CHAPTER 4

RESULTS

4. Results

4.1 Identification and nomenclature of candidate polyamine biosynthetic genes in tomato

Identification of candidate polyamine biosynthetic genes from tomato *(S. lycopersicum* L.*)* genome was done by bioinformatics analysis. Known gene and protein sequences of polyamine biosynthetic genes from *Arabidopsis thaliana* obtained from TAIR (https://www.arabidopsis.org/) were used to identify the gene sequences of candidate polyamine biosynthetic 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 eighteen (18) genes were identified associated with polyamines biosynthesis in tomato genome (Table 4.1). The identified genes were namely arginine decarboxylase (ADC), ornithine decarboxylase (ODC), spermidine synthase (SPDS), S-adenosyl methionine decarboxylase (SAMDC), spermine synthase (SPMS) and thermospermine synthase (ACL5).

Identified genes in tomato genome were Solyc10g054440.1, Solyc01g110440.2, Solyc04g082030.1, Solyc03g098300.2, Solyc05g005710.2, Solyc04g026030.2, Solyc08g014310.2, Solyc06g053510.2, Solyc06g053520.2, Solyc05g010420.1, Solyc1g010050.2, Solyc02g089610.1, Solyc06g054460.1, Solyc01g080380.3, Solyc03g007240.2, Solyc08g061970.2, Solyc09g075900.2 and Solyc07g041300.1 and based on homology with *Arabidopsis* sqeuences, the candidate genes were named as SolycADC1, SolycADC2, SolycODC1, SolycODC2, SolycSPDS1, SolycSPDS2, SolycSPDS3, SolycSPDS4, SolycSPDS5, SolycSAMDC1, SolycSAMDC2, SolycSAMDC3, SolycSAMDC4, SolycSAMDC5, SolycSPMS, SolycACL5, SolycACL5-Like1, SolycACL5-Like2, respectively.

We have found that there were two homologs of *Arabidopsis* ADC in tomato. Interestingly, *Arabidopsis* genome does not contain any gene for ODC (Hanfrey et al., 2001), whereas ODC is encoded by two genes in tomato. In *A. thaliana*, putrescine is synthesized via the activity of ADC only. Furthermore, tomato genome has five homologs of SPDS compared with single gene in *Arabidopsis*. Similarily, SAMDC is also encoded by five genes in tomato, whereas tomato genome only has single gene for SPMS. In tomato, thermospermine synthase is encoded by three ACL5 homologs whereas *Arabidopsis* has single gene for ACL5.

4.1.1 Conserved motifs and phylogenetic analysis

Conserved domins analysis using InterProScan database revealed the conserved domains and structural features of all identified polyamine biosynthetic genes. By multiple sequence alignment of *Arabidopsis* and tomato polyamine biosynthetic genes, we found that there was about 80% of sequences were conserved between tomato and *Arabidopsis* ADC genes. Similarily, SAMDC genes showed 60-70% conserved sequences, SPDS exhibited 80-90%, while SPMS revealed 90% and ACL5 showed around 80-85 % conserved sequences among both the plants (Supplementary Figure 1). Among the eighteen (18) identified genes, proteins of SolycADC1 and SolycADC2 contains single domain namely Orn/DAP/Arg decarboxylase2, N-terminal (*De-COase2_N*). While SolycODC1 and SolycODC2 contains additionally Orn/DAP/Arg decarboxylase2, C-terminal *(De-COase2_C*) domain besides Orn/DAP/Arg decarboxylase2, N-terminal. SolycSPDS1, SolycSPDS2, SolycSPDS3, SolycSPDS4 and SolycSPDS5, SolycSPMS, SolycACL5 and SolycACL5-Like1 and SolycACL5-Like2 contain two domains which are polyamine biosynthesis domain (*PABS*) and spermidine synthase, tetramerisation domain (*Spermidine_synt_N*) (Figure 4.1). The results indicate that the polyamine biosynthetic genes of same family have conserved domain struture similar to found in *Arabidopsis*.

The gene network involved in polyamine biosynthesis are composed of number of genes from different gene families, namely arginine/ ornithine decarboxylase family, S-adenosyl methionine decarboxylase family, spermidine/ spermine synthases family, which have been characterized at the genome-wide level mainly in *Arabidopsis*. Early steps polyamine biosynthetic genes ADC/ODC and SAMDC belong to arginine decarboxylase and S-adenosyl methionine decarboxylase family, respectively. Whereas,SPDS, SPMS and ACL5 belongs to spermidine/ spermine synthases family.

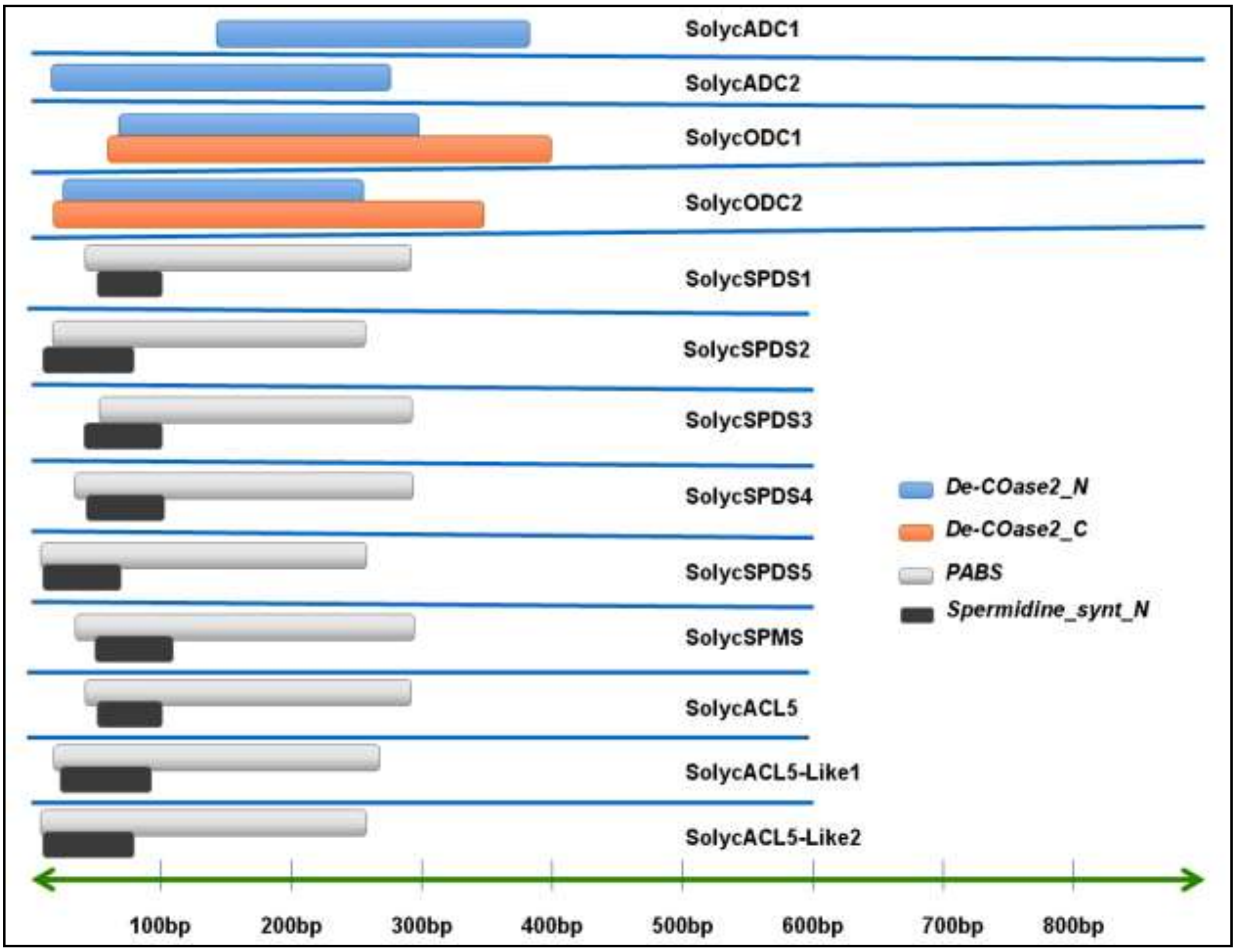


Figure 4.1: Conserved domain structure of polyamine biosynthetic enzymes. Each conserved domain is represented by a colour box.De-Coase2_N for Orn/DAP/Arg decarboxylase2, N-terminal; De-Coase2_C for Orn/DAP/Arg decarboxylase2, C-terminal; PABS for Polyamine biosynthesis domain; Spermidine_synt_N for Spermidine synthase, tetramerisation domain.

To further explore the phylogenetic relationships of the polyamine biosynthetic genes of tomato and *Arabidopsis*, phylogenetic tree was constructed with the polyamines proteins of tomato and *Arabidopsis* (Figure 4.2). The phylogenetic analysis revealed that all the tomato polyamine biosynthetic genes have high sequence similarity with *Arabidopsis* sequences.

Phylogenetic analysis revealed that unlike *Arabidopsis*, tomato genome has two genes encoding ODC. Also additional homolog of ACL5 was found in tomato. Presence of additional homolog of ACL5 in tomato genome may be a consequence of species-specific gene duplication event during evolution after the separation of the ancestors of tomato (Rosids) and *Arabidopsis* (Asterids) about 120 Ma (Consortium 2012).

We observed that identified SolycADC1 and SolycADC2 were showing about 70% sequence similarity with *Arabidopsis* ADC proteins. As there is no ODC reported in *Arabidopsis* yet, we can not find out the sequence similarity of tomato ODC proteins. SolycSAMDC proteins shares about 60% sequence similarity with *Arabidopsis* SAMDC proteins. SolycSPDS showed 60-80% homolgy with *Arabidopsis* SPDS proteins. Similarily SolycSPMS had 80% sequence similarity with *Arabidopsis* SPMS. Whereas, SolycACL5, SolycACL5-Like1 and SolycACL5-Like2 showed 72%, 63% and 58% sequence similarity, respectively, with *Arabidopsis* ACL5 at the protein level.

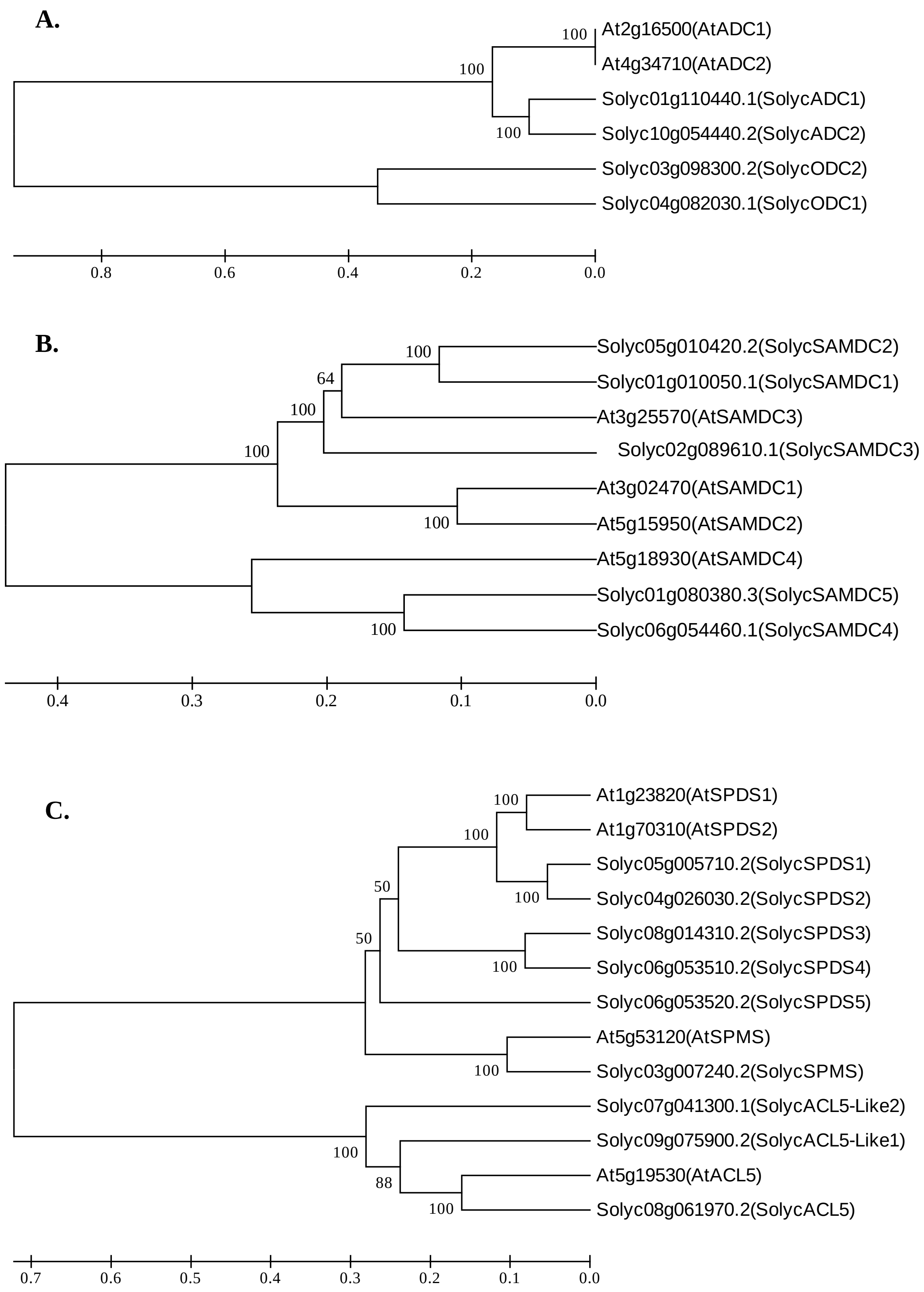


Figure 4.2: Phylogenetic relationships of polyamine biosynthetic genes of tomato (Solyc) with *Arabidopsis* (At). A). Phylogenetic tree of arginine/ ornithine decarboxylase family. B) Phylogenetic tree of S-adenosyl methionine decarboxylase family. C) Phylogenetic tree of spermidine/ spermine synthases family. Phylogenetic tree was constructed using MEGA6 by the Maximum Likelihood method and bootstrap values from 100 replicates are indicated at each node.

4.1.2 Gene structure analysis of candidate polyamine biosynthetic genes

To understand the structural diversity of polyamine biosynthetic genes, we determined the exon-intron boundary of candidate genes by Gene Structure Display Server tool (Hu et al., 2014) (Figure 4.3). Polyamine biosynthetic genes SolycADC1 and SolycADC2, SolycODC1 and SolycODC2 and SolycSAMDC1, SolycSAMDC2, SolycSAMDC3, SolycSAMDC4 and SolycSAMDC5 consisted of one exon and are not interrupted by any intron. SolycSPDS1, SolycSPDS2, SolycSPDS3, SolycSPDS4 and SolycSPDS5 exhibit nine exons and eight introns structure, SolycSPMS, SolycACL5,SolycACL5-Like1 and SolycACL5-Like2 have ten exons and nine introns.

We also analysed the distribution of these genes on different chromosomes (Figure 4.4). SolycADC1, SolycSAMDC1 and SolycSAMDC5 genes were positioned on chromosome1. Gene SolycSAMDC3 was found to be positioned on chromosome 2. SolycODC2 and SolycSPMS genes were located on chromosome 3. SolycODC1 and SolycSPDS2 were located on chromosome 4. SolycSAMDC2 and SolycSPDS1 genes were found on chromosome 5. Genes SolycSPDS4, SolycSPDS5 and SolycSAMDC4 were located on chromosome 6. SolycACL5-Like2 was situated on chromosome 7. SolycSPDS3 and SolycACL5 were located on chromosome 8. Genes SolycACL5-Like1 and SolycADC2 were positioned on chromosome 9 and 10, respectively. Some of the characteristic features of the identified candidate polyamine biosynthetic genes are listed in Table 4.1.

The open reading frame (ORF) length of candidate polyamine biosynthetic genes in tomato ranged from 960bp (SolycACL5-Like2) to 3558bp (SolycADC2), encoding polypeptides ranging from 310 amino acid (SolycSPDS2) to 707 amino acids (SolycADC1). The molecular weight of these proteins ranged from 33.81kDa (SolycSPDS2) to 76.92 kDa (SolycADC1) and the predicted pI varied from 4.81(SolycSPDS1) to 6.40 (SolycODC2).

Table 4.1: Characteristics of polyamine biosynthetic genes in tomato

Gene name	Locus name	Genomic location	ORF length (bp)	Protein		
				Length (aa)	MW (kDa)	pI
SolycADC1	Solyc10g054440.1	SL2.50Ch10:55417815..55419938	2124	707	76.92	5.08
SolycADC2	Solyc01g110440.2	SL2.50Ch01:97145565..97149122	3558	608	66.17	4.95
SolycODC1	Solyc04g082030.1	SL2.50Ch04:65834452..65835747	1296	432	46.65	5.50
SolycODC2	Solyc03g098300.2	SL2.50Ch03:60643404..60644570	1167	389	42.78	6.40
SolycSPDS1	Solyc05g005710.2	SL2.50Ch05:526630..530399	1319	343	37.68	4.81
SolycSPDS2	Solyc04g026030.2	SL2.50Ch04:19662555..19666009	1240	310	33.81	5.14
SolycSPDS3	Solyc08g014310.2	SL2.50ch08:4125709..4129406	1192	340	37.57	6.07
SolycSPDS4	Solyc06g053510.2	SL2.50ch06:36306186..36310983	1337	340	37.48	6.16
SolycSPDS5	Solyc06g053520.2	SL2.50ch06:36312412..36317010	1161	316	35.10	5.09
SolycSAMDC1	Solyc05g010420.1	SL2.50Ch05:4654381..4655463	1083	361	39.75	5.09
SolycSAMDC2	Solyc01g010050.2	SL2.50Ch01:4683610..4687162	1843	365	40.20	5.32
SolycSAMDC3	Solyc02g089610.1	SL2.50ch02:51368627..51369715	1089	362	39.49	4.68
SolycSAMDC4	Solyc06g054460.1	SL2.50ch06:37225779..37226795	1017	338	37.84	5.30
SolycSAMDC5	Solyc01g080380.3	SL2.50ch01:79625044..79626143	1100	330	37.32	5.13
SolycSPMS	Solyc03g007240.2	SL2.50Ch03:1795042..1800986	1708	357	39.27	5.62
SolycACL5	Solyc08g061970.2	SL2.50Ch08:50190804..50195709	1267	350	39.28	5.54
SolycACL5-Like1	Solyc09g075900.2	SL2.50Ch09:67537185..67540591	1232	332	37.39	5.30
SolycACL5-Like2	Solyc07g041300.1	SL2.50ch07:52235923..52239600	960	320	35.96	5.58

ORF**:** Open reading frame; bp:base pair; aa:amino acid; MW: molecular weight; pI: Isoelectric point

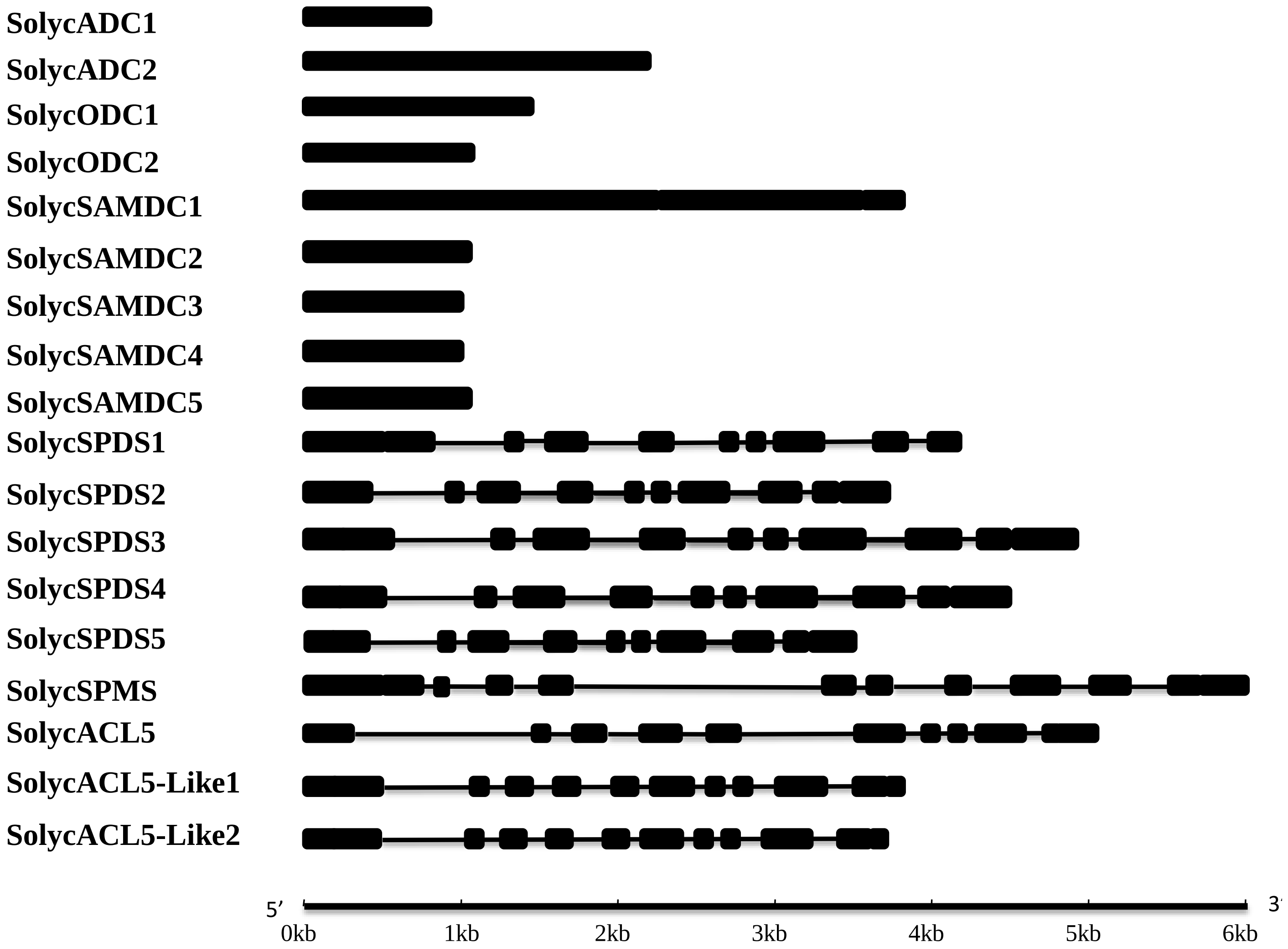


Figure 4.3: Exon-intron structure of tomato polyamine biosynthetic genes. Black boxes and lines indicate exons and intron, respectively.

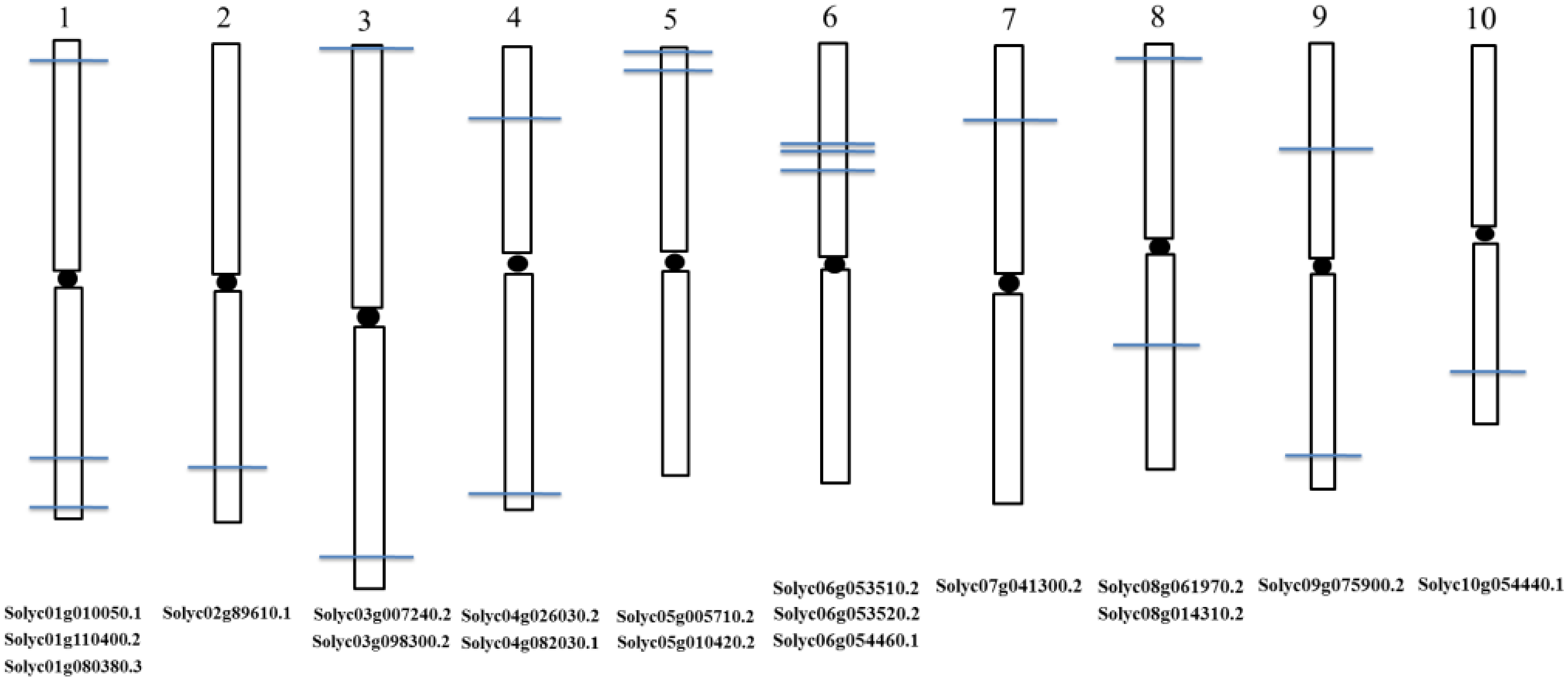


Figure 4.4: Chromosomal distribution of tomato polyamine biosynthetic genes. Vertical bars indicate locus of polyamine biosynthetic genes on tomato chromosomes. Black dots at the center of the bars represent centromere. Blue horizontal lines on the bars indicate position of each gene (gene ID written at the bottom of each bar) in the chromosome. Chromosome number is mentioned at the top of each chromosome.

