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Genetic and amino-acid analysis of two maize threonine-overproducing, lysine-insensitive aspartate kinase mutants

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Abstract The aspartate-derived amino-acid pathway leads to the production of the essential amino-acids lysine, methionine, threonine and isoleucine. Aspartate kinase (AK) is the first enzyme in this pathway and exists in isoforms that are feedback inhibited by lysine and threonine. Two maize (*Zea mays* L.) threonine-overproducing, lysine-insensitive AK mutants (*Ask1-LT19* and *Ask2-LT20*) were previously isolated. The present study was conducted to determine the map location of *Ask2* and to examine the amino-acid profiles of the *Ask* mutants. The threonine-overproducing trait conferred by *Ask2-LT20* was mapped to the long arm of chromosome 2. Both mutants exhibited increased free threonine concentrations (nmol/mg dry weight) over wild-type. The percent free threonine increased from approximately 2% in wild-type kernels to 37–54% of the total free amino-acid pool in homozygous mutant kernels. Free methionine concentrations also increased significantly in homozygous mutants. Free lysine concentrations were increased but to a much lesser extent than threonine or methionine. In contrast to previous studies, free aspartate concentrations were observed to decrease, indicating a possible limiting factor in threonine synthesis. Total (free plus protein-bound) amino-acid analyses demonstrated a consistent, significant increase in threonine, methionine and lysine concentrations in the homozygous mutants. Significant increases in protein-bound (total minus free) threonine, methionine and lysine were observed in the *Ask* mutants, indicating adequate protein sinks to incorporate the increased free amino-acid concentrations. Total amino-acid contents (nmol/kernel) were approximately the same for mutant and wild-type kernels. In five inbred lines both *Ask* mutations conferred the threonine-overproducing phenotype, indicating high expressivity in different genetic backgrounds. These analyses are discussed in the context of the regulation of the aspartate-derived amino-acid pathway.

Key words *Zea mays* · Aspartate kinase
Threonine-overproducing mutants · Lysine · Methionine

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Introduction

Cereals, including maize (*Zea mays* L.), when fed to non-ruminant animals and humans, contain nutritionally-low levels of several essential amino-acids, particularly lysine, threonine, methionine and tryptophan. In plants and bacteria, lysine, threonine, methionine and isoleucine are derived from aspartate (Cohen and Saint-Girons 1987; Bryan 1990 a) (Fig. 1). Regulation of aspartate-derived amino-acid biosynthesis is mediated through endproduct feedback-inhibition of regulatory enzymes (Bryan 1990a) which include aspartate kinase (AK; EC 2.7.2.4), homoserine dehydrogenase (HSDH; EC 1.1.1.3) and dihydrodipicolinate synthase (DHPS; EC 4.2.1.52). AK is the first pathway enzyme and catalyzes the conversion of aspartate to aspartyl-phosphate. AK forms inhibited by lysine, lysine plus S-adenosylmethionine (SAM), and threonine, have been identified in plants (Bryan 1990 a).

Threonine-sensitive AK exists as a bifunctional enzyme with HSDH activity in carrot (Wilson et al. 1991; Weisemann and Matthews 1993) and with threonine-sensitive HSDH in maize (Azevedo et al. 1992; Muehlbauer et al., manuscript submitted). Maize also has a monofunctional threonine-insensitive HSDH form (Walter et al. 1979). HSDH catalyzes the reduction of aspartate semialdehyde to homoserine, which is the first committed step in the synthesis of threonine. DHPS, the branch enzyme in the synthesis of lysine appears to be solely feedback-inhibited by lysine (Bryan 1990 a; Frisch et al. 1991). Most enzymes in this pathway function in the chloroplast with a notable exception of threonine-insensitive HSDH, which is active

