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A STRATEGY OF *TADOF1* CASSETTE DEVELOPMENT IN PLANT EXPRESSION VECTOR TO ENHANCE NITROGEN ASSIMILATION IN WHEAT

A. Hasnain, A. Maqbool* and Kauser A. Malik

Department of Biological Sciences, Forman Christian College (A Chartered University) Ferozpur Road, Lahore 54600, Pakistan

*Corresponding author's email: asmamaqbool@fccollege.edu.pk

ABSTRACT

TaDof1 gene is involved in enhanced nitrogen assimilation in plants. Nitrogen assimilation is essential to the growth and development of plants as it produces large quantities of organic nitrogen including proteins, amino acids and nucleic acids. In plants functional genomic studies, gene cloning and vector construction for transformation are common procedures. The availability of effective transformation vector is one of the pre-requisites for plant transformation studies. Current research depicts the cloning of complete cassette of *TaDof1* in a binary monocot expression vector (pSB219) using basic cloning strategy. The digested fragments of the vector and insert were ligated followed by transformation of ligated product in *E. coli* (strain *DH10α*). The synthetic plasmid was successfully co-transformed along with the helper plasmid pAL154 in *Agrobacterium tumefaciens* (strain AGL1). The plasmid constructed in this study is suitable for *Agrobacterium*-mediated transformation of elite wheat varieties. The study paves the way of developing transgenic *TaDof1* wheat cultivars exhibiting enhanced nitrogen assimilation.

Keywords: Nitrogen use efficiency, Gene cloning, Single-gene cassette construction, *Agrobacterium*-mediated transformation, Wheat transformation.

INTRODUCTION

Wheat is providing the greatest part of daily nutritional requirement for human diet. Annual world production of wheat was around 685 million tons in 2009. In order to meet the increasing need for wheat, production should be raised to an annual rate of 2% without any additional land (Sparks *et al.,* 2014). Traditional breeding methods have reached a plateau, where increase in nitrogen application does not improve productivity. The use of biotechnological approach certainly holds a great promise to increase wheat production (Sparks and Jones, 2014).

Agrobacterium tumefaciens naturally infects the wound sites in plant causing the formation of crown gall tumors. *Agrobacterium*-mediated transformation in plants has become the most commonly used method for the introduction of foreign genes into plant cells and subsequent regeneration of transgenic plants (Sparks *et al.,* 2014). Nevertheless, because of lower transformation efficiencies and genotype dependence, the transformation of wheat still presents more difficulties than transformation of other cereals such as rice and maize (Sparks and Jones, 2014).

Nitrogen assimilation is crucial to growth and development of plants. An enormous amount of nitrogen fertilizers is added to fields to increase crop yield (Nosengo, 2003). Due to excessive fertilizing regimes, the adverse effects of nitrogen in the environment include algal blooms (Vitousek *et al.,* 2009; Wuebbles, 2009), stratospheric ozone depletion and global warming (Coruzzi and Zhou, 2001). Increasing nitrogen fertilizer cost has resulted in demand for more nitrogen use efficient (NUE) crops i.e., crops that are better able to uptake, assimilate and remobilize the nitrogen available to them (Yanagisawa and Sheen, 1998).

Since a single transcription factor affects multiple genes in a metabolic pathway, it is possible to modulate the pathway using transcription factors (Yanagisawa *et al.,* 2004). *Dof1* transcription factor, unique to plants, is involved in enhanced nitrogen use efficiency in plants. *Dof1* over-expressing in rice and *Arabidopsis* showed increased expression of the gene encoding phosphoenol pyruvate carboxylase (PEPC). PEPC is involved in increased carbon flow towards nitrogen assimilation pathway (Kurai *et al.,* 2011). *Triticum aestivum Dof1* (*TaDof1*) transcription factor is involved in enhanced nitrogen use efficiency in plants (Kumar *et al.,* 2009).

Gene cloning is a technique used for getting high copy number of a specific DNA fragment, recovering large quantity of protein produced by the particular gene. In the present study, *TaDof1* was cloned in vector pSB219 using basic cloning strategy. The development of plant expression vectors with a gene of interest has been extensively exercised over the last few decades (Dafny-Yelin and Tzfira, 2007). Cloning of the desired gene into plasmid vectors involves several steps. Initially, DNA and plasmid vector are digested with the same restriction enzymes. Then, ligation of digested products is done using ligation enzyme. Finally, transformation of ligation product into competent *E. coli*

cells is performed via different transformation methods followed by selection and screening of the desired recombinants.

