Molecular cloning and functional identification of a plant ornithine decarboxylase cDNA

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A cDNA for a plant ornithine decarboxylase (ODC), a key enzyme in putrescine and polyamine biosynthesis, has been isolated from root cultures of the solanaceous plant *Datura stramonium*. Reverse transcription–PCR employing degenerate oligonucleotide primers representing conserved motifs from other eukaryotic ODCs was used to isolate the cDNA. The longest open reading frame potentially encodes a peptide of 431 amino acids and exhibits similarity to other eukaryotic ODCs, prokaryotic and eukaryotic arginine decarboxylases (ADCs), prokaryotic *meso*-diaminopimelate decarboxylases and the product of the *tab*A gene of *Pseudomonas syringae* cv. tabaci. Residues involved at the active site of the mouse ODC are conserved in the plant enzyme. The plant ODC does not possess the C-terminal extension found in the mammalian enzyme, implicated in rapid turnover of the protein, suggesting that the plant ODC may have a longer half-life. Expression of the plant ODC in *Escherichia coli* and demonstration of ODC activity confirmed that the cDNA encodes an active ODC enzyme. This is the first description of the primary structure of a eukaryotic ODC isolated from an organism where the alternative ADC route to putrescine is present.

INTRODUCTION

In animals and fungi, ornithine decarboxylase (ODC) catalyses the first and rate-limiting step in polyamine biosynthesis [1–3] producing the diamine putrescine directly from ornithine. Putrescine is the precursor of the polyamines spermidine and spermine [1]. ODC activity is closely associated with cellular proliferation and is essential for normal cell growth [4,5]. The human ODC promoter is directly regulated by the product of c-*myc* [6,7] and overexpression of human ODC in NIH3T3 cells resulted in cell transformation [8,9], suggesting that ODC may be a proto-oncogene. ODC has also been directly implicated in the c-*myc*-mediated accelerated cell death of IL-3 deprived, c-*myc* overexpressing cell lines [10]. Activity of ODC is regulated at the transcriptional, translational and post-translational levels and polyamine feedback repression is mediated by an ODC antizyme [2,11].

Eukaryotic ODCs require pyridoxal 5′-phosphate (PLP) as a cofactor and the Lys^{69} residue of the mouse ODC has been shown to be an important residue for the binding of PLP [12]. Poulin et al. [12] also demonstrated that Cys^{360} is the main residue that binds difluoromethylornithine (DFMO, a specific 'suicide' inhibitor of ODC [13]); thus Lys^{69} and Cys^{360} are critical residues at the active site of ODC. Other residues such as Lys^{115}, Lys^{169} and His^{197} have been shown by site-directed mutagenesis to be important for ODC activity and probably form part of the active site [14–16]. The enzyme functions as a homodimer, with the Lys^{69} of one subunit forming an active site *in trans* with the Cys^{360} of the other subunit [17,18].

Plants provide an interesting eukaryotic system for investigating the role of ODC and polyamines because an additional route to putrescine via arginine decarboxylase (ADC) is present [19], involving production of the intermediates agmatine and *N*-carbamoylputrescine. In *Escherichia coli*, where both routes are present, the biosynthetic ODC and ADC genes have been cloned and sequenced. The bacterial ODC gene is unrelated to the animal and fungal ODCs that synthesize putrescine in the absence of the additional ADC pathway [20], whereas the primary structure of the *E. coli* and plant ADCs are similar [21]. We were interested in knowing whether the plant ODC would be similar to other eukaryotic ODCs or whether the presence of the additional ADC pathway meant that the plant ODC would have been under less selective pressure to conserve its primary structure. Biochemical evidence, based on cofactor requirement and activity regulation by spermidine and spermine, suggests that the tomato and tobacco ODCs are similar to those of mammalian origin [22].

An additional point of interest for polyamine metabolism is that plants are apparently unique in accumulating conjugated forms of the polyamines, where an amide bond is formed with hydroxycinnamic acids [19], and in transformed root cultures the conjugates are the predominant form [23]. Transformed root cultures are derived from the infection of plants by the soil bacterium *Agrobacterium rhizogenes*, which transfers a segment of DNA to the plant genome and initiates root growth by *de novo* production of bacterial enzymes in the plant that modify plant hormone metabolism [24].

Several vertebrate, invertebrate and fungal ODCs have been

Abbreviations used: ADC, arginine decarboxylase; DapDC, *meso*-diaminopimelate decarboxylase; DFMO, difluoromethylornithine; DTT, dithiothreitol; ODC, ornithine decarboxylase; PLP, pyridoxal 5′-phosphate; RT, reverse transcription; SAMDC, *S*-adenosylmethionine decarboxylase.

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cloned and sequenced and conserved residues are present throughout the length of the protein. We designed degenerate oligonucleotide primers encoding conserved motifs found in diverse eukaryotic ODCs and employed RT–PCR to clone the first plant ODC cDNA from transformed root cultures [23] of *Datura*.

METHODS

Plant material

Transformed root cultures of *Datura stramonium* D15/5 were propagated in liquid culture medium as previously described [23]. New cultures were initiated by subculturing 0.5 g of 10-day-old root culture material into 50 ml of fresh B50 medium [23] in 250 ml Erlenmeyer flasks; cultures were grown at 25 °C.

RNA and DNA extraction

Total RNA was prepared from 7-day-old root cultures by using a modified version of the protocol described by Verwoerd et al. [25]. About 2–3 g of of roots were ground in liquid nitrogen. Some of the frozen powder was used for RNA and DNA preparation, and the rest was used for polyamine and enzyme activity analysis. For RNA and DNA preparation, frozen powder was placed in 5 ml of citrate-buffered phenol, pH 4.3 (Sigma) and 5 ml of extraction buffer [0.1 M Tris/HCl, pH 8.0, 0.1 M LiCl, 10 mM EDTA, 1.0% (w/v) SDS] in a 50 ml tube, then mixed on a vortex mixer for 30 s. Immediately 5 ml of chloroform/isoamyl alcohol (24:1, v/v) was added and vortexed for 30 s. The sample was centrifuged at 2500 rev./min for 10 min and the supernatant transferred to a baked 30 ml Corex tube. An equal volume of 4.0 M LiCl was added to the supernatant and kept at 4 °C overnight to precipitate the RNA. The sample was centrifuged at 12100 ***g*** on a Beckman JA20 rotor and the DNA-containing supernatant transferred to a new tube. The RNA pellet was resuspended in 2.0 ml of water and reprecipitated with ethanol and after centrifugation the RNA pellet was resuspended in 1.0 ml of water. Two volumes of ethanol were used to precipitate the chromosomal DNA in the first supernatant, which was then gently spooled onto a Pasteur pipette and resuspended in 2.0 ml of water.

