Winter 2009

Regulation of polyamine metabolism in transgenic poplar cell cultures

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Regulation of polyamine metabolism in transgenic poplar cell cultures

Abstract
Polyamines (PA) are naturally occurring low molecular weight aliphatic amines found in all living organisms and essential for their growth and development. The present study uses the tool of transgenic manipulation to elucidate the regulation of PA pathway in poplar cells. This study was divided into two segments; the first segment provides insight into biochemistry of a putrescine overproducing cell line (HP) that overexpresses a mouse ornithine decarboxylase (mODC) under 35S-CaMV promoter, with respect to different treatments. The second segment focused on creation and biochemical characterization of a cell line (SOS1) that overexpresses ODC/SAMDC from Plasmodium falciparum controlled by the same 35S-CaMV promoter producing a bifunctional protein.

From the results it is concluded that: (i) nutrient stress in the form of Ca deficiency in the growth medium is more detrimental to HP cells than control cells, (ii) the tested PA analogues and inhibitors at the tested concentrations have no major observable effect on either HP or the control cells, (iii) Cysteine and methionine although consumed faster in HP cells, are not limiting either for protein synthesis or for growth, (iv) uptake of sucrose is higher in the control cells but its incorporation into PAs is higher in HP cells, (v) SOS1 cells after stable integration of PfODC/SAMDC produce significantly increased amounts of Spd and Spm than control cells, and (vi) the simultaneous expression of both SAMDC and ODC from Plasmodium has greater impact on production of Spd/Spm than on Put in SOS1 cells.

Keywords
Biology, Botany, Chemistry, Biochemistry, Biology, Cell
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REGULATION OF POLYAMINE METABOLISM IN TRANSGENIC POPLAR CELL CULTURES

BY

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THESIS
Submitted To the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of

Master of Science

In

Plant Biology

December, 2009
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Date: 10, 2009
DEDICATION

This dissertation is dedicated to my family and God.
ACKNOWLEDGEMENTS

It is a great pleasure to thank all those who made this thesis possible.

First and foremost I offer my sincerest gratitude to my advisor Dr. Subhash C. Minocha and my co-advisor, Dr. Rakesh Minocha for giving me this research opportunity, for their constant guidance, support and motivation and for helping me understand the finer aspects of the subject with their immense knowledge.

My sincere thanks go to my Master’s committee members Dr. Curtis Givan and Dr. Lee Jahnke for their guidance and helpful suggestions. I would also like to thank Dr. Estelle Hrabak for her valuable advice and for letting me use instruments in her lab when needed. Stephanie Long from USDA- Forest Service Lab deserves special thanks for helping me with several analyses and for being patient and helpful with my numerous technical queries. I am grateful to Dr. Carl Vaughan for guiding and helping me with my responsibilities as a graduate teaching assistant.

It is indeed a great pleasure to thank all my labmates who helped me in my research from time to time and made the life at UNH a lot more fun. I would like to thank Dr. Sridev Mohapatra for teaching me several lab techniques and for providing constant support, help and guidance. I also extend my thanks to Dr. Andrew Page for creating the plasmid used in my transformation experiments and helping me with my molecular work. My thanks go to Rajtilak Majumdar for helping me with my calcium experiments and Dr. Sridevi Ganapathi for her suggestions and troubleshooting tips for my molecular work. Thanks to Charles Rice, Lin Shao and the undergraduate lab members for their help,
support and lovely lab time. Thanks to all the faculty members and students of Department of Plant Biology for making the graduate life easier and enjoyable.

Thanks to my family and all my friends for always being there by my side and believing in me. My heartfelt thanks to my husband, Alaap Cherry, for being an inspiration, for sharing my anxieties and pleasures of thesis writing and for loving and supporting me.

Finally, thanks to God for the wonderful life and the strength that He has provided, making my faith in Him stronger.

This research was co-funded by the USDA-NRI award # 2002-35318-12674, the NH Agricultural Experiment Station, the USDA-Forest Service, and Department of Plant Biology, UNH.
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ABBREVIATIONS

ABA= abscisic acid; ACC= 1-aminocyclopropane-1-carboxylic acid; ADC= arginine decarboxylase; Agm= Agmatine; APCHA= N-(3-aminopropyl) cyclohexylamine; Arg= arginine; Asp= aspartate; ATP= adenosine triphosphate; BSA= bovine serum albumin; Cad= Cadaverine; CaMV= cauliflower mosaic virus; cDNA= complementary DNA; CHA= cyclohexylamine; CHAP= cyclohexylammonium phosphate; CHAS= Cyclohexylammonium sulfate; CIP= calf intestinal phosphatase; CTAB= hexadecyltrimethylammonium bromide; Cys= cysteine; 2,4-D= 2, 4-dichlorophenoxyacetic acid; DAO= diamine oxidase; DCHA= dicyclohexylamine; dcSAM= decarboxylated S-adenosylmethionine; DFMA= α-DL-difluoromethylarginine; DFMO= α-DL-difluoromethylornithine; DHFR= dihydrofolate reductase; DNA= deoxyribonucleic acid; DPM= Disintegrations per minute; DTT= dithiothreitol; EDTA= ethylenediamine tetraacetic acid; EGTA= ethylene glycol tetraacetic acid; GABA= γ-aminobutyric acid; GAD= glutamate decarboxylase; Gln= glutamine; Glu= glutamate; Gly= glycine; GOGAT= glutamate-2-oxoglutarate aminotransferase; GR= glutathione reductase; GSH= reduced glutathione; GUS= β- glucuronidase; HD= heptanediamine; HEH= β-hydroxyethylhydrazine; HP= high putrescine; HPLC= High Performance Liquid Chromatography; IAA= Indole-3-acetic acid; α-KG= α-ketoglutarate; MCHA= 4-methylcyclohexylamine; MDHAR= monodehydroascorbate reductase; Met= methionine; MGBG= methylglyoxal bis(guanyl)hydrazone; MTA= methylthioadenosine; MTT= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NADP=...
nicotinamide adenine dinucleotide phosphate; NPT = neomycin phosphotransferase; OAS = O-acetyl-serine; OAT = ornithine aminotransferase; ODC = ornithine decarboxylase; Orn = ornithine; P5CR = Δ1-pyrroline-5-carboxylate reductase; PA = polyamines; PAO = polyamine oxidase; PCA = perchloric acid; PCR = polymerase chain reaction; PDH = proline dehydrogenase; PLP = pyridoxal phosphate; Pro = proline; Put = putrescine; RNA = ribonucleic acid; ROS = reactive oxygen species; SAM = S-adenosyl methionine; SAMDC = S-adenosyl methionine decarboxylase; SDS = sodium dodecyl sulphate; SE = standard error; Ser = serine; Spd = spermidine; SPDS = spermidine synthase; Spm = spermine; SPMS = spermine synthase; SSAT = Spd/Spm Δ1-acetyltransferase; TCA = tricarboxylic acid; Tet = tetracycline; TS = thymidylate synthase
ABSTRACT

REGULATION OF POLYAMINE METABOLISM IN TRANSGENIC POPLAR CELL CULTURES

By

Smita Cherry

University of New Hampshire, December, 2009

Polyamines (PA) are naturally occurring low molecular weight aliphatic amines found in all living organisms and essential for their growth and development. The present study uses the tool of transgenic manipulation to elucidate the regulation of PA pathway in poplar cells. This study was divided into two segments; the first segment provides insight into biochemistry of a putrescine overproducing cell line (HP) that overexpresses a mouse ornithine decarboxylase (mODC) under 35S-CaMV promoter, with respect to different treatments. The second segment focused on creation and biochemical characterization of a cell line (SOS1) that overexpresses ODC/SAMDC from Plasmodium falciparum controlled by the same 35S-CaMV promoter producing a bifunctional protein.

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cells after stable integration of *PfODC/SAMDC* produce significantly increased amounts of Spd and Spm than control cells, and vi) the simultaneous expression of both *SAMDC* and *ODC* from *Plasmodium* has greater impact on production of Spd/Spm than on Put in SOS1 cells.
INTRODUCTION

Polyamines

Polyamines (PA) are naturally occurring low molecular weight aliphatic amines found in all living organisms. They are important cellular constituents that are required for the growth and development in all plants and animals. The three common PA are diamine putrescine (Put, \( \text{NH}_2-(\text{CH}_2)_4-\text{NH}_3^+ \)), triamine spermidine (Spd, \( \text{NH}_2-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_3^+ \)), and tetraamine spermine (Spm, \( \text{NH}_2-(\text{CH}_2)_3-\text{NH}-(\text{CH}_2)_4-\text{NH}-(\text{CH}_2)_3-\text{NH}_3^+ \)). PAs are important polycations as they carry positive charges at physiological pH (Wallace et al. 2003). Some of the less commonly known PAs include 1,3-diaminopropane and cadaverine (Cad), which are diamines; norspermidine, aminopropylcadaverine, and homospermidine, which are triamines; and norspermine, thermospermine, and canavalmine, which are tetraamines. Higher PAs such as pentaamines (caldopentamine, homocaldopentamine) and hexaamines (caldohexamine, homocaldohexamine) are also known (Kuehn et al. 1990). It has been suggested that these uncommon PAs are meant to help the organism (mostly lower organisms such as bacteria, algae and mosses) survive and adapt under extreme conditions such as high temperature or high salt environment (Kuehn et al. 1990).

Polyamines are present in two forms, bound and free. The bound forms are acid-insoluble and are higher in molecular weight as compared to free forms, which are acid soluble (Santanen and Simola 2007). Polyamines are known also to form conjugates with compounds like hydroxycinnamic acids (Martin-Tanguy 1997) or be methylated, for the synthesis of nicotine and tropane alkaloids (Hashimoto et al. 1989). These conjugates...
have role in plant defense against pathogen infection (Martin-Tanguy 1997, Walters 2000). The ratio of free to conjugated PAs depends on the tissue and the developmental stage of the organism (Torrigiani et al. 1987a). The transport of PAs has been a challenge to study owing to its interactive properties with cell wall and membranes, although it has been established that the transport is bidirectional (into and out of the cells) and involves dependency on energy, Ca$^{2+}$, pH, and hormones. The transport is also intercellular and interorgan (Edreva 1996).

Functions

Polyamines are known for their role in stabilization of polyanionic macromolecules which can be attributed to the positive charges carried by them. The interaction between PAs and negatively charged macromolecules such as nucleic acids (DNA, RNA, phospholipids) enables them to affect chromatin structure, transcription and translation (Marton and Morris 1987, Pollard et al. 1999, Bachrach 2005), and aid in the stabilization of membranes (Schuber 1989, Thomas and Thomas 2001). Polyamines are also required for ribosomal aggregation, protection of ribosomal RNA against enzymatic digestion and stability of tRNA conformation, thus playing important roles in protein synthesis (Bachrach 2005). Furthermore, PAs regulate the activity of protein kinases, thus playing a role in growth, differentiation and signal transduction (Bachrach 2005). This activity also includes their role in regulation of cell cycle, making them responsible for promoting malignant cell/tissue growth. In the presence of PA inhibitors, dividing cells get arrested at the G1 phase and their transition to S phase is made possible by exogenous PAs (Galston and Sawhney 1990).
Although PAs are essential for the survival of all life forms, studies also reveal that their higher concentrations in cells might be toxic in certain cases (Poulin et al. 1995, Stefanelli et al. 1998). Cancer cells have been reported to have higher levels of PAs and their derivatives (Pegg 1988, Wallace et al. 2003), thus their biosynthesis has become an important target in the chemotherapeutic treatment of cancer (Davidson et al. 1999, Thomas and Thomas 2003, Boncher et al. 2007). Phenotypic abnormalities like reduction in root and shoot length, leaf necrosis and severe leaf curling have been reported in tobacco plants that were constitutively over-producing Put (DeScenzo and Minocha 1993, Masgrau et al. 1997, Panicot et al. 2002). Reduction in seed germination percentage and inhibition of root growth by Spd accumulation has been reported in Arabidopsis (Mirza and Rehman 1998, Tassoni et al. 2000). Poulin et al. (1995) suggested over-accumulation of Spd in mouse leukaemia cells to induce apoptosis, as seen by chromatin condensation, and DNA and nuclear fragmentation.

Many researchers have reported the importance of PAs in somatic and/or zygotic embryogenesis (Robie and Minocha 1989, Minocha et al. 1991, 1993, 1999, 2004b, Minocha and Minocha 1995, Singh and Rajam 1998, Martinez et al. 2000, Shoeb et al. 2001, Puga-Hermida et al. 2003). Effects of exogenous PAs on the endogenous contents of PAs and plant growth regulators (IAA, ABA) in embryogenic cultures of Araucaria angustifolia were studied by Steiner et al. (2007). In the absence of plant growth regulators in the culture medium, exogenous PAs had a positive effect on growth of embryogenic cultures as well as endogenous IAA and ABA levels. Similar report on increase in endogenous PA content resulting in increase in number of somatic embryos in Solanum melongena leaf discs was submitted by Yadav and Rajam (1998). Addition of
PA inhibitors to the culture medium resulted in inhibition of shoot bud induction in seedling explants of *Capsicum frutescens*, which was reversed to some extent by exogenous PA addition (Kumar et al. 2007). Chiancone et al. (2006) reported positive stimulation of gametic (anther) embryogenesis in *Citrus clementina* upon exogenous addition of Spd. Enhanced embryogenesis by PA addition has also been observed in *Gossypium hirsutum* (Sakhanokho et al. 2005) and *Hevea brasiliensis* (El Hadrami and D’Auzac 1992). Over-production of Put by transgenic expression of mouse ornithine decarboxylase (mODC) in carrot cells promoted somatic embryogenesis (Bastola and Minocha 1995). Imai et al. (2004) showed that the double mutant of Spd synthase (SPDS1 and SPDS2) was lethal at the embryonic stage in *Arabidopsis*.

Polyamines have been of great interest to plant scientists due to their association with biotic and abiotic stresses (Kurepa et al. 1998, Bouchereau et al. 1999, Perez-Amador et al. 2002, Navakoudis et al. 2003). Mo and Pua (2002) reported up-regulation of arginine decarboxylase (ADC) in response to salt and chilling stress accompanied by increased accumulation of PAs in *Brassica juncea*. Difluoromethyl ornithine (DFMO), an inhibitor of PA biosynthesis increased electrolyte leakage in tomato leaves, which was decreased by exogenous Put under cold stress (Kim et al. 2002). Higher S-adenosylmethionine decarboxylase (SAMDC) transcript levels were observed in cold-stressed rice in resistant varieties compared to the susceptible ones, along with elevated Spd content (Pillai and Akiyama 2004). Increases in PA contents and activities of ADC (EC 4.1.1.19), SAMDC (EC 4.1.1.50) and SPDS (EC 2.5.1.16) were also observed in rice leaves when subjected to drought (Yang et al. 2007). Polyamines have also been associated with oxidative stress in both positive and negative ways (Wojtaszek 1997, Apel and Hirt 2004, Nayyar
and Chander 2004, Papadakis and Roubelakis-Angelakis 2005, Mohapatra et al. 2009a). H$_2$O$_2$ generated by PA catabolism is an important cause of oxidative burst (Wojtaszek 1997, Apel and Hirt 2004). An increase in Spd was recorded in chilling tolerant cucumber, although none of the PAs showed increase in the sensitive type (Shen et al. 2000). The chill-induced H$_2$O$_2$ production was reduced with exogenous Spd treatment simultaneously reducing the NADPH-oxidase activity and superoxide generation via NADPH pathway in the sensitive type. Inhibiting PA synthesis in the tolerant type enhanced chilling injury. Polyamines under stress may play a direct role as reactive oxygen species (ROS) scavengers and membrane protecting agents (Roberts et al. 1986, Lovaas 1991) or an indirect role as signaling molecules (Kasukabe et al. 2004). Up-regulation of DREB genes (stress-responsive genes) has been reported in Arabidopsis transformed with SPDS cDNA from Cucurbita ficifolia under stress (Kasukabe et al. 2004). On the other hand, enhanced PA production accompanied by increased PA catabolism could also cause oxidative damage in the cells (Mohapatra et al. 2009a).

Increased Put content and elevated ADC activity were seen in Cd$^{2+}$ treated oat and bean leaves, although no appreciable change was observed in Spd and Spm content (Weinstein et al. 1986). Although accumulation of perchloric acid (PCA) soluble conjugates was observed in androgenic embryos of carrot on treatment with copper sulfate, the total PA content decreased due to decline in free PAs and PCA insoluble conjugates (Görecka et al. 2007). Among the major PAs, Spm has been suggested to play an important protective role against salt stress (Sanchez et al. 2005, Yamaguchi et al. 2006), although Put has also been reported to reduce the damage due to salinity (Liu et al. 2006). An Arabidopsis double knockout mutant, unable to produce Spm showed
increased sensitivity to high salinity, which was reversed by exogenous application of Spm but not by Put or Spd (Yamaguchi et al. 2006).

Hamasaki-Katagiri et al. (1997) showed that the null mutant of SPE3 gene coding SPDS in *Saccharomyces cerevisiae* required Spd or Spm for growth. Later Chattopadhyay et al. (2003) showed that Spd is specifically required for growth in yeast. Similar results were obtained in *Dictyostelium discoideum*, (Guo et al. 1999), *Leishmania donovani* (Roberts et al. 2001) and *Aspergillus nidulans* (Jin et al. 2002).

