Health Biotechnology and Biopharma (2019), 3(1): 19-28

Original Research Article

Replication of pgR107 binary Ti vector without pSoup helper plasmid in disarmed *Agrobacterium tumefaciens* LBA4404

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DOI: 10.22034/HBB.2019.09

Received: March 5, 2019; **Accepted:** April 16, 2019

ABSTRACT

Nowadays the dual binary vector system pGreen/pSoup is used for *Agrobacterium* transformation. The pGreen vector uses the pSa replication locus composed of the pSa ori and the pSa replicase gene (repA). The repA gene is resident on a pSoup and provides replication functions *in Trans* for pGreen. Therefore, the present study was a preliminary attempt to investigate how pgR107 binary vector replicates without subsidiary plasmid pSoup, in disarmed *Agrobacterium* LBA4404. So, human Nerve Growth Factor gene was cloned in a binary pgR107 vector based on pGreen. Recombinant pgR107-*hNGF* plasmid was introduced in competent *A. tumefaciens* strain LBA4404 using a freeze-thaw technique. Transformed colonies containing pgR107-*hNGF* grown on selective medium was approved by colony PCR using *hNGF* specific primers. Examination of *A. tumefaciens* LBA4404 genome for presence of pSa Rep (RepA) gene was done via basic local alignment search tool (BLAST). There was no homology observed between RepA sequence and *A. tumefaciens* strain LBA4404 genome. Our findings show although *A. tumefaciens* LBA4404 does not contain pSoup plasmid, binary vector could interestingly replicate in such strain. But, the mechanism of replication is unknown and being to be investigated.

***Keywords*:** *Agrobacterium tumefaciens* LBA4404, binary Ti vector, RepA, pgR107, pSoup

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INTRODUCTION

Agrobacterium-based plasmid vectors represent a promising strategy for the transformation of a wide range of plant species by introducing DNA into the nuclear genome and heterologous protein production in plants [1]. *Agrobacterium* transfers a piece of its DNA (T-DNA) into the nuclear genome [2]. T-DNA is a distinct part of Ti plasmid. Ti plasmid includes the T-DNA flanked by two 25 bp repeats, the right and left borders, and ~35 virulence (vir) genes, clustered together into a vir region. The combined action of the vir genes ensures the processing and delivery of the T-DNA into the plant nuclear Genome [3]. One of the obstacles in using *A. tumefaciens* for transfer of gene of interest was presence of genes necessary for this T-DNA transfer on a tumour-inducing (Ti) plasmid [4]. To overcome this problem a number of disarmed *A. tumefaciens* strains have been developed by removal of all the genes within the T-DNA. These strains which are no longer oncogenic include LBA4404 [5], C58C1 [6], GV3101 [7], EHA101 [8] and EHA105 [9].

It would appear that two primarily advances have made *Agrobacterium* suitable for gene transfer; development of binary Ti vectors and a range of disarmed *Agrobacterium* strains [2].

In 1983, two groups determined that the T-DNA and vir regions of Ti-plasmids could be split onto two separate plasmids within the same *Agrobacterium* cell. These form the basis of modern binary Ti vectors [5,10]; Which results in the efficiency of recombination procedures by reduce the size of the plasmid DNA [11]. As a broad-host-range replication origin (ori) is often used in binary Ti vectors, these plasmids are able to replicate in a wide range of gram-negative bacteria including *Escherichia coli* and *Agrobacterium* [12].

Since the 1980s, binary Ti vectors have been developed to contain an extensive range of selectable marker and also reporter genes [13-15], as well as improvement of transformation efficiency with the addition of vir genes [3,16]. Binary Ti vectors with tandem border repeats [17,18], and or with small T-DNAs [19,20] have also been designed to minimize the insertion of unnecessary DNA sequences into the plant genome. BiBAC binary Ti vectors have been engineered that permit the insertion of large DNA segments (up to at least 150 kb) and their transfer to *Agrobacterium* [21-25]. Binary Ti plasmids are also available for promoter fusions to reporter gene, such as pBIN19 plasmids that harbour a GUS coding sequence [26,27].

In this report, we used the dual binary vector system pGreen/pSoup [28] for *Agrobacterium* transformation. pGreen is a small binary Ti vector able to replicate in *E. coli* but unable to replicate in *Agrobacterium* without the presence of the helper plasmid, pSoup, in the same strain [3]. The pGreen vector uses the small pSa replication locus, which has been divided into the smaller portions; the pSa ori and the pSa replicase gene (repA). The repA gene is resident on a pSoup, a compatible plasmid, in *Agrobacterium* and provides replication functions *intrans* for pGreen [28].

