A T-DNA insertion knockout of the bifunctional lysine-ketoglutarate reductase/saccharopine dehydrogenase gene elevates lysine levels in Arabidopsis seeds

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A T-DNA Insertion Knockout of the Bifunctional Lysine-Ketoglutarate Reductase/Saccharopine Dehydrogenase Gene Elevates Lysine Levels in Arabidopsis Seeds[1]

Xiaohong Zhu, Guiliang Tang, Fabienne Granier, David Bouchez, and Gad Galili*

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100 Israel (X.Z., G.T., G.G.); and Laboratoire de Biologie Cellulaire, Institut National de la Recherche Agronomique, 78026 Versailles, France (F.G., D.B.)

Plants possess both anabolic and catabolic pathways for the essential amino acid lysine (Lys). However, although the biosynthetic pathway was clearly shown to regulate Lys accumulation in plants, the functional significance of Lys catabolism has not been experimentally elucidated. To address this issue, we have isolated an Arabidopsis knockout mutant with a T-DNA inserted into exon 13 of the gene encoding Lys ketoglutarate reductase/saccharopine dehydrogenase. This bifunctional enzyme controls the first two steps of Lys catabolism. The phenotype of the *LKR/SDH* knockout was indistinguishable from wild-type plants under normal growth conditions, suggesting that Lys catabolism is not an essential pathway under standard growth conditions. However, mature seeds of the knockout mutant over-accumulated Lys compared with wild-type plants. This report provides the first direct evidence for the functional significance of Lys catabolism in regulating Lys accumulation in seeds. Such a knockout mutant may also provide new perspectives to improve the level of the essential amino acid Lys in plant seeds.

Lys is an essential amino acid that is present in limiting amounts in seeds of many crop plants (Galili et al., 1994). In plants, Lys is synthesized from Asp via diaminopimlate, and its synthesis is regulated primarily by the sensitivity of its biosynthetic enzyme dihydrodipicolinate synthase (DHPS) to feedback inhibition by Lys (Galili, 1995). However, the steady-state level of Lys in plants, particularly in plant seeds, may be regulated in a concerted manner both by its synthesis and catabolism. Plants, like animals, catabolize Lys into α-amino adipic acid and Glu (Fig. 1) (Arruda et al., 2000). Two enzymes linked on a single bifunctional polypeptide control the first two steps of this pathway. Lys ketoglutarate reductase (LKR) first combines Lys and α-ketoglutarate into saccharopine, and saccharopine dehydrogenase (SDH) then converts saccharopine into α-aminoadipic semi-aldehyde and Glu (Fig. 1). α-Amino adipic acid is further into acetyl-coenzyme A and several additional molecules of Glu (Fig. 1) (Arruda et al., 2000). Based on expressed sequence tag and genomic sequencing databases, Arabidopsis possesses only a single copy *LKR/SDH* gene, and LKR/SDH homologs have been also identified in a number of other plant species.

The physiological significance of Lys catabolism in plants is not clear, but a number of studies provided indirect evidence, suggesting that this pathway may be important for the regulation of Lys homeostasis in developing seeds. Expression of a bacterial feedback-insensitive DHPS, the major rate-limiting enzyme for Lys biosynthesis, in transgenic tobacco plants resulted in a dramatic increase of free Lys levels in vegetative tissues but not in seeds (Shaul and Galili, 1992, 1993; Karchi et al., 1994). The lack of increase in seed Lys was associated with a significant Lys-dependent stimulation of LKR activity, suggesting that the α-amino adipic acid pathway may function specifically in seeds as a mechanism to prevent over-accumulation of free Lys (Karchi et al., 1994, 1995). Yet, in contrast to tobacco, expression of a bacterial feedback-insensitive DHPS in transgenic soybean, canola, and maize resulted in a dramatic over-accumulation of free Lys with no major effect on seed development and germination (Falco et al., 1995; Mazur et al., 1999). Seeds from all of these plants also over-accumulated several catabolic products of Lys (Falco et al., 1995; Mazur et al., 1999).