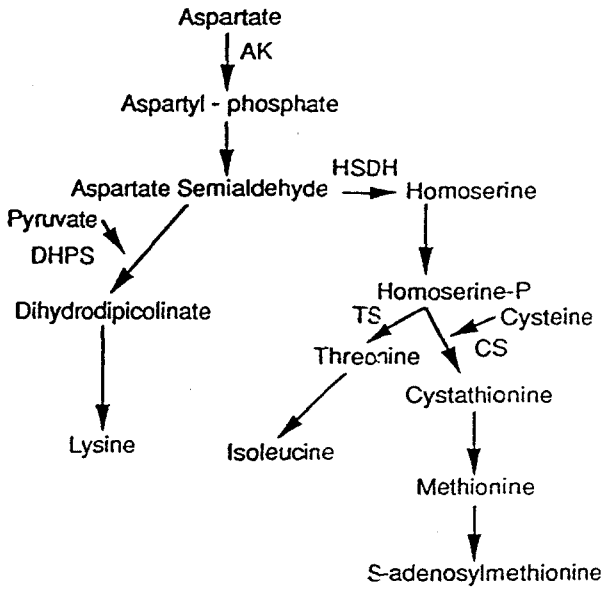

Fig. 1 Aspartate-derived amino-acid pathway. The pathway leading to the production of the essential amino-acids lysine, threonine, methionine and isoleucine. *AK*, aspartate kinase; *HSDH*, homoserine dehydrogenase; *DHPS*, dihydrodipicolinate synthase; *CS*, cystathionine synthase; *TS*, threonine synthase

in the cytoplasm (Bryan et al. 1977; Wallsgrove et al. 1983).

Addition of lysine plus threonine (LT) to plant tissue-cultures inhibits cell growth by starving cells for methionine, presumably due to inhibition of lysine- and threonine-sensitive AKs. By exploiting this characteristic, LT-resistant mutants were isolated in this pathway in barley (Bright et al. 1982 a, b; Rognes et al. 1983), carrot (Cattoir-Reynaerts et al. 1983), tobacco (Bourgin et al. 1982; Frankard et al. 1991) and maize (Hibberd et al. 1980; Hibberd and Green 1982; Miao et al. 1988; Diedrick et al. 1990). LT-resistance in maize and carrot-tissue cultures (Hibberd et al. 1980; Cattoir-Reynaerts et al. 1983), and barley (Bright et al. 1982 b), tobacco (Frankard et al. 1991) and maize plants (Dotson et al. 1990), is a consequence of lysine-insensitive AK which further results in overproduction of free threonine. Three maize threonine-overproducing, lysine-insensitive AK mutants (*Ask1-LT19*, *Ask2-LT20* and *LTR-3*) have been isolated (Hibberd and Green 1982, Miao et al. 1988; Diedrick et al. 1990). *Ask1* and *Ask2* were shown to be partially-dominant nonallelic genes (Diedrick et al. 1990). *Ask1* was positioned on chromosome 7S linked to *opaque-2* (Azevedo et al. 1990), whereas, *Ask2* has not been mapped.

Free amino-acid concentrations of the *Ask* mutants were determined on limited material (Hibberd and Green 1982; Diedrick et al. 1990) and demonstrated that free threonine was overproduced 77- and 15-fold in homozygous *Ask1-LT19* and heterozygous *Ask2-LT20* mutant kernels, respectively. Free methionine concentrations increased slightly in both mutants. Protein-bound threonine, methionine, lysine and glycine concentrations also were shown to increase in heterozygous *Ask2-LT20* mutants (Diedrick et al. 1990).

In the present study, a more extensive genetic approach was used to characterize the amino-acid concentrations of the maize *Ask1-LT19* and *Ask2-LT20* mutants and to map the *Ask2* locus. Wild-type and homozygous mutant kernels were analyzed for free and total (free plus total) amino-acid concentrations to obtain a general understanding of the *Ask* phenotype. Analyses of free amino-acid concentrations were conducted on both mutants in five inbred lines to determine penetrance and expressivity of the trait across genotypes and, thereby, the utility of *Ask* alleles in maize improvement.

Materials and Methods

Plant material

F_3 lines from crosses between inbred line B73 and the *Ask2-LT20* mutant (Diedrick et al. 1990) were used to map the *Ask2* locus. Sixteen F_3 lines were grown in the field at St. Paul in 1992 and five immature ears from each F_3 line were combined for DNA isolations. Remnant kernels from each F_3 line were used for free amino-acid analysis.

Genetic materials to compare amino-acid concentrations in wild-type and homozygous *Ask* mutants were derived with *Ask1-LT19* in inbred line A661 and *Ask2-LT20* in inbred line A619. A661 *Ask1-LT19/Ask1-LT19*, A661 +/+, A619 *Ask2-LT20/Ask2-LT20* and A619 +/+ plants were selfed to derive ears produced in the same field environment. Ten kernels from two or three ears from each line were analyzed for free and total amino-acid concentrations.

To examine expressivity of the threonine-overproducing *Ask* trait in different genetic backgrounds, inbred lines A554, A661, W64A, Va26 and B37 were crossed with pollen from A661 *Ask1-LT19/Ask1-LT19* and A619 *Ask2-LT20/Ask2-LT20*. Control crosses were made with pollen from wild-type A661 and A619. Ten F_1 kernels from two ears of each cross were analyzed separately.

