Theor Appl Genet (1996) 92:70–77

D. C. Bittel · J. M. Shaver · D. A. Somers
B. G. Gengenbach

Lysine accumulation in maize cell cultures transformed with a lysine-insensitive form of maize dihydrodipicolinate synthase

Received: 12 April 1995 / Accepted: 23 June 1995

Abstract Lysine is one of the nutritionally limiting amino acids in food and feed products made from maize (*Zea mays* L.). Two enzymes in the lysine biosynthesis pathway, aspartate kinase (AK) and dihydrodipicolinate synthase (DHPS), have primary roles in regulating the level of lysine accumulation in plant cells because both enzymes are feedback-inhibited by lysine. An isolated cDNA clone for maize DHPS was modified to encode a DHPS much less sensitive to lysine inhibition. The altered DHPS cDNA was transformed into maize cell suspension cultures to determine the effect on DHPS activity and lysine accumulation. Partially purified DHPS (wildtype plus mutant) from transformed cultures was less sensitive to lysine inhibition than wildtype DHPS from nontransformed cultures. Transformed cultures had cellular free lysine levels as much as four times higher than those of nontransformed controls. Thus, we have shown that reducing the feedback inhibition of DHPS by lysine can lead to increased lysine accumulation in maize cells. Increasing the capacity for lysine synthesis may be an important step in improving the nutritional quality of food and feed products made from maize.

Key words Maize · Transformation · Lysine · Dihydrodipicolinate synthase · Aspartate kinase

Introduction

Most crop seeds are deficient in one or more of the essential amino acids; lysine is especially limiting in cereals, including maize. Low levels of lysine in food and feed products made from cereals prevent the efficient utilization of plant protein by monogastric animals. Lysine, threonine, methionine and isoleucine are synthesized from a common precursor, aspartate, in higher plants (Bryan 1990). The first step in the aspartate pathway (Fig. 1) is catalyzed by aspartate kinase (AK), which generally appears in two isoforms in plants. The predominant form of AK in maize is feedback-inhibited by lysine, while the minor form is inhibited by threonine (Azevedo et al. 1992). The minor form occurs as a bifunctional enzyme that also exhibits homoserine dehydrogenase activity (Azevedo et al. 1992; Muehlbauer et al. 1994 b). The first committed step in the lysine branch of the pathway is catalyzed by dihydrodipicolinate synthase (DHPS), which is subject to feedback inhibition by lysine alone (Bryan 1990; Kumpaisal et al. 1987). In maize, mature DHPS is a homotetramer that is 50% inhibited by 23 μM lysine (Frisch et al. 1991 a). The proenzyme of maize DHPS contains a transit sequence that directs DHPS to the chloroplast (Muehlbauer and Bittel unpublished) where amino acid synthesis occurs.

Attempts to produce plants which have an increased capacity for lysine synthesis using tissue culture have had varying levels of success. Mutagenesis and tissue culture selection allowed the recovery of lysine and threonine overproducers in *Nicotiana sylvestris* (Frankard et al. 1991; Negrutiu et al. 1984). The bases for increased lysine and increased threonine were determined to be mutations in DHPS and AK, respectively, which made the enzymes less sensitive to lysine inhibition. Maize tissue culture selection following mutagenesis led to the recovery of plants containing lysine-insensitive AK (Diedrick et al. 1990; Dotson et al. 1990; Hibberd and Green 1982). Homozygous mutant kernels accumulated 30- to 175-fold more free threonine than control kernels (Diedrick et al. 1990; Dotson et al. 1990; Muehlbauer et al. 1994 a; Gengenbach and Diedrick 1994). Tissue culture selection strategies, however, have not resulted in a lysine-over-producing mutant in maize.

Because the aspartate pathway is similar in plants and bacteria (Bryan 1990; Umbarger 1978), alternative molecular strategies to increase lysine synthesis in plants are possible. It has recently been shown that tobacco

Communicated by F. Salamini

D. C. Bittel · J. M. Shaver · D. A. Somers
B. G. Gengenbach (✉)
Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108, USA

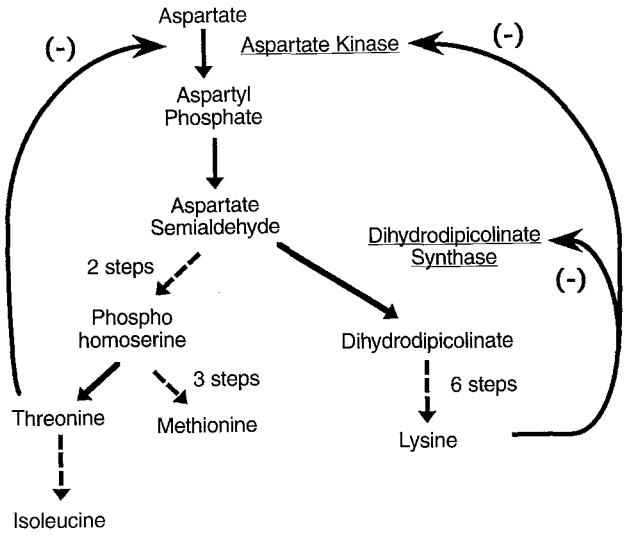


Fig. 1 Simplified diagram of the aspartate family amino acid biosynthesis pathway. Major enzymes and products are shown. *Curved lines* represent feedback inhibition

(*Nicotiana tobaccum*) and potato (*Solanum tuberosum*) transformed with an *Escherichia coli* form of DHPS, which is less sensitive to lysine inhibition than the endogenous plant enzyme, increased free lysine accumulation (Shaul and Galili 1992; Perl et al. 1992). Although several clones encoding plant DHPS have been identified (Kaneko et al. 1990; Frisch et al. 1991 b; Vauterin and Jacobs, 1994) to date there have been no reports of attempts to use plant-derived DHPS genes to modify plant lysine biosynthesis.