There are different strategies that can be adopted for cloning. Blunt-end DNA cloning introduces the insertion of blunt-ended DNA or 5′-end phosphorylated PCR product into a linearized blunt-ended vector (Upcroft and Healey, 1987). The limitations of blunt-end cloning are non-directional ligation and self-ligation of vector (An *et al.,* 2010). TA cloning is achieved by *Taq* DNA polymerase which has non-template-dependent terminal transferase activity which helps adding a single deoxyadenosine (A) to 3′ ends of PCR products. The PCR product can be directly cloned into a linearized T-vector that has a single base 3′-T overhang on each end (Zhou *et al.,* 1995). The major drawback of this strategy is non-directional cloning, the insert (DNA or PCR product) is ligated in linearized T-vector in both the orientations. Among gene cloning methods, sticky-end cloning is the most efficient and widely used method (Conze *et al.,* 2009). In order to produce sticky or complementary ends, insert DNA and vector are separately cut with the same restriction endonuclease enzymes. The insert DNA is ligated into plasmid vector by DNA ligase (An *et al*., 2010).

In this study, a gene cloning strategy was used to clone a single transgene cassette in a binary monocot expression vector pSB219. *TaDof1* was cloned under *CaMV35S* promoter and *Nos* terminator. The synthetic plasmid containing the complete *TaDof1* cassette was subsequently transformed in *A. tumefaciens* for wheat transformation.

MATERIALS AND METHODS

Gene and promoter resources: *TaDof1* accession number AY955493.2 from *Triticum aestivum* codes for transcription factor (Yanagisawa *et al.,* 2004). The nucleotide sequence was retrieved from GenBank (accession number AY955493.2). The coded protein comprises of 291 amino acids. *TaDof1* transcription factor gene was got synthesized from Operon Technologies, USA. Cauliflower mosaic virus 35S (*CaMV35S*) promoter was amplified from pGR187. The amplified *CaMV35S* promoter comprised of 438 bp.

Bacterial strains: The binary monocot expression vector pSB219 was obtained from Leibniz Institute of Plant Genetics and Crop Plant Research, Germany. The expression vector was maintained in *E. coli* strain DH10α. *TaDof1* construct was developed in pSB219. *TaDof1* cassette was co-transformed with pAL154 into AGLI strain of *A. tumefaciens* having C58 chromosome background. The pAL154 provides replication function in trans to pSB219. It has a 15 kb Komari fragment having additional virulence genes (virB, virC and virG542) for efficient plant transformation (Wu *et al.,* 2008). The pAL154 and *A. tumefaciens* were kindly provided by Ms. Caroline Sparks, Rothamsted Research, UK. The *TaDof1* construct developed in pSB219 was used for *A. tumefaciens* (AgL1) transformation.

Plasmid construction: The GFP expression cassette in pSB219 was replaced with *TaDof1* cassette (Fig. 1). For promoter insertion, *CaMV35S* was PCR amplified from already available vector using primers (35S-HinF-1 and 35S-AscR-1) with the restriction sites *HindIII* and *Asc1* (Table 1). *Pfu* DNA Polymerase (Cat# EP0502) which is highly thermostable polymerase was used for amplification of genes. The PCR product was digested and cleaned (Favorgen PCR purification kit Cat# FAPCK001-1). The vector pSB219 was digested with the respective restriction enzymes (*HindIII* and *Asc1*) and purified.

The vector was purified by gel extraction and the PCR product was purified through a column (Favorgen PCR purification kit Cat# FAPCK001-1). The ligated product was transformed into *E. coli DH10α* by electroporation method (EppendorfEporator®, Hamburg, Germany) set (Yanagisawa *et al.,* 2004). *Nos* terminator was PCR amplified from already available vector using Nos-specific primers *Nos*-Eco8-F2 and *Nos*-Sda-R2 with restriction sites *Eco*81I and *Sda1* (Table 1). The PCR amplified product and the plasmid were double digested with *Eco81I* and *Sda1* and purified.

After ligation, the product was transformed in *E. coli DH10α* by electroporation method. Synthetic *TaDof1* was PCR amplified using primers Dof-Asc-F2 and Dof-Eco8-R2 with the restriction sites (*Asc1* and *Eco81I*) (Table 1). The PCR product and the plasmid were double digested with *Asc1* and *Eco81I* and purified. *E. coli DH10α* was transformed with the ligated product by electroporation method.

