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# CLONING THE COTTON rrn23–rrn5 REGION FOR DEVELOPING A UNIVERSAL INTERFAMILY PLASTIDIAL VECTOR

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***Abstract***. Although plastid transformation has attractive advantages in plant biotechnology, it has been highly proficient only in tobacco. The lack of efficient semi lethal selection procedure along with the inefficient recombination through heterologous flanking regions used in transformation vectors prevented this technology to major crops. However, due to the published plastidial genomic sequences and the distinct features of their genomic parts lead the scientists to use of specific and new regions in transformation vectors. In this work, we worked out rrn23–rrn5 fragment as new flanking regions for Malvaceae, Caricaseae, Rutaceae and Solanaceae families by nucleotide homology search. Therefore, the PCR–amplified rrn23–rrn5 region was cloned into *the* cloning vector using competent cells of *E.coli* strains DH5α. The sequencing results showed the high homology compared to chloroplast genome of *Gossypium sp.* with identities of 99 % values.
***Keyword***: Malvaceae, flanking region, plastid, *trnI, trnA,* vector.

## Introduction

Plastid Engineering has become a promising technology at unicellular as well as higher plants, and require to be extended on more species and exploited efficiently in basic and applied biotechnology [BOCK, 2010].

In light of all the enumerated findings on plastomic nucleotides sequences, construction of large sets of transformation vectors becomes a prerequisite for effective modification at the future.

Taken together with the inherent requirement to replace and shuffle flanking sequences necessary for homologous recombination and integration of the transgene cassettes into the genome of a given plant at a specific situation, the cited considerations point to extensive and oftentimes hefty cloning procedures as the critical impediment to dynamic development and far–reaching application of plastid genome engineering.

Consequently, an extensive array of expression vectors have been developed and made available to the research community during recent years [LUTZ *et al.*, 2007, GOTTSCHAMEL *et al.*, 2013].

In general, two distinct components are required to construct the final plastid transformation vector system: a vector containing the flanking sequences, left flanking sequence, and right flanking sequence, and the sequences essential for efficient transgene expression (expression cassette).

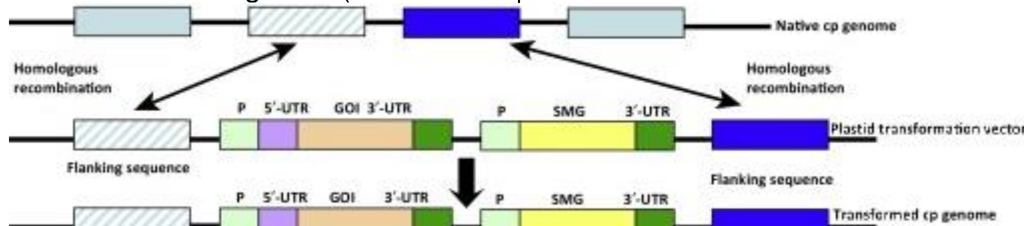
Flanking sequences are the DNA sequences from the plastid genome, which are homologous to the desired site of integration.

Their function is to simplify the site–specific recombination and define the integration site of the transgene (Figure 1A).

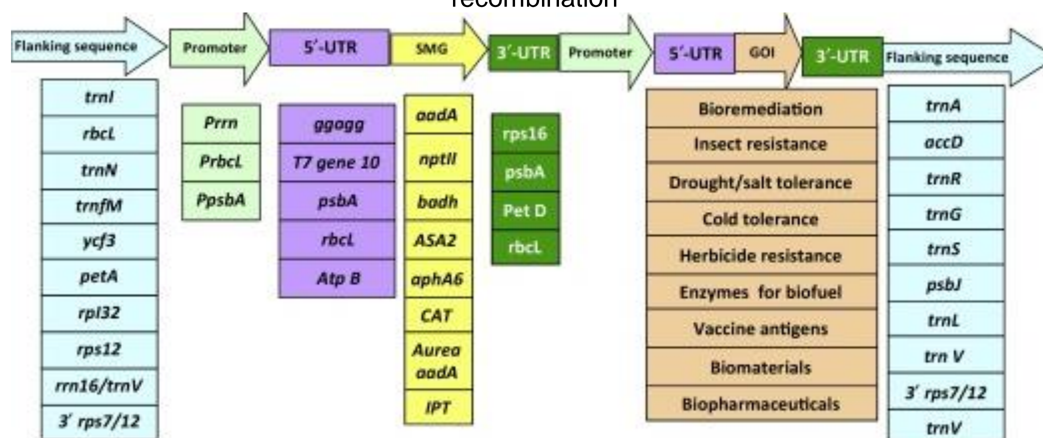
Therefore, two segments of chloroplast DNA are used as flanking sequences in chloroplast vectors to insert the transgene cassette into an intergenic spacer region, without disrupting any functional genes; these sequences are used in length of about 1 kb at either side of the expression cassette [JELLI VENKATESH, 2012]. Earlier, plastid transformations were accomplished with vectors designed for tobacco transformation, as the tobacco homologous flanking sequences present in these transformation vectors share a very high homology to the relevant

