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Regulation of Aspartate Kinase Isoenzymes in Barley Mutants Resistant to Lysine plus Threonine[1]

CONSTRUCTION AND ANALYSIS OF COMBINATIONS OF THE *Lt1a*, *Lt1b*, AND *Lt2* MUTANT GENES

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PAULO ARRUDA[2], SIMON W. J. BRIGHT*, JOSEPH S. H. KUEH, PETER J. LEA, AND SVEN E. ROGNES[3]
Biochemistry Department, Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ, United Kingdom

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ABSTRACT

Two homozygous mutant lines of barley (*Hordeum vulgare* L.) R3202 (*Lt1b*/*Lt1b*) and R3004 (*Lt2*/*Lt2*), are resistant to lysine plus threonine. They contain aspartate kinase isoenzymes with lost or decreased feedback sensitivity to lysine in either isoenzyme AKII (R3202) or isoenzyme AKIII (R3004). A homozygous double mutant line (*Lt1b*/*Lt1b*, *Lt2*/*Lt2*) has now been constructed that grows vigorously on 8 millimolar lysine, 8 millimolar threonine, and 1 millimolar arginine. Both AKII and AKIII from the double mutant have altered lysine sensitivities, identical to those previously observed in R3202 and R3004, respectively. Aspartate kinase activity in extracts of leaves, roots, and the maturing endosperm of the double mutant was much less sensitive to lysine inhibition than the enzyme in comparable extracts of the parent cv Bomi, suggesting that aspartate kinase is expressed in a similar manner in different tissues of barley.

A further mutant, R2501, resistant to lysine plus threonine has now given rise to a homozygous line (*Lt1a*/*Lt1a*), which had previously not been possible. AKII isolated from the homozygous line was completely insensitive to 10 millimolar lysine; however, the combined action of 10 millimolar lysine and 0.8 millimolar *S*-adenosylmethionine inhibited it by 60%, demonstrating the retention of some of the regulatory characteristics of the wild type enzyme.

Mutants of higher plants resistant to the toxic combination of lysine plus threonine have been identified in barley (3, 4, 11, 12), maize (8), tobacco (1), and carrot (7). A number of resistant cell lines of various plant species have also been identified. Such mutants may accumulate threonine in the soluble amino acid fraction of leaves or seeds in nutritionally significant amounts (3, 4, 7, 8). Where an enzymic basis for the mutant phenotype has been established, AK[4] (EC 2.7.2.4), the first enzyme in the branched pathway of lysine, threonine and methionine biosynthesis, has been found to have all or part of its activity insensitive to feedback regulation by lysine (4, 7, 9, 11, 12). In barley, three mutants with dominant mutations at either of the two unlinked loci *lt1* (mutant R2501 contains gene *Lt1a*; mutant R3202, gene *Lt1b*) or *lt2* (mutant R3004, gene *Lt2*) have been identified (3, 4). These mutants have been used to demonstrate the presence of three putative isoenzymes of AK designated AKI, AKII, and AKIII in order of elution from an ion exchange column (4, 12). The AKI activity is a minor, threonine-sensitive component (up to 18%) of the total activity. The AKII and AKIII isoenzymes have similar properties in wild type barley, both being inhibited by lysine or lysine plus AdoMet (4, 12, 13).

We have now crossed the mutants R3004 and R3202 and identified the double mutant pure line (*Lt1b*/*Lt1b*, *Lt2*/*Lt2*). We present here the enzymic characterization of both this and the doubly heterozygous F_1 (*Lt1b*/+, *Lt2*/+). In addition, the mutant R2501 was previously isolated as a heterozygous individual and homozygous plants were not viable (4). By backcrossing to wild type plants we have now produced a homozygous line and we present an enzymic comparison of the heterozygous and homozygous plants.

