Physiologia Plantarum 129: 707–724. 2007

The effects of genetic manipulation of putrescine biosynthesis on transcription and activities of the other polyamine biosynthetic enzymes[1]

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Received 9 October 2006;
revised 9 November 2006

doi: 10.1111/j.1399-3054.2007.00860.x

We have studied the effects of overproduction of putrescine (Put) via transgenic expression of a mouse ornithine decarboxylase (ODC) gene on the expression of native genes for four enzymes involved in polyamine biosynthesis in hybrid poplar (*Populus nigra* × *maximowiczii*) cells. An examination of the transcript levels of arginine decarboxylase (ADC), ODC, S-adenosylmethionine decarboxylase (SAMDC) and spermidine synthase (SPDS), as well as their enzyme activities (except SPDS), showed that the expression of different members of the SAMDC and SPDS gene families was affected differently in response to alteration of the cellular Put content. It was further observed that there was a strong correlation between transcript levels and the activity of the respective enzyme in the cells. Moreover, there was no feedback inhibition of the expression of the native ODC or the ADC genes or their enzyme activities by increased Put in the cells.

Introduction

As the field of plant genetic manipulation moves toward modulation of cellular metabolism, it has become apparent that it is not sufficient to ensure that an introduced gene is functioning; the effect of altering a single reaction on the regulation of an entire pathway, and also of other related pathways, must be critically examined (Blakeley 1997, Dixon 2005, Hitz and Pierce 1997, Kinney 1998, Nuccio et al. 1999, Ohlrogge 1999). One of the major applications of genetic manipulation in plants is as a tool to reveal information about the regulation of metabolism so that strategies can be developed to achieve optimal levels of desired cellular metabolites. This not only requires identification of rate-limiting steps in a pathway but also understanding of how the pathway is regulated, particularly whether regulation occurs at the transcriptional, translational or post-translational levels. The transgenic approach can reveal mechanisms of metabolic regulation that may not be seen by mutant analyses and inhibitor studies alone. Some examples of novel information that has come from the transgenic approach include the complex regulation of Lys, Thr and Met biosynthetic pathways (Galili 1995, Lee et al. 2005, Tzchori et al. 1996, Zhu and Galili 2004), the complexity

Abbreviations – ADC, arginine decarboxylase; dcSAM, decarboxylated SAM; DNAse, deoxyribonuclease; FW, fresh weight; *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; *GUS*, β-glucuronidase gene; HP, high Put; m*ODC*, mouse *ODC* gene; NCBI, National Center for Biotechnology Information; ODC, ornithine decarboxylase; ORFs, open reading frames; PA, polyamines; pADC, poplar ADC; PCA, perchloric acid; pODC, poplar ODC; pSAMDC, poplar SAMDC; pSPDS, poplar SPDS; Put, putrescine; QRT-PCR, quantitative reverse–transcriptase polymerase chain reaction; SAM, S-adenosylmethionine; SAMDC, S-adenosylmethionine decarboxylase; Spd, spermidine; SPDS, spermidine synthase; Spm, spermine; SPMS, spermine synthase; UTR, untranslated region.

[1]Scientific contribution No. 2289 from the New Hampshire Agricultural Experiment Station. This work was partially supported by the USDA-NRI award 2002-35318-12674 and the USDA Forest Service, Northeastern Experiment Station.

of glycolysis regulation by phosphofructokinase (Thomas et al. 1997) and the regulation of secondary metabolism in plants (Dixon 2005).

Polyamines (PAs), which are low–molecular weight polycations, are found in all living cells. The common PAs in plants are spermidine (Spd), spermine (Spm) and their diamine precursor, putrescine (Put). They often exist in free, bound and conjugated forms, and in some cases, also serve as precursors for secondary metabolites such as nicotine (Bagni and Tassoni 2001, Ghosh 2000, Martin-Tanguy 1997). Rates of PA biosynthesis, degradation, conjugation with phenolic acids and intercellular transport all contribute to cellular PA content (Bhatnagar et al. 2001, 2002). PAs have been implicated in a variety of physiological responses and molecular interactions; however, neither the regulation of their biosynthesis nor the mechanisms of their action are clearly understood. The roles of PAs in plant growth/development and their interactions with cellular macromolecules have been reviewed (Cohen 1998, D'Agostino and Di Luccia 2002, Kakkar and Sawhney 2002, Minocha and Minocha 1995, Watson and Malmberg 1996). PAs, particularly Put, have been implicated in alleviating Ca^{2+} deficiency, and due to their richness in amine groups and their presence in millimolar quantities in plants, PAs also modulate reduced nitrogen and help sequester NH_3 produced within cells, thus preventing NH_3 toxicity (Lovatt 1990, Minocha et al. 1997, 2000, Slocum and Weinstein 1990).

In spite of numerous publications on the importance of PAs in plant growth, development and stress responses, only limited experimental evidence for metabolic regulation of PA biosynthesis has been forthcoming. The major enzymes involved in PA biosynthesis in plants are arginine decarboxylase (ADC), ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (SAMDC), spermidine synthase (SPDS) and spermine synthase (SPMS) (Fig. 1). For decades, the most common approach to modulate cellular PAs was the use of inhibitors of these enzymes, a strategy hindered by severe limitations (e.g. differential rates of uptake, metabolic conversions, deleterious side effects on membrane characteristics and the lack of specificity) for correct interpretation of results (McCann et al. 1987, Nissen and Minocha 1993, Robie and Minocha 1989). Since the cloning of genes for the key enzymes in PA metabolism, the genetic manipulation of specific enzymes has become feasible (Alcazar et al. 2005, Bhatnagar et al. 2001, 2002, Capell et al. 2004, Franceschetti et al. 2004, Kasukabe et al. 2004, Kumar and Minocha 1998, Minocha et al. 2004, Roy and Wu 2002). The use of genetic manipulation alleviates problems associated with the use of inhibitors and also allows the upregulation of specific steps in a pathway, which is generally not feasible with inhibitors.

Our lab has studied the regulation of PA metabolism in tobacco, carrot, poplar and red spruce using inhibitors

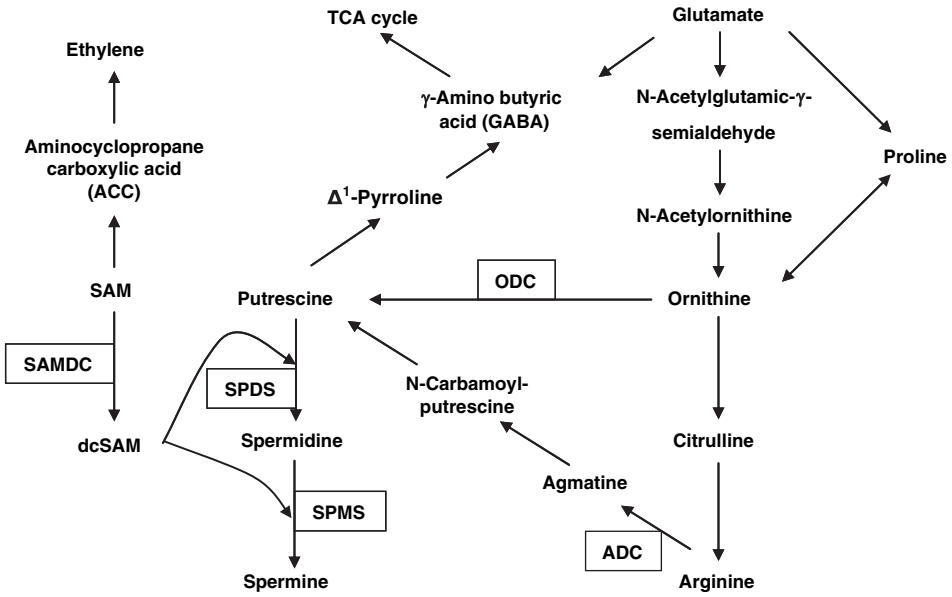

Fig. 1. The abbreviated polyamine biosynthetic pathway and the related pathways with which it interacts, highlighting only the enzymes studied in this paper. The enzymes are ODC, ornithine decarboxylase; ADC, arginine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase.

as well as genetic manipulation (Andersen et al. 1998, Bastola and Minocha 1995, Bhatnagar et al. 2001, 2002, DeScenzo and Minocha 1993, Minocha et al. 2004, Quan et al. 2002). The results of these studies, while providing considerable insight into the regulation of PA metabolism, have provoked several questions regarding the regulation of cellular Put content and its role as a regulator of the expression of other genes involved in PA metabolism: (1) If an alternate source of increased Put production was available to the cells that use ADC as the primary pathway (e.g. a transgenic *ODC*), how will it affect the native ADC and ODC enzyme activities and the expression of their genes? (2) What is the effect of increased Put accumulation on the activity of SAMDC and the expression of genes encoding *SAMDC* and *SPDS*? (c) Is the expression of the introduced transgenic *ODC* under the control of a 35S promoter stable in the cells, or does it vary with their metabolic state; if so, does the expression of other genes involved in PA biosynthesis vary in relation to this?

