Annals of Agri-Bio Research **28** (2): 178-182, 2023 **Impact Score : 0.28 (Scopus)**

Cloning of CTB-*proinsulin* Gene in Binary Vector pBin*Gy1Red2* for *Agrobacterium*-mediated Plant Transformation

SAPNA BHORIA* AND POOJA MALIK

Centre for Biotechnology, M. D. University, Rohtak-124 001 (Haryana), India
**(e-mail: bhoriasapna@gmail.com; Mobile: 94665 32143)*

(Received: January 20, 2023; Accepted: February 24, 2023)

ABSTRACT

The inefficient production and insensitivity of human body to insulin have caused diabetes in a large section of human population worldwide. In this study, CTB was fused with proinsulin gene and cloned downstream of the soybean seed-specific glycinin (Gy1) promoter in binary vectors, pBinGy1red2 with a *DsRed2* reporter gene. CTB was conjugated at the N-terminus of the *proinsulin* gene to prevent degradation and also to facilitate purification and uptake into the gut- associated lymphoid tissue (GALT) via oral administration. The *CTB.pro-insulin* also contained three furin cleavage sites that made possible for its *in vivo* processing (outside the pancreas) in most of the mammalian body cells. The cloned binary vectors were transferred into *E. coli* (for plasmid maintenance) and *Agrobacterium tumefaciens* (for plant transformation).

Key words: CTB-proinsulin, binary vector, cloning, *Agrobacterium*, heat shock

INTRODUCTION

Diabetes mellitus is a rapidly increasing metabolic disorder with 537 million affected people and caused 6.7 million deaths in 2021 (IDF, 2021). It is expected to affect 79.4 million individuals by 2030 in India alone. Diabetes, being the prime cause of death world over, necessitates efficient production of human insulin to meet the growing demands (Hirsch and Gaudiani, 2021). Insulin, after its discovery in 1921, was isolated from animals' pancreas to treat the diabetic patients, but with advent of rDNA technology, recombinant human insulin was successfully produced in the bacterial (*E. coli*) or yeast (*Saccharomyces cerevisiae*) cells. Initially, both the A-and B-chains were produced separately in *E. coli*. Subsequently, the production of active and mature insulin was completed by the *in vitro* chain combination (Baeshen *et al.*, 2014). In the second approach, the post-translated proinsulin was isolated and purified; this method was selected for insulin analogue Insuman® production in *E. coli* (Sandow *et al.*, 2015). The previous studies revealed that insulin production by individual expression of the two chains was a lengthy and less efficient process. Therefore, for cost-effective insulin production, the expression of proinsulin as a single chain is preferred. Additionally, the C-peptide in proinsulin helps in the correct folding of insulin (Khan *et al.*, 2020). In the present study, a single chain CTB-fused *proinsulin* gene was cloned into binary vector pBinGyl.red2 to yield pBin*Gy1red2-pin* vector, and was transformed into *E. coli* (plasmid maintenance) and *Agrobacterium tumefaciens* (plant transformation).

MATERIALS AND METHODS

All the chemicals used for designing the gene constructs were either analytical or molecular grade and were arranged from the authorized dealers. Restriction enzymes, PCR master mix, T4-DNA ligase, DNA markers, DNA loading dye and antibiotics (kanamycin and rifampicin) were purchased from the Fermentas Pvt. Ltd., India. The bacterial media (Himedia Pvt. Ltd., India), buffers and other chemicals were prepared in distilled water and autoclaved at 121°C for 20 min. Plasmid isolation, DNA extraction and purification kits were arranged from the Thermo Fisher Scientific Pvt. Ltd., USA. The primers were designed by using the software Primer 3 (version 0.4.0.) and synthesized by Eurofins Analytical Services India Pvt. Ltd. The chemically synthesized, CTB-*proinsulin* gene with three furin cleavage sites was amplified from pBI121.pin binary vector after using gene-specific primers. The CTB conjugated with *proinsulin* was cloned into the binary vector, pBinGylRed2 at *EcoR*I and

*Xho*I restriction enzyme sites to yield pBin*Gy1red2-pin*. The CTB.*pro-insulin* gene was under the control of seed-specific glycinin (Gy1) promoter for their expression only in seeds. The T-DNA of binary vector pBin*Gy1red2-pin* also contained *DsRed2* as a plant selectable and reporter markers, respectively and this vector also contained *Kan*^{R} gene for their selection in bacteria. The binary vector was mobilized into chemically competent *E. coli* strain DH5α and in *A. tumefaciens* strain EHA105 by freeze and thaw method.

