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DELETION ANALYSIS OF SUSY-Sl PROMOTER FOR THE IDENTIFICATION OF OPTIMAL PROMOTER SEQUENCE

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Abstract

The promoter region of *sucrose synthase* (susy-Sl) was identified and isolated from tomato. The 5′ deletion analysis was carried out for the identification of minimum optimal promoter. Transgenic lines of *Arabidopsis thaliana* were developed by floral dip method incorporating various promoter deletion cassettes controlling *GUS* reporter gene. *GUS* assay of transgenic tissues indicated that full length susy-Sl promoter and its deletion mutants were constitutively expressed in vegetative and floral tissues of *A. thaliana*. The expression was observed in roots, shoots and flowers of *A. thaliana*. Analysis of 5′ deletion series of susy-Sl promoter showed that a minimum of 679 bp fragment of the promoter was sufficient to drive expression of *GUS* reporter gene in the major tissues of transgenic *A. thaliana*.

Key words: *Sucrose synthase*, susy-Sl, Deletion, Optimal promoter, *GUS*, *A. thaliana*.

Introduction

The promoter sequence is basically the regulatory region of a gene which controls gene expression under various circumstances (Shinwari *et al*., 1998). The cauliflower mosaic virus promoter (35S) is the most commonly used constitutive promoter (Klaas *et al*., 2006) in plant research for the expression of transgenes. The plants react to these viral sequences antagonistically (Elmayan & Vaucheret, 1996), which results in transcriptional gene silencing. The promoters isolated from plants are effective because they are compatible with the plants genetic system (Nakashima *et al*., 2000). The *FSltp4* promoter belongs to cotton lipid transfer protein gene (Delaney *et al*., 2007) and *GhSCFP* promoter related to cotton fiber development have previously been reported (Lei *et al*., 2008). The promoter region consists of various *cis*-regulatory elements that are responsible for coping the various developmental and environmental responses (Narusaka *et al*., 2003). The inducible promoters respond to hormonal stress due to the presence of hormone inducible elements such as ABRE (Simpson *et al*., 2003; Kidokoro *et al*., 2009). The z-element with consensus nucleotide sequence (GCACATACGT) identified from *nopaline synthase* promoter was found to be auxin responsive (Gynheung *et al*., 1990). The web based databases of *cis*-acting regulatory elements provide information about these regulatory motifs and can help in the *in silico* analysis of promoter sequences.

The research work presented here is related to the characterization of deletion mutants of susy-Sl promoter isolated from tomato. The deletion studies of the full length promoter can be useful in the identification of minimum promoter sequence that retains the complete promoter activity. The susy-Sl promoter regulates *sucrose synthase* gene by converting sucrose and UDP to UDP-glucose and fructose (Komatsu *et al*., 2002). This promoter is generally expressed under low-oxygen conditions and during cell wall synthesis when UDP-glucose is available (Delmer & Amor, 1995).

Materials and Methods

Analysis of susy-Sl promoter sequence in plant PAN promoter navigator tool: Three deletions (D1, D2, D3) of full length (2.7kb) susy-Sl promoter were designed on the basis of plantPAN motifs. The D1 was designed by deleting the 5′ 1027bp including the binding site of CACGTG motif and the total promoter size was reduced to from 2.7kb to1673bp. The 5′ 123 bp having binding site for CIACADIANLELHC transcription factor were removed in D2 deletion and the promoter size was reduced to from 1673 to 1550bp. Similarly D3 deletion was designed by removing 5′ 871bp including the binding site (GAAWTTGTGA) for 5659BOXLELAT5659 motif and the size was further reduced from 1550bp to 679bp.

PCR amplification of deletion mutants and construction of plant expression vectors: Different forward primers were designed on each deletion mutant of the promoter sequence by incorporating 5′*Sac*I site, while same reverse primer having 5′*Hin*dIII site was used for the PCR amplification of deletion mutants. The forward (F) and reverse (R) primer sequences are given below:

F1 5′ TACGAGCTCTACGAGCTCTATCTTATTGATTTATTG 3′;
F2 5′ TACGAGCTCTACGAGCTCCGTGAATCAGTGATTG 3′;
F3 5′ TACGAGCTCTACGAGCTCATTTTGTATTTCTATAAAAAG 3′;
R3 5′ TACAAGCTTATTAAGCTTTGCACCTGCAAATCCTC 3′.

