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Single-amino acid substitutions eliminate lysine inhibition of maize dihydrodipicolinate synthase

(lysine biosynthesis/genetic complementation/mutagenesis/*Zea mays* L./feedback inhibition)

JONATHAN M. SHAVER, DOUGLAS C. BITTEL, JANITA M. SELLNER, DAVID A. FRISCH*, DAVID A. SOMERS, AND BURLE G. GENGENBACH†

Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN 55108

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ABSTRACT **Dihydrodipicolinate synthase (DHPS; EC 4.2.1.52) catalyzes the first step in biosynthesis of lysine in plants and bacteria. DHPS in plants is highly sensitive to end-product inhibition by lysine and, therefore, has an important role in regulating metabolite flux into lysine. To better understand the feedback inhibition properties of the plant enzyme, we transformed a maize cDNA for lysine-sensitive DHPS into an *Escherichia coli* strain lacking DHPS activity. Cells were mutagenized with ethylmethanesulfonate, and potential DHPS mutants were selected by growth on minimal medium containing the inhibitory lysine analogue *S*-2-aminoethyl-L-cysteine. DHPS assays identified surviving colonies expressing lysine-insensitive DHPS activity. Ten single-base-pair mutations were identified in the maize DHPS cDNA sequence; these mutations were specific to one of three amino acid residues (amino acids 157, 162, and 166) localized within a short region of the polypeptide. No other mutations were present in the remaining DHPS cDNA sequence, indicating that altering only one of the three residues suffices to eliminate lysine inhibition of maize DHPS. Identification of these specific mutations that change the highly sensitive maize DHPS to a lysine-insensitive isoform will help resolve the lysine-binding mechanism and the resultant conformational changes involved in inhibition of DHPS activity. The plant-derived mutant DHPS genes may also be used to improve nutritional quality of maize or other cereal grains that have inadequate lysine content when fed to animals such as poultry, swine, or humans.**

Plants and bacteria have similar pathways for amino acid biosynthesis (1, 2). Information gained from bacterial studies has often guided the isolation and characterization of corresponding enzymes from plants. Sufficient similarities exist to provide a means to identify plant cDNA clones by direct selection in bacterial auxotrophic mutants. Plant genes for enzymes such as glutamine synthetase (3), aspartate aminotransferase (4), dihydrodipicolinate synthase (DHPS) (5, 6), Δ^1-pyrroline-5-carboxylate reductase (7), Δ^1-pyrroline-5-carboxylate synthase (8), ornithine aminotransferase (8), acyltransferase (9), cysteine synthase (10), and superoxide dismutase (11) have been selected directly or their identity has been confirmed by restoration of enzyme activity to the corresponding auxotrophic *Escherichia coli* cell lines.

Because auxotroph survival depends on the expression and function of the enzyme encoded by the plant cDNA, transformed bacterial auxotroph cells also can provide a convenient means to select for altered characteristics in plant enzymes. For example, enzymes affected by naturally occurring inhibitors such as pathway products or intermediates, by artificial compounds such as herbicides, or by other metabolite or physical stresses may be amenable to modification by mutagenesis and selection under the appropriate inhibitory or stress condition. Molecular and biochemical analysis of selected mutants can then identify functional or regulatory changes in the enzyme and relate site-specific mutations to specific amino acid or motif changes in the polypeptide product. We have taken this approach to modify a plant gene involved in regulating lysine biosynthesis.

In plants and bacteria, lysine is produced via a branched biosynthetic pathway starting from aspartate (1, 2). DHPS (EC 4.2.1.52) catalyzes the first reaction in the lysine-specific branch and is feedback-inhibited by lysine and analogs of lysine. Plant DHPS mutants with reduced sensitivity to lysine have been sought to better understand the regulatory properties of the enzyme and its role in controlling lysine concentrations in plant cells. Direct selection of tobacco (*Nicotiana sylvestris*) tissue cultures for resistance to the lysine analog *S*-2-aminoethyl-L-cysteine (AEC) resulted in a dominant mutation that reduced lysine inhibition of DHPS and increased the concentration of free lysine in leaves and seeds (12). No other plants with altered DHPS inhibition have been obtained by AEC selection. Bacteria also express DHPS with reduced lysine sensitivity. As examples, the wild-type *dapA* gene of *Corynebacterium glutamicum* encodes a lysine-insensitive DHPS (13, 14) and the *E. coli dapA* gene codes for DHPS that is approximately 40 times less sensitive to lysine inhibition than maize (*Zea mays* L.) DHPS (I_{50} = 1 mM vs. 25 μM) (15, 16).

