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Genetic engineering of the polyamine biosynthetic pathway and somatic embryogenesis in carrot (Daucus carota L)

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GENETIC ENGINEERING OF THE POLYAMINE BIOSYNTHETIC PATHWAY AND SOMATIC EMBRYOGENESIS IN CARROT (*DAUCUS CAROTA* L.)

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DEDICATION

Dedicated to all the parents with disabled children

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ABBREVIATIONS

ABA= abscisic acid; ACC= 1-aminocyclopropane carboxylic acid ; ADC= arginine decarboxylase; APCHA= aurintricarboxylic acid [N-(3-aminopropyl)cyclohexylamine]; AT35SODC^{t}= *Agrobacterium tumefaciens* strain containing a 3'-truncated sequence of mouse ODC cDNA; AT35S-SAM= Agrobacterium strain containing a human SAMDC cDNA; CaMV= cauliflower mosaic virus; CHAP= cyclohexylamine phosphate; DAO= diamine oxidase; DEPC= diethylpyrocarbonate; DFMA= α-difluoromethylarginine; DFMO= α-difluoromethyl-ornithine; DTT= dithiothreitol; GABA= γ-amino butyric acid ; MCHA= Trans-4-methylcyclohexylamine; MGBG= methylglyoxal bis(guanylhydrazone); NBT= nitroblue tetrazolium; NPT II= neomycin phosphotransferase II, ODC= ornithine decarboxylase; PAO= polyamine oxidase; PCA= perchloric acid; PEMS= proembryogenic masses; PVP= polyvinylpyrrolidone; SA/AP= streptavidin-conjugated alkaline phosphatase; SAM= S-adenosylemethionine; SAMDC= S-adenosylemethionine decarboxylase; IAA= Indole-3-acetic acid.

ABSTRACT

GENETIC ENGINEERING OF THE POLYAMINE BIOSYNTHETIC PATHWAY AND SOMATIC EMBRYOGENESIS IN CARROT (*DAUCUS CAROTA* L.)

BY

Dhundy R. Bastola
University of New Hampshire, December, 1994

Ornithine decarboxylase (ODC), arginine decarboxylase (ADC), and S-adenosylmethionine decarboxylase (SAMDC) are three of the key regulatory enzymes involved in the biosynthesis of polyamines (putrescine, spermidine and spermine). To modulate the biosynthesis of putrescine, carrot (*Daucus carota* L.) cells were transformed with *Agrobacterium tumefaciens* strains containing 3'-truncated mouse ornithine decarboxylase (ODC) cDNA under the control of a CaMV 35S promoter. Neomycin phosphotransferase gene linked with nopaline synthase promoter was used to select transformed cell lines on kanamycin. While the non-transformed cells contained no ODC, high levels of ODC activity were observed in the transformed cells. A significant increase in the cellular levels of putrescine in transgenic cells as compared to control cells was observed.

Spermidine levels, however, remained unaffected. Not only did the transformed cells exhibit improved somatic embryogenesis in the auxin-free medium, they also regenerated embryos in the presence of inhibitory levels of 2,4-D. These cells acquired tolerance to α-difluoromethylarginine (a potent inhibitor of arginine decarboxylase) at concentrations that inhibit growth as well as embryogenesis in non-transformed carrot cells.

Transformation of carrot cells with a human SAMDC cDNA lead to increased production of SAMDC enzyme. This increase in the biosynthesis of SAMDC translated to an increase in the cellular levels of spermidine and a decrease of putrescine. The transgenic cells were highly embryogenic and also tolerant to low levels of methylglyoxal bis(guanylhydrazone).

INTRODUCTION

Somatic Embryogenesis

In most animals, cells that are destined for sexual reproduction (called germline cells) are set aside early in development into specialized tissues distinct from somatic cells. While the germline cells produce gametes that after fertilization develop into whole organisms, neither the germline cells nor the somatic cells have the capacity to directly develop into a complete organism. In contrast, most somatic plant cells demonstrate totipotency, i.e. the ability to develop directly into adult plants.

Wound parenchyma tissue of plants commonly called callus, can be maintained more or less indefinitely on solid and/or liquid media. These calli can be forced to undergo changes which lead to the production of: (1) a unipolar structure with a shoot or a root primodium by the process of organogenesis, or (2) a bipolar structure consisting of both shoot and root axis called somatic embryo, through somatic embryogenesis[1]. The earliest reports on controlled somatic embryogenesis *in vitro* were published in 1958 with carrot

[1] Somatic embryogenesis is defined here as the appearance of at least the globular stage embryos (Schiavone and Cook, 1985).

(Reinert, 1958; Steward et al., 1958). To date, about 150 species from both angiosperms and gymnosperms have been reported to undergo somatic embryogenesis (Thorpe, 1993).

In carrot (*Daucus carota*. L), embryogenic tissue is induced in the presence of auxin and maintained as callus until auxin is removed from the medium. The callus can be successfully initiated from any part of the carrot plant, although petiole and tap-root segments are the most commonly used explants (Ammirato, 1984). Calli are also easily obtained from hypocotyl segments excised from aseptically germinated seeds. When these calli are transferred to auxin free medium and the cells diluted to a relatively low density, embryogenic cell masses develop into somatic embryos. It is believed that most of these embryos, like the zygotic embryos originate from single cells (Backs-Hüsemann and Reinert, 1970; Komamine et al., 1992). During this process, some cells undergo changes and divide, leading to the formation of clusters of small cells with dense cytoplasm. These cell masses appear to have no well-defined epidermis (Sung, 1985) and are generally termed as proembryogenic masses (PEMS). These aggregates separate from the highly vacoulated cells, continue to divide and give rise to heart shaped embryos which then produce torpedo-shaped embryos (Schianov and Cook, 1985) through localized meristematic activity and cell enlargement. These embryos subsequently develop into small germlings (also

called emblings) capable of growing into whole plants in the field.

Somatic embryogenesis in carrot is an excellent system to study early physiological and morphological events in the normal development of plants because: (1) structures similar to globular, heart and torpedo stage embryos are found in both zygotic as well as somatic embryos, and (2) unlike zygotic embryos, somatic embryos develop completely outside the maternal tissue in the presence of defined medium. Since the appearance of an organ or an embryo marks the end point of a series of developmental steps involving dedifferentiation, cellular determination and differentiation (Christianson, 1987), study of physiological events and regulated gene expression during this period may be an important gateway to understanding plant development.

Physiological Aspects of Somatic Embryogenesis

At the physiological, biochemical and structural levels, *de novo* organized development, whether organogenesis or embryogenesis, has certain features in common. Presence of a physiological gradient of materials from the medium to tissue has been suggested as a requirement for initiating organized development *in vitro* (Ross and Thorpe, 1973). Initiation of differentiation in the sub-epidermal cell layers of the surface of radiata pine (*Pinus radiata*) cotyledons in contact with the medium supports this

physiological gradient concept (Yeung et al., 1981; Villalobos et al., 1985). However, this mechanism cannot explain how individual cells become activated and induced to undergo *de novo* organogenesis. Skoog and Miller (1957) suggested that organized development arises through quantitative interactions between diverse growth factors including phytohormones and metabolites. While this concept is supported by several examples of *in vitro* organogenesis, the explanation is by no means generally applicable to somatic embryogenesis.

Auxins, cytokinins, gibberellins and abscisic acid are known to regulate plant growth and development. It has been shown that synthetic auxin, such as 2,4-D actually stimulates the accumulation of large amounts of endogenous IAA in carrot, which is suggested to maintain callus proliferation and prevent the development of somatic embryos (Michalczuk et al., 1992a). Other classes of molecules such as polyamines, lectins, flavonoids, oligosaccharides and salicylic acid (Tran Thanh Van et al., 1985; Fry, 1986; Lerouge et al., 1990; Raskin, 1992) have also been implicated in the regulation of such development. It is suggested that these chemicals affect organogenesis/ somatic embryogenesis by modifying cell wall structure and properties via induction or activation of cell wall enzymes (Gendy, 1991).

Control of organogenesis by endogenous auxin/cytokinin has been well documented through the use of disarmed Ti plasmids of *Agrobacterium tumefaciens* (Thompson and Trope, 1990). Auxin has been shown to play important roles both in the induction and subsequent morphogenesis of somatic embryos in carrot (Schiavone and Cooke, 1987; Michalczuk et al., 1992a,b). Although the role of auxin in somatic embryogenesis is complex (Zimmerman, 1993), its effect through biosynthesis of ethylene has been suggested (Minocha, 1988; Robie and Minocha, 1989; Minocha et al., 1991a).

Reduced nitrogen in the form of ammonium has been shown to be required for the initiation of embryogenesis in carrot cell cultures, since nitrate alone was insufficient (Halperin and Wetherell, 1964; Reinert and Tazawa, 1969). Among the amino acids that have been tested along with 20 mM KNO_3, alanine was found to be particularly effective. It was seen that alanine was rapidly transformed to glutamate via alanine aminotransferase and utilized as a nitrogen source (Kamada and Harada, 1984).

The importance of extracellular matrix and its role in development and differentiation is becoming clearer (Knox, 1990). Kreuger and Holst (1993) showed that carrot cells in culture secrete a characteristic set of arabinogalactan proteins (AGPs) into the medium. They also demonstrated that addition of carrot-seed AGPs significantly increased

the total number of dense and highly cytoplasmic cells in carrot cell culture. More recently, extracellular glycoproteins have been shown to be involved in somatic embryogenesis (De Jong et al., 1992; LoSchiavo et al., 1990). De Jong et al. (1992), showed that somatic embryogenesis in a temperature-sensitive carrot mutant ts 11, that did not grow past the globular stage at a relatively higher temperature, was rescued by the addition of 32 kD glycoprotein EP3 in the medium.

It therefore seems that many factors, exogenous and endogenous, which influence the physiology of cultured cells have effects on organogenesis and somatic embryogenesis.

Biochemical and Molecular Aspects of Somatic Embryogenesis

Cells undergoing somatic embryogenesis have a high energy requirement and are metabolically very active. Polymeric compounds such as starch and lipids are mobilized during the process. Street and Withers (1974) observed a decrease in starch accumulation during somatic embryo formation in carrot cells. Thorpe (1993) studied the fate of inorganic nitrogen by feeding $^{15}NH_4^+$ and NO_3^- to carrot and white spruce cells in culture. It was reported that inorganic nitrogen principally gets incorporated into glutamine and glutamate via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathways and subsequently into the ornithine cycle and the polyamines,

urea and γ-amino butyric acid (GABA) during embryo development.

Although plant embryogenesis is morphologically well described (Johri et al., 1984; Steeves and Sussex, 1989; Mayer et al., 1991), little is known about the molecular events that generate plant embryo from a single cell. Analysis of abundant proteins were initially used as molecular markers of embryogenesis (Sung and Okimoto, 1983). They showed the presence of two embryogenic proteins in somatic embryos that were not seen in the callus cells. With the mutant line ts11 where embryogenesis was inhibited at high temperature but reversed by addition of a glycoprotein, somatic embryogenesis was shown to be controlled by specific proteins whose putative functions were related to changes in cell wall properties (De Jong et al., 1992).

Twenty-one genes have been isolated and cloned from carrot somatic embryos (Zimmerman, 1993). Although not all the gene products have been characterized, a group of genes preferentially expressed in somatic embryos have been reported to have characteristics of a class of proteins called LEA proteins (Dure et al., 1981, 1989; Galau et al., 1986). These proteins are abundantly expressed late in zygotic embryogenesis in many plant species (Dure et al., 1989). The LEA genes are ABA inducible and are generally associated with desiccation tolerance. Although somatic

embryos do not have to desiccate and become dormant like the zygotic embryos in a seed, the LEA mRNAs are shown to increase significantly in somatic embryos at the heart shape stage (Choi et al., 1987; Wilde et al., 1988; Franz et al., 1989; Kiyosue et al., 1992, 1993, Wurtele et al., 1993). It has also been established that the expression pattern of a number of cloned genes are identical in somatic and zygotic embryos (Perez-Grau and Goldberg, 1989).

Various genes cloned from carrot somatic embryos with a known gene product are listed in Table 1. Some of these genes that have been shown to be preferentially expressed during somatic embryogenesis are:(1) DC59, that encodes a lipid body membrane protein called oleosin; (2) a series of DC clones isolated by Aleith and Richter (1990) that are found to encode glycine and proline rich proteins; and (3) genes that are translationally enhanced during somatic embryogenesis such as the EF1-α and ATP-2.

Much research has been focused at identifying some biochemical markers that characterize the switch from unorganized to organized growth. Nucleic acid, amino acid and protein metabolism are centrally involved in the differentiation process (Thorpe, 1993). Patel and Thorpe (1984) showed increased histochemical staining for both DNA and RNA as well as proteins in the shoot forming regions of radiata pine. It was shown that the accumulation of

Table 1. List of various genes cloned from carrot somatic embryos with a known gene product (modified from Zimmerman, 1993).

Gene	Gene product	Characteristics of the gene
DC8	LEA (Grp3) located in cytoplasm and cell wall	Found both in somatic and zygotic embryos; ABA inducible; trace amount of mRNA were also detected in callus (except the ECPs). Borkird et al. (1986), Franz et al. (1989), Hatzopoulos et al. (1990a,b), Goupil et al. (1992), Choi et al. (1987)
DC59	Oleosin	
ECP31	LEA (Grp4)	Kiyosue et al. (1992, 1993)
ECP40	LEA (Grp2)	
DC3	LEA (Grp3)	Same as above but not determined in zygotic embryos
EMB-1	LEA (Grp1)	Expression pattern in somatic embryos is analogous to the zygotic embryos (Wurtele et al., 1993)
EP-2	Lipid transfer protein	Proteins secreted by somatic embryos; Expression of EP2 not embryo specific and spatially regulated; suggestive role in the regulation of cell expansion. (De Vries et al. 1988), Sterk et al. (1991)
EP-3	Chitinase	De Jong et al. (1992)
DC2.15	Polypeptide with proline rich domain	Early-induced embryogenic genes; show resemblance to certain cell wall proteins. Aleith and Richter (1990)
DC7.1 and DC9.1	Glycine rich proteins	
EF1-α	Elongation factor	Translationally enhanced in somatic embryogenesis. Apuya and Zimmerman (1992)
ATP-2	ATPase	
The gene products of other genes such as DC49, DC5, DC13, DC1.2, DC2.26, DC3.1, DC4.2 and DC10.1 not characterized.		

macromolecules is due to synthesis and not reduced degradation (Villalobos et al., 1984a). Fujimura and Komamine (1980) showed that a high turnover rate of RNA and protein preceded DNA synthesis in carrot cells. As auxin transport inhibitors prevent polarization of the globular embryo (Schiavone, 1988; Michalczuk et al., 1992a,b; Nissen and Minocha, 1993; Liu et al., 1993), polar auxin transport is believed to play a key role during the transformation of embryos from globular to the heart stage (Liu et al., 1993). However, De Jong et al. (1993a) hold the view that in the *in vitro* formation of embryogenic plant cells, both asymmetric cell division and control of cell expansion are important mechanisms. Among the biochemical markers considered, polyamines have also been assigned an important role in embryo induction and development (Minocha, 1988; Galston and Flores, 1991; Bagni et al., 1993; Thorpe, 1993; Minocha and Minocha, 1994).

Polyamine Biosynthesis

Polyamines are aliphatic amines of low molecular weight. They are biologically ubiquitous and remain protonated at physiological pH. In the cell, polyamines can be found as (1) free molecules, (2) bound to macromolecules and cellular components through hydrogen, ionic and covalent linkages, and (3) as conjugates. Diamine putrescine and polyamines spermidine and spermine are the commonly found

free polyamines that have been studied in relation to growth and development, stress, embryogenesis and cell division. In addition to the diamine putrescine, another diamine cadaverine is less widely distributed in multicellular organisms (Hamana and Matsuzaki, 1985a).

While acetyl derivatives of polyamines are commonly found in animals, hydroxycinnamic acid amides are the most common polyamine conjugates found in plants (Tiburcio et al., 1990). Metabolic significance of these conjugates has been suggested to be (1) a storage reservoir (Tiburcio et al., 1990a); (2) means of polyamine transport (Martin-Tanguy, 1985); and (3) precursors of polyamine derived alkaloids (Tiburcio et al., 1985a).

In animals, putrescine is synthesized by decarboxylation of ornithine by ODC (Fig. 1). Ornithine is synthesized from glutamate and is an intermediate in arginine biosynthesis. It can also be produced during the catabolism of arginine by the enzyme arginase. While ODC is the only known pathway to produce putrescine in animals, plants and microorganisms have two parallel pathways which use either ODC or ADC as the biosynthetic enzymes (Slocum et al., 1984; Tabor and Tabor, 1984; Pegg, 1986) (Fig. 1). These two pathways reportedly have different tissue distribution (Reviewed in Smith, 1985; Evans and Malmberg, 1989) and differential regulation (Hiatt and Malmberg, 1988). ADC has been linked to stress responses (Flores and Galston, 1982;

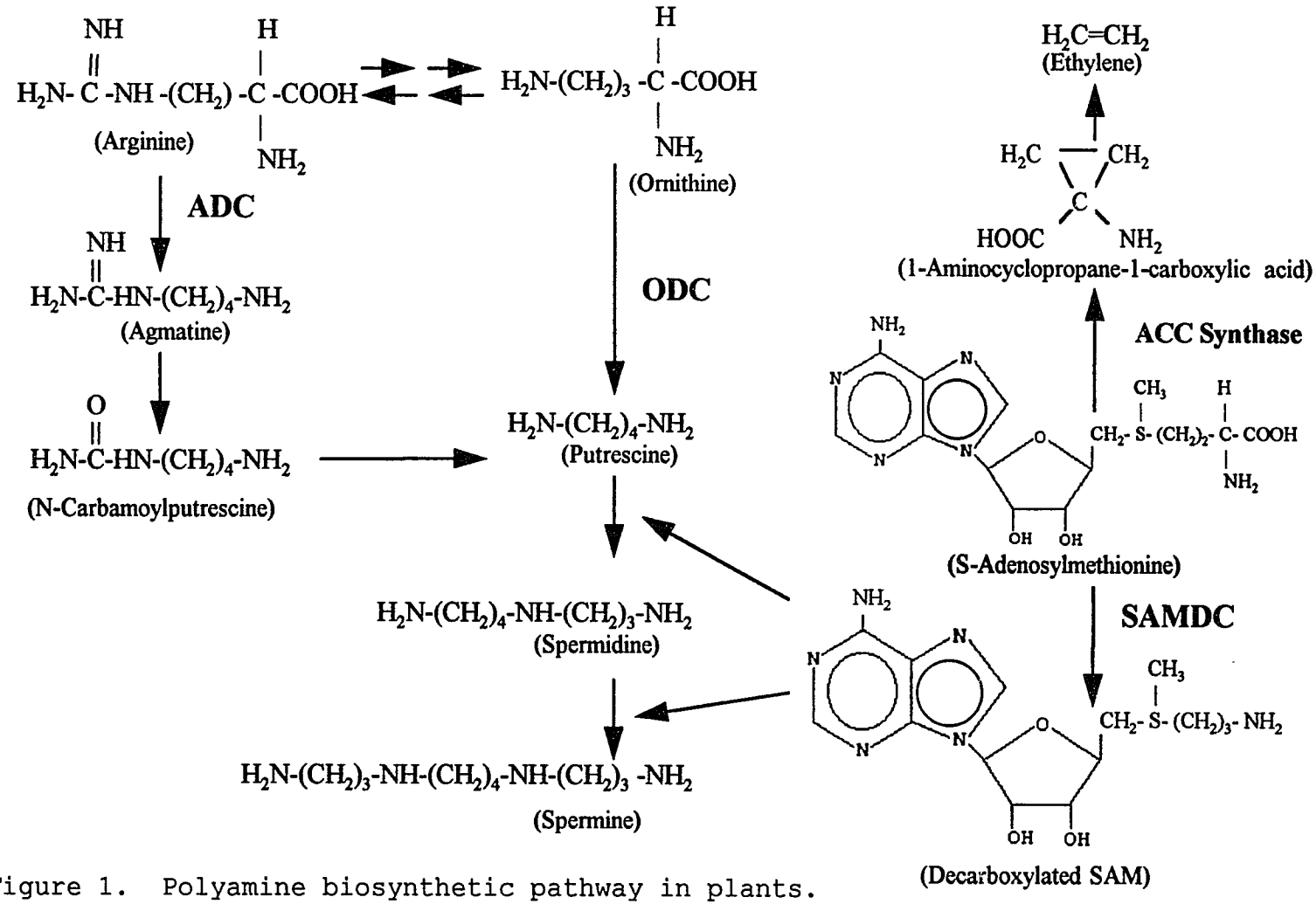

Figure 1. Polyamine biosynthetic pathway in plants.

Yong and Galston, 1983; Young and Galston, 1984), whereas ODC is very often linked to the cell cycle and rapid cell division (Heimer et al., 1979; Cohen et al., 1982a; Heimer and Mizrahi, 1982). It is controversial as to which of the two pathways contribute more to the synthesis of putrescine while both the pathways are active in different plant tissues (Tiburcio et al., 1990a). Cadaverine is synthesized from lysine by its decarboxylation using lysine decarboxylse.

Like most other organisms, plants synthesize spermidine and spermine by sequential transfer of aminopropyl groups from decarboxylated SAM to the terminal aminogroups of putrescine and spermidine, respectively (Tabor and Tabor, 1984, 1985; Pegg, 1986; Tiburcio et al., 1990a; Slocum, 1991). In addition to being a principal source of biological methylation reactions, SAM is an important intermediate in both polyamine and ethylene biosynthesis. It is a substrate for both 1-aminocyclopropane carboxylic acid (ACC) synthase and S-adenosylmethionine decarboxylase; where former leads to ethylene and the latter to polyamine biosynthesis. Once decarboxylated, SAM is committed to polyamine production. It donates aminopropyl group to putrescine and/or spermidine. However if ACC is produced, this product is finally cleaved oxidatively into ethylene, HCN and CO_2.

While no other physiologically significant reactions are known to utilize decarboxylated SAM (Pegg and McCann, 1982) other than to donate aminopropyl group to putrescine and spermidine, production of ethylene via ACC has been extensively studied. ACC synthase is a cytoplasmic enzyme. It forms ACC from SAM and is believed to be the rate-limiting enzyme in ethylene biosynthesis (Yang and Hoffman, 1984; Yip et al., 1992; Mattoo and White, 1991). It is reported to be developmentally regulated (Kushad and Dumbroff, 1991) and it can be induced by different types of stimuli (Nakagawa et al., 1991) such as chemical (auxin; Yoshii and Imaseki, 1982), physiological (Fruit ripening; Kende and Boller 1981) and physical (wounding; Kende and Boller 1981). There is evidence that the ACC synthase is regulated primarily at the transcriptional level; with post-transcriptional regulation providing an additional mechanism for its regulation. It is now known that more than one genes encode ACC synthase (Van Der Streaten et al., 1990; Hwang et al., 1991). However, it is seen that the primary structures of ACC synthases are quite similar. There are seven regions of high homology among ACC synthases from different sources and the homology is concentrated around the active site of the enzymes (Kende, 1993).

Using radiolabelling and immunological techniques it has been shown that the key enzymes ODC and SAMDC have a very short half life of 60 min or less (Pegg and Williams-

Ashman, 1981; Seely and Pegg, 1982; Pegg, 1986). In most organism, ODC is a dimer of 52 to 55 kD subunits. In animals this enzyme is found to be highly regulated in response to growth stimuli and by the polyamines themselves (Hayashi, 1989; Pegg, 1986; Tabor and Tabor, 1984; Tabor and Tabor 1985 of Williams et al., 1992). It has been argued that the regulation of ODC activity by polyamines *in vivo* includes production of a protein called an antizyme (Fong et al., 1976; Heller et al., 1976) which both inhibits ODC activity and accelerates its turnover (Murakami et al., 1985, 1992).

The rapid turnover of ODC enzyme is believed to be a means of functional control of this enzyme *in vivo* (Schimke, 1973). Changes in the rates of gene transcription and mRNA translation, and in the stabilities of mRNA and protein, have been suggested to influence expression (Manzella and Blackshear, 1990). While post-transcriptional regulation of rat ODC due the GC-rich 5′ untranslated regions (capable of forming a stable stem-loop structure) has been suggested (Grens and Scheffler, 1990; Williams et al., 1992), another school of thought suggest the control to be post-translational (Ghoda et al., 1992; Li and Coffino, 1992, Li and Coffino, 1993). As with other rapidly degraded proteins (Rogers et al., 1986), it was shown that mouse ODC contains two regions rich in PEST amino acids (single-letter code for proline, glutamate, serine, and threonine) in their carboxy

terminus (Ghoda et al., 1989). The first region started at the amino acid residues 298 to 333 and a second at amino acids residues 423-449. Recently, using chimeric ODCs containing internally located PEST regions, it was shown that the second region was largely responsible for the constitutive degradation of ODC enzyme *in vivo* (Ghoda et al., 1992). However, polyamine-mediated degradation required a distinct region outside the carboxy terminus.

The cDNA for arginine decarboxylase was isolated from *E. coli* (Moore and Boyle, 1990; GenBank accession number M31770), oat (Bell and Malmberg, 1990), and recently from tomato (Rastogi et al., 1993). The open reading frame in the oat cDNA encoded a 66 kD protein, but the purified protein had a molecular weight of 24 kD. It was therefore suggested that maturation of arginine decarboxylase in oats include processing of precursor protein. While Fleming et al. (1993) showed a tissue specific expression of a partial cDNA clone isolated from a tomato meristem library that showed homology to the oat ADC, Rastogi et al. (1993) isolated and characterized ADC gene from tomato fruit and examined its expression during ripening.

SAMDC from plants has been isolated from corn (Suzuki and Hirawawa, 1980) and Chinese cabbage (Yamanoha and Cohen, 1985) and found to be of 25-35 kD in size. This enzyme is not stimulated by putrescine but is competitively inhibited by dcSAM (Yamanoha and Cohen, 1985). Recently, using a

differential screening of a potato tuber cDNA library with probes against leaf and tuber poly(A)$^+$ RNA, two putative genes (TUB13 and TUB8) were isolated. The deduced amino acid sequence of one of the genes (TUB13) was shown to be homologous to human SAMDC gene (Taylor et al., 1992).

SAMDC is the only mammalian example of the few enzymes to contain bound pyruvate (Heby and Persson, 1990). The mammalian SAMDC is synthesized as an inactive 38 kD proenzyme containing 333-334 amino acids which is cleaved into two polypeptides of 32 and 6 kD sizes (Pajunen et al., 1988). The mammalian SAMDC also contains a PEST sequence at residue 243-269 with in the large subunit and may be responsible for a fast turn over of the enzyme (Rogers et al., 1986).

Polyamine Metabolism

Polyamines are metabolically related to the basic amino acids arginine and ornithine and therefore also to glutamic acid, a key intermediate in nitrogen metabolism (Thompson, 1980). In some plant systems polyamines can serve as the sole source of nitrogen (Bagni et al., 1978; Flores and Filner, 1985a). Once formed and protonated at cellular pH, polyamines can be N-acetylated, as in animals, or conjugated by various hydroxycinnamic acids in plants (Smith et al., 1983; Tiburcio et al., 1990). They can be ionically coupled to phosphate or other anionic groups on macromolecules or

can be covalently linked to some proteins through transglutaminase activity. In plants, putrescine and other polyamines can also be transformed into alkaloids after methylation, oxidation, and cyclization (Walton et al., 1990; Robins et al., 1991).

In addition to biosynthesis and conjugation, polyamines can also be oxidized. Diamine oxidase (DAO; EC 1.4.3.6) and polyamine oxidase (PAO; EC 1.4.3.4) catalyze oxidative deamination of putrescine and spermidine to γ-aminobutyraldehyde (which is then converted to pyrroline) and aminopropylpyrroline, respectively. Ammonia and hydrogen peroxide are common byproducts of the reaction (Santanen and Simola, 1994). Both DAO and PAO are usually found in the cell wall but there are reports in lentil tissue (Federico et al., 1985) and barley, for example, (Li and McClure, 1989) suggesting a cytosolic location of DAO and PAO. These enzymes have also been suggested to participate in lignin biosynthesis (Federico and Angelini, 1991) and auxin oxidation (Park and Park, 1987).

Inhibitors of Polyamine Biosynthesis and Metabolism

In the absence of molecular approach, such as the availability of mutants lacking the putrescine biosynthetic pathways, the only other possibility of establishing their specific role has been through the use of chemical inhibitors of polyamine biosynthetic enzymes. These

chemical inhibitors like DFMA, DFMO, MGBG, CHA, MCHA and APCHA (see Table 2 for abbreviations) are classically used to demonstrate the requirements of polyamines in normal development, metabolism and physiology.

DFMO and DFMA are two of the well-known suicide inhibitors of ODC and ADC, respectively. Five more suicide inhibitors of ADC are known (Bitonti et al., 1987). DFMA and DFMO have the α-carbon of the substrate molecule, (arginine and ornithine), substituted with a difluoromethyl group. These inhibitors bind covalently to the enzyme's active site (Metcalf et al., 1978; Kallio et al., 1981). Unlike other competitive and reversible inhibitors of ADC and ODC, DFMA and DFMO are catalytic and irreversible inhibitors. However, it has been seen that DFMO does not inhibit ODC in all plant species (Galston, 1983; Flores and Galston, 1984a,b, Slocum and Galston, 1985; Koromilas and Kyriakidis, 1988; Pandit and Ghosh, 1988; Robie and Minocha, 1989), while DFMA is always effective in inhibiting ADC *in vitro*. It has been shown that DFMA can be hydrolyzed to DFMO by arginase *in vivo*, in plants containing high arginase activity (Slocum et al., 1988; Slocum, 1991).

By using inhibitors of polyamine biosynthetic pathway, the requirement for polyamines during adventitious root formation has been confirmed (Friedman et al., 1982; Jarvis et al., 1983; Biondi et al., 1990; Altmura et al., 1991). DFMA, DFMO, and MGBG have been shown to repress root growth

Table 2. Inhibitors of enzymes involved in polyamine and ethylene biosynthesis.

	Enzyme	Inhibitor	Remarks
1.	Ornithine decarboxylase (ODC)	DL-α-difluoromethylornithine (DFMO)	Suicide inhibitor; Metcalf et al., 1978
		L-Canaline	Structural analog of ornithine; Tiburcio et al., 1990
		DL-α-Methylornithine	Competitive and reversible inhibitor
2.	Arginine decarboxylase (ADC)	DL-α-difluoromethylarginine (DFMA)	Suicide inhibitor; Kallio et al., 1981
		L-Canavaline	Structural analog of arginine; Tiburcio et al., 1990
		DL-α-Methylarginine	
		α-monofluoromethyl-dehydroarginine	More potent than DFMA
		α-monofluoromethyl-arginine	
		α-monofluoromethyl-agmatine	
		α-ethynylagmatine	
		α-allenylagmatine	
3.	S-adenosylmethionine decarboxylase (SAMDC)	Methylglyoxal bis (guanylhydrazone) (MGBG)	Competitive and reversible; Williams-Ashman and Schenone, 1972
		[(1,1'-methylethanediylidine) dinitrilo]-bis(3-aminoguanidine)	Irreversible inhibitor of mammalian SAMDC but has not been tested in plant systems. Tiburcio et al., 1990
4.	Spermidine synthase	Cyclohexylamine phosphate (CHAP) or (DCHA)	Hibasami et al., 1980a; Slocum, 1991
		Trans-4-methylcyclohexyl-amine (MCHA)	
		S-adenosyl-1,8-diamino-3-thiooctane	Coward et al., 1983
5.	Spermine synthase	5' - methylthioadenosine	Inhibits both spermidine and spermine synthase.
		5' - methylthiotubercidin and aurintricarboxylic acid	
		[N-(3-aminopropyl)cyclo-hexylamine] (APCHA)	Shirahata et al., 1993
6.	1-aminocyclopropane-1-carboxylic acid (ACC) synthase	Amino-ethoxyvinylglycine (AVG)	Yang and Hoffman, 1984

in hairy root cultures of *Hyoscyamus muticus* transformed with *Agrobacterium rhizogenes*. However, DFMO increased elongation growth and inhibited root hair formation (Biondi et al., 1993). It has been suggested that DFMO inhibited cell division and not cell expansion. Similar observations were reported earlier by Berlin and Forche (1981).

Methylglyoxal bis-(guanylhydrazone) is a potent inhibitor of SAMDC from many sources. However, varying degrees of inhibition of SAMDC from mammalian and plant sources are reported (William-Ashman and Schenone, 1972; Pegg, 1989; Slocum, 1991). It inhibits accumulation of both spermidine and spermine in carrot (Minocha et al., 1990b). While MGBG completely inhibited somatic embryogenesis, inhibition of spermidine synthase by CHAP was found to reduce cellular spermidine and only delay somatic embryogenesis (Khan and Minocha, 1991).

