Cloning and Characterization of Beta tubulin Gene Promoter from *Brassica Rapa* and its Expression Analysis

Hira Mubeen[1], Aftab Bashir[2], Muhammad Waseem Shoaib[3], Shahid Raza[4], Naveed Hussains[5]

Abstract— In the present study, we report the cloning and characterization of abundantly expressed Beta tubulin (*BT*) gene promoter from the dicot plant Brassica rapa. Isolation and characterization of useful promoters is routinely required for genetic manipulation of plants and is important in achieving controlled gene expression in transgenic plants. The major constraint regarding the utilization of commercially available promoters is the intellectual property right (IPR), therefore, discovery of efficient new promoters is important considering the IPR issues. The theme of this research was focused on stable transformation, using *GUS* expression cassette under selected Beta tubulin gene promoter sequence, in *Nicotiana tabacum* via *Agrobacterium*-mediated transformation and perform expression analysis via histochemical *GUS* assay. The selected *BT* promoter sequence was cloned in plant expression vector (pGA482). The expression cassette was constructed in a modified vector derived from pJIT166 (pGR1) that contains *GUS* with intron under 2X35S promoter followed by CaMV terminator. The selected *GUS* expression cassette was developed by replacing 2X 35S promoter in pGR1, with the selected *BT* promoter. Further, *GUS* expression cassette was verified through restriction analysis and PCR amplifications. The PCR positive tobacco transgenic for *BT* expression cassettes was obtained and stained to check the *GUS* expression levels. The results are presented along with the proposed utilization of the studied promoter.

Index Terms— Beta tubulin, Brassica rapa, Transgenic plants, Promoter, Histochemical *GUS* Staining.

INTRODUCTION

TUBULIN is one of several members of a small family of globular proteins. The most common members of the tubulin family are α-tubulin and β-tubulin, the proteins that make up microtubules. The initial cloning of tubulin was achieved from chicken cDNA during 1980s (Cleveland *et al*., 1980). Tubulin genes of a wide variety of organisms have been isolated and characterized so far. The basic unit of microtubules is a heterodimer protein composed of α and β-tubulin polypeptides. It is puzzling that why there are large number of tubulin genes in eukaryotes, Each has a molecular weight of approximately 55 kilo Daltons. Microtubules are assembled from dimers of α and β-tubulin. These subunits are slightly acidic with an isoelectric point between 5.2 and 5.8.Tubulin was long thought to be specific to eukaryotes.while a single type of α and β-tubulin would be sufficient to fulfill the polymerization of microtubules. In the higher plants, both α and β-tubulin genes form multigene families. Some of these tubulin genes are expressed constitutively, while many others exhibit tissue, organ, or cell specific expression patterns. Studies have indicated that the β-tubulin promoter contains a GC-rich region between the TATA box and the transcription initiation site, with 7 copies of 10 bp sequence motifs called tub box. These tub box motifs are involved in the induction of transcription. Indeed, removing 4 or 5 tub box motifs prevents transcriptional increase but it does not significantly affect the transcription level (Davies and Grossman, 1994).

[1]Lecturer/Researcher, Faculty of Biotechnology and Agro- Sciences, University of South Asia, Lahore/National institute for Biotechnology and genetic engineering, Faisalabad, Pakistan *PH-92-3007943269. E-mail: hira_sh@hotmail.com*

[2]*Principle Scientisit*, National institute for Biotechnology and genetic engineering, Faisalabad, Pakistan. *PH-92-3206687953. E-mail: aftabb_pk@yahoo.com*

[3]Resident/Researcher, Hera General Hospital, Makkah, Kingdom of Saudi Arabia/ National institute for Biotechnology and genetic engineering, Faisalabad, Pakistan.