**Biosynthesis and Catabolism**

The biosynthesis of PAs involves decarboxylation of amino acids ornithine (Orn) and arginine (Arg) by specific decarboxylases. All eukaryotic organisms are known to possess Orn decarboxylase (ODC, EC 4.1.1.17) pathway for Put production from Orn except the obligate protozoan *Trypanosoma cruzi* and the plant *Arabidopsis thaliana* (Hanfrey et al. 2001). In plants and bacteria an additional pathway for Put synthesis is also present which utilizes Arg as the substrate; the first reaction in this case is regulated by ADC (Hanfrey et al. 2001). An aminopropyl group derived from decarboxylated S-adenosylmethionine (dcSAM) is transferred to Put to produce Spd; transfer of another aminopropyl moiety to Spd forms Spm. The two reactions are catalyzed by aminopropyl transferases, SPDS and Spm synthase (SPMS, EC 2.2.1.22), respectively (Tabor and Tabor 1984, Pegg 1986, Cohen 1998). The action of a decarboxylase (SAMDC) produces dcSAM required for these reactions from SAM. The enzymes ODC/ADC and SAMDC are considered rate limiting enzymes for the entire pathway and are known to be highly regulated at transcriptional, translational and post-translational levels for PA biosynthesis (Müller et al. 2000). The pathway is summarized in Fig. 1.
Polyamine catabolism is an important process for living organisms as it helps not just to get rid of the excess PAs and modulate cellular PA contents but it also serves as a link to several important metabolic pathways, e.g., TCA cycle, Urea cycle, amino acid biosynthesis, and recycling of carbon and nitrogen of the PAs (Mohapatra et al. 2009b). Cellular contents of PAs are additionally regulated by their excretion, recycling and/or movement in/out of cellular organelles (Casero and Pegg 1993). Polyamines are catabolized by enzymes diamine oxidases (DAO, EC 1.4.3.6) and PA oxidases (PAO, EC 1.5.3.3), there is presumably specific oxidase for each PA (Kaur-Sawhney et al. 2003).
Both DAO and PAO work on different parts of the molecules breaking PAs into aminoaldehydes, simultaneously producing ammonia and $\text{H}_2\text{O}_2$. The aminoaldehydes are cyclized into $\Delta^1$-pyrroline and 1,5-diazabicyclononane (Federico and Angelini 1991, Edreva 1996); the former is converted to $\gamma$-aminobutyric acid (GABA) which then is converted to succinate via succinic semialdehyde, linking the PA catabolism with TCA cycle. In animals, Spd and Spm catabolism involves first an acetylation step by Spd/Spm $N^1$-acetyltransferase (SSAT, EC 2.3.1.57) which is followed by the action of PAO (Casero and Pegg 1993). Ultimately Spd gets converted to Put and Spm to Spd. The SSAT reaction is a rate limiting step and thus highly regulated. This back conversion of PAs through PAO was initially thought to be present only in animals, but recently it has been shown to take place also in plants, although no SSAT activity has yet been demonstrated in plants (Bhatnagar 2002, Tavladoraki et al. 2006, Moschou et al. 2008). Plant PAOs, unlike animals, act directly on PAs. Recently, one of the identified PAOs, AtPAO3, was found to be localized in peroxisomes similar to the situation in mammals (Moschou et al. 2008). In plants, PAO has been reported mostly in the apoplast and in barley also in the vacuole (Moschou et al. 2008).

**Transgenic manipulation**

Transgenic manipulation of single or multiples genes has been extensively used in order to elucidate the regulation of PA metabolism in living organisms and also to investigate the role played by PAs in developmental processes. By transgenic manipulation, it is possible to target a specific step in a given pathway and study the effect of its up- or down-regulation on the pathway itself and the overall global impact on the system. The choice of promoter provides additional flexibility to this tool. The

Burtin and Michael (1997) overexpressed oat ADC cDNA in tobacco under CaMV 35S promoter with duplicated enhancer sequences and observed high accumulation (10-20 fold) of agmatine in the plants as a result of elevated ADC activity. This accumulation, however, had no effect on the pool of PAs in the plant, neither was there an effect on activities of ODC, SAMDC or DAO. In the same year, another group of scientists, Masgrau et al. (1997) also overexpressed oat ADC under the tetracycline-inducible promoter in tobacco. This manipulation resulted in the accumulation of Put to a toxic level during vegetative phase of growth producing altered phenotypes. The flowering stage, in contrast, did not show any phenotypic effects. Capell et al. (1998) introduced oat ADC cDNA with CaMV 35S promoter in rice cell lines and plants using particle bombardment and observed higher ADC transcript level, higher ADC activity and about 2 fold high Put content. They also suggested that amount of Put higher than a certain threshold had a negative impact on in-vitro development of callus. No increase in Spd and Spm concentrations was observed. Capell et al. (2000) created another transgenic rice, this time expressing antisense oat ADC, which resulted in down-regulation of both native ADC and ODC. A significant decrease (up to 95%) in Put and Spd contents was reported in these transformed callus lines as compared to controls. Decrease in Spm was less (68%) and was observed in only 20% of the transgenic lines.
Roy and Wu (2001) used the *Agrobacterium* mediated transformation technique to produce transgenic rice with oat ADC cDNA. The transgene was under the control of an ABA-inducible promoter. Under conditions of salinity (NaCl) stress the transgenic plants showed a very high accumulation of Put along with substantially high ADC activity. The plants had different copy numbers of the transgene (1 to 5), but this did not affect the PA content or the ADC activity significantly.

Bastola and Minocha (1995) created transgenic carrot suspension cultures constitutively expressing mouse *ODC* and observed enhanced Put production by the cells. These transgenic cells exhibited improved somatic embryogenesis when grown in auxin-free medium and were also able to grow and produce embryos in the presence of α-difluoromethylarginine (DFMA), a known inhibitor of ADC. In 1998, Andersen et al. characterized this cell line further. According to them, introduction of m*ODC* into the cells resulted in overall higher ODC activity without a significant change in ADC activity. Higher Put production also resulted in higher degradation of Put and its conversion to Spd and Spm. Similar results with respect to ADC activity were reported by DeScenzo and Minocha (1993) in transgenic tobacco expressing m*ODC*.

A transgenic cell line of *Populus nigra × maximoviczii* overexpressing m*ODC* (called HP) was created and described by Bhatnagar et al. (2001) with respect to role played by Orn, Arg, Urea, and glutamine (Gln) in the PA pathway. Since the Put over-producing cell line utilized Orn in much greater amounts than the control cell line, it was speculated that the production of Orn from Gln or glutamate (Glu) might become a limiting factor in these cells. Over-production of Put via ODC pathway either did not affect the native ADC activity or the enzyme transcript levels or increased it (Bhatnagar
et al. 2001, Page et al. 2007). [14C]Orn, when fed to these mODC transgenic cells was rapidly metabolized into Put (Bhatnagar et al. 2002). Furthermore, the amount of Put degradation observed in these cells was proportionate to the total Put present in the cells, thus being higher in ODC transgenics than non-transgenics (NT). The catabolic breakdown of diamine constituted the major part of Put degradation process, conversion to Spd and secretion into the medium being of minor importance. Surprisingly, the activity of DAO in both transgenic cells and NT cells was found to be similar. Since manipulation of a single step in PA pathway can lead towards changes in several connecting pathways or parts of the pathways, Page et al. (2007) suggested existence of coregulation of genes of the PA pathway. According to them, native ADC and ODC expression and activities were not under the control of feedback mechanism although high Put did affect SAMDC expression and activity. Overexpression of mODC in HP cells not only affected cellular Orn concentration but also the cellular content of almost all amino acids (Mohapatra et al. 2009b).

Transgenic tobacco plants expressing a human SAMDC cDNA under CaMV 35S promoter created by Noh and Minocha (1994) showed 2 to 4 fold increase in SAMDC activity, with a concomitant decrease in Put and increase in Spd content of the cells; Spm remained unaffected in these cells. While the transgenic plants exhibited a higher regeneration potential in vitro (a cytokinin treatment characteristic), no major phenotypic changes were observed in these plants. Expression of the same transgene in carrot resulted in production of somewhat thickened/stout somatic embryos in the transgenic cell lines (Bastola 1994). Transgenic potato plants constitutively expressing antisense SAMDC showed abnormal phenotypes such as stunted growth, small leaves, and profuse
branching and root growth inhibition. Plants expressing sense and antisense \textit{SAMDC} under the control of inducible promoter (Tetracycline) showed changes in the transcript level and activity of \textit{SAMDC} along with changed PA levels (Kumar et al. 1996). The Tet/\textit{SAMDC} sense transgenic plants showed increase in PA pool on treatment with tetracycline with Spd increasing up to 7 fold than control, Spm increasing 3 fold than control and although Put content increased, the increase was not consistent. Tetracycline treatment on Tet/\textit{SAMDC} antisense transgenic plants resulted in reduction of PA content although the decrease was small. Another use of sense and antisense \textit{SAMDC} transformation in potato was seen in the work of Pedros et al. (1999) where they used a tuber-specific patatin promoter to drive the expression of the gene. \textit{SAMDC} transcript level and enzyme activity was higher in developing tubers from plants that were sense-transformed as compared to controls. The content of Spd was also significantly higher in these tubers. The antisense transformants, on the other hand, showed reduction in \textit{SAMDC} transcript level, \textit{SAMDC} activity and total PA content.

A SPDS cDNA from \textit{Cucurbita ficifolia} was expressed in \textit{Arabidopsis thaliana} under the control of CaMV 35S promoter and an increase in SPDS activity and Spd content was observed in leaves with increased stress tolerance (Kasukabe et al. 2004). Over expression of SPDS cDNA from \textit{Datura stramonium} in transgenic tobacco plants showed higher Spd to Put ratio, although the total PA pool remained the same suggesting tight regulation of PA biosynthesis (Franceschetti et al. 2004). Recent developments in the transgenic manipulation of PAs in plants are summarized in Table 1.
Table 1. List of recent transgenic experiments in plants with genes encoding polyamine biosynthetic enzymes (2004-2009)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Gene</th>
<th>Promoter-Orientation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>Datura <em>ADC</em></td>
<td>Maize ubiquitin- sense</td>
<td>Capell et al. 2004</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Arabidopsis <em>ADC</em></td>
<td>CaMV- sense</td>
<td>Alcázar et al. 2005</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Tobacco <em>ADC</em></td>
<td>CaMV- antisense</td>
<td>Chintapakorn and Hamill 2007</td>
</tr>
<tr>
<td>Eggplant</td>
<td>Oat <em>ADC</em></td>
<td>CaMV- sense</td>
<td>Prabhavathi and Rajam 2007</td>
</tr>
<tr>
<td>Wheat</td>
<td>Oat <em>ADC</em></td>
<td>Maize ubiquitin- sense</td>
<td>Bassie et al. 2008</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Datura <em>SAMDC</em></td>
<td>CaMV- antisense</td>
<td>Torrigiani et al. 2005</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>Arabidopsis <em>SAMDC</em></td>
<td>CaMV- sense</td>
<td>Hu et al. 2006</td>
</tr>
<tr>
<td>Tomato</td>
<td>Yeast <em>SAMDC</em></td>
<td>E8 promoter- sense</td>
<td>Mattoo et al. 2006</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Carnation <em>SAMDC</em></td>
<td>CaMV- sense</td>
<td>Wi et al. 2006</td>
</tr>
<tr>
<td>Rice</td>
<td>Datura <em>SAMDC</em></td>
<td>Maize ubiquitin- sense</td>
<td>Peremarti et al. 2009</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Datura <em>SPDS</em></td>
<td>CaMV- sense</td>
<td>Franceschetti et al. 2004</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td><em>Cucurbita ficifolia</em> <em>SPDS</em></td>
<td>CaMV- sense</td>
<td>Kasukabe et al. 2004</td>
</tr>
<tr>
<td>Pear</td>
<td>Apple <em>SPDS</em></td>
<td>CaMV- sense</td>
<td>He et al. 2008</td>
</tr>
<tr>
<td>Pear</td>
<td>Apple <em>SPDS</em></td>
<td>CaMV- sense</td>
<td>Wen et al. 2008</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Maize or pea <em>PAO</em></td>
<td>CaMV- sense</td>
<td>Rea et al. 2004</td>
</tr>
</tbody>
</table>
Bifunctional *Plasmodium falciparum* ODC/SAMDC

Until recently, transgenic manipulations in plants were mostly restricted to only one functional gene (in addition to selection/marker genes) at a time, although several studies have recently demonstrated the simultaneous transgenic expression of two or more genes each controlled independently by its own promoter (Ye et al. 2000, Bohmert et al. 2002, El Amrani et al. 2004, Lücker et al. 2004, Aluru et al. 2008). Certain bifunctional proteins have been identified in some protozoans in different metabolic pathways, one of them being the PA pathway. *Plasmodium falciparum* produces a bifunctional protein ODC/SAMDC where a single polypeptide possesses two kinds of activities. Its N-terminus has SAMDC activity and C-terminus has ODC activity (Müller et al. 2000, Krause et al. 2000). The ODC part which is a homodimer, and SAMDC, a heterotetramer, form a heterotetrameric complex together. Other examples include the folate pathway that possesses a bifunctional protein with N-terminus having the dihydrofolate reductase (DHFR) activity and C-terminus having thymidylate synthase (TS) activity. Dihydropteroate synthetase (N-terminus) and dihydro-6-hydroxymethylpterin pyrophosphokinase (C-terminus) also exist as a single bifunctional protein (Müller et al. 2000). Based on sequence similarities to different organisms, the ODC/SAMDC of *Plasmodium* was deduced to be 1419 amino acids long and made of 3 regions (Fig. 2): SAMDC (residues 1-529), connecting or hinge region (residues 530-804) and ODC (residues 805-1419).
Such a bifunctional protein in the PA pathway appears to be unique to Plasmodium. Although the degree of similarity between amino acid sequence of P. falciparum ODC/SAMDC and its mammalian counterpart is low (Fig. 3), the essential regions responsible for enzyme structure and functioning are conserved; for example, there is only 21% similarity between mODC and PfODC, but the essential catalytic domains are conserved in the two genes (Müller et al. 2000). There are large parasite-specific inserts in the region of amino acid homology that make the protein very large as compared to its mammalian counterpart. These inserts are suggested to have role in intra- and inter-domain interactions for optimal catalytic activity (Birkholtz et al. 2004), although the catalytic sites of the two domains can act independently as determined by mutagenic analyses (Wrenger et al. 2001). Birkholtz et al. (2004) showed that deletion mutagenesis of one or more of these inserts not only had negative effect on their respective domains but also on the neighboring domain. Putrescine in mammals has stimulatory effect on SAMDC and exerts feedback control over ODC; in Plasmodium it has no similar effect on SAMDC but the ODC feedback is even stronger (Müller et al. 2001). The suggested rationale behind the presence of this bifunctional protein is that these two enzymes are the most important in the PA pathway being the rate-limiting steps and this feature provides coordinated transcription and translation (Müller et al. 2001).
Figure 3. Alignment of the deduced amino acid sequences of the bifunctional ODC/SAMDC from *P. falciparum* (Pf) with the respective SAMDC from human (Hs) and ODC from mouse (Mm). Identical amino acid residues have been highlighted. Gaps (-) were introduced into sequences to maximize homology. (Adapted from Müller et al. 2000)
Calcium and Polyamines

Calcium (Ca\(^{2+}\)) is an essential nutrient and is a crucial regulator of growth and development in plants (Hepler 2005). It maintains ionic balance in the cells and provides rigidity to the cell wall. It controls several physiological processes in plants by functioning as a second messenger (White and Broadley 2003). The role of Ca\(^{2+}\) in signaling has been reported and reviewed by several authors (Bush 1995, Clapham 1995, 2007, Knight 2000, Anil and Sankara Rao 2001, Plieth 2001, Reddy and Reddy 2004). The cytoplasmic Ca\(^{2+}\) concentration increases in response to various stimuli but is not kept at high levels for longer time since it might become toxic (Lecourieux et al. 2002, Broadley et al. 2003). Ca\(^{2+}\) is moved from the cytoplasm to the apoplast, vacuole or endoplasmic reticulum. Ca\(^{2+}\) chelating organic compounds maintain the submicromolar concentration of cytoplasmic Ca\(^{2+}\) (Broadley et al. 2003, White and Broadley 2003). About 60% of the plant cell Ca\(^{2+}\) is present bound to cell walls (Rengel 1992).

The requirement of Ca\(^{2+}\) varies with different plant species. Several Ca\(^{2+}\) deficiency diseases are known in horticulture. As the deficiency of Ca\(^{2+}\) can be harmful for the plants, so is the excess of it. Excess Ca\(^{2+}\) in the soil may reach toxic levels causing inhibition of seed germination, reduced plant growth and symptoms like 'gold spot' (accumulated calcium oxalate crystals) in tomatoes (White and Broadley 2003).

Cell cultures are a sensitive system and respond to slightest change in the medium contents (Giinter and Ovodov 2005). The effect of manipulation of medium nutrients on growth and cell biochemistry has been studied by several researchers. Calcium deficiency led to significant reduction in growth of carrot cells (Konno et al. 1999). The yield of pectic polysaccharides extracted from cell walls did not change significantly in Ca\(^{2+}\)
deficient cells when compared to controls. However, Ca\textsuperscript{2+} deficient cells were found to contain less galacturonic acid in their pectic fractions and released more acidic extracellular polysaccharides into the medium. Thus Konno et al. (1999) suggested that Ca\textsuperscript{2+} deprivation impedes interaction of pectic polysaccharides and cell wall. Günter and Ovodov (2005) studied the effect of change in Ca\textsuperscript{2+}, phosphate and nitrogen concentration on growth of *Silene vulgaris* callus and also on amount and composition of the polysaccharides, arabinogalactan and pectin (silenan) produced by the callus. Accumulation of polysaccharides resembling those of cell walls has been observed in culture media of plant cells during growth (Konno et al. 1999). Their results suggested that Ca\textsuperscript{2+} was required for growth of the callus and polysaccharide synthesis and in its absence cell growth reduced significantly. Calcium absence also affected the composition of silenan. There was a 54% reduction of the galacturonic acid residues in silenan and silenan production decreased in absence of Ca\textsuperscript{2+} as compared to control treatment. The arabinogalactan composition did not change with varying Ca\textsuperscript{2+}. Similar results of decrease in growth and reduction in pectic polysaccharides were reported earlier by Konno et al. (1984) for cucumber roots.

Borgatto et al. (2002) treated *Chrysanthemum* plants to different calcium, potassium and magnesium concentrations and studied their effect on callogenesis from leaf and stem explants. Callogenesis by leaf explants was found to be proportional to Ca\textsuperscript{2+} concentration whereas it remained largely unaffected in stem explants. Low Ca\textsuperscript{2+} treatments for longer periods resulted in reduced callogenesis for both explant types.

