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Evaluation of the grain methionine, lysine and tryptophan contents of maize (*Zea mays* L.) germplasm in the Germplasm Enhancement of Maize Project

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Abstract

The Germplasm Enhancement of Maize (GEM) Project is a cooperative effort between the USDA-ARS, private industry and public researchers to broaden and enhance the germplasm base of maize. In this program, selected accessions from the Latin American Maize Project, and seven tropical hybrids donated by DeKalb to the GEM Project, were crossed to elite proprietary inbred lines contributed by commercial plant breeding programs. In most cases, the resulting hybrids were crossed to a second commercial inbred line and the resulting 25% exotic hybrids were used as breeding populations for further development. To identify GEM germplasm with value to protein quality breeding programs, we developed a process for evaluating the content of the essential amino acids methionine, lysine and tryptophan in the grain of GEM germplasm that balances the need for multiple-year evaluations with the constantly changing entry list of this germplasm screening program. This process involves annual field trials with common checks. Weak entries are dropped from the trial each year to make room for new entries, while strong entries are retained. Methionine exhibited the most significant variation, followed by lysine and then tryptophan. A number of GEM lines had methionine or lysine levels that were significantly better than Corn Belt checks and some were competitive with high-amino acid checks.

Keywords: amino acid; germplasm enhancement; nutrition

Introduction

Since the majority of maize produced in the world is used for food or feed, nutritional quality is an important trait. While lysine is the nutritionally limiting essential amino acid, supplements are available and relatively inexpensive. Methionine and tryptophan can also be limiting and supplements for these amino acids are more expensive. Breeders have therefore focused considerable attention on improving the lysine, methionine and tryptophan concentration of maize grain. A mutation called *dzr*1 that results in increased methionine content has been used to develop inbred lines with improved grain methionine content (Olsen *et al*., 2003). The *opaque2* (*o2*) mutation results in increased levels of tryptophan and lysine in grain, and has been used to develop nutritionally enhanced, agronomically acceptable germplasm (Prassana *et al*., 2001).

Transgenic approaches have been employed successfully to improve amino acid balance as well. One such approach utilized a molecular mechanism similar to that occurring in *dzr*1 and resulted in an increase in methionine content of maize grain (Lai and Messing, 2002). Lysine

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concentration was increased by manipulation of enzymes involved in lysine metabolism in transgenic plants (Huang *et al.*, 2005; Houmard *et al.*, 2007; Frizzi *et al.*, 2008) or by the addition of a high lysine protein to the grain (Bicar *et al.*, 2008).

Novel sources of genetic diversity are critical for making progress in crop improvement. In the US Corn Belt, relatively little germplasm is derived from tropical sources (Nelson *et al.*, 2006). Tropical germplasm is therefore a potentially valuable source of genetic diversity for the improvement of US Corn Belt varieties but integration of this germplasm into Corn Belt breeding programs has been hindered by lack of adaptation of this germplasm to Corn Belt environments. To facilitate the use of tropical germplasm in Corn Belt breeding programs, the Germplasm Enhancement of Maize (GEM) Project was developed as a cooperative effort between the USDA-ARS, private industry and public researchers. In this program, selected accessions from the Latin American Maize Project were crossed to elite inbred lines contributed by commercial plant breeding programs. In most cases, the resulting hybrids were crossed to a second commercial inbred line and the resulting hybrids were used as breeding populations. GEM lines are developed by repeated cycles of self-pollination and selection from GEM breeding populations to the S3 level, whereupon the lines are maintained as bulks to preserve the diversity within them.

The GEM Project has been successful in developing and utilizing exotic germplasm for the enhancement of value-added grain traits (protein >13%, oil >5% or unique starch thermal properties), sources of disease and insect resistance, reduced mycotoxins and yield (Pollak, 2003; Blanco *et al.*, 2005). Recently, a source of germplasm with modifier genes that enhance amylose content above 70% was released (Campbell *et al.*, 2007).

