The Plant Cell, Vol. 9, 1305–1316, August 1997 © 1997 American Society of Plant Physiologists

Regulation of Lysine Catabolism through Lysine–Ketoglutarate Reductase and Saccharopine Dehydrogenase in Arabidopsis

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In plant and mammalian cells, excess lysine is catabolized by a pathway that is initiated by two enzymes, namely, lysine–ketoglutarate reductase and saccharopine dehydrogenase. In this study, we report the cloning of an Arabidopsis cDNA encoding a bifunctional polypeptide that contains both of these enzyme activities linked to each other. RNA gel blot analysis identified two mRNA bands—a large mRNA containing both lysine–ketoglutarate reductase and saccharopine dehydrogenase sequences and a smaller mRNA containing only the saccharopine dehydrogenase sequence. However, DNA gel blot hybridization using either the lysine–ketoglutarate reductase or the saccharopine dehydrogenase cDNA sequence as a probe suggested that the two mRNA populations apparently are encoded by the same gene. To test whether these two mRNAs are functional, protein extracts from Arabidopsis cells were fractionated by anion exchange chromatography. This fractionation revealed two separate peaks—one containing both coeluted lysine–ketoglutarate reductase and saccharopine dehydrogenase activities and the second containing only saccharopine dehydrogenase activity. RNA gel blot analysis and in situ hybridization showed that the gene encoding lysine–ketoglutarate reductase and saccharopine dehydrogenase is significantly upregulated in floral organs and in embryonic tissues of developing seeds. Our results suggest that lysine catabolism is subject to complex developmental and physiological regulation, which may operate at gene expression as well as post-translational levels.

INTRODUCTION

In the cell, the level of the essential amino acid lysine is subject to tight regulation in both mammals and plants. In both types of organisms, excess lysine is catabolized via saccharopine and α-aminoadipic semialdehyde into α-aminoadipic acid and glutamate (Moller, 1976; Bryan, 1980; Markovitz et al., 1984; Galili et al., 1994; Galili, 1995; Goncalves-Butruille et al., 1996). The first enzyme in the lysine catabolic pathway is lysine–ketoglutarate reductase (LKR), which condenses lysine and α-ketoglutarate into saccharopine and uses the cofactor NADPH (Figure 1, reaction 1). The second enzyme, saccharopine dehydrogenase (SDH), converts saccharopine into α-aminoadipic semialdehyde and glutamate (Figure 1, reaction 2). This enzyme uses NAD^+ or, much less efficiently, $NADP^+$ as a cofactor (Markovitz et al., 1984; Goncalves-Butruille et al., 1996).

The molecular and biochemical regulation of lysine catabolism is still not clearly understood. Feeding lysine to rats or applying it to tobacco plants stimulated the activity of LKR in rat livers or in tobacco seeds, respectively (Foster et al., 1993; Karchi et al., 1994). Stimulation of this enzyme has also been observed in transgenic tobacco seeds overproducing lysine because of expression of a feedback-insensitive bacterial dihydrodipicolinate synthase (Karchi et al., 1995). This suggests that in both mammalian and plant cells, lysine may autoregulate its own catabolism. In addition, recent studies have shown that in tobacco seeds, the lysine-dependent stimulation of LKR activity is mediated by an intracellular signaling cascade requiring Ca^{2+} and protein phosphorylation (Karchi et al., 1995). The control of LKR activity in plants may be even more complex. In developing maize seeds, LKR activity was found to be reduced by two- to threefold in the high-lysine *opaque2* mutant, as compared with wild-type plants (Brochetto-Braga et al., 1992). *Opaque 2* is a transcription factor that regulates the expression of seed storage proteins (Shotwell and Larkins, 1988). This transcription factor could also complement the yeast GCN4 transcription factor that regulates the expression of many yeast genes encoding enzymes involved in amino acid metabolism (Hinnebusch, 1988).

Although LKR and SDH appear to control important processes, their structural aspects and cellular functions differ among various eukaryotic species. In yeast cells, in which lysine is synthesized via α-aminoadipate (Bhattacharjee, 1985), LKR and SDH play essential roles in lysine biosynthesis, and they appear as two separate polypeptides (Ogawa and Fujioka, 1978). In mammalian cells, which cannot synthesize lysine, LKR (LYS1) and SDH (LYS9) play an essential role in the catabolism of excess cellular lysine (Dancis et al., 1969), but their structural aspects may vary among species.

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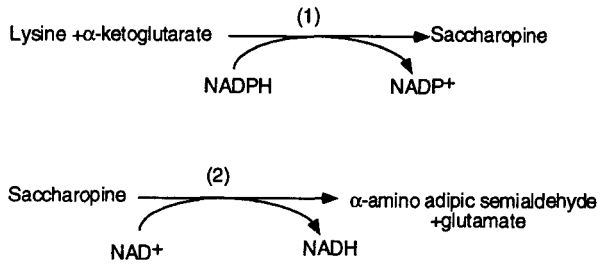

Figure 1. Lysine Catabolism via the Saccharopine Pathway.

Reaction 1 is catalyzed by LKR, which condenses L-lysine and α-ketoglutarate into saccharopine. Reaction 2 is catalyzed by SDH, which hydrolyzes saccharopine into α-aminoadipic semialdehyde and glutamic acid.

In rat liver, LKR and SDH were shown to be distinct monofunctional enzymes (Noda and Ichihara, 1978); however, human placenta (Fjellstedt and Robinson, 1975) and bovine liver (Markovitz et al., 1984) possess these two enzyme activities on a single bifunctional protein. In plants, which synthesize lysine via diaminopimelate, LKR and SDH also function in lysine catabolism (Arruda and da Silva, 1983), and recently, a bifunctional LKR/SDH enzyme has been purified from developing maize seeds (Brochetto-Braga et al., 1992). Moreover, in plants, LKR and SDH activities have been detected only in developing seeds to date (Arruda and da Silva, 1983; Karchi et al., 1994).

To elucidate further the regulatory role of LKR and SDH in lysine catabolism, we have cloned and characterized two cDNAs encoding a bifunctional LKR/SDH and a monofunctional SDH from Arabidopsis. We also show that Arabidopsis cells contain an mRNA species encoding a bifunctional LKR/SDH and another mRNA encoding a monofunctional SDH and that these are likely to be transcribed from a single gene. In addition, we have determined that expression of the Arabidopsis LKR/SDH gene is subject to spatial and developmental controls.

RESULTS

Identification and Characterization of an Arabidopsis cDNA Encoding a Monofunctional SDH

