

The Role of *Opaque2* in the Control of Lysine-Degrading Activities in Developing Maize Endosperm

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We have isolated a cDNA clone, designated *ZLKRSDH*, encoding the bifunctional enzyme lysine–ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) from maize. The predicted polypeptide has an N-terminal LKR domain and a C-terminal SDH domain that are similar to the yeast *LYS1* and *LYS9* monofunctional proteins, respectively. The maize LKR/SDH protein is located in the cytoplasm of subaleurone endosperm cell layers. Transcripts and polypeptides as well as enzyme activities showed an upregulation and downregulation during endosperm development. The developmental expression of *ZLKRSDH* was examined in normal and *opaque2* seeds. In the mutant endosperm, mRNA levels were reduced by >90%, with concomitant reductions in polypeptide levels and LKR/SDH activity. These results suggest that lysine levels in the endosperm are likely to be controlled at the transcriptional level by the *Opaque2* transcription factor.

INTRODUCTION

Since the discovery of the high-lysine *opaque2* (*o2*) maize mutant (Mertz et al., 1964), research efforts have been directed toward understanding the biochemical and molecular mechanisms leading to the increase in lysine content in the endosperm. Studies conducted during the past 30 years revealed that the homozygous *o2* mutation causes an ~70% reduction in zein content and affects the content of a number of proteins and enzymes related to nitrogen and sugar metabolism in the maize endosperm (Habben et al., 1993; Giroux et al., 1994; Gallusci et al., 1996). The cloning of the *O2* gene revealed that it encodes a basic leucine zipper protein transcription factor involved in the transcriptional control of the zein genes, the b-32 ribosome-inactivating protein, and cytoplasmic pyruvate orthophosphate dikinase (cyPPDK), an enzyme involved in carbon partitioning (Lohmer et al., 1991; Bass et al., 1992; Schmidt et al., 1992; Cord Neto et al., 1995; Gallusci et al., 1996). Lysine catabolism also is affected in the *o2* mutant. ¹⁴C-lysine feeding experiments revealed that lysine was converted to glutamic acid and proline but to a much lesser extent in *o2* than in normal endosperm (Sodek and Wilson, 1970).

Despite the observation of extensive lysine degradation in maize seeds (Sodek and Wilson, 1970; da Silva and Arruda,

1979), there have been several attempts to establish positive selection strategies to isolate mutants with aspartate kinase (AK) insensitivity to feedback inhibition caused by lysine. AK is the first regulatory enzyme of the aspartate pathway, which leads to the biosynthesis of lysine, methionine, threonine, and isoleucine. Thus, the derepression of AK would result in an overproduction of these amino acids (reviewed in Azevedo et al., 1997). AK-insensitive mutants that overproduce threonine have been described in barley (Bright et al., 1982), maize (Diedrick et al., 1990), and tobacco (Frankard et al., 1992), but they exhibit only marginal effects on lysine accumulation. The absence of lysine overproduction in these mutants was attributed to the second regulatory enzyme, dihydrodipicolinate synthase (DHDPS), which is the first enzyme of the lysine branch of the aspartate pathway. This enzyme is much more sensitive to lysine feedback inhibition than is AK (reviewed in Azevedo et al., 1997). A few experiments succeeded in isolating lysine-insensitive DHDPS mutants, but the seeds from these mutants did not show a significant increase in lysine (Negrutiu et al., 1984). AK/DHDPS-insensitive double mutants did not have a significant increase in lysine content either (Frankard et al., 1991).

Recently, transgenic tobacco and canola plants expressing lysine-insensitive AK or DHDPS or both enzymes were produced. These transgenic plants showed threonine overproduction and appreciable free lysine increments in young leaves and seeds. These mutants, however, displayed severe

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phenotypic alterations (Karchi et al., 1993; Shaul and Galili, 1993; Falco et al., 1995; Pederson et al., 1996), and lysine overproduction was followed by increased lysine degradation (Karchi et al., 1994; Falco et al., 1995).

We have postulated that lysine catabolism is an important mechanism for the control of free lysine levels in maize endosperm cells (Arruda and da Silva, 1979; da Silva and Arruda, 1979). Lysine degradation in maize endosperm is catalyzed by lysine-ketoglutarate reductase (LKR; Arruda et al., 1982). During kernel development, LKR activity is coordinated with nitrogen input and zein protein synthesis (Arruda and da Silva, 1983). Further analysis of the developmental pattern of LKR activity in normal and *o2* endosperm showed that enzyme activity is decreased in the mutant, suggesting that reduced lysine degradation in the *o2* endosperm could be due to a lower content of LKR polypeptides (Brochetto-Braga et al., 1992).

Maize LKR was purified to homogeneity and demonstrated to be associated with saccharopine dehydrogenase (SDH) in a bifunctional 125-kD polypeptide (Gonçalves-Butruille et al., 1996), similar to the previously isolated mammalian enzyme (Markovitz and Chuang, 1987). The complete genomic and cDNA sequences recently reported for the Arabidopsis LKR/SDH gene confirm the bifunctional nature of the enzyme (Epelbaum et al., 1997; Tang et al., 1997).

To further study the influence of the *o2* mutation on lysine accumulation in the developing maize endosperm, we cloned the gene encoding the maize bifunctional LKR/SDH (designated *ZLKRSDH* for *Zea mays* LKR and SDH) and examined its spatial and temporal patterns of expression. We found that the maize LKR/SDH is a cytosolic enzyme encoded by a single gene that is highly expressed in the subaleurone cell layers of the distal part of the developing endosperm. In the *o2* mutant, *LKR/SDH* mRNA and protein quantities are severely reduced, and the expression pattern during kernel development is markedly modified.

RESULTS

Isolation of a cDNA Encoding the Maize LKR/SDH Bifunctional Enzyme

LKR/SDH was purified to homogeneity from 17-days after pollination (DAP) maize endosperm, and the N-terminal sequences of four tryptic peptides were used to design a set of 21- to 23-mer degenerate oligonucleotides. These primers and the cDNA produced from reverse-transcribed 17-DAP total endosperm RNA were used in reverse transcription-polymerase chain reactions (RT-PCRs). A 1.2-kb DNA fragment was amplified and confirmed to be part of the *ZLKRSDH* gene by comparing its sequence with the nucleotide sequences of the yeast *Lys1* and *Lys9* genes, which encode LKR and SDH, respectively. This fragment was used as a probe to screen an immature endosperm cDNA library

(Aukerman et al., 1991). The nucleotide sequences from 20 positive clones were analyzed, but they either contained mostly the SDH domain or were chimeric at their 5' ends. To obtain a full-length clone, we isolated the 5' end of the cDNA by using rapid amplification of cDNA ends (RACE), and the 3.5-kb cDNA was further amplified using primers annealing to the 5' and 3' untranslated regions.

As shown in Figure 1A, the amplified cDNA encodes an open reading frame of 1064 amino acids, which predicts a protein of 116.8 kD, close to the maize 125-kD polypeptide observed after SDS-PAGE (Gonçalves-Butruille et al., 1996). The 5' untranslated region is 178 bp long and presents two in-frame ATG codons. The first one does not present a good initiation context and is followed by a stop codon. On the other hand, the second ATG codon is in a favorable context for translation initiation (Luehrsen and Walbot, 1994). The 3' untranslated region is 136 bp long and has two putative polyadenylation signals (AATAAA) located 93 and 102 bp downstream from the TAG stop codon (data not shown). Residues H-110, K-113, and R-146 of the maize LKR domain (Figure 1B) are conserved in all LKR sequences obtained to date. These residues have been shown to be essential for substrate binding in the yeast LYS1 protein (Ogawa et al., 1979; Fujioka et al., 1980; Ogawa and Fujioka, 1980; Fujioka and Takata, 1981).

Figure 2 shows a schematic representation of similarities between the predicted maize polypeptide and other known monofunctional and bifunctional LKR/SDH enzymes. A SDH C-terminal domain sharing 42% similarity with the yeast LYS9 protein and an N-terminal domain sharing 27% similarity with the yeast LYS1 protein constitute the bifunctional maize enzyme. Similarities to the related mouse (F. Papes, E.L. Kemper, G. Cord Neto, F. Langone, and P. Arruda, submitted manuscript), *Caenorhabditis elegans*, and Arabidopsis proteins are significantly higher (44, 46, and 72%, respectively). The interdomain region (Figure 1A), which is ~106 residues long in maize, is 57% identical to the corresponding Arabidopsis sequence. Interestingly, this region seems to be absent in *C. elegans* and mouse LKR/SDH proteins (Figure 2).

Comparisons of primary protein structures also reveal several conserved motifs (Figure 2). The initiation ATG and stop codons, as well as some motifs with a high degree of similarity, appear at comparable sites in the open reading frame of all compared proteins.

To confirm the structure of the LKR/SDH bifunctional polypeptide, we separated the LKR and SDH domains by limited proteolysis (Figure 3). A partially purified preparation of LKR/SDH protein was chromatographed on an ion exchange column (Figure 3A) or digested with elastase before chromatography (Figure 3B). The most active fractions of LKR and SDH peaks from Figure 3B were immunoblotted with anti-LKR/SDH polyclonal antibodies (Figure 3C). Functionally isolated LKR and SDH domains were recovered, confirming the bifunctional enzyme structure (Gonçalves-Butruille et al., 1996; Kemper et al., 1998).

