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Tissue culture isolation of a second mutant locus for increased threonine accumulation in maize

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Summary. Regenerable maize (*Zea mays* L.) tissue cultures were selected for ability to grow in the presence of inhibitory (1.0–1.5 m*M*) concentrations of L-lysine plus L-threonine. Testcross kernels from one regenerated plant (LT20) segregated for wild-type and high free threonine concentration in a 1:1 ratio consistent with a single dominant gene for high free threonine. Free threonine concentrations (nmol/mg dry weight) increased an average of 29-fold in bulked F_2 kernel samples from heterozygous mutant plants, and the total (free plus protein-bound) threonine concentration increased 68%. Increases in protein-bound methionine, lysine and glycine concentrations were also noted, suggesting a possible effect of the mutation on protein concentration and composition. Allelism tests with a previously selected mutant line, *Ltr*19*, showed that two unlinked, codominant genes conditioned the high free threonine phenotype. Based on a separate study of aspartate kinase feedback inhibition characteristics in the two mutant lines, we propose that the mutant alleles [gene and allele designations are according to guidelines for maize genetic nomenclature (Burnham et al. 1975)] be designated *Ask-LT19* and *Ask2-LT20* for the *Ltr*19* and *LT20* mutants, respectively.

Key words: Tissue culture mutant selection – Amino acid biosynthesis – Lysine plus threonine resistance – Aspartate kinase – Maize

Introduction

Mutations affecting amino acid biosynthesis have been obtained by selection in plant tissue culture systems for resistance to amino acid analogues and/or combinations of amino acids (Jacobs et al. 1987). The aspartate family amino acid biosynthetic pathway (Bryan 1980) is a focal point for such tissue culture selection research, because the end products are the nutritionally important amino acids, lysine, threonine and methionine.

For selection purposes, the combination of two pathway end products, lysine and threonine (LT), has been shown to inhibit growth of cells and tissue cultures of a number of plants (Green and Phillips 1974; Gengenbach 1984). Inhibition is due to insufficient methionine for growth (Henke and Wilson 1974), presumably because of end product feedback inhibition of a regulatory enzyme(s) in the pathway prior to homoserine, a direct precursor of methionine. Aspartate kinase is the first enzyme in the aspartate family pathway and is subject to feedback inhibition by lysine, threonine or both, depending on the species (Bryan 1980). Selection for LT resistance, therefore, was proposed by Green and Phillips (1974) as a potential means to obtain mutants with aspartate kinase that is less sensitive to feedback inhibition. Altered regulation might then lead to changes in synthesis and/or accumulation of end product amino acids.

LT-resistant mutants have been selected in barley (*Hordeum vulgare* L.), carrot (*Daucus carota* L.), maize (*Zea mays* L.) and tobacco (*Nicotiana tabacum* L.) (Bright et al. 1982; Cattoir-Reynaerts et al. 1983; Hibberd et al. 1980; Hibberd and Green 1982; Miao et al. 1988; Bourgin et al. 1982). Progeny from regenerated tobacco and maize usually exhibited an inheritance pattern consistent with a single dominant gene for growth of seedlings or excised root tips in the presence of LT (Bourgin et al. 1982; Hibberd and Green 1982; Miao et al. 1988). Barley mutants obtained from screening excised embryos were also conditioned by dominant alleles at two unlinked loci (Bright et al. 1982). Enzyme analyses

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of LT-resistant maize and carrot cultures (Hibberd et al. 1980; Cattoir-Reynaerts et al. 1983) and barley seedlings (Arruda et al. 1984; Rognes et al. 1983) have shown that aspartate kinase with reduced sensitivity to lysine feedback inhibition is associated with LT resistance. Changes in free amino acid pools have also been demonstrated in LT-resistant mutants. The concentration of free threonine in seeds and vegetative tissues increased 10- to 77-fold, with smaller increases in other amino acids such as serine, methionine and lysine (Bright et al. 1982; Hibberd and Green 1982; Miao et al. 1988).