4.1.3 Identification of putative cis-regulatory elements in the promoter sequences of candidate polyamine biosynthetic genes

To gain further insights into the transcriptional regulations and potential functions of candidate polyamine biosynthetic genes, putative cis-regulatory elements in the promoter sequences were analyzed using Plant CARE database (Lescot et al., 2002). A total of eighty two (82) cis-regulatory elements were identified in the 1kb of genomic sequences upstream of 5' UTR of polyamine biosynthetic genes (Supplementary Table 4.1). 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, Box4, 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 polyamine 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 candidate gene promoters detected to have light and hormone 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 cross-talk with hormones. Presence of several lights and circadian responsive elements suggest that polyamines may also involve in regulation of light responsiveness, photomorphogenetic responses and circadian rhythms. SolycACL5, SolycACL5-Like1 and SolycACL5-Like2 genes promoter have HSE and TC rich repeat 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 (Table 4.2).

Table 4.2: Putative cis-regulatory elements identified in the promoter sequences of tomato polyamine biosynthetic genes

Gene	**Plant developmentrelated cis-elements**	**Stress and hormonal related cis-elements**
SolycADC1	Skn-1-motif;GCN4-motif; Box4	ARE^{4}; HSE; TC-rich repeats; TCA-element; ; LTR; ELI-box3
SolycADC2	Skn-1-motif; GCN4-motif; Box4	ARE^{4} ; HSE; TC-rich repeats; TCA-element; ; LTR; ELI-box3
SolycODC1	Skn-1-motif^{2}; GCN4-motif; Box4^{7}	ABRE; ARE; Box-W1; CGTCA-motif^{2}; HSE; MBS; HSE; TC-rich repeats; ; LTR;TGACG-motif^{2}
SolycODC2	as-2-box ^{2}; O_2-site; Box4	ABRE; Box-W1^{2}; CGTCA-motif; TC-rich repeats ; GARE-motif; TC-rich repeats;
SolycSAMDC1	GCN4-motif; circadian; Box4	ARE; CGTCA-motif; TCA-element^{3}; GARE-motif; GC-motif; HSE; MBS; TC-rich repeats; TGACG-motif; GARE-motif; EIRE
SolycSAMDC2	Skn-1-motif; GCN4-motif;CAT-box; Box4^{3}	CGTCA-motif^{2}; MBS; TGACG-motif^{2}; TC-rich repeats^{2} ; TCA-element^{2}; ; LTR
SolycSAMDC3	Skn-1-motif;GCN4-motif^{2}	ARE^{4}; MBS; P-box; TC-rich repeats; GARE-motif; EIRE
SolycSAMDC4	GCN4-motif; circadian^{2} ; as-2-box	ARE; CGTCA-motif; HSE^{3}; MBS; TGACG-motif;TC-rich repeats^{2}; TCA-element; GARE-motif; EIRE
SolycSAMDC5	Skn-1-motif;	ARE; Box-W1^{2}; CGTCA-motif; MBS$^{2\,;}$ TGACG-motif; TC-rich repeats; TCA-element; ELI-box3
SolycSPDS1	Skn-1-motif; circadian; Box4	Box-W1^{2} ; HSE^{3}; TC-rich repeats; TCA-element
SolycSPDS2	Skn-1-motif^{2} ; circadian; O_2-site; CAT-box;	ABRE; ARE; ERE; TC-rich repeats^{2}; TCA-element

	Box4	
SolycSPDS3	Skn-1-motif^{2} ;CCGTCC –box; circadian; O_2-site; Box4^{4}	TC-rich repeats
SolycSPDS4	Skn-1-motif ;CCGTCC –box; HD-Zip1,HD-Zip2; circadian^{2}; O_2-site; Box4	ABRE; CGTCA-motif; HSE^{2};TGACG-motif;
SolycSPDS5	Skn-1-motif; Box4^{4}	MBS; ERE; TC-rich repeats^{3};TCA-element
SolycSPMS	Skn-1-motif; circadian; Box4^{2}	ABRE; ARE; TCA-element
SolycACL5	Skn-1-motif; circadian; Box4^{3}	ARE; HSE^{2} ; TC-rich repeats; LTR
SolycACL5-Like1	Skn-1-motif^{5} ; circadian ; O_2-site; CAT-box; Box4^{4}	HSE^{2}; MBS; TC-rich repeats
SolycACL5-Like2	Skn-1-motif^{2}; Box4^{4}	ARE; HSE; MBS;TC-rich repeats; GARE-motif
Note: Number shown in superscript is total number of each cis-element present, whereas cis-elements without any superscript indicate only one copy of corresponding cis-element. Skn-1-motif and GCN-4-motif, involved in endosperm expression; as-2-box, involved in shoot specific expression and responsiveness; O_2-site, involved in zein metabolism regulation; circadian, responsible for circadian control; CAT-box, related to meristem expression; ACE- light responsive element. ABRE, abscisic acid responsive element; ARE, essential for anaerobic induction; Box-W1, fungal elicitor responsive element; CGTCA-motif, involved in methyl-jasmonic acid (MeJA) response; HSE, heat stress responsive element; MBS, involved in drought inducibility; MBSI, MYB binding site involved in flavonoid biosynthetic gene regulation; TGACG-motif, involved in MeJA responsiveness; ERE, Ethylene responsive element; TCA-element, salicylic acid responsive element; TC-rich repeats, involved in defense and stress responsiveness; GARE- motif, gibberellin responsive element; LTR, low temperature responsive element; GC-motif, involved in anoxic specific inducibility; P-box, gibberellin responsive element; EIRE, elicitor responsive element.		

4.2 Expression patterns of candidate polyamine biosynthetic genes during development of tomato plants

To gain insights into the function of these genes in plant develoment, their relative expression pattern was analyzed in different tissues of tomato at different developmental stages using qRT-PCR. Tissue-specific expression pattern was analysed in vegetative tissues such as roots, cotyledons, hypocotyl of 15 dag young plants and both vegetative and reproductive tissues such as stems, leaves, flowers and fruits of 60 dag old mature tomato plants. For expression pattern analysis, we have taken tissue samples at different developmental stages, viz., four stages of leaves, Immature leaves (IL), Young leaves (YL), Mature leaves (ML) and Senescent leaves (SL); two layers of stem, Outer layer of stem (OL of St) and Inner layer of stem (IL of St); Abscission zone of flowers (AZ); eight developmental stages of flowers, viz., Developing flower bud 1 (FL1), Developing flower bud 2 (FL2), Developing flower bud 3 (FL3), Immature flower 1 (FL4), Immature flower 2 (FL5), Immature flower 3 (FL6), Mature flower (FL7), Senescent flower (FL8) and eight different developmental stages of fruits, namely Immature fruit 1 (FR1), Immature fruit 2 (FR2), Immature fruit3 (FR3), Mature green fruit (FR4), Mature breakerfruit (FR5), Mature turning fruit (FR6), Mature pink fruit (FR7), Mature red fruit (FR8). Our qRT-PCR analyses showed that all the candidate genes have considerably distinct expression patterns in different tissues of both vegetative and reproductive developmental stages of tomato plants.

SolycADC1 showed substantial expression in roots, hypocotyl and cotyledons of 15 dag old plants. In the case of leaf development, it had lower expression during the immature and young leaves, while its expression reached to highest level in mature leaves and then it decreased gradually in leaves undergoing senescence, suggesting that SolycADC1 may have some important role during the maturation of leaves. SolycADC1 also showed higher expression in the outer layer of stem (OL of St) which include cortex and phloem than the inner layer of stem (IL of St:woody) (Figure 4.5A). At the time of reproductive tissues development, it had very high expression level during flower abscission zone formation. Developing flower bud (FL1) has the highest expression which decreased slowly during the successive stages of flower development till immature flower formation (FL6). After that its expression again increased to some extent when the flower undergone maturation and senescence. Thus, it is presumed that SolycADC1 may be involved in different stages of flower development. In the case of fruit tissues, it has considerable expression level during immature fruit formation stages (FR1 and FR2). Subsequently, it has very low expression level during the maturation stages of fruit development (FR3-FR8), suggesting that the SolycADC1 gene might play some role in developing fruits (Figure 4.5B).

In contrast, SolycADC2 has relatively very low expression level in roots, hypocotyl and cotyledons. Moreover, it has highest expression during immature leaf stage and its expression gradually decreased from immature leaf stage to leaves undergoing senescence. So, it can be assumed that SolycADC2 has some role during the early developmental stages of leaves. Also, it has more expression in outer layer of stem (OL of St) than the inner layer (Figure 4.5C). During reproductive tissue development, it has differential expression patterns in different stages of flower development. Abscission zone has slightly higher expression whereas during various flower developmental stages from flower bud formation to flower maturation its expression was increased and also decreased in few stage ssuggesting SolycADC2 might be participating in flower development. In fruit developmental stages, SolycADC2 has highest expression during the immature fruit formation stage (FR1). While, in the other developental stages of fruits (FR2-FR8), it has lower expression indicating that SolycADC2 is not likely to be involved in fruit development (Figure 4.5D).

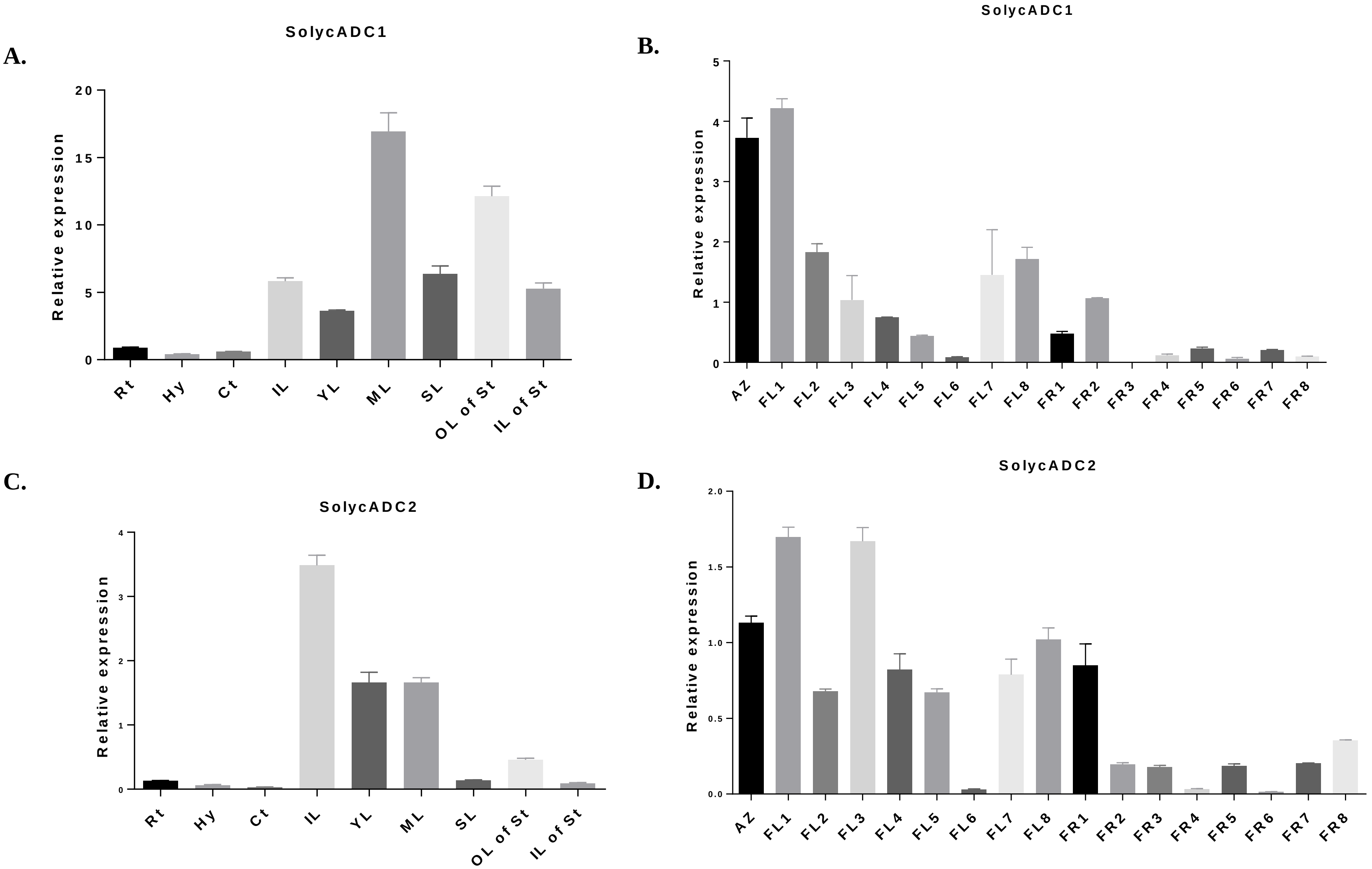


Figure 4.5: **Expression pattern analysis of SolycADC1 (A and B) and SolycADC2 (C and D) expression in different vegetative and reproductive tissues of tomato plants, respectively.** Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

Expression analysis of SolycODC1 revealed that it has very high expression in the root tissues as compared to hypocotyl and cotyledons, suggesting, it might have some role during root development. In the case of various leaf developmental stages, SolycODC1 has highest expression in immature leaves which was decreased in young leaves. Whereas, its expression was not detected in mature and senescence stages of leaves showing its role during early stages of leaf developmental. SolycODC1 also displayed higher expression in both the layers of stem suggesting it might be involved in the development of stem (Figure 4.6A).

During the reproductive development, SolycODC1 has very low expression level at the time of abscission zone formation. Qunatitaive RT-PCR analysis of various flower developmental stages showed differential expression pattern having highest expression at the time of immature flower development (FL6). So, it can be speculated that SolycODC1 does not play any role during early flower development whereas during later flower developmental stages it might be invoved. In contrast to flower development, SolycODC1 displayed higher expression during early stages of fruit development as compared to the later stages, so, it might be possible that this gene play some role during initial stages of fruit development (Figure 4.6B).

Unlike SolycODC1, SolycODC2 did not show considerable expression in roots. Also, it did not show any expression in hypocotyl and cotyledons. In the case of leaf developmental stages, immature and young leaves has very low expression level of SolycODC2, whereas mature and leaves undergoing senescence did not show any expression of SolycODC2. As compared to other vegetative tissues, it show some expression in the outer layer of stem, suggesting SolycODC2 might not be involved in vegetative tissues development (Figure 4.6C).

In reproductive tissues, SolycODC2 showed increasing expression during the immature flower developmental stages (FL1-FL3), then it expression drastically reduced during the successive developmental stages of flower (FL4-5) and again it increased at the stage of mature flower (FL6) and then again it reduced to very low level at the time of senescence stage (FL7-8). Contrary to this, SolycODC2 showed highest expression level only during the initial immature fruit stage (FR1). Whereas, it did not show any considerable expression in other developmental stages of fruit, suggesting it might not involved in the later stages of fruit development (Figure 4.6D).

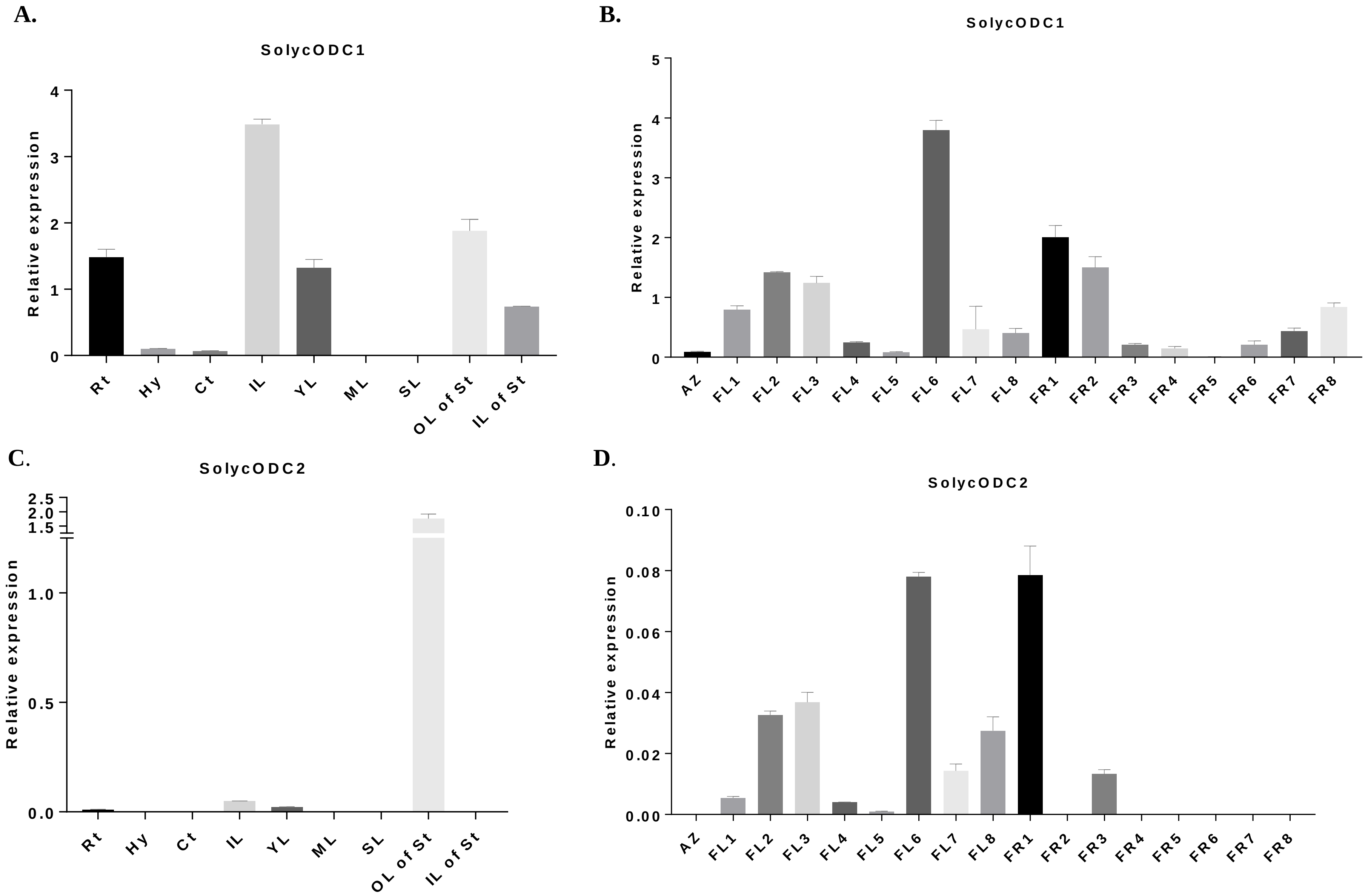


Figure 4.6: Quantitative RT-PCR analysis showing expression of SolycODC1 (A and B) and SolycODC2 (C and D) in vegetative and reproductive tissues of tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

Amongst the memebers of S-adenosyl methionine decarboxylase gene family SolycSAMDC1 showed relatively much higher expression level than any other memebrs of the family. SolycSAMDC1 showed relatively lower expression in roots, hypocotyl and cotyledons of 15 dag old tomato seedlings, whereas in leaf, it showed highest expression in immature leaves compared to other developmental stages of leaves where its showed somewhat similar expression level. Also SolycSAMDC1 strongly expressed in both the inner and outer layer of stem tissues (Figure 4.7A). In reproductive tissues it showed considerable expression level in abscission zone of flower pedicel. While its expression decreased from developing flower bud (FL1) to immature flower stage (FL6). After that its expression again increased to some extent during later stages of flower development. During fruit development, SolycSAMDC1 showed highest expression in mature breaker fruit stage (FR5). During other fruit developmental stages it also showed higher expression, signifying that SolycSAMDC1 might be playing some role during vegetative tisssues development in plants (Figure 4.7B).

As compared to SolycSAMDC1, SolycSAMDC2 showed very little expression in roots, hypocotyl and cotyledons. During leaf development expression of SolycSAMDC2 increased from immature to mature leaves and then again decreased in leaves undergone senescence. This indicates that SolycSAMDC2 is involved in development of leaves. In the case of stem, Inner layer has higher expression as compared to outer layer, in fact it showed highest expression among all the vegetative tissues analyzed. So, it could be predicted that SolycSAMDC2 might be involved in stem tissues development (Figure 4.7C).

SolycSAMDC2 showed very little expression in abscission zone. Also during flower developmental stages, SolycSAMDC2 showed decreasing expression from developing flowering bud stage (FL1) to immature flower stage (FL6). Whereas, its expression relatively increased at the stage of flower senescence (FL8). Furthermore SolycSAMDC2 showed differential expression pattern during the development of fruits. It was observed to be increased and then decreased at the different developmental stages of fruits (Figure 4.7D).

During vegetative development, SolycSAMDC3 showed expression in stem tissues only. Outer layer of stem showed more expression than the inner layer, suggesting its role during stem tissues development. Other vegetative tissues did not show any expression for SolycSAMDC3 (Figure 4.8A). Similarly, in reproductive tissues, high expression of SolycSAMDC3 was only obserevd at the immature fruit stage (FR1). After that it did not show any significant expression in other developing fruit stages (Figure 4.8B).

Similar to SolycSAMDC3, SolycSAMDC4 also showed expression in stem tissues only, but in this case inner layer of stem has higher expression as compared to outer layer. In other vegetative tissues, SolycSAMDC4 did not show any expression suggesting that it may not be important in these tissues development (Figure 4.8C). Also in reproductive tissues, SolycSAMDC4 showed some expression during later stages of flower development i.e., immature and mature stages (FR5-FR7). It did not show any expression during the fruit development stages (Figure 4.8D).

Similarily, SolycSAMDC5 also showed some expression in the inner layer of stem, whereas in other vegetative tissues its expression could not be detected (Figure 4.9A). In case of reproductive tissues, SolycSAMDC5 was specifically expressed during developing flower bud stage (FL2), suggesting its specific role only in stem tissues and early stages of flower development (Figure 4.9B).

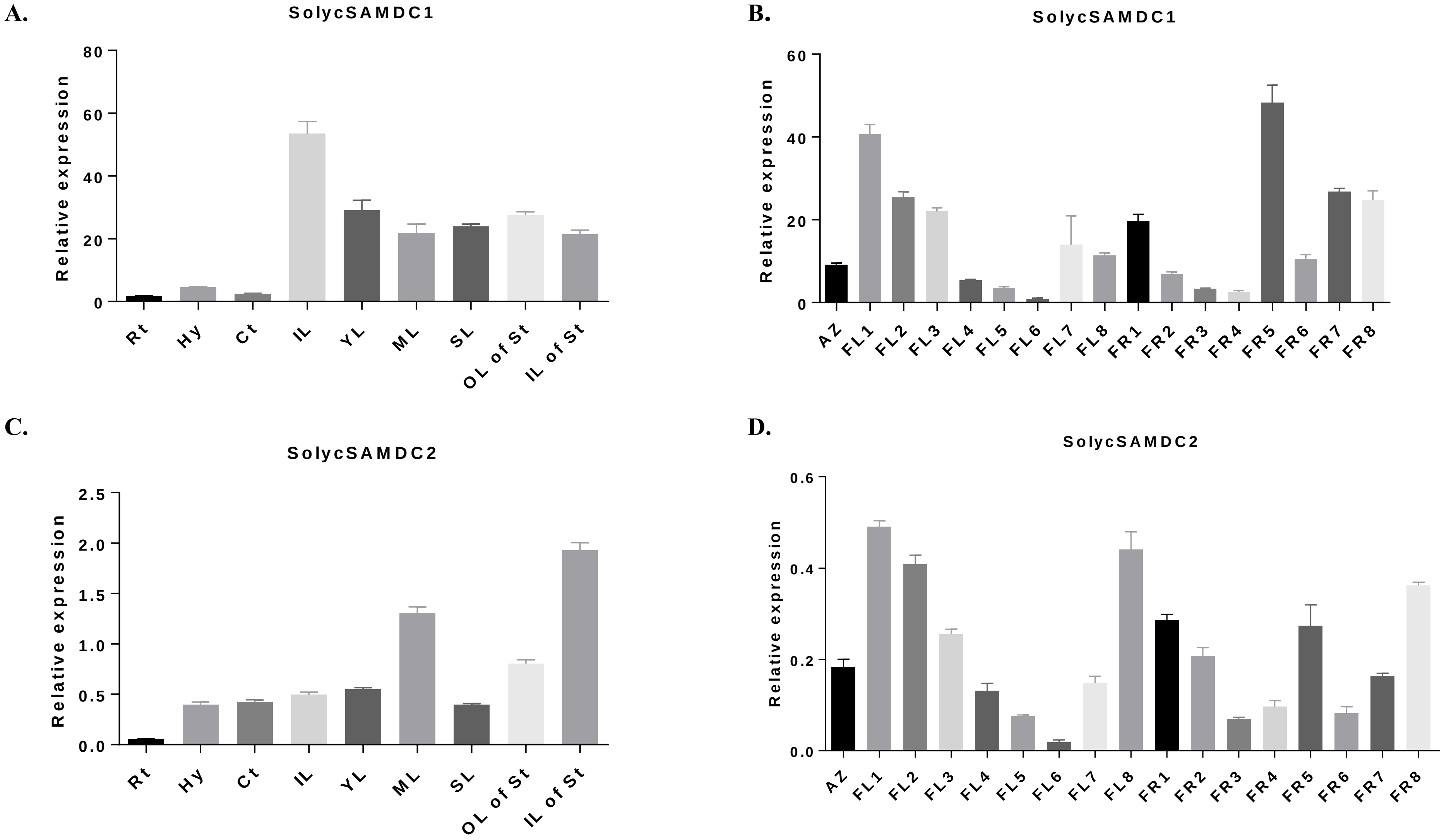


Figure 4.7: **Real time expression analysis of SolycSAMDC1 (A and B) and SolycSAMDC2 (C and D) in vegetative and reproductivetissues, respectively.** Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

