Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 2577–2581, March 1994
Plant Biology

Lysine synthesis and catabolism are coordinately regulated during tobacco seed development

(essential amino acids/lysine/dihydrodipicoliante synthase/lysine ketoglutarate reductase/transgenic plants)

HAGAI KARCHI, ORIT SHAUL, AND GAD GALILI*

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Communicated by J. Schell, November 8, 1993

ABSTRACT **The regulation of synthesis and accumulation of the essential amino acid lysine was studied in seeds of transgenic tobacco plants expressing, in a seed-specific manner, two feedback-insensitive bacterial enzymes: dihydrodipicolinate synthase (EC 4.2.1.52) and aspartate kinase (EC 2.7.2.4). High-level expression of the two bacterial enzymes resulted in only a slight increase in free lysine accumulation at intermediate stages of seed development, while free lysine declined to the low level of control plants toward maturity. To test whether enhanced catabolism may have contributed to the failure of free lysine to accumulate in seeds of transgenic plants, we analyzed the activity of lysine–ketoglutarate reductase (EC 1.5.1.7), an enzyme that catabolizes lysine into saccharopine. In both the control and the transgenic plants, the timing of appearance of lysine–ketoglutarate reductase activity correlated very closely with that of dihydrodipicolinate synthase activity, suggesting that lysine synthesis and catabolism were coordinately regulated during seed development. Notably, the activity of lysine–ketoglutarate reductase was significantly higher in seeds of the transgenic plants than in the controls. Coexpression of both bacterial enzymes in the same plant resulted in a significant increase in the proportions of lysine and threonine in seed albumins. Apparently, the normal low steady-state levels of free lysine and threonine in tobacco seeds may be rate limiting for the synthesis of seed proteins, which are relatively rich in these amino acids.**

Like many bacterial species, higher plants synthesize the essential amino acid lysine from aspartate by a specific branch of the aspartate-family pathway (1, 2). Biochemical studies demonstrated that lysine feedback inhibits the activities of two key enzymes in its pathway; aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS) (1, 2). Yet, analyses of mutant and transgenic plants, carrying enzymes with reduced sensitivity to feedback inhibition, showed that lysine synthesis is regulated primarily by DHPS (3–5).

The regulation of synthesis and accumulation of lysine in seeds is poorly understood. Plant seeds generally contain low levels of free threonine and only trace amounts of free lysine (6). In addition, these amino acids are present in very low amounts in many seed proteins (6). Previous studies suggested that free lysine may be catabolized rather efficiently in developing plant seeds (7–10). Yet the role of such processes in the accumulation of lysine in seeds has not been elucidated.

To study the regulation of lysine synthesis and accumulation in plant seeds, we have expressed a chimeric gene encoding a bacterial DHPS, either by itself or together with another chimeric gene encoding a bacterial insensitive AK (11), in a seed-specific manner in transgenic tobacco plants. Although this expression resulted in increased lysine synthesis during seed development, free lysine failed to accumulate to high level in mature seeds. Notably, the transient increase of free lysine in tobacco seeds caused an induction in the activity of lysine–ketoglutarate reductase (LKR), an enzyme involved in lysine catabolism.

MATERIALS AND METHODS

Construction of the Chimeric Phaseolin–DHPS Gene. The chimeric phaseolin–DHPS gene used in the present study is shown in Fig. 1. The starting plasmid used for its construction contained a cauliflower mosaic virus 35S promoter instead of the phaseolin promoter (5). The phaseolin promoter was excised from the plasmid pGZ322 (12) digested with a *Sma* I and *Sal* I. A *Bam*HI linker was then added at the 5′ end of the promoter, and the resulting *Bam*HI–*Sal* I DNA fragment was used to replace the *Bam*HI–*Sal* I DNA fragment containing the 35S promoter in the plasmid containing the chimeric 35S promoter–DHPS gene (5). The chimeric phaseolin–DHPS gene was inserted into the polylinker of the binary Ti vector pGA492 of *Agrobacterium tumefaciens* (13) and introduced into *Nicotiana tabacum* cv. Samsun NN by the leaf-disk protocol (14). Growth conditions of the plants were as described (11).