Agrobacterium tumefaciens strain EHA105 was transformed with binary vector pBin*Gy1red2-pin* grown on semi-solid yeast extract peptone (YEP) medium at 28°C. *E. coli* DH5α strain was used for gene cloning and maintenance of the binary plasmid. *E. coli* was grown in Luria-Bertani broth (LB) at 37°C on a rotary shaker. A single colony of *E. coli* strain DH5α and *A. tumefaciens* strain EHA105 were individually grown in 5 ml of liquid YEP medium on an orbital shaker at 150 rpm for overnight at 37 and 28°C, respectively. One hundred microlitre of overnight grown cultures were transferred to fresh 20 ml LB (Lauria-Bertini) medium and incubated at 37 and 28°C with vigorous shaking at 180 rpm till O.D. reached to 0.6 to 0.8.The bacterial cells were later placed on ice for 30 min and centrifuged at 6000 rpm for 10 min at 4°C. The pellets were re-suspended in 2.5 ml ice-cold 20 mM $CaCl_2$ solution and aliquot of 100 µl in quantities were transferred into chilled sterile eppendorf tubes which were quickly frozen in liquid"N_2 and kept at -80°C.

The reaction mixture (20 ml) containing 1.0 µg of vector, pBinGy1Red2, 1X reaction buffer and 1U restriction enzymes *EcoR*1 and *Xho*1 per microgram of the plasmid DNA were incubated at 16°C for overnight. The digested vector, pBinGy1Red2 and insert (CTB.pro-insulin) were resolved on 1% agarose gel (w/v). The double-digested vector and insert (pro-insulin) were extracted from the gel using a Thermo Scientific gel extraction kit (Thermo Fischer Scientific, USA) as per the manufacturer's protocol. The concentration of the extracted vector and insert was determined using nanodrop spectrophotometer (Bio-rad, USA) and used further for ligation reaction.

The double restriction digested *CTB.pro-insulin* gene was ligated with vector pBinGy1Red2 using a ligation kit (Thermo Fischer Scientific, USA). The double-digested insert *CTB.pro-insulin* and vectors were mixed in 3:1 ratio. In a total volume of 20 ml ligation reaction mixture for each digested vector, the following components: 100 ng digested vector, 300 ng digested insert (*CTB.pro-insulin*) DNA, 1U T_4 DNA ligase and 1 µl 10x T_4 DNA ligase buffer were mixed and the ligation reaction was performed overnight at 16°C. On the next day, the ligated vector and *pBinGy1Red2.pin* was transformed into freshly prepared competent *E. coli* DH5α (100 µl) cells at 42°C for 90 sec. Eight hundred microlitre LB medium was added in transformed cells and allowed to grow for 45 min at 37°C on an orbital shaker. The cells were harvested by centrifugation and resuspended in 100 µl LB medium and streaked on solid LB medium containing kanamycin antibiotic. The streaked Petri plates were placed at 37°C for overnight.

Transformed colonies of *E. coli* strainDH5α were screened for the successful ligation by direct colony PCR method. Colonies appeared on kanamycin containing medium were marked and re-suspended in 50 µl of autoclaved water. The resuspended cells were used as a DNA sample for polymerase chain reaction. A PCR reaction (with a total volume of 25 µl) contained 3 µl of DNA template, 2 X master mix (Fermentas), 0.5 µM of *CTB.pro-insulin* specific forward 5'GAATTCATGACACCTCAA 3' and reverse 5'CTCGAGTTAGTTGCAGTAGTTC 3' primers and the final volume was raised with nuclease-free water. PCR was carried out with the reaction cycles of 95°C (5 min) followed by 30 cycles of 95°C (45 s), 51°C (45 s), 72°C (45 s) and ended with the extension of 72°C for 10 min. The PCR products were resolved on 1.0% agarose gel electrophoresis.