Mutant cell lines, regenerated plants and progeny derived from them provide the necessary material to examine relationships between the "resistance" trait and changes in the accumulation of specific amino acids, feedback inhibition properties of enzymes regulating amino acid biosynthesis and other characteristics of cell and plant growth.

The study reported here was conducted to select additional LT-resistant mutants of maize and to determine the genetic relationship with a previously selected mutant that had been provisionally designated *Ltr*19* (Hibberd and Green 1982). We recovered one new mutant line from selected tissue cultures and determined that it had characteristics similar to *Ltr*19* but was controlled by a different, unlinked genetic locus. We have also recently determined that both mutants have altered aspartate kinase activity and, therefore, propose that the two genes be designated *Ask-LT19* and *Ask2-LT20* (representing *as*partate *k*inase) in place of the provisional *Ltr*19* mutant and the new mutant line, respectively.

Materials and methods

Culture initiation and maintenance

Tissue cultures of maize (*Zea mays* L.) inbred line A188 were initiated from immature embryos (Green and Phillips 1975) on Murashige and Skoog medium (1962) containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 m*M* L-asparagine. Separate embryo-derived culture lines were increased and maintained on the same media.

Culture mutagenesis and selection

Four hundred pieces of calli (ca. 20 mg each) from 18 embryo-derived culture lines were each treated with one drop of filter-sterilized 1 m*M* sodium azide in 0.1 *M* phosphate buffer (pH 3). After 5 days, cultures were transferred to maintenance media containing 1 m*M* L-lysine and 1 m*M* L-threonine (LT). Actively growing tissue was subsequently transferred four times at 3-week intervals to selection media containing 1.0, 1.25, 1.5 and 1.5 m*M* LT, respectively, in succession. Tissue still actively growing after five selection intervals was transferred to LT-free media without 2,4-D to promote leaf, shoot and root formation and to initiate plant regeneration (Green and Phillips 1975).

Crosses and progeny analysis

Nine plants were regenerated from four cell lines identified during the fourth selection period and grown to maturity. Three plants produced viable pollen and were crossed to wild-type inbred line Wf9 used as the female parent. Progeny were obtained from two regenerated plants that traced back to the same callus piece in the fourth selection period. Segregation for high free threonine concentration was tested by amino acid analysis of extracts of individual kernels from the initial testcrosses to Wf9. Two crosses from the same regenerated plant (LT20) segregated for kernels with wild-type (0.2 nmol/mg dry weight) and high (6.9 nmol/mg dry weight) free threonine concentrations. The mutant *Ask2-LT20* allele and other related materials used in this study were derived from plant LT20.

Analysis of free amino acids in kernels

Dried kernels were ground individually or as bulked samples in a Wig-L-Bug mixer/grinder (Crescent Dental Mfg. Co., Chicago/IL). The dry meal and 0.5 µmol of norleucine for an internal standard were combined and extracted overnight with 5 ml of 5% (w/v) trichloroacetic acid (TCA). After centrifugation the pellet was re-extracted with 5 ml of 5% TCA for 4 h. Combined supernatants were passed through an AG50W cation exchange column and amino acids eluted with 20 ml of 0.6 *M* NH_4OH. The eluants were frozen, lyophilized, rehydrated with 2.5 ml water and then frozen and lyophilized again. The residue was dissolved in 0.01 *N* HCl and aliquots were analyzed on an amino acid analyzer (Dionex Corp., Sunnydale/CA). Methionine was determined as the sum of the methionine plus methionine sulfoximine peaks.

Analysis of total amino acids

For analysis of total (free + protein-bound) amino acids, 26.5 mg of kernel meal was hydrolyzed in 1 ml of distilled 6.05 *N* HCl at 100 °C for 24 h, 0.5 µmol norleucine was added and the contents were dried under vacuum. The residue was rehydrated, filtered and dried under vacuum. The samples were rehydrated, applied to ion exchange columns and processed the same as TCA extracts.

