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IDENTIFICATION, ISOLATION AND EVALUATION OF A CONSTITUTIVE SUCROSE PHOSPHATE SYNTHASE GENE PROMOTER FROM TOMATO

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Abstract

Sucrose phosphate synthase (*SPS*) is one of the abundantly expressed genes in plants. The promoters of *SPS* gene was identified, analyzed and retrieved from high throughput genomic sequence (HTGS) database. The cis-acting regulatory elements and transcription start sites of promoter were identified through different bioinformatics tools. The *SPS* promoter was isolated from *Solanum lycopersicum* and was initially cloned in TA vector (pTZ57R/T). Later on this promoter was transferred to a plant expression binary vector, pGR1 (pGR*SPS*) that was used for the transient GUS expression studies in various tissues of *Nicotiana tabacum*. *SPS* promoter was also cloned in plant stable expression vector pGA482 (pGA*SPS*) and was transformed in *Nicotiana tabacum* through *Agrobacterium*-mediated transformation method. The histochemical *GUS* expression analysis of both transient and stable transgenic plants for this promoter indicated its functional importance in regulating gene expression in a constitutive manner. It was concluded that *SPS* promoter is constitutively expressed with a strength equivalent to CaMV 2X35S promoter. The promoter isolated through these studies may be effectively substituted in plant genetic engineering with other constitutive promoter for transgene expression in economically important agricultural crops.

Key words: Promoter, Sucrose Phosphate Synthase, GUS, PCR.

Introduction

Promoters are non coding DNA sequences which occur upstream of the coding regions of genes. The promoter plays an important role in the process of plant gene expression and regulation (Dare *et al*., 2008). To introduce desirable traits for improving the productivity of agricultural crops like cotton, wheat, rice etc., suitable sets of promoter: gene pairs are the necessity. Some already reported and most commonly used plant gene promoters that have constitutive type expression includes CaMV 35S, *rbcs* promoter, *actin* promoter from rice, the maize and soybean polyubiquitin promoters (Jani *et al*., 2002; Christensen *et al*., 1992; Chiera *et al*., 2007). However, to expand the availability of promoters for driving effective constitutive gene expression more promoter sequences are required to be explored for utilization in transgene development programs.

Sucrose is the central and widely distributed compound used by higher plants to remobilize reserves from storage tissues during plant germination. It plays a pivotal role in plant metabolism, plant growth and development. It is a major end product of photosynthesis and it functions in translocation (Winter & Huber, 2000), storage and in some cases also serves as a signal molecule regulating gene expression (Smeekens, 2000, Wiese *et al.*, 2004). Sucrose phosphate synthase (*SPS*) catalyzes the first step in the synthesis of sucrose in photosynthetic tissues. *SPS* activity has been shown to be highly regulated both at the transcriptional and post-transcriptional levels. *SPS* is also active in other sucrose synthesizing organs or tissues including those adapting to cold or drought, fruits, etiolated cotyledons, germinating seeds, sugarcane (*Saccharum officinarum*) stems, beet (*Beta vulgaris*) and roots (Huber & Huber, 1996; Winter and Huber, 2000). However, only a few studies of *SPS* activity in heterotrophic tissues have any possible relationship to secondary wall deposition or cellulose synthesis. *SPS* is not confined to photosynthetic tissues, but also occurs in non-photosynthetic tissues that are active in sucrose biosynthesis, e.g ripening fruits. The role of *SPS* in non-photosynthetic tissues is in sucrose import and degradation (Park *et al*., 2007). Furthermore, *SPS* is the most important regulatory component in sucrose formation during fruit ripening in banana. The promoter region of *SPS* gene in banana fruit have some *cis*-acting regulatory elements such as ARE motifs (TGTCTC), LTRE (CCGAA), ERE (Ethylene responsive Element), GAGA box, GATA box along with TATA and CAAT boxes (Choudhury *et al*., 2008). For the reasons mentioned above, *SPS* promoter was selected as a constitutive promoter and determination of its strength relative to 2X 35S promoter. The present study was focused on identification, isolation, and evaluation of promoter region associated with the *SPS* gene and its characterization by transiently and stably transforming expression cassettes of selected promoter sequences in *Nicotiana tabacum* via particle bombardment and agrobacterium mediated plant transformation, respectively.

Materials and Methods

Retrieval and evaluation of the promoters via Bioinformatics approach: The regulatory regions controlling the query gene, sucrose phosphate synthase (*SPS*) was obtained and evaluated using bioinformatics approaches. The nucleotide sequence of the selected gene was taken from Uniprot (www.expasy.ch) based on its high expression level in respective dicots. The selected gene sequence was searched in BLAST against HTGS database to identify their promoter regions. The query sequence of *SPS* gene matched an HTGS reported for *Solanum lycopersicum*. The subject HTGS was taken and the start codon of *SPS* gene was located. About 3 Kb upstream region of *SPS* gene was picked and analyzed using different bioinformatics tools i.e. at http://expasy.ch/tools/dna.html. BlastX and BlastP were performed to eliminate any coding sequences. The remaining sequence was then analyzed for transcription initiation site (TSS) using the software, BDGP

(http://www.fruitfly.org/seq_tools/promoter.html). This regulatory sequence of *SPS* was BLAST searched against the patent sequences to verify that it is not regulated by any filed patent.