Plasmid DNA with full length susy-Sl promoter was used as template for the PCR amplification of various deletions. The PCR products were then cloned in pGR1, a plant transient expression vector. The deletion cassettes in pGR1 were then cloned in binary vector pGA482 for *Agrobacterium* mediated transformation in *A. thaliana*. The plant expression cassettes were constructed by ligating deletion mutants of the full length promoter (D1, D2 and D3) individually upstream to *GUS* gene in binary vectors (pCX*GUS*p, pGA482). The full length promoter expression cassette was made by fusing susy-Sl promoter upstream to *GUS* gene and a downstream CaMV terminator in pGA482. The pGA482 cassette containing

GUS gene under the CaMV 35S promoter was kept as positive control, while promoter less pGA482 construct was considered as negative controls.

***Agrobacterium*-mediated transformation of *A. thaliana* with promoter cassettes:** The full length promoter cassette with *GUS* reporter gene under the susy-Sl promoter and deletion cassettes with *GUS* gene under each deletion fragment were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Fiedle & wirth, 1988) using the protocol adopted by Clough & Bent, 1998. The *A. thaliana* plants were grown in controlled conditions. The immature siliques were removed. The culture of *Agrobacterium* was incubated at 28°C for 8 hours. The *Agrobacterium* culture was diluted 1/200 in 100 ml LB media and kept at 28°C for 14 hours with constant shaking. The culture was then centrifuged and the pellet was dissolved in 5 percent sucrose and 1X MS Macro solution. 100 ml of *Agrobacterium* culture was poured in 15 cm diameter petri dishes and aerial plant parts were dipped in *Agrobacterium* solution for 30 seconds. Silwet L-77(0.05%) was added to the culture at the time of infection. The extra liquid was dried with tissue paper and the flowers were left a little wet. The plants were kept under humid conditions for 24 hours and grown in growth chamber under controlled environment. The plants were then exposed to second infection after one week. The seeds were collected and regenerated on *Kanamycin* and *Hygromycin* media. The antibiotic resistant plants were grown in green house and confirmed by PCR. *GUS* expression was checked after one month stage.

Localization of *GUS* in transgenic *A. thaliana* plant tissues: The expression of *GUS* enzyme activity was carried out using (*X-Gluc*). The transgenic plant tissues were dipped into *X-gluc* staining solution and they were infiltrated under vacuum. The infiltrated *A. thaliana* tissues were kept at 37°C in dark for 12h. Serial dilutions of ethanol were used to remove green pigments from the incubated plant tissues. The light microscope was used for observing the expression of *GUS* gene in various tissues. The microscope attached to a high resolution digital camera was used for photography of plant tissues.

Results

Analysis of susy-Sl promoter sequence and its deletions: The full length susy-Sl promoter consisted of 139 tissue specific motifs, while there were 77, 71 and 36 tissue specific motifs in first (D1), second (D2) and third (D3) deletions respectively (Table 1). The DNA sequence of the smallest (679bp) deletion (D3) has been shown in figure 1 and its TSS, ATG, salt inducing, root specific, pollen specific and chloroplast specific motifs have been highlighted.

PCR amplification of deletion mutants and making expression cassettes: The various deletion mutants of susy-Sl promoter were amplified from the plasmid template having full length promoter using the specific primers designed on the basis of PlantPAN analysis. The PCR results are shown in figure 2A. Each of the deletion fragments was successfully cloned upstream to *GUS* gene separately in plant expression vector pCX*GUS*-p and pGA482 (Fig. 2B).

Table 1. Tissue specific motifs detected in full length susy-Sl promoter and its deletion mutants.