A

1 MGSAAATEGNDTLLGNGVVGILAEETCNMWER
 31 RAPLTPSHCARLLGGGKNGPRVNR IIVQP
 61 STRRIHHD AQYEDAGCEI SEDLSECGLIIG
 91 IKQPKLQ MILSDRAYAFFS**HTHKAQ**KENMP
 121 LLDKILEERVSLFDYELIVGDDGKRSLAFG
 151 KFAGRAGLIDFLHGLGQRYLSLGYSTPFLS
 181 LGQSHMYPSLAAAKAAVIVVAEEIATFGLP
 211 SGICPIVVFVTG VGNVSQGAQE IFKLLPHT
 241 FVDAEKLP EIQARNLSKQSQSTKR V FQLY
 271 GCVVTSRDMVSHKDPTRQFDKGDYYAHPEH
 301 YTPVFHERIAPYASVIVN C MYWEKRFPPL
 331 NMDQLQQLMETGCP LVGVCDITCDIGGSIE
 361 FINKSTSIERPFFRYDPSKNSYHDDMEGAG
 391 VVCLAVDILPTEFSKEASQHF GNILSRLVA
 421 SLASVKQPAELPSYLR RACIAHAGRLTPLY
 451 EYIPRMRNTMIDLAPAKTNPLPDKKYSTLV
 481 SLSGHLFDKFLINEALDIETAGGSFHLVR
 511 CEVGQSTDDMSYSELEVGADDTATLDKIID
 541 SLTSLANEHGGDHDAGQEIELALKIGK VNE
 571 YETDVTIDKGGPKILILGAGRVC RPAAEFL
 601 ASYDICTYGVDDHDADQIHVIVASLYQKD
 631 AEETVDGIEN TTATQLDVADIGSLSDLVSQ
 661 VEVVISLLPASFHAAIAGVCI ELKHKM VTA
 691 SYVDESMSNLSQA AKDAGVTILCEMGLDPG
 721 IDHLSM MKMIDEAHARKGKIKAF TSYCGGL
 751 PSPAAANNPLAYKFSWNPAGALRS GKNPAV
 781 YKFLGETI HV DGHNLYESAKRLRLRELPAF
 811 ALEHLPNRNSLIYGDLYGISKEASTIYRAT
 841 LRYEGFSEIMVTL SKTGFFDAANHPLLQDT
 871 SRPTYKGFLELLNNISTINTDL D IEASGG
 901 YDDDLIARLLKLGCCCKNKEIAVKT V KTIKF
 931 LGLHEETQIPKGCSSPFDVICQRMEQRMAF
 961 GHNEQDMVLLHHEVEVEY PDGQPAEKHQAT
 991 LLEFGKVENGRSTTAMALTVGIPAAIGALL
 1021 LLKNKVQTKGVIRPLQPEIYVPALEI LESS
 1051 GIKLVEKVETKFPE

B

ZEA FFS**HTHKAQ**KENMPLLDKILEERVSLFDYELIVGDDGKRSLAFG
 ATH FFS**HTHKAQ**KENMPLLDKILSERVTLCDYELIVGDHGKRLLAFG
 CEL FFS**HTIKAQ**QDNMEMLDITLQRNIRLLDYEKICDDKGRRLVMFG
 CAL QFAHCYKDQAGWQDVLKRFPQGNIGILYDLEFLENDQGRRVAAFG
 YLI QFAHCYKDQGGWKDVLRSFPAGNGTLYDLEFLEDDNGRRVAAFG
 SCE QFAHCYKDQAGWQNVLMRFIKGHGTLYDLEFLENDQGRRVAAFG
 *

Figure 1. Predicted Sequence of the *ZLKRSDH* cDNA-Encoded Protein.

(A) The protein sequence encoded by the 3.5-kb *ZLKRSDH* cDNA is shown. Underlined residues highlight the interdomain region. Conserved residues shown in (B) are in boldface. The GenBank accession number is AF003551.

(B) Alignment of the LKR subdomain that is homologous to the putative substrate binding site previously assigned to the yeast LYS1 protein. GenBank accession numbers are listed in the legend to Fig-

ZLKRSDH Is Present in Maize and Related Species

We investigated the presence of the *ZLKRSDH* gene in maize and related cereals. The 1.2-kb RT-PCR DNA fragment, comprising part of the LKR domain, the interdomain region, and part of the SDH domain (residues 444 through 849; Figure 1), was hybridized to genomic DNA from maize, Coix, and sorghum. As shown in Figure 4, both BamHI and EcoRV digests gave single, strong hybridizing bands, suggesting that *ZLKRSDH* is likely to be present as a single-copy gene in *Andropogoneae* sp.

Analysis of *ZLKRSDH* mRNA Levels in Different Maize Tissues

Total RNA extracted from roots, leaves, coleoptiles, embryos, and endosperm was hybridized with either LKR- or SDH-specific probes. As shown in Figure 5, *ZLKRSDH* mRNA was detected mainly in the endosperm. No signal was detected in embryos, and only marginal amounts of expression were detected in roots, leaves, and coleoptiles. Five different transcripts were detected in 20-DAP developing endosperm with both probes. Along with the major 3.5-kb band, which corresponds to >90% of the total hybridizing mRNA, alternate transcripts of 4.1, 2.6, 2.4, and 1.5 kb were detected. The 4.1- and 3.5-kb transcripts were detected equally by both probes. The 2.4- and 2.6-kb species seemed to contain mainly SDH sequences (Figure 5B), because they hybridized only weakly with the LKR-specific probe (Figure 5A), whereas the 1.5-kb transcript seemed to contain only LKR sequences (Figure 5A). The nature and function of these alternate transcripts are not known, but they certainly do not encode monofunctional LKR or SDH enzymes because in the results shown in Figure 3, only one polypeptide product was detected in the endosperm by the anti-LKR/SDH polyclonal antibodies. This polypeptide is able to recognize with similar efficacy both the LKR and SDH domains.

Developmental Expression of *ZLKRSDH* in Normal and $\alpha 2$ Mutant Endosperm

We showed previously that LKR activity is reduced two- to threefold in the $\alpha 2$ endosperm (Brochetto-Braga et al., 1992). To test whether this lower enzyme activity was due to

ure 2. The essential conserved residues H-110, K-113, and R-146 of the maize protein are shown in boldface characters. Identical residues are marked by asterisks. ZEA, *Z. mays*; ATH, *Arabidopsis thaliana*; CEL, *C. elegans*; CAL, *Candida albicans*; YLI, *Yarrowia lipolytica*; SCE, *Saccharomyces cerevisiae*.

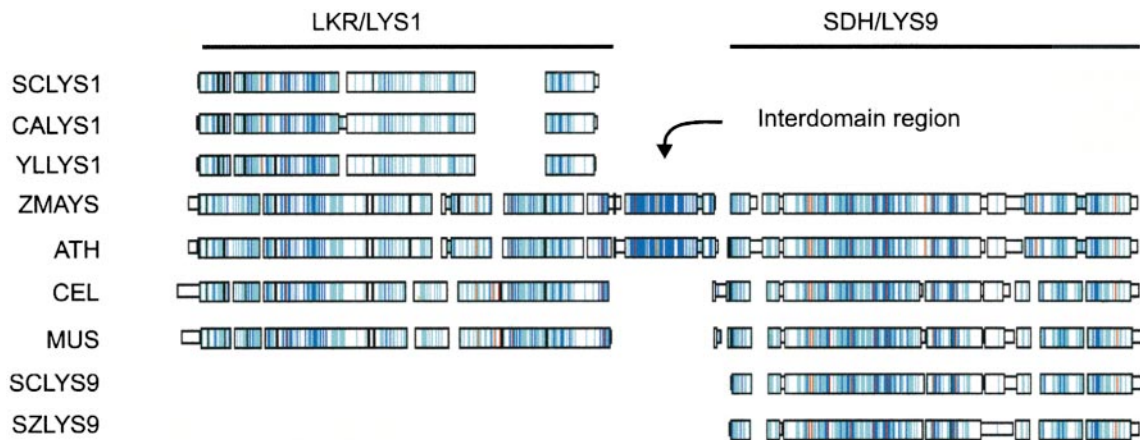


Figure 2. Schematic Diagram of Similarities among Monofunctional and Bifunctional LKR/SDH Polypeptides from Yeast, Plants, and Animals.

The predicted amino acid sequences for monofunctional saccharopine dehydrogenase (LYS1, lysine forming) and saccharopine dehydrogenase (LYS9, glutamate forming) from yeast were aligned to plant, nematode, and mammalian bifunctional LKR/SDH polypeptides. Sequence alignments and similarity analysis were performed using the MACAW program applying the BLOSUM 62 matrix. The similarity at a given position is designated by the following color codes: red, >90%; blue, 50 to 90%; cyan, 10 to 49%; and white, <10%. Similarity at a given position means the percentage of sequences being compared that match the consensus sequence. The LKR and SDH domains, corresponding to the yeast LYS1 and LYS9 proteins, respectively, as well as the unique interdomain region present in plant bifunctional polypeptides, are indicated. Blank spaces indicate sequence gaps introduced to maximize the alignment. Nomenclature and GenBank accession numbers are as follows: SCLYS1, *S. cerevisiae* (P38998); CALYS1, *C. albicans* (P43065); YLLYS1, *Y. lipolytica* (P38997); ZMAYS, *Z. mays* (AF003551); ATH, *A. thaliana* (U95759); CEL, *C. elegans* (AF038615); MUS, *Mus musculus* (AJ224761); SCLYS9, *S. cerevisiae* (P38999); and SZLYS9, *Schizosaccharomyces pombe* (AL022244).

reduced expression of the *ZLKRSDH* gene, we assayed normal and *o2* seeds, harvested at 10, 15, 20, 25, 30, 35, and 40 DAP, for enzymatic activities and analyzed them by RNA gel blotting and immunoblotting using anti-LKR/SDH polyclonal antibodies (Kemper et al., 1998).