From the Arabidopsis sequence databases, we have identified an expressed sequence tag (EST) clone showing significant homology with SDH from yeast (clone 23A3T7). The complete nucleotide sequence of this cDNA was determined. As shown in Figure 2, this ~1.5-kb cDNA (designated cAt-SDH) contains an open reading frame initiated by an ATG codon with a consensus (G/A) at position −3 (Joshi, 1987) and encodes a putative protein of 482 amino acids. In addition, this cDNA contains a 5′ noncoding sequence of 51 nucleotides and a 3′ noncoding sequence of 80 nucleotides that is ended by a poly(A) tail. As shown in Figure 3, the open reading frame of cAt-SDH has significant homology with yeast SDH (LYS9), sharing 36.1% identity and 56.4% similarity. The initiation ATG and the stop codons of both yeast and the putative Arabidopsis SDH also appeared at

```
   1 CTGAAGATTGGTAAAGTCCAGCAAGAAAATGAGATAAAAGAGAAGCCTGAAATGACGAAA
                                                   M  T  K

  61 AAATCAGGTGTTTTGATTCTTGGTGCTGGACGTGTGTGTCGCCCAGCTGCTGATTTCCTA
     K  S  G  V  L  I  L  G  A  G  R  V  C  R  P  A  A  D  F  L

 121 GCTTCAGTTAGAACCATTTCGTCACAGCAATGGTACAAAACATATTTCGGAGCAGACTCT
     A  S  V  R  T  I  S  S  Q  Q  W  Y  K  T  Y  F  G  A  D  S

 181 GAAGAGAAAACAGATGTTCATGTGATTGTCGCGTCTCTGTATCTTAAGGATGCCAAAGAG
     E  E  K  T  D  V  H  V  I  V  A  S  L  Y  L  K  D  A  K  E

 241 ACGGTTGAAGGTATTTCAGATGTAGAAGCAGTTCGGCTAGATGTATCTGATAGTGAAAGT
     T  V  E  G  I  S  D  V  E  A  V  R  L  D  V  S  D  S  E  S

 301 CTCCTTAAGTATGTTTCTCAGGTTGATGTTGTCCTAAGTTTATTACCTGCAAGTTGTCAT
     L  L  K  Y  V  S  Q  V  D  V  V  L  S  L  L  P  A  S  C  H

 361 GCTGTTGTAGCAAAGACATGCATTGAGCTGAAGAAGCATCTCGTCACTGCTAGCTATGTT
     A  V  V  A  K  T  C  I  E  L  K  K  H  L  V  T  A  S  Y  V

 421 GATGATGAAACGTCCATGTTACATGAGAACGCTAAGAGTGCTGGGATAACGATTCTAGGC
     D  D  E  T  S  M  L  H  E  N  A  K  S  A  G  I  T  I  L  G

 481 GAAATGGGACTGGACCCTGGAATCGATCCCATGATGGCGAAGAAAATGATCAACGATGCT
     E  M  G  L  D  P  G  I  D  P  M  M  A  K  K  M  I  N  D  A

 541 CCTATCACAAAAGGGAAAGTGAAGTCTTTTACCTCTTATTGTGGAGGGCTTCCCTCTCGT
     P  I  T  K  G  K  V  K  S  F  T  S  Y  C  G  G  L  P  S  R

 601 GCTGCAGCAAATAATCCATTAGCATATAAATTTAGCTGGAACCCTGCTGGAGCAATTCGA
     A  A  A  N  N  P  L  A  Y  K  F  S  W  N  P  A  G  A  I  R

 661 GCTGGTCAAAACCCCGCCAAATACAAAAGCAACGGCGACATAATACATGTTGATGGGAAG
     A  G  Q  N  P  A  K  Y  K  S  N  G  D  I  I  H  V  D  G  K

 721 AATCTCTATGATTCCGCGGCAAGATTCCGAGTACCTAATCTTCCAGCTTTTGCATTGGAG
     N  L  Y  D  S  A  A  R  F  R  V  P  N  L  P  A  F  A  L  E

 781 TGTTTTCCAAATCGTGACTCCTTGGTTTACGGGGAACATTATGGCATCGAGAGCGAAGCA
     C  F  P  N  R  D  S  L  V  Y  G  E  H  Y  G  I  E  S  E  A

 841 ACAACGATATTTCGTGGAACACTCAGATATGAAGGGTTTAGTATGATAATGGCAACACTT
     T  T  I  F  R  G  T  L  R  Y  E  G  F  S  M  I  M  A  T  L

 901 TCGAAACTTGGATTCTTTGACAGTGAAGCAAATCAAGTACTCTCCACTGGAAAGAGGATT
     S  K  L  G  F  F  D  S  E  A  N  Q  V  L  S  T  G  K  R  I

 961 ACGTTTGGTGCTCTTTTAAGTAACATTCTAAATAAGGATGCAGACAATGAATCAGAGCCC
     T  F  G  A  L  L  S  N  I  L  N  K  D  A  D  N  E  S  E  P

1021 CTAGCGGGAGAAGAAGAGATAAGCAAGAGAATTATCAAGCTTGGACATTCCAAGGAGACT
     L  A  G  E  E  E  I  S  K  R  I  I  K  L  G  H  S  K  E  T

1081 GCAGCCAAAGCTGCCAAAACAATTGTATTCTTGGGGTTCAACGAAGAGAGGGAGGTTCCA
     A  A  K  A  A  K  T  I  V  F  L  G  F  N  E  E  R  E  V  P

1141 TCACTGTGTAAAAGCGTATTTGATGCAACTTGTTACCTAATGGAAGAGAAACTAGCTTAT
     S  L  C  K  S  V  F  D  A  T  C  Y  L  M  E  E  K  L  A  Y

1201 TCCGGAAATGAACAGGACATGGTGCTTTTGCATCACGAAGTAGAAGTGGAATTCCTTGAA
     S  G  N  E  Q  D  M  V  L  L  H  H  E  V  E  V  E  F  L  E

1261 AGCAAACGTATAGAGAAGCACACTGCGACTCTTTTGGAATTCGGGGACATCAAGAATGGA
     S  K  R  I  E  K  H  T  A  T  L  L  E  F  G  D  I  K  N  G

1321 CAAACAACAACCGCTATGGCCAAGACTGTTGGGATCCCTGCAGCCATTGGAGCTCTGCTG
     Q  T  T  T  A  M  A  K  T  V  G  I  P  A  A  I  G  A  L  L

1381 TTAATTGAAGACAAGATCAAGACAAGAGGAGTCTTAAGGCCTCTCGAAGCAGAGGTGTAT
     L  I  E  D  K  I  K  T  R  G  V  L  R  P  L  E  A  E  V  Y

1441 TTGCCAGCTTTGGATATATTGCAAGCATATGGTATAAAGCTGATGGAGAAGGCAGAATGA
     L  P  A  L  D  I  L  Q  A  Y  G  I  K  L  M  E  K  A  E  *

1501 TCAAAGAACTCTGTATATTGTTTCTCTCTATAACTTGGAGTTGGAGACAAAGCTGAAGAA

1561 GACAGAGACATTAGACCAGCAAAAAAAAAAAAAAAA
```

Figure 2. Nucleotide and Deduced Amino Acid Sequence of cAt-SDH.

The ATG and TAG initiation and stop codons of the open reading frame encoding the putative SDH protein are in boldface and underlined. The asterisk indicates the protein termination site. The GenBank accession number is U90523.

```
  1 MTKKSGVLILGAGRVCRPAADFLASVRTISSQQWYKTYFGADSEEKTDVH 50
    . ..|:||.|.| |..|. | ||.                      ..|::
  1 ..MGKNVLLLGSGFVAQPVIDTLAA.....................NDDIN 28

 51 VIVASLYLKDAKETVEGISDVEAVRLDVSDSESLLKYVSQVDVVLSLLPA 100
    |.|||:  | :| :.:.  |: .|:.|||.|...| | :.: |||:||:|
 29 VTVACRTLANA.QALAKPSGSKAISLDVTDDSALDKVLADNDVVISLIPY 77

101 SCHAVVAKTCIELKKHLVTASYVDDETSMLHENAKSAGITILGEMGLDPG 150
    . |: |.|..|  |..:||.||:... . |..:  .||||::.|:|||||
 78 TFHPNVVKSAIRTKTDVVTSSYISPALRELEPEIVKAGITVMNEIGLDPG 127

151 IDPMMAKKMINDAPITKGKVKSFTSYCGGLPSRAAANNPLAYKFSWNPAG 200
    ||.: | | |::.. . ||:||| |||||||.....:|||:|||||.. |
128 IDHLYAVKTIDEVHRAGGKLKSFLSYCGGLPAPEDSDNPLGYKFSWSSRG 177

201 AIRAGQNPAKYKSNGDIIHVDGKNLYDSAARFRVPNLPAFALECFPNRDS 250
    .: | .|.|||..:|.|  |.:.:| ..| .: :  .|::|: |:||||
178 VLLALRNSAKYWKDGKIETVSSEDLMATAKPYFI..YPGYAFVCYPNRDS 225

251 LVYGEHYGIESEATTIFRGTLRYEGFSMIMATLSKLGFFDSEANQVLSTG 300
    :: : | | .||.|::|||||:||. :: .| .:|:...:|||::|
226 TLFKDLYHI.PEAETVIRGTLRYQGFPEFVKALVDMGMLKDDANEIFS.. 272

301 KRITFGALLSNILNKDADNESEPLAGEEEISKRIIKLGHSKETAAKAAKT 350
    |.|.:.. |...|. ......: :|: :. ..:     ...|. .: ..
273 KPIAWNEALKQYLGAKSTSKEDLIASIDSKATW.....KDDEDRERILSG 317

351 IVFLGFNEEREVPSLCKSVFDATCYLMEEKLAYSGNEQDMVLLHHEVEVE 400
    :.:||: .: .:.. ... :|. |  :|| :.|.:||.|||:|:|..::|
318 FAWLGLFSDAKITPRGNA.LDTLCARLEELMQYEDNERDMVVLQHKFGIE 366

401 FLESKRIEKHTATLLEFGDIKNGQTTTAMAKTVGIPAAIGALLLIEDKIK 450
    : ::. .|.:|.||:::|.:   .. ..|| |||.|.||:. :::::.||
367 WADGT.TETRTSTLVDYGKV...GGYSSMAATVGYPVAIATKFVLDGTIK 412

451 TRGVLRPLEAEVYLPALDIL.QAYGIKLMEKAE* 482
    ..|:| |...|:  | :. | : ||| |.||.
413 GPGLLAPYSPEINDPIMKELKDKYGIYLKEKTVA* 446
```

Figure 3. Comparison of the Deduced Amino Acid Sequence of cAt-SDH with the Yeast SDH.

