GENETIC MANIPULATION OF POLYAMINE METABOLISM IN POPLAR[1]

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INTRODUCTION

Using a variety of gene transfer techniques, it is now possible to introduce foreign genes into plant cells, which can be used for analysis of expression of the foreign genes. In addition to transferring novel genes that do not directly affect plant metabolism (Bt gene, viral coat protein genes, etc.), a number of steps in the primary and secondary metabolism of plants have been targeted for genetic manipulation in order to understand the regulation of cellular metabolism or to produce plants with modified metabolite contents (reviewed in, Hitz and Pierce, 1997; Blakeley, 1997; Lindsey, 1998; Kinney, 1998; Nuccio *et al.*, 1999; Ohlrogge, 1999). As we move towards modulation of cellular metabolism in plants through transgenic approach, the impact of manipulating a simple reaction on the regulation of the whole pathway, and also of other related pathways that interact with the intermediates of this pathway, must be studied. The transgenic approach can also reveal mechanisms of metabolic regulation that may not be seen simply by mutant analysis and inhibitor studies. Some examples of such novel information include revelation of a complex regulation of the lysine and threonine biosynthetic pathway (Galili, 1995; Tzchori *et al.*, 1996), and the complexity of regulation of glycolysis by phosphofructokinase (Thomas *et al.*, 1997) using the transgenic approach. The major goal of research in our laboratory is to delineate the regulation

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[2] Abbreviations: ACC, 1-Aminocyclopropane-1-carboxylic acid; Arg, arginine, ADC, Arg decarboxylase; DAO, diamine oxidase, Gln, glutamine; Glu, glutamate; GDH, Glu dehydrogenase; GOGAT, Glu synthase; GS, Gln synthetase; Met, methionine; NT, non-transformed; Orn, Ornithine; Put, putrescine; ODC, Orn decarboxylase; PA, polyamines; PAO, polyamine oxidase; SAM, S-adenosylmethionine; SAMDC, SAM decarboxylase; Spd, spermidine; Spm, spermine.

of the metabolism of polyamines (PAs)[2], and analysis of the impact of altered PA metabolism on the metabolism of related metabolites in the ethylene, Arg, Orn and Pro pathways in cell cultures of poplar (*Populus nigra x maximowiczii*).

Polyamines (Put, Spd and Spm) are open-chained aliphatic amines found in all living organisms (Cohen, 1998). In addition to their role in the growth and development of all organisms, an increase in cellular PAs in response to abiotic stress has been suggested to play an important role in stress tolerance in plants (Flores, 1991; Bouchereau *et al.*, 1999). At the molecular level, PAs stabilize nucleic acids and cell membranes, stimulate protein synthesis, retard cell senescence, and help activate specific enzymes. Unique to plants is the suggestion that the pathway for PA biosynthesis competes with the biosynthesis of ethylene, which may explain some of the antagonistic effects of PAs and ethylene in many developmental processes (Even-Chen *et al.*, 1982; De Rueda *et al.*, 1994; Gallardo *et al.*, 1995, 1996; Bleecker and Kende, 2000).

In spite of numerous publications on the importance of PAs in the growth, development and stress response in plants (Slocum, 1991; Evans and Malmberg, 1989; Walden *et al.*, 1997; Cohen, 1998; Bouchereau *et al.*, 1999), little experimental evidence for the metabolic regulation of PA biosynthesis has been forthcoming. The most common approaches to modulate cellular PAs involve the use of inhibitors and exogenous supply of PA, both of which suffer from severe limitations for correct interpretation of results. Some problems associated with the use of inhibitors are: differential rates of uptake, metabolic conversion, deleterious effects of some inhibitors on membrane characteristics, and the lack of specificity (reviewed in McCann *et al.*, 1987; Birecka *et al.*, 1985; Nissen and Minocha, 1993). Genetic manipulation by transfer and expression of genes for specific enzymes helps alleviate most of these problems.

The genetic manipulation of a metabolic pathway is a complex process often because of the metabolic interactions among the different pathways. The PA biosynthetic pathway has some unique features that make it an excellent model for delineating the metabolic interactions among the network of a limited number of pathways in plants. All key enzymes in the biosynthesis as well as catabolism of PAs have been well characterized and their genes have been cloned from several plants (Kumar and Minocha, 1998). Transgenic expression of foreign genes has opened up new ways to analyze and understand cellular metabolism of PAs in prokaryotic as well eukaryotic organisms. Our laboratory has taken a lead both in demonstrating the role of PAs in growth and development and in genetically manipulating PA metabolism in plants (Minocha and Minocha, 1995; Minocha *et al.*, 1993, 1995, 1999b; DeScenzo and Minocha, 1993; Noh and Minocha, 1994; Bastola and Minocha, 1995; Andersen *et al.* 1998; Bhatnagar *et al.*, 2001, 2002; Quan *et al.* 2002). Several other laboratories have also demonstrated genetic manipulation of the PA content of plants, however, few have studied PA metabolism to the extent that we have done (see Kumar and Minocha, 1998 for ref. before 1998; Capell *et al.*,1998, 2000; Rafart-Pedros *et al.*,1999; Bassie, *et al.*, 2000 a, b). In addition to being ubiquitous, thus evolutionarily highly conserved, the PA biosynthetic pathway directly involves only a few enzymes (each one of them also highly conserved) but interacts with several key metabolic pathways (e.g. biosynthesis of ethylene, Arg, Pro and GABA) in plants. As discussed below, SAM, in addition to being used in a variety of methylation reactions, is a common precursor for both ethylene and PA biosynthesis, thus leading to speculation of a metabolically important competition between PAs and ethylene biosynthesis. This competition becomes highly significant when one considers the opposite physiological roles of PAs and ethylene. Likewise, Orn not only is a precursor for Put, but also for Pro and Arg biosynthesis. Thus PA metabolic

pathway has a potential impact on the metabolism of some of the key metabolites that are crucial players in nitrogen metabolism and stress response in plants. Genetic manipulation of the PA pathway, therefore, can be envisioned to have a profound effect on this part of the competing pathway.

The studies in our laboratory are aimed at elucidation of interactions among the biosynthesis of PA, ethylene and some amino acids that share the same pathway, e.g. Arg, Orn, Pro, and GABA. The primary hypotheses that we are testing are: (i) major changes in the metabolism of PAs in the transgenic cells must cause concomitant and correlative changes in the metabolism of precursor biosynthesis, and if the precursor biosynthesis is a part of a branched pathway, it may cause compensatory changes in the related pathways; (ii) increased production of PAs must also lead to an induction of the metabolic reactions involved in the sequestration (e.g. into bound forms) or the catabolism of PA. It is envisioned that answers to some of the specific questions posed in our study will lead to the development of functional models for the regulation of the metabolism of PAs and related compounds, and also aid in achieving metabolic control of these compounds using the transgenic approach. The models developed through this research will help us distinguish between important rate-limiting steps in the pathway and the role of the flux of metabolites through shared pathways showing either competition or independent regulation.

