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ASPARTATE KINASE IN THE MAIZE MUTANTS *ASK1-LT19* AND *OPAQUE-2*

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Key Word Index—*Zea mays*, Gramineae; maize; aspartate kinase; lysine; threonine; mutants.

Abstract—The A*sk1*-LT19 mutant of maize, which contains a lysine insensitive aspartate kinase was repeatedly backcrossed to the mutant *opaque-2* and its wild type counterpart, the inbred line Cat100-1. Aspartate kinase was partially purified and characterized from seedlings, anthers and 20- to 25-day old endosperm tissue isolated from the wild type and *Ask1*-LT19 mutant. In addition, aspartate kinase was also characterized from 20- to 25-day old endosperms isolated from the *opaque-2* mutant and the double mutant *Ask1*-LT19/*opaque-2*. No major variation was detected in the sensitivity of aspartate kinase to feedback inhibition by threonine in the three tissues of all the genotypes tested. On the other hand, there was clear evidence of a reduction in sensitivity of aspartate kinase to inhibition by lysine, when the wild type was compared with the *Ask1*-LT19 mutant. The reductions in lysine sensitivity in the *Ask1*-LT19 seedlings, anthers and endosperm were 37, 41 and 25%, respectively. When the *opaque-2* mutant was compared with the double mutant *Ask1*-LT19/*opaque-2*, there was a 54% reduction in the sensitivity of the double mutant endosperm aspartate kinase to lysine. The data suggest that the gene encoding the lysine-sensitive aspartate kinase (*Ask*1) may be regulated by the *opaque*-2 mutation in maize.

INTRODUCTION

The seeds of cereals are nutritionally deficient in certain amino acids. The goal of improving the nutritive value of cereals and elucidating the molecular mechanisms that regulate the synthesis of these amino acids has led to an examination of a range of genotypes and mutants for altered regulatory mechanisms. Aspartic acid serves as a common precursor of several nutritionally essential amino acids, including lysine, threonine, methionine and isoleucine, with most of the enzymes required for their synthesis being localized in the chloroplasts of higher plants [1]. Multiple forms of the enzymes that catalyse identical chemical reactions have been identified, purified and characterized in many organisms [2–7]. These isoenzymes, which exhibit differences in their regulatory or catalytic properties, are of particular interest due to their potential involvement in the regulation of specific pathways. Aspartate kinase (EC 2.7.2.4) is the first enzyme of the aspartic acid metabolic pathway. Different isoenzymic forms of the enzyme showing inhibition by lysine, threonine and lysine plus *S*-adenosylmethionine have been identified in higher plants [4, 6–9]. In maize cell cultures, the threonine-sensitive aspartate kinase isoenzyme and the threonine-sensitive homoserine dehydrogenase (EC 1.1.1.3) co-purified [10], and a bifunctional protein showing both aspartate kinase and homoserine dehydrogenase activities, have been observed in carrot [11], soybean [12], *Arabidopsis thaliana* [13] and maize [14].

The growth of cell cultures and seedlings of many plants can be inhibited by the combined action of amino acid end products, or their analogues, due to feedback inhibition at one or more steps in the aspartic acid metabolic pathway [15, 16]. Mutants showing resistance to the inhibition caused by lysine and threonine have been shown to accumulate high concentrations of soluble threonine [17], whereas mutants resistant to *S*-2-aminoethyl-L-cysteine do not always lead to the overproduction of lysine [16, 18]. In barley, lysine plus threonine resistant mutants, contained altered forms of aspartate kinase isoenzymes, that were less sensitive to lysine feedback inhibition [19, 20]. In *Nicotiana sylvestris*, one lysine plus threonine-resistant mutant exhibited overproduction of soluble threonine in leaves and seeds associated with an aspartate kinase less sensitive to feedback inhibition by lysine [21]. In maize mutants resistant to lysine plus threonine inhibition [17, 22], isoenzymes of aspartate kinase extracted from ears and cell cultures have been shown to be altered in their sensitivity to lysine [23]. The maize mutant used in this study was originally

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designated as *Ltr*1* [15, 17]. It has since been renamed as *Ask1*-LT19 and has been shown to contain a lysine-insensitive aspartate kinase due to a mutation in the *Ask1* gene [23]. The *Ask1* gene, which is the structural gene for one of the aspartate kinase isoenzymes sensitive to lysine in maize, has been mapped on the short arm of chromosome 7 at 10.6 centimorgans from the *opaque-2* gene and shown to be regulated by the *opaque-2* gene, when soluble and total amino acids were analysed [24]. Furthermore, it was observed that the *Ask1*-LT19 mutation intensified the effects of the *opaque-2* mutation on storage protein synthesis in maize endosperm [24].

