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REVIEW ARTICLE

Polyamines and cellular metabolism in plants: transgenic approaches reveal different responses to diamine putrescine versus higher polyamines spermidine and spermine

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Abstract Distribution of biogenic amines—the diamine putrescine (Put), triamine spermidine (Spd), and tetraamine spermine (Spm)—differs between species with Put and Spd being particularly abundant and Spm the least abundant in plant cells. These amines are important for cell viability and their intracellular levels are tightly regulated, which have made it difficult to characterize individual effects of Put, Spd and Spm on plant growth and developmental processes. The recent transgenic intervention and mutational genetics have made it possible to stably alter levels of naturally occurring polyamines and study their biological effects. We bring together an analysis of certain metabolic changes, particularly in amino acids, to infer the responsive regulation brought about by increased diamine or polyamine levels in actively growing poplar cell cultures (transformed with mouse *ornithine decarboxylase* gene to accumulate high Put levels) and ripening tomato pericarp (transformed with yeast S-adenosylmethionine decarboxylase gene to accumulate high Spd and Spm levels at the cost of Put). Our analysis indicates that increased Put has little effect on increasing the levels of Spd and Spm, while Spd and Spm levels are interdependent. Further, Put levels were positively associated with Ala (α and β), Ile and GABA and negatively correlated with Gln and Glu in both actively growing poplar cell cultures and non-dividing tomato pericarp tissue. Most amino acids showed positive correlations with Spd and Spm levels in actively growing cells. Collectively these results suggest that Put is a negative regulator while Spd–Spm are positive regulators of cellular amino acid metabolism.

Keywords Amino acids · Biogenic amines · Metabolome · Poplar cell cultures · Tomato

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Abbreviations

DAO	Diamine oxidase
eIF5A	Eukaryotic initiation factor 5A
HP	High putrescine
NAGS	N-acetyl glutamate synthase
P5CS	Δ1-Pyrroline-5-carboxylate synthase
PAO	Polyamine oxidase
Put	Putrescine
Spd	Spermidine
Spm	Spermine
ySAMDC	Yeast S-adenosylmethionine decarboxylase

Introduction

Polyamines are a class of biogenic amines with multiple in vitro effects on cellular processes of most organisms.

The three ubiquitous amines that have long attracted the attention of researchers are the diamine putrescine (Put) and higher polyamines, spermidine (Spd) and spermine (Spm) (reviewed in Cohen 1998). These three amines have often been grouped together and generally assumed to have similar biological effects albeit with differing amplitudes. This assumption stems from classifying them together as nitrogenous substances with increased amine strength from Put (di-amine) to Spd (tri-amine) to Spm (tetra-amine). Thus, together as 'polyamines', they constitute a large body of literature on possible roles in myriad biological processes including cell division and differentiation, cell proliferation, homeostasis, gene expression, macromolecular synthesis, and apoptosis (Cohen 1998; Jänne et al. 2004). The potential of polyamine research is vast with greater implications in such disciplines as oncology (Jänne et al. 2004; Agostinelli et al. 2006; Casero and Marton 2007); obesity (Pfeffer et al. 2001; Jell et al. 2007), cerebral stroke and related disorders (Tomitori et al. 2005), parasitology (Bacchi and Yarlett 2002), oxidative stress (Bachrach and Wang 2002; Paschalidis and Roubelakis-Angelakis 2005; Agostinelli et al. 2006; Mohapatra et al. 2009a), and apoptosis (Tome and Gerner 1997; Li et al. 2004; Pignatti et al. 2004). Added to this wide list of roles are their plant-specific functions in more diverse processes like: cell elongation, somatic and zygotic embryogenesis, root formation, floral initiation and development, fruit development and ripening, pollen tube growth and senescence, and biotic and abiotic stress responses (Slocum and Flores 1991; Mengoli et al. 1992; Minocha et al. 2004b; Fluhr and Mattoo 1996; Del Duca et al. 1997; Bagni and Tassoni 2001; Kaur-Sawhney et al. 2003; Cassol and Mattoo, 2003; Kusano et al. 2007; Mattoo and Handa 2008). Although long known for their pharmacological effects, we are only now beginning to understand their role in plant growth, development and senescence, and responses to abiotic and biotic stresses through molecular genetics and modern biochemical approaches (Mehta et al. 2002; Minocha and Minocha 2005; Yamaguchi et al. 2006, 2007; Kusano et al. 2007; Mattoo and Handa 2008; Moschou et al. 2008).

