Construction of Expression Vector pVBG2307 by adding Transcription Initiation and Termination Elements

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Abstract

Cloned elements of expression vector pBI121 carrying transcription initiation codon namely cauliflower mosaic virus section 35S and a nucleotide sequence NOS functioning transcription termination codon of cloning plasmid pBI221 were inserted into a precursor cloning plasmid pCAMBIA2300 to construct an intermediate vector termed as pVBG2307. Resultant vector pVBG2307 offers vast multiple cloning site with an adjacent imported CaMV35S promoter and transcription stop codon sequences on its left border. This vector allows controlled transformation and regulation of nucleic acids in both E. coli and Agrobacterium tumefaciens. Many cloned CaPG, orf456, ipt genes and E8 (a fruiting promoter), were amplified from cDNA libraries of sweet pepper (Capsicum annum) and tomato (Lycopersicon esculentum) and were then transferred into vector pVBG2307. The viability of this vector was demonstrated, as it regulated CaPG, orf456, ipt and E8 genes in Escherichia coli and could be transferred into tumor inducing Agrobacterium strain EHA105-4.

Key words: pBI121, pBI221, pCAMBIA2300, pVBG2307, *CaPG* gene, *E8* promoter

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INTRODUCTION

Many important genetic programs regarding crop improvement against physiological aging were taken around the world and considered on top priority in GMOs. Various genetically improved horticultural commodities are not only fulfilling food demands but also alleviating the poverty among people dwelling in many poor parts of the entire world (AVRDC, 2005). Sweet peppers and tomatoes are gaining values and earning high capital among many other horticultural commodities all over the world, where not only involved human diet and health but also serving in culinary, nutritional, medicinal, pharmaceutical and therapeutic uses and many other food processing industries (Pruthi and Sharma, 1998; Anon, 2003; Kothari *et al.,* 2009).

An ethylene biosynthesis-related gene named as E8 was first dealt and cloned by Lincoln with collaboration of many other colleagues from freshly harvested tomato (*Solanum lycopersicum* cv. VFNT Cherry) (Broglie *et al*., 1986; Holdsworth *et al*., 1987; Lincoln *et al*., 1987; McGarvey *et al*., 1992; Zhao *et al*., 2009). Ethylene is induced first and the transcription of the *E8* gene was activated at the beginning of fruit setting and ripening. With regard of E8 expression which exhibited aberrant behavior as spatially and temporally regulated in growing fruits of tomatoes (Deikman and Fischer, 1988). Smith *et al*. (1990) and Wang *et al*. (2000) demonstrated that fruit ripening is a complex and genetically programmed process which directly and indirectly related to increases in respiration and ethylene production in many parts of growing tissues and hence resulting into changes in color, flavor, and softening. Fischer and Bennett (1991); and Ahmed *et al*. (2011) reviewed the phenomenon of fruit

softening which is associated with structural changes in the construction of cell wall, including reduction in the size of polysaccharides hemicellulose, loss of galactose side-chains, and solubilization and depolymerization of pectin by the influence of one of the hydrolytic enzymes, polygalacturonase (PG).

With regard of cytoplasmic male sterility (CMS) in sweet peppers which is actually a parental and maternally inherited trait that does not allow the fruits to produce functional pollen. Schnable and Wise (1998) identified that the first candidate gene associated with the CMS trait in the mitochondrial genome of sweet pepper. Some years later, Kim *et al*. (2007) reported that *orf456* is a strong candidate gene for determining the male-sterile phenotype of CMS in chili pepper.

One of the paramount plant hormones named as cytokinin was first noted as able to promote plant cell division by (Miller *et al*., 1955; Guivarch *et al*., 2002). It is therefore considered important to check the function of cyokinins related genes like isopentenyl trnasferase (*ipt)* and one approach to investigating the function of cytokinins is the generation of transgenic plants overproducing cytokinins by expressing the Agrobacterium T-DNA-derived *ipt* gene, which encodes an isopentenyl transferase that catalyzes the rate-limiting step of cytokinin biosynthesis (Akiyoshi *et al*., 1984; Barry *et al*., 1984).

In the current time crop improvement by means of genetic engineering has become the most authentic, efficient and robust approach for the production of higher and preserved food yield (Kahl and Winter, 1995). Among many choices of gene transformation *Agrobacterium-mediated transformation* has become most outstanding and favorite choice for the researchers to establish control over different genes.

The present studies aimed at establishing efficient protocol for constructing different cloning and expression vectors possessing some vital cloned DNA like *CaPG*, *orf456*, *ipt* and fruit promoters like E8 in a novel expression vector, pVBG2307, which may help to establish control over all of them.

MATERIALS AND METHODS

Plant material

Freshly harvested fruits at different stages of Tomato (*Lycopersicon esculentum*) and sweet pepper (*Capsicum annum*) were collected with care of approximately uniform size and color and free from foreign contamination and under gone for total RNA extraction followed by the construction of *cDNA* libraries.

