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Constructional and Functional Evaluation of Two New Plant Expression Vectors—pBI121^{gus-6} and pBI121^{5+1}

DOI: 10.7904/2068–4738–IX(17)–60

Reza MOHAMMADHASSAN[1], Kasra ESFAHANI[2]*, Bahareh KASHEFI[3]

[1]*College of Agriculture, Damghan Branch, Islamic Azad University, Damghan, IRAN*
[2]*National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, IRAN*
[3]*College of Agriculture, Damghan Branch, Islamic Azad University, Damghan, IRAN*
Corresponding Author: kasra13@gmail.com, Phone: +982144787451

Abstract. Binary vectors are widely used in Agrobacterium gene transfer in plants. Although new plant expression vectors have been designed, pBI121–based vectors are still more common. The availability of fewer numbers of restriction enzyme sites is the most important drawback in pBI121. In this article, two new vectors–based on pB121, pBI121^{5+1}, and pBI121^{GUS-6}– were introduced. The construction of new vectors was confirmed by PCR and digestion pattern analysis. Furthermore, the reporter gene (*gus*) was cloned in these vectors. The T–DNA transformation ability of the new vectors and pBI121 (as the control sample) to tobacco via *Agrobacterium tumefaciens* strain LBA4404 was evaluated. Transgenic plants were regenerated with BAP and NAA in a selective medium. Thereafter, plant DNA was extracted and successful gene transformation was confirmed by PCR analysis. The GUS assay also confirmed gene expression in transgenic leaves. The results indicate that the vectors are totally efficient in plant transformation.

Keyword: Agrobacterium–mediated transformation, binary vector, Plant expression vector, pBI121, pBI121^{5+1}, pBI121^{GUS-6}.

Introduction

Binary vectors are widely used in gene transformation into plants via the Agrobacterium–mediated transformation method [GELVIN, 2003]. The genetic manipulation facility of binary vectors leads to pervasive usage [LEE and GELVIN, 2008]. Furthermore, the possibility of separating transferred region (T–DNA) into the plant genome, genetic factors of the transfer (*vir* genes), and placing them on separated replicons were discovered.

This turned out to be the key point in designing the vectors. The discovery led to a reduction in vectors' size, and the gene transformation into the plant was facilitated [MOHAMMADHASSAN *et al*., 2014]. In several studies, many binary vectors were introduced [KARIMI *et al*., 2013; SRIPRIYA *et al*., 2011; NAKAMURA *et al*., 2010]. Additionally, the features and benefits of these new vectors–including the high copy number in *E. coli*, the possibility of cloning long DNA fragments and the improved compatibility with different strains of *A. tumefaciens* were mentioned [KARIMI *et al*., 2013]. Despite great achievements of several new and improved constructions of vectors, some well–known vectors are still widely used. One of the classic vectors is pBI121, which is derived from pBIN19 [JEFFERSON, 1987]. This vector can replicate in *A. tumefaciens* and *E. coli* [LIM *et al.*, 1999].

Immediately after its introduction, pBI121 became popular among molecular biologists. According to studies, pBI121 has been used in 40 % of 180 articles on Agrobacterium–mediated transformations [KOMORI *et al*., 2007]. The size of the vector and its T–DNA region are 14 758 bp and 6 193 bp, respectively [LEE *et al*., 2013].

The T–DNA region of pBI121 includes left and right borders and the expression cassette of neomycin phosphotransferase II (*npt*II) as selectable markers. Multiple cloning sites contain *CaMV*35S promoter, recognition sites of few restriction enzymes, β–glucuronidase gene as a reporter gene, and NOS terminator [CHEN *et al*., 2003]. Although pBI121 is widely used and popular, there are some deficiencies in it, including less numbers of restriction enzyme sites in the downstream of *CaMV*35S promoter (*Xba*I,

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*Bam*HI and *Sma*I) and existence of a mere restriction enzyme site (*Sac*I) in the upstream of NOS terminator [KOMORI *et al.*, 2007]. In the article, two new binary vectors are introduced, which contain two more restriction enzyme sites (*Kpn*I and *Xho*I) as compared to pBI121 in their multiple cloning sites. The accuracy of vector construction, cloning, the ability of gene transformation, and expression of a reporter gene (*gus*) were evaluated.

