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Increased Putrescine Biosynthesis through Transfer of Mouse Ornithine Decarboxylase cDNA in Carrot Promotes Somatic Embryogenesis

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Increased Putrescine Biosynthesis through Transfer of Mouse Ornithine Decarboxylase cDNA in Carrot Promotes Somatic Embryogenesis
Increased Putrescine Biosynthesis through Transfer of Mouse Ornithine Decarboxylase cDNA in Carrot Promotes Somatic Embryogenesis

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Carrot (Daucus carota L.) cells were transformed with Agrobacterium tumefaciens strains containing 3'-truncated mouse ornithine decarboxylase (ODC) cDNA under the control of a cauliflower mosaic virus 35S promoter. A neomycin phosphotransferase gene linked with a nopaline synthase promoter was used to select transformed cells on kanamycin. Although the nontransformed cells contained no ODC, high amounts of mouse-specific ODC activity were observed in the transformed cells. Transgenic cells showed a significant increase in the cellular content of putrescine compared to control cells. Spermidine, however, remained unaffected. Not only did the transformed cells exhibit improved somatic embryogenesis in the auxin-free medium, they also regenerated some embryos in the presence of inhibitory concentrations of 2,4-dichlorophenoxyacetic acid. These cells acquired tolerance to dL-α-difluoromethylarginine (a potent inhibitor of arginine decarboxylase) at concentrations that inhibit growth as well as embryogenesis in nontransformed carrot cells, showing that the mouse ODC can replace the carrot arginine decarboxylase for putrescine biosynthesis in the transgenic cells.

The polyamines (putrescine, spermidine, and spermine) play an important role in a variety of metabolic and developmental processes in plants (Minocha, 1988; Evans and Malmberg, 1989; Galston and Flores, 1991; Slocum and Flores, 1991). Plants possess two parallel pathways to synthesize putrescine, i.e. via the enzymes ODC and ADC, whereas animals have only the ODC pathway. dcsSAM is the aminopropyl donor to putrescine and spermidine to produce spermidine and spermine, respectively. Although one or both of the pathways may be active in different plant tissues, their relative contribution to the biosynthesis of putrescine in higher plants is controversial (Tiburcio et al., 1990). High concentrations of polyamines are commonly observed in tissues undergoing rapid cell division, active metabolism, growth, and somatic embryogenesis (Montague et al., 1978, 1979; Heimer and Mizrahi, 1982; Feirer et al., 1984; Fienberg et al., 1984; Walker et al., 1985; Robie and Minocha, 1989; Khan and Minocha, 1991; Minocha et al., 1991; Minocha and Minocha, 1995).

Most of the functions attributed to polyamines are based on studies using inhibitors of their biosynthesis. In these studies, a partial or complete inhibition of enzyme activity is generally correlated with a physiological or a morphological response (McCann et al., 1987; Evans and Malmberg, 1989). A common problem with many studies using these inhibitors is the inability to ascertain if an observed developmental response is a direct result of reduced polyamine biosynthesis or if the reduced polyamine biosynthesis is the result of some effect of the inhibitor on development. This difficulty in establishing a cause-and-effect relationship between polyamine biosynthesis and a particular plant response has at times complicated our understanding of the role of polyamines during plant development (Evans and Malmberg, 1989). To overcome some of the limitations and ambiguities associated with the use of chemical inhibitors, the approach of transgenic expression of foreign genes to modulate the cellular content of polyamines in plants was employed in these studies. It had been demonstrated earlier that mammalian cDNAs for ODC and SAMDC, when expressed in tobacco plants under the control of plant-specific promoters, caused a significant increase in cellular putrescine and spermidine, respectively (DeScenzo and Minocha, 1993; Noh and Minocha, 1994).