Cloning of *proinsulin* gene into pBinGy1Red2 to form pBin*Gy1red2.pin* was confirmed by a combination of restriction digestion reaction using different enzymes. Plasmid DNA (1.0 µg) was restricted with the fast digest restriction enzymes, *EcoRI and XhoI* at 37°C for a specific time period. The digested DNA fragments were fractionated on 1% agarose gel electrophoresis.

The newly constructed plasmid was isolated from *E. coli* and gently mixed with freshly prepared competent"cells of *A. tumefaciens* EHA105 and frozen in liquid N_2"for 30 s, thawed" at 37°C "up to" 5 min. Twenty microlitre of culture were then transferred into the 200 µl of antibiotic free LB medium and incubated for 1 h at 28°C on an orbital shaker

at 160 rpm."Thereafter, 100 µl of the suspension was spread on the LB plates containing 25 mg/l rifampicin and 50 mg/l kanamycin. The plates were incubated at 28°C for overnight. The developed colonies were evaluated for the presence of *pro-insulin* gene by the colony PCR.

RESULTS AND DISCUSSION

The CTB fused *proinsulin* gene was cloned into disarmed binary vector; pBinGy1Red2, one with a visual reporter gene. The native vector, pBinGy1Red2 (10.9 kb in size) in its T-DNA had *gus* gene under the control of glycinin promoter and glycinin terminator and a reporter gene *DsRed2* under control of CvMV (Cassava vein mosaic virus) promoter and *nos* terminator. The native vector (pBinGy1Red2) on digestion with *EcoR*1 and *Xho*1 restriction enzymes produced two DNA fragments of size 9.3 and 1.6 kb (*gus* gene) for pBinGy1Red2 (Fig. 1). The *gus* (1.6 kb) genes were replaced from their respective vector with *CTB-proinsulin* gene which was cloned into *EcoR*1 and *Xho*1 sites downstream of seed specific promoter glycinin resulted into pBin*Gy1Red2*-pin (9.9 kb) (Fig. 2). The ligated vector and insert was transformed into *E. coli* strain DH5α and *Agrobacterium* strain EHA105 as confirmed by colony PCR using primers specific to proinsulin. The

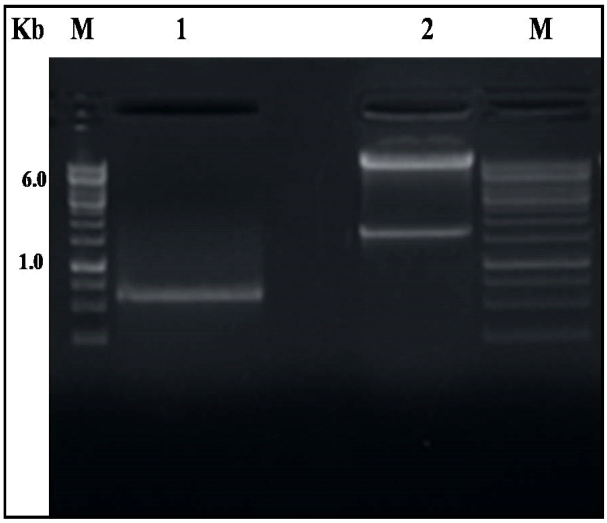

Fig. 1. Double digestion of insert (CTB-*proinsulin*) and native vector pBinGlyRed2 with *EcoR*1and *Xho*1 Restriction enzyme. Lane M: 1 kb DNA marker, Lane 1: Double digestion of insert (pro-insulin) (0.6 kb) and Lane 2: Double digestion of vector pBinGlyRed-2 (fragment size: 9.3 and 1.6 kb).