Analysis of free threonine by thin layer chromatography

Individual kernels were crushed and the total meal was extracted overnight with 5% TCA. The extract was centrifuged and 10 µl was spotted onto cellulose thin layer chromatography (TLC) plates (Kodak No. 13255 or Analtech No. 05011). Spots were dried and plates were developed in sealed chromatography tanks containing an acetone : n-butanol : ammonium hydroxide : water solvent (10 : 10 : 5 : 2, v/v) until the solvent front migrated 16 cm. Plates were dried overnight and sprayed with ninhydrin (2 g/l) in acetone to visualize the pattern of separated amino acids. The threonine spot size and intensity were compared to a wild-type control on the same plate and each sample was scored as wild-type (+/+) or mutant (high threonine = *Ask2-LT20/Ask2-LT20* or *Ask2-LT20*/+).

Genetic materials for allelism test

A homozygous *Ask2-LT20/Ask2-LT20* plant was crossed to a homozygous *Ask-LT19/Ask-LT19* plant that had been derived from the source of the *Ask-LT19* allele [(A188 × LT19-2), Table 1 in Hibberd and Green 1982] by self-pollination for three generations. The double mutant *Ask-LT19*/+;*Ask2-LT20*/+ F_1 plants were self-pollinated or crossed as males with wild-type inbred line A619. F_2 plants were self-pollinated and testcrossed to wild-type A619 female plants.

Results

Selection of LT-resistant maize tissue cultures

Because the A188 callus cultures used for selection were highly differentiated and contained organogenic tissues, the selection pressure in the first two cycles was kept low so resistant cells or small groups of cells could be retrieved from large masses of susceptible cells. Tissue growth during the first cycle of selection of 1 m*M* LT was not severely inhibited. The level of LT was not increased in cycle 2, but the size of the pieces transferred was considerably smaller than in cycle 1. Growth of the smaller inocula was still not severely inhibited on 1 m*M* LT, but necrosis was more evident after 3 weeks on 1.25 m*M* LT (cycle 3). Only 45 callus pieces out of 400 survived 1.5 m*M* LT (cycle 4) and were subdivided into individual cell lines for cycle 5 (1.5 m*M* LT). At the end of cycle 5, 16 pieces of callus, all tracing back to the same plate in cycle 4, were still growing and were transferred to regeneration medium without LT.

Progeny analysis

Nine plants were regenerated from the 16 callus pieces; three plants were grown to anthesis and crossed to wild-type Wf9 plants. Four crosses involving two regenerated plants produced seed. To determine whether free amino acid concentrations were altered in the progeny, five kernels from two crosses with regenerated plant LT20 were analyzed individually. Two kernels had wild-type free threonine concentrations (average = 0.2 nmol/g dry wt.) and three kernels had high free threonine concentrations (average = 6.9 nmol/mg dry wt.). The apparent segregation of a high threonine phenotype suggested that the regenerated plant LT20 was heterozygous for a dominant gene for threonine overproduction.

Several progeny lines were derived from the initial crosses of LT20 and Wf9. TLC analyses to detect segregation of wild-type and high free threonine levels in kernels (Fig. 1), and occasionally in anthers (data not shown), of testcross and selfed progeny were employed to determine the genotype of the plants and to assure that the high threonine trait was being maintained in each generation. A BCS_4 (fourth generation of self-pollination after the first testcross to Wf9) plant was determined to be homozygous for the high threonine trait based on production of only high threonine progeny in the BCS_5 generation. Two homozygous BCS_5 plants, 3253-3 and 3253-4, were used in crosses with wild-type A188 and A619, respectively. F_1 plants from those crosses were testcrossed to A619, and individual kernels were scored for high and wild-type free threonine by TLC analysis (Table 1). Data from two testcross ears showed excellent fit to a 1:1 segregation, indicating that the mutant phenotype was most likely caused by a single dominant gene.

