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Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture

(mutants/amino acid overproduction)

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ABSTRACT **Selection for resistance to growth inhibition by lysine plus threonine in equimolar concentrations (LT) in tissue cultures of maize yielded a stable resistant line, LT19. Genetic analysis of progeny of plants regenerated from LT19 showed that LT resistance was inherited as a single dominant nuclear gene, temporarily designated *Ltr**-19. Tissue cultures initiated from resistant embryos required 5–10 times higher levels of LT to inhibit growth than did cultures from LT-sensitive embryos. LT resistance in *Ltr**-19 was expressed as much reduced inhibition of root and shoot growth in the presence of LT. The free pool of threonine was increased 6 times in cultures initiated from immature embryos of LT-resistant plants, and 75–100 times in kernels homozygous for *Ltr**-19, as compared to cultures and kernels from LT-sensitive embryos and plants, respectively. Overproduction of free threonine increased the total threonine content in homozygous *Ltr**-19 kernels by 33–59%. The results demonstrate that LT resistance selected with tissue culture methods is heritable and is expressed in cultures, seedlings, and kernels. Furthermore, they demonstrate a method to obtain amino acid-overproducer mutants in maize, which have the potential to increase substantially specific amino acids in kernels. The capability to increase specifically the nutritionally limiting amino acid(s) could have important nutritional implications for the grain of cereals and other crops.**

The heritability and expression in plants of traits selected in tissue culture are of particular concern to those using these methods for genetic modification of plants. Only a few studies have demonstrated expression in regenerated plants and sexual transmission of selected traits to progeny. These include resistance to methionine sulfoximine (1), streptomycin (2, 3), *Helminthosporium maydis* race T toxin (4), picloram (5), and valine (6).

Selection for resistance to growth inhibition by lysine plus threonine in equimolar concentrations (LT) has been proposed as a means of obtaining feedback-resistant mutants in the lysine-threonine-methionine-isoleucine (aspartate family) biosynthetic pathway (7). Reversal of LT inhibition by the addition of methionine or the methionine precursor, homoserine, indicates that inhibition is the result of methionine starvation (8, 9) and that altered phenotypes which increase methionine synthesis should provide resistance to LT inhibition. Aspartokinase and homoserine dehydrogenase, in the aspartate pathway, are subject to feedback inhibition by lysine and threonine, respectively, in maize (10, 11).

In a recent report from this laboratory, we described the selection from tissue cultures of maize of a stable LT-resistant line (D33) that contained aspartokinase with decreased sensitivity to lysine inhibition (12). Analysis of free amino acid pools in D33 cultures showed substantial increases in methionine and threonine and small increases for lysine and isoleucine. Attempts to determine the inheritance of LT resistance failed because of complete sterility in plants regenerated from D33 cultures.

Additional LT-resistant lines have now been selected from which we have obtained regenerated plants and progeny. In this report, we describe the inheritance of LT resistance and the expression of this trait in tissue cultures and progeny of regenerated plants.

METHODS

The initiation, maintenance, and incubation of tissue cultures from immature embryos were as described (13, 14). Immature embryos, 1–2 mm in length, were obtained from a cross between inbred lines A188 and W22. Cultures were grown on Murashige and Skoog (MS) medium (50 ml per 25 × 100 mm Petri dish) containing 0.5 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) per liter. The cultures were transferred to fresh medium after 1 month, and after 2 months they were subdivided into pieces of *ca.* 20 mg of fresh weight and placed on 50 ml of MS medium. One drop of 0.1 M phosphate buffer (pH 3.0) containing 0, 0.1, 1, or 10 mM sodium azide was applied to each explant. This solution was completely absorbed into the tissue or growth medium after 1 hr. Eight days after azide treatment, lysine and threonine were added to the culture plates to give a final concentration of 2 mM for each. At monthly intervals, sectors of cultures which appeared to be alive (nonnecrotic) were transferred to fresh MS medium containing 2 mM LT.

Plant regeneration was initiated by transferring pieces of *ca.* 50 mg fresh weight of LT-resistant and unselected control cultures to MS medium lacking 2,4-D (14). Regenerated control and putative LT-resistant plants were grown to maturity in the greenhouse.