Calcium has also received a lot of attention because of its ability to ameliorate aluminum (Al), Na and heavy metal toxicity in plants (Rengel 1992, Rengel and Zhang
Aluminum presumably blocks Ca\(^{2+}\) channels, thus limiting its uptake and thereby reducing Ca\(^{2+}\) accumulation in plant tissues. Treatment with Al resulted in reduction of [Ca\(^{2+}\)]\(_{cyt}\) accompanied by reduction in growth in BY-2 tobacco cells. Also, treatment of cells with EGTA (chelating external Ca\(^{2+}\)) and with La\(^{3+}\) (blocking Ca\(^{2+}\) channels) led to reduced [Ca\(^{2+}\)]\(_{cyt}\) and growth inhibition in these cells (Jones et al. 1998). Earlier it was thought that Al binds to calmodulin, one of the most important proteins in Ca\(^{2+}\) signaling, changing its conformation and affecting its functioning, but later this idea was discarded (Rehgel and Zhang 2003). It appears that Al toxicity is dependent on the Al/Ca activities ratio rather than Al alone. Ca\(^{2+}\) and Al\(^{3+}\) are suggested to compete with each other for binding sites on plasma membrane or cell walls thus justifying role of Ca\(^{2+}\) in reducing Al toxicity (Rengel 1992). Inhibition of Ca\(^{2+}\) uptake in response to Al treatment was also reported by Zhou et al. (1995) in *Catharanthus roseus* and Minocha et al. (1996, 1997) in red spruce. Also, high Al/Ca ratio has been reported to cause increase in cellular PA content, particularly Put in red spruce cell cultures as well as mature trees (Minocha et al. 1996, 1997), although contrasting results of decrease in Put content in response to Al were observed with *Catharanthus roseus* and hybrid poplar (Zhou et al. 1995, Mohapatra 2008).

Preliminary investigations on the role of PAs in relation to Al toxicity in cell cultures of poplar were conducted in our lab by Mohapatra (2008). The cells accumulated higher Ca\(^{2+}\), which as discussed before, is known for its role in ameliorating Al toxicity. The results on Al:Ca interaction were not clear; therefore, some further experimentations on the variation in Ca concentration in the medium were undertaken by me.
Polyamine analogues and inhibitors

Use of inhibitors in understanding PA metabolism, functions and applications has been invaluable. The site of action of some of the PA inhibitors is depicted in Fig. 4.

Figure 4. Site of action of inhibitors of PA pathway.

Burtin et al. (1991) treated tobacco plants with DFMA and DFMO, and studied their effects on the phenotype. DFMO treatment, inhibiting ODC pathway of Put synthesis, produced a phenotype that mimicked the altered phenotype (wrinkled leaves, reduced internodal length and reduced apical dominance) of tobacco by root-inducing, left-hand, transferred DNA (Ri T-DNA) of Agrobacterium rhizogenes. The degree of phenotypic abnormality was found to be proportional to the effects on PA concentration in the plants. DFMA (inhibiting ADC) had no significant effect on either PA content or the phenotype. This also indicated that perhaps ODC was the major route by which Put was made in this plant. Inhibitors of Spd synthesis, cyclohexylammonium sulfate (CHAS - inhibits SPDS) and methylglyoxal bis(guanyl)hydrazone (MGBG, inhibits SAMDC) lowered Spd.
content and had a negative effect on sexual differentiation; however, the plants did not produce a phenotype mimicking Ri T-DNA transformed phenotype. These data suggested that action of Ri T-DNA possibly involved interference with PA pathway.

Gallardo et al. (1994) reported an increase in radicle emergence in chick-pea (*Cicer arietinum* L. cv. Castellana) seeds on addition of cyclohexylamine (CHA). This was correlated to the increase in Put and Cad content and decrease in Spd and Spm content accompanied by increased ethylene production via activation of ACC synthase (EC 4.4.1.14) and ACC oxidase (EC 1.14.17.4). Similar increase in ODC activity on inhibition of Spd synthesis via addition of 4-methylcyclohexylamine (MCHA, inhibitor of SPDS) and MGBG was also reported by Theiss et al. (2002). Loss of cell division occurred in *Chlamydomonas reinhardtii* on treatment with MCHA, MGBG or DFMO plus MGBG (Theiss et al. 2002). Addition of DFMO alone, however, did not stop cell division.

Role of PAs in flowering has been demonstrated by several researchers using inhibitors. Kaur-Sawhney et al. (1988) showed that addition of CHA to culture medium reduced flower formation in tobacco and instead supported the formation of vegetative buds. Floral initiation was increased by exogenous Spd. Similarly, bolting and flower formation were inhibited in *A. thaliana* when its seeds were grown on a medium supplemented with CHA plus DFMO (Applewhite et al. 2000). Flowering under short-day conditions is usually delayed but the percentage of plants flowering under short-day conditions increased significantly with exogenous Spd. Addition of Spd was also able to accelerate flowering in late-flowering mutant of *Arabidopsis*. This emphasizes the role of Spd in flowering. Role of PAs in flowering was also demonstrated by Aziz et al. (2001)
in grapevine. Fruitlet abscission in grapevine increased with DFMA treatment accompanied by reduction in free PA content in all plant parts. Abscission remained unaffected by DFMO although it did decrease Put content in leaves and increased Spd, Spm and Agmatine (Agm) in roots and inflorescences. This result suggested that PA synthesis before anthesis possibly occurs via ADC and not ODC pathway. Abscission of floral organs also increased in response to CHA and β-hydroxyethylhydrazine (HEH) addition that block SPDS and PAO respectively, suggesting a requirement for optimal concentration of Spd. CHA treatment led to a decrease in Spd and Spm levels in all organs whereas Put and Agm either increased or remained unchanged in different organs. Put also increased with HEH treatment and Spm reduced, but Spd remained unchanged. CHA and HEH treatments also decreased the soluble sugar content in all organs of this plant (Aziz 2003). However, they caused an increase in total amino acid content, including proline (Pro), in the inflorescences, emphasizing the role of Spd in fruitlet abscission.

Growth inhibiting effects by reducing the PA accumulation have also been reported in several fungi such as Botrytis sp, B. cinerea, Rhizoctonia solani, Monilinia fructicola and Colletotrichum truncatum (Rajam and Galston 1985, Gamarnik et al. 1994). In these cases the inhibition of mycelial growth was reversed by addition of Put or Spd, suggesting absolute requirement of PAs for growth. Rajam and Galston (1985) also suggested a possible conversion of DFMA to DFMO by the action of enzyme arginase (EC 3.5.3.1).

Inhibitors have also been used in determining the role of PAs in stress. Under osmotic stress conditions rape leaf discs (Brassica napus) accumulated high Put and Cad
whereas Spd was reduced significantly. Addition of DFMA and DFMO reduced PA accumulation in osmotically stressed leaf discs. Cad content also was reduced significantly in DFMA treated stressed leaf discs (Aziz et al. 1997). The results from CHA, aminoguanidine (DAO inhibitor) and GABA treatments suggested Spd oxidation rather than inhibition of Spd biosynthesis to be responsible for low Spd in tomato leaf discs (Aziz et al. 1998). Experiments with DFMA and DFMO demonstrated that ADC was acting both under normal and stressed conditions, whereas ODC pathway in tomato operated mostly under stress. DFMA also inhibited Pro accumulation in stressed leaf discs. Treatment with aminoguanidine too prevented Pro accumulation, thus suggesting that Pro produced under stress was affected by changes in PA metabolism. Also, MGBG and CHA treatments showed that SAMDC and SPDS activities were not inhibited under stress (Aziz et al. 1998). Spermidine accumulated more in a chilling tolerant variety of cucumber in response to cold stress. This accumulation was reduced on treatment with MGBG leading to increased chilling injury, which was alleviated by exogenous Spd (Shen et al. 2000). Although Put has been known for providing chilling tolerance in many plants (Bouchereau et al. 1999), through the use of MGBG, it was shown that Put was not playing a protective role in this case as its concentration increased on MGBG treatment, and so did the injury; external Put did not reduce the injury. It was also shown that Spd suppressed superoxide generation by inhibiting the activation of NADPH oxidase and thus reducing chilling induced membrane injury (Shen et al. 2000).

Over the years, DFMO has received considerable attention as a possible antitumor agent in animals. Its inhibitory action on ODC reduced Put and Spd content in cells, thus becoming even more important to malignant cell treatment (Seiler 2003a). But it had its
own shortcomings; for example, it had no effect on Spm, was required in very high amounts since its uptake was slow, and it did not stop the uptake of PAs from diet by the cells (Wallace et al. 2003). With this, the attention moved to PA analogues, derivatives of PAs, as possible agents for reducing cellular PA content (Seiler 2003b, Wallace et al. 2003).

Polyamine analogues resemble in structure to PAs and this provides these compounds the ability to serve as better anticancer drugs. Most of these compounds owing to their PA like structure are easily transported by the PA uptake system. Their increase in cells down regulates major PA synthesizing enzymes, ODC and SAMDC. However, these analogues are not able to replace PAs in functionality (Wallace et al. 2003). These analogues based on their nature may or may not reduce PA content of the cell, but are able to reduce cell growth.

As mentioned earlier, PA catabolism in animals involves the activity of inducible enzyme SSAT, which produces acetyl derivatives of Spd or Spm. These derivatives are subjected to degradation by PAO. Some PA analogues are known to induce SSAT (Wallace et al. 2003). Although SSAT activity has not yet been detected in plants, some acetyl derivatives of PAs have been reported in Helianthus tuberosus and Arabidopsis (Del Duca et al. 1995, Tassoni et al. 2000). AtPAO1 was able to oxidize N\textsuperscript{\text{1}}-acetyl-Spm but with a very low efficiency than Spm (Tavladoraki et al. 2006). The acetyl derivatives are not taken up by the cells readily as compared to their parent molecules, thus keeping the PA pool controlled. Also, these derivatives do not accumulate within the cells as they are usually exported out, but their amount is higher in cancerous cells (Wallace et al. 2003). The acylation of PAs leads to reduction in total number of positive charges held
by the parent molecule at physiological pH, prevents the acylated amino group from donating electrons, and also introduces steric hindrance (Karahalios et al. 1998, Seiler 2005), thus inhibiting its polycationic properties. Karalahios et al. (1998) studied the effect of acetyl derivatives of PAs (e.g. acetyl-Spd and acetyl-Spm) on E. coli, but saw no impact on the growth of bacteria. Some of the analogues were taken up by the cells and some were not. The accumulated analogues did not change the cellular PA pool significantly.

The effect of inhibitors like DFMA and DFMO on HP cells was tested by Bhatnagar (2002). DFMA reduced Put content in both HP and non-transgenic control cells. DFMO on the other hand, being specific for mouse ODC had no effect on NT cells. The results established ADC to be the predominant pathway for Put production in NT cells. Here, I have tested some other PA inhibitors and analogues for their effect on cellular PA content in HP cells.

**Sulfur and sulfur amino acids**

Sulfur is one of the essential macronutrients in living organisms. However, being the least abundant of all the required macronutrients in plants, its metabolism has not been studied much. Sulfur is usually taken up by plants in the form of sulfate which can be then either stored or assimilated (Bick and Leustek 1998, Tabe and Droux 2001). Assimilation of sulfate involves its reduction to sulfite and then to sulfide which then combined with the amino acid skeleton of O-acetyl- serine (OAS), produces cysteine (Cys), the first stable organic compound of sulfur assimilation. Cysteine serves as the starting point for the synthesis of some very important metabolites and biomolecules as it is the only donor of sulfide for cell constituents containing reduced sulfur (Hell et al.
2002, Kopriva 2006). Cysteine provides the sulfur atom for methionine (Met) synthesis, the carbon backbone coming from aspartate (Asp), and serine (Ser) providing the methyl group (Ravanel et al. 1998). Cysteine along with Glu and glycine (Gly) forms the tripeptide glutathione, which is one of the thiol compounds involved in maintaining the redox balance of the cell and thus important in oxidative stress defense (Leustek and Saito 1999, Mohapatra et al. 2009a). Cysteine and Met are also protein constituents; Met being the first amino acid during protein synthesis. Cysteine in proteins has multiple roles such as maintaining protein structure, and providing catalytic and metal ion binding properties (Droux 2004). Methionine has also been suggested to be important for maintaining the structural and functional integrity of proteins. Most of the cellular Met (≥80%) is involved in the synthesis of SAM (Ravanel et al. 1998, Hesse and Hoefgen 2003), which is involved in a variety of transmethylation reactions in the cell, in addition to being a precursor of ethylene in plants and a donor of aminopropyl groups for PA biosynthesis (after decarboxylation by SAMDC) in all organisms. Methionine after transferring its methyl group to various acceptors is regenerated via a series of reactions constituting methylthioadenosine (MTA) cycle (Ravanel et al. 1998, Sauter et al. 2004, Bürstenbinder et al. 2007). Thus Met becomes a central regulator in a range of interconnected reactions controlling cellular metabolism.

The inter-connection of sulfate and carbon metabolism was shown by Kopriva et al. (2002). Their experiments with *Lemna minor* using $^{35}\text{SO}_4^{2-}$ showed that sulfur assimilation was completely inhibited in the absence of CO$_2$ although the sulfate pool itself was not affected. This inhibition was overcome by resupply of normal air or other carbon sources.
The role of sulfur amino acids in PA metabolism has not received much attention, although there have been a few reports in the past two decades. Turano et al. (1997) reported an elevation in Put (5 fold) after treatment with Met in detached soybean leaves. This elevation was greater (28-fold) when the leaves were fed Met plus citrulline. When given alone, citrulline raised Put concentrations higher than those with Met (16-fold with citrulline), but it was lower than that obtained by Met plus citrulline. While Spd also increased in response to these treatments, however, the increase was not as high as Put (2 fold with Met plus citrulline). Spermine was unaffected.

In a study from our lab, the cellular content of the two sulfur containing amino acids, Cys and Met, were found to be significantly lower in the Put overproducing cell line of poplar (HP) as compared to control cell line (Mohapatra et al. 2009b). The content of Cys and Met decreased during the first four days of growth after transfer to fresh medium and then recovered on the later days of growth in both cell lines. The increased protein synthesis was suggested to be responsible for low Cys and Met content in the HP cells. Additionally, since Met is produced from Cys, low Cys in HP cells was also suggested to be a reason for low Met in this line. Also, as mentioned earlier, most of the Met is utilized for SAM production and since HP cells produce high Put and Spd, increased utilization of SAM by SPDS may also be responsible for reduction in Met content.

Thus it can be seen that these amino acids not only participate in protein synthesis but also have interaction with PA and ethylene pathways through SAM, maintain the redox state of the cell through GSH, protect against metal detoxification by metal chelating properties, maintain structural integrity of cells and interconnect sulfate, carbon and nitrogen assimilation. Having seen extremely low accumulation of these two amino
acids in the HP cells, I tested if they were limiting for either PA biosynthesis or for growth of cells.

**Sucrose: the ultimate source of carbon for PA synthesis**

Plant photosynthesis produces sugars, which depending on the physiological demand may be transported to sink tissues (mostly in the form of sucrose), metabolized for energy production (respiration) and generation of various cellular metabolites or stored in various forms (Sturm 1999). The organic carbon supply of a cell is shared by both carbon and nitrogen metabolic pathways, thus providing a link between the two (Huppe and Turpin 1994). Increase in carbohydrate content of a cell may under some circumstances stimulate nitrogen assimilation. At the same time, increased nitrogen assimilation may lead to reduction in carbohydrate synthesis and in some cases to a breakdown of stored carbohydrates to flux more carbon into amino acid synthesis (Champigny and Foyer 1992, Huppe and Turpin 1994) redirecting the carbon flow towards amino acid synthesis. The carbon skeleton for synthesis of several amino acids in nitrogen assimilation process is supplied by keto-acids via the respiratory pathway, and requires energy. In the transamination reactions of ketoacids for amino acid biosynthesis, Glu serves as a major donor of amino groups (Huppe and Turpin 1994). Glutamate synthesis requires the transfer of amide group from Gln to \( \alpha \)-ketoglutarate (\( \alpha \)KG) (Turpin et al. 1997). This reaction is catalyzed by glutamate synthase (GOGAT, E.C. 1.4.7.1). The increased demand of ketoacids for amino acid synthesis is met by directing the carbon flow into the respiratory pathway.

Glutamate through a multistep process involving acetyl derivatives produces Arg, generating Orn as an intermediate. Ornithine can also be produced from Arg by the action
of arginase; as mentioned earlier, both Arg and Orn serve as precursors in PA biosynthesis (Forde and Lea 2007). Mattoo et al. (2006) suggested a possible regulation of carbon metabolism by PAs based on their observation of reduction in sucrose content during ripening in tomato fruits which was more pronounced in the transgenic fruits (having yeast SAMDC fused to a ripening specific promoter) accumulating PAs than non-transgenic, control fruit. A higher rate of respiration was observed in ripened transgenic fruits than control implying increased carbohydrate catabolism. Glucose level declined and an increase in TCA cycle intermediates was observed suggestive of increased metabolism in transgenic tomato. As mentioned previously, GABA provides a link between PA catabolism and TCA cycle via succinate. GABA is also produced by decarboxylation of Glu catalyzed by Glu decarboxylase (GAD, EC 4.1.1.15). Thus Glu plays a central role in N assimilation, synthesis of PAs and several important metabolites and thus maintaining the C-N balance in the cell.

In our experimental poplar cell cultures, the organic carbon is supplied in the form of sucrose added to the medium. The HP transgenic cells overproduce Put and thus it seems obvious that it would utilize more Orn and ultimately more Glu than the control cells. In fact, Glu and Orn were significantly lower on all seven days of growth in the HP cells as compared to controls (Mohapatra et al. 2009a, b). The results of 14C-sucrose feeding experiment showed that carbon assimilation was higher in HP cells than control cells, and the total cellular C content was higher too.
Goals and Objectives

Several transgenic lines of poplar (*Populus nigra x maximowiczii*) have been created in our laboratory. One such cell-line which over-expresses a mouse *ODC* (m*ODC*) gene under the control of 2x35S CaMV promoter has been extensively used to study several aspects of the PA metabolic pathway in response to transgenic modulation (Bhatnagar et al. 2001, 2002, Quan et al. 2002, Minocha et al. 2004a, Page et al. 2007, Mohapatra et al. 2009a, b). This cell line is called high putrescine (HP) line (previously called 2E). A cell line expressing β-glucuronidase (*GUS*) gene has been used as control in most of these studies (Page et al. 2007, Mohapatra et al. 2009a, b). Several cell lines having a carrot *SAMDC* have also been produced, but these lines have not been biochemically characterized.

