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Histochemical GUS expression of beta tubulin promoter in transgenic tobacco

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Agrobacterium **mediated plant transformation is a useful technique for stable transformation of plants. This study aimed at stable transformation of the beta tubulin (β) gene promoter and its expression analysis by histochemical GUS assay. The beta tubulin promoter sequence was cloned in plant expression vector (pGA482) and transformed stably in tobacco through *Agrobacterium* mediated transformation. The stable GUS expression assays for this promoter in various tissues of *Nicotiana tabacum* indicated its functional importance in regulating gene expression in a constitutive manner. It was concluded that the β tubulin promoter is constitutively expressed with a strength equivalent to CaMV 2X35S promoter. The isolated promoter can be used in plant genetic engineering for crop improvement in future.**

Key words: Constitutive, gene expression, *GUS,* transformation.

INTRODUCTION

Transgenic plants contain genes which are genetically modified by using genetic engineering techniques. The mode of insertion of transgene is completely artificial. The modified genes can be obtained from completely different species, for example transgenic Bt corn contains a gene from a bacterium and produces its own insecticide. The aim is to introduce a new trait to the plant and this trait does not occur naturally in the species. The production of transgenic plants is a useful process. Transgenic plants can express foreign proteins with industrial and pharmaceutical value. This will help to increase shelf life, quality and resistance against biotic and abiotic stresses. Several new methods of gene transformation has been used for improvement of certain crops. A very common technique known as “Particle Bombardment” is used for transformation of foreign genes in plants. It uses biolistic approach for shooting a piece of DNA into the recipient plant tissue. Gene gun is used in which gold particles are coated with the plasmid vector having gene of interest. These coated particles are accelerated by helium gas and bombarded on particular plant tissues. The gold particles pass through external barrier, that is, the cell

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wall of plant and most of them may enter the nucleus under suitable conditions. The major drawback of this technique is tissue damage, copy number and low transformation efficiency. This method can be used on both monocotyledonous and dicotyledonous species.

Dicotyledonous plants develop from two cotyledons in the seed. They can be recognized by the branching veins in their leaves. Dicots of commercial value include many horticultural plants such as petunias, and crops such as tobacco, tomatoes, cotton, soybean and potatoes. The other method of great importance for gene transformation is electroporation. It works by using electric field that generates holes in the plasma membrane allowing DNA to be taken up by the cell. According to some studies, the reported mortality rate of cells is much high, almost 25 to 50% survival. This technique can be used for variety of species and tissue types. The related drawback is that it needs an established protoplast regeneration system for the plant species being manipulated with foreign DNA.

Agrobacterium-mediated gene transfer method has also gained much importance for production of transgenic plants. *Agrobacterium* is a Gram negative soil pathogen used to transfer DNA to plant cells for the purposes of plant genetic engineering. A number of different reports related to gene transfer through *Agrobacterium* mediated transformation have been reported. Examples in monocotyledonous plant species include rice, barley, maize and wheat (Cheng et al., 1997; Hiei et al., 1994; Rashid et al., 1996; Tingay et al., 1997; Ishida et al., 1996). *Agrobacterium tumefaciens* is a Gram-negative soil pathogen which can naturally infects the wounded sites in plants. The infection results in the formation of crown gall tumors under natural conditions (Smith and Townsend, 1907). The bacterium transfers mobile DNA segment also called T-DNA into the nucleus of infected cells along with DNA coded by some virulence proteins. The transferred DNA is then stably integrated into the host genome and transcribed (Nester et al., 1984; Binns and Thomashaw, 1988). The bacterium is involved in transformation of several dicots (DeCleene and DeLey, 1976). It may even transform various fungal species and human cells (Bundock et al., 1995; De Groot et al., 1998; Gouka et al., 1999; Kunik et al., 2001).