Material and methods

Cloning Site Fragment Design. The restriction enzymes, the recognition sites of which are not available on the backbone of pBI121, were recognized by the MapDraw software (DNASTAR software series) and NEB cutter V2.0 online software (http://tools.neb.com/NEBcutter2).

According to the price and availability of the enzymes, two enzymes (*Kpn*I and *Xho*I)–the recognition sites of which are not present on the backbone of the vector–were chosen.

The arrangement of the enzyme recognition site sequences have been provided in Figure 1.

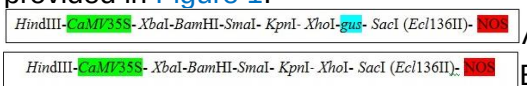

Figure 1. Restriction enzymes recognition sites in designed fragments; A) multiple cloning site of pBI121^{5+1}, B) multiple cloning site of pBI121 $^{GUS-6}$

pBI121^{5+1} Vector Construction. Two new restriction enzyme sites were added by PCR. UP35S and DOWN35S primers were designed and synthesized by TAG Copenhagen. The UP35S primer was designed to anneal with upstream sequence of *CaMV*35S.

The sequence of DOWN35S primer includes multiple cloning sites of pBI121 (from downstream of *CaMV*35S to upstream of gus) and the sequence of three recognition sites of *Kpn*I, *Xho*I, and *Sac*I (*Ecl136*II) enzymes. Sequences of the primers are mentioned in Table 1.

Table 1.

Names and Sequences of Used Primers

No.	Noun	Sequence (5'–3')	Nucleotides
1	UP35SF*	CCAGGCTTTACACTTTATGC	20
2	DOWN35S**	CGAGCTCGAGGTACCCGGGGATCCTCTAGAGTCC	34
3	35S–seqF*	GCACAATCCCACTATCCTTC	20
4	GUSF*	GGTGGTCAGTCCCTTATGTTACG	23
5	GUSR**	CCGGCATAGTTAAAGAAATCATG	23
6	VirGF*	ATGATTGTACATCCTTCACG	20
7	VirGR**	TGCTGTTTTTATCAGTTGAG	20
8	RENOS**	CCAGTGAATTCCCGATCTAGTAAC	24

* Forward, **Reverse

The PCR product and pBI121 were respectively digested by *Hind*III and *Ecl136*II, and *Hind*III and *Sma*I. Each digested PCR fragment separately replaced the corresponding part of T–DNA of pBI121 and the upstream of β–glucuronidase gene.

Different stages of cloning and transformation of *E. coli* competent cells and DH5α strain were performed by standard methods [SAMBROOK and RUSSELL, 2001].

E. coli grown colonies on selective medium (kanamycin 50 mg/L) were evaluated by colony PCR, and the new vector was extracted by plasmid extraction kit (high pure plasmid isolation kit, Roche, German). The PCR and digestion pattern analysis were evaluated by the construction of the vector.

pBI121^{GUS-6} Vector Construction. The DOWN35S2 primer contains the sequence of multiple cloning site of pBI121 (between promoter and *gus* gene) and recognition sites of *Kpn*I, *Xho*I, and *Sac*I enzymes at its 5' overhang end (Table 1).

The PCR product was amplified by using UP35S and Down35S primers. Further, the PCR fragment and pBI121 were digested by *Hind*III and *Sac*I. The digested PCR segment (only promoter and new multiple cloning sites) was then replaced with promoter and β–glucuronidase gene of pBI121.

In the following, the possibility of cloning a reporter gene in pBI121^{GUS-6} and pBI121^{5+1} was evaluated. β–glucuronidase gene was isolated from pBI121 by *XbaI* and *Sac*I in order to clone in both vectors. Cloning was confirmed by PCR and digestion pattern analysis.

Agrobacterium Transformation. The vectors were transformed to *A. tumefaciens* strain LBA4404 by the freezing and thawing method [HOLSTERS *et al*., 1978]. First, the recombinant vectors were extracted from *E. coli* cells (DH5α strain).

The colonies appeared after 48 h of transferring the plasmids to *A. tumefaciens*. In addition to the recombinant vectors, pBI121 was transformed to *A. tumefaciens* by the same method. The transformed colonies were recognized by colony PCR with 35S–seqF and GUSR primers.