MATERIALS AND METHODS

Plant Material. The derivation of the barley (*Hordeum vulgare* L.) mutants and their mutant gene designations R2501 (*Lt1a*), R3202 (*Lt1b*), and R3004 (*Lt2*) in the background of cv Bomi has been described (3, 4). R3004, R3202, and Bomi were maintained as pure breeding, self-fertilizing lines in a glasshouse. Resistant plants derived from the originally selected R2501 plant were normally heterozygous. Plants were crossed among themselves or to the normal cultivar Golden Promise in a glasshouse as described (3). Mature embryos from seed were excised, dried, surface sterilized, and grown on agar-solidified basal medium with various amino acid additions under lights as previously described (5). Growth was measured as the length of the shoot and the longest root. Plants were grown for initial enzyme assays in a standard potting compost in a growth chamber (20°/16°C, 12 h day with lights 450 $\mu E\ m^{-2}\ s^{-1}$ from mixed fluorescent and tungsten bulbs) for 15 to 20 d until the emergence of the first tiller. After removal of the main stem into liquid nitrogen for enzyme assay, the plants were grown to maturity in the glasshouse.

Aspartate Kinase Assay and Separation of Isoenzymes. The extraction, assay, and separation of the isoenzymes of barley AK were carried out essentially as previously described (12). Leaves were ground in extraction buffer A (25 mM K-phosphate [pH 7.5]; 2 mM $MgCl_2$; 2 mM EDTA; 15% [v/v] glycerol and 0.2%

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[2] Present address: Depto. Genetica e Evolucao, Instituto de Biologia, Universidade Estadual de Campinas, 13100 Campinas S.P., Brazil.

[3] Present address: Department of Botany, P.O. Box 1045, University of Oslo, Blindern, Oslo 3, Norway.

[4] Abbreviations: AK, aspartate kinase; AdoMet, *S*-adenosyl-L-methionine.

[v/v] 2-mercaptoethanol) plus 50 mM KCl at a ratio of 3 ml g^{-1} fresh weight. Solid $(NH_4)_2SO_4$ was added to the centrifuged extracts to give 65% saturation. The precipitated protein was collected by centrifugation, redissolved in buffer A plus 50 mM KCl, and passed over Sephadex G-75. The desalted extracts were applied in buffer A plus 50 mM KCl to 1 × 11 cm columns of DEAE-Sephacel and the columns were washed with 30 ml of buffer A plus 75 mM KCl. AKII was first eluted with 15 ml of buffer A plus 120 mM KCl and AKIII was then eluted with 20 ml buffer A plus 220 mM KCl. The feedback sensitivity of the two isoenzymes was determined using pooled fractions from each peak.

Crude extracts of roots and shoots were prepared by extracting 50 7-d-old seedlings (weights as shown in Table IV) in 20 ml of buffer A plus 50 mM KCl followed by two further extractions of 10 ml each. Combined extracts were treated with $(NH_4)_2SO_4$ and desalted on Sephadex G-75 as described above. Immature seeds ranging from 20 to 28 d after anthesis were collected from glasshouse grown plants and stored in liquid nitrogen. Endosperm material (weight as shown in Table IV) was extracted in 200 ml of buffer A plus 50 mM KCl and treated as described for roots and shoots.

AK was assayed by the production of radioactive aspartyl hydroxamate from aspartic acid (12, 13). Reaction mixtures (0.1 ml, pH 7.5) contained 12 mM L-[U-^{14}C]aspartic acid (26 MBq $mmol^{-1}$); 18 mM $MgCl_2$; 15 mM ATP; 400 mM NH_2OH-HCl adjusted to pH 7.5 with KOH; 1 mM DTT; 10 to 100 µg enzyme protein. Labeled aspartic acid was purified over a Dowex 1-X8 column before use. Incubations were for 1 to 2 h at 30°C. Labeled aspartyl hydroxamate was separated by high-voltage paper electrophoresis at pH 5.0.

Protein Estimation. Protein was assayed by the method of Bradford (2) using BSA as a standard.

