

Original article

Genetic manipulation of polyamine metabolism in poplar II: effects on ethylene biosynthesis

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Received 2 November 2001; accepted 6 June 2002

Abstract

Possible competition between polyamine and ethylene metabolisms was studied in two types of transgenic poplar (*Populus nigra* × *maximowiczii*) cells: (a) constitutively expressing a mouse ornithine decarboxylase (ODC, EC 4.1.1.17) cDNA under the control of double 35S cauliflower mosaic virus (CaMV) promoter (cell line 2E), and (b) constitutively expressing a *Datura* *S*-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50) cDNA under the control of a single 35S CaMV promoter (line PS-18). The 2E cells contained significantly higher putrescine (Put) as well as spermidine (Spd) contents than the non-transgenic (NT) cells. The PS-18 cells contained three- to five-fold lower amounts of Put than the NT cells; their Spd content was either comparable to NT cells (at 3 d of culture) or it was higher than the NT cells (at 6 d of culture). The production of ethylene in the 2E cells was generally higher than in the NT cells throughout the 7-d culture period. Ethylene production in the PS-18 cells was comparable to NT cells. The cellular content of 1-aminocyclo-propane-1-carboxylic acid in the NT and 2E cells was quite similar, while it was slightly lower in the PS-18 cells. It is concluded that in poplar cells the cellular pool of *S*-adenosylmethionine is probably large enough to satisfy the demand for both polyamine and ethylene production and no competition between the two pathways is apparent. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: Ethylene; Ornithine decarboxylase; Polyamines; Transgenic cells; *S*-adenosylmethionine decarboxylase

1. Introduction

The diamine putrescine (Put) and the polyamines spermidine (Spd) and spermine (Spm) occur universally in all plants, animals and microorganisms and are referred to as common polyamines [9]. Polyamines and their precursors are considered essential for the proper growth and development of plants, animals and microorganisms [6,9,32,38]. The pathway for the biosynthesis of polyamines is well established [9,39]. The first step in polyamine biosynthesis in higher plants and bacteria is the decarboxylation of either

arginine or ornithine, which is catalyzed by arginine decarboxylase (ADC, EC 4.1.1.19) and ornithine decarboxylase (ODC, EC 4.1.1.17), respectively (Fig. 1). While the latter reaction directly produces Put, agmatine formed by the ADC is hydrolyzed and deaminated by agmatine iminohydrolase, resulting in the formation of *N*-carbamoyl-putrescine, which is then converted to Put by *N*-carbamoylputrescine amidohydrolase. Spd and Spm are synthesized by sequential additions of an aminopropyl moiety to the terminal amino groups of Put and Spd; the reactions being catalyzed by Spd synthase (EC 2.5.1.16) and Spm synthase (EC 2.2.1.22), respectively. The aminopropyl moiety is derived from decarboxylated *S*-adenosylmethionine (dcSAM), produced from *S*-adenosylmethionine (SAM) by the enzyme SAM decarboxylase (SAMDC, EC 4.1.1.50). SAM is synthesized from L-methionine by SAM synthetase.

Ethylene is a gaseous plant growth regulator that affects a variety of plant growth and developmental processes including fruit ripening, seed germination, abscission,

Abbreviations: ACC, 1-aminocyclo-propane-1-carboxylic acid; AVG, amino-oxyvinylglycine; ADC, arginine decarboxylase; CaMV, cauliflower mosaic virus; dcSAM, decarboxylated *S*-adenosylmethionine; GC, gas chromatography; MACC, *n*-malonyl-1-aminocyclo-propane-1-carboxylic acid; NT, non-transgenic; ODC, ornithine decarboxylase; Put, putrescine; SAM, *S*-adenosylmethionine; SAMDC, *S*-adenosylmethionine decarboxylase; Spd, spermidine; Spm, spermine; SSA, sulfosalicylic acid

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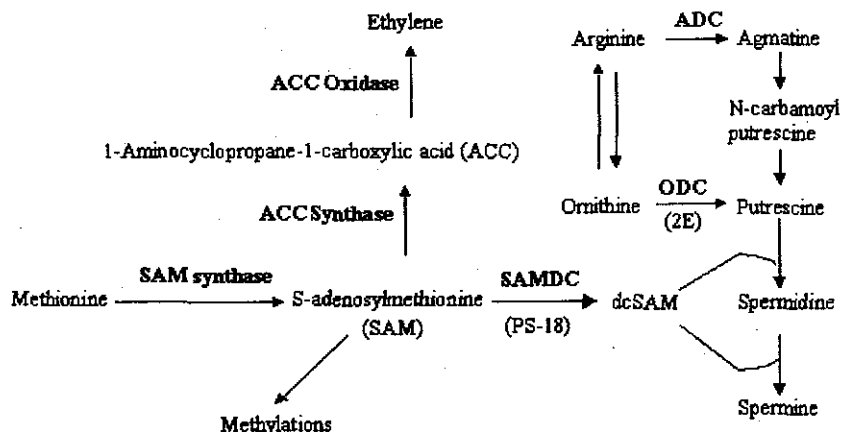


Fig. 1. The biosynthetic pathway of polyamines and ethylene in plants. The 2E cells are transgenic for a mouse *odc* cDNA while the PS-18 cells harbor a *Datura samdc* cDNA.

flower senescence, wound response and environmental stress responses [5,20–22,25]. The biosynthesis of ethylene begins with the conversion of SAM into 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase (Fig. 1). Besides ACC, this reaction also produces 5'-methylthioadenosine, which is recycled for the synthesis of methionine. This salvage pathway preserves the methylthio group at every revolution of the cycle at the cost of one molecule of ATP [reviewed in 5,21]. Thus high rates of ethylene biosynthesis can be maintained even when the pool of free methionine is small.