Selection of bacterial cells was performed on spectinomycin (100mg/L). Screening was done by colony PCR with the respective primers of promoter, gene and terminator. The cloned plasmids were confirmed with restriction digestion (Fig. 3) and sequencing analysis (Fig. 5).

DNA Ligation Kit (*Thermo Scientific*, EU, Lithuania) was used for ligation reactions. Concentration of the plasmid and DNA insert was determined by using nanodrop spectrophotometer (*Thermo Scientific*). The ligation reaction mixture contained 1:4 ratio of plasmid and DNA insert. T4 DNA Ligase (5u/µl), 5X Rapid ligation Buffer and nuclease-free water were also added in ligation reaction.

The ligation mixture was incubated at 22°C for 1 hour and then at 16°C overnight. The ligation mixture was added in *E. coli DH10α* competent cells and then electroporated with electroporator (Eppendorf Eporator®, Hamburg, Germany) set at 2.4 kV using cuvette with

2mm gap width (Yanagisawa *et al.,* 2004). In one vial of competent cells (100µl), 1µl of ligation mixture was added and incubated on ice for 30 minutes. After electroporation, 800µl of LB broth was added and the samples were incubated at 37ºC for one hour. Transformation mixture (120µl) was spread on LB agar plates containing spectinomycin (100mg/L) and incubated at 37ºC overnight.

Detection of recombinants: To analyze the presence and orientation of the DNA insert in recombinant clones, colony PCR, restriction digestion and sequencing analyses were performed. The colony PCR method was firstly applied for the detection of recombinants. Individual colony was picked and re-suspended in 25 µl of PCR master mix. Restriction analysis was also done using appropriate restriction endonuclease enzymes. Plasmid DNA was isolated from an overnight bacterial culture and cut with restriction endonucleases *HindIII, AscI, Eco8I1 and SdaI*. These enzymes were found on the map of cloning vector. If the transformed colony carried right orientation of the DNA insert, plasmid was sequenced with forward and reverse sequencing primers.

Strain construction: *A. tumefaciens* transformation was done by electroporation method as mentioned previously (Yanagisawa *et al.,* 2004). The *TaDof1* cassette was co-transformed with pAL154 into AGLI strain of *A. tumefaciens.* Bacterial cells were grown on media containing rifampicin (50mg/L), spectinomycin (100mg/L) and tetracyclin (2mg/L). Screening was done on the basis of colony PCR using gene junction primers PGF2 junc (ATCCTTCGCAAGACCCTTCC), PGR2 junc (TGGAGTTGGAGTTGGACGAC), GTF2 junc (ATGACGAACTACCCCTTCGC) and GTR2 junc (TAATCATCGCAAGACCGGCA) (Table 1).

RESULTS AND DISCUSSION

Development of *TaDof1* cassette in pSB219 vector: Cloning of gene is a basic technique widely used in molecular biology in order to get large number of copies of the DNA fragment in host cells that is finally translated into the desired protein (Tulpova *et al*., 2018). In the present study, the transgene delivered was under the control of *CaMV35S* promoter since it is a strong and constitutive promoter (Seternes *et al.,* 2016). For transcription of gene, *Nos* terminator was used as it is extensively used as a stop signal in transgenic organisms. *Nos* terminator was for the first time identified by Lipp *et al.* (1999). The *TaDof1* cassette was developed in a binary monocot transformation vector pSB219. The vector had two selectable markers; aadA gene that confers resistance to aminoglycosides spectinomycin and streptomycin in *E. coli* and a *bar* gene (*hpt*) that induces resistance against BASTA (Table 2). The *bar* gene, under the control of maize ubiquitin promoter, makes this vector a reliable candidate to be used in further studies for screening of putative transgenic wheat plants residing pSB219.

TaDof1 construct comprised of 1,602 bp which include *CaMV35S* promoter (having 99% identity to NCBI accession number AB863158.1), synthetic *TaDof1*, the stop codon and the *Nos* terminator (Fig. 1, Table 2). The insert was cloned between left and right T-DNA borders of pSB219. The cassette was cloned using *HindIII, AscI, Eco8I1* and *SdaI* in the multiple cloning site (MCS) of pSB219. The physical map of MCS is shown in Fig. 2.