Free amino-acid analysis

Free amino-acid analyses were conducted according to Knecht and Chang (1986). Ten mature kernels from each ear were ground to meal in a cyclone sample mill. Ground samples were dried in an oven at 60°C for 16–24 h. Dry meal (500 mg) was extracted in 25% CH_3CN overnight with constant agitation. Samples were centrifuged at 3000 *g* for 10 min, filtered through a 0.45-µM filter and dried at 70°C with an air flow. Samples were dissolved in 50 mM of $NaHCO_3$ (40 µl), derivatized with (Dimethylamino)azobenzenesulfonyl chloride (80 µl), heated at 70°C for 10 min, and diluted to 1 ml with 50 mM of Na_2HPO_4 (pH 7.0)/95% EtOH (1:1). Derivatized samples were analyzed by high-performance liquid chromatography.

Total amino-acid analysis

Kernel meal samples were also used for total (free plus protein-bound) amino-acid analyses (Knecht and Chang 1986). Meal (500 mg) was defatted by suspending in petroleum ether with agitation for 1 h, centrifuging for 15 min at 3000 *g* and discarding the supernatant. The pellet was resuspended in petroleum ether, agitated for 1 h, collected onto filter paper and dried overnight at room temperature. For hydrolysis, 6N HCl (2 ml) was added to 35 mg of dried defatted meal; tubes were flushed with nitrogen for 3 min, capped and heated at 110°C for 24 h. Samples were dried in a vacuum centrifuge, dissolved in 0.1N HCl and centrifuged for 5 min at 15 000 *g*. Derivatization and quantitation were conducted as for free amino-acid analyses.

High-performance liquid chromatography

Amino-acid analyses were conducted on a Spectraphysics HPLC using a phase-separation spherisorb S5 ODS2 25×4.6 mm column. Tryptophan was unstable under the conditions used for both the free and total analyses. The hydrolysis procedure employed for the total amino-acid analyses converted glutamine and asparagine to glutamate and aspartate, respectively. In these analyses, proline and valine were not consistently resolved from one another so their combined quantities were recorded.

DNA isolation and DNA gel-blot analysis

DNA was isolated (Saghai-Maroof et al. 1984) from freeze-dried immature ear tissue, digested with restriction endonucleases, electrophoresed on 0.8% agarose gels and then transferred overnight onto Immobilon-N membrane. The blots were prehybridized and hybridized overnight at 65°C using standard conditions (Sambrook et al. 1989). Final post-hybridization washes were at 65°C with 0.1×SSC and 0.1% SDS.

DNA probes and labelling reactions

DNA probes from various clones were isolated as restriction fragments or as polymerase chain reaction products from agarose gels following the procedure accompanying the Geneclean kit (Bio 101, LaJolla, Calif.). The probes were random-primer labelled with α-^{32}P dCTP (Feinberg and Vogelstein 1983).

Amino-acid analyses of lines segregating for *Ask2*

Free threonine concentrations (nmol/mg dry weight) were determined for ten-kernel samples from each of 16 *Ask2-LT20* F_3 lines. Compared to B73, F_3 lines with 1–5-fold, 5–20-fold and 20–150-fold higher free threonine concentrations were classified as homozygous wild-type, heterozygous and homozygous *Ask2* mutants, respectively.

Linkage calculations and statistical analysis

The *P* values for tests of cosegregation and recombination distances between chromosome-2L pAKHSDH2, UMC55 and UMC5 (Gardiner et al. 1993) DNA markers and *Ask2* were calculated using the Linkage-1 computer program (Suiter et al. 1983). Analysis of variance from the Statview computer program was used to calculate Fisher's protected LSD on amino-acid and kernel weight data.

Results

Ask2 map location

The *Ask1-LT19* threonine-overproducing trait was previously mapped to chromosome 7S (Azevedo et al. 1990) and shown to be nonallelic to *Ask2-LT20* (Diedrick et al. 1990). To locate the *Ask2-LT20* threonine-overproducing trait in the maize genome, a cross was made to wild-type B73 and F_3 lines were derived. Free threonine concentrations were measured in bulked samples of ten F_3 kernels to determine the *Ask2* genotype of 16 individual F_2 plants. DNA-blot analysis was conducted on bulked DNA from five F_3 plants for each of the 16 F_3 lines. These blots were hybridized with a chromosome-2L marker from a cDNA