The importance of polyamines during somatic embryogenesis in carrot (Daucus carota L.) has been studied by many workers (Feirer et al., 1984; Fienberg et al., 1984; Robie and Minocha, 1989; Khan and Minocha, 1991; Minocha et al., 1991; Nissen and Minocha, 1993; Minocha and Minocha, 1995). It has been established that undifferentiated carrot cells lack ODC activity and use the ADC pathway exclusively to produce putrescine (Montague et al., 1978, 1979; Robie and Minocha, 1989). The inhibition of the ADC pathway by high concentrations of DFMA causes a complete inhibition of somatic embryogenesis as well as growth. It has also been observed that DFMO, an inhibitor of ODC in animals, promotes somatic embryogenesis in carrot cell cultures (Robie and Minocha, 1989; Nissen and Minocha, 1993). This is accompanied by an increased bio-

Abbreviations: ADC, Arg decarboxylase; CaMV, cauliflower mosaic virus; dcsSAM, decarboxylated S-adenosylmethionine; DFMA, dL-α-difluoromethylarginine; DFMO, dL-α-difluoromethylornithine; NPT-II, neomycin phosphotransferase; ODC, ornithine decarboxylase; PCA, perchloric acid; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase.

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synthesis of putrescine, presumably via the promotion of the ADC pathway by some unknown mechanism. To further determine if an increased biosynthesis of putrescine plays a critical role in somatic embryogenesis in carrot cells, an approach involving the transgenic expression of mammalian ODC cDNA was employed. Results presented here demonstrate that: (a) cellular putrescine concentration can be increased through the transfer and expression of a mouse ODC cDNA, and (b) increased production of putrescine results in an increased efficiency of somatic embryogenesis in the absence of 2,4-D as well as the production of somatic embryos in the presence of otherwise inhibitory concentrations of 2,4-D in some of the transgenic cell lines.

MATERIALS AND METHODS

Culture and Transformation of Carrot Cells

Agrobacterium tumefaciens strain AT35SODC© (DeScenzo and Minocha, 1993) containing a 3‘-truncated sequence of mouse ODC cDNA under the control of the CaMV 35S promoter and a selection marker gene (NPT-II, linked with the nopaline synthase promoter) was used as a vector for transformation. The truncated ODC cDNA consisted of 69 nucleotides of the 5’ untranslated leader sequence and an open reading frame of 1273 bp. Truncation had removed, along with all the 3’ noncoding sequence, 110 bp of the 3’ coding sequence corresponding to the “PEST” amino acid sequence at the C terminus of ODC (Kahana and Nathans, 1985; Ghoda et al., 1989; DeScenzo and Minocha, 1993). The construction of plasmids and A. tumefaciens strain AT35SODC© is described by DeScenzo and Minocha (1993).

Suspension cultures of carrot (Daucus carota L.) were maintained in B5 medium (Gamborg et al., 1968) supplemented with 2% Suc and 0.45 µM 2,4-D. The cells were subcultured in fresh medium at a 1:20 dilution at weekly intervals. On alternate weeks, the cultures were passed through a 250-µm sieve before subculture. This treatment prevented the formation of large tissue clumps. Unless otherwise stated, all liquid cultures were kept on a gyratory shaker (150 rpm) under 16 h of light (80 ± 10 µmol m⁻² s⁻¹) at 25 ± 2°C. Somatic embryogenesis was initiated either on a solid (0.8% agar) or a liquid medium that was free of 2,4-D (called the embryogenic medium) using cell clumps between 100 and 250 µm in size. For somatic embryogenesis studies with both the transformed and the nontransformed cell lines, 5 mL of a low-density (100–200 clumps/mL) suspension of cells were grown in 50-mm-diameter Petri dishes. All additives in the experimental media except for 2,4-D were adjusted to pH 5.6, filter-sterilized, and added to the autoclaved medium cooled to room temperature. Embryos at different stages of development were counted under a microscope on different days. Three random fields of view were counted for cell clumps and embryos for the data in Figure 6, whereas only embryos that were 1 mm or larger in size were counted in the Petri dish after 10 d for the data in Table II. Single cells and small cell clumps (fewer than five cells) were excluded.

For transformation, 0.5-cm hypocotyl sections from in vitro-grown somatic embryos were precultured in 2,4-D-supplemented solid medium for 2 to 4 d. These explants were removed from the plates, wounded at many places using a sterile needle, and co-cultured with A. tumefaciens AT35SODC© suspended in 20 mL of sterile 14.5 mM NaCl. Acetosyringone, dissolved in DMSO, was added to the suspension to achieve a final concentration of 100 µM. After co-cultivation for 1 h, the explants were rinsed with sterile distilled water, patted dry between three to four layers of sterile 3MM Whatman filter paper, and returned to solid medium containing 2,4-D. After 24 to 48 h, the explants were transferred to fresh medium supplemented with 250 mg/L ceftaxime and 300 mg/L kanamycin and incubated for 3 weeks. The resistant calli were subcultured on solid medium with 300 mg/L kanamycin on a monthly routine. Suspension cultures of transformed cell lines were initiated by transferring approximately 1 g fresh weight of callus to 50 mL of liquid medium containing 2,4-D. The suspensions were sieved like the control cells and maintained on a weekly subculture routine in the liquid medium containing 300 mg/L kanamycin.