To study the functional significance of Lys catabolism in plants, we have isolated an Arabidopsis knockout mutant with a T-DNA inserted into exon 13 of the *LKR/SDH* gene. As compared with wild-type plants, the knockout mutant exhibits no morphologically distinguishable phenotype under regular growth conditions, but possesses significantly higher free and protein-incorporated Lys in its seeds com-

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* Corresponding author; e-mail gad.galili@weizmann.ac.il; fax 972–8–9344181.

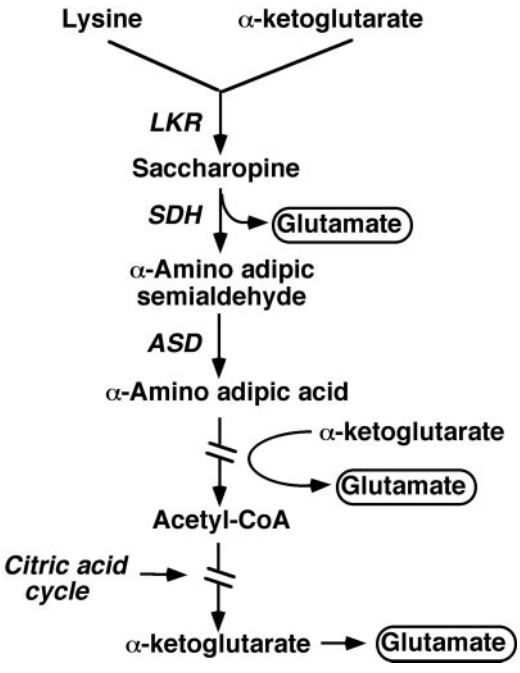


Figure 1. The Lys catabolism pathway and metabolites derived from it. LKR, Lys ketoglutarate reductase; SDH, saccharopine dehydrogenase; ASD, aminoadipic semialdehyde dehydrogenase. Broken arrows represent several non-specified enzymatic reactions. Glu residues are situated inside large boxes.

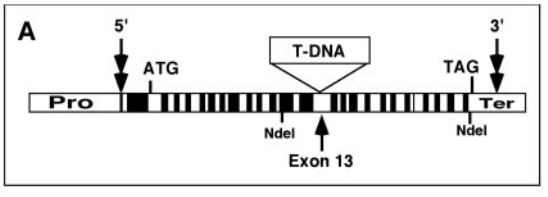
pared with wild-type Arabidopsis. These results provide the first direct evidence for the functional significance of Lys catabolism in regulating Lys accumulation in plant seeds. They also offer a new tool to improve the nutritional quality of plants.

RESULTS

Isolation of a Homozygous Arabidopsis LKR/SDH Knockout Mutant

To obtain an Arabidopsis *LKR/SDH* knockout mutant, we screened a T-DNA insertion population (Bechtold et al., 1993) by PCR analysis of DNA pools with specific sets of primers derived from the *LKR/SDH* gene and the T-DNA. One candidate knockout line was obtained and the genomic region of the *LKR/SDH* locus was characterized by DNA sequence analysis. As shown in Figure 2A, the *LKR/SDH* locus in this line possessed a T-DNA insert within exon 13. Insertion of the T-DNA created an addition of five bases (CCTATA) at the junction between the T-DNA left border and the LKR/SDH sequence (Fig. 2B).