Cyclohexylamine is the commonly used inhibitor of spermidine synthase. Plant tissues treated with the inhibitor of spermidine/ spermine synthase (MGBG and CHA) show an increase in putrescine level (Altamura et al., 1991, Biondi et al., 1986; Minocha et al., 1991a, Khan and Minocha, 1991). This probably is not only because the conversion of putrescine to spermidine and spermine is inhibited, but also because MGBG inhibits diamine oxidase activity (Yanagisawa et al., 1981).

Polyamine Functions

Polyamines have been thought to play an important role in a variety of metabolic and developmental processes (Galston, 1983; Evans and Malmberg, 1989; Minocha, 1988; Minocha and Minocha, 1994). The exact mechanism of their involvement in the growth and development is not understood. Several reviews have described a multitude of functions of polyamines in plants (Slocum et al., 1988; Smith, 1985, 1990; Evans and Malmberg, 1989; Slocum and Flores, 1991).

Many of the biological functions of polyamines are generally related to their cationic nature at physiological pH. This cationic property enables them to bind to negatively charged phospholipid groups in membranes and, therefore, affect the stability and permeability of membranes (Srivastava and Smith, 1982). For example, exogenous polyamines stabilized protoplasts isolated from oat leaves like the divalent Ca^{+2} ions (Altman et al., 1977; Galston et al., 1978; Kaur-Sawhney et al., 1980). In the absence of polyamines or Ca^{+2}, the protoplasts senesced with a progressive loss of ability for the synthesis of DNA, RNA and proteins, and a rise in RNase. Similarly it has been reported that di- and polyamines are capable of electrostatic interactions with macromolecules such as the nucleic acids and negatively charged functional groups of enzymatic or structural proteins in the cell (Slocum et al., 1984; Tiburcio et al., 1993). It is suggested that the

charge distribution in spermidine, for example, makes it bind strongly to phosphate groups in each strand of the DNA double helix, spanning the minor and/or the major groove and stabilizing the helix by binding its two strands together (Tiburcio et al., 1993). Similarly, polyamines can stabilize other double-helical structures in rRNA and mRNA and the conformation of tRNA by binding to specific sites. Heby and Persson (1990) suggested that this ability of polyamines could be the basis for their effect on DNA, RNA and protein synthesis.

The finding that small amounts of spermine could stabilize osmotically fragile bacteria was the earliest evidence that polyamines interacted with either the cell wall or the cell membrane (reviews in Bachrach, 1973). Tabor and Tabor (1984) postulated that spermine does not act through the "osmotic mechanism" to stabilize membrane but forms complexes with acidic groups in the cell membrane thereby reducing the repulsive forces without diminishing cohesive forces. More recently, based on their immunological evidence, Besford et al. (1993) suggested that polyamines stabilize the composition of the chloroplast thylakoid membranes by: (1) direct binding to the membrane and preventing lipid peroxidation and proteolytic attack, and (2) inhibition of ethylene synthesis through inhibition of ACC synthase and conversion of ACC to ethylene (Winer and Apelbaum, 1986).

Polyamines are in general found to stimulate the synthesis of proteins of high-molecular-weight to a greater extent than the ones with low-molecular-weight (Tiburcio et al., 1993). Direct evidence that polyamines are functionally involved in protein synthesis comes from studies where polyamines were able to substitute for Mg^{+2} in the *in vitro* translation reactions. The polyamines not only substituted for the metal but also increased the rate of synthesis of the product by several fold (Tabor and Tabor, 1984; Marton and Morris, 1987).

Polyamine binding is also known to affect membrane fluidity and indirectly modulate the activities of membrane-associated enzymes (Slocum et al., 1984). Using microsomal membranes from *Phaseolus*, Roberts et al. (1986) reported that exogenously applied polyamines were able to interact with membrane lipids and thus reduce membrane fluidity. Apelbaum et al. (1981) related the inhibition of ethylene biosynthesis in apple tissue by spermidine, to decreased viscosity of membrane and its effect on the conformation of membrane bound ethylene-synthesizing complex.

Polyamines in Plant Growth and Development

Investigation into the physiology and biochemistry of polyamines in plants have made it clear that polyamines are involved in cell division (Bagni et al., 1981; Seraffini-Fracassini et al., 1980, 1984; Serafini-Fracassini, 1991;

Huhtinen et al., 1983), organ differentiation (Cohen et al., 1982a,b; Goren et al., 1982; Slocum and Galston, 1985; Tiburcio et al., 1989; Tran Thanh Van and Gendy, 1993); senescence (Kaur-Sawhney and Galston, 1979; Altman and Bachrach, 1981; Kaur-Sawhney et al., 1982a,b), stress (Flores and Galston, 1982; 1984, Flores, 1991; Young and Galston, 1983), and somatic embryogenesis (Montague et.al., 1978,1979; Feirer et al., 1984; Fienberg et al., 1984; Robie and Minocha, 1989; Minocha and Minocha, 1994). However, the mechanism of polyamine action is still unclear.

Serafini-Fracassini (1991) showed that polyamine biosynthesis in Jerusalem artichoke tuber tissue occurs during early part of cell division. In fact, in addition to their action on cell division, which is similar to that of auxins and cytokinins, polyamines share with auxins their action on the differentiation of vascular strands (Serafini-Fracassini and Alessandri, 1983).

Galston and Flores (1991) summarized the roles of polyamines in the growth of shoot apex, root formation and tuber formation in plants. Root differentiation in tobacco thin cell layers has been correlated with a peak of putrescine synthesis (Tiburcio et al., 1990). It was therefore suggested that putrescine is a good marker for root differentiation. This increase was inhibited more by DFMA than equal concentrations of DFMO (Tran Thanh Van and Gendy, 1993). This not only implies a role for putrescine

but also signifies the importance of the pathway of putrescine biosynthesis during root differentiation. Similarly, Altamura et al. (1991) also showed that *de novo* root formation from tobacco thin cell layers was affected by the inhibition of polyamine biosynthesis.

While abnormal flowering pattern in tobacco has been related to an abnormal metabolism of polyamines (Malmberg and McIndoo, 1983; Evans and Malmberg, 1989), other evidence correlating polyamine metabolism and reproductive development exists in *Spirodela punctata* (Bendeck de Cantu and Kandeler, 1989), *Zea mays* (Martin-Tanguy et al., 1982), and various members of the *Araceae* (Ponchet et al., 1982). More recently, DeScenzo and Minocha (1993) reported abnormal floral phenotype in tobacco plants transformed with mouse ODC that produced high levels of putrescine. Similar abnormal flowers were also observed by Rastogi and Sawhney (1990) in tomato that produced high levels of putrescine.

Polyamines and Ethylene

Among other effects of ethylene in growth and development such as fruit ripening, seed germination, abscission and flower senescence, a number of physiological process such as wound response (Abeles et al., 1992), and environmental stresses (Abeles et al., 1992; Lieberman, 1979; Yang and Hoffman, 1984) have been found to be regulated by this gas. Recent cloning of the genes encoding

ACC synthase (Sato and Theologis, 1989) and ethylene forming enzyme (Hamilton et al., 1991) have led to studies designed to block the endogenous ethylene biosynthesis to study the role of ethylene in development. Transgenic plants with reduced ethylene levels have been seen to be unaffected phenotypically except in the ripening of the fruit (Klee et al., 1991).

Auxin is found to stimulate ethylene biosynthesis in many tissues (Sakai and Imaseki 1971; Kang et al., 1971, Imaseki et al., 1975). Its endogenous concentration is seen to regulate the biosynthetic rate of ethylene (Kondo et al., 1975; Imaseki et al., 1975, 1989). The effect of auxin in ethylene biosynthesis has been studied in etiolated mung bean and pea seedlings. There was neither an effect on the rate of conversion of methionine to SAM nor on the conversion of ACC to ethylene. This indicated that IAA stimulates ethylene production by inducing the synthesis of ACC from SAM (Imaseki, 1983).

Like auxin, ethylene is shown to have a strong inhibitory effect on somatic embryogenesis (Roustan et al., 1989a,b, 1990). However, Nissen and Minocha (1993) showed that low levels of exogenously supplied ethylene are actually stimulatory to embryogenesis under certain conditions. The fact that SAM is the major source of methyl groups in most of the biological methylation reactions and at the same time it is a common substrate for both polyamine

and ethylene biosynthesis demands that the availability of SAM for these two pathways be properly regulated. The antisenescence properties of polyamines and their ability to directly antagonize many ethylene-mediated responses are good indicators that sharing SAM as a substrate by these two important biosynthetic pathways may have physiological significance in this regard.

It has been suggested that the dynamic equilibrium between polyamines and ethylene in cells may be regulated through the availability of SAM (Roberts et al., 1984). More recently Roustan et al. (1992) showed that in the absence of 2,4-D, higher levels of radioactivity were recovered in SAM and spermidine than in ACC or ethylene, when the cells were supplied with ^{14}C-labeled methionine. Although polyamine-mediated regulation of ethylene biosynthesis is known and has been found to affect both the formation of ACC (Evan-Chen et al., 1982; Fuirer et al., 1982) and its subsequent conversion to ethylene (Suttle, 1981; Apelbam et al., 1981), the mechanism by which ethylene inhibits SAMDC activity (Icekson et al., 1986) and hence affect the formation of dcSAM is still unknown. Many reports are available showing an increase in polyamine levels by the inhibitors of ethylene biosynthesis (Park and Lee, 1990; Samuelsen, 1990), and also conversely, increasing the biosynthesis of ethylene by the inhibitors of polyamine biosynthesis such as MGBG, DFMA, DFMO, and CHAP (Lee and

Park, 1988; Park and Lee, 1990; Samuelsen, 1990). Furthermore, it has been shown that exposing the apical meristems of pea plants to ethylene decreased the levels of ADC and SAMDC activity (Apelbaum et al., 1985, Icekson et al., 1985, 1986).

Polyamines and Somatic Embryogenesis

Polyamine biosynthesis during organogenesis has been studied in a number of plants (Slocum et al., 1984; Kaur-Sawhney et el., 1985, Tiburcio et al., 1987; Torrigiani et al., 1987, Leshem et al., 1991; Bagni et al., 1993). The importance of polyamines during somatic embryogenesis has been primarily studied in carrot (Montague et al., 1978, 1979; Feirer et al., 1984; Fienberg et al., 1984; Robie and Minocha, 1989; Khan and Minocha, 1991; Minocha et al., 1991a). A few studies showing the relationship between polyamine metabolism and somatic embryogenesis have also used celery (Denin et al., 1993), mango (Litz and Schaffer, 1987), clover (Meijer and Simonds, 1988), eggplant (Fobert and Webb, 1988) and more recently grape (Faure et al., 1991), Norway spruce (Santanen and Simola, 1992), rubber (El Hadrami and D'Auzac, 1992), rice (Koetje et al., 1993), and maize (Santos et al., 1993).

The role of polyamines in somatic embryogenesis has been demonstrated by correlating the activities of key enzymes in the polyamine biosynthetic pathway and the

cellular levels of free polyamines (putrescine, spermidine and spermine) with somatic embryogenesis. In most cases, a positive correlation is observed. Still the data obtained so far seem inadequate to answer the question "How do polyamines function in plant growth and development?" One of the main reasons could be the ubiquitous nature of polyamines in plant tissues and a tight link that exists between growth and morphogenesis (Galston and Flores, 1991).

Montague et al. (1978) reported an increase in the cellular levels of three major polyamines when carrot cells were transferred to embryogenic medium (i.e. absence of auxin). Later Fienberg et al. (1984) showed that the enzymes for putrescine and spermidine biosynthesis generally increased under such embryogenic conditions. Koetje et al. (1993) found a ten-fold increase of polyamine levels during embryogenic callus induction and rapid growth of cell suspension cultures of indica rice. Santanen and Simola (1992) showed a decrease in putrescine but a four-fold increase in the spermidine level when embryogenic tissue was transferred to ABA containing medium that supported the maturation of globular embryos of Norway spruce. In rubber plant, El Hadrami and D'Auzac (1989) found that exogenous supply of polyamines in the culture medium increased the embryogenic potential of calli that were otherwise known to be recalcitrant with regard to somatic embryogenesis.

In some earlier reports it was shown that the presence of certain amino acids with ammonium ions in the medium enhanced the development of somatic embryos in cultures of *Medicago sativa* (Stuart and Strickland, 1984; Stuart et al., 1985). L-proline and L-serine when added singly before the omission of auxin from the medium significantly enhanced the number of somatic embryos formed in suspension cultures of carrot (Nuti-Ronchi et al., 1984). Addition of L-proline also supported embryogenic callus of maize (Armstrong and Green, 1985). Recently in an auxin-established maize culture system, addition of arginine and ornithine were found to improve the rate of embryogenic callus production (Santos et al., 1993). It is often seen in the literature that the inhibitory effects of the polyamine biosynthetic inhibitors can be reversed to some extent by addition of the respective polyamines whose synthesis was blocked (Koetje, 1993; El Hadrami and D'Auzac, 1992; Minocha et al., 1991a). However, addition of polyamines alone or in combination has enhanced somatic embryogenesis in relatively few cases (Altman et al., 1989; Danin et al., 1993). In *Vitis vinifera* (Faure et al., 1991), addition of spermidine has been shown to increase the conversion rate of abnormal somatic embryos to normal ones. However, in carrot, exogenous supply of spermidine and spermine show a strong inhibition of somatic embryogenesis (Minocha, unpublished data).

In their studies on a comparison of polyamine biosynthesis in zygotic and somatic embryos in grape, Faure et al. (1991) reported high levels of polyamines in the somatic embryos. The torpedo stage of somatic embryos and stratified zygotic embryos, which were morphologically at the same developmental stages, showed 22-fold increase in putrescine, 2.5-fold increase in spermidine and 1.5-fold higher level of spermine. Unlike the situation in *Vitis*, Mengoli et al. (1989) found more spermidine than putrescine in the torpedo stage embryos in carrot, however, embryos in the heart stage showed increased putrescine. In the embryogenic carrot cells, the presence of higher levels of putrescine than spermidine is the usual observation (Feirer et al., 1984, Montague 1979, Mengoli et al., 1989, Minocha et al., 1991a). As it is difficult to get synchronous cell cultures in carrot, cellular levels of polyamines have been seldom determined in the tissue samples from different stages of embryogenic development.

Danin et al. (1993) compared several developmental stages of somatic embryos of celery for biosynthesis and cellular levels of polyamines. In celery, like in carrot, somatic embryos are produced in auxin free medium when preceded by culture in auxin containing maintenance medium (Altman et al., 1990). Incorporation of ^{14}C-arginine and ornithine showed that polyamine biosynthesis was required for embryo growth and plantlet organization. As in carrot

and grapes, celery also showed high putrescine content and biosynthesis relative to spermidine and spermine during early stages of somatic embryos. Elevated levels of spermidine and spermine biosynthesis were observed during embryo maturation.

In addition to the change in polyamine content, and the activity of polyamine biosynthetic enzymes, the role of polyamines in somatic embryogenesis has also been inferred from studies using the inhibitors of polyamine biosynthesis especially DFMA and MGBG. Inhibition of somatic embryogenesis through inhibition of putrescine biosynthesis using DFMA (Feirer et al., 1984; Fienberg et al., 1984; Robie and Minocha, 1989) or canavanine (Mengoli et al., 1989) has been reported in carrot. Similarly, Koetje et al. (1993) observed a significant decrease in the number of meristematic cells and embryo-like structure in rice by DFMA. The effect of DFMA was reversed by the addition of exogenous agmatine and putrescine in rice and carrot respectively (Fienberg et al., 1984; Feirer et al., 1985; Robie and Minocha, 1989; Koetje et al., 1993)

Conflicting results are often found in the literature regarding the effect of DFMO on somatic embryogenesis. Therefore, the interpretation of the effect of DFMO has been rather difficult and confusing. In carrot, DFMO did not inhibit cell growth or embryo development (Mengoli et al., 1989; Robie and Minocha, 1989). While Mengoli et al. (1989)

reported a decrease in putrescine accumulation by DFMO in the different stages of somatic embryos, Robie and Minocha (1989) found an increase in ADC activity that correlated with increased accumulation of polyamines. Moreover, Robie and Minocha (1989), and Nissen and Minocha (1993) showed that DFMO promotes somatic embryogenesis in carrot and it is also capable of partially counteracting the inhibitory effect of auxin and inhibitors of polar auxin transport on somatic embryogenesis. In *Hevea*, DFMO inhibited somatic embryogenesis (El Hadrami and D'Auzac, 1992).

The significance of SAMDC during somatic embryogenesis is evident from the studies in carrot (Minocha and Khan, 1991; Minocha et al., 1991b), Norway spruce (Santanen and Simola, 1992), and celery (Danin et al., 1993). While carrot and celery show a correlation between inhibition of SAMDC and somatic embryogenesis, only higher levels of MGBG were effective in inhibiting somatic embryogenesis in Norway spruce (Santanen and Simola, 1992). Inhibition of SAMDC by MGBG was also found to be accompanied by decrease in ADC activity, and an increase in accumulation of putrescine and ACC in carrot (Minocha et al., 1991a).

Considering the fact that both polyamine and ethylene biosynthesis share the common precursor SAM, together with the results from the studies with DFMA and MGBG suggest that somatic embryogenesis in carrot may be affected by a competition between the two metabolic pathways (Minocha and

Minocha, 1994). This is also consistent with the observation that AVG, an inhibitor of ACC synthase, not only promotes spermidine and spermine biosynthesis but also promotes somatic embryogenesis at low concentration.

It is evident that much of research in carrot has focused in correlating cellular levels of polyamines and/or the activities of polyamine biosynthetic enzymes to somatic embryogenesis. Recently, the use of molecular techniques in conjunction with genetic transformation has been employed as an approach to genetically modulate cellular levels of polyamines. Hamill et al. (1990) observed an increase in the cellular levels of putrescine and nicotine in the roots of transgenic *Nicotiana rustica* by over-expressing yeast ODC. DeScenzo and Minocha (1993) successfully transformed *Nicotiana tabacum* with mouse ODC and observed a significant increase in the biosynthesis of putrescine. Noh and Minocha (1993) observed a 2-3 fold increase in the levels of spermidine and significantly low levels of putrescine in transgenic tobacco expressing human SAMDC. Spermine levels in these plants either increased slightly or remained unchanged. Similarly, an increase in the levels of cadaverine and anabasine in tobacco root cultures has been achieved by expressing a bacterial lysine decarboxylase gene (Fecker et al., 1993). These studies provide an alternative powerful tool to modulate cellular polyamines without the

complicating effects of inhibition of the polyamine biosynthetic pathway.

Objectives of This Study

In relation to the regulation of somatic embryogenesis in carrot cell cultures, previous studies in our laboratory have focused on the role of auxin, polyamines and ethylene (Robie, 1987; Papa, 1988; Robie and Minocha, 1989; Samuelsen, 1990; Minocha et al., 1990a,b; 1991a,b; Khan and Minocha, 1991; Minocha and Khan, 1991, Nissen and Minocha, 1993). From these studies using inhibitors of the enzymes in polyamine and ethylene biosynthesis, it has been shown that: (1) carrot cells in culture produce putrescine through the ADC pathway and ODC activity is significant only in fully developed somatic embryos; (2) inhibition of polyamine biosynthesis causes an increased accumulation of ACC in the cells; (3) more ethylene is produced in the presence of auxin than in its absence; (4) inhibition of ADC and SAMDC causes an inhibition of somatic embryogenesis; (5) promotion of polyamine biosynthesis promotes somatic embryogenesis; and (6) high levels of ACC/ethylene are associated with low levels of somatic embryogenesis. It is also known that upon removal of auxin from the medium, the activities of SAMDC and SAM synthetase increase, leading to increased production of polyamines. Thus, it has been hypothesized that (i)

auxin may inhibit somatic embryogenesis through increased biosynthesis of ACC/ethylene, and (ii) differentiation of cells into embryos could be possibly regulated by the capacity of the cells to divert higher amounts of SAM towards the formation of polyamines rather than ethylene.

The approach of using chemical inhibitors, although useful, suffers from a number of several drawbacks to specifically modulate polyamine biosynthetic pathway. Therefore, alternative approaches such as the genetic manipulation of the metabolic pathway using genetic engineering techniques were successfully used in generating transgenic tobacco plants expressing a mouse ODC and a human SAMDC cDNA (DeScenzo, 1991; Noh, 1991; DeScenzo and Minocha, 1993; Noh and Minocha, 1993).

The present research was aimed at studying the effect(s) of expression of a mouse ODC and a human SAMDC cDNA on polyamine metabolism and its relationship to the development of somatic embryos in transgenic carrot (*Daucus carota*. L) cell cultures.

Two specific objectives of the study were:

(1) to modulate the cellular levels of polyamines by regulated expression of a mouse ODC and a human SAMDC cDNA in carrot cells; and

(2) to determine the effect(s) of this modulation on the development of somatic embryos in the transgenic cell lines.

The results of this research were expected: (1) to elucidate the role of polyamine/ethylene biosynthesis in somatic embryogenesis in carrot cell cultures; (2) to enable us to analyze the competition between the polyamine and the ethylene biosynthetic pathways; and (3) to demonstrate the usefulness of the approach of regulated gene expression to study physiologically important biochemical pathways where specific inhibitor(s) for the pathway may be lacking.

The study involved: (1) transformation of carrot cells with *Agrobacterium tumefaciens* strains containing a mouse ODC or a human SAMDC cDNA under the control of a cauliflower mosaic virus 35S promoter along with a selection marker gene namely neomycin phosphotransferase under the control of *Agrobacterium* nopaline synthase (nos) promoter, (2) selection of transformed cells, (3) testing for the presence and expression of the NPT, ODC and SAMDC genes, (4) analysis of cellular polyamines, and (5) analysis of the development of somatic embryos in transgenic cell lines.

MATERIALS AND METHODS

Bacterial Culture

Agrobacterium tumefaciens strain AT35SODC^{t} (DeScenzo and Minocha, 1993; Fig. 2) containing a 3'-truncated sequence of mouse ODC cDNA under the control of CaMV 35S promoter and a selectable marker gene (NPT-II) linked with NOS 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^{t} is described in DeScenzo and Minocha (1993). Agrobacterium strain containing a human SAMDC (Noh and Minocha, 1993) cDNA (AT35S-SAM) was used as a vector to obtain transgenic SAMDC carrot cells. This bacterium is identical to AT35SODC^{t} in all aspects except it contained human SAMDC in place of mouse ODC cDNA.

To start fresh cultures of Agrobacterium, one mL of the bacterial suspension that had been stored in the presence of

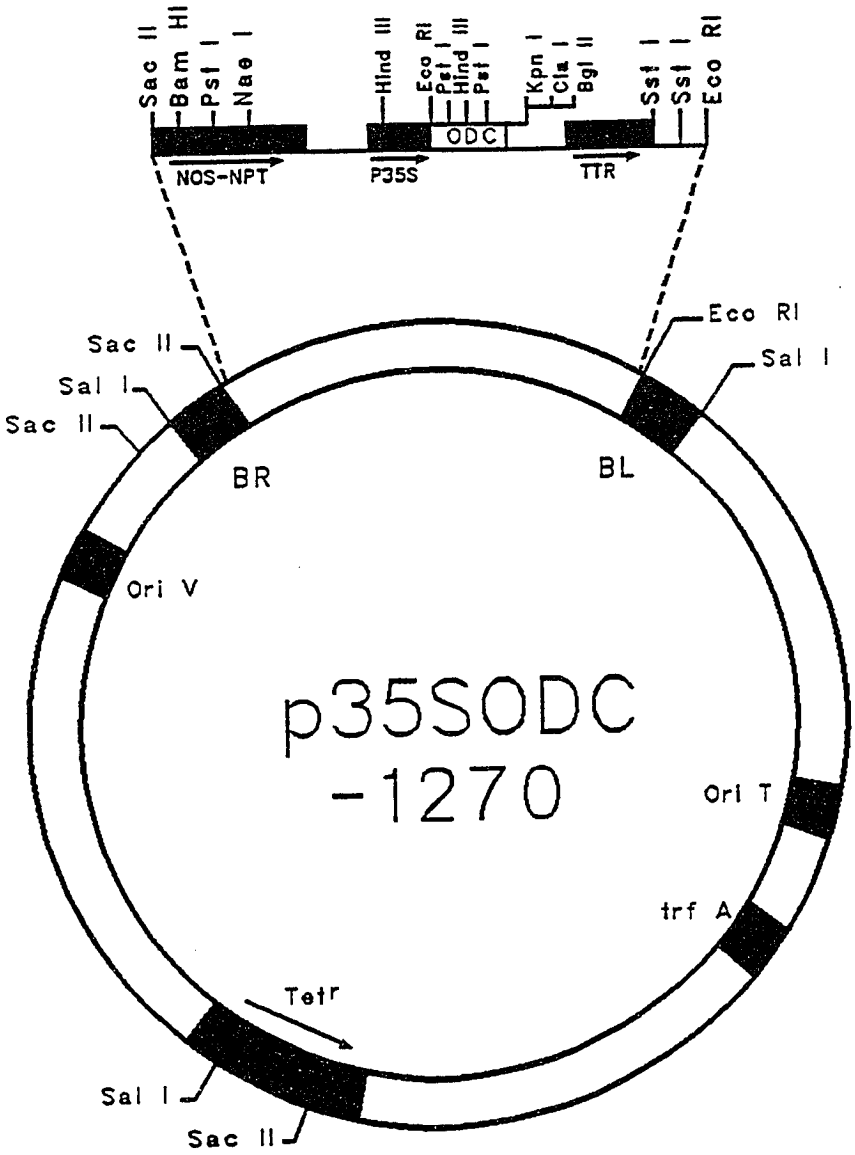

Figure 2. Plasmid p35ODC1270 present in AT35SODC^t (DeScenzo, 1991)

Table 3. Constituents of YEB medium (Kao et al., 1982)

Constituent	Amount (g/L)
Bacto beef extract	5.0
Bacto yeast extract	1.0
Peptone	5.0
Sucrose	5.0
Magnesium sulfate	0.5
Bacto Agar(for solid medium)	7.0

Medium was adjusted to pH 7.0 prior to autoclaving

15% (v/v) glycerol; 850 µL of bacterial suspension in 150 µL of glycerol, was transferred to 50 mL of YEB medium (Kao et al,. 1982; Table 3) containing 50 µg/mL rifampicine and 12 µg/mL of tetracycline. After 16 h incubation at 25°C with constant shaking (150 rpm), 1 mL of this culture was again transferred to fresh medium and similarly cultured for overnight (18-24h). Following this, the bacterial culture was (1) streaked on minimal agar medium to select Agrobacterium colonies, (2) used for transformation work, and/or (3) stored in glycerol for later use. For the selection of Agrobacterium colonies, individual colonies were picked from the minimal agar medium and used to inoculate fresh YEB liquid medium with appropriate antibiotics. For storage, after 16 h at 25°C with shaking, (150 rpm) 850 µl of this bacterial solution was added to 150 µl of sterile (autoclaved) glycerol in a microfuge tube. The microfuge tubes were then vortexed and stored at -70°C. When these bacterial cultures were required for transformation work, each tube was thawed on ice and then transferred to fresh YEB medium and cultured overnight as described earlier.

Carrot Cell Culture

Suspension cultures of carrot (*Daucus carota L.*) were maintained in B5 medium (Gamborg et al., 1968; Table 4) supplemented with 2% sucrose and 0.45 µM 2,4-D. A pre-

mixed powder of Gamborg's B5 basal medium with minimal organics (Sigma Chemical CO., St Louis, MO ; Cat. # G-5893) was used. The cells were subcultured in fresh medium at a 1:20 dilution at weekly intervals. Every month, those cell lines that were maintained for experimentation were forced through a 250 μm sieve. The cell clumps were broken by gently crushing them with a sterile spatula. Cell clumps passing through the sieve were collected in a beaker and washed by decantation with fresh medium containing 2,4-D (B5+). The cell clumps were re-suspended in fresh B5+ and transferred to 50 mL of medium (B5+) in a 125 mL Erlenmeyer flask. For the maintenance of transgenic cells, 300 mg/L kanamycin was also added to the media. Unless otherwise stated, all liquid cultures were kept on a gyratory shaker (150 rpm) under 16 h 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 (embryogenic medium) using cell clumps between 100 and 250 μm in size. Cell clumps of this size were obtained by collecting the cells that passed through the 250 μm sieve but were retained by 100 μm sieve. These cells were washed 3-4 times with auxin free medium by decantation and centrifugation (200 rpm, 2 min). The cells were re-suspended in B5- medium such that 100 clumps were present per mL of the medium to give a low density suspension of cells. For somatic embryogenesis studies with both the transformed and the non-transformed cell lines, 5 mL of low density suspension cells were grown

Table 4. Constituents of B5 medium (Gamborg et al., 1968)
(Sigma Chemical CO., Catalog # G-5893)

Constituent	Amount (mg/L)
Ammonium sulfate	134.0
Calcium chloride (anhydrous)	113.24
EDTA.Na_2.$2H_2O$	37.25
Ferrous sulfate.$7H_2O$	27.85
Magnesium sulfate	122.09
Manganese sulfate	10.0
Potassium nitrate	2500.0
Sodium phosphate	130.5
Boric acid	3.0
Cobalt chloride.$6H_2O$	0.025
Cupric sulfate.$5H_2O$	0.025
Molybdic acid (Na-salt).$2H_2O$	0.25
Potassium iodide	0.75
Zinc sulfate $7H_2O$	2.0
myo-inositol	100.0
Nicotinic acid	1.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0

in 50 mm Petri dishes. All additives in the experimental media (except for 2,4-D that was added before autoclaving) were adjusted to pH 5.6, filter-sterilized, and added to the autoclaved medium cooled to room temperature. All the embryos, 1 mm or larger in size, in the petri plates were counted after ten days in the embryogenic medium using a dissecting microscope (25X).

Transformation of Carrot Cells

For transformation, 0.5 cm hypocotyl sections from in vitro-grown embryos (4-6 weeks) were precultured in 2,4-D-supplemented solid medium for 2-4 days. These explants were removed from the plates, injured at many places using a sterile needle, and co-cultured with *A. tumefaciens* AT35SODC^{t} (obtained from 50 mL culture grown overnight in YEB medium, re-suspended in 20 mL of sterile 0.85% (w/v) sodium chloride solution). Acetosyringone, dissolved in DMSO, was added to the suspension at a final concentration of 100 μM. Following co-cultivation for 1 h, the explants were rinsed with sterile distilled water, pat-dried between 3-4 layers of sterile 3 MM Whatman filter paper, and returned to solid medium containing 2,4-D. After 24-48 h, the explants were transferred to fresh medium supplemented with 250 mg/L of mefoxime (Merck & CO, Inc, West Point, PA) 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 FW of the 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 liquid medium.

Neomycin Phosphotransferase (NPT-II) Assay

The amount of NPT protein in transgenic cells was determined by using the NPT-II ELISA kit (5 Prime → 3 Prime Inc., West Chester, PA) following manufacturer's suggested protocol. Briefly, 100 mg of cells were homogenized in a microfuge tube with a tight fitting plastic homogenizer in 200 μL of freshly prepared extraction buffer [62.5 mM Tris-HCl, 10 % (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 0.1 % (w/v) sodium dodecyl sulfate] at 4°C. The homogenate was centrifuged at 4°C for 3 min at 14,000 x g and the supernatant used for ELISA and for determination of total protein (Bradford, 1976).

