I.5 Role of Polyamines in Somatic Embryogenesis*

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1 Introduction

The aliphatic amines putrescine, spermidine, and spermine are present in all living organisms. Since the demonstration of "an essential nutritional function" for putrescine in the bacterium *Hemophilus parainfluenzae* (Herbst and Snell 1948), polyamines have attracted a great deal of attention from workers in diverse fields of the life sciences. The first reports of the existence of putrescine in plants date back to 1911 (see Smith 1991 for a historical summary).

Several physiological functions of polyamines in plants have been reviewed (Slocum et al. 1984; Smith 1985, 1990; Evans and Malmberg 1989; Slocum and Flores 1991). While no hypothesis on the specific mode(s) of polyamine action has been advanced, their positive charges at cellular pH and ability to bind (interact) with several cellular macromolecules are obviously important for their functions. It has been suggested that polyamine metabolism plays an important role in growth, development, and stress responses in plants (Evans and Malmberg 1989; Slocum and Flores 1991). Most arguments regarding their putative functions are based upon two types of experimental observations: (1) correlative changes in cellular polyamine levels during and prior to the growth and developmental process in question; and (2) chemical inhibition of the biosynthesis of polyamines and its effects on the growth and developmental process. Among the developmental processes in which a major role for polyamines has been proposed in cell and tissue cultures are cell division and morphogenesis. The following discussion is focused primarily on the current status of our knowledge on the metabolism of polyamines in relation to somatic embryogenesis in plants.

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Biotechnology in Agriculture and Forestry, Vol. 30
Somatic Embryogenesis and Synthetic Seed I (ed. by Y.P.S. Bajaj)

2 Polyamine Metabolism

Biosynthesis of polyamines has been studied in a number of organisms (Pegg 1986; Smith 1990; Slocum and Flores 1991). In animals, the primary pathway for putrescine biosynthesis involves decarboxylation of ornithine by ODC[3] (Fig. 1). Plants and microorganisms utilize an additional pathway for putrescine biosynthesis via the enzyme ADC which converts arginine into agmatine, which is subsequently metabolized into putrescine in two steps. The distribution of the two pathways seems to be tissue-, organ-, and species-specific.

Spermidine and spermine are synthesized by the sequential addition of an aminopropyl moiety to putrescine, using the enzymes spermidine synthase and spermine synthase, respectively. Decarboxylated SAM, which is the product of SAMDC reaction, donates the aminopropyl groups. ADC, ODC, and SAMDC are considered to be key regulatory enzymes for the biosynthesis of polyamines. Each of these enzymes has a relatively short half-life (20–120 min) and its activity generally shows a positive correlation with cellular levels of polyamines. The catabolism of polyamines is regulated by diamine and polyamine oxidases (Federico and Angelini 1991). Since polyamines can also serve as precursors for a number of secondary metabolites, including nicotine, anabasine, and other

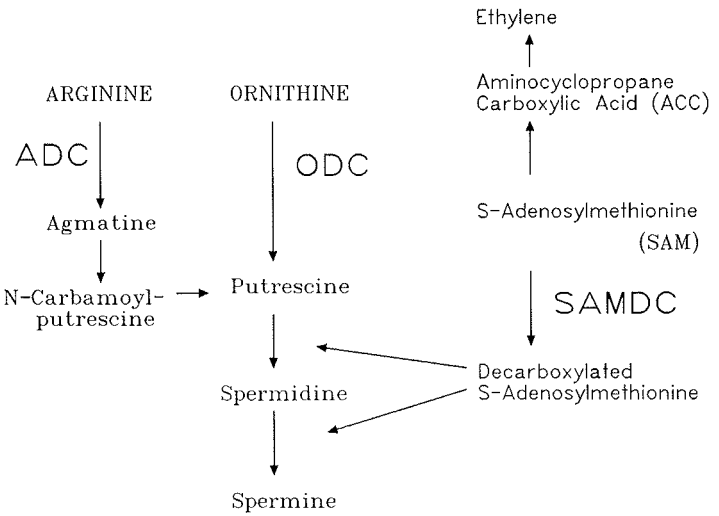

Fig. 1. Pathway for the biosynthesis of polyamines and ethylene in plants. The enzymes listed are: ADC, ODC, and SAMDC

[3] Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; ADC, arginine decarboxylase; AOA, aminooxyacetic acid; AVG, aminoethoxyvinylglycine; ODC, ornithine decarboxylase; CHAP, cyclohexylammonium phosphate (same as DCHA); DFMA, α-difluoromethylarginine; DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis (guanylhydrazone); SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase.

alkaloids (Flores and Martin-Tanguy 1991), their cellular pools are subject to modulation via biosynthesis, interconversion, degradation, and conversion to secondary compounds. Total cellular polyamine levels are further subject to changes in the soluble, bound, and conjugated forms of each polyamine (Smith 1985, 1990). In addition to the three common polyamines mentioned above, some plants contain other polyamines, e.g., cadaverine, nor-spermidine, nor-spermine, canavanine, etc.