The top line indicates the amino acid sequence of cAt-SDH; the bottom line is that of the yeast SDH (LYS9). Identical amino acids are indicated by bars; highly similar amino acids are indicated by colons; and similar amino acids are indicated by a single dot. The asterisks indicate the protein termination sites.

very similar positions along the open reading frames. However, the Arabidopsis SDH has a short amino acid sequence (Figure 3, positions 26 to 45) that is not present in the yeast SDH. The 5′ noncoding region of cAt-SDH contains an additional ATG consensus codon in a coding frame different from that of the putative SDH open reading frame and is immediately followed by a stop codon. Whether this ATG has any functional role is still not known.

To test whether cAt-SDH encodes an SDH enzyme, the entire coding sequence of this cDNA was subcloned into either the pUC18 or pET-15b bacterial expression vectors and used to transform *Escherichia coli* cells. As shown in Figure 4, bacterial cells harboring either of these plasmids containing the cAt-SDH insert have significantly elevated levels of SDH activity, as compared with control bacteria harboring the expression plasmids with no inserts.

Analysis of SDH mRNA Levels in Different Arabidopsis Tissues

To test the expression of the Arabidopsis SDH gene in different tissues, total RNA was extracted from cell cultures, leaves, stems, roots, flowers, and young seedlings, and the levels of SDH mRNA were analyzed by hybridization with the cAt-SDH DNA as a probe. As shown in Figure 5, two major cross-hybridizing mRNA bands were detected. One had the expected size of ~1.5 kb corresponding to cAt-SDH, and a second larger mRNA was ~3.5 kb. Both mRNA bands were detected in all tissues after a long exposure time (data not shown) and were most intense in flowers.

Cloning and Characterization of an Arabidopsis cDNA Encoding a Putative Bifunctional LKR/SDH Polypeptide

Previous studies have shown that plants, like mammals, may have bifunctional LKR/SDH enzymes (Goncalves-Butruille et al., 1996). Therefore, we hypothesized that the ~3.5-kb mRNA band, shown in Figure 5, encodes a bifunctional LKR/SDH in which the SDH region is highly homologous to the monofunctional SDH mRNA. To examine this possibility, we screened an Arabidopsis cDNA library with the 1.5-kb SDH cDNA as a probe. Several positive clones were rescued, and the longest (~3.2-kb insert, designated cAt-LKR/SDH) was sequenced. As shown in Figure 6, cAt-LKR/SDH contains a long open reading frame of 3195 nucleotides encoding a protein of 117 kD. The size of the encoded protein is similar to the size of the bifunctional LKR/SDH recently purified from

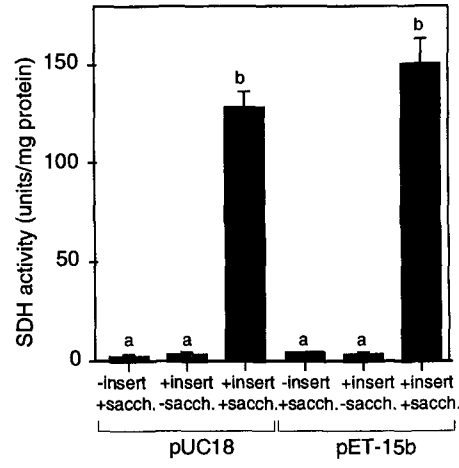

Figure 4. cAt-SDH Encodes a Functional SDH Enzyme.

The coding sequence of cAt-SDH was subcloned into two different bacterial expression vectors and transformed into *E. coli*. Protein extracts from bacteria harboring the plasmids containing cAt-SDH, as well as control bacteria transformed with the expression vector lacking cAt-SDH, were analyzed for SDH activity with (+) or without (−) the substrate saccharopine (sacch.). Each histogram represents an average of three separate activity tests ±SE. Letters above error bars represent significant differences at the 5% level, as determined by an ANOVA test. −insert, the empty vector not containing cAt-SDH; +insert, the vector with cAt-SDH.

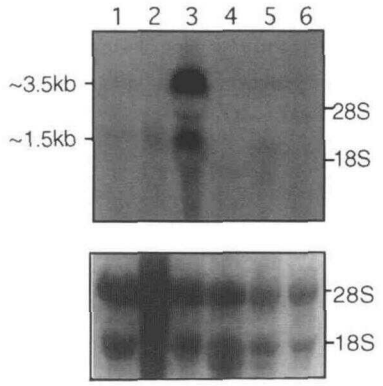

Figure 5. RNA Gel Blot Analysis of cAt-SDH mRNA.

Twenty micrograms of total RNA from cell cultures (lane 1), young seedlings (lane 2), floral organs (lane 3), leaves (lane 4), stems (lane 5), and roots (lane 6) was fractionated by gel electrophoresis and either hybridized on an RNA gel blot with cAt-SDH used as a probe (top) or stained with ethidium bromide as a control (bottom). The migration of the 18S and 28S rRNAs is shown at right. The positions of the monofunctional SDH mRNA (~1.5 kb) and the bifunctional LKR/SDH mRNA (~3.5 kb) are indicated at left.

maize (125 kD; Goncalves-Butruille et al., 1996) and from soybean (123 kD; D. Miron, S. Ben-Yaacov, D. Reches, and G. Galili, manuscript in preparation). This open reading frame is flanked by a 5′ noncoding sequence of 62 nucleotides and a 3′ noncoding sequence of 10 nucleotides. The cAt-LKR/SDH cDNA also lacks a 3′ poly(A) tail, suggesting that its 3′ region is not complete. Interestingly, the 3′ 1510 nucleotides of cAt-LKR/SDH are 100% homologous to nucleotides 1 to 1510 of cAt-SDH encoding the monofunctional SDH (cf. Figure 2).

As shown in Figure 7, the N-terminal part of the putative protein encoded by cAt-LKR/SDH (460 amino acids) exhibits significant homology to the yeast monofunctional LKR, with 24.9% identity and 52.1% similarity. The ATG initiation and stop codons of the yeast and the putative Arabidopsis LKR proteins also appear at comparable places along the open reading frame (Figure 7). However, the Arabidopsis LKR also has several small amino acid sequences that are not present in the yeast LKR (Figure 7).

The 5′ noncoding region of cAt-LKR/SDH contains three ATG triplets located seven to 41 nucleotides upstream of the presumed ATG translation initiation codon of the LKR/SDH open reading frame. These ATG codons form small open reading frames of nine to 15 amino acids, and none of these ATG codons contains the (A/G) consensus at position −3, which is generally found before eukaryotic translation initiation codons (Joshi, 1987), suggesting that these ATG triplets may have limited if any function in translational initiation.