Genetic manipulation of various steps in the polyamine biosynthetic pathways has been accomplished through both mutagenesis and up- or down-regulation of the key biosynthetic genes. We summarize here the metabolic consequences of engineering the cellular contents of either Put and/or Spd and Spm via expression of key biosynthetic genes, which pinpoint contrasting physiological functions for diamine Put versus tri- and tetra-amines (Spd and Spm) in plants. We have largely drawn from studies conducted with in vitro-grown transgenic cells of poplar (*Populus nigra* × *maximowiczii*) transformed to constitutively express a mouse ODC (*mODC*) gene under the control of CaMV 35S constitutive promoter, and the transgenic tomato (*Solanum lycopersicum*) transformed with a yeast S-adenosylmethionine decarboxylase (ySAMDC) gene regulated by a fruit ripening-specific promoter.

Biosynthesis of polyamines and its regulation

Put is the smallest and the most ubiquitous biological amine, and is synthesized in plants from either ornithine (Orn) by Orn decarboxylase (ODC) or arginine (Arg) by Arg decarboxylase (ADC) (Fig. 1). Other than its terminal conversion into phenolic derivatives in highly differentiated tissues or its conversion into alkaloids in some plants, Put is a primary substrate for Spd and Spm biosynthesis (Slocum and Flores 1991; Cohen 1998). The cellular Put content in different plant tissues widely fluctuates during normal development and in response to environmental perturbations. Spd and Spm are synthesized from Put and decarboxylated SAM (dcSAM) by Spd and Spm synthases, respectively; dcSAM is produced from SAM by SAMDC. The key precursor SAM (AdoMet) is a molecule central to 1C metabolism and, in plants, also for ethylene biosynthesis (Fluhr and Mattoo 1996; Cohen 1998). A new development in the polyamine biosynthesis arena is the elucidation of an alternative pathway in bacteria such as *Vibrio cholerae*, which uses aspartate β-semialdehyde as a source of aminopropyl group to produce *sym*-norSpd (Lee et al. 2009a, b). Diaminopropane, another substrate formed from aspartate β-semialdehyde, and Put together are used for a reaction that produces Spd.

Put content has been the target of successful genetic manipulation in numerous studies, and as much as 10 to 20-fold increases have been achieved (DeScenzo and Minocha 1993; Bastola and Minocha 1995; Capell et al. 1998; Bhatnagar et al. 2001). Often increased Put accumulation is accompanied by a concomitant increase in its catabolism or secretion with only small amounts being converted to Spd (Bhatnagar et al. 2002). Its catabolic breakdown recycles both the N and the C moieties of Put via diamine oxidase (DAO) and the γ-aminobutyric acid (GABA) shunt. The homeostatic regulation of Spd and Spm in plants is more effective than of Put, whose concentrations in plants (as well as animals) may fluctuate more widely as compared to the two higher polyamines, in particular under conditions of environmental extremes. Several mechanisms are employed to achieve homeostasis of intracellular polyamine levels including: (1) transcriptional and translational regulation of enzymes ADC, ODC and SAMDC, all of which turnover rapidly; (2) regulation of catabolism by amine oxidases DAO and PAO; (3) conjugation (mostly Put) with phenolics; and (4) sequestration by transport to vacuoles or extracellularly.