The enzyme ACC oxidase (also called ethylene-forming enzyme) converts ACC into ethylene, CO₂ and HCN. Alternatively, ACC can be conjugated with malonate to form *n*-malonyl-ACC (MACC) [42]. The conversion of ACC to MACC is essentially irreversible; MACC is thus considered to be a biologically inactive end product of ACC. Another minor conjugate of ACC in plants is γ -glutamyl-ACC, whose fate in the cell has not been characterized.

The induction of ethylene biosynthesis in most instances is based on increase in ACC synthase activity, while ACC oxidase is often considered to be constitutive. There is, however, mounting evidence that the transcript levels and enzyme activity of ACC oxidase also increase in some plants in response to internal or external factors that induce ethylene formation [5,21].

In addition to being a precursor for ethylene and polyamines, SAM is the primary donor of methyl groups in transmethylation reactions for a number of substrates, and also an allosteric activator of threonine synthase [26]. In some plants, the methyl moiety of SAM can be transferred to Put, via Put-*N*-methyltransferase, to form *N*-methyl-Put, which serves as a precursor for the biosynthesis of nicotine and other related alkaloids [18].

Delaying fruit ripening and flower fading by reducing ethylene biosynthesis has been a major goal of post-harvest physiologists [16, and references therein]. Two strategies of genetic engineering have been employed to achieve this goal: (a) inhibiting the expression of genes encoding ethyl-

ene biosynthetic enzymes by transformation with the respective antisense genes; and (b) lowering the cellular level of ACC by introduction of a bacterial gene encoding ACC deaminase. The use of transgenic approach can eliminate the toxic side effects inherent to the chemical control of ethylene biosynthesis.

Since SAM is a precursor for the biosynthesis of both ethylene and polyamines, and its utilization into both pathways is nonreversible, a competition between polyamine and ethylene biosynthesis for utilization of SAM has been postulated to explain some of their antagonistic relationships in plant growth and development [31]. It has been hypothesized that increased polyamine production in plants may limit the amount of available precursor allocated for ethylene biosynthesis or vice versa. The levels of polyamines and the activity of their biosynthetic enzymes increase during many growth processes in plants, whereas, for most part, ethylene production increases during senescence of tissues or in association with respiratory climax in fruits. The nature and extent of the competition between the polyamine and the ethylene biosynthetic pathways is at least partly a function of tissue type and its physiological stage of development [10,13–15,36,41].

In order to understand fully the role played by polyamines and ethylene in plant development, and the metabolic relationships between the two biosynthetic pathways, genetic manipulation of the two pathways has been a useful approach [3,5,23]. Thus far, however, neither the transgenic plants expressing polyamine biosynthetic genes have been analyzed for ethylene production, nor have those in which ethylene was modulated been analyzed for polyamine metabolism. In the present study, biochemical interactions between polyamine and ethylene metabolism were investigated using two transgenic cell lines of poplar, one expressing a mouse *odc* cDNA and the other expressing a *Datura samdc* cDNA. The results demonstrate that the overproduction of polyamines in poplar cells did not significantly affect either the cellular ACC levels or the production of ethylene.

2. Results

2.1. Polyamines in poplar NT and 2E cells

Poplar (*Populus nigra* × *maximowiczii*) cells transformed with plasmid *pcw122-odc* containing a mouse *odc* cDNA under the control of a double 35S CaMV promoter, along with a bacterial *nptII* selection gene [3,4] were used for the first part of this study. As reported earlier [3,4], the transgenic cells of line 2E contain several-fold elevated amounts of Put as compared to the nontransformed (NT) cells. During the period studied, Put content of the 2E cells was two- to four-fold higher than the NT cells. The cellular levels of Spd were also significantly higher in the transgenic cells than the NT cells. In both the NT and the 2E cells, Put increased with time during the 1-week cell culture cycle reaching a peak around the 5th–6th d, then decreasing slightly on 7th d (data not shown, see [3] for details). Overall, Spd showed a steady decline during the culture period from day 3 to day 7 in both cell lines. While Put was the predominant polyamine, Spm was found in the least amount, and was generally higher in the NT cells than the 2E cells on any given day of analysis (data not shown; see [3,4] for details).

2.2. Ethylene production in NT and 2E cells

Production of ethylene in the NT and 2E cells was compared using either 3- or 6-d-old cells over a period of 25 h of accumulation. The transgenic 2E cells produced significantly higher amounts of ethylene than the NT cells over the entire 3–25 h incubation periods for both 3- and 6-d-old cultures (Fig. 2). For the 6-d-old cell cultures, ethylene production by the 2E cells was almost twice the amount of that produced by the NT cells for any period of accumulation. Ethylene production by the 3-d-old NT as well as 2E cells was always higher than that by the 6-d-old cultures in the respective cell line. However, the difference in ethylene production between 6- and 3-d-old cells was more pronounced for the NT cells than for 2E cells.