Detection of cis-regulatory elements within the promoters: The cis-regulatory elements within the selected promoter sequence were screened using PlantCARE software (http://bioinformatics.psb.ugent.be /webtools/ plantcare /html/).

Isolation of *SPS* promoter: The genomic DNA from the young and fresh leaves of *Solanum lycopersicum* (tomato) was isolated using CTAB method (Rogers & Bendich, 1985). The full-length *SPS* promoter was amplified from the genomic DNA of *Solanum lycopersicum*, by conventional PCR. Four sets of primer pairs were designed on the selected *SPS* promoter sequences, to pick full length promoter from the genomic DNA. According to the cloning strategy, SacI restriction site (GAGCTC) was added to forward primer and HindIII site (AAGCTT) to reverse primer. A gradient PCR was run initially to optimize the annealing temperature and selection of best primer pair for the amplification of promoter region. After this optimization, best primer pair and annealing temperature was selected to get the appropriate fragment size of the promoters. The primer pairs *SPS*LyPrF-4 **(5'GATGAGCTCCTTTTATATAATCAAATAAGA GCA 3'),** and *SPS*LyPrR-1 **(5' TACAAGCTTCTCCTCCGCCGCACT 3')** were found to amplify a 1.5Kb *SPS* promoter fragment without any non-specific amplification product by polymerase chain reaction (1 reaction: 4 µL Taq Polymerase buffer (10X), 4 µL MgCl2 (25 mM), 1 µL dNTP (10 mM), 1 µL each primer (50 ng/µl), 1 µL Taq DNA polymerase (2.5 unit/µl), 5 µL DNA (1:40 dilution), 32 µL H2O) under the following conditions: initial denaturation 94°C for 5 min; followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 49°C for 1 min, and extension at 72°C for 2 min and a final extension at 72°C for 10 minutes.

Cloning of *SPS* promoter in TA (pTZ57R/T): PCR product was purified and ligated to the general purpose cloning vector TA (pTZ57R/T) and was transformed in E.coli (TOP10) cells. After transformation TA Clones of *SPS* (TA-*SPS*) promoter was confirmed via restriction digestion, PCR, and sequencing techniques.

Cloning of *SPS* promoter in plant transient expression vector (pGR1): The promoter was transferred to an independent transient expression vector (pGR1 *i.e.* a modified pJITT166 having GUS with intron) for the expression of a reporter gene (GUS). The plasmid (pGR1) is 5.8 kb in size and contains GUS with intron under 2X35S promoter followed by CaMV terminator. The pGR1 was used to construct expression cassette by replacing its 2X35S promoter with *SPS* promoter. The 2X35S promoter is 750bp and digestion of pGR1 with SacI and HindIII released the 2X35S promoter. Double digested PCR product of promoter with SacI and HindIII was ligated to SacI and HindIII digested pGR1 and transformed in E.coli (TOP10) cells. After transformation the clones of *SPS* (pGR*SPS*) promoter were confirmed via restriction digestion, PCR, and sequencing techniques.

Cloning of *SPS* in plant transformation vector (pGA482): The pGR1 vector having the promoter cassette (pGR*SPS*) was first digested with *Xho*I. The sticky ends generated by *Xho*I were blunt ended by end filling reaction. A second digestion with *Sac*I was performed to release the expression cassette from the parent vector. However, the pGA482 was digested with *Sac*I and *Hpa*I to obtain sites complementary to the ends of Promoter-Gene-Terminator Cassette. DNA was purified by phenol treatment followed by precipitation and ethanol washing. Furthermore, the ligation of digested DNA was done. After transformation the clones of *SPS* (pGA*SPS*) promoter were confirmed via restriction digestion, PCR, and sequencing techniques.

***Agrobacterium*-mediated tobacco transformation:** To start an *In vitro* tissue culture experiment, highly sterile conditions are required. All reagents and chemicals used in this protocol were of high purity and analytical grade, meeting the standards of plant cell tissue culture applications. The *SPS* plant expression construct was transformed in tobacco through *Agrobacterium* mediated transformation using LBA4404 as a carrier strain. The experiment was performed under ultra-sterile conditions. Plasmid DNA of the individual pGA482 clone having *SPS* promoter cassette was isolated from overnight cultures and plasmid miniprepkit. The isolated plasmids were transformed into electrocompetant cells of agrobacterium strain LBA4404 strain via an electric shock adjusted at 1.44 KV by electroporation method. The confirmed clone was then preserved in 30% glycerol for future use. Surface sterilized Tobacco (*Nicotiana tabacum* L.) seeds were germinated and grown *In vitro*.

Molecular analysis of putative transgenic plants: Young leaves from putative transgenic plants and negative control tobacco plant were selected for DNA isolation by CTAB method. The isolated DNA was re-suspended in 50µl of ultrapure sterile H_2O and stored at -20°C. Positive control plasmid DNA for each construct was isolated by using miniprep plasmid isolation kit.