My research was aimed at developing a better understanding of the metabolism of PAs and its regulation using a combination of biochemical and molecular tools. There were two goals of the study: (1) Further biochemical characterization of transgenic poplar cell lines already created in our lab, and (2) creating new transgenic poplar cell lines using the bifunctional *ODC/SAMDC* from *Plasmodium falciparum* and their characterization. The specific objectives were as follows:

Goal 1: Specific Objectives

i. To investigate the effects of changes in calcium concentration in the medium on free polyamines, cell viability, and mitochondrial activity in control and HP cells.

ii. To investigate the effect of PA analogues and inhibitors of polyamine biosynthetic enzymes on free PA content and mitochondrial activity in control and HP cells.
iii. To study the effect of exogenous sulfur and sulfur-containing amino acids on free polyamine content of control and HP cells.

iv. To study the uptake of carbon from sucrose and its incorporation into polyamines in control and HP cells.

**Goal 2: Specific Objectives**

i. To create a transgenic cell lines with the bifunctional $ODC/SAMDC$ from *Plasmodium falciparum*.

ii. To characterize the newly created cell lines with respect to the PA content and activity of enzymes ODC, ADC and SAMDC.
MATERIALS AND METHODS

Cell lines

The study was divided into two major objectives based on experiments that involved transgenic cell lines already created in our lab and newly generated cell lines of poplar (Populus nigra × maximowiczii). The lines that have been described earlier (Bhatnagar et al. 2001, 2002, Page et al. 2007) include a high putrescine cell line (called HP; a.k.a. 2E) and two control lines (wild type – NT and GUS-transformed – control). HP cells over express a mouse ornithine decarboxylase (mODC) gene under the control of a CaMV-35S promoter, producing high-putrescine and hence the name. Another transgenic cell line expressing the β-glucuronidase (GUS) gene was used as control. The newly created cell line (called SOS1) expresses a bifunctional Plasmodium falciparum ODC/SAMDC gene (Müller et al. 2000) under the control of the same 35S promoter. A Neomycin phosphotransferase (NPTII) was used as the selectable marker gene for all transgenic cell lines. All transgenes were under the control of CaMV-35S promoter.

Cell cultures

All suspension cultures were maintained by a weekly subculture routine on MS-medium (Murashige and Skoog, 1962; Sigma-Aldrich, St. Louis, MO, Cat # M-5524), pH 5.7 containing B-5 vitamin (Gamborg et al. 1968; Sigma, G1019), sucrose (2 %) and 2, 4-dichlorophenoxy-acetic acid (2, 4-D) (0.5 mg/L; Sigma, D7299). Suspension cultures were started by transferring 7 mL of the 7 d old cell suspensions to 50 mL of fresh medium in 125 mL Erlenmeyer flasks and kept on a gyratory shaker at 150 rpm. Stock
cultures were maintained on solid MS medium (0.72% agar type A, Sigma, A-4550) on a four-week culture cycle. Medium for transgenic cell lines was supplemented with kanamycin (0.1 mg/mL, Genlantis, Cat # M150025), although the experimental cells were grown in antibiotic free medium for at least two weeks. All cultures were kept at 25±2 °C under a 12 h photoperiod (80±10. μE.m².s⁻¹). Cells were harvested by vacuum filtration through Miracloth (Calbiochem, La Jolla, CA, Cat # 475855).

**Effects of change in calcium concentration in the medium**

This experiment was designed to study the effect of change in calcium concentration in the growth medium on HP and control cells. As mentioned earlier, liquid cell cultures of both cell lines were routinely maintained on MS-medium containing 0.1 mg/mL kanamycin. The cultures were made free of kanamycin two weeks before the experiment. MS medium for the experimental treatments was prepared by mixing 1.65 g/L NH₄NO₃, 1.9 g/L KNO₃, 0.181 g/L MgSO₄.7H₂O, 0.17 g/L KH₂PO₄ (monobasic), 36.7 mg/L Iron-EDTA, 16.9 mg/L MnSO₄.H₂O, 8.6 mg/L ZnSO₄.7H₂O, 0.83 mg/L KI, 6.2 mg/L Boric acid, 0.25 mg/L Sodium molybdate (dihydrate), 0.025 mg/L CoCl₂, 0.016 mg/L CuSO₄, B-5 vitamin, sucrose (2 %) and 2, 4-D (0.5 mg/L). Different concentrations (0.0, 0.05x, 0.2x, 0.5x, 1.0x and 2.0x of the normal concentration) of calcium in the form of CaCl₂.2H₂O were added to the medium (using a stock of 10X CaCl₂.2H₂O = 3.32 g/L). To check the effect of laboratory prepared medium from individual components, another control was added to the experiment (N) which was the regular MS medium prepared from commercially available pre-mix MS powder (described under “Cell culture” of Materials and Methods). For experimental cultures, 7 d old cell cultures were transferred into autoclaved Florence flask and washed three times with washing medium. Washing
medium was prepared in a similar way as experimental ones, except that it had no Ca, sucrose and 2,4-D. The cells were then resuspended in the same washing medium. For the experimental cultures, 30 mL of experimental medium was inoculated with 5 mL of washed resuspended cells. All cultures were placed on gyratory shaker at 150 rpm. Cells were collected on d 3 and 5 by vacuum filtration on to Miracloth and taken for analysis. Cells from 2x Ca treatment were collected only on d 3.

**Effect of addition of polyamine analogues and inhibitors**

The effect of polyamine analogues, N-acetyl-Put, N-acetyl-Spd and N-acetyl-Spm along with N-acetyl-Orn was studied in 3 d old HP and control cells. The experimental cultures were started by adding 5 mL of 7 d old suspension culture into 30 mL fresh medium in 125 mL Erlenmeyer flasks. The treatments were given on d 3 of the growth cycle in volume ranging from 15 μL– 30 μL per flask. The polyamine analogues (N-acetyl-Put and N-acetyl-Spd) and N-acetyl-Orn were added in the final concentration of 0.2 mM. The concentration of N-acetyl-Spm was 0.1 mM. Cells were collected at 24, 48 and 72 h (i.e. days 4, 5, & 6 from the start of the culture cycle) after adding the treatments by vacuum filtration method.

Inhibitor study was performed by testing the effect of APCHA (N-(3-aminopropyl) cyclohexylamine) and MCHA on 3 d old HP and control cells. The experimental set up was similar to the above mentioned PA analogue experiment. Again 30 mL fresh medium was inoculated with 5 mL of 7 d old suspension culture and the respective treatments were added on d 3 in 30 μL volume per flask. The concentration of inhibitors used was 0.1 mM. Cells were collected at 24 h, 48 h and 72 h. Control (N, no analogue or inhibitor) was kept for each cell line in each experiment.
Effect of addition of sulfur or sulfur amino acids

As mentioned under introduction, it was shown previously that concentrations of some of the amino acids, especially the sulfur containing Cys and Met reduced to a great extent in the HP cells (Mohapatra et al. 2009b). It was suggested that this was due to the increased PA and protein synthesis in HP cells during the early days of growth. The effects of exogenous sulfur amino acids, Cys and Met were studied in HP and control cells. Also, to check whether sulfur was the essential element affecting polyamine concentrations, additional sulfur in the form of potassium sulfate was added to the growth medium. For Cys and Met experiments, cell cultures were started by adding 7 mL of 7 d old culture into 50 mL fresh medium. Cysteine (Sigma, C7755) and Met (Sigma, M9625) were given at a concentration of 0.2 mM and 1 mM on d 3 in volumes stock solutions ranging from 50-250 μL depending upon the desired concentration. Cells were collected at 24 h, 48 h and 72 h post treatment by vacuum filtration. Cell cultures for experiment where inorganic sulfur was added were prepared in the same way as for Cys/Met experiment. Sulfur was added in the form of potassium sulfate to double the concentration of sulfur in the medium. The addition was done at zero time or on the 3rd day of growth. Cells were collected after every 24 h through out the 7 d growth period. Control having no treatment was kept for each of these experiments for each cell line.

14C sucrose feeding and PA analysis of radioactive samples

For the first set of experiments, 3 d old cell cultures were transferred into an autoclaved Florence flask and allowed to settle. Volume of the supernatant medium equal to half the total volume of the suspension culture in the flask was removed. To the remaining concentrated cell suspension, 1 μCi [U-14C]-sucrose (ICN, Cat # 1113783) per
10 mL of cell suspension was added. The suspension was transferred into 50 mL autoclaved Erlenmeyer flasks (10 mL per flask) and placed in culture room. Cells were collected at 4, 8, 24, 48 and 72 h by vacuum filtration on to Miracloth and used for polyamine analysis.

For the second set of experiments, 3 d old cells were incubated with 0.5 μCi [U-14C]sucrose per 10 mL culture for 4 h and then washed twice with MS medium. After the second wash, cells were resuspended in the same (i.e. original) volume of fresh medium. Cells were collected at 0, 4, 24, 48 and 72 h. All analyses were done in duplicates following the procedure of Bhatnagar et al. (2002) with minor modifications as summarized below.

For polyamine analysis, 500 mg FW of cells were collected in 1 mL of 7.5% PCA and frozen (-20 °C) and thawed (room temperature) three times before dansylation. Samples were vortexed for 1 min and then centrifuged for 5 min. All centrifugations throughout this experiment were done at 13,000 x g. To 500 μL of the supernatant, 500 μL of saturated sodium carbonate solution was added followed by 250 μL of a 40 mg/mL solution of dansyl chloride in acetone. The samples were incubated for 2 h in water bath at 60 °C, following which 100 μL of amino acid solution (20 mg/mL L-asparagine and 200 mg/mL proline (Sigma, P0380)) were added to react with the excess dansyl chloride. After incubating the samples again at 60 °C for 30 min, 300 μL Photrex grade toluene was added to separate polyamines from amino acids followed by brief vortexing and centrifugation. From the top organic layer, 250 μL was removed and the process was repeated with 200 μL toluene in which the entire 200 μL was removed. The toluene, aqueous and PCA fractions (50 μL each) were mixed with 5 mL Econo-safe
biodegradable counting cocktail (Research Products International Corp., IL, USA) and counted for radioactivity in a scintillation counter (Beckman, Fullerton, CA; LS 6000SC).

**Free polyamine analysis**

Cells (200 ± 20 mg FW) after vacuum filtration were taken mixed with cold 5% perchloric acid (PCA; Fisher Scientific, A-228; 4 μL of 5% PCA per mg cells) on ice. Samples were then frozen (-20 °C) and thawed (room temperature) three times before dansylation (Minocha et al. 1994). Samples were vortexed and centrifuged for 5-7 min. All centrifugations were done at 13,000 x g in a microfuge. A mixture of three polyamines Put-diHCl (Sigma, D1-320-8), Spd-triHCl (Sigma, S2501) and Spm-tetraHCl (Sigma, S2876) was used to create a standard curve. Dilutions prepared for the standard curve had final concentrations of the individual PAs ranging from 0.002 mM Put, 0.001 mM Spd and Spm to 0.04 mM Put, 0.02 mM Spd and Spm. A 100 μL volume of each sample or the standards was mixed with 20 μL of 10 mM heptanediamine (HD) which was used as the internal standard. To this mix, 100 μL of a saturated solution of sodium carbonate and 100 μL of 2% (w:v in acetone) dansyl chloride (Fluka, Milwaukee, WI, Cat # 39220) were added and the tubes were vortexed for 30 s. The tubes were incubated in water bath at 60°C for 1 h. The reaction was stopped by adding 50 μL of 2% L-asparagine (Sigma, A0884). The tubes were vortexed for 30 s and again incubated at 60 °C for 30 min. Acetone was evaporated in a SpeedVac (Savant Instruments Inc., Farmingdale, NY) under vacuum. Then 400 μL toluene (Photrex grade; J.T. Baker, Phillipsburg, Cat # 9456-03) were added to extract the dansyl-polyamines. The samples were vortexed for a minute and allowed to sit for 5 min to separate the lower aqueous and upper organic (toluene) phases. After centrifugation for another minute, 200 μL of the top
layer was transferred to a new microfuge tube, and placed in SpeedVac for 15 min to evaporate toluene. 1 mL of methanol (Fisher Scientific, Fair Lawn, NJ, Lot # 970153) was added to each tube followed by vortexing and centrifuging for 2 min. Methanol extract (500 μL) was then transferred into autosampler vials. The polyamines were analyzed by HPLC using a gradient of acetonitrile (40-100%; EMD Chemicals, Gibbstown, NJ, Cat # AX0145-1) and 10 mM heptanesulfonic acid (Fisher, O-3013), pH 3.4 on a reversed phase C18 column (4.6 x 33 mm, 3 μm; PE) using the Perkin-Elmer (PE) HPLC system (Minocha et al. 1990, Bhatnagar et al. 2001). The system included a PE series 200 autosampler fitted with a 200 μL loop (sample volume was 10 μL), a PE series 200 gradient pump at a flow rate of 2.5 mL min⁻¹. For detection and quantitation, a series 200 fluorescence detector (Perkin-Elmer) with excitation and emission wavelengths set at 340 and 515 nm, respectively, was used. The PE TotalChrom software (Version 328 6.2.1) was used to integrate data. A multiplication factor was incorporated into the software and data were calculated either as nmol mL⁻¹ PCA or nmol g⁻¹ FW.

**Measurement of mitochondrial activity and membrane integrity**

Mitochondrial activity and membrane integrity were measured using the method of Ikegawa et al. (1998) as modified by Minocha et al. (2001). Mitochondrial activity was measured by mixing 100 mg FW of cells with 1 mL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma, M2128] solution in MS medium (250μg/mL). The samples were incubated by shaking for 1 hr at room temperature on a thermomixer at 600 rpm. The supernatant was discarded after centrifugation for 10 min at 13,000 x g and 1 mL acid-propanol (0.04 N HCl in isopropanol) was added to the cells. The tubes were vortexed and centrifuged for 5 min and the absorbance of the supernatant
was recorded at 590 nm (Hitachi U-2000 spectrophotometer, Hitachi Instruments, San Jose, CA).

Membrane integrity was measured by adding 1 mL of 0.05 % Evans Blue (Sigma, E2129) solution to 100 mg FW of cells. The samples were incubated for 15 min at room temperature followed by gentle mixing and centrifugation for 15 min at 13,000 x g. The cells were washed four times with distilled water by adding 1 mL water each time, followed by mixing and then centrifugation for 10 min. After the last wash, the cells were resuspended in 1 mL of 1 % sodium dodecylsulfate (SDS; Sigma L4390). The samples were frozen (-20 °C) and thawed (room temperature) once, mixed and then centrifuged for 10 min. The absorbance of the supernatant at 600 nm was recorded.

Measurement of total protein content

Total soluble proteins were estimated using Bradford assay (1976). The protein extract was prepared by suspending 100 mg FW of cells in 200 μL 0.1 M potassium phosphate buffer, pH 8.0. The samples were frozen and thawed once. The extract was diluted 2x and 50 μL of the diluted extract was added to 1.5 mL Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA, Cat # 500-0006) or Sigma Bradford reagent (Sigma, B6916). Bio-Rad dye reagent was prepared by diluting the dye concentrate to 5x with distilled water. The samples were incubated for 30 min and the absorbance of the blue colored solution was read at 595 nm in a “Spectronic® 20 Genesys™” spectrophotometer (Spectronic Instruments Inc., Rochester, NY). Protein standards were prepared using known concentrations of bovine serum albumin (BSA; Sigma, A4503) in distilled water or buffer used in samples. All standards were prepared in duplicate.
Plasmid construction

The plasmid (pCW122-PfODC/SAMDC) used in my study was prepared by Dr. Andrew Page as follows. The donor vector pASK-IBA3 containing the bifunctional *Plasmodium falciparum ODC/SAMDC* gene (Accession number AF094833, Müller et al. 2000) was obtained from Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany. It was digested using *XbaI*, *AfeI* and *BglII*. The pCW122 expression vector (Walter et al. 1998) was cut with *BamHI* to remove the *GUS* gene, religated and then cut open using *HindIII*. All restriction enzymes were from New England Biolabs, Beverly, MA. All the fragments were made blunt ended using a filling-in reaction (Klenow polymerase) and then pCW122 (-minus *GUS*) was dephosphorylated using calf intestinal phosphatase (CIP). All the fragments were mixed together and ligated by incubating the mixture overnight with T4 ligase at 16 °C. Electrocompetent *Escherichia coli* SURE cells (Stratagene) were transformed with the ligated DNA and selected on ampicillin, and tested for correct orientation of the *Plasmodium* cDNA by restriction analysis. The reconstituted plasmid, called pCW122-PfODC/SAMDC, contains the *PfODC/SAMDC* cDNA regulated by a 2X 35S CaMV promoter and a CaMV 3'-termination sequence. The plasmid also contains an *NPTII* gene under the control of a single 35S CaMV promoter for selection of transgenic plant cells on kanamycin (Fig. 5).
Biolistic bombardment technique

The transgenic lines were created using the particle/biolistic bombardment or 'gene gun' technique. The method of Walter et al. (1998) was modified as described by Bhatnagar et al. (2001). Gold particles (<10 μm, Aldrich, Cat # 326585) were coated with the plasmid pCW122-SOS (*PfODC/SAMDC* + *NPTII*) DNA (2 μg DNA per shot) in the presence of 1 M CaCl2 and 15 mM spermidine using the protocol described below. Cells were prepared by spreading 1-2 mL of the suspension culture (2d, 3d or 4d old) onto...
sterile filter papers (55 mm, Whatman #1) placed atop the shooting medium (MS medium as above, supplemented with 0.25 M sorbitol and 7.2 g/L agar) in 100 x 15 mm Petri-plates and left overnight. Rupture discs of 1350 psi were used for bombardment. After two days of shooting, the filter discs with cells were transferred to the selection medium (MS medium supplemented with 7.2 g/L agar and 100 mg/L kanamycin) in 100 x 20 mm Petri-plates. The cells were transferred to fresh selection medium every 4-5 weeks. The plates with cells were monitored regularly for signs of growth. Following several subcultures, cell clumps from solid medium were transferred to liquid suspension culture medium with kanamycin and maintained routinely.