Amino acid sequence alignment of the deduced polypeptide product of cAt-LKR/SDH with the yeast monofunctional LKR and SDH (Figures 2 and 6) shows that the putative cAt-LKR/SDH–encoded protein contains an intermediate region (amino acids 462 to 582, shown in boldface letters in Figure 6) that is not present in either the yeast LKR or the SDH enzymes. Although the functional significance of this region is still not known, intermediate regions previously have been found in other bifunctional polypeptides, such as the aspartate kinase/homoserine dehydrogenase isozyme of the aspartate family pathway (Ghislain et al., 1994; Galili, 1995).

To test whether the ~3.5-kb mRNA detected on the RNA gel blot shown in Figure 5 is related to cAt-LKR/SDH, the same blot was washed to remove the cAt-SDH probe and rehybridized with the putative LKR domain of cAt-LKR/SDH. As shown in Figure 8, this hybridization detected the ~3.5-kb mRNA band corresponding to cAt-LKR/SDH but not the ~1.5-kb mRNA band corresponding to cAt-SDH.

To determine further whether the N-terminal part of cAt-LKR/SDH encodes an LKR enzyme, the entire coding sequence of this cDNA was subcloned into the bacterial expression vector pET-15b and used to transform *E. coli* cells. Bacterial cells harboring this plasmid had SDH but no LKR activity (data not shown). Because bacterial cells did not produce an active LKR, we attempted to express the Arabidopsis LKR protein in yeast cells. Yeast has a monofunctional LKR enzyme, so we subcloned the N terminus of the presumed LKR domain of cAt-LKR/SDH into the yeast expression vector pVT-102u and transformed this plasmid into the yeast *Lys1* mutant. As shown in Figure 9, yeast cells harboring this plasmid have significantly higher LKR activity than do control cells transformed with the same plasmid without the LKR insert, thereby confirming our supposition that cAt-LKR/SDH indeed encodes a bifunctional LKR/SDH enzyme.

Organization of the LKR and SDH Genes in Arabidopsis

Based on the DNA sequence identity between cAt-SDH and the 3′ half of cAt-LKR/SDH (cf. Figures 2 and 6) and the presence of two mRNA species, corresponding in sizes to both cAt-SDH and cAt-LKR/SDH (Figure 5), we wanted to determine whether these two cDNAs are clustered within a single locus. To investigate whether the two cDNAs were derived from a single gene, Arabidopsis DNA was digested with several restriction enzymes, fractionated by agarose gel electrophoresis, and hybridized on DNA gel blots by using the SDH cDNA as a probe. After 1 week of autoradiography, the membrane was stripped and rehybridized with the LKR probe. As illustrated in Figures 10A and 10B, a comparison of the two autoradiographies shows that a signal appeared at exactly the same position when digested with both EcoRI and BamHI. These results suggest that the cAt-SDH/LKR and cAt-SDH are derived from a single gene. In the HindIII and BglII digests, the LKR and SDH probes highlighted different bands, apparently because the Arabidopsis LKR/SDH gene has a number of introns (G. Tang and G. Galili, unpublished data) that contain single or multiple sites for some of the restriction enzymes used for digestions.

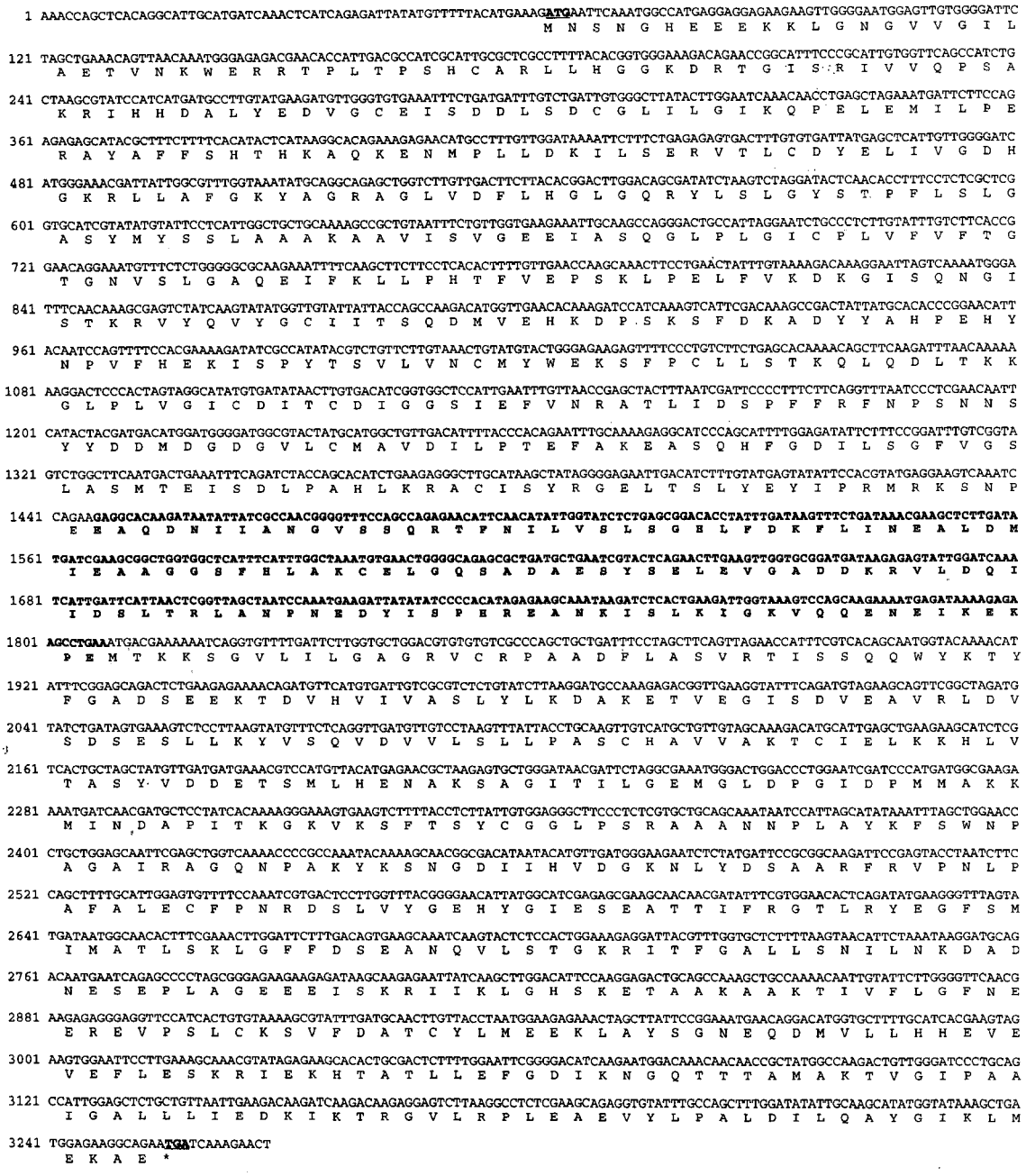

Figure 6. Nucleotide and Deduced Amino Acid Sequences of cAt-LKR/SDH.

The ATG and TAG translation initiation and stop codons of the open reading frame encoding the putative LKR/SDH protein are in boldface and underlined. The boldface region in the middle of the cDNA represents an intermediate region not present in either of the monofunctional LKR and SDH proteins of yeast. The asterisk indicates the protein termination site. The GenBank accession number is U90522.