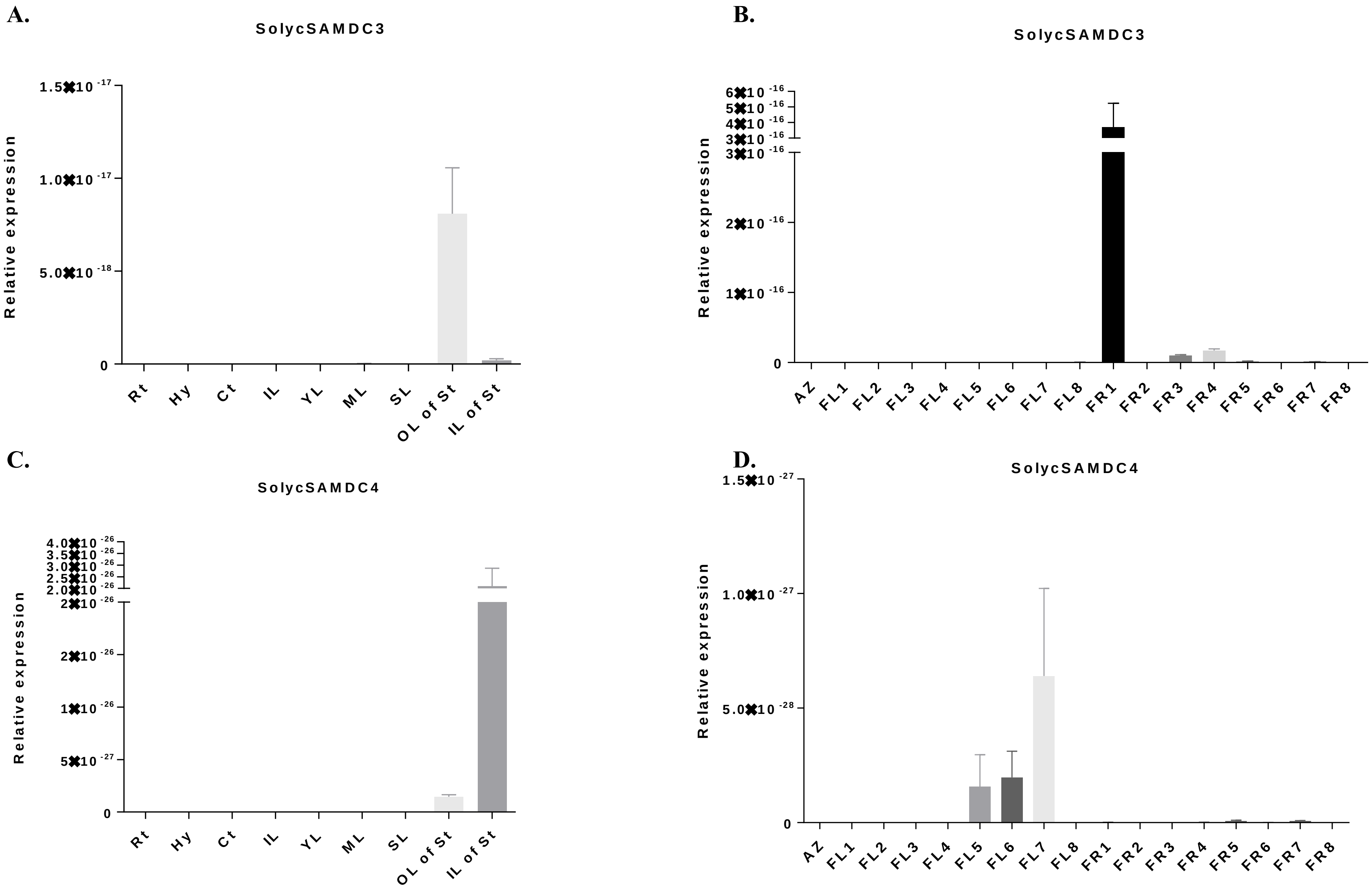


Figure 4.8: Real time expression pattern analysis of SolycSAMDC3 (A and B) and SolycSAMDC4 (C and D) F) during vegetative and reproductive tissues development in tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

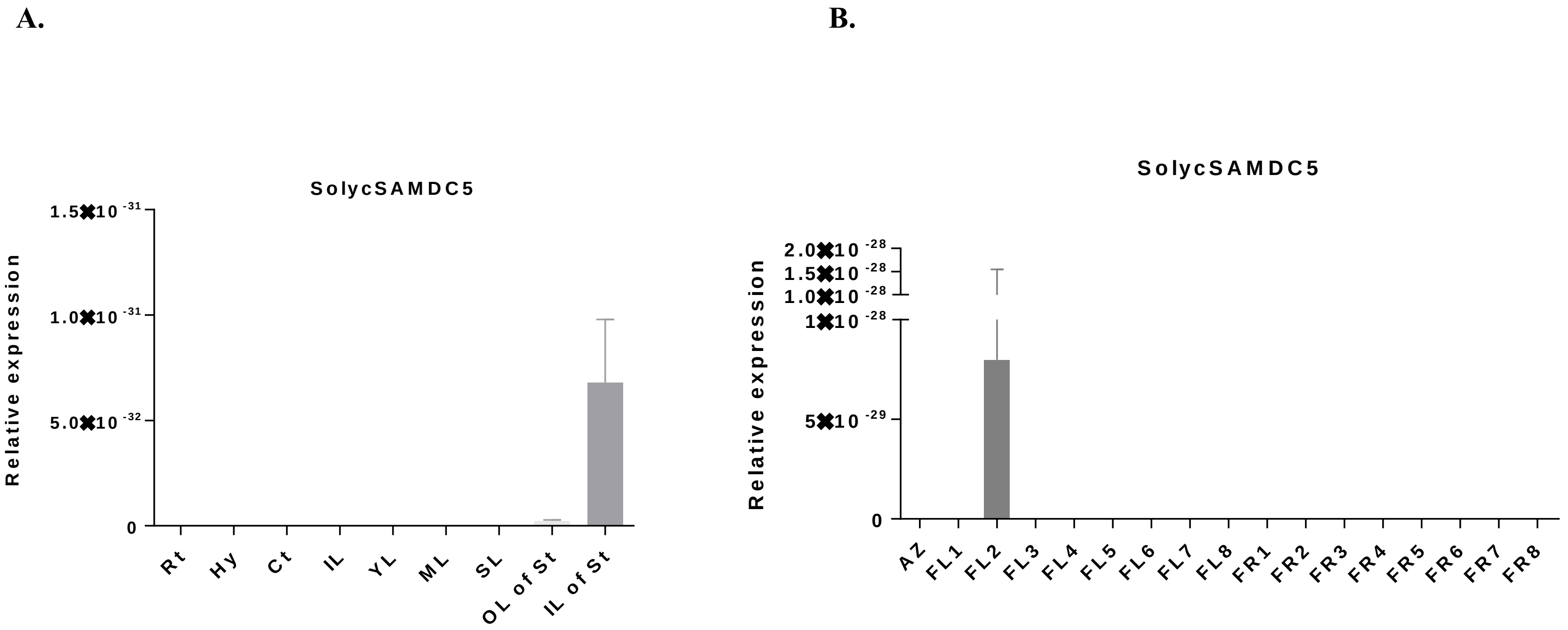


Figure 4.9: Real time expression pattern analysis of SolycSAMDC5 (A and B) during vegetative and reproductive tissues development in tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

Candidate spermidine biosynthetic gene, SolycSPDS1 displayed strong expression in vegetative tissues like root, hypocotyl and cotyledons. Whereas, in leaf tissues, it was highly expressed in immature leaves as compared to other stages of leaves. Stem tissues also have its stronger expression, where outer layer of stem showed relatively higher expression than inner layer (Figure 4.10A). Therefore, it is likely that SolycSPDS1 may be involved in development of vegetative tissues of tomato. Additionally, SolycSPDS1 also showed higher expression in the abscission zone. During the flower development, higher expression of SolycSPDS1 was observed at early stages of flower development i.e., developing flower bud stages (FL1-2). After that it decreased in subsequent stages indicating its role during early stages of flower development. At the time of fruit development, SolycSPDS1 has consistent expression throughout all the stages except in mature red fruit stage (FR8), where its much higher expression was obserevd indicating its involvement in maturation or ripening of fruits (Figure 4.10B).

SolycSPDS2 also has the substantial expression in the root, hypocotyl and cotyledons. Also, in the leaf samples, it showed higher expression in the immature leaves compared to other stages of leaf developmental. In the case of stem tissues, both the layer has strong expression but outer layer displayed relatively more expression of SolycSPDS2 (Figure 4.10C). In flower tissues, SolycSPDS2 displayed the similar pattern as that of SolycSPDS1. Developing flower buds (FL1-2) has the higher expression level among all stages of flower development. In fruit tissues, initial stages of fruit development i.e., immature fruit stages (FR1-2) has the higher expression than that of other stages, indicating SolycSPDS2 might have some role during the initial stages of both flowers and fruits development (Figure 4.10D).

Another member of spermidine/ spermine synthases gene family, SolycSPDS3 showed expression only in some of the vegetative and reproductive tissues during development. Cotyledons and inner layer of stem displayed some level of expression of SolycSPDS3 in vegetative tissues (Figure 4.11A), however in the case of reproductive tissues, some late stages of flowers and initial stages of fruit development showed some expression of SolycSPDS3 (Figure 4.11B).

Two other candidate spermidne biosynthetic genes, SolycSPDS4 (Figure 4.11 C-D) and SolycSPDS5 did not show any significant expression during vegetative and reproductive tissues development in tomato plant (Figure 4.12A-B), indicating that they might not be involved in development of tomato plants.

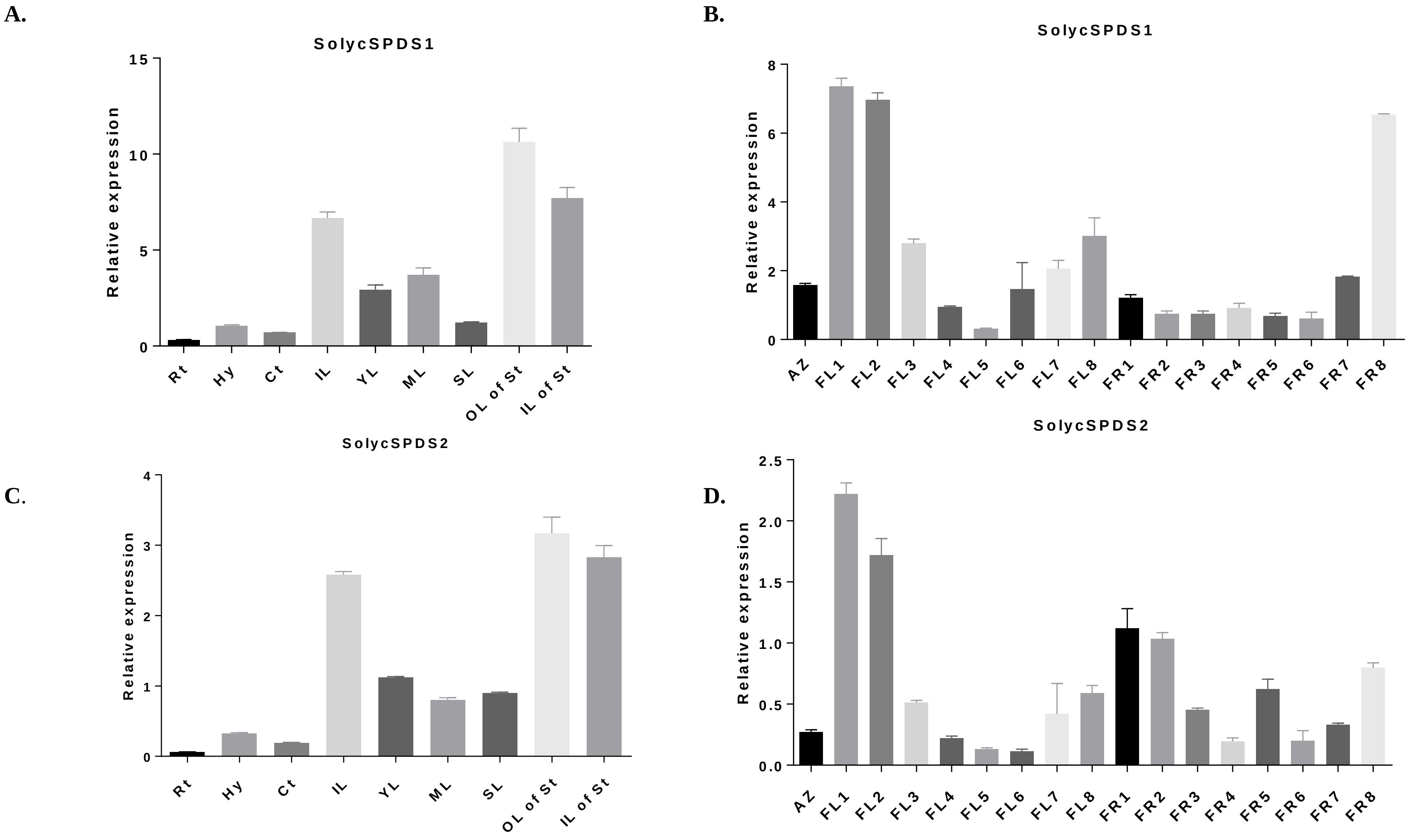


Figure 4.10: Quantitative RT-PCR analysis showing expression of SolycSPDS1 (A and B) and SolycSPDS2 (C and D) in vegetative and reproductive tissues development of tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

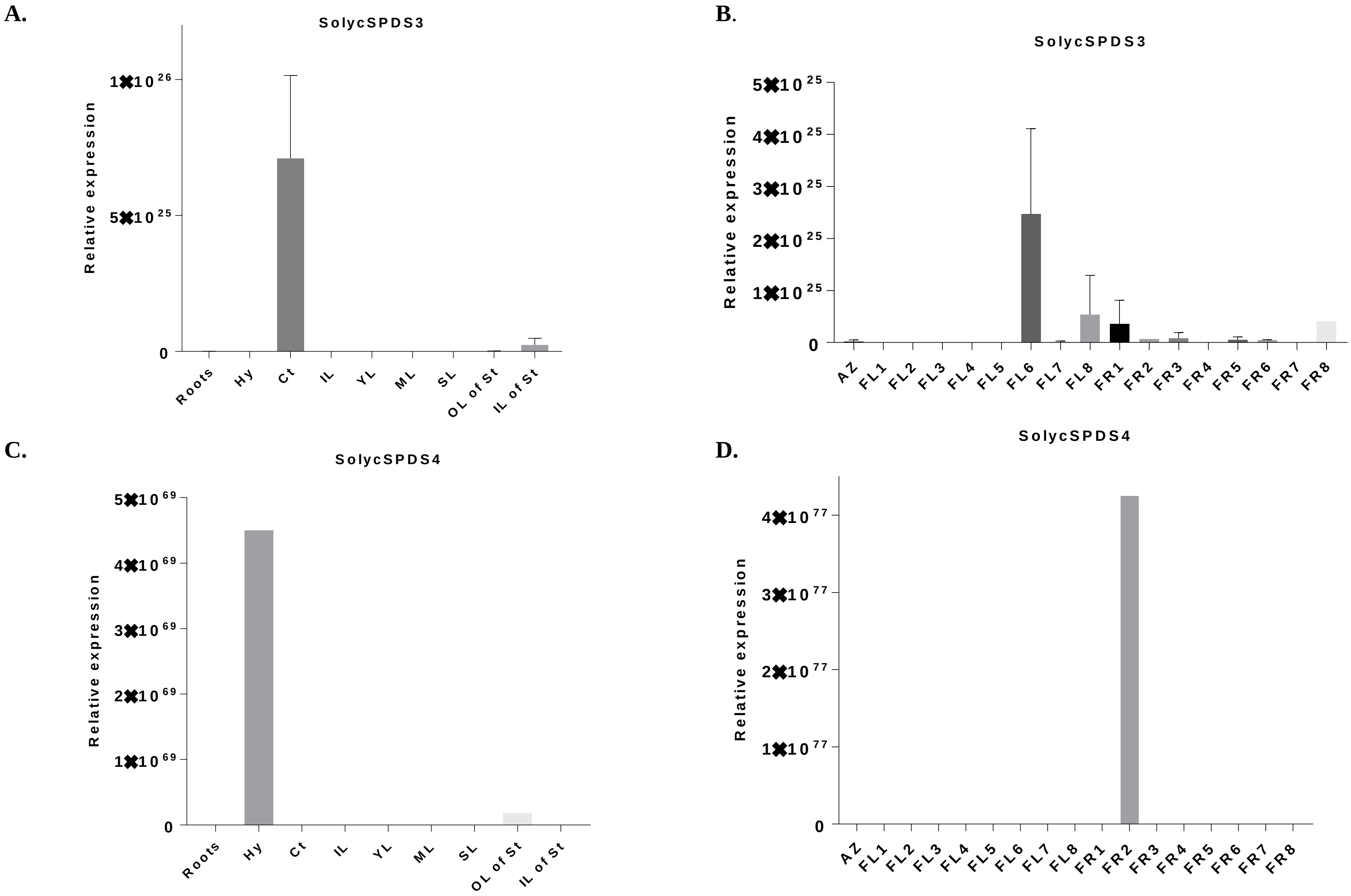


Figure 4.11: Expression patterns of SolycSPDS3 (A and B) and SolycSPDS4 (C and D) in developing vegetative and reproductive tissues of tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

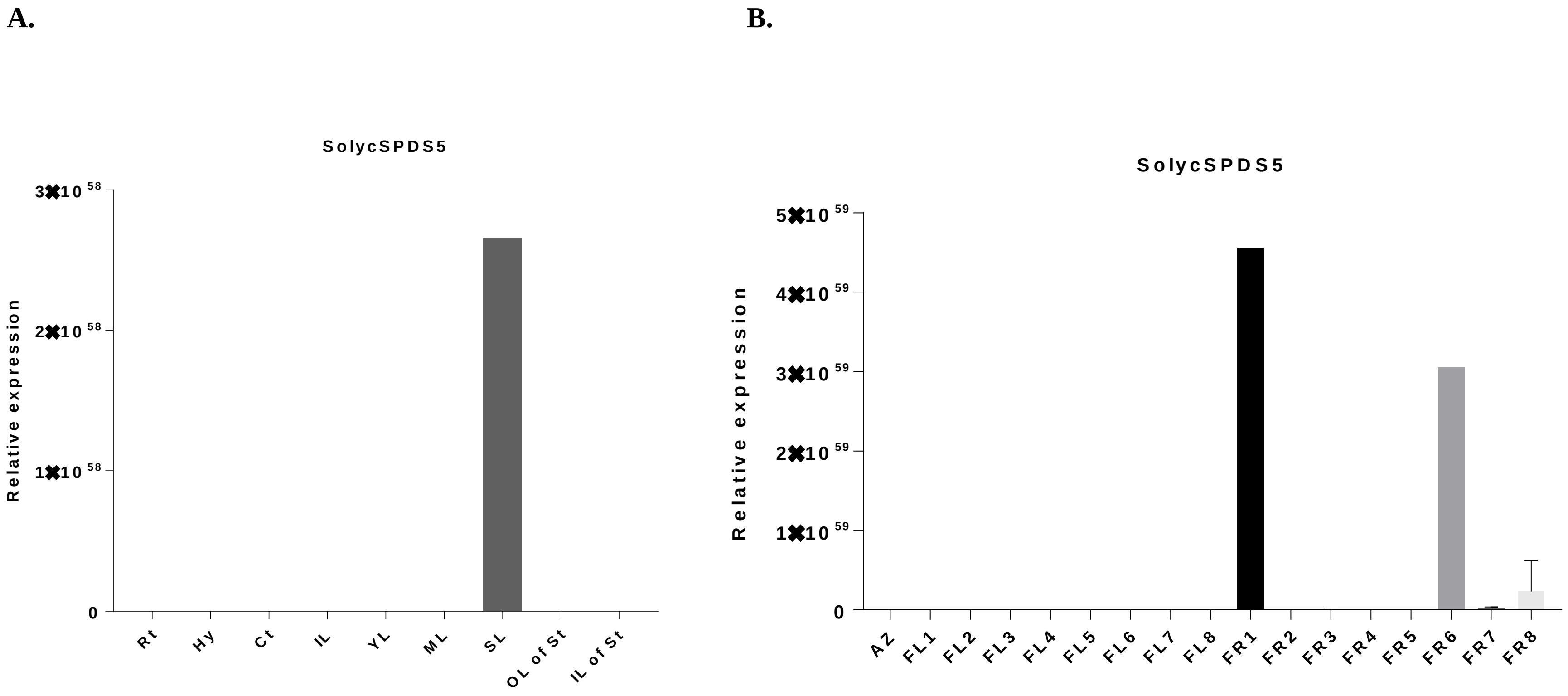


Figure 4.12: Expression patterns of SolycSPDS5 (A and B) in developing vegetative and reproductive tissues of tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

Spermine biosynthetic gene, SolycSPMS displayed significant expression in roots, however, hypocotyl and cotyledons relatively have lower expression. During the developmental stages of leaves, comparativeley higher expression of SolycSPMS was observed in the immature leaves followed by in young leaves and least in mature leaves. Interestingly, its expression was increased in leaves undergoing senescence. In stem tissues its strong expression was noticed in both the layers having sligtly more in inner layer (Figure 4.13A). Reproductive tissues showed very low expression of SolycSPMS during the initial stages of flower development, whereas in fruit tissues its highest expression was onserved in mature turning fruit stage (FR6) (Figure 4.13B). Our expression analysis suggest that probably SolycSPMS involved in vegetative tissues development and in some late stages of fruit development.

Candidate thermosperime synthase gene, SolycACL5 showed its highest expression in roots among all vegetative tissues. Besides these, hypocotyl and cotyledons also showed higher expression of SolycACL5. Whereas, its expression in leaves was observed to be gradually decreasing as the development of leaves progresses from immature stage to senescence stage. In case of stem, expression of SolycACL5 was observed more in inner layer of stem (Figure 4.13C). This indicates that SolycACL5 may play important roles in development of roots, leaves and stem tissues. Furthermore, in abscission zone of flower pedicel also it has stronger expression. During reproductive tissues development, its expression was obseved to be higher in flower bud development stages (FL1-3). After that its expression gradually decreased during later stages of flower development. A comparatively lower but consistent expression of SolycACL5 was observed in all developmental stages of fruits (Figure 4.13D).

Paralog of SolycACL5, SolycACL5-Like1 also showed strong expression in roots and hypocotyl. In leaves, its higher expression was observed in immature leaves as compared to other leaf development stages. Like SolycACL5, its expression was also observed to be gradually decreasing from immature leaf stage to leaves undergoing senescence. A similar expression pattern to that of SolycACL5 was also seen in stem tissues, inner woody layer has higher expression level of SolycACL5-Like1 than the outer layer (Figure 4.14A). In the case of reproductive tissues, SolycACL5-Like1 showed considerable expression in the abscission zone of flower pedicel.Whereas, its expression was found to be gradually decreasing from the developing flower bud stage (FL1) to immature flower stage (FL6), suggesting its role during initial stages of flower development. Like SolyACL5, it also has almost similar expression pattern during different fruit developmental stages (Figure 4.14B). Expression patterns indicate that both the close paralogs might be participating in vegetative tissues devlopment of tomato plants.

Another paralog of SolycACL5, SolycACL5-Like2 has relatively higher expression in roots rather than hypocotyl and cotyledons. Developmental stages of leaves showed highest expression of SolycACL5-Like2 during the mature stage of leaves. Whereas, in stem tissues it expression was not detectable suggesting its lesser involvement in vegetative tissues development (Figure 4.14C). In developing reproductive tissues, it has higher expression during initial stages of flowers (FL1 and FL2) and fruits development (FR2) (Figure 4.14D).

Thus, the real time expression analysis shows that most of the identified candidate polyamine biosynthetic genes are differentially expressed in different developing vegetative and reproductive tissues of tomato plant.