To better understand the basis for lysine feedback inhibition, we have directly selected mutations that confer lysine insensitivity to enzyme activity expressed by a cloned plant DHPS gene. We previously obtained a maize lysine-sensitive wild-type DHPS cDNA‡ by genetic selection in an *E. coli* DHPS$^-$ (*dapA*$^-$) auxotroph (5). Herein we describe the selection of maize lysine-insensitive DHPS cDNAs by using the *E. coli dapA*$^-$ auxotroph. We found that several single nucleotide changes resulting in amino acid replacements in the mutant maize DHPS polypeptide sequence caused nearly complete lack of lysine inhibition. All the selected mutations were located within a 10-amino acid region that presumably identifies the lysine-binding site of this plant DHPS.

MATERIALS AND METHODS

Mutagenesis of Maize DHPS cDNA. The pZMDHPS5 plasmid (5) encodes a fusion protein containing, in order from the amino terminus, the following sequences: 5 amino acids of β-galactosidase, 18 amino acids due to the multiple cloning

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Abbreviations: AEC, *S*-2-aminoethyl-L-cysteine; DHPS, dihydrodipicolinate synthase; EMS, ethylmethanesulfonate.

*Present address: DeKalb Plant Genetics, 62 Maritime Drive, Mystic, CT 06355-1958.
†To whom reprint requests should be addressed.

‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. X52850 for wild-type maize DHPS).

sequence in λgt11, 13 amino acids from the normally untranslated 5′ region of the DHPS cDNA, 54 amino acids of the chloroplast-specific transit peptide of DHPS, and 326 amino acids of the mature DHPS enzyme. This entire coding sequence was cloned into the *Eco*RI site of pUC119 plasmid and transferred by electroporation (BTX Electro Cell Manipulator 6000 electroporator; 2.45 kV, 129 Ω) into cells of *E. coli* K-12 *dapA*$^-$ auxotroph AT997 (Genetics Stock Center, Yale University). Nontransformed AT997 cells were maintained on M9 minimal medium (17) supplemented with 100 μM diaminopimelate.

Prior to ethylmethanesulfonate (EMS) mutagenesis (18), 50 ml of transformed AT997 cells were grown to 2 to 3 × 10^8 cells per ml in 1 × A medium (18). Cells were recovered by centrifugation (5000 × *g*, 10 min, 4°C), washed in 1 × A medium plus 0.2 M Tris·HCl (pH 7.5), and resuspended in 25 ml of 1 × A medium (minus glucose) plus 0.2 M Tris·HCl (pH 7.5). The cells were divided into 12 populations in 12 30-ml tubes, 30 μl of EMS was added to a final concentration of 18 mM, and the tubes were incubated with shaking for 2 h at 37°C. Cells were washed twice with 15 ml of 1 × A medium, resuspended in 4 ml of M9 medium containing ampicillin (50 μg/ml), and grown 8–10 h at 37°C. From each tube, 1 ml of cells was plated onto solid M9 medium containing ampicillin (50 μg/ml) and 0, 5, or 10 mM AEC and incubated for 72 h at 37°C. A total of 720 colonies from the AEC selection plates were restreaked onto fresh plates of the same medium and 146 fast-growing colonies were chosen for assays of DHPS activity.

DHPS Extraction and Activity Assays. For DHPS assays of lysine inhibition, 50 ml of culture was centrifuged, resuspended in 5 ml of buffer (150 mM NaCl/16 mM Na_2HPO_4/4 mM NaH_2PO_4, pH 7.3/20 mM pyruvate), chilled on ice, subjected twice to lysis in a French press (1.15 × 10^5 kg/m^2, 4°C), and centrifuged (6000 × g, 15 min, 4°C). The supernatant was heated to 60°C for 15 min, the precipitate was removed by centrifugation, and the supernatant was desalted on a Sephadex G-25 column. Removal of pyruvate by desalting required enzyme assays to be conducted the same day. DHPS assays were conducted as described by Frisch *et al.* (16), and lysine inhibition was determined by adding 50, 100, 500, 1000, or 5000 μM L-lysine to the assay medium.