As shown in Figure 6, transcripts and polypeptides as well as enzyme activities showed an upregulation and a down-regulation during endosperm development. In normal endosperm, LKR and SDH activities and LKR/SDH protein levels reached a maximum at 20 DAP, whereas in the mutant endosperm, maximum polypeptide levels and enzyme activities were observed at 25 DAP. *ZLKRSDH* mRNA amounts in normal endosperm peaked at 15 DAP, whereas in *o2* endosperm, the mRNA amount peaked at 20 to 25 DAP.

At 20 DAP, LKR and SDH activities in the mutant were reduced to ~2 and 18%, respectively, of the values in normal seeds (Figure 6A). This was accompanied by an ~90% reduction in the amounts of transcript and polypeptide (Figures 6B and 6C). At 25 DAP, the polypeptide amount increased in the mutant in comparison to the wild type, with a concomitant increase in LKR and SDH activities to ~9 and 50%, respectively.

The correlation between enzyme activities and quantitative immunoblot band intensities, for both normal and *o2* endosperm, was 94% for SDH and 81% for LKR activity (data not shown). This indicates that decreased enzyme activity is due to reduced polypeptide levels resulting from a reduced

transcript accumulation from the *ZLKRSDH* gene in the mutant endosperm.

LKR/SDH Is Located in the Cytosol of Subaleurone Cell Layers

Histochemical staining for SDH activity was used to locate the LKR/SDH protein within the endosperm. SDH activity was detected in the peripheral distal part of the endosperm (Figures 7A and 7B). An immunological study of LKR/SDH was conducted to further characterize the cells currently expressing the bifunctional enzyme (Figures 7C to 7F). The immunolabeled region was similar to that stained for SDH activity. Expression of LKR/SDH is most intense in the subaleurone layers and surrounding the nuclei and starch grains. In cells far away from the subaleurone cell layers, the immunostaining was weak and tended to be near the peripheral cell regions, adjacent to cell walls. No immunolabeling was detected in the aleurone layer.

Subcellular fractionation was used to determine the cell compartmentalization of LKR/SDH. As shown in Figure 8, LKR and SDH activities were only detected in the cytosol, whereas the activities of the marker enzymes, cytochrome *c* oxidase and cytochrome *c* reductase, were observed in the endoplasmic reticulum and mitochondria, respectively.

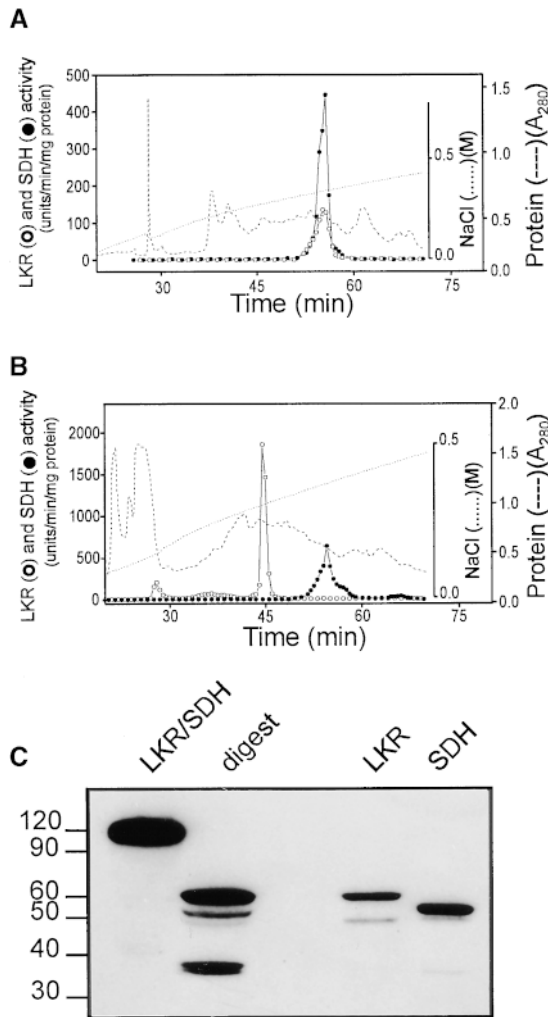


Figure 3. Delimitation of Maize Bifunctional LKR and SDH Domains.

(A) and (B) Elution profiles for LKR and SDH activities before (A) and after (B) digestion with elastase. A partially purified DEAE-Sepharose preparation of LKR/SDH was digested with elastase, applied to a Protein-Pak Q 8HR column, and eluted with a linear gradient of 0 to 5 M NaCl in buffer B at a flow rate of 1.5 mL/min (see Methods for further details). Fractions of 0.75 mL were collected and assayed for LKR and SDH activities. Absorbance at 280 nm is indicated at right.

(C) Immunoblot of native and elastase-digested LKR/SDH. Equivalent aliquots of the most active fractions of LKR and SDH peaks were applied to a 6 to 18% gradient SDS-polyacrylamide gel. The separated proteins were blotted onto nylon membranes and incubated with anti-LKR/SDH polyclonal antibodies. Numbers at left correspond to the molecular mass markers of the 10-kD protein ladder (Gibco BRL). The lane labeled LKR/SDH contains native enzyme; digest, elastase digestion products; LKR, LKR peak; SDH, SDH peak.

DISCUSSION

The biochemical and molecular mechanisms by which the $\alpha 2$ mutation increases lysine content in the endosperm have been investigated during the past 30 years. Both lysine synthesis and degradation as well as its incorporation into lysine-rich proteins should contribute to the final content of this amino acid in the seed. In this report, we describe the isolation of a maize cDNA clone encoding the lysine-degrading enzyme LKR/SDH and present evidence on how lysine levels may be controlled in the endosperm. A conspicuous correlation between lysine-degrading activities and transcriptional regulation by the Opaque2 transcription factor was observed. LKR/SDH seems to be located within cells in which zein protein is being actively synthesized.

The Bifunctional Maize LKR/SDH Enzyme

A cDNA encoding the bifunctional enzyme LKR/SDH was isolated from the immature maize endosperm mRNA pool. The cDNA predicts a 117-kD protein (Figure 1) bearing distinct N- and C-terminal domains identified, respectively, as LKR and SDH due to similarities to yeast monofunctional enzymes (SDH lysine- and glutamate-forming enzymes encoded by the *Lys1* and *Lys9* genes, respectively). Further confirmation of the identity of the maize cDNA was obtained by comparing sequences from similar genes recently isolated from Arabidopsis (Epelbaum et al., 1997; Tang et al., 1997) and mouse (F. Papes, E.L. Kemper, G. Cord Neto, F. Langone, and P. Arruda, submitted manuscript).

In plants and mammals, the saccharopine pathway is used for lysine catabolism, whereas in yeast and other fungi, this pathway is used in the reverse order for lysine biosynthesis. Either bifunctional (maize, soybean, Arabidopsis, bovine, murine, and human) or monofunctional (yeast, rat, and Arabidopsis) polypeptides operate in this pathway. As illustrated in Figure 2, the amino acid sequences from these enzymes share a high degree of similarity. Nevertheless, there are marked differences between bifunctional LKR/SDH polypeptides from plants and animals.

First, the enzyme seems to be expressed in different cell compartments. The mammalian LKR and SDH enzymes have been located in the mitochondrial fraction (Markovitz et al., 1984; Ameen et al., 1987; Blemings et al., 1994), whereas the maize enzyme seems to be located in the cytosol (Figure 8). It is interesting that neither the predicted maize LKR/SDH (Figure 1) nor the Arabidopsis protein (Epelbaum et al., 1997) contains putative mitochondrial or chloroplast targeting sequences.

Second, the interdomain region is present in plants but seems to be absent from the animal enzymes (Figure 2). This region is 57% identical in maize and Arabidopsis, but its functional role remains unknown. Limited proteolysis studies with the maize LKR/SDH suggest that it might be involved in

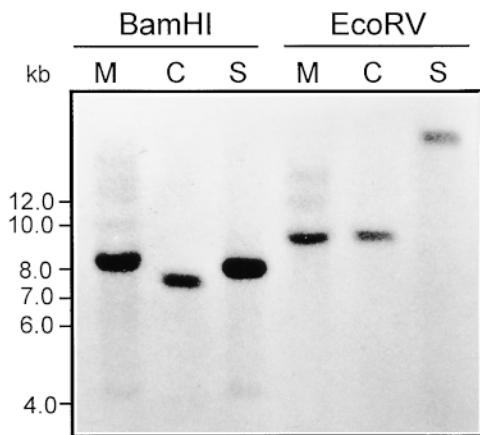


Figure 4. DNA Gel Blot Analysis of *ZLKRSDH* in Maize, Coix, and Sorghum.

