Plant Physiol. (1993) 103: 829–834

Cloning of Tomato (*Lycopersicon esculentum* Mill.) Arginine Decarboxylase Gene and Its Expression during Fruit Ripening[1]

Rajeev Rastogi*, Jacqueline Dulson, and Steven J. Rothstein

Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Arginine decarboxylase (ADC) is the first enzyme in one of the two pathways of putrescine biosynthesis in plants. The genes encoding ADC have previously been cloned from oat and *Escherichia coli*. Degenerate oligonucleotides corresponding to two conserved regions of ADC were used as primers in polymerase chain reaction amplification of tomato (*Lycopersicon esculentum* Mill.) genomic DNA, and a 1.05-kb fragment was obtained. This genomic DNA fragment encodes an open reading frame of 350 amino acids showing about 50% identity with the oat ADC protein. Using this fragment as a probe, we isolated several partial ADC cDNA clones from a tomato pericarp cDNA library. The 5′ end of the coding region was subsequently obtained from a genomic clone containing the entire ADC gene. The tomato ADC gene contains an open reading frame encoding a polypeptide of 502 amino acids and a predicted molecular mass of about 55 kD. The predicted amino acid sequence exhibits 47 and 38% identity with oat and *E. coli* ADCs, respectively. Gel blot hybridization experiments show that, in tomato, ADC is encoded by a single gene and is expressed as a transcript of approximately 2.2 kb in the fruit pericarp and leaf tissues. During fruit ripening the amount of ADC transcript appeared to peak at the breaker stage. No significant differences were seen when steady-state ADC mRNA levels were compared between normal versus long-keeping Alcobaca (*alc*) fruit, although *alc* fruit contain elevated putrescine levels and ADC activity at the ripe stage. The lack of correlation between ADC activity and steady-state mRNA levels in *alc* fruit suggests a translational and/or posttranslational regulation of ADC gene expression during tomato fruit ripening.

The diamine putrescine and the polyamines spermidine and spermine are apparently of ubiquitous occurrence in plants, and changes in their levels and biosynthesis have been correlated with a variety of plant developmental processes (Evans and Malmberg, 1989; Slocum and Flores, 1991). However, the specific physiological role(s) of polyamines in the various plant processes with which they have been associated remains unclear. Furthermore, very little is known about either of the mechanisms that regulate polyamine biosynthesis or their subcellular localization, two aspects critical to understanding their role in plant growth and development. In animal systems, the role of polyamines in cell division, growth, and differentiation and the mechanisms that regulate their intracellular levels are better understood (Heby and Persson, 1990; Auvinen et al., 1992). This has largely been facilitated by the isolation of polyamine mutants and application of molecular biology techniques.

In plants and bacteria, putrescine, also a precursor for the polyamines spermidine and spermine, is synthesized via one of two pathways. Putrescine can be formed directly from L-Orn by ODC; this pathway represents the only route to putrescine biosynthesis in animals and most fungi (Tabor and Tabor, 1984; Pegg, 1986). Alternatively, putrescine may be produced from L-Arg by ADC via agmatine (Tabor and Tabor, 1984). In plants, these two pathways appear to have specific roles in growth and development. For example, in nondividing mature tissues and in plant tissues subjected to environmental stress, ADC appears to be the primary enzyme for putrescine synthesis, whereas in meristematic and reproductive tissues and other actively dividing cells, ODC activity seems to correlate with changes in polyamine levels (Slocum et al., 1984; Tabor and Tabor, 1984; Evans and Malmberg, 1989).

Recently, molecular analysis of polyamine biosynthesis in plants has been initiated. Bell and Malmberg (1990) reported the cloning of oat ADC cDNA. Hammill et al. (1990) produced transgenic tobacco roots overexpressing yeast ODC. Recently, a partial cDNA clone, isolated from a tomato meristem library and showing homology to the oat ADC, was reported to be expressed in the meristem in a tissue-specific manner (Fleming et al., 1993). The use of molecular approaches including the cloning of polyamine biosynthetic enzymes, production of transgenic plants over- and underexpressing these enzymes, and analysis of gene promoters fused with reporter genes should allow a better understanding of the function of polyamines in plant growth and development.

We have investigated the role of polyamines in tomato (*Lycopersicon esculentum* Mill.) fruit ripening and storage using the Alcobaca (*alc*) ripening mutant. The fruit of this line ripen more slowly than the standard commercial varieties, and if picked ripe they can be kept four times longer (Mutschler, 1984b). The delayed overripening characteristic is conferred by the single recessive gene *alc*, whose inheritance, linkage, and effects on ripening-related mRNAs have

[1] Supported by an operating grant from Natural Sciences and Engineering Research Council of Canada to S.J.R.

* Corresponding author; fax 1–519–837–2075.

Abbreviations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; PCR, polymerase chain reaction; SAMDC, *S*-adenosylmethionine decarboxylase.

been previously described (Mutschler, 1984a; Mutschler et al., 1988). The *alc* fruit contain three times as much putrescine as the normal variety at the ripe stage (Dibble et al., 1988), and it has been suggested that the enhanced putrescine levels in this line may be responsible for its ripening and storage features (Davies et al., 1990). It was further shown that the elevated putrescine levels in *alc* fruit are not due to changes in putrescine conjugation or metabolism but are, instead, due to an increase in ADC activity (Rastogi and Davies, 1991). In this paper, we report the isolation and characterization of the ADC gene from tomato and examine the expression of ADC during ripening of normal and *alc* fruit.