In another set of experiments, ethylene production over an accumulation period of 24 h was analyzed at different times after transfer of cells to fresh medium. During the week-long culture period, ethylene production on any given day was generally higher in the 2E cells than the NT cells, particularly between the 2nd and the 4th d of culture (Fig. 3A). Ethylene production by both the NT and 2E cells showed a similar trend during the week-long culture period, i.e. it increased during the first 3 d of culture and then decreased during the latter half of the week.

2.3. Cellular ACC content in NT and 2E cells

Following ethylene analysis, the same cells were collected for determination of ACC. Data presented in Fig. 3B show

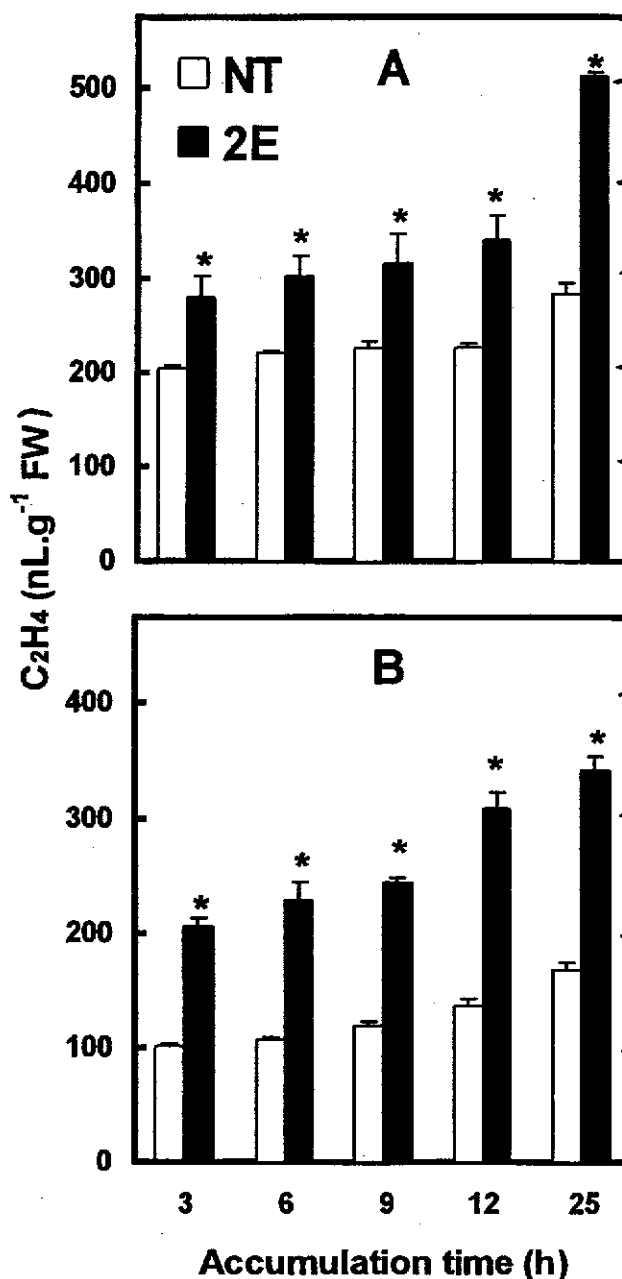


Fig. 2. Ethylene production by 3- and 6-d-old poplar NT and 2E cells at 3–25 h of accumulation period. (A) 3-d-old culture; (B) 6-d-old culture. Data presented are mean ± S.E. of four replicates. Statistically significant differences ($P < 0.05$) between the two cell lines on a given day are indicated by ‘*’.

the cellular ACC content in both cell lines on different days of culture after transfer to fresh medium. A similar trend was observed in the changes of cellular ACC contents in both cell lines as that seen for ethylene production, i.e. an increase up to 3rd or 4th d, and then a decrease during the later part of the week. Cellular ACC contents in the 2E cells were slightly lower (often not statistically significant) than the NT cells on most of the days, except on the 1st and 2nd d of culture, when the differences were statistically significant.

