Journal of Advances in Biology & Biotechnology
8(1): 1-8, 2016; Article no.JABB.27071
ISSN: 2394-1081

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Histochemical GUS Expression of Cotton SPS Promoter in Transgenic Tobacco

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Authors' contributions

Present work was conducted in collaboration between all authors. Authors NI and AM conducted the experiments and managed the analyses of the study. Author AB designed the study and experimental protocol. Authors NI and AB wrote the first draft of the manuscript. Author MA managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JABB/2016/27071
Editor(s):
(1) Anil Kumar, Professor & Head (Chair), School of Biotechnology, Devi Ahilya University, Madhya Pradesh, India.
Reviewers:
(1) Yongfei Wang, Jinan University, The People's Republic of China.
(2) Kiri H. Jaryum, University of Jos, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/15553

Short Research Article

Received 17th May 2016
Accepted 26th June 2016
Published 28th July 2016

ABSTRACT

Sucrose phosphate synthase has role in synthesis of sucrose and cellulose in higher plants. A 2 kb cotton sucrose phosphate synthase (SPS) promoter and its 1.5 kb deletion fragment were cloned in plant expression vector pGA482 respectively. Both promoter fragments were transformed stably in tobacco through *Agrobacterium* mediated transformation. SPS promoter was found to express in tobacco plants. Full length 2 kb promoter showed high expression as compared to 1.5 kb fragment. The study revealed that SPS promoter was active in heterologous system and may be used to transform transgenes in dicots.

Keywords: Agrobacterium mediated transformation; sucrose phosphate synthase; promoter; histochemical GUS expression; Tobacco.

1. INTRODUCTION

Promoters are DNA sequences that regulate gene expression. Various regulatory motifs are present within the promoters that determine their constitutive, temporal and spatial expression [1]. Promoters are components of expression cassette to express foreign genes in any

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organisms. Most of promoters used in biotechnology to generate transgenic plants are from viral origin like 35S and 19S [2,3]. However, some of viral promoters are unable to express foreign gene due to post transcriptional gene silencing [4]. Moreover, these are already patented and scientists have to address IPR issues to use in research. So, there is need to explore novels to express in dicot plants to over rule patency issues. A number of promoters have been isolated from dicot plants. Previously a cotton lipid transfer protein promoter showed expression in tobacco trichomes [5]. *Arabidopsis* pGc1 promoter exhibited in tobacco and *Arabidopsis* guard cells [6]. A cotton α globulin gene promoter showed seed specific expression [7].

In this study upstream region of cotton SPS gene was expressed stably in tobacco. Sucrose phosphate synthase (SPS) is an important enzyme that has role in carbohydrate metabolism and carbon partitioning [8]. This enzyme catalyses the synthesis of sucrose, which is most widely transported material in plants. Sucrose has crucial roles in plants including increase plant growth as well as increase in plant biomass [9]. Sucrose is degraded to UDP-glucose and fructose of which UDP-glucose is used as for cellulose synthesis in cotton [10]. Over expression of SPS in higher plants has resulted in production of more sucrose as compared to cellulose [11]. Cotton SPS gene promoter was retrieved through high through put genomic sequences (HTGS) and its sequence was analyzed through available databases. Promoter showed expression in cotton fiber (unpublished data).

In the present study, we cloned 2 kb SPS promoter and its deletion fragment in plant expression pGA482. Both promoter fragments were transformed in model plant tobacco through *Agrobacterium* mediated transformation. Stable GUS expression in tobacco revealed that promoter showed expression in tobacco leaves. However, expression was weak in 1.5 kb deletion fragment as compared to 2 kb fragment. The results showed that cotton SPS promoter is able to derive GUS expression in tobacco tissues and may be used to transform foreign genes in dicot plants.