Transient GUS Assay: Transient expression studies were carried out to evaluate the activity of *SPS* promoter by using reporter gene (GUS) expression in the model plant tobacco. Biolistic particle delivery system (PDS1000 He) was used for the bombardment of selected constructs in various tobacco tissues. The stopping screen, rupture discs and macrocarriers were treated with ethanol and dried. pGR1 having GUS gene under the control of CaMV 35S promoter (control) and pGR1 having GUS gene under the control of *SPS* promoter were selected for bombardment. Tobacco leaf, stem, and roots were used for the bombardments.

Bombardment by PDS-1000/biolistic gun: The fresh samples of tobacco leaves, stems and roots were collected from the green house. They were rinsed with sterilized water and allowed to dry. Each sample was placed in the center of petri plate containing MS medium (Murashige & Skoog, 1962). Single leaves but multiple numbers of stem and root tissues were used for maximum exposure of the tissues to the coated gold particles. The coating of gold particles with pGR*SPS* construct was done (Battraw & Hall, 1990). The tobacco leaf, stem, and root tissues were bombarded with a 9 cm target distance at 27 inch Hg vacuum using 1100 psi rupture disks. After bombardment, a single layer of parafilm was wrapped around each dish and the dishes were incubated at 30 °C for 24 hours.

Analysis of ß-glucuronidase expression: Histochemical localization of *GUS* enzyme activity was carried out using 5- bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Glc). All types of tissues were covered with X-gluc staining solution, and vacuum infiltrated. The samples were incubated at 37ºC in dark for overnight. At the completion of incubation time, the leaves, stems, and roots were treated with serial dilutions of ethanol (20%, 30%, 40%, 50%, 60% and 70%) to remove the pigmentation due to chlorophyll. The localized expression of *GUS* in all the tissues was intermittently monitored. The detailed observation was carried out with light microscope. Histochemically stained tissues i.e. control and positive transformants were photographed using a microscope attached to a digital microscope camera.

Results

Detection and retrieval of the promoter sequence: *SPS* gene sequence was taken from Genbank at NCBI and was searched in BLAST against HTGS. The query gene sequence for *SPS* matched to the HTGS of *Solanum lycopersicum* (accession number AC210351). About 3 kb of the nucleotide sequences upstream to each of the *SPS* gene was selected. Based on the regulatory regions analysis a 1500bp promoter region of *SPS* gene was selected.

Detection of Transcription Start Site (TSS) in promoter sequences: Transcription start site is a sequence in a promoter that functions as the RNA polymerase binding site. Its identification assures that the sequence is truly a promoter region. The BDGP promoter analysis revealed following sequence with a probability of 60% for the *SPS* promoter.

AGATCTCCTATAAATTAGCATATTAGCTTTCCATT
CTATT**G**GTTAGTTAG

The capital G in above sequence represents the predicted transcription start site (Fig. 1).

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TGGTAATTTTTGAAAAAAAATTTTTATTTTATTTTATACAACTCTAAAAATACTTTTATATAA   - 1284
TCAAATAAGAGCAATATTTTAAACAAATTTTTCCACTAATAAATATTGGAAACACAAATTTT   - 1221
AGAAAAAAAAAATATTGAAAATGCTTCTAAATATGATGTAAATTATAATCTTTGTTCCCGAA   - 1159
TTATTAATTTTACCGAATAATAAATATATAATTATTCTCATTTATTTAGATTATTTTTATTGATA - 1097
TATATTATATTTAAAAAATCATTAAAATTTCTTATGAATAAATTAAAACACTACTTTTTTATTA - 1032
GGGCAACAAGTCCGAGTTGTTTTTATAAATTATACAGTCAGAAAATCAGTTCTTGTAGTTTTC - 968
TTTGACCGCCTCTAAAGTTTTTTTCCCGTCCATTGATTCCGATTCAACAGATGAACACTCATCC - 905
TTTTGTAGTCATGAATAGTATAATGGTAAGCTCACATTCCTTCTGAATCGATATCATATATAT - 841
TAAAAAGACATTTTTTTGCATTTGATATTTGTGCAACGTCTTATATTAACTTGAAGAAACTGA - 778
TTAATTTAGGCGCTTCACCAATCTATGATAAAAGATCTGATATTTTAATAGTTTTTGCAAGTA - 715
TTGATGACTAGCGGCGATCATCAAATAATTTTTCGATCCAAATTAGGTTAAAAAAATACATAC - 652
ACATATTTGTATTCATCGTTTTCTAATAAAGTAACAGTTGTTATAACATCTCATTTCATATTCG - 590
ACGATTCTCATTCTAACACATATAATTTTATATAACATGTTTATTGAATGTATCCGAACCATC - 526
ATCGTCTCCAAATAAAATATATTCTACCGTAAAATCACGAATAAGTACTTTCCACGTGTTAA - 463
TTCTCGGTCAAACACATTCAGTTTATACAGTTAACTTGATCGACATATTTACTAATTATATTT - 401
ACCGTTTTACCATCATCGTCTCCAAATAAACTATTTTCTAGAGTAACATCATAAAAATCACAA - 338
TTCACGCTTACCAATTTTCAGTCGAACACATAAATTTTTACTGATTATATCTGCCGTATATATC - 275
ATCATCATTTTACATTGACATAAACACAGTTAATTTGACATCTTTACTGATTATATCTTTCGTA - 211
TACACCATTATCTTCTCCAAATAAAATATTTTCTACCTAGTGACTACTTGTCAATTCTTAGTCT - 147
ATCACTTTCAAATTTCAATTATAAAATCAATTTAATATATTTTCTTATTATTATAAAAGTTTGA - 83
GTGATTTTACTCTATTTTTTTTTTACAAATATTTAAAGAGAAGATCTCCTATAAATTAGCATAT - 19
TAGCTTTCCATTCTATTGGTTAGTTAGAATCTCTGTATTTTTAGCTTCTTTTTTTTTTTCTCTAA  46
ATTCTCTCTCACTGTCCTTATCATTTCACCACCTCCATAAATCTAGAAACATCTTTTCTACTCCG  111
TTAATCGCTCTAGCACACGGCGGAGGAGTGCGGCGGAGGAGATG                       155
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Fig. 1. Sequence of *SPS* promoter from *Solanum Lycopersicum*. ATG showing start of SPS gene and capital G represents transcription start site.

