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Understanding polyamine metabolism through transgenic manipulation in poplar suspension cultures

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UNDERSTANDING POLYAMINE METABOLISM THROUGH TRANSGENIC MANIPULATION IN POPLAR SUSPENSION CULTURES

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DEDICATION

This dissertation is dedicated to my family: my grandfather, my parents, my brother, my sister-in-law, my wife and my little niece.

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TABLE OF CONTENTS

DEDICATION iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES xi

LIST OF FIGURES xii

ABBREVIATIONS xvi

ABSTRACT xviii

GENERAL INTRODUCTION 1

What are polyamines? 1
Why study polyamine metabolism? 2
Metabolism of polyamines and related amino acids 5
Genetics of polyamine metabolism in plants 7
Genetic manipulation of polyamine metabolism 9
Functions of polyamines 17
Polyamines in growth and development 17
Polyamines and stress 20
Polyamines as indicators of stress in trees 22
Current status of polyamine research in our laboratory 24
Objectives of the present study 25
Suspension culture vs. intact tissues 26

GENERAL MATERIALS AND METHODS 28

Cell culture and harvest 28
Free polyamine analysis 29
Amino acid analysis 30
Determination of reduced glutathione (GSH), the phytochelatin PC_2 and γ-glutamylcysteine (γ-EC) 31
Ion analysis 32
Measurement of mitochondrial activity and membrane integrity 33

Measurement of total protein content 33
Genomic DNA isolation and quantification 34
Polymerase Chain Reaction (PCR) 35
Statistical Analysis 36

CHAPTER I 37
PUTRESCINE OVERPRODUCTION AND ACTIVITIES OF THE POLYAMINE BIOSYNTHETIC ENZYMES 37

Introduction 37

Materials and Methods 39
ODC, ADC and SAMDC enzyme assays 39

Results 41
Confirmation of stable transformation 41
Cellular contents of polyamines over the 7 d culture cycle 41
Cellular contents of soluble proteins over the 7 d culture cycle 44
Activities of ODC, ADC and SAMDC over the 7 d culture cycle 45
Expression of different genes over the 7 d culture cycle 48

Discussion 48

Conclusions 58

CHAPTER II 59
ENHANCED PUTRESCINE METABOLISM AND SOLUBLE AMINO ACIDS.. 59

Introduction 59

Materials and Methods 63
Total organic carbon and nitrogen 63

Results 63
Amino acids derived from α-ketoglutarate (the Glutamate family) 63
Amino acids derived from 3-phosphoglycerate (3-PGA) 66
The aromatic amino acids (derived from phospho*enol*pyruvate) 66
Amino acids derived from pyruvate (the pyruvate family) 68
Amino acids derived from oxaloacetate (the aspartate family) 70
Gamma-aminobutyric acid (GABA) 73
Total carbon and nitrogen 73

Discussion 73

Conclusions 87

CHAPTER III **89**
POLYAMINES AND OXIDATIVE STRESS **89**

Introduction **90**

Materials and Methods **96**
Glutathione Reductase (EC 1.6.4.2) Assay 96
Ascorbate Peroxidase (EC 1.11.1.11) Assay 97
Monodehydroascorbate Reductase (EC 1.6.5.4) Assay 97

Results **98**
Total Soluble Protein 98
Standardization of Glutathione Reductase activity 99
Glutathione Reductase (GR) 99
Ascorbate Peroxidase (APX) 103
Monodehydroascorbate Reductase (MDHAR) 103
Glutathione 103
Accumulation of Ca and K 105
Mitochondrial Activity and Membrane Function 105

Discussion **107**

Conclusions **116**

CHAPTER IV **117**
POLYAMINES AND ALUMINUM TOXICITY **117**

Introduction **117**

Materials and Methods **120**
Cell growth and harvest 120

Results **121**
Effect of Al and Ca on cell growth 121
Effect of Al and Ca on mitochondrial activity and membrane permeability 121
Effect of Al and Ca on cellular polyamines 127
Effect of Al and Ca on accumulation of inorganic ions 127
Effect of Al on GSH, γ-EC and PC_2 133
Effect of Al and Ca on cellular free amino acids 133

Discussion **143**

Conclusions **152**

CHAPTER V **153**
POLYAMINES, GAD AND GABA **153**

Introduction **153**

Materials and Methods **159**
Characterization of glutamate decarboxylase (GAD) activity 159

Results **160**
Glutamate decarboxylase (GAD) 160
Effects of 3-MPA on growth and fresh weight of cells 160
Effects of 3-MPA on mitochondrial activity and membrane function 161
Effects of 3-MPA on polyamines, GABA, and glutathione 162

Discussion **162**

Conclusions **171**

CHAPTER V1 ..... **172**
PUTRESCINE OVERPRODUCTION AND CARBON ASSIMILATION **172**

Introduction **173**

Materials and Methods **175**
^{14}C sucrose feeding 175
^{14}C arginine feeding 176

Results **176**
^{14}C-sucrose feeding 176
^{14}C arginine feeding 178

Discussion **178**

Conclusions **181**

CHAPTER VII **183**
PUTRESCINE OVERPRODUCTION AND THE PROLINE PATHWAY **183**

Introduction **183**

Materials and Methods **186**
OAT assay 186
PCR amplification 187
TOPO-ligations and transformation of *Escherichia coli* 188
Plasmid DNA isolation 189
Analysis of ligated products by restriction digestion 189

DNA Sequencing and sequence analysis 189
RNA Extraction 190
cDNA synthesis 191
Normalized gene expression 191

Results **192**
OAT activity and total protein content 192
Normalized gene expression 192
Celluar proline content 198

Discussion **198**

Conclusions **200**

GENERAL CONCLUSIONS **202**

APPENDICES **208**

APPENDIX A **209**
A STRUCTURAL REPRESENTATION OF METABOLISM OF POLYAMINES AND RELATED AMINO ACIDS **209**

APPENDIX B **210**
ANALYSIS OF GENE EXPRESSION (Results of Dr. Andrew Page; Page et al., 2007) **210**

APPENDIX C **215**
EFFECTS OF Al AND Ca ON CELLULAR AMINO ACIDS **215**

APPENDIX D **218**
ENHANCED PUT METABOLISM AND CELLULAR INORGANIC IONS **218**

LITERATURE CITED **223**

LIST OF TABLES

Table 1. Partial list of genes of the polyamine metabolic pathway and the plants from which they have been cloned 11

Table 2. Partial list of genes of the ornithine and arginine biosynthetic pathway and the plants from which they have been cloned 13

Table 3. Partial list of genes of the proline biosynthetic pathway and the plants from which they have been cloned 15

Table 4. List of transgenic experiments in plants with genes encoding polyamine metabolic enzymes 18

LIST OF FIGURES

Figure 1. Structures of the three common polyamines 1

Figure 2. Polyamine biosynthesis and related nitrogen metabolism 4

Figure 3. Control and HP cells as seen under the light microscope 27

Figure 4. PCR amplification of (A) a 0.8 Kb m*ODC* fragment in HP cells and (B) a 1 Kb *GUS* fragment in control cells 42

Figure 5. PCR amplification of *NPT*II in transgenic poplar cells 43

Figure 6. Cellular contents of (A, B) Put, (C, D) Spd and (E, F) Spm on (A, C, E) g^{-1} FW and (B, D, F) mg^{-1} protein basis, in control and HP cells over the 7 d culture cycle 46

Figure 7. Cellular contents of total proteins in the control and HP cells over the 7 d culture cycle 47

Figure 8. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of ODC in control and HP cells over the 7 d culture cycle ..... .49

Figure 9. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of ADC in control and HP cells over the 7-d culture cycle 49

Figure 10. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of SAMDC in control and HP cells over the 7 d culture cycle. 50

Figure 11. Role of Glu as a precursor of several important metabolites. 62

Figure 12. The biosynthesis of amino acids in plants 64

Figure 13. Cellular contents of the glutamate family of amino acids (derived from α-ketoglutarate): (A) glutamate (B) ornithine (C) arginine (D) proline (E) glutamine (F) histidine in control and HP cells ..... .67

Figure 14. Cellular contents of amino acids derived from 3-phosphoglycerate: (A) serine (B) glycine (C) cysteine+cystine in control and HP cells 69

Figure 15. Cellular contents of amino acids derived from Phospho*enol*pyryvate: (A) Phenylalanine (B) Tryptophan in control and HP cells 71

Figure 16. Cellular contents of amino acids derived from pyruvate: (A) leucine (B) valine (C) alanine in control and HP cells 72

Figure 17. Cellular contents of amino acids derived from oxaloacetate: (A) aspartate, (B) threonine (C) lysine (D) methionine (E) isoleucine in control and HP cells...74

Figure 18. Cellular contents of GABA in control and HP cells 75

Figure 19. Total C and N content in control and HP cells 75

Figure 20. The ascorbate-glutathione cycle 93

Figure 21. Cellular contents of total protein content in control and HP cells..... 100

Figure 22. Standardization of GR activity in the control and HP cells. (A) Change in absorbance with time. (B) The slope of Absorbance vs. Time. (C) Change in absorbance with time in 4-day-old HP cells at different concentrations of the cell extract..... 101

Figure 23. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of GR over the 7-d culture cycle in control and HP cell lines..... 102

Figure 24. (A) Activity and (B) specific activity of APX over the 7-d culture cycle in control and HP cell lines. 104

Figure 25. (A) Activity and (B) specific activity of MDHAR over the 7-d culture cycle in control and HP cell lines. 104

Figure 26. Cellular contents of GSH in control and HP cells..... 106

Figure 27. Cellular contents of (A) Ca and (B) K in the control and HP cells over the 7-d culture cycle..... 106

Figure 28. (A) Mitochondrial activity as measured by MTT reduction and (B) cell viability as measured by Evans blue retention in the control and HP cells.. 108

Figure 29. Fresh weight of control and HP cells growing in different concentrations of (A) and Al (B) Al and Ca 6, 24 and 48 h after Al treatment.....123

Figure 30. Absorbance of (A) MTT reaction products and (B) Evans blue retention in control and HP cells 6, 24 and 48 h after Al treatment..... 124

Figure 31. Absorbance of (A) MTT reaction products and (B) Evans blue retention in control and HP cells 3 and 5 days after transfer to media with different concentrations of Ca. 125

Figure 32. Absorbance of MTT reaction products in control and HP cells growing in normal and reduced Ca, 6, 24 and 48 h after Al addition 126

Figure 33. Cellular contents of (A) Put and (B) Spd in control and HP cells 6, 24 and 48 h after Al addition to the media. 129

Figure 34. Cellular contents of (A) Put and (B) Spd in control and HP cells on days 3 and 5 after transfer to media containing different concentrations of Ca. 130

Figure 35. Cellular contents of (A) Put and (B) Spd in control and HP cells growing in normal and reduced Ca, 6, 24 and 48 h after Al addition to the media..... 131

Figure 36. Cellular contents of (A, B) Ca and (C, D) Mn in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca.....135

Figure 37. Cellular contents of (A, B) Al and (C, D) Mg in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca.....136

Figure 38. Cellular contents of (A, B) Fe and (C, D) P in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca..... 137

Figure 39. Cellular contents of (A, B) K and (C, D) Zn in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca.....138

Figure 40. Cellular contents of (A) GSH, (B) PC_2, and (D) γ-EC in the control and HP cells due to different concentrations of Al 6, 24 and 48 h after treatment.... 140

Figure 41. Cellular contents of (A, B) Glu, (C, D) Gln, and (E, F) Arg in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment..... 141

Figure 42. Cellular contents of (A, B) Pro, (C, D) GABA and (E, F) His in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment..... 142

Figure 43. The GABA shunt..... 154

Figure 44. The inhibition of GAD activity by 3-MPA. 163

Figure 45. (A) Fresh weight (B) Mitochondrial activity as measured by MTT reduction and (C) cell viability as measured by Evans blue retention in the control and HP cells, 6, 24 and 48 h after 3-MPA treatment..... 164

Figure 46. Cellular content of (A) Put and (B) Spd in the control and HP cells, 6, 24 and 48 h after 3-MPA treatment. 165

Figure 47. Cellular content of GABA 24 h after 3-MPA treatment to the control and HP cells. 166

Figure 48. Cellular content of GSH 24 h after 3-MPA treatment to the control and HP cells. 167

Figure 49. Radioactivity recovered in the (A) toluene (B) aqueous and (C) PCA extracts at different time periods after [U-^{14}C]sucrose feeding to control and HP cells. 177

Figure 50. Radioactivity recovered in total PAs at different time periods after [U-C^{14}]Arg feeding and subsequently growing the cells with or without sucrose. 179

Figure 51. (A) Activity (g^{-1} FW), (B) specific activity (mg^{-1} protein) of OAT and (C) total protein content during analysis of enzyme activity in the control and HP cells over the 7-d culture cycle. 194

Figure 52. (A) PCR amplification of the 0.7 Kb *P5CR* and *PDH*1 gene fragments from a poplar cDNA library. (B) PCR amplification of a genomic 1.1 Kb *PDH*2 fragment. (C) Gel 1 represents PCR amplification of 0.7 Kb *PDH*1 fragment and gel-2 represents the 0.58 Kb *PDH*2 fragment from control and HP cDNA from all days of the 7 d culture cycle.....195

Figure 53. *Eco*RI restriction digestion of several clones of (A) TOPO-*P5CR* (B) TOPO-*PDH*1 and (C) TOPO-*PDH*2 showing fragments of the desired lengths i.e. single fragments of approximately 700 bp in (A) and (B) and two fragments (200 bp and 900 (C).....196

Figure 54. Gene expression (normalized to *G3PDH*) of (A) *PDH*1, (B) *P5CR* and (C) *OAT* in the control and HP cells over the 7-d culture cycle..... 197

Figure 55. Major conclusions from (A) chapter I and (B) chapter II.....204

Figure 56. Major conclusions from (A) chapter III and (B) chapter IV.....205

Figure 57. Major conclusions from (A) chapter V, (B) chapter VI and (C) chapter VII.....206

Figure 58. Summary of major conclusions from the present study 207

Figure A1. Metabolism of polyamines and related metabolites (structural) 209

Figure B1. Normalized gene expression of (A) m*ODC* (B) *ADC* and (C) *SAMDC*1 in the control and HP cells on d 1, 3, 5 and 7 of subculture. 213

Figure B2. Normalized gene expression of (A) *SAMDC*2 (B) *SAMDC*3 (C) *SPDS*1 and (D) *SPDS*2 in the control and HP cells over the 7 d culture period. 214

Figure C1. Cellular contents of (A, B) Thr, (C, D) Val and (E, F) Lys in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment..... 215

Figure C2. Cellular contents of (A, B) Ser, (C, D) Cys + cysteine and (E, F) Gly in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment. 216

Figure C3. Cellular contents of (A, B) Phe, and (C, D) Trp in the control and HP cells due to different concentrations of Al (A, C) or Al and Ca (B, D), 48 h after treatment. 217

Figure D1. Cellular contents of (A) Ca, (B) K, (C) Mg and (D) Mn in the control and HP cells over the 7-d culture cycle.....221

Figure D2. Cellular contents of (A) Fe, (B) P and (C) Zn in the control and HP cells over the 7-d culture cycle. 222

ABBREVIATIONS

ABA= abscisic acid; ACC= 1-aminocyclopropane-1-carboxylic acid; ADC= arginine decarboxylase; ADP= adenosine diphosphate; AL= argininosuccinate lyase; Ala= alanine; AO= ascorbate oxidase; AOS= active oxygen species; APX= ascorbate peroxidase; Arg= arginine; AS= argininosuccinate synthase; AsA= ascorbic acid; Asp= aspartate; AT= alanine aminotransferase; ATP= adenosine triphosphate; CaMV= cauliflower mosaic virus; CCD= charged couple device; cDNA= complementary DNA; CGS= cystathionine γ-synthase; CoA= coenzyme A; CPS= carbamoyl-phosphate synthase; CTAB= hexadecyletrimethylammonium bromide; Cys= cysteine; 2,4-D= 2, 4-dichlorophenoxyacetic acid; DAO= diamine oxidase; dcSAM= decarboxylated S-adenosylmethionine; DFMA= α-DL-difluoromethylarginine; DFMO= α-DL-difluoromethylornithine; DHA= dehydroxyascorbate; DHAR= dehydroxyascorbate reductase; DNA= deoxyribonucleic acid; DTPA= diethyltriamine pentaacetic acid; DTT= dithiothreitol; DW= dry weight; EDTA= ethylinediamine tetraacetic acid; GABA= γ-aminobutyric acid; GABA-T= γ-aminobutyric acid transaminase; GAD= glutamate decarboxylase; GHB= γ-hydrobutyric acid; GHBDH= γ-hydrobutyric dehydrogenase; Gln= glutamine; Glu= glutamate, Gly= glycine; GOGAT= glutamate-2-oxoglutarate aminotransferase; GR= glutathione reductase; GS= glutamine synthetase; GSH= reduced glutathione; GSSG= oxidized glutathione; GUS= β- glucuronidase; HerP= O-phospho-L-homoserine; His= histidine; HP= high putrescine; HPLC= High Performance Liquid Chromatography; ICP= inductively coupled plasma; Ile= isoleucine; α-ketoglutarate; LDC= lysine decarboxylase; Leu= leucine; Lys= lysine; MDHA= Monodehydroxy-

ascorbate; MDHAR= monodehydroxyascorbate reductase; Met= methionine; mRNA= messenger RNA; MPA= mercaptopropaonic acid; MSX= methionine sulfoxinine; MTT= 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; NADP= nicotinamide adenine dinucleotide phosphate; NAGK= *N*-acetyl-glutamate kinase; NAGS= *N*-acetylglutamate synthase; NAOAT= *N*-acetyl-ornithine acetyltransferase; NAOD= *N*-acetylornithine deacetylase; NAOG*Ac*T= *N*-acetylornithine: glutamate acetyltransferase; NPT= neomycin phosphotransferase; NR= nitrate reductase; OAA= oxaloacetic acid; OAT= ornithine aminotransferase; ODC= ornithine decarboxylase; Orn= ornithine; OTC= ornithine carbamoyltransferase; P5C= Δ^1-pyrroline-5-carboxylate; P5CDH= Δ^1-pyrroline-5-carboxylate dehydrogenase; P5CR= Δ^1-pyrroline-5-carboxylate reductase; P5CS= Δ^1-pyrroline-5-carboxylate synthase; PA= polyamines; PAO= polyamine oxidase; PC= phytochelatin; PCA= perchloric acid; PCR= polymerase chain reaction; PDH= proline dehydrogenase; PEP= phospho*enol*pyruvate; PGA= phosphoglyceric acid; Phe= phenylalanine; PLP= pyridoxal phosphate; Pro= proline; Put= putrescine; PyDH= pyrroline dehydrogenase; Pyr= pyruvate; QRT-PCR= quantitative real time polymerase chain reaction; RNA= ribonucleic acid; ROS= reactive oxygen species; SAM= S-adenosyl methionine; SAMDC= S-adenosyl methionine decarboxylase; SDS= sodium dodecyl sulphate; SE= standard error; Ser= serine, Spd= spermidine; SPDS= spermidine synthase; Spm= spermine; SPMS= spermine synthase; SSA= succinic semialdehyde; SSADH= succinic semialdehyde dehydrogenase; TCA= tricarboxylic acid; TD= threonine deaminase; Tet= tetracycline, TFA= trifluoroacetic acid; Thr= threonine; Trp= tryptophan; TS= threonine synthase; Val= valine.

ABSTRACT

UNDERSTANDING POLYAMINE METABOLISM THROUGH TRANSGENIC MANIPULATION IN POPLAR SUSPENSION CULTURES

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University of New Hampshire; May 2008

Polyamines are low molecular weight aliphatic amines that are obligatory requirements for cell survival and growth. The commonly occurring polyamines in plants are putrescine, spermidine, and spermine. Suspension cultures of poplar (*Populus nigra x maximowiczii*), transformed with a mouse ornithine decarboxylase gene (under the control of a 2X 35S CaMV promoter) were used to study the impact of up-regulation of putrescine biosynthesis (and consequent enhanced catabolism) on several aspects of cellular metabolism. The transgenic cells were compared with a control cell line that was transformed with the *beta-glucuronidase* (*GUS*) gene.

It was observed that enhanced putrescine metabolism resulted in: (i) increased expression of arginine decarboxylase gene, along with enhanced activity of the corresponding enzyme, (ii) decreased expression of *S*-adenosylmethionine gene and activity of the enzyme, (iii) changes in the cellular contents of almost all amino acids, (iv) a compromise in cell health due to increased oxidative stress, (v) better tolerance towards Aluminum toxicity, (vi) increased susceptibility to glutamate decarboxylase inhibition,

(vii) greater assimilation of carbon from sucrose in the growth medium, and (viii) small changes in the expression of ornithine aminotransferase, proline dehydrogenase and Δ^1-pyrroline-5-carboxylate reductase genes, and an increase in ornithine aminotransferase activity.

GENERAL INTRODUCTION

What are polyamines?

Polyamines (PAs) are low molecular weight aliphatic amines that are obligatory requirements for cell survival and growth (reviewed by Bais and Ravishankar, 2002; Kusano et al., 2007). Polyamines are known to have a role in plant stress response (Alcázar et al., 2006, and references therein) and development (Wallace et al., 2003; Kaur-Sawhney et al., 2003), although the exact mechanisms by which they operate are still under scrutiny. The commonly occurring PAs in plants are putrescine (Put), spermidine (Spd), and spermine (Spm); their structures are shown in Fig. 1.

Putrescine: NH_2-$(CH_2)_4$-NH_3^+

Spermidine: NH_2-$(CH_2)_4$-NH-$(CH_2)_3$-NH_3^+

Spermine: NH_2-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_3^+

Figure 1. Structures of the three common polyamines

Putrescine is the diamine precursor to the triamine Spd, which in turn produces the tetraamine Spm. Other than these three PAs, several uncommon PAs also exist in plants: namely, norspermidine (caldine), norspermine (thermine), pentamine, hexamine, homospermidine and cadaverine (Kuehn et al., 1990; Hamana et al., 1992). Kuehn et al. (1990) reported that uncommon PAs are found mostly in organisms that have to adapt to environmental extremes such as halophilic and thermophilic bacteria. Bagga et al. (1997) reported the occurrence of uncommon PAs in osmotic stress tolerant alfalfa.

While norspermidine and norspermine have been found in mosses, homospermidine is present in ferns and algae. Cadaverine, formed from lysine is found in legumes and serves as a precursor of quinolizidine alkaloids (Smith and Wilshire, 1975; Schoofs et al., 1983). A recent report by Knott et al. (2007) states that spermine synthases from the diatom *Thalassiosira pseudonana* and from *Arabidopsis thaliana* are actually thermospermine synthases.

Polyamines exist in free, bound and conjugated forms (Paschalidis and Roubelakis-Angelakis, 2005). Free PAs are soluble in dilute perchloric acid (PCA) whereas bound forms are not. Polyamines are generally conjugated with hydroxycinnamic acid, fatty acids or alkaloids to produce plant defense-related compounds (Flores and Filner, 1985; Martin-Tanguy, 1997; Ghosh, 2000; Bagni and Tassoni, 2001). Polyamines also serve as precursors for secondary metabolites such as nicotine (Martin-Tanguy, 1997). Paschalidis and Roubelakis-Angelakis (2005) have recently reported on the spatial and temporal distribution of PAs in tobacco; they observed a decreasing trend in PA titers and biosynthesis along the plant axis. They showed that while Spd and Spm are predominantly synthesized in the shoot apical meristems, Put is the predominant PA produced in the hypogeal tissues.

Why study polyamine metabolism?

There are several important reasons why the PA metabolic pathway is of particular interest to scientists:

1. Polyamines are important cations and because of the presence of positive charges on them, they can interact with macromolecules like DNA and RNA (Reviewed by

Lomozik et al., 2005). They are believed to be involved in stabilizing nucleic acids against methylation and denaturation induced by heat (Oshima, 2007) or by X-rays (Baeza et al., 1992). Polyamines affect DNA, RNA and protein synthesis (Cohen, 1998; Bachrach, 2005). Besides, as mentioned earlier, PAs are ubiquitous and are considered indispensable for cell survival, growth and development

2. The PA metabolic pathway can be considered a simple pathway (cf. photosynthesis and respiration), but is linked to some very important metabolites like glutamate (Glu), proline (Pro), ethylene and γ-aminobutyric acid (GABA), and also serves the role of recycling the carbon and nitrogen moieties of key amino acids like Glu, arginine (Arg) and ornithine (Orn) (Fig. 2; also see Fig. A1 for details).

3. The molecular and biochemical properties of the enzymes involved in PA biosynthesis are a combination of features rarely found together in most pathways. These include: long UTRs (untranslated regions; Wallace et al., 2003; Kusano et al., 2007), sometimes with open reading frames (ORFs) in the UTRs (Hanfrey et al., 2005), absence of introns within most genes, rapid turnover of the protein, etc. (Wallace et al., 2003).

4. Since they are indispensable for growth of any cell, their biosynthesis is the target of many chemotherapeutic treatments against cancer as well as against human parasites (Bachrach, 2004, 2005; Saunders and Wallace, 2007).

For reasons outlined above, the PA metabolic pathway can serve as an excellent system for manipulation to modulate one or more steps in the same and study its metabolic regulation. It is expected that such a transgenic manipulation, either by over-

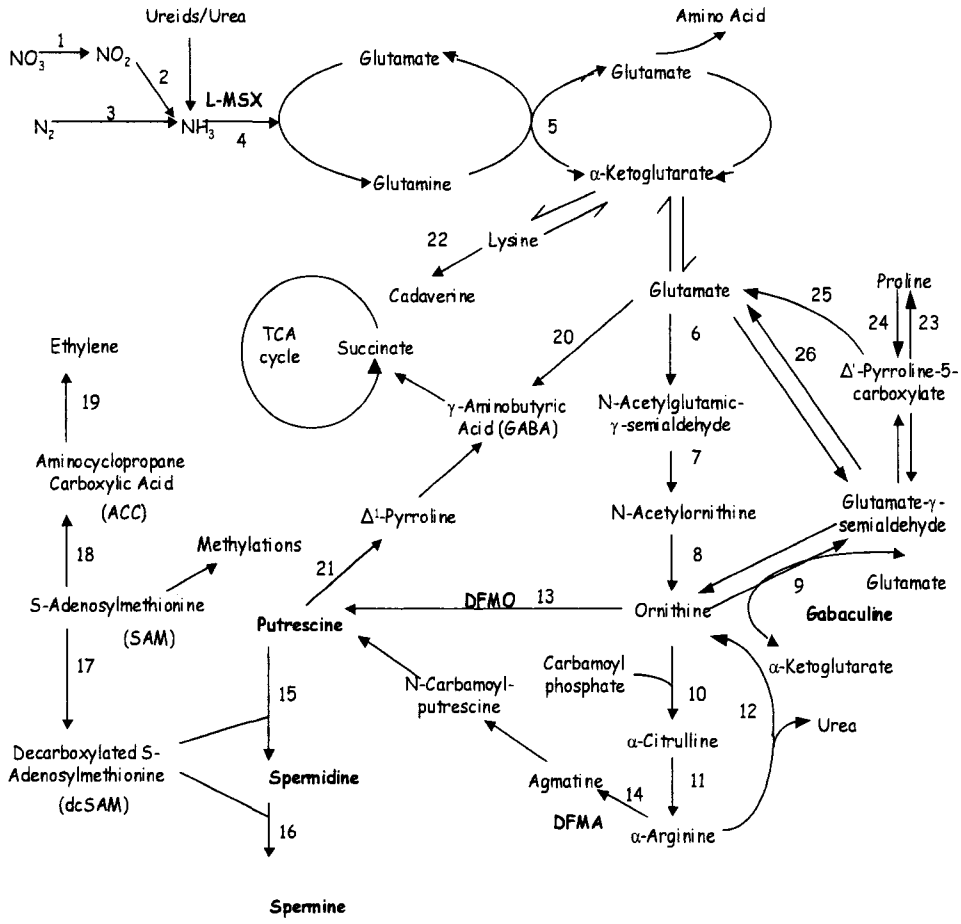

Figure 2. Polyamine biosynthesis and related nitrogen metabolism. The enzymes are: 1. Nitrate reductase, 2. Nitrite reductase, 3. Nitrogenase, 4. Glutamine synthetase (GS), 5. Glutamate synthase (GOGAT), 6. Glutamate reductase, 7. Acetylglutamic-γ-semialdehyde transaminase, 8. Acetylornithinase, 9. Ornithine aminotransferase (OAT), 10. Ornithine transcarbamylase, 11. Arginine synthase, 12. Arginase, 13. Ornithine decarboxylase, (ODC) 14. Arginine decarboxylase (ADC), 15. Spermidine synthase, 16. Spermine synthase, 17. SAM decarboxylase (SAMDC), 18. ACC synthase, 19. ACC oxidase, 20. Glutamate decarboxylase (GAD), 21. Diamine oxidase (DAO), 22. Lysine decarboxylase (LDC), 23. P5C Reductase, 24. Proline oxidase, 25. P5C Dehydrogenase. 26. P5C synthase.

expressing a gene or suppressing its expression can lead to pleiotropic changes in cellular metabolism affecting those of several other important metabolites, either directly or indirectly related to PA metabolism.

Metabolism of polyamines and related amino acids

Polyamines are present in millimolar quantities in plant cells. This property, coupled with their richness in amine groups makes them useful in modulation of reduced nitrogen (N) and in the sequestration of free, cellular NH_3 (Lovatt, 1990; Slocum and Weinstein, 1990; Forde and Lea, 2007). The biosynthetic and catabolic pathways of PAs have been well described although details on the regulation of various steps in them are lacking. Parallels have been drawn with animal cells; however, at times, differences are significant (Slocum and Flores, 1991; Cohen, 1998; Bhatnagar et al., 2001, 2002; Page et al., 2007; Paschalidis and Roubelakis-Angelakis, 2005). Polyamine metabolism is intricately connected to the metabolism of several amino acids and other important metabolites that either serve as precursors to the biosynthesis of PAs, or are the products of their catabolism, or just intermediates in the process of PA metabolism (Fig. 2).

The primary source of all PA biosynthesis is the amino acid Glu, whose conversion into the immediate precursors of Put, i.e. Orn or Arg is a multi-step process, which is poorly understood at present (Slocum, 2005). While Orn is the direct precursor to Put biosynthesis in almost all organisms, in plants, bacteria and fungi, Put us often produced from Arg. The former reaction involves a single step catalyzed by Orn decarboxylase (ODC; EC 4.1.1.17), while the latter involves two intermediates, agmatine (Agm) and N-carbamoylputrescine, and is presumably regulated by Arg decarboxylase (ADC; EC 4.1.1.19). The product of ADC (i.e. Agm) is converted by Agm iminohydrolase (EC

3.5.3.1.2) to N-carbamoylputrescine, which in turn is converted to Put by N-carbamoylputrescine amidohydrolase (EC 3.5.1.53). The biosynthesis of Orn/Arg begins with N-assimilation into Gln by Gln synthetase (GS, EC 6.3.1.2) and Glu by Glu synthase, *a.k.a.* Gln-2-oxoglutarate aminotransferase (GOGAT; EC 1.4.7.1). The primary source of carbon for these reactions is α-ketoglutarate (α-KG) derived from the TCA cycle (Coruzzi and Last, 2000; Forde and Lea, 2007; and references therein). Glu, through a series of intermediates produces Orn (Fig. 2) which then produces Arg via citrulline using the enzymes Orn transcarbamoylase (OTC, EC 2.1.3.3) and Arg synthase (EC 6.3.4.5). Arginine can be converted back to Orn by arginase (EC 3.5.3.1), which is a part of the urea cycle. The other two amino acids that are intricately connected to the PA metabolic pathway are Pro and GABA.

The Pro metabolic pathway has been subjected to intense scrutiny owing to proposed roles of Pro in stress tolerance (Kocsy et al., 2005). Proline can be synthesized either from Glu or from Orn (Roosens et al., 1998, 2002). In both cases, the initial product formed is Glu-γ-semialdehyde, a compound that non-enzymatically cyclizes to form Δ^1-pyrroline-5-carboxylate (P5C). The synthesis of Glu-γ-semialdehyde is aided by the enzyme ornithine aminotransferase (OAT, EC 2.6.1.13) from Orn and by P5C synthase (P5CS; EC 2.7.2.11, 2.7.2.4.1) from Glu. The reaction from P5C to Pro is catalyzed by P5C reductase (P5CR; EC 1.5.1.2). For more details on Pro metabolism, see chapter VII.

Another important amino acid in this pathway that plays parallel roles to PAs and Pro is GABA, the catabolic (oxidation) product of Put (by diamine oxidase – DAO, EC 1.4.3.6) and the decarboxylation product of Glu brought about by Glu decarboxylase (GAD, EC 4.1.1.15). Putrescine is also the precursor of the other two common PAs; i.e.

Spd and Spm, which are synthesized by Spd synthase (SPDS, EC 2.5.1.16) and Spm synthase (SPMS, EC 2.2.1.22), respectively. For each of these reactions, an aminopropyl moiety is required which is provided by decarboxylated *S*-adenosylmethionine (dcSAM), a product formed from the decarboxylation of SAM by SAM decarboxylase (SAMDC, EC 4.1.1.50). In plants, SAM is also the precursor of ethylene. Hence, it is clear that the PA metabolic pathway interacts with the biosynthetic and catabolic pathways of several important metabolites.

In summary, not only are PAs ubiquitous molecules that are considered obligate requirements for cell survival and growth because of their involvement in several important biochemical processes, but their biosynthesis and catabolism are closely related to the metabolism of several other important metabolites like Pro, ethylene, GABA etc. Hence, delineating the regulation of PA metabolic pathway and its interaction with the interacting pathways of Pro, GABA and ethylene biosynthesis is of particular interest.

Genetics of polyamine metabolism in plants

The generation or selection of mutants in plants to understand the functions and metabolism of PAs has a long history. In 1983, Malmberg and McIndoo produced tobacco lines that were resistant to difluoromethylornithine (DFMO; an inhibitor of ODC) and methylglyoxal (bis)guanylhydrazone (MGBG; an inhibitor of SAMDC). Similar lines were also produced by Malmberg and Rose (1987), Hiatt and Malmberg (1988), Trull et al. (1992), and Fritze et al. (1995). Some common observations in these mutants included: inhibitor resistance, elevated PAs, and a variety of phenotypic changes e.g. dwarfism, alterations in floral morphology, etc. Martin-Tanguy (1985) reported the generation of a non-flowering mutant (*RMB7*) in tobacco which lacked the production of

PA conjugates in leaves and their subsequent transportation to the shoot apex, thus suggesting a potential role of PA conjugates in flowering. Gerats et al. (1988) reported higher Put content and ADC activity associated with alterations in floral morphology in a floral mutant of *Petunia*. Rastogi and Sawhney (1990) described a male-sterile *stamenless-2* mutant in tomato, in which the mutated floral parts had elevated contents of PAs and higher activities of the PA biosynthetic enzymes, ODC and SAMDC.

Mirza and Iqbal (1997) and Mirza and Rehman (1998) obtained two Spm resistant mutants in *Arabidopsis, spr*1-1 and *spr*1-2, each with a set of phenotypic peculiarities like longitudinally folded-in cauline leaves, vigorous growth, prominent flowers etc. Arabidopsis mutants deficient in ADC have been characterized by Watson et al. (1997) and Soyka and Heyer (1999). Two *ADC*1 mutants (*spe*1 and *spe*2) showed an enhancement in root growth and a decrease (but not complete abolition) in ADC activity accompanied by a reduction in PA content by about 10-20%. The other *(spe*2 *or adc*2) while showing no clear phenotype, had lower (by about 40%) activity of ADC. Hanzawa et al. (1997, 2000) yet again showed a possible connection between PAs and flowering in a SPDS3-deficient mutant *acl*5 (*ACAULIS5*) that showed "early proliferative arrest" of apical inflorescence meristems, resulting in reduced stem internode length and the number of flowers.

Bagni et al. (1997) had earlier shown that in the *Flacca*-ABA deficient mutant in tomato, there was higher ADC than ODC activity in the older plants, with a decrease in the total PA content. The CS3123-late flowering mutant and 35S::*APETALA*1 early flowering transgenic produced in *Arabidopsis* by Applewhite et al. (2000) showed that Spd promoted flowering in the former but not in the later. The double knock-out mutant

acl5/spms in *Arabidopsis*, with mutations in *SPDS*3 and *SPMS* genes (Imai et al., 2004A) had shorter stems but was able to complete its life-cycle normally. Double knock-out mutants *adc1/adc2* and *spds1/spds2* in *Arabidopsis* with defects in seed development have been reported by Imai et al. (2004B) and Urano et al. (2005).

Several PA biosynthetic and catabolic genes have been cloned and characterized from a variety of plants as shown in Table 1; Tables 2 and 3 list genes of the Orn, Arg and Pro biosynthetic pathways, which have been cloned and characterized in a variety of plant tissues.

Genetic manipulation of polyamine metabolism

Since most of the genes for PA biosynthetic enzymes are present in plants in multiple copies (Kusano et al., 2007), mutants have not been very useful in delineating either the regulation of PA metabolism or their specific developmental roles in plants. The genetic manipulation techniques with heterologous genes under the control of either a native or a CAMV-35S promoter have been used in many studies to alter the PA contents in several plants and to study their effects on plant development or their stress response (Kumar and Minocha, 1998; Kakkar and Sawhney, 2002; Kaur-sawhney et al., 2003; Liu et al., 2007). Hamill et al. (1990) reported the first successful overexpression in tobacco of a yeast (*Saccharomyces cerevisiae*) *ODC* under the control of a CaMV 35S promoter. They observed an increase in not only ODC activity and Put content, but nicotine content as well. Herminghaus et al. (1991, 1996) and Fecker et al. (1993) used a 35S::*LDC* gene construct to transform tobacco with increased cadaverine content.

DeScenzo and Minocha (1993) used both a full length and a truncated mouse *ODC* cDNA under the control of a 35S promoter to transform tobacco. Plants with very high

Put content had crumpled leaves and some were male-sterile. Noh and Minocha (1994) used a human *SAMDC* cDNA to transform tobacco and reported an increase in SAMDC activity by about 9-fold. While Put content decreased; both Spd and Spm increased, sometimes as much as 2- to 3-fold. The transformants in this study were all phenotypically normal. Masgrau et al. (1997) reported the transformation of tobacco with an oat *ADC* cDNA with a tetracycline (*tet*)-inducible promoter. Substantial increases in ADC activity accompanied by phenotypic abnormalities like growth inhibition, leaf necrosis, etc. were observed; no phenotypic change was observed when *tet*-induction preceded floral growth initiation. Burtin and Michael (1997) used the same oat *ADC* cDNA (with CaMV 35S promoter) to transform tobacco and found that: while ADC activity increased along with enhanced Agm content, the contents of Put, Spd and Spm remained unchanged; and so did the activities of ODC and SAMDC. Bastola and Minocha (1995) again used the truncated mouse *ODC* to transform carrot cells and reported significant increases in Put content and ODC activity in the transgenics; concomitantly there was an improvement in somatic embryogenesis and tolerance to DFMA. Andersen et al. (1998) later demonstrated increased Put catabolism in the above transgenic carrot cells. Kumar et al. (1996) transformed potato cells with a 'sense' as well as an 'anti-sense' construct of *SAMDC* with both the 35S and a *tet*-inducible promoter. Whereas the constitutively expressing sense transformants did not survive beyond the microcallus stage, the *tet*- inducible sense transformants showed increases in *SAMDC* transcripts and PA contents after induction. Both kinds of anti-sense constructs had lower *SAMDC* activity in the transgenics. Pedros et al. (1999) also described potato transformants with sense and anti-sense constructs of *SAMDC*, driven by a tuber-specific

Table 1. Partial list of genes of the polyamine metabolic pathway and the plants from which they have been cloned. Updated from Kakkar and Sawhney, 2002.

Gene	EC	Plant	Reference
ADC	4.1.1.9	Oat	Bell and Malmberg, 1990
		Tomato	Rastogi et al., 1993
		Pea	Perez-Amador et al., 1995
		Arabidopsis	Watson and Malmberg, 1996
		Soybean	Nam et al., 1997
		Grapevine	Pimikirios and Roubelakis-Angelakis., 1999
		Carnation	Chang et al., 2000
		Tobacco	Bortolotti et al., 2004
		Apple	Hao et al., 2005A
		Wheat	Bassie et al., 2008
		Rice	Akiyama and Jin, 2007
ODC	4.1.1.7	Datura	Michael et al., 1996
		Tomato	Kwak and Lee, 2001
		Apple	Hao et al., 2005A
SAMDC	4.1.1.50	Potato	Mad-Arif et al., 1994
		Spinach	Bolle et al., 1995
		Catharanthus roseus	Schroder and Schroder., 1995
		Tritordeum	Dresselhaus et al., 1996
		Arabidopsis, rice, maize	Franceschetti et al., 2001
		Carnation	Lee et al., 1997
		Apple	Hao et al., 2005B
		Wheat	Bassie et al., 2008

Continued on page 12

Table 1- continued....

Gene	EC	Plant	Reference
***SAM* Synthase**	2.5.1.6	Arabidopsis	Peleman et al., 1989
		Carnation	Larsen and Woodson, 1991
		Parsley	Kawalleck et al., 1992
		Tomato	Espartero et al., 1994
		Poplar	Doorsselaere et al., 1993
		Rice	Van Breusegem et al., 1994
		Petunia	Izhaki et al., 1995
		Pea	Gomez-Gomez and Carrasco, 1998
		Flax	Lamblin et al., 2001
***Spd/Spm* synthase**	2.5.1.16	Arabidopsis	Hashimoto et al., 1998 Hanzawa et al., 2000
		Apple	Kitashiba, 2005
DAO	1.4.3.6	Lentil	Angelini et al., 1996
PAO	1.5.3.3	*Medicago sativa*, *Avena sativa*	Koc et al., 1995

Table 2. Partial list of genes of the ornithine and arginine biosynthetic pathway and the plants from which they have been cloned. Updated from Slocum, 2005.

Gene	EC	Plant	Reference
NAGS	2.3.1.1	Soybean	Jain et al., 1987
NAGK	2.7.2.2	Soybean	Jain et al., 1987
	2.7.2.8	Carrot	Lohmeier-Vogel et al., 2005
NAOAT	1.2.1.38	Soybean	Shargool et al., 1978
		Pea	Taylor and Stewart, 1981
		Pea	deRuiter and Kollöffel, 1985
		Soybean	Jain et al., 1987
		Arabidopsis	Kleffmann et al., 2004
NAOGAcT	2.3.1.35	Soybean	Shargool et al., 1978
		Pea	Taylor and Stewart, 1981
		Pea	deRuiter and Kollöffel, 1985
		Soybean	Jain et al., 1987
NAOD	3.5.1.16	Watermelon	Yokota et al., 2002
GAT	2.3.1.35	Watermelon	Takahara et al., 2005

Continued on page 14

Table 2-continued...

Gene	EC	Plant	Reference
CPS	6.3.5.5	Soybean	Shargool et al., 1978
		Pea	Taylor and Stewart, 1981
		Pea	deRuiter and Kollöffel, 1985
		Soybean	Jain et al., 1987
OTC	2.1.3.3	Soybean	Shargool et al., 1978
		Pea	Taylor and Stewart, 1981
		Pea	deRuiter and Kollöffel, 1985
		Soybean	Jain et al., 1987
		Arabidopsis	Slocum et al., 2000
		Spinach	Bellocco et al., 2002
		Soybean	Shargool et al., 1978
AS	6.3.4.5	Pea	Taylor and Stewart, 1981
		Soybean	Jain et al., 1987
		Arabidopsis	Kleffmann et al., 2004
AL	4.3.2.1	Soybean	Shargool et al., 1978
		Pea	Taylor and Stewart, 1981

Table 3. Partial list of genes of the proline biosynthetic pathway and the plants from which they have been cloned.

Gene	EC	Plant	Reference
OAT	2.6.1.13	Arabidopsis	Roosens et al., 1998
P5CS	2.7.2.11	Alfalfa	Ginzberg et al., 1998
		Glycine max	Porcel et al., 2005
		Lactuca sativa	
P5CDH	1.5.1.12	Alfalfa	Miller et al., 2005
		Wheat, barley, maize, rice	Ayliffe et al., 2005
P5CR	1.5.1.2	Soybean	Delauney and Verma, 1990
PDH	1.5.9.9.8	Alfalfa	Miller et al., 2005
		Tobacco	Ribarits et al., 2007

patatin promoter. Although the sense transformants produced more *SAMDC* transcripts and a higher Spd content, and the anti-sense constructs resulted in a decrease in *SAMDC* transcripts and activity; neither affected the tuber number.

Bhatnagar et al. (2001) used biolistic bombardment to transform poplar (*Populus nigra* x *maximowiczii*) cell suspensions with the same 35S::m*ODC* construct as the one used by DeScenzo and Minocha (1993) and Bastola and Minocha (1995), resulting in several-folds increased production of Put. They and others since then have found several metabolic changes in these cells associated with enhanced PA metabolism (see more details below). Wisniewski and Brewin (2000) used the coding sequence of a pea *DAO* in both sense and anti-sense orientations using tissue-specific promoter to transform peas. They concluded that DAO does not have an essential role in the initiation of root nodules and that products resulting from DAO activity on Put could adversely affect the nodulation process.

An oat *ADC* cDNA under the control of a CaMV 35S promoter was used to transform rice (Capell et al., 1998) in which Put content almost doubled in the leaves of transgenic plants, but a similar increase was not seen in the seeds. Later, the same Oat *ADC* cDNA in the anti-sense orientation was used in rice by the same group (Capell et al., 2000); they observed lowering of Put and Spd content, but not Spm. Bassie et al. (2000) and Noury et al. (2000) also reported the transformation of rice with an anti-sense oat *ADC* cDNA with the result that both ODC and ADC activities were down-regulated. Roy and Wu (2001) used the oat *ADC* cDNA under the control of an ABA-inducible promoter, again in rice, and found that the transgenic plants had greater Put and ADC

than the wild type plants; the second generation transgenic plants exhibited enhanced biomass production under conditions of salt stress.

In addition to using specific PA biosynthetic genes, several studies have used non-specific genes such as *Agrobacterium rhizogenes rol* genes; e.g. *rolA,* and *rolC* (Martin-Tanguy et al., 1990, 1993; Mengoli et al., 1992; Michael et al., 1990; Burtin et al., 1991; Altabella et al., 1995; Benhayyim et al., 1996), with interesting and sometimes contradictory effects on PA content and the phenotype. Table 4 represents some additional transgenic experiments in plants with genes encoding polyamine biosynthetic enzymes (updated from Kumar and Minocha, 1998).

Functions of polyamines

Polyamines in growth and development

The role of PAs in cell growth and development has been a matter of major interest over the years (Reviewed by Bais and Ravishankar, 2002; Kakkar and Sawhney, 2002; Kaur-Sawhney et al., 2003; Kusano et al., 2007). Generally, the PAs seem to promote regeneration and somatic embryogenesis (Robie and Minocha, 1989; Minocha et al., 1991, 1993, 1999, 2004B; Minocha and Minocha, 1995; Singh and Rajam, 1998; Martinez et al., 2000; Shoeb et al., 2001; Puga-Hermida et al., 2003; Sakhanokho et al., 2005). More recently, Geoffriau et al. (2006) observed that in onion, specific proportions of the PAs were required for successful gynogenesis, and also that in the in-vitro cultures, addition of Spd or Spm promoted embryogenesis. Chiancone et al. (2006) reported that Spd, but not Put stimulated gametic embryogenesis in *Citrus Clementine*. Vuosku et al. (2006) reported that in Scots pine, while PAs typically increased during the early stages of zygotic embryogenesis, they showed a decrease towards later stages.

Table 4. List of transgenic experiments in plants with genes encoding polyamine metabolic enzymes (updated from Kumar and Minocha, 1998).

Gene	Promoter-orientation	Plant	Reference
Yeast *ODC*	35S CaMV-sense	Tobacco	Hamill et al., 1990
Mouse *ODC*	35S CaMV-sense	Tobacco	DeScenzo and Minocha, 1993
Mouse *ODC*	35S CaMV-sense	Carrot	Bastola and Minocha, 1995
Mouse *ODC*	2X 35S CaMV-sense	Poplar	Bhatnagar et al., 2001
Human *SAMDC*	35S CaMV-sense	Tobacco	Noh and Minocha, 1994
Potato *SAMDC*	35S CaMV-sense	Potato	Kumar et al., 1996
Arabidopsis SAMDC	CaMV-sense CaMV- antisense	Arabidopsis	Hu et al., 2006
Carnation *SAMDC*	CaMV- sense	Tobacco	Wi et al., 2006
Oat *ADC*	Tet-inducible-sense	Tobacco	Masgrau et al., 1997
Oat *ADC*	Maize ubiquitin-sense	Rice	Noury et al., 2000
Oat *ADC*	CaMV- antisense	Rice	Capell et al., 1998
Oat *ADC*	ABA inducible-sense	Rice	Roy and Wu, 2001
Oat *ADC*	35S CaMV-sense	Tobacco	Burtin and Michael, 1997
Oat *ADC*	Maize ubiquitin	Wheat	Bassie et al., 2008
Oat *ADC*	35S CaMV-sense	*Cajanus cajan*	Sivamani et al., 2001
Toabcco *ADC*	CaMV- antisense	Tobacco	Chinatapakron and Hamill., 2007
Datura *ADC*	Maize ubiquitin- sense	Rice	Capell et al., 2004
Oat *ADC*	Tet-inducible- sense	Tobacco	Panicot et al., 2002
Arabidopsis *ADC*	CaMV- sense	Arabidopsis	Alcázar et al., 2005
Oat *ADC*	CaMV-antisense	Rice	Trung-Nghia et al., 2003
Bacterial *LDC*	35S CaMV-sense	Tobacco	Fecker et al., 1993
Bacterial *LDC*	35S CaMV-sense	Tobacco	Herminghaus et al., 1991, 1996
Pea *DAO*	35S CaMV-sense 35S CaMV-antisense	Pea	Wisniewski and Brown, 2000
Pea *DAO*	pENOD 12-inducible-sense	Rice	Bassie et al., 2000
Pea *DAO*	35S CaMV-antisense	Rice	Bassie et al., 2000

Kumar et al. (2007) found that application of PA inhibitors to the culture medium inhibited shoot bud formation in *Capsicum frutescens* cultures. Steiner et al. (2007) reported that the addition of exogenous PAs to culture medium devoid of plant growth regulators, promoted growth of embryogenic cultures of *Araucaria augustifolia.*

The role of PAs in flowering and fruit development has also been studied by several workers (Slocum and Flores, 1991; Rodriguez et al., 1999; Applewhite et al., 2000; Kaur-Sawheny et al., 2003; Liu and Moriguchi, 2007). Polyamines have been implicated in regulating cell cycle (Galston and Kaur-Sawheny, 1995; Kaur-Sawheny et al., 2003) in that dividing cells contain high PA contents, synthesized mostly by ODC, while those undergoing elongation and expansion show low PA content, synthesized by ADC. However, little ODC activity was detected in cell cultures of *Catharanthus roseus*, carrot, and poplar; all had ADC as the key enzyme involved in Put biosynthesis during cell division (Minocha et al., 1991, 1995, Bastola and Minocha, 1995, Bhatnagar et al., 2001). The role of PA metabolism in mediating the hormonal regulation of plant growth processes has been reviewed by Galston and Kaur-Sawheny (1995). It has been proposed that since Spd and ethylene share a common precursor, i.e. SAM, they may have antagonistic roles with respect to some physiological processes in plants (Kaur-Sawheny et al., 2003); e.g. PAs inhibit leaf senescence while ethylene promotes the same. This has lead to a suggestion that the metabolism of PAs may compete with the metabolism of ethylene; however, contradictory results have been reported depending upon the tissue and the plant species used (Chen et al., 1991; Liu et al., 2006).

Polyamines and stress

The roles of PAs in plant stress response has been widely studied and has been reviewed by Bouchereau et al. (1999), Alcázar et al. (2006) and Liu et al. (2007). Although conjugated PAs had been implicated to have a role in plant defense against pathogen attack (Martin-Tanguy, 1997; Walters, 2000), Walters (2003) suggested a role for free PA catabolism in generating hypersensitive response (HR) to plant pathogen attack. The catabolism of PAs generates H_2O_2, a major component of the group of molecules collectively known as Reactive Oxygen Species (ROS) or Active Oxygen Species (AOS) that are responsible for causing oxidative burst (Wojtaszek 1997; Apel and Hirtz, 2004), possibly a form of HR.

As early as 1952, Richards and Coleman reported that Put content of oat leaves increased in response to K deficiency. The expression of several genes involved in the PA biosynthetic pathway has been shown to change in *Arabidopsis* in response to salt (NaCl) treatment. Urano et al. (2003) and Zimmermann et al. (2004) reported that *ADC*1, *ADC*2, *SAMDC*1, *SAMDC*2, *SPDS*1, *SPDS*2 as well as *SPMS* were all induced in response to NaCl treatment. Increased salt tolerance has been achieved by increasing Put production by overexpression of a mouse *ODC* gene in rice (Kumria and Rajam, 2002). Yamaguchi et al. (2006) have investigated the protective role of Spm in salt stress of *Arabidopsis*. The importance of ADC route to PA biosynthesis under salt stress was reinforced by Liu et al. (2006) in apple callus. Simon-Sarkadi et al. (2007) reported that deletions of chromosome 5A, that houses several genes affecting abiotic stress in wheat, caused changes in PA content under salt stress. In rice, the overexpression of a

Tritordeum SAMDC, under the control of an ABA-inducible promoter, caused increased Spd and Spm accumulation and conferred salt tolerance (Roy and Wu, 2002).

Nayyar and Chander (2004) demonstrated that water stressed chick pea plants showed increased accumulation of Spd and Spm and concluded that PAs had a protective role against drought stress in this species. They also studied the role of PA accumulation under cold stress in chick pea and reported an enhancement in Put content in response to the same. Capell et al. (2004) generated transgenic rice transformed with a *Datura stramonium ADC* gene and observed increased production of Put and improved response to drought stress. They concluded that increase in Put content, consequently also increasing Spd and Spm content, protected the plants against drought stress. Yang et al. (2007) reported that in rice, water deprivation led to higher activities of ADC, SAMDC and SPDS in the leaves, leading to an enhancement in the contents of Put, Spd and Spm.

Kim et al. (2002) studied the role of ABA and PAs in cold-stressed leaves of tomato and observed that in both, the wild type and the ABA-deficient mutants, Put reduced electrolyte leakage induced by cold stress, while DFMO application increased this leakage. Pillai and Akiyama (2004) reported that in the cold tolerant varieties of rice, *SAMDC* transcripts increased for up to 72 h after stress treatment, while the susceptible variety showed no change. Spermidine content also increased in the cold resistant variety.

Lomozik et al. (2005) have reviewed the chemistry behind the formation of complexes between PAs and metal ions in solution. Depending on the nature of the amine (-NHx) group, interactions between the N atom (containing a lone pair of electrons) and metal ions are possible. Since metal toxicity is a widely studied area of plant physiology and PAs are considered important molecules modulating stress response in plants, the

relationship between metal toxicity and PA metabolism has been investigated by several groups. Recently, Majerus et al. (2007) reported that in the African rice *Oryza glaberrima*, that shows varying degrees of resistance to iron toxicity, PA contents were always higher in a tolerant cultivar, in all organs of the plant and during all time periods of treatment. The concentration of Pro and soluble sugars increased in the sensitive cultivar, while remaining unaffected in the resistant one. Elevated Mn concentrations have been reported to increase PA and amino acid accumulation in *Populus catahayana* (Lei et al., 2007). Increased PA concentrations were also reported in tobacco (Kuthanová, 2004) and sunflower (Groppa et al., 2007) due to Cd treatment.