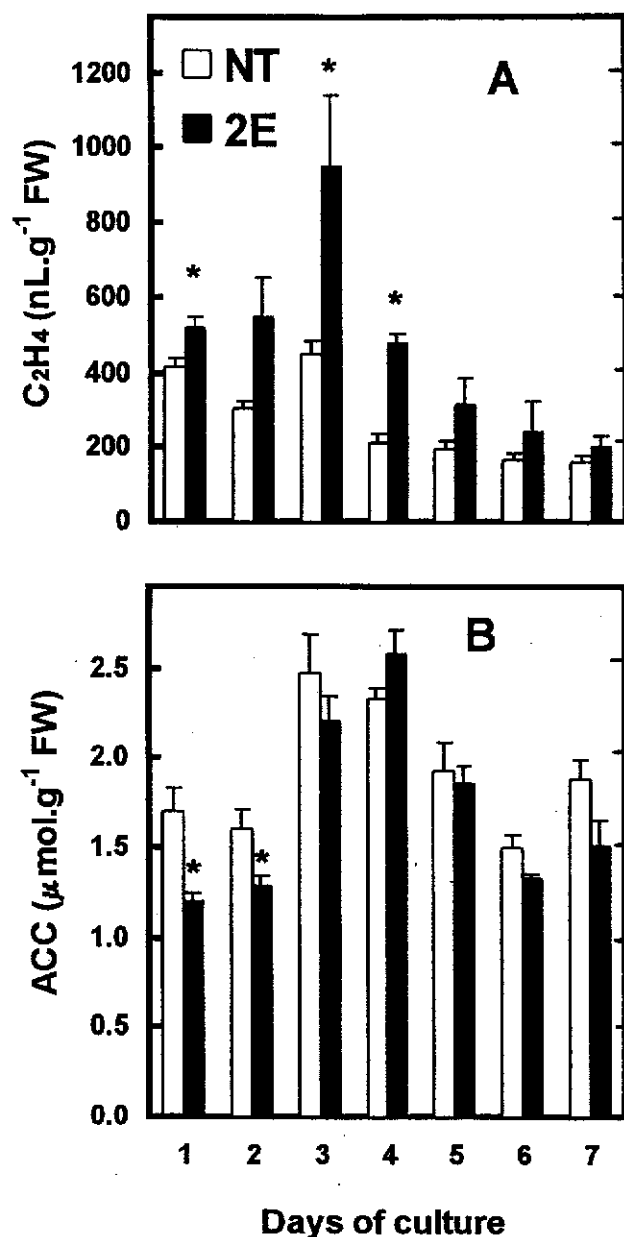


Fig. 3. Ethylene production over 24 h accumulation period by poplar NT and 2E cells on different days after transfer to fresh medium (A) and cellular ACC content in poplar NT and 2E cells at the end of ethylene production analysis in the above experiment (B). Data presented are mean $\pm$ S.E. of four replicates. Statistically significant differences ($P < 0.05$) between the two cell lines on a given day of analysis are indicated by '*'.

2.4. Polyamines, ethylene production, and ACC content in NT and PS-18 cells

The transgenic poplar line PS-18 was produced in our laboratory by transferring a *Datura samdc* cDNA along with a bacterial *hptII* gene (both were under the control of a single 35S CaMV promoter) into poplar cells by biolistic bombardment (J.S. Lee, R. Minocha, and S.C. Minocha, unpublished data). The cells selected on hygromycin after biolistic bombardment were characterized with respect to

the presence of the transgene by PCR. The transgenic cells showed the presence of the *Datura samdc* gene (data not presented). Polyamine analysis showed that Put content in the PS-18 cells was significantly lower than the NT cells in both 3- and 6-d-old cultures (Fig. 4A). Spd contents of the two cell lines were similar for the 3-d-old cultures; however, Spd in the PS-18 cells was significantly higher than the NT cells on the 6th d (Fig. 4B). Spm content for the NT and PS-18 cells was also similar on 3rd d, but was higher in the PS-18 cells on the 6th d (Fig. 4C). Both the NT and the PS-18 cells had higher level of Put on the 6th d than the 3rd d of culture. The contents of Spd and Spm in both the NT and the PS-18 cells were lower on the 6th d as compared to 3rd d within the respective line.

The 6-d-old cells of both lines produced less ethylene than the 3-d-old cells whether the ethylene measurements were done over 6- or 24-h periods of accumulation (Fig. 5A). For 3-d-old cells, no significant differences in ethylene production were observed between the two cell lines, although the amounts were slightly lower for the PS-18 cells than the NT cells. Ethylene production by the 6-d-old PS-18 cells was somewhat higher than that by 6-d-old NT cells, but the differences were small and statistically insignificant. Cellular ACC levels did not change much between 3rd and 6th d of culture in either the NT or the PS-18 cells (Fig. 5B). At both times, cellular ACC in the PS-18 cells was slightly (but not statistically significant) lower than that in the NT cells.

3. Discussion

Due to the commonality of the precursor SAM [9,38,42], a competition between polyamine and ethylene biosynthesis has often been envisioned [1,26–28,31]. Two types of observations have supported this idea: (a) the apparently contrasting roles of polyamines and ethylene in plant growth and development, and (b) a reverse correlation between cellular polyamine content and ethylene production under certain growth conditions in several plant tissues. The experimental evidence on the existence of such a competition and its physiological significance in plant growth and development is still very mixed, and sometimes contradictory. The present study was aimed at analyzing the existence of such a competition between the two pathways using transgenic cell lines in which cellular polyamines had been genetically manipulated. Our results using cell lines that either overproduce Put and Spd, or have reduced levels of Put, do not support the idea of a competition between the two pathways in poplar cells. The following is a brief discussion of the contrasting evidence in relation to this competition.