In this study, we characterized germplasm from the GEM program with regard to its content of the essential amino acids methionine, tryptophan and lysine in order to identify additional sources of genetic variation for these traits. One challenge to screening germplasm for amino acid levels is to develop and implement an alternative method to the standard analytical method (AOAC International, 2002), which is time consuming and expensive. For evaluating amino acid levels in breeding programs, microbial methods with high throughput and high precision have been shown to be effective. A microbial method for grain methionine concentration determination was shown to be an effective tool for the selection of varieties with altered methionine content in a recurrent selection program, and the results of this method correlated very well with those of the standard analytical method (Scott *et al.*, 2008). Our second challenge was that we had a constantly changing set of germplasm to be evaluated, but we needed multiple-year evaluations to obtain reliable data. The objective of this work, then, was to develop a sustainable process for an evaluation of germplasm that would accommodate a changing set of entries, while allowing multiple-year testing for the content of the amino acids tryptophan, methionine and lysine in the grain. We met this objective by an ongoing evaluation of entries with good performance in multiple-year field trials that included a common set of check varieties.

Materials and methods

Grain production

The GEM Project currently evaluates over 1500 germplasm sources per year in top cross yield trials with elite testers provided by GEM commercial co-operators. First year trials are conducted on S2 lines. Lines that meet benchmark standards of yield (within 10% of commercial check hybrids), lodging resistance and maturity are further studied for resistance to disease, insects, reduced mycotoxins and value-added grain traits, and these lines are advanced in the breeding program. The selected lines are subsequently evaluated for a second year of yield trials. Thus, there is a constant turnover of lines in the evaluation process for yield and traits, with selected lines continued, new lines added and other lines deleted after each year of evaluation.

For an evaluation of amino acid content, a group of check samples were included in the field trial every year. As a group, these check samples have a wide range of content of the amino acids of interest. Standard Corn Belt checks included the inbreds B73, Mo17 and the F_1 hybrid, B73 × Mo17. Two high-amino acid inbreds included B45 *o2* for lysine and tryptophan and B101, which is high in methionine (Hallauer and Wright, 1995). In addition to the checks, a number of GEM lines were included to bring the total number of samples analyzed each year to about 90. About half of these GEM lines were selected as lines that performed well in previous evaluations and about half are new entries. Selected inbreds were advanced by self-pollination of eight to ten plants planted in our nursery in single-row plots of 365 cm length and 76 cm width. Fertilizer was applied as a dry custom mix of 30–10–10 with actual N of 135 kg per hectare, and P and K at 45 kg per hectare.

Amino acid analysis

Hand-pollinated (self-pollinated) ears were harvested from each nursery row. Forty randomly selected kernels

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from a bulked sample of eight ears of each line were dried and ground to a fine powder. The amino acids methionine and tryptophan were analyzed using the microbial methods previously described (Scott *et al.*, 2004). In this method, ground grain was treated with pepsin in acidic solution to hydrolyze proteins and extract amino acids. The resulting extract was added to bacterial culture systems designed, so that culture growth was limited by the availability of an amino acid of interest. For example, the cultures used to assay lysine lacked lysine in the culture medium and contained *Escherichia coli* strain KL334, a strain that is auxotrophic for lysine (Birge and Low, 1974). Following culture growth, the turbidity of each culture was measured using a microplate spectrophotometer. These optical density measurements were converted to g of each amino acid per 100 g of grain using standards containing pure amino acids in known concentrations. Each year, about 90 samples were analyzed in triplicate with each replication treated as a block in a randomized complete block design. In 2004, seven samples were selected for amino acid analysis by the AOAC official method (AOAC International, 2002).

Statistical analysis

To determine performance across years, the amino acid values for each genotype in each year were analyzed using a fixed effects model using the standard least-squares fitting method.

