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Molecular Analysis of a New Member of the Opium Poppy Tyrosine/3,4-Dihydroxyphenylalanine Decarboxylase Gene Family[1]

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An aromatic amino acid decarboxylase DNA fragment was generated from opium poppy (*Papaver somniferum* L.) genomic DNA by the PCR using primers designed from conserved amino acid sequences of other aromatic amino acid decarboxylase genes. Using this fragment as a probe, a genomic clone was isolated that encodes a new member of the opium poppy tyrosine/3,4-dihydroxyphenylalanine decarboxylase gene family (*TyDC5*). The predicted *TyDC5* amino acid sequence shares extensive identity with other opium poppy tyrosine/3,4-dihydroxyphenylalanine decarboxylases (84%), and when expressed in *Escherichia coli*, it is active against tyrosine and to a lesser extent against 3,4-dihydroxyphenylalanine. Ribonuclease protection assays indicate that *TyDC5* is expressed primarily in the roots of mature poppy plants. A peak of *TyDC5* expression was also observed during germination, coincident with the emergence of the radicle from the seed coat. Parallel results were obtained in transgenic tobacco using a *TyDC5* promoter fragment (−2060) translationally fused to the β-glucuronidase reporter gene (*GUS*). In *TyDC5::GUS* tobacco, GUS activity transiently appeared in all parts of the seedling during germination, but was limited to the roots in older plants. These results indicate that *TyDC5* expression is transcriptionally regulated and suggest that the *TyDC5* enzyme may play an important role in providing precursors for alkaloid synthesis in the roots and germinating seedlings of opium poppy.

Opium poppy (*Papaver somniferum* L.) is the sole commercial source of several important medicinal alkaloids, including morphine, codeine, and thebaine. Biogenesis of these and other isoquinoline alkaloids begins with the modification of two molecules of Tyr to form dopamine and 4-hydroxyphenylacetaldehyde (Loeffler et al., 1987; Stadler et al., 1988). The stereospecific condensation of dopamine and 4-hydroxyphenylacetaldehyde produces norcoclaurine, a key intermediate in the synthesis of protoberberine, benzophenanthridine, and morphinandienone alkaloids (Stadler et al., 1987).

Although the biosynthesis of the morphinane alkaloids has received considerable attention, the enzymes involved in the early steps of the pathway have not been fully characterized. For example, although dopamine is clearly an abundant constituent of the poppy latex (Roberts et al., 1983), it is not certain whether dopamine is formed from the decarboxylation of DOPA or from the hydroxylation of tyramine, the decarboxylation product of Tyr, or perhaps by both routes.

Opium poppy is reported to have both TyDC and DODC activities. Seedling homogenates are capable of catalyzing the decarboxylation of Phe, Tyr, DOPA, and Glu (Jindra et al., 1966), although later workers were unable to detect DODC activity in seedlings (Ashgar and Sidiqi, 1970). In contrast, isolated latex is reported to have DODC activity as well as low but measurable activity against Phe, Tyr, and His (Roberts and Antoun, 1978).

Recently, two independent cDNAs and two related genomic clones encoding ADCs were isolated from opium poppy (Facchini and De Luca, 1994). When expressed as fusion proteins, the enzymes encoded by both cDNAs showed their highest activities against DOPA, but also accepted Tyr as a substrate (Facchini and De Luca, 1994, 1995).

Here we describe a new member of the opium poppy TyDC/DODC gene family (*TyDC5*), characterize its substrate specificity, and analyze its expression in opium poppy tissues. We also use a *TyDC5* promoter-reporter gene construct to study its expression in transgenic tobacco. The correlation between the results obtained in both the homologous and the heterologous systems show that the *TyDC5* gene is preferentially expressed in roots and during seedling development.