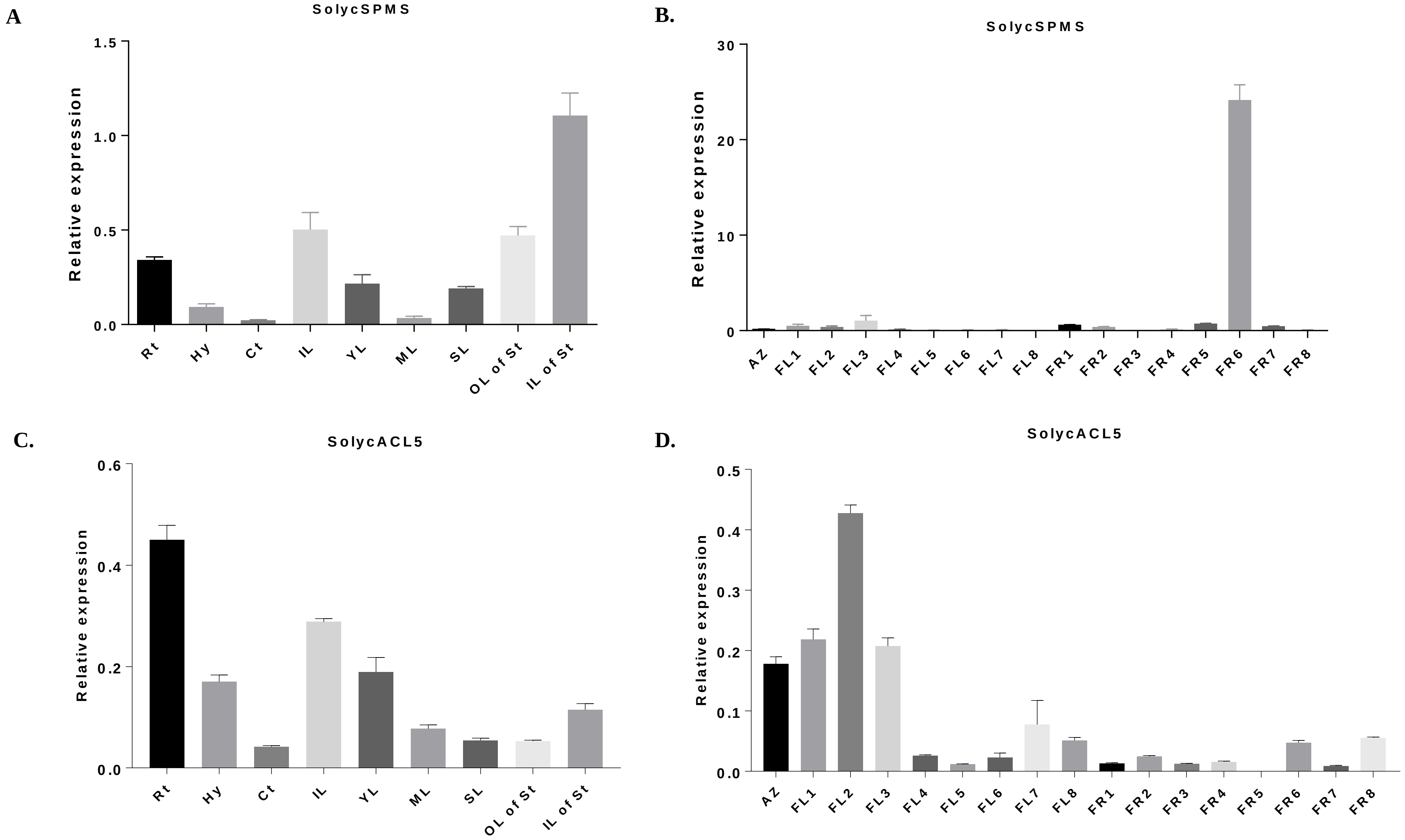


Figure 4.13: Real time expression analysis of SolycSPMS (A and B) and SolycACL5 (C and D) in developing vegetative and reproductive tissues of tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

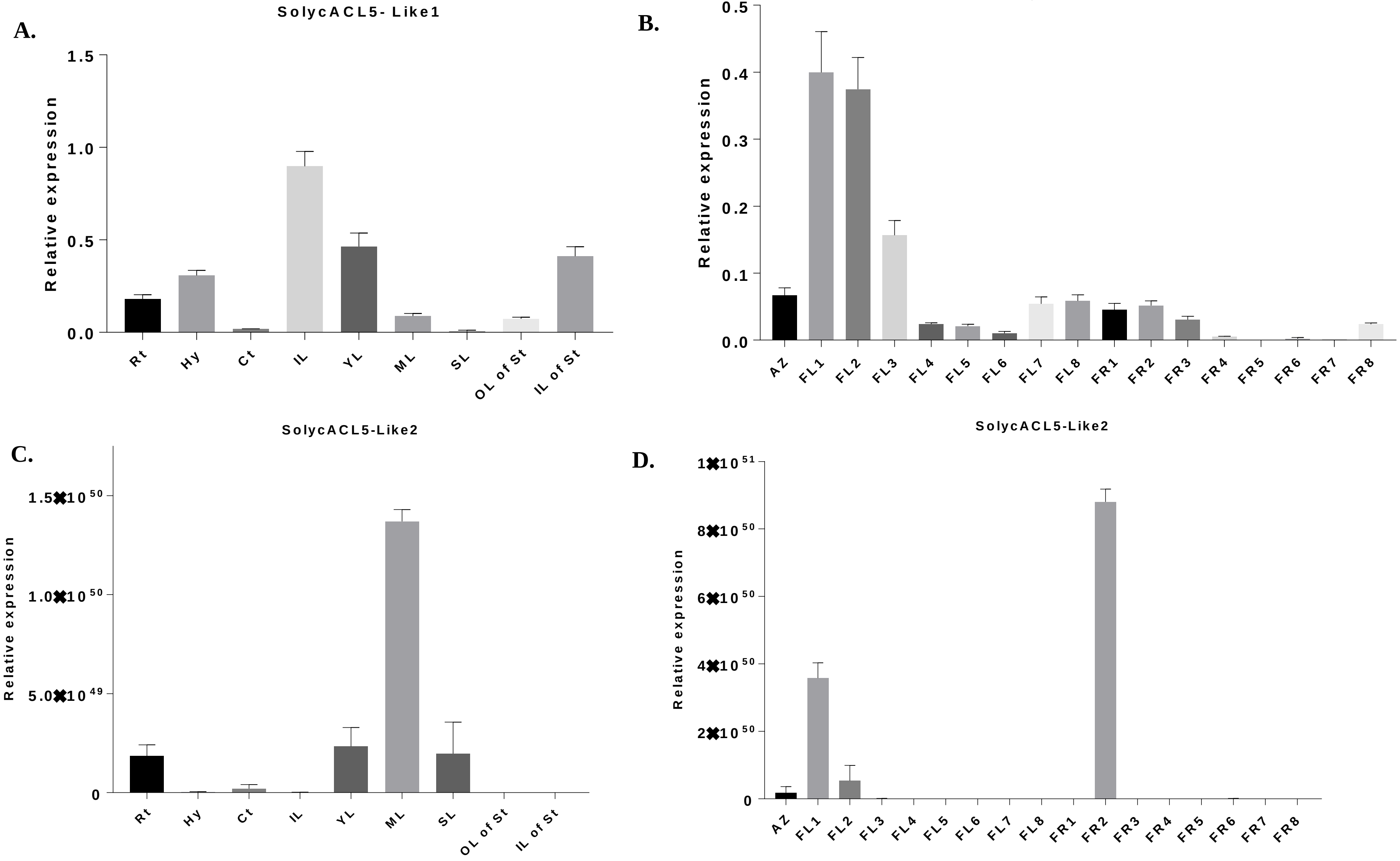


Figure 4.14: Real time expression analysis of SolycACL5-Like-1 (A and B) and SolycACL5-Like2 (C and D) in developing vegetative and reproductive tissues of tomato, respectively. Values are mean± SEM of three biological replicates. Rt, root; Hy, hypocotyl; Ct, cotyledon; IL, immature leaf; YL, young leaf; ML, mature leaf; SL, senescent leaf; OL of St, Outer layer of stem; IL of St, Inner layer of stem; AZ, abscission zone; FL1, developing flower bud 1; FL2, developing flower bud 2; FL3, developing flower bud 3; FL4, immature flower 1; FL5, immature flower 2; FL6, immature flower 3; FL7, mature flower; FL8, senescent flower; (FR1), immature fruit 1; FR2, immature fruit 2; FR3, immature fruit 3; FR4, mature green fruit; FR5, mature breaker fruit; FR6, mature turning fruit; FR7, mature pink fruit; FR8, mature red fruit.

4.3 Expression patterns of candidate polyamine biosynthetic genes during stress responses in tomato

To understand the roles of candidate polyamine biosynthetic genes during different stresses, expression pattern of these genes in response to various stress conditions have been analysed. We have divided stress treatments in to three categories namely, physical stress, hormone treatmentsand chemical treatements. In the physical stress we have grown the tomato plants under heat, cold, UV-C, drought and flood conditions. For wounding, plants leaves were mechanically injured with needles. In plants, abscissic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) acts both as plant growth regulators as well stress responsive phytohormones. Therefore to see their effects on expression of polyamine biosynthetic genes we have treated the plants with these phytohormones in certain concentrations. Under the chemical treatments, we have used certain conentrations of NaCl, mannitol, methylviologen (MV), rosebengal (RB) and fumonisin B1 (FB1) and grown the tomato plants for 12 and 24 hours durations. It is known that mannitol induces osmotic stress, NaCl induces salt stress, methyl viologen and rose bengal generates oxidative stress to the plants. Wounding induces herbvory respones, while Fumonisin B1 is a known fungal toxin cause toxicity in plants.We performed the quantitative real time expression analysis to understand the effects of these stresses on the expression of polyamine biosynthetic genes and observed that most of the candidate genes were differentially expressed.

Quantitative real time expression analysis showed that SolycADC1 was significantly upregulated by heat, cold, UV-C, flood, drought and wounding. While there was no significant change in expression was obsereved by the hormone treatments. SolycADC1 expression was downregulated by mannitol treatment for both 12 and 24 h,wheras its expression was upregulated by RB and FB1 treatments for 24 h duration (Figure 4.15). SolycADC2 also displayed significant upregulation during heat, cold, UV-C, drought, flood, wounding and FB1 treatment for 12 h.Whereas it expression was found to be downregulated by ABA, JA, SA, NaCl and mannitol treatments for 12 h and mannitol and MV treatments for 24 h (Figure 4.16).

Expression of SolycODC1 was significantly upregulated by heat, cold, UV-C, drought, flood and wounding. Its expression was significantly downregulated by all the hormonal treatments but reduced significantly to very low level by JA. Also its downregulated expression was observed by mannitol treatment for 12 h and 24 h as well MV and RB treatment for 24 h, whereas its expression was significantly upregulated by MV, RB and FB1 treatments for 12 h. In contrast to SolycODC1, SolycODC2 expression was highly downregulated by all the physical treatments and highly upregulated by FB1 treatment for 24 h, while it remain unaffected by hormone treatments (Figure 4.17 and 4.18).

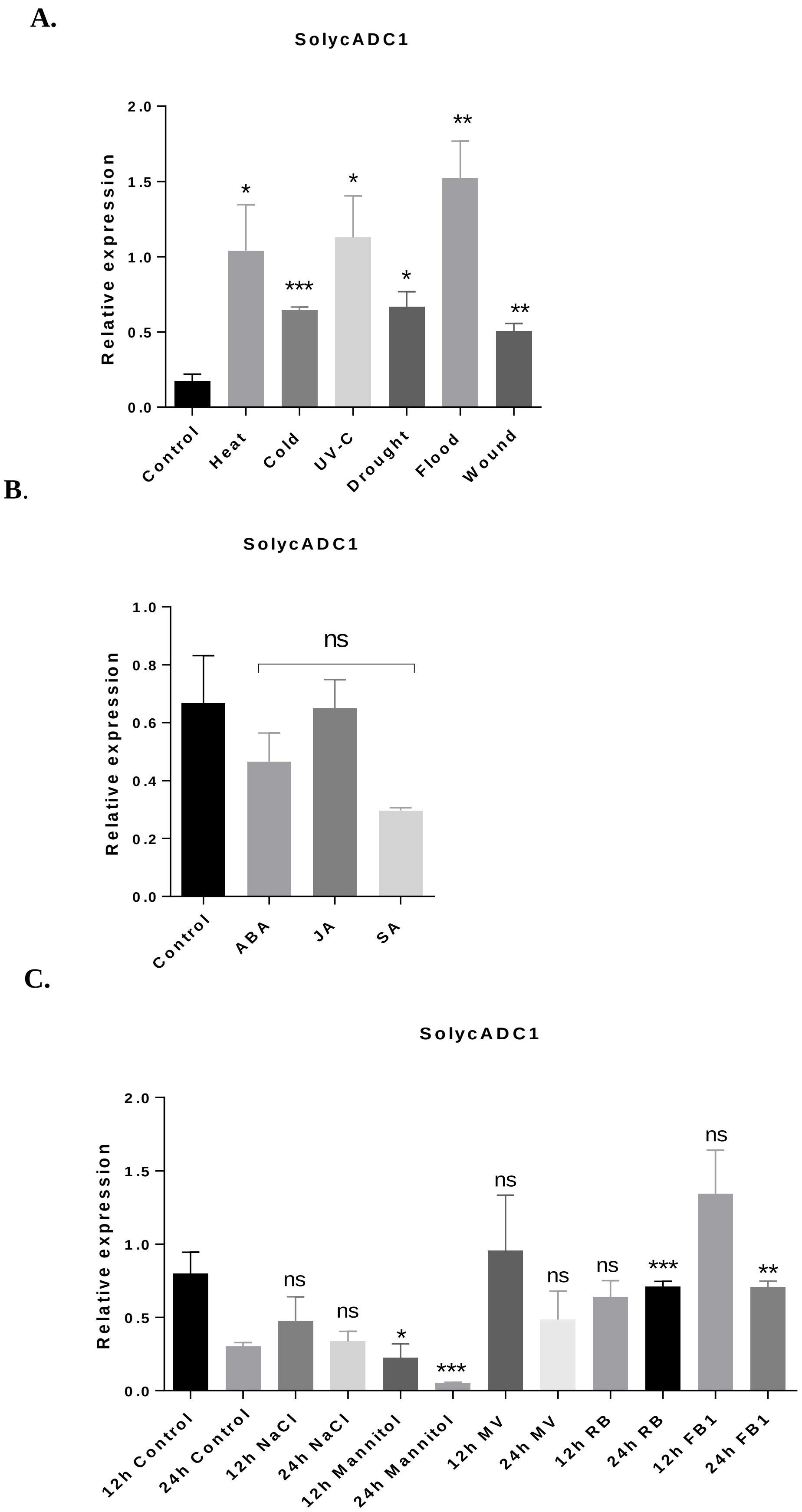


Figure 4.15: Expression patterns of SolycADC1 (A, B and C) in tomato plants treated with various physical, chemical and hormonal stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, P<0.05; **, P<0.01, t-test) compared with the corresponding controls.

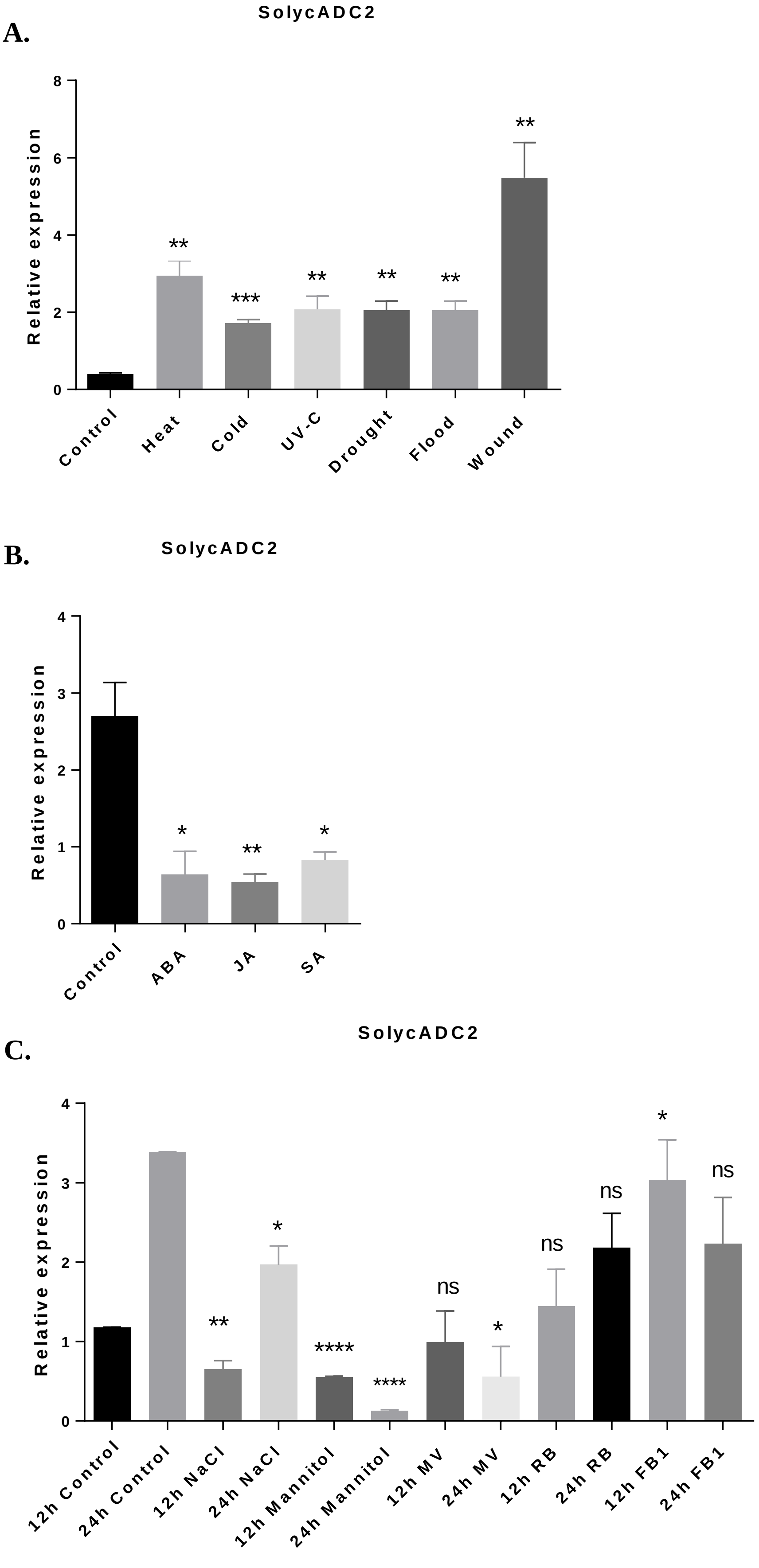


Figure 4.16: Expression patterns of SolycADC2 (A, B and C) in tomato plants treated with various physical, chemical and hormonal stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

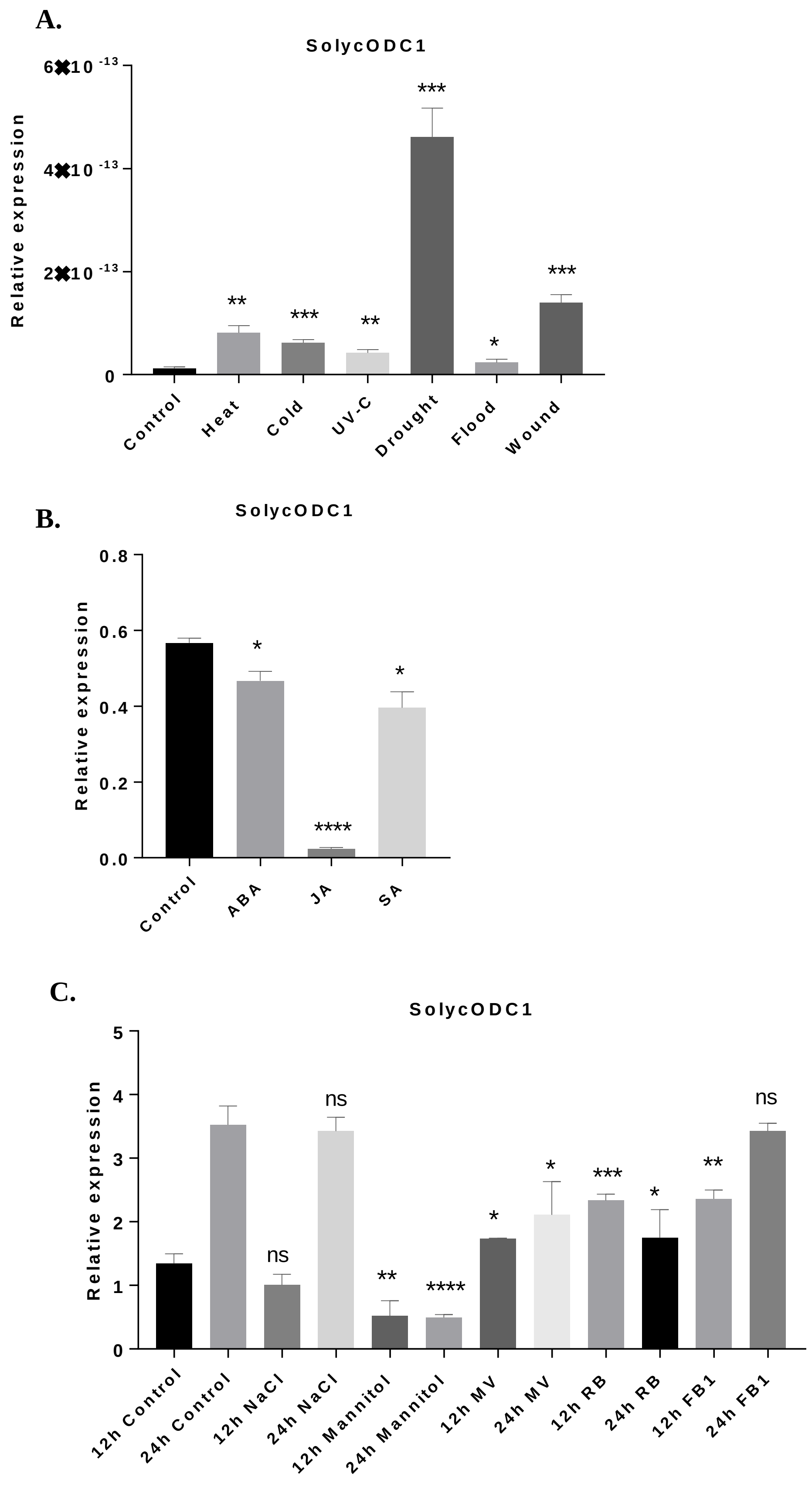


Figure 4.17: Real time expression analysis of SolycODC1 (A, B and C) in tomato plants grown under different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

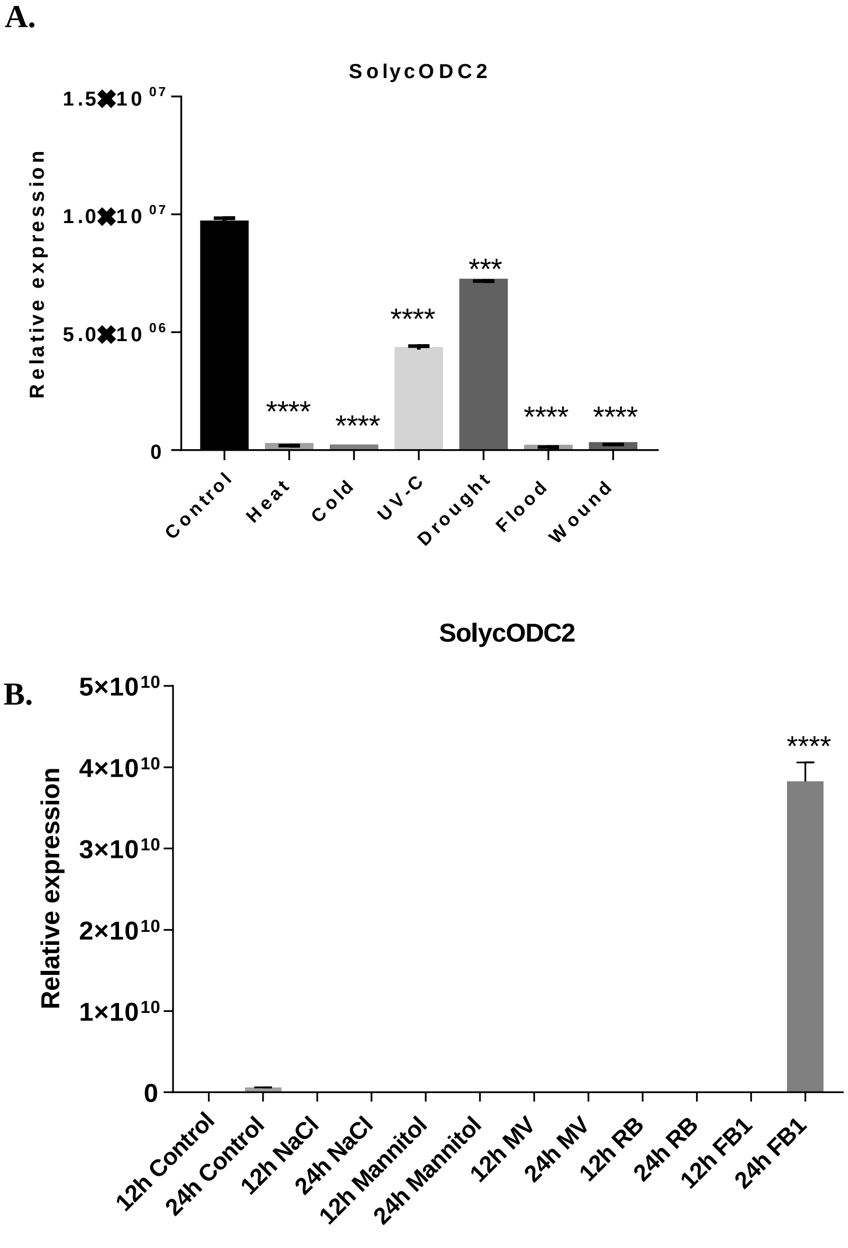


Figure 4.18: Real time expression analysis of SolycODC2 (A and B) in tomato plants grown under different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (****, $p < 0.0001$, t test) compared with the corresponding controls.

Candidate polyamine biosynthetic gene, SolycSAMDC1 showed significant upregulated expression by heat and cold, while its expression was downgulated under drought condition. ABA, NaCl and mannitol treatments for 24 h and MV for 12 h, also showed significant downregulated expression of SolycSAMDC1 (Figure 4.19). Wheras, SolycSAMDC2 did not show any significant change in expression under any physical stress treatments. Highly significant upregulated expression of SolycSAMDC2 was observed by JA and SA treatments. It showed highly downregulated expression by NaCl, mannitol, MV, RB and FB1 treatments for 24 h as comapred to controls (Figure 4.20).

Expression of SolycSAMDC3 was significantly downregulated by cold and UV-C, while its expression was upregulated by heat and drought treatments. Its expression was drastically upregulated by SA, while downregulated by NaCl, mannitol, MV, RB and FB1 treatments for 12 h (Figure 4.21).

