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RESEARCH

Interaction of Genetic Mechanisms Regulating Methionine Concentration in Maize Grain

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ABSTRACT

Methionine is a limiting amino acid in poultry diets, so methionine supplementation is typically required to meet nutritional demands. Maize (*Zea mays* L.) varieties with increased methionine levels have been developed using three different approaches: (i) increased levels of the methionine-rich 10-kDa zein, (ii) disruption of protein deposition using the *floury-2* allele, and (iii) recurrent selection. The goal of this study was to characterize the interactions of these three mechanisms for increasing methionine to develop optimal breeding strategies for this limiting amino acid. A complete diallel mating design was used to produce all possible hybrid combinations, which were analyzed by Griffing's experimental Method 3, Model 1. Grain samples were analyzed for methionine concentration using a microbial method. The significantly negative general combining ability (GCA) for inbred RS2 suggests it did not perform well in hybrid combination, while the significant specific combining abilities (SCAs) suggest that some specific combinations of mechanisms worked well together in this germplasm. Analysis of grain quality traits by near-infrared spectroscopy (NIRS) revealed that the high-methionine hybrid combinations had starch and oil concentrations similar to all other hybrids but had elevated protein concentrations. In some hybrids in this study, *dzr1* and recurrent selection were effective mechanisms to elevate methionine in hybrid combination and did not have an associated yield penalty relative to other hybrids produced in the study, which supports their use in a high-methionine maize breeding program.

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Abbreviations: GCA, general combining ability; NIRS, near-infrared spectroscopy; SCA, specific combining ability.

Poultry diets based on corn and soybean [*Glycine max* (L.) Merr.] require methionine supplementation because both grains have inadequate methionine concentrations. Feed supplementation with commercially produced methionine is used to alleviate these deficiencies but also increases poultry production costs (Waldroup et al., 1981). Breeding new maize varieties with improved methionine levels would mitigate the high cost associated with methionine supplementation and provide a more nutritionally balanced feed source.

At least three approaches have been found to be effective for increasing grain methionine concentration. The first is exemplified in the inbred line B101, which was developed from the Iowa stiff stalk synthetic population (Hallauer and Wright, 1995). The methionine concentration of this line was reported to exceed related inbred lines by 20% (Phillips et al., 1981). Early literature suggested an overexpression of the methionine-rich 10-kDa delta zein (*dzs10*) was responsible for the increase in total grain methionine concentration (Kirihara et al., 1988). It was later revealed that *dzs10* transcripts are regulated posttranscriptionally by *delta zein regulator1* (*dzr1*), which encodes a *trans*-acting factor. Thus, B101 has an increased methionine concentration because *dzr1*

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stabilizes *dzs10* messenger RNA levels, resulting in higher expression of a methionine-rich protein (Cruz-Alvarez et al., 1991). Furthermore, a transgenic approach to increase methionine levels uses this knowledge to increase *dzs10* transcript stability by modifying the 5′ untranslated region, promoter, and 3′ regions around the *dzs10* coding region (Lai and Messing, 2002).

A second method for increasing methionine concentration is use of another naturally occurring mutation. The *floury-2* (*fl2*) mutant leads to an increased methionine concentration resulting in a more balanced amino acid pattern (Nelson et al., 1965). The *fl2* mutation is reported to be codominant, which might be advantageous in breeding programs because heterozygous hybrids may express the beneficial effects of the mutation. *fl2* encodes an unusual form of a major α zein (Coleman et al., 1995), which causes aberrant protein body formation resulting in kernels with an opaque phenotype instead of the wild-type vitreous phenotype (Lending and Larkins, 1992). It is not clear how this results in elevated methionine levels.

Numerous studies have determined the extent of variation in amino acid profiles for various germplasm (Doty et al., 1946; Aguirre et al., 1953; Scott et al., 2004). The results of Reynolds et al. (2005) suggest the variability in amino acid levels among commercial hybrids is controlled by the genetic background and growing conditions. Taken together, these studies suggest it should be possible to use plant breeding methods to alter the methionine concentration in breeding populations. Recurrent selection is a plant breeding approach that has been widely used to improve quantitative traits in maize. The Illinois long-term selection experiment for grain composition (reviewed in Moose et al., 2004) altered protein and oil concentrations to produce populations with trait values representing the known extreme values for maize. Furthermore, Scott et al. (2008) altered grain composition by selecting for either high or low methionine concentrations. Three cycles of selection were sufficient to cause a significant difference in methionine concentration between populations selected for high and low values, respectively, illustrating that the methionine concentration of maize grain can be altered by traditional breeding and providing a third approach to increasing methionine concentration. While the molecular mechanism for increasing methionine concentration is not well understood, in this study we refer to this mechanism throughout the manuscript as recurrent selection.

The diallel mating design involves production of all possible crosses among a set of inbred lines and allows for evaluation of the genetic effects of these lines and their resulting hybrid combinations. Using this mating design with a statistical model outlined by Griffing (1956), information regarding GCA, SCA, and reciprocal effects can be determined. The general definitions provided by Sprague and Tatum (1942) for GCA and SCA are the average performance of an inbred line in hybrid combination and the expected performance of a hybrid combination compared with the average performance of inbred lines involved, respectively. Previously, this approach has been used to understand the genetic effects controlling methionine concentration in lines with normal methionine levels (Darrigues et al., 2005).

An additional advantage of this mating design is that it allows statistical tests of hypotheses about interactions of specific genetic mechanisms to be tested. Midparent heterosis can be evaluated by calculating the difference between a hybrid and the mean of the two inbred parents used in its production (Hallauer and Miranda, 1981). The absence of significant SCA or midparent heterosis would be interpreted as no evidence for interaction between the genetic mechanisms combined. Reciprocal effects in F_2 plants could be due to maternal inheritance and can be important for assigning genders to the parents of a hybrid. Thus, a diallel mating design can provide guidance to breeders developing hybrids with high-methionine grain and provide clues about how different genetic mechanisms interact to determine methionine concentration in grain.

The objectives of this study are (i) to determine the effects of combining inbreds with different genetic mechanisms for increasing methionine concentration in hybrid combination and (ii) to estimate the genetic effects regulating methionine concentration in resulting hybrids. Results from this study will provide breeders with additional information they should consider when breeding high-methionine corn with regards to each method used to increase methionine levels as well as any interactions between them.