MATERIALS AND METHODS

Plant Materials

Seeds of *Papaver somniferum* L. cv UNL186 were surface sterilized with a 20% Clorox solution for 15 min, rinsed with sterile, distilled water, and then placed into 80 × 100 mm storage dishes containing 100 mL of sterile one-half-

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Abbreviations: ADC, aromatic amino acid decarboxylase; DODC, 3,4-dihydroxyphenylalanine decarboxylase; DOPA, 3,4-dihydroxyphenylalanine; PI, postimbibition; RPA, ribonuclease protection assay; TDC, tryptophan decarboxylase; TyDC, tyrosine decarboxylase.

strength Murashige and Skoog (Murashige and Skoog, 1962) inorganic salts solidified with 0.25% agar. The dishes were sealed with Parafilm (American National Can, Neenah, WI) and placed in a growth chamber with 165 μE m^{-2} s^{-1} illumination by fluorescent lights. Photoperiod was 16 h d/8 h night at 25°C d/20°C night. Seedlings were harvested at 0, 2, 4, 6, 10, and 14 d postimbibition and frozen in liquid nitrogen for RPA.

Opium poppy plants were grown from seed under controlled environmental conditions as reported earlier (Nessler, 1988). Developing flower buds (5–7 d preanthesis), leaves, and stems were frozen in liquid nitrogen and stored at −70°C. Latex was collected from unripe capsules 2 d after petals fell.

PCR of a Poppy Decarboxylase

An ADC probe was generated from opium poppy genomic DNA by PCR. Two degenerate oligonucleotide primers were constructed based on conserved amino acid sequences of parsley *TyDC2* (Kawalleck et al., 1993) and TDC of *Catharanthus roseus* (De Luca et al., 1989) and *Camptotheca acuminata* (R.J. Burnett, M. López-Meyer, and C.L. Nessler, unpublished data). The 5′ primer, CACTTGA(AG)CCNGA(AG)GA(AG) TTCCGAA, was designed to prime sequences encoding the residues PLEPEEFR. The 3′ primer, TTCC(AC)GC(AG)TANGC(AT)GC(AG)TCCAC(AG)TG, was designed to prime the reverse complement of the region encoding the conserved pyridoxal phosphate-binding domain HVDAAYAG.

PCR was performed as previously described (Nessler and Vonder Haar, 1990) using 100 ng of opium poppy genomic DNA as template. The predicted 826-bp PCR fragment was subcloned into pBluescript KS^+ (Stratagene) and sequenced to confirm its homology to other ADCs (data not shown).

Library Screening and Sequence Analysis

A ^{32}P-labeled decarboxylase probe was made from the cloned PCR fragment and used to screen an opium poppy λEMBL3 genomic library as previously described (Nessler et al., 1990). Six independent clones were isolated in a screen of 400,000 recombinant plaques. One of these, designated *TyDC5,* was selected for further study.

Both strands of a 3.75-kb *Pst*I-*Hin*dIII fragment of the *TyDC5* gene (see "Results") were sequenced by the dideoxy chain termination method (Sanger et al., 1977). Sequence analyses were performed using the University of Wisconsin Genetics Computer Group (Madison, WI) software package version 7.3 and GeneWorks version 2.3.1 (IntelliGenetics, Mountain View, CA).

Mapping the Transcription Start and Expression Analysis

The site of transcript initiation for the *TyDC5* gene was determined using the RPA. Antisense transcripts were made in vitro in the presence of [^{32}P]UTP with a MAXIscript Kit (Ambion, Austin, TX). This riboprobe spanned a region 440 nucleotides 5′ of the translational start. Tissues were frozen in liquid N_2, ground into a fine powder, and further processed using the Direct Protect Kit (Ambion). The size of the protected fragment was determined by fractionation of the reaction products on a 6% denaturing polyacrylamide gel adjacent to a sequencing ladder. The transcription start site mapped 34 bp upstream of the translational start in two independent experiments.

Tissue-specific expression of the *TyDC5* gene was examined by RPA using a 300-nucleotide riboprobe. The probe included a region of 130 nucleotides of 3′ untranslated sequence downstream of the translational stop, which is not conserved among different gene members in this family. Even if the probe hybridized with other TyDC genes, the hybrids formed will contain multiple mismatches producing protected fragments that will be cleaved during the subsequent RNase digestion step, resulting in shorter fragments than those seen with the completely homologous RNA. Frozen tissues and latex from a mature flowering plant were processed using the Ambion Direct Protect Kit. A 28S rRNA riboprobe, prepared from a *Camptotheca acuminata* cDNA, was used as an internal RNA standard for each sample. Relative amounts of RNA were quantitated with a Fujix (Stamford, CT) BAS2000 Bio-Image Analyzer.