Binary cloning and expression vectors

Various grades of binary and cloning expression vectors named as pBI121 and pBI221 (Chen *et al*. 2003) were manipulated into the cloning vector pCAMBIA2300 (Xu *et al*. 2010) towards developing the novel vector pVBG2307. Initially it was evident from the genomic map of cloning vector pBI221 that elements like *GFP* and *GUS* reporter genes present along with ampicillin resistance gene, furthermore it also possesses different range of restriction endonucleases like Apa*L*I, Kpn*I*, Pst*I*, Sac*I*, Sca*I* and Xba*I* loci at its cloning site. On the other hand, the cloning vector pBI121 is devoid of these elements. Precursor vector pCAMBIA2300 was used to equip with many vital elements like CaMV35S and NOS terminal, which it did not possess initially.

cDNA libraries

It is proven over a significant period of time that the cloning of functional genes must be cloned from the *cDNA* libraries of relevant organisms to investigate their functional characteristics as more number of transcripts may obtain from such experiments, therefore fruit pericarp of capsicum and tomato were thoroughly ground in liquid nitrogen at -80 and total RNA was extracted by the protocol mentioned in the manufacturer kits. Extraction of total RNA was carried by the help of an Invisorb-Spin Plant-RNA Mini kit (Invitek, Berlin, Germany). All the extracted RNAs were added with RNase-free DNase-I (Xue and Loveridge, 2004) to remove possible contaminating DNA and other unwanted proteins. All the samples originated at different growth stages were reverse-transcribed by using oligo (dT) 18 primers

and a RevertAid First Strand cDNA Synthesis kit protocol mentioned by the manufacturer. Possible *cDNA* libraries of different samples were obtained using the M-MLV reverse transcriptase kit (Promega, Madison,WI, USA). All the samples were reverse-transcribed in situ by adding a real-time mix to give a total volume of 20µL. Each tube contained 4.5µL (1µg) RNA and 0.5 µg/µL oligo (dT) 18 primer. Reverse transcription proceeded for 60 min at 42°C before the reaction was terminated by heating to 72°C for 10 min.

Amplification of *CaPG*

Cloning of *CaPG* gene was carried from capsicum *cDNA* library in both forward (5'-3') and reverse (5'-3') directions by the help of specially designed primers including appropriate restriction enzyme cutting sites of Sma*I* and Xba*I* (Table 1). Constituent composition PCR system of reaction mixture was approximately 25 µL (pepper *cDNA* template, 0.5 µL; 10× PCR buffer, 2.5 µL; 5 U/µL *Taq* DNA polymerase, 0.2 µL; 10 mM dNTP mixture, 0.5 µL; ddH_2O, 19.3 µL; and PG-F and PG-R, 1 µL each). Amplification consisted of 30 cycles of 45 s at 94°C (denaturation), 60 s at 54°C (primer annealing) and 120 s at 72°C (extension) (Giorno *et al*., 2010). The optical density of the electrophoretic bands of *CaPG* genes were determined with a gel-imaging analysis system and photo spectrometry where, the formula for DNA concentration (ng/µL) = 50 x dilution factor x OD (Syngene, Cambridge, U.K.). All the treatments were replicated 3 times and the primer sequences of the genes and their expected amplified product or open reading frame (orf) size are given in Table 1.

Amplification of *orf456*

Cloning of *orf456* gene was carried from both capsicum and tomato *cDNA* libraries by the help of specially designed primers including appropriate restriction enzyme cutting sites of Kpn*I* and Sac*I* (Table 1). Constituent composition PCR system of reaction mixture was approximately 25 µL (pepper and tomato *cDNA* template, 0.5 µL; 10× PCR buffer, 2.5 µL; 5 U/µL *Taq* DNA polymerase, 0.2 µL; 10 mM dNTP mixture, 0.5 µL; ddH_2O, 19.3 µL; and PG-F and PG-R, 1 µL each). Amplification consisted of 30 cycles of 45 s at 94°C (denaturation), 60 s at 54°C (primer annealing) and 120 s at 72°C (extension) (Giorno *et al*., 2010). The optical density of the electrophoretic bands of *orf456* gene was determined with a gel-imaging analysis system and photo spectrometry where, the formula for DNA concentration (ng/µL) = 50 x dilution factor x OD (Syngene, Cambridge, U.K.). All the treatments were replicated 3 times and the primer sequences of the genes and their expected amplified product or open reading frame (orf) size are given in Table 1.

