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IDENTIFICATION AND ANALYSIS OF AN EFFICIENT DICOT CONSTITUTIVE PROMOTER FROM TOMATO

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Abstract

The regulatory sequence of *sucrose synthase* (*susy*) was explored in HTGS and screened using various bioinformatics tools for promoter prediction and identification of functional regulatory motifs. Transcription start site (TSS) was predicted in the promoter sequence. Species specific motifs were identified by using Plant PAN database. The Plant Care predicted various light responsive, hormone inducible and tissue specific motifs in the full length promoter which may be essential for the constitutive expression governed by this promoter. Full length *susy* promoter isolated from tomato (*Solanum lycopersicum*) drove maximum transient expression of *GUS* gene in various tissues of tobacco, cotton and peas. The *susy* promoter identified and analyzed in this study is suitable for transgene expression in economically important agricultural crops, especially to avoid strong over-expression.

Key words: Dicot, Promoter, *Sucrose synthase*, Tomato, TSS

Introduction

Promoter is the regulatory sequence containing transcription factor binding sites (TFBS) for the specific expression of genes under various developmental and environmental conditions. The most commonly used promoters for the transgene expression analysis include *Cauliflower mosaic* virus (35S) promoter and *nopalin synthase* promoter (Klaas *et al*., 2006). Most of the commercially available promoters are of viral nature and therefore, the plant cells recognize these promoters as foreign sequences and make them silenced (Elmayan & Vaucheret, 1996). This transcriptional gene silencing can be alleviated by using promoters of plant origin. Many promoters have been discovered from dicot plants and they are being utilized for the tissue specific and constitutive expression of foreign genes in agricultural crops. The promoter of wax producing *eceriferum* gene (CER6) in *Arabidopsis thaliana* flower petals has showed high flower specific expression in *Chrysanthemum* (Hannoufa *et al*., 1996). The promoter of a cotton lipid transfer protein gene (*FSltp4*) was found responsible for the transcription of fiber-specific mRNA in cotton (Delaney *et al*., 2007). Another novel fiber specific promoter, *GhSCFP* isolated from cotton fiber cDNA library has been found useful in the molecular research on fiber cell development and in cotton fiber improvement (Lei *et al.,* 2008).

Plant CARE is a web based database of plant *cis*-acting regulatory elements and it provides a tool for the *in silico* analysis of promoter sequences. Promoters contain various *cis*-regulatory elements which are 5-15 nucleotides long and they are activated under various environmental stimuli (Picot *et al*., 2010). Hormone-inducible promoters consist of conserved elements such as ABRE (Simpson *et al*., 2003). The small auxin-up RNA soybean (*SAUR*) gene promoter consists of auxin responsive motifs (TCTCTC and CCTCCCAT) which response to exogenous auxins (Li *et al*., 1994). The *nopaline synthase* promoter consists of z-element (GCACATACGT) which also responses to auxin (Gynheug *et al*., 1990).

The *susy* is an important enzyme for the mobilization of sucrose into various metabolic pathways and energy metabolism by converting sucrose and UDP to UDP-glucose and fructose (Komatsu *et al*., 2002). *Susy* belongs to a small multigene family and it have been found both in monocot and dicot species (Komatsu *et al*., 2002). It has been found that *susy* plays a role in structural and storage tissues of plants (Hesse & Willmitzer, 1996). It is up regulated under low-oxygen conditions. Recent studies have shown that *susy* directs carbon towards the pathways of polysaccharide biosynthesis. The UDP glucose is a product of *susy*. It is utilized in the formation of starch and also the synthesis of callose and various cell wall polysaccharides. The endogenous oxygen levels are generally reduced to varying degrees in active sinks such as potato tubers and developing seeds, and also in the phloem. The *susy* gene can work under such conditions, and can overcome the oxygen deficient conditions. Although *susy* is an enzyme but sometimes it works as a substrate for respiration in vascular tissues (Fu & Park, 1995). UDP-glucose becomes available for cell wall synthesis due to the enzymatic activity of *susy* (Delmer & Amor, 1995). Due to its importance, the present study was designed to analyze the promoter region of *susy* gene in tomato and identify its functional regulatory motifs.