Primer Extension Analysis of the Monofunctional SDH mRNA

To characterize further the putative monofunctional SDH mRNA observed on the RNA gel blots, we synthesized a 26-bp antisense DNA primer homologous to a region located 20 nucleotides downstream of the ATG translation initiation codon of cAt-SDH (Figure 2, nucleotides 75 to 100). This primer was then hybridized with total RNA from Arabidopsis flowers, and the hybrid molecules were used as templates for

```
  1 MNSNGHEEEKKLGNGVVGILAETVNKWERRTPLTPSHCARLLHGGKDRTG 50
              ::...: : ||| ..:| |.:|||. . :|:  |
  1 ..........MAAVTLHLRAET.KPLEARAALTPTTVKKLIAKG..... 33
                .         .         .         .
 51 ISRIVVQPSAKRIHHDALYEDVGCEISDDLS.....DCGLILGIKQ.PEL 94
      :| |:.|:... : . | :.|. | .. |        :|:|:|: ||
 34 .FKIYVEDSPQSTFNINEYRQAGAIIVPAGSWKTAPRDRIIIGLKEMPET 82
                .         .         .         .
 95 EMILPERAYAFFSHTHKAQKENMPLLDKILSERVTLCDYELIVGDHGKRL 144
    : :   :..  |.|..|.| :   :| :::.::.||:|.|:: .|:|:|:
 83 DTFPLVHEHIQFAHCYKDQAGWQNVLMRFIKGHGTLYDLEFLENDQGRRV 132
                .         .         .         .
145 LAFGKYAGRAGLVDFLHGLGQRYLSLGYSTPFLSLGASYMYSSLAAAKAA 194
     ||| ||| || .    |: :: :. ..|..  .|.|    |..:..||
133 AAFGFYAGFAGAA...LGVRDWAFKQTHSDD.EDLPA...VSPYPNEKAL 175
                .         .         .         .
195 VISVGEEIASQGLPLGICPLVFVFTGTGNVSLGAQEIFKLLPHTFVEPSK 244
    | .|..:. ..  . :  | |:::.: |..: || ::: :
176 VKDVTKDYKEALATGARKPTVLIIGALGRCGSGAIDLLHKV......... 216
                .         .         .         .
245 LPELFVKDKGISQNGISTKRVYQVYGCIITSQDMVEHKDPSKSFDKADYY 294
              ||.:..| .. :                |:.|::
217 .........GIPDANILKWDI................KETSRG....... 234
                .         .         .         .
295 AHPEHYNPVFHEKISPYTSVLVNCMYWEKSFPCLLSTKQLQDLTKKGLPL 344
      :.:::.:      | ..:::||:|:.|.:::. . ..|.: .::  .:
235 ...GPFDEI......PQADIFINCIYLSKPIAPFTNMEKLNNPNRRLRTV 275
                .         .         .         .
345 VGICDITCDIGGSIEFVNRATLIDSPFFRFNPSNNSYYDDMDGDGVLCMA 394
    |:::. |.:  ..|.: . ||:::.|        . . ..  |. : .:.
276 VDVSADTTNPHNPIPIYTVATVFNKP........TVLVPTTVGPKLSVIS 317
                .         .         .         .
384 VDILPTEFAKEASQHFGDILSGFVGSLASMTEISDLPAHLKRACISYRGE 444
    :| ||. :::|||: |:. |      |:|:    :| :: | |.:. |:.
318 IDHLPSLLPREASEFFSHDL......LPSL....ELLPQRKTAPVWVRAK 357
                .
445 LTSLYE.YIPRMRKSNPE 461
      .|:: ..:|:::|..
358 ..KLFDRHCARVKRSSRL* 373
```

Figure 7. Comparison of the Deduced Amino Acid Sequence of cAt-LKR/SDH with the Yeast LKR.

The top line indicates the amino acid sequence of the LKR domain of the cAt-LKR/SDH; the bottom line is that of the yeast LKR (LYS1). Identical amino acids are indicated by bars; highly similar amino acids are indicated by colons; and similar amino acids are indicated by a single dot. The asterisk indicates the protein termination site.

reverse transcription in a primer extension reaction. As shown in Figure 11, this reaction generated a DNA band of 54 nucleotides that was extended approximately five nucleotides upstream of the cAt-SDH translation initiation ATG codon.

Arabidopsis Cells Contain Bifunctional LKR/SDH and Monofunctional SDH Isozymes

To determine whether the two mRNAs derived from the Arabidopsis LKR/SDH gene were functional in translating bifunctional LKR/SDH and monofunctional SDH isozymes, we partially purified LKR and SDH from an Arabidopsis cell culture by using an anion exchange column, after polyethylene glycol (PEG) fractionation. As shown in Figure 12, elution from the anion exchange column resolved two distinct SDH peaks. The first was eluted at ~90 mM KCl and contained only SDH activity, whereas the second peak was eluted at ~190 mM KCl and had both SDH and LKR activities. The level of SDH activity in the peak that did not show LKR activity was ~3.5-fold higher than the level in the peak containing both coeluted SDH and LKR activities. Moreover, under the excess substrate concentrations that were used in the enzymatic assays (D. Miron, S. Ben-Yaacov, D. Reches, and G. Galili, manuscript in preparation), LKR activity in this peak was approximately fourfold higher than was SDH activity.

In Situ Hybridization with the SDH and LKR mRNAs as Probes

We have shown that cAt-SDH mRNA is expressed to a high level in floral tissues of Arabidopsis (Figure 5). To determine whether the expression of both LKR/SDH and SDH mRNAs in Arabidopsis tissues is subject to developmental regulation, particularly in reproductive organs, we used LKR/SDH RNA probes for in situ hybridization analysis of Arabidopsis flowers and seeds. Digoxigenin-labeled RNA probes from both LKR (Figures 13A, 13D, and 13G) and SDH (Figures 13B, 13E, and 13H) domains of the Arabidopsis LKR/SDH cDNA were used in this analysis. As shown in Figures 13A and 13B, the LKR and SDH mRNA was highly abundant in the ovules and vascular tissue of anther filaments but not in pollen grains. In developing and mature seeds, hybridization signals were found in the embryo (at either the globular [Figures 13G and 13H] or torpedo [Figures 13D and 13E] stages) and in the outer layers of the endosperm (Figures 13G and 13H). No signal was detected in the control sections reacted with either the LKR (Figure 13C) or SDH (Figure 13F) sense probes. The somewhat lower intensity of signal obtained with the SDH probe compared with that of the LKR probe was probably due to a lower amount of the SDH probe and possibly the lower incorporation of digoxigenin during in vitro transcription used during hybridization. This result indicates that the expression of both SDH and LKR/SDH genes is regulated in a tissue-specific manner during plant development.

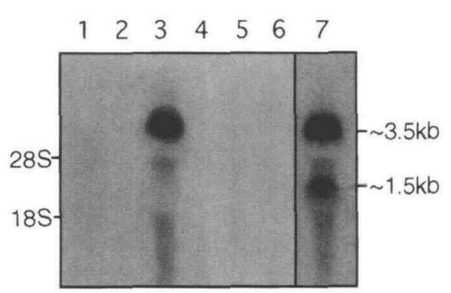

Figure 8. RNA Gel Blot Analysis of cAt-LKR/SDH.

The same blot as shown in Figure 5 was stripped to remove the cAt-SDH probe and rehybridized, with the LKR coding region of cAt-LKR/SDH as a probe. Lane 1 contains RNA from cell cultures; lane 2, young seedlings; lane 3, floral organs; lane 4, leaves; lane 5, stems; and lane 6, roots. Lane 7 is the same as lane 3 shown in Figure 5 containing RNA from floral organs hybridized with cAt-SDH as a probe. The migration of the 18S and 28S rRNAs is shown at left. The positions of the monofunctional SDH mRNA (~1.5 kb) and the bifunctional LKR/SDH mRNA (~3.5 kb) are shown at right.

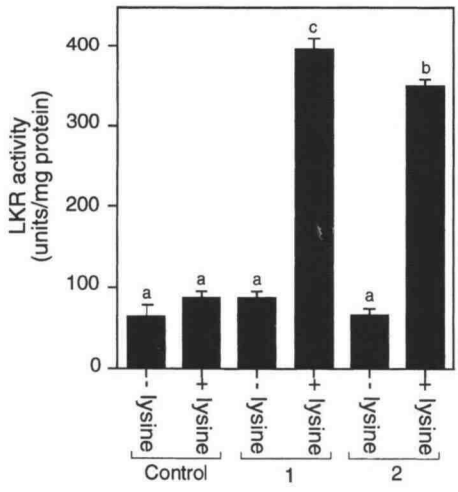

Figure 9. The LKR Domain of cAt-LKR/SDH Encodes a Functional LKR Enzyme in Yeast Cells.