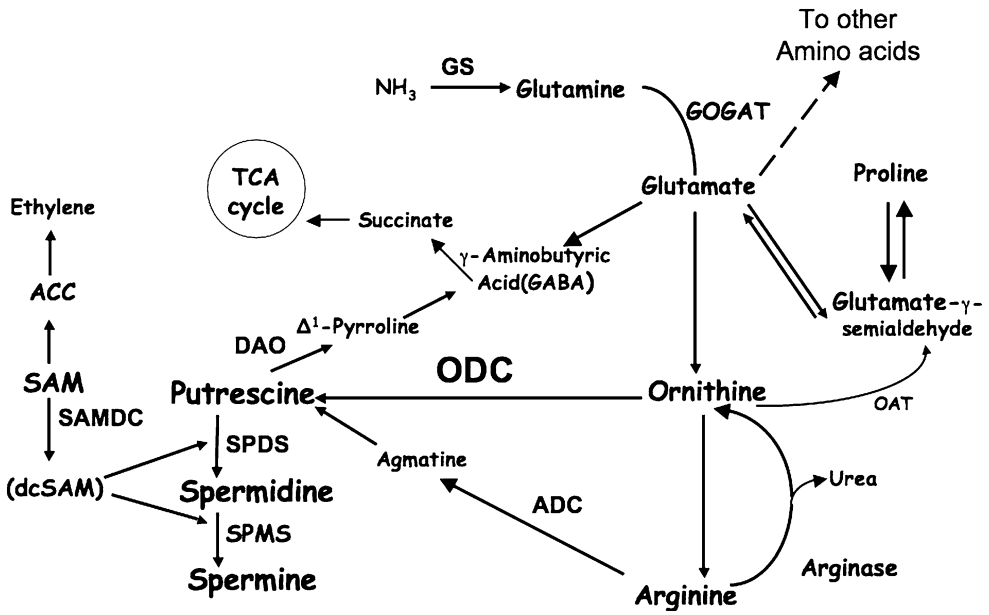

Fig. 1 Biosynthesis pathways showing interconnections between biogenic amines, amino acids, TCA cycle and ethylene

One of the crystallized functions of Spd is in its being a substrate for a two-step posttranslational modification of the eukaryotic translation initiation factor 5A (eIF5A) (Park et al. 1981; Mehta et al. 1991; Chou et al. 2004; Wolff et al. 1997, 2007; Park et al. 2006). Hypusine modification of eIF5A enables the protein to be confined to cytoplasm where it functions in protein synthesis (Lee et al. 2009a, b). The eIF5A has been implicated in apoptosis in several organisms, including plants (Tome and Gerner 1997; Wang et al. 2003; Li et al. 2004; Taylor et al. 2007). Cellular Spd concentration is therefore a determinant for eIF5A function, which involves recruiting and impacting translation of mRNAs (Jao and Chen 2005; Zanelli and Valentini 2007).

Relative to Put and Spd, the cellular Spm content is often small. This may reflect on the necessity of plant cells to maintain Spm below a certain threshold concentration. Interest in function and role of Spm in plants has recently intensified with the genetic evidence that: (1) Spm provides plant tolerance against salinity (Krishnamurthy and Bhagwat 1989; Yamaguchi et al. 2006; Kusano et al. 2007) and drought (Capell et al. 2004; Yamaguchi et al. 2007), (2) Spm is back converted to Spd (Tavladoraki et al. 2006) and thence Spd to Put (Moschou et al. 2008; Kamada-Nobusada et al. 2008). The identity, cellular localization and role of polyamine oxidases responsible for the back conversion pathway of higher polyamines have been summarized in an elegant review (Moschou et al. 2008).

A unique feature of plant polyamine metabolism is that Put, proline (Pro) and GABA are all synthesized from a common substrate, glutamate (Glu), and all these N-rich metabolites often respond similarly to abiotic stresses (Aziz and Larher 1995; Aziz et al. 1998; Houdusse et al. 2005; Simon-Sarkadi et al. 2005, 2006; Sharma and Dietz 2006; Seki et al. 2007). The mechanism of their coordinated accumulation in plant cells is largely unknown. Since they all are derived from Glu, it is possible that a common signal triggers all three sub-pathways in a coordinated manner (Mohapatra et al. 2009b). Glu, along with arginine (Arg), glutamine (Gln) and asparagine (Asn), is not only a substrate and a major organic N-form in plants, it may also be a cellular sensor of N status (Corruzi and Zhou 2001; Glass et al. 2002; Stitt et al. 2002). In this context, Glu, Arg, Gln and Asn, and its associated organic acids respond together to cellular/environmental N status (Urbanczyk and Fernie 2005). Likewise, transgenic tomatoes engineered to accumulate Spd and Spm, at the cost of Put, showed corresponding increases in Glu, Gln, Asn, fumarate and succinate (Mattoo et al. 2006, 2007), while poplar cells transformed with ODC, which produced increased Put, had reduced contents of Glu and Gln (Mohapatra et al. 2009b).