Since, most types of stresses, whether induced by temperature, drought or chemicals, are responsible for ultimately affecting the oxidative stress machinery by inducing the generation of ROS; several groups have studied stress response of PA metabolism in the light of oxidative stress (Nayyar and Chander, 2004). Also, the catabolism of PAs generates H_2O_2 which is a part of the ROS system (Papadakis and Roubelakis-Angelakis, 2005). Despite this direct correlation between PA catabolism and oxidative stress, a role of PAs in ameliorating the harmful effects of ROS has been suggested. PAs can do so either by direct interaction with the ROS (Løvaas, 1997) or indirectly; e.g. by inhibiting the generation of NADPH-oxidase meditated ROS in membranes (Papadakis and Roubelakis-Angelakis, 2005). This area of research has been followed both in plants as well as in animals (Papadakis and Roubelakis-Angelakis, 2005 and references therein)

Polyamines as indicators of stress in trees

The role of foliar PA concentration as early indicators of abiotic stress in trees has been proposed by several authors (Dohmen et al., 1990; Hauschild, 1993; Minocha et al.,

1997; Sanchez et al., 2005), and has been investigated in detail by R. Minocha et al. Earlier, Minocha et al. (1992, 1996) observed an inverse relationship between Al addition and Put content in suspension cultures of periwinkle (*Catharanthus roseus*) and red spruce (*Picea rubens*). A similar trend was seen later in mature trees of several species subjected to increased Al exposure due to acid precipitation (Minocha et al., 1997, 2000). Based on extensive analyses at several sites affected by a variety of abiotic stressors, this group has proposed that foliar PA (particularly Put) content can be used as an early indicator of abiotic stress in trees even before the appearance of visible symptoms of stress injury or loss of growth and productivity. For example, in response to acidic soil conditions (due to acid precipitation and Al solubilization), several-fold increase in the accumulation of foliar PAs (PCA-soluble) and inorganic ions in red spruce trees was observed; there was also an inverse relationship with Ca accumulation. A higher Ca:Al ratio in the soil resulted in lower Put and Spd content in the needles, as opposed to the contents of the same in trees growing on soil with lower Ca (Minocha et al., 1997). Minocha et al. (2000) also studied the relationship between free PA, inorganic ions and chronic N deposition in the soil, in pine and hardwood trees. They observed that chronic addition of N to the soil increased foliar Put content. In the hardwood trees, a strong negative correlation was again seen between foliar PAs and total Ca, Mg and Mn in the soil. Negative correlations were also seen among foliar PA s and exchangeable K and P in the soil. The amelioration of stress (e.g. addition of Ca in Ca-depleted soils) reversed the profile of both the Put content as well as cellular Ca in sugar maple (Wargo et al., 2002).

Current status of polyamine research in our laboratory

For over 20 years, research in our laboratory has focused on understanding the intricacies of the PA metabolic pathway and its physiological significance in plants. A variety of plant materials (carrot, tobacco, Arabidopsis, and poplar) and experimental approaches (use of inhibitors, enzyme activities, cloning of genes, analysis of gene expression, and genetic manipulation) have been used to understand the regulation of their metabolism and delineate their roles in embryogenesis and stress. Following earlier work with carrot (Robie and Minocha, 1989; Minocha et al., 1991; Bastola and Minocha, 1995; Andersen et al., 1998) and tobacco (DeScenzo and Minocha, 1993; Noh and Minocha, 1994), recent efforts have concentrated on the use of cell cultures of poplar (*Populus nigra x maximowiczii*) transformed with a truncated version of mouse *ODC* (Bhatnagar et al., 2001, 2002; Quan et al., 2002; Page et al., 2007). The activities of key biosynthetic and catabolic enzymes have been studied in response to transgenic manipulation of a specific step in the PA metabolic pathway, i.e. up-regulation of Put biosynthesis. A summary of important findings with the transgenic poplar cells, which have been used in our research presented here, is given below:

- Transgenic expression of m*ODC* causes several-fold increase in Put production and accumulation, without negative effects on the native ADC activity and its contribution to Put production
- The rate of Put catabolism is enhanced proportionate to its biosynthesis
- The source of Orn production in these cells is primarily from Glu/Gln and not from Arg

- Ornithine biosynthesis becomes limiting for Put biosynthesis in the Put-overproducing cells
- The production of ethylene in these cells is not affected by increased PA metabolism

One of the major applications of genetic manipulation in plants is as a tool to reveal information about the regulation of metabolism so that strategies can be developed to achieve optimal levels of desired cellular metabolites. This not only requires identification of rate-limiting steps in a pathway, but also understanding of how the pathway is regulated, particularly whether regulation occurs at the transcriptional, translational, post-translational or metabolite (substrates, cofactors, etc.) levels. The transgenic approach can help us reveal mechanisms of metabolic regulation that may not be seen through mutant analyses and/or inhibitor studies alone. Some examples of novel information that has come from the transgenic approach include the complex regulation of lysine (Lys), threonine (Thr) and methionine (Met) biosynthetic pathways (Galili, 1995; Tzchori et al., 1996; Zhu and Galili, 2004; Lee et al., 2005), the complexity of glycolysis regulation by phosphofructokinase (Thomas et al., 1997), and the regulation of secondary metabolism in plants (Dixon, 2005).

Objectives of the present study

We hypothesized that:

a) *Put overproduction affects the metabolism of Pro, Arg and GABA, metabolites which are produced from intermediates of the PA metabolic pathway, and*

b) *Enhanced PA metabolism has pleiotropic effects on the physiology of cells in terms of stress response, ionic balance and gene expression.*

The specific objectives of my research were to investigate the effects of enhanced metabolism of Put in transgenic poplar cells on:

- the activities of enzymes (and their transcripts) involved in PA biosynthesis;
- the metabolism of amino acids like proline, arginine, ornithine, and GABA (for which Glu is a precursor) as well as other amino acids;
- the oxidative state of cells;
- the response to Al and its interaction with Ca in the culture medium; and
- the assimilation of carbon into polyamines

Suspension cultures vs. intact tissues

Non-regenerating, suspension cultures of a cell line of hybrid poplar (*Populus nigra x maximowiczii*) were used in this study in contrast to the embryogenic cultures of carrot or intact plants of tobacco used in our lab in the past. Cell suspensions are excellent model systems to study cellular metabolism, particularly if they are comprised of non-nodular clumps, which is the case with the cultures used here (Fig. 3). Not only are they easy to handle, they can be maintained under defined growth conditions year round. One of the major hindrances to working with biochemical pathways in intact plants is the complexity encountered with isotope feeding experiments due to heterogeneity of tissue types and the problem of uptake and distant transport. Generally cell suspension cultures allow better distribution of the label than in an intact plant organ, analogous to the microbial and animal cell culture systems that have been extensively used for numerous metabolic studies. Also, the growth conditions, including the growth medium and the physical environment, can be better controlled for cell cultures than for intact plants.

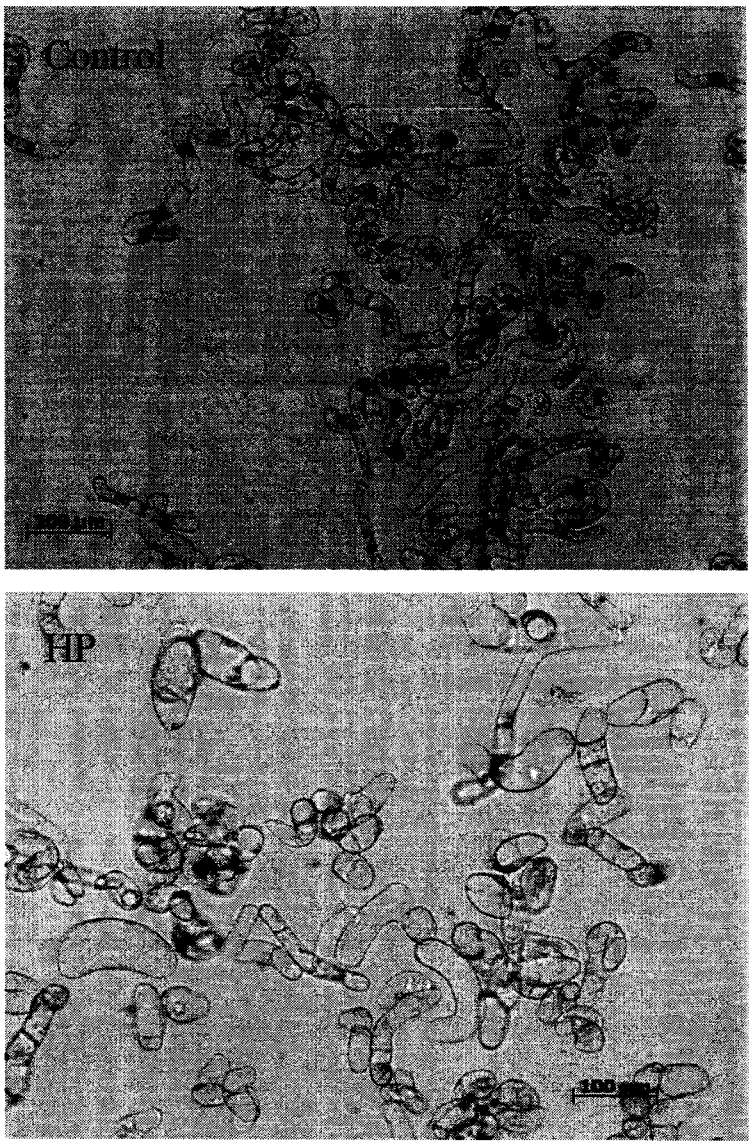

Figure 3. Control and HP cells as seen under light microscope.

GENERAL MATERIALS AND METHODS

Cell culture and harvest

The high putrescine cell line (called HP for "High Put") and the control cell lines of *Populus nigra* × *maximowiczii* used in this study have been previously described (Bhatnagar et al. 2001, 2002; Page et al. 2007). The former (previously called 2E – Bhatnagar et al. 2001, 2002, Quan et al., 2002) expresses a mouse *ornithine decarboxylase* (m*ODC*) gene. The control cells have either been non-transgenic or a line that expresses the *β-glucuronidase* (*GUS*) gene; both transgenic cell lines also express *neomycin phosphotransferase* (*NPT*II) selectable marker gene. All three transgenes are controlled by variations of the 35S CaMV promoter. By using a transgenic control cell line in my study, the effect of kanamycin in the culture medium during maintenance of stock cultures gets neutralized.

All cell cultures were maintained in MS medium (Murashige and Skoog, 1962; Sigma-Aldrich, St. Louis, MO, Cat # M-5524), containing vitamins of B5 medium (Gamborg et al. 1968; Sigma, G1019), 2% sucrose, 0.5 mg L^{-1} 2, 4-dichlorophenoxy-acetic acid (2, 4-D; Sigma, D7299) and 100 mg L^{-1} kanamycin, except the non-transgenic cells (Genlantis, San Diego, CA, Cat # M150025). Liquid suspensions were subcultured weekly by adding 7 mL of 7-d-old suspension to 50 mL of fresh medium in 125 mL Erlenmeyer flasks. Kanamycin was added after cooling the medium to about 55 C; the antibiotic was not added for at least two weeks before experimentation. Stock cultures were maintained on solid medium with kanamycin with a 4 week culture cycle. All cultures were kept at 25±2 °C under a 12 h photoperiod (80±10. $\mu em^2.s^{-1}$); the liquid

cultures were kept on a gyratory shaker at 150 rpm. Cells were harvested by vacuum filtration through Miracloth (Calbiochem, La Jolla, CA, Cat # 475855).

Free polyamine analysis

Following vacuum filtration, 200 ± 20 mg (FW) of cells were mixed with 4× volume (800 ± 80 μL of 5% (v/v) perchloric acid (PCA) and frozen (at –20 °C) and thawed (at room temperature) three times (Minocha et al., 1994) before dansylation and quantification of PAs by HPLC (Minocha et al., 1990; Bhatnagar et al., 2001; Page et al., 2007). Bhatnagar et al (2002) demonstrated that the freeze-thaw method enabled the extraction of the PCA soluble PAs, almost entirely. The thawed samples were vortexed until they were well mixed and centrifuged for 10-15 min (13,000 xg). Supernatant (100 μL) was used for dansylation. Parallel to the samples, 100 μL of a mixture of 3 PAs and the internal standard heptanediamine was dansylated to be used as standards. The PAs used as standards were Put-diHCl (Sigma, D1-320-8), Spd-triHCl (Sigma, S2501) and Spm-tetraHCl (Sigma, S2876). The concentrations of the individual PAs in the mixture ranged from 0.002 mM Put, 0.001 mM Spd or Spm to 0.04 mM Put, 0.02 mM Spd or Spm. Twenty μL of 0.1 mM heptanediamine (Sigma, D3266), used as an internal standard was added to both the standards and the samples. This was followed by the addition of 100 μL of saturated Na_2CO_3 solution, and 100 μL of 20 mg mL^{-1} (in acetone) dansyl chloride (Fluka, Milwaukee, WI, Cat # 39220). Following incubation for 1 h at 60 °C, 50 μL of 100 mg mL^{-1} L-alanine (Sigma, A7469) or 20 mg mL^{-1} L-asparagine (Sigma, A0884), made in distilled water, was added and the samples were vortexed briefly. After additional 30 min incubation at 60 °C, acetone was evaporated under vacuum (5 min) using a Speed-Vac (Savant Instruments Inc., Farmingdale, NY). Dansyl-

PAs were recovered in 400 μL of toluene (Photrex grade; J.T. Baker, Phillipsburg, Cat # 9456-03). Following toluene addition, the standards and the samples were vortexed and allowed to stand undisturbed for 5 min to facilitate the separation of the organic and the aqueous phases. Following centrifugation at 13000 xg for 1 min, 200 μL of the organic (toluene) phase was transferred to a new microfuge tube and dried under vacuum. The pellet was reconstituted in 1 mL methanol (Fisher Scientific, Fair Lawn, NJ, Lot # 970153) by vortexing for 2 min, followed by a 2 min centrifugation. The methanol extracts (500 μL) were transferred to autosampler vials and analyzed for free PAs by HPLC using a gradient of acetonitrile (40-100%; Burdick and Jackson, Muskegon, MI, Cat # AH015-4 or EMD Chemicals, Gibbstown, NJ, Cat # AX0145-1) and 10 mM heptanesulfonic acid (Fisher, O-3013), pH 3.4 on a reversed phase C18 column (4.6 x 33 mm, 3 μm) using the Perkin-Elmer (PE) HPLC system (Minocha et al., 1990; Bhatnagar et al., 2001). The system included a PE series 200 autosampler fitted with a 200 μL loop (sample volume was 10 μL), a PE series 200 gradient pump at a flow rate of 2.5 mL min^{-1}. For detection and quantitation, a series 200 fluorescence detector (Perkin-Elmer) with excitation and emission wavelengths set at 340 and 515 nm, respectively, was used The PE TotalChrom software (Version 328 6.2.1) was used to integrate data. A multiplication factor was incorporated into the software and data were calculated either as nmol mL^{-1} PCA or nmol g^{-1} FW.

Amino acid analysis

The aqueous fraction, obtained after partitioning the samples with toluene (described above) was used for free amino acid analysis by HPLC (Minocha and Long, 2004B). To 730 μL methanol, 135 μL of the aqueous fraction was added along with 135 μL of 2.9 M

acetic acid. After leaving the tubes open for 10-12 min to remove excess CO_2, the solution was filtered through a 0.45 μ nylon syringe filter (Pall-Gelman Labs., Ann Arbor, MI). Quantitation was done against an external standard curve made by using a mix of 23 amino acids standard (21 L-amino acids + glycine- Fluka, 9416; GABA - Sigma, A5835; Orn- Sigma, O2375). Dansylation of standards was done similar to the samples. A Hydro-RP, 4 μm, 100 mm x 4.6 mm i.d. column (Phenomenex, Torrance, CA) in a column heater (Bio-Rad labs, Hercules, CA) set at 40 °C was used for separation of amino acids. A C_{18} security guard column (5 μm, 4 mm x 3 mm i.d.; Phenomenex) and a C_{18} Scavenger column (10 μm, 33 mm x 4.6 mm i.d. cartridge; Perkin-Elmer) were also used. Amino acid analyses were done by Stephanie Long in Dr. Rakesh Minocha's lab at the USDA Forest Service, NRS, Durham, NH.

Determination of reduced glutathione (GSH), the phytochelatin PC_2 and γ-glutamylcysteine (γ-EC)

About 100 mg of cells were collected in 500 μL of 6.3 mM diethyltriamine pentaacetic acid (DTPA; Fluka, 3238) containing 0.1% trifluoroacetic acid (TFA; Sigma, 30248-1), derivatized and analyzed for GSH, PC_2 and their common precursor γ-EC by HPLC according to the method of Thangavel et al. (2007). After freezing and thawing the samples three times, the supernatant was collected by centrifugation at 13,000x g for 10 min. To a mixture of 615 μL of 200 mM 4-(2, hydroxyethyl)-piperazine-1-propane sulfonic acid (Sigma, E-1894) buffer containing 6.3 mM DTPA, pH 8.2 and 25 μL of 20 mM tris (2-carboxyethyl) phosphine hydrochloride (Sigma, C-4706), 250 μL mix of standards [γ-glutamyl-Cys (Sigma, G-0903) + phytochelatins (PC_2, PC_3, PC_4 and PC_5; custom ordered from Anaspec, San Jose, CA) + GSH (Sigma, G-6529)] or sample

extract was added. Following incubation of the reaction mix for 10 min at 45 °C, 10 µL of 0.5 mM N-acetyl-L-Cys (Sigma, A-8199) was added which acted as the internal standard. Derivatization was done by the addition of 10 µL of 50 mM monobromobimane (Molecular Probes, Eugene, OR; M-20381) and subsequent incubation at 45°C in the dark, for 30 min; the reaction was terminated by adding 100 µL 1 M methanesulfonic acid (Fluka, 64280). Following filtration through a 0.45 µm nylon syringe filter (Pall-Gelman Labs, New York, NY); the samples were separated by HPLC as described above. The excitation and emission wavelengths were 380 and 470 nm, respectively. The PE TotalChrom software (PerkinElmer) was used to integrate data. Glutathione and phytochelatins analysis were done by Dr. Thangavel Palaniswamy in Dr. Rakesh Minocha's lab at the USDA Forest Service, NRS, Durham, NH.

Ion analysis

The method of Minocha and Long (2004A) was used to quantify cellular contents of inorganic ions; the analyses were done by Stephanie Long in Dr. Rakesh Minocha's lab at the USDA Forest Service, NRS, Durham, NH. The cell extracts (100 µL) in PCA (described under "free polyamine analysis") were diluted 50x with 4.9 mL H_2O and analyzed for inorganic ions using a Varian Vista charged couple device (CCD) simultaneous Inductively Coupled Plasma-axial emission spectrometer (ICP-AES; Varian Inc., Melbourne, Australia). The ICP software (version 4.0; Varian Inc.) was used for data integration. Quantitation was done against an external standard curve made by using a mix of 8 elements (Al- ICP-013-5, Ca- ICP-020-5, K- ICP-019-5, Mg- ICP-012-5, Mn-ICP-025-5, Ultra Scientific, North Kingstown, RI; Fe- PLFE2-2X, P- PLP9-2X, Spex Certiprep, Metuchen, NJ; Zn- 140-051-301, SCP Science, Baie D'Urfé, Quebec, Canada).

Measurement of mitochondrial activity and membrane integrity

The method of Ikewaga et al. (1998) as modified by Minocha et al. (2001) was used to measure mitochondrial activity and membrane integrity in these cells. For measurement of mitochondrial activity, about 100 mg (FW) cells were placed in 1 mL MS medium containing 250 µg MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma, M2128]. Following gentle mixing at room temperature for an hour, the cells were collected by centrifugation at 16,000 xg (10 min); the supernatant was discarded. Following resuspension in 1 mL of 0.04 M acid propanol (0.04 M HCL in isopropanol), the cells were centrifuged at 16,000 xg for 5 min and the supernatant analyzed for absorbance at 590 nm (Hitachi U-2000 spectrophotometer, Hitachi Instruments, San Jose, CA).

For measurement of membrane integrity, about 100 mg of cells were incubated for 15 min in 1 mL of 0.05 % (w/v) Evans blue dye (Sigma, E2129), centrifuged at 16,000 xg for 15 min and the supernatant discarded. The pellet was washed (5x – 6x) with distilled water by centrifugation (16,000 xg) until the supernatant was clear. Cells were then resuspended in 1 mL 1% (w/v) sodium dodecyl sulfate (SDS; Sigma L4390), frozen and thawed once, centrifuged at 16,000 xg and the absorbance of supernatant measured at 600 nm (Hitachi U-2000 spectrophotometer).

Measurement of total protein content

Soluble protein content was measured by the method of Bradford (1976). Cells (100 mg) were collected in either 200 or 250 µL Tris-EDTA (0.1 M Tris-HCl; J.T. Baker, Cat # 4103-02 + 0.1 mM ethylenediamine tetraacetic acid) buffer, pH 6.8 or K-Pi buffer (0.1 M KH_2PO_4 + 0.1 M K_2HPO_4), pH 7.0. Cells were frozen and thawed once, vortexed, and

centrifuged at 16,000 xg for 5 min. The supernatant (25 μL) was diluted 2x (with either distilled water or buffer) and incubated with 1.5 mL Bio-Rad protein assay dye (Bio-Rad Laboratories, Hercules, CA, Cat # 500-0006) or Sigma Bradford reagent (Sigma, B6916), for 15 min. If the Bio-Rad dye was used, it was diluted 5x with distilled water prior to incubation with cell extract. Absorbance of the resulting solution was measured at 595 nm in a "Spectronic® 20 Genesys™" spectrophotometer (Spectronic Instruments Inc., Rochester, NY). Known concentrations of bovine serum albumin (BSA; Sigma, A4503) dissolved in buffer or distilled water, were used as standards.

Genomic DNA isolation and quantification

Genomic DNA was isolated using a modified protocol from Murray and Thompson (1980). Approximately 100 mg of cells were ground in liquid nitrogen and incubated with 500 μl of pre-heated (60 °C) CTAB buffer [2% (w/v) Hexadecyltrimethyl-ammonium bromide (Sigma, H6269), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, and 0.2% (w/v) β-mercaptoethanol (J.T. Baker, Lot # 208358) added just before use] in 1.5mL microfuge tubes. The ground tissue was incubated at 60° C for 30 min with gentle agitation. An equal volume of chloroform (Sigma, C2432) : isoamyl alcohol (Fisher, A 393-500) (24:1) was added, the samples mixed by inversion, and centrifuged at 14,000 xg for 5 min. The upper aqueous layer was removed and mixed with an equal volume of cold isopropanol. The tubes were incubated at -20° C for 15-30 min. The precipitated DNA was pelleted by centrifugation at 14,000 xg at 4° C for 15 min. The pellet was washed with 70% ethanol/10 mM ammonium acetate (Sigma, A1542) and re-centrifuged for 5 min at 14,000 xg. The pellet was dried in a Speed-Vac and resuspended in 20-50 μl of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) or DNAse-free water. The

quality of DNA was checked by A_{260}/A_{280} ratio ($\geq$ 2), and DNA quantified spectrophotometrically using the following formula:

Concentration of DNA= $O.D._{260\ nm}$ x 50 ng/µL x dilution factor, where 50 µg/mL is the concentration of DNA where $O.D._{260\ nm}$= 1 and the dilution factor is 200x.

Polymerase Chain Reaction (PCR)

In order to confirm the presence of the m*ODC* and *GUS* genes in the HP and control cell lines, respectively, and the *NPT*II gene in both, PCR was performed using either Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Piscataway, NJ, Lot # 6630), or Quick Load Taq Polymerase Master Mix (New England Biolabs, Ipswich, MA, Cat # M0271L). A typical reaction was performed in a total volume of 25 µl using 300-500 ng genomic DNA as template. Primers were added at 10 pmol per reaction. Buffer and dNTP's were a part of the PCR beads and Master Mix. Reactions were run in a PTC 100 Programmable Thermocycler (MJ Research, Waltham, MA) with a heated lid. The PCR primers used were 5'ATG GGC AGC TTT ACT AAG GAC3' (forward) and 5'CAT GGC TCT GGA TCT GTT TCA3' (reverse) for m*ODC*; 5'TAT GCG GGC AAC GTC TGG TAT CA3'(forward) and 5'ACG CTT GGG TGG TTT TTG TCA3'(reverse) for *GUS*; and 5'GAG GCT ATT CGG CTA TGA CT3' (forward) and 5'TCG GGA GCG GCG ATA CCG TA3'(reverse) for *NPT*II. The samples were subjected to initial denaturation at 93 °C for 1 min and another denaturation step at 94 °C for 30 sec; the annealing temperature was 55 °C, followed by an extension temperature of 72 °C for 1 min. The reaction was repeated for 35-40 cycles, followed by extension at 72 °C for 5 min and hold at 4 °C. The PCR products were separated on 1% Seakem LE agarose (Cambrex Bio Science, Rockland, ME, Cat # 50000), in TAE (40 mM Tris-acetate, 1 mM

EDTA) buffer. Samples were mixed with 6x loading dye; TriDye 2-Log DNA ladder (NEB, N3270S) was used as marker. Electrophoresis was run at 5 V/cm for approximately 1 h. Gels were stained with 0.5 μg/ml ethidium bromide for 10 min followed by destaining in distilled water for 10 min. Gels were visualized on a UV trans-illuminator and digitally photographed using Nucleotech Gel Expert (version 3.5) software and the Nucleotech gel-documentation system (Nucleotech, San Mateo, CA). For each gene, a negative and a positive control reaction was set up parallel to the samples.

Statistical analysis

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

CHAPTER I

PUTRESCINE OVERPRODUCTION AND ACTIVITIES OF THE POLYAMINE BIOSYNTHETIC ENZYMES

In this chapter, data are presented on the comparison of cellular contents of PAs, activities of key PA biosynthetic enzymes, and changes in the transcripts of these enzymes over the 7 day culture period. This study was conducted in collaboration with Dr. Andrew Page, and the results have been published as Page et al. (2007). Whereas Dr. Page did most of the molecular analysis, I did all the enzyme assays and maintained cell cultures for this part.

Introduction

In spite of numerous publications on the importance of PAs in plant growth, development and stress responses, only limited experimental evidence for metabolic regulation of PA biosynthesis has been forthcoming. The major enzymes involved in PA biosynthesis in plants are ADC, ODC, SAMDC, SPDS and SPMS (Fig. 2). For decades, the most common approach to modulate cellular PAs was the use of inhibitors of these enzymes, a strategy hindered by severe limitations (e.g. differential rates of uptake, metabolic conversions, deleterious side effects on membrane characteristics, and the lack of specificity) for correct interpretation of results (McCann et al., 1987; Robie and Minocha, 1989; Nissen and Minocha 1993). Since the cloning of genes for the key enzymes in PA metabolism, the genetic manipulation of specific enzymes has become feasible (Kumar and Minocha, 1998; Bhatnagar et al., 2001, 2002; Roy and Wu, 2002;

Capell et al., 2004; Franceschetti et al., 2001; Kasukabe et al. 2004; Minocha et al. 2004A; Alcázar et al. 2005., Imai et al., 2006; Kusano et al., 2007; Bassie et al., 2008). The use of genetic manipulation alleviates problems associated with the use of inhibitors and also allows the up-regulation of specific steps in a pathway, which is generally not feasible with inhibitors.

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

The main hypothesis for the work presented in this chapter is that transgenic manipulation of a specific step in the PA biosynthetic pathway will cause concomitant changes in expression of the native genes encoding enzymes that regulate that step and

also the other reactions downstream of the manipulated step. As mentioned under "General Introduction", the PA metabolic pathway is a branched pathway which interacts with a limited number of adjacent pathways (Fig. 2), all of which are important in plants, rendering the study of these interactions important. Examining the effects of modulating a single step in PA metabolism on other branches of the pathway will help us in developing functional models for the regulation of PA metabolism and the metabolism of related compounds; e.g. Pro, Arg, γ-aminobutyric acid (GABA), and ethylene (Fig. 2). This will also aid in achieving desirable manipulation of these compounds using the transgenic approach. Manipulation of PA metabolism in plants will potentially have far-reaching implications, including some in the field of oncology, where foods with reduced PA content are deemed desirable to retard tumor growth (Quemener et al., 1994), particularly in combination with a strategy of chemotherapeutic use of PA inhibitors in cancer patients (Catros-Quemener et al., 1999; Stoneham et al., 2000; Milovic, 2001; Kalac and Krausova, 2005).

The use of Quantitative Reverse-Transcriptase PCR (QRT-PCR) has enabled us to examine the expression of individual paralogues of genes and assess their relative contributions to PA metabolism in poplar cells. The gene expression data for this part of the study were generated by Dr. Andrew Page, using the same cells that were collected for enzyme activities and polyamine measurements.

Materials and Methods

ODC, ADC and SAMDC enzyme assays

The activities of ODC, ADC and SAMDC were measured daily during the 7 d culture cycle using slight modification of the method of Minocha et al. (1999). Cells collected

by vacuum filtration (100 ± 5 mg FW for ODC and ADC, and 200 ± 5 mg FW for SAMDC) were placed in assay buffer in 16×100 mm glass test tubes [for mODC: 250 μL 0.1 M Tris, 0.1 mM EDTA; pH 6.8, 0.5 mM pyridoxal phosphate (Sigma, P3657), 1.0 mM DTT (Sigma, D9779); for poplar ODC and ADC: the same as for mODC but at pH 8.4; for SAMDC: 350 μL 0.1 mM potassium phosphate buffer, pH 7.5, 3.0 mM putrescine-diHCl (Sigma, D1-320-8), 1.0 mM DTT(Sigma, D9779)] and frozen for 2-4 h. After thawing, 50 μL of the appropriate labeled substrate [for ODC: 0.05 μCi of [1-^{14}C]Orn, specific activity 58 mCi mmol^{-1} (Moravek Biochemicals, Brea, CA; Cat # 142-173-953) plus 12 mM unlabeled L-Orn (Sigma, O2375); for ADC: 0.1 μCi of [1-^{14}C]Arg, specific activity 57 mCi mmol^{-1} (Amersham Life Sciences, Elk Grove, IL; Cat # CFA.434) plus 12 mM unlabeled L-Arg (Sigma, A5006); for SAMDC: 0.1 μCi of [1-^{14}C]SAM, specific activity 58 mCi mmol^{-1} (Moravek, Cat # 127-279-056) plus 4.0 mM unlabeled SAM (Sigma- A2408] was added to each tube, and a 2 cm^2 Whatman 3 MM filter paper soaked with 50 μL Scintigest (Fisher Scientific, Lot # 872729) was placed in a polypropylene well (Kontes, Vineland, NJ) suspended from a rubber stopper. The tubes were incubated in a water bath (37 °C) for 60 min. Reactions were terminated by injecting 1.0 mL of 0.5 N H_2SO_4 into each tube through the rubber stopper. Following additional incubation of 30 min, the filter papers were removed and counted for radioactivity in 10 mL Scintilene (Fisher Scientific, Lot # 980805) in a LSC-6000 liquid scintillation counter (Beckman, Fullerton, CA).

Enzyme activity was calculated as nmol $CO_2.h^{-1}.g^{-1}$ FW of cells as well as nmol $CO_2.h^{-1}.mg^{-1}$ soluble protein. The cellular contents of PAs and total proteins were

analyzed as mentioned under "General Materials and Methods". Details of quantification of gene expression are presented in Page et al. (2007).

Results

Confirmation of stable transformation

In order to confirm the presence of the m*ODC* gene in the HP cells, the *GUS* gene in the control cells and the *NPT*II gene in both, PCR amplifications were done. The expected fragment sizes of 0.8 Kb for m*ODC*, 1 Kb for *GUS* and 0.7 Kb for *NPT*II were observed, confirming the presence of the respective genes in these transgenic cell lines (Fig. 4, 5).

Cellular contents of polyamines over the 7 d culture cycle

The cellular contents of PAs (Fig. 6) were analyzed in the same batch of cells as the protein contents (Fig. 7) and the enzyme activities (Fig. 8-10) on a given day. Since the protein content varied over the 7 d culture period, the cellular contents of the three major PAs were also calculated both as nmol.g^{-1} FW and nmol.mg^{-1} protein. In either case, Put content of the HP cells was several-fold higher than the control cells on any given day of analysis (Fig. 6A, B); on some days the differences were 8 to 9 fold on g^{-1} FW basis. The trend in changes with time was different in the two cell lines. On FW basis, the control cells showed a small increase in Put around days 5 and 6; for HP cells, the peak of Put content was seen around days 2 to 4. Since the protein content of the latter was higher at days 2 to 4, Put content mg^{-1} protein was actually the lowest on these days and highest on days 1 and 7. Spermidine (g^{-1} FW) increased significantly in the HP cells between days 1 and 2, rising to a peak on day 3, and then dropping to the lowest amount on day 7 (Fig.6C). In control cells, on the other hand, the increase was much smaller and occurred

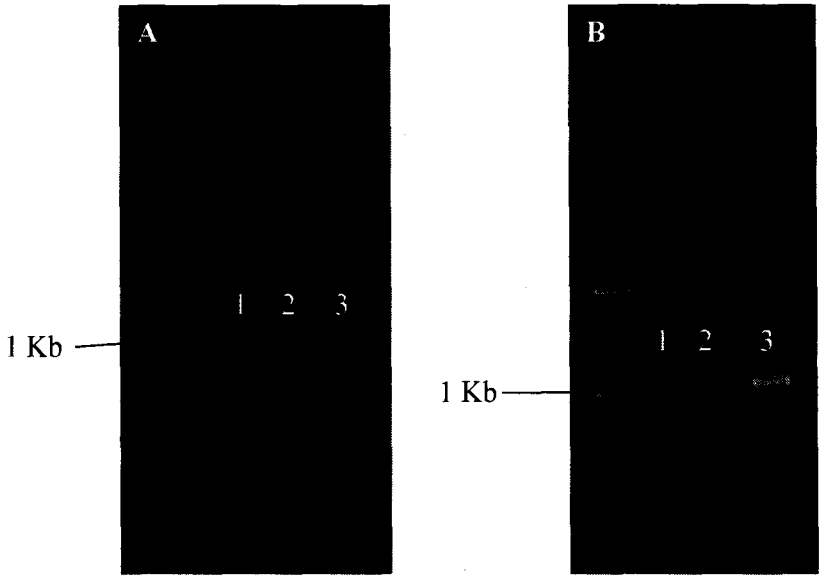

Figure 4. PCR amplification of (A) a 0.8 Kb m*ODC* fragment in HP cells and (B) a 1 Kb *GUS* fragment in control cells. In both gel pictures, lane 1 represents the respective cell line, lane 2 represents a water control and lane 3, a plasmid (positive) control. The NEB 2-Log DNA ladder was used as the marker.

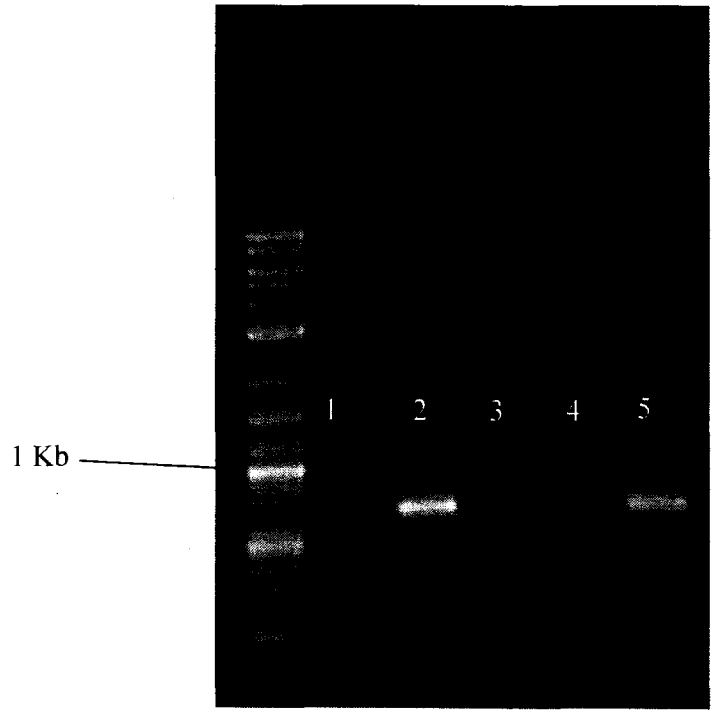

Figure 5. PCR amplified 0.7 Kb fragments of *NPTII* in HP and control cells. Lanes 1 and 4 represent replicates of the HP cell line, lane 5, the control cell line, lane 2, a plasmid control and lane 3, a water control. The NEB 2-Log DNA ladder was used as the marker.

only around days 3 to 4.

The Spd content in HP cells was higher around 2 to 3 d and somewhat lower on days 5 and 6. Only a small increase in Spd was seen in either cell line within 1 d of transfer to fresh medium. When the data were normalized to protein content, both lines showed a small increase in Spd within a day of transfer to fresh medium followed by a significant decline in its content by day 2 (Fig. 6D). Thereafter, only small increase in Spd content mg^{-1} protein was seen in the two cell lines over the next 4-5 d. Overall, Spd content (g^{-1} FW as well as mg^{-1} protein) was higher on days 4, 5 and 6 in the control cells (Fig. 6C, D). Significant differences in the Spm contents (g^{-1} FW) between control and HP cell lines were seen on days 4, 5 and 6 of the week (Fig. 6E) and on mg^{-1} protein basis, significant differences were seen between the two cell lines on days 2-6 (Fig. 6F), where the Spm content was lower in the HP cells than the control cells.

Cellular contents of soluble proteins over the 7 d culture cycle

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

Activities of ODC, ADC and SAMDC over the 7 d culture cycle

The poplar and mouse ODC activities were distinguished from each other by using extraction and assay buffers of appropriate pH for each; i.e. 6.8 for mODC in HP and 8.2 for pODC in control cells (DeScenzo and Minocha 1993).

As reported earlier (Bhatnagar et al. 2001), ODC activity (g^{-1} FW) was rather low in the control cells as compared with the m*ODC*-transgenic HP cells (Fig. 8A); nevertheless, it varied somewhat over the 7 d culture period (Fig. 8A) with an increase being seen during the first 3 d on transfer of cells to fresh medium and a decline after that. The specific activity ($CO_2.mg^{-1}$ protein) of pODC remained rather constant over the 7 d culture period (Fig. 8B), due to changes in protein content of the cells. The HP cells, where the total ODC activity was almost 25-fold higher than the pODC activity in the control cells on d 1, exhibited a similar trend for change of enzyme activity over the 7 d culture period; the peak of activity being seen on d 4 (Fig. 8A). The specific activity of ODC in HP cells, however, exhibited a different trend in that there was a significant decrease between d 1 and 2, after which there was no change, when it again increased significantly after d 6 (Fig. 8B). The pODC activity in HP cells could not be measured accurately because mODC activity still persists at pH 6.8, albeit at low levels (DeScenzo and Minocha, 1993); therefore the data on pODC in these cells are not presented. The activity of ADC (g^{-1} FW) was significantly higher in the HP cells than the control cells on any day of analysis (Fig. 9A). Whereas the HP cells showed a peak of ADC activity around d 2 to 4 and a decline thereafter, changes in ADC activity in the control cells were small and statistically insignificant over the 7 d culture period. On transfer to fresh medium on the 7^{th} day, an increase in activity was seen only in the HP cells.

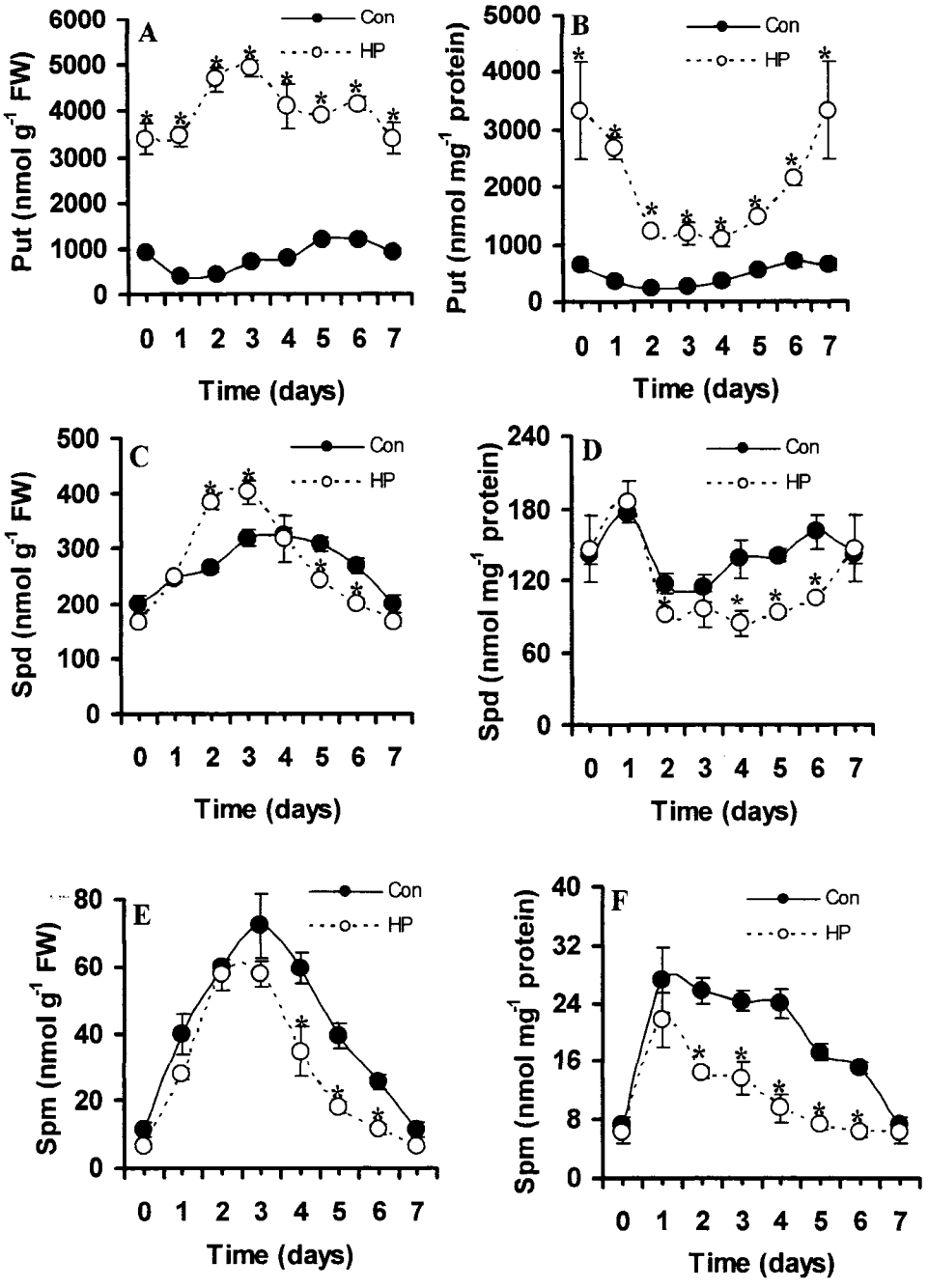

Figure 6. Cellular contents of (A, B) Put, (C, D) Spd and (E, F) Spm on (A, C, E) g^{-1} FW and (B, D, F) mg^{-1} protein basis, in control and HP cells over the 7 d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) between the cellular PA content between the control and HP cells on a given day of the 7 d culture cycle.

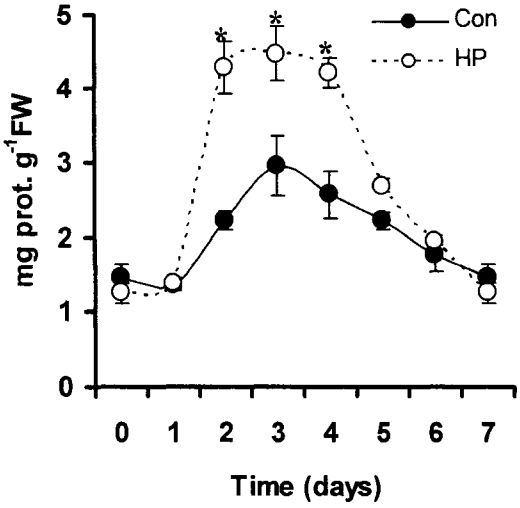

Figure 7. Cellular contents of total proteins in the control and HP cells over the 7 d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments An * indicates a significant difference ($P \leq 0.05$) in the cellular protein content between the control and HP cells on a given day of the 7 d culture cycle.

When ADC specific activity was compared, the differences between the two cell lines over time were smaller, and also, the peak of ADC activity observed in the HP cells between d 2 and 4 was not apparent (Fig. 9B). In fact, highest ADC activity in these cells was seen on the 7^{th} and the 1^{st} d of culture, a situation similar to that for mODC activity.

The activity of SAMDC (g^{-1} FW) was significantly lower in the HP cells than the control cells on all but the first 3 d of culture (Fig. 10A); both cell lines showed a decrease in enzyme activity over the course of the experiment after 2 d of culture in HP and after 4 d in the control cells. On transfer of cells to fresh medium, a small but significant increase in SAMDC activity was seen in both cell lines. When calculated as specific activity, differences between the two cell lines were seen over the entire 7 d period, the enzyme activity being always lower in the HP cells (Fig. 10B). It should be pointed out that the enzyme activity measurements did not distinguish between the products of various *SAMDC* genes.

Expression of different genes over the 7 d culture cycle

As mentioned before, gene expression data was generated by **Dr. Andrew Page** (Page et al., 2007) and are presented in the Appendix B.

Discussion

The calculation of enzyme activity and PA content on the basis of g^{-1} FW vs. mg^{-1} protein may lead to somewhat different interpretations of data concerning changes in cellular metabolism with time during the 7 d culture cycle. Several of the genes tested in the present study showed a peak in expression and enzyme activity on g^{-1}FW basis during the first 2 to 3 d of culture (e.g. ADC and ODC), suggesting an association with rapid growth/cell division occurring in cell cultures around this period. However, due to higher

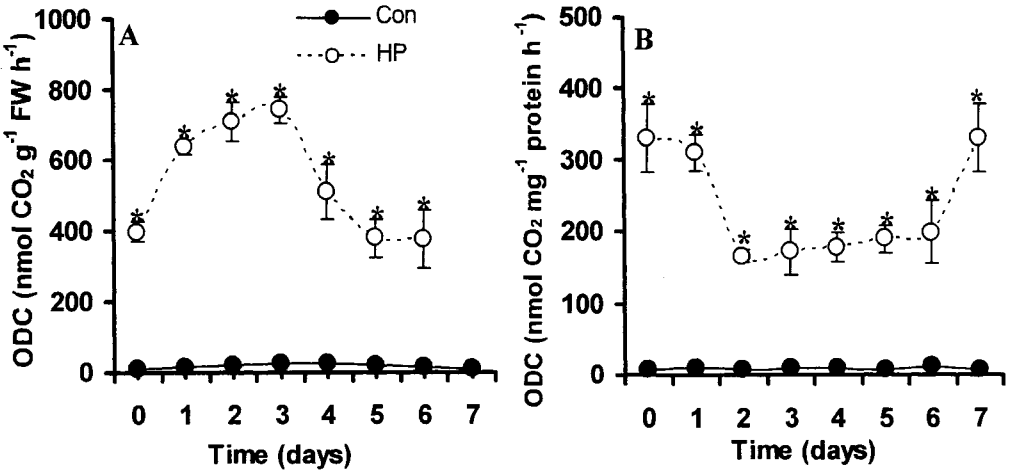

Figure 8. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of ODC in control and HP cells over the 7 d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the enzyme activity between the control and HP cells on a given day of the 7 d culture cycle.

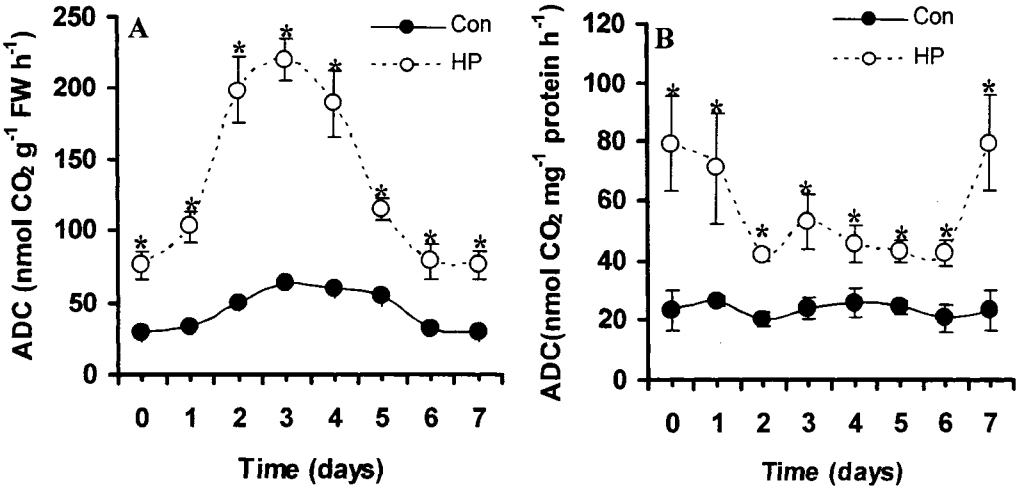

Figure 9. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of ADC in control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the enzyme activity between the control and HP cells on a given day of the 7 d culture cycle.

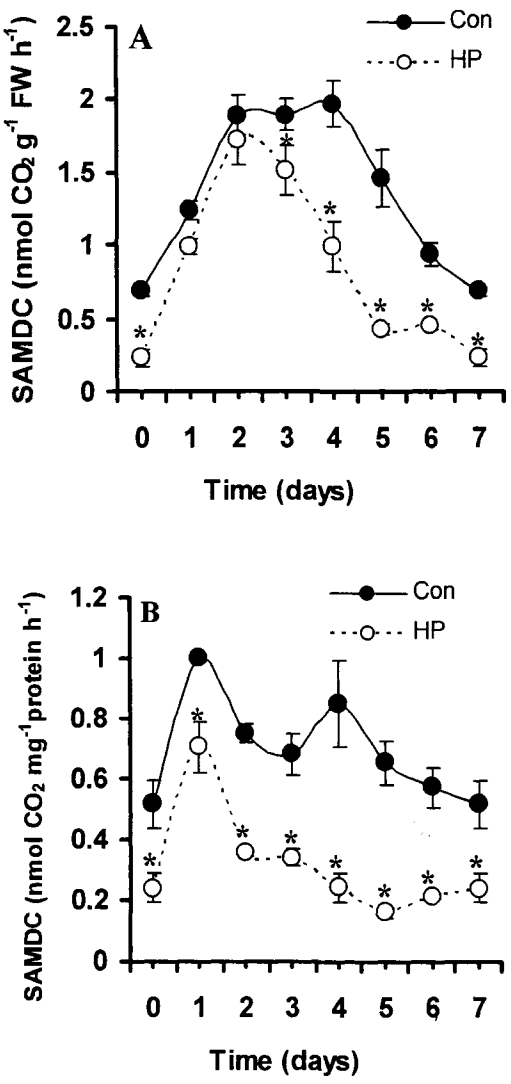

Figure 10. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of SAMDC in control and HP cells over the 7 d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the enzyme activity between the control and HP cells on a given day of the 7 d culture cycle.

protein content during this period (Fig. 7), perhaps again due to rapid growth, differences over time in specific activity, particularly that of ADC, during the 7 d culture period became less apparent (Fig. 9B). Thus the data suggest that some of the changes with time are perhaps a reflection of general metabolic status of the cells during the 7 d culture cycle, including changes in total protein content and mitochondrial activity.

In HP cells, the expression of m*ODC* (Fig. B1A), the activity of ODC g^{-1}FW (Fig. 8A), and the content of Put (g^{-1}FW; Fig. 7A) all showed a similar trend of an initial steady rise from the 1st to the 3rd or 4th d, which was concomitant with an increase in cellular protein content. In control cells, although the activity of pODC remained low throughout the culture period, an increase during the first 3 d was observed. It should be pointed out that a portion of $^{14}CO_2$ released from [^{14}C]Orn in the control cells could also have come from its conversion into [^{14}C]Arg and subsequent decarboxylation by ADC. The lack of change in specific activity during the 2nd to 6th d is due to changes in protein content of cells which was higher during this period as compared to days 1 or 7. The increases in Put in both the control and the HP cells following transfer to fresh medium are consistent with the observed changes in ODC and ADC activities. The change in m*ODC* expression (and resultant change in enzyme activity and Put production) is interesting in light of the fact that the transgene is under the control of supposedly a constitutive promoter (for review see Yoshida and Shinmyo, 2000). However, many studies have shown that the 35S CaMV-regulated expression is not entirely constant and varies according to tissue type as well as developmental stage (e.g. Sunilkumar et al., 2002); a situation similar to that seen for ubiquitin-regulated promoter, another

commonly used constitutive promoter (Capell et al., 2004). Hence, m*ODC* expression and enzyme activities vary with the metabolic state of cells.

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

The QRT-PCR results revealed that not only does the transcript abundance of the three *SAMDC* genes in cultured poplar cells vary independently of each other over the 7 d

cycle but also that the three genes respond differently to increased accumulation of Put in the HP cells. The most significant and rather unexpected observation is that the m*ODC* transgenic cells show a reduced expression of the predominant *SAMDC*1 as well as the least expressed *SAMDC*3, with little effect on *SAMDC*2. Although the decrease in *SAMDC*1 and *SAMDC*3 transcripts in HP cells is accompanied by a concomitant decrease in SAMDC activity; this apparently does not affect the rates of biosynthesis and accumulation of Spd in these cells (Bhatnagar et al., 2001). In fact, the HP cells generally produce and accumulate more Spd than the control cells, at least during the first few days of the culture period (P. Bhatnagar, R. Minocha and S.C. Minocha, unpublished data). In a study similar to ours, Capell et al. (2004) reported that overexpression of a *Datura ADC* in rice caused an increase in both Put and Spd. Although the authors pointed to a positive relationship between *SAMDC* transcripts and tissue Spd content in both wild type and transgenic plants on certain days in response to stress, this positive relationship between *SAMDC* transcripts and tissue Spd was not seen in the untreated tissue. Since they used gel blots for transcript analysis, no distinction was made among different paralogues of SAMDC; also, no enzyme activity data were presented to correlate them with the PA contents. On the other hand, an inverse relationship between cellular Put and *SAMDC* transcripts in both wild type and transgenic rice was observed on several days of stress treatment (Capell et al., 2004), a situation similar to our results with poplar. Of course, an increased production of Spd by SAMDC overexpression, such as that seen in tobacco by Noh and Minocha (1994) and in tomato by Mehta et al. (2002) would indicate that SAMDC alone may be sufficient to affect the cellular contents of Spd. In both these studies, as a consequence of increased utilization of Put as a substrate, its content in the

transgenic cells was actually lower. The normal control of *SAMDC* expression by Put in these cases was of course absent. These observations raise some interesting questions about the role of SAMDC in regulation of this part of the pathway as well as its own regulation by Put. For example: (a) how does a lower activity of SAMDC in HP cells sustain a higher rate of dcSAM production for increased Spd biosynthesis? (b) Is there a common mechanism by which high Put regulates expression of the two *SAMDC* genes (but not the third one), or is the reduction in transcripts of the two genes a reflection of their increased turnover? Although nothing is known about promoters of the three *SAMDC* genes in poplar, promoters of the two Arabidopsis *SAMDC* genes (*AtSAMDC*1 and *AtSAMDC*2) show almost 50% sequence identity and possess several common motifs; such as, IBOX (light regulated), DRE Core (drought responsive), Myb-binding protein (abiotic stress), and GAREAT (GA responsive element) (C.F. Rice and S.C. Minocha, unpublished). It is conceivable that some common Put-sensory element(s) are present in the promoters of the p*SAMDC*1 and p*SAMDC*3 genes which regulate their response to Put.