The main hypothesis for the work presented here was that transgenic manipulation of a specific step in the PA biosynthetic pathway will cause concomitant changes in expression of the native genes encoding enzymes that regulate that step and also the other reactions downstream of the manipulated step. The PA metabolic pathway is a branched pathway, which interacts with a limited number of adjacent pathways (Fig. 1), all of which are important in plants, rendering the study of these interactions important. Examining the effects of modulating a single step in PA metabolism on other branches of the pathway will help us in developing functional models for the regulation of PA metabolism and the metabolism of related compounds; e.g. Pro, Arg, γ-aminobutyric acid and ethylene (Fig. 1). This will also aid in achieving desirable manipulations of these compounds using the transgenic approach. Manipulation of PA metabolism in plants will potentially have far-reaching implications, including some in the field of oncology, where foods with reduced PA content are deemed desirable to retard tumor growth (Quemener et al. 1994), particularly in combination with a strategy of chemotherapeutic use of PA inhibitors in cancer patients (Catros-Quemener et al. 1999, Kalac and Krausova 2005, Milovic 2001, Stoneham et al. 2000).

This study presents comprehensive quantitative analyses of gene expression and activities of key enzymes involved in regulating PA biosynthesis in response to manipulation of a single step, i.e. Put overproduction. Use of quantitative reverse–transcriptase polymerase chain reaction (QRT-PCR) has enabled us to examine the expression of individual paralogues of genes and assess their relative contributions to PA metabolism in poplar cells. We examined the expression of different paralogues of the native *ADC, SAMDC* and *SPDS* genes, as well as that of the introduced mouse *ODC* (m*ODC*) gene, over a 7-day growth cycle between subculturing. We present evidence for concomitant changes in the expression of several genes coding for PA biosynthetic enzymes and demonstrate that different paralogues of these genes may have differing roles in poplar cell cultures.

Materials and methods

Cell growth and harvest

The high Put (HP) and the control cell lines of *Populus nigra* × *maximowiczii* used here have been described (Bhatnagar et al. 2001, 2002); the former (previously called 2E) expresses a m*ODC* gene, while the latter expresses the β-glucuronidase (*GUS*) gene (and serves as a control); both cell lines also express the *npt*II selectable marker. All three transgenes are controlled by 35S CaMV promoters. By using a transgenic control line, we neutralized the effect of kanamycin in the culture medium during maintenance of stock cultures.

Cell lines were maintained in Murashige and Skoog medium (Murashige and Skoog 1962) containing B5 vitamins (Gamborg et al. 1968), 2% sucrose, 0.5 mg l^{-1} 2,4-D and 100 mg l^{-1} kanamycin (Bhatnagar et al. 2002). Cells were grown either on solid medium (1% type A agar, w/v) and subcultured monthly, or in liquid cultures, which were subcultured weekly by adding 7 ml of 7-day-old culture to 50 ml fresh medium in 125-ml Erlenmeyer flasks. While the stock cultures were maintained in the presence of 100 mg l^{-1} kanamycin, the antibiotic was absent for at least 2 weeks before experimentation. All liquid cultures were kept at 150 rpm on a gyratory shaker at 25 ± 2°C under a 12-h photoperiod (80 ± 10 μEm^{-2} s^{-1}).

Harvesting of cells was done by vacuum filtration through Miracloth at 1, 3, 5 and 7 days after subculturing for molecular analyses, and daily for biochemical analyses. For RNA isolation, cells [50–55 mg fresh weight (FW)] were immediately frozen in liquid nitrogen and stored at −80°C. For PA analysis, the cells were frozen (−20°C) in 5% (v/v) perchloric acid (PCA), and for soluble protein and enzyme analyses, they were frozen in the respective enzyme assay buffer for 2–4 h.

RNA extraction and cDNA synthesis

Cell samples were removed from storage (−80°C), and total RNA was isolated using the RNeasy Plant Mini Kit

(Qiagen, Valencia, CA). Following quantification, 3 μg of the RNA was treated with 3 units of deoxyribonuclease (DNAse) (RQ1 RNase-Free DNase; Promega, Madison, WI) in a total volume of 30 μl. Samples were incubated at 37°C for 30 min before removal of protein by phenol: chloroform:isoamyl alcohol (25:24:1). Traces of phenol were removed by partitioning against chloroform:isoamyl alcohol (24:1), precipitation with an equal volume of isopropanol (20 min, −20°C), centrifugation (13 000 *g*, 15 min), 80% ethanol wash, drying and resuspension in 10 μl ribonuclease-free water. Samples were reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, using oligo $(dT)_{22}$, 7.5 μl RNA and a reverse transcription time of 60 min at 50°C. Resultant cDNA was stored at −20°C before QRT-PCR analysis.

Quantitative polymerase chain reaction

Reactions were set up using two master mixes for each reaction in order to maintain intersample consistency. The first mix contained Taqman® Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and template (0.5 μl), while the second mix contained forward and reverse primers (0.3 μM final concentration) and the probe (0.2 μM final concentration). QRT-PCR was performed in a GeneAmp 5700 Sequence Detection System (Applied Biosystems) using 40 cycles of 95°C for 15 s, 55°C for 30 s and 65°C for 45 s. For all experiments, baseline cycles were set at 6–15 and threshold at 0.05; validation of this was performed by inspection of a plot of cycle number vs fluorescence using both log and linear axes for fluorescence. Critical cycle number (C_t) was determined as the point where fluorescence exceeded the threshold; lower C_t values, therefore, indicate higher quantities of template and thus more expression of that particular gene.

To confirm absence of genomic DNA contamination in DNase-treated RNA, reactions were set up using DNase-treated RNA (prereverse transcription) as a template and glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) primers and probe. Lack of amplification in these reactions (data not presented) showed the absence of DNA prior to reverse transcription.

Data analysis

Relative gene expression was determined for each gene using the formula:

$$\frac{T_i}{T_{con}} = \frac{(1 + E_i)^{C_{ti}}}{(1 + E_{con})^{C_{tcon}}},$$

where, T_i/T_{con} is the expression of the gene of interest scaled by the expression of the internal control gene (*G3PDH*), E is the PCR efficiency for each gene and C_t is the critical cycle number. PCR efficiency for each gene was determined from a dilution series (10 dilutions) of matching template over three orders of magnitude. Each experiment was repeated in its entirety at least thrice. Data were analyzed by repeated measures analysis of variance, using Newman–Keuls post hoc test.

Sequence acquisition

Following PCR amplifications using primers for *ODC*, *ADC*, *SAMDC*, *SPDS* and *G3PDH*, the resulting products were cloned and sequenced (at the UNH Hubbard Center for Genome Studies) to confirm their identity, then used to make probes for screening a cDNA library made from our poplar suspension cultures using a Creator™ SMART™ cDNA Library Construction Kit (BD Biosciences, Palo Alto, CA). Additional sequences were obtained by searching the *Populus trichocarpa* database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and used to design additional primers for amplification, cloning and sequencing of the corresponding gene fragments in our species. The sequences were deposited in the GenBank database (accession nos. are given where appropriate). These approaches identified a total of one poplar (p) *ADC*, two *SPDS*, three *ODC* and four *SAMDC* paralogues; one of the *SAMDC* genes was shown by RT-PCR not to be expressed in the poplar cells (data not shown). The m*ODC* sequence (accession no. M10624) has been described previously (Bhatnagar et al. 2001, DeScenzo and Minocha 1993).

Primer and probe design and testing

Taqman® probes were designed using Primer Express™ (Applied Biosystems) in such a way that each primer/probe combination would amplify only the desired paralogue of the gene. Because of the lack of introns in these sequences, we could not use exon–exon boundaries to avoid signal from genomic DNA contamination, hence we relied on DNase treatment of RNA as described above. Probes were 5′ labeled with 6′ fluorescein and 3′ labeled with carboxytetramethylrhodamine and were obtained from Applied Biosystems. Primers were made by Integrated DNA Technologies (Coralville, IA). Each primer/probe combination was tested using templates for all of the genes studied to ensure specificity and satisfactory amplification (details not presented). For p*ODC*, only semiquantitative PCR using four sets of

cycles (20, 25, 30 and 35) was used to estimate relative abundance of transcripts on different days.

