Synopsis of the thesis on

Understanding the Functional Role of Some Polyamine Biosynthetic Genes in Tomato

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Introduction

Polyamines (PAs) are low molecular weight polycations containing two or more primary amino groups ($-NH_2$) [1]. They are essential molecules for all living organisms including bacteria, fungi, plant, and animals [2]. They are present in both free as well as in conjugated forms with phenolic compounds, proteins and nucleic acids [2]. Common PAs exist amongst all organisms are diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm) [3]. Some unusual PAs such as thermospermine, homospermidine, norspermidine, homospermine, norspermine have limited distribution and reported in certain microorganisms and plants [4].

In plants PAs are mostly present in actively growing tissues and under stress conditions [5-8]. Because of their cationic nature they are involved in various cellular processes including transcriptional and translational regulation, membrane stabilization, ion channels maintenance, cell differentiation, cell signaling [9-11]. They may also regulate cell death pathways [12, 13]. In case of plants they are shown to be involved in several developmental processes including embryogenesis, vascular tissues formation, senescence and in abiotic and biotic stress responses [14-17].

Although the mechanism of action of PAs remains elusive but it can be speculated that uniform distribution of positive charge under physiological pH plays a very important role. Due to this ability they can bind to almost all negatively charged macromolecules such as nucleic acids, phospholipids and proteins and alters their structure and functions, moreover protect them from degradation and modifications [18]. The simplicity in their structure, consistent distribution in all the cellular compartments and involvement in various developmental and physiological processes make PAs an interesting group of compounds for investigation.

There are enough evidences about the involvement of PAs in plants during abiotic and biotic stresses. Also fluctuations in their cellular contents are reported in plants in response to different stresses [19]. Among the polyamines, spermine is reported to have an assured role against salt and drought stress in Arabidopsis. Arabidopsis spm mutant (Atspm) plants lacking spermine were observed to hypersensitive to those stresses [20]. Similarly, PAs roles during plant pathogen interactions are well studied. Several reports demonstrated participation of spermine in plant defense against pathogens [21-24]. However the role of its isomer thermospermine in plant defense has not been investigated. It is possible that thermospermine may also carry out the similar protective role as spermine plays. A recent report suggested that exogenous application of thermospermine decreases CMV infection and increases the expression of defense related genes in Arabidopsis [25]. However the basic mechanism how thermospermine is protective against CMV has yet to be elucidated further.

Thermospermine is a structural isomer of tetraamine spermine. It was first reported from extreme thermophile *Thermus thermophilus* where it is shown to protect thermophiles under extreme growth conditions by stabilizing their nucleic acids [26]. In Arabidopsis thermospermine is synthesized by thermospermine synthase which is encoded by a single gene ACAULIS5 (ACL5) [21]. It is shown to be involved in prevention of premature cell death of xylem cells in Arabidopsis [21, 27, 28]. Recently thermospermine has been reported from wide range of plants [29].

Using genome wide analysis we have identified several PA biosynthetic genes including three ACL5 like genes in tomato genome. We aim to characterize the role of some of these candidate genes in tomato. Therefore, in present study we will investigate the possible role of ACL5 like genes and some other identified polyamine biosynthetic genes during plant development and stresses in tomato using molecular genetic and biochemical approaches.

Aim and Objectives

Aim of the proposed work is to study the role of tomato ACL5 like genes and some other polyamine biosynthetic genes using molecular and biochemical based approaches in plants.

The objectives of proposed work are:

1. Identification of candidate polyamine biosynthetic genes in tomato.
2. Expression analysis of candidate polyamine biosynthetic genes during plant development and stresses.
3. Characterization of tomato ACL5 like genes function by gene silencing and/or overexpression in plants.