While the transcript levels or enzyme activities for the key regulatory PA biosynthetic enzymes have been studied separately in a few cases, a direct correlation between the two has not been clearly demonstrated. Among the main reasons for lack of a positive relationship between transcript abundance and enzyme activity are translational controls, transcript turnover rates, enzyme turnover rates, availability of cofactors and other cellular metabolites that affect enzyme activity, and, finally, the processing and activation of the proenzyme. A strong temporal correlation between the transcript levels and enzyme activities (g^{-1} FW) of the transgenic m*ODC* as well as the native *ADC* and

SAMDC were seen in poplar cells over the entire 7 d culture cycle. For example, HP cells which have higher *ADC* and lower *SAMDC* transcripts compared to the control cells (Fig. B1) also have higher ADC and a lower SAMDC activity (Fig. 9A, 10A). An increase in m*ODC* transcripts in HP cells between day 2 and 3 (Fig. B1A) is accompanied by a similar increase in ODC activity around days 2 to 4 (Fig. 8A), and a decrease in SAMDC activity (Fig. 10A) after day 2 follows a decrease in its transcripts (Fig. B1, B2). Changes in ADC activity and its transcript during the first 4 d also parallel each other.

A positive relationship between cellular PAs and respective enzyme activities responsible for their biosynthesis is readily apparent, whether the data calculations are done on g^{-1}FW basis or mg^{-1} protein basis. Both ODC and ADC increase during early days of growth in the fresh medium (Fig. 8, 9), and this is accompanied by an increase in cellular Put (Fig. 6). Likewise, an increase in SAMDC during the first 3 to 4 days of culture in the control cells parallels changes in cellular Spd and Spm. As mentioned above, the main discrepancy in this respect is that in spite of lower SAMDC g^{-1}FW, the HP cells maintain a slightly higher amount of Spd (also g^{-1}FW). The apparent lack of a large increase in Spd in the HP cells which have several-fold higher Put content indicates the lack of stimulation of SAMDC activity by Put. This is in contrast to the demonstrated up-regulation of animal SAMDC activity by cellular Put (Stanley et al., 1994, Ruan et al., 1996, Xiong et al., 1997); but is consistent with our current knowledge that plant SAMDC is not activated by Put (Xiong et al., 1997; Park and Cho, 1999; Bennett et al., 2002). An alternate explanation would be that Spd biosynthesis is regulated more by SPDS than by SAMDC; which is in contrast to what is generally believed (Ruan et al., 1996, Thu-Hang et al. 2002). As discussed above, it is conceivable that reductions in

SAMDC transcripts as well as SAMDC activity in HP cells are actually caused by the increased Put (or total PA) content of these cells.

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

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

The use of QRT-PCR has permitted us greater precision in measuring gene expression than that afforded by any other method (Gachon et al., 2004) and has allowed greater insight into the regulation of PA metabolism than was previously known in plants.

Not only does it appear that PA metabolism is regulated, at least in part, at the

transcriptional level, but also that (as expected) different paralogues of a gene have differing roles in the maintenance of steady state enzyme activities and PA levels. This is in contrast to past publications based on alternate techniques which failed to reveal such precise metabolic regulation. For example, Trung-Nghia et al. (2003) found that over-expressing an oat (*Avena sativa*) *ADC* in anti-sense mode resulted in a decrease in Put and Spd in rice (*Oryza sativa*) and concluded that there was no effect on the expression of downstream genes. However, the changes that we were able to detect using QRT-PCR could not have been detected using northern blotting or RT-PCR (the techniques used by Trung-Nghia et al., 2003) due to the relative insensitivity and difficulty in quantifying results with these techniques. Similarly, Primikirios and Roubelakis-Angelakis (1999) did not see a change in *ADC* expression upon exogenous application of Put in *Vitis vinifera* suspension cultures although a decrease in ADC specific activity was observed. Watson and Malmberg (1996) found a ten-fold increase in ADC activity and a twenty-fold increase in Put in response to high K^+ stress in Arabidopsis, but again using northern blotting, found no change in *ADC* transcripts. Likewise, many studies that have shown an increase in ADC activity in response to abiotic stress have not made a distinction between the two or more paralogues of this gene that are often found in plants.

Although the results presented here show differential regulation of the three *SAMDC* genes, it is still not clear as to what the relative contribution of each to the final SAMDC activity is, and if there are differences in the properties of the three isoforms of the enzyme that may affect their contribution to the final reaction. This is due to the fact that each transcript and protein is subject to its own translational and post-translational controls, including possible variation in substrate affinities and pH responses (Kauppinen,

1995; Watson and Malmberg, 1996; Primikirios and Roubelakis-Angelakis, 1999; Trung-Nghia et al., 2003). Little is known about the subcellular localization of the different SAMDC proteins as well. This indeed may be the challenge to deal with in metabolic engineering via genetic manipulation because, while changes in gene transcription, translation, enzyme kinetics, etc. can be quantified *in vitro*, their relative importance to the total reaction *in vivo* is often hard to assess.

Conclusions

This study provides an insight into the regulation of transcription and activities of enzymes that regulate PA metabolism in poplar cells with a combined breadth and accuracy previously not reported for any tissue. The results reveal that poplar *ADC* and *ODC* expression and enzyme activities are not subject to feedback regulation, while increased accumulation of Put may inhibit expression of some members of the *SAMDC* family, leading to lower SAMDC activity. This happens even though the biosynthesis and the accumulation of Spd is equal or greater in the HP cells. Furthermore, a quick response is seen for transcription as well as enzyme activities on transfer of cells to fresh medium.

CHAPTER II

ENHANCED PUTRESCINE METABOLISM AND SOLUBLE AMINO ACIDS

As can be seen from Fig. 2, the metabolism of PAs is intricately connected to the metabolism of several amino acids like Orn, Arg, Pro, GABA and Glu. While Orn is the direct precursor of Put, the latter can also be synthesized from Arg, both amino acids being ultimately formed from Glu. Glutamate is also a precursor of Pro, an important metabolite under stress conditions (reviewed by Kavi Kishor et al., 2005), that can also be synthesized from Orn in an alternate pathway. Both Put and Glu form GABA, another important signaling molecule under stress (Bown et al., 2006; Mazucotelli et al., 2006). Thus, PA metabolism, from several directions, is linked to the metabolism of Glu, an amino acid whose importance as a central molecule in cellular metabolism has gained considerable attention in recent years (reviewed by Forde and Lea, 2007). Glutamate not only serves as a precursor of the above-mentioned metabolites, but also as an important donor of amine groups to keto acids in several transaminase reactions, leading to the biosynthesis of several other amino acids. Thus, it was considered important and necessary to study the impact of over consumption of Orn and ultimately, Glu in our transgenic poplar cells, on the metabolism of other amino acids.

Introduction

The importance of Glu as a key intermediate in N metabolism cannot be over-emphasized (Forde and Lea, 2007). Glutamate serves as a key intermediate for not only N assimilation via the GS-GOGAT cycle (Lam et al., 1996; Coruzzi and Last, 2000; Weber

and Flügge, 2002; Miflin and Habash, 2002; Foyer et al., 2003), but also serves as a substrate for the biosyntheses of several important metabolites, thus playing an important role in the C-N balance in cells (Fig. 11). It acts as a direct precursor to the biosynthesis of amino acids through aminotransferase/transaminase reactions (Coruzzi and Last, 2000; Forde and Lea, 2007; Ferrario-méry et al., 2000); its role in the biosynthesis of Orn, Arg, Pro and PAs has been discussed under "General Introduction" and represented in Fig. 2. Putrescine catabolism produces GABA, an important metabolite in stress response (Mazzucotelli et al., 2006) and signaling (Bouché and Fromm, 2004) that finally enters the TCA cycle through succinate, thus recycling both the N and the C component of Glu and Put (Bouché and Fromm, 2004). This important metabolite can also be produced directly from Glu by GAD (Bouché and Fromm, 2004). Thus, Glu is linked to not only the biosynthesis of several amino acids, but PAs as well.

Amino acid metabolism is intricately connected to glycolysis and the TCA cycle in that these two pathways supply the carbon skeletons required for the biosynthesis of various amino acids (Fig. 12; Coruzzi and Last, 2000). Important glycolysis intermediates are 3-phosphoglycerate (3-PGA), phosphoenol pyruvate (PEP) and pyruvate (Pyr), while the TCA cycle contributes oxaloacetate (OAA) and α-KG (Coruzzi and Last, 2000). For most of these biosynthetic reactions, Glu serves as the amino group donor. As mentioned earlier, GS assimilates NH_4^+, converting Glu into Gln. The other important enzyme in the N-assimilation pathway is GOGAT which converts Gln back to Glu, by transferring its amide amino group to α-KG. One form of this enzyme (EC 1.4.7.1) uses reduced ferredoxin (Fd) and the other (EC 1.4.1.14) uses NADH as the electron donor (Forde and Lea, 2007). Glutamine, formed from Glu is responsible for the synthesis of histidine

(His). Aspartate (Asp) is formed by the transfer of an amino group from Glu to OAA; the latter is in turn the precursor for asparagine (Asn) synthesis by an aminotransferase reaction, in which the amino group is donated by Gln, converting Asp to Asn and itself getting deaminated to Glu, the reaction is catalyzed by Asn synthetase (EC 6.3.5.4). Aspartate can also react with NH_4^+ to directly produce Asn. Aspartate is also the source of threonine (Thr), methionine (Met) and lysine (Lys); Thr then forms isoleucine (Iso). Met is the substrate for SAM, which is formed by SAM synthetase; the S component of SAM is recycled to Met through a series of intermediates.

The aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) are synthesized via a series of intermediates from chorismate, again the aminotransferase reaction uses Glu as the amino group donor. Synthesis of Trp on the other hand utilizes NH_4^+ for a direct amination. Chorismate, the common branch point for the synthesis of these three amino acids is synthesized via two other intermediates from the glycolytic pathway intermediate; PEP 3-phosphoglycerate forms serine (Ser), which in turn gives rise to glycine (Gly) and cysteine (Cys). Pyruvate is the substrate for alanine (Ala), leucine (Leu) and valine (Val).

Bhatnagar et al. (2002) showed that in HP cells Put catabolism is able to keep pace with its biosynthesis, leading to enhanced Put oxidation, which will in turn lead to an increase in GABA biosynthesis. Therefore, an increased consumption of Orn which occurs in the HP cells due to its over-utilization by mODC must be accompanied by its increased biosynthesis from Glu; this in turn will significantly affect Glu pool in these cells. We hypothesize that reduced Glu levels in the cells will affect the biosynthesis and

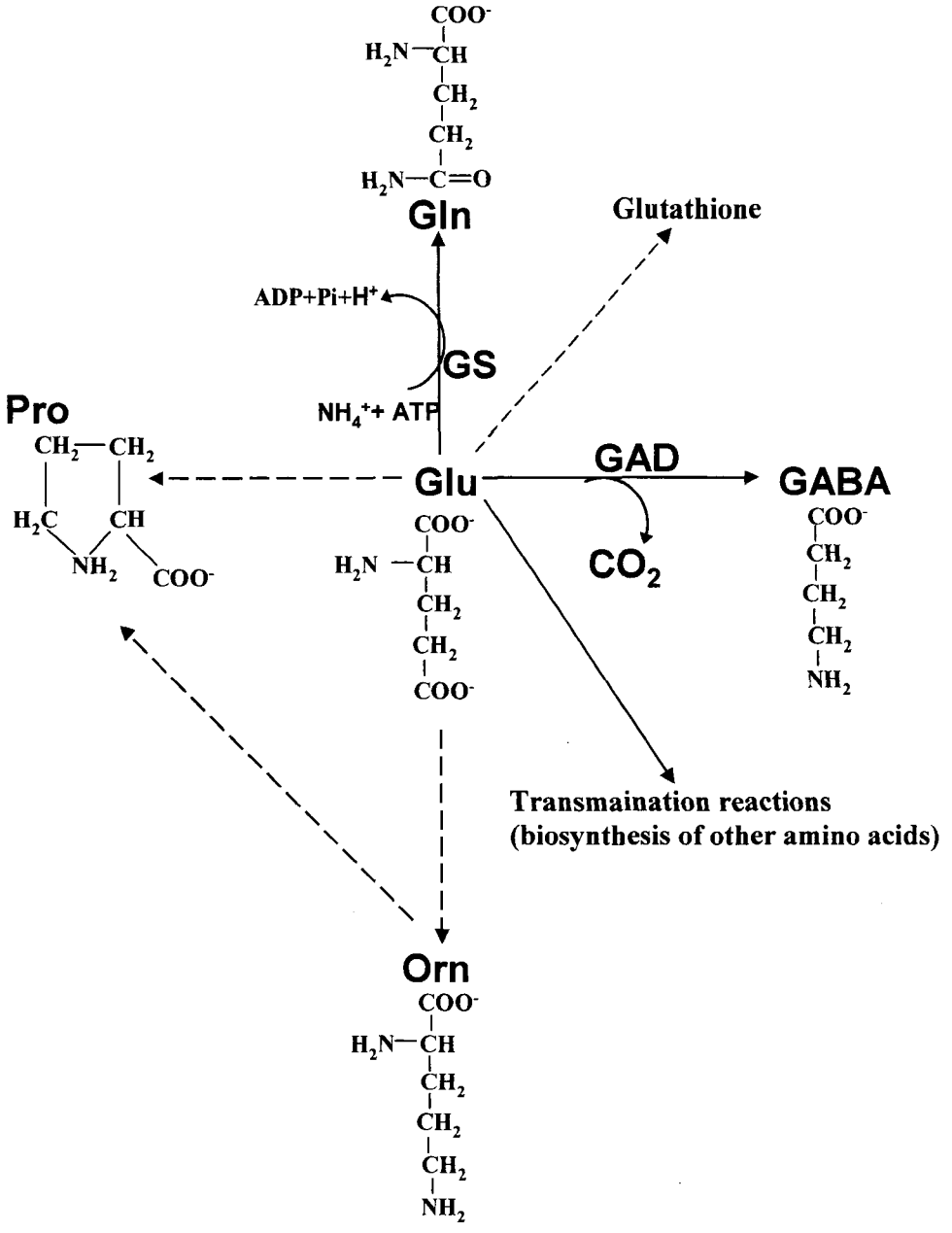

Figure 11. Role of Glu as a precursor of several important metabolites (Adapted from Coruzzi and Last, 2000).

accumulation of other amino acids which are derived directly or indirectly from Glu. Thus we analyzed the cellular contents of all 20 protein amino acids along with two non-protein amino acids, namely GABA and Orn, in the two cell lines on each of the seven days of the culture period.

Materials and Methods

Amino acid analysis was done as described under "General Materials and Methods".

Total organic carbon and nitrogen

The total organic C and N was measured in 4-d-old control and HP cells. Cells (200±20 mg) were harvested, dried at 70 ^{0}C and analyzed for C and N content using a CHNS analyzer (Perkin-Elmer series 2-2400).

Results

Amino acids derived from α-ketoglutarate (the glutamate family)

Glutamate content was significantly lower in the HP cells than in the control cells on all 7 days of the week (Fig. 13A). Fresh medium effect on Glu content was seen in both cell lines; i.e. there was almost a doubling of cellular Glu within 24 h after transfer to fresh medium, followed by a similar decline within the next 24 h. The HP cells showed a gradual decrease in Glu content up to day 3, followed by a slight increase up to day 6. In the control cells, Glu did not change much after day 2 or 3. Cellular Orn, the precursor of the transgenic ODC, was present in relatively small amounts (typically 30 to 40 folds less than Glu) at any time; its amounts were always lower in the HP than the control cells on any day of analysis (Fig. 13B). While there was a significant increase in Orn in the HP cells on transfer to fresh medium, highest amounts of Orn in the control cells were found between days 4 and 6.

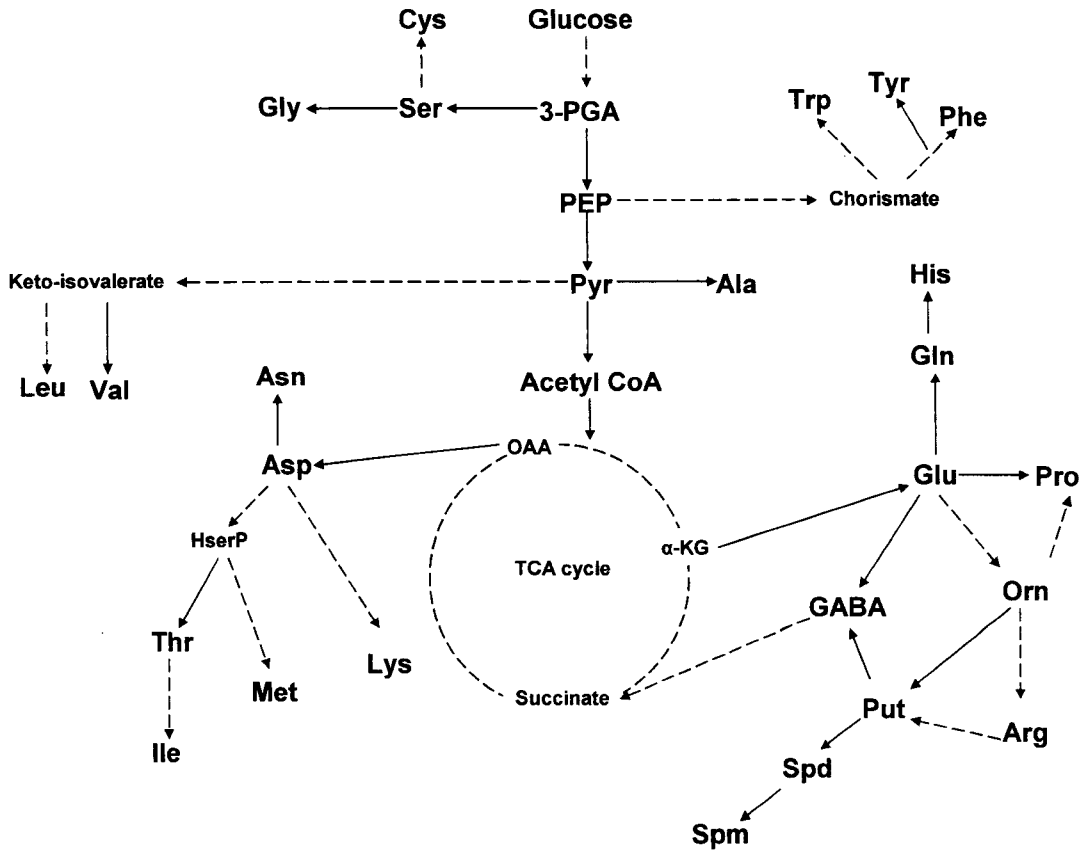

Figure 12. The biosynthesis of amino acids in plants (adapted from Coruzzi and Last; 2000). A dashed line represents a multi-step process.

Although in the control cells, Arg content remained fairly stable throughout the week; in the HP cells, a sharp increase in cellular Arg was seen during the two days following transfer to fresh medium (Fig. 13C). Thereafter, a gradual decline in Arg was observed in these cells. Arginine is the primary source of PAs in the control cells and its utilization for Put biosynthesis in the HP cells is not affected by the presence of transgenic ODC (Bhatnagar et al., 2001). Changes in the cellular content of Pro were parallel to those in Arg while its amounts were higher than Arg in both cell lines (Fig. 13D). There was a significant increase in Pro content of cells in response to fresh medium, particularly in the HP cells followed by a decline; only small changes with time in the control cells were seen. Pro was significantly higher in the HP cells on days two and three only.

Glutamine was higher in the control cells as compared to the HP cells both at the beginning and towards the end of the culture cycle; the differences being several-folds on days 1, 6 and 7 (the same as day zero in Fig. 13E). In control cells, Gln content showed a trend that was somewhat opposite to that of Glu on transfer to fresh medium; i.e. a decline was seen within 24 h of transfer and a gradual increase during the latter half of the growth cycle (i.e. days 5 to 7). The Gln content of HP cells remained rather low and unchanged during the entire 7 d culture period.

Finally His, which is the most basic of this group of amino acids, showed small but significant differences between the two cell lines on days 4, 5 and 6 (Fig. 13F), with the HP cells having lower His content on these days. Its content showed an increase on transfer to fresh medium with a peak on day 2 in both cell lines.

Amino acids derived from 3-phosphoglycerate (3-PGA)

Serine and Gly, which are readily interconvertible, are derived largely from 3-PGA as shown in Fig. 12 (although Gly can also be made by direct transamination of glyoxalate); both pathways use Glu as the donor of the amide group. Along with Cys, which is made from Ser, all three amino acids of this group showed a similar trend of changes in both cell lines during the week; there was a decline (up to three-folds) in their cellular contents during the first 2 to 4 days on transfer to fresh medium, followed by a recovery during the latter half of the week (Fig. 14). While the cellular contents of Ser and Gly were comparable (Fig. 14A, B), Cys was present only in small amounts (20-30 folds lower than either) quantities on any given day (Fig. 14 C). On most of the days, all three were higher in the control than in the HP cells, the difference being most pronounced for Cys + cystine (Fig. 14C).

The aromatic amino acids (derived from phospho*enol*pyruvate)

Of the three aromatic amino acids, two (Tyr and Phe) use Glu as the amide group donor, while Trp takes its amino group from Ser (Siehl, 1999). The carbon skeleton for these amino acids comes from phosphoenolpyruvate (PEP) via the intermediate chorismate (Fig. 12). The contents of Phe and Trp varied parallel to each other during the week in the two cell lines (Fig. 15); the changes were also similar to those seen for the three amino acids derived from 3-PGA. Cellular contents of Phe (Fig. 15A) were almost twice as much as those of Trp (Fig. 15B) on a given day; both showed a decline on transfer to fresh medium and an increase during the latter half of the week. Differences between the two cell lines were significant both for Trp and Phe on most days of culture;

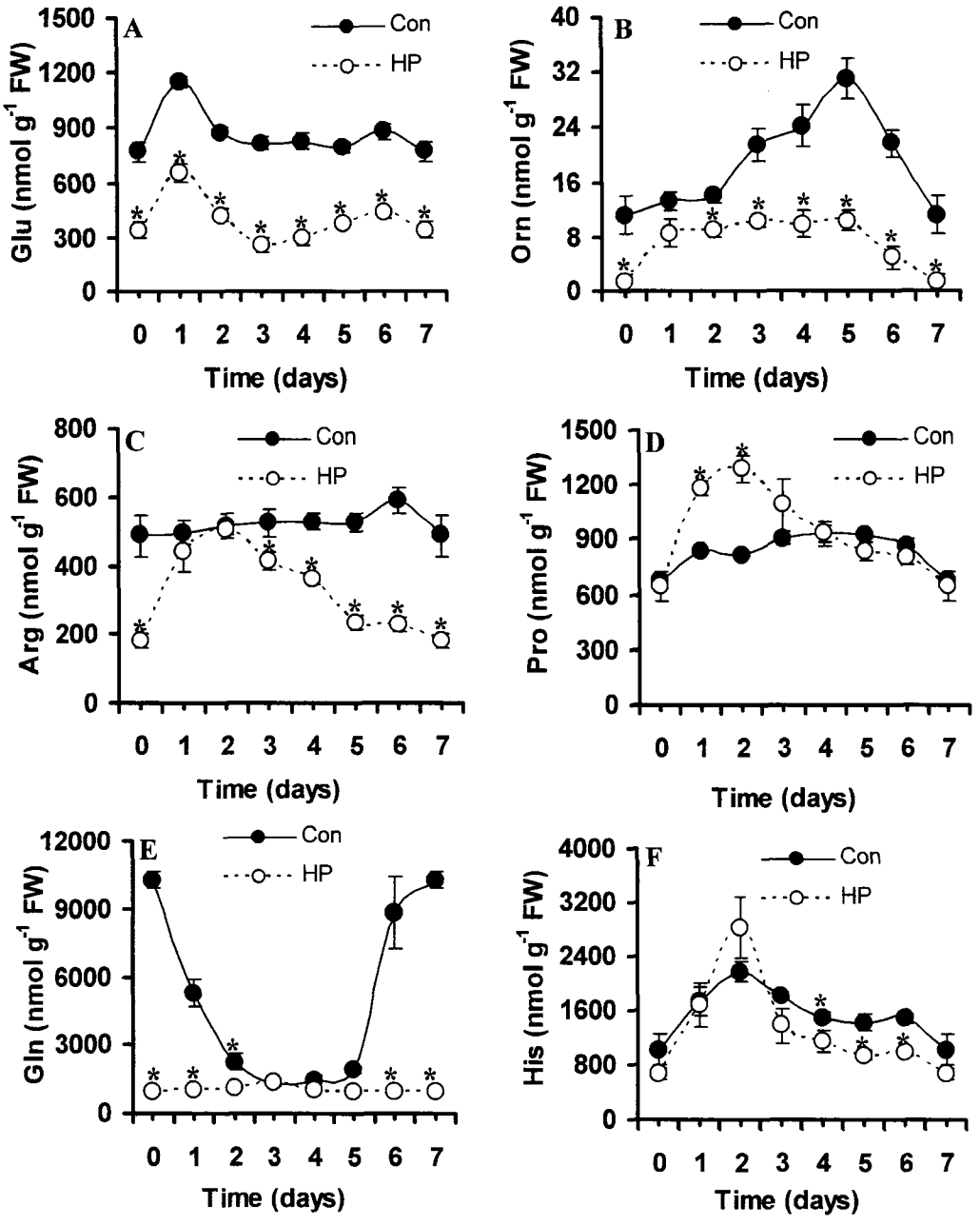

Figure 13. Cellular contents of the glutamate family of amino acids (derived from α-ketoglutarate): (A) glutamate (B) ornithine (C) arginine (D) proline (E) glutamine (F) histidine in control and HP cells. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the cellular content of an amino acid between control and HP cells on a given day.

both being lower in the HP cells than the control cells. Tyrosine was not resolved in the HPLC system used here; thus data for this amino acid are not presented.

Amino acids derived from pyruvate (the pyruvate family)

Leucine, Val and Ile constitute a group of branched-chain amino acids; the former two being derived from pyruvate and the third from 2-ketogluterate (Singh, 1999). However, the enzymes involved in biosynthesis of Val and Ile are similar in that they perform parallel reactions but use different substrates. Alanine is also derived from pyruvate by a direct aminotransferase reaction from Glu. Of the three amino acids of this group, Ala was the dominant amino acid in control cells, followed by Leu and Val. The Leu content was at least three-folds lower in the HP cells than the control cells on almost all days of the week (Fig. 16A). While in the control cells, there was a rapid (more than two-fold) decline in Leu during the first two days of culture in the fresh medium, which was followed by consistent increase during the next four days; changes in Leu content of HP cells were much smaller in magnitude, although similar response to fresh medium was seen in these cells. In contrast to Leu, both Val and Ala were higher in the HP cells than the control cells during several days of culture (Fig. 16B, C); while the former showed an increase in HP cells on transfer to fresh medium, the latter did not change much on most days. In the control cells, a significant decrease in Ala was seen during the first two days of culture; its content recovered again during the last two days of the 7 d cycle.

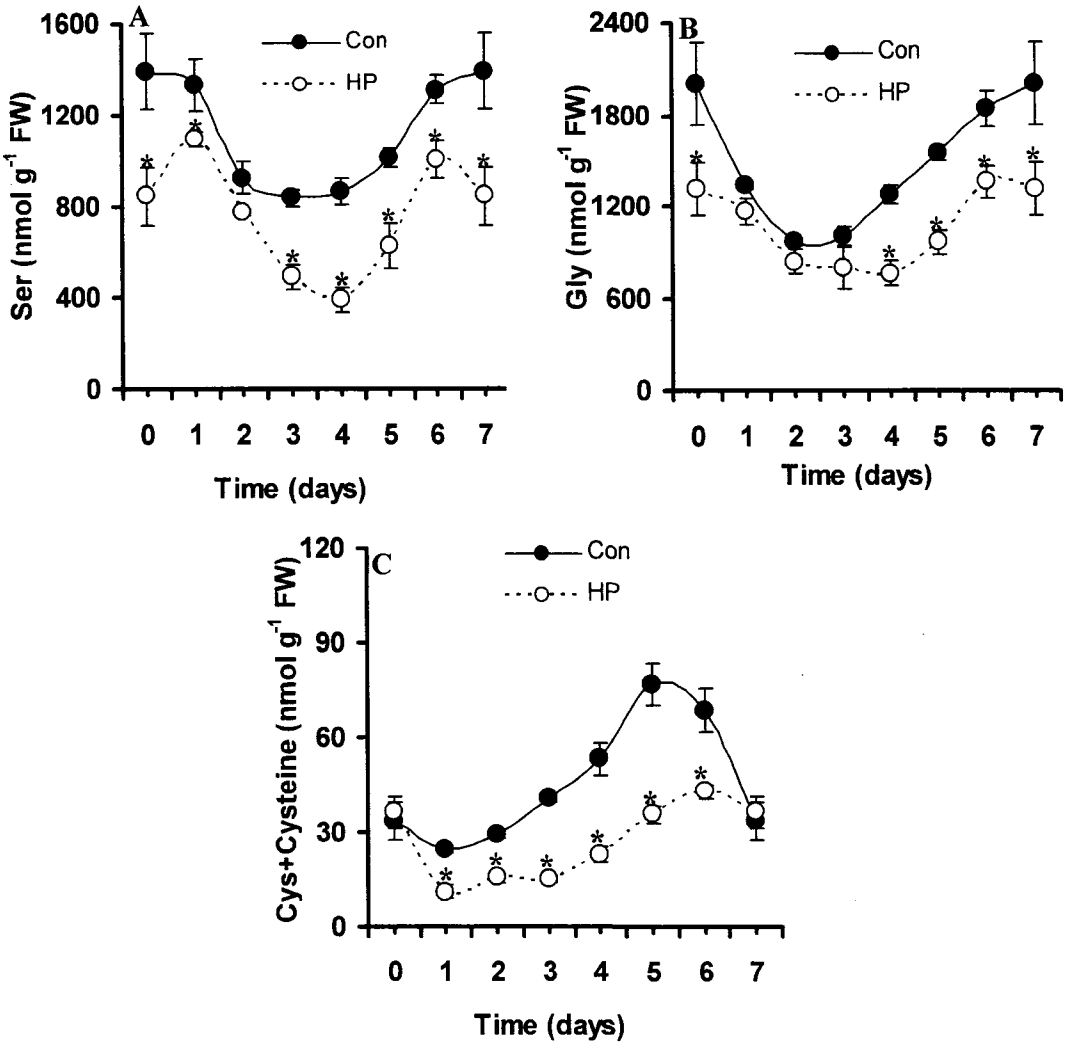

Figure 14. Cellular contents of amino acids derived from 3-phosphoglycerate: (A) serine (B) glycine (C) cysteine+cystine in control and HP cells. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the cellular content of an amino acid between control and HP cells on a given day.

Amino acids derived from oxaloacetate (the aspartate family)

There are six amino acids that constitute the aspartate family and are derived from oxaloacetate (OA) as the source of carbon skeleton; five of these were analyzed in the present study. Each showed a somewhat different pattern of change during the week, and each showed different response to high PA metabolism in the HP cells (Fig. 17). The biosynthesis of both Asp and Asn can utilize free ammonia rather than depending entirely on transamination from Glu or Gln. Aspartate and Thr, the two dominant amino acids in this group showed a similar pattern of changes during the week in the HP cells but there were differences in their relative contents in the two cell lines (Fig. 17A, B). Both amino acids showed a rapid but transient increase (more than two-fold for Asp and four-fold for Thr) in response to fresh medium effect in the HP cells; only a small increase was seen in Thr in the control cells at this time. After two days, the cellular contents of both these amino acids declined to their original levels (of day 0) and stayed there for the remainder of the week. Moreover, while Asp was higher in the control cells, Thr was higher in the HP cells, at least for the first four days of culture.

Lysine, Met and Ile, all showed a decrease in cellular content in both cell lines on transfer to fresh medium followed by an increase during second half of the week (Figs. 17C, D, E). Methionine was the lowest in amount of this group of amino acids, and remained higher in the control cells throughout the week-long culture period. While Ile was higher in the HP cells during the first 2 to 3 days, Lys was higher in the control cells during the latter part of the growth cycle. Threonine is among the few amino acids whose content increased the most in response to Put overproduction (i.e. in HP cells) on the first three days of culture (Fig. 17B).

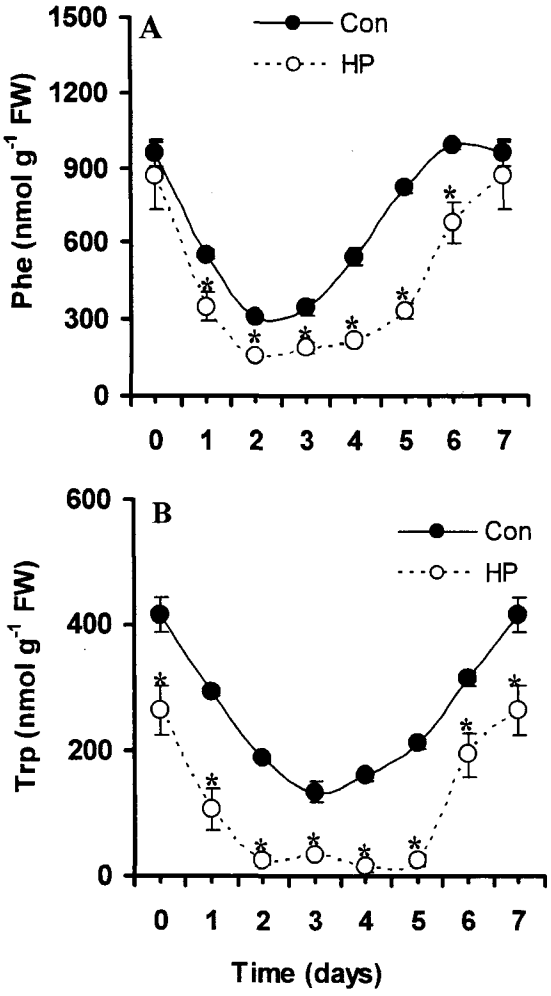

Figure 15. Cellular contents of amino acids derived from Phospho*enol*pyryvate: (A) Phenylalanine (B) Tryptophan in control and HP cells. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the cellular content of an amino acid between control and HP cells on a given day.

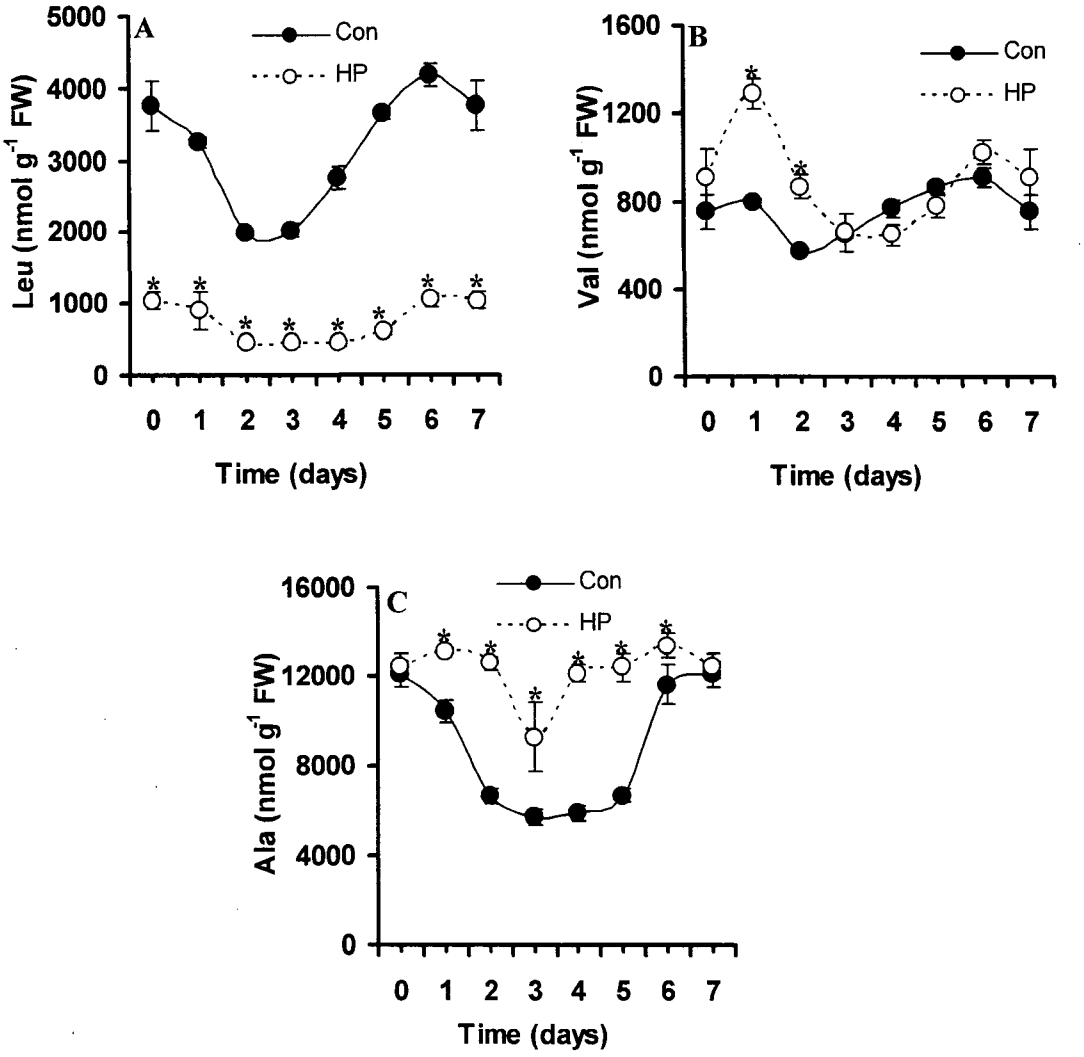

Figure 16. Cellular contents of amino acids derived from pyruvate: (A) leucine (B) valine (C) alanine in control and HP cells. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the cellular content of an amino acid between control and HP cells on a given day.

Gamma-aminobutyric acid (GABA)

Gamma-aminobutyric acid (GABA), is a non-protein amino acid which is produced both directly from Glu by glutamate decarboxylase (GAD) and from the catabolism of Put by diamine oxidase (Fig.12; Rhodes et al., 1999). It serves as an intermediate in the recycling of C of Glu as well as the diamine Put, and has been implicated in some of the same roles as Put; e.g. response to low Ca and abiotic stressors. A distinct fresh medium effect was seen for GABA content in both cell lines (Fig. 18), resulting in a sharp and almost a three-fold increase from day 0 to day 1. Thereafter, the cellular content of GABA decreased in the control cells within the next day (from day 1 to day 2) and in the HP cell two days later (between days 3 and 5). Thus in the HP cells, GABA content was higher for at least two to three days of the 7 d culture period.

Total carbon and nitrogen

Both total C and total N were significantly higher in the HP cells than in the control cells on day-4 of the 7-day culture cycle when this analysis was done (Fig. 19). The total C content was several-fold higher than the total N content in both cell lines.

Discussion

While Glu is a precursor of a large number of nitrogenous compounds in plants, as well as a source of N for most other amino acids, large amounts of this amino acid are utilized in the production of Arg, Pro and GABA (Singh, 1999). Of course, Glu is also a common constituent of cellular proteins, and in whole plants, it can be transported between different tissues and organs. Hence, the cellular contents of Glu are subject to numerous regulatory signals, both on the production side as well as the consumption side

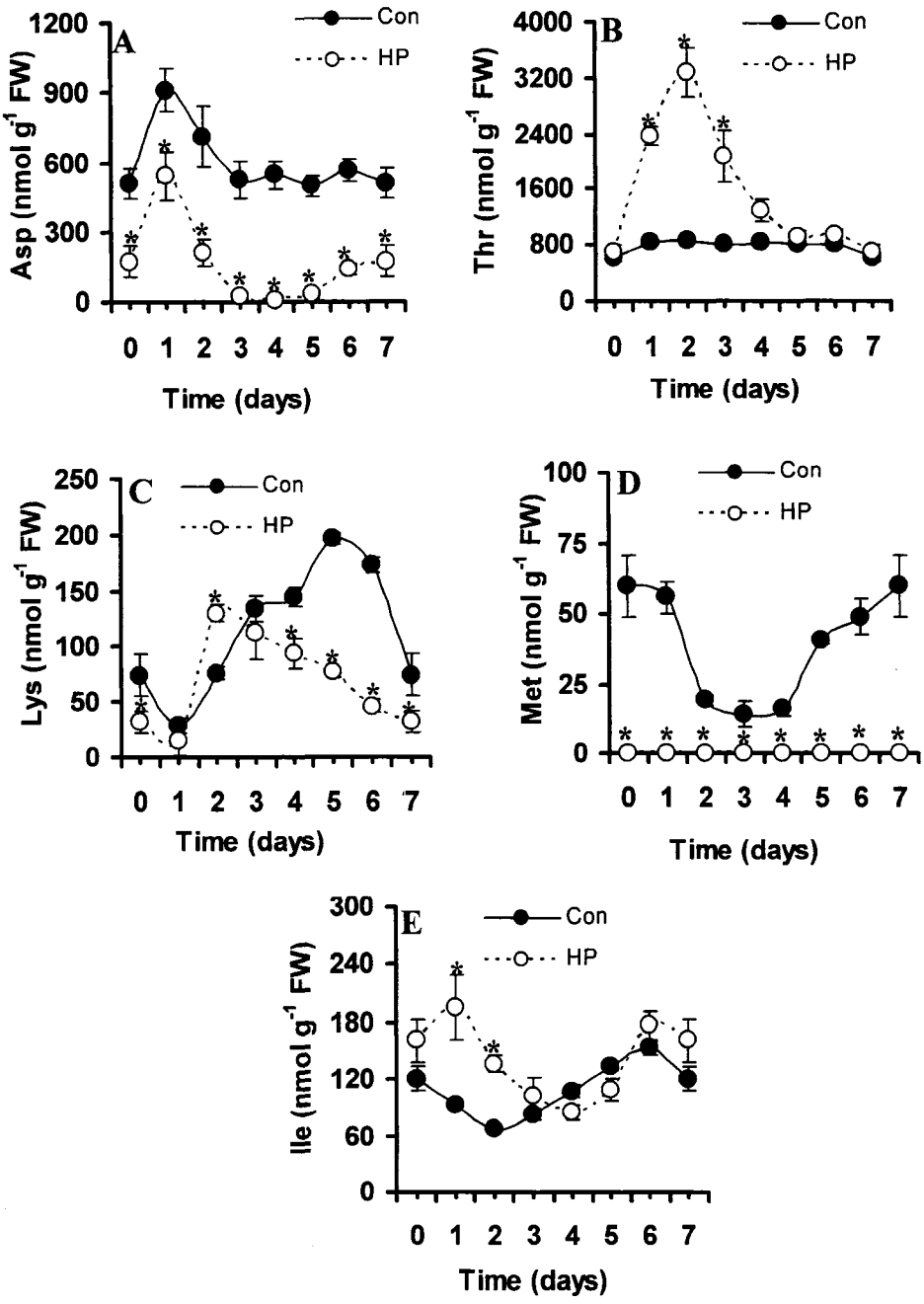

Figure 17: Cellular contents of amino acids derived from oxaloacetate: (A) aspartate, (B) threonine (C) lysine (D) methionine (E) isoleucine in control and HP cells. Data are mean (±) SE of 6 replicates from 2 experiments (3 replicates only for Asp). An * indicates a significant difference ($P \leq 0.05$) in the cellular content of an amino acid between control and HP cells on a given day.

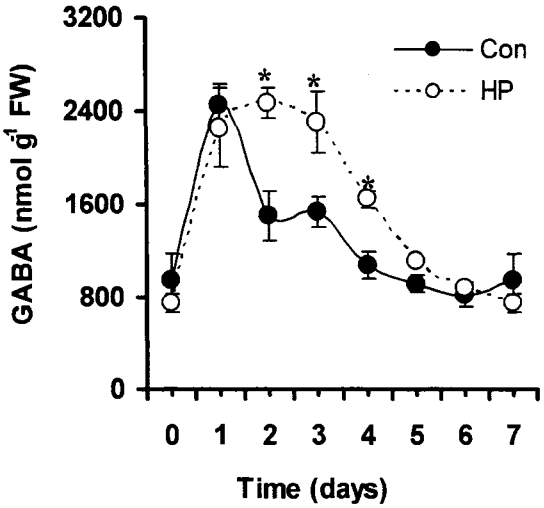

Figure 18: Cellular contents of GABA in control and HP cells. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) in the cellular content of an amino acid between control and HP cells on a given day.

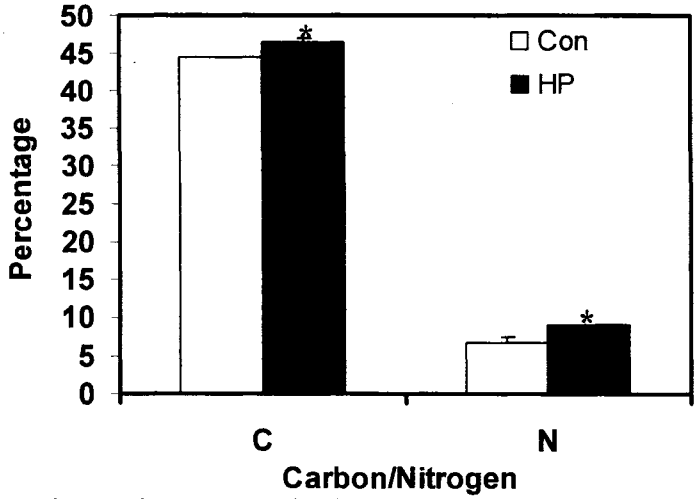

Figure 19: Total C and N content in the control and HP cells on d- 4 of the 7-d culture cycle. Data are mean (±) SE of 6 replicates. An * indicates a significant difference ($P \leq 0.05$) in the cellular content of total C/N between the two cell lines.

(Forde and Lea, 2007). How quickly do plant cells respond to the demand of Glu for variousmetabolic pathways, is not yet understood.Since (a) Orn is the primary substrate of the transgenic ODC in the present study, and its quantity in the cells is rather low (cf. many other amino acids; almost a 100-fold less in the present situation); and (b) Orn is also used in the formation of Arg (Slocum, 2005), and presumably Pro (Roosens et al., 2002), both of which are produced in large quantities; its biosynthesis from Glu must respond quickly to the demand on its utilization imposed by the transgenic ODC. Thus it can be argued that the reactions that produce Orn keep pace with its utilization in the formation of Put, Arg, and Pro. What regulates these biosynthetic steps under normal conditions as well as under the conditions of its increased utilization is not known. A combination of microarray and QRT-PCR analyses of the genes for various enzymes involved in the biosynthesis of these three metabolites showed no significant change in transcript levels of any of these enzyme genes in response to up-regulation of Put biosynthesis in the HP cells (A. Page, S.C. Minocha, et al., unpublished data). This would indicate that the regulation probably occurs either at the level of translation or involves biochemical mechanisms that affect enzyme activities; e.g. cofactors, substrate availability, etc. The molecular analysis by QRT-PCR further shows that the transcription of the genes of all these enzymes is highly coordinated during the 7 d culture cycle (A. Page, S.C. Minocha, et al., unpublished data).

A logical hypothesis would also involve some type of a sensing mechanism for monitoring Orn content in the cells and then triggering the pathway (at the level of translation or post-translational modifications, including the biochemical needs for the substrates and cofactors) for its biosynthesis from Glu. Moreover, since changes in Glu

concentration in the cells are smaller than its utilization in Put production; it can also be hypothesized that the biosynthesis of Glu (i.e. N assimilation) is also enhanced in the HP cells. Slocum (2005) has summarized various steps in the pathway for the biosynthesis of Orn and Arg from Glu (Fig. A1). However, no regulatory mechanism has been proposed for the entire pathway.

Similarities between the postulated and documented roles of Pro and Put, and also GABA, particularly under conditions of abiotic stress conditions are remarkable. While no specific mechanisms for the roles of these metabolites have been suggested, they all appear to respond to the same treatments in a similar (and coordinated?) manner (Aziz and Larher, 1995; Aziz et al., 1998; Houdusse et al. 2005; Simon-Sarkadi et al., 2005, 2006). While they are all rich in N, a major biochemical difference among them is the charges they carry under physiological conditions, which may call for different roles of these metabolites of the triangular pathways of Put, Pro and GABA (Fig. 2). Since their biosynthesis utilizes the same substrate, i.e. Glu, it is possible that a common signal transduction mechanism (molecule) triggers all three sub-pathways in a coordinated manner. While the nature of this mechanism is not known, Orn itself could play a major role in this pathway, acting both as a sensory as well as a regulatory molecule (S. C. Minocha, Personal communication). If that was the case, the most likely site of action will be the initial reactions that direct Glu into the three different but interacting pathways; i.e. the steps involving NAGS, P5CS, and GAD.

Houdusse et al. (2005) have shown that the cellular contents of Pro were more correlated with Put than with Spd or Spm in wheat when different sources of N were used. With a 2-3 fold increase in Put with increased N supply, a similar 3 to 4 fold

increase was seen in Pro in all conditions of N, whether the growth was promoted or inhibited. Amounts of Pro g^{-1}DW were 50-100 folds higher than Put or Spd in the foliage and about 10 fold higher in the roots. In pepper, the ratios of Pro to Put ranged from 2:1 to about 5:1. In poplar cell cultures used here, Pro was never more than 2-3 fold higher than Put in control cells and almost equal to Put in the HP cells.

Houdusse et al. (2005) also observed a strong negative correlation between Put and growth in pepper and wheat (r^2 = 0.9478 and 0.6134, respectively) with different forms of N, i.e. N treatments that caused the accumulation of Put were negatively correlated with growth; in other words, the higher the Put index, the lower was the growth. The lower growth index was associated with high organic N treatment with lower nitrate. In poplar cells the growth index is positively correlated with Put as well as Pro; however, the cell health (membrane integrity and mitochondrial activity) is negatively correlated with this PA. The design of the present study does not permit us to determine the cause and effect relationship between Pro, Put, and growth vs. health of cells. The above authors made an interesting suggestion that increased Pro may be the source of Orn for Put biosynthesis; however, no experimental data were presented. According to them, the increased Put production from Orn acts as a trigger to make more Orn via P5CS and Pro. When P5CS activity is increased, and more Pro is made; NH_4 toxicity is decreased and also there is more Orn/Arg available for Put production.

We further extend this hypothesis and suggest that increased Put production (thus a resulting reduction in Orn) in the HP cells starts the futile pathway involving all three metabolites whose amounts in the mean time are elevated. An additional trigger must also enhance the uptake and assimilation of extra nitrogen (perhaps also Carbon) from the

medium, which compensates for the utilization of Glu in these three pathways. Our data on the observed increase in total N and C in HP cells (Figs. 19, 49A) support this contention.

The production of Glu depends on nitrogen assimilation via the GS-GOGAT pathway (Lam et al., 1996; Coruzzi and Last, 2000; Weber and Flügge, 2002; Miflin and Habash, 2002; Foyer et al., 2003). Another important enzyme in Glu metabolism is NADH dependent glutamate dehydrogenase (GDH; EC 1.4.1.2), whose primary role is to re-assimilate NH_3 produced within the cells. Glutamine, formed from Glu is responsible for the synthesis of His. All Glu-derived amino acids, except Pro, were lower in the HP cells; perhaps a consequence of increased utilization of Glu for Put biosynthesis. However, each had a somewhat different profile of variation with time of culture, particularly with respect to the change in response to fresh medium effect. While Glu, Orn, Arg, Pro and His all increased on transfer to fresh medium, Gln in the control cells declined within a day of transfer to fresh medium. The changes with time in control cells were less pronounced for Arg and Pro, and in HP cells for Orn and Gln. Lower concentration of Gln in the HP cells is a predictable consequence consistent with its role as the source of Glu. The lower content of Arg in the HP cells is probably due to a combination of its reduced production from Orn as well as its increased utilization by ADC as shown earlier and also discussed above in Chapter I in that the HP cells have enhanced ADC activity (Bhatnagar et al., 2001). Histidine, the most basic amino acid in this group changes with time following a parallel course on different days in the two cell lines; more consistent with the fresh medium effect rather than the PA levels in the cells.

The aromatic amino acids Phe, Tyr and Trp are synthesized from phospho*enol*-pyruvate (Fig. 12) and chorismate, with Glu being the amino group donor for the former two. Unfortunately, the amino acid analysis technique used here was unable to resolve Tyr; therefore, it is not discussed further. Synthesis of Trp on the other hand utilizes NH_4^+ for a direct amination reaction. Chorismate, the common precursor for the synthesis of these amino acids is synthesized via 2 intermediates from the glycolytic pathway (Coruzzi and Last, 2000). Since Glu is the amino-group donor in these reactions, it is not surprising that lower contents of these two aromatic amino acids are coincident with the lowering of Glu in the cells. The changes with time in Phe and Trp are parallel, suggesting their common mode of regulation.

Although Val, Leu and Ile are synthesized in two different pathways (Fig. 12), they make up a functional group called branched-chain amino acids. The biosyntheses of Val and Ile follow a parallel path using different substrates but identical enzymes (reviewed in Binder et al., 2007). Leucine is then made from ketoisovalerate, the immediary precursor of Val biosynthesis. The branch point for Leu synthesis is presumably regulated by the enzyme isopropylmalate synthase (EC 2.2.3.13), which is feedback inhibited by Leu. The overall pathway for Ile biosynthesis is regulated at the first step of Thr deaminase (TD; EC 4.2.1.16) via feedback inhibition by Ile; Val on the other hand can overcome this feedback inhibition by Ile (Halgand et al., 2002).

A comparison of the profile of changes in Val and Ile accumulation in the HP cells shows an identical pattern; both rise during the first 24 h, decline during the next three days and rise again during the last three days of the culture period. A similar pattern of changes in these two amino acids is seen in the control cells; however, the two cell lines

differ from each other in their Val and Ile contents during the first three days of culture (Figs. 16B and 17E). Val is 5 to 6 fold higher than Ile at any given time, which is consistent with the argument that its biosynthesis is less subject to feedback inhibition by Ile than its own (Halgand et al., 2002). The observed changes with time are consistent with the explanation of Binder et al. (2007), in that a surge in the accumulation of Ile would inhibit TD; this will cause a reduction in its biosynthesis for the next few days until the inhibition is relieved and another surge in its accumulation can occur.

A similar explanation applies to changes observed in Leu, if one assumes feedback regulation at the first step starting at the branch point of ketoisovalerate as discussed above. While initial changes in Leu vs. Val/Ile are in opposite direction, from days 3 to 6, all three amino acids follow a similar pattern. In actively growing cells, the actual changes in the cellular amino acids are perhaps regulated by a combination of their biosynthesis and utilization in protein synthesis and in secondary metabolic reactions. It is important to point out that there is a strong regulation of the degradation pathway as well. With respect to the branched-chain amino acids, the biosynthesis and degradation are spatially separated into chloroplasts and mitochondria, respectively (Binder et al. 2007). Since most of these steps involve enzymes, each of them encoded by several genes, regulation of these reactions by substrates and products is probably an efficient way of metabolic homeostasis. Precise information about the number of genes encoding most of these enzymes and their spatial and temporal expression patterns in plants are not known; therefore, it is difficult to invoke the specific role of transcriptional and translational regulation of these enzymes.