Individual plants, germinated from different seeds of the original Arabidopsis LKR/SDH knockout line, were all resistant to kanamycin and contained three independent T-DNA insertions, as determined by Southern-blot analysis using the T-DNA as a probe (Fig. 3B, lanes g and i). To screen for homozygous *LKR/SDH* knockout plants, the original line was back-crossed twice to wild-type Arabidopsis, and selection for the *LKR/SDH* knockout allele was performed by PCR analysis (data not shown). Following the second back-cross, progenies containing the *LKR/SDH* knockout allele were selfed. Some of these lines segregated 3:1 for kanamycin resistance, suggesting that they contained only a single T-DNA insertion in the *LKR/SDH* locus. To select plants homozygous to the *LKR/SDH* knockout locus, DNAs from individual plants from these selfed lines were reacted in a Southern blot with a *Nde*I fragment from the Arabidopsis *LKR/SDH* genomic DNA (shown in Fig. 2) as a probe. As shown in Figure 3A, three types of hybridization patterns were observed. Some plants possessed two hybridized bands, one corresponding to the native *LKR/SDH* locus and the second larger band corresponding to the *LKR/SDH* knockout allele containing the T-DNA insert (lanes a and b). These plants were heterozygous for the *LKR/SDH* knockout allele. The second type contained only the larger hybridized band, suggesting that these plants are homozygous *LKR/SDH* knockout mutants (lanes c and d). The third type contained only the smaller hybridizing band, suggesting they were wild-type plants possessing only the wild-type *LKR/SDH* locus (lane e). To confirm that the two homozygous lines from Figure 3A (lanes c and d) contained only one



B

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 51  GGTAATAGGA  CACTGGGATT  CGTCTTGGAC  AACTTTCCTT  CTCATCTAAG
101  CGTAGACAAC  CCTCAACTGG  AAACGGGCCG  GACTCCAGGG  CGTGTGCCAG
151  GTGCCCACGG  AATAGTTTTG  GCCAGACCCT  TGAAAATCCG  ATTCAGTACA
     ▼
201  ACCTATATCA  GGTGTTTTGA  TTCTTGGTGC  TGGACGTGTG  TGTCGCCCAG
251  CTGCTGATTT  CCTAGCTTCA  GTTAGAACCA  TTTCGTCACA  GCAATGGTAC
301  AAAACATATT  TCGGAGCAGA  CTCTGAAGAG  AAAACAGATG  TTCATGTGAT
351  TGTCGCGTCT  CTGTATCTTA  AGGATGCCAA  AGAGGTAGGA  GAAGCCTTTG
401  GGCTTCATCT  GAGTAATTCA  GTGTATACGA  TGAACTATCA  ATCTTTTCAA
451  GTTTTACTGA  TGATCAAATT  TTCCGCAGAC  GGTTGAAGGT  ATTTCAGATG
551  GTTTCTCAGG  TATTTTCCTA  ACTTCTCTGT  TCTTAGATCA  CCTTTACTTC
501  TAGAAGCAGT  TCGGCTAGAT  GTATCTGATA  GTGAAAGTCT  CCTTAAGTAT
601  AAACTCCACT  GTTCTCCATG  ATCTTATATT  TTTTTTTCAT  TGCACGCAGG
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Figure 2. Localization of the T-DNA insert in the Arabidopsis *LKR/SDH* locus. A, Schematic diagram showing the insertion of the T-DNA in exon 13 of the *LKR/SDH* locus. The initiator ATG and terminator TAG codons, as well as the promoter (Pro) and terminator (Ter) are illustrated. The 5′ (cap) and 3′ (poly A) boundaries of the LKR/SDH mRNA are indicated by double headed arrows. The DNA fragment between the two *Nde*I sites shown below was used as a probe to select the homozygous *LKR/SDH* knockout mutants. B, DNA sequence around the 3′-insertion site of the T-DNA into the *LKR/SDH* locus. The T-DNA sequence ranges between nucleotides 1 and 201, whereas the LKR/SDH sequence begins at nucleotide 208. An additional non-related CCTATA sequence (nucleotides 202–207 shown in bold) was created during the T-DNA insertion. The end of the T-DNA is marked by a vertical arrow.