MATERIALS AND METHODS

Plant Material

The plants of tomato (*Lycopersicon esculentum* Mill.) isogenic lines Alcobaca (*alc*) versus Alcobaca-Red (*Alc*-Red: a revertant of *alc*), near-isogenic lines Rutgers versus Rutgers-*alc* (*alc* backcrossed into cv Rutgers), and those of cv Jumbo were grown as described previously (Rastogi and Davies, 1990). Leaf samples for isolation of genomic DNA for PCR amplification and genomic blot analysis were collected from cv Jumbo plants. The fruit from the above-described isogenic lines, collected at four ripening stages, immature green, mature green, breaker (streaks of orange at the distal end), and ripe, were used for isolation of total RNA for northern blot analysis.

Extraction of Genomic DNA

Genomic DNA was isolated using a modified version of the method described by Fedoroff et al. (1983). Leaf tissue (1 g) was powdered in liquid nitrogen and mixed with 6 mL of lysis buffer and 0.3 mL of 20% SDS. The mixture was extracted once with 75:24:1 phenol:chloroform:isoamyl alcohol, once with 25:24:1 phenol:chloroform:isoamyl alcohol, and finally once with 24:1 chloroform:isoamyl alcohol. The nucleic acids were precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol, resuspended in 2 mL of 10 mM Tris-HCl, 45 mM EDTA (pH 8.0), and treated with RNase A. The DNA was ethanol precipitated and resuspended in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).

PCR Amplification of Genomic DNA

Genomic DNA (1 μg) was used in a 100-μL PCR amplification containing 150 pM of each primer (AD1 and AD2; Fig. 1), 200 μM each deoxyribonucleotide triphosphates, and 5 units of *Taq* polymerase, using a Coy Tempcycler. The PCR conditions were 94°C for 1.5 min for denaturation, 40°C for 2 min for primer annealing, and 72°C for 1 min for synthesis, for a total of 30 cycles. The PCR products were cloned into pBluescript (Stratagene, La Jolla, CA).

Screening of cDNA and Genomic Libraries

A λgt11 library constructed from poly(A)$^+$ RNA isolated from tomato fruit pericarp (breaker stage) and a λ Fix II (Stratagene) tomato genomic library were screened to isolate tomato ADC cDNA and genomic clones, respectively. Approximately 300,000 recombinant plaques for each library were screened with the 1.05-kb tomato ADC PCR fragment (labeled with α-^{32}P by random priming; Feinberg and Vogelstein, 1983) using standard plaque lift methods (Sambrook et al., 1989). Filters were prehybridized at 42°C in 5× SSPE, 10× Denhardt's solution, 0.5% SDS for 2 to 3 h and then hybridized overnight at 42°C in 50% formamide, 5× SSPE, 0.5% SDS. Filters were washed at room temperature for 15 min each with 2× SSC, 0.1% SDS and 0.1× SSC, 0.1% SDS and then at 68°C for 1 h in 0.1× SSC, 0.1% SDS. Five positive cDNA clones and three positive genomic clones were identified. *Not*I cDNA inserts from the positive clones were subcloned into pBluescript. The three genomic clones were restriction mapped, and one of them was found to contain the entire tomato ADC gene. A 1.74-kb *Hin*dIII fragment from this clone, containing about 500 bp of the 5′ end of the coding region and upstream sequences, was also subcloned in pBluescript.

DNA Sequencing

The PCR products, cDNA clones, and the 5′ end genomic fragment were partially sequenced using dideoxy sequencing (Sanger et al., 1977) and Sequenase version 1.0 (United States Biochemical) to confirm their identity. The complete sequence of the various inserts in both directions was obtained by sequencing the restriction fragments and/or exonuclease III/mung bean nuclease-generated deletion clones and by using specific primers. All DNA and protein sequence analyses were performed using the DNASIS and PROSIS software (Hitachi America Ltd., San Bruno, CA).

Genomic DNA Blot Analysis

Genomic DNA (10 μg) was digested with *Hin*dIII and *Bgl*II, separated by electrophoresis on a 0.7% agarose gel, and transferred to a Zetabind membrane in 20× SSC. The membranes were prehybridized and hybridized as described above with the addition of 10% dextran sulfate and 50 μg μL^{-1} of salmon sperm DNA. Blots were washed in 0.1× SSC, 0.1% SDS either at 50°C for low-stringency washes or at 68°C for high-stringency washes. For the probes, the 1.05-kb PCR product and a 1.4-kb cDNA fragment were gel purified and labeled by random priming (Feinberg and Vogelstein, 1983).

RNA Extraction and Blot Analysis

Total RNA was extracted from leaf and pericarp tissues according to the method of Jones et al. (1985), except the extraction buffer was replaced with 50 mM Tris-HCl (pH 8.0) containing 4% *p*-aminosalicylic acid. RNA samples (20 μg each) were fractionated through a 1.2% agarose-formaldehyde gel and transferred to a Zetabind membrane. Hybridization conditions were the same as used for the genomic blots. The probe was the gel purified 1.05-kb PCR product. Washing was done in 0.1× SSC, 0.1% SDS at 68°C. To verify loading of equal amounts of RNA in each lane, blots were reprobed with an *Arabidopsis* actin clone. RNA extractions and blot analysis were repeated once.