Table 1. Primer sequences of the genes used for PCR amplification along with expected amplified bands

Gene	Primer	Primer sequence	Amplified size (bp)
CaPG-F	CaPG-F1 CaPG-R1	5'-CTAGTCTAGAATTATCATGTCTATCCAAAAGATTA-3' 5'-TCCCCCGGGCACCACATTTTTCACTTTAACT-3'	1143
CaPg-R	CaPG-F2 CaPG-R2	5'-TCCCCCGGGATTATCATGTCTATCCAAAAGATTA-3' 5'-CTAGTCTAGACACCACATTTTTCACTTTAACT-3'	1143
E8	E8-F E8-R	5'-GACCTTCTTTTGCACTGTGAATGATT-3' 5'-CTAGAAGGAATTTCACGAAATCGGC-3'	1074
Orf456	Orf-F Orf-R	5'-ATGCCCAAAAGTCCCATGTAT-3' 5'-TTACTCGGTTGCTCCATTGT-3'	450
ipt	Ipt-F Ipt-R	5'-ATGGATCTTAGACTTATTTTTGGAC-3' 5'-CTAATACATTCCAAATGGATGTCC-3'	750

Amplification of *ipt*

Cloning of *ipt* gene was carried from both capsicum and tomato *cDNA* libraries by the help of specially designed primers including appropriate restriction enzyme cutting sites of BamH*I* and *SacI* (Table 1). Constituent composition PCR system of reaction mixture was approximately 25 µL (pepper and tomato *cDNA* template, 0.5 µL; 10× PCR buffer, 2.5 µL; 5 U/µL *Taq* DNA polymerase, 0.2 µL; 10 mM dNTP mixture, 0.5 µL; ddH_2O, 19.3 µL; and PG-F and PG-R, 1 µL each). Amplification consisted of 30 cycles of 45 s at 94°C (denaturation), 60 s at 54°C (primer annealing) and 120 s at 72°C (extension) (Giorno *et al*., 2010). The optical density of the electrophoretic bands of *ipt* gene was determined with a gel-imaging analysis system and photo spectrometry where, the formula for DNA concentration (ng/µL) = 50 x dilution factor x OD (Syngene, Cambridge, U.K.). All the treatments were replicated 3 times and the primer sequences of the genes and their expected amplified product or open reading frame (orf) size are given in Table 1.

Amplification of *E8* promoter

Primarily, total concentration of extracted tomato genomic *cDNA* was adjusted to 20 ng/µL for the PCR template, and PCR was then performed using *ExTaq* DNA polymerase (Takara, Japan). Constituent composition PCR system of reaction mixture was approximately 25 µL containing 2µL capsicum and tomato *cDNA* templates, 2.5µL 10× PCR buffer (100 mM Tris-HCl buffer, 500 mM KCl, 0.01% gelatin), 1.5µL 25 mM $MgCl_2$, 1.5µL 2.5 mM dNTPs (Takara, Japan), 1µL PCR primers (*E8*-F and *E8*-R, 10 µM), 0.3µL *ExTaq* DNA polymerase (5 U/µL) and 15.2µL sterilized ddH_2O. The program was initiated with a hot start at 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 54°C for 45 s and 72°C for 1.5 min, and finally 72°C for 8 min. PCR products were electrophoresed on a 2.0% (w/v) agarose gel with ethidium bromide (EB) in 1× TAE buffer and photographs were taken under ultraviolet light. All the treatments were replicated 3 times and the primer sequences of the genes and their expected amplified product or open reading frame (orf) size are given in Table 1.

Cloning and sequencing of *CaPG-F, CaPG-R, orf456* and *ipt* genes and *E8* promoter

PCR products of all the mentioned nucleic acids were purified periodically using a gel extraction kit (Watson Biotechnologies, Inc, Shanghai, China) and linked into pGEM-T Easy (Promega, Madison, WI, USA) and sequenced with an ABI Sequencer 3700 (Shanghai, China). Sequencing results were analyzed by the help of software BioEdit version 5.0.6 and the correct clones were subsequently transformed into *E. coli* by means of an improved freezing and thawing protocol as described by (Deikman *et al*., 1998; Xu *et al*., 2005).

pVBG2307 and other allied vectors

Many plasmid constructs possessing *CaPG, orf456* and *ipt* coding sequences controlled under the influence of CaMV35S and *E8* promoters were constructed at different times and validated accordingly. Initially, CaMV35S segment was cleaved from vector pBI121 by the digestion of Hind*III* and Xba*I* and ligated into vector pCAMBIA2300 (Xu *et al*. 2010), the resulted plasmid was confirmed by sequencing and enzyme digestion (Figure 1).

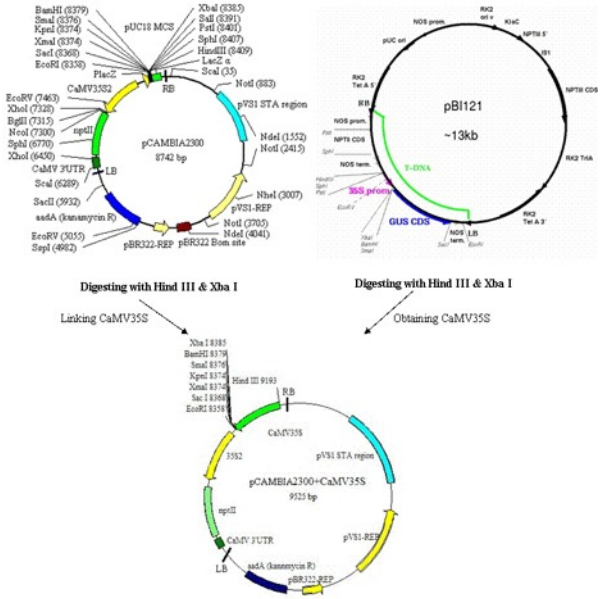

Figure 1: Cloning vector pCAMBIA2300 used as a precursor of expression vector pVBG2307, as the cleaved CaMV35S promoter from pBI121was linked using endonucleases named as Hind*III* and Xba*I*.