Confirmation and DNA Analysis of Mutagenized DHPS cDNA. Plasmid DNA from each transformed cell line selected on the basis of DHPS lysine insensitivity was transformed into nonmutagenized AT997 cells and DHPS assays were repeated to confirm the stability of the mutation. Plasmids were subsequently transformed into *E. coli* DH5α cells for DNA sequencing either with Sequenase 2.0 (United States Biochemical) using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate or with fmolDNA Sequencing System (Promega) using [γ-^{32}P]dCTP according to manufacturers' instructions.

RESULTS

Selection of Maize cDNAs Encoding Lysine-Insensitive DHPS. Auxotrophic AT997 *dapA*$^-$ cells require a functional DHPS gene product for survival on minimal medium, which can be supplied by complementation with the wild-type maize DHPS cDNA (5). Bacterial-expressed maize DHPS is highly inhibited by lysine (Fig. 1) and analogs such as AEC. Survival of transformed AT997 cells on high levels of AEC, therefore, requires a further alteration such as a mutation to lysine insensitivity within the maize DHPS cDNA. Approximately 80,000 viable EMS-treated AT997 cells transformed with the wild-type DHPS coding sequence were incubated in the presence of 5 or 10 mM AEC for 2 or 3 days before 720 putative AEC-resistant colonies were visible. DHPS in crude extracts from 146 of the faster growing colonies was assayed for inhibition by lysine, and 16 colonies that retained significant activity at 500 μM lysine were chosen for further analysis.

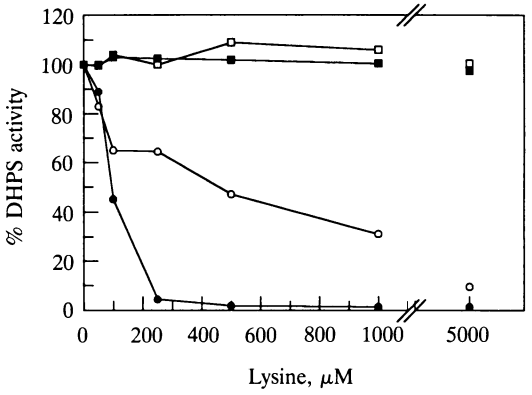

FIG. 1. Lysine inhibition of mutant and wild-type maize DHPS expressed by *E. coli dapA*$^-$ auxotrophic cells. □, Maize mutant mDHPS166av, 9 units; ■, maize mutant mDHPS157sn, 6 units; ●, wild-type maize, 10 units; ○, wild-type *E. coli*, 4 units (1 unit = 0.001 OD_{520} min^{-1}).

Plasmid DNAs from the 16 cell lines were isolated and transferred into nonmutagenized AT997 cells, and the DHPS activity of the maize DHPS again exhibited lysine insensitivity, confirming the plasmid-specific transmissibility and stability of altered DHPS expression.

Lysine inhibition curves representative of the 16 mutants are illustrated in Fig. 1. Partially purified mutant DHPS activity typically exhibited little or no inhibition at L-lysine concentrations ranging from 50 μM to 5 mM. In contrast, activity from control AT997 cells containing the wild-type maize DHPS cDNA was significantly inhibited in a concentration-dependent manner starting at 50 μM lysine (Fig. 1). Bacterial DHPS from nontransformed wild-type *E. coli* DH5α in these assays showed 50% inhibition at 500 μM lysine and 90% inhibition at 5 mM lysine. These assays showed that lysine-insensitive activity was retained by the 16 maize DHPS mutants even at 5 mM lysine, which is much higher than the concentration required to inhibit wild-type maize DHPS or the bacterial enzyme from wild-type *E. coli*.

The inhibition curves for DHPS activity from two of the other initially selected 130 colonies were indistinguishable from that of wild-type maize DHPS (data not shown), indicating these colonies were not bacterial genome revertants of the *dapA*$^-$ mutation. These colonies were not studied further, but they likely survived the AEC selection by alternative mechanisms unrelated to DHPS modification, such as an uptake mutation in the bacterial genome that prevented AEC from reaching an inhibitory concentration within the AT997 cells.