MATERIALS AND METHODS

Field Procedures

Two separate experiments were conducted in this study with grain composition (especially methionine concentration) and grain yield being of interest for the first and second experiments, respectively. In the first experiment, seven different inbred lines, shown in Table 1, were selected based on previous information about grain methionine concentration. Prior to this study, these lines had not been evaluated for their methionine concentration relative to each other, and in this study, we found that assignment of inbreds to high or low methionine classes was not always correct. All lines were mated in a complete seven-by-seven diallel mating design to produce 49 entries (including inbreds and hybrids). Each of the 49 entries was planted in the summers of 2010 and 2011 at the Iowa State University Agronomy Farm near Ames, IA. Each field contained two replications planted in a randomized complete block design with all entries grown in one-row plots 5.5 m long with 0.76 m between rows. Four ears per plot were self-pollinated, harvested, dried to ~12% moisture, and shelled individually. The second experiment included a yield trial for 42 hybrid combinations (excluding inbred parents) produced in the seven-by-seven diallel mating. Each of the 42 F_1 hybrids were planted in the summer of 2010 in Ames, IA, and 2011 in Hampton, Ames, Eldora, and Cresco, IA, for a total of five locations. Each location contained

Table 1. Inbred lines used in the seven-by-seven diallel study.

Name of parent	Characteristics
B101	High grain methionine concentration attributed to increased levels of the 10-kDa zein conferred by the *dzr1* gene and derived from the Iowa stiff stalk synthetic population.
RS2	Generated from BS31 following two generations of recurrent selection (RS) for high methionine prior to self-pollination.
RS3	Generated from BS31 following three generations of recurrent selection (RS) for methionine prior to self-pollination.
*fl2*Oh43	*floury-2* allele fixed in Oh43 genetic background. The number of backcrosses is unknown but the mutant line is phenotypically similar to Oh43.
*fl2*W64A	*floury-2* allele fixed in W64A genetic background. The number of backcrosses is unknown but the mutant line is phenotypically similar to W64A.
Oh43	Normal methionine control.
W64A	Normal methionine control.

two replications planted in a randomized complete block design with each entry grown in a two-row plot 5.5 m long with 0.76 m between rows. Each plot was harvested using a combine equipped with a weighing unit and moisture meter. Plot yield was calculated from the grain weight of each plot following harvest and adjusted to 15.5% grain moisture.

Grain Composition Analysis

Methionine concentrations were measured on four ears from each plot using a high-throughput microbial method outlined by Scott et al. (2004). This method is based on measurement of turbidity of bacterial cultures that are auxotrophic for methionine. Throughout the manuscript, references to methionine concentration reflect the results of this assay. Grain samples from each ear were measured in quadruplicate and averaged to produce an average methionine concentration for each ear. Briefly, 10 mg of ground samples were randomly weighed in a 96-well, V-bottom plate. Protein extraction and hydrolysis used 0.2 mg of pepsin suspended in 50 mM KCl buffer adjusted to pH 2.0 with HCl. Plates were then centrifuged for 20 min at 1600 *g* following a 16-h shaking incubation at 37°C. Five μL of the resulting hydrolysate were transferred to a second 96-well, flat-bottom plate and inoculated with 100 μL of an auxotrophic *E. coli* strain in M9 minimal medium (P4X, Jacob and Wollman, 1961). This strain has a genetic lesion in the methionine biosynthetic pathway so that culture growth is limited by the amount of methionine in the medium. Following incubation for 8 h at 37°C, the growth of each culture was determined by measuring its turbidity at 595 nm using a spectrophotometer. Culture turbidity measurements have been shown to be a reliable estimate for methionine concentration compared with standard AOAC determinations with turbidity measurements being proportional to amino acid concentrations (Wright and Orman, 1995). Each of the grain samples produced in 2010 and 2011 at Ames, IA, were analyzed for methionine concentration along with protein, starch, and oil concentrations predicted by NIRS using a Foss Infratec 1241 grain analyzer (Foss NIR Systems, Inc.).

Statistical Analysis

Experimental Method 3, Model 1 (Griffing, 1956) was fit to the data to calculate GCA, SCA, and reciprocal effects for the set of parents included in this study and for all traits of interest using Diallel-SAS05 with SAS version 9.4 (Zhang et al., 2005). Method 3, Model 1 (Griffing, 1956) includes all F_1 hybrids (including reciprocal crosses) but does not include parents. The model contains restrictions such that GCA effects and SCA effects sum to zero.

The linear model used for each trait (yield, methionine, protein, oil, and starch concentration) was as follows:

$$Y_{ijkl} = \mu + \alpha_l + \nu_{ij} + b_{k(l)} + (\alpha\nu)_{ijl} + e_{ijkl}$$

$$\nu_{ij} = g_i + g_j + s_{ij} + r_{ij}$$

in which Y_{ijkl} is the observed trait value for each experimental unit, μ is the grand mean, α_l is the environment effect, ν_{ij} is the F_1 hybrid effect which equals the GCA for the *i*th and *j*th parent plus the SCA for the *ij*th F_1 hybrid plus the reciprocal effect of the *ij*th cross, $b_{k(l)}$ is the replication effect nested within each environment, $(\alpha\nu)_{ijl}$ is the interaction effect between the *ij*th F_1 hybrid and environment, and e_{ijkl} is the residual error. All effects in the model were fit as fixed effects. Studentized residuals [(observed value − predicted value)/standard error)] were evaluated for their fit to a normal distribution by visual examination of quantile–quantile plots. Significance of all factors included in the model was tested by an analysis of variance.

Determination of the effectiveness of each mechanism to increase methionine concentration and protein quality (percentage methionine/percentage protein) used several contrasts to compare average methionine concentrations and protein quality. These contrasts, performed using JMP Pro 11.0 (SAS Institute, 2013), included normal methionine hybrids vs. hybrids with each mechanism individually, hybrids with one mechanism vs. two mechanisms, hybrids with two copies of *fl2* vs. hybrids with one copy of *fl2*, hybrids with two copies of *fl2* vs. normal methionine hybrids and, hybrids with one copy of *fl2* vs. normal methionine hybrids.