sequences of plastid DNA in other *Solanaceae* crops [VLADIMIR *et al.,* 1999, IRVING JOSEPH BERGER, *et al.,* 2005, THANH *et al.,* 2005].

To date, one of the most commonly used sites of transgene integration is the transcriptionally–active intergenic region between the *trnI–trnA* genes (in the rrn operon) located within the inverted repeat regions on the chloroplast genome [DHEERAJ *et al.,* 2007, GRAY *et al.,* 2009, MAUREEN *et al.,* 2013, DHEERAJ VERMA *et al.,* 2008], although several other sites have been explored [SHUANGXIA *et al.,* 2015] (Figure 1B).




Schematic representation of the transgene sequence integration into plastid genomeby homologos recombination




Chloroplast vectors systems for biotechnology applications

**Figure 1**. (A) Schematic representation of the chloroplast vector. The vector includes two chloroplast DNA fragments as flanking sequences to facilitate insertion by double homologous recombination, a selectable marker gene, and regulatory elements. (B) Examples of components used in chloroplast vectors. Abbreviations: cp, chloroplast; GOI, gene of interest; P, promoter; SMG, selective marker gene. 3′–UTR, 3′ untranslated region; 5′–UTR, 5′ untranslated region [12].

In this research, we selected the rrn23–rrn5 region in the IRs of plastidial genome of cotton as a new flanking region to develop a new plastidial transformation vector specific to Malvaceae, Caricaseae, Rutaceae and Solanaceae families.

## Material and methods

### Materials

Cotton (*Gossypium hirsutum* L. var.varamin) seeds were planted in a pot and let to grow to 2–3 leaf stage for extracting their total cellular genomic DNA.

In this study, the *E. coli* strain of DH5a (Invitrogen, USA) was used in the cloning steps. The used restriction/cutting enzymes, *Taq* DNA polymerase were from Thermo Corporation.

Plasmid DNA extraction kits were provided from Bioneer Corporation (South Korea).

The sequencing of recombinant plasmids were carried out by Bioneer Corporation too.

### DNA extraction

To prepare high–quality DNA, plants were incubated in a dark place for 24 hours for the full breakdown of most likely cellular starch content and oil bodies.

Total DNA was extracted from the developing leaf sample at 2–3 leaves stage using CTAB method [SAGHAI–MAROOF *et al.,* 1984].

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Quality, quantity and concentration of the extracted DNA were evaluated by 0.8 % agarose gel electrophoresis using uncut λDNA Weight Marker (SinaClon).

**Designing the primers**

The nucleotide sequence of the chloroplast genome of cotton (*Gossypium hirsutum* L.) with the accession number of NC–007944.1 was downloaded and used for designing the specific primers of F:5'CTGCAGCTTAGGTGGAAGGCGAA–3´ and R:5'GGGCCCTACAGTAT CGTCACCGC–3' by the online software of Primer–Blast.

The cleavage sites of *Apa*I and *Pst*I were embedded in the 5' end of the forward and reverse primers respectively to ensure the cloning procedure.

**PCR amplification and Bacterial Transformation**

Total Genomic DNA was used as a template for amplification of target fragment at the concentration of 5 ng/μL.