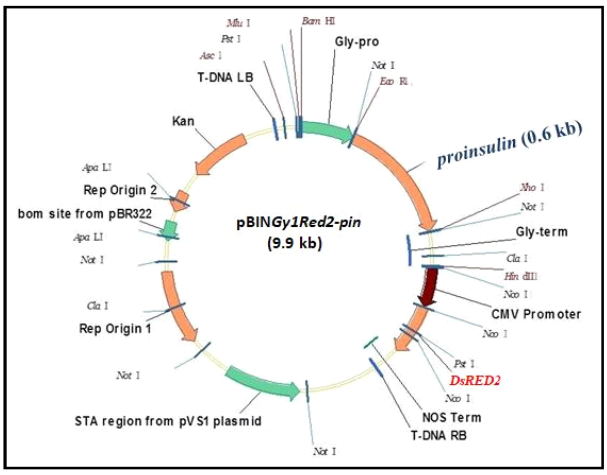

Fig. 2. Map of newly constructed binary vector pBin*Gy1red2-pin* with restriction sites.

amplification of 0.6 kb expected size band corresponding to *proinsulin* gene was observed confirming ligation of insert with vector (Fig. 3A)**.**

The constructed pBin*Gy1Red2.pin* plasmids were isolated from *E. coli* DH5α and digested products with single (with *EcoR*1) and double (*EcoR*1 and *Xho1*) restriction enzymes, were resolved on 1% agarose gel. The double digestion of vector, *pBinGy1Red2.pin* showed the presence of bands of expected size 9.3 and 0.6 kb (Fig. 3B), respectively. The presence of a 0.6 kb band confirmed the cloning of CTB. *pro-insulin* gene into the binary vectors.

The numbers of diabetic patients are increasing rapidly and the present production systems (*E. coli* and yeasts) are trying hard to combat the increasing insulin demand (Bhoria *et al.*, 2022). Advent of rDNA technology and the successful transformation of plants through *Agrobaterium*-mediated approach opened up the way for the expression of novel genes in plant genomes. Many researchers also tried to express insulin and its precursor into plants (Yarbakht *et al.*, 2015; Tang *et al.*, 2015), but the success has been limited yet. So, in the present study, constructed an efficient binary vector pBin*Gy1Red2-pin,* for *Agrobacterium*-mediated plant transformation. The *Agrobacterium* strain EHA105 used here showed good transformation efficiency in many plants (Kumar *et al.*, 2021). The literature showed that binary vector pBinGlyred2 was used for many vector designs for the expression of *AtKCS, AtKCR, AtHCD* and *AtECR* (Huai *et al.*, 2015), *FAD2/FAE1* (Nguyen *et al.*, 2013), *B.pPAD* genes (Menard *et al.,* 2022) in oilseed

