Molecular analysis of polyamine biosynthesis in higher plants

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The polyamines are low molecular weight, polycationic, nitrogenous compounds, and normal constituents of prokaryotic and eukaryotic cells. They have been implicated in a wide range of metabolic processes in plants ranging from cell division and organogenesis to protection against stress. Genes for several key biosynthetic enzymes like arginine decarboxylase, ornithine decarboxylase and S-adenosyl methionine decarboxylase have been cloned from different plant species. They have been found to be regulated by developmental cues and also by different kinds of stress. Both over expressed and antisense transgenic approaches of polyamine biosynthetic genes have further supported that these compounds are essential for normal plant growth and development. Recent generation of polyamine biosynthetic mutants of *Arabidopsis* also suggests the importance of polyamines as cellular metabolites. The current development in the knowledge of polyamine biosynthesis, particularly molecular genetic analysis, has been discussed in this review that will help to open up new avenues of investigation for the researchers.

POLYAMINES (PAs) (putrescine, spermidine and spermine), a class of aliphatic amines are ubiquitous, non-protein, straight chain, polycationic metabolites present in both prokaryotic and eukaryotic cells. They have been implicated in several important cellular processes like replication, cell division, protein synthesis[1], protein–DNA interactions[2], and plant responses to abiotic stress[3–8]. Polyamines can form conjugates that can account for 90% of the total PA[9]. PAs have been localized in various organelles including the vacuole, mitochondria, nuclei and chloroplast[10]. In the latter they have been detected from thylakoid membranes associated with PS-II and light-harvesting complex in spinach[11]. The apoproteins of the chlorophyll *a/b* antena complex and RuBISCO (ribulose-bis-phosphate carboxylase) large subunit have been identified as substrates for a plastidic transglutaminase, which catalyses the incorporation of PAs into both thylakoid membrane and stromal proteins in *Helianthus tuberosus*[12]. The biosynthesis and regulation of PA level in animal cells are well characterized and are controlled primarily by two enzymes: ornithine decarboxylase (ODC), responsible for the direct production of putrescine (Put), and S-adenosyl-l-methionine decarboxylase (SAMDC), for the synthesis of spermidine (Spd) and spermine (Spm)[13]. The activities of Spd-synthase and Spm-synthase are rather constitutive, depending on the availability of precursor molecules. The decarboxylating enzymes are remarkable for their rapid rate of turn over. Their activities are modulated by a wide array of hormonal, developmental and cell growth-related factors[14,15]. In higher plants and some bacteria, there is an additional biosynthetic enzyme, arginine decarboxylase (ADC), which represents an alternate, indirect pathway to ODC for the synthesis of Put, through agmatine[14,16]. A variety of physiological and stress stimuli are known to affect the activity of polyamine biosynthetic enzymes in plants and the two pathways are regulated quite differently[16,17–19].

The information related to the mechanism of regulation of PA biosynthesis or the subcellular localization of their biosynthetic enzymes is limited[8,20,21]. This knowledge is critical to understand the various functions of PAs in the physiology of higher plants. Although studies of various inhibitors of PA biosynthesis have provided some useful information regarding plant growth and development, some of the inhibitors are non-specific, and not well characterized for their permeability and stability in cells. Figure 1 shows that the enzymes ADC and ODC can be irreversibly inhibited by the inhibitors including DL-α-difluromethyl arginine (DFMA) and DL-α-difluromethyl ornithine (DFMO), respectively. Methylglyoxal-bis-guanil hydrazone (MGBG) and cyclohexylamine (CHA) are reversible inhibitors of SAMDC and spermidine synthase, respectively[16,20,22]. They are generally effective only in reducing the cellular levels of PAs. The use of molecular approach including the cloning of genes for PA biosynthetic enzymes, production of transgenic plants, isolation and characterization of mutants defective in PA biosynthesis will provide a better understanding of the role of PAs in higher plants. New impetus to polyamine research has come with the cloning of many PA biosynthetic genes and the cellular perturbation of PA levels and studying its effect on plant developmental processes and stress using transgenic and molecular genetic approaches.

The present review briefly updates our knowledge of cellular and molecular biology of PA biosynthesis in higher plants.