RT–PCR

Poly(A)$^+$ RNA was produced from total RNA by two passages over oligo(dT)-cellulose. Reverse transcription of 0.5 μg of poly(A)$^+$ RNA was achieved by using 12.6 units of AMV reverse transcriptase (Boehringer Mannheim), 0.5 μg of a d(T)17 adapter primer [26], 1.0 mM of each dNTP and 50 mM Tris/HCl, pH 8.0, 6 mM $MgCl_2$, 40 mM KCl and 10 mM dithiothreitol (DTT) in a total volume of 50 μl. The reaction was incubated at 42 °C for 1 h, then at 52 °C for 30 min and the reaction was stopped by diluting the sample to 1.0 ml with water. The first-strand cDNA solution was stored at 4 °C. For the PCR, 2.5 ng of first-strand cDNA was used in a 'hot-start' reaction. The PCR conditions were 94 °C for 5 min, 75 °C for 5 min, then 35 cycles of 94 °C for 1 min, 52 °C for 2 min and 72 °C for 3 min. The degenerate oligonucleotide primers were used at a final concentration of 3 μM. One-hundredth of the first-round products were used in the second-round PCR with the inner pair of nested primers, under identical conditions.

Oligonucleotide primers

The outer pair of primers were the 5′ primer [5′-CCNTT(T/C)-TA(T/C)GCNGTNAA(A/G)TG(T/C)AA-3′], representing the amino acid sequence PFYAVKCN, and the 3′ primer [5′-CC(A/G)TC(A/G)CANGTNGGNCCCCA-3′], representing the sequence WGPTCDG. The second-round internal pair of primers were the 5′ primer [5′-ATNT(T/A)(T/C)GC-NAA(T/C)CCNTG-3′], representing the amino acid sequence I(F/Y)ANPC, and the 3′ primer [5′-TNCC(A/G)TANACNCC-(A/G)TC(A/G)TT-3′], representing the sequence NDGVYG-(S/N).

Cloning of PCR products

The products of several PCRs were pooled and size-fractionated on a 2.0% (w/v) agarose gel. After the second round of PCR, only one distinct band was visible after ethidium bromide staining and DNA from that band was isolated, blunt-ended with T4 DNA polymerase and cloned into the *Eco*RV site of the vector pK8.2 (a gift from P. Jones; see [27]). The identity of the cloned fragment was determined by sequencing the PCR product.

cDNA library screening

A cDNA library of poly(A)$^+$ RNA from 7-day-old root cultures was made by using a Stratagene Uni-ZAP cDNA synthesis kit and phage vector kit (also Stratgene). One microgram of poly(A)$^+$ RNA generated 1.8×10^6 independent plaques in the unamplified library. The ODC PCR product was radioactively labelled by using a Stratagene Prime-it kit, and single plaques were isolated from the *Datura* transformed root library after three rounds of screening. Bluescript phagemids containing putative ODC cDNAs were excised from the Uni-ZAP XR vector by using the Exassist phage supplied by the manufacturer.

Sequencing

All sequencing reactions were performed on double-stranded plasmid DNA by using an Applied Biosystems Prism Sequencing kit. The sequence of both strands of the ODC cDNA was determined by a combination of oligonucleotide primer walking and subcloning into pBluescript. Sequence determination was carried out with an ABI373A automated sequencer.

Northern and Southern analysis

Standard techniques [28] were employed. Prehybridization and hybridization were carried out in QuickHyb solution (Stratagene). All hybridization washes were at high stringency: $0.1 \times$ SSC and 0.1% SDS at 65 °C (two 30 min washes).

Free and conjugated polyamine analysis

Samples were ground in liquid N_2 and the frozen powder was homogenized in 5% (w/v) cold trichloroacetic acid and allowed to stand on ice for 2 h. Homogenates were centrifuged at 20000 ***g*** for 20 min. Part of the supernatant was mixed 1:1 (by vol.) with 12 M HCl and hydrolysed for 18 h at 110 °C. The hydrolysates were evaporated to dryness under a stream of nitrogen and resuspended in trichloroacetic acid. Aliquots of the supernatant and hydrolysed supernatant were analysed by HPLC as their dansylated derivatives [29]. The internal standard was 1,7-diaminoheptane.

ODC, ADC and *S*-adenosylmethionine decarboxylase (SAMDC) assays

Frozen powder was homogenized in 0.1 M Hepes buffer containing 10 mM DTT, 10 mM EDTA, 0.1 mM PLP and 0.5% (w/v) ascorbic acid in the presence of 100 mg/g insoluble

poly(vinylpyrrolidone). The homogenates were centrifuged for 25 min at 20000 *g* and the supernatants loaded onto a PD10 column (Pharmacia) and eluted with the same buffer minus the ascorbic acid. The eluates were used for the enzyme activity analysis. The enzyme reaction mixture contained 100 μl of extract and 20 μl of radiolabelled substrate. The ODC assay contained 0.1 μCi L-[1-^{14}C]ornithine (57 mCi/mmol) and 2.5 mM unlabelled ornithine, and L-[U-^{14}C]arginine (0.1 μCi) and unlabelled arginine (1.25 mM final concentration) were added for the analysis of ADC. For SAMDC activity, 20 μl of 5 μCi/ml *S*-adenosyl-L-[^{14}C]methionine (56 mCi/mmol) diluted in 4.5 mM unlabelled *S*-adenosylmethionine was used. Each reaction was carried out in duplicate at 37 °C for 45 min. The activities of ODC, ADC and SAMDC were determined by measurement of $^{14}CO_2$ release from L-[1-^{14}C]ornithine, L-[U-^{14}C]arginine and *S*-adenosyl-L-[^{14}C]methionine respectively, by the method of Robins et al. [30]. Protein concentration was determined by the Bradford [31] method with a Bio-Rad kit.

Expression of the *Datura* ODC in *E. coli*

Single colonies of *E. coli* XL1-Blue containing the *Datura* ODC cDNA in pBluescript were grown overnight in 2 ml of Luria broth containing 50 mg/ml ampicillin. Overnight culture (1 ml) was used to inoculate 9 ml of prewarmed LB medium and then grown for 30 min at 37 °C with vigorous shaking. Expression was induced by addition of isopropylthiogalactoside to a final concentration of 2 mM. After a further 5 h at 37 °C, the induced and non-induced cells were harvested and disrupted by the method of [32]. Cell extracts were resuspended in 0.1 M Hepes buffer, pH 8.0, containing 10 mM EDTA, 10 mM DTT and 0.1 mM PLP. Enzyme activities were determined as above. The specific enzyme-activated 'suicide' inhibitor DFMO [13] was used at a concentration of 1.0 mM.