Precursor vector pCABMIA2300 was also incorporated with NOS terminal on its right border as it was missing in its earlier appearance, a foreign NOS terminal was cleaved from cloning vector pBI221 with the digestion of EcoR*I* and Sac*I*. Ultimately the resultant plasmid was validated by sequencing and endonucleases digestion and the name was given as pVBG2307 (Plant Vegetable Breeding and Genetics) (Figures 2 and 3). Many other allied vectors were developed and validated, pVBG2307+PG-F, pVBG2307+PG-R, pVBG2307+E8+PG-F and pVBG2307+E8+PG-R (Figures 4-7), all of these vectors were further incorporated with *E8* promoter (which alter the choice of CaMV35S and offers broader expression of genes at particular fruit development stages of capsicum and tomato) and *CaPG* gene fragments from pGEM-T into pBluescript *II* SK and then pCAMBIA2300 by using different endonucleases like EcoR*I*, Pst*I*, Sal*I*, Sma*I* and Xba*I* sites following the downstream pathway of different appropriate restriction enzymes. Many functional plasmids possessing *orf456* and *ipt* genes were also developed by the digestion of Kpn*I*, Sac*I*, and BamH*I* enzymes (figures not provided). On each step all the plasmids were confirmed and validated by performing PCR, sequencing and restriction endonuclease digestion.

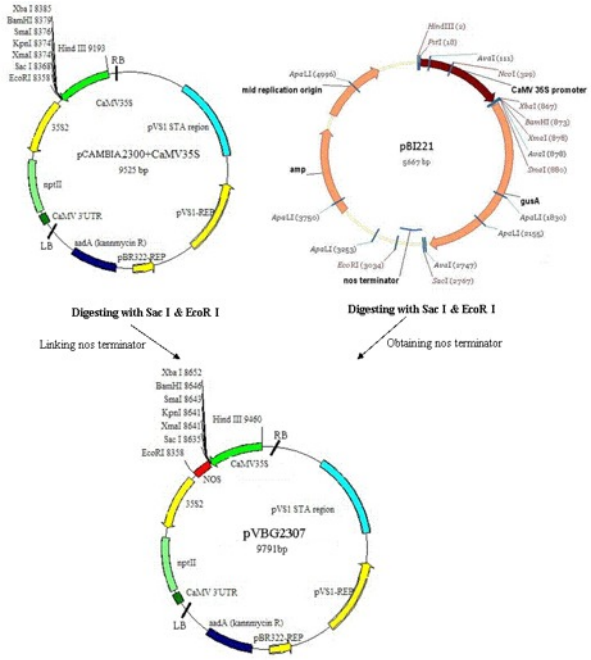

Figure 2: Final construct of Expression vector pVBG2307 was obtained by adding translation stop codon named as NOS terminal from pBI221 by the help of Sac*I* and Eco*RI* endonucleases

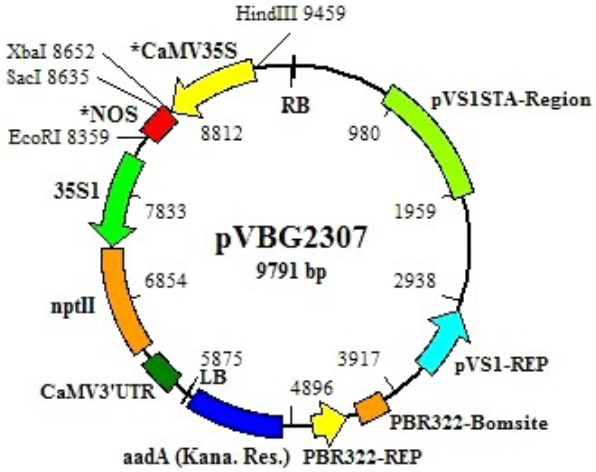

Figure 3: Final construct of Expression vector pVBG2307.

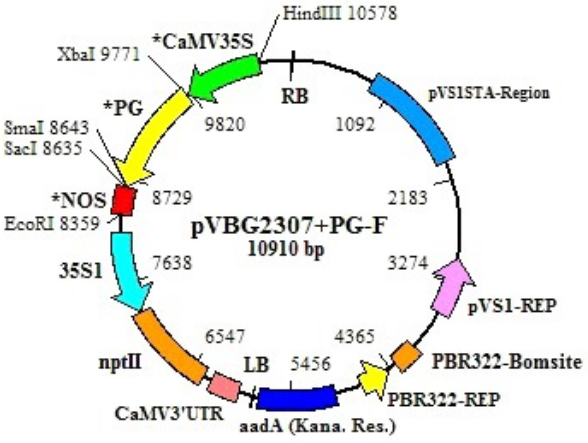

Figure 4: Successful linkage of *Ca*PG gene in forward orientation with vector pVBG2307.