The putative LKR domain of cAt-SDH was subcloned into a yeast expression vector and transformed into yeast. Protein extracts from two different yeast colonies (marked 1 and 2) harboring the plasmid containing the Arabidopsis LKR, as well as control yeast cells transformed with the expression vector without the insert, were then analyzed for LKR activity in reactions containing (+lysine) or lacking (−lysine) the substrate lysine. Letters above error bars represent significant differences at the 5% level, as determined by an ANOVA test. Each histogram is an average of three separate activity determinations ±SE.

DISCUSSION

Arabidopsis Contains Bifunctional LKR/SDH and Monofunctional SDH Isozymes, Which May Be Derived from a Single Gene

This report describes the cloning of LKR and SDH cDNAs from Arabidopsis and shows that the structural and regulatory aspects of LKR and SDH in plants are much more complex than what has been previously elucidated for yeast and mammals (Bhattacharjee, 1985; Feller et al., 1994). To date, either single LKR or SDH (yeast and rat) or bifunctional LKR/SDH (human, bovine, maize, and soybean) has been shown to exist within a given species; however, in this study, we show that Arabidopsis cells contain two isozymic peaks, as deduced from anion exchange chromatography. One of these peaks contains both LKR and SDH activities, which presumably are located on a bifunctional polypeptide encoded by cAt-LKR/SDH, and the other contains only SDH activity. Although a bifunctional LKR/SDH enzyme has been reported previously in maize, our results show that plant cells may also contain a monofunctional SDH. In fact, we have recently purified the SDH protein (shown in Figure 12 as the first SDH activity peak) to homogeneity and found that it is a 53-kD protein, in agreement with the expected size of a monofunctional SDH (data not shown).

Our results also strongly suggest that these two isozymes of LKR/SDH and monofunctional SDH are translated from two distinct mRNAs, which are produced from a single gene. We reached this conclusion based on several lines of evidence: (1) detection of two mRNA bands with the expected sizes of the isozymes (~1.5 and ~3.5 kb) on RNA gel blots hybridized with the monofunctional SDH cDNA as a probe under high-stringency conditions; (2) the presence of an in-frame "plant" ATG consensus codon at the initiation of the SDH coding sequence (as deduced from amino acid sequence homology with the yeast SDH), which also gave rise to the production of an active recombinant monofunctional SDH in bacteria; and (3) DNA gel blot analysis, which suggested the presence of only a single gene in Arabidopsis that hybridized with either the LKR or the SDH domains of cAt-LKR/SDH as probes.

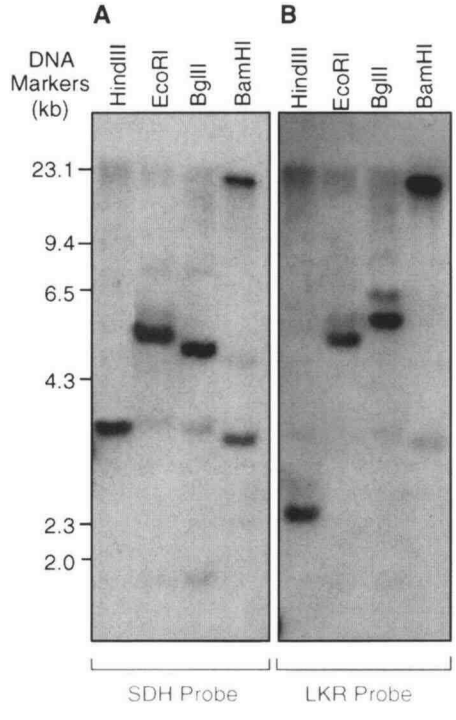

Figure 10. DNA Gel Blot Hybridization Pattern of cAt-SDH and cAt-LKR/SDH.

(A) Genomic DNA was digested with several restriction enzymes as indicated above the gel. Ten micrograms of digested DNA was separated on a gel, transferred to a membrane, and hybridized under high-stringency conditions with cAt-SDH.

(B) The same blot as shown in **(A)** was stripped and hybridized under high-stringency conditions with the LKR domain of cAt-LKR/SDH as probes.

The migration of the molecular length markers is indicated at left, and their lengths are given in kilobases.

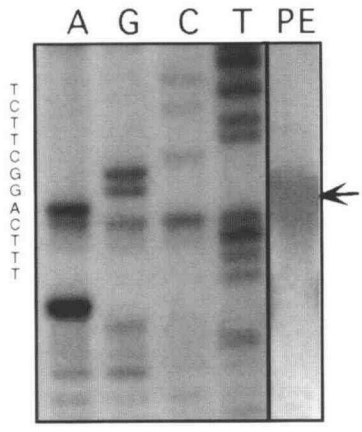

Figure 11. Primer Extension Reaction of Total Arabidopsis Flower RNA with the Antisense Primer Located 20 to 46 Nucleotides Downstream of the cAt-SDH ATG Initiation Codon.

The primer extension (PE) reaction product is indicated by an arrow; A, G, C, and T indicate sequencing ladders of the same primer annealed to the relevant genomic fragment. The sequence around the extended product is indicated at left.

The presence of an mRNA encoding a monofunctional SDH was also supported by the primer extension analysis shown in Figure 11. However, the primer extension band was shorter than expected, based on the 5′ noncoding sequence of cAt-SDH, and terminated approximately five nucleotides upstream of the translation initiation ATG of this cDNA. The reason for the shorter than expected primer extension fragment is still not known. However, computer analysis predicted that the 5′ noncoding region of cAt-SDH may contain a relatively stable stem and loop structure (data not shown). Experiments are now in progress in our laboratory to analyze whether this region may indeed form stable secondary structures in vivo and whether these structures may function in the regulation of the LKR/SDH gene expression. Nevertheless, based on the primer extension results, we cannot yet affirm whether cAt-SDH was derived from the monofunctional SDH mRNA or is a truncated form of cAt-LKR/SDH.

Structural and Functional Properties of the Bifunctional LKR/SDH Enzyme

Amino acid sequence alignment of cAt-LKR/SDH with the yeast monofunctional LKR and SDH isozymes revealed that the plant bifunctional enzyme possesses an intermediate region between the two enzyme domains that was not present in any of the yeast enzymes. Similar intermediate regions were also reported for other bifunctional enzymes, such as bacterial and plant aspartate kinase/homoserine dehydrogenase (Kalinowski et al., 1991; Ghislain et al., 1994). The functional role of this intermediate region is still not known. However, the fact that the LKR and SDH domains of the bifunctional LKR/SDH can be dissected into single functional enzymes (Figures 4 and 9; Markovitz and Chuang, 1987; Goncalves-Butruille et al., 1996) suggests that this region may enable independent folding of the two domains. In addition, because bifunctional LKR/SDH are generally homooligomers (Markovitz et al., 1984; Goncalves-Butruille et al., 1996), the intermediate domain may also function in its assembly, as was previously reported for the bacterial bifunctional aspartate kinase/homoserine dehydrogenase enzyme (Kalinowski et al., 1991).

Another interesting issue is whether the linkage between the LKR and SDH domains has a regulatory significance, which may result from "cross-talk" between the two domains. Although this issue is still not solved, our study indicates that such cross-talk may indeed occur. Upon fractionation on the anion exchange column and analysis under conditions of excess substrates of LKR and SDH (D. Miron, S. Ben-Yaacov, D. Reches, and G. Galili, manuscript in preparation), the specific activity of SDH in the monofunctional SDH peak was much higher than that in the bifunctional LKR/SDH peak. This difference could not be explained by the differential degree of purification of the two peaks because both peaks contained comparable levels of total protein. The differences in SDH activity between the two isozymes also could not be explained by differences in mRNA levels because the intensity of the LKR/SDH mRNA band was slightly higher than that of the monofunctional SDH mRNA (Figure 5). Thus, although we cannot yet rule out the possibility of variation in translational efficiency or protein stability, it is tempting to hypothesize that the activity of SDH may be negatively reg-

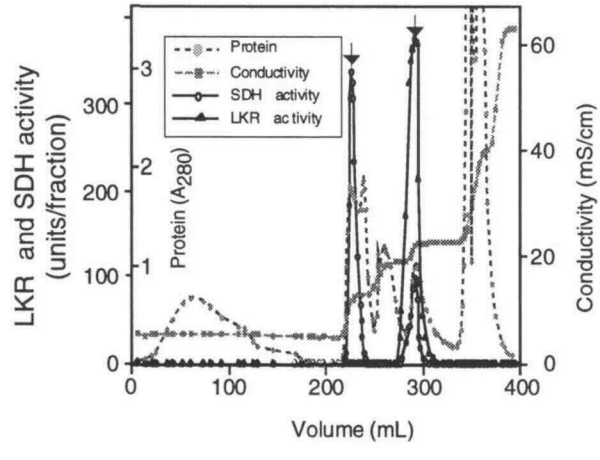

Figure 12. Fractionation of LKR and SDH Activities from Arabidopsis Cell Culture on an Anion Exchange Column.