2. MATERIALS AND METHODS

2.1 Cloning of SPS Promoter in pGA482

A cotton SPS promoter cloned in pGR1 (an expression vector) was granted by plant genome resource lab, gene isolation group, NIBGE Faisalabad, Pakistan. Expression cassette containing 2 kb SPS promoter followed by GUS gene and CaMV terminator was excised from pGR1 and cloned in plant expression vector pGA482. Expression vector pGR1 having 2 kb SPS promoter cassette was linearized with *Sph*I and purified through salt precipitation method. This vector was end filled to remove overhangs produced by *Sph*I. Plasmid DNA was then restricted with S*ac*I and purified again. Plant expression vector pGA482 was digested with *Sac*I and *Hpa*I to produce one blunt by *Hpa*I and other *Sac*I overhang complementary to linearized pGR1 vector. Both DNA fragments were ligated and transformed in bacterial cells. Clones containing expression cassette having 2 kb SPS promoter in pGA482 were confirmed through digestion with *Hind*III. A 1.5 kb deletion fragment of SPS promoter was also cloned using same method. PCR analysis using promoter specific primer was also performed to confirm promoter clones (Table 1).

Table 1. Primers used for PCR analysis

Promoter	Primer name	Sequence (5´-3´)
2 kb SPS	SPSGhV2F-5	TATGAGCTCGAGTGCAATATTGGCTTACC
	SPSGhR-4	GACAAGCTTGACTGAAAAAATCCACCAAAC
1.5 kb SPS	SPSGhV1F-1	GACAAGCTTGACTGAAAAAATCCACCAAAC
	SPSGhR-4	GATGAGCTCTCAATTCATAAAGGGATAAATC
SPS	BDSPSGUSF	TGCTACACCGCCATAGACTCTG
(gene junction)	BDSPSGUSR	GCTTTCCCACCAACGCTGATC
35S	BD35SGUSF	CCTCCTCGGATTCCATTGC
(gene junction)	BD35SGUSR	GTCGGTTCTGTAACTATCATCATC

2.2 *Agrobacterium* Mediated Transformation in Tobacco

Plasmid constructs having 2 kb SPS and 1.5 kb SPS promoter were transformed in *Agrobacterium* strain LBA4404 through electroporation. Colonies were selected in antibiotic media containing tetracycline (15 µg/l) and Rifampicin (12.5 µg/l). PCR *analysis* using promoter specific primers were used to confirm transformation of recombinant plasmids in *Agrobacterium*.

2.3 Cocultivation of Tobacco Leaf Discs and Regeneration of Tobacco Plants

Tobacco variety (*Nicotiana tabacum* L., Samsun) was used in the study. Tobacco plants were grown in glass jars containing MS media [13]. Tobacco leaf discs were obtained from 5-6 weeks plants grown in the sterilized conditions. Leaves were cut into about 1 cm^2 pieces after removing midrib and margins. For inoculation of each construct 15-20 leaf discs were soaked for 45 minutes in 10-15 ml culture of *Agrobacterium* having recombinant plasmids. For positive and negative control plasmids having 35S promoter and promoterless constructs were used. These leaf discs were shifted to MS media and placed at dark at 25°C. After two days, these leaf discs were shifted to regeneration media at 25ºC and 16/8 hour day/night length. As callus began to appear, these leaf discs were moved to selection media. After two weeks, tiny plants were separated and transferred to jars to develop adventitious roots. These rooted plants were shifted to pot in green house at 16 hour day length at 25°C.

2.4 Analysis of Transgenic Plants

Chromosomal DNA of putative transgenic plants was used for PCR amplification using gene junction primers designed through Beacon Designer software (Table 1). PCR analysis of positive control (plasmid DNA) and non transgenic plants transformed with promoterless construct were also performed. PCR was performed at initial denaturation at 94°C, followed by denaturation at 94°C, annealing at 55°C, extension at 72°C (one minute each, 40 cycles) followed by final extension at 72°C for 10 minutes.