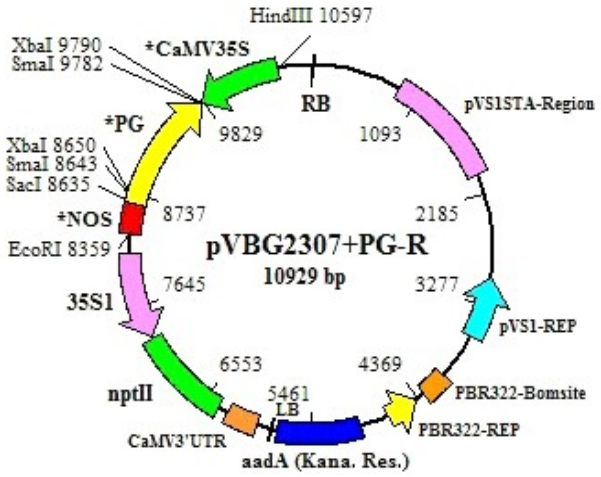

Figure 5: Successful linkage of *Ca*PG gene in reverse orientation with vector pVBG2307.

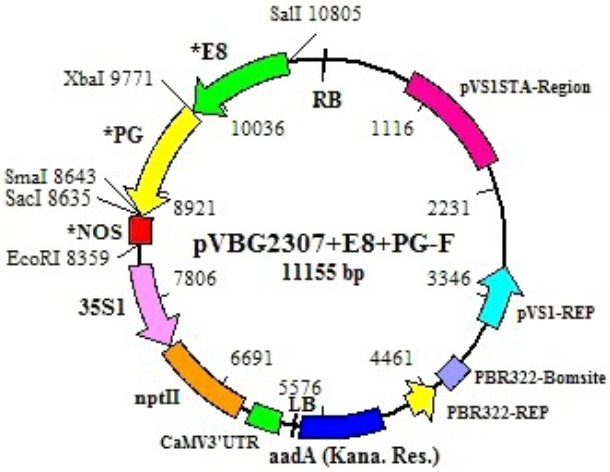

Figure 6: Replacing CaMV35S promoter with ethylene inducing promoter named as E8 with the final construct possessing *CaPG* gene in forward orientation.

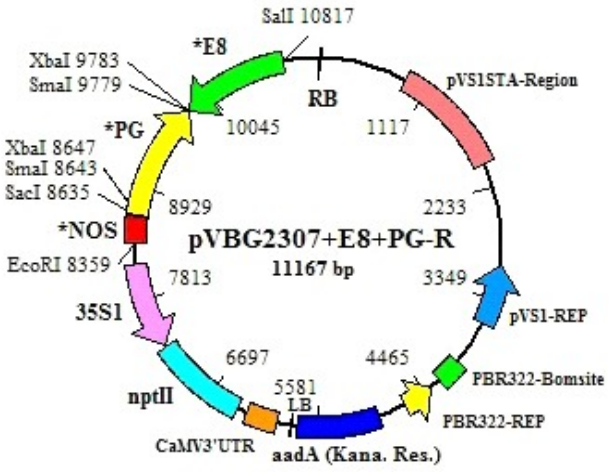

Figure 7: Replacing CaMV35S promoter with ethylene inducing promoter named as E8 with the final construct possessing *CaPG* gene in reverse orientation.

RESULTS AND DISCUSSION

Amplification of *CaPG*, *orf456*, *ipt* genes and *E8* promoter

After polymerase chain reactions of *orf456* gene was separated on agarose gel and the PCR product of the *orf456* gene of approximately 0.45 kb was (Kim *et al*., 2007) obtained and then cloned into pGEM-T Easy (Promega, Madison, WI, USA). After recycling and sequencing analysis *orf456* gene cassette was inserted into pVBG2307, and the resultant plasmid was validated by performing PCR and endonucleases digestion (Figure 8; PIC. 5).

After polymerase chain reactions of *E8* promoter was separated on agarose gel and the PCR product of the *E8* gene of approximately 1.07 kb was (Zhao *et al*. 2009) obtained and then cloned into pGEM-T Easy (Promega, Madison, WI, USA). After recycling and sequencing analysis *E8* gene cassette was inserted into pBluescript *II* SK and pVBG2307, and the resultant plasmid was validated by performing PCR and endonucleases digestion (Figure 8; PIC. 3).