RESULTS

RT–PCR cloning of a *Datura* ODC fragment

Transformed root cultures of *Datura* grew rapidly and exhibited frequent branching; a large number of meristems, regions of cell proliferation, were present. Such cultures were a good source for cloning a plant ornithine decarboxylase (ODC) cDNA because of the abundance of root apical meristems containing rapidly dividing cells. First-strand cDNA, synthesized from mRNA from 7-day-old root cultures, was used for the PCR. Two rounds of PCR (nested PCR) with external and internal pairs of nested degenerate oligonucleotide primers representing conserved amino acid motifs in the different ODCs yielded a single product band of 603 bp. The external pair of degenerate primers represented the conserved motifs containing the lysine residue shown to bind PLP and the cysteine that binds DFMO in the mouse ODC [12]. DNA sequencing of the PCR product indicated that the amino acid sequence of the deduced open reading frame was similar to known ODC sequences.

Isolation of cDNA clones

A lambda Uni-ZAP XR cDNA library of mRNA from 7-day-old root cultures was screened with the cloned *Datura* ODC PCR product. Screening of 2×10^5 plaques provided approximately 100 positive signals. Four of the primary plaques were purified by two further rounds of screening, and sequence analysis indicated that the entire open reading frame was present in one of the cDNA inserts. In 7-day-old *Datura* root cultures, ODC represents 0.05% of the derived cDNA library. The longest

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1     GCACGAGGAAACACTCAATATTCATATATACATATATATTTGAATCCCTAATTTCTTTGT   60
61    TTTCCTTTGATTCCTCCTCTCTTTGGATGGCCGGCCAGACAGTCATCGTTTCCGGGTTGA  120
                              M  A  G  Q  T  V  I  V  S  G  L  N
121   ACCCGGCGGCCATCCTTCAGTCCACAATCGGCGGAGCTACTCCTGCACCGGCGGCGGAGA  180
      P  A  A  I  L  Q  S  T  I  G  G  A  T  P  A  P  A  A  E  N
181   ACGACCACACCAGAAAAGTTGTTCCCCTCTCAAGAGATGCCCTTCAGGATTTCATGGTTT  240
      D  H  T  R  K  V  V  P  L  S  R  D  A  L  Q  D  F  M  V  S
241   CAATCATCACCCAGAAATTACAAGATGAAAAGCAGCCCTTTTATGTGCTGGATTTGGGAG  300
      I  I  T  Q  K  L  Q  D  E  K  Q  P  F  Y  V  L  D  L  G  E
301   AAGTTGTCTCCCTTATGGATCAATGGAACGCCGGTTTACCAAATATCCGTCCATTTTATG  360
      V  V  S  L  M  D  Q  W  N  A  G  L  P  N  I  R  P  F  Y  A
361   CTGTTAAGTGTAACCCAGAACCTTCATTTCTGTCCATGTTGTCTGCTATGGGTTCCAATT  420
      V  K  C  N  P  E  P  S  F  L  S  M  L  S  A  M  G  S  N  F
421   TTGATTGTGCTAGCCGTGCTGAAATTGAGTATGTTTTGTCACTCGGTATCTCACCGGACC  480
      D  C  A  S  R  A  E  I  E  Y  V  L  S  L  G  I  S  P  D  R
481   GTATCGTTTTCGCCAACCCATGTAAGCCGGAATCCGACATAATTTTCGCCGCAAAAGTTG  540
      I  V  F  A  N  P  C  K  P  E  S  D  I  I  F  A  A  K  V  G
541   GGGTTAATCTCACGACCTTCGATTCCGAGGACGAGGTTTACAAGATCCGTAAGCATCACC  600
      V  N  L  T  T  F  D  S  E  D  E  V  Y  K  I  R  K  H  H  P
601   CGAAATGCGAGCTGTTGCTCCGAATCAAACCCATGGACGACGGCAATGCAAGATGCCCTA  660
      K  C  E  L  L  L  R  I  K  P  M  D  D  G  N  A  R  C  P  M
661   TGGGACCTAAATACGGGGCACTGCCTGAAGAAGTCGAGCCCCTGCTCCGGACCGCTCAAG  720
      G  P  K  Y  G  A  L  P  E  E  V  E  P  L  L  R  T  A  Q  A
721   CCGCCAGGCTAACCGTCTCTGGCGTTTCCTTCCACATCGGCAGCGGAGACGCCGATTCCA  780
      A  R  L  T  V  S  G  V  S  F  H  I  G  S  G  D  A  D  S  K
781   AAGCTTATTTAGGAGCCATAGCTGCGGCGAAGGGAGTTTTCGAAACAGCGGCTCGGTTCG  840
      A  Y  L  G  A  I  A  A  A  K  G  V  F  E  T  A  A  R  F  G
841   GTATGTCCAAAATGACGGTGTTAGACATCGGCGGTGGATTTACCTCCGGCCACCAATTCA  900
      M  S  K  M  T  V  L  D  I  G  G  G  F  T  S  G  H  Q  F  T
901   CCACCGCCTCCGCCGCTGTCAGATCAGCCCTGGAGCAACACTTCCATGACGAACAAGAGC  960
      T  A  S  A  A  V  R  S  A  L  E  Q  H  F  H  D  E  Q  E  L
961   TAACAATCATCGCCGAACCGGGTCGGTTCTTTGCAGAGACGGCGTTCACTTTAGCCACAA 1020
      T  I  I  A  E  P  G  R  F  F  A  E  T  A  F  T  L  A  T  T
1021  CCATTATCGGAAAAAGAGTGAGGGGAGAATTAAGAGAATACTGGATCAACGACGGCTTGT 1080
      I  I  G  K  R  V  R  G  E  L  R  E  Y  W  I  N  D  G  L  Y
1081  ACGGATCGATGAACTGTGTACTTTACGACCACGCGACGGTGAACGCGACGCCGTTAGCGT 1140
      G  S  M  N  C  V  L  Y  D  H  A  T  V  N  A  T  P  L  A  C
1141  GCATGTCGAATCGTAGTAACCTCAACTGCGGCGGGTCGAAAACGTTTCCGTCGACTGTAT 1200
      M  S  N  R  S  N  L  N  C  G  G  S  K  T  F  P  S  T  V  F
1201  TCGGGCCCACCTGTGATGCACTTGATACTGTGTTAAGGGATTACCAGTTACCGGAGCTAC 1260
      G  P  T  C  D  A  L  D  T  V  L  R  D  Y  Q  L  P  E  L  Q
1261  AGGTTAATGACTGGCTGATTTTTCCTAATATGGGTGCTTACACAAAGGCTGCTGGGTCCA 1320
      V  N  D  W  L  I  F  P  N  M  G  A  Y  T  K  A  A  G  S  N
1321  ACTTCAATGGATTCAACACATCCGCCATTGTTACTCACCTTGCTTATGCTTATCCTAGCT 1380
      F  N  G  F  N  T  S  A  I  V  T  H  L  A  Y  A  Y  P  S
1381  GATAAAATCACCACCAATTAGAATCTAATTAGCAGCGTGGTTGTAATTTTTTCCTTTTTT 1440
1441  GGGGTTTTTTTTCTAATTTGTTTCTTTTTTTTTTGTAATTTATTTTTCCAGTCAGCTTGT 1500
1501  AGTAGTTCTCCTGTGTCTCATAAGAAAAGCAAGGATTTGCTAATTGGATTTGCTCTTAAT 1560
1561  TAGAAGTCATTTAATTAAAAAAAAAAA 1587
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Figure 1 Nucleotide sequence and predicted amino acid sequence of the *Datura* ODC