Motif sequence	Motif specificity	Full length	D 1	D 2	D 3
TATTCT	Chloroplast	4	3	3	3
TGACG	Root	2	2	2	0
ATAGAA	Plastid	1	1	1	0
CACGTG	Leaf shoot	1	0	0	0
GATA	Leaf shoot, chlorophyll	15	7	7	4
GGTTAA	Leaf shoot	1	1	1	0
GATAA	Leaf shoot	5	3	3	2
GTGA	Pollen	16	10	6	3
AAAGAT	Nodule, root	1	0	0	0
CTCTT	Nodule, root	6	5	5	1
ACTTTA	Root, shoot, meristem, vascular	3	2	2	1
AGAAA	Pollen	6	3	3	0
CAACA	Root, leaf, shoot	2	0	0	0
ATATT	Root	21	11	10	7
TAAAG	Guard cell	5	0	0	0
GAAAAA	Salt induced	4	2	2	1
AATAT	Root	18	11	10	7
TATAAAT	5′ UTR region of pea legA gene	1	0	0	0
TTATTT	5′ UTR region of pea glutamine synthetase gene	11	6	6	4
TAAAATAT	chlo a/b binding protein the promoter of Petunia LECPLEACS2	3	2	2	2
CAAT	pea legumin gene CAAT BOX1	13	8	8	1
Total motifs		**139**	**77**	**71**	**36**

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                                                        AAATTTTGTTGATAT  -282
TTACTAAATTCTAGATGGCATATTCTTTCACTTTATTTCTTTTGAAATATAATATTTGAA  -222
GGGTACCTATTGATCATATAGTATATAGGGAGTATGTCATAGGTGTTTTTTATTTATTTA  -162
TTGGTTCAATTATTGGTGTGGTTTAACTAAAACAGTGAAATACGACTGCTAACACCTATT  -102
TTTTAATAAATATATAATAGGAAGATCAGTGCAAGTTCTCTCTGTTGTGTTATTATTGGG   18
CCACATTTCTTTTGGTCGTTTACGTATCCTATTCTGCATTTATTTGATAACTCTCATGAC   78
ATTATAATATTGGGTAGGTCTGCTAGTTTTTATTATTCCGTCCGTGTTAGTTTGATCGGA  138
CGAGTTGATGAATTAAAATAAAAAATTTATGATTTTAAATTATCAGTTAGATATTTGAAT  198
TGAAAAAACTTATAAAATATAAAAAATACTTTTTTTTTTTTGGAAAATATAGTAGAGCTAG  258
GTGGGGTTGAAAATTTGTGCTAGTATATTCTAGATAATTGGAAAATTAGGTGATTAATTA  318
GGTGACACGATCTCATCTCTTTGAATAGCAGTAATGTGCTAAAATATTTTTATGATTTTT  378
GAGTTAACAGTATTTTGTTTGTTTATTTTTACAGTTGAAAGTCATCTGAGGATTTGCAGG  438
TGCAATG
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Fig. 1. The nucleotide sequence of susy-Sl promoter deletion (D3). The TSS (+1) is indicated by green colour while translation start site (ATG) is indicated by red colour and underlined. The 441bp region between TSS and ATG indicates 5′ UTR sequence. The root specific motifs have been shown in turquoise colour. The salt inducing element is represented by gray colour. The pollen specific motifs have been shown by dark yellow colour. The chloroplast specific motifs have been shown by yellow colour.