Materials and Methods

Retrieval of *susy* promoter sequence: The gene sequence of *susy* (Accession No. AJ011319) at NCBI was BLAST searched in high throughput genome sequences (*HTGS*) and the upstream 2-3kb of matched *HTGS* sequence (Accession No. AC212655.2) was picked for promoter identification. The DNA sequence

of *susy* gene was matched to the *HTGS* of tomato. The starting codon for the tomato *HTGS* was found by BLAST search in translation tool at http.www.expasy.ch and coding frame was identified. The upstream sequence was also BLAST searched in translation tools to remove the coding region and the non-coding part was selected for promoter prediction and identification of various regulatory motifs. PlantCare was used for the identification of light responsive and hormone inducible motifs while PlantPAN was used for the identification of species specific motifs.

Isolation of *susy* promoter region through PCR: Genomic DNA was extracted (Doyle & Doyle, 1990) from tomato (*Solanum lycopersicum*) for PCR based isolation of *susy* promoter. PCR was carried out to amplify the full length promoter region of *susy* gene using specific oligos having *Sac*I site in forward primer (TACGAGCTCTCATAGCAACTGATATATAAAAC AAAAC) and reverse primer with *Hind*III site (ATTAAGCTTTGCACCTGCAAATCCTCAGA). The PCR product was cloned in TA cloning vector for sequence verification and then fused with an intron containing *Gus* gene in plant expression vector pGR1.

Plant expression vector construction: The expression cassette *susy*pGR1 was constructed by ligating *susy* promoter upstream to an intron-containing *GUS* gene in plant expression vector pGR1. The CaMV promoter was excised by *Sac*I/*Hind*III. The original pGR1 with *GUS* gene driven by CaMV 35S promoter was used as a positive control while promoter less construct of pGR1 was used as a negative control. The plant expression cassette (*susy*pGR1) consisting of *GUS* gene under *susy* promoter was used for transient *GUS* assay.

Transient *GUS* assay: Transient *GUS* assay was performed for *susy*pGR1 cassette of full length susy promoter. The standard protocol for transient assay was used (Battraw & Hall, 1990) and followed. Gold microcarriers (size 1.6 µm) were prepared for all bombardments using 500 µg of the microcarrier per bombardment. The microcarriers were washed with ethanol and sterile water. Gold particles were coated with plasmid DNA (1.0 µg/µl). For transient *GUS* assay tobacco leaf, cotton leaf, cotton fiber and pea cotyledon were bombarded at 9 cm target distance and 27 inch Hg vacuum using 1100 psi rupture disks. The petri dishes were then wrapped with a single layer of parafilm and the plates were incubated at 27°C for 24 hours. *X-gluc* (5- bromo-4-chloro-3-indolyl-b-D-glucuronide) was used for the localization of *GUS* enzyme expression. The plant tissues were dipped into staining solution of *X-gluc* under vacuum for infiltration was applied. The infiltrated tissue samples were incubated at 37°C in dark for 24h. Serial dilutions of ethanol were used for the removal of chlorophyll pigments. The tissues were then observed under the light microscope for the *GUS* expression.

Results

Isolation and sequence analysis of *susy* promoter: The transcription initiation nucleotide predicted in *susy* promoter sequence was T as given in the sequence (TTAATAAATATATAATAGGAAGATCAGTGCAAG TTCTCTC**T**GTTGTGTTA).