**Preparation of microcarriers for shooting**

Microcarriers or gold particles (Aldrich Chemicals, St. Louis) were washed using the following procedure. Gold particles (60 mg) were vortexed for 5 min with sterile 1 mL 70% ethanol and then incubated at room temperature for 15 min. The mixture was centrifuged for 5 s and the liquid was discarded. The gold pellet obtained was washed twice with 1 mL sterile ddH₂O by vortexing for a min, settling for a min, centrifuging for 5 s and removing the wash liquid. After the second wash the pellet was resuspended in 1 mL sterile 50% glycerol and stored at 4 °C.

The above prepared gold was coated with DNA of interest (pCW122-*PfODC/SAMDC*) as detailed here. The glycerol-gold suspension was vortexed for 5 min and 50 μL of it was withdrawn in microfuge tube. To the gold, 12 μL DNA (concentration approximately 1 mg/mL) was added and vortexed for 15 sec. To the DNA gold mix, 50 μL of sterile 2.5 M CaCl₂ and 20 μL of 0.1 M Spd (filter sterilized) were added, vortexing for 15 sec after each addition. The final suspension was vortexed for 3
min, settled for 1 min and then centrifuged for 5 sec. The supernatant was discarded and the pellet was washed with first 150 μL of sterile 70% ethanol and then with 150 μL absolute ethanol by simply adding the ethanol and removing it without disturbing the pellet. Finally the pellet was resuspended by vortexing vigorously in 70 μL absolute ethanol. While vortexing, 10 μL of the suspension was removed and plated on macrocarriers. The macrocarriers with gold were dried in the laminar flow hood before using for shooting.

Genomic DNA isolation and quantification

A modified Murray and Thompson (1980) CTAB method was used for genomic DNA isolation. CTAB buffer [2% (w/v) Hexadecyltrimethyl-ammonium bromide (Sigma, H6269), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 with freshly added 0.2% (w/v) β-mercaptoethanol ( J.T. Baker, Lot # 208358)] was pre-heated to 60 °C. Cells (100 mg FW) were ground in liquid nitrogen and incubated in 500 μL CTAB buffer at 60 °C for 30 min with gentle shaking. The sample was extracted with an equal volume of chloroform (Sigma, C2432): isoamyl alcohol (Fisher, A 393-500) (24:1) by inversion, and centrifuged at 13,000 x g for 5 min. The upper aqueous layer was mixed with an equal volume of cold isopropanol and incubated at -20 °C for 15-30 min. Following centrifugation at 13,000 x g at 4 °C for 15 min, the pellet was washed with 70% ethanol/10 mM ammonium acetate (Sigma, A1542) and again centrifuged for 5 min at 13,000 x g. The pellet was placed in a SpeedVac until dried and resuspended in 20-50 μL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) or DNase-free water. The quality of DNA was checked by A_{260}/A_{280} ratio (≥ 2), and DNA quantified using NanoDrop Nd-1000 UV/Vis 1 μL Spectrophotometer.
Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* cultures using the Wizard® Plus Maxiprep DNA Purification Kit (Promega, Madison, WI, Cat # A7270) following manufacturer's instructions as described here. A 400 mL bacterial culture was centrifuged for 10 min at 5,000 x g. All centrifugations were done at room temperature. The pellet was resuspended in 15 mL cell resuspension solution and mixed with 15 mL of cell lysis solution. The contents were mixed with 15 mL neutralization solution and centrifuged for 15 min at 14,000 x g. The clear supernatant was mixed with 0.5 volume isopropanol and centrifuged at 14,000 x g for 15 min. The pellet was resuspended in 2 mL TE buffer and mixed with 10 mL Wizard® Maxiprep DNA purification resin. The DNA/resin mix was poured into the Maxicolumn and a vacuum was applied to pack the resin. The resin was washed with 25 mL column wash solution and then rinsed with 5 mL 80% ethanol. The resin filled base of Maxicolumn was centrifuged to remove traces of column wash solution at 1300 x g for 5 min and then vacuum dried for 5 min. DNA was eluted by adding 1.5 mL preheated (65 °C) nuclease free water and centrifuging at 1300 x g for 5 min. The eluted DNA was filtered through the supplied syringe and collected by centrifuging at 14,000 x g for 1 min and stored at -20 °C. The concentration of DNA was determined using NanoDrop Nd-1000 UV/Vis 1 μL Spectrophotometer.

Restriction Enzyme Digests

The plasmid pCW122-*PfODC/SAMDC* was digested with different restriction enzymes (New England Biolabs, Beverly, MA). The reaction mixture for each digestion consisted of 1X enzyme buffer (optimized for activity of the restriction enzyme), 1X acetylated bovine serum albumin (if required), 150-200 ng plasmid DNA and 10 units
restriction enzyme per µg DNA. The reactions (10 µL) were incubated for 2-4 h at 37 °C or the temperature specified by the manufacturer and then stored at 4 °C.

**Polymerase Chain Reaction (PCR)**

Successful incorporation of *PfODC/SAMDC* in poplar cell DNA was confirmed by PCR. DNA was amplified using forward 5'-ATT TAC CGA ACG AAC GGT TG-3' and reverse 5'-CCA TTT TCA TCA TCA CTG TTC C -3' primers (SOS1 primers). The PCR mixture (25 µL) was prepared by mixing Quick Load Taq Polymerase Master Mix (New England Biolabs, Ipswich, MA, Cat # M0271L) with 0.2 µM forward primer, 0.2 µM reverse primer, DNA template (400-500 ng genomic DNA or 1 ng plasmid DNA). A negative and a positive control were prepared in parallel. Samples were denatured by heating to 94 °C for 2 min followed by 35 cycles consisting of 30 s at 94 °C (denaturation), 30s at 55 °C (annealing) and 1 min elongation at 72 °C. Final elongation was done at 72 °C for 5 min. Cycling was carried out in a PTC-100 (MJ Research Inc., MA) thermocycler with heated lid enabled. The PCR products were stored at 4 °C.

For confirming the presence of *NPTII* gene, the primers used were forward 5'-GAG GCT ATT CGG CTA TGA CT-3' and reverse 5'-TCG GGA GCG GCG ATA CCG TA-3'. Rest of the conditions were the same as that for *PfODC/SAMDC*.

Following amplification, PCR products were electrophoresed on 1% Seakem LE agarose (Cambrex Bio Science, Rockland, ME, Cat # 50000) in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA) along with TriDye 2-Log DNA ladder (NEB, N3270S). The gel was run in 1X TAE buffer for 45-60 min at 5 V/cm, then stained with ethidium bromide (0.5 µg/mL) for 15 min and destained with water for 5 min. The DNA was visualized on a UV trans-illuminator and digitally photographed using Nucleotech Gel
RNA Extraction

Total RNA was isolated using the RNeasy® Plant Mini Kit (Qiagen, Valencia, CA; Cat # 74904). Cell samples (100 mg FW) ground in liquid nitrogen were mixed with 450 μL RLT buffer (containing freshly added 10 μL β-mercaptoethanol/mL buffer). The samples were vortexed and incubated at 56 °C for 3 min to lyse the cells. The lysate was then transferred to a QIAshredder spin column placed in 2 mL collection tube and centrifuged for 2 min at 13,000 x g. The supernatant was mixed with 0.5 volume of 100% ethanol and transferred to an RNeasy spin column and centrifuged for 15 s at 10,000 x g. The column was washed with 700 μL Buffer RW1 followed by 500 μL Buffer RPE, centrifuging each time at 10,000 x g for 15 s. After another wash with 500 μL Buffer RPE, the column was centrifuged for 2 min to remove any ethanol. RNA was eluted with 30 μL of RNase-free water.

The eluted RNA was purified by treatment with DNase. A 30 μL reaction was set up containing 3 μg RNA, 3 μl RQ-1 DNase Buffer (Promega, M198A), and 3 μL RQ-1 DNase enzyme (Promega, M 610A). Following incubation at 37 °C for 30 min, the reaction volume was brought up to 100 μL with RNase-free water and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to it. The reaction was centrifuged at 4 °C for 5 min and the upper aqueous layer was extracted with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifuging for 5 min at 4 °C, the top aqueous layer was precipitated with an equal volume of isopropanol and incubated at -20 °C for 30 min. The reactions were then centrifuged at 4 °C for 15 min. The
supernatant was discarded and the pellet washed with 80% EtOH by centrifugation at 4 °C for 5 min. The pellet was vacuum dried and resuspended in 10 μL RNase-free water.

cDNA synthesis

cDNA was prepared from DNase-treated RNA using Roche Transcriptor First Strand cDNA synthesis Kit (Roche Applied Science, Cat # 04 379012001). A 13 μL template-primer mixture was prepared by mixing 1-2 μg total RNA, 1 μL of 2.5 μM Anchored-oligo(dT)₁₈ primer and RNase free water. The reaction was heated at 65 °C for 10 min in a thermal block cycler with a heated lid and then immediately cooled on ice. To the tubes was then added 4 μL Transcriptor Reverse Transcriptase Reaction buffer (5x), 0.5 μL of 40 units μL⁻¹ Protector RNase Inhibitor, 2 μL of 10 mM deoxynucleotide mix and 0.5 μL of 20 units μL⁻¹ Transcriptor Reverse Transcriptase. The mixture was incubated at 50 °C for 60 min in a thermal block cycler with a heated lid followed by enzyme inactivation at 85 °C for 5 min. Reaction was cooled on ice and stored at -20 °C.

ODC, ADC and SAMDC enzyme assays

The enzyme activities were measured using a modified protocol of Minocha et al. (1999). For ODC and ADC, 100 mg FW of cells were collected in 250 μL Tris-EDTA buffer at pH 7.5 (0.1 M Tris, 0.1 mM EDTA) with freshly added 0.5 mM pyridoxal phosphate (PLP, Sigma, P3657) and 1.0 mM DTT (Sigma, D9779) in 16×100 mm glass test tubes. For SAMDC, 200 mg FW of cells were mixed with 350 μL of 0.1 M potassium phosphate buffer (pH 7.5) containing freshly added 3 mM putrescine-diHCl (Sigma, D1-320-8) and 1 mM DTT. The cells were then frozen for 3 hrs (-20 °C) and thawed at room temperature. The respective substrates (50 μL) were then added to the
samples [for ODC: 0.1 μCi of [1-14C]Orn, specific activity 56 mCi mmol⁻¹ (Moravek Biochemicals, Brea, CA; Cat # 142-173-953) with 2 mM unlabeled L-Orn (Sigma, O2375); for ADC: 0.1 μCi of [1-14C]Arg, specific activity 56 mCi mmol⁻¹ (Amersham Life Sciences, Elk Grove, IL; Cat # CFA.434) plus 2 mM unlabeled L-Arg (Sigma, A5006); for SAMDC: 0.1 μCi of [1-14C]SAM, specific activity 55 mCi mmol⁻¹ (Moravek, Cat # 127-279-056) plus 0.3 mM unlabeled SAM (Sigma- A2408)]. A 2 cm² Whatman 3 MM filter paper placed in a polypropylene well (Kontes, Vineland, NJ) was immediately soaked with 50 μL Scintigest (Fisher Scientific, Lot # 872729) and placed in the glass tubes with rubber stoppers. The tubes were incubated in shaking water bath (37 °C, 60 rpm) for 60 min (ODC, ADC) or 30 min (SAMDC). The reaction was stopped by injecting 0.5 mL 0.5 N sulfuric acid through the stopper. The tubes were incubated again in the shaking water bath for 30 min, after which the filter papers were removed and counted for radioactivity in 10 mL Scintilene (Fisher Scientific, Lot # 980805) in a LSC-6000 liquid scintillation counter (Beckman, Fullerton, CA).

Enzyme activity was calculated as nmol CO₂.h⁻¹.g⁻¹ FW of cells as well as nmol CO₂.h⁻¹.mg⁻¹ soluble protein as shown below:

\[
\text{DPM of sample- DPM blank} = \text{DPM recovered}
\]

\[
\text{DPM added} = 0.1 \mu\text{Ci} \sim 2.2 \times 10^5 \text{dpm (actually counted)}
\]

\[
(DPM \text{ recovered}/ DPM \text{ added}) \times (\text{final substrate concentration in reaction/ time in h}) = \text{nmol CO}_2 \text{ h}^{-1}
\]

\[
\text{nmol CO}_2 \text{ h}^{-1}/ \text{amount of tissue in g} = \text{nmol CO}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ FW}
\]

\[
\text{nmol CO}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ FW/ mg protein g}^{-1} \text{ FW} = \text{nmol CO}_2 \text{ h}^{-1} . \text{mg}^{-1} \text{ soluble protein}
\]
Statistical analysis

For all experiments, unless stated otherwise, three replicates were used per treatment and per time period. Data were combined from two or more separate experiments and subjected to analysis of variance (ANOVA) using SYSTAT, version 10.2. Significance at $P \leq 0.05$ was determined using Tukeys test.
RESULTS

Effects of altering calcium concentration in the medium

Fresh weight yield

Growth of cells (HP and control) as determined by fresh weight of cells per flask was not affected by lower Ca concentrations in the medium by d 3 (Fig. 6). Lowering Ca to 0.05x and 0.2x of the normal concentration caused a significant reduction in fresh weight of HP cells by d 5. In control cells, a reduction in growth was seen only at 0.05x Ca.

Figure 6. Yield of fresh mass per flask of control and HP cells grown in different concentrations of Ca from time zero. Each bar represents Mean + SE of four replicates from two experiments. N= control treatment with MS medium made from premix MS powder. An * indicates a significant difference (P<0.05) in the fresh mass between the cells growing in normal Ca and those growing in reduced Ca within the same cell line and time period.
Polyamines

The Put content, as expected, was much higher in HP cells than control cells on both d 3 and d 5 (Fig. 7A). The effect of varying Ca concentration was more visible in HP cells than the control cells, and only on d 5. Control cells did not show any significant change in any of the PAs in response to variation in Ca content of the medium except on d 5 where Put was lower in 0.05x Ca (Fig. 7A). Growth in 0.05x and 0.2x Ca caused a significant reduction in Put in HP cells on d 5 as compared to normal Ca. The differences were due to a significant decrease in Put content from d 3 to d 5 in 0.05x and 0.2x Ca treatments. The 0.0x Ca treated cells did not survive after day 1, thus no data on PA contents are shown. There was no significant difference in the amount of Spd (Fig. 7B) in either cell line with different concentrations of Ca in the medium. As reported earlier (Bhatnagar et al. 2001), Spd content in the normal Ca concentration in the medium was somewhat higher in the HP cells than the control cells; this difference was seen for each concentration of Ca. Cellular Spm did not show many significant changes in its content either (Fig. 7C). At 0.05x Ca concentration, HP cells on d 5 showed higher Spm content than that of normal Ca. There was no significant difference in PA content of 1x and normal Ca treatments in either cell line on any day.
Figure 7. Cellular polyamine content (A- Put, B- Spd, C- Spm) in 3 d and 5 d old control and HP cells grown in MS medium with different concentrations of Ca. Each bar represents Mean + SE of four replicates from two experiments. N= control treatment with MS medium made from premix MS powder. Data for concentrations 0.0x and 2x are from a separate set of experiments. An * indicates a significant difference (P<0.05) in the polyamine content between the cells growing in normal Ca and those growing in reduced Ca within the same cell line and time period.
Mitochondrial activity and membrane permeability

As seen for PA content, the mitochondrial activity was affected more in HP cells than control cells with respect to lowering of Ca in the medium (Fig. 8). On d 3, HP cells grown in 0.05x and 0.2x Ca showed significantly lower mitochondrial activity than those grown in normal concentration of Ca (treatment N); the effect was even greater by d 5. In the control cells there was no significant difference in mitochondrial activity on d 3; only 0.05x treatment showed a significant decrease when compared to the corresponding value of d 3 and also with respect to 0.2x and 1x treatments of d 5.

The membrane damage as indicated by retention of Evans blue dye was again more apparent in HP cells than control cells when Ca content of the growth medium was lowered (Fig. 9). At 0.05x and 0.2x concentrations of Ca, the control cells showed significantly higher retention of Evans blue compared to normal Ca, indicating loss of membrane integrity at lower Ca concentrations. The HP cells retained more Evans blue than the control cells in normal Ca conditions on d 3 but on d 5 the two cell types had similar amounts of dye retention g⁻¹ FW. The adverse effects of low Ca were visible in both cell types on d 5. By d 5, growth in normal Ca showed significant improvement in membrane integrity in both cell lines.
Figure 8. Absorbance per gram fresh weight of MTT reaction product (Formazan) for 3 d and 5 d old control and HP cells grown in MS medium with different concentrations of Ca. Each bar represents Mean ± SE of four replicates from two experiments. N= control treatment with MS medium made from premix MS powder. An * indicates a significant difference (P<0.05) in the absorbance g⁻¹ FW between the cells growing in normal Ca and those growing in reduced Ca within the same cell line and time period.