As with the branched-chain amino acids, the two sulfur containing amino acids, Met and Cys are also synthesized in two separate pathways; the latter acts as the primary source of S for the former. While the biosynthesis of Cys is dependent on a series of S assimilatory reactions (Hoefgen and Hesse, 2007 and references therein), Met is a part of the Asp family of four amino acids, which is itself synthesized by transfer of NH_3 from Glu. Thus it can be argued that a reduction in the cellular content of Glu would affect this part of the pathway with three branches leading to the synthesis of Lys, Met, Thr and Ile (Fig. 12). The branching for Lys synthesis occurs early, and is controlled by dihyrodipicolinate synthase (EC 4.2.1.52) via feedback regulation (Coruzzi and Last, 2000). The lower Lys content in the HP cells is thus consistent with the possibility of Asp becoming a limiting factor for its biosynthesis (Fig. 17A). However, the regulation of Thr and Met accumulations is rather complex and depends on two enzymes (Thr synthase; EC 4.2.99.2 and Cystathionine γ-synthase - CGS; EC 4.2.99.9) with contrasting properties (Amir et al., 2002; Lee et al., 2005). The common substrate O-phospho-L-homoserine (HserP) plays a crucial role in the process as well. Since CGS has more than 200-fold lower affinity for HserP than TS, and TS is regulated mostly at the enzymatic level (rather than at transcription level – Casazza et al., 2000), it is not surprising that Thr biosynthesis continues while Met biosynthesis is drastically reduced. The first step of the entire pathway which produces HserP, is controlled by Asp kinase (EC 2.7.2.4), which is subject to feedback regulation by Lys as well as Thr. In the poplar cells used here, since the amounts of Thr are more than 10-folds greater than Lys, it is possible that the pathway is regulated more by Thr than by Lys. A rapid increase in Thr in the HP cells (from day 0 to day 2) was followed by a concomitant reduction in both Thr and Lys

between day 3 and day 7 of culture (Fig. 17); Met was always low (near the detection limits) in the HP cells.

Alanine, one of the simplest of the 20 amino acids, is a product of reductive amination of pyruvate, catalyzed by Ala aminotransferase (AT, EC 2.7.6.1) in a reaction where the amino group is donated by Glu (Coruzzi and Last, 2000). On several days of the week, Ala was the most abundant amino acid in both the control and HP cells. According to de Sousa and Sodek (2003), Ala is a major product of anaerobic metabolism in plants, caused by the induction of AT under conditions of low oxygen. Since the poplar cells used in this study are grown in liquid medium, perhaps under limited availability of oxygen, it is not surprising that Ala is the most abundant amino acid in these cells on most days of the week (Fig. 16C). Alanine naturally occurs in two forms: α and β form. While α -Ala is synthesized directly from pyruvate by AT (Coruzzi and Last, 2000), β-Ala in plants is believed to be produced by the degradation of Spd, propionate or uracil (Raman and Rathinasabapathi, 2004); its production from Spd/Spm also occurs in yeast (White et al., 2001) and animals (Urdiales et al., 2001). As early as 1978, Terano and Suzuki reported the conversion of Spd and Spm into β-Ala in maize. The fact that HP cells have higher Spd content on some days of the week (Fig. 6C) may explain the enhanced production of Ala in the HP cells (Fig. 16C).

Using a combination of mutants and transgenic plants of Arabidopsis, Lee et al. (2005) have further demonstrated that both CGS and TS are substrate-limited. A lower amount of Met in the HP cells is most likely due to the reduced availability of Cys, which is also required for Met biosynthesis. Since a major use of Met is in the production of SAM (Hoefgen and Hesse, 2007), the increased use of SAM in Spd production in the HP

cells may also have contributed to its low levels in these cell. While this argument is consistent with the observations of Aubert et al. (1998) with sycamore (*Platanus occidentalis*) and *Echinochloa* cell cultures, it is in contrast to the results of Lee et al. (2005) with Arabidopsis. Whether the differences are species-specific or due to the types of tissues being used in these studies (whole plants for *Arabidopsis* vs. cell cultures for others) is subject to speculation. Manipulation of both Lys and Met metabolism are the target of many studies, therefore, an answer to this question may determine the success of these attempts. Lee et al. (2005) have proposed that genetic manipulation of Met by overexpression of *CGS* may be more successful if it was accompanied by the presence of a feedback-insensitive Asp kinase.

The two sulfur amino acids (Cys and Met), although synthesized in two separate pathways, responded in a similar way to high Put production in the HP cells, indicating a potential common regulation, probably because Cys is a precursor of Met for the S moiety. It is therefore quite conceivable that reduction in Met in HP cells is at least partially due to the limitation of Cys. It is noteworthy that all three amino acids (Ser, Cys and Gly) which are produced from 3-PGA were reduced in the HP cells; again pointing to a common signal for their production/accumulation. It is possible that the increased flux of 3-PGA towards the TCA cycle (because α-ketogluterate is being excessively used for Glu production) affects not only the biosynthesis of these three amino acids but also those of Leu, Phe and Trp, which are produced from the downstream intermediates PEP and pyruvate. On the other hand, both Cys and Met may be lower simply due to their increased utilization in protein synthesis, particularly during the first 2-3 days of culture

in the fresh medium. Cysteine content recovers during the latter half of the week probably due to protein degradation.

Genetic manipulation of the biosynthesis of a single amino acid causing a change in the cellular content of almost all other amino acids has been previously reported. Simon-Sarkadi et al. (2005, 2006) showed that genetic manipulation to alter Pro in soybean subjected to simultaneous drought and heat stress altered the concentrations of several other amino acids, whether or not directly related to Pro metabolism. In their study, increase in Pro concentration as a result of simultaneous drought and heat stress was accompanied by concomitant increase in Glu, but a reduction in Arg. They concluded that Arg content decreased because of the higher utilization of Orn in making Pro. A simultaneous decrease in GABA content was also attributed to the increased utilization of Glu for Pro biosynthesis. These observations point to a competition among these subpathways for Glu, which does not appear to be the case in poplar cells. These authors also observed an increase in Asp in response to water deprivation in the Pro-overproducing transformants; which in turn resulted in enhanced accumulation of Lys, Met, Thr and Ile; all of them share Asp as an indirect precursor. Unfortunately, no data on PA content was provided in this study.

It is interesting to note that the soluble protein content of HP cells is significantly higher than in the control cells during the first few days after transfer to fresh medium. This will also affect the cellular contents of the various amino acids. Following the rapid loss of cellular proteins in the HP cells during the latter part of the week, the cellular content of many of these amino acids would increase. Alternatively, the enhancement in Put accumulation in the HP cells could significantly reduce the availability of the carbon

skeleton which is also required for amino acid production. The data presented here show that there indeed is a change in the total C:N ratio in the HP cells as compared to the control cells. While most of the carbon skeleton comes from glycolysis and TCA cycle, the recycling of Put through its increased catabolism in the HP cells (Bhatnagar et al., 2002) will also contribute carbon through succinate, which enters the TCA cycle following the breakdown of GABA (Figs. 12, 43).

Foyer et al. (2003) have reviewed the existence of signals regulating the organic C and N metabolism in plants and have pointed out that primary C and N assimilation in leaves is controlled by two important metabolic checkpoints, nitrate reductase (NR) and PEP carboxylase (PEPC). While, NR brings about the reduction of nitrate, one of the very first steps in N-assimilation, PEPC brings about the carboxylation of PEP to form OAA, feeding the anapleurotic TCA cycle that recycles the C-skeleton in the form of organic acids that provide precursors for amino acid biosynthesis. In HP cells, there is a higher utilization of both the available C and N for enhanced Put biosynthesis. Higher Pro content in the HP cells, when both its precursors (Glu and Orn) are being directed towards Put production, is enigmatic. However, it is consistent with the observation that both these metabolites often increase concurrently in response to a variety of abiotic stresses (Aziz and Larher, 1995; Aziz et al., 1998; Kocsy et al., 2005; Tonon et al., 2004). It has been argued in Chapter III that increased Put metabolism in HP cells puts them under a state of higher oxidative stress than the control cells.

A comment must be made with respect to the use of cell cultures vs. intact plants in terms of the consequences of genetic manipulation of biochemical steps of branched pathways. In most cell cultures which are subcultured into fresh medium over a short

regime, the changes in metabolites being measured are more dynamic and show almost daily variation depending on the nutrient supply and the growth phase of cells. In contrast, in terminal (non-growing) tissues and organs (e.g. endosperm, storage tissue in tubers, or mature leaves), steady state accumulations of metabolites could be quite different. Nevertheless, numerous studies have shown that the enzymatic properties and the regulatory mechanisms are often cell specific and under genetic control; thus cell cultures are highly suitable in revealing them, particularly if a combination of metabolite measurements, enzyme activities and gene expression analyses are combined to delineate these controls. It must be kept in mind that all interpretations are based on the assumption that in these cells, all substrates are freely available to the enzymes, while in reality; compartmentation of the different metabolites within a cell may be a hindrance to such free interactions.

In our cell cultures, we have observed through microarray analysis that there are only minor changes in the expression of genes (i.e. transcript levels) related to amino acid metabolism due to Put overproduction even though several hundred unrelated genes are up-or down-regulated (Page et al., unpublished data). Hence, it is proposed that most of the changes that we observed in amino acid content in relation to Put overproduction must be regulated at the enzymatic activity and/or metabolite levels, or by the changes in protein synthesis and degradation.

Conclusions

Although the pathways for the biosynthesis of various amino acids are regulated somewhat differently, each responding to a complex interaction of feedback inhibitory mechanisms and the availability of either inducible or constitutive enzymes, perturbation

in the biosynthesis or consumption of a single substrate like Orn has pleiotropic effects on the entire set of amino acid biosynthetic pathways. This is particularly intriguing since the amounts of Orn in the cell are rather low as compared to any other amino acid, although its consumption, therefore flux rate, is very high. Since the primary target of this study was the increased utilization of Orn in the *mODC* transgenic cells; it is remarkable that the accumulation of the entire set of amino acids in the cells was affected. Of particular interest are the concomitant increases in all amino acids that involve Orn as a potential substrate. We propose that cellular Orn levels may actually play a role as a sensor in the signal transduction pathway that regulates its biosynthesis from Glu. In turn, there may also be an increase in overall N assimilation in the plant cells.

CHAPTER III

POLYAMINES AND OXIDATIVE STRESS

There are numerous references in the literature emphasizing a role for PAs in aiding the cells overcome various forms of stress including oxidative stress (Løvaas, 1997; Nayyar and Chander, 2004; Foyer and Noctor, 2005; Papadakis and Roubelakis-Angelakis, 2005; Shevyakova et al., 2006). Arguments have been made in favor of the ability of PAs to scavenge free radicals and experimental demonstrations of the effects of exogenous PAs on reducing or combating oxidative stress have been reported (Løvaas, 1997; Papadakis and Roubelakis-Angelakis, 2005). There are few examples that directly demonstrate that increased PA biosynthesis increases the ability of plant or animal cells to overcome oxidative damage. While on one hand, PAs are believed to prevent the synthesis of ROS generation or ameliorate their presence, on the other, it is also known that the catabolism of PAs via DAO and PAOs itself generates H_2O_2, a common component of the oxidative damage machinery (Foyer and Noctor, 2005; Papadakis and Roubelakis-Angelakis, 2005; Shevyakova et al., 2006).

We have already seen that the modulation of Put metabolism has effects on the metabolism of compounds like Pro, whose cellular concentration has been known to increase under oxidative stress; and Glu, a precursor to the synthesis of Pro, Put and glutathione (GSH - an ROS scavenging molecule; Kocsy et al., 2005). This chapter includes results of the analysis of a series of enzymes that are characteristic of the

oxidative stress pathway in plants and the general health of the cells as studied by measurement of their mitochondrial activity and membrane integrity. The results presented here lead us to conclude that enhanced Put metabolism adversely affects the oxidative state of poplar cells in culture.

Introduction

Although ROS are deemed important for their roles as second messengers in signal transduction cascades, their adverse effects on cells are also well known (Navrot et al., 2007, and references therein). Termed also as AOS, they encompass superoxide (O_2^-) and hydroxyl radicals ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2) (Noctor and Foyer, 1998; Navrot et al., 2007). Reactive oxygen species are produced as byproducts of metabolic reactions in all parts of the cell, particularly in organelles like chloroplasts, mitochondria and peroxisomes. Each compartment has its own mechanism of regulating the synthesis of these compounds so as to prevent 'oxidative damage', a term used to define the collective harmful effects of these molecules (Apel and Hirtz, 2004). The antioxidative system within plant cells is comprised of several antioxygenic enzymes and metabolites that aid in processing reactions to rapidly break down ROS and protect the cell from oxidative damage.

As mentioned above, ROS play important roles in cell signaling, e.g. root gravitropism (Joo et al., 2001), regulation of plant cell growth (Foreman et al., 2003), defense reaction to pathogen attack, by what is known as the "oxidative burst" (Wojtaszek, 1997; Apel and Hirtz, 2004), and cellular responses to abiotic stress and in programmed cell death (Noctor and Foyer, 1998; Mittler, 2002; Navrot et al., 2007). The

generation of ROS in response to environmental changes has been widely studied (Mittler, 2002; Blokhina et al., 2003). Although ROS are postulated to be indicators of abiotic stress and to aid in defense against pathogens, their over-production can be harmful, causing oxidative stress in cells. The major damaging effect of most superoxide radicals involves the initiation of reactions resulting in the production of molecules such as lipid peroxidases. Oxidative stress can be induced by various factors, such as, drought (Moran et al., 1994), salinity (Hernández et al., 2001), light (Fryer et al., 2002), aluminum (Yamamoto et al., 2002), etc.

Several antioxygenic enzymes work in tandem in order to destroy toxic molecules like O_2^- and H_2O_2; these are glutathione reductase (GR), ascorbate peroxidase (APX), ascorbate oxidase (AO), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), superoxide dismutase (SOD) and catalase (CAT). Their roles in various reactions are shown in Fig. 20. Hydrogen peroxide oxidizes thiol groups rather rapidly, creating a disruption in the photosynthetic machinery by breaking down the thiol-regulated photosynthetic enzymes; hence its accumulation in the chloroplast must be prevented (Noctor and Foyer, 1998). Although CAT converts H_2O_2 into H_2O and O_2, H_2O_2 can also be broken down by peroxidases, especially in the absence of CAT (e.g. in the chloroplasts). Peroxidases need a reductant like reduced glutathione (GSH), reduced ascorbate (AsA), NADH or NADPH to reduce H_2O_2. Both GSH and AsA have been known to act as reductants in the ROS scavenging pathway in plant cells (Noctor and Foyer, 1998). The ascorbate-GSH cycle functions efficiently to bring about reactions that prevent oxidative stress in plants (Fig. 20). The reduction of H_2O_2 to H_2O and O_2 by APX generates monodehydroascorbate (MDHA), which gets (non-enzymatically) converted to

dehydroascorbate (DHA), which is then reduced to ascorbate by DHAR, using GSH as the reductant. Oxidized glutathione (GSSG), produced as a result is reduced to GSH by GR (Noctor and Foyer, 1998). Superoxide dismutase is present in chloroplasts, cytoplasm, and mitochondria and in peroxisomes (Mittler, 2002). While APX is believed to be present in these cellular compartments as well, catalase is localized to the peroxisomes (Mittler, 2002). Glutathione reductase is reported to be present in the cytoplasm, chloroplasts and mitochondria (Andersen et al., 1995). Dehydroascorbate reductase and MDHAR have been reported mostly in chloroplasts (Chen and Gallie, 2006).

There are several places where PAs have been suggested to interact with ROS generation or amelioration. This area of research has been followed both in plants as well as in animals (Papadakis and Roubelakis-Angelakis, 2005 and references therein). While on one hand, PAs are believed to prevent the synthesis of ROS generation, on the other, through their catabolism they potentially generate large quantities of these harmful oxidative molecules (Foyer and Noctor, 2005; Papadakis and Roubelakis-Angelakis, 2005; Shevyakova et al., 2006). Over the past 30 years, there have been several reports linking PAs with antioxidative properties. As early as 1979, Kitada et al. discovered that PAs inhibited lipid peroxidation in rat liver microsomes. Verma et al. (1979) reported that oxidative stress induced by UVB irradiation in mice led to an increase in activities of ODC and SAMDC. Hillebrand et al. (1990) also reported an increase in epidermal ODC in mice following UV radiation. Several members of ROS have been known to increase

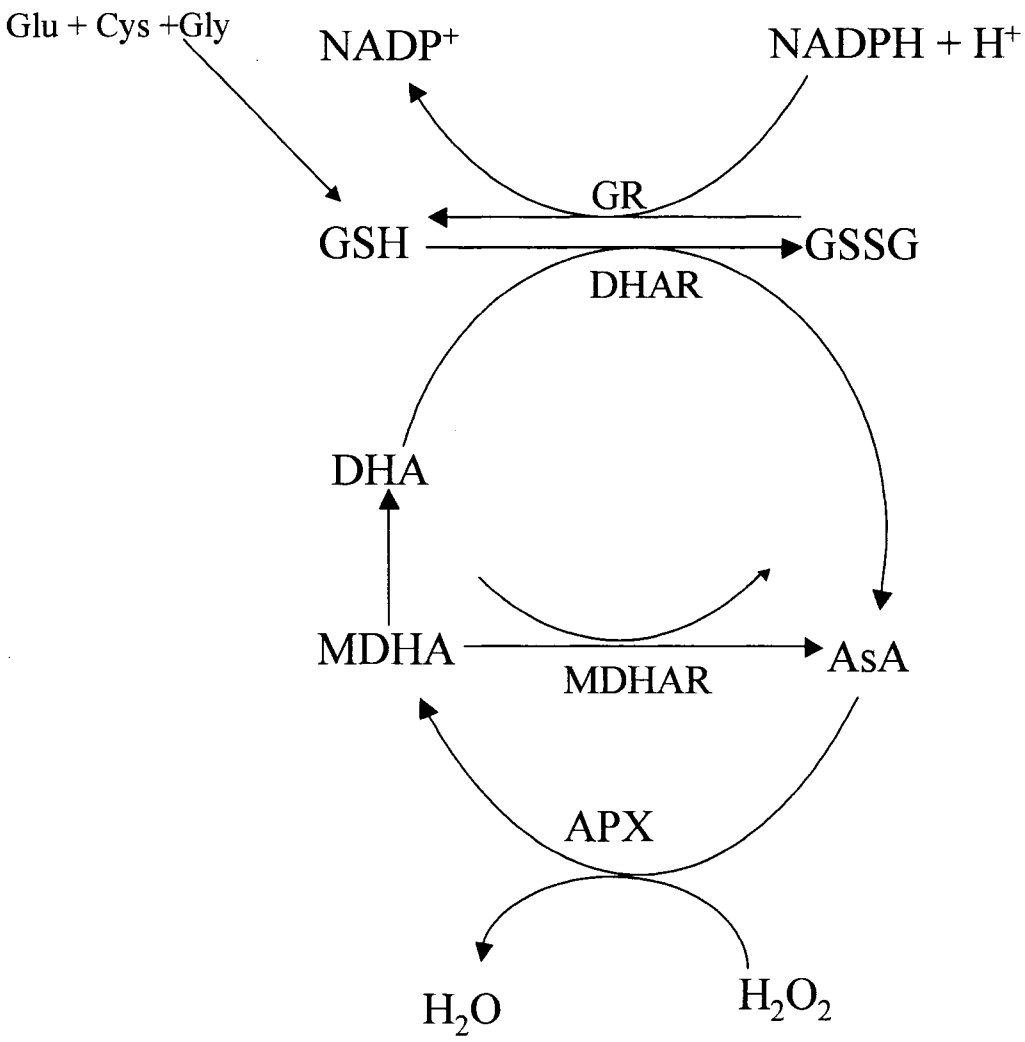

Figure 20. The ascorbate-glutathione cycle (adapted from Noctor and Foyer, 1998).

PA biosynthesis. These include $O_2^{\cdot-}$ (Fischer et al., 1988), O_3 (Langebartels et al., 1991) and peroxides (Binder et al., 1989; Harari et al., 1989).

Reports mentioning an uptake of PAs in response to oxidative stress have been made by Byers and Pegg (1989) in Chinese hamster ovary (CHO) cells and Toninello et al. (1992) in rat liver cells. Minton et al. (1990) and Balasundaram et al. (1993) reported that cells defective in PA biosynthesis are exceedingly sensitive to oxygen. Khan et al. (1992) and Muscari et al. (1995) observed that PAs play a role in protection of isolated DNA against oxidative damage. Løvaas (1997) has elucidated the chemistry which may make PAs potential scavengers of ROS, although he points out that PAs are inefficient scavengers of ROS as compared to other antioxidants like Asc. He states that among the different oxidants of the ROS family of molecules, PAs can effectively scavenge only O_3.

Borell et al. (1997) studied the inhibitory effect of PAs on lipid peroxidation in senescing oat leaves. They found that exogenously supplied diaminopropane, Spd, Spm and guazatine (an inhibitor of PAO) inhibited the loss chlorophyll and also decreased the levels of malondialdehyde (a product of lipid peroxide decomposition) in dark-incubated and osmotically stressed oat leaves. Spermine also decreased the activity of the enzyme lipoxygenase (EC 1.13.11.12) which is believed to be involved in membrane lipid peroxidation during plant senescence. They suggested that the anti-senescence effects of PAs may be occurring through the inhibition of lipid-peroxidation. As mentioned in general introduction, most types of stresses, induced by temperature, drought or chemicals, are responsible for ultimately affecting the antioxidative machinery by inducing the generation of ROS. Stress response of PA metabolism in light of oxidative stress has been studied by Nayyar and Chander (2004) who studied the role of PA

accumulation under cold stress in chick pea and reported an enhancement in Put content in response to the same.

The catabolism of PAs generates H_2O_2 which can act as a potential oxidant (Pappadakis and Roubelakis-Angelakis, 2005). Despite this direct correlation between PA catabolism and oxidative stress, a role of PAs in ameliorating the harmful effects of ROS has been suggested. Polyamines can do so either by direct interaction with the oxidative species (Løvaas, 1997) or indirectly, e.g. by inhibiting the generation of NADPH-oxidase meditated ROS in membranes (Papadakis and Roubelakis-Angelakis, 2005 and references therein). Kubiś (2005) studied the effects of exogenous Spd on activity of SOD and levels of oxidants like H_2O_2 and superoxide radical in barley leaves subjected to drought stress and observed that PA treatment caused a decrease in the contents of the two toxic molecules. Tang and Newton (2005) observed that PAs decreased salt-induced oxidative damage by increasing the activities of antioxidative enzymes and decreasing lipid peroxidation in Virginia pine. Verma and Mishra (2005) observed that Put alleviated growth in salt stressed *Brassica juncea* by stimulating an antioxidative defense system.

In the high Put (HP) poplar cells used here, Put catabolism via diamine oxidase (DAO), which generates H_2O_2, has been shown to keep pace with its increased biosynthesis (Bhatnagar et al., 2002). It was concluded that in these cells the rate of Put catabolism is proportional to the rate of its biosynthesis and increased Put degradation occurs without significant changes in the extractable DAO activity. It is known that cells in culture are exposed to oxidative stress by what has been termed by Halliwell (2003) as "culture shock". We hypothesize that modulation of Put metabolism also has an effect on

the metabolism of related compounds like Pro, whose cellular concentration has been known to increase under oxidative stress; and Glu, a precursor to the synthesis of Pro, Put and GSH (Kocsy et al., 2005). This would in turn affect the oxidative state of the cells. Thus there is a strong rationale for investigating the effects of enhanced putrescine turnover on the activities of ROS scavenging enzymes and related effects of ROS production. The results presented here lead us to conclude that enhanced Put metabolism changes the oxidative state of poplar cells in culture.

Materials and Methods

Glutathione reductase (EC 1.6.4.2) assay

The method of Schaedle and Bassham (1977) as described by Jahnke et al. (1991) was slightly modified to extract and assay this enzyme. The technique involves measuring oxidation of NADPH by the enzyme, consequently resulting in the reduction of the substrate, glutathione disulphide (oxidized glutathione or GSSG). For enzyme extraction, 100 mg (FW) of cells were collected in 200 µL of 50 mM potassium phosphate (K-Pi) buffer (pH 7.0) containing 0.2 mM diethylenetriamine pentaacetic acid (DTPA). After a round of freezing (-20 °C) for 2 h and thawing (on ice) for 30 min, the mixture was vortexed for 5 min and centrifuged at 16,000 xg for 10 min. To 50 µL of the supernatant, 850 µL of K-Pi buffer (25 mM, pH 7.8 with 0.2 mM DTPA) was added. Then, 50 µL of 3 mM NADPH (Sigma, N7505) made in 3 mM NAOH was added. Change in absorbance with time due to oxidation of NADPH was monitored for 30 to 50 sec with U-2000 Spectrometer (Hitachi Instruments Inc., Schaumburg, IL). This was followed by addition of 50 µL of 10 mM GSSG (Sigma, G4376) and the rate of its reduction (to GSH) was monitored by measuring the change in absorbance again for 30

sec. This rate of change of absorbance was subtracted from the one determined in the absence of GSSG. Enzyme activity was expressed as μmol NADPH oxidized min^{-1} g^{-1} FW; calculations were done by using the millimolar extinction coefficient of NADPH (millimolar ε_{340} = 6.2; Jahnke and White, 2003). Specific activity of the enzyme is expressed in terms of μmol NADPH oxidized min^{-1} mg^{-1} protein.

Ascorbate peroxidase (EC 1.11.1.11) assay

The method of Nakano and Asada (1981) as described by Jahnke et al. (1991) was modified to assay this enzyme. Briefly, 100 mg cells were collected in 400 μL of 50 mM K-Pi buffer (pH 7.0) containing 0.2 mM DTPA. After freezing, thawing, and centrifugation as above, 50 μL of the supernatant was mixed with 850 μL K-Pi buffer. After zeroing the spectrophotometer at 290 nm, 25 μL of 10 mM ascorbic acid (Sigma, A7506) was added, followed by 50 μL of 10 mM H_2O_2. The decrease in absorbance with time due to oxidation of ascorbate was monitored for 30 sec. Enzyme activity was expressed in terms of μmol ascorbate oxidized min^{-1} g^{-1} FW and calculations were done using the millimolar extinction coefficient of ascorbate (millimolar ε_{290} = 2.8; Jahnke and White, 2003). Specific activity of the enzyme is expressed in terms of μmol ascorbate oxidized min^{-1} mg^{-1} protein.

Monodehydroascorbate reductase (EC 1.6.5.4) assay

Cell free extracts were made from 100 mg cells in 200 μL of 50 mM K-Pi buffer (pH 7.0) containing 0.2 mM DTPA as described above. To 850 μL of K-Pi buffer (25 mM, pH 7.8 with 0.2 mM DTPA), 25 μL of this extract was added, followed by 50 μL 50 mM ascorbic acid. This was used to blank the spectrophotometer. Following the addition of 50 μL of 3 mM NADH (Sigma, N8129) made in 3mM NaOH, the absorbance at 340 nm

was measured for 30 sec (modified from Hossain et al., 1984). This was followed by the addition of 25 µL of ascorbate oxidase (12 U mL^{-1}; Sigma, A0157). The slope of change in absorbance was subtracted from the slope with the addition of NADH alone. Activity was expressed in terms of µmol NADH oxidized min^{-1} g^{-1} FW and calculations were done using the millimolar extinction coefficient of NADH at 340 nm (millimolar ε_{340} = 6.2; Matsushita et al., 1987). Specific activity was calculated in terms of µmol NADH oxidized min^{-1} mg^{-1} protein.

Measurement of mitochondrial activity, cell viability (membrane integrity) and cellular contents of PAs, amino acids, GSH and ions were done as described under "General Materials and Methods".

Results

The cellular PA contents over the 7 day culture period are presented in Fig. 6 (For details, see Chapter I).

Total soluble protein

Cellular contents of total soluble (buffer extractable) protein (g^{-1}FW) were significantly higher in the HP than in the control cells on days 1 through 4 (Fig. 21). Starting with similar amounts of soluble protein on day 0 (the same as day 7), the HP cells showed a rapid and significant increase in protein content with a peak on day 2, followed by a rapid decline during the next 3 days. On the contrary, in the control cells, there was only a small fluctuation in protein content over the 7 day culture cycle. For the last 3 days of culture, protein content g^{-1}FW was similar in the two cell lines.

Standardization of glutathione reductase activity

Before starting experiments involving the 7-day activity profile of GR in the control and HP cells, its activity was characterized in 4-day-old cells. Decrease in absorbance due to oxidation of NADPH was measured after 5 min of incubating the cells with the buffer and the substrate and the result is represented in Fig. 22A. The rate of change of absorbance was several-fold higher in the HP cells than the control cells. For determining the linear range of the slope due to decrease in absorbance (caused by the oxidation of NADPH by GR), a time-course scan was performed in both cell lines for 250 sec and it was determined that for ~50 sec, the slope was perfectly linear in the HP cells while the linearity was maintained for as long as ~200 sec in the control cells (Fig. 22B). Diluting the HP cell extract caused a proportionate decrease in the rate of change in absorbance (Fig. 22C).

Glutathione reductase (GR)

The activity of GR was several folds higher in the HP cells than the control cells on the first four days of the week (when calculated as units.g^{-1} FW) and on all seven days when calculated as specific activity (units.mg^{-1} protein) (Fig. 23A, B). The data were statistically significant for all days of the week. The peak of enzyme activity (g^{-1}FW) was seen on day 3 following transfer of cells to fresh medium. The lowest amount of activity calculated either way was seen on day 6 or 7. The HP cells showed a significant surge in enzyme activity within 24 h of transfer to fresh medium; on the other hand, the control cells showed little variation in GR activity over the 7 day culture cycle.

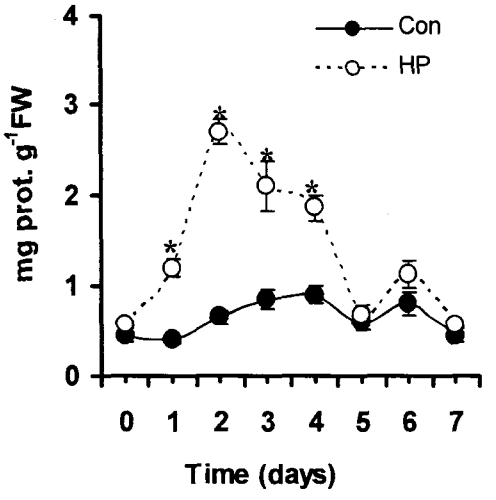

Figure 21. Cellular contents of total proteins in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P \leq 0.05$) between the cellular protein content between the control and HP cells on a given day of the 7-d culture cycle.

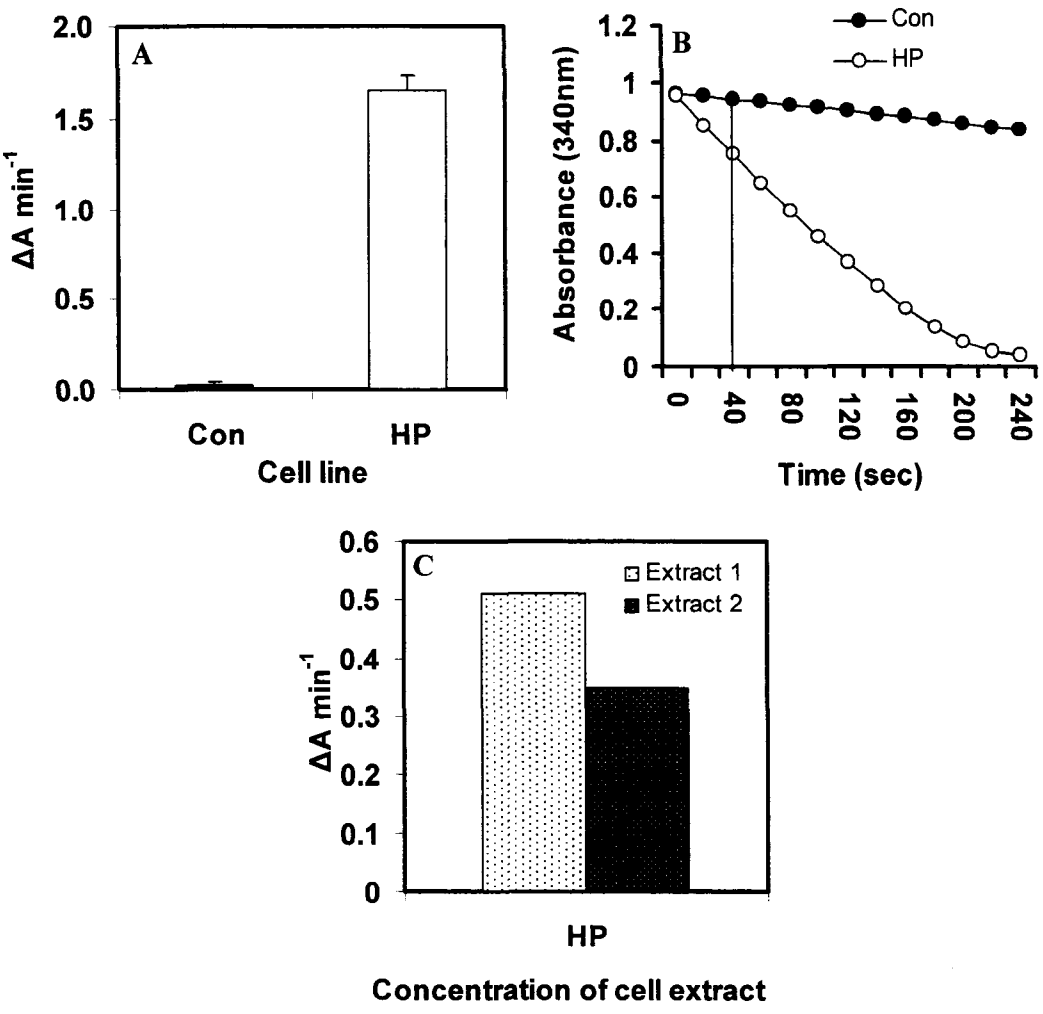

Figure 22. Standardization of GR activity in the control and HP cells. (A) Change in absorbance with time (measured for 5 min) in the control and HP cells on day 4 of the 7-d culture cycle. (B) The slope of Absorbance vs. Time in the HP cells was found to be perfectly linear for ~50 sec. (C) Change in absorbance with time in 4-day-old HP cells at different concentrations of the cell extract where "Extract 2" is twice as dilute as "Extract 1".

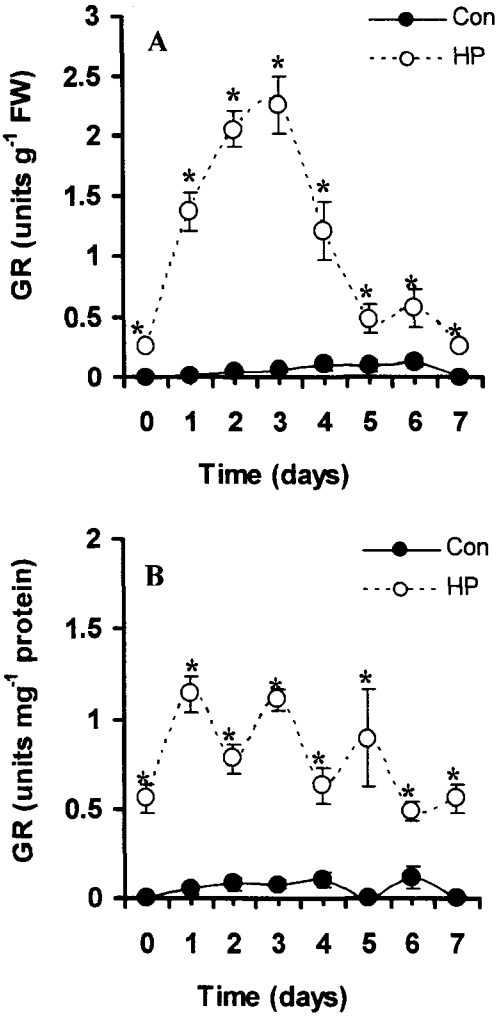

Figure 23. (A) Activity (g^{-1} FW) and (B) specific activity (mg^{-1} protein) of GR over the 7-d culture cycle in control and HP cell lines. Data are mean (±) SE of 6 replicates from 2 experiments. One unit is defined as 1 µmol NADPH oxidized min^{-1}. An * indicates a significant difference ($P<0.05$) in the enzyme activity (g^{-1} FW) or specific activity (mg^{-1} protein) between the 2

Ascorbate peroxidase (APX)

Significant difference in the activity of APX (units.g^{-1} FW) was seen between the HP and the control cells on some days of the 7 day culture cycle (Fig. 24A); in HP cells, a decrease in APX activity was seen after day 3. In the control cells, enzyme activity remained higher than in the HP cells during the last 3 to 4 days of the week (Fig. 24A). Due to significant differences in the protein contents of the two cell lines (Fig. 21), statistically significant differences in the specific activity of APX were seen on all days of the 7 day culture cycle; the specific activity of APX being higher in the control than the HP cells (Fig. 24B). A several fold increase in APX activity (g^{-1} FW) was seen within 48 h of transfer to fresh medium in both cell lines. Coincident with the increase in soluble protein content, HP cells showed a small decline in APX specific activity within a day after transfer to fresh medium.

Monodehydroascorbate reductase (MDHAR)

As with GR, MDHAR activity was also higher in the HP than the control cells, although significantly only on days 2, 3 and 4 (Fig. 25A). In both cell lines a peak of enzyme activity (units.g^{-1} FW) was seen around midweek. When MDHAR activity (g^{-1} FW) was normalized to specific activity (Fig. 25B), the variation over time in both cell lines over the entire week of study was smaller and so were the differences between the two cell lines on days 2 to 4. This was obviously due to the peak of MDHAR activity in HP cells coinciding with the peak of soluble protein content in these cells.

Glutathione

Biosynthesis of GSH uses the amino acids Glu, Cys and Gly (Noctor et al., 1998); the first two combine to form γ-glutamylcysteine (γ-EC) in an ATP dependent reaction

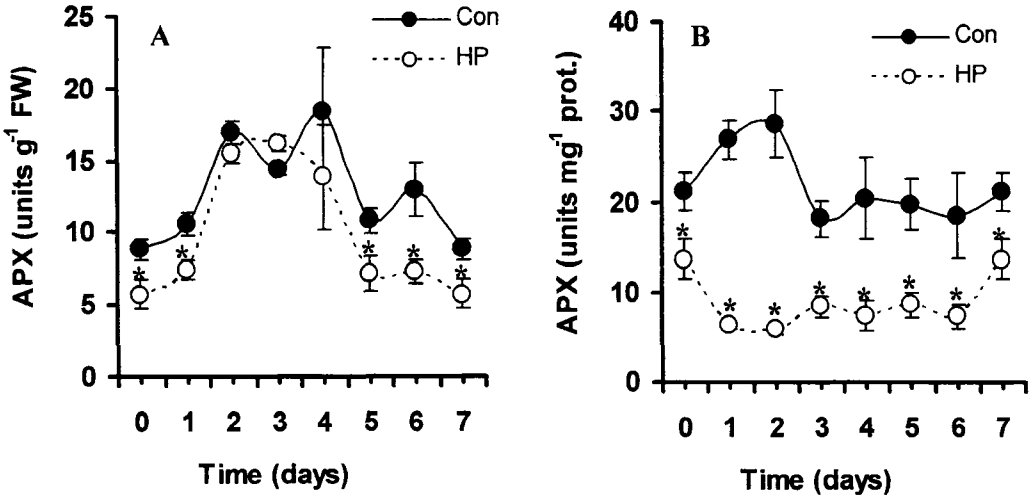

Figure 24. (A) Activity and (B) specific activity of APX over the 7-d culture cycle in control and HP cell lines. Data are mean (±) SE of 6 replicates from 2 experiments. One unit is defined as 1 µmol ascorbic acid oxidized min^{-1}. An * indicates a significant difference ($P<0.05$) in the enzyme activity (g^{-1} FW) or specific activity (mg^{-1} protein) between the 2 cell lines on a given day.

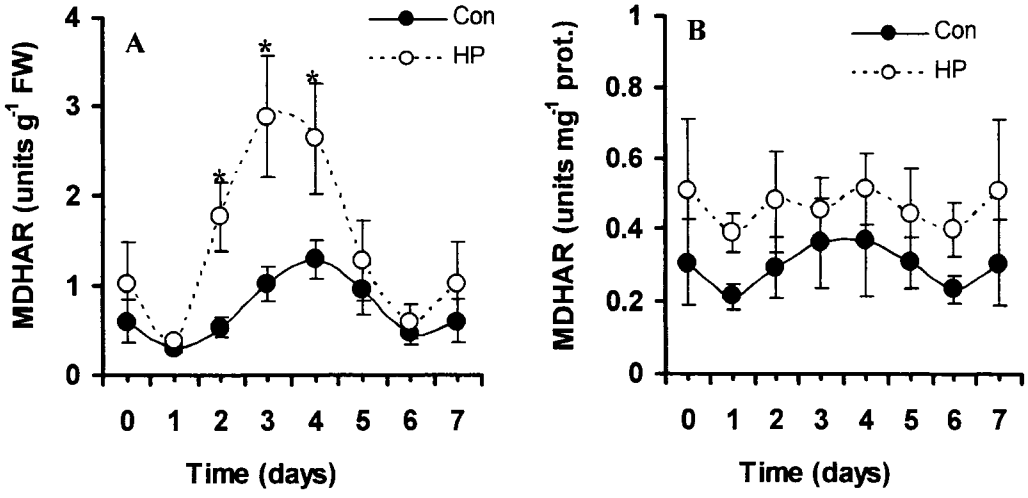

Figure 25. (A) Activity and (B) specific activity of MDHAR over the 7-d culture cycle in control and HP cell lines. Data are mean (±) SE of 6 replicates from 2 experiments. One unit is defined as 1 µmol NADH oxidized min^{-1}. An * indicates a significant difference ($P<0.05$) in the enzyme activity (g^{-1} FW) or specific activity (mg^{-1} protein) between the 2 cell lines on a given day.

catalyzed by γ-EC synthetase. Following this, γ-EC combines with Gly in another ATP dependent reaction catalyzed by the enzyme GSH synthetase to produce GSH. We measured the cellular contents of GSH over the entire 7 day culture cycle. Cellular contents of GSH were significantly lower in the HP than in the control cells on each of the 7 days (Fig. 26). There was no effect of transfer to fresh medium and the GSH amounts did not change appreciably with time over the 7 day culture cycle.

Accumulation of Ca and K

Figures 27A and B compare the accumulation of PCA soluble Ca and K in the control and the HP cells over the 7 day culture period. The accumulation of Ca in the HP cells was several-fold higher in the HP cells as opposed to the control cells for most of the 7 day culture cycle, being significantly so on days 1, 2, 3, 4 and 7 days. On the other hand, an opposite response was seen for the accumulation of K in these cells in that, it was significantly lower in the HP cells as compared to the control cells for the entire culture period. A distinct and significant fresh medium effect on the uptake of K was seen in both cell lines within 24 h of transfer, with a gradual decline in the same thereafter; i.e. from day 1 through day 7.

Mitochondrial activity and membrane function

Colorless MTT interacts with the mitochondrial electron transport chain and gets reduced to form a blue colored product called formazan (Mosmann, 1983; Minocha et al., 2001). Thus, when the overall mitochondrial oxido-reductase activity is higher, the intensity of blue color is proportionately higher. As shown in Fig. 28A, the mitochondrial activity was generally comparable in the two cell lines, it being slightly lower (but not significantly so) in HP cells than in the control cells on days 3 and 4 of culture.

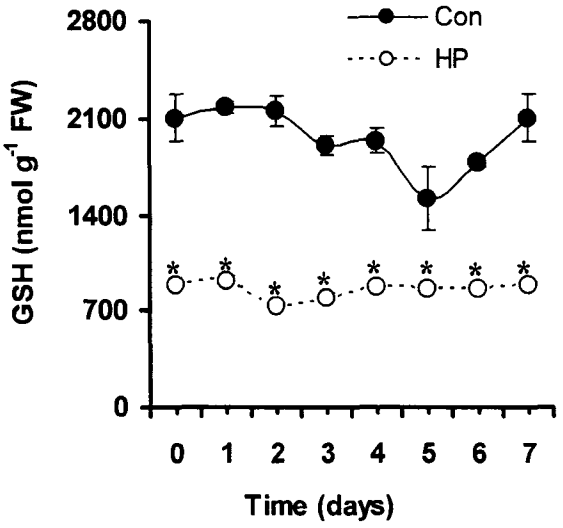

Figure 26. Cellular contents of GSH in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the GSH content between the 2 cell lines on a given day.

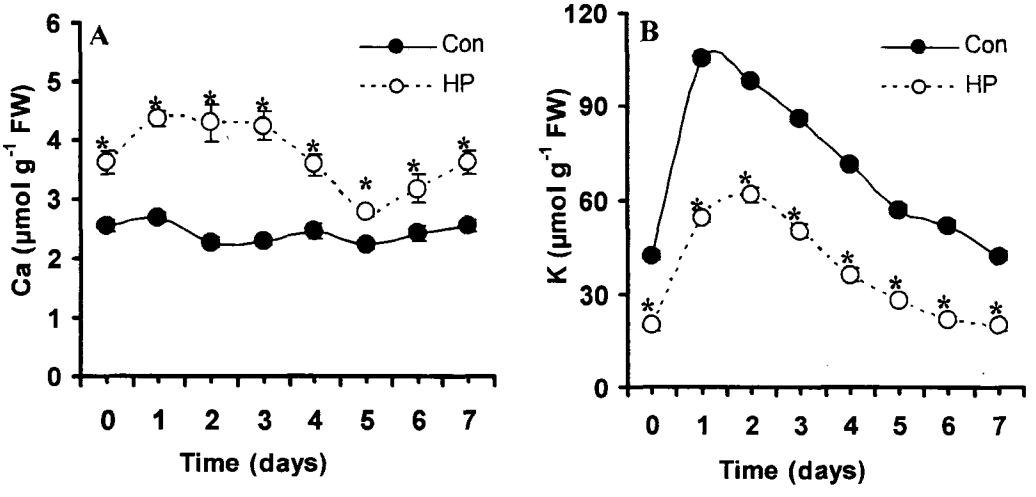

Figure 27. Cellular contents of (A) Ca and (B) K in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the ion content between the 2 cell lines on a given day.

Evans Blue is a non-permeating dye that can enter the cells only through a damaged (depolarized) plasma membrane, thus staining the contents of membrane-compromised cells (Mosmann, 1983; Minocha et al. 2001). Hence, higher the number of membrane-compromised cells, greater is the absorbance of the supernatant obtained after allowing the cells to absorb the dye and then releasing it with SDS. Evans Blue retention (absorbance g^{-1} FW) was significantly higher in the HP cells than the control cells on all three days of analysis (Fig. 28B), indicating a significantly higher number of membrane-compromised HP cells in the culture. But the membrane integrity of HP cells appears to improve with time between day 3 and 5, as seen by lowering of the dye retention by the cells. Evans Blue retention in the control cells remained practically unchanged from day 3 to day 5.

Discussion

The postulated association of PAs with oxidative stress has a long history (Kitada et al., 1979; Russo et al., 1985) and has received considerable attention in recent years (Walters, 2003; Nayyar and Chander, 2004; Papadakis and Roubelakis-Angelakis, 2005; Shevyakova et al., 2006). Papadakis and Roubelakis-Angelakis (2005) found that all three major PAs are responsible for suppressing ROS generation in tobacco cultures, possibly by inhibiting the microsomal membrane NADPH oxidase-mediated generation of $O_2^{\cdot-}$. They also found that a supply of exogenous Put ameliorated the toxic effects of H_2O_2 generated via increased PA catabolism. Nayyar and Chander (2004) found an increase in PAs as a response to cold and water stress in chickpea (*Cicer arietinum* L.), along with an enhanced accumulation of ROS scavenging metabolites like AsA and GSH on different days of the week.

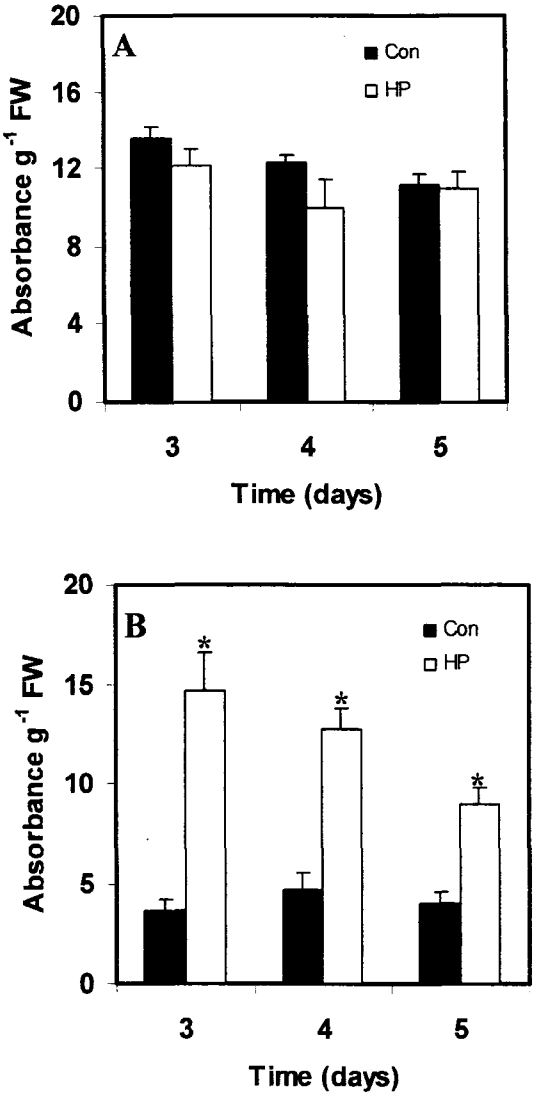

Figure 28. (A) Mitochondrial activity as measured by MTT reduction and (B) cell viability as measured by Evans blue retention in the control and HP cells. Each bar represents mean (±) SE of 24 replicates from 8 experiments in (A) and 9 replicates from 3 experiments in (B). An * indicates a significant difference ($P<0.05$) in the absorbance g^{-1} FW between the 2 cell lines on a given day.

Polyamines have been known to mediate programmed cell death and oxidative burst in plant cells as a response to several environmental factors like pathogen attack. A constitutive increase in Put accumulation as well as catabolism in the HP cells (Bhatnagar et al., 2001, 2002) has probably made them prone to greater damage by enhanced oxidative stress, but as pointed out above, high Put could also be instrumental in ameliorating its own adverse effects. Therefore, it was deemed important to examine the level of oxidative stress in poplar cells whose PA content has been altered through genetic manipulation.

Biochemical indicators of greater ROS generation (and therefore higher oxidative stress) in plant cells include a higher activity of ROS scavenging enzymes such as GR, MDHAR, APX, CAT, etc. (Kocsy et al., 2005). The redox state of a cell is further indicated by pools of oxidized and reduced forms of antioxidants like GSH, ascorbate, Pro, and PAs. The presence of large pools of these antioxidants is responsible for the regulation of redox homeostasis in the cells (Foyer and Noctor, 2005). Increased accumulation of the reduced metabolites like AsA and GSH also points to lower oxidative stress and thus, better cell health. From the data presented here, it is apparent that not only are the activities of antioxidative enzymes GR and MDHAR higher in the HP cells as opposed to the control cells, but also that the cellular content of the ROS scavenger GSH is lower than the control cells on all days of culture. These data, coupled with slightly lower mitochondrial activity (Fig. 28A) and increased membrane damage (Fig. 28B) in the HP cells, point to a state of higher oxidative stress in these cells as opposed to the control cells.

In general, biosynthetic metabolism of HP cells is much higher during the first two days after transfer to fresh medium as exemplified by rapid increase in protein biosynthesis (Fig. 7, 21, 51C), the accumulation of Pro (Fig. 13D), the expression of several PA biosynthetic enzymes (Fig. 8-10) and the accumulation of PAs (Fig. 6); most of them showing a rapid decline afterwards. As mentioned above, Evans Blue is a non-permeating dye that can enter a cell only through a damaged plasma membrane. The absorbance of Evans Blue by the cells significantly decreased from day 3 to day 5 in the HP cells, indicating a recovery in cell health with time. This can be attributed to a higher production of ROS in the HP cells on day 3 as opposed to day 5. The activities of enzymes GR and MDHAR are not only higher in the HP cells than the control cells (for most of the 7-day culture cycle), but also within the HP cells, the activities of these enzymes increase significantly from day 1 to day 3. These data correlate well with the Evans Blue retention data to possibly suggest that these cells experience the highest level of oxidative stress between days 2 and 4. This is followed by a rapid decline in soluble protein content in the HP cells. It is postulated that this decrease in soluble proteins is due to increased proteolysis. The fact that the HP cells are experiencing higher growth and metabolism during days 2 to 4; this could lead to the generation of higher amounts of ROS as by-products of several of these metabolic reactions, including an enhanced turnover of PAs. But there is also a higher scavenging of these molecules during this period as indicated by the higher activities of GR and MDHAR. These patterns are not seen in the control cells on different days of the week; they maintain a rather steady state of these molecules, and also of total soluble proteins. Whether or not higher fluctuations in soluble proteins and the other metabolites on different days of culture are a

consequence of enhanced biosynthesis and turnover of PAs is not clear. While it is not plausible at present to suggest a mechanistic model of signal transduction for the rapid changes in protein content of HP cells, it can be argued, however, that a major difference between the two cell lines is that on transfer to fresh medium, the HP cells undergo a flurry of metabolic activity as a result of increased activity of ODC (due to transcription as well as translation of the m*ODC* gene). Consequently, increased Put production is accompanied by its increased turnover, resulting in a situation where a protector molecule (i.e. Put) actually becomes a contributor to the oxidative stress in these cell; and hence an enhancement of protein degradation.

Although AsA has a direct role in scavenging reactive oxygen species, it is the cascade of reactions following oxidation of AsA that reduces GSSG to GSH (Fig. 20). While GR uses NADPH to reduce GSSG to GSH, various free radicals and oxidants are able to oxidize GSH back to GSSG (Noctor et al., 1998). Also, as a response to high oxidative stress, greater recycling of AsA in the HP cells might be responsible for the lower level of GSH in these cells. This causes a change in the normal redox state of the cells, leading to decline in cell health in the HP cells as opposed to the controls. Glutathione and AsA are important metabolic indicators of the oxidative state of a cell. As mentioned above, APX activity (gFW^{-1}) was significantly higher in the control cells on some days of the week (Fig. 24A), while both the GR (Fig. 22A) and MDHAR (Fig. 25A) activities were higher in the HP cells on almost all days of the week. This shows the rate of H_2O_2 scavenging via APX is higher in the control cells than in the HP cells. But, AsA recycling from MDHA is higher in HP cells, potentially resulting in higher GSH metabolism. Hence the question arises; *what happens to the excess AsA in HP cells if it is*

not being used up to scavenge H_2O_2 via APX? Pignocchi et al. (2003) have argued that in plant cells, although AsA is localized mostly in the cytoplasm, a portion of it is transported to the apoplast where the first line of defense against antioxidants is generated. This AsA is then oxidized in the apoplast to MDHA by ascorbate oxidase (AO); the unstable MDHA being rapidly converted into DHA and AsA. Thus, in the HP cells, the high MDHAR activity could be a consequence of high AO activity. Furthermore, it is possible that, although in the control as well as HP cells a similar amount of AsA is being oxidized by APX to produce MDHA, there is higher MDHA production in the apoplast of HP cells by AO, thus providing additional substrate for MDHAR activity, ultimately resulting in the higher recycling of AsA as explained below.