An additional calculation included midparent heterosis for methionine concentration. Midparent heterosis was calculated using the following formula {midparent = [(mean of F_1 − mean of parents)/mean of parents] × 100}. Specific contrasts were used to determine the significance of this effect.

Several additional contrasts were performed to determine if differences in grain quality traits (oil, starch, and protein) were found between hybrids with significantly positive SCAs and all other hybrid combinations or inbred parents.

RESULTS

Evaluation of Methionine Concentration

Mean methionine concentrations are presented in Table 2 for all 49 entries in Exp. 1. Mean values for self-pollinated inbred parents and F_1 hybrid crosses are shown in this table, where row and column means do not include inbred values.

Table 2. Mean methionine (Met) concentration (g 100 g^{-1} dry wt.), starch, protein, and oil concentration (% dry matter) for all 49 entries produced in the diallel study.

		♂							
♀		**B101**	**RS2**	**RS3**	***fl2*Oh43**	***fl2*W64A**	**Oh43**	**W64A**	**Mean†**
B101	Met	**0.1904**	0.1799	0.1854	0.1953	0.1847	0.1729	0.1900	0.1847
	Protein	**16.5545**	12.5521	12.9602	13.1131	12.6111	10.4566	11.5722	12.2109
	Starch	**65.4036**	66.3341	66.2130	70.3420	68.7117	69.9176	69.7210	68.5399
	Oil	**4.5888**	5.4890	5.5187	4.5313	4.4684	4.5828	5.3760	4.9944
RS2	Met	0.1631	**0.1928**	0.1778	0.1756	0.1714	0.1743	0.1686	0.1718
	Protein	11.5873	**15.5657**	11.5581	11.3926	12.4272	13.4072	11.9192	12.0486
	Starch	69.3677	**68.4952**	70.4323	70.6702	69.5059	67.6263	69.3920	69.4991
	Oil	4.6681	**4.5404**	4.4366	4.4879	4.5997	4.3719	4.4811	4.5076
RS3	Met	0.2005	0.1788	**0.1995**	0.1717	0.1897	0.1765	0.1832	0.1834
	Protein	14.3986	10.8497	**15.4257**	10.6368	10.6483	12.6773	13.5017	12.1187
	Starch	70.3838	71.5236	**65.4286**	71.4074	71.6318	69.5237	68.6215	70.5153
	Oil	4.4519	4.1923	**4.4264**	4.3908	3.6496	3.9037	4.5354	4.1873
*fl2*Oh43	Met	0.1774	0.1594	0.1756	**0.1961**	0.1909	0.1904	0.1742	0.1780
	Protein	13.4658	13.6369	11.4463	**16.3166**	12.9107	12.4999	12.4415	12.7335
	Starch	68.8086	68.4782	70.3272	**65.1626**	66.5454	69.2232	67.0766	68.4099
	Oil	4.3774	4.5557	4.5391	**5.5887**	5.3065	4.3623	5.3911	4.7553
*fl2*W64A	Met	0.1770	0.1469	0.1919	0.1920	**0.2041**	0.1768	0.1918	0.1794
	Protein	9.6652	11.5377	12.9703	13.4148	**15.1082**	12.5291	12.3301	12.0745
	Starch	71.4334	68.4797	70.0580	67.6496	**67.8241**	65.3109	67.4000	68.3886
	Oil	4.6223	4.6035	5.5301	5.1787	**5.5716**	5.2590	5.2232	5.0694
Oh43	Met	0.1844	0.1843	0.1844	0.1865	0.1820	**0.1929**	0.1854	0.1845
	Protein	13.4841	11.7148	14.6192	12.3252	13.4427	**14.0263**	12.4367	13.0038
	Starch	64.4983	66.1482	65.1550	69.6313	67.5042	**69.6635**	69.5636	67.0834
	Oil	5.0269	4.7505	4.9383	4.5571	4.7785	**3.8079**	4.5403	4.7653
W64A	Met	0.1701	0.1701	0.1696	0.1863	0.1949	0.1407	**0.1868**	0.1720
	Protein	12.7023	11.6306	12.6395	13.8255	13.8358	12.4794	**13.5024**	12.8522
	Starch	69.3737	70.5424	65.1112	66.2850	69.6169	66.5807	**66.3693**	67.9183
	Oil	3.8467	4.5950	5.6685	4.4345	4.5275	4.6227	**4.5113**	4.6158
Mean†	Met	0.1788	0.1699	0.1808	0.1846	0.1856	0.1719	0.1828	–
	Protein	12.5506	11.9870	12.6989	12.4513	12.6459	12.3416	12.3669	–
	Starch	68.9776	68.5844	67.8828	69.3309	68.9193	68.0304	68.6291	–
	Oil	4.4989	4.6976	5.1052	4.5967	4.5550	4.5171	4.9245	–

	Met	**Starch**	**Protein**	**Oil**
F_1 Hybrid mean†	0.1791	68.6221	12.4346	4.6993
F_1 Hybrid SD	0.0156	1.9846	1.1758	0.5361
Inbred mean‡	0.1947	66.9067	15.2142	4.7193
Inbred SD	0.0134	1.7509	1.2082	0.6581
LSD	0.0197	0.7661	0.7747	0.4309

† Mean does not include inbred values in boldface text.

‡ Mean only includes inbred values in boldface text.

For the four grain-quality traits analyzed, all genetic sources of variation (GCA, SCA, reciprocal effects, heterosis, and variation among inbreds) were significant except inbred variation for methionine and heterosis for oil concentration (Table 3). Inbred RS2 had the only significant ($p = 0.001$) estimate for the GCA effect and it was negative (Table 4). Since Griffing (1956) constrains the individual SCA effects to zero, positive and significant estimates of genetic effects indicate hybrid combinations of interest. RS3/B101, Oh43/RS2, and W64A/*fl2*W64A all had positive and significant SCA estimates with RS3/B101 being the only one exhibiting a synergistic effect between two different mechanisms for increasing methionine concentration. Conversely, *fl2*Oh43/RS3 had a significant negative SCA and was the only example of an antagonistic interaction between genetic mechanisms. Some of the hybrids with high methionine had one normal methionine parent. We conclude that while high-methionine hybrids can be produced when only one inbred parent contains a mechanism to increase methionine concentration, it is also possible to produce low methionine hybrids in this way as well.

