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*Short Communication*

# Characterization of chloroplast region *rrn16-rrn23S* from the tropical timber tree *Cedrela odorata* L. and *de novo* construction of a transplastomic expression vector suitable for Meliaceae trees and other economically important crops

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**ABSTRACT.** The forest tree Spanish cedar (*Cedrela odorata* L.) is well-known for its high-value timber; however, this species is attacked by the shoot borer (*Hypsipyla grandella*) during its early years of development, resulting in branched stems and making the plants useless for high-quality wood production. The generation of resistant varieties expressing entomotoxic proteins may be an alternative to pesticide treatments. The use of plastid transformation rather than nuclear transformation should be used because it reduces the risk of