Enzyme assays for ODC, ADC and SAMDC

The activities of ODC, ADC and SAMDC were measured daily during the 7-day culture cycle using slight modification of the method of Minocha et al. (1999). Cells collected by vacuum filtration (100 ± 5 mg FW for ODC and ADC, and 200 ± 5 mg FW for SAMDC) were placed in assay buffer in 16- ×100-mm glass test tubes (for mODC: 250 μl 0.1 *M* Tris, 0.1 m*M* EDTA, pH 6.8, 0.5 m*M* pyridoxal phosphate, 1.0 m*M* DTT; for pODC and pADC: the same as for mODC but at pH 8.4; for SAMDC: 350 μl 0.1 m*M* potassium phosphate buffer, pH 7.5, 3.0 m*M* Put-diHCl, 1.0 m*M* DTT) and were frozen for 2–4 h. After thawing, 50 μl of the appropriate labeled substrate [for ODC: 0.05 μCi of [1-^{14}C]Orn, specific activity 58 mCi mmol^{-1} (Moravek Biochemicals, Brea, CA) plus 12 m*M* unlabeled L-Orn; for ADC: 0.1 μCi of [1-^{14}C]Arg, specific activity 57 mCi mmol^{-1} (Amersham Life Sciences, Elk Grove, IL) plus 12 m*M* unlabeled L-Arg; for SAMDC: 0.1 μCi of [1-^{14}C]S-adenosylmethionine (SAM), specific activity 58 mCi mmol^{-1} (Moravek) plus 4.0 m*M* unlabeled SAM] was added to each tube, and a 2-cm^2 Whatman 3 MM filter paper soaked with 50 μl Scintigest (Fisher Scientific, Pittsburg, PA) was placed in a polypropylene well (Kontes, Vineland, NJ) suspended from a rubber stopper. The tubes were incubated in a water bath (37°C) for 60 min for ODC and ADC, and 30 min for SAMDC. Reactions were terminated by injecting 1.0 ml of 0.5 N H_2SO_4 into each tube through the rubber stopper. Following additional incubation of 30 min, the filter papers were removed and counted for radioactivity in 10 ml Scintilene (Fisher Scientific) in an LSC-6000 liquid scintillation counter (Beckman, Fullerton, CA).

Enzyme activity was calculated as nmol CO_2 h^{-1} g^{-1} FW of cells as well as nmol CO_2 h^{-1} mg^{-1} soluble protein. The daily enzyme assays and protein estimations were repeated over 2 weeks with three replicates at each time. Protein content was determined using Bio-Rad (Hercules, CA) dye (Bradford 1976).

PA analysis

Following collection, 200 ± 20 mg (FW) cells were mixed with four times the volume of 5% PCA and frozen and thawed three times before dansylation and quantification of PAs by HPLC (Minocha et al. 1990, 1994). Briefly, the thawed samples were vortexed and centrifuged for 10 min (13 000 *g*). Supernatant (100 μl) was mixed with 20 μl of 0.1 m*M* heptanediamine (internal standard), 100 μl of a saturated solution of Na_2CO_3 and 100 μl of 20 mg ml^{-1} (in acetone) solution of dansylchloride (Sigma, St Louis, MO). Following incubation for 1 h at 60°C, 50 μl of a 100 mg ml^{-1} L-Ala or L-Asn solution was added. After additional 30-min incubation at 60°C, acetone was removed by vacuum centrifugation (5 min). Dansyl-PAs were extracted in 400 μl toluene, 200 μl of which was transferred to a fresh microfuge tube and dried under vacuum. The dansyl-PAs were redissolved in 1 ml methanol and analyzed by HPLC using a gradient of acetonitrile (40–100%) and 10 m*M* heptanesulfonic acid, pH 3.4, on a reversed-phase C18 column. Quantification was done by a fluorescence detector.

Results

There are multiple copies of genes for PA biosynthetic enzymes in poplar

We amplified and cloned four different *SAMDC* fragments of 509, 510, 536 and 736 bp, which were individually compared with the *P. trichocarpa* and other *SAMDC* sequences in the National Center for Biotechnology Information (NCBI) database. The *SAMDC*1[2] fragment (accession no. DQ173764, 509 bp) differed from its closest match in the *P. trichocarpa* genomic DNA database (LG_IV8847666-8848174) by only four bases and showed 82% identity over its entire length with the closest NCBI match of a *Vitis vinifera SAMDC* cDNA (accession no. AJ567368). The p*SAMDC*2 fragment (510 bp; accession no. DQ173765) showed five-base difference from a second *P. trichocarpa SAMDC* (LG_X13181376-13181884) and a 79% identity over its entire length with the closest NCBI match, *Nicotiana tabacum* (accession no. AF033100). The third fragment, p*SAMDC*3 (536 bp; accession no. DQ173766) differed by four bases from a third *P. trichocarpa SAMDC* sequence (LG_X3566516-3567051), while the 736-bp fragment of p*SAMDC*4 (accession no. DQ173767) revealed four bases different from the fourth *P. trichocarpa SAMDC* (LG_XVIII10349303-10350038).

The two *SPDS* sequences amplified from hybrid poplar cDNA library were 433 bp (p*SPDS*1) and 473 bp (p*SPDS*2) long and showed over 70% sequence identity with each other. The p*SPDS*1 cDNA fragment (accession no. DQ173768) was identical to a *SPDS* sequence of *P. trichocarpa* (LG_XII1389869-1391611) and showed

[2]The numbering of different genes coding for the same enzyme follows an arbitrary system based on the chronology of their cloning; thus, there is no correspondence between, for example, the *SAMDC*1 of poplar and the annotated *SAMDC*1 of Arabidopsis or other species in which more than one *SAMDC* has been reported.

84% identity with the closest NCBI match of tomato (*Lycopersicon esculentum*) *SPDS* (accession no. BT014416). The p*SPDS*2 fragment (accession no. DQ173769) differed from the second *P. trichocarpa SPDS* (LG_VIII9924958-9926472) by only two bases.

Cloning of PCR-amplified fragments of p*ODC* yielded three clones containing inserts of approximately 625 bp each. In contrast to p*ODC*, p*SAMDC* and p*SPDS*, only one 584-bp-long p*ADC* fragment (accession no. Q173763) was amplified by the primers used in this study; its sequence was identical to the single match from *P. trichocarpa* genomic DNA database over the entire region, and it differed from a *P. nigra ADC* expressed sequence tag (accession no. AJ849361) by a single base over the 286-bp overlapping region.

The poplar *G3PDH* sequence (accession no. DQ173770, 1350bp) used as an internal standard for QRT-PCR differed from that of its *P. trichocarpa* counterpart (LG_X6168259-6170692) by only seven bases and showed 85% identity with the *G3PDH* sequence of *Solanum tuberosum* (accession no. AF527779).

Normalization of QRT-PCR data

Normalization of QRT-PCR data is a universal problem when using this technique for comparing the transcript levels of different genes: adjustments must be made to compensate for variation between samples in amount of starting RNA, or later in amount of cDNA used (Gachon et al. 2004, Gonçalves et al. 2005, Herrera et al. 2005). A common approach is to use one of the several housekeeping genes, e.g. 18S rRNA, *G3PDH*, *β-actin*, etc., to normalize the transcript levels of the gene of interest (Bustin 2005, Hashimoto et al. 2004, Vandesompele et al. 2002). The expression of these genes is assumed not to be influenced by experimental manipulation and therefore be a suitable standard against which to scale data. The use of normalization gene(s) is not without flaws, however, since it is now known that the expression of many of these genes is not constitutive (Dheda et al. 2005, Gonçalves et al. 2005, Herrera et al. 2005). Nevertheless, in the absence of a better alternative, we used *G3PDH* as a normalization gene. Since our experimental system consists of liquid suspensions that are subcultured every 7 days, they exhibit standard dynamics of lag phase, logarithmic growth and a stationary phase. It is, therefore, possible that normally more consistent aspects of central metabolism (including *G3PDH*) may vary as the cells transition from rapid division around day 2 to day 4 through to eventual cessation of growth. This is no different from the variation in the expression of housekeeping genes during development and maturation of whole plant organs and tissues.