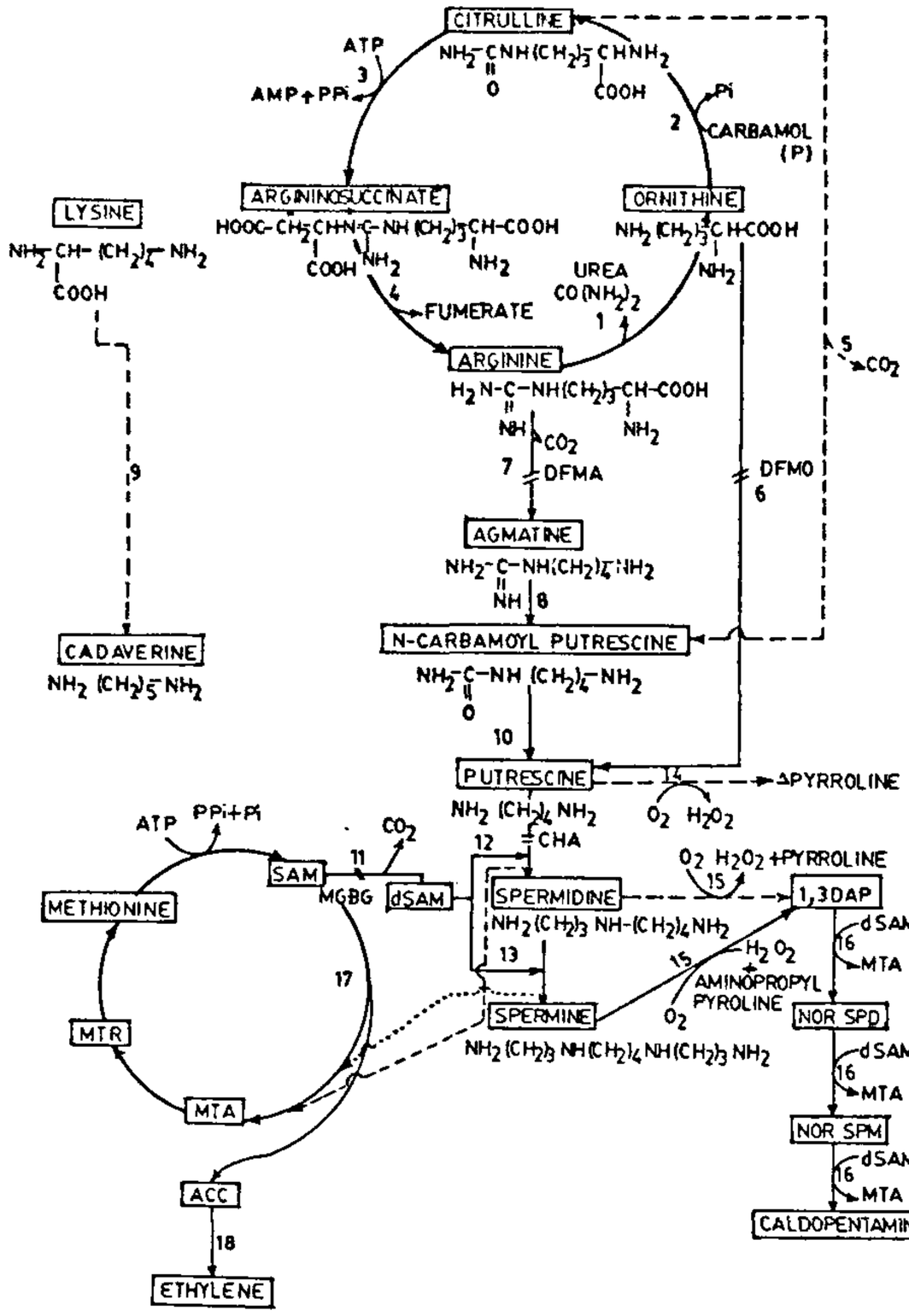

Figure 1. Schematic representation of polyamine metabolism and their interrelationships with the ethylene biosynthesis in higher plants. Inhibitors are indicated in abbreviated forms. DFMO, α-difluromethyl ornithine; DFMA, α-difluromethyl arginine; MGBG, methylglyoxal-bis-(guanylhydrazone); CHA, cycloheximine. The enzymes involved in the metabolic pathways are indicated in numbers (1–18). (1) Arginase, (2) Ornithine carbomoyl transferase, (3) Arginino succinate synthase, (4) Arginino succinate lyase, (5) Citrulline decarboxylase, (6) Ornithine decarboxylase, (7) Arginine decarboxylase, (8) Agmatine iminohydrolase, (9) Lysine decarboxylase, (10) N-carbamoyl putrescine amidohydrolase, (11) S-adenosyl methionine decarboxylase, (12) Spermidine synthase, (13) Spermine synthase, (14) Diamine oxidase, (15) Polyamine oxidase, (16) Aminopropyl transferase, (17) 1,amino 1-cyclopropane 1-carboxylic acid synthase, (18) Ethylene forming enzyme. The major pathway is indicated by continuous lines, while the side branches are indicated by broken and dotted lines.