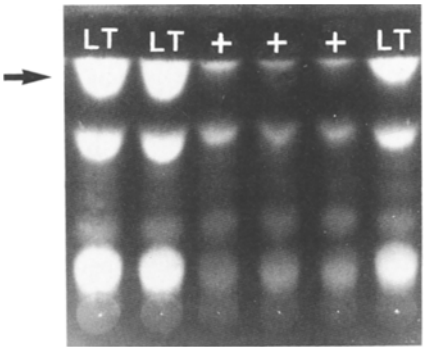

Fig. 1. Thin layer chromatographic analysis of free amino acids illustrating the segregation of wild-type (+) and high free threonine (LT) kernels. The threonine spot is indicated by the *arrow*

Table 1. Segregation for high (LT) and wild-type (+/+) free threonine concentrations by TLC analysis of single kernels from (A) testcrosses between A619(+/+) female and *Ask2-LT20*/+ or *Ask-LT19*/+ F_1 plants, and (B) the F_2 generation and testcrosses between A619(+/+) females and F_1 plants of a cross between homozygous *Ask-LT19*/*Ask-LT19* and *Ask2-LT20*/*Ask2-LT20* plants

F_1 plant tested	LT	+/+	χ^2 1:1	*P*
A Single mutant F_1				
Ask2-LT20/+				
3151-12 × 3253-3	44	36	0.80	>0.35
3108-9 × 3253-4	21	29	1.28	>0.25
Pooled	65	65	0.00	>0.99
Ask-LT19/+				
3106-51 × 3105-11	45	45	0.00	>0.99
3103-49 × 3104-2	55	45	1.00	>0.30
Pooled	100	90	0.53	>0.45
B Double mutant F_1				
F_2 progeny		15:1 ratio		
3126-1	72	3	0.65	>0.4
-2	154	11	0.05	>0.8
-6	34	2	0.03	>0.85
-7	71	4	0.11	>0.7
-8	17	3	2.61	>0.1
-14	8	1	0.36	>0.5
-23	44	1	1.25	>0.25
-27	122	5	1.16	>0.25
Pooled	522	30	0.63	>0.4
Testcross progeny		3:1 ratio		
3126-7	27	13	1.20	>0.25
-11	22	18	8.53	<0.005
-13	27	13	1.20	>0.25
-15	18	12	3.60	>0.05
-27	27	13	1.20	>0.25
Pooled	121	69	12.98	<0.001

This mutant allele has been designated *Ask2-LT20*. Segregation of similar testcross progeny from F_1 plants of *Ask-LT19* (formerly *Ltr*19*) also fit a 1:1 ratio (Table 1) as reported previously for LT resistance tests of seedlings (Hibberd and Green 1982). Both mutant lines thus condi-

tioned accumulation of high concentrations of free threonine in mature kernels, and the phenotype was inherited as though controlled by a single dominant gene.

Allelism tests

The similarity of expression and inheritance raised the question of whether the two mutant lines represented mutations in different genes or were independent isolations of mutations at the same locus. Homozygous plants of *Ask-LT19/Ask-LT19 and Ask2-LT20/Ask2-LT20* were crossed, and kernels from the F_2 and testcross generations were scored for segregation of high and wild-type free threonine concentrations. F_2 and testcross kernels segregated for high and low free threonine classes as shown in Fig. 1 and Table 1. If the mutations were allelic, segregation should not have occurred, and only kernels with high free threonine would have been expected in the two generations.

The F_2 segregation pattern obtained fit a 15:1 ratio for high free threonine versus wild-type kernels, which is the ratio expected for separate, unlinked dominant genes (Table 1). Each F_2 family tested contained wild-type kernels and exhibited a reasonable fit to a 15:1 ratio, as did the F_2 pooled data. Segregation for wild-type kernels should have occurred only if separate genes were involved.