RESULTS

PCR Amplification

Two degenerate oligonucleotides (Fig. 1), corresponding to two conserved regions in the oat and *E. coli* ADC proteins (Bell and Malmberg, 1990), were designed. PCR amplification of genomic DNA with these primers generated a single band of about 1.0 kb. The PCR product was cloned and sequenced; the sequence analysis revealed that this 1.05-kb fragment contained an open reading frame encoding 350 amino acids, which showed approximately 50% identity with the deduced amino acid sequence of the oat ADC (Bell and Malmberg, 1990). In addition, it contained all of the corresponding conserved regions found in the oat and *E. coli* ADC gene products and hybridized to the tomato nuclear DNA under high-stringency conditions, thus confirming that this amplified genomic fragment indeed represented the tomato ADC sequence.

ADC Gene Cloning and Sequence Analysis

The 1.05-kb PCR product was used as a hybridization probe to screen a tomato pericarp (breaker stage) cDNA library constructed in λgt11. Five positive clones with insert sizes ranging from 1 to 1.5 kb were isolated. Hybridization of the PCR product to an RNA blot of fruit and leaf RNA, however, indicated that the ADC transcript is approximately 2.2 kb; thus, the isolated cDNAs were not full-length ADC clones. Partial sequence of the cDNAs revealed that their 3′ ends with the poly(A) tails were identical, and their 5′ ends overlapped with the PCR product showing 100% DNA identity. This indicated that all of the cDNAs were derived from the tomato ADC gene but lacked the 5′ end of the coding region. To obtain the 5′ end of the ADC coding region, a tomato genomic library was screened, and a genomic clone containing the entire tomato ADC gene was isolated. A *Hind*III fragment of 1.74 kb that contains the 5′ end of the ADC gene, overlapping with the PCR product and the 1.5-kb cDNA, was subcloned from this genomic clone.

The nucleotide and derived amino acid sequences of the tomato ADC-coding region are presented in Figure 2. The ATG start site is at position 25 (Fig. 2), given that there are nonsense codons upstream of this position in the same reading frame. The tomato ADC sequence has an open reading frame of 1506 bp encoding a polypeptide of 502 amino acids, a stop codon, and a 530-bp-long 3′ untranslated region before the start of the poly(A) segment. The derived amino acid sequence of 502 amino acids has a calculated molecular mass of 54.4 kD.

The predicted amino acid sequence of the tomato ADC shows strong similarity to the oat (Bell and Malmberg, 1990) and *E. coli* (Moore and Boyle, 1990) ADC proteins (Fig. 3), with 47 and 38% identity, respectively. A sequence similarity of 34% is also seen between the oat and *E. coli* ADCs. Therefore, at the amino acid level, the tomato and oat ADCs are more similar to each other than to the *E. coli* ADC. Among the three sequences, there are several regions of striking similarity that are completely conserved in both the amino acid sequence and spacing. These conserved regions are denoted by shaded areas in Figure 3.

```
                    V  C  N  G  Y  K  D
AD1 (sense)      5'-GTTTGTAATGGTTATAAAGAT
                     C  C  C  A  C  G  C
                     G

                    G  D  S  D  C  T
AD2 (antisense)  5'-ACCATCACTATCACAAGT
                     T  G  G  G  G  G
                                   T
```