The *opaque-2* mutation is regulated by a well characterized endosperm gene, which in its recessive form (*o2o2*) reduced the zein content of the endosperm by up to 70% [25, 26]. The reduction of zein synthesis in the *opaque-2* endosperm caused an accumulation of the soluble protein fraction including albumins, globulins, glutelins and soluble amino acids, which led to an increase in the total level of lysine [27, 28]. Furthermore, the activity of lysine-ketoglutarate reductase, the first enzyme in the catabolism of lysine, was greatly reduced in activity in the endosperm tissue of *opaque-2* [29, 30] and may have been under the control of the *opaque-2* gene. Therefore, we have used the *opaque-2* mutant to examine the effect of combination with the *Ask1*-LT19 mutant on the regulation of aspartate kinase activity.

In the present paper, we have determined the distribution of aspartate kinase activity during endosperm development. The enzyme has been partially purified and the feedback regulation by lysine and threonine examined in seedlings, anthers and endosperms of wild type (*Ask1*Ask*1*/*O2O2*), *Ask1*-LT19 (*ASK1ASK1*/*O2O2*), *opaque-2* (*Ask1*Ask*1*/*o2o2*) single mutants and the double mutant *Ask1*-LT19/*opaque-2* (*ASK1ASK1*/*o2o2*).

RESULTS

Aspartate kinase was extracted from developing seeds, and a peak of maximum activity was detected *ca* 20–25 days after pollination for the inbred line Cat100-1. Both threonine and lysine alone, or in combination, inhibited the activity of the enzyme, with lysine showing the highest inhibitory action (Fig. 1). Developing seeds, 22 days after pollination, were selected for aspartate kinase extraction for the genotypes tested, since the activity was at the highest level. Aspartate kinase activity was also determined in endosperm and scutellum separately, where the activity detected in the scutellum was 90% higher than that observed in endosperm (Table 1). The levels of inhibition of aspartate kinase activity caused by threonine and lysine were slightly different for each tissue. The inhibition by threonine was higher in the scutellum (32%), than in endosperm (21%), while lysine inhibition was higher in the endosperm (53%) than in the scutellum (47%).

The extraction of soluble amino acids from anther spikelets and their identification by TLC, confirmed the presence of the *Ask1*-LT19 mutation in the homozygous dominant form (*ASK1ASK1*, which accumulates

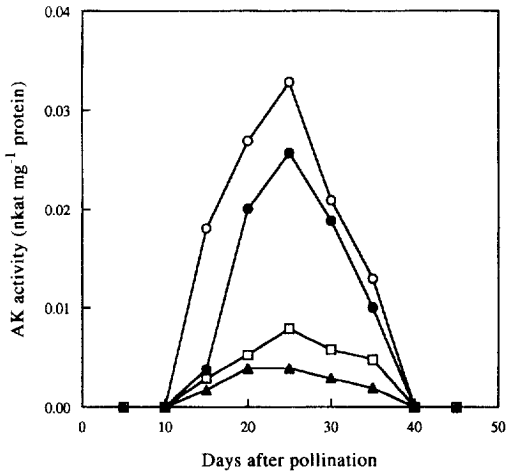

Fig. 1. Distribution of aspartate kinase activity during the development of maize endosperm. Asparate kinase activity (○), aspartate kinase activity in the presence of 5 mM lysine (□), 5 mM threonine (●) and 5 mM lysine plus 5 mM threonine (▲).

threonine and contains a lysine-insensitive aspartate kinase), and recessive form (*Ask1*Ask*1*, which does not accumulate threonine and contains a lysine-sensitive aspartate kinase). The single mutant *Ask1*-LT19 (*ASK1ASK1*/*O2O2*) accumulates threonine and has a normal endosperm. The wild type (*Ask1*Ask*1*/*O2O2*) does not accumulate threonine and has a normal endosperm. The double mutant *Ask1*-LT19/*opaque-2* (*ASK1ASK1*/*o2o2*) accumulates threonine and has an *opaque-2* endosperm. The single mutant *opaque-2* (*Ask1*Ask*1*/*o2o2*) does not accumulate threonine and has an *opaque-2* endosperm.