Microtubules occur as a result of cell division and cell elongation. They are mainly composed of α- and β-tubulin having some evolutionary conserved genes (Little and Seehaus, 1988). Simply, tubulin are globular proteins that make up microtubules. Many plant tubulin genes are found as a part of multigene family. However, almost six tubulin genes exist in Arabidopsis and about seven in maize. All tubulin genes works differently in a different manner. Some of them work in a constitutive manner and few are tissue specific genes (Ludwig et al., 1988; Kim and An, 1992; Kopczak et al., 1992). More generally, the expression of tubulin genes in plants can be best studied in dividing tissues.

Studies reported the cloning of tubulin which was achieved from chicken cDNA during 1980s (Cleveland et al., 1980). However, a number of different tubulin genes from different organisms have been isolated and characterized. Tubulin was long thought to be specific to eukaryotes. The basic unit of microtubules is a heterodimer protein composed of α and β-tubulin polypeptides. It is surprising why a large number of tubulin genes in eukaryotes and a single type of α and β-tubulin would be sufficient to fulfill the polymerization of microtubules. In the higher plants, both alpha 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. The isotype-specific expression of tubulins suggests some functional distinction among these proteins. 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).

In order to develop transgenic plants with specialized characteristics and properties, it is important to study and understand the functions of dicot plant promoters for gene expression studies. The present study was planned to characterize the dicot promoter from tubulin family. The β-tubulin promoter, therefore, 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.

MATERIALS AND METHODS

A variety of different tobacco leaves was selected to perform a tissue culture experiment. 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 antibiotic solutions were filter sterilized, while the equipment's were sterilized by autoclaving.

Transformation in *Agrobacterium*

The β tubulin construct was transformed in tobacco through *Agrobacterium* mediated transformation method. For this, the *Agrobacterium* strain LBA4404 was used as a carrier strain. The plasmid DNA of the pGA482 clone having beta tubulin promoter cassette was isolated from overnight culture. The isolated plasmids were further transformed in electrocompetant cells of *Agrobacterium* strain LBA4404 by electroporation. This method works by applying electric shock. The agar plates containing antibiotics were used to spread the transformed cells. These plates were prepared by adding Rifampicin (25 µg/ml) and Kanamycin (50 µg/ml) and were incubated at 28°C for 48 h. Moreover, for *Agrobacterium* growth, single colonies were cultured in LB broth containing antibiotics with relative concentrations. Finally, plasmid were isolated after 48 h by using classical alkaline lysis technique and amplified by PCR with repeated cycles of denaturation,

Table 1. PCR profile for amplification of promoter fragment.

1	Initial denaturing temperature (one cycle)	94°C	4 min
2	Denaturing temperature	94°C	1 min
3	Annealing temperature	46-52°C	1 min
4	Extension temperature	72°C	2 min
	40 cycles from step 2 to step 4		
5	Final extension temperature (one cycle)	74°C	10 min

annealing and extension. The amplified plasmids was further verified by agarose gel electrophoresis for clear visualization of bands. The confirmed clones were then preserved as glycerol stock prepared with 30% concentration for future use.

The seeds of tobacco (*Nicotiana tabaccum* L.) were germinated and grown *in vitro*. For this purpose, surface sterilized tobacco seeds were used. About 1 g of tobacco seeds were soaked in 10 ml bleach prepared with 15% concentration for about 15 min, rinsed in deionized water five times and dried on sterile Whatman filter paper. Ten seeds were planted on Petri dish containing the MS medium. Seeds were germinated and grown in growth room under 16 h constant light at 25°C. The stems with single node and two leaves were cut out from grown plantlets and then transplanted to fresh MS medium.