A wealth of information has accumulated on the genetics, physiology, molecular biology, genetic transformation, regeneration in tissue culture, and siliviculture of *Populus* (reviewed in Han *et al.* 1997; Ahuja, 1997; Tzfira *et al.*, 2000). Poplar cell lines used in the present study are comprised of non-regenerating, fast growing, small filamentous suspensions that are easy to maintain under controlled conditions. The cells lack visible (green) chloroplasts. The suspensions are uniform enough to subdivide into aliquots for experimental treatments. The cells are easily transformed by biolistic bombardment and show excellent selection on either kanamycin or hygromycin. While whole plant model systems like Arabidopsis, are uniquely suited for molecular genetic and developmental studies, these systems suffer from critical deficiencies for metabolic pathway studies for the following reasons: (i) short term analysis of metabolic flux of radio-labeled precursors in specific cell types is complicated by the difficulties of uptake, transport across multiple tissues, and the longer term analysis is complicated by transport across organs; (ii) each organ is comprised of a heterogeneous group of cell types; and (iii) uniform material at a particular stage of development is not easily available for the numbers of replicates needed for most experiments. The cell suspensions provide an experimental model system that is analogous to microbial and yeast systems which have been extensively used for biochemical and molecular studies of this type.

PHYSIOLOGICAL IMPORTANCE OF POLYAMINES

The variety of physiological responses in which a role of PAs has been implicated is large, however, the regulation of their biosynthesis and the mechanisms of their action are still in question. Obviously, a single mode of action seems unlikely. Their role in embryogenesis and growth/development of plants has been reviewed by Minocha and Minocha (1995), Watson and Malmberg (1996), Walden *et al.* (1997), Cohen (1998). A number of molecular interactions of PAs

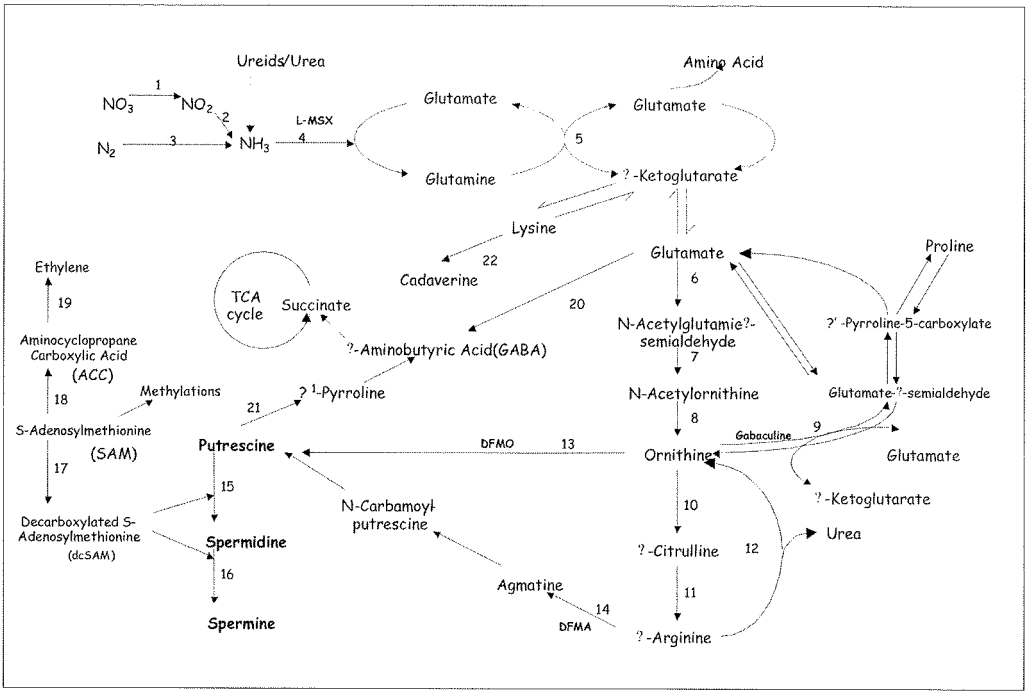

Fig. 1. Polyamine biosynthesis and related nitrogen metabolism. The enzymes are: 1. Nitrate reductase, 2. Nitrite reductase, 3. Nitrogenase, 4. Glutamine synthetase (GS), 5. Glutamate synthase (GOGAT), 6. Glutamate reductase, 7. Acetylglutamic-?-semialdehyde transaminase, 8. Acetylornithinase, 9. Ornithine aminotransferase (OAT), 10. Ornithine transcarbamylase, 11. Arginine synthase, 12. Arginase, 13. Ornithine de carboxylase, (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, 22. Lysine decarboxylase (LDC).

with cellular macromolecules could account for some of their functions. For example, it is known that PAs stabilize nucleic acids against enzymatic degradation, methylation and thermal or X-ray-induced denaturation, and also affect DNA and RNA synthesis and the process of translation (reviewed in Walden *et al.*, 1997; Cohen, 1998; Ha *et al.*, 1998). Additionally, PAs are thought to regulate cellular nitrogen metabolism in plants through sequestration of excess ammonia (Slocum and Weinstein, 1990; Cohen, 1998; Minocha *et al.*, 2000).

In recent years, considerable attention has been paid to changes in PA metabolism in plants in response to various forms of abiotic stress (Flores, 1991; Rajam, 1997; Bouchereau *et al.*, 1999). Cellular Put increases under conditions of Ca^{+2} and Mg^{+2} deprivation, exposure to increased Al, low external pH, high salinity levels, high ammonia concentration, osmotic stress, ozone and SO_2 fumigation (reviewed in Bouchereau *et al.*, 1999). The enhancement of Put levels under these conditions is often many-fold and has been observed in cell cultures as well as mature plants belonging to diverse taxa (Minocha *et al.*, 1992, 1996, 1997, 2000; Wargo *et al.*, 2002).

POLYAMINE METABOLISM

Pathways for the biosynthesis of three major PAs are well established (Pegg, 1986, Slocum, 1991; Minocha and Minocha, 1995; Tiburcio *et al.*, 1997; Cohen, 1998). While ODC is the primary pathway for Put biosynthesis in animals, higher plants and prokaryotes have two routes for Put production. These organisms can either produce Put by decarboxylation of Orn by ODC as in the animals, or they can use ADC to produce agmatine from Arg, which is then converted into Put in two steps (Fig. 1). Spermidine and Spm are synthesized by sequential addition of one or two aminopropyl groups to Put; the aminopropyl moieties being donated by decarboxylated SAM which is produced from SAM by SAMDC. The reactions are brought about by Spd synthase and Spm synthase. While SAM is used in a variety of methylation reactions as well as for ethylene biosynthesis, decarboxylation of SAM commits it exclusively to PA biosynthesis. All three decarboxylases have extremely short half lives.