Cellular levels of polyamines respond to a variety of external stimuli, e.g., physical stress, chemical stress, biological stress, plant growth regulators, etc. In recent years, a number of specific inhibitors have been used to successfully modulate the biosynthesis of polyamines (Pegg 1986; McCann et al. 1987). The activities of ADC and ODC can be inhibited by the substrate analogs DFMA and DFMO, respectively. Both are considered to be irreversible, or so-called suicide inhibitors. While DFMA is almost universally effective in the inhibition of ADC, DFMO does not inhibit ODC in all plants (Galston 1983; Flores and Galston 1984; Slocum and Galston 1985; Robie and Minocha 1989). Other inhibitors of ODC and ADC include methylornithine, monofluoromethylornithine, D-arginine, and canavanine. Methylglyoxal bis (guanylhydrazone) (MGBG) is a commonly used inhibitor of SAMDC, which causes a decrease in the biosynthesis of both spermidine and spermine. MGBG also causes stabilization of SAMDC in certain tissues, thus long-term use of MGBG may actually result in elevated levels of these two polyamines (Malmberg and Hiatt 1989). A number of somewhat specific inhibitors of spermidine and spermine synthases have also become available but only cyclohexylamine has been tested for inhibition of spermidine in plants. Recent studies from our laboratory show that MCHA (*trans*-4-methylcyclohexylamine) is an effective inhibitor of spermidine synthase in carrot tissue, causing a reduction in cellular levels of spermidine and an increase in putrescine. Spermine levels are either unaffected or show a slight increase during the 96 h of treatment (Andersen and Minocha, unpubl.). APCHA [N-(3-aminopropyl) cyclohexylamine], which is an inhibitor of spermine synthase in rat tissue (Shirahata et al. 1993), also shows similar effects on carrot cells, i.e., it causes a reduction in cellular spermine and a concomitant increase in spermidine. Putrescine is not affected by APCHA in carrot cells.

Most enzymes involved in polyamine biosynthesis (Fig. 1) have been purified and characterized in several microbial and animal systems (Tabor and Tabor 1984; Pegg 1986; Boyle et al. 1989). The genes for ADC, ODC, SAMDC, plus spermidine and spermine synthases have been isolated, cloned, and characterized from *E. coli*, yeast, and several mammalian tissues (Boyle et al. 1984, 1989; Kontula et al. 1984; McConlogue et al. 1984; Glass et al. 1987; van Kranen et al. 1987; Pajunen et al. 1988; Kahana 1989; Tabor and Tabor 1989; Xie et al. 1989). With the exception of preliminary reports on the cloning of ADC and SAMDC genes from plants (Bell and Malmberg 1990; Taylor et al. 1992; Rastogi et al. 1993), not much is known about genes coding for other enzymes of the polyamine biosynthetic pathway.

It is also obvious from Fig. 1 that the pathway for spermidine and spermine biosynthesis shares a common precursor with the biosynthesis of ethylene in

plants. Whereas for polyamine biosynthesis SAM is irreversibly decarboxylated by SAMDC, ACC synthase utilizes SAM for the production of ethylene. The fact that many of the physiological functions of polyamines and ethylene are antagonistic to each other has led to speculation that regulation of polyamine biosynthesis may have a physiological function via its effects on ethylene biosynthesis (Apelbaum et al. 1981, 1985; Even-Chen et al. 1982; Minocha 1988; Flores et al. 1990). As described later in this chapter, several laboratories have demonstrated that ethylene may be inhibitory to somatic embryogenesis in carrot; therefore, it is conceivable that increased polyamine biosynthesis could promote somatic embryogenesis via reduction in ethylene production (Minocha 1988; see also Nissen and Minocha 1993).

3 Polyamines and Somatic Embryogenesis

The roles of polyamines in the growth of shoot apex, root formation, and tuber formation in plants have been summarized by Galston and Flores (1991). The first report of a critical role of polyamines in somatic embryogenesis was given by Montague et al. (1978). Since then, several studies have been aimed at the changes in cellular levels of polyamines during somatic embryogenesis in plant tissues.

Analysis of polyamines in several tissues of mango (*Mangifera indica*) revealed no positive correlation with either the development of nucellar embryos or the effect of 2,4-D (Litz and Schaffer 1987). Polyamine levels were higher in nonembryogenic than embryogenic calli, and the addition of polyamines to the medium had either no effect or suppressed the formation of nucellar callus in most cultivars. Likewise, exogenous polyamines did not affect the development of somatic embryos from nucellus or nucellar callus. It was concluded by the authors that polyamine levels in these tissues were correlated more with increased growth rate than with morphogenesis.