Figure 9. Absorbance per gram fresh weight for Evans blue retention in 3 d and 5 d old control and HP cells grown in MS medium with different concentrations of Ca. Each bar represents Mean ± SE of four replicates from two experiments. N= control treatment with MS medium made from premix powder. An * indicates a significant difference (P<0.05) in the absorbance g⁻¹ FW between the cells growing in normal Ca and those growing in reduced Ca within the same cell line and time period.
Effect of PA analogues on PA content and mitochondrial activity

When the cells were treated with two PA analogues (N-acetyl-Spd and N-acetyl-Orn) on d 3 of growth, there was no significant effect on the PA content of either cell line within 72 h of treatment (Fig. 10). Likewise, neither acetyl-Put nor acetyl-Spm had a significant effect on cellular PAs during the next 3 d (Fig. 11). The mitochondrial activity of either control or HP cells was not affected by these analogues (Fig. 12).
Figure 10. Cellular polyamine content (A- Put, B- Spd, C- Spm) in control and HP cells treated with 0.2 mM N-acetyl-Spd and N-acetyl-Orn. Each bar represents Mean ± SE of three replicates. Three-day old cells were treated with the inhibitor and samples collected at 24h, 48h and 72h thereafter. N = no treatment. An * indicates a significant difference (P<0.05) in the polyamine content between the cells treated with PA analogues and untreated cells within the same cell line and time period.
Figure 11. Cellular polyamine content (A- Put, B- Spd, C- Spm) in control and HP cells treated with 0.2mM N-acetyl-Put and 0.1 mM N-acetyl-Spm. Each bar represents Mean ± SE of three replicates. Cells were collected at 24h, 48h and 72h. N = No treatment. An * indicates a significant difference (P<0.05) in the polyamine content between the cells treated with PA analogues and untreated cells within the same cell line and time period.
Figure 12. Absorbance per gram fresh weight of MTT reaction product for control and HP cells treated with (A) 0.2mM N-acetyl-Spd and N-acetyl-Orn and (B) 0.2mM N-acetyl-Put and 0.1 mM N-acetyl-Spm. Each bar represents Mean ± SE of three replicates. Cells were collected at 24h, 48h and 72h. N= No treatment. An * indicates a significant difference (P<0.05) in the absorbance g⁻¹ FW between the cells treated with PA analogues and untreated cells within the same cell line and time period.
Effect of inhibitors on PA content and mitochondrial activity

Two inhibitors of PA biosynthesis, APCHA (N-(3-aminopropyl) cyclohexylamine, inhibits Spm synthase) and MCHA (4-methylcyclohexylamine, inhibits Spd synthase), were added to 3 d old HP and control cells and their effects on PAs and mitochondrial activity were analyzed at 24, 48 and 72 h post treatment. Although, Put in HP cells increased somewhat from 24 h to 72 h in all treatments, the increase in APCHA or MCHA treated cells was less than that of untreated cells (Fig. 13A). Spermidine content, in general, did not differ much between HP and control cells on any given day of analysis (Fig. 13B). Cells treated with APCHA showed greater Spd accumulation than untreated cells of both lines at 48 and 72 h after treatment. While Spd content decreased with time of culture in untreated and MCHA treated cells; however, APCHA treated cells did not show much decrease in Spd with time. Spermine content did not show any difference in response to any treatment at any given time in either cell line (Fig. 13C).

Neither APCHA nor MCHA had a significant effect on mitochondrial activity at any given time, in either of the cell lines (Fig. 14).
Figure 13. Cellular polyamine content (A- Put, B- Spd, C- Spm) in control and HP cells treated with 0.1mM APCHA and MCHA. Each bar represents Mean ± SE of three replicates. Cells were collected at 24h, 48h and 72h. N = Control treatment. An * indicates a significant difference (P<0.05) in the polyamine content between the cells treated with inhibitors and untreated cells within the same cell line and time period.
Figure 14. Absorbance per gram fresh weight of MTT reaction product for control and HP cells treated with 0.1 mM APCHA and MCHA. Each bar represents Mean ± SE of three replicates. Cells were collected at 24h, 48h and 72h. N= Control treatment.
Effect of adding sulfur amino acids or inorganic sulfur

It has been shown earlier (Mohapatra et al. 2009b) that cellular contents of the two sulfur-containing amino acids (Cys and Met) were significantly lower in the HP cells vs. the control cells on most days of culture. These two amino acids are also among the least abundant of the amino acids in the cells. This was coincident with the increase in soluble protein content in the HP cells during the first half of the week and decrease during the latter. Thus it was postulated that soluble protein content may be related to some critical amino acid, particularly Cys or Met, since both are found in extremely low concentrations in these cells. Thus the effect of Cys and Met as well as inorganic sulfur (sulfate) was tested in HP and control cells at various times. The exogenous addition of either Cys or Met at two different concentrations on day 3 of culture did not cause any change in the cellular PA content over the next 72 h (Fig. 15, 16). Likewise, supplementation of the medium with Cys or Met did not change the mitochondrial activity of control or the HP cells (Fig. 17).
Figure 15. Cellular polyamine content (A- Put, B- Spd, C- Spm) in control and HP cells treated with different concentrations of Cys. Each bar represents Mean ± SE of six replicates from two experiments. Cells were collected at 24h, 48h and 72h.
Figure 16. Cellular polyamine content (A- Put, B- Spd, C- Spm) in control and HP cells treated with different concentrations of Met. Each bar represents Mean ± SE of six replicates from two experiments. Cells were collected at 24h, 48h and 72h.
Figure 17. Absorbance per gram fresh weight of MTT reaction product for control and HP cells treated with different concentrations of (A) Cys and (B) Met. Each bar represents Mean ± SE of six replicates from two experiments. Cells were collected at 24h, 48h and 72h.
The amount of inorganic sulfur in the medium was doubled to test its effects on PAs, cellular protein content and the mitochondrial activity; extra sulfate was added at time zero or on d 3. Cell collection was done every 24 h post treatment till the end of 7 d growth cycle. No effect of adding extra sulfur on cellular content of any PA was seen in either cell line at any given time (Fig. 18).

Total soluble protein content in HP cells increased from d 1 through d 3 and then declined (Fig. 19) as has been reported earlier (Page et al. 2007, Mohapatra et al. 2009a). Protein content on d 2, 3 and 4 was significantly higher in HP cells than the control cells but no effect of added sulfur was seen in any case at any time. In HP cells where additional sulfate was added on d 3 of growth (HP-3dS), protein was highest on d 4 after which it declined gradually resulting in significantly low value by d 7. Protein content in the two cell lines was similar on most days except d 2, 3 and 4 where HP cells had significantly high protein than control cells. The mitochondrial activity did not show any significant response to sulfur supplementation either at time zero or at 3 d in either cell line (Fig. 20).
Figure 18. Cellular polyamine content (A- Put, B- Spd, C- Spm) in control and HP cells grown in medium with 2x sulfur. Each bar represents Mean ± SE of six replicates from two experiments. Cells were collected after every 24h through out the 7 days growth period. N = control treatment with regular amount of sulfur.
Figure 19. Milligram protein per gram fresh weight in control and HP cells grown in medium with 2x sulfur. Each bar represents Mean ± SE of six replicates from two experiments. Cells were collected after every 24h throughout the 7 days growth period. N = Control treatment.

Figure 20. Absorbance per gram fresh weight of MTT reaction product for control and HP cells grown in medium with 2x sulfur. Each bar represents Mean ± SE of six replicates from two experiments. Cells were collected after every 24h throughout the 7 days growth period. N = Control treatment.
Assimilation of $^{14}$C sucrose into polyamines

Mohapatra et al. (2009b) has reported that the total N as well as total C in the HP cells was higher than the control cells on most days of culture. In order to test if this increase in total C was due to increased C uptake, both cell lines were treated with $^{14}$C sucrose and its accumulation in various fractions in the cells was followed for up to 3 d. The three fractions that were analyzed included PCA soluble fraction, the aqueous fraction following dansylation of PCA extracts and the toluene fraction following dansylation; the latter would contain mostly the dansyl-PAs.

Two different types of experiments were conducted: In the first set of experiments, 1 μCi U$^{14}$C-sucrose was added to 3 d old cell suspensions and samples were collected at 4, 8, 24, 48 and 72 h after sucrose feeding. In the second set of experiments (called pulse experiments), 3 d old cells were incubated with U$^{14}$C sucrose for 4 h, and after thorough washing with sucrose-free MS medium, were resuspended in the original volume of fresh medium with normal amounts of sucrose. Cells were again collected at 0, 4, 24, 48 and 72 h. In both sets of experiments, the amount of radioactivity incorporated into the three fractions (PCA extract, and aqueous and toluene fractions) was measured.

The first set of experiments, where $^{14}$C-sucrose was not expected to be limiting, radioactivity in all three fractions showed a gradual increase in both cell lines until 48 h after which there was a small decline by 72 h (Fig. 21). $^{14}$C accumulation was significantly lower in HP cells at all times up to 48; at 72 h, HP cells had more $^{14}$C than control cells (Fig. 21A). The aqueous fraction, which represents radioactivity in unused sucrose, total free amino acids, and other metabolites, also followed the same pattern of $^{14}$C accumulation as the total $^{14}$C in the PCA extract from 4 h to 48 h (Fig. 21B).
actual amount of radioactivity in this fraction was almost equal to that in the PCA extract, thus the $^{14}$C in HP cells was lower than that in the control cells at all times except at 72 h. In the toluene fraction (representing PAs), which also showed a gradual increase with time in both cell lines, there was a higher amount of $^{14}$C in the HP than the control cells at 48 and 72 h (Fig. 21C). Total radioactivity going into the toluene fraction was very low; never exceeding 2% of the total $^{14}$C at a given time.

In the pulse experiments, there was a significant decrease in the total radioactivity retained by the cells from zero time to 48 h in all three fractions (Fig. 22) in both cell lines; at 72 h, however, $^{14}$C amounts were higher than 48 h, particularly in the control cells. The amount of $^{14}$C in the PCA extract and the aqueous fractions were rather similar, with less than 2% of the total radioactivity being present in the toluene fraction at any given time. In contrast to the previous experiment, the amount of total $^{14}$C incorporated into the PAs in the HP cells was slightly lower or equal to that in the control cells at any given time (Fig. 22C). Although, the total radioactivity taken up by the cells was higher in control cells, its percentage incorporation into total free PAs, was higher in HP cells.
Figure 21. The amount of radioactivity incorporated into (A) PCA extracts, (B) Aqueous fractions and (C) Toluene fractions of control and HP cell lines at different time periods on incubation with $^{14}$C sucrose. Cells were grown in the culture medium supplemented with $^{14}$C-sucrose after 3 days of growth in regular MS medium. Each bar represents mean (±) SE of three replicates. An * indicates a significant difference (P<0.05) in $^{14}$C assimilation between the control and HP cells at any one time of analysis.
Figure 22. The amount of radioactivity incorporated into (A) PCA extracts, (B) Aqueous fractions and (C) Toluene fractions of control and HP cell lines at different time periods on incubation with $^{14}C$ sucrose. Cells were incubated with $^{14}C$ sucrose for 4 h after 3 days of growth and were then transferred into regular MS medium. Each bar represents mean (±) SE of three replicates. An * indicates a significant difference (P<0.05) in $^{14}C$ assimilation between the control and HP cells at any one time of analysis.
Characterization of pCW122-PfODC/SAMDC

The plasmids pCW122 and pCW122-PfODC/SAMDC were digested separately with EcoRI, SmaI and EcoRV and subjected to electrophoresis on 0.9 % agarose gel (Fig. 23). In both plasmids the expected band sizes (Table 2) were obtained indicating the correctness of insertion. The final plasmid is shown in Fig. 5.

![Plasmid restriction enzyme digested samples separated on 0.9 % agarose gel. pCW122 (lanes 1, 3, 5), pCW122-PfODC-SAMDC (lanes 2, 4, 6). EcoRI (lanes 1, 2), SmaI (lanes 3, 4), EcoRV (lanes 5, 6), NEB 2-Log DNA ladder was used as the marker.](image-url)

**Figure 23.** Plasmid restriction enzyme digested samples separated on 0.9 % agarose gel. pCW122 (lanes 1, 3, 5), pCW122-PfODC-SAMDC (lanes 2, 4, 6). EcoRI (lanes 1, 2), SmaI (lanes 3, 4), EcoRV (lanes 5, 6), NEB 2-Log DNA ladder was used as the marker.
Table 2. The predicted fragment sizes for the plasmid DNA restriction enzyme digest.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Expected fragment size (bp) pCW122</th>
<th>Expected fragment size (bp) pCW122-PfODC/SAMDC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EcoRI</em></td>
<td>2725, 5367</td>
<td>1187, 1718, 2179, 5362</td>
</tr>
<tr>
<td><em>SmaI</em></td>
<td>784, 1926, 5382</td>
<td>5068, 5378</td>
</tr>
<tr>
<td><em>EcoRV</em></td>
<td>200, 1840, 6052</td>
<td>1018, 1889, 7539</td>
</tr>
</tbody>
</table>

Characterization of transgenic cell line “SOS1”

Poplar cells were transformed with pCW122-*PfODC/SAMDC* containing the *PfODC/SAMDC* gene using biolistic bombardment and putative transformed cell clumps were selected on 100 mg/L kanamycin. Only one cell clump was able to grow on the selection medium. To check for the presence of transgene in this cell line, genomic DNA was isolated and used for PCR analysis. The primers used were expected to produce a 740 bp fragment representing a part of the hinge region between the *SAMDC* and *ODC* domains of the gene. The transformed line (named SOS1) showed the presence of the expected fragment (Fig. 24), while the non-transgenic line (PBNT) lacked it (data not shown).
Figure 24. PCR amplification product of 0.74 Kb from hinge region of *PfODC/SAMDC* using genomic DNA from SOS1 cells. SOS1 genomic DNA (lanes 2,3), negative control (water, lane 4), positive control (plasmid, lane 5); Lane 1 = NEB 2-Log DNA ladder.
To check if the inserted DNA was being expressed, cDNA was prepared from DNase-treated RNA and used as a template for PCR amplification using the same primers as for genomic DNA PCR. As shown in Fig. 25, the presence of expected 740 bp fragment was confirmed. cDNA amplified using $P5CR$ primers (designed for poplar cDNA, Mohapatra 2008) was the positive control for cDNA template, since $P5CR$ ($\Delta^1$-pyrroline-5-carboxylate reductase) is constitutively expressed in poplar cells. This generated the expected 700 bp fragment.

Figure 25. PCR amplification product of 0.7 Kb from hinge region of $PfODC/SAMDC$ using cDNA from SOS1 cells. SOS1 cDNA (lanes 2,3), positive control (plasmid, lane 4), negative control (water, lane 5). Lanes 2-5 show samples amplified using SOS1 primers, lane 6 shows SOS1 cDNA amplified using $P5CR$ primers (positive control for cDNA template), Lane 1 = NEB 2-Log DNA ladder used as marker.
As mentioned above, the absence of introns in *PfODC/SAMDC* causes the amplification product of SOS1 genomic DNA and cDNA to be of the same size. Thus to overrule genomic DNA contamination in cDNA, leading to false interpretation of gene expression, another primer set was used for amplification of both genomic DNA and cDNA. The primers used, produce a 700 bp amplification product of *PDH1* (proline dehydrogenase) with poplar cDNA and 1.1 Kb with genomic DNA (Fig. 26).

**Figure 26.** PCR amplification of 0.7 Kb *PfODC/SAMDC* (lane 2), 1.1 Kb *PDH1* gene fragments (lane 3) from genomic DNA and 0.7 Kb *PfODC/SAMDC* (lane 4) and *PDH1* (lane 5) gene fragments from cDNA. Lane 6 = negative control (water) and lane 7 = positive control (plasmid), lane 1 = NEB 2-Log DNA ladder.
Biochemical characterization of SOS1 cells

After confirming the newly created transgenic cell line for stable transformation, it was characterized for its biochemical properties. A 7 d profile for PA content, total soluble proteins and enzyme activity of ODC, ADC and SAMDC in SOS1 cell line was created. The non-transgenic cell line, PBNT, was used as control in all these experiments. The cells when visualized under a light microscope at 200X magnification did not seem much different from the progenitor line except being somewhat shorter in length (Fig. 27).

**Figure 27.** Poplar cells: SOS1 (*PfODC/SAMDC* transgenic) and PBNT (non-transgenic) under the light microscope (200X) Magnification
Cellular polyamine content of SOS1 cells

The SOS1 cells contained significantly higher amounts of Spd and Spm on all 7 days of culture, while Put content was similar in the two cell lines on most days (Fig. 28). Putrescine content in both cell lines decreased on transfer to fresh medium during the first two days in SOS1 cells and only the first day in control cells; there was a gradual increase in Put thereafter. The control cells also showed an increase, but only until d 5. The two lines showed significant difference in Put content only on the first and last day of growth (Fig. 28A). Both Spd and Spm contents increased during the first 2 to 4 d in both cell lines; the amounts of both these PAs were significantly higher in SOS1 cells than control cells on all seven days (Fig. 28B, C).
Figure 28. Cellular polyamine content (A- Put, B- Spd, C- Spm) in transgenic SOS1 and non-transgenic PBNT cell lines on all 7 days of growth. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates a significant difference (P≤0.05) in the cellular PA content between PBNT and SOS1 cells on a given day of the 7 d culture cycle.
Soluble protein content

The protein content (g\textsuperscript{-1} FW) in the two cell lines followed similar trend of increase up to d 3 and a decline thereafter until d 7 (Fig. 29). A significant difference in soluble protein content of the two cell lines was visible only on d 1 and d 6. However, SOS1 cells had higher protein than controls on all days of culture.

**Figure 29.** Milligram protein per gram fresh weight in SOS1 and PBNT cells. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates a significant difference (P<0.05) in the protein content between PBNT and SOS1 cells on a given day of the 7 d culture cycle.
Activities of ADC, ODC and SAMDC

The enzyme activities of ADC, ODC and SAMDC were compared between the two cell lines over the 7 d culture period both in terms of nmol CO_2.gFW^{-1} and as specific activity (nmol CO_2.mg protein^{-1}). The activity of ADC was higher in SOS1 cells on most days although significant differences were seen only earlier in the week (Fig. 30A). The peak of ADC activity was seen on d 2 in control and d 3 in SOS1 cells. The specific activity of ADC on the other hand showed smaller differences due to the concomitant increases seen in protein content of the two cell lines (Fig. 30B). A sharp increase in ADC specific activity was seen on d 1 after transfer of cells to fresh medium; this also being the result of lowered soluble protein in both cases.