Table 1. *Cis*-regulatory motifs in *SPS* promoter.

Site name	Organism	Position	Sequence	function
5UTR Py-rich stretch	*Lycopersicon esculentum*	1382	TTTCTTCTCT	*cis*-acting element conferring high transcription levels
A-box	*Petroselinum crispum*	404	CCGTCC	*cis*-acting regulatory element
ABRE	*Arabidopsis thaliana*	873	CACGTG	*cis*-acting element involved in the abs*cis*ic acid responsiveness
ACE	*Petroselinum hortense*	871	ACGTGGA	*cis*-acting element involved in light responsiveness
AE-box	*Arabidopsis thaliana*	1438	AGAAACAT	part of a module for light response
AT1-motif	*Solanum tuberosum*	189	ATTAATTTTACA	part of a light responsive module
Box I	*Pisum sativum*	8	TTTCAAA	light responsive element
Box I	*Pisum sativum*	1206	TTTCAAA	light responsive element
Box-W1	*Petroselinum crispum*	380	TTGACC	fungal elicitor responsive element
Box-W1	*Petroselinum crispum*	888	TTGACC	fungal elicitor responsive element
CAAT-box	*Brassica rapa*	1290	CAAAT	common *cis*-acting element in promoter and enhancer regions
CAAT-box	*Arabidopsis thaliana*	1343	CCAAT	common *cis*-acting element in promoter and enhancer regions
CCGTCC-box	*Arabidopsis thaliana*	404	CCGTCC	*cis*-acting regulatory element related to meristem specific activation
G-box	*Brassica napus*	872	CACGTGG	*cis*-acting regulatory element involved in light responsiveness
G-box	*Arabidopsis thaliana*	873	CACGTG	*cis*-acting regulatory element involved in light responsiveness
GARE-motif	*Brassica oleracea*	423	TCTGTTG	gibberellin-responsive element
GATA-motif	*Solanum tuberosum*	1409	AAGGATAAGG	part of a light responsive element
GT1-motif	*Arabidopsis thaliana*	677	GGTTAA	light responsive element
HSE	*Brassica oleracea*	14	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
HSE	*Brassica oleracea*	512	AAAAAATTTC	*cis*-acting element involved in heat stress responsiveness
I-box	*Solanum tuberosum*	977	TATTATCTAGA	part of a light responsive element
I-box	*Helianthus annuus*	1407	atGATAAGGTC	part of a light responsive element
MBS	*Arabidopsis thaliana*	1099	TAACTG	MYB binding site involved in drought-inducibility
TATA-box	*Arabidopsis thaliana*	1294	TATTTAAA	core promoter element around -30 of transcription start
TATA-box	*Daucus carota*	1312	ccTATAAATT	core promoter element around -30 of transcription start
TC-rich repeats	*Nicotiana tabacum*	557	ATTTTCTTCA	*cis*-acting element involved in defense and stress responsiveness
TC-rich repeats	*Nicotiana tabacum*	1146	ATTTTCTCCA	*cis*-acting element involved in defense and stress responsiveness
as-2-box	*Nicotiana tabacum*	1139	GATAatGATG	involved in shoot-specific expression and light responsiveness

***Cis*-regulatory elements associated with the promoter:** PlantCARE software was used for the evaluation of the *cis*-regulatory elements in the *SPS* promoter. Several types of motifs were found dispersed over the entire promoter. The most frequent motif was observed to be light responsive element, while stress, infection and hormone response motifs were also found scattered throughout the promoter region. The predicted regulatory elements are given in Table 1.

Isolation of promoters from respective plant species: The genomic DNA was isolated from the fresh leaves of *Solanum lycopersicum* and a 1.5Kb *SPS* promoter fragment was amplified without any non-specific amplification product at 49°C annealing temperature (Fig. 2a).

Cloning of promoters in TA vector: PCR products of *SPS* promoter was ligated separately in TA (pTZ57R/T) vector and after transformation the clones of *SPS* promoter was confirmed in TA (pTZ57R/T) vector. TA Clones of *SPS* (TA-*SPS*) were confirmed via restriction (Fig. 2b) and PCR (Fig. 2c). Clones were also confirmed by DNA sequencing on an ABI3100 DNA sequencer using BigDye Terminator sequencing kit version 3.1.