Meijer and Simmonds (1988) demonstrated a sharp increase in putrescine during the callus induction phase in petiole explants of two cultivars of clover (*Medicago sativa*). This was followed by a decrease in putrescine upon transfer to embryogenic media. DFMA and DFMO inhibited putrescine accumulation in both cultivars, while embryo development was only inhibited in one cultivar. The effect of inhibitors was not reversed by exogenous putrescine. In the same year, Altman et al. (1988) reported a promotion of somatic embryo development by spermidine and its inhibition by MGBG. Unfortunately, no data on cellular polyamines were presented.

Fobert and Webb (1988) studied the effects of exogenous polyamines and enzyme-activated inhibitors of putrescine biosynthesis on somatic embryogenesis from cotyledons of eggplant (*Solanum melongena*). The addition of arginine, ornithine, agmatine, putrescine, spermidine, or spermine generally had no significant effect on somatic embryogenesis, except that high concentrations (10 mM or more) were inhibitory. DFMO was more potent than

DFMA in inhibiting somatic embryogenesis. Exogenous putrescine restored the inhibition by DFMO. Explants growing on auxin-containing medium that produced somatic embryos had higher levels of putrescine than those not producing somatic embryos (minus auxin medium). DFMO inhibited cellular putrescine as well as spermidine.

El Hadrami and D'Auzac (1992) observed an increase in embryogenic potential of callus in the rubber tree (*Hevea brasiliensis*) through the addition of arginine, spermidine, or a combination of putrescine, spermidine, and spermine. The maximum effect was seen with the combination of polyamines. DFMA, DFMO, and MGBG, while having no effect on callus formation, inhibited somatic embryogenesis. The inhibition was partially reversed by spermidine. During the early period of growth (0-20 days), neither DFMO nor MGBG had much effect on cellular polyamines. However, between 40 and 60 days of growth, MGBG caused a decrease in all three polyamines, while DFMO caused a decrease in spermidine and spermine but a small increase in putrescine. DFMA caused a decrease in putrescine but an increase in spermidine and spermine. Although no direct enzyme assays were performed, it was concluded that ADC was probably the major pathway for putrescine biosynthesis in this tissue.

Proembryogenic tissues of Norway spruce (*Picea abies*) were used by Santanen and Simola (1992) to study changes in polyamines during the maturation of somatic embryos. The results showed a substantial decrease in polyamines in tissue grown on the maturation medium. The results were further confirmed by Minocha et al. (1993) in both Norway spruce and red spruce (*Picea rubens*). In contrast to the tissue on maturation medium, that on the proliferation medium always had two- to threefold higher levels of putrescine and spermidine. Neither DFMO nor DFMA showed an appreciable effect on somatic embryogenesis. However, it should be pointed out that the development of somatic embryos in conifers follows a very different pattern compared to that in herbaceous angiosperms. In conifers, the proliferation of proembryogenic masses involves relatively fast growth on a medium containing an auxin and a cytokinin. The maturation of somatic embryos that is accompanied by slow growth and senescence of a large part of the callus mass occurs in the presence of abscisic acid (Tautorus et al. 1991). Thus, the decrease in polyamines in the tissue grown on the maturation medium may reflect the physiological status of the whole tissue rather than the developing/maturing somatic embryos. In contrast to conifers, the development of somatic embryos in most angiosperms entails a continued fast growth.

It is obvious from the above discussion that for most species only preliminary data are available on the metabolisms of polyamines in relation to somatic embryogenesis. No common pattern seems to emerge from these reports. Sufficiently detailed studies have been conducted only with carrot cell cultures; therefore, the remainder of this chapter is devoted primarily to this tissue.

4 Polyamines in Carrot

In carrot cell cultures, either putrescine or spermidine is the predominant polyamine depending upon the time of analysis; spermine being present only in limited amounts based upon fresh weight. Trace amounts of cadaverine have also been reported (Baker and Yon 1983). Furthermore, it is known that polyamines in carrot cell cultures are largely present in the soluble form; conjugated polyamines being generally less than 10% of the total. Arginine decarboxylase is the major regulatory enzyme for putrescine biosynthesis in most carrot cell cultures (Montague et al. 1979; Feirer et al. 1984; Robie and Minocha 1989; also see Mengoli et al. 1989 for an exception). However, in green somatic embryos, as well as in leaf and floral tissues of mature plants, high levels of ODC are present. Since ODC is absent from both cell cultures and the root tissue of mature plants, it is conceivable that ODC activity in carrot may be localized in the chloroplast. Carrot ADC is sensitive to DFMA and canavanine (Feirer et al. 1984; Robie and Minocha 1989; Mengoli et al. 1989). On the other hand, both the mature plant ODC as well as the ODC in cell cultures (where present) are not inhibited by DFMO (Mengoli et al. 1989; Robie and Minocha 1989).