PEG-fractionated Arabidopsis cell culture extract was loaded onto a DEAE–Sepharose column, washed, and eluted with a step gradient of 0 to 1 M KCl. The protein level, conductivity, and LKR and SDH activities in each fraction are presented. mS, millisiemens.

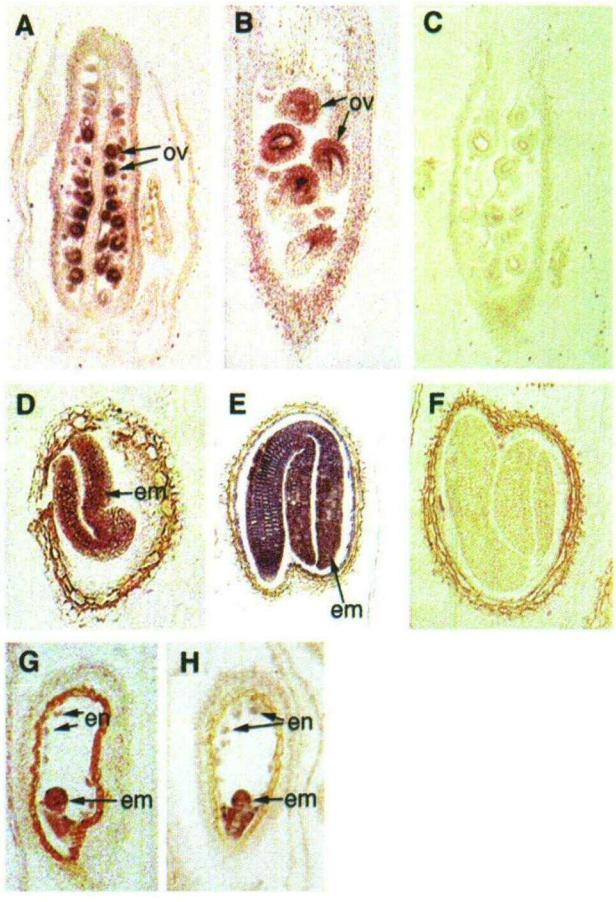

Figure 13. In Situ Hybridization of Arabidopsis Flower and Seed Tissues with LKR and SDH Antisense Probes.

(A), **(D)**, and **(G)** LKR probe. **(A)** shows a longitudinal section of an Arabidopsis flower. **(D)** shows cross-sections of seeds with a torpedo-shaped embryo. **(G)** depicts cross-sections of seeds with a globular-shaped embryo.

(B), **(E)**, and **(H)** SDH probe. **(B)** shows a longitudinal section of an Arabidopsis flower. **(E)** shows cross-sections of seeds with a torpedo-shaped embryo. **(H)** shows cross-sections of seeds with a globular-shaped embryo.

(C) and **(F)** Negative controls with LKR and SDH sense probes, respectively. **(C)** shows a longitudinal section of an Arabidopsis flower. **(F)** shows cross-sections of seeds with a torpedo-shaped embryo.

em, embryo; en, endosperm; ov, ovules.

ulated by its linked LKR domain. If indeed such a control occurs in vivo, it is expected that plant species producing only a single bifunctional LKR/SDH will accumulate saccharopine (the product of LKR and the substrate of SDH; see Figure 1), whereas those producing both isozymes will accumulate a downstream metabolite of the catabolic pathway. Interestingly, whereas lysine-overproducing transgenic soybean seeds, expressing a bacterial dihydrodipicolinate synthase, were shown to accumulate saccharopine, transgenic tobacco and canola expressing the same bacterial enzyme accumulated the downstream metabolite α-aminoadipic acid (Falco et al., 1995). Whether the differential accumulation of saccharopine and α-aminoadipic acid in these plant species is related to differential expression of the LKR/SDH and SDH isozymes still remains to be demonstrated.

Expression of the LKR/SDH Gene Is Developmentally Regulated

Although the LKR/SDH and monofunctional SDH mRNAs were detected in all tissues tested, their levels varied among the different tissues. Both mRNAs were significantly higher in floral organs than in vegetative tissues (Figure 5). In addition, in situ mRNA hybridization using reproductive organs showed that these mRNAs were most abundant in the ovaries of developing flowers as well as in the embryos but not in the endosperm tissues of developing and mature seeds. The spatial pattern of LKR/SDH gene expression in developing flowers and seeds appears very similar to that of the Arabidopsis gene encoding the bifunctional aspartate kinase/homoserine dehydrogenase that leads to the synthesis of lysine as well as threonine, methionine, and isoleucine (Zhu-Shimoni et al., 1997). These results support our previous hypothesis (Karchi et al., 1994) that expression of genes encoding enzymes in lysine biosynthesis and catabolism may be coordinately expressed during plant development. We have also previously shown that the presence of excess cellular lysine caused the stimulation of LKR activity in developing tobacco seeds (Karchi et al., 1995). Therefore, it will be interesting to test whether the coordinated expression of the LKR/SDH gene with other genes encoding enzymes in lysine biosynthesis is due to common transcriptional elements in their promoters or to a special regulation of LKR/SDH gene expression by sensing the relatively high lysine levels in cells in which lysine biosynthesis is upregulated.

Post-Transcriptional Regulation of LKR

The Arabidopsis SDH was active when expressed in bacterial cells; however, LKR was not. This was not due to lack of expression, because the LKR/SDH construct leads to the production of SDH but not LKR activity in bacteria. Moreover, the lack of production of active Arabidopsis LKR in bacteria was not due to a mutation in its sequence, because the same DNA produced active LKR when expressed in yeast cells. These results suggest that LKR may be activated by post-translational modification, which does not operate in prokaryotes. Indeed, we have recently found that the active LKR enzyme from soybean is a phosphoprotein and that removal of its phosphate residue(s) by alkaline phosphatase knocked out LKR activity in vitro (D. Miron, S. Ben-Yaacov, H. Karchi, and G. Galili, submitted manuscript).

METHODS

Plant Material

Arabidopsis thaliana var C24 plants were grown in a greenhouse, and different tissues were collected from the developing plants for the isolation of the total RNA and in situ hybridization.

The cell culture of Arabidopsis ecotype Landsberg *erecta* was kindly provided by M.J. May (University of Oxford, Oxford, UK; May and Leaver, 1993). This culture was grown in MSMO liquid medium (Sigma), pH 5.7, containing 3% sucrose, 0.05 mg/L kinetin, and 0.5 mg/L naphthaleneacetic acid. The culture was placed on a rotary shaker at 110 rpm at 22°C in continuous fluorescent white light.

Cloning of the Full-Length cAt-LKR/SDH and cAt-SDH and Subcloning Them into Expression Vectors

The expressed sequence tag (EST) clone 23A3T7 and the λZAP II cDNA library (Kieber et al., 1993) were kindly provided by the Arabidopsis Biological Resource Center (Columbus, OH). To clone the full-length cAt-LKR/SDH from the λZAP II library, the cDNA from the EST clone was used as a probe to screen the library, as previously described (Sambrook et al., 1989). The plasmid containing the full-length cAt-LKR/SDH was excised from the λZAP II by using a helper phage, and its DNA sequence was determined by an automatic sequencer (model 373A, version 1.2.0; Applied Biosystems, Foster City, CA).