The testcrosses involving wild-type A619 female plants segregated for a high proportion of wild-type kernels (Table 1). For separate, independent dominant genes, a testcross ratio of 3 high threonine to 1 wild-type kernel was expected; however, all families had more than 25% wild-type kernels. For two of the five families the ratio was significantly different than 3:1. Deviation from the expected ratio could not be attributed to linkage. Because the F_1 was in repulsion phase, linkage would reduce the frequency of wild-type kernels, not increase it. The testcross ratios had a better fit to a 2:1 ratio (Chi-square values not shown), and the average frequency of wild-type gametes from the F_1 plants was 0.36 instead of 0.25. An increased wild-type frequency might have arisen from poor pollen transmission of a mutant gamete type, such as *Ask-LT19;Ask2-LT20*. The wild-type frequency (0.054) in F_2 progeny, however, did not indicate reduced transmission of mutant gametes. If mutant gamete transmission had been reduced through the pollen only or through both pollen and egg, the expected wild-type F_2 frequencies would have been 0.09 (11:1 ratio) or 0.13 (8:1 ratio), respectively, instead of 0.06 (15:1 ratio). None of the F_2 families had a significant excess of wild-type kernel phenotypes based on TLC analysis of free threonine concentrations. A potential counteracting influence on the F_2 ratios could have been the occasional misclassification of genetically wild-type F_2 kernels as the high threonine phenotype, due to the contribution of increased threonine from the maternal F_1 *Ask-Lt19/+; Ask2-LT20/+* plant to all F_2 kernels on the ear.

To confirm the F_2 segregation of wild-type and high threonine kernel phenotypes, sibling F_2 plants were grown from remnant 3126-8 and 3126-27 F_2 seed, and F_3 and F_2 testcross progenies were obtained (Table 2). Four F_2 plants produced only wild-type kernels in testcrosses to A619 (Table 2) and in their F_3 progeny (data not shown), confirming that homozygous wild-type genotypes were recovered by segregation in the F_2. Two testcross families had only high threonine kernels in samples of 20 kernels and presumably were homozygous for one or both mutant genes (probability was >0.99 for observing at least one wild-type in testcross progeny of double mutant).

The remaining 21 F_2 plants tested produced segregating testcross progeny and were assigned either to a 1:1 or a 3:1 segregation class, which were the two types of segregating progeny expected for two independent dom-

Table 2. Segregation for high (LT) and wild-type (+/+) free threonine concentrations by TLC analysis of single kernels from testcrosses between A619 (+/+) females and F_2 plants of a cross between homozygous *Ask-LT19/Ask-LT19* and *Ask2-LT20/Ask2-LT20* plants

F_2 plant tested	LT	+/+	Most likely segregation	F_2 genotypes
29005-1	0	40	All wild-type	+/+; +/+
29013-2	0	20		
29013-10	0	20		
29015-1	0	20		
29009-1	9	11	1LT: 1+/+	*Ask-LT19/+; +/+* or
29013-3	11	9		*+/+; Ask2-LT20/+*
29013-4	10	10		
29013-5	11	9		
29013-6	9	11		
29013-7	12	8		
29013-9	7	13		
29015-4	9	11		
29015-6	10	10		
29015-10	3	17		
29017-2	10	10		
29017-4	10	10		
29017-5	4	16		
29017-6	7	13		
29017-7	4	16		
29015-2	15	5	3LT: 1+/+	*Ask-LT19/+; Ask2-LT20/+*
29015-3	14	6		
29015-5	16	4		
29017-1	15	5		
29017-3	14	6		
29017-9	19	1		
29007-1	20	0	All LT	*Ask-LT19/Ask-LT19;*
29013-1	20	0		−/− or −/−; *Ask2-LT20/Ask2-LT20* or *Ask-LT19/Ask-LT19; Ask2-LT20/Ask2-LT20*

inant genes for high free threonine. According to the minimax procedure described by Steel and Torrie (1960) and for a sample size of 20, progeny containing 13 or more high threonine kernels were assigned to the 3:1 segregation class and progeny containing 12 or fewer high threonine kernels were assigned to the 1:1 segregation class. The probability of assigning the wrong testcross segregation class was 0.23 for each family, but this probability was less than the error associated with choosing any other breakpoint for the assignment.