The activity of SAMDC is correlated with the cellular levels of spermidine and spermine (Roustan et al. 1989b; Minocha et al. 1991b). This enzyme is inhibited by MGBG, which also causes a reduction in cellular spermidine and spermine levels (Minocha et al. 1991a). Cyclohexylammonium phosphate (CHAP), a potent inhibitor of spermidine synthase, causes a significant decrease in spermidine and a concomitant increase in both putrescine and spermine (Khan and Minocha 1991). A combination of CHAP + MGBG causes a synergistic increase in putrescine. On the other hand, the increase in spermine with CHAP treatment is completely counteracted by MGBG (Minocha and Khan 1991).

4.1 Polyamines and Somatic Embryogenesis in Carrot

In earlier studies on the metabolism of polyamines in carrot cell cultures, Montague et al. (1978) reported that putrescine levels increased sharply when cells were transferred to fresh media, regardless of the presence or absence of 2,4-D. However, embryogenic cultures maintained a higher level of putrescine than nondifferentiating cells. The rate of biosynthesis of putrescine in the absence of 2,4-D was almost double that in its presence. While spermidine showed no significant difference in response to treatment with auxin, spermine levels were actually lower in cultures undergoing somatic embryogenesis. The amounts of radioactive putrescine and spermidine synthesized from ^{14}C-arginine remained higher in the absence of auxin throughout the 72 h of study as compared to those in the presence of auxin. During the same period, incorporation of a label into spermine was lower in the differentiating cultures. However, in pulse-labeling studies, the label disappeared from putrescine at a faster rate than from spermidine, whereas spermine was quite stable during the 24–72 h of incubation. Based on these results, it was concluded that the metabolism of polyamines was different in the presence and absence of 2,4-D.

In a later study by Montague et al. (1979), it was demonstrated that increases in putrescine synthesis were correlated with increased cellular activity of ADC. Within 6 h of incubation, ADC levels were higher in the absence of 2,4-D vs +2,4-D cultures, and this difference was sustained for at least 3 days. It was further shown that increase in ADC activity was dependent upon the biosynthesis of both the mRNA as well as the ADC protein. Following inhibition of protein synthesis by cycloheximide, the half-life of ADC in carrot cell cultures was calculated to be about 30 min. While SAMDC activity also increased sharply on transfer of cells to fresh medium, there was no major difference in SAMDC activity in the presence or absence of auxin.

The role of ADC in the biosynthesis of putrescine in cultured cells of carrot was further documented by the studies of Feirer et al. (1984). Using specific inhibitors of ADC and ODC, it was shown that ADC was the predominant pathway for putrescine biosynthesis in these cells. Both putrescine biosynthesis and somatic embryogenesis were inhibited by DFMA. Furthermore, the effect of DFMA was reversible by the addition of exogenous polyamines. DFMO had little effect on either cellular putrescine or somatic embryogenesis.

A parallel study by Fienberg et al. (1984) confirmed the importance of polyamine biosynthesis during the differentiation of somatic embryos in carrot cell cultures. They compared changes in the cellular content of soluble polyamines in an embryogenic and a nonembryogenic cell line of carrot grown in the presence or absence of 2,4-D. It was seen that both the embryogenic cultures grown in the presence of auxin and the nonembryogenic cultures grown in (+) or (–) auxin media had similar profiles of changes in putrescine, spermidine and spermine. This involved the following: (1) a sharp decrease in putrescine within 24 – 48 h followed by low levels of this diamine until 8–10 days, and then an increase by 12–14 days; (2) little changes in spermidine levels during the first 8 days, followed by a slow increase during the next 4 – 6 days; and (3) only small fluctuations in spermine during most of the culture period. In contrast, the embryogenic cultures grown in the absence of auxin showed significantly higher levels of putrescine during the period of 4 – 8 days, a sharp increase in spermidine during the first 2 days, maintenance of higher levels of this polyamine through most of the 14 day culture period, and a peak of spermine during the first 3 days of culture. No significant differences in growth rates of the two lines were observed under the two growth conditions. These results appeared to contradict the observations of Montague et al. (1978), but the differences could be attributed to differences in cell density and the method of somatic embryo induction (see Fienberg et al. 1984 for further discussion). Whereas a sharp decrease in putrescine was seen in embryogenic cultures grown either in plus or minus auxin medium, a substantial increase in ADC activity was evident during the same period; the latter treatment (minus auxin) sustained higher ADC activity during the first 4 days as compared to the former treatment. The apparent contradiction between putrescine levels and ADC activity, which is not unique to carrot (similar observations have been made with *Picea* tissue; R. Minocha and S.C. Minocha, unpubl.), may be attributed to a rapid metabolism of putrescine in the absence of 2,4-D. The activity of ADC was always lower in the nondifferentiating cell line regardless of the presence of 2,4-D.