In addition to free PA, several conjugated forms of PAs are found in many plants, some that are soluble in PCA, others conjugated with macromolecules, therefore, are PCA insoluble. The soluble fraction of conjugates includes various derivatives of hydroxy-cinnamic acids (Martin-Tanguy, 1997). The combined proportion of conjugated PAs may be as high as 60-70 % of the total PAs in some plants (Martin-Tanguy *et al*., 1997; Bouchereau *et al.*, 1999), while in others (e.g. carrot and poplar), they are less than 10% of the total PAs (personal observations).

Putrescine is catabolized largely by DAO, yielding ?¹-pyrroline which is oxidized to GABA, which then enters the TCA cycle through succinate, thus releasing CO_2 (Fig. 1). PAO bring about analogous reactions with Spd and Spm, producing aminopropyl pyrroline. While the conversion of Spd and Spm to Put via PAO is common in animals, it is rare in plants (Bhatnagar, Ph.D. Thesis). H_2O_2, a byproduct of DAO and PAO action, is presumably used in oxidative polymerization of lignin precursors in the cell wall. In some plants, e.g. in Solanaceae, Put is also a precursor for alkaloid biosynthesis (Smith, 1990; Suzuki *et al.*, 1990; Galston and Kaur-Sawhney, 1995). The biosynthesis and the role of GABA in plant growth and development have been reviewed by Scott-Taggart et al. (1999) and Shelp *et al.* (1999). Its production is considered to be primarily from decarboxylation of Glu, however, GABA is also a major degradation product of Put. Thus, an upward manipulation of Put production and its degradation could negatively affect direct production of GABA from Glu by Glu decarboxylase.

The biosynthesis of PAs also shares a common precursor (i.e. SAM) with the biosynthesis of ethylene (Fig. 1). While PA biosynthesis is increased in tissues showing active normal growth, increased tolerance to stress (Rajam, 1997; Bouchereau *et al.*, 1999), organogenesis and somatic embryogenesis, increased ethylene synthesis follows just the opposite situations, i.e. growth inhibition, senescence, stress damage, wounding, etc. While PAs stabilize cell membranes and macromolecules, play a role in DNA, RNA and protein synthesis, and scavenge free radicals, ethylene plays a role in tissue damage and inhibits protein synthesis (Kushad and Dumbroff, 1991; Cohen, 1998; Bleecker and Kende, 2000). Supporting evidence for this competition comes from experiments showing opposite trends in the biosynthesis of PAs and ethylene in plant tissues, inhibition of ACC and ethylene synthesis by PAs and *vice versa*, and the effects of inhibition of one pathway on the other (Flores *et al.*, 1990; Apelbaum, 1990; Jiang and Chen, 1995; De Rueda *et al.*, 1994; Gállardo *et al.*, 1995, 1996).

ARGININE AND ORNITHINE BIOSYNTHESIS AND AMMONIA ASSIMILATION

The biosynthesis of Arg and Orn begins with acetylation of Glu by Glu acetyltransferase (Fig. 1 - Wu and Morris, 1998; Coruzzi and Last, 2000). The resulting N-acetyl-Glu is phosphorylated by a kinase and then metabolized to Orn via N-acetylGlu-?-semialdehyde and N-acetyl-Orn. Ornithine is used both as a precursor for Put biosynthesis via ODC and for Arg biosynthesis via Orn transcarbamylase (OTC) and Arg synthase. In addition, Orn is a precursor for Pro synthesis, the first step being catalyzed by Orn aminotransferase (OAT). Pro biosynthesis often competes with Arg biosynthesis, particularly under stress conditions (Thompson, 1980; Ugalde *et al.*, 1995). Arginase can reconvert Arg into Orn, the byproduct urea being catabolized into ammonia and CO_2 by urease (urea cycle). For actively dividing cells, actions of arginase/urease are considered wasteful in terms of energy (Polacco and Holland, 1993; Stebbins and Polacco, 1995). Our observations confirm that there is little conversion of Arg back into Orn in the poplar cells (Bhatnagar *et al.*, 2001). Arg levels can be as high as 20-50% of total soluble amino acid nitrogen in developing seeds, the highest concentration of any amino acid in plants (Van Etten *et al.*, 1967; Pallacco and Holland, 1993). However, actively growing poplar cells and the foliage of many hardwood and conifer trees do not accumulate much of Arg (R. Minocha, in preparation). Although the GS-GOGAT cycle is the primary entry point for all inorganic NH_3 in higher plants (Ireland, 1997; Coruzzi and Last, 2000), in the presence of abundant NH_4^+, GDH may play a significant role in its assimilation. GOGAT is the primary step by which the amide nitrogen of Gln is transferred to ?-ketogluterate to generate ?-amino position of amino acids. Further distribution of nitrogen into different pathways is regulated by various enzymes, many of which show feedback inhibition (Forde and Clarkson, 1999).

TRANSGENIC MANIPULATION OF POLYAMINE BIOSYNTHESIS

The analyses of the regulation of PA biosynthesis in plants have been hampered by several factors, the most important of which is the lack of mutants deficient in specific steps of the pathway (Walden et al., 1997). The presence of two alternate pathways for Put biosynthesis (ADC and ODC) in most plants further complicates the situation, particularly for the production of knockout mutants. While a few polyamine biosynthetic mutants have been identified in Arabidopsis, and it is believed that Arabidopsis may possess only the ADC pathway for Put biosynthesis (Watson *et al.*, 1998; Hanazawa *et al.*, 2000; Hanfrey *et al.*, 2001), little metabolic work has been reported that could shed light on the regulation of PA metabolism.

In recent years, genes coding for key enzymes of PA biosynthesis (ADC, ODC, SAMDC, Spd synthase, and Spm synthase) have been cloned and sequenced from a variety of organisms, including several plants (reviewed in Kumar *et al.* 1997; Kumar and Minocha, 1998). This has allowed studies on genetic manipulation of PA metabolism in both animals and plants alleviating the problems associated with the inhibitors. Since the first report of Hamill *et al.* (1990) on genetic manipulation of Put by a yeast ODC cDNA, our laboratory has taken a lead in this field and several papers have recently been published on the metabolic aspects of PA metabolism in transgenic cells of tobacco, carrot and poplar. DeScenzo and Minocha (1993) demonstrated increased Put production in tobacco, and Bastola and Minocha (1995) in carrot, by overexpres-

sion of a mouse *odc* cDNA under the control of a CaMV 35S promoter. While most of the transgenic tobacco plants were phenotypically normal, carrot cultures exhibited significantly increased frequency of somatic embryogenesis. It was later demonstrated that not only were the rates of Put biosynthesis higher in the transgenic carrot cells, the catabolism of Put was also enhanced in them as compared to the NT cells (Andersen *et al.*, 1998). This is similar to the situation seen with genetic manipulation of lysine biosynthesis (Kinney, 1998). Noh and Minocha (1994) produced phenotypically normal transgenic tobacco plants expressing a human *samdc* cDNA which contained significantly higher levels of Spd and reduced levels of Put. Unfortunately, neither tobacco nor carrot plants are suitable for detailed biochemical analysis of PA metabolism due to the difficulties of feeding labeled precursors in short term studies. The incorporation of radioactive precursors in these tissues will be subject to complications of intercellular transport following uptake from the medium. Therefore, we changed our experimental material to poplar cell suspension cultures, which are made of single cells or small multicellular (2-5 cells) filaments.