RESULTS

Production, Identification, and Growth Characteristics of a Double Mutant. The mutants R3202 and R3004 were crossed together as male and female parents and F_1 seeds were either grown to produce the F_2 generation, or used to produce F_1 plants from embryos for enzyme assay. The segregation of resistance to lysine plus threonine in the selfed F_2 generation was examined. In basal medium plus 2 mM lysine and 2 mM threonine, there was a ratio of 174 resistant to 10 sensitive plants. It was not possible unequivocally to identify double mutant plants, as the growth of embryos of the single mutant R3202 in these conditions was 80% of growth in the absence of lysine and threonine. However, the 12 largest plants from this screen were retained and self-fertilized for progeny analysis. Lysine alone can inhibit growth by interference with arginine metabolism (6, 10), so F_2 progeny of the same cross were tested in basal medium containing 8 mM lysine, 8 mM threonine, and 1 mM arginine. In this treatment, a number of different phenotypes were distinguished (Fig. 1). Wild type (sensitive) plants possessed short roots, dark pigmentation of the scutellum and leaf base and short shoots; R3004 types also had short roots but had pale scutella and longer shoots; R3202 types had long roots, pale scutella, and long, green shoots. Finally, there were a few plants that grew better than the R3202 controls (Table I).

Selfed progeny seedlings from 10 F_2 plants selected for good growth in the presence of 2 mM lysine plus 2 mM threonine were grown in pots until the emergence of the first tiller. The main shoots of individual plants were then harvested, extracted, and assayed for aspartate kinase activity, in crude extracts and after separation of the AKII and AKIII isoenzymes. One plant possessed AK activity with the characteristic insensitivity to feedback inhibition by lysine of both R3202 and R3004 (Table II). The remaining portions of this plant were grown to maturity in a glasshouse and selfed progeny embryos tested for growth in the presence of 8 mM lysine, 8 mM threonine, and 1 mM arginine (Table III). The double mutant plants had uniformly long roots and superior shoot growth to both R3004 and R3202 plants.

Characterization of Aspartate Kinase from the Double Mutant R3004 × R3202. The two lysine-sensitive isoenzymes (AKII and AKIII) were well separated under the conditions used (Fig. 2). A comparison of the lysine sensitivity of AKII from the wild type, the two single mutants (R3004 and R3202), the F_1 (R3004 × R3202), and the double mutant is shown in Figure 3. AKII from R3004 had normal, wild type, sensitivity to lysine and AKII from R3202 was completely insensitive to feedback inhibition as found previously (12). The double mutant also had a completely lysine-insensitive AKII. The isoenzyme from the F_1 showed an intermediate degree of inhibition upon the addition of lysine. Addition of AdoMet to AKII from R3202 (12) or the double mutant did not increase the inhibition at 10 mM lysine.

The AKIII isoenzyme isolated from R3202 showed a similar sensitivity to lysine as the wild type (Fig. 4). AKIII from R3004 was inhibited 20% by 0.6 mM lysine, but over 50% of the activity remained in the presence of 10 mM lysine. The double mutant isoenzyme was slightly more sensitive to lysine than that of R3004; and AKIII from the F_1 plant was still a little more inhibited by lysine. AKIII from all sources was strongly inhibited by the combined action of 10 mM lysine and 0.8 mM AdoMet.

AK activity was detected in the roots, leaves, and developing endosperm of cv Bomi and the double mutant (Table IV). The specific activity of the enzyme on a protein basis was highest in shoots and roots and lower in the endosperm samples. When calculated on the basis of fresh weight, shoots had a much higher relative activity. It was not possible to separate out sufficient