Two hundred microliters of coating antibody (1:900 dilution of the stock in coating buffer) was added to each well in the polystyrene microwell strips provided with the NPT-II ELISA kit. The polystyrene strips containing the diluted coating antibody were wrapped in saran wrap and incubated at 37°C for 2 h. At the end of 2 h, the wells were emptied by inverting the plate and shaking several

times with a sharp downward motion to completely empty each well (referred to as emptying of well). Using a squirt bottle, each well was washed with 1X PBS (Phosphate Buffered Saline, Azide) by flooding with the buffer followed by emptying in the sink. The wells were washed for a total of three times. Four hundred µL of 1X "Blocking and Dilution (B&D) Buffer" were added to each well and incubated at room temperature for 30 min. Meanwhile, the NPT enzyme standard concentrate (970 µg/mL) was first diluted to make a 1µg/mL working solution. Using this working stock, four dilutions i.e.(750, 500, 250 and 100 pg/mL) of the NPT protein standards were prepared. At the end of half hour incubation with the B&D Buffer, the wells were emptied and washed 5X with 1X washing buffer (WB). Finally 200 µL each of NPT protein standards serial dilution, negative control (1X B&D Buffer) and the cell extracts were added to the wells. The polystyrene strips were wrapped with saran wrap and incubated at room temperature for 2 h. While the reaction was in progress, a 1:1250 dilution of biotinylated antibody to NPT (Biot-α-NPTII) was made in 1X B&D buffer. At 2 h, the wells were emptied and washed 5X with 1X WB. To each well, 200 µL of the Biot-α-NPTII was added and the polystyrene plates incubated for an hour. This was followed by washing 5X with 1X WB. Next, 200 µL of a diluted Streptavidin-conjugated alkaline phosphatase (SA/AP; 1:750 in B&D Buffer) were added to each well and incubated at room temperature for 30 min. Color development substrate

solution (2 mg p-nitrophenyl phosphate in 1 mL diethanolamine buffer) was prepared 5 min before the end of the incubation with SA/AP. The SA/AP solution was discarded and the wells were washed 5 X with 1X WB. Finally, 200 µL of substrate solution were added to each well and incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 µL of Stop Solution. The solution was then diluted 1:1 with 0.4N sodium hydroxide (5X dilution of Stop Solution with water) and the absorbance measured at 405 nm using Lambda 3B, UV/Vis spectrophotometer (Perkin Elmer, Norwalk, CT). Each well was read against the reagent blank.

Ornithine Decarboxylase (ODC) Assay

Mouse ornithine decarboxylase activity was determined by the procedure modified from Robie and Minocha (1989) as described by DeScenzo and Minocha (1993). One-half gram FW of cells were homogenized in 1 mL of cold extraction buffer (50 mM Tris-HCl, 0.1 mM Na_2EDTA, 0.5 mM pyridoxal 5'-phosphate, and 0.05 mM dithiothreitol; TAB) using a Brinkmann Polytron at 2 x 10^4 rpm for 90 s. The homogenate was centrifuged at 18,000 x g for 30 min at 4°C, and the supernatant fraction used for enzyme assay and quantification of total protein. To determine mouse and plant ODC enzyme activities, pH of the extraction buffer was adjusted at 25°C to 6.8 and 8.4, respectively.

Enzyme activity was determined by measuring the amount of $^{14}CO_2$ released from L-[1-^{14}C] ornithine. The reaction was carried out in 15 mL disposable glass test tubes fitted with a rubber cap holding a plastic center well containing a 3 cm^2 Whatman 3MM filter paper soaked with 50 µL of ScintiGest (Fisher Scientific, Fair Lawn, NJ). The reaction mixture consisted of 50 µL of substrate solution (0.1 µCi L-1-^{14}C-ornithine, NEN DuPont, Boston, MA; Sp. Act. 60 mCi/mmol in 2 µM cold L-ornithine), 200 µL of crude enzyme extract and 50 µL of extraction buffer. The reaction was carried out in a water bath at 37°C for 60 min. However, in experiments using DFMO, the buffer was substituted with 50 µL of the inhibitor solution. Furthermore, the crude extract along with the inhibitor was preincubated at 37°C for 10 min prior to adding the substrate. The assay was then completed as per the standard protocol. The reaction was terminated by injecting 200 µL of 0.5N H_2SO_4 through the rubber stopper. The tubes were incubated for an additional 30 min before the filter papers were removed and counted for radioactivity in 10 mL of OCS scintillation fluid (Amersham Corp., Arlington Heights, IL).

Parallel to the procedure described for determining ODC enzyme activities, ADC enzyme activity was determined by measuring the amount of $^{14}CO_2$ released from L-[1-^{14}C] arginine (Moravek Biochemicals Inc. Brea, CA). The cells were homogenized in the TAB extraction buffer (pH 8.4),

centrifuged and the supernatant fractions used for enzyme assays.

For the determination of SAMDC enzyme activity, the cells (500 mg fresh weight) were homogenized in phosphate buffer (0.1 M potassium phosphate, pH 7.5, 3 mM putrescine, and 1 mM DTT). The homogenates were centrifuged as described earlier and the supernatant used for enzyme assays. The enzyme activity was determined by measuring the amount of $^{14}CO_2$ released from S-[carboxyl-^{14}C]-adenosyl-L-methionine (Amersham Corp., Arlington Heights, IL). The reaction mixture in the tubes were incubated at 37°C for 30 min, following which the reaction were stopped with acid and further incubated for 30 min. The filter papers were then removed and counted for radioactivity as described earlier.

Quantification of Polyamines

Cells (100 mg FW) were collected in 400 mL of 5% (v/v) PCA. The PCA-soluble polyamines were extracted by freeze-thawing three times before centrifugation at 14,000 x g for 10 min at 4°C. Heptanediamine was used as an internal standard (30 µL of 0.4 mM HD plus 270 µL of PCA extract) at a final concentration of 0.04 mM and the samples were stored at-70°C until ready for dansylation. The polyamines were dansylated and quantified by HPLC as described in Minocha et al.(1990a). The HPLC system consisted of a Perkin-Elmer

series 400 or 410 gradient pump, a Rheodyne injection valve fitted with a 10 µL loop, a Perkin-Elmer Pecosphere-3x3 CR C_{18}, 33 x 46 mm I.D. cartridge column (3 µm particle size), and a fluorescence detector (LS-1, Perkin Elmer). Dansylated polyamines dissolved in methanol were injected using a Hitachi AS 4000 autoinjector (Hitachi Instruments Inc. San Jose, CA). A linear gradient of acetonitrile and heptanesulfonate (10 mM, pH 3.4), at a flow rate of 2.5 mL/ min was used to separate dansylated polyamines. The details of the gradient are given in Table 5. The results were integrated using Gilson 712 HPLC System Controller Software (Gilson Medical Electronics Inc. Middleton, WI). A typical chromatograph for the separation of polyamine standards is shown in Fig. 3.

All the data for somatic embryogenesis, exzyme activity and polyamine levels were analyzed using Tucky HSD multiple comparisons, SYSTAT 5.01, SYSTAT Inc. Evanston, IL.

Isolation of Plant Genomic DNA

DNA was isolated by the procedure of Thomas et al. (1989). Five grams of cells were pulverized in liquid nitrogen, and placed in a 30 mL Corex centrifuge tube containing 15 mL of extraction buffer [1 % (w/v) CTAB, 0.7 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM sodium metabisulfite, 1% (w/v) polyvinylpyrrolidone, 40 mM

Table 5. Gradient profile of acetonitrile and heptanesulfonic acid used in the separation of dansyl polyamines.

Step	Time	Running Time	Flow rate (mL/min)	%Solvent (A)	%Solvent (B)
0	0.1	0.1	2.5	40	60
1	1.5	1.6	2.5	70	30
2	2.5	4.1	2.5	100	0
3	3.0	7.1	2.5	100	0
4	0.1	7.2	2.5	40	60
5	1.5	8.7	2.5	40	60

A= 100% acetonitrile; B= 10 mM heptanesulfonate, pH 3.4, contains 10% acetonitrile to inhibit fungal and bacterial growth

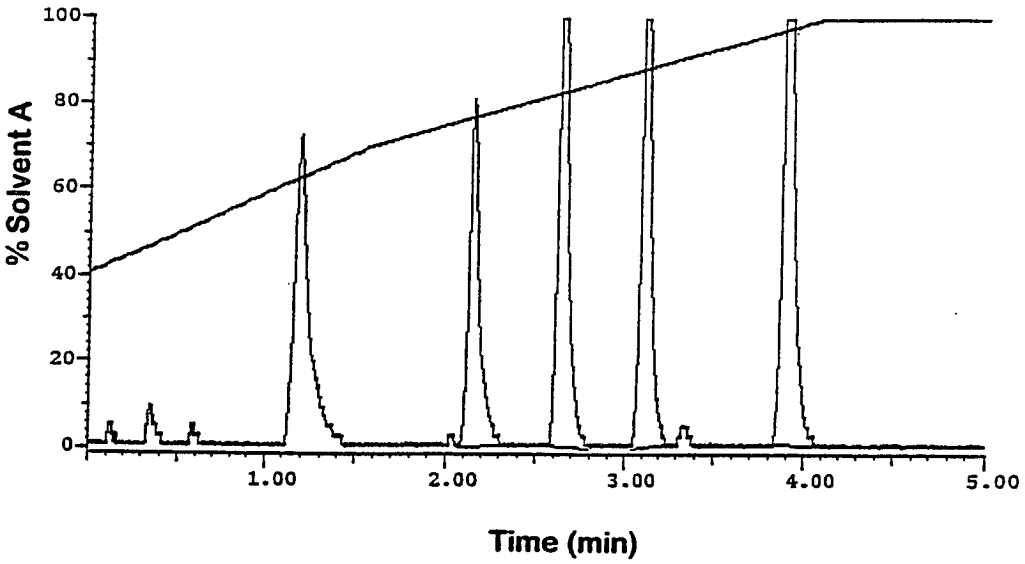

Figure 3. Chromatographic separation of dansylated polyamine standards. Put= putrescine, Hda= heptanediamine, Spd= spermidine, and Spm= spermine. Gradient profile described in Table 5 was used for separation.

dithiothreitol, and 10 mg/ mL of proteinase K] at 55-60°C. Just after the tissue had thawed, 10 mL of chloroform:isoamyl alcohol (24:1) were added and the solution mixed by inverting the tube. The slurry was incubated at 55-60°C for 60 min, centrifuged (500 x g, 5 min at room temperature), and the aqueous phase reextracted with equal volume of chloroform:isoamyl alcohol. Solid ammonium acetate was added to the aqueous fraction at a final concentration of 2.5 M. After the salt was completely dissolved, DNA was precipitated with an equal volume of isopropanol, centrifuged, and washed with 70% (v/v) ethanol containing 60 mM sodium acetate. The DNA was dissolved in sterile distilled water, precipitated twice as an ammonium salt, and quantified using TKO 100 Fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

Isolation of Plasmid DNA

One mL of bacteria pUO-1 (DeScenzo, 1991), stored at -70°C were thawed on ice and transferred to 50 mL of LB medium (Table 6) containing 50 mg/L ampicillin in a 125 mL flask. The culture was placed on a shaker (150 rpm) and incubated overnight (16 h) at 37°C. The next day, the suspension was centrifuged at 2000 x g for 5 min in a 50 mL polypropylene tube. The resulting pellet was suspended in 1 mL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and transferred to a 1.5 mL microfuge tube. The tube was

centrifuged in the microcentrifuge for 30 s and the pellet resuspended in 0.45 mL of STE buffer (18% sucrose, 35 mM Tris, 100 mM EDTA, pH 8.0). One-hundred and fifty microliter of 10 mg/mL of freshly prepared lysozyme (Sigma Chemical Co., St. Louis, MO) was added to the tube. Following a thorough mixing by vortexing, the tube was incubated on ice for 15 min. Five hundred µL of TET buffer (1% Triton X-100, 15 mM EDTA and 50 mM Tris, pH 8.5) was added to the tube and mixed by inverting and shaking. After 15 min of incubation on ice, the tube was heated to 80°C in a water bath for 5 min and rapidly cooled by incubating on ice for 10 min. The contents were centrifuged at 14,300 x g for 30 min and the supernatant transferred to a new tube. Equal volumes of phenol and Chloroform:Isoamyl alcohol (24:1) were added to the plasmid solution and mixed by vortexing briefly. The solution was centrifuged and the aqueous layer transferred to a new tube. The procedure was repeated with Chloroform:Isoamyl alcohol until no white residue was seen in the interface. Finally, the plasmid DNA was precipitated with 0.1 volume of 3.3 M sodium acetate and 2 volumes of 100% ethanol. The microfuge tube was kept at -20°C for at least 2 h or at -70°C for 15 min before centrifugation at 14,300 x g for 30 min. The pellet was washed with 0.5 mL of cold 70% ethanol and dried in the Savant speedvac for 2 min before reconstituting in 100 µl of water.

Table 6. Constituents of LB medium (Sambrook et al., 1989).

Constituent	Amount (g/L)
Bacto tryptone	10.0
Bacto yeast extract	5.0
Sodium chloride	10.0
Bacto agar (for solid medium)	7.0

Medium was adjusted to pH 7.2 prior to autoclaving

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was carried out in 25 µL total reaction volume containing 65 ng of genomic DNA, 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100), 100 mM deoxynucleoside triphosphates, 0.6 mM each of the forward and the reverse sequence (NPT or ODC) primers (Keystone Laboratories, Menlo Park, CA), and 0.125 µL (500 units) of *Taq* polymerase (Promega Corp., Madison, WI). Reaction mixture was overlaid with 50 µL of autoclaved light mineral oil with no stabilizer added. Thermocycler was programmed for denaturation temperature of 94°C for 2 min, annealing temperature of 50°C (ODC) or 60°C (NPT) for 90 sec, and 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→220 in the NPT-II gene (5'GAGGCTATTCGGCTATGACT3') and the reverse primer was from position 898→879 (5'TCGGGAGCGGCGATACCGTA3'), giving an expected 700 bp amplification product (Beck et al., 1982; Accision # J01834). The ODC forward sequencing primer was from position 324→343 in the mouse ODC cDNA (5'TATCTATGCAAATCCTTGTA3') and the reverse primer was from position 862→843 (5'ACTGCAAGCGTGAAAGCTGA3') giving a PCR product of 540 bp (Kahana and Nathans, 1985; Accession # M10624).

Southern Hybridization

Isolating DNA Fragment from pUO-1

Homologous probe for mouse ODC cDNA was obtained from *E.Coli* containing the plasmid pUO-1 (Fig. 4) as a 1.8 kb EcoRI/XbaI (BamHI) fragment (DeScenzo, 1991). Five μg of plasmid DNA was digested overnight with 30 units of EcoRI and XbaI using 10X buffer for EcoRI. The reaction was stopped by adding 1 μL of EDTA (200 μM, pH 8). To the reaction mixture was added 3 μL of gel loading buffer (15% Ficoll type 400, 0.25% xylene cyanol, 0.25% bromophenol blue) and the DNA was subjected to electrophoresis in a minigel. Standard gel consisted of 1% of GTG SeaPlaque Agarose in 1X TAE (40 mM Tris, 40 mM acetic acid, 1 mM EDTA). The electrophoresis was done at 40 V for 3-4 h. The gel was stained with ethidium bromide (0.5 mg/L) for 5 min and destained for 1 h with water. After destaining, the 1.8 kb fragment was excised while visualizing under UV with a clean razor blade. It was made sure that the gel was exposed to UV for minimum amount of time. The gel fragments were stored at -20°C until ready to be purified using the manufacturer's suggested protocol for Geneclean Kit (Bio 101 Inc., La Jolla, CA). Approximate volumes of gel slices were determined by melting it at 55°C in a waterbath for 10 min in microfuge tubes. To this agarose containing DNA, 2.5 volumes of NaI stock solutions were added from the kit giving a final concentration of 4 M. The microfuge tubes

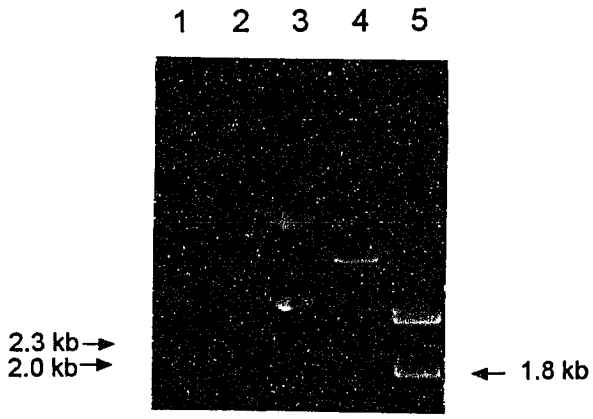

Figure 4. Restriction digest of pUO-1 with EcoRI/XbaI using buffer for XbaI. Lanes 1-5 are: 1= λ HindIII digest; 2= blank; 3= uncut plasmid; 4= double digest for 1 h; and 5= double digest overnight.

were then incubated in a 55°C waterbath for 5 min and mixed to make sure the agarose was completely mixed. The 'glassmilk' suspensions containing silica matrix, were added to each microfuge tube containing the DNA solution at a rate of 5 µL of the suspension per 5 µg of DNA digested. One µL of the 'glass milk' suspension was added for each additional 0.5 µg of DNA. The contents were incubated at room temperature for 5-10 min to allow binding of the DNA to the silica matrix, mixing every 1-2 min to ensure that glassmilk stayed suspended. The silica matrix with DNA bound to it was pelleted by spinning for approximately five sec in a microcentrifuge. The NaI supernatant solutions were discarded and the pellets resuspended in 400 µL of NEW (Geneclean Kit, Bio 101 Inc., La Jolla, CA) by repeated pipetting while digging into the pellet with the pipet tip. The contents centrifuged briefly (5-10 sec) in a microcentrofuge. The pellets were washed three times in this manner. After the supernatant from the third wash had been removed, the tubes were spun again for 5-10 seconds and the last bit of the liquid was removed with a fine tipped pipet. Fifty µL of water were added to each tube containing the silica matrix with the bound DNA and incubated at 55°C for 3 min in a water bath. Finally they were centrifuged for 30 sec to obtain a solid pellet. The supernatant containing the eluted DNA was carefully removed and placed in a new tube. The eluted DNA was quantified and stored at

5°C until required for use in making a probe or using the DNA as a positive control in Southern hybridization.

Southern Hybridization of PCR Products

One µL of the PCR products were subjected to electrophoresis in 1.2% agarose gel (12 x 14 cm) at 80 V for 2 h. The nucleic acids were vacuum transferred to GeneScreen Plus membrane (New England Nuclear, Boston, MA) for 2 h using 10X SSC. Southern analysis was carried out using PhotoGene Nucleic Acid Detection system (Chromogenic system; BRL Life Technologies, Inc. MD). The biotin labeled probe for Southern hybridization was prepared by PCR as described above but using approximately 1 ng of template DNA (1.8 Kb EcoRI/XbaI fragment from the plasmid pUO-1), 0.2 mM each of dATP, dGTP, dCTP, 0.15 mM dTTP and 0.033 mM biotin dUTP. Hybridization reaction was carried out overnight at 42°C in hybridization solution consisting of 20% dextran sulfate in formamide, 10X Denhardt (1X contains 0.02% Ficoll, 0.02% PVP, 0.02% BSA), 1.8 M NaCl, 0.12 M sodium dihydrogen phosphate and 0.012 M $Na_2EDTA.2H_2O$, 2% SDS and 400 µg/mL of sheared and denatured Salmon sperm DNA. The membrane was washed twice for 5 min in 5X SSC and 0.5% SDS at 65°C; once for 30 min in 0.1X SSC 1% SDS at 50°C; and once for 5 min in 2X SSC at room temperature. The membrane was incubated with 3% BSA in TBS-Tween 20 (100 mM Tris, pH 7.5; 150 mM NaCl and 0.05% Tween 20) at 65°C for 1 h and was followed by treatment with streptavidin alkaline phosphatase

(SA-AP) for 10 min. The membrane was washed twice for 15 min each, with TBS-Tween 20 and then once with 1X "Final wash buffer" for 1 h at room temperature and dried between the folds of Whatman 3MM filter paper; treated with 0.01 mL/cm^2 detection reagent and finally incubated at 25°C for 3 h and exposed to Kodak X-Omat AR film for 15 min.

Southern Hybridization of Genomic DNA

For genomic Southern, 5-10 μg of genomic DNA was restricted overnight with appropriate endonucleases (5 units of enzyme per μg of DNA). The standard gel running conditions for Southern blotting consisted of using TBE buffer with a 0.8% (w/v) agarose in a 8 x 7 cm gel box (Hoefer Scientific Instruments, San Francisco, CA). The electrophoresis was done at 40 V for 4 h, the gel was stained with ethidium bromide (0.5 mg/L) for 5 min and destained with water for 1 h. The gel was first treated with 250 mL of 0.25 M HCl for 10 min, when the blue tracking dye turned yellow. The gel was rinsed with water and treated with 250 mL of denaturing solution (1.5 M NaCl + 0.5M NaOH) twice, for 15 min each, with gentle shaking. It was then rinsed and treated with neutralization solution (1M Tris-HCl, pH 8) for 15 min. Meanwhile, a 8 x 7 cm piece of GeneScreen Plus (Dupont NEN, Boston, MA) nylon membrane was prepared for use in the transfer of DNA by soaking it in water for 1 min and then in 20X SSC for 5 min. The DNA was transferred to the nylon membrane using a TurboBlotter

(Schleicher & Schuell, Keen, NH), a rapid downward transfer systems, for 4-6 h. The TurboBlotter consisted of a Stack tray, Buffer tray , wick cover and two types of blotting papers (GB004 and GB002). For the transfer of DNA, 20 sheets of dry GB004 blotting paper were first stacked on the 'stack tray' followed by four sheets of dry GB002 and one sheet of GB002 blotting paper, wetted in transfer buffer on the top. Finally the prepared nylon membrane was placed over this stack of paper. The membrane was covered with agarose gel making sure no air bubbles were trapped between the gel and the membrane. The top of the gel was wet with transfer buffer and 3 sheets of GB002 blotting paper presoaked in transfer buffer were placed on top of the gel. The buffer tray was attached to the stacking tray by using the circular alignment buttons on the Stacking and Buffer trays. The Buffer tray was filled with transfer buffer (20X SSC). The transfer was started by connecting the gel stack with the buffer tray using the pre-cut "buffer wick", included in the blotter stack, presoaked in transfer buffer. The wick was placed across the stack so that the short dimension of the wick completely covered the blotting stack and both ends of the long dimension extended into the buffer tray. The wick cover was finally placed on top of the stack to prevent evaporation.

Following the transfer, nylon membrane was rinsed with 2X SSC buffer and allowed to dry such that excess buffer

could not be seen on the membrane but the membrane was still wet. The DNA in the membrane was then crosslinked by exposing the membrane to UV light for 2 min. The membrane was stored between two sheets of Whatman 3MM filter paper in a box containing desiccant until ready for pre-hybridization

Random-prime Labeling of DNA

The 1.8 Kb EcoRI/BamHI fragment of ODC cDNA from pUO-1 (DeScenzo and Minocha, 1993) was labeled by random-primed incorporation of digoxigenin-labeled deoxyuridine-triphosphate (DIG-11-dUTP). The standard procedure for labeling reactions using Genius kit (Boehringer Mannheim, Indianapolis, IN) consisted of a 20 µL reaction. The following reagents were added in the order:

Reagent	Volume	Final Concentration
DNA template	variable (10 ng-1µg)	0.5-50 µg/mL
Hexanucleotide mix (10X)	2µL	1X
dNTP labeling mixture (10X)	2µL	1X
Water	to 19µL	
Klenow enzyme	1µl	100 units/mL

The reaction tube was incubated at 37°C for 20-24 h and the reaction terminated by adding 2 µL of EDTA (200 mM, pH 8). The labeled DNA was precipitated with 0.1 volume of LiCl and 2.5 volumes of chilled ethanol. The microfuge tube containing the precipitate was incubated at -70°C for 30 min and finally centrifuged at 13,000 x g for 15 min following a brief thawing period at room temperature. The pellet was washed with 70% ethanol and resuspended in 50 µL of TE/SDS (10 mM Tris-HCl, 1 mM EDTA; pH 8; containing 0.1 % SDS). The labeled probe was stored at -20°C until ready for use.

Estimating the Yield of DIG-labeled DNA

A serial dilution of the labeled control DNA in DNA dilution buffer was made as follows:

Labeled Control DNA Starting Concentration	Stepwise dilution	Final concentration (Dilution name)	Total Dilution
5ng/µL (S)	2 µL S/8 µL dil. buffer	1 ng/µL (A)	1:5
1ng/µL (dilution A)	2 µL A/18µL dil. buffer	100 pg/µL (B)	1:50
100 pg/µL (dilution B)	2 µL B/18 µL dil. buffer	10 pg/µL (C)	1:500
10 pg/µL (dilution C)	2 µL C/18 µL dil. buffer	1 pg/µL (D)	1:5,000
1 pg/µL (dilution D)	2 µL D/18 µL dil. buffer	0.1 pg/µL (E)	1:50,000

Similarly a serial ten-fold dilution of the labeled experimental DNA probe of unknown starting concentration was made in the DNA dilution buffer until a 1:10,000 dilution was made. One µL of dilution B-E and the 10^{-1}-10^{-4} dilution

from the experimental DNA were spotted onto a nylon membrane. The position of each spot of diluted DNA was marked and noted. The DNAs were crosslinked to the membrane by exposing it to UV for 2 min and detected immunologically using colorimetric detection with NBT (50 mg/mL nitroblue tetrazolium salt in 70% v/v dimethylformamide) and X-Phosphate (50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate salt in 100 % dimethylformamide) (Boehringer Mannheim, Indianapolis, IN).

Prehybridization and Hybridization of GeneScreen-bound DNA

The membrane was prepared for hybridization by blocking non-specific nucleic acid-binding sites on the membrane during pre-hybridization. The membrane was placed in a hybridization container with 20 mL standard prehybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1.0% (w/v) blocking reagent for nucleic acid hybridization) and incubated at 60°C for 2-4 h with a gentle shaking.

The standard prehybridization solution was discarded and replaced with hybridization solution containing the DIG-labeled probe. Hybridization of the DIG-labeled probe was carried out at 60°C for 14-16h. At the end of hybridization, the hybridization solution was saved for future use at -20°C in a 15 mL polypropylene tube. The membrane was washed twice for 5 min each in excess amount of 2X wash solution (2X SSC, 0.1% SDS) at room temperature.

Then the membrane was washed twice, again 15 min per wash in 0.5X wash solution pre-heated to 60°C with a gentle shaking. Following the final wash with 0.5X wash solution, the membrane was either saved in a box containing desiccant between folds of Whatman 3 MM filter paper or proceeded to immunological detection step. The labeled probe was then detected colorimetrically with NBT and X-phosphate.

Immunological Detection of DIG-labeled Probe

All the steps involved were carried out at room temperature. The dried membrane containing (1) serial dilution of labeled probe and the standards for estimating the yield or(2) DIG-labeled probe hybridized to restricted DNA, or (3) RNA, was placed in a clean tray with 1.5 mL/100 cm^2 membrane area, malate buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5) and washed for one min. The membrane was then blocked for 1 h in 1.5 mL/100 cm^2 of blocking solution (2% w/v Blocking reagent for nucleic acid hybridization in 0.1 M sodium maleate, pH 7.5; Genius system, Boehringer Mannheim, Indianapolis, IN). After completion of the blocking step, the membrane was further incubated for 30 min in 1:5000 dilution of anti-DIG-alkaline phosphatase in blocking solution with a gentle shaking. The membrane was rinsed briefly with 20 mL of malate buffer and then transferred to a clean dish and washed twice, 15 min per wash, with 50 mL malate buffer. Finally the membrane was equilibrated for 5

min in 25 mL of Genius buffer 3 (100 mM Tris-HCl, 100 mM NaCl, pH 9.5 and 50 mM $MgCl_2$) and then transferred to another clean dish containing 10 mL of color substrate solution (45 µL NBT and 35µL of X-phosphate in 10 mL of Genius buffer 3). The membrane was incubated for 3 h (usually for spot test of probes) to 16 h. The membrane was washed with water once the bands were visible and allowed to dry. The membrane was wet with water when needed for photodocumentation.

Isolation of Plant RNA

All the glassware, plasticware and solutions used for RNA work were treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma, St. Lous, MO). Total RNA was isolated by modifying the DNA isolation method of Thomas et al. (1989). Five grams of cells from suspension culture were pulverized in liquid nitrogen and placed in a 50 mL Nalgene centrifuge tube containing 15 mL of extraction buffer (1% (w/v) CTAB, 0.7 M NaCl, 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 mM sodium metabisulfite, 1% (w/v) polyvinylpyrrolidone, 40 mM dithiothreitol, and 10 mg/mL of proteinase K) at 55-60°C. Just after the tissue had thawed, 10 mL of chloroform:isoamyl alcohol (24:1) were added and the solution mixed by inverting the tube. The slurry was incubated at 55-60°C for 60 min, centrifuged (500 x g, 5 min at room temperature), and the aqueous phase re-extracted

with equal volume of chloroform:isoamyl alcohol. Lithium chloride was added to the aqueous fraction at a final concentration of 0.8 M and incubated on ice for 4-8 h. The nucleic acids were separated by centrifugation at 16,000 x g for 30 min. The pellet was resuspended in 2.6 mL of ice cold water in the ice bucket and further precipitated with 0.8 M lithium chloride. Finally the pellet was washed with 70% (v/v) ethanol, allowed to dry in air and resuspended in 90 µL of TMN (40 mM Tris, pH 7.9; 6 mM $MgCl_2$, 10 mM NaCl; 0.1 mM $CaCl_2$). RQ1 RNAse free DNAse (Promega, Madison, WI) was added to a final concentration of 1 unit/µL and incubated at 37°C for 15 min. Equal volume of phenol saturated with 0.1 M citrate buffer (pH 4.3; Sigma, St. Louis, MO) was added and mixed thoroughly. The mixture was centrifuged at 5°C for 5 min and the aqueous layer transferred to a clean microfuge tube containing equal volume of chloroform:isoamyl alcohol (24:1 v/v). It was centrifuged again under identical conditions for 5 min and the aqueous fraction transferred to a clean microfuge tube. To this aqueous fraction, 0.1 volume of 3.3 M Sodium acetate (pH 5.3) was added and mixed thoroughly, and finally, 2.5 volumes of ethanol were added and the sample stored at -70°C for 15 min. The RNA was pelleted by centrifugation at 16,000 x g for 30 min at 5°C, the pellet washed with 70% ethanol and dried in air. The pellet was resuspended in 100-200 µL of water and the RNA quantified using 1 µL of the sample by UV spectrophotometer at 260 nm, assume A_{260} = 1.0

for a solution containing 40 µg/mL of RNA (Sambrook et al., 1989).

Northern Blotting

All solutions and apparatus used for Northern hybridization analysis were treated with 0.1% DEPC. Fifty mL of 1.2% formaldehyde agarose denaturing gel was prepared as follows: 0.6 g of agarose was boiled in 36 mL of water and the solution cooled in a water bath maintained at 60°C. Five mL of 10X MOPS (0.4 morpholinopropane sulfonic acid, 100 mM sodium acetate, 10 mM EDTA, pH 8.0) running buffer and 5 mL of 37% formaldehyde were added and mixed well. The gel was poured in a horizontal gel running apparatus and allowed to stand for 1-2 h. Thirty minutes prior to the running of gel, 1X MOPS running buffer was poured in the gel apparatus.

RNA samples were prepared for electrophoresis by adding 40 µg of total RNA (11 µL max volume) to 2 µL of 10X MOPS; 6.6 µL of 37% formaldehyde; 25 µL of formamide and water to 50 µL. The samples were mixed well and incubated at 60°C for 15 min. Two µL of gel loading buffer (1mM EDTA, pH 8; 0.25% Bromophenol Blue; 0.25% xylene cyanol; 50% glycerol) were added to the sample before loading to the gel. The gel was run for 3 h at 40 V and washed twice, 15 min per wash, with 200 mL of water. The gel was then equilibrated for 45 min in 250 mL of 20X SSC with a gentle shaking and

transferred to GeneScreen nylon membrane by capillary blotting using TurboBlotter (Schleicher and Schuell, Keen, NH). Alternatively, nylon membrane was prepared for slot blot filtration manifolds by first soaking in water and then in 5X SSC for 5 min. The wet membrane was placed within the depression aligning the clipped corner of the membrane with the clipped corner of the depression, sandwiched between the two blocks of the slot blot apparatus (Hoefer Scientific Ins., San Francisco, CA). Twenty µg of total RNA sample was loaded (50 mL in volume) into each well and a vacuum of 40 mm Hg applied. Following the transfer, membrane was rinsed with 5X SSC and the RNA crosslinked to the membrane by exposing it to UV for 2 min. It was then baked at 80°C for 2 h prior to prehybridization.

The membrane was prehybridized for 2 h as described for Southern hybridization. However, the prehybridization was carried out at 37°C in the presence of 50% formamide in addition to 5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS and 1.0% (w/v) blocking reagent for nucleic acid hybridization in the prehybridization buffer. Hybridization was similarly carried out at 40°C for 14 h in the presence of 50 ng of DIG-labeled probe in 10 mL of prehybridization solution containing 50% formamide. Post hybridization washing, blocking and immunological detection were carried out as described earlier for Southern hybridization but with DEPC treated solutions and apparatus.

Western blotting

Protein Extraction and Separation by Electrophoresis

One gram FW of cells were frozen with liquid nitrogen and pulverized in a mortar and pestle. One mL of freshly prepared extraction buffer [62.5 mM Tris-HCl, 10% (v/v) glycerol, 1% (v/v) β-mercaptoethanol, 0.1% (w/v) sodium dodecylsulfate] was added to the frozen sample and allowed to thaw on ice. The homogenate was centrifuged at 4°C for 5 min at 14,000 x g and the supernatant used for determining total protein (Bradford, 1976). One hundred µg of total protein was mixed with equal volume of 2X Maizel sample buffer (20% sucrose, 8% SDS, 67 mM Tris, pH 6.8, 10% β-mercaptoethanol).