The PCR program was consisted of an initial denaturing at 94°C for 5 min, continued by 35 cycles of 94°C for 60 s, 60.4°C for 30s and 72°C for 60s, with a final extension step at 72°C for 5 min.

The quality and quantity of PCR amplificants were evaluated by electrophoresis on 0.8 % agarose gel using 1Kb weight marker DNA (SinaClon).

Target amplificant was eluted using extraction kit and used at concentration of 38 ng/μL in ligation reaction with *p*TG19–T vector at 4°C for 24 hrs. *E.* coli Competent bacterial cells were prepared using TSS protocol and transformation were carried out by 5 μL of ligation reaction using heat shock procedure.

*p*TG19T plasmid contains an ampicillin resistance gene, so the plasmid–free bacteria do not live on medium containing ampicillin antibiotic plus x–gal and IPTG, in contrast to white recombinant colonies.

The resultant white colonies on media containing x–gal and IPTG were further confirmed by direct colony PCR technique before inoculation of liquid bacterial culture.

The plasmid DNA was extracted from the liquid culture of PCR positive colonies using plasmid extraction kit.

Finally, the recombinant plasmid DNA was reconfirmed with *Eco*RI digestion and was sent for sequencing to Bioneer Co, South Korea.

## Results and discussion

**Cloning of rrn23–rrn5 region**

To amplify the rrn2–rrn5 region with designed specific primers, the lengths and nucleotide composition as well as Tm of the primers was considered to ensure the efficiency of the PCR.

The length of the predestinated fragment was 1227 nts, which resulted as such and was shown in Figure 2.

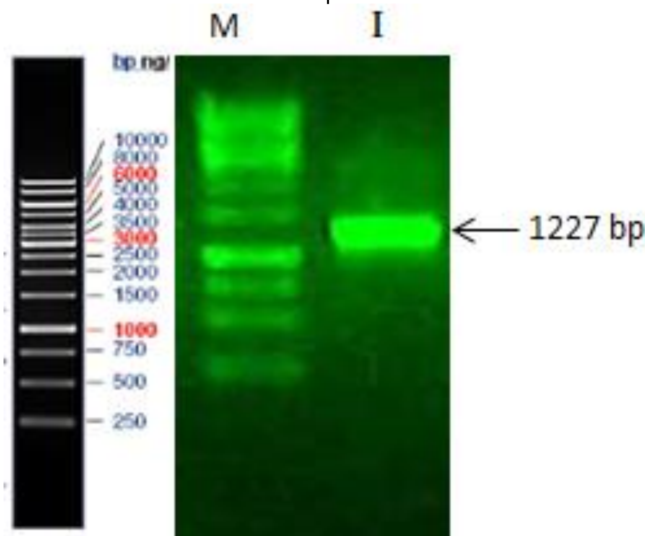


**Figure 2.** PCR, product run on an agarose gel.

Where: M) 1 KbDNA marker, Sinaclon, I) Amplified fragments. The fragments were ligated to the cloning vector of *p*TG19–T illustrated in Figure 3.

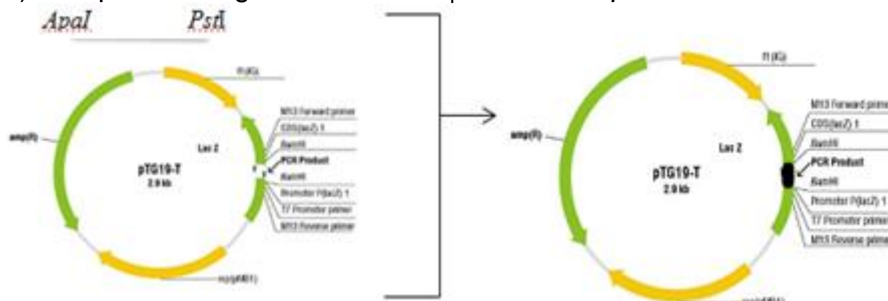


**Figure 3**. Illustration of Ligation reaction. The *p*TG19–T is a linear–type vector which takes a circular form after ligation.