Regeneration of transformed callus

The prepared glycerol stock was streaked on LB agar plates containing Rifampicin and Kanamycin antibiotics to get true recombinant colonies. The single colonies were cultured in LB media. The cultures were incubated at 28°C with 130 rpm for 48 h. The overall objective of tobacco leaf discs preparation was to maximize wounded surface area for *Agrobacterium* attachment and maintaining tissue health for efficient regeneration of cells. Sterile tobacco leaves were cut from plantlets and soaked in MS liquid medium in a sterile Petri dish to avoid dehydration. After two days, 10 to 20 ml *Agrobacterium* inoculum of each of the four clones was poured in separate petri dishes. About 20 to 30 leaf disks were added per Petri plate, covered and placed at room temperature to allow physical attachment of *Agrobacterium* to plant tissues for about half an hour. After co-cultivation, 5 to 7 leaf disks were placed per petri dish on the co-cultivation media to physical transfer of genetic material by *Agrobacterium* causing virulence. Petri dishes were covered and sealed well with PVC cling film and incubated at 26°C for almost 36 to 42 h.

The leaf disks co-cultivated with *A. tumefaciens* were collected, washed 4 to 5 times with MS liquid medium containing Cefotaxime (250 µg/ml) to remove extra growth of *Agrobacterium* and blotted to remove excessive water. About 4 to 5 leaf disks were placed per Petri dish on the shoot selection medium for induction of callus formation. Regenerated plants were carefully transferred to the pots containing mixture of loamy soil and sand (1:1), covered with water soaked polythene bags and placed at 25°C in the green house. Plants were lightly irrigated after every 2 days interval. When plants attained a height of about 5-inches, they were shifted to soil pots till maturity and collection of seeds. The putative transgenic and control tobacco plants were routinely observed and noted for their morphological appearance during developmental stages.

Molecular analysis of transgenic plants

Young leaves of putative transgenic plants and negative control tobacco plant were selected for DNA isolation by CTAB method. The isolated DNA was re-suspended in 50 µl 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 Fermantas. The primers for transgene analysis were designed at specific sites inside the promoter and gene for amplification of junction regions near promoter. The PCR products for transgene analysis were analyzed by electrophoresis on agarose gel prepared with 1% concentration containing 0.05% concentrated EtBr along with standard 1 kb DNA ladder. Leaves of young plants containing transgenes was selected for DNA isolation by CTAB method with a negative control. The isolated DNA was re-suspended in 30 µl of ultrapure sterile H_2O and stored at -20°C. Gene specific primers for transgene analysis were designed for amplification of junction regions near gene. Finally, the presence of transgenes was confirmed by PCR reaction. The PCR profile is shown in Table 1.

β-Glucuronidase expression

The expression of transgenes was studied by using histochemical *GUS* assay. The tissues of selected plants were covered with 20 µl of 0.1 M X-gluc staining solution. The samples were incubated at 37°C in dark overnight and vacuum infiltrated. At the completion of incubation time, the leaves, stems and roots were treated with different serial dilutions of ethanol. The expression of *GUS* in all the tissues was recurrently monitored. The detailed observation was carried out with light microscope and photographed using a camera.

RESULTS

Agrobacterium-mediated tobacco transformation

The construct for β tubulin was transformed into the *Agrobacterium* LBA4404 strain by electroporation. The clones were confirmed by PCR using reverse and forward promoter specific primers to amplify the selected gene promoter. The results are shown in Figure 1.

Tobacco transformation

A number of different leaf discs were cut and co-cultivated with the cultured *Agrobacteroium* containing plant expression vector. This gives a fair chance to the *Agrobacterium* to transform the gene of interest into the plant causing virulence. Leaf disc were placed on solidified MS0 medium till shooting and rooting for proper growth. Cultivated tobacco plants at different stages are shown in Figure 2.