Again-of-function achieved by overexpression of a transgene provides an excellent method of manipulating plant metabolism. Following the earlier reports cited above, several laboratories have achieved transformation in tobacco and rice with heterologous *adc, odc or SAMDC* cDNAs (Kumar *et al.*, 1996; Burtin and Michael, 1997; Capell *et al.*, 1998, 2000; Rafart-Pedros *et al.*, 1999; Bassie, et al., 2000 a, b; Roy and Wu, 2000). While most studies show increased production of Put and/or Spd in the transgenic cells/plants, none have involved detailed analysis of PA metabolism in the transgenic cells. A summary of results from our laboratory on several metabolic aspects of PA metabolism in poplar cells using the transgenic approach with heterologous genes for *odc* and *samdc* are summarized below:

Several transgenic cell lines of poplar (*Populus nigra x maximowiczii*) using plasmids containing either a mouse *odc* cDNA or a Datura *samdc* cDNA under the control of a 2x35S CaMV promoter were produced by the biolistic bombardment (gene gun) technique (Bhatnagar, 2002; Bhatnagar *et al.*, 2001, 2002; Quan *et al.*, 2002; Lee, Minocha and Minocha, unpublished). Transformed cell lines were selected on kanamycin and analyzed for the presence of the transgene sequences by PCR and Southern hybridization, and for cellular polyamines and enzyme activities (Minocha *et al.*, 1994, 1999a). While total cellular PA contents of different *odc*-transgenic cell lines varied on different days of analysis, Put contents were 3-10-fold higher in the selected transgenic cell lines on any given day as compared to the NT and *gus*-transgenic cell lines (Fig. 2). The maximum Put concentration in some of the transgenic cells was as high as 6.5 ?mol.g^{-1}.FW. There was often an increase in the contents of Spd also but no change in Spm was seen in the transgenic cells.

The mouse ODC activity was distinguished from the native plant ODC by its sensitivity to DFMO and its pH optima at 6.8 (vs. 8.2 for the plant ODC - DeScenzo and Minocha, 1993; Bhatnagar *et al.*, 2001). The native ODC activity is completely lacking in both the NT and the transgenic poplar cells, thus it is established that the former exclusively utilize ADC pathway for Put biosynthesis. High activity of mouse ODC was seen only in the *odc*-transgenic cells (Fig. 3A). This activity was almost completely inhibited by 2 μM DFMO.

The activity of ADC was either comparable in all cell lines (transgenic or NT) or was higher in the transgenic cells (Fig. 3B), showing that there was no feedback inhibition of ADC by elevated levels of Put. The ADC activity was strongly inhibited by DFMA. These results are in

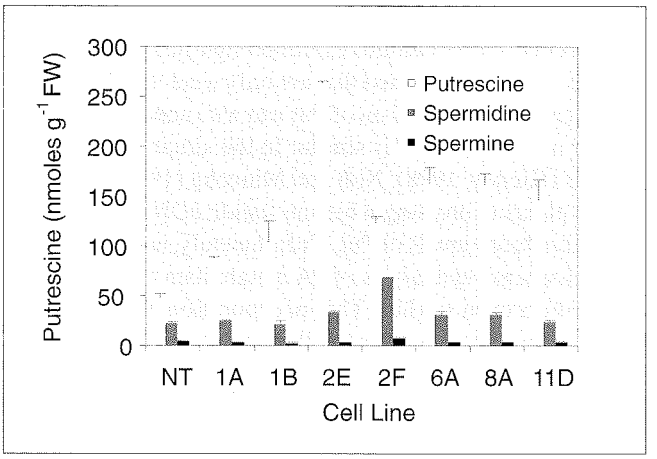

Fig. 2. Cellular contents of polyamines in NT and several transgenic cell lines of poplar grown for 7 days on solid medium. Each bar represents mean ± SE of four replicates.

contrast to the general belief (based upon limited experimental evidence) that ADC is feed-back inhibited in plants (Borell *et al.*, 1996; Primikirios and Roubelakis-Angelakis, 1999). The incorporation of label from U-^{14}C-Orn into Put was significantly higher in the *odc*-transgenic cells as compared to the NT cells (Fig. 4). The amounts of label in Spd and Spm were also generally higher in the transgenic cells as compared to the NT cells (data not shown).

Birecka *et al.* (1985) had shown the presence of relatively high arginase/urease activities in cell extracts of plants, while our studies with *in vivo* incorporation of ^{14}C-Arg clearly demonstrate that arginase/urease cycle is not a significant source of Orn production from Arg in poplar cells. Instead, Orn is produced solely from Glu/Gln as shown by a significant reduction in Put content in both the NT and the transgenic cells in the presence of methionine sulfoximine (an inhibitor of glutamine synthase), and its reversal only by Orn and not by Arg (Bhatnagar *et al.*, 2001).

Using extensive analysis of the fate of ^{14}C-Put and ^{14}C-Spd in the *odc*-transgenic cells, we have calculated the half-life of Put turnover to be around 5-6 h in contrast to that of Spd, whose half-life is calculated to be 30-40 h (Bhatnagar, 2002; Bhatnagar *et al.*, 2002). We have also demonstrated that Put catabolism in transgenic cells is 3-4 fold higher than the NT cells; its conversion into Spd or its excretion into the medium constituting a much smaller (only about 10% of Put) component of Put turnover (Bhatnagar *et al.*, 2002). This situation is strikingly similar to the results with transgenic mice overexpressing an *odc* transgene, where increased Put content was accompanied by little change in Spd and Spm, and a five-fold increase in PA catabolism via Spd/Spm acetyltransferase (SSAT - Suppola *et al.*, 2001). These authors concluded that the major aim of the machinery that regulates polyamine metabolism is to prevent an over-accumulation of the higher polyamines. We believe that a similar situation may exist in plants.