Although many studies on the development and use of binary vectors has been done. But, to the best of our knowledge there is no research about the possibility of pGreen plasmid replication without presence of helper plasmid, pSoup.

So, the dual binary vector system pGreen/pSoup is used for *Agrobacterium* transformation nowadays. The pGreen vector uses the pSa replication locus composed of the pSa ori and the pSa replicase gene (repA). The repA gene is resident on a pSoup and provides replication functions *in Trans* for pGreen. Therefore, the present study was a preliminary attempt to investigate how pgR107 binary vector replicates without subsidiary plasmid pSoup, in disarmed *Agrobacterium* LBA4404.

Therefore, the present study was aimed to investigate how pgR107 Binary Ti Vector based on pGreen replicates without subsidiary pSoup Helper Plasmid which provides replication functions *in Trans* for pGreen in disarmed *Agrobacterium* LBA4404.

MATERIALS AND METHODS

Bacteria strains and culture conditions

E. coli strain DH5 was grown on Luria Bertani Agar (LBA) medium at 37 °C. *A. tumefaciens* strain LBA404 was grown on LBA medium at 28 °C. Plasmids were transformed into bacteria using a freeze-thaw technique [11].

Construction of recombinant pgR107-rhNGF vector

As described in our previously work, human *NGF* gene sequence was optimized according to *N. benthamiana* codon usage using Leto software ver. 1.0.11 (Entelechon, Germany). The *rhNGF* gene was synthesized by GenScript (USA).

pgR107 vector was kindly provided by Cristiano Lacorteh, Wageningen University. Synthesized *rhNGF* gene has been isolated and cloned into the ClaI and SalI restriction sites of pgR107 containing the CaMV 35S promoter and a nos terminator. Resulted recombinant viral vector, pgR107-*rhNGF*, was transferred into *E. coli* DH5α competent

cells. Grown colonies were screened on selective media containing 50 µg/ml Kanamycin. Finally, Pgr107-*rhNGF* construct was confirmed by digestion with restriction enzymes and sequencing [29]. DNA restriction and ligation were done according to standard methods [11].

Transformation of A. tumefaciens and detection of transformed agrobacteria by the polymerase chain reaction (PCR)

Recombinant pgR107-*hNGF* plasmids were introduced in competent *A. tumefaciens* strain LBA4404 using a freeze-thaw technique. The transformed agrobacteria were selected on LBA medium selective media containing Rifampicin (50 mg/l), Streptomycin (100 mg/l) and Kanamycin (50 mg/l); and finally the positive agrobacteria colony was confirmed by PCR using primers mhNGF-F (Forward: 5' ACACATATGTCATCATCCCATCCCAT CTTC 3') and mhNGF-R (Reverse:5' AAGGATCCCTAGGCTCTTCTCACAGC 3'); just to be sure the *rhNGF* gene insert is still there.

A typical PCR reaction carried out contained in the final volume of 25 µl containing Taq buffer (10 mM Tris–HCl, pH: 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 1 unit Taq DNA Polymerase (Fermentas, Maryland, NY, USA), and 0.5 µM of each primer. The thermal profile for the reaction included pre-PCR denaturation at 94 °C for 5 minutes followed by 30 cycles of denaturing at 94 °C for 30 seconds, annealing at 60 °C for 30 sec and extension at 72 °C for 40 sec, and a final extension at 72 °C for 10 min. After PCR, products were electrophoresed on 1 % agarose gel in TBE buffer (0.09 M Tris, 0.09 M boric acid, pH: 8.0/2.0 mM EDTA), stained with ethidium bromide and visualized on a UV transilluminator.

Examination of *A. tumefaciens* LBA4404 genome for presence of pSa Rep (RepA) gene

To investigate whether there is the sequence of pSa Rep (RepA) gene nucleotide in the *A. tumefaciens* LBA4404 genome, the nucleotide sequence of this gene was extracted from the pSoup nucleotide sequence and examined for any similarity with *A. tumefaciens* LBA4404 genome or other strains being closely related to this bacteria via Basic Local Alignment Search Tool.