NPT-II Assay

The amount of NPT-II protein in transgenic cells was determined by using the NPT-II ELISA kit (5 Prime ® 3 Prime Inc., West Chester, PA) following the manufacturer’s suggested protocol. Briefly, 100 mg of cells were homogenized in a microfuge tube with a tight-fitting plastic homogenizer in 100 µL of freshly prepared extraction buffer (62.5 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 1% [v/v] β-mercaptoethanol, 0.1% [w/v] SDS) at 4°C. The homogenate was centrifuged at 4°C for 3 min at 14,000g and the supernatant fraction was used for ELISA and for determination of total protein.

ODC

Mouse ODC activity was determined by measuring the amount of 14CO² released from L-[1-14C]Orn (Robie and Minocha, 1989; DeScenzo and Minocha, 1993). One-half gram fresh weight of cells was homogenized in 1 mL of cold extraction buffer (50 mM Tris-HCl, pH 6.8 or 8.4, 0.1 mM Na₂EDTA, 0.5 mM pyridoxal 5’-phosphate, and 0.05 mM DTT) using a Brinkmann Polytron at 2 × 10⁴ rpm for 90 s. The homogenate was centrifuged at 18,000g for 30 min at 4°C, and the supernatant fraction was used for enzyme assay and quantification of total protein. The enzyme activity of mouse and plant ODC was determined at pH 6.8 and 8.4 (adjusted at 25°C), respectively. For the determination of the pH optima, both the extraction and the assay buffers were adjusted to appropriate pH1 values. Total protein was determined by the method of Bradford (1976).

Quantification of Polyamines

Cells were collected on Miracloth (Calbiochem) by vacuum filtration and transferred to 5% (v/v) PCA (200 mg fresh weight of cells in 800 µL of PCA). The PCA-soluble polyamines were extracted by freeze-thawing three times (Minocha et al., 1994) before centrifugation (14,000g, 10 min, 4°C). Heptanedianine was added as an internal stan-
dard, and the samples were stored at -70°C until ready for dansylation (2–10 d). The polyamines were dansylated and quantified by HPLC as described by Minocha et al. (1990).

**DNA Isolation and PCR**

DNA was isolated by the procedure of Thomas et al. (1989) using 5 g fresh weight of cells. After precipitation with isopropanol and a wash with 70% ethanol, the DNA was dissolved in sterile deionized water, precipitated twice as the sodium salt, and quantified using a TKO100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

PCR was carried out in a 25-μL total reaction volume containing 65 ng of genomic DNA, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 100 μM deoxynucleoside triphosphates, 0.6 μM each of the forward and the reverse sequence (NPT II or ODC) primers (Keystone Laboratories, Menlo Park, CA), and 0.125 μL of Taq polymerase (Promega). The reaction mixture was overlaid with 50 μL of autoclaved light mineral oil without stabilizer. The thermocycler was programmed for a denaturation temperature of 94°C for 2 min, an annealing temperature of 50°C (ODC) or 60°C (NPT II) for 90 s, and an extension temperature of 72°C for 2 min. The reaction was carried out for 35 cycles. An additional extension at 72°C followed for 7 min after completion of the final cycle. The NPT-II forward sequence primer was from position 201 to 220 in the NPT-I1 gene, and the reverse primer was from position 898 to 879, to yield an amplification product of approximately 700 bp (Beck et al., 1982). The ODC forward sequence primer was from position 321 to 340 in the mouse ODC cDNA, and the reverse primer was from position 860 to 841, giving a PCR product of about 540 bp (Kahana and Nathans, 1985). For PCR of virA gene products, the primers (JDH 6 and JDH 10) and the protocol suggested by Drs. Joe Don Heath and Eugene Nester (University of Washington, Seattle, personal communication) were used.