Tissue cultures, established from immature embryos of first (R_1) and second (R_2) generation progeny of regenerated plants (R_0) were used as one bioassay to determine inheritance of LT resistance. Two months after initiation, *ca.* 30-mg (fresh weight) pieces of these cultures were tested for growth on 0, 1.25, and 2.5 mm LT. Inheritance of LT resistance also was determined by using a bioassay based on seedling root length (9). Seeds sterilized for 20 min in 2.5% (wt/vol) sodium hypochlorite were soaked overnight in Orthocide (1 mg/ml; Chevron, San Francisco). Embryos removed from the seed by dissection were placed in storage dishes (80 × 100 mm) containing 50 ml of MS medium lacking 2,4-D and with or without 2 mM LT. The length (cm) of the primary root of each seedling was measured after 7 days.

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Abbreviations: LT, lysine plus threonine in equimolar concentrations; MS medium, Murashige and Skoog medium; 2,4-D, dichlorophenoxyacetic acid.

Levels of free amino acid in cultures were determined with a Beckman 119 automatic analyzer (see ref. 12). Mature seeds were individually ground in a Torit amalgamator, and the free amino acids were extracted from the meal with the solvents of Bielinski and Turner (15). These extracts were passed through Bio-Rad AG 50W-X8 and analyzed as described (12). The protein content of seeds was calculated from the nitrogen content determined by micro-Kjeldahl analysis. The amino acid composition of total kernel proteins was determined by extracting the proteins with the Osborne procedure, followed by acid hydrolysis of the protein and amino acid analysis of the hydrolysate (16).

RESULTS

Sodium azide, a potent seed mutagen in several cereal species (17, 18), was applied to the tissue cultures prior to selection as a possible means of increasing the likelihood of obtaining LT resistance. A total of 600 inocula, each weighing *ca.* 20 mg, were treated with various NaN_3 concentrations. Growth was substantially retarded in the 200 cultures treated with 1 mM NaN_3 when compared to equal numbers of cultures treated with 0 or 0.1 mM NaN_3. Concentrations higher than 1 mM NaN_3 were lethal.

After sodium azide treatment, selection for resistance to 2 mM LT was initiated. Sectors of cultures that appeared viable were transferred to fresh MS medium containing 2 mM LT at monthly intervals. Three presumptive LT-resistant lines, LT6, LT2, and LT19, were isolated from the 0, 0.1, and 1 mM NaN_3 treatments, respectively, at the end of the third selection cycle (3 months). One of these lines, LT19, was highly resistant to LT inhibition, whereas the two others, LT2 and LT6, were marginally resistant. Plant regeneration was begun from these LT-resistant lines and unselected control cultures after the third selection cycle. Regenerated plants were self-pollinated when possible or crossed to A188 plants grown from seed. Progeny seeds were obtained from six unselected controls, two LT2-, one LT6-, and five LT19-regenerated plants.

Inheritance. Analysis of LT resistance in the progeny of selected lines was carried out by using the tissue culture or seedling bioassay methods. Of the three presumptive LT-resistant lines, only one—LT19 from the 1 mM NaN_3 treatment—proved to contain a transmissible resistance trait. A detailed description of LT19 cultures, regenerated plants, and their progeny follows.

Tissue cultures were initiated from immature embryos from self-pollinated regenerated control (A188 × W22) plants and from regenerated LT19 plants crossed to A188. Growth of all 20 cultures initiated from control (A188 × W22) ⊗ plants was severely inhibited with LT in MS medium (factors of 15.6 ± 2.3, 2.6 ± 0.6, and 0.6 ± 0.1 fresh weight increase at 0, 1.25, and 2.5 mM LT, respectively). The response to the same LT concentrations of cultures initiated from A188 × LT19-2 embryos fell into two distinct classes. One class of 17 cultures was as susceptible to LT as the control (17.5 ± 1.7, 3.0 ± 0.9, and 0.4 ± 0.1), whereas the second class of seven cultures grew well (21.9 ± 2.7, 15.3 ± 3.1, and 11.3 ± 2.6) and these responses were deemed to indicate LT resistance.

Progeny from two regenerated plants, LT19-2 and LT19-3, crossed to A188 were examined for the frequency of LT resistance using the tissue culture bioassay. This analysis established that LT resistance was expressed in 40% (25 of 62 cultures) of the progeny, that resistance was a pollen-transmissible trait, and that it was expressed in the heterozygous condition. This information indicates that LT resistance is conditioned by a dominant nuclear gene; this is temporarily designated as *Ltr**-19, until the biochemical basis of the mutation is determined.