The activity of ODC, measured either way, was much less than that of ADC, and was somewhat higher in SOS1 cells than control cells on most days but difference was significant only on a few days (Fig. 30C, D). The activity of ODC fluctuated widely during the week.

The most significant difference between the two cell lines was observed for the SAMDC activity (Fig. 30E). It was significantly higher in SOS1 than control cells on most days of the week. There was several-fold increase in SAMDC activity on transfer to fresh medium in both cell lines; the increase being several-folds higher in the SOS1 cells. After d 1, there was a steady decline in SAMDC activity until d 7. When compensated for differences in soluble protein content, the differences between the two cell lines became smaller on most days (Fig. 30F).
Figure 30. Activity (g\textsuperscript{-1} FW) of ADC (A), ODC (C), SAMDC (E) and specific activity (mg\textsuperscript{-1} protein) of ADC (B), ODC (D), SAMDC (F) in PBNT and SOS1 cells over the 7-d culture cycle. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates a significant difference (P<0.05) in the enzyme activity between PBNT and SOS1 cells on a given day of the 7 d culture cycle.
DISCUSSION

The present study was aimed at furthering our understanding of the regulation of polyamine biosynthetic pathway using the approach of transgenic manipulation. The study was done on one previously described (Bhatnagar et al. 2001, 2002) transgenic poplar cell line, called HP that over-expresses a mouse ODC gene and a newly created cell line called SOS1 that expresses a bifunctional ODC/SAMDC gene from the malarial parasite *Plasmodium falciparum*. While the transgene in HP cells largely affected Put biosynthesis, the new cell line SOS1 was created in order to affect the biosynthesis of higher PAs, Spd and Spm. Studies with HP cells involved biochemical characterization of these cells with respect to various treatments while the work on SOS1 cells included its production and its preliminary biochemical and molecular characterization.

**Effects of altering calcium concentration in the medium**

Calcium is an essential macronutrient playing important roles in physiological processes such as maintaining structural integrity and ionic balance of cells, cell signaling, and metal stress (reviewed in Hepler 2005). The involvement of Ca in ameliorating Al-related stress has been well documented in natural forest systems (Minocha et al. 1997, Wargo et al. 2002) as well as in crop plants (Wang and Kao 2004, Hossain et al. 2005). It was also observed that the Put overproducing cell line, HP, naturally accumulated higher amount of Ca which was further enhanced in response to Al addition (Mohapatra 2008). Further, it has been shown that under oxidative stress conditions, there is increased Ca influx and K efflux (Pei et al. 2000, Demidchik et al. 2003, Foreman et al. 2003). Pei et al. (2000) demonstrated the increase in cytosolic Ca in response to H₂O₂ through activation of Ca²⁺-
permeable channels in the plasma membrane of *Arabidopsis* guard cells. Similarly, Foreman et al. (2003) demonstrated that ROS (OH\(^{-}\)) elevated cytoplasmic free Ca ions by activating plasma membrane Ca influx pathway required for root-hair cell elongation in *Arabidopsis*. Mohapatra et al. (2009a) concluded that HP cells, perhaps due to their higher catabolism of PAs, were under a greater oxidative stress as compared to control cells, and that is why high Ca content was accompanied by low K accumulation in these cells.

In my study, modulation of Ca concentration in the growth medium had a significant effect on growth. It can be argued that since HP cells accumulate more Ca than the control cells, the Ca in the medium will be used up faster by HP cells and hence the Ca deficiency will be more detrimental to them with increase in time; the data presented here support this argument. Control cells on the other hand, seem to be less affected by reduction in Ca in the medium. HP cells, as expected, accumulated higher amounts of Put on both days of observation (d 3 and d 5) as compared to control cells, and the effects of lowering Ca in the medium were clearly seen (Fig. 7A). In control cells, from d 3 to d 5, a reduction in Put content was only seen when the Ca was reduced 20 fold (0.05x) that of normal concentration; the decrease in Put accumulation was rather small. A 5-fold reduction (0.2x) in Ca did not have any negative effect on Put content. The relatively small changes in Put may be due to a reduction of Put catabolism. According to Bouchereau et al. (1999), nutrient deficiency can be associated with increase in cellular Put, although it is difficult to say that this is what is happening here as this increase in Put was not seen in all treatments or at all times. In HP cells, the effect of low Ca was more prominent by d 5 where cells from both 0.05x and 0.2x treatments had significantly low
Put than the cells grown in normal Ca and also lower than the corresponding treatments from d 3. This decrease could be due to reduced Put biosynthesis, increased Put catabolism towards production of GABA, increased conversion of Put into Spd, conjugation of Put or excretion of Put from the cells. An exchange of Ca (in) with Put (out) is also a possibility, which has not been experimentally tested.

Accumulation of PAs as well as decrease in PA content have been reported under various stress conditions indicating a complex regulation of cellular PA content which not only depends on the type of stress but also on the duration of stress (Liu et al. 2008 and references therein). The type of plant species/tissue and their developmental stage are also important at the same time. Whereas an increase in Put was observed in *Picea rubens* cell cultures and foliage of forest tree (Minocha et al. 1996, 1997), a decrease was observed in *Catharanthus roseus* cell cultures (Minocha et al. 1992). A decrease in PA content (Put + Spd) was observed by Aziz et al. (1999) under high salt (NaCl) conditions in tomato leaf discs. A decrease was also seen in response to high Ca and K in the medium with or without addition of salt. Similarly, with long-term salt stress in *Fraxinus angustifolia* callus, a decrease in PA accumulation was observed, while short-term treatment led to an increase in free PAs (Tonon et al. 2004). Legocka and Kluk (2005) demonstrated an organ specific change in PAs with salt and osmotic stress. A short-term (4 h) treatment caused an increase in PAs in leaves and roots of drought tolerant *Lupinus luteus*, whereas, a longer-term treatment (24 h) led to a decrease in PAs in roots and an increase in leaves. An interesting finding in this study was that ADC activity was higher in roots and similar to control treatments in leaves, contrary to Put contents in these two organs suggesting a translocation of PAs from roots to leaves. Liu et al. (2008) reported a
decrease in free Put in apple shoots treated with salt for 10 days. Despite these findings, it is difficult to make a generalization for regulation of PA content during stress related to inorganic ions.

Bouchereau et al. (1999) suggested an increased conversion of Put into Spd/Spm to impart resistance/tolerance against stress as high Put itself has been mentioned to be harmful for plants and Spd/Spm have been known to confer stress tolerance in many cases (Shen et al. 2000, Pillai and Akiyama 2004, Sanchez et al. 2005, Yamaguchi et al. 2006). Plants that accumulate Put without production of higher PAs are suggested to be more sensitive to stress. In the present study an increase in Spd content was observed in HP cells with low Ca on d 5, although it was significant only in 0.05x Ca concentration, suggesting a slight shift of PA pathway towards synthesis of higher PAs in response to nutrient stress. But this increase in Spd is not able to account for all the decrease in Put seen in low Ca HP cells. There is thus a possibility that PA biosynthetic pathway in HP cells is more sensitive to Ca deficiency and shifts towards oxidative degradation. It has been demonstrated that the rate of Put catabolism is higher in HP cells than control cells and is proportional to the rate of its biosynthesis (Bhatnagar et al. 2002). Furthermore, this increase in catabolism is not accompanied by increase in DAO activity. Although decrease in PA content due to reduced ADC activity or possible increase in DAO/PAO activity has been reported in the presence of high Ca and K (Nam et al. 1997, Aziz et al. 1999); whether the rate of catabolism is affected by low Ca, remains to be determined.

It has been shown earlier that HP cells perhaps due to high oxidative state experience increased membrane damage and reduced mitochondrial activity (Mohapatra et al. 2009a). Both cell lines experienced greater membrane damage during their first 2-3 days
of growth where there is culture shock due to transfer to fresh medium and also increased metabolism. They recovered during the later days of growth. The activities of ROS scavenging enzymes, such as glutathione reductase (GR) and monodehydroxyascorbate reductase (MDHAR), which are known biochemical indicators of high oxidative stress, were found to be higher in HP cells than the control cells during the first 3-4 days of transfer to fresh medium. The increased generation of ROS leading to lipid peroxidation in the membranes is likely to be among the factors responsible for membrane damage (Stark 2005, Cuin and Shabala 2007 and references therein). At the same time, Ca has also been known to control membrane permeability and hence low Ca is associated with leakage of ions and metabolites (Hepler 2005). Calcium also imparts selectivity to ion transport process. Thus, one of the reasons for low Put in HP cells with low Ca on d 5 could be the leakage of Put through the membranes, since these cells have reduced ability to retain the solutes. A recovery in membrane damage was seen in HP cells with normal Ca by d 5 and hence the chances of Put leakage should be low.

At a first glance, the results of the present study are somewhat in contrast to the studies with forest trees whose foliage repeatedly showed an inverse relationship with the availability of Ca in the soil (Minocha et al. 1996, 1997, 2000). In several species of hardwoods and conifers, it has been observed that reduced availability of Ca in the soil (due to acid precipitation and/or Al solubilization) leads to enhanced Put levels in the foliage, leading to the suggestion that cellular Put in combination with some amino acids could be used as a valuable biochemical marker of abiotic stress in forest trees (Minocha et al. 1996, 1997, 2000, Wargo et al. 2002). The apparent discrepancy may lie in the fact that poplar cells in culture are undergoing high rate of cell division vs. the foliage of
mature trees; and their (poplar cells') ability to take up Ca from the surrounding environment may be high. Alternatively, lower cellular Ca may induce secretion of Put through damage to the cellular membranes; both these possibilities are experimentally testable but have not been studied. Another possible explanation could be the known increased uptake of Ca in response to constitutive expression of ODC gene and related increase in oxidative stress in poplar cells (Mohapatra et al. 2009a).

Thus, it can be concluded that HP cells producing high Put are more susceptible to low Ca availability than control cells as supported by the data on mitochondrial activity and membrane damage, along with increased oxidative stress in these cells. Even the control cells did not respond to lower Ca by increasing Put production, showing that even lowered concentrations of Ca in the medium may be sufficient to meet their metabolic demand. Although several possible explanations could be suggested, the exact reason for the observed decrease in Put in HP cells with low Ca treatment remains unknown. Since no change in cellular PAs was seen in response to elevated Ca in the medium in either case, it is apparent that Ca levels in the normal growth medium are not limiting.

**Effect of exogenous PA analogues**

Although the back conversion of Spm into Spd, and that of Spd into Put, has been widely reported in animals (Wu et al. 2003, Schrader and Fahimi 2004, Pledgie et al. 2005), and similar reports have been published with respect to plants (Torrigiani et al. 1993, Del Duca et al. 1995, de Agazio et al. 1996, Tassoni et al. 2000, Tavladoraki et al. 2006, Moschou et al. 2008); the mechanisms of this back-conversion are different in the two kingdoms. In animals, back conversion is largely mediated through SSAT (Spd/Spm acetyl transferase) that produces acetyl derivatives of the parent PA which serve as
substrates for oxidation by PAOs (except Spm oxidase, which directly converts Spm to Spd). In plants this is presumed to be via direct oxidation by PAOs without intermediary acetyl derivatives (Casero and Pegg 1993, Tavladoraki et al. 2006, Moschou et al. 2008). Although the role of PAO in terminal catabolism of PAs was established, its involvement in back conversion of PAs was only hypothesized (Del Duca et al. 1995, Tassoni et al. 2000). The first report in this regard was published by Tavladoraki et al. (2006), who demonstrated the conversion of Spm to Spd and norspermine to norspermidine by recombinant AtPAO1. Later, Moschou et al. (2008) identified another PAO, AtPAO3 that was able to oxidize Spm into Spd and Spd into Put. The acetyl derivatives of Spm or Spd were poor substrates for both AtPAO1 and AtPAO3, suggesting SSAT independent pathway in plants.

The presence of SSAT pathway in animals is also believed to regulate the cellular PA content via secretion of acetylated forms of higher PAs or as Put (which can be degraded or secreted), depending on the availability of PAOs (Casero and Pegg 1993). Although, plants generally do not possess the SSAT pathway, the presence of acetyl-PAs has been reported in some species (Del Duca et al. 1995, Tassoni et al. 2000). Although maize root seedlings treated with acetyl-Spm showed the presence of acetyl-Spd (de Agazio et al. 1996), it was suggested that acetyl-Spd was not involved in the back conversion of PAs. This was because feeding $^{14}$C-Spd did not label acetyl-Spd while radioactivity was detected in Put. It was also concluded that acetyl-Spd degradation by PAO does occur in higher plants (also see, Federico et al. 1996). While $N^7$-acetyl-Spm was oxidized readily by maize PAO, $N^7$- and $N^8$-acetyl-Spd degraded slowly and acetyl-Put and acetyl-Cad were not oxidized.
Back conversion of PAs has also been reported from our lab in the poplar cells used here (Bhatnagar 2002). Poplar cells incubated with \([^{14}C]\text{Spd}\) and \([^{14}C]\text{Spm}\) showed presence of radioactivity in lower PAs; no acetyl derivatives of Spd and Spm were detected in poplar cells. I tested the effects of exogenously-supplied PA analogues (N-acetyl-Put, N-acetyl-Spd and N-acetyl-Spm) on cellular PAs and found no effect of these analogues on either cellular PA content or their mitochondrial activity. This behavior might be due to non-accumulation of these analogues in the cells or their physiological inefficacy to produce changes in the PA pool, if they did accumulate. Accumulation of these analogues depends on their uptake by the PA carriers in the cells. Different analogues have different affinities for uptake by these carriers as demonstrated by Karahalios et al. (1998) in \textit{E. coli} where N\(^4\),N\(^9\)-diacetyl-SPM and N\(^1\),N\(^4\)-bis(β-alanyl)diaminobutane did not enter the cells, but N\(^1\)-acetyl-Spm and, to a lesser degree, N\(^1\),N\(^12\)-diacetyl-Spm, did accumulate. In either case there was no effect on cellular PA content or on growth of cells. No direct evidence for the existence of PAO activity oxidizing acetyl-PAs in the poplar cells is presently known. Also, no difference in physiological behavior of the two cell lines towards these analogues was observed.

**Effect of inhibitors**

Like the PA analogues, reports on a large number of other PA biosynthetic inhibitors are available largely from animals as compared to plants; an important reason being the extensive research carried out in animals targeting PA pathway for cancer chemotherapy (Seiler 2003a, Gerner and Meyskens 2004, Casero and Marton 2007). Spermidine synthase inhibitors like CHA, MCHA, cyclohexylammonium phosphate (CHAP) and dicyclohexylamine (DCHA) have been tested in several plants (Biondi et al. 1986, 1988,
Torrigiani et al. 1987b, Minocha and Khan 1991, Minocha and Minocha 1995, Berta et al. 1997, Aziz et al. 2001, Aziz 2003), animals (Hibasami et al. 1980, Beppu et al. 1995, He et al. 1995, Kobayashi et al. 2006) and bacteria (Bitonti et al. 1982, Mattila et al. 1984, Burtin et al. 1991). Although these inhibitors have been shown to cause a reduction in Spd and increase in Put in many cases; MCHA did not significantly affect Spd in poplar cells. It is important to mention here that the effects of these inhibitors are concentration-dependent and also vary with the tissue/cell type. In animal cells these inhibitors are effective at much lower concentration than in plants (Torrigiani et al. 1987b). DCHA tested in various bacterial species showed different results on their growth (Mattila et al. 1984); some were highly sensitive to low inhibitor concentrations (1.25 to 5 mM), while growth in others was either reduced at higher inhibitor concentration (10 mM) or not affected at all. In plants too, similar results have been seen. In excised cotyledons of radiata pine, DCHA effect was not only concentration dependent but was also modified by the presence of cytokinin (Biondi et al. 1986). Effect of DCHA was also studied by Torrigiani et al. (1987b) in *Helianthus tuberosus* tuber slices; no effect on PA content was seen with 1 mM DCHA while 5 mM concentration was able to reduce Spd content slightly after 12 h. Putrescine almost doubled after 18 h and Spm was largely unaffected. However, according to Batchelor et al. (1986) DCHA used in these studies was actually CHA at twice the quoted molarity. The concentration of MCHA used in my study was less compared to what has been used for other similar inhibitors previously in plant studies and perhaps was too low to produce any change in PAs.

In contrast to MCHA, 0.1 mM APCHA, a Spm synthase inhibitor did result in altered PA levels in poplar cells. Both cell lines showed significant increase in Spd
content at 48 h of treatment, with control cells having higher Spd even at 72 h, although there was no corresponding decrease in Spm content. A decrease in Spm was only seen in HP cells at 24 h. Treatment of rat hepatoma cells (HTC) with APCHA caused a decrease in Spm with a compensatory increase in Spd. However, the total PA content did not change and also there was no significant change in the growth rate of cells (Beppu et al. 1995). In my experiments, an increase in Spd without a decrease in Spm suggests that Spm biosynthesis is even more tightly regulated than Spd and the cells are still able to keep the optimum level of Spm in them. The lack of decrease in Spm in the presence of the inhibitor may also be due to its slow turnover rates ($t_{1/2} = 40$ to 44 h) in these cells (Bhatnagar 2002). These inhibitors had no noticeable effect on the mitochondrial activity of the cells either. The control and HP cell lines responded in a similar way to the inhibitor treatments.

**Effect of adding sulfur amino acids or inorganic sulfur**

The cellular contents of the two sulfur containing amino acids, Cys and Met, were found to be significantly lower in HP cells as compared to control cells on all days of growth (Mohapatra et al. 2009b). Their content was especially low during the first half of the week (days 2, 3 and 4) in both cell lines when their protein contents were high. Furthermore, HP cells had significantly higher protein content than the control cells during the early 2-4 d of growth and also had overall lower levels of free amino acids during this period compared to control cells. During the latter part of the week, there was accumulation of several amino acids, either due to a decline in protein synthesis or increase in protein degradation. The protein content in HP cells declined and by d 6 the level of proteins in the two cell lines was similar. Since Cys and Met were significantly
lower in HP cells, it was hypothesized that their addition to the medium might help maintain higher soluble protein levels in the cells and possibly maintain continued high growth during the second half of the growth period in culture.