Cloning the *SPS* promoter in a derivative of pJIT166 (pGR1): The 2X35S promoter in pGR1 was replaced successfully with *SPS* promoter using SacI and HindIII sites (Fig. 3a). Clones of *SPS* (pGR*SPS*) were confirmed via restriction (Fig. 3b) and PCR (Fig. 3c). The physical map of the final vector pGR*SPS* is shown in Fig. 4.

Cloning of *SPS* gene promoters in plant transformation vector pGA482: The complete expression cassette was obtained from previous clone in pGR1 (pGR*SPS*P) through and was successfully ligated to pGA482 (pGA*SPS*P) Figs. (5 & 6).

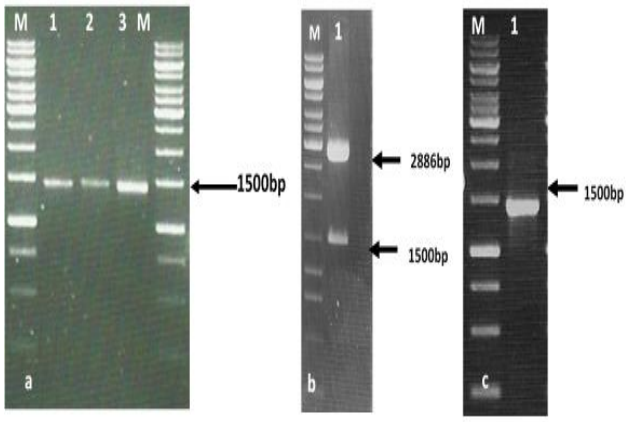

Fig. 2. PCR amplification of *SPS* and confirmation of *SPS* clones. a) PCR amplification of *SPS* promoter from *Solanum lycopersicum*. M: 1Kb Ladder, Lane 1,2,3: Showing PCR amplification of *SPS* promoter from the genomic DNA of *Solanum lycopersicum* at different annealing temperatures (46°C, 48°C, 49°C). b) Verification of promoter clones in TA (pTZ57R/T). M: 1Kb DNA ladder, Lane 1: Digestion of selected *SPS* promoter clone with *Sac*I and *Hind*III indicating a 1.5 kb insert, c) Confirmation of promoter clones in TA (pTZ57R/T) by PCR. M: 1Kb DNA ladder, Lane 1: PCR amplification of *SPS* promoter.

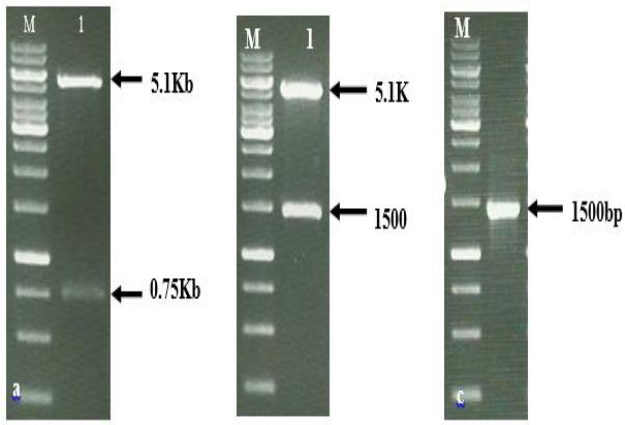

Fig. 3. Cloning confirmation of *SPS* promoter in pGR1. a) Restriction digestion of pGR1-35S vector. M: 1Kb DNA ladder, Lane 1: Digestion of pGR1 vector with *Sac*I and *Hind*III released about 0.75 kb fragment., b) -Confirmation of promoter clones in pGR1-SPS vector, M: 1Kb DNA ladder, Lane 1: Digestion of *SPS* promoter clone with *Sac*I and *Hind*III released a 1500bp fragment, c) Confirmation of promoter clone in pGR1 by PCR, M: 1Kb DNA ladder, Lane 1: PCR amplification of *SPS* promoter.