Table 1. Frequency of resistance to LT inhibition in segregating R_2 seedling populations

Genotype	Susceptible†	Resistant†	*P*
(A188 × LT19-2)⊗	40	71	<0.01‡
A188 × (A188 × LT19-2)	56	47	0.3–0.4§

† Classification as resistant or susceptible was based on the growth of seedling roots in the presence of 2 mM LT.
‡ χ^2 analysis based on a 3:1 resistant/susceptible segregation frequency.
§ χ^2 analysis based on a 1:3 resistant/susceptible segregation frequency.

The seedling root bioassay permits progeny to be screened more conveniently than the tissue culture method, and it also indicates whether or not LT resistance is expressed during seedling growth. In the presence of 2 mM LT, the root growth of all control seedlings was severely inhibited as compared to growth in the absence of LT (2.1 ± 0.2 vs. 6.7 ± 0.4 cm, respectively). Seedlings from crosses of LT19-2 and LT19-3 to A188 segregated into a class with short (1.6 ± 0.2 cm) and one with long (5.7 ± 0.5 cm) roots when grown in the presence of 2 mM LT. The short class was classified as susceptible (+/+) and the long was resistant (*Ltr**-19/+). The frequency of LT resistance using the root bioassay was 38% (26 of 69 seedlings). The validity of the root bioassay method was substantiated by testing seedling progeny from plants classified as resistant and susceptible. In all cases, self-pollinated resistant plants gave at least 60% resistant progeny, and susceptible plants gave only susceptible progeny according to the seedling root assay.

Using the seedling root assay, the frequency of LT-resistant plants was also examined in R_2 progeny produced from selfed or crossed R_1 plants. As in the R_1 generation, R_2 controls contained only susceptible seedlings. Self- or cross-pollination of R_1 LT-resistant plants yielded both susceptible (short roots) and resistant (long roots) R_2 seedlings. A summary of the observed segregation frequencies is shown in Table 1. The χ^2 test for a 1:1 segregation of LT resistance in progeny of A188 × (A188 × LT19-2) crosses was not significant, supporting the idea of a single dominant nuclear gene. The χ^2 test for a 3:1 segregation in progeny of (A188 × LT19-2)⊗ crosses was significant, however.

Expression of LT Resistance. Growth of tissue cultures initiated from immature embryos of R_2 control and LT19 plants was measured in the presence of 0–8 mM LT (Fig. 1). Differences between the resistant cultures LT19-R2 and LT19-R12

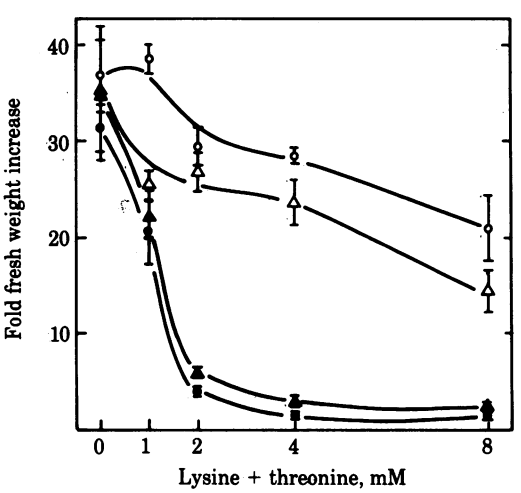

FIG. 1. Effect of equimolar concentrations of lysine plus threonine on 30-day growth of cultures from immature embryos of the R_2 generation. ○, LT19-R2; △, LT19-R12; ▲, control A; ●, control B.

