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Review

Higher polyamines restore and enhance metabolic memory in ripening fruit

Autar K. Mattoo [a,*], Avtar K. Handa [b]

[a] *Sustainable Agricultural Systems Laboratory, The Henry A. Wallace Beltsville Agricultural Research Center, Building 001, USDA-ARS, Beltsville, MD 20705-2350, USA*
[b] *Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN 47907, USA*

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Abstract

Polyamines are ubiquitous, biogenic amines that have been implicated in diverse cellular functions in most living organisms. Ever since spermine phosphate crystals were isolated over three centuries ago, scientists have kept busy in unraveling the mystery behind biological roles of spermine and other known polyamines, viz., putrescine and spermidine. Although the pathway of polyamine biosynthesis has been elucidated, the molecular basis of their *in vivo* function is far from being understood. Molecular biology tools have provided a promising avenue in this direction, with success achieved in altering endogenous polyamines in plants by over-expression and knock-out of the genes responsible for polyamine biosynthesis. Such transgenic material has become a good genetic resource to learn about the biological effects of polyamines and their interaction with other signaling molecules. Interestingly, engineered accumulation of higher polyamines, spermidine and spermine, in tomato in a fruit-specific manner restored metabolic activity even at late developmental stages of fruit ripening, reviving cellular programs underlying N:C signaling, energy and glucose metabolism. Along with these, a wide array of genes regulating transcription, translation, signal transduction, chaperone activity, stress proteins, amino acid biosynthesis, ethylene biosynthesis and action, polyamine biosynthesis, isoprenoid pathway and flavonoid biosynthesis was activated. Based on various reports and our results, we suggest that polyamines act as 'surrogate messengers' and nudge other signaling molecules, such as plant hormones and NO, to activate a vast genetic network to regulate growth, development and senescence.
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Keywords: Spermidine; Spermine; Transcriptome; Metabolome; Fruit ripening; Nitrogen:carbon signaling

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1. Introduction

Polyamines are ubiquitous, aliphatic polycation class of biogenic amines with essential functions in living organisms. The most common plant polyamines are the diamine putrescine and the higher polyamines spermidine and spermine. Anton Leeuwenhoek was the first to observe crystals of spermine phosphate in human semen as early as 1678, predating the discoveries of the defense signaling molecule nitric oxide (1772) and plant hormone ethylene (1794) (see [1] for historical time line). Putrescine was identified more than two centuries later while spermidine was discovered only in 1927. Spermine and spermidine were early examples of trimethylenediamine

* Corresponding author. Tel.: +1 301 504 6622; fax: +1 301 504 6491.
E-mail addresses: autar.mattoo@ars.usda.gov (A.K. Mattoo), ahanda@purdue.edu (A.K. Handa).

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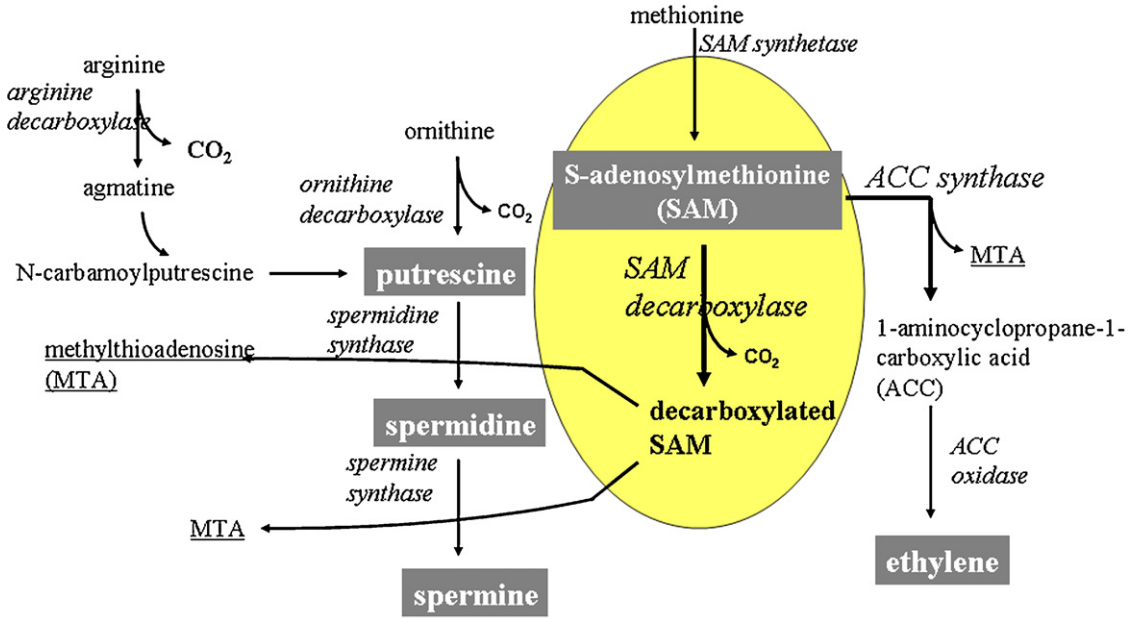