Ascorbic acid plays a multitude of roles in plant cells. Not only is it a major contributor to scavenging of ROS by its rapid conversion into MDHA via APX or AO, but it also acts as a cofactor in the hydroxylation of prolyl and lysil-residues by peptidyl-prolyl and -lysil hydroxylases, playing a role in cell wall synthesis and in cell division (Conklin, 2001). Smirnoff (1996) and Conklin (2001) have suggested a role of AsA and AO in the regulation of cell expansion. There is evidence that the cell wall generates MDHA via AO and reduces it back to AsA by a plasma membrane-bound, NADPH-requiring cytochrome b (Horemans et al., 2000). Ascorbic acid thus produced is transported to the apoplastic free space, a process that has been reported to aid in cell expansion. Smirnoff (1996) also suggested that AsA and MDHA in the cell wall aid in controlling cell expansion by helping in regulation of the cross-linking of cell wall proteins and polysaccharides, lignification and Ca levels. The fact that MDHAR activity is mostly high in the HP cells on days 2 and 3 of the 7-day culture cycle, a period when

cell division is in the logarithmic phase, supports the proposal that increased MDHAR activity is a consequence of increased AO activity in the cell walls of HP cells. It has been shown in tobacco cells that during cell elongation, AsA and the apoplastic activity of AO increase (Kato and Esaka, 1999). It is unclear as to what other factors may stimulate the production of MDHA via AO as opposed to APX in the HP cells. The oxidation of AsA by AO in the apoplast and by APX and AO in the cytoplasm suggests that the reduced and oxidized forms of ascorbate are transported between the two compartments so as to keep a balance in the redox state of the cell. It is possible that in the HP cells, greater recycling of AsA occurs in order to increase the redox state, thus compensating for the lower accumulation of GSH as a means to minimize the damage caused due to high oxidative stress.

Biosythesis of GSH occurs by an ATP utilizing reaction which combines γ–glutamyl cysteine (γ-EC) with Gly, brought about by the enzyme GSH synthetase (Noctor et al., 1998). Biosynthesis of γ-EC is catalyzed by γ-EC synthetase which involves the condensation of Glu with Cys. As pointed out earlier, the cellular contents of Glu (Fig. 13A), Cys (Fig. 14C) and Gly (Fig. 14B) are different in the two cell lines on all days of the week. As discussed previously, the difference in Glu in the two cell lines is perhaps a consequence of its increased utilization in the production of Put in HP cells. The low cellular content of GSH in the HP cells (Fig. 26) could then also be the result of a reduction in the availability of its precursor molecules, i.e. Glu, and also, Cys and Gly. Lower Cys and Gly in HP cells may be the consequence of either a reduction in their biosynthesis or utilization in increased protein synthesis soon after transfer to fresh medium. Higher GR activity in the HP cells points towards a greater requirement of the

cell to recycle GSH from GSSG, probably in an attempt to scavenge the higher quantities of ROS generated in these cells. To further complicate this interaction, it has been shown that stress-induced alterations in Pro may also influence the amount of GSH, and hence, the activity of GR, since Glu is a common precursor for both (Kocsy et al., 2005). The HP cells during the first few days do have higher amounts of Pro as well. While a probable reason for higher amounts of Pro in the HP cells during the first two days is an overall increase in biosynthetic metabolism on transfer to fresh medium, this time is also coincident with the increase in protein accumulation and inversely related to decrease in Glu, which is its precursor.

The observed increase in cellular Ca and a decrease in K are additional indicators of heightened oxidative state of the HP cells. Foreman et al. (2003) demonstrated that an inwardly rectifying Ca^{2+} channel was activated by membrane-associated NADPH oxidase-generated $OH^{\cdot}$ radicals; this activation did not occur in response to treatments with either H_2O_2 or Cu^{2+} or ascorbate. This activation caused increased Ca accumulation in root cells in the elongation zone. Pei et al. (2000) demonstrated that the Ca^{2+} channel can actually be activated by H_2O_2 in the guard cells of *Vicia faba*. Bowler and Fluhr (2000) had earlier suggested a connection between the ROS and the signal transduction pathways involving cytosolic Ca. Demidchik et al. (2003) observed a simultaneous activation of the Ca_{in} and K_{out} channels in response to ROS and postulated that the activation of these channels was regulatory in nature and not due to a loss of membrane permeability. A similar activation of Ca and K channels by ROS has been demonstrated in animal cells as well (Kourie, 1998). Thus it can be argued that the loss of membrane integrity and a concomitant increase in Ca and a decrease in K accumulation in the HP

cells observed here are not merely the harmful effects of increased ROS activity; rather these effects are mediated by independent mechanisms. Moreover, since $OH^{\cdot}$ itself is not membrane permeable (Demidchik et al., 2003), it is possible that either these radicals are being produced in the apoplast or it is the H_2O_2 produced by PA oxidation that is responsible for this response (Pei et al., 2000).

It can further be suggested that while the Ca influx and K efflux may be due to the activation of specific channels, the loss of membrane integrity as seen by Evans blue retention in HP cells may be due to the harmful effects of ROS on lipid peroxidation in the membrane, which is a well known phenomenon both in animal and plant cells (Stark, 2005). Stark has further pointed out that the increased accumulation of Ca may also be due to depolarization of the membrane potential, and may actually be the cause of cell death. While an interaction of ROS with plasma membrane Ca^{2+} channels seems widely documented, only a few reports have explored a strong and specific interaction of ROS with voltage-sensitive outward rectifying K^+ efflux channels (Demidchik et al., 2003; Shabala, 2006; Cuin and Shabala, 2007). This interaction leads to an efflux of K from the cells; our data on lower accumulation of K in the HP cells vs. the control cells is consistent with this argument. A further effect of ROS that would non-specifically influence the cellular ionic imbalance as well as result in increased permeability to the Evans blue dye could be due to the known effects of ROS on membrane lipid peroxidation (Kourie, 1998; Stark, 2005; Cuin and Shabala, 2007, and references therein).

Conclusions

Based on the vast amount of literature pointing to contrasting roles of ROS in plants (i.e. a key function in signal transduction, opposite effects on Ca^{2+} and K^{+} transport, and harmful effects on membrane peroxidation), it is not surprising that a somewhat contradictory effect of enhanced PA metabolism is seen in poplar cells. While the past discussion on PAs has generally emphasized their positive role in ROS scavenging and increasing plant tolerance to a variety of abiotic stress responses in plants (Foyer and Noctor, 2005; Papadakis and Roubelakis-Angelakis, 2005; Shevyakova et al., 2006), we found that an overproduction of PAs could actually be detrimental to the cells, if it was accompanied by their enhanced catabolism. It is shown here that Put overproduction (accompanied by increased catabolism) changes the oxidative state of poplar cells in culture; the HP cells exhibiting several of the biochemical effects of increased ROS, including enhanced activity of ROS scavenging enzymes, a reduction in K accumulation, a boost in Ca accumulation and increased membrane damage. While acting as protectants against oxidative stress, and in turn other forms of abiotic stress, in moderate to high quantities, the enhanced production of PAs in these cells may actually become detrimental due to a concomitant increase in their catabolism which results in increased ROS production. It is the balance of the two contrasting phenomena that determines the overall health of cells.

CHAPTER IV

POLYAMINES AND ALUMINUM TOXICITY

As described earlier, PAs have been implicated to play a role in imparting tolerance to a variety of abiotic stress conditions, including those related to fluctuations (both high and low concentrations) in essential (e.g. K) or harmful (e.g. heavy metals) inorganic ions in the soil. Aluminum (Al) toxicity in plants has received considerable attention in recent years (reviewed by Rout et al., 2001; Kochian et al., 2004, 2005; Vitorello et al., 2005). Since the HP cells have substantially higher amounts of Put, and cellular Put has been suggested to play a role in Al toxicity via interaction with cellular Ca, we tested the effects of Al, in the presence of normal and lower amounts of Ca in the medium, on the physiology of HP and control cells.

Introduction

Aluminum toxicity in plants is often manifested in acidic soils, below a pH of either 5.0 (Kochian et al., 2004; Sharma and Dubey, 2007) or 5.5 (Rout et al., 2001, Kochian et al., 2004; Vitorello et al., 2005). The harmful effects of Al include reduced DNA replication and restriction of cell division, resulting in decreased growth and development, particularly in the roots (Minocha et al., 1992; Zhou et al., 1995; Jones et al., 1998; Rout et al., 2001; Yamamoto et al., 2002; Minocha and Long, 2004A; Wang and Kao, 2006). The toxic effects of Al vary with the tissue and its uptake depends upon an interaction with other ions in the soil, particularly Ca (reviewed by Rengel, 1992;

Rengel and Zhang, 2003). It is known that Ca greatly ameliorates the negative impacts of Al (Rengel, 1992; Rout et al., 2001; Rengel and Zhang, 2003; Hossain et al., 2005). One of the proposed ways in which Al is cytotoxic in plants is by blocking Ca^{2+} channels in the plasma membrane. There have been several reports on interference of Al with cellular Ca homeostasis consequently impairing the Ca-dependent signal transduction cascades that may be essential for cell growth and division (Rengel, 1992; Jones et al., 1998; Rengel and Zhang, 2003; Kochian et al., 2004, 2005). Studies with mature trees as well as cell cultures have shown that Al causes changes in Ca uptake. While Zhou et al. (1995), Minocha et al. (1996, 1997), and Jones et al. (1998) showed that Al inhibited Ca uptake in plant cell cultures, Sivaguru et al. (2005) reported a moderate increase in intracellular Ca content due to Al treatment in tobacco cells. In addition to Ca, Al also interferes with the uptake of other essential inorganic ions like Mg, Mn and P (Rengel, 1992; Zhou et al., 1995; Jones et al., 1998; Rout et al., 2001). Aluminum inhibition of P uptake may result in P deficiency in plants growing in the acidic soils (Rout et al., 2001). Studies of Al tolerance in rice by Sivaguru and Paliwal (1994) demonstrated that the tolerant cultivars were more efficient in uptake and utilization of Ca and P than the Al sensitive varieties.

Typically, a several-fold increase in the accumulation of Put has been observed in response to high Al and low Ca in the soil; leading to the suggestion that this response can actually be used as a biochemical marker of Ca deficiency in forest trees (Minocha et al., 1996, 1997; Wargo et al., 2002; Minocha and Long, 2004A). Along with changes in the uptake of other inorganic ions, cell growth, cell viability and mitochondrial activity, Put content has been shown to fluctuate in plant cell cultures as well in response to Al

treatment (Minocha et al., 1992; Zhou et al., 1995). In cultures of *Catharanthus roseus*, Minocha et al. (1992) found a significant increase in cellular Put content 4 h after Al addition, but a decline was observed in the same thereafter, up to 32 h. They also observed an increase in the Spm content at 24 and 48 h after Al addition. These observations were accompanied by inhibition of the activities of several important enzymes of the PA metabolic pathway at various time periods after Al addition. Similar observations were reported by Zhou et al. (1995) who found that the largest increase in Put was observed at 6 h after Al addition. Minocha and Long (2004A) observed an enhancement in cellular Put content due to Al addition to red spruce suspension cultures.

More recently, Wang and Kao (2006) have reported that the inhibition of root growth in rice by Al is mediated by enhanced Put content. They observed that treatment with Al increased the Put content concomitant with decreases in Spd and Spm contents in rice roots. While treatment with Al resulted in marked inhibition of root growth, addition of known inhibitors of Put biosynthesis such as D-Arg and α-methyl-Orn caused a recovery in the growth of roots.

Since Put has been implicated to be an indicator of abiotic stress (including Al stress) in trees and the Put content of cell cultures has also been shown to be affected by Al stress, we wanted to study the effect of enhanced Put accumulation on several aspects of cell metabolism due to Al addition to the HP cells. Also, as stated before, Ca has been suggested to alleviate the deleterious effects of Al (Rengel, 1992). The rationale behind the experiments including Al addition to low Ca poplar cultures was to see if there was any difference in the uptake and effect of Al due to lowering of Ca (to 0.8 mM) in the medium as the culture medium normally contains 4 mM Ca.

Materials and Methods

Cell growth and harvest

As described earlier, for routine subculture, 7 mL of 7-d old cells were added to 50 mL fresh medium. With experiments involving treatments with Al alone, 0.1 or 0.25 mM $AlCl_3$ was added to the cells 3 d after subculture. Subsequently, cells were collected at 6, 24 and 48 h after addition of Al for various analyses. With experiments involving cells growing in different concentrations of Ca, 7-d old cells were subcultured into medium with either normal (1x, i.e. 4 mM), 0.2x (0.8 mM) or 0.05x (0.2 mM) concentrations of Ca. Medium for these experiments was prepared by mixing individual constituents of the MS medium rather than using a pre-mixed powder as used in experiments involving Al alone. The amount of $CaCl_2$ was varied with treatment. Cells were also grown in commercially available MS medium mix (Sigma) for comparison of results.

In experiments involving Ca and Al interaction, a similar set-up (as above) was used in terms of subculturing the cells into medium with varying Ca concentration, except that only 1x and 0.2x concentrations of Ca were used. After 3 days of growth, they were either left untreated or were treated with 0.1 mM $AlCl_3$. Collections were done 6, 24 and 48 h after adding Al.

Determinations of mitochondrial activity, cell viability (membrane integrity) and cellular contents of PAs, amino acids, inorganic ions, GSH, PC_2 and γ-EC were done as described under "General Materials and Methods".

Results

Effect of Al and Ca on cell growth

Starting with similar size of the inoculum (as indicated by the fresh weight at 6h after Al addition), the fresh weight of the harvested pellet was comparable in the two cell lines at given time of analysis (Fig. 29). In response to Al addition, slight increase in pellet fresh weight was seen in the HP cells at 24 h, with the 0.1 mM concentration vs. no Al. The fresh weight of untreated as well as the Al-treated cells in both cell lines increased from 6 h to 48 h. While lowering the Ca concentration in the medium caused a reduction in the fresh weight of HP cells at 24 and 48 h, adding Al to low Ca cultures reversed this effect (Fig. 29B). There was no effect of either lowering the Ca concentration or the addition of Al to low- or normal-Ca medium in the control cells.

Effect of Al and Ca on mitochondrial activity and membrane permeability

The mitochondrial activity of untreated cells of the two lines was quite comparable and showed only small changes at different times of analysis (Fig. 30A). Whereas both cell lines showed a significant reduction in mitochondrial activity when treated with 0.25 mM Al within 6 h; in both cell lines, the effect was reversed by 24 and 48 h. In fact, a small but significant increase in mitochondrial activity was seen in HP cells at both these times in the presence of Al (at both concentrations at 24 h and in 0.1 mM Al at 48 h).

In general, the untreated HP cells retained higher amounts of Evans blue showing more compromised cell membranes than the control cells at any time of analysis; the change with time in either case was small (Fig. 30B). While the membrane permeability of control cells was not affected by Al, the response of HP cells to Al was variable with concentration and time of treatment. These cells showed a small but significant

improvement in membrane integrity in response to treatment with Al, as shown by a decrease in Evans blue retention at 48 h in lower Al concentration.

As mentioned earlier, the damaging effects of Al are typically apparent when Ca supply is limited. In order to test the possibility that the culture medium was Ca saturated, and therefore, Al effects may be masked; the cells were grown in a medium with reduced amounts of Ca. The two media prepared differently (one from the pre-mix powder – marked as "C" and the other made by combining individual components – marked as 1x Ca) showed no significant difference in mitochondrial activity (Fig. 31A) or Evans blue retention (Fig. 31B) in either of the cell line at any time. While in the control cells, the mitochondrial activity was adversely affected only after lowering the Ca content twenty-fold (to 0.05x of normal Ca) at 5 days after subculture, the HP cells showed a reduction in mitochondrial activity even at five-fold (0.2x of normal Ca) reduction in Ca, both at days 3 and 5 of analysis (Fig. 31A); the effect increased with time but was not affected by further lowering of Ca concentration in the medium. The Evans blue retention data (Fig. 31B) show that a reduction of Ca concentration in the medium had an adverse effect on membrane integrity of both cell lines on day 5 but only in the control cells on day 3. Addition of Al to the complete growth medium (with normal amounts of Ca) only affected the mitochondrial activity of HP cells, but not the control cells (Fig. 32) as in the previous experiment. In the medium with low Ca, however, where the mitochondrial activity of HP cells was significantly reduced, an increase in the same was seen on Al addition to low Ca cultures (Fig.32).

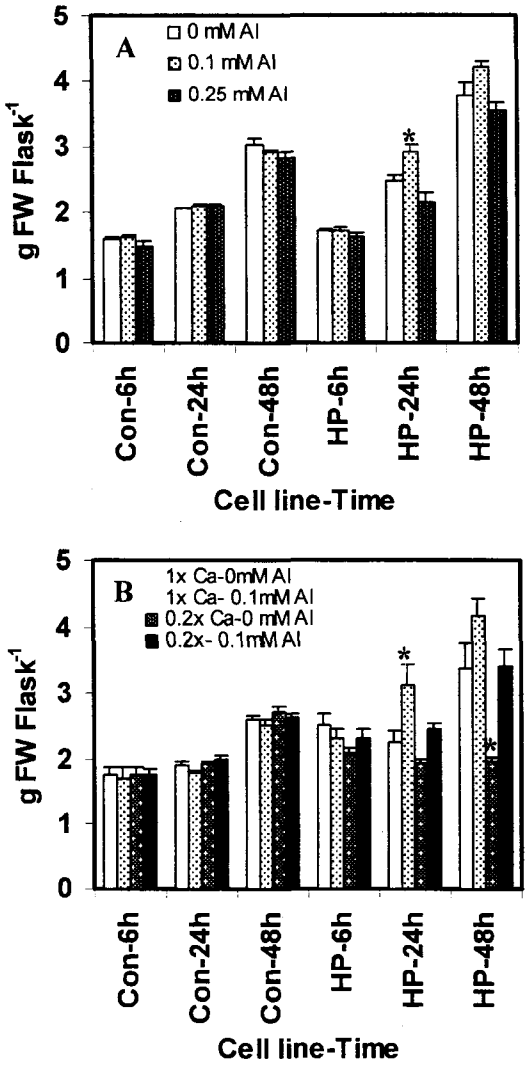

Figure 29. Fresh weight of control and HP cells growing in different concentrations of (A) and Al (B) Al and Ca 6, 24 and 48 h after Al treatment. Each bar represents mean (±) SE of 6 replicates from 2 experiments in (A) and 9 replicates from 3 experiments in (B). An * indicates a significant difference ($P<0.05$) in the g FW between the untreated and Al treated cells in (A) and between the cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca in (B) within the same cell line and time period.

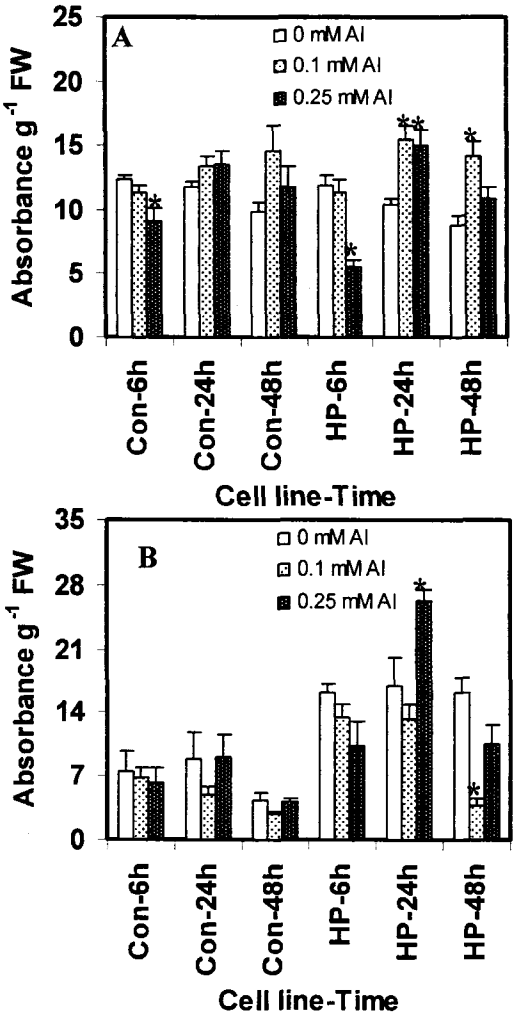

Figure 30. Absorbance of (A) MTT reaction products and (B) Evans blue retention in control and HP cells 6, 24 and 48 h after Al treatment. Each bar represents mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the absorbance g^{-1} FW between the untreated and Al treated cells within the same cell line and time period.

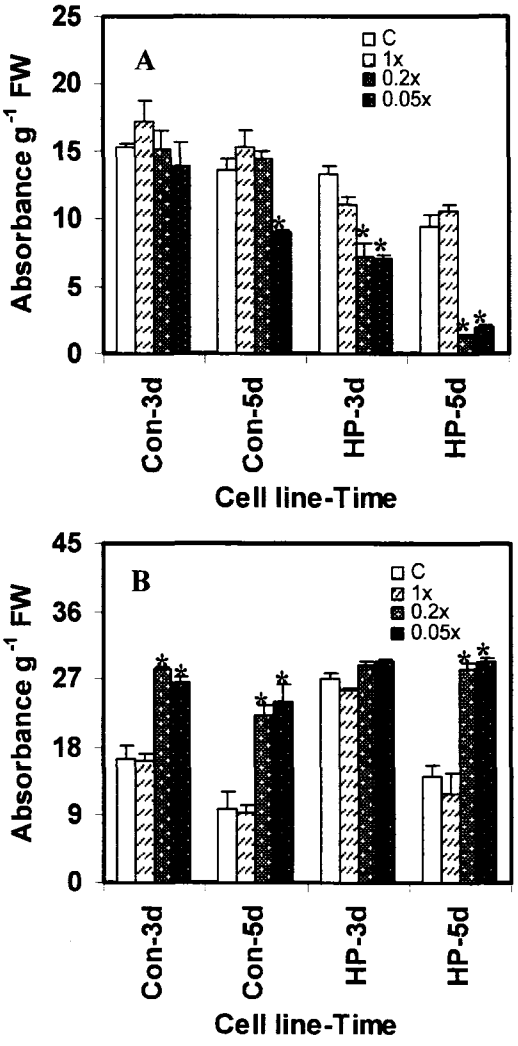

Figure 31. Absorbance of (A) MTT reaction products and (B) Evans blue retention in control and HP cells 3 and 5 days after transfer to media with different concentrations of Ca. Each bar represents mean (±) SE of 4 replicates from 2 experiments. C refers to cells growing in commercially available MS medium. An * indicates a significant difference ($P<0.05$) in the absorbance g^{-1} FW between the cells growing in normal Ca and those growing in reduced Ca within the same cell line and time period.

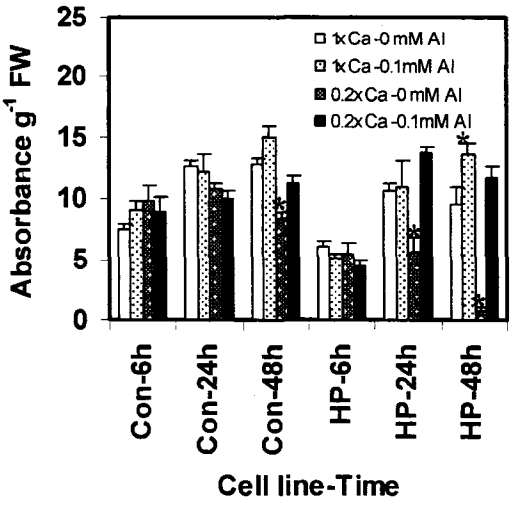

Figure 32. Absorbance of MTT reaction products in control and HP cells growing in normal and reduced Ca, 6, 24 and 48 h after Al addition. Each bar represents mean (±) SE of six replicates from two experiments in (A) and nine replicates from three experiments in (B). An * indicates a significant difference ($P<0.05$) in the absorbance g^{-1} FW between the cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca within the same cell line and time period.

Effect of Al and Ca on cellular polyamines

As expected, cellular Put content was higher (by as much as 8 to 10 folds) in the HP cells than in the control cells; the differences in Spd were much smaller (Figs. 33). Cellular Put was lowered by more than 50% in response to Al treatment in both cell lines, the decrease being significant at 24 and 48 h in both cases (Fig. 33A). The effects of Al were not concentration dependent. Cellular Spd concentrations only showed a small decrease in the control cells at 48 h and the HP cells at 24 h after Al addition (Fig. 33B). Overall changes in Put and Spd were relatively minor during the period of this study.

Lowering the amount of Ca in the growth medium by five- or twenty-folds caused a significant decrease in the cellular Put content in HP cells at 5 d after transfer to low Ca medium (Fig. 34A); only the lowest concentration of Ca had an effect on Put in the control cells. No significant change was seen in cellular Spd content as a result of lowering the amount of Ca in the medium, except for a small increase in the HP cells on day 5, with a twenty-fold reduction in Ca (Fig. 34B). Adding Al to medium with low Ca further lowered the Put content in both cell lines at 24 and 48 h (Fig. 35A). Again, only minor changes were seen in cellular Spd with the addition of Al to medium with low Ca (Fig 35B).

Effect of Al and Ca on accumulation of inorganic ions

The HP cells accumulated somewhat higher amounts of Ca than the control cells on any given day regardless of the presence or absence of Al in the medium (Fig. 36A, B); the differences were not always statistically significant. No change in Ca accumulation was seen in the control cells in response to Al treatment at any time of analysis. In the HP cells, however, both concentrations of Al caused a significant increase in Ca

accumulation at 48 h, although the enhancement was not concentration dependent (Fig. 36A). By this time, the untreated cells had lower Ca than the day before in both cell lines, indicating a loss of accumulated Ca during this period. In other words, the presence of Al prevented the loss of Ca from the HP cells. Lowering the amount of Ca (to 0.2 x) in the medium resulted in significantly lower Ca accumulation in both the control and the HP cells (Fig. 36B). However, there was no further change in cellular Ca in response to Al in the low Ca cultures (Fig. 36B). As in the previous experiment, increased Ca accumulation in the HP cells due to 0.1 mM Al was seen at 48 h with normal amounts of Ca in the medium. It must be kept in mind that the compartmentation of Ca within the cell may be a hindrance to its extraction.

Cellular contents of Mn, which were similar in the two cell lines at most times of analyses, were enhanced in the HP cells by the presence of Al in a time dependent manner; there was no dependence on concentration of Al (Fig. 36C). Lowering the amount of Ca in the medium by itself caused an increase in Mn accumulation in both cell lines except in the control cells at 24 h (Fig. 36D). Adding Al to low Ca cultures caused no further change in Mn content in either cell line. At 0.1 mM concentration, the uptake of Al was lower in the HP cells than the control cells at all three time periods (Fig. 37A). At 0.25 mM, Al accumulation increased drastically in the control cells at 24 and 48 h and in the HP cells at 6 h. However, in the HP cells there was a significant decrease in the accumulation of Al with time after 6 h. In other words, with time, while the control cells retained higher amount of Al, the HP cells lost more than 50% of accumulated Al by 48 h of treatment. Decreasing the concentration of Ca in the medium did not have a significant effect on Al uptake (Fig. 37B) at its lower concentration (the higher concentration was

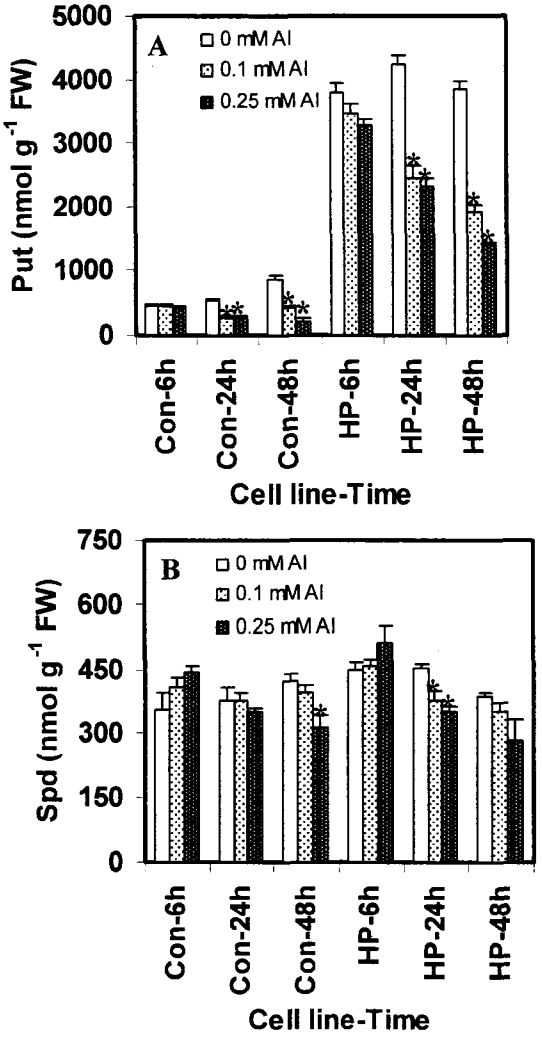

Figure 33. Cellular contents of (A) Put and (B) Spd in control and HP cells 6, 24 and 48 h after Al addition to the media. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in the polyamine content between the untreated and Al treated cells within the same cell line and time period.

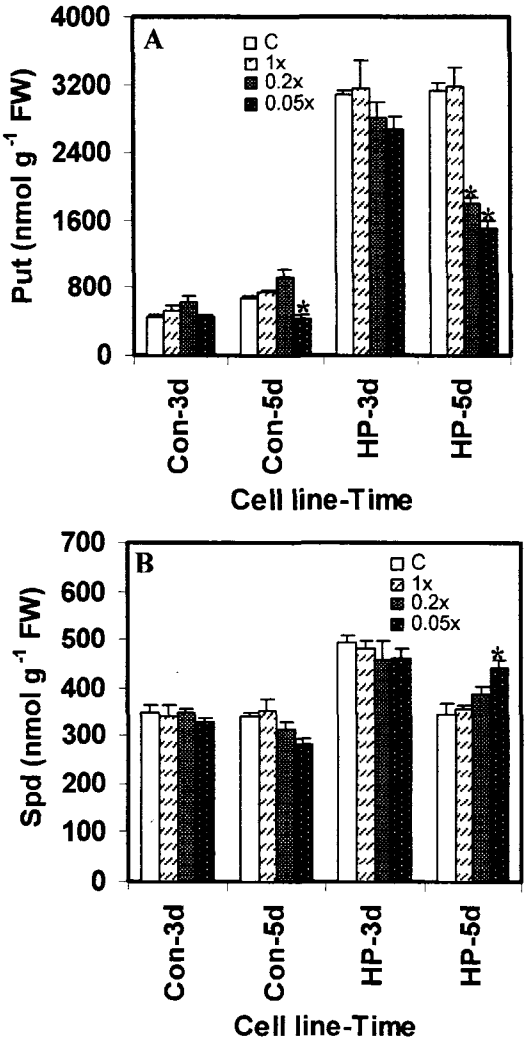

Figure 34. Cellular contents of (A) Put and (B) Spd in control and HP cells on days 3 and 5 after transfer to media containing different concentrations of Ca. Each bar represents mean (±) SE of four replicates from two experiments. C refers to cells growing in commercially available MS medium. An * indicates a significant difference ($P<0.05$) in the polyamine content between the cells growing in normal Ca and those growing in reduced Ca within the same cell line and time period.

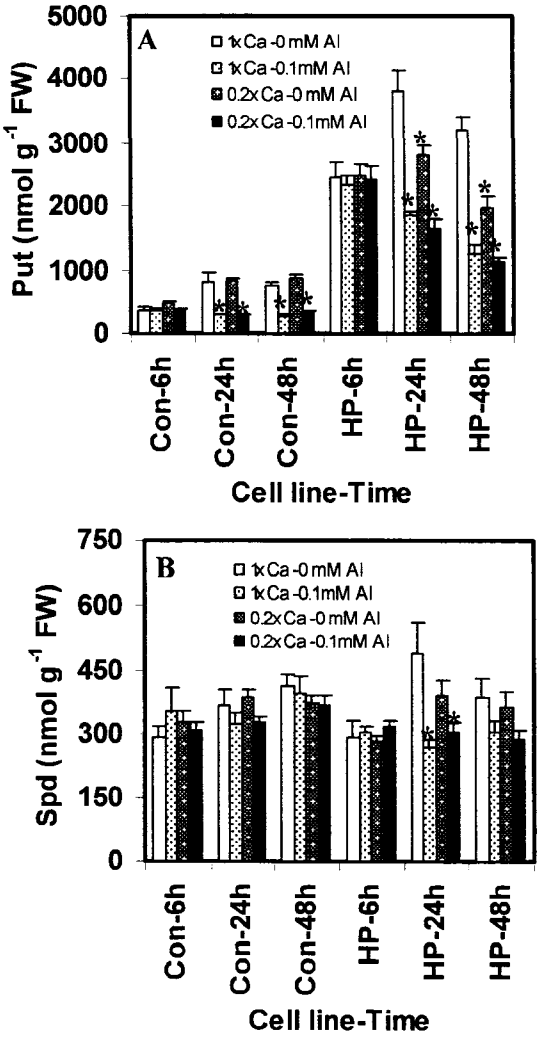

Figure 35. Cellular contents of (A) Put and (B) Spd in control and HP cells growing in normal and reduced Ca, 6, 24 and 48 h after Al addition to the media. Each bar represents mean (±) SE of nine replicates from three experiments. An * indicates a significant difference ($P<0.05$) in polyamine content between the cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca within the same cell line and time period.

not tested in these experiments); the HP cells did show a leakage of Al from the cells at 48 h.

Cellular content of Mg was similar in the untreated control as well as HP cells at all time periods (Fig. 37C); the presence of Al did cause a small dose-dependent decrease in Mg accumulation at 6 h in both cell lines, although this decrease was statistically significant only in the HP cells. Lower Ca in the medium caused a significant increase in Mg content in both cell lines at 6h, while no such effect was seen in either cell line at 24 and 48 h (Fig. 37D). Adding Al to low Ca cultures decreased the Mg content in both cell lines at 6h, while causing an increase in the Mg content of HP cells 48 h after treatment.

The accumulation of Fe was significantly higher in the HP cells (and to some extent in control cells as well) with the addition of 0.25 mM Al (Fig. 38A). Lowering the concentration of Ca in the medium had an effect similar to that of the addition of Al; i.e. higher accumulation of Fe in the HP cells (Fig. 38B). Adding Al to the low Ca medium did not further affect Fe accumulation in either cell line. The accumulation of P, which was similar in the two cell lines, was not affected either by the addition of Al or the reduction in Ca (Fig. 38C, D); P accumulation in the two cell lines on any given day was quite comparable.

While cellular content of K was higher in the HP cells than the control cells at any given time of analysis (Fig. 39A, B), adding Al mostly caused an increase in K content at 24 and 48 h, at both concentrations of Al in the HP cells and mostly at the higher concentration in the control cells. Lowering Ca alone decreased the cellular content of K in both cell lines at 24 h, while Al addition to low Ca cultures increased the K content of the HP cells at 24 and 48 h (Fig. 39B). The cellular content of Zn mostly increased with

the addition of Al in both cell lines (Fig. 39C, D). Also, lowering Ca in the culture medium significantly increased the Zn content in both cell lines at 6 h and also in the HP cells at 48 h. Adding Al to low Ca cultures did not significantly affect the accumulation of Zn in either cell line (Fig. 39D).

Effect of Al on GSH, γ-EC and PC_2

Since Al toxicity is known to cause oxidative stress (Yamamoto et al., 2002; Kochian et al., 2004, 2005) and because GSH is a known scavenger of ROS (Noctor and Foyer, 1998; Foyer and Noctor, 2005), we studied the effects of Al addition on cellular GSH content in the control and HP cells. The HPLC method used to quantify GSH also enabled the quantification of PC_2 (a known chelator of heavy metals like Zn, Cd, etc.) as well as their common precursor, γ-EC. As seen shown in Fig. 40, the cellular contents of all three sulfur metabolites in the HP cells were lower than those in the control cells on any day of analysis; the differences were at least 4-5 folds for PC_2 and γ-EC and about two-folds for GSH. The presence of Al caused a small but significant increase in the accumulation of PC_2 in the control cells (Fig. 40 B) and a significant decrease in GSH in the HP cells (Fig. 40A); otherwise, there was no effect of Al on these metabolites at any time in either cell line. The cellular contents of these metabolites did not change much with time during the experimental period.

Effect of Al and Ca on cellular free amino acids

Changes in the cellular content of amino acids, particularly those which are directly involved in PA biosynthesis, were analyzed in the control and the HP cells in response to treatments with 0.1 mM and 0.25 mM Al in the presence of normal (1x) Ca and 0.1 mM Al in the presence of both normal and five-fold reduced concentration (0.2x) of Ca.

Although in the HP cells, Orn is the major contributor to Put biosynthesis, its cellular content was below detection limits of the HPLC system in all cases; therefore data on Orn are not presented. As stated earlier (Chapter II), the content of Glu in the HP cells was about half that in the control cells. Addition of Al significantly increased the cellular content of this amino acid in both cell lines (Fig. 41A, B); almost doubling it in the control cells and tripling it in the HP cells (with 0.1 mM Al). Lowering Ca content of the medium did not significantly affect Glu content in control cells but reduced it in the HP cells. The presence of Al even in low Ca medium reversed the effects of low Ca in these cells; Glu content of Al-treated cells was similar whether Ca content of the medium was normal or lower (Fig. 41B).

Glutamine was also lower in the HP cells than the control cells; the reduction being greater than two-fold. Gln content was not significantly affected by Al treatment in the presence of normal amounts of Ca in the medium, except for a reduction in control cells at 0.25 mM Al (Fig. 41C). Lowering the Ca content in the medium caused a significant increase in cellular free Gln in the control cells, but a reduction in the HP cells (Fig. 41D). Adding Al to low Ca cultures restored the Gln content in both cases to their respective amounts in the untreated cells.

No significant effects of Al or Ca were seen on the cellular contents of Arg (Fig. 41E, F), while Pro significantly increased in the HP cells in response to 0.25 mM Al (42A). GABA, which is made directly from Glu by decarboxylation as well as from Put by its oxidation, was higher in the HP cells as compared to the control cells, and its content was enhanced by the presence of Al (Fig. 42C) as well as the lowering of Ca in

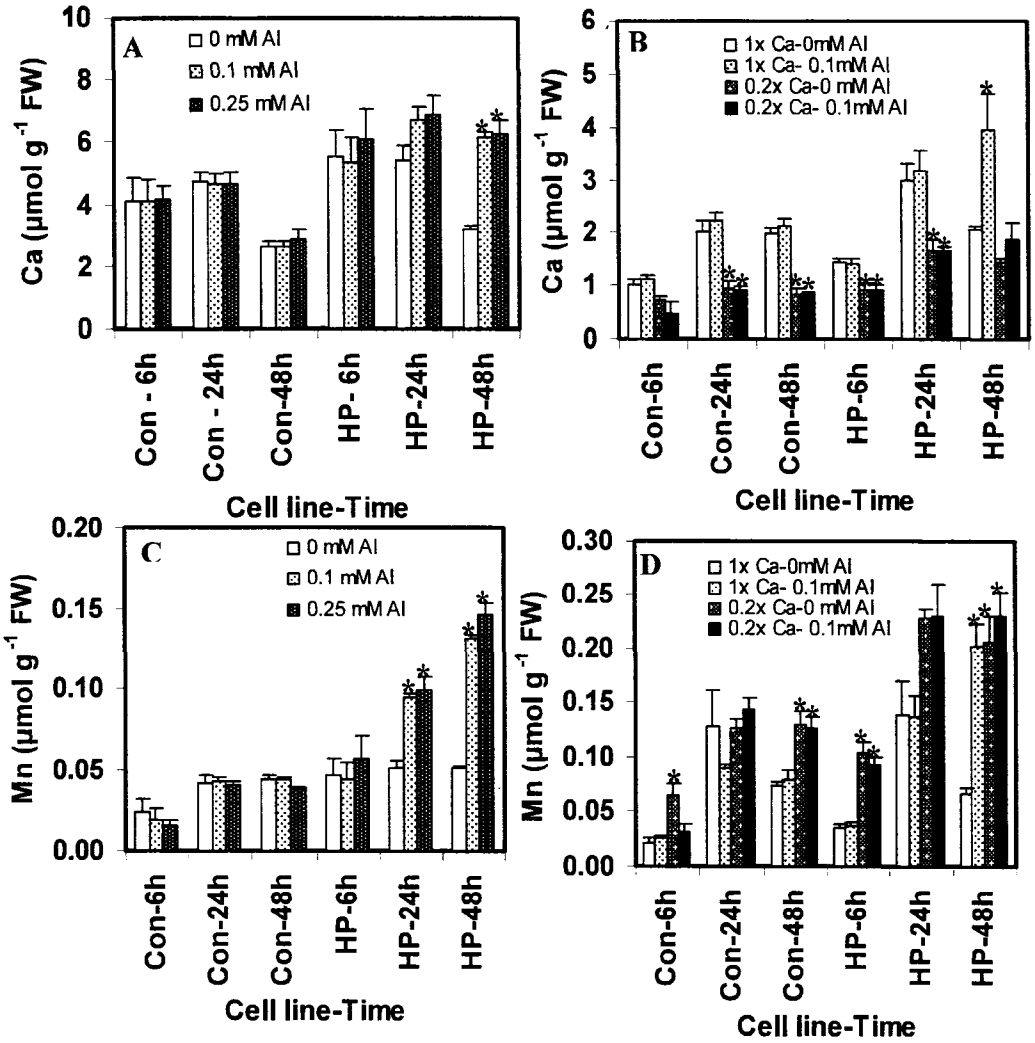

Figure 36. Cellular contents of (A, B) Ca and (C, D) Mn in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in ion content between the untreated cells and cells growing in Al in (A) and (C) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca in (B) and (D), within the same cell line and time period.

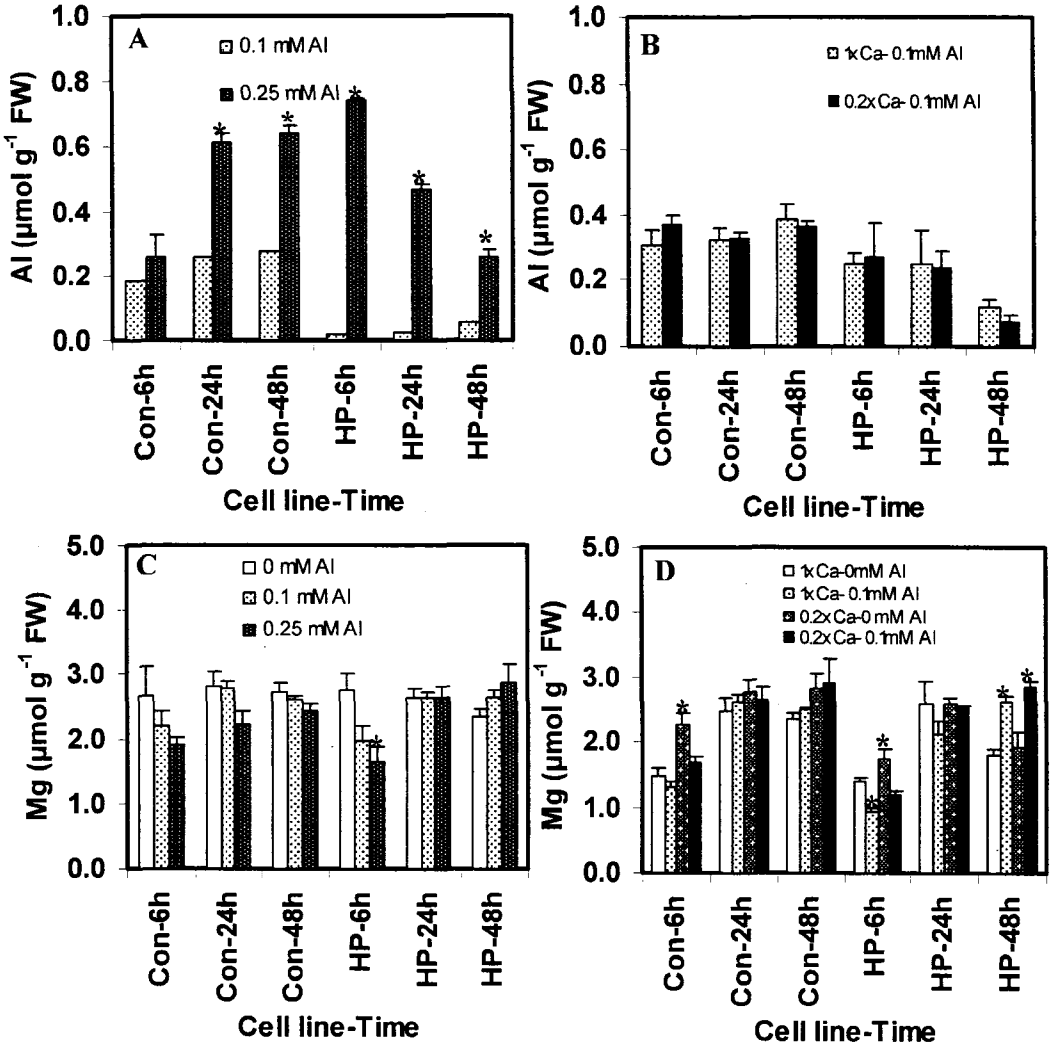

Figure 37. Cellular contents of (A, B) Al and (C, D) Mg in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in ion content between the untreated cells and cells growing in Al in (A) and (C) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca in (B) and (D), within the same cell line and time period.

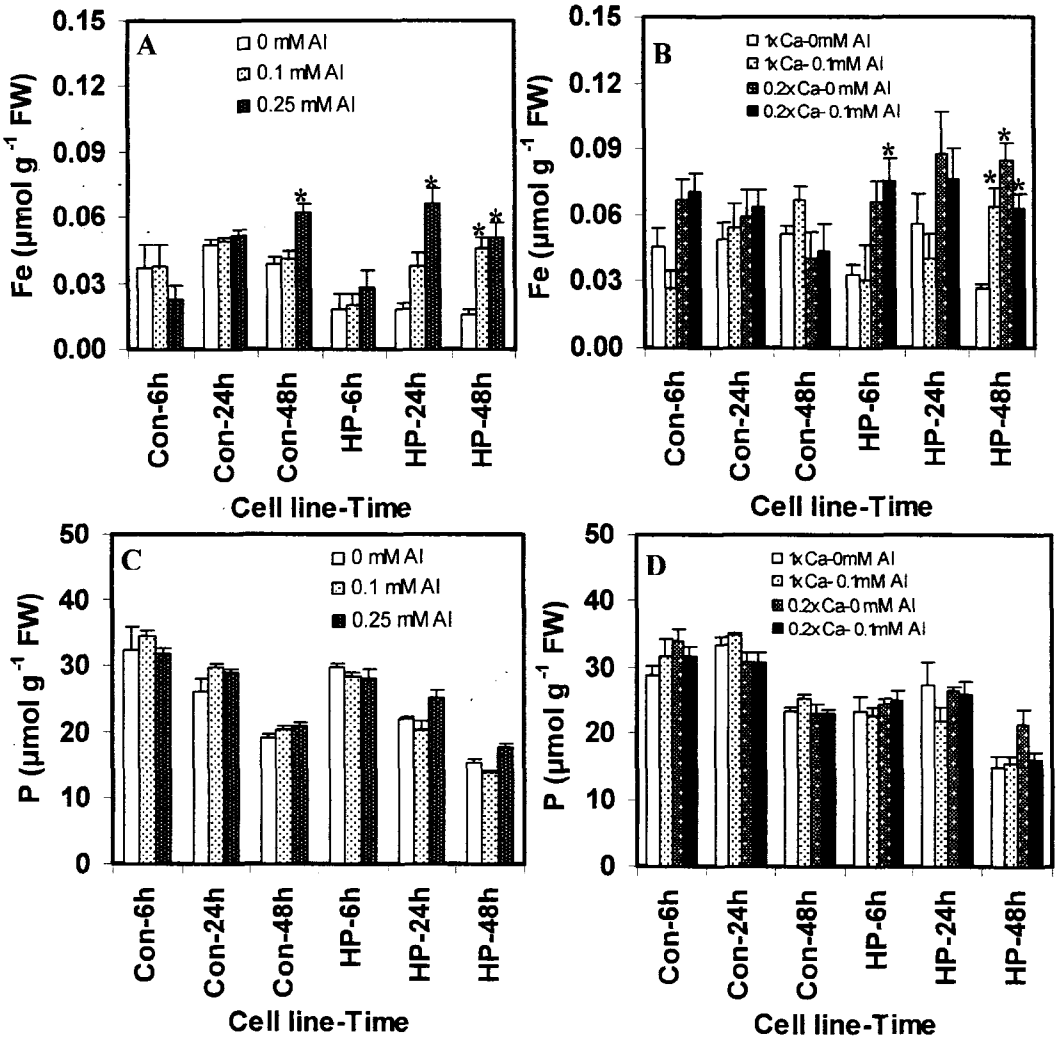

Figure 38. Cellular contents of (A, B) Fe and (C, D) P in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in ion content between the untreated cells and cells growing in Al in (A) and (C) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca in (B) and (D), within the same cell line and time period.

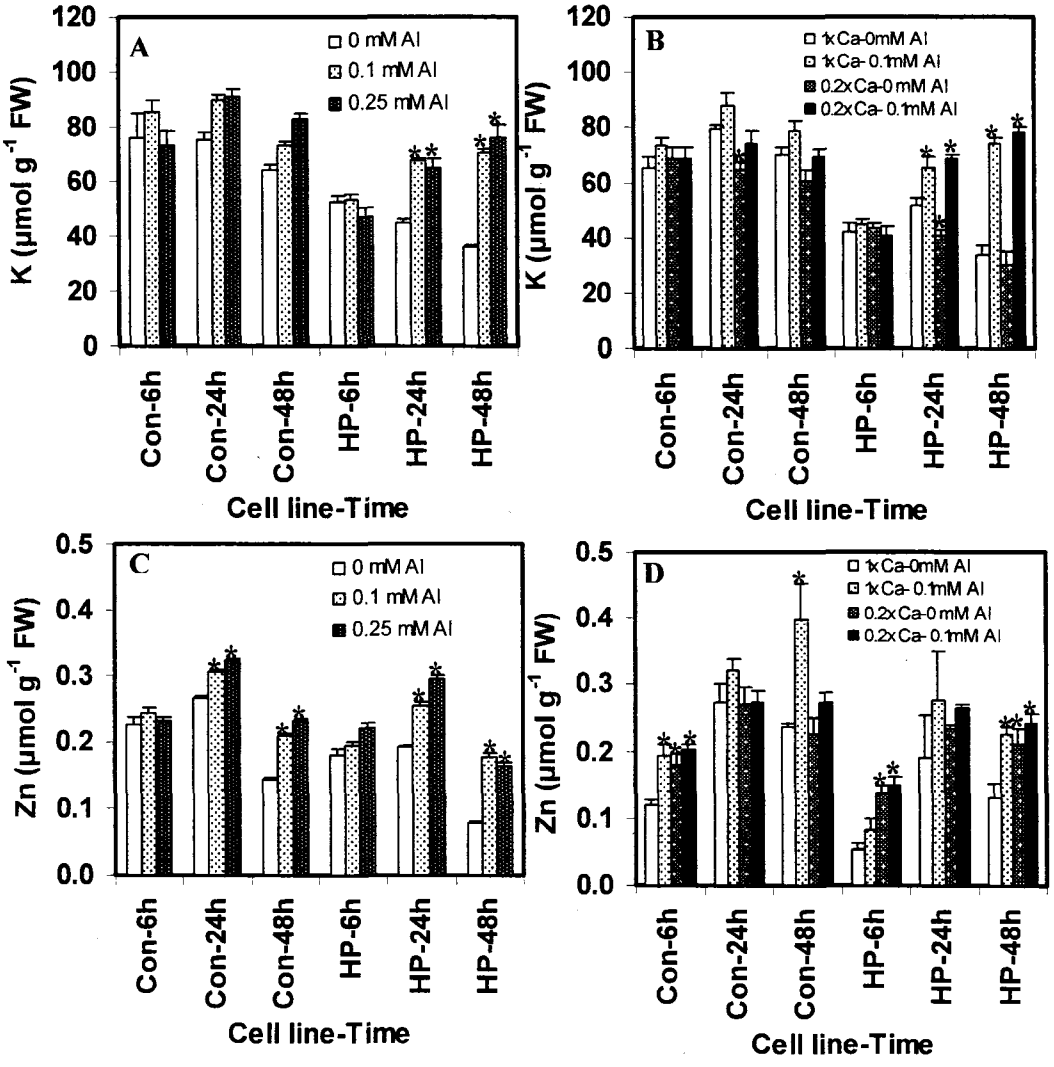

Figure 39. Cellular contents of (A, B) K and (C, D) Zn in control and HP cells growing in different concentrations of (A, C) Al and (B, D) Al and Ca. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in ion content between the untreated cells and cells growing in Al in (A) and (C) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca in (B) and (D), within the same cell line and time period.

the medium in both cell lines (Fig. 42D). The presence of Al in the low-Ca medium further enhanced GABA content in control cells while lowering it in the HP cells. Cellular content of His increased significantly in the HP cells with the 0.25 mM concentration of Al and on lowering Ca in the control cells (Fig. 42E, F).

Addition of Al mostly caused an increase in the cellular content of Thr, especially at the higher concentration in both cell lines (Fig. C1A). No significant changes were observed in Thr content in either cell line either on lowering Ca or adding Al to low Ca cultures (Fig. C1B). Cellular content of Val was comparable in both cell lines with or without Al treatment (Fig. C1C). Lowering Ca significantly increased cellular Val content only in the control cells, while adding Al to low Ca cultures caused a reduction in the same. No such fluctuations were seen in the HP cells (Fig. C1D). Addition of Al caused an increase in the cellular Lys content only in the HP cells at 0.25 mM concentration (Fig. C1E). Lowering Ca in the medium caused an increase in the Lys content only in the Con cells. Adding Al to low Ca cultures did not cause any further change to the Lys content (Fig. C1F).