Plant Transformation and Regeneration. *Nicotiana tobaccum* L. cv. Samsun was transformed by using tobacco transformation optimized method [GALLOIS and MARINHO 1996]. First, the recombinant bacteria were incubated in LB media at 28 ºC for 12–18 h, which contain 50 mg/L Kanamycin and 300 mg/L Rifampicin at 150 rpm to OD=0.5.

The incubated bacteria were centrifuged at 4 ºC and 3 000 rpm for 10 min. The supernatant was removed and 5–10 mL of inoculation medium (MS solution, 50 g/L sucrose, pH=5.5) was added. The 1×1 slices of tobacco leaves were shacked gently for a minute in this medium. The infected leaves were dried on filter papers and cultured on MS media, which include proper hormones and antibiotics. The Petri dishes were put under 25 ºC and in darkness for 48 h.

The transformed leaves were then washed twice in the 500 mg/L cefotaxime solution. They were cultured on regeneration and selection medium, which contains MS with 20 g/L sucrose, 2 mg/L BAP, 100 µg/L NAA, 50 mg/L kanamycin, and 300 mg/L cefotaxime (pH=5.8). The cultured samples were regenerated at 25 ºC and a photoperiod of 16 h light and 8 h darkness was conducted. After two months, the regenerated plantlets were transferred to the root induction medium (MS with 20 g/L sucrose, 200 µg/L BAP, and 10 µg/L NAA, with 80 mg/L kanamycin and 300 mg/L cefotaxime [pH= 5.8]).

Analysis of Transformed Plant. The plant genomic DNA was extracted from the leaves of transformed plants by the quick extraction method. First, a small leaf was crushed by two glass slides and washed by 500 µL extraction buffers (100 µL LiCl 2M, 25 µL EDTA 500 mM [pH=8], 100 µL Tris–HCl 1 M [pH=8], 50 µL SDS 10 %, and 225 µL deionized water at final volume of 500 µL) into 1.5 µL vial.

The mixture was centrifuged at 13 000 rpm for 5 min at room temperature. Then 350 µL of chilled supernatant was transferred into new vial and 350 µL isopropanol was added into it.

The solution was centrifuged at 13 000 rpm for 10 min at room temperature. The supernatant was removed and the sediment was resolved in 100 µL deionized water by gentle inverting.

PCR analysis with GUSF and GUSR primers were used to confirm successful plant transformation. The lack of Agrobacterium contamination was evaluated by PCR with VirGF and VirGR primers. Furthermore, a 737 bp fragments, related to VirG gene, were replicated in the presence of *Agrobacterium* contamination.

GUS Activity Assay. The GUS activity was assayed by the histochemical method [JEFFERSON *et al*., 1987]. The fresh leaves of putative transgenic plants were soaked in the GUS assay buffer including 50 mM phosphate buffer (pH 7.0), 1 mM 5–Bromo–4–chloro–3–indolyl–β–D–glucuronide (X Gluc), 1 mM EDTA, 0.001 % Triton X–100, and 10 mM β–mercaptoethanol, and then incubated overnight at 37 ºC. To enhance the GUS staining contrast, the chlorophyll of leaves was eliminated by 70 % ethanol. The activity of β–glucuronidase and blue color were observed by microscope.

Results and discussion

pBI121^{5+1} Vector Construction. The construction of pBI121 $^{5+1}$ vector (Figure 2) was confirmed by the colony PCR of transformed *E. coli* colonies with

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35S–SEQF and RENOS primers (Figure 3). This was after the extraction of recombinant vector from incubated colonies on medium, which contains proper antibiotic. Digestion pattern analysis was performed by two enzyme pairs–*Xba*I/ *Sac*I and *Hin*dIII/ *Eco*RI. The results confirmed the accuracy of the construction of pBI121^{5+1} (Figure 8). Sequencing analysis also verified accurate construction of the new vector (data was not shown).