**Southern Hybridization**

Following digestion with restriction enzymes and electrophoresis, genomic DNA was vacuum transferred to GeneScreen Plus membrane (New England Nuclear, Boston, MA) and fixed by UV cross-linking. The 1.8-kb EcoRI/BamHI fragment of ODC cDNA from pU0-1 (DeScenzo and Minocha, 1993) was labeled by random-primed incorporation of digoxigenin-labeled deoxyuridine triphosphate. Southern hybridization was carried out at 60°C for 16 h using digoxigenin-labeled probe and the labeled probe was detected colorimetrically with (nitroblue tetrazolium salt) and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim). The PCR products were also transferred to the membrane and treated in the same manner for hybridization with the ODC probe.

**RNA Isolation and Northern Hybridization**

All the glassware, plasticware, and solutions used for RNA work were treated with 0.1% diethylpyrocarbonate (Sigma). Total RNA was isolated by modification of the DNA isolation method of Thomas et al. (1989). Following two extractions with chloroform:isoamyl alcohol, lithium chloride was added to the aqueous fraction at a final concentration of 0.8 M and incubated on ice for 4 to 8 h. After centrifugation at 16,000g for 30 min, the pellet was resuspended in 2.6 mL of ice-cold water and again precipitated with 0.8 M lithium chloride. Finally, the pellet was washed with 70% (v/v) ethanol, allowed to dry in air, and dissolved in 90 μL of 40 mM Tris, pH 7.9, 6 mM MgCl2, 10 mM NaCl, 0.1 mM CaCl2. RQ1 RNase-free DNase (Promega) treatment was given at a final concentration of 1 unit/μL at 37°C for 15 min. RNA was reprecipitated with 0.33 M sodium acetate and ethanol following phenol and chloroform:isoamyl alcohol extractions. RNA was collected by centrifugation at 16,000g for 30 min at 4°C and the pellet was washed with 70% ethanol, dried in air, and dissolved in 100 to 200 μL of deionized water. RNA was quantified spectrophotometrically at 260 nm.

For slot-blot hybridization of RNA, the nylon membrane was prepared by first soaking it in water and then in 5× SSC for 5 min. The membrane was placed between the blocks of the blotting apparatus (Hoefer Scientific Instruments). Twenty micrograms of total RNA sample was loaded into each well, and a vacuum of 40 mm Hg was applied. Following the transfer, the membrane was rinsed with 5× SSC and RNA was cross-linked to the membrane by UV for 2 min. The membrane was then baked at 80°C for 2 h prior to prehybridization and hybridization as described above in “Southern Hybridization.”

**RESULTS**

Nontransformed carrot cells did not grow in the presence of 300 mg/L kanamycin in the medium. After co-culture of hypocotyl segments with *A. tumefaciens* and selection on kanamycin, resistant calli were obtained in 6 to 7 weeks of culture. These calli exhibited normal growth after subculture on both solid and liquid media containing 300 mg/L kanamycin. All of the cell lines that were resistant to kanamycin showed the presence of varying amounts of NPT-II protein, whereas none of the nontransformed control lines showed the presence of this protein (Bastola, 1994; data not shown). These putative transgenic cell lines (labeled as N1, N2, N3, etc.) were further screened by PCR using oligonucleotide primers that could amplify a 540-bp fragment of the mouse ODC cDNA and a 700-bp fragment of the NPTII gene. Although PCR products for both of the genes were observed from the DNA of all of the transgenic cell lines, the nontransformed control cells did not yield any positive amplification signals (Fig. 1). Southern hybridization of the PCR amplification product for ODC showed a positive hybridization signal with the labeled probe (Fig. 2A). Similarly, the 700-bp PCR amplification product for NPT-II hybridized to the labeled NPT-II probe, confirming the presence of the NPT-II gene in the transgenic cells (data not shown).

To further confirm stable transformation of these cells, genomic DNA digested separately with EcoRI, HindIII, or XbaI was hybridized with the labeled mouse ODC probe. The control DNA showed three bands in the EcoRI digest,
the genomic DNA from plant cells using virA-specific primers. Although total *A. tumefaciens* DNA produced the expected 630-bp amplification product, DNA from neither nontransformed cells nor from any of the transgenic cell lines produced such a signal (data not shown).

The northern slot-blot analysis showed no RNA signal in the nontransformed cell lines. The transgenic ODC cell lines that contained mouse ODC cDNA showed the presence of RNA that hybridized with the mouse cDNA probe (Fig. 3).