The full length 2.7kb *susy* promoter (Fig. 1) consists of 41 root specific motifs (with consensus nucleotide sequences ATATT, TGACG and AATAT), and 13 CAAT motifs. There were 4 salt inducible motifs (GAAAAA), 11 motifs with consensus nucleotide sequence TTATTT, 27 motifs for plastid, chlorophyll, and leaf and shoot development with consensus nucleotide sequences TATTCT, ATAGAA, CACGTG, GATA, GGTTAA and GATAA. Seven motifs with consensus nucleotide sequences (AAAGAT and CTCTT) were nodule specific, 22 pollen specific motifs with consensus nucleotide sequences AGAAA and GTGA, 5 motifs for guard cell specificity with consensus nucleotide sequence TAAAG and 3 LECPLEACS2 motifs with consensus nucleotide sequence TAAAATAT for chlorophyll binding protein were identified. The *susy* promoter also contains a number of light response motifs. There were 12 light responsive motifs, 2 auxin and gibberellin motifs, 10 defense and stress motifs and 8 species specific motifs in *susy* promoter. The putative TATA box was located at nucleotide -598 which was reported to be involved in RNA-PolII recognition. BoxII (CATTTTCAC), G-box (CACGTC), GATA-motif (GATAGGG), GT1-motif, I-box, GA-motif and P1BS has also been detected in *susy* promoter sequence. The as-1 motif (TGACG) that was found in other *Caulimovirus* promoters such as CaMV, FMV and MMV has been detected in *susy* promoter. The PCR amplification results of full length (2.7kb) *susy* promoter have been shown in Fig. 2A. The *susy* promoter was cloned in TA vector (Fig. 2B) for sequence verification.

Construction of plant expression cassette: The full length promoter sequence of *susy* was PCR amplified from genomic DNA of tomato using the specific primers and cloned into pGR1. The restriction analysis of pGR1, a plant expression vector (Fig. 2C) and pGA482, a binary vector (Fig. 2D) with *Sac*I/*Hind*III confirmed cloning of *susy* promoter in these vectors.

***GUS* expression assay of full length *susy* promoter:** Activity of full length *susy* promoter was checked by transient *GUS* expression analysis in various tissues of tobacco, cotton and pea (Fig. 3). Histochemical analysis of different tissues indicated that the promoter expressed *GUS* gene in tobacco leaf (Fig. 3A), cotton leaf (Fig. 3B), cotton fiber (Fig. 3C) and pea cotyledon (Fig. 3D). The activity of *susy* promoter was compared with 2X 35S promoter (positive control) and promoter less pGR1 (negative control). The promoter gave excellent expression in cotton and pea cotyledon.