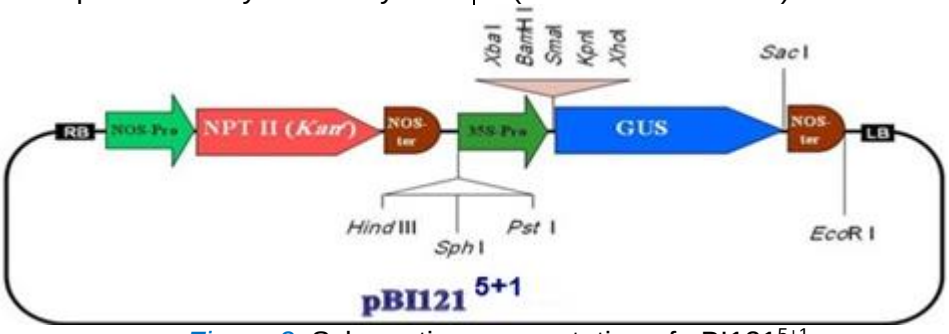

Figure 2. Schematic representation of pBI121^{5+1}

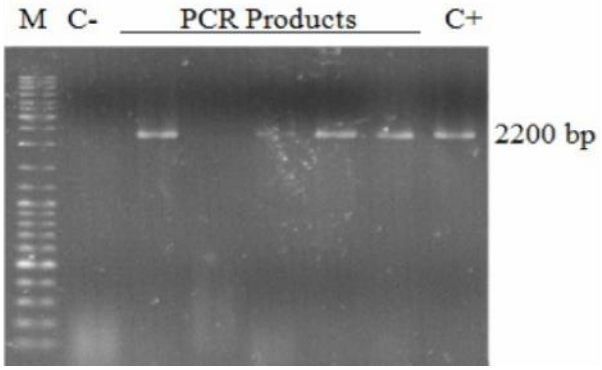

Figure 3. Colony PCR with 35S–SEQF and RENOS; about 2200bp fragments were amplified from positive colonies, C+) pBI121, M) DNA ladder Mix (Fermentas)

pBI121$^{GUS–6}$ Vector Construction. Cloning of the designed fragment includes new restriction enzyme recognition sites (*Kpn*I, *Xho*I, and *Sac*I) and was confirmed by PCR analysis with four primer pairs including NTPF/ RENOS, GUSF/ GUSR, 35S–seq/ RENOS, and UP35SF/ RENOS (Figure 4). Accurate construction of pBI121$^{GUS–6}$ vector (Figure 5) was confirmed by digestion pattern analysis with two enzyme pairs–*Hin*dIII/ *Sac*I and *Hin*dIII/ *Eco*RI (Figure 6). Finally, sequencing analysis verified the results (data was not shown).

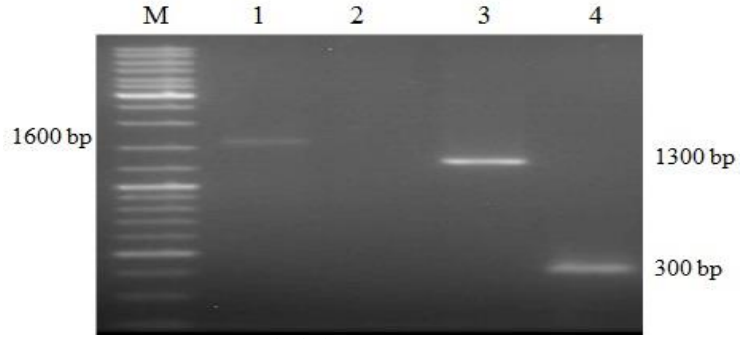

Figure 4. PCR analysis of pBI121$^{GUS–6}$ construct; 1) PCR product by NTPF/ RENOS primers, 2) No PCR product by GUSF/ GUSR primers, 3) PCR product by UP35SF/ RENOS primers, 4) PCR product by 35S–seqF/ RENOS, M) DNA ladder Mix (Fermentas)