Ornithine is a key intermediate in the biosynthesis of both Put and Arg (Slocum 2005), and perhaps Pro (Coruzzi and Last 2000; Roosens et al. 2002). It is also one of the least abundant amino acids in plants, and a possible candidate that plays a regulatory role in coordinating biosyntheses of these metabolites (Mohapatra et al. 2009b). A likely site of Orn regulation could be the initial reactions that direct the flux of Glu into the three interacting pathways (Fig. 1), i.e., the steps involving N-acetyl-Glu synthase (NAGS; Slocum 2005), Δ1-pyrroline-5-carboxylate synthase (P5CS; Roosens et al. 2002; Székely et al. 2008), and Glu decarboxylase (GAD) (Bouché and Fromm 2004). Arg has been suggested to be a sensor molecule to regulate its own content in animals also (Morris 2007). It is noted that in animals dietary Arg is the starting point for the

biosynthesis of Orn, Pro, Glu and polyamines, a role taken by the N-assimilatory product Glu in plants.

Another key component of the tripartite pathway is GABA, which can be synthesized directly from Glu by GAD and via Put catabolism by DAO, and its cellular concentration is also elevated in response to abiotic stress (Coruzzi and Last 2000). It has been suggested that the cellular Orn concentration may play an important role and regulate not only its own biosynthesis but also that of Arg, Pro and GABA from Glu (Mohapatra et al. 2009b). Thus, polyamines interact with critical processes that affect N assimilation and metabolism as well as stress responses in plants.

Metabolic consequences of manipulating putrescine in poplar cell culture system

Poplar (*Populus nigra* × *maximowiczii*) cell lines constitute rapidly dividing, small filamentous suspensions that are non-regenerating in the sense that they do not produce shoots or somatic embryos. The suspensions are homogeneous in consistency and represent an experimental system that is analogous to microbial and animal cell cultures. Detailed comparisons of isogenic cell lines differing only in one gene are ideal to find out the effects of constitutive over-expression of a gene, in this instance mouse *ODC*. Thus, a transgenic poplar cell line over-expressing a *mODC* gene was obtained and named 'high putrescine' (HP) line (previously described as 2E line by Bhatnagar et al. 2001). It accumulates several-fold higher amounts of Put, and has been extensively characterized (Bhatnagar et al. 2001, 2002; Quan et al. 2002; Minocha et al. 2004a; Page et al. 2007; Mohapatra et al. 2009a, b).

One of the profound effects of *mODC* expression in poplar cell culture was on the steady state cellular concentrations of all amino acids (Fig. 2), presumably a response to up-regulation of Put biosynthesis from Orn in poplar. Notably, in the HP cells, down-regulation was apparent in the cellular contents of acidic amino acids Glu and Asp, basic amino acids Arg, His and Lys, polar amino acids Cys, Gly, Gln, Orn and Ser, and non-polar amino acids Phe, Trp, Leu and Met. However, the contents of non-polar amino acids Ala, Val, Ile and Pro and two polar amino acids, GABA and Thr, showed increased accumulation (Mohapatra et al. 2009b). The most abundant amino acid in both cell lines was Ala ($\alpha + \beta$); the other amino acids that were present in high relative concentrations were Gln, Leu, His and GABA in control cells, and GABA and Thr in the HP cells. Among the least abundant amino acids in both cell lines were Met, Cys and Orn. It is plausible that these changes are a consequence of reduction in Glu availability in response to its increased utilization for Orn production. Thus, the genetic manipulation of Put biosynthesis, which directly affected Orn consumption, caused a major change in the accumulation pattern of many other amino acids.