Point Mutations in cDNAs Encoding Lysine-Insensitive Maize DHPS. The initial DNA sequence analysis of the DHPS cDNA from one of the 16 mutant clones revealed a single point mutation at amino acid residue 166 and nucleotide 497 relative to the ATG start codon (Fig. 2*A*). The remaining 1139 nucleotides were identical to those in the wild-type maize DHPS cDNA sequence. To facilitate subsequent sequence analysis of the 15 remaining putative mutant cDNAs, each was first sequenced by using sequencing primers that covered the region around nucleotide 497. All 16 lysine-insensitive lines were found to have a single nucleotide mutation in the region bounded by nucleotides 470–497. As shown in Fig. 2*A*, these point mutations were clustered within a 10-amino acid region from residues 157 to 166 relative to the first methionine. The mutations affected only residues 157, 162, and 166; however, two different amino acid substitutions were found at residue 166. The mutation at residue 157 (designated mDHPS157sn) caused a Ser → Asn substitution and the mutation (mDHPS162ek) at residue 162 caused a Glu → Lys substitution. Of the two mutations at residue 166, one

A Maize wild-type and mutant sequences

Nucleotide #			470					484			496,497		
Residue #			157					162			166		
Mutation			A					A			AT		
Wildtype	ACA	GGA	AGC	AAC	TCA	ACC	AGA	GAA	GCC	GTC	CAC	GCA	ACA	GAA
	Thr	**Gly**	Ser	**Asn**	Ser	**Thr**	Arg	Glu	Ala	Val	His	Ala	Thr	Glu
Substitution			Asn					Lys			Thr,Val			

B Wild-type sequences from bacteria, wheat and soybean

	78	80	82										
E. coli	Thr	**Gly**	Ala	**Asn**	Ala	**Thr**	Ala	Glu	Ala	Ile	Ser	Leu	Thr	Gln
C. glut.	Val	**Gly**	Thr	**Asn**	Asn	**Thr**	Arg	Thr	Ser	Val	Glu	Leu	Ala	Glu
B. lact.	Val	**Gly**	Thr	**Asn**	Asn	**Thr**	Arg	Thr	Ser	Val	Glu	Leu	Ala	Glu
Wheat	Thr	**Gly**	Ser	**Asn**	Ser	**Thr**	Arg	Glu	Ala	Ile	His	Ala	Ser	Glu
Soybean	Thr	**Gly**	Ser	**Asn**	Ser	**Thr**	Arg	Glu	Ala	Ile	His	Ala	Thr	Glu
Conserved		*		*		*								

FIG. 2. (*A*) Four single nucleotide mutations identified in lysine-insensitive DHPS cDNA clones of maize expressed by EMS-mutagenized *E. coli dapA*$^-$ auxotrophic cells. The nucleotide and amino acid sequences shown are positions 463–504 and 155–168, respectively, relative to the maize DHPS initiator methionine (5). (*B*) Alignment of the corresponding amino acid sequences from *E. coli* (19), *C. glutamicum* (14), *Brevibacterium lactofermentum* (20), wheat (6), and soybean (21). Numbers 78, 80, and 82 correspond to the *E. coli* residues.

(mDHPS166at) caused an Ala → Thr substitution, and the other (mDHPS166av) resulted in an Ala → Val substitution.

The frequencies of the four mutations isolated from independently mutagenized *E. coli* populations are shown in Table 1. The mDHPS166av mutation was found in only one mutagenized cell population; in contrast, the mDHPS166at mutation was isolated a total of 11 times from five populations. The mDHPS157sn mutation occurred in one population that also included a second mDHPS166at mutation, and the mDHPS162ek mutation was isolated in two populations that included independent mDHPS166at mutations. Multiple isolations of the same mutation within the same mutagenized population were assumed to originate from one mutagenic event. Thus, a total of 10 mutations were obtained for the four mutation sites; five of these were the mDHPS166at mutation. Although this conservative estimate of independent mutations shows a decided tendency for the mDHPS166at site, the distribution of 1:2:2:5 is not statistically different ($\chi^2 = 3.6$; $P = 0.25$ to 0.50) from a random distribution.

In addition to the first clone (mDHPS166av), complete sequence analysis of clones representing each of the other three mutant classes showed that they contained no other mutations outside the region bounded by amino acids 157 and 166. These analyses confirmed that several different single amino acid substitutions within a limited region of the DHPS polypeptide sequence could result in complete loss of lysine inhibition. Although the remaining 12 mutant clones were not sequenced completely, it does not seem likely that other, as yet unidentified, mutations outside this region, either alone or in combinations, would be required to have a significant impact on lysine feedback inhibition of maize DHPS.

