Polyamine metabolism in Arabidopsis: Transgenic manipulation and gene expression

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POLYAMINE METABOLISM IN ARABIDOPSIS: TRANSGENIC MANIPULATION AND GENE EXPRESSION

By

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Doctor of Philosophy

In

Plant Biology

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DEDICATION

This dissertation is dedicated to my family: my father late Sarojaksha Majumdar, my parents, my uncle, my aunt, my wife, my sisters and brother in-laws, my cousins, and my in-laws. I would also like to dedicate this dissertation to the memory of late Prof. Edward Herbst, a pioneering polyamine researcher at the University of New Hampshire.
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ABBREVIATIONS

ABA = abscisic acid; Ala = alanine; Arg = arginine; Asp = aspartate; Cad = cadaverine; cDNA = complementary DNA; Cys = cysteine; dcSAM = decarboxylated S-adenosylmethionine; DW = dry weight; EDTA = ethylenediamine tetraacetic acid; FW = fresh weight; GABA = γ-aminobutyric acid; Gln = glutamine; Glu = glutamate; Gly = glycine; h = hour; His = histidine; HPLC = high performance liquid chromatography; Ile = isoleucine; Leu = leucine; Lys = lysine; Met = methionine; mRNA = messenger RNA; MS = murashige and skoog; NADP = nicotinamide adenine dinucleotide phosphate; Orn = ornithine; PAO = polyamine oxidase; PCA = perchloric acid; Phe = phenylalanine; Pro = proline; RNA = ribonucleic acid; SAM = S-adenosyl methionine; SE = standard error; Ser = serine; TCA = tricarboxylic acid; Thr = threonine; Trp = tryptophan; tSPMS = thermospermine synthase; Val = valine; WT = wild type.
ABSTRACT

POLYAMINE METABOLISM IN ARABIDOPSIS: TRANSGENIC MANIPULATION AND GENE EXPRESSION

By
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University of New Hampshire; December 2011

The metabolism of polyamines (Putrescine, Spermidine and Spermine) in wild type and transgenic Arabidopsis thaliana (Col 0 ecotype) plants was studied using the techniques of transgenic manipulation and gene expression. Two specific objectives were:

1. To study the effects of inducible and constitutive transgenic manipulation of polyamines via a mouse ornithine decarboxylase (mODC) gene on plant metabolism.

2. To analyze the spatial and temporal expression patterns of Arabidopsis thaliana S-adenosylmethionine decarboxylase genes (AtSAMDC3, AtSAMDC4 and AtSAMDC5) during its life cycle.

The major findings are: (i) Orn becomes a limiting substrate for Put biosynthesis in transgenic Arabidopsis expressing mODC gene, (ii) Put over-production cause delayed flowering, increased FW and DW, higher siliqule number and higher chlorophyll content, (iii) the cellular contents of several amino acids change under constitutive as well as short term induction of mODC, (iv) there is a greater utilization and assimilation of carbon and nitrogen into polyamines and amino acids by the mODC-transgenic plants, (v) different members of the AtSAMDC gene family are expressed differently in different tissues/organs during development (vi) expression of AtSAMDC4 is much higher than AtSAMDC3 and AtSAMDC5, (vii) the promoter regions of all AtSAMDC genes contain common cis-regulatory elements that are associated with stress responses, developmental regulation, and hormone responses.
INTRODUCTION

Polyamines (PAs; putrescine – Put, spermidine – Spd, and spermine – Spm) are organic polycations found in all living organisms. They are implicated in many physiological phenomena in plants relating to growth and development, stress responses and are involved in a multitude of cellular functions, e.g. regulation of transcription and translation, nucleic acid and membrane stabilization, and regulation of reduced N pool in cells (Minocha and Minocha 1995; Bouchereau et al. 1999; Hyvonen et al. 2006; Peremarti et al. 2009; Landau et al. 2010; Mattoo et al. 2010a; Mohapatra et al. 2010a; Quinet et al. 2010). The diamine Put, is synthesized either by direct decarboxylation of ornithine (Orn) by Orn decarboxylase (ODC; EC 4.1.1.17) or indirectly from arginine (Arg) by Arg decarboxylase (ADC; EC 4.1.1.19). The product of ADC (Agmatine – Agm) is then converted into Put via one or two steps (Fig. 1). Putrescine is sequentially converted into Spd and Spm by combined actions of S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) and two different aminopropyltransferases (APTs; Spd synthase – SPDS; EC 2.5.1.16 and Spm synthase – SPMS; EC 2.5.1.16).

Involvement of PAs in the process of somatic embryogenesis has been well known (Minocha and Minocha 1995; Minocha et al. 1995) and still remains a topic of interest (Meskaoui and Trembaly 2009). Their involvement has been demonstrated by a variety of experimental approaches; e.g. showing changes in PAs during embryogenesis, inducing embryogenesis by exogenous application of PAs, inhibiting embryogenesis using inhibitors, and inducing embryogenesis via genetic manipulation of PAs. There still remains the question of the mechanism(s) by which PAs help regulate and promote
Figure 1. Pathway for the biosynthesis of polyamines and amino acids (adapted from Mohapatra et al. 2010b); Abbreviations (PA enzymes): ODC = ornithine decarboxylase; ADC = Arginine decarboxylase; SAMDC = S-adenosylmethionine decarboxylase; SPDS = spermidine synthase; SPMS = spermine synthase. Dashed lines = multiple steps.
somatic embryogenesis in numerous plant species. Unfortunately, little progress has been made in this direction in recent years.

In addition to developmental roles of PAs, the relationship of PAs with abiotic stress has a long history of discussion and reviews (Kumar et al. 1997; Bouchereau et al. 1999; Kasukabe et al. 2004; Duan et al. 2008; Ozawa et al. 2009; Chai et al. 2010; Ding et al. 2010; Shevyakova et al. 2010; Quinet et al. 2010; Alsokari 2011; Hussain et al. 2011). Due to their physiological importance and the diversity of roles in cellular processes, there is widespread interest in understanding the regulation of PA metabolism at the level of gene expression as well as enzymatic reactions. The latter is especially important since the key enzymes involved in PA biosynthesis are often short-lived, and precursors of their biosynthesis are involved in several key metabolic pathways (Cohen 1998). Although many enzymes, and hence genes, participate in PA metabolism, the activities of ADC, ODC, SAMDC, and diamine/PA oxidases (DAO/PAO) are considered crucial for homeostatic regulation of the PA metabolic pathway and the steady state of cellular contents. While most plants have multiple copies of genes for each enzyme, including Arabidopsis thaliana (Table 1), there apparently is no ODC gene in this species (Hanfrey et al. 2001); hence Put biosynthesis in Arabidopsis occurs entirely by ADC pathway.

Application of exogenous PAs and the use of PA biosynthetic inhibitors have been common approaches to analyze some of the developmental roles of PAs in plants (Mengoli et al. 1989; Minocha and Minocha 1995; Minocha et al. 1995; Rajyalakshmi et al. 1995; Bagni and Tassoni 2001; Couée et al. 2004). Some recent examples of exogenous PA applications to study physiological and biochemical processes in plants under normal conditions and under different abiotic and biotic stresses include: Khan et
Table 1. The principle polyamine biosynthetic genes annotated in the *Arabidopsis thaliana* genomic databases (TAIR and NCBI)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>E.C.</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AtADC1</em></td>
<td>At2g16500</td>
<td>4.1.1.19</td>
<td>NM_127204</td>
</tr>
<tr>
<td><em>AtADC2</em></td>
<td>At4g34710</td>
<td>4.1.1.19</td>
<td>NM_119637</td>
</tr>
<tr>
<td><em>ODC</em></td>
<td>Not applicable</td>
<td>Not applicable</td>
<td>Not applicable</td>
</tr>
<tr>
<td><em>AtSAMDC1</em></td>
<td>At3g02470</td>
<td>4.1.1.50</td>
<td>NM_111114</td>
</tr>
<tr>
<td><em>AtSAMDC2</em></td>
<td>At5g15950</td>
<td>4.1.1.50</td>
<td>NM_121600</td>
</tr>
<tr>
<td><em>AtSAMDC3</em></td>
<td>At3g25570</td>
<td>4.1.1.50</td>
<td>NM_113454</td>
</tr>
<tr>
<td><em>AtSAMDC4</em></td>
<td>At5g18930</td>
<td>4.1.1.50</td>
<td>NM_121898</td>
</tr>
<tr>
<td><em>AtSAMDC5</em></td>
<td>At3g17715</td>
<td>4.1.1.50</td>
<td>Not available</td>
</tr>
<tr>
<td><em>AtSPDS1</em></td>
<td>At1g23820</td>
<td>2.5.1.16</td>
<td>NM_102230</td>
</tr>
<tr>
<td><em>AtSPDS2</em></td>
<td>At1g70310</td>
<td>2.5.1.16</td>
<td>NM_105699</td>
</tr>
<tr>
<td><em>AtSPMS</em></td>
<td>At5g53120</td>
<td>2.5.1.16</td>
<td>NM_124691</td>
</tr>
<tr>
<td><em>AtACL5</em></td>
<td>At5g19530</td>
<td>2.5.1.16</td>
<td>NM_121958</td>
</tr>
<tr>
<td>(tSPMS)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
al. (2008); Khosroshahi and Esna-Ashari (2008); Khan and Singh (2010); Bibi et al. (2010). Two types of genetic approaches that have been used to experimentally alter PA metabolism in plants in addition to physiological manipulations using PA biosynthesis inhibitors are mutants and genetic engineering. The former approach was tested first by Malmberg and McIndoo (1983), and since been used with almost all PA biosynthetic genes to manipulate PA metabolism in plants more so than in animals.

**Manipulation of polyamine metabolism in plants via mutants**

Several mutants of PA biosynthetic genes are reported in plants. Mutants of *AtADC1* and *AtADC2* in *A. thaliana* have been well characterized and their phenotypes are summarized in Table 2. Watson et al. (1998) reported ADC-deficient mutants in Arabidopsis generated through ethylmethane sulfonate (EMS) mutagenesis. Reduced activities of ADC were observed in the *adc1* (*spe1*; 23-36% reduction of ADC activity) and *adc2* (*spe2*; 39-50% reduction of ADC activity) mutant plants. Double mutants (*spe1-l/spe2-l*) showed somewhat higher reduction in ADC activity than either of the single mutants; this was accompanied by reduced Put and Spb contents in the roots and shoots as in *spe2-l* alone. The single mutants exhibited higher lateral root number and root growth as compared to the WT plants, whereas the double mutants exhibited a highly kinked and compact root pattern. The single mutants did not show any change in whole plant morphology except for a few lines with occasional increase in the numbers of sepals and petals in the flowers. The *spe1-l/spe2-l* plants showed narrow and twisted leaves, narrow sepals and petals, and delayed flowering (by a week) as compared to the WT or the single mutant plants.
Table 2. Characterization of Arabidopsis mutants of *ADC*, *SAMDC*, *SPDS*, *SPMS* and *ACL5* genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant</th>
<th>Mutagen</th>
<th>Mutation loci/type</th>
<th>Phenotype/Biochemical alteration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine deacarboxylase</td>
<td>spe1-1, spe2-1, spe1-1/spe2-1</td>
<td>Ethylmethane sulfonate</td>
<td>ADC-deficient mutant</td>
<td>Decrease in Put and Spd; single mutant had higher lateral root initiation and growth; double mutants were viable with kinked and compact roots</td>
<td>Watson et al. 1998</td>
</tr>
<tr>
<td>adc2</td>
<td>En-1 insertion</td>
<td>Insertion at 1508 bp of intron-less coding region</td>
<td>Lower ADC activity with no phenotype; little induction of ADC in the transgenic plants under osmotic stress in comparison to WT plants</td>
<td>Soyka and Heyer, 1999</td>
<td></td>
</tr>
<tr>
<td>adc1-1, adc2-2 and adc1-1/adc2-2</td>
<td>T-DNA insertion</td>
<td>Insertion at 1141 bp (adc1-1) and 1364 bp (adc2-2) of coding regions</td>
<td>Lower Put and Spd. Double mutants showed narrow and twisted leaves, narrow sepals, petals, delay in flowering and embryo lethality</td>
<td>Urano et al. 2005</td>
<td></td>
</tr>
<tr>
<td>S-adenosyl-methionine deacarboxylase</td>
<td>bud2-1, samdc1-1 and bud2-1/samdc1-1</td>
<td>T-DNA insertion</td>
<td>Insertion at coding region of bud2-1 and at 3'UTR of samdc1-1</td>
<td>Bushy and dwarf with increased lateral roots in bud2-1; samdc1-1 similar to bud2-1 but even severer; bud2-1/samdc1-1 was embryo lethal</td>
<td>Ge et al. 2006</td>
</tr>
<tr>
<td>Spermidine synthase</td>
<td>spds1-1</td>
<td>T-DNA insertion</td>
<td>Insertion in exon 7</td>
<td>No phenotypic change, no alteration of PAs</td>
<td>Imai et al. 2004b</td>
</tr>
<tr>
<td></td>
<td>spds2-1</td>
<td>T-DNA insertion</td>
<td>Insertion in intron 3</td>
<td>Same as spds1-1</td>
<td>Imai et al. 2004b</td>
</tr>
<tr>
<td>Spermidine synthase</td>
<td>spds2-2</td>
<td>T-DNA insertion</td>
<td>Insertion in exon 7</td>
<td>Same as spds1-1</td>
<td>Imai et al. 2004b</td>
</tr>
<tr>
<td></td>
<td>spds1-1/spds2-1</td>
<td>T-DNA insertion</td>
<td>See spds1-1 and spds2-1</td>
<td>Embryo lethal; greatly increased Put, decreased Spd and Spm</td>
<td>Imai et al. 2004b</td>
</tr>
<tr>
<td>Spermine synthase</td>
<td>spms-1</td>
<td>T-DNA insertion</td>
<td>Insertion in intron 1</td>
<td>No phenotypic change; increased Spd, decreased Spm</td>
<td>Imai et al. 2004a</td>
</tr>
<tr>
<td>Acaulis 5</td>
<td>acl5-1</td>
<td>Methane-sulfonate</td>
<td>1 bp substitution in exon 4</td>
<td>Dwarf inflorescence stems, incomplete xylem development; Lack of tSpd higher Put, Spd and Spm</td>
<td>Hanzawa et al. 2000; Imai et al. 2004a; Kakehi et al. 2008; Rambla et al. 2010</td>
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<td>---</td>
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</tr>
<tr>
<td>acl5-3</td>
<td>Ds insertion</td>
<td>Insertion in intron 6</td>
<td>Dwarfism phenotype same as acl5-1</td>
<td>Hanzawa et al. 2000</td>
<td></td>
</tr>
<tr>
<td>acl5-4</td>
<td>Ds insertion</td>
<td>Large deletion from the excision of acl5-3 Ds transposon</td>
<td>Dwarfism phenotype same as acl5-1</td>
<td>Hanzawa et al. 2000; Muñiz et al. (2008)</td>
<td></td>
</tr>
<tr>
<td>Spermine synthase and acaulis 5</td>
<td>spms-1/acl5-1</td>
<td>T-DNA insertion and Methanesulfonate</td>
<td>See spms-1 and acl5-1</td>
<td>Dwarfism phenotype same as acl5-1, hypersensitive to salinity and drought; Lack of tSpm, decreased Spm, increased Spd</td>
<td>Imai et al. 2004a; Yamaguchi et al. 2006, 2007; Rambla et al. 2010</td>
</tr>
<tr>
<td>Acaulis 5</td>
<td>Thickvein</td>
<td>Diepoxybutane</td>
<td>1 bp deletion in exon 7 of ACL5</td>
<td>Dwarfism, thicker veins, disruption of auxin polar transport</td>
<td>Clay and Nelson 2005</td>
</tr>
</tbody>
</table>
Soyka and Heyer (1999) reported Arabidopsis adc2 mutant generated through insertion of a maize transposable element En-1. The mutant plants showed no detectable $AtADC2$ transcripts in different organs and a 44% reduction in ADC activity with no obvious phenotypic changes. In a detached leaf assay under osmotic stress, WT plants showed 18-fold induction of ADC activity whereas the mutant showed only a 2-fold induction of ADC activity vs. their corresponding controls. In another study involving T-DNA insertion mutants of $adcl$ and $adc2$, there was a 70% reduction in Put and 35% reduction in Spd in the latter with no significant change in PAs in the former (Urano et al. 2005). The $ADC$ double mutant ($adcl/adc2$) showed embryo lethality, while $adcl^{+/−}/adc2^{+/−}$ or $adcl^{+/−}/adc2^{−/−}$ mutants showed abnormal seeds in the developing siliques.

Several mutants of $ADC$ have also been used to study stress responses in relation to reduced level of Put. In a study with $adc2$ mutant of Arabidopsis, Urano et al. (2004) saw a decrease in Put content and reduced tolerance to salt stress. Up-regulation of Put biosynthesis under low temperature and the role of Put in alleviating cold stress in concert with ABA have also been implicated in plants. Arabidopsis mutants ($adcl$-3 and $adc2$-3) with lower Put levels, showed less tolerance to low temperature (Cuevas et al. 2008). There was also a down-regulation of $NCED3$ expression (a key gene involved in ABA biosynthesis) and other ABA-related genes, as well as lower ABA content. Complementation and reciprocal complementation of $adc$ and $aba2$-3 mutants with ABA and Put, respectively, restored cold tolerance in the mutants. Transgenic expression of a Poncirus trifoliata ADC ($PtADC$) under 35S promoter in $adcl$-1 resulted in higher Put content (in the transgenic mutant lines) with a recovery of stomatal frequency (i.e. stomata/unit leaf area comparable to the WT plants; Wang et al. (2011). Transgenic
plants showed greater tolerance to drought, high osmoticum, and low temperature stresses in comparison to WT and *adc1-1* plants. Mutant transgenic plants showed longer roots under normal as well as stress conditions, greater cell number in roots, longer root meristematic zone, and displayed less accumulation of reactive oxygen species under stress conditions compared to WT plants.

Changes in gene expression and associated metabolic changes in mutants of PA biosynthetic enzymes have also been reported occasionally but not studied systematically. For example, microarray analysis of *SPDS* and *Spm oxidase* double mutant (Δspe3Δfms1) in yeast showed differential responses to exogenous Spd or Spm vs. the WT (Chattopadhyay et al. 2009); Spd affected a higher number of genes. Other mutants of *SAMDC*, *SPDS*, *SPMS* and *tSPMS* are described by Shao et al. (2011) and summarized in Table 2. No ODC mutant has been reported in plants.

**Manipulation of polyamine metabolism in plants via transgene expression**

Manipulations of PA metabolism through transgenic expression (genetic engineering) of *ODC*, *ADC*, *SAMDC*, *SPDS* and *PAO* have often been reported in plants and most studies have used constitutive expression (Thu-Hang et al. 2002; Mayer and Michael 2003; Rea et al. 2004; Alcázar et al. 2005; Wen et al. 2008; Nölke et al. 2008). In some cases, the transgenic plants have also been tested for the roles of PAs in developmental and stress responses. The first report of transgenic manipulation of Put involved the expression of a yeast *ODC* gene under the control of Cauliflower Mosaic Virus 35S promoter (*CaMV 35S*; henceforth called 35S) which caused a significant increase in ODC activity and Put content (by 2-fold), accompanied by a 2-fold increase in Put-derived alkaloid nicotine in transgenic tobacco (*Nicotiana rustica*) root cultures (Hamill et al. 9
1990). In a later study with *N. tabacum*, DeScenzo and Minocha (1993) expressed a mouse *ODC* (m*ODC*) under 35S promoter, achieving a 2.2-fold increase in Put in the leaves with the accompanying phenotype of male sterility in mature plants. Further studies with wild carrot (*Daucus carota* var. Queen Anne’s Lace) using the same gene-promoter combination found 4- to 12-fold higher Put accumulation and a promotion of somatic embryogenesis in transgenic suspension cultures (Bastola and Minocha 1995). The transgenic cells were able to produce somatic embryos even in the presence of difluoromethylarginine (DFMA - an inhibitor of ADC), showing that Put produced by mODC substituted for native ADC function. It was further shown that ADC activity was not affected by increased ODC production, and higher Put production was accompanied by higher rate of Put conversion into Spd and Spm with increased catabolism of Put in the cells (Andersen et al. 1998).

Kumria and Rajam (2002) also reported increased Put production in tobacco using the same m*ODC* cDNA as used by the Minocha lab. Regeneration efficiency of the transgenic plants correlated with Put to Spd ratio; e.g. low Put to Spd ratio in the transgenic plants resulted in lower responses to the regeneration medium and vice versa. Transgenic lines with elevated Put showed higher germination rates and overall seedling performance under salt stress. Transgenic expression of a human *ODC* (h*ODC*) under the control of maize *Ubi-1* promoter in rice while causing a ~2 to 6-fold increase in Put also resulted in significant increases in Spd and Spm (Lepri et al. 2001). Mayer and Michael (2003) reported that transgenic expression of a *Datura stramonium ODC* (Ds*ODC*) cDNA in tobacco under 35S promoter resulted in 25-fold higher ODC activity in leaves and a five-fold increase in flower buds but there was <2-fold increase in Put in these
tissues. There was no significant change in Spd, Spm and soluble or insoluble hydroxycinnamic acid-conjugated PAs. Feeding of 10 mM Orn to cell cultures derived from control and transgenic plants showed 1.5 to 2.5-fold increase in Put after 7 h.

A transgenic (*mODC* gene under 2x35S promoter) cell line of poplar (*Populus nigra x maximowiczii*) in our lab showed 3- to 7-fold increase in Put content with a small increase in Spd and a decrease in Spm; increased biosynthesis of Put was accompanied by a parallel increase in its catabolism (Bhatnagar et al. 2001, 2002). Contrary to the expectation of feedback regulation, there actually was an increase in the relative expression of *ADC* in the high Put (called HP) cell line. Further, the expression of three *SAMDCs* and two *SPDSs* was affected differently (Page et al. 2007). Total soluble protein (g⁻¹ FW) was significantly higher in the HP cells. Increased production of Put was accompanied by a significant reduction in Glu with accompanying pleiotropic effects on several other amino acids, changes in the oxidative state of the cells, and altered carbon (C) and nitrogen (N) balance in the transgenic cells (Mohapatra et al. 2009, 2010b). It was later shown (Page et al. 2010) that the expression of Glu-Orn-Arg pathway genes was highly coordinated and their expression was not affected by the increased flux of metabolites through this pathway due to increased utilization of Orn by mODC.

Inhibition of ODC activity through RNAi in hairy root cultures of *N. tabacum* showed down-regulation of both *ODC* transcripts and ODC activity (DeBoer et al. 2011). A slight increase of *ADC* transcripts and increase in ADC activity were also seen. Decreased ODC activity altered alkaloid composition in the transgenic plants where major alkaloid nicotine decreased, with a concomitant increase in anatabine both in the roots and leaves as well as hairy root cultures. Methyl jasmonate (MeJ), which typically stimulates
nicotine biosynthesis in tobacco, did not have any effect in the ODC RNAi plants; however, there was a further increase in anatabine content upon MeJ treatment. Several other studies on genetic manipulation of Put in plants through transgenic expression of ODC are summarized in Table 3A.

There are several examples of genetic manipulation of Put via transgenic expression of ADC in plants (Table 3B). In contrast to ODC over-expression, in most cases small only changes in PAs are often seen. Most studies targeting Put overproduction have found that increased Put production/accumulation is not accompanied by parallel increases in either Spd or Spm, suggesting that the cellular contents of these PAs are more tightly regulated than the diamine Put. A part of the explanation may lie in the fact that SAMDC, whose product decarboxylated SAM (dcSAM) is a co-substrate for APTs, is a highly regulated enzyme of the pathway (Pegg et al. 1986; Slocum 1991). Also, the added complexity of APT requirement also contributes to limiting the production of higher PAs. The third factor that could prohibit parallel increases in Spd and Spm would be the PAOs, which catabolize these PAs. Thus there have been attempts to directly manipulate cellular Spd and Spm through genetic manipulation of SAMDC and/or SPDS in plants (Table 3C). A summary of information on SAMDC expression, its regulation, its genetic manipulation, and responses to abiotic stresses and phytohormones in plants is covered in the SAMDC section of this thesis. The current status of research on plants genetically manipulated with APTs has been reviewed by Shao et al. (2011). Rarely, if ever, have more than two-to-three-fold increases in Spd and Spm have been achieved. On the other hand, even with these relatively small increases, the stress tolerance of these plants has been variously demonstrated (Shao et al. 2011 and references therein).
Table 3A. Genetic manipulations of ODC activity in plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Transgene source</th>
<th>Approach</th>
<th>Biochemical alteration/ Phenotype if any</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiana rustica</em></td>
<td>Yeast ODC</td>
<td>35S promoter</td>
<td>High ODC activity, high Put and Put-derived alkaloid nicotine in root culture</td>
<td>Hamill et al. 1990</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Mouse ODC</td>
<td>35S promoter</td>
<td>Increase in Put in the leaves with accompanying male sterility</td>
<td>DeScenzo and Minocha 1993</td>
</tr>
<tr>
<td><em>Daucus carota</em></td>
<td>Mouse ODC</td>
<td>35S promoter</td>
<td>High Put content and a decrease in Spm with a promotion of somatic embryogenesis</td>
<td>Bastola and Minocha 1995</td>
</tr>
<tr>
<td><em>Daucus carota</em></td>
<td>Mouse ODC</td>
<td>35S promoter</td>
<td>Increased Put content via ODC, while ADC activity not changed; higher Put catabolism and conversion to Spd and Spm</td>
<td>Andersen et al. 1998</td>
</tr>
<tr>
<td><em>Populus nigra x maximowiczii</em></td>
<td>Mouse ODC</td>
<td>35S promoter</td>
<td>Higher Put content, with a small increase in Spd and decrease in Spm</td>
<td>Bhatnagar et al. 2001</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>Human ODC</td>
<td>Maize Ubi-1 promoter</td>
<td>Higher Put, Spd and Spm in the leaves, seeds and roots</td>
<td>Lepri et al. 2001</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Mouse ODC</td>
<td>35S promoter</td>
<td>Higher ODC activity, increase in Put and Spd contents; greater tolerance to salt stress</td>
<td>Kumria and Rajam 2002</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Datura ODC</td>
<td>35S promoter</td>
<td>Higher activity of ODC in leaves and flower buds; small increase in Put; no morphological phenotype</td>
<td>Mayer et al. 2003</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Human ODC</td>
<td>Cytosolic and apoplastic expression via N-terminal signal peptide; 35S promoter</td>
<td>Increase in ODC activity and Put content; no change in Spd and Spm</td>
<td>Nölke et al. 2008</td>
</tr>
<tr>
<td><em>Datura innoxia</em></td>
<td>Mouse ODC</td>
<td>35S promoter</td>
<td>Increase in PAs and the alkaloid scopolamine</td>
<td>Singh et al. 2011</td>
</tr>
<tr>
<td><em>N. tabacum</em></td>
<td><em>N. glauca</em> ODC</td>
<td>RNAi</td>
<td>Lower ODC activity; lower nicotine and increase in anatabine</td>
<td>DeBoer et al. 2011</td>
</tr>
</tbody>
</table>
Table 3B. Genetic manipulations of ADC activity in plants.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Transgene source</th>
<th>Approach</th>
<th>Biochemical alteration/ Phenotype if any</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum</td>
<td>Avena sativa ADC</td>
<td>35S promoter</td>
<td>Higher ADC activity and Agm content without change in Put, Spd and Spm; no phenotypic change observed</td>
<td>Burtin and Michael 1997</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>Avena sativa ADC</td>
<td>tetracycle inducible promoter</td>
<td>Higher Put and Spd, short internodes, thin stems and leaves, leaf chlorosis and necrosis, reduced root growth</td>
<td>Masgrau et al. 1997</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Avena sativa ADC</td>
<td>35S promoter</td>
<td>Higher Put with a small decrease in Spm in calli and regenerated plants; lower regeneration efficiency</td>
<td>Capell et al. 1998</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Avena sativa ADC</td>
<td>maize Ubi-1 promoter</td>
<td>Higher ADC activity with high or low Put, Spd and Spm based on developmental stages of calli</td>
<td>Bassie et al. 2000</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Avena sativa ADC</td>
<td>maize Ubi-1 promoter</td>
<td>Small increase in Put, Spd and Spm in the leaves without much change in the seeds</td>
<td>Noury et al. 2000</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Avena sativa ADC</td>
<td>ABA-inducible promoter</td>
<td>Increased Put in the leaves, increased biomass and greater salt tolerance</td>
<td>Roy and Wu 2001</td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>Datura stramonium ADC</td>
<td>35S promoter</td>
<td>Increase in Put and Spd, little delay in flowering and greater tolerance to drought</td>
<td>Capell et al. 2004</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>A. thaliana ADC2</td>
<td>35S promoter</td>
<td>Put content increased in the leaves, dwarf and late flowering that was restored by GA3</td>
<td>Alcázar et al. 2005</td>
</tr>
<tr>
<td>Nicotiana tabacum</td>
<td>Nicotiana tabacum ADC</td>
<td>Antisense - 35S promoter</td>
<td>Decrease in nicotine and increase in anatabine contents in hairy root cultures</td>
<td>Chintapakorn and Hamill 2007</td>
</tr>
<tr>
<td>Solanum melongena</td>
<td>Avena sativa ADC</td>
<td>35S promoter</td>
<td>Increase in ADC and ODC activities, increase in Put and Spd; greater tolerance to abiotic stresses and Fusarium wilt disease</td>
<td>Prabhavathi and Rajam 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Avena Sativa ADC</td>
<td>pRD29A promoter</td>
<td>Increased Put content under low temperature and dehydration stresses; greater tolerance</td>
<td>Alet et al. 2011</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>Poncirus trifoliate ADC</td>
<td>35S promoter</td>
<td>Higher Put content in the mutant, restoration of normal stomatal frequency (comparable to WT) in the mutant</td>
<td>Wang et al. 2011</td>
</tr>
</tbody>
</table>
Table 3C. Genetic manipulations of SAMDC, SPDS and SPMS genes in plants.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene in Vector</th>
<th>Promoter</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Human SAMDC</td>
<td>35S promoter</td>
<td>Transgenic leaf discs produced shoots in the CIM; no phenotypic changes in mature plants</td>
<td>Noh and Minocha 1994</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td><em>Solanum tuberosum</em> SAMDC</td>
<td>35S promoter</td>
<td>High SAMDC activity with high Put, Spd and Spm in the leaves. Transgenic plants failed to regenerate from callus</td>
<td>Kumar et al. 1996</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td><em>Solanum tuberosum</em> SAMDC</td>
<td>Antisense SAMDC - 35S promoter</td>
<td>Decrease in SAMDC activity accompanied by a decrease in Put, Spd and Spm contents; stunted growth, higher branching, smaller leaves, poor root growth and early senescence</td>
<td>Kumar et al. 1996</td>
</tr>
<tr>
<td><em>Solanum tuberosum</em></td>
<td><em>Solanum tuberosum</em> SAMDC</td>
<td>Tuber specific patatin promoter</td>
<td>High SAMDC activity and increased Spd content in the tubers, larger numbers of smaller tubers at maturity stage in transgenic plants</td>
<td>Pedros et al. 1999</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>Yeast SAMDC</td>
<td>Tomato fruit ripening specific E8 promoter</td>
<td>High Spd and Spm in ripe fruits, high lycopene content in the juice and increased vine life of the fruits</td>
<td>Mehta et al. 2002</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>Tritordeum SAMDC</td>
<td>ABA-inducible promoter</td>
<td>Increase in Spd and Spm contents and decrease in shoot length and fresh weight upon salt stress vs. WT plants</td>
<td>Roy and Wu 2002</td>
</tr>
<tr>
<td><em>Oryza sativa</em></td>
<td>Datura stramonium SAMDC</td>
<td>Maize Ubi promoter</td>
<td>High Spd content in the leaves and increase in Spd and Spm contents in the seeds with no phenotypic changes in mature plants</td>
<td>Thu-Hang et al. 2002</td>
</tr>
<tr>
<td><em>Daucus carota</em></td>
<td>Human SAMDC</td>
<td>35S promoter</td>
<td>Increased SAMDC activity along with higher Spd; higher no of somatic embryos and morphologically stouter embryos</td>
<td>Bastola 1994</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Human SAMDC</td>
<td>35S promoter</td>
<td>Increase in Spd and Put contents; delayed regeneration, slower growth and shorter internode without any abnormalities in flowers and fruits</td>
<td>Waie and Rajam 2003</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Datura stramonium SAMDC</td>
<td>Antisense SAMDC - 35S promoter</td>
<td>Decreased SAMDC activity with an increase of Put to Spd ratio. Decrease in rhizogenic potential of leaf explants from transgenic plants</td>
<td>Torrigiani et al. 2005</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>Dianthus caryophyllus SAMDC</td>
<td>Over-expression under 35S promoter</td>
<td>Increase in total PAs, total seed number and seed weight, net photosynthetic rate and greater tolerance to salt and oxidative stresses</td>
<td>Wi et al. 2006</td>
</tr>
<tr>
<td>Plant</td>
<td>Organism /_modifier</td>
<td>Promoter/Expression</td>
<td>Phenotype</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------------------</td>
<td>-----------------------</td>
<td>---------------------------------------------------------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td><em>Nicotiana tabacum</em></td>
<td>RNAi</td>
<td>Lower SAMDC activity accompanied by a decrease in Put, Spd and Spm; greater susceptibility to salt stress and decreased biomass upon salt treatment</td>
<td>Moschou et al. 2008b</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>Yeast SAMDC</td>
<td>35S promoter</td>
<td>Increase in Spd and Spm; greater tolerance to high temperature stress</td>
<td>Cheng et al. 2009</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em></td>
<td>Yeast SAMDC</td>
<td>tomato fruit ripening specific E8 promoter</td>
<td>Increased Omega-3 fatty acids in the fruits; changes in gene expression associated with carbohydrate, amino acid and protein metabolism, and up-regulation of stress related genes in the fruit</td>
<td>Kolotilin et al. 2011</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td><em>Datura stramonium</em> SPDS</td>
<td>35S promoter</td>
<td>Lower Put/Spd; with no change in Spm and total PAs. SPDS and SAMDC activity increased in leaves; plants were shorter with fewer internodes; delayed flowering</td>
<td>Franceschetti et al. 2004</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>Antisense A. thaliana SPDS1</td>
<td>35S promoter</td>
<td>No PA or phenotypic change in WT background. Resembled spds1-1/spds2-1 double mutant phenotype (seen in table 2) when expressed in spds2 mutant.</td>
<td>Imai et al. 2004b</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td><em>Cucurbita ficifolia</em> SPDS</td>
<td>35S promoter</td>
<td>Increased SPDS activity, Spd and Spm in leaves; enhanced tolerance to low temperature, salinity, hyperosmosis, drought and oxidative stresses</td>
<td>Kasukabe et al. 2004</td>
</tr>
<tr>
<td><em>Ipomoea batatas</em></td>
<td><em>Cucurbita ficifolia</em> SPDS</td>
<td>35S promoter</td>
<td>Increased Spd content in leaves and storage roots; less affected by salinity, drought and week light, less damaged to photosynthesis by chilling and heat stresses</td>
<td>Kasukabe et al. 2006</td>
</tr>
<tr>
<td><em>Pyrus communis</em> L. 'Ballad'</td>
<td>Malus sylvestris var. domestica SPDS</td>
<td>35S promoter</td>
<td>Reduced shoot heights of seedlings, elevated Spd; enhanced tolerance to salt, osmosis and heavy metal stresses along with increased antioxidant activity</td>
<td>He et al. 2008; Wen et al. 2008, 2009, 2010</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em> acl5 mutant</td>
<td><em>Arabidopsis thaliana</em> ACL5</td>
<td>Heat shock- inducible promoter</td>
<td>Restore the dwarfism phenotype of acl5 mutant, produced detectable tSpm absent in mutant</td>
<td>Hanzawa et al. 2000; Kakechi et al. 2008</td>
</tr>
<tr>
<td><em>Arabidopsis thaliana</em></td>
<td>A. thaliana SPDS2</td>
<td>35S promoter</td>
<td>Increased susceptibility to cyst nematode <em>H. schachtii</em> infection</td>
<td>Hewezi et al. 2010</td>
</tr>
<tr>
<td>Plant Species</td>
<td>Organism</td>
<td>Promoter</td>
<td>Phenomenon</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>----------</td>
<td>----------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>Solanum lycopersicum</strong>&lt;br&gt;(Malus sylvestris var. domestica)&lt;br&gt;SPDS</td>
<td>35S promoter</td>
<td>Higher Put, Spd and Spm in fruits; higher carotenoid contents especially lycopene. Primary metabolism altered.</td>
<td>Neily et al. 2010</td>
<td></td>
</tr>
<tr>
<td><strong>Solanum lycopersicum</strong>&lt;br&gt;Yeast SPDS</td>
<td>35S promoter</td>
<td>Increased Spd, decreased Put and Spm in leaves and developing fruits; fruits had higher lycopene content, delayed ripening, longer shelf life and reduced shriveling.</td>
<td>Nambeesan et al. 2010</td>
<td></td>
</tr>
<tr>
<td><strong>Solanum lycopersicum</strong>&lt;br&gt;Yeast SPDS</td>
<td>fruit-ripening specific promoter E8</td>
<td>Increased Spd, Spm in developing fruits; fruits had increased lycopene content, delayed ripening, longer shelf life and reduced shriveling.</td>
<td>Nambeesan et al. 2010</td>
<td></td>
</tr>
<tr>
<td>Marsilea vestita&lt;br&gt;(M. vestita) SPDS</td>
<td>RNAi by double stranded RNA in male gametophyte</td>
<td>Reduction of Spd, development arrest at cell division stage, defects in microtubules, base bodies and spermatid chromatin condensation</td>
<td>Deeb et al. 2010</td>
<td></td>
</tr>
</tbody>
</table>
In several cases, the effects of genetic manipulation of PAs on the expression of other genes have also been reported; e.g. down-regulation of GA oxidases in ADC2 over-expressing lines of Arabidopsis (Alcázar et al. 2005). These changes were accompanied by delayed flowering in the transgenic plants, which was overcome by GA3 treatment. Expression of AtGA2ox1 was higher in the transgenic lines as compared to the WT plants. In Arabidopsis expressing the Cucurbita ficifolia SPDS (Kasukabe et al. 2004), under low temperature stress conditions, there was an up-regulation of 33 genes (0.24% of total 13,704 genes analyzed by cDNA microarrays) in the leaves of transgenic plants as compared to the WT plants. The genes that were up-regulated in response to low temperature treatment in the transgenic plants were those encoding WRKY transcription factors, DREB2B, MYB, and NAC domain proteins, NAM proteins, B-box zinc finger proteins, low-temperature induced protein 78 (LTI78), calmodulin-related proteins, cytochrome P450, peroxidases and protein kinases.