Fig. 1. The biosynthetic pathways of polyamines and ethylene in plants. Shown are precursors, intermediates and enzymes (in italics) involved. S-adenosylmethionine (SAM), formed from methionine by SAM synthetase, is decarboxylated by SAM decarboxylase. Decarboxylated SAM provides aminopropyl groups that are used by spermidine synthase and spermine synthase to synthesize, respectively, spermidine from putrescine and spermine from spermidine, and a common product methylthioadenosine (MTA; underlined). Putrescine is formed from arginine and/or ornithine by arginine decarboxylase and ornithine decarboxylase, respectively. SAM is also a substrate for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, forming ACC and MTA. ACC is then converted to ethylene by ACC oxidase. Both these pathways, therefore, use SAM as a substrate and MTA as a product. MTA is readily metabolized and recycled to methionine.

derivatives among natural products. We are only now beginning to understand their role in growth, development and senescence through molecular genetics and modern biochemical approaches. Polyamines have been implicated in a myriad of biological processes including cell proliferation, cell division and differentiation, apoptosis, homeostasis, gene expression, protein and DNA synthesis [1–5]. The list gets longer when processes implicated in plants are considered: cell division, cell elongation, embryogenesis, root formation, floral initiation and development, fruit development and ripening, pollen tube growth and senescence, and in response to biotic and abiotic stress [1–4]. Interest in polyamine research has further intensified because of their (and polyamine analogs') potential involvement and implications in such diverse disciplines as oncology [6], obesity [7], gastroenterology [8], cerebral stroke and other disorders [9], parasitology [10], oxidative stress [9,11,12], apoptosis (programmed cell death) [8,13], and plant developmental processes [2–4,12,14]. Applications in pharmacology and medicine, including cancer therapy and as anticancer agents [6,11,15] are exciting new avenues for polyamine research. Likewise, the elucidation of the roles polyamines play in modulating pre- and postharvest biology will contribute to the development of functional foods using modern biotechnology [16,17].

The biosynthesis pathway and genes encoding the enzymes catalyzing these pathway reactions have been elucidated and identified in both prokaryotes and eukaryotes, as reviewed in [1–4,10,18]. In addition to putrescine, spermidine and spermine, flowering plants also synthesize cadaverine, 1,3-diaminopropane, and other modified forms [1]. An isomer of spermine, thermospermine, has been found widespread in bacteria and higher plants [19]. In plants, putrescine is formed from either arginine via an intermediate agmatine, a reaction catalyzed by arginine decarboxylase, or from ornithine by ornithine decarboxylase. Spermidine is synthesized from putrescine and the aminopropyl group donated by decarboxylated S-adenosylmethionine (SAM), which is a product of SAM decarboxylation (Fig. 1). In turn, spermidine incorporates another aminopropyl group (from decarboxylated SAM) to form spermine. SAM is a key intermediate for another plant growth regulator that controls ripening of fruits, ethylene [20–22]. Ethylene is synthesized from SAM by a sequential action of two ethylene-biosynthesis enzymes, 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase (Fig. 1). It would appear that a living cell has the potential to commit the flux of SAM either into polyamine biosynthesis, ethylene biosynthesis, or both. Our foray into polyamine research was catalyzed by studies, first published in early 1980s, which showed that polyamines inhibit ethylene biosynthesis in a variety of fruit and vegetative tissues [23–26]. These studies brought to light a possible temporal relationship between polyamines and ethylene during plant development, which led to suggestions that changes in the levels of polyamines and ethylene may influence specific physiological processes in plants [1–4]. One of these papers, published in Plant Science Letters in 1982 [24], reported that Ca^{2+} and spermine inhibited ethylene biosynthesis in a temperature-dependent manner which was related to the prevention of decreased membrane microviscosity associated with ripening and senescence. A quarter century later, down regulation of spermine in a mutant of *Arabidopsis* was found to cause hypersensitivity to NaCl possibly via Ca^{2+}-homeostasis impairment [27]. Salt tolerance

in plants had previously been shown to correlate with sustained endogenous levels of spermidine and spermine in salt-tolerant rice varieties [28].