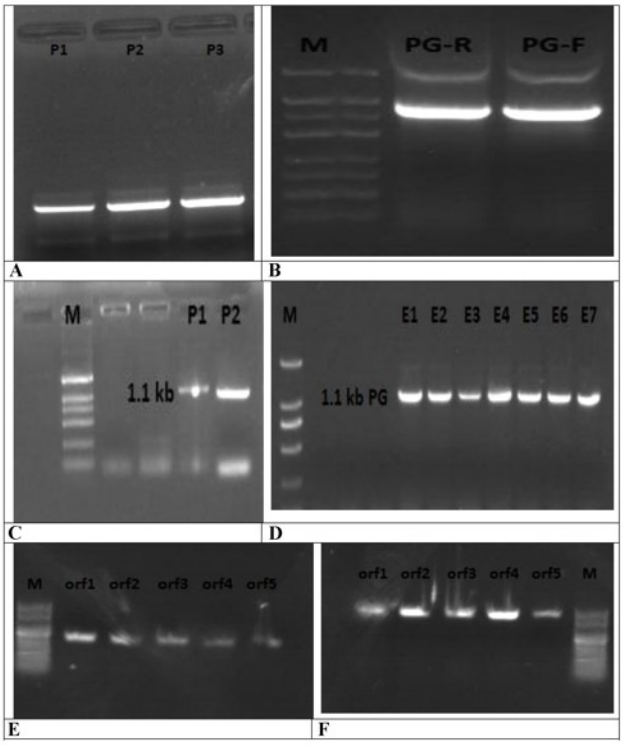

Figure 8: Picture **A.** and **B.** PCR product of the PG gene. **C.** PCR product of the E8 promoter. **D.** Amplified *PG-F* + pVBG2307 in EHA105-4. **E.** PCR of the *orf456* gene. **F.** Amplified *orf456* + pVBG2307 from EHA105-4.

After polymerase chain reactions of *CaPG* genes in both directions were separated on agarose gel and the PCR product of the *CaPG* gene of approximately 1.1 kb was (Chen *et al*., 2010) obtained at ripening stages of pepper (Figure 5; PIC. 1 & 2) and latterly cloned into pGEM-T Easy (Promega, Madison, WI, USA). After recycling and sequencing analysis *CaPG* gene cassette was inserted into pVBG2307, and the resultant plasmid was validated by performing PCR and endonucleases digestion (Figure 9; PIC. 1).

After polymerase chain reactions of *ipt* gene was separated on agarose gel and the PCR product of the *ipt* gene of approximately 0.75 kb was (Guivarch *et al*., 2002) obtained and then cloned into pGEM-T Easy (Promega, Madison, WI, USA). After recycling and sequencing analysis *orf456* gene cassette was inserted into pVBG2307, and the resultant plasmid was validated by performing PCR and endonucleases digestion (Figure 9; PIC. 5).

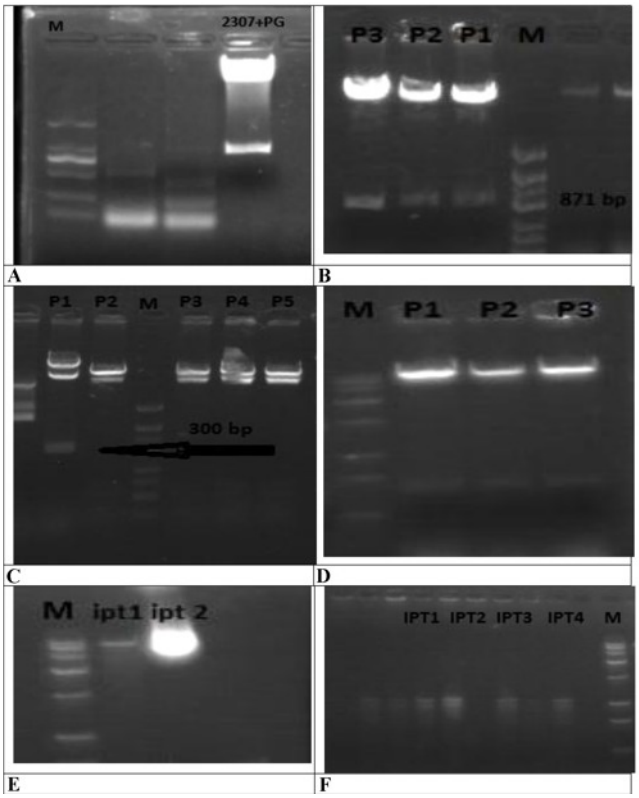

Figure 9: Picture **A.** Confirmation of *PG* ligation in pVBG2307 with the digestion of enzymes *Sma*I and *Xba*I. **B.** Confirmation of ligated CaMV35S from pVBG2307 by double digestion with *Hin*dIII and *Xba*I. **C.** Confirmation of ligated NOS terminal from pVBG2307. **D.** Cleavage of CaMV35S from pBI121 with *Hin*dIII and *Xba*I. **E.** PCR product of the *ipt* gene. **F.** Amplified *ipt* + pVBG2307 from EHA105-4.