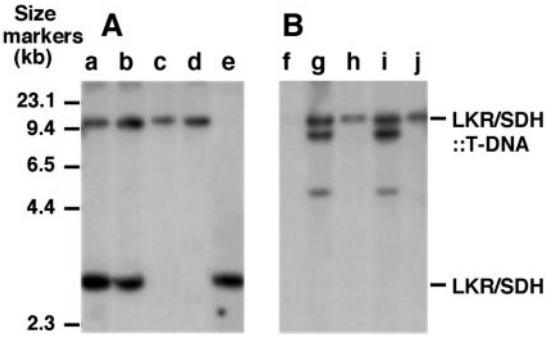

Figure 3. Southern-blot analysis of LKR/SDH and T-DNA-containing sequences in progenies derived from the initial line containing the *LKR/SDH* knockout allele. Genomic DNA from the different lines was digested with *Nde*I and reacted in a Southern blot either with the *Nde*I probe of *LKR/SDH* locus, shown in Figure 2A, or with a probe derived from the T-DNA (Fig. 2B). A, Includes plants heterozygous (lanes a and b), homozygous (lanes c and d), or lacking the *LKR/SDH* knockout allele (lane e). B, Includes wild-type Arabidopsis (lane f), the original *LKR/SDH* knockout line with three T-DNA insertions (lanes g and i), and the final homozygous plants for the *LKR/SDH* knockout allele (lanes h and j). The position of the bands representing the native *LKR/SDH* locus and *LKR/SDH* allele containing a T-DNA insertion are shown on the right. The migration of DNA size markers is shown on the left.

T-DNA insert, the same DNA from these lines was reacted in a Southern blot with the T-DNA as a probe. As opposed to the original *LKR/SDH* knockout line or wild-type Arabidopsis, which contained respectively either three positive bands (Fig. 3B, lanes g and i) or no band (Fig. 3B, lane f), these two lines possessed only one band (Fig. 3B, lanes h and j). This band had the expected size for the LKR/SDH knockout allele.

The Homozygous LKR/SDH Knockout Plants Possess No Detectable LKR/SDH mRNA and Protein

To test whether insertion of the T-DNA into exon 13 of the *LKR/SDH* gene affects its expression, we analyzed the LKR/SDH mRNA and protein levels in the heterozygous and homozygous *LKR/SDH* knockout plants compared with control wild-type Arabidopsis. First, total RNA was extracted from stem sections containing inflorescence and developing siliques of all three plant types and subjected to northern-blot analysis, using the LKR domain of *LKR/SDH* as a probe. As shown in Figure 4 lane a, wild-type plants possessed a positively hybridized mRNA band of approximately 3.5 kb, which is the expected size of the LKR/SDH mRNA. Plants heterozygous for the *LKR/SDH* knockout possessed the same band, but its intensity was lower than that of the wild-type plants (Fig. 4, compare with lanes a and c). Plants homozygous for the *LKR/SDH* knockout possessed no detectable LKR/SDH mRNA band (lanes b and d).

To analyze the effect of the T-DNA insertion on the production of the LKR/SDH polypeptide, proteins

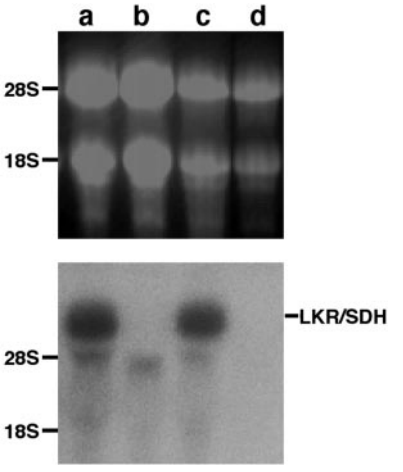

Figure 4. Relative levels of LKR/SDH mRNA in the different *LKR/SDH* knockout lines. Northern-blot analysis of total RNA from stem sections containing inflorescence and developing siliques of wild-type Arabidopsis (lane a), homozygous LKR/SDH knockout mutants (lanes b and d), and an heterozygous LKR/SDH knockout mutant (lane c). Top, The ethydium bromide staining pattern of the ribosomal RNAs. Bottom, The northern-blot hybridization pattern with the LKR domain of *LKR/SDH* as a probe. The position of the LKR/SDH mRNA is marked on the right. The migration of the 28S and 18S marker RNAs are indicated on the left.