It is known that polyamines retard the senescence of detached leaves and flower petals, and inhibit the production of ethylene in auxin-treated tissues [40]. Polyamines also inhibit ethylene biosynthesis and other degradative processes associated with senescence including chlorophyll

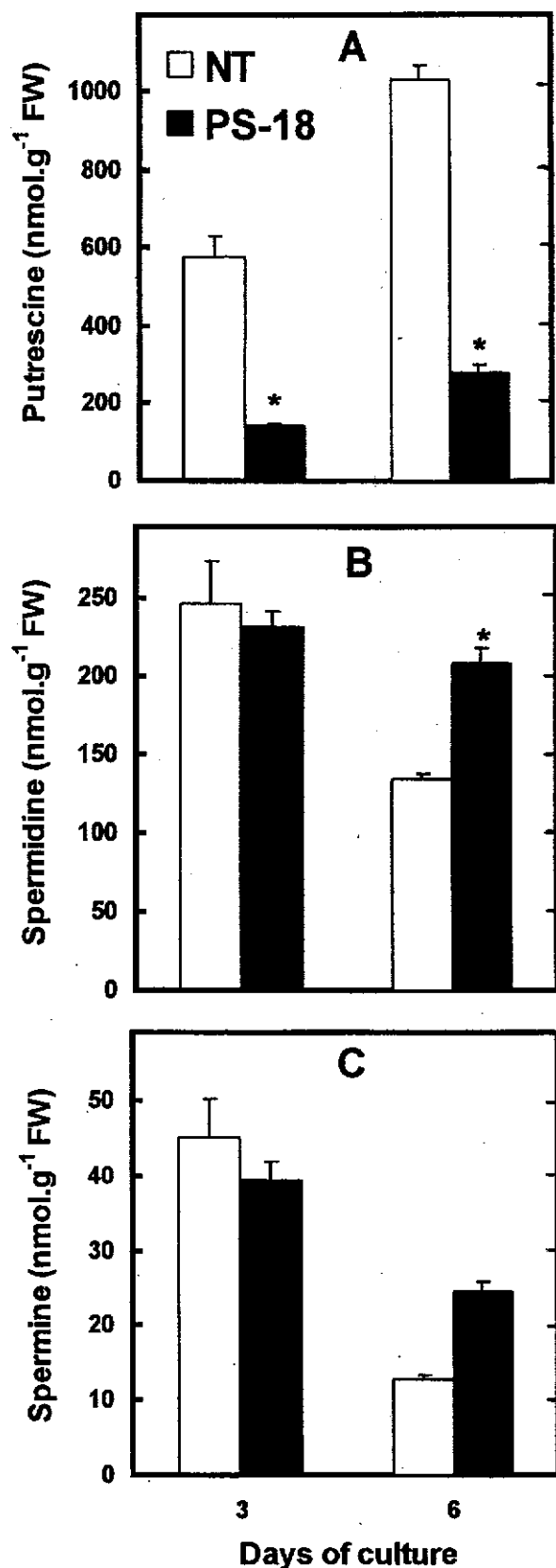


Fig. 4. Cellular free polyamine content in 3- and 6-d-old poplar NT and PS-18 cells. (A) Put; (B) Spd; (C) Spm. Data presented are mean $\pm$ S.E. of four replicates. Statistically significant differences ($P < 0.05$) between the two cell lines on a given day of analysis are indicated by '*'.

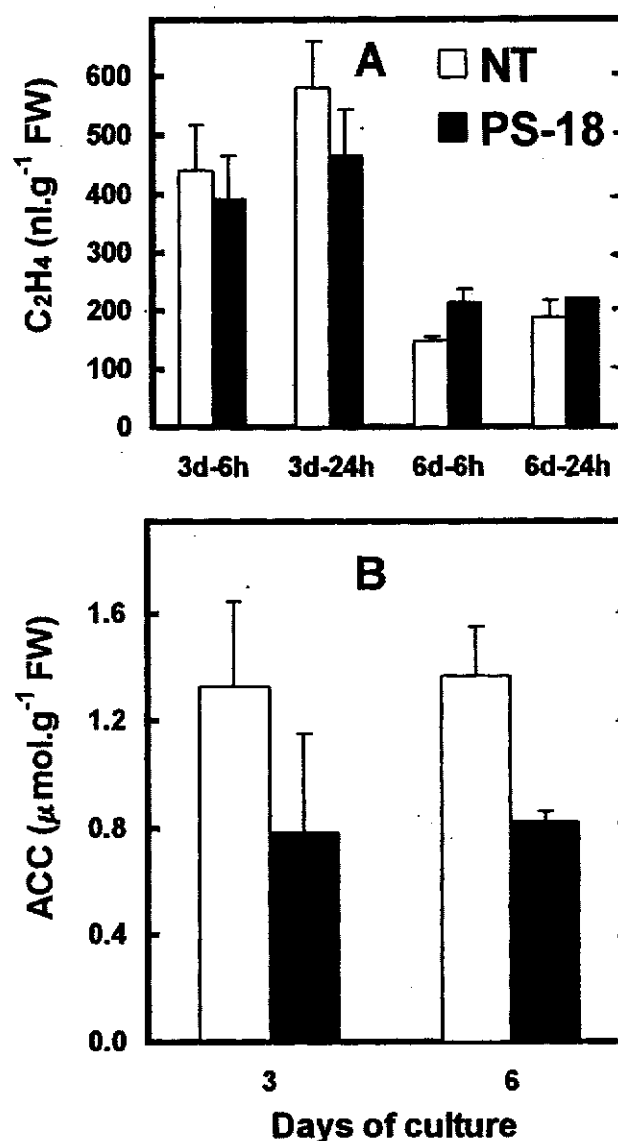


Fig. 5. Ethylene production by 3- and 6-d-old poplar NT and PS-18 cells over 6 and 24 h accumulation periods (A) and cellular ACC content in 3- and 6-d-old poplar NT and PS-18 cells (B). Data presented are mean $\pm$ S.E. of three or four replicates. There were no statistically significant differences ($P < 0.05$) between the two cell lines on any given day of analysis.