Based upon analysis of the effects of exogenous supply of nitrogen in the medium, we have proposed that increased utilization of Orn in the transgenic cells must be accompanied by a concomitant increase in the assimilation of NO_3 and NH_4, and stimulation of key steps in the Orn/Arg biosynthetic pathway (Minocha *et al.*, 2003). Similar results have been reported in mice stimulated to produce high amounts of ODC (Han *et al.*, 2001).

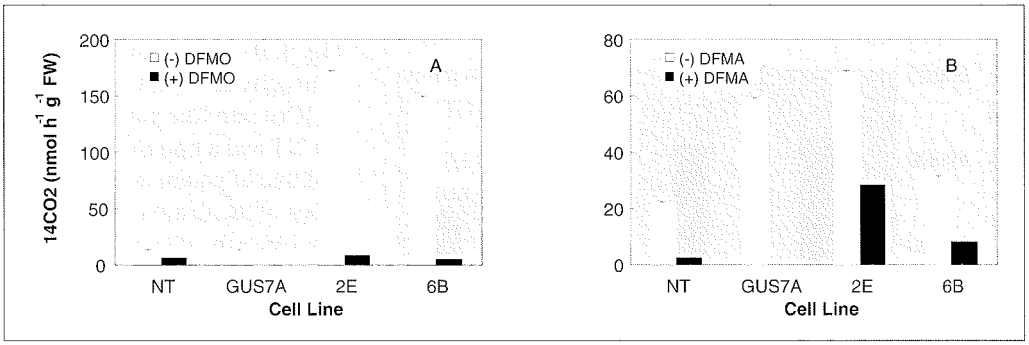

Fig. 3. The rate of $^{14}CO_2$ production from L-[1-^{14}C]Orn (A) and DL-[1-^{14}C]Arg (B) by NT and transgenic (2E) cells of poplar. Data in Figs. 3Aand 3B are from standard enzyme assays using intact cells (Minocha *et al.*, 1999a) in the absence or presence of DFMO or DFMA. Each Bar represents mean ± SE of two replicates.

While the *odc* transgenic cells have been well characterized (Bhatnagar *et al.*; 2001, 2002 and Ph.D. Thesis) and summarized above, we have also produced several transgenic lines of poplar cells with a Datura *samdc* cDNA. These cell lines have been characterized only with respect to the presence of the transgene and its effects on cellular polyamines. Analyses of the production rates of ethylene and ACC in the NT and the *samdc*-transgenic cells shows no difference in ethylene metabolism in two types of cells (Quan *et al.*, 2002).

We have also prepared several additional plasmid constructs with genes of other key enzymes involved in PA metabolism for transgenic expression in the poplar cells. Availability of diverse transgenic cell lines of poplar expressing genes for all of the PA biosynthetic enzymes will open up new avenues for physiological and biochemical dissection of this pathway and the associated nitrogen metabolism. The results obtained so far with the transgenic cells of carrot and poplar have laid a solid foundation for the suitability of these cell lines and this approach for the proposed studies on ethylene and amino acid metabolism in relation to PA metabolism, and the expression of genes involved in regulating this metabolism in plant cell cultures.

CONCLUSIONS AND FUTURE PERSPECTIVES

The results obtained so far show that: (a) transgenic expression of an *odc* gene can be used to modulate Put metabolism in poplar; (b) overproduction of Put does not affect the native ADC enzyme activity; (c) ornithine biosynthesis occurs from Glu and not via catabolic breakdown of Arg; (d) there is no competition for Orn between Put and Pro biosynthesis; (e) the rate of Put degradation is coupled to the rate of its biosynthesis and accumulation; (f) the rate of conversion of Put to Spd is 3X higher in the 2E than in the NT cells; (g) half-life of Put is similar in the two cell lines; (h) Spd is turned over more slowly than Put – the half-life for Spd turnover being 36-42 h as compared to about 6 h for Put; (i) only 2-3% of Spd is converted into Spm, and a similar amount is converted back into Put.

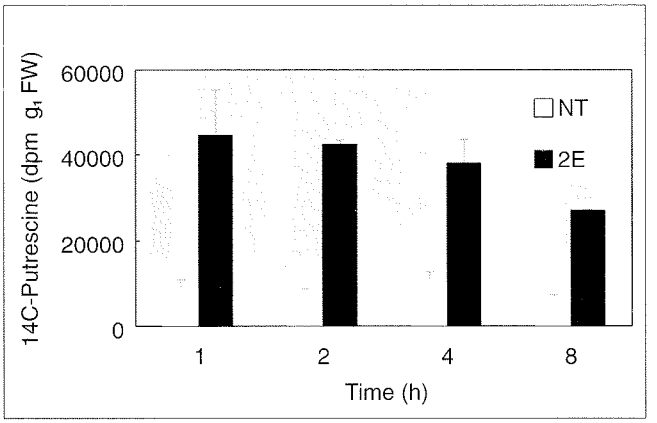

Fig. 4. Incorporation of radioactivity from L-[U-^{14}C]Orn into free putrescine in the NT and a transgenic (2E) cell line of poplar at different times of incubation. Three-day-old cells (about 1 g in 10 ml) were incubated with 0.2 ?Ci of L-[U-^{14}C]Orn for various time periods. Each bar represents mean ± SE of two replicates.

The major goal of the future research is to profile the metabolic impact of genetically manipulating PA metabolism in plant cells by comparing the expression of different genes in the transgenic and the nontransgenic cells. Using combined biochemical, molecular and radio-labeled precursor studies, we eventually hope to develop a mathematical model of metabolic flux in the interacting pathways of PA, Pro, and ethylene metabolism (see Mudd and Datko, 1990; Ranocha *et al.*, 2001 for guidelines). Such a model for PA metabolic pathway should allow simulation of interactions among the related pathways, leading to predictions that can be experimentally tested via genetic or mutational manipulations. In order to understand how metabolic regulation of the PA, ethylene and Pro pathways is brought about, we must study them in a systemic way. At the same time, in order to experimentally manipulate such pathways, we must dissect individual components of the branching pathways and identify steps that involve regulatory enzymes.

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REFERENCES

Ahuja, M.R. 1997. Transgenes and genetic instability. In: Klopfenstein, N.B., Chun, Y.W., Kim, M.-S., Ahuja, M.R. (eds.), Micropropagation, Genetic Engineering, and Molecular Biology of Populus, pp. 90-100. USDA, Forest Service, Rocky Mountain Research Station, Fort Collins.

Andersen, S.C., Bastola, D.R., Minocha, S.C. 1998. Metabolism of polyamines in transgenic cells of carrot expressing a mouse ornithine decarboxylase cDNA. Plant Physiol 116: 299-307.

Apelbaum, A. 1990. Interrelationship between polyamines and ethylene and its implication for plant growth and fruit ripening. In: Flores, H.F., Arteca, R.N., Shannon, J.C. (eds.), Polyamine

and Ethylene: Biochemistry, Physiology, and Interaction, pp. 278-294, Amer. Soc. Plant Physiologists.