No in-frame upstream termination codons are present in the first 86 bp of the sequence. The open reading frame presented is thus the longest frame possible from the first ATG in a good translation initiation consensus context [49,50], which in effect is the first ATG in the sequence. The underlined sequences indicate the position of the first and second rounds of nested PCR primers, and the arrowheads show the direction of polymerization from the primers.

cDNA insert contained 1576 bp, excluding the poly(A) tail, with 86 bp of 5′ untranslated leader sequence (Figure 1). The Trp codon used at the 3′ end of the 3′ external degenerate primer in the first round of the nested PCR reaction is represented by a Phe in the *Datura* ODC sequence. Thus, even though the second and third nucleotides of the 3′ outer PCR primer were mismatched with respect to the template, the PCR still amplified efficiently.

The predicted coding sequence

The deduced amino acid sequence encoded by the open reading frame of the *Datura* ODC cDNA consists of 431 amino acids. Figure 2 shows that in addition to homology with other eukaryotic ODCs, the *Datura* ODC sequence and the other ODCs exhibit similarity to ADCs, *meso*-diaminopimelate decarboxylases (DapDCs) and the product of the *tabA* gene of *Pseudomonas syringae* cv. tabaci. ADC, ODC and DapDCs form

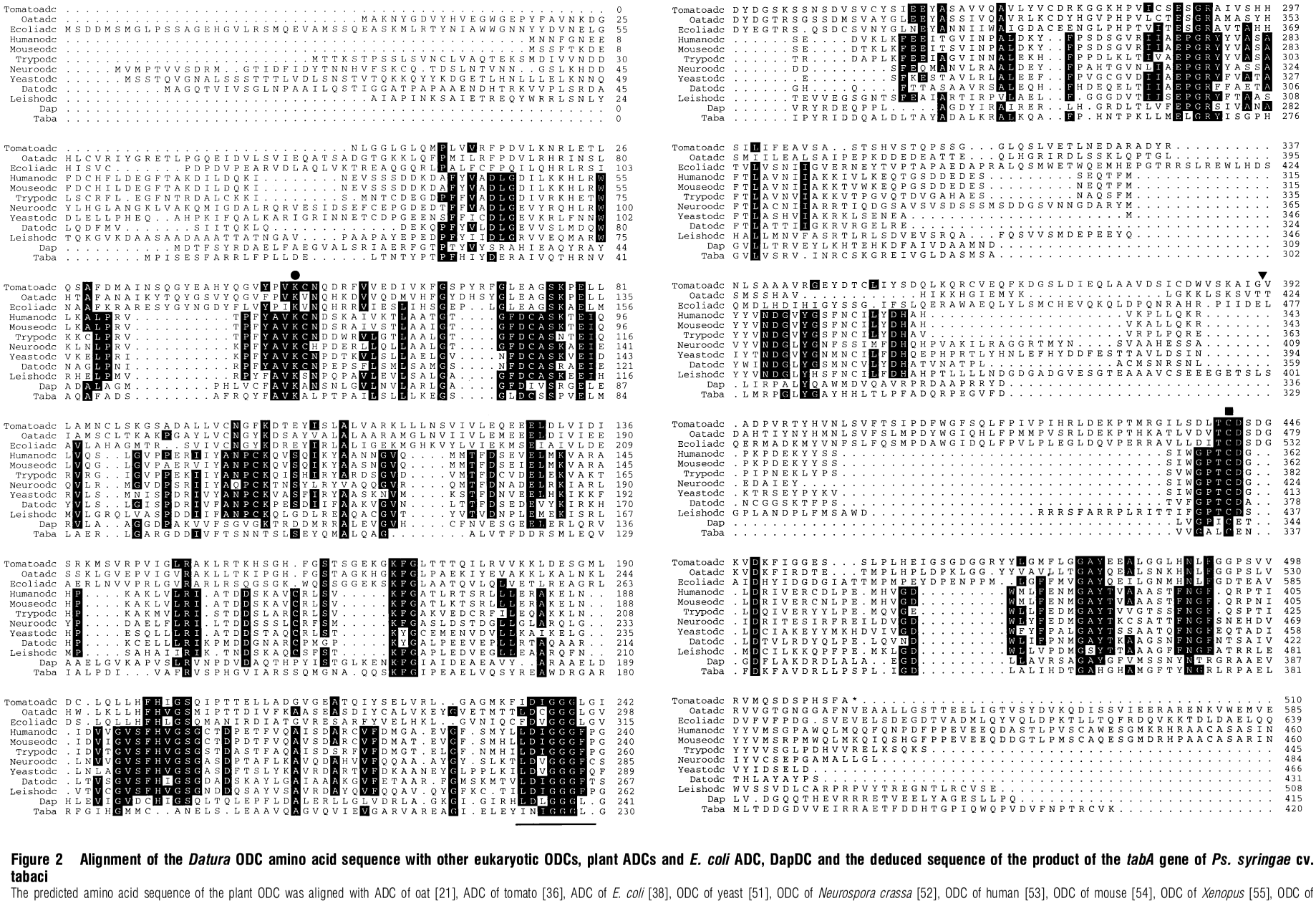

Figure 2 Alignment of the *Datura* ODC amino acid sequence with other eukaryotic ODCs, plant ADCs and *E. coli* ADC, DapDC and the deduced sequence of the product of the *tabA* gene of *Ps. syringae* cv. tabaci

The predicted amino acid sequence of the plant ODC was aligned with ADC of oat [21], ADC of tomato [36], ADC of *E. coli* [38], ODC of yeast [51], ODC of *Neurospora crassa* [52], ODC of human [53], ODC of mouse [54], ODC of *Xenopus* [55], ODC of *Trypanosoma brucei* [56], ODC of *Drosophila* [57], ODC of *Leishmania donovanii* [58], DapDC of *Pseudomonas aeruginosa* [59] and the product of the *tabA* gene of *Pseudomonas syringae* cv. tabaci [41]. The first N-terminal 200 amino acids of the leishmanial ODC and the last 22 of the oat and last 19 amino acids of the *E. coli* ADCs have been removed to facilitate the comparison. The black circle marks the Lys shown to be the main PLP-binding site and the black square marks the Cys shown to be the DFMO binding site in the mouse ODC [12]. The black triangle marks the cleavage site for the processing of the oat ADC [37] and the underlined sequence is the motif resembling a sequence conserved in methyltransferases and spermidine synthases. Only the DapDC with the greatest similarity to the *Datura* ODC is presented. The sequence comparison was generated by the PILEUP program of the University of Wisconsin GCG package [33].