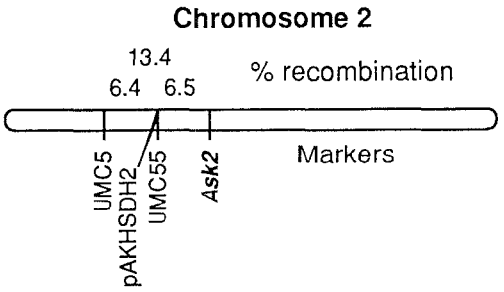

Fig. 2 Maize chromosome-2 location of *Ask2* relative to pAKHSDH2, UMC55 and UMC5. Recombination percentages were: *Ask2*/pAKHSDH2, 6.5±4.5; *Ask2*/UMC55, 6.5±4.5; *Ask2*/UMC5, 13.4±9.1; UMC5/pAKHSDH2, 6.4±6.3. *P* values for chi-square tests of linkage were all < 0.02

encoding an aspartate kinase-homoserine dehydrogenase bifunctional enzyme (pAKHSDH2) (Muehlbauer et al., manuscript submitted). Linkage analysis demonstrated that pAKHSDH2 was positioned 6.5 cM from *Ask2* (Fig. 2). Additional chromosome-2L DNA markers, UMC55 and UMC5 (Gardiner et al. 1993), were used to confirm the position of *Ask2*. UMC55 exhibited no recombination with pAKHSDH2 in this population and was positioned 6.5 cM from *Ask2*; UMC5 was positioned 13.4 cM from *Ask2*. The order of the UMC markers and pAKHSDH2 in this population were consistent with the published maize map (Gardiner et al. 1993). These data demonstrated that the threonine-overproducing trait conferred by *Ask2* is located on chromosome 2L.

Free and total amino-acid analysis

Free amino-acid concentrations were determined for homozygous *Ask1-LT19*, *Ask2-LT20* and wild-type kernels (Table 1). Free threonine concentrations increased 56- and 41-fold for *Ask1-LT19* and *Ask2-LT20*, respectively, and threonine comprised 37 and 54% of the respective total free amino-acid pools. Concentrations of free methionine increased significantly in the *Ask* mutants, resulting in 2.7% and 3.3% of the total free amino-acid content for *Ask1-LT19* and *Ask2-LT20*, respectively. Only *Ask2-LT20* showed a significant increase in free lysine concentrations, but other analyses (unpublished data) of F_3, F_4 and F_5 generations of wild-type and homozygous *Ask1-LT19* kernels also demonstrated significant increases in free lysine concentrations for *Ask1-LT19*.

Free aspartate concentrations decreased in both mutants possibly due to increased production of pathway endproducts. Free glutamate concentrations also decreased in both mutants possibly because glutamate can serve as a precurser to aspartate.

Some non-aspartate-derived amino-acids, namely serine and glycine, increased significantly in the mutants. Total free amino-acid concentrations increased two-fold in *Ask1-LT19* and *Ask2-LT20*. On a per kernel basis, total free amino-acid content increased significantly in both mu-

Table 1 Free amino-acid concentrations (nmol/mg dry weight) in kernels of wild-type, *Ask1-LT19/Ask1-LT19*, and *Ask2-LT20/Ask2-LT20* genotypes[a]

Amino-acid	A661 +/+	A661 *Ask1-LT19/ Ask1-LT19*	A619 +/+	A619 *Ask2-LT20/ Ask2-LT20*
Threonine	0.21	11.82** (56)[b]	0.43	17.73** (41)
Methionine	0.03	0.63** (21)	0.05	1.07** (21)
Lysine	0.78	0.99	0.24	0.73* (3)
Isoleucine	0.07	0.51	0.06	0.15
Aspartate	1.9	0.45**	0.55	0.14*
Asparagine	2.19	1.73	1.57	1.03
Glutamate	2.2	0.69**	1.31	0.58**
Glutamine	0.34	0.36	0.20	0.23
Serine	0.31	2.47**	0.27	3.01**
Glycine	0.16	1.3*	0.20	1.76**
Alanine	1.1	0.75	0.62	0.54
Proline/Valine	4.7	8.95**	8.67	4.88*
Arginine	0.45	0.87	0.32	0.38
Leucine	0.10	0.24*	0.30	0.13*
Phenylalanine	0.54	0.13**	ND	0.11**
Cysteine	0.08	0.10	0.28	0.13*
Histidine	0.16	0.11	0.10	0.17
Tyrosine	0.17	0.09	0.12	0.05
Total free pool	15.44	32.17** (2)	15.28	32.80** (2)
% Threonine	1.4	37.0	2.8	54.0
% Methionine	0.20	2.7	0.33	3.3
% Lysine	5.1	3.1	1.6	2.2
% Isoleucine	0.45	1.6	0.39	0.46
100 kernel weight	29.7	23.37*	28.63	22.95
Total/kernel[c]	4.6	7.1**	4.4	7.5**