2.5 Expression of GUS Gene in Transgenic Tobacco Plants

GUS expression analysis of PCR confirmed transgenic plants was carried out as reported by Jafferson [12]. Tobacco tissues were dipped in freshly prepared GUS staining buffer containing 0.1M X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) and vacuum infiltered for 3 minutes. Soaked tissues were kept in dark for 2 to 16 hrs at 37°C. Histochemical GUS expression was performed in thoroughly stained tobacco tissues and photographed using digital camera attached with microscope.

3. RESULTS

Cotton sucrose phosphate synthase promoter was used to study its expression in model dicot plant tobacco. Expression cassette containing 2 kb and 1.5 kb SPS promoter fragments followed by GUS gene ad CaMV terminator were cloned in plant expression vector pGA482. Clones were confirmed using *Hind*III excising promoter fragment (Fig. 1A). For positive and negative control 35S promoter and pormoterless constructs were also cloned in pGA482 respectively. PCR analysis also confirmed the desired promoter fragments cloned in pGA482 (Figs. 1B and 1C). Final clones containing 2 kb and 1.5 kb SPS promoter were named pGASPS2 and pGASPS1.5 respectively as shown by physical maps (Fig. 2A and 2B). GUS expression was determined in stably transformed tobacco plants using *Agrobacterium* mediated transformation (Figs. 3A-3D). Transgenic plants were confirmed through PCR analysis (Fig. 3E). Expression analysis revealed that 2 kb SPS promoter showed expression in tobacco leaves in the form of blue spots (Figs. 4A-4C). On the other hand, there was weak GUS expression driven by 1.5 kb SPS promoter (Figs. 4D-4F). There was no GUS expression detected in other tobacco tissue. Strongest expression was exhibited by CaMV 35 S promoter and was detected in all tobacco tissue (only leaves shown) (Fig. 4G). On the other hand, no expression was detected in negative controls under same conditions (Fig. 4H). Magnified view of tobacco leaves show GUS expression driven by 2 kb SPS promoter in tobacco leaves in sharp blue spots. However, blue stain was relatively weak in leaves transformed with 1.5 kb promoter (Figs. 5A and 5B). Leaves of positive control tobacco plant show intense blue colour while negative control leaves remained without stain (Figs. 5C and 5D).

4. DISCUSSION

Cotton SPS promoter was cloned in plant expression pGA482 to determine its expression in dicot plants. SPS promoter was retrieved from high through put genomic sequences and its sequence was analyzed through databases. Cotton SPS promoter was isolated from cotton genomic DNA and cloned in expression vector upstream of GUS reporter gene. Through transient GUS expression analysis SPS promoter exhibited expression in elongating cotton fibers (unpublished data). A 2 kb SPS promoter and its 1.5 kb deletion fragment respectively were cloned in pGA482 vector respectively (Fig. 1).