Transgenic expression of PAOs has also been studied in plants since these enzymes play a significant role in the catabolism of PAs (Rea et al. 2004; Moschou et al. 2008a, 2008b). A review by Cona et al. (2006) has covered this aspect nicely.

Polyamines and the related biochemical pathways

Transgenic expression of PA biosynthetic genes often results in elevated cellular concentrations of PAs in transgenic plants and cells, yet the regulation of the homeostatic levels of PAs in the transgenic (as well as the WT) cells, the metabolic (and other) limits on their flux rates, effects on their catabolism, and the metabolic adjustments that the transgenic cells have to make to elevated PAs are poorly understood. The role of substrates of the transgene products (i.e. the enzymes) in controlling metabolic fluxes of
PAs, the related amino acids, the organic acids, and the assimilated N on one side, and the need for substrates like Orn and SAM have rarely been studied. For example, Orn is often present in plant cells in extremely low quantities but may be overutilized in transgenics by an enzyme like ODC. Some progress in analyzing the biochemical impacts of genetic manipulation in plant cells has been made in our lab using transgenic cells of poplar (*Populus nigra x maximowiczii*). In summary, the results show that:

- Transgenic expression of m*ODC* showed 3-10 fold increase in Put content with a small increase in Spd and a decrease in Spm contents (Bhatnagar et al. 2001).

- Increased Put biosynthesis in the HP (high Put) cells was accompanied by increased rate of its catabolism without a major change in DAO activity (Bhatnagar et al. 2002).

- The HP cells and the control (*GUS*-transformed) cells had similar levels of ACC and produced similar amounts of ethylene (Quan et al. 2002)

- Up-regulation of Put biosynthesis in the HP cell line up-regulated the expressions of *ADC, SAMDC2* and *SPDS2* genes, while the other paralogues of these genes were down-regulated (Page et al. 2007).

- Put over-production in the HP cell line increased H$_2$O$_2$ production, increased the activities of oxidative stress related enzymes (e.g. glutathione reductase and monodehydroascorbate reductase), and negatively affected the oxidative state of the transgenic cells (Mohapatra et al. 2009).

- Increased production of Put in the HP cells was accompanied by an increase in GABA and significant reduction in Glu, Gln, His, Arg, Ser, Gly, Phe, Trp, Asp,
Lys, Leu, Cys, and Met, and already low Orn; carbon (C) and nitrogen (N) balance in the transgenic cells was also altered (Mohapatra et al. 2010b).

- Higher accumulation of Put in the HP cells increased ROS production and compromised their tolerance to low Ca (Mohapatra et al. 2010a).

- Increased utilization of Orn by mODC, which would have resulted in increased flux rate through the Glu-Orn-Arg pathway, occurred without major changes in the expression of the genes of this pathway (Page et al. 2010).

- Metabolome and transcriptome analyses of the HP cell line showed significant increase in carbohydrates and other related metabolites (e.g. organic acids and amines) along with changes in expressions of the genes associated with 'C' and 'N' metabolism (unpublished by our lab).

**S-Adenosylmethionine decarboxylase: a brief background**

*S-adenosylmethionine decarboxylase (SAMDC, a.k.a AdoMetDC)* is a multigene family in most angiosperms encoding the enzyme SAMDC (Franceschetti et al. 2001; Hu et al. 2005a, 2005b; Hao et al. 2005; Tassoni et al. 2007). The enzyme carries out the important rate-limiting step of the biosynthesis of dcSAM (Greenburg and Cohen 1985; Slocum 1991), which serves as donor of the aminopropyl moiety for the biosynthesis of Spd and Spm by the enzymes Spd synthase and Spm synthase, respectively (Fig. 1).

*Arabidopsis thaliana* (*A. thaliana*) apparently has five SAMDC genes (Table 1 and Table 4), which show a high degree of sequence similarity amongst them (Table 5). The two commonly studied paralogues of *AtSAMDC*, i.e. *AtSAMDC1* and *AtSAMDC2*, have been well characterized with respect to their expression as well as the regulation of their
translation via 5' untranslated region (5' UTR) sequences. In each case, the 5' UTR contains within it two upstream open reading frames (uORFs) which are translated in PA-dependent manner and they (or their products) control \textit{SAMDC} mRNA translation (Franceschetti \textit{et al.} 2001; Hanfrey \textit{et al.} 2005; Ivanov \textit{et al.} 2010). No information is available on the role of 5' UTR in regulation of \textit{AtSAMDC3}, \textit{AtSAMDC4} and \textit{AtSAMDC5} mRNA translation.

The transcripts of \textit{AtSAMDC1}, \textit{AtSAMDC2}, \textit{AtSAMDC3} and \textit{AtSAMDC4} have been detected (using northern blots, RT-PCR and microarrays) in vegetative as well as in the reproductive organs (Franceschetti \textit{et al.} 2001; Urano \textit{et al.} 2003; Ge \textit{et al.} 2006; Jumtee \textit{et al.} 2008). However, the studies are limited in the number of developmental stages that were analyzed (except those using microarrays), and no information is available on characterization of the promoters of these paralogues in regulating spatial and temporal pattern of gene expression during development, and in response to phytohormones or other abiotic stresses. No data are currently available on the expression and the role of \textit{AtSAMDC5}, which was believed to be transcriptionally inactive (Franceschetti \textit{et al.} 2001). The published information on the biochemical properties of SAMDC, genomic organization of its genes, loss-of-function mutants, transgenic manipulation of PAs via \textit{SAMDC}, the expression of \textit{SAMDC} during development and in responses to abiotic stresses and phytohormones in plants is summarized here.
Table 4. Details of the SAMDC genes annotated in the Arabidopsis thaliana database (www.arabidopsis.org).

<table>
<thead>
<tr>
<th>Gene</th>
<th>ESTs</th>
<th>5'UTR</th>
<th>ORF (bp)</th>
<th>3'UTR (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtSAMDC1</td>
<td>1247</td>
<td>1-1106 Introns: 680, 837-931</td>
<td>284-2207 (1101)</td>
<td>2208-2445 (238)</td>
</tr>
<tr>
<td>AtSAMDC2</td>
<td>83</td>
<td>1-837 Introns: 215, 286-541</td>
<td>838-1926 (1089)</td>
<td>1927-2119 (193)</td>
</tr>
<tr>
<td>AtSAMDC3</td>
<td>6</td>
<td>1-876 Introns: 90-187, 258-423, 612-723</td>
<td>877-1926 (1050)</td>
<td>1927-2106 (180)</td>
</tr>
<tr>
<td>AtSAMDC4</td>
<td>1</td>
<td>1-108</td>
<td>109-1152 (1044)</td>
<td>1153-1599 (447)</td>
</tr>
<tr>
<td>AtSAMDC5</td>
<td>6</td>
<td>1-12</td>
<td>13-1176 (1164)</td>
<td>1177-1228 (52)</td>
</tr>
</tbody>
</table>

Table 5. Comparison of the % sequence identities among different members of Arabidopsis SAMDC gene family (sequences analyzed by BioEdit software).

<table>
<thead>
<tr>
<th>Genes compared</th>
<th>Promoter</th>
<th>5'UTR</th>
<th>Coding sequence</th>
<th>3'UTR</th>
<th>Genomic (5'UTR+ORF+3'UTR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtSAMDC1/AtSAMDC2</td>
<td>46.2</td>
<td>46.5</td>
<td>78.1</td>
<td>44.1</td>
<td>61.6</td>
</tr>
<tr>
<td>AtSAMDC3/AtSAMDC4</td>
<td>44.0</td>
<td>7.2</td>
<td>57.5</td>
<td>25.4</td>
<td>33.7</td>
</tr>
<tr>
<td>AtSAMDC1/AtSAMDC3</td>
<td>49.0</td>
<td>47.4</td>
<td>63.1</td>
<td>46.5</td>
<td>54.3</td>
</tr>
<tr>
<td>AtSAMDC2/AtSAMDC3</td>
<td>42.4</td>
<td>48.2</td>
<td>63.9</td>
<td>49.2</td>
<td>57.9</td>
</tr>
<tr>
<td>AtSAMDC1/AtSAMDC4</td>
<td>42.7</td>
<td>6.1</td>
<td>53.4</td>
<td>30.4</td>
<td>31.5</td>
</tr>
<tr>
<td>AtSAMDC2/AtSAMDC4</td>
<td>44.9</td>
<td>7.9</td>
<td>52.9</td>
<td>25.5</td>
<td>35.3</td>
</tr>
<tr>
<td>AtSAMDC1/AtSAMDC5</td>
<td>48.2</td>
<td>0.8</td>
<td>50.8</td>
<td>18.6</td>
<td>27.9</td>
</tr>
<tr>
<td>AtSAMDC2/AtSAMDC5</td>
<td>39.1</td>
<td>1.0</td>
<td>50.2</td>
<td>22.0</td>
<td>32.6</td>
</tr>
<tr>
<td>AtSAMDC3/AtSAMDC5</td>
<td>50.3</td>
<td>1.1</td>
<td>45.6</td>
<td>24.8</td>
<td>33.1</td>
</tr>
<tr>
<td>AtSAMDC4/AtSAMDC5</td>
<td>42.1</td>
<td>7.4</td>
<td>48.3</td>
<td>10.3</td>
<td>43.1</td>
</tr>
</tbody>
</table>
Structure and biochemical properties of SAMDCs

Plants as well as mammalian SAMDCs are characterized by the presence of several highly conserved amino acid sequences related to the pro-enzyme cleavage, rapid turnover of the enzyme, and Put activation of the SAMDC pro-enzyme. It should be pointed out that plant SAMDCs are not activated by Put in majority of cases except for a few (Suresh and Adiga 1977; Yang and Cho 1991; Stanley et al. 1994; Xiong et al. 1997). Several plant SAMDCs including potato, spinach, Tritordeum (amphiploid between *Hordeum chilense* and *Triticum turgidum* var. *durum*), carnation, rice, wheat, Arabidopsis, soybean, carrot, grapevine and others have been characterized to varying degrees (Mad Arif et al. 1994; Bolle et al. 1995; Dresselhaus et al. 1996; Lee et al. 1997; Li and Chen 2000a, 2000b; Franceschetti et al. 2001; Tian et al. 2004; Varma 2003; Tassoni et al. 2007).

Cloning and characterization of a full length spinach (*Spinacia Oleracea*) SAMDC cDNA (Bolle et al. 1995) revealed the presence of certain signature sequences that are specific to SAMDC, e.g. putative PEST sequence (TIHITPEGFSYASFE) required for rapid turnover of the enzyme found at residues 243-258, pro-enzyme cleavage site (LSESSLFI) at residues 66-73 and catalytic site having residues Glu9, Glu12, Cys83, besides other conserved sequences observed in yeast or mammalian SAMDC (Pajunen et al. 1988; Kashiwagi et al. 1990; Bale and Ealick 2010). A comparison of potato SAMDC amino acid sequences with human, golden hamster and bovine showed sequence identities ranging between 35-36% only. However, several highly conserved regions including pro-enzyme cleavage site (LSESSLFI), and putative PEST sequence were identified by the authors (Mad Arif et al. 1994). The Km value of carnation (*Dianthus*
caryophyllus) SAMDC for SAM was found to be 26.3 μM (Lee et al. 1997). The optimum temperature and pH for this enzyme are 35°C and 8.0, respectively. The Km values reported for other SAMDCs e.g. corn, Chinese cabbage, soybean SAMDC1 and SAMDC2 are 5 μM, 38 μM, 8.1 μM and 16.0 μM, respectively (Suzuki and Hirasawa 1980; Yamonoha and Cohen 1985; Yang and Cho 1991; Choi and Cho 1994).

Arabidopsis SAMDC1, a heterotetramer (α2β2) with a molecular mass of 40.3 kDa, is comprised of 366 residues (Park and Cho 1999). The observed Km value for SAM was 23.1 μM and Ki for methylglyoxal bis-guanylhydrazone (MGBG; an inhibitor of SAMDC) was 0.15 μM. A comparison of all AtSAMDC proteins in relation to location of different residues responsible for their processing and functionality is given in Table 6.

Two wild carrot (Daucus carota - Queen Anne’s Lace) SAMDCs that have been cloned and sequenced show 98% identity of nucleotides and 100% identity of amino acids with a molecular mass of 40 kDa (Varma 2003). The residues 1-70 constitute the small subunit (β) and residues 71-361 form the large subunit (α) in the DcSAMDC. Three dimensional modeling (Sayle 1995; http://molvis.sdsc.edu/protexpl/frntdoor.htm) of DcSAMDC shows it to be a monomeric enzyme just like the potato SAMDC. The active site of the enzyme includes residues Glu11, Glu14, Ser71, Cys85, Ser234 and His247 as per the report of Xiong et al. (1997) and Tolbert et al. (2001), who showed that these residues were critical for catalytic activity of human SAMDC. The derived sequence of DcSAMDC showed the presence of highly conserved motifs, including the residues YVLSE↓SS (position 66-72), which is considered to be a signature pattern for SAMDC and is the putative cleavage site for processing. The highly conserved PEST sequence was found at residues 245-260 in carrot.
<table>
<thead>
<tr>
<th>SAMDCs</th>
<th>PEST site (bp in parenthesis indicate corresponding ORF sequence)</th>
<th>Putative cleavage site (bp in parenthesis indicate corresponding ORF sequence)</th>
<th>Catalytic site conserved residue</th>
<th>Putative active cleft residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtSAMDC1</td>
<td>TIHVT PEDGFSY ASFE (244-259 bp)</td>
<td>YVL SEJ SS (64-70 bp)</td>
<td>Glu9, Glu12, Cys83</td>
<td>Phe8, Glu9, Glu12, Leu66, Glu68, Ser69, Ser70, Cys83, Phe227, Cys230, Ser233, His246, Thr248, Glu250</td>
</tr>
<tr>
<td>AtSAMDC2</td>
<td>TIHVT PEDGFSY ASFE (243-258 bp)</td>
<td>YVL SEJ SS (64-70 bp)</td>
<td>Glu9, Glu12, Cys83</td>
<td>Phe8, Glu9, Glu12, Leu66, Glu68, Ser69, Ser70, Cys83, Phe226, Cys229, Ser232, His245, Thr247 and Glu249</td>
</tr>
<tr>
<td>AtSAMDC3</td>
<td>TIHVT PEDGFSY ASFE (246-261 bp)</td>
<td>YVL SEJ SS (64-70 bp)</td>
<td>Glu9, Glu12, Cys83</td>
<td>Phe8, Glu9, Glu12, Leu66, Glu68, Ser69, Ser70, Cys83, Phe229, Cys232, Ser235, His248, Thr250 and Glu252</td>
</tr>
<tr>
<td>AtSAMDC4</td>
<td>TIHVT PEDGFSY ASFE (248-263 bp)</td>
<td>YVL SEJ SS (62-68 bp)</td>
<td>Glu7, Glu10, Cys81</td>
<td>Phe6, Glu7, Glu10, Leu64, Glu66, Ser67, Ser68, Cys81, Phe231, Cys234, Ser237, His250, Thr252 and Glu254</td>
</tr>
<tr>
<td>AtSAMDC5</td>
<td>none</td>
<td>YLLSA J SS (56-62 bp)</td>
<td>Glu9, Glu12, Cys83</td>
<td>none</td>
</tr>
</tbody>
</table>
Four substitutions in potato vs. hSAMDC (Arg18/Leu13, Arg114/Phe111, Val118/Asp174 and His294/Phe285) and carrot vs. hSAMDC (Arg16/Leu13, Arg112/Phe111, Val 179/Asp174 and Gln292/Phe285) were observed. Several highly conserved residues (Phe10, Glu11, Glu14, Leu68, Glu70, Ser71, Ser72, Cys85, Phe228, Cys231, Ser234, His247, Thr249 and Glu251) were identified in the protein structure model of DcSAMDC at the active site. Other conserved residues (Asp174, Glu178 and Glu256) involved in Put binding in hSAMDC (Stanley and Pegg 1991) were also found in DcSAMDC, e.g. Asp177, Glu183 and Glu260.

The two different apple SAMDCs (MdSAMDCl and MdSAMDC2) are 39.8 kDa and 40.7 kDa molecular mass proteins and share 70% similarity in amino acid sequences (Hao et al. 2005). Several conserved sequences associated with turnover, processing and catalytic activity of SAMDC are conserved in MdSAMDCs. High degree of sequence identities and similarities in SAMDC structure and presence of conserved amino acid sequences were also observed in a study of Vitis SAMDC by Tassoni et al. (2007).

Current status of research on microbial and animal SAMDCs is summarized by Bale and Ealick (2010).

**Genomic organization of SAMDC genes**

Most plants contain multiple copies of SAMDC genes, whose coding sequences show a high degree of homology with each other, but little is known about their expression in various tissues/cells in the plant and about the regulation of their expression, translation of their mRNAs, and turnover of these proteins. A major difference in gene organization between mammalian and plant SAMDCs is the presence of introns in the former and their absence in the latter (Larsson and Rasmuson-Lestander 1997; Nishimura et al. 1999).
The length of 5′UTRs (based on genomic sequences) in plant SAMDCs varies between 366 and 1570 bp with a few exceptions where a very small 5′UTR was discernible (Table 7). The longest 5′UTR for a plant SAMDC reported is 1570 bp in potato, the smallest being 12 bp in spinach (Spinacia oleracea) and AtSAMDC5. The ORFs of different SAMDCs show a high degree of sequence identities at DNA level as well as in the sequences of amino acids (Franceschetti et al. 2001; Hao et al. 2005; Tassoni et al. 2007). The length of the 3′UTR varies between 117-304 bp in majority of the plant species studied, except for a few where relatively small 3′UTRs were observed without any ORF (Table 7).

Overlapping highly conserved ‘small’ and ‘tiny’ upstream ORFs (uORFs) have been considered as characteristic components of 5′UTRs in most plant SAMDCs (Table 7) and are presumed to regulate the translation of SAMDC mRNAs, and perhaps also the expression of SAMDC genes in plants. Presence of an uORF has also been observed in the 5′UTR of mammalian SAMDCs that is apparently involved in the regulation of its mRNA translation in response to elevated levels of PAs. Unlike plants, mammalian SAMDC has only one uORF that is located close (14 bp downstream) to the 5′ cap and encodes for a hexapeptide MAGDIS (Law et al. 2001). Similar uORFs have also been reported from other organisms including metazoa, Pezizomycotina fungi and Chlamydomonas but not observed in Chlorella and prasinophyte algae (Ivanov et al. 2010). The authors also reported the presence of longer tiny uORF in the green algae with a greater overlap (as compared to higher plants) with the small uORF and lack of terminal Pro residue in case of mammalian and sea squirt SAMDC.
Table 7. Genomic organization of SAMDC genes from different plant species those are phylogenetically related to Arabidopsis thaliana SAMDC (Urano et al. 2003; Tassoni et al. 2008). Abbreviations used: **At**= Arabidopsis thaliana; **Os**= Oryza sativa; **St**= Solanum tuberosum; **Dc**= Daucus carota; **Zm**= Zea mays; **Bj**= Brassica juncea; **Dec**= Dendrobium crumenatum; **Ps**= Pisum sativum; **Vf**= Vicia faba; **So**= Spinacia oleracea; **Dic** = Dianthus caryophyllus; **Gm** = Glycine max; **Hv**= Hordeum vulgare.

<table>
<thead>
<tr>
<th>Genus</th>
<th>5'UTR (bp)</th>
<th>Tiny uORF (bp)</th>
<th>Small uORF (bp)</th>
<th>ORF (bp)</th>
<th>3'UTR (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtSAMDC1</td>
<td>1106</td>
<td>691-702 (12)</td>
<td>702-865 (156)</td>
<td>1101</td>
<td>238</td>
</tr>
<tr>
<td>AtSAMDC2</td>
<td>837</td>
<td>553-564 (12)</td>
<td>564-719 (156)</td>
<td>1089</td>
<td>193</td>
</tr>
<tr>
<td>AtSAMDC3</td>
<td>876</td>
<td>none</td>
<td>468-635 (168)</td>
<td>1050</td>
<td>180</td>
</tr>
<tr>
<td>AtSAMDC4</td>
<td>108</td>
<td>none</td>
<td>none</td>
<td>1044</td>
<td>447</td>
</tr>
<tr>
<td>AtSAMDC5</td>
<td>12</td>
<td>none</td>
<td>none</td>
<td>1164</td>
<td>52</td>
</tr>
<tr>
<td>OsSAMDC1</td>
<td>539</td>
<td>197-205 (9)</td>
<td>205-360 (156)</td>
<td>1197</td>
<td>300</td>
</tr>
<tr>
<td>OsSAMDC2</td>
<td>536</td>
<td>212-220 (9)</td>
<td>220-378 (156)</td>
<td>1188</td>
<td>247</td>
</tr>
<tr>
<td>StSAMDC</td>
<td>1570</td>
<td>1105-113 (9)</td>
<td>1113-1248, 1354-1374 (156)</td>
<td>1083</td>
<td>251</td>
</tr>
<tr>
<td>DcSAMDC</td>
<td>466</td>
<td>142-150 (9)</td>
<td>150-311 (162)</td>
<td>1086</td>
<td>209</td>
</tr>
<tr>
<td>ZmSAMDC</td>
<td>366</td>
<td>38-46 (9)</td>
<td>46-198 (153)</td>
<td>1203</td>
<td>304</td>
</tr>
<tr>
<td>BjSAMDC1</td>
<td>397</td>
<td>139-150 (12)</td>
<td>150-305 (156)</td>
<td>1107</td>
<td>149</td>
</tr>
<tr>
<td>BjSAMDC2</td>
<td>411</td>
<td>129-140 (12)</td>
<td>140-298 (159)</td>
<td>1110</td>
<td>155</td>
</tr>
<tr>
<td>DecSAMDC</td>
<td>538</td>
<td>216-224 (9)</td>
<td>224-376 (153)</td>
<td>1110</td>
<td>246</td>
</tr>
<tr>
<td>PsSAMDC</td>
<td>548</td>
<td>194-202 (9)</td>
<td>202-366 (165)</td>
<td>1062</td>
<td>219</td>
</tr>
<tr>
<td>VfSAMDC</td>
<td>562</td>
<td>210-218 (9)</td>
<td>218-382 (165)</td>
<td>1062</td>
<td>171</td>
</tr>
<tr>
<td>SoSAMDC</td>
<td>6</td>
<td>none</td>
<td>none</td>
<td>1092</td>
<td>81</td>
</tr>
<tr>
<td>DicSAMDC1</td>
<td>472</td>
<td>144-152 (9)</td>
<td>152-316 (165)</td>
<td>1146</td>
<td>117</td>
</tr>
<tr>
<td>DicSAMDC2</td>
<td>502</td>
<td>148-156 (9)</td>
<td>156-314 (159)</td>
<td>1134</td>
<td>193</td>
</tr>
<tr>
<td>GmSAMDC</td>
<td>556</td>
<td>213-221 (9)</td>
<td>221-382 (162)</td>
<td>1068</td>
<td>200</td>
</tr>
<tr>
<td>HvSAMDC</td>
<td>512</td>
<td>187-195 (9)</td>
<td>195-344 (150)</td>
<td>1182</td>
<td>199</td>
</tr>
</tbody>
</table>
Analysis of cDNA sequences of plant and human *SAMDCs* shows the presence of an extended C-terminus of the monocot enzyme as compared to the dicot and the human SAMDCs. Sequence analyses of the 5’UTRs of *SAMDCs* from different plant species reveal that the lengths of the tiny and small uORF vary between 9-12 bp and 150-168 bp, respectively. Distribution of tiny and small uORFs can range from 38-865 bp upstream of the translation start site among different species depending on the lengths of 5’UTRs. The tiny uORF codes for a peptide of 2-3 amino acids whereas the small uORF codes for a protein 48-55 amino acids long (Franceschetti et al. 2001). The last base of the tiny uORF overlaps with the first base of the small uORF.

Phylogenetic analysis (based on Urano et al. 2003; Tassoni et al. 2007; Wang et al. 2010) of Arabidopsis *SAMDC* gene family and its closest homologues reveals that the length of the *SAMDC* 5’UTRs in Arabidopsis varies between 12 to 1106 bp (Table 7). While *AtSAMDC1*, *AtSAMDC2* and *AtSAMDC3* have relatively long 5’UTRs (876 to 1106 bp), *AtSAMDC4* has a much shorter 5’UTR (108 bp) and *AtSAMDC5* has the smallest 5’UTR of 12 bp. While *AtSAMDC3* contains only the small uORF, *AtSAMDC4* and *AtSAMDC5* do not have any uORFs in their 5’UTRs. The length of 3’UTRs of *AtSAMDC1*, *AtSAMDC2* and *AtSAMDC3* ranges from 180 to 238 bp, whereas *AtSAMDC4* has a 3’UTR of 446 bp and *AtSAMDC5* has the shortest 3’UTR (68 bp).

In Arabidopsis, the involvement of *SAMDC* 5’UTR uORFs in translational regulation of SAMDC was experimentally demonstrated by Hanfrey et al. (2005) and confirmed later by Ivanov et al. (2010). The translational control of the main ORF of *SAMDC* by tiny and small uORFs of 5’UTR is PA dependent. Under low PA conditions, the AUG of the tiny uORF is recognized by scanning 43S pre-initiation complex, which results in
ribosome binding at this site and its translation. After the translation termination, 43S pre-initiation complex can reinitiate translation of the main ORF of the \textit{SAMDC} mRNA, thus up-regulating the cellular PAs. Under higher cellular PA concentration, the scanning 43S pre-initiation complex skips the AUG of the tiny uORF and binds to the AUG of the small uORF. Translation of the small uORF in turn represses the translation of the main \textit{SAMDC} ORF; this results in down regulation of SAMDC production and cellular PAs.

The role of introns within the 5'UTR in the regulation of \textit{SAMDC} gene expression and its post transcriptional modification have been demonstrated in Arabidopsis transformed with different mutation or deletion constructs of \textit{Brassica juncea} (\textit{BjSAMDC2}) 5'UTR fused with the reporter gene 35S::\textit{GUS} gene (Hu et al. 2005b). Absence of introns in the 5'UTR negatively affected \textit{GUS} transcripts as well as GUS activity, and differentially regulated \textit{GUS} in response to exogenous PAs and abiotic stresses. The role of introns in regulating gene expression for other plant genes has been reported by other workers, and it is believed that the introns are involved in RNA processing and escape of mRNA from translational repression pathway in the nucleus (Braddock et al. 1994; Jeong et al. 2008; Rose 2008). Deletion of 5'UTR of \textit{BjSAMDC2} increased \textit{GUS} transcripts as well as the presence of GUS activity in transgenic Arabidopsis using the native \textit{BjSAMDC2} promoter. Particularly, mutation of the tiny uORF within the 5'UTR abolished translational repression of GUS. Recent sequence analysis of a wide range of organisms having uORF in their 5'UTR reveal that the tiny uORF most likely originated from chlorophytan algae where such uORFs are longer and have greater overlaps with small uORFs (Ivanov et al. 2010). Deletion of tiny uORF was also shown to increase SAMDC
activity significantly, resulting in disruption of PA homeostasis affecting plant growth and development (Hanfrey et al. 2002; Franceschetti et al. 2004; Tassoni et al. 2007).

The uORFs in the 5'UTR of eukaryotic mRNA can also play an important role in post-transcriptional gene regulation. Many eukaryotic mRNAs (20-40%) have been found to have uORF (Hayden and Bosco 2008; Tran et al. 2008) and can negatively regulate the expression of the main ORF either directly inhibiting translation or transcript destabilization. The uORF can also act in nonsense-mediated decay (NMD) of aberrant mRNAs containing premature termination codons (Muhlemann 2008; Rebbapragada and Lykke-Anderson 2009; Silva and Romao 2009); the induction of NMD by uORFs is dependent on the size of the uORF; >50 amino acids is more active (Nyiko et al. 2009).

The length of the promoter region necessary to drive the expression of different members of the AtSAMDC gene family in relation to development or stress responses is not fully understood. No direct studies have been reported on the characterization of the AtSAMDC promoters, while bioinformatics information about the presence of regulatory motifs of different types has been discussed (Urano et al. 2003). Based on the genomic map of Arabidopsis available from NCBI or TAIR, the neighboring genes of AtSAMDC1, AtSAMDC2 and AtSAMDC4 are close to 700-800 bp upstream. For AtSAMDC3 and AtSAMDC5 these genes are >3 kb bp upstream of the transcription start site.

In other plants, only a few studies have been published about the sequences that constitute a functional SAMDC promoter or its regulation during development. A 2322 bp sequence (upstream of transcription initiation site) including the putative promoter region and the 5'UTR of BjSAMDC2 fused with the reporter gene GUS showed moderate expression of GUS in the rosette leaves of 3 week old Arabidopsis plants (Hu et al. 2003).
2005b). These authors did not define the precise length of the promoter. In carnation (*Dianthus caryophyllus*), the *SAMDC* promoter has been studied extensively in relation to developmental expression and also in response to diurnal changes. When an 1821 bp upstream (of transcription start site) promoter sequence + 5'UTR was fused with *GUS* and used to transform tobacco (*N. tabacum*), transgenic plants showed high activity of GUS in different flower parts including stamens, pollen, stigma and petals (Kim et al. 2004). Several *cis*-regulatory elements (e.g. ABA, GA, auxin-responsive elements and others associated with abiotic and biotic stresses) located upstream region around -273 and -158 bp were identified in the promoter for pollen specific expression. Analysis of this region also showed presence of other putative *cis*-regulatory elements related to hormonal responses. In another study, Kim et al. (2006) showed that the promoter region of carnation *SAMDC* contains certain sequences e.g. ‘GGTAAT’ (GT-1 consensus sequence), ‘CAACTTCATC’ that positively regulates and the sequence ‘TAATAATTA’ (located between -754 and -274 bp upstream of transcription start site) that negatively regulates the circadian expression of carnation *SAMDC*. In tobacco, a 1824 bp upstream promoter + 5'UTR sequence of *SAMDC* fused with *GUS* showed low level of GUS activity throughout the (4-week-old) transgenic seedlings, but was highly induced in the roots and leaves upon exposure to salt and acidic stresses (Wi et al. 2006).

**Mutants of *S*-adenosylmethionine decarboxylase**

Mutants are useful tools to study physiological and developmental roles of genes in plants and other organisms. In this regard while the mutants of ADC and APS have been well characterized, those of *SAMDC* have not been well studied in plants. A part of the reason may be the number of genes that encode a functional SAMDC enzyme. Reported
information on a few *SAMDC* mutants is presented in Table 2; the relevant work on Arabidopsis is summarized here.

An Arabidopsis *SAMDC4* mutant called *bud2* was identified by Ge et al. (2006) that had somewhat higher Put and lower Spd and Spm than the WT plants. Besides altered PA homeostasis, the mutant had bushy and dwarf phenotype with curly leaves in seedlings, altered petiole length, and higher number of lateral roots. Enlarged vasculature was also observed in the roots, petioles and inflorescences with higher lignin deposition. The *bud2* mutant plants exhibited altered hypocotyl elongation and lateral bud outgrowth as compared to the WT plants; this was explained later as an effect of altered auxin-mediated response in this mutant (Cui et al. 2010). A knockdown mutant of *AtSAMDC1* showed similar phenotype to that of *samdc4*; whereas the phenotype was more severe, there was a compensatory increase in the expression of *AtSAMDC4* and vice versa (Ge et al. 2006). Although knockdown mutant of either *samdc4* or *samdc1* individually did not result in embryo lethality; double mutant *samdc4/samdc1* was embryo lethal.