[a] Means from two ears of A619 *Ask2-LT20/Ask2-LT20*; all others from three ears
[b] Parentheses indicate fold increase over wild-type
[c] nmole free amino-acids per kernel
ND, not detected
*,** Significantly different from wild type (+/+) at 5 and 1% levels of probability, respectively, according to Fisher's protected LSD

Table 2 Total (free plus protein-bound) amino-acid concentrations (nmol/mg dry weight) in kernels of wild-type, *Ask1-LT19/Ask1-LT19*, and *Ask2-LT20/Ask2-LT20* genotypes[a]

Amino-acid	A661 +/+	A661 *Ask1-LT19/ Ask1-LT19*	A619 +/+	A619 *Ask2-LT20/ Ask2-LT20*
Threonine	20.0	39.8** (2)[b]	22.6	52.4** (2)
Methionine	11.8	25.4** (2)	12.8	26.6** (2)
Lysine	16.2	20.4**	18.8	25.1**
Isoleucine	32.5	38.9**	33.4	45.6*
Aspartate + Asparagine	19.5	21.4	26.6	14.8**
Glutamate + Glutamine	64.4	81.2**	90.9	59.2**
Serine	32.4	42.4**	35.9	39.7
Glycine	41.1	51.6**	44.5	67.8**
Alanine	70.6	87.1**	77.7	84.9
Proline/Valine	125.9	153.0**	127.9	182.2**
Arginine	24.4	23.0	19.5	25.2**
Leucine	112.6	132.3*	121.3	135.2
Phenylalanine	30.5	42.0**	39.8	46.3
Cysteine	2.1	3.1*	3.1	2.5
Histidine	9.5	12.3**	12.5	13.8
Tyrosine	5.6	8.3**	11.0	5.9**
Total	618.9	782.2**	698.3	827.1*
% Threonine	3.2	5.1	3.2	6.3
% Methionine	1.9	3.2	1.8	3.2
% Lysine	2.6	2.6	2.7	3.0
% Isoleucine	5.3	5.0	4.8	5.5
100 kernel weight (g)	29.7	22.4*	28.6	23.0*
Total/kernel[c]	184	175	186	190

[a] Means from two ears of A619 *Ask2-LT20/Ask2-LT20*; all others from three ears
[b] Parentheses indicate fold increase over wild-type
[c] nmole total amino-acids per kernel
*,** Significantly different from wild-type (+/+) at 5 and 1% levels of probability, respectively, according to Fisher's protected LSD

tants. Overproduction of free threonine could account for the entire increase in total free amino-acid concentration in *Ask2-LT20* but not in *Ask1-LT19*.

Total (free plus protein-bound) amino-acid concentrations for *Ask1-LT19* and *Ask2-LT20* are presented in Table 2. Total threonine concentrations increased two-fold or greater to comprise 5.1% and 6.3% of the total amino-acid concentration in *Ask1-LT19* and *Ask2-LT20*, respectively. Protein-bound threonine (total minus free) was 70% and 66% of the total threonine pool for *Ask1-LT19* and *Ask2-LT20*, respectively (Table 3). Total methionine concentrations increased from approximately 1.8% in wild-type to 3.2% in the mutants (Table 2) but 96% was protein-bound, indicating a significant effect on protein composition (Table 3). Total lysine concentrations were increased significantly in both mutants but exhibited no increase in the percentage of total amino-acids (Table 2). However, protein-bound lysine increased significantly with 95 and 97% incorporated into protein for *Ask1-LT19* and *Ask2-LT20*, respectively (Table 3). These results demonstrated that substantial percentages of the total amino-acid concentrations of threonine, methionine and lysine in the *Ask* mutants were incorporated into protein.

Total aspartate plus asparagine concentrations decreased significantly in *Ask2-LT20* kernels but not in *Ask1-LT19*. In other analyses of wild-type and *Ask1-LT19* material, total aspartate plus asparagine concentrations also decreased significantly in two of three comparisons (unpublished data).

The total amino-acid concentrations on a dry weight basis were 26 and 18% higher in *Ask1-LT19* and *Ask2-LT20*, respectively, than in wild-type (Table 2). Free threonine overproduction in these mutants does not account for the entire increase in total amino-acid concentrations, suggesting a general increase in amino-acid biosynthesis in these mutants. However, *Ask1-LT19* and *Ask2-LT20* kernel weights were 25 and 20% lower than wild-type, respectively, resulting in approximately the same total amino-acid content per kernel for the mutants and wild-type (Table 2).