PH-92-3006630086. E-mail: mwshoaib@hotmail.com

[4]Dean, Faculty of Biotechnology and Agro- Sciences, University of South Asia, Lahore, Pakistan. .*PH-92-3004238303. E-mail: mianrs@yahoo.com*

[5]Scholar, Institute of Biochemistry & Biotechnology, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. *PH-92-3314786351. E-mail: faranians4all@gmail.com*

Therefore, the *BT* tubulin promoter was selected in this study to figure out, if it really represents a constitutive promoter and can substitute the equivalent promoters for construction of multiple gene expression cassettes. Promoters are important in controlling

overall expression profile of a gene and can drive or prevent transcription at specific developmental stages. The promoters can be roughly divided in two parts. The first part is referred as the core/proximal promoter, which is the region within 100-250bp around the transcription start site. The proximal part is believed to be responsible for correctly assembling the RNA polymerase II complex at the right position and for directing a basal level of transcription (Nikolov *et al*., 1996; Nikolov and Burley, 1997; Berk, 1999; Rombauts *et al*., 2003). It is mediated by elements, such as TATA and initiator boxes through the binding of the TATA box-binding protein and other general TFs (Featherstone, 2002). The second part comprises of distal part of the promoter. This region contains elements that regulate the spatio-temporal expression (Tjian and Maniatis, 1994; Fessele *et al*., 2002).

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 (Rushton *et al*., 2002). 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; Potenza *et al*. 2004) for driving gene expression in soybean (Hernandez-Garcia *et al*., 2009). The *pGC1* promoter from *Arabidopsis* is strongly expressed in guard cells of *Arabidopsis* and tobacco plants. It provides an essential research tool for targeted guard cell expression or gene silencing (Yang *et al*., 2008). Promoters typically have a modular structure consisting of several *cis*-acting regulatory elements.

MATERIAL AND METHODS

Bioinformatics approach to characterize the promoters

The objective of this research was to identify and characterize the promoter sequence isolated from HTGS sequence database. The regulatory sequence was isolated from highly expressed gene in dicots. The studies were carried out to determine the analysis of selected promoter. Bioinformatics provides various tools and software's to analyze promoter sequences from highly expressed dicot genes. The *cis*-regulatory elements within the selected promoter sequence was analyzed using PlantCARE software (http://oberon.rug.ac.be:8080/ identified using PlantPAN (www.plantpan.mbc.nctu.edu.tw/. These elements are usually dispersed over the promoter region and most of them lie within the 1kb region upstream the transcription start site (TSS). Different bioinformatics tools can be used for identifying potential cis-regulatory elements. CAREs, another class of cis-acting regulatory elements, are short conserved motifs of approximately 5 to 20 nucleotides expected to occur at random every few hundred base pairs and are hard to differentiate from other usual sequences (Blanchette and Sinha, 2001). Comparison with the detection of unknown motifs and that 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 (Wingender *et al*., 1996). In the present study, the *BT* constitutive promoter selected from its source plant Brassica rapa was named according to its associated gene. Isolation and characterization of useful promoters is routinely required for genetic manipulation of plants and is important in achieving controlled gene expression in transgenic plant development programs. The *β-tubulin* gene promoters are abundantly expressed in dicot plants. This study was focused on stably transforming expression cassette of selected *BT gene* promoter sequence in *Nicotiana tabacum* via *Agrobacterium*-mediated transformation and to study its expression via histochemical *GUS* assay.

Cloning of expression cassettes in plant transformation vector (pGA482)

Construct having the promoter for *BT* was provided by the Gene Isolation Group, National institute for biotechnology and genetic engineering, Faisalabad. The construct was cloned in a modified vector derived from pJIT166 (pGR1) that contains *GUS* with intron under 2X35S promoter followed by CaMV terminator. Glycerol stock of the *BT* construct was streaked on LB agar plates having ampicilin. The single colonies were cultured to isolate the plasmid. The construct was further verified by restriction digestion with respective enzymes and PCR amplification. The construct for *BT* promoter was verified by restriction digestion with *Kpn*I and *Hind*III for further analysis.