Figure 1. Primers used for PCR amplification of tomato ADC genomic fragment. The corresponding peptide sequences (in single-letter code) are shown above each oligonucleotide sequence. Primer AD1 corresponds to amino acid residues 151 to 157 of the oat ADC (Bell and Malmberg, 1990) and 170 to 176 of the *E. coli* ADC (Moore and Boyle, 1990). Primer AD2 corresponds to amino acid residues 474 to 479 of the oat ADC and 527 to 532 of the *E.coli* ADC.

```
   1                                   Met Pro Leu Val Val Arg Phe Pro Asp Val Leu Lys
   1 AAT TTG GGT GGA CTT GGG CTC CAG ATG CCT CTT GTT GTC CGT TTT CCT GAT GTT CTG AAG

  13 Asn Arg Leu Glu Thr Leu Gln Ser Ala Phe Asp Met Ala Ile Asn Ser Gln Gly Tyr Glu
  61 AAC CGT TTG GAG ACT CTG CAA TCG GCT TTT GAC ATG GCG ATT AAT TCT CAA GGC TAT GAG

  33 Ala His Tyr Gln Gly Val Tyr Pro Val Lys Cys Asn Gln Asp Arg Phe Val Val Glu Asp
 121 GCT CAC TAT CAA GGT GTT TAT CCG GTG AAA TGC AAT CAA GAT AGG TTC GTG GTG GAG GAT

  53 Ile Val Lys Phe Gly Ser Pro Tyr Arg Phe Gly Leu Glu Ala Gly Ser Lys Pro Glu Leu
 181 ATC GTG AAA TTC GGG TCG CCA TAC CGA TTC GGG CTG GAA GCC GGG TCT AAA CCG GAG CTC

  73 Leu Leu Ala Met Asn Cys Leu Ser Lys Gly Ser Ala Asp Ala Leu Leu Val Cys Asn Gly
 241 CTG TTG GCG ATG AAC TGT CTG TCA AAG GGC AGT GCT GAT GCT CTT CTT GTT TGC AAT GGT

  93 Phe Lys Asp Thr Glu Tyr Ile Ser Leu Ala Leu Val Ala Arg Lys Leu Leu Leu Asn Ser
 301 TTC AAG GAC ACT GAG TAT ATT TCG CTT GCT TTG GTC GCA AGA AAG CTC CTT TTG AAC AGT

 113 Val Ile Val Leu Glu Gln Glu Glu Glu Leu Asp Leu Val Ile Asp Ile Ser Arg Lys Met
 361 GTG ATT GTG CTT GAA CAA GAG GAG GAG CTT GAC CTG GTG ATT GAT ATC AGC CGT AAG ATG

 133 Ser Val Arg Pro Val Ile Gly Leu Arg Ala Lys Leu Arg Thr Lys His Ser Gly His Phe
 421 TCT GTC CGG CCT GTA ATT GGA CTT CGT GCT AAG CTC AGG ACA AAG CAT TCT GGC CAT TTT

 153 Gly Ser Thr Ser Gly Glu Lys Gly Lys Phe Gly Leu Thr Thr Thr Gln Ile Leu Arg Val
 481 GGA TCC ACT TCT GGT GAA AAG GGT AAG TTT GGG TTG ACA ACA ACC CAG ATT CTT CGT GTA

 173 Val Lys Lys Leu Asp Glu Ser Gly Met Leu Asp Cys Leu Gln Leu Leu His Phe His Ile
 541 GTG AAG AAG CTT GAT GAA TCT GGA ATG CTG GAT TGT CTC CAG TTA TTG CAT TTT CAC ATT

 193 Gly Ser Gln Ile Pro Thr Thr Glu Leu Leu Ala Asp Gly Val Gly Glu Ala Thr Gln Ile
 601 GGA TCT CAG ATC CCC ACA ACA GAG TTG CTT GCT GAT GGT GTT GGT GAG GCC ACT CAG ATT

 213 Tyr Ser Glu Leu Val Arg Leu Gly Ala Gly Met Lys Phe Ile Asp Ile Gly Gly Gly Leu
 661 TAC TCT GAA TTA GTC CGT CTT GGA GCT GGT ATG AAA TTC ATT GAT ATC GGA GGG GGG CTT

 233 Gly Ile Asp Tyr Asp Gly Ser Lys Ser Ser Asn Ser Asp Val Ser Val Cys Tyr Ser Ile
 721 GGA ATC GAC TAT GAC GGT TCT AAA TCA AGC AAT TCT GAT GTC TCT GTT TGC TAT AGC ATT

 253 Glu Glu Tyr Ala Ser Ala Val Val Gln Ala Val Leu Tyr Val Cys Asp Arg Lys Gly Gly
 781 GAA GAA TAT GCC TCT GCT GTT GTC CAA GCG GTC CTC TAT GTC TGT GAT CGT AAG GGC GGA

 273 Lys His Pro Val Ile Cys Ser Glu Ser Gly Arg Ala Ile Val Ser His His Ser Ile Leu
 841 AAG CAT CCA GTG ATT TGC AGC GAA AGT GGC AGG GCA ATT GTT TCT CAC CAT TCA ATT CTG

 293 Ile Phe Glu Ala Val Ser Ala Ser Thr Ser His Val Ser Thr Gln Pro Ser Ser Gly Gly
 901 ATT TTT GAA GCC GTG TCT GCT TCT ACT AGT CAT GTT TCT ACA CAG CCA TCT TCG GGT GGT

 313 Leu Gln Ser Leu Val Glu Thr Leu Asn Glu Asp Ala Arg Ala Asp Tyr Arg Asn Leu Ser
 961 TTA CAA TCC TTG GTG GAG ACT CTC AAT GAA GAT GCC CGT GCT GAC TAC AGA AAC TTA TCT

 333 Ala Ala Ala Val Arg Gly Glu Tyr Asp Thr Cys Leu Ile Tyr Ser Asp Gln Leu Lys Gln
1021 GCT GCT GCT GTC CGT GGA GAA TAT GAT ACA TGT CTC ATC TAT TCT GAT CAG TTG AAA CAG

 353 Arg Cys Val Glu Gln Phe Lys Asp Gly Ser Leu Asp Ile Glu Gln Leu Ala Ala Val Asp
1081 AGA TGT GTT GAA CAG TTC AAA GAT GGG TCC TTG GAT ATT GAG CAG CTC GCT GCA GTG GAT

 373 Ser Ile Cys Asp Trp Val Ser Lys Ala Ile Gly Val Ala Asp Pro Val Arg Thr Tyr His
1141 AGC ATT TGT GAT TGG GTG TCG AAG GCT ATC GGG GTT GCT GAT CCT GTC CGC ACT TAC CAT

 393 Val Asn Leu Ser Val Phe Thr Ser Ile Pro Asp Phe Trp Gly Phe Ser Gln Leu Phe Pro
1201 GTG AAT CTG TCA GTT TTC ACC TCA ATC CCT GAT TTT TGG GGC TTC AGC CAA TTG TTT CCT

 413 Ile Val Pro Ile His Arg Leu Asp Glu Lys Pro Thr Met Arg Gly Ile Leu Ser Asp Leu
1261 ATT GTT CCA ATT CAC CGT CTG GAT GAA AAG CCT ACA ATG AGA GGA ATA CTG TCT GAC CTG

 433 Thr Cys Asp Ser Asp Gly Lys Val Asp Lys Phe Ile Gly Gly Glu Ser Ser Leu Pro Leu
1321 ACG TGT GAC AGT GAT GGA AAG GTT GAT AAG TTC ATT GGG GGC GAA TCA AGC TTG CCG CTC

 453 His Glu Ile Gly Ser Gly Asp Gly Gly Arg Tyr Tyr Leu Gly Met Phe Leu Gly Gly Ala
1381 CAT GAA ATT GGA AGT GGT GAT GGT GGG CGG TAT TAT CTG GGG ATG TTT TTG GGT GGG GCT

 473 Tyr Glu Glu Ala Leu Gly Gly Leu His Asn Leu Phe Gly Gly Pro Ser Val Val Arg Val
1441 TAT GAG GAG GCG CTC GGA GGA CTC CAC AAT CTA TTT GGT GGA CCA AGC GTT GTT CGG GTG

 493 Met Gln Ser Asp Ser Pro His Ser Phe Ala ■■■
1501 ATG CAG AGC GAT AGC CCT CAC AGC TTT GCG TGA CTC GCT CTG TCC CTG GTC CAT CGT GTG
1561 CTG ATG TGC TCC GGG CGA TGC AGT TTG AGC CTG AAC TCA TGT TCG AGA CTC TCA AGC ACC
1621 GTG CAG AGG AAT CCT TGG AAC AAG GAG AAG GAG AAG GCG AAG GTG TTG CCT TTG GAT CTT
1681 TGA CCA GCA GCT TAG CTC AGT CCT TCC ACA ACA TGC CTT ACC TTT CGT CTT GCT GCT TCA
1741 CTG CAG AAG CCA CTG CCA ATG CCA ATA CCA ATA CCA ATA ATG GTG GCT ATT ACT ATT ACA
1801 GTG AAG ACA ATG CTG CAG CAG AGG AAG ATG AGA TTT GGT CCT ACT AAA CTC TGC TTG AAG
1861 TGT CTC TTG TTA GCA TCT CCA GTT TGT TTT AGT TTG TGG TCG AGG TCG TCT GTT TTT TTA
1921 TAA TAA TCC CAC CCC TTA GTT TGG GTG CAT GTT AAT TAC TTT TGT TTG CAA TAG ATG CAG
1981 TAG ACT GTC ATC TCC TAT TGC AAC TAA GCT TAT GTT ATG ACC GCA ATC AGT TTT ATA TTA
2041 ATG CTG TCT TTT TTT GTT TC
```