**ODC**

As previously reported (Feirer et al., 1984; Robie and Minocha, 1989), little ODC activity was observed in the nontransformed cultures grown in the presence of 2,4-D. However, all of the transgenic cell lines that tested positive by PCR amplification of mouse ODC cDNA contained substantial amounts of ODC activity (Fig. 4). With the exception of one cell line, N1, that lost most of the ODC activity after about 1 year of subculture, all others have

Figure 1. Agarose gel electrophoresis of PCR products obtained by using ODC primers (A and B) and NPT-II primers (C) with the genomic DNA from different sources. M, Molecular size markers; P1, mouse ODC cDNA in pUC19; P2, AT35SODC; P3, PBI121; N, nontransformed cells; T, transgenic cell lines. Lanes from left to right are: in A, M, P1, N, N1, N2, N10, N11, N12, N14, and N16; in B, M, P1, P2, N10, N11, N12, N19, N20, N23, N25, N26, N28, N9, N13, N, N21, N24, N1, N2, and N3; and in C, M, N, N13, N3, P1, N1, N2, P2, N10, N15, N25, and N26.

all of which were larger than 2.1 kb (Fig. 2B). The same bands were also present in all of the transgenic cell lines. However, only the transgenic cell lines showed an additional 2.1-kb band from mouse ODC, as expected, with EcoRI digestion. A HindIII digest of plant DNA transformed with AT35SODC would be expected to show a DNA fragment of approximately 0.8 kb in addition to a large fragment of variable size depending on the site of insertion of the T-DNA in the plant genome. As expected, all of the putative transformants that were examined showed the 0.8-kb internal HindIII fragment (Fig. 2C). Additional fragments of variable length but all larger than 1.3 kb represented the ODC cDNA adjacent to the right border and a part of the plant DNA.

The fact that the transgenic cultures did not show growth of *A. tumefaciens* in either liquid or solid medium during the 2-year period of subculture (up to 4 weeks of growth each time) indicates that the cultures did not harbor live bacteria. The B5 medium used for subculture is adequate to support the growth of these bacteria. The absence of live or dead bacteria was further confirmed by performing a PCR amplification reaction with total *A. tumefaciens* DNA and the genomic DNA from plant cells using virA-specific primers. Although total *A. tumefaciens* DNA produced the expected 630-bp amplification product, DNA from neither nontransformed cells nor from any of the transgenic cell lines produced such a signal (data not shown).

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Figure 2. Southern hybridization with labeled ODC cDNA probe of PCR products in Figure 1A (A), genomic DNA digested with EcoRI (B), and genomic DNA digested with HindIII (C). P1, Mouse ODC cDNA in pUC19; P2, 1.8-kb mouse ODC DNA fragment; N, nontransformed cells; T, transgenic cell lines. Lanes from left to right are: in A, the same as in Figure 1A; in B, P2, N, N1, N10, N14, N12, and N19; and in C, P2, N, N, N10, N12, N14, N19, and N26 greenhouse-grown plant.
maintained high levels of ODC for more than 2 years. The enzyme activity varied in these cell lines when tested at different times during the 2-year period, yet it was consistently higher in some of the cell lines (e.g. N14) than others on any given day.

Although the mere presence of high ODC activity in transgenic carrot cell cultures indicates transcription and translation of the mouse ODC cDNA, the enzyme was further characterized in several ways and found to be mouse ODC and not plant ODC. It is known that the mouse enzyme differs from the native plant ODC (from leaf tissue) in at least three important characteristics: (a) the optimum pH for mouse ODC is around 7, whereas carrot ODC has a pH optimum of 8.2 to 8.4; (b) the mouse ODC activity is extremely sensitive to DFMO, whereas the carrot ODC is not; and, finally, (c) the mouse ODC activity can be inhibited by treatment with a specific anti-mouse ODC antiserum. The enzyme activity in transgenic cells was maximum in the pH range 6.8 to 7.2, and it decreased substantially at pH 8.2 (data not presented). ODC in transgenic cells also exhibited sensitivity to the suicide inhibitor DFMO (Table I). In all cases, the enzyme activity was inhibited by 83 to 100% by treatment with 2.00 mM DFMO. Likewise, the activity of ODC was strongly inhibited by anti-mouse ODC antiserum (obtained from Dr. Lo Persson, University of Lund, Sweden) in all of the transgenic cell lines tested (data not presented).