The activity of SAMDC was substantially higher in differentiating than in nondifferentiating cultures. Both DFMA and DCHA inhibited cellular polyamines in a predictable manner and also inhibited somatic embryogenesis. The effects of inhibitors were reversed by the addition of spermidine to the medium. Exogenous spermine was generally inhibitory to somatic embryogenesis. DFMO had no effect.

Bradley et al. (1984) published a brief report on the effects of exogenously supplied arginine and putrescine on somatic embryogenesis in carrot. When cells were grown for 40 days in the presence of 0.03 μM putrescine in a medium containing 2 mg/l 2,4-D and a PRL-4-DM amino acid mixture which included 40 mg/l L-arginine (Gamborg 1966), and then transferred to a medium lacking 2,4-D and putrescine, but containing arginine, only globular stage embryos were seen and no mature embryos were formed. If the later growth was allowed to occur in the absence of arginine, about half of the globular embryos matured within 4 days. Control cultures that were not treated with putrescine produced normal embryos within 3 weeks of transfer to 2,4-D-free medium. While this work was done with the same cell line (W001C) used by Fienberg et al. (1984), the results are, unfortunately, difficult to compare with most other published data for several reasons: (1) the culture period was relatively long (i.e., 40-60 days); (2) no rationales for the selection of medium supplements and concentration of putrescine and arginine nor for the duration of treatment were provided; (3) no information on scoring of somatic embryogenesis was given; and (4) cellular polyamine levels were not measured.

In a study of the long-term effects of DFMO, Mengoli et al. (1987) demonstrated that repeated culture of carrot cells in the presence of 5 mM DFMO had no effect on growth rate, but caused an increase in cellular protein content and polyamines and a decrease in ADC and ODC activities. In this study, the control and DFMO-treated cells did not differ in their ability to form somatic embryos (cf. Robie and Minocha 1989).

In a later study, Mengoli et al. (1989) reported that DFMO supplied in the presence of 2,4-D prolonged the commitment of cells to embryogenesis on transfer to an auxin-free medium. Canavanine, an inhibitor of ADC, inhibited both the growth of cells and (consequently) their ability to form somatic embryos. In contrast to the results of Montague et al. (1979), Feirer et al. (1984), Robie and Minocha (1989), and Roustan et al. (1992), they observed a higher ODC activity than ADC during the preembryogenic phase in their cells. They also found DFMO to have no effect on ODC activity in vivo, while it inhibited ODC activity in vitro (cf. Robie and Minocha 1989).

During the past several years, research in our laboratory has focused on the effects of several inhibitors of polyamine biosynthetic enzymes on the metabolism of polyamines and ethylene and the activities of ODC, ADC, SAMDC, and SAM synthetase in carrot cell cultures. A series of papers have been published (Robie 1987; Minocha 1988; Papa 1988; Robie and Minocha 1989; Minocha et al. 1990a,b, 1991a,b; Samuelsen 1990; Khan and Minocha 1991; Minocha and Khan 1991; Nissen and Minocha 1993) in which we have shown that:

1. The only polyamines seen in carrot cells are putrescine, spermidine, and spermine.
2. Whereas ADC is the primary enzyme for putrescine biosynthesis in undifferentiated cell suspensions, ODC activity is relatively high in fully developed somatic embryos and in leaves and flowers of mature plants.
3. Inhibition of ADC by DFMA significantly inhibits polyamine biosynthesis and also inhibits somatic embryogenesis.
4. DFMO, which does not inhibit ODC (from mature plants), promotes ADC-derived polyamine biosynthesis and also inhibits the production of ethylene.
5. DFMO promotes somatic embryogenesis in the absence of auxin and allows some somatic embryogenesis even in the presence of auxin.
6. MGBG is a potent inhibitor of SAMDC and thus inhibits spermidine and spermine biosynthesis while promoting the accumulation of putrescine as well as ACC.
7. MGBG inhibits somatic embryogenesis.
8. Cyclohexylammonium phosphate (CHAP), an inhibitor of spermidine biosynthesis, while promoting the accumulation of putrescine, has no effect on cellular ACC or the production of ethylene from cells.
9. CHAP does not inhibit somatic embryogenesis but only retards the development of somatic embryos.
10. The activity of SAM synthetase increases rapidly (up to 25-fold) within 5 days and is not significantly affected by any of the inhibitors except MGBG.
11. AVG strongly inhibits ACC and ethylene biosynthesis and promotes spermidine and spermine biosynthesis.
12. At low concentrations (5–20 μM), AVG and $AgNO_3$ promote somatic embryogenesis.
13. Exogenously supplied spermidine and spermine (1–10 mM but not lower concentrations) inhibit somatic embryogenesis and promote ACC and ethylene production, probably through a feedback inhibition of their own biosynthesis in the cells. These results are in general agreement with earlier work, except for the DFMO effects on somatic embryogenesis.

