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IDENTIFICATION, ANALYSIS AND CLONING OF SUCROSE SYNTHASE GENE PROMOTER IN PLANT EXPRESSION VECTOR

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ABSTRACT

The study involves identification and analyses of constitutive dicot gene promoter from Solanum lycopersicum isolated through High throughput Genomic Sequences (HTGS). High throughput Genomic Sequences (HTGS) in the GenBank provide one of the information databases for utilizing bioinformatics approaches to identify promoter regions of different genes. The regulatory regions of a particular gene detected on an HTGS can be further screened by different bioinformatics tools to detect cis- regulatory elements, transcription start sites and transcription factor binding sites. A variety of promoters is necessary at all levels of genetic engineering in plants to regulate gene expression. The plant promoters fall into various categories including constitutive, tissue specific, inducible and differentially regulated. The expression of transgenes is regulated by the promoter attached upstream to the gene. The selected SUS promoter sequence was cloned in plant expression vector pGA482. It is proposed that the promoter identified through this study may be utilized to overcome the gene silencing problem by reducing the foreign gene expression to a lower level than the 2X35S.

KEYWORDS: Vector, HTGS, promoter, tissue specific, transcription factor binding sites.

INTRODUCTION

Sucrose synthase (*SUS*) 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. It belongs to a small multigene family and it have been found both in monocot and dicot species.[1] It has been found to play a role in structural and storage tissues of plants.[2] It is upregulated under low-oxygen conditions. Recent studies have shown that *SUS* directs carbon towards the pathways of polysaccharide biosynthesis. 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 sucrose synthase gene can work under such conditions, and can overcome the oxygen deficient conditions.

A variety of promoters is necessary at all levels of genetic engineering in plants from basic research to the development of economically viable crops. The plant promoters fall into various categories including constitutive, tissue specific, inducible and differentially regulated. Moreover, different promoters in the same category may have different strengths for the expression of a gene being regulated by them.[3] Gene promoters that direct high levels of constitutive gene expression are important for crop biotechnology applications.

Currently, the most widely used plant promoters for gene expression in plants include 2X35S, ubiquitin and RolC etc. The correct regulatory sequences are added to the gene of interest and the cassette in a transformation vector is transferred to the plant tissues using an appropriate methodology. The 35S promoter and its derivatives can drive high levels of transgene expression in dicotyledonous plants but their activities are substantially lower in monocotyledonous plants.[4-7] These promoters are very useful but are patented. Moreover, additional promoters are always required to clone multiple genes in a vector, specifically when each of the gene is targeted to different tissues for appropriate expression.

Promoters are a set of transcription control modules clustered around the initiation site of RNA polymerase II.[8] They are important in the control of the overall expression profile of a gene, either driving or preventing transcription at appropriate times and places.[9] The most commonly used promoters for the transgene expression analysis include cauliflower mosaic virus (35S) promoter and nopalin synthase promoter.[10] 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.[11] The promoter of a cotton lipid transfer protein gene *(FSltp4)* is responsible for the transcription of fiber-specific

mRNA in cotton.[12] 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.[13] A large number of constitutive, tissue-specific and inducible promoters have been characterized and reported in dicot plants. Hence, these plant-derived promoters can be characterized and used for developing transgenic plants.[14] An example of constitutive dicot gene promoter is polyubiquitin promoter (Gmubi). This promoter from soybean (*Glycine max*) showed high levels of constitutive expression and was used as an alternative to viral promoters (CaMV35S) for driving gene expression in soybean.[15- 16] The characterization and understanding the functions of other dicot plant promoters is essential to enhance the understanding of gene expression and to develop transgenic plants with specialized traits. The present study was planned to analyze and clone the *SUS* gene promoter in plant expression vector pGA482.

Role of Cis- regulatory elements in transcription regulation: Different experimental and bioinformatics approaches can be used for identifying potential cis-regulatory elements. Transcriptional regulatory networks that drive organ-specific and cell-specific patterns of gene expression also mediate interactions with the environment and represent one aspect of plant cell signaling. Essentially, the transcriptional regulation of gene expression in eukaryotes is mediated by the recruitment of transcription factors (TFs) to cis regulatory elements. Multiple cis-elements comprise cis-regulatory modules (CRMs) which integrate signals from multiple TFs, resulting in combinatorial control and highly specific patterns of gene expression. Therefore, identifying and understanding the functions of cis-elements and their role in CRMs is essential for elucidating the mechanisms by which cells perceive and correctly respond to their environment and participate in the development of an organism.

MATERIALS AND METHODS

The objective of this research was to analyze and clone the promoter sequence isolated from High throughput genome sequence (HTGS) database. The regulatory sequence was isolated from highly expressed gene in dicots. The studies were carried out to analyze one of the selected *SUS* promoter through various bioinformatics tools.

Promoter analyses through Bioinformatics tools
Bioinformatics provides many tools and software's to analyze highly expressed dicot gene promoters. The cis-regulatory elements within the selected promoter sequences were analyzed using PlantCARE software (http://oberon.rug.ac.be:8080/ PlantCARE/index.html). The transcription start site (TSS) indicates the transcription starting position in a sequence. It was obtained by using BDGP software. PlantPAN and PLACE (www.plantpan.mbc.nctu.edu.tw/) identifies the transcription factors that are key regulators of gene expression.

