Chapter 1

Polyamines in the Context of Metabolic Networks

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Abstract

Polyamines (PAs) are essential biomolecules that are known to be involved in the regulation of many plant developmental and growth processes as well as their response to different environmental stimuli. Maintaining the cellular pools of PAs or their metabolic precursors and by-products is critical to accomplish their normal functions. Therefore, the titre of PAs in the cells must be under tight regulation to enable cellular PA homeostasis. Polyamine homeostasis is hence achieved by the regulation of their input into the cellular PA pool, their conversion into secondary metabolites, their transport to other issues/organs, and their catabolism or turnover. The major contributors of input to the PA pools are their in vivo biosynthesis, interconversion between different PAs, and transport from other tissues/organs; while the output or turnover of PAs is facilitated by transport, conjugation and catabolism. Polyamine metabolic pathways including the biosynthesis, catabolism/turnover and conjugation with various organic molecules have been widely studied in all kingdoms. Discoveries on the molecular transporters facilitating the intracellular and intercellular translocation of PAs have also been reported. Numerous recent studies using transgenic approaches and mutagenesis have shown that plants can tolerate quite large concentrations of PAs in the cells; even though, at times, high cellular accumulation of PAs is quite detrimental, and so is high rate of catabolism. The mechanism by which plants tolerate such large quantities of PAs is still unclear. Interestingly, enhanced PA biosynthesis via manipulation of the PA metabolic networks has been suggested to contribute directly to increased growth and improvements in plant abiotic and biotic stress responses; hence greater biomass and productivity. Genetic manipulation of the PA metabolic networks has also been shown to improve plant nitrogen assimilation capacity, which may in turn lead to enhanced carbon assimilation. These potential benefits on top of the widely accepted role of PAs in improving plants' tolerance to biotic and abiotic stressors are invaluable tools for future plant improvement strategies.

Key words Putrescine, Spermidine, Spermine, Nitrogen, NO, Glutamate, Proline, TCA, Transport

1 Introduction

Polyamines (PAs) are polycationic amines found in all living organisms, and even viruses. Major free PAs in plants include putrescine (Put), spermidine (Spd), spermine (Spm), and its structural isomer thermospermine (tSPM). Polyamines play important physiological roles in a broad range of biological functions including transcription and translation, the stabilization of nucleic acids and cell membranes, regulation of cell division and growth, organogenesis,

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embryogenesis, leaf senescence, flower and fruit development as well as in responses to abiotic and biotic stressors [1–3]. Whereas some of these PA functions are common to those in animals, others are somewhat unique to plants. The cellular concentrations of PAs often fluctuate depending on the stage of plant development and growth, the type of nitrogen (N) nutrition as well as in response to various internal (i.e., developmental) and environmental signals, e.g., those induced by biotic or abiotic stressors. In general, plants have evolved robust control mechanisms for keeping PA homeostasis both to accomplish the aforementioned physiological functions and also to maintain the balance of C: N ratio in plant cells [4]. This homeostasis control is achieved through regulation of biosynthesis, interconversion among various PAs, catabolism, their conjugation with phenolic compounds and other macromolecules, and transport to other cells, tissues, and organs [5, 6]. This chapter summarizes metabolic interactions of PAs with a variety of metabolic pathways in relation to their biological significance in plants.

1.1 Polyamine Biosynthesis

One of the major players in the regulation of PA homeostasis is their biosynthesis, which in most plants occurs via two pathways, namely the arginine (Arg) decarboxylase (ADC) pathway and the ornithine (Orn) decarboxylase (ODC) pathway (Fig. 1). However, a major exception to this is the commonly used model plant *Arabidopsis thaliana*, whose genome lacks a gene encoding an ODC-like protein [8, 9]. In the ADC pathway, PA biosynthesis starts with the conversion of Arg to agmatine (Agm), then into Put, which is the precursor of all higher PAs (Fig. 1). The ODC pathway on the other hand, involves direct conversion of Orn (also an important intermediate in Arg biosynthesis) into Put. Higher PAs, namely Spd and Spm/tSpm are synthesised from Put by sequential actions of aminopropyltransferases [10], Spd synthase (SPDS), and Spm/tSpm synthase (SPMS) or tSpm synthase (tSPMS), respectively via addition of an aminopropyl moiety from decarboxylated S-adenosylmethionine (dcSAM). The enzyme SAM decarboxylase (SAMDC) produces dcSAM from SAM; this reaction is often believed to be a rate-limiting step in the biosynthesis of the higher PAs [11]. Methionine is the immediate precursor of SAM. Some plants as well as animals also produce and accumulate cadavarine (Cad), another diamine made by direct decarboxylation of Lys by Lys decarboxylase (LCD) [12] or in mammals by ODC [13]. In some plants, Cad is used for the production of speciality alkaloids [12].

1.2 Polyamine Catabolism

Catabolism of PAs is the most significant player (other than synthesis) in regulating dynamic equilibrium of cellular PA concentrations in all organisms. It also contributes to the production of several growth and development regulatory molecules in plants. The turnover of cellular PAs is rather rapid, with a half-life ($t_{½}$) for Put being around 6–7 h; for Spd and Spm the half-life is >20 h [14, 15]. The