Variance was partitioned among years, genotypes and error. Outliers from the model were identified as those data points with studentized residuals greater than 3.5 or less than -3.5. Fewer than 3% of the measurements were identified as outliers. Least-squares mean values predicted by the model are reported for each genotype in Tables 2, 3 and 4. Significance groupings were assigned using a Student's t test of the least-squares mean values. Because the prediction of each genotype least-squares mean value had a different amount of error, it is possible for two genotypes to have the same least-squares mean value and belong to different significance groups. The least-squares mean values for each genotype were used to calculate Pearson product moment correlations and amino acid distributions. The broad-sense heritability (H^2) was calculated from the estimated variances of the genotype, year and error effects.

Results

An outcome of the germplasm enhancement program used by GEM is that new germplasm is continually being developed and this germplasm is in need of testing. We developed an evaluation program that involved the evaluation of a set of GEM lines each year using a microbial amino acid analysis method. Over the course of 5 years of evaluation (2003–2007), we have evaluated 160 lines, although some lines appear in many of the years and some lines appear in only a few years of the trial. For all amino acids, both genotype and year contributed significantly to the variation observed in the data (Table 1), although the genotypic variation was much more prominent for methionine than for tryptophan and lysine. This resulted in the broad-sense heritability (H^2) of methionine concentration being much higher than those of the other two amino acids. The heritabilities of methionine, lysine and tryptophan concentration were 0.70, 0.10 and 0.05, respectively (Table 1). Methionine concentration for the genotypes in the study is summarized in Table 2. While the high methionine check B101 was the highest in the test, three GEM lines were not significantly different from B101. The three lines were derived from 50% exotic breeding populations that originated from the Brazilian tropical hybrids DKXL370A and DKXL380. These hybrids were crossed to an adapted proprietary stiff stalk line designated as S11. The three GEM lines are publicly available from the North Central Regional Plant Introduction (NCRPIS) at www.ars-grin.gov. Two of the three lines were registered in Crop Science (Balint-Kurti *et al.*, 2006), and are designated as PI 639055 (2258-03 XL380 S11 in Table 2), and PI 639056 (2282-01 XL380 S11 in Table 2). The third line

Table 1. ANOVA of microbial amino acid assay results

		Sums of squares		
Effect	DF	Met	Trp	Lys
Genotype	44	0.067**	0.019**	0.009**
Year	4	0.002*	0.089**	0.015**
Error (met/trp/lys)	114/110/109	0.015	0.004	0.003
Model R^2		0.82	0.96	0.89
H^2		0.70	0.10	0.05

* Statistically significant at $P < 0.05$, ** Statistically significant at $P < 0.01$.

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Table 2. Methionine content of GEM lines evaluated for 3 or more years