As expected for EMS mutagenesis, three of the four nucleotide substitutions (Fig. 2*A*) were G → A transitions in the coding strand and one was in the noncoding strand that resulted in a C → T transition in the coding strand. Two mutations were found at the first position within the codon and two were in the second position. Between nucleotides 470 and 498, there are three other guanosines subject to G → A transitions and 10 other cytosines subject to C → T transitions. Of these 13 potential mutation sites, 7 are in the first or second position and would result in amino acid substitutions. Alignment of the amino acid sequence of maize and five other DHPS genes (Fig. 2*B*) shows that this region contains three amino acids completely conserved among bacteria and plants. The failure to recover mutations at the other potential sites in this region may indicate either that the contribution of these amino acid residues to lysine binding is less critical than amino acid residues 157, 162, and 166 or that they are required to maintain enzyme conformation and activity.

Functionality of Maize DHPS Fusion Protein in *E. coli*. The DHPS cDNA (pZMDHPS5) encodes a fusion protein that contains an extra 90 amino acids at the amino terminus (5). For this cDNA to complement the *dapA*$^-$ auxotrophic cells, the expressed polypeptide need only confer sufficient enzyme activity to support lysine synthesis for growth. DHPS assays of crude extracts showed that AT997 cells transformed with wild-type or mutant maize DHPS cDNAs had about 12.5-fold more DHPS activity on a cell fresh weight basis than nontransformed wild-type cells (1.1 units/mg vs. 13.8 units/mg). Kinetic analyses indicated that the affinities for the substrates, pyruvate and aspartate semialdehyde, were similar for the wild-type and mutant maize DHPS fusion proteins expressed by AT997 cells (Table 2). Comparisons with data obtained for wild-type maize DHPS purified from maize cells (ref. 16 and D.A.F., unpublished data) also indicated that, except for lysine affinity, the properties of these DHPS fusion proteins expressed in *E. coli* were not much different from those of wild-type DHPS purified from maize cells.

Table 1. Number and distribution of four lysine-insensitive maize DHPS mutations recovered from EMS-mutagenized populations of *E. coli dapA*$^-$ auxotrophic cells

	No. of mutations			
Cell population	157sn	162ek	166at	166av
1	—	—	—	1
2	—	—	3	—
7	1	—	2	—
8	—	1	1	—
10	—	1	4	—
11	1	—	1	—
Total	2	2	11	1
No. independent	2	2	5	1

157sn, Ser-157 → Asn; 162ek, Glu-162 → Lys; 166at, Ala-166 → Thr; 166av, Ala-166 → Val.

Table 2. Comparison of kinetic properties of wild-type and mutant (mDHPS166av) maize DHPS fusion proteins partially purified from *E. coli dapA*$^-$ auxotrophic cells

	E. coli-derived		
Kinetic parameter	Wild-type DHPS	Mutant DHPS	Purified maize DHPS
K_m(Pyr), mM	6.0	4.5	12.2†
K_m(ASA), mM	0.6	0.9	0.8*
K_i(Lys), μM	<100	>5 × 10^3	<25*

K_m(Pyr), kinetic constant for pyruvate; K_m(ASA), kinetic constant for aspartate semialdehyde; K_i(Lys), inhibitor constant for L-lysine.
*Data from Frisch *et al.* (16).
†D.A.F., unpublished data.

DISCUSSION

DHPS catalyzes the condensation of pyruvate with aspartate semialdehyde to form dihydrodipicolinate, which is the initial metabolite unique to lysine biosynthesis in higher plants (1). Regulation of plant DHPS activity appears to primarily involve endproduct inhibition by lysine with concentrations required for half-maximal inhibition of purified enzyme reported in the low micromolar range (16, 22–24).

This report demonstrates that random mutagenesis and selection in an *E. coli* DHPS auxotroph is an effective strategy for isolating feedback-insensitive mutations in a plant DHPS cDNA. Although the colonies obtained in the initial AEC selection contained DHPS mutants, they also likely included mutants that had reduced AEC uptake. It was readily feasible, however, to screen selected colonies to identify those with reduced lysine inhibition of DHPS activity. The maize DHPS cDNA, which encoded a fusion protein with an extra 90 amino acids at the amino terminus, was expressed as an active enzyme. Our initial comparisons indicated that the maize wild-type and mutant DHPS fusion proteins expressed by the AT997 auxotroph did not differ significantly in their substrate affinities (Table 2). Because we were most interested in identifying the sites of mutations and their effect on lysine inhibition, further purification and detailed kinetic studies were not conducted with these fusion protein versions of maize DHPS. However, analyses of maize cDNAs constructed to allow recovery of only the mature DHPS polypeptide showed that the K_m value for pyruvate was about 60% higher for the mDHPS166av mutant than for wild-type maize DHPS (J.M.S., unpublished data). Except for lysine binding, the other kinetic parameters appeared unchanged.