Expression of different genes varies on different days of culture

As expected, the control (*GUS* transformed) cells showed no signal corresponding to the transcripts of m*ODC* on any day of analysis; the expression of m*ODC* in the HP cells varied on different days. The m*ODC* transcripts increased slightly between days 1 and 3 and declined to almost 50% between days 3 and 5 (Fig. 2A). The m*ODC* transcripts again showed a small but statistically insignificant increase on day 7. Comparing the transcript data for days 7, 1 and 3, it is clear that following transfer to fresh medium, there was a lag of about 3 days before an increase in m*ODC* transcript was seen. The p*ODC* transcripts were analyzed in a semiquantitative manner (using a pair of primers that amplified all three pODCs) by band density analysis of the PCR products at 20, 25, 30 and 35 cycles of amplification; no difference in the transcripts of p*ODC* was seen between the two cell lines on any day of analysis (data not presented).

The p*ADC* transcripts in the m*ODC*-transformed HP cells were higher than those in the control cells on any given day (Fig. 2B); however, differences between the two cell lines were not statistically significant ($P < 0.05$). A small increase in p*ADC* transcript was seen on day 3 followed by a decline thereafter in both cell lines. A fresh medium effect on p*ADC* transcript abundance was clearly visible in both cell lines within 1 day of transfer; the effect continued until 3 days in the fresh medium.

Out of the three p*SAMDC* genes whose transcripts were quantified by QRT-PCR, p*SAMDC*1 showed by far the greatest expression; its transcripts in the control cells being almost 20 and 200 times greater than those of p*SAMDC*2 and p*SAMDC*3, respectively on day 1 (Fig. 3). In control cells, the p*SAMDC*1 transcript levels fell almost three-fold between days 1 and 5, after which there was no significant change (Fig. 3A). On transfer to fresh medium on day 7, a significant increase in the transcripts of this gene occurred within 1 day. In the HP cells, there was neither a significant difference in p*SAMDC*1 expression between any of the time points nor was there a fresh medium effect apparent from comparison of data for days 7 and 1. Furthermore, p*SAMDC*1 transcripts were significantly lower in the HP cells vs the control cells on any day of analysis.

Transcript levels of p*SAMDC*2 were not significantly different between the two cell lines on any of the days tested (Fig. 3B). Both lines exhibited a significant

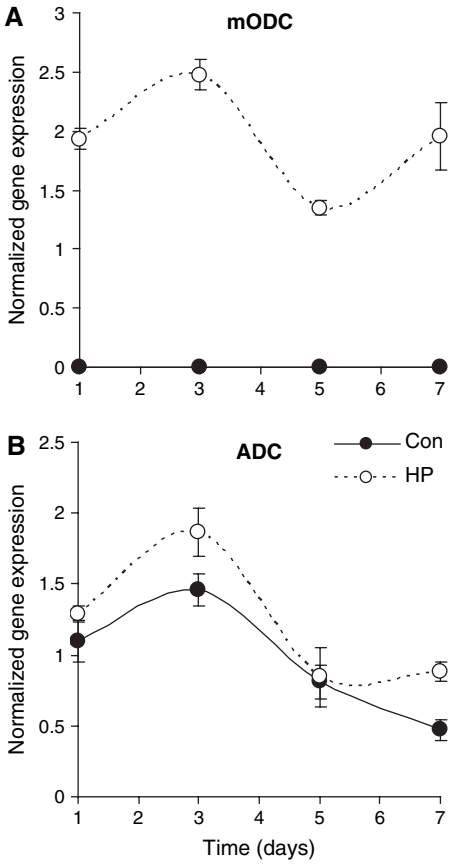

Fig. 2. Expression profiles (relative transcript levels normalized to *G3PDH*) of (A) m*ODC* and (B) p*ADC* in the control and mODC transgenic cells (high putrescine) over the 7-day culture period as determined by quantitative reverse–transcriptase polymerase chain reaction (n = 3, bars = SE. *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; m*ODC*, mouse *ODC* gene; ODC, ornithine decarboxylase; p*ADC*, poplar arginine decarboxylase.

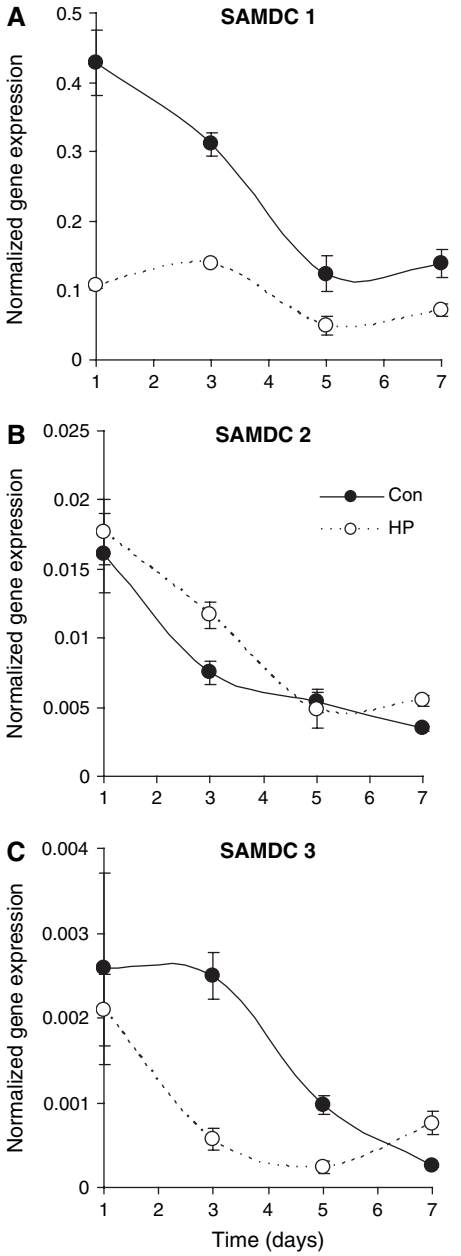

Fig. 3. Expression profile (relative transcript levels normalized to *G3PDH*) of (A) *SAMDC*1, (B) *SAMDC*2, and (C) *SAMDC*3 in the control and mODC transgenic cells (high putrescine) over the 7-day culture period as determined by quantitative reverse–transcriptase polymerase chain reaction (n = 3, bars = SE). *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; mODC, mouse ornithine decarboxylase; *SAMDC*, S-adenosylmethionine decarboxylase.

($P < 0.05$) decrease in p*SAMDC*2 transcripts between days 1 and 5, after which there was no further loss of transcripts by day 7. In contrast to p*SAMDC*1 transcription, which did not show a fresh medium effect in HP cells, a comparison of p*SAMDC*2 transcripts on days 7 and 1 showed that there was a significant increase in transcription of this gene on transfer to fresh medium in both the cell lines.

The transcripts of p*SAMDC*3 fell during the course of the 7-day culture period in both cell lines; its transcript abundance in HP cells was consistently lower than the control cells (Fig. 3C). As with p*SAMDC*2, there was an increase in transcripts of this gene within 1 day of transfer to fresh medium.

Of the two p*SPDS* genes, the maximum expression of p*SPDS*1 was almost five-fold lower than that of p*SPDS*2 on day 1 of culture (Fig. 4). The two cell lines on any given day had similar levels of the p*SPDS*1 transcripts (Fig. 4A); the same was true of p*SPDS*2 (Fig. 4B). Transcripts of both genes showed a significant increase on transfer to fresh medium (day 7 vs day 1); the

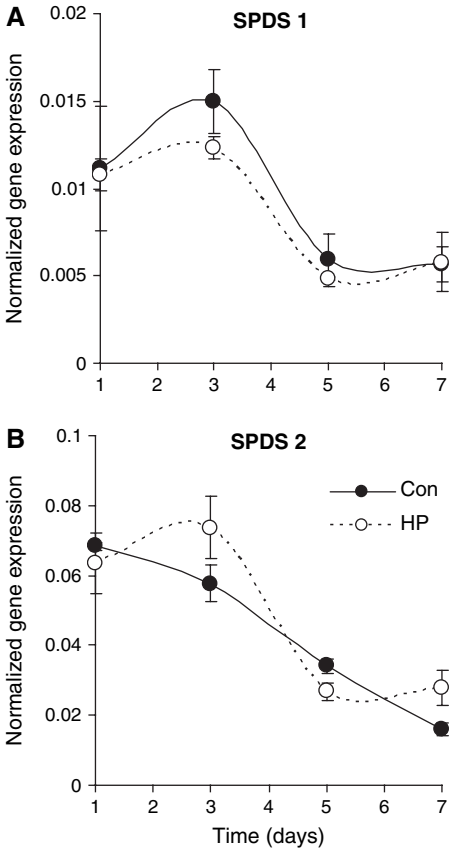

Fig. 4. Expression profile (relative transcript levels normalized to *G3PDH*) of (A) *SPDS*1 and (B) *SPDS*2 in the control and mODC transgenic cells (high putrescine) over the 7-day culture period as determined by quantitative reverse–transcriptase polymerase chain reaction (n = 3, bars = SE). *G3PDH*, glyceraldehyde-3-phosphate dehydrogenase; mODC, mouse ornithine decarboxylase; *SPDS*, spermidine synthase.