Ask1-LT19 and *Ask2-LT20* expression in different backgrounds

Homozygous *Ask1-LT19* and *Ask2-LT20* plants of A661 and A619 lines, respectively, were used as pollen sources in crosses to five wild-type inbred lines to assess the expression of heterozygous mutants in different genetic backgrounds. Crosses with wild-type A661 and A619 were analyzed as a comparison. Aspartate-derived amino-acid concentrations of kernels heterozygous for *Ask1-LT19* and *Ask2-LT20* in hybrids with A554, A661, W64A, Va26 and B37 are presented in Tables 4 and 5. Free threonine concentrations of *Ask1-LT19* increased between seven-fold (B37) to 12-fold (Va26), whereas, *Ask2-LT20* exhibited between a four-fold (B37) to 11-fold (A554) increase. In crosses with *Ask1-LT19*, free threonine concentrations were increased from a low of 8% (W64A) to a high of 21% (Va26) of the total free amino-acids. Concentrations of free threonine from *Ask2-LT20* crosses were increased from a low of 5.9% (B37) to a high of 14.3% (A554) of the total free amino-acid content. No other aspartate-derived amino-acid was consistently different in *Ask1-LT19* or *Ask2-LT20* compared to wild-type kernels. Interestingly, threonine overproduction in heterozygous *Ask* lines did not result in a significant increase in total kernel free amino-acid concentration. These results demonstrated high expressivity by both *Ask* loci and the general applicability of either mutant allele for increasing threonine production in maize kernels.

Table 3 Free, total and protein-bound[a] threonine, methionine and lysine concentrations (nmol/mg dry weight) in kernels of wild-type and *Ask1-LT19* and *Ask2-LT20*[b]

Amino-acid	A661 +/+	A661 *Ask1-LT19/ Ask1-LT19*	A619 +/+	A619 *Ask2-LT20/ Ask2-LT20*
Threonine				
Total	20.01	39.8**	22.6	52.4**
Free	0.21	11.8**	0.43	17.7**
Protein-bound	19.8	28.0*	22.1	34.7**
% Incorporated into protein	99	70	98	66
Methionine				
Total	11.82	25.4**	12.8	26.6**
Free	0.03	0.63**	0.05	1.07**
Protein-bound	11.8	24.5**	12.8	25.5**
% Incorporated into protein	99	98	99	96
Lysine				
Total	16.2	20.4**	18.8	25.1**
Free	0.78	0.99	0.24	0.73*
Protein-bound	15.4	19.4**	18.6	24.4**
% Incorporated into protein	95	95	99	97

[a] Protein-bound indicates total amino-acid concentrations minus free amino-acid concentrations
[b] Means from two ears of A619 *Ask2-LT20/Ask2-LT20*; all others from three ears
*,**Significantly different from wild-type (+/+) at 5 and 1% levels of probability, respectively, according to Fisher's protected LSD

Discussion

Overproduction of free threonine is a characteristic phenotype of maize *Ask1-LT19* and *Ask2-LT20* mutants and the corresponding mutants in barley (Bright et al. 1982 a,b; Rognes et al. 1983), tobacco (Frankard et al. 1991) and carrot (Cattoir-Reynaerts et al. 1983). *Ask* mutants encode a lysine-insensitive AK (Dotson et al. 1990), indicating that AK regulates the carbon flux into the pathway generally and into threonine specifically. For free threonine to accumulate, threonine-sensitive HSDH must either convert to a threonine-insensitive form as previously shown (Krishnaswamy and Bryan 1983 a,b; Bryan 1990 b) or the cytoplasm-localized, threonine-insensitive HSDH must play an

Table 4 Free amino-acid concentrations (nmol/mg dry weight) in kernels of heterozygous *Ask1-LT19*/+[a] and homozygous +/+[b] in different genetic backgrounds[c]

Amino-acid	A554		A661		W64A		Va26		B37	
	+/+	*Ask1-LT19*/+	+/+	*Ask1-LT19*/+	+/+	*Ask1-LT19*/+	+/+	*Ask1-LT19*/+	+/+	*Ask1-LT19*/+
Threonine	0.15	1.26** (8)[d]	0.11	0.95** (9)	0.11	0.93** (9)	0.38	4.4** (12)	0.24	1.55** (7)
Methionine	0.08	0.23	0.12	0.11	0.04	0.08	ND	ND	0.11	0.06
Lysine	0.29	0.32	0.39	0.38	0.43	0.41	2.40	2.61	0.29	0.54
Isoleucine	0.06	0.07	0.03	0.09	0.08	0.08	ND	ND	0.15	0.11
Aspartate	1.28	0.91	0.76	0.49	0.86	1.03	1.21	1.74*	1.06	0.77
Total	11.91	12.89	9.91	9.56	10.85	11.56	12.28	20.97**	14.2	13.0
% Threonine	1.3	9.8	1.1	9.9	1.0	8.0	3.1	21.0	1.7	12.0
100 kernel weight (g)	30.6	27.8	29.1	28.3	24.1	24.3	31.4	29.1	32.0	29.2