Several other mutants show altered SAMDC activity but are not direct mutants of *SAMDC*. For example, a tobacco mutant cell line resistant to SAMDC inhibitor MGBG was produced by Malmberg and McIndoo (1983). Regenerated plants produced from this cell line showed significantly higher Spd (4.7-fold) and Spm (~7-fold) contents along with a small increase in Put content in the leaves. The flowers of these plants though had morphology to WT lacked pollen grains in the anther and showed male sterility; the gynoecia were fertile. In a later analysis of a male sterile stamenless-2 (*sl-2/sl-2*) mutant of tomato, higher activity of SAMDC was observed in sepals, petals and stamens as compared to the WT plants (Rastogi and Sawhney 1990). In both lines, SAMDC activity
was higher in the stamens in comparison to other floral parts. Polyamine contents varied among different floral organs within the same line and among control and the mutant in different organs. Highest amount of Put was observed in the gynoecium for both WT and mutant plants. A T-DNA insertion mutants of MGBG-resistant tobacco cell lines showed significantly higher SAMDC activity (by 100-138%) and higher Put (2-fold) and Spd (~1.5-fold) in regenerated plants vs. the WT plants (Fritze et al. 1995). Plantlets regenerated from the MGBG resistant calli showed shorter internodes, reduced petal numbers, and altered petal shape of the flowers; and they were male sterile.

**Transgenic manipulation of polyamines via S-adenosylmethionine decarboxylase**

While mutants provide a useful tool to study physiological and developmental roles of genes in plants and other organisms; they generally do not allow experimental manipulations under the investigator’s control. Moreover, most mutants involve down-regulation of a step; few being the up-regulation type. In this regard, genetic manipulation involving constitutive or inducible/tissue-specific promoters provides complementary approach to regulate specific steps in metabolism and correlate them with a phenotype. Several attempts have been made over the years to genetically manipulate PA metabolism in plants using homologous or heterologous *SAMDC* genes under the control of both constitutive and inducible promoters (see summary in Table 3C). It should however be kept in mind that any change in PAs in response to SAMDC up or down regulation must depend upon the activity of APTs, which in most of these studies was not experimentally measured or manipulated. Furthermore, most studies targeting Put overproduction do not show parallel increases in either Spd or Spm, suggesting that the cellular contents of these PAs are more tightly regulated than that of the diamine Put. A
part of the explanation may lie in the fact that SAMDC itself is a highly regulated enzyme of the PA biosynthetic pathway.

Transgenic expression of a human *SAMDC* (*hSAMDC*) under 35S promoter in tobacco showed 2- to 4-fold increase in SAMDC activity accompanied by a 2- to 3-fold increase in Spd with a concomitant reduction in Put in the transgenic plants (Noh and Minocha 1994). Leaf discs of the transgenic plants produced shoots in addition to callus when cultured in a callus induction medium containing 0.5 μM BA + 0.5 μM NAA, which was not observed in WT plants. Mature plants had no noticeable change in phenotype. Later studies with carrot (*Daucus carota*) using the same promoter::*hSAMDC* gene combination showed that the transgenic cell cultures had significantly higher SAMDC activity (6- to 15-fold) and Spd content (by 1.5- to 2.0-fold) accompanied by a slightly elevated Put level without any significant change in Spm content (Bastola 1994). The frequency of somatic embryogenesis in transgenic cells was much higher than the control cells, and the transgenic somatic embryos were morphologically stouter (thicker hypocotyl and cotyledons) than the WT controls; several of them developed into normal plants that produced viable seeds.

Transgenic expression of a homologous *StSAMDC* cDNA under the control of 35S promoter in antisense orientation in potato (*Solanum tuberosum*) showed reduction of SAMDC activity by 10-28% accompanied by a decrease in Put, Spd, and Spm contents in the transgenic plants. Transgenic plants displayed abnormal phenotypes which included stunted growth, more branching, smaller leaves, poor root growth, and early senescence both in vitro and under glass house conditions (Kumar et al. 1996). An increase in ethylene production was also observed in these plants. In potato leaf explants, induction
of *SsSAMDC* with tetracycline-inducible promoter showed 2- to 6-fold increase in *SAMDC* transcripts upon induction with a concomitant increase in SAMDC activity and the contents of Spd, Spm, and Put (Kumar et al. 1996). Transgenic leaf explants over-expressing the same gene under 35S promoter failed to regenerate plantlets, while antisense lines regenerated a few plants with severe abnormalities (Kumar et al. 1996).

Over-expression of the same potato *SAMDC* under the control of a tuber-specific (patatin) promoter showed increase in *SAMDC* transcripts, SAMDC activity, and Spd content (by 85%) in the transgenic potato tubers (Pedros et al. 1999). Larger numbers of smaller tubers were observed in the transgenic lines at maturity stage as compared to the WT plants. Antisense expression of *SAMDC* under the same promoter showed reduced *SAMDC* transcripts and SAMDC enzyme activity accompanied by a decrease in total PAs (by 30-40%) in the tubers without any phenotypic changes in the plants and tubers.

Heterologous expression of a *Datura stramonium* *SAMDC* (*DsSAMDC*) cDNA under the control of maize *Ubi-1* promoter in rice (*Oryza sativa*) resulted in small (1.5- to 2.5-fold) increase in Spd concentration in the leaves, with no apparent phenotypic changes (Thu-Hang et al. 2002). A maximum increase of 3-fold activity of SAMDC was observed in the transgenic plants. The native transcripts of rice *SAMDC* or *SPDS* maintained a steady-state level, indicating the lack of feedback regulation of *SAMDC* expression in rice leaves; the seeds also accumulated more Spd (2.5-fold) and Spm (2-fold) without any observed change in phenotype in the transgenic plants compared to WT plants.

Tomato (*Lycopersicon esculentum*) plants have been the target of several studies on transgenic expression of a heterologous *SAMDC* under different promoters in order to analyze both metabolic and developmental changes in the plants and their fruits. Mehta et
al. (2002) found that transgenic expression of yeast *SAMDC* under fruit specific promoter E8 resulted in higher accumulation of Spd and Spm in the ripened fruits whereas all three PAs (Put, Spd and Spm) decreased in the ripened WT fruits. Transgenic fruits showed a considerable increase in lycopene content, prolonged vine life, and an overall improvement in juice quality vs. the WT fruits.

In another study using *E8* promoter and yeast *SAMDC*, Mattoo et al. (2006) observed a greater accumulation of Spd and Spm and several other metabolites (such as Gln, Asn, choline, citrate, fumarate, malate, etc.) in transgenic tomato fruits ripened off the vines as compared to the WT red fruits that ripened on the vine. A significant decrease in Val, Asp, sucrose and glucose was observed in the vine-ripened fruits vs. the WT fruits at the same ripening stage. A positive correlation (p>0.8) was observed between intracellular concentrations of Spd-Spm and several metabolites including Gln, Asn, Ala, Thr, Ile, choline, citrate, fumarate and malate. Alternatively, intracellular Put showed a negative correlation (p<0.8) with these metabolites in the transgenic fruits (Handa and Mattoo 2010). Put positively affected the activity of ACC oxidase, whereas Spd-Spm had negative effect on this enzyme that was reflected accordingly at the transcript level. Several heat-shock proteins showed a positive correlation with Spd-Spm and a negative correlation with Put, which was similar at the transcript level. An overall activation of N:C sensing/signaling genes, e.g. NADP-dependent isocitrate dehydrogenase and phosphoenolpyruvate carboxylase, were observed concomitant with higher Spd and Spm in the transgenic fruits in comparison to the WT fruits.

In a more recent study in transgenic tomato with E8 promoter::*SAMDC* combination (same as Mehta et al. 2002), alterations of fatty acid metabolism with a significant
increase in omega-3 fatty acids at the expense of other lipids in the ripening transgenic fruits was reported (Kolotilin et al. 2011). Transcript analysis of the fruits revealed significant changes in gene expression mainly associated with carbohydrate, amino acid, and protein metabolism. This observation was similar to what was observed by Mattoo et al. (2006). Other stress related genes were also up-regulated in the transgenic fruits. Cheng et al. (2009) used the same yeast SAMDC gene but constitutively expressed it under 35S promoter in tomato and found a 2.4-fold higher content of Spd and 1.7-fold higher Spm in the leaves of transgenic plants. When subjected to high temperature (38°C) stress, transgenic plants showed greater resistance than the WT plants.

Waie and Rajam (2003) reported heterologous expression of a hSAMDC gene under 35S promoter in tobacco (N. tabacum). Transgenic plants showed a significant increase in total SAMDC activity (50-200%), 21-240% increase of Spd in the conjugated fraction (with a small but significant increase in free Spd only in a few lines) and 50-360% increase of Put in free, conjugated and bound fractions of leaf samples. Regeneration of shoots from leaf explants in the transgenics showed a 40-45 d delay as compared to the untransformed control plants and an overall slow growth with phenotypic abnormalities including shorter internode was observed in the transgenic plants. When subjected to 200 mM NaCl stress, transgenic plants showed higher germination percentage and better seedling growth compared to the WT plants.

In a more recent study, transgenic tobacco plants expressing an antisense cDNA of DsSAMDC (Torrigiani et al. 2005) under 35S promoter showed a decrease in SAMDC activity in the shoots with increased ratio of Put to Spd in the T1 transformants. A significant decrease in rhizogenic potential of leaf explants obtained from antisense
plants was observed as compared to the WT plants. Wi et al. (2006) reported a 2- to 3-fold increase in total PAs in response to transgenic expression of a carnation SAMDC (under 35S promoter) in tobacco; total number of seeds and seed weight, and net photosynthetic rates were higher in the transgenic plants. Whereas there were no morphological changes, the transgenic plants showed greater tolerance to salt and oxidative stresses; also, APX, manganese superoxide dismutase (Mn-SOD) and glutathione S-transferase (GST) genes were highly up regulated in response to oxidative stress. In mature plants, 200 mM NaCl treatment for 8 wk caused severe reduction in the size of leaves accompanied by leaf chlorosis and overall growth inhibition in WT tobacco plants. Higher net leaf photosynthesis was observed in the transgenic plants in the presence of NaCl. At higher concentration of salt (400 mM), WT plants died, whereas transgenic plants survived but their overall health was severely affected. Cold treatment (4°C) for 24 h followed by subsequent recovery showed greater chilling tolerance in the transgenic plants vs. the WT plants.

Down-regulating the expression of SAMDC in N. tabacum via SAMDC-RNAi resulted in a >10-fold decrease in the expression and activity of SAMDC (Moschou et al. 2008b). This was accompanied by a significant increase in ADC activity and reduction of Put (5-30%), Spd (33-73%) and Spm (65-100%). The SAMDC-RNAi plants when grown in vitro and supplemented with 200 mM NaCl showed lesser tolerance and decreased biomass as compared to the WT plants. No significant changes were observed in the catabolic activity of PAs or the levels of Spd in the apoplast upon salt stress.
Besides plants, transgenic animals over-expressing homologous or heterologous \textit{SAMDC} have also been produced to study the regulation and role of higher PAs in metabolic and developmental regulation (reviewed in Alhonen et al. 2009).

\textbf{Expression patterns of S-adenosylmethionine decarboxylase genes in plants}

\textit{Organ and tissue specific expression}

Five different approaches, namely northern hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR), quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR), in situ hybridization, and promoter::reporter fusion have been employed to study gene expression of the four \textit{AtSAMDC} genes in Arabidopsis. Each shows different level of specificity for expression at tissue, organ and cell levels. In addition, microarray data on the expression of some of these genes are available. Still, the information is quite sporadic and inconsistent, thus leading to inconclusive assessment of their role during development or in response to stress. A part of the problem lies in the high degree of sequence similarity among the \textit{AtSAMDC} family members (Table 5).

Northern blot analysis of \textit{StSAMDC} transcripts in potato showed highest level of transcripts in the younger and actively dividing tissues as compared to the mature and non-dividing vegetative and reproductive tissues (Mad Arif et al. 1994). Higher \textit{SAMDC} transcripts were also observed in petals and stems of carnation plants (Lee et al. 1997). In pea (\textit{Pisum sativum}) plants, northern blot analysis of \textit{SAMDC} transcripts showed higher accumulation in young seedlings in comparison to its lower level in older senescing tissues (Marco and Carrasco 2002).

Expression of \textit{SAMDC} and the role of higher PAs in somatic embryogenesis was studied in our lab using carrot (\textit{D. carota}) suspension cultures by QRT-PCR. Higher
expression was observed in cultures undergoing somatic embryogenesis (Chretien 2003); moreover, the expression was greater at early stages of embryo development vs. the later stages. When embryos at different stages of development collected from the same culture at day 24 were analyzed for DcSAMDC1 and DcSAMDC2 transcripts, both genes were expressed similarly. The late stage embryos (larger than 1.7 mm size) showed a decline in SAMDC expression. The relative amount of ADC transcripts was lower than SAMDC transcripts at all stages of embryo development; in the mature plants, the highest levels of DcSAMDC transcripts were found in the leaves and the lowest in the callus.

Using the approach of promoter::GUS fusion, Kim et al. (2004) showed that a 1821-bp upstream promoter region and 5'UTR sequence of carnation SAMDC showed moderate GUS activity in the stems and cotyledonary veins of young tobacco seedlings. In mature plants, high activity of GUS was observed in the stamens, pollen, stigma and petals of open flowers besides moderate expression in the ovaries. Several pollen-specific regulatory elements e.g. AGAAA and GTGA located in the promoter region between -273 bp and -158 bp upstream of transcription start site were identified; also some putative intron sequences present in the 5'UTR of the gene were suggested to regulate pollen-specific expression of GUS. Other features of the promoter region and their role are described above by Kim et al. (2006) and Wi et al. (2006).

The expression of the two known apple [Malus sylvestris (L.) Mill. var. domestica] SAMDCs (MdSAMDCs) in all tissues was studied by RNA gel blot analysis with higher expression being seen in reproductive organs vs. the vegetative organs (samples collected between 19-174 days after flowering) of mature plants (Hao et al. 2005). Expression of MdSAMDC1 was higher in the leaves as compared to MdSAMDC2; similar was the case
in fruits at early developmental stages. It was also reported that \textit{MdSAMDC1} expression was differentially regulated on different days of suspension cultures. In another study in bean (\textit{Phaseolus vulgaris}), high level of \textit{SAMDC} transcripts were observed ubiquitously in mature and young leaves, stems, and roots with relatively lower expressions in petals, stigma and filaments, and least in anthers and ovaries as seen by semi-quantitative RT-PCR (Jiménez-Bremont et al. 2006).

Expression of three members of the poplar (\textit{Populus nigra x maximowiczii}) \textit{pSAMDC} gene family was studied by QRT-PCR in our lab (Page et al. 2007) in two transgenic cell lines; one constitutively over-expressing a \textit{mODC} gene and the other a \textit{GUS} gene. While \textit{pSAMDC1} and \textit{pSAMDC3} were down regulated, expression of \textit{SAMDC2} showed up-regulation in the high putrescine (HP) cells as compared to the \textit{GUS}-transformed control cells. Overall activity of \textit{SAMDC} was also lower in HP cells vs. the control cells.

More recently, the potential role of \textit{SAMDC} in flower and early fruit development in olive (\textit{Olea europaea}) was studied by Gomez-Jimenez (2010a); open flowers showed 8-fold higher level of \textit{SAMDC} transcripts as compared to the closed ones. Leaves and shoots showed similar levels of transcripts as in fruits. A positive correlation was found between \textit{OeSAMDC} gene expression and cell division. Fluorescence \textit{in situ} hybridization showed high expression of \textit{OeSAMDC} in gynoecial tissue with no significant signal being seen in sepals, petals or stamens. Integuments, nucellus and inner epidermal tissues of ovules had higher \textit{OeSAMDC} transcripts. High level of \textit{OeSAMDC} and \textit{OeSPDS} transcripts were found to be co-localized in cells of fruit mesocarp and exocarp, irrespective of the fruit developmental stages, whereas no significant signal was detected in the endocarp or vascular bundles at any stage of fruit development. \textit{OeSAMDC} and
OeSPDS genes showed similar pattern of spatial and temporal expressions, although expression of OeSAMDC was relatively higher. High activities of SAMDC and ADC were also observed in open flowers followed by gradual decline after pollination; SAMDC activity was positively correlated with OeSAMDC transcripts. No significant change in SAMDC activity was observed at the early fruit development in the abscission zone (AZ) but SAMDC activity increased significantly in the AZ at later stage of fruit development. Activity of ODC was highest in the AZ of early fruit as compared to ADC or SAMDC, and ODC and ADC activities gradually declined at later stages (Gomez-Jimenez et al. 2010b). The content of free Put and Spd was higher in open flowers as vs. the closed ones; Spd being the highest in open flowers followed by Put and Spd. Changes in expressions of the SAMDC genes in GA3-induced fruit development has been studied in citrus (Citrus clementina) by Trénor et al. (2010); the expression of different members CcSAMDC gene family varied during the 21 d period of fruit development.

In Arabidopsis thaliana, the expression of AtSAMDC1 and AtSAMDC2 was first reported by Franceschetti et al. (2001). Semi-quantitative RT-PCR analysis showed high ubiquitous expression of AtSAMDC1 in all organs of mature plants, whereas expression of AtSAMDC2 was much lower compared to AtSAMDC1, and was mainly localized in the inflorescences and leaves of mature plants. Later, Urano et al. (2003), using semi-QRT-PCR showed high ubiquitous expression of AtSAMDC1 in all organs, whereas expression of AtSAMDC2 was mainly localized in the flowers, roots and cauline leaves. Semi-quantitative analysis of AtSAMDC genes through RNA gel blot analysis showed spatial variations in the expression of different members of the AtSAMDC gene family in mature plants (Ge et al. 2006). Ubiquitous expression of AtSAMDC1 was seen in all organs of
mature plants with relatively higher expression in the siliques. Expression of \textit{AtSAMDC2} was high in roots, leaves and flowers. Whereas \textit{AtSAMDC3} showed weaker expressions in almost all organs except for the siliques; expression of \textit{AtSAMDC4} was ubiquitous in all organs but at lower level than other \textit{AtSAMDCs}.

Microarray data (Genevestigator - www.genevestigator.com) mostly corroborate the constitutive presence of \textit{AtSAMDC1} transcripts in all organs and tissues of Arabidopsis with relatively higher expression in the roots and cotyledons of younger seedlings and in siliques; the highest expression is seen in flowers, especially in the pollen. Overall expression of \textit{AtSAMDC2} was relatively lower as compared to \textit{AtSAMDC1}; with highest expression in the pollen besides the cotyledons. On the other hand, expression of \textit{AtSAMDC3} seems to be much lower as compared to \textit{AtSAMDC1} and \textit{AtSAMDC2} with relatively higher expression in the stamens and cotyledons. \textit{AtSAMDC4} appears to be ubiquitously expressed, with higher expression in roots, pedicel and vascular tissues of rosette leaves but the signal intensities are much lower as compared to \textit{AtSAMDC1} and \textit{AtSAMDC2}. No data are available on the expression of \textit{AtSAMDC5} in the database.

\textit{Expression of SAMDC in response to abiotic stress and phytohormones}

The expression of \textit{SAMDC} has been shown to be regulated by different environmental stimuli. Light mediated up-regulation of \textit{PnSAMDC} was seen in the seedlings of \textit{Pharbitis nil} (Yoshida et al. 1998). Seedlings grown in dark when exposed to light showed up-regulation of \textit{SAMDC} expression within 15 min, reaching a peak by 45 min and gradually declining after 60 min. Further analysis showed similar results on exposure (from dark) to blue, green, red and UV light but not to far-red (FR) light (Yoshida et al. 1999). Expression of \textit{SAMDC} was also affected by blue and red light in the seedlings in
the presence of inhibitors associated with signaling pathways (Yoshida et al. 2002). Northern blot analysis showed up-regulation of \textit{PnSAMDC} by \textit{Ca}^{1+2} and calmodulin in red light whereas in blue light it was down-regulated. Changes in expression of PA biosynthetic genes in response to FR have also been studied by Jumtee et al. (2008) in phytochrome ‘A’ \textit{(phyA)} mutant plants. The different \textit{AtSAMDC}s showed differential responses upon exposure to FR in WT and the mutant plants. Expression of \textit{AtSAMDC2} significantly increased upon exposure to FR at 6 h with gradual decline thereafter, whereas \textit{phyA} plants did not show any change in \textit{AtSAMDC2} expression. On the other hand, expression of \textit{AtSAMDC4} decreased upon exposure to FR in WT and \textit{phyA} plants without change in the expression of \textit{AtSAMDC1} and \textit{AtSAMDC3}.

Song et al. (2001) reported high activity of SAMDC during pollen germination in tomato \textit{(Lycopersicon esculentum)} at 25°C which was significantly inhibited at 38°C (Song et al. 2002). On the other hand, activity of ADC increased at initial pollen germination stage but remained unaltered at higher temperature. Among the soluble PAs, Put content increased during the first 2 h of pollen germination and remained high thereafter; Spd and Spm also showed transient increments during the first hour of incubation. Elevated temperature did not affect Put content, but Spd and Spm content did not increase in either case. Conjugated PAs remained unaltered between control and high temperature treatments. Exogenous application of Spd and Spm were partially able to recover pollen germination and tube growth at 38°C, whereas Put was ineffective. Application of transcription inhibitor actinomycin D did not affect SAMDC activity or pollen germination, but cycloheximide (translation inhibitor) significantly inhibited SAMDC activity, pollen germination and tube growth. These findings demonstrate an
active synthesis of SAMDC during pollen germination. Reduction of pollen viability in tomato upon long term storage at -30°C has been suggested to be due to reduced activities of SAMDC and ADC (Song and Tachibana 2007) and lower PA biosynthesis.

Low temperature induction of SAMDC activity and cold tolerance has been reported in cucumber (Cucumis sativus) by Shen et al. (2000), more so in the chilling tolerant (T) cultivar at 12 h after chilling treatment. Activity of ADC was slightly induced at 12 h in the ‘T’ variety as compared to the chilling sensitive (S) cultivar where a small increase in ODC was observed at 12 h after stress. Upon rewarming, activities of ADC and ODC were higher in the ‘T’ variety without major changes in the ‘S’ variety. Cellular Spd increased upon chilling and Spd and Put upon rewarming in the ‘T’ variety without any significant changes in PA contents in the ‘S’ variety under similar treatments.

Positive effects of low temperature (4°C) and salt-induced up-regulation of SAMDC transcripts were also observed in in-vitro grown apple shoot cultures by Hao et al. (2005). RNA gel blot analysis of apple MdSAMDC1 showed up-regulation within 6 h of low temperature treatment and a gradual decrease thereafter exhibiting lower expression than control at 24 h to 120 h of treatment. Induction of MdSAMDC2 by low temperature was much greater than that of MdSAMDC1. At elevated temperature (37°C), both MdSAMDC1 and MdSAMDC2 transcripts were down regulated.

QRT-PCR analysis of SAMDC transcripts in Theobroma cacao exhibited higher expression in mature leaves and open flowers in response to drought (Bae et al. 2008). Induction of SAMDC was also seen in the roots of seedlings at 7 d after drought that reached the peak at 10 d. Induction of ODC showed a similar pattern, whereas ADC reached near maximum by 7 d. Induction of SPDS and SPMS on the other hand was
faster than other PA genes, and showed increase immediately after water was withheld; there was a positive correlation with leaf water potential. Expression of \textit{SPDS} and \textit{SPMS} was unaltered in response to drought stress in the leaves. Intracellular concentrations of Put and Spd significantly increased in the leaves upon drought stress, with a small increase in Spm. Expression of \textit{SAMDC} was also up-regulated by ABA.

\textit{Arabidopsis samdc4} mutant, \textit{bud2}, exhibits altered hypocotyl elongation and lateral bud outgrowth as an effect of altered auxin-mediated response in the mutant line vs. the WT plants (Cui et al. 2010). Application of exogenous IAA showed a rapid accumulation of \textit{BUD2} transcripts within 30 min reaching a maximum level by 2 h in the WT seedlings. Furthermore, a 2.5-fold increase in the expression of this gene was observed in response to exogenous IAA treatment along with \textit{AtSAMDC2} and \textit{AtSAMDC3}, but not \textit{AtSAMDC1}. On the other hand, cytokinin did not cause any significant change in the expression of \textit{BUD2}. Altered PA biosynthesis in the \textit{bud2} mutant resulted in altered levels of endogenous cytokinins, among which some were increased and some decreased. The \textit{bud2} plants exhibited hypersensitivity to exogenous cytokinin treatment. Callus induction in \textit{bud2} hypocotyl explants was better than the WT plants; the best results were obtained from low cytokinin and high auxin concentrations. These results together show complicated effects of \textit{bud2} mutation on alteration of PA levels through imbalance of auxin and cytokinin homeostasis in plants resulting in morphological changes.

Another plant hormone methyl jasmonate (MeJ), which is mainly associated with wounding and pathogenesis, has been shown to affect the expression of \textit{SAMDC} and other PA biosynthetic genes in different plant species. In tobacco BY-2 cells where \textit{ADC} and \textit{ODC} were induced by MeJ, there was also an increase in Put content concomitant
with an increase in nicotine (Imanishi et al. 1998). In contrast Arabidopsis did not show any change in \textit{SAMDC} and \textit{SPDS} expression when subjected to Mej treatment (Peréz-Amador et al. 2002). In 2 month old rice (\textit{Oryza sativa}) plants, exogenous spraying of 200 \( \mu \text{M} \) Mej resulted in a reduction of both rice \textit{ADC} (\textit{OsADC}) and \textit{SAMDC} (\textit{OsSAMDC}) mRNAs (semi-QRT-PCR) in the leaves of WT type as well as transgenic plants expressing \textit{D. stramonium ADC} cDNA under \textit{Ubi} promoter (Peremarti et al. 2010). A 75\% reduction in Put content of WT plants was observed in the leaves but no change in Spd or Spm content was observed in the WT in response to Mej treatment.

The transcripts of \textit{AtSAMDC2} were up regulated by ABA treatment (Urano et al. 2003). As mentioned earlier, Wi et al. (2006) had seen higher expression of \textit{SAMDC}::\textit{GUS} in response to exogenous ABA treatment in transgenic tobacco. Urano et al. (2009) have also reported increased expressions of PA biosynthetic genes and increased contents of PAs and other metabolites in ABA-treated Arabidopsis plants.

In grape vine (\textit{Vitis vinifera}) leaf disc assay, exogenous treatment with 10 \( \mu \text{M} \) ABA showed a significant increase in the activities of SAMDC, ADC and ODC by 15-, 30- and 10-fold, respectively, at 3 h in the drought tolerant (T) genotype (Toumi et al. 2010). In the drought sensitive (S) genotype, changes in the activities of these enzymes were seen only at 24 h post-treatment. A 2- and 4-fold increase in Put and Spm contents was observed in the T genotype at 1 h post-treatment. In the S genotype, Put and Spm were elevated after longer treatment (at least 6 h); cellular Spm decreased in both T and S genotypes. Increases in the activities of DAO and PAO were observed in both T and S genotypes in response to exogenous ABA treatment, although the peak activities and magnitude were higher and attained sooner in the T vs. the S genotype. In intact plants,
activities of SAMDC and ODC were induced and doubled at 8 d post-drought in the T genotype, whereas SAMDC and ODC were reduced in the S genotype. Total PAs were 2-fold higher in the S than the T genotype but the trend was reverse in total PAs (a 7- and 3-fold increase in Put and Spd contents in the T genotype) when subjected to drought.

Effects of abiotic stresses on the expression of SAMDC and associated changes in PAs have been extensively studied in Arabidopsis thaliana. In the rosette leaves of 4 week-old unbolted plants, AtSAMDC2 transcripts were up-regulated by 10 h after treatment with 250 mM NaCl, whereas expression of AtSAMDC1 remained unchanged (Urano et al. 2003). The same authors reported that 4°C treatment of the same age plants showed significant up-regulation of AtSAMDC2 in the rosette leaves at 5 h; the induced level of expression was seen up to 24 h (semi-QRT-PCR). Analysis of PAs upon salt stress treatments showed a ~2-fold increase in Put content and a ~3-fold increase in Spm content with only a small increase in Spd at 10 h. On the other hand, low temperature reduced Spm without significant changes in Put and Spd. In another study, Bagni et al. (2006) showed induced expression of AtSAMDC1 by salt (75 mM NaCl) stress was higher than AtSAMDC2; their expression remained almost unchanged in rosette leaves at vegetative stage but at reproductive stage the same leaves had increased (by 40-50%) expression of AtSAMDC1 but not AtSAMDC2. Expression of both AtADC1 and AtADC2 was highly induced in the rosette leaves by NaCl stress both at vegetative and reproductive stages; AtADC2 showed higher expression than AtADC1. Expression of AtSPDS genes in the rosette leaves was induced by salt only at vegetative stage, and the expression of AtSPDS1 was higher than AtSPDS2. Expression of AtSPMS was also induced both at vegetative and reproductive stages. The activities of SAMDC and other PA biosynthetic enzymes showed
corresponding increases in response to salt stress at different stages studied. With respect to soluble PAs, Put did not show any significant change upon NaCl stress in the rosette leaves whereas Spd was lower (76%) and Spm increased by small (14%) amount. Similar trend of changes in PA levels was observed upon 75 mM NaCl stress at reproductive stage. This study was later followed by a study by Tassoni et al. (2008) in Arabidopsis flowers in plants subjected to NaCl stress. Expression of $AtSAMDC1$ was higher than $AtSAMDC2$ in the flowers under normal conditions; NaCl treatment (75 mM) showed a slight increase in the expression of both genes. Among other PA genes, the expression of $AtADC2$ was higher than $AtADC1$ and both of them were induced upon exposure to salt. The $AtSPDS$, $AtSPMS$ and $AtACL5$ genes were highly induced by salt. Among different PAs, Spd and Spm showed <10% increase whereas Put showed a similar decrease.

In summary, a comprehensive analysis of expression of different members of the $SAMDC$ gene family using a common experimental approach is lacking in any plant species. In most cases, data on correlations among transcripts, enzyme activity, and the amounts of PAs produced by the action of SAMDC are lacking. In addition, the regulation of promoters of the different $SAMDC$ gene family members with respect to their functional redundancy or unique functions is not known. A part of my research was thus focused on a comprehensive analysis of gene expression of three members of the $SAMDC$ gene family ($AtSAMDC3$, $AtSAMDC4$ and $AtSAMDC5$) that have thus far not been studied in details. Careful analysis of the current status of research involving transgenic manipulation of PAs in Arabidopsis and in other plant species reveals that there is a lack of information on the role of substrates; i.e. Orn, Arg and Glu in regulating metabolic fluxes of PAs. The pleiotropic effects of PA manipulation on other associated
pathways that share common substrates with the PA biosynthetic pathway, especially amino acid metabolism have not been thoroughly analyzed in any intact plant. Similarly effects of altered PA metabolism on amino acid metabolism in relation to increased C and N availability have not been analyzed. With respect to the regulation of gene expression of the five *AtSAMDC* genes, bioinformatics analyses of their promoters and the 5’UTRs have not been reported. Taking advantage of recent advances in genomic, transcriptomic and proteomic research with *A. thaliana*, and the previous work on the expression analysis of *AtSAMDC1* and *AtSAMDC2* in our lab, I undertook the present study with the goals of (1) analyzing the biochemical consequences of genetic manipulation of PAs and (2) comprehensively analyzing the expression of the three remaining *AtSAMDC* genes, in this model species. The specific objectives of my research were:

1. To study the effects of inducible and constitutive transgenic manipulation of PAs via a mouse ornithine decarboxylase (mODC) gene on plant development and metabolism.

2. To analyze the spatial and temporal expression patterns of *Arabidopsis thaliana* *S*-adenosylmethionine decarboxylase genes (*AtSAMDC3*, *AtSAMDC4* and *AtSAMDC5*) during its life cycle.
MATERIALS AND METHODS

Bacterial culture and plasmid isolation

Liquid cultures of *Escherichia coli* (*E. coli*) and *Agrobacterium tumefaciens* were grown in Luria Broth medium (10 g L\(^{-1}\) Bacto Tryptone, 5 g L\(^{-1}\) Bacto Yeast Extract, and 10 g L\(^{-1}\) NaCl - Maniatis et al. 1982) containing appropriate antibiotics (ampicillin 100 \(\mu\)g mL\(^{-1}\) or spectinomycin 100 \(\mu\)g mL\(^{-1}\) or kanamycin 50 \(\mu\)g mL\(^{-1}\) or gentamycin 50 \(\mu\)g mL\(^{-1}\)). Sterile pipette tips or applicators were used to start a 3 mL liquid culture of *E. coli* or 200 mL liquid culture of *Agrobacterium* from glycerol stocks or plates. For solid cultures, 1.3% Bacto agar was added to the medium before autoclaving. Solid or liquid cultures of *E. coli* and *A. tumefaciens* were incubated at 37°C for 18 h and 28°C for 24 to 48 h, respectively. Liquid cultures were grown on a shaker at 250 rpm.

Plasmid DNA was isolated from *E. coli* using Zyppy™ Plasmid Miniprep Kit from Zymo Research (Irvine, CA, #D4020), 3 ml liquid culture (grown overnight at 37°C) was pelleted at 10,000 xg for 30 s in a 1.5 mL microcentrifuge tube. The pellet was re-suspended in sterile water followed by the addition of 100 \(\mu\)L of Lysis Buffer and 350 \(\mu\)L of Neutralization Buffer, sequentially. The tubes were inverted to mix thoroughly and centrifuged for 4 min at 14,000 xg. The supernatant was then transferred into the Zymo-Spin™ column attached to a collection tube. The column assembly was centrifuged for 15 s and the flow-through was discarded. This was followed by two washes, each of 200 \(\mu\)L of Endo-Wash Buffer for 15 s and 400 \(\mu\)L of Zyppy™ Wash Buffer for 30 s, respectively (by spinning at 14,000 xg). The column was then transferred into a clean 1.5 mL microcentrifuge tube, 30 \(\mu\)L of Zyppy™ Elution Buffer was added to the column.
matrix and the tubes were let stand for one minute at room temperature. The plasmid DNA was eluted by centrifugation for 15 s at 14,000 xg, quantified with Nanodrop spectrometer (Fisher Scientific), and was stored at -20°C. Alternate kits were used occasionally following the manufacturer’s protocols.

**Genomic DNA isolation**

Genomic DNA was isolated from plant tissues using modified Murray and Thompson (1980) protocol. Approximately 100-150 mg of plant tissue was ground in liquid nitrogen with 500 μL of pre-heated (60°C) CTAB buffer [2% (w/v) Hexadecyltrimethylammonium bromide (Sigma, H6269), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 0.2% (w/v) β-mercaptoethanol added just before use] in a microcentrifuge tube. The ground tissue was incubated for 30 min at 60°C with gentle agitation, followed by addition of equal volume of chloroform:isoamyl alcohol (Fisher, A393-500) (24:1). The sample was centrifuged for 5 min at 14,000 xg, the upper aqueous phase removed into a new tube, followed by addition of equal volume of cold isopropanol. The tubes were incubated for 30 min at -20°C. The DNA was pelleted by centrifugation for 15 min at 14,000 xg at 4°C. The pellet was washed with 70% ethanol containing 10 mM ammonium acetate (Sigma, A1542) by centrifugation. The pellet was vacuum-dried in a Speed-Vac and resuspended in 30 μL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA). Nucleic acid was quantified spectrophotometrically (Nano-drop) and its quality assessed by A_{260}/A_{280} ratio (≥ 1.8).