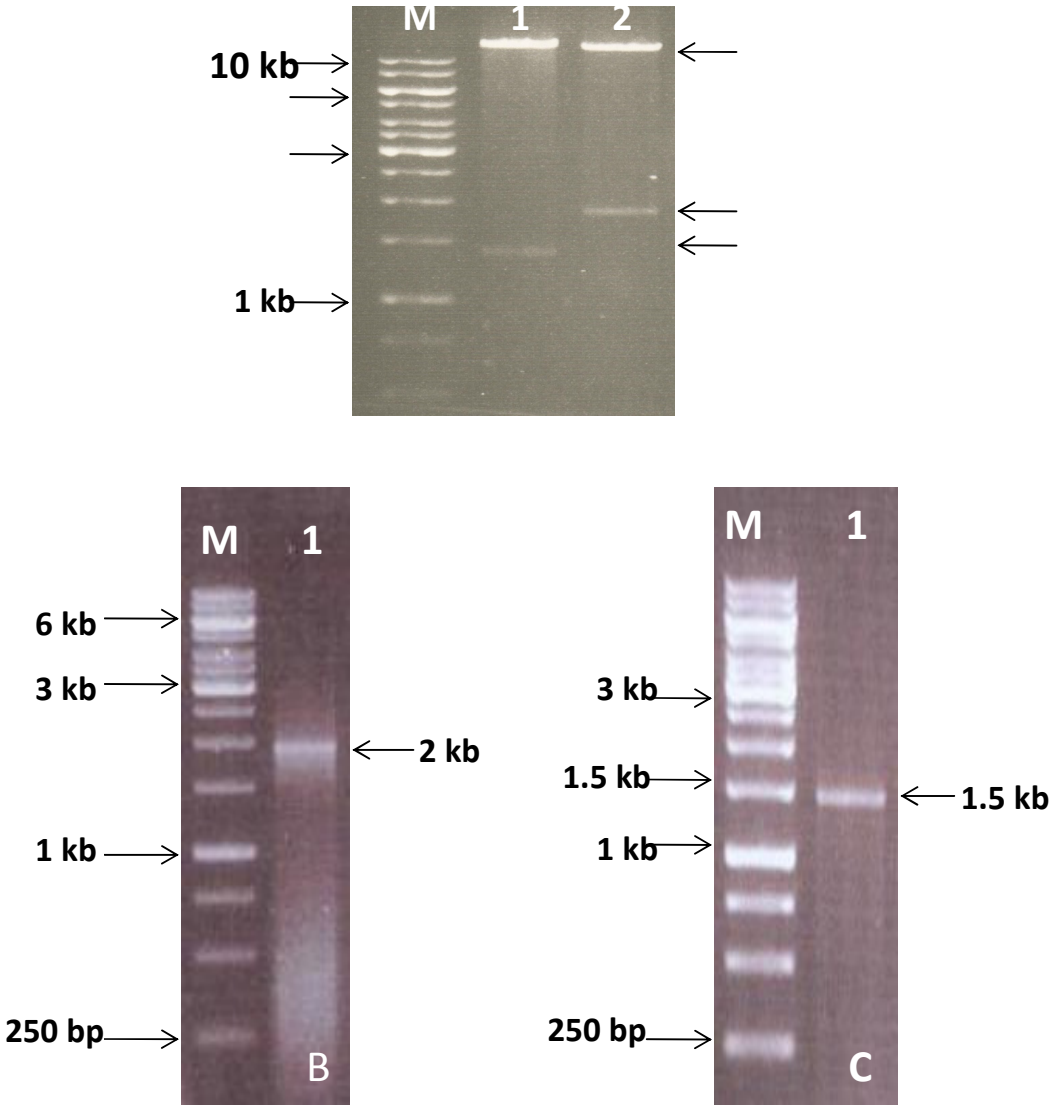

Fig. 1. Cloning of SPS promoter in pGA482. A) Restriction analysis of promoter clones with *Hind*III. M: 1Kbp DNA ladder, Lanes 1and 2 represent restriction digestion with *Hind*III showing 1.5 kb and 2 kb promoter along with 13.2 kb vector backbone B) PCR amplification to confirm promoter clone. M: 1 kb DNA ladder, Lane 1; PCR amplification of 2 kb SPS promoter with promoter specific primers. C) PCR amplification to confirm promoter clone. M: 1Kbp DNA ladder, Lane 1; Amplification of 1.5 kb SPS promoter with promoter specific primers