Molecular biology and expression of PA biosynthesizing enzymes

Arginine decarboxylase

Arginine decarboxylase (ADC), the first key enzyme in the alternate pathway, plays a pivotal role in the biogenesis of precursor Put. The enzyme has been purified and characterized from several systems like oat[23–25], rice[26,27] *Lathyrus*[28], mung bean[29] and *Brassica*[30]. It requires a thiol compound and pyridoxal phosphate for its activity but metal dependency has not been detected.

Earlier reports indicate that ADC is a cytosolic enzyme[4,23]. However, Borrell and coworkers[31] report its specific association with the thylakoid membranes of the oat chloroplast by cell fractionation and immunocytochemistry. In recent years, ADC gene has been cloned from oat[25], tomato[32], pea[33], *Arabidopsis*[34] and soybean[35]. Each of the cloned ADC genes is similar to the biosynthetic *Escherichia coli* ADC and encodes proteins containing the conserved, putative substrate-binding site that has been found in all eukaryotic ADCs and ODCs reported so far. This fact suggests a similar catalytic mechanism[36]. It has been observed from phylogenetic distance analysis that the *Arabidopsis* gene is more closely related to tomato or pea than *E. coli* or oat[34].

The regulations of ADC gene expression are rather complicated and require *de novo* protein synthesis[37].

Bell and Malmberg[25] first obtained the cDNA clone of ADC from oat. The open reading frame (ORF) encodes a 66 kDa protein, but the purified ADC polypeptide has an apparent molecular weight of 24 kDa and is encoded by the carboxy-terminal region of the ORF. Subsequently, Malmberg and coworkers[38,39] demonstrated that the enzyme is post-translationally cleaved and processed into N-terminal 42 kDa and 24 kDa C-terminal fragments and this step is necessary as a part of the activation of ADC enzyme activity in oat. These two are linked together to form the 66 kDa protein by a disulphide linkage. The 24 kDa fragment consists of the DFMA binding site and may contain at least part of the active site of the enzyme which is prevalent in the soluble fraction[38,39].

In tomato, ADC mRNA levels increase from the immature green stage to the breaker stage[32]. Whereas in pea, ADC expression is higher in young developing tissues and early stages of fruit growth than in mature organs[33]. However, in these two cases, ADC activity does not appear to correlate with the mRNA levels, suggesting that expression may be subject to translational and/or post-translational regulation. In fact, pea and soybean mRNAs have long 5′-untranslated regions, which may be involved in the processing[33].

In K^+-deficient *A. thaliana*, Watson and Malmberg[34] could not detect any change in ADC mRNA level or protein level correlating with the increase in ADC enzyme activity. They cannot detect any evidence of regulation by post-translational proteolysis, either by existence of pre-protein or clipped polypeptides. In our experiments[40], 498 bp ADC, PCR amplified oat genomic DNA (OAD1) shows strong hybridization with different rice cultivars by Southern blot analysis and reveals the presence of a single copy gene and restriction fragment length polymorphism (RFLP) of ADC gene. A comparison of Northern blot analysis in salt-tolerant and salt-sensitive cultivars shows differential accumulation of ADC mRNA level during salinity stress, correlating well with the differences in ADC enzyme activity.

The most interesting results are obtained by Borrell and coworkers[31,41,42] in a series of experiments with osmotically stressed, dark induced senescing oat protoplasts. Under senescence-inducing conditions, in presence of 0.6 M sorbitol and in absence of exogenous Spm, the ADC mRNA level shows a significant increase after 1 h, then a rapid decrease after 2 h of incubation in dark. But under presence of Spm, there is a more marked increase in ADC mRNA level and the level remains high even after 2 h. Western blot analysis with the polyclonal antibodies from the C-terminal region, reveals a significant increase in the level of 24-kDa polypeptide (ADC processed and active form) in the soluble fraction of the osmotically stressed oat leaves. However, the presence of exogenous Spm leads to a dramatic decrease in the level of 24 kDa polypeptide after 4–24 h of incubation and it cannot be detected at all after 24 h of incubation. But an unprocessed polypeptide of 66 kDa mol.wt. appears which corresponds to the decrease in ADC enzyme activity.