Cellular content of Ser was not affected by Al addition to control cells (Fig. C2A). The HP cells showed a small increase in Ser content due to 0.1 mM Al addition. Lowering Ca caused a surge in the cellular Ser content of the control cells, while decreasing the same in the HP cells (Fig. C2B). Adding Al to low Ca cultures restored the cellular content of Ser in both cell lines. Cellular contents of Cys + cysteine decreased significantly on Al addition in both cell lines, the decrease being somewhat dose dependent (Fig. C2C). Decreasing Ca caused a significant surge in Cys + cysteine content only in the control cells (Fig. C2D). Adding Al to low Ca

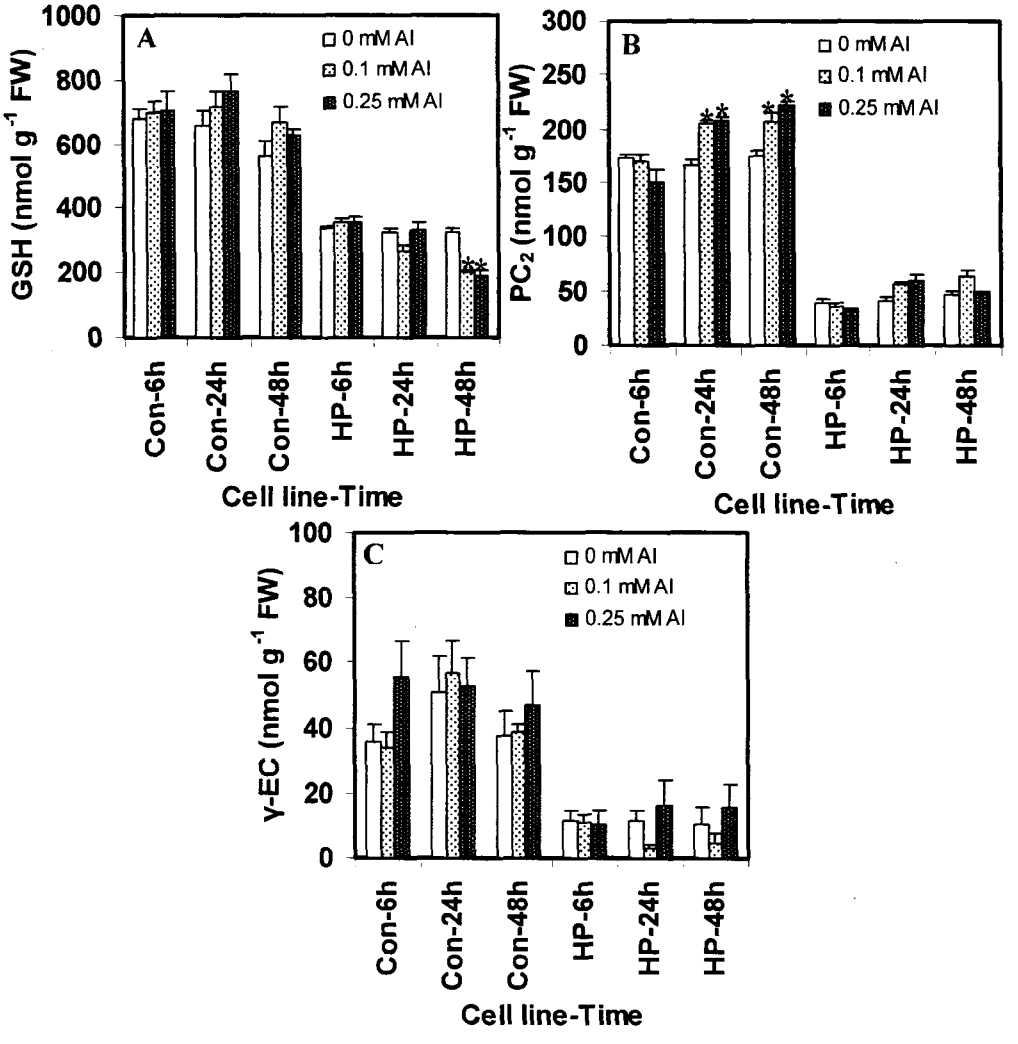

Figure 40. Cellular contents of (A) GSH, (B) PC_2, and (D) γ-EC in the control and HP cells due to different concentrations of Al 6, 24 and 48 h after treatment. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in metabolite content between the untreated cells and cells growing in Al.

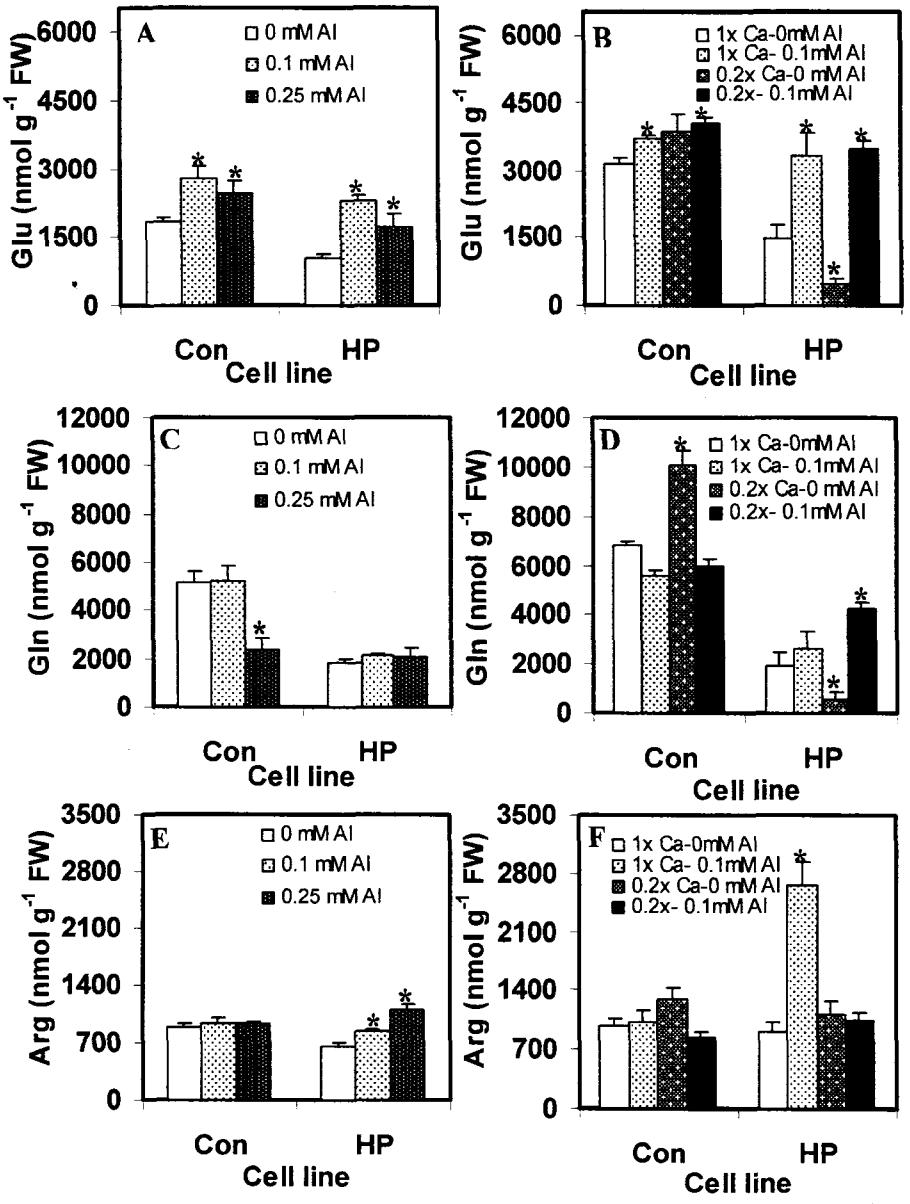

Figure 41. Cellular contents of (A, B) Glu, (C, D) Gln, and (E, F) Arg in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in amino acid content between the untreated cells and cells growing in Al (in A, C, E) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca (in B, D, F) within the same cell line.

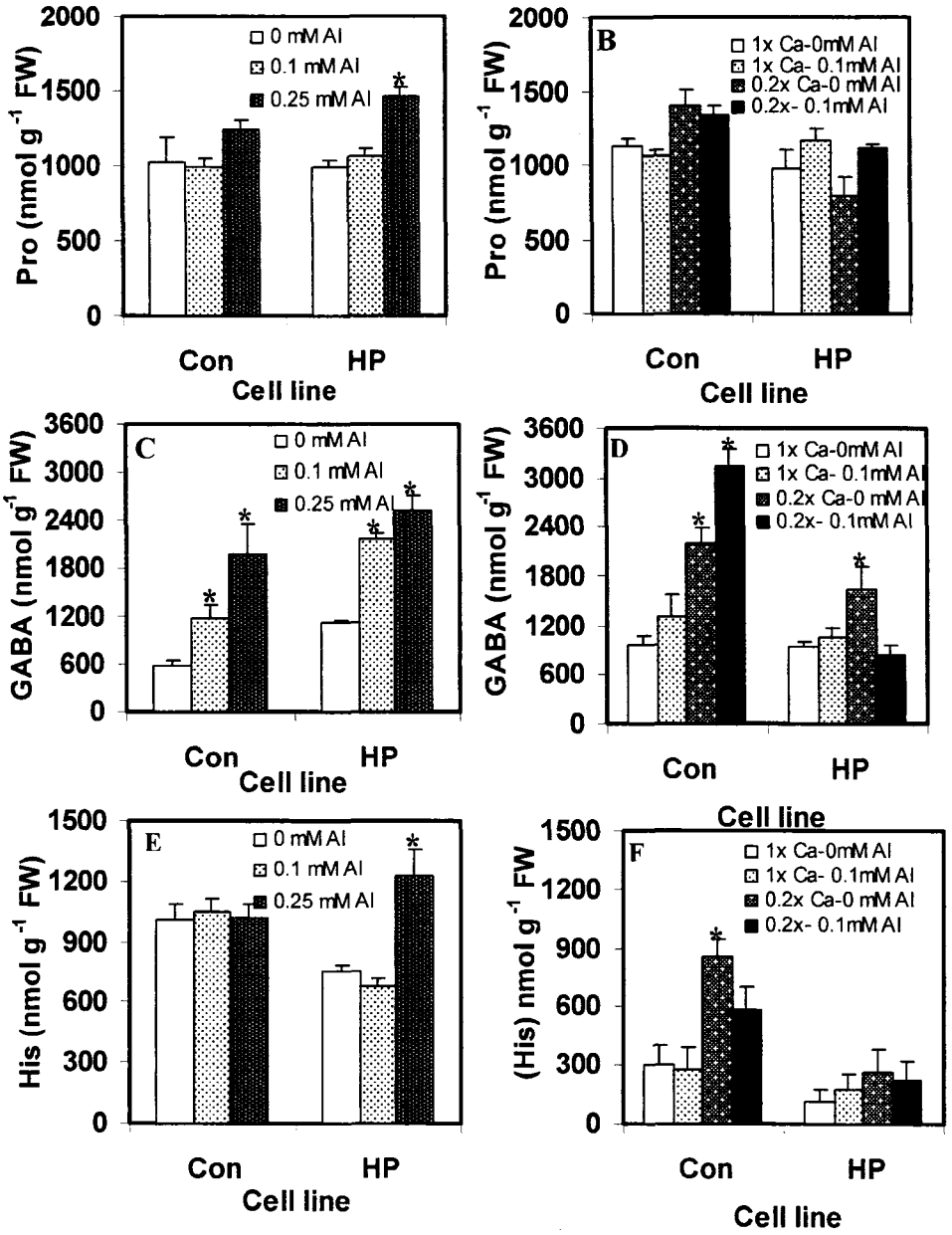

Figure 42. Cellular contents of (A, B) Pro, (C, D) GABA and (E, F) His in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in amino acid content between the untreated cells and cells growing in Al (in A, C, E) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca (in B, D, F) within the same cell line.

cultures decreased the cellular content of these amino acids in these cell lines. While addition of Al did not cause much change in the cellular Gly content in either cell line, lowering Ca concentration in the medium caused a surge in Gly in the control cells only (Fig. C2E, F). No such fluctuations were seen in the HP cells.

Cellular content of Phe decreased in both cell lines on Al treatment, especially in the highest concentration (Fig. C3A). Lowering Ca content in the medium increased the cellular content of Phe only in the control cells (Fig. C3B). Cellular Trp did not change much on Al addition to either cell line (Fig. C3C). Lowering Ca in the medium caused an increase in the Trp content of control cells and adding Al to low Ca cultures, restored the Trp concentration (Fig. C3D).

Discussion

As mentioned before, the negative effects of Al in plants involve restriction of cell division, reduction in DNA replication and decrease in cellular respiration; it also affects the uptake, transport and utilization of mineral nutrients like Ca, Mg, P and Fe (Minocha et al., 1992, 2001; Zhou et al., 1995; Rout et al., 2001). That Al exposure stimulates the generation of ROS, induces the expression of several genes of the ROS scavenging machinery, and causes peroxidative damage to membranes are also well known (Yamamoto et al., 2002; Kochian et al., 2004, 2005). The effects of Al on mitochondrial dysfunction and membrane damage are additional factors contributing to its harmful effects (Minocha et al., 2001; Yamamoto et al., 2002).

Yamamoto et al. (2002) reported a rapid suppression of mitochondrial activity within 6 h of Al addition to cultured tobacco cells, which was followed by an increase in mitochondrial activity for up to 12 h; Al also impaired the growth capabilities of these

cells. Based on several parameters, the authors proposed that Al caused damage to the mitochondrial electron transport chain, leading to an increased production of O_2^- by the leakage of electrons directly to O_2. We found a similar response of poplar cells with higher amounts of Put (i.e. the HP cells) to Al addition. At 0.25 mM concentration, Al caused a reduction in mitochondrial activity within 6 h; the effect was fully reversed by 24 h (Fig. 30A).

The Evans blue retention data show that despite a transient reduction in mitochondrial activity, which could be responsible for enhanced generation of ROS, and therefore, potential membrane damage, (Chapter I), the plasma membrane integrity in these cells was still maintained (Fig. 30B). However, by 24 h after 0.25 mM Al treatment, when the effects on mitochondrial activity were fully reversed, there now was a noticeable increase in membrane disruption. Interestingly enough though, this condition was reversed at 48 h, i.e. the membrane integrity in the HP cells was restored. The timings of the two events indicate a lag between when effects on mitochondrial activity are seen and when the resulting damage to the membrane integrity occurs. This is further borne out from the results of the effects of 0.1 mM Al, which caused an increase in mitochondrial activity at 24 h without short term (6h) adverse effects; this treatment caused a recovery in membrane damage at 48 h. It is noteworthy that the uptake of Al at its lower concentration was several-fold lower in the HP cells as compared to the control cells, and the accumulation of Ca in the former (i.e. HP cells) was higher than that in the control cells. It can therefore be argued that maintenance of higher amounts of Put in the HP cells increases their uptake of Ca and inhibits the uptake of Al; thus preventing the damage due to Al (at least at its lower concentrations). Under low Ca conditions, when

Ca accumulation is lower (Fig. 36B), the response in these (HP) cells is reversed in that both the mitochondrial activity as well as the membrane integrity are adversely affected (Fig. 31, 32). Still having higher amounts of cellular Ca in the HP cells growing in the low-Ca medium, provides some protection from Al damage in these cells (Fig. 36 HP at 24 and 48 h). Therefore, as mentioned earlier by Minocha et al. (1997), it is the ratio of Al: Ca in the medium that is a predictor of Al toxicity and not Al concentrations alone.

As expected, the Put content was always higher in the HP cells than in the control cells during this part of the study. In response to Al treatment, cellular Put decreased significantly in both the control and the HP cells (Fig 33A). This is consistent with an earlier report involving *Catharanthus roseus* cell cultures, which also showed a reduction of Put accumulation in response to Al treatment (Zhou et al., 1995), but in contrast to other reports (Minocha et al., 1996, 1997). What is not clear is if reduced accumulation of Put is due to lower biosynthesis, increased catabolism and/or leakage due to disruption of plasma membrane integrity. Knowing that: (a) the half-life of Put in these cells is about 6-7 h (Bhatnagar et al., 2002), (b) the decrease in Put is not seen until 24 h after Al treatment, and (c) Al has little effect on membrane integrity by this time; it can be hypothesized that the effect of Al is exerted through inhibition of Put biosynthesis or enhancement of its catabolism, and not through increased leakage of Put from the cells.

The effects of Al on cellular Spd were rather small; so were the effects of reduced Ca or a combination of Al and lower Ca. This is consistent with numerous observations, in cell cultures as well as mature plants, on the changes in Spd in response to a variety of treatments, including abiotic stress and genetic manipulation (Kasukabe et al., 2004; Sanchez et al., 2005).

The adverse effect of Al on cellular Put is in contrast to the repeatedly observed effects of Al on Put accumulation in mature trees subjected to Al solubilization in the soil due to acidic deposition (Minocha et al, 1997); in that situation, the uptake/accumulation of Ca ions was adversely affected by Al (Minocha et al., 1996; Rout et al., 2001). Minocha and Long (2004A) have suggested that the symptoms of Al toxicity and Ca deficiency in plants are similar. The accumulation of Mn, Mg, Fe and P also decreases in roots and shoots exposed to increased Al supply (Rout et al., 2001). Increased availability of divalent cations like Ca and Mg in the soil ameliorates rhizotoxicity and other adverse effects of Al in crop plants (Rengel, 1992; Rout et al., 2001) as well as in forest trees (Minocha et al., 1997; Wargo et al., 2002). The data presented here show that the accumulation of Ca, Mn and Fe actually increased in the presence of Al in the HP cells, although such a trend was not seen in the control cells (Fig. 36, Fig. 38A, B). While the increased uptake of these ions, especially Ca, may have a protective role in HP cells against Al toxicity, it may also be related to enhanced activity of ROS in the HP cells due to high PA turnover, whose byproduct is H_2O_2 (See Chapter III).

The relationship between ROS and the accumulation of Ca and K has been discussed in Chapter III. Increased ROS, which can be induced by Al, have been shown to activate the inwardly rectifying Ca^{2+} channels in plant cells (Pei et al., 2000; Bowler and Fluhr, 2000; Foreman et al., 2003). Demidchik et al. (2003) observed a simultaneous activation of the Ca_{in} and K_{out} channels in response to ROS and postulated that the activation of these channels was regulatory in nature and not due to general membrane depolarization. It can further be argued that while the Ca influx may be due to the activation of specific channels, the loss of membrane integrity as seen by Evans blue retention in HP cells, may

be due to the harmful effects of ROS (produced in the apoplast) on lipid peroxidation in the membrane, which is a well known phenomenon in both animal and plant cells (Stark, 2005). In line with the suggestion of Stark (2005), it is also conceivable that the increased accumulation of Ca and Mn may in reality be the cause of cell damage in the HP cells.

Surprisingly, however, the accumulation of Al itself was substantially lower in the HP cells than the control cells at 0.1 mM concentration of Al. At 0.25 mM Al, however, membrane depolarization may have resulted in a burst of Al uptake, followed by its slow release, thus producing a completely different profile of accumulated Al in these cells. Kochian et al. (2004) described two distinct classes of Al tolerance mechanisms in plants, one that works towards excluding Al from the root apex and one that allows the plant to tolerate Al accumulation in the root and shoot symplasm. The pattern of rapid Al uptake (at 6 h), followed by its efflux with time is similar to the profile of K^+ accumulation in these cells without Al addition (Fig. 27B, Fig. 39A). Although the efflux of K^+ is suggested to be regulated by activated K_{out} channels (Demidchik et al., 2003), the cause of Al leakage in HP cells but not in the control cells (Fig. 37A) is not known.

The cellular contents of phytochelatins like PC_2 (Fig. 40B), and its precursor γ-EC (Fig. 40C), have been shown to increase in response to metal toxicity in plant cells (Hirata et al., 2005; Thangavel et al., 2007). Although, it is common knowledge that phytochelatins bind heavy metals, thus playing a role in ameliorating metal toxicity; Vitorello et al. (2005), who reviewed the mechanisms of Al toxicity and resistance in higher plants, argued that phytochelatins and metallothioneins do not play any major role in binding of Al. The HP cells always showed significantly lower contents not only of GSH and PC_2, but their common precursor γ-EC as well (Fig. 40). This was not

unexpected because γ-EC is synthesized by condensation reactions involving the amino acids Glu, Cys and Gly; we have seen that the cellular contents of these three amino acids are lower in the HP cells than the control cells (Figs. 13A, 14B, C). Although, the cellular contents of PC_2 increased significantly with Al addition to the control cells at 24 and 48 h, no such change in PC_2 was observed in HP cells (Fig. 40B). As mentioned above, Al uptake is much lower in the HP cells than the control cells at the 0.1 mM concentration, while there is a slow time-dependent release of Al from these cells at higher Al concentration (Fig. 37A). This low accumulation and gradual release could be a reason for the lack of a stimulatory effect of Al on PC_2 content of HP cells. Hence, it can be postulated that while both cell lines are responding to Al treatment in an attempt to minimize its toxic effects, the HP cells are doing so at the level of Al uptake, while the control cells, that probably lack the ability to regulate its entry into them, are doing so post-uptake by enhanced PC_2 biosynthesis. The content of GSH decreased significantly in the HP cells at 48 h, in response to Al addition (Fig. 40A), being indicative of a low redox state of the cells, and hence, probably higher oxidative stress (see also Chapter III).

It was deemed important to study the effects of Al toxicity on the cellular contents of amino acids for three reasons: (i) Along with other N containing compounds like PAs, betaine and nicotinamine, the accumulation of amino acids like Pro and His have been shown to increase by metal toxicity (Sharma and Dietz, 2006). (ii) since the cellular content of Put decreased due to both Al addition and Ca deprivation in the two cell lines (Figs. 29, 30, 31), we were interested to see if the metabolism of amino acids related to the PA metabolic pathway was affected; and (iii) organic acids like citrate and malate have been shown to be secreted from the cells in response to Al treatment and also bind

to Al (Pellet et al., 1995; Ma et al., 2001; Minocha and Long, 2004B; Kochian et al., 2005); thus their decreased availability should affect the amino acids derived from them.

The response of the poplar cells to Al treatment in terms of changes in the contents of free amino acids that were quantified (Fig. 41, 42) were not very different in the two cell lines except for Gln, the cellular content of which significantly decreased in the presence of 0.25 mM Al in the control cells (Fig. 41C), and Pro, which significantly increased in response to 0.25 mM Al in the HP cells. Since Pro has been postulated to play a protective role against metal toxicity because of its complex-forming ability with metals (Sharma and Dietz, 2006), a significant increase in the Pro content of HP cells in response to 0.25 mM Al can be considered as a protective mechanism to deal with Al toxicity. Sharma and Deitz (2006) also mentioned His to be another amino acid whose content increases under conditions of metal toxicity. The cellular content of His, which was lower in the HP cells as compared to the control cells, also increased at the 0.25 mM Al concentration at 48 h after treatment in the HP cells (Fig. 42E). Decreased Ca in the medium had a similar effect on His in the control cells (Fig. 42F).

Significant increases in GABA content in response to Al and low Ca treatments in both cell lines can be correlated with enhanced Put catabolism (GABA is an oxidative product of Put), which may also be the cause of the reduction in Put content in response to Al treatment in these cells. It is surprising then that despite greater utilization of Put and higher accumulation of Pro and GABA in response to Al treatment, the cellular content of Glu in these cells was maintained at a higher level in the presence of Al (Fig. 41A); Glu is an indirect precursor of Put and Pro as well as that of GABA. For more discussion on the regulation of GABA, see Chapter V.

Calcium, an essential plant nutrient, is required for various physiological and regulatory functions in cells, acts as a counter ion for anions in the cell, and also acts as an intracellular messenger in the cytosol (White and Broadley, 2003; Hepler, 2005). Hence, it is understandable that decreasing Ca concentration in the growth medium would adversely affect both cell lines in terms of mitochondrial activity as well as membrane integrity (Fig. 31, 32). The rationale behind the experiments of lowering the amounts of Ca in the medium to which Al was added was to see if saturating amounts of Ca were actually minimizing the Al effects in the normal MS medium which contains 4 mM Ca. It would be expected that adding Al to low Ca cultures, could cause further respiratory stress leading to cytotoxicity. On the contrary, mitochondrial activity increased in the HP cells on addition of Al to low Ca cultures, pointing to tolerance of Al by these cells (perhaps via reduced Al uptake at this concentration). However, since cellular Put in HP cells decreased on addition of Al to low Ca medium, it is apparent that whatever Al was taken up, it did have a significant metabolic effect on cells. These observations support the argument of a protective role of high Put in poplar cells against Al toxicity. As stated before, Ca has been known to alleviate the deleterious effects of Al (Rengel, 1992, Rengel and Zhang, 2003). Lower Ca in the medium did not seem to have an impact on the uptake of Al in either cell line (Fig. 36B); even though it seems that we may have reached limiting levels of Ca in the medium for reactions that affect mitochondrial activity, membrane integrity, ion uptake, and PA metabolism.

Minocha et al. (2001) studied the effect of Al in red spruce cultures on several parameters including intracellular localization of Al. They reported the presence of Al in cell walls, cytoplasm, plastids and vacuoles of red spruce cells, 48 h after Al treatment.

Movement of Al across the plasma membrane did not cause any substantial cellular disruption. Measurement of cell growth and cell viability (membrane function) indicate that a 48 h exposure to different concentrations of Al resulted in growth inhibition, decrease in mitochondrial activity and membrane damage.

There was an obvious leakage of Al from the HP cells into the growth medium at 0.25 mM Al concentration. A higher uptake of Ca and a decrease in the uptake of Al with time as a response to Al addition in the HP cells can be perceived as an attempt by these cells to minimize the toxic effects of Al. Hence, these events were accompanied by a simultaneous increase in mitochondrial activity and cell viability, 48 h after Al addition. The fact that these repercussions of Al addition were also accompanied by a decrease in Put content in the HP cells raises the question: *Is the over-produced Put getting used up (catabolized) faster to trigger a cascade of reactions in several different pathways ultimately preparing the HP cells to fight against Al toxicity better?* Based on the above arguments, we hypothesize that in response to Al addition, a series of reactions from several different pathways is triggered in the HP cells to protect them against Al toxicity. These include, but are not limited to, reduced Al uptake at lower concentration of Al, increased Al efflux, enhancement of mitochondrial activity, improved membrane integrity, and enhanced accumulation of Ca. Although it can be argued that the influx of cations such as Ca^{2+} and Mg^{2+} may be indicative of increased oxidative stress in the HP cells, due to Al addition, a recovery in both mitochondrial activity and membrane integrity between 24 and 48 h of Al addition to HP cells suggests a protective role of high Put against Al toxicity. This hypothesis is further supported by the observation that in the

HP cells although lowering Ca content in the medium caused a severe impairment in mitochondrial activity, adding Al to the low Ca cultures significantly restored the same.

Conclusions

Although both the control and the HP cells showed a decrease in cellular Put content in response to Al addition, several responses in the HP cells suggest a protective role of the enhanced Put metabolism in them against Al toxicity. Not only did Al addition result in lower Al accumulation and greater efflux in these cells as opposed to the control cells, but it also lead to greater accumulation of Ca, a potential alleviator of Al toxicity. Both membrane integrity and mitochondrial activity also improved in these cells at some Al concentrations and at certain time periods. While lowering Ca proved detrimental to both cell lines, adding Al to low Ca cultures recovered normal conditions in the HP cells alone.

CHAPTER V

POLYAMINES, GAD AND GABA

As pointed out earlier, the biosynthesis and catabolism of PAs are metabolically linked with the biosynthesis of GABA and Pro since they all utilize a common substrate, i.e. Glu. Also, their accumulation in the cells occurs under similar conditions involving responses to a variety of abiotic stresses (Aziz et al., 1998; Shelp et al., 1999; Kinnersley and Turano, 2000; Mazucotelli et al., 2006; Simon-Sarkadi et al., 2005, 2006). The biosynthesis of GABA occurs by an anabolic decarboxylation of Glu as well as by catabolic oxidation of Put (Fig. 2, Fig. 43). Since the HP cells being used here have an elevated rate of Put catabolism (Bhatnagar et al., 2002), potentially providing a major source of GABA production, it was hypothesized that the production of large amounts of GABA via this route may affect its production via GAD. Thus the activity of GAD and the expression of its genes were investigated in the control and the HP cells on different days of the 7 d culture cycle. The effect of an inhibitor of GAD on the production of GABA from the two pathways was also investigated.

Introduction

The non-protein amino acid GABA, discovered in plants about half a century ago, has gained considerable attention as not only an important neurotransmitter in mammals (Bouché and Fromm, 2004, Mazzucotelli et al., 2006), but also as a signaling molecule in plant cells (Bouché and Fromm, 2004; Beuve et al., 2004; Bown et al., 2006).

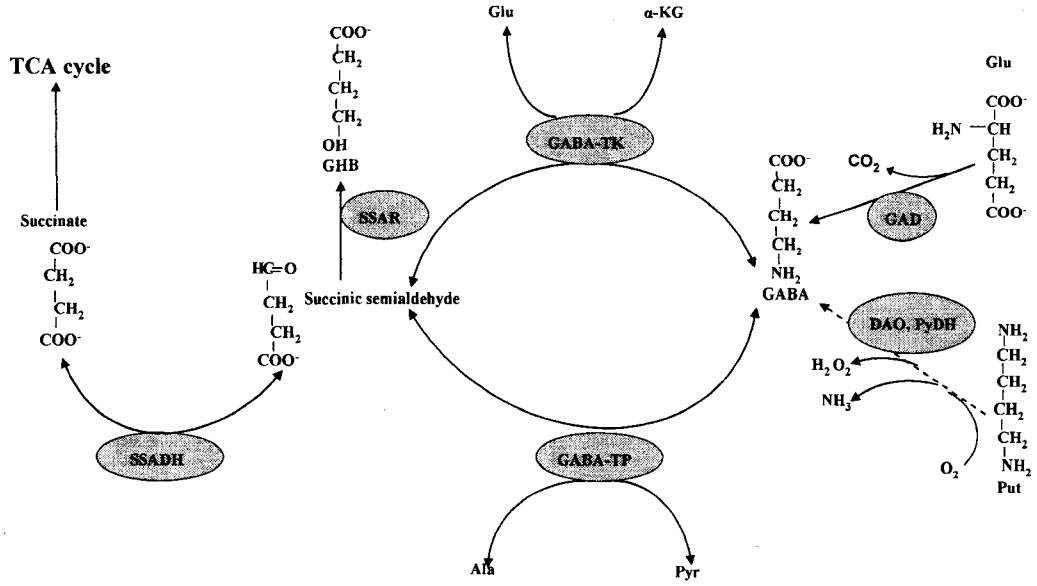

Figure 43. The GABA shunt (adapted from Bouché and Fromm, 2004).

A short pathway known as GABA-shunt (Fig. 43) is responsible for the metabolism of GABA in both plants and animals (Kinnersley and Turano, 2000; Bouché and Fromm, 2004; Mazzucotelli et al., 2006). The three important enzymes making up this pathway are the cytosolic glutamate decarboxylase (GAD, EC 4.1.1.15), and the mitochondrial GABA transaminase (GABA-T, EC 2.6.1.19) and succinic semialdehyde dehydrogenase (SSADH, EC 1.2.1.16; also mentioned as 1.2.1.24; Busch and Fromm, 1999). The irreversible decarboxylation of Glu to produce GABA is catalyzed by GAD which is apparently regulated by the Ca^{2+}-calmodulin (CAM) complex (Akama and Takaiwa, 2007). GABA is then transported into mitochondria where a transaminase reaction converts it into succinic semialdehyde (SSA). This reaction is catalyzed by GABA-T which uses either α-ketoglutarate (α-KG; GABA-TK) or pyruvate (Pyr; GABA-TP) as an amino group acceptor. The enzyme SSADH reduces SSA to succinate which is then catabolized via the TCA cycle. The activity of SSADH can be inhibited by both ATP and NADH. This pathway of GABA catabolism (the GABA-shunt) bypasses two steps of the regular TCA cycle, namely the conversion of α-KG to succinyl-CoA by α-KG dehydrogenase (α-KGDH; EC 1.2.4.2) and succinyl CoA to succinate by succinyl-CoA ligase (SCoAL; EC 6.2.1.5). Alternatively, especially under conditions of hypoxia, SSA can be reduced to γ-hydroxybutyric acid (GHB) via succinic semialdehyde reductase (SSAR, EC 1.1.1.61), also called GHB dehydrogenase (GHBDH; Breitkreuz et al., 2003). This reduction product of SSA is a potential neurotransmitter in animals, while its production and its role in plants are still unknown (Bouché and Fromm, 2004).

The other route for the synthesis of GABA in plants is via the oxidation of Put by DAO; the product Δ^1-pyrroline gets converted stoichiometrically to GABA by Δ^1-

pyrroline dehydrogenase (PyDH; EC 1.5.1.35) (Bhatnagar et al., 2002; Cona et al., 2006). While the pathway for the origin of GABA from Put is well known, most of the prominent articles on GABA do not give it a major importance while discussing GABA metabolism (Shelp et al., 1999; Bouché and Fromm, 2004). Cona et al., 2006 have reviewed the functions of amine oxidases in plant defense and development. Diamine oxidase is a Cu-amine oxidase which has been isolated from a variety of plants including rice (Chaudhri and Ghosh, 1984), barley (Cogoni et al., 1990), maize (Suzuki and Hagiwara, 1993) and wheat (Suzuki, 1996).

The importance of GABA shunt has been established through observations that GABA accumulates in response to a variety of abiotic stresses (Shelp et al., 1999; Kinnersley and Turano, 2000, Mazucotelli et al., 2006). Rolin et al. (2000) observed that GABA contents reached as much as 50% of the free amino acid pool in cherry tomato fruits with pH fluctuations during development. Breitkreuz et al. (1999) found that growth of *Arabidopsis* was quite efficient on medium containing GABA as the only source of nitrogen, showing that its catabolism could provide sufficient amounts of N to regenerate Glu and other amino acids. Bouché and Fromm (2004) pointed out a role of GABA in storage and transport of nitrogen; they stated that the GABA shunt was a pathway that assimilated carbon to generate C:N fluxes for the TCA cycle. This was further confirmed by Studart-Guimarães et al. (2005), who demonstrated that a reduction in the expression of SCoAL (by antisense expression of this gene), which reduces the production of succinate in the TCA cycle, can be compensated by upregulation of the GABA shunt thus providing an alternate source of succinate in tomato leaves. This was ascribed to an increase in the activity of GAD. Both SCoAL and GABA-shunt provide

succinate to the TCA cycle. This enhanced participation of the GABA-shunt in feeding the TCA cycle resulted in mild phenotypic changes even in transgenic lines where the SCoAL activity had been strongly inhibited. Their study not only points to the importance of succinate as a metabolite, but also towards importance of the alternate route to its production, i.e. via the GABA shunt. Unfortunately, these authors did not study PA metabolism in the antisense transgenic plants. Castanie-Cornet et al. (1999) and Ma et al. (2002) have mentioned a role of GABA shunt in acid resistance in bacteria, thus indicating a role of GABA in regulating cytosolic pH. The activity of GAD and accumulation of GABA have been known to increase in plants by acidic pH (Snedden et al., 1995, 1996; Shelp et al., 1999).

A role of GABA shunt has also been suggested in protection of plants against oxidative stress (Bouché et al., 2003; Bouché and Fromm, 2004). Bouché et al. (2003) reported that *Arabidopsis* mutants under-expressing *SSADH* lacked the ability to scavenge H_2O_2, thus being more susceptible to oxidative stress. Based on the fact that the conversion of SSAD to succinate generates both succinate and NADH to the respiratory chain, they hypothesized that the utilization of GABA for production of succinate may limit the accumulation of reactive oxygen intermediates during oxidative stress when some TCA cycle enzymes are inhibited. Coleman et al. (2001) reported that yeast knock-out mutants of GABA-shunt genes were more susceptible to injuries caused by H_2O_2.

The property of GABA to act as a neurotransmitter in animals prompted the idea that it might be a potential deterrent towards insects feeding in plants (Bouché and Fromm, 2004). Ramputh and Bown (1996) demonstrated that an enhancement of GABA biosynthesis in plants inhibited the growth and development of certain types of insect

larvae. Bown et al. (2002) found that "insect footsteps" on leaves stimulated the synthesis of GABA. Over-expression of GAD has not only been suggested to make tobacco plants resistant to the root-knot nematode (McLean et al., 2003), but also to prevent their feeding by phytophagous insect larvae (MacGregor et al., 2003). Janzen et al. (2001) noted an enhancement in GABA synthesis during oxidative burst in the mesophyll cells of *Asparagus sprengeri*. The role of GABA as an osmoregulator has been studied by Rentsch et al. (1996) who reported that an *Arabidopsis* Pro transporter, which also transports GABA, is induced during water or salt stress.

As mentioned above, GABA is not only a decarboxylation product of Glu, but also an oxidation product of Put (Cona et al., 2006). As mentioned in Chapter III, Bhatnagar et al. (2002) reported that in the HP cells, the rate of Put catabolism keeps pace with its biosynthesis. Greater catabolism of Put occurs without significant increase in the activity of DAO. Thus, we were interested to test how the inhibition of GAD by 3-mercaptopropionic acid (3-MPA), an established inhibitor of GAD (Martyniuk et al., 2007) affected the metabolism of GABA and that of PAs in the HP cells. As described earlier, the catabolism of Put releases H_2O_2 (Bhatnagar et al., 2002), the accumulation of which has been correlated with cell death (Pellinen et al., 2002, Gechev and Hille, 2005). Thus, the inhibition of GABA synthesis by GAD could have an accelerating effect on Put catabolism by DAO to compensate for the loss of GABA in both the HP and control cells, a condition that may lead to an even greater accumulation of H_2O_2 in the HP cells, causing greater cell damage than in the control cells. Therefore, we also studied the viability of these cells and their mitochondrial activity in response to treatments that inhibited GAD activity.

Materials and Methods

Three-day-old suspension cultures of control and HP cells were used for analysis of the effects of 3-MPA on growth, PAs, MTT activity and Evans blue retention. In the first experiment, 50, 100, and 250 μL of a 100 mM stock solution (in water) of 3-MPA were added to 50 mL suspension cultures giving final concentrations of 0.1, 0.25 and 0.5 mM. For further analysis, only 0.1 mM 3-MPA (Sigma, M6760) was used, and cells were harvested by vacuum filtration (as described before) at 6, 24 and 48 h after treatment. Cells were analyzed for mitochondrial activity (by MTT assay), membrane damage (by Evans blue retention), cellular contents of PAs, GABA, GSH and Ca as described under "General Materials and Methods".

Characterization of glutamate decarboxylase (GAD) activity

Three-day-old control and HP cells (not treated with 3-MPA) were harvested by vacuum filtration (200 ± 5 mg FW) and placed in assay buffer (350 μL 0.1 M Tris, pH 7.3, 0.1 mM PLP, 10% glycerol, 1 mM DTT) in 16×100 mm glass test tubes and frozen (-20 °C) overnight. After thawing on ice for 1 h, 50 μL of labeled substrate (0.1 μCi [1-^{14}C]Glu (Cat. # ARC0240; American Radiolabeled Chemicals, Inc., St. Louis, MO; sp. Act. 50-60 mCi mmol^{-1}) in 5.0 mM unlabelled Glu (Sigma, G8415) was added to each tube. A 2 cm^2 Whatman 3 MM filter paper (Whatman, Maidstone, UK) soaked with 50 μL Scintigest (Fisher Scientific, Lot # 882757) was placed in a polypropylene well (Kontes, Vineland, NJ; Cat # 88230-0000) suspended from a rubber stopper as for the set up of ODC activity. In order to test for the in vitro inhibitory effects of 3-MPA on enzyme activity, either 1 or 5 mM 3-MPA was added to the tubes. The tubes were incubated in a water bath (30 °C) for 60 min at 60 rpm. Reactions were terminated by

injecting 1.0 mL of 0.5 N H_2SO_4 into each tube through the rubber stopper. Following additional incubation for 30 min, the filter papers were removed and counted for radioactivity in 10 mL Scintiverse (Fisher Scientific, Lot # 036130) in a liquid scintillation counter (LSC-6000; Beckman, Fullerton, CA). Enzyme activity was calculated as nmol $CO_2.h^{-1}.g^{-1}$ FW. This experiment was done twice and each time, two replicates per cell line, per concentration of inhibitor were used.

Results

Glutamate decarboxylase (GAD)

Activity (g^{-1} FW) of GAD was almost 40% higher in the untreated HP cells than the control cells (Fig. 44). Addition of 1 or 5 mM 3-MPA to the reaction mix caused 80-90% reduction in enzyme activity in extracts of both cell lines showing that 3-MPA was an effective inhibitor of GAD.

Effects of 3-MPA on growth and fresh weight of cells

In a preliminary experiment, three different concentrations (0.1, 0.2 and 0.5 mM) of 3-MPA were used for treatment with three day old cells. Within 24 h, the HP cells appeared sticky and unhealthy in the two higher concentrations; they died by 48 h. However, the control cells appeared healthier at 0.1 and 0.2 mM concentrations. Based on these observations, only the 0.1 mM concentration was chosen for subsequent experiments. The fresh weights of the harvested pellets of the untreated control and HP cells per flask were comparable at any given time of analysis (Fig. 45A). Significant decrease in fresh weight was observed in both control and HP cells at 24 and 48 h after addition of 0.1 mM 3-MPA, although this decrease was much higher in the HP cells than

in the control cells .

Effects of 3-MPA on mitochondrial activity and membrane function

As mentioned before, colorless MTT interacts with the mitochondrial electron transport chain and gets reduced to form a blue colored product called formazan. Thus, when the overall mitochondrial oxido-reductase activity is higher, the intensity of blue color is proportionately higher. As seen in earlier experiments, the mitochondrial activity in the two cell lines was quite comparable at a given time. Both, the control and the HP cells responded quickly to treatment with 3-MPA in that they showed a significant reduction in mitochondrial activity within 6 h (Fig. 45B). During the next 42 h, the response of the two cell lines was quite different; while in the HP cells, a further decrease in mitochondrial activity was observed at 24 h, in the control cells, there was a small (but statistically significant) increase in mitochondrial activity at 24 after addition of 3-MPA. By 48 h, the HP cells had almost entirely lost their mitochondrial activity; however, the control cells seemed to maintain it at a still higher level in the MPA-treated cells than the untreated cells.

Evans Blue retention, which indicates depolarization of the plasma membrane, showed (as before) that there were more membrane-compromised HP cells than control cells at each time of analysis (Fig. 45C). A significant increase in membrane damage in response to 3-MPA treatment was seen within 6 h in both types of cells; however, the control cells showed a recovery from the effect, while in the HP cells the adverse effect continued until 48 h of treatment.

Effects of 3-MPA on polyamines, GABA and glutathione

As expected, the Put content was several-fold higher in the untreated HP cells than the control cells at all times during the period of this experimentation (Fig. 46A). A significant decrease in Put content on addition of 3-MPA was seen at 24 and 48 h in both cell lines, although this decrease was much greater (as a percentage of the original amount) in the HP cells than the control cells; little effect on Put content was seen at 6 h in either cell line. No significant changes in cellular Spd was seen in the control cell line on treatment with 3-MPA (Fig. 46B); however, in the HP cells 30 to 40% reduction in Spd content was seen with 3-MPA treatment.

The effect of the GAD inhibitor 3-MPA on GABA and GSH content was studied only at 24 h after 3-MPA addition. It was seen that the presence of 3-MPA caused a significant increase in GABA content in the control cells, while a significant decrease in GABA was seen in the HP cells under the same conditions (Fig. 47). As with GABA, the addition of 3-MPA caused no significant change in the GSH content of control cells (Fig. 48); in the HP cells, an almost 70% reduction was seen in GSH content in response to 3-MPA addition.

Discussion

As mentioned earlier, the decarboxylation of Glu and oxidation of Put, both lead to the production of GABA which then enters the TCA cycle via succinate and the GABA shunt. It is however not known if the two pools of GABA ever mix or perform separate functions; even though their catabolic fate is the same, i.e. entry into the TCA cycle via succinate (Fig. 43). Putrescine catabolism is thus not only instrumental in recycling the

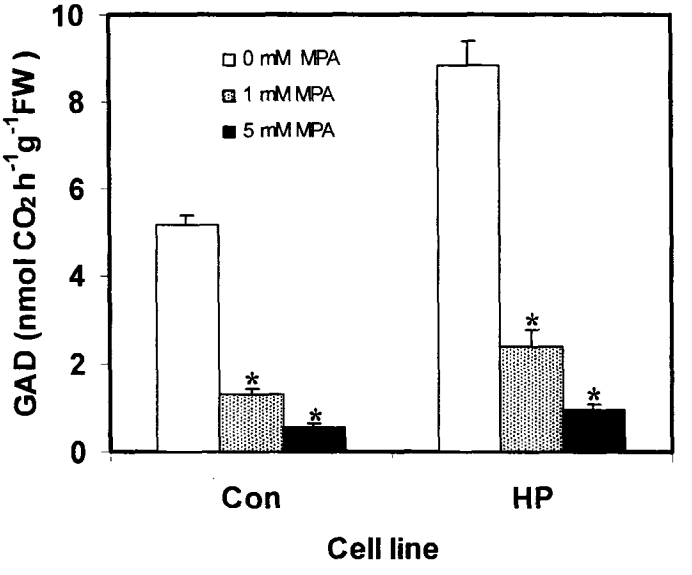

Figure 44. The inhibition of GAD activity by 3-MPA. Extracts of frozen and thawed control and HP cells were incubated in the reaction mix in the presence or absence of 3-MPA and radioactive $^{14}CO_2$ was collected on filter papers. Each bar is mean (±) SE of 4 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in enzyme activity due to 3-MPA.

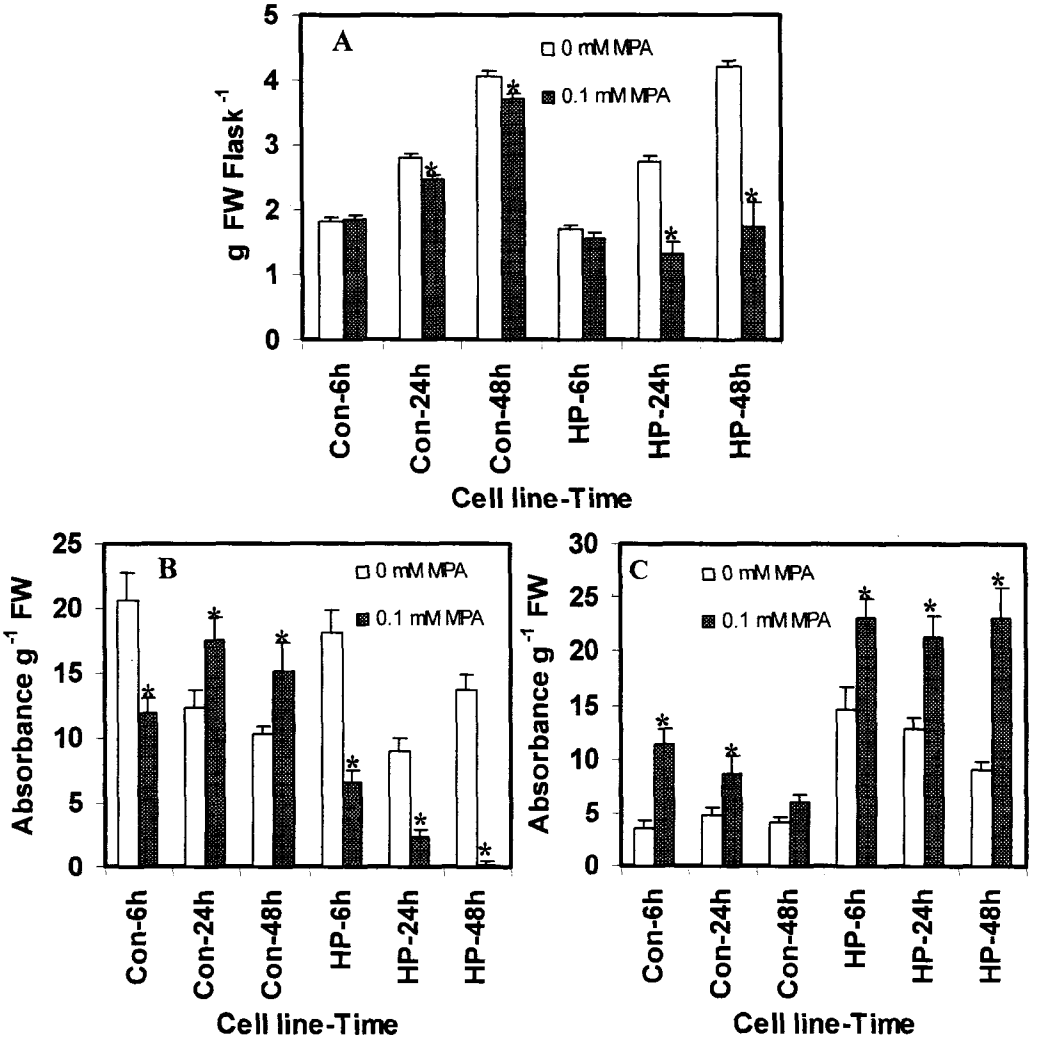

Figure 45. (A) Fresh weight (B) Mitochondrial activity as measured by MTT reduction and (C) cell viability as measured by Evans blue retention in the control and HP cells, 6, 24 and 48 h after 3-MPA treatment. Each bar represents mean (±) SE of 9 replicates from 3 experiments. An * indicates a significant difference ($P<0.05$) between untreated and 3-MPA treated cells within the same cell-line and a given time.

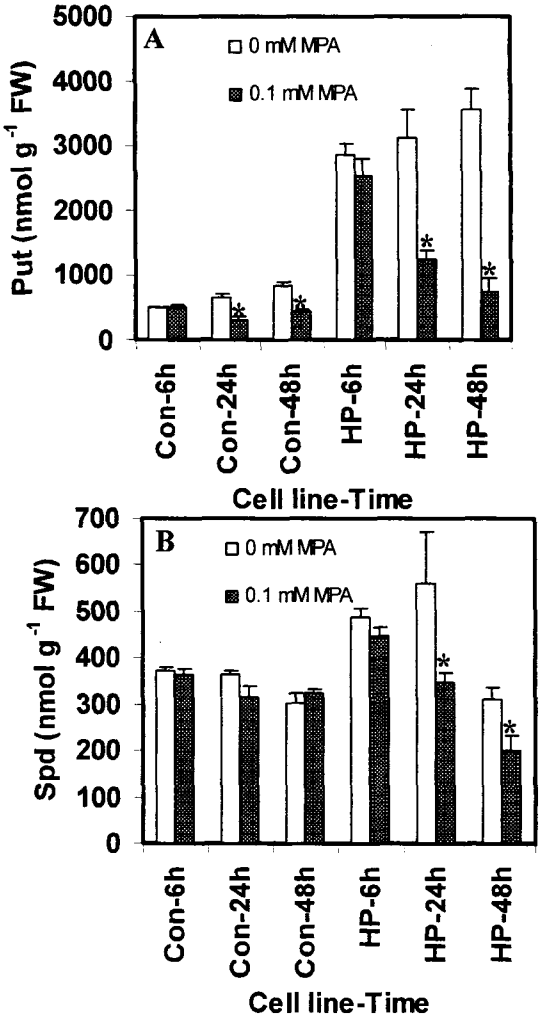

Figure 46. Cellular content of (A) Put and (B) Spd in the control and HP cells, 6, 24 and 48 h after 3-MPA treatment. Each bar represents mean (±) SE of 9 replicates from 3 experiments. An * indicates a significant difference ($P<0.05$) in the PA content g^{-1} FW between the untreated and 3-MPA treated cells within the same cell-line and time period.

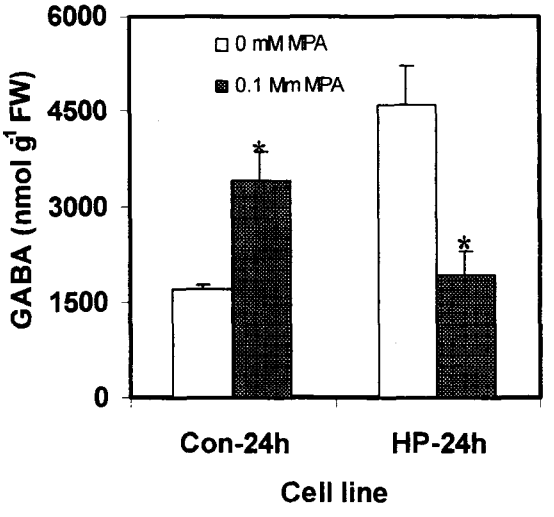

Figure 47. Cellular content of GABA 24 h after 3-MPA treatment to the control and HP cells. Each bar represents mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the GABA content g $^{-1}$ FW between the untreated and 3-MPA treated cells within the same cell-line and time period.

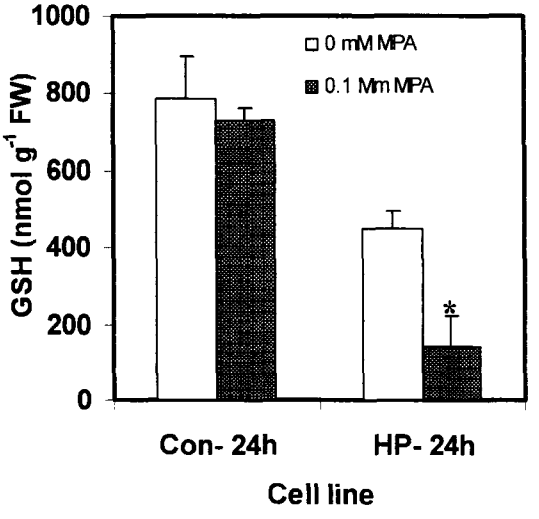

Figure 48. Cellular content of GSH 24 h after 3-MPA treatment to the control and HP cells. Each bar represents mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the GSH content g^{-1} FW between the untreated and 3-MPA treated cells within the same cell-line and time period.

carbon skeleton of this PA, and, thus furnishing some important TCA cycle intermediates with its carbon skeleton, but also aiding in the recycling of the nitrogen component of Put. Since GABA is an important intermediate, which is often accumulated in response to a wide variety of abiotic stress factors, its cellular content at a given time is regulated by: (a) its biosynthesis from Glu directly by GAD or indirectly via Orn and/or Arg by ODC and/or ADC and DAO, and (b) its recycling via GABA-T and SSADH. It has already been established that the HP cells have both a higher rate of Put production as well as its catabolism (Bhatnagar et al., 2002), thus the contribution of this pathway is several-fold higher in these cells than the control cells. The higher amount of GABA in the HP cells observed here on a given day, and that observed earlier in our lab on several days of the 7 day culture cycle (M. Serapiglia, R. Minocha and S.C. Minocha, unpublished data), is consistent with this argument.

It has been suggested that high Ca content stimulates calmodulin-mediated GAD activity (Kinnersley and Turano, 2000). As can be seen from Fig. 27A, HP cells have significantly higher Ca content than the control cells on almost all days of the week. This may explain as to why GAD activity is several-fold higher in the untreated HP cells as opposed to the untreated control cells (Fig. 44). This observation is also consistent with the detailed analysis of GAD activity by M. Serapiglia in the two cell lines. Serapiglia also observed that there was no major difference in the mRNA of GAD between the two types of cells on several different days of analysis during the 7 day culture cycle. These observations lead us to conclude that either the biosynthesis of GABA by GAD is not feed-back regulated at transcriptional or enzyme activity levels, or the two pools of GABA are compartmentalized separately in the cells. The use of 3-MPA as an inhibitor

of GAD, has been reported in animals (Martyniuk et al., 2007); it acts as a competitive inhibitor of the enzyme. It is evident from Fig. 44 that 3-MPA was quite effective in inhibiting GAD activity in poplar cells.

Based on several parameters, we found that the response of the two cell lines to the GAD inhibitor 3-MPA was quite different. A sharp decrease in cell fresh weight was seen in the HP cells, 24 and 48 h after addition of the inhibitor, indicating a strong decline in growth (Fig. 45A). While the mitochondrial activity (Fig. 45B) was significantly affected in the control cells only at 6h, a decrease in mitochondrial activity in HP cells was seen up until 48 h by 3-MPA treatment. These data correlate well with the data from Evans blue retention assay which measures damage to the cell membrane (Fig. 45C); membrane damage due to 3-MPA treatment was greater and longer-lived in the HP cells than the control cells. This may cause more Put (and perhaps GABA and GSH as well) to be lost from the HP cells than the control cells in response to 3-MPA treatment, especially at 24 and 48 h after treatment.