[a] *Ask1-LT19* is in inbred line A661 and was crossed to the indicated inbred line
[b] +/+ indicates A661 crossed to the indicated inbred line
[c] Means of two ears
[d] Parentheses indicate fold increase over wild-type
*,** Significantly different from wild-type (+/+) at 5 and 1% levels of probability, respectively, according to Fisher's protected LSD
ND, not detected

Table 5 Free amino-acid concentrations (nmol/mg dry weight) in heterozygous *Ask2-LT20*/+[a] and homozygous +/+[b] kernels in different genetic backgrounds[c]

Amino-acid	A554		A661		W64A		Va26		B37	
	+/+	*Ask2-LT20*/+	+/+	*Ask2-LT20*/+	+/+	*Ask2-LT20*/+	+/+	*Ask2-LT20*/+	+/+	*Ask2-LT20*/+
Threonine	0.22	2.36** (11)[d]	0.12	1.23** (10)	0.14	1.25** (9)	0.25	1.8** (7)	0.21	0.82* (4)
Methionine	0.02	0.13*	0.05	0.07	0.06	0.04	ND	ND	0.04	0.08
Lysine	0.33	0.26	0.34	0.37	0.3	0.34	1.41	1.85*	0.18	0.28
Isoleucine	0.05	0.07	0.03	0.08	0.06	0.12	ND	ND	0.20	0.09*
Aspartate	0.93	1.04	0.86	0.50*	1.0	0.76	0.69	0.48	1.0	0.99
Total	16.58	16.46	12.03	10.37	10.81	11.54	11.52	12.89	15.2	14.0
% Threonine	1.3	14.3	0.9	12.0	1.3	11.0	2.2	14.0	1.4	5.9
100 kernel weight (g)	25.3	26.2	24.5	20.2	21.8	20.45	25.60	21.65	27.3	29.0

[a] *Ask2-LT20* is in inbred line A619 and was crossed to the indicated inbred line
[b] +/+ indicates A619 crossed to the indicated inbred line
[c] Means of two ears
[d] Parentheses indicate fold increase over wild-type
*,** Significantly different from wild-type (+/+) at 5 and 1% levels of probability, respectively, according to Fisher's protected LSD
ND, not detected

important role. The potential role of AK in the regulation of amino-acid biosynthesis has been discussed previously. Giovanelli et al. (1989) proposed that AK activity levels vary in *Lemna* with no apparent difference in flux through AK, indicating a minor role for AK in the regulation of threonine, lysine, methionine and isoleucine biosynthesis. In contrast, transgenic tobacco plants containing a lysine-insensitive AK from *Escherichia coli* exhibited the threonine-overproducing phenotype, providing additional support for the hypothesis that AK is a regulatory enzyme in this pathway (Shaul and Galili 1992 a; Karchi et al. 1993). Our data also indicate that the concentration of available free aspartate may influence threonine concentration. Aspartate concentrations were reduced in homozygous *Ask* mutants, in contrast to previous studies of tobacco lysine-insensitive AK mutants (Frankard et al. 1991), suggesting that aspartate concentrations along with AK may regulate threonine concentrations in maize.

Free methionine concentrations also increased in homozygous mutant maize kernels, indicating that AK also regulates methionine biosynthesis. One form of AK is feedback-inhibited by lysine plus SAM, a metabolite synthesized from methionine (Rognes et al. 1980); therefore, alteration of this form of AK might also deregulate the control of methionine synthesis. It is not known whether either of the *Ask* loci defined by the *Ask1-LT19* and *Ask2-LT20* mutations encode the lysine-plus-SAM-regulated AK in maize. Dotson et al. (1990) proposed that lysine-sensitive AK is a heterotetramer composed of subunits from *Ask1* and *Ask2*. Similarity in methionine concentrations between *Ask1-LT19* and *Ask2-LT20* (Tables 1 and 2) suggests that relaxation of lysine-sensitive AK also relaxes the AK isoform sensitive to lysine plus SAM. The fact that free methionine concentrations are not equal to free threonine, however, suggests either a higher metabolic demand for the methionine pool or further regulation by another enzyme in the pathway, possibly cystathionine synthase. Another possibility for lower methionine accumulation compared to threonine could be that threonine synthase is stimulated by SAM (Madison and Thompson 1976; Thoen et al. 1978), preferentially routing carbon into threonine production.