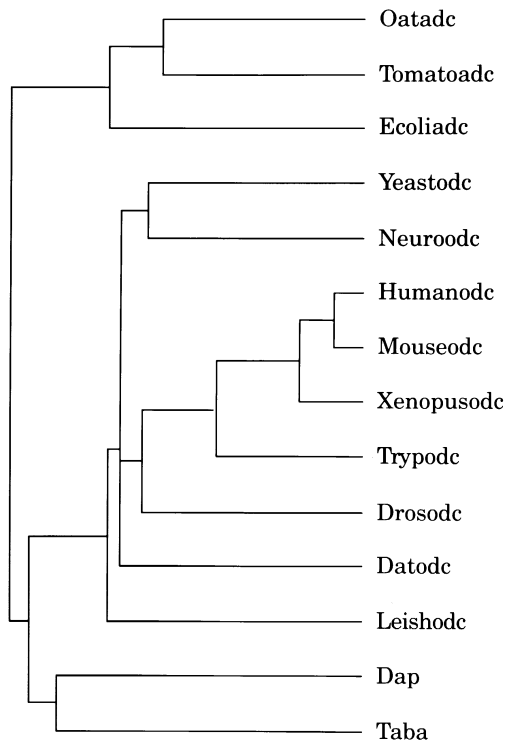

Figure 3 Evolutionary relationship of the *Datura* ODC to other Group IV decarboxylases

A dendrogram depicting the relationship, based on sequence alignment using the GCG PILEUP program of the Wisconsin package [33], of the *Datura* ODC to other Group IV decarboxylases [40]. See legend to Figure 2 for explanation of sequences.

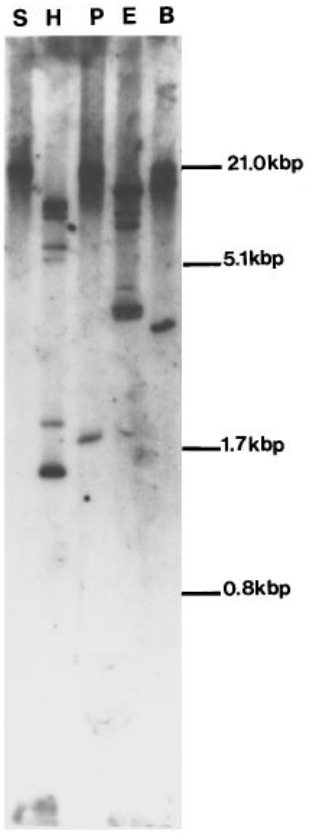

Figure 4 Southern analysis of the *Datura* ODC genomic sequences

Each track of the 1.0% agarose gel was loaded with 30 μg of *Datura* genomic DNA digested with *Sal*1 (S), *Hin*dIII (H), *Pst*1 (P), *Eco*R1 (E) and *Bam*H1 (B). The entire ODC cDNA was used as the probe.

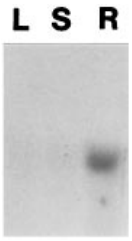

Figure 5 ODC transcript levels in *Datura* organs

Each track of the 1.0% agarose formaldehyde gel was loaded with 15 μg of total RNA from *Datura* leaves (L), stems (S) and 7-day-old transformed root cultures (R). The full-length *Datura* ODC cDNA was hybridized with the RNA gel blot.

group IV of the PLP-dependent decarboxylases [40]. ODC itself falls into four subgroups represented by the animal, fungal, plant and leishmanial enzymes (Figure 3). Amino acid residues that have been shown to be important for the activity of the mouse ODC are conserved in the plant enzyme. Particularly relevant are the *Datura* ODC residues equivalent to mouse Lys^{69}, Lys^{169}, His^{197} and Cys^{360} (Figure 2). In total, 23 residues are conserved between all the decarboxylase sequences. Using the coordinates of the mouse ODC sequence, the absolutely conserved residues are: Lys^{69}, Gly^{84}, Ser^{91}, Glu^{94}, Glu^{138}, Arg^{154}, Lys^{169}, Gly^{171}, His^{197}, Gly^{199}, Ser^{200}, Asp^{233}, Gly^{235}, Gly^{236}, Gly^{237}, Glu^{274}, Gly^{276}, Arg^{277}, Cys^{360}, Asp^{364}, Gly^{381}, Tyr^{383} and Asn^{398}. The Glu^{94} equivalent is replaced by a Ser and the Gly^{381} equivalent is replaced by an Arg in the *Bacillus subtilis* DapDC sequence [39], which is not shown in Figure 2. The *Datura* ODC C-terminus does not contain the conserved region shared in the mammalian and *Xenopus* enzymes, thought to be implicated in rapid degradation of the protein.

One of the conserved motifs found in all of these decarboxylases is similar to a motif found in *N*- and *O*-methyltransferases and spermidine synthases. The decarboxylase motif has a consensus of hh(D/N)hGGGh(G/T), where h represents a small hydrophobic residue, and this is similar to the consensus motif hh(D/E)hGGG(P/T)G found in at least 69 methyltransferases [34]. In the spermidine synthases (which are closely related to the methyltransferases at the primary sequence level and by binding decarboxylated *S*-adenosylmethionine and *S*-adenosylmethionine respectively [35]), the motif is represented by hhhhGGGDG.