To further explore the impact of including specific mechanisms for increasing methionine in hybrids, comparisons were made between various hybrid combinations containing *dzr1*, recurrent selection, *fl2*, or more than one of the previously mentioned mechanisms to wild-type hybrids lacking any mechanism to elevate methionine. As shown in Table 5, most of these comparisons were not significant. Thus, it appears the effectiveness of different

Table 3. Analysis of variance (ANOVA) for the methionine (g 100 g^{-1} dry wt.), oil, protein, and starch concentration (% dry matter) linear model.

		Mean Squares			
Source of variation	df	Methionine	Oil	Protein	Starch
Environment	1	0.00003	1.3357***	4.065***	10.5625***
Replication	2	0.00132***	0.7883***	1.5474**	1.0072*
Average heterosis	1	0.00444***	0.0096	185.4271***	70.6191***
Inbreds	6	0.00015	1.6608***	5.0218***	12.4928***
General combining ability	6	0.00054**	0.3466***	2.8323***	17.7242***
Specific combining ability	14	0.00037*	0.6594***	5.9450***	17.9192***
Reciprocal effects	21	0.00031*	1.1637***	4.5131***	12.5802***
Genotype × environment	48	0.00025*	0.1184***	0.2587	0.1986
Experimental error	96	0.00015	0.0561	0.2666	0.2299
CV†		6.27%	5.29%	3.56%	0.63%

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

† Average coefficient of variation of treatment means.

Table 4. Estimates for the genetic effects for methionine (Met) (g 100 g^{-1} dry wt.), starch, protein, and oil concentration (% dry matter). The estimates for general combining ability (GCA) are included in the main diagonal of the table (boldface type). The estimates for specific combining ability (SCA) are included below the diagonal. The estimates for reciprocal effects are included above the diagonal.

		♂						
♀		B101	RS2	RS3	*fl2*Oh43	*fl2*W64A	Oh43	W64A
B101	Met	**0.00198**	0.00839	−0.0076	0.01089*	0.00385	−0.0026	0.00995*
	Starch	**0.164**	−1.5168*	−2.0854**	0.7667	−1.3609	2.7097**	0.1737
	Protein	**−0.0647**	0.4824	−0.7192	−0.1763	1.4729*	−1.5138**	−0.565
	Oil	**0.0568**	0.4104*	0.5334*	0.077	−0.077	−0.2221	0.7646**
RS2	Met	−0.0027	**−0.00766*** **	−0.0005	0.00741	−0.0002	−0.0071	−0.0007
	Starch	−1.4388*	**0.5036**	−0.5457	1.096	0.5131	0.739	−0.5752
	Protein	0.1999	**−0.5002***	0.3542	−1.1221*	0.4447	0.8462	0.1443
	Oil	0.4385*	**−0.1160***	0.1222	−0.0339	−0.0019	−0.1893	−0.0569
RS3	Met	0.00868*	0.00364	**0.00242**	−0.0026	−0.0011	−0.0039	0.00679
	Starch	−1.1801	1.16	**0.6924**	0.5401	0.7869	2.1844*	1.7552
	Protein	1.3404*	−0.6996	**−0.0309**	−0.4047	−1.1610*	−0.9709*	0.4311
	Oil	0.2929	−0.2052	**−0.0637**	−0.0741	−0.9402**	−0.5173*	−0.5666*
*fl2*Oh43	Met	0.00686	−0.0029	−0.00941*	**−0.0004**	0.00756	0.00395	−0.006
	Starch	0.4912	0.1506	1.2549	**0.298**	−0.5521	−0.204	0.3958
	Protein	0.7301	0.391	−1.5515**	**0.1894**	−0.252	0.0874	−0.692
	Oil	−0.2738	−0.0335	−0.1428	**−0.0279**	0.0639	−0.0974	0.4783*
*fl2*W64A	Met	−0.0045	−0.0041	0.00498	−0.0021	**0.00351**	−0.0026	−0.0016
	Starch	1.2482	−0.1711	1.4922*	−1.8609*	**0.0383**	−1.0966	−1.1084
	Protein	−1.1425*	0.1373	−0.5052	0.628	**−0.0892**	−0.4568	−0.7528
	Oil	−0.3463	−0.1172	−0.1813	0.4357*	**0.1355***	0.2402	0.3479
Oh43	Met	−0.0068	0.00866*	−0.0023	0.00653	−0.0068	**0.00046**	0.01281*
	Starch	−0.2999	−0.9602	−0.6969	1.7854*	−1.6715*	**−1.2782***	1.4914*
	Protein	−0.6853	0.3409	0.9589*	−0.4971	1.3137*	**0.2857**	−0.0214
	Oil	0.1185	0.0477	−0.1449	−0.1419	0.1088	**−0.0698**	−0.0412
W64A	Met	−0.0015	−0.0026	−0.0056	0.00106	0.01024*	−0.0016	**−0.0003**
	Starch	1.1793	1.2595	−2.0301*	−1.8212*	0.2661	1.1463	**−0.418**
	Protein	−0.4426	−0.3694	0.457	0.2996	0.5276	−0.4722	**0.2099**
	Oil	−0.2298	−0.1302	0.3813*	0.1564	−0.0445	−0.1331	**0.085**

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

mechanisms conferring high methionine in hybrid combinations depends on the mechanism and probably on the specific hybrid as well.

Unlike the SCA effect, the estimates for reciprocal effects are not constrained to zero. A positive estimate denotes a hybrid combination has methionine concentration that is higher than the reciprocal hybrid produced

Table 5. Methionine concentration (g 100 g^{-1} dry wt.) comparisons for hybrid combinations with different methods to increase methionine.