In addition to the use of inhibitors, the modulation of cellular polyamines in tobacco and carrot was achieved by the expression of mammalian genes for ODC and SAMDC (De Scenzo and Minocha 1993; Noh and Minocha 1993; Bastola 1994). Transgenic carrot cell lines that overexpress a mouse ODC cDNA show several-fold higher levels of putrescine, with little or no change in spermidine and spermine. These cell lines are highly embryogenic in the absence of auxin and show substantial somatic embryogenesis even in the presence of auxin in the medium at concentrations that normally inhibit somatic embryogenesis. Furthermore, these cell lines are highly tolerant to DFMA both for growth as well as for somatic embryogenesis. Transgenic cell lines expressing a human SAMDC cDNA show significantly higher levels of spermidine and also produce somatic embryos at a high frequency (Bastola 1994).

4.2 Polyamines and Ethylene in Carrot Somatic Embryogenesis

Another aspect of somatic embryogenesis that is influenced by polyamine metabolism is the role of ethylene. Like auxin, ethylene has been implicated as a requirement for somatic embryogenesis and as having a strong inhibitory effect. Tisserat and Murashige (1977) observed only a slight effect of exogenously applied ethylene on somatic embryogenesis in carrot. Roustan et al. (1989a,b) reported that inhibitors of ethylene biosynthesis (salicylic acid and acetylsalicylic acid) and ethylene action (Co^{2+} and Ni^{2+}) had a strong stimulatory effect on somatic embryogenesis in carrot. They emphasized that the effects of these inhibitors were mediated through ethylene synthesis and action. Exogenously supplied ethylene (by the addition of ethephon in the medium) caused a substantial decrease in somatic embryogenesis in this tissue (Roustan et al. 1990). In an unrelated study, Kiyosue et al. (1990) demonstrated that a number of heavy metal ions including Co^{2+}, Ni^{2+} and Cd^{2+} were capable of inducing direct somatic embryogenesis in apical tips of carrot seedlings without the formation of callus.

Keeping in mind that the polyamine and ethylene biosynthetic pathways share a common precursor (Fig. 1), Robie and Minocha (1989) studied the production of ethylene by carrot cell cultures under embryogenic and nonembryogenic conditions. It was observed that significantly more ethylene was generated by cells grown in the presence of 2,4-D. Furthermore, the promotion of polyamine biosynthesis by DFMO was accompanied by a reduction in ethylene accumulation. Aminoethoxyvinylglycine (AVG) was found to stimulate somatic embryogenesis. It was suggested that auxin-induced ethylene biosynthesis may play an important role in the inhibition of somatic embryogenesis by 2,4-D (Minocha 1988; Robie and Minocha 1989; Minocha et al. 1991a).

In line with this suggestion are the results of Roustan et al. (1992) who studied the incorporation of 3,4-[^{14}C]-methionine into polyamines and ethylene during somatic embryogenesis in carrot. They showed that in control cultures (–2,4-D), the incorporation of radioactive methionine into SAM and spermidine was about eight to nine times higher than that recovered in ACC or ethylene. Furthermore, incorporation into spermine was almost twice as high as in spermidine. Treatments that promoted somatic embryogenesis, including cobalt chloride and salicylic acid (Roustan et al. 1989a,b), stimulated the incorporation of methionine into spermidine and spermine while reducing its incorporation into ethylene. The amount of radioactivity present in ACC was also higher in the presence of $CoCl_2$ and salicylic acid (probably due to its reduced utilization in ethylene synthesis). It should be pointed out that the release of $^{14}CO_2$ was not affected under these conditions, nor was there any effect on the uptake of methionine. Exogenously supplied ethylene (as ethephon) caused an inhibition of somatic embryogenesis and a reduction in the incorporation of methionine into spermidine and spermine. Activities of ADC as well as SAMDC were higher in the presence of either $CoCl_2$ or salicylic acid than in the control cultures. Ethephon caused a reduction in the measurable activity of both these enzymes. It was concluded that the reduction in polyamine biosynthesis could be due to the effects of ethylene on ADC and SAMDC activities. Their results are in agreement with the work of Even-Chen et al. (1982), who had observed an increased flux of

radioactive methionine into spermidine in orange peel tissue in response to treatment with aminoethoxyvinylglycine (AVG). Biondi et al. (1990) have also reported a similar effect of AVG on the incorporation of radioactive methionine into spermidine in *Prunus avium* shoot cultures.

The interaction of auxin, ethylene, and polyamines has been discussed by Nissen and Minocha (1993) in the light of some recent results from our laboratory. However, ethylene has also been found to be suboptimal for somatic embryogenesis in a different cell line of carrot where addition of low concentrations of ACC or ethephon actually stimulated somatic embryogenesis (Nissen 1993). For this cell line the inhibitors of ethylene biosynthesis actually caused a slight inhibition rather than a promotion of somatic embryogenesis. The observation that the carrot cell cultures can be suboptimal with respect to ethylene under certain conditions has obvious implications for the mechanism of action of 2,4-D in the inhibition of somatic embryogenesis.