loss, increase in membrane leakiness, and rise in RNase and protease activities [28]. A potent inhibitor of SAMDC activity, methylglyoxal bis-guanyldiazide (MGBG), caused an elevation of ethylene production, increases in the activities and amount of transcripts of ACC synthase and ACC oxidase, and brought the ethylene production pattern forward by 1 d in cut carnation flowers. The authors suggested that endogenous polyamines might possibly suppress ethylene production. On the other hand, using the same plant material, Serrano et al. [37] found that when cut flowers were treated with 50 mM aminotriazole, an inhibitor of ethylene biosynthesis, senescence was retarded and the climacteric peak of ethylene was inhibited without any effect on the levels of Put and Spd. The latter data suggest

that inhibition of ethylene biosynthesis does not affect polyamine biosynthesis in this tissue.

In Litchi fruit during storage, the contents of Put, Spd and Spm decreased gradually, whereas membrane permeability and ethylene evolution increased. Treatment with Spd delayed the increase of membrane permeability without an effect on ethylene evolution. These data suggest that Spd could regulate senescence through an effect on the membrane system and then perhaps affect ethylene evolution indirectly [19].

Roustan et al. [36] showed that the presence of high concentrations of ethylene at the beginning of carrot cell culture in auxin-free fresh medium reduced the formation of polyamines from [^{14}C] arginine by decreasing the activities of ADC and SAMDC. On the contrary, the inhibition of ethylene biosynthesis caused an increase in the incorporation of label from methionine into Spd and Spm. Investigations of polyamine and ethylene metabolism in apple fruit discs and mature tomato fruit showed that polyamines incorporated into the incubation medium markedly inhibited ethylene production [1].

The ability of polyamines to suppress the action of ACC oxidase may be due to their ability to scavenge the superoxide radical that is required for the conversion of ACC to ethylene [12]. The efficiency of scavenging free radicals by polyamines is concentration dependent and positively correlated with the number of amine groups in the molecule, which means that Spd and Spm are more effective than Put and cadaverine.

In a study by Tian et al. [41], polyamine and ethylene biosynthetic pathways were shown to compete for SAM during the development of panicles in cytoplasmic male sterile (CMS) rice and its maintainer line. Ethylene release in the panicles of CMS line was higher than its maintainer line, while polyamine content in the CMS line was lower. Inhibition of SAMDC by MGBG caused a decrease in Spd and Spm levels and promoted ethylene production, and amino-oxyvinylglycine (AVG) inhibited ethylene production and promoted polyamine synthesis. Consistent with these observations, it has also been found that inhibition of ethylene formation by AVG in aged orange peel caused a large increase in incorporation of radio-labeled SAM into Spd [13].

In another test of this hypothesis, the fate of labeled methionine was compared in healthy and virus-infected Chinese cabbage-leaf protoplasts [17]. While isotope incorporation into both ACC and Spd from labeled methionine was similar in the uninfected cells, the infected cells tripled their incorporation of methionine into ACC and halved its incorporation into Spd. Cyclohexylamine, which inhibited Spd synthase, caused a reduction in the synthesis of Spd and Spm in germinating chickpea seeds, and an increase in the production of ACC and ethylene [14]. Conversely, inhibition of the ethylene biosynthesis in this system by norbornadiene, resulted in an accumulation of free and bound polyamines [10].

In contrast to the above reports, there is evidence suggesting a lack of competition between polyamines and ethylene in some plants. In water-stressed apple leaves, both ACC and ethylene levels rose sharply but SAM and polyamine pools remained essentially unchanged [26]. In young, rapidly growing rice internodes, ethylene stimulated the activity of SAMDC and ADC, preventing a decline in polyamine pool [8]. These observations suggest that the size of the pool of SAM may not limit the biosynthesis of polyamines and ethylene.

Transgenic manipulation of ACC synthase and ACC oxidase genes in sense or antisense orientations has been used extensively to alter ethylene biosynthesis and analyze its roles in different physiological processes, particularly senescence and fruit ripening [16, and references therein]. Transgenic manipulation of ACC deaminase is another way to modulate ethylene biosynthesis in plants [22]. Several studies on the transgenic expression of homologous and heterologous sequences of *odc* and *adc* genes in plants have also been reported [reviewed in 23]. A significant increase in the content of Put in transgenic plant tissues or cells has been observed with the expression of either *odc* or *adc* gene [2–4,7,11]. The competition between the two pathways has not been analyzed in any of these studies.