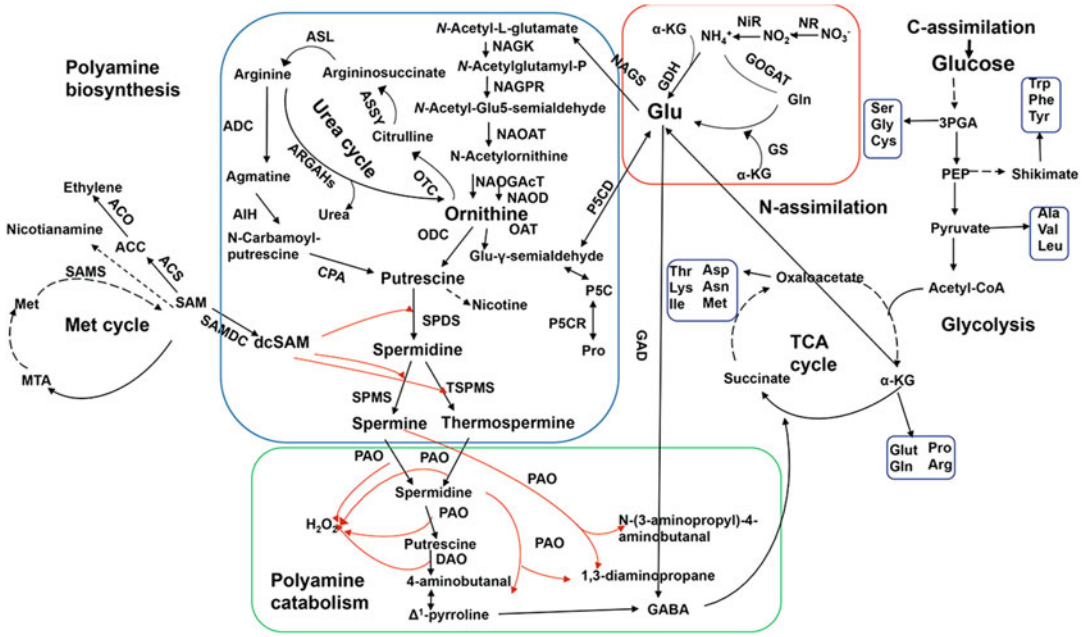

Fig. 1 Networks of polyamine metabolic pathways from N-assimilation to PA catabolism along with its interacting pathways such as TCA cycle, Glycolysis, Urea cycle, and others. Abbreviations of the enzymes and metabolites involved in the interacting pathways are as follows. *GS* Glutamine synthetase, *GOGAT* Glutamate synthase, *NAGS* *N*-acetylglutamate synthase, *NAGK* *N*-acetylglutamate kinase, *NAGPR* *N*-acetylglutamatyl-5-P reductase, *NAOAT* *N*-acetylornithine aminotransferase, *NAOGAcT* *N*-acetylornithine-glutamate acetyltransferase, *NAOD* *N*-acetylornithine deacetylase, *OTC* Ornithine transcarbamylase, *ASSY* Argininosuccinate synthase, *ASL* Argininosuccinate lyase, *CoA* Coenzyme A, *CPS* Carbamoyl phosphate synthetase, *δ-OAT* ornithine-δ-aminotransferase, *P5CR* Δ^1 pyrroline-5-carboxylate reductase, *P5CD* Δ^1 pyrroline-5-carboxylate dehydrogenase, *ACC* 1-Aminocyclopropane-1-carboxylic acid oxidase, *ADC* Arginine decarboxylase, *DAO* Diamine oxidase, *GAD* Glutamate decarboxylase, *GOGAT* Glutamate synthase, *GS* Glutamine synthetase, *LysDC* Lysine decarboxylase, *NAGK* N-Acetylglutamate kinase, *NAGPR* N-Acetylglutamate-5-P reductase, *NAGS* N-acetyl-glutamate synthase, *NAOAT* N2-acetyl-Orn aminotransferase, *NAOD* N2-acetyl-Orn deacetylase, *NIR* Nitrite reductase, *NR* Nitrate reductase, *OAT* Ornithine aminotransferase, *ODC* Ornithine decarboxylase, *OTC* Ornithine transcarbamylase, *SAMDC* S-adenosylmethionine decarboxylase, *SPDS* spermidine synthase, *SMPS* spermine synthase. (Adapted with modification from [7])

PA turnover is catalysed by copper amine oxidases (CuAOs) and the Flavin-dependent PA oxidases (PAOs) [3, 4, 16]; the former are homodimeric enzymes that catalyze breakdown of Put or Cad to γ-aminobutyric acid (GABA) via the intermediates 4-aminobutanal and Δ^1-pyrroline (Fig. 1). These enzymes can also catalyze the oxidation of Spd and Spm although with a lower affinity. On the other hand, the monomeric PAOs catabolize either Spd, Spm/tSpm (and their acetylated derivatives in animals) or back-convert them into respective lower amines (Put and Spd, respectively). Polyamine oxidation results in the formation of (a) H_2O_2, which is a signaling molecule that is perhaps a major player in PA-induced regulation of various biological processes [17, 18]; and (b) GABA,

another very important molecule in numerous physiological functions in all organisms [19–21]. Besides the terminal degradation of PAs to recycle C and N, thereby bridging PA metabolism with the tricarboxylic Acid (TCA) cycle, it has now become clear that PA catabolism serves a vital role in the regulation of plant growth and development [3, 22].

1.3 Polyamine Conjugation

In plants, PAs may accumulate as free molecules or be conjugated with phenolic acids, such as hydroxycinnamic acid (HCA), coumaric acid, caffeic acid, and ferulic acid, or be bound to various macromolecules (e.g., proteins), thus significantly affecting their cellular titres. Many PA conjugates, and the enzymes catalysing their biosynthesis, have been identified in different plant species [2]. Some important PA-conjugates are the HCA amides (HCAA), also known as phenolamides, produced by hydroxycinnamoyl acylation of the mono-, di-, or tri-phenolic acid substitutions of PAs [23] by enzymes called hydroxycinnamoyl transferases belonging to the BAHD *N*-acyltransferase group [24]. Conjugation of PAs to proteins on the other hand occurs via covalent linkage to the glutamyl residues to produce either mono-γ-glutamyl-PAs or bis-γ-glutamyl-PAs; transglutaminases catalyze these reactions [25, 26]. The less polar and hydrophobic nature of PA conjugates as opposed to PAs, may favor their intercellular transport and long term storage [23]. The proportion of conjugated vs. free PAs ranges widely in different species, depending on growth and developmental status of different organs. Although some interconversion between conjugated and free PAs has also been reported [27]; it apparently is not a major contributor to the cellular PA content. The PA conjugates participate in various biological processes, e.g., pollen development, plant defense responses to pathogens and insects, and response to abiotic stresses [3, 25, 28].