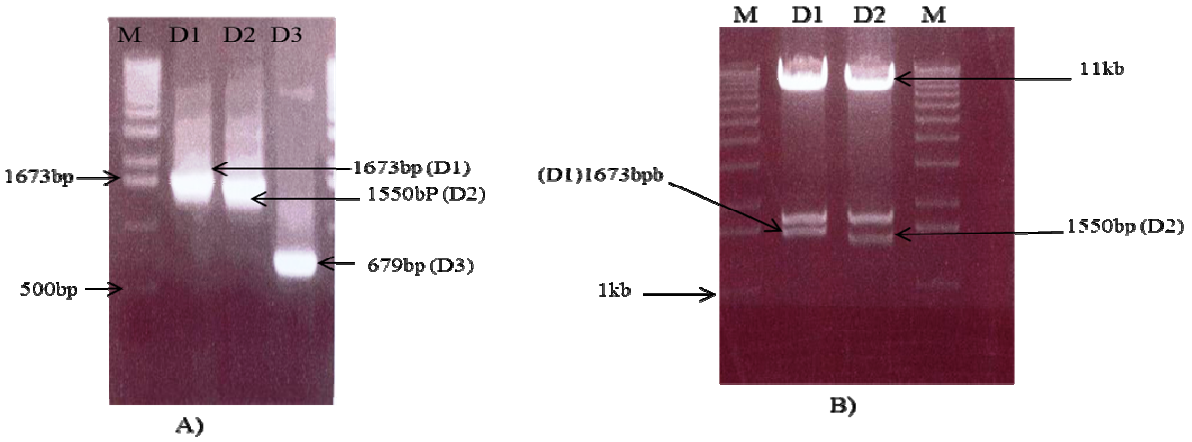

Fig. 2. PCR amplification of susy-Sl promoter deletion mutants and their cloning in pCX*GUS*-p. A) M: 1Kb DNA ladder, Lines D1, D2 and D3: PCR amplification of deletion mutants of *susy* promoter. The size of D1 is 1673bp and size of D2 is 1550bp while size of D3 is 679bp. B) M: 1Kb DNA ladder, Lines D1, D2; Restriction analysis of pCX*GUS*-p with *Sac*1/*Hind*III released 1673bp and 1550bp deletion fragments.

***Agrobacterium* mediated transformation of *A. thaliana*:** The *GUS* expression under the full length susy-Sl promoter was observed in roots, stem, flowers and siliques of *A. thaliana* (Fig. 3) indicating the constitutive behavior of the promoter. The expression of *GUS* gene under the deletion mutants (D1, D2) was also observed in the same tissues of transgenic *A. thaliana* indicating the constitutive expression of *GUS* for the deletion mutants (Fig. 4). The small deletion of susy-Sl promoter (D1) showed good expression in almost all the tissues of *A. thaliana* (Fig. 4), while the expression of *GUS* gene under the 679bp promoter was observed in leaves, stem, trichomes and flowers (Fig. 5).

Analysis of 5′ deletion fragments of susy-Sl promoter: The D3 deletion consisted of 3 motifs with consensus nucleotide sequence (TATTCT) of chloroplast specific and 4 motifs with consensus nucleotide sequence (GATA) for leaf and chlorophyll specific expression. It had 2 motifs with nucleotide sequence (GATAA) for leaf and shoot specific expression and 3 motifs with nucleotide sequence (GTGA) for pollen specific expression. There were 7 motifs with consensus nucleotide sequence (ATATT) and 7 motifs with the nucleotide sequence (AATAT) for root specific expression. A single motif with consensus nucleotide sequence (ACTTTA) for meristem and vascular specific expression was also detected in D3 of susy-Sl promoter. Additionally, there were 4 motifs with nucleotide sequence (TTATTT) and 2 motifs with consensus nucleotide sequence (TAAAATAT) for chl a/b binding protein which is the binding site for *Cys-protease*. A single motif with nucleotide sequence (CAAT) for pea *legumin* gene specificity was also detected in the 679bp (D3) *susy* deleted promoter.