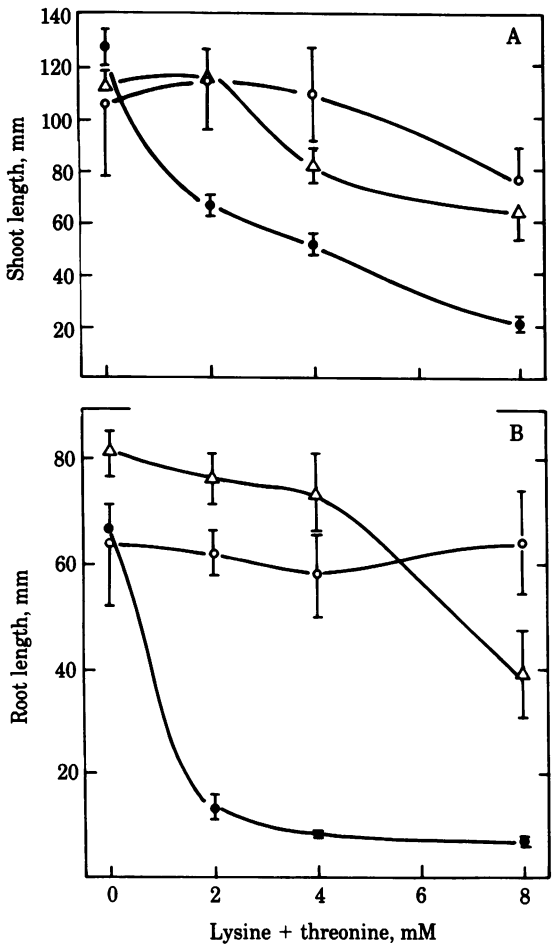

FIG. 2. Growth of shoots (*A*) and roots (*B*) of mature embryos isolated from *Ltr**-19/*Ltr**-19 (○), *Ltr**-19/+ (△), and nonselected control (●) progeny in the presence of LT.

and the susceptible cultures were highly significant at LT concentrations of 2 mM and higher. Between 5- and 10-fold higher levels of LT were required to inhibit growth by 50% in the resistant cultures compared with the susceptible cultures. The growth response differences to LT between the two resistant cultures might reflect the number of copies of *Ltr**-19. LT19-R2 was from a self pollinated R_1 LT-resistant plant and could have been homozygous for *Ltr**-19. LT19-R12 was from an R_1-resistant plant crossed to a susceptible control and could only have been heterozygous for *Ltr**-19, assuming that our hypothesis is correct.

The expression of LT resistance on the growth of developing seedlings is shown in Fig. 2 *A* and *B*. Families of homozygous and heterozygous seedlings were obtained by selfing LT-resistant plants in a segregating R_2 family and by backcrossing them to a susceptible A188 control. Families homozygous for *Ltr**-19 were identified by the lack of segregation of LT resistance in corresponding backcrosses. Root development of susceptible seedlings was severely inhibited at concentrations of 2 mM LT and above (Fig. 2*B*). Shoot development of susceptible seedlings also was inhibited by increasing concentrations of LT (Fig. 2*A*) but to a lesser degree than was root development. Roots and shoots of *Ltr**-19/+ and *Ltr**-19/*Ltr**-19 seedlings grew significantly better than the controls did at all levels of LT tested. Root growth of the homozygote appeared to be at a slight disadvantage compared to the heterozygote, except at 8 mM LT.

Analysis of Free Amino Acids. The free amino acid levels were examined in tissue cultures initiated from immature embryos of two susceptible and two LT-resistant R_2 plants (Table 2). Free threonine in the resistant cultures LT19-R2 and LT19-R12 was increased 4- to 7-fold over the level found in the susceptible cultures. Isoleucine also was increased 1.5- to 2-fold in the resistant cultures. The remaining amino acids were unchanged between control and resistant cultures grown on 0 mM LT. In the presence of 2 mM LT, however, the resistant cultures maintained the free methionine pool at levels similar to cultures grown in the absence of LT. It is interesting to note that high levels of free arginine were found in all tissue cultures regardless of genotype, whereas very little was found in kernels (Table 3).

Free amino acid levels were examined also in +/+, *Ltr**-19/+, and *Ltr**-19/*Ltr**-19 kernels (Table 3). Of particular interest is the marked increase in the level of free threonine in *Ltr**-19/+ and *Ltr**-19/*Ltr**-19 kernels as compared to normal. The dominance and gene dosage effect of *Ltr**-19 is shown by the 30-fold increase in free threonine in heterozygous kernels and the 77-fold increase in homozygous kernels. Amino acid analyses of other *Ltr**-19/*Ltr**-19 kernels showed increases in free threonine as high as 109-fold. The levels of free methionine, serine, and proline were increased 3- to 4-fold in *Ltr**-19/*Ltr**-19 kernels, but not in *Ltr**-19/+ kernels, with the exception of proline, which was substantially higher. The significance of this is doubtful, however, because the increase was the consequence of high free proline in only one of four kernels in the analysis. The total free amino acid level was doubled in *Ltr**-19/+ kernels and tripled in *Ltr**-19/*Ltr**-19 kernels. The data in Table 3 indicate that the increase in threonine, methionine, serine, and proline did not dramatically alter the free pools of the remaining amino acids.