Inserted fragment in the vector *p*TG19–T was further corroborated by PCR and digestion by restriction enzyme of *Eco*RI (Figure 4).

When the extracted plasmids were digested with the enzyme *Eco*RI, three fragments were produced due to presence of three restriction sites within plasmid DNA.

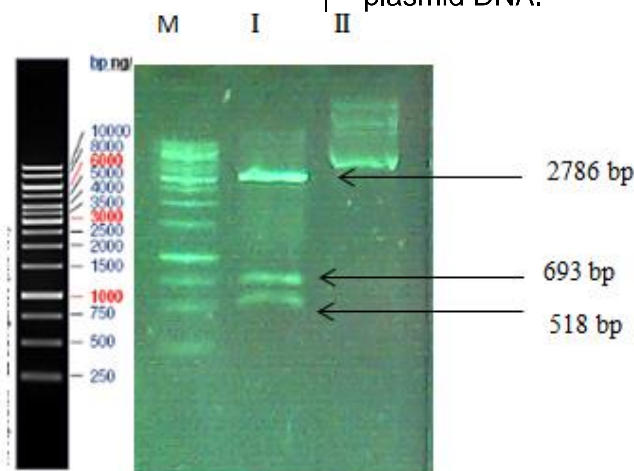


**Figure 4**. Gel agarose of digested plasmid DNA by *Eco*RI. M) 1KbDNA weight marker Sinaclon, I) cut and II) uncut plasmid.

Using the online BLAST software and aligning by CLUSTAL Omega program, The sequencing result of target fragments from cotton was compared with reference accession (NC–007944.1) and some other plants that are shown in Table 1 with highest similarity with *Gossyoium sp* (99 %), and followed by 97 % with potato, tomato, papaya, cacao, orange and nicotiana.

Moreover, some variations were observed between the target sequence and other sequences within the chloroplast genome of this species (Table 1). Plastid transformation requires double homologous recombination [DHEERAJ VERMA *et al.*, 2007, DHEERAJ VERMA *et al.*, 2008].

Therefore, two regions of chloroplast DNA are used as flanking sequences in pastid vectors.

The discussion in this field was to find the beau–ideal site for transgene integration.

Here, two dissident theories were transpired: based on the Maliga concept, insertion of foreign sequence into transcriptionally silent spacer regions in which plastid genes are located on inverted DNA strands in inverted orientations and according to the Daniell concept, insertion into transcriptionally active spacer regions within chloroplast operons.

The benefits of each idea were experimented recently by insertion of the lux operon with an same expression cassette at both sites and the transcriptionally–active spacer region was found to suggestion a 25–fold higher level of expression [KRICHEVSKY *et al.,* 2010], and authors ascribed this to higher read–through transcriptional activity.

In this work, we report rrn23–rrn5 region as a new insertion site that located within the inverted repeat region (IR) of the chloroplast genome and could be driven by the internal 16S rrn promoter.

**Table 1.**

Percent identity and number of base mutations after alignment of various species and cotton *rrn23–rrn5* region used in plastid transformation vector.

| Species | Family | Identity (%) | Mutation(bp) Substitution | Omission | Addition |
|---|---|---|---|---|---|
| *Gossypium hirsutum* | Malvaseae | 99 | 12 | 3 | – |
| *G.barbadence* | Malvaseae | 99 | 13 | 3 | – |
| *G.herbaceum* | Malvaseae | 99 | 11 | 3 | – |
| *Talipariti hamabo* | Malvaseae | 99 | 12 | 3 | – |
| *Hibiscus syriacus* | Malvaseae | 99 | 14 | 3 | – |
| *Theobroma cacao* | Malvaseae | 98 | 16 | 3 | – |
| *Tilia oliveri* | Malvaseae | 99 | 14 | 3 | – |
| *Carica papaya* | Caricaseae | 97 | 23 | 3 | 6 |
| *Citrus sinensis* | Rutaceae | 97 | 27 | 3 | 6 |
| *Nicotiana tabacum* | Solanaceae | 97 | 26 | 6 | 5 |
| *Solanum lycopersicum* | Solanaceae | 97 | 31 | 6 | 6 |
| *Solanum tuberosum* | Solanaceae | 97 | 25 | 6 | 6 |

Transgene expression at this insertion site in the IR of the p benefits from replication of the gene copy number and from increased mRNA levels due to read–through transcription from the upstream rrn operon promoter.