Results

1. Identification of candidate polyamines biosynthetic genes in tomato

Identification of candidate PA biosynthetic genes from tomato *(Solanum lycopersicum* L.*)* genome was done by bioinformatics analysis. Known gene and protein sequences of PAs biosynthetic genes from *Arabidopsis thaliana* obtained from TAIR (https://www.arabidopsis.org/) were used to identify the gene sequences of candidate PA genes in tomato. These sequences were blast searched against the tomato genome in the Sol genomics network (https://solgenomics.net/). On the basis of sequence similarity a total of 18 genes were identified associated with PAs biosynthesis in tomato genome. Identified genes were named as SolycADC1 and SolycADC2, SolycODC1 and SolycODC2, SolySPDS1, SolycSPDS2, SolycSPDS3, SolycSPDS4 and SolycSPDS5, SolycSAMDC1, SolycSAMDC2, SolycSAMDC3, SolycSAMDC4 and SolycSAMDC5, SolycSPMS and SolycACL5, SolycACL5-Like1 and SolycACL5-Like2 based on their sequence homology with Arabidopsis PAs biosynthetic genes.

A. Conserved Motifs and Phylogenetic Analysis

Multiple sequence alignment of Arabidopsis and tomato PAs revealed the conserved domains and structural features of all identified PA biosynthetic genes. Among the 18 identified SolycPAs proteins, SolycADC1, SolycADC2 and SolycODC1, SolycODC2 contains Orn/DAP/Arg decarboxylase2, N-terminal (De-COase2_N) and Orn/DAP/Arg decarboxylase2, C-terminal (De-COase2_C) domains. Additionaly SolycADC1 contains Alanine racemase/group IV decarboxylase, C-terminal domain (Ala_racemase/Decarboxylase_C). SolycSAMDC1, SolycSAMDC2, SAMDC3, SAMDC4 and SAMDC5 have only one domain namely S-adenosylmethionine decarboxylase (S-AdoMet_deCO2ase_core). Besides these SolycSPDS1, SolycSPDS2, SolycSPDS3, SolycSPDS4 and SolycSPDS5, SolycSPMS, SolycACL5 and SolycACL5- Like1 and SolycACL5-Like2 also contain single domain which is Polyamine biosynthesis domain (PABS). The results indicate that the SolycPAs genes of same family have conserved domain struture similar to found in Arabidopsis. To further explore the phylogenetic relationships of the PA biosynthetic genes of tomato and arabidopsis, phylogenetic tree was constructed with the PAs proteins of tomato and arabidopsis. The phylogenetic analysis revealed that all the tomato PAs biosynthetic genes have high sequence similarity with Arabidopsis sequences. We observed that identified SolycACL5, SolycACL5-Like1 and SolycACL5-Like2 shows 83%, 80% and 76% sequence similarity, respectively, with Arabidopsis ACL5 at the protein level.

B. Gene structure analysis of candidate PA biosynthetic genes

To understand the structural diversity of SolycPAs genes, we determined the exon-intron boundary of SolycPAs genes by aligning ORF sequences of SolycPAs and compared with their respective Arabidopsis gene sequences. Also we analysed the distribution of SolycPAs genes on different chromosomes. SolycADC1, SolycSAMDC1 and SolycSAMDC5 were positioned on chromosome1. SolycSAMDC3 was found to be positioned on chromosome 2. SolycODC2 and SolycSPMS were located on chromosome 3. SolycODC1 and SolycSPDS2 were situated at chromosome 4. SolycSAMDC2 and SolycSPDS1 were found on chromosome 5. SolycSPDS4, SolycSPDS5 and SolycSAMDC4 were located on chromosome 6. SolycACL5-Like2 was situated on chromosome 7. SolycSPDS3 and SolycACL5 were located on chromosome 8. SolycACL5-Like1 and SolycADC2 were positioned on chromosome 9 and 10 respectively.

