, 3.9

†Yield and moisture recorded in CS only.

‡Value reflecting the level of amino acid per mass of bacterial tissue.

and 20 h for lysine. Each plate was measured in a microplate reader at 595 nm and the light scattered by each well gave a value reflecting the level of amino acid per mass of bacterial tissue. Amino acid content was also adjusted for variable protein content by dividing the amino acid concentration by total protein concentration, and multiplying that value by 1000.

Statistical Analysis

SAS 9.2 (SAS Institute, Cary, NC) 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 Supplemental Table S2. General combining ability (GCA) was estimated for lines and specific combining ability (SCA) was estimated for A × B group line interactions using fixed effects. PROC CORR was used for Pearson correlations across traits and PROC GLM was used for means separation analysis. To account for variation across the different 96-well plates used in the amino acid assay where plate, row, and column were confounded, 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 3). These results were similar but improved (less residual error) compared to a model without the 'plate' analysis.

RESULTS AND DISCUSSION

Agronomic traits (e.g., days to anthesis, days to silk, and height) were significantly and substantially different in AM than in CS or WE (Table 2). Shortened maturity in WE is often seen due to rapid growing degree day accumulation and photo-period sensitivity. The increase in height in AM (approx. 0.7 m) compared to WE and CS demonstrated a lack of adaptation also likely caused by photoperiod sensitivity. It was important to have a test in the midwestern Corn Belt to evaluate Texas material adaptation and to determine how agronomic and kernel composition traits were affected by an extreme environment for which they were not selected. Although not a central target of this study, 2011 grain yields (Table 2) were limited to CS. Yield data for WE was accidentally lost and yield was not measured in AM because the plants were still at very high harvest moisture, another indication that some of the Texas germplasm was unadapted to the Midwest climate.

Among the four seed NIRS composition traits (i.e., fat, starch, crude protein, and phosphorous) the largest differences were observed for crude protein in AM (Table 2). Lysine and tryptophan estimates were relatively stable across environments and all three amino acids had the greatest range in AM, while methionine content was slightly lower in AM. Methionine and lysine content as a proportion of kernel protein was higher in AM, an effect of lower overall protein content. Lower protein concentrations may be due to immaturity at harvest, which did not allow for the late-developing starch matrices to be filled with protein bodies (Khoo and Wolf, 1970), as well as less available protein bodies due to the reduced zein content in QPM material.

Table 3. The percentage of observed variation across College Station and Weslaco, TX and the broad (H^2) and narrow (h^2) sense heritabilities (plot basis). Each table value sums to 100% for each trait, and nonsignificant values were omitted so that heritability estimates were not skewed. Environment (Env), replicate (Rep), specific combining ability (SCA), general combining ability (GCA), general combining ability × environment (GCA × Env), specific combining ability × environment (SCA × Env), residual error (Res).

			Gen			Gen × Env					
				GCA		SCA ×	GCA × Env				
Traits	Env	Rep	SCA	Group A	Group B	Env	Group A	Group B	Res	H^2	h^2
Grain yield kg ha^{-1}†			11*	34**	11*				44	0.56	0.45
Grain moisture g kg^{-1}† (harvested)			9*	12**	32**				47	0.53	0.44
500 kernel weight		1*	6**		56**		5**	5**	28	0.62	0.56
Days to silk† (50%)			8**	9.0**	63**				20	0.80	0.72
Days to anthesis† (50%)			10**	10**	54**				26	0.74	0.64
Plant height (cm)	31**	1*	9**	7**	21**				32	0.54	0.42
Ear height (cm)	25**			12*	30**	7**			27	0.55	0.55
Endosperm opacity			6**	9**	66**				20	0.80	0.74
Lysine‡			10**		12**				78	0.22	0.12
Tryptophan‡			1**		58**				41	0.59	0.58
Methionine‡				2**	22*			8**	68	0.24	0.24
Methionine (protein adjusted)				3*				18**	79	0.03	0.03
Lysine (protein adjusted)					25**	13**			62	0.25	0.25
Tryptophan (protein adjusted)	1*	1*	8**	7**	48**				35	0.64	0.56
Crude protein g kg^{-1}	11**	2**	8**	3*	39**				37	0.57	0.48
Starch g kg^{-1}	22**		9**	14**	22**				33	0.58	0.46
Fat g kg^{-1}	4**	2**	6**	23**	52**				14	0.85	0.79
Phosphorus g kg^{-1}	37*			5.0**					55	0.08	0.08

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

† Traits analyzed in CS only.