The samples were prepared for electrophoresis by boiling the contents in a microfuge tube in a water bath for 4-5 min and then transferring to ice until ready to load on to the gel. Two µL of Bromophenol Blue in methanol was added to the sample just before loading on to the gel. The standard gel running conditions consisted of 10% polyacrylamide running gel and 4% polyacrylamide stacking gel in the presence of 0.1% SDS (Hoefer Model SE-400 Vertical Slab Apparatus). The samples were subjected to electrophoresis for 8 h at 20 mA constant current in a 4°C cold room.

A Bio-Rad Trans Blot Cell (Bio Rad, Rockville Centre, NY) electroblotter was used for the transfer of protein from the gel to the membrane. The apparatus consisted of a 2.5 L tank and a electroblotter cassette containing two Scotch-Brite pads. Following electrophoresis, the gel was removed from the apparatus and equilibrated in the transfer buffer (25 mM Tris-glycine, pH 8.3, 25% v/v methanol) three times each for 20 min prior to transfer. The GeneScreen nylon membrane, cut to the size of the gel, was also equilibrated with the transfer buffer for a total of 20 min and then placed on top of two pieces of Whatman 3 MM filter paper prewet with transfer buffer. The gel was layered on the membrane, making sure that no air bubbles were trapped. Finally another two pieces of prewet Whatman 3MM paper were placed on the gel and the whole transfer stack was sandwiched between two Scotch-Brite pads and placed in the electroblotter cassette. The electroblotter was set up as per the manufacturer's instructions. The transfer of protein was done overnight at 20 V in the cold room. Following 14-16 h, the nylon membrane was removed from the electroblotter and air dried at room temperature.

Immunological Detection of ODC Protein

The ODC protein was detected in the Western blot colorimetrically using NBT and X-phosphate. All the steps were carried out at room temperature. The dried membrane

containing the protein was soaked in maleate buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5 using 10 N NaOH) for 2 min and transferred to a clean container containing 20 mL of blocking solution (2% Genius blocking reagent for nucleic acid hybridization in maleate buffer). The membrane in the blocking solution was gently shaken for 2 h. At the end of two hour, the blocking solution was poured out and replaced with 10 mL of primary incubation solution (1:10,000 dilution of Rabbit-anti-Mouse ODC antibody (Dr. Lo Persson, Dept. of Physiology, University of Lund, S-22362 Lund, Sweden) with blocking solution). The membrane was incubated in the primary incubation solution for 1 h. The membrane was then removed from the container and rinsed with maleate buffer twice and washed 2X, 15 min each, with excess amount of maleate buffer. The final washing was followed by incubation of the membrane in secondary incubation solution [1:5000 dilution of Goat-α-Rabbit IgG (Sigma chemicals) in blocking solution] with a gentle shaking for 30 min. The membrane was similarly rinsed and washed 2X with maleate buffer and transferred to a clean container. The membrane was equilibrated with 20 mL of Genius buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH adjusted to 9.5 and filtered with 0.44 μm membrane before use) for 5 min and placed on a clean Whatman 3 MM filter paper to soak excess buffer. Finally the membrane was transferred to color substrate solution (45 μL of NBT solution and 35 μL of X-phosphate in 10 mL of Genius buffer 3). Once the band was

visible and the color developed to satisfactory intensity, the membrane was washed with water to prevent over-development. The membrane was air dried before storing away for later photodocumentation.

RESULTS

Transformation of Carrot Cells

Nontransformed carrot hypocotyl segments tolerated the presence of 100, 150, 200 and 250 mg/L of kanamycin in the solid medium but did not show any growth in the presence of 300 mg/L kanamycin in the culture medium. After co-culture of hypocotyl segments with *A. tumefaciens* and incubation on the medium containing kanamycin, resistant calli were obtained in 6 to 7 weeks of culture. Only the hypocotyl segments precultured in 2,4-D-supplemented solid medium for 2-4 days showed any success (approximately 1-5%) in producing resistant calli. As shown in Fig. 5, resistant calli were generally produced at the cut ends of a hypocotyl segment. A callus clump obtained from one hypocotyl explant was maintained as a cell line and labeled as ODC-N1 or ODC-N2 or SAM-N1 etc. (depending upon the Agrobacterium strain used for co-culture). These calli exhibited normal growth after subculture on both solid and liquid media supplemented with 300 mg/L kanamycin. They also produced somatic embryos like the nontransformed cells that grew well both in the liquid and solid media. A few of the somatic embryos were removed from the medium and grown into whole plants in the

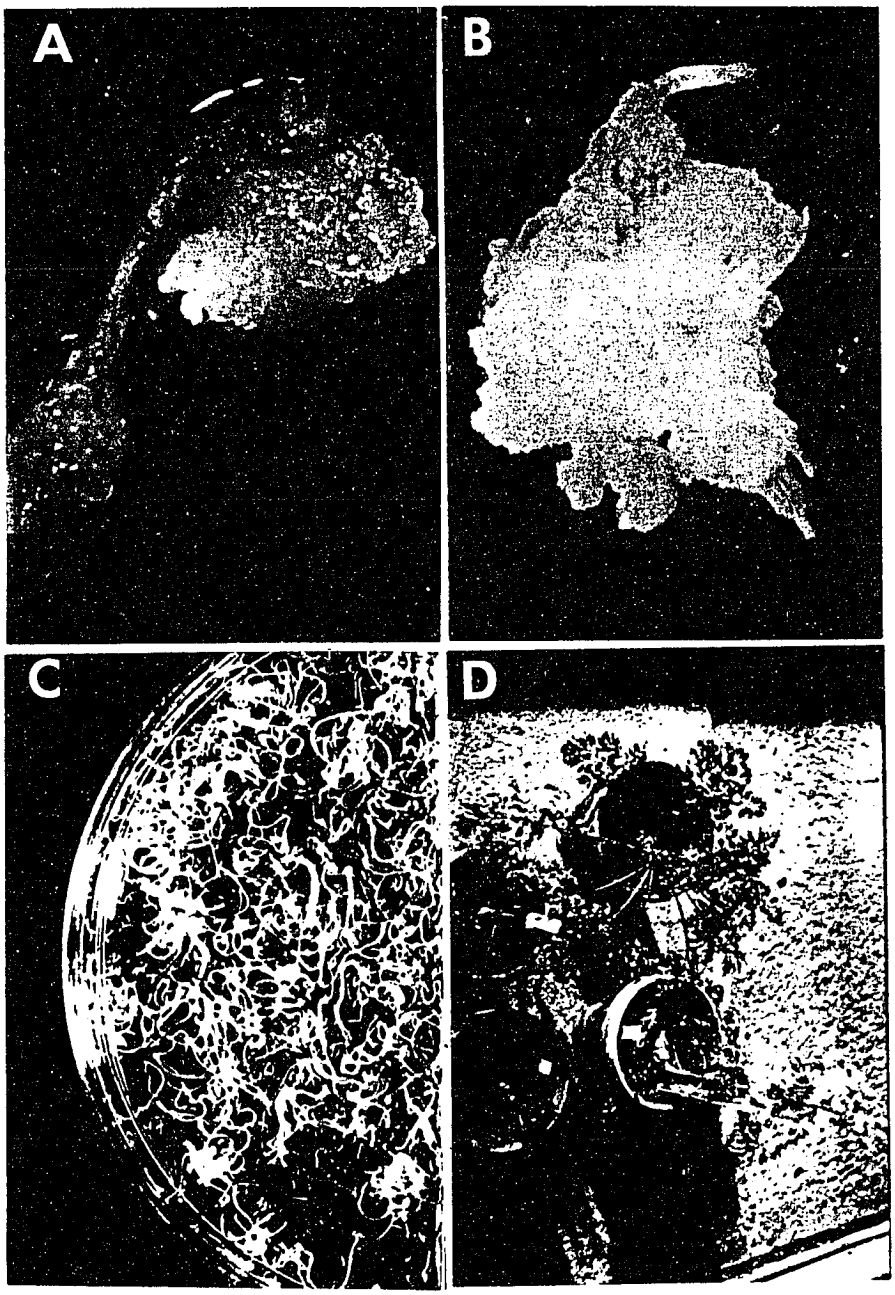

Figure 5. Different stages of development of the transgenic carrot plants. (A) callus originating from the cut ends of a hypocotyl co-cultured with *Agrobacterium tumefaciens;* (B) ODC transformed callus growing on a medium containing 300 mg/L kanamycin; (C) somatic embryos growing in auxin free medium; and (D) mature transgenic ODC plants.

greenhouse. Various developmental stages of *in vitro* grown embryos, from the time of callus induction to a mature flowering plant are shown in Fig. 5.

Neomycin Phosphotransferase (NPT-II) Assay

All of the ODC cell lines resistant to kanamycin showed the presence of varying levels of NPT-II protein while none of the nontransformed control lines showed the presence of this protein (Fig. 6). This protein was also found to be present in all the mature plants produced from the transformed cells (Fig. 7). The amount of detectable NPTII protein varied in different cell lines on different days of analysis but the protein was always present.

Confirmation of Stable Transformation

The putative ODC transgenic cells that were resistant to kanamycin were 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 NPT-II gene. While all of the transgenic cell lines showed the amplified PCR products for ODC as well as NPT (Fig. 8) in agarose gels, the nontransformed control cells did not yield any positive amplification signal. Southern hybridization of the amplification product for ODC showed hybridization of the

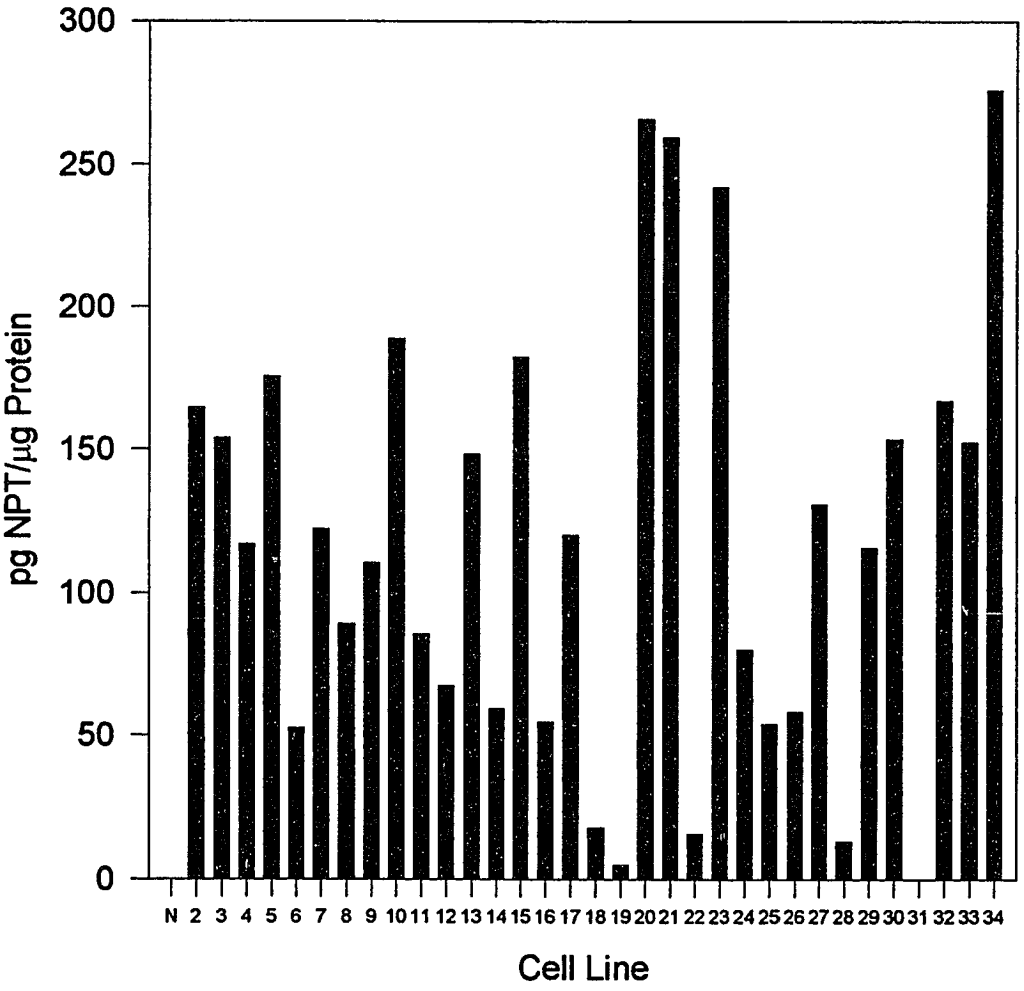

Figure 6. Neomycin phosphotransferase (NPT) protein levels in the nontransformed and several transgenic carrot cell lines; N= nontransformed cells; 2-30= transgenic ODC cells; 31= blank; 32= ODC-N1 embryos; 33= SAM1 cells; 34= SAM1 embryos.

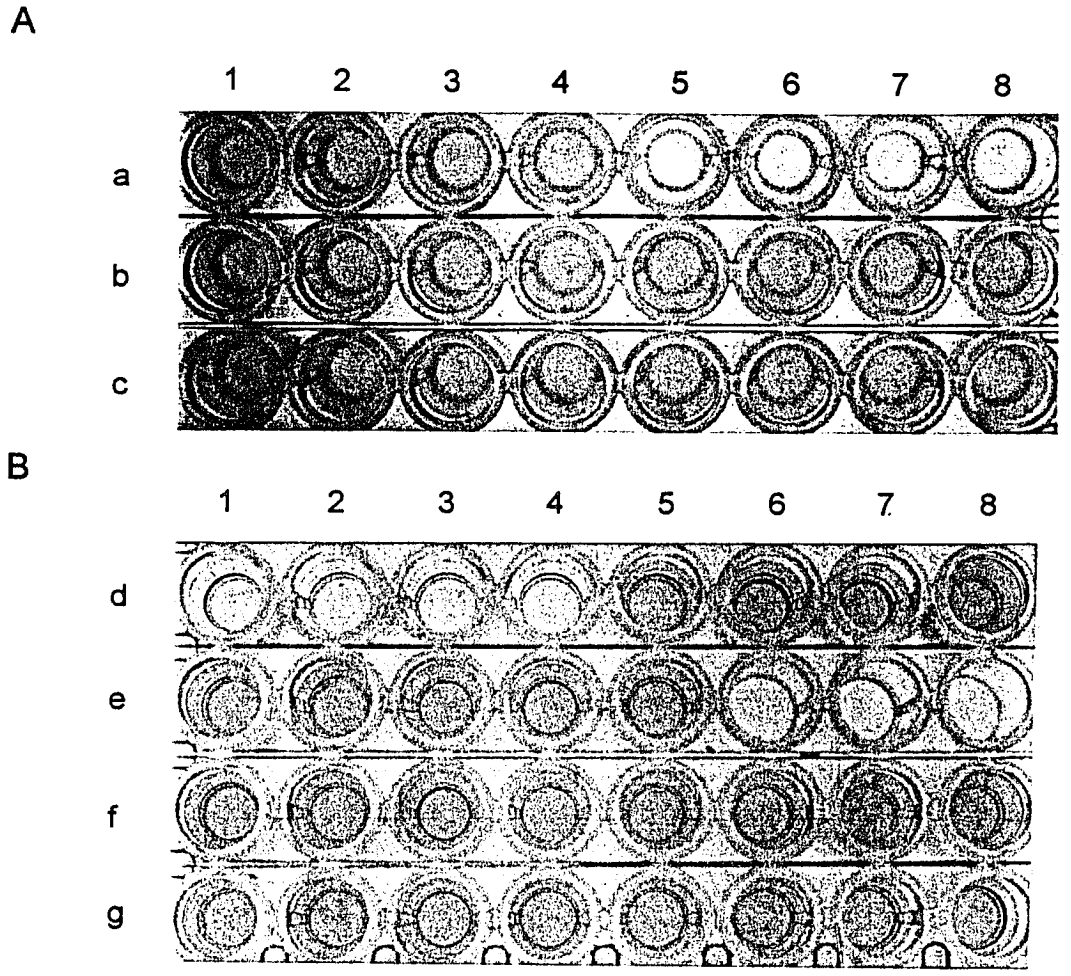

Figure 7. A photographic representation of ELISA for the detection of neomycin phosphotransferase (NPT) in transgenic carrot cells in culture and greenhouse grown plants. (A) transgenic ODC cell lines (B) transgenic SAMDC cell lines. a1-4= 150, 100, 50 and 20 pg standard NPT protein; a5-6= blank; a7-8= nontransformed control; b1-4= N1, N26, N12(G) and N10 plants from green house; b5-8= N1, N10, N12, N14 from *in vitro* cultures; c1-4= replicate of b1-4; c5-8= N12, N18, N19 and N20 *in vitro* cultures; d1-2= nontransformed control; d3-4= blank; d5-8= 20, 50, 100 and 150 pg standard NPT protein; e1-5= SAM-19, SAM19-1, SAM10-2, SAM1-0, SAM10-4; e6-8= empty; f1-8= SAM10-3, SAM1-2, SAM4, SAM1-1, SAM3-0, SAM2-0, SAM4-1, SAM4-2; g1-8= SAM19-3, SAM2-3, SAM19-4 plants, SAM1, SAM2, SAM3, SAM4, SAM5 *in vitro* cultures.

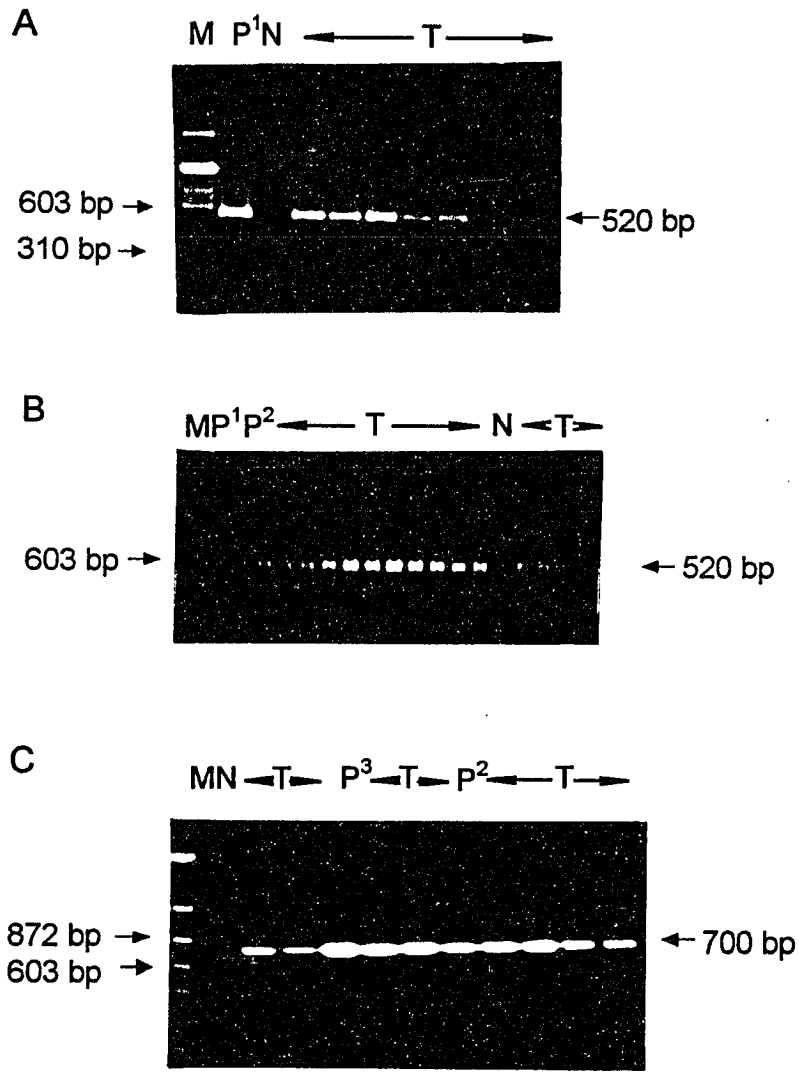

Figure 8. Agarose gel electrophoresis of PCR products obtained by using (A,B) ODC and (C) NPT primers with the genomic DNA from various transgenic ODC cell lines. M= MW marker (ϕX174 HaeIII digest); P^1= pUO-1; P^2= AT35SODC^t; P^3= pBI121; N= nontransformed cells; T= transgenic cells; Lanes from left to right are: (A) Marker, P^1, N, N1, N2, N10, N11, N12, N14 and N16; (B) Marker, P^1, P^2, N10, N11, N12, N19, N20, N23, N25, N26, N28, N9, N13, N, N21, N24, N1, N2 and N3; (C) Marker, N, N13, N3, P^3, N1, N2, P^2, N10, N12, N15 and N25.

labeled probe to the PCR product (Fig. 9). The amplification by PCR using ODC and/or NPT-primers were done using template DNA isolated at more than 3 different times during a 3 year period.

In order 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 DNA from nontransformed carrot cells was used as control. All of the samples of DNA obtained from control nontransformed cells (more than three separate DNA isolations) showed three bands in the EcoRI digest that were larger than 4 kb in size (Fig. 9). The same bands were also present in all the transgenic cell lines. However, only the transgenic cell lines showed an additional 2.1 kb band of mouse ODC, as expected (Fig. 9) with EcoRI digest. These bands of higher molecular weight disappear if the hybridization was carried out at 42°C in a hybridization buffer containing 50% formamide or the post hybridization wash was done with 0.5X wash solution pre-heated to 65°C.

HindIII digest of transformed plant DNA would be expected to show a DNA fragment approximately 0.8 kb in size in addition to a large fragment of variable size depending upon the site of integration of T-DNA in the plant genome (Fig 9). As expected, all of the putative transformants

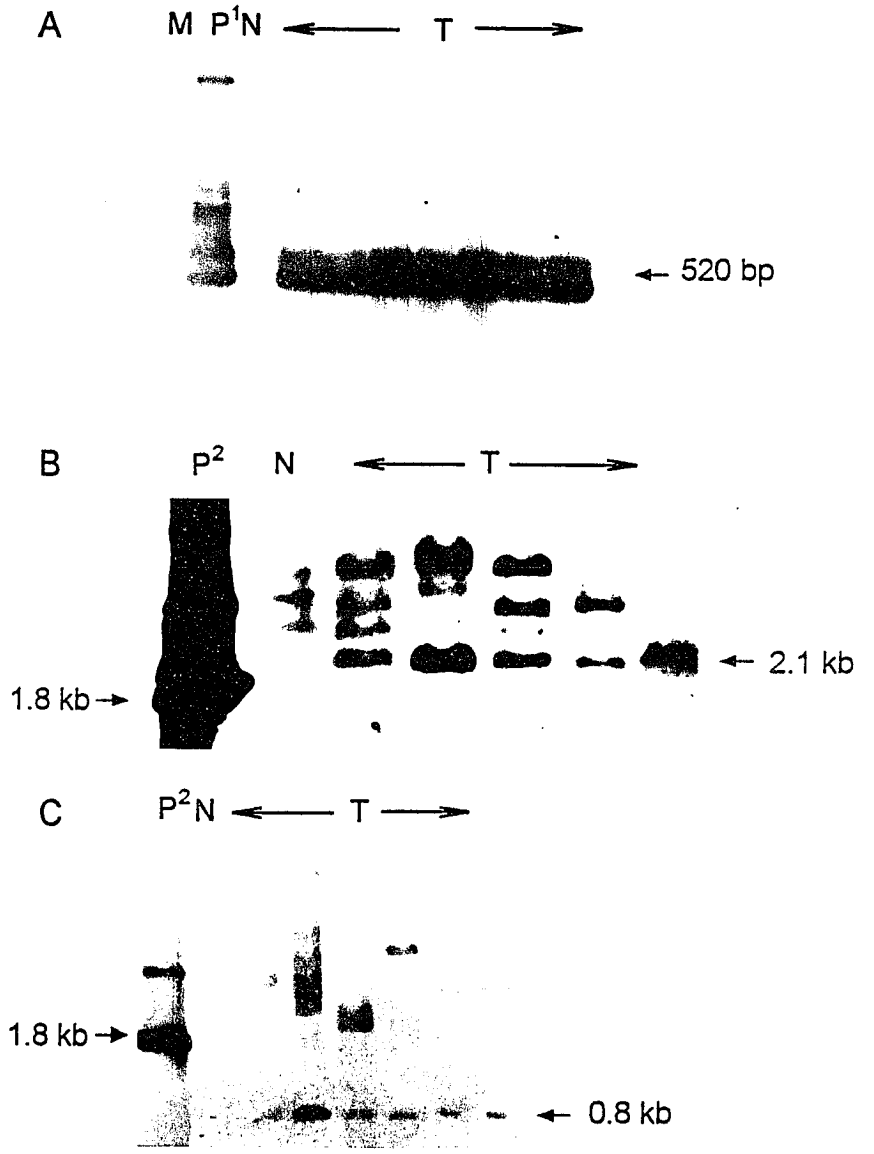

Figure 9. Southern hybridization of (A) PCR products in Figure 8A using biotin-labeled ODC probe; Southern hybridization of genomic DNA digested with (B) EcoRI; and (C) HindIII using DIG-labeled ODC probe. 10 µg of DNA was digested and loaded in each well. M= MW marker (ϕX174 HaeIII digest); P^1= pUO-1; P^2= 1.8 kb DNA fragment (EcoRI/XbaI digest of pU0-1); N= nontransformed cells; T= transgenic ODC cell lines. Lanes from left to right are: (A) Marker, P^1, N, N1, N2, N10, N11, N12, N14 and N16; (B) P^2, N, N1, N10, N14, N12, N19; (C) P^2, N, N1, N10, N12, N14, N19, N26 (plant grown in green house).

that were examined showed the 0.8 kb internal HindIII fragment (Fig. 9). Additional fragments larger than 1.3 kb represent the ODC cDNA adjacent to the right border and a part of the plant DNA.

More than three separate isolations of DNA from the cell lines N1, N10, N12, N14, N19 and N20 have been analyzed by Southern hybridization. However, DNA from the transgenic ODC-N12(G) and N26 plants has been analyzed only once by this method. Both of these plants showed the 2.1 kb fragment in the EcoRI digest of the total DNA.

The Northern slot blot analysis showed no RNA signal in the nontransformed cell lines. Several transgenic ODC cell lines that contained mouse ODC cDNA showed the presence of RNA that hybridized with the mouse cDNA probe (Fig. 10). Similarly, analysis of protein extracts by western blotting on the SDS-PAGE (Fig. 10) showed three strong immuno-reactive signals that were unique to transgenic cell lines.

Ornithine Decarboxylase Activity

It has previously been reported that nondifferentiating carrot cell cultures do not contain ODC activity (Feirer et al., 1984; Robie and Minocha, 1989). In the present study also, little ODC activity was observed in the nontransformed

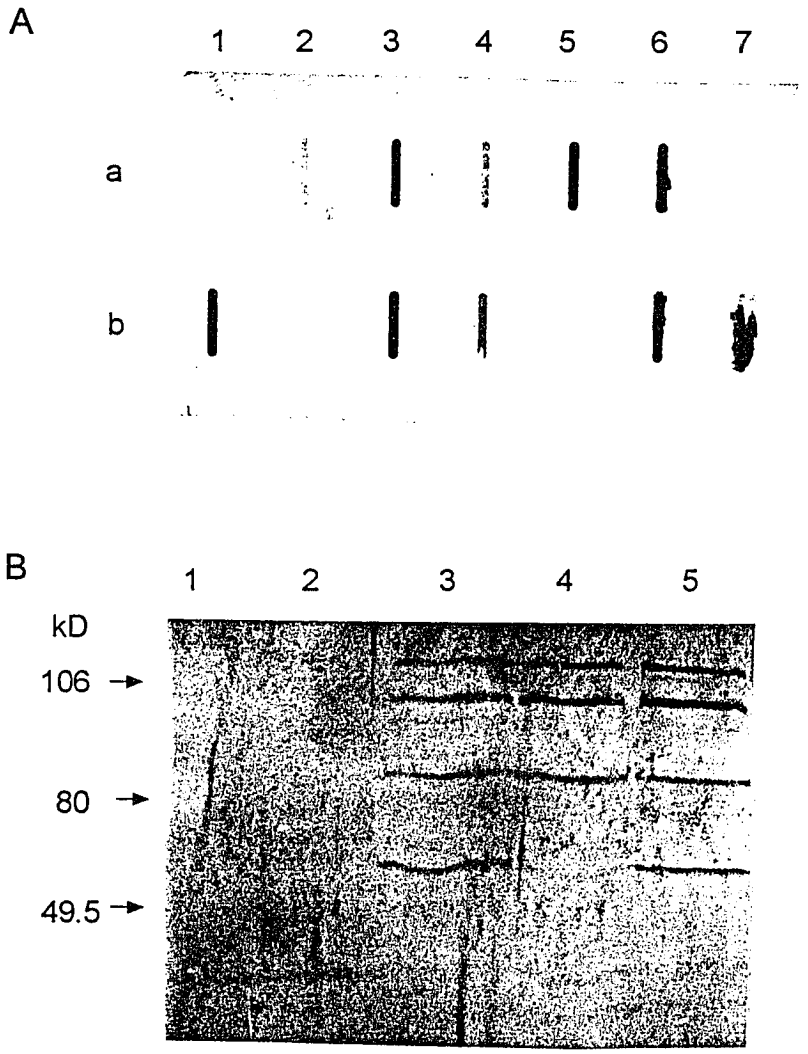

Figure 10. A= Northern hybridization of 20 µg of total RNA from nontransformed and several transgenic ODC cell lines. a1 and b2= nontransformed cells; a2-a7= N1, N10, N12, N14, N19, blank; b1= N20; b3-b7= N10, N12, N1, N14, N19. B= Western blot analysis of nontransformed and several transgenic ODC cell lines using rabbit-anti-mouse ODC antibody. Lanes are: 1= MW marker; 2= nontransformed cells; 3-5= N1, N10 and N14.

cultures grown in the presence of 2,4-D. However, all of the transgenic cell lines that tested positive by PCR amplification contained substantial amounts of ODC activity (Fig. 11). Many of these cell lines have been analyzed for ODC activity several times over a period of more than 2 years. With the exception of one cell line (ODC-N1) that lost most of the ODC activity after about one year of subculture, all others have maintained high levels of ODC. The enzyme activity varied in different cell lines when tested at different times during the 2-year period.