Genotype	Grouping[a] (based on microbial method)	Met content (g/100 g grain)	
		Microbial	AOAC
B101	A	0.188	
2228-03_DK370A_S11_F2S4_3358	AB	0.178	
2282-01_XL380_S11_F2S4_9226	ABC	0.174	0.31
2258-03_XL380_S11_F2S4_71/97	ABC	0.173	0.33
CHIS740:S1411a-783-2	BCD	0.169	0.32
CUBA164:S2012-235-1	BCDE	0.164	
2011-01_SE32_S17_F2S4_9148	BCDEF	0.161	
DKXL212:N11a-139-1-1	CDEFG	0.158	0.25
CUBA164:S1517-163-1	CDEFGH	0.156	
CUBA164:S1511b-325-1	DEFGHI	0.154	
CUBA164:S2012-459-1	DEFGHIJ	0.153	
CUBA117:S15-372-1	EFGHIJ	0.153	
CUBA164:S2012-313	EFGHIJK	0.152	
CUBA164:S2012-606	EFGHIJKL	0.150	
B73	EFGHIJK	0.150	
CH05015:N15-003-1	EFGHIJKLM	0.149	
2253-01_XL370A_S11_F2S4_9220	FGHIJKLMN	0.145	
FS8B(S):S0316-814-1	FGHIJKLM	0.145	
AR16035:S02-450-1	FGHIJKLMN	0.145	
CUBA164:S2012-488-1	FGHIJKLMN	0.144	
2088-01_DK212T_S11_F2S4_9157	FGHIJKLMNO	0.143	
2084-02_DK212T_S11_F2S4_9151	GHIJKLMNO	0.142	0.23
CUBA164:S2012-444-1	HIJKLMNO	0.141	
AR16035:S19-161-1	HIJKLMNOP	0.140	
DREP150:N2011d-624-1	HIJKLMNOPQ	0.140	
2152-02_DK888_S11_F2S4_9202	HIJKLMNOPQ	0.140	
UR13085:N0215-014-1	HIJKLMNOPQR	0.138	
DKXL370:N11a20-322-1	IJKLMNOPQRS	0.136	
GUAT209:N1925-081-1	KLMNOPQRST	0.134	0.34
AR16026:S1704-167-2	KLMNOPQRST	0.133	
AR16026:S1704-153-1	MNOPQRS	0.133	
UR10001:S1813-257-1	LMNOPQRST	0.133	
CUBA164:S2012-966-1	JKLMNOPQRSTU	0.133	
CHIS775:N1912-651-1	NOPQRSTUV	0.128	
CHIS775:S1911b-120-1	OPQRSTUV	0.125	
2142-01_DK888_S11_F2S4_9190	PQRSTUV	0.122	
BR52051:N04-070-1	QRSTUVW	0.121	
DKB844:N11b-118-1	RSTUVW	0.120	
AR16026:S1704-058-3	STUVW	0.118	
B45 o2	TUVW	0.118	
2112-02_DK212T_S11_F2S4_9169	STUVW	0.118	
B73 × Mo17	UVW	0.116	
FS8B(T):N1802-382-1	VW	0.113	0.18
BR52051:N04-076-1	WX	0.103	
Mo17	X	0.096	

[a] Genotypes not connected by the same letter are significantly different ($P < 0.05$).

'2228-03 DK370A' has not been registered but is also publicly available as Ames 26 501 from the NCRPIS.

Tryptophan content for the genotypes in the study is summarized in Table 3. The most notable feature of these data is that with the exception of the high tryptophan check, B45 *o2*, there is very little significant variation among the samples. A similar result was obtained for lysine (Table 4), although for lysine, one GEM line was not different from the high lysine check, B45 *o2*.

It is most desirable to improve multiple nutritionally limiting amino acids simultaneously. For this reason, it is important to examine the relationship between the different amino acids. We determined Pearson product-moment correlations for the three amino acids examined in this study. Tryptophan and lysine exhibited a significant correlation of 0.44, while methionine was not significantly correlated with the other two amino acids. These data suggest that it should be possible to

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Table 3. Tryptophan content of GEM lines evaluated for 3 or more years