Table 2 shows the results obtained in seedlings when aspartate kinase activity was determined in the wild type and in the *Ask1*-LT19 mutant. The activity of aspartate kinase was only slightly higher in the single mutant than in the wild type seedlings, but variation in the inhibition caused by lysine was observed. Lysine (5 mM) caused a 65% inhibition of aspartate kinase activity in the wild type, while the same concentration of lysine only inhibited aspartate kinase activity by 41% in the *Ask1*-LT19 mutant. Threonine, on the other hand, did not show any variation, causing identical levels of inhibition of aspartate kinase activity in the two genotypes tested. The combined action of lysine and threonine showed a clear additive effect of inhibition on aspartate kinase activity. In the *Ask1*-LT19 mutant, the addition of lysine and threonine together did not produce as strong an inhibition of aspartate kinase activity as observed for the wild type.

The results obtained for aspartate kinase isolated from anthers (Table 3) were very similar to those obtained for aspartate kinase extracted from seedlings. However, the specific activity of aspartate kinase was lower in anthers than in seedlings. Aspartate kinase extracted from endosperm tissue showed different pattern of inhibition by lysine and threonine (Table 4). In the endosperm tissue,

Table 1. The effect of lysine and threonine on aspartate kinase activity extracted from wild type maize (Cat100-1)

	Tissue			
	Endosperm		Scutellum	
Treatment	Activity*	Inhibition (%)	Activity*	Inhibition (%)
Control	0.028	—	0.053	—
+ 5 mM threonine	0.022	21	0.036	32
+ 5 mM lysine	0.013	53	0.028	47
+ 5 mM lysine + 5 mM threonine	0.009	86	0.010	81

*nkat mg^{-1} protein.

Table 2. The effect of lysine and threonine on aspartate kinase activity isolated from seedlings of wild type maize (Cat100-1) and the *Ask1*-LT19 mutant

	Genotypes			
	Wild type *Ask1Ask1/O2O2*		*Ask1*-LT19 *ASK1ASK1/O2O2*	
Treatment	Activity*	Inhibition (%)	Activity*	Inhibition (%)
Control	0.0141	—	0.0153	—
+ 5 mM threonine	0.0097	31	0.0109	29
+ 5 mM lysine	0.0047	65	0.0090	41
+ 5 mM lysine + 5 mM threonine	0.0015	89	0.0067	56

*nkat mg^{-1} protein.

Table 3. The effect of lysine and threonine on aspartate kinase activity isolated from anthers of wild type maize (Cat100-1) and the *Ask1*-LT19 mutant

	Genotypes			
	Wild type *Ask1Ask1/O2O2*		*Ask1*-LT19 *ASK1ASK1/O2O2*	
Treatment	Activity*	Inhibition (%)	Activity*	Inhibition (%)
Control	0.0111	—	0.0124	—
+ 5 mM threonine	0.0074	33	0.0087	30
+ 5 mM lysine	0.0038	66	0.0075	39
+ 5 mM lysine + 5 mM threonine	0.0010	91	0.0050	60

*nkat mg^{-1} protein.

threonine inhibition was reduced to *ca* 20%, which was lower than the inhibition observed in seedlings and anthers.

The inhibition by lysine, on the other hand, was reduced by 25% in the endosperm of the single mutant *Ask1*-LT19 in relation to the wild type and by 54% in the endosperm of the double mutant in relation to the single *opaque-2* mutant.

DISCUSSION

The extraction of high concentrations of soluble threonine from anther spikelets, identified by TLC separation, has proved to be a reliable indicator of the presence of the *Ask1*-LT19 mutation, since the overproduction of soluble threonine is the main effect of the altered aspartate kinase on amino acid metabolism [24].