Expression of *TyDC5* in *Escherichia coli*

Bacterial expression of the *TyDC5* gene product was accomplished using a modified pTrc99A expression vector (Pharmacia LKB Technology, Piscataway, NJ) to which sequences encoding six His residues were added upstream of the multiple cloning site (p6HTrc99A). The six-His tag was added to the N terminus of the recombinant enzyme to permit direct purification by nickel affinity chromatography (Hochuli et al., 1987).

The *TyDC5* coding region was isolated by PCR of opium poppy genomic DNA and cloned into p6HTrc99A. To facilitate translational fusion with the His tag, an in-frame *Nco*I site was added to the 5′ primer replacing the TTATGG of the wild-type gene with CCATGG. An artificial *Sac*I site was added to the 3′ primer downstream of the translational stop.

Overnight cultures of *Escherichia coli* (DH5-α) containing *TyDC5*-p6HTrc99A or *TyDC5*-p6HTrc99A as a control were diluted 1:50 and grown to an A_{600} of approximately 0.8. After the addition of 1 mM isopropyl-β-D-thiogalactopyranoside, the cultures were grown for an additional 4 h, pelleted by microcentrifugation, and lysed under nondenaturing conditions (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mg/mL lysozyme, 1 mM PMSF).

Decarboxylase Activity Assay

Decarboxylase activity assays were performed under conditions outlined by Kawalleck et al. (1993) in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 μM pyridoxal-1-phosphate, 0.01% (w/v) NaN_3, 10 mM β-mercaptoethanol, 10% glycerol (v/v), 20 μM (4.6 kBq) radiolabeled aromatic substrate with 100 μL of protein extract in a final volume of 120 μL. Substrate specificity of the bacterially expressed *TyDC5* gene product was assayed by measuring the amount of $^{14}CO_2$ released from L-carboxyl-^{14}C-labeled dihydroxyphenylalanine, Phe, and Tyr. TDC activity was measured by monitoring tryptamine production by TLC using uniformly [14]C-labeled Trp as substrate (De Luca et al., 1988). Quantitation of the reaction products was performed with a Fujix BAS2000 Bio-Image Analyzer.

Plasmid Construction and Plant Transformation

A *TyDC5::GUS* translational fusion was made by ligating a 2060-bp *Pst*I-*Nco*I fragment from the *TyDC5* promoter to a *GUS* reporter gene (pRAJ275; Jefferson et al., 1987) containing the nopaline synthase polyadenylation signal at its 3′ end. *TyDC5::GUS* constructs were assembled in pUC18 and then transferred to the binary vector pBin19 (Bevan, 1984). Constructs were electroporated into *Agrobacterium tumefaciens* LBA 4404 and used to transform tobacco leaf discs (Horsch et al., 1985).

GUS Assays and Histochemistry

Quantitative GUS assays were performed as described by Jefferson et al. (1987). Fluorescence of methylumbelliferone cleaved from methyl-umbelliferyl-β-D-glucuronide was measured in a fluorometer (Hoefer Scientific, San Francisco, CA) and expressed as nmol of methyl-umbelliferyl-β-D-glucuronide min^{-1} mg^{-1} protein. Protein was measured by the method of Bradford (1976). GUS expression was histochemically localized by incubating tissues in the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronide acid cyclohexylammonium salt for 3 to 16 h at 37°C as described by Jefferson (1987). Stained tissues were bleached for 30 to 60 min in 10% Clorox at 37°C and hand-sectioned for photomicrography.

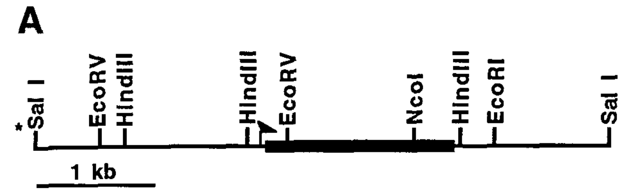
RESULTS

Isolation of an Opium Poppy ADC Gene

ADCs share significant blocks of amino acid identity, particularly in their pyridoxal phosphate-binding domains (De Luca et al., 1988; Kawalleck et al., 1993). This sequence conservation was used to develop two degenerate oligonucleotide primers to obtain an opium poppy decarboxylase PCR probe (see "Materials and Methods").