As part of an ongoing effort to increase lysine biosynthesis in maize, we have mutagenized the cDNA encoding maize DHPS isolated by Frisch et al. (1991 b). We have subsequently selected mutant forms of DHPS which are less sensitive to lysine inhibition yet still retain enzymatic activity (Shaver, Sellner and Gengenbach, unpublished data). In the study presented here, we used a mutant cDNA of maize DHPS coupled to a constitutive promoter to transform maize suspension cell cultures to determine whether the presence of lysine-insensitive maize DHPS would allow the accumulation of excess free lysine in maize cells.

Maize AK is also sensitive to lysine inhibition and may limit lysine accumulation even in the presence of lysine-insensitive DHPS. Plant AK genes with reduced sensitivity to lysine inhibition have not been isolated; therefore, a construct containing the *E. coli LysC* gene for AK, which is less sensitive to lysine inhibition than maize AK, was cotransformed into maize cells along with lysine-insensitive DHPS to determine whether both enzymes must be lysine insensitive to increase lysine accumulation. This study contributes to our effort to improve the nutritional quality of maize and to determine the molecular and biochemical regulation of lysine biosynthesis in maize.

Materials and methods

Construction of transformation vectors

DHPS

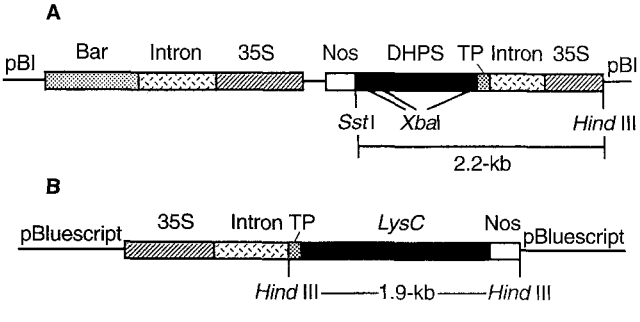
The cDNA encoding lysine-insensitive maize DHPS (DHPS166 av), differs from wildtype DHPS by the substitution of valine for alanine at amino acid 166 relative to the translation initiation start codon (Shaver, Sellner and Gengenbach, unpublished data). The mutant enzyme, partially purified from the *E. coli* auxotroph AT997, shows no inhibition by lysine concentrations as high as 10 m*M*, which compares to I_{50} values of 35 μ*M* for the wildtype maize enzyme purified from the auxotroph and 1 m*M* for the *E. coli* enzyme isolated from wildtype bacterial cells. The activity of both the maize enzyme expressed in the *E. coli* auxotroph and the *E. coli* wildtype enzyme is completely inhibited by 10 m*M* lysine.

The cDNA encoding lysine-insensitive maize DHPS166 av and the transit peptide sequence (Frisch et al. 1991b) were amplified by polymerase chain reaction (PCR) using an upstream primer containing a *Bam*HI restriction site (CGCGGGATCCATGATTTC-GCCGACGAATGTCC) and a downstream primer containing an *Sst*I site (CGCGGAGCTCCTAGTACCTACTGATCAACACG). The 1.2-kb amplified segment was digested with *Sst*I and *Bam*HI and ligated into the corresponding sites in plasmid pBI221 (Clontech), replacing the β-glucuronidase (GUS) gene to produce the plasmid pBI35/166 av. The 1-kb sequence encoding the cauliflower mosaic virus 35 S promoter plus the alcohol dehydrogenase (Adh) intron 1 was removed from plasmid pH24 (gift from M. Fromm) by digestion with *Hin*dIII and *Bam*HI. This sequence was ligated into the corresponding sites in plasmid pBI35/166 av, replacing the 35 S promoter to produce p35Adh/166 av. The BAR cassette (35 S promoter plus Adh intron and the BAR gene, which confers resistance to the herbicide Basta) was released from the plasmid pBARGUS (gift from M. Fromm) with *Not*I, and the ends were filled in by the action of the large subunit of *E. coli* DNA polymerase I (*Klenow* enzyme). The plasmid p35Adh/166 av was linearized with *Nar*I, the ends filled in with *Klenow* enzyme, purified on silica resin (Geneclean, Bio 101, La Jolla, Calif.) and the BAR cassette blunt-end ligated into p35Adh/166 av to produce the plasmid p35Adh/166 av/B (Fig. 2A).

LysC

A 1.9-kb *Hin*dIII fragment containing the *E. coli LysC* gene attached to a DNA sequence encoding a chloroplast transit peptide (gift from K. Glassman, Plant Science Research) was ligated into the *Hin*dIII site of pBluescript (Stratagene, La Jolla, Calif.) to produce the plasmid pBS/LysC. The plasmid pBARGUS was cut with *Bam*HI and filled in with *Klenow* enzyme, purified on silica resin and cut with *Not*I to release a 1-kb fragment containing the 35S promoter plus the Adh

Fig. 2A,B Constructs used for transformation. **A** p35Adh/166av/B, **B** p35Adh/LysC. *35S* Cauliflower Mosaic Virus 35S promoter, *Intron* alcohol dehydrogenase Intron 1, *TP* chloroplast transit peptide, *Bar* BAR gene, confers Basta resistance, *Nos* 3′ terminus of nopaline synthase gene



intron. The plasmid pBS/LysC was cut with *Xba*I, filled in with *Klenow* enzyme, purified on silica resin and cut with *Not*I. The 35S promoter plus Adh intron fragment was directionally ligated into pBS/LysC using the *Not*I site to produce plasmid p35Adh/LysC (Fig. 2B).