1.4 Polyamine Transport

Uptake of PAs from external sources and their organellar transport contribute to the regulation of PA homeostasis in many tissues. Translocation in microorganisms is mainly facilitated by the activities of PA transporters [29]; only recently have they been reported in plants [30, 31]. The first plant PA transporter to be functionally characterized was the rice PA uptake transporter (*Os*PUT1) [31]. This protein is produced in most tissues except seeds and roots; and, was shown to be a high affinity PA transporter preferentially facilitating the import of Spd. Recent studies have identified additional proteins belonging to an L-type amino acid transporter (LAT) family of proteins in rice and Arabidopsis as transporters of PAs, and their analog paraquat (PQ—*a.k.a.* methyl viologen) [30, 32, 33]. Three of the five Arabidopsis LATs that exhibit PA transport activity displayed different subcellular localization indicating that they may be involved in different cellular activities, e.g., *At*LAT1/PUT3 responsible for resistance to PQ (e.g., RMV1 or

LAT1/PUT3) has been shown to localize in the plasma membrane and hence may be involved in the intercellular transport of PAs and PQ. *At*LAT3 and *At*LAT4, on the other hand, were shown to localize in the endoplasmic reticulum and Golgi apparatus, respectively [34]. Specific molecular functions to these putative PA transporters are only now becoming assigned, one being the Organic Cation Transporter (*At*CAT1) that is involved in Cad transport, and another *At*LAT1 in stabilizing mRNA under heat stress conditions [35, 36]. The most important missing information currently is: what signals regulate the partitioning of PAs between the cytoplasm and the vacuoles, and transport from one cell type to the other? Also, it is not clear as to whether the three common PAs are translocated to the same compartment or some are preferentially transported over the others, and perhaps into different compartments.

2 Networks of Metabolic Pathways Interacting with Polyamines and Their Metabolism

As shown in Fig. 1, PAs are synthesized from glutamate (Glu) as the starting material in plants, which is also the source of amide group for the biosynthesis of most other amino acids as well as other nitrogenous compounds in plants. The other substrate of importance for PA biosynthesis is Met, which is the primary source of SAM biosynthesis; SAM, like Glu, is also required for numerous methylation reactions in the cell. As outlined above, PAs can be accumulated in plant cells in rather high (up to mmolar) quantities, thus affecting the total N pool; their reactions with phenolic acids also contribute to the flux of C into PA conjugates. Then there are the catabolic products of PAs, including metabolites like GABA (also present in high concentrations in plants) on one side, and H_2O_2 and nitric oxide (NO) on the other. It is, therefore, not surprising that PAs play diverse physiological roles in plants, both directly and indirectly as their metabolism is intertwined in a complex network of metabolic pathways in the cell.

Some of the major interacting metabolic pathways or metabolites of considerable importance to understand the nature of regulation of these metabolic networks involving PAs include: (1) the initial N assimilation pathway (NH_4^+ and NO_3^-) leading to Glu formation; (2) the role of Glu in the biosynthesis of other amino acids by transamination reactions; (3) the biosynthetic subpathways from Glu to PAs via Orn (Glu → Orn → Put/PAs) or via Arg (Glu → Orn → Arg → Put/PAs); (4) Pro biosynthetic pathways from Glu (Glu → Pro and Orn → Pro); and (5) Arg → Orn reverse pathway involving the production of NO and urea. In addition, there is the pathway for SAM biosynthesis from Met and the

metabolic pathways utilizing SAM as precursor for higher PAs, a myriad of methylation reactions that use SAM as a precursor; plus, the unique pathway of ethylene biosynthesis in plants. On the other hand, PA catabolism and its reaction products—the Reactive Oxygen Species (ROS) like H_2O_2, and GABA are major signaling molecules in plants. These reactions are then directly connected to the TCA cycle via GABA shunt. Finally, there are the PA conjugation pathways involving a variety of phenolic derivatives like HCAs and coumaric acid. The products of these pathways are involved in storage of organic N to essential signaling reactions, to interactions with cellular macromolecules ranging from DNA, RNA and proteins, and to cellular membranes. Signaling molecules derived from these pathways contribute to metabolic crosstalk with major phytohormones such as ethylene, abscisic acid (ABA), and jasmonic acid (JA). To understand the regulatory mechanism of these complex networks, it is essential to critically evaluate the individual metabolites along with the set of pathway(s) leading to the formation of individual PAs. Numerous studies have dealt with the consequences of perturbation of one subpathway on some of the related metabolic pathways in the networks, and related signaling molecules as well as the metabolites therein. A group of such interactions in plants are summarized here:

2.1 Polyamine Metabolism and Nitrogen Assimilation Pathway (NO_3^-/NH_4^+ → Glu)

Plants can assimilate different forms of N including inorganic forms such as NO_3^- and NH_4^+ and organic forms such as urea and amino acids. Via reduction of NO_3^-, all N assimilated by plants ends up in three main amino acids—asparagine (Asn), glutamine (Gln), and Glu. The first step in N assimilation is the conversion of NO_3^- to NH_4^+ by the successive actions of NO_3^- reductase (NR) and NO_2^- reductase (NiR) or urea to NH_4^+ by urease. Subsequently, NH_4^+ is assimilated to Glu and Gln via the Gln synthetase-Glu synthase (GS/GOGAT) pathway. The product of this pathway is Glu, which in addition to being the precursor for biosynthesis of PAs and several related metabolites like Orn, Arg, Pro and GABA, is the major donor of amine groups for most other amino acids in plants. Therefore, the form of N absorbed by plants (NO_3^- or NH_4^+) may have major effects on the metabolism of PAs.

Since cells cannot store reduced N as NH_3 or NH_4^+, PAs can be important N storage compounds in plants, their biosynthesis also performs the function of alleviating the NH_3/NH_4^+-induced toxicity in the cells [4]. In agreement with this, an increase in the cellular concentrations of Put to a larger extent, and in some cases also Spd and Spm, were reported under relatively higher NH_4^+ fertilization vs. NO_3^- feeding conditions [37]. Moreover, an increase in Put biosynthesis from radiolabeled Orn in NH_4^+-fed soybean seedlings was accompanied by reduced activities of the PA catabolic CuAOs [38]. Similarly, NH_4^+ nutrition, and also urea to a lesser extent, were found to increase Put biosynthesis leading to

reduced plant growth in wheat and pepper; supplementing NH_4^+ and urea with NO_3^- ameliorated this effect.