were extracted from stem sections containing inflorescence and developing siliques of the wild-type plants as well as the heterozygous and homozygous *LKR/SDH* knockouts. These proteins were reacted in a western blot with monoclonal antibodies that specifically recognize the LKR domain of LKR/SDH. As shown in Figure 5, the monoclonal antibodies recognized an intense LKR/SDH protein band in the wild-type plants (lane d). Heterozygous plants for the

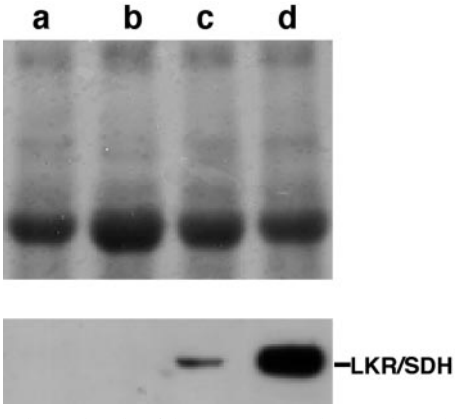

Figure 5. Relative levels of the LKR/SDH polypeptide in the different *LKR/SDH* knockout lines. Western-blot analysis of proteins extracted from stem sections containing inflorescence and developing siliques of homozygous LKR/SDH knockout mutants (lanes a and b), an heterozygous LKR/SDH knockout mutant (lane c), and wild-type Arabidopsis (lane d). Top, The Coomassie Blue staining pattern of the protein extracts. Bottom, The western-blot pattern following treatment with the anti-Arabidopsis LKR monoclonal antibodies. The position of the LKR/SDH polypeptide is marked on the right.

LKR/SDH knockout possessed the same band, but its intensity was reduced compared with the wild-type plants (Fig. 5, compare with lanes c and d). No detectable LKR/SDH polypeptide was observed in the homozygous LKR/SDH knockout mutants (lanes a and b).

The LKR/SDH Knockout Mutant Possesses Indistinguishable Phenotype from the Wild Type under Normal Growth Conditions

The phenotype of the homozygous *LKR/SDH* knockout mutant was carefully inspected, compared with the wild-type, under standard greenhouse growth conditions. No detectable difference was observed at any growth stage, including seed germination, plant morphology and growth, flowering time, fertility, silique development, and seed dormancy (data not shown).

Effect of the LKR/SDH Knockout Mutant on Lys Accumulation

Using northern-blot analysis, we have previously shown that the Arabidopsis LKR/SDH mRNA level is quite abundant in inflorescence and developing siliques, whereas it is significantly lower in vegetative tissues (Tang et al., 1997). It was therefore interesting to test the effect on the *LKR/SDH* knockout on Lys accumulation in leaves and seeds. The effect of LKR/SDH on Lys accumulation in seeds is also an issue of significant nutritional importance since Lys is an essential amino acid for human and livestock. To address this issue, we analyzed the free amino acids profiles from leaves and mature seeds of wild-type Arabidopsis and the homozygous *LKR/SDH* knockout mutant. As shown in Figure 6, the relative level of free Lys in leaves (mol % of total free amino acids) was similar between the wild-type and LKR/SDH knockout mutant. However, in mature seeds, the relative level of free Lys was significantly higher in the knockout mutant than in the wild-type plants. No major difference was observed between leaves and seeds of the wild type and *LKR/SDH* knockout mutant in the relative levels of other free amino acids (data not shown).

The majority of the Lys-rich proteins in plant seeds belongs to the class of water-soluble albumins. This class contains a diverse group of proteins that regulate seed development and response to environmental stimuli. In most dicot plants, a second class of seed salt-soluble globulins contains a much less diverse family of seed storage proteins. To test whether the free Lys level in Arabidopsis seeds limits its incorporation into seed proteins, we analyzed the proportion of Lys in seed albumins and globulins derived from the wild-type and *LKR/SDH* knockout mutant. As shown in Figure 7, the proportion of Lys in seed albumins of the homozygous *LKR/SDH* knockout mutant was slightly but significantly higher (approximately 6%) than that in the wild-type plants. No statistically significant difference (*t* test, see "Materials and Methods") was observed in Lys proportion of the seed globulins between the *LKR/SDH* knockout and wild-type lines.