Table 4. The effect of lysine and threonine on aspartate kinase activity isolated from the endosperm of wild type maize (Cat100-1), the *Ask1*-LT19 mutant, the *opaque-2* mutant and the double mutant

Treatment	Genotypes							
	Wild type *Ask1Ask1/O2O2*		*Ask1*-LT19 *ASK1ASK1/O2O2*		*Opaque-2* *Ask1Ask1/o2o2*		Double mutant *ASK1ASK1/o2o2*	
	Activity*	Inhibition (%)	Activity*	Inhibition (%)	Activity*	Inhibition (%)	Activity*	Inhibition (%)
Control	0.0312	0	0.0331	0	0.0265	—	0.0305	—
+ 5 mM threonine	0.0247	21	0.0257	22	0.0239	10	0.0237	22
+ 5 mM lysine	0.0171	45	0.0219	34	0.0106	60	0.0220	28
+ 5 mM lysine + 5 mM threonine	0.0091	71	0.0146	56	0.0074	72	0.0158	48

*nkat mg^{-1} protein.

The results obtained in seedlings, anthers and endosperms showed that lysine was the major inhibitor of aspartate kinase activity and confirmed the results reported previously by Dotson *et al.* [23], who analysed the effect of two mutations, in the *Ask1* and *Ask2* genes, on aspartate kinase isolated and purified from immature maize ears. Dotson *et al.* [23] analysed the *Ask1*-LT19 and *Ask2*-LT20 mutations in the heterozygous form, which made analysis difficult, since lysine-sensitive and insensitive forms of aspartate kinase were present. Moreover, the genetic background and the tissue used were different, which might have altered the effect of the end product amino acids on enzyme activity. The reduced inhibitory effect of lysine on aspartate kinase activity confirmed that the *Ask1* gene encodes one of the isoenzymes of lysine-sensitive aspartate kinase in maize. Although Dotson *et al.* [23] were not able to identify a threonine-sensitive aspartate kinase, the data presented in this paper indicate that the inhibition caused by threonine was not altered in all tissues tested and was thus independent of the presence of a mutation in the *Ask1* gene. Other genes must, therefore, be responsible for the threonine-sensitive aspartate kinase in maize.

The additive effect on the inhibition of aspartate kinase by lysine and threonine confirmed the results obtained in previous reports for maize aspartate kinase [7, 10]. The reduced inhibition by lysine and threonine observed in the mutant *Ask1*-LT19 was due to a reduced sensitivity of the lysine-sensitive aspartate kinase. Of particular interest is the extent of the reduced inhibitory effect of lysine in the double mutant (*ASK1ASK1/o2o2*). This result indicates that the lysine-sensitive aspartate kinase isoenzyme coded by the *Ask1* gene may be regulated by the *opaque-2* gene. This suggestion is also supported by the effect of lysine on aspartate kinase from the endosperm of the single mutant *Ask1*-LT19 (*ASK1ASK1/O2O2*), in which the reduction of lysine inhibition with the introduction of the mutated *Ask1* gene was not as strong (Table 4). These results support, at the enzyme level, the interaction between the *Ask1* and *opaque-2* genes that was previously proposed, based on the regulatory effect of the *Ask1*-LT19 mutation by the *opaque-2* mutation, when amino acids (soluble threonine and total soluble amino acids) and storage protein were analysed [24]. Moreover, the *Ask1* gene was mapped on chromosome 7 linked to the *opaque-2* gene and possibly linked to zein polypeptides, which are regulated by the *opaque-2* gene [24].

EXPERIMENTAL

Plant material. The maize inbred line Cat100-1 of the maize collection of the Departamento de Genética, Universidade Estadual de Campinas and the *opaque-2* mutant were donated by Dr P. Arruda. The *Ask1*-LT19 mutant (previously designated *Ltr***1*) introduced in the inbred line Cat100-1 was kindly donated by Dr K. A. Hibberd.

Identification of aspartate kinase activity during seed development Cat100-1 maize plants were self-pollinated

and maize ears collected every 5 days and used for aspartate kinase extraction. Endosperm and scutellum tissues were sepd and both used for aspartate kinase extraction.