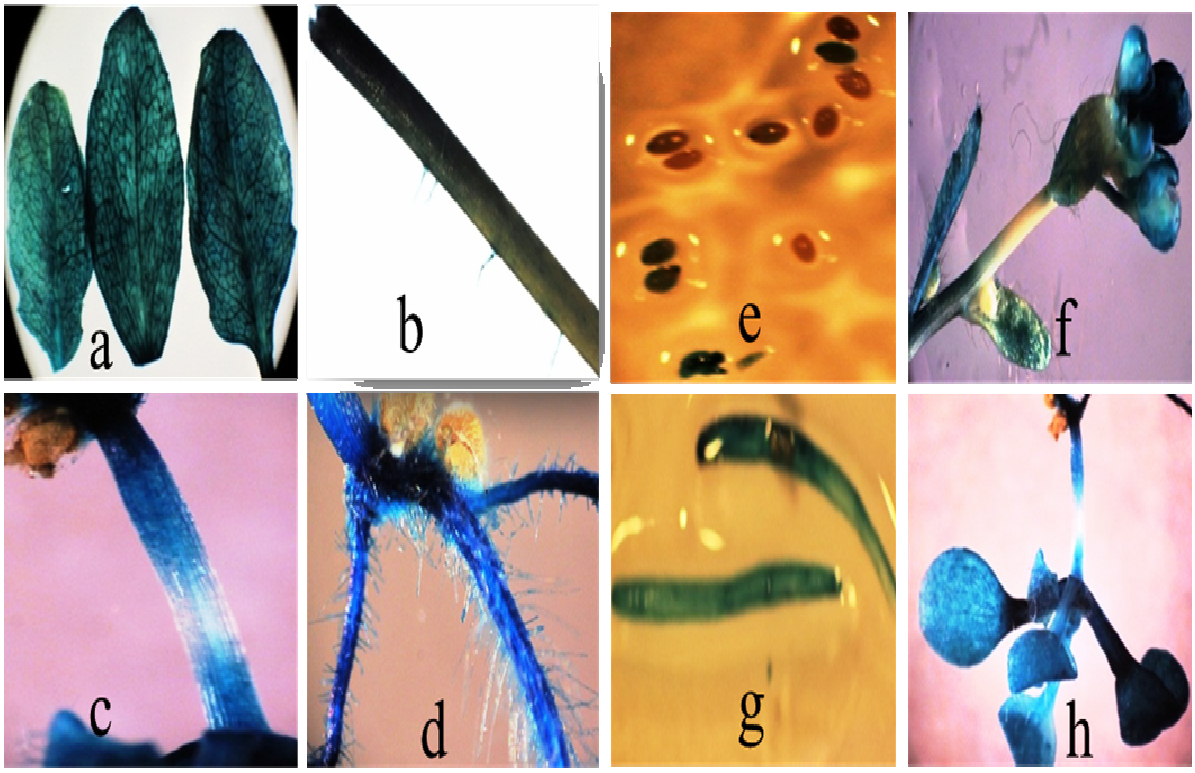

Fig. 3. Histochemical localization of *GUS* reporter gene driven by full length susy-Sl promoter in various tissues of *A. thaliana*. (a) leaves, (b) stem trichome, (c) stem, (d) roots, (e) seeds, (f) flower (g) siliques and (h) seedling.

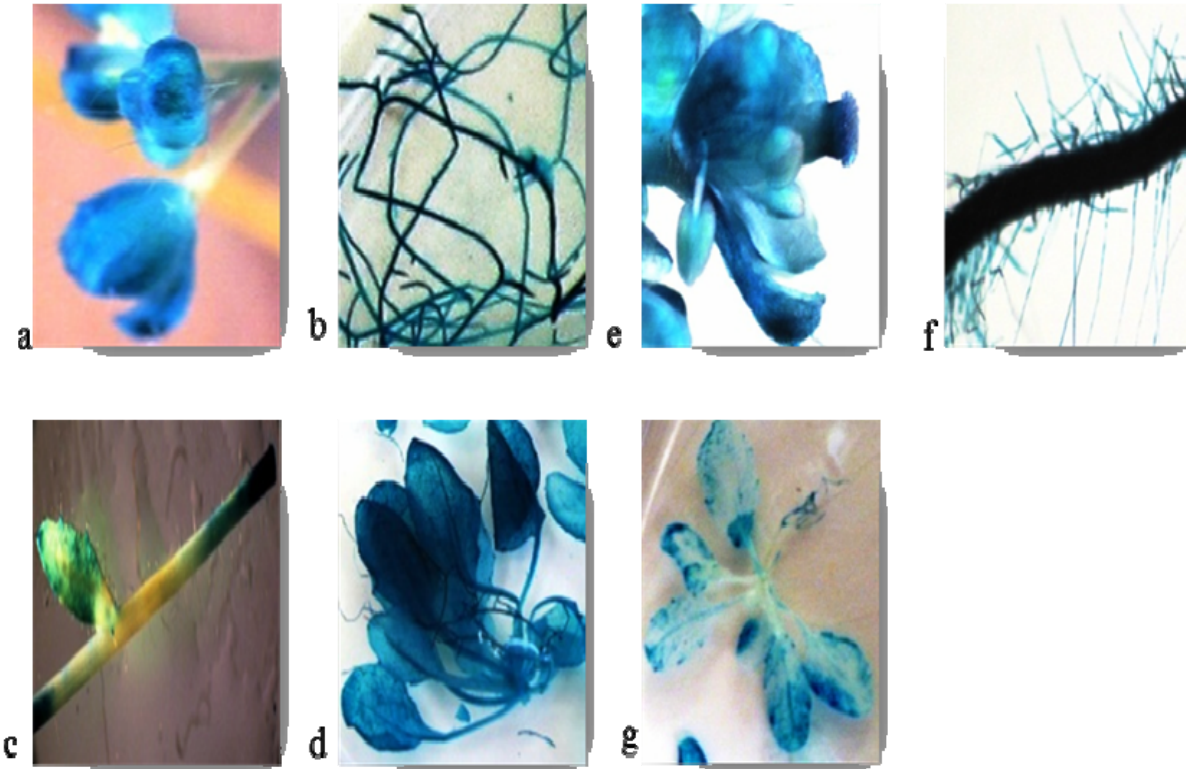

Fig. 4. *GUS* expression under the D1 deletion of susy-Sl promoter in various tissues of transgenic *A. thaliana*. a) Flowers, (b) roots, (c) stem, (d) seedling and *GUS* expression under the D2 deletion of susy-Sl promoter in various tissues of transgenic *A. thaliana*. (e) flower, (f) roots, (g) seedling.