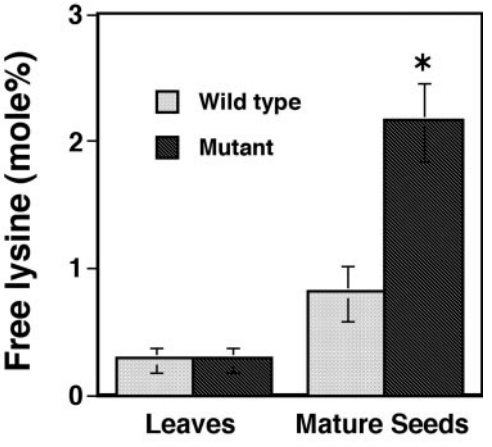

Figure 6. The relative level of free Lys in leaves and mature seeds of wild-type Arabidopsis and homozygous *LKR/SDH* knockout lines. Relative Lys levels are given in mol % of the total free amino acids. Bars on top represent the SD of five independent repeats for each histogram. Statistically significant differences ($P < 0.01$) are marked by an asterisk on top of the histogram.

DISCUSSION

Lys Catabolism Negatively Regulates Free Lys Accumulation in Plant Seeds

Although Lys catabolism has been previously implied to affect Lys accumulation in plant seeds, this has been based only on indirect evidence. *LKR/SDH* gene expression is up-regulated in plant seeds, and the activity of LKR is stimulated by Lys (Karchi et al., 1994, 1995; Tang et al., 1997, 2000). In addition, seeds of transgenic tobacco plants, expressing a bacterial feedback-insensitive DHPS in a seed-specific manner, failed to over-accumulate free Lys in mature seeds (Karchi et al., 1994). In contrast to tobacco, seed-specific expression of a bacterial feedback-insensitive DHPS in soybean, canola, and maize caused a significant over-accumulation of free Lys, but this was associated with enhanced accumulation of Lys catabolic products (Falco et al., 1995; Mazur et al., 1999). Our observation that knocking out the expression of the *LKR/SDH* locus in Arabidopsis results in increased free Lys levels in the seeds provides the first direct support for the significance of Lys catabolism in regulating Lys accumulation in plant seeds. Although free Lys accumulation in mature seeds of the *LKR/SDH* knockout mutant was significantly higher than in wild-type plants, it still amounted only to approximately 2% of total free amino acids. This is apparently due to a tight biochemical regulation of Lys synthesis due to the high

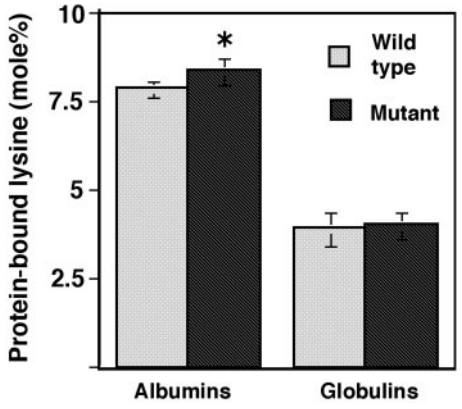

Figure 7. The relative level of Lys in the albumin and globulin fractions of mature seeds derived from wild-type Arabidopsis and the homozygous *LKR/SDH* knockout line. Relative Lys levels are given in mol % of the total amino acids in the two fractions. Bars on top represent the SD of five independent repeats for each histogram. Statistically significant differences ($P < 0.05$) are marked by an asterisk on top of the histogram.

sensitivity of the natural Arabidopsis DHPS to feedback inhibition by Lys (Galili, 1995; Ben Tzvi-Tzchori et al., 1996).