Tomato model: fruit-specific expression of yeast SAMDC

The tomato (*S. lycopersicum*) fruit 'model' presents a developmental stage independent of cell division, growth and cell expansion slated for the terminal stage in plant development, ripening and senescence. During the ripening process, the levels of Spd and Spm decline in fruits during ripening (Mattoo and Handa 2008). Thus, transgenic tomato lines were developed by transformation with a yeast *SAMDC* gene fused to the ripening-specific promoter E8. These transgenic tomatoes over-accumulate Spd and Spm at the cost of Put (Mehta et al. 2002). This model represents a resource akin to a 'gain-of-function' mutant, and is being used to define Spd–Spm mediated alterations at the transcriptome and the metabolome levels (Mehta et al. 2002; Mattoo et al. 2006, 2007; Srivastava et al. 2007; Neelam

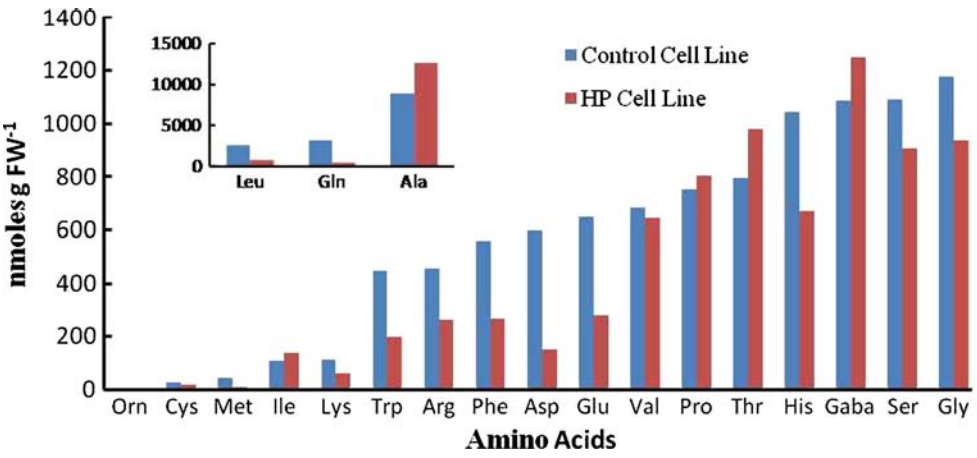

Fig. 2 Differences in the amounts of individual amino acids in the HP and control poplar cells. Combined data of samples from 1 to 7 days of growth ($n = 6$–12) (Mohapatra et al. 2009b)

et al. 2008). Metabolite profiling revealed that higher polyamines influence multiple cellular pathways in tomato fruit during ripening (Mattoo et al. 2006). Prominent changes included: increased levels of choline, Glu, Gln, Asn, citrate, malate and fumarate and decrease in Asp, Thr, Val, glucose and sucrose in the transgenic fruits compared to the wild type and azygous control lines. The cellular contents of Ile, GABA, Phe and fructose were similar in the transgenic and the non-transgenic fruits. Thus, processes in diverse subcellular compartments such as mitochondria, cytoplasm, chloroplasts and chromoplasts seem to be responsive to Spd and Spm accumulation. Also, Spd and Spm accumulation leads to specific metabolic fluxes that result in up-regulating nitrogen (N) and carbon (C) interactions (Mattoo et al. 2006; Neelam et al. 2008). The high Spd–Spm lines showed responses that share common features with responses of plant roots, leaves and trees to exogenous additions of N (Rennenberg et al. 1998; Foyer and Noctor 2002; Bauer et al. 2004). Based on these data, it was proposed that the fruit cells sense Spd and Spm as forms of organic N which then signals corresponding changes in a number of amino acids, notable among which were increases in Glu, Gln and Asn (Mattoo et al. 2006). Bagni et al. (1978) had previously shown that Put is utilized as a sole nitrogen source by tuber explants in vitro. Interestingly, the transgenic tomato lines had phenotypes of agro-economical importance such as the enhanced phytonutrient content and fruit quality (Mehta et al. 2002; Mattoo and Handa 2008). Thus, higher polyamines regulate the developmental processes in tomato fruit.

Are levels of different polyamines tightly regulated in actively dividing and non-dividing cells?