Bassie, L., Noury, M., Lepri, O., Lahaye, T., Christou, P. Capell, T. 2000a. Promoter strength influences polyamine metabolism and morphogenic capacity in transgenic rice tissues expressing the oat *adc* cDNA constitutively. Transgen. Res. 9: 33-42.

Bassie, L., Noury, M., Wisniewski, J.P., Topsom, L., Christou, P. Capell, T. 2000b. Transgenic cell lines as useful tools to study the biochemistry of down-regulation of an endogenous rice gene using a heterologous diamine-oxidase cDNA. Plant Physiol. Biochem. 38: 729-737.

Bastola, D.R., Minocha, S.C. 1995. Increased putrescine biosynthesis through transfer of mouse ornithine decarboxylase cDNA in carrot (*Daucus carota* L.) promotes somatic embryogenesis. Plant Physiol. 109: 63-71.

Bhatnagar, P. 2002. Transgenic Manipulation of the Metabolism of Polyamines in Poplar. Ph.D. Thesis, Univ. of New Hampshire, Durham, NH, pp. 248.

Bhatnagar, P., Glasheen, B.M., Bains, S.K., Long, S.L., Minocha, R. Walter, C., Minocha S.C. 2001. Transgenic manipulation of the metabolism of polyamines in poplar cells. Plant Physiol. 125: 2139-2153.

Bhatnagar, P., Minocha, R. Minocha S.C. 2002. Transgenic manipulation of the metabolism of polyamines in poplar cells: The catabolism of putrescine. Plant Physiol. 128: 1455-1469.

Birecka, H., Bitonti, A.J., McCann, P. P. 1985. Activities of arginine and ornithine decarboxylases in various plant species. Plant Physiol. 79: 515-519.

Blakeley, S. 1997. The manipulation of resource allocation in plants. In: Dennis, D.T. H.,Turpin, D.H., Lefebvre, D.D., Layzell, D.B. Plant Metabolism pp. 580-591 Addison Wesley.

Bleecker, A.B., Kende, H. 2000. Ethylene: A gaseous signal molecule in plants, Annu. Rev. Cell Dev. Biol. 16: 1-18.

Borrell, A., Besford, R.T., Altabella, T., Masgrau, C., Tiburcio, A.F. 1996. Regulation of arginine decarboxylase by spermine in osmotically-stressed oat leaves. Physiol. Plant. 98: 105-110.

Bouchereau, A., Aziz, A., Larher, F., Martin-Tanguy, J. 1999. Polyamines and environmental challenges: recent developments. Plant Sci. 140: 103-125.

Burtin, D., Michael, A. J. 1997. Overexpression of arginine decarboxylase in transgenic plants. Biochem. J. 325: 331-7

Capell, T. Escobar, C., Lui, H., Burtin, D., Lepri, O. Christou, P. 1998. Over-expression of oat arginine decarboxylase cDNA in transgenic rice (Oryza sativa L.) Affects normal developmental patterns in vitro and results in putrescine accumulation in transgenic plants. Theor. Appl. Gen. 97: 246-254.

Capell, T., Bassi, L., Topsom, L., Hitchin, E., Christou, P. 2000. Simultaneous downregulation of two unrelated enzymes in early steps of the polyamie biosynthetic pathway in transgenic rice by a single antisense mRNA species. Mol. Gen. Genet. 264: 470-476

Cohen, S. S. 1998. A Guide to the Polyamines New York: Oxford University Press. 595 pp.

Coruzzi, G., Last., R. 2000. In: Buchanan, B.B., Gruissem, W., Jones, R.L., (eds.), Biochemistry and Molecular Biology of Plants. Amer Soc Plant Biol, Rockville, MD, pp.358-410.

De Rueda, P.M., Gállardo, M., Sánchez-Calle, I.M., Matilla, A.J., 1994. Germination of chick-pea seeds in relation to manipulation of the ethylene pathway and polyamine biosynthesis by inhibitors. Plant Sci. 97: 31-37.

DeScenzo, R.A., Minocha, S.C. 1993. Modulation of cellular polyamines in tobacco by transfer and expression of mouse ornithine decarboxylase cDNA. Plant Mol. Biol. 22: 113-27

Evans, P. T., Malmberg, R. L. 1989. Do polyamines have roles in plant development? Annu. Rev. Plant Physiol. Mol. Biol. 40: 235-269

Even-Chen, Z., Mattoo, A.K., Goren, R. 1982. Inhibition of ethylene biosynthesis by aminoethoxyvinylglycine and by polyamines shunts label from 3,4-[^{14}C]methionine into spermidine in aged orange peel discs, Plant Physiol. 69: 385-388

Flores, H.E. 1991. Changes in polyamine metabolism in response to abiotic stress. In: Slocum, R., Flores, H.E. (eds.), The Biochemistry and Physiology of Polyamines in Plants, pp. 214-225. CRC Press, Boca Raton, FL.

Flores, H.E., Arteca, R.N., Shannon, J.C. 1990. Polyamines and Ethylene: Biochemistry, Physiology, and Interactions, Rockville, MD: Amer. Soc. Plant Physiol. 425 pp.

Forde, B.G, Clarkson, D.T. 1999. Nitrate and ammonium nutrition of plants: Physiological and molecular perspectives. Adv. Bot. Res. 30: 1-90

Galili, G. 1995. Regulation of lysine and threonine synthesis. Plant Cell 7: 899-906

Gallardo, M., de Rueda, P.M , Matilla, A.J. Sánchez-Calle, I.M 1995. Alterations of the ethylene pathway in germinating thermoinhibited chick-pea seeds caused by the inhibition of polyamine biosynthesis. Plant Sci. 104: 169-175.

Gallardo, M., Sánchez-Calle, I.M., Munoz, D.R.P., Matilla, A.J. 1996. Alleviation of thermoinhibition in chickpea seeds by putrescine involves the ethylene pathway. Austral. J. Plant Physiol. 23: 479-487.

Galston, A. W., Kaur-Sawhney, R. 1995. Polyamines as endogenous growth regulators. In: Davies, P. J. (ed.) Plant Hormones, pp. 158-178, Kluwer Acad. Publ., The Netherlands..

Ha, H.C., Sirisoma, N.S., Kuppusamy, P., Zweier, J.L., Woster, P.M., Casero Jr., R.A. 1998. The natural polyamine spermine functions directly as a free radical scavenger. Biochemistry 95: 11140-11145.

Hamill, J.D., Robins, R.J., Parr, A.J., Evans, D.M., Furze, J.M., Rhodes, M.J. 1990. Over-expressing a yeast ornithine decarboxylase gene in transgenic roots of *Nicotiana rustica* can lead to enhanced nicotine accumulation. Plant Mol. Biol. 15: 27-38.