Molecular characterization of transgenic plants

The transgenic plants were grown in a growth chamber to

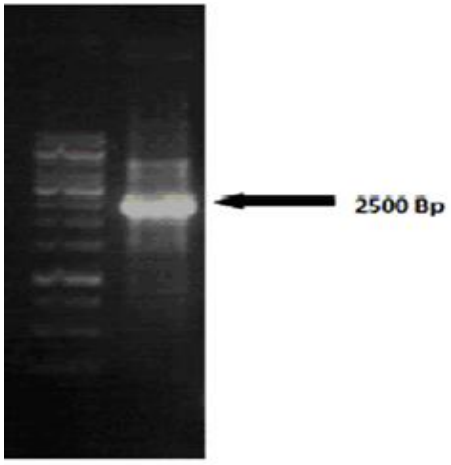

Figure 1. Confirmation of clones in *Agrobacterium*. Lane M: 1kb DNA ladder, PCR amplification of beta tubulin gene promoter of *Agrobacterium* clone.

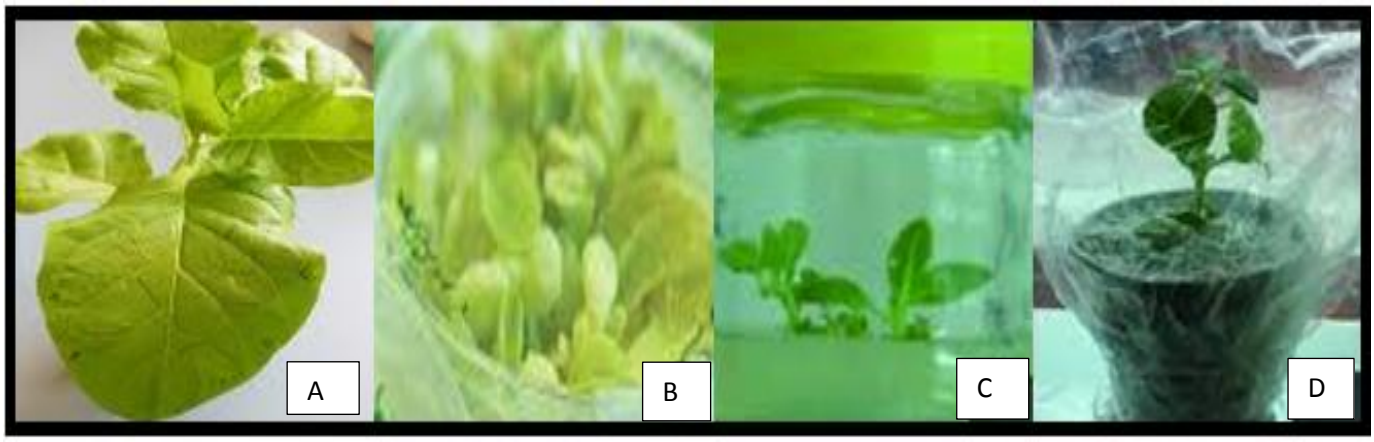

Figure 2. Stages of *Agrobacterium*-mediated tobacco transformation. (A) Tobacco leaf discs for co-cultivation with *Agrobacterium* inoculums. (B) Tiny plantlets of tobacco leaf discs co-cultivation with *Agrobacterium* inoculum. (C) Excised 7 days old plantlets which survived on Kanamycin selection media shifted to jar. (D) Mature plantlets shifted to soil.

study their morphology. More than 100 events were established in the soil. The maximum number of transformed plants was fertile and produced many seeds. All the transgenic plants were analyzed by polymerase chain reaction (PCR) to check the presence of tubulin gene. The analysis showed positive incorporation of tubulin gene in various examined tobacco plants. Out of 5 experiments, the average transformation efficiency of callus producing plants was found to be highest in experiment no. 2 with approximately 79.5% average. The transformation efficiency was calculated by dividing total number of explants showing transformation with number of explants inoculated. The results obtained are shown in Table 1 and Figure 3.

Evaluation of transgenic plants

The insecticidal activity in all 5 experiments was performed to measure the resistance levels. Results showed different resistance levels including high and low levels with variation in all experiments. Screening of plants with bioassays was useful to measure the different resistance levels in the selected plants. The obtained results are presented in Table 2 and Figure 4.