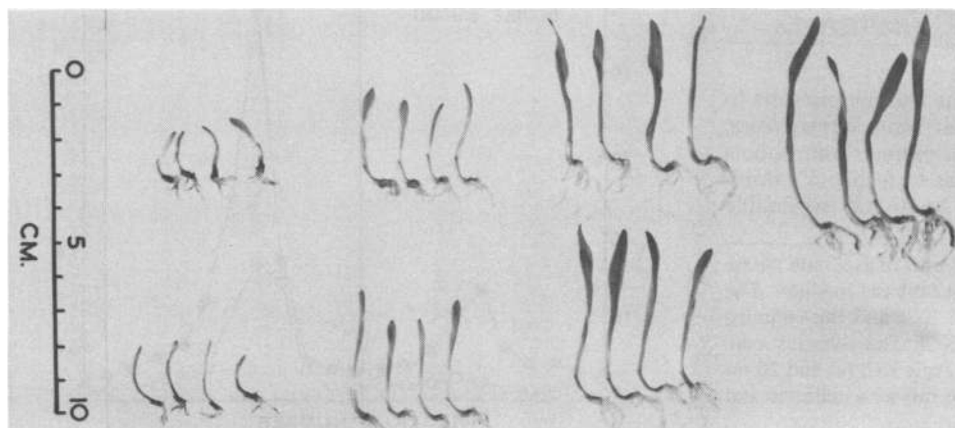

FIG. 1. Growth of isolated 7-d-old F_2 barley plants obtained from crossing the two mutant lines R3202 and R3004. Embryos were germinated and grown on a medium containing 8 mM lysine, 8 mM threonine, and 1 mM arginine. F_2 plants (upper row) were classified by comparison with plants of known genotype (lower row left, wild type; middle, R3004; right, R3202).

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Table I. *Segregation of Plants in the F_2 Generation of the Cross R3202 × R3004 into Four Phenotypes when Grown on Medium Containing 8 mM Lysine, 8 mM Threonine, and 1 mM Arginine.*

Pooled results from F_2 progeny taken from five separate F_1 plants, (three with R3004 as female parent and two with R3202 as female parent). Plants were classified as wild type, R3004, R3202 types by comparison with plants of known genotype grown at the same time. Plants growing better than R3202 were scored as >R3202.

Growth Phenotype	No. of Plants		Suggested Genotype at	
	Observed	Expected	*lt1*	*lt2*
Wild type	10	8.5	+/+	+/+
R3004 type	27	25.5	+/+	*Lt2*/+, *Lt2*/*Lt2*
R3202 type	91	93.5	*Lt1b*/+	+/+, *Lt2*/+, *Lt2*/*Lt2*
			Lt1b/*Lt1b*	+/+, *Lt2*/+
>R3202	8	8.5	*Lt1b*/*Lt1b*	*Lt2*/*Lt2*

Table II. *Lysine Sensitivity of Aspartate Kinase Isoenzymes of Single and Double Mutants*

Main shoots of 15 to 20 d plants were assayed. The activity of the uninhibited enzyme in each fraction in pkat mg^{-1} protein is shown in parentheses.

Plant	Genotype at		[Lysine]	Inhibition of Enzyme Activity	
	lt1	*lt2*		AKII	AKIII
			mM	%	
Bomi	+/+	+/+	1	72 (13)	73 (9)
			5	79	81
R3004	+/+	*Lt2*/*Lt2*	1	62 (15)	37 (8)
			5	72	48
R3202	*Lt1b*/*Lt1b*	+/+	1	0 (7)	71 (6)
			5	0	79
Double mutant	*Lt1b*/*Lt1b*	*Lt2*/*Lt2*	1	0 (6)	28 (6)
			5	0	39

Table III. *Growth of Single and Double Mutants on Medium Containing 8 mM Lysine, 8 mM Threonine, and 1 mM Arginine*

Fourteen to 28 embryos were plated and plants grown for 7 d. Control plants (Bomi, no added amino acids) had shoot length of 62.1 ± 7.2 and root length 26.4 ± 6.2 mm. Values followed by different letters were significantly different at $p < 0.05$ when tested by paired *t* tests.