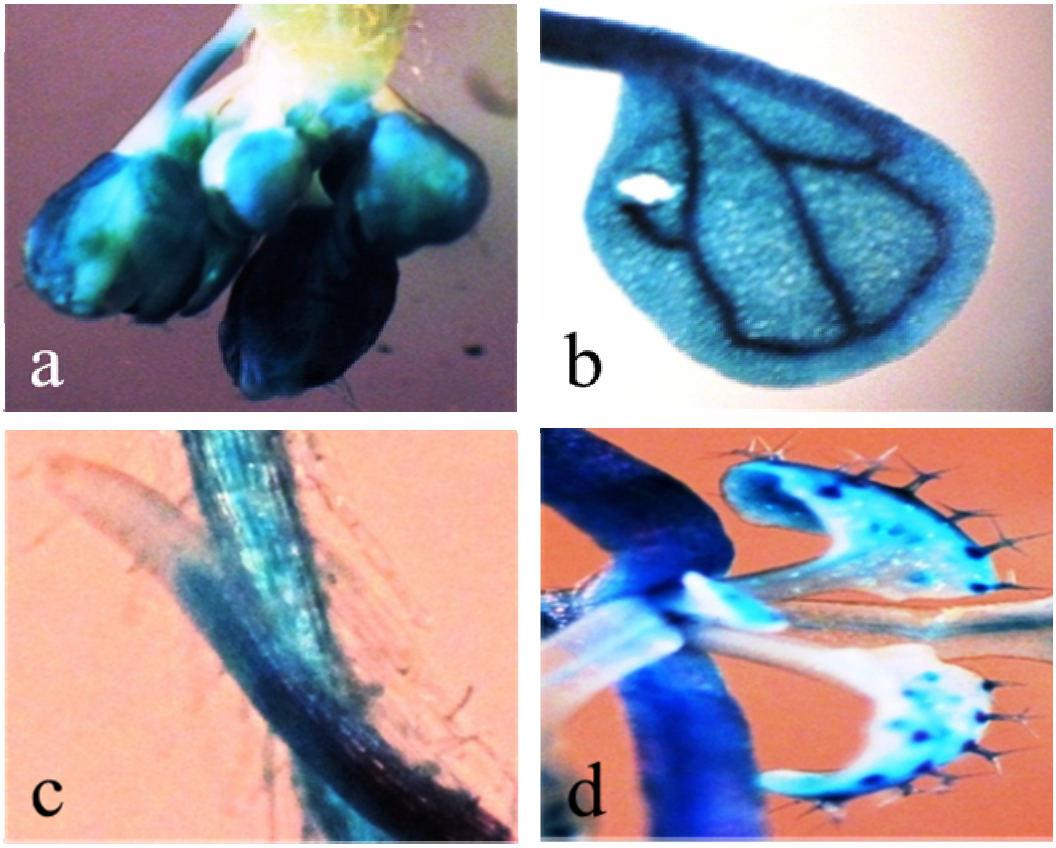

Fig. 5. Histochemical localization of *GUS* reporter gene driven by 679bp deletion mutant (D3) of susy-Sl promoter in various tissues of *A. thaliana*. (a) flowers, (b) leaf and vascular bundles, (c) roots and (d) leaf trichomes.

The various deletions of full length susy-Sl promoter showed constitutive expression of *GUS* gene in *A. thaliana*. The expression of *GUS* gene under the D1 and D2 promoter deletions (Fig. 4) indicate that these deletions do not harm the constitutive expression of *GUS* gene in *A. thaliana*. Similarly, the expression of *GUS* gene under the 679bp (D3) promoter (Fig. 5) also indicates the promoter remains active for the constitutive expression of *GUS* gene in leaves, vascular tissues, stems, roots, trichomes and flowers of transgenic *A .thaliana*.