The 27 F_2 plants giving the four testcross segregation patterns (Table 2) occurred in a 2:6:15:4 ratio, which was significantly different than the 7:4:4:1 ratio expected for the all high threonine, 3:1, 1:1, and all wild-type progeny classes, respectively. These data show that F_2 plants with zero or one *Ask* mutant allele were more frequent than double heterozygous (*Ask-LT19/+; Ask2-LT20/+*) or homozygous (*Ask-LT19/Ask-LT19; +/+, +/+; Ask2-LT20/Ask2-LT20, Ask-LT19/Ask-LT19; Ask2-LT20/Ask2-LT20*) plants. The low frequency of homozygous mutant F_2 plants (nonsegregating, high free threonine testcross progeny) was most likely a consequence of poor germination of F_2 seed of certain genotypes. Seeds produced on known homozygous *Ask-LT19/Ask-LT19; +/+*, and *+/+; Ask2-LT20/Ask2-LT20* plants usually have exhibited low germination when planted in the field or in laboratory germination tests (data not shown). Seeds heterozygous for either or both mutant genes have germinated normally, thus segregation tests that rely on growth of homozygous and heterozygous mutant and wild-type plants may often be biased against the homozygous mutant. Assays that do not rely on germination, such as the TLC analysis of free threonine in kernels, should provide a better assessment of segregation.

Amino acid concentrations in Ask2-LT20 kernels

To obtain a preliminary determination of the effects of the *Ask2-LT20* mutation on kernel amino acids, two sets of material were analyzed. The first set of kernels was produced by backcrossing heterozygous *Ask2-LT20/+* plants to A619+/+ female plants. Free amino acid differences between kernels segregating on backcross ears reflect the expression of a single dose of the mutant in the kernel tissue with no interference from *Ask2-LT20* expression in vegetative tissues. Segregating kernels from backcross ears were randomly selected for analysis of individual kernels. The +/+ and *Ask2-LT20/+* genotypes were classified after amino acid analysis on the basis of threonine mole percent (percent threonine in total free amino acid pool). Nine kernels classified as wild-type had an average threonine concentration of 0.13 nmol/mg dry wt. or 1.4 mol percent. Threonine values for two *Ask2-LT20/+* kernels were 6.7 and 2.5 nmol/mg dry wt. or 29 and 30 mol percent, respectively. These values indicated a free threonine increase of 20- to 52-fold for individual heterozygous kernels. Serine, glycine and methionine were also two- to three-fold higher in the *Ask2-LT20/+* kernels.

The second set of materials was obtained from bulked, 40-kernel samples of four self-pollinated ears of each of two genotypes. Four ears were from known wild-type +/+ plants and the other four ears were from heterozygous *Ask2-LT20/+* plants in the BCS_4 generation. Free amino acid concentrations in the samples from the *Ask2-LT20/+* selfed plants reflected a weighted average of the expression of the segregating kernel genotypes including endosperm dosages. Kernel amino acid profiles could also have been affected by changes in metabolism due to expression of *Ask2-LT20* in the vegetative portion of the plant. The TCA-soluble free pools of several amino acids were significantly higher for ears of *Ask2-LT20/+* plants compared to +/+ plants (Table 3). Threonine increased 29-fold from 0.5 nmol/mg dry wt. to 14.5 nmol/mg dry wt. Serine and glycine increased about four-fold and cystine, histidine and glutamine increased less, but significantly. The total free amino acids increased by 90%, although aspartate and tyrosine concentrations decreased slightly.

Total amino acids (free + protein-bound) were determined in acid hydrolysates of the same kernel samples used for free amino acid analysis. Total threonine concentrations (nmol/mg dry wt.) were 68% higher in ears of *Ask2-LT20/+* plants (Table 3). The increase in the free pool accounted for 68% of the increase in total threonine concentration. The increase in protein-bound threonine, determined from the difference between total and free amino acid concentrations, could have been due to higher protein concentration and/or a higher proportion of threonine in the protein. The threonine mole percent values for both genotypes were essentially equal, suggesting that the mutant had higher protein concentration on a dry-weight basis.