The Level of Free Lys in Arabidopsis Seeds May Represent a Rate-Limiting Factor for Synthesis of Lys-Rich Seed Proteins

Our amino acid analysis showed that the proportion of Lys in seed albumins, but not globulins was slightly but significantly higher in the *LKR/SDH* knockout plants than in the wild-type plants. A comparable slight and specific increase in the proportion of Lys and Thr in seed albumins, but not globulins, was previously reported in transgenic tobacco plants expressing feedback insensitive bacterial DHPS and Asp kinase (Karchi et al., 1994). The reason for the specific increase in Lys proportion in seed albumins and not globulins is still not clearly understood. It may be due to the fact that the increased free Lys level in the *LKR/SDH* knockout mutant may improve the translational efficiency of Lys-rich proteins due to the potential availability of higher concentrations of acylated lysyl tRNAs. Since the albumins (but not the globulins) consist of a large and diverse group of proteins, a slight change in their relative levels may cause a noticeable shift in the total Lys proportion in this fraction. Such a mechanism may also be supported by molecular analysis of the Lys-rich opaque2 mutants of maize. Larkins and associates (Habben et al., 1993) have shown that the relative levels of specific Lys-rich albumins, like the translational elongation factor EF1α, are preferentially increased in the opaque2 lines compared with wild-type plants. Moreover, a highly significant positive correlation between the relative level of EF1α and seed Lys level was reported in different maize lines (Habben et al., 1995).

Functional Significance of Lys Catabolism in Plants

The indistinguishable phenotype of the *LKR/SDH* knockout mutant from the wild-type plants under normal growth conditions suggests that Lys catabolism is not essential for normal plant growth as well as for seed development. This observation is also in agreement with previous reports showing that developing embryos of transgenic soybean, canola, and maize seeds, expressing a feedback-insensitive DHPS, can accumulate significantly higher levels of free Lys than wild-type plants without interference of seed development and germination (Falco et al., 1995; Mazur et al., 1999). It is thus likely that Lys catabolism acts as one of a number of regulatory networks that control the balance of free amino acids in plants seeds rather than as a specific mechanism to prevent Lys over-accumulation due to a potential toxicity of this amino acid. Such a delicate balance of free amino acids in the seed may be important for efficient incorporation of the free amino acids into seed proteins.

Although humans cannot synthesize Lys, they do possess an active of LKR/SDH-dependent Lys catabolism pathway, similar to the pathway existing in plants. In humans, defects in LKR or SDH activities are not lethal, but they do cause genetic disorders called familial hyperlysinemias, which are associated with increased plasma Lys levels (Woody, 1964; Markovitz et al., 1984). Some of the familial hyperlysinemias patients suffer from mental retardation, and it has been suggested that the major function of Lys catabolism in humans is to generate Glu that serves as a brain fuel operating via Glu receptors (Rao et al., 1992). Plants also possess homologs of the human Glu receptors, which regulate plant growth in response to light (Lam et al., 1998; Brenner et al., 2000), and it will be interesting to elucidate whether Lys catabolism in plants also functions as a catabolic pathway to generate Glu. Besides its concerted function with Glu receptors, Glu is also an important donor for a variety of regulatory metabolites, such as the stress associated compounds γ-amino butyric acid and Pro (Baum et al., 1996; Nuccio et al., 1999). Glu is also the direct precursor for Arg, which is metabolized into additional regulatory compounds such as polyamines and nitric oxide (Slocum et al., 1984; Walden et al., 1997; Klessig et al., 2000). It is notable that LKR/SDH gene expression was recently found to be significantly up-regulated in rapeseed leaves upon osmotic stress (Deleu et al., 1999). Our Arabidopsis *LKR/SDH* knockout mutant may be a highly suitable system for future dissection of the functional significance of Lys catabolism in the response of plants to stress and environmental factors.