Some laboratories have inserted transgenes between the trnI and trnA genes in the inverted repeat region of plastome.

These two tRNAs listed above are placed between the small and large rRNA subunit genes (rrn16–rrn23) and this operon is transcribed from promoters upstream of rrn16 [JELLI VENKATESH, *et al.,* 2012, DHEERAJ VERMA, *et al.,* 2007, DHEERAJ VERMA *et al.,* 2008, VERA *et al.,* 1995, SUZUKI *et al.,* 2003, DANIELL *et al.,* 2005, YU *et al.,* 2007, HANSON and AHNER *et al.,* 2013, GRAY *et al.,* 2009, XIE *et al.,* 2014].

To attain efficient foreign sequence integration by homologous recombination transplastomic approaches have used endogenous ptDNA sequences to target insertions.

Thus, similarity between flanking region used in transformation vectors and plastomes is another important issue, which must be to observe in the design of transgenic vectors.

Recently, it was approved that the existence of high similarity among many homologous region in plastome and transplastomic vectors will increase the transformation greatly [RUHLMAN *et al.,* 2010].

Studies illustrate a positive correlation between both the length and degree of sequence similarity and the rate of recombination in prokaryotes [SHEN, 1986, FUJITANI and KOBAYASHI, 1995].

Although the size of flanking region did not study carefully, however, about 1 to 2 kilo base is used [JELLI VENKATESH, 2012].

It seems possibly the more length of the flank region causes the more increase in abundant in homologous recombination [SHASHI KUMAR *et al.,* 2004].

In the plastid genomes of seed plants, gene content and gene order are well conserved [DROUIN and XIA, 2008, RUHLMAN, 2014], usually this high degree of genome conservation allows the use of plastid transformation vectors for closely related species [KAVANAGH *et al.,* 1999, RUF *et al.,* 2001], avoiding the need to construct species–specific vectors.

The similarity of other sequences (e.g., trnN–trnR, psaB–trnS and rrn16–rps7/12), earlier employed in plastid transformation tests showed a higher homology between potato and tobacco (both of Solanaceae) than the other plastid regions investigated in various studies suggesting the need for suitable sequence analysis and precise choice of

flanking sequences before attempting to use vectors developed in non–target species.

Decreasing identity between transformation vectors and target plastome regions, using *Nicotiana tabacum* flanking sequences to transform different species, yields much lower transformation efficiencies [RUHLMAN *et al.*, 2010, SIDOROV *et al.*, 1999].

In another study, The replacement of tobacco flanking sequences with homologous potato sequences in transformation vectors produced a more increase in transformation efficiency, giving approximately one shoot per bombardment that corresponds to 15–18–fold improvement [VALKOV *et al.*, 2011].

The rbcL–accD region used in Valkov study [VALKOV *et al.*, 2011] was 95.6 % identical between tobacco and potato considering the length of sequence fragments enough large to allow the beginning of recombination.

Genome organization of cotton is more like to other angiosperm chloroplast genomes of other species [SEUNG–BUM LEE *et al.*, 2006].

According to our survey a higher similarity is shown between the rrn23–rrn5 region from cotton and the chloroplast genome of other species and families (table1).

Species specific plastid vector design and construction for every species is difficult and take long time [LUTZ *et al.*, 2004] and we might suggest that cotton *rrn23–rrn5* region vectors developed here can be likely used interchangeably between Malvaceae, Caricaseae, Rutaceae and Solanaceae families.

## Conclusions

The aim of this study was to clone and characterize rrn23–rrn5 region of cotton plastid genome for construction a novel chloroplast transformation vector.

The results of the survey showed that this region can be used in specific vector for cotton plastid engineering and universal vector for some Malvaceae, Caricaseae, Rutaceae and Solanaceae families, including potato, tomato, cacao, papaya and orange plants as a high–performance vector.

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