Analyses of DHPS and LKR Activity in Tobacco Seeds. Seeds were homogenized at 4°C in the following buffers: (*i*) for DHPS activity, 100 mM Tris·HCl, pH 7.5/2 mM EDTA/1.4% sodium ascorbate/1 mM phenylmethylsulphonyl fluoride with leupeptin at 0.5 μg/ml, and (*ii*) for LKR activity, 100 mM potassium phosphate, pH 7.0/1 mM EDTA/1 mM dithiothreitol/15% (vol/vol) glycerol with leupeptin at 0.5 μg/ml. The homogenates were centrifuged for 15 min at 21,000 × *g* at 4°C, and the supernatant was collected and analyzed for protein concentration according to Bradford (15). DHPS activity was assayed in crude seed extracts containing 40 μg (transgenic plants) or 160 μg (control plants) of protein, by using the *o*-aminobenzaldehyde method (16). LKR activity was assayed in crude seed extracts containing 150 μg of protein, as described (7).

Measurements of Free and Protein-Bound Amino Acids in Tobacco Seeds. Extraction of free amino acids, as well as albumins and globulins, and subsequent analyses of the amino acid composition of these fractions were described previously (11).

RESULTS

Seed-Specific Expression of the Bacterial DHPS in T_0 and Selfed T_1 Progenies of the Transgenic Plants. To study the role of DHPS in the synthesis and accumulation of lysine in plant seeds, tobacco plants were transformed with a chimeric gene encoding a bacterial DHPS under the regulation of the

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Abbreviations: AK, aspartate kinase; DHPS, dihydrodipicolinate synthase; LKR, lysine–ketoglutarate reductase.

*To whom reprint requests should be addressed.

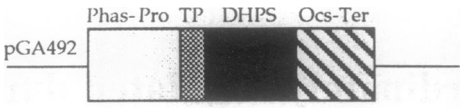

FIG. 1. The chimeric gene utilized in the present study. Phas-Pro, the bean phaseolin promoter; TP, the DNA coding for the pea *rbcS-3A* plastid transit peptide; DHPS, the coding DNA sequence of the mutant *Escherichia coli dapA* gene encoding DHPS; Ocs-Ter, the DNA sequence of the octopine synthase 3′ terminator.

seed-specific promoter of the bean phaseolin gene (Fig. 1). This promoter is active both in the endosperm and embryo of tobacco seeds (H. Levanony and G.G., unpublished data). A DNA encoding a pea *rbcS* plastid transit peptide was used to direct the bacterial enzyme to the organelle where the aspartate-family pathway is located (5). The expression of the chimeric DHPS gene was assayed in mature seeds derived from 15 separately transformed heterozygous T_0 plants as well as from 3 control nontransformed plants. DHPS activities in seeds of the transgenic plants ranged from about 1- to 140-fold higher than in seeds of control nontransformed plants. No change in DHPS activity was evident in leaves, stems, and flowers (data not shown), indicating that the bacterial DHPS was expressed in a seed-specific manner. Two transgenic genotypes, AE26 and AE30, expressing relatively medium and high levels of the bacterial DHPS, were selected for further analyses. Following self-pollination, ≈20 T_1 plants were grown in the greenhouse to maturity. These T_1 progenies were divided into three classes, carrying either zero (control), one (heterozygous), or two (homozygous) doses of the chimeric DHPS gene, based on the germination of their selfed T_2 seeds on medium with kanamycin (the gene for kanamycin resistance is tightly linked to the chimeric DHPS gene on the T-DNA). Whereas all T_2 seeds from control plants were sensitive to kanamycin, all T_2 seeds of the homozygous progenies were kanamycin resistant. T_2 seeds of heterozygous plants segregated as 3/4 resistant and 1/4 sensitive to kanamycin. The segregation frequency of the T_1 plants also indicated that the T-DNA behaved as a single Mendelian unit in both AE26 and AE30 plants. The average DHPS activity (±SE) in mature seeds of the heterozygous T_1 progenies was increased by a factor of 80 ± 10.6 (AE30) or 124 ± 2.4 (AE26) compared with control plants. This activity was approximately twice as high in the homozygous T_1 progenies. In addition, while 50% inhibition (I_{50}) of the endogenous DHPS activity in seeds of control plants was about 20 μM lysine, the I_{50} of DHPS activity in seeds of the transgenic plant was about 1 mM lysine (data not shown), as expected for the bacterial enzyme (2, 5, 16).