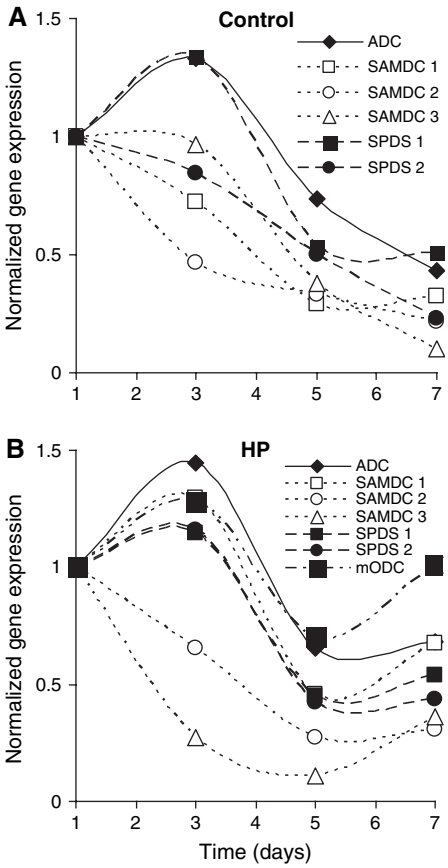

Fig. 5. Temporal changes in the expression profile of all genes studied normalized to their expression on day 1 of culture in (A) control and (B) mODC transgenic high-putrescine cells measured over the 7-day culture period as determined by quantitative reverse–transcriptase polymerase chain reaction (n = 3, error bars not shown for clarity). mODC, mouse ornithine decarboxylase.

increase for p*SPDS*1 transcripts continued in both cell lines until day 3, but p*SPDS*2 increased only in HP; thereafter a decrease in transcripts of both genes was seen in both cell lines.

When relative amounts of transcripts for all genes (except p*ODC*) were plotted on the same scale and normalized to their respective amount on day 1 of culture (Fig. 5), a positive fresh medium effect (i.e. upregulation of transcription) was seen for all in both cell lines except the m*ODC* in HP cells (Fig. 5B), where this response was delayed to day 3. While only *ADC*1 and *SPDS*1 transcripts showed further increases beyond day 1 in the control cells (Fig. 5A), for HP cells, an increase was seen for transcripts of all genes except p*SAMDC*2 and p*SAMDC*3 (Fig. 5B). A small increase in m*ODC* was also seen in the HP cells between days 5 and 7. In all cases, a decrease in transcripts of all genes was observed between days 3 and 5.

Activities of ODC, ADC and SAMDC vary over the 7-day culture period

In order to establish a correlation between transcript levels of a gene and the corresponding enzyme activity of its product, the activities of ODC, ADC and SAMDC were measured in both cell lines over the 7-day culture period. The pODC and mODC activities were distinguished from each other by using extraction and assay buffers of appropriate pH for each; i.e. 6.8 for mODC in HP and 8.2 for pODC in control cells (DeScenzo and Minocha 1993).

As reported earlier (Bhatnagar et al. 2001), ODC activity (g^{-1} FW) was rather low in the control cells as compared with the m*ODC* transgenic HP cells (Fig. 6A); nevertheless, it varied somewhat over the 7-day culture period (inset, Fig. 6A), with an increase being seen during

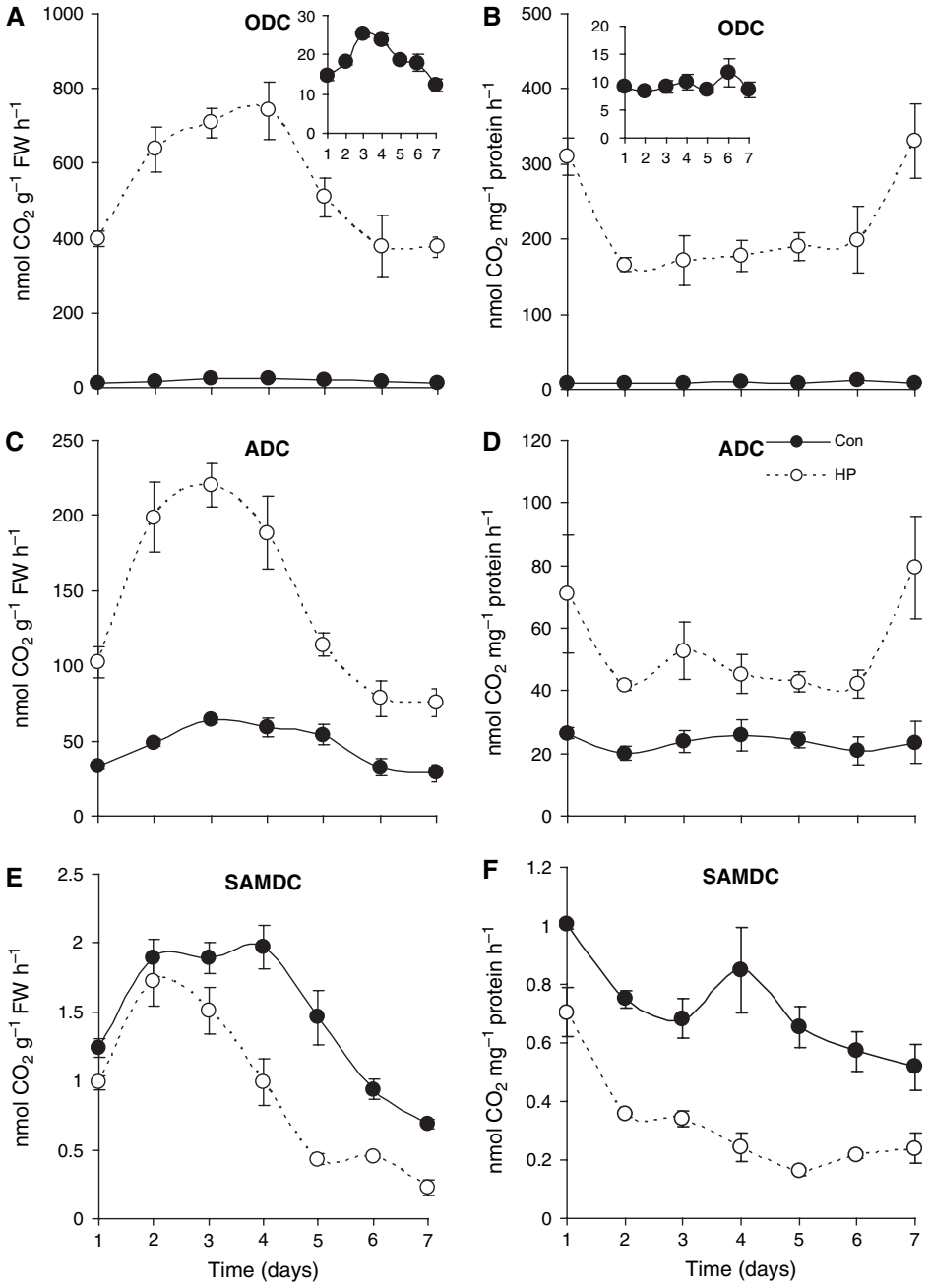

Fig. 6. Enzyme activities g^{-1} fresh weight (A, C, E) and specific activities (B, D, F) of mODC (A, B), ADC (C, D) and SAMDC (E, F) in control and mODC transgenic cells (high putrescine) over the 7-day culture period. Insets in A and B show expansion of the data for control cells. Data are combined from two separate experiments each with three replicates (n = 6 for each point, bars = SE). mODC, mouse ornithine decarboxylase; ADC, arginine decarboxylase; S-adenosylmethionine decarboxylase.

the first 3 days on transfer of cells to fresh medium and a decline after that. The specific activity (CO_2 mg^{-1} protein) of pODC remained rather constant over the 7-day culture period (inset, Fig. 6B), due to changes in protein content of the cells. The HP cells, where the total ODC activity was almost 25-fold higher than the pODC activity in the control cells on day 1, exhibited a similar trend for change in enzyme activity over the 7-day culture period; the peak of activity being seen on day 4 (Fig. 6A). The specific activity of ODC in HP cells, however, exhibited a different trend in that there was a significant decrease between days 1 and 2 ($P < 0.05$), after which

there was no change until day 7, when it again increased significantly over day 6 (Fig. 6B). The pODC activity in HP cells could not be measured accurately because mODC activity still persisted at pH 6.8, albeit at low levels (DeScenzo and Minocha, 1993); therefore the data on pODC in these cells are not presented.