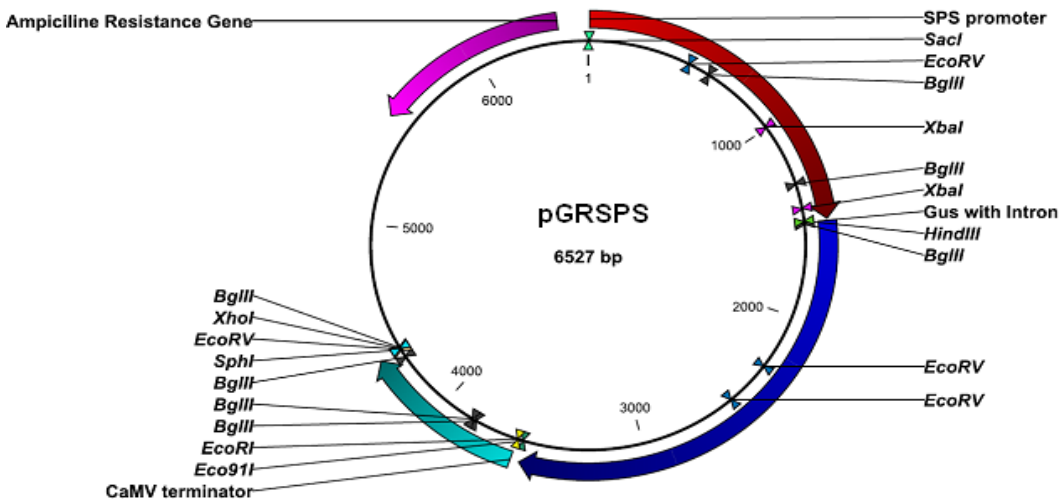

Fig. 4. Construct pGR*SPS*: *SPS* promoter controlling *GUS* expression in pGR1.

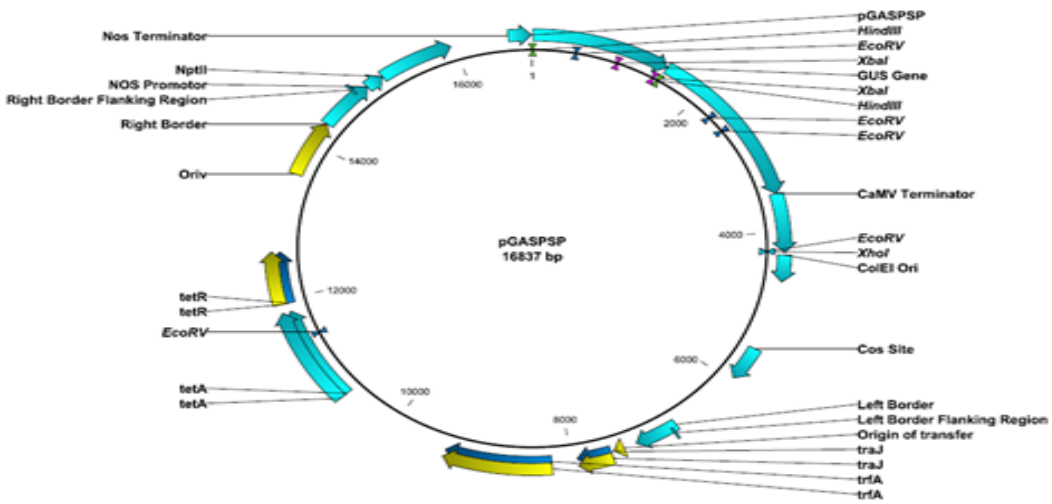

Fig. 5. Physical map of pGA*SPS*P.

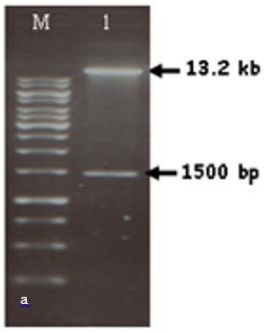

Fig. 6. Confirmation of *SPS* expression cassette in pGA482. Confirmation of *SPS* expression cassette in pGA482 by digestion with *Sac*I, M: 1Kb Ladder, Lane 1: Restriction of pGA*SPS* with *SacI* and *Hpa*I.

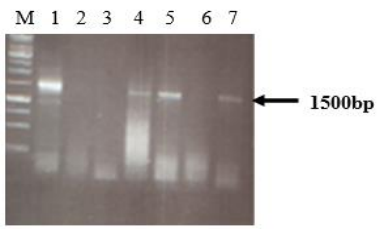

Fig. 7. PCR analysis of putative transgenic tobacco plants for *SPS* promoter.
M: 1kb DNA ladder, Lane1: PCR of positive control using plasmid DNA as a template, Lane2: Negative control of PCR master mix, Lane3: Negative control of tobacco, Lane 4-7: PCR analysis of 4 randomly selected putative transgenic plants using promoter specific primers, showing expected amplification product of 1500bp.

Tobacco transformation and transgenic analysis: The construct (pGA*SPS*P) was transformed independently into the *Agrobacterium* (LBA4404) elcetrocompetent cells by electroporation. The clones were confirmed by PCR using reverse and forward promoter specific primers. Leaf discs of *Nicotiana tabacum* were cut and co-cultivated with the cultured *Agrobacteroium* containing plant expression vectors. This gives a fair chance to the *Agrobacterium* to transform the gene of interest into the plant. Leaf disc were placed on solidified MS0 medium**.** Many putative transgenic plants were obtained through *Agrobacterium*-mediated transformation. However, a few plants for each construct were selected randomly for transgene analysis. The total genomic DNA was isolated from the putative transgenic and non-transformed negative control plants using CTAB method. The transgenics were confirmed by PCR using promoter specific primers. Results of PCR analysis are shown in Fig. 7. The results indicate amplification of expected fragments from the transgenics for each construct. The amplifications in the transgenics yielded identical size of amplified DNA fragment that could also be seen in the positive control. However, no amplification was observed in the genomic DNA of control plant.