For each fragment, specific primers with appropriate restriction sites were designed and used. The restriction sites were added to primers in order to obtain directional cloning. For *CaMV35S* promoter, *HindIII* and *AscI* sites were added; for *TaDof1* gene*, AscI* and *Eco81I* sites were added and for *Nos* terminator, *Eco81I* and *SdaI* sites were added to the primers. In order to create sticky ends compatible with vector sticky ends, each PCR amplified fragment and vector were cut with the same restriction enzymes. Firstly, *HindIII* and *AscI* were used to digest promoter and vector, secondly, *AscI* and *Eco81I* were employed to cut gene and vector followed by using *Eco81I* and *SdaI* to digest terminator and vector. The digestion of each fragment with respective enzymes created sticky ends having 5′end overhangs. For successful cloning of Dof1 gene in a plant vector, Kurai *et al.* (2011) added *BglII* and *EcoRI* restriction sites to forward and reverse primers of Dof1 gene. An effector plasmid was constructed in which *PstI-HincII* insert of Dof1 cDNA was cloned in plant expression vector (Yanagisawa, 2000). In another study by Yanagisawa and Sheen (1998), *NcoI-PstI* insert of Dof1 was cloned in a plant expression vector between 35SC4PPDK promoter and *Nos* terminator.

In our experiment, the digested products were ligated using ligation enzyme. DNA ligase enzyme is used to ligate the insert DNA into plasmid vector (An *et al*., 2010). Different parameters affect ligation reaction which include ratio of DNA insert and plasmid vector, temperature and components of buffer (Costa and Weiner, 1994). In the current investigation, the ligation reaction was set under optimum conditions using T4 DNA ligase enzyme. The cloning of each fragment in pSB219 was confirmed by restriction digestion analysis (Fig. 3). In addition to these molecular detection methods, sequence analyses of the positive colonies were also performed. The sequence analyses confirmed that full-length error free genes were cloned in monocot expression vector (Fig. 5). Previously, a linear gene cassette (35S-phytase gene-*nos*) having T-DNA borders was introduced in a plant transformation vector (Gao *et al.,* 2007).

The ligated product was transformed in *E. coli DH10α* competent cells. Abid *et al*. (2017) transformed

E. coli with a monocot expression vector in which phytase gene cassette was cloned. After transformation, screening of transformed colonies was done by PCR amplification of CaMV35S promoter, *TaDof1* and *Nos* terminator.

***Agrobacterium tumefaciens* transformation:** *A. tumefaciens* (strain AGL1) was employed to transform the confirmed recombinant vector using electroporation method. Electroporation involves the use of high voltage current to open pores found in the cell membrane. As a result, proteins, nucleic acids and membrane-impermeable molecules easily enter the cells. To make the cell membrane permeable, the electric pulses must be strong but not so intense that result in cell death (Yang *et al*., 2011). The results of electroporation vary according to the size and type of cells, size of plasmid and temperature. In the current study, a high voltage of 2.4 kV was used because the size of the vector pSB219 was large. In a study, a strong influence of increasing pulse number on electroporation was observed in *Agrobacterium*-mediated transformation (Mahmood *et al*., 2008).

Wheat embryogenic calli were infected with *A. tumefaciens* (AGL1 strain) harboring plasmid with the gene of interest (Habib *et al.,* 2014). In the current investigation, the plasmid with complete *TaDof1* cassette and the helper plasmid pAL154 were co-transformed in AGL1 strain (*A. tumefaciens)*. Marker-free transgenic wheat developed by *Agrobacterium*-mediated transformation harbored the helper plasmid pAL154 (Wang *et al*., 2016). The plasmid pAL154 facilitates transformation as it contains Komari fragment. The results were verified by colony PCR using gene junction primers (Table 1). PCR amplification results using gene junction primers are shown in Fig. 4. The transformed *A. tumefaciens* can be used to express the transgene in elite wheat cultivars in future studies.

Table 1. Primer sequences used in study.