A decrease in the GABA content of HP cells in response to 3-MPA shows that GAD activity in these cells is a major contributor to GABA, despite the fact that Put catabolism in these cells is several-folds elevated. This is consistent with a higher GAD activity in the HP cells seen here (and also earlier on all days of the 7-d culture cycle by M. Serapiglia, R. Minocha and S.C. Minocha; unpublished data). This observation suggests that there probably is no feed-back regulation of GAD by GABA. However, the increase in GABA in control cells in the presence of 3-MPA is an unexpected observation, unless 3-MPA has some role in stimulating the activity of DAO in these cells. Perhaps DAO activity is subject to stimulation by reduced GABA, which will be the case with control

cells in the presence of 3-MPA. There is currently no evidence in favor of or contradictory to this argument. An alternate explanation would be that the observed decrease in mitochondrial activity and increase in membrane damage to HP cells due to 3-MPA (as mentioned above) result in the loss of GABA from HP cells. It should be pointed out that the mitochondrial activity and membrane damage are differentially affected in the control and the HP cells; the former recovering by 24 h and the latter continuing to show the effect. The difference in the reduction in cellular Put in two cell lines is consistent with this explanation; the HP cells lose more Put than the control cells, assuming its production is not affected by 3-MPA. This may also be an explanation for lower cellular contents of GSH in these cells.

This increase in GABA content in the control cells on 3-MPA addition could be attributed to either a higher catabolism of Put or a lower catabolism of GABA, resulting in its greater accumulation. The latter seems more probable since Put content does not decrease significantly in the control cells on addition of 3-MPA. Thus, inhibiting GABA synthesis from Glu could be acting as a signal to inhibit its utilization in downstream processes, leading to its greater accumulation. In the HP cells, however, a significant decrease in Put content due to GAD inhibition points towards an even greater catabolism of Put to compensate for the loss of GABA from Glu, resulting in greater generation of H_2O_2 . This could explain the greater loss in membrane integrity and mitochondrial activity in these cells on GAD inhibition.

As stated before, a role of GABA has been suggested in preventing oxidative stress in plants (Bouché and Fromm, 2004). It was observed that *Arabidopsis* mutants, exhibiting limited expression of *SSADH* lacked the ability to scavenge H_2O_2, thus being

more susceptible towards the toxic effects of oxidative stress (Bouché et al., 2003). Since the conversion of SSAD to succinate supplies both succinate and NADH to the respiratory chain, Bouché et al. (2003) hypothesized that the utilization of GABA towards production of succinate may limit the accumulation of reactive oxygen intermediates during oxidative stress when some TCA cycle enzymes are inhibited. Also, as stated before, Coleman et al. (2001) reported that yeast knock-outs in GABA shunt-genes were more susceptible to injuries caused by H_2O_2. Hence, it is possible that inhibiting GABA production from Glu in the HP cells leads not only to greater catabolism of Put, leading to higher generation of H_2O_2, but also to enhanced utilization of GABA itself, so as to scavenge the same. The GSH content does not change much due to 3-MPA addition in the control cells, while in the HP cells, it decreases significantly (Fig. 46). This is further indicative of enhanced oxidative stress in HP cells due to GAD inhibition, since the lower content of GSH could mean a possible utilization of the same as a reductant to ameliorate the toxic effects of H_2O_2 generation.

Conclusions

Data presented here show qualitatively different responses of the HP and control cells towards GAD inhibition by 3-MPA. While, the already stressed HP cells show a general decline in cell health, Put and GABA content, the control cells exhibit a better tolerance towards the possible negative impacts of GAD inhibition by 3-MPA.

CHAPTER V1

PUTRESCINE OVERPRODUCTION AND CARBON ASSIMILATION

The carbon skeleton of PAs is derived primarily from α-ketogluterate (α-KG), an intermediate of the TCA cycle and the primary precursor of Glu. In poplar cell cultures used here, the ultimate source of carbon is sucrose in the medium. Sucrose is taken up by the cells either by symplastic transport or by diffusion through the plasma membrane (Dennis and Blakely, 2000). Depending upon the biochemical needs in non-photosynthetic tissues, sucrose is channeled along a variety of pathways into different sub-cellular locations (Sturm, 1999). The catabolism of sucrose occurs by its conversion into hexoses either by sucrose synthase (EC 2.4.1.1.3) or invertase (EC 3.2.1.26). While sucrose synthase, in an UDP utilizing reaction, converts sucrose into UDP-glucose and fructose; invertase produces the monosaccharides glucose and fructose. As mentioned earlier, the TCA cycle intermediate α-KG provides the carbon skeleton for biosynthesis of amino acids like Glu, the amine group being derived from assimilated N via Gln. Since the increased production of Put in the HP cells will utilize large amounts of Glu, it was considered prudent to study the incorporation and assimilation of ^{14}C-sucrose from the medium into PAs. We wanted to test if the increased utilization of carbon into PAs in these cells results in higher uptake and assimilation of sucrose or there only is a shift in the metabolism of carbon without effects on sucrose uptake; thus we studied the uptake and incorporation of ^{14}C from sucrose into PAs in the control and the HP cells during the 7 d culture cycle.

Introduction

As described above, the pathway for the biosynthesis of PAs has been well established in a variety of organisms. While changes in PAs during development as well as in response to various environmental conditions have been described in detail; very few attempts have been made to relate these changes to the availability of precursors and their effect on carbon and nitrogen metabolism in the cell. Little information is currently available on the incorporation of carbon from the primary carbohydrates (sucrose, glucose) into PAs when their cellular concentrations fluctuate either during development or in response to abiotic stress, although the study of incorporation of labeled carbon into amino acids has a long history. As early as 1955, Koeppe and Hill elucidated the incorporation of carboxyl and bicarboxylate carbon into Glu in rat. Hill et al. (1958) suggested that labeling patterns in Glu, Ala, Ser and Asp could be used to differentiate between most of the known routes of metabolism of non-carboxyl carbons in rats. Gaitonde et al. (1964) studied the incorporation of carbon from glucose into amino acids in rat brain and liver and observed that about 30 min after intravenous injection with [^{14}C]glucose, brain Glu incorporated 37% of the label and liver Glu, 5.2%. They also made some important observations about different pools of amino acids present in rat brain. Stone et al. (1972) found that the incorporation of carbon from [^{14}C]glucose into several amino acids was greater in hypoglycaemic dogs as opposed to the control animals. Vuorinen (1992) studied the metabolism of inorganic carbon in willow roots and observed that 1 h after feeding the cuttings with ^{14}C-labelled $NaHCO_3$, most of the ^{14}C was fixed into organic acids and amino acids both in light and in darkness in all parts of the plants. More recently Chikov et al. (2005) studied the metabolism of carbon from

labeled glucose and made several interesting observations on the incorporation of label into metabolites like sucrose and amino acids in different tissues and organs of the plant.

The incorporation of labeled carbon from [^{14}C]Orn and [^{14}C]Arg into PAs in the HP cells was studied by Bhatnagar et al. (2001, 2002). They observed that the incorporation of label into any of the three PAs was significantly higher in the HP cells than the non-transformed controls at all time periods tested. The amount of label in the Put fraction was seen to decline slightly after the first 4 h of incubation in response to Put overproduction. Total amount of label recovered in Put was several-fold higher than that in the other two PAs, while the uptake of both labeled amino acids was similar in the two cell lines. Bhatnagar et al. (2002) also studied the regulation of Put catabolism in the HP cells by feeding them with [^{14}C]Orn and [^{14}C]Put and following the loss of [^{14}C] Put in them at various times after transfer to label-free medium. They concluded that in these cells the rate of Put catabolism was proportional to the rate of its biosynthesis; the increased Put degradation occurred without significant changes in the DAO activity.

Earlier studies suggest that the turnover of Glu in plants is rapid (Forde and Lea, 2007). This observation combined with the fact that the TCA cycle intermediates also cycle the carbon rapidly, one can assume that changes in PA biosynthesis will directly affect the pool of Glu and in turn that of α-KG, its direct precursor. Therefore, we investigated the incorporation of ^{14}C from labeled sucrose into PAs in the control and the HP cells. We also studied the effect of limiting carbon supply in the medium on the direct utilization of Arg into PA biosynthesis in the two cell lines.

Materials and Methods

^{14}C sucrose feeding

Three days after transfer to fresh medium, the contents of several flasks of control and the HP cells were pooled separately into 500 mL Florence flasks and mixed by slow stirring on a magnetic stirrer. The cells were then aliquoted into 10 mL portions in 50 mL Erlenmeyer flasks, to which 1 μCi [U-^{14}C]sucrose (ICN, Cat # 1113783) was added. The cells were collected by vacuum filtration on Miracloth at 8, 24, 48 and 72 h after incubation with radioactivity. In a separate experiment, collections were also done at 2, 4, 6 and 8 h. The cells were washed with ~20 mL 2% sucrose solution after filtration. Free polyamines were extracted with 1 mL of 7.5 % perchloric acid (PCA; Fisher scientific, A-228) per 500 mg FW of cells and processed for analysis of PAs by slight modification of the procedure of Bhatnagar et al. (2002). Following freezing and thawing three times, the cells in PCA were mixed by vortexing for 1-4 min and centrifuged for 10 min at high speed. To 500 μL of the supernatant (in a disposable glass test-tube), equal amounts of saturated sodium carbonate was added. The mixture was then transferred to a 2 mL microfuge tube and 250 μL of a 40 mg/mL solution of dansyl chloride in acetone were added. Following 1 h incubation in a water bath at 60^0 C, 100 μL of 100 mg/mL Ala and 200 mg/mL Pro (Sigma, P0380) were added. This was followed by 30 min incubation at 60^0 C, after which PAs were separated from amino acids by addition of 300 μL photrex toluene. Following vortexing and centrifugation, 250 μL of the top organic layer was removed to a fresh 1.5 mL microfuge tube. The remaining layer was re-extracted with additional 200 μL toluene. The toluene and aqueous fractions, as well as the original PCA fraction (50 μL each) were counted for radioactivity in 5 mL scintillene (for toluene

fraction; Fisher SX 2-4) or scintiverse (for aqueous and PCA fractions; Fischer 12-4) using a liquid scientific counter (Beckman, Fullerton, CA; LS 6000SC).

^{14}C arginine feeding

For ^{14}C-Arg feeding experiments, two flasks of either control or HP cells (3-day old) growing under normal conditions were pooled and ~ 50% of the spent medium was removed. Each flask was then incubated with 1 μCi [U-^{14}C-D, L]Arg for 2 h. Cells were then washed with label-free medium and transferred to fresh medium with or without 2% sucrose. Cells were harvested at 0, 8, 24, 48 and 72 h after transfer to fresh medium and analyzed for incorporation of radioactivity into PAs as described above.

Results

^{14}C-sucrose feeding

The radioactivity recovered in the toluene phase accounted for the recovery of label in total free PAs and is presented in Fig. 49A. The assimilation of labeled carbon into PAs was significantly higher in the HP cells than in the controls, at all time periods of analysis after 8 h of incubation; small increase was visible as early as 2 h of incubation. The assimilation of ^{14}C into PAs could be detected as early as 2 h after feeding the cells with ^{14}C-sucrose. While there was a steady increase in ^{14}C assimilation into PAs in the HP cells for at least up to 72 h after feeding, in the control cells, this assimilation seemed to change much less after 24 h.

The radioactivity in the aqueous fraction of the dansylation reaction mix contains both unused sucrose and its various metabolites, including total free amino acids (Fig. 49B). Up until 24 h, the radioactivity recovered in the aqueous phase was almost the

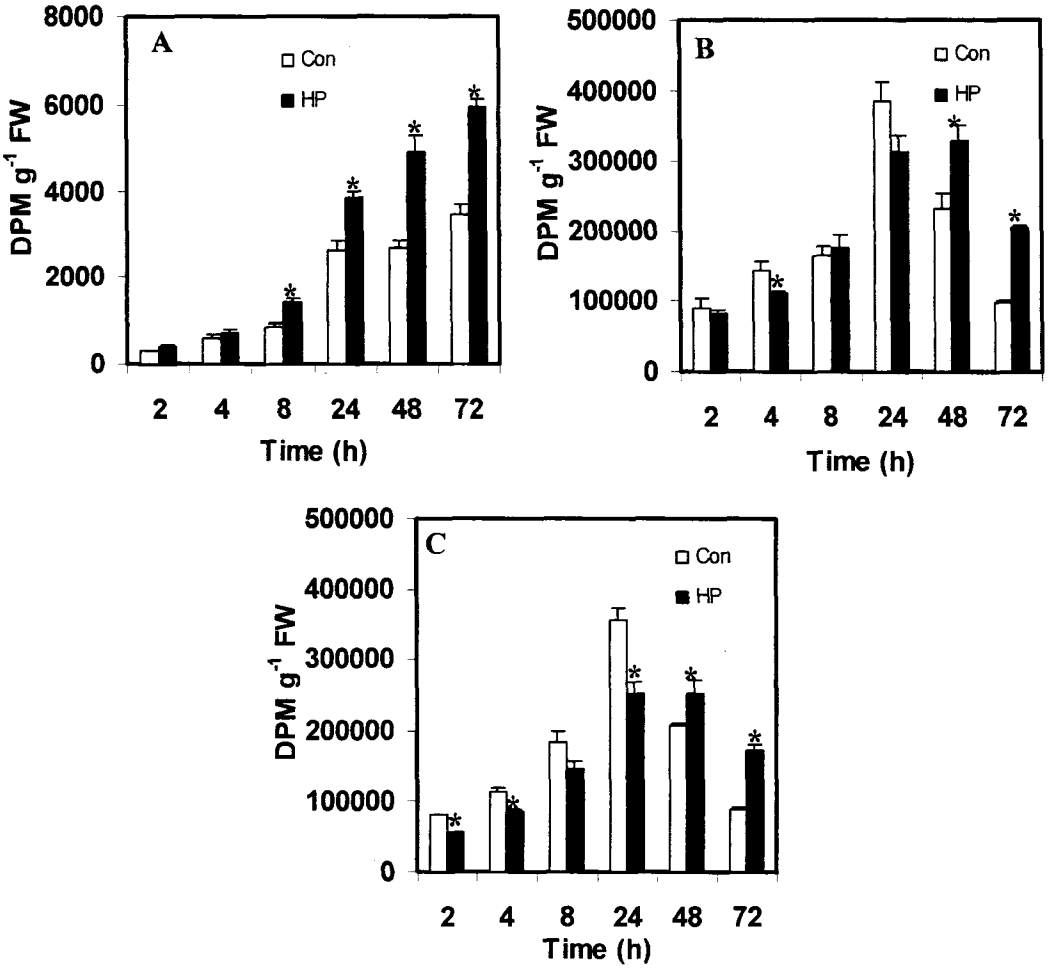

Figure 49. Radioactivity recovered in the (A) toluene (B) aqueous and (C) PCA extracts at different time periods after [U-^{14}C]sucrose feeding to control and HP cells. Each bar represents mean (±) SE of three replicates for 2, 4 and 72 h and six replicates from two experiments for 8, 24 and 48 h. An * indicates a significant difference ($P<0.05$) in ^{14}C assimilation between the control and HP cells at any one time of analysis.

same in both cell lines, perhaps due to the fact that this fraction also contains ^{14}C-sucrose. At 48 and 72 h, a greater amount of radioactivity was seen in the HP cells than in the control cells. At these times, there was also a net loss of ^{14}C content in both the cell lines; the loss being greater in the control than the HP cells. The maximum assimilation of ^{14}C in the aqueous fraction was at 24 h in the control cells and at 24 and 48 h in the HP cells.

Radioactivity in the PCA extract (Fig. 49C) was a measure of the total radioactivity that had entered the cells from the culture medium and it followed trend similar to that for the aqueous fraction, since the percentage of radioactivity entering the PAs (and hence the toluene phase) was much smaller than the amount recovered in the total amino acids (i.e. the aqueous phase).

^{14}C arginine feeding

The recovery of label in the toluene fraction was, as expected (Bhatnagar et al., 2001), higher in the HP cells than in the controls (Fig. 50). For most of the time-periods, in both cell lines, there was a decrease in ^{14}C content in PAs with deprivation of carbon, although these numbers were still higher in carbon deprived HP than in the control cells growing in regular (sucrose supplemented) medium. The difference between plus and minus sucrose cultures increased with the time of culture. Also, with time the total amount of radioactivity in the PA fraction decreased in all cases.

Discussion

During glycolysis, one molecule of sucrose (2 hexose molecules) produces 4 molecules of Pyr, which, through acetyl CoA (a 2-C compound) enter the TCA cycle. Acetyl CoA is formed from Pyr in a decarboxylation reaction (with the release of a single

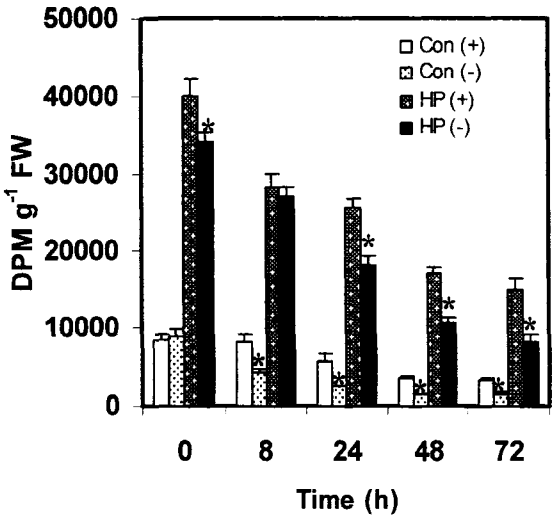

Figure 50. Radioactivity recovered in total PAs at different time periods after [U-C^{14}]Arg feeding and subsequently growing the cells with or without sucrose. Each bar represents mean (±) SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in ^{14}C assimilation between the cells supplemented with sucrose and the ones without it within the same cell line and time period.

CO_2 molecule), brought about by the enzyme Pyr dehydrogenase. Since sucrose that was fed to the cells was uniformly labeled, a $^{14}CO_2$ molecule was released during the conversion of Pyr to acetyl CoA. Two more molecules of CO_2 are released during TCA cycle, one during the conversion of isocitrate to α-KG and the other one, in the subsequent step, i.e. during the conversion of α-KG to succinyl-CoA, although, the carbon molecules lost through CO_2 generation in these two steps are not the ones derived from acetyl CoA (which carries the labeled C from Pyr), hence the label gets carried through to the next intermediate.

The 5-C compound α-KG is responsible for the biosynthesis of amino acids of the Glu family, including Orn and Arg, the two precursors of Put. Both ^{14}C atoms, per molecule of α-KG get carried to Glu and subsequently to Orn. The CO_2 released during decarboxylation of Orn to yield Put, does not carry the label, hence, both ^{14}C atoms get transferred to a molecule of Put. The data presented in Figs. 49 B & C show that the uptake of sucrose during the first 24 h period was somewhat lower in the HP cells than the control cells. After a peak in ^{14}C accumulation at 24 h, both cell lines lost the label; the loss being slower in the HP than the control cells. Thus at 48 and 72 h of incubation in ^{14}C-sucrose, the amount of label in HP cells was greater than that in the control cells. This loss of radioactivity includes all metabolites derived from sucrose as well as its respiratory loss as $^{14}CO_2$. However, the incorporation of ^{14}C into PAs in the HP cells was greater than that in the control cells from the very beginning; small difference being visible within two h and the difference being sustained through out the study period (Fig. 49A). This shows that the flux of sucrose into PAs was increased without additional uptake; which is not surprising since the total fraction of radioactivity incorporated from

sucrose into the PAs was about 1-2% of the sucrose taken up by the cells. The data also indicate that the arte of C in the PAs was turned over slower than in other metabolites (combined) because while the total amount of radioactivity in the cells decreased after 24 h; the fraction in the PAs actually increased with time up to 72 h in both cell lines.

The ^{14}C-Arg feeding experiment was conducted to compare the conversion of ^{14}C-Arg to ^{14}C-Put between the two cell lines under conditions of carbon deprivation. We have reported earlier that the activity of ADC in our suspension cultures is higher in the HP cells than in the controls on almost all days of the 7-d culture cycle (Chapter III). It has been suggested that Orn biosynthesis in the HP cells is enhanced due to its enhanced utilization in Put biosynthesis. Thus the increased incorporation of Arg into PAs is in line with earlier results of Bhatnagar et al. (2001, 2002) and Page et al. (2007). Since the absence of sucrose in the medium caused a reduction in radioactivity in the PA fraction that was derived from Arg; it can be argued that this reduction is the result of a faster turn over of Put in the cells. This may be due to the fact that the cells start using PAs as a source of carbon under conditions of carbon deprivation in the medium.

Conclusions

The hypothesis governing the ^{14}C-sucrose feeding experiment was that the assimilation of carbon into PAs should be higher in the HP cells than in the control cells. As expected, the recovery of label in total PAs was higher in the HP cells than the control cells. It is also interesting to note that while the assimilation of radioactive carbon into control cells increases by more than 50% between 8 and 24 h, it does not increase significantly after that. This is consistent with more than 75% loss of radioactivity from the cells during the period of 24 to 72 h in the control cells. In the HP cells, however, this

assimilation maintains an upward trend up to 72 h after feeding, consistent with the profile of total radioactivity in these cells.

CHAPTER VII

PUTRESCINE OVERPRODUCTION AND THE PROLINE PATHWAY

The PA metabolic pathway is intricately connected to Pro metabolism (Fig. 2, Fig. A1) in that they both share common precursors Glu and Orn. The greater utilization of both Glu and Orn towards Put biosynthesis and at the same time, an increase in Pro accumulation in response to enhanced Put metabolism made it necessary for us to study the effect of such a genetic manipulation on the metabolism of Pro. Thus in addition to the cellular contents of Pro, we analyzed the enzyme activity and the transcript levels of two key enzymes in Pro biosynthesis in the two cell lines on several days of culture.

Introduction

Proline is one of the most widely studied molecules in plant cells under abiotic stress conditions (Madan et al., 1995, Kavi Kishor et al., 2005; Kocsy et al., 2005). Among common responses in all organisms for regulating internal osmolarity is the accumulation of compatible solutes such as sugars and neutral amino acids. Proline accumulation in stressed plants, with salt stress being the most widely studied, confers enhanced osmotolerance to the plant. The enzymes mainly responsible for Pro biosynthesis in plants are OAT, P5CS and P5CR (Kocsy et al., 2005; Kavi Kishor et al., 2005). Proline can be made either from Glu or from Orn. The former occurs via the enzyme P5CS and the latter via OAT (Fig. 2). Proline is catabolized by its conversion to P5C, the reaction being catalyzed by PDH or to Glu by P5CDH (Hare et al., 1999). The cellular contents of

Pro are regulated by a combination of its biosynthesis and degradation as well as its utilization in protein synthesis (Kavi Kishor et al., 2005). The role of the Glu pathway in Pro accumulation under stress conditions has been well established (Kavi Kishor et al., 1995, 2005; Vendruscolo et al., 2007). The role of Orn pathway in Pro accumulation during stress has been a matter of debate (Roosens et al., 1998, 2002; Kavi Kishor et al., 2005). The results of transgenic manipulation of the PA metabolic pathway in poplar cells to overproduce Put also showed an increase in the accumulation of Pro. The fact that both share common precursors for their biosynthesis, prompted us to investigate the role of OAT, which aids in the synthesis of Pro from Orn in the transgenic poplar cells.

Genetic manipulation of tobacco to alter Pro metabolism has also been studied previously (Kavi Kishor et al., 2005). The transformation of tobacco was done with the *V. aconitifolia P5CS* gene regulated by the 35S promoter. The transgenics were reported to produce a high level of the enzyme and accumulated 10 to 18-fold more Pro than the corresponding wild type plants. Overproduction of Pro also enhanced root biomass and the plants tolerated NaCl stress under glasshouse conditions. Although several reports have been published on the transformation of plants to independently manipulate either Pro or PA metabolism, the effect of such a manipulation of one metabolite over the metabolism of the other has not been published.

As mentioned earlier, the levels of cellular Put increase in plants under conditions of abiotic stress. It has been seen that both PA and Pro levels were affected by cadmium stress in soybean root nodules (Balestrasse et al., 2005); i.e. increased concentrations of Cd increased levels of Put as well as Pro in some of the plant organs. Also, it has been known that both Put and Pro accumulate under conditions of high nitrogen nutrition, thus

acting towards the sequestration of excess nitrogen (Minocha et al., 2000; Bauer et al., 2004). So intimately are the pathways for synthesis and degradation of Pro and Put related that it becomes essential to study the impact of over-production and in turn, catabolism of Put through genetic manipulation on the pathway of Pro biosynthesis.

Ornithine aminotransferase is a pyridoxal phosphate-dependent enzyme that utilizes L-Orn and α-kG to produce glutamic-γ-semialdehyde; the latter cyclizes non-enzymatically to form P5C (Kim et al., 1994). Proline can be produced either from Glu or from Orn, with glutamic-γ-semialdehyde being an intermediate in both pathways (Bhatngar et al., 2001). It has been known from labeling experiments that Orn can serve as a precursor to Pro in microorganisms, mammals as well as higher plants (Roosens et al., 1998). Although in bacteria, Orn is converted to α-keto- δ- aminovalerate by α-OAT, followed by a spontaneous cyclization of the product to pyrroline 2-carboxylate (P2C), in plants δ-OAT is responsible for the conversion of Orn to P5C (Kavi Kishor et al., 2005). The *δ* –OAT gene in plants has been studied by several groups (Delauney et al., 1993; Roosens et al., 1998, 2002; Armengaud et. al., 2004). Subcellular localization of OAT has been mostly indicated to be mitochondrial (Kim et. al. 1994; Armengaud et. al., 2004). It has been reported from several sources that OAT activity does indeed increase as a prerequisite for Pro levels to increase (Hervieu et al. 1995; Madan et al., 1995; Roosens et al. 1998, 2002). The other genes of the Pro metabolic pathway, namely, *P5CS, P5CR, PDH, P5CDH* (Fig. 2), have been cloned and characterized in a variety of organisms (Delauney and Verma, 1990; Ginzberg et al., 1998; Miller et al., 2005).

Materials and Methods

OAT assay

Ornithine-δ-aminotransferase was assayed by slightly modifying the method of Kim et al. (1994). Cells (200 ± 5) mg were collected by vacuum filtration on Miracloth and placed in 400 µL extraction buffer in 1.5 mL microfuge tubes. The extraction buffer consisted of 100 mM potassium phosphate (K-Pi) buffer, pH 8.0, 0.2 mM pyridoxal 5-phosphate (PLP) and 0.2 % triton-X 100 (Sigma, T9284). The cells were frozen (at -20^0 C) and thawedg on ice; they were vortexed until thoroughly mixed and centrifuged at 14,000 rpm in a microfuge for 10 min. The supernatant (200 µL) was used for enzyme assay.

The assay mixture (in 2 mL microfuge tubes) in a final volume of 1 mL consisted of 80 mM K-Pi buffer, pH 8.0, 35 mM L-ornithine (Sigma, O2375), 5 mM α-ketoglutarate (Calbiochem, 4210), 0.05 mM PLP, 5 mM o-aminobenzaldehyde (Sigma, A9628) (a 100 mM stock of o-aminobenzaldehyde was made in 95% ethanol). The tubes were incubated in a 37 °C water-bath for 45 min. Following incubation, the reaction was stopped by adding 500 µL 10% trichloroacetic acid (Sigma, T6399). After additional incubation at room temperature for 30 min, absorbance was measured at 440 nm using a Hitachi U-2000 spectrophotometer.

Enzyme activity was expressed as µmol P5C produced per g^{-1} FW h^{-1}, which was calculated using the millimolar extinction coefficient (2.71) for the product of this enzymatic reaction with o-aminobenzaldehyde as mentioned by Kim et al. (1994). Specific activity was expressed as µmol P5C produced mg^{-1} protein h^{-1}. Protein content was determined as mentioned in "General Materials and Methods".

PCR amplification

Potential coding sequences in poplar for the genes of interest were determined by searching the *Populus trichocarpa* database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) using the known sequences from several other plants. Based on the aligned sequences, primers were designed using the Primer-3 software (version 0.4.0) to PCR-amplify the corresponding gene fragments from the cDNA library from our cells (constructed by Dr. Andrew Page from our poplar suspension cultures, using a Creator™ SMART™ cDNA Library Construction Kit; BD Biosciences, Palo Alto, CA) or from the genomic DNA. The annealing temperatures were set at 56 °C for both genes; the rest of the PCR profile was the same as described under "General Materials and Methods". This approach identified one *P5CR* and two *PDH* paralogues (named *PDH*1 and *PDH*2). The PCR primers used were:

P5CR: Fwd (PP5CR-F) 5'CAA TCC ACT CAA ATC CTG CT 3'

Rev (PP5CR-R) 5' GCA GCA ACA ACA GCA TTC AT 3'

*PDH*1: Fwd (PPDH-F) 5' CAG TCT CCC CTC TAA A-3'

Rev (PPDH-FR) 5' CCA CAA TCT CTC CTG GCC TA-3'

*PDH*2: Fwd (PPDH-99F) 5' CGC CGC CTC TCC TCT AAA T-3'

Rev (PPDH-680R) 5' CAT GGA AGG TTG AAG GAA GGA TC-3'

While the *PDH*1 and *P5CR* gene fragments were amplified from the cDNA library, genomic DNA from the poplar suspension cultures was used to amplify *PDH*2 as it could not be amplified from the cDNA library. Details of the PCR reaction and agarose gel electrophoresis have been described under "General Materials and Methods".

An OAT fragment had been previously amplified and cloned in our lab from the cDNA library by Dr. Sridevi Ganapathi, using the following primers. The PCR primers used for *OAT* were:

OAT: Fwd 5' GAG GAG TTC TTG CTA AGC CTA CAC A-3'

Rev 5' CGA GCT GAT GTG GTG TAC TCT GAT-3'

TOPO-ligations and transformation of *Escherichia coli*

Following PCR amplification, the gene fragments were ligated to the TOPO vector using an Invitrogen TOPO TA cloning® kit (Invitrogen, Carlsbad, CA, Lot # 1401099). The ligation reaction, in a final volume of 6 μL, contained up to 4 μL of the fresh PCR product, 1 μL salt solution, and 1 μL TOPO® vector. The reaction was incubated at room temperature for 5 min. The ligated products (2 μL) were used to transform OneShot® TOP10 chemically competent *Escherichia coli* cells according to the manufacturer's instructions (Invitrogen, Lot # 1402704). Following a 30 sec heat-shock at 42 °C, the cells were immediately transferred to ice and treated with 250 μL of S.O.C medium (2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The vials were placed horizontally in an incubator shaker (New Brunswick Scientific, Edison, NJ) at 200 rpm and 37 °C for an hour.

Cells were plated on LB medium supplemented with 50 mg L^{-1} ampicillin and 20 mg mL^{-1} X-gal in dimethylformamide (to enable blue/white screening) and incubated overnight at 37 °C. Following this, white colonies (transformed) were isolated and grown overnight in liquid LB medium (containing 50 mg L^{-1} ampicillin) at 37 °C and 250 rpm.

Plasmid DNA isolation

Plasmid DNA was isolated from *E. coli* cultures by using the FastPlasmid® Mini Kit (Eppendorf, Hamburg, Germany; Cat # 95150619). Approximately 3 mL of culture was centrifuged at 12,000 xg for 1 min and the supernatant discarded. The pellet was mixed thoroughly with 400 µL ice-cold Complete Lysis Solution by vortexing for 1 min. Following incubation at room temperature for 5 min, the lysate was transferred to a Spin Column Assembly and centrifuged for 1 min at 16,000 xg. The DNA was washed with 400 µL of Wash Buffer and eluted with 50 µL Elution Buffer. The concentration of DNA and its quality were measured by UV spectrometry (Abs_{260} and $A_{260:280}$, respectively).

Analysis of ligated products by restriction digestion

Restriction digestion was performed using *Eco*RI (New England Biolabs, Ipswich, MA; Lot # 32) to confirm the presence of the insert in the TOPO vector. Reaction mix contained 1x buffer (NEB, Lot # B306), approximately 2 U/µg DNA of restriction enzyme, 150-200 ng of template DNA (for analysis), brought to a volume of 10 µL with sterile distilled water. Reactions were incubated for 2 to 6 h at 37 °C, and the products were separated by electrophoresis on 1% agarose gel and analyzed as described earlier.

DNA Sequencing and sequence analysis

A typical 20 µL reactions included 8 µL of sequencing premix, 66-132 ng DNA, 5 pmol primer (either T7 or M13), and brought to volume with sterile water. Reactions were cycled 20-30 times followed by ethanol precipitation and sent to the UNH Hubbard Genome Center for sequence analysis. DNA sequences were analyzed and aligned using the BioEdit Sequence Alignment Editor (Hall, 1999).

RNA Extraction

Total RNA was isolated from cell samples using the RNeasy® Plant Mini Kit (Qiagen, Valencia, CA; Cat # 74904). Cell samples (100 mg) were ground in liquid nitrogen and mixed (by vortexing) with 450 µL RLT buffer. The lysate was then transferred to a QIAshredder spin column placed in 2mL collection tube and centrifuged for 2 min at 16,000 xg. The flow-through was transferred to a new microfuge tube and mixed with half the volume 100% ethanol. The sample was then applied to an RNeasy minicolumn placed in a 2 mL microfuge tube and centrifuged for 15 sec at 10,000 xg. The RNA was washed with 700 µL of Buffer RW1, followed by two washes with 500 µL of Buffer RPE and eluted with 50 µL of RNAse free water.

For DNase treatment, RNA was quantified (Hitachi U-2000 spectrophotometer) and approximately 3 µg RNA was used in a 30 µl DNase reaction containing 3 µl RQ-1 DNAse Buffer (Promega, M198A), and 3 µl RQ-1 DNase enzyme (Promega, M 610A). The reaction was incubated at 37°C for 30 min. Protein was removed by bringing the volume to 100 µl with RNase-free water and adding an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The reaction was centrifuged at 4°C for 5 min at top speed. The upper aqueous layer was transferred to a new tube and an equal volume of chloroform:isoamyl alcohol (24:1) added, followed by 5 min centrifugation at 4°C at top speed. RNA was precipitated with an equal volume of isopropanol and incubation at -20°C for 20 min, followed by a 15 min centrifugation at 4°C. The supernatant was discarded and the pellet washed with 80% EtOH by centrifugation at 4°C for 5 min. The pellet was dried in a vacuum centrifuge and resuspended in 10 µL RNase-free water.

cDNA synthesis

DNAse-treated RNA was reverse-transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen, Cat # 18080-044). In a total volume of 13 µL, about 5 µg total RNA was pre-incubated with 1µL of 50 mM oligo-$(dT)_{20}$ (Promega, Cat # 18418-020), 1 µL of 10 mM dNTP mix and an appropriate volume of RNAse free water at 65 °C for 5 min. Following that, the mixture was treated with 4 µL 5x First-Strand Buffer, 1 µL 0.1 M DTT, 1 µL of 40 units μL^{-1} RNAseOUT™ of Recombinant RNAse Inhibitor (Cat # 10777-019) and 1 µL of SuperScript™ III Reverse Transcriptase. The mixture was incubated at 50 °C for 60 min. Resultant cDNA was stored at -20 °C before QRT-PCR.

Normalized gene expression

A SYBR-green dye based assay was performed to quantify gene expression. Typical 30 µL reactions contained 15 µL of Absolute SYBR Green Rox mix (Thermo-Fisher, Cat # AB-1162/A) in a final concentration of 1x, 50 nM each of the forward and reverse primers, an appropriate amount of cDNA (upto 500 ng), and an appropriate volume of DNAse free water. Samples were transferred to a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems, Foster City, CA; Part # 4346906) and subjected to quantitative real-time QRT-PCR using the Applied Biosystems 7500-Fast Real-Time PCR System. The thermocycling conditions included a pre-incubation at 50 °C for 2 min, dye activation at 96 °C for 15 sec, denaturation at 95 °C for 15 sec, primer annealing at 56 °C for 30 sec, and extension at 72 °C for 1 min. A dissociation curve (60 °C to 95 °C) confirmed that majority of the amplification signal was due to interaction of the SYBR-green dye with the specific amplicon and not the primer dimers. Known amounts of cDNA were used as standards and the expression of the gene of interest was extrapolated

from a standard curve. This expression was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*) gene used as an internal control.

Results

OAT activity and total protein content

The activity of δ-OAT g FW^{-1} fluctuated during the 7-d culture cycle in both cell lines (Fig.51A); the changes being much less in control cells than the HP cells. Activity was found to be consistently higher on days 2, 3 and 4 than on days 1, 5 and 7 in both cell lines. Activity of δ-OAT in HP cells was 4 to 5 fold higher than the control cells on days 2, 3 and 4, largely due to an increase from day 1 to day 2 on transfer to fresh medium; this increase was rather small in the control cells. The enzyme activity in both cell lines peaked on days 2 and 3 of the week and declined sharply from day 4 onwards.

The δ-OAT specific activity pattern over the seven-day culture cycle was similar to its activity based on FW basis (Fig.51B), with the HP cells showing significantly higher specific activity than the control cells on days 2,3 and 4.

The total protein content of both cell lines showed an increasing trend from the first to the third days after which they started to decline; the results being similar to those seen in other experiments where soluble protein content was measured (Fig. 51C). Protein content in the HP cells was significantly higher than in the control cells on days 1 to 4.

In summary, the HP cells showed a greater fresh medium effect than the control cells for all three of the parameters studied.

Normalized gene expression

As mentioned previously, one copy of *P5CR* and 2 copies of *PDH* were identified in

poplar cells used here. Following PCR amplification of the gene fragments (Fig. 52) and their ligation into the TOPO vector, the ligated products were analyzed by restriction digestion and subsequent electrophoresis on 1% agarose gel which showed the expected ~700 bp insert in *P5CR*-TOPO and *PDH1*-TOPO and the 3.9 Kb vector (Fig. 53A, B). Since the 1.1 Kb insert in *PDH2*-TOPO (Fig. 46B) had a ~200 bp *Eco*RI fragment, restriction digestion produced a ~200 bp product, a ~900 bp product and the 3.9 Kb vector (Fig. 47B).

The expression of *PDH*1 was declined significantly with time soon after transfer of cells to fresh medium but remained higher in the HP cells than in the control cells on day one (Fig. 54A). Both cell lines showed an increase in *PDH*1 expression thereafter with the expression in HP cells being several-fold higher on day 4. For the remainder of the week, no significant difference was seen in the expression of this gene between the two cell lines.

No significant differences in the expression of *P5CR* were seen between the two cell lines on any given day (Fig. 54B). The expression of *P5CR* followed a similar pattern in both cell lines, showing an increase on transfer to fresh medium up until day 2 followed by a small decline. Over the entire period of 7 days, the maximum fluctuation was about 3 fold in either cell line. The expression of *PDH*2 was much lower as compared to *PDH*1 and could not be accurately measured by the SYBR-green QRT-PCR assay used here. Figure 52 (C) represents PCR-amplified *PDH*1 and *PDH*2 fragments from control and HP cDNA, extracted each day of the week. The extremely faint bands of *PDH*2 are indicative of much lower transcript levels as compared to expression level as compared to *PDH*1. Normalized expression of *OAT* also showed considerable variation on different

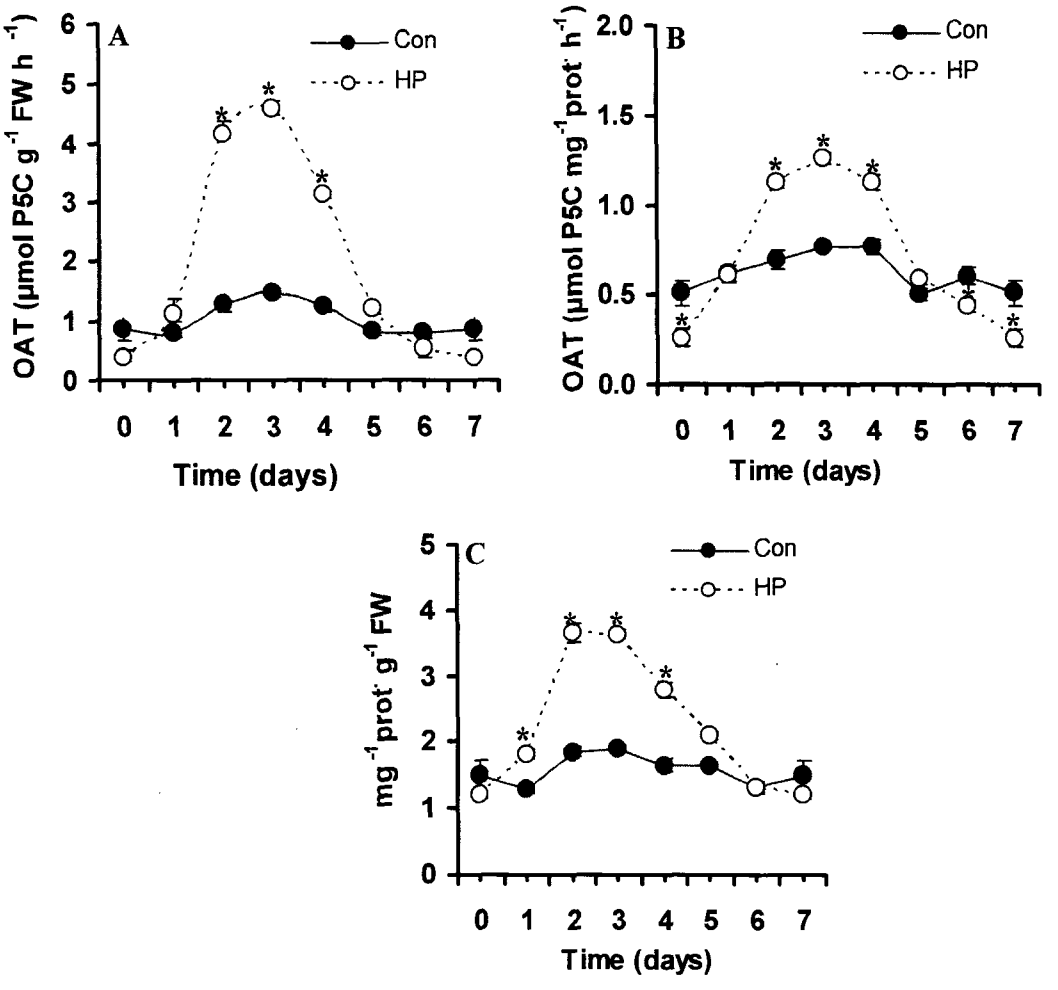

Figure 51. (A) Activity (g^{-1} FW), (B) specific activity (mg^{-1} protein) of OAT and (C) total protein content during analysis of enzyme activity in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the enzyme activity (g^{-1} FW), specific activity (mg^{-1} protein) or protein content between the 2 cell lines on a given day.

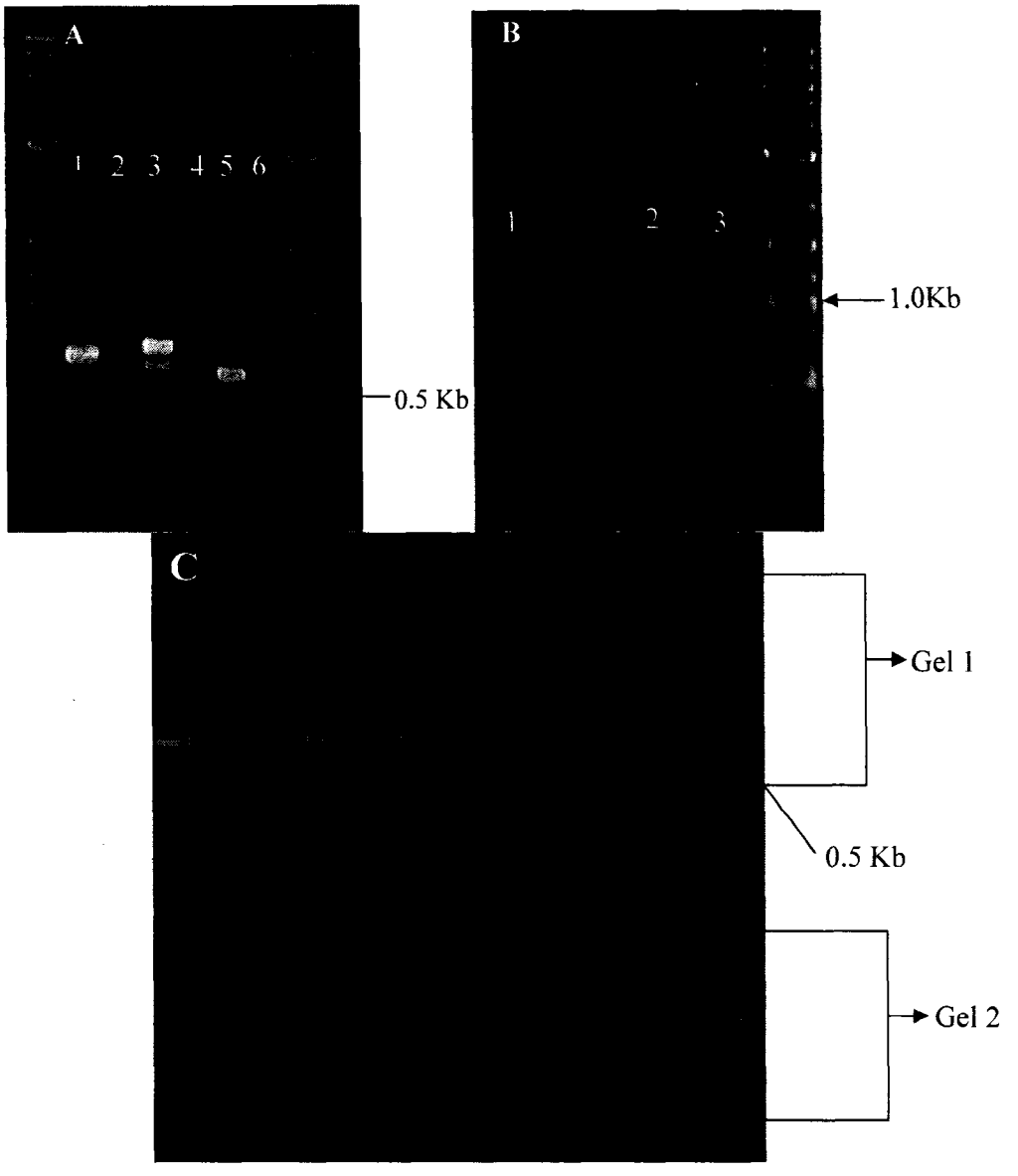

Figure 52. (A) PCR amplification of the 0.7 Kb *P5CR* (lanes 1 and 2) and *PDH1* (lane 3) gene fragments from a poplar cDNA library. Lanes 4 and 6 represent the no-template controls and lane 5 has a 0.6 Kb *ADC* fragment used as positive control. (B) PCR amplification of a genomic 1.1 Kb *PDH2* fragment (lane 1). Lane 2 represents a 1.1 Kb genomic fragment of *PDH1* as positive control and lane 3 represents a no template control. (C) Gel 1 represents PCR amplification of 0.7 Kb *PDH1* fragment and gel-2 represents the 0.58 Kb *PDH2* fragment from control and HP cDNA from all days of the 7 d culture cycle. The NEB 2-Log DNA ladder was used as the marker.

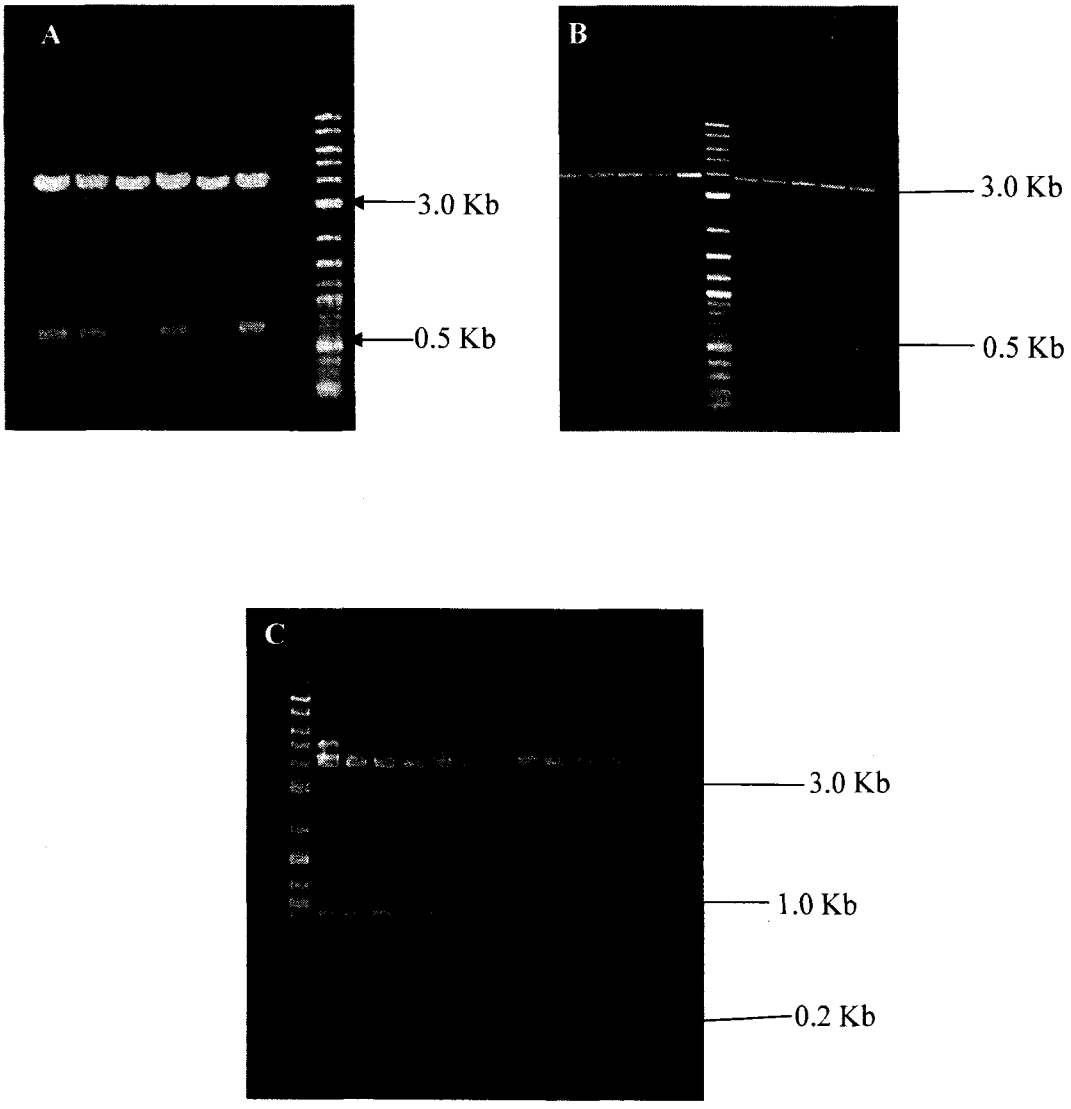

Figure 53. *Eco*RI restriction digestion of several clones of (A) TOPO-*P5CR* (B) TOPO-*PDH*1 and (C) TOPO-*PDH*2 showing fragments of the desired lengths i.e. single fragments of approximately 700 bp in (A) and (B) and two fragments (200 bp and 900 bp) in (C). The NEB 2-Log DNA ladder was used

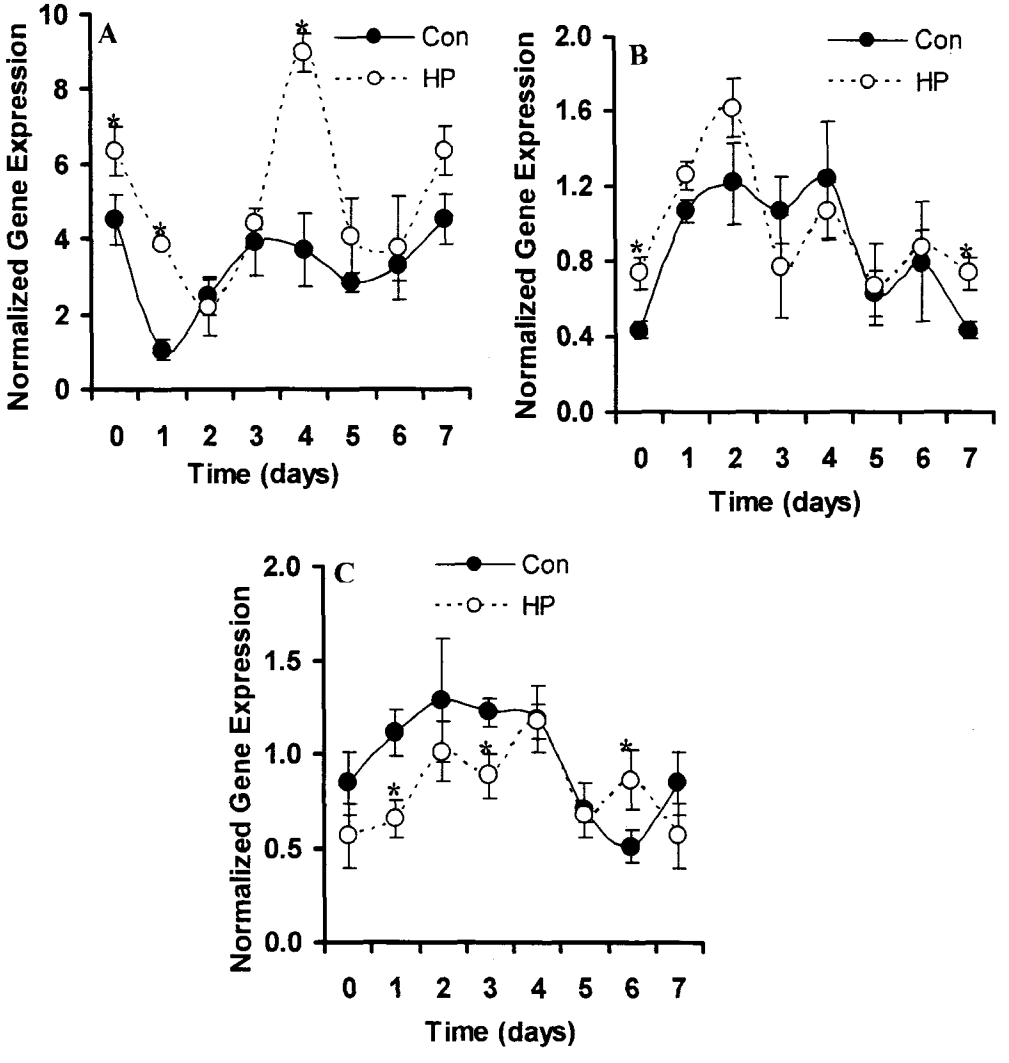

Figure 54. Gene expression (normalized to *G3PDH*) of (A) *PDH*1, (B) *P5CR* and (C) *OAT* in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 3 replicates from 3 experiments. An * indicates a significant difference ($P<0.05$) in gene expression between the control and HP cells on a given day.

days of the week in both cell lines. Small but significant differences in the normalized expression of *OAT* were seen between the two cell lines on some days but the differences were always less than 2-folds.

Celluar proline content

As mentioned before (Fig. 13D), Pro was significantly higher in the HP cells on days two and three. A significant increase was seen in the content of this amino acid in response to fresh medium in the HP cells followed by a decline, while only small changes were seen with time in the control cells.