Discussion

The full length susy-Sl promoter isolated from tomato was characterized through deletion analysis and further *GUS* assay in transgenic *A. thaliana*. The various tissue specific motifs identified in this promoter include chlorophyll a/b binding sites, pea legumin gene promoter motif, 5′ UTR sequence of pea legA gene, specific motifs for root expression, motifs for leaf and shoot specific expression, motifs for plastid, chloroplast and nodule specific expression. The binding site (TCACAATTTC) for pollen specific transcription factor 5659BOXLELAT5659, embryo specific motif (CACGTGMOTIF), CIACADIANLELHC transcription factor binding site (GATGGTGTTG), ERELEE4 binding site (TTTGAAAT) specific for ethylene inducing, binding site for LECPLEACS2 transcription factor responsible for cysteine protease response, MYB1LEPR binding site (GTTAGTT) which is defense responsive and P1BS with consensus sequence (GCATATTC) for phosphate starvation response were detected in susy-Sl promoter.

There was no effect on the constitutive expression of susy-Sl promoter by deleting the species specific motif (CACGTG) that is binding site for transcription factor (CACGTG MOTIF). This motif is necessary for binding with tomato Pti49(ERF) which acts as defense responsive element (Chandrasekharan *et al*., 2003) and the deletion mutant (D1) expressed *GUS* gene in all tissues of *A. thaliana*. The CAANNNNATC motif which is binding site for CIACADIANLELHC transcription factor and necessary for circadian regulation of tomato (Piechulla *et al*., 1998) was deleted in second deletion (D2) but the expression of *GUS* under this deletion mutant was the same as the full length *susy* promoter and there was no negative effect on promoter activity. The third deletion (D3) was designed by removing the GAAWTTGTGA motif that is binding site for the transcription factor (5659BOXLELAT5659) required for the expression of *LAT* gene promoters in pollen (Twell *et al*., 1991). The deletion of GAAWTTGTGA motif did not affect the promoter activity of 679bp (D3) promoter and *GUS* expression was found in major tissues of *A. thaliana*. The expression results indicated that the deletion mutants of full length susy-Sl promoter expressed *GUS* gene constitutively and the 679bp promoter (D3) derived from susy-Sl promoter expressed *GUS* gene in all tissues of *A. thaliana*.

The 679bp D3 deletion consisted of chloroplast, leaf and chlorophyll specific (TATTCT, GATA) motifs. The novel transcription factor (MYBST1) binds with DNA (Baranowskij *et al*., 1994), which further binds to GATA motif for transcription regulation. The small deletion of susy-Sl promoter consists of leaf and shoots specific motifs (GATAA) and anther box (GTGA). The anther box is pollen specific and it is found in pollen-specific promoters of tomato and tobacco (Rogers *et al.,* 2001). The D3 deletion mutant also consisted of ATATT and AATAT motifs, which play active role in root specific expression. The root specific motifs ATATT or AATAT have been reported in AM-responsive PiT promoters (Chen *et al*., 2010). The TAPOX1 with consensus nucleotide sequence (ATATT) detected in D3 have been reported in root specific promoters *rolD* and wheat *peroxidase* gene promoter (Elmayan & Tepfer, 1995). The motif (ACTTTA) for meristem and vascular specific expression was predicted in 679bp (D3) deletion mutant. This deletion also consists of TTATTT motif which has already been reported in pea *glutamine synthetase* gene specific expression, promoters of meiotically-active genes as well as cold inducible genes of rice and *Arabidopsis* (Angelica *et al*., 2008). The TAAAATAT motif is the binding site for *Cys protease* in tomato and active in the biosynthesis of ethylene (Lingxia *et al*., 2009). The pea *legumin* gene specific motif (CAAT) is also present in D3 which has been reported to be involved in seed storage (Shirsat *et al.*, 1989).

Conclusions

The deletion analysis of dicot promoter isolated from tomato showed that the 679bp (D3) is sufficient to drive the expression of transgene due to the presence of key motifs. This deletion fragment can be utilized for the constitutive expression of genes in transgenic plants, especially, when a gene expression lesser than 35S is required. The small size of the promoter is important for making plant expression cassettes and is an excellent candidate when multiple genes are targeted for expression in a transgenic plant.

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