**Polymerase Chain Reaction (PCR)**

A typical PCR was performed using Takara *Ex Taq™* Polymerase (Takara Bio Inc., #TAK RR001A). A 50 μL PCR was performed that contained total 1.25 units of Takara *Ex Taq* DNA polymerase, 1X final concentration of buffer (Mg^{2+}), 200 μM final
concentration of dNTP mix, 0.2 μM final concentration each of the forward and the reverse primers, and 100 ng of total plasmid DNA or 100-150 ng of genomic DNA. Reactions were run in a PTC™ 100 Programmable Thermal Controller (MJ Research Inc., Waltham, MA). The PCR conditions were activation at 94°C for 1 min, followed by 30 cycles of denaturation at 94°C for 30 sec, variable annealing temperatures (Table 8) for 1 min and elongation at 72°C, followed by a final extension at 72°C for 2 min. The reaction tubes were stored at 4°C. Other Taq polymerases and thermocyclers were also used with similar reaction conditions.

Restriction Digestion and Agarose Gel Electrophoresis

Typical restriction digestion was performed using enzymes from New England Biolabs (Ipswich, MA). A 10 μL reaction mix contained a final concentration of 1x buffer, 1x bovine serum albumin (if required), 2 units/μg DNA of the enzyme, 150-200 ng of template DNA all brought to the volume by sterile distilled water. The reaction was incubated for 2 h at specified temperature according to the manufacturer’s recommendation and used immediately or stored at -20°C before gel electrophoresis.

Agarose gel electrophoresis was performed to analyze DNA using a 1% Seakem GTG (Lonza, #50070) or LE agarose (Lonza, #50000) dissolved in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Prior to loading, samples were mixed with 6x loading dye and then electrophoresed at 5 V/cm for 1 h along with appropriate DNA size standards (e.g. NEB 2-log DNA Ladder or 1 kb DNA Ladder or Low Molecular Weight DNA Ladder). Electrophoresis was followed by staining of the gel for 15 min in 0.5 μg mL⁻¹ of ethidium bromide and subsequent destaining for 10 min in distilled water. The gel was visualized and photographed using Nucleotech gel-documentation system (Nucleotech,
Table 8. Primers used for cloning, sequencing and (Q)RT-PCR.; the (Q)RT-PCR primers are adapted from Jumtee et al. 2008.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Td (°C)</th>
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<tr>
<td>35S-mODC</td>
<td>GAACCATGGGGAGCTTTTAC</td>
<td>CTACTACATGGCTCTTGGA</td>
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<td>Inducible-mODC</td>
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<td>CTACTACATGGCTCTTGGA</td>
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<td>SAMDC3-A</td>
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<td>TTTTCAAAGGCGAAGAGACT</td>
<td>59.0</td>
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<tr>
<td>SAMDC3-C</td>
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<td>TGAAAGACAGCAATAAAAGG</td>
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<tr>
<td>SAMDC3-D</td>
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<td>TGAAGAACAGCAATAAAAGG</td>
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<tr>
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<td>GTTGCAACAAATTGAGCAATGC</td>
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</table>
San Mateo, CA) or Fotodyne gel-documentation system (Fotodyne Incorporated, Hartland, WI). The DNA fragments were analyzed with reference to an appropriate DNA size standard.

**DNA sequencing and sequence analysis**

A 6 μL sequencing reaction was performed that contained total 300 ng of DNA and 5 pmol of either forward or the reverse sequencing primer. The sequencing was done at UNH Hubbard Genome Centre using a ABI 3130 DNA Analyzer (Foster City, CA) and the results obtained from sequencing were aligned and analyzed with the target sequences using BioEdit Sequence Alignment Editor (Hall 1999).

**Electroporation**

Electroporation of TOP10 *E. coli* (Invitrogen, Carlsbad, CA, #C404003) and GV3101 *A. tumefaciens* was performed using an Eppendorf model 2510 electroporator. A 1-2 μL LR clonase product was added to 50 μL aliquot of competent bacterial cells thawed on ice. The mixture was transferred to prechilled cuvettes (1 mm gap) and electroporated at 1800 V. This was followed by addition of fresh LB and incubation at 37°C for 1 h for *E. coli* and 28°C for 2 h for *A. tumefaciens*. The cells were plated on solid LB medium with appropriate antibiotic and incubated overnight. The resulting colonies were screened by plasmid isolation, restriction analyses and sequencing or PCR.

**Glycerol stocks**

Liquid cultures (3 mL) in LB medium with appropriate antibiotics from desired colonies were grown overnight at 37°C or 28°C and 250 rpm. Glycerol stocks were made by mixing thoroughly 85% of the bacterial culture with 15% of sterile glycerol (by
volume) in cryo-vials. The stocks were incubated in dry ice for 15 min and cryo-preserved at -80°C.

**Cloning of mODC and AtSAMDC promoter regions**

A 1190 bp sequence representing the ORF of mouse *ODC* (mODC) gene (Accession #M10624 Kahana and Nathans 1985)) was PCR amplified from pCW122-*ODC* (Bhatnagar et al. 2001) using sequence specific primers (Table 8). The amplified PCR product was cloned into pENTR™/D-TOPO® vector (Invitrogen, Carlsbad, CA, #45-0218), confirmed through restriction digestion, electrophoresis and sequencing of the TOPO clone and subsequently transferred into estradiol inducible Gateway compatible pMDC7 destination vector (Curtis and Grossniklaus 2003) using LR clonase reaction according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, #11791-020). For constitutive over-expression studies, mODC was cloned into pCR8.0/GW/TOPO vector (Invitrogen, Carlsbad, CA, #45-0642), confirmed through restriction digestion, electrophoresis and sequencing of the clone and then transferred into pMDC32 destination vector (Curtis and Grossniklaus 2003) containing double 35S CaMV promoter using LR clonase reaction. For promoter analyses (using *GUS* reporter gene), respective fragments of *SAMDC* constructs (summarized in Fig. 36 and described in results section) representing the putative promoter regions were PCR amplified from *A. thaliana* (Col 0) genomic DNA (gDNA) and cloned into pCR8.0/GW/TOPO vector (Invitrogen, Carlsbad, CA, #45-0642). Following confirmation through restriction digestion, gel electrophoresis and sequencing of the TOPO clones, the PCR fragments were then moved into the final destination vector pMDC163 (Curtis and Grossniklaus 2003) containing *GUS*, through LR clonase reaction.
Plant transformation

*Arabidopsis thaliana* (ecotype Columbia-0 – Col-0) plants were transformed by *Agrobacterium tumefaciens* (strain GV3101) using modified floral dip method (Clough and Bent 1998). For each transformation, 3 pots were prepared one week prior to dipping by clipping the primary bolt to encourage synchrony in branching and flowering. A 500 mL culture of *Agrobacterium* was started in LB with kanamycin (50 µg mL\(^{-1}\)) and gentamycin (50 µg mL\(^{-1}\)) and grown for 24 h at 28°C on a 250 rpm shaker. The entire culture was pelleted at 5,000 xg for 10 min and the pellet was resuspended in 50 ml of 5% (w/v) sucrose. This culture was further diluted with 5% (w/v) sucrose containing 0.05% final concentration of L-77 Silwet (Lehle Seeds, TX #VIS-02) to adjust the OD\(_{600}\) to 0.8±0.2 before dipping. The unopened flower buds along with flowers (4-5 week old plants) were dipped into this bacterial suspension for 8-10 s avoiding contact with the basal leaves and soil. The pots were laid overnight on their sides on a flat and covered with a clear plastic to prevent dessication. The next morning, dipped plants were rinsed thoroughly under cold water and moved to their normal growth conditions. The same plants were re-dipped in a similar way after a week. T1 seeds were harvested from each pot separately after the siliques matured. Seeds were desiccated at room temperature for 5-7 d followed by sterilization and storage at 4°C.

Transient expression in poplar

The biolistics bombardment protocol for transient GUS activity in poplar (*Populus nigra x maximowiczii*) cells was as described in Bhatnagar et al. (2001). The plasmid DNA (approximately 1.5 µg DNA/mg gold particles) was coated onto 1.5-3 µm gold particles (Aldrich, Milwaukee, WI) in the presence of 1.0 M CaCl\(_2\) and 16.7 mM Spd and
bombarded by Bio-Rad (Hercules, CA) PDS 1000/He gene gun, with either 1100 or 1350 psi rupture discs. Approximately 48 h after bombardment, the cells were treated with X-Gluc (Jefferson et al. 1986), incubated overnight at 37° C, and numbers of blue cells (showing GUS activity) were counted.

Screening for homozygous transgenic lines

Transgenic lines (for \( mODC \) from pre-screened (tolerant to hygromycin 70 \( \mu \)g mL\(^{-1} \) or kanamycin 50 \( \mu \)g mL\(^{-1} \)) T1 generation were reconfirmed by PCR for the presence of \( mODC \) or the desired gene sequence, depending on the construct. Confirmed T1 plants were grown to obtain T2 generation seeds. Five independent T2 transgenic lines were selected that had a single insertion of the \( mODC \) gene (tested by observing 3:1 segregation of live:dead plants on hygromycin plates) and showed significantly higher amount of Put either under inducible or constitutive conditions as compared to the WT controls; these lines were grown to obtain T3 generation seeds/plants. For most experiments T3 or T4 generation plants have been used. For screening of transgenic GUS plants, plant parts were subjected to GUS staining and plants that showed blue color were used for subsequent studies. Screened independent 3-5 homozygous T3 lines were used for all of our subsequent experiments.

Qualitative Assay of β-Glucuronidase (GUS) during Development

Histochemical assay of GUS was performed using GUS reaction mix containing 1 mM of 5-bromo-4-chloro-3-indonyl-β-D-glucuronide (X-Gluc; Research Products International Corp.; #16831), 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 100 mM sodium phosphate buffer pH 7.0, 5 mM EDTA, 0.1% Triton X-100, and 20% methanol. Plant samples collected at different developmental stages were
submerged in GUS reaction mix and vacuum infiltrated for 5 min. Following incubation at 37°C for 18-24 h, the reaction mix was removed and the samples were stored in 70% ethanol (for decolorization of chlorophyll background) at 4°C until analysis and photography (Martin et al. 1992). Representative photographs were taken using an Olympus C650 digital camera (www.olympusamerica.com) mounted on a Olympus SZX9 dissecting microscope.

Seed sterilization and growth

Seeds of Arabidopsis were surface-sterilized in microfuge tubes. Approximately 40 mg of seeds were mixed with 1 mL of 70% ethanol and a drop of 10% Triton X-100 (v/v) and gently agitated for 5 min. Following brief centrifugation, the supernatant was removed and 1 mL of 100% ethanol with one drop of 10% Triton X-100 (v/v) was added with another 5 min of incubation and gentle agitation. This was followed by another wash with 100% ethanol for 5 min. The seeds were dried in the laminar flow hood for 7-8 h and plated on solid germination medium or GM (4.3 g L^{-1} MS salts, 0.5 g L^{-1} MES, 1 g L^{-1} sucrose, and 0.8% type A agar; pH adjusted to 5.7 by 4 M KOH). The plates were covered with aluminum foil and the seeds were stratified for 48 h in dark at 4°C. Following that, the foil was removed and the plates moved to a growth chamber.

Arabidopsis seedlings were typically grown at 25±1°C under 12 h photoperiod (80±10 μE.m^{-2}.s^{-1}). For gene induction experiments, batches of 8-10 seedlings (12-d old) grown in solid GM were transferred into liquid GM in 12 well plates; each well contained 1 mL of the medium. Each treatment was assigned 4 wells where samples from each well were considered as one replicate. A final concentration of 5 μM estradiol (Sigma, #E2758; from a stock of 10 mM of estradiol in dimethyl sulfoxide) solution with or without 0.1
mM L-Orn or 1 mM Glu or 0.5 mM Arg (final concentration) were applied in the liquid medium; the medium without treatment is called control. The plates were then left in the growth chamber (conditions described above) and samples were collected at 12 h and 24 h, soaked dry to remove excess medium, and collected for PA and amino acid analyses. To study the effects of exogenous PAs, WT seedlings were grown for 7 d in solid GM supplemented with 1.0 mM of Put or Spd or 0.5 mM Spm or no PAs (control). For PA and amino acid analyses of 35S-mODC lines and WT plants, seedlings were typically grown in 1x solid MS medium for 14 d before collection. Amino acid and PA analyses were also performed in different tissues collected from 5 week old mature plants grown in pots. For extra N and sucrose treatments, seeds were germinated in GM containing either 30 mM or 60 mM KNO₃ or N-free medium (PhytoTechnology Laboratories, #M531) and sucrose (0, 50 mM, 100 mM). The seedlings were collected for fresh weight, dry weight, PA and amino acid analyses. Seedlings from different treatments, lines and also tissues from mature plants were collected in 5% HClO₄ (v/v from a stock of 60%; approx. 0.77N) and stored at -20°C prior to analysis of PAs and amino acids. The ratio of plant material to HClO₄ was typically 1:9 (i.e. 100 mg FW tissue in 900 µL 5% HClO₄).

Plant growth in soil

Arabidopsis seeds were sown in moist soil mix containing 3 parts Scott's 360 Metro-Mix (Scotts Company, Marysville, OH) and 1 part perlite in 3” pots. In each pot, 10-15 seeds were sown and the pots were placed on a flat covered with a clear plastic lid; the flats were kept for 48 h in dark at 4°C. Thereafter, the flats were moved to growth chamber at 21°C under 18 h photoperiod (80±10µE.m⁻².s⁻¹). The plastic lid remained on for 7 d followed by a 3 d hardening off period after which the lid was removed. After 10
to 12 d of germination the pots were thinned with 4-5 plants/pot. Plants were watered on alternate days and fertilized with ¼ strength Miracle-Gro (Scotts Company) synthetic fertilizer applied with irrigation water every 5th day. For recovery of transgenic plants selected on antibiotics, 12-14 d old seedlings were transferred to the pots using forceps.

**Incorporation of labeled precursors**

For incorporation of labeled precursors, two-week old inducible mODC transgenic seedlings grown in solid GM were transferred to 50 mL conical flasks containing 10 mL of liquid GM. The flasks were incubated at 25°C±1°C on a gyratory shaker at 150 rpm. Inducer (5 μM estradiol – final concentration) was added to the liquid GM immediately after transfer of the seedlings. Two hours later, 0.5 μCi of either L-[U-14C]Orn (specific activity 257 mCi/mmol, Amersham Pharmacia Biotech) along with 0.1 mM cold Orn (final conc.) or 0.5 μCi L-[U-14C]Arg (specific activity 272 mCi/mmol, Moravek Biochemicals, St. Louis, MO) along with 0.5 mM cold Arg (final conc.) were added to each flask and incubated for 8 h. Samples were washed 3 times either with 2 mM cold Orn or 10 mM cold Arg, followed by 3 consecutive washes of de-ionized water. The samples were collected in 5% PCA and stored at -20°C before processing to determine 14C incorporation into PAs. Following 3 cycles of freezing and thawing, the samples were dansylated (Bhatnagar et al. 2001) and 300 μL aliquot of toluene and 50 μL of the aqueous phase were counted for radioactivity in 10 mL Scintilene (Fisher Scientific, Lot #980805) in a LSC-6000 liquid scintillation counter (Beckman, Fullerton, CA).

**Quantification of polyamines**

Wild type and transgenic seedlings were grown in solid GM for the number of days specified for each experiment. Seedlings were collected in 9x volume of 5% HClO4 and
frozen and thawed 3x before dansylation and quantification of PAs by HPLC (Bhatnagar et al. 2001; Mohapatra et al. 2010b). The thawed samples were vortexed for 5 min, centrifuged (13,000 xg) for 10-15 min, and 100 µL of the supernatant was used for dansylation. For standards, a 100 µL mixture of 3 PAs (0.004 mM Put, 0.002 mM Spd or Spm, 0.08 mM Put, and 0.04 mM Spd and Spm); heptanediamine was used as internal standard. To 100 µL of either the sample or the standard mix, 20 µL of 0.1 mM heptanediamine (Sigma, #D3266) was added. This was followed by the addition of 100 µL of saturated Na₂CO₃ solution and 100 µL of 20 mg mL⁻¹ (in acetone) dansyl chloride (Fluka, Milwaukee, WI, #39220). The mixture was vortexed for 30 s and incubated for 1 h at 60°C. Then 20 µL of 20 mg mL⁻¹ L-asparagine was added to the samples and the standards, and the tubes were vortexed for 30 s. Following incubated for 30 min at 60°C, acetone was vacuum evaporated for 5 min in a Speed-Vac (Savant Instruments Inc., Farmingdale, NY) and dansyl-PAs were recovered in 400 µL of toluene (Phortex grade; Fisher, #9456-03). Following toluene addition, the samples and the standards were vortexed for 1 min, left undisturbed for 5 min to separate the organic and the aqueous phases, and centrifuged for 1 min at 13,000 xg. An aliquot of 200 µL of the toluene phase was transferred to a new microfuge tube and vacuum dried in Speedvac. The dansyl-PAs were reconstituted in 500 µL of methanol by vortexing for 2 min, followed by centrifugation for 2 min. A 250 µL aliquot of methanol extracts was transferred to autosampler vials for analysis of PAs using a gradient of acetonitrile (Fisher, #75-05-8) and 10 mM heptanesulfonic acid (Fisher, #300111), pH 2.8 on a reverse phase column (4.6 x 33 mm, 3 µm) using Perkin-Elmer (PE) HPLC system (Bhatnagar et al. 2001). The system consisted of a PE series 200 autosampler fitted with a 200 µL loop (10 µL of
sample volume injected) and a series 200 gradient pump at a flow rate of 2.5 mL min\(^{-1}\). A series 200a fluorescence detector (Perkin Elmer) set at 340 nm and 515 nm for excitation and emission, respectively, was used for detection and quantitation of PAs. The PE TotalChrom software (Version 328 6.2.1) was used to integrate data, and a multiplication factor incorporated into the software was used to obtain the amounts as nmol mL\(^{-1}\) PCA or nmol g\(^{-1}\) FW.

**Amino acid analysis**

The aqueous fraction from toluene partitioning (described above) of the dansylated samples was used for amino acid analysis by HPLC (Minocha and Long 2004). A 135 \(\mu\)L aliquot of the aqueous fraction along with 135 \(\mu\)L of 2.9 M acetic acid was added to 730 \(\mu\)L of methanol. The tubes with the solution mixture were left open for 10-12 min to remove excess CO\(_2\) and the mixture was filtered through a 0.45 \(\mu\) nylon syringe filter (Pall-Gelman Labs. Ann Arbor, MI). A mixture of 22 amino acids (20 L-amino acids + GABA - Sigma, #A5835 + Orn - Sigma, #O2375) was used to make a standard curve for quantitation of amino acids. A Hydro-RP, 4 \(\mu\)m particles, 100 mm x 4.6 mm i.d. column (Phenomenex, Torrance, CA) and a C\(_{18}\) security guard column (5 \(\mu\)m, 4 mm x 3 mm i.d.; Phenomenex) in a column heater (Bio-Rad labs, Hercules, CA) set at 40\(^{\circ}\)C were used for amino acid separation. The amino acids were analyzed at the USDA Forest Service (NRS) Laboratory, Durham, NH.

**Total soluble protein measurement**

Total soluble protein was measured using Bradford method (1976) using the Biorad protein assay dye (Biorad Labs). Whole seedlings or plant leaves (20-25 mg) were collected in 100 \(\mu\)L of K-Pi buffer (0.1 M KH\(_2\)PO\(_4\); 0.1 M K\(_2\)HPO\(_4\)), pH 7.0. Samples
were frozen and thawed once, vortexed and centrifuged for 5 min at 16,000 xg. A 10 μL of plant extract diluted 4x with k-Pi buffer was mixed with 1.5 mL of diluted Bio-Rad protein assay dye. Following incubation in the dark for 15 min, A_{595} was measured in a Spectronic® 20 Genesys™ spectrophotometer (Spectronic Instruments Inc., Rochester, NY). Known concentrations of bovine serum albumin (Sigma, #A4503) were used for a standard curve which was used to calculate the protein concentration in the extract.

Chlorophyll analysis

Chlorophyll analysis was performed using modified protocol from Gitelson et al. (2009). Two to three oldest rosette leaves of 4 week-old-plants were used for pigment extraction. About 200-250 mg FW of leaf lamina were ground using a mortar and pestle with 200 μL of methanol mg^{-1} FW of leaf tissue. A pinch of CaCO₃ was added to the samples while grinding to prevent pheophytization of chlorophyll. Homogenates were centrifuged for 5 min at 10,000 xg and the A_{665} and A_{652} values of the supernatants were measured using a Spectronic® 20 Genesys™ spectrometer Total chlorophyll was calculated using as per Lichtenthaler and Buschmann (1987) [chlorophyll a; c_a (μg/ml) = 16.72A_{665.2}-9.16A_{652.4} and chlorophyll b; c_b (μg/ml) = 34.09A_{652.4}-15.28A_{665.2}]

Total percent carbon and nitrogen

For total C and N contents, individual plants were collected separately, dried at 70°C and analyzed by a CHNS analyzer (Thermo Scientific CE Elantech Flash EA1112 Soil) at the USDA Forest Service Lab. National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) samples 1515 (Apple Std) and 1547 (Peach Std) were used for procedure verification (Mohapatra et al. 2010b).
RNA isolation and cDNA synthesis

Plant samples stored at -80°C were used for total RNA extraction using the ZR Plant RNA MiniPrep™ Kit (Irvine, CA, #R2024). Frozen samples were removed from liquid nitrogen, ground quickly in 800 µl of RNA lysis buffer, and centrifuged for 1 min at 12,000 xg. The supernatant (400 µl) was transferred to Zymo-Spin™ IIIC column in a collection tube and centrifuged for 30 s at 8,000 xg. This was followed by addition of 0.8 volume of 100% ethanol to the flow-through, transfer of the mixture to a Zymo-Spin™ IIC column, and centrifugation for 30 s at 12,000 xg. The flow-through was discarded and in-column DNase treatment was performed using RQ1 RNase-Free DNase (Promega, Madison, WI, #M610A), and incubation at 37°C for 20 min. The column was centrifuged for 30 s at 12,000 xg, washed 2x with 400 µl of RNA Prep buffer for 60 s, and 800 µl of RNA wash buffer for 30 s by centrifugation (12,000 xg). The RNA wash buffer step was repeated and the column was transferred to a DNase/RNase-free collection tube. DNase/RNase-free water (25 µl) was added to the column matrix and the column was let stand at room temperature for 30 s. RNA was eluted by centrifugation for 30 s at 10,000 xg and cDNA synthesized immediately from the RNA.

RNA samples were reverse transcribed to first strand cDNA using qScript™ cDNA SuperMix kit (Quanta Biosciences, Gaithersburg, MD, #95048-100). A 20 µl cDNA synthesis reaction mix was made to contain a final concentration of 1X cDNA SuperMix, 1 µg of total RNA, and RNase/DNase-free water. The reaction was run in a PTC™ 100 Programmable Thermal Controller. The reaction conditions were 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The resultant cDNA was stored at -20°C before QRT-PCR.
Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR)

Relative gene expression was quantified by SYBR-green dye based assay. A 20 μl reaction mix containing 1X final concentration SYBR-Green FastMix, Low ROX (Quanta Biosciences, Gaithersburg, MD, #95074-250) was set up with a final concentration of 50 nmole each of the forward and reverse gene specific primers (Table 8) and an appropriate amount of cDNA (up to 500 ng). The reactions were run in MicroAmp™ Fast Optical 96-Well reaction Plate (Applied Biosystems, Foster City, CA, #4346906) in Applied Biosystems 7500- Fast Real-Time PCR machine (Applied Biosystems, Foster City, CA). The cycle conditions included a pre-incubation at 50°C for 2 min, dye activation at 96°C for 15 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min. A dissociation curve around 60°C to 95°C confirmed that majority of the signal was due to the interaction between SYBR-green and the specific amplicon, and not due to the primer dimers. A standard curve was prepared from serial dilutions of cDNA; the value for specific gene expression was extrapolated from the standard curve and expressed as a ratio of the value of the gene of interest to the internal control gene TIP-41 (At4g34270) (Czechowski et al. 2005; Page et al. 2007).

Statistical analyses

For all experiments three to six replicates were used for each treatment. Each experiment was repeated at least twice and data from a single representative experiment are presented here in most cases. The data were subjected to analysis of variance (ANOVA) using SYSTAT version 10.2. Significance at P ≤ 0.05 was determined using Tukey’s t-test. Specific analyses are described in the Figure and Table legends, where applicable.
Analysis of cis elements

For analysis of putative cis elements in the promoter regions Athena promoter analysis software was used (www.bioinformatics2.wsu.edu/cgi-bin/Athena/; O’Connor et al. 2005). The locus IDs of different SAMDC genes of Arabidopsis were used to obtain the putative cis elements available in the promoter regions.
OBJECTIVE 1. Transgenic manipulation of PAs

The mODC ORF (1190 bp) gene without PEST sequence (responsible for fast turnover) was PCR amplified from the pCW122-mODC plasmid (Bhatnagar et al. 2001) and cloned into pENTR™/D-TOPO® (for induction) and pCR8.0/GW/TOPO (for constitutive expression) vectors. The cloned mODC in the TOPO vectors was then transferred into vector pMDC7 (estradiol inducible promoter) and pMDC32 (constitutive promoter), by LR clonase reaction (see materials and methods), and tested by PCR and sequencing for accuracy. The primers used for the cloning of mODC are listed in Table 8. The different steps, including maps of the plasmids, PCR amplification, confirmation through restriction digestion and gel electrophoresis of the entry clones and final destination vectors, are shown in Figs. 2 and 3. Finally, they were transferred into Agrobacterium tumefaciens GV3101 in two formats – one constitutive (35S) and the other inducible by estradiol as per the guidelines from Curtis and Grossniklaus (2003). Parallel to the mODC vectors, an inducible GUS gene vector was also created (Fig. 4). This served as a control for testing the inducibility of the gene by estradiol. The results indicated that 5 μM of estradiol was sufficient to induce the GUS gene (Fig. 5). There was no visible GUS expression without estradiol treatment.

Using these vectors for floral dip transformation, several T1 transgenic lines (both inducible and constitutive) of Arabidopsis were selected on hygromycin and grown to obtain T2 seeds. Individual T2 lines were tested for the presence of the transgene by PCR (Fig. 6) using mODC specific primers; 4 lines were found to be PCR positive in the case
Figure 2. Plasmid maps (A and B) and gel analysis results (C) of inducible mODC constructs (i) PCR product, (ii) pENTR™/D-TOPO® digests (HincII; 1051 bp, 2805 bp) with mODC insert, and (iii) pMDC7 digests (NcoI; 1739 bp, 2648 bp, 8599 bp) with mODC insert. NEB 2-Log DNA ladder.
Figure 3. Plasmid maps (A, B) and gel analysis results (C) of constitutive mODC construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (HincII; 1051 bp, 2805 bp) with mODC insert, and (iii) pMDC32 digests (HindIII; 1598 bp, 9913 bp) with mODC insert. NEB 2-Log ladder except for the PCR product where 1 kb DNA ladder was used.
Figure 4. Plasmid maps (A, B) and gel analysis results (C) of inducible GUS construct (i) PCR product, (ii) pENTR™/D-TOPO® digests (HindIII; 534 bp, 793 bp, 3065 bp) with GUS insert, and (iii) pMDC7 digests (EcoRV; 231 bp, 4719 bp, 8465 bp) with GUS insert. NEB 2-Log DNA ladder.
Figure 5. Estradiol inducible transgenic seedlings containing GUS; (a) un-induced and (b) 24 h after induction by 5 μM estradiol.
Figure 6. PCR screening of T2 inducible mODC (a) and 35S-mODC (b) plants. NEB 2-Log DNA ladder (The numbers on the top denote individual plant lines).
of inducible mODC, and 10 lines were positive for the constitutive mODC transgene construct. Some of these T2 lines were used to produce T3 homozygous plants, which were tested for induction by estradiol at the seedling stage. Two lines (named mODC10-1 and mODC1-14) that showed high Put upon induction were selected for further analysis at the biochemical level (for the induction work). Likewise three lines (1-7, 4-11, 18-2) of transgenic plants were selected for physiological and developmental analysis for the constitutive expression.

**Inducible expression of mODC: Associated biochemical changes**

As described above, a maximum increase in Put was often seen at 12 h after estradiol treatment, with only small increases being seen thereafter. Role of substrates, especially Orn, Arg and Glu, in modulating PA and amino acid metabolism is not fully understood. As Orn occupies a pivotal position for biosynthesis of Put, Arg or Pro, we have previously hypothesized (Mohapatra et al. 2009, 2010b; Page et al. 2010) that Orn (as the substrate of mODC) rather than Glu could be a limiting factor for Put biosynthesis. We applied several treatments to further test this hypothesis. Note that Put is solely produced in WT Arabidopsis via Arg as it lacks a functional ODC gene; thus the mODC introduces a shunt to siphon away the cellular Orn and (potentially) depriving the pathway to make Arg. Moreover, in Arabidopsis, as in most plants, the cellular contents of free Orn are very low, and it is a critical substrate for Arg biosynthesis. In order to test whether Orn in transgenic Arabidopsis seedlings could become limiting for additional production of Put by mODC, we added the three amino acids (Orn, Glu and Arg) during the period of induction of mODC and monitored the PA contents at two different times after induction. We also studied the contents of all soluble amino acids in these plants. Plants were also
fed with U$^{14}$C-Orn and U$^{14}$C-Arg with or without induction of mODC to test the incorporation of $^{14}$C into PAs in response to induction of mODC. The PA and amino acid content of the WT and inducible mODC transgenic plants (in the absence of the inducer) were similar, thus the inducible mODC lines without the inducer were treated as controls.

**Inducible expression of mODC: Changes in polyamine content**

The different PAs changed differently in response to induction in the presence of exogenously supplied Orn, Arg and Glu in seedlings. At 12 h of induction of mODC by estradiol, Put content increased in plants by >10 fold (Fig. 7a). Addition of Orn concomitant with induction caused a further 4-fold increase (total up to 39-fold), whereas the presence of Glu and Arg during the induction period had little positive effect. Exogenous supply of Orn, Glu or Arg alone had no effect on Put content in the un-induced (control) transgenic plants. Increased accumulation of Put continued at 24 h after induction but not in the un-induced and untreated control plants, which only showed a doubling of Put at 24 h as compared to 12 h. The corresponding numbers in estradiol + Orn were >44-fold than time zero but only 5- to 6-fold higher with Glu and Arg.

Spermidine content was up slightly upon induction of mODC at 12 h both in induced (1.4-fold) and E+O (1.5-fold) plants and was comparable in un-induced plants treated with Orn alone (Fig. 7b). At 24 h of induction, no significant change in Spd contents was observed among different treatments except for those supplemented with Arg.

Cellular content of Spm increased about 1.3-fold at 12 h in plants treated with exogenous Orn, with little effect of induction itself (Fig. 7c). At 24 h of induction, a small increase was seen again. In no case was more than doubling of Spm seen. In seedlings treated with estradiol, a significant amount of Cad (Figs. 7d & S1) was seen in all cases.
Figure 7. Cellular contents of PCA soluble polyamines in 12 h and 24 h induced (+E) and un-induced mODC-10-1 transgenic plants with or without 0.1 mM Orn, 0.5 mM Arg or 1 mM Glu. N= 4; each replicate consists of 6-7 seedlings. * = P≤ 0.05 for significant difference between treatment and control at a given time.
within 12 h with or without the presence of Orn, Glu or Arg. The amount of Cad increased further by 2- to 3-fold at 24 h with E+Arg, E+Glu and E as compared to their 12 h corresponding values with the highest effect being seen by estradiol treatment only.

**Inducible expression of mODC: Changes in amino acids**

Since (i) the biosynthesis of PAs begins directly with the substrates Orn (by ODC) and Arg (by ADC), (ii) these two amino acids are derived from Glu, and (iii) Glu serves as a substrate for biosynthesis of most other amino acids (Fig. 1); it is logical to postulate that increased utilization of Orn by mODC will affect the connected pathways in which Orn, Arg or Glu are involved. Thus we analyzed not only the contents of PAs in response to induction and exogenous supply of Orn, Arg and Glu, but also the soluble amino acids in the cells. The data are presented in Figures 8 to 12.