An 826-bp PCR product was isolated and sequenced to confirm its identity as a decarboxylase gene fragment (data not shown). The PCR product was then used to screen a λEMBL3 opium poppy genomic library (Nessler et al., 1990). Six different recombinants were isolated in this screen and one, designated *TyDC5*, was selected for more complete analysis.

A 3.75-kb *Pst*I-*Hin*dIII fragment of the *TyDC5* gene (Fig. 1A) was sequenced and found to contain a single, uninterrupted open-reading frame of 523 amino acids. The putative *TyDC5* protein has a calculated M_r of 57,304 and a pI of 5.96. Predictably, amino acid sequence alignment with other plant TyDCs/DODCs (Fig. 1B) shows highest identities to related opium poppy genes: 86% identity to *TyDC1* and 75% to *TyDC2* (Facchini and De Luca, 1994), and only a 63% identity to the most similar parsley *TyDC2* gene (Kawalleck et al., 1993).



B

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TyDC5 MGSLPTDN-LE-SMSICSQNPLDPDEFRRQGHMIIDFLADYYKNV-KVSSRSQANPG-SQ
TyDC1 .....AN--F.-...L............................E.YPV.T.VD..YLK
TyDC2 ....N.EDV..N.SAFGVT.....E.................RD.E.YPV...VE..YLR
P.c.  ...IDNLT--.KLA.QFPM.T.E.E........M........RK.ENYPV...VS..YLR

TyDC5 QTLPETAPNHSESIETILQDVQNDIIPGITHWQSPNYFAYFPSSGSVAGFLGEMLSSGFN
TyDC1 KR...S..YNP.......E..T......L.................I.........T...
TyDC2 KR......YNP..........TTE....L........Y..................T...
P.c.  EI...S..YNP..L........TK............F.........T.........T...

TyDC5 VVGFNWMSSPAATELESIVMNWLGQMLNLPKSFLFSSDDNAGSSGGGVLQGTTCEAILCT
TyDC1 ..........................T..........GSS.--................
TyDC2 ................V..D.F.K.....E.....GSG------.......S.......
P.c.  .......V........NV.TD.F.K..Q........GGG------...............

TyDC5 LTASRDKMLNKIGRENINKLVVYASDQTHCALQKAAQIAGINPKNFRAIATSKATDFGLS
TyDC1 ...A.......................LS................L........N....
TyDC2 ...A..RK.......H.GR....G.............V...........K.F.ENS....
TyDC3 ...A..RK.......H.GR....G........................VK.F..NS...A
P.c.  .V.A...N.RQH.MD..G.....C.....S......K....D.......E.T.SS..Q.C

TyDC5 PQALLSTILADIESGLVPLFLCATVGTTSSTAVDPIGPLCEVAKQFGIWVHVDAAYAGSA
TyDC1 .NS.Q..................................A...LH.....I........
TyDC2 AAT.REV..E...A..I...V.P.............S.I.....EYEM...........
TyDC3 AST.REV..E...A..I...V.P................I.....EYEM...I........
P.c.  .KR.E.A..H.LQN..I..Y..........T...LPA.T....KYDL............

TyDC5 CICPEFRHFIDGVEEADSFSLNAHKWFFTTLDCCCLWVKDSNALVKALSTSPEYLKNKAT
TyDC1 ..............D.........................DS................
TyDC2 ......................................PS........N....R....
TyDC3 ......................................PSS.......N....R....
P.c.  .......QYL....N............L..........RNPS..I.S...Y..F...N.S

TyDC5 DSKQVIDYKDWQIALSRRFRSMKLWLVLRSYGVANLRSFLRSHVKMAKHFDGLIAMDKRF
TyDC1 ................................I....T.............Q...G..N..
TyDC2 E.R..V...............L...M.......T...N...........T.E...C..G..
TyDC3 E.R..V.........I.........M.......T...N......R...T.E..VGA.R..
P.c.  ETNK.V.......M......AL...F.......GQ..E.I.G..G...Y.E..VN.....