CaMV35S linkage
CaMV35S (Tanaka *et al*. 2011) was cleaved from cloning vector pBI121 by the digestion of endonucleases designated as Hind*III* and Xba*I* (Figure 6; PIC. 4) later it was ligated into cloning plasmid pCAMBIA2300 (Figure 9; PIC. 1).

NOS linkage
NOS terminal (Ishikawa *et al*. 2011) was absent on the right border of pCAMBIA2300 and therefore it was cleaved from cloning vector pBI221 by the digestion of EcoR*I* and Sac*I* endonuclease later it was ligated into pCAMBIA2300 (Figure 9; PIC. 3).

pVBG2307 Validation
On the basis of DNA cloning with ample number of polymerase reactions with appropriate primers as well as double and sequential digestion by the action of respective endonuclease proved that the utility of vector pVBG2307 is enormous and can be used for multiple choice of endonucleases at its cloning site. We have demonstrated successful cloning of various nucleic acids like promoterless *CaPG, orf456, ipt* and *E8* from pGEM-T and pBluescript *II* SK downstream of the imported CaMV35S promoter element among the BamH*I*, Kpn*I*, Sac*I*, Sma*I* and Xba*I* sites of vector pVBG2307. Moreover we have also developed certain allied vectors on the basis of precursor pCAMBIA2300 by incorporating mentioned molecular chaperons and these are termed pVBG2307+*PG-F*, pVBG2307+*PG-R*, pVBG2307+*E8*+*PG-F*, pVBG2307+*E8*+*PG-R*, pVBG2307+*orf456* and pVBG2307+*ipt*. Protocol of improved freezing and thawing method as elaborated by Chen *et al*. (2003) and Xu *et al*. (2005) was used for successful regulation and transformation of these plasmids into *Escherichia coli* and *Agrobacterium tumefaciens* strain EHA105-4. On the basis of PCR and restriction endonuclease digestion experiments we came to know that pVBG2307 not only employ for successful cloning of genes but also used for their transcription and translation in many organisms and we have demonstrated these results in *Escherichia coli* and *Agrobacterium tumefaciens* strain EHA105-4 (Sambrook and Russell, 2000); (Figure 9; PIC. 1; PIC. 2; PIC. 3; PIC. 4; PIC. 6 and Figure 8; PIC. 4; PIC. 6). Additionally expression vector pVBG2307 offers more number of recognition sites in the MCS for 13 different endonucleases to allow higher manipulation of nucleic acid fragments for cloning and expression. Approaches like direct analysis of genes such as *CaPG, orf456* and *ipt* and *E8* promoter without the need for additional sub-cloning in *Escherichia coli* and *Agrobacterium tumefaciens* strain EHA105-4 (Sambrook and Russell, 2000) provide an excellent example of its significance. Similar kind of approach for gene validation analysis in usefulness of plasmids were demonstrated by Watson *et al*. (1996) and Chen *et al*. (2003. Such attempts for gene cloning and construction of novel plasmids leads to successful and efficient gene transcription and translation like Agrobacterium-mediated transformation in many interested organisms.

REFERENCES

- Ahmed SS, Gong ZH, Khan MA, Yin YX, Guo WL and Imran J. (2011). Activity and expression of polygalacturonase vary at different fruit ripening stages of sweet pepper cultivars. Genet. Mol. Res. 10 (4): 3275-3290.
- Akiyoshi DE, Klee H, Amasino RM, Nester EW and Gordon MP. (1984). T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. PNAS, USA. 81: 5994-5998.
- AVRDC, 2005. The world vegetable centre.www.avrdc.org/pdf/brief-poverty.pdf
- Anonymous. (2003). The production and post-harvest handling of sweet pepper in Kenya (HCDA).
- Barry GF, Rogers SG, Flarley RT and Brand L. (1984). Identification of a cloned cytokinin biosynthetic gene. PNAS, USA. 81: 4776-4780.

• Broglie KE, Gaynort JJ and Broglie RM. (1986). Ethylene-regulated gene expression: molecular cloning of the genes encoding an endochitinase from *Phaseolus vulgaris*; Proc. Natl. Acad. Sci. USA. 83: 6820–6824.

• Chen PY, Wang CK, Soong SC and Ying K. (2003). Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Molecular Breeding 11 (4): 287-293.

• Chen RG, Yang RP, Gong ZH, Li DW and Ahmed SS. (2010). Cloning and Sequence Analysis of the Polygalacturonase Gene *CaPG* in Pepper. Acta Botanica Boreal-Occidentalia Sinica. 30: 1941-1945.

• Deikman J and Fischer RL. (1988). Interaction of a DNA binding factor with the 5'-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J. 7: 3315–3320.

• Deikman J, Xu R, Kheissl ML, Ciardi JA, Kim KN and Pelah D. (1998). Separation of *cis* elements responsive to ethylene, fruit development, and ripening in the 5´-flanking region of the ripening-related *E8* gene. J. Plant Mol. Biol. 37: 1001–1011.

• Fischer RL and Bennett AB. (1991). Role of cell wall hydrolases in fruit ripening. Annu. Rev. Plant Physiol. 42: 675-703.