For expression of the putative monofunctional SDH in bacteria, an SmaI to XbaI DNA fragment containing the entire coding sequence of cAt-SDH was subcloned by a translational fusion into EcoRI (blunt ended with the Klenow fragment of DNA polymerase I) and XbaI sites of pUC18. For subcloning into the bacterial expression vector pET-15b, the coding sequence of cAt-SDH was excised with XbaI (blunt ended with the Klenow fragment) and SalI and subcloned as a translational fusion into the BamHI (blunt ended with the Klenow fragment) and XhoI sites of pET-15b to form the plasmid pET-15b-SDH.

For expression of the LKR/SDH sequence in bacteria, cAt-LKR/SDH was digested with EcoRI, which cleaves immediately after the LKR translation initiation codon (ATGAATTC). The plasmid was then blunt ended with the Klenow fragment, digested with NheI, which cleaves in the SDH domain, and subcloned into the NcoI (blunt ended with the Klenow fragment) and NheI sites of pET-15b-SDH, resulting in the plasmid pET-15b-cAt-LKR/SDH.

For expression in yeast, pET-LKR/SDH was digested with XbaI, which cleaves immediately upstream of the LKR translation initiation ATG codon, and PstI, which cleaves in the SDH domain. The insert was then inserted into the XbaI and PstI sites of pVT-102u, resulting in the plasmid pVT-102u-LKR.

Production of Recombinant Proteins in Bacteria and Yeast

The expression plasmids were transformed into *Escherichia coli* (Sambrook et al., 1989) and yeast cells (Ito et al., 1983) by using general heat shock and LiOAc transformation methods, respectively. Transformed bacterial cells were grown to mid-exponential phase (A_{600} of ~0.5 to 0.8) and then induced with 0.4 mM isopropyl β-D-thiogalactopyranoside for an additional 4 hr. Transformed yeast cells (mutant 8973b from A. Pierre, Université Libre de Bruxelles, Brussels, Belgium; Ramos et al., 1988) were grown to mid-log phase in liquid SC medium (Sherman et al., 1983) lacking uracyl.

Processing of Bacteria and Yeast for Analysis of LKR and SDH Activities

E. coli cells were precipitated, dissolved in one-tenth of buffer A (25 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, and 10 μg/mL leupeptin), and sonicated. The total lysate was precipitated at top speed (16,000*g*) in a tabletop centrifuge for 10 min at 4°C, and the supernatant was used for activity assays. Yeast cells were precipitated, redissolved in one-tenth of buffer A, and broken by vortexing with glass beads for half an hour at 4°C. The lysate was precipitated again, and the supernatant was used for activity assays.

DNA Gel Blot Analysis

Extraction of genomic DNA was performed according to the procedure in Sambrook et al. (1989). DNA samples (10 μg) were electrophoresed in a 1% agarose gel and transferred to a Hybond N^+ (Amersham) nylon membrane. The blots were hybridized for 12 to 16 hr at 65°C with ^{32}P-labeled probes containing either the LKR or SDH domain of cAt-LKR/SDH. Hybridization was performed in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 × Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 1% SDS. Blots were washed twice for 10 min at 65°C in 1 × SSPE (1 × SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.5) and 0.1% SDS, followed by another wash in 0.1 × SSPE and 0.1% SDS. Radioactive bands were detected by autoradiography. The hybridization probes included either the 1454-bp SalI-NdeI fragment of cAt-SDH (SDH probe) or a 771-bp NotI-HindIII fragment from cAt-LKR/SDH in pBluescript SK− (Stratagene, La Jolla, CA; LKR probe).

RNA Gel Blot Analysis

Total RNA was extracted from various tissues by using Tri-Reagent (MRC, Inc., Cincinnati, OH), according to the protocol provided by the manufacturer. RNA samples (20 μg) were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and 50 mM 3-(*N*-morpholino) propanesulfonic acid, pH 7.0, and transferred to a Hybond N nylon membrane. Probe utilization, hybridization, and washing were as described above for the DNA gels blots. The migration of the 28S and 18S rRNAs was visualized by ethidium bromide staining of the gel before transfer to a membrane.

Partial Purification of the LKR and SDH from Arabidopsis Cell Culture

A 1-week-old cell culture was filtered, and the resulting cell pellet was frozen in liquid nitrogen and kept at −80°C until used. For purification, the frozen pellet was ground with a mortar and pestle and then homogenized using an Ultraturax (Ystral GmbH, Dottingen, Germany) in an equal volume of buffer A. After centrifugation at 25,000*g* for 15 min, the pH of the supernatant was brought to pH 5.6 with solid KH_2PO_4 and then fractionated with polyethylene glycol (PEG) 8000 between 7 and 14%. After fractionation with 14% PEG, the pellet was resuspended in one-tenth the initial volume of buffer A and loaded

onto an anion exchange DEAE–Sepharose column (Pharmacia). After washing the unbound protein, the column was eluted with a step gradient of 0 to 1 M KCl in buffer A.

Analysis of LKR and SDH Activities

The kinetics of LKR activity was measured spectrophotometrically by determining the rate of NADPH oxidation at 340 nm for 10 min at 30°C. The activity assays included 50 μg of protein extract in 0.3 mL of 0.1 M Tris-HCl, pH 7.4, 20 mM lysine, 14 mM α-ketoglutarate, and 0.4 mM NADPH. Each reaction also included a control lacking the substrate lysine. One unit of LKR was defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per min at 30°C.

The kinetics of SDH activity was measured spectrophotometrically by determining the rate of NAD^+ reduction at 340 nm for 10 min at 30°C. The activity assay included 50 μg of protein extract in 0.3 mL of 0.1 M Tris-HCl, pH 8.5, 2 mM saccharopine, and 2 mM NAD^+. Each reaction also included a control lacking the substrate saccharopine. One unit of SDH was defined as the amount of enzyme that catalyzes the reduction of 1 nmol of NAD^+ per min at 30°C.

Protein Determination

Protein levels were determined by the method of Bradford (1976), using the Bio-Rad protein assay kit.

In Situ Hybridization

For preparation of the hybridization probe, the LKR and SDH domains of cAt-LKR/SDH were subcloned separately into the pBluescript SK− plasmids. Digoxigenin-labeled sense and antisense probes were obtained by in vitro transcription using the digoxigenin RNA labeling kit (Boehringer Mannheim). Tissue preparation and in situ hybridization were conducted as described by Drews (1995). An antisense probe and the corresponding sense control probe were used in each experiment.

Primer Extension

Primer extension analysis was performed according to Sambrook et al. (1989), with several modifications. Total RNA (10 μg) from flowers was mixed with ^{32}P–end-labeled antisense primer located 20 to 46 nucleotides downstream of the transcription initiation ATG codon of cAt-SDH. The reaction was then incubated at 80°C for 10 min and cooled slowly to room temperature for annealing. Reverse transcription was conducted at 42°C for 1.5 hr. The reaction was stopped by boiling for 10 min and cooling on ice, and the mixture was then treated with RNase free of DNase for 30 min at 37°C. After ethanol precipitation, the primer extension product was analyzed on a sequencing gel along with a sequencing ladder of the same primer annealed to the relevant genomic fragment. Radioactive bands were detected by autoradiography.

Computer Analysis

DNA sequence analyses were performed using the Genetics Computer Group (Madison, WI) software package (version 8).

ACKNOWLEDGMENTS

We are indebted to Hua Zhang for excellent technical assistance. We also thank Dr. Hillel Fromm for critical reading of the manuscript and helpful comments; Dr. Shula Michaeli for help with the primer extension experiment; Dr. Joseph K. Ecker for making available the λZAP II cDNA library; the Arabidopsis Biological Resource Center for providing this library and the EST clone 23A3T7; Dr. Mike J. May for providing the Arabidopsis cell culture; and Dr. André Pierard for providing us with the yeast mutant 8973b. This work was supported in part by grants from the Israel Academy of Sciences and Humanities, National Council for Research and Development, Israel, and the Leo and Julia Forchheimer Center for Molecular Genetics. G.G. is an incumbent of the Bronfman Chair of Plant Sciences.

Received May 5, 1997; accepted May 19, 1997.

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