Transformation

Microprojectile bombardment followed the procedure of Gordon-Kamm et al. (1990) with slight modification. DNA from p35Adh/166av/B and p35Adh/LysC plasmids (25 µg each) were co-precipitated onto 2 mg of sterile 1.5-µm diameter tungsten particles with 250 µl 2.5 *M* $CaCl_2$, 50 µl 0.1 *M* spermidine and placed on ice for 10 min. The suspension was microfuged for 5 min, and all but 10 µl of the supernatant was removed. The particles were resuspended in the remaining solution, and 1 µl of DNA/particles was pipetted onto the macrocarrier of the Biolistics Model PDS-1000 (E.I. du Pont de Nemours & Co.) powered by 0.22-caliber blanks. The macrocarrier was loaded into the firing chamber and accelerated toward Black Mexican Sweet (BMS) maize cells (approximately 500 mg fresh weight) slightly mounded on Whatman 3-mm filter discs saturated with MS2-D media [MS salts (Murashige and Skoog 1962) supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid, 0.5 mg/l thiamine and 20 g/l sucrose, pH 5.8]. One milliliter of MS2-D media was added to the plates, the cells allowed to recover for 2 days and the cells were plated on solid MS2-D media containing 3 g/l gelrite and 3 mg/l Basta. Transformant selection and maintenance followed the procedure detailed in Kaepler et al. (1990). Cell lines resistant to Basta (presumably containing the BAR gene) were kept individually isolated, subcultured every 2–4 weeks and maintained continuously on solid media containing Basta.

DNA gel blots

DNA was extracted from BMS cell cultures following standard techniques (Saghai-Maroof et al. 1984) and cut with *Hin*dIII and *Sst*I. The two enzymes in combination released a 2.2-kb fragment containing the 35S promoter and DHPS166av from p35Adh/166av/B. *Hin*dIII released a 1.9-kb *LysC* fragment from p35Adh/LysC. The DNA was elecctrophoresed and blotted to nitrocellulose membranes (Micron Separations) using standard practices (Maniatis et al. 1984). The membranes were hybridized with radiolabeled probes using the 0.65-kb *Xba*I fragment of DHPS (Fig. 2A) or the 1.9-kb *Hin*dIII fragment containing the *LysC* gene (Fig. 2B), washed in 0.2% SSC, 0.1% SDS at 65 °C for 30 min prior to exposure to X-ray film (Kodak) and then stripped according to manufacturer's instructions prior to rehybridization with the second probe, *LysC*.

Enzyme assays

DHPS

Approximately 100 mg of cells (fresh weight) were ground with a Kontes pestle in a 1.5-ml microcentrifuge tube in PBS buffer (150 m*M* NaCl, 16 m*M* Na_2HPO_4, 4 m*M* $Na_2H_2PO_4$, pH 8.0) containing 50 m*M* pyruvate. Debris was spun down, and the supernatant was brought to 20% (w/v) with PEG (MW 4,000) and centrifuged 15 min at 15,000 *g*. The supernatant was removed, brought to 10% (w/v) with ammonium sulfate and centrifuged at 10,000 *g* for 15 min. allowing two layers to form. The lower protein-enriched phase was removed and desalted over a Sephadex S25 column equilibrated with PBS extraction buffer. The desalted extract was assayed as described by Frisch et al. (1991 a). Briefly, 100 µl of extract was added to a reaction mix containing 10 m*M* pyruvate, 1.4 m*M* aspartate semialdehyde and 0.1 m*M* Tris-HCl, pH 8.0, brought to 250 µl with water and incubated for 30 min at 37 °C. The reaction was stopped by adding 1 ml of stop buffer (0.22 *M* sodium citrate, 0.55 *M* sodium phosphate pH 5.0 and 0.25 mg/ml *o*-aminobenzaldehyde). Color was allowed to develop for 3 h, and absorbance readings were taken at 520 nm.

AK

Approximately 3 g of cells (fresh weight) were suspended in extraction buffer [25 m*M* Tris-HCl pH 7.4, 2 m*M* lysine, 2 m*M* threonine, 200 m*M* KCl, 1 m*M* DTT, 2 m*M* EDTA, 10% (v/v) polyethylene glycol], and the cells were disrupted with a tissue homogenizer (Brinkman). Debris was pelleted and the supernatant transferred to a fresh tube. Ammonium sulfate was added to 60% (w/v) and the proteins pelleted at 15,000 *g* for 20 min. The pellet was resuspended in 500 µl of buffer B [25 m*M* Tris-HCl pH 7.4, 1 m*M* DTT, 0.1 m*M* lysine, 0.1 m*M* threonine, 75 m*M* KCl, 10% (v/v) polyethylene glycol] and desalted over a Sephadex S25 column equilibrated with buffer C [25 m*M* Tris-HCl pH 7.4, 1 m*M* DTT, 75 m*M* KCl, 10% (v/v) polyethylene glycol]. The assay contained 400 µl buffer D (0.5 m*M* Tris-HCl pH 8.0, 2 m*M* KCl), 200 µl 4 *M* NH_2OH (pH to 8.0 with KOH), 100 µl 0.1 *M* ATP, 0.1 *M* $MgCl_2$ (pH 8.0) and 100 µl 0.2 *M* aspartate (pH 8.0) and 200 µl enzyme. The assay was incubated at 37 °C for 60 min and stopped with the addition of 1.5 ml of 10% (w/v) $FeCl_3$, 3.3% (w/v) TCA in 0.7 *M* HCl. The solution was spun down and absorbance at 540 nm was recorded. For each reaction, paired blanks were prepared consisting of all components except aspartate, which was added with the stop mix. The blank absorbance value was subtracted from the reaction absorbance to obtain the AK activity.