Primer	Sequence	Application/notes
35S-HinF-1	5′-CCCAAGCTTAACATGGTGGAGCAC-3′	Amplification of *CaMV35S* promoter from plasmid pGR187
35S-AscR-1	5′-TTGGCGCGCCGTCCTCTCC-3′	
Nos-Eco8-F2	5′-AATCCTTAGGGATCGTTCAAACATT-3′	Amplification of *Nos* terminator from plasmid pBRACT404
Nos-Sda-R2	5′-AATCCTGCAGGGATCTAGTAACATA-3′	
Dof-Asc-F2	5′-TTGGCGCGCCACCATGC-3′	Amplification of dof1
Dof-Eco8-R2	5′-AATCCTTAGGCTAGGGTAGGTA-3′	
PG F1 junc	5′-GACGTAAGGGATGACGCACA-3′	Amplification of promoter gene junction region
PG R1 junc	5′-ACTTGGTGTTGGTGGACTCG-3′	
GT F2 junc	5′- ATGACGAACTACCCCTTCGC-3′	Amplification of gene terminator junction region
GT R2 junc	5′-TAATCATCGCAAGACCGGCA-3′	

Table 2. Details of plasmid vector.

Plasmid	Insert	Unique RE sites	Selection marker	GenBank accession
pSB219	*CaMV 35S* promoter	*HindIII, Asc1*	aadA	AB863158.1
pSB219	*Dof1* transcription factor	*Asc1, Eco81I*	aadA	AY955493.2
pSB219	*Nos* terminator	*Eco81I, Sda1*	aadA	LC221392.1

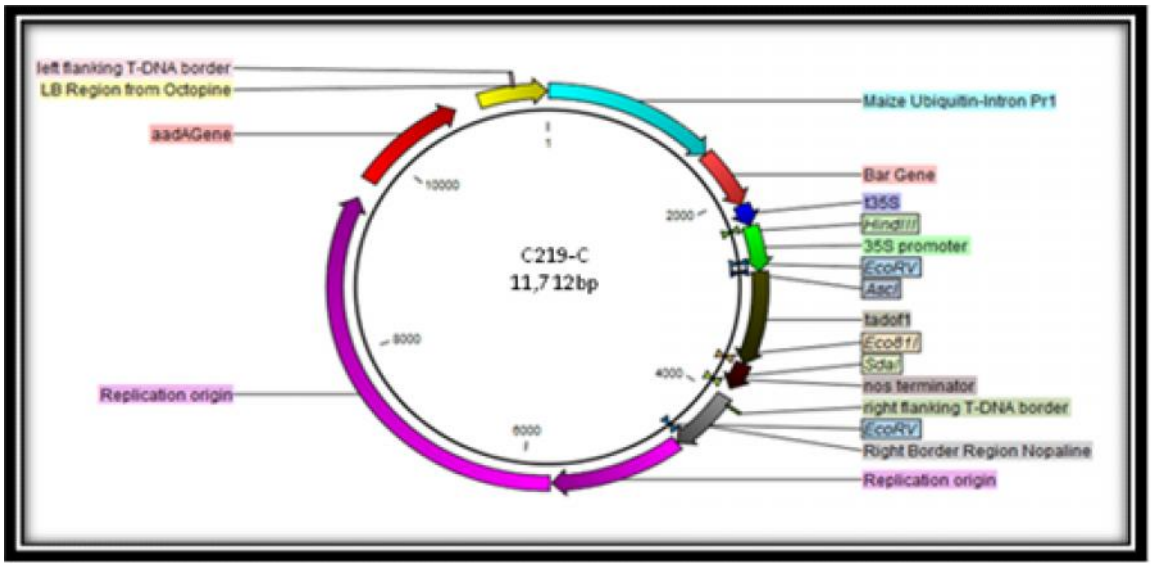

Fig.1: Generic plasmid vector map for *TaDof1* cassette expression; the physical map of the *TaDof1* cassette between left and right T-DNA borders of pSB219 vector. The cassette comprises of *CaMV35S* promoter, *TaDof1* gene followed by a *Nos* terminator. The restriction enzymes to join the DNA fragments are shown at the junctions of promoter-CDS and CDS-terminator sequences. The *bar* gene is controlled by maize ubiquitin promoter and t35S terminator. Bacterial selection can be performed by using spectinomycin resistance gene (aadA)