It appears that PA biosynthesis via the Glu-Orn pathway is favoured under high NH_4^+ nutrition [39, 40]. On the other hand, studies involving poplar and red spruce cell cultures have shown that higher than optimal total N (NH_4^+ + NO_3^-) in the growth medium does not increase PA concentrations [41, 42]. However, low N concentration and lower NO_3^-: NH_4^+ ratio than the normal (2:1) ratio in the medium, led to reduced concentrations of all three major PAs [42]. Thus, the type of N fertilizer impacts the PA metabolic pathway through its effect on the rate of NH_4^+-assimilation via the GS/GOGAT pathway.

A recent study using transgenic Arabidopsis seedlings harboring a mouse *ODC* gene to channel large amounts of N assimilates to Put via Orn showed that concentrations of most amino acids and all PAs were reduced in the N deficient medium both in transgenic and wild-type seedlings [7]. High NO_3^- in the growth medium had rather negative effect on Put and Spd concentrations in transgenic seedlings probably due to depletion of the substrate Orn. The results suggest that while N availability may not be a limiting factor for PA biosynthesis, additional N and C do have positive effects on PA metabolic pathway highlighting the dynamic coordination between N and C assimilation pathways. Moreover, most reactions in the Glu → PA pathway seem to be regulated posttranscriptionally as deduced from the results of m*ODC* expressing transgenic lines showing insignificant changes in the expression of most genes coding for the PA pathway enzymes compared to the nontransgenic controls [43, 44].

Since the precursors of PA biosynthesis (Orn and Arg) are derived from Glu produced largely by the GS/GOGAT pathway, the cellular concentrations of PAs presumably depend on the concentration of Glu being made from the N assimilated via the GS/GOGAT pathway. Consistent with this is the report that inhibition of GS activity with methionine sulfoximine caused a reduction in PA concentrations in poplar cells [39]. On the other hand, studies with poplar and red spruce cell cultures also showed that higher than optimal N availability does not affect the activity of GS, whereas low N and lower NO_3^-: NH_4^+ ratio than 2:1 led to a reduction in the activity of GS [42]. Though GOGAT plays a critical role in N and C metabolism [45], its direct connection with the metabolism of PAs has not been addressed.

2.2 Pathways of Glutamate to Polyamines (Glu → Orn → Put/PAs and Glu → Orn → Arg → Put/PAs)

In addition to abiotic and biotic stresses, three approaches (i.e., chemical inhibitors, genetic mutants, and transgenic manipulations) have been used to manipulate cellular PA contents to understand the regulation of PA biosynthesis in plants. Most abiotic stress responses have shown a rapid increase in PAs, particularly Put, which is often related to increase in ADC activity with or without increase in transcription of a specific member of the ADC gene family. There are several hypotheses regarding mechanisms of signal transduction from the moment of perception of stress to the various biochemical responses; however, the signaling pathway for the induction of PA biosynthesis has not been elucidated [46]. One thing consistent in this regard is that under stress conditions, several metabolites of the PA interacting pathways (Pro, GABA, Arg, and H_2O_2—Fig. 1) are often elevated concurrent with increase in PAs. However, the physiological importance of increase in PAs is not clearly understood; neither is the role of any of the other metabolites related to these interacting pathways. Two contrasting possibilities have been repeatedly mentioned in the literature; these are a protective role vs. being the cause of stress damage. While the enzymology, and in many cases, gene expression (measurements of the transcript and analysis via promoter::reporter approach) have been variously discussed, no clear hypothesis regarding the role of increased metabolic flux into the PA pathway, and its consequences on the remainder of the pathways shown in Fig. 1 are discussed.

The use of a variety of biochemical inhibitors of the PA biosynthetic enzymes has been a common tool to lower the cellular contents of PAs to study their role in a number of growth and developmental processes in plants. Although the expected results in terms of lowering PA contents are often seen, and the specific inhibitors do lead to a better knowledge of the active pathways in various cells/tissues of plants, the complex interactions of inhibitors with other metabolic pathways (i.e., their side effects) have not been reported in most cases. The lack of this information often interferes with getting a clear understanding of the role of PAs in specific functions. Thus, the use of inhibitors is less favored than molecular approaches, which allow more precise regulation of the biosynthetic pathways.

Specific gene mutagenesis (chemical mutagen treatment or producing knockouts using T-DNA insertions) has yielded highly useful information with respect to the physiological and developmental roles of specific genes regarding the tissue- and organ-specific expression of different members of a gene family producing a functional enzyme. Once again, its usefulness in delineating metabolic consequences of manipulating PAs in an organ/tissue-specific manner with this approach has been rather limited.

Genetic engineering approach using PA metabolic pathway genes, which targets the cellular concentration of PAs as opposed

to the activities of genes as the selection criterion, has been used in numerous studies both to understand PA metabolism as well as their physiological and developmental roles. Our laboratory has studied the effects of genetic manipulation of the Orn → Put step by expression of a mouse (*Mus musculus*) cDNA coding for ODC driven by either a constitutive 35S promoter or an estradiol-inducible promoter on the metabolism of PAs and related subpathways in several different plant systems including tobacco, carrot, poplar and Arabidopsis. Detailed studies on long-term, nondifferentiating, and rapidly growing cell cultures of poplar (*Populus nigra* × *maximowiczii*) and seedlings of *A. thaliana* showed several-fold increase in cellular concentration of Put but only minor changes in the concentrations of Spd and Spm as compared to nontransgenic control cells [39]. Similar results were reported earlier when m*ODC* cDNA was overexpressed in tobacco [47] and carrot [48]. Since increased Put production in m*ODC*-expressing poplar cells was due to rapid conversion of the substrate Orn into Put, it was shown to be a limiting factor in controlling overall PA production in the transgenic cells [39, 49]. However, an interesting observation is that, in spite of its low cellular concentration, large amounts of Orn (to produce up to mmolar concentrations of Put in the cells) were sustainably produced. This led to the conclusion that Orn production (i.e., the Glu → Orn part of the pathway) responds largely to its rate of utilization (i.e., Orn → Put step) with its accumulation remaining very low. In other words, the flux of Glu → Orn increased several-fold under increased Orn → Put conversion. We further observed that this change in Glu → Orn flux in these cells/plants did not require increased expression of genes whose products code for various steps in this part of the pathway (Fig. 1). Based on these studies, we postulate that Orn, which is centrally located in the Glu/Pro, Put and Arg pathways, may not only be a key regulator of the flux of Glu into these metabolites but also influence the biosynthesis of Glu via concurrent assimilation of N and C [43, 44, 50].