Amino acid analysis

Free amino acids were extracted overnight by shaking 100 mg of cells (fresh weight) in 800 µl of 25% (v/v) acetonitrile. Extracts were spun down, and 125 µl of supernatent was dried in HPLC vials at 70 °C with air flow. For culture media analysis, the media was spun down and 125 µl was dried directly. Dried samples were dissolved in 40 µl of 50 m*M* Na_2HPO_4 (pH 7.0), derivatized with the addition of 80 µl 0.2% (w/v) (dimethyl) aminoazobenzensyulfonyl chloride in acetonitrile and heated to 70 °C for 10 min. Samples were cooled to room temperature, diluted with 880 µl of Na_2HPO_4 (pH 7.0)/95% ethanol (1:1), and 20 µl of each sample was injected into a Spectraphysics HPLC using a phase separation spherisorb S5 ODS2 25 × 4.6 mm column. Amino acids were eluted from the column in a gradient of acetonitrile and 2 m*M* sodium acetate (pH 6.5) plus 2% (v/v) dimethyl formamide, and absorption at 436 nm was recorded continuously. Peak areas of individual amino acids were converted to nanomoles according to an amino acid standard included with each set of samples. Amino acid concentrations were determined at least three times for each cell line examined.

Results

Selection and confirmation of transformants

Twelve culture plates containing BMS cells were bombarded with tungsten particles coated with DNA from both plasmids p35Adh/166av/B and p35Adh/LysC. After 4–5 weeks 91 Basta-resistant calli, 2–3 mm in diameter, were transferred to fresh plates containing Basta.

DNA, extracted from Basta-resistant calli, was restricted with *Hin*dIII and *Sst*I. The two enzymes released a 2.2-kb fragment (Fig. 2A) from p35Adh/166av/B that was resolved independently of the endogenous DHPS gene(s) (Fig. 3, BMS lane). Sixty-nine lines (76%) contained a 2.2-kb fragment that hybridized to the labeled DHPS cDNA probe (Fig. 3). The A-type hybridization pattern (Fig. 3) is representative of the lines characterized in this report, differing from the nontransformed BMS pattern by a single 2.2-kb band.

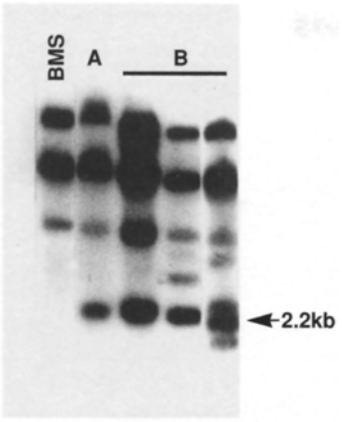
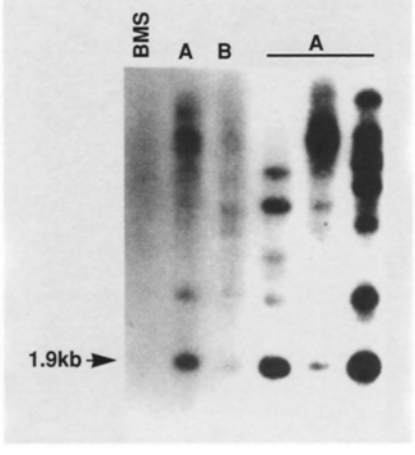


Fig. 3 DNA blot hybridized with the 0.65-kb *Xba*I DHPS fragment. *Arrow* indicates 2.2-kb fragment released from the p35Adh/166av/B plasmid when cut with *Hin*dIII and *Sst*I. *Type A* indicates the pattern expected for integration of intact plasmid DNA, *Type B* patterns may result from insertions of rearranged plasmid DNA. *BMS* Nontransformed control

Three bands hybridizing to the probe were present in the nontransformed BMS pattern, suggesting that there may be more than one copy of the DHPS gene present in the BMS genome. The B-type patterns (Fig. 3) contained extra bands that may have been due to incomplete digestion. The constructs contained two or more copies of several elements, including the promoter, which could potentially lead to rearrangements within the vector also producing extra bands. Digestion with *Hin*dIII released a 1.9-kb fragment from p35Adh/LysC (Fig. 2B) encoding the *LysC* gene. The *LysC* sequence did not hybridize to DNA from nontransformed cells (Fig. 4, BMS lane); therefore, any lanes with clear hybridization (Fig. 4, B lanes) were *LysC*-negative. Fifty-nine of the Basta-resistant lines (65%) were found to contain the *LysC* gene; 47 resistant lines (52%) contained both the DHPS166av sequence and the *LysC* gene. The 69 lines containing the DHPS transgene were analyzed by HPLC for changes in amino acid levels. Seven lines with free lysine concentrations 2 to 4 times higher than that of the controls and two lines that contained only the *LysC* sequence were characterized more extensively.