Hybrid comparisons†	Hybrid type†	*N*	Mean	Hybrid type†	*N*	Mean	*p*-value
WT vs. 1 system	WT	2	0.1755	1 system	20	0.1803	0.3172
WT vs. 2 systems	WT	2	0.1755	2 systems	20	0.1807	0.2721
1 system vs. 2 systems	1 system	20	0.1803	2 systems	20	0.1807	0.8165
WT vs. *dzr1*	WT	2	0.1755	*dzr1*	4	0.1778	0.6847
WT vs. RS	WT	2	0.1755	RS	8	0.1769	0.7866
	WT	2	0.1755	RS2	4	0.1753	0.9714
	WT	2	0.1755	RS3	4	0.1784	0.5964
WT vs. *fl2*/+	WT	2	0.1755	*fl2*/+	8	0.1809	0.0655
WT vs. *fl2*/*fl2*	WT	2	0.1755	*fl2*/*fl2*	2	0.1915	0.0134
fl2 vs. *fl2*/*fl2*	*fl2*/+	8	0.1809	*fl2*/*fl2*	2	0.1915	0.0328

† WT, hybrids produced using two normal methionine inbreds; 1 system, hybrids produced using one normal methionine inbred and one containing a mechanism to increase methionine; 2 systems, hybrids containing two different mechanisms to increase methionine; *dzr1*, hybrids produced using B101 and one normal methionine inbred; RS, hybrids produced using one recurrently selected inbred and one normal methionine inbred; *fl2*/+, hybrid produced using one *fl2* inbred and one normal methionine inbred; *fl2*/*fl2*, hybrids produced using two *fl2* inbreds.

using the same inbred parent pair. The converse situation is also true for defining a negative reciprocal effect estimate. Three significantly positive reciprocal effects were found with B101/*fl2*Oh43 and B101/W64A, being of interest because they contained at least one high-methionine parent. Our results suggest that when the selected inbreds are crossed, the directionality of a hybrid cross can be important. We did not observe enough reciprocal effects draw a conclusion about the interaction of genetic mechanisms regulating grain methionine concentration.

With the diallel containing two pairs of *fl2* mutants and their corresponding wild-type genetic background, we were able to completely evaluate the performance of *fl2* as a mechanism to increase methionine concentration by comparing *fl2*- and non-*fl2*-containing lines. A significant difference was found between homozygous *fl2* hybrids and heterozygous *fl2* hybrids ($p = 0.0328$) as well as between homozygous *fl2* hybrids vs. wild-type hybrids ($p = 0.0134$) (Table 5). A significant difference was not found between heterozygous *fl2* hybrids and wild-type hybrids. In summary, we observed *fl2* to only have a beneficial effect on methionine concentration in a homozygous state as heterozygous *fl2* hybrid combinations have similar methionine concentrations to wild-type hybrids. This is apparently inconsistent with observations of the *fl2* mutation, which exhibits a codominant phenotype, but these reports were based on the kernel opacity phenotype and not on methionine concentration (Emerson et al., 1935).

To analyze each hybrid combination further, midparent heterosis for methionine concentration was calculated. Of the 42 hybrid combinations, 20 were found to have significantly lower methionine concentrations than their midparent, while none were found to be significantly higher (Table 6). Additionally, 12 of the 20 hybrid combinations contained two high-methionine parents. However, the comparison of all inbreds to all hybrids suggests an explanation. The prevalence of negative midparent heterosis for methionine concentration (Table 6) together with the highly significant difference between inbred and hybrids overall (Table 3) can be explained by the common observation that hybrids have higher starch and lower protein concentration than inbreds. The low hybrid methionine concentrations could be due to protein dilution of methionine by additional starch in hybrids. To determine if this was the case in this study, we next evaluated protein, oil, and starch concentrations in the samples that were analyzed for methionine concentration.

Evaluation of Grain Quality Traits

Near-infrared spectroscopy was used to predict grain quality traits (protein, starch, and oil) for all 49 entries produced by the seven-by-seven diallel (Table 2). On average, inbreds were found to have significantly ($p = 0.001$) higher protein than hybrid combinations, while hybrids contained significantly ($p = 0.001$) higher starch than inbred lines (Table 2). In relation to methionine, inbreds were also found to have higher methionine concentrations, on average, than grain produced by hybrids. Oil concentrations were not found to be significantly different between inbred and hybrid combinations. These data revealed an inverse relationship existing between protein and starch when comparing inbred parents and resulting hybrid crosses, supporting the idea that methionine concentrations are lower in hybrids because hybrids contain more starch.

Select hybrid combinations with significantly positive SCAs, such as RS3/B101, were compared with all other hybrids or inbred parents with regards to their grain quality traits of starch, protein, and oil concentration. Although these hybrids were found to have significantly ($p = 0.0069$) higher protein levels than all other hybrid combinations, their average was still significantly ($p = 0.001$) less than the inbred mean in Table 2 (Table 7). Conversely, the average starch concentration of these select hybrids was not significantly different from all other hybrid combinations. Additionally, the oil concentrations of high-methionine hybrid combinations were not significantly different than either the inbred mean or all other hybrid combinations. These results suggest mechanisms

Table 6. Single-factor analysis of variances (ANOVAs) between hybrid combinations and midparent (MP) methionine concentration (g 100 g^{-1} dry wt.).

F_1 hybrid	Hybrid mean	MP	MP heterosis
			%
B101/RS2	0.1799	0.1916	−6.09
B101/RS3	0.1854	0.1916	−3.22
B101/*fl2*Oh43	0.1953	0.1933	1.04
B101/*fl2*W64A	0.1847	0.1972	−6.35
B101/Oh43	0.1729	0.1916	−9.80
B101/W64A	0.1900	0.1860	2.15
RS2/B101	0.1598	0.1916	−16.57***
RS2/RS3	0.1785	0.1971	−9.43*
RS2/*fl2*Oh43	0.1756	0.1944	−9.69*
RS2/*fl2*W64A	0.1714	0.1984	−13.62***
RS2/Oh43	0.1743	0.1928	−9.61
RS2/W64A	0.1686	0.1872	−9.92**
RS3/B101	0.2005	0.1959	2.33
RS3/RS2	0.1788	0.1971	−9.28*
RS3/*fl2*Oh43	0.1734	0.1988	−12.79***
RS3/*fl2*W64A	0.1897	0.2028	−6.45
RS3/Oh43	0.1765	0.1972	−10.46*
RS3/W64A	0.1832	0.1915	−4.32
*fl2*Oh43/B101	0.1774	0.1933	−8.20*
*fl2*Oh43/RS2	0.1573	0.1944	−19.11***
*fl2*Oh43/RS3	0.1773	0.1988	−10.82**
*fl2*Oh43/*fl2*W64A	0.1884	0.2001	−5.85
*fl2*Oh43/Oh43	0.1904	0.1945	−2.10
*fl2*Oh43/W64A	0.1742	0.1889	−7.75*
*fl2*W64A/B101	0.1770	0.1972	−10.26**
*fl2*W64A/RS2	0.1469	0.1984	−25.98***
*fl2*W64A/RS3	0.1919	0.2028	−5.38
*fl2*W64A/*fl2*Oh43	0.1733	0.2001	−13.41***
*fl2*W64A/Oh43	0.1768	0.1985	−10.94*
*fl2*W64A/W64A	0.1918	0.1928	−0.55
Oh43/B101	0.1844	0.1916	−3.79
Oh43/RS2	0.1843	0.1928	−4.42
Oh43/RS3	0.1844	0.1972	−6.49
Oh43/*fl2*Oh43	0.1865	0.1945	−4.11
Oh43/*fl2*W64A	0.1820	0.1985	−8.31
Oh43/W64A	0.1913	0.1872	2.18
W64A/B101	0.1701	0.1860	−8.55*
W64A/RS2	0.1701	0.1872	−9.13**
W64A/RS3	0.1696	0.1915	−11.42**
W64A/*fl2*Oh43	0.1863	0.1889	−1.38
W64A/*fl2*W64A	0.1949	0.1928	1.09
W64A/Oh43	0.1407	0.1872	−24.86**