While the mere presence of high ODC activity in transgenic carrot cell cultures indicates expression of the mouse ODC cDNA, the enzyme was further characterized in several ways to be specifically of mouse type and not plant type. It is known that the mouse enzyme differs from the native plant ODC (leaf tissue) in at least three important characteristics (Robie, 1987). These are: (1) the optimum pH for mouse ODC is around 7.0 whereas carrot ODC has a pH optimum of 8.2 to 8.4; (2) the mouse ODC activity is extremely sensitive to DFMO while the carrot ODC is not; and (3) the mouse ODC activity can be eliminated by treatment with anti-mouse ODC antiserum. Results presented in Fig. 12 clearly show that the enzyme activity observed in transgenic cells was maximum at pH 6.8 to 7.2, and it decreased substantially at pH 8.2. The low levels of ODC in control

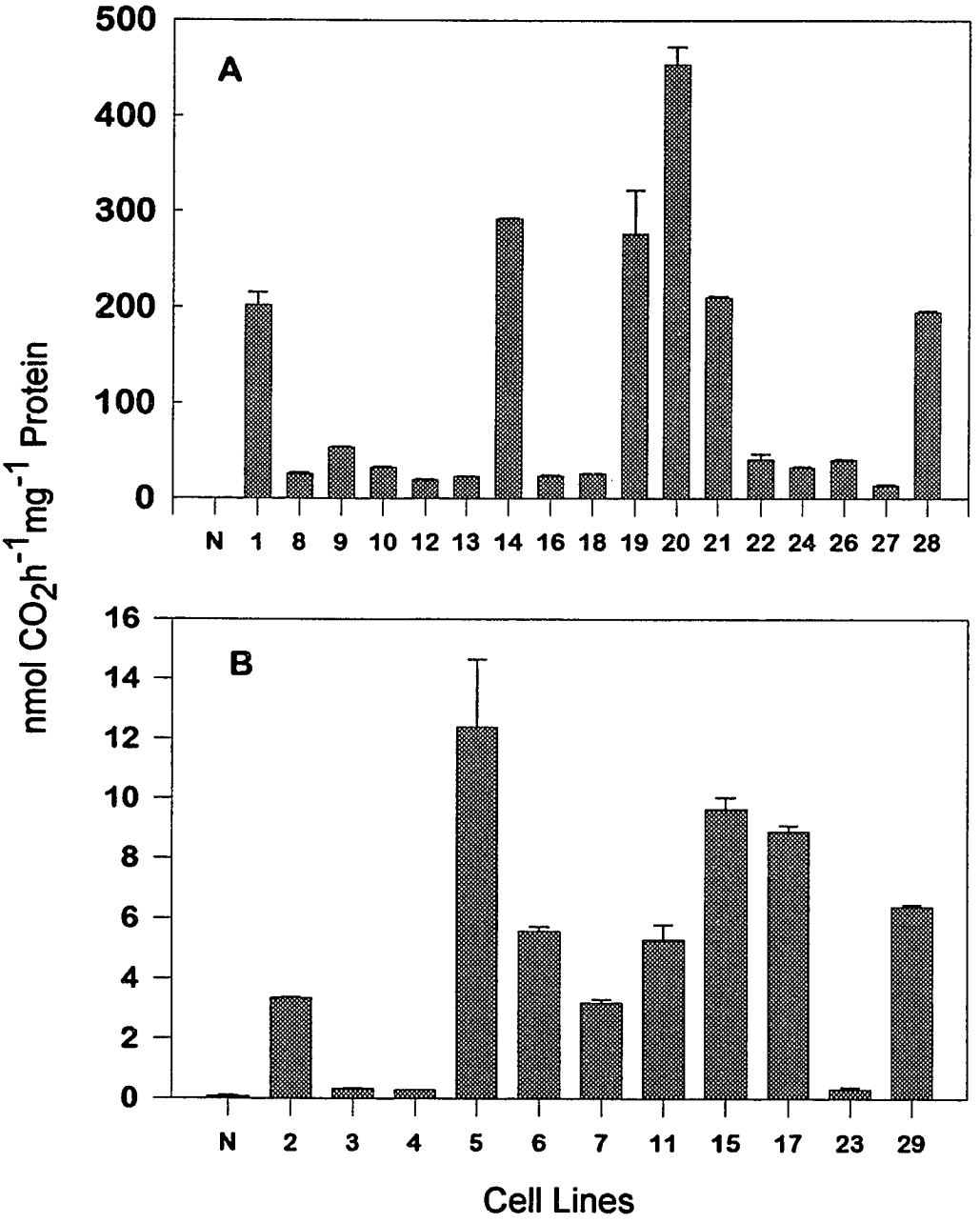

Figure 11. (A-B) ODC enzyme activity in nontransformed control (N) and different transgenic carrot cell lines grown in the absence of 2,4-D for three days. The enzyme was extracted and assayed at pH 6.8. No ODC activity was detected in nontransformed control (N) at this pH. Values are mean ±SE of three replicates.

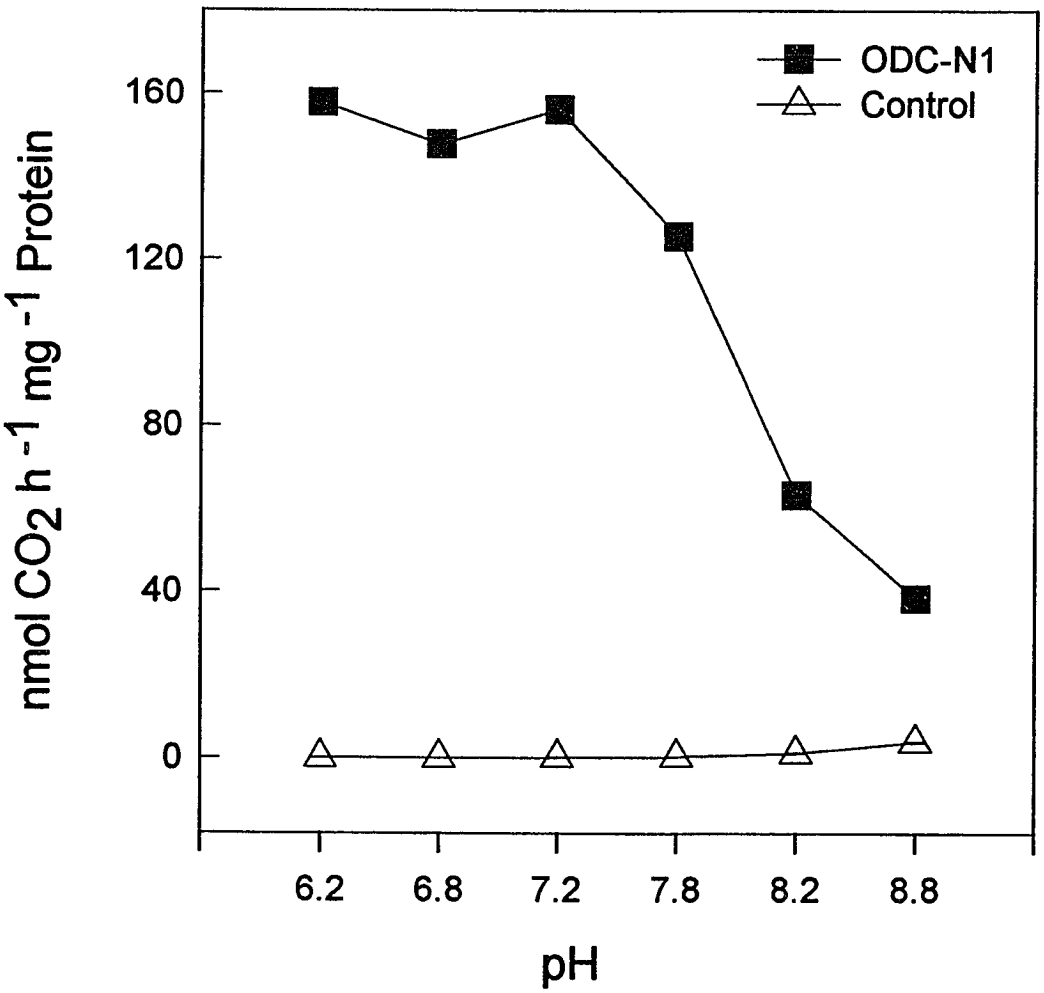

Figure 12. The effect of pH on ODC enzyme activity in nontransformed control (N) and transgenic ODC cells (N1) grown in the absence of 2,4-D for three days. Cells were homogenized as well as assayed in the same buffer adjusted to different pH in each case. Values are mean ±SE for three replicates. The SE bars are all smaller than the symbols.

cells did not allow an accurate testing of the pH optimum of plant ODC. The effects of DFMO on ODC activity in transgenic cells also show its sensitivity to this suicide inhibitor. As shown in Table 7, the ODC activity in all transgenic cell lines was completely inhibited by 2 mM DFMO. Likewise, the activity of ODC was largely inhibited by anti-mouse ODC antiserum in all the transgenic cell lines.

Cellular Polyamine Levels

Other than the three major polyamines (putrescine, spermidine and spermine), no other PCA soluble derivatives of polyamines were observed in control cells. All transformed cell lines showing high levels of ODC enzyme activity also exhibited increased levels of cellular putrescine (Fig. 13). A ten-to-twenty fold increase in putrescine was often observed in different transgenic lines as compared to the nontransformed control cells on any given day. As with ODC activity, the cellular putrescine levels were quite variable in different transgenic cells when tested on different days during the 2 year study period. Although putrescine increased significantly, the level of spermidine remained unaffected, and that of spermine was generally lower as compared to nontransformed cells (Fig. 14). Several transgenic lines that showed high levels of ODC activity and higher levels of putrescine, also showed

Table 7. Effect of 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. No activity was detected at pH 8.4. Values are mean ±SE for three replicates.

Cell Line	DFMO [mM]		% inhibition
	0	2	
Control (N)	0	0	0
N1	139.76 ±3.48	0.60 ±0.01	99.6
N9	13.39 ±0.15	2.25 ±0.15	83.2
N10	49.28 ±0.43	0.20 ±0.02	99.6
N12	4.48 ±0.02	-0.14	100.0
N14	66.85 ±4.19	1.72 ±0.09	97.4
N19	54.22 ±0.78	2.65 ±0.21	95.1
N20	222.99 ±2.61	0.39 ±0.02	99.8
N22	119.28 ±0.81	0.40 ±0.02	99.7
N26	63.29 ±0.15	-0.04	100.0

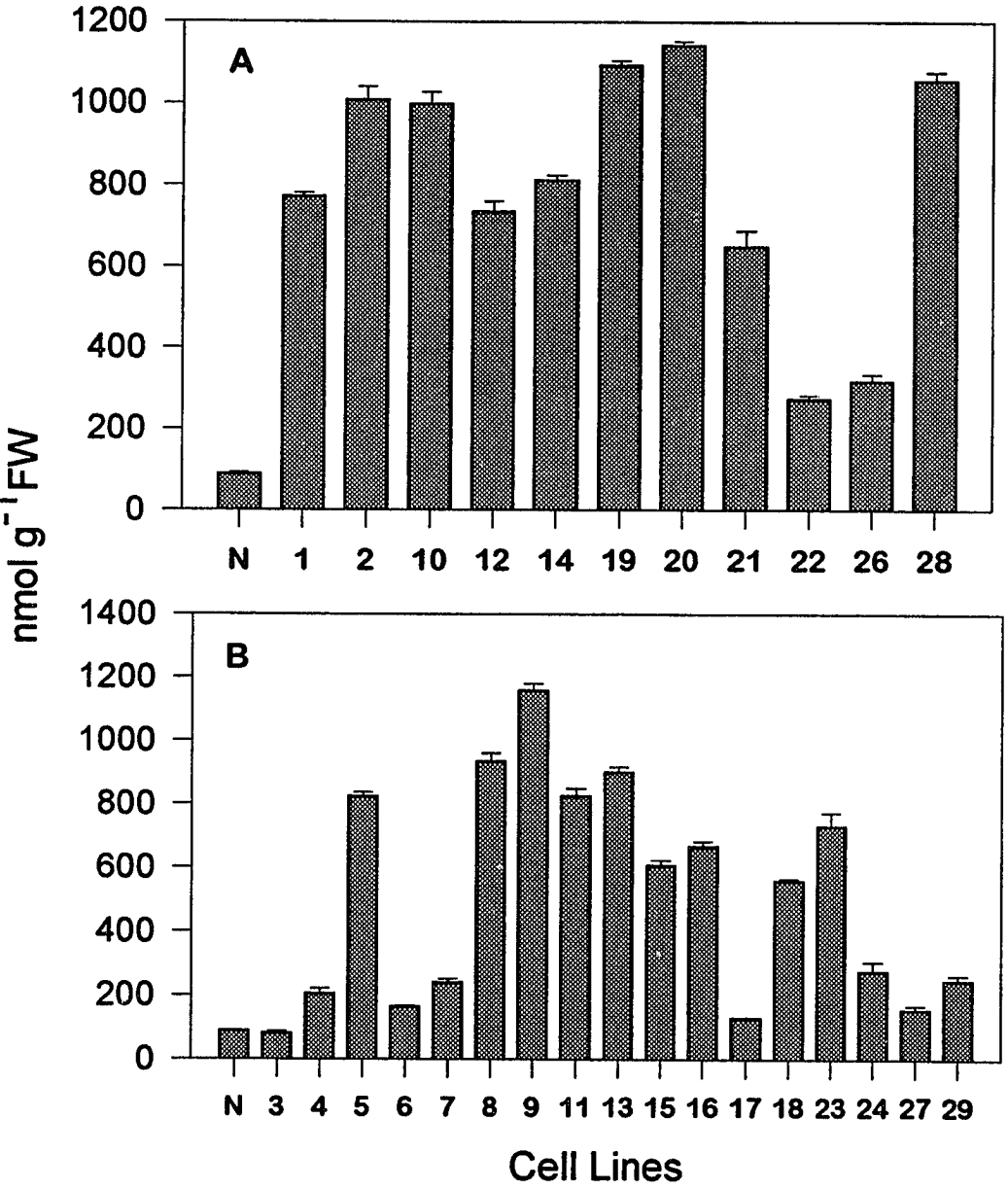

Figure 13. (A-B) Cellular levels of putrescine in nontransformed control (N) and several transgenic ODC cell lines. The cells were grown in the absence of 2,4-D for three days. Values are means ±SE of three replicates.

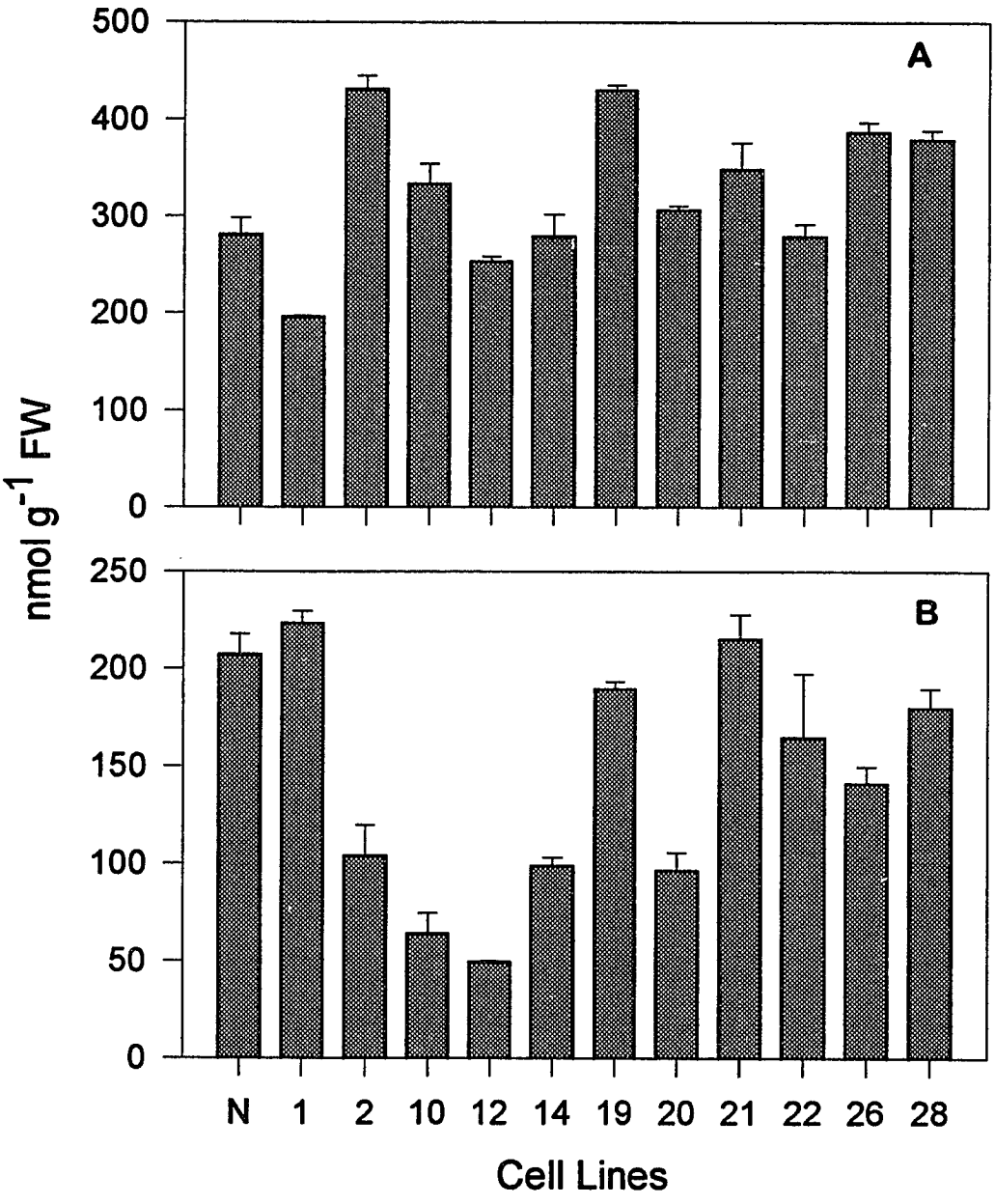

Figure 14. Cellular levels of (A) spermidine and (B) spermine in nontransformed control (N) and several transgenic ODC cell lines. The cells were grown in the absence of 2,4-D for three days. Values are means ±SE of three replicates.

the presence of an additional dansylamine immediately following putrescine in the HPLC chromatograms. This peak was identified to be cadaverine based on its elution time and internal spiking in HPLC (Fig. 15). This peak was never observed in the nontransformed cells and a few of the transgenic cell lines (Fig. 16).

Primarily due to increased putrescine, total polyamines were significantly higher in all of the transgenic cell lines than those in the control cells (Table 8, Fig. 16a). The proportion of putrescine as a percentage of total polyamines varied from about 11% to as high as 74%. For spermidine this range was 12-58% and for spermine 4-34%.

Time Course Studies on the Cellular Polyamine Levels

Some of the transgenic cell lines (N1, N10, N12, N14 and N19) were analyzed several times for cellular polyamine levels during a week long culture period. In these representative cell lines, the cells were collected at different times (0 h-5 d) after 3 d old cells were washed and transferred to 2,4-D free medium as described in materials and methods.

For all the short-term treatments, samples were collected at the time of transfer to fresh media, and 3, 6

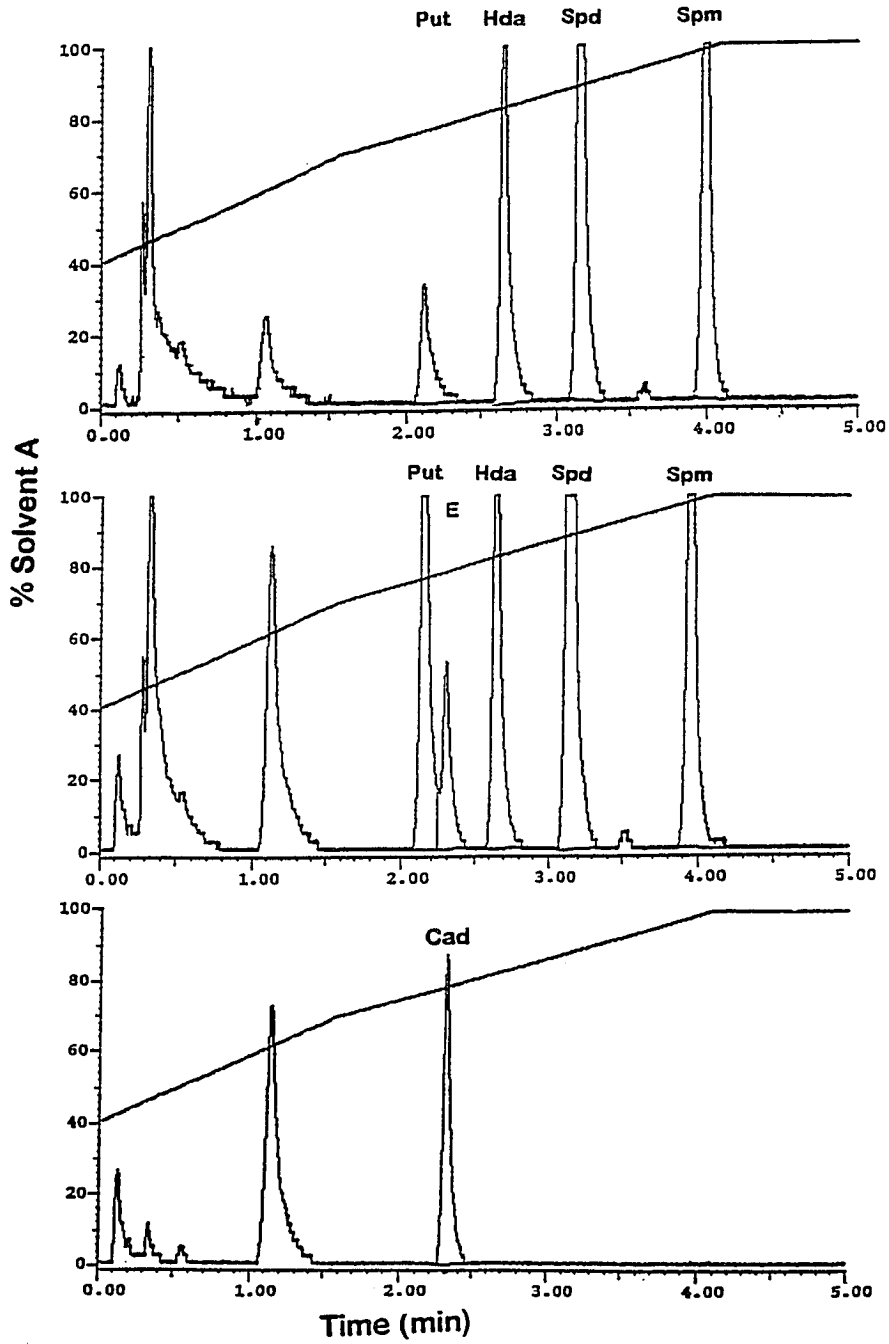

Figure 15. (A-C) Representative HPLC chromatographs showing separation of dansylpolyamines in different cell lines of carrot. (A) nontransformed cells; (B) transgenic cells (N14); and (C) dansylated cadaverine. Put= putrescine, Hda= heptanediamine, Spd= spermidine, Spm= spermine, E= extra peak. Gradient profile described in Table 5 was used for separation.

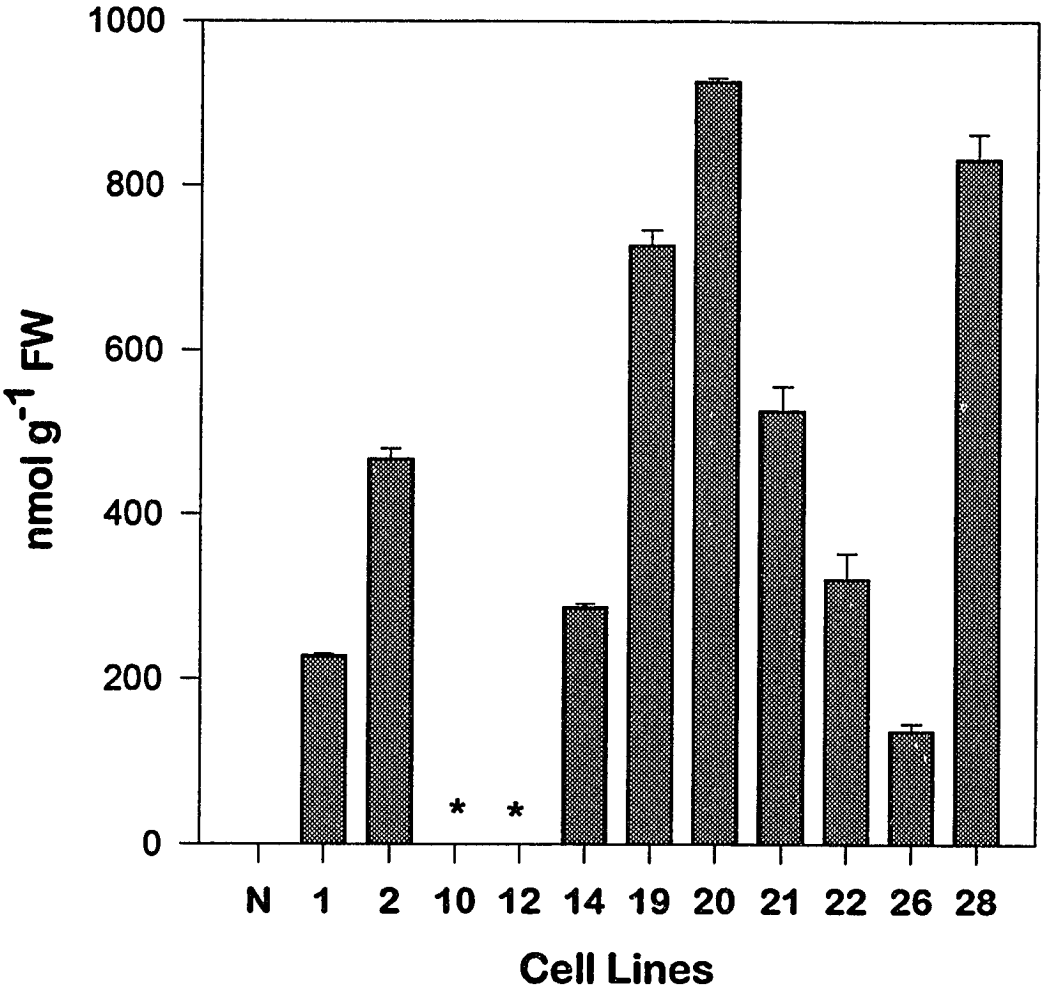

Figure 16. Cellular levels of cadaverine in various transgenic ODC cell lines. (*) = trace amount. The cells were grown in the absence of 2,4-D for three days. Values are means ±SE of three replicates.

Table 8. Summary of the proportion (% of total) of different polyamines in nontransformed (N) and several transgenic cell lines. (A) cell lines that produce cadaverine; (B) cell lines that do not produce cadaverine. (*) produced trace amount of cadaverine, ND= not detected

(A)

Cell Line	Total Polyamines (nmol/g FW)	% due to			
		Putrescine	Spermidine	Spermine	Cadaverine
N	567	15	49	36	0
N1	1418	54	14	16	16
N2	917	31	12	2	55
N6	736	22	45	20	13
N7	1186	20	36	16	28
N10	1394	71	24	5	*
N12	1035	71	24	5	*
N14	1475	55	19	7	19
N18	802	70	25	5	*
N19	2438	45	18	8	30
N20	2513	47	12	4	37
N21	1738	37	20	12	30
N22	1036	26	27	16	31
N24	673	41	39	8	12
N26	1016	35	38	14	13
N27	855	18	51	26	5
N28	2321	42	16	8	34
N29	796	31	43	17	9

(B)

N	567	15	49	36	ND
N3	711	11	55	34	ND
N4	992	21	58	21	ND
N5	1220	67	27	5	ND
N8	1285	73	23	4	ND
N9	1558	74	22	4	ND
N11	1300	63	31	6	ND
N13	1449	62	23	15	ND
N15	1278	47	47	6	ND
N16	1113	60	32	8	ND
N17	441	29	54	17	ND
N23	1175	62	31	7	ND

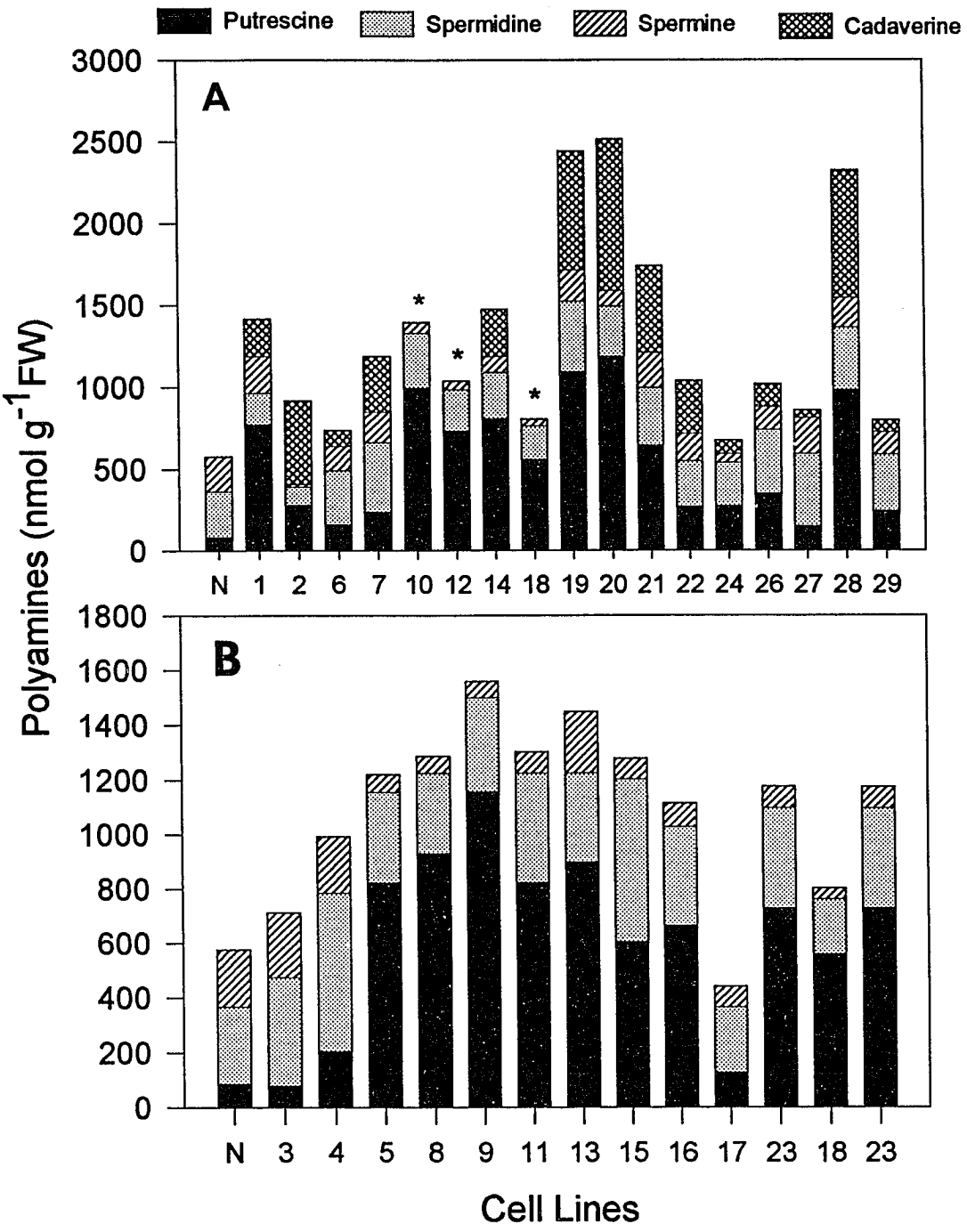

Figure 16a. Cellular polyamine levels in (N) nontransformed and several transgenic ODC cell lines. (A)= transgenic ODC cell lines that produce cadaverine; (B)= transgenic ODC cell lines that do not produce cadaverine. (*) produced trace amount of cadaverine.

and 12 h thereafter. The cellular putrescine as well as total polyamine levels were significantly lower in nontransformed cells at any given time. Polyamine levels showed only small changes during this period.

In experiments involving long-term cultures, samples were collected at 0, 1, 3 and 5 d following the transfer of 3 d old cells to fresh embryogenic media. The nontransformed cells generally showed a decrease in cellular putrescine levels and also total polyamines during the 5 d of the experiment. However, all of the transgenic cell lines that were analyzed showed significant increase in putrescine levels after one day in fresh medium. Thereafter, a decrease in total polyamines was observed in these cells also. Once again total polyamines were always higher in transgenic cells as compared to control cells on any given day.