In the present study, ethylene production and changes of cellular ACC levels in the NT and two types of transgenic cells were investigated at the same time as the analysis of free polyamines. The two types of transgenic cells include one (2E) that produces several-fold higher levels of Put and significantly higher amounts of Spd, and the other (PS-18) that produces significantly lower levels of Put but similar levels of Spd as the NT cells. It has been shown earlier using the same (2E) cell line that these cells had elevated levels of the mouse ODC enzyme activity, and the conversion of radio-labeled Put into Spd was significantly higher in them than the NT cells [3,4]. The two cell lines have also been characterized with respect to their growth (changes in fresh weight, over most of the growth period), response to extra nitrogen in the medium, mitochondrial activity, polyamine content, etc. and found to show a similar pattern on different days of culture (P. Bhatnagar, J.-S. Lee, S. Long, R. Minocha, S.C. Minocha, unpublished data). The results presented here show that the production of ethylene was generally higher in the 2E cells as compared with the NT cells on any given day of the 7 d culture cycle. This difference was observed both in short-term (3–6 h) and long-term (25 h) periods of ethylene accumulation. Thus, it appears that there was no negative impact of the overproduction of Put and its high rate of conversion into Spd on the rates of ethylene production in the 2E cells.

During the 7 d culture period, cellular ACC contents in both cell lines followed a trend similar to that seen for ethylene production. The changes in the cellular ACC and ethylene production on different days of culture indicate that the production of ACC kept pace with the production of ethylene. During this period, cellular ACC content in the

transgenic 2E cells was either similar to the NT cells, or was somewhat lower than the NT cells. We believe that the decline in ethylene production after the 3rd d of culture may be due to a decrease in ACC oxidase activity, and not to reduced ACC synthesis. This conclusion is based upon the observation that the decrease in ethylene production preceded the changes in cellular ACC content. As a consequence, ACC content in 4- and 5-d-old cultures was still high when ethylene production had been reduced by almost 50%. The lower ACC content observed in the 2E cells may be due to its somewhat higher consumption for ethylene production. The data suggest that ethylene production in these cells may be regulated more by ACC oxidase than by ACC synthase, the latter only keeping up with its demand for use in order to maintain a steady state level.

The enzyme SAMDC catalyzes decarboxylation of SAM, with the product (dcSAM) donating the aminopropyl moieties needed for the biosynthesis of Spd and Spm. Several reports have been published where transgenic expression of a *samdc* cDNA has been used to modulate Spd and Spm levels in plants [24,34,35]. In most cases while the content of Put is significantly reduced, there is only a relatively small increase in cellular Spd and Spm. These observations, combined with the finding that Put is often the only polyamine that shows high degree of fluctuation in response to abiotic stress in plants [6], indicate a tight regulation of Spd and Spm biosynthesis and/or concentration in plants. In the PS-18 cells, which were transformed with a *Datura samdc* cDNA, Put content was much lower than that in the NT cells at both 3 and 6 d of culture, however, Spd and Spm levels were similar in the two cell lines. Lower Put levels in PS-18 cells may reflect its increased utilization for Spd and Spm production. However, relatively small changes seen in Spd and Spm levels indicate, once again, the existence of a tight regulation of Spd and Spm contents in the cells, probably via increased turnover of these polyamines.

While ethylene production by 3-d-old PS-18 cells was slightly but not significantly lower than that by the NT cells, and the ACC levels in the former were also slightly lower than those in the latter cells, no difference was evident for the 6-d-old cultures. If anything, at day 6, the transgenic cells produced slightly more ethylene than the NT cells. Thus we feel that the present data do not support the conclusion of a competition between ethylene and polyamine biosynthesis. It is, however, conceivable that because of only small effects of *samdc* expression on Spd and Spm levels in these cells, the effect on ACC and ethylene is also relatively small. It can further be argued that Spd and Spm synthase may play a bigger role in the regulation of Spd and Spm production in these cells than does SAMDC. However, this requires direct experimental evidence. Further work involving a simultaneous co-expression of the *samdc* and the Spd and Spm synthase transgenes in combination with increased production of Put

to substantially enhance Spd and Spm production should help better clarify this situation.

In conclusion, the limited evidence currently available on the transgenic cell lines showing high levels of Put production and moderate levels of increase in Spd production do not support the notion of a strong competition between the polyamine and the ethylene biosynthetic pathways for the precursor SAM. A simple explanation for the lack of competition for this intermediate may be that there is a large pool of SAM in the cells to meet the needs for all the metabolic reactions that SAM is used for and the amounts needed for the two pathways are relatively small. An alternate possibility is that there are several independent pools of SAM in the cell that are not shared by the various metabolic pathways in which SAM is used. No experimental evidence for the existence of multiple pools of SAM in plant cells is currently available.