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

used to increase the methionine concentration result in hybrid grain having similar levels of starch and oil as all other hybrid combinations tested.

Methionine concentration in units of mass of methionine per total dry matter describes the absolute amount of methionine present in maize grain. Another measure of interest is the amount mass of methionine per unit mass of protein, a quantity that we consider to be a measure of protein quality. By analyzing the percentage of protein composed of methionine, changes in protein quality could be detected (Table 8). On average, hybrid combinations had a higher (p = 0.0256) percentage methionine than the inbred parents. This indicates that although the hybrid protein concentration was lower than the inbred parents, the quality of protein was improved. Similar to methionine concentration, several comparisons were made between the different methods to increase methionine for protein quality (Table 9). The protein quality between hybrids with only one method to increase methionine was found to be significantly (p = 0.0007) lower than hybrids containing two different methods. Taken together with hybrids having less protein than inbred parents, the presence of two different methods to increase methionine concentration has a beneficial effect on the nutritional quality of maize hybrids.

Evaluation of Grain Yield

In addition to grain composition analysis, a separate yield trial experiment was completed using all 42 hybrid combinations. Two hybrids, *fl2*Oh43/RS2 and RS2/*fl2*Oh43, did not have yield data collected because of low germination and were left out of the analysis. The genetic effects for yield were estimated in a similar fashion to methionine concentration. Mean values for all hybrid combinations across the five tested environments are shown in Table 10.

All genetic factors in the ANOVA, GCA, SCA, and reciprocal effects were significant for grain yield (Table 11). Similar to methionine analysis, further partitioning of the GCAs, SCAs, and reciprocal effects revealed significant combining abilities for individual lines and crosses. Evaluation of GCAs revealed that B101, RS3, and *fl2*W64A were all significant, with RS3 having the only positive effect and contributing to some of the highest yielding hybrid combinations (Table 12). Following the previous individual SCA effect constraints, positive and significant estimates of genetic effects indicate hybrid combinations of interest. Nine significant SCA effects were found, with five of them being positive. All five with positive estimates involved at least one mechanism to increase methionine concentration: *fl2*Oh43/B101, W64A/B101, *fl2*W64A/RS2, Oh43/RS2, and Oh43/*fl2*W64A. Using the same criteria as used previously to define the estimates for reciprocal effects, three significant effects for yield were found. All included inbred line B101 with two of the three being negative and the high methionine × high methionine cross of B101/*fl2*W64A having the only positive effect. In summary, hybrids containing RS3 performed well in yield trials, while the presence of other mechanisms used to increase methionine concentration may result in yield reductions, although some combinations of these genetic mechanisms performed well.

Lastly, to determine whether the marginal increases in methionine concentration produced by *fl2* had an associated yield penalty, hybrid combinations of similar genetic backgrounds were analyzed. A significant (p = 0.001) difference

Table 7. Grain quality trait (oil, starch, and protein [% dry matter]) comparisons for hybrids with significant specific combining abilities (SCAs) to all other hybrid combinations and inbred parents.

Trait	Comparison group	*N*	Mean	Comparison Group	*N*	Mean	*p*-value
Oil	Significantly positive SCAs	3	4.5766	All other hybrids	39	4.7087	0.0658
Oil	Significantly positive SCAs	3	4.5766	Inbred parents	7	4.7193	0.0842
Starch	Significantly positive SCAs	3	68.7163	All other hybrids	39	68.6148	0.4817
Protein	Significantly positive SCAs	3	13.3164	All other hybrids	39	12.3668	0.0069
Protein	Significantly positive SCAs	3	13.3164	Inbred parents	7	15.2142	0.0001

Table 8. Protein quality (% methionine/% total protein) for all 49 entries produced in the diallel study.

	♂							
♀	B101	RS2	RS3	*fl2*Oh43	*fl2*W64A	Oh43	W64A	Mean†
B101	**0.0115**	0.0143	0.0145	0.0152	0.0147	0.0166	0.0164	0.0153
RS2	0.0141	**0.0124**	0.0154	0.0155	0.0138	0.0130	0.0142	0.0143
RS3	0.0139	0.0165	**0.0130**	0.0160	0.0178	0.0139	0.0136	0.0153
*fl2*Oh43	0.0132	0.0119	0.0153	**0.0120**	0.0148	0.0152	0.0140	0.0141
*fl2*W64A	0.0184	0.0149	0.0148	0.0143	**0.0135**	0.0141	0.0156	0.0154
Oh43	0.0132	0.0162	0.0126	0.0148	0.0135	**0.0132**	0.0149	0.0142
W64A	0.0134	0.0147	0.0135	0.0135	0.0141	0.0133	**0.0139**	0.0137
Mean†	0.0144	0.0147	0.0144	0.0149	0.0148	0.0143	0.0148	
							F_1 hybrid mean†	0.0146
							F_1 hybrid SD	0.0018
							Inbred mean‡	0.0128
							Inbred SD	0.0013
							LSD	0.0018

† Mean does not include inbred values in boldface text.