Effect of DFMO

The effects of DFMO on cellular polyamine levels in nontransformed and transgenic cell lines (N14) are shown in Fig. 17. An increase in putrescine levels was observed in the presence of 1 mM DFMO in nontransformed cells at 24h. Transgenic cells on the other hand, showed inhibition by DFMO at all times. The effects were more pronounced during

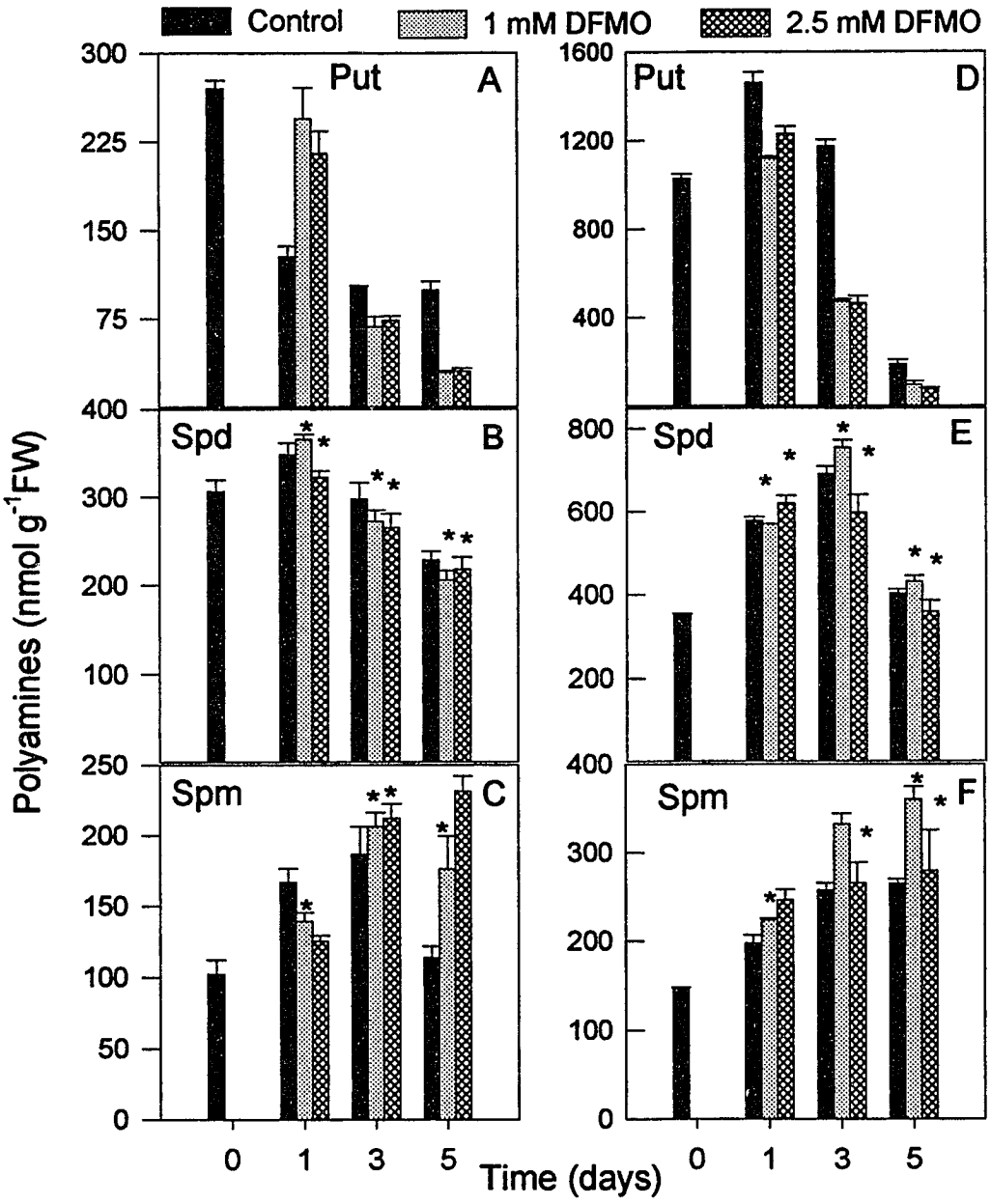

Figure 17. Effect of DFMO on cellular polyamine levels in (A-C) nontransformed and (D-F) transgenic ODC-N14 carrot cells. 7 d old carrot cells were washed with 2,4-D free B5 medium and cultured for 3 d prior to treatment with DFMO and aliquots collected at different times as indicated above. Time 0 refers to the time of DFMO addition. Values are means ±SE of three replicates. All the values for DFMO treatment except those with * are significantly different ($p \leq 0.05$) from the control cells at a given time.

the long-term treatments. It is also observed that the inhibitory effect of DFMO on transgenic cells was independent of the concentration of DFMO in the medium. While no significant changes were seen in the levels of spermidine, the levels of spermine were slightly higher in DFMO-treated nontransformed cells. In transgenic cells spermine was unaffected.

Effect of DFMA

The effects of two different concentrations of DFMA (0.05 and 0.1 mM) on cellular levels of polyamines are shown in Fig. 18. Nontransformed cells showed a significant reduction in putrescine concentration within 6 h of treatment. However, total polyamine levels were reduced significantly only after 12 h of treatment in these cells. Putrescine levels in the transgenic cells were also significantly decreased within 3 h. This decrease in putrescine level also contributed to the decrease in total polyamines in these cells. In longer term treatments, DFMA caused almost a complete loss of putrescine in the non-transgenic cells by day 3 and 5. Similarly, by day 5 in transgenic cells also, the putrescine levels were very low but still significantly higher than those in control cells. Cellular spermidine levels were also lowered by DFMA in all

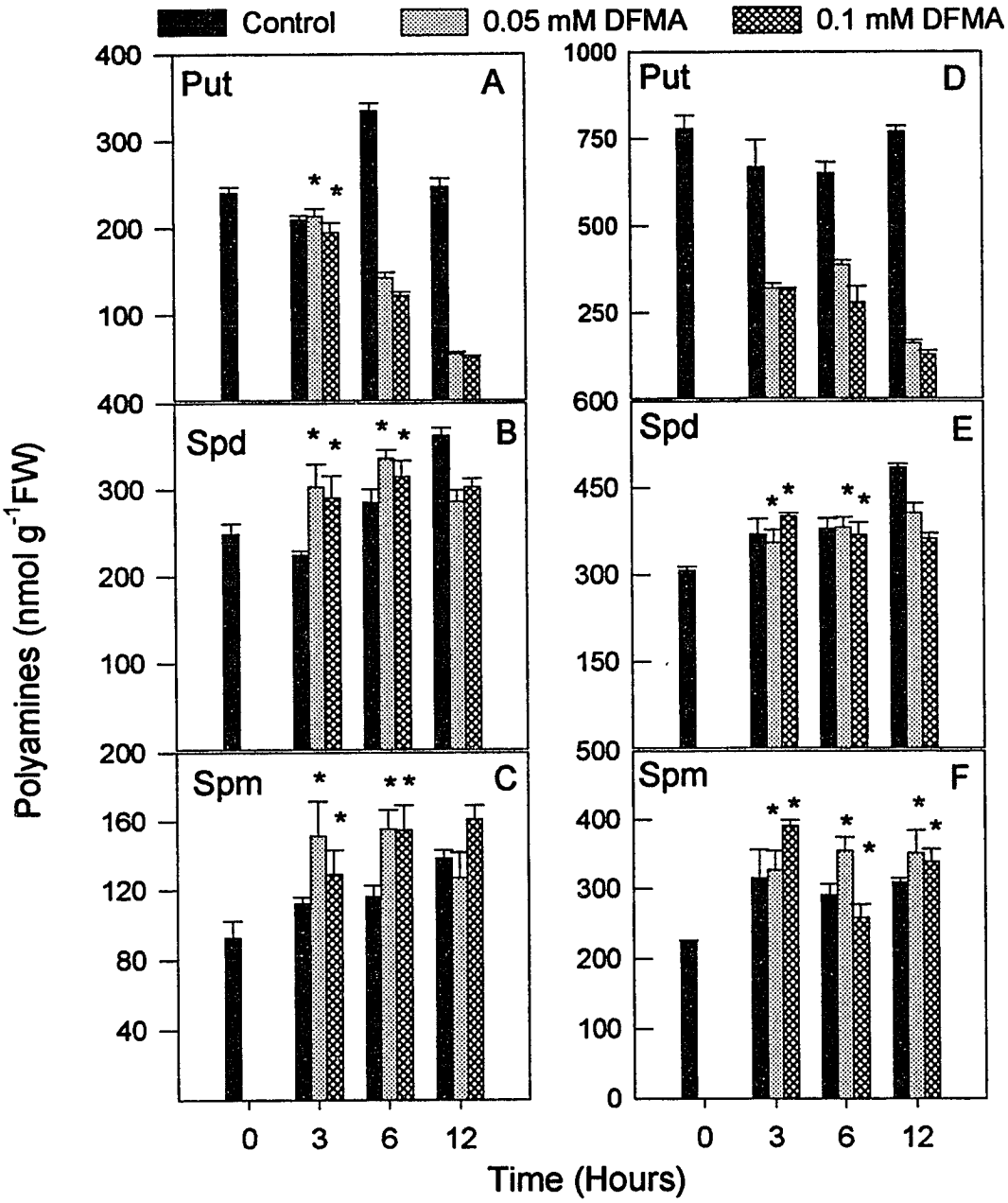

Figure 18. Effect of DFMA on cellular polyamine levels in (A-C) nontransformed and (D-F) transgenic ODC-N14 carrot cells. 7 d old carrot cells were washed with 2,4-D free B5 medium and cultured for 3 d prior to treatment with DFMA and aliquots collected at different times as indicated above. Time 0 refers to the time of DFMA addition. Values are means ±SE of three replicates. All the values for DFMA treatment except those with * are significantly different ($p \leq 0.05$) from the control cells at a given time.

cases beyond 12 h of treatment. Spermine was not significantly affected. Both the concentrations of DFMA used in these experiments had significant effects.

Addition of 1 mM DFMO in the presence of 0.5 mM DFMA in the medium seemed to partially counteract the inhibitory effects of DFMA on the cellular levels of putrescine in the nontransformed cells. Similar treatment on the transgenic cells showed an additive effect leading to a further decrease in putrescine concentration (Fig. 19)

Effect of MGBG

The effects of two different concentrations of MGBG on the cellular polyamine levels in nontransformed and transgenic cell lines are shown in Fig. 20. Both types of cells showed a significant increase in the level of putrescine in the presence of MGBG. Generally the effect was greater with the higher concentration of MGBG. Higher concentration of MGBG also slightly inhibited spermidine and spermine levels.

Effect of L-Ornithine

The effects of exogenous ornithine on the cellular polyamine concentration are shown in Fig. 21 and Fig. 22.

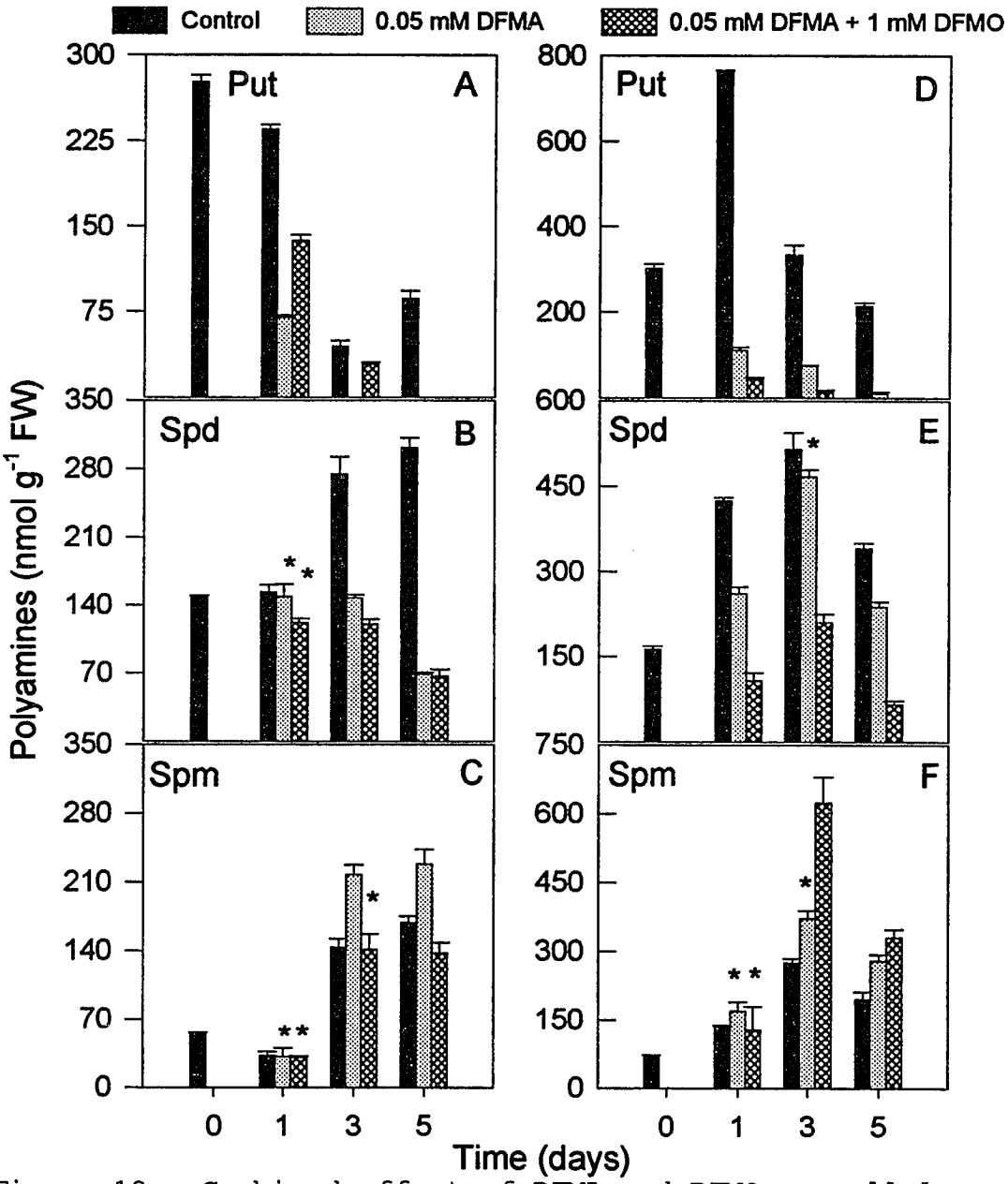

Figure 19. Combined effect of DFMA and DFMO on cellular polyamine levels in (A-C) nontransformed and (D-F) transgenic ODC-N14 carrot cells. 7 d old carrot cells were washed with 2,4-D free B5 medium and cultured for 3 d prior to treatment with the inhibitors and aliquots collected at different times as indicated above. Time 0 refers to the time of inhibitor addition. Values are means ±SE of three replicates. All the values for inhibitor treatment except those with * are significantly different ($p \leq 0.05$) from the control cells at a given time.

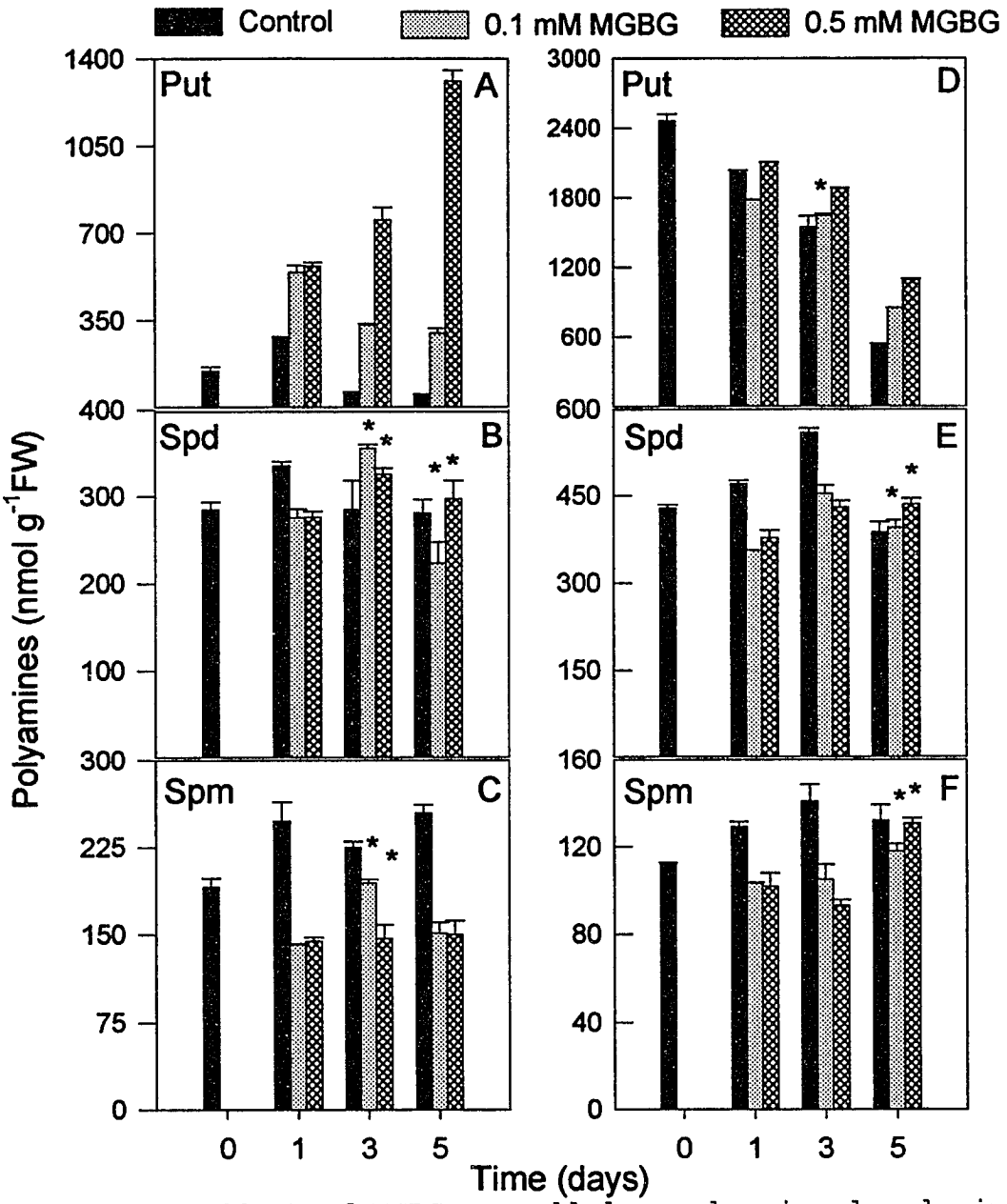

Figure 20. Effect of MGBG on cellular polyamine levels in (A-C) nontransformed and (D-F) transgenic (ODC-N19) carrot cells. 7 d old carrot cells were washed with 2,4-D free B5 medium and cultured for 3 d prior to treatment with MGBG and aliquots collected at different times as indicated above. Time 0 refers to the time of MGBG addition. Values are means ±SE of three replicates. All the values for MGBG treatment except those with * are significantly different ($p \leq 0.05$) from the control cells at a given time.

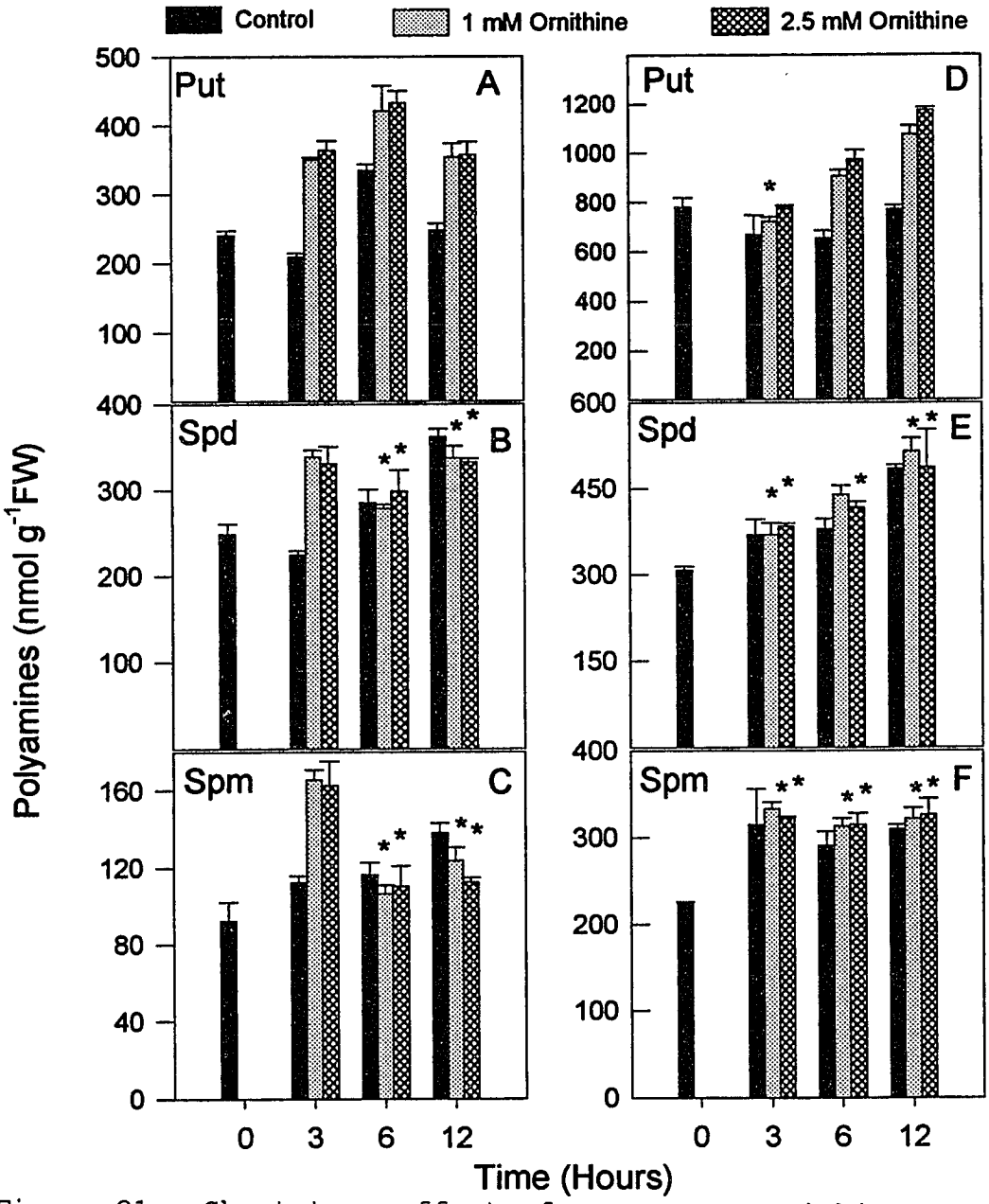

Figure 21. Short term effect of exogenous ornithine on cellular polyamine levels in (A-C) nontransformed and (D-F) transgenic (ODC-N14) carrot cells. 7 d old carrot cells were washed with 2,4-D free B5 medium and cultured for 3 d prior to treatment with ornithine and aliquots collected at different times as indicated above. Time 0 refers to the time of ornithine addition. Values are means ±SE of three replicates. All the values for ornithine treatment except those with * are significantly different ($p \leq 0.05$) from the control cells at a given time..

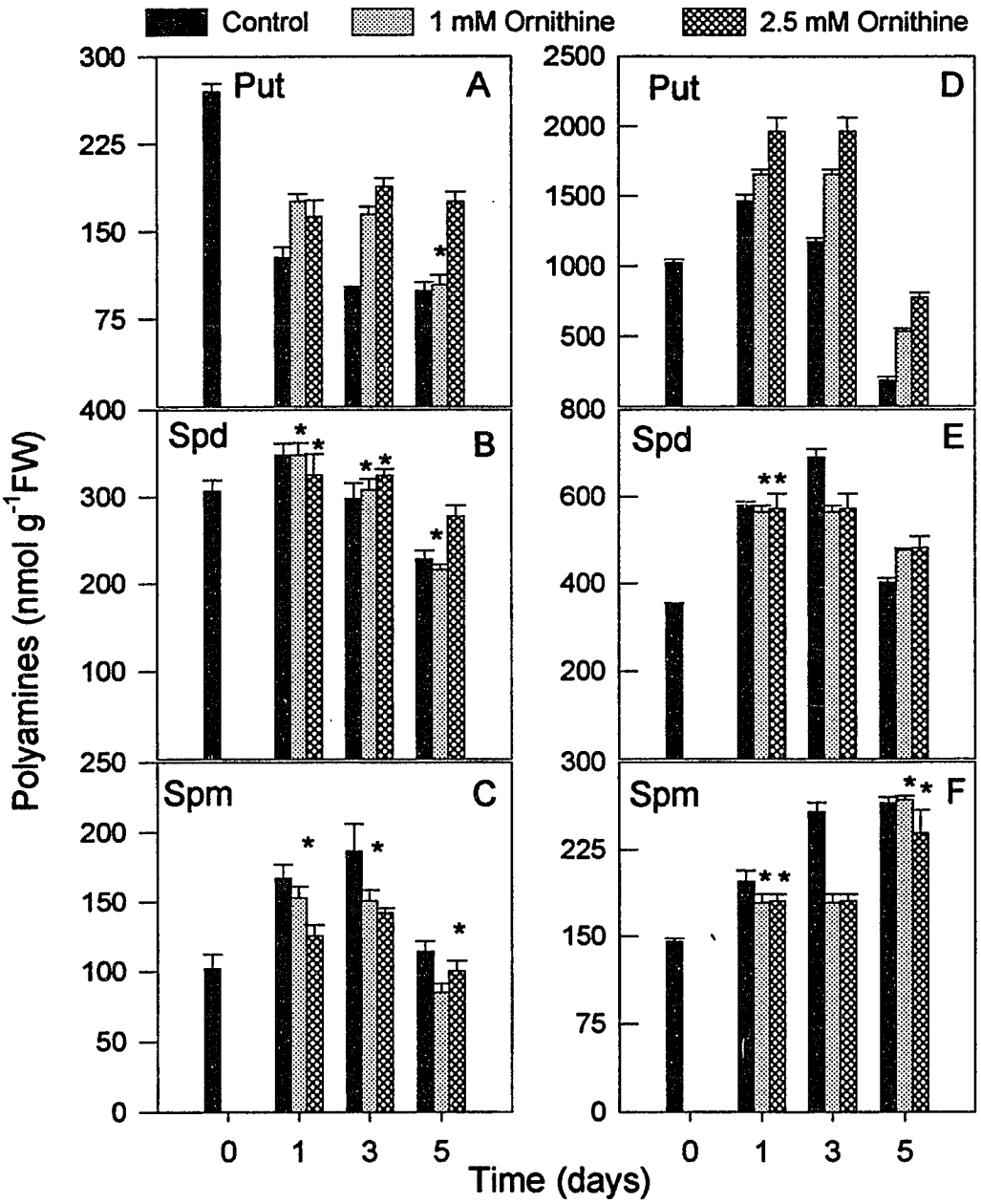

Figure 22. Long term effect of exogenous ornithine on cellular polyamine levels in (A-C) nontransformed and (D-F) transgenic (ODC-N14) carrot cells. 7 d old carrot cells were washed with 2,4-D free B5 medium and cultured for 3 d prior to treatment with ornithine and aliquots collected at different times as indicated above. Time 0 refers to the time of ornithine addition. Values are means ±SE of three replicates. All the values for ornithine treatment except those with * are significantly different ($p \leq 0.05$) from the control cells at a given time..

Putrescine levels significantly increased in the presence of exogenous ornithine in all cases. The effects on putrescine accumulation persisted throughout the 5 d treatment period in both the cell types, the effect increasing with the time of culture. However, the effect was not concentration dependent in short-term treatments. While spermidine and spermine levels were not affected in the short-term, spermidine level increased significantly during the long-term treatment. Spermine levels were generally not affected by the presence of ornithine.

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 torpedo and fully grown somatic embryos within 12 to 14 d. All of the transgenic cell lines that showed higher ODC activity and high levels of putrescine, developed somatic embryos at heart and torpedo stages as early as 3-4 d and always within a week on transfer to 2,4-D-free medium. Transgenic cell lines showed significantly higher numbers of somatic embryos on any given day (Table 9, Fig. 23). Morphologically, the embryos produced from transgenic lines had a relatively longer root and were much further developed than nontransformed embryos on any given day (Fig. 23a).

Table 9. (A) Effect of inhibitors of polyamine biosynthetic enzymes on somatic embryogenesis in nontransformed (N) and several transgenic ODC cell lines. Only embryos equal to or longer than 1 mm were counted. Values are mean of three replicates. Means with the same letters in a row are not significantly different ($p \leq 0.05$) as determined by Tukey HSD multiple comparisons. Values in columns 3-6 were compared with each other. Comparisons of the values in column 7 were made only with the values in column 3. (B) Number of two different stages of somatic embryos in nontransformed and transgenic ODC-N14 cells. Values are means of three fields of view. G= globular stage; T= torpedo stage.

(A)

Exp. Replicate	Cell Line	Control	1 mM DFMA	1 mM DFMA +2.5 mM Orn	1 mM DFMA +2.5 mM Put	2.5 mM DFMO
1	N	2 ^{a}	0 ^{a}	0 ^{a}	11 ^{b}	21 ^{b}
2	N	4 ^{a}	0 ^{b}	2 ^{a}	-	5 ^{a}
3	N	8 ^{a}	0 ^{b}	0 ^{b}	-	4 ^{a}
4	N	3 ^{a}	0 ^{b}	1 ^{ab}	0 ^{ab}	3 ^{a}
1	N1	38 ^{a}	8 ^{b}	20 ^{a}	-	26 ^{a}
2		30 ^{a}	4 ^{b}	8 ^{b}	17 ^{a}	28 ^{a}
1	N10	31 ^{ab}	20 ^{ab}	39 ^{a}	-	39 ^{a}
2		33 ^{a}	8 ^{b}	21 ^{c}	25 ^{ac}	24 ^{b}
1	N12	110 ^{a}	56 ^{b}	69 ^{b}	67 ^{b}	128 ^{a}
2		34 ^{a}	6 ^{b}	7 ^{b}	-	25 ^{b}
1	N14	70 ^{a}	41 ^{b}	68 ^{a}	53 ^{a}	77 ^{a}
2		57 ^{a}	8 ^{b}	31 ^{c}	37 ^{ac}	52 ^{a}

(B)

Experiment Number	Time (Days)	Number of embyros/Total cell clumps counted	
		Nontransformed	Transgenic
1	4	7G, 0T/47	10G, 7T/47
	7	10G, 7T/43	21G, 9T/43
	15	11G, 2T/39	0G, 10T/10
2	3	3G, 0T/71	13G, 5T/62
	5	10G, 3T/60	35G, 11T/65
	9	16G, 2T/40	10G, 26T/40

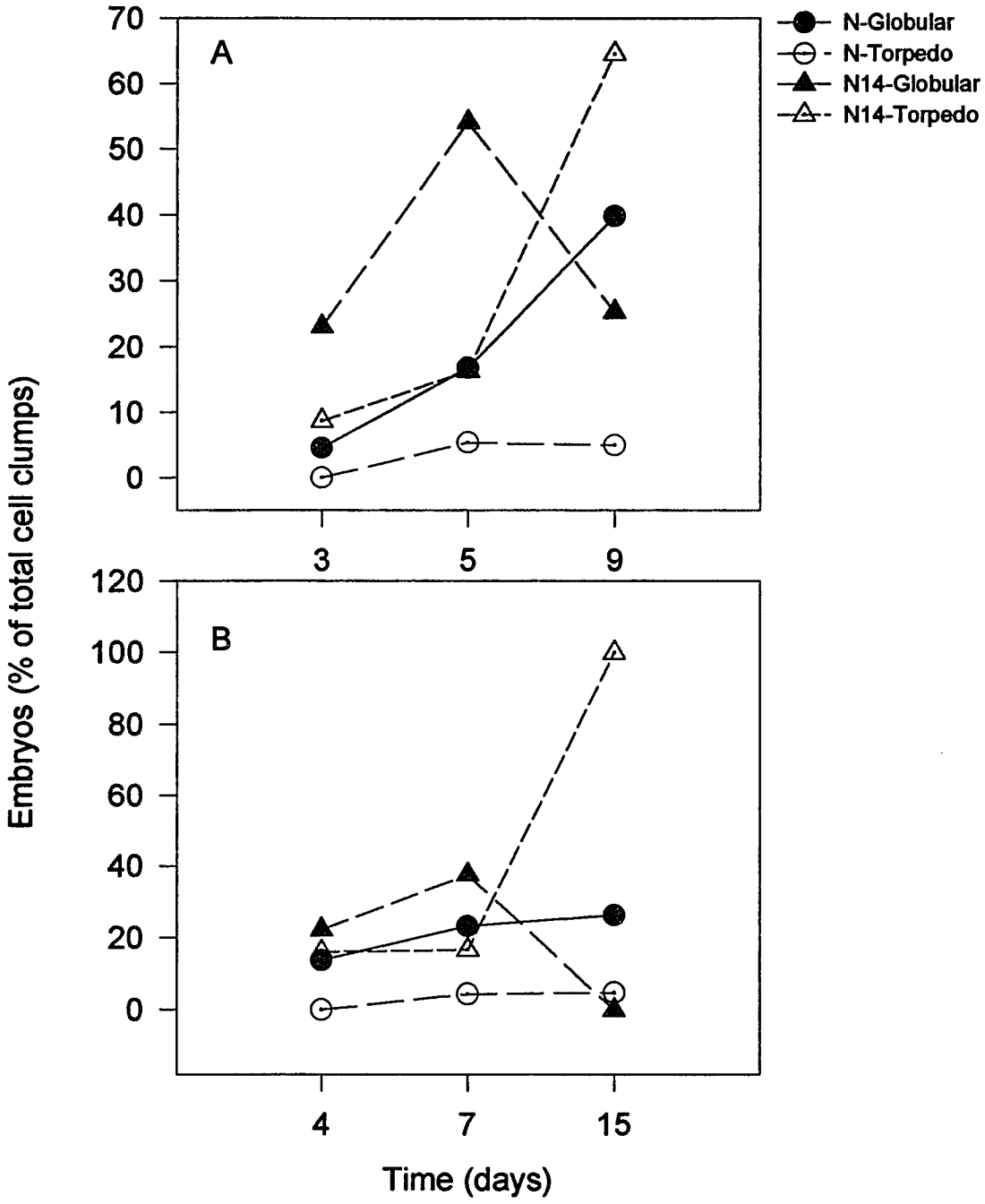

Figure 23. Two different stages of somatic embryos in nontransformed (N) and transgenic ODC-N14 cells. The cells were grown in the absence of 2,4-D in petri dishes in a liquid medium. Values are means of three fields of view.