In the previous two sections, we have focused on some of the metabolic changes that occur in both actively growing poplar cell cultures and non-growing, ripening tomato fruit cells in response to, respectively, increases in Put and/or Spd–Spm. Actively growing poplar HP cells exhibited up to a tenfold increase in Put, about 50% increase in Spd and no change in Spm during the growth cycle. Also, the total polyamine (Put + Spd + Spm) content of all the three biogenic amines increased about sixfold in transgenic lines compared to the non-transgenic cell line (Page et al. 2007). The results clearly showed that Put accumulation resulted in a net gain of total polyamines, likely at the expense of Glu and perhaps other amino acids (Mohapatra et al. 2009b). One can speculate that accompanying these changes would include an increased flux to produce Orn and/or Arg to meet the demand created by Put biosynthesis. In contrast, transgenic tomato fruit cells expressing *ySAMDC* showed no major increase in the total amounts of polyamines; these fluctuated between 66 and 110% of the wild type ripening fruits (Mehta et al. 2002). However, dramatic increases in the cellular concentrations of Spd (600% increase) and Spm (900% increase) were obtained (Mehta et al. 2002). These results were the first demonstration that the levels of Spd and Spm are under the control of SAMDC activity.

A possible explanation for the opposite results on total polyamine changes in the two types of tissues (poplar cells versus tomato fruit) may lie in the fact that increased utilization of Orn in the poplar cells can be compensated for by increased production of Orn from Glu, which in turn can be synthesized as needed from additional N assimilation. Indeed, the HP cells accumulated more N (as well as C) than the control cells (Mohapatra et al. 2009b). Thus, a sink for substrates of polyamine biosynthesis can increase the total flux of N and C into polyamines depending upon continued supply of these elements. This is consistent with the demonstration that lysine content in Arabidopsis increased several-fold by manipulating lysine degradation (Zhu and Galili 2003).

Correlation coefficient analysis between the levels of Put, Spd and Spm were also determined. A weak positive correlation between Put and Spd levels and a weak negative correlation between Put and Spm levels were observed in poplar cell culture while a strong positive correlation was seen between Spd and Spm levels in poplar cell culture (Fig. 3). Similar results were obtained in ripening fruit cells as Put levels showed a weak correlation with Spd and Spm, while and Spd and Spm levels had a correlation coefficient of over 0.8 among each other. Collectively, these data indicate that increased Put, while influencing the reactions involved in the biosynthesis of its substrates, has little effect on increasing the levels of its products Spd and Spm, but Spd and Spm levels are inter-dependent. These results further support the argument that SAMDC catalyzes the rate-limiting step in higher polyamine biosynthesis.

Differential effects of Put, Spd and Spm on cellular metabolism

As discussed above, the synthesis of Put, Spd and Spm is intimately linked with metabolism of other amino acids as well as organic acids, which provide their carbon skeleton. Also, it was described as to what alterations these biogenic amines cause in the steady state levels of the soluble amino acids in both model systems. Thus, a multitude of targets were revealed in these analyses. We sought to use these datasets to determine statistically the correlation coefficients between and among these metabolites. The results of this analysis are presented in (Fig. 3).