‡ Mean only includes inbred values in boldface text.

Table 9. Protein quality (% methionine/% protein) comparisons for hybrid combinations with different methods to increase methionine.

Hybrid comparisons†	Hybrid type†	*N*	Mean	Hybrid type†	*N*	Mean	*p*-value
WT vs. 1 system	WT	2	0.0141	1 system	20	0.0143	0.6306
WT vs. 2 systems	WT	2	0.0141	2 systems	20	0.0150	0.0518
1 system vs. 2 systems	1 system	20	0.0143	2 systems	20	0.0150	0.0007
WT vs. *dzr1*	WT	2	0.0141	*dzr1*	4	0.0149	0.1237
WT vs. RS	WT	2	0.0141	RS	8	0.0140	0.7742
	WT	2	0.0141	RS2	4	0.0145	0.4339
	WT	2	0.0141	RS3	4	0.0134	0.1930
WT vs. *fl2*/+	WT	2	0.0141	*fl2*/+	8	0.0144	0.5712
WT vs. *fl2*/*fl2*	WT	2	0.0141	*fl2*/*fl2*	2	0.0146	0.4325
fl2/+ vs. *fl2*/*fl2*	*fl2*/+	8	0.0144	*fl2*/*fl2*	2	0.0146	0.6690

† WT, hybrids produced using two normal methionine inbreds; 1 system, hybrids produced using one normal methionine inbred and one containing a mechanism to increase methionine; 2 systems, hybrids containing two different mechanisms to increase methionine; *dzr1*, hybrids produced using B101 and one normal methionine inbred; RS, hybrids produced using one recurrently selected inbred and one normal methionine inbred; *fl2*/+, hybrid produced using one *fl2* inbred and one normal methionine inbred; *fl2*/*fl2*, hybrids produced using two *fl2* inbreds.

in yield was found between all *fl2*-containing hybrids and all wild-type-containing hybrids. To determine if a difference between genetic backgrounds was present, each was analyzed individually. When all *fl2*Oh43-containing hybrids were compared with all Oh43-containing hybrids, a significant difference in yield was found ($p = 0.0102$), with *fl2*Oh43-containing hybrids yielding on average ~4% less than Oh43-containing hybrids. Similarly, when all *fl2*W64A-containing hybrids were compared with all W64A-containing hybrids, a significant ($p = 0.001$) difference in yield was found with *fl2*W64A-containing hybrids yielding on average ~11% less than W64A-containing hybrids. Based on these findings, we conclude that *fl2* causes lower yields when used in these hybrid combinations.

DISCUSSION

Producing new maize varieties with increased methionine levels is of great interest because of the importance of this amino acid as a supplement in poultry diets. Several mechanisms exist to improve methionine concentrations, while information regarding how these mechanisms act in combination is not available. In the present study, inbred lines with three distinct genetic mechanisms for increasing methionine concentration were crossed to produce F_1

Table 10. Mean yield (kg ha^{-1}) for all 42 hybrid combinations produced in the diallel study.

	♂							
♀	B101	RS2	RS3	*fl2*Oh43	*fl2*W64A	Oh43	W64A	Mean
B101	–	2002.99	6398.77	6055.42	5916.70	3815.79	6136.40	5054.24
RS2	5821.29	–	4819.48	–	6243.73	7699.37	6415.72	6199.79
RS3	5580.88	4809.44	–	7150.76	6693.79	7345.35	7361.67	6490.42
*fl2*Oh43	5753.50	–	7976.18	–	5634.86	3364.47	6592.73	5864.60
*fl2*W64A	3480.60	6278.26	5957.50	6217.37	–	5903.52	3296.05	5189.20
Oh43	5665.62	7659.20	8220.36	3751.76	5990.77	–	6782.30	6344.79
W64A	6051.66	6536.24	7567.55	6337.26	3122.81	6751.54	–	6061.07
Mean	5392.57	5457.22	6823.10	5902.89	5600.34	5813.13	6097.48	–
							Grand mean†	5877.78
							LSD	1882.47

† Mean of all F_1 hybrid crosses.

Table 11. Analysis of variance (ANOVA) for the yield (kg ha^{-1}) linear model.

Source of variation	df	Mean squares
Environment	4	257,517,953.30***
Replication	5	2,924,966.90*
GCA	6	32,436,323.02***
SCA	13	35,128,434.92***
Reciprocal effects	20	6,511,335.38***
Genotype × environment	156	2,633,432.06***
Experimental error	195	998,092.00
CV†		17.95%

* Significant at the 0.05 probability level.

*** Significant at the 0.001 probability level.

† Average coefficient of variation of treatment means.

hybrids. The diallel mating design allowed us to investigate how methionine concentration is controlled at the genetic level. Understanding the interactions of genetic mechanisms to increase methionine concentration was of primary interest; however, because only one or two varieties represent each genetic mechanism, caution should be used when drawing conclusions about these interactions. It is possible that these mechanisms perform differently in different genetic backgrounds.

The inclusion of two *fl2* inbreds and their wild-type counterparts allowed us to evaluate the impact of *fl2* on methionine concentration and yield. Heterozygous *fl2* hybrids were expected to have higher methionine concentrations than their normal methionine counterparts, but an increase was only found between homozygous *fl2* hybrids and homozygous wild-type hybrids. We concluded from our results that the *fl2* mutation by itself has, at best, marginal value for increasing methionine concentration in this germplasm. Given that the yield GCAs for both *fl2*-containing lines were either not significant or significantly negative, producing hybrids with the *fl2* allele may result in a yield penalty. It is of note that the benefits of *fl2* introgression are dependent on the genetic background as the two lines tested in the present study behaved differently. Although *fl2* has been previously characterized as being a codominant gene, our data suggests it behaves similar to a recessive gene, at least in the genetic backgrounds used in this study. This conclusion was based on comparison of hybrids with a single copy of *fl2*, which did not differ from wild-type controls, while hybrids with two copies of *fl2* had increased the methionine concentration. Taken together, these data suggest the use of *fl2* is not an effective approach to produce high methionine in heterozygous maize hybrids but is beneficial in homozygous maize hybrids if the added value of increased methionine is sufficient to make up for the yield penalty.