Polyamine catabolism mediated by amine oxidases has attracted considerable attention largely because polyamine catabolism generates a secondary messenger and a signaling molecule H_2O_2 as one of the products, with a potential to provide defense against biotic and abiotic stresses [12,16]. In spite of significant progress made in understanding aspects of polyamine metabolism and transport, we know little about the *in vivo* role(s) of polyamines in cellular metabolism [1–5,12,17].

Several studies have succeeded in altering endogenous polyamines in plants by over-expression and knock-out of genes of polyamine biosynthesis, in an effort to gain insight into the role of polyamines in growth and development [29–31]. Such studies on transgenics should provide new knowledge towards our understanding of how polyamine biosynthesis is controlled and the processes that polyamines regulate. Thus, molecular genetics approaches have led to confirmation of previous reports on the role of polyamines in abiotic stresses including drought and salinity, and provided possible mechanisms [12,18,32].

It is desirable to develop transgenic plants or cell lines using regulatable promoters such that the transgene expression can be spatially or temporally controlled in a tissue-specific manner. Plants have two pathways to synthesize the diamine putrescine, which adds complexity: the arginine decarboxylase pathway that uses arginine as a substrate, and the ornithine decarboxylase pathway that uses ornithine. The fact that arginine and ornithine are inter-convertible adds even more complexity (Fig. 1; [1–4]). Here, we focus on our recent data on the processes regulated by higher polyamines, spermidine and spermine, in tomato fruit. We chose to transform tomato plants in a fruit-specific manner, introducing yeast S-adenosylmethionine (SAM) decarboxylase gene fused to a ripening-specific E8 promoter [31]. The advantage of using a fruit lies in the fact that mature fruit has arrived at a developmental stage where it can only ripen, independent of cell division, growth and cell expansion. The levels of spermidine and spermine are on the decline during ripening of pear [33], avocado [34] and tomato [31] fruits. Therefore, tomato fruits over-accumulating spermidine and spermine at the cost of putrescine during ripening are analogous to a 'gain-of-function' mutant, providing a model system to define alterations at the transcriptome level and to ascertain the effects of higher polyamines on the metabolome [31,35–37]. Importantly, harvested mature green fruit of these transgenics can be ripened off the plant, thus allowing evaluation of the effects of high levels of polyamines in the absence of any perturbation from the parent plant. The information thus generated has enabled a synthesis of the information on the coordination of higher polyamine-mediated processes in a highly regulated fashion, which eventually impacts nutritional attributes and stress response of the tomato fruit. Profiling of metabolites and engineering their pathways are tools to assist understanding how plants regulate cellular processes and to reveal intracellular networks [35,38–40].

2. Higher polyamine-mediated N:C signaling is sustained till late in ripening fruit

Metabolite profiling analysis of fruit from transgenic tomato lines transformed with yeast SAM decarboxylase, carried out using high-resolution NMR spectroscopy, revealed that these higher polyamines influence multiple cellular pathways in tomato fruit during ripening [35]. These transgenic tomatoes accumulate spermidine and spermine in the fruit during ripening compared to the wild type and azygous controls [31]. Prominent changes were found: levels of choline, Glu, Gln, Asn, citrate, malate and fumarate increased and those of Asp, Thr, Val, glucose and sucrose decreased in the transgenics compared to the wild type and azygous control lines [35]. The levels of Ileu, GABA, Phe and fructose were similar in the transgenic and non-transgenic fruits. The metabolic pathways linking the polyamine-regulated, identified metabolites are illustrated in Fig. 2. Thus, spermidine and spermine accumulation impacts processes in diverse subcellular compartments such as mitochondria, cytoplasm, chloroplasts and chromoplasts.

