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Preliminary Genetic Studies of the Phenotype of Betaine Deficiency in *Zea mays* L.[1]

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ABSTRACT

Glycinebetaine-deficient inbreds of *Zea mays* do not exhibit a general deficiency of nitrogenous solutes; the total free amino acid levels of betaine-deficient lines are not significantly less than those of inbreds which exhibit >100-fold higher betaine levels. Betaine-deficient inbreds are characterized by extremely low betaine: total free amino acid ratios (<0.0015). Highly significant correlations are demonstrated between the expected mid-parent and observed betaine:amino acid ratios of 30 hybrids of known pedigree. In 12 hybrids constructed from a betaine-deficient male parent (inbred 1506), the observed betaine:amino acid ratios of the hybrids are proportional to the betaine:amino acid ratios of the female parents (r = 0.83). Two hybrids, 1146 × 1074 and 1146 × 1506, were chosen for further genetic analysis. The common female parent (1146) and inbred 1074 both exhibit betaine:amino acid ratios of 0.090, a value which is approximately 90-fold greater than the betaine:amino acid ratio of inbred 1506. Hybrid 1146 × 1074 exhibits almost exactly twice the betaine:amino acid ratio of hybrid 1146 × 1506. If inbred 1506 is homozygous recessive for a single nuclear gene responsible for the phenotype of betaine deficiency, and if inbreds 1146 and 1074 are homozygous dominant for this allele, then this twofold difference in betaine:amino acid ratio must be associated with the homozygous dominant and heterozygous conditions, respectively, for 1146 × 1074 and 1146 × 1506. Evidence is presented from both greenhouse and field evaluations of F_2 populations of these hybrids that a single nuclear recessive gene is most likely responsible for the phenotype of betaine-deficiency in inbred 1506. Approximately 25% of the F_2 segregants from 1146 × 1506 exhibited extremely low betaine:amino acid ratios (<0.0015), whereas 0% of the F_2 segregants from 1146 × 1074 exhibited this phenotype. The segregation patterns with respect to betaine:amino acid ratio suggest a 1:2:1 segregation ratio for homozygous recessive:heterozygous:homozygous dominant individuals within the 1146 × 1506-F_2 population.

Glycinebetaine (*N,N,N*-trimethylglycine; betaine) is thought to play a major role as a compatible cytoplasmic osmotic solute and/or osmoprotectant in certain higher plant species (most notably members of the Chenopodiaceae and Gramineae) (7, 9, 24, 26, 27) and bacteria (2, 3, 15, 16, 23, 25). Recent evidence suggests that in spinach glycinebetaine may represent a major osmotic solute of the chloroplast in salinized leaves (22), where the chloroplast represents the principal site of betaine biosynthesis (10). In barley, genetic evidence has been acquired for an intimate link between betaine accumulation and osmoregulation (5). Thus, genotypes of barley selected for a high betaine level tend to maintain a lower solute potential at all levels of osmotic stress (5, 6).

The nonperturbing or favorable effects of osmolytes such as betaine on macromolecular-solvent interactions are proposed to result in 'compatibility' (28). Betaine appears to be far less inhibitory to protein synthesis *in vitro* in comparison to inorganic ions of equivalent concentration (1, 4), and this type of evidence supports the notion that betaine accumulation is more compatible with cytoplasmic functions than accumulation of inorganic ions as cytoplasmic osmotica (27). Glycinebetaine is reported to have stabilizing effects on proteins at high temperatures (12, 18).

Identification and detailed characterization of betaine-null mutants or naturally occurring variants of betaine-accumulating plant species might be useful in developing further genetic tests of the adaptive value of betaine accumulation in environmental stress tolerance in higher plants. Several betaine-deficient inbreds and hybrids of maize have recently been identified (21), and this has prompted preliminary genetic analysis of the phenotype of betaine deficiency in maize. At the outset, we emphasize that nothing is yet known about the relationship between this phenotype of betaine deficiency and stress susceptibility in maize, and indeed it is unlikely that such relationships could be definitively established without substantial efforts first devoted to creating isogenic lines or isopopulations differing solely for this metabolic character (8, 9). Our initial emphasis has focused on establishing the heritability of this characteristic in maize and initiating breeding programs which might ultimately address the issue of stress susceptibility/tolerance relationships in a manner that is not confounded by genetic differences among breeding lines for numerous unknown biochemical and morphological characters unrelated to glycinebetaine accumulation (for review see Ref. 9).

MATERIALS AND METHODS

Sampling for Betaine and Amino Acids from Plant Tissues (California Trial, 1984). Leaf tissue of *Zea mays* inbreds[2] and hybrids (all of Northrup King Co., Stanton, MN) was excised from rows (23 ft rows; 40 plants per row) grown at the Northrup King field station at Woodland, CA, in the summer of 1984. Each sample was comprised of a random sample of 5 leaves per row (the youngest fully expanded leaf from 5 individual plants per row) from which a subsample of 37.5 cm^2 of leaf tissue (5 ×

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[2] Maize inbreds used in the experiments described in this report are the proprietary property of Northrup King Co., MN, and cannot be released by the authors. However, F_2 and subsequent progeny from two hybrids employed in this investigation (1146 × 1506 and 1146 × 1074) are available from the authors upon request.