Synthesis and Accumulation of Lysine in Seeds of T_1 Progenies of AE26 and AE30 Plants. The composition of free amino acids was studied in mature seeds of T_1 progenies of the AE26 and AE30 plants. Despite the high activity of the bacterial DHPS, no change in the level of free lysine, or any other free amino acid, was evident in mature seeds of the transgenic compared with the control plants (data not shown). To detect any changes in the pattern of lysine accumulation during seed development, we measured DHPS activity and free lysine accumulation in developing seeds of the control and homozygous AE26 plants. In the control plants, the endogenous DHPS activity was first detected at 14 days after anthesis, reaching the highest level at 18 days, and then declining toward seed maturity (Fig. 2*A*). DHPS activity in the homozygous transgenic plant AE26 was also first detected at 14 days after anthesis but continued to rise dramatically until maturity (Fig. 2*A*), as expected from its regulation by the phaseolin promoter (17). Free lysine accumulation in seeds of the control plants correlated with the activity of DHPS, reaching a maximum at 18–22 days after anthesis and then declining to its lowest level in mature seeds (Fig. 2 *B* and *C*).

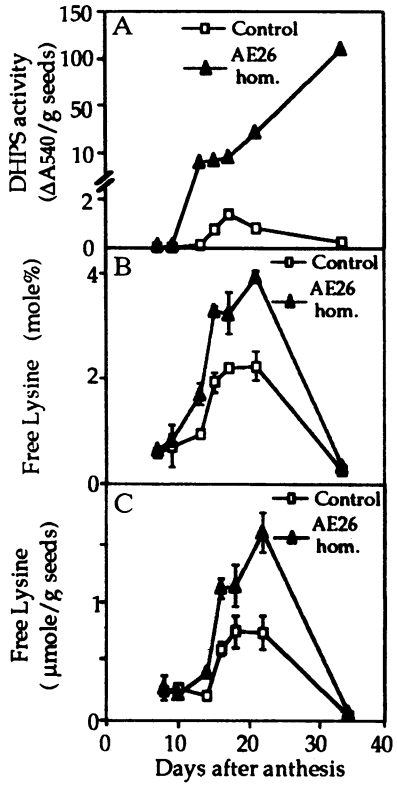

FIG. 2. Relations between DHPS activity and free lysine content during development of seeds from control (□) and homozygous AE26 transgenic (▲) plants. (*A*) DHPS activity. (*B*) Proportion of free lysine from total free amino acids. (*C*) Actual level of free lysine. Data in *B* and *C* include at least two measurements of different plants at each developmental stage for the control and transgenic genotypes. Bars represent standard errors of the means.

The level of free lysine was about twice as high in the transgenic AE26 plant, compared with control plants between 14 and 22 days after anthesis. However, toward maturity, free lysine declined drastically to a level as low as that of the control plants, even though DHPS activity in AE26 was highest in mature seeds (Fig. 2). Thus, although lysine synthesis was enhanced in the seeds of the transgenic plant, it did not accumulate to a high steady-state level. The transient increase in free lysine accumulation at 14–22 days in both control and transgenic AE26 plants was accompanied by a parallel transient decrease in free asparagine and a transient parallel increase in free aspartate, the precursor of lysine (Fig. 3). This suggested that asparagine was converted into aspartate, which, in turn, was further converted into aspartate-family amino acids. Still, the reduction in free asparagine was significantly greater in the homozygous transgenic AE26 plant than in the control plant (Fig. 3*A*).