Han, K.H., Gordon, M.P., Strauss, S.H. 1997. High frequency transformation of cottonwoods (genus *Populus*) by *Agrobacterium rhizogenes*. Can. J. For. Res. 27: 464-470.

Hanazawa, Y., Takahashi, T., Michael, A.J., Burtin, D., Long, D., Pineiro, M., Coupland, G., Komeda, Y. 2000. ACAULIS5, an Arabidopsis gene required for stem elongation, encodes a spermine synthase. EMBO J. 19: 4248-4256.

Hanfrey, C., Sommer, S., Mayer, M.J., Burtin, D., Michael, A.J. 2001. Arabidopsis polyamine biosynthesis: absence of ornithine decarboxylase and the mechanism of arginine decarboxylase activity. Plant J. 27: 651-660.

Hitz, W.D., Pierce, J.W. 1997. The biochemical basis for crop improvement. In: Dennis, D.T. Turpin, H., Lefebvre, D.D., Layzell, B. (eds.) Plant Metabolism, pp. 592-605 Addison Wesley.

Ireland, R. 1997. Amino acid and ureide biosynthesis. In: Dennis, D.T. Turpin, H., Lefebvre, D.D., Layzell, B. (eds.), Plant Metabolism, pp. 478-494. Addison Wesley.

Jiang, Y.M., Chen, F., 1995. Effect of spermine in the regulation of senescence of litchi fruit and its regulation to ethylene. Chin. J. Bot. 7: 121-125.

Kinney, A.J. 1998. Manipulating flux through plant metabolic pathways. Curr. Opin. Plant Biol. 1: 173-178.

Kumar, A., Altabella, T., Taylor, M.R., Tiburcio, A.T. 1997. Recent advances in polyamine research. Trends Plant Sci. 2: 124-130.

Kumar, A., Minocha, S.C. 1998. Transgenic manipulation of polyamine metabolism. In: Lindsey, K. (ed.) Transgenic Research in Plants, pp. 187-199. Harwood Acad. Publ. U.K.

Kumar, A., Taylor, M.R., Mad Arif, S.A., Davies, H. 1996. Potato plants expressing antisense and sense S-adenosylmethionine decarboxylase (SAMDC) transgene show altered levels of polyamines and ethylene: antisense plants display abnormal phenotypes. Plant J. 9: 147-158.

Kushad, M.M., Dumbroff, E.B. 1991. Metabolic and physiological relationship between the polyamine and ethylene biosynthetic pathways, in: Slocum, R., Flores, H. (eds.), Biochemistry and Physiology of Polyamines in Plants, pp. 78-89, CRC Press, Boca Raton.

Lindsey, K., (ed.) 1998. Transgenic Research in Plants, Harwood Acad. Publ. U.K. 286 pp.

Martin-Tanguy, J. 1997. Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. Physiol Plant. 100: 675-688.

McCann, P.P., Pegg, A.E., Sjoerdsma, A. 1987. Inhibition of Polyamine Metabolism: Biological Significance and Basis for New Therapies Academic Press. 371 pp.

Minocha, R., Kvaalen, H., Minocha, S.C., Long, S. 1993. Polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*). Tree Physiol.13: 365-377.

Minocha, R., Lee, J.S., Long, S., Bhatnagar, P. Minocha, S.C. 2003. Physiological responses of wild type and putrescine-overproducing transgenic cells of poplar to variations in the form and content of nitrogen in the medium. Tree Physiol. In Press.

Minocha, R., Long, S., Magill, A., Aber, J., McDowell, W.H. 2000. Foliar free polyamines and inorganic ion content in relation to soil and soil solution chemistry in two fertilized forest stands at the Harvard Forest, Massachusetts. Plant Soil. 222: 119-137.

Minocha, R., Long, S., Maki, H., Minocha, S.C. 1999a. Assays for the activities of polyamine biosynthetic enzymes using intact tissues. Plant Physiol. Biochem. 37: 597-603.

Minocha, R., Minocha, S.C., Long, S.L., Shortle, W.C. 1992. Effects of aluminum on DNA synthesis, cellular polyamines, polyamine biosynthetic enzymes, and inorganic ions in cell suspension cultures of a woody plant, *Catharanthus roseus*. Physiol Plant 85: 417-424.

Minocha, R., Minocha, S.C., Simola, L.K. 1995. Somatic embryogenesis and polyamines in woody plants. In: Jain, S.M., Gupta, P.K., Newton, R.J. (eds.), Somatic Embryogenesis in Woody Plants, Vol. 1. pp. 337-359. Kluwer Sci. Publ. Dordrecht, The Netherlands.

Minocha, R., Shortle, W.C., Coughlin, D.J.,Jr., Minocha, S.C. 1996. Effects of Al on growth, polyamine metabolism, and inorganic ions in suspension cultures of red spruce. Can. J. For. Res. 26: 550-559.

Minocha, R., Shortle, W.C., Lawrence, G.B., David, M.B., Minocha, S.C. 1997. A relationship among foliar chemistry, foliar polyamines and soil chemistry in red spruce trees growing across the northeastern United States. Plant Soil 191: 109-122.

Minocha, R., Shortle, W.C., Long, S.L., Minocha, S.C. 1994. A rapid and reliable procedure for extraction of polyamines and inorganic cations from plant tissues. J. Plant Gr. Regul. 13: 187-193.

Minocha, R., Smith, D.R., Stewart, C., Steele, K.D., Minocha, S.C. 1999b. Polyamine levels during the development of zygotic and somatic embryos of *Pinus radiata* D Don. Physiol Plant. 105: 155-164.

Minocha, S.C., Minocha, R. 1995. Role of polyamines in somatic embryogenesis. In: Bajaj, Y. P. S. (ed.), Biotechnology in Agriculture and Forestry: Somatic Embryogenesis and Synthetic Seed I, Vol. 30, pp. 55-72. Springer-Verlag, Berlin.

Mudd, S.H., Datko, A.H. 1990. The S-methylmethionine cycle in *Lemna paucicostata*. Plant Physiol. 93: 623-630.

Nissen, P., Minocha, S. C. 1993. Inhibition by 2,4-D of somatic embryogenesis in carrot as explored by its reversal by difluoromethylornithine. Physiol Plant 89: 673-680.

Noh, E.W., Minocha, S.C. 1994. Expression of a human S-adenosylmethionine decarboxylase cDNA in transgenic tobacco and its effects on polyamine biosynthesis. Transgen. Res. 3: 26-35.

Nuccio, M.L., Rhodes, D., McNeil, S.C., Hanson, A.D. 1999. Metabolic engineering of plants for osmotic stress resistance. Curr. Opin. Plant Biol. 2: 128-134.