Since the main source of Orn in these cells is Glu, which is also a precursor for Pro and Arg production (the latter entirely via Orn), both of which concurrently accumulate in large quantities; it was postulated that the total amount of Glu production in the transgenic cells and plants must be greatly increased. Moreover, as described above, since in actively growing cells/plants, all Glu must come from N assimilation, it was suggested that the increased conversion of Orn to Put must be accompanied by increased production of Glu from assimilation of inorganic N (NH_4^+ and/or NO_3^-) from the growth medium [43]. It also follows that competition for the Orn → Put step catalysed by mODC, and for δ-Orn aminotransferase (OAT) resulting in Orn to Δ^1-pyrroline-5-carboxylate (P5C) for Pro production, must be minimal, as its inhibition did not affect Put production in poplar cells [39, 49]. Based on

the rather limited effects of *mODC* expression on Spd and Spm in the presence of higher cellular Put, it has been suggested that these metabolites are subject to stricter homeostatic regulation as compared to Put.

As Glu is the primary source of the amine group for most physiologically important amino acids, the reduction in its cellular concentration due to increased flux into Orn/Put in transgenic cells resulted in significant changes in the concentration of several amino acids, as well as many related N metabolites in the mODC-transgenic cells [50]. Also, since metabolic regulatory mechanisms for various N metabolites in a cell are quite variable, and often independent; the reduction in Glu concentration did not negatively impact all amino acids. Amino acids like Gln, His, and Orn, and others that use Glu/Gln as an amine donor including members of the serine family (Ser, Gly, and Cys), shikimate family (Phe and Trp), and some of those in Asp family (Asp, Met and Lys) were found to be significantly reduced [44, 50]. In contrast, concentrations of amino acids closely associated with the PA, Pro, and Arg pathway, metabolites associated with PA catabolism (GABA, succinate, and Ala), and some amino acids like Thr, Val, and Ile were higher in the transgenic cells expressing *mODC* [44, 50].

Based on the results with poplar cells and Arabidopsis seedlings, it was postulated that increased utilization of Glu in reactions involving Orn, Pro, Arg, Put, and GABA, the increased flux of Glu into Orn/Put must be accompanied by replenishment of cellular Glu content via its increased biosynthesis directly from increased N uptake. The facts (1) that key N rich metabolites did not decrease with high flux of Orn into Put, and (2) that sustained N assimilation is intimately dependent upon C availability; it was further suggested that the transgenic cells/plants must also absorb more C from the growth medium concurrent with N. If this holds true for enhanced N assimilation by the roots from the soil and by increased C assimilation from the environment via photosynthesis; manipulation of Put biosynthesis should provide a novel approach to increase both N and C assimilation (and in turn increased biomass production) in plants. Thus, this approach would help us tackle two of our important environmental goals—namely (1) increased N use efficiency of the plants, and decreasing N fertilizer loss from soil, and (2) increasing C assimilation to help reduce atmospheric CO_2 accumulation. There is, however, an important consideration when applying this technology to design food crops vs. biomass producing nonedible crops. While in the latter, high Put/PAs can be tolerated, the PA contents in foods are known to enhance tumor growth in animals and humans (http://www.cancernetwork.com/colorectal-cancer/high-dietary-polyamines-may-foster-colorectal-adenomas). Therefore, it will be prudent to apply this approach cautiously by using tissue/organ-specific promoters

in food/feed crops, where the edible part of the plant does not accumulate high concentrations of PAs.

Important prerequisites for the above chain of events (Orn depletion leading to increased flux of Glu → Orn → increased uptake of N and C) are: (1) a mechanism for cells to sense and monitor Orn concentration, and (2) a signal transduction mechanism to regulate N and C assimilation. Neither a reliable mechanism for sensing cellular Orn concentration nor the signaling pathway from Orn to increased Glu biosynthesis and uptake of extra N and C is known. Of course, there is also the need for a mechanism for increased production of Orn itself. Since Orn is an intermediate for several products of this group of interacting pathways (including Arg and Pro—Fig. 1), and its cellular concentration is rather low as compared to all end products, it is likely that stimulation of Glu biosynthesis may be affected by significant increases in the biosynthesis of any or all these metabolites. This situation raises some very interesting challenges to speculate one or more mechanisms, and then to design experiments to test them.

The results with Arabidopsis, where we used both an inducible and a constitutive promoter showed that the two systems had similar responses indicating that the role of Orn is similar under long-term as well as short-term changes in its consumption. As pointed out earlier, *A. thaliana* does not directly convert Orn to Put because it lacks a functional ODC, thus m*ODC* transgene adds a new short-cut to Put production. The inducible expression system was valuable to study the effect of short-term induction of m*ODC* (i.e., increased utilization of Orn) on the regulation of PA biosynthesis [43], which would be expected to mimic the natural conditions that plants face when they are in the phase of rapid growth or being subjected to environmental stress—abiotic or biotic.

Dalton et al. [51] have recently reported that manipulation of the Orn-Put pathway by RNAi silencing of the *ODC* gene in tobacco caused a significant reduction in the concentration of all three major PAs (Put, Spd, and Spm) along with the transcript levels of genes encoding SAMDC, SAMS, and SPDS. The transgenic plants also displayed an increase in the concentrations of downstream metabolites including Orn, Arg, Asp, Glu, and Gln. These results clearly demonstrate a coordinated regulation of this entire group of pathways (Fig. 1). The expression levels of genes coding for the alternative pathway of Put biosynthesis (ADC) was elevated, along with concentration of Arg in the RNAi plants suggesting the presence of compensation for the reduced ODC activity, thereby maintaining the baseline levels of PAs for essential metabolic functions. These observation are consistent with the postulated role of Orn in regulating the entry of Glu into the Orn/Pro/Arg/Put pathway.