Figure 2. Nucleotide sequence and deduced amino acid sequence of coding region of the tomato ADC gene. The underlined regions correspond to the primers used for PCR amplification.

```
TOMATO    1  MPLVVRFPDVLKNRLETLQSAFDMAINSQGYEAHYQGVYPVKCNQDRFVVEDIVKFGSPY   60
OAT      63  .PMILRFPDVLRHRINSLHTAFA.AIK...YGS.YQGVFPVK.NQHK.VVQDMVHFG..H  122
E.COLI   86  LP.L..FPQILQHRL.SINAAFKRA.ES.GYNGDY..VYPIK.NQHR.VIESLIH.GEP-  144

TOMATO   61  RFGLRAGSKPELLLAMNCLSKGSADALLVCNGFKDTEYISLALVARKLLLNSVIVLEQEE  120
OAT     123  SYGLRAGSKPELLIAMSCLTKAKPGA.LVCNGYKDSAYVALALAAR.M.LN.IIVLE.EE  182
E.COLI  145  -LGLRAGSKAELM.VLA--..G.T.SVIVCNGYKD.EYIRLALIG.KM..K..LVIEK.S  201

TOMATO  121  ELDLVIDISRKMSVRPVIGLRAKLRTKHSGHFGSTSGEKGKPGLTTTQILRVVKKLDESG  180
OAT     183  ELDIVIE.SSKLGV.PVIGVRAKL.TK.PGHFGSTAGKHGKPGLPAEKI..VAKKLKA.N  242
E.COLI  202  EIAIVLD.A.RLNV.P.LGVRARLRSQ.SGKW.SSGGEKSKPGLAATQVLQLVETL.EAG  261

TOMATO  181  MLDCLQLLEFHIGSQIPTTELLADGVGEATQIYSELVR-LG-AGMKFIDIGGGLGIDYDG  238
OAT     243  KLH.LKLLEFHVGS.IPTTDIV.KAASEASDIYCALVK..G.ETMT.LD.GGGLGVDYDG  303
E.COLI  262  RLDSLQLLEFHLGSQMANI..IATGV.ESARFY.EL.K-LG-VNIQ.FDVGGGLGVDYEG  319

TOMATO  239  SKSSNEDVSVCYSIEEYASAVVQAVLYVCDRKGGKHPVICSESGRAIVSHHSILIFEAVS  298
OAT     304  TRSGSSDMSV.YGLEEYASSIVQAV...CD.HG.PHPVLCTESGRAMASYHSMILEALS  362
E.COLI  320  TR-SQSD.SV.YGLNEYANNII.AI..ACE.NG..HPTV.TESGRAVTAHHTVLV.N.IG  378

TOMATO  299  ASTSH--VSTQPSSGG---LQSLVET---LNE-DARADYR------NLSAAAVRGEYDTC  343
OAT     363  A.PKD--.EDEATTE.---LHG.I.D---LSS-K.QPT..------SMSS.AVH.K.HG.  409
E.COLI  379  VE.NE..VPTAPAEDA...LQSM.ET...MHE.GTR.S.R......QMD..DIH.GYSS.  437

TOMATO  344  LIYSDQLKQRCVEQFKDGSLDIE-QLAAVDSICDWVSKAIGVADPVRTYHVNLSVFTSIP  402
OAT     410  .MY.....---------------.----------LSKSVTTAHTI.NYHMNLSVFS.MP  443
E.COLI  438  -IFS.Q.RAW..Q.Y.S.C.EVQ.QLDP.NR....I.DEL....A.K.Y-VNFLSF.SMP  496

TOMATO  403  DFWGFSQLFPIVPIHRLDEKPTMRGILSDLTCDSDGKVDKFIGGESSLPLHEIGSGDGG  461
OAT     444  DYWGI.HLFPMMPV.RLDEKPT.KATLVDVTCDSDGKVDKFI.DTETMPLH.LDP..GG  502
E.COLI  497  D.WGIDQLFPVLPLE.LDQ.PERRAVL.DITCDSDG.IDHYIDGDG..TMP..E.D.EN  556

TOMATO  462  RYYLGMFLGGAYEAALGGLAWLFGGPSVVRVMQSDSPHSFA  502
OAT     503  -YYVAVLLTGATQEALSN.AWLFGGPSLVRVV.TGN..AF.  542
E.COLI  557  P..LGFFM.GATQE.LGNMAWLFGDTEAV.V.........  597
```