3 × 2.5 cm) was cut from the leaf lamina at a distance of 15 to 20 cm from the leaf tip. The leaf tissue was cut into 15 × 1 cm × 2.5 cm sections and immediately placed in vials containing 10 mL methanol. Sampling generally took less than 2 min. per row, facilitating screening of all 52 genotypes in the trial in triplicate (i.e. from three replicate rows) in less than 3 h at mid-day (11:00 AM–2:00 PM).

Samples for betaine analyses (methanol extracts) were taken approximately 8 weeks after planting (planting date = 4/23/84, sampling date = 6/20/84) from 2 replicate rows of each genotype grown in randomized, bordered blocks. Inbreds, non-heterotic hybrids, and heterotic hybrids were each grown in separate blocks. This trial, used specifically for betaine determination, was furrow-irrigated on 4/24/84, 5/08/84, 5/29/84, 6/19/84, 7/06/84, and 7/24/84.

A separate replicate trial, under a different irrigation regime, was sampled for free amino acids. Samples for amino acid analyses (methanol extracts) were taken on 5/30/84, 6/27/84, 6/29/84, and 7/09/84 in triplicate from each genotype on each sampling date. This trial was planted on 4/23/84 and received furrow-irrigations on 4/24/84, 5/08/84, 5/29/84, 6/17/84, 6/28/84, 7/10/84, 7/20/84, and 8/01/84. Again, inbreds, nonheterotic hybrids and heterotic hybrids were grown in separate blocks.

Sampling for Betaine and Amino Acids from Plant Tissues (Indiana Trial, 1986 and Greenhouse Trial, 1986). Two hybrids, 1146 × 1074 and 1146 × 1506, were grown in adjacent 15 ft rows (20 plants/row) in West Lafayette, IN, in the summer of 1986 (planting date = 5/11/86; sampling date = 6/24/86). Leaf tissue (the youngest most fully expanded leaf) was taken from 10 individual plants per row (1.0 to 2.0 gfw[3] per leaf) from the leaf lamina at a distance of about 15 to 20 cm from the leaf tip of each individual plant. Leaf tissue was sliced into approximately 1 cm × 2.5 cm sections and placed in preweighed vials containing 10 mL methanol. Vial weights before and after tissue addition were used to determine fresh weight of tissue extracted. This trial was grown with supplemental sprinkler irrigation through the growth period prior to sampling. Each plant sampled was selfed using standard silk bagging techniques to avoid cross pollination. F_2 seeds from 1146 × 1074 and 1146 × 1506 were grown in the Purdue University, Department of Horticulture, greenhouse in late fall (1986) in 15 cm diameter pots (depth = 15 cm) containing 20% soil, 40% sphagnum peat, and 40% perlite, with the soil mixture amended with (per m^3): 744. 1-g $3Ca(H_2PO_4)_2$, 496.1 g KNO_3, 496.1 g $MgSO_4$, 3.97 kg $CaCO_3$, and 62 g fritted trace elements (3.0% Cu [Cu_2O], 7.5% Mn [MnO_2], 18.0% Fe [Fe_2O_3],1.5% B [$Na_2B_4O_7 \cdot 10H_2O$], 0.2% Mo [MoO_3], and 7.0% Zn [ZnO]). After steam sterilization, the soil pH was 6.2. Plants were irrigated daily to soil capacity with water containing 516.6 mg/L KNO_3, 367.6 mg/L NH_4NO_3, and 0.124 mL/L 75% H_3PO_4, pH 6.3. Individual plants were monitored for growth rate by measuring plant height (cm) (distance from soil level to the tip of the most fully expanded leaf when held vertical) at 10, 13, 17, 20, and 34 d after planting. Leaf tissue (from the youngest most fully expanded leaf of each plant) was taken 34 d after planting from the leaf lamina at approximately 15 to 20 cm from the leaf tip and cut into 1 cm × 2.5 cm sections and immediately extracted in preweighed vials containing 10 mL methanol (0.6–1.2 gfw per sample).

Sampling for Betaine and Amino Acids from Plant Tissues (Indiana Trial, 1987). Selfed seed from hybrid 1146 × 1506 and 1146 × 1074 was planted at the Purdue University O'Neall farm (W. Lafayette, IN) on 5/15/87 in alternate rows (30 plants per row). After 49 days (7/3/87), samples of leaf tissue were taken from immature leaves from individual plants (0.6–1.0 gfw per sample) and immediately extracted in preweighed vials containing 10 mL methanol. Thirty individuals of the 1146 × 1074-F_2 population and 45 individuals of the 1146 × 1506-F_2 population were screened. Each individual sampled was tassel and silk bagged and selfed to derive F_3 seed for subsequent genetic evaluations.