Analysis of transgene and *GUS* expression

The genomic DNA was isolated by using CTAB method

Table 2. Different resistance levels in the selected transgenic tobacco plants.

Experiment	Number of Plants used	Resistance Level
1.	5	Highly resistant
2.	4	Less resistant
3.	6	Moderately resistant
4.	3	Less resistant
5.	4	Highly resistance

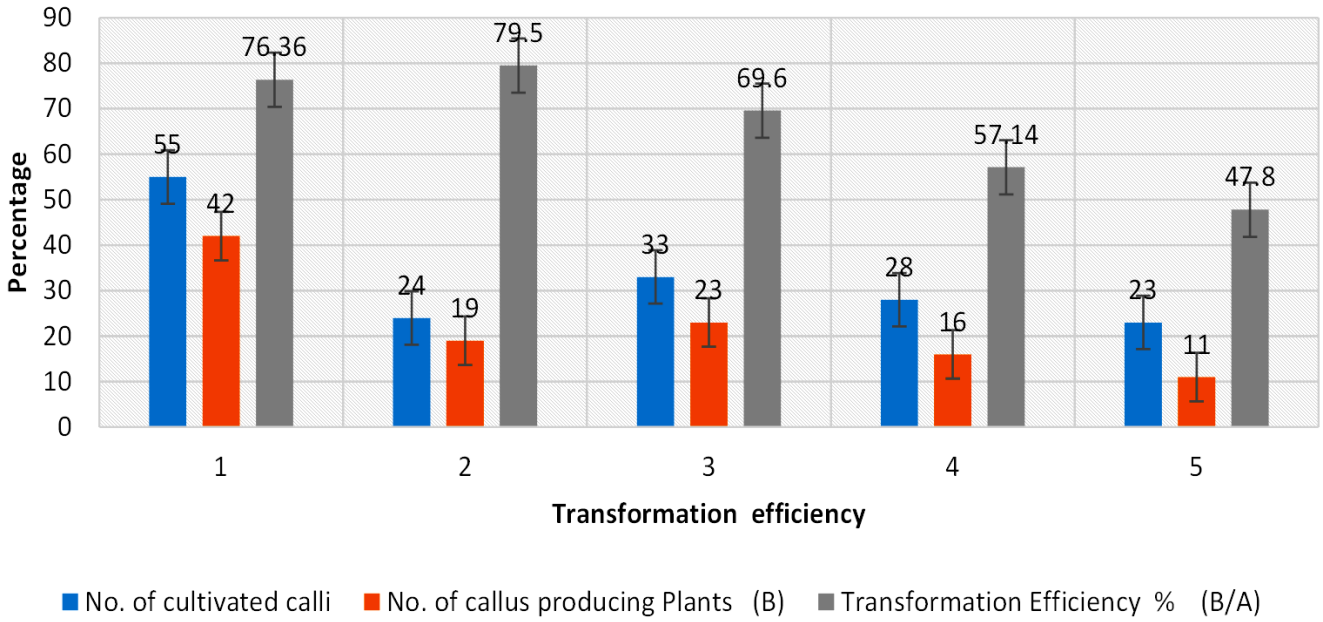

Figure 3. The average transformation efficiency of transgenic plants.

from the putative transgenic and non-transformed negative control plants. The transgenic plants were confirmed by PCR using promoter specific primers. Results of PCR analysis are shown in Figure 5. The results show amplification of expected fragments from the transgenics for β tubulin construct. The amplifications in the transgenic 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. Further, the selected leave tissues of beta tubulin transgenic plants were stained for *GUS* activity. Staining patterns of representative leaf tissues are shown in Figure 6. All the leaf tissues were dipped in staining solution to monitor color intensity of expression levels in different transgenic events. The plants tissues expressing *GUS* using *2X35S* and beta tubulin promoter was stained for 24 h. The staining reaction showed that beta tubulin promoter was constitutively expressed in leaves. The staining of leaves from non-transgenic plants did not reveal any expression.