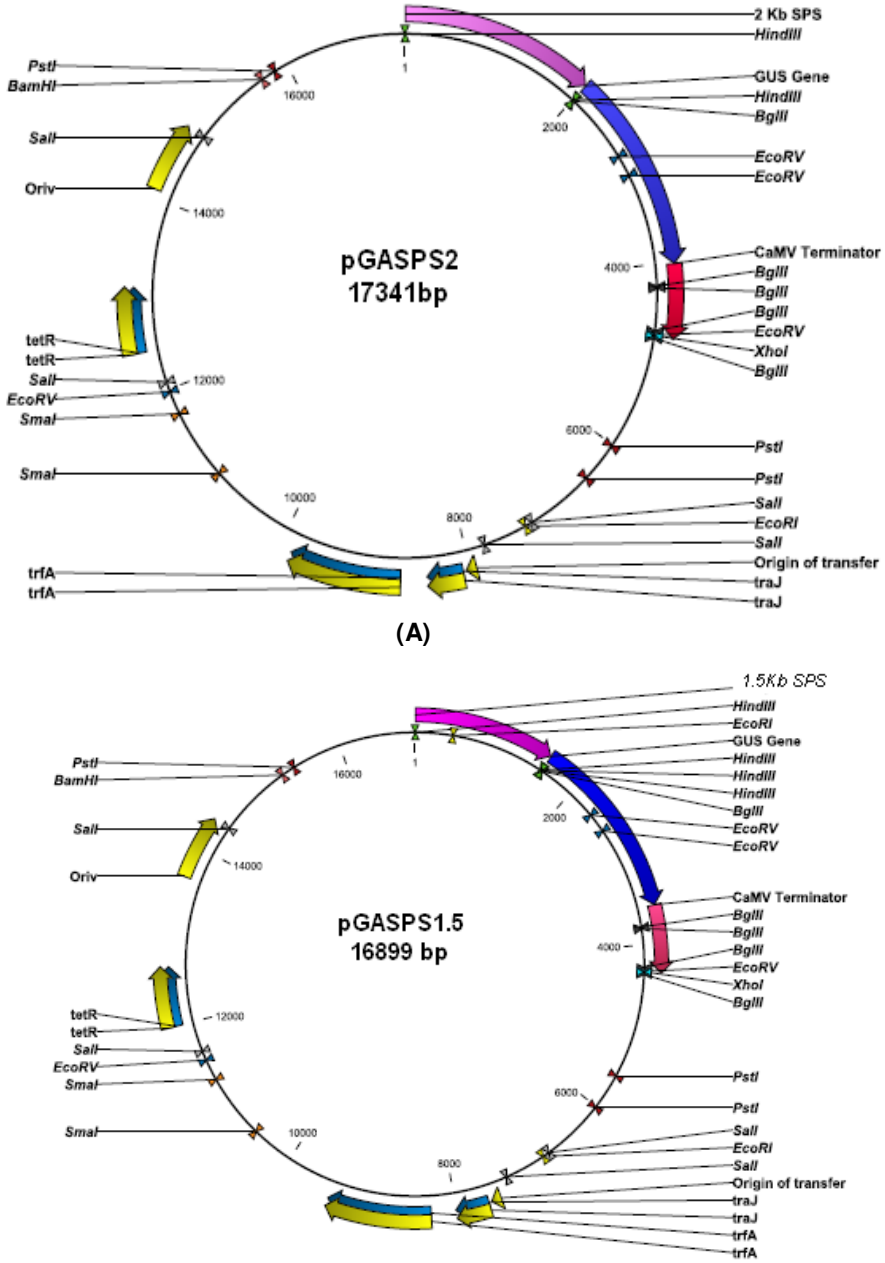

Fig. 2. Physical map of plasmid constructs having SPS promoter in pGA482. A) Expression cassette having 2 kb SPS promoter, GUS gene followed by CaMV terminator (pGASPS2).B) Expression cassette having 1.5 kb SPS promoter, GUS gene followed by CaMV terminator (pGASPS1.5)

Both promoter fragments were transformed stably in tobacco (Fig. 3). Expression study demonstrated that 2 kb SPS promoter showed strong expression in tobacco leaves as compared to its 1.5 kb fragment (Fig. 4). Although both promoter fragments exhibited GUS expression in tobacco leaves but there was considerable difference in expression level (Fig. 5).

The difference in the expression level might be due to deletion of some regulatory motif related to promoter activity. Previously sequence analysis of SPS promoter revealed many crucial motifs. These included 5´UTR py-rich stretch (TTTCTTCTCT), MYBST1 (GGATA), GATA box (TATC), AGL3 (CTATTTATGG), INRNTPSADB (TTCAGTTC) and ARRIAT (aataatcACG Taggttgg).