Glutamate is a key intermediate in cellular N metabolism being involved in N assimilation, serving as substrate for biosynthesis of numerous metabolites including key amino acids, and being involved in the biosynthesis of PAs. Its content was not affected following induction by estradiol, nor was any other amino acid of this family (Fig. 8a-i). However, in the presence of Orn, induced plants had more than twice the content of Glu whether or not they were treated with estradiol (Fig. 8a). Exogenous Glu or Arg did not cause a similar effect, showing that extra Glu was quickly utilized in other reactions. Glutamine content was not affected either by induction or by exogenous supply of Orn, Glu or Arg (Fig. 8b). Overall, Gln constituted about 50% of the total content of soluble amino acids in the plants (Tables 9A, 9B). Among different treatments Glu, E+Arg and E produced small increases in Gln at 24 h corresponding to their respective 12 h values.
Figure 8. Cellular contents of PCA soluble amino acids in induced (+E) and un-induced mODC-10-1 transgenic plants with or without 0.1 mM Orn, 0.5 mM Arg or 1 mM Glu. N= 4; each replicate consists of 6-7 seedlings. * = P≤ 0.05 for significant difference between treatment and control at a given time.
Figure 8. Continued
Figure 8. Continued
Table 9A. Contents of individual amino acids at 12 h post induction (as % of total PCA-soluble amino acids) in 12 d old seedlings of mODC-10-1 grown in control (C), Induced (E), Orn (0.1 mM) + E, Glu (1 mM) + E, Arg (0.5 mM) + E, Orn (0.1 mM), Glu (1 mM) and Arg (0.5 mM) in liquid MS media. Numbers in red and blue (bold denotes >50% change and regular between <50% change) denote amino acids that are up-regulated and down-regulated respectively (N= 4 and each replicate consists of 6-7 seedlings).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C</th>
<th>E</th>
<th>E + Orn</th>
<th>E + Glu</th>
<th>E + Arg</th>
<th>Orn</th>
<th>Glu</th>
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<tr>
<td>Glu</td>
<td>5.34 ± 0.12</td>
<td>5.36 ± 0.13</td>
<td>10.50 ± 0.13</td>
<td>5.12 ± 0.02</td>
<td>4.39 ± 0.13</td>
<td>9.98 ± 0.26</td>
<td>5.18 ± 0.14</td>
<td>4.52 ± 0.14</td>
</tr>
<tr>
<td>Gin</td>
<td>45.06 ± 1.38</td>
<td>48.57 ± 2.55</td>
<td>50.06 ± 0.66</td>
<td>50.43 ± 2.07</td>
<td>47.21 ± 1.66</td>
<td>49.97 ± 0.48</td>
<td>50.49 ± 1.46</td>
<td>47.77 ± 0.81</td>
</tr>
<tr>
<td>Ser</td>
<td>1.66 ± 0.10</td>
<td>1.57 ± 0.08</td>
<td>1.88 ± 0.08</td>
<td>1.53 ± 0.06</td>
<td>1.65 ± 0.05</td>
<td>2.21 ± 0.03</td>
<td>1.42 ± 0.06</td>
<td>1.54 ± 0.05</td>
</tr>
<tr>
<td>Arg, Thr, Gly</td>
<td>16.23 ± 0.41</td>
<td>15.95 ± 0.34</td>
<td>12.52 ± 0.39</td>
<td>15.18 ± 0.49</td>
<td>17.6 ± 0.44</td>
<td>12.83 ± 0.64</td>
<td>14.12 ± 0.18</td>
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</tr>
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<td>4.04 ± 0.38</td>
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<td>3.43 ± 0.37</td>
<td>0.73 ± 0.05</td>
<td>4.57 ± 0.41</td>
<td>3.16 ± 0.42</td>
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<tr>
<td>Pro</td>
<td>0.29 ± 0.01</td>
<td>0.31 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>0.21 ± 0.04</td>
<td>0.33 ± 0.01</td>
<td>0.26 ± 0.00</td>
</tr>
<tr>
<td>Gaba</td>
<td>0.32 ± 0.01</td>
<td>0.44 ± 0.02</td>
<td>0.43 ± 0.01</td>
<td>0.52 ± 0.03</td>
<td>0.35 ± 0.02</td>
<td>0.34 ± 0.06</td>
<td>0.60 ± 0.03</td>
<td>0.29 ± 0.02</td>
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<tr>
<td>Val</td>
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<td>0.31 ± 0.01</td>
<td>0.40 ± 0.03</td>
<td>0.49 ± 0.02</td>
<td>0.29 ± 0.01</td>
<td>0.52 ± 0.02</td>
<td>0.41 ± 0.03</td>
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<tr>
<td>Met</td>
<td>0.07 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.01</td>
<td>0.07 ± 0.01</td>
<td>0.06 ± 0.00</td>
<td>0.07 ± 0.00</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>Ile</td>
<td>0.23 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.19 ± 0.00</td>
<td>0.23 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>0.26 ± 0.01</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>Leu + Trp</td>
<td>21.47 ± 0.77</td>
<td>19.12 ± 1.53</td>
<td>19.37 ± 0.12</td>
<td>19.53 ± 1.10</td>
<td>20.19 ± 1.37</td>
<td>18.65 ± 0.46</td>
<td>18.18 ± 0.76</td>
<td>19.69 ± 0.60</td>
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<tr>
<td>Phe</td>
<td>2.35 ± 0.18</td>
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<td>1.96 ± 0.12</td>
<td>2.05 ± 0.11</td>
<td>1.75 ± 0.10</td>
<td>2.00 ± 0.37</td>
<td>1.77 ± 0.03</td>
<td>2.11 ± 0.27</td>
</tr>
<tr>
<td>Cys</td>
<td>0.54 ± 0.03</td>
<td>0.61 ± 0.00</td>
<td>0.32 ± 0.01</td>
<td>0.50 ± 0.05</td>
<td>0.45 ± 0.02</td>
<td>0.28 ± 0.02</td>
<td>0.44 ± 0.04</td>
<td>0.46 ± 0.03</td>
</tr>
<tr>
<td>Orn</td>
<td>0.59 ± 0.02</td>
<td>0.44 ± 0.05</td>
<td>0.39 ± 0.05</td>
<td>0.39 ± 0.05</td>
<td>0.52 ± 0.02</td>
<td>1.43 ± 0.21</td>
<td>0.69 ± 0.10</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>Lys</td>
<td>0.43 ± 0.01</td>
<td>0.38 ± 0.05</td>
<td>0.26 ± 0.03</td>
<td>0.33 ± 0.03</td>
<td>0.36 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.49 ± 0.05</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>His</td>
<td>0.88 ± 0.07</td>
<td>0.84 ± 0.15</td>
<td>0.70 ± 0.03</td>
<td>0.74 ± 0.14</td>
<td>0.90 ± 0.06</td>
<td>0.53 ± 0.03</td>
<td>0.87 ± 0.07</td>
<td>0.63 ± 0.08</td>
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Table 9B. Contents of individual amino acids at 24 h post induction (as % of total PCA-soluble amino acids) in 12 d old seedlings of mODC-10-1 grown in control (C), Induced (E), Orn (0.1 mM) + E, Glu (1 mM) + E, Arg (0.5 mM) + E, Orn (0.1 mM), Glu (1 mM) and Arg (0.5 mM) in liquid MS media. Numbers in red and blue (bold denotes >50% change and regular between <50% change) denote amino acids that are up-regulated and down-regulated respectively (N= 4 and each replicate consists of 6-7 seedlings).

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C</th>
<th>E</th>
<th>E + Orn</th>
<th>E + Glu</th>
<th>E + Arg</th>
<th>Orn</th>
<th>Glu</th>
<th>Arg</th>
</tr>
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<tr>
<td>Glu</td>
<td>4.93 ± 0.06</td>
<td>4.87 ± 0.13</td>
<td>10.97 ± 0.44</td>
<td>5.25 ± 0.18</td>
<td>4.38 ± 0.08</td>
<td>9.50 ± 0.17</td>
<td>5.09 ± 0.05</td>
<td>5.98 ± 0.26</td>
</tr>
<tr>
<td>Gln</td>
<td>55.28 ± 1.72</td>
<td>54.91 ± 0.99</td>
<td>49.94 ± 0.71</td>
<td>53.46 ± 1.52</td>
<td>49.77 ± 1.53</td>
<td>48.86 ± 1.42</td>
<td>54.09 ± 1.07</td>
<td>40.63 ± 2.10</td>
</tr>
<tr>
<td>Ser</td>
<td>1.53 ± 0.08</td>
<td>1.83 ± 0.09</td>
<td>2.68 ± 0.07</td>
<td>1.69 ± 0.08</td>
<td>1.54 ± 0.09</td>
<td>2.91 ± 0.05</td>
<td>1.67 ± 0.05</td>
<td>1.28 ± 0.06</td>
</tr>
<tr>
<td>Arg + Thr + Gly</td>
<td>13.32 ± 0.65</td>
<td>14.29 ± 0.22</td>
<td>13.32 ± 0.18</td>
<td>13.71 ± 0.35</td>
<td>19.18 ± 0.26</td>
<td>14.31 ± 0.12</td>
<td>14.16 ± 0.12</td>
<td>20.28 ± 0.80</td>
</tr>
<tr>
<td>Ala</td>
<td>6.54 ± 0.57</td>
<td>7.39 ± 0.99</td>
<td>2.08 ± 0.14</td>
<td>7.60 ± 0.77</td>
<td>7.63 ± 0.68</td>
<td>1.04 ± 0.03</td>
<td>8.31 ± 0.42</td>
<td>9.59 ± 0.91</td>
</tr>
<tr>
<td>Pro</td>
<td>0.23 ± 0.01</td>
<td>0.34 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.46 ± 0.12</td>
<td>0.37 ± 0.01</td>
<td>0.19 ± 0.03</td>
<td>0.34 ± 0.01</td>
<td>0.46 ± 0.01</td>
</tr>
<tr>
<td>Gaba</td>
<td>0.20 ± 0.01</td>
<td>0.33 ± 0.02</td>
<td>0.31 ± 0.02</td>
<td>0.47 ± 0.01</td>
<td>0.27 ± 0.00</td>
<td>0.37 ± 0.08</td>
<td>0.44 ± 0.03</td>
<td>0.73 ± 0.15</td>
</tr>
<tr>
<td>Val</td>
<td>0.41 ± 0.01</td>
<td>0.44 ± 0.01</td>
<td>0.32 ± 0.00</td>
<td>0.45 ± 0.02</td>
<td>0.47 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>0.47 ± 0.00</td>
<td>0.65 ± 0.04</td>
</tr>
<tr>
<td>Met</td>
<td>0.05 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.06 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.00</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Ile</td>
<td>0.17 ± 0.01</td>
<td>0.22 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.00</td>
<td>0.16 ± 0.00</td>
<td>0.15 ± 0.01</td>
<td>0.35 ± 0.04</td>
</tr>
<tr>
<td>Leu + Trp</td>
<td>14.08 ± 0.43</td>
<td>12.48 ± 0.35</td>
<td>17.22 ± 0.42</td>
<td>13.76 ± 0.90</td>
<td>13.41 ± 0.75</td>
<td>17.89 ± 1.38</td>
<td>11.81 ± 0.66</td>
<td>16.14 ± 1.84</td>
</tr>
<tr>
<td>Phe</td>
<td>1.63 ± 0.24</td>
<td>1.50 ± 0.10</td>
<td>1.38 ± 0.12</td>
<td>1.52 ± 0.03</td>
<td>1.39 ± 0.07</td>
<td>1.86 ± 0.34</td>
<td>1.75 ± 0.05</td>
<td>1.92 ± 0.10</td>
</tr>
<tr>
<td>Cys</td>
<td>0.37 ± 0.02</td>
<td>0.35 ± 0.02</td>
<td>0.29 ± 0.02</td>
<td>0.31 ± 0.03</td>
<td>0.38 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.39 ± 0.01</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>Orn</td>
<td>0.23 ± 0.01</td>
<td>0.16 ± 0.03</td>
<td>0.26 ± 0.03</td>
<td>0.20 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>1.43 ± 0.23</td>
<td>0.34 ± 0.03</td>
<td>0.49 ± 0.11</td>
</tr>
<tr>
<td>Lys</td>
<td>0.28 ± 0.01</td>
<td>0.22 ± 0.03</td>
<td>0.20 ± 0.01</td>
<td>0.24 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.29 ± 0.04</td>
<td>0.31 ± 0.02</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>His</td>
<td>0.74 ± 0.06</td>
<td>0.63 ± 0.11</td>
<td>0.59 ± 0.09</td>
<td>0.66 ± 0.03</td>
<td>0.60 ± 0.06</td>
<td>0.56 ± 0.09</td>
<td>0.64 ± 0.07</td>
<td>0.73 ± 0.06</td>
</tr>
</tbody>
</table>
Proline and His contents did not change significantly during the 12 h period of the induction experiment; however, higher amounts of Pro were seen at 24 h in plants treated with E or E+Glu, and/or Arg alone (Fig. 8c,d). The peaks of Arg, Thr and Gly did not separate well in our HPLC system (Fig. S2 and Table S1); thus the combined values of these three amino acids are presented here. It should, however, be pointed out that the combined peak represents largely (>90%) of Arg (Data not shown). No significant changes were seen among different treatments except when exogenous Arg was supplied; in this case cellular Arg+Thr+Gly were significantly higher (Fig. 8e).

Ornithine is present in relatively small amounts (≤0.5% of total soluble amino acids) as compared to Arg, Glu or Gln in these plants (Tables 9A, 9B). The presence of Orn in the medium did cause a significant increase in the accumulation of this amino acid under un-induced conditions (Fig. 8f). However, on induction of mODC, Orn did not accumulate in the plants, showing its continued utilization by mODC. Exogenous Arg or Glu did not affect Orn accumulation in the cells even in control plants.

Non-protein amino acid GABA can be produced directly from Glu by GAD or from Put by DAO (Fig. 1). The carbon skeleton of Glu and Put is recycled through the reactions of the GABA shunt via succinate and the TCA cycle. The cellular content of GABA increased on induction of mODC (Fig. 8g). Its content was also higher when the un-induced plants were supplied with exogenous Arg and Glu (at 24 h). The results indicate that Put overproduction may result in concomitant increase in its catabolism to produce additional GABA, which is not processed fast enough via the TCA cycle.

The cellular content of Cys (including cystine) was not affected (Fig. 8h) in a major way by any treatment, except that it was somewhat lower in the presence of Orn.
regardless of induction) vs. the other treatments both at 12 h and 24 h. Serine was higher at 24 h in plants treated with E, E+Orn, Orn and Glu; the maximum difference was about a 40% increase over the control plants (Fig. 8i).

Neither Phe nor Trp (a small component close to the peak of Leu) was affected by any treatment; Tyr was not resolved in any of our samples by the HPLC method used here (Fig. 9a, 9b). Of the three branched chain amino acids, Ala was about 8- to 10-fold higher than Val and 15- to 40-fold higher than Ile in the control plants (Fig. 10a-c). The content of Ala increased significantly during the experimental period of 12 to 24 h in most cases, except in the presence of Orn. Even at 12 h, exogenous Orn caused a reduction in Ala both in the induced and the un-induced plants. All other treatments showed similar amounts of Ala at a given time. Valine was also lower in the presence of Orn (plus or minus E) 12 h after induction, but higher in Glu and Arg treatments at 24 h; other treatments were not different (Fig. 10b). Isoleucine contents were significantly higher than controls in the presence of E or Arg (un-induced) at 24 h (Fig. 10c).

The Asp family contains six amino acids including Ile (discussed above) that utilize OAA as the C skeleton (Fig. 1); only four of them could be quantified here. Cellular Lys, while lower in the presence of Orn (+E) or Arg (-E), showed a small increase in the presence of Glu; there was no major change over time between 12 h and 24 h (Fig. 11a). Methionine, whose content was the lowest (<0.1% of total) among the PCA-soluble amino acids, did not change much either with time or with treatment (Fig. 11b). Since Asn was added to remove excess dansyl chloride in the dansylation reaction, it could not be correctly quantified; Asp did not get resolved and Thr was a part of the Arg peak.
Figure 9. Cellular contents of PCA soluble amino acids in 12 h and 24 h induced (+E) and un-induced mODC-10-1 transgenic plants with or without 0.1 mM Orn, 0.5 mM Arg or 1 mM Glu. N= 4; each replicate consists of 6-7 seedlings. * = P ≤ 0.05 for significant difference between treatment and control at a given time.
Figure 10. Cellular contents of PCA soluble amino acids in 12 h and 24 h induced (+E) and un-induced mODC-10-1 transgenic plants with or without 0.1 mM Orn, 0.5 mM Arg or 1 mM Glu. N= 4; each replicate consists of 6-7 seedlings. * = P≤ 0.05 for significant difference between treatment and control at a given time.
Figure 11. Cellular contents of (a) lysine and (b) methionine in 12 h and 24 h induced (E) and un-induced mODC-10-1 transgenic plants with or without 0.1 mM Orn, 0.5 mM Arg or 1 mM Glu. N = 4; each replicate consists of 6-7 seedlings. * = P ≤ 0.05 for significant difference between treatment and control at a given time.
Figure 12. Cellular contents of total PCA soluble amino acids in 12 h and 24 h induced (E) and un-induced mODC-10-1 transgenic plants with or without 0.1 mM Orn, 0.5 mM Arg or 1 mM Glu. N= 4; each replicate consists of 6-7 seedlings. * = P≤ 0.05 for significant difference between treatment and control at a given time.
Total soluble amino acids were slightly higher (1.2-fold) in the induced plants at 12 h of induction as compared to the control plants (Fig. 12). No significant changes were observed among different treatments and control plants at this time. At 24 h, induced plants maintained relatively higher (1.2-fold) amounts of total soluble amino acids as compared to the control. Interestingly, Arg-treated plants showed higher amounts of total soluble amino acids (1.3-fold), although Orn and E+Orn treated plants showed slightly lower amounts of soluble amino acids as compared to the control plants. On the basis of their abundance, the major amino acids in the PCA-soluble fraction were Gln, Arg (+Thr and Gly), Leu (+Trp), Glu, Phe and Ser; all others constituted less than 2% of the total fraction. Among those that varied with Orn treatment were Glu (which increased) and Arg, Ala, Pro, Val, Cys, and Lys whose relative concentrations were lowered (Tables 9A and 9B). At 24 h, often the changes were more pronounced but showed the same trends.

**Inducible expression of mODC: Uptake and incorporation of $^{14}$C into PAs**

In order to directly measure the activity of mODC in the transgenic plants, and its possible compensatory effects on ADC activity, 14 d-old seedlings were induced with estradiol and immediately supplied with U$^{14}$C-Orn or U$^{14}$C-Arg, and the incorporation of $^{14}$C into the PAs was followed by dansylation and partitioning into toluene (Bhatnagar et al. 2001). The data in Fig. 13a clearly show that induction of mODC had no effect on the incorporation of U$^{14}$C-Arg into total PAs, indicating no reduction in Put biosynthesis via the ADC pathway. However, the incorporation of U$^{14}$C-Orn was several-fold higher in the induced transgenic plants. Some of the isotope incorporation into Pu in the absence of induction is perhaps due to its entry into Put via its conversion into Arg and the ADC pathway. Figure 13b represents the amount of radioactivity retained in the aqueous
fraction, which includes unused $^{14}\text{C}-\text{Orn}$ as well as that which may have been incorporated into amino acids, and its catabolic products. Radioactivity in this fraction was generally inversely related to its incorporation into the PA fraction.

While it may appear that in the absence of induction more $^{14}\text{C}-\text{Orn}$ was taken up by the seedlings (Fig. 13c), it is also plausible that some of it was incorporated into PCA-insoluble fraction, which was not counted. This radioactivity could be in proteins or in PA conjugates. No significant difference in radioactivity present in this fraction was observed for $^{14}\text{C}-\text{Arg}$ in the induced vs. the un-induced plants.

**Constitutive expression of mODC: Polyamines and amino acids in seedlings**

The effect of Orn utilization and the accumulation of Put in transgenic plants constitutively expressing the mODC gene (under 35S promoter) were analyzed at the seedling stage as well as in different organs at maturity. Cellular Put content was significantly higher in different transgenic lines but to a variable extent (mODC-1-7 by ~39-fold, mODC-4-11 by ~50-fold, and mODC-18-2 by ~24-fold) as compared to the WT seedlings at 14 d of growth (Fig. 14a). While Cad did not always separate well from Put (Fig. S1), its peak was obvious in the transgenic plants; Fig. 14a includes combined amounts of Put + Cad. However, Put contributed > 80% of the total area of the Put+Cad peak. No Cad peak was observed in the WT seedlings. Spermidine content did not show a significant change in two of the three lines (Fig. 14b); it was somewhat lower in the third. Spermine was significantly higher (but to a much lesser extent than Put; up to 1.3-fold) in the line mODC-1-7 but lower in the line mODC-18-2 as compared to the WT seedlings (Fig. 14c). A 7.3-fold and 4.8-fold increase in total PAs was observed in the lines mODC-1-7 and mODC-18-2, respectively vs. the WT seedlings (Fig. 14d).
Figure 13. Distribution of $^{14}$C into polyamines (a), the aqueous fraction (b), and the PCA extract (c) from $^{14}$C-Orn or $^{14}$C-Arg (given for 8 h following 2 h of induction) in 14 d old mODC-10-1 transgenic seedlings N= 3; each replicate consists of 12-14 plants. * = P≤ 0.05 for significant difference between un-induced (C) and induced (+E) samples.
Figure 14. Cellular contents of PCA soluble polyamines in 14 d old WT and 35S::mODC transgenic plants grown in solid germination medium N= 3; each replicate consists of 7-8 seedlings. * = P< 0.05 for significant difference between WT and mODC transgenic plants; (a) Put+Cad in case of mODC plants.
Glutamate was the third most abundant amino acid (after Gln and Arg) found in both the WT and the 35S::mODC transgenic seedlings. Of the two transgenic lines analyzed, Glu was somewhat lower (P=0.06) in the m mODC-18-2 vs. the WT seedlings (Fig. 15a), no significant difference was observed between the WT and those of transgenic line 1-7. It should be pointed out that line 1-7 had 1.6-fold more Put than the line 18-2. Glutamine was the most abundant amino acid in the seedlings, and its content was relatively lower in both the transgenic lines (P= 0.07 for mODC-1-7 and P= 0.09 for mODC-18-2) as compared to the WT (Fig. 15b). Ornithine, which again was among the least abundant of the amino acids, was several-fold lower in both of the mODC transgenic seedlings vs. the WT plants (Fig. 15c); cellular content of Pro was somewhat higher in the line 1-7 (Fig. 15d). No major differences in His and Arg contents were observed between WT and mODC transgenic seedlings; although both were slightly lower in the transgenic lines (Figs. 15e, 15f). While the cellular concentration of GABA was significantly higher in the m mODC-1-7 line (Fig. 15g) and Cys was generally lower, Ser and Gly were similar in all three genotypes (Fig. 15g-j). With the exception of Met (which was not detected in WT – Fig. 16a), and Lys and Phe, which were lower in the transgenic plants (Fig. 16b, 16c); all other amino acids only showed minor differences between the transgenic and the WT plants (Figs. 16d-i).

A small reduction (by ~20%) in total soluble amino acids was observed in both of the transgenic lines (Fig. 17). In relative amounts, Gln constituted about 60% of the total soluble amino acid pool in both the WT and the transgenic seedlings, followed by Arg and Glu, respectively; Met and Trp were the least abundant (Table 10).
Figure 15. Cellular contents of PCA soluble amino acids in 14 d old WT and 35S::mODC transgenic plants grown in solid germination medium. Replicates and statistics are similar to Fig. 14.
Figure 16. Cellular contents of PCA soluble amino acids in 14 d old WT and 35S::mODC transgenic plants grown in solid germination medium. Replicates and statistics are similar to Fig. 14.
Table 10. Contents of different amino acids (as % of total PCA-soluble amino acids) in 14 d old seedlings of WT, mODC-1-7, and mODC-18-2 grown in solid MS medium (N= 3; each replicate consists of 7-8 seedlings). Numbers (bold= >50% change, others= 40-50% change) denote amino acids that are up-regulated and down-regulated respectively.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>WT</th>
<th>mODC1-7</th>
<th>mODC18-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>7.09 ± 0.27</td>
<td>8.60 ± 0.95</td>
<td>7.46 ± 0.10</td>
</tr>
<tr>
<td>Gln</td>
<td>64.41 ± 3.17</td>
<td>58.20 ± 7.16</td>
<td>62.47 ± 2.47</td>
</tr>
<tr>
<td>Ser</td>
<td>3.94 ± 0.24</td>
<td>4.71 ± 0.26</td>
<td>3.97 ± 0.02</td>
</tr>
<tr>
<td>Arg</td>
<td>10.68 ± 1.48</td>
<td>9.20 ± 1.04</td>
<td>10.16 ± 0.58</td>
</tr>
<tr>
<td>Thr</td>
<td>0.85 ± 0.08</td>
<td>1.42 ± 0.08</td>
<td>1.15 ± 0.04</td>
</tr>
<tr>
<td>Gly</td>
<td>2.92 ± 0.51</td>
<td>4.93 ± 0.46</td>
<td>3.90 ± 0.12</td>
</tr>
<tr>
<td>Ala</td>
<td>1.87 ± 0.16</td>
<td>3.07 ± 0.26</td>
<td>2.67 ± 0.07</td>
</tr>
<tr>
<td>Pro</td>
<td>2.88 ± 0.74</td>
<td>5.03 ± 0.95</td>
<td>3.77 ± 0.63</td>
</tr>
<tr>
<td>GABA</td>
<td>0.61 ± 0.09</td>
<td>1.23 ± 0.11</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>Val</td>
<td>0.41 ± 0.02</td>
<td>0.64 ± 0.04</td>
<td>0.55 ± 0.01</td>
</tr>
<tr>
<td>Met</td>
<td>0.00 ± 0.00</td>
<td>2.79 ± 1.13</td>
<td>0.93 ± 0.08</td>
</tr>
<tr>
<td>Ile</td>
<td>0.11 ± 0.00</td>
<td>0.17 ± 0.02</td>
<td>0.15 ± 0.00</td>
</tr>
<tr>
<td>Leu</td>
<td>0.42 ± 0.02</td>
<td>0.54 ± 0.06</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>Trp</td>
<td>0.02 ± 0.02</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Phe</td>
<td>0.34 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.33 ± 0.01</td>
</tr>
<tr>
<td>Cys</td>
<td>0.25 ± 0.03</td>
<td>0.22 ± 0.03</td>
<td>0.20 ± 0.05</td>
</tr>
<tr>
<td>Orn</td>
<td>1.43 ± 0.31</td>
<td>0.07 ± 0.01</td>
<td>0.31 ± 0.05</td>
</tr>
<tr>
<td>Lys</td>
<td>0.41 ± 0.08</td>
<td>0.30 ± 0.02</td>
<td>0.29 ± 0.02</td>
</tr>
<tr>
<td>His</td>
<td>1.35 ± 0.14</td>
<td>1.29 ± 0.03</td>
<td>1.30 ± 0.03</td>
</tr>
</tbody>
</table>

Figure 17. Total PCA soluble amino acids in 14 d old WT and 35S::mODC transgenic plants grown in germination medium. Replicates and statistics are similar to Fig. 14.
Constitutive expression of mODC: Polyamines and amino acids in mature plants

Constitutive transgenic lines over-producing Put showed delay in flowering as compared to the WT plants. A 7-8 day delay in flowering was observed in the lines 1-7 and 4-11 containing a relatively higher amount of Put, whereas line 18-2 with relatively lower Put content flowered at the same time as the WT plants (Fig. 18). While ~90% of the WT and mODC 18-2 plants were flowering by the end of 4-5 weeks, less than 50% of the transgenic plants of the other two lines flowered at 5 weeks. The initial delay in flowering was overcome in the course of time and the transgenic lines 1-7 and 4-11 showed significant increase in vegetative as well as reproductive growth. Both fresh weight (FW) and dry weight (DW) per plant were significantly higher in the transgenic line 4-11 (Figs. 19a, 19b). Moisture (FW-DW) content was also higher in the transgenic lines as compared to the WT plants (Fig. 19a), still an increase in DW was still visible. The height of the plant, the number of siliques per plant, and the number of branches per plant were significantly greater in both 1-7 and 4-11 lines (Fig. 19c-e).

While significantly higher chlorophyll a and chlorophyll b contents per mg protein were observed in the line 1-7; a smaller increase (P= 0.07 for chlorophyll a P= 0.19 in chlorophyll b) was observed in the line 4-11 (Fig. 20). Small but significant (P<0.05) increases in total C as well as total N content per plant were observed in the line 4-11, but the differences were not statistically significant (Fig. 21).

Since mODC-1-7 line had higher amount of Put in the seedlings, the T3 plants of this line were grown to maturity and various plant organs were analyzed for their PA and amino acid contents. Whereas the cauline leaves and the siliques showed two-to-three fold higher amounts of Put in the transgenic plants (Fig. 22a), rosette leaves, flower buds
Figure 18. Progression of flowering with time in wild type (WT) and three different transgenic lines of 35S::mODC plants.
Figure 19. Comparison of different phenotypic parameters between WT and mODC transgenic plants (a, b= 4 week old plants; c, d, e= 7 week old plants). * = P≤ 0.05 for significant difference between WT and mODC transgenic plants (N= 9-10).
Figure 20. Cellular contents of chlorophyll a and b in rosette leaves of 4 wk-old WT and mODC transgenic plants grown in pots. N= 4; * = P≤ 0.05 for significant difference between WT and mODC transgenic plants.

Figure 21. Cellular contents of total carbon (C) and nitrogen (N) in 4 wk-old WT and mODC transgenic plants grown in pots. N=10; * = P≤ 0.05 for significant difference between WT and transgenic plants.
Figure 22. Cellular contents of PCA soluble polyamines in different organs of 6 wk-old WT (□) and mODC-1-7 (■) transgenic plants. N= 3; each replicate consists of 2-3 individual plants. * = P≤ 0.05 for significant difference between WT and mODC transgenic plants.
and mature flowers did not show significant differences in their Put contents vs. the WT counterparts. Likewise, in most tissues, Spm was higher in the transgenic plants (vs. the WT) but no difference in Spd was seen (Figs. 22b, 22c). All of the mature organs that were tested contained significant amounts of Cad, which was actually even higher than Put in almost all organs (Fig. 22d); WT plants had no Cad in any organ. Among the different organs, flower buds and mature flowers had the greatest amount of all four PAs, and the transgenic buds (P=0.10), cauline leaves (P=0.07), rosette leaves (P=0.02), and flowers (P=0.04) had significantly higher total PAs as compared to the WT plants.

Whereas the total soluble amino acid contents were comparable in the leaves, flowers and siliques of the WT and the transgenic plants, total amino acids pool of flower buds was greater in the latter. As with the seedlings, Gln was the most abundant amino acid in all organs but its relative content was generally <40% (Table 11). Glutamate made up almost twice the relative content vs. the seedlings; moreover, while Arg was reduced tremendously, Thr was much greater in mature parts than the seedlings.

The amino acids whose contents were different in transgenic vs. the WT plants in different organs are summarized in Figs. 23 and 24; they include increases in Glu, Gln, Leu, Val and His in the buds, and Pro and Ile in the rosette leaves. On the other hand, Glu and Thr in the leaves, Ala in the buds, Orn in the flowers, and Arg, Orn, and His in the siliques were somewhat higher in the WT than the transgenic plants. Notably, Glu content was significantly higher in the buds of the transgenic plants vs. the buds of WT plants (Fig. 23a), but it was significantly lower (than WT) in rosette leaves and siliques of the transgenic plants. Also, while Orn was absent in many organs except siliques and flowers, His was not detected in the rosette leaves and buds of WT plants.
Table 11. Contents of amino acids (as % of total PCA-soluble amino acids) in different tissues of 6-week old WT and mODC-7 plants grown in pots (N= 3; Each replicate consists of tissues from 2-3 plants). Numbers in bold denotes >50% change, and others 40-50% change.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>WT (Siliques)</th>
<th>mODC (Siliques)</th>
<th>WT (Flowers)</th>
<th>mODC (Flowers)</th>
<th>WT (Buds)</th>
<th>mODC (Buds)</th>
<th>WT (Rosette leaves)</th>
<th>mODC (Rosette Leaves)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>20.47 ± 2.31</td>
<td>19.09 ± 1.72</td>
<td>11.91 ± 1.32</td>
<td>11.17 ± 0.33</td>
<td>18.07 ± 0.50</td>
<td>13.12 ± 0.85</td>
<td>32.35 ± 2.60</td>
<td>28.11 ± 2.78</td>
</tr>
<tr>
<td>Gln</td>
<td>36.03 ± 4.22</td>
<td>37.43 ± 3.34</td>
<td>34.40 ± 2.31</td>
<td>41.94 ± 1.42</td>
<td>36.77 ± 2.55</td>
<td>52.38 ± 0.73</td>
<td>16.57 ± 1.41</td>
<td>16.76 ± 3.59</td>
</tr>
<tr>
<td>Ser</td>
<td>6.01 ± 1.35</td>
<td>6.94 ± 0.26</td>
<td>6.39 ± 0.46</td>
<td>5.35 ± 0.14</td>
<td>6.06 ± 0.44</td>
<td>3.95 ± 0.22</td>
<td>11.14 ± 3.04</td>
<td>21.22 ± 1.79</td>
</tr>
<tr>
<td>Arg</td>
<td>2.68 ± 1.40</td>
<td>0.12 ± 0.07</td>
<td>0.71 ± 0.36</td>
<td>1.44 ± 0.07</td>
<td>0.80 ± 0.27</td>
<td>2.52 ± 1.21</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Thr</td>
<td>11.41 ± 2.16</td>
<td>14.69 ± 1.43</td>
<td>6.79 ± 0.33</td>
<td>6.38 ± 0.07</td>
<td>6.00 ± 0.36</td>
<td>3.70 ± 0.49</td>
<td>16.82 ± 2.17</td>
<td>9.75 ± 0.58</td>
</tr>
<tr>
<td>Gly</td>
<td>1.57 ± 0.28</td>
<td>2.31 ± 0.37</td>
<td>5.47 ± 0.21</td>
<td>4.66 ± 0.20</td>
<td>2.51 ± 0.26</td>
<td>2.03 ± 0.14</td>
<td>1.41 ± 0.50</td>
<td>1.57 ± 0.37</td>
</tr>
<tr>
<td>Ala</td>
<td>6.40 ± 0.99</td>
<td>7.35 ± 0.41</td>
<td>8.72 ± 1.41</td>
<td>7.48 ± 0.42</td>
<td>11.17 ± 0.42</td>
<td>5.85 ± 0.29</td>
<td>8.84 ± 1.76</td>
<td>5.22 ± 0.34</td>
</tr>
<tr>
<td>Pro</td>
<td>2.82 ± 0.56</td>
<td>1.76 ± 0.30</td>
<td>6.75 ± 0.69</td>
<td>4.42 ± 0.34</td>
<td>7.29 ± 0.76</td>
<td>4.55 ± 0.67</td>
<td>1.88 ± 0.39</td>
<td>3.78 ± 0.59</td>
</tr>
<tr>
<td>Gaba</td>
<td>2.11 ± 0.73</td>
<td>3.09 ± 0.17</td>
<td>3.87 ± 0.44</td>
<td>3.46 ± 0.42</td>
<td>4.51 ± 0.41</td>
<td>2.68 ± 0.05</td>
<td>3.08 ± 0.50</td>
<td>2.21 ± 0.50</td>
</tr>
<tr>
<td>Val</td>
<td>2.81 ± 0.23</td>
<td>2.61 ± 0.33</td>
<td>3.51 ± 0.24</td>
<td>3.39 ± 0.04</td>
<td>2.51 ± 0.16</td>
<td>2.32 ± 0.15</td>
<td>1.89 ± 0.48</td>
<td>3.09 ± 0.14</td>
</tr>
<tr>
<td>Met</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.00</td>
<td>0.14 ± 0.04</td>
<td>0.12 ± 0.02</td>
<td>0.08 ± 0.00</td>
<td>0.07 ± 0.02</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Ile</td>
<td>1.79 ± 0.22</td>
<td>1.59 ± 0.37</td>
<td>2.32 ± 0.17</td>
<td>2.24 ± 0.03</td>
<td>1.03 ± 0.22</td>
<td>1.36 ± 0.33</td>
<td>0.71 ± 0.21</td>
<td>1.23 ± 0.20</td>
</tr>
<tr>
<td>Leu</td>
<td>1.57 ± 0.06</td>
<td>1.43 ± 0.24</td>
<td>3.80 ± 0.23</td>
<td>3.44 ± 0.06</td>
<td>1.47 ± 0.23</td>
<td>1.59 ± 0.07</td>
<td>1.51 ± 0.39</td>
<td>2.50 ± 0.32</td>
</tr>
<tr>
<td>Trp</td>
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<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
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<td>nd</td>
</tr>
<tr>
<td>Phe</td>
<td>0.71 ± 0.04</td>
<td>0.83 ± 0.06</td>
<td>1.20 ± 0.08</td>
<td>1.23 ± 0.07</td>
<td>0.85 ± 0.03</td>
<td>0.62 ± 0.02</td>
<td>2.93 ± 0.29</td>
<td>3.32 ± 0.19</td>
</tr>
<tr>
<td>Orn</td>
<td>0.12 ± 0.02</td>
<td>nd</td>
<td>0.17 ± 0.04</td>
<td>nd</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Lys</td>
<td>0.88 ± 0.02</td>
<td>0.69 ± 0.06</td>
<td>1.17 ± 0.05</td>
<td>0.94 ± 0.08</td>
<td>0.85 ± 0.01</td>
<td>0.64 ± 0.05</td>
<td>1.07 ± 0.07</td>
<td>1.22 ± 0.11</td>
</tr>
<tr>
<td>His</td>
<td>3.86 ± 0.06</td>
<td>nd</td>
<td>3.47 ± 0.39</td>
<td>2.33 ± 0.22</td>
<td>0.00 ± 0.00</td>
<td>2.60 ± 0.44</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>
Figure 23. Cellular contents of amino acids in different organs of 6 wk-old WT (■) and mODC-1-7 (■) transgenic plants. N= 3; each replicate consists of 2-3 individual plants. * = P≤ 0.05 for significant difference between WT and mODC transgenic plants.
Figure 24. Cellular contents of amino acids in different organs of 6 wk-old WT (□) and mODC-1-7 (■) transgenic plants. N= 3; each replicate consists of 2-3 individual plants. * = P ≤ 0.05 for significant difference between WT and mODC transgenic plants.
Total soluble amino acids were higher in the reproductive organs among which flowers recorded the highest amount as compared to the rosette leaves (Fig. 25). No significant change in total soluble amino acids was observed among different organs except for the buds of mODC transgenic plants that showed significantly higher amount of total soluble amino acids as compared the WT buds.