Ten micrograms of DNA isolated from maize, Coix, and sorghum was digested with BamHI and EcoRV, resolved in a 1% agarose gel, transferred onto a nylon membrane, and hybridized under high-stringency conditions with the LKR/SDH 1.2-kb RT-PCR cDNA fragment as a probe. Restriction fragments hybridizing to the probe are present in maize (M), Coix (C), and sorghum (S). The migration of molecular length markers is indicated at left in kilobases.

the modulation of LKR activity (Kemper et al., 1998). Other evidence for the role of the interdomain region on the modulation of LKR activity came from studies of the effect of Ca^{2+} and ionic strength on maize and bovine enzymes. Maize LKR is activated by Ca^{2+} and high salt concentrations, whereas bovine LKR is insensitive to these modulators (Kemper et al., 1998).

Tissue-Specific and Developmental Expression of *ZLKRSDH*

RNA gel blot analysis of total RNA from 20-DAP endosperm revealed multiple hybridizing bands ranging in size from 1.5 to 4.1 kb (Figure 5). The 3.5-kb transcript, which accounts for >90% of total *ZLKRSDH* transcribed mRNA, has the expected size of the isolated cDNA shown in Figure 1. The alternate transcripts may originate from a single gene, and whether they have any functional relevance is still unknown. Tang et al. (1997) have shown that Arabidopsis cells contain mRNA species, encoding bifunctional LKR/SDH and monofunctional SDH, likely to be transcribed from a single gene. Bifunctional LKR/SDH and monofunctional SDH also were observed in mouse (F. Papes, E.L. Kemper, G. Cord Neto, F. Langone, and P. Arruda, submitted manuscript). In maize, however, the sole protein product detected corresponds to a single protein band of 125 kD exhibiting both LKR and SDH activities (Brochetto-Braga et al., 1992; Gonçalves-Butruille et al., 1996; Kemper et al., 1998). In addition, the

immunoblot results shown in Figures 3 and 6 clearly demonstrate the presence of a single 125-kD polypeptide in the maize endosperm cells, and the results shown in Figure 3 clearly demonstrate equal affinity of the LKR/SDH polyclonal serum for the LKR and SDH domains.

Detection of LKR and SDH activities in plants has been reported only for developing seeds (Arruda and da Silva, 1983; Karchi et al., 1994; Gaziola et al., 1997) and Arabidopsis cell suspension cultures (Tang et al., 1997). Although LKR/SDH-encoding transcripts were detected in several organs in Arabidopsis, they were most abundant in the ovary and the em-

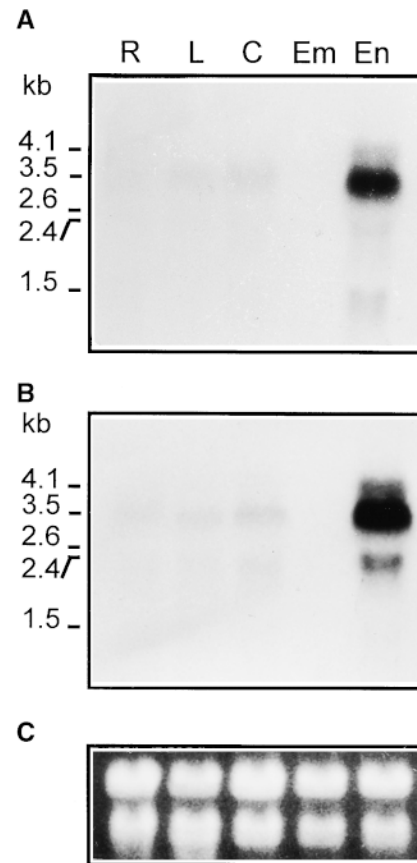


Figure 5. RNA Gel Blot Analysis of LKR/SDH-Endcoding mRNA in Different Maize Tissues.

(A) and (B) Twenty micrograms of total RNA from roots (R), leaves (L), coleoptiles (C), embryos (Em), and 20-DAP normal endosperm (En) was fractionated in a 1% agarose gel containing 0.66 M formaldehyde, transferred to a nylon membrane, and hybridized with an LKR domain probe (A). After 48 hr of exposure, the membrane was stripped and hybridized against an SDH domain probe (B). The lengths of the detected LKR/SDH transcripts are shown at left in kilobases. A better resolution of the 2.4- and 2.6-kb bands is shown in Figure 6C.

(C) Loading control performed by ethidium bromide staining.

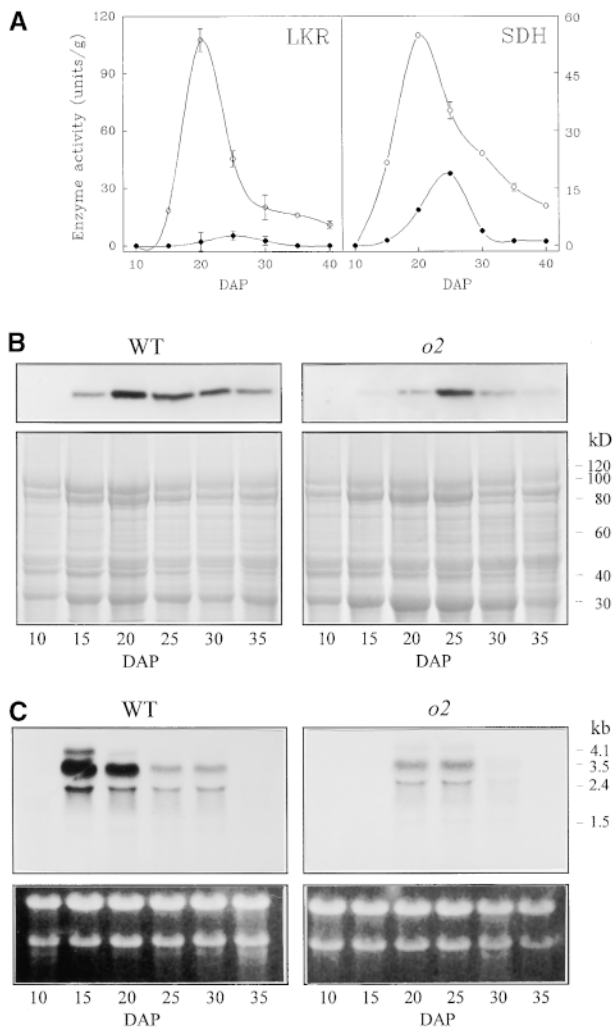


Figure 6. Developmental Expression of LKR/SDH in Wild-Type and *o2* Endosperm.

(A) Temporal patterns of LKR and SDH activities in normal and *o2* endosperm. Seeds of wild type (open circles) and *o2* (closed circles) were harvested from 10 to 40 DAP at 5-day intervals. Equal amounts of endosperm were homogenized in buffer A and fractionated with ammonium sulfate. The fractions collected between 35 and 60% saturation were assayed for LKR and SDH activities. Each point is the average of duplicate assays, and the bar indicates the standard deviation.

(B) Immunoblot showing LKR/SDH protein temporal profile in normal and *o2* endosperms. Aliquots of wild-type (WT) and *o2* ammonium sulfate-fractionated extracts from **(A)** were dialyzed against buffer A, separated in a 7% SDS-polyacrylamide gel, blotted onto nylon membranes, and incubated with anti-LKR/SDH polyclonal antibodies at a 1:10,000 (v/v) dilution. At top are the immunoblots, and at bottom are the protein loading controls stained with Coomassie blue. Numbers at right correspond to the molecular mass markers of the 10-kD protein ladder (Gibco BRL).

(C) *ZLKRSDH* gene expression during development of normal and *o2* endosperm. Fifteen-microgram samples of total RNA extracted

bryo (Tang et al., 1997). In maize, *ZLKRSDH* mRNA is abundant in the endosperm but is completely absent in the embryo and scarcely detectable in roots, leaves, and coleoptiles (Figure 5). Enzymatic activity, indeed, was not detected in those organs. Furthermore, endosperm specific activity is ~30-fold higher in maize than in soybean and common bean (data not shown), suggesting that LKR/SDH may play an important physiological role in maize endosperm.

Histochemical and immunological assays revealed that LKR/SDH is highly expressed in the subaleurone cell layers (Figure 7). This region comprises cells that are actively expressing zein genes at a high rate (Dolfini et al., 1992). These cells probably have a low lysine requirement because zein proteins, which represent >70% of total endosperm protein, are devoid of lysine residues. Thus, we suggest that LKR/SDH activity is important to regulate lysine levels in this narrow area of the endosperm.

In addition, it could be possible that some product arising from lysine degradation could regulate cellular and developmental processes operating in subaleurone cells. Interestingly, it has been suggested that lysine degradation may have an influence on the growth of the mammalian brain, because LKR is highly active during embryonic rat brain development (Rao et al., 1992).

Effects of the *o2* Mutation on *ZLKRSDH* mRNA Amounts

Altered lysine catabolism (Sodek and Wilson, 1970; Arruda and da Silva, 1979) may be one of the mechanisms by which the *o2* mutation creates a high-lysine maize phenotype. Most of the lysine in the endosperm seems to originate from lysine-rich proteins, which are more abundant in *o2* than in normal endosperm (Habben et al., 1993). Synthesis of lysine-rich proteins presumes the existence of increased availability of free lysine to be incorporated. We previously observed increased contents of lysine-rich proteins in the double homozygous mutant *o2 Ask1* (Azevedo et al., 1990). This was attributed to increased lysine availability due to the feedback-insensitive AK encoded by *Ask1* and to reduced lysine degradation determined by *o2*.