Evaluation of *SPS* promoter by *GUS* assay: The specificity and efficiency of the *SPS* promoter was evaluated by transient GUS assay. The clone (pGR*SPS*) was used in particle bombardment experiments on leave, stem and root tissue of tobacco. The positive and negative controls were included in each biolistic experiment. The bombarded tissues were incubated and observed under the microscope as described earlier. The stained tissues were photographed with a Polaroid digital camera as shown in Fig. 8. The microscopic studies revealed that the *SPS* promoter constitutively expresses in all the selected tissues. In tobacco tissues the GUS activity under *SPS* promoter was very strong and comparable with the positive control expression (expression under 2X35S promoter). The GUS activity assays in leaf, stem and root tissues are indicated by figure 6.

Histochemical *GUS* staining: The leaf tissues from PCR selected transgenic plants were stained for *GUS* activity. *GUS* assay was carried out on leaf tissues. The plants tissues expressing *GUS* using *2X35S* and the selected *SPS* promoter was stained for 24 hours (Fig. 9).

Discussion

Cis-regulatory elements work synergistically with the other promoter elements to confer a given function and are generally conserved between species. The *cis*-regulatory elements were analyzed in the isolated promoters. Analysis of the regulatory elements in *SPS* promoter identified 5' UTR pyrimidin-rich stretch along with the core promoter element, TATA-box and enhancer region (CAAT-box). The 5' UTR stretch confers high transcription levels for the expression of many dicot genes. ABRE and HSE elements observed in the *SPS* promoter have been studied in the promoter regions of many *Arabidopsis* genes that are involved in oxidative stress response (Rizhsky *et al*., 2004). This *cis*-regulatory element has been reported earlier in the promoters of many wound inducible genes from *Arabidopsis*. The presence of I and G boxes in the *SPS* promoter can be necessary for the expression in photosynthetic tissues as has been demonstrated in *rbcs* and *cab* genes (Arguello-Astorga *et al*., 1996; Gilmartin *et al*., 1990). Some *cis*-regulatory elements like ERE, GATA and LRE responsible for the stress induction found in *SPS* promoter have already been characterized in the *SPS* promoter from banana (Choudhury *et al*., 2008).

Other motifs located in *SPS* promoter region include ACE, AE-box, AT1-motif, I-box, GATA-motif, GT1-motif and G-Box. These are the *cis*-regulatory motifs found in *rbcS* and other light-regulated promoters of many dicot plant genes for the expression in photosynthetic tissues as has been demonstrated in *rbcs* and *cab* genes (Arguello-Astorga *et al*., 1996). The MYB binding site (MBS), involved in drought-inducibility, is also present in *SPS* promoter. This motif is reported in stress inducible genes of *Arabidopsis*. MBS and G-box are the coordinated motifs which combine ethylene or abscisic acid and other stresses with photoperiod (Thomashow, 1999; Mongkolsiriwatana *et al*., 2009). Thus, all the motifs mentioned above (GARE, ACGT, G box and MBS) work as a complex to respond not only to light responsiveness but also to hormonal and other stress responses. The TC-rich repeats represent the *cis*-acting elements involved in defense and stress responsiveness in *Arabidopsis* and rice. These repeats have also been found in the *SPS* promoter along with the fungal elicitor responsive motif Box-W1, which have been reported in pathogen inducible promoters of many plant genes (Bernard *et al*., 2010). The study on rice SPS promoters

suggest that these promoters contain motifs that are controlled by light illumination and as well as by circadian clock (Yonekura *et al*., 2013). Some of the previous studies showed, the importance of light and circadian rhythms in the transcriptional regulation of the SPS promoter. The transgenic plants were measured under a light-dark cycle with an observation with low promoter activity in dark and increased activity after the beginning of light period. However, these studies indicated that light was essential for expression of SPS and also for regulation of post transcriptional regulation (Winter & Huber, 2000). Moreover, some clear circadian rhythms were detected after exposure of some transgenic plants to light and dark cycles. The circadian rhythms of promoter activities were monitored for plants being studied for expression. Several changes were observed in plants after exposure to light and dark cycles, which depicted the role and existence of circadian rhythm.

The presently characterized tomato *SPS* promoter which has a wide variety of *cis*-regulatory motifs. The analyses of most of the motifs associated with the *SPS* promoter indicate that this promoter is expressed in all the tissues in *Solanum lycopersicum* and is regulated by light, stress as well as hormonal response. Therefore, it can be supposed that this promoter is rather having a constitutive response. This assumption is supported by the fact that *GUS* expression is detected in all the selected plant tissues both in transient as well as stable transformation studies.