Coexpression of the Insensitive Bacterial AK and DHPS in Seeds of Transgenic Plants. Compared with their parents, which possessed either one of the insensitive enzymes AK or DHPS, tobacco leaves possessing both insensitive enzymes were previously shown to produce more lysine at the expense of threonine (18, 19). We therefore wished to study the concerted effect of AK and DHPS on lysine and threonine synthesis and accumulation in tobacco seeds. The T_0 heterozygous transgenic tobacco plant AE30 was crossed with two other T_0 transgenic plants, AN5 and AN20, which expressed an insensitive bacterial AK using the same phaseolin promoter and overproduced free threonine in mature seeds (11). T_1 plants from these crosses were grown to maturity and classified into genotypes expressing (*i*) no bacterial enzyme, (*ii*) only the bacterial DHPS, (*iii*) only the bacterial AK, or

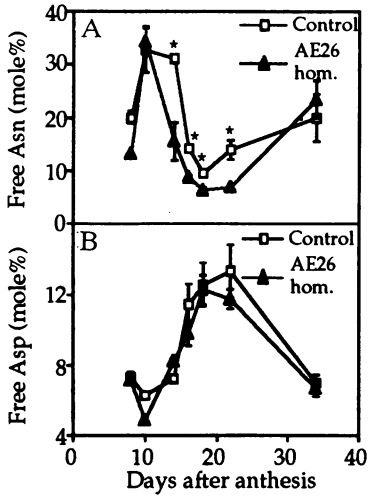

FIG. 3. Changes in free asparagine and aspartate during development of seeds from control (□) and homozygous AE26 transgenic (▲) plants. Proportion of free asparagine (*A*) and the proportion of free aspartate (*B*) from the total free amino acids are shown. Data include at least two measurements of different plants at each developmental stage for the control and transgenic genotypes. Bars represent the standard error of the means. Stars above the points in *A* represent significant differences between the control and transgenic plants at the 5% level as determined by a Duncan test.

(*iv*) both bacterial enzymes, based on the activities of AK and DHPS in their T_2 seeds. The four genotypes were also confirmed by PCR analysis as well as by their segregation for kanamycin resistance (data not shown).

Free amino acid composition was then analyzed in mature seeds derived from the four segregating genotypes of the T_2 progenies of the crosses AE30 × AN5 and AE30 × AN20. The level of free seed threonine in the T_2 genotypes expressing only the bacterial DHPS was similar to that of control plants (Fig. 4, DHPS het.), as expected from the specificity of DHPS for lysine synthesis. In addition, free seed threonine in the T_2 genotypes expressing only the bacterial AK was increased by about 6-fold compared with the control plants

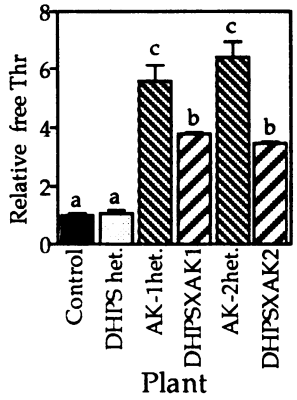

FIG. 4. Free threonine accumulation in mature tobacco seeds in response to the bacterial DHPS and AK. Control, control plants; DHPS het., heterozygous transgenic AE30 plants expressing the *E. coli* DHPS; AK-1het. and AK-2het., heterozygous transgenic plants AN5 and AN20, expressing the *E. coli* AK; DHPSxAK1 and DHPSxAK2, transgenic plants AE30 × AN5 and AE30 × AN20, coexpressing both bacterial enzymes. The level of free threonine in each transgenic genotype (average of at least three measurements of different plants for each genotype) is plotted relative to the average level of free threonine in six control plants, which was given a value of 1. Error bars represent the standard errors of the means. Different letters above the bars represent significant differences at the 5% level as determined by a Duncan test.

(Fig. 4, AK-1het. and AK-2het.), confirming our previous observations on the role of AK in the synthesis of seed threonine (11). Overproduction of free seed threonine was about 50% lower in the T_2 progenies coexpressing the two bacterial enzymes than in transgenic plants expressing the bacterial AK by itself (Fig. 4), suggesting that in seeds of the coexpressing plants, lysine synthesis was enhanced at the expense of threonine. Nevertheless, no increase in free lysine was evident in mature seeds of these plants, compared either with the control plants or with transgenic plants expressing only the bacterial DHPS (data not shown). The level of free lysine in developing seeds (14–22 days after anthesis) of transgenic plants coexpressing both bacterial enzymes was very similar to that of developing seeds in transgenic plants expressing only the bacterial DHPS (data not shown; see Fig. 2 *B* and *C*).