Ornithine decarboxylase

Ornithine decarboxylase (ODC) is also a pyridoxal-phosphate-dependent enzyme and has been purified from cytoplasm and nucleus of germinating barley seed where it is tightly bound to chromatin[43,44]. It has been partially purified from wheat[45] and jute embryo[46]. The regulation of ODC in higher plant system has not been studied in detail. In animals and yeast, it is regulated in a complex manner by antizymes and proteosomes and there are reports of polyamine dependent translational frame shifting of ODC antizyme[47,48]. In animal systems, ODC has been reported to be under transcriptional[49,50] as well as translational control[51]. The turnover rate of ODC is highly variable with a half-life ranging from as short as 5 min to several hours; this is at least partially due to the PEST (Pro–Glu–Ser–Thr) sequence in the C-terminal region[52]. Phosphorylation may also play a role in the regulation of ODC by PAs[53,54]. Role of antizyme in the control of ODC from jute has also been revealed by Pandit and Ghosh[55].

The cDNA clone representing ODC from *Datura* has been obtained recently by Michael and coworkers[56]. It is similar to other eukaryotic ODCs, but lacks long 3′ and 5′ untranslated regions that are present in mammalian ODC mRNAs, and are probably involved in translational efficiency. A decarboxylase amino acid motif with a consensus sequence, XX [D/N] X GGG X [G/T], where X represents a small hydrophobic residue, is found in all eukaryotic ADCs and ODCs cloned so far (between 345–353 nt in pea sequence)[33]. The putative pyridoxal phosphate binding domain from nt position 161 and 552 in pea ADC sequence has also been detected[33].

S-adenosyl methionine decarboxylase

S-adenosyl methionine decarboxylase (SAMDC) is probably the rate-limiting enzyme that provides the aminopropyl moiety used by Spd and Spm synthases to convert Put to Spd and Spm respectively. SAM is also a precursor for ethylene biosynthesis. SAMDC has been observed in carrot[57], oat[58] and tobacco[59]. In tobacco it is a 35 kDa polypeptide as revealed by SDS-PAGE analysis[60].

Genes for SAMDC have been cloned from potato[61], spinach[62], periwinkle[63], carnation[64] and *Tritordeum*[65]. There is a strong (70%) sequence similarity, at the level of deduced amino acid sequences among the plant SAMDC genes, but they have only 30–35% and 20–26% sequence identity with the mammalian and bacterial

SAMDC[53,63–66]. Inspite of a low overall sequence similarity, plants share some regions of similarity between all SAMDC genes analysed so far including a putative PEST sequence, characteristic of peptides having high turnover rates and a pre-enzyme cleavage site. Indeed, SAMDC in potato and periwinkle is synthesized as a precursor molecule or as a pre-protein and it is post-translationally self-processed to form two subunits (α, β), which then together constitute the functional enzyme. As a result of this modification, a covalently linked prosthetic pyruvate group is generated from the N-terminal Ser-residue at the cleavage site[61,67–69]. A third important feature is their long (approximately 500 bp) 5′ untranslated leader sequence which contains untranslated open reading frame[63]. Potato SAMDC is highly expressed in actively dividing and differentiating tissues of both vegetative and reproductive organs[61]. In *Tritordeum* SAMDC mRNA accumulates in wounded tissues and the level follows a circadian rhythm, suggesting its expression in the absence of cell division[65]. Genes encoding spermidine and spermine synthase have not been cloned from plant system till date.

Transgenic plants expressing PA biosynthesizing genes

Initially, due to the non-availability of native genes from plant sources, the polyamine biosynthesis genes from non-plant sources like yeast, mice and human are engineered in transgenic plants. The genes are linked to cauliflower mosaic virus 35S RNA promoter, thus producing high levels of constitutive expression. The yeast ODC gene overexpressed in tobacco hairy root cultivar produces up to 3-fold increase in ODC activity and doubles the nicotine content[69]. DeScenzo and Minocha[70] transform tobacco with mouse ODC and obtain 2–3 fold increase in Put in the leaves and 4–12 fold increase in the callus cells. Most of the transformants appear normal, although the ones having highest ODC and Put levels display stunted growth, wrinkled leaves and flowers with reduced stems. The tobacco transgenic with the human SAMDC[71] shows a 2–4 fold increase in SAMDC activity, reduced Put levels, and 2–3 fold increase in Spd level.