Table 2. Free amino acids in LT-resistant and -susceptible tissue cultures†

	Susceptible controls§		LT19-R2¶		LT19-R12¶	
Amino acid‡	A	B	0 mM LT	2 mM LT	0 mM LT	2 mM LT
Threonine	0.86 ± 0.13	0.95 ± 0.22	5.88 ± 1.98	NA	4.07 ± 0.93	NA
Methionine	0.10 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.11 ± 0.01	0.10 ± 0.01
Lysine	0.25 ± 0.02	0.31 ± 0.02	0.41 ± 0.01	6.50 ± 0.51	0.25 ± 0.03	4.62 ± 0.01
Arginine	4.00 ± 1.60	3.69 ± 0.64	3.40 ± 0.77	3.98 ± 1.90	2.57 ± 0.23	3.90 ± 1.35

† Each value in μmol/g fresh weight is the mean ± SEM of four replications. NA, not analyzed.

‡ Resistance or susceptibility had little effect on other amino acids assayed: aspartic acid, 1.76–2.11; μmol/g fresh weight; valine, 1.17–1.67; isoleucine, 0.21–0.37; leucine, 0.25–0.39; tyrosine, 0.35–0.65; phenylalanine, 0.09–0.18; histidine, 0.50–0.75.

§ Control A is from a nonselected, regenerated control plant crossed to A188; control B is from an LT-susceptible segregate from the progeny of a self-pollinated, first generation LT-resistant (*Ltr**-19/+) plant.

¶ LT19-R2 was from a self-pollinated, first generation LT-resistant (*Ltr**-19/+) plant; LT19-R12 was from a first generation LT-resistant (*Ltr**-19/+) plant crossed to A188 (+/+).

Table 3. Free amino acids in heterozygous (*Ltr**-19/+) and homozygous (*Ltr**-19/*Ltr**-19) LT-resistant and -susceptible (+/+) seed†

Amino acid‡	Genotypes,§ μmol/g dry weight +/+	*Ltr**-19/+	*Ltr**-19/*Ltr**-19	*Ltr**-19/*Ltr**-19 to +/+ ratio
Threonine	0.15 ± 0.01	4.35 ± 0.35	11.15 ± 1.33	76.8
Serine	0.74 ± 0.10	0.81 ± 0.10	2.37 ± 0.25	3.2
Proline	0.35 ± 0.09	2.63 ± 0.80	1.39 ± 0.19	4.0
Methionine	0.10 ± 0.03	0.05 ± 0.01	0.36 ± 0.09	3.7
Lysine	0.15 ± 0.04	0.16 ± 0.03	0.19 ± 0.01	1.3
Arginine	Trace	Trace	Trace	—
Total	6.74	12.08	19.70	
Total kernel	0.84	1.62	2.54	

† Each value represents the mean ± SEM of four replications.
‡ Genotype had little affect on the level of other amino acids assayed: aspartic acid, 1.1–1.7 μmol/g dry weight; glutamic acid, 1.0–1.6; glycine, 0.23–0.35; alanine, 0.35–0.63; valine, 0.35–0.49; isoleucine 0.10–0.19; leucine, 0.07–0.08; tyrosine, 0.18–0.21; histidine, 0.16–0.27.
§ *Ltr**-19/*Ltr**-19 seed was obtained by self-pollination of a third generation *Ltr**-19/*Ltr**-19 plant, which was also crossed to +/+ to produce the *Ltr**-19/+ seed; +/+ seed was from an unselected regenerated control plant crossed to A188 and then backcrossed to A188 in the second generation.

The effect of threonine overproduction in *Ltr**-19/+ and *Ltr**-19/*Ltr**-19 on total kernel threonine is assessed in Table 4. These data show that the mutant kernels are at least as large as normal and that the protein content in kernels of all genotypes was not greatly diffferent. The threonine content of the kernel proteins also was very near 4% in all genotypes. The large increase in free threonine in *Ltr**-19/+ and *Ltr*-19/*Ltr**-19 kernels contributed to substantial increases in total threonine. The elevated free threonine present in *Ltr**-19/*Ltr**-19 increased the total threonine in these kernels by 33–59%.