**Exogenous supply of polyamines: Polyamines and amino acids**

Since significant differences in some amino acids were seen between the WT and the mODC transgenic plants, it was hypothesized that if the changes in amino acids were due to the presence of high Put, it should be possible to mimic these changes in WT plants by treatment with exogenous PAs. In other words, the question was: will exogenous PA treatments achieve the same effects as the metabolic up-regulation of Put via genetic manipulation? Seeds of WT plants were germinated in the presence of Put, Spd or Spm and seedlings collected after 7 d for analyses of PAs and amino acids.

As shown in Fig. 26a, exogenous Put was accumulated in the WT seedlings up to a level about 50% of that seen in mODC transgenic plants; however, there was no significant change in the cellular Spd content (Fig. 26b) and there was a small reduction in Spm content (Fig. 26c). Treatments with either exogenous Spd or Spm on the other hand resulted in the accumulation of not only the respective PA but also higher Put was seen in the seedlings. Whether or not it was due to back-conversion of the higher PAs into Put or a reduction in the use of Put to make higher PAs was not investigated.

Among the amino acids in which differences were observed between the control and Put treated seedlings were decreases in Pro, GABA, Phe and Ala and increases in Gln (\(P = 0.10\)), Orn, Trp and to some extent in Cys+cystine (Figs. 27, 28). Exogenous Spd had
Figure 25. Total soluble amino acids in different organs of 6 wk-old WT (□) and mODC-1-7 (■) transgenic plants. N= 3; each replicate consists of 2-3 individual plants. * = P≤ 0.05 for significant difference between WT and mODC transgenic plants.
Figure 26. Cellular contents of PCA soluble polyamines in 7 d old wild type (WT) Arabidopsis plants grown in solid germination medium without any treatment (control; C) and medium supplemented with exogenous 1 mM Put, 1 mM Spd or 0.5 mM Spm. N= 6 (pooled from two independent experiments); each replicate consists of 7-8 seedlings. * = P≤ 0.05 for significant difference between control and different treatments.
Figure 27. Cellular contents of soluble amino acids in 7 d old wild type (WT) Arabidopsis plants grown in solid germination medium without any treatment (control; C) and medium supplemented with exogenous 1 mM Put, 1 mM Spd or 0.5 mM Spm. N= 3; each replicate consists of 7-8 seedlings. * = P≤ 0.05 for significant difference between control and different treatments.
Figure 28. Cellular contents of soluble amino acids in 7 d old wild type (WT) Arabidopsis plants grown in solid germination medium without any treatment (control; C) and medium supplemented with exogenous 1 mM Put, 1 mM Spd or 0.5 mM Spm. N= 3; each replicate consists of 7-8 seedlings. * = P ≤ 0.05 for significant difference between control and different treatments.
little effect on most amino acids, except Orn, Arg+Thr+Gly and Val/Ile were higher in its presence. The effects of Spm were somewhat similar to those of Spd. Exogenous Put increased the amount of total amino acids by ~1.3-fold (FW basis; Fig. 29) whereas Spd and Spm did not have similar effects. As a percentage of the total amino acids content, exogenous Put increased Trp and decreased Pro and Phe (>50%), whereas exogenous Spm increased the proportion (>50%) of Arg+Thr+Gly, Pro, Leu, Orn and Lys; Spd had a minimal effect on proportions of different amino acids (Table 12).

**Constitutive expression of mODC: Extra nitrogen and carbon in the medium**

As described earlier, it can be argued that Orn is a limiting metabolite for the continued production of Put in the mODC-transgenic plants. Since Orn is largely produced from Glu, whose production requires the continued supply/assimilation of N and C, the following question could be raised: “Will an increased supply of N and/or C in the growth medium allow extra Put to be produced in either the WT or the transgenic plants?” Furthermore, “Will the variation in either N or C availability affect the amino acids pool in the seedlings which are not fully photosynthetic?” Thus an experiment was set up in which both N and C was varied independently in the medium on which seeds of WT and a transgenic (mODC-1-7) line were germinated and grown for 12 d, at which time the seedlings were collected and analyzed for PAs and soluble amino acids.

The seeds germinated and the seedlings grew quite well on all media except those growing without N in the medium where both the WT and the transgenic seedlings looked pale and weak. Seedlings grown in high sucrose concentrations were darker green as compared to the control seedlings (Fig. 30). The FW and DW of the seedlings were higher with increase in nitrate and sucrose in the medium for both genotypes (Figs. 31).
Table 12. Contents of amino acids (as % of total PCA-soluble amino acids) in 7-d old WT seedlings grown in control (C), 1 mM Put, 1 mM Spd and 0.5 mM Spm treated solid GM (N= 3; each replicate consists of 7-8 seedlings). Red= increased, blue= decreased, bold = >50% change.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>C</th>
<th>1 mM Put</th>
<th>1 mM Spd</th>
<th>0.5 mM Spm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu</td>
<td>3.53 ± 0.20</td>
<td>2.74 ± 0.29</td>
<td>3.12 ± 0.34</td>
<td>3.14 ± 0.39</td>
</tr>
<tr>
<td>Gln</td>
<td>79.50 ± 1.07</td>
<td>83.78 ± 7.95</td>
<td>74.99 ± 5.03</td>
<td>72.12 ± 7.39</td>
</tr>
<tr>
<td>Ser</td>
<td>2.88 ± 0.14</td>
<td>2.89 ± 0.19</td>
<td>3.45 ± 0.11</td>
<td>4.15 ± 0.48</td>
</tr>
<tr>
<td>Arg+Thr+Gly</td>
<td>9.71 ± 0.45</td>
<td>7.71 ± 0.57</td>
<td>13.56 ± 0.38</td>
<td>14.80 ± 1.64</td>
</tr>
<tr>
<td>Ala</td>
<td>1.23 ± 0.08</td>
<td>0.67 ± 0.04</td>
<td>1.12 ± 0.20</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>Pro</td>
<td>0.53 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.63 ± 0.07</td>
<td>0.82 ± 0.06</td>
</tr>
<tr>
<td>GABA</td>
<td>0.62 ± 0.06</td>
<td>0.37 ± 0.03</td>
<td>0.77 ± 0.11</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>Val</td>
<td>0.30 ± 0.00</td>
<td>0.22 ± 0.02</td>
<td>0.36 ± 0.01</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Met</td>
<td>0.07 ± 0.00</td>
<td>0.05 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>Ile</td>
<td>0.29 ± 0.01</td>
<td>0.30 ± 0.03</td>
<td>0.38 ± 0.01</td>
<td>0.40 ± 0.02</td>
</tr>
<tr>
<td>Leu</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Trp</td>
<td>0.09 ± 0.01</td>
<td>0.16 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>Phe</td>
<td>0.47 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.42 ± 0.04</td>
<td>0.55 ± 0.03</td>
</tr>
<tr>
<td>Cys</td>
<td>0.20 ± 0.02</td>
<td>0.27 ± 0.06</td>
<td>0.22 ± 0.03</td>
<td>0.28 ± 0.08</td>
</tr>
<tr>
<td>Orn</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.37 ± 0.05</td>
<td>0.55 ± 0.09</td>
</tr>
<tr>
<td>Lys</td>
<td>0.13 ± 0.01</td>
<td>0.07 ± 0.00</td>
<td>0.16 ± 0.01</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>His</td>
<td>0.23 ± 0.03</td>
<td>0.19 ± 0.03</td>
<td>0.22 ± 0.04</td>
<td>0.32 ± 0.04</td>
</tr>
</tbody>
</table>
Figure 29. Total soluble amino acids in 7 d old WT seedlings grown in solid GM without any treatment (control; C) and GM supplemented with 1 mM Put, 1 mM Spd and 0.5 mM Spm. N= 3; each replicate consists of 7-8 seedlings. *=P≤ 0.05 for significant difference between control and different treatments.
Figure 30. Representative samples of 12 d old WT seedlings grown in GM (C), GM supplemented with 50 mM sucrose and GM without N (w/o N). Similar phenotypes were observed for mODC-1-7 transgenic line in different media.
Figure 31. Biomass (fresh weight – FW, dry weight - DW and DW as % of FW) parameters of 12 d old WT and mODC1-7 transgenic seedlings in response to different concentrations of sucrose and nitrate. * = P ≤ 0.05 for significant difference between treatments and corresponding control within WT and mODC plants (N= 4; each replicate consists of 40-50 seedlings).
Sucrose had greater effect on DW than nitrate although the increment of DW (as % of FW) was slightly higher in the mODC seedlings as compared to the WT seedlings (Fig. 31c). Nitrogen limitation significantly reduced FW and DW in both genotypes as compared to the corresponding controls (GM medium); the mODC seedlings had higher FW and DW (P= 0.09) than the WT seedlings under N limitation.

As expected, the ODC transgenic line had almost 50-fold higher amount of Put with little change in Spd and Spm (Fig. 32). In the complete absence of N, Put was barely detectable, whereas, Spd and Spm were reduced only by about 50-60% each. Additional nitrate at 30 mM did not affect Put or Spm in the WT seedlings, while there was a significant reduction in Put as well as Spd in both genotypes at 60 mM nitrate; the effect being more pronounced in the transgenic plants. Spermine was either not altered (WT) or increased slightly (transgenics) with increasing N in the medium. In the absence of sucrose, Put was reduced only in the transgenic plants; other PAs showed just small changes if any. However, additional sucrose enhanced the accumulation of PAs in most cases, except at 100 mM in the transgenic seedlings where Put was reduced by >50%.

Significant differences were seen in the amino acids content of the WT and the transgenic plants on the control medium as well as in response to different treatments within each genotype. Among the abundant (>500 nmol.g⁻¹FW) amino acids were Glu, Gln, Arg, Ser, Ala, and Pro; the ones present in low amounts (<100 nmol.g⁻¹FW) were Leu, Met, Lys, His, Trp, and Orn (Figs. 33A, 33B, 34A, 34B). Ornithine, which was always present in the WT plants, was conspicuously absent in the transgenic plants under almost all conditions. In the absence of N in the medium, most amino acids were present in very small amounts although many plants seemed to have been alive.
Figure 32. Effects of different concentrations of nitrate and sucrose on PCA soluble polyamines in 12 d old WT and mODC Arabidopsis seedlings grown in solid GM. N= 4; each replicate consists of 7-8 seedlings. * as in Fig. 27.
Figure 32 (continued).
As shown earlier, cellular concentrations of Glu and Gln were somewhat lower in the transgenic plants vs. the WT plants, but the differences were often not statistically significant. Higher nitrate in the medium increased Glu content in both genotypes; again the differences were not significant and the transgenic plants had lower Glu. On the other hand higher sucrose concentration in the medium significantly increased Glu and Gln content in the transgenic plants; in the absence of sucrose, a significant decrease in these amino acids was seen (Figs. 33A.a, 33A.b). Ornithine was often below detection limits in the transgenic plants, and changed erratically in the WT plants (up in one experiment and down in the other) with either extra nitrate or sucrose. Histidine was also very low and did not show a major change in response to various treatments (Fig. 33A.d). Cellular Pro, whose content was similar in the two genotypes grown in the control medium, increased with increasing concentrations of both nitrate and sucrose; its relative concentration was slightly higher in the mODC seedlings vs. their WT counterparts (Fig. 33B.a). Also, sucrose had a greater effect on Pro than nitrate especially in the transgenic seedlings. Either N or sucrose limitation in the medium significantly reduced Pro in both genotypes.

The combined contents of Arg+Thr+Gly were comparable in the two genotypes and varied similarly in response to experimental treatments. While N limitation significantly reduced the amounts of these amino acids in both genotypes; 60 mM additional nitrate also caused a small reduction. Extra sucrose had no major effect on these amino acids except for a small increase in the WT plants. Reduced N and higher sucrose had similar effects on GABA and Ser contents, in that lower N reduced them and higher sucrose enhanced these amino acids in both genotypes (Figs. 33B.c, 33B.d).
Figure 33A. Effects of different concentrations of nitrate and sucrose on PCA soluble amino acids in 12 d old WT (□) and mODC-1-7 (△) plants. *= P < 0.05 for significant difference between treatments and control within WT and mODC plants; + represents P < 0.05 for significant difference between WT and mODC plants (N = 3; each replicate= 7-8 seedlings).
Figure 33B. Effects of different concentrations of nitrate and sucrose on PCA soluble amino acids in 12 d old WT (■) and mODC-1-7 (□) plants. For explanation of statistics, see Fig. 33A.
Tryptophan was not detected in most cases except for a substantial amount in the 60 mM nitrate treatment in both genotypes (Fig. 34A.a). The presence of extra N in the medium significantly increased Phe content and its absence lowered it in both genotypes (Fig. 34A.b); sucrose had little effect on this amino acid. Higher sucrose as well as nitrate in the medium significantly increased Ala and Val contents in both WT and mODC plants, and lower N negatively affected them (Figs. 34B.a, 34B.b). While Ala content was more than double in the mODC plants than the WT, and remained higher in all treatments; Val showed less difference between the two genotypes. Extra sucrose positively affected Ile in both genotypes (Fig. 34B.c). Cellular contents of Met, Lys and Leu were generally low, and showed smaller variations among the treatments except that Lys was always lower in the mODC plants (Figs. 34A.c, 34A.d, 34B.d).
Figure 34A. See details for Fig. 33A
Figure 34B. See details for Fig. 33A
OBJECTIVE 2. The spatial and temporal expression patterns of AtSAMDCs

Cloning of promoter regions

In order to identify the putative promoter region of each of the three AtSAMDCs, the expression profile of these genes, and the role of their 5'UTRs and ORFs in the regulation of their expression, the technique of promoter:reporter gene fusion was used. Three or four different promoter::GUS fusion constructs were made for each gene that contained the promoter+5'UTR+ORF (translational fusion) or promoter+5'UTR (transcriptional fusion) or just the promoter or its 5' truncated version of the promoter. The length of the putative promoter sequence cloned for each gene varied and was based on the location of the neighboring genes upstream of the gene of interest. Selection of deletion at the 5' end of the putative promoter was guided by the presence of cis regulatory elements that were identified by bioinformatics analysis of the putative promoter sequence (Table 15-17). For example, four different fragments of AtSAMDC3 promoter, named SAMDC3-A, SAMDC3-B, SAMDC3-C and SAMDC3-D (Fig. 35), were PCR amplified from A. thaliana gDNA using sequence specific primers (Table 8). The products were cloned into pCR8.0/GW/TOPO vector and the insertions were confirmed first by restriction digestion and gel electrophoresis and then by sequencing for correct orientation (Figs. 36-39). They were then moved into the destination vector pMDC163 (containing the reporter GUS gene) through LR clonase reaction (details under Materials and Methods). The final constructs in the destination vector were also confirmed similarly. Agrobacterium tumefaciens strain GV3101 was transformed with the selected plasmids for transformation of A. thaliana plants. Similarly four constructs for AtSAMDC4 and three for AtSAMDC5 were prepared and analyzed before transformation.
Figure 35. Different constructs of *AtSAMDC3*, *AtSAMDC4* and *AtSAMDC5* promoters::*GUS* fusion made for analysis of gene expression.
Figure 36. Plasmid maps (A, B) and gel analysis results (C) of SAMDC3-A construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (XbaI; 719 bp, 6923 bp) with SAMDC3-A insert, and (iii) pMDC163 digests (XbaI; 538 bp, 15492 bp) with SAMDC3-A insert. NEB 2-Log DNA ladder.

Figure 37. Plasmid maps (A, B) and gel analysis results (C) of SAMDC3-B construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (BglII+BglII; 1516 bp, 5083 bp) with SAMDC3-B insert, and (iii) pMDC163 digests (SacI; 4148 bp, 10838 bp) with SAMDC3-B insert. NEB 2-Log DNA ladder.
Figure 38. Plasmid maps (A, B) and gel analysis results (C) of SAMDC3-C construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (HpaI; 819 bp, 2862 bp) with SAMDC3-C insert and (iii) pMDC163 digests (HindIII+XbaI; 845 bp, 11224 bp) with SAMDC3-C insert. NEB 2-Log DNA ladder.

Figure 39. Plasmid maps (A, B) and gel analysis results (C) of SAMDC3-D construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (HindII; 339 bp, 2862 bp) with SAMDC3-D insert, and (iii) pMDC163 digests (HpaI+SphI; 551 bp, 11038 bp) with SAMDC3-D insert. NEB 2-Log DNA ladder.
of *A. thaliana* plants by *A. tumefaciens*; the details of these constructs and their confirmation results are shown in Figures Figs. 40-46. A summary of these constructs is shown in Fig. 35. Each of these plasmids was tested for transient expression of *GUS* in poplar (*Populus nigra x maximowiczi*) cells (Bhatnagar et al. 2001) to see if it would produce a functionally active GUS protein.

Following transformation of *A. thaliana*, the T1 seeds were collected and the putative transformed seedlings selected on hygromycin before being transferred to pots. Following GUS analysis in excised leaves, several independent T2 transgenic lines were tested for the presence of a single copy of the T-DNA insert (by observing 3:1 segregation of live : dead plants on hygromycin); five of these lines were selected for the production of T3 generation plants. Each line was further tested for homozygosity (segregation analysis) before further experimental use.

**Transient expression**

All promoter::*GUS* constructs shown in Figure 35 along with pCAM-35S::*GUS* (CaMV35S::*GUS*; used as control) were tested for transient expression of *GUS* using biolistic bombardment (Bhatnagar et al. 2001) in poplar cells. The data summarized in Fig. 47 show that *SAMDC4*-D plasmid, with the shortest putative promoter region of all constructs, showed the highest transient expression of GUS as compared to the other plasmids used in this experiment; its activity was only slightly less than the control 35S promoter. Overall, all *SAMDC4::*GUS constructs had higher activity than the other promoters. Activity of *SAMDC3* promoter was lower than *SAMDC4* promoter; but considerably higher than *SAMDC5* promoter constructs.
Figure 40. Plasmid maps (A, B) and gel analysis results (C) of *SAMDC4*-A construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (*EcoRV*; 342 bp, 4488 bp) with *SAMDC4*-A insert, and (iii) pMDC163 digests (*BglII+XhoI*; 1094 bp, 4699 bp, 7425 bp) with *SAMDC4*-A insert. NEB 2-Log DNA ladder.

Figure 41. Plasmid maps (A, B) and gel analysis results (C) of *SAMDC4*-B construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (*BglII*; 1625 bp, 2164 bp) with *SAMDC4*-B insert, and (iii) pMDC163 digests (*BsaI*; 4490 bp, 7687 bp) with *SAMDC4*-B insert. NEB 2-Log DNA ladder.
Figure 42. Plasmid maps (A, B) and gel analysis results (C) of SAMDC4-C construct (i) PCR product, (ii) pCR8.0/GW/TOPO (BglI+BglII; 1517 bp, 2164 bp) with SAMDC4-C insert, and (iii) pMDC163 digests (BglII+XhoI; 1094 bp, 3550 bp, 7425 bp) with SAMDC4-C insert. NEB 2-Log DNA ladder.

Figure 43. Plasmid maps (A, B) and gel analysis results (C) of SAMDC4-D construct (i) PCR product and (ii) pMDC163 digests (BsaI; 3909 bp, 7579 bp) with SAMDC4-D insert. NEB 2-Log DNA ladder. The pCR8.0/GW/TOPO insert did not have appropriate sites for orientation check, hence was confirmed by sequencing.
Figure 44. Plasmid maps (A, B) and gel analysis results (C) of SAMDC5-A construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (BglI+NcoI; 2429 bp, 3546 bp) with SAMDC5-A insert, and (iii) pMDC163 digests (Hpal+SphI; 1044 bp, 13319 bp) with SAMDC5-A insert. NEB 2-Log DNA ladder except for iii, where it is 1 kb DNA ladder.

Figure 45. Plasmid maps (A, B) and gel analysis results (C) of SAMDC5-B construct (i) PCR product, (ii) pCR8.0/GW/TOPO digests (XbaI; 1703 bp, 3127 bp) with SAMDC5-B insert and (iii) pMDC163 digests (SpeI+SphI; 919 bp, 12299 bp) with SAMDC5-B insert. NEB 2-Log DNA ladder except for iii, where it is 1 kb ladder.
Figure 46. Plasmid maps (A, B) and gel analysis (C) of SAMDC5-C PCR product. The pCR8.0/GW/TOPO and pMDC163 vectors with SAMDC5-C insert did not have appropriate sites for orientation check, and were confirmed by sequencing. 1 kb ladder.

Figure 47. Transient expression of different constructs of AtSAMDC3, AtSAMDC4 and AtSAMDC5: pCAM-35S as control in poplar cells (C). Each bar represents the Mean ± SE number of blue cells from 6 different plates.
Within each promoter, different constructs having/missing different regions of the promoter sequence show that the presence of the 5' UTR or the SAMDC-ORF had rather variable effects in different promoters. For example, SAMDC4-D with the shortest promoter sequence, showed maximum transient expression of GUS in poplar; and the presence or absence of the 5' UTR and/or the SAMDC-4 ORF did not significantly affect transient expression. The expression under two segments of the SAMDC5 promoter was very low as compared to the SAMDC4 promoter fragments. The absence of the ORF increased GUS expression in both SAMDC3 and SAMDC5 and the absence of 5' UTR increased expression in SAMDC3 promoter. The CaMV35S::GUS construct (used as a control), showed the highest expression of all. Although the absolute numbers of blue spots varied among plates; overall the relative expression of different constructs within a promoter was consistent with GUS activity later seen in seedlings and mature plants.

**Expression of AtSAMDC3, AtSAMDC4 and AtSAMDC5 in seedlings**

Five independently transformed (T3 generation) lines were selected for each construct in order to compensate for the bias due to site of integration of T-DNA or any other factors that may affect gene expression. Starting at germination, 10 seedlings grown on GM from each of the 5 transformed lines (in some cases only 3 lines were used) were collected on days 1, 2, 3, 5, 7, 9, 11, 13, and 15 d and assayed for GUS activity (staining); this provided a total of 30-50 samples for each construct for the first 15 days.

Different translational and transcriptional fusion plants of all three SAMDC genes were used to analyze for the presence of GUS activity at 24 h and 48 h of incubation. While detailed photographs were taken on all days of analysis (in each case several plants were photographed), only representative pictures are shown here. At 24 h of incubation
of the seeds on GM plates in the dark at 4°C, no GUS activity was detected for *SAMDC3-A* seeds (Fig. 48). On the other hand, *SAMDC3-B* seeds showed some GUS staining in the cotyledons at 24 h which spread throughout the germinating seedlings at 48 h except for the roots where only the basal half showed GUS activity. On the other hand, high activity of GUS was seen in *SAMDC4-A* seedlings (germinating embryos) at the root tip, emerging cotyledons and hypocotyls, and in the seed coat (around the area of radicle emergence) at 24 h (Fig. 48). By 48 h, staining for GUS activity was intense and spread throughout the entire root, cotyledonary veins and surrounding tissues, and also the entire hypocotyl. For *SAMDC4-B* construct, similar pattern (like *SAMDC4-A*) of GUS staining was observed at 24 h and a significant increase in GUS activity at 48 h throughout the seedlings without any activity in the root tips. For *SAMDC5-A*, no activity of GUS was observed at 24 h, although slight blue color was detected in the cotyledons of emerging seedlings at 48 h (Fig. 48). High activity of GUS was observed at 24 h in the seed coat, cotyledons and root vascular tissue with no expression at the root tips for both *SAMDC5-B* and *SAMDC5-C* seedlings. The intensity of GUS staining was higher throughout the seedlings at 48 h for both *SAMDC5-B* and *SAMDC5-C* constructs (*SAMDC5-B > SAMDC5-C*). Overall the activity of GUS was the highest for *AtSAMDC4* translational fusion (*SAMDC4-A*) as compared to the translational fusions of *AtSAMDC3* and *AtSAMDC5*. High expression of GUS was seen for different transcriptional fusions for all *SAMDC* promoters studied here, *AtSAMDC3* showing the lowest activity.

In one-day old seedlings (actual emergence of the radicle at 25°C) of *SAMDC3-A*, low activity of GUS was localized in the cotyledon veins (Fig. 49). For this construct, the trend of GUS staining was similar during the 15 d growth period where localized activity
Figure 48. Activity of GUS at early germination stages (24 h and 48 h) in different constructs of AtSAMDC3, AtSAMDC4 and AtSAMDC5 transgenic Arabidopsis seeds.
Figure 49. Activity of GUS in 1 d (A1-A4), 3 d (B1-B4), 7 d (C1-C4) and 11 d (D1-D4) old seedlings of *SAMDC3*-A, *SAMDC3*-B, *SAMDC3*-C and *SAMDC3*-D plants.
of GUS was detected mainly in the veins and petioles of the emerging leaves. Removal of ORF from the SAMDC3-B promoter constructs significantly increased GUS activity in all organs except the roots that showed little or no activity throughout the 15 days of analysis. Deletion of the 5'UTR, also deletion at the 5' end of the promoter, did not further affect GUS staining except that in SAMDC3-C the root vascular tissue showed significant staining.

High activity of GUS was observed in the cotyledons, hypocotyls and root vascular tissue except for root tips of 1 d old (Fig. 50) SAMDC4-A seedlings (translational fusion of AtSAMDC4). Similar staining was seen during the first 7 d of growth which continued until day 15 d with relatively high expression also being seen in the emerging leaves and shoot apices, lower part of the roots, and secondary root junctions. Deletion of the ORF sequence in the SAMDC4 constructs lowered GUS activity mainly localizing it as patches in the cotyledons and hypocotyls without much activity in the roots (Figs. 50 A2, B2, C2, D2). At later stages of growth, higher activity of GUS was observed mainly in the newly emerging leaves and secondary root junctions. Removal of the 5'UTR and further deletion of the 5' end of the putative promoter increased GUS activity throughout the seedlings (SAMDC4-C and SAMDC4-D plants), respectively; the activity was higher during the entire 15 d period of seedling growth.

Activity of GUS for AtSAMDC5 promoter low activity in the cotyledons at early stages with gradual increase in activity being localized in the newly emerging leaves, shoot apices, but little activity in the hypocotyls (particularly the upper half), and even less in the roots during the 15 d period of growth (Fig. 51). Removal of ORF (SAMDC5-B) and 5' deletion of the promoter region (SAMDC5-C) exhibited higher GUS activity in
Figure 50. Activity of GUS in 1 d (A1-A4), 3 d (B1-B4), 7 d (C1-C4) and 11 d (D1-D4) old seedlings of $SAMDC4$-A, $SAMDC4$-B, $SAMDC4$-C and $SAMDC4$-D plants.
Figure 51. Activity of GUS in 1 d (A1-A3), 3 d (B1-B3), 7 d (C1-C3) and 11 d (D1-D3) old seedlings of *SAMDC5-A*, *SAMDC5-B* and *SAMDC5-C* plants.
the entire seedlings throughout the 15 d growth period. For translational fusion, while *AtSAMDC4* showed high activity of GUS throughout the seedlings, similar constructs of *AtSAMDC3* and *AtSAMDC5* showed lower activity; and it was mainly localized in the cotyledons and veins with little or no expression in the roots. Transcriptional fusions on the other hand had higher GUS activity in most cases except for *AtSAMDC4*. Deletion of the 5' regions of the promoters did not equally/similarly affect GUS activity in all cases at the seedling stage.

**Expression of *AtSAMDC3*, *AtSAMDC4* and *AtSAMDC5* in mature vegetative organs**

Typically different organs of mature plants at 5 weeks of age were analyzed for presence of GUS activity by reacting with X-Gluc reaction mix. At least three independently selected transgenic lines (with 4-5 plants from each line) per construct were used. Pictures of representative data are shown in Figs. 52-54.

In 5-week old transgenic *SAMDC3-A* plants, little or no GUS activity was observed in the roots, with low staining in the rosette or cauline leaves, which was largely restricted to the leaf or petiole vasculature (Fig. 52). However, there was relatively high GUS activity at the rosette junctions (Fig. 52 B1). Relatively high staining for GUS activity was observed in majority of the organs of *SAMDC3-B* plants, showing that the presence of the ORF had a negative effect on GUS expression/activity. Removal of the 5'UTR in *SAMDC3-C* and *SAMDC3-D* (also partial deletion of the promoter sequence) resulted in substantial increase in GUS staining in the leaves besides high activity in the roots. Rosette leaf junctions always had high GUS activity.

The *SAMDC4-A::GUS* plants showed high GUS activity in the rosette leaves (particularly in the veins of both the lamina and the petioles), rosette leaf junctions, roots
Figure 52. Activity of GUS in different vegetative organs of 5 wk old SAMDC3-A, SAMDC3-B, SAMDC3-C and SAMDC3-D transgenic plants.
and rather low activity in the cauline leaves (Fig. 53). The staining for GUS was relatively low in the majority of the organs of \textit{SAMDC4-B} plants as compared to \textit{SAMDC4-A} plants except for the cauline leaves where GUS activity was relatively higher than \textit{SAMDC4-A} cauline leaves. Removal of 5'UTR and partial deletion of the 5' end of the promoter (\textit{SAMDC4-C} and D) increased GUS activity in leaves and roots in addition to high staining at the rosette junctions.

Activity of GUS in \textit{SAMDC5-A} plants was low in the roots and none in the cauline leaves; but staining was seen in the veins of rosette leaves (more so in the younger leaves) and at the rosette junctions (Fig. 54). The staining of GUS was higher in all organs (particularly in the roots for SAMDC5-B, and rosette leaves, petiole veins and rosette junctions for both \textit{SAMDC5-B} and \textit{SAMDC5-C} plants; also some activity in the cauline leaves) when 5'UTR and/or 5'UTR+ORF were deleted.

\textbf{Expression of At\textit{SAMDC3}, At\textit{SAMDC4} and At\textit{SAMDC5} in reproductive parts}

The activity of GUS was very low in the \textit{SAMDC3-A} flowers overall; it was mainly localized in sepal veins, petals, the filaments and the receptacle (Fig. 55). In the flowers of \textit{SAMDC3-B} (plus 5'UTR but no ORF) plants GUS activity increased significantly in the same organs, still with no expression in the anther sacs or the ovary wall. In \textit{SAMDC3-C} and \textit{SAMDC-D} constructs, further (than \textit{SAMDC3-A} and B) increase in GUS staining was observed, again without change in the organ type. In the developing siliques, GUS was mainly localized in the pedicel and valve junction of \textit{SAMDC3-A} and \textit{SAMDC3-D} plants, whereas for \textit{SAMDC3-B} and \textit{SAMDC3-C} considerable GUS activity was seen in the valves and pedicels of developing siliques. No activity of GUS was detected in the developing embryos of \textit{SAMDC3-A} and \textit{SAMDC3-D} plants, whereas light
Figure 53. Activity of GUS in different vegetative organs of 5 wk old *SAMDC4*-A, *SAMDC4*-B, *SAMDC4*-C and *SAMDC4*-D transgenic plants.
Figure 54. Activity of GUS in different vegetative organs of 5 wk old *SAMDC5-A*, *SAMDC5-B* and *SAMDC5-C* transgenic plants.
Figure 55. Activity of GUS in reproductive parts of SAMDC3-A, SAMDC3-B, SAMDC3-C and SAMDC3-D plants.
GUS staining was observed in the cotyledons of developing embryos. High GUS activity was also seen in the upper part of the ovary including the base of the stigma, except in *SAMDC3-A* plants.

Reproductive tissues and organs showed high activity of GUS in the inflorescence stalks and unopened flower buds of *AtSAMDC4::GUS* plants. In *SAMDC4-A* mature flowers, the intensity of GUS staining was highest in the stamens particularly in the filaments (with only a little expression in the anther sacs/pollen) and in the sepals; (Fig. 56). Stigma and receptacle also showed higher GUS activity but ovary wall had little staining. In sepals, the vascular tissue stained darker than the surrounding cells; in contrast, very low activity of GUS was detected in the petal veins. In *SAMDC4-B* flowers high activity of GUS was observed in all flower parts with some activity being seen in the anther sac cells but not in pollen. Removal of 5′UTR increased overall GUS activity in *SAMDC4-B* and *SAMDC4-C* flowers with the presence of GUS activity in the pollen grains/microspores. In the developing siliques, GUS activity was mainly localized in the valves and pedicels. High activity of GUS was observed for all *SAMDC4* constructs throughout the developing embryos cotyledons. Overall, the GUS expression/activity for *SAMDC4::GUS* constructs was relatively more pronounced and widespread than the *SAMDC3::GUS* constructs.

Unlike the *SAMDC4::GUS* plants, the flowers of *SAMDC5-A::GUS* plants showed only a little or no GUS staining in any part (Fig. 57); the intensity of GUS staining was higher in the other two constructs of this promoter (*SAMDC5-B* and *SAMDC5-C*). The *SAMDC5-B::GUS* and *SAMDC5-C::GUS* flowers mainly showed staining in the sepals, filaments, stigma, petals and receptacle, with very little expression in the petals or pollen.
Figure 56. Activity of GUS in reproductive parts of *SAMDC4-A*, *SAMDC4-B*, *SAMDC4-C* and *SAMDC4-D* plants.
Figure 57. Activity of GUS in reproductive parts of *SAMDC5*-A, *SAMDC5*-B and *SAMDC5*-C plants.
grains. No activity of GUS was detected in the siliques or embryos of $SAMDC5$-A plants, whereas relatively high activity was seen in the valves, receptacle as well as upper part of the ovary and the base of stigma in $SAMDC5$-B and C plants. Low levels of GUS activity were also seen in the developing embryos in $SAMDC5$-B and $SAMDC5$-C plants.

**Bioinformatics analysis of regulatory motifs in the promoter regions of $AtSAMDCs$**

Bioinformatics analysis of the cloned putative promoter regions of all five $SAMDC$ genes in Arabidopsis revealed the presence of several commonly recognized cis-regulatory motifs (transcription factor binding sites) in most of them; the details of their specific names, sequences of these motifs, location within the promoter region, and their general regulatory functions are listed in Tables 13-17. The promoters of $AtSAMDC1$ and $AtSAMDC2$ (not studied for expression here) contained GAREAT and GADOWNAT (GA responsive elements) motifs, CACGTG, MYB1AT and MYB4, and Box II motifs (light responsive element). Presence of several common stress-response motifs; e.g. ABRE-like, cold stress, and MYB1AT (dehydration response) were also found in these genes. The unique motifs in $AtSAMDC1$ promoter sequence are $W$ box (pathogenic response and wounding), $Z$ box (developmental expression) and MYB binding site (developmental and stress). On the other hand promoter sequences of $AtSAMDC2$ shows presence of CARGCW8GAT (AGL-15 site, regulating embryogenesis), $I$ box (light response), RAV1-B (DNA-binding protein), MYCATERD1 (early response to dehydration) and AtMYC2 BS in RD22 (dehydration responsive element). Besides sharing common motifs in the promoter regions associated with abiotic stresses and developmental regulations among different SAMDCs, $AtSAMDC4$ and $AtSAMDC5$ showed presence of auxin binding site factors motif (ARF).
Table 13. Putative *cis* elements in the *AtSAMDC1* promoter sequence (analyzed using Athena promoter analysis software, O'Connor et al. 2005) showing consensus sequences, binding sites, and physiological responses of the identified elements.