‡Value reflecting the level of amino acid per mass of bacterial tissue.

Sources of Observed Variation

Due to the large climatic differences between Iowa and the Texas locations, two separate analyses of variance were performed for trait analysis. Table 3 presents the two Texas locations and Supplemental Table S3 presents all three locations. References to variance components of traits refer to CS and WE only (Table 3), unless otherwise noted. Although slight differences were observed, environment did not significantly affect amino acid or opacity, but did affect ear height, starch, and phosphorus. The hybrid genetic component was highly significant for all traits and the design-II mating scheme allowed the genetic component to be divided into group A and group B GCA as well as SCA. Across all traits, the additive component (GCA) explained a higher percentage of variation than SCA, as is expected in a mating design, even though parents were labeled as 'group A' and 'group B' without regard to heterotic grouping. It was encouraging that similar genetic-component (SCA and GCA) variation was observed for most traits in the 2010 pilot study (data not shown).

More trait variation was attributable to group B additive effects. This occurred for numerous reasons including unequal distribution of parents (five in group A, nine in group B), and distribution of the germplasm diversity. The five group A parents were all developed by Texas AgriLife research, but included CIMMYT, Texas, and Tennessee germplasm in the pedigree. The nine group B lines were derived from multiple breeding programs including two U. S. commercial non-QPM lines.

Genotype × environment interactions were significant for few traits in Table 3, illustrating the relative similarity between CS and WE, especially for composition traits. Importantly, G×E effects were generally a small portion of total trait variation for our amino-acid microbial assay estimates across all three locations. This was especially encouraging considering the AM location had substantial G×E effects on most other traits (Supplemental Table S3). A large amount of genetic variation allowed for better separation of genotypes based on amino acid concentrations across conditions to which the varieties are marginally adapted and despite relatively high residuals for the trait. A lack of G×E variation, as well as similar genotype variation from Table 3 and Supplemental Table S3 suggests that endosperm modification is relatively stable across a wide variety of environments. A small G×E effect had been previously reported, but not across such diverse environments and large number of genotypes (Gutierrez-Rojas et al., 2008). This is an important finding, as visual recognition of opaqueness has traditionally been used to verify the presence of *o2o2* genes (Mertz, 1992; Vasal, 2002). Low G×E effects for amino acid

Table 4. Hybrid grouping by 'group A', quality protein maize (QPM) × commercial inbred cross, and commercial check. Lysine (Lys), Tryptophan (Trp), Methionine (Met) content and protein adjusted content.

Hybrid group	Entries†	Lys‡	Lys (protein)	Trp‡	Trp (protein)	Met‡	Met (protein)	Endosperm opacity	Grain yield
Comm. checks	4	0.076 B§	0.654 C	0.084 C	0.742 D	0.137 B	1.181 AB	3.99A	9415 A
QPM × Comm.	17	0.077 B	0.666 C	0.091 B	0.792 D	0.143 A	1.213 A	3.55B	7846 B
Tx830 crosses	10	0.087 A	0.741 B	0.103 A	0.889 C	0.119 D	0.996 BC	2.66E	7218 B
Tx831 crosses	12	0.083 A	0.751 B	0.105 A	0.951 AB	0.103 F	0.931 C	2.57E	7784 B
Tx832 crosses	12	0.09 A	0.835 A	0.103 A	0.952 AB	0.127 C	1.151 AB	3.08D	8033 B
Tx833 crosses	10	0.085 A	0.799A B	0.104 A	0.984 A	0.118 D	1.085 ABC	3.23C	7596 B
Tx834 crosses	8	0.087 A	0.758 B	0.105 A	0.918 BC	0.106 E	0.92 C	2.32E	5775 C

† Total number of entries within each hybrid group.

‡ Value reflecting the level of amino acid per mass of bacterial tissue.

§ Different letters represent significant differences by Duncan's Multiple Range test at $P = 0.01$.

content and endosperm modification suggest that selection and improvement can be achieved with a minimal number of locations and replications.