Figure 3. Comparison of the derived amino acid sequences of tomato, oat (Bell and Malmberg, 1990; GenBank accession No. X56802), and *E. coli* (Moore and Boyle, 1990; GenBank accession No. M31770) ADCs. The three sequences were compared using the PROSIS software (Hitachi America), which uses the algorithm of Lipman and Pearson (1985). The numbers indicate the position of the residues from the N terminus of the protein. Complete amino acid sequence is shown for the tomato ADC, and for oat and *E. coli* only amino acids that match the tomato sequence (identical and conserved substitutions) are shown. Boldface letters indicate amino acids that are absolutely conserved in all three sequences. Periods represent amino acids in the oat and *E. coli* sequences that are different from those in tomato. Gaps, indicated by dashes, were introduced for maximum alignment. Shaded areas denote stretches of absolutely conserved amino acid residues in the three sequences.

The N- and the C-terminal regions of the ADC proteins appear to be the least conserved in the three species. Compared with the oat and *E. coli* ADC proteins, the tomato ADC polypeptide is shorter at both the N and the C termini, and this accounts for its overall smaller size. Another divergent region in the tomato ADC corresponds to amino acid residues between positions 285 and 400. It is interesting that this region is also divergent when the oat and *E. coli* sequences are compared. For example, in this region oat ADC has 23 fewer and *E. coli* ADC has 14 extra amino acids relative to that in the tomato protein (Fig. 3).

Genomic Organization

Southern blot analysis of genomic DNA was performed to determine the number of ADC genes in the tomato genome. Genomic DNA digested with either *Hin*dIII or *Bgl*II was hybridized to either the 1.05-kb PCR product or the 1.4-kb ADC cDNA fragment. In both cases, the pattern of hybridizing bands observed was consistent with a single tomato ADC gene based on the restriction map of the gene (Fig. 4C). The 1.05-kb probe that contains one *Hin*dIII site and no *Bgl*II sites hybridized to two *Hin*d III fragments of 0.82 and 1.74 kb and to a *Bgl*II fragment of approximately 6.0 kb (Fig. 4A). The 1.4-kb cDNA probe, which has two *Hin*dIII sites [the second site located only 53 bp upstream from the poly(A) tract] and no *Bgl*II sites, hybridized to two *Hin*dIII fragments of 0.82 and 0.64 kb and to a 6.0-kb *Bgl*II fragment (Fig. 4B). The 6.0-kb *Bgl*II band is the same as that detected by the 1.05-kb PCR product (Fig. 4A). When blots were washed under lower stringency conditions (50°C), identical results were obtained (data not shown).

ADC Expression during Tomato Fruit Ripening

To determine the pattern of ADC expression during tomato fruit ripening, gel blot analysis of total RNA isolated from normal and *alc* fruit at four ripening stages, i.e. immature green, mature green, breaker, and ripe, and from leaf was performed. Figure 5 shows an RNA blot (Rutgers versus Rutgers-*alc* in this case) probed with the 1.05-kb PCR product. In all samples, a single transcript of approximately 2.2 kb was observed. In both genotypes, the level of ADC transcript in the fruit pericarp appeared to increase from immature green to breaker stage and then decrease at the ripe stage (Fig. 5). There were, however, no significant differences between the normal and *alc* fruit at any given