Plant	Shoot Length	Root Length
	mm	*mm*
Bomi	6.9 (a) ± 1.5	4.0 (e) ± 0.8
R3004	16.6 (b) ± 2.9	4.4 (e) ± 0.6
R3202	40.1 (c) ± 11.8	15.9 (f) ± 2.9
Double mutant	47.7 (d) ± 11.6	18.9 (g) ± 2.8

activity of either AKII or AKIII from roots and endosperms to carry out accurate determinations of their lysine sensitivities. However, total AK activity assayed in crude extracts from shoots and endosperm showed similar sensitivities to feedback inhibitors. The root enzyme activity from both Bomi and the double

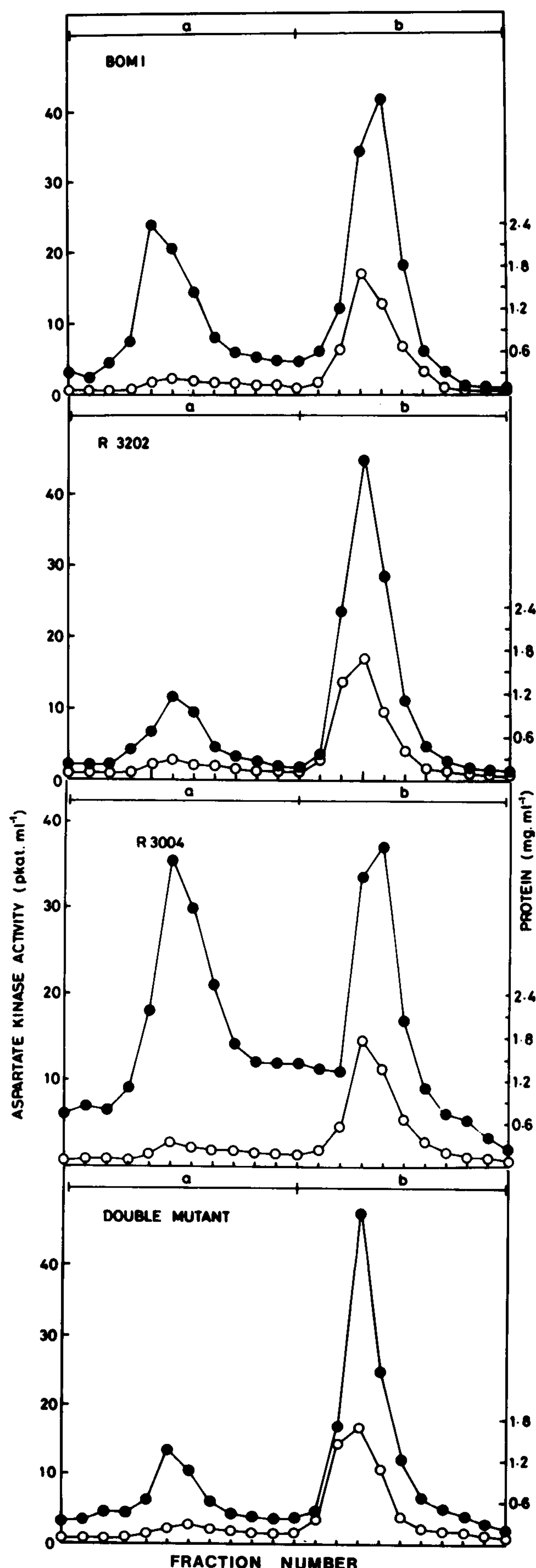

FIG. 2. Separation on DEAE-Sephacel of two forms of aspartate kinase from 7-d-old barley plants grown from embryos on basal medium. The extracts were loaded in buffer A plus 50 mM KCl and the columns washed with 30 ml of buffer A plus 75 mM KCl. The columns were eluted stepwise with 15 ml of buffer A plus 120 mM KCl (a) and 20 ml of buffer A plus 220 mM KCl (b). Fractions (1.6 ml) were collected and assayed for AK (●) and protein (○) content.