<table>
<thead>
<tr>
<th>Motif name</th>
<th>Consensus sequence</th>
<th>Binding sites</th>
<th>Physiological responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABRE-like binding site motif</td>
<td>CACGTGTC</td>
<td>-433 to -428</td>
<td>Dehydration, low temperature</td>
</tr>
<tr>
<td>ACGTABREMOTIFA2OSEM</td>
<td>ACGTGTC</td>
<td>-432 to -426</td>
<td>ABA responsive expression</td>
</tr>
<tr>
<td>BoxII promoter motif</td>
<td>GGTTAA</td>
<td>-414 to -409</td>
<td>light activation</td>
</tr>
<tr>
<td>CACGTGMOTIF</td>
<td>CACGTG</td>
<td>-433 to -428</td>
<td>essential for beta phaseolin gene expression during embryogenesis</td>
</tr>
<tr>
<td>GADOWNAT</td>
<td>ACGTGTC</td>
<td>-432 to -426</td>
<td>GA down regulated expression during seed germination</td>
</tr>
<tr>
<td>GAREAT</td>
<td>TTTGTTA</td>
<td>-282 to -276</td>
<td>GA induced seed germination</td>
</tr>
<tr>
<td>MYB binding site promoter</td>
<td>CACCTACC</td>
<td>-230 to -223</td>
<td>Flower specific motif</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>TGGTTA</td>
<td>-415 to -410, -644 to -639</td>
<td>drought responsive element</td>
</tr>
<tr>
<td>MYB4 binding site motif</td>
<td>ACCTACC</td>
<td>-229 to -223</td>
<td>drought, salt, cold, wounding</td>
</tr>
<tr>
<td>TATA-box Motif</td>
<td>TATAAA</td>
<td>-127 to -122, -66 to -61, -384 to -379</td>
<td>Transcription</td>
</tr>
<tr>
<td>W-box promoter motif</td>
<td>GGCTCA</td>
<td>-108 to -103, -657 to -652</td>
<td>Wounding response</td>
</tr>
<tr>
<td>Z-box promoter motif</td>
<td>ACACGTAT</td>
<td>-449 to -442</td>
<td>light independent developmental expression</td>
</tr>
</tbody>
</table>

Table 14. Putative *cis* elements in the *AtSAMDC2* promoter sequence (analyzed using Athena promoter analysis software, O'Connor et al. 2005) showing consensus sequences, binding sites, and physiological responses of the identified elements.

<table>
<thead>
<tr>
<th>Motif name</th>
<th>Consensus sequence</th>
<th>Binding sites</th>
<th>Physiological responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABRE-like binding site motif</td>
<td>CACGTGTA and TCCACGTG</td>
<td>-458 to -451, -267 to -260, -269 to -262</td>
<td>dehydration, low temperature</td>
</tr>
<tr>
<td>ACGTABREMOTIFA2OSEM</td>
<td>ACGTGTC</td>
<td>-478 to -472, -288 to -282, -266 to -260</td>
<td>ABA responsive expression</td>
</tr>
<tr>
<td>CACGTGMOTIF</td>
<td>CACGTG</td>
<td>-458 to -453, -267 to -262</td>
<td>essential for beta phaseolin gene expression during embryogenesis</td>
</tr>
<tr>
<td>GADOWNAT</td>
<td>ACGTGTC</td>
<td>-478 to -472, -296 to -290</td>
<td>GA down regulated expression during seed germination</td>
</tr>
<tr>
<td>GAREAT</td>
<td>TAACAA</td>
<td>-665 to -659, -621 to -615</td>
<td>GA induced seed germination</td>
</tr>
<tr>
<td>BoxII promoter motif</td>
<td>CTCTAC</td>
<td>-333 to -328</td>
<td>light regulated expression</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>TGGTTA</td>
<td>-729 to -724</td>
<td>dehydration</td>
</tr>
<tr>
<td>MYB4 binding site motif</td>
<td>ACCAAAC</td>
<td>-687 to -681</td>
<td>drought, salt, cold, wounding</td>
</tr>
<tr>
<td>RAV1-B binding site motif</td>
<td>CACCTG</td>
<td>-513 to -508</td>
<td>domain for DNA binding protein</td>
</tr>
<tr>
<td>TATA-box Motif</td>
<td>TATAAA</td>
<td>-31 to -26</td>
<td>transcription</td>
</tr>
</tbody>
</table>
### Table 15. Putative cis elements in the *AtSAMDC3* promoter sequence (analyzed using Athena promoter analysis software, O’Connor et al. 2005) showing consensus sequences, binding sites, and physiological responses of the identified elements.

<table>
<thead>
<tr>
<th>Motif name</th>
<th>Consensus sequence</th>
<th>Binding sites</th>
<th>Physiological responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtMYB2 BS in RD22</td>
<td>CTAACCA</td>
<td>-665 to -659</td>
<td>drought and ABA</td>
</tr>
<tr>
<td>MYB binding site promoter</td>
<td>AACCTAAC GTTTGGTT</td>
<td>-2655 to -2648</td>
<td>flower specific motif</td>
</tr>
<tr>
<td>SV40 core promoter motif</td>
<td>CAATCCAC CTAACCAC</td>
<td>-503 to -496</td>
<td>enhancer</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>TAAACCA TGCTTT TGTTA TGTTT</td>
<td>664 to -659, -715 to -710, -1485 to -1480, -2376 to -2371</td>
<td>drought responsive element</td>
</tr>
<tr>
<td>GAREAT</td>
<td>TTTGTTATTGTA</td>
<td>-1195 to -1189, -2271 to -2265</td>
<td>GA induced seed germination</td>
</tr>
<tr>
<td>TATA-box Motif</td>
<td>TATAAA TATAAA TTATA TTATA TTATA TTATA TTATA</td>
<td>-2343 to -2338, -484 to -479, -408 to 403, -555 to -550, -1336 to -1331, -1840 to -1835, -2692 to -2687</td>
<td>transcription</td>
</tr>
<tr>
<td>DREB1A/CBF3</td>
<td>GCCGACTTT</td>
<td>-2970 to -2963</td>
<td>drought response</td>
</tr>
<tr>
<td>MYB2AT</td>
<td>TAACTG TAACTG CAGTTA</td>
<td>-2894 to -2889, -2310 to -2305, -1473 to -1468</td>
<td>drought responsive element</td>
</tr>
<tr>
<td>ATHB2 binding site motif</td>
<td>TAATTATTATA</td>
<td>-1905 to -1897</td>
<td>environmental stresses</td>
</tr>
<tr>
<td>TELO-box promoter motif</td>
<td>AAACCTAA TTAGGGTT TTAGGGTT</td>
<td>-1328 to -1320, -127 to -119, -161 to -153</td>
<td>root specific expression</td>
</tr>
<tr>
<td>CARGC8GAT</td>
<td>CAATAAAATAGCTA TTATATGCTAATTT AGCTTTTATTGTT CTTTTTATGGCTAAT TTAAAGCTTTAT ATGCAATAAATG</td>
<td>-2571 to -2562, -975 to -966, -877 to -868, -25 to -16, -877 to -868, -975 to -966, -2571 to -2562</td>
<td>ABA mediated inhibition of seed germination</td>
</tr>
<tr>
<td>DRE core motif</td>
<td>GCCGACGCCGAC</td>
<td>-2970 to -2965, -1627 to -1622</td>
<td>drought, salt and freezing</td>
</tr>
<tr>
<td>Motif name</td>
<td>Consensus sequence</td>
<td>Binding sites</td>
<td>Physiological responses</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------------</td>
<td>---------------------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>MYB4 binding site motif</td>
<td>ACCTAAC</td>
<td>-2654 to -2648</td>
<td>drought, salt, cold, wounding</td>
</tr>
<tr>
<td></td>
<td>AACAAC</td>
<td>-1722 to -1716</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACAAAAC</td>
<td>-1105 to -1099</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AACAAAC</td>
<td>-729 to -723</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTGTT</td>
<td>-156 to -150</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTGGT</td>
<td>-238 to -232</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTGTT</td>
<td>-281 to -275</td>
<td></td>
</tr>
<tr>
<td>CCA1 motif1 BS in CAB1</td>
<td>TAGATTGTTT</td>
<td>-563 to -554</td>
<td>phytochrome regulation</td>
</tr>
<tr>
<td>W-box promoter motif</td>
<td>TTAGACTAGTCAA</td>
<td>-206 to -201</td>
<td>wounding response</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2739 to -2734</td>
<td></td>
</tr>
<tr>
<td>CCA1 binding site motif</td>
<td>AGATTGTAGATTGTT</td>
<td>-243 to -236</td>
<td>phytochrome regulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-562 to -555</td>
<td></td>
</tr>
<tr>
<td>L1-box promoter motif</td>
<td>TAAATGTA</td>
<td>-588 to -581</td>
<td>layer specific gene expression</td>
</tr>
<tr>
<td>I-box promoter motif</td>
<td>GATAAG</td>
<td>-1408 to -1403</td>
<td>light regulated expression</td>
</tr>
<tr>
<td>GCC-box promoter motif</td>
<td>GCCGCC</td>
<td>-1630 to -1625</td>
<td>dehydration and low temperature</td>
</tr>
<tr>
<td>RY-repeat promoter motif</td>
<td>CATGCATG</td>
<td>-572 to -565</td>
<td>seed protein related</td>
</tr>
<tr>
<td></td>
<td>CATGCATG</td>
<td>-572 to -565</td>
<td></td>
</tr>
<tr>
<td>Hexamer promoter motif</td>
<td>CGTGCAGCGACGG</td>
<td>-2975 to -2970</td>
<td>histone protein related</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2888 to -2883</td>
<td></td>
</tr>
<tr>
<td>CACGTG-motif</td>
<td>CACGTG</td>
<td>-1206 to -1201</td>
<td>essential for beta phaseolin gene expression during embryo</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>genesis</td>
</tr>
</tbody>
</table>
Table 16. Putative cis elements in the *AtSAMDC4* promoter sequence (analyzed using Athena promoter analysis software, O'Connor et al. 2005) showing consensus sequences, binding sites, and physiological responses of the identified elements.

<table>
<thead>
<tr>
<th>Motif name</th>
<th>Consensus sequence</th>
<th>Binding sites</th>
<th>Physiological responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF binding site motif</td>
<td>GAGACA</td>
<td>-720 to -715</td>
<td>auxin responsive element</td>
</tr>
<tr>
<td>BoxII promoter motif</td>
<td>TTAACC</td>
<td>182 to -177</td>
<td>Transcriptional activator</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>AAACCA</td>
<td>-980 to -975</td>
<td>drought responsive element</td>
</tr>
<tr>
<td></td>
<td>AAACCA</td>
<td>-515 to -510</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAACCA</td>
<td>-360 to -355</td>
<td></td>
</tr>
<tr>
<td>MYB1LEPR</td>
<td>AACTAAC</td>
<td>-434 to -428</td>
<td>defense related gene expression</td>
</tr>
<tr>
<td>MYB4 binding site motif</td>
<td>AACTAAC</td>
<td>-434 to -428</td>
<td>drought, salt, cold, wounding</td>
</tr>
<tr>
<td>RAV1-B binding site motif</td>
<td>CACCTG</td>
<td>-423 to -418</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>T-box promoter motif</td>
<td>ACTTTG</td>
<td>-564 to -559</td>
<td>transcriptional activator</td>
</tr>
<tr>
<td>TATA-box Motif</td>
<td>TATAAA</td>
<td>402 to -397</td>
<td>transcription</td>
</tr>
<tr>
<td></td>
<td>TATAAA</td>
<td>-139 to -134</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTTATA</td>
<td>-684 to -679</td>
<td></td>
</tr>
<tr>
<td>W-box promoter motif</td>
<td>AGTCAA</td>
<td>-528 to -523</td>
<td>wound response</td>
</tr>
</tbody>
</table>
Table 17. Putative cis elements in the *AtSAMDC5* promoter sequence (analyzed using Athena promoter analysis software, O’Connor et al. 2005) showing consensus sequences, binding sites, and physiological responses of the identified elements.

<table>
<thead>
<tr>
<th>Motif name</th>
<th>Consensus sequence</th>
<th>Binding sites</th>
<th>Physiological responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARF binding site motif</td>
<td>TGTCTC</td>
<td>-531 to -526</td>
<td>auxin responsive element</td>
</tr>
<tr>
<td>AtMYC2 BS in RD22</td>
<td>CATGTG CATGTG</td>
<td>-862 to -857 -1359 to -1354</td>
<td>transcriptional activator</td>
</tr>
<tr>
<td>BoxII promoter motif</td>
<td>GGTTAA GGTTAA TTAACC TTAACC</td>
<td>-1672 to -1667 -1354 to -1349 -382 to -377 -1352 to -1347</td>
<td>transcriptional activator</td>
</tr>
<tr>
<td>CARGCW8GAT</td>
<td>CATTTATTCG CAATTTTTTG CTTTTATAAG CTTTTATAAG CAATTTTTTG CTTTTATTG</td>
<td>-2222 to -2213 -780 to -771 -96 to -87 -96 to -87 -780 to -771 -2222 to -2213</td>
<td>ABA-mediated inhibition of seed germination</td>
</tr>
<tr>
<td>CCA1 binding site motif</td>
<td>AAAATCT</td>
<td>-187 to -180</td>
<td>phytochrome regulation</td>
</tr>
<tr>
<td>E2F binding site motif</td>
<td>TTTCCCGC</td>
<td>-933 to -926</td>
<td>cell cycle regulation</td>
</tr>
<tr>
<td>GAREAT</td>
<td>TAAACAA CTGTGTA</td>
<td>-877 to -871 -899 to -893</td>
<td>GA induced seed germination</td>
</tr>
<tr>
<td>l-box promoter motif</td>
<td>GATAAG CTTATC CTTATC CTTATC</td>
<td>-2354 to -2349 -996 to -991 -1209 to -1204 -1383 to -1378</td>
<td>light regulated expression</td>
</tr>
<tr>
<td>MYB binding site promoter</td>
<td>GTTAGGTT GTTTGTTG</td>
<td>-493 to -486 -2304 to -2297</td>
<td>flower specific motif</td>
</tr>
<tr>
<td>MYB1AT</td>
<td>TGGTTA TGGTTT TGGTTA</td>
<td>-1355 to -1350 -1644 to -1639 -2104 to -2099</td>
<td>drought responsive element</td>
</tr>
<tr>
<td>MYB1LEPR</td>
<td>GTTAGTT</td>
<td>-599 to -593</td>
<td>defense related gene expression</td>
</tr>
<tr>
<td>MYB2AT</td>
<td>TAACTG TAACTG</td>
<td>-2359 to -2354 -2030 to -2025</td>
<td>drought responsive element</td>
</tr>
<tr>
<td>MYB4 binding site motif</td>
<td>GTTAGGT GTTAGTT GTTTGTT GTTTGTT</td>
<td>-493 to -487 -599 to -593 -1548 to -1542 -2304 to -2298</td>
<td>drought, salt, cold, wounding</td>
</tr>
<tr>
<td>MYCATERD1</td>
<td>CATGTG CATGTG</td>
<td>-1359 to -1354 -862 to -857</td>
<td>signal transduction</td>
</tr>
<tr>
<td>RAV1-B binding site motif</td>
<td>CAGGTG</td>
<td>-2205 to -2200</td>
<td>DNA-binding</td>
</tr>
<tr>
<td>RY-repeat promoter motif</td>
<td>CATGATG CATGATG</td>
<td>-1363 to -1356 -1363 to -1356</td>
<td>seed protein related</td>
</tr>
</tbody>
</table>
Table 17. (continued)

<table>
<thead>
<tr>
<th>Motif name</th>
<th>Consensus sequence</th>
<th>Binding sites</th>
<th>Physiological responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-box promoter motif</td>
<td>ACTTTG CAAAGT</td>
<td>-702 to -697</td>
<td>transcriptional activator</td>
</tr>
<tr>
<td></td>
<td>CAAAGT</td>
<td>-553 to -548</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-2456 to -2451</td>
<td></td>
</tr>
<tr>
<td>TATA-box Motif</td>
<td>TATAAA</td>
<td>-1251 to -1246</td>
<td>transcription</td>
</tr>
<tr>
<td></td>
<td>TTTATA</td>
<td>-94 to -89</td>
<td></td>
</tr>
<tr>
<td>W-box promoter motif</td>
<td>AGTCAA</td>
<td>-1614 to -1609</td>
<td>wound response</td>
</tr>
</tbody>
</table>
Comparison of 5'UTR among different SAMDC genes in Arabidopsis

Sequence analyses of Arabidopsis SAMDC cDNAs/genes show that AtSAMDC1, AtSAMDC2 and AtSAMDC3 have relatively larger 5'UTR ranging from 876-1106 bp, whereas AtSAMDC4 has shorter 5'UTR of 108 bp in length and AtSAMDC5 has extremely small 5'UTR of 12 bp long (Table 4). The presence of highly conserved overlapping 'tiny' and 'small' uORFs, an important characteristic of plant SAMDCs (Franceschetti et al. 2001; Hao et al. 2005; Tassoni et al. 2007; Wang et al. 2010), is seen in the 5'UTRs of AtSAMDC1 and AtSAMDC2; the last base of the stop codon of the tiny uORF is the first base pair of the start codon of the small uORF (Franceschetti et al. 2001; Hanfrey et al. 2005). Sequence analyses of the 5'UTRs of different Arabidopsis SAMDCs reveal that while the length of the tiny uORFs is 12 bp, and the small uORF length varies from 156 to 168 bp (Table 7). The location of the tiny and small uORFs varies between 553-865 bp downstream from the transcription start site in AtSAMDC1, AtSAMDC2 and AtSAMDC3 genes. AtSAMDC3 contains only small uORF in its 5'UTR, whereas AtSAMDC4 and AtSAMDC5 do not have a uORF in their 5'UTRs. Although AtSAMDC4 and AtSAMDC5 promoters do not have a uORF, yet removal of 5'UTR increased GUS expression by them.

QRT-PCR of AtSAMDC gene expression

In order to establish (1) if the promoter::GUS fusion results revealed a similarity with the actual transcript levels of the respective genes in the plants for various SAMDC genes, and (2) to select the construct(s) that would mimic the actual expression of a particular SAMDC gene, QRT-PCR analysis was done for each gene in selected organs of WT plants. Relatively high and comparable levels of transcripts were observed in seedlings at
initial stages (e.g. 2 d after germination) for all four $SAMDC$ genes (Fig. 58A); however by 10 d after germination, the seedlings started to show differences, particularly for the $AtSAMDC2$ gene, whose relative expression was about half that of the other three (Fig. 58B). In 5-week old plants, relatively high expression of $AtSAMDC1$ was observed in all organs with slightly higher expression in the siliques and lower stems (Fig. 58C). The expression of $AtSAMDC2$ was also relatively high in the buds, siliques and lower part of the stem, and low in the upper part of the stem. The highest relative expression among the four genes was observed for $AtSAMDC4$ whose transcripts were 2 to 7-fold higher than other $SAMDC$ genes, particularly in the root and the lower stem.
Figure 58. Relative expression of the four *AtSAMDC* genes in WT Arabidopsis (A = 2 d, B = 10 d and C = 5 week) plants. The expression of target gene was normalized to *AtTIP41*. RL = rosette leaf, CL = cauline leaf, LS = lower stem, US = upper stem (N=4).
DISCUSSION

Objective 1 - Transgenic manipulation of PAs

Genetic manipulation of PA metabolism has often been targeted to understand their role in growth and development, and stress tolerance. Mutations (to some extent) as well as transgene expression (more often) have been used for this purpose. Most studies on transgene expression have used constitutive promoters. A major limitation in studies that employ constitutive over-expression of transgenes is that the metabolic system under investigation is often homoeostatically regulated even under elevated condition of the product, e.g. Put in the present case. Thus it is not feasible to analyze the impact of short-term metabolic perturbations, and responses of the cells to increased production of the metabolite. On the other hand, in most cases of natural responses of a plant to environmental or developmental stimuli, short-term changes are perhaps more common, and probably the most critical events in its life. In this regard inducible transgenic expression systems permit us to mimic turning on a gene as it would happen in nature; this control should provide us a deeper insight regarding the effect of transient (short term) expression of genes, and consequently, quick changes in cellular metabolic fluxes.

For example, over-expression of a number of heterologous genes encoding regulatory enzymes has been used to achieve genetic manipulation of plant metabolism with the assumption that their substrates may not be limiting (Galili and Hofgen 2002; Goff and Klee 2006; Schnee et al. 2006, Bartels and Hussain 2008). With respect to PAs,
expression of \textit{ODC} has often resulted in small (often <5 fold) increase in Put content in transgenic plants; rarely has it been considered that the results may be regulated more by the substrate (Orn) than the expression of the transgene. Moreover, we do not understand the regulation of Orn metabolism and its role in controlling the biosynthesis of PAs and other amino acids in plants, while a number of studies have been reported in animals (Morris 2006, 2007). Since the PA biosynthetic pathway is intricately tied to amino acid biosynthesis, it can be argued that overutilization of Orn by transgenic ODC may have major effects on amino acids, particularly those whose biosynthesis depends upon Glu (Page et al. 2010; Mohapatra et al. 2010b). Under these circumstances, the effects of short-term induction of \textit{ODC} may be substantially different from those of constitutive overexpression due to homeostatic regulation of the amino acid biosynthetic pathways. A major objective of the current study was to examine the role of substrates like Orn and Arg, and their precursor Glu, in regulating PA metabolism as well as the metabolism of amino acids. The effects of additional supply of C and N on PA and amino acid metabolism under conditions of constitutive \textit{ODC} expression were also analyzed.

Up-regulation of Put biosynthesis through over-expression of \textit{ODC} under 35S promoter has been reported in many plant species (Table 3A); another constitutive promoter that has been used for transgenic \textit{ODC} or \textit{ADC} expression is maize \textit{Ubi-1} promoter (Noury et al. 2000; Capell et al. 2004). In most cases ~2- to 3-fold increase in Put content, with or without significant changes in Spd and Spm, were seen except for a 6- and 10-fold increase of Put in rice seeds (\textit{ODC} and \textit{ADC} expression, respectively), and a 4- to 12-fold increase in Arabidopsis with \textit{AtADC2} overexpression. Masgrau et al. (1997) demonstrated that transgenic expression of an oat \textit{ADC} in tobacco under the
control of a tetracycline-inducible promoter increased cellular content of Put only by 16 to 46% upon induction, with little change in Spd and Spm. The induced plants showed leaf chlorosis and necrosis, short internodes, thinner stem, and reduced root growth. The severity in phenotype was correlated with the amount of Put accumulation upon ADC induction. On the other hand, in animals, inducible (tetracycline) transgenic expression of mODC in HEK293 embryonic kidney cells and LNCaP prostate cells showed >100-fold increase in ODC activity in the latter, with >10-fold increase in Put (Wilson et al. 2005). Highest level of ODC induction was accompanied by a decrease in Spd and Spm levels with a concomitant increase of Spd/Spm N¹-acetyltransferase (SSAT) activity by >40-fold. Growth of the cells was unaffected during the 12 h period of ODC induction. In the present study, up to 50-fold increase in Put was observed in the transgenic plants both under constitutive and inducible expression conditions. Surprisingly, no major phenotypic changes were observed except for a few discussed below.

Since A. thaliana does not have its own ODC gene (Hanfrey et al. 2001), one can hypothesize that Arg may be a limiting substrate for Put biosynthesis in this plant. The two ADCs (AtADC1 and AtADC2) seem to contribute equally to the production of Put in different parts of the plant, except for a few situations where the expression of one is dominant over the other. However, my results clearly show that in the mODC transgenic plants, large amounts of Put are produced from Orn by mODC without impacting its production from Arg by ADC. Moreover, Orn is present in rather small amounts in the seedlings and quickly becomes limiting for still further increases in Put since exogenous supply of Orn stimulated Put accumulation. At the same time, it was found that Glu is not a limiting for Orn production, and its conversion (through Orn) into Arg also continues to
occur unabated. Two additional metabolic revelations are that Put does not show a feedback inhibition of its biosynthesis via the native ADC pathway, and that the limitation of Arg itself is not responsible for low Put content in the normal life of the plant. These conclusions are consistent with earlier reports from our lab with mODC transgenic poplar cells (Bhatnagar et al. 2001; Mohapatra et al. 2010a,b). Overall, the elevation of cellular Put (which does not have concomitant effects on Spd and Spm accumulation) has rather small effects on plant morphology, development and flowering pattern (see discussion below). These observations show a remarkable homeostatic tolerance for high Put in Arabidopsis, and are in line with previous reports of similar tolerance for high Put in plants (Mayer and Michael 2003; Alcázar et al. 2005; Nölke et al. 2008). Bhatnagar et al. (2001) had reported that increased Put production via transgenic ODC in poplar cell cultures was accompanied by its increased catabolism (Bhatnagar et al. 2002), which ultimately limited its infinite increase. A similar situation is envisioned for Arabidopsis although it has yet to be experimentally confirmed. In poplar cells also, (i) Orn was present in extremely low concentrations, (ii) ADC pathway was not negatively impacted by high Put, and (iii) neither Glu nor Arg were limiting for Put production from Orn by ODC (Page et al. 2010, Mohapatra et al. 2010a, b).

The conclusion that Orn availability is the limiting factor as opposed to Arg or Glu is directly supported by the observed increase in Put production on induction and supply of Orn, beyond induction alone; this was not the case for un-induced seedlings (Fig. 7) or for seedlings that were exogenously supplied with Arg or Glu. Similar increases were seen in the case of constitutive over-expression of mODC. The fact that increase in Put varied with developmental stages and organ type, shows that the amount of available Orn
may be different in different tissues/organs and or the transgene expression itself was tissue/organ dependent, even though it was regulated by a constitutive promoter. Under conditions of induction with estradiol, a ~10-fold increase in Put was seen, whereas up to a 40-fold increase in Put was seen in the presence of the inducer + Orn. The effect of Orn was more pronounced at 24 h than at 12 h after induction of mODC; this indicates the possibility of homeostatic upward adjustment in Orn production in the transgenic plants with time as the demand for Orn increased or its cellular content decreased. In addition, the transgenic plants always had substantial amounts of Cad both under constitutive and inducible expression. This observation further supports the argument that Orn is not available in sufficient quantities to transgenic mODC, which then uses Lys as a substrate (albeit it's high Km for Lys), which is well documented for mammalian ODC (Pegg and McGill 1979). Further investigations involving the feeding of $^{14}$C-Orn and $^{14}$C-Arg showed significant incorporation of $^{14}$C in PAs upon induction, leading us to deduce that the two substrates were being used independently by mODC and AtADC, respectively. It can further be argued that there is little conversion of Arg into Orn in Arabidopsis as was as was the case in poplar cells (Bhatnagar et al. 2001; Mohapatra et al. 2010b).

Constitutive as well as inducible expression of mODC in seedlings showed significant increase in Spm (but rather low magnitude vs. that for Put) but no increase in Spd; actually it was often lower in the transgenic plants. This is quite intriguing but may be related more to a decreased catabolism of Spm (by a specific Spm oxidase) rather than its increased production; since Spd is an intermediate for Spm production and both are co-regulated by SAMDC and the APTs (Shao et al. 2011). Alternatively, lower Spd may
perhaps be due to its increased catabolism by Spd oxidase. It is also noteworthy that
decrease in Spd in the transgenic plants was inversely related to increase in Put.

In mature transgenic plants with constitutive expression of mODC, the trend of
changes in PAs (very high Put, somewhat higher Spm and slightly lower Spd) was
similar to the seedlings but the magnitude of increase in Put was much smaller than in the
seedlings, and was organ/tissue dependent. The Spm content was higher in vegetative
tissues (>4-fold) but less so in the reproductive tissues (~1.8- to 2-fold). Cadaverine was
also observed in all organs of mature plants, with the highest amount in the reproductive
tissues. Overall, the reproductive organs had higher amounts of total PAs than the
vegetative organs, both in the WT as well as in the transgenic plants; this is in agreement
with the results of Urano et al. (2003). These results reflect developmental regulation of
PAs in different tissues/organs of both the WT and the transgenic plants, which is
consistent with the previous reports from our lab as well as from other labs.

Exogenous supply of all three PAs to WT plants expectedly increased the
corresponding PA level, while Spd and Spm also caused an increase in Put content; the
latter could possibly be due to back-conversion of Spd or Spm to Put. There was little
effect of exogenous Put on cellular Spd and Spm, again confirming a tighter regulation of
of higher PAs in plant cells. Back conversion of PAs (from Spd to Put or Spm to Spd and
Put) through the actions of PAOs is common in animals (Seiler 2004; Zahedi et al. 2010),
and has been shown in plants as well (Tavladoraki et al. 2006; Kamada-Nobusada et al.
2008; Moschou et al. 2008c; Fincato et al. 2011).

The presence of additional nitrate in the medium significantly reduced Put as well as
Spd contents in both the WT and the transgenic plants; the effects on Spm were different
in the two genotypes. While the transgenic plants had significantly higher Spm, the WT plants had lower Spm in the presence of high nitrate. These results are consistent with the earlier observation of Minocha et al. (2004), and show that N availability in the medium is not limiting in either case. Garnica et al. (2009) also found in wheat seedlings treated with ammonium, that there was a significant decrease in Put and an increase in Spd both in roots and shoots. In the present study, lack of N in the medium also affected Put more than the higher PAs, again showing a strong regulation of higher PA accumulation.

Absence of sucrose in the medium preferentially affected Put accumulation in the transgenic plants, but the higher PAs were not affected in either case. These results indicate that while C may not be limiting in the WT plants (all plants are photosynthetic), its need in making Put in the transgenic plants (perhaps also in keeping up with increased biomass production in these plants – see later) is not met by photosynthesis alone. However, the positive effects of increased sucrose availability in the medium on cellular Put accumulation in both genotypes (transgenic and WT) show that C may actually be limiting for the production of Put even in the WT plants. It is worth noting that the lower concentration of sucrose in the medium produced a greater increase in Put in the transgenic plants, whereas Put in WT plants was not much affected at lower sucrose concentration. Higher concentration of sucrose showed completely an inverse trend for Put accumulation in the two genotypes; while Put was higher in the WT plants, it was lower in the transgenic plants. A positive correlation between cellular Put and sucrose content was seen in transgenic tomato fruits with high PAs (Handa and Mattoo 2010). A similar positive effect of high sucrose (300 mM) on Put was also reported by Kim et al. (2011) in lemon balm (Melissa officinalis).
Phenotypic changes in mODC transgenic plants

While the transgenic plants constitutively over-expressing mODC were phenotypically similar to the WT plants; there were subtle developmental differences between the two genotypes. The transgenic plants had higher fresh biomass as well as dry mass per plant just before bolting, which was delayed by at least a week as compared to the WT plants. Transgenic plants also had more branches per plant and siliques per plant. Similar effects of Put over-production (2- to 4-fold increase) on flowering (delay by 4-5 d) were reported in transgenic rice plants over-expressing D. stramonium ADC gene under Ubi-1 promoter (Capell et al. 2004). My results are also consistent with a study of transgenic Arabidopsis plants constitutively over-expressing a native AtADC gene, where Alcázar et al. (2005) found that higher accumulation of Put (4- to 12-fold) was accompanied by a greater leaf number at the time of flowering, an overall dwarfism, shorter stamen length, and partial sterility. This phenotype was attributed to apparent alteration of GA metabolism, since it was reversed by external application of GA3. Effect of elevated Put on biomass increase is also reported in other plants; e.g. under salinity stress conditions (Roy and Wu 2001).

Ability of the mODC transgenic plants to maintain higher water content might be a useful trait against drought; elevated Put and greater tolerance to drought stress are often reported to co-occur in plants (Capell et al. 2004; Alcázar et al. 2010). Higher chlorophyll content in the transgenic plants, and greater number of branches and siliques, all seem to indicate positive effects of high Put production in the present study. These effects are consistent with the observed increase in total C and N in the transgenic plants (also seen in high putrescine producing poplar cells – Mohapatra et al. 2010b), which may be related to higher chlorophyll (photosynthesis?) in the leaves of these plants. The diamine
Put (vs. the higher PAs) has been shown to promote light reactions of photosynthesis through increased photophosphorylation (Ioannidis et al. 2006). Also, among the three major PAs, Put is the best stimulator of ATP synthesis as compared to Spd and Spm (Ioannidis et al. 2007). Higher PA concentrations were also shown to restore the efficiency of photosystem II in the chloroplast membrane (Ioannidis et al. 2006, 2007).

**Ornithine as a key player in controlling PA, Pro and GABA biosynthesis**

Understanding the regulation of PA and amino acid metabolism remains a key interest both in plants and animals (Sinclair et al. 2004; Rees et al. 2009; Takahashi et al. 2010) as it occupies a central position in connecting with N metabolism, C fixation, and several pathways associated with secondary metabolism. In this regard, as mentioned above, we have argued that Orn plays a more important role than is recognized (in the literature) in regulating PA, Pro and GABA metabolism. Not only that, we have also demonstrated that metabolic flux through the interacting pathways of Glu-Arg-Pro-GABA biosynthesis affects most other amino acids in the cell. While Orn by itself is not used directly for Put biosynthesis in *A. thaliana* (since this species does not have an *ODC* gene); on the whole in the plant kingdom, Orn metabolism and its role deserve serious attention (Shargool et al. 1988; Slocum 2005). The data presented here and in earlier studies (Bhatnagar et al. 2001; Mohapatra et al. 2010b) with high-putrescine producing poplar cells clearly show that cellular Orn level is probably monitored very tightly since its biosynthesis is largely driven by its use (by ODC and perhaps other pathways) without significant accumulation at any time. How this monitoring and coordination is achieved in the plants can only be speculated at present. With respect to similar monitoring of Arg levels in animals, Morris (2007) has implicated a key role for the ratio of charged to uncharged tRNA$^{Arg}$ and G-
A parallel mechanism for Orn monitoring via tRNA cannot however be envisioned because it is a non-protein amino acid; and the latter mechanism has not been investigated. Whatever the mechanism(s) of monitoring, there must exist a coordination of the pathway from Glu to Orn and a system to up regulate this pathway when demand for Orn is increased. Mohapatra et al. (2010b) and Page et al. (2010) have suggested that this seems to occur more at the biochemical level by modulation of enzyme activity rather than by induction of genes (transcription) for the enzymes in the pathway. Regulation at the biochemical level (e.g. increased Orn production in response to its increased consumption) is analogous to the situation found for Lys biosynthesis by Zhu and Galili (2003).

Ornithine is present in very small quantities as compared to Arg, Pro and Glu; it is synthesized from Glu by the action of several enzymes (Slocum 2005). It is also involved (directly or indirectly) in alkaloid biosynthesis in plants (reviewed by Shargool et al. 1988). The first (regulatory?) step in Orn biosynthesis in plants is from Glu via N-acetyl-L-Glu synthase (NAGS); this is in contrast to animals where nutritional Arg is the primary source of Orn, and the reaction is controlled by arginase (Morris 2006, 2007). Constitutive over-expression of a tomato \textit{NAGS1} gene in Arabidopsis led to higher accumulation of Orn and citrulline in the rosette leaves without significant increase in Arg content (Kalamaki et al. 2009). Transgenic plants showed greater retention of chlorophyll under salt and drought stresses and higher tolerance against these stresses. Accumulation of different protein and non-protein amino acids e.g. Ala, Arg, citrulline, Gly, Leu, Orn, Pro, Ser, Val have been reported under salt stress conditions suggesting
possible link of these amino acids with salt tolerance in higher plants (Mansour 2000; Ashraf and Harris 2004). Unfortunately, no data on PAs were reported in these studies.