The amount of LKR activity in the *o2* endosperm is reduced in comparison to the wild type (Brochetto-Braga et al., 1992). In this work, we calculated LKR and SDH activities

from the same lots of wild-type (WT) and *o2* endosperm shown in **(A)** were fractionated in a formaldehyde-containing 1.0% agarose gel, transferred to a nylon membrane, and hybridized with the 1.2-kb RT-PCR cDNA fragment as a probe. The lengths of the detected LKR/SDH-encoding transcripts are shown at right. Loading controls, representing an ethidium bromide-stained gel, are shown at bottom.

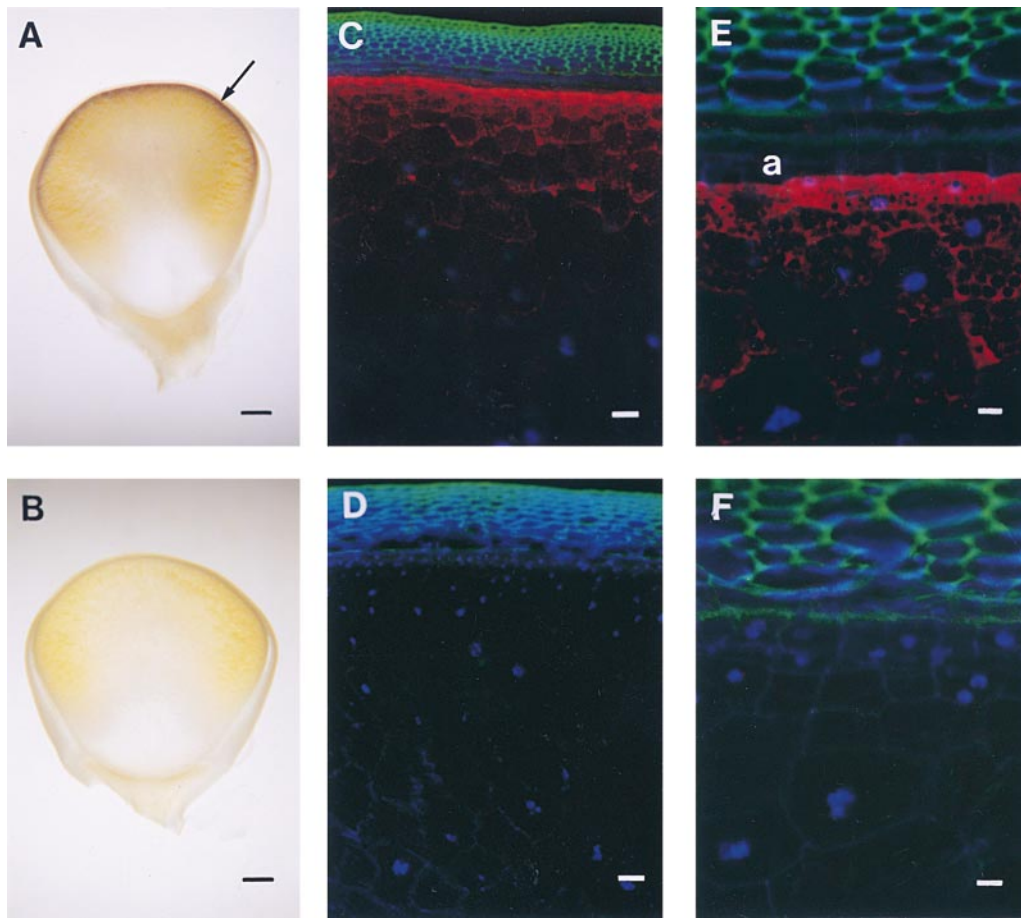


Figure 7. Localization of LKR/SDH in Normal Endosperm through SDH in Situ Activity and Immunocytochemistry.

(A) Seeds 17 DAP were longitudinally sectioned and incubated in an SDH reaction mixture, as described in Methods. An arrow indicates the positive colorimetric reaction for SDH activity.

(B) Endosperm of a tissue section incubated in the absence of saccharopine, the substrate for the SDH reaction.

(C) Immunolocalization of LKR/SDH from endosperm sections visualized by using a confocal laser scanning microscope. A positive reaction is shown in red. The images include nuclei stained blue by 4',6-diamidino-2-phenylindole. The autofluorescence of the cell walls appears in green and in blue. Immunolabeling was most intense in the subaleurone cell layers. The immunocytochemical reaction for LKR/SDH was barely detected in cells farther away from the first several subaleurone cell layers.

(D) and (F) Tissue sections incubated with nonimmune serum followed by treatment with the secondary antibody.

(E) Higher magnification of (C). LKR/SDH labeling is found around the starch granules and tended to be at the cell periphery, close to the cell wall in cells far away from the subaleurone. a, aleurone layer.

Bars in (A) and (B) = 1 mm; bars in (C) and (D) = 50 μm ; bars in (E) and (F) = 12.8 μm .

per amount of endosperm and found 36-fold and fourfold reductions in LKR and SDH activities, respectively, in the mutant endosperm (Figure 6A). Between 15 and 20 DAP, this decrease correlates well with reduced amounts of both the LKR/SDH polypeptide (Figure 6B) and the *ZLKRSDH* mRNA (Figure 6C).

Interestingly, in 25-DAP *o2* endosperm, LKR/SDH protein accumulated to levels comparable to those in the normal endosperm, but LKR and SDH activities did not reach normal levels. In addition, the high accumulation of the LKR/

SDH polypeptide in the 25-DAP *o2* endosperm was not accompanied by a proportional accumulation of *ZLKRSDH* mRNA, suggesting post-transcriptional regulation possibly due to stabilization of *ZLKRSDH* mRNA by translation (Gallie et al., 1991; Sullivan and Green, 1993; Jacobson and Peltz, 1996; Petracek et al., 1998). LKR activity seems to be differentially modulated in *o2* when compared with the normal endosperm. The enzyme is hypophosphorylated in *o2*, probably due to the absence of a casein kinase II in the mutant endosperm. Curiously, casein kinase II was able to restore LKR

activity in a lysine-dependent manner, after in vitro dephosphorylation by alkaline phosphatase (E.L. Kemper, F. Papes, A.C. da Silva, A. Leite, and P. Arruda, submitted manuscript).

At 20 DAP, the *o2* mutation causes a 90% reduction in *ZLKRSDH* steady state mRNA levels. This effect was observed in two near-isogenic mutant lines derived from alleles *Oh43o2* (Figure 6) and *W64Ao2* (data not shown). The severe reduction caused by the *o2* mutation on *ZLKRSDH* mRNA accumulation is comparable to the reduction observed for α -zein in the same genetic background (Cord Neto et al., 1995). The O2 protein is a transcriptional activator of the basic domain/leucine-zipper family that is specifically expressed in the nonaleuronic endosperm and recognizes different binding sites in the promoters of several endosperm-specific genes (Lohmer et al., 1991; Varagona et al., 1991; Schmidt et al., 1992; Cord Neto et al., 1995; Gallusci et al., 1996). Recent evidence suggests that O2 is involved in the coordinated regulation of storage protein synthesis and nitrogen and sugar metabolism during seed development (Giroux et al., 1994; Gallusci et al., 1996). Therefore, the involvement of O2 in the transcriptional regulation of lysine catabolism is in keeping with its role of controlling nitrogen metabolism in the seed.

O2 regulates cyPPDK, in an expression pattern similar to that observed for LKR/SDH. cyPPDK is localized in the sub-aleurone cell layers, with a weak signal spreading toward the central endosperm cells (Gallusci et al., 1996). The en-

dosperm region, where LKR/SDH and cyPPDK are localized, is also the region actively expressing zein genes and the O2 protein (Varagona et al., 1991; Dolfini et al., 1992). This observation correlates with the hypothesis of transcriptional control of LKR/SDH by O2 protein and supports the idea of a general control of amino acid metabolism and protein synthesis by O2.

In yeast, the GCN4 factor is one of the major determinants of the regulatory system termed general control of nitrogen (GCN). In this organism, the synthesis of lysine and many other amino acids is coordinately regulated by nutritional conditions (Hinnebusch, 1988). In addition, it has been demonstrated that the GCN4-like box may be a key element in regulating the response of a barley storage protein to nitrogen (Müller and Knudsen, 1993). Also, the prolamin composition of maize seeds seems to be dictated by nitrogen status (Singleton et al., 1990). The effects of the *o2* mutation on enzymes participating in amino acid metabolism and carbon partitioning, coupled with structural (Lohmer et al., 1993) and functional (Mauri et al., 1993) similarities between O2 and the yeast GCN4 factor, suggest that the O2 protein may be involved in similar general amino acid control in the maize endosperm.

METHODS

Plant Material

The commercial maize (*Zea mays* L.) hybrid F-352 from Agrocere SA (São Paulo, Brazil) was used. To obtain near-isogenic lines exhibiting normal phenotypes and the phenotype of the *opaque2* (*o2*) mutant, we crossed either *Oh43o2* or *W64Ao2* mutant lines to the F-352 hybrid. The resulting F₁ progeny was self-pollinated, and normal seeds from ears segregating for normal and *o2*-conferred phenotypes were planted. Plants were self-pollinated, and again normal seeds from ears segregating for normal and *o2*-conferred phenotypes were planted. Six successive rounds of self-pollination were performed. After the sixth generation, seeds from homozygous normal and *o2* ears were planted, and the resulting ears were collected for the experiments. Ears were harvested either at 17 days after pollination (DAP) or at distinct developmental stages and stored frozen at -70°C .

Roots, leaves, and coleoptiles from 4-day-old seedlings, grown in vermiculite under controlled conditions, were used for the isolation of total RNA.