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TCATAGCAACTGATATATAAAACAAAACAACCTATATATATCCCTATTTATCCTATATAA -2262
ATCCTAATTAAACGAGCTTACAATATATACCAAAATATAGATATTATTTTCATGGTATTA -2202
TTTTTCTGTCATGTCAAACCCAAGGTCTTTGAAAATGTGAACTTAGTTTTCTTTCTTTTC -2142
AAAAGAAGAAAATTGGACAAGAGGTAATTGGTTATTCAATGAACTCAAAGGCCAACAACT -2082
ACCACAATTACTGTGAGATGAGATTTGTACCAAAAAAGTTAAATGGATAAATAATTTAAA -2022
GGTTTAAATAGTTATTAATTCTATATCATAAAGTTGAAGTGAATCTTAAAGTAAAAATAA -1962
TTTTATCTTTACATAATTTATTAGTTATGAGTTGGAACTTAGAATAATAAGATAATATTT -1902
ATCTAATAAGTGCAATCATTTCCTAAAATTTGCGTGAACAACATAAAGTACATCAAATTA -1842
TCCTGTTTTGTAGGGTGTGTTTGGTAGTATGGAGGAAAGTTAACATTTTCTTATTTTCTT -1782
TTCCATGTTCAATTGATGAATCTTTTTGAAAAAAAATAAATTTTCTTAAAAAATAAAGAT -1722
ATATATCTTTTTCTTCCTGAATATAGAAAAAATAAATTTTATCAGTGACATTTTACATTG -1662
ATCGTGATATTCTATCATTTTTTTTTATATCTTTAGCACACTTCATTTTTTATCCACATG -1602
CCTCAACTCTCATATTATTTATTTAAATACATAAAATATTTTAATATTATATTTTTTTCT -1542
TAGATATATCTCTTGTTTACTATTAAACATGATGAAATAAGAAATTTACTATTTTCGCAT -1482
AAAAAAGTAATAAACATTTTTCTTGGATGGATACACCCATAATTATTAAATTATGTTTCT -1422
AAGAGAATTTTTTTTTTTTATCTTTTGTTATTATATTATAATTTAATTAATTTAAATTCG -1362
CGTTATATATATTAAGAATATTTTCATGTTTAATTTTAGAATCTAAAATCGATGAATAAC -1302
TTTATTTATCACGTGAATCAGTGATTGTGATGGTGTTGCTTAAAAGAGCTTAGCAGGCAA -1242
ACAAGAATTAATTAATATTCATAGCATTTTGTTTGTATAATTAAAATGTGATTCACTTGA -1182
GAAGACACGTACACATTTTGTATTTCTATAAAAAGGGACCTCTACCATTCCATTTTTTCT -1122
CATCACCATTCATAAGCAATACTCTTTCATTTCCATCTTTGTGTTAATTTCCTCCATCCA -1062
TTACTTCCCTCTATTTATTATTATTCCTTTCAATATTCCTCTCTTTCCATTTTATTCATA -1002
AAAAATAAAAACTAAAAAAAGGTAAAACCTGCGACTCTAATTTGTTTATGTTATGTCGGT -942
GCGTAAGAATTTTGTTGTTTCATCCCTATCTCATTCGTACATATAAAAAAAAAGGAATAA -882
TTTTCAGTAGCATTTGCTTGCACTACTACTCTTGTTCATGTACCTTTCGTTTTCAGAGTA -822
GTTCTTTCTTTTTTTTTTAACAAAAAAAAACATTTTAATATTCATTTTTGTTGACTGATA -762
AAAAGAAGAGATTGTGAGATTTTTTTTTTGTTATACTCTGCAAATTCTAACTTTACTTTC -702
TTTTTTTTTTCATTTTTTTGACAATATTGACAGAAAAATCTATTTATAACCGTCACGGAT -642
AGAAAACAATGTATTATACATTTGACGAGAAGAAACAAAAATCCTCACATCTCTTTTCAT -582
CTTTTTCTCCTGTTAGTATTGCTCTGTTTTTCTTTCCTCTGTTTTTTTATTTTTTTCAAT -522
TCTAGCTATTTAGATGTTGCTTTGTGTGGGGTTGAATGGGTTAAAATCATTTTATTTTTA -462
CTTGATTTAGTTTGACGTGAGCTTCAGATATCGAATGATTAAAAAAAATAGTTTCCAATC -402
AAGACATCTTTTATCTTCATAATGTTTTAGTATTTGATTTTGTCACAATTTCTTTAATTT -342
CTTCCCAGTTTTTTGTTTTCGAGTGAAACGTTGAGGAAAACGGGGAAATTTTGTTGATAT -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. Sequence of *susy* promoter isolated from tomato. Start codon (ATG) of *susy* gene is highlighted with red color, putative TATA box (-598) and G-box (-1233) are highlighted with pink and sky blue colors respectively. Position of transcription initiation site (+1) has been indicated with bold letter (T) and green color.