Genotype	Grouping[a] (based on microbial method)	Trp content (g/100 g grain)	
		Microbial	AOAC
B45 o2	A	0.173	
FS8B(S):S0316-814-1	B	0.115	
FS8B(T):N1802-382-1	B	0.115	0.07
B101	C	0.107	
DKXL212:N11a-139-1	CD	0.106	0.07
2258-03_XL380_S11_F2S4_71/97	CDE	0.106	0.07
BR52051:N04-070-1	CDEFG	0.105	
AR16026:S1704-167-2	CDEFG	0.105	
B73	CDEF	0.105	0.08
GUAT209:N1925-081-1	CDEFGH	0.105	
BR52051:N04-076-1	CDEFGHI	0.104	
2011-01_SE32_S17_F2S4_9148	CDEFGHIJ	0.103	
AR16035:S02-450-1	CDEFGHI	0.103	
2152-02_DK888_S11_F2S4_9202	CDEFGHIJ	0.102	
DREP150:N2011d-624-1	CDEFGHIJ	0.102	
CUBA164:S2012-313-1	CDEFGHIJ	0.102	
CH05015:N15-003-1	CDEFGHIJ	0.102	
DKB844:N11b-118-1	CDEFGHIJK	0.101	
AR16026:S1704-153-1	CDEFGHIJ	0.101	
CUBA164:S2012-444-1	CDEFGHIJK	0.100	
CUBA164:S2012-488-1	CDEFGHIJK	0.100	
CUBA117:S15-372-1	CDEFGHIJK	0.100	
CHIS740:S1411a-783-2	CDEFGHIJK	0.100	
Mo17	CDEFGHIJK	0.100	0.08
B73 x Mo17	CDEFGHIJK	0.099	
2228-03_DK370A_S11_F2S4_3358	CDEFGHIJK	0.099	
CUBA164:S2012-966-1	CDEFGHIJK	0.099	
2084-02_DK212T_S11_F2S4_9151	CDEFGHIJK	0.099	0.08
DKXL370:N11a20-322-1	CDEFGHIJKL	0.099	
CUBA164:S2012-459-1	CDEFGHIJKL	0.098	
UR10001:S1813-257-1	CDEFGHIJKL	0.098	0.08
2282-01_XL380_S11_F2S4_9226	CDEFGHIJKL	0.098	
CUBA164:S1511b-325-1	EFGHIJKL	0.098	
CHIS775:N1912-651-1	FGHIJKL	0.097	
2112-02_DK212T_S11_F2S4_9169	FGHIJKL	0.097	
AR16026:S1704-058-3	GHIJKL	0.096	
CUBA164:S2012-606-1	HIJKL	0.096	
AR16035:S19-161-1	HIJKL	0.095	
CHIS775:S1911b-120-1	IJKL	0.095	
2088-01_DK212T_S11_F2S4_9157	IJKL	0.095	
2142-01_DK888_S11_F2S4_9190	IJKL	0.094	
CUBA164:S1517-163-1	JKL	0.093	
CUBA164:S2012-235-1	JKL	0.093	
UR13085:N0215-014-1	KL	0.092	
2253-01_XL370A_S11_F2S4_9220	L	0.089	

[a] Genotypes not connected by the same letter are significantly different ($P < 0.05$).

improve multiple amino acids simultaneously, although it may be easier to improve lysine and tryptophan together than in combinations involving methionine.

Discussion

Our objective was to develop a procedure for obtaining reliable grain amino acid content data in the course of development of germplasm by the GEM program. We accomplished this with an evaluation plan that included evaluation of germplasm with a high-throughput amino microbial assay for the nutritionally limiting amino acids, lysine, methionine and tryptophan. Yearly evaluations were carried out, with common checks included in the trial in each year. Germplasm that performed well in the trial was retained in subsequent years and germplasm that did poorly was removed

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Table 4. Lysine content of GEM lines evaluated for 3 or more years