Enzyme Purification

Lysine-ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) was purified from 17-DAP F-352 hybrid endosperms, as described by Gonçalves-Butruille et al. (1996), with minor modifications. All steps were performed at 4°C . Tissues were homogenized in buffer A (25 mM sodium phosphate, pH 7.4, 1 mM DTT, 1 mM EDTA, and 5 mM benzamidine) and centrifuged at $20,000g$ for 10 min, and the supernatant was brought to pH 5.5 by adding solid NaH_2PO_4 .

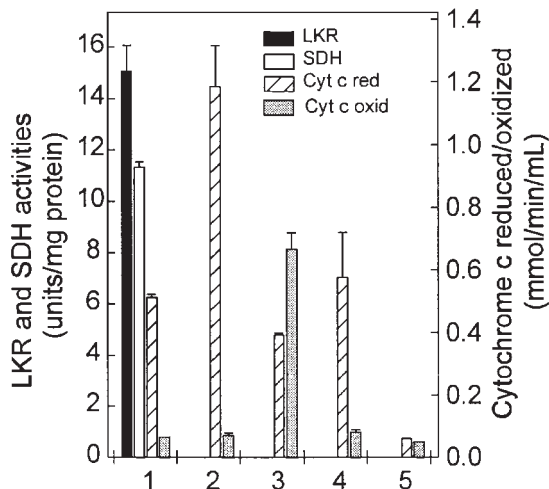


Figure 8. Subcellular Localization of LKR/SDH.

A homogenate from 20-DAP endosperm was separated by sucrose gradient centrifugation, and the fractions corresponding to the cytoplasm (1), endoplasmic reticulum (2), mitochondria (3), peroxisomes (4), and protein bodies (5) were assayed for LKR and SDH activities. Marker enzymes cytochrome *c* oxidase (Cyt *c* oxid) and cytochrome *c* reductase (Cyt *c* red) were assayed for resolution of organellar components.

Polyethylene glycol 8000 at 50% (w/v) was added to the homogenate to obtain a final concentration of 7.5%. The mixture was centrifuged at 20,000g for 10 min, and the supernatant was brought to a 15% (w/v) polyethylene glycol concentration and centrifuged again at 20,000g for 10 min. The pellet was resuspended in buffer B (50 mM Tris-HCl, pH 8.5, 1 mM DTT, and 1 mM EDTA) and dialyzed overnight against the same buffer. The dialyzed sample was applied to a DEAE-Sepharose column (2.5 × 40 cm) previously equilibrated with buffer B. The enzyme was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000g for 10 min. The pellet was resuspended in buffer B, dialyzed against the same buffer, and applied to a Protein-Pak Q 8HR (Waters, Milford, MA) column. The enzyme was eluted from the column with a linear gradient from 0 to 0.5 M NaCl in buffer B. Fractions containing enzyme activity were combined, brought to 70% saturation with solid ammonium sulfate, and centrifuged at 20,000g for 10 min. The pellet was resuspended in buffer B and applied to a Superdex 200 HR (Pharmacia) column previously equilibrated with buffer C (buffer B containing 0.3 M NaCl). The enzyme was eluted from the Superdex column with buffer C and stored at -70°C.

N-Terminal Protein Sequencing

The purified LKR/SDH was separated in by SDS-PAGE in a 7% gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R 250 and sent to the protein sequencing facility of the Weizmann Institute of Science (Rehovot, Israel). The protein band was eluted from the gel and digested with trypsin, and the major peaks were sequenced. Four internal peptide sequences were obtained: (1) GLIDFLHGL, (2) RYEGFSEIMVTL, (3) RLTPLEYEI, and (4) RELPAFALEHLPNR.

cDNA Cloning

Cloning of a full-length maize LKR/SDH-encoding cDNA was completed by using a combination of three procedures: (1) reverse transcription-polymerase chain reaction (RT-PCR), (2) cDNA library screening, and (3) 5' rapid amplification of cDNA ends (5' RACE).

After protein sequencing, a set of degenerate oligonucleotides was synthesized based on the tryptic peptide sequences and used in RT-PCR experiments. One microgram of total RNA extracted from 17-DAP endosperm was used in an RT reaction by using an RT-PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A 1.2-kb cDNA fragment was amplified by subsequent PCR reactions (2 μL of template cDNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate mix, 100 pmol of each primer, and 2.5 units of *Taq* DNA polymerase in a final volume of 50 μL) by using primers derived from the above-described peptide sequences 2 (5'-ISWIARIGTIACCATDATTTC-3') and 3 (5'-CTIACICCICTITAYGARTATAT-3'). S (G or C), W (A or T), R (A or G), D (A, G, or T), and Y (C or T) designate IUB codes for variable nucleotide sites, and I denotes inosine.

Amplification was performed on a thermal cycler (model 480; Perkin-Elmer) as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 42°C, 2 min at 72°C; and 5 min at 72°C. After sequencing, the 1.2-kb amplified cDNA fragment was confirmed to be part of the maize LKR/SDH-encoding gene by sequence comparison to the yeast *Lys1* and *Lys9* genes. This fragment then was used as a probe to

screen a maize endosperm cDNA library constructed from RNA extracted from 25-DAP seeds of the R-802 inbred line (Aukerman et al., 1991). Twenty clones were isolated and sequenced, but all of them were incomplete. The 5' cDNA end was cloned by using the RACE system (Gibco BRL), according to the manufacturer's instructions, with the primers designed on the basis of the cDNA sequences (5'-TATCAAATAGGTGCCAC-3' and 5'-GTGTGGGAAAAGAAG-GCGTA-3').

The full-length maize *ZLKRSDH* cDNA clone was finally isolated from F-352 total RNA extracted from 17-DAP endosperms. Primers annealing to the 5' untranslated region (5'-TTCAACTCTCCACTTTCT-CAACCA-3') and 3' untranslated region (5'-CTCGTCCGTCTCCGT-TCCGTC-3') of the cDNA were used to obtain a 3.5-kb complete cDNA in RT-PCR reactions catalyzed by Pfu polymerase (Stratagene) according to the manufacturer's instructions. This fragment subsequently was cloned into pBluescript KS+ and sequenced in an automatic ABI377 DNA sequencer (Perkin-Elmer).

DNA Gel Blot Analysis

Genomic DNA was extracted from maize, Coix, and sorghum seedlings as described by Rivin et al. (1982). Ten micrograms of genomic DNA was digested to completion with BamHI or EcoRV. The digests were ethanol precipitated and loaded onto a 0.7% agarose gel, blotted onto a nylon membrane (Hybond-N; Amersham), and hybridized with the ³²P-labeled (Megaprime DNA labeling system; Amersham) 1.2-kb RT-PCR fragment. Hybridization was conducted at 65°C in SSPE buffer (5 × SSPE [1 × SSPE is 150 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4], 5 × Denhardt's solution [1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA], and 0.5% SDS), and washes were performed twice at room temperature in 2 × SSPE 0.1% SDS solution for 20 min and repeated twice at 65°C in 0.1 × SSPE 0.1% SDS. Autoradiography was performed at -70°C for 72 hr by using intensifying screens.

Total RNA Isolation and RNA Gel Blot Analysis

Total RNA used in both RT reactions or RNA gel blot analysis was extracted from developing seeds and seedling tissues, according to the procedures described by Prescott and Martin (1987). For RNA gel blot experiments, 20 μg of total RNA was electrophoresed in 1.0 or 1.5% agarose-formaldehyde gels (0.66 M formaldehyde, 50 mM Mops). After electrophoresis, gels were stained with ethidium bromide as a loading control. The RNA then was transferred from the gel onto a nylon membrane (Hybond-N⁺; Amersham). The probes used were either the 1.2-kb RT-PCR fragment or specific LKR or SDH domain probes. The LKR probe consisted of a 0.55-kb cDNA fragment corresponding to the region spanning residues 96 through 284, and the SDH probe (0.49 kb) encompassed residues 652 to 814 (Figure 1). DNA labeling, hybridization, and washing were as described above for the DNA gel blots. For quantitative experiments, pre-flashed x-ray films were exposed to the radioactive membrane, according to the manufacturer's instructions (Amersham). Relative amounts of hybridized mRNA were analyzed through scanning with a laser densitometer (LKB UltraScan XL, Bromma, Sweden).

Immunoblotting

Immunoblotting was performed as described by Kemper et al. (1998), with minor modifications. After electrophoresis, the gel was

soaked in 25 mM Tris-base, 190 mM glycine, and 20% methanol for 10 min. Proteins were transferred to nylon membranes (Hybond-N; Amersham) in a semidry electroblotting apparatus (Pharmacia). Membranes were blocked overnight at 4°C in 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.1% (v/v) Tween 20, and 5% (w/v) nonfat dry milk and then incubated with anti-LKR polyclonal antibodies. After incubation with anti-rabbit IgG alkaline phosphatase conjugate, the membranes were incubated for 20 min in the dark in a developing mixture containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and a 1:1000 solution of CSPD (Tropix, Bedford, MA). Bands were detected by exposure on a preflashed chemiluminescence-sensitive film (Amersham) and quantified by using laser densitometry.