Physical map of pGABTP

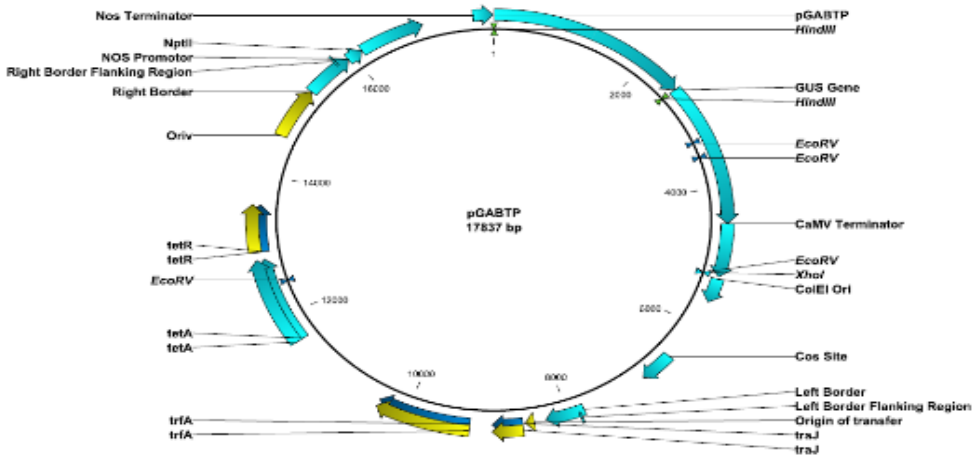

Fig. 1.1 Physical map of pGABTP vector.

Cloning of expression cassetes of *BT* in pGA482

The Promoter-Gene-terminator cassette having *BT* promoter was first digested with *Xho*I. The sticky ends generated by *Xho*I were blunt ended by end filling reaction. The reaction was stopped by heating at 75ºC for 10 minutes. The DNA was precipitated, ethanol washed and resuspended in 20µl of H_2O. A second digestion with *Kpn*I was performed to release the expression cassette from the parent vector. The *Kpn*I digestion mixture was phenol treated and the digested DNA was precipitated as described earlier.

Ligation of promoter-vector fragments

The pGA482 was digested with *Kpn*I and *Hpa*I to obtain sites complementary to the ends of Promoter-Gene-Terminator Cassette. 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 as described above using 1kb DNA ladder as a marker.

Transformation of ligation and transformant analysis

The ligation mixtures, 1µl each, were then transformed into *E.coli* competent cells (DH10) separately by heat shock method. About 50µl of the transformation mix for slected promoter cassette was 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.

***Agrobacterium*-mediated tobacco transformation**

To start an *in-vitro* tissue culture experiment, highly sterile conditions are required. The antibiotic solutions were filter sterilized, while the equipments were sterilized by autoclaving. The *BT* plant expression construct was transformed in tobacco through *Agrobacterium* mediated transformation using LBA4404 as a carrier strain.

RESULTS

The objective of this study was identification and cloning of an important dicot plant promoter sequence from constitutive and highly expressed gene. The promoter sequence was previously retrieved from High Throughput Genomic Sequence Database (HTGS) in the lab. This promoter sequence was analyzed using bioinformatics approaches. The results of experiments are elaborated below:

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 95% for *BT* promoter respectively. ATTATTTTCTTAAAAAATGGTGTTAGTACCTTAATGTGTG**A**GAAGTTCGA. The capital A in *BT* sequence represents the predicted transcription start site. The transcription start site for *BT* promoter was detected at nucleotide position 1962 of the 2500bp promoter.

Promoter sequence analysis through PlantCARE

PlantCARE software was used for the evaluation of the *cis*-regulatory elements in selected promoter sequence. Several types of motifs were found dispersed over the entire promoter sequence. The most frequent motifs observed in *BT* gene promoters were identified to be light responsive elements. Stress and hormone response motifs were also found scattered throughout the promoter region (Tab. 1.1).

Table 1.1 *Cis*-regulatory elements in *BT* gene promoter.