The morphogenetic role of 2,4-D, DFMO, and ethylene in somatic embryogenesis is complicated by the observations that: (1) inhibitors of ACC biosynthesis (AOA and AVG), while promoting embryogenesis at lower concentrations, actually inhibit this process when used at higher concentrations (Nissen 1993; Minocha, unpubl.); (2) low levels of exogenously supplied ethylene are actually stimulatory to embryogenesis under certain conditions (Nissen 1993); (3) while some of the inhibitors of ethylene biosynthesis or action promote somatic embryogenesis in the absence of auxin, none are able to reverse the inhibitory effect of 2,4-D; and (4) DFMO can restore embryogenesis even in the presence of relatively high concentrations of exogenously applied ethylene in the form of ethephon.

The situation becomes even more complex when one compares the studies of Robie and Minocha (1989) and Roustan et al. (1992) with the data obtained by Samuelsen (1990) who found that the inhibition of spermidine biosynthesis by MGBG was accompanied by a decrease in ethylene biosynthesis and a concomitant inhibition of somatic embryogenesis. Detailed analysis showed, however, that ACC levels were significantly higher in MGBG-treated cells than in controls. The inhibition of ethylene production in this case could be due to some direct effects of MGBG on ethylene-forming enzyme (ACC oxidase). It is, therefore, quite possible that not only ethylene but also ACC itself could play a morphogenetic role in carrot cell cultures. This is in agreement with the findings that moderate to high levels of ACC (50–100 μM) inhibit somatic embryogenesis in carrot (Verma and Tarka 1984; Minocha, unpubl.). Since there is currently no information available on the effects of salicylic acid, Co^{2+}, Ag^{+}, and exogenously applied ethylene (ethephon) on the cellular levels of ACC, the role of ethylene vs ACC cannot be easily resolved. Moreover, the data obtained from ethylene measurement in the culture vessel do not necessarily represent the actual cellular levels of ethylene. Being a gas, the cellular effects of ethylene must be exerted largely at the time and site of production. Undoubtedly, the physiological functions of ethylene in somatic embryogenesis and its competitive interaction with polyamines deserve further analysis. Studies are currently underway to accurately measure the rates of biosynthesis of ACC and ethylene in response to treatments with the various inhibitors and promoters of somatic embryogenesis.

Any explanation for the role of polyamines in somatic embryogenesis is further complicated by the following apparently contradictory observations: While DFMO-promoted somatic embryogenesis is accompanied by increased cellular polyamine levels and inhibition of their synthesis inhibits somatic embryogenesis (Robie and Minocha 1989; Nissen and Minocha 1993), an exogenous supply of either spermidine or spermine (1–10 mM) actually inhibits somatic embryogenesis (Minocha, unpubl.). Since polyamines are rapidly taken up by carrot cells (Pistocchi et al. 1988; Kanchanapoom et al. 1991), it can be argued that the inhibitory effects of spermidine and spermine and the promotory effects of DFMO are mediated through cellular metabolism and are not due to the mere presence of these compounds in the medium. Moreover, the fact that DFMO effects cannot be mimicked by other analogs (e.g., methylornithine, D-ornithine, L-ornithine, and putrescine) points to a specific physiological effect of DFMO. Whether this effect is mediated through the DFMO effects on cellular polyamine metabolism or through an independent mechanism is still not clear and needs further investigation (see Nissen and Minocha 1993, 1994). The arguments in favor of the former mechanism are: (1) DFMO causes a significant increase in putrescine biosynthesis in the cells both in the presence and absence of auxin; (2) this increase in putrescine biosynthesis is correlated with an increase in ADC activity; and (3) the increased rate of putrescine biosynthesis by the overexpression of a mouse ODC cDNA induces a behavior that mimics the DFMO effects on the untransformed cells. On the other hand, an equally strong argument can be made against the idea that DFMO effects are mediated largely through polyamines. The observations that the inhibitory effects of DFMA and MGBG can be counteracted by an exogenous supply of putrescine and spermidine, but the same polyamines cannot replace DFMO for promotion of somatic embryogenesis, indicate that the need for polyamines during somatic embryogenesis is independent of the stimulation caused by DFMO.

A third possibility that is presently being explored in our laboratory is that it is not merely the presence of high(er) concentrations of putrescine and spermidine that is sufficient for somatic embryogenesis, but their rapid turnover may even be more critical for the development of somatic embryos. It is envisioned that increased turnover of polyamines may somehow regulate nitrogen balance in the cell and also affect ethylene biosynthesis through the increased consumption of SAM. This hypothesis reconciles the contradiction between the effects of increase in cellular polyamines via increased synthesis (by DFMO treatment or transgene expression) and those due to an exogenous supply in the medium. The latter could cause a decrease in polyamine turnover by feedback inhibition and thus inhibit somatic embryogenesis.