Heritability

Traits with high heritability are desirable since a large portion of the variation is inherited in subsequent generations. Broad-sense heritability (H^2) is based on dominant and additive genetic variance (Sleper and Poehlman, 2006). In contrast to most traits, endosperm opacity observed an improved H^2 (plot based) when AM was included (Supplemental Table S3), showing its stability and lack of environmental effect. Estimates of H^2 for lysine, tryptophan, and methionine were 0.59, 0.22, and 0.24, respectively (Table 3), higher than previously reported in normal maize using the same microbial assay (Scott and Blanco, 2009). When compared to other traits, and to composition traits in particular, analysis of amino acid concentration across all three environments minimally altered the analysis, and sufficient variation existed to clearly separate genotypes based on amino acid concentration. Other estimates of heritability have ranged from 0.17 to 0.72 (Dudley et al., 1971) for lysine 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 the possibility for simultaneous improvement of both amino acid and overall protein content.

The microbial amino acid assay values are estimates that serve to rank genotypes and the moderate heritability estimates here and in past studies (Gutierrez-Rojas et al., 2008; Scott et al., 2004, 2008;) suggest this method is suited to the high-throughput needs of modern breeding programs. Amino acid quantification is the costliest aspect of the CIMMYT QPM breeding program at $7 per sample (Atlin et al., 2011) while the microbial method is estimated at $5 per sample (labor included).

Quality Protein Maize Group A Performance

To take advantage of the group structure of the design-II mating scheme and to more effectively characterize our group A QPM lines of interest, means separations were performed based on seven groups (Table 4). Five groups represented each of the QPM lines, and the remaining two represented commercial parent × QPM parent crosses and commercial checks, respectively.

For lysine and tryptophan estimates, there was no significant variation between sets of group A hybrids but they were all significantly higher than the two commercial groups. Although means across hybrid groups were not significant, individual hybrid combinations were significant for all traits (Supplemental Table S4). Adjusting amino acid estimates for protein content created significant differences among group A lines due to variation in total protein among hybrid combinations (Table 4). Increased variation and subsequent rank changes suggests that simultaneous breeding for amino acid and total protein content should continue to be of priority in future QPM development. Important to future temperate QPM endeavors specifically, Tx832 crosses were among the highest for lysine and tryptophan content, and also highest among group A groups for methionine. Decreasing methionine concentrations in QPM material as compared to normal maize are concerning (Atlin et al., 2011) and have been previously reported (Scott et al., 2004). Although available in larger quantities than lysine and tryptophan, methionine remains important to human health and can be deficient in cereal-based diets (Atlin et al., 2011). It is also important to note that commercial inbreds crossed to QPM lines significantly outperformed commercial checks for methionine concentration.

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 low yielding (5774 kg ha^{-1}).

Hybrid Mean Separation for Amino Acids, Endosperm Modification, and Yield

There were no commercial hybrids or inbreds in the top 10 for lysine or tryptophan (Supplemental Table S4), reinforcing that these QPM hybrids can improve amino acid

content over commercially-available hybrids. The top QPM hybrid in this study outperformed the top commercial hybrid by 35 and 30% for lysine and tryptophan, respectively, less than previously reported (48%) by Pixley and Bjarnason (1993).

Two commercial hybrids had the highest endosperm opacity score, as is the case with most non-QPM hybrids. Five QPM × QPM hybrids outperformed at least one commercial hybrid for endosperm opacity, displaying the kernel hardness of this new QPM germplasm as well as the variation for endosperm opacity among elite genotypes.

Among the top five grain yielding hybrids were DKC67–23 and BH9014VT3 (commercial checks), Tx831 × LH195, Tx832 × B73 *o2o2*, and Tx832 × LH195. Although limited to one location, it was promising that four QPM × QPM hybrids outyielded two of the commercial checks in the test (Supplemental Table S4). While improved nutrition drives QPM research, improved yields drive general maize research. It was encouraging then that the top-yielding QPM hybrid (Tx832 × B73 *o2o2*) was also in the top five for lysine and tryptophan (Supplemental Table S4). For QPM to gain popularity among growers, especially food-grade maize producers, the nutritional benefits of enhanced amino acid profiles must also be coupled with consistently competitive yields.