What does the metabolite profiling analysis tell us? Spermidine and spermine accumulation leads to specific metabolic fluxes that result in emphasizing nitrogen (N) and carbon (C) metabolism. Leaf amino acids and organic acids, which included Glu, Gln, and citramalate, change in tomatoes in response to N status [41]. Glu, Gln and Asn are the major forms of N in plant leaves and have been suggested to be sensors of nitrogen status [42–44]. The ratio of asparagine to glutamine has been predicted as a sensor of N-status in maize, involving aspartate aminotransferase, Gln synthetase and Asn synthetase as indicators of plant N status during maize kernel development [45]. The changed metabolic flux in the transgenic fruit transformed with yeast SAM decarboxylase, which accumulate spermidine and spermine, is similar (but not identical) to leaves of plants grown on increasing N concentrations as indicated above. We suggest that tomato fruit may sense spermidine and spermine as N-forms and correspondingly signal increases in the other N forms such as Glu, Gln and Asn. The fruit sensing and signaling responses to higher polyamines have common features with responses of plant roots, leaves and trees to exogenous additions of N [46–48]. These metabolic shifts were observed postharvest without any interaction with the rest of the parent [35,37]. Thus, a reproductive organ, such as the tomato fruit contains and maintains an organic-N (spermidine and spermine) sensing and signaling machinery late into ripening.

In leaves, C and N assimilation seems coordinated by complex signaling networks that are initiated by metabolites including nitrate, sugars, glutamine, 2-oxoglutarate and malate [42,46]. We hypothesized that if the spermidine and spermine accumulating fruit follows similar N regulatory aspects as in roots or leaves [42,46], it should cause a coordinated signaling of the carbon metabolism to optimize C and N budgets. The lower levels of sucrose and glucose, higher levels of citrate and fumarate, and increased respiratory activity in the red ripe transgenic fruit, suggest a more active metabolic status of these

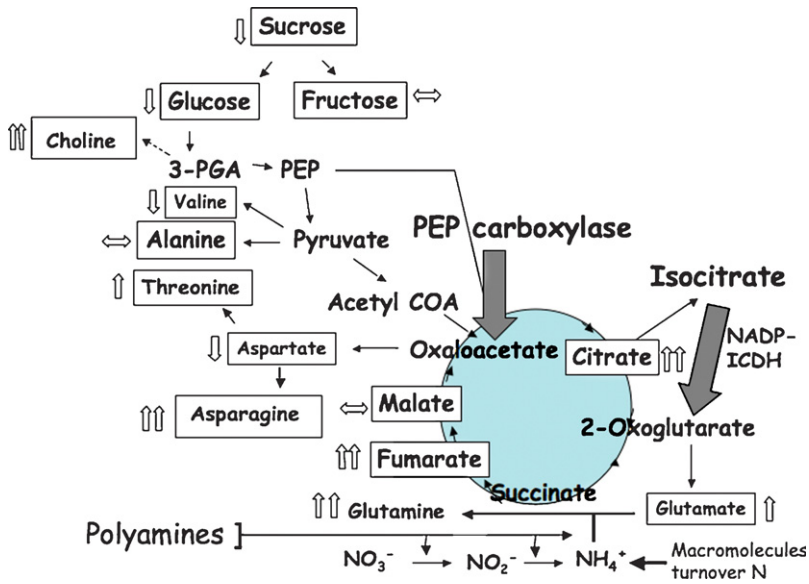

Fig. 2. Inter-related metabolic pathways that lead to the steady accumulation of nutrients in spermidine and spermine accumulating tomato fruit during ripening and involve N:C signaling. The large arrows pinpoint the target reactions catalyzed by PEP carboxylase and cytosolic NADP-dependent isocitrate dehydrogenase whose transcripts are upregulated in spermidine and spermine fruit. Metabolites identified and quantified by NMR spectroscopy [35] are boxed. Open arrows represent high (one upward arrow), higher (two upward arrows), lower (single downward arrow), or no change (one horizontal arrow) in the concentration of the metabolite between the transgenic, high polyamine fruit as compared to the wild type and/or azygous control fruit.

fruits [35]. In the absence of nutrient supply from other parts of the plant, the detached fruits from yeast SAM decarboxylase transgenic plants signal activated sugar metabolism and the Krebs cycle yielding more fumarate and citrate, which can generate more 2-oxoglutarate, which in turn becomes a substrate to produce glutamate family of amino acids. Organic acid metabolism and respiration is highly dynamic and dependent on the kind of fruit [49,50]. The higher respiration found in the spermidine and spermine accumulating tomato fruit reveals an *in vivo* role of polyamines in mitochondrial metabolism, as previously shown for spermine effects on rat liver mitochondria [51].