***Cis*-regulatory element**	**Organism**	**Sequence**	**Function**
5UTR Py-rich stretch	*Lycopersicon esculentum*	TTTCTTCTCT	cis-acting element conferring high transcription levels
ACE	*Petroselinum crispum*	CTAACGTATT	cis-acting element involved in light responsiveness
Box 4	*Petroselinum crispum*	ATTAAT	part of a conserved DNA module involved in light responsiveness
Box I	*Pisum sativum*	TTTCAAA	light responsive element
CAAT-box	*Brassica rapa*	CAAAT	common cis-acting element in promoter and enhancer regions
G-Box	*Pisum sativum*	CACGTT	cis-acting regulatory element involved inlight responsiveness
G-Box	*Solanum tuberosum*	CACATGG	cis-acting regulatory element involved in light responsiveness
GAG-motif	*Arabidopsis thaliana*	AGAGAGT	part of a light responsive element
I-box	*Solanum tuberosum*	TATTATCTAGA	part of a light responsive element
TATA-box	*Arabidopsis thaliana*	TATAAA	core promoter element around -30 of transcription start
TATA-box	*Brassica napus*	ATATAT	core promoter element around -30 of transcription start
TC-rich repeats	*Nicotiana tabacum*	ATTTTCTTCA	cis-acting element involved in defense and stress responsiveness
TCA-element	*Brassica oleracea*	GAGAAGAATA	cis-acting element involved in salicylic acid responsiveness
TGACG-motif	*Hordeum vulgare*	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness

Identification of transcription factor binding site through PlantPAN

PlantPAN software identifies the transcription factors that are key regulators of gene expression. The putative transcription factor binding sites (TFBs) of *BT* gene promoter are illustrated in table 1.2.

Table 1.2: Transcription factor binding site in *Beta tubulin* promoter

Transcription Factor Binding Sites in "BT promoter"
Sequence Length=2500

Factor	Site	Strand	Seq	Species	Source
AGL3	881	-	ctagaaaacTATGGtttt	Arabidopsis	TRANSFAC
AGL3	986	-	gtatttgagTATGGacca	Arabidopsis	TRANSFAC
AGL3	1087	-	acgtattcaTATGGatac	Arabidopsis	TRANSFAC
AGL3	1582	-	acttattttTATGGttcg	Arabidopsis	TRANSFAC
AGL3	2092	+	tgaaCCATAattttttt	Arabidopsis	TRANSFAC
AG	887	-	aactatggtTTTGGggtt	Arabidopsis	TRANSFAC
AG	1364	+	agatCCAAAaaacaatcg	Arabidopsis	TRANSFAC
AG	1929	-	acaaagattTTTGGtgag	Arabidopsis	TRANSFAC
ANT	221	+	taactgaCCCGAtt	Arabidopsis	TRANSFAC
Athb-1	117	-	gtgcATAATaagga	Arabidopsis	TRANSFAC
Athb-1	174	-	gctgATAATatcaa	Arabidopsis	TRANSFAC
Athb-1	258	-	ttggATAATtcaag	Arabidopsis	TRANSFAC
Athb-1	288	-	tttcATAATtagaa	Arabidopsis	TRANSFAC
Athb-1	313	+	gtatgATTATcctt	Arabidopsis	TRANSFAC
Athb-1	527	+	cgatgATTATatgt	Arabidopsis	TRANSFAC
Athb-1	558	-	aagtATAATgatac	Arabidopsis	TRANSFAC
Athb-1	577	-	actaATAATgatga	Arabidopsis	TRANSFAC
Athb-1	647	+	tggatATTATcatc	Arabidopsis	TRANSFAC
Athb-1	1200	+	gttcaATTATtgat	Arabidopsis	TRANSFAC
Athb-1	1250	-	tgacATAATaataa	Arabidopsis	TRANSFAC
Athb-1	1253	-	cataATAATaaaaa	Arabidopsis	TRANSFAC
Athb-1	1309	+	atgacATTATtatt	Arabidopsis	TRANSFAC
Athb-1	1312	+	acattATTATtttt	Arabidopsis	TRANSFAC

Cloning of *BT* gene promoters in plant transformation vector pGA482

The vector pGA482 was digested with *Kpn*I and *Hpa*I. To obtain the complete expression cassettes for *BT* gene promoter, *Hind*III and *Kpn*I was used to clone the expression cassettes in pGA482. The resultant pGA482 construct was named as (pGABTP). The construct was digested with *Hind*III for clone confirmation. A 2500bp *BT* gene promoter fragment was obtained along with vector backbone (Fig.1.2).