DISCUSSION

Transformation of plants by *Agrobacterium*-mediated gene transfer is the most commonly used method for transferring gene of interest in plants. According to some recent reports, the method of *Agrobacterium*-mediated transformation has been applied in a number of monocotyledonous plant species (Hiei et al., 1994; Rashid et al., 1996), barley (Tingay et al., 1997), maize (Ishida et al., 1996) and wheat (Cheng et al., 1997). The integration of T-DNA into host plant can infer resistance against disease.

Stress tolerance in plants can be improved by using several enzymes of biosynthetic pathways (Lata et al., 2011). The *Agrobacterium* mediated plant transformation can be used for genetic improvements. This is due to small copy number of T-DNA and variable expression of transferred genes (Murai, 2013). Gene regulation plays a vital role in controlling genetic modification of plants. Cellular homeostasis can be maintained with proper

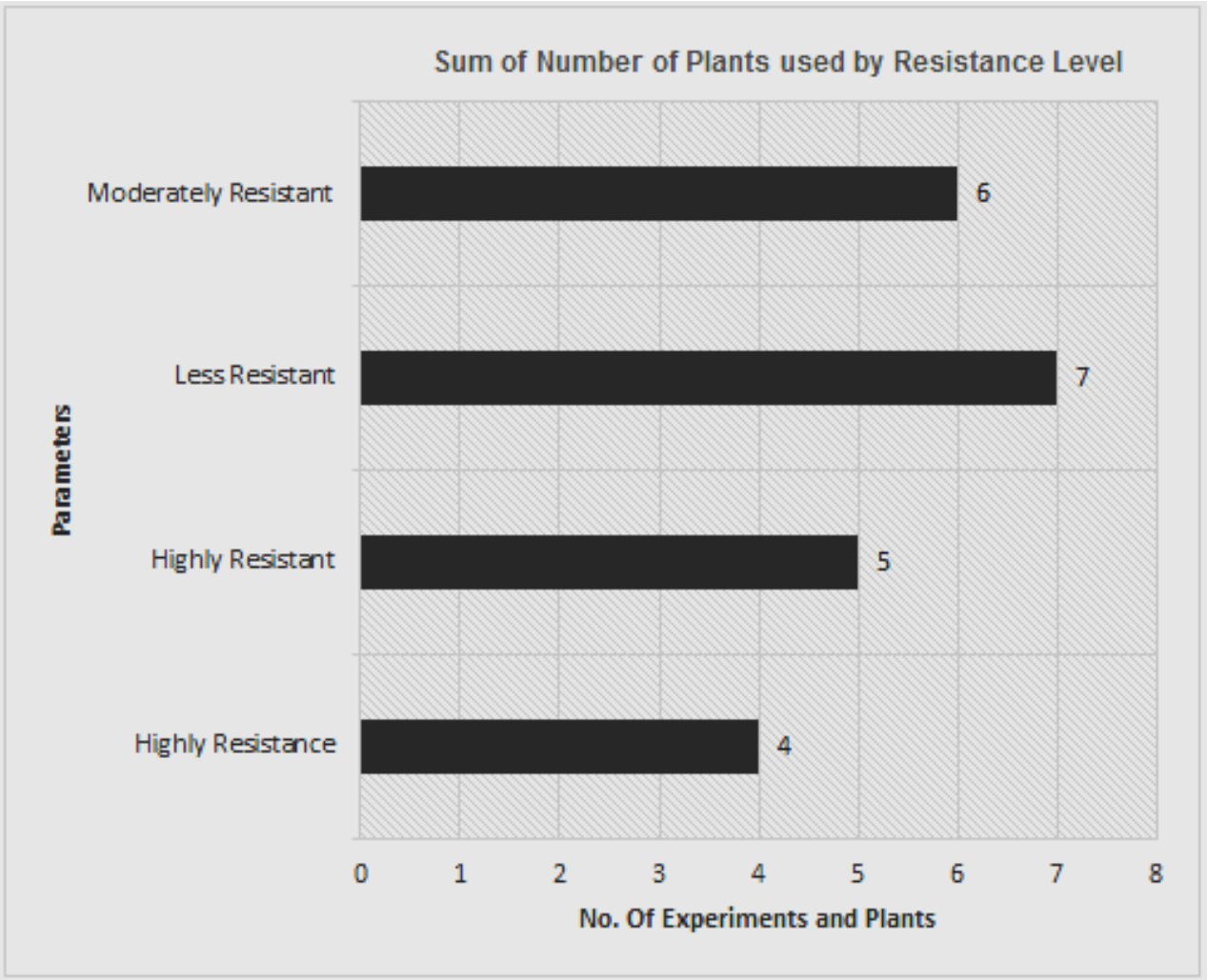

Figure 4. Resistance levels in different plants with variation of resistance.

regulation and growth (Arie, 2002; Mizoi and Yamaguchi-Shinozaki, 2013). Transcriptional regulation involves regulation of all genes involved in regulatory pathways (Julieta, 2014). Transcription factors are key factors in transcription regulatory mechanism acting as gene switches.

A number of different factors exists and can limit this transformation efficiency in both monocots and dicot plants. This can also affect plant regeneration process. Mostly, the regeneration rates are poor with monocotyledonous plants. *Gus* is a very useful reporter gene expression system used in plant transformation. The histochemical GUS assay has been used for subcellular localization of *GUS* associated fusion proteins. However, efficient