TyDC5 EIVVPNTFAMVCFRLKPA---AIF-NGKLGENGVDYNC-IEEKTNE--INSKL----LES
TyDC1 .....R.............---...-RK.IV.D----DH-..AQ.....V.A..----...
TyDC2 ..T..R.........L.PKTIKVY-DNGVHQ..NGVVP-LRDENENLVLAN..NQVY..T
TyDC3 ..T..R.........L.PTTVKVCGENGVHQ..NGVIAVLRNENE.LVLAN..NQVY.RQ
P.c.  .V.A.RL.S.....I..--------SAMI.K.D-------.DEV......R..----...

TyDC5 VNASGSIYMTHAVVGGVYMIRFAVGATLTEERHVSMAWKVIQEHTDAIL-GTVDD-SVVA
TyDC1 .....K..........................TG....V........-.ALG.-D.C-
TyDC2 ...T..V.................S........IY...IL...A.L..-.K.S.ADFSS
TyDC3 .K.T..V.................S........IH..E.L...A.L..-SK...ANFSS
P.c.  ..D..R..VS.T.L..I.V....I.G...DIN...A....L.D.AG.L.DD..TSNKL.E

TyDC5 ---   523
TyDC1 ---   518
TyDC2 ---   531
TyDC3 ---   359
P.c.  VLS   514
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Figure 1. Restriction map of *TyDC5* and amino acid sequence comparison of *TyDC* genes. A, *TyDC5* coding region is represented by the filled box. The arrowhead indicates transcription start site. The *Sal*I site labeled *Sal I is from the EMBL3 vector. B, Comparison of deduced amino acid sequence of *TyDC5* with sequences from three other opium poppy *TyDC* genes (*TyDC1*, *TyDC2*, and *TyDC3*) (Facchini and De Luca, 1994) and a parsley *TyDC2* gene (P.c.) (Kawalleck et al., 1993). Dots denote residues identical to *TyDC5*. Dashes indicate spaces introduced to maximize alignment.

Substrate Specificity of the *TyDC5* Gene Product

The coding region of the *TyDC5* gene was isolated by PCR and cloned into the modified vector p6HTrc99A for expression in *E. coli*. Under nondenaturing conditions the His-tagged *TyDC5* protein did not efficiently bind to the

Ni-nitrilotriacetic acid resin, and thus this affinity system could not be used to purify nondenatured, active enzyme. Nevertheless, the tagged protein was highly active in whole-cell lysates, which were used to determine its substrate specificity.

Activity of the recombinant *TyDC5* enzyme was highest against the Tyr substrate (Table I). The relative rate of DOPA decarboxylation was 64% of the TyDC activity, whereas the *TyDC5* enzyme showed a measurable but extremely low activity against Phe. No activity was detected against Trp. Isopropyl-β-D-thiogalactopyranoside-induced bacterial extracts containing the p6HTrc99A alone showed no activity against any of the radiolabeled substrates.

Tissue Differences in Expression of *TyDC5* in Opium Poppy

Tissue-specific expression of the *TyDC5* gene was examined by the RPA. To ensure accurate quantitation, a 28S ribosomal RNA probe was used as an internal standard. Results from the RPA indicate that the *TyDC5* gene is expressed in the roots of mature poppy plants and is virtually undetectable in any other tissues (Table II).

Expression of the *TyDC5* gene during germination of opium poppy seedlings was also analyzed by the RPA (Table II). In duplicate experiments a transient induction of *TyDC5* gene expression was observed at d 4 PI, which corresponds to the beginning of the emergence of the radicle from the seed coat. *TyDC5* mRNA levels then returned to their original levels by d 6 PI and gradually increased during the development of the seedling root system.

Expression of *TyDC5::GUS* in Transgenic Tobacco

Regulation of the *TyDC5* gene was studied in transgenic tobacco using a 2060-bp promoter fragment transcriptionally fused to the *GUS* reporter gene. Table III shows the quantitative pattern of GUS expression in plants containing the *TyDC5* promoter-*GUS* fusion as measured by fluorometric assays. As in the opium poppy RPA analysis, expression of the *TyDC5::GUS* gene construct was restricted to the roots of soil-grown plants.