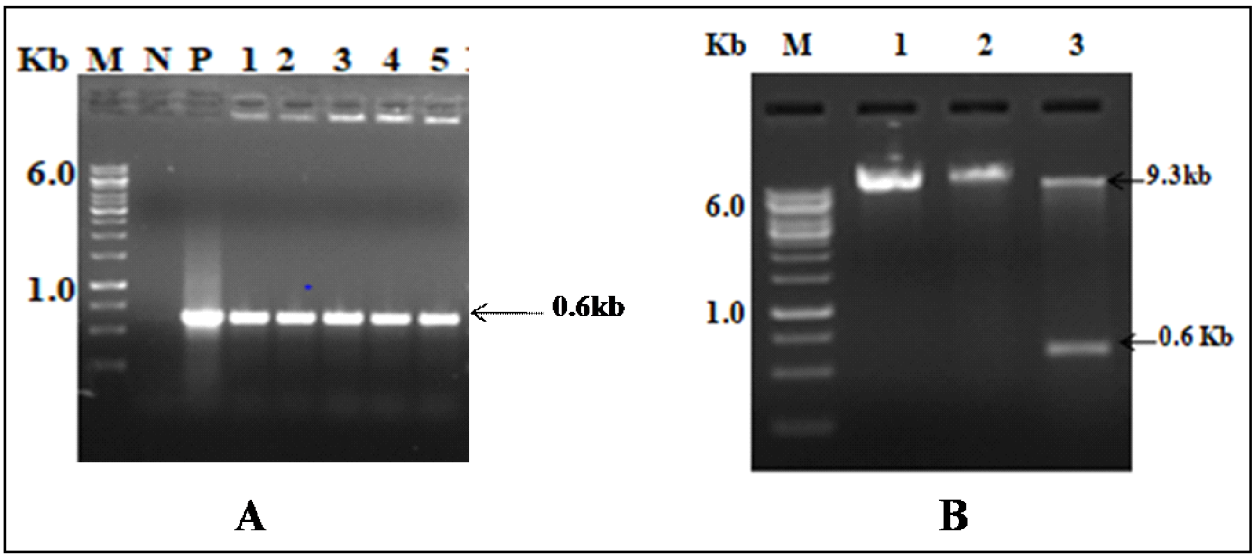

Fig. 3.(A) Colony PCR confirmation of DH5α *(E. coli)*) and Agrobacterium EHA105 harboring a newly constructed binary vector pBin*Gy1red2-pin*. Lane M–1 kb DNA marker, Lanes 2, 3–Colony PCR from DH5α harboring pBin*Gy1red2-pin*, Lanes 4, 5–Colony PCR from *Agrobacterium* harboring pBin*Gy1red2-pin,* Lane N–Negative control, Lane P–Positive control. (B) Restriction digestion confirmation of ligated vector pBin*Gy1red2-pin,* Lane M–1 kb DNA marker, Lane 1–Linear recombinant pBin*Gy1red2-pin* vector digested with *EcoR*1 restriction enzyme, Lane 2–Undigested pBin*Gy1red2-pin* vector (9.9 kb) and Lane 3–Double digested recombinant pBin*Gy1red2-pin* vector with *EcoR*1 and *Xho*1 restriction enzymes.

crop, *Camelina sativa*. The popularity of these two binary vectors was due to the presence of seed-specific glycinin promoter, which helped in the localization of expressed products in plant seeds (Sánchez-Álvarez *et al.*, 2019) and availability of *dsRed2* gene. The red fluorescent gene (DsRed) was isolated from the marine organism, *Discosoma striata*. The presence of these proteins offered the ability to visualize, track, and quantify molecules and events in plant cells. Its red fluorescence would provide a distinct label for multicolour tracking of fusion proteins alone (Okada *et al.*, 2019) and together with GFP (Wang *et al.*, 2018) in plants.

CONCLUSION

The *CTB-proinsulin* gene was successfully cloned into disarmed binary vector pBin*Gy1Red2* under the downstream of a seed-specific glycinin promoter and terminator. The vectors were transformed into *E. coli* (*DH5α*) for plasmid maintenance and in *Agrobacterium tumefaciens* (EHA10) for *Agrobaterium*-mediated transformation of plants for proinsulin protein production.

ACKNOWLEDGEMENTS

The authors are thankful to the University Grants Commission, New Delhi, for the award of Senior Research Fellowship and Dr. Edgar Cahoon, USA for providing native vectors.

REFERENCES

Baeshen, N. A., Baeshen, M. N., Sheikh, A., Bora, R. S., Ahmed, M. M. M., Ramadan, H. A. and Redwan, E. M. (2014). Cell factories for insulin production. *Microbial Cell Factories* **13**: 01-09. *https://doi.org/10.1186/s12934-014-0141-0.*

Bhoria, S., Yadav, J., Yadav, H., Chaudhary, D., Jaiwal, R. and Jaiwal, P. K. (2022). Current advances and future prospects in production of recombinant insulin and other proteins to treat diabetes mellitus. *Biotechnology Letters* **44**: 643-669. *https://doi.org/10.1007/s10529-022-03247-w.*

Hirsch, I. B. and Gaudiani, L. M. (2021). A new look at brittle diabetes. *J. Diabetes Compl.* **35**, 107646. *https://doi.org/10.1016/j.jdiacomp.2020.107646.*