• Giorno F, Wolters AM, Grillo S, Scharf KD, Vriezen WH and Mariani C. (2010). Developmental and heat stress-regulated expression of HsfA2 and small heat shock proteins in tomato anthers. J. Exp. Bot. 61: 453-462.

• Guivarch A, Rembur J, Goetz M, Roistsch T, Noin M, Schmulling T and Chriqui D. (2002). Local expression of the *ipt* gene in transgenic tobacco (*Nicotiana tabacum* L. cv. SRI) axillary buds establishes a role for cytokinins in tuberization and sink formation. J. Exp. Bot. 53 (369): 621-629.

• Holdsworth MJ, Bird CR, Ray J, Schuch W and Grierson D. (1987). Structure and expression of an ethylene-related mRNA from tomato. Nucleic Acids Res. 15: 731–739.

• Ishikawa M, Murata T, Sato Y, Nishiyama T, Hiwatashi Y, Imai A, Kimura M, Sugimoto N, Akita A, Oguri Y, Friedman WE, Hasebe M and Kubo M. (2011). Physcomitrella cyclin-Dependent Kinase A links Cell cycle Reactivation to other Cellular Changes during Reprogramming of Leaf Cells. *Plant Cell.* 23 (8): 2924-2938.

• Kahl G and Winter P. (1995). Plant genetic engineering for crop improvement. World J. Microbiol. Biotech. 11: 449-460.

• Kim DH, Kang JG and Kim BD. (2007). Isolation and characterization of the cytoplasmic male sterility associated orf456 gene of chili pepper (*Capsicum annuum* L.). Plant Mol. Biol. 63: 519-532.

• Kothari SL, Joshi A, Kachhwaha S and Ochoa Alejo N. (2009). Chili peppers – A review on tissue culture and transgenesis. J. Biotechnol Adv.

• Lincoln JE, Cordes S, Read E and Fischer RL. (1987). Regulation of gene expression by ethylene during *Lycopersicon esculentum* (tomato) fruit ripening. PNAS, USA. 84: 2793–2797.

• McGarvey DJ, Sirevag R and Christoffersen RE. (1992). Ripening related gene from avocado fruit: ethylene-inducible expression of the mRNA and polypeptide. Plant Physiol. 98: 554–559.

• Miller CO, Skoog F, Okamura FS, Von-Salza MH and Strong FM. (1955). Isolation, structure and synthesis of kinetin, a substance promoting cell division. J. American Chem. Soc. 78: 1375-1380.

• Pruti J and Sharma RP. (1998). Major spices of India crop management and

post harvest technology. Chapter IV: Chilies or Capsicum. Indian council of agricultural research publications and information division.

- Schnable PS and Wise RP. (1998). The molecular basis of cytoplasmic male sterility and fertility restoration. Trends Plant Sci. 3: 175–180.

- Sambrook J and Russell DW. (2000). Molecular Cloning: A Laboratory Manual Third Edition. CSHL Press, NY. USA.

- Smith CJS, Watson CF, Bird CR, Ray J, Schuch W and Grierson D. (1990). Expression of a truncated tomato polygalacturonase gene inhibits expression of the endogenous gene in transgenic plants. Mol. Gen. Genet. 224: 477-481.

- Tanaka Y, Nakamura S, Kawamukai M, Koizumi N and Nakagawa T. (2011). Development of a series of gateway binary Vectors possessing a tunicamycin resistance gene as a marker for the transformation of *Arabidopsis thaliana*. Biosci. Biotechnol. Biochem. 75 (4): 804-807.

- Wang ZY, MacRae EA, Wright MA, Bolitho KM, Ross G.S. and Atkinson RG. (2000). Polygalacturonase gene expression in kiwifruit: relationship to fruit softening and ethylene production. Plant Mol. Biol. 42: 317-328.

- Watson AA, Alm R.A. and Mattick JS. (1996). Construction of improved vectors for protein production in *Pseudomonas aeruginosa*. Gene, 172: 163-164.

- Xue GP and Loveridge CW. (2004). HvDRF1 is involved in abscisic acid-mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. Plant J., 37: 326–339.

- Xu WR, Wang YJ, Wang XP, Hao W and Sun M. (2005). Construction of the Plant Expression Vectors Carrying Resistant Genes to Powdery Mildew and Adversities in Wild Species of *Vitis* in China. Acta Bot. Boreal.-Occident . Sin. 25 (5): 851—857.

- Xu G, Sui N, Tang Y, Xie K, Lai Y and Liu Y. (2010). One-step, Zero-background ligation-independent cloning intro-containing hairpin RNA constructs for RNAi in plants. *New Phytol.* 187 (1): 240-250.

- Zhao LX, Lu L, Zhang L, Wang A, Wang N, Liang Z, Lu X and Tang K. (2009). Molecular evolution of the E8 promoter in tomato and some of its relative wild species. J. Biosci. 34: 71-83.