ODC may be represented by more than one gene copy in *Datura*

The *Datura* cDNA does not contain a recognition site for *Eco*R1, *Bam*H1 or *Pst*1 yet at least five *Eco*R1-digested DNA bands hybridized to the *Datura* ODC cDNA probe when the gel blot was washed at high stringency (Figure 4). At least three *Bam*H1- and *Pst*1-digested DNA bands hybridized. Fainter bands could also be detected after high-stringency washes that may represent partial digests or related sequences. The Southern blot data are

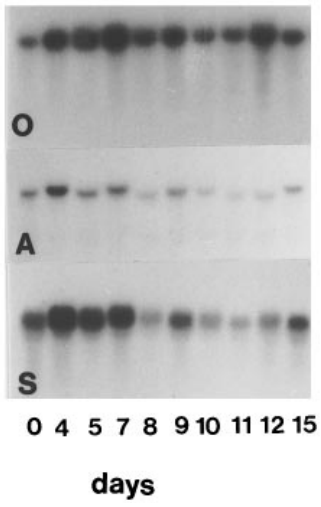

Figure 6 Time course of ODC, ADC and SAMDC transcript accumulation during growth of *Datura* transformed root cultures

RNA samples for the time course of each gene were run on parallel 1.0% agarose formaldehyde gels with 15 μg of total RNA loaded on each track. Each individual RNA sample was derived from the pooling of four or five flasks containing replicate root cultures grown and harvested in parallel. The probes used were the *Datura* ODC cDNA (O), a PCR fragment of *Datura* ADC (A) and a PCR fragment of *Datura* SAMDC (S).

consistent with the possibility of there being more than one ODC gene, in contrast with the situation reported for ADC in tomato, which may be present in only one copy [36].

ODC transcript accumulation, enzyme activity and free and conjugated polyamine accumulation during the growth of *Datura* transformed root cultures

More ODC mRNA accumulated in rapidly growing 7-day-old transformed root cultures of *Datura* than in either leaves or stems of mature plants (Figure 5), consistent with the association of ODC activity with cellular proliferation. The accumulation of ODC mRNA during the time course of transformed root growth is compared with that of ADC and SAMDC mRNAs in Figure 6. PCR-derived probes for ADC and SAMDC were obtained by using the same PCR procedure employed for isolating the ODC PCR fragment. The general pattern of a relatively sharp increase during the first few days of subculture, a maximum at days 4–5 followed by a decline is seen for the *Datura* ODC, ADC and SAMDC transcripts. This expression profile is reflected by the ODC, ADC and SAMDC enzyme activities and the accumulation of free and polar conjugated polyamines (except for the free and conjugated spermine levels) (Figure 7). ODC was approximately three times more active than ADC in the transformed root cultures and ODC and ADC activities were both induced approximately 5-fold by subculturing, whereas the increase in free and conjugated polyamine levels was less than 50%. The fresh weight of the root cultures increased throughout the time course (Figure 7). It is noteworthy that spermidine was the most abundant free polyamine, with a maximal concentration nearly three times that of putrescine but putrescine conjugates were

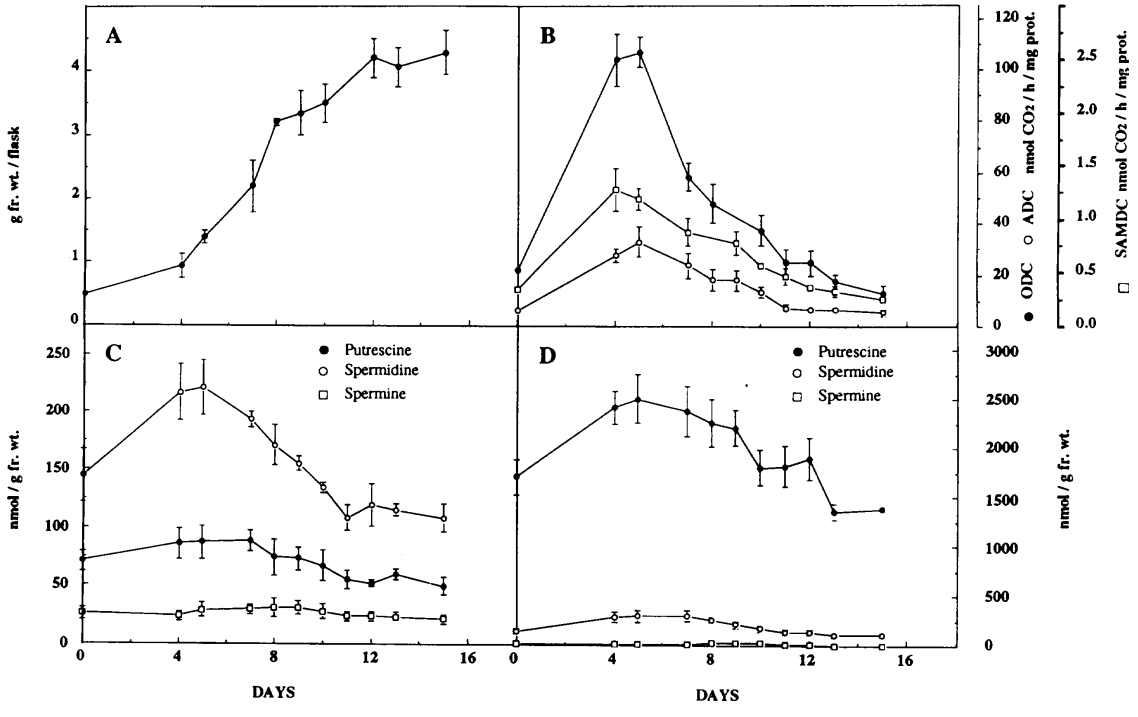

Figure 7 Time course of enzyme activity, free and conjugated polyamine (polar conjugates) accumulation and fresh weight increase of transformed root cultures

(**A**) Average fresh weight increase of root cultures for four or five replicate flasks; (**B**) enzyme activities of ODC, ADC and SAMDC; (**C**) accumulation of free polyamines; (**D**) accumulation of conjugated polyamines. Each time point represents four or five pooled replicate flasks.

Table 1 *Datura* ODC expression in *E. coli*

Two independent cultures were used for both the control plasmid (pBluescript plus control cDNA insert) and the *Datura* ODC in pBluescript. Values are given in nmol/h per mg of protein.

	Uninduced		Induced	
	− DFMO	+ DFMO	− DFMO	+ DFMO
Control	65 ± 3	64 ± 7	55 ± 6	64 ± 6
ODC	347 ± 37	73 ± 2	833 ± 13	70 ± 2

nearly ten times more abundant than those of spermidine. The total concentration of polyamine conjugates was about ten times higher than that of the total free polyamines. The initial increase in free putrescine during the time course of root growth was 20 nmol/g fresh weight; however, the increase in putrescine linked to hydroxycinnamic acids (conjugated putrescine) was at least 800 nmol/g fresh weight. Thus although free spermidine exhibited a steeper induced accumulation than free putrescine, the increase in conjugated putrescine was an order of magnitude greater than that of free spermidine.