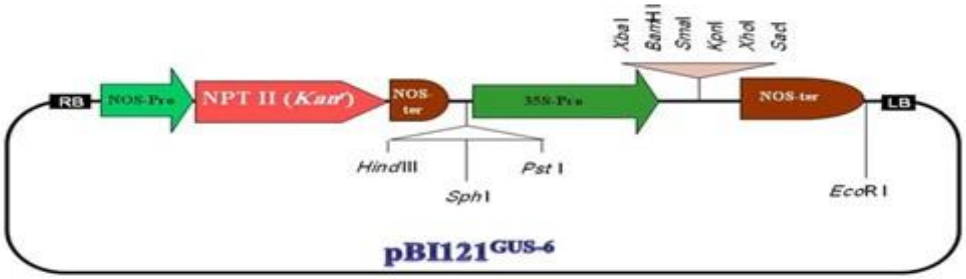

Figure 5. Schematic representation of pBI121$^{GUS–6}$

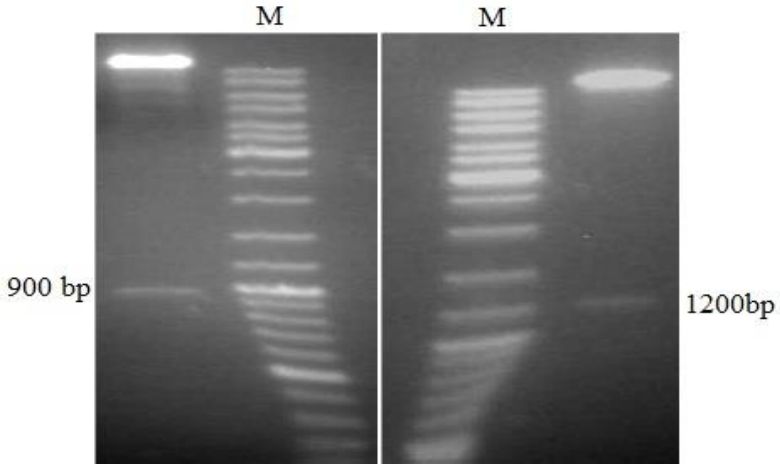

Figure 6. Digestion pattern analysis of pBI121GUS–6; A) *Hind*III/ *Sac*I; about 900bp fragment was isolated from vector as expected. B) *Hind*III/ *Eco*RI; about 1200bp fragment was isolated from vector as expected. M) DNA ladder Mix (Fermentas)

The ability of the new recombinant vectors to express transgene in the plant was evaluated by the cloning of *β–glucuronidase* gene as a reporter gene.

The cloning of *gus* was confirmed by PCR with a primer pair (35S–seqF/ RENOS) (Figure 7). However, the digestion pattern analysis was confirmed by two restriction enzyme pairs (*Xba*I/ *Sac*I and *Hind*III/ *Eco*RI) (Figure 8) and sequencing analysis (data was not shown).

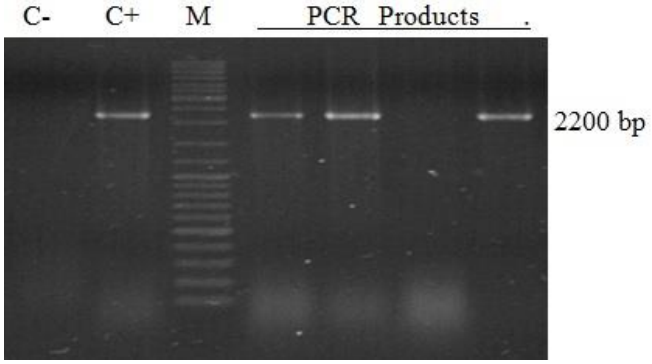

Figure 7. PCR with 35S–seqF and RENOS; about 2200bp fragments were amplified in positive samples. C+) pBI121, M) DNA ladder Mix (Fermentas)

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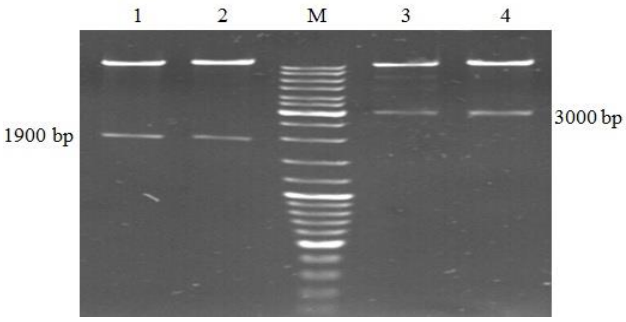

Figure 8. Successful cloning of *β– glucuronidase* (*gus*) in new vectors; 1) digestion of *gus*inpBI121^{GUS-6} by *Xba*I/ *Sac*I, 2) digestion of pBI121^{5+1} by *Xba*I/ *Sac*I, 3) digestion of *gus*inpBI121^{GUS-6} by *Hin*dIII/ *Eco*RI, 4) digestion of pBI121^{5+1} by *Hin*dIII/ *Eco*RI, M) DNA ladder Mix (Fermentas)

Agrobacterium Transformation. Presence of the new vectors (containing *gus* as a reporter gene) in the bacteria colonies, which were cultured on selective medium, was confirmed by PCR with 35S–seqF and GUSR primers (Figure 9).