On the Put catabolic side of the pathway, the turnover rate (half-life) of this diamine (which includes its conversion to Spd + Spm, catabolic breakdown, and conjugation) in both poplar and Arabidopsis was not affected by its enhanced biosynthesis [14, 49]. Likewise, the $t_{1/2}$ of Spd and Spm turnover also remained unchanged [15]. The observation that increased Put catabolism was accompanied by increased GABA accumulation in the transgenic cells/plants, suggests the absence of a feedback mechanism for GABA production from Glu by GAD. Later studies [52] support these conclusions in that there was increased Put catabolism (lower Put concentration) accompanying GABA accumulation under salinity, and a reduction in GABA concentration followed by increased Put concentrations during recovery from salinity in soybean.

2.3 Polyamines and Proline Pathways Interactions (Glu → Pro ↔ Orn ← Glu)

The first step in Pro biosynthesis from Glu is catalysed by P5C synthetase (P5CS) to form Glu-γ-semialdehyde (GSA), that subsequently cyclizes to P5C [53] (Fig. 1). In the final step, P5C reductase (P5CR) converts P5C into Pro. Alternatively, Pro can be made from Orn by OAT, which transaminates GSA joining the route shown above to Pro [53]. Like the Glu↔Pro steps, the Pro↔Orn subpathway is also reversible, using the same enzyme OAT. Proline turnover on the other hand, involves its oxidation back to Glu by Pro dehydrogenase (PRODH) and P5C dehydrogenase (P5CDH) [54]. As discussed above, since the cellular concentration of Pro does not change significantly under conditions of high Put production in the m*ODC*-transgenic cells, the reactions involving Glu↔Pro and Pro↔Glu must not be affected by the increased flux of Glu into Orn/Put. The transcriptomic analysis in poplar and Arabidopsis also show the lack of significant changes in the expression of P5CS, P5CR, OAT, and ProDH [7, 44].

2.4 Interactions Between Polyamines and S-Adenosylmethionine (SAM) Metabolism

S-Adenosylmethionine (SAM) is a common source of numerous methylation, transsulfuration, and aminopropylation reactions to various substrates such as nucleic acids, proteins, lipids and secondary metabolites. In addition, significant amounts of SAM are used in the biosyntheses of ethylene and the higher PAs (Spd, Spm and tSpm); it is also used as a substrate for nicotianamine (NA) in plants [55, 56]. Consequently, the cellular concentrations of Spd and Spm are affected by the rate of dcSAM production from SAM, the use of SAM for various methylation reactions, and the biosynthesis of ethylene [57, 58]. The production of dcSAM is regulated by SAMDC, an enzyme with some unique properties [59], e.g., (1) a long 5′ UTR with two uORFs (called "small" and "tiny"), whose translation is regulated by PAs; (2) the ribosomal binding to the small uORF further regulates SAMDC translation; (3) the last codon of small uORF is the first nucleotide of the tiny uORF; and (4) the enzyme has a rather short half-life of <30 min. This

monofunctional enzyme is coded by up to five genes in *A. thaliana*, some of which are expressed constitutively, and others in a cell/tissue/organ-specific manner [60]. Most of the SAM reactions regenerate Met through the Yang cycle [61].

A study involving Arabidopsis mutants of the two genes encoding 5'methylthioadenosine[1] nucleosidase (MTN) namely *mtn1* and *mtn2* confirmed that the Met cycle is linked to the regulation of PA homeostasis [62, 63]. The concentrations of Put and Spd were higher in the *mtn1-1mtn1* double mutant with reduced MTN activity concomitant with increased concentration of MTA and SAM/dcSAM [63]; however, the production of ethylene and nicotianamine was unchanged [62]. The *mtn2*-1 and *mtn2*-2 double mutants, had only a small reduction in MTN activity; their SAM/dcSAM, MTA and PA contents were similar to wild-type plants. A mutant for both MTN genes (*mtn*1-1/*mtn*2-1) was sterile; the sterility was partially reversed by PAs [63]. Decreases in the cellular contents of Spd and Spm in one of the MTN mutants was shown to be due to MTA-mediated feedback inhibition of the activities of SPDS and SPMS [63]. These results demonstrate that MTA is a crucial regulatory metabolite acting as a link between the Yang cycle and biosynthesis of PAs, and that MTN is a crucial enzyme that regulates this metabolism. The authors suggested that key targets affected by increased MTA content were tSPM and Spd-dependent hypusine formation in the eukaryotic initiation factor elF5A.

Earlier studies in potato had shown that downregulation of SAMDC activity results in reduced concentrations of not only Spd and Spm but also Put while significantly increasing the release of ethylene resulting in abnormal plant/tuber phenotypes [64]. Studies using m*ODC* transgenic poplar cell cultures overproducing Put or in tomato fruits expressing a transgenic yeast SAMDC gene did not show notable competition between ethylene and PA biosynthetic pathways for SAM [14, 65]. Similar results (i.e., the lack of competition between PAs and ethylene for SAM) were reported in tomato during climacteric ripening [66]. Consistent with this are the results of [15], who found that elevated PAs due to transgenic expression of yeast SAMDC to channel most of the SAM to PAs did not alter the rate of ethylene synthesis in tomato fruit. All these studies together indicate that the cellular pool of SAM might be sufficient in these cells/tissues to meet the concurrent demands of the two pathways.

On the other hand, expression of sense or antisense strands of *SAMDC* cDNA for up- or down-regulation of *SAMDC* expression in Arabidopsis showed competitive relationships between the two pathways [11]. A competition between the two pathways was also observed in the developing kernels of maize [67] in response to

[1] Methylthioadenosine (MTA) is a byproduct of ethylene, PA, and nicotianamine biosynthesis

drought at grain filling stage in rice [68] and wheat [61]. Taken together, interaction between PAs and ethylene seems essential for the regulation of certain developmental processes in plants and the utilization of SAM in these pathways may be subjected to different regulatory mechanisms. Recently, a study involving miRNA396b from trifoliate orange (*Poncirus trifoliate* L.) showed that this miRNA positively affects cold tolerance via the modulation of ethylene-PA homeostasis; i.e., by inhibiting the evolution of ethylene while increasing the production of PAs [69]. The involvement of miRNA in the regulation of ethylene-PA interaction highlights the functional complexity of the PA metabolic pathway in terms of going beyond the Glu-Orn-Put-Arg-GABA-amino acids pathway.