Histochemical Staining

Histochemical staining of SDH activity on maize kernel sections was based on the gel staining reaction described by Gonçalves-Butruille et al. (1996). Seventeen-DAP maize kernels were longitudinally hand sectioned every 2 mm with a razor blade. Sections were fixed in 4% formalin, pH 7.0, for 30 min, rinsed 10 times in water over 18 hr at 4°C to remove endogenous substrates, and then incubated for 10 min at room temperature in a reaction mixture containing 2 mM saccharopine, 1 mM NAD, 0.5% nitro blue tetrazolium, 0.1 mM phenazine methasulfate, and 100 mM Tris-HCl, pH 8.5, with rocking. Control sections were incubated in the absence of saccharopine. The reaction was stopped by rinsing the sections in double distilled water.

Immunohistochemistry

Indirect immunofluorescence and confocal laser scanning microscopy were used to visualize LKR/SDH in frozen sections of maize endosperm. Seeds at 17 DAP were dissected from cobs, immersed in ice-cold PBS (140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4, and 0.02% [w/v] of sodium azide) containing 4% (w/v) paraformaldehyde and 0.1 M sucrose, and cut into 2- to 3-mm-thick longitudinal slices. After overnight fixation at 4°C, the slices were rinsed three times in PBS containing 0.5 M sucrose for 20 min each and frozen in liquid nitrogen. The frozen slices were mounted in specimen supports with Cryo-embedding compound (Microm Laborgerate, Waldorf, Germany) and sectioned at 16 μm in a Micron cryostat (Microm Laborgerate). Sections were collected on albumin-coated slides, postfixated with acetone for 15 min, and air dried.

Sections were blocked with 2% (w/v) BSA and 0.3% (v/v) Tween 20 in PBS (PBS-T-B) for 30 min in a 200-mL coupling jar before incubation with the primary antibody. Sections then were incubated with a polyclonal antibody raised in rabbit against LKR/SDH (Kemper et al., 1998) diluted at 1:200 (v/v) in PBS-T-B overnight at room temperature in a 50-mL coupling jar. The control consisted of incubation with an unrelated rabbit serum at the same dilution. The slides were washed three times for 20 min in PBS-T in a 200-mL coupling jar. Sections were covered with a rhodamine-conjugated goat anti-rabbit immunoglobulin (Calbiochem-Novabiochem, La Jolla, CA) diluted 1:30 (v/v) in PBS-T-B and incubated for 1 hr at room temperature. After washing, the sections were counterstained with 4',6-diamidino-2-phenylindole (0.8 $\mu\text{g}/\text{mL}$ VectaShield; Vector Laboratories, Burlingame, CA). Sections were analyzed by using a confocal laser scanning microscope (model MRC 1024UV; Bio-Rad Life Sciences, Richmond, CA). Filter sets for rhodamine (immunolabeling), fluores-

cein (natural cell wall fluorescence), and 4',6-diamidino-2-phenylindole (nuclear staining plus natural cell wall fluorescence) were used. Immunolabeled and control sections were analyzed using the same laser power, iris, and gain settings as their experimental counterparts. This treatment was performed twice with kernels from different field seasons. Photographs were taken using Focus Graphics (Focus Graphics Inc., Plymouth, MN) equipment with Kodak ISO 100 film and processed commercially.

Subcellular Localization of the LKR/SDH Enzyme

Subcellular fractionation was conducted according to Habben et al. (1993), with the following modifications. Buffer A contained 10 mM Hepes, pH 7.5, 1 mM EDTA, 10 mM KCl, 200 mM sucrose, 1 mM DTT, and 5 mM benzamide. After centrifugation in a discontinuous sucrose gradient, the interface fractions were collected by lateral puncture through the centrifuge tube wall and assayed for LKR and SDH activities. The activities of the marker enzymes cytochrome *c* oxidase and cytochrome *c* reductase were assayed for resolution of organellar components, as described by Tolbert (1974) and Larkins and Hurkman (1978).

Separation of Maize LKR and SDH Domains by Limited Proteolysis

Aliquots of LKR/SDH partially purified by chromatography on DEAE-Sepharose were incubated with elastase (Sigma). Enzyme/protease proportions and proteolysis conditions are indicated in the legend to Figure 3. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. The digest was applied to a Protein-Pak Q 8HR column (Waters) previously equilibrated with buffer B. The column was washed with buffer B and then eluted with a linear gradient from 0 to 0.5 M NaCl in buffer B. The fractions containing separated LKR and SDH domains were used for enzyme assays and immunoblotting analysis.

Enzyme Assays

LKR and SDH activities were measured spectrophotometrically by following the oxidation of NADPH and reduction of NAD^+ , respectively, at 30°C. LKR assays contained 20 mM L-lysine, 10 mM α -ketoglutaric acid (neutralized to pH 7.0 with potassium hydroxide), 0.1 mM NADPH, 150 mM Tris-HCl buffer, pH 7.4, and 0.04 to 0.1 mg of protein in a final volume of 0.3 mL. SDH assays contained 2 mM L-saccharopine, 2 mM NAD^+ , and 100 mM Tris-HCl buffer, pH 8.5, in a final volume of 0.3 mL. Oxidation of NADPH and reduction of NAD^+ were monitored at 340 nm in a spectrophotometer (model DU-65; Beckman). One unit of enzyme activity is defined as 1 nmol of NADPH oxidized or NAD^+ reduced per min at 30°C. The protein concentration in the enzyme extracts was determined by the method of Bradford (1976), using the Bio-Rad protein assay dye reagent.

Computer Analysis

Amino acid sequence alignments and similarity analysis were performed using the MACAW (Schuler et al., 1991) and BOXSHADE (FTP downloaded from Vax0.biomed.uni-koeln.de) programs.

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REFERENCES

- Ameen, M., Palmer, T., and Oberholzer, V.G. (1987). Inhibition of bovine liver lysine-ketoglutarate reductase by urea cycle metabolites and saccharopine. *Biochem. Int.* **14**, 589–595.
- Arruda, P., and da Silva, W.J. (1979). Amino acid composition of vascular sap of maize ear peduncle. *Phytochemistry* **18**, 409–410.
- Arruda, P., and da Silva, W.J. (1983). Lysine-ketoglutarate reductase activity in maize. Its possible role in lysine metabolism of developing endosperm. *Phytochemistry* **22**, 206–208.
- Arruda, P., Sodek, L., and da Silva, W.J. (1982). Lysine-ketoglutarate reductase activity in developing maize endosperm. *Plant Physiol.* **69**, 988–989.
- Aukerman, M.J., Schmidt, R.J., Burr, B., and Burr, F.A. (1991). An arginine to lysine substitution in the bZIP domain of an *opaque2* mutant in maize abolishes specific DNA binding. *Genes Dev.* **5**, 310–320.
- Azevedo, R.A., Arana, J.L., and Arruda, P. (1990). Biochemical genetics of the interaction of the lysine plus threonine resistant mutant *Ltr*1* with *opaque-2* maize mutant. *Plant Sci.* **70**, 81–90.
- Azevedo, R.A., Arruda, P., Turner, W.L., and Lea, P.J. (1997). The biosynthesis and metabolism of the aspartate derived amino acids in higher plants. *Phytochemistry* **46**, 395–419.
- Bass, H.W., Webster, C., O'Brian, G.R., Roberts, J.K.M., and Boston, R.S. (1992). A maize ribosome-inactivating protein is controlled by the transcriptional activator *Opaque2*. *Plant Cell* **4**, 225–234.
- Blemings, K.P., Crenshaw, T.D., Swick, R.W., and Benevenga, N.J. (1994). Lysine- α -ketoglutarate reductase and saccharopine dehydrogenase are located only in the mitochondrial matrix in rat liver. *J. Nutr.* **124**, 1215–1221.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Bright, S.W.J., Mifflin, B.J., and Rognes, S.E. (1982). Threonine accumulation in the seeds of a barley mutant with an altered aspartate kinase. *Biochem. Genet.* **20**, 229–243.
- Brochetto-Braga, M.R., Leite, A., and Arruda, P. (1992). Partial purification and characterization of lysine-ketoglutarate reductase activity in normal and *opaque-2* maize endosperms. *Plant Physiol.* **98**, 1139–1147.
- Cord Neto, G., Yunes, J.A., Vettore, A.L., da Silva, W.J., Arruda, P., and Leite, A. (1995). The involvement of *Opaque2* in β -prolamin gene regulation in maize and *Coix* suggests a more general role for this transcriptional activator. *Plant Mol. Biol.* **27**, 1015–1029.
- da Silva, W.J., and Arruda, P. (1979). Evidence for the genetic control of lysine catabolism in maize endosperm. *Phytochemistry* **18**, 1803–1805.
- Diedrick, T.J., Frisch, D.A., and Gegenbach, B.G. (1990). Tissue culture isolation of a second mutant locus for increased threonine accumulation in maize. *Theor. Appl. Genet.* **79**, 209–215.
- Dolfini, S.F., Landoni, M., Tonelli, C., Bernard, L., and Viotti, A. (1992). Spatial regulation in the expression of structural and regulatory storage-protein genes in *Zea mays* endosperm. *Dev. Genet.* **13**, 264–276.
- Epelbaum, S., McDevitt, R., and Falco, S.C. (1997). Lysine-ketoglutarate reductase and saccharopine dehydrogenase from *Arabidopsis thaliana*: Nucleotide sequence and characterization. *Plant Mol. Biol.* **35**, 735–748.
- Falco, S.C., Guida, T., Locke, M., Mauvais, J., Sanders, C., Ward, R.T., and Webb, P. (1995). Transgenic canola and soybean seeds with increased lysine. *Bio/Technology* **13**, 577–582.
- Frankard, V., Ghislain, M., and Jacobs, M. (1991). High threonine producer mutant in *Nicotiana sylvestris* (Spegg. and Comes). *Theor. Appl. Genet.* **82**, 273–282.
- Frankard, V., Ghislain, M., and Jacobs, M. (1992). Two feedback-insensitive enzymes of the aspartate pathway in *Nicotiana sylvestris*. *Plant Physiol.* **99**, 1285–1293.
- Fujioka, M., and Takata, Y. (1981). Role of arginine residue in saccharopine dehydrogenase (L-lysine forming) from baker's yeast. *Biochemistry* **20**, 468–472.
- Fujioka, M., Takata, Y., Ogawa, H., and Okamoto, M. (1980). The inactivation of saccharopine dehydrogenase (L-lysine forming) by diethyl pyrocarbonate. *J. Biol. Chem.* **255**, 937–942.
- Gallie, D.R., Feder, J.N., Schimke, R.T., and Walbot, V. (1991). Post-transcriptional regulation in higher eukaryotes: The role of the reporter gene in controlling expression. *Mol. Gen. Genet.* **228**, 258–264.
- Gallusci, P., Varott, S., Matsuoko, M., Maddaloni, M., and Thompson R.D. (1996). Regulation of cytosolic pyruvate, orthophosphate dikinase expression in developing maize endosperm. *Plant Mol. Biol.* **31**, 45–55.
- Gaziola, S.A., Teixeira, C.M., Lugli, J., Sodek, L., and Azevedo, R.A. (1997). The enzymology of lysine catabolism in rice seeds—Isolation, characterization, and regulatory properties of a lysine 2-oxoglutarate reductase/saccharopine dehydrogenase bifunctional polypeptide. *Eur. J. Biochem.* **247**, 364–371.
- Giroux, M.J., Boyer, C., Feix, G., and Hannah, C. (1994). Coordinated transcriptional regulation of storage product genes in the maize endosperm. *Plant Physiol.* **106**, 713–722.
- Gonçalves-Butruille, M., Szajner, P., Torigoi, E., Leite, A., and Arruda, P. (1996). Purification and characterization of the bifunc-