Cloning of expression cassettes for *SUS* promoter in pGA482
The Promoter-Gene-Terminator cassette for *SUS* could be picked by digestion with the restriction enzymes *Sac*I and *Xho*I. The pGR1 vector having the promoter cassette was first digested with *Xho*I. The sticky ends generated by *Xho*I were blunt ended by end filling reaction. The end filling reaction was stopped by heating at 75ºC for 10 minutes. The DNA was precipitated, washed with ethanol and resuspended in 20µL of H_2O. A second digestion with *Sac*I was performed to release the expression cassette from the parent vector. The *Sac*I digestion mixture was treated with phenol and the digested DNA was precipitated as described earlier.

Ligation and Transformation of promoter-vector fragments: The pGA482 was digested with *Sac*I and *Hpa*I to obtain sites complementary to the ends of Promoter-Gene-Terminator cassettes. The digested DNA was purified by phenol treatment followed by precipitation and ethanol washing. The ligation reaction was incubated at 16°C overnight and ligation monitoring was performed at the completion of ligation reaction by resolving the ligation mix on 1% agarose gel containing 0.05% ethidium bromide. The 1kb DNA ladder was used as size standard. The ligation mixtures, 1µL each, were then transformed into *E.coli* competent cells (DH10) separately by heat shock method. About 50 µL of the transformation mix for each promoter cassette was then spread on LB plates containing tetracycline (100µg/mL). The plates were incubated overnight at 37˚C. Colonies were cultured and the plasmids were isolated for screening the cloned promoter cassettes. The selected transformants were verified on the basis of single digestion i.e when digested with *Hind*III, it released specific sizes of promoter fragment and pGA482 vector backbone along with gene-terminator cassette. The clones with expected sizes were isolated and were finally confirmed through conventional PCR.

RESULTS

Bioinformatics approach for promoter sequence analysis

Sequence retrieval of sucrose synthase gene promoter through HTGS database

Table 1: Transcription factor binding site in sucrose synthase promoter

Factor	Site	Sequence	Species
AGL3	534	gtttggtagTATGGagga	Arabidopsis
AG	121	acacCCATAattattaaa	Arabidopsis
AG	301	tgtaCCAAAaaagttaaa	Arabidopsis
Athb-1	133	tagatATTATtttc	Arabidopsis
AGL3	908	acacCCATAattattaaa	Arabidopsis

Fig. 1. Sequence of *SUS* promoter isolated through HTGS database. Start codon (ATG) of *SUS* gene is highlighted with red color, putative TATA box (-598) and G-box (-1233) are highlighted with pink and sky blue colors respectively.

Analysis of transcription start site (TSS) through BDGP: Transcription start site of sucrose synthase gene promoter sequence was detected using BDGP promoter analysis. Its identification assures that the sequence is truly a promoter region. The BDGP promoter analysis revealed the following sequence with a probability of 82% for *SUS* promoter respectively.

TSS
TTAATAAATATATAATAGGAAGATCAGTGCAAG TTCTCTC**T**GTTGTGTTA

Identification of transcription factor binding site through PlantPAN
PlantPAN software (www.plantpan.mbc.nctu.edu.tw/) identifies the transcription factors that are key regulators of gene expression. The putative transcription factor binding sites (TFBs) of *SUS* gene promoter are illustrated in table below.

Construction of physical map for *SUS* promoter using CLC Bio work bench
Physical map for selected promoter was designed using CLC Bio software having complete expression cassette (i.e, promoter, gene, terminator).

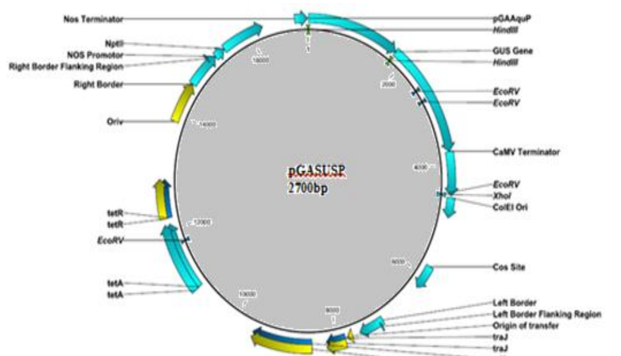

Fig 4: Physical map of pGASUSP vector.

Cloning of *SUS* gene promoter in plant transformation vector pGA482: The vector pGA482 was digested with *Sac*1 and *Hpa*I. The enzyme *Sac*I produces cohesive ends, while *Hpa*1 produces blunt ends. The complete expression cassette was obtained from clone in pGR1 (pGRSUSP) through digestion with *Sac*I and *Hind*III for *SUS* gene promoter. The resultant pGA482 construct was named as (pGASUSP). The construct was digested with *Hind*III for clone confirmation. In case of *SUS* gene promoter (2769bp) fragment was obtained along with vector backbone.