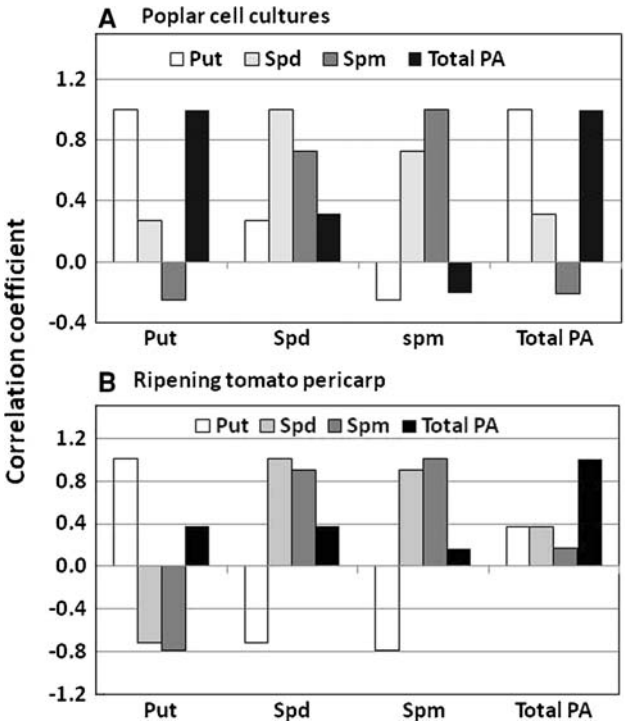

Fig. 3 Correlation between steady state levels of Put, Spd, Spm and total (Put + Spd + Spm) polyamines with each of them in poplar cell cultures (**a**) and ripening tomato pericarp (**b**). Correlation coefficients for each system were determined by pooling data from both wild-type and transgenic genotypes. Polyamine levels for tomato pericarp were from Mehta et al. (2002) and the poplar cell cultures from Page et al. (2007)

In actively growing poplar cell culture, Ala showed a strong positive correlation with Put, intermediate correlation with Spd, and a weaker but positive correlation with Spm. The other amino acids that showed weak positive correlation with Put were Thr, Pro, Ile and GABA. All other amino acids examined in the poplar cell cultures showed negative correlation with Put levels. Most amino acids except Asp and Ile showed positive correlation with Spd in the poplar cells. Strong positive correlation was observed for a majority of amino acids (11 out of 20) with intracellular steady state Spm levels; only Orn showed a negative to neutral correlation with Spm.

As compared to poplar cell culture analysis, only limited metabolite profiles of amino acids have been analyzed in transgenic and non-transgenic tomato lines. However, the datasets were still sufficient to obtain correlation coefficient values (Fig. 3). In the ripening fruits, Put exhibited positive correlation with Ala, Phe, Val, Ile and GABA and negative correlation with Gln, Thr and Glu. Spd showed positive correlation with Gln, Glu and Asp and negative correlation with Ala, Phe, Thr, Val, Ile and GABA. Spm demonstrated associations similar to that observed with Spd, exhibiting positive correlations with Gln, Glu and Asp and negative correlations with Ile, Val, Thr, Ala, Gln, Phe, and GABA (Fig. 3).

Comparison of the effects of transgenic alteration of Put, Spd and Spm levels on the steady state levels of amino acids in actively growing poplar cell cultures and non-dividing fruit cell revealed several interesting features. First, Put seems to act as a negative regulator whereas Spd and Spm as positive regulators of accumulation of many amino acids in both systems. Although the effects of altering polyamine levels differ between the two systems, some similarities are apparent. For example, Put negatively regulates Glu levels in both systems. Further, Put exhibited negative correlations with most amino acid levels in the cell cultures but was positively correlated with several amino acids in the ripening tomato pericarp tissue. Steady state levels of most amino acids were positively correlated with Spd and Spm in actively growing cell cultures but not necessarily in the case of tomato pericarp. In fruit tissue, the levels of Ala, Phe, Thr, Val, Ile, and GABA were negatively associated with Spd and Spm levels (Fig. 4).

Put was negatively correlated with the methyl donor choline and three unidentified compounds A, B and C, whereas they exhibited positive correlation with Spd and Spm levels. Sugars, glucose, fructose, and sucrose exhibited positive correlation with Put levels but were negatively associated with Spd and Spm. The organic acids evaluated in this study showed mixed patterns. Whereas fumarate and malate were negatively correlated with Put and Spd, citrate showed negative correlation with Put but positive with Spd and Spm (Fig. 3). We interpret these data to suggest that non-dividing tomato pericarp metabolism is contrastingly associated with the internal levels of diamine and tri and tetra-amine.

The two systems discussed here differ in a number of parameters and the association analysis therefore should be interpreted with caution. The two systems represent different alterations in metabolism: m*ODC* is expressed in poplar and y*SAMDC* in the tomato fruit. Additionally, the former is an actively growing cell system whereas the latter is at the terminal step of development. Finally, the two systems represent very different genotypes. It is tempting to speculate that Put acts as a nitrogenous source and its levels increase under conditions of low metabolic activity such as environmental induced stresses. As the growth inhibition is reduced or eliminated its levels go down as observed by the negative correlation with various amino acids. Spd and Spm, on the other hand, act as growth simulators either by providing environments to maintain the active configurations of macromolecules or by stabilizing the homeostasis by maintaining integrity of cell membranes. Elevated levels of these polyamines help maintain cellular vitality as observed in longer vine life of ripening tomato. The different roles of Put versus Spd and