DISCUSSION

Our data clearly indicate a mutational origin for LT resistance in the LT19 line. Resistance is expressed both in tissue cultures and seedlings of progeny from plants carrying *Ltr**-19. Pollen transmission of LT resistance indicates that the trait is coded by a nuclear gene. χ^2 analysis of progeny from cross-pollinations showed that the segregation of LT resistance was not significantly different from a single dominant gene (Table 1). However, segregation in self-pollinations of *Ltr**-19 plants did deviate significantly from the expected 3:1 resistant/susceptible frequency. The reduced frequency could be accounted for by reduced transmission of gametes carrying the *Ltr**-19 gene. Data from reciprocal crosses showed that when *Ltr**-19/+ plants were used as male parents, LT-carrying pollen was reduced in competitive effectiveness. Cytological analysis of meiosis in LT-resistant plants has shown the probable presence of a duplication that could account for the reduced transmission of LT resistance in certain crosses if the duplication and the *Ltr**-19 gene were linked.

The LT selection procedure in this study was modified from previous work (12) to include the use of recently initiated cultures for mutant selection and the regeneration of plants from resistant cultures as soon as possible after they were identified. Selection was completed in 3 months instead of 6 and was carried out at a higher concentration of LT. Sodium azide also was applied to the cultures to increase possibly the mutation frequency and, thus, the likelihood of obtaining LT-resistant cultures. Because only one mutant was obtained, no conclusions can be made about the effectiveness of azide as a mutagen in maize tissue cultures.

LT resistance has not resulted in any immediately visible changes in the plants during development and maturation in the greenhouse or field. Plants carrying the *Ltr**-19 gene appear normal and as vigorous as comparable control plants. Because LT resistance is strongly expressed in the roots (Fig. 2*B*), it can be detected routinely with the seedling root bioassay. Shoot development appears less responsive to LT inhibition (Fig. 2*A*), and consequently the expression of LT resistance in this organ is less clear. It is not known whether LT directly inhibits amino acid synthesis and growth in shoots or whether shoots are affected indirectly through the effect of LT on roots.

The increased concentration of free threonine in kernels carrying *Ltr**-19 (Table 3) corresponds to the increased free threonine observed in LT-resistant tissue cultures (Table 2). The magnitude of the increase indicated the possibility that total threonine content of the kernel might be increased. This is borne out by the data in Table 4, which show that total threonine in *Ltr**-19/*Ltr**-19 kernels was increased by 33–59%. This ta-

Table 4. Effect of free threonine overproduction in various genotypes on total threonine in seed†

Analysis	+/+ 1	+/+ 2	*Ltr**-19/+ 1	*Ltr**-19/*Ltr**-19 1	*Ltr**-19/*Ltr**-19 2
Kernel weight	116 ± 5	123 ± 9	154 ± 14	122 ± 9	134 ± 10
Protein/100 mg of meal	9.4 ± 0.15	10.1 ± 0.18	8.9 ± 0.11	10.4 ± 0.14	10.2 ± 0.09
Threonine/100 mg of protein	4.05 ± 0.49	3.95 ± 0.41	3.81 ± 0.29	3.99 ± 0.42	3.90 ± 0.44
Protein threonine/kernel	0.44 ± 0.05	0.49 ± 0.05	0.52 ± 0.06	0.51 ± 0.06	0.53 ± 0.04
Free threonine/kernel	<0.01 ± <<0.01	<0.01 ± <<0.01	0.07 ± 0.01	0.17 ± 0.02	0.31 ± 0.03
Total threonine/kernel	0.44	0.50	0.59	0.68	0.84
Free threonine as % of protein threonine	0.4%	0.6%	13%	33%	59%

† Each value (in mg except last item) represents the mean ± SEM of three replications.

ble also indicates that kernel weight, total protein, and threonine content of protein were not altered appreciably between the +/+, *Ltr**-19/+, and *Ltr**-19/*Ltr**-19 genotypes.

In conclusion, a mutation, *Ltr**-19, has been isolated from tissue culture that conditions the overproduction of a nutritionally essential amino acid in tissue cultures and, most importantly, in the seed of regenerated plants and subsequent generations. The magnitude of threonine overproduction in seed carrying *Ltr**-19 offers the opportunity to isolate and use amino acid-overproducer mutants to increase selectively the levels of specific nutritionally limiting amino acids in the seeds of crops.

Nutritionally, threonine is the third most limiting of the essential amino acids in maize, but in several other cereals, particularly wheat and rice, it is the second limiting amino acid (19). The more serious threonine deficiency in these species, coupled with the results of our study, demonstrate the need to initiate selections for LT resistance. The results of our study also clearly indicate the desirability of isolating lysine-, tryptophan-, and methionine-overproducer mutants in various crops.

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