The activity of ADC (g^{-1} FW) was significantly higher ($P < 0.05$) in the HP cells than in the control cells on any day of analysis (Fig. 6C). Whereas the HP cells showed a peak of ADC activity around days 2–4 and a decline thereafter, changes in ADC activity in the control cells were small and statistically insignificant over the 7-day culture period. On transfer to fresh medium on day 7, an increase in activity was seen only in the HP cells. When ADC specific activity was compared, the differences between the two cell lines over time were smaller, and also the peak of ADC activity observed in the HP cells between days 2 and 4 was not apparent (Fig. 6D). In fact, highest ADC activity in these cells was seen on the seventh and the first day of culture, a situation similar to that for mODC activity.

The activity of SAMDC (g^{-1} FW) was significantly ($P < 0.05$) lower in the HP cells than in the control cells on all but the first 3 days of culture (Fig. 6E); both cell lines showed a decrease in enzyme activity over the course of the experiment, after 2 days of culture in HP and after 4 days in the control cells. On transfer of cells to fresh medium, a small but significant increase in SAMDC activity was seen in both cell lines. When calculated as specific activity, differences between the two cell lines were seen over the entire 7-day period, the enzyme activity being always lower in the HP cells (Fig. 6F). It should be pointed out that the enzyme activity measurements did not distinguish between the products of various *SAMDC* genes.

Soluble protein content differs in the two cell lines and changes with time of culture

Buffer-soluble protein content of the cells varied over the 7-day culture cycle (Fig. 7), rising to a peak on days 2–4, then falling throughout the remainder of the week. On days 2–4, the protein content (g^{-1} FW) in the HP cells was significantly higher than in the control cells; however, on other days, no significant differences were observed. The apparent discrepancy between the enzyme activity data calculated on g^{-1} FW basis and as specific activity is obviously due to changes in the protein content of cells, reflecting changes in overall metabolism over the weeklong culture period. Which of the two measurements more accurately reflects changes in enzyme activity that is important for regulation of PA biosynthesis is difficult to assess from these data.

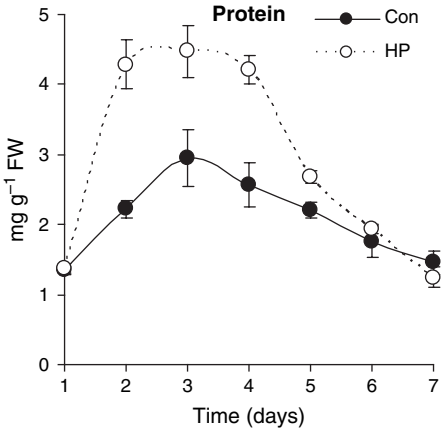

Fig. 7. Soluble (buffer extractable) protein content in control and mODC transgenic cells (high putrescine) over the 7-day culture cycle (n = 3 for days 1 and 2; n = 6 for days 3–7, bars = SE). mODC, mouse ornithine decarboxylase.

Different PA vary independently over the 7-day culture period

The cellular contents of PAs were analyzed in the same batch of cells as the protein contents and the enzyme activities on a given day. Since the protein content varied over the 7-day culture period, the cellular contents of the three major PAs were also calculated both as nmol g^{-1} FW and nmol mg^{-1} protein. In either case, Put content of the HP cells was several-fold higher than of the control cells on any given day of analysis (Fig. 8A, B); on some days, the differences were eight- to nine-fold on g^{-1} FW basis. The trend in changes with time was different in the two cell lines. On FW basis, the control cells showed a small increase in Put around days 5 and 6; for HP cells, the peak of Put content was seen around days 2–4. Since the protein content of the latter was higher on days 2–4, Put content mg^{-1} protein was actually the lowest on these days and highest on days 1 and 7.

Spd (g^{-1} FW) increased significantly ($P < 0.05$) in the HP cells between days 1 and 2, rising to a peak on day 3, and then dropping to the lowest amount on day 7 (Fig. 8C). In control cells, on the other hand, the increase was much smaller and occurred only around days 3–4. The Spd content in HP cells was higher around 2–3 days and somewhat lower on days 5, 6 and 7. Only a small increase in Spd was seen in either cell line within 1 day of transfer to fresh medium. When the data were normalized to protein content, both lines showed a small increase in Spd within a day of transfer to fresh medium followed by a significant decline in its content by day 2 (Fig. 8D). Thereafter, only small increase in Spd contents mg^{-1} protein was seen in the

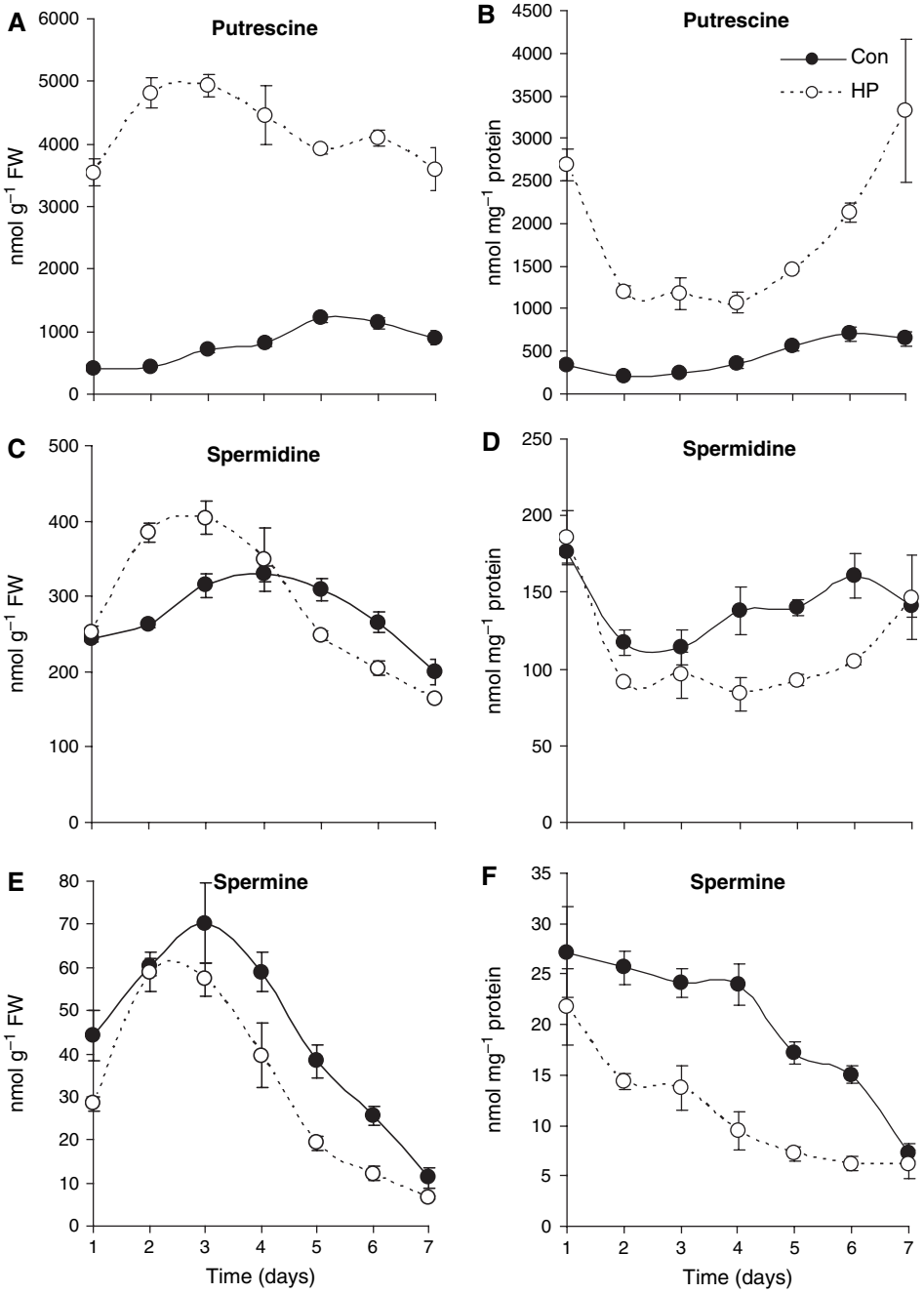

Fig. 8. Cellular contents of perchloric acid–soluble polyamines expressed as nmol g^{-1} fresh weight (A, C, E) and as nmol mg^{-1} protein (B, D, F) over the 7-day culture cycle in the control and mODC transgenic (high putrescine) cell lines. (A, B) = putrescine, (C, D) = spermidine and (E, F) = spermine. Data are combined from two separate experiments each with three replicates. For data points n = 6, bars = SE. mODC, mouse ornithine decarboxylase.

two cell lines over the next 4–5 days. Overall, Spd content (g^{-1} FW as well as mg^{-1} protein) was higher on days 4, 5 and 6 in the control cells (Fig. 8C, D).