RESULTS

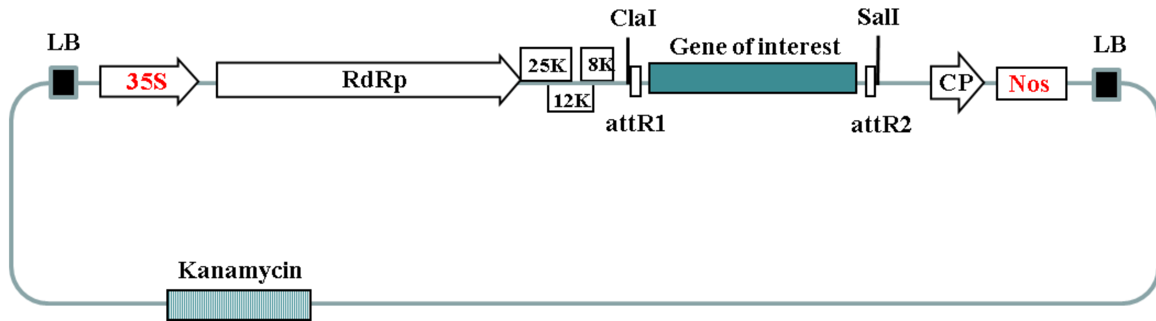
Construction of expression vectors

The purified *rhNGF* fragment was cloned in pgR107 binary vector (Figure 1). The

sequence of the insert was confirmed by restriction enzyme digestion and sequencing.

Detection of transformed Agrobacterium by polymerase chain reaction (PCR)

The positive clones were transferred into agrobacteria and verified for the presence of the *rhNGF* using colony PCR (Figure 2a, b).



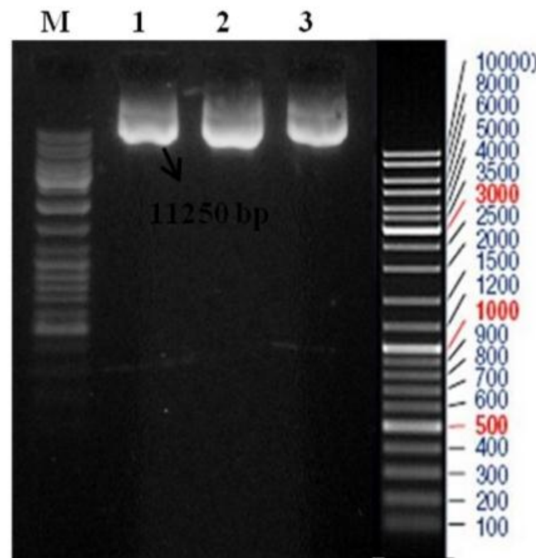


Figure 1. a) Schematic design of pgR107 vector containing gene of interest. 35s, CaMV35s promoter; RdRp, RNA dependent RNA polymerase; 25 K, 12 K, 8 K, triple gene box; CP, coat protein; Nos, Nos terminator; LB and RB, left and right border of T-DNA. **b)** Lanes 1-3; Recombinant pgR107-*rhNGF* plasmids, Lane M; 10,000 bp molecular weight marker.

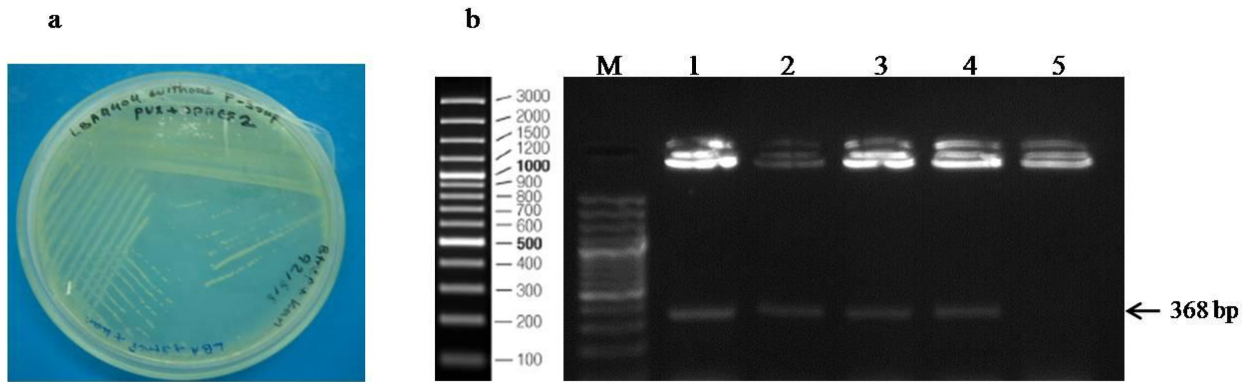


Figure 2. a) The positive *Agrobacterium* colonies. **b)** Colony PCR of positive agrobacteria. Lanes 1- 4; positive colonies (the observed 368 bp band is related to *hNGF* gene), Lane 5; negative control, Lane M; DNA molecular weight marker.