Further support for a role of spermidine and spermine in controlling N:C interactions is apparent from upregulation of gene transcripts of phospho*enol*pyruvate (PEP) carboxylase and cytosolic NADP-dependent isocitrate dehydrogenase in spermidine and spermine accumulating fruits, both of which link carbon metabolism with N sensing in leaves [46,52,53]. These findings are consistent with results obtained in transgenic potato plants over-expressing PEP carboxylase: the flux of soluble sugars and starch was directed to organic acids such as malate and amino acids glutamate and glutamine [54]. Thus, the sensing-signaling mechanism and gene players involved in N assimilation and carbon metabolism in different organs of a plant seem conserved—likely involving metabolic memory and being the reason behind the totipotent nature of plant cells, and spermidine and spermine may mediate these. The fruit cells can thus be activated by spermidine and spermine to revive the developmental program involving metabolic memory linked to N:C interactions. Metabolic memory is used here in relation to the developmental history of the organ, the fruit cells normally originate when leaf cells get committed and differentiate. Our interpretations of the results discussed above are consistent with the suggestion of a possible role for spermidine and spermine in down-regulating the pathway for assimilating nitrate-derived N [55,56], supporting the idea that polyamines may act as signals of nitrogen-replete conditions. One way of achieving this regulation is by stimulating specific protein–protein interactions [55–57]. A highly conserved class of eukaryotic proteins, 14-3-3, binds to other (protein) targets regulating processes of cell division, cell cycle, differentiation and apoptosis [58,59]. In one such interaction, 14-3-3 protein binds to N responsive NADH:nitrate reductase in a Mg^{2+} dependent manner, leading to repression of nitrate reductase [55]. Spermidine and spermine at physiological concentrations were found to substitute for Mg^{2+} in stimulating this binding [55,56], and the authors attributed it to the polycationic nature of these polyamines. In another instance where 14-3-3 protein was shown to interact with proton ATPase, spermine was found to have a stimulatory effect, which was, however, independent of Mg^{2+} [57]. Spermine effect was stronger and different than that by Mg^{2+} since it was found that spermine, and not Mg^{2+}, induced interaction of 14-3-3 protein with an unphosphorylated target [57], suggesting that polyamines impact cellular processes also by other mechanisms, independent of their polycationic nature.

Another linkage unraveled by metabolite profiling analysis of the polyamine accumulating transgenic tomato fruit is the accumulation of choline. Choline is a ‘vital amine’ for human health and an essential micronutrient required for brain development [60,61]. Choline is a precursor of membrane phospholipids, intracellular messengers diacylglycerol and ceramide, signaling lipids platelet-activating factor and sphingosylphosphorylcholine, and neurotransmitter acetylcho-

line [60,62]. It is also a precursor of methyl donor betaine and an osmoprotectant glycine betaine [63,64]. We speculate that spermidine and spermine increase the metabolic flux towards choline that would normally metabolize to glycine betaine except in tomato where this reaction was not detected [65]. Glycine betaine is known to confer tolerance to environmental stresses such as salinity and drought in plants [63,66]. The activation of SAM decarboxylase in transgenic rice resulted in higher levels of spermidine and spermine and the plants were drought tolerant [32], while a spermine mutant of *Arabidopsis* was found to be salt sensitive [18,27]. Thus, we gauge that in addition to their role in stabilizing membranes some of the polyamine-regulated stress responses may occur via an effect on the biosynthesis of choline and its conversion to osmoprotectants such as glycine betaine [64].

These developments in polyamine research are very recent. It remains to be determined if changes in metabolism as discussed here and revealed by metabolic profiling analysis of the SAM decarboxylase transformed tomato fruit [31,35] also occur in other plants similarly transformed including high spermidine and spermine accumulating transgenic rice [32] and negated in the *Arabidopsis* polyamine mutants [18,27]. It will also be of interest to ascertain the metabolic profiles and transcriptome of slow ripening and long-keeping fruits of tomato landrace having the recessive allele *alc*, Alcobaca [67], and Liberty cultivar of tomato [68] both of which accumulate different polyamines during fruit ripening as compared to their wild types.