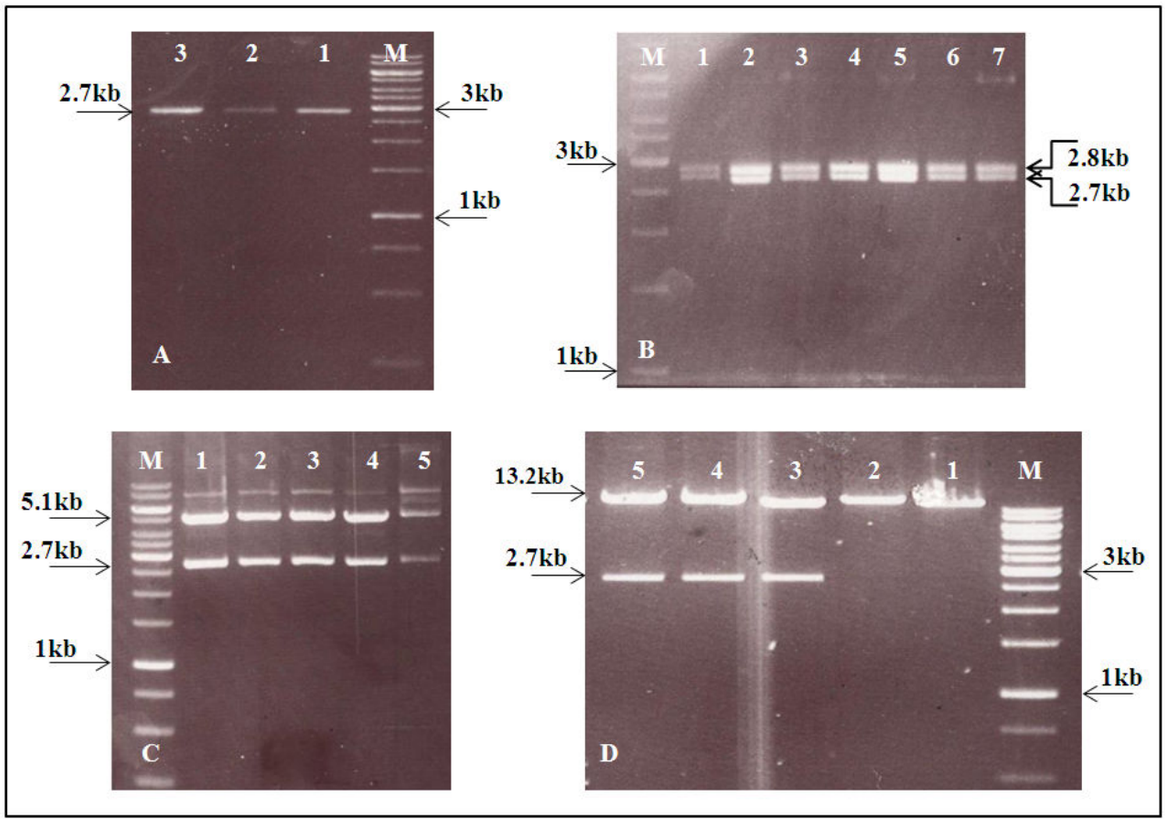

Fig. 2. PCR amplification, cloning and restriction analysis of *susy* promoter. A) M: 1Kb DNA ladder, 1 to 3: PCR amplification of full length (2.7kb) *susy* promoter from three samples of tomato genomic DNA. B) M: 1Kb DNA ladder, 1 to 7: Seven clones of *susy* promoter (cloned in TA) digested with *Sac*I/*Hind*III and releasing the 2.7kb *susy* promoter and 2.8 Kb vector backbone. C) M: 1Kb DNA ladder, 1 to 5: Five clones of *susy* promoter (cloned in pGR1) digested with *Sac*1/*Hind*III and releasing 2.7Kb *susy* promoter fragment and 5.1Kb vector backbone. D) M: 1Kb DNA ladder, 1-2: undigested plasmid, 3 to 5: Three clones of *susy* promoter (cloned in pGA482) digested with *Sac*1/*Hind*III and releasing 2.7Kb *susy* promoter fragment and 13.2Kb vector backbone.