Ohlrogge, J. 1999. Plant metabolic engineering: are we ready for phase two? Curr. Opin Plant Biol. 2: 121-122.

Pegg, A.E. 1986. Recent advances in the biochemistry of polyamines in eukaryotes. Biochem. J. 234: 249-62

Polacco, J.C., Holland, M. A. 1993. Roles of urease in plant cells. Intern. Rev. Cytol. 145: 65-103.

Primikirios, N. I., Roubelakis-Angelakis, K. A. 1999. Cloning and expression of an arginine decarboxylase cDNA from *Vitis vinifera* L. cell-suspension cultures. Planta 208: 574-582.

Quan, Y., Minocha, R., Minocha, S.C. 2002. Genetic manipulation of polyamine metabolism in poplar II: Effects on ethylene biosynthesis. Plant Physiol. Biochem. 40: 929-937.

Rajam, M.V. 1997. Polyamines. In: Prasad, M.N.V. Plant Ecophysiology, pp. 343-374, John Wiley & Sons.

Ranocha, P. McNeil, S.D., Ziemak, M.J., Li, C., Tarczynski, M.C., Hanson, A.D. 2001. The S-methyl-methionine cycle in angiosperms: ubiquity, antiquity and activity. Plant J. 25: 575-584.

Rafart-Pedros, A., MacLeod, M.R., Ross, H.A., McRae, D., Tiburcio, A.F., Davies, H.D., Taylor, M.A. 1999. Manipulation of S-adenosylmethionine decarboxylase activity in potato tubers, Planta 209: 153-160.

Roy, M., Wu, R. 2001. Arginine decarboxylase transgene expression and analysis of environmental stress tolerance in transgenic rice. Plant Sci. 160: 869-875.

Scott-Taggart, C.P., Van Cauwenberghe, O.R., McLean, M.D., Shelp, B.J. 1999. Regulation of ?-amino-butyric acid synthesis *in situ* by glutamate availability. Physiol. Plant. 106: 363-369

Shelp, B.J., Bown, A.W., McLean, M.D. 1999. Metabolism and functions of gamma-aminobutyric acid. Trends Plant Sci. 4: 446-452

Slocum, R.D. 1991. Polyamine biosynthesis in plants. In: Slocum, R.D., Flores, H.E. (eds.) The Biochemistry and Physiology of Polyamines in Plants, pp. 23-40. CRC Press, Boca Raton, FL.

Slocum, R.D., Weinstein, L.H. 1990. Stress-induced putrescine accumulation as a mechanism of ammonia detoxification in cereal leaves. In: Flores, H.E., Arteca, R.N., Shannon, J.C. (eds.), Polyamines and Ethylene: Biochemistry, Physiology, and Interactions, pp. 157-165. American Society of Plant Physiologists, Rockville, MD.

Smith, T.A. 1990. Plant polyamines - metabolism and function. In: Flores, H.E., Arteca, R.N., Shannon, J.C. (eds.), Polyamines and Ethylene: Biochemistry, Physiology, and Interactions, pp. 1-23. American Society of Plant Physiologists, Rockville, MD.

Stebbins, N.E., Polacco, J.C. 1995. Urease is not essential for ureide degradation in soybean. Plant Physiol. 109: 169-175

Suppola, S., Heikkinen, S., Parkkinen, J.J., Uusi-Oukari, M., Korhonen, V.-P., Keinänen, T., Alhonen, L., Jänne, J. 2001. Concurrent overexpression of ornithine decarboxylase and spermidine/spermine N^1-acetyltransferase further accelerates the catabolism of hepatic polyamines in transgenic mice. Biochem. J. 358: 343-348.

Suzuki, Y., Hirasawa, E., Yanagisawa, H., Matsuda, H. 1990. The enzymes of polyamine metabolisms in higher plants. In: Flores, H.E., Arteca, R.N., Shannon, J.C. (eds.), Polyamines and Ethylene: Biochemistry, Physiology, and Interactions, pp. 73-90. American Society of Plant Physiologists, Rockville, MD.

Thomas, S., Mooney, P.J.F., Burrell, M.M., Fell, D.A. 1997. Finite change analysis of glycolytic intermediates in tuber tissue lines of transgenic potato overexpressing phosphofructokinase. Biochem. J. 322: 111-117.

Thompson, J.F. 1980. Arginine synthesis, proline synthesis, and related processes. In: Miflin, B.J. (ed.) The Biochemistry of Plants, Vol. 5, pp. 375-402. Academic Press, New York.

Tiburcio, A.F., Altabella, T., Borrell, A., Masgrau, C. 1997. Polyamine metabolism and its regulation. Physiol Plant 100: 664-674

Tzchori, I. B.-T., Perl, A., Galili, G. 1996. Lysine and threonine metabolism are subject to complex patterns of regulation in *Arabidopsis*. Plant Mol. Biol. 32: 727-734

Tzfira, T., Wang, W., Altman, A. 2000. Genetic transformation of *Populus* toward improving plant performance and drought tolerance. In: Jain, S.M., Minocha, S.C. (eds.), Molecular Biology of Woody Plants, Vol. 2, pp. Kluwer Academic Publishers, Dordrecht.

Ugalde, T.D., Maher, S.E., Nardell, N.E., Wallgrove, R.M. 1995. Amino acid metabolism and protein deposition in the endosperm of wheat; synthesis of proline via ornithine. In: Wallgrove, R.M. (ed.) Amino Acids and Their Derivatives in Higher Plants, pp. 7-86, Cambridge Univ. Press.

Van Etten, C.H., Kwolek, W.F., Peters, I.E., Barclay, A.S. 1967. Plant seeds as protein sources for food or feed. J. Agric. Food Chem. 15: 1077-1085.

Walden, R., Cordeiro, A., Tiburcio, A.F. 1997. Polyamines: small molecules triggering pathways in plant growth and development. Plant Physiol 113: 1009-1013.

Wargo, P.M., Minocha, R., Wong B., Long, R.P., Horsley, S.B., and Hall, T.J. 2002. Measuring stress and recovery in lime fertilized sugar maple in the Allegheny Plateau area of Northwestern Pennsylvania. Can. J. For. Res. 32: 629—641.

Watson, M. B., Malmberg, R. L. 1996. Regulation of *Arabidopsis thaliana* (L.) Heynh arginine decarboxylase by potassium deficiency stress. Plant Physiol 111: 1077-83.

Watson, M.B., Emory, K.K., Piatak, R.M., Malmberg, R.L. 1998. Arginine decarboxylase (polyamine synthesis) mutants of *Arabidopsis thaliana* exhibit altered root growth. Plant J. 13: 231-239.

Wu, W. H., Morris, D. R. 1998. Arginine metabolism. Biochem. J. 336: 1-17.