Huai, D., Zhang, Y., Zhang, C., Cahoon, E. B. and Zhou, Y. (2015). Combinatorial effects of fatty acid elongase enzymes on nervonic acid production in *Camelina sativa*. *PloS One* **10**: e0131755. *https://doi.org/10.1371/journal.pone.0131755.*

Khan, M. A. B., Hashim, M. J., King, J. K., Govender, R. D., Mustafa, H. and Al Kaabi, J. (2020). Epidemiology of type 2 diabetes–Global burden of disease and forecasted

trends. *J. Epidem. Global Health* **10**: 107. *https://doi.org/10.2991%2Fjegh.k.191028.001.*

Kumar, A., Sainger, M., Jaiwal, R., Chaudhary, D. and Jaiwal, P.K. (2021). Tissue culture- and selection-independent *Agrobacterium tumefaciens*-mediated transformation of a recalcitrant grain legume, cowpea [*Vigna unguiculata* (L.) Walp.]. *Mol. Biotechnol.* **63**: 710-718. *https://doi.org/10.1007/s12033-021-00333-8.*

Menard, G. N., Langdon, M., Bhunia, R. K., Shankhapal, A. R., Noleto-Dias, C., Lomax, C. and Eastmond, P. J. (2022). Diverting phenylpropanoid pathway flux from sinapine to produce industrially useful 4-vinyl derivatives of hydroxycinnamic acids in Brassicaceous oilseeds. *Metab. Engine.* **70**: 196-205. *https://doi.org/10.1016/j.ymben.2022.01.016.*

Nguyen, H. T., Silva, J. E., Podicheti, R., Macrander, J., Yang, W., Nazarenus, T. J. and Cahoon, E. B. (2013). Camelina seed transcriptome: A tool for meal and oil improvement and translational research. *Plant Biotech. J.* **11**: 759-769. *https://doi.org/10.1111/pbi.12068.*

Okada, A., Arndell, T., Borisjuk, N., Sharma, N., Watson-Haigh, N. S., Tucker, E. J. and Whitford, R. (2019). CRISPR/Cas9-mediated knockout of Ms1 enables the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. *Plant Biotech. J.* **17**: 1905-1913. *https://doi.org/10.1111/pbi.13106.*

Sánchez-Álvarez, A., Ruíz-López, N., Moreno-Pérez, A. J., Martínez-Force, E., Garcés, R. and Salas, J. J. (2019). Agrobacterium-mediated transient gene expression in developing *Ricinus communis* seeds: A first step in making the castor oil plant a chemical biofactory. *Front. Plant Sci.* **10**: 1410. *https://doi.org/10.3389/fpls.2019.01410.*

Sandow, J., Landgraf, W., Becker, R. and Seipke, G. (2015). Equivalent recombinant human insulin preparations and their place in therapy. *Eur. Endocrin.* **11**: 10. *https://doi.org/10.17925%2FEE.2015.11.01.10.*

Tang, Y., Yu, Y. and Zhao, Y. (2015). Construction of seed protein body targeted expression vector and transformation rice for oral recombinant proinsulin. *Chinese J. Rice Sci.* **29**: 481-489. *http://www.ricesci.cn/EN/abstract/abs.*

Wang, P., Zhang, J., Sun, L., Ma, Y., Xu, J., Liang, S., Deng, J., Tan, J., Zhang, Q., Tu, L., Daniell, H., Jin, S. and Zhang, X. (2018). High efficient multisites genome editing in allotetraploid cotton (*Gossypium hirsutum*) using CRISPR/Cas9 system. *Plant Biotech. J.* **16**: 137-150. *https://doi.org/10.1111/pbi.12755.*

Yarbakht, M., Jalali-Javaran, M., Nikkhah, M. and Mohebodini, M. (2015). Dicistronic expression of human proinsulin–Protein A fusion in tobacco chloroplast. *Biotech. Appl. Biochem.* **62**: 55-63. *https://doi.org/10.1002/bab.1230.s*