Expression of *Datura* ODC in *E. coli*

The *Datura* ODC cDNA in pBluescript contained 86 bp of 5′ untranslated leader sequence before the first ATG of the open reading frame. Although the coding sequence was in the same orientation as the β-galactosidase promoter it was not in-frame with the β-galactosidase ATG. Nevertheless we were able to demonstrate induction and over-expression of the *Datura* ODC in *E. coli* as well as selective inhibition by DFMO. Table 1 shows that the induced ODC activity in *E. coli* XL1-Blue cells containing the *Datura* ODC cDNA was more than 10-fold greater than in cells containing a control plasmid. In two separate cultures the uninduced *Datura* ODC activity was also approximately 5-fold higher than in the control cells. Further evidence that the increased ODC activity was from the *Datura* cDNA was provided by the sensitivity of the *Datura* ODC activity to the presence of 1.0 mM DFMO, whereas the background activity represented by the endogenous *E. coli* ODC was resistant to DFMO as previously described [37].

DISCUSSION

The predicted amino acid sequence of the *Datura* ODC exhibits extensive similarity to other eukaryotic ODCs. The sequence similarity between ODC, ADC and DapDcs has been described previously [38–40]. Eukaryotic ODCs, the plant and bacterial ADCs and the bacterial DapDCs belong to group IV of the PLP-dependent decarboxylases [40]. In addition, the *tabA* gene of *Pseudomonas syringae* pv. *tabaci* involved in tabtoxin production [41] encodes a protein that 'utilises a substrate analogue of a compound in the lysine biosynthesis pathway' [41] and also exhibits sequence similarity with ODC, ADC and DapDC. Unlike the mammalian ODC sequences, the *Datura* ODC does not possess the 3′ terminal extension thought to be involved in rapid turnover of the ODC protein. It is therefore possible that the plant enzyme may be relatively stable *in vivo* in comparison with the mammalian proteins.

The similarity between the *Datura* and mouse ODC amino acid sequences permits a tentative assignment of the PLP binding site as Lys^{94} and the DFMO binding site as Cys^{376} on the *Datura* sequence. Similarly, by comparing the *Datura* ODC and oat ADC sequences, the PLP binding site and DFMA binding sites of the oat ADC can be tentatively assigned as Lys^{104} and Cys^{475} respectively on the oat ADC sequence.

Certain residues are conserved in all the eukaryotic ODCs, *E. coli* and plant ADCs, and the bacterial DapDCs [39]. The conservation of these residues across the three PLP-dependent enzymes from Gram-positive and Gram-negative bacterial, plant, animal, protozoan and fungal sources indicates their importance in the enzyme's function. The residues might be conserved because they bind PLP and common substructures in the different substrates, and there could be conformational constraints in the enzyme structures that require the small size of the eight conserved glycine residues.

One particular motif of the decarboxylases resembles motif I of methyltransferases, which is thought to comprise part of the *S*-adenosylmethionine binding pocket [34] and a similar motif in an equivalent position in spermidine synthases [35]. The decarboxylase motif also resembles the nucleotide binding site of FAD-binding flavoproteins [42] and the P region of GTP-binding proteins, probably involved in the hydrolysis of GTP [43]. Whether the decarboxylase motif binds decarboxylated *S*-adenosylmethionine is unknown. We are not aware of any reports of ODC being regulated by decarboxylated *S*-adenosylmethionine.

Not surprisingly the ODC mRNA accumulates to a greater extent in highly branching and fast growing transformed root cultures than in leaves or stems, where cell division is much more limited. The accumulation of the ODC mRNA during the time course of root culture growth is essentially the same as that of SAMDC and ADC and seems to be tightly linked to the induction of growth and cell division due to subculturing into new growth medium. The kinetics and relative levels of free and conjugated polyamine accumulation that we report here are in agreement with those of Robins et al. [23], who used the same root line D15/5; however, the peak of enzyme activity we report (day 4–5) is earlier. This may reflect faster growth of our root cultures. The transformed root system is like an organized tumour because the bacterially derived *rol* oncogenes that cause the transformed phenotype [44,45] maintain a constant growth signal resulting in branching and growth.

The increase in free putrescine accumulation due to subculturing was only in the region of 20 nmol/g fresh weight whereas the increase in conjugated putrescine was in the region of 800 nmol/g fresh weight, indicating that most of the free putrescine synthesized was then conjugated to hydroxycinnamic acids.

The activity of the *Datura* ODC in *E. coli* was surprising because of the presence of some of the untranslated leader sequence and the fact that the ODC ATG was out of frame with respect to the β-galactosidase ATG, suggesting that a sequence resembling a ribosome binding site is present in the ODC leader. A similar phenomenon was reported for the expression of the aspen lignin-bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase in pBluescript, also expressed in *E. coli* XL-1 Blue [46].

The isolation of a plant ODC will help address a fundamental enigma in plant polyamine biochemistry: why are two routes to putrescine (ODC and ADC) present? However, it is not certain that the sole purpose of ADC is to produce putrescine; for example, in mammals agmatine has been implicated in physiological roles in the brain [47]. ADC produces putrescine via agmatine and *N*-carbamoylputrescine in plants [19].

The conservation of the plant ODC primary sequence has occurred in spite of the presence of an additional biosynthetic route to putrescine. There is conservation between the bacterial

and plant ADC amino acid sequences but not between the bacterial and plant ODC sequences. Conservation of the plant ODC amino acid sequence with other eukaryotic ODCs suggests that the ODC and ADC pathways have important and different functions in plants, as has been suggested by differential effects of ODC and ADC inhibition in tobacco suspension cultures [60] and on tobacco plant development [61].