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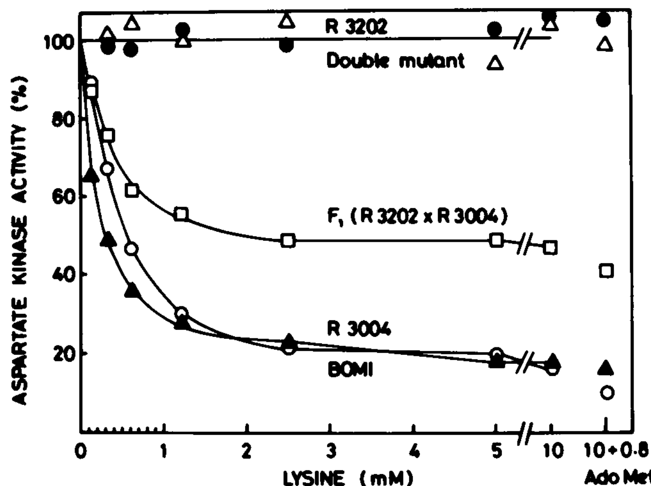

FIG. 3. The effect of lysine on partially purified AK isoenzymes (AKII) from Bomi (O), R3004 (▲), R3202 (●), F_1 (R3202 × R3004) (□), double mutant pure line isolated from the cross R3202 × R3004 (Δ). Activity in the presence of 10 mM lysine plus 0.8 mM AdoMet was also determined.

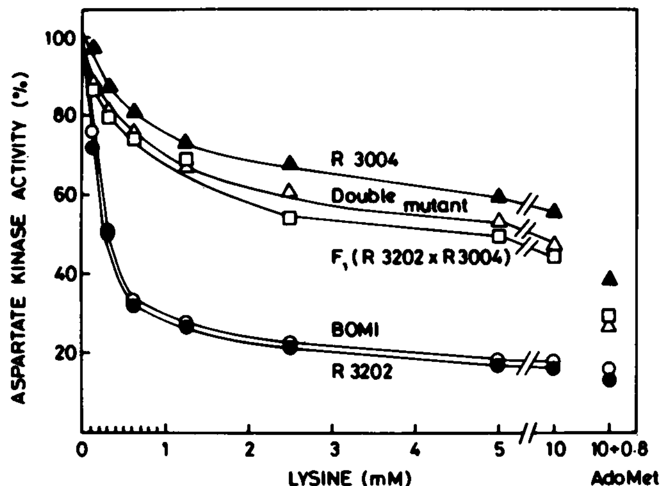

FIG. 4. The effect of lysine on partially purified aspartate kinase isoenzymes (AKIII) from Bomi (O), R3004 (▲), R3202 (●), F_1 (R3202 × R3004) (□), double mutant pure line isolated from the cross R3202 × R3004 (Δ). Activity in the presence of 10 mM lysine plus 0.8 mM AdoMet was also determined.

mutant was slightly less sensitive to lysine or lysine plus AdoMet than the shoot activity. A marked decrease in the sensitivity to lysine was noted in crude extracts of all three tissues of the double mutant (7–18% inhibition at 1.25 mM) as compared to the wild type (55–65% inhibition at 1.25 mM). AdoMet (0.8 mM) caused further inhibition of the Bomi enzyme activity in the presence of lysine and markedly increased the inhibition of the double mutant activity.

Characterization of Aspartate Kinase from Homozygous Mutant Plants Derived from R2501. Attempts to isolate a homozygous pure breeding and vigorous resistant line from selfed progeny of the mutant R2501 had been unsuccessful. However, a single, slow growing and pale green homozygous plant was identified and crossed with the normal barley cv Golden Promise. In the F_2 population there was a 3:1 ratio of resistant-to-sensitive plants, suggesting that the homozygous plants were viable. Selfed progeny embryos from these F_2 plants were tested and resistant F_3 progeny were harvested from a homozygous F_2 plant (which gave rise only to resistant progeny) and a heterozygous one (which produced both sensitive and resistant types). The lysine-sensitive isoenzymes of aspartate kinase from these plants were separated on DEAE-Sephacel and from the parent line cv Bomi. There was an increased proportion of AKII activity in both sets of mutant plants. The ratio of AKII/AKIII activities in both was determined as 1.8 as compared to 0.67 in Bomi; the same ratios in R3004 and R3202 were 1.2 and 0.38, respectively.