Under conditions of its over-consumption in mODC transgenic plants, what turns on the Orn biosynthesis is not yet understood. In this regard the data presented here support the earlier hypothesis from our lab (Mohapatra et al. 2010b; Page et al. 2010) that Orn acts as a sensor molecule to regulate its own cellular content, and is involved in some (as yet unknown) signal transduction pathway that in turn activates (mostly biochemically) its biosynthesis from Glu. This would in turn reduce the availability of Glu and other associated amino acids (Fig. 1); however, due to the preferred biosynthesis of Glu, it does not become limiting unless N and/or C become limiting. This argument is supported by the data presented here as well as the earlier results with mODC transgenic poplar cells.

Further support for this argument comes from the observation that exogenous Orn allowed Glu content to stay high on short-term (12 h) induction of mODC. We do not envision the increase in Glu to have happened by back conversion of Orn into Glu; rather we believe that it occurs via the regulatory role of Orn. There are two arguments that favor this idea: first the amount of Orn supplied in the medium was not sufficient to produce the amount of Glu seen/retained in the transgenic cells; second, large amount of Put was still produced on induction (of mODC) from exogenous Orn (corroborated by data from 14C-Orn incorporation into PAs) that would directly utilize this substrate. The increase in Glu seen in response to exogenous Orn treatment in the un-induced plants was also perhaps due to underutilization of Glu to enter the Glu-Orn-Arg pathway rather than back conversion of Orn; again supporting the proposed regulatory role of Orn in controlling this pathway.
In animals, nutritional Arg is involved in the production of PAs, Orn, Pro and Glu, whereas in plants it is Glu that performs this role. Another important non-protein amino acid, GABA, is known to accumulate in higher amounts in response to cold, drought, salinity, hypoxia, hormonal changes, and pH change; it also plays an important role in N storage and development in plants (Kinnersley and Turano 2000; Mazzucotelli et al. 2006; Allan et al. 2008; Shi et al. 2010). Induction of *ODC* for 12 h slightly increased soluble Arg (+Thr+Gly) content (g\(^{-1}\)FW), but decreased its relative content (as % of total soluble amino acid pool). Its relative content significantly increased (>50% of soluble pool) at 24 h when plants were supplied with exogenous Arg or E+Arg. The increase in GABA in the induced plants (with or without Orn) is understandable since it is a direct product of Put degradation (in addition to its biosynthesis from Glu by GAD). While cellular contents of GABA were typically higher in the transgenic plants, how much of it was being produced by GAD vs. by Put degradation by DAO has not been determined. Application of exogenous Glu also resulted in a significant rise of the content of GABA, perhaps due to its direct conversion via GAD. This could be experimentally tested by designing experiments using stable and radio-isotopes of C and N to delineate the metabolic flux of Glu into GABA via the two pathways.

Proline biosynthesis from Orn in plants is not regulated by feedback inhibition as the enzymes involved in this pathway, (i.e. 8-OAT and pyrroline-5-carboxylate reductase - P5CR), are not regulated by Pro (LaRosa et al. 1991). Induction of *ODC* resulted in up-regulation of Pro (as also seen in HP poplar cell by Mohapatra et al. 2010b) and further on supply of exogenous Glu or Arg. Also, in seedlings and in rosette leaves of mature plants, constitutively over-expressing m*ODC*, there was increased Pro accumulation.
While exogenous Put had a completely reverse effect on Pro content (i.e. it caused a
decrease); higher PAs, especially Spm caused an increase in Pro content (>53% of total
soluble amino acids). Taken together, these data indicate a complex triangular
relationship between PAs and Glu on one side and PAs and GABA on the other; there is
a similar three-way relationship among Glu, Orn and Pro. Higher accumulation of GABA
and Pro both under inducible and constitutive mODC expression conditions in the
seedlings and mature plants indicate that these plants might be useful tools to study a
wide a range of stress responses, although this was not done in the present case.

Sucrose affected Pro, GABA and Arg but the responses were different in the
transgenic as compared to the WT seedlings. Higher concentration of sucrose in the
medium significantly increased Pro content both types of seedlings, yet the fold increase
in Pro was much higher in the transgenic seedlings. Sucrose limitation on the other hand
negatively impacted Pro only in the WT plants without affecting transgenic plants that
showed relatively higher amount of Pro than WT plants even in the control medium.
Higher sucrose concentration (100 mM) significantly increased cellular GABA
accumulation. On the other hand Arg showed an increase only in the WT plants in
response to lower concentration of sucrose (50 mM). Once again, these data reflect the
complex interaction between C and N metabolism in plants.

Changes in other amino acids

Besides PA metabolism, I also investigated if the constitutive expression and the
inducible expression of mODC in the presence or absence of Orn affected the cellular
soluble amino acids pool in a similar way. In case the results were similar, it would
indicate a homeostatic role for high Put biosynthesis from Orn and/or its accumulation on
the amino acids. Otherwise it could be argued that short-term changes in Orn availability or Put biosynthesis were different from those that involved homeostatic stabilization of Orn to Put metabolism. Amino acids that changed significantly within 12/24 h in response to externally supplied Orn with (+E) or without induction of mODC are: Ala (decreased ~80%), Val (decreased ~40%), and Cys (decreased 40-50%); Glu and GABA contents were almost doubled during (Tables 9A and 9B). Exogenous supply of Put, on the other hand, had quite different consequences; i.e. Glu did not change, GABA, Phe and Pro were 50-70% lower, and Trp was somewhat higher (Table 10). Thus it appears that exogenous Put and metabolically synthesized Put have different effects on amino acids related to Glu and Orn. It should be kept in mind that the induction experiment was shorter in duration (12-24 h) than the constitutive expression experiment (7 d).

Differences in PCA-soluble amino acids were also observed between the seedlings and the mature plants in the constitutive over-expression lines. Constitutive presence of ODC caused >50% increase in the proportions of Thr, Gly, Ala, Pro, Val, Met, Ile and GABA, and a similar decrease in the proportions of Trp and Orn in the soluble amino acid pool in seedling (Table 10). The trend of changes in amino acids as % of total soluble amino acid pool was similar in the transgenic line 18-2. Mature plants on the other hand showed tighter regulation of the amino acids; of course changes in Put biosynthesis and accumulation were also different in the young vs. the mature plants. This was also true in the reproductive organs where there were much greater changes in PAs. Effects of genetic manipulation of PAs (yeast SPDS under fruit specific promoter) on amino acids have also been studied in tomato fruits; the results were quite different in that case. Whereas Put showed a negative correlation (p<0.8) with amino acids like Gln, Asn, Ala,
Thr, Ile; Spd-Spm showed a positive correlation (p>0.8) with the same amino acids (Handa and Mattoo 2010; Mattoo et al. 2011). Changes in amino acid pool in response to mODC over-expression in poplar showed a decrease in relative concentrations in several amino acids including Gln, Leu, His, Glu, Arg, and Orn; there was an increase in Ala, Thr, GABA, and occasionally Pro (Mohapatra et al. 2010b).

It was not surprising that the lack of N (nitrate) availability in the medium had greater effects on amino acids than the lack of C (sucrose) because the seedlings are photosynthetic; thus they have a source of C even in the absence of sucrose. Higher nitrate concentration in the medium increased cellular Pro, Trp, Phe, Ala, Val, and Ile, both in WT and mODC transgenic seedlings, showing that additional N was being sequestered into amino acids but not into PAs. Sucrose availability in the medium affected a greater number of amino acids that showed differential responses in WT and mODC transgenic seedlings, confirming postulated the interaction of PAs with amino acid biosynthesis. Higher concentration of sucrose (100 mM) significantly increased cellular Glu, Gln, Pro, Ser, Phe, Ala and Val, and lowered cellular Lys, more so in the ODC transgenics than the WT seedlings (Figs. 33A, 33B, 34A, 34B). Almost a doubling of Lys at lower sucrose concentration was seen in the WT plants but a decrease was apparent in the mODC plants. The latter was perhaps due to the ability of mODC to use this amino acid as a substrate to make Cad. This is consistent with the proposed Orn deficiency syndrome in the transgenic plants. Ornithine increased significantly in the WT plants in response to low levels of additional sucrose (50 mM) but decreased at higher sucrose concentration; the transgenic plants did not show any change in Orn in response to sucrose supplementation in the medium. This is also consistent with a severe Orn
limitation in the transgenic plants, and further shows a link between available C and the 
regulation of cellular Orn. In lemon grass (*Melissa officinalis*), high sucrose 
supplementation (300 mM) significantly increased cellular contents of Pro and decreased 
Glu, Ala, Arg, Gly, Asp, Asn, and Thr (Kim et al. 2011). The same amino acids that 
showed decrease in higher concentration of sucrose were significantly increased by 50 
mM of sucrose.

**Total cellular C and N in relation to increase in polyamines**

The metabolism of C and N are inter-dependent in all organisms whether 
photosynthetic or not; however the interactions between the two are rather complicated. It 
is well known that in plants either C or N can become limiting for growth/biomass in 
short term (e.g. day vs. night) as well as long term (young vs. old plants), and in an organ 
and developmental stage specific manner. Other factors such as temperature, O2 and CO2 
supply, pathogens, and abiotic stress all affect the assimilation of C and N independently 
as well as coordinately. The effects of air pollution and climate change on the total 
biomass of individual plants as well as populations (e.g. aquatic systems, croplands and 
forests), the mode of N assimilation (uptake vs. N fixation), the form of N (nitrate vs. 
ammonium), water supply, root morphology, etc. are complex and interactive (McCarthy 
et al. 2010; Melillo et al. 2011). With increased interest in biofuels and renewable energy, 
in addition to food production, this interaction has taken on a new dimension.

In tobacco leaves, N assimilation has been linked to activation of specific enzymes 
like phosphoenolpyruvate kinase (PEPC) and isocitrate dehydrogenase (ICDhc), and up-
regulation of organic acids (Scheible et al. 2000). Roles of PEPC and other Krebs cycle 
enzymes in providing C skeletons in leaves in relation to assimilation of N has also been
discussed in other higher plants (Foyer and Noctor 2000). Transgenic expression of PEPC in potato showed higher organic acids content along with Glu and Gln content (Rademacher et al. 2002). Likewise, up-regulation of N:C indicator genes (e.g. PEPC and ICDH) in transgenic tomato fruits having elevated level of Spd/Spm indicates PA mediated changes in C and N metabolism (Mattoo et al. 2006). In cell cultures, higher Put biosynthesis has been shown to increase total C and N (Mohapatra et al. 2010b).

In plant leaves Glu, Gln and Asn are the major form of storage N (Corruzi and Zhou 2001; Stitt et al. 2002). As discussed above, metabolic alterations have been reported in tomato fruit in response to fruit specific transgenic manipulation of higher PAs. Transgenic expression of yeast SAMDC under the control of fruit ripening specific promoter in transgenic tomato fruit showed significant changes in Gln, Val, Asn, Asp and several TCA cycle metabolites including citrate, fumarate and malate. Significantly higher ratios of fructose to glucose, and organic acids to sugars (glucose+fructose+sucrose) were observed in the transgenic fruits in response to higher levels of Spd/Spm (Mattoo et al. 2006). It is well known that the primary N assimilation in plants is controlled negatively by the total N status, but in a complex way involving soluble protein as well as total soluble amino acid pool (Foyer et al. 2003; Miller et al. 2007). Foyer et al. (2003) have also discussed possible roles of certain minor amino acids in regulating the soluble amino acid pool and N and C assimilation by the cells. Of course, changes in the levels of Gln, Asn and other abundant amino acids play a major role in determining N status in plants (Glass et al. 2002; Stitt et al. 2002). Metabolome analysis of high-Put poplar cells showed significant increases in soluble sugars and
organic acids, besides changes of other metabolites (unpublished data from Minocha lab) in response to the elevated level of the diamine Put.

Transgenic mODC plants with elevated Put (and Spm) in the current study not only showed increase in cellular contents of C as well as N on per plant basis; there also was increase in percentage C (conversely perhaps a decrease in % N) in the transgenic plants as compared to the WT plants. Increase in C and N on per plant basis was accompanied by an increase in DW of the plants, showing that the overall productivity of the plants was also increased in response to genetic manipulation of Put. A delay in flowering seen in these (transgenic) plants, which might be detrimental in some cases, could be construed as a positive feature for plants where biomass may be more important than its utility in organ-dependent food value (i.e. foliage vs. the seed or fruit). This is of particular value in light of the recent interests in developing high biomass plants for use in biofuel production.

In this regard it is difficult to decide whether this increase in DW is due to the direct effect (at physiological and biochemical level) of elevated levels of Put, Spm or both in the mODC transgenic plants in accumulating biomass or an indirect effect of PAs in regulating genes that are involved in C and N metabolism. As mentioned above, involvement of higher PAs in regulating genes associated with C and N metabolism has been suggested by Mattoo et al. (2010a). These results together highlight the importance of the diamine Put and the higher PAs, and their overlapping functions in coordinating genes that are involved in C metabolism via N assimilation in higher plants both in cell cultures as level as well as in mature plants.
Conclusions and future perspectives – Objective 1

Over the years, metabolic engineering in plants has gained a central focus in transgenic research to enhance the nutritional value of food/feed crops and to improve the abiotic stress tolerance of plants (Mattoo et al. 2010b; Hussain et al. 2011 and references therein). Metabolic engineering in plants has often been targeted to achieve different goals; some examples are: increase in plant volatiles for increased plant defense (Schnee et al. 2006), stress tolerance (Chen and Murata 2002; reviewed by Bartels and Hussain 2008), improved quality of flowers and fruits (Lücker et al. 2001; Goff and Klee 2006), increased production of secondary metabolites (Pilate et al. 2002; Verhoeyen et al. 2002), and increase in essential amino acids (Lys, Met, Trp etc.) in food and forage crops from a nutritional perspective (Galili and Hofgen 2002). Current advances in genomics, proteomics and metabolomics have opened up new avenues to precisely evaluate the impact of single step metabolic engineering on the entire cellular system. Various mechanisms have been postulated in controlling metabolic fluxes of connected pathways that share a common substrate (Palsson 2009; Allen et al. 2009; Grüning et al. 2010). One mechanism of increasing certain metabolite contents without increases in transcription or translation of the related enzymes can be through increased demand of the product (Zhu and Galili 2003), i.e. the metabolic pull. While most studies have lacked experimental evidence involving detailed analysis of the pleiotropic effects of such metabolic manipulations, Mohapatra et al. (2010b) and Page et al. (2007, 2010, and AF Page, RMinocha and SC Minocha, unpublished) have demonstrated that manipulation of a single step in the PA biosynthetic pathway (i.e. increased Put production via transgenic ODC) causes a major redirection of the cell’s transcriptome and the metabolome. Results
presented here confirm the earlier conclusions of such a major metabolic shift in the overall cellular metabolism by \textit{mODC} over-expression in cultured cells, and extend the evidence in support of this to intact plants. These results also caution us to carefully ascertain the pleiotropic effects of genetic engineering of a single metabolite on causing a disturbance in the homeostatic regulation of several key metabolites in plants. In many cases, such perturbations of a beneficial metabolite may actually result in a reduction in the overall nutritional value of the transgenic food/feed crops. On the other hand, cases where plant biomass is intended to be used for industrial purposes (e.g. bioenergy, biofuels, enzyme catalysts, etc.), such metabolic engineering may be totally acceptable. In fact, the observed increase in N assimilation, and the resulting/accompanying C sequestration due to metabolic engineering of PAs, may provide a useful tool in not only producing greater biomass under the conditions of optimal growth but also under the conditions of stress because of the beneficial effects of higher PAs in improving the stress response of plants. In addition, of course, there is tremendous interest in regulating PA levels in human food because of the use of PA inhibitors for cancer and antiparasitic chemotherapy (Kalač and Krausová 2005; Kalač 2009).

**Objective 2 - Expression of \textit{AtSAMDC} genes**

Microarrays, northern blots, in situ hybridization, and qualitative and quantitative or Real Time (RT)-PCR, have all been used to assess gene expression, and they all have their pros and cons for such analyses. While providing reasonably reliable data on most genes in most tissues/organisms, they are often unsuitable for obtaining precise information about the cell and tissue-specific expression of individual genes, particularly members of a gene family which have a high degree of sequence homology amongst them. This is
precisely the situation with the \textit{SAMDC} gene family. Promoter::reporter fusion approach, in which the putative promoter region of a gene is fused with a reporter gene whose expression is then monitored in the transgenic plants carrying this construct, while having some of its own limitations (e.g. the assumption that an isolated promoter, which is not clearly defined, will behave the same way as the native gene promoter); nevertheless, offers two principal advantages over the other techniques: (1) the reporter gene expression is controlled by the promoter of the specific gene of interest, thus eliminating the problems associated with sequence similarity between two or more genes; and (2) the gene expression can be studied \textit{in vivo} (even non-destructively, e.g. by using Green Fluorescent Protein or luciferase), in all tissues and cells of the plant at the same time without cumbersome RNA preparations (Mantis and Tague 2000; Almoguera et al. 2002; Curtis and Grossniklaus 2003; Martin et al. 2009; Xiao et al. 2010). It should, however, be pointed out that this approach shows the results of a combination of transcriptional plus translational regulation of gene expression, while the earlier-mentioned methods measure transcript levels only. The \textit{GUS} reporter gene system has been employed routinely to study developmentally regulated gene expression, inducible gene expression, and to characterize different regulatory motifs in the promoter region of a gene (Godard et al. 2000; Curtis and Grossniklaus 2003; Malnoy et al. 2003, Furtado et al. 2009). In the present study, the expression of three \textit{SAMDC} genes in the PA biosynthesis pathway, namely \textit{AtSAMDC3, AtSAMDC4} and \textit{AtSAMDC5} were profiled during the life cycle of Arabidopsis. The expression of the other two \textit{SAMDC} genes (viz. \textit{SAMDC1} and \textit{SAMDC2}) has been studied previously in our lab (Mitchell 2004).
Defining the promoter

Numerous attempts have been made to define the promoter region of a gene (Yamamoto and Obokata 2008; Smirnova et al. 2011; Yilmaz et al. 2011); none have given a precise definition of a promoter. In a recent article, Balasubramanian et al. (2009) reported that the promoter region of the majority of genes in the human genome contain a rather high degree of GC rich quadruplexes, which may aid in better defining a promoter. While the 3' end of a promoter can be more easily defined (based on its proximity to the transcription initiation site), the 5' end is ill-defined in most cases. A common approach to isolate/clone the promoter sequences is to identify a fragment up to 2 kb or to the next known gene, if this information is available. We have used a combination of these two approaches (depending on the location of the neighboring gene at the 5' end of the putative promoter) to identify the promoters of the three \textit{SAMDC} genes studied here.

The length of the promoter region necessary to drive sufficient expression of the different members of \textit{AtSAMDC} gene family in relation to development or stress responses is not fully understood. No direct studies are reported on \textit{AtSAMDC} promoter characterizations, while bioinformatics information about the presence of regulatory motifs of different types has been discussed (Urano et al. 2003). Based on the genomic map of Arabidopsis available from NCBI (www.ncbi.nlm.nih.gov) or TAIR (www.arabidopsis.org), while the neighboring gene of \textit{AtSAMDC4} is ~900 bp upstream; the neighboring genes in the case of \textit{AtSAMDC3} and \textit{AtSAMDC5} are >3 kb bp upstream of their respective transcription start site; thus the precise length of promoter in each case is a matter of speculation. We therefore cloned 864 bp fragment for the promoter of \textit{AtSAMDC4}, 2905 bp for \textit{AtSAMDC3}, and 2001 bp in case of the \textit{AtSAMDC5} promoter.
(Fig. 35) with or without the inclusion of the 5'UTR and/or the ORF to obtain different transcriptional and translational fusions with GUS along with partial deletion of the 5' end of the promoter in each case. The goal was to study the role of the promoter in regulation of gene expression in coordination with the 5'UTR and/or the ORF in all three SAMDCs. In order to check if the promoter::GUS fusions represented the normal state of affairs for gene expression, we also tested the transcript levels of the four genes by qPCR.

**Transient Expression vs. *in vivo* expression**

A common approach to test the efficacy of a promoter, and in many cases to delineate the role of various cis-regulatory elements within it, is to use transient expression assays in homologous (Schenk et al. 1999; Park et al. 1999; Zhang et al. 2002; Qu and Takaiwa 2004; Martin et al. 2009) or heterologous tissues (Agius et al. 2005; Zhang et al. 2008). While often the results of transient and stable expression assays driven by a cloned promoter match with each other, in most cases the comparisons are never made. Most studies assume that the observed reporter gene expression is representative of the real gene expression without experimentally verifying it. We used transient expression of the various constructs not only to test that the promoter::GUS fusions produced a functional GUS protein but also found a strong correlation between transient GUS expression in poplar cells and stable GUS expression in Arabidopsis. For example, the activity of GUS was much higher in the SAMDC4 translational fusion plasmids than SAMDC3 or SAMDC5 translational fusion plasmids. Activity of SAMDC3 promoter was lower than SAMDC4 promoter; but considerably higher than SAMDC5 promoter constructs. We further tested if the promoter::GUS fusion assays were consistent with expression of the native gene and our expression data based on the two techniques (at the organ level) were
highly consistent with each other; so were the results from qPCR analysis of 2 d and 10 d old seedlings. It was also revealed by these assays that the minimal functional promoter in my experiments was 283 bp for SAMDC4, which showed very high activity both in transient as well as stable (integrated) gene expression assays. Studies on the responses of these constructs in the transgenic plants to various growth regulators and stress responses are currently underway. Preliminary studies on the response of promoter::GUS fusion transgenic plants to high osmoticum, low temperature and wounding show differential responses of the different constructs/gene promoters (data not included here).

Expression during early development

The expression data of the three AtSAMDC genes studied here, and the other two AtSAMDC genes studied earlier in our lab, during early development show that there probably is a differential role of the various SAMDC gene family members in different tissues and organs. While all of them appear to be transcribed early in germination, their tissue/organ specificity of expression starts to differ as the seedlings grow older, and it gets maximized in the mature plants. Furthermore, the published data (and also preliminary data from our lab) on SAMDC expression in response to abiotic stress treatments confirms that not only they may play differential roles during development, they also respond differently to environmental factors. These conclusions are consistent with the evolutionary changes in different members of large gene families where they may have arisen from gene duplication and rearrangements (Flagel and Wendel 2009; Lan et al. 2009). The greater differences in the sequences of the different promoters vs. the coding sequences further lead us to propose that the promoters perhaps evolved faster than the genes, which maintained highly conserved sequences.
The different translational and transcriptional fusions give us a deeper insight into the regulation of SAMDC genes (in terms of the actual production of SAMDC protein) at different levels of gene expression and at different stages of development. Higher activity of GUS for the translational fusion of AtSAMDC4 was observed during the first 48 h of seed germination; this activity was present in the emerging radicle as well as the cotyledons. On the other hand, little or no GUS activity for AtSAMDC3::GUS was observed, and AtSAMDC5::GUS showed only limited activity in the cotyledons. This might suggest a prominent role of native AtSAMDC4 enzyme at early stages of seedling development; and a differential regulation of its activity at the translational level by the ORF. This is borne out from the observation that transcriptional fusions of all SAMDCs showed higher activity of GUS throughout the germinating seedlings. Increased expression on deletion of the 5’UTRs in all three SAMDCs further confirms the involvement of 5’UTR in translational regulation of the SAMDC genes studied earlier (Hanfrey et al. 2002, 2003, 2005); even though the size of the 5’UTR varies considerably among them. The 5’UTRs of AtSAMDC1 and AtSAMDC2 contain both tiny and small uORFs. It has been demonstrated that under low intracellular PA conditions, the tiny uORF is translated, which in turn allow the binding of ribosomes to the main SAMDC ORF and augments its translation. In the presence of excess intracellular PAs, the tiny uORF is not translated and the ribosomes bind to the main small 5’uORF; their binding to the main ORF is reduced and its translation is inhibited. Only one uORF in the 5’UTR of AtSAMDC3 and no uORFs in the 5’UTRs of AtSAMDC4 and AtSAMDC5 were observed. While, our data show an overall consistency with the results of Franceschetti et al. (2001) and Hanfrey et al. (2002) on the role of uORFs within the SAMDC mRNAs; removal of
5'UTR and increased GUS activity in all three SAMDCs seen here provides further arguments for the importance of the 5'UTR regardless of the presence of uORFs.

High activity of GUS (in plants without the 5'UTR constructs) observed in the roots of AtSAMDC3 and AtSAMDC5 seedlings would suggest negative regulation of translation by the 5'UTR in the roots; again showing consistency with the role of uORFs and/or UTRs in regulating gene transcription and/or translation. Deletion of a major 5' portion (up to 80% in some cases) of the putative promoter in all three SAMDCs (down to 384 bp in case of AtSAMDC3 and AtSAMDC5 and 283 bp in AtSAMDC4) did not negatively impact GUS activity; rather it maintained high activity or enhanced the GUS activity both in vegetative and reproductive organs. This might suggest that the core promoters for all SAMDCs are much smaller to drive sufficient spatio-temporal pattern of gene expression. Thus the remaining 5' part of the promoter with several common motifs might be associated either with developmental and environmental responses or with fine-tuning of gene expression in different cell types. The presence of GAREAT (GA Response Element) motifs in the promoter of AtSAMDC3 and AtSAMDC5 genes and their higher expression in the transcriptional fusions is consistent with the role of GA in seed germination. Although no such element was observed in the promoter of AtSAMDC4, yet higher expression both at translational and transcription level might suggest involvement of other regulatory elements present either in the promoter or the ORF itself stabilizing the expression. The expression data during early development for AtSAMDC3 and AtSAMDC4 are consistent with the reported patterns through microarray analysis (Genevestigator- www.genevestigator.com). No expression data are available on
AtSAMDC5; as stated as earlier, and confirmed by the lack of its ESTs, it is possible that this gene might not be transcriptionally or translationally active.

This study shows for the first time that the AtSAMDC5 promoter is transcriptionally active; however, however, its activity could be low due to its extremely short 5'UTR (12 bp) or the ORF negatively effecting translation; removing the 5'UTR increased GUS expression. The GUS expression with AtSAMDC1 and AtSAMDC2 promoters also show that there is a differential role of these two genes in different tissues during development, although both are expressed often in the same tissues (Mitchell 2004). For example, the expression of AtSAMDC1 started at the root tip of germinating seedlings and gradually proceeded towards the cotyledons; the reverse was the case for AtSAMDC2 during seed germination. No AtSAMDC2 expression was seen in the root tips where AtSAMDC1 was strongly expressed. Their promoter sequences reveal the presence of specific regulatory motifs (e.g. ABRE, WRKY elements) that might explain this differential expression pattern during development, and also in relation to different hormonal signals in the root tip vs. the base of the root and the hypocotyl. The AtSMADC3 promoter has root-specific regulatory motifs in its 5' region, which is consistent with its high expression in the roots. Higher degree of sequence identities with respect to ORF of all the SAMDC genes as compared to their promoters might explain that the promoters might have evolved independently than their coding regions, and the functional diversity of the different members of this gene family is due to the diversification of the promoters.

Mature plants

High activity of GUS was observed in the vascular tissues of leaves, roots and rosette junctions in translational fusion of AtSAMDC4, whereas AtSAMDC3 and AtSAMDC5
fusions showed very low GUS activity which was localized in the vascular tissues. Again at the organ levels, the results are in agreement with the earlier reports on the expression of at least three AtSAMDCs (Franceschetti et al. 2001, Urano et al. 2003, Ge et al. 2006) and the microarray data (except for AtSAMDC5) for several organs in A. thaliana.

Together these data are also consistent with the situation of other genes with multiple family members. Transcriptional fusions showed higher GUS activity in AtSAMDC3 and AtSAMDC5 promoter::GUS fusions but lower activity of GUS in AtSAMDC4::GUS fusions; this observation suggests the involvement of regulatory elements in the SAMDC-ORF in positively affecting the expression of this gene. Removal of the 5'UTR, on the other hand, significantly increased GUS activity in the leaves; this again points to a negative control of the gene product, most likely via translational regulation.

In mature plants, constitutively high GUS activity was observed in the leaves for AtSAMDC1 promoter; the veins and the hydathodes staining darker than the surrounding tissues. In contrast, AtSAMDC2 promoter showed localized GUS expression, limited largely to leaf veins and hydathodes. As with the vegetative organs, GUS activity profiles for both AtSAMDC1 and AtSAMDC2 (Mitchell 2004) in flowers are consistent with the available microarray data, where highest signal values were obtained for pollen followed by sepals, petals, stigma and ovary, respectively, for both these genes; and, overall expression of AtSAMDC1::GUS was much greater than AtSAMDC2::GUS. The GUS analyses data are also in line with earlier observations of Franceschetti et al. (2001) and Urano et al. (2003), where high ubiquitous expression of AtSAMDC1 was observed in all organs (with highest expression in flowers) of mature plants. Expression of AtSAMDC2
(as seen by GUS activity) was much lower compared to \textit{AtSAMDC1}, and was mainly localized in the inflorescences and leaves of mature plants.

The present study, combined with the earlier study by Mitchell (2004) provides the most comprehensive analyses for expression of all five members of the \textit{AtSAMDC} gene family in terms of tissue specificity during flower development. For example: (1) within the stamens, high expression was mainly localized in the pollen and less so in the anther sac cells; (2) in the filament, expression was localized more in vascular tissues than the rest of the filament; (3) higher expression in the sepal and petal veins as compared to the lamina; and (4) restricted expression in the peduncle, receptacle and sepal junctions and not the entire ovary. Such details, as mentioned above, are not discernible from studies using other techniques for gene expression. The data also show a stronger expression \textit{AtSAMDC1} compared with \textit{AtSAMDC2} during the entire flower development process.

The lack of GUS activity in parts of the developing ovary and the siliques might suggest that PAs required for these tissues either are transported from neighboring cells; alternatively, this could be due to poor infiltration of the GUS stain in these tissues. High activity of GUS in developing embryos of \textit{AtSAMDC1}, \textit{AtSAMDC2} and \textit{AtSAMDC4} when isolated but no visual GUS staining in the developing seeds within the siliques \textit{in vivo} again could be due to poor infiltration of the stain.

In contrast to \textit{AtSAMDC1} and \textit{AtSAMDC2}, no activity of GUS for \textit{AtSAMDC3}, \textit{AtSAMDC4} and \textit{AtSAMDC5} was detected in the pollen which clearly points to the differential role of different members of this gene family in flower parts. High level of expression of \textit{SAMDC} genes in the vascular tissue perhaps is related to intense cell division activity associated with this tissue. It is quite possible that large amounts of PAs
are actually produced in the vascular tissue and transported to many cells/tissues that do not show \textit{SAMDC} expression. This argument is consistent with the notion that all living cells require PAs to perform necessary functions, including transcription and translation (Cohen 1998). On the other hand, most of the published studies on promoter::GUS fusions often show high expression in the vascular tissue.

A role of PAs in plant vasculature development is implicated in several plant species. High concentrations of PAs were seen in the xylem sap of mungbean, sunflower and orange (Friedman et al. 1986). Such correlations among PA concentrations in the plant axis and the size and age of tissues have also been reported in other plants (Paschalidis and Roubelakis-Angelakis 2005). A possible explanation for PA role in vasculature differentiation and ligification is through the production of H$_2$O$_2$ via PA oxidation by DAOs and PAOs (Cona et al. 2003; Passardi et al. 2005). The involvement of \textit{SAMDC} in the development of proper vasculature was demonstrated in \textit{bud2} (a \textit{samdc4} mutant), which had limited dcSAM availability and showed increased vascular bundle size (Ge et al. 2006). A mutant of Spm/tSPM synthase \textit{(aclS)}, which is deficient in tSpm, had a severe phenotype including defective stem elongation, smaller mature leaves and enlarged xylem vessels in the inflorescence stalks; this mutation could not be rescued by common plant hormones (Hanzawa et al. 1997; Muñiz et al. 2008). Higher activity of GUS in the roots of \textit{AtSAMDC4} translational fusion (in comparison to \textit{AtSAMDC3} and \textit{AtSAMDC5}) also supports the study with \textit{bud2} mutant that showed altered seedling root growth with higher number of lateral roots, enlarged vasculature, and higher lignin deposition (Ge et al. 2006). Altered hypocotyl elongation and lateral bud growth in \textit{bud2} was later explained as an effect of altered auxin-mediated response in this mutant (Cui et
al. 2010). Bioinformatics analyses of the promoters of all SAMDC genes further reinforce the role of auxin involvement (Cui et al. 2010) with \textit{AtSAMDC4}, as \textit{AtSAMDC4} and \textit{AtSAMDC5} promoters show auxin responsive elements in their promoter regions.

**Conclusions – Objective 2**

The results of this part of the study confirm the differential role of different SAMDCs during development, and perhaps in response to different environmental conditions. It is envisioned that the different \textit{cis}-regulatory elements in the promoter region regulate the expression of these genes through involvement of different signal transduction pathways, many of which include plant hormones. The presence of several stress, hormone, and ABA-response elements in the promoters of various SAMDC genes, and the repeated demonstration of changes in cellular PAs and the expression of PA-biosynthetic genes during development and stress response, re-enforce the suggested role of PAs in almost all aspects of plant life. My study specifically reveals that \textit{AtSAMDC5} is transcriptionally active as opposed to previous assumption that it is not transcribed or translated.

Having covered almost all organs of \textit{A. thaliana} for the expression of all five members of the SAMDC gene family, it is evident that there is no organ/tissue where at least one of these five genes is not expressed, showing the indispensability of dcSAM production in almost every plant cell. On the contrary, several cells/tissues appear to express several if not all members of this gene family, confirming an enormous degree of redundancy in the expression of these genes. This observation also leads us to conclude that the SAMDC enzyme is very critical for cell’s survival, and the functional redundancy has been a selective feature for plants during evolution.


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Figure S1. Representative HPLC chromatograms of WT (a) and mODC1-7 (b) plants showing separation of dansyl-polyamines. Heptanediamine (Hd) was used as an internal standard.
Figure S2. A representative HPLC chromatogram of dansylated amino acids. Note the lack of clear separation of Arg, Thr and Gly at retention time around 13.12 min. For retention times of other amino acids, see Table S1.
Table S1. Typical Retention times of dansyl-amino acids eluted in the HPLC system.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Retention time (min)</th>
</tr>
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<tbody>
<tr>
<td>Asp</td>
<td>7.06</td>
</tr>
<tr>
<td>Glu</td>
<td>7.40</td>
</tr>
<tr>
<td>Gln</td>
<td>11.06</td>
</tr>
<tr>
<td>Ser</td>
<td>12.12</td>
</tr>
<tr>
<td>Arg</td>
<td>12.92</td>
</tr>
<tr>
<td>Thr</td>
<td>13.57</td>
</tr>
<tr>
<td>Gly</td>
<td>13.90</td>
</tr>
<tr>
<td>Ala</td>
<td>14.89</td>
</tr>
<tr>
<td>Pro</td>
<td>16.93</td>
</tr>
<tr>
<td>GABA</td>
<td>17.89</td>
</tr>
<tr>
<td>Val</td>
<td>19.47</td>
</tr>
<tr>
<td>Met</td>
<td>20.26</td>
</tr>
<tr>
<td>Ile</td>
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<tr>
<td>Leu</td>
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</tr>
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</tr>
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