5´UTR py-rich stretch is an important motif associated with high level of gene expression [14]. In 1.5 kb fragment, one of 5´UTR py-rich stretch was deleted. A transcription factor ARRAIT which has role as activator of transcription was also reduced from 20 to 17. Some of INRNTPSADB and GATA boxes that have role in imitation and activation of transcription respectively [2] were also deleted in 1.5 kb SPS promoter. Likewise, transcription factor binding sites for ARRIAT, MYBST1 and AGL3 (all are regulators of transcription) were also eliminated in 1.5 kb fragment.

There may be some negative regulatory or repressor region in 1.5 kb fragment which might be activated upon deletion of 500 bases at 5´ end. The results demonstrate that region away from transcription initiation site and core promoter elements may exert influence on promoter activity.

A fragment of 1.5 was generated to examine the role crucial elements in promoter activity and minimized the promoter size to facilitate transformation. But its expression was reduced to great extent. Therefore, 2 kb fragment may be more useful to use as component of expression cassette in dicot system. SPS gene promoter have isolated previously in higher plants controlled by light [15]. SPS promoter isolated in this study expressed under normal environmental conditions. So, it may also be used as one of promoters in multiple expression cassettes. Multiple promoters from diverse sources may help to control gene silencing due to sequence homology of promoters.

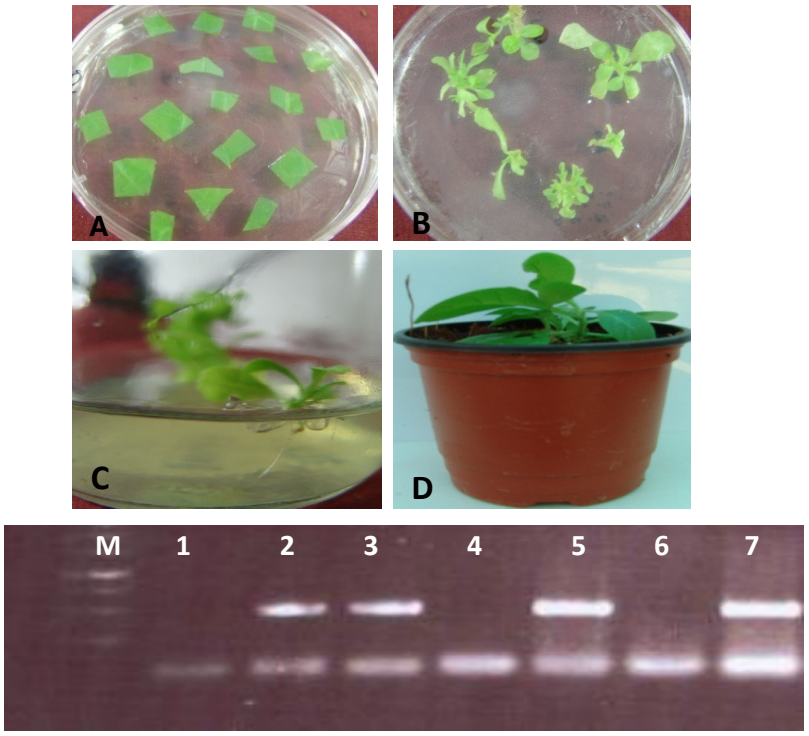

Fig. 3. *Agro bacterium* mediated transformation in tobacco. A) Inoculation of tobacco leaf discs with *Agrobactreium* having recombinant plasmids. B) Regeneration of plantlets on selection media. C) Rooting of transgenic plants. D) Mature plants in pots. E) PCR analysis of transgenic tobacco plants. Lane 1 DNA marker, Lane 2, 3, 5 show putative transgenic plants, Lane 6; Negative control (non transgenic plant DNA), Lane 7; positive control (plasmid DNA)