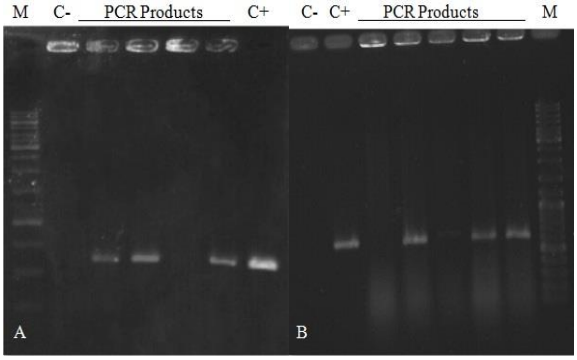

Figure 9. Colony PCR by 355S–seqF and GUSR primers; A) transformed Agrobacteriums by *gus*inpBI121^{GUS-6}; C+) *gus*inpBI121^{GUS-6}, M) 1Kb DNA ladder (Fermentas); B) transformed Agrobacteriums by pBI121^{5+1}, C+) pBI121^{5+1}, M) 1Kb DNA ladder (Fermentas)

Plant Transformation and Regeneration. The leaves of *Nicotiana tobaccum*, which were infected by Agrobacterium bacteria, were transferred to the co–culture medium and incubated at 25 ^{o}C in dark for three days. The explants were cultured on selective regeneration medium, which contains 300 and 500 mg/L concentration of Cefotaxime and Kanamycin. After two weeks, calli were formed and were transferred to the new medium, respectively. The regenerated shoots from calli were transferred to selective medium and sub–cultured to new medium every two weeks when Kanamycin concentration was regularly added to select transgenic explant. After two months, the shoots were cultured on a selective (Kan) root induction medium. There are four steps of the regeneration, during the period as Figure 10.

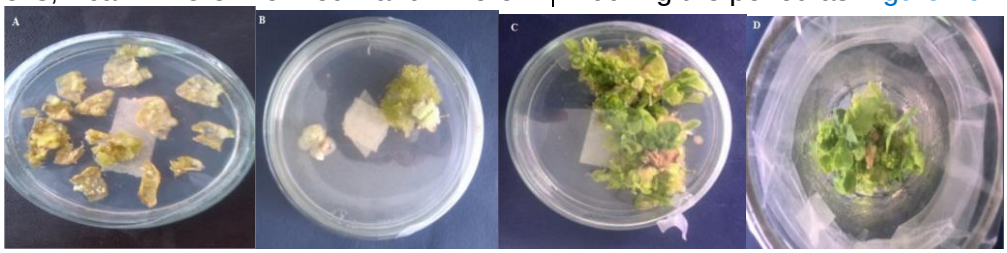

Figure 10. Transformation and regeneration of tobacco; A) callus formation at the edge of explants, B) explants were transferred to new selective medium containing higher concentration of antibiotic. C) Growing of explants and shoot formation D) separating of explants and culture on root induction medium.

The genomic DNA of plants, which was resistant to selectable agent, was extracted.

The presence of T–DNA fragment and β–glucuronidase gene were verified by PCR with 35S–seqF and GUSR primers.Therefore, the results demonstrate that the new vectors are capable of integrating reporter gene to plant genome (Figure 11).

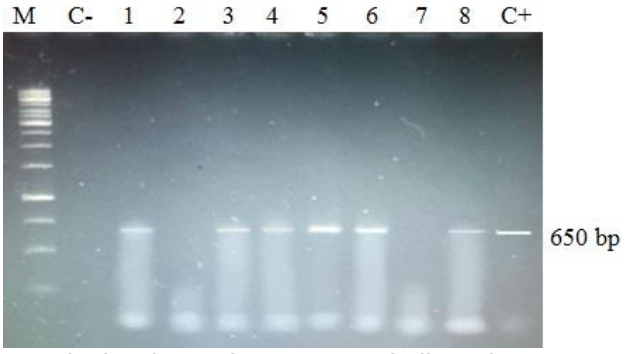

Figure 11. PCR analysis of putative transgenic lines by 35S–seqF/GUSR; 1–3) transformed plants by gus in pBI121GUS–6, 4–5) transformed plant by pBI121, 6–8) transformed plants by pBI1215+1, C+) pBI121, M) 1Kb DNA ladder (Fermentas)

Moreover, the probability of *Agrobacterium* contamination was evaluated by PCR with VirGF and VirGR primer pairs. There was no band on the gel except for the C+ band. The result did not support Agrobacterium contamination (data has not been shown).