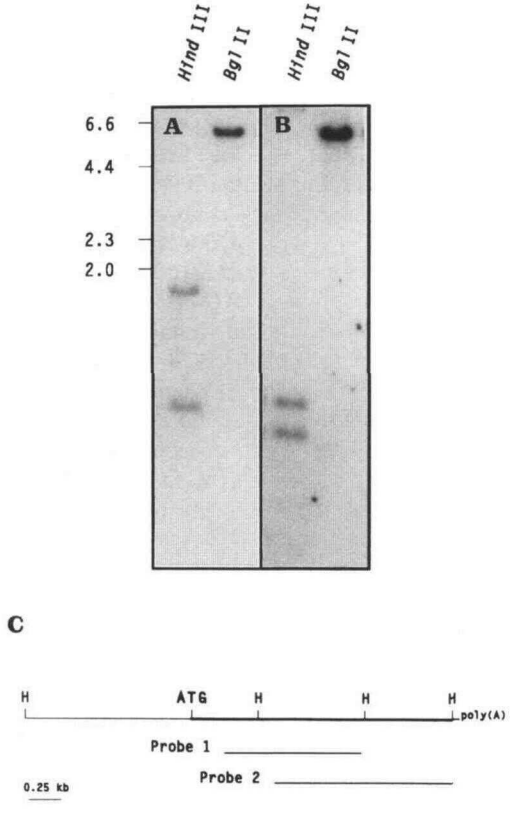

Figure 4. Southern blot analysis of tomato genomic DNA. DNA (10 μg) was digested with either *Hin*dIII or *Bgl*II. The blot was hybridized with the 1.05-kb tomato ADC PCR product (probe 1, A) or a 1.4-kb fragment of tomato ADC cDNA (probe 2, B). Size markers (kb, *Hin*dIII-digested λ-DNA) are indicated on the left. A restriction map of the ADC coding region (thick line), upstream region, and the probes used is shown in C. H, *Hin*dIII.

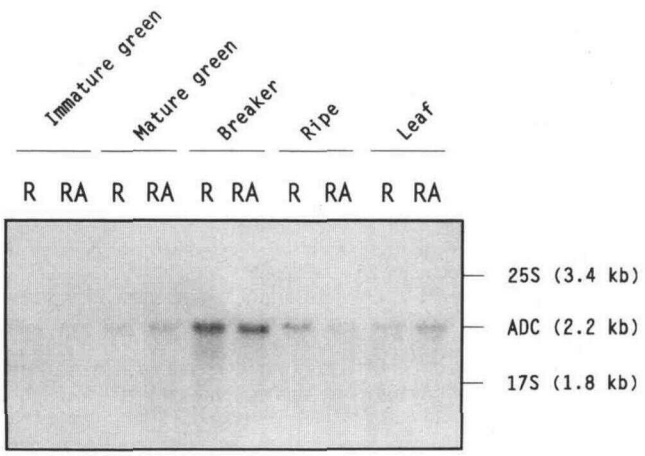

Figure 5. RNA blot analysis of ADC expression in fruit pericarp of Rutgers (R) and Rutgers-*alc* (RA) at four different stages of ripening. Total RNA (20 μg) was loaded in each lane, and the blot was hybridized with the 1.05-kb ADC PCR product. The positions of 25S (3.4 kb) and 17S (1.8 kb) rRNAs are indicated on the right.

ripening stage (Fig. 5). The level of ADC expression in the leaves of the two genotypes was similar to that in the mature green fruit (Fig. 5). The RNA blots were reprobed with an *Arabidopsis* actin clone, and an equivalent hybridization signal was detected for all RNA samples (not shown). Similar results were obtained with RNA samples isolated from fruit of the *Alc-Red* and *alc* isogenic pair (data not presented).

DISCUSSION

In this report we have described the cloning and nucleotide sequence of the ADC gene from tomato. Two lines of evidence confirm the identity of the clone. First, the overall amino acid similarity to the oat and *E. coli* sequences is very high. Second, a number of different regions conserved in the oat and *E.coli* ADCs were identified in the tomato ADC sequence. The tomato ADC gene contains an open reading frame encoding a protein of 502 amino acids. Gel blot analysis of RNA isolated from fruit and leaf indicated that the tomato ADC gene is expressed as a transcript of approximately 2.2 kb, which is in agreement with the size of the ADC transcript detected in tomato apex and root tissues (Fleming et al., 1993). Analysis of genomic sequences indicated that the tomato genome contains a single ADC gene. Comparison of the predicted amino acid sequence of tomato ADC revealed striking similarity to the oat and *E. coli* ADCs (47 and 38% identities, respectively). As one might expect, the tomato and oat ADC proteins are more closely related to each other than to the *E. coli* ADC, because there is only 34% amino acid identity between the oat and the *E. coli* sequences.

The most noteworthy feature is that, among the three sequences, there are several distinct regions that are scattered throughout the proteins and are conserved in not only amino acid sequence but also in spacing. It is likely that the identical amino sequences in tomato, oat, and *E. coli* may be involved in catalytic function. Bell and Malmberg (1990) suggested that the three conserved regions toward the C terminus of the protein may be functionally associated with the active site of the enzyme. Because most decarboxylases require pyridoxal phosphate as a cofactor, it is possible that one of these conserved regions is involved in the binding of pyridoxal phosphate.

During tomato fruit ripening, the steady-state levels of ADC mRNA appear to increase from immature green to breaker stage, with the ripe stage showing levels similar to that at the mature green stage. Furthermore, there appear to be no significant differences in ADC mRNA levels between the normal and *alc* fruit at a given ripening stage. The pattern of ADC expression during fruit ripening in *alc* fruit, therefore, appears to be different from that observed for putrescine levels and ADC activity. In both normal and *alc* fruit, putrescine levels are high at the immature green stage and decline at the mature green stage. In normal fruit, this decline in putrescine levels persists, but in *alc* fruit, putrescine levels increase during ripening to a level similar to that at the immature green stage; the ripe *alc* fruit contain approximately three times as much putrescine as the normal fruit (Dibble et al., 1988). The activity of ADC in normal and *alc* fruit during ripening showed a pattern similar to that of putrescine (Rastogi and Davies, 1991).