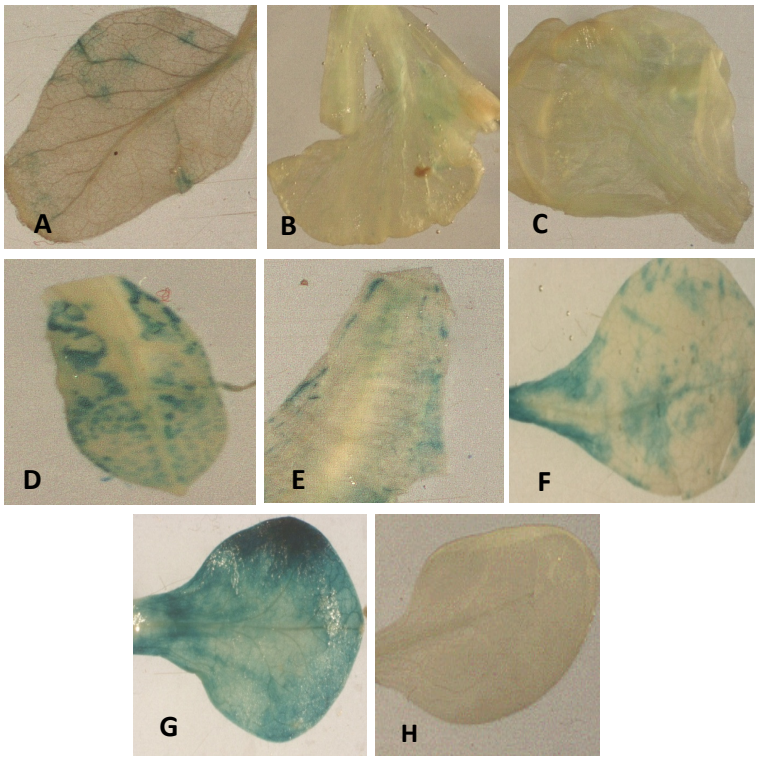

Fig. 4. Histochemical GUS expression in tobacco leaves. Panel A-C shows GUS expression in tobacco under control of 1.5 kb SPS promoter. Panel D-F shows GUS expression in tobacco under control of 2 kb SPS promoter. G and H show +ve control (35 S promoter) and –ve control (promoterless construct) respectively

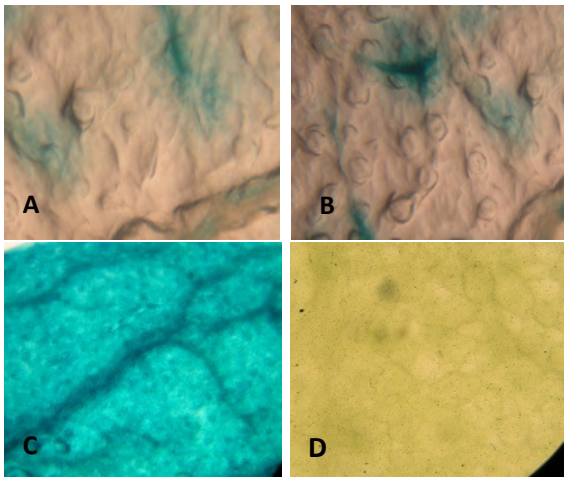

Fig. 5. GUS expression in tobacco leaves (larger view). A) 1.5 kb SPS promoter, B) 2 kb SPS promoter, C) 35 S promoter, D) Pormoterless construct

5. CONCLUSION

From the above study it is summed that cotton SPS promoter was found to express in tobacco leaves and it may be used in biotechnology to transform gene in dicot plants. A 1.5 kb fragment was cloned in expression vector but it exhibited reduced expression. Therefore, 2 kb promoter was more reliable to use in expression vector instead of 1.5 kb fragment. In agriculture based country like Pakistan, exploration of novel promoter is highly desirable to improve agronomic traits without IPR issues.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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