Purification of Betaines and Amino Acids. Methanol extracts were phase-separated by addition of 5 mL chloroform + 6 mL H_2O. The upper aqueous phase was removed and concentrated to dryness under a stream of dry air, at which point known amounts of internal standards were added (250 nmol of L-α-amino-n-butyrate for amino acid analyses of Tables I and II, and Figure 1, and 1125 or 500 nmol d_9-glycine betaine for the betaine determinations [21]). Samples for betaine analysis spiked with internal standard were then redissolved in 2 mL H_2O and applied to 1 cm × 2 cm columns of Dowex-1-OH^- resin, and betaines were eluted with 6 mL H_2O. This aqueous eluant was then applied to 1 cm × 2 cm columns of Dowex-50-H^+, columns were washed with a further 8 mL H_2O, and betaines were eluted with 6 mL 6 M NH_4OH. The latter fraction was then concentrated to dryness under a stream of dry air. When amino acids were determined in the same extracts (Tables I and II, and Fig. 1), amino acids were eluted from the Dowex-1-OH^- resin with 6 mL 2.5 N HCl, concentrated to dryness in a rotary evaporator at 35°C, redissolved in 2 mL H_2O, and applied to 1 cm × 2 cm columns of Dowex-50-H^+. Columns were washed with 8 mL H_2O, and amino acids were eluted with 6 mL 6 M NH_4OH and concentrated to dryness under a stream of dry air. When amino acids were determined in separate extracts, (Tables III, IV, and V) the aqueous extracts after methanol:chloroform:H_2O phase-separation were applied directly to Dowex-50-H^+, and amino

Table I. *Glycinebetaine and Total Free Amino Acid Levels of F_1 Hybrids 1146 × 1074 and 1146 × 1506 Observed in Field Trial in Indiana (1986)*

Hybrid	Mean Glycinebetaine Level[a]	Mean Total Free Amino Acid Level[a]	Mean Betaine: Amino Acid Ratio
	nmol/gfw	*nmol/gfw*	
1146 × 1074	4549 (1020)	16730 (3023)	0.278 (0.064)
1146 × 1506	2429 (440)	18114 (3046)	0.137 (0.032)
ANOVA[b]	**	NSD	**

[a] Means of 10 individual plants sampled from the youngest, most fully expanded leaf as described in "Materials and Methods." Standard deviations are shown in parentheses. [b] Analysis of variance for significant differences between the means; ** = significantly different at the P = 0.01 level; NSD = not significantly different.

Table II. *Frequency Distributions of Betaine:Amino Acid Ratios in 30 F_2 Segregants of Hybrids 1146 × 1506 and 1146 × 1074 under Greenhouse, Well-Irrigated Growth Conditions.*

Betaine:Amino Acid Ratio Class	No. of Individuals in Each Class	
	1146 × 1506-F_2	1146 × 1074-F_2
0 to 0.0015	6	0
0.0015 to 0.020	0	0
0.020 to 0.040	7	1
0.040 to 0.060	8	1
0.060 to 0.100	3	6
0.100 to 0.150	4	7
0.150 to 0.200	1	8
0.200 to 0.250	0	6
0.250 to 0.300	1	0
0.300 to 0.400	0	1
>0.400	0	0

[3] Abbreviations: gfw, gram fresh weight.

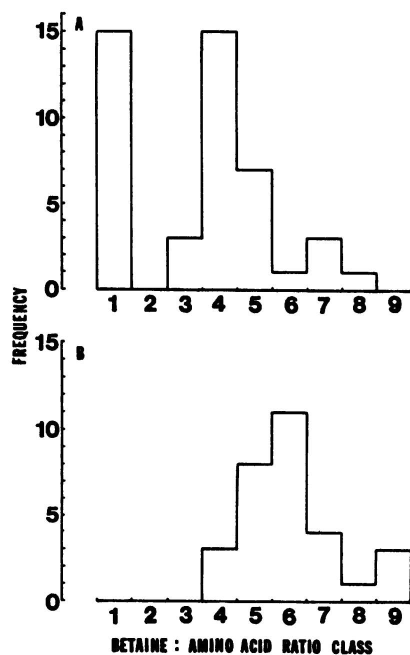

FIG. 1. Frequency distributions of individuals in the 1146 × 1506-F_2 (A) and 1146 × 1074-F_2 (B) populations with respect to betaine:amino acid ratio observed in the Indiana (1987) trial. Betaine:amino acid ratio classes were as follows. Class 1, 0 to 0.0015; class 2, 0.0015 to 0.050; class 3, 0.050 to 0.10; class 4, 0.10 to 0.20; class 5, 0.20 to 0.30; class 6, 0.30 to 0.40; class 7, 0.40 to 0.50; class 8, 0.50 to 0.60; class 9, 0.60 to 0.70.