3. Polyamine-mediated revival of metabolic memory occurs in concert with activation of anabolism-related genes

How do plant cells sense threshold levels of polyamines and what downstream signaling pathways are involved is a matter of conjecture at this stage. While the sensing machinery may be plant cell-specific, not a single but several different signaling pathways are probably invoked. Our analysis suggests that the processes regulated by higher threshold levels of the diamine putrescine are different from the ones regulated by higher polyamines, spermidine and spermine. This is apparent from the type of major changes in gene expression that occur in response to putrescine accumulation [69,70] versus those that occur in response to higher spermidine and spermine levels [36,37,71]. Putrescine may downregulate biosynthesis of gibberellins (GAs), thereby impacting GA signaling, and cause dwarfism and late flowering [69] or be oxidized by amine oxidases to produce a signaling molecule, H_2O_2, and induce defense responses to environmental stresses and programmed cell death [12,72]. Conversely, spermidine and spermine may prime organogenesis possibly via interactions with other plant hormones such as auxins, cytokinins and ethylene [73]. Would this mean that polyamines are 'surrogate messengers' and nudge other plant hormones to act? If so, it is clear that transcription/translation of a number of genes is correlated with spermidine and spermine-mediated changes in the metabolite profiles, suggesting that activation and sustenance of transcription is one means by which spermidine and spermine regulate cellular metabolism. An interesting cross talk between spermidine (and spermine) and nitric oxide (NO) biosynthesis has also been unearthed [74,75].

Transcriptome analysis of tomato fruit accumulating spermidine and spermine in a ripening-specific manner [31], performed with a custom array containing 1067 unique fruit cDNAs, showed that about one-quarter of these genes were differentially regulated between the transgenic and non-transgenic control. Genes thus identified represent functional categories including transcription, translation, signal transduction, chaperone family, stress related, amino acid biosynthesis, ethylene biosynthesis and action, polyamine biosynthesis, isoprenoid pathway, and flavonoid biosynthesis [36,37]. The differentially up-regulated genes were twice as abundant as down-regulated genes in the high spermidine and spermine accumulating tomato fruit. Nearly half of the differentially expressed cDNAs represented genes as yet not classified for purported functional attributes. The changes in transcripts for a few selected genes were validated by mRNA expression analysis by northerns. Transcript accumulation correlated with the accumulation of protein products in most instances [37]. The nature of the genes identified and their function suggests that spermidine and spermine act as growth regulators of anabolic processes.

The polyamine-mediated circuitry that regulates transgene-activated N:C signaling responses remain to be determined but it can be surmised that distinct metabolic pathways are targets of polyamine action. The huge number of processes potentially influenced by one or all of the polyamines is summarized in Fig. 3. It illustrates the diversity of processes from cell division, to differentiation, to development, in addition to the responses to abiotic and biotic stresses that have been described in the literature as being modulated by polyamines. Various mechanisms that have been invoked to explain the effects of polyamines are also shown, from chromatin remodeling to metabolic buffering to gene expression to hormone-like regulation. Based on literature, some of these are inter-related and have cross talk with one another, as shown by double arrow lines, regulating a vast network by which polyamines control plant growth, development and senescence.

4. Consequences to fruit biotechnology

Tomato is used as a model crop whose genome sequencing is anticipated to be completed by 2008–2009. Tomato transformation has become a routine and therefore is used to address important questions in functional genetics related to crop quality improvement [76]. Tomato fruit and its processed products are the dietary source of antioxidants, vitamins and minerals. It has become a fruit/vegetable of choice to improve nutritional quality including carotenoid levels and other quality antioxidants via genetic manipulation [77]. Fruit ripening is a senescence program set into motion by the plant hormone ethylene [20–22]. It is thought of as a plant developmental stage at a point of no return because of enhanced degradation of cellular and sub-cellular processes.