GUS Activity Assay. The leaves of PCR positive transgenic plants were tested in β–glucuronidase activity assay. The GUS appeared blue in color and, therefore, its activity was observed in all samples which have been transformed by new plant expression vectors.

The results of β–glucuronidase activity assay indicated significant expression of this reporter gene in the leaf tissue of transgenic plants in comparison with the control plant leaves. There are qualitative comparison of gene expression of new vectors and pBI121 and non–transgenic explant (Figure 12).

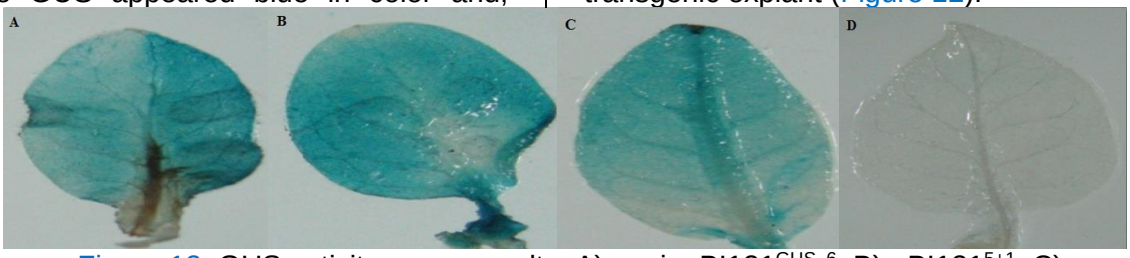

Figure 12. GUS activity assay results; A) *gus*inpBI121$^{GUS–6}$, B) pBI121^{5+1}, C) pBI121(C+), D) non–transgenic tobacco leaf (C–)

The reasons behind the popularity of pBI121 are probably its extensiveness in laboratories, the high number of published articles (which used pBI121), and its derivations that encourage researchers to use this vector.

In addition, the simplicity of pBI121, the facility of replacing the intended gene instead of the *gus* gene, and the appropriate expression of the transferred gene by *CaMV* 35S as an acceptable promoter are other reasons behind pBI121 acceptance.

Nevertheless, the limited number of enzyme–recognition sites in the vector leads to difficulty in cloning; especially when the enzymes present in the pBI121 multiple cloning sites exist in the sequence of gene of interest [KOMORI *et al*., 2007]. To fix the problem and the development of the pBI121 multiple cloning site, this study chose two new recognition sites of enzymes, which are available and inexpensive, do not digest pBI121 as observed in the other regions.

The enzyme arrangement was somehow considered in a way that enzymes, which are suitable for upstream of the gene and recognizing translation, start codon [LUTCKE *et al*., 1987] such as *Kpn*I

placed at first half of multiple cloning sites. The application of *Kpn*I in the intended gene upstream makes an ACC site that is suitable for recognizing the translation start codon of most genes [KOZAK, 1984].

The presence of transferred gene in their genomic DNA was confirmed in most of the transformed explants produced by new vectors (pBI121$^{GUS–6}$ and pBI121^{5+1}). These are similar to the control plants (transformed explants by pBI121).

The results indicate the effective transformation of intended gene by new vectors.

Moreover, the results of GUS assay demonstrate β–glucuronidase activity in all the transformed explants.

The observation of blue color in all transformed explants compared with the control indicates the gene expression ability of pBI121$^{GUS–6}$ and pBI121^{5+1} as well as routine vector pBI121.

Conclusions

Multiple cloning site developments of pBI121$^{GUS–6}$ and pBI121^{5+1} do not have negative effects on the function of vectors.

According to the results, and the advantage of pBI121$^{GUS–6}$ and pBI121^{5+1} vectors (developed multiple cloning site), they can be used instead of pBI121 in the gene transformation projects.

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Received: April 5, 2017
Article in Press: April 30, 2018
Accepted: Last modified on: May 28, 2018