Fig. 4 DNA blot hybridized with the 1.9-kb *Hin*dIII *LysC* fragment. *Arrow* indicates the 1.9-kb fragment corresponding to that released from the p35Adh/LysC plasmid when cut with *Hin*dIII. The lanes labeled *A* represent cultures with integrated plasmid DNA and were designated *LysC*-positive. Higher molecular weight bands are probably a consequence of incomplete digestion or possibly of rearranged plasmid insertions. The lane labeled *B* was designated *LysC*-negative, corresponding to the nontransformed BMS control



Growth of transformants on selective media

Cultures containing DHPS166av and/or *LysC* were transferred to media containing 2 m*M* lysine plus threonine (LT), which prevents the growth of wildtype BMS maize cells by inhibiting AK activity (Table 1). Culture 63 was Basta-resistant, but contained neither DHPS166av nor *LysC* and did not differ from nontransformed cells in response to LT. Cultures 29, 53 and 66 grew on 2 m*M* LT, suggesting that the *LysC* gene produced enough lysine-insensitive AK to allow the cultures to grow in the presence of the inhibitors. Cultures 7 and 69 failed to grow on 2 m*M* LT, apparently because the incorporated *LysC* gene was either inactive or produced too little enzyme to overcome LT inhibition.

The cultures were tested on media containing the lysine analog, *S*-(2-aminoethyl) cysteine (AEC), which prevents the growth of wildtype cells by inhibiting DHPS and possibly AK activity and by substituting for lysine in protein synthesis. Cultures 7, 29, 66 and 70 were capable of growing on 1 m*M* AEC, indicating that the DHPS166av transgene was active (Table 1). Cultures 29 and 66, which contained both DHPS166av and *LysC*, grew faster on 1 m*M* AEC than culture 70, which contained only DHPS166av. Furthermore, culture 29 was able to grow on 2 m*M* AEC, which prevented the growth of all other lines. No cultures grew on 3 m*M* AEC, and all cultures grew on 0.5 m*M* AEC, including the nontransformed control BMS cells (data not shown). Cultures without the DHPS166av transgene failed to grow on media containing 1 m*M* AEC even when they contained the *LysC* transgene.

The cultures were also placed on media containing 2.5–5 m*M* threonine. Threonine had no effect on the growth of wildtype BMS cells or on any cultures containing the *LysC* gene, even at concentrations as high as 5 m*M*. However, 2.5 m*M* threonine inhibited the growth of culture 70, which contained only the DHPS166av transgene, possibly as a consequence of high endogenous free lysine concentrations inhibiting wildtype AK activity in concert with the exogenous threonine. In contrast to culture 70, culture 7 was inhibited by 2 m*M* LT but was not affected by 2.5 m*M* threonine in the media. This suggests that the *LysC* gene may have been active in culture 7 but at levels too low to restore growth in the presence of 2 m*M* LT.

Table 1 Culture genotype and growth characteristics of maize cell culture lines transformed with plasmids containing DHPS166av and/or the AK (*LysC*) genes (*LT* lysine plus threonine, *T* threonine, *AEC* S-(2-aminoethyl) cysteine)

Cell line	DHPS-166av	*LysC*	Basta	2 m*M* LT	2.5 m*M* T	1 m*M* AEC	2 m*M* AEC
BMS[a]	No	No	–[c]	–	+	–	–
63	No	No	+	–	+	–	–
70	Yes	No	+	–	–	+	–
7	Yes	ia[b]	+	–	+	+	–
29	Yes	Yes	+	+	+	+	+
66	Yes	Yes	+	+	+	+	–
53	No	Yes	+	+	+	–	–
69	No	ia	+	–	+	–	–

[a] Nontransformed
[b] ia, Inactive; gene was present, but there was no evidence of modified AK activity
[c] +, Growth; –, no growth

Enzyme activity and sensitivity to lysine inhibition

The specific activity of total DHPS from the three transformed cultures, 7, 29 and 70, was not significantly different from that of the nontransformed BMS control (Table 2), although the mean value of culture 29 was about 50% higher than that for BMS.

Likewise, western blotting using a polyclonal antibody against maize DHPS indicated only minor changes in the amount of DHPS relative to the amount of total protein in comparisons between transformants and nontransformed controls (data not shown). However, there was a clear reduction in sensitivity to lysine inhibition of total DHPS from transgenic cultures (Fig. 5). Wildtype DHPS isolated from nontransformed cultures was inhibited 50% at approximately 35 μ*M* lysine, whereas the three transformant lines tested were 50% inhibited at concentrations of lysine greater than 80 μ*M* (Table 3). At 500 μ*M* lysine, the three transformed cultures retained 20–50% DHPS activity, while DHPS activity from BMS controls was totally inhibited (Fig. 5). The DHPS activity remaining at 500 μ*M* lysine may represent the proportion of total DHPS activity attributable to the DHPS166av transgene.