Significant upregulated expression of SolycSAMDC4 was observed in heat, drought, wounding. SA and MV treatment for 12 h (Figure 4.22). Whereas the expression pattern of SolycSAMDC5 did not show any detectable or significant change in expression by any chemical treatments. However, heat, wounding and SA treatments showed significant upregulated expression of SolycSAMDC5 in tomato (Figure 4.23)

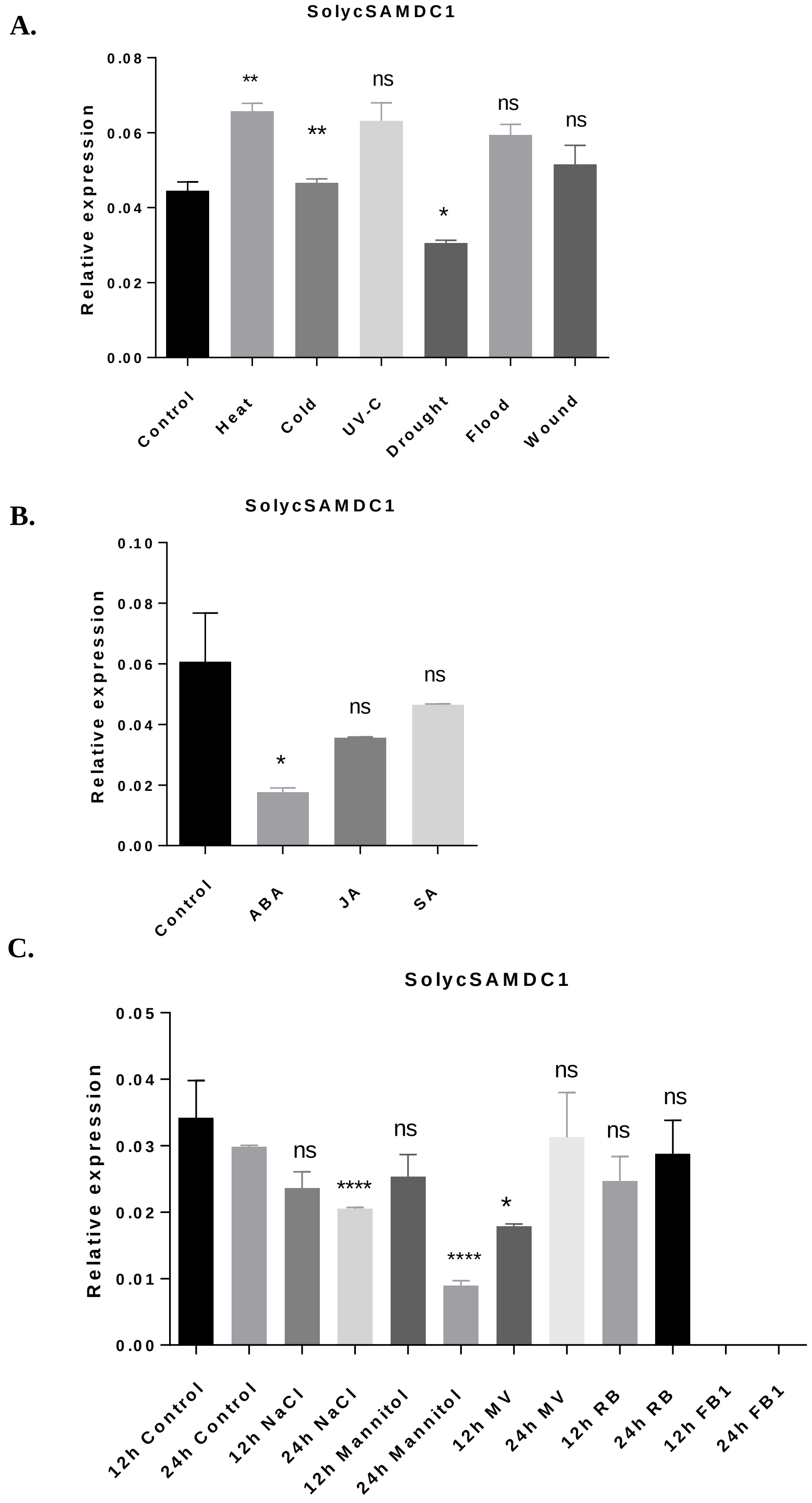


Figure 4.19: Expression pattern of SolycSAMDC1 (A, B and C) in tomato under different stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

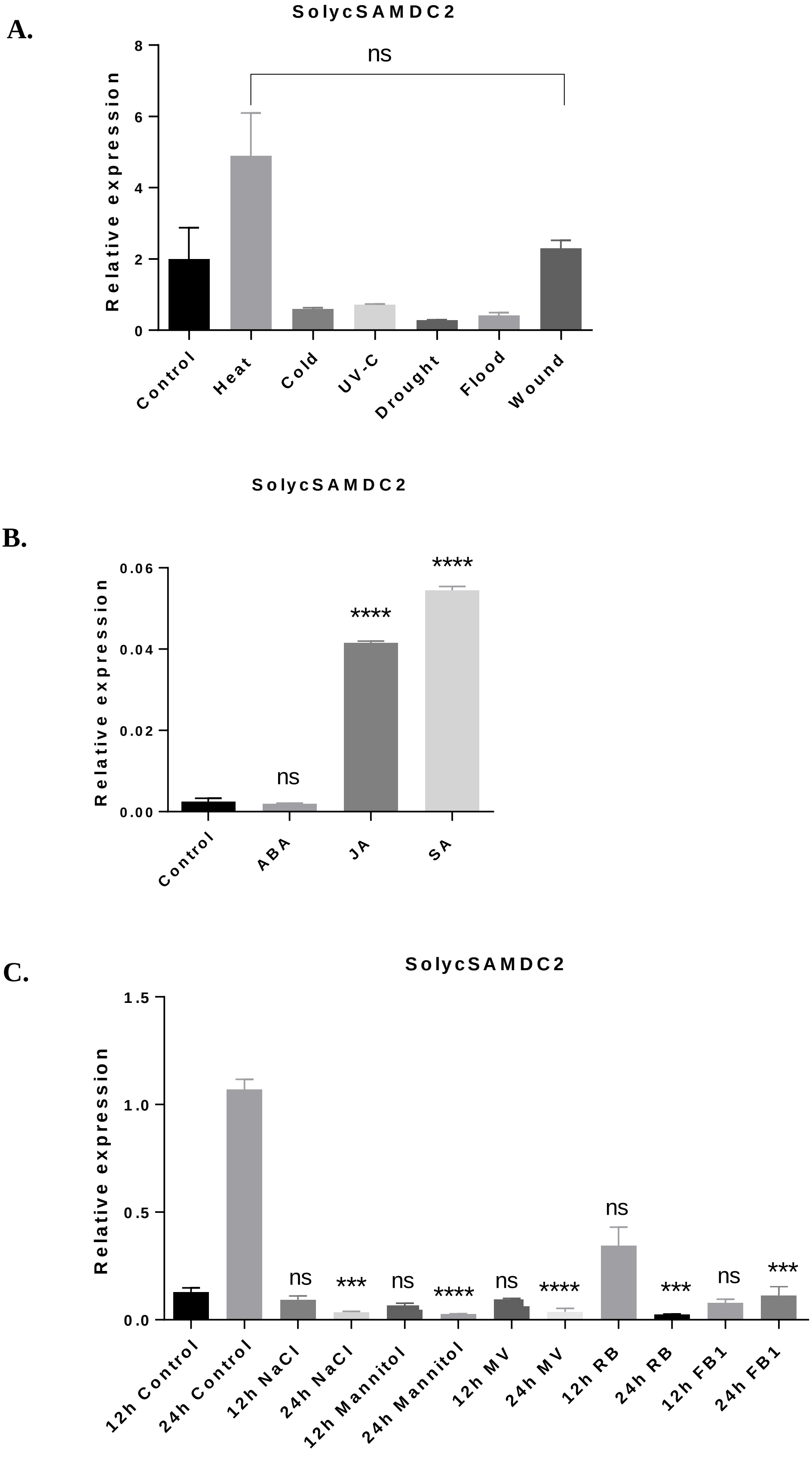


Figure 4.20: Expression pattern of SolycSAMDC2 (A, B and C) in tomato under different stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

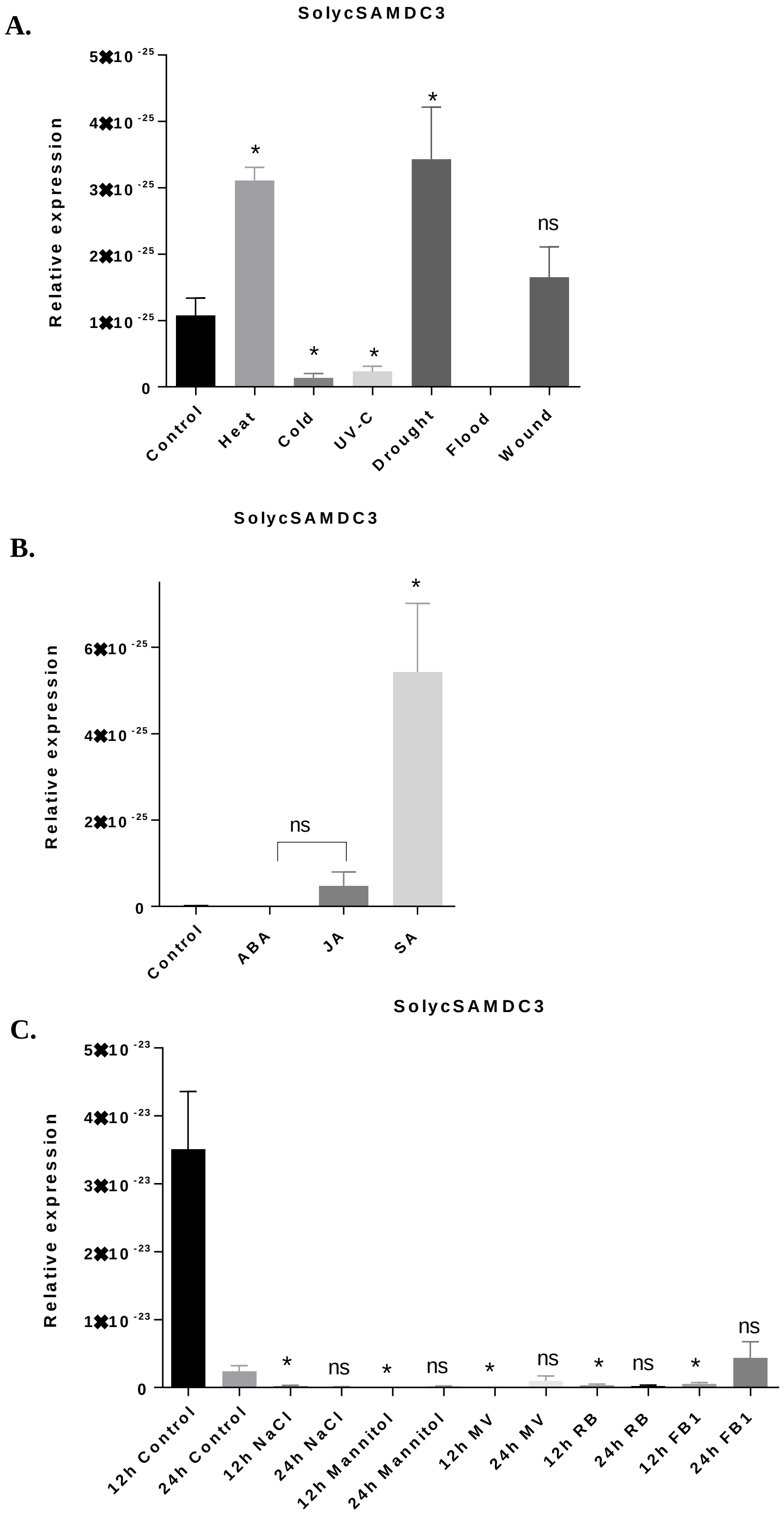


Figure 4.21: Expression pattern of SolycSAMDC3 (A, B and C) in tomato under different stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, p<0.05; **, p<0.01, t-test) compared with the corresponding controls.

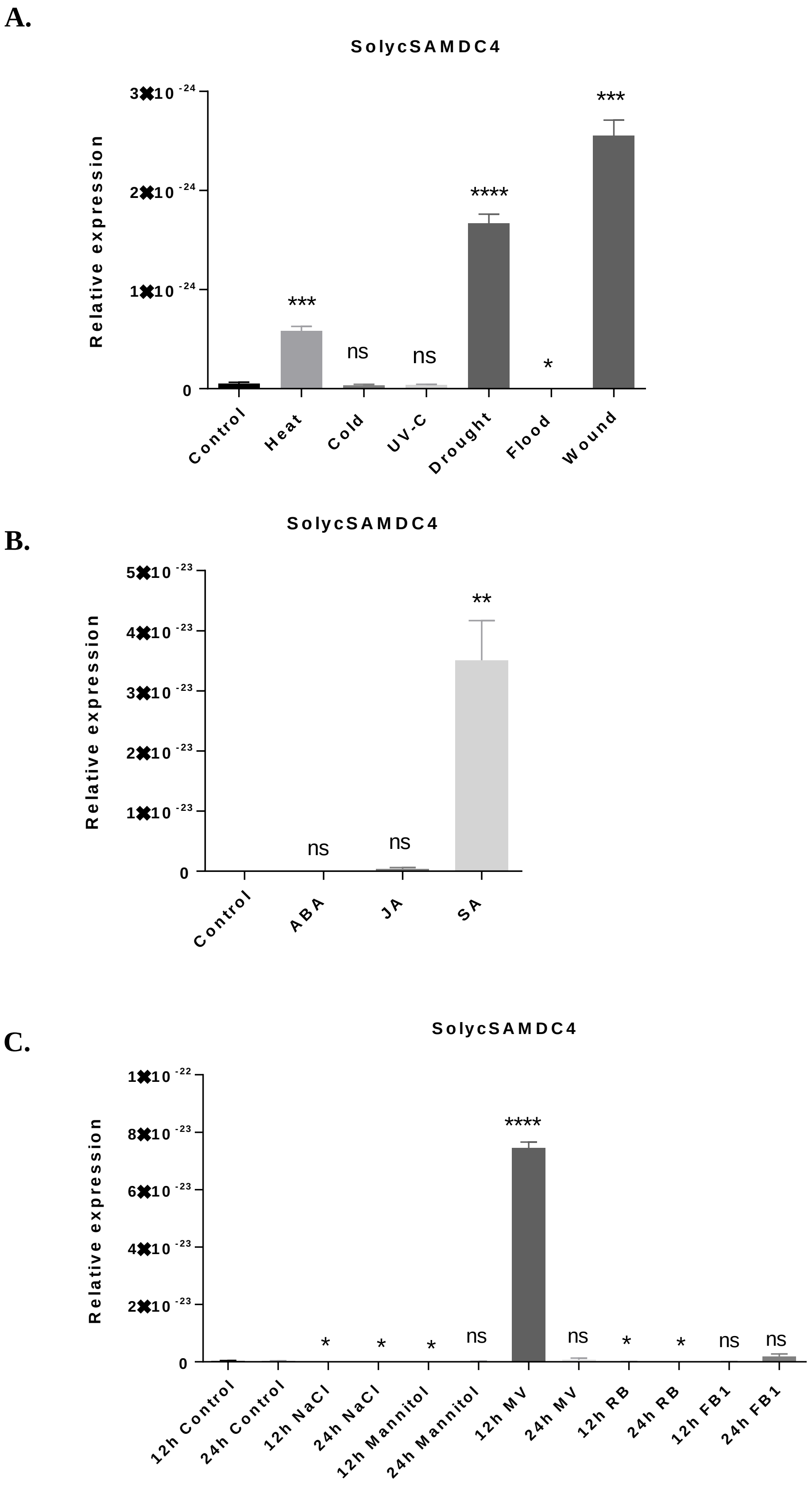


Figure 4.22: Real time expression analysis of SolycSAMDC4 (A, B and C) genes in tomato treated with various stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

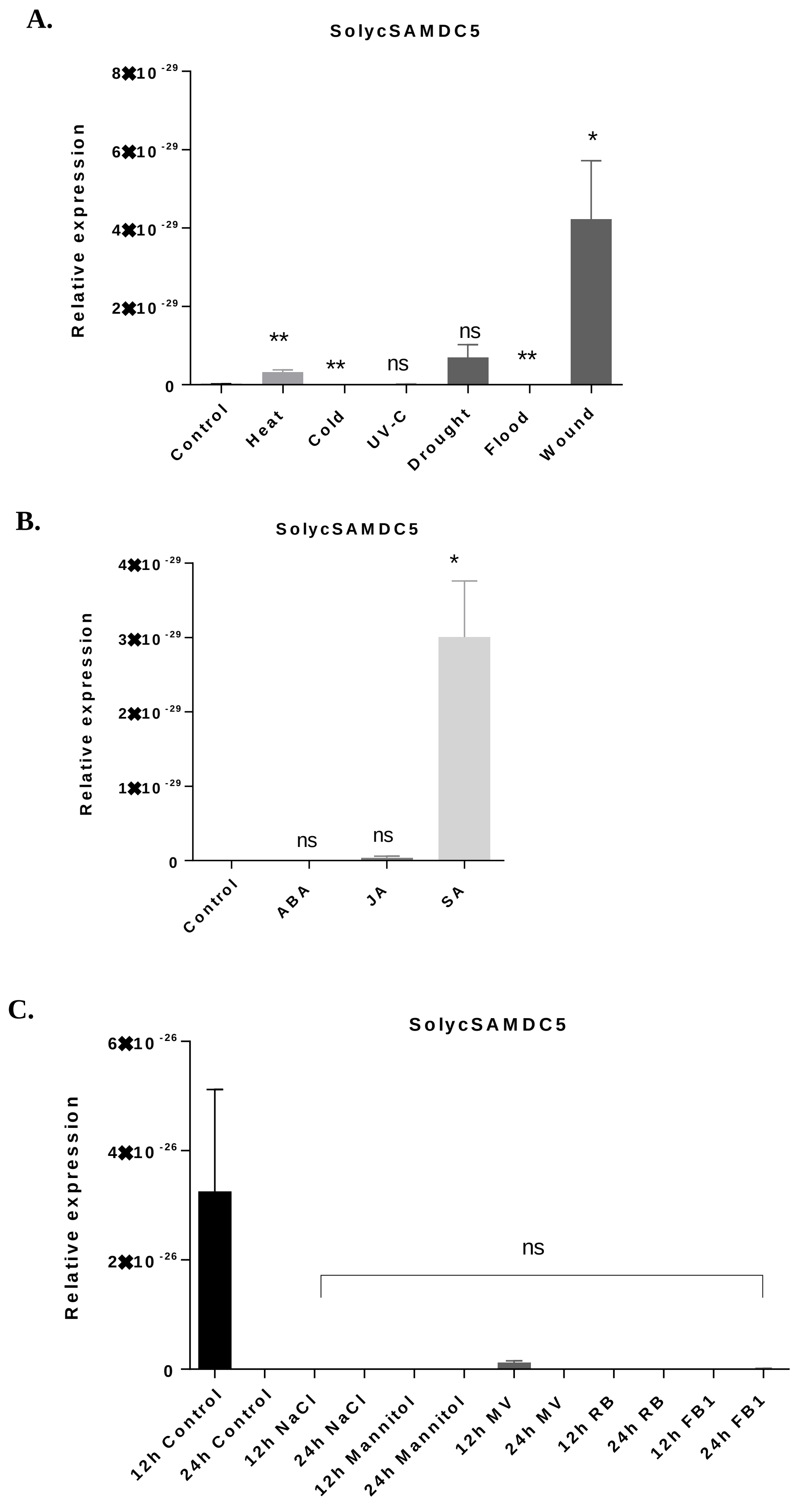


Figure 4.23: Expression patterns of candidate genes SolycSAMDC5 (A, B and C) in tomato during different stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, t-test) compared with the corresponding controls.

Our qRT-analysis showed that expression of SolycSPDS1 was significant upregulated by UV-C, whereas its expression was downregulated by wounding. Significant downregulated expression of SolycSPDS1 was observed in ABA and JA treatments. Its expression was highly downregulated by NaCl and mannitol treatments for 12 h and 24 h, and also by MV and RB and FB1 treatments for 12h (Figure 4.24). Expression of candidate spermidine synthase gene, SolycSPDS2 was significantly downregulated by flooding and mannitol treatment for 24 h. However it showed significant upregulation by JA, SA and FB1 treatment for 24 h (Figure 4.25).

Our expression analysis revealed that candidate gene SolycSPDS3 did not show any significant change in expression during various physical, chamical and hormonal treatments (Figure 4.26). However, SolycSPDS4 showed upregulation only in flood and downregulated expression by wounding (Figure 4.27). Whereas SolycSPDS5 was highly downregulated by FB1 treatment for 24 h only (Figure 4.28). These results showed that expression of SolycSPDS2, SolycSPDS3 and SolycSPDS4 was not influenced by most the physical, chemical and hormonal treatments indicating that these genes are not involved in stress responses in tomato.

Expression of candidate spermine synthase gene SolycSPMS showed significant upregulation during heat, cold, UV-C, SA, RB for 12 h and 24 h and FB1 for 24 h treatments, whereas it was significantly downregulated during drought, flood, NaCl and mannitol treatments for 12 h and 24 h (Figure 4.29).

Expression of SolycACL5 was highly upregulated by heat, wounding, JA, SA and also by MV treatment for 12 h though it was downregulated by cold, UV-C and flooding. Also its expression was downregulated by mannitol treatment for 12 h (Figure 4.30).

Whereas its close paralog, SolycACL5-Like1 showed highly upregulated expression in heat, drought and wounding treatments. Similarily, JA and SA treatments also showed its significant upregulation whereas ABA caused downregulation in its expression. Also, a significant downregulated expression of SolycACL5-Like1 was obserevd in mannitol and MV treatments for 24 h (Figure 2.31). Expression analysis also revealed that SolycACL5-Like2 was highly upregulated by flooding, ABA and mannitol treatment for 24 h.Whereas its expression was significantly downregulated by heat, cold, drought, wounding and MV treatment for 12 h (Figure 4.32).

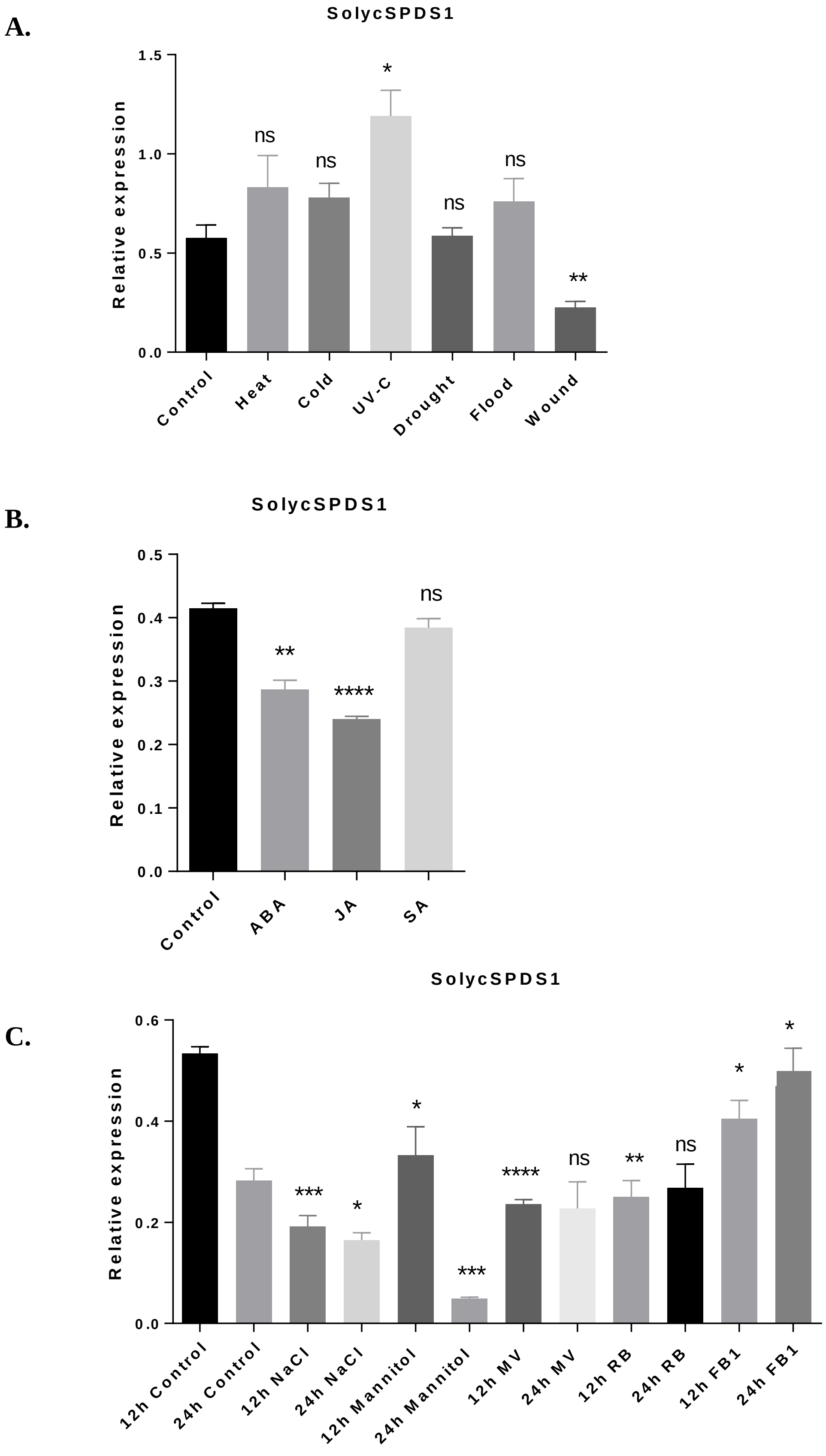


Figure 4.24: Expression patterns of candidate genes SolycSPDS1 (A, B and C) in tomato during different stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

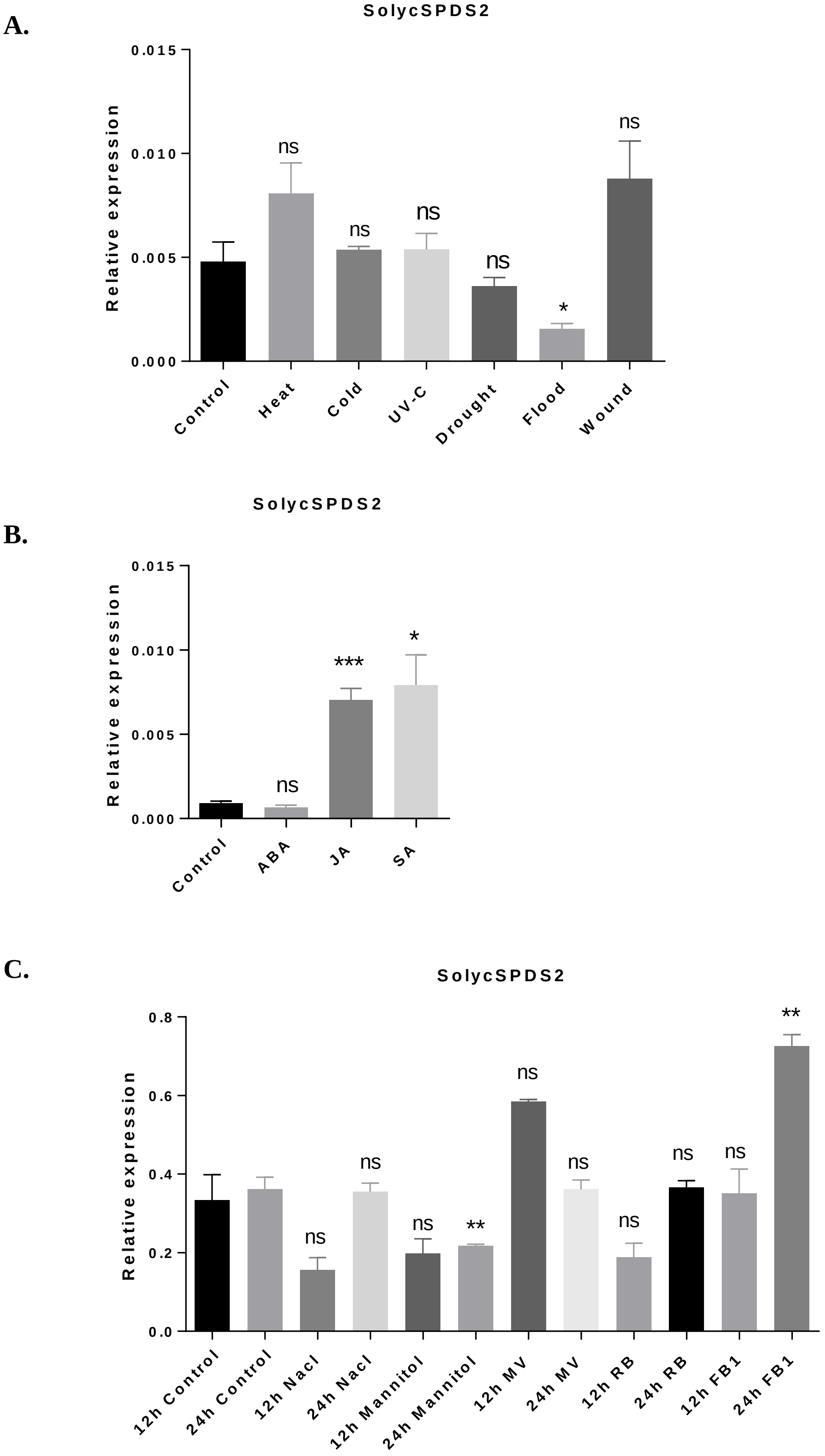


Figure 4.25: Expression patterns of candidate genes SolycPSDS2 (A, B and C) in tomato during different stress conditions. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, t-test) compared with the corresponding controls.