- tional enzyme lysine-ketoglutarate reductase-saccharopine dehydrogenase from maize. *Plant Physiol.* **110**, 765-771.
- Habben, J.E., Kirleis, A.W., and Larkins, B.A.** (1993). The origin of lysine-containing proteins in the *opaque-2* maize endosperm. *Plant Mol. Biol.* **23**, 825-838.
- Hinnebusch, A.G.** (1988). Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **52**, 248-273.
- Jacobson, A., and Peltz, S.W.**, (1996). Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu. Rev. Biochem.* **65**, 693-739.
- Karchi, H., Shaul, O., and Galili, G.** (1993). Seed specific expression of a bacterial desensitized aspartate kinase increases the production of seed threonine and methionine in transgenic tobacco. *Plant J.* **3**, 721-727.
- Karchi, H., Shaul, O., and Galili, G.** (1994). Lysine synthesis and catabolism are coordinately regulated during tobacco seed development. *Proc. Natl. Acad. Sci. USA* **91**, 2577-2581.
- Kemper, E.L., Cord Neto, G., Capella, A.N., Gonçalves-Butruille, M., Azevedo, R.A., and Arruda, P.** (1998). Structure and regulation of the bifunctional enzyme lysine-oxoglutarate reductase-saccharopine dehydrogenase in maize. *Eur. J. Biochem.* **253**, 720-729.
- Larkins, B.A., and Hurkman, W.J.** (1978). Synthesis and deposition of zein in protein bodies of maize endosperm. *Plant Physiol.* **62**, 256-263.
- Lohmer, S., Maddaloni, M., Motto, M., Di Fonzo, N., Hartings, H., Salamini, F., and Thompson, R.D.** (1991). The maize regulatory locus *Opaque-2* encodes a DNA-binding protein which activates the transcription of the *b-32* gene. *EMBO J.* **10**, 617-624.
- Lohmer, S., Maddaloni, M., Motto, M., Salamini, F., and Thompson, R.D.** (1993). Translation of the mRNA of the maize transcriptional activator *Opaque-2* is inhibited by upstream open reading frames present in the leader sequence. *Plant Cell* **5**, 65-73.
- Luehrens, K.R., and Walbot, V.** (1994). The impact of AUG start codon context on maize gene expression in vivo. *Plant Cell Rep.* **13**, 454-458.
- Markovitz, D.J., and Chuang, D.T.** (1987). The bifunctional amino-adipic semialdehyde synthetase in lysine degradation. *J. Biol. Chem.* **262**, 9353-9358.
- Markovitz, D.J., Chuang, D.T., and Cox, R.P.** (1984). Familial hyperlysinemias: Purification and characterization of the bifunctional amino-adipic semialdehyde synthase with LKR and SDH activities. *J. Biol. Chem.* **259**, 11643-11646.
- Mauri, I., Maddaloni, M., Lohmer, S., Motto, M., Salamini, F., Thompson, R.D., and Martegani, E.** (1993). Functional expression of the transcriptional activator *Opaque-2* in yeast. *Mol. Gen. Genet.* **341**, 319-326.
- Mertz, E.T., Bates, L.S., and Nelson, O.E.** (1964). Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science* **145**, 279-280.
- Müller, M., and Knudsen, S.** (1993). The nitrogen response of a barley C-hordein promoter is controlled by positive and negative regulation of the GCN4 and endosperm box. *Plant J.* **4**, 343-355.
- Negrutiu, I., Cattoir-Reynaerts, A., Verbruggen, I., and 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.
- Ogawa, H., and Fujioka, M.** (1980). The reaction of pyridoxal 5'-phosphate with an essential lysine residue of saccharopine dehydrogenase (L-lysine forming). *J. Biol. Chem.* **255**, 7420-7425.
- Ogawa, H., Okamoto, M., and Fujioka, M.** (1979). Chemical modification of the active site sulfhydryl group of saccharopine dehydrogenase (L-lysine-forming). *J. Biol. Chem.* **254**, 7030-7035.
- Pederson, H.B., Galili, G., Knudsen, G., and Holm, P.B.** (1996). Engineering of the aspartate family biosynthetic pathway in barley (*Hordeum vulgare* L.) by transformation with heterologous genes encoding feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. *Plant Mol. Biol.* **32**, 611-620.
- Petracek, M.E., Dickey, L.F., Hansen, E.R., Sowinski, D.A., Nguyen, T.-T., Allen, G.C., and Thompson, W.F.** (1998). Dependence of Fed-1 light regulation on translation. In *A Look beyond Transcription: Mechanisms Determining mRNA Stability and Translation in Plants*, J. Bailey-Serres and D.R. Gallie, eds (Rockville, MD: American Association of Plant Physiologists), pp. 96-101.
- Prescott, A., and Martin, C.** (1987). Rapid method for quantitative assessment of levels of specific mRNAs. *Plant Mol. Biol. Rep.* **4**, 219-224.
- Rao, V.V., Pan, X., and Chang, Y.-F.** (1992). Developmental changes of L-lysine-ketoglutarate reductase in rat brain and liver. *Comp. Biochem. Physiol.* **103B**, 221-224.
- Rivin, C.J., Zimmer, E.A., and Walbot, V.** (1982). Isolation of DNA and DNA recombinants from maize. In *Maize for Biological Research*, W.F. Sheridan, ed (Grand Forks, ND: University Press), pp. 161-164.
- Schmidt, R.J., Ketudat, M., Aukerman, M.J., and Hoschek, G.** (1992). *Opaque-2* is a transcriptional activator that recognizes a specific target site in 22-kD zein genes. *Plant Cell* **4**, 689-700.
- Schuler, G.D., Altschul, S.F., and Lipman, D.J.** (1991). A workbook for multiple alignment construction and analysis. *Proteins Struct. Funct. Genet.* **9**, 180-190.
- Shaul, O., and Galili, G.** (1993). Concerted regulation of lysine and threonine synthesis in tobacco plants expressing bacterial feedback-insensitive aspartate kinase and dihydrodipicolinate synthase. *Plant Mol. Biol.* **23**, 759-768.
- Singletary, G.W., Doehlert, D.C., Wilson, C.M., Muhitch, M.J., and Below, F.E.** (1990). Response of enzymes and storage proteins of maize endosperm to nitrogen supply. *Plant Physiol.* **94**, 858-864.
- Sodek, L., and Wilson, C.M.** (1970). Incorporation of leucine-C¹⁴ and lysine-C¹⁴ into protein in the developing endosperm of normal and *opaque-2* corn. *Arch. Biochem. Biophys.* **140**, 29-38.
- Sullivan, M.L., and Green, P.J.** (1993). Post-transcriptional regulation of nuclear-encoded genes in higher plants—The roles of messenger RNA stability and translation. *Plant Mol. Biol.* **23**, 1091-1104.
- Tang, G., Miron, D., Zhu-Shimoni, J.X., and Galili, G.** (1997). Regulation of lysine catabolism through lysine-ketoglutarate reductase and saccharopine dehydrogenase in Arabidopsis. *Plant Cell* **9**, 1305-1316.
- Tolbert, N.E.** (1974). Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. *Methods Enzymol.* **31**, 734-746.
- Varagona, M.J., Schmidt, R.J., and Raikhel, N.V.** (1991). Monocot regulatory protein *Opaque-2* is localized in the nucleus of maize endosperm and transformed tobacco plants. *Plant Cell* **3**, 105-113.