Discussion

The full length *susy* promoter isolated from tomato was predicted using bioinfromatic tools and analyzed through *GUS* transient assay in various plant tissues. The *cis*-regulatory elements (TSS, TATA box and various transcription factor binding sites) were identified in *susy* promoter. The *susy* promoter sequence was identified to have light responsive, pollen specific, embryo specific, ethylene inducing, defense responsive, phosphate starvation responsive and hormone inducible elements. The tissue specific motifs found in this promoter include chlorophyll a/b binding motifs, motif for pea *legumin* gene, sequence found in 5′ UTR region of pea *legA* gene, root specific motifs, leaf and shoot specific motifs, plastid and chloroplast specific motifs and nodule specific motifs. The hormones play a very important role in cell signaling and the gene promoters contain hormone responding motifs. The amylase box (seed specific), GARE (seed germination), sugar responsive motif (W-box) were found in *susy* promoter. The CARE and GARE boxes were reported to be required during seed germination in *Arabidopsis* and rice (Skriver *et al.*, 1991). Flower specific motif was detected in *susy* promoter. The TGACG motif, also called the *as*-1 or *ocs*-element detected in *susy* promoter has also been reported in viral, bacterial and plant promoters (Ellis *et al*., 1987). There are five nodule specific CTCTT motifs in *susy* promoter which are involved in symbiosis (Ramlov *et al*., 1993).

The *susy* promoter consists of E-box which was reported to activate transcription synergistically with the G-box in *phaseolin* gene expression (Kawagoe *et al*., 1994). The light responsive motifs (ATCT-motif, GAG-motif, BoxI, I-box, BoxII, G-box, TCT-motif and GATA-motif) predicted in *susy* promoter sequence have been previously reported in *Pt-RbcS* promoter which acts as light responsive *cis*-acting regulatory sequences (Like *et al*., 2013). The light responsive motifs, Box-II and GATA-box act as phloem specific expression of *RTBV* promoter (Yin *et al*., 1997). The GATA motif has earlier been identified in highly expressed genes like *cab* (Gilmartin *et al*., 1990) in several plant species and in the *CaMV 35S* promoter (Benfey *et al*., 1990). The *susy* promoter also consists of defense responsive MYB1LEPR (GTTAGTT) motif. The MYB elements belong to regulatory factors of plants which control development and determination of cell fate and identity (Stracke *et al*., 2001). The root specific motif with consensus nucleotide sequence ATATT predicted in *susy* promoter has also been reported in root specific tobacco *rolD* promoter and

this motif has been identified in root-specific wheat *peroxidase* gene promoter (Elmayan & Tepfer, 1995). The *susy* promoter consists of G-box (CACGTG) which is essential for *JA*-inducible genes in various plant species including potato (Kim *et al*., 1992) and *Arabidopsis* (Guerineau *et al*., 2003). The AT-1-box, 3-AF1 binding site and Box-II present in *susy* promoter sequence have been identified in the *PNZIP* promoter.

The root specific motif (TGACG) predicted in *susy* promoter sequence has also been detected in constitutive root promoters in association with *as-1* element (Krawczyk *et al*., 2002). The root specific motifs (ATATT or AATAT) which are found in AM-responsive PiT promoters (Chen *et al*., 2010) have also been detected in *susy* promoter. The *susy* promoter consists of *cis*-acting elements (AGAAA and GTGA) which have been detected in the promoter region of pollen-specific gene *TaPSG719* obtained from wheat (Ling *et al*., 2010). The binding site for *Cys protease* detected in *susy* promoter functions as an important element in biosynthesis of ethylene in tomato fruit (Matarasso *et al*., 2005).

The *susy* promoter showed *GUS* expression in various plant tissues of tobacco, cotton and pea. The *susy* promoter was found to be highly constitutive dicot promoter for expression of transgenes in dicot plants. Full length promoter of *susy* characterized in the current studies has been found to be a constitutive and highly expressed dicot promoter that can be utilized for the transgene expression in economically important agricultural crops.