Another common product made from SAM in plants is nicotianamine (NA), a metal chelator involved in the intra and intercellular transport and accumulation of cations, like Fe^{2+}, Fe^{3+} and Zn^{2+} [55, 56, 70, 71]. The enzyme NA synthase (NAS) trimerizes SAM to form NA. Since SAM is present in large quantities in the presence of these metal ions, it is likely to affect the availability of SAM for PA biosynthesis as well.

2.5 Polyamine Catabolism and the Regulation of PA Metabolic Pathway

Hydrogen peroxide and NO are two major signaling molecules that play critical roles in PA-induced regulation of various biological processes and responses to biotic and abiotic stresses [18]. While PAs have been advocated to protect the cells against oxidative damage by reactive oxygen species (ROS), their catabolism makes them susceptible to oxidative damage by generation of H_2O_2 and NO as well [72–74]. The formation of H_2O_2 from PA catabolism in plants is well established as opposed to PA-induced NO production; neither however is exclusively produced directly from the PAs. The catabolic breakdown of PAs generally produces H_2O_2 and 4-aminobutanal, which is the precursor of GABA (Fig. 1). Similarly, in high-Put transgenic poplar cells (from constitutive *mODC* expression), high concentration of both H_2O_2 and GABA were reported [49, 73]. The increase in H_2O_2 accumulation in transgenic cells was accompanied by increased activities of enzymes responsible for ROS scavenging, e.g., glutathione reductase (GR) and monodehydroascorbate reductase (MDHAR) [73]. Likewise, the concentration of ROS scavenging metabolites including reduced glutathione (GSH) was significantly decreased in transgenic cells indicating the creation of an elevated state of oxidative stress in the cells, which eventually led to a substantial damage to the cell membranes [73].

Nitric oxide plays a vital role in plant growth and development as well as plant immunity to pathogens. Several pathways have been suggested as routes for NO production in plants, including the metabolism of PAs [75]. Among the enzyme-based pathways that contribute to NO biosynthesis in plant cells are one involving nitrate reductase (NR) and the nitric oxide-associated-1 (NOA1)

protein [76, 77]. The experimental evidence for NO production from this pathway involved the use of triple mutants of two NR structural genes (NIA1 and NIA2 and NOA1) to demonstrate the presence of an alternative pathway for NO biosynthesis [77]. Polyamine catabolism also produces NO via protein-nitrosylation [74, 78]. The latter study suggests that CuAO may be directly involved in the biosynthesis of NO from PAs. The Arabidopsis mutants of gene coding for this enzyme displayed reduced PA-induced production of NO suggesting that CuAOs might be a putative candidate that plays a role in the biosynthesis of NO from PAs. This study also reported that CuAO participates in ABA signaling. The PA-dependent signal transduction system involving NO has been suggested to regulate plant development including root growth, defense responses and abiotic stress responses. Therefore, the mechanism by which PAs regulate different responses might be via cross-linking the signaling intermediates (H_2O_2 or NO) and hormonal signaling pathways including ABA and methyl jasmonate; the latter is well known to initiate downstream response machinery of stress response.

2.6 Interaction Between Polyamines and the TCA Cycle

The TCA cycle is the primary supplier of the C backbones for assimilation of N. Biosynthesis of Glu, the primary product of N assimilation, requires α-ketoglutarate (α-KG) as a substrate for the synthesis of all amino acids in the Glu family plus the main components of the PA biosynthetic pathway including Orn and Arg. Oxaloacetate (OAA) is another TCA cycle intermediate that is important for the biosynthesis of Asp family amino acids including Met, which as discussed above, supplies dcSAM for synthesis of higher PAs. The catabolism of PAs as well as decarboxylation of Glu contribute succinate to the TCA cycle via what is known as the GABA shunt. Thus the GABA shunt lies at the interface of the C and N metabolic interactions in recycling of C and N from the PAs [75]. GABA is also an essential component of plant responses to stress conditions, and plays a signaling role in plants for other physiological processes [19–21]. Its biosynthesis occurs mainly from Glu and PA catabolism though it has been recently indicated that it could also be synthesized nonenzymatically from Pro under oxidative stress conditions. This happens when Pro acting as a hydroxyl radicals (OH) scavenger removes H from amine group leading to the spontaneous decarboxylation of Pro resulting in the formation of Δ^1-pyrroline, a precursor of GABA [79]. Besides creating a crucial link with the TCA cycle, GABA catabolism indirectly contributes to the sustainable regeneration of Glu (via supplying α-KG for its biosynthesis), and also, other amino acids [50]. These interactions between TCA cycle and both the anabolic and the catabolic pathways of PAs and amino acids signify the important contribution of these networks to maintain intracellular homeostatic equilibrium of N and C metabolites in plants.

2.7 Interaction Between Metabolic Pathways of Free and Conjugated PAs

In addition to the biosynthetic and catabolic pathways of PAs, there also are reactions of PAs with several phenolic compounds (phenolamides) in plants to produce PA conjugates, which not only produce some important secondary metabolites but also contribute to PA homeostasis [23, 80]. Additional metabolic aspects of PA conjugation are the regulation of C-rich phenolic intermediates and the end products, which play important roles in plant biotic interactions ranging from bacterial and fungal pathogenesis to herbivore avoidance [81–83]. Thus, the cellular concentrations of phenolamides and PAs may be interdependent as the biosynthesis of the former depends on the latter. Whether conjugated PAs contribute (through their reversal to free PAs) to the physiologically active cellular PA pool is still unclear. A recent study involving RNAi-mediated silencing of tobacco *ODC* gene showed a significant reduction in the concentration of phenolamides (caffeoyl-Put—CP and dicaffeoyl-Spd—DCP) along with the corresponding PAs, indicating that ODC may play an essential role in the regulation of phenolamide biosynthesis [51].