Discussion

The effects of transgenic manipulation of the PA metabolic pathway on Pro biosynthesis and *vice versa*, have not been studied in details although several reports have been published on independent manipulation of each pathway (Roosens et al., 1998, 2002; Kavi Kishor et al., 2005; Bhatnagar et al., 2001, 2002). This is particularly interesting since both pathways share a number of common regulatory aspects: both share common precursors Glu and Orn and both are upregulated by a variety of abiotic stress responses. The results from my experiments also show clearly that the upregulation of Put causes a concomitant increase in Pro accumulation. What we see here is that the activity of OAT, the enzyme presumably functioning to convert Orn into P5C is significantly higher in the HP cells than the control at a time that is coincident with higher Pro levels in the cells. While the role of this enzyme in Pro biosynthesis is not clearly understood (Roosens et al., 1998), this observation is rather surprising in light of the fact that orn levels in these cells are already low, perhaps due to its over utilization by

the transgenic ODC. Increases in the expression of *P5CR,* although small, are consistent with the increase in Pro seen in these cells at this time. No major change in the expression of *OAT* was seen on any day of the week. As stated earlier, the role of the Glu pathway in Pro accumulation via *PDH* and *P5CR* has been well established.

Considering the response of *δ-OAT* in osmotic stress conditions, there has been a discrepancy between results that indicate an increase in enzymatic activity occurring in many plants (Kandpal and Rao, 1982; Hervieu et al., 1995) and the decrease in mRNA level observed in others (Delauney et al., 1993). Roosens et al. (1998) showed that salt stress induced a higher OAT activity in *Arabidopsis thaliana* seedlings while the adult plants did not show much increase. Levels of *δ-OAT* mRNA also increased in these plantlets under salt stress conditions. Recently, Armengaud et al. (2004) showed a positive correlation between the accumulation of proline and *OAT* transcripts in *Medicago truncatula.*

Since the HP cells overproduce Put from Orn, it can be expected that Put overproduction makes Orn limiting for the synthesis for Pro; i.e. if Orn is a source of Pro in these cells, the latter should decrease in response to the overproduction of Put. This can also be expected while considering the other pathway for Pro biosynthesis; i.e. directly from Glu. But what was instead found with the trends in OAT activity was a higher activity and specific activity of OAT in the HP cells for most of the 7 d culture period. The activity of OAT in the HP cells peaked on the second or third day and was almost at par with the control cells on the first and fifth days of the week and even lower than that in the control lines on the sixth and the seventh days. The trend in OAT activity over the 7 d culture cycle in the HP cells resembled the trend in activity of ODC

(Fig.7A). Thus, by up-regulating the Put biosynthesis, Orn probably does not become limiting for the synthesis of Pro, but rather, the whole pathway gets up-regulated in the forward direction. An alternate explanation would be that OAT instead of functioning in the forward reaction for P5C production acts to in the reverse direction to produce Orn from P5C, thus compensating for its over utilization in Put production; a suggestion that has been made earlier for animal cells (Haslett et al., 2004). This is further supported by the observed increase in the expression of P5CR and *PDH*, which would provide the substrate for this reaction.

Gabaculine and 4-amino-5-hexyonic acid have been used as inhibitors for OAT (Hervieu et al., 1993) and 5-fluoromethylornithine has been reported as a specific, irreversible inhibitor of the same (Daune et al., 1988). Gabaculine has been previously used in our lab to study the inhibition of OAT on polyamine levels (Bhatnagar, 2002: Ph.D thesis) and it was found that at a concentration of 1 mM, gabaculine increased Put and Spd production significantly in the HP cells, 72 h after treatment, while only decreasing the Put content in the non-transformed controls. The increase in PA levels in the HP cells on inhibiting OAT can be explained on the basis of more Orn being available for its conversion to Put.

Conclusions

Cellular content of Pro has been observed to be higher in the HP than the controls in the first three days of the week (Fig. 13D). This raises the question: is there a common regulation of the pathway for the synthesis of Pro and Put, even though the regulatory point for Put production is ODC? Increased expression of *PDH* and *P5CR* and increased OAT activity seem to support the above argument. Although there is a lack of

information in literature regarding the conversion of Pro to Orn in plants; i.e. of the reversibility of OAT, this might well be the case in the HP cells, considering the fact that there is an enhanced consumption of Orn in them, and yet a higher activity of OAT. In animals OAT is known to be a reversible enzyme (Haslett et al., 2004), although its reversibility in plant has not been demonstrated. Since P5CR leads to the biosynthesis of Pro, while PDH leads to its degradation, it is possible that in these cells Pro is acting as a substrate to synthesize Orn. Biochemical assays for the enzymes other than OAT and measurement of their expression levels (including that of *OAT*) will provide more insight into this debate.

GENERAL CONCLUSIONS

Data presented here clearly demonstrate that manipulation of a single step in a metabolic pathway has far-reaching consequences for several reactions within the pathway (Fig. 55-58). Enhanced Put metabolism caused by genetically modifying poplar cells to over express a mouse *ODC* gene resulting in several-fold higher production of Put not only caused concomitant changes in the accumulation of several related metabolites but also affected the physiological responses of these cells to abiotic stress treatments. This study provides an insight into PA metabolism in poplar cells with a combined breadth and accuracy previously unseen in any tissue. The results reveal a complex homeostatic mechanism at work for PA metabolism (as well as the metabolism of pro and GABA; also that of ethylene as shown earlier by Quan et al., 2002) involving several parts of the pathway operating in a coordinated manner. The use of transgenic cells in which a single step in the PA metabolism has been manipulated in a constitutive manner provides evidence for co-regulation of the expression of several genes that control this pathway, and shows that the PA content of cells is indeed under a complex regulation involving multiple layers of control. Specifically, we see that *ADC* and *ODC* expression and enzyme activities are not subject to feedback regulation, while increased accumulation of Put may inhibit expression of some members of the *SAMDC* family, leading to decreased SAMDC activity.

Suspension cultures such as those used here offer a unique opportunity to study the effects of modulating a specific step in a metabolic pathway without complications of different tissue/organ types, translocation of the metabolites from one part of the plant to the other, or intercellular variations in biochemistry. It is, however, possible that different

genes, and in particular different members of the same gene family, may be regulated differently in mature plants in response to different stimuli. Global analysis of the transcripts (by microarrays), combined with complete metabolic profiling, should reveal much more information than the present study has done (A. Page, S.C. Minocha, and R. Minocha - unpublished). Thus far we have seen that upregulation of Put biosynthesis causes: (i) Increased expression of *ODC* and *ADC* genes, along with enhanced activities of the corresponding enzymes; (ii) Decrease in expression of *SAMDC* and activity of the enzyme; (iii) Changes in the cellular contents of almost all amino acids; (iv) A compromise in cell health due to increased oxidative stress; (v) Better tolerance towards Al toxicity; (vi) Increased susceptibility to GAD inhibition; (vii) Greater assimilation of carbon from sucrose in the growth medium; and (viii) Small changes in the expression of *PDH* and *P5CR* and an increase in OAT activity.

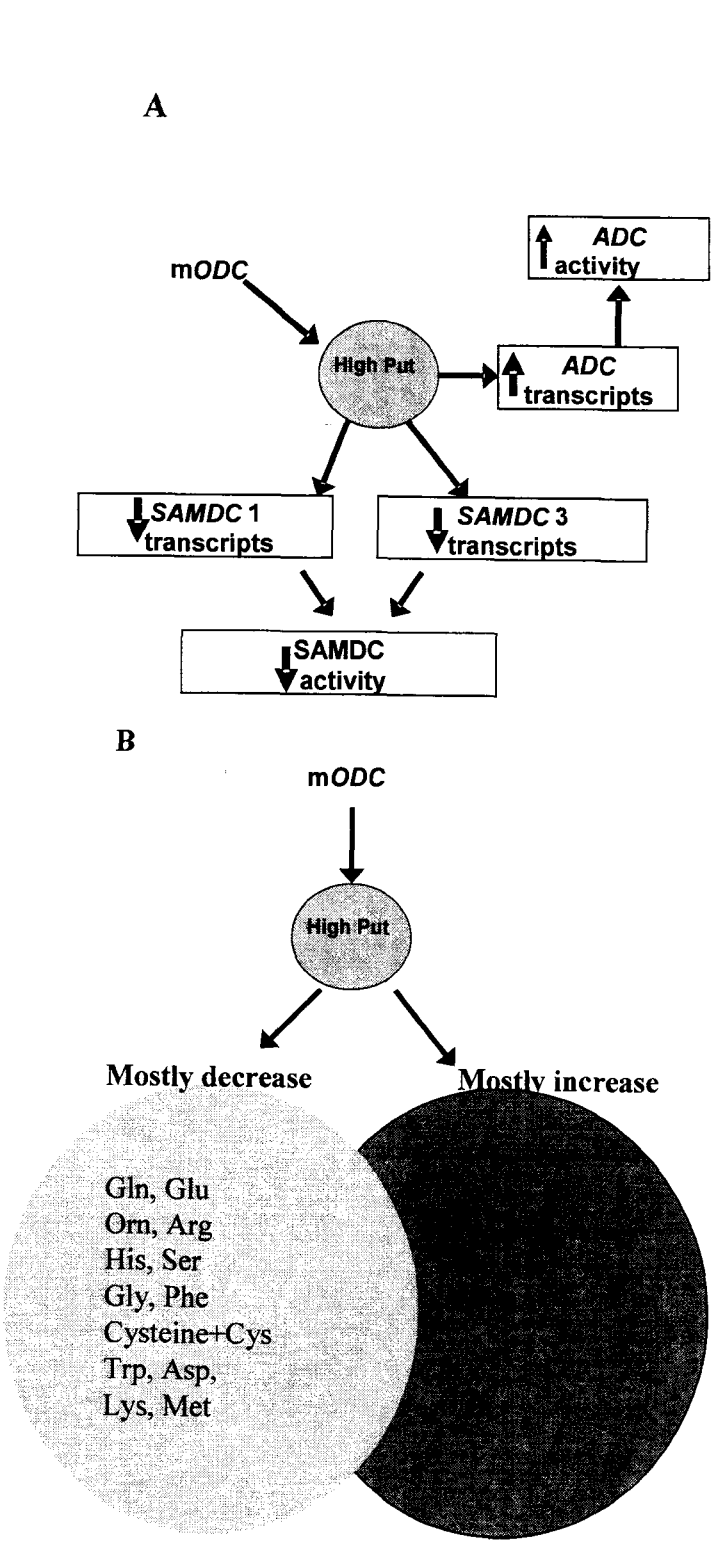

Figure 55. Major conclusions from (A) chapter I and (B) chapter II

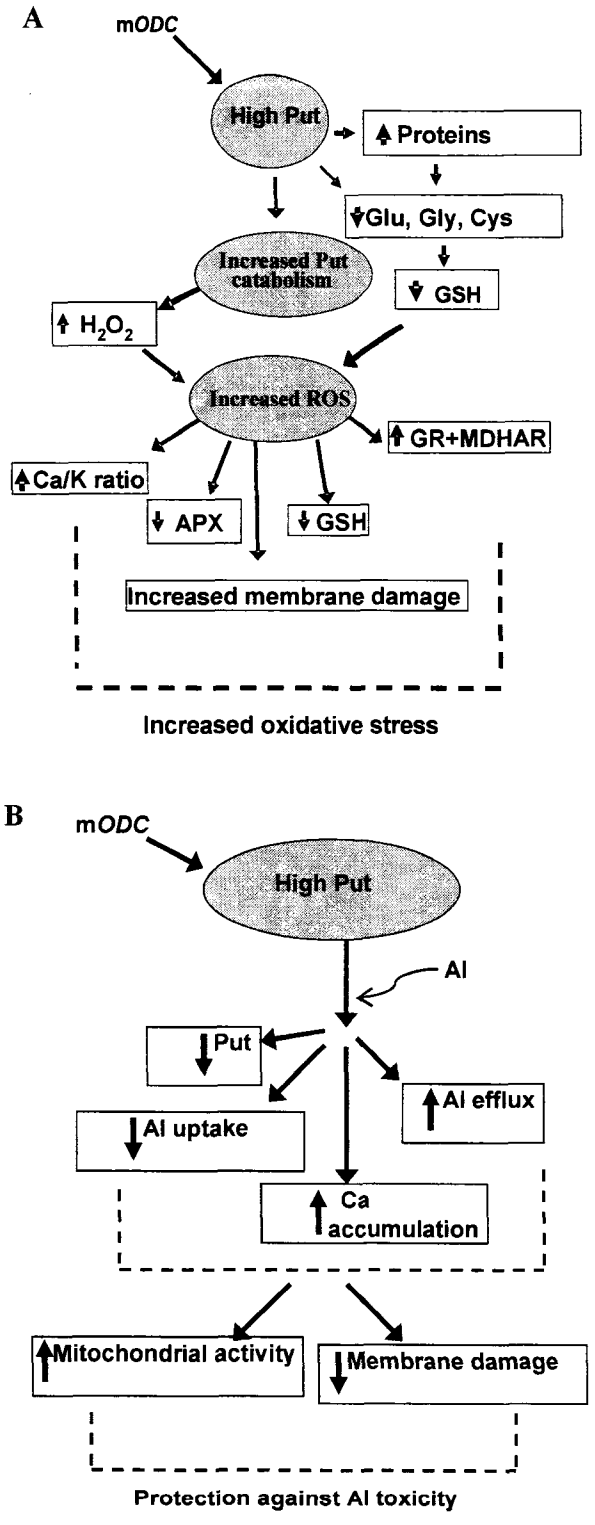

Figure 56. Major conclusions from (A) chapter III and (B) chapter IV

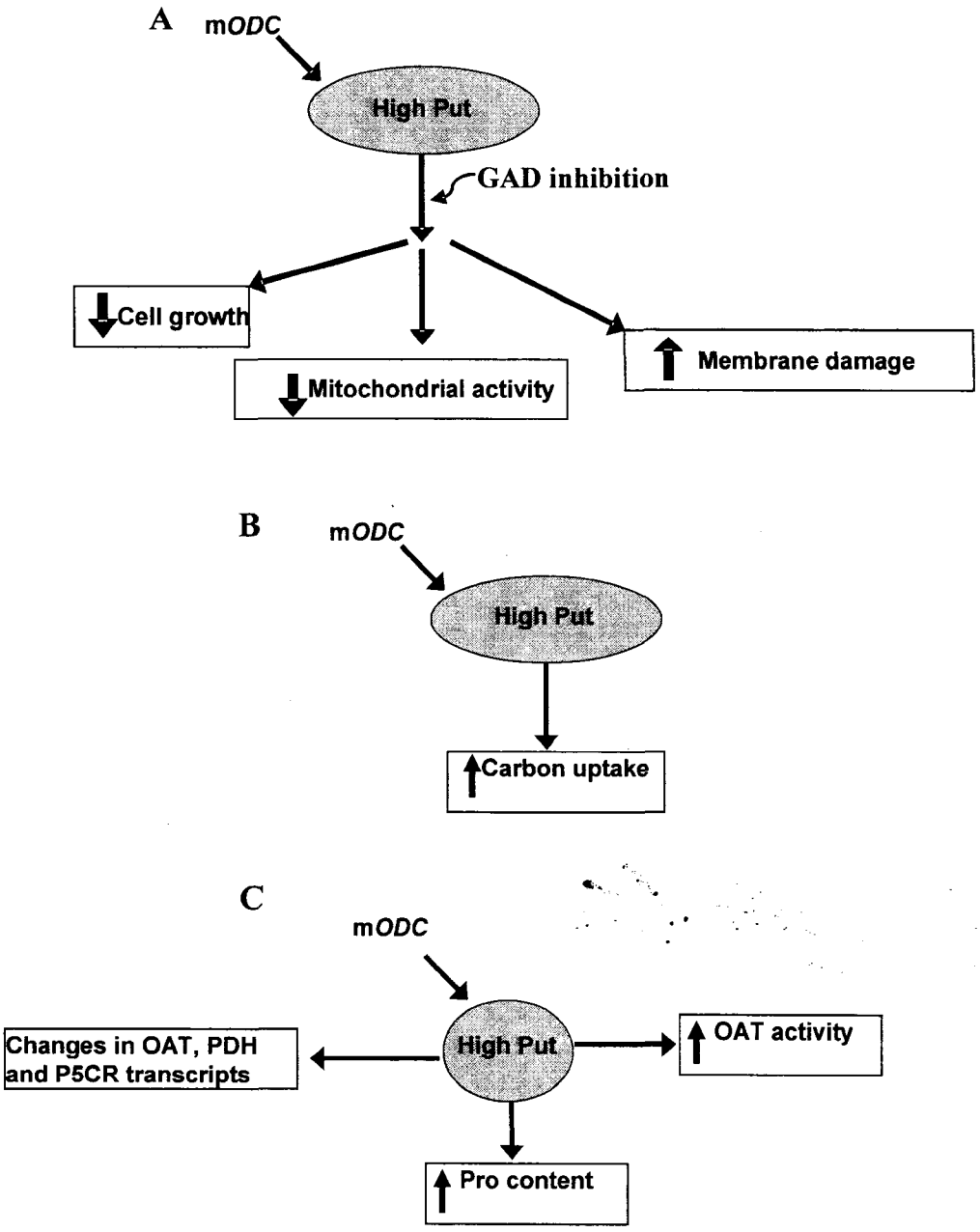

Figure 57. Major conclusions from (A) chapter V, (B) chapter VI and (C) chapter VII

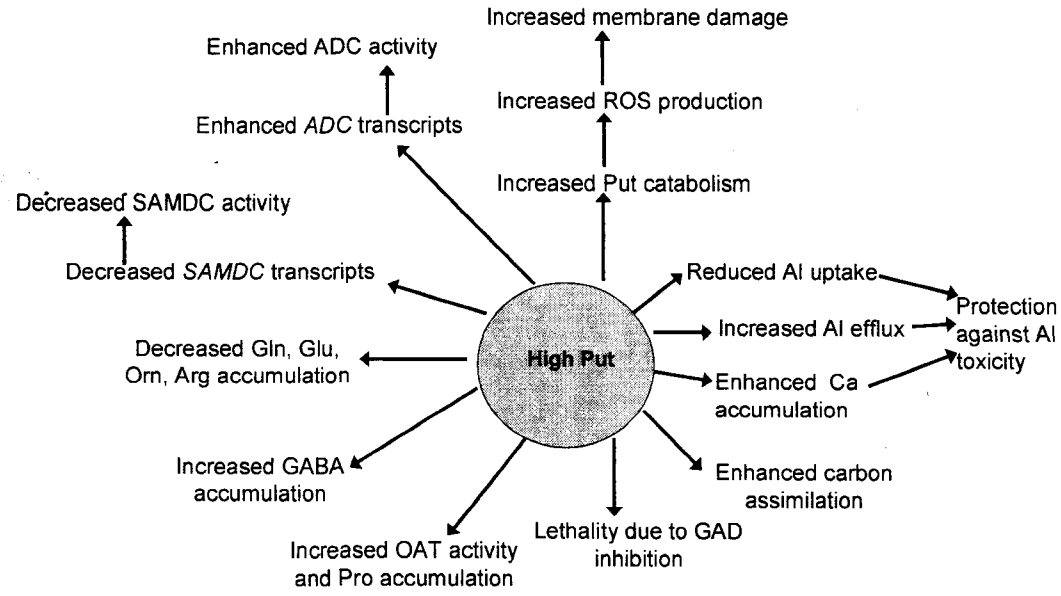

Figure 58. A summary of the major conclusions from the present study.

APPENDICES

APPENDIX A

A STRUCTURAL REPRESENTATION OF THE METABOLISM OF POLYAMINES AND RELATED AMINO ACIDS

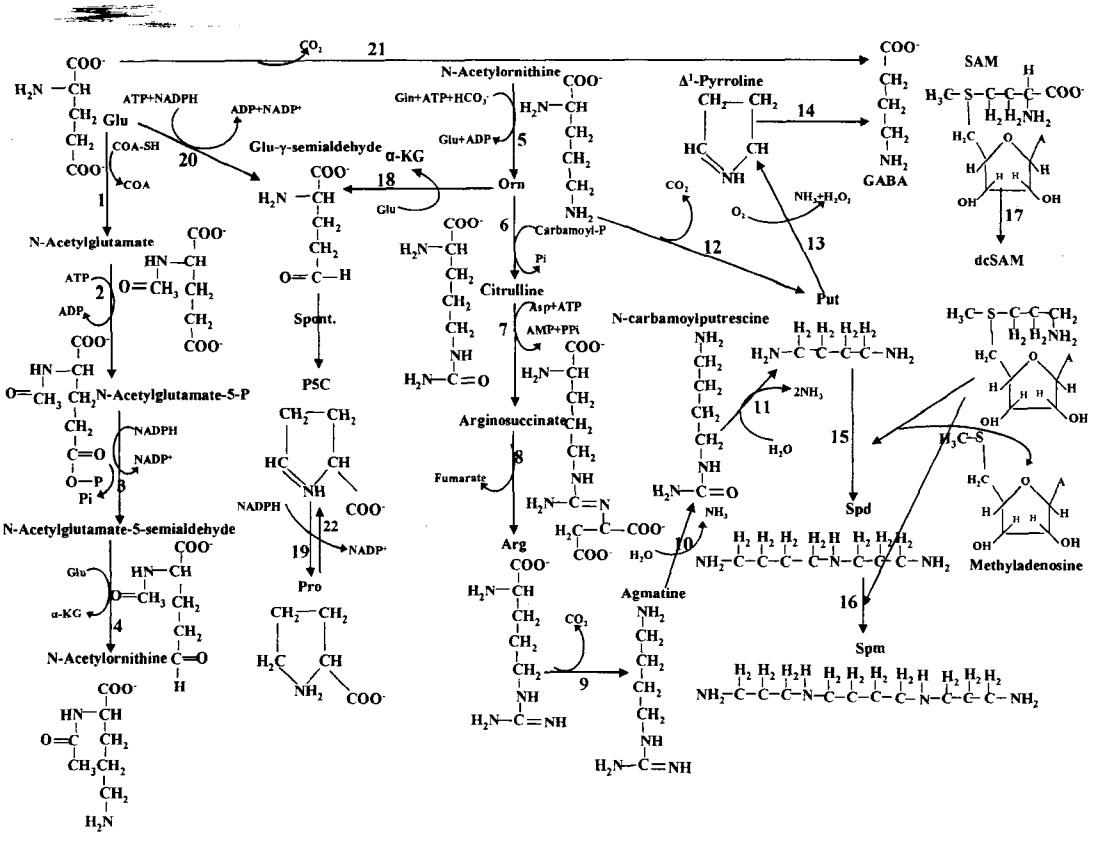

Figure A1. Metabolism of polyamines and related metabolites. The enzymes are: **1**- N-acetylglutamate synthase (NAGS); **2**- *N*-acetyl-glutamate kinase (NAGK); **3**- *N*-acetylglutamate-P Reductase (NAGPR); **4**- *N*2-Acetylornithine aminotransferase (NAOAT); **5**- *N*2-Acetyl-ornithine:glutamate acetyltransferase (NAOGAcT); **6**- *N*2-Acetylornithine deacetylase (NAOD); **7**- Ornithine transcarbamoylase (OTC); **8**- Arginine synthase (AS); **9**- Arginine decarboxylase (ADC), **10**-Agmatine imino-hydrolase (AIH); **11**- *N*-carbamoylputrescine amidohydrolase (CPA); **12**- Ornithine decarboxylase (ODC); **13**- Diamine oxidase (DAO); **14**-Pyrroline dehydrogenase (PDH); **15**- Spermidine synthase (SPDS); **16**- Spermine synthase (SPMS); **17**- SAM decarboxylase (SAMDC); **18**- Ornithine aminotransferase (OAT); **19**- P5C reductase (P5CR); **20**- P5C synthase (P5CS); **21**- Glutamate decarboxylase (GAD); **22**- Proline dehydrogenase (PDH).

APPENDIX B

ANALYSIS OF GENE EXPRESSION

(Results of Dr. Andrew Page; Page et al., 2007)

As expected, the control (*GUS*-transformed) cells showed no signal corresponding to the transcripts of m*ODC* on any day of analysis; the expression of m*ODC* in the HP cells varied on different days (Fig. B1A). The m*ODC* transcripts increased slightly between days 1 and 3, and declined to almost 50% between days 3 and 5. The m*ODC* transcripts again showed a small but statistically insignificant increase on day 7. Comparing the transcript data for days 7, 1 and 3, it is clear that following transfer to fresh medium, there was a lag of about 3 d before an increase in m*ODC* transcript was seen (Fig. B1A).

The p*ODC* transcripts were analyzed in a semi-quantitative manner (using a pair of primers that amplified all three pODCs) by band density analysis of the PCR products at 20, 25, 30 and 35 cycles of amplification; no difference in the transcripts of p*ODC* was seen between the two cell lines on any day of analysis (data not presented).

The p*ADC* transcripts in the m*ODC* transformed HP cells were higher than those in the control cells on any given day (Fig. B1B); however, differences between the two cell lines were not statistically significant ($P<0.05$). A small increase in p*ADC* transcript was seen on day 3 followed by a decline thereafter in both cell lines. A fresh medium effect on p*ADC* transcript abundance was clearly visible in both cell lines within 1 d of transfer; the effect continued until 3 d in the fresh medium.

Out of the three p*SAMDC* genes whose transcripts were quantified by QRT-PCR, p*SAMDC*1 showed by far the greatest expression (Fig. B1C); its transcripts in the control cells being almost 20 and 200 times greater than those of p*SAMDC*2 and p*SAMDC*3 (Fig.

B2A, B), respectively on d ay1. In control cells, the p*SAMDC*1 transcript levels fell almost three-fold between days 1 and 5, after which there was no significant change. On transfer to fresh medium on day 7, a significant increase in the transcripts of this gene occurred within 1 d. In the HP cells, there was neither a significant difference in p*SAMDC*1 expression between any of the time points, nor was there a fresh medium effect apparent from comparison of data for days 7 and 1. Furthermore, p*SAMDC*1 transcripts were significantly lower in the HP cells vs. the control cells on any day of analysis.

Transcript levels of p*SAMDC*2 were not significantly different between the two cell lines on any of the days tested (Fig. B2A). Both lines exhibited a significant ($P<0.05$) decrease in p*SAMDC*2 transcripts between days 1 and 5, after which there was no further loss of transcripts by day 7. In contrast to p*SAMDC*1 transcription, which did not change on transfer to fresh medium in HP cells, a comparison of p*SAMDC*2 transcripts on days 7 and 1 showed a significant increase in transcription of this gene on transfer to fresh medium in both cell lines.

The transcripts of p*SAMDC*3 (Fig. B2B) fell during the course of the 7 d culture period in both cell lines; its transcript abundance in HP cells was consistently lower than the control cells. As with p*SAMDC*2, there was an increase in transcripts of this gene within 1 d of transfer to fresh medium.

Of the two p*SPDS* genes, the maximum expression of p*SPDS*1 (Fig. B2C) was almost 5-fold lower than that of p*SPDS*2 (Fig. B2D) on day 1 of culture. The two cell lines on any given day had similar amounts of p*SPDS*1 transcripts; the same was true of p*SPDS*2. Transcripts of both genes showed a significant increase on transfer to fresh medium (day

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

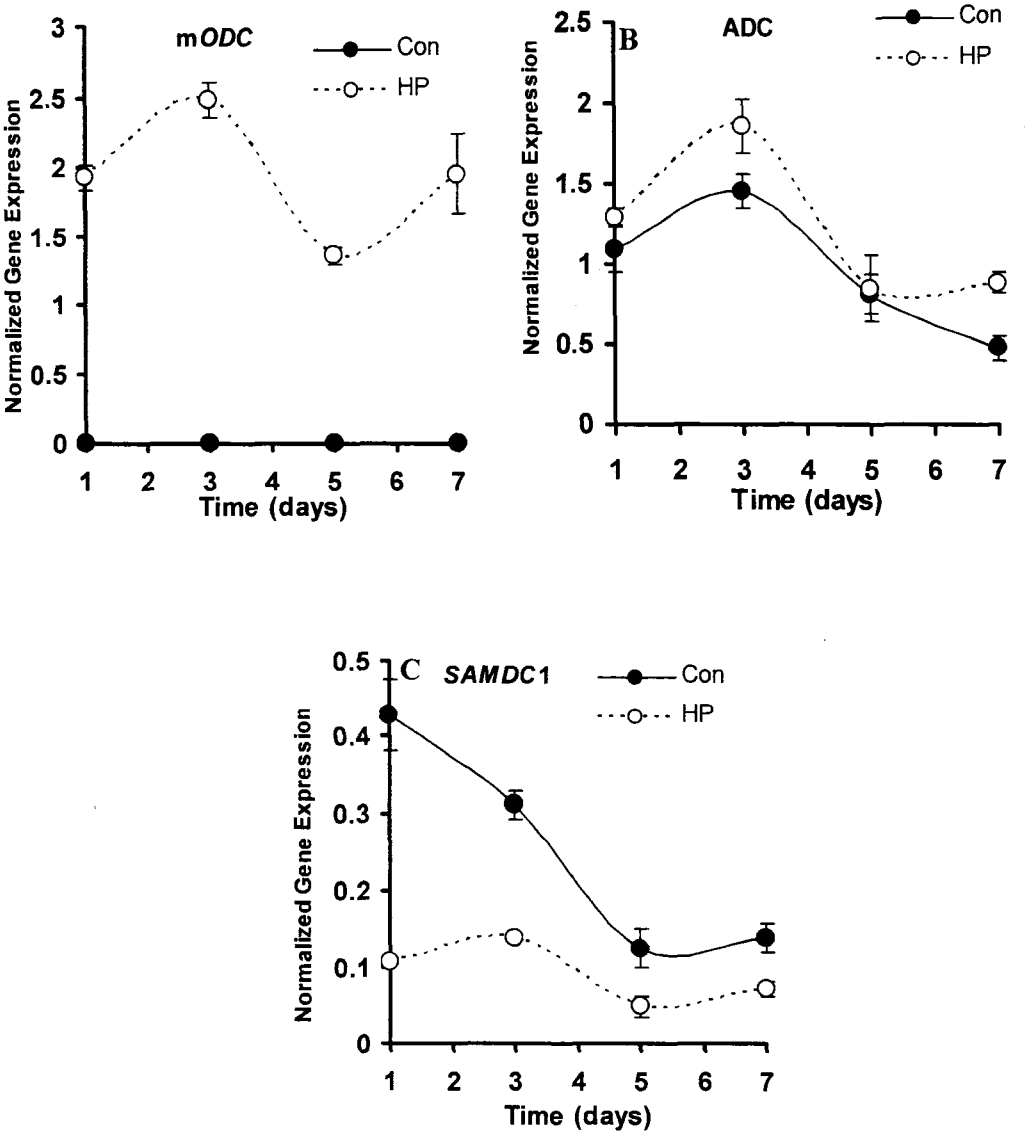

Figure B1. Normalized gene expression of (A) m*ODC* (B) *ADC* and (C) *SAMDC*1 in the control and HP cells on d 1, 3, 5 and 7 of subculture. Data generated by **Dr. Andrew Page**.

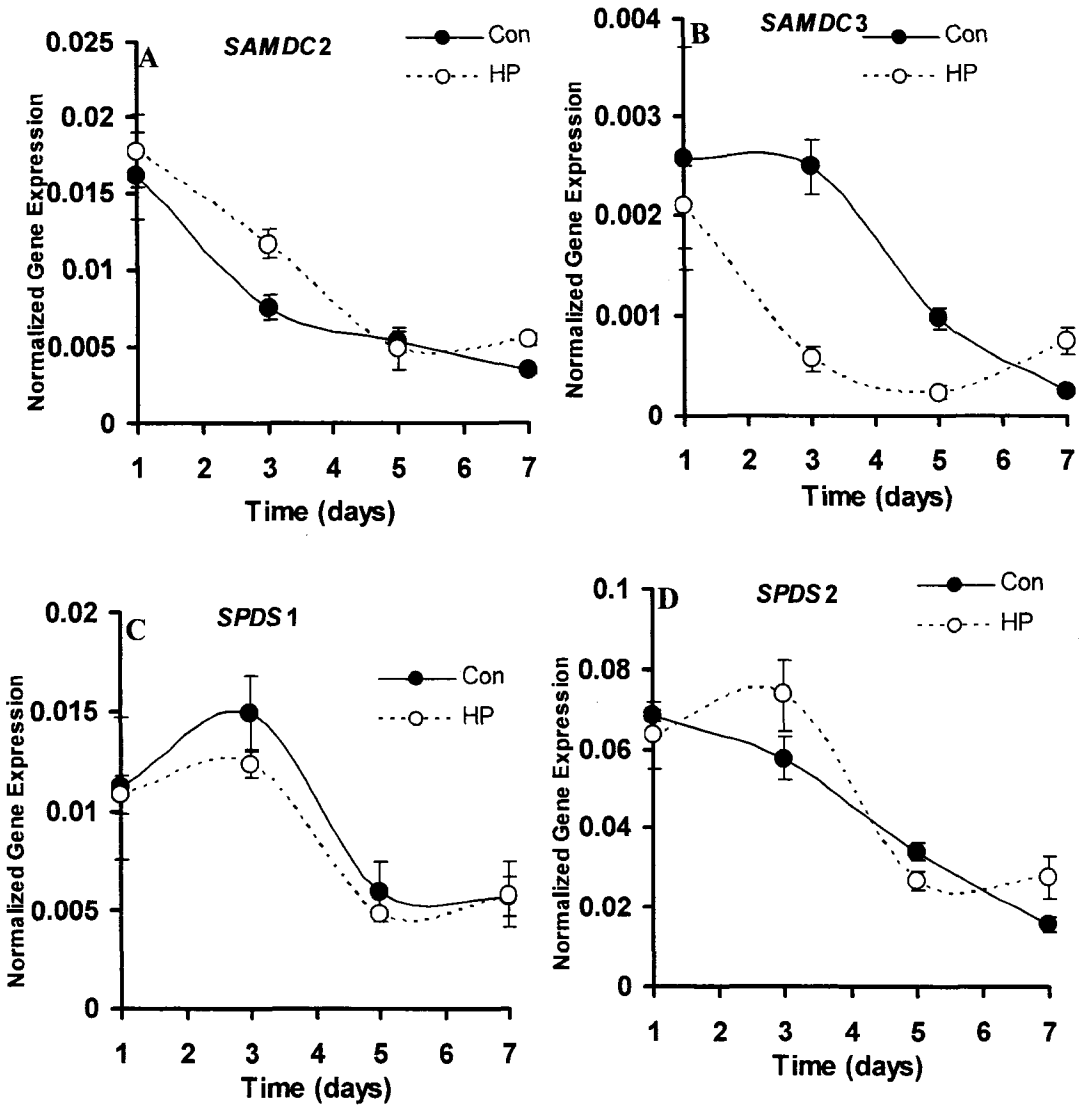

Figure B2. Normalized gene expression of (A) *SAMDC*2 (B) *SAMDC*3 (C) *SPDS*1 and (D) *SPDS*2 in the control and HP cells over the 7 d culture period. Data generated by **Dr. Andrew Page**.

APPENDIX C

EFFECTS OF Al AND Ca ON CELLULAR AMINO ACIDS

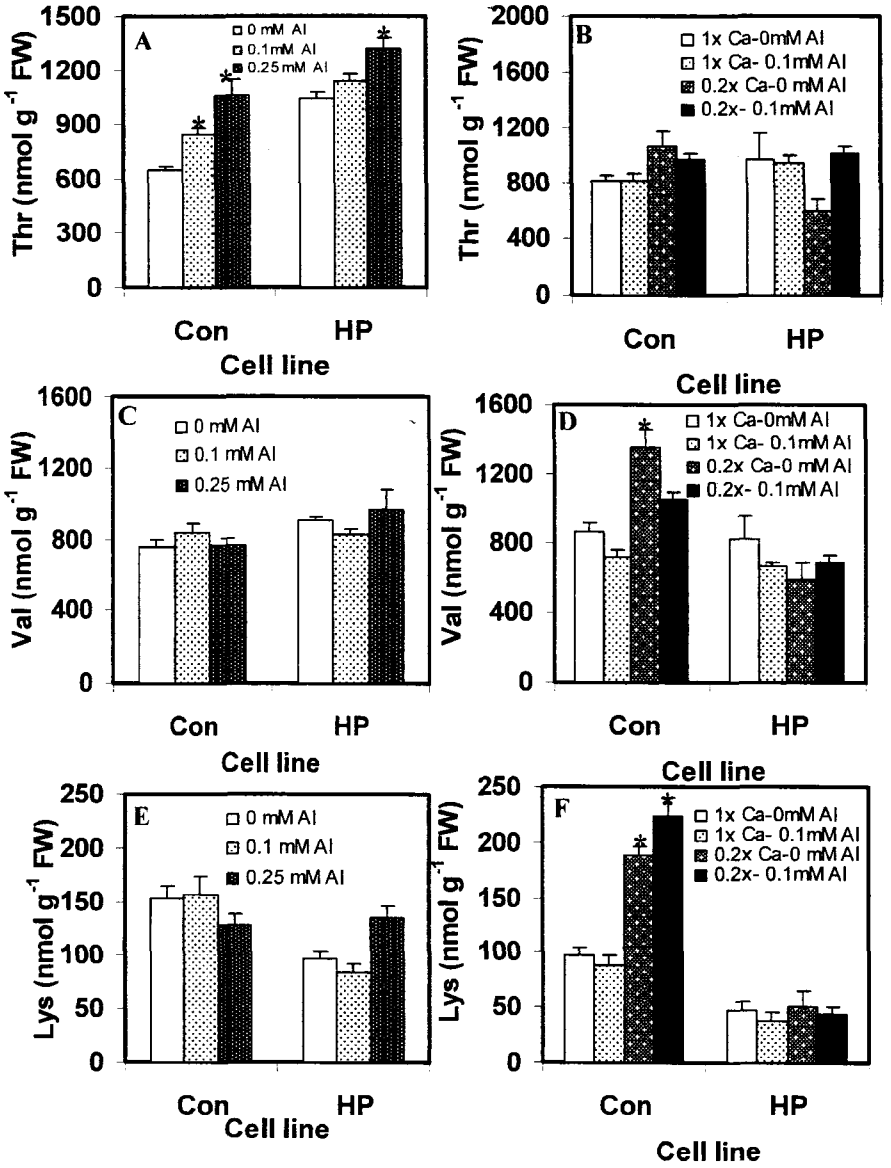

Figure C1. Cellular contents of (A, B) Thr, (C, D) Val and (E, F) Lys in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in amino acid content between the untreated cells and cells growing in Al (in A, C, E) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca (in B, D, F) within the same cell line.

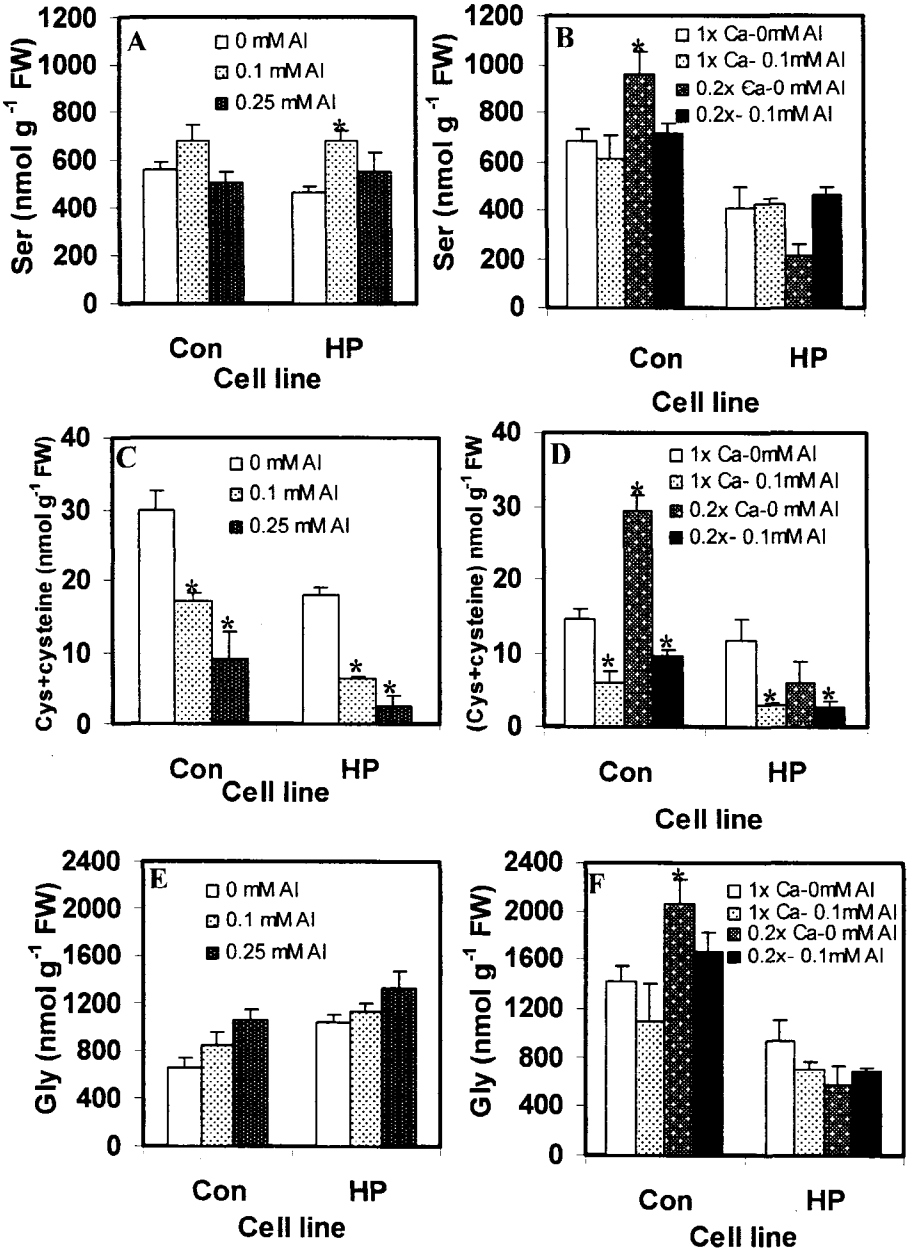

Figure C2. Cellular contents of (A, B) Ser, (C, D) Cys + cysteine and (E, F) Gly in the control and HP cells due to different concentrations of Al (A, C, E) or Al and Ca (B, D, F), 48 h after treatment. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in amino acid content between the untreated cells and cells growing in Al (in A, C, E) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca (in B, D, F) within the same cell line.

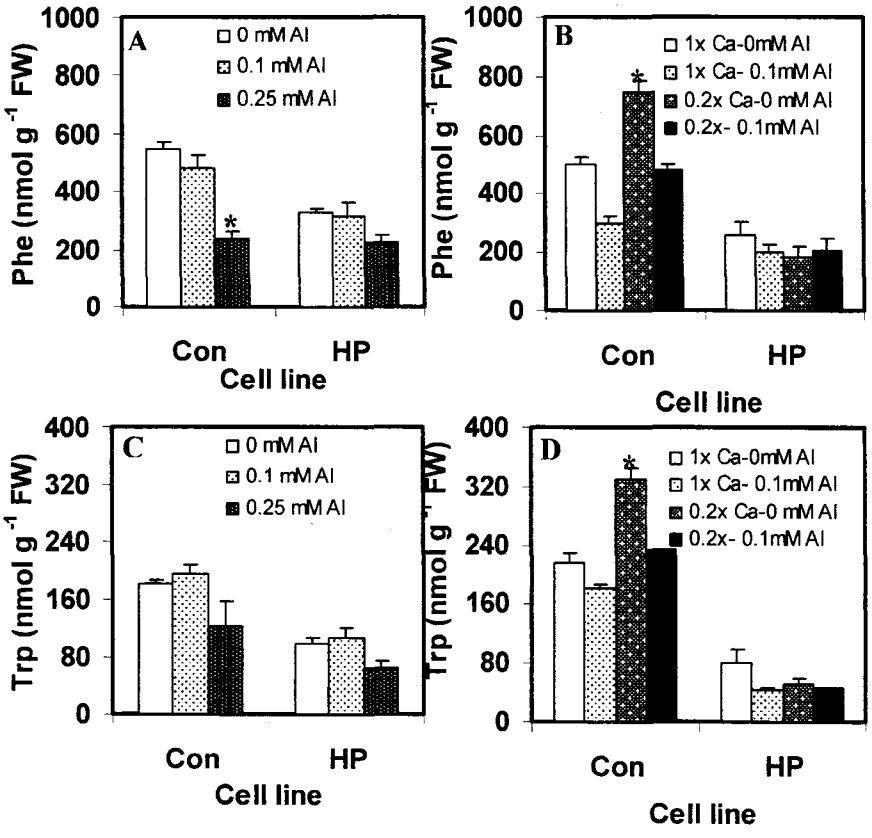

Figure C3. Cellular contents of (A, B) Phe, and (C, D) Trp in the control and HP cells due to different concentrations of Al (A, C) or Al and Ca (B, D), 48 h after treatment. Each bar represents Mean ± SE of six replicates from two experiments. An * indicates a significant difference ($P<0.05$) in amino acid content between the untreated cells and cells growing in Al (in A, C, E) and between cells growing without Al in normal Ca and those growing in different concentrations of Al and Ca (in B, D, F) within the same cell line.

APPENDIX D

ENHANCED PUTRESCINE METABOLISM AND CELLULAR INORGANIC IONS

The relationship between PAs and inorganic ions has been studied in both plants and animals (Minocha et al., 1997, 2000; Williams., 1997; Oliver et al., 2000). Williams (1997) reviewed the interaction of PAs with ion channels in animal membranes and mentioned that intracellular Spm is responsible for blocking inward rectifier K^+ channels by directly "plugging" the pore of the ion channel. A similar review by Oliver et al. (2000) emphasized the roles of PAs as "gating molecules of inward rectifier K^+ channels. Minocha et al (1997) studied the relationship between foliar PAs with foliar soil inorganic ions in red spruce (*Picea rubens* Sarg.) trees across northeastern United States. They found an inverse correlation of foliar Put and Spd with foliar Ca and Mg content, while no such significant correlation was observed with Al, K, P and Mn. They also observed negative correlations between soil exchangeable Ca and Mg and foliar PAs, while a positive correlation of the later was seen with soil exchangeable Al. Minocha et al. (2000) also studied the impact of "chronic N additions" to pine and hardwood forests on the contents of foliar PA and inorganic ions. They found a negative correlation between foliar Put and most cations in the soil. Foliar Put content was also negatively correlated with foliar Ca, Mg and Mn in hardwoods.

As mentioned in Chapter IV, the cellular contents of both PAs and inorganic ions changed in response to Al treatment to our poplar cells. Changes in the contents of inorganic ions in response to Al addition have also been previously seen in cell cultures

of *Catharanthus roseus* (Minocha et al., 1992; Zhou et al., 1995) and red spruce (Minocha et al., 1996, 2001). These changes in the inorganic ion content were either positively or negatively correlated with cellular PA content. Cellular contents of PCA soluble inorganic ions were quantified in both control and HP cells on all 7 days, as described in "General Materials and Methods".

Fluctuations in the cellular contents of K and Ca (Fig. D1A, B) in response to enhanced Put metabolism have been described Chapter III. The accumulation of Ca in the HP cells was several-fold higher in the HP cells as opposed to the control cells for most of the 7 day culture cycle, being significantly so on days 1, 2, 3, 4 and 7 days. On the other hand, an opposite response was seen for the accumulation of K in these cells in that, it was significantly lower in the HP cells as compared to the control cells for the entire culture period. A distinct and significant fresh medium effect on the uptake of K was seen in both cell lines within 24 h of transfer, with a gradual decline in the same thereafter; i.e. from day 1 through day 7. As discussed in Chapter III, the observed increase in cellular Ca and a decrease in K are additional indicators of heightened oxidative state of the HP cells.

Cellular content of Mg saw a fresh medium effect in both cell lines 24 h after subculture (Fig. D1C). Significant differences were seen between the two cell lines on days 1, 2, 4, 5 and 6 in that the cellular Mg content was higher in the HP cells on days 1 and 2 and lower on days 4, 5 and 6. From day 2 onwards, a gradual decline in the cellular Mg content was seen in both cell lines, this decrease being somewhat sharper in the HP cells than the control cells. Cellular Mn content was significantly higher in the HP cells than in the control cells on all days of the week (Fig. D1D). Its content remained fairly

constant in both cell lines, except for a rise on day 4 in the control cells and a sharp decline on day 5 in the HP cells.

Cellular Fe content was significantly higher in the HP cells on days 1, 2, 4 and 7 while being lower on day 4 (Fig. D2A). In both cell lines, Fe content first decreased for the first 3-4 days and then gradually increased. Phosphorous was significantly lower in the HP cells than the control cells on almost all days of the week (Fig. D2B). In both cell lines, a sharp increase in the cellular P content was seen 24 h after transfer to fresh medium, followed by a gradual decline for the rest of the week. Cellular content of Zn was higher in the control cells than in the HP cells on almost all days of the week (Fig. D2C).

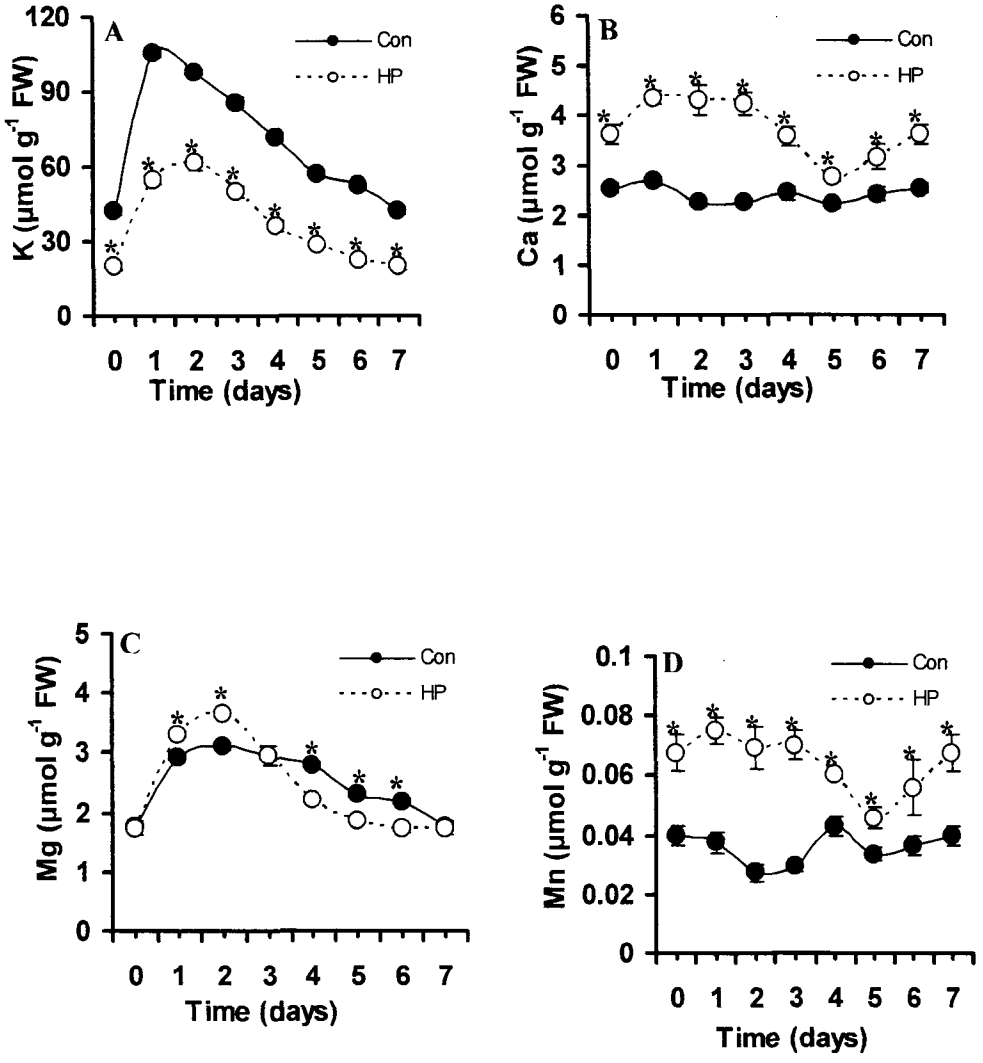

Figure D1. Cellular contents of (A) Ca, (B) K, (C) Mg and (D) Mn in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the ion content between the 2 cell lines on a given day.

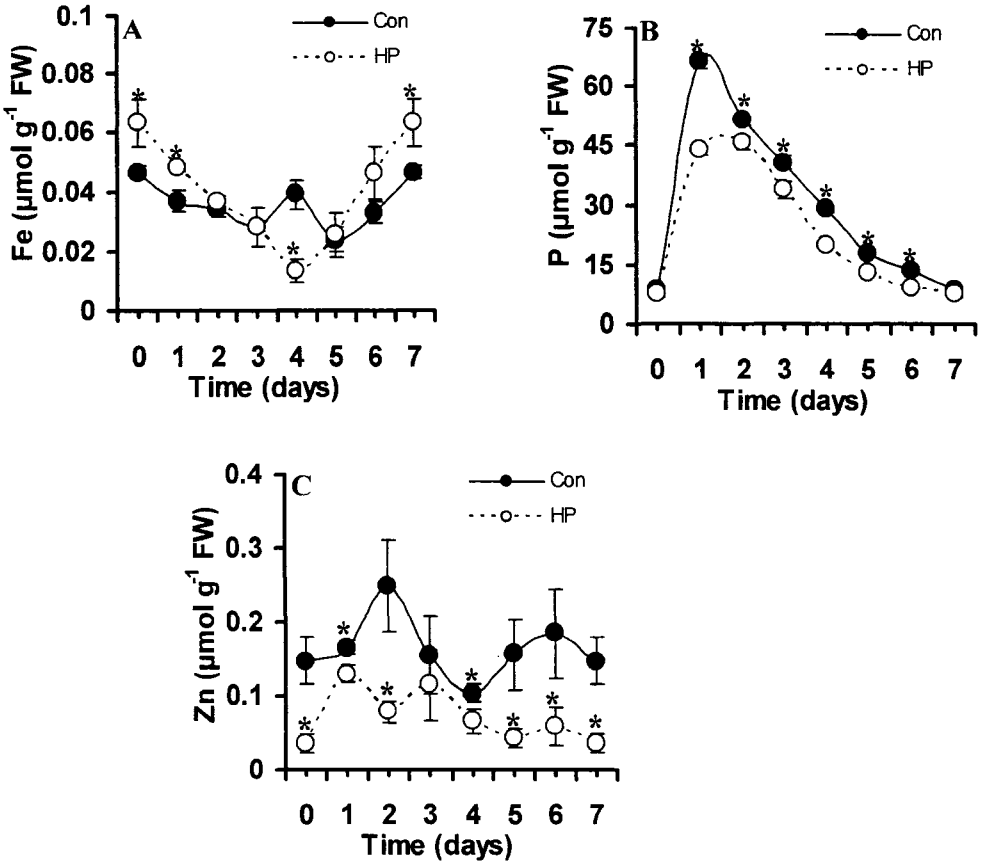

Figure D2. Cellular contents of (A) Fe, (B) P and (C) Zn in the control and HP cells over the 7-d culture cycle. Data are mean (±) SE of 6 replicates from 2 experiments. An * indicates a significant difference ($P<0.05$) in the ion content between the 2 cell lines on a given day.

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