The GUS staining patterns of germinating *TyDC5::GUS* seeds also paralleled the RPA results obtained from developing opium poppy seedlings. No GUS staining was observed during the first day of imbibition. At d 2 to 3 PI, dark-blue staining was observed at the site of radicle emergence (Fig. 2A). Initially, young seedlings (4–5 d PI) were uniformly stained; however, GUS staining became progressively localized to the cotyledons and elongating hypocotyls as the seedlings grew (Fig. 2B). Once the cotyledons were green and fully expanded, GUS expression was limited to the roots, primarily in the region of elongation (Fig.

Table I. *Specific decarboxylase activities and relative conversion rates of different ADCs by whole-cell isopropyl-β-D-thiogalactopyranoside-induced E. coli expressing opium poppy TyDC5*

These results represent the means of two independent experiments with four replicates per experiment.

Substrate	Specific Activity	Relative Conversion Rate
	µkat/kg protein	%
Tyr	6.50 ± 0.24	100
DOPA	4.16 ± 0.51	64
Phe	0.103 ± 0.047	1.6
Trp[a]	0.0	0

[a] No TDC activity was observed by TLC analysis.

Table II. *Expression of TyDC5 in opium poppy tissues and during poppy seedling development*

Relative levels of RNase-protected *TyDC5* transcript are expressed as a percentage of protected message in root (100%). Relative levels of RNase-protected *TyDC5* transcript are expressed as a percentage of protected message at d 14 PI (100%). This value represents approximately 6% of the message expressed in root tissues. The same patterns were observed in two independent RPA experiments.

Tissue/Days	Percent of mRNA Expression
Tissue	
Root	100
Stem	2.3
Leaf	0.3
Sepal	1.0
Stamen	1.2
Petal	0.9
Capsule	1.2
Latex	1.7
Cell-suspension cultures	2.7
Days PI	
0	11.9
2	17.1
4	73.9
6	18.9
10	45.8
14	100

Table III. *Quantitative TyDC5::GUS activity in transgenic tobacco*

Tissues analyzed by fluorometric GUS assays. Young root, First 3 cm from the root apex; Old root, root tissue above young root; Young stem, first 10 cm from the shoot apex; Mature stem, stem tissue below young stem; Mature leaves, 10 to 14 cm long; Middle leaves, 3 to 6 cm long; Young leaves, 1.5 to 3 cm long; Apex, 0.5 to 1 cm of vegetative shoot meristem; Flower bud, stages 1 to 3 (Koltunow et al., 1990); Open flower, stage 6 (Koltunow et al., 1990). Results represent the average of three replicates from one transformant. Three independent transformants were analyzed and each showed the same relative levels of tissue-specific GUS activity. MUG, Methylumbelliferyl-β-D-glucuronide.

Tissue	nmol MUG min^{-1} mg^{-1} protein
Young root	1,249.80 ± 176.47
Old root	703.50 ± 234.81
Young stem	3.51 ± 0.46
Mature stem	5.88 ± 1.05
Mature leaves	0.20 ± 0.13
Middle leaves	0.58 ± 0.13
Young leaves	1.21 ± 0.26
Apex	3.24 ± 1.09
Flower bud	24.33 ± 11.29
Open flower	2.03 ± 0.65

Figure 2. 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide acid cyclohexylammonium salt localization of GUS activity in *TyDC5*::*GUS* transgenic tobacco. A, Transgenic tobacco seeds at 2 d PI showing GUS activity at the point of radicle emergence. B, Transgenic tobacco seedlings of various ages showing uniform GUS staining early in development and staining restricted to the cotyledons and hypocotyls in older seedlings. C, Seven-day-old seedling (10 d PI) with GUS activity stain localized to the region of elongation in the primary root. D, Seedling root with GUS staining in the epidermis and cortex of the elongation zone, but absent from the root cap and root apical meristem. E, Older roots showing GUS staining in the vascular tissue.

2C). GUS staining patterns in tissues of the root were not consistent. Staining appeared in the epidermis and cortex (Fig. 2D), but occasionally concentrated in the vascular tissue (Fig. 2E). Staining was never observed in the root apical meristem or in the root cap. Similar histochemical results were obtained from 20 independent transformants.