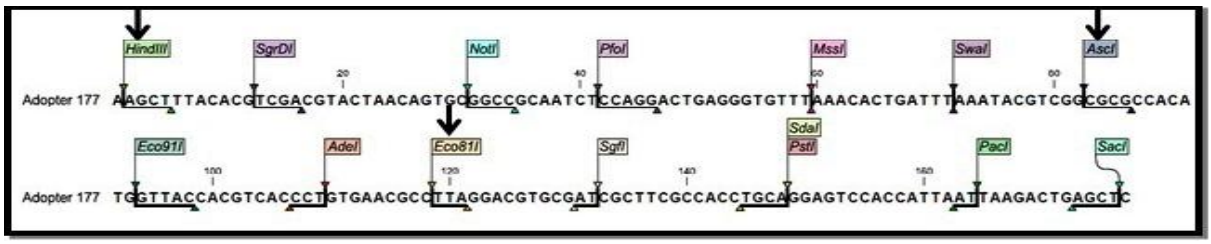

Fig. 2: A detailed map of unique restriction sites (MCS) in pSB219; the restriction enzymes used for the *TaDof1* cassette constructions are marked by arrows

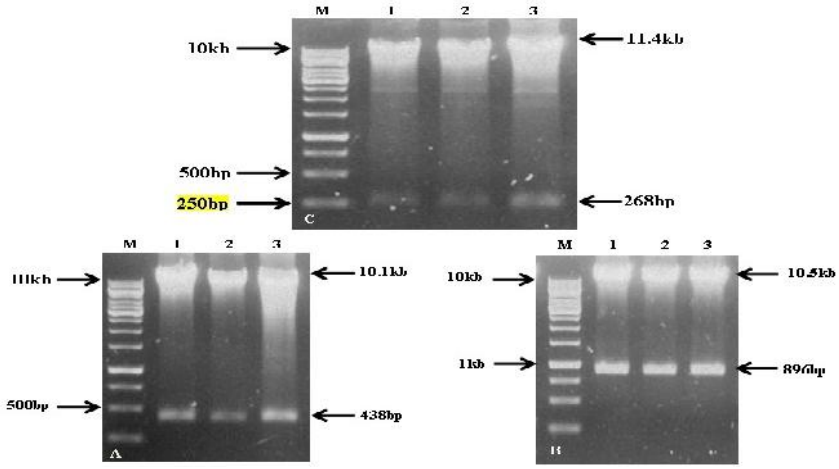

Fig. 3: Restriction digestion confirmation by unique restriction enzymes (A) Restriction digestion confirmation by *HindIII* and *Asc1.* M, 1 kb ladder. Lane 1-3, digested clones. (B) Restriction digestion confirmation by *Asc1* and *Eco81I.* M, 1 kb Ladder. Lane 1-3, digested clones. (C) Restriction digestion confirmation by *Eco81I* and *Sda1.* M, 1 kb Ladder. Lane 1-3, digested clones

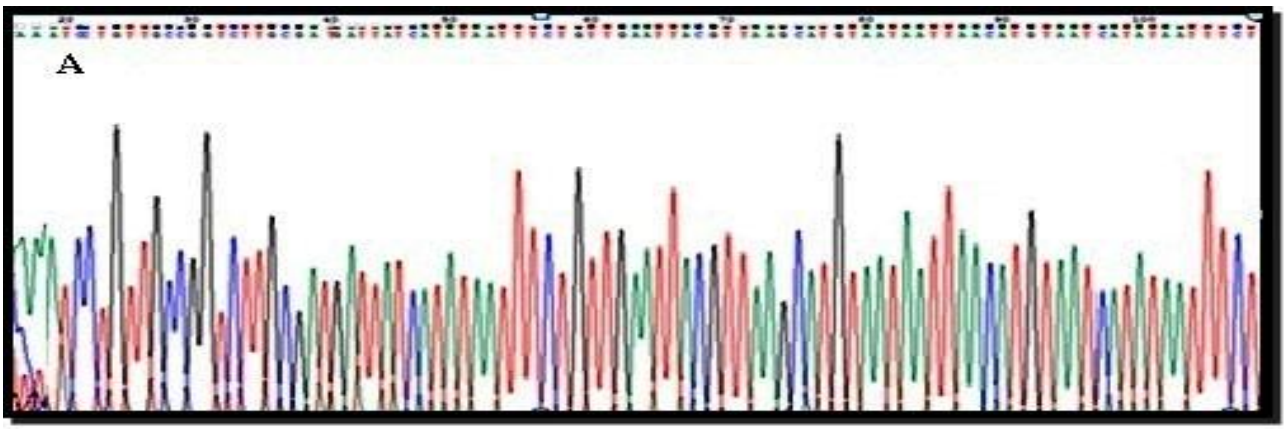

Fig. 4: PCR results using gene junction primers (A) PCR result using primers GTF2R2. M, 1 kb Ladder. Lane 1, -ve control. Lane 2-10, PCR amplified product (B) PCR result using primers PGF1R1. M, 1 kb ladder. Lane 1, -ive control. Lane 2 -10, PCR amplified product