5 Concluding Remarks

A few generally accepted conclusions with respect to somatic embryogenesis in carrot are:

1. Tissue explants must be treated with auxin to produce organized proembryogenic cell masses.
2. On transfer to an auxin-free medium, these proembryogenic cell masses undergo a programmed sequence of cell divisions and cell enlargement to produce heart- and torpedo-stage embryos that continue to grow into small plantlets.
3. The development of somatic embryos can be halted, and reversion to the proembryogenic stage is seen if auxin is added back to the medium at any time.
4. While a number of physical and chemical treatments can suppress the development of somatic embryos in the auxin-free medium, until recently nothing was known to overcome the inhibitory effect of auxin.
5. Biochemical and molecular analyses of cell clumps grown in the presence and the absence of auxin reveal only minor differences, indicating that gross changes in gene expression are probably not involved in early stages of somatic embryogenesis.

While a few authors have speculated on the acquisition of embryogenic potential by carrot cells and the role of auxin in this process (de Vries et al. 1988; Chasan 1993; Cooke et al. 1993; De Jong et al. 1993; and references therein), many more hypotheses have been advanced to explain the role of auxin in the suppression of somatic embryo development beyond the globular stage (Nissen and Minocha 1993 and references therein). Many of these suggestions are based upon correlative changes in the physiology of cells and involve either the production of some inhibitory compounds (e.g., ethanol, acetaldehyde, ethylene, etc.) or the production of certain promotory factors. Some of these suggestions have recently been experimentally tested in the light of the ability of DFMO to counteract the auxin effects (Nissen and Minocha 1993).

A strong argument has been made for the need of an auxin gradient to establish a bilateral symmetry (polarity) in the developing proembryo (Liu et al. 1993; see also comments in Cooke et al. 1993 and references therein). The question as to whether this polarity is already present in the proembryogenic cell masses growing in the presence of auxin, or if it is generated subsequent to the transfer of these cell masses to auxin-free medium, still remains to be answered. It has been suggested that the bilateral symmetry may involve (or depend upon) the polar transport of auxin itself. However, we must be cautious in our assumptions with respect to the nature of the auxin responsible for this polarity, i.e., endogenously produced auxin vs the exogenously supplied auxin. There is ample evidence to show that carrot cells growing in the presence of exogenously supplied synthetic or natural auxin (i.e., 2,4-D or IAA) do produce endogenous auxin (Michalczuk et al. 1992a,b). Furthermore, it is quite apparent that the polar transport of auxin is essential for the development of somatic embryos. It is possible that the globular stage proembryogenic masses present in the 2,4-D-containing medium already possess polarity with respect to the distribution and polar transport of auxin and the exogenous auxin simply overwhelms the endogenous gradient. Thus, the removal of auxin from the medium permits the auxin gradient to influence further development. Alternatively, it is also

conceivable that the globular masses do not actually possess an auxin gradient and it is only after removal of the exogenous auxin that a gradient is formed.

Having established that: (1) the presence of DFMO in the medium promotes polyamine biosynthesis and can counteract the auxin effect, and (2) the overexpression of mouse ODC cDNA in carrot also has the same two effects; the question that can be asked is: Does the increased production (see discussion above for rate of synthesis vs cellular content) of polyamines have something to do with the establishment or maintenance of the endogenous auxin gradient, or are the two observations a consequence of different mechanisms for counteracting the auxin effects? In the first case, a possibility exists that DFMO has some direct effects on auxin metabolism or transport that are unrelated to cellular polyamine metabolism. The latter situation is, however, more difficult to reconcile with the above explanation because the transgenic cells are grown under the same set of conditions as the controls. The reversal of the effects of TIBA, CPIB and other auxin transport inhibitors by DFMO (Nissen, pers. commun.) suggests that DFMO is either able to protect the endogenous gradient of IAA directly or the increased production of polyamines somehow helps to maintain or (re)establish the auxin gradient.

A provocative suggestion that can be made to centralize the role of polyamines in in vitro morphogenesis is as follows:

Polyamines being positively charged at cellular pH could contribute to: (1) either the establishment of polarity through their own unequal distribution in the cell, thus causing the unequal distribution of some other negatively charged macromolecule(s); or (2) the maintenance of an asymmetrical distribution of a negatively charged (macro)molecule by neutralizing its charge. In either case an asymmetrical distribution of polyamines would be required. At present, however, nothing is known about the cellular compartmentation/distribution of polyamines in plant cells.

Acknowledgments. The authors are thankful to Drs. Arthur Mathieson and Curtis Givan for useful suggestions on the manuscript and to Nancy Jackson for word processing. Research discussed in this chapter was partially supported by National Science Foundation Grant DCB-8615915.

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