The lack of correlation between ADC activity and ADC mRNA levels in ripening *alc* fruit suggests translational and/or posttranslational regulation of ADC expression in tomato fruit. In animal systems, the regulation of ODC and SAMDC expression at translational/posttranslational level has been well documented, and polyamines themselves have been shown to exert control over rates of translation of ODC and SAMDC mRNAs as well degradation of ODC and SAMDC proteins (Heby and Persson, 1990). In oat, it has been shown that the ADC polypeptide is posttranslationally processed (Malmberg et al., 1992), but whether this processing is related to enzyme activation is not clear. Whether the tomato ADC protein, like the oat protein, is also processed in vivo is not known at this stage, and further work is needed to address this question.

ACKNOWLEDGMENTS

We would like to thank Bonnie Ferrie and Nathalie Beaudoin for the tomato pericarp cDNA and genomic libraries, respectively, Dr. Martha Mutschler, Cornell University, for seed of the various Alcobaca lines, and Dr. Richard Meagher, University of Georgia, for the *Arabidopsis* actin clone.

Received May 13, 1993; accepted July 12, 1993.
Copyright Clearance Center: 0032-0889/93/103/0829/06.
The GenBank accession number for the nucleotide sequence reported here is L16582.

LITERATURE CITED

Auvinen M, Paasinen A, Andersson LC, Holtta E (1992) Ornithine decarboxylase activity is critical for cell transformation. Nature **360:** 355–358

Bell E, Malmberg RL (1990) Analysis of a cDNA encoding arginine decarboxylase from oat reveals similarity to the *Escherichia coli* arginine decarboxylase and evidence of protein processing. Mol Gen Genet **224:** 431–436

Davies PJ, Rastogi R, Law DM (1990) Polyamines and their metabolism in ripening tomato fruit. *In* HE Flores, RN Arteca, JC Shan-

non, eds, Polyamines and Ethylene: Biochemistry, Physiology and Interactions. American Society of Plant Physiologists, Rockville, MD, pp 112–125

Dibble ARG, Davies PJ, Mutschler MA (1988) Polyamine content of long-keeping alcobaca fruit. Plant Physiol **86:** 338–340

Evans PT, Malmberg RL (1989) Do polyamines have roles in plant development? Annu Rev Plant Physiol Plant Mol Biol **40:** 235–269

Fedoroff N, Mauvais J, Chaleff D (1983) Molecular studies on mutations at the *Shrunken* locus in maize caused by the controlling element *Ds*. J Mol Appl Genet **2:** 11–29

Feinberg AP, Vogelstein B (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specificity. Anal Biochem **132:** 6–13

Fleming AJ, Mandel T, Roth T, Kuhlemeier C (1993) The patterns of gene expression in the tomato shoot apical meristem. Plant Cell **5:** 297–309

Hammill JD, Robins RJ, Parr AJ, Evans DM, Furze JM, Rhodes MJC (1990) Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. Plant Mol Biol **15:** 27–38

Heby O, Persson L (1990) Molecular genetics of polyamine synthesis in eukaryotic cells. Trends Biochem Sci **15:** 153–158

Jones JDG, Dunsmuir P, Bedbrook J (1985) High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J **4:** 2411–2418

Lipman DJ, Pearson WR (1985) Rapid and sensitive protein similarity searches. Science **227:** 1435–1441

Malmberg RL, Smith KE, Bell E, Cellino ML (1992) Arginine decarboxylase of oats is clipped from a precursor into two polypeptides found in the soluble enzyme. Plant Physiol **100:** 146–152

Moore RC, Boyle SM (1990) Nucleotide sequence and analysis of the *speA* gene encoding biosynthetic arginine decarboxylase in *Escherichia coli*. J Bacteriol **172:** 4631–4640

Mutschler M, Guttieri M, Kinzer S, Grierson D, Tucker G (1988) Changes in ripening-related processes conditioned by the *alc* mutant. Theor Appl Genet **76:** 285–292

Mutschler MA (1984a) Inheritance and linkage of the alcobaca ripening mutant in tomato. J Am Soc Hort Sci **109:** 500–503

Mutschler MA (1984b) Ripening and storage characteristics of the alcobaca ripening mutant in tomato. J Am Soc Hort Sci **109:** 504–507

Pegg AE (1986) Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J **234:** 249–262

Rastogi R, Davies PJ (1990) Polyamine metabolism in ripening tomato fruit. I. Identification of metabolites of putrescine and spermidine. Plant Physiol **94:** 1449–1455

Rastogi R, Davies PJ (1991) Polyamine metabolism in ripening tomato fruit. II. Polyamine metabolism and synthesis in relation to enhanced putrescine content and storage life of *alc* tomato fruit. Plant Physiol **95:** 41–45

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Slocum RD, Flores HE, eds (1991) Biochemistry and Physiology of Polyamines in Plants. CRC Press, Boca Raton, FL

Slocum RD, Kaur-Sawhney R, Galston AW (1984) The physiology and biochemistry of polyamines in plants. Arch Biochem Biophys **235:** 283–303

Tabor CW, Tabor H (1984) Polyamines. Annu Rev Biochem **53:** 749–790