methods of *Agrobacterium* mediated gene transfer have been established mainly for dicotyledoneous plants. The optimization of *Agrobacterium tumefaciens* for plant transformation is possibly the most important aspect to be considered. Expression of transgenes in tobacco leaves and callus was determined using histochemical GUS assay. Some of the relevant findings about *Gus*-reporter gene have been studied in tobacco plant (Nakashima et al., 2014).

To evaluate the accuracy of tubulin promoter that has been transformed through stable transformation, it is preferred to generate its large number of transformants. Out of which few was screened for gene expression analysis. Therefore, important elements that control the tubulin transcriptional expression may lie in both the 5′- and 3′-flanking regions of the genes (Stotz and Long, 1999; Doyle and Han, 2001). Moreover, it was found that 5′-flanking regions are more commonly involved in this process. Some of the previous studies predicted, to increase the level of GUS expression of rice tubulin genes, the first intron of *tubulin-α1* was sufficient (Jeon et al., 2000). This was observed in many transformation experiments performed with the use of *GUS* as the reporter gene. The expression of the reporter gene was controlled by the tubulin sequences 5′-upstream of the

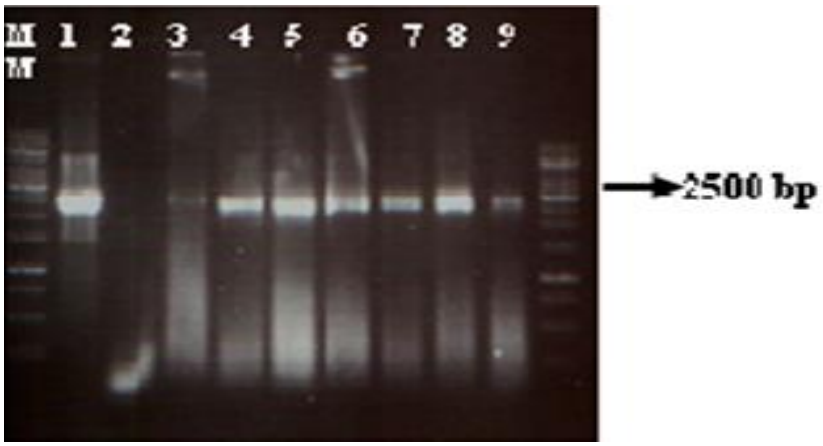

Figure 5. PCR analysis of putative transgenic tobacco plants for β tubulin promoter. M: 1 kb DNA ladder, Lane 1: PCR of positive control using plasmid DNA as a template, Lane 2: Negative control of PCR master mix, Lane 3: Negative control of tobacco Lanes 4 to 8: PCR analysis of 5 randomly selected putative transgenic plants using promoter specific primers, showing expected amplification product of 2500 bp.