**Polyamines**

All transformed cell lines showing high ODC enzyme activity also exhibited increased amounts of cellular putrescine (Fig. 5A). A 10- to 20-fold increase in putrescine was often observed in different transgenic lines compared to the nontransformed control cells. Although putrescine increased significantly, the amounts of spermidine did not exhibit consistent or significant elevations, and spermine tended to be lower compared to that in the nontransformed cells (Fig. 5, B and C). Total polyamines were substantially higher in all of the transgenic cell lines than those in the control cells, primarily due to increased putrescine. Cell lines that maintained a higher ODC activity also had the highest putrescine concentrations as determined on several occasions over the 2 years of culture. Several transgenic lines that showed high ODC activity and higher amounts of putrescine also showed the presence of an additional dansylamine that eluted immediately after putrescine in the HPLC chromatograms. This peak has been tentatively identified as cadaverine based on its elution time and internal spiking in HPLC. This peak was never observed in the nontransformed cells.

**Somatic Embryogenesis**

Nontransformed cells always remained undifferentiated in the presence of 0.45 to 2 μM 2,4-D. In the absence of 2,4-D, these cells differentiated into somatic embryos within 7 to 14 d. Although a few globular-stage embryos were visible by d 3, no torpedo-stage embryos were seen until 5 to 7 d of culture in the auxin-free medium. Most of the transgenic cell lines that exhibited higher ODC activity and increased cellular putrescine contained large numbers of globular- as well as torpedo-stage somatic embryos as early as 3 to 4 d after transfer to 2,4-D-free medium (Fig. 6). On any given day, transgenic cell lines generally showed significantly higher numbers of somatic embryos than the control cells. Figure 7 shows that the ODC activity (growing in 2,4-D-free B5 medium for 3 d) maintained high levels of ODC for more than 2 years. The enzyme activity varied in these cell lines when tested at different times during the 2-year period, yet it was consistently higher in some of the cell lines (e.g. N14) than others on any given day.

**Table I. Effect of 2.0 mm DFMO on ODC enzyme activity at pH 6.8 in different cell lines of carrot growing in 2,4-D-free B5 medium for 3 d**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>ODC Specific Activity (nmol CO₂ h⁻¹ mg⁻¹ protein)</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N1</td>
<td>139.76 ± 3.48</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>N9</td>
<td>13.39 ± 0.15</td>
<td>2.25 ± 0.15</td>
</tr>
<tr>
<td>N10</td>
<td>49.28 ± 0.43</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>N14</td>
<td>66.85 ± 4.19</td>
<td>1.72 ± 0.09</td>
</tr>
<tr>
<td>N19</td>
<td>54.22 ± 0.78</td>
<td>2.65 ± 0.21</td>
</tr>
<tr>
<td>N20</td>
<td>222.99 ± 2.61</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>N22</td>
<td>119.28 ± 0.81</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>N26</td>
<td>63.29 ± 0.15</td>
<td>-0.04</td>
</tr>
</tbody>
</table>

No activity was detected at pH 8.4. Specific activity of ODC is defined as nmol ¹⁴CO₂ h⁻¹ mg⁻¹ protein. Values are means ± SE of three replicates.
Morphologically, the embryos produced from transgenic lines had a relatively longer root and were much further developed than nontransformed embryos on any given day.

Some of the transgenic cell lines showing high amounts of ODC activity and putrescine produced somatic embryos even in the presence of 0.45 μM 2,4-D when grown on a solid medium (Fig. 7A). Normal development of fully mature embryos in the presence of 2,4-D was not observed in liquid cultures; however, numerous torpedo-stage embryos were routinely seen (Fig. 7B). This is a highly unusual observation and represents a unique case of somatic embryo development in the presence of such an inhibitory concentration of 2,4-D. The effects of DFMA (a potent inhibitor of putrescine biosynthesis via ADC) on somatic embryogenesis were examined for both the transformed and the nontransformed cells to determine if the alternative pathway for putrescine biosynthesis (i.e. via the mouse ODC) was sufficient by itself to support growth and morphogenesis in transgenic cells. The inhibitor was added at the time of transfer of the cell suspension to the Petri dishes at low density. As shown in Figure 7C and Table II, addition of 1 mM DFMA caused a complete inhibition of somatic embryogenesis as well as of growth of nontransformed cells. Microscopic observation showed no organized embryo-like structures after 10 to 12 d. This effect of DFMA was partially reversed by the presence of 2.5 mM exogenous putrescine in the medium (data not presented). Transgenic cells in the presence of DFMA (Fig. 7D) showed an abundance of organized early-stage embryos that later developed into complete embryos similar to those observed in the absence of the inhibitor (Fig. 7D; Table II). Thus, in contrast to the control cells, which depend entirely on the ADC pathway for putrescine biosynthesis and are sensitive to DFMA, the transgenic cell lines exhibited a high degree of tolerance to otherwise toxic levels of DFMA.