Table 2 Total DHPS and AK activities in BMS and transformed maize cell culture lines (*NA* not assayed)

Cell line[a]	Enzyme activity[b]	
	DHPS	AK
BMS	0.091 (0.015)	4.91 (0.01)
70	0.112 (0.028)	NA
7	0.081 (0.029)	NA
29	0.157 (0.032)	7.65 (0.25)
53	NA	7.35 (0.25)

[a] BMS, Nontransformed control; culture 70 contained DHPS166av; cultures 7 and 29 contained both DHPS166av and *LysC*; culture 53 contained *LysC*
[b] Units/mg protein. Mean of 3 assays +/– (SE)

The specific activity of total AK in extracts from cultures 29 and 53 was significantly higher (ca. 50%) than that of extracts from nontransformed BMS cells (Table 2). Lysine inhibition of AK activity was not examined. Both cultures 29 and 53 grew on 2 m*M* LT as expected if they had an active *LysC* gene producing an AK less sensitive to lysine inhibition than the endogenous maize AK. Culture 7 also contained the *LysC* transgene but did not grow on LT media, and the

Table 3 Summary of characteristics exhibited by maize cell culture lines containing DHPS166av and/or *LysC* transgenes (*NA* not assayed)

Cell line and transgene	Modification of DHPS	Modification of AK	Free amino acid	
			Lysine (pmol/mg fresh wt)[b]	Threonine
No DHPS166av, No LysC				
BMS	I_{50}[a] = 35 μM	Endogenous AK	103 (8)	173 (44)
63	NA	NA	128 (2)	167 (13)
+ DHPS166av, No LysC				
70	I_{50} > 80 μM	NA	418 (26)**	163 (22)
+ DHPS166av, + LysC				
7	I_{50} > 100 μM	LysC inactive	422 (31)**	186 (16)
29	I_{50} > 80 μM	LysC active	398 (31)**	214 (13)
66	NA	LysC active	295 (19)*	192 (35)
No DHPS166av, + LysC				
53	NA	LysC active	283 (56)*	256 (7)*

*,** Significantly different from control, $P \leq 0.05$, 0.01 respectively
[a] I_{50} = lysine concentration which inhibits enzyme activity 50%
[b] Mean of 3 assays +/– (SE)

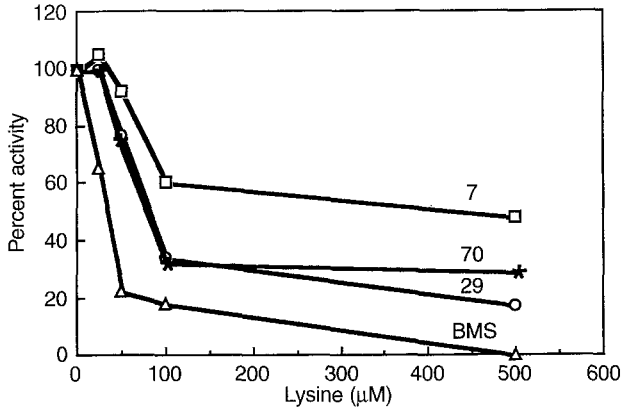


Fig. 5 Lysine inhibition of total DHPS activity from BMS control and transformed maize cells expressing the DHPS166av transgene. Plots show percent change relative to activity without added lysine for each line

specific activity of AK was not different from the nontransformed control. This suggests there was little AK activity due to the *LysC* transgene in culture 7.

Amino acid analysis

Free amino acid concentrations from transformed lines were determined by HPLC and compared to those of nontransformed controls (Table 3). Wildtype BMS cells contained an average of 103 (+/− 8) pmoles of lysine per milligram of tissue. The cultures expressing transgenic DHPS had up to fourfold more free lysine than the nontransformed control BMS cells (Fig. 6). Significantly increased lysine indicated that the lysine-insensitive fraction of total DHPS functioned in these cultures and allowed them to grow in the presence of normally inhibiting levels of AEC (Table 1). Free lysine concentrations were similar whether the cell line lacked the *LysC* transgene (no. 70) or whether *LysC* was expressed (no. 29) based on an increase in AK specific activity. Culture 53, which had an active *LysC* transgene but lacked DHPS166av, also had about 2.5-fold more free lysine than controls, indicating that modifying either AK or DHPS could affect lysine accumulation in BMS maize cells. Culture 53 also exhibited a modest but significant increase in free threonine, which is consistent with an increase in carbon flow through the pathway due to modified AK.

Secretion of lysine by lysine-over-producing cells

Several transformed cultures were transferred to nonselective liquid media and allowed to grow for 3 days. Media in which transgenic lines 70, 7, 29 and 53 had grown contained lysine up to 5.2 times that of media in which nontransformed controls and culture 63 had grown (Fig. 7). This suggests that in addition to increasing the intracellular free lysine pool, the transgenic lines secreted some excess lysine. Cultures 29 and 53, which contained the *LysC* transgene, also secreted more threonine than the controls (Figure 7). No other cultures had significant changes in free threonine in the media.

Discussion

Previous studies have demonstrated that it is possible to increase the accumulation of the endproducts of the aspartate pathway in plants by transformation using bacterial genes encoding enzymes that are less sensitive to feedback regulation. Tissue culture selection strategies resulting in altered regulatory enzymes have also proven effective in increasing free threonine in tobacco (Frankard et al. 1991) and maize (Diedrick et al. 1990;

Fig. 6 Cellular free lysine and free threonine in various transformed maize cell lines relative to nontransformed control. *BMS* Nontransformed control. *,** Significantly different from control, $P \leq 0.05$, 0.01, respectively