Cysteine is required for the synthesis of Met (Ravanel et al. 1998), which is then used to produce higher PAs via SAM. Although Cys and Met were very low in HP cells, their additional supply in the medium did not have any effect on cellular PAs. This indicates that even though the two amino acids are consumed at a higher rate in HP cells, they probably are not limiting either for protein synthesis or growth. The use of Met in the biosynthesis of SAM (AdoMet) is believed to be in the range of 80%; however, most of Met is regenerated (recycled) during the metabolism of AdoMet via the MTA cycle without necessitating the need for additional S (Ravanel et al. 1998, Sauter et al. 2004, Bürstenbinder et al. 2007). Thus besides getting consumed faster in Spd biosynthesis, Met is also regenerated, enough to keep the cycle going.

In order to check if sulfur was becoming limiting in HP cells, inorganic sulfur was also added to the growth medium. Like, sulfur amino acids, inorganic sulfur too had no effect on cellular PAs. Additional sulfur did not change the cellular protein or PA contents in either of the cell lines, thus suggesting that sulfur was either not limiting in these cells or its assimilation was dependent on other factors such as nitrogen conditions or availability of sulfur assimilating enzymes. It has been shown that maximal accumulation of soluble protein in cells is dependent on sulfate as well as nitrate concentrations and that sulfate assimilation and nitrate reduction show regulatory interactions (Reuveny et al. 1980, Koprivova et al. 2000). For example, low nitrogen negatively regulates both nitrogen and sulfate assimilation (Brunold and Suter 1984,
Koprivova et al. 2000). Increased availability of amino acids by addition of ammonium to the medium increased the incorporation of sulfur into proteins in *Lemna minor* (Brunold and Suter 1984). Also, sulfate assimilation is tightly controlled based on demand and it is positively regulated by sulfur deprivation. In fact, in excess sulfate OAS becomes limiting for Cys synthesis and can thus lead to low uptake and assimilation (Kopriva et al. 2002, Kopriva and Rennenberg 2004).

In a study involving changes in the nitrogen content of the medium, Minocha et al. (2004a) showed that the PA content of poplar cells declined in the absence of N in the medium. An excess of N in the form of supplemental NH$_4$NO$_3$ and KNO$_3$ also led to a decline in PA concentration. Additional NH$_4$NO$_3$ at some concentrations (20, 40, 60 mM) did however cause a small but significant increase in soluble proteins. Thus it would be interesting to analyze the effect of additional sulfur or sulfur amino acids simultaneously with modulation of nitrogen supply in the medium to get a better picture of the role of sulfur in PA metabolism of HP cells.

**Sucrose uptake and assimilation**

The assimilation of N is dependent on carbon flow through the respiratory pathways (glycolysis and the TCA cycle) that provide the carbon skeleton for amino acid biosynthesis. In the absence of photosynthesis, poplar cells mostly get their carbon supply from sucrose in the medium. It was observed that HP cells had significantly higher cellular C and N than control cells and C/N ratio was high in both cell lines (Mohapatra 2008, Mohapatra et al. 2009b). To analyze whether the high C and N in HP cells was associated with increased carbon (sucrose) uptake or increased C metabolism, the cells were fed with $^{14}$C-labeled sucrose and the assimilation of radioactivity into PAs was
analyzed. The results, when sucrose was not limiting, showed a gradual increase in sucrose uptake by both cell lines until 48 h, after which a loss in radioactivity was observed. One would expect that cells with higher C content (i.e. HP cells) would take up more sucrose from the medium. However, during the 48 h period of study, sucrose uptake surprisingly was significantly lower in the HP cells than the control cells. After 48 h there was a loss in radioactivity in both cell lines and this loss was slower in HP cells as indicated by higher amount of radioactivity at 72 h in HP cells compared to control cells. In a similar experiment by Mohapatra (2008), the peak of sucrose uptake was observed at 24 h in both cell lines after which there was a loss of radioactivity. As seen in present study, Mohapatra (2008) too observed lower sucrose uptake and slower loss of label by HP cells. The incorporation of $^{14}$C into PAs, although similar initially until 24 h in both cell lines, increased significantly in HP cells thereafter. Thus, although control cells showed higher sucrose uptake, the percent radioactivity going into PAs in HP cells was significantly higher. Of the total $^{14}$C-sucrose taken up, one to two percent was incorporated into the PA fraction in HP cells.

In the short term pulse experiment, where cells were incubated with $^{14}$C sucrose for only 4 h, again there was a higher sucrose uptake by control cells until initial 4 h after which the differences were not significant, except for unexplained increase in radioactivity at 72 h in control cells. Following removal of labeled sucrose from the medium, there was a decrease in radioactivity with time in all fractions. The incorporation of $^{14}$C into PAs in both cell lines appeared similar despite control cells taking up more sucrose, thus, in this case too, the fraction of radioactive sucrose going into PAs was higher in HP cells. These experiments suggest that the increased C
assimilation in HP cells takes place without increase in sucrose uptake and is dependent on increased metabolism.

Transformation with bifunctional PfODC/SAMDC

The malarial parasite Plasmodium falciparum is the causative agent of malaria. It was discovered that the human erythrocytes, which lack nuclei and do not possess any ODC or SAMDC activity, show increased activity of these enzymes and also increased levels of PAs on infection with Plasmodium (Assaraf et al. 1984). Spermidine synthesis via SPDS has also been found in Plasmodium (Müller et al. 2001). Since PAs are essential for the survival of the parasite, this pathway has become an important target for development of drugs for treatment of parasitic diseases (Heby et al. 2003, Clark et al. 2008). It was shown that drugs causing a depletion of PAs had antiproliferative effect on parasitic cells (Müller et al. 2001, 2008, Das Gupta et al. 2005).

Enzymes ODC and SAMDC are usually synthesized as two separate proteins from two separate genes and act as monofunctional units in most eukaryotes. In P. falciparum, however, these two enzymes are part of a bifunctional protein where both enzyme activities are located on a single polypeptide. The N-terminus of the polypeptide possesses SAMDC activity while the C-terminus has ODC activity. These two protein domains are separated by a connecting hinge region (Müller et al. 2000, Krause et al. 2000). It was found that the SAMDC and ODC domains in Plasmodium contained large inserts and were hence bigger than their mammalian counterparts. The SAMDC domain contains 529 amino acids compared to the human SAMDC with 334 residues. Similarly ODC in Plasmodium consists of 614 residues, which is larger than 461-residue long mouse ODC. All inserts are predicted to be surface localized and not associated with core
structure (Birkholtz et al. 2004). Studies involving deletion mutagenesis have shown that these inserts are important for the activity of their respective domains and also affect the activity or conformation of the neighboring domains (Birkholtz et al. 2004). Deletion of specific insert(s) in a domain reduced the activity of the respective enzyme by ≈95% in the bifunctional PfODC/SAMDC. The residual decarboxylase activities were also significantly reduced in the deletion mutants of the monofunctional domains. The results also suggest that although both domains could be expressed in active monofunctional forms, the physical interactions between the two are important for optimal catalytic activity of ODC. However, there is no suggestion for existence of any regulatory influence of one domain on the other (Krause et al. 2000). Another bifunctional protein (DHFR-TS) in *P. falciparum* however, behaves in a contrasting manner, in that it was not possible to express functional TS domain alone to obtain a functional enzyme. Insert O₁ (residues 1047-1085 in ODC domain) is highly conserved among *Plasmodium* species and, along with the hinge region, is considered important for the formation of the hybrid complex (Birkholtz et al. 2004). Although deletion mutants of bifunctional PfODC/SAMDC showed reduced enzyme activities, these mutants were able to form heterotetrameric protein complexes of ≈330 kDa. Inhibition of one domain by using specific inhibitors did not affect the activity of the other domain suggesting independent functioning of both active sites (Wrenger et al. 2001).

Bifunctional proteins are not unique to protozoa and have been reported in bacteria (Feucht et al. 1996, Wann et al. 2000), animals (Venkatachalam et al. 1998, Chaouki and Salz 2006) as well as in plants (Wilson et al. 1991, Rébeillé et al. 1997, Storozhenko et al. 2007). Origin of bifunctional arrangement can be attributed to gene-fusion or
duplication events and is thought to provide distinct evolutionary advantage (Birkholtz et al. 2004). The suggested advantage of bifunctional arrangement in substrate channeling for optimized product formation as seen in DHFR-TS assembly, is not accepted in PfODC/SAMDC complex. The benefit of bifunctional arrangement here is likely to enable regulation of PA turnover at different development stages of the parasite in different host environments. The parasite specific inserts probably enable beneficial interdomain interactions. The two decarboxylase domains in PfODC/SAMDC show moderate identity with the respective decarboxylases from other organisms; however the essential amino acid regions responsible for protein formation and functioning are conserved (Müller et al. 2000). The SAMDC domain of *P. falciparum* contains the consensus sequence LSESS, which is the cleavage site for processing of proenzyme into its active form in the mammalian protein. The consensus sequence PFYAVKCN, which contains the binding site for cofactor pyridoxal 5-phosphate (PLP) in mammalian ODC, is also present in the ODC domain of the parasite with modifications (PFYSVKSN). Similarly, the DFMO binding site in mammalian ODC (GPTCDGLD) is also conserved in the plasmodium ODC with some modifications (GQSCDGLD).

**Polyamines in transgenic SOS1 cells**

Up- and down-regulation of a specific step in the PA biosynthetic pathway has been accomplished in a number of plant species using transgenic approach (Noh and Minocha 1994, Bastola and Minocha 1995, Kumar et al. 1996, Capell et al. 2000, Bhatnagar et al. 2001, 2002, Roy and Wu 2001, Kasukabe et al. 2004). In all of these cases, a single gene of the pathway was expressed either constitutively or under the control of an inducible/tissue specific promoter. Noh and Minocha (1994) created transgenic tobacco
plants with elevated SAMDC activity (2-4 folds) by expressing human SAMDC cDNA under CaMV 35S promoter. Increase or decrease in PA content of potato plants was achieved by the use of sense and antisense SAMDC expressed under inducible (Kumar et al. 1996) or tissue specific (Pedros et al. 1999) promoter. Several such examples have been mentioned under “Introduction”. In SOS1 cell line that was produced in the present study, PfODC/SAMDC was expressed under the control of CaMV-35S promoter similar to the one used for mODC expression in poplar cells (Bhatnagar et al. 2001). The constitutive expression of two genes at the same time was expected to affect both Put and higher PA biosynthesis since the two enzymes are known to be rate-limiting enzymes in PA pathway. Simultaneous over-expression of SAMDC and ODC has been demonstrated in mice, where the double transgenic animals were obtained by cross breeding mice lines overexpressing human ODC and a rat SAMDC (Heljasvaara et al. 1997). Similar approach was used earlier by Kauppinen et al. (1993) to produce hybrid mice overexpressing human SPDS and human ODC and later by Suppola et al. (2001) to produce hybrid mice lines overexpressing mouse SSAT and human ODC. In hybrid transgenic mice overexpressing both ODC and SAMDC, the changes in Spd and Spm levels remained insignificant. The concentration of Put in selected tissues was lower as compared to that of lines expressing ODC alone (Heljasvaara et al. 1997). The combined overexpression of SPDS and ODC in transgenic mice also did not produce any significant change in tissue Spd and Spm concentrations (Kauppinen et al. 1993).

Over-expression of PfODC/SAMDC led to a significant increase in Spd and Spm levels in SOS1 cells compared to control cells on all seven days of growth, while Put was higher in SOS1 only at the beginning and end of the 7 d culture cycle. Increase in Put
content has been reported in tobacco plants (4-12 fold; DeScenzo and Minocha 1993), carrot cells (10-20 fold; Bastola and Minocha 1995) and poplar cells (2-10 fold; Bhatnagar et al. 2001) in response to over-expression of mODC under an identical CaMV-35S promoter. This increase in Put was accompanied by only small increases in Spd and Spm. Over-expression of human SAMDC under CaMV-35S promoter resulted in increased Spd content in tobacco plants (2-3 fold; Noh and Minocha 1994). Spermidine was also elevated in potato tubers with over-expressed SAMDC under the control of a tuber specific patatin promoter (Pedros et al. 1999) and in tomato fruit expressing a yeast SAMDC regulated by a fruit-specific promoter (Mehta et al. 2002). Other reports showing increased production of Spd via genetic manipulation of SAMDC include transgenic rice overexpressing SAMDC cDNA from Datura stramonium under Ubi-1 promoter (Thu-Hang et al. 2002), ABA-inducible SAMDC cDNA from Tritordeum introduced in rice expressing under NaCl stress (Roy and Wu 2002) and transgenic tobacco expressing SAMDC cDNA from carnation flower under the control of CaMV 35S promoter (Wi et al. 2006). In most cases there was 2-4 folds increase in Spd; this was accompanied by either an increase in Spm or Spm remained unchanged. Putrescine in these reports showed varied responses. A reduction in Put content was observed by Noh and Minocha (1994), and also by Mehta et al. (2002), whereas an increase was reported by Thu-Hang et al. (2002) and Wi et al. (2006). In the present study, the simultaneous over-expression of both ODC and SAMDC appears to shift the biosynthetic pathway towards higher PAs. With respect to Put, only small changes were seen during the 7 d culture period, and the difference between the two lines became apparent only on days 1, 6 and 7 when Spd and Spm content difference in the two lines is not so high. This small increase in Put despite
over-expression of *ODC* indicates increased utilization of Put for synthesis of Spd; this however, needs to be experimentally verified. This also suggests that SPDS and SPMS are probably not limiting in the cells and the synthesis of Spd and Spm is accelerated by over-expression of *SAMDC*, which is the rate-limiting enzyme.

Putrescine in poplar cells is produced mostly by ADC with some contribution from ODC (Page et al. 2007). The high level of Put in SOS1 cells on transfer to fresh medium is consistent with the increased ADC and ODC activities, which are higher as well on d 1. The differences seen between enzyme activities of various enzymes shown here and their specific activities are perhaps due to variations in protein content of the cells on different days of growth. The gradual increase in Put after d 1 is however inconsistent with the two enzyme activities. It is known that there is a strong feedback inhibition of *PfODC* by Put (Müller et al. 2001) and thus despite of being constitutively expressed the ODC activity declines through the 7 d growth cycle.

Unlike in mammals, Put in plants and in *Plasmodium* has no stimulatory effect on SAMDC activity (Xiong et al. 1997, Müller et al. 2001). Although most of the acidic residues involved in Put activation of SAMDC as found in mammals are also present in plants, no similar role of Put in proenzyme processing and activation of this enzyme has been reported (Xiong et al. 1997). The absence of Asp-174, found in human SAMDC, from plant sequences is suggested to be responsible for this behavior. However, other translational regulations of SAMDC are present in plants (Hanfrey et al. 2003). SAMDC activity in SOS1 cells is significantly higher than control cells on 5 out of 7 days. By d 7 the activities become similar in the two cell lines.
Mammalian ODC as well as SAMDC have a very short half-life and are thus unstable. A PEST region, having high abundance of Pro, Glu, Ser and Thr at the C-terminal of mammalian ODC, is responsible for the rapid degradation of this enzyme (Ghoda et al. 1989, Müller et al. 2000, Theiss et al. 2002). PyODC domain at the C-terminus of the bifunctional enzyme does possess abundant residues similar to the PEST region; however PfODC is known to have a longer half-life. *Plasmodium* ODC-SAMDC has a half-life of over 2 h which is greater than 15 min half-life of mammalian ODC or 35 min of SAMDC (Müller et al. 2001).

Thus this transgenic overexpression of two genes simultaneously in the form of bifunctional *ODC/SAMDC* from *Plasmodium* was able to produce significant changes in the PA content and the activities of enzyme of PA biosynthetic pathway in poplar cells.
CONCLUSIONS

The elucidation of the PA pathway intricacies has been made easier by the use of genetic manipulation tools. In our lab the Put overproducing HP cell line was created by Bhatnagar et al. (2001) by overexpressing mODC under 35S-CaMV promoter. Previous works on this cell line provided some deeper insight into the regulation of PA pathway (Bhatnagar et al. 2001, 2002, Minocha et al. 2004a, Page et al. 2007, Mohapatra et al. 2008, 2009a, b). The effect of Put overproduction via mODC overexpression in poplar cells was studied both at molecular and biochemical levels. At molecular level the gene expression of ADC, SPDS, mODC, SAMDC, GAD, PDH, P5CR and OAT (Bhatnagar 2002, Page et al. 2007, Mohapatra 2008) was studied. At biochemical level, the activities of several enzymes of PA or related pathways, turnover rate of PAs, carbon and nitrogen assimilation into PAs, content of protein and non-protein amino acids, response to Al toxicity and level of oxidative stress in HP cells have been analyzed (Bhatnagar 2002, Minocha et al. 2004a, Page et al. 2007, Mohapatra et al. 2008, 2009a, b). This study provides further insight into biochemistry of HP cells with respect to supplementation of growth medium with different treatments or removal of medium nutrients. Another transgenic cell line created, SOS1, overexpresses both SAMDC and ODC simultaneously in poplar cells. This cell line was created with the objective of increasing the content of higher PAs (Spd/Spm) in poplar cells and analyzing the affect of this manipulation on PA pathway. Unfortunately, only one successful transgenic line was produced during this study; thus the results need to be verified with more transgenic cell lines with this gene of dual function.
The major conclusions drawn from this study are:

1. Nutrient stress in the form of Ca deficiency in the growth medium is more detrimental to HP cells than control cells.

2. The tested PA analogues and inhibitors at the tested concentrations have no major observable impact on either HP or control cell line.

3. Cysteine and Met although are consumed faster in HP cells, they probably are not limiting either for protein synthesis or for growth.

4. Uptake of sucrose is higher in control cells but its incorporation into PAs is higher in HP cells.

5. SOS1 cells after stable integration of PfODC/SAMDC produced significantly increased amounts of Spd and Spm than control cells.

6. The simultaneous expression of both SAMDC and ODC from Plasmodium has greater impact on production of Spd/Spm than Put in SOS1 cells.
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