4. Materials and methods

4.1. Poplar cell culture

Poplar (*P. nigra* × *maximowiczii*) cells were transformed by biolistic bombardment using plasmids containing either a mouse *odc* [3] or a *Datura samdc* cDNA (J.-S. Lee, R. Minocha, S.C. Minocha, unpublished) along with the *npIII* or the *hptII* gene, respectively. Following selection, the transgenic cells with the mouse *odc* gene (cell line called 2E) were grown for over a year in the presence of 100 mg l⁻¹ kanamycin sulfate, while those with the *Datura samdc* gene (cell line PS-18) were grown in the presence of 20 mg l⁻¹ hygromycin B. Non-transgenic (NT) cells were grown in the absence of antibiotics. During the experimental treatments, however, the antibiotics were excluded from the medium. The transgenic cells were characterized with respect to the presence and expression of the transgene by PCR, Southern hybridization and northern hybridization [3]. Suspension cultures were maintained in a liquid medium supplemented with 2% (w/v) sucrose and 0.5 mg l⁻¹ 2,4-D in 125 ml Erlenmeyer flasks [3]. The medium was adjusted to pH 5.7 before autoclaving. Stock cultures were maintained by transferring 7 ml of 7-d-old culture into 50 ml of fresh medium. Suspension cultures were placed on gyratory shakers (155 rpm) at 25 ± 1 °C with a 12 h daily photoperiod (80 ± 10 µmol m⁻² s⁻¹) provided by fluorescent bulbs.

4.2. Ethylene measurement

Ethylene was measured by gas chromatography (GC, Hewlett-Packard HP 6980 system, Wilmington, DE; equipped with a Chromosorb 102, 80/100 column, Supelco, Bellefonte, PA), using the software of GC ChemStation (Hewlett-Packard). The column was preheated to 200 °C for cleaning. Carrier gases for ethylene quantification were pure N₂, H₂, and compressed air. The GC running conditions

were: oven temperature 110 °C, inlet temperature 170 °C, detector temperature 190 °C, flow rate 30 ml min⁻¹. For each measurement, 0.4 ml of gas was injected manually. Pure ethylene (Alltech, Deerfield, IL) was used as a standard. Each run took about 3 min. Retention time for ethylene peak was 1.38–1.39 min. For the quantification of ethylene production from cells, 10 ml aliquots of cell suspension on different days of culture were capped in 25 ml glass vials. After appropriate periods of incubation, 0.4 ml of the head space gas was injected into the gas chromatograph.

4.3. ACC determination

The cellular ACC levels were determined by minor modifications of the procedure of Lizada and Yang [29]. A standard curve was constructed using different concentrations (0.01–10 mM) of ACC (Sigma, St Louis, MO) dissolved in 5% (v/v) sulfosalicylic acid (SSA). Two hundred microliters of the standard solution were mixed with 600 ml of water and 100 ml of cold 10 mM mercuric chloride in 10 or 25 ml glass vial, which were sealed with a serum stopper and stored on ice. One hundred microliters of a mix of Clorox bleach (5.25% w/v NaClO) and saturated NaOH (2/1, v/v) were injected into the vial through the stopper to release ethylene. The mixture was vortexed for 5 s, incubated on ice for 2.5 min and vortexed again for 5 s. After incubation at room temperature for 5 min, 0.4 ml gas sample was injected into the GC to measure ethylene production. A regression equation between ethylene production and ACC amount was generated ($R^2 = 0.9964$), which allowed conversion of ACC in the cells into ethylene gas evolved. For cellular ACC determination, the cells were collected by vacuum filtration on Miracloth (Calbiochem, La Jolla, CA), and 500 mg FW of cells were placed in a 2 ml microfuge tube containing 1.0 ml of cold 5% (v/v) SSA. The cell suspension was frozen (–20 °C) and thawed (room temperature) three times. After centrifugation at 10 000 × g (at 4 °C) for 10 min, 200 ml of the supernatant fraction was mixed with 600 ml of water and 100 ml of 10 mM mercuric chloride in a 10 or 25 ml glass vial, which was then sealed with a serum stopper and stored on ice. The remaining procedure was the same as for the standard curve of ACC determination. The ACC amount in the sample was calculated from the regression equation, and cellular ACC concentration represented as μmol ACC per g FW.

4.4. Analysis of free polyamines

Poplar cells were collected on Miracloth by vacuum filtration as for ACC experiments. Cells were mixed with cold 5% (v/v) perchloric acid (PCA) at a ratio of 1:4 (0.2 g FW cells in 0.8 ml PCA) on ice. The PCA suspension was frozen (–20 °C) and thawed (room temperature) three times,

then centrifuged at 13 000 × g for 4 min. One hundred microliters of the supernatant was dansylated, and the dansyl-polyamines were quantified by high performance liquid chromatography (HPLC) according to the procedure of Minocha et al. [30,33].

4.5. Statistical analysis

For all experiments two to four replicates were used for each treatment. Most experiments were repeated two to four times. Data from a single representative experiment are presented here. The data were subjected to one-way analysis of variance (ANOVA) using SYSTAT version 9. Tukey's test was used to determine significance at $P < 0.05$.

Acknowledgements

The authors thank Dr. A. Michael for providing the *Datura samdc* cDNA, Dr. Jae Soon Lee for the production of the PS-18 cell line, Stephanie Long and Benjamin Mayer for help in analysis of polyamines, Robert Mooney for help in the analysis of ethylene, and Dr. Curtis Givan and Dr. John Wallace for useful comments on the manuscript. Partial support for the study was provided by the USDA Forest Service, Northeastern Experiment Station. Scientific contribution No. 2109 from the NH Agricultural Experiment Station.

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