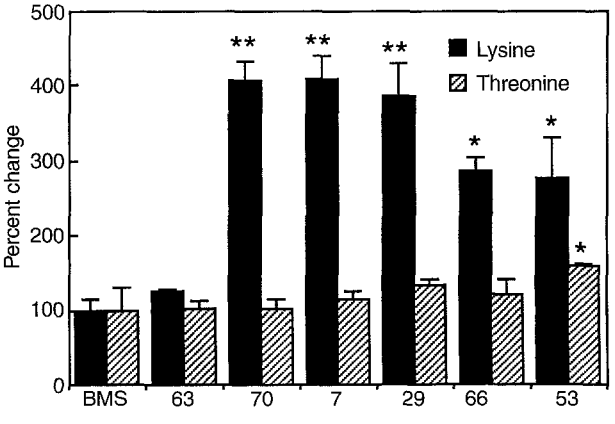
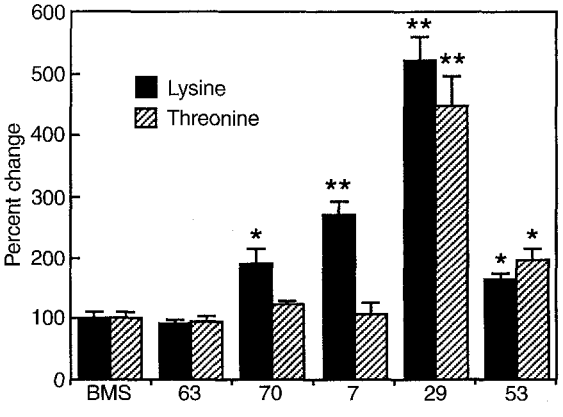


Fig. 7 Lysine and threonine excreted into culture media by various transformed maize cell lines relative to nontransformed control. *BMS* Nontransformed control. The concentrations of lysine and threonine in media from BMS cultures were 2.6 (+/− 0.25) and 15.5 (+/− 1.25) pmol/μl, respectively. *,** Significantly different from control, $P \leq 0.05$, 0.01, respectively



Dotson et al. 1990; Hibberd and Green 1982; Miao et al. 1988) and free lysine in tobacco (Negrutiu et al. 1984). However, attempts to deregulate the aspartate pathway in order to increase lysine synthesis in maize have not succeeded to date. We have cloned a maize DHPS cDNA and introduced a mutation in the sequence that confers upon the enzyme reduced sensitivity to lysine feedback inhibition (Shaver, Sellner and Gengenbach unpublished). In this paper we show that the mutant DHPS166av sequence is functional when reintroduced into maize cells and, furthermore, that molecular genetic deregulation of two enzymatic steps in the aspartate pathway in maize cells can lead to the accumulation of excess cellular free lysine.

The ability of the transgenic cultures to grow on inhibitory concentrations of AEC or LT media (Table 1) clearly demonstrates that the transgenes were expressed, resulting in enzymes less sensitive to AEC (DHPS166av) or LT (*LysC*). The cultures containing the DHPS166av transgene had a greater accumulation of free lysine than nontransformed controls (Fig. 6), suggesting that the presence of the altered DHPS enzyme (Fig. 5) led to increased lysine biosynthesis. There was little change in total DHPS activity or protein, indicating that modest amounts of deregulated enzyme can lead to significant increases in free lysine accumulation.

The mature DHPS enzyme in plants is a homotetramer (Kumpaisal et al. 1987; Frisch et al. 1991a), and the mutant and wildtype subunits likely can combine to form mixed tetramers. The resulting mature enzyme could contain from zero to four mutant subunits. The effect that mixed subunits may have on lysine sensitivity is difficult to predict. Negrutiu et al. (1984) employed mutagenesis and tissue culture selection to obtain a tobacco plant containing a lysine-insensitive DHPS isoform. In a heterozygous state the mutant monomer would probably combine with the wildtype monomer in a manner similar to what is expected for hemizygous transgenic maize cells. Negrutiu et al. (1984) found a significant increase in free lysine in the heterozygous plants, suggesting that mixed tetramers had either reduced lysine sensitivity or that enough of the homomeric lysine-insensitive tetramer was produced to effect an increase in free lysine. Our results agree with those of Negrutiu et al. (1984) in that the transformants had increased concentrations of lysine regardless of the *in vivo* interaction of the two isomers.

The effect of increased lysine accumulation is highlighted by the sensitivity of culture 70, which contains DHPS166av and not the *LysC* transgene, to threonine added to the culture media (Table 1). The growth of control BMS cells was unaffected by high levels of threonine in the media. Culture 70, however, failed to grow on media containing 2.5 m*M* threonine. Apparently, free lysine accumulated to high enough levels so that, in concert with the threonine added to the media, maize AK activity was inhibited, thereby preventing carbon flow into the pathway and resulting in methionine starvation. Cultures containing the *LysC* transgene were unaffected by threonine in the media regardless of the presence of DHPS166av. It is likely that the cellular free lysine concentration did not reach levels that were inhibitory to bacterial AK encoded by the *LysC* transgene.

In addition to DHPS, AK plays a key role in regulating carbon flow through this pathway. The predominant form of maize AK is highly sensitive to lysine inhibition, while the minor form is sensitive to threonine inhibition (Azevedo et al. 1992; Frisch et al. 1991a). It is likely that as lysine builds up in the transformed cultures, AK activity may decline and reduce the flux through the pathway. Culture 29 contained both the *LysC* and the DHPS166av transgenes and was the only culture tested that grew on media supplemented with 2 m*M* AEC (Table 1). No increase in cellular free lysine was observed in culture 29 compared to culture 70, which did not contain *LysC* (Table 1). However, culture 29 had nearly twice as much lysine secreted into the media as the other cultures examined, suggesting that AK is important for determining the overall capacity for synthesis of lysine. This result might also indicate that maize suspension cells have a tolerance threshold to cellular free lysine, above which lysine must be secreted for the cells to remain viable. If high free lysine is inhibitory to growing maize cells, this would have implications for genetic engineering strategies to modify this pathway in order to boost lysine in maize. It may be necessary to confine increased lysine synthetic capacity to the kernel to reduce impairment of normal metabolic functions in vegetative tissues. Deregulating the lysine biosynthetic pathway in maize may prove to be particularly effective if done in conjunction with the addition of high-lysine seed storage proteins.