Sequence similarity between RepA nucleotide sequence and A. tumefaciens strain LBA4404

As we extracted the RepA sequence (Figure 3) and search Sequence similarity using basic local alignment search tool, it was not observed any homology between RepA sequence and *A. Tumefaciens* strain LBA4404 genome.

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tgcctaagaacaacaaagcccccggccatcgtatcaacgagatcatcaagacgagcctcgcgctcgaaatggaggatgcccgcgaagctggcttagtcggctacatg
gcccgttgccttgtgcaagcgaccatgccccacaccgaccccaagaccagctactttgagcgcaccaatggcatcgtcaccttgtcgatcatgggcaagccgagcatc
ggcctgccctacggttctatgccgcgcaccttgcttgcttggatatgcaccgaggccgtgcgaacgaaagaccccgtgttgaaccttggccggtcgcaatcggaatttct
acaaaggctcggaatgcacaccgatggccgttacacggccacccttcgcaatcaggcgcaacgcctgttttcatccatgatttcgcttgccggcgagcaaggcaatga
cttcggcattgagaacgtcgtcattgccaagcgcgcttttctattctggaatcccaagcggccagaagatcgggcgctatgggatagcaccctcaccctcacaggcgat
ttcttcgaggaagtcacccgctcaccggttcctatccgaatcgactacctgcacgccttgcggcagtctccgcttgcgatggacatttacacgtggctgacctatcgcgt
gttcctgttgcgggccaagggccgccccttcgtgcaaatcccttgggtcgccctgcaagcgcaattcggctcatcctatggcagccgcgcacgcaactcgcccgaactg
gacgataaggcccgagagcgggcagagcgggcagcactcgccagcttcaaatacaacttcaaaaagcgcctacgcgaagtgttgattgtctatcccgaggcaagcg
actgcatcgaagatgacggcgaatgcctgcgcatcaaatccacacgcctgcatgtcacccgcgcacccggcaagggcgctcgcatcggccccccctccgacttg
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Figure 3. Nucleotide sequence of RepA gene.

DISCUSSION

As outlined in the Introduction, our intention was find out the reason for the replication of the pgR107 vector without the helper plasmid, pSoup, in disarmed *A. tumefaciens*LBA4404.

In this study, the *rhNGF* gene was inserted into both nos and CaMV 35S expression cassette of pgR107 vector. pgR107 is a binary vector based on pGreen0000. pgR107 contains CalI-SmalI-SalI restriction sites. The selection marker for pgR107 is Kanamycin antibiotic (50 µg/ml). It can replicate to high copy number in *E. coli*. But this vector need helper plasmid pJICSa_Rep for replicating in *Agrobaterium* strains [30]. This helper plasmid carries tetracycline as selection marker (5 µg/ml). The expression of the pgR107 sequence which consists of replicase, triple block and coat protein genes is motivated by the 35S promoter [30].

To be sure that the target gene is inserted, after ligation reaction; the positive *Agrobacterium* colony was confirmed by PCR (Figure 2b).

This paper takes a new look at the subject by matching *A. Tumefaciens* LBA4404 genome and pSa Rep gene via nucleotide Blast (BLAST) to examine whether there is any similarity between these sequences or not. By separation of the repA nucleotide sequence of the pSa locus from pSoup vector which is necessary for replication in *Agrobacterium*, we have been able to comparise this gene against *A. tumefaciens* LBA4404 genome.

As a result, it was no homology observed between RepA sequence and *A. tumefaciens* strain LBA4404 genome. Although the used *A. tumefaciens* LBA4404 does not contain pSoup plasmid, pgR107 binary vector could interestingly replicate in such strain. The mechanism of replication is unknown and being to be investigated.

In the history of binary vectors, to the best of our knowledge, the focus has always been on the development of its performance in order to increase the efficiency [3,31-33].

As far as we know what has been proposed in the literature was that pGreen is not able to replicate in *Agrobacterium* species lacking the pSoup helper plasmid [3,28,34] and there was no report about replication of binary pgR107 vector without Soup in *A. tumefaciens* LBA4404.

These findings can add to a growing body of literature on T-DNA binary systems.

CONCLUSION

Our findings can act as a platform for additional enhancements to *Agrobacterium* technology. As it show that within a single *Agrobacterium* a binary pgR107 vector can exist and replicate without supplementary pSoup plasmid. This new report will permit the development of pSoup helper plasmid-free transgenic protocols and should greatly increase the quality of *Agrobacterium* strains-mediated transformation events.

ACKNOWLEDGMENT

This research was financially supported by Iranian council for Stem Cell Sciences and Technologies.

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