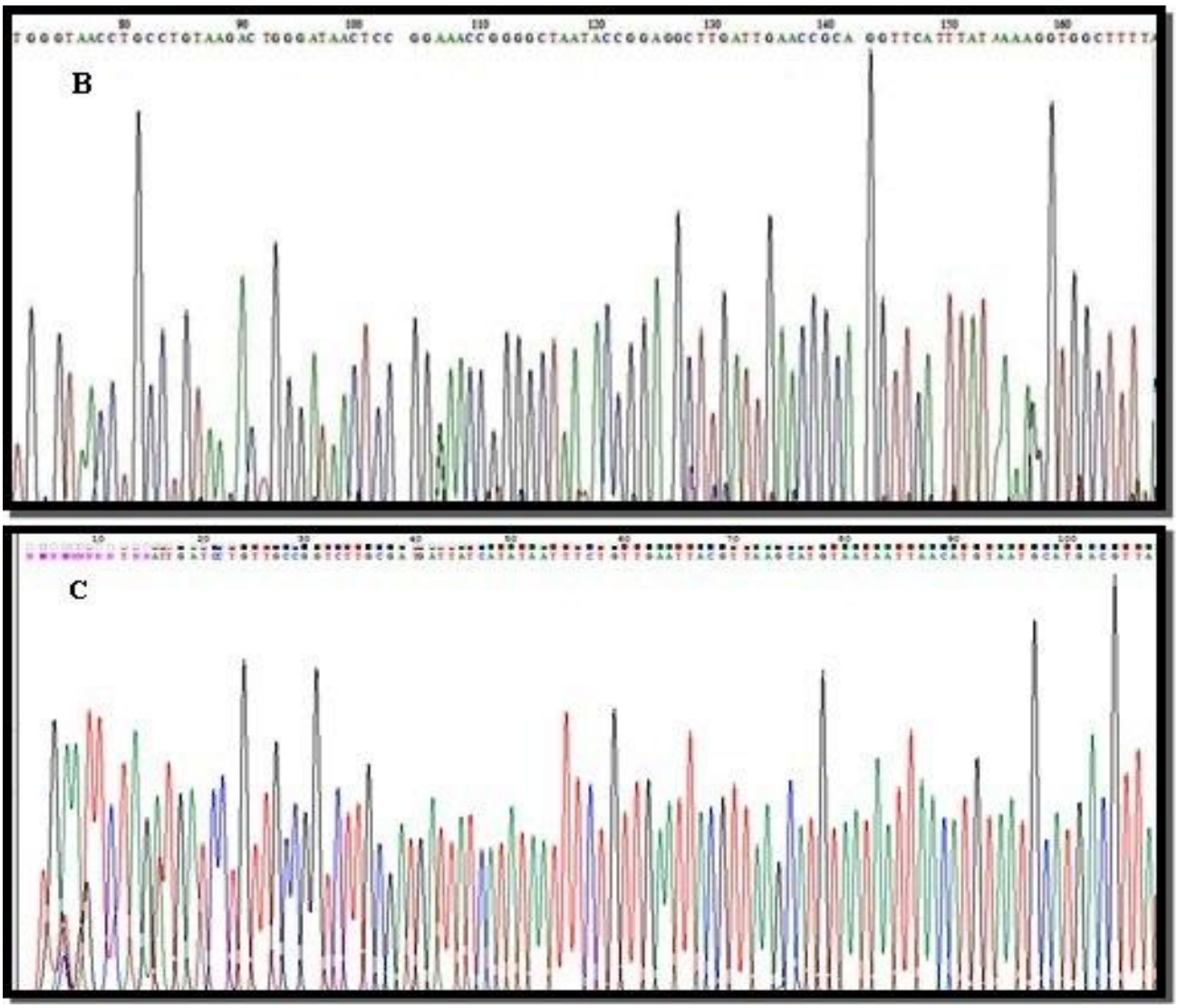

Fig. 5: Sequencing results (A) *CaMV 35S* promoter in pSB219 (B) *TaDof1* in pSB219 (C) *Nos* terminator in pSB219

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