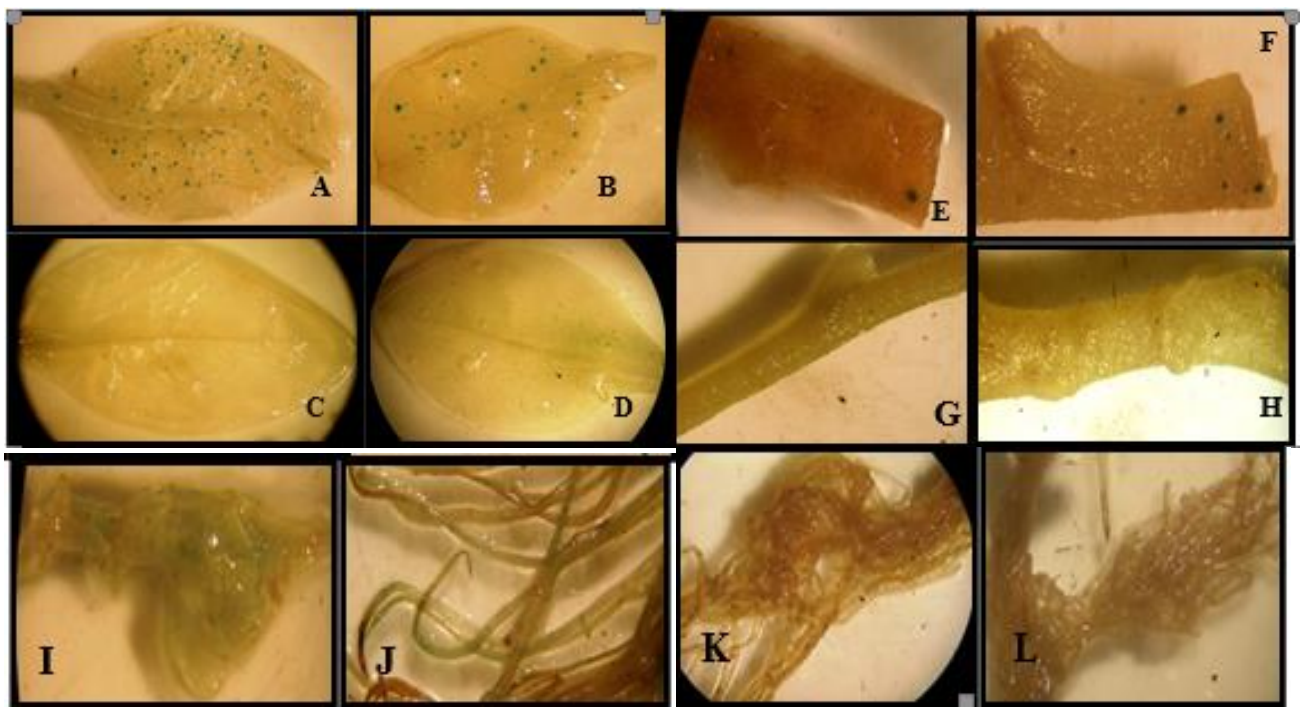

Fig. 8. Transient GUS expression by *SPS* promoter in tobacco leaf, stem and roots.
A) GUS activity under *SPS* promoter, B) Positive control: GUS activity under 2X35S promoter, C) Negative control: tissue bombarded with uncoated gold particles, D) Negative control: Un-bombarded but GUS stained leaf. E) GUS activity under *SPS* promoter, F) Positive control: GUS activity under 2X35S promoter, G) Negative control: tissue bombarded with uncoated gold particles, H) Negative control: Un-bombarded but GUS stained stem. I) GUS activity under *SPS* promoter, J) Positive control: GUS activity under 2X35S promoter, K) Negative control: tissue bombarded with uncoated gold particles, L) Negative control: Un-bombarded but GUS stained roots.

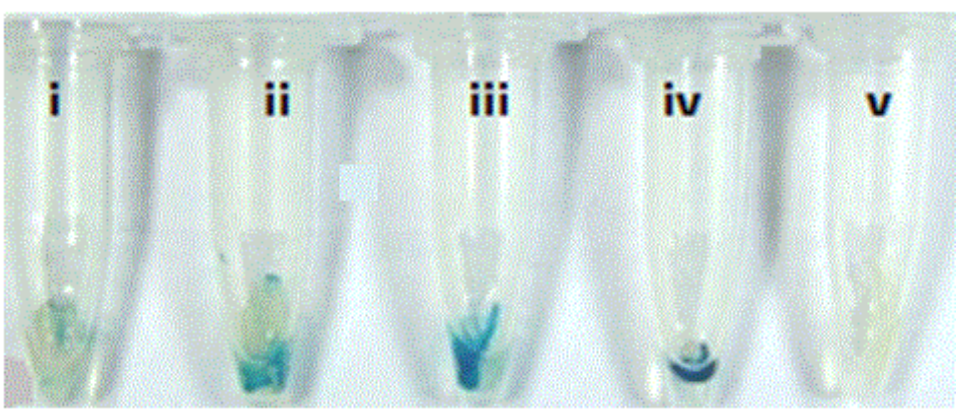

Fig. 9. Histochemical assay for *GUS* activity in stably transformed tobacco plants. (i-iii) Leaf tissue stained in different transgenic events for GUS activity controlled by *SPS* promoter: (iv) shows *GUS* activity controlled by 2X35S: (v) shows non-transgenic i.e negative control.

Conclusion

The transient and stable expression studies revealed that the selected promoter viably controlled *GUS* expression. *SPS* promoter has shown a strong *GUS* activity in all the selected tissues (leaf, stem, roots) of the model plant. The *GUS* staining indicated that the *SPS* promoter had comparable expression in different transgenic events, the staining of leaves from non-transgenic plants did not reveal the development of *GUS* stain. Therefore, it is concluded that the *SPS* promoter can be utilized to confer a constitutive gene expression in all plant tissues.

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