To study whether enhanced lysine catabolism may have contributed to the failure of free lysine accumulation in seeds

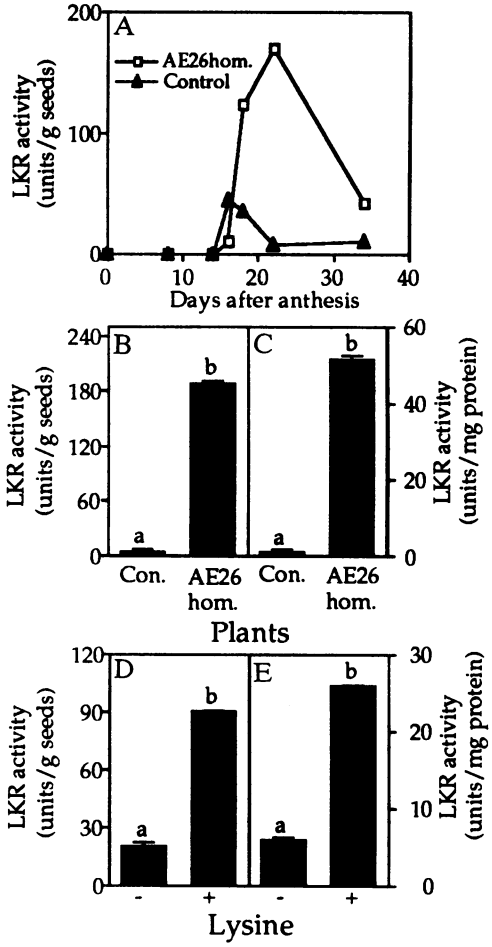

FIG. 5. Effect of increased lysine synthesis on LKR activity in tobacco seeds. (*A*) LKR activity was determined during seed development and is plotted in units per g of seeds. (*B* and *C*) LKR activity in control (Con.) and homozygous AE30 plant expressing the bacterial DHPS (AE30 hom.) was measured at 22 days after anthesis. (*D* and *E*) Developing pods at 20 days after anthesis from wild-type plants were injected with water (−) or with 1 mM lysine (+). Seeds were harvested 44 hr after injection and analyzed for LKR activity. LKR activity was normalized either to g of seeds (*B* and *D*) or to mg of protein (*C* and *E*). The analyses in *B–E* included at least three measurements of different plants for the control and transgenic AE30 hom. Error bars represent standard errors of the means. Different letters above bars represent significant differences at the 5% level as measured by a Duncan test. One unit of LKR was defined as the amount of enzyme required to oxidize one nmol of NADPH per min at 28°C.

of the transgenic plants, we compared the activity of LKR during seed development of the homozygous transgenic AE26 plants and control nontransformed plants. LKR activity was first detected in seeds of both of these plants at about 14 days after anthesis, coinciding with the transient activity of DHPS and the transient accumulation of free lysine (Fig. 5A, compare with Fig. 2). Yet the level of LKR activity in the homozygous AE26 plants continued to rise considerably up to 22 days (Fig. 5A), reaching about 20-fold higher values than the control plants (Fig. 5 B and C). To study more directly whether the increase in LKR activity in the transgenic plants resulted from elevated free lysine levels, developing pods of wild-type plants at 20 days after anthesis were injected with a solution containing 1 mM lysine or with water as a control. Seeds were harvested 44 hr after injection and analyzed for LKR activity. Injection of lysine caused a significant increase in the activity of LKR (Fig. 5 D and E).

Accumulation of Lysine and Threonine in Seed Proteins. We next tested the amino acid composition of seed proteins from transgenic plants expressing each of the bacterial enzymes alone and those coexpressing the two bacterial enzymes. No significant change was observed in the proportion of any amino acid in the globulin fraction from all of the transgenic plants (data not shown) or in the albumin fraction from plants expressing each of the bacterial enzymes alone (Table 1). Yet the proportions of lysine and threonine in the albumin fraction of plants coexpressing both bacterial enzymes were significantly higher than in control plants or than in transgenic plants expressing either of the bacterial enzymes alone (Table 1). Notable changes were also observed in the proportions of glutamate plus glutamine, isoleucine, leucine, and arginine in the albumin fractions of plants coexpressing both bacterial enzymes, but these differences were small and insignificant at the 5% level from the control plants (Table 1).