Identification of the Ask1 gene. The *Ask1*-LT19 mutant was backcrossed to near isogenic condition to the maize mutant *opaque-2* of the inbred line Cat100-1 and its wild type counterpart. Single and double mutants were selected on the basis of endosperm phenotype and accumulation of soluble threonine as detected by TLC. Seeds from maize ears containing the two single mutations, the double mutant and the wild type were planted and grown to maturity. Prior to pollination, the presence of the *Ask1*-LT19 mutation was detected by the overproduction of soluble threonine in anther spikelets.

Soluble threonine extraction and TLC. Anther spikelets were collected from each plant before anthesis and used for threonine extraction. One anther spikelet from each plant was homogenized with 30 μl distilled–deionized H_2O in 0.5 ml microfuge tubes using a glass rod. The homogenates were centrifuged in a microcentrifuge at 16000 rpm and the supernatant used for amino acid analysis. Aliquots of 5 μl were applied to cellulose TLC plates. The chromatograms were developed in *n*-BuOH–Me_2CO–NH_4OH (33%, w/v)–H_2O (5:5:2.5:1) for 90 min. After sepn of the amino acids, the plates were dried for 3 days at room temp. and sprayed with 0.2% (w/v) ninhydrin in Me_2CO. Threonine (1%, w/v) soln in H_2O was used as a standard to identify the amino acid. Anthers and immature endosperms (30 days after pollination) for each genotype were harvested and kept at $-80°$ for further analysis. Mature seeds were planted and grown at 24° in a 16:8 hr light:dark period for 10 days and watered at 48 hr intervals. At the end of this period (seeds normally showing 5 leaves), leaves were harvested and fr. wt recorded. The leaf samples were immediately frozen in liquid N_2 and used for aspartate kinase extraction.

Enzyme extraction. All procedures were carried out at 4° unless state otherwise. Aspartate kinase was extracted and partially purified from 10 g tissue for seedlings, anthers, immature endosperms and scutellum. Samples were ground with liquid N_2 in an ice-cold pestle and mortar with 5:1 (v/w) extraction buffer [50 mM Tris–HCl (pH 7.4) with 50 mM KCl, 2 mM lysine, 2 mM threonine, 1mM DTT, 0.1 mM PMSF, 15% (v/v) glycerol and 5% (v/w) insoluble PVP]. The extract was filtered through several layers of miracloth (Calbiochem) and the filtrate was centrifuged at 10000 rpm for 30 min. $(NH_4)_2SO_4$ (35–60% satn) was added to the supernatant and the protein pellet collected by centrifugation at 16000 rpm for 30 min. Pellets were resuspended and desalted on Sephadex G25 columns (1.8 × 10 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.4) containing 50 mM KCl, 0.1 mM lysine, 0.1 mM threonine and 10% (v/v) glycerol.

Aspartate kinase assay. The hydroxamate assay described in ref. [31] was used with some modifications. The assay was performed in 1.5 ml microfuge tubes and the assay mixt. comprised: 100 μl 250 mM aspartic acid (pH 7.4); 100 μl 100 mM Tris–HCl (pH 7.4) containing 1 mM DTT and 20% (v/v) glycerol; 50 μl 125 mM $MgSO_4$; 50 μl 200 mM ATP (pH 7.4); 50 μl 4 M hydroxylamine (pH 7.4), and 50 μl H_2O. The assay was started by the addition of 100 μl enzyme, incubated at 35° for 1 hr and terminated by addition of 500 μl of 0.67 M $FeCl_3$ containing 0.37 M HCl and 20% (w/v) TCA.

The microfuge tubes were centrifuged for 5 min at 16000 rpm to remove pptd protein and the *A* of the supernatant read at 505 nm. The extinction coefficient for DL-aspartic acid hydroxamate at 505 nm was 750 $cm^{-1} M^{-1}$. The assay solns were read against a blank containing all the components minus the substrate. Lysine (10 mM) and threonine (10 mM) were added substituting the H_2O component of the assay.

Protein determination. Protein was estimated by the dye-binding method of ref. [32] supplied as a kit by Bio-Rad. BSA was used as standard.

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