The 16 maize DHPS mutants obtained in this study are highly insensitive to lysine inhibition and, thus, are similar to the wild-type *C. glutamicum* DHPS (13) but differ from wild-type *E. coli* DHPS, which is inhibited about 50% by 0.5–1.0 mM lysine (Fig. 1) (15). The fact that these mutations affected amino acid substitutions at only three residues (residues 157, 162, and 166) within a 10-amino acid region (Fig. 2*A*) indicates that this region is essential for feedback inhibition by lysine. Identifying these specific mutations that change the highly sensitive maize DHPS to a lysine-insensitive isoform should lead to resolution of the lysine-binding mechanism and to the resultant conformational changes involved in inhibition of DHPS activity.

Purified *E. coli* DHPS has been crystallized and the three-dimensional conformation of the homotetramer has been described (25). Each monomer is composed of an α/β-barrel and a carboxyl-terminal α-helical domain; and each of the four monomers interacts differentially with two other monomers to form a planar homotetramer. Maize and *E. coli* DHPS have 32% amino acid identity (5, 19). Alignment of DHPS sequences from *E. coli* (19), *C. glutamicum* (14), *B. lactofermentum* (20), wheat (6), soybean (21), and maize (5), however, revealed a number of conserved amino acids in the α/β-barrel for plants and bacteria (25). The invariant lysine residue at position 161 in *E. coli* (position 237 in maize) is required for binding pyruvate (26); the binding domain for aspartate semialdehyde has not been identified. The *E. coli* DHPS $\beta 3$–$\alpha 3$ loop (25) contains a conserved glycine at position 78 and a conserved asparagine at position 80 (residues 156 and 158 in maize); a conserved threonine is located at position 82 at the start of the $\alpha 3$-helix (position 160 in maize) (Fig. 2*B*). Thus, the maize mutations at residues 157 and 162 are between or adjacent to three conserved amino acid residues. The conserved glycine and threonine would have been amenable to EMS-induced transitional mutations; perhaps the lack of recovery of such mutations indicates a functional requirement for these residues that might override any influence on lysine binding. The crystallographic structural analysis showed that the glycine residue (position 78) participates in the formation of a channel from the outside of the tetramer to the pyruvate-binding site (25).

The availability of several different point mutations in the maize DHPS gene provides new options for gene transfer experiments to modify lysine synthesis in plants. As the initial step in determining whether a modified maize DHPS enzyme could alter lysine synthesis, we have produced transgenic maize cell cultures containing the mutant mDHPS166av gene (27). Based on the reduction in lysine inhibition of total DHPS activity and analyses of free lysine concentrations in verified transgenic cell lines, we concluded that the modified maize gene was expressed and resulted in moderately higher concentrations of free lysine (27). This result is consistent with reports of increases in free lysine or total lysine concentrations in tobacco, potato, soybean, and canola plants transformed with the *E. coli* or *C. glutamicum dapA* genes (13, 28–31).

The physiological consequences of increasing the capacity for lysine synthesis and accumulation are not yet known for transgenic maize plants. In future applications of genetic modification of maize via transformation with a maize mutant DHPS gene, it is likely that attention will need to be given to a seed-specific promoter and to the possibility of increased lysine catabolism due to a parallel induction of higher levels of lysine ketoglutarate reductase (32, 33). The derivation of maize DHPS genes with specific single-amino acid substitutions affecting feedback regulation in the otherwise wild-type DHPS, as described in this study, will make possible a number of additional studies ranging from inhibitor binding and x-ray crystallographic analysis to genetic improvement of lysine biosynthesis capacity in maize and other agriculturally significant crops.

We dedicate this publication to the memory of Janita M. Sellner (1967–1990) in recognition of her brief, but productive and promising, scientific career. Cooperative investigation of the Minnesota Agricultural Experiment Station Projects 4813-32 and 4813-56. This material is based on work supported by the Cooperative State Research Service, U.S. Department of Agriculture, under Agreement 89-37262-4360 and Purdue/593-0038-06, by NATO Collaborative Research Grants Programme 900601, and by Pioneer Hi-Bred International, Inc. Minnesota Agricultural Experiment Station Publication 21,988.

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