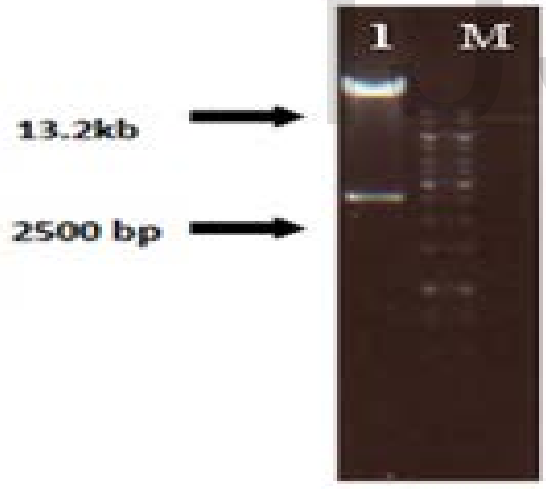

Fig.1.2: M: 1kb DNA ladder, **Lane 1:** Confirmation of BT expression cassette in pGA482 by digestion with *KpnI* and *XhoI*.

***Agrobacterium*-mediated tobacco transformation and confirmation of clones in LBA4404 strain**

Agrobacterium mediated tobacco transformation of *BT* gene promoters was carried out using LBA4404 strain of *Agrobacterium tumefacians*. The construct (pGABTP) were transformed independently into the *Agrobacterium* (LBA4404) elcetrocompetent cells by electroporation. The clones were confirmed by PCR using reverse and forward promoter specific primers to amplify the selected gene promoter.

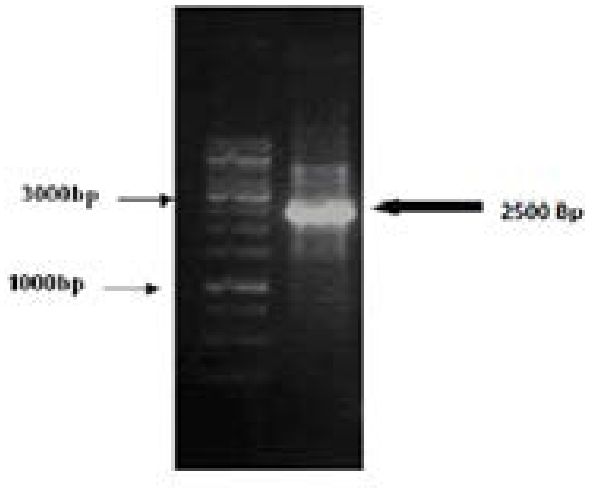

Fig 1.3: Confirmation of clone in *Agrobacterium*. *Lane* **M:** 1kb DNA ladder **(a)** PCR amplification of *BT* gene promoter of *Agrobacterium* clone.

Tobacco Transformation

Leaf discs of *Nicotiana tabacum* were cut and co-cultivated with the cultured *Agrobacterium* containing plant expression vectors. This gives a fair chance to the *Agrobacterium* to transform the gene of interest into the plant. Leaf discs were placed on solidified MS0 medium**.**

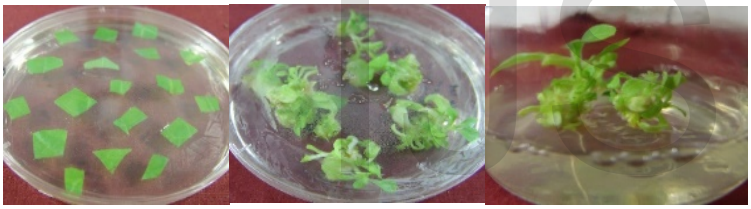

Fig 1.4: Different stages of *Agrobacterium*-mediated tobacco transformation (a): Tobacco leaf discs for co-cultivation with *Agrobacterium* inoculums. (b) Tiny plantlets appearing after 7-10 days of tobacco leaf discs co-cultivation with *Agrobacterium* inoculum. **(c)** Excised 7 days old plantlets which survived on Kanamycin selection media shifted to jar.

Transgenic Analysis

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. The results indicate amplification of expected fragments from the transgenics for each construct. The amplifications in the transgenics yield 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.