C. Identification of Putative Cis-Regulatory Elements in the Promoter Sequences of candidate PA Biosynthetic Genes

To gain further insights into the transcriptional regulations and potential functions of SolycPAs, putative cis-regulatory elements in the promoter sequences were analyzed using Plant CARE database [30]. A total of 82 cis-regulatory elements were identified in the 1kb of genomic sequences upstream of 5’ UTR of SolycPAs genes. As expected, the conventional promoter elements like TATA box and CAAT box were found in all the promoters. Besides these, some specific *cis*- element, like, Unnamed_4 was identified in all the promoters, while some were very specific to only few promoters of polyamine genes. Identified *cis*-elements were divided in to different categories, like, light responsive which includes AAAC-motif, Box 4, GATA-motif, GT1-motif, I-box, TCT-motif, chs-CMA1a, ACE, ATCT-motif, AE-box, Box I, MNF1, TCCC-motif, as-2-box, CATT-motif, GA-motif, GTGGC-motif, chs-CMA2a, AT1, Sp1, ATCT-motif, chs-CMA2b, GAG-motif, 3-AF1 binding site and circadian. Some of the other identified *cis*-elements found in promoters were hormone responsive, like, TGA-element (auxin), AuxRR-core (auxin), ABRE (abscisic acid), GARE-motif, P box and TATC-box (gibberellin) and ERE (ethylene). Some of the well known stress responsive elements, like, ELI-box3 (elicitor), HSE (heat), LTR (low temperature), TC-rich repeats (defense), Box-W1 (fungal elicitor), MBS (drought), CGTCA-motif and TGACG-motif (MeJA), WUN-motif (wound), and TCA-element (salicylic acid) were also identified in promoter sequences. Besides these, *cis*-element like Skn-1_motif was also found in promoter of all PA biosynthetic genes except SolycODC2, SolycACL5-Like2, SolycSAMDC1 and SolycSAMDC4 which known to have endosperm specific expression. Cis elements like HD-Zip1 and HD-Zip2 which are involved in regulating leaf morphology were present in SolycSPDS4 promoter only. Also the cis element related to meristem specific development namely CCGTCC-box present in SolycSPDS3 and SolycSPDS4 promoter only. Most of the SolycPA gene promoters detected to have light and hormone responsive elements. Based on the identified *cis*-elements it could be suggested that PA biosynthetic genes might play important roles during plant development, abiotic and biotic stresses and also involved in cross-talk with hormones. Presence of several lights and circadian responsive elements suggest that PAs may also involve in regulation of photomorphogensis and circadian rhythms. As SolycACL5, SolycACL5-Like1 and SolycACL5-Like2 genes promoter have HSE and TC rich repeats cis elements, suggests that these genes are induced under heat stress and defense responses. The *cis*-elements like BOX4, G-box and Box-1 were also found in the promoter regions of SolycACL5, SolycACL5-Like1 and SolycACL5-Like2 genes which are responsible for light responsiveness.

2. Expression pattern analysis of candidate polyamine biosynthetic genes in tomato

Quantitative RT-PCR was performed to analyze the detail expression pattern of candidate SolycPA genes in different tissues such as root, stem, leaves, flowers and fruits at different developmental stages of tomato plants. Expresssion analysis showed that all the SolycPA genes were diffrentially expressed in developmental tissues of tomato plants. SolycADC1and SolycADC2 show high expression during leaf maturation. SolycADC2 also has significant expression during flower development. While SolycSAMDC1 shows high expression in immature leaves and during initial stages of flower development. Its expression was also observed in fruits undergoing ripening. Whereas SolycSAMDC2 has comparative low expression in hypocotyl, cotyledons, flower and fruit developmental stages, while mature leaves and xylem layer of stem show its higher expression. PA biosynthetic gene SolycSPDS1 and SolycSPDS2 displayed higher expression in immature leaf and stem tissues, whereas expression of SolycSPDS2 was decreased during different stages of flower development. Both SolycSPDS1 and SolycSPDS2 also show significant expression during different development stages of fruits. While SolycSPMS displayed a very high expression during fruit ripening stages. However, SolycACL5 has very high expression in root tissues, whereas its expression in immature leaves was observed to be much lower. During reproductive stages of plants, its expression was highest during flower development. A comparatively lower expression of SolycACL5 was also observed in different developmental stages of fruits. In contrast, SolycACL5-Like1 displayed high expression in immature leaves and xylem layer of stem development.