Biotechnological Implications of Plant LKR/SDH Knockout Mutants

Due to the nutritional importance of the essential amino acids Lys for human and livestock, increasing

Lys production in plants is considered to be biotechnologically important. Breeding programs for high-Lys plants should manipulate Lys catabolism not only because it interferes with Lys accumulation. Some of Lys catabolic products, whose seed levels are concomitantly increased with increasing seed Lys levels (Falco et al., 1995; Mazur et al., 1999), are toxic to mammals (Karlsen et al., 1982; Welinder et al., 1982; Bonaventure et al., 1985; Reichenbach and Wohlrab, 1985). Manipulations of Lys synthesis in *LKR/SDH* knockout plants may provide an important way to increase seed Lys levels, while maintaining its toxic catabolic products at marginal levels. Experiments are in progress in our laboratory to test this hypothesis.

MATERIALS AND METHODS

Materials

Arabidopsis plants were grown in pots under a greenhouse condition (12-h photoperiod at 25°C ± 5°C). Long template PCR amplification *Taq* polymerase and SuperTherm DNA Polymerase used for PCR screening were purchased respectively from Roche Diagnostics (Mannheim, Germany) and JMR Holdings (London).

Isolation of T-DNA Insertion Line of LKR/SDH

DNA pools of the Arabidopsis T-DNA insertion lines from Versailles collection were screened for T-DNA insertion in the *LKR/SDH* locus. Forward and reverse primers from the coding sequence of the *LKR/SDH* locus were designed for PCR screening of the DNA pools by the combination of T-DNA left and right border-specific primers. PCR products were analyzed by southern hybridization of duplicate membranes to both the LKR/SDH gene probe generated from LKR/SDH cDNA fragment and the T-DNA probe. A positive PCR product was identified from Superpool-7 and further amplified positively in primary pool 26A by *LKR/SDH* locus primer P5 (5′-GATGAAAATGATCAACGATGCT-3′) and T-DNA primers TAG5 (5′-CTACAAATTGCCTTTTCTTATCGAC-3′) or TAG6 (5′-CACTCAGTCTTTCATCTCGGCA-3′). T-DNA insertion in the LKR/SDH gene was confirmed by sequencing the resulting positive PCR fragment. The 48 lines from the primary pool 26A were further screened, and line 41 was identified for T-DNA insertion in the LKR/SDH gene. Homozygous mutant plants were isolated from line 41 by PCR using two sets of primers. One set included the LKR/SDH gene primer P5 and the T-DNA primer TAG5, whereas the other set included the LKR/SDH gene primers P5 and P9: 5′-CTCGGTTAGCTAATCCAAATG-3′ on the opposite border of the T-DNA. Homozygous mutant plants were confirmed by Southern-blot analysis using the *LKR/SDH* gene probe generated from 3.2-kb NdeI cutting fragment of LKR/SDH gnomic DNA (Fig. 2).

DNA Sequence Analysis

Sequence analysis was performed by an automatic sequencer (model 373A, Version 1.2.0, Applied Biosystems, Foster City, CA).

DNA and RNA Gel-Blot Analysis

Extraction of total DNA and RNA as well as Southern- and northern-blot analyses were performed as previously described (Tang et al., 1997).

Western-Blot Analysis of the LKR/SDH Protein

A recombinant LKR domain of an Arabidopsis LKR/SDH cDNA, fused at its N terminus to an epitope tag of six histidines (His tag), was expressed in yeast and purified on a nickel column as previously described (Zhu et al., 2000). This protein was used for immunizing mice and preparing hybridomas to obtain anti-LKR monoclonal antibody.

Protein extraction from Arabidopsis plants, fractionation on SDS PAGE, transfer to PVDF membrane, and western-blot analysis were performed essentially as previously described (Zhu et al., 2000). Anti-LKR monoclonal antibody was used as primary antibodies for detecting LKR/SDH expression in plants.

Amino Acid Analysis

Rosette leaves and mature seeds from homozygous mutant plants and wild-type plants were harvested. Extraction of free amino acids as well as albumin and globulin, and subsequent analyses of the amino acid composition of these fractions were described previously (Karchi et al., 1993). Five sample preparations for each genotype plant organs were followed by amino acid measurements.

Statistic Analysis

The JMP-4 statistics program (Student's paired *t* test) was used to compare the relative levels of amino acids between the wild-type and *LKR/SDH* knockout plants.

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