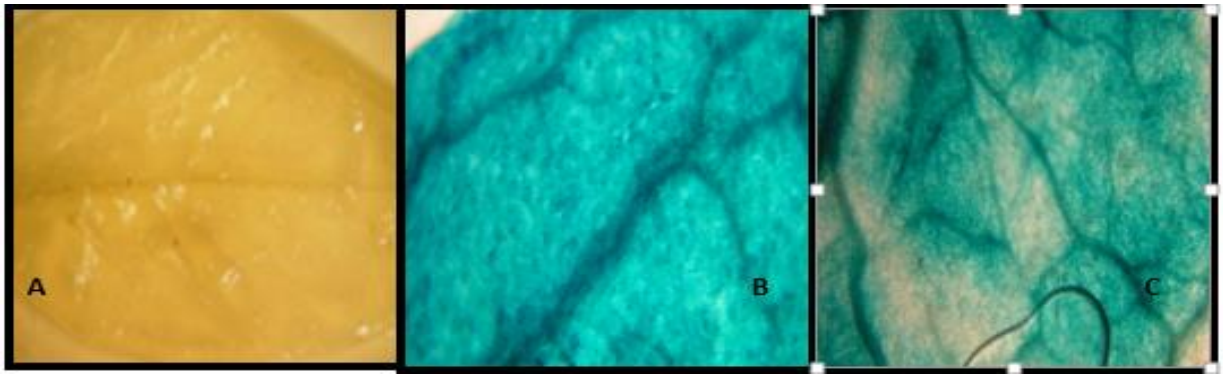

Figure 6. Histochemical assay for *GUS* activity in stably transformed tobacco plants. A: negative control of tobacco, B: *GUS* activity in leaves controlled by 2X35S, C: Close view of leaf tissue stained for *GUS* activity controlled by β tubulin promoter.

ATG in construct being used (Carpenter et al., 1992). Therefore, *GUS* expression will be high in these experiments (Uribe et al., 1998; Stotz and Long, 1999; Cheng et al., 2001). The study was designed to characterize and analyze the expression of the tubulin promoter in tobacco (*Nicotiana tabacum* L.) by using *Agrobacterium*-mediated transformation. The tubulin gene from beta family was selected and cloned in plant expression vector (pGA482) for stable transformation. Beta tubulin promoter was retrieved from high throughput genomic sequences and its sequence was analyzed through databases. The transgenic plants were obtained and *GUS* activity in each of the transgenic was determined through *GUS* staining. The selected tubulin promoter showed GUS expression in various plant tissues of tobacco and cotton. Also, it was found to highly constitutive dicot promoter for expression of transgenes in various dicot plants. The *GUS* expression studies revealed that the selected beta tubulin promoter actively control *GUS* expression in different transgenic events of the stably transformed tobacco plants. *In situ GUS* activity on leaf tissues of transgenic plants showed, the β tubulin promoter was active with different levels of expression.

Conclusions

It is concluded that β tubulin promoter can be utilized to confer a constitutive gene expression in all plant tissues. The *2X35S* promoter has been reported to exhibit strong constitutive activity in different plant species and the

same was observed in the current experiments. It is proposed that the promoter identified through this study may be utilized in future to overcome the major issues related to gene silencing and to ravel novel mechanisms of different plants. Moreover, highly expressed constitutive dicot promoters can be utilized for the transgene expression in economically important agricultural crops in future.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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