Free lysine concentrations were increased significantly in the homozygous mutants but at considerably lower concentrations than for free threonine. Dihydrodipicolinate synthase (DHPS) is feedback-inhibited by low concentrations of lysine (Ghislain et al. 1990; Frisch et al. 1991) which provides a lysine branch-specific regulation of lysine production. Additional support for the role of DHPS in lysine regulation was obtained from a lysine-overproducing tobacco mutant that contained a lysine-insensitive DHPS (Negrutiu et al. 1984). Moreover, transformation of tobacco and potato with a lysine-insensitive DHPS from *E. coli* also resulted in an increase in free lysine (Perl et al. 1992; Shaul and Galili 1992 b). A tobacco double mutant with lysine-insensitive AK and lysine-insensitive DHPS resulted in overproduction of lysine (Frankard et al. 1992). Our data and the available literature indicate that although AK regulates the carbon flux into the pathway, DHPS specifically regulates the biosynthesis of lysine. This model will become increasingly important as strategies for engineering maize for increased lysine production are designed. Transgenic maize plants containing a lysine-insensitive DHPS might need to be coupled with an *Ask* mutant or a deregulated AK transgene to obtain a relaxed AK and, theoretically, more carbon available for lysine biosynthesis.

Overproduction of free threonine in homozygous *Ask* mutant kernels ranged from 41- to 56-fold. This large increase in free threonine is generally associated with 100 to 150% increases in total threonine

of which approximately 70% was protein-bound threonine. In contrast, higher proportions of total lysine (87 and 97%) and total methionine (greater than 96%) in *Ask1-LT19* and *Ask2-LT20* were incorporated into protein. These data indicate that *Ask* maize kernels have substantial protein sinks for free lysine, threonine and methionine. Therefore, it may be of interest to examine *Ask1-LT19* and *Ask2-LT20* mutations in genetic backgrounds that contain altered kernel protein profiles to investigate the relationships between free pool amino-acids and protein sinks. The combination of *Ask1-LT19* with opaque-2 resulted in elevated free amino-acid concentrations and decreased zein synthesis beyond that of *opaque-2* alone (Azevedo et al. 1990), but the implications of this interaction are not understood.

Analyses of embryos and endosperm tissue separated from wild-type, *Ask1-LT19* and *Ask2-LT20* kernels showed higher free amino-acid concentrations in embryos of all genotypes (unpublished data), suggesting that amino-acid biosynthesis may be higher in embryos compared to endosperm tissue. It would be of considerable interest to examine the fate of free amino-acids in the embryo and endosperm by determining whether specific proteins increase or decrease in these tissues in concert with changes in total amino-acid concentrations.

Decreased kernel weight or plant vigor is characteristic of the maize *Ask* mutants (Table 1), the corresponding tobacco mutants (Frankard et al. 1991), and transgenic tobacco plants containing a lysine-insensitive *E. coli* AK (Shaul and Galili 1992 a). Reduction in kernel weight may be the result of the drastic changes in amino-acid production or an altered kinetic mechanism of the deregulated AK. Both *Ask* mutants increase total amino-acid concentrations on a dry weight basis; however, total amino-acid content per kernel is approximately the same as for wild-type kernels. These data may indicate an upper limit on kernel amino-acid concentrations beyond which dry matter accumulation is impaired. It is interesting to note that development of transgenic tobacco plants was normal when they expressed a seed-specific, deregulated AK that conferred threonine-overproduction only in the seed (Karchi et al. 1993). This result suggests that altered amino-acid production throughout the plant may cause poor seed development.

The data in this study demonstrated that single genes, *Ask1* and *Ask2*, play an important regulatory role in aspartate-derived amino-acid biosynthesis. In a related study of F_3, F_4 and F_5 lines, the threonine-overproducing *Ask1-LT19* trait was shown to be stable over three generations of selfing and in different field environments (unpublished results), suggesting that *Ask1-LT19* and *Ask2-LT20* could be used in breeding programs. Locating *Ask1* on chromosome 7S (Azevedo et al. 1990) and *Ask2* on chromosome 2L (this study) will facilitate marker-assisted incorporation of one or both genes controlling the threonine-overproducing trait into elite maize lines.

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