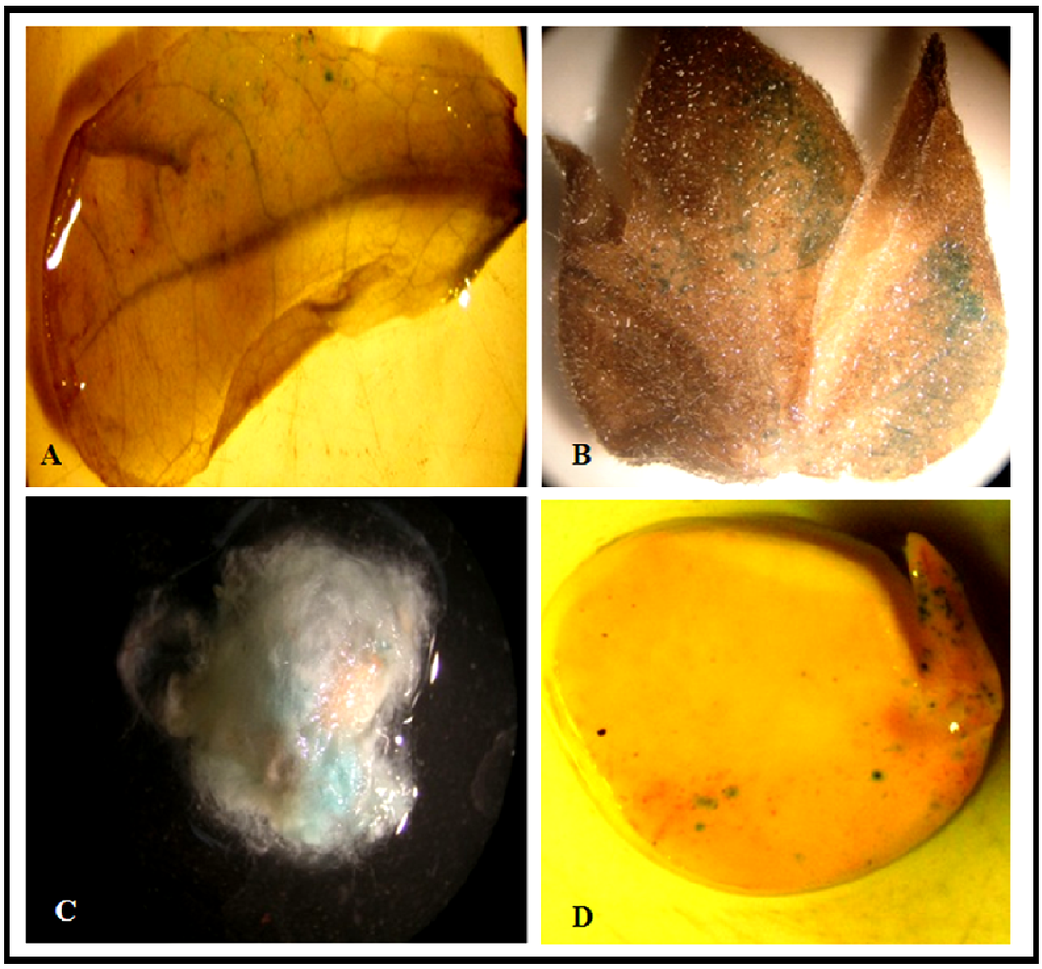

Fig. 3. Transient expression assay of *GUS* reporter gene under the full length *susy* promoter in various tissues of dicot plants. (A) tobacco leaf, (B) cotton leaf and leaf trichomes(C) cotton fiber and (D) pea cotyledon.

Conclusions

The dicot promoter of *susy* gene isolated from tomato was found a highly constitutive promoter for the expression of transgenes. The full length promoter expressed *GUS* gene in various tissues of tobacco, cotton and peas due to various light responsive, tissue specific and hormone inducible motifs predicted in the promoter region. The promoter can also express under phosphate deficient and nitrogen deficient conditions due to the presence of highly conserved motifs.

It is postulated that the reported promoter is of equal importance for nitrogen fixing or non-nitrogen fixing dicot plants for low to moderate expression of transgenes.

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