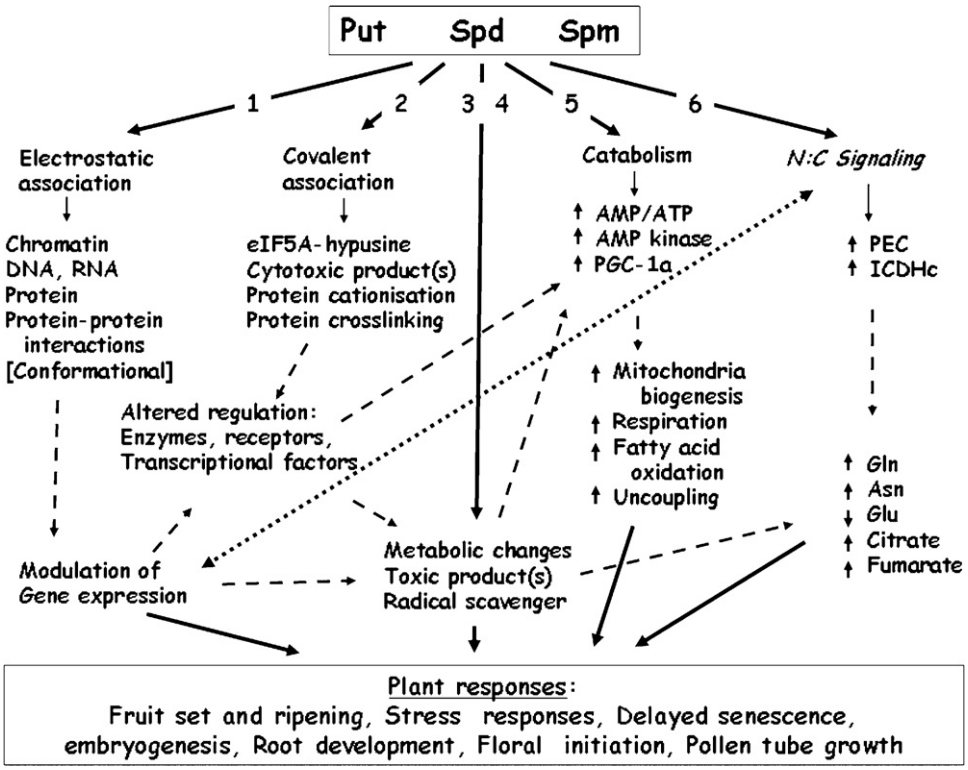

Fig. 3. Multiple routes that polyamines use to regulate physiological functions in living cells. Various interactive modes of polyamines with other cellular components are shown which result in various plants phenotypes: (1) Conformational changes resulting from association of polyamines with macromolecular components, such as chromatin, DNA, RNA and proteins, can induce massive shift in gene expression in plants and other organisms [36,37,69,71,80], some involving polyamine stimulation of protein-protein interactions [55–57]; (2) Covalent binding by enzyme-catalyzed reactions allow protein cationisation and crosslinking, synthesis of hypusine (a cofactor of eIF-5A) and cytotoxic, lipophilic polyamine derivatives (see [80]); (3) Scavenging of free radicals affects redox balance [81–83]; (4) Apoptogenic effects of cytotoxic aldehydes and reactive oxygen species produced after oxidative deaminations of polyamines, with the toxicity of the products increasing in the sequence putrescine < spermidine < spermine [84,85]; (5) Evidence for the role of polyamines in the regulation of energy and glucose metabolism is based on work carried out with transgenic mice overexpressing spermidine/spermine N(1)-acetyltransferase (SSAT) [86] and increased respiration in transgenic tomatoes overexpressing SAM decarboxylase [35]. PGC-1a, peroxisome proliferator-activated receptor coactivator 1a; AMPK, AMP kinase; (6) Role of polyamines in nitrogen:carbon (N:C) signaling is based on metabolic profiling of transgenic tomatoes expressing SAM decarboxylase and upregulation of PEPC (phospho*enol*pyruvate carboxylase) and ICDHc (cytosolic NADP-dependent isocitrate dehydrogenase) [35,37]. Solid arrows emanating from top emphasize the major modes and candidates involved in polyamine action; the dashed arrows indicate the interactive modes of regulation and possible cross talks within modes linking the indicated processes; the dotted arrow is the information generated by metabolic profiling [35] and gene expression [36,37] of higher polyamine accumulating tomato fruit; the solid arrows at the bottom of the figure point to the plant responses invoked by one or more polyamines.

However, it is clear from the discussion above that a ripening fruit can retain its metabolic memory and signaling cascades till late into ripening (see also [37]). This is consistent with recent developments showing intricate regulation and interconnected networks that function to ensure ripening of fruits [20,21,78,79]. The active response of tomato fruit to engineered high levels of polyamines predicates that it will be possible to modulate ripening and influence nutrient levels of the fruit by rational design of genes with precision-based and ripening stage-specific promoters.

Acknowledgments

We dedicate this article to all the past, present and future researchers who have contributed or will contribute in the future to define the function of polyamines in living cells, particularly plants. We would like also to acknowledge the contributions of Prof. Arthur Galston and his laboratory to the field of polyamine biology in plants. Finally, we thank our students, postdoctoral fellows, visiting scientists and collaborators for their contributions to our research programs.

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