A study with Arabidopsis stamens showed that knockout of the Spd-HCA transferase (SHT), responsible for the biosynthesis of HCA conjugates of Spd did not affect the free Put, Spd and Spm concentrations, despite a drastic reduction in the concentration of HCA-bound Spd as compared to the wild-type plants [80]. The lack of increase in the substrate of SHT (Spd) in the *sht* knockouts was suggested to be due to a tight regulation of intracellular titers of free PAs. This is in line with the conclusions of our studies (cited earlier) with Arabidopsis, poplar, carrot and tobacco, which also concluded that higher PA titers in plants were more tightly regulated than Put. It was further indicated that this regulation did not operate at the level of transcription of the PA biosynthetic genes but perhaps involved posttranscriptional/translational regulation at the biochemical level.

Polyamine conjugates like CP and DCP were found to increase in response to either actual herbivore attack or simulations of it [84]. Such increases in the accumulation of PA conjugates appeared to be regulated by a transcriptional master regulator, MYB8 in *Nicotiana attenuata* based on the results from a microarray analysis, showing increased transcripts of several key enzyme genes downstream of the PA and phenylpropanoid biosynthetic pathways in a MYB8-dependent manner [85]. However, the concentrations of free PAs were not determined in this study, and hence it is difficult to discern what impact the metabolic pathway of PA-conjugates has on the metabolism of its free PA counterpart.

2.8 Interaction Between PA Metabolic Pathway and Plant Hormones

As much as the PAs are associated with stress response, ABA is implicated as one of the major players in plant response to stress. Coordination between PAs and ABA in the regulation of response to environmental stressors has been reported by several studies; however, little details are available on the molecular mechanism of their interactions and crosstalk in most cases. The involvement of ABA in transcriptional upregulation of genes coding for *SPDS1*, *SPMS* and *ADC2* in Arabidopsis has been suggested concurrent with changes in Put concentration under drought conditions [86]. Conversely, Cuevas et al. [87] showed that Put can modulate transcriptional upregulation of an ABA biosynthetic gene, *9-cis-epoxycarotenoid dioxygenase-3* (*NCED3*) and other ABA-regulated genes thereby increasing the concentration of ABA during cold stress. These findings signify the existence of positive feedback loop between ABA and Put in response to cold stress [2]. Furthermore, ABA signaling has also been shown to induce accumulation of PAs as well as its catabolism, thereby increasing the rate of H_2O_2 generation leading to activated stress responses in grape [88]. A crosstalk between NO and ABA has been suggested to play a role during Arabidopsis seed germination with endogenous increase in NO ameliorating the inhibitory effect of ABA on seed germination via induction of its catabolism [89]. Therefore, the molecular mechanism of PA-induced regulation of stress responses may likely involve its interaction with ABA via the signaling molecules H_2O_2 and NO.

Other phytohormones, such as auxins and cytokinins, have also been suggested to interact with PAs to regulate certain physiological functions. Previous studies suggested that auxin could stimulate PA biosynthesis especially during early root initiation stage [90, 91]. The involvement of PAs in modulation of cytokinin-regulated physiological phenomena via its effect on the expression of cytokinin response genes is also known [92, 93].

3 Methods Used for Studying PA Interacting Pathways and the Future

It is apparent from the above discussion that PAs themselves and their metabolic pathway substrates and products interact with a wide network of cellular metabolic pathways, such as the TCA cycle, GABA shunt, N assimilation, modulation of phenolics through conjugation and binding, modulating SAM synthesis and its utilization, signaling pathways involving phytohormones, stress and senescence related metabolism, and oxidative stress pathways to facilitate the regulation of various biological functions in which they play vital roles. Due to their accumulation in relatively large quantities (up to mmolar in concentrations), PAs are also capable of affecting total soluble N balance in the cells; i.e., at times they act as N storage compounds and at other times, they release the stored N.

These interactions may be manifested by changes in the transcription of genes that code for PA metabolic enzymes, the level of active proteins/enzymes, and the substrates used for PA biosynthesis. They also influence directly (through hypusilation of elF5) or indirectly (by interactions with mRNA) the process of translation, including that of their own biosynthetic enzyme (e.g., interaction with the uORF in the 5′UTR of SAMDC). To this end, some of the recent studies have highlighted substantial changes in the transcriptome and metabolome of the interacting pathways, resulting from genetic perturbation of a single step in the PA biosynthetic pathway [7, 44]. The reverse is also true where "Omics" studies targeted at other genotypic or phenotypic variations in plants have revealed significant changes in plant PA metabolism where the transcripts of PA metabolic genes, as well as precursors and intermediates of the PA metabolic pathway were related to specific growth and development phenomena being investigated. It is time that we use the combined approaches of next-generation sequencing (NGS) of DNA and RNA, and biochemical analyses using tools of proteomics and metabolomics (GC-MS-MS, LC-MS-MS, NMR, stable- and radio-isotope labeling, etc.) to unravel biochemical mechanisms of the complex cellular responses accompanying these alterations in the PA metabolic network. These approaches can help discern changes in the rates of biosynthesis of thousands of genes and metabolites of not only the interacting pathways but also other previously considered unrelated pathways; and hence, may lead to the discovery of some novel relationships among PA and other pathways.

While the mutants and constitutive transgene expression approaches have been commonly used, and provide excellent tools to delineate metabolic interactions of the PA metabolic pathways and other pathways, a major drawback of these two approaches is that they don't often mimic the real-life dynamic and transient changes in cellular metabolite concentrations during short-term responses of plants to internal (e.g., growth, development, and differentiation), and to external stimuli (abiotic and biotic stresses, diurnal fluctuations of C and N availability, etc.). In this regard, studies involving well-designed regulated promoters (inducible, cell, tissue, and organ specific, and developmentally regulated) must be planned in conjunction with the modern tools of transcriptomic, proteomic, and metabolomics analyses. Furthermore, the aspects of PA metabolism related to the flux rates of various metabolites in the PA associated pathways, have received minimal attention. These studies capable of monitoring small changes in N- and C-rich metabolites together would contribute immensely to our understanding of the flow of various metabolites and intermediates into different interactive and branched chain pathways where a metabolite (e.g., Glu, Orn, Arg, and SAM) serves as a common precursor for several pathways that produce products

with either competing (e.g., SAM for PAs and ethylene) or complementary biological functions (e.g., H_2O_2, ABA, NO). These advances, combined with improvements in bioinformatics tools for analysis of large datasets, could be utilized to better understand the complexities of PA metabolic networks.

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