Genetic Trends of Endosperm Modification

We sought to investigate whether different endosperm modifiers of *o2o2* exist due to the diverse genetic backgrounds of parents included in this study. Expected midparent endosperm opacity was determined for each hybrid combination using the endosperm opacity ratings for each individual parent (Table 1). Comparing the calculated midparent endosperm opacity with the actual ratings of the hybrids, we found a linear relationship (0.74 R^2) between the midparent values and observed hybrid endosperm opacity (Fig. 1). Since parent endosperm opacity scores were taken on seed from a different year (2010) than the hybrid test year (2011), endosperm opacity scores were also taken on parent seed from a 2012 increase and these results were averaged with the original endosperm opacity scores and the results were very similar (Supplemental Fig. S1). While not definitive, this moderately strong relationship suggests that these genetic modifiers for endosperm opacity behaved largely in an additive manner. These observations were supported by much higher GCA than SCA variation for endosperm opacity (Table 3 and Supplemental Table S3). However, some lines deviated from a linear model suggesting some nonadditive variation was present. Across the 69 hybrids, 17 were substantially below (≤ -0.50) their expected endosperm opacity rating and 8 were above their expected rating (≥ 0.50). Upon analysis of the pedigrees of the hybrids which failed to reach their midparent rating, source germplasm diversity appeared to play a role. Hallauer parents were in 10 of the hybrids and CIMMYT parents were in three of the hybrids that deviated substantially from the expected endosperm opacity.

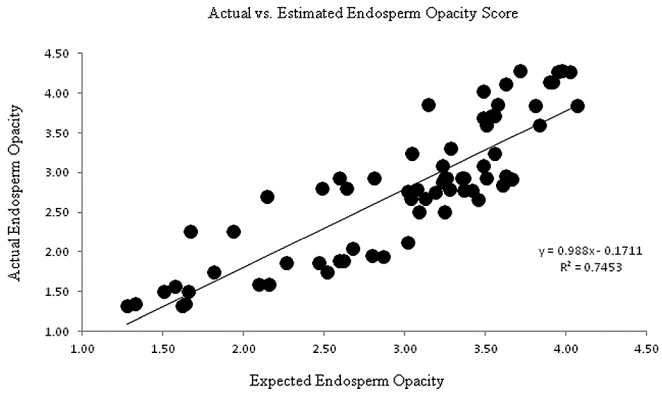

Figure 1. Plot of actual endosperm opacity score against midparent endosperm opacity score across three environments (College Station and Weslaco, TX, and Ames, IA).

Correlations Among Traits

Correlation between traits is important to identify broad generalizations that might confound our primary estimates as well as determining secondary traits that may be good selection criteria. Overall there were many significant correlations between traits (Table 5).

Methionine (0.95), lysine (0.76), and tryptophan (0.69) had strong Pearson correlations between amino acid estimates and those adjusted for protein. The extremely high correlation for methionine, coupled with the poor heritability for the protein-adjusted methionine value (Table 5), suggests the bulk 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 high as reported by Hernandez and Bates, (1969) (0.85). Methionine (−0.21, −0.23) and tryptophan (0.13, 0.16) were significantly correlated with silking and anthesis. 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 these QPM hybrids had little yield drag.

Softer, starchy endosperm has an opaque phenotype while hard, vitreous endosperm is typically denser and heavier; thus a substantial, significantly-positive correlation (0.31) was observed between starch and endosperm opacity. Endosperm opacity was negatively correlated with tryptophan (−0.41). As endosperm becomes increasingly more vitreous (modified), concentrations of lysine and tryptophan can decrease as a result of moving further away from the original *o2o2* phenotype, which is why

Table 5. Pearson's correlations for primary and secondary traits measured in 'group A' × 'group B' combinations.