We have demonstrated that a maize DHPS cDNA encoding a lysine-insensitive enzyme was functional when coupled to a constitutive promoter and transformed into maize cells. Furthermore, the presence of the mutant enzyme was associated with the accumulation of excess free lysine. The data demonstrate that the metabolic pathway leading to the synthesis of lysine can be deregulated in maize cells, resulting in the increased accumulation of free lysine. These data suggest that the molecular genetic modification of the lysine synthetic pathway is a viable approach to aid in the effort to increase lysine levels in important crop species such as maize.

References

Azevedo RA, Blackwell RD, Smith RJ, Lea PJ (1992) Three aspartate kinase enzymes from maize. Phytochemistry 31:3725–3730

Bryan JK (1990) Advances in the biochemistry of amino-acid biosynthesis. In: Miflin BJ, Lea PJ (eds) The biochemistry of plants, vol 16: Intermediary nitrogen metabolism. Academic Press, New York, pp 161–195

Diedrick TJ, Frisch DA, Gengenbach BG (1990) Tissue culture selection of a second mutant locus for increased threonine accumulation in maize. Theor Appl Genet 79:209–215

Dotson SB, Frisch DA, Somers DA, Gengenbach BG (1990) Lysine-insensitive aspartate kinase in two threonine-overproducing mutants of maize. Planta 182:546–552

Frankard V, Ghislain M, Negrutiu I, Jacobs M (1991) High threonine producer mutant of *Nicotiana sylvestris* (Spegg. and Comes). Theor Appl Genet 81:273–282

Frisch DA, Gengenbach BG, Tommey AM, Sellner JM, Somers DA, Myers DE (1991a) Isolation and characterization of dihydrodipicolinate synthase from maize. Plant Physiol 96:444–452

Frisch DA, Tommey AM, Gengenbach BG, Somers DA (1991b) Direct genetic selection of a maize cDNA for dihydrodipicolinate synthase in an *Escherichia coli* dapA$^-$ auxotroph. Mol Gen Genet 228:287–293

Gengenbach BG, Diedrick TJ (1994) Alterations in the biosynthesis of lysine, threonine and methionine by tissue culture approaches in maize. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 25:Maize. Springer, Berlin Heidelberg New York, pp 587–603

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willetts NG, Rice TB, Mackey CJ, Kreuger RW, Kausch AP, Lemaux PG (1990) Transformation of maize cells and regeneration of fertile plants. Plant Cell 2:603–618

Hibberd KA, Green CE (1982) Inheritance and expression of lysine plus threonine resistance selected in maize tissue culture. Proc Natl Acad Sci USA 79:559–563

Kaepler HF, Somers DA, Rines HW, Cockburn AF (1992) Silicon carbon fiber-mediated stable transformation of plant cells. Theor Appl Genet 84:560–566

Kaneko T, Hashimoto T, Kumpaisal R, Yamada Y (1990) Molecular cloning of wheat dihydrodipicolinate synthase. J Biol Chem 265:17451–17455

Kumpaisal R, Hashimoto T, Yasuyuki Y (1987) Purification and characterization of dihydrodipicolinate synthase from wheat suspension cultures. Plant Physiol 85:145–151

Maniatis T, Fritsch EF, Sambrook J (1982). Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Miao S, Duncan DR, Widholm J (1988) Selection of regenerable maize callus cultures resistant to methyl-*DL*-tryptophan, S-2-aminoethyl-L-cysteine and high levels of *L*-lysine plus *L*-threonine. Plant Cell Tiss Org Cult 14:3–14

Muehlbauer GM, Gengenbach BG, Somers DA, Donovan CM (1994 a) Genetics and amino acid analysis of two maize threonine-overproducing, lysine-insensitive aspartate kinase mutants. Theor Appl Genet 89:767–774

Muehlbauer GM, Somers DA, Matthews BF, Gengenbach BG (1994 b) Molecular genetics of the maize (*Zea mays* L.) aspartate kinase-homoserine dehydrogenase gene family. Plant Physiol 106:1303–1312

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture cells. Physiol Plant 15:473–497

Negrutiu I, Cattoir-Reynearts A, Verbruggen I, Jacobs M (1984) Lysine overproducer mutants with an altered dihydrodipicolinate synthase from protoplast culture of *Nicotiana sylvestris* (Spegazzini and Comes). Theor Appl Genet 68:11–20

Perl A, Shaul O, Galili G (1992) Regulation of lysine synthesis in transgenic potato plants expressing a bacterial dihydrodipicolinate synthase in their chloroplasts. Plant Mol Biol 19:815–823

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8019

Shaul O, Galili G (1992) Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts. Plant J 2:203–209

Umbarger HE (1978) Amino acid biosynthesis and its regulation. Annu Rev Biochem 47:533–606

Vauterin M, Jacobs M (1994) Isolation of a poplar and an *Arabidopsis thaliana* dihydrodipicolinate synthase cDNA clone. Plant Mol Biol 25:545–550