A.

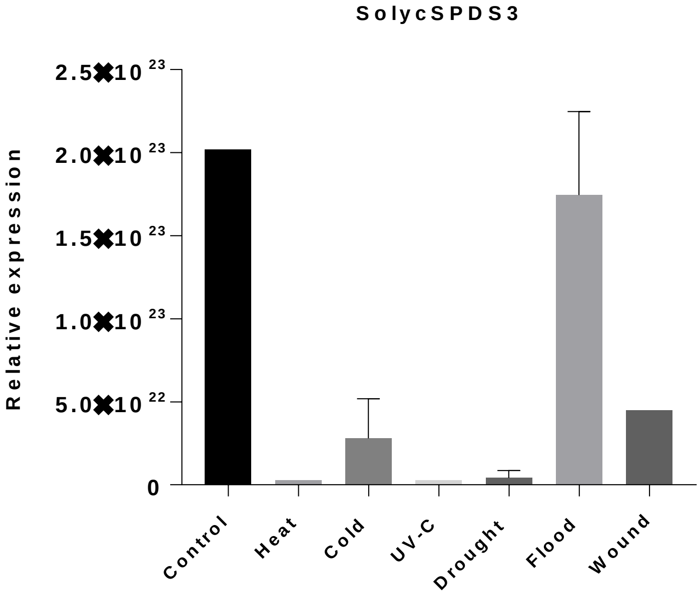


Figure 4.26: Expression patterns of SolycSPDS3 in tomato plants during different stresses (A). Values are mean± SEM of three biological replicates.

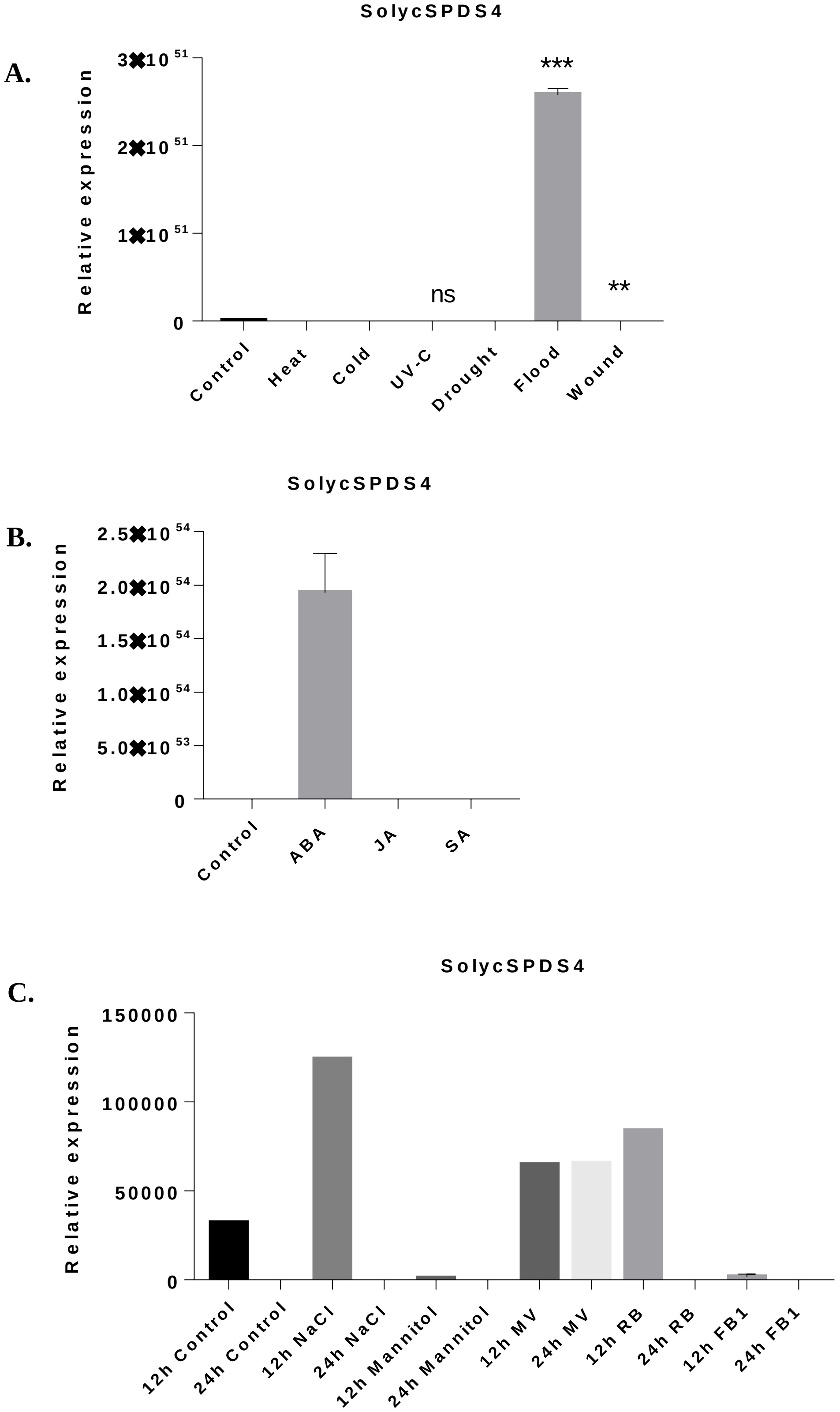


Figure 4.27: Expression patterns of SolycSPDS4 (A, B and C) in tomato plants during different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, t-test) compared with the corresponding controls.

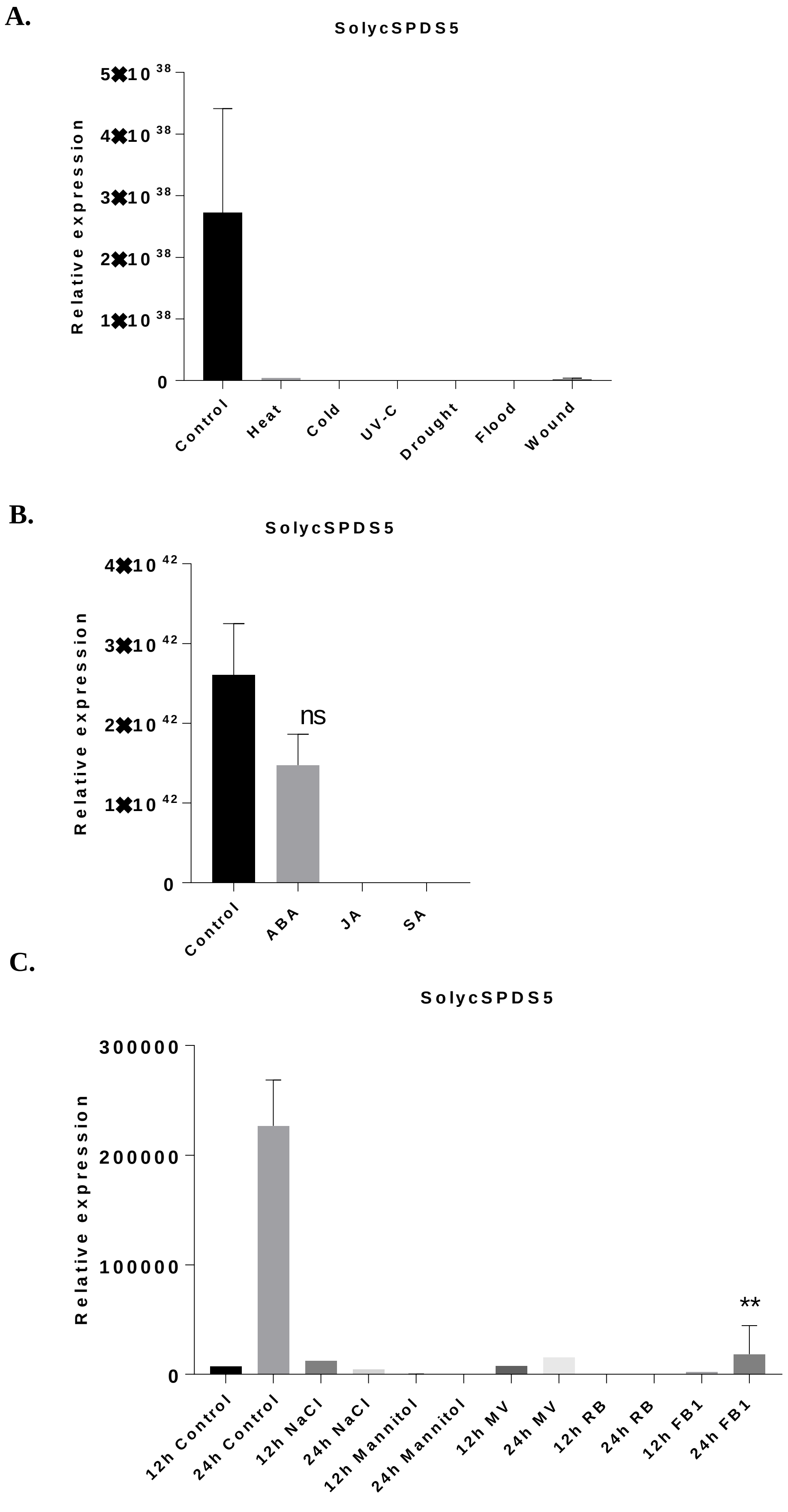


Figure 4.28: Expression patterns of SolycSPDS5 (A, B and C) in tomato plants during different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, p<0.05; **, P<0.01, t-test) compared with the corresponding controls.

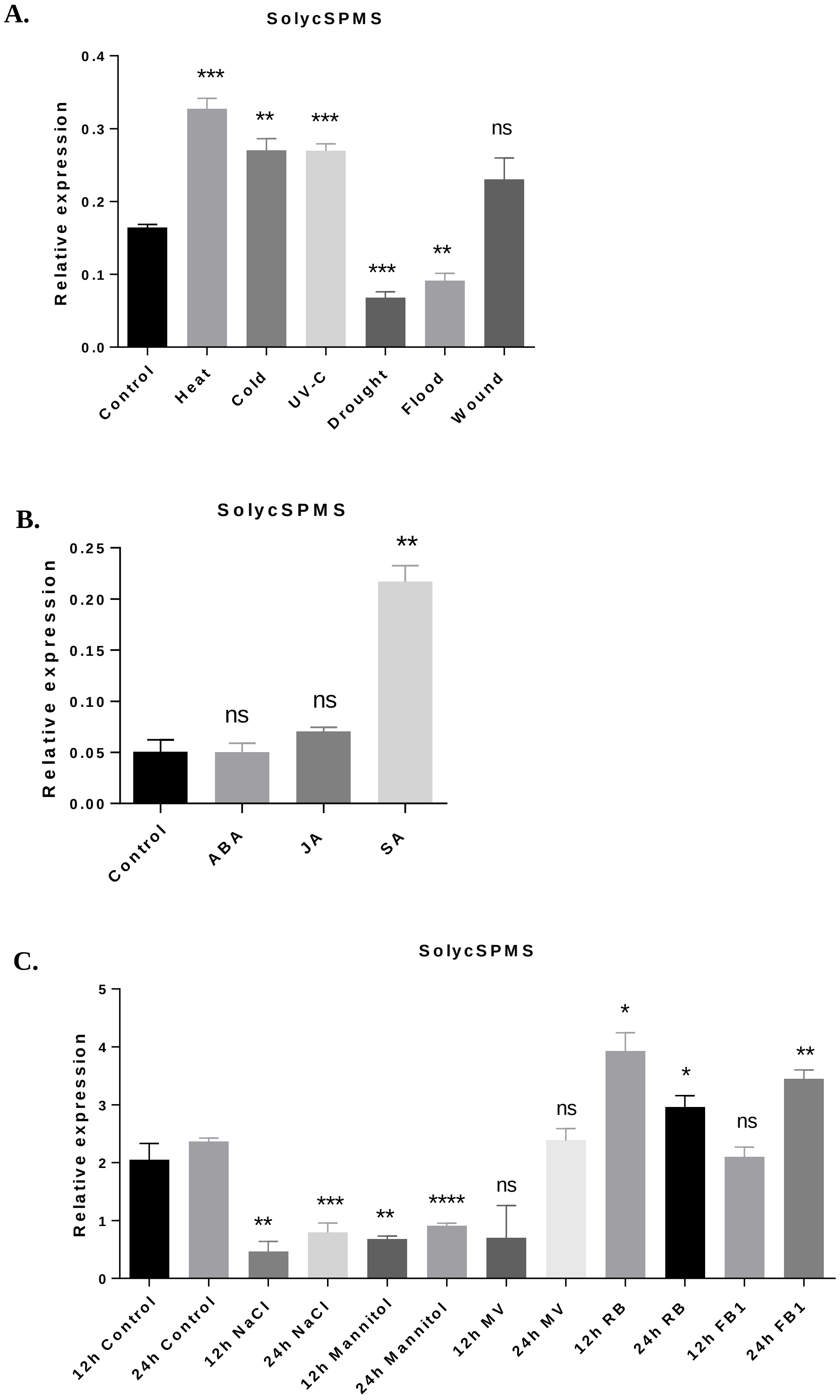


Figure 4.29: Expression patterns of SolycSPMS (A, B and C) in tomato plants during different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, p<0.05; **, p<0.01, ****, p <0.0001, t-test) compared with the corresponding controls.

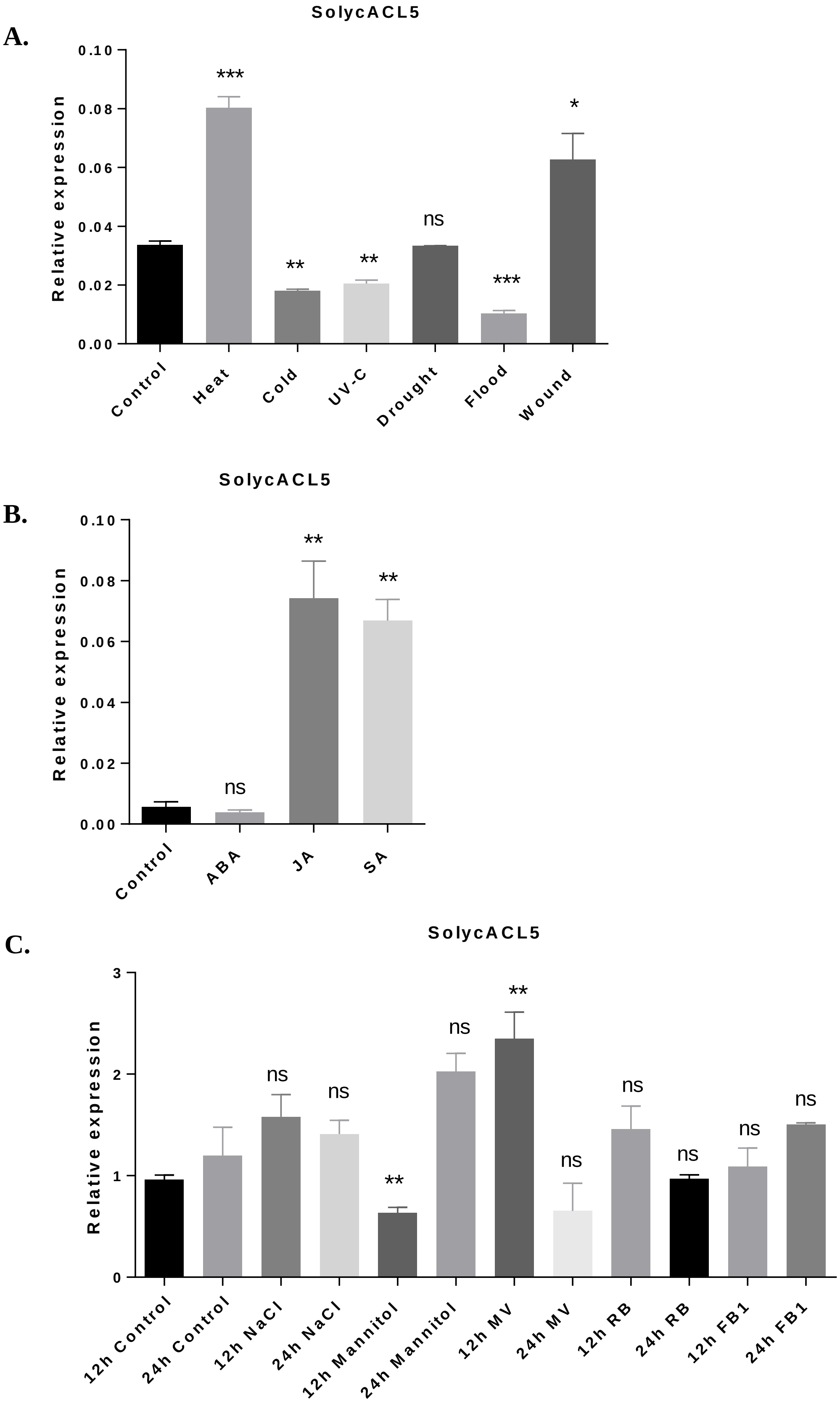


Figure 4.30: Expression patterns of SolycACL5 (A, B and C) in tomato plants during different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, t-test) compared with the corresponding controls.

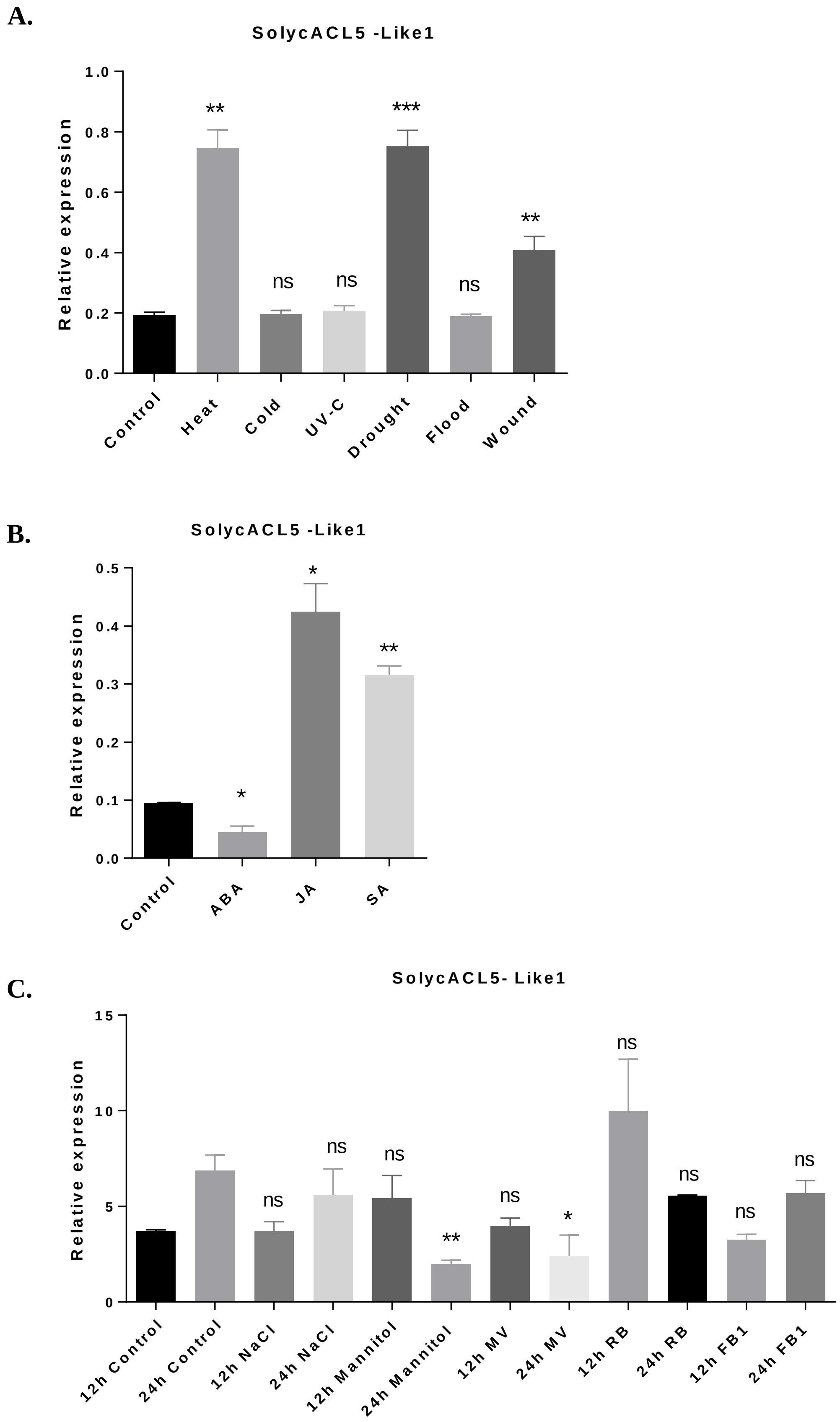


Figure 4.31: Expression patterns of SolycACL5-Like 1 (A, B and C) in tomato plants during different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, t-test) compared with the corresponding controls.

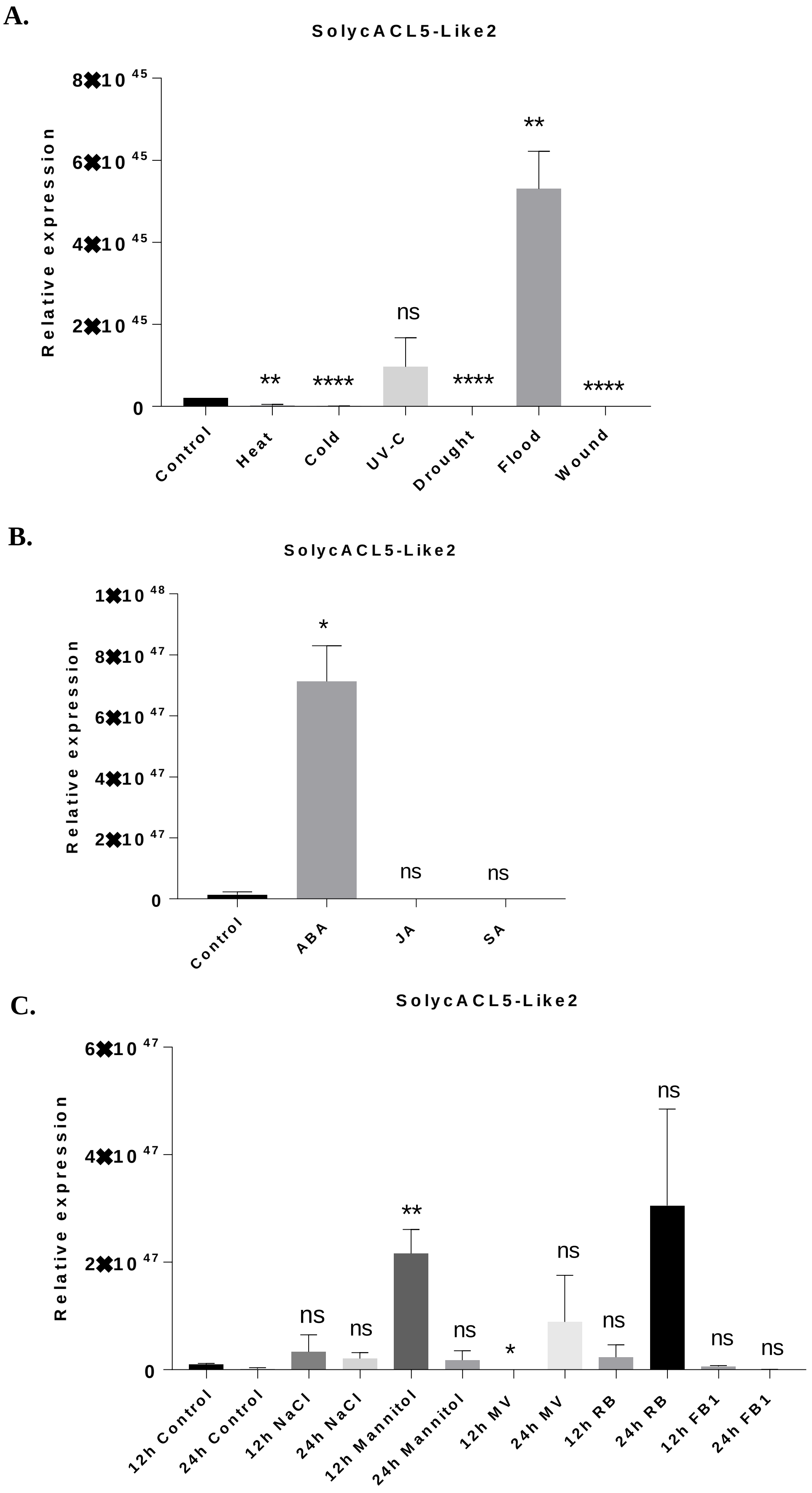


Figure 4.32: Expression patterns of SolycACL5-Like 2 (A, B and C) in tomato plants during different stresses. Values are mean± SEM of three biological replicates. Asterisks indicate a significant difference (*, $p<0.05$; **, $p<0.01$, ****, $p<0.0001$, t-test) compared with the corresponding controls.

4.4 Functional analysis of tomato SolycACL5 in plants

On the basis of expression analysis, we selected SolycACL5 (Solyc08g061970.2) gene for its functional characterization in plants. Quantitative RT-PCR analysis showed that during vegetative tissues development, differential expression of SolycACL5 was observed. In leaves, its expression was observed to be highest in immature leaves followed by in mature leaves and also as development of leaves progresses from immature to senescence stage, its expression gradually decreased. It has higher expression in roots and in the inner woody layer (xylem) of the stem as compared to other vegetative tissues (Figure 4.13C). These results are suggesting that SolycACL5 may involve in leaves, root and stem development.

4.4.1 Silencing of tomato SolycACL5 in plants

For gene silencing of SolycACL5, we designed and cloned amiRNAs specific to SolycACL5 by the method described in material and method section 3.7.2.1. Gene specific amiRNA was constructed by overlapping PCR a, b, c and d. Gene specific amiRNA was then cloned into donor vector pDONR207 and transformed in to *E.coli*. Isolated entry clones were then validated by PCR with the respective primers. The isolated plasmids were then sequenced. Further the entry clone was subsequently recombined to destination vector pMDC32 and transformed to *E.coli* to generate the expression clones. The expression clone formed was further transformed to *Agrobacterium tumefaciens* strain GV3101pMP90 for genetic transformation of plants (Figure 4.33). To study the effects of gene silencing, we transformed thegene construct to generate gene silencing in tomato.