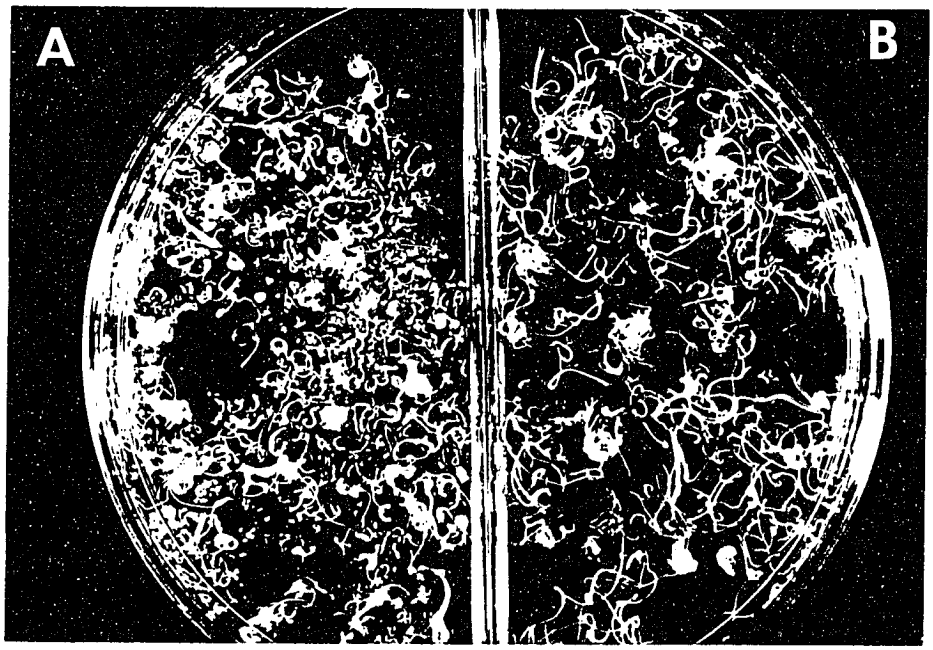

Figure 23a. Representative 3 week old somatic embryos in (A) nontransformed and (B) transgenic ODC-N14. Only about half of the total embryos produced by transgenic cells are shown. The cells were grown in the absence of 2,4-D in petri dishes in a liquid medium.

Some of the transgenic cell lines showing high levels of ODC activity and putrescine also produced somatic embryos even in the presence of 0.45 μM 2,4-D when grown on a solid medium (Fig. 24). 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. 25). 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.

Two of the transformed cell lines were remarkably different from the others in that part of the callus clump as shown in Fig. 25 turned dark green and showed high numbers of organized embryo-like structures in the callusing medium. One of these green calli containing embryo-like structures was separated and has been maintained with a label of ODC-N12(G) since then. Green color of the cells was stable through several subcultures in the liquid maintenance medium. Under microscopic observation these cells showed the presence of numerous well-developed chloroplasts, which are not usually observed in the nondifferentiating carrot cells (Fig. 26).

The effects of DFMA (a potent inhibitor of putrescine biosynthesis via ADC) on somatic embryogenesis were

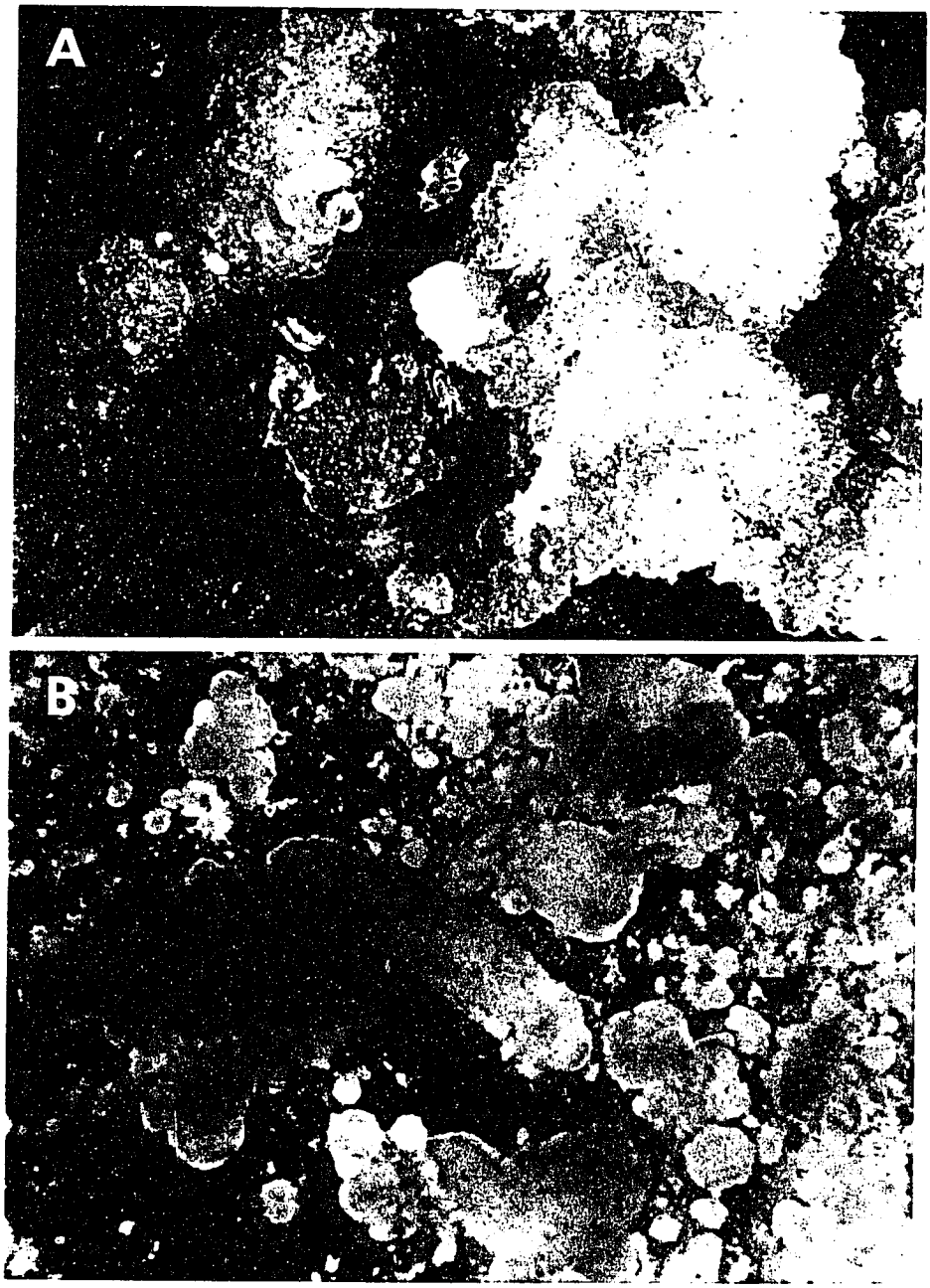

Figure 24. Somatic embryos produced by transgenic ODC cells in the presence of 0.1 mg/L 2,4-D in (A) N23 on solid medium and (B) N12G in liquid medium.

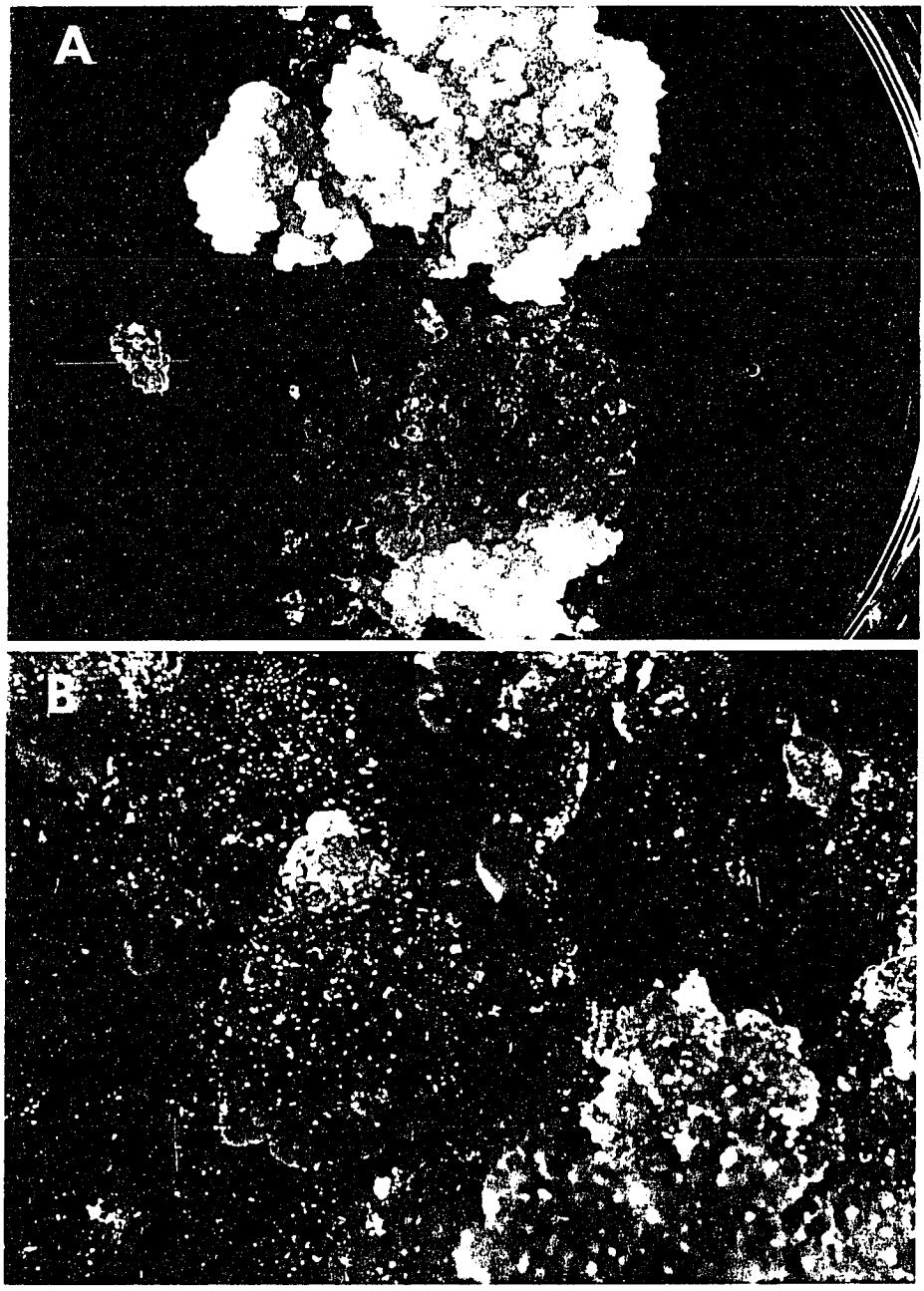

Figure 25. A= The origin of green cell line from transgenic ODC cells (N12). The green cells were subcultured separately and labeled as N12G. B= The green cell line showing somatic embryos in the presence of 0.1 mg/L 2,4-D.

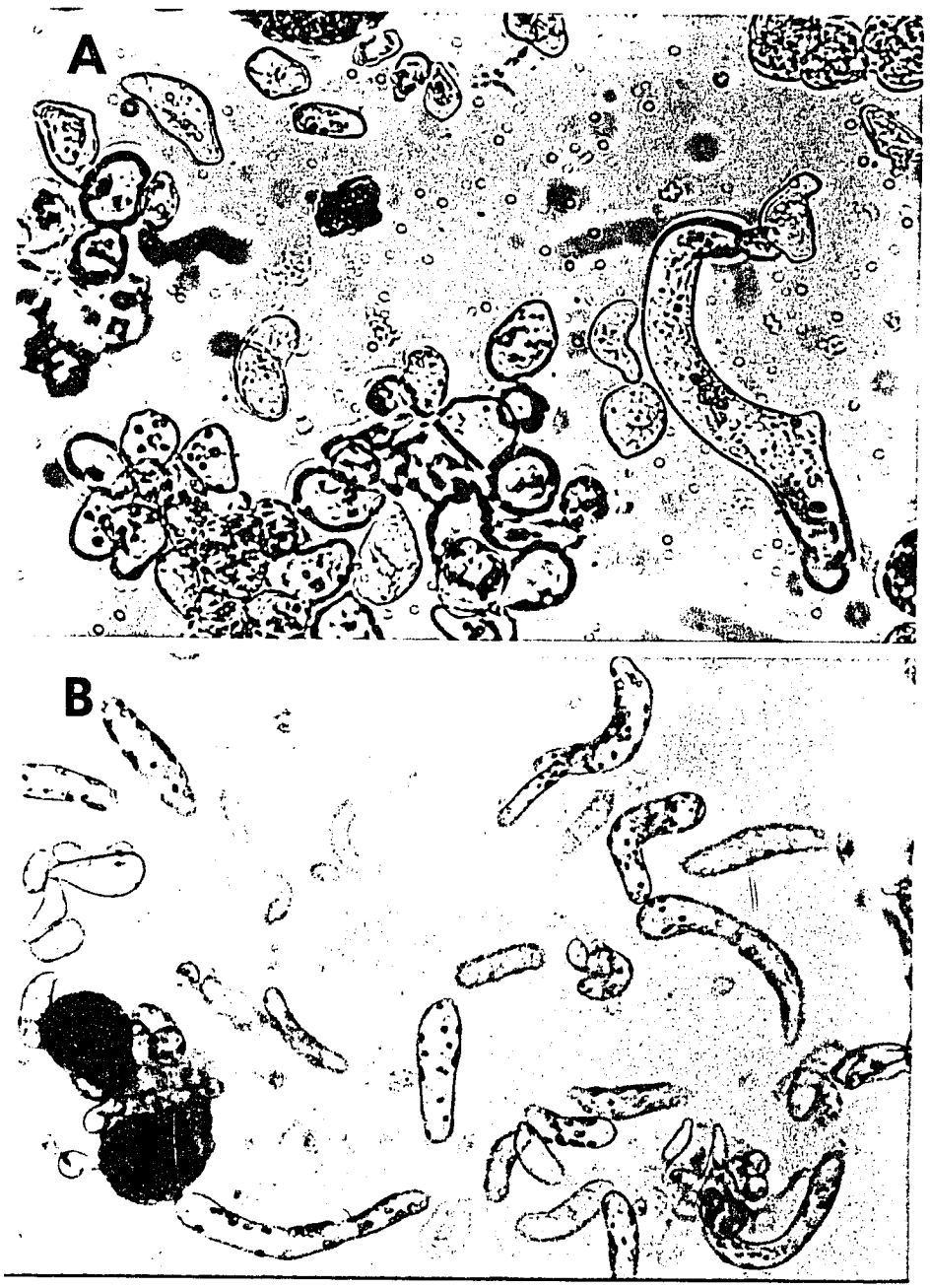

Figure 26. Actively growing (A) nontransformed and (B) transgenic ODC carrot cells and cell clumps (N12G) in liquid medium containing 0.1 mg/L 2,4-D.

examined for both the transformed and the nontransformed cells. The inhibitor was added at the time of transfer of cell suspension to the petri dishes at low density i.e. following 3 d preculture in the absence of 2,4-D. As shown in Fig. 27, addition of 1 mM DFMA caused a complete inhibition of somatic embryogenesis as well as growth of nontransformed cells. Microscopic observation showed no organized embryo-like structures after 14 d. Lower concentrations of DFMA partially inhibited the development of somatic embryos. This effect of DFMA was somewhat reversed by the presence of 2.5 mM exogenous putrescine in the medium (Table 9). The transgenic cells in the presence of DFMA 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. 28). The development of these embryos was, however, considerably slower than those in the absence of DFMA. The transgenic cell lines in general exhibited a high degree of tolerance to otherwise toxic levels of DFMA. Similarly, 2.5 mM exogenous ornithine in the medium significantly reversed the inhibitory effects of DFMA on somatic embryogenesis in the transgenic cell lines (Fig. 28).

The effects of MGBG, a potent inhibitor of SAMDC, were also examined on somatic embryogenesis (Fig. 29). It was observed that transgenic cell lines were tolerant to 0.1 mM

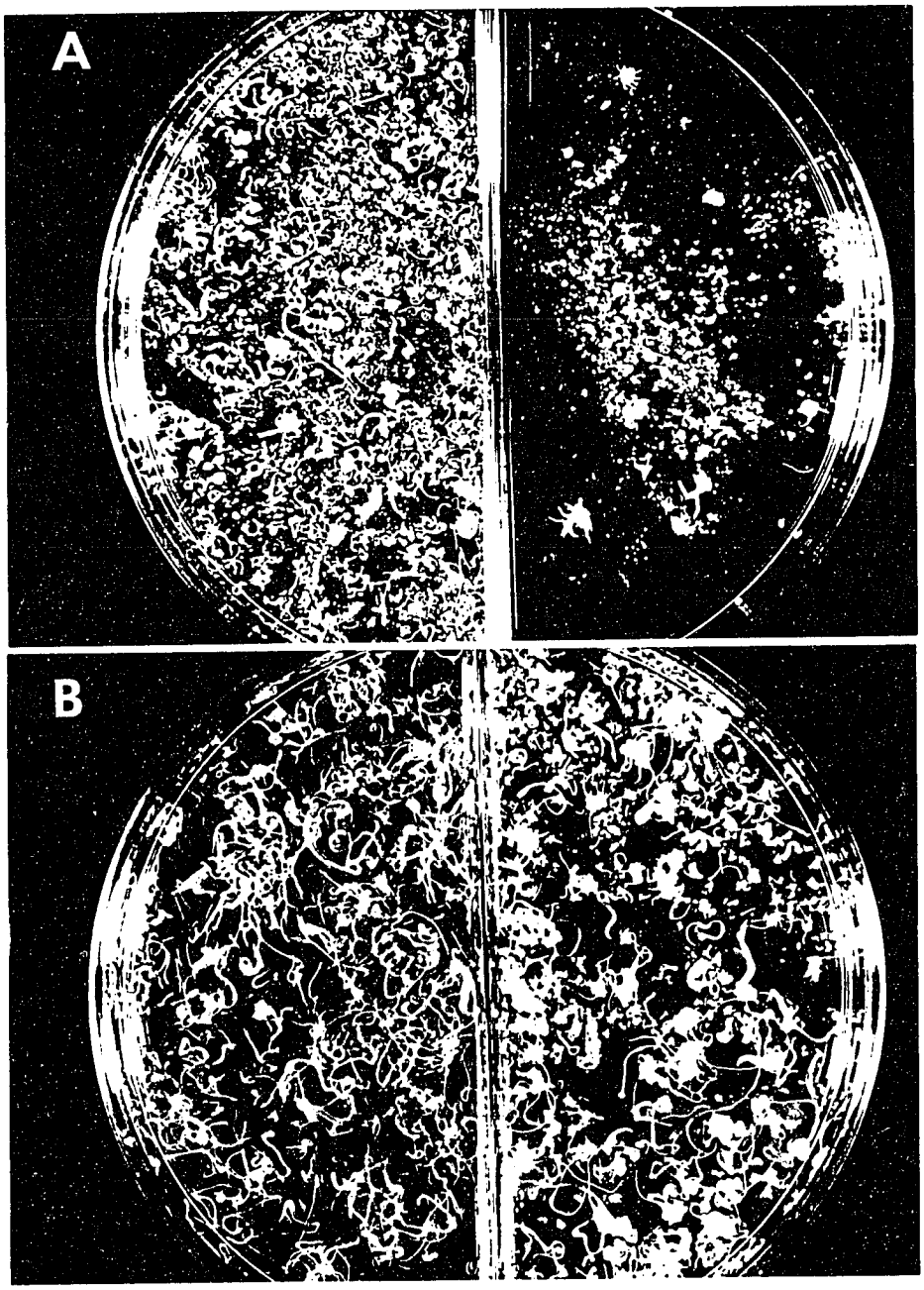

Figure 27. Effect of 1 mM DFMA on somatic embryogenesis in nontransformed and transgenic cells. A= nontransformed control cells in the absence (left) or presence (right) of 1 mM DFMA; B= transgenic ODC cells (N14) in the absence (left) or presence (right) of 1 mM DFMA. Left half of (B) contains about half of the total embryos produced. All cultures were 3 week old when photographed.

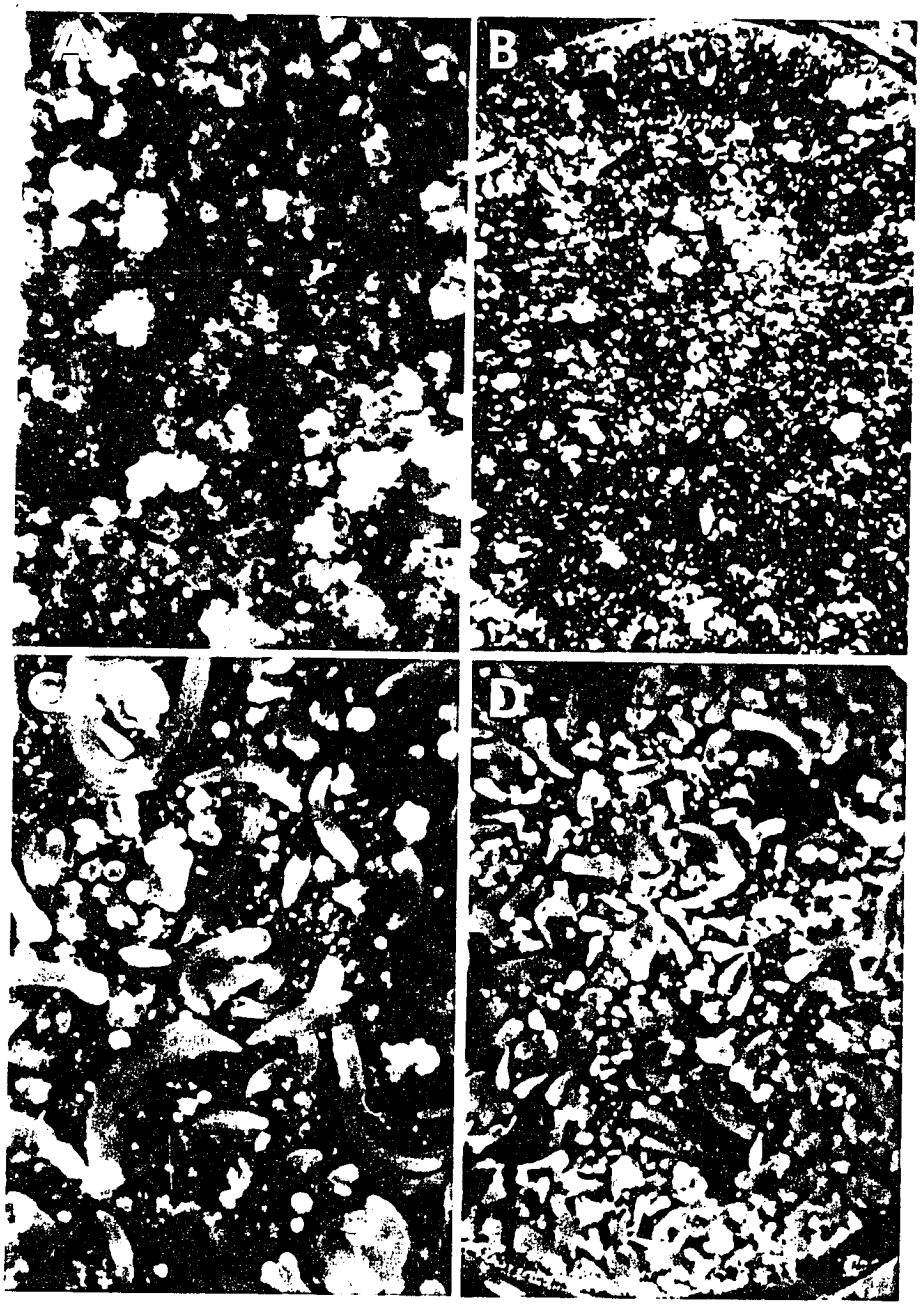

Figure 28. Reversal of the DFMA-effect on somatic embryogenesis by exogenous ornithine. A= nontransformed cells in 1 mM DFMA; B= nontransformed cells in 1 mM DFMA and 2.5 mM L-ornithine; C= transgenic ODC cells (N10) in 1 mM DFMA; D= transgenic ODC cells (N10) in 1 mM DFMA and 2.5 mM L-ornithine. (A and C= 20.8x; B and D= 12.3x)

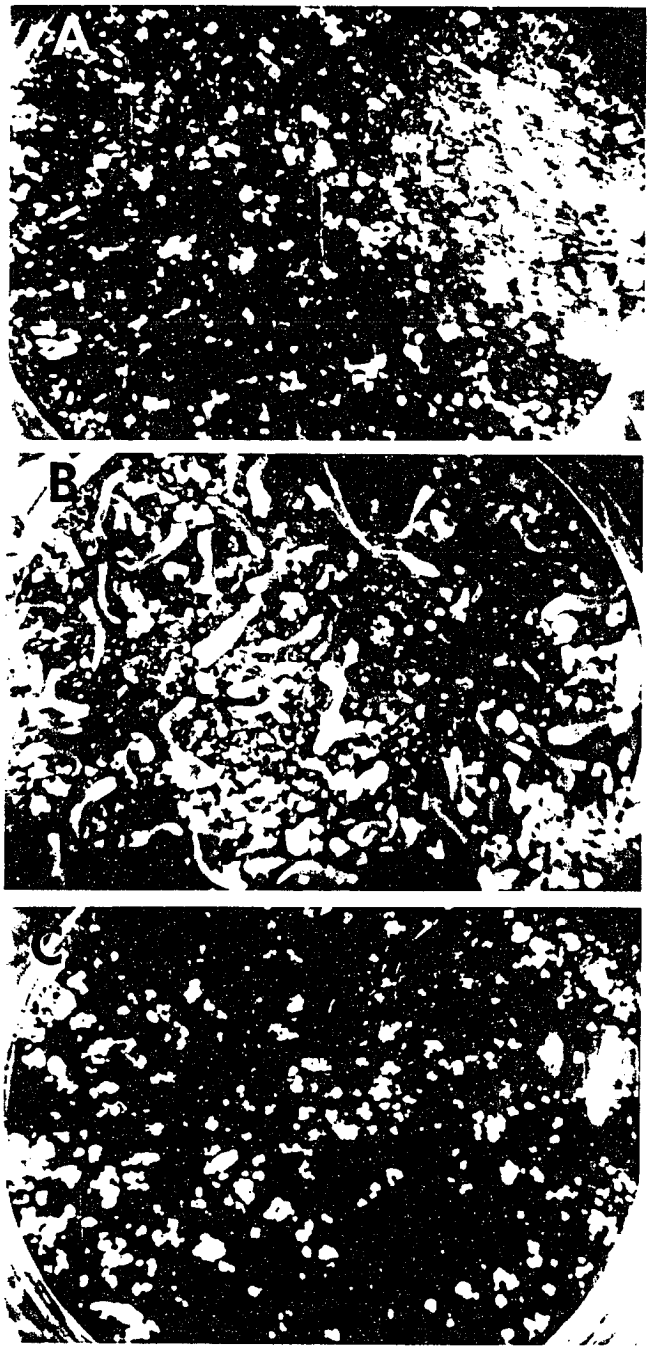

Figure 29. Effect of MGBG on somatic embryogenesis. A= nontransformed cells in the presence of 0.1 mM MGBG; B= transgenic ODC cells (N10) in 0.1 mM MGBG; C= transgenic ODC cells (N10) in 0.5 mM MGBG. (12.3x).

MGBG compared to the nontransformed control. However, relatively higher levels of MGBG (0.5 mM) caused a complete inhibition of somatic embryo development in all cases and the addition of exogenous 2.5 mM spermidine could not reverse this inhibitory effect on somatic embryogenesis (Table 10).

ADC Enzyme Activity in Transgenic Cells

Arginine decarboxylase activity was determined in the transgenic cells which repeatedly showed elevated levels of mouse ODC and cellular putrescine. Consistent with earlier observations (Robie and Minocha, 1989), nontransformed cells showed some ADC activity which was severely inhibited by DFMA (Table 11). The ADC activity in the transgenic cells was surprisingly several-fold higher than in the nontransformed cells. Moreover, ADC activity was further enhanced by the presence of DFMA in the reaction mixture.

Transgenic Cells with Human SAMDC cDNA

Twenty of the SAMDC cell lines were selected and maintained as calli on solid medium containing 2,4-D. Many of these cell lines were tested for the presence of NPT gene by PCR amplification and all were found to contain the gene

Table 10. Effect of various concentrations of MGBG on somatic embryogenesis in nontransformed (N) and several transgenic ODC cell lines. Only embryos equal to or longer than 1 mm were counted. Values are mean of three replicates. Means with the same letters in a row are not significantly different ($p \leq 0.05$) as determined by Tukey HSD multiple comparisons.

Exp. Replicate	Cell Line	0	0.1 mM MGBG	0.5 mM MGBG	0.5 mM MGBG + 2.5 mM Spd
1	N	2 [a]	-	0 [a]	-
2	N	4 [a]	0 [b]	0 [b]	-
3	N	8 [a]	0 [b]	0 [b]	-
4	N	3 [a]	0 [b]	0 [b]	0 [b]
1	N1	38 [a]	5 [b]	1 [b]	-
2		30 [a]	7 [c]	0 [b]	0 [b]
1	N10	31 [a]	9 [b]	0 [b]	-
2		33 [a]	6 [c]	0 [b]	0 [b]
1	N12	110 [a]	-	4 [b]	-
2		34 [a]	11 [c]	0 [b]	-
1	N14	70 [a]	83 [c]	9 [b]	3 [b]
2		57 [a]	8 [b]	0 [b]	0 [b]

Table 11. Arginine decarboxylase (ADC) activity in nontransformed and three of the transgenic cell lines. The enzyme activities were measured at pH 8.4 in the presence or absence of different concentrations of DFMA. Values are the mean of three replicates. Values in a column for each experiment were compared with each other. All values were significantly different ($p \leq 0.05$) except those marked with * as determined by Tukey HSD multiple comparisons.