Total serine, glycine, methionine, tyrosine, histidine, lysine and arginine concentrations increased by 33%, 36%, 81%, 22%, 33%, 30% and 37%, respectively. Increases in the free amino acid concentrations were not sufficient to account for all of the increase in the total concentration. Examination of protein-bound amino acid levels revealed that significant increases in the protein concentrations were responsible for the major portion of the increases in total levels. Assuming equal efficiency in protein extraction and recovery, the data indicated a higher protein concentration in HCl hydrolysates of *Ask2-LT20/+* ears compared to +/+ ears.

Discussion

The gradual stepwise enrichment selection protocol was effective in producing LT-resistant maize tissue cultures.

Table 3. Free, protein-bound and total amino acid concentrations in self-pollinated ears of +/+ and *Ask2-LT20*/+ plants[a]

Amino acid	Free		Protein-bound		Total	
	+/+	*Ask2-LT20*/+	+/+	*Ask2-LT20*/+	+/+	*Ask2-LT20*/+
	nmol/mg dry wt. or (mol percent)					
Aspartate	4.5*	3.1	48.5 (7.0)	58.2 (7.1)	55.8	64.9
+Asparagine	2.8	3.6				
Threonine	0.5**	14.5	29.5 (4.3)	36.0 (4.4)	30.0**	50.5
Serine	0.8*	3.5	36.1 (5.2)	45.5 (5.6)	36.9*	49.0
Homoserine	0.1	0.5	–	–	–	–
Glutamate	2.3	2.1	133.4 (19.3)	152.7 (18.7)	136.9	156.8
+Glutamine	1.2*	2.1				
Glycine	0.5**	2.1	57.1 (8.3)**	76.3 (9.3)	57.6**	78.4
Alanine	2.7	1.4	82.2 (12.3)	91.3 (11.2)	87.9	92.7
Cystine	0.1*	0.2	–	–	–	–
Valine	0.3	0.4	42.3 (6.1)	51.2 (6.3)	42.6	51.6
Methionine	0.5	1.0	12.1 (1.8)**	21.8 (2.7)	12.6*	22.8
Isoleucine	0.3	0.6	26.8 (3.9)	29.8 (3.7)	27.1	30.4
Leucine	0.1	0.1	99.9 (14.5)	104.4 (12.8)	100.0	104.5
Tyrosine	0.5*	0.3	15.0 (2.2)*	18.6 (2.3)	15.5*	18.9
Phenylalanine	0.2	0.2	32.6 (4.7)	34.6 (4.2)	32.8	34.8
Histidine	0.5*	0.7	20.3 (2.9)	26.9 (3.3)	20.8*	27.6
Lysine	1.4	1.7	24.0 (3.5)**	31.2 (3.8)	25.4**	32.9
Arginine	1.2	1.4	27.7 (4.0)*	38.2 (4.7)	28.9**	39.6
Total	20.5**	39.5	690.5	816.7	710.8	855.4

[a] Mean of four bulk samples comprised of 40 randomly selected kernels per ear for each genotype. Free amino acid concentrations in TCA extracts and total amino acid concentrations in HCl hydrolysates were determined first and then protein-bound concentrations were obtained by difference

* Significant at 5% level of probability according to *t*-test

** Significant at 1% level of probability according to *t*-test

Several regenerated plants were obtained and testcross progeny were produced from one regenerated plant, LT20. Inheritance tests based on limited quantiative analysis and then on extensive qualitative TLC analysis (Table 1) for free threonine in kernels demonstrated the presence of a dominant, single-gene mutation for increased free threonine. The increases in free and total threonine concentrations (Table 3) were comparable to those reported for the first LT-resistant mutant (*Ltr*19*) selected in maize (Hibberd and Green 1982).