To generate gene silencing lines of SolycACL5 in plants, we transformed amiRNA-SolycACL5 gene silencing construct under control of constitutive promoter 35S in tomato. We successfully generated several putatively transformed shoots on hygromycin selection plates. The generated transformed shoot buds grown up to 3-5 mm long shoots after few weeks of growth but were failed to develop further and finally died (Figure 4.34). The putatively transformed small shoots obtained were further genotyped using genotyping primers A and B by PCR for the presence of transgene. PCR results showed that they all were positively transformed (Figure 4.35A). We also performed the qRT-PCR analysis to confirm the expression level of SolycACL5 in transformed shoots and observed that expression of SolycACL5 in tranformed shoots was severely reduced relative to control (Figure 4.35B). We speculated that this severely abnormal growth of shoot was due to the silencing of solycACL5 in the plants. A more or less similar observation was also reported in case of *Arabidopsis*, where knockout of ACL5 gene resulted in severely dwarf plants (Imai et al., 2006) . Therefore, due to the lethality or abnormality of shoots of SolycACL5 silencing lines in tomato we could not performed any further experiments using these lines.

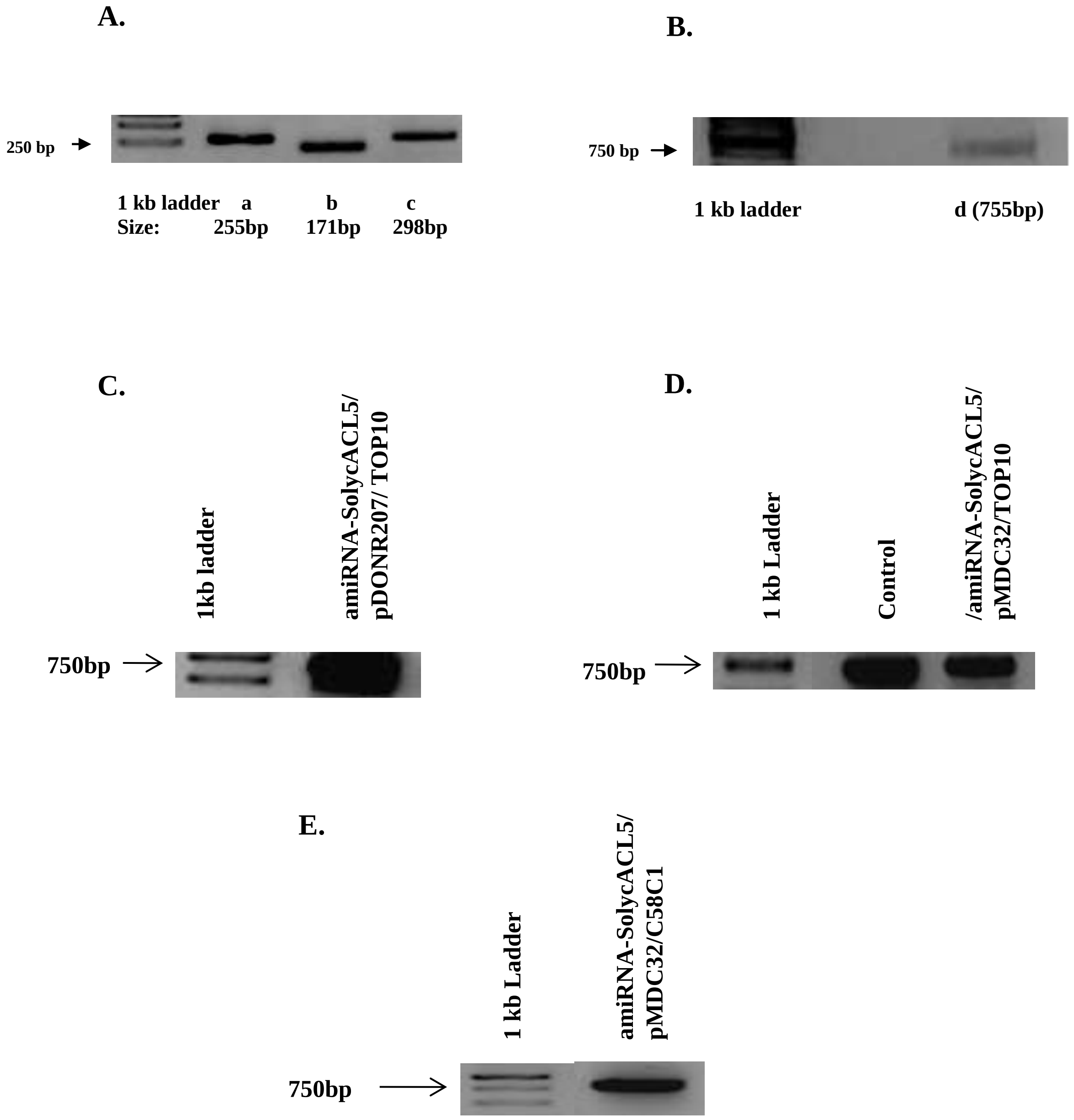
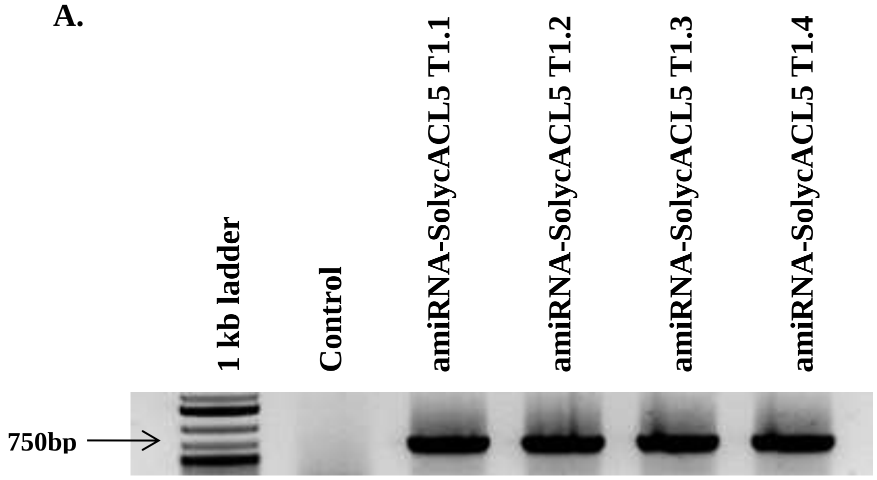


Figure 4.33: Cloning of amiRNA-SolycACL5 for generation of SolycACL5 silencing construct. A) and B) Amplification of amiRNA-SolycACL5 by overlapping PCR. C), D) and E) PCR verification of amiRNA-SolycACL5 gene insert in pDONR207, pMDC32 and *Agrobacterium* C58C1, respectively.

Figure 4.34 Regeneration of amiRNA-SolycACL5 expressing SolycACL5 gene silencing lines in tomato. Regeneration on hygromycin selection plate (A). Transformed shoot buds which failed to grow beyond 3-5mm (B).



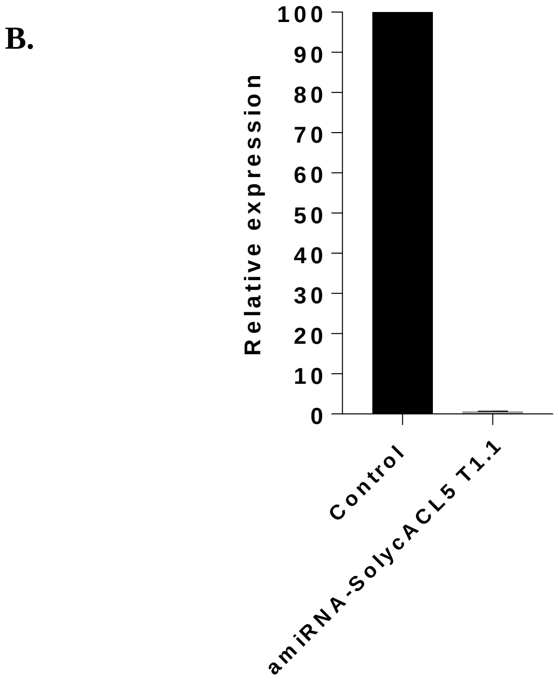


Figure 4.35: A). PCR verification of putative amiRNA-SolycACL5 expressing SolycACL5 gene silencing tomato lines using primers A and B. B). Quantitative RT-PCR analysis showing expression of SolycACL5 in selected amiRNA-SolycACL5 expressing gene silencing line T1.1 and control tomato.

4.4.2 Overexpression of tomato SolycACL5 in tobacco

To generate overexpresser construct, gene coding sequence of SolycACL5 was PCR amplified using gene specific primers containing attB1 and attB2 sites. Further cloning was done by Gateway cloning described in section 3.7.2.1. pDONR207 vector was used to generate entry clone. The entry clone was then PCR verified and sequenced and further cloned to destination vector pMDC32 to generate expression clone. The SolycACL5 expression clone was further transformed to *Agrobacterium* strain GV3101pMP90 as described in section 3.7.2.6 (Figure 4.36).

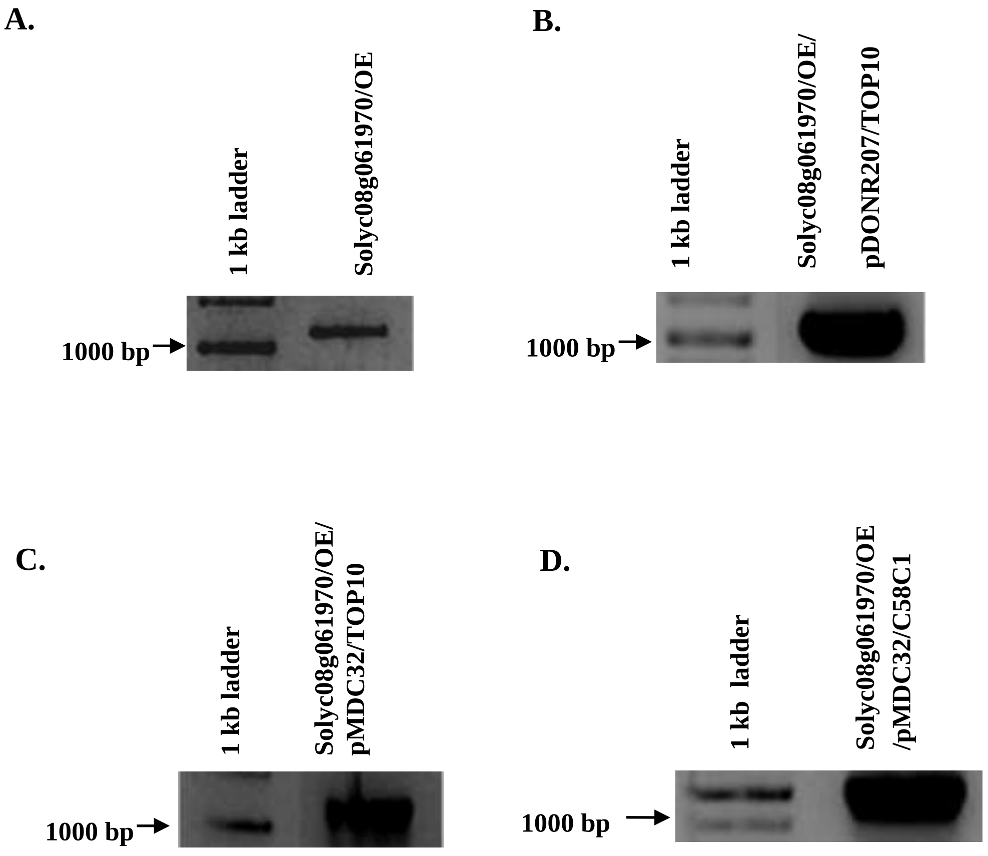


Figure 4.36. A) PCR amplification of SolycACL5. B), C) and D) Cloning of overexpresser construct of SolycACL5 in pDONR207, pMDC32 and C58C1, respectively.

To obtain overexpressor plants, tobacco leaf explants were co-cultivated with the *A. tumefaciens* strain GV3101 carrying overexpresser construct CaMV35S::SolycACL5. The transgenic tobacco lines carrying the overexpresser construct were selected on the selection medium containing 50 mg/l hygromycin. These lines were multiplied and maintained as shoot culture in tissue culture. For rooting, 2 cm long shoots were isolated and transferred to root induction medium to get rooting plantlets. After rooting the plantlets were acclimatized and transferred to soil pots and grown in culture room for further analysis.

Putatively transformed lines were PCR genotyped using SolycACL5, 35S promoter, and NosT specific primers to verify the integration of SolycACL5 in the regenerated plants. The PCR amplification with specific primers showed that expected DNA fragments with the same size as that of plasmids were produced in all of the tested lines, indicating that they were transformants with SolycACL5 gene overexpresser construct (Figure 4.37). Further overexpression of SolycACL5 gene transcript was analyzed in 35S::SolycACL5 overexpressing tobacco lines (OE) by qRT-PCR and on the basis of transcript overxpression level three lines, OE1, OE7, and OE9 were selected for further studies (Figure 4.38).

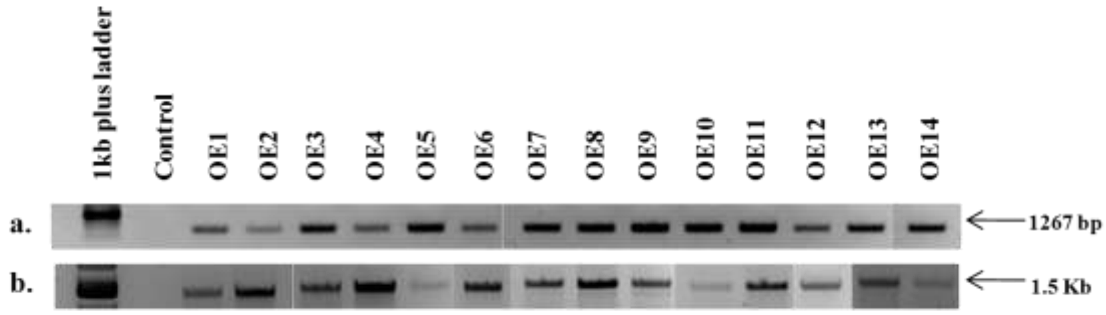


Figure 4.37: Genotyping of SolycACL5 overexpresser lines. PCR amplification of transgenic plants using gene specific primers of SolycACL5 (a) and CaMV35S -NosT (b).

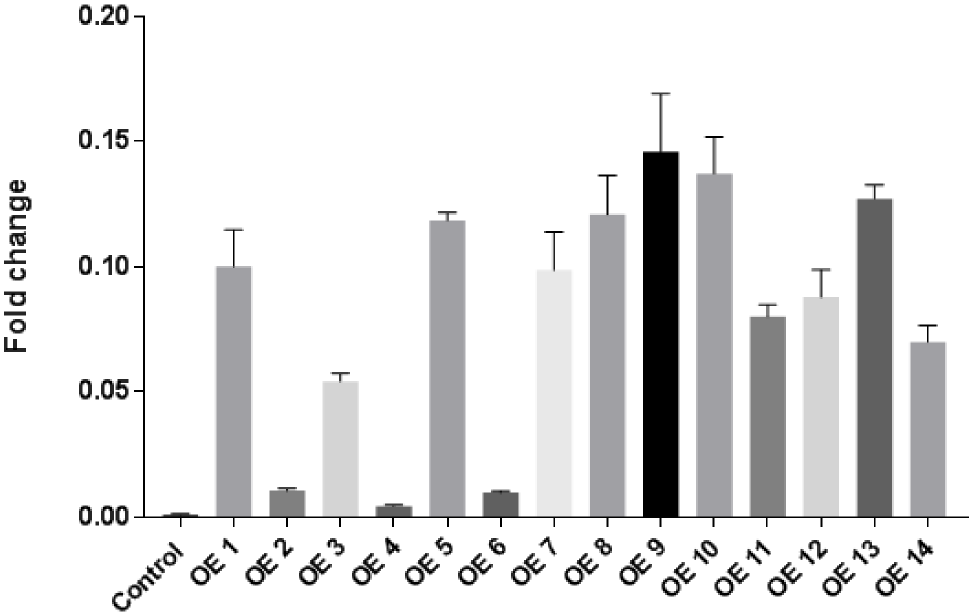


Figure 4.38: Expression analysis of overexpresser lines. qRT-PCR expression analysis of SolycACL5 in control and in 35S::SolycACL5 overexpressing tobacco lines.

4.4.2.1 Phenotyping of selected SolycACL5 overexpresser (OE) lines

For phenotypic characterization, the selected SolycACL5 OE tobacco lines were grown *in vitro* and in growth room and scored for various phenotypic traits. When the plants grown *in vitro*, OE lines were showing stronger growth phenotype (Figure 4.39). They have sturdier growth than the control plants. Similar growth patterns of SolycACL5 OE lines were also observed when grown in pots under growth room conditions. Our analysis showed that the length of the SolycACL5 OE lines was more than those of control tobacco plants. We observed that the length of 60 dag old OE1 plants was approximately 27.6 ± 2.5 cm and length of OE-09 plants was approximately 25.6 ± 2.5 cm, while control was 17.3±1.5 cm in length. The difference in length of SolycACL5 OE lines was approximately 40% to 60% more than the control plants. Furthermore, the morphology of leaves was also altered, as they appeared to be larger in OE lines. We observed that length of mature leaves of 60dag old OE1 and OE9 lines was approximately 11.9 ± 0.9 cm and 12.3 ± 0.7 cm, respectively, while control was approximately 9.8 ± 0.15 cm in length. We also found that ratio of more numbers of green leaves and fewer numbers of senescent leaves per plants were higher in OE lines than the control plants. Number of green and senescent leaves in OE1 plants was approximately 9.6 ± 0.5 and 4.6 ± 1.15 respectively; in OE9 plants was approximately 11.0 ± 1.0 and 5.3 ± 0.57 respectively, while control was 6.6 ± 1.15 and 8.6 ± 0.5 respectively (Figure 4.40). All the measurements were done in triplicates. One-way ANOVA was performed for statistical analysis.

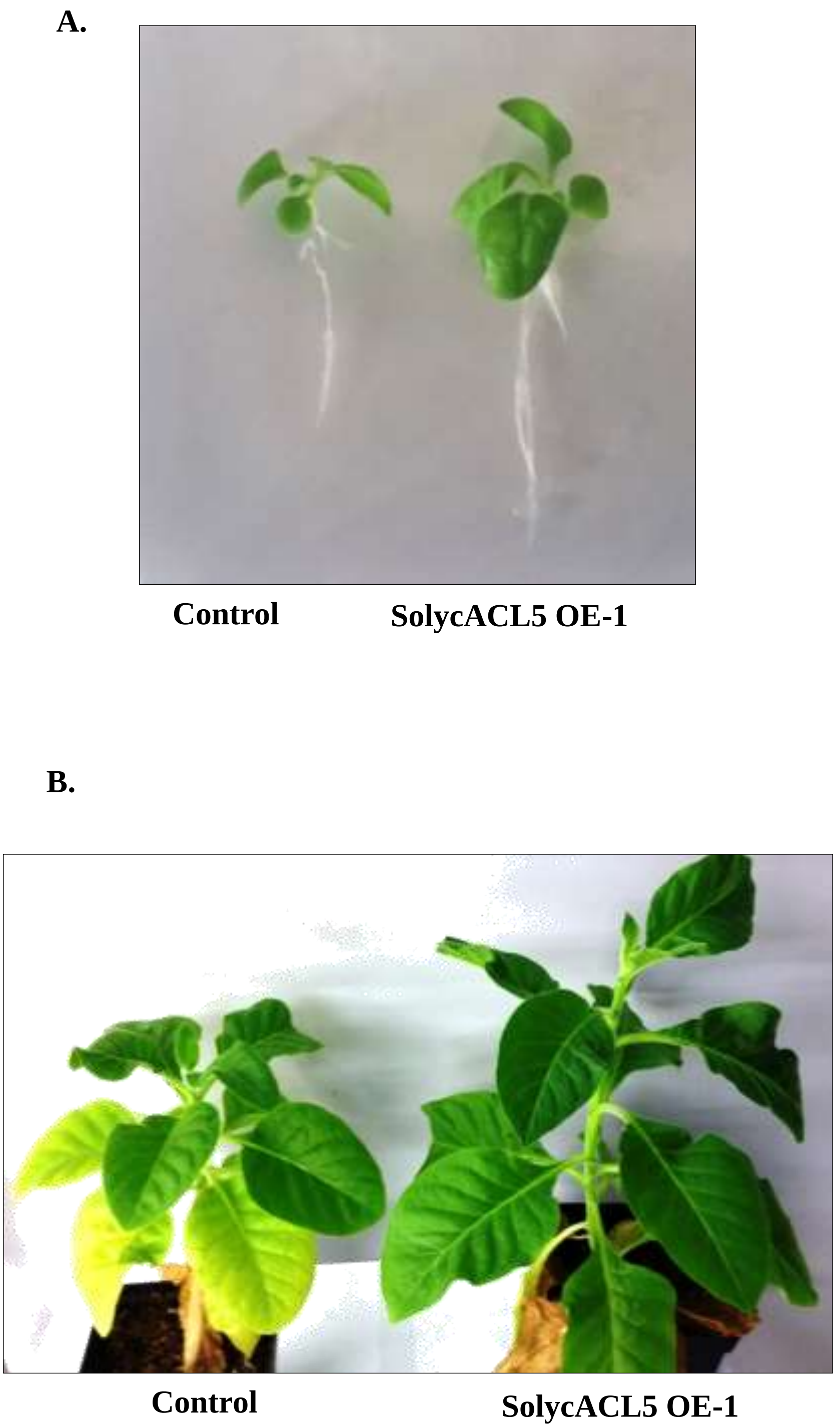


Figure 4.39: Control and 35S::SolycACL5 overexpressing OE-1 tobacco plant. (A). 15 dag seedlings grown on plates. (B). Two month old plant showing vigorous growth phenotype.

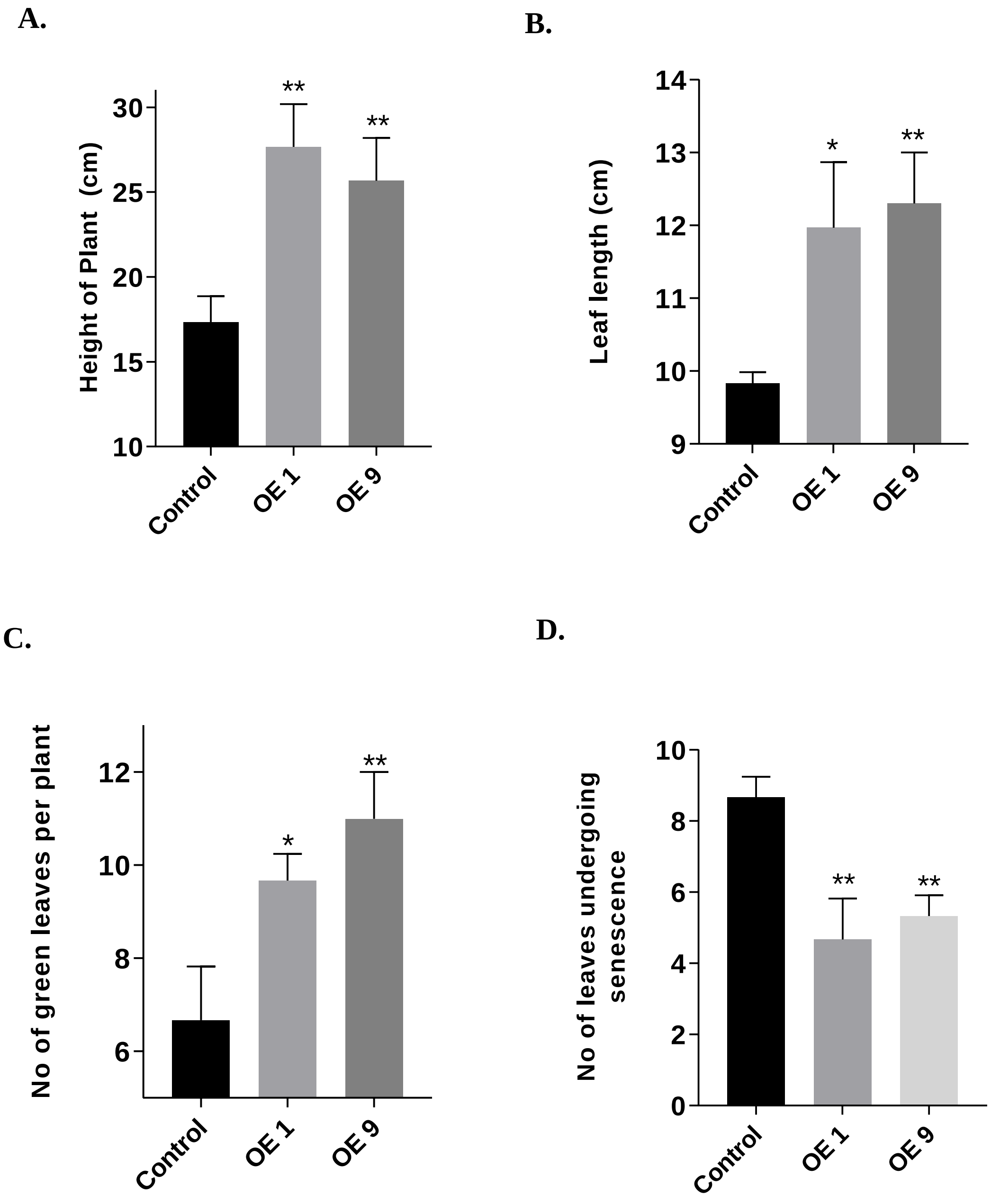


Figure 4.40: Phenotypic characteristics of control and SolycACL5 OE tobacco lines. A). Length of the control and OE-1 and OE-9 plants. B). Length of control and OE-1 and OE-9 tobacco plant leaves. C). Number of green leaves per plant. D). Number of leaves undergoing senescence. *, ** indicate values are significantly different compared with control at significance level of $p<0.05$.

4.4.2.2 Biochemical characterization of SolycACL5 OE lines

Further we did the biochemical characterization of selected OE lines. As the polyamines acts as ROS scavenger but their oxidation also leads to reactive oxygen species generation mainly hydrogen peroxide (H_2O_2), we performed various biochemical assays to check whether the overexpression of SolycACL5 had any effect on the ROS scavenging enzymes activity and also if there was any influence on the ROS production especially on H_2O_2 in the OE lines. Also, chlorophyll, anthocyanin, proline and MDA contents were measured in the SolycACL5 OE lines and control tobacco plants. Through the phenotypic analysis, we have observed some delayed senescence of leaves of OE plants as compared to control, so we also performed real time expression analysis of selected senescence associated marker genes such as SGR1 and SAG12. Expression of some chloroplast specific marker genes was also analyzed using qRT-PCR to confirm delayed senescence in OE plants.