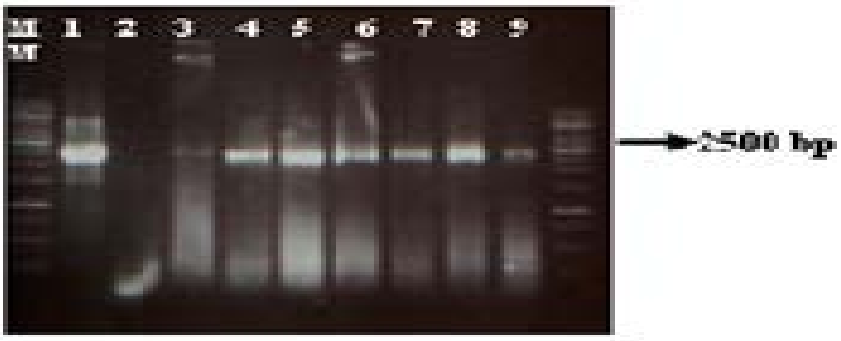

Fig 1.5: PCR analysis of putative transgenic tobacco plants for *BT* promoter. (a) M: 1kb DNA ladder, Lane1: PCR of positive control using plasmid DNA as a template, Lane2: Negative control of PCR master mix, Lane 3: PCR analysis of 7 randomly selected putative transgenic plants using promoter specific primers, showing expected amplification product of 2500bp.

Histochemical *GUS* staining

The leaf tissues from PCR selected transgenic plants were stained for *GUS* activity. Staining patterns of representative leaf tissues for stably transformed construct in different transgenic events are shown in Figure. *GUS* assay was carried out on leaf tissues. For this, leaf tissues were dipped in staining solution to monitor color intensity of expression levels in different transgenic events for the selected promoters. The plant tissues expressing *GUS* using *2X35S* and the selected promoter were stained for 24 hours. The staining reaction showed that the *BT* promoter was found to be constitutively expressed in leaves. Longer incubation in the staining solution led to diffusion of the stain but did not reveal any increase of *GUS* activity or spreading of stain in additional tissues. The *GUS* staining indicated that the *BT* promoter showed comparable expression in different transgenic events (Fig. 1.6). The staining of leaves from non-transgenic plants did not reveal the development of *GUS* stain.

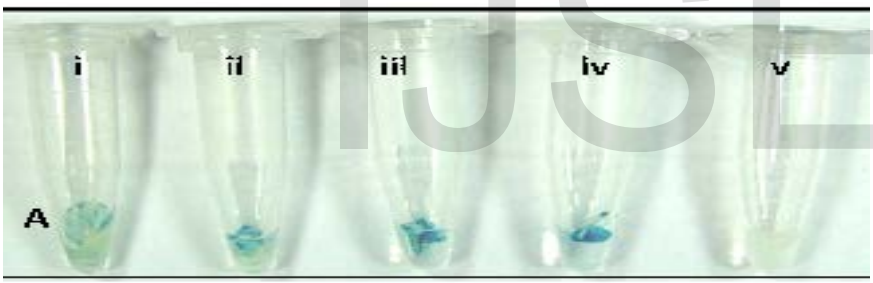

Fig 1.6: Histochemical assay for *GUS* activity in stably transformed tobacco plants. **Panel A** (i-iii) Leaf tissue stained in different transgenic events for GUS activity controlled by *BT* promoter: (iv) shows *GUS* activity controlled by 2X35S: (v) shows non transgenic i.e negative control.