Spm, which was the least abundant of the three PAs, showed a steady decline between days 2 or 3 and 7, regardless of how the amounts were calculated (Fig. 8E, F). There were no differences in Spm content of the two cell lines on g^{-1} FW basis, but the control cells seemingly had higher Spm mg^{-1} protein for days 1–6.

Thus, the PA analysis shows that, as expected, cells overexpressing mODC have several-fold higher Put but do not show a corresponding increase in Spd or Spm as

compared with the control cells. Therefore, the levels of different PAs must be regulated independently.

Discussion

ODC and ADC expression and enzyme activities vary with the metabolic state of cells

The calculation of enzyme activity and PA content on the basis of g^{-1} FW vs mg^{-1} protein may lead to somewhat different interpretations of data concerning changes in cellular metabolism with time during the 7-day culture cycle. An analysis of mitochondrial activity based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Ikegawa et al. 2000) on different days of the 7-day culture period showed a higher activity during the first few days of culture compared with the latter half of the week (R. Majumdar, S. Singh, S.C. Minocha and R. Minocha, unpublished data), a trend supporting the argument about changing metabolism of cell cultures. Several of the genes tested in the present study showed a peak in expression and enzyme activity on a g^{-1} FW basis during the first 2–3 days of culture (e.g. ADC and ODC), suggesting an association with rapid growth/cell division occurring in cell cultures around this time. However, due to higher protein content during this period (Fig. 7), differences over time in specific activity, particularly that of ADC, during the 7-day culture period became less apparent (Fig. 6D). Thus, the data point to some of the changes with time being a reflection of general metabolic status of the cells during the 7-day culture cycle, including changes in total protein content and mitochondrial activity.

In the HP cells, expression of m*ODC* (Fig. 2), the activity of ODC g^{-1} FW (Fig. 6) and the Put content (g^{-1} FW; Fig. 8) all showed a similar trend of an initial steady rise from the first to the third or fourth day, which was concomitant with an increase in cellular protein content. In control cells, although the activity of pODC remained low throughout the culture period, an increase during the first 3 days was observed. It should be pointed out that a portion of $^{14}CO_2$ released from [^{14}C]Orn in the control cells could also have come from its conversion into [^{14}C]Arg and subsequent decarboxylation by ADC. The lack of change in specific activity during the second to sixth day is due to changes in protein content of cells, which was higher during this period as compared with day 1 or 7. The increases in Put in both the control and the HP cells following transfer to fresh medium are consistent with the observed changes in ODC and ADC activities. The change in m*ODC* expression (and resultant change in enzyme activity and Put production) is interesting in light of the fact that the transgene is under the control of the supposedly constitutive 35S CaMV promoter (for review, see Yoshida and Shinmyo 2000). However, many studies have shown that 35S CaMV-regulated expression is not entirely constant and varies according to tissue type and developmental stage (e.g. Sunilkumar et al. 2002), a situation similar to that seen for ubiquitin-regulated promoter, another commonly used constitutive promoter (Capell et al. 2004).

Different SAMDC genes are regulated differently

SAMDC is considered to be a regulatory enzyme for the biosynthesis of both Spd and Spm since it controls the production of decarboxylated SAM (dcSAM), the primary donor of aminopropyl moieties for SPDS and SPMS (Cohen 1998, Evans and Malmberg 1989, Pegg et al. 1998). Most plants have two *SAMDC* genes, sometimes even more (Franceschetti et al. 2001, Tian et al. 2004). The *SAMDC* transcripts in both animals and plants have some unusual features such as a long (400–700 nucleotide) 5′ untranslated region (UTR), which contains one or more translatable open reading frames (ORFs); there may also be additional nontranslated ORFs (Franceschetti et al. 2001, Hanfrey et al. 2002, Law et al. 2001, Thu-Hang et al. 2002). The main *SAMDC* coding sequence in plants does not contain introns, but the 5′UTR typically contains two or more highly conserved introns. The situation in animals is just the opposite; i.e. no introns are present in the 5′UTR, but several may be present in the ORF. Our present knowledge about *SAMDC* genes indicates that (1) *SAMDC* transcription and translation are subject to regulation by Put as well as by other PAs; (2) the different *SAMDC* genes are expressed differentially in different tissues; (3) SAMDC is an unstable enzyme with a half-life of 20–60 min; (4) the coding sequence and the 5′UTR of *SAMDC* among different plants are highly conserved; and (5) while Put is an activator of SAMDC activity in animals, plant SAMDCs lack a Put-binding site for stimulation. No information on the expression patterns of various paralogues of *SAMDC* in response to cellular Put content is currently available in plants.

The use of QRT-PCR has revealed that not only does the transcript abundance of the three *SAMDC* genes in cultured poplar cells vary independently of each other over the 7-day cycle but also that the three genes respond differently to increased accumulation of Put in the HP cells. The most significant and rather unexpected observation is that the m*ODC* transgenic cells show a reduced expression of the predominant *SAMDC*1 as well as the least expressed *SAMDC*3, with little effect on *SAMDC*2 (Fig. 3). Although the decrease in *SAMDC*1 and *SAMDC*3 transcripts in HP cells is accompanied by a concomitant decrease in SAMDC activity, this

apparently does not affect the rates of biosynthesis and accumulation of Spd in these cells (Bhatnagar et al. 2001). In fact, the HP cells generally produce and accumulate more Spd than the control cells, at least during the first few days of the culture period (P. Bhatnagar, R. Minocha and S.C. Minocha, unpublished data). In a study similar to ours, Capell et al. (2004) reported that overexpression of a Datura ADC in rice caused an increase in both Put and Spd. Although the authors pointed to a positive relationship between *SAMDC* transcripts and tissue Spd content in both wild-type and transgenic plants on certain days in response to stress, this positive relationship between *SAMDC* transcripts and tissue Spd was not seen in the untreated tissue (days 0 in fig. 4A, C in Capell et al. 2004). Since they used gel blots for transcript analysis, no distinction was made among different paralogues of SAMDC; also, no enzyme activity data were presented to correlate them with the PA contents. On the other hand, an inverse relationship between cellular Put and *SAMDC* transcripts in both wild-type and transgenic rice was observed on several days of stress treatment (figs 3D and 4A in Capell et al. 2004), a situation similar to our results with poplar. Of course, an increased production of Spd by SAMDC overexpression, such as that seen in tobacco by Noh and Minocha (1994) and in tomato by Mehta et al. (2002), would indicate that SAMDC alone may be sufficient to affect the cellular contents of Spd. In both these studies, as a consequence of increased utilization of Put as a substrate, its content in the transgenic cells was actually lower. The normal control of SAMDC expression by Put in these cases was of course absent. These observations raise some interesting questions about the role of SAMDC in regulation of this part of the pathway as well as its own regulation by Put. For example (1) how does a lower activity of SAMDC in HP cells sustain a higher rate of dcSAM production for increased Spd biosynthesis? (2) Is there a common mechanism by which HP regulates expression of the two *SAMDC* genes (but not the third one), or is the reduction in transcripts of the two genes a reflection of their increased turnover? Although nothing is known about promoters of the three *SAMDC* genes in poplar, promoters of the two Arabidopsis *SAMDC* genes (*AtSAMDC*1 and *AtSAMDC*2) show almost 50% sequence identity and possess several common motifs, such as IBOX (light regulated), DRE Core (drought responsive), Myb-binding protein (abiotic stress), and GAREAT (GA responsive element) (C.F. Rice and S.C. Minocha, unpublished). It is conceivable that some common Put-sensory elements are present in the promoters of the p*SAMDC*1 and p*SAMDC*3 genes, which regulate their response to Put.