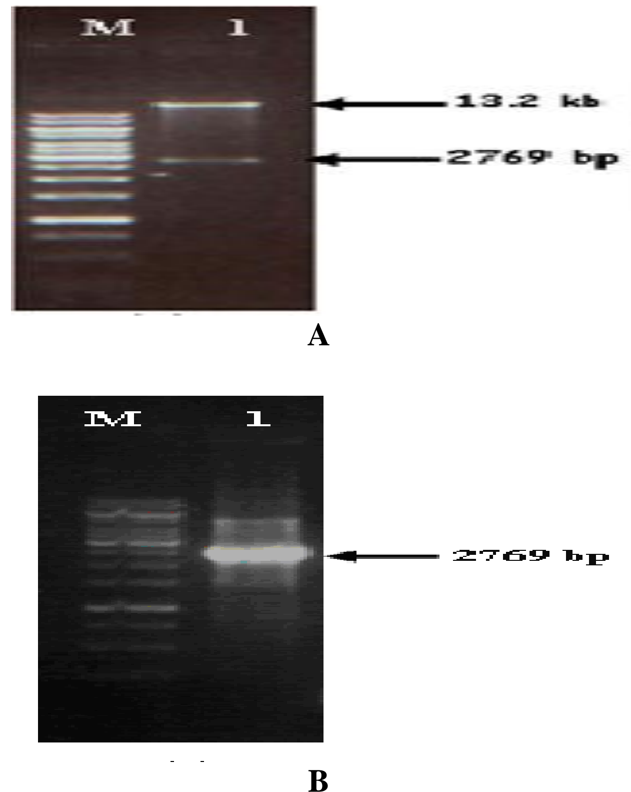

Fig 3: Confirmation of *SUS* expression cassette. Lane M: 1kb DNA ladder, Lane 1: (a) Digestion of pGA482 with *Sac*I and *Xho*I. (b) PCR amplification of *SUS* gene promoter of *Agrobacterium* clone.

DISCUSSION

The present study focused on idenitification, analysis and cloning of constitutive gene promoter in plant transformation vector (pGA482). The promoter region from highly expressed constitutive gene; sucrose synthase (*SUS*) was selected and cloned in the plant expression vector (pGA482). The promoter is an important component in a plant transformation vector and is generally patented after its discovery and usefulness. To develop indigenous promoters free from IPR issues, it is a prerequisite to characterize them by expressing in a model plant system. A wider range of effective promoters would also make it possible to introduce multiple transgenes into plant cells, while still avoiding the risk of homology-dependent gene silencing.[17] The selected promoter sequence was also characterized with respect to specific motifs. A large number of putative *cis*-acting regulatory elements were detected in the promoter region of the selected gene. Variations in the number of regulatory motifs play an important role in the expression studies. Analysis of *SUS* promoter indicated various motifs involved in light responsiveness like 3-AF1 binding site, A-box *cis*-acting regulatory element, Box I and TCT-motif of *Arabidopsis thaliana*. TC-rich repeats are also an important *cis*-acting elements involved in defense and stress responsiveness. Analysis of *SUS* promoter depicted many transcription factor binding motifs. Transcription factors are highly specific in nature for interacting with proteins in order to modulate transcription. Transcription factor binding sites (TFBSs) are short sequences located near transcription start sites (TSSs) and recognized by respective transcription factors (TFs) for gene regulation. TFBSs are recognized by the same TF and usually show a conserved pattern, which is often called a TF binding motif (TFBM). The ability to determine the location and relative strength of all transcription-factor binding sites is important both for a comprehensive understanding of gene regulation and for effective promoter activity. Promoter of *SUS* genes was enriched with conserved transcription binding site motifs AGL3, AG, Athb-1 and ANT. These motifs are located in sense (+) and (-) strands of promoter sequences. The transcription factor AGL3 is expressed in all above-ground vegetative organs; AGL3 may be involved in the transcriptional regulation of genes. The conserved AGL3 binding site motif was identified *SUS* promoter at nucleotide position 534 in (-) strand and 908 in (+) strand. Comparison with the detection of unknown motifs, the detection of known motifs is fairly straightforward and is performed by the scanning of DNA sequence with a given motif via specialized databases such as TRANSFAC.[18] It is proposed that the promoters identified through these studies may be utilized to overcome the gene silencing problem by reducing the foreign gene expression to a lower level than the *2X35S*. The over-expression of the desired genes usually results in gene silencing.[19]

CONCLUSION

The novel constitutive gene promoters provide great asset for improvement and advancement in crop biotechnology applications. Isolation and identification of useful plant promoters is routinely required for genetic manipulation of plants and is important in achieving controlled gene expression in transgenic plant development programs. These constitutive cloned promoters like *SUS* can then be used for developing transgenic plants with controlled expression of desired genes. Hence, it is advantageous to have the choice of a variety of different promoters so that the most suitable promoter may be selected for a particular gene expression, according to cell, tissue, plant or environment. The use of alternative promoters with similar characteristics is essential for the stacking of several transgenes in order to avoid homology-dependent gene silencing, a phenomenon which often occurs in transgenic plants with multiple copies of the same promoter.

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