Exp.	Cell Line	mM DFMA		
		0	0.1	1
1	N	1.39		
	N1	6.55		
	N10	39.74		
	N14	15.19		
2	N	1.54	0.00	
	N10	13.92	28.93	
3	N	1.93	0.07	
	N10	26.61	34.32	
	N14	14.72	5.64	
4	N	1.35		0.00
	N10	4.32		6.96
5	N	6.3		0.78
	N1	18.05*		45.93
	N14	17.90*		32.89

(Fig. 30). Likewise, seven of the SAMDC transformants plants grown in the greenhouse were found to be positive for the presence of NPT gene. Amplification by the SAMDC primers has given unreliable results due to the production of multiple bands in the PCR reaction. However, Southern hybridization of the plant genomic DNA from plants in the greenhouse shows the presence of human SAMDC cDNA in their genome (Fig. 31)

SAMDC enzyme assays were done once on each of the cell lines. Preliminary results show a 6-10 fold increase in the SAMDC enzyme activity in these putative transgenic cell lines (Table 12). Determination of cellular levels of polyamines showed at least a two fold increase in the spermidine whereas elevated levels of spermine were seen in only few of the cell lines tested (Table 13). Similar to the transgenic ODC cells, transgenic SAMDC cells were also highly embryogenic and were found to be tolerant to low levels of MGBG (Fig. 32)

Transfer of *in vitro* Grown Plantlets to Greenhouse

Acclimation of the *in vitro* grown plantlets in the pots was especially difficult with the transgenic ODC plants compared to the SAMDC. Few of the ODC plants that survived through the acclimation phase died earlier than SAMDC

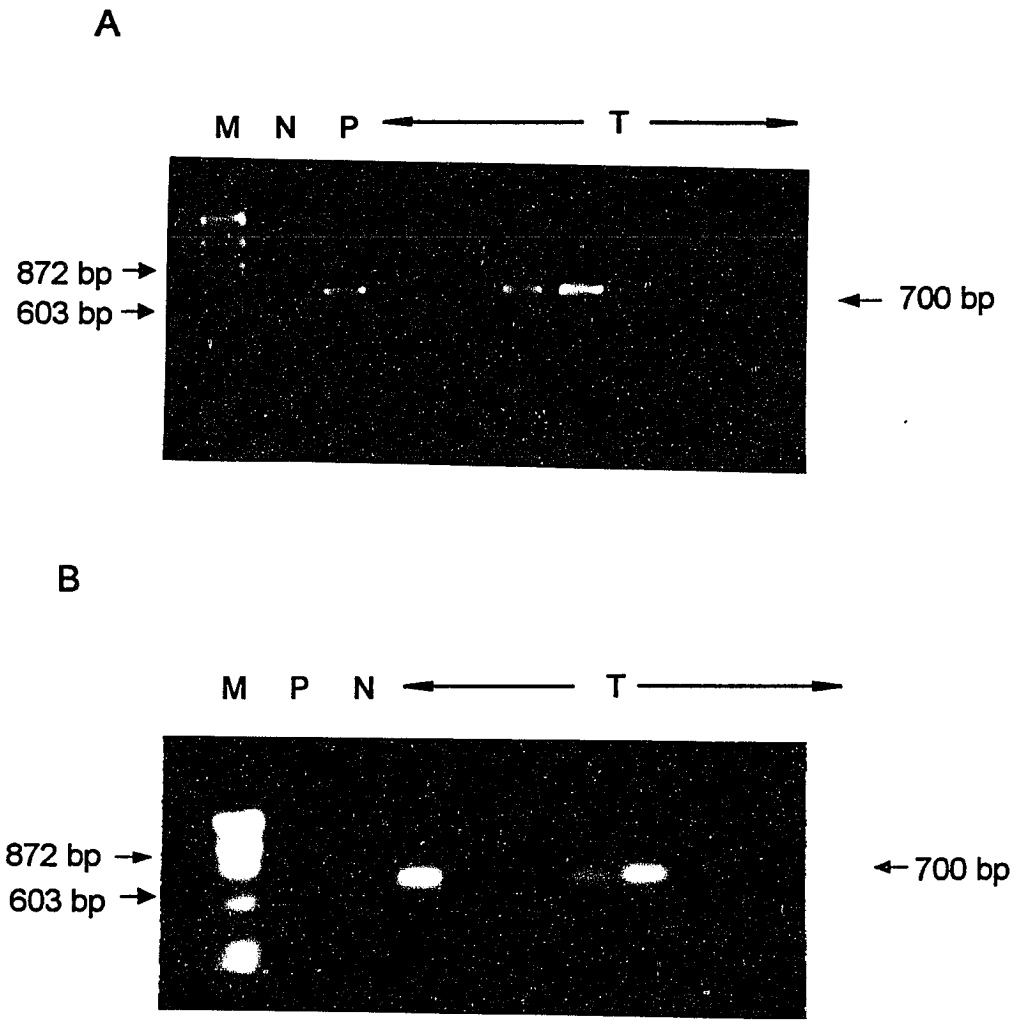

Figure 30. Agarose gel electrophoresis of PCR products obtained by using NPT primers on the genomic DNA of SAMDC transformants. A= transgenic SAMDC plants. B= transgenic cells in culture. M= MW marker (ϕX174 HaeIII digest); P= AT35S-SAM; N= nontransformed cells; Lanes from left to right are: (A) marker, N, P, SAM1-0, SAM4-1, SAM10-2, SAM12-0, SAM15-0, blank, SAM19-4 and (B) marker, P, N, SAM1, SAM4, SAM10, SAM12, SAM15, blank, SAM19

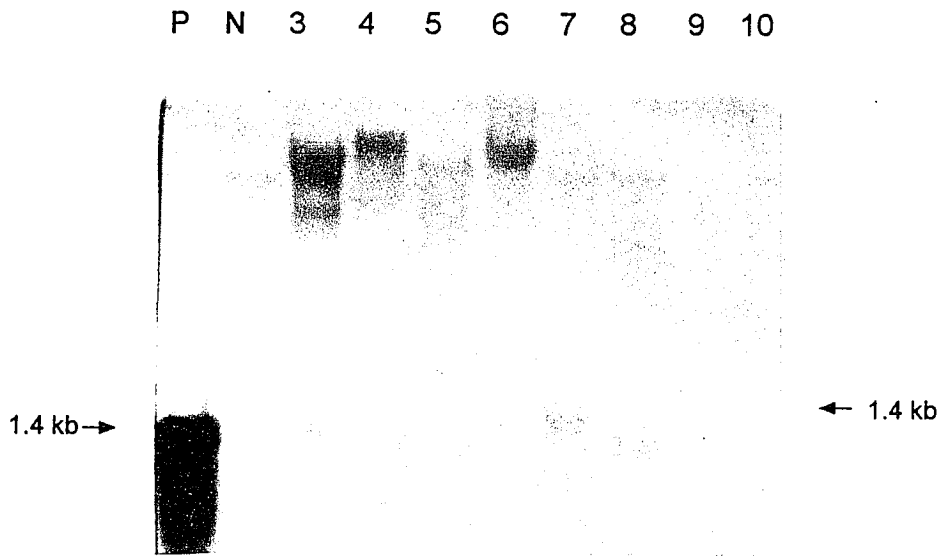

Figure 31. Southern hybridization of genomic DNA from several transgenic SAMDC cells digested with XbaI. 10 µg of DNA was digested and loaded in each well. Hybridization was done using DIG-labeled probe (1.4 kb fragment from pUCSAM-B digested with XbaI). P= plasmid pUCSAM-B digested with XbaI; N= nontransformed; Lanes 3-10 are: SAM1, SAM4-1, SAM10-2, SAM12, SAM14-1, SAM15, ODC-N10 and SAM19-4.

Table 12. S-adenosylmethionine decarboxylase (SAMDC) activities in nontransformed (N) and several transgenic SAMDC cell lines. ND= not determined. Values are the mean of three replicates. Means with the same letters in a column are not significantly different ($p \leq 0.05$) as determined by Tukey HSD multiple comparisions.

Cell Line	nmol CO2/h/mg protein	
	exp.1	exp.2
N	0.43 ^{e}	0.51 ^{c}
SAM-N1	2.82 ^{c}	6.06 ^{d}
SAM-N2	2.61 ^{bc}	4.77 ^{e}
SAM-N3	2.76 ^{c}	2.85 ^{a}
SAM-N4	6.36 ^{d}	3.60 ^{b}
SAM-N5	2.25 ^{bc}	ND
SAM-N7	3.90 ^{a}	3.87 ^{b}
SAM-N8	4.52 ^{ad}	2.56 ^{a}
SAM-N10	5.14 ^{d}	2.01 ^{a}
SAM-N14	3.92 ^{a}	ND
SAM-N16	2.04 ^{b}	2.35 ^{a}
SAM-N19	3.58 ^{a}	3.79 ^{b}

Table 13. Cellular levels of polyamines in nontransformed (N) and several transgenic SAMDC cell lines. Samples were collected from the cells grown in 2,4-D free medium for three days. Means with the same letters in a column are not significantly different ($p \leq 0.05$) as determined by Tukey HSD multiple comparisions.

Cell Line	nmol/g FW		
	Putrescine	Spermidine	Spermine
N	79.87 [a]	295.60 [a]	222.20 [a]
SAM-N1	222.27 [b]	443.43 [b]	239.63 [a]
SAM-N3	193.70 [bc]	408.90 [b]	204.77 [a]
SAM-N4	160.27 [d]	433.67 [b]	148.67 [a]
SAM-N5	371.73 [e]	458.73 [b]	152.90 [a]
SAM-N14	122.20 [bd]	681.70 [c]	688.87 [b]

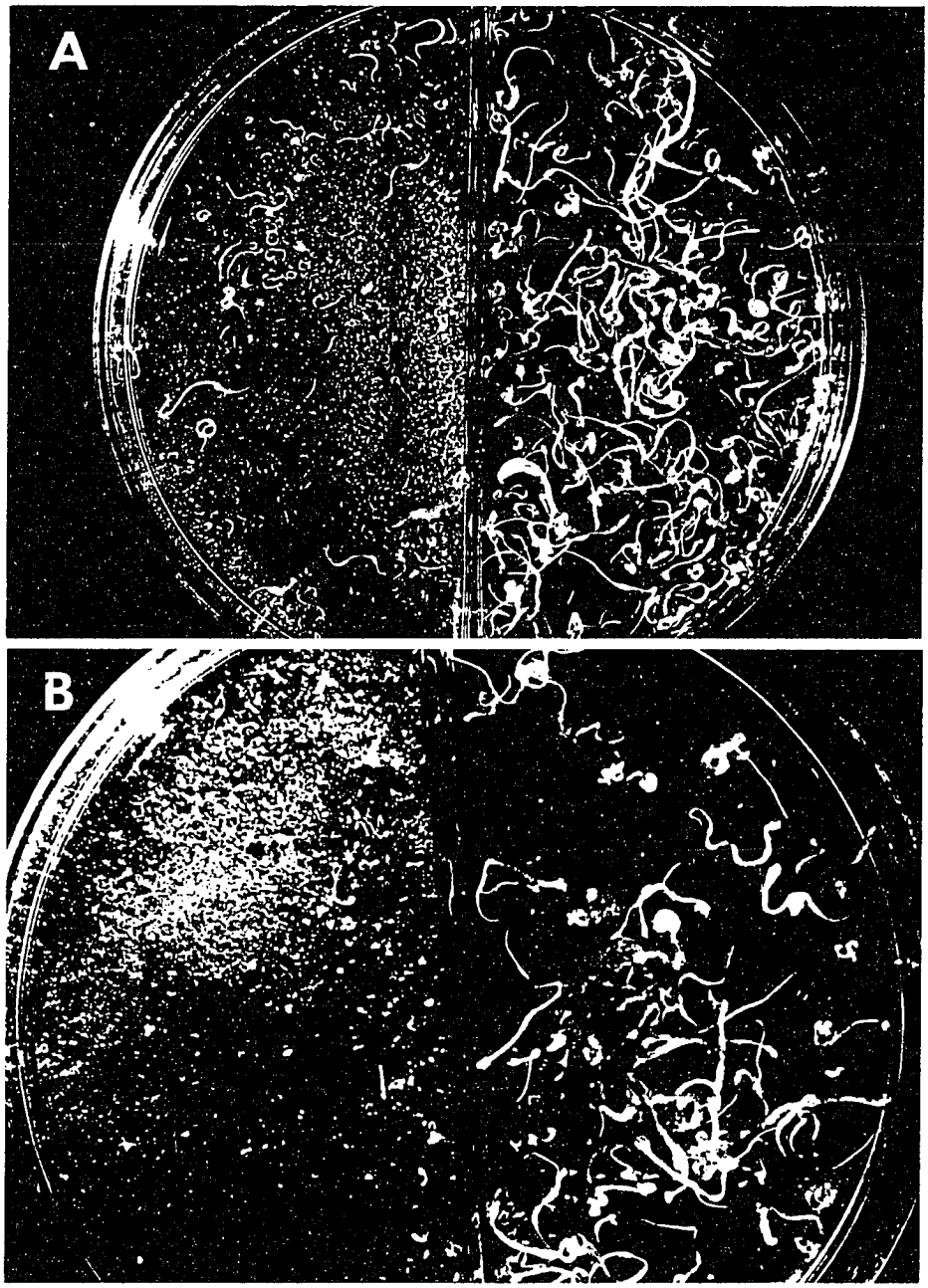

Figure 32. Effect of MGBG on somatic embryogenesis in nontransformed and transgenic SAMDC cells (SAM4). A= nontransformed control (left) transgenic (right) cells in the absence of MGBG (1.14x); B= nontransformed control (left) transgenic (right) cells in the presence of 0.1 mM MGBG(1.42x). All cultures were 3 week old when photographed.

plants. Plants grew better when potted in regular soil than promix, peat:vermiculite (1:3). or soil:sand(1:3).

The ODC transformed plants were morphologically similar to control plants (Fig. 33). However, the SAMDC plants were quite different from the ODC or the nontransformed control plants. The SAMDC plants typically showed multiple crowns and shoots (Fig. 34). Most of the SAMDC plants produced flowers during summer months whereas none of the transgenic ODC-plants (N1, N10, N12, N19, N26 and N28) except ODC-N12, produced flowers. As shown in the Fig. 35, some of the flowers from the SAMDC plants matured to produce seeds. The ODC plant that had flowered died before any seeds were formed. These seeds were collected and stored.

Figure 33. Mature carrot plants in the green house.
A= nontransformed (left) and transgenic ODC-N2 (right);
B= nontransformed (left) and transgenic SAM19-1 (right). The nontransformed plant is 8 months old and both the transgenic plants are approximately 16 months old.

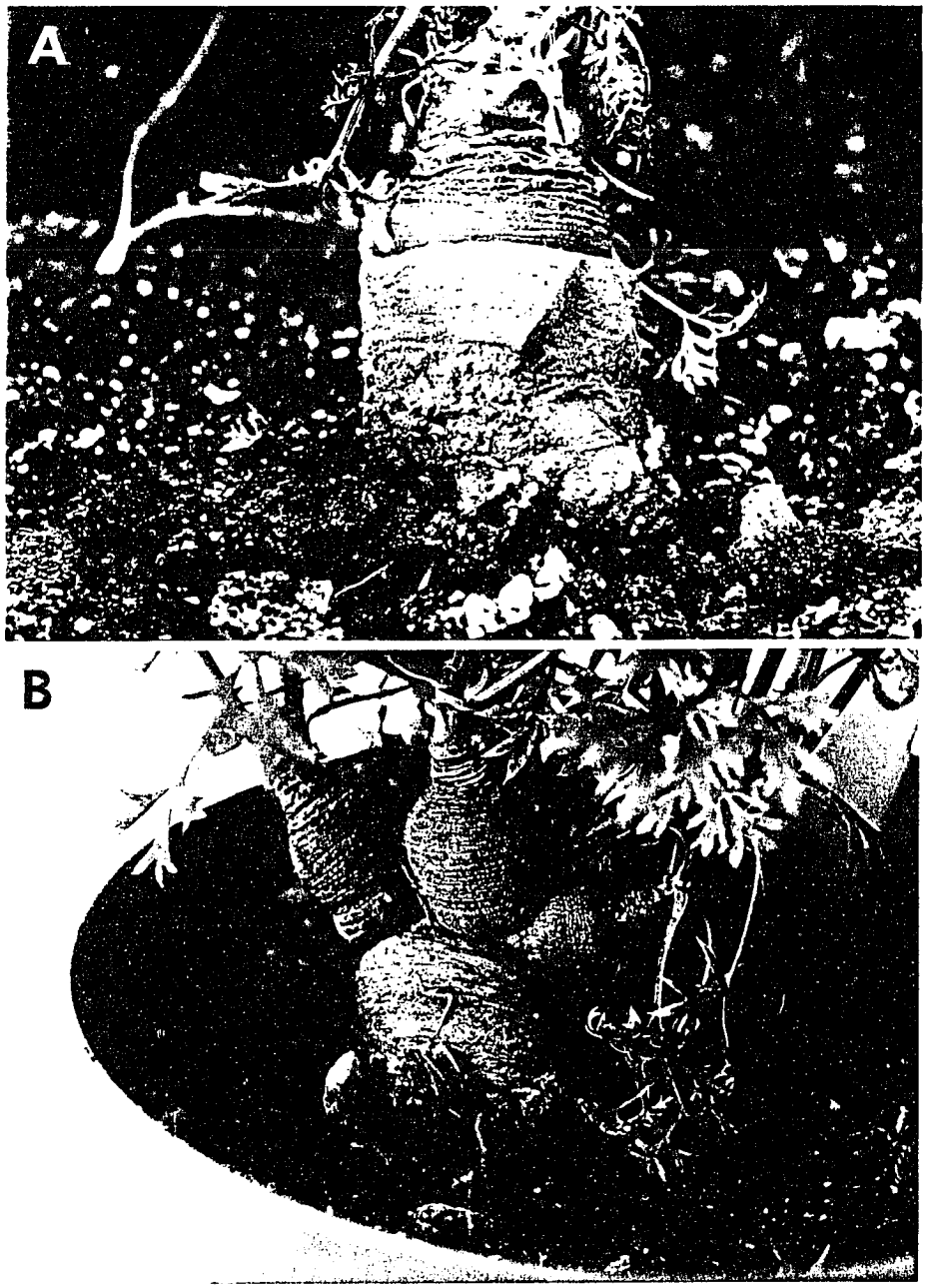

Figure 34. A comparison of the morphological appearance of transgenic plants. Both the plants were about 16 months old. A= transgenic ODC-N1; B= transgenic SAM1-0 plants.

Figure 35. Transgenic SAM4-1 plants showing (A) influorescence and (B) maturing seeds in one of the influorescence. The plant is about 16 months old.

DISCUSSION

Cellular levels of the diamine putrescine can be increased: (i) by increase in its biosynthesis through either the ADC or the ODC pathway, (ii) by decrease in its catabolism through oxidation, and/or (iii) by its decreased utilization for spermidine biosynthesis. Previously, an increase in putrescine level by treatment with DFMO in carrot cells has been reported (Robie and Minocha, 1989). Similarly, putrescine accumulation has been demonstrated by inhibiting SAMDC (Papa, 1988; Minocha et al., 1990b; Minocha et al., 1991), another key regulatory enzyme in the polyamine biosynthetic pathway that inhibits the utilization of putrescine into spermidine. Promotion of cellular levels of putrescine in carrot has also been demonstrated by inhibiting spermidine synthase (Khan and Minocha, 1991). The inhibitors, with a few exceptions, have been extremely useful in reducing the biosynthesis of polyamines but not for the promotion of any specific steps. The results presented here and those published earlier from this laboratory (DeScenzo and Minocha, 1993; Noh and Minocha, 1993) clearly demonstrate that the approach of modulating polyamine biosynthesis through gene transfer is a practical alternative in a situation where (1) polyamine overproducing

mutants are lacking, and (2) promotion of a specific step in a biosynthetic pathway is required. A similar approach was also demonstrated by Hamill et al. (1990).

The use of specific inhibitors and the availability of mutant cell lines lacking ODC has helped in elucidation of the role of putrescine in cell division and development in animal and microbial cells (Heby, 1989). Because of the existence of two pathways for the biosynthesis of putrescine, and non-specificity of inhibitors like DFMO in plants, it has not been possible to establish a similar causative role of polyamines in plant growth and development. While the mechanism of promotion of somatic embryogenesis due to increased production of putrescine in transgenic cell lines is still unclear, the approach of genetic manipulation alleviates problems associated with the interpretation of data from the compounding effects of chemical inhibitors and other treatments that may affect growth and development.

The presence of NPT-II reporter gene in the genetically engineered *Agrobacterium* construct was helpful in the selection of putative transformed carrot cells. The ability of several of the hypocotyl segments, co-cultured with genetically engineered *Agrobacterium*, to produce callus in the presence of lethal levels of kanamycin showed that it is

an effective means of selecting putative transformed cells. The inhibitory concentration of kanamycin used here seems relatively higher than what is commonly used in other plants (Weising et al., 1990). While determining a dosage curve for toxicity of kanamycin, it was observed that growth of callus was reduced only with concentrations of kanamycin higher than 150 mg/L in the liquid medium. However, callus growth still occurred in the solid medium with kanamycin levels lower than 300 mg/L. Since co-cultured hypocotyl segments had to be placed on a solid selection medium a higher concentration of kanamycin was used in the selection of transgenic cells.

The use of PCR to amplify a known DNA sequence is an effective method for screening a large number of samples to demonstrate the presence of the foreign DNA fragments. However, this method does not distinguish between foreign DNA that is integrated into the host genome and that may be located somewhere else, including in the bacteria carried through the routine subculture. Since the cells had been subcultured in solid and liquid media that generally allow rapid proliferation of *A. tumefaciens* it is unlikely that the bacteria would have remained at visually undetectable levels in the medium. Furthermore, the results on Southern hybridization, the presence of mouse ODC specific

transcripts, the western blots showing the presence of ODC protein, and the high levels of enzyme activity characteristic of the mouse ODC leave little doubt that the transgenic cells had indeed cointegrated the T-DNA that was being transcribed and translated. The existence of live bacteria in the culture was ruled out by performing PCR reactions with total bacterial DNA and the plant genomic DNA using vir A specific primers obtained from Dr. Eugene Nester, University of Washington, Seattle, WA. While the total *A. tumefaciens* DNA produced an expected 630 bp amplification product, none of the control or transgenic cell lines produced such a signal. Furthermore, the ODC cDNA is under the control of a 35S CaMV promoter, it is specific only to eukaryotic cells and should be expressed only in the plant cell and not in free-living *Agrobacterium*. Likewise, all of the cell lines that showed the presence of NPT-II gene were also found to have increased NPT activity showing the expression of the transferred gene. This gene is also under the control of a eukaryotic type promoter that has never been shown to be expressed in *Agrobacterium*.

Southern blotting of the genomic DNA digested with EcoRI indicated some homology of nucleotide sequences between the mouse and the plant DNA. However, the absence of an amplification signal in PCR makes it difficult to

ascertain what the homologous segment of plant DNA is. Similar bands of homologous DNA from nontransformed and transgenic tobacco were observed by DeScenzo and Minocha (1993). No RNA signal was seen in the nontransformed plants by Northern blotting in either tobacco (DeScenzo and Minocha, 1993) or carrot. Therefore, it is unlikely that these bands correspond to active ODC gene(s) in plants. The absence of any such signal in the HindIII digest is intriguing and remains unexplained.

The expression of mouse ODC cDNA to produce a functional ODC enzyme in the transgenic cell lines was confirmed by: (1) the analysis of ODC protein by Western blotting, (2) determination of the enzyme activity at pH optimum for mouse ODC, (3) inhibition of the high enzyme activity (at pH optimum for mouse ODC) by DFMO and monoclonal antibodies against ODC protein, and (4) increase in the cellular levels of putrescine. It is known that mouse ODC is a dimer of 100 kD with a subunit M_r of about 53 kD (Seely et al., 1982). Among the three unique bands seen in Fig 10B, the smallest one corresponds to the expected size for the mouse ODC.

While higher levels of ODC activity were always observed in transgenic cell lines compared to nontransformed controls, the enzyme activity varied considerably on

different days in different transgenic lines. The intra-clonal variation in gene activity regulated by the CaMV 35S-promoter is quite common in transgenic plants (Nagata et al., 1987; Hamill et al., 1990; Weising et al., 1990; Fecker et al., 1993; Stefanov et al., 1994). Both the activity of mouse ODC as well as cellular putrescine also varied at different times of analysis in the same line. This could potentially be related to the growth cycle of the cultures. Variation in 35S promoter-regulated expression of yeast ODC during growth cycle in transgenic *Nicotiana rustica* roots was also noted by Hamill et al. (1990). The difference in the site of integration of the foreign gene in the plant genome and its copy number may also be responsible for the observed variation in ODC activity in different transgenic lines. Increase in the copy numbers of GUS and the linked NPT gene by simply selecting the cells at high concentrations of kanamycin was shown in transgenic *Petunia* cells carrying genes encoding for NPT-II and β-glucuronidase (Jones et al., 1994). Although the restriction digest of genomic DNA in the Southern blot (Fig 9) was not designed to specifically evaluate the copy numbers, it does show a variation in the site of integration in the genomic DNA and probably difference in copy number as well. Despite all the observed variations, the transgenic cell lines always showed higher levels of mouse enzyme activity; whereas, the non-

transgenic controls had no detectable ODC activity. This increased enzyme activity translates into the observed increase in cellular levels of putrescine.

Two observations stand out from the results with transgenic cell lines of tobacco and carrot. While the cellular levels of putrescine can vary widely in response to overexpression of the ODC gene, the cellular levels of spermidine vary only within a narrow range in both putrescine overproducing cell lines as well as in SAMDC transgenics. This indicates a much tighter control over the biosynthesis of spermidine and spermine. These observations are also consistent with a number of observation on changes in cellular polyamines during development, cell division cycle and in response to stress (Minocha et al., 1991a). We know that synthesis of spermidine and spermine requires dcSAM as an aminopropyl donor in addition to the two aminotransferases, i.e. spermidine synthase and spermine synthase. The dcSAM is obtained from SAM which sits at the cross-roads of ethylene and polyamine biosynthesis. The production of dcSAM is known to be highly regulated and is the rate limiting factor in the synthesis of spermidine and spermine (Pegg and McCann, 1982; Greenberg and Cohen, 1985). Unlike the SAMDC from animal tissues, the plant SAMDCs are not stimulated by putrescine (Tiburcio et al., 1990). The cellular levels of dcSAM in the transgenic ODC cell lines

may be limiting despite the elevated levels of putrescine, therefore, the cellular levels of higher polyamines are not affected. The increased putrescine may also be used in the biosynthesis of secondary metabolites as polyamines can occur in plant cells not only as free bases (that are extractable with PCA or TCA) but also as conjugates (Flores and Martin-Tanguy, 1991). The most widespread polyamine-derived secondary metabolites found in higher plants are the hydroxycinnamic acid amides which have been show to have biological significance in flowering, seed and fruit development, and in response to viral or fungal infection. Among the putrescine-derived alkaloids, the pyrrolidine and tropane alkaloids of the Solanaceae, especially nicotine in tobacco, are well studied. Studies by Hamill et al. (1990) using a transgenic *Nicotiana rustica* roots containing a yeast ODC show a positive relationship between higher levels of free putrescine and increase in cellular nicotin. Furthermore, putrescine can also be metabolized to γ-aminobutyric acid (GABA) via a diamine oxidase and a NAD-dependent γ-aminobutyraldehyde dehydrogenase (Flores and Martin-Tanguy, 1991). An alternative possibility is that excess putrescine may simply be secreted out of the cells into the growth medium.

Whether it is zygotic or somatic embryogenesis, we know that two fundamental things occur during embryonic development: (1) polar axis of the plant is established, and (2) primary tissue and organ systems are determined. The question as to how an undifferentiated mass of cells takes on a different pattern where the developmental fate of the cells is determined through a regulated pattern of cell divisions is still an open question in developmental biology.

In carrot somatic embryos, and perhaps also in the zygotic embryos of carrot and other plants, localized distribution of auxin is suggested to mediate the induction of bilateral symmetry (Liu et al., 1993). Earlier studies have clearly demonstrated the requirement for elevated levels of polyamines and the importance of ADC pathway during somatic embryogenesis in carrot cell cultures (Montague et al., 1979; Feirer et al., 1984; Robie and Minocha, 1989; Minocha and Minocha, 1994). A decrease in cellular putrescine by treatment with DFMA was accompanied by loss of somatic embryogenesis. In 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 question of whether the increase in cellular putrescine (on removal of 2,4-D from the medium) is merely a consequence of embryo

growth and development or a crucial pre-requisite for embryo development has remained unanswered. The increased production of somatic embryos in the 2,4-D-free medium and the development of somatic embryos (albeit at low frequency) in the presence of 2,4-D in the transgenic cell lines suggest a developmental role for putrescine. However, the fact that neither exogenous supply nor a decreased utilization of putrescine (for spermidine biosynthesis) substitute for the increased endogenous production (achieved either by DFMO treatment or by the expression of mouse ODC), suggests that the turnover of putrescine and/or ornithine may be more critical than the mere presence of high putrescine in the cells for somatic embryogenesis to proceed. This may in turn affect the overall metabolism of reduced nitrogen in the cells (Altman and Levin, 1993).

The results strongly demonstrate that despite ADC being the primary (perhaps the only) enzyme responsible for putrescine biosynthesis in undifferentiated carrot cells, overproduction of mouse ODC enzyme provides an effective alternative to ADC. As a consequence, the cells acquire a high degree of tolerance to toxic levels of DFMA.

The number of somatic embryos reported here in control cell lines is lower than that reported by Nissen and Minocha (1993). This is because of the difference in the criteria

for scoring of somatic embryos. In the studies of Nissen and Minocha (1993), 0.5 mm or larger embryos were counted, whereas in the present study only embryos equal to or larger than 1 mm were counted. This was done because of the ability of transgenic cells to differentiate into somatic embryo far earlier than the nontransformed control cells.

Consistent with the earlier reports (Minocha et al., 1991a), MGBG strongly inhibited somatic embryogenesis. However transgenic cells were somewhat tolerant to lower levels of MGBG. Although it is not know as to how MGBG inhibits somatic embryogenesis, it has been demonstrated that MGBG treated cells show increased levels of ACC. It was, therefore, suggested that polyamine and ethylene biosynthesis probably compete for the common precursor S-adenosylmethionine (Minocha et al., 1991a, Minocha & Minocha 1994).

It is sometimes difficult to interpret the endogenous polyamine levels since they can be stored in the vacuoles or cell walls where they cannot interact with the metabolic pathways occurring in the cytoplasm or other organelles. It was observed that L-ornithine (Fig. 21 and 22), unlike D- and methyl ornithine, supplied exogenously is metabolized to putrescine by both nontransformed and transgenic cells. The levels of putrescine in the transgenic cell lines were

always higher than those seen in nontransformed cells. This observation suggests that: (1) transgenic cells have the capacity to use relatively higher amounts of cellular ornithine than the nontransformed cells because of the presence of mouse ODC, and (2) in the absence of exogenous ornithine, transgenic cells may be in a state of reduced cellular levels of ornithine. Recent results from our laboratory (Scott Andersen, personal communication) indicate that the metabolism of U-^{14}C-ornithine by transgenic cells is several-fold higher than the control cells.

As reported by DeScenzo and Minocha (1993) in transgenic tobacco, many of the transgenic carrot cell lines also produced large quantities of cadaverine (Fig. 15). As mouse ODC is known to be able to use lysine as a substrate, production of cadaverine by transgenic cells was no surprise. In general, only those cell lines that showed high mouse ODC activity produced cadaverine. In the plant cells where compartmentalization of intermediates or enzymes is possible, deprivation of ornithine pools is highly probable. Since mouse ODC is known to have a low affinity for lysine, this could explain the production of cadaverine in high putrescine producing cell lines. The possibility of deprivation of ornithine in the cells could also be the reason for (1) interclonal and intraclonal variation in

cellular putrescine in transgenic cells, and (2) a significant decrease in putrescine levels with the age of the culture.

As shown in Fig. 1, arginine is produced in the cells either by the breakdown of proteins or *de novo* from ornithine, whereas ornithine is produced from arginine through arginase activity or from glutamate. It has been suggested that arginase is possibly responsible for the biosynthesis of putrescine that is derived from ornithine (Kang and Cho, 1990). It is demonstrated in soybean that arginase is activated by putrescine in a concentration-dependent manner (Kang and Cho, 1990). In the embryogenic medium, mobilization of proteins with high content of L-arginine can be expected like in an actively growing embryo in germinating seeds. Furthermore, in the transgenic cells with elevated levels of cellular putrescine, this endogenous putrescine could increase *in vivo* arginase activity. It has recently been shown in the *Nostoc* PCC 73102, a free-living cyanobacterium, that the addition of the intermediates of ornithine cycle significantly increases arginase and other enzymes involved in the cycle (Martel et al., 1993).

The question as to why do the nontransformed undifferentiated carrot cells favor the ADC over the ODC pathway for putrescine biosynthesis still remains

unanswered. The data presented here on the effect of DFMA in nontransformed cells is consistent with earlier report (Robie and Minocha, 1989) where cellular putrescine production was inhibited by DFMA. This observation suggests that nontransformed cells use the ADC pathway to produce putrescine. However, the results from the transgenic cells raise the question as to why putrescine levels are significantly reduced in the presence of DFMA when the increase in putrescine levels in transgenic cells are due to the overexpression of mouse ODC? While this observation, on the one hand, may seem to contradict a casual relationship between overexpression of mouse ODC and increase in putrescine level, it is a clear example of the limitation of the inhibitor studies in polyamine metabolism research. Although DFMA is highly specific for ADC *in vitro*, it has been found that radiolabeled DFMA is metabolized to DFMO *in vivo* (Slocum et al., 1988). It is very likely that the decrease in putrescine level seen in the transgenic cells is because of the combined effects of DFMA and DFMO, as DFMA is the inhibitor of plant ADC and DFMO an inhibitor of mouse ODC.

In conclusion, data presented here demonstrate that: (1) mouse ODC cDNA is stably integrated in the plant genome, (2) it is expressed to produce an active mouse ODC enzyme,

and (3) transgenic cells are highly embryogenic. Although the native ODC gene(s) is/are present in the cells (since green tissues show high ODC activity), carrot cells predominantly use the ADC pathway to produce putrescine in the callus state. It is not known at this point as to how the regulation of native plant ODC occurs and what effect, if any, the expression of mouse ODC has on the expression of the native plant ODC. If it is assumed that the cells have an abundance of active mouse ODC, the transgenic cells should become low in cellular ornithine. However, it would be premature to make a confirmatory statement in this context. I believe the ongoing research using radiolabeled substrates for ODC and ADC, i.e. ornithine and arginine, respectively, in the nontransformed and transgenic cells will address many of the unanswered questions from this present work. This study should yield a complete picture as to how polyamine biosynthesis may be involved in somatic embryogenesis.

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