

SUMMARY

Summary

Polyamines are low molecular weight polycations, necessary for all living organisms including bacteria, fungi, plants and animals (Takahashi and Kakehi 2010, Yatin 2002). They exist in both free and conjugated forms with phenolic compounds, proteins and nucleic acids (Yatin 2002). Putrescine, spermidine and spermine are the most common polyamines present amongst all the organisms (Kumar et al., 1997). Besides these, thermospermine is also present in plants as tetraamine (Takano et al., 2012).

Polyamines are shown to be involved in several developmental processes including embryogenesis, fruits development, vascular tissues formation, senescence and in abiotic and biotic stress responses in plants (Evans 1989, Galston and Sawhney 1990, Kusano et al., 2008, Slocum and Flores 1991). Due to the importance, genes involved in polyamines biosynthesis have also been identified and characterized in some plants (Hu et al., 2016, Sagor et al., 2016, Sekula et al., 2016, Sequera-Mutiozabal et al., 2016). Various mechanisms regulating intracellular polyamines are also being elucidated in some details, however an overall understanding of the roles of polyamines and their regulatory gene network during development is still needs to be elucidated in plants. Therefore, in present investigation, we aimed to characterize the possible role of some of the genes involved in polyamine biosynthetic pathway in tomato using molecular genetics and biochemical approaches.

Using genome-wide analysis, we have identified eighteen polyamine biosynthetic genes and a comparative multiple sequence alignment and phylogenetic analysis revealed that all the identified genes were closely related to *Arabidopsis* polyamine biosynthetic genes. 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* polyamine biosynthetic genes.

Our *in silico* analysis revealed that among the all identified genes, SolycADC1, SolycADC2 and SolycODC1, SolycODC2 belonged to Arginine/ Ornithine decarboxylase family containing Orn/DAP/Arg decarboxylase2, N-terminal and Orn/DAP/Arg decarboxylase2, C-terminal domains. Besides these SolycSPDS1, SolycSPDS2, SolycSPDS3, SolycSPDS4 and SolycSPDS5, SolycSPMS, SolycACL5 and SolycACL5-Like1 and SolycACL5-Like2 belonged to Spermidine/ Spermine synthases family

containing polyamine biosynthesis domain and spermidine synthase, tetramerisation domains. Further the phylogenetic analysis revealed that all the tomato polyamine biosynthetic genes have high sequence similarity with homologous *Arabidopsis* polyamine biosynthetic genes and their protein sequences were highly conserved between them. ADC genes of tomato and *Arabidopsis* shared 70% sequence similarity, SAMDC genes have around 60%, SPDS genes have 60-80% and SPMS has 80% and ACL5 members shared about 60-70% sequence similarity with *Arabidopsis* homologs.

To understand the structural diversity of polyamine biosynthetic genes we determined the exon-intron boundary of all the identified genes and also analysed the distribution of these genes on different chromosomes of tomato. To know the transcriptional regulations and potential functions of these genes, we have also analyzed the putative cis-regulatory elements in the 1kb promoter sequences of all the identified genes. A total of 82 cis-regulatory elements were identified and as expected, the conventional promoter elements like TATA box and CAAT box were found in all the promoters. Promoter sequence analysis showed presence of a relatively large number of hormone and stress responsive cis-regulatory elements in promoters of candidate genes. Besides these, *cis*-element like Skn-1_motif was also found in promoter of all polyamine biosynthetic genes except few which known to have endosperm specific expression. Cis elements like HD-Zip1 and HD-Zip2 which are involved in regulating leaf morphology and the cis element related to meristem specific development namely CCGTCC-box were present in specific gene promoters only. Most of the candidate gene promoters detected to have light responsive elements. Based on the identified *cis*-elements it could be suggested that polyamine biosynthetic genes might play important roles during plant development, abiotic and biotic stresses and also involved in interactions with hormones. As some of the genes promoter have HSE and TC rich repeats cis elements, suggesting that these genes are induced under heat stress and defense responses.

Quantitative RT-PCR was performed to analyze the detail expression patterns of candidate genes in different tissues such as hypocotyl, cotyledons, root, stem, leaves, flowers and fruits at different developmental stages of tomato plants. Expression analysis showed that all the polyamine biosynthetic genes were differentially expressed in developmental tissues of tomato plants. SolycADC1 and SolycADC2 were highly expressed during initial stages of flower and fruit development. Also, they showed higher expression during stem development. Whereas, SolycODC genes showed distinct expression pattern during tomato development. SolycSAMDC1 and SolycSAMDC2 had high expression during