DISCUSSION

Synthesis and Accumulation of Free Lysine and Threonine in Tobacco Seeds. As demonstrated in this work, seed-specific expression of a bacterial DHPS in transgenic tobacco plants brought about an increased synthesis of free seed lysine, whereas coexpression of this enzyme with bacterial feedback-insensitive AK caused increased synthesis of both free seed lysine and free seed threonine. These results support our previous report (11) and indicate further the efficient operation of both branches of the aspartate-family pathway in plant seeds. In addition, in plants coexpressing the two bacterial enzymes, compared with those expressing either one of them, lysine synthesis was apparently increased further at the expense of threonine. This supports previous analyses of tobacco leaves possessing feedback-insensitive DHPS and AK enzymes (18, 19), suggesting that the concerted regulatory role of these two key enzymes is very similar in leaves and seeds.

Although lysine and threonine are synthesized by a common pathway, their accumulation in tobacco seeds varied considerably. As the level of free threonine was much higher in mature seeds of the transgenic plants, compared with the controls, it is suggested that threonine accumulation in tobacco seeds is regulated primarily by the rate of its own synthesis. In contrast, free lysine accumulated transiently at intermediate stages of seed development and declined to its lowest level in mature seeds in both the control and the transgenic plants. The transient accumulation of free lysine in the control plants could be explained by the concomitant transient appearance of the activity of the endogenous DHPS, which is the major limiting enzyme for lysine biosynthesis. However, a limiting level of DHPS activity could not explain the pattern of lysine accumulation in the transgenic plants, in which DHPS activity was highest in mature seeds. The limiting factor for free lysine accumulation in seeds is still unknown. However, several lines of evidence suggest that lysine catabolism plays at least a partial role in preventing lysine accumulation in maturing seeds. (*i*) The more pronounced decrease in free lysine level in maturing seeds of the transgenic plants, compared with control plants, was positively correlated with increased LKR activity in the transgenic plants. (*ii*) In tobacco leaves, where free lysine could accumulate to very high levels (4, 5, 19), no LKR activity was detected either in control plants or in transgenic plants expressing the bacterial DHPS constitutively (data not shown). (*iii*) Feeding of radioactive lysine into cereal grains has shown that lysine is efficiently catabolized in this organ via saccharopine into α-aminoadipic acid and glutamate (8–10). This conclusion was supported by the demonstration that developing cereal grains also exhibited relatively high activity of the enzyme LKR, which converts lysine into saccharopine (7). (*iv*) Developing grains of mutant opaque-2

Table 1. Amino acid composition in albumins of mature seeds from control plants and from transgenic plants expressing the bacterial AK and DHPS

	mol % (mean ± SE)				
Amino acid	Wild-type	DHPS	AK	DHPS × AK1	DHPS × AK2
Asx	7.62 ± 0.03	7.46 ± 0.05	7.46 ± 0.08	7.57 ± 0.12	7.85 ± 0.14
Thr	4.72 ± 0.06	4.61 ± 0.10	4.69 ± 0.12	5.53 ± 0.27*	5.31 ± 0.07*
Ser	5.33 ± 0.07	5.08 ± 0.05	5.38 ± 0.08	5.32 ± 0.11	5.33 ± 0.03
Glx	19.1 ± 0.10	19.2 ± 0.22	19.1 ± 0.21	18.7 ± 0.67	18.2 ± 0.51
Pro	4.51 ± 0.07	4.44 ± 0.05	4.50 ± 0.06	4.41 ± 0.10	4.47 ± 0.05
Gly	9.88 ± 0.13	9.80 ± 0.09	9.62 ± 0.11	9.66 ± 0.03	9.84 ± 0.20
Ala	8.66 ± 0.10	8.67 ± 0.08	8.45 ± 0.14	8.30 ± 0.20	8.82 ± 0.22
Val	7.08 ± 0.11	7.20 ± 0.07	7.09 ± 0.12	7.00 ± 0.19	7.17 ± 0.21
Met	1.25 ± 0.05	1.23 ± 0.05	1.41 ± 0.03	1.31 ± 0.03	1.35 ± 0.07
Ile	4.81 ± 0.07	4.96 ± 0.06	4.89 ± 0.08	4.94 ± 0.06	5.10 ± 0.09
Leu	8.26 ± 0.07	8.31 ± 0.18	8.23 ± 0.09	8.51 ± 0.05	8.55 ± 0.11
Phe	3.84 ± 0.10	3.77 ± 0.05	3.90 ± 0.05	3.87 ± 0.09	3.88 ± 0.06
Lys	4.64 ± 0.10	4.51 ± 0.10	4.26 ± 0.09	5.25 ± 0.01*	5.35 ± 0.10*
His	2.00 ± 0.04	1.90 ± 0.03	1.96 ± 0.04	2.08 ± 0.03	2.12 ± 0.02
Arg	8.12 ± 0.46	7.64 ± 0.27	8.10 ± 0.26	7.64 ± 0.13	7.36 ± 0.27