3. Expression pattern analysis of candidate polyamine biosynthetic genes during different stress in tomato

To understand the role of SolycPA genes during different stresses, 15 days old tomato “Money Maker” seedlings were treated with various stresses. Expression pattern of all SolyPA genes was analysed in response to heat, cold, UV-C, drought, flood, wounding, salt, mannitol, methylviologen (MV), rosebengal (RB), salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and Fumonisin B1 (FB1) treatments.We observed significantly differential expression of all SolycPA genes in all the treatments.We observed that expression of SolycADC1, SolycADC2, SolycACL5, SolycACL5-Like1 was highly upregulated by heat, drought and wounding. SolycSPDS1 was observed to have a significant induced expression in FB1 treatment, whileSolycSPDS2 was upregulated by MV treatment. When plants were treated with JA, only SolycACL5and SolycACL5-Like1 were showing significant upregulation. Thus on the basis of stress induced expression analysis results it can be suggested that PA genes also play important roles at the time of stresses.

4. Characterization of SolycACL5 function by gene silencing and/ or overexpression in plants

On the basis of expression analysis, we selected SolycACL5 gene for their functional characterization in plants. For gene silencing, we designed and cloned amiRNAs specific to SolycACL5 in the destination vector pMDC32. Whereas for overexpression, gene coding sequences of SolycACL5 were PCR amplified and cloned in the destination vector pMDC32. Both the gene constructs were transformed in *Agrobacterium tumefaciens* strain GV3101 for genetic transformation of plants. To study the effects of gene silencing and overexpression, we transformed these constructs to generate gene silencing and overexpresser lines in tomato and tobacco, respectively.

A. Silencing of SolycACL5 in tomato:

We transformed amiRNA-SolycACL5 gene silencing construct under control of constitutive 35S promoter to generate gene silencing lines in tomato. We generated transformed shoot buds of 2-4 mm long which were failed to develop further. The shoot buds were also genotyped positively by PCR for presence of transgene.We speculate that this lethality or abnormality of shoots was may be due to the effects of silencing of solycACL5 in plants. A more or less similar observation was also reported in case of Arabidopsis, where knockout of ACL5 gene resulted in severely dwarf plants [31]. Therefore in the present investigation we could not performed further experiments using gene silencing lines due to the lethality or abnormality of trangenic shoot buds.

B. Overexpression of SolycACL5 in tobacco

For functional analysis, 35S::SolycACL5 construct was transformed in tobacco and several overexpresser lines were generated. These overexpresser lines were further genotyped by PCR using gene specific primers. Quantitative RT-PCR analysis was also performed to quantify the overexpression of SolycACL5 in tobacco. For further characterization, selected lines were grown in soil under controlled growth room conditions. We observed that there was higher accumulation of chlorophyll pigments and delayed senescnece of leaves in overexpresser lines. Also the length of the overexpresser plants was more than the control plants. Also length of leaves and more number of green leaves were observed in overexpresser plants.

PA metabolism also known to influence the ROS generation and activity of ROS scavenging enzymes [32]. Generally, ROS generation is counterbalanced by the activity of ROS scavenging enzymes. Therefore, to know whether overexpression of SolycACL5 could also leads to modulation in the activity of various antioxidant enzymes such as Catalase (CAT), Superoxide Dismutase (SOD), Guaiacol Peroxidase (GP) and Ascorbate Peroxidase (APX), biochemical enzymatic assays were carried out to monitor the activity of these enzymes in SolycACL5 overexresser and control plants. We observed that there was comparatively lower enzymatic activity of APX in the overexpresser lines as compared to the control plants. In addition, MDA assay was also performed to measure lipid peroxidation which is indicator of oxidative degradation of lipids in cells. We observed that lipid peroxidation was less in the overexpresser lines. Therefore a lower activity of antioxidant enzyme and lower lipid peroxidation could be due to less generation of ROS in SolycACL5 overexpresser lines. In order to know if overexpression of SolycACL5 could leads to higher accumulation of chlorophyll and anthocyanin pigments in plants, we quantified the total chlorophyll and anthocyanin content in leaves of overexpresser lines and control plants. We observed that chlorophyll and anthocyanin contents were higher in overexpresser plants. We concluded that overexpression of SolycACL5 in plants may leads to higher accumulation of anthocyanin and chlorophyll pigments in cells.