various stages of leaves development, initial stages of flower development and fruits undergoing ripening. Also, xylem layer of stem showed higher expression of SolycSAMDC2 suggesting their role during plant development. Other paralogs of SAMDC in tomato showed very insignificant expression. Polyamine biosynthetic gene SolycSPDS1 and SolycSPDS2 displayed higher expression in immature leaf and stem tissues, whereas their expression in flower developmental stages was progressively in decreasing pattern. Both SolycSPDS1 and SolycSPDS2 also showed consistent expression during different development stages of fruits. Whereas, SolycSPDS3, SolycSPDS4 and SolycSPDS5 did not show any significant expression in the vegetative and reproductive tissues of tomato. SolycSPMS displayed considerable expression in the vegetative tissues of tomato, while a very high expression was observed during fruit ripening stages. However, SolycACL5 has very high expression in root tissues, whereas SolycACL5 and SolycACL5-Like1 showed similar expression pattern during leaf developmental stages, which was gradually decreasing from immature leaf stage to leaf undergoing senescence. During reproductive stages of plants, SolycACL5 expression was highest during initial stages of flower development. However, a comparatively lower expression of SolycACL5 was observed in different developmental stages of fruits. In contrast, SolycACL5-Like2 displayed high expression in mature leaves and initial stage of fruit development. Differential and higher expression of tomato polyamine biosynthetic genes in specific developmental tissues revealed that these genes might play important roles in the corresponding tissues development.

Expression pattern of all the identified polyamine genes was analysed in response to heat, cold, UV-C, drought, flood, wounding, salt, mannitol, methyl viologen, rose bengal, Fumonisin B1, JA, SA and ABA treatments. We observed significantly differential expression of all the candidate genes in all the treatments. We observed that expression of SolycADC1 and SolycADC2 had substantial expression during various stresses. SolycODC1 displayed significant expression by different treatments, whereas SolycODC2 expression was observed only during physical stresses. Among the SAMDC paralogs, SolycSAMDC1, SolycSAMDC3 and SolycSAMDC4 showed noteworthy expression during physical stress signifying their role during these unfavourable conditions. However members of Spermidine/spermine synthase family, SolycSPDS1 and SolycSPDS2 only showed their expression during some particular chemical treatments, whereas its another three paralogs SolycSPDS3, SolycSPDS4 and SolycSPDS5 did not show any expression by various treatments. Another member of this family SolycSPMS exhibited strong

upregulation during heat and SA treatments. SolycACL5, SolycACL5-Like1 was highly upregulated by heat, drought and wounding. When plants were treated with hormones, only SolycACL5 and SolycACL5-Like1 were showing significant upregulation by JA and SA treatment, suggesting that all the candidate genes might be playing important roles at the time of a number of stresses.

On the basis of expression analysis, we selected SolycACL5 gene for its functional characterization in plants. For gene silencing, we designed and cloned amiRNAs specific to SolycACL5 in the destination vector pMDC32 under the control of 35S promoter using Gateway cloning technology. Whereas, for overexpression, gene coding sequences of SolycACL5 were PCR amplified and cloned in the destination vector pMDC32 again under the control of constitutive 35S promoter. Both the gene constructs were transformed in *Agrobacterium tumefaciens* strain GV3101pMP90 for genetic transformation of plants.

We transformed amiRNA-SolycACL5 gene silencing construct to generate gene silencing lines in tomato. We generated transformed shoots of 3-5 mm long which were failed to develop further due to lethality of shoots. The shoot buds were also genotyped positively by PCR for presence of transgene. The expression level of SolycACL5 gene in silencing lines was also analysed which showed almost 99% decreased expression of SolycACL5 in the silencing lines. Thus, we speculated that this lethality of shoots was may be due to the effects of silencing of SolycACL5 in plants. A similar observation was also reported in case of *Arabidopsis*, where knockout of ACL5 gene resulted in severely dwarf plants (Imai et al., 2006). Therefore, in the present study, we could not performed any further experiment due to the severely dwarf shoot bud phenotype of tomato.

For further functional analysis, 35S::SolycACL5 overexpression construct was transformed in tobacco and several overexpresser lines were generated. These OE lines were further genotyped by PCR using gene specific primers. We also performed qRT-PCR to quantify the overexpression of SolycACL5 in tobacco. For additional characterization, selected lines were grown in pots under controlled growth room conditions. Our phenotypic analysis showed that overexpression of SolycACL5 in tobacco led to vigorous growth of plants which also showed delayed senescence of their leaves. We observed that OE plants were taller than the control plants. Also, larger leaves and more number of green leaves and less number of senescent leaves per plants were observed in OE lines.

SolycACL5 OE lines also observed to accumulate more chlorophylls and anthocyanin pigments in plants. Modulation in the enzymatic activity of ROS scavenging enzymes such as CAT, SOD, APX and GP and low MDA content in the OE lines suggested that

due to the overexpression of SolyACL5 in tobacco, generation of ROS specifically H₂O₂ was significantly reduced. A comparatively much lower expression level of senescence associated genes SGR1 and SAG12 and higher transcript level of chloroplast encoded genes namely rpoA, rbcL and petB was also observed in the OE lines proposing that overexpression of SolyACL5 leads to delayed leaf senescence process in plants.

This study provides insights in to the characteristics of polyamine biosynthetic genes which could be a valuable resource to further explore their potential roles during development and various stresses in plants. In addition, we also characterized the SolyACL5 gene function using molecular genetics and biochemical based approaches. The amiRNA based silencing of SolyACL5 produced severely dwarf shoot phenotype, whereas its overexpression resulted in vigouros plants growth, higher accumulation of chlorophyll and anthocyanin in leaves and also delayed progression of leaf scenescence.