DISCUSSION

The study was focused on cloning and characterization of dicot gene promoter for its expression analysis in tobacco (*Nicotiana tabacum* L.). The promoter region from highly expressed constitutive gene-Beta tubulin (*BT*) was selected. The selected promoter sequence was also characterized with respect to specific motifs, which are discussed below.The promoter is an important component in a plant transformation vector and is generally patented after its discovery and usefulness. Primary components of promoter are the *cis*-acting regulatory regions. 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 plays an important role in the expression studies. Many *cis*-regulatory motifs were detected in the *BT* promoter along with CAAT-box and TATA-box ACE motif include Box I, G-Box involved in light responsiveness. TC-rich repeats are important cis-acting element involved in defense response. Analysis of *BT* promoter depicted many transcription factor binding motifs. Transcription factor binding sites (TFBSs) are short sequences located near genes transcription start sites (TSSs) and recognized by respective transcription factors (TFs) for gene regulation. TFBSs are recognized by the same TF 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. The selected Beta tubulin (*BT*) promoter was found to be enriched with conserved Transcription binding site motifs AGL3, AG, Athb-1, ANT. These motifs are located in sense (+) and (-) strands of promoter sequences. Major transcription binding sites of *BT* promoter are AGL3, AG, Athb-1, ANT. The transcription factor AGL3 is expressed in all above-ground vegetative organs; AGL3 may be involved the transcriptional regulation of genes. Other motifs AG, ANT and Athb-1 sequence-specific DNA-binding protein motifs

are found in *BT* promoter.

ACKNOWLEDGMENT

We are thankful to Dr. Sohail Hameed (Director, NIBGE) for full support and also members of our group for fruitful discussions and suggestions. We apologize to all colleagues whose valuable work could not be cited due to space limitations.

REFERENCES

[1] Berk, A. J., 1999, Activation of RNA polymerase II transcription. Curr. Opin. Cell Biol. 11 (3), 330-335.
[2] Blanchette M., Sinha S., 2001.Separating real motifs from their artifacts.Bioinformatics. 17, 30–38.
[3] Cleveland L, MacDonald C, Rutter K (1980). Number and evolutionary conservation of a- and beta-tubulin and cytoplasmic beta- and gamma-actin genes using specific cloned cDNA probes. Cell. 20: 95-105.
[4] Davies G (1994). Sequences controlling transcription of the Chlamydomonas reinhardtii beta 2-tubulin gene after deflagellation and during the cell cycle. Mol. Cell Biol. 14: 5165-5174
[5] Featherstone, M., 2002. Coactivators in transcription initiation: here are your orders. Curr.Opin. Genet. Dev. 12 (2), 149-155.
[6] Fessele, S., Maier, H., Zischek, C., Nelson, P. J., Werner, T., 2002. Regulatory context is a crucial part of gene function. Trends Genet. 18 (2), 60-63.
[7] Hernandez-Garcia, C.M., Martinelli, A.P., Bouchard, R.A., Finer, J.J., 2009. A soybean (Glycine max) polyubiquitin promoter gives strong constitutive expression in transgenic soybean. Plant Cell Rep. 28(5), 837-49.
[8] Nikolov, D. B., Burley, S. K., 1997. RNA polymerase II transcription initiation. A structural view.Proc. Nat. Acad. Sci. 94(1), 15-22.
[9] Nikolov, D.B., Chen, H., Halay, E.D., Hoffman, A., Roeder, R.G., Burley, S.K., 1996. Crystal structure of a human TATA box-binding protein/TATA element complex. Proc. Natl. Acad. Sci. USA 93, 4862-4867.
[10] Potenza, C., Aleman, L., Sengupta-Gopalan, C., 2004. Targeting transgene expression in research agricultural, and environmental applications: promoters used in plant transformation. In Vitro Cell. Dev. Biol. Plant 40, 1-22.
[11] Rombauts, S., Florquin, K., Lescot, M., Marchal, K., Rouze, P., Van de Peer, Y., 2003. Computational approaches to identify promoters and cis-regulatory elements in plant genomes. Plant Physiol. 132, 1162-1176.
[12] Rushton, P.J., Reinstadler, A., Lipka, V., Lippok, B., Somssich, I.E., 2002. Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signaling. Plant cell 14, 749-762.
[13] Tjian, R., Maniatis, T., 1994. Transcriptional activation: A complex puzzle with few easy pieces. Cell. 77 (1), 5-8.
[14] Wingender E., Dietze P., Karas H., Knuppel R., 1996. TRANSFAC: a database on transcription factors and their DNA binding sites. Nucleic Acids Res.24, 238–241.
[15] Yang, Y., Costa, A., Leonhardt, N., Siegel, R.S., Schroeder, J.I., 2008. Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. Plant Methods. 4, 6.