The recovery of two maize mutants following LT selection raised the question of the number of genes that might control these similar phenotypes. Segregation of homozygous recessive wild-type plants in the F_2 generation of crosses between the two different homozygous mutants clearly established that separate genetic loci were involved (Table 1). F_2 segregation was also consistent with an independent, codominant two-gene model, although reduced recovery of mutant gametes occurred in testcross progeny.

Additional characterization of the two mutants has revealed that both have altered aspartate kinase activity (S. B. Dotson, D. A. Frisch, D. A. Somers and B. G. Gengenbach, unpublished results). Aspartate kinase from wild-type A619 was shown to require only 10 μM L-lysine for 50% inhibition, but aspartate kinase from the homozygous mutant isolated in this study required 760 μM for 50% inhibition. Aspartate kinase from heterozygous mutants (*Ltr*19*/+) obtained from the previous selection study (Hibberd and Green 1982) also required 25–38 μM lysine for similar inhibition. These biochemical analyses indicated that the LT-resistant mutants contain alleles of structural genes for aspartate kinase. Therefore, we propose that the mutant alleles be designated *Ask-LT19*, in place of the provisional *Ltr19* designation for the mutant selected by Hibberd and Green (1982), and *Ask2-LT20* for the mutant obtained in this study.

The genetic control of lysine-sensitive aspartate kinase in maize appears to be similar, but not identical, to that in barley (Arruda et al. 1984; Bright et al. 1982; Rognes et al. 1983). Enzyme analyses established that the barley loci controlled two distinct aspartate kinase isozymes separable by ion exchange chromatography. Each LT resistance mutation decreased the lysine sensitivity of a different aspartate kinase isozyme and did not affect the sensitivity of the isozyme controlled by the other locus. In contrast, all the aspartate kinase activity from the maize mutants exhibited reduced feedback inhibition by lysine, and a separable wild-type aspartate kinase was not detected. Additional biochemical characterization of the mutant and wild-type maize aspartate

kinases should elucidate the relationship between the *Ask* and *Ask2* alleles and subunit composition and structure of the holoenzyme.

This study and that of S. B. Dotson, D. A. Frisch, D. A. Somers and B. G. Gengenbach (unpublished data) demonstrate the substantial effect that an alteration in the feedback inhibition properties of a regulatory enzyme can have on pathway end products. Free threonine exhibited the most pronounced change in the mutants relative to wild-type (Table 3; Hibberd and Green 1982), but other free pool amino acids such as serine and glycine, which are not aspartate pathway end products, may also be altered (Table 3). We have found that free amino acid changes differ in extent and composition depending on the mutant genotype, genetic background and tissue source (e.g. embryo versus endosperm) (T. J. Diedrick and B. G. Gengenbach, unpublished data). Free lysine concentrations were not significantly different as expected, given that the lysine-specific branch enzyme, dihydrodipicolinate synthase, is also feedback-inhibited by lysine. Dihydrodipicolinate synthase activities in both LT-resistant mutants were equally sensitive to lysine inhibition as wild-type (D. A. Frisch and B. G. Gengenbach, unpublished data). Although free methionine has shown modest and variable increases in mutant kernels (Table 3; Hibberd and Green 1982), the 80% increase in the concentration of protein-bound methionine shown in Table 3 has been observed in other genetic backgrounds for both *Ask-LT19* and *Ask2-LT20* mutants (T. J. Diedrick and B. G. Gengenbach, unpublished data). A more thorough analysis of the effects of both mutations on free and protein-bound amino acid composition is nearly complete.

The availability of mutant alleles for genetically distinct loci provides valuable germ plasm, not only for biochemical and molecular biology studies but also for continued assessment of their role in improving the nutritional value of maize. Generation of additional mutant alleles of *Ask* and *Ask2* by LT selection and mutants for other enzymes, such as dihydrodipicolinate synthase, would also be of interest for the studies mentioned above. Access to a number of mutations from the same selection system provides an opportunity to identify mutants with the most favorable expression and to test new genetic combinations.

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