Genotype	Grouping[a] (based on microbial method)	Lys content (g/100 g grain)	
		Microbial	AOAC
DREP150:N2011d-624-1	A	0.106	
B45 o2	A	0.104	
Mo17	AB	0.099	0.32
DKXL212:N11a-139-1-1	BC	0.096	
2253-01_XL370A_S11_F2S4_9220	BCDEF	0.091	
2258-03_XL380_S11_F2S4_71/97	CDE	0.091	0.30
FS8B(T):N1802-382-1	CD	0.091	0.32
CHIS740:S1411a-783-2	CDEF	0.089	0.32
2084-02_DK212T_S11_F2S4_9151	CDEFG	0.088	0.32
2228-03_DK370A_S11_F2S4_3358	DEFGHI	0.086	
B73 x Mo17	DEFGH	0.086	
2088-01_DK212T_S11_F2S4_9157	DEFGHIJ	0.086	
B101	DEFGHI	0.085	
2282-01_XL380_S11_F2S4_9226	DEFGHIJK	0.085	0.32
BR52051:N04-070-1	DEFGHIJKL	0.084	
CUBA117:S15-372-1	EFGHIJKLM	0.083	
CUBA164:S2012-459-1	FGHIJKLMN	0.082	
GUAT209:N1925-081-1	GHIJKLMNO	0.080	0.32
CUBA164:S2012-313-1	GHIJKLMNO	0.080	
DKB844:N11b-118-1	GHIJKLMNO	0.080	
BR52051:N04-076-1	GHIJKLMNO	0.080	
DKXL370:N11a20-322-1	GHIJKLMNO	0.080	
CHIS775:N1912-651-1	GHIJKLMNO	0.079	
CUBA164:S2012-235-1	HIJKLMNOP	0.079	
AR16035:S02-450-1	IJKLMNO	0.079	
2011-01_SE32_S17_F2S4_9148	HIJKLMNOP	0.079	
CUBA164:S2012-488-1	HIJKLMNOP	0.078	
FS8B(S):S0316-814-1	JKLMNOP	0.078	
2152-02_DK888_S11_F2S4_9202	IJKLMNOP	0.078	
CHIS775:S1911b-120-1	JKLMNOP	0.077	
CUBA164:S2012-966-1	JKLMNOP	0.077	
2142-01_DK888_S11_F2S4_9190	JKLMNOP	0.077	
CUBA164:S1511b-325-1	JKLMNOP	0.077	
B73	KLMNOP	0.077	
AR16026:S1704-153-1	KLMNOP	0.076	
UR10001:S1813-257-1	KLMNOP	0.076	
AR16026:S1704-058-3	KLMNOP	0.076	
AR16035:S19-161-1	LMNOP	0.075	
UR13085:N0215-014-1	MNOP	0.075	
AR16026:S1704-167-2	KLMNOP	0.075	
CUBA164:S2012-444-1	NOP	0.074	
CUBA164:S1517-163-1	NOP	0.074	
CUBA164:S2012-606-1	OP	0.073	
2112-02_DK212T_S11_F2S4_9169	OP	0.072	
CH05015:N15-003-1	P	0.070	

[a] Genotypes not connected by the same letter are significantly different ($P < 0.05$).

from the trial, so new germplasm could be added. By changing a portion of the entries in the study each year, we reduced our power to precisely determine amino acid concentration, but it is important to keep in mind that the GEM program produces new germplasm sources for evaluation each year, and this system provides a rigorous, multiple-year and cost-effective evaluation method that accommodates the addition of new germplasm.

We observed more variation for methionine than for tryptophan and lysine in this study. The reduced variation for tryptophan and lysine is likely to slow the progress in breeding for these two amino acids. By contrast, the relatively high variability for methionine suggests that recurrent selection may be an effective way to improve this amino acid. This variability may be a consequence of the multiple roles it serves in plant metabolism. In addition to being a component of

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proteins, methionine is involved in one-carbon metabolism in the form of S-adenosyl methionine. It would be interesting to determine whether the variation observed in methionine is primarily in methionine contained in proteins or whether it is in free methionine involved in other aspects of metabolism.

The values produced by the microbial method, while very reproducible, are known to be lower than those produced using the standard AOAC method, so these values should not be used for comparisons across studies but rather for making comparisons within this study. In addition, the correlation between the AOAC method and the microbial method is low for lysine and tryptophan. One explanation for this is that there is very little variation for these amino acids in the germplasm examined. A second possible explanation is that the AOAC data were obtained only in one year of the study, while the microbial data were obtained in at least 3 years.

The significant correlation between lysine and tryptophan is consistent with earlier observations (Vivek *et al.*, 2008). As suggested in this earlier study, it may be possible to improve both lysine and tryptophan by selecting for one or the other of these amino acids. The lack of strong, negative correlations between the three amino acids suggest that it should be possible to improve all three amino acids simultaneously; however, the low heritability of tryptophan and lysine suggest that breeding progress for these traits will be slow and mutation breeding or transgenic approaches might be more appropriate for these traits.

A great deal of effort has been devoted for improving the amino acid balance of grain, with the majority of the effort being devoted to mutation breeding and transgenic approaches. The agronomically superior germplasm with good amino acid content identified by this program provides a foundation to which these other breeding methods can be applied. We conclude that GEM germplasm is a valuable resource for breeding for methionine content.

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