The controlled expression of a transgene is always better in comparison with constitutive over-expression. For example, potential over-expression of a homologous SAMDC gene in potato being reported to be lethal. The Tet repressor system has been designed to regulate the gene in sense and antisense orientation[72]. In the sense orientation, SAMDC/Tet construct results in a 2–6 fold increase in the SAMDC transcript level, following Tet induction in leaf explants, along with an increase in SAMDC activity and Spd, Spm and intriguingly Put levels. The plants having antisense SAMDC construct display a variety of phenotypes including stunted growth, short internodes, stem branching and small leaves, concomitant with the reduction in the levels of SAMDC mRNA and activity and Put, Spd and Spm content, along with a 46-fold increase in ethylene content.

Recently, ADC gene of oat has been over-expressed in tobacco under the control of an inducible promoter; the Tet-repressor system and this shows a significant change in the levels of ADC transcript and activity, and the Put content upon tetracycline-induction[73]. Some transgenic plants display abnormal phenotypes including short internodes, small stems and leaves, chlorosis and advancement in the flowering period[73].

The root induction and morphological abnormalities, due to the presence and expression of the Ri TL-DNA (*Agrobacterium rhizogenes*, T-DNA left border) are linked to an altered metabolism of polyamines[74,75]. A range of phenotypes including short internodes, highly branched stems, with small leaves have been obtained in plants transformed with RiTL-DNA of *Agrobacterium rhizogenes*[74], this correlates well with the reduced PA level, suggesting that the products of the Ri TL-DNA encoding genes may be involved in repressing PA biosynthesis. In tobacco root, a correlation exists between the expression of the *rol* genes from the TL-DNA (*rol*A, *rol*B, *rol*C) of *A. rhizogenes* and PA metabolism. The higher PA contents found in roots transformed by *rol*A parallel with higher ODC and ADC activities as well as higher nicotine contents[76,77].

More recently, activation T-DNA tagging has been used to create MGBG-resistant tobacco cell lines[77]. Regenerated plants display characteristic leaf and floral malformation as well as parthenocarpy. One line shows increase in activity of SAMDC whereas in another line this is coupled with elevated levels of Put and Spd although transcript levels of the respective enzymes remain unchanged. The tagged plant genomic DNA is isolated and has been used to detect a functional cDNA[78].

Mutational analysis

Several efforts have been made to select polyamine mutants. In petunia and tomato, floral-morphology mutants have been shown to have increased Put content and ADC activity; however, the molecular analysis of the mutants has not been carried out in detail[79,80]. The mutants from MGBG and DFMO-resistant tobacco show an array of phenotypic characteristics including dwarfing and floral abberations. Recently, Malmberg and Watson[81] after screening 25,000 EMS-M_2 seedlings of *Arabidopsis* for ADC and ODC mutants have identified a number of alleles defective in ADC, which fell into two loci. One of these, Spe 1, represents the ADC

structural gene and the other represents a regulatory locus involved in post-translational regulation at the protein level (Spe 10). These mutants show minor root, stem and floral morphogenesis. The allele deficient in ODC activity (Spc 2) is generally hypomorphs, with low but non-zero levels of enzyme activity and the Put content. In another genetic approach, the SAMDC gene of potato has been mapped to chromosome 5 and shows genetic linkage with the tuberization process[21].

Concluding remarks

From the above discussion, it can be concluded that the molecular evidence essentially supports the biochemical data. Although mRNA accumulation seems to be important in the control of polyamine biosynthesis pathway, it is quite clear in all the cases other regulatory mechanisms also exist, which function at the level of translation, enzyme processing and assembly levels. Accumulating evidences from the analysis of the transgenic plants show that PA metabolism can be manipulated as well as changes in PA biosynthesis appear to have a reciprocal effect on ethylene biosynthesis. The analysis of transgenic plants, together with results from mutant analysis, indicates that changes in levels of PAs can affect leaf and floral morphology. Finally the availability of molecular probes for the biosynthetic enzymes, coupled with mutational analysis to knockout genes, the use of antisense technology and activation T-DNA tagging shall prove to be useful in understanding the role of polyamines in plant metabolism and control of dormancy, fruit ripening, senescence and responses of plants to stress.

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Received 4 August 1997; revised accepted 27 January 1998

MEETINGS/SYMPOSIA/SEMINARS

Short Course on Active Tectonics and Paleoseismology

Date: 14–19 September 1998
Place: Bangalore

The course will present the major trends and techniques in paleoseismology and active tectonics. It is open to postgraduate students, researchers and teachers with background in geology/geophysics.

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