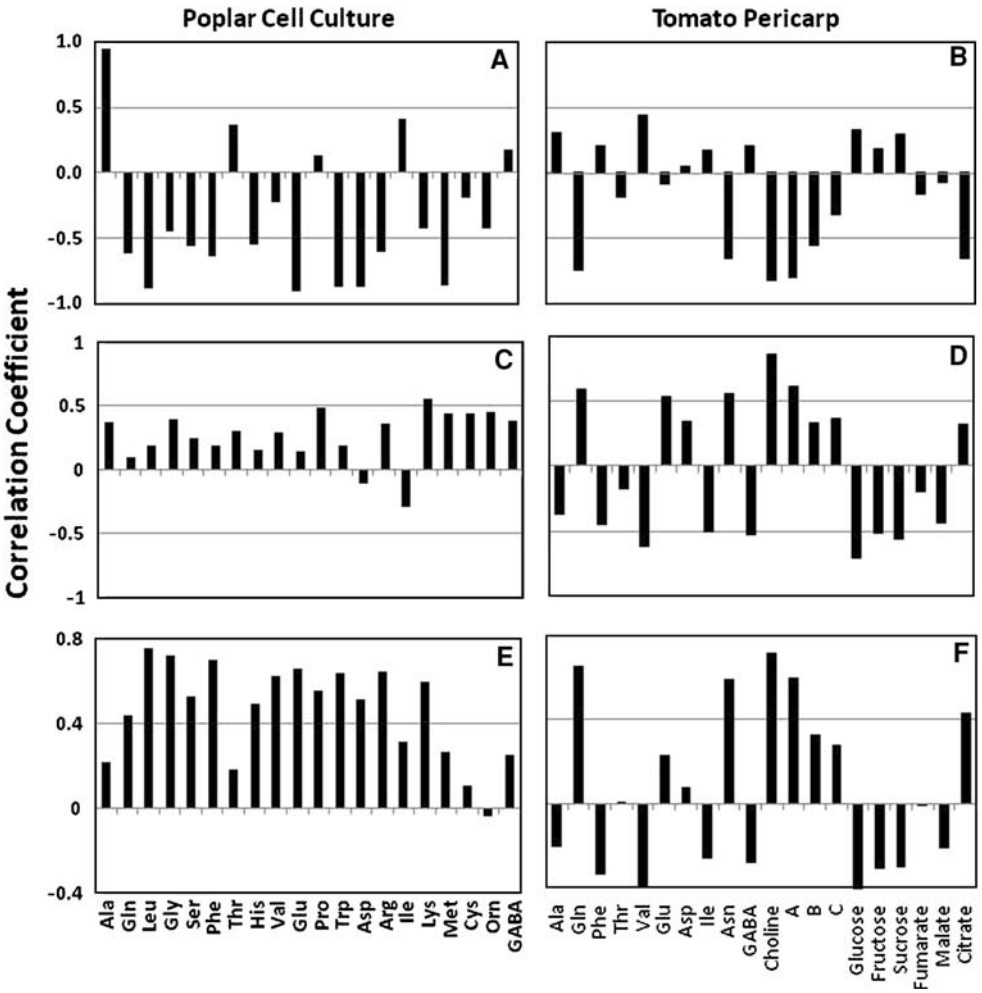

Fig. 4 Correlation between steady state levels of Put (**a**, **b**), Spd (**c**, **d**) and Spm (**e**, **f**) with the indicated metabolites. Datasets used for the analysis were taken from previously published papers (Mehta et al. 2002; Mattoo et al. 2006; Page et al. 2007; Mohapatra et al. 2009b)

Spm were illustrated several years ago in light of salt tolerance of rice cultivars (Krishnamurthy and Bhagwat 1989) and recently in relation to their effects on the function of photosynthetic membranes (Ioannidis and Kotzabasis 2007). The themes drawn in this review exemplify the importance of looking at the role(s) of diamine Put and polyamines Spd and Spm not just as nitrogenous compounds but also as signaling molecules with specific biological functions.

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