Of two remaining mechanisms used to increase methionine concentration, both may be useful approaches to include in a breeding program. While the effectiveness of *dzr1* and recurrent selection were shown by Olsen et al. (2003) and Scott et al. (2008), respectively, the present study suggests there may be a benefit of having either or both methods in hybrid combination. Current data suggests synergism may exist between *dzr1* and recurrent selection because of the significant SCA for methionine concentration in the RS3/B101 hybrid. Further evaluation of hybrid combinations including both mechanisms is needed as differing genetic backgrounds have been shown to alter the inheritance of *dzr1* and its ability to increase methionine levels (Chaudhuri and Messing, 1994).

Estimates for the genetic effects regulating all traits of interest revealed the significance of both GCAs and SCAs. Since the GCA is associated with additive genetic effects, selection of lines with positive values would pass favorable alleles on to progeny. Conversely, related to GCA, the SCA reflects nonadditive genetic effects such as dominance and epistasis. The calculated GCA/SCA ratio was >1 for methionine concentration (1.46), suggesting additive gene action has a higher importance than nonadditive gene action in its inheritance. Conversely, calculated ratios for protein (0.48), oil (0.53), starch (0.99), and yield (0.86) were less than one suggesting nonadditive gene action has a higher importance on the inheritance of these traits. With methionine concentration of interest, a higher presence of additive gene action is favorable as genes regulating this trait can be selected for and fixed before being passed onto progeny.

Table 12. Estimates for the genetic effects for yield (kg ha^{-1}). The estimates for general combining ability (GCA) are included in the main diagonal of the table (boldface type). The estimates for specific combining ability (SCA) are included below the diagonal. The estimates for reciprocal effects are included above the diagonal.

	♂						
♀	B101	RS2	RS3	*fl2*Oh43	*fl2*W64A	Oh43	W64A
B101	**−786.78*****	−1909.36***	408.97	150.99	1217.91**	−924.93*	42.50
RS2	−1129.45**	**−50.49**	5.00	–	−17.50	20.00	−60.50
RS3	−35.98	−1947.19***	**933.39*****	−412.97	367.97	−437.47	−102.99
*fl2*Oh43	808.11*	–	746.82	**4.30**	−291.48	−193.49	127.49
*fl2*W64A	187.70	1013.80**	94.41	624.02	**−581.20****	−43.50	86.49
Oh43	−591.84	1610.65***	730.26	−2565.34***	1139.24**	**240.34**	15.50
W64A	761.46*	407.14	411.68	341.35	−2328.91***	407.28	**240.44**

* Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.001 probability level.

Analysis of grain quality traits from the diallel study produced several results of interest. Although hybrid combinations generally had lower protein than inbred parents, they had higher starch levels than the inbred parents. When comparing those results with the select few hybrid combinations having elevated methionine concentrations, the hybrids with significantly positive SCAs had comparable starch and oil concentrations to all other hybrid combinations, but they had higher protein. In summary, producing high-methionine hybrids with acceptable or improved methionine concentration without altering the balance of protein, oil, and starch is possible.

Producing maize hybrids with increased methionine levels and increased protein quality was also of interest in the present study. When each mechanism used to increase methionine concentrations were compared with wild-type hybrids, two copies of the *fl2* allele were able to increase methionine level over wild-type hybrids. Direct analysis of protein quality revealed that hybrids with two different methods to increase methionine had higher protein quality over hybrids only containing one mechanism. These results suggest that it is possible to increase methionine per protein concentration using the genetic mechanisms identified to increase methionine concentration.

Methionine is an interesting amino acid because, unlike lysine and tryptophan, one of the main pools of methionine is in the zein seed storage proteins. In the Illinois long-term selection experiment, selection for increased protein content resulted in preferential accumulation of zeins (Below et al., 2004). It would therefore be reasonable to expect methionine concentration to be related to total protein concentration, making it difficult to increase grain quality defined as methionine per protein. Another way that methionine is different than other amino acids is that it has an unusually large free-amino-acid pool because of its roles in sulfur assimilation, S-adenosyl methionine metabolism, and redox regulation. It would be reasonable to expect this pool to vary independently of total protein content facilitating improvements in protein quality. Our data had fewer significant effects for protein quality than methionine concentration, and this is likely a result of the many cellular roles played by methionine in seeds.

One important consideration is that some inbred lines that were selected for their potential to have high methionine did not have higher methionine concentration than both control inbreds (Table 2). One possible explanation for this observation is that the method used to quantify amino acids is not accurate. It has been shown that there is a high correlation between the AOAC standard method (AOAC International, 1995) for methionine analysis and the bacterial assay used in this study (Scott et al., 2008). In addition, it has been shown that selection for methionine using the microbial growth method results in populations with altered methionine concentration as measured by the AOAC standard high-performance-liquid-chromatograph-based method (Newell et al., 2014). A second possible explanation is that the different mechanisms for controlling methionine concentration are present in different genetic backgrounds than the control lines. Genetic background effects may influence methionine concentration. One limitation of this study is that all genetic mechanisms are not in the same genetic background. The study would have also been improved by including lines with higher methionine concentration.

Reciprocal effects for grain yield in F_2 plants were observed. Three crosses were found to have significantly different yields than their reciprocal combinations. Differences in pollen production and ear traits could cause some hybrids to produce significantly different yields depending on parent designation. Maternal effect or parent-of-origin effects could be involved as well. Interestingly, both hybrids exhibiting reciprocal effects for grain yield involved the inbred line B101, which carries the *dzr1* gene. This gene has been shown to have parent-of-origin effects for methionine content (Chaudhuri and Messing, 1994).

In conclusion, breeders should consider each method to increase methionine and their interactions with each other when breeding for high-methionine corn. Both methionine concentration and yield are complex traits and important factors to consider in a breeding program.

The significance of a genotype × environment interaction effect for both traits adds further complexity into any analysis as it indicates the lack of stability across tested environments. Crosses combining *dzr1* and recurrent selection in hybrid combination can elevate methionine concentration to increase grain nutritional quality but may result in slightly lower yields based on the germplasm examined here. As a result, we found crosses with *dzr1* and lines derived from recurrent selection were superior to crosses containing a single copy of *fl2*.

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