Traits	2	3	4	5	6	7	8	9	10
1: Grain moisture	0.09	0.21*	0.13	−0.04	−0.24**	0.22**	0.05	0.01	0.24**
2: Grain yield		0.23**	0.01	0.12	−0.17*	−0.01	0.39**	0.44**	0.18*
3: Endosperm opacity			0.18**	−0.18**	−0.53**	0.29**	−0.03	−0.02	−0.09
4: Methionine				−0.05	−0.19**	0.38**	−0.21**	−0.23**	−0.40**
5: Lysine					0.41**	−0.13*	0.06	0.07	−0.06
6: Tryptophan						−0.37**	0.13*	0.16**	0.20**
7: 500 Kernel weight							−0.33**	−0.40**	−0.53**
8: Days to silk								0.88**	0.36**
9: Days to anthesis									0.46**
10: Plant height									
11: Ear height									
12: Crude protein									
13: Fat									
14: Starch									
15: Phosphorous									
16: Methionine (protein)									
17: Lysine (protein)									
18: Tryptophan (protein)									

Traits	11	12	13	14	15	16	17	18
1: Grain moisture	0.28**	0.02	0	−0.03	0.03	0.13	−0.01	−0.18*
2: Grain yield	0.25**	−0.27**	0.13	0.06	−0.07	0.1	0.27**	0.05
3: Endosperm opacity	−0.12*	−0.03	−0.31**	0.13*	−0.11*	0.21**	−0.14**	−0.37**
4: Methionine	−0.43**	0.44**	−0.19**	−0.21**	−0.04	0.95**	−0.32**	−0.46**
5: Lysine	−0.1	0.1	0.22**	−0.16**	0.11*	−0.1	0.76**	0.20**
6: Tryptophan	0.22**	0.01	0.31**	−0.21**	0.14**	−0.23**	0.32**	0.69**
7: 500 Kernel weight	−0.55**	0.35**	−0.27**	0.02	−0.11*	0.31**	−0.34**	−0.52**
8: Days to silk	0.34**	−0.19**	0.27**	−0.04	−0.01	−0.17**	0.22**	0.27**
9: Days to anthesis	0.43**	−0.26**	0.27**	−0.05	0.15**	−0.18**	0.26**	0.33**
10: Plant height	0.95**	−0.46**	0.04	0.11*	0.09	−0.34**	0.27**	0.53**
11: Ear height		−0.49**	0.06	0.13**	0.09	−0.33**	0.28**	0.57**
12: Crude protein			−0.10*	−0.70**	0.15**	0.17**	−0.55**	−0.71**
13: Fat				−0.32**	0.22**	−0.18**	0.23**	0.27**
14: Starch					−0.39**	0	0.31**	0.35**
15: Phosphorous						−0.10*	−0.01	0.01
16: Methionine (protein)							−0.17**	−0.28**
17: Lysine (protein)								0.63**
18: Tryptophan (protein)								

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

periodic quantification of amino acids in QPM programs is vital. Days to silk and anthesis, as well as plant and ear height, were highly correlated with one another as previously reported (Buckler et al., 2009; Meghji et al., 1984), providing confidence in phenotyping.

CONCLUSION

The microbial method for amino acid–concentration estimation proved effective to separate genotypes and estimate genetic variance parameters across diverse environments. Of the three amino acids examined, normal maize contained larger quantities of methionine, while QPM contained larger quantities of lysine and tryptophan. Among the new lines, Tx832 shows the potential of QPM germplasm to be bred for elevated lysine and tryptophan content as well as elevated methionine concentrations (compared to other QPM), which is an area in need of improvement within QPM breeding. QPM hybrid Tx832 × B73*o2o2* displayed stability across the 2010 pilot study and the primary 2011 test. This combination and future combinations with Tx832 have potential for further improvement of temperate QPM hybrids which produce elevated levels of essential amino acids lysine and tryptophan and also yield competitively.

Variation in protein quantity resulted in substantial rank changes for lysine and tryptophan, displaying the importance of selecting for protein quantity and amino acid quality. However, top hybrids for amino acid

estimation were similar to their protein-adjusted counterparts, thus a two-trait selection breeding process seems useful. Endosperm modification, while not affected by the diverse environments in this study, also followed a primarily additive, midparent trend, with some hybrids deviating from that trend displaying the complexity and recessive nature of multiple modifier loci as well as the effect diverse genetic backgrounds have on modifier expression and genetic effects. The Iowa location provided an extreme environment to which most hybrid combinations were found to be unadapted, significantly altering most traits, although amino acid estimation and endosperm opacity were found to be substantially less affected.

Supplemental Information Available

Supplemental information is included with this article.

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