**DISCUSSION**

The diamine putrescine can be increased in the cells by (a) an increase in its biosynthesis through either the ADC or the ODC pathway, (b) a decrease in its catabolism through oxidation, or (c) its decreased utilization for spermidine biosynthesis. Previously, an increase in putrescine by treatment with DFMO in carrot cells was reported (Robie and Minocha, 1989). The results presented here

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**Figure 5.** Cellular polyamine levels in nontransformed control (N) and different transgenic cell lines (N1, N2, N10, etc.) grown in the absence of 2,4-D for 3 d. The polyamines were extracted in 5% PCA, dansylated, and separated in an acetonitrile/heptanesulfonic acid gradient by HPLC. Values are means ± se of three replicates. A, Putrescine; B, spermidine; C, spermine.

**Figure 6.** Number of globular- and torpedo-stage embryos in control (N) and the transgenic line N14 at different times after transfer of cells to 2,4-D-free medium. The cells were grown in Petri dishes in liquid medium. Values are means of three fields of view under the microscope; all cell clumps with more than five cells were counted.

**Table II.** Effect of 1.0 mM DFMA on somatic embryogenesis in nontransformed (N) and several transgenic cell lines

About 500 to 1000 cell clumps ranging in size from 100 to 250 μm were grown in Petri dishes in the absence of 2,4-D for 10 d. Only embryos equal to or larger than 1 mm were counted. Values are means ± se of three replicates.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Number of Embryos/Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−DFMA</td>
</tr>
<tr>
<td>N</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>N1</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>N10</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>N12</td>
<td>110 ± 2</td>
</tr>
<tr>
<td>N14</td>
<td>70 ± 1</td>
</tr>
</tbody>
</table>
Increased Putrescine Biosynthesis Promotes Embryogenesis

Figure 7. A and B, Somatic embryos produced in transgenic cells in the presence of 2,4-D in solid medium (A) and liquid medium (B). C, Three-week-old cultures of nontransformed control cells in the absence (left) or presence (right) of 1 mM DFMA. D, Three-week-old cultures of transformed cells in the absence (left) or presence (right) of 1 mM DFMA. The cell clumps ranged in size from 100 to 250 μm, and approximately 120 cell clumps/mL were grown in 2,4-D-free medium. The left half of D contains about half of the total embryos produced.

demonstrate that the approach of modulating putrescine biosynthesis through gene transfer is a practical alternative in a situation in which polyamine-overproducing mutants are lacking. This approach alleviates ambiguities associated with the interpretation of data from the compounding effects of chemical inhibitors and other treatments that are not highly specific and may directly affect growth and development.

Although the EcoRI digests in the Southern blot indicated some homology of nucleotide sequence between the mouse ODC and the control plant DNA, the absence of an amplification signal in PCR with control plant DNA and the absence of a hybridization signal in HindIII-digested genomic DNA make it difficult to identify the homologous segment found in the control plant DNA. Similar bands of homologous DNA from nontransformed tobacco were observed by DeScenzo and Minocha (1993). However, as with carrot, no RNA signal was seen in the nontransformed plants by northern blotting. Therefore, it is unlikely that these bands correspond to active ODC gene(s) in plants.