The sensitivity to lysine of AKII from Bomi and the two mutant populations derived from R2501 is shown in Figure 5. The AKII isoenzyme in homozygous resistant plants was completely insensitive to 10 mM lysine alone, but strongly inhibited in the presence of 10 mM lysine plus 0.8 mM AdoMet. This result was confirmed by testing the inhibitory action of lysine in the presence of 1.6 mM AdoMet. Maximum inhibition of AKII activity was 60% in the presence of both amino acids with half-maximal inhibition being obtained with 2.3 mM lysine (data not shown). AKII from the progeny of the heterozygous plants showed an intermediate lysine inhibition, which was enhanced by addition of AdoMet. AKIII isoenzymes from Bomi and mutant types derived from R2501 were inhibited to the same extent by lysine or lysine plus AdoMet.

DISCUSSION

The routine screening procedure of growing embryos in the presence of 2 mM lysine plus 2 mM threonine did not lead to identification of double mutant plants in the F_2 generation of the cross between R3202 and R3004, due to the vigorous growth of R3202 in this medium. However, if the embryos were germinated on 8 mM lysine, 8 mM threonine plus 1 mM arginine (6, 10), it was possible to identify plants that grew better than R3202 (Fig. 1; Table I). One F_3 plant was identified as being a potential double mutant by showing the characteristic lack of sensitivity to lysine in both isoenzymes of aspartate kinase, AKII and AKIII (Table II). All the F_4 generation embryos from this plant grew well in the presence of 8 mM lysine, 8 mM threonine, and 1 mM arginine.

The separation of AKII and AKIII achieved using stepwise elution from DEAE-Sephacel has allowed us to carry out a reliable comparison of the two lysine-sensitive AK isoenzymes from the various mutant lines without any confusion with the threonine-sensitive form, AKI. The profiles of elution of AKII and AKIII from DEAE-Sephacel of Bomi, R3004 and R3202 (Fig. 2) are very similar to those previously reported from DEAE-cellulose (12), with the characteristic increase in the proportion of AKII in R3004 and decrease in R3202 being observed. The double mutant showed an elution profile very similar to R3202, whereas the F_1 was intermediate between the two mutants. Alterations in proportions of enzyme activities could result from changes in synthesis, stability, or specific activity of the mutant enzyme.

The isoenzyme AKII from both R3202 and the double mutant was completely insensitive to lysine and lysine plus AdoMet (Fig. 3), clearly indicating that the double mutant is homozygous at the *lt1* locus. AKII from the F_1 line showed intermediate inhibition by lysine with similar kinetics as the wild type isoenzyme. At present no detailed information is available about the structure of AK from any plant source. It is not possible from the inhibition survey shown in Figure 3 to state whether AKII in the heterozygous F_1 line is a mixture of two different enzyme species, one of normal lysine sensitivity and one completely insensitive, or is a single enzyme with an equal number of two different subunits. However, it does indicate that both alleles at the *lt1* locus in the heterozygote (*Lt1b*/+) are expressed.

The lysine inhibition curve of the isoenzyme AKIII isolated from R3004 shown in Figure 4 is very similar to that previously reported (12) and clearly shows that unlike AKII in R3202, the isoenzyme is still sensitive to high concentrations of lysine and the synergistic action of lysine plus AdoMet. The lysine inhibi-

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Table IV. *Aspartate Kinase Activity in Different Tissues of Wild Type Barley (cv Bomi) and the Double Mutant*

Plant	Tissue	Fresh Wt	Protein Concentration	AK Activity
		g	*mg g⁻¹ fresh wt*	*p kat mg⁻¹ protein*
	Root	5.19	1.3	44.0
Bomi	Shoot	4.04	7.7	41.5
	Endosperm	40.0	4.4	19.2
	Root	4.83	1.5	17.9
Double mutant	Shoot	4.23	7.7	24.1
	Endosperm	53.0	4.1	11.7