We further observed that senescence of leaves was delayed in overexpression lines. In order to know whether developmental senescence is affected, we further analyzed the expression of senescence markers, SGR1 (Stay Green 1) and SAG12 (Senescence associated gene12) in overexpression lines. Total RNA was isolated from immature, young, mature and senescent leaves and used for expression analysis. Expression was normalized using NtUBQ5 gene. Results showed that overexpresser lines comparatively have lower expression of SGR1 and SAG12 than the control leaves. We concluded that delayed senescence of leaves of SolyACL5 expressing plants is due to late initiation of senescence program of their leaves.

Conclusions

In the present study, we performed genome wide analysis and identified 18 genes involved in PAs biosynthetic pathway in tomato. A comparative multiple sequence alignment and phylogenetic analysis revealed that all the identified genes were closely related to Arabidopsis PA biosynthetic genes of Arginine/ Ornithine decarboxylase, S-adenosyl methionine decarboxylase and Spermidine/ Spermine synthases gene families. We identified three close homologs of Arabiodpsis ACL5 genes in tomato genome.

Cis-regulatory elements play important roles in regulation of gene expression. In the present study, a series of well-known stress responsive elements such as HSE, LTR, TC-rich repeats, Box-W1, MBS, WUN-motif, and TCA-element were detected in the promoters of several candidate genes. These elements may play important roles in regulating the expression of these genes in a range of stress conditions.

Expression pattern analysis revealed that most of the candidate genes were differentially expressed in various developmental tissues. We also analyzed the expression of all identified biosynthetic genes in responses to various stresses and defense hormones in tomato. Our results shows that a number of genes were upregulated during heat, cold, UV-C and drought treatments, whereas expression of some of the genes were either downregulated or remain unchanged in response to phytohormones JA, SA and ABA treatments.

Gene silencing of SolycACL5 in tomato leads to abnormal shoot buds formation which failed to grow beyond 2-4 mm in length. Ectopic overexpression of SolycACL5 in tobacco leads to vigorous plants which also show delayed senescence of their leaves. Overexpresser lines also observed to accumulate more chlorophylls and anthocyanin pigments in plants. A lower enzymatic activity of ROS scavenging enzymes such as APX in transgenic plants was observed which suggest that due to the overexpression of SolycACL5 in tobacco, generation of ROS was significantly reduced. However, comparatively lower expression level of SGR1 and SAG12 in overexpresser lines suggest that overexpression of SolycACL5 leads to delayed leaf senescence process. Our results demonstrate that SolycACL5 is differentially expressed in plants and is involved in plant development as its overexpression leads to alteration in senescence process and higher accumulation of chlorophyll and anthocyanin pigments and reduced activity of antioxidant enzymes.

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Publications

In Peer reviewed Journals:

1. Farsodia, M. and Singh S.K. (2017). Genome-wide identification and expression analysis of the Polyamine biosynthetic genes in tomato. (Communicated to JPR).
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3. Dubey, N., Trivedi, M., Varsani, S., Vyas, V., Farsodia, M and Singh, S.K. (2017). Genome-wide analysis and expression profiling of Metacaspases during development in potato. (Communicated to Gene).
4. Farsodia, M. and Singh S.K. (2017). Ectopic expression of tomato ACL5 in tobacco leads to higher pigmentation and delayed senescence of leaves. (Manuscript in preparation).

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