Expression of different genes in the PA biosynthetic pathway is coordinated

Fig. 5 shows variation in expression of the different known paralogues of the three key PA biosynthetic genes over the 7-day culture period in the two cell lines relative to their expression on day 1 of culture. This profile reveals that (1) all genes, with the exception of m*ODC,* show a fresh medium effect of increased expression within 24 h of transfer; (2) the fresh medium effect on transcript levels is concomitant with changes in protein contents of the two cell lines over the 7-day culture period; (3) by day 5, the expression of all genes is highly reduced; (4) the two cell lines reveal important differences in that in the HP cells the decrease in transcripts of all genes except *SAMDC*2 and *SAMDC*3 is delayed to day 5, whereas in the control cells the decrease is visible by day 3 except for *ADC* and *SPDS*1. The combined data point to a coordinated expression of all these genes with the growth phase of cells.

Transcript levels, enzyme activities and PA contents in the cells are coordinated

While the transcript levels or enzyme activities for the key regulatory PA biosynthetic enzymes have been studied separately in a few cases, a direct correlation between the two has not been clearly demonstrated. Among the main reasons for lack of a positive relationship between transcript abundance and enzyme activity are translational controls, transcript turnover rates, enzyme turnover rates, availability of cofactors and other cellular metabolites that affect enzyme activity and, finally, the processing and activation of the proenzyme. A strong temporal correlation between the transcript levels and enzyme activities (g^{-1} FW) of the transgenic m*ODC* as well as that of the native *ADC* and *SAMDC* was seen in poplar cells over the entire 7-day culture cycle. For example, HP cells that have higher *ADC* and lower *SAMDC* transcripts compared with the control cells (Figs 2 and 3) also have higher ADC and a lower SAMDC activity (Fig. 6). An increase in m*ODC* transcripts in HP cells between days 2 and 3 (Fig. 2) is accompanied by a similar increase in ODC activity around days 2–4, and a decrease in SAMDC activity after day 2 follows a decrease in its transcripts (Fig. 6). Changes in ADC activity and its transcript during the first 4 days also parallel each other.

A positive relationship between cellular PAs and respective enzyme activities responsible for their biosynthesis is readily apparent, whether the data are calculated on g^{-1} FW basis or mg^{-1} protein basis. Both ODC and ADC increase during the early days of growth in

the fresh medium (Fig. 6), and this is accompanied by an increase in Put levels (Fig. 8). Likewise, an increase in SAMDC during the first 3–4 days of culture in the control cells parallels changes in cellular Spd and Spm. As mentioned above, the main discrepancy in this respect is that in spite of lower SAMDC g^{-1} FW, the HP cells maintain a slightly higher amount of Spd (also g^{-1} FW). The apparent lack of a large increase in Spd in the HP cells, which have several-fold higher Put content, indicates the lack of stimulation of SAMDC activity by Put. This is in contrast to the demonstrated upregulation of animal SAMDC activity by cellular Put (Ruan et al. 1996, Stanley et al. 1994, Xiong et al. 1997) but is consistent with our current knowledge that plant SAMDC is not activated by Put (Bennett et al. 2002, Park and Cho 1999, Xiong et al. 1997). An alternate explanation would be that the regulation of Spd biosynthesis occurs more by SPDS than by SAMDC, in contrast to what is generally believed (Ruan et al. 1996, Thu-Hang et al. 2002). As discussed above, it is quite possible that reductions in *SAMDC* transcripts as well as in its enzyme activity in HP cells are actually caused by the increased Put content of these cells.

The regulation of mammalian ODC is achieved by a complex mechanism involving an ODC antizyme, which responds to cellular PA levels and helps its subsequent degradation by the 26S proteasome (Hoyt et al. 2003). The presence of an ODC antizyme in plants that is active against mODC has not been demonstrated; thus, its turnover must be regulated by a different mechanism. It should be pointed out that the m*ODC* gene used here has been modified to render it more stable by deletion of the PEST sequence at the C-terminus (Bhatnagar et al. 2001, DeScenzo and Minocha 1993), which is responsible for its rapid turnover (Ghoda et al. 1989).

Several studies have shown that dcSAM (the product of SAMDC) is required by SPDS not only as a substrate (it donates the aminopropyl group) but also for regulation of its activity (Jänne et al. 2004, Kauppinen 1995, Pegg et al. 1986, 1998). Kauppinen (1995) also found that SPDS is a stable enzyme and its activity is not correlated with mRNA levels; it was further concluded that regulation of its translation was mediated by its 5′UTR. These findings would explain the observation in the present study that there was no difference between the two cell lines in *SPDS* expression. The enzyme activity of SPDS was not measured in the present study. These findings support the idea of a strong homeostatic control of Spd levels in the cells.

The use of QRT-PCR has permitted us greater precision in measuring gene expression than that afforded by any other method (Gachon et al. 2004) and has allowed greater insight into the regulation of PA metabolism than was previously known in plants. Not only does it appear that PA metabolism is regulated, at least in part, at the transcriptional level, but also that (as expected) different paralogues of the same gene have differing roles in the maintenance of steady-state enzyme activities and PA levels. This is in contrast to past publications based on alternate techniques, which failed to reveal such precise metabolic regulation. For example, Trung-Nghia et al. (2003) found that overexpressing an oat (*Avena sativa*) *ADC* in antisense mode resulted in a decrease in Put and Spd in rice (*Oryza sativa*) and concluded that there was no effect on the expression of downstream genes. However, the changes that we were able to detect using QRT-PCR could not have been detected using northern blotting or RT-PCR (the techniques used by Trung-Nghia et al. 2003) due to the relative insensitivity and difficulty in quantifying results with these techniques. Similarly, Primikirios and Roubelakis-Angelakis (1999) did not see any change in *ADC* expression upon exogenous application of Put in *V. vinifera* suspension cultures although a decrease in ADC specific activity was observed. Watson and Malmberg (1996) found a 10-fold increase in ADC activity and a 20-fold increase in Put in response to high K^+ stress in Arabidopsis, but again using northern blotting, found no change in *ADC* transcripts. Likewise, many studies that have shown an increase in ADC activity in response to abiotic stress have not made a distinction between the two or more paralogues of this gene that are often found in plants.

Although the results presented here show differential regulation of the three *SAMDC* genes, it is still not clear what the relative contribution of each to the final SAMDC activity is, and if there are differences in the properties of the three isoforms of the enzyme that may affect their contribution to the final reaction. This is due to the fact that each transcript and protein is subject to its own translational and post-translational controls, including possible variation in substrate affinities and pH responses (Kauppinen 1995, Primikirios and Roubelakis-Angelakis 1999, Trung-Nghia et al. 2003, Watson and Malmberg 1996). Very little is known about the subcellular localization of the different SAMDC proteins as well. This indeed may be the challenge to deal with in metabolic engineering via genetic manipulation because while changes in gene transcription, translation, enzyme kinetics, etc. can be quantified in vitro, their relative importance to the total reaction in vivo is often hard to assess.

Conclusions

Data presented here clearly demonstrate that manipulation of a single step in a metabolic pathway has far-reaching consequences for several reactions within

the pathway. This study provides an insight into PA metabolism in poplar cells, with a combined breadth and accuracy previously unseen in any tissue. The results reveal a complex homeostatic mechanism at work for PA metabolism involving several parts of the pathway operating in a coordinated manner. The use of transgenic cells in which a single step in the PA metabolism has been manipulated in a constitutive manner has provided evidence for coregulation of the expression of several genes that control this pathway, and has shown that the PA content of cells is indeed under a complex regulation involving multiple layers of control. Specifically, we show that *ADC* and *ODC* expression and enzyme activities are not subject to feedback regulation, while increased accumulation of Put may inhibit expression of some members of the *SAMDC* family, leading to decreased SAMDC activity. Suspension cultures such as those used here offer a unique opportunity to study the effects of modulating a specific step in a metabolic pathway without complications of different tissue/organ types, translocation of the metabolites from one part of the plant to another or intercellular variations in biochemistry. It is, however, possible that different genes, and in particular different members of the same gene family, may be regulated differently in mature plants in response to different stimuli. As a next step, global analyses of the transcripts (e.g. by microarrays), combined with complete metabolic profiling, should reveal much more information than the present study has done.

Acknowledgements – The authors are thankful to Dr Robert Kenefick for help with statistical analysis, Dr Karen Carlton for help with QRT-PCR analysis, Stephanie Long for help in PA analysis and Drs Estelle Hrabak and Curtis Givan for valuable suggestions in improving the manuscript. Useful comments from the anonymous reviewers who reviewed an earlier version of the manuscript are also acknowledged.

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Edited by A. Crozier