4.4.2.2.1 Hydrogen peroxide concentration in the OE lines

As SolycACL5 overexpression lead to better growth phenotype than the control plants, we further analyzed ROS status of SolycACL5 OE lines and control plants. We did the DAB (3,3'-diaminobenzidine) staining of leaves of control and OE lines which revealed that there was a lesser accumulation of H_2O_2 in the leaves of overexpresser plants than the control. The calorimetric assay was also performed using the method mentioned by Junglee et al. (Junglee et al., 2014) which showed the comparatively lower H_2O_2 level in OE-1 and OE-9 (30.78 ± 2.62 and 27.53 ± 0.86 µg of H_2O_2/mg FW of leaf, respectively) with respect to control plants (44.63 ± 7.20 µg of H_2O_2/mg FW of leaf). Low titre (~34% decrease) of H_2O_2 in the OE plant leaves as compared to the control, suggested that there was less ROS generation in OE lines (Figure 4.41).

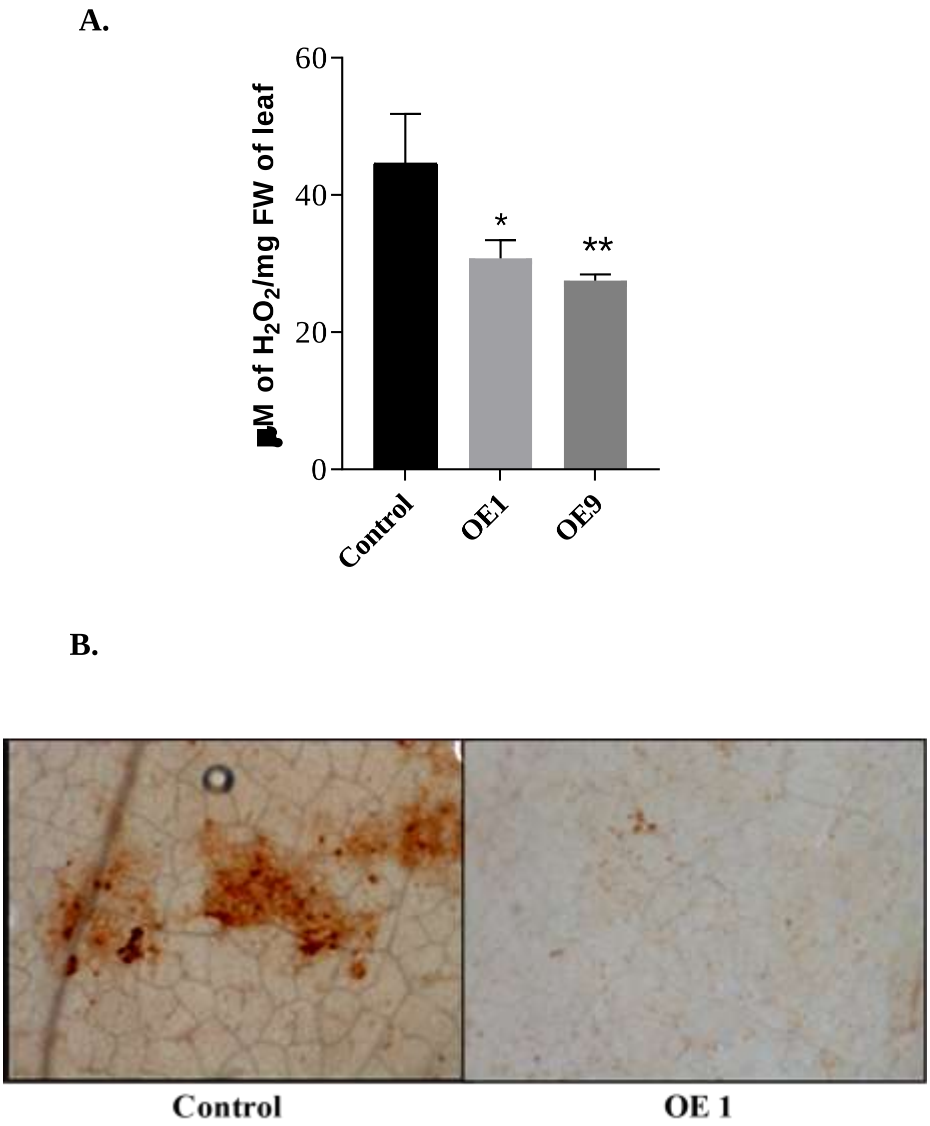


Figure 4.41: Calorimetric determination of H_2O_2 in the leaves of control and OE1 plants (A). DAB staining showing H_2O_2 accumulation (B). * and ** indicates values are significantly different compared with control at significance level of $p<0.05$, One-way ANOVA test.

4.4.2.2.2 Antioxidant enzymes activity in the OE lines

As the ROS production is controlled by the antioxidant enzymes, to know, whether the activity of antioxidant enzymes got altered by the overexpression of SolycACL5 in the plants biochemical enzymatic assays were carried out to monitor the activity of these enzymes in OE lines and control plants. We verified the activity of various antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), guaiacol peroxidase (GP) and ascorbate peroxidase (APX). Young leaf (YL), mature leaf (ML) and senescent leaf (SL) samples of mature plants were taken for the assays. We observed that there was 10% - 44% and 20% - 34% increase in the enzymatic activities of CAT and GP in the OE lines than the control plants, respectively. However, APX activity was found to be reduced in OE plants by 39% - 43% (Figure 4.42). Our analyses suggested that there are some changes in the activity of some of the cellular antioxidant enzymes which resulted in reduced ROS contents in the OE plants.

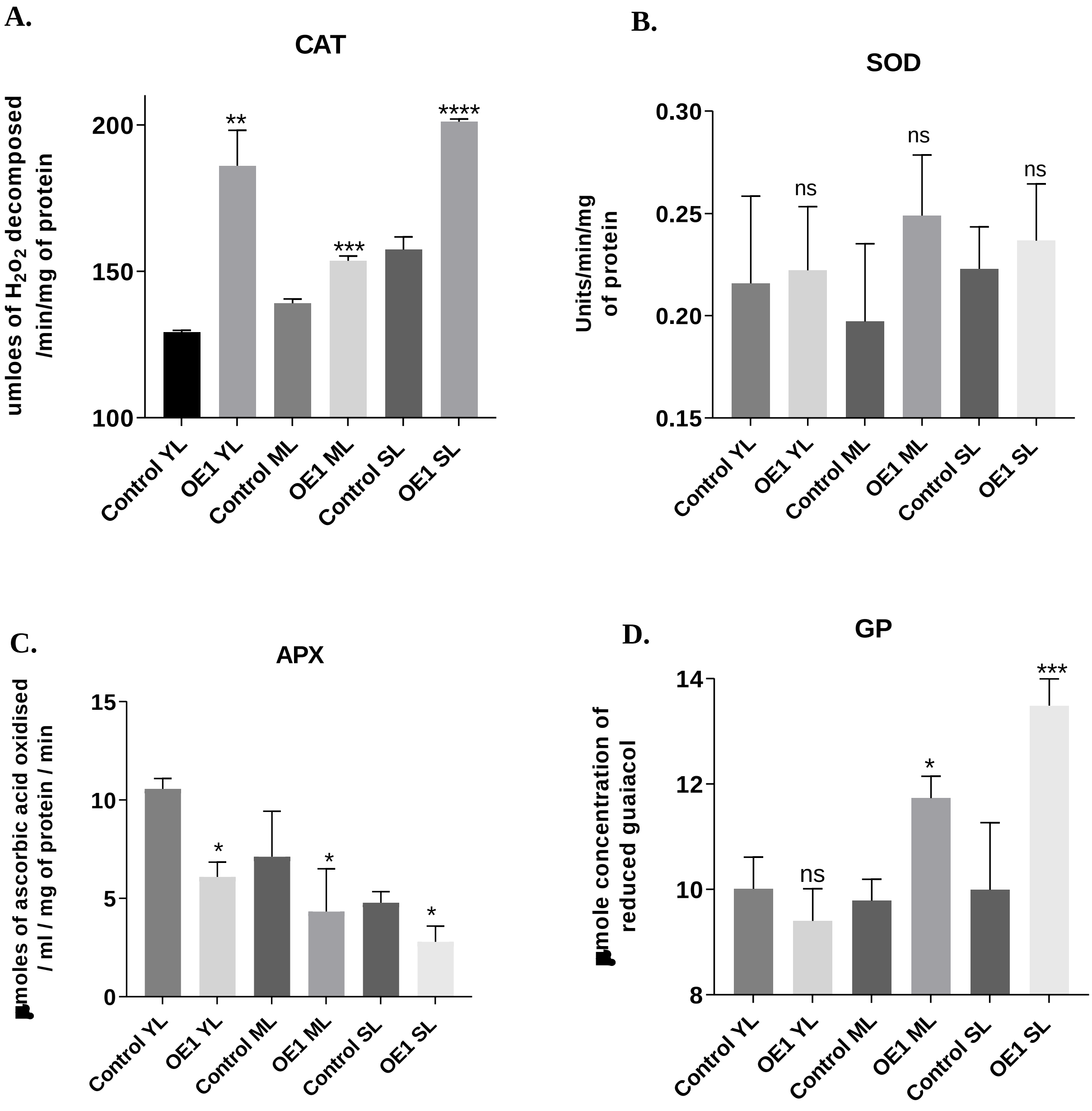


Figure 4.42: Analysis of CAT (A), SOD (B), APX (C) and GP (D) activity in the control and OE plant leaves. YL; young leaf; ML; mature leaf, SL; senescent leaf. *, **, ***, **** indicates values are significantly different compared with control at significance level of $p< 0.05$, $p<0.01$, $p<0.0003$ $p<0.0001$, respectively, t-test.

4.4.2.2.3 Chlorophyll content in the OE lines

We observed that chlorophyll content was about two-fold higher in the mature leaves of OE plants as compared to the control that is in support of our phenotypic observations (Figure 4.43). Hence, we can conclude that overexpression of SolycACL5 may resulted in the higher chlorophyll content in the plant cells of OE lines.

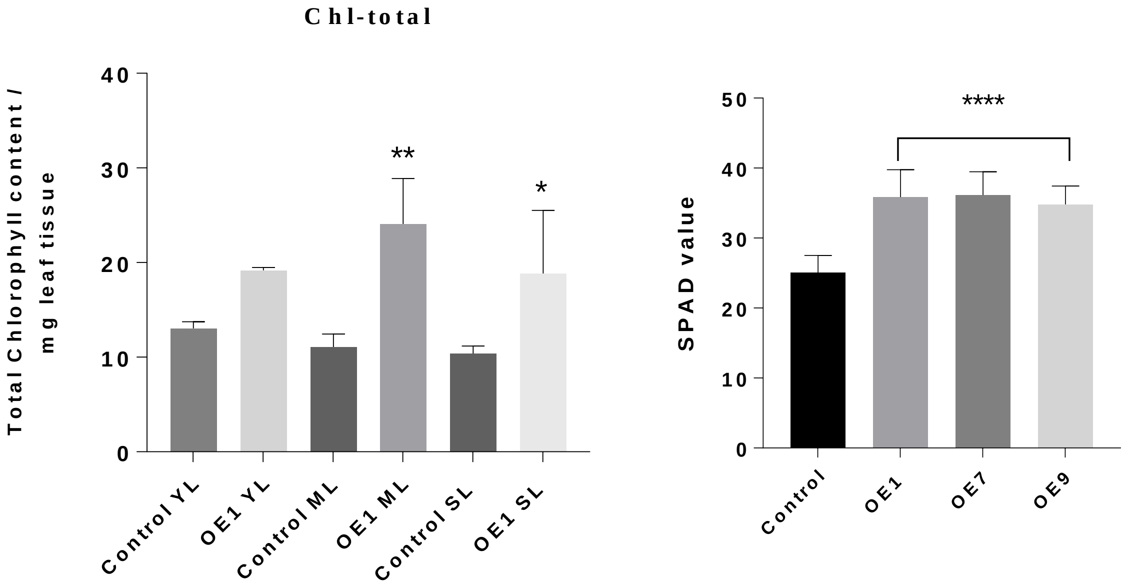


Figure 4.43: Total chlorophyll content in the leaves of control and OE lines.
(A) Calorimetric assay (B) SPAD analysis. YL; young leaf; ML; mature leaf, SL; senescent leaf. *, ** and **** indicates values are significantly different compared with control at significance level of $p<0.05$, $p<0.01$and $p<0.0001$, respectively, One-way ANOVA test.

4.4.2.2.4 Anthocyanin content in OE lines

In order to know whether overexpression of SolycACL5 leads to increase in the anthocyanin content in the plants, we have measured anthocyanin concentrations calorimetrically in the excised mature leaves of OE and control plants (Figure 4.44). We observed that there was 2% - 3 % higher accumulation of anthocyanin in the leaves of SolycACL5 OE plants, suggesting that polyamine may also regulate anthocyanin accumulation in the plants.

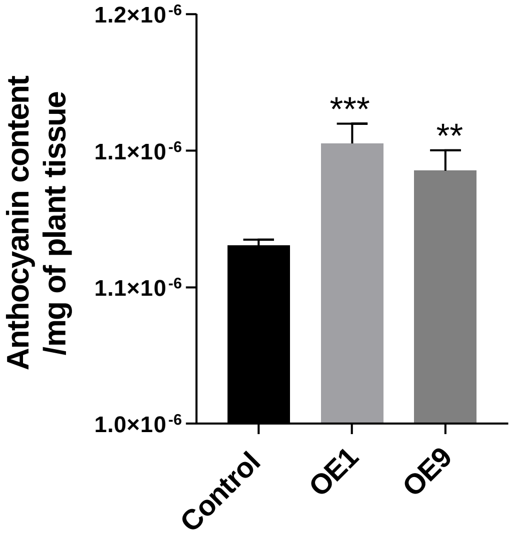


Figure 4.44: Anthocyanin content of control and SolyACL5 OE tobacco plants. **, *** indicates values are significantly different compared with control at significance level of $p<0.05$, One-way ANOVA test.

4.4.2.2.5 MDA content in SolycACL5 OE lines

We have also observed there was less ROS production and modulated antioxidant enzyme activities in OE lines. So, MDA assay was also performed to measure lipid peroxidation which is an indicator of oxidative degradation of lipids in cells. Our analysis showed that there was a decrease in MDA by 10% in the OE plants (Figure 4.45). This suggested that lipid peroxidation was significantly reduced in the OE lines which could be due to less generation of ROS in SolycACL5 overexpression in OE lines.

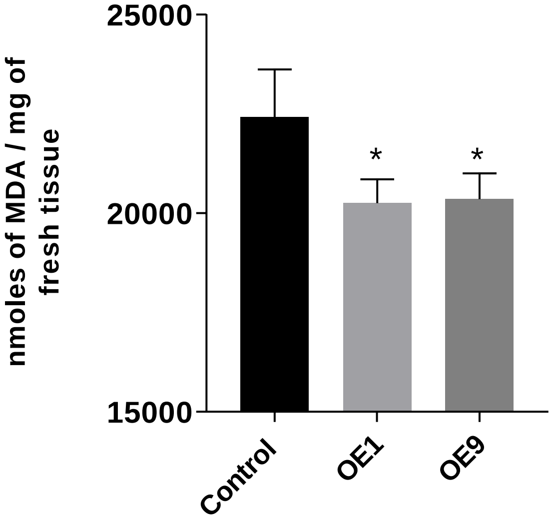


Figure 4.45: MDA content of control and SolycACL5 OE plants.* indicates values are significantly different compared with control at significance level of $p < 0.05$, One-way ANOVA test.

4.4.2.2.6 Expression analysis of senescence associated marker genes in OE lines

Leaf senescence is marked by yellowing of leaves and is commonly found associated with loss of total chlorophyll. It has been already reported that polyamines play important role in suppressing leaf senescence (Bais et al., 2002). Moreover, it was noticed that overexpression of SPDS in tomato lead to increased its shelf life and also delayed leaf senescence (Nambeesan et al., 2010). We observed that in SolycACL5 OE lines there was higher chlorophyll content and yellowing of leaves was delayed as compared to control plants (Figure 4.43). We also observed that there were more numbers of green leaves and less number of yellow leaves in OE plants (Figure 4.40 D and E.). This observation suggested that there was a delay in initiation of senescence program in the OE lines. To determine whether developmental senescence program is affected in OE lines, we analyzed the expression of known early and late stage senescence marker genes SGR1 (*Stay green 1*) and SAG12 (*Senescence-associated gene 12*) in excised leaves of different maturity level of both OE lines and control plants (Figure 4.46A) using qRT-PCR analysis. Expression results showed that 30-80% reduced level of SGR1 expression and 60-80% decreased the level of SAG12 expression in OE lines as compared to control plants (Figure 4.46B and C), suggesting that leaf senescence processes in OE plants are delayed due to overexpression of SolycACL5 in plants.

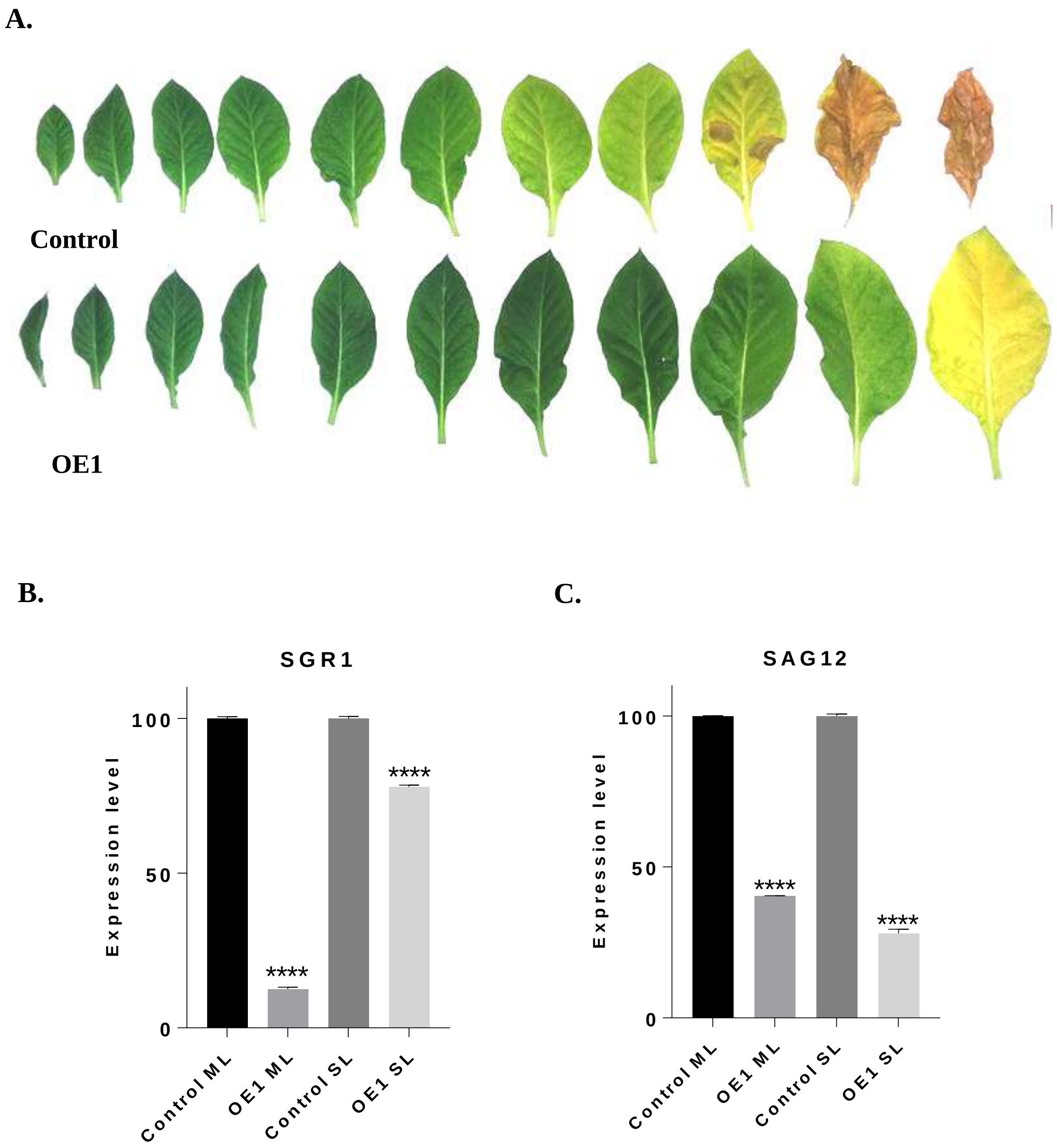


Figure 4.46: A). Developmental progression of leaf senescence in control and OE plant leaves. B). and C). Expression pattern of senescence associated marker genes, SGR1 and SAG12 in different developmental stages of leaves of control and OE-1 line, respectively. ML; mature leaf, SL; senescent leaf. ****indicates values are significantly different compared with control at significance level of $p<0.0001$, One-way ANOVA test.

4.4.2.2.7 Expression of chloroplast-encoded genes in OE plants

To know whether chloroplast activity in OE lines was modified, we analyzed the expression of some chloroplast-encoded genes rpoA, rbcL, and petB by qPCR. rpoA encodes plastid specific alpha subunit of RNA polymerase; rbcL encodes a large subunit of RUBISCO protein located in the stroma, whereas petB encodes cytochrome B6 which is a component of Cytochrome b-f complex of photosynthetic electron transfer system. Expression of these genes is essential for chloroplast development and their normal function. Therefore, it may be used as markers to infer the chloroplast gene expression activity and function. We observed a highly significant increase in expression of rpoA (10000-20000 fold), rbcL (1000-2000 fold) and petB (1000-3000 fold) in OE lines compared to control plants (Figure 4.47A).

4.4.2.2.8 Total cPDNA content in OE plants

Presence and abundance of DNA in plastids were checked by qPCR of rpoA, rbcL, and petB which showed copy number of chloroplast. Increased genomic DNA content of rpoA (1.7-2.2 fold), rbcL (2.5-3 fold) and petB (1.8-2 fold) indicated increased cpDNA copy numbers (Figure 4.47B). Thus, it can be concluded that SolyACL5 OE plants have higher chloroplast number and activity which resulted in delayed senescence.

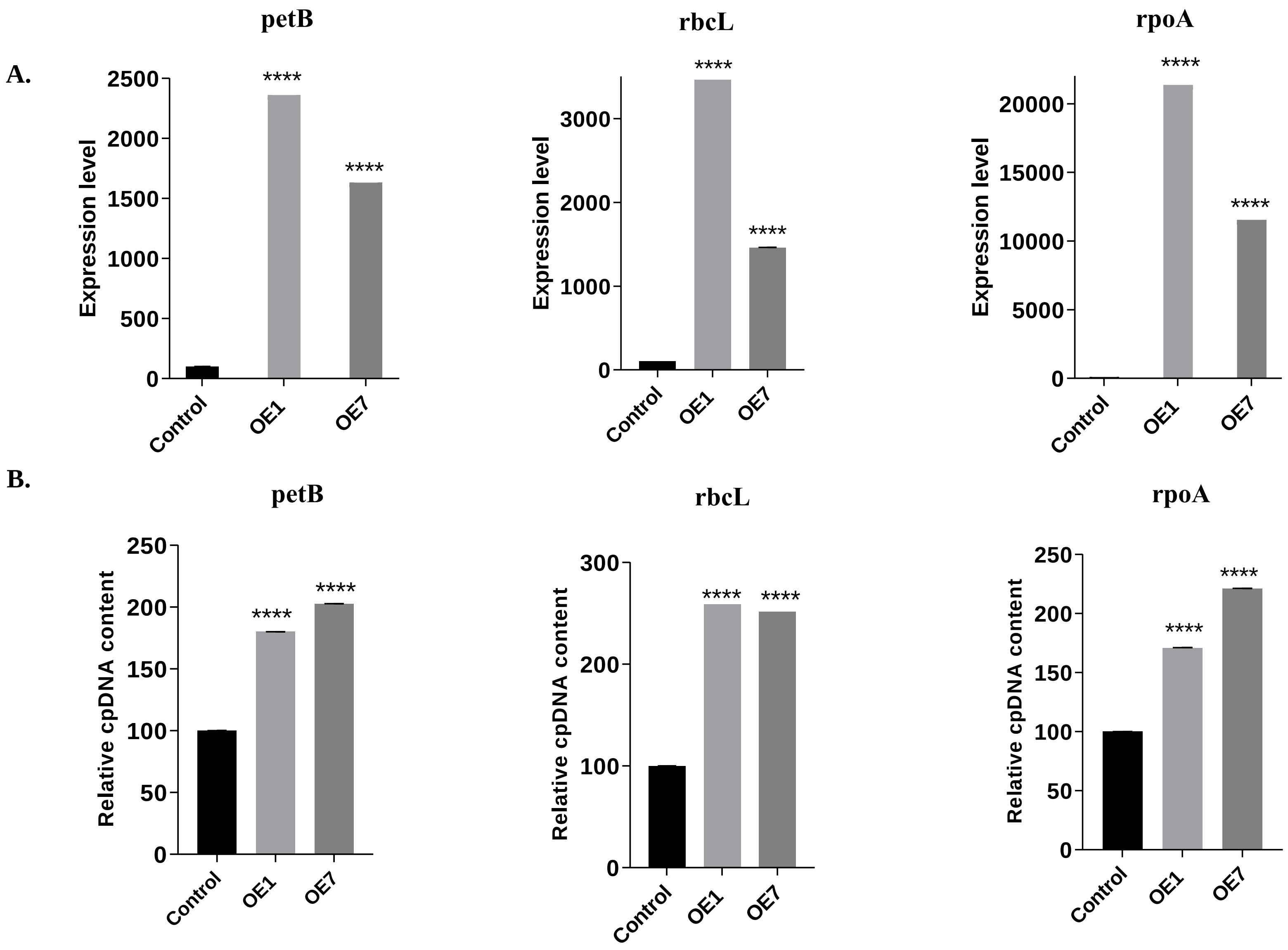


Figure 4.47: Expression pattern of chloroplast associated marker genes petB, rbcL and rpoA in control and SolycACL5 OE lines. A).Expression level and B. cPDNA content **** indicates values are significantly different compared with control at significance level of $p<0.0001$, One-way ANOVA test.