Although higher amounts of ODC activity were always observed in the transgenic cell lines compared to the nontransformed controls, the enzyme activity varied in different transgenic lines on different days. Such variations in transgene activity regulated by the CaMV 35S promoter are quite common (Nagata et al., 1987; Hamill et al., 1990; Fecker et al., 1993; Stefanov et al., 1994). Variations in ODC activity in different transgenic lines could be due to differences in the site of integration of the foreign gene in the plant genome and in its copy number. The variation in the activity of ODC and putrescine on different days of culture in the same transgenic cell line complicates the specific correlation of the number of somatic embryos present on a given day with the absolute amounts of putrescine. However, cell lines (e.g. N14) that consistently showed high ODC activity and high putrescine always exhibited a high degree of somatic embryogenesis in any experiment compared to those cell lines that showed relatively lower amounts of ODC and putrescine.

Earlier studies have clearly demonstrated the requirement for increased biosynthesis of polyamines and the importance of the ADC pathway during somatic embryogenesis in carrot cell cultures (Montague et al., 1979; Feirer et al., 1984; Robie and Minocha, 1989; Minocha and
A decrease in cellular putrescine by treatment with DFMA is often accompanied by loss of somatic embryogenesis. By contrast, an increase in putrescine in response to DFMO treatment was positively correlated with increased somatic embryogenesis (Robie and Minocha, 1989; Nissen and Minocha, 1993). The potential ambiguity due to opposite double effects of DFMO precludes its use as an inhibitor of putrescine biosynthesis in these cells. However, the fact that exogenously supplied putrescine does not substitute for its increased endogenous production (achieved either by DFMO treatment or by the expression of mouse ODC) further suggests that the turnover of putrescine and/or Orn may be more critical for somatic embryogenesis than the mere presence of high putrescine in the cells. This may in turn affect the overall metabolism of reduced nitrogen in the cells (Altman and Levin, 1993).

The results presented here demonstrate that despite ADC being the primary enzyme responsible for putrescine biosynthesis in undifferentiated carrot cells, production of mouse ODC enzyme provides an effective alternative biosynthetic pathway in the transgenic cells. Consequently, the cells acquire a high degree of tolerance to toxic concentrations of DFMA. Although it may seem logical to test the role of mouse ODC in the promotion of somatic embryogenesis in the transgenic cells by treatment with DFMO (which should effectively inhibit the mouse enzyme), the data from such experiments would be difficult to interpret.

The reason for this is that DFMO treatment itself promotes putrescine biosynthesis as well as somatic embryogenesis in nontransformed carrot cells (Robie and Minocha, 1989; Nissen and Minocha, 1993). This potential ambiguity due to opposite double effects of DFMO precludes its use as an inhibitor of putrescine biosynthesis in these cells.

It should be pointed out that the number of somatic embryos reported here in control cell lines (Table II) is lower than that previously reported (Nissen and Minocha, 1993). In the previous studies, 0.5 mm or larger embryos were counted, whereas in the present study only embryos equal to or larger than 1 mm were counted.

It is apparent from the results that increased putrescine in transgenic cells does not result in increased spermidine or spermine. This is consistent with the results from transgenic tobacco, where spermidine and spermine also did not increase (DeScenzo and Minocha, 1993). This may be due to the fact that spermidine biosynthesis requires not only putrescine but also (a) deSAM, which is produced by SAMDC, and (b) the enzymes spermidine synthase and spermine synthase. As reported by DeScenzo and Minocha (1993) in transformed tobacco, transgenic carrot cells also produce large quantities of cadaverine. This is probably due to a reduction in the cellular Orn and the use of Lys as an alternate substrate by the mouse ODC (Pegg, 1989). The question of why the nontransformed undifferentiated carrot cells favor the ADC over the ODC pathway for putrescine biosynthesis still remains unanswered, as does the ultimate fate of putrescine.

In conclusion, data presented here demonstrate that: (a) the mouse ODC cDNA is stably integrated in the carrot cell genome, (b) it is expressed and produces an active mouse-specific ODC enzyme, (c) the increased enzyme activity results in increased cellular putrescine, and (d) the transgenic cell lines show a high degree of somatic embryogenesis. It is not known at present how the regulation of the native plant ODC occurs or what effects, if any, the expression of the mouse ODC has on the expression of plant ODC in whole plants. It is also premature to speculate on the mechanism(s) by which increased production of putrescine causes the promotion in somatic embryogenesis in transgenic cells. Ongoing research using radiolabeled substrates (Arg, Orn, Met, and putrescine) to analyze the rates of metabolism of polyamines and the cellular localization of polyamines in carrot cells will address many of these questions.

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