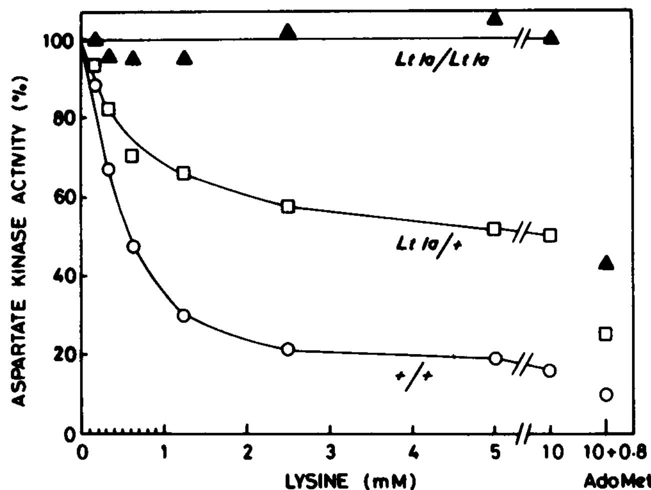

FIG. 5. The effect of lysine on the AK isoenzyme AKII partially purified from the resistant progeny of (a) a homozygous mutant plant derived from R2501 (*Lt1a/Lt1a*) (▲), (b) a heterozygous mutant plant derived from R2501, (*Lt1a*/+) (□), as well as from (c) the parent cv Bomi (+/+) (O). Activity in the presence of 10 mM lysine plus 0.8 mM AdoMet was also determined.

tion curve obtained with AKIII isolated from the double mutant was similar to that obtained from R3004. The curve obtained with AKIII isolated from the F_1 plants was not exactly intermediate between the curves obtained from R3004 and Bomi, possibly because the difference between mutant and wild type was less dramatic than for AKII (Fig. 3). However, the lysine sensitivity, expressed as the concentration giving half-maximal inhibition, was intermediate (1.2 mM) for the F_1 line AKIII as compared to 0.3 mM Bomi, 0.3 mM R3202, and 2 mM R3004.

AK activity was detected in the roots and developing endosperm of barley as well as in the leaves (Table IV). AK activity expressed on a fresh weight basis was markedly higher in leaves although the activities expressed on a protein basis were similar. In crude extracts, the lysine inhibition curves of AK isolated from shoots and endosperm were very similar, but the root enzyme was consistently less inhibited (approximately 10%).

Thus, a double mutant line (*Lt1b/Lt1b, Lt2/Lt2*) that shows vigorous growth on increased levels of lysine and threonine (8 mM) in the presence of 1 mM arginine has been constructed. Both AKII and AKIII were less sensitive to lysine in the same manner as in R3202 and R3004, respectively. From the data obtained with crude extracts, we suggest that aspartate kinase is expressed in a similar manner in the leaves, roots, and developing endosperm of barley. This accounts for the ability of R3004 and R2501 to accumulate soluble threonine in the maturing grain (3).

By outcrossing and reselection it has been possible to obtain a homozygous line of R2501 (*Lt1a/Lt1a*). One or a few deleterious mutations present in the original selected plant have presumably been removed by the outcrossing. Nevertheless the homozygous plants of R2501, unlike those of R3202 and R3004, are still not as vigorous as the parent. AKII isolated from the homozygous line was completely insensitive to lysine (Fig. 5), whereas the enzyme from the progeny of a heterozygous plant showed an intermediate sensitivity as shown originally (4). There are two major differences between the mutation affecting AKII in R2501 and in R3202: the activity of this isoenzyme is much higher in R2501, and the activity in R2501 still maintains a marked sensitivity to the synergistic inhibitory action of lysine and AdoMet, *i.e.* it is still potentially subject to regulatory control.

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