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REFERENCES

1 Tabor, C. W. and Tabor, H. (1984) Annu. Rev. Biochem. **53**, 749–790
2 Heby, O. and Persson, L. (1990) Trends Biochem. Sci. **15**, 153–158
3 Pegg, A. E. (1986) Biochem J. **234**, 249–262
4 Pegg, A. E. (1988) Cancer Res. **48**, 759–774
5 Pegg, A. E. and McCann, P. P. (1992) Pharmacol. Ther. **56**, 359–377
6 Bello-Fernandez, C., Packham, G. and Cleveland, J. L. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7804–7808
7 Wagner, A. J., Meyers, C., Laimins, A. L. and Hay, N. (1993) Cell Growth Differ. **4**, 879–883
8 Auvinen, M., Paasinen, A. and Hölttä, E. (1992) Nature (London) **360**, 355–358
9 Moshier, J. S., Dosescu, J., Skunca, M. and Luk, G. (1993) Cancer Res. **53**, 2618–2622
10 Packham, G. and Cleveland, J. L. (1994) Mol. Cell. Biol. **14**, 5741–5747
11 Hayashi, S. and Murakami, Y. (1995) Biochem. J. **306**, 1–10
12 Poulin, R., Lu, L., Ackerman B., Bey, P. and Pegg, A. E. (1992) J. Biol. Chem. **267**, 150–158
13 Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. and Vevert, J. P. (1978) J. Am. Chem. Soc. **100**, 2551–2553
14 Lu, L., Stanley, B. and Pegg, A. E. (1991) Biochem. J. **277**, 671–675
15 Tsirka, S. E. and Coffino, P. (1992) J. Biol. Chem. **267**, 23057–23062
16 Tsirka, S. E., Turck, C. W. and Coffino, P. (1993) Biochem. J. **293**, 289–295
17 Tobias, K. E. and Khahana, C. (1993) Biochemistry **32**, 5842–5847
18 Coleman, C. S., Stanley, B. A. and Pegg, A. E. (1993) J. Biol. Chem. **268**, 24572–24579
19 Smith, T. A. (1985) Annu. Rev. Plant Physiol. **36**, 117–143
20 Boyle, S. M., Barroso, L., Moore, R. C., Wright, J. M. and Patel, T. (1994) Gene **151**, 157–160
21 Bell, E. and Malmberg, R. L. (1990) Mol. Gen. Genet. **224**, 431–436
22 Heimer, Y. M. and Mizrah, Y. (1982) Biochem. J. **201**, 373–376
23 Robins, R. J., Parr, A. J., Bent, E. G. and Rhodes, M. J. C. (1991) Planta **183**, 185–195
24 Michael, A. and Spena, A. (1995) Methods Mol. Biol. **44**, 207–222
25 Verwoerd, T. C., Dekker, B. M. M. and Hoekma, A. (1989) Nucleic Acids Res. **17**, 2362
26 Frohman, M. A. (1990) in PCR Protocols, a Guide to Methods and Applications (Innis, M. A. Gelfand, D. H., Sninsky, J. J. and White, T. J., eds.), pp. 28–38, Academic Press, San Diego
27 Waye, M. M. Y., Verhoeyen, M. E., Jones, P. T. and Winter, G. (1985) Nucleic Acids Res. **13**, 8561–8571
28 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning; a Laboratory manual, 2nd edn, Cold Spring Harbour Press, New York
29 Smith, M. A. and Davies, P. J. (1985) Plant Physiol. **78**, 89–91
30 Robins, R. J., Parr, A. J., Payne, J., Walton, N. J. and Rhodes, M. J. C. (1990) Planta **181**, 414–422
31 Bradford, M. (1976) Anal. Biochem. **72**, 248–254
32 Szumanski, M. B. W. and Boyle, S. M. (1990) J. Bacteriol. **172**, 538–547
33 Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395
34 Kagan, R. M. and Clarke, S. (1994) Arch. Biochem. Biophys. **310**, 417–427
35 Hibi, N., Higashiguchi, S., Hashimoto, T. and Yamada, Y. (1994) Plant Cell **6**, 723–735
36 Rastogi, R., Dulson, J. and Rothstein, S. J. (1993) Plant Physiol. **103**, 829–834
37 Kallio, A. and McCann, P. P. (1981) Biochem. J. **200**, 69–75
38 Moore, R. C. and Boyle, S. M. (1990) J. Bacteriol. **172**, 4631–4640
39 Mills, D. A. and Flickner, M. C. (1993) Appl. Env. Microbiol. **59**, 2927–2937
40 Sandmeier, E., Hale, T. I. and Christen, P. (1994) Eur. J. Biochem. **221**, 997–1002
41 Engst, K. and Shaw, P. D. (1992) Mol. Plant–Microbe Interact. **4**, 322–329
42 Van Beeuman, J. J., Demol, H., Samyn, B., Bartsch, R. G., Meyer, T. E., Dolata, M. M. and Gusanovich, M. A. (1991) J. Biol. Chem. **266**, 12921–12931
43 Kaziro, Y., Itoh, H., Kozasa, T., Nafafuku, M. and Satoh, T. (1991) Annu. Rev. Biochem. **60**, 349–400
44 Spena, A., Estruch, J. J., Hansen, G., Langenkemper, K., Berger, S. and Schell, J. (1992) in Advances in Molecular Genetics of Plant–Microbe Interactions (Nester, E. W. and Verma, D. P. S., eds.), pp. 109–112, Kluwer, Dordrecht
45 Klee, H. (1992) Curr. Biol. **2**, 271–272
46 Bugos, R. C., Chiang, V. L. C. and Campbell, W. H. (1991) Plant Mol. Biol. **17**, 1203–1215
47 Li, G., Regunathan, S., Barrow, C. J., Eshraghi, J., Cooper, R. and Reis, D. J. (1994) Science **263**, 966–969
48 Wahlfors, J., Alhonen, L., Kauppinen, L., Hyvönen, T., Jänne, J. and Eloranta, T. O. (1990) DNA Cell Biol. **2**, 103–110
49 Kozak, M. (1984) Nucleic Acids Res. **12**, 857–872
50 Kozak, M. (1986) Cell **44**, 283–292
51 Fonzi, W. A. and Sypherd, P. S. (1987) J. Biol. Chem. **262**, 10127–10133
52 Williams, L. J., Barnett, G. R., Ristow, J. L., Pitkin, J., Perriere, M. and Davis, R. H. (1992) Mol. Cell. Biol. **12**, 347–359
53 Hickok, N., Seppänen, P. J., Gunsalus, G. L. and Jänne, O. A. (1987) DNA **6**, 179–187
54 Gupta, M. and Coffino, P. (1985) J. Biol. Chem. **260**, 2941–2944
55 Bassez, T., Paris, J., Omilli, F., Dorel, C. and Osbourne, H. B. (1990) Development **110**, 955–962
56 Phillips, M. A., Coffino, P. and Wang, C. C. (1987) J. Biol. Chem. **262**, 8721–8727
57 Rom, E. and Kahana, C. (1993) DNA Cell Biol. **12**, 499–508
58 Hanson, S., Adelman, J. and Ullman, B. (1992) J. Biol. Chem. **267**, 2350–2359
59 Martin, C. Cami, B., Yeh, P., Stagier, P., Parsot, C. and Patte, J.-C. (1988) Mol. Biol. Evol. **5**, 549–559
60 Hiatt, A. C., McIndoo, J. and Malmberg, R. L. (1986) J. Biol. Chem. **261**, 1293–1298
61 Burtin, D., Martin-Tanguy, J. and Tepfer, D. (1991) Plant Physiol. **95**, 461–468

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