DISCUSSION

In contrast to more general animal ADCs, plant ADCs often show significant substrate specificity. For example, TDC from *Catharanthus roseus* is highly specific for Trp (Noé et al., 1984) and DODC from *Cytisus scoparius* can only decarboxylate DOPA (Tocher and Tocher, 1972). Other plant species, such as *Syringa vulgaris*, *Thalictrum rugosum*, and *Eschscholtzia californica*, appear to have dual TyDC/DODC activities that accept both Tyr and DOPA as substrates, but not Trp or Phe (Marques and Brodelius, 1988).

Recent cloning of plant ADC genes has permitted the controlled expression of individual genes and determination of their enzyme substrate specificities without potential contamination from related isozymes with different activities. This approach has been particularly useful in analyzing the TyDC multigene families of parsley (Kawalleck et al., 1993) and opium poppy (Facchini and De Luca, 1994).

As with other TyDC genes, the opium poppy *TyDC5* gene described here lacks introns, a feature that facilitated its cloning and expression in *E. coli*. *TyDC5* recombinant protein, as well as other TyDC gene products reported before (Kawalleck et al., 1993; Facchini and De Luca, 1994), are able to decarboxylate both DOPA and Tyr. Although it is possible that the His tag in the *TyDC5* recombinant protein could modify its substrate specificity, it is unlikely due to the fact that this system has been extensively used to purify a variety of proteins, including enzymes (Döbeli et al., 1990), transcription factors (Jankench et al., 1991), and antigens (Stüber et al., 1990; Takacs and Girard, 1991) among many others, and it has not been found to interfere with the structure or function of the purified protein.

The substrate specificity of *TyDC5* is slightly different from that of *TyDC1* and *TyDC2*, two other opium poppy TyDC genes recently described (Facchini and De Luca, 1994). *TyDC1* and *TyDC2* were more active against DOPA than Tyr (90 and 65% of DODC activity, respectively), whereas the relative rate of DOPA decarboxylation for *TyDC5* was 64% of the TyDC activity. These substrate preferences may simply reflect the heterogeneity within members of the TyDC gene family in their ability to decarboxylate both Tyr and DOPA or may have some undiscovered biological significance.

The transient increase in expression of the *TyDC5* gene in germinating poppy seeds and in GUS staining seen in the emerging radicles of *TyDC5*::*GUS* tobacco seeds suggests that *TyDC5* expression might be important for providing additional alkaloids during this vulnerable stage of the life cycle. High levels of tyramine, a product of TyDC/DODC activity, have also been found in cell walls of wounded plant tissues (Borg-Olivier and Monties, 1993). After oxidative polymerization, tyramine or its derivatives may function to reinforce cell walls, making them less susceptible to penetration by pathogens. These observations, along with the fact that TyDC genes have been isolated from plants that do not produce isoquinoline alkaloids, such as *Arabidopsis thaliana* (Trezzini et al., 1993) and parsley (Kawalleck et al., 1993), indicate that TyDC genes have additional roles in plants beyond providing alkaloid precursors.

TyDC5 expression in opium poppy and the spatial distribution of GUS activity directed by the *TyDC5* promoter in the transgenic tobacco were both localized to the root. These results indicate that the developmental regulation of the *TyDC5* promoter is similar in both species.

Recently, several TyDC genes have been shown to be transcriptionally activated upon fungal infection or elicitor treatment (Kawalleck et al., 1993; Trezzini et al., 1993). It should be noted, however, that both the poppy and tobacco germination experiments were conducted under axenic conditions. Thus, the observed increases in *TyDC5* expression were not the result of elicitation by soil microbes but reflect developmental regulation. The possibility that the *TyDC5* promoter can be induced with microbial elicitors is currently under investigation in both species.

The opium poppy TyDC gene family consists of at least 10 to 14 members (Facchini and De Luca, 1994), which are likely to be organized in distinct subfamilies with divergent activities and expression patterns. The deduced amino acid sequence and root-specific expression pattern of *TyDC5* are most similar to those of *TyDC1* (Facchini and De Luca, 1994), suggesting that they are members of a common TyDC/DODC gene subfamily. The exact role of each one of the opium poppy TyDC genes identified so far remains unknown. Isolation and characterization of additional TyDC/DODC family members from opium poppy should provide important insights into the evolution and regulation of this complex gene family.

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