The transgenic plants analyzed in this experiment are AE30 (DHPS), AN5 (AK), AE30 × AN5 (DHPS × AK1), and AE30 × AN20 (DHPS × AK2). Values represent means and SE of the means from at least four plants per genotype. Asx and Glx represent Asp + Asn and Glu + Gln, respectively. Cys, Tyr, and Trp were not determined. Asterisks represent significant differences from the control at the 5% level as measured by a Duncan test. Analysis was done on a log transformation to stabilize variances within different transgenic genotypes.

maize plants, which possess lower LKR activity than wild-type plants (7), also contain higher lysine levels (6, 20).

Notably, at the latest stages of seed development (>22 days after anthesis, when seeds turned brown) the activity of LKR decreased sharply (Fig. 5*A*), but still lysine did not accumulate in seeds of the transgenic plants (Fig. 2). This observation suggests that at these late stages of seed development, lysine accumulation is determined by other factors such as shortage of enzymes or precursors of the aspartate pathway.

Regulation of LKR Expression During Tobacco Seed Development. Both in the control and in the transgenic plants, the timing of appearance of LKR activity correlated very closely with the transient increases in the endogenous DHPS activity and free lysine accumulation. This suggests that enzymes involved in both lysine synthesis and lysine catabolism are coordinately regulated during tobacco seed development. Interestingly, increasing the levels of free lysine in the seeds, either by expression of the bacterial DHPS or by injection of lysine into developing pods, was accompanied by a significant increase in LKR activity compared with the controls. Understanding the mechanism of this induction process in seeds is a subject of a continuous interest in our laboratory.

Notably, the timing of the apparent conversion of asparagine into aspartate (Fig. 3) correlated very closely with the timing of the transient elevation in free lysine levels and the transient increase in the activity of the endogenous DHPS and LKR (Figs. 2 and 5), all being initiated at about 14 days after anthesis. These results may suggest the presence of a general control mechanism of amino acid metabolism during tobacco seed development.

Incorporation of Lysine and Threonine into Seed Proteins. We also questioned whether the low pools of free seed lysine and threonine were limiting factors for their incorporation into proteins. Our results indicate that the mechanism of incorporation of lysine and threonine into seed proteins is complex. When the synthesis of either lysine or threonine was increased by itself, no elevation was evident in the proportion of these amino acids in seed proteins. However, when the synthesis of both of these amino acids was increased, their proportions were significantly increased in seed albumin, but not in the globulin fraction. Although the molecular basis for this increase is unknown, it implies that the elevated synthesis of both free lysine and free threonine in tobacco seeds may have caused an increased synthesis of specific lysine- and threonine-rich albumins.

We thank Prof. N. Kedar, Prof. J. Gressel, Dr. A. A. Levy, and Mr. Y. Avivi for critical reading of the manuscript; Dr. L. M. Hoffman for the bean phaseolin promoter; Prof. N.-H. Chua for the pea *rbcS-3A* gene; Prof. J. C. Patte for the *E. coli dapA* gene; and Dr. G. An for pGA492. We thank Ms. E. Rojanski for outstanding technical assistance. This work was supported by a grant from the National Council for Research and Development (Israel), and the Gesellschaft für Biotechnologische Forschung MBH (Braunschweig, Germany). G.G. is an incumbent of the Abraham and Jenny Fialkow Career Development Chair in Biology.

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