Table III. *Glycinebetaine and Total Free Amino Acid Levels of Inbreds*

Inbred	Mean Glycinebetaine Level[a]	Mean Total Free Amino Acid Level[b]	Betaine:Amino Acid Ratio
	nmol/cm²	*nmol/cm²*	
1564	28.99	412.3 (74.4)	0.070
1588	57.6	493.8 (137.8)	0.117
1528	39.1	441.0 (118.6)	0.089
1244	74.4	490.3 (105.3)	0.152
1508	71.9	579.8 (50.1)	0.124
1506	0.61	611.3 (110.9)	0.0010
1522	25.0	651.5 (143.8)	0.038
1146	46.2	522.0 (135.3)	0.089
1500	65.7	452.0 (93.6)	0.145
1572	101.3	452.3 (113.7)	0.224
1562	23.4	492.0 (96.9)	0.048
1530	64.1	435.0 (99.6)	0.147
1158	34.7	494.3 (118.9)	0.070
1422	64.5	571.3 (38.3)	0.113
1504	70.4	557.0 (133.9)	0.126
864	64.9	460.5 (103.5)	0.141
644	0.70	468.3 (75.3)	0.0015
338	0.76	575.0 (78.0)	0.0013
672	35.9	414.8 (48.7)	0.087
1566	49.3	432.5 (72.3)	0.114
1944	72.5	532.8 (86.6)	0.136
1074	54.9	607.8 (98.9)	0.090

[a] Derived from Rhodes *et al.* (21); means of two replicate analyses. [b] Means of 12 independent analyses; SD are shown in parentheses; 1 cm² = 0.018 gfw.

Table IV. *Glycinebetaine and Total Free Amino Acid Levels of Nonheterotic Hybrids*

Nonheterotic Hybrid (Female × Male)	Mean Glycinebetaine Level[a]	Mean Total Free Amino Acid Level[b]	Betaine:Amino Acid Ratio	
			Observed	Expected[c]
	nmol/cm²	*nmol/cm²*		
864 × 1158	64.2	513.0 (106.4)	0.125	0.101
1500 × 1562	29.9	430.8 (98.5)	0.069	0.097
1944 × 1500	57.8	506.5 (106.9)	0.114	0.141
1146 × 1564	62.2	431.0 (139.1)	0.144	0.079
1944 × 672	59.0	513.5 (128.4)	0.115	0.112
1158 × 1562	39.2	522.5 (127.9)	0.075	0.059
338 × 644[2]	0.6	610.5 (179.1)	0.0010	0.0014
1504 × 1074	65.9	525.5 (88.2)	0.125	0.108
1944 × 1158	64.0	540.8 (112.5)	0.118	0.103
1422 × 1158	46.4	553.0 (152.0)	0.084	0.092

[a] Means of two replicate analyses. [b] Means of 12 replicate analyses; SD are shown in parentheses. [c] Expected mid-parent betaine:amino acid ratios calculated from the inbred betaine:amino acid ratios of Table III as described in the text.

acids + betaines were eluted with 6 M NH_4OH (*i.e.* the Dowex-1-OH^- step was eliminated).

Derivatization and Quantification of Betaines and Amino Acids. Betaine fractions (only those which had free amino acids removed on Dowex-1-OH^-) were derivatized either with *n*-propanol:acetyl chloride (5:1 v/v) at 120°C for 20 min. or with *n*-butanol:acetyl chloride (5:1 v/v) (Tables I and II, and Fig. 1) as described previously (21) and then were analyzed by fast atom bombardment MS as described previously (21), monitoring the intensity of ions 160 and 169 (for *n*-propanol-derivatized samples) or 174 and 183 (for *n*-butanol-derivatized samples).

Amino acids were derivatized as *N(O,S)*-heptafluorobutyryl isobutyl esters essentially as described previously (20), except that the first derivatization step used 200 µL isobutanol:acetyl chloride (5:1 v/v) 120°C, 20 min, and the second derivatization step used 100 µL heptafluorobutyric anhydride 120°C, 10 min. In the results of Tables III, IV, and V amino acid derivatives in ethyl acetate:acetic anhydride (1:1 v/v) were analyzed on a 30 m × 0.22 mn DB5-30N fused silica capillary column using a Varian 3700 gas chromatograph equipped with a Varian 8000 autosampler interfaced to a HP3354 data system via a HP18652A A/D converter and a teletype model 43 (20). Gas chromatographic conditions were as follows: injector temp, 250°C; detector temperature, 290°C; oven temperature program, 90°C (6 min) to 265°C at 6°C per min; carrier gas, helium (20 psi at injector port); column carrier gas flow rate, 1 mL/min; split ratio at injector port, 40:1; air flow rate, 200 mL/min, H_2 flow rate, 30 mL/min; helium sweep gas flow rate, 40 mL/min; detector, flame ionization.

In the results of Tables I and II and Figure 1, amino acid derivatives in ethyl acetate:acetic anhydride (1:1 v/v) were analyzed on a 30 m × 0.25 mm fused silica capillary column (SE.30) using a HP5790A gas chromatograph equipped with a HP7671A autosampler and HP3390A computing integrator. Gas chromatographic conditions were as follows: injector temperature, 250°C; detector temperature, 280°C; oven temperature program, 100°C (4 min) to 260°C at 6°C/min; carrier gas, N_2 (20 psi at injector port); column carrier gas flow rate, 1 mL/min; split ratio at injector port, 20:1; air flow rate, 222 mL/min; H_2 flow rate, 35 mL/min; N_2 sweep gas flow rate, 20 mL/min; detector, flame ionization (see Ref. 19 for details of amino acid retention times on the latter system).

In the California trial (Tables III, IV, and V) no internal standard was used. Amino acids were quantified by reference to the response factors (area counts/nmol) of amino acid standard

Table V. *Glycinebetaine and Total Free Amino Acid Levels of Heterotic Hybrids*

Heterotic Hybrid (Female × Male)	Mean Glycinebetaine Level[a]	Mean Total Free Amino Acid Level[b]	Betaine:Amino Acid Ratio	
			Observed	Expected[c]
	nmol/cm²	*nmol/cm²*		
1500 × 1508	125.2	502.5 (138.6)	0.249	0.135
1530 × 1566	107.0	522.5 (105.7)	0.205	0.131
864 × 1506	75.4	560.0 (133.6)	0.135	0.071
	91.1	535.3 (106.2)	0.170	0.074
1562 × 1506	28.9	576.5 (115.6)	0.050	0.024
1572 × 1506	86.0	581.5 (100.6)	0.148	0.113
1146 × 1506	58.3	597.0 (128.9)	0.098	0.045
1522 × 1506	39.4	647.0 (150.8)	0.061	0.020
1528 × 1506	59.6	604.0 (162.9)	0.099	0.045
1146 × 1522	50.7	582.3 (161.2)	0.087	0.064
1500 × 1506	90.6	590.5 (129.6)	0.153	0.073
(1146 × 1564) × 1506	59.4	587.8 (64.7)	0.101	0.073
(1158 × 1562) × 1506	50.7	605.3 (187.1)	0.084	0.030
(1944 × 672) × 1506	68.1	648.5 (184.2)	0.105	0.056
(1944 × 1500) × 1506	70.8	609.5 (164.5)	0.116	0.071
(1944 × 672) × 1074	77.1	542.8 (125.8)	0.142	0.101
(864 × 1158) × 1522	48.3	562.0 (133.5)	0.086	0.072
(1944 × 1158) × 1522	109.0	621.3 (187.7)	0.175	0.071
(1422 × 1158) × 1244	109.5	498.5 (99.1)	0.220	0.122
864 × (338 × 644²)	85.6	540.5 (114.6)	0.158	0.071

[a] Means of two replicate analyses. [b] Means of 12 replicate analyses; SD are shown in parentheses.
[c] Expected mid-parent betaine:amino acid ratios calculated from the inbred betaine:amino acid ratios of Table III as described in the text.

mixtures, which were derivatized and analyzed under identical conditions, assuming 100% recovery of amino acids in the phase-separation and Dowex-50-H^+ ion exchange steps. In the results of Tables I and II and Figure 1, where amino acids were processed via two ion exchange columns, an internal standard (α-amino-*n*-butyrate) was found to be necessary for accurate quantification since recoveries ranged from 75 to 95%. In the former trial (Tables III, IV, and V) no α-amino-*n*-butyrate was detectable in any of the genotypes evaluated; hence, the choice of this internal standard in subsequent work (but see Ref. 19 for evidence for endogenous accumulation of this amino acid in response to certain herbicides). Where an internal standard was included, amino acids were quantified by reference to the area of the internal standard and its reponse factors relative to the individual components of amino acid standard mixtures of known concentration. In this paper, only the total free amino acid pools are reported; the compositions of the free amino acid pools of this germplasm will be discussed in a separate paper (see also Ref. 17 for a discussion of genotypic variation for free amino acid composition of maize).

RESULTS

Inbred Glycinebetaine and Total Amino Acid Levels. The betaine and total free amino acid levels of the youngest most fully expanded leaf of 22 inbred lines of *Zea mays* (Northrup King Co. germ plasm) grown under field conditions (Woodland, CA; 1984) are summarized in Table III. These results establish that betaine deficiency in inbreds, 1506, 644, and 338 is not associated with a general deficiency of nitrogenous solutes. Total free amino acid levels of these inbreds are not significantly less (or greater) than several inbreds which exhibit >100-fold higher betaine levels. The betaine-deficient inbreds are characterized by betaine:total amino acid ratios of < 0.0015. This ratio becomes particularly useful when applied to the screening of individuals in an F_2 segregating population, as will be demonstrated subsequently in this manuscript, since it can correct for differences in the general nitrogen status of individuals in such a population. However, we wish to note that in this trial (Woodland, CA) amino acids levels and betaines were determined on separate extracts from several plants per row and grown in separate trials, rather than from individual plants (see "Materials and Methods"). Assuming that these average values are representative of the range and extent of genotypic variation for betaine and total amino acid levels within this germ plasm, we will first consider the heritability of these metabolic traits in a range of hybrids derived from these inbred parents. At a later stage, the F_2 segregation patterns of two contrasting F_1 hybrids (1146 × 1074 and 1146 × 1506) will be considered with respect to betaine and total free amino acid levels.

Hybrid Glycinebetaine and Total Amino Acid Levels. Tables IV and V list the observed mean betaine and total free amino acid levels of 10 nonheterotic and 20 heterotic hybrids, respectively, constructed from the inbreds listed in Table III. The hybrids were evaluated in the same trial at the same times (days after planting) as the inbreds, under the same irrigation regimes (see "Materials and Methods" for details). The nonheterotic hybrids (Table IV) represent 'sister-line' hybrids derived from inbred parents which are somewhat related to one another (Northrup King Co., personal communication). These sister-line hybrids appear in several of the three-way heterotic hybrids listed in Table V. Because of the different 'vigor' of these materials, the inbreds, sister-line (nonheterotic) hybrids and heterotic hybrids were grown in separate blocks in the trials to avoid shading of the less vigorous material by the heterotic hybrids. Yields of the nonheterotic hybrids ranged from 24 to 100 bushels/acre, whereas yields of the heterotic hybrids ranged from 109 to 179 bushels/acre in this specific trial (results not shown).

It can be seen from these results that, of the 30 hybrids evaluated, only one was comprised of two betaine-deficient inbred parents (*i.e.* hybrid 338 × 644²; Table IV), and this was the only hybrid to exhibit a betaine:amino acid ratio of < 0.0015 (Table IV). For each hybrid an expected mid-parent be-

taine:amino acid ratio has been calculated (Tables IV and V). These expected values are derived from the observed betaine:amino acid ratios of the inbred parents (Table III). For a two-way hybrid ($A \times B$) the expected mid-parent betaine:amino acid ratio = $(A' + B')/2$, where A' and B' are the observed betaine:amino acid ratios of the inbred parents. For a three-way hybrid $[(A \times B) \times C]$, the expected mid-parent betaine:amino acid ratio = $([(A' + B')/2] + C')/2$, where C' = the observed betaine:amino acid ratio of the third (non-sister-line) inbred in the cross.

Linear regression analyses of observed versus expected betaine:amino acid ratios of these hybrids are summarized in Table VI. In general, it appears that there is a significant correlation between the observed and expected values for these 30 hybrids of distinct pedigree ($r = 0.69$). This suggests that the betaine:amino acid ratio is a heritable characteristic, perhaps under the control of genes that are largely additive in their effects. When the heterotic hybrids as a group are considered separately from the nonheterotic hybrids as a group, much higher correlation coefficients ($r = 0.87$ and 0.77, respectively) between observed and expected betaine:amino acid ratios are obtained (Table VI). It appears that the slope of the regression line of best-fit to the nonheterotic hybrids is substantially less than the slope of the regression line of best-fit to the heterotic hybrids (slopes = 0.85 and 1.40, respectively) (Table VI), suggesting that the heterotic hybrids tend to give consistently higher than expected betaine:amino acid ratios. Since betaine does not appear to be catabolized in plants (9, 11, 14) and betaine synthesis rate is modulated by water deficits (9, 11, 14), the betaine level can reflect the specific water stress history of the individual (or population) sampled (14). It is possible that the different growth rates and hence water consumption rates of heterotic *versus* nonheterotic hybrids might produce different patterns of betaine accumulation, especially when integrated over the entire growth period preceding sampling. For this reason, we believe that it is appropriate to consider the heterotic and nonheterotic hybrids as separate groups in evaluating the heritability of this metabolic trait.

Because one betaine-deficient inbred (1506) was used as a male parent for 12 of the heterotic hybrids (see pedigrees of Table V), it is particularly useful to consider the observed and expected betaine:amino acid ratios of these 1506-derived hybrids. As shown in Table VI, the highly significant correlation coefficient ($r = 0.83$) between the observed and expected betaine:amino acid ratios of heterotic hybrids derived from inbred 1506, strongly suggests that when the male parent is betaine-deficient, the betaine titer of the hybrid is proportional to the betaine titer of the female parent in the cross.

Two hybrids were chosen for further genetic studies, hybrids 1146 × 1074 and 1146 × 1506. These were chosen for the following specific reasons: (a) they share a common female parent (1146), and thus differences in segregation patterns in their F_2's can be attributed to inheritance of nuclear encoded genes of the male parents which differ by a factor of 90-fold in their betaine:amino acid ratios (Table III); (b) inbreds 1146 and 1074 have virtually identical betaine:amino acid ratios (Table III), and thus the expected mid-parent betaine:amino acid ratio for hybrid 1146 × 1074 is almost exactly twice that of hybrid 1146 × 1506 (expected betaine:amino acid ratios = 0.090 and 0.045, respectively) (Table III); and (c) these two hybrids exhibit similar vigor, yield potential, and maturity (Northrup King Co., personal communication), so that differences between the F_1 hybrids *per se* cannot be attributed to large differences in growth and water consumption rates when screened in the same environment at the same developmental stage.

When these two hybrids were grown in adjacent rows in Indiana in the summer of 1986, and 10 individual plants of each hybrid were screened (6 weeks after planting) for betaine and free amino acid levels in the youngest, most fully expanded leaf, a significant two-fold difference in betaine:amino acid ratio was indeed observed for these hybrids (Table I). This was due specifically to betaine; the hybrids were not significantly different with respect to total free amino acid levels. As expected, hybrid 1146 × 1074 exhibited a betaine:amino acid ratio of almost exactly twice that of hybrid 1146 × 1506. This represents an important point, since if betaine deficiency in inbred 1506 is due to a single nuclear gene in the homozygous recessive condition, and inbreds 1146 and 1074 are homozygous dominant for this allele, then these results can be interpreted in terms of semidominance, *i.e.* heterozygotes may, on average, exhibit half the betaine titer of homozygous dominant individuals. This information is useful in interpreting the F_2 segregation patterns below.

F_2 Population Betaine and Amino Acid Levels. In order to determine whether betaine deficiency in inbred 1506 can be attributed to a single nuclear gene in the homozygous recessive condition, F_2 populations of each of these two hybrids were screened for betaine and amino acid levels of the youngest most fully expanded leaf 5 weeks after planting under greenhouse, well-irrigated growth conditions. In principle, a frequency of 25% betaine-deficient individuals (*i.e.* individuals with betaine:amino acid ratios of < 0.0015) should be observed in the 1146 × 1506-F_2 and a frequency of 0% betaine-deficient individuals in the control 1146 × 1074-F_2 population. Moreover, we might expect to see approximately 50% of the segregants in the 1146 × 1506-F_2 population that exhibit an intermediate betaine:amino acid ratio relative to the control 1146 × 1074-F_2 population (*i.e.* a 50% frequency of heterozygotes in the 1146 × 1506-F_2).

The results of Tables II show the frequency distributions with respect to betaine:amino acid ratio observed in each segregating

Table VI. *Linear Regression Analyses of Observed versus Expected Betaine:Amino Acid Ratios of Hybrids*

Variables[a]		Linear Equation of Best Fit [$Y = mX + b$]			
X Expected betaine: amino acid ratio	Y Observed betaine: amino acid ratio	N No. of pairs of X and Y	m Slope	b Intercept	r Correlation coefficient
All hybrids		30	1.024	0.040	0.6916**
Heterotic hybrids alone		20	1.394	0.030	0.869**
Nonheterotic hybrids alone		10	0.853	0.021	0.771**
Heterotic hybrids with 1506 as male-parent alone		12	1.142	0.044	0.834**

[a] Derived from Tables IV and V. ** Significant at the P = 0.01 level.

population. A frequency of 6 out of 30 individuals with betaine:amino acid ratios of < 0.0015 was observed in the 1146 × 1506-F_2 and a frequency of 0 out of 30 individuals in this class for the 1146 × 1074-F_2 (Table II). These results strongly support the hypothesis that betaine deficiency in inbred 1506 is due to a single nuclear encoded homozygous recessive gene. A frequency of 6/30 betaine-deficient individuals in the 1146 × 1506-F_2 is not significantly different from an expected frequency of 7.5/30 assuming a single gene model. Whereas over 94% of the 1146 × 1074-F_2 segregants exhibited betaine:amino acid ratios of >0.06 in this environment, only 30% of the 1146 × 1506-F_2 segregants fell into this category (Table II). About 50% of the individuals in the 1146 × 1506-F_2 population exhibited intermediate betaine:amino acid ratios of between 0.02 and 0.06 (Table II). Such a result appears consistent with a 50% frequency of heterozygotes in the 1146 × 1506-F_2 expected for segregation for a single nuclear gene. However, the wide range of betaine:amino acid ratios in the 1146 × 1074-F_2 population (0.03 to 0.39) (Table II) suggests the possibility that inbreds 1146 and 1074 may not be isogenic for all genes that can influence this metabolite ratio; there may be segregation for new gene combinations that markedly affect betaine and/or amino acid accumulation independently of the 1506-specific recessive gene and its dominant allele. The occurrence of such segregation for other genes of unknown inheritance and number in the 1146 × 1506-F_2 population cannot yet be precluded, and such a phenomenon might tend to confound a simple 1:2:1 segregation pattern (with respect to the 1506-specific recessive gene) by substantially increasing the standard deviations for the putative heterozygous and homozygous dominant classes of individuals. Nevertheless, it is encouraging that these F_2 populations yield an extremely wide range of segregants with respect to betaine:amino acid ratios (>400-fold in this specific environment at this specific developmental stage). This holds significant promise for developing breeding lines with a wide range of betaine levels required to test whether there is any relationship between environmental stress resistance/tolerance and capacity to accumulate betaine in maize.

Similar segregation patterns to those observed in the greenhouse trials above, were obtained in evaluations of betaine and amino acid levels of field grown F_2 populations of these hybrids (Indiana trial, 1987) (Fig. 1). Of a total of 45 individuals in the 1146 × 1506-F_2 population sampled, 15 individuals exhibited the phenotype of betaine deficiency (Fig. 1A). None of the 30 individuals of the 1146 × 1074-F_2 sampled exhibited this phenotype (Fig. 1B). The wide range of betaine:amino acid ratios observed in the latter population (0.13 to 0.69) again suggests segregation for gene(s) within the 1146 × 1074-F_2, which can have a marked effect on this metabolite ratio independently of the 1506-specific recessive allele and its dominant counterpart. In general, the betaine:amino acid ratios of the field-grown materials were substantially greater than those of the greenhouse grown materials. We await the evaluation of F_3 populations derived from the field-grown materials to test whether high and low betaine:amino acid ratio segregants in the 1146 × 1074-F_2 populations breed true for this character, to test whether the intermediate betaine:amino acid ratio segregants in the 1146 × 1506-F_2 exhibit 1:2:1 segregation patterns in the F_3 (*i.e.* to confirm that these intermediate individuals are indeed heterozygous), and to test whether betaine-deficient and relatively high betaine:amino acid ratio segregants from the 1146 × 1506-F_2 breed true as homozygous recessive and dominant respectively.

A final point of interest in relation to the phenotype of glycinebetaine deficiency in maize is that this phenotype is not associated with "abnormal" growth under well-irrigated conditions. From measurements of plant height with respect to time of each of the 60 individuals evaluated in the greenhouse trial of Table II, there was no significant relationship between betaine deficiency and growth rate (*i.e.* plant height doubling time) in either F_2 population (results not shown).

DISCUSSION

The results presented here indicate that the phenotype of glycinebetaine deficiency in at least one inbred line of *Zea mays* (inbred 1506), is due to a single, nuclear gene in the homozygous recessive condition. We have not yet established whether betaine deficiency in inbreds 338 and 644 is governed by the same locus (gene). However, since the hybrid 338 × 664[2] is betaine-deficient, it seems likely that inbreds 338 and 644 share a common recessive gene (or genes). This conclusion is not surprising since inbreds 338 and 644 are related to one another (Northrup King Co., personal communication).

The available data support the notion that the dominant allele (of the recessive gene) of inbred 1506 exerts a strong dosage effect on betaine level; thus, heterozygotes appear to exhibit half the betaine:amino acid ratio of homozygous dominant individuals. The heterotic hybrids derived from 1506 as male parent (all presumably heterozygous for the 1506-specific recessive gene) exhibit betaine:amino acid ratios that are proportional to the betaine:amino acid ratios of the female parent. There may be significant genetic variability for other nuclear encoded or cytoplasmic encoded genes that can affect betaine and/or amino acid levels severalfold within this germ plasm. Precedents for such nuclear genes influencing betaine level three- to four-fold have been set for barley (5, 6, 8, 13), and it is not unreasonable to suppose that such genetic differences may also exist in maize. Random segregation for the 1506-specific recessive gene (and its dominant allele) and other genes which may influence betaine and/or amino acid levels an additional three- to four-fold might account for the striking 400-fold range of betaine:amino acid ratios observed in the 1146 × 1074- and 1146 × 1506-F_2 populations of rather modest size (<140 individuals total).

Further work is clearly now required: (a) to establish that the putative homozygous segregants breed true, and (b) to test for relationships between betaine accumulation and environmental stress tolerance/resistance in maize (*cf.* barley [5]). In barley it appears that high betaine selections maintain a higher betaine level and concomitantly lower solute potential then low betaine selections across a range of osmotic stress environments (5). An important question that has not yet been addressed in these investigations with maize is whether the betaine-deficient genotypes maintain the phenotype of betaine deficiency at all developmental stages, in all plant parts, and at all levels of imposed osmotic stress. Our current studies have been confined to the youngest most fully expanded leaf of plants which have not been deliberately exposed to severe water deficits and/or salinity stresses. We await the development of true-breeding F_3 and F_4 selections to rigorously address these questions. At present, very little is known about the molecular and biochemical basis of the phenotype of betaine deficiency in this germ plasm. Rigorous studies of the underlying biochemistry and enzymology of the deficiency must rely to a large extent on progress in both the genetic and physiological characterizations, in particular the development of true-breeding lines with known differences in developmental and/or osmotic stress modulated expression of this metabolic trait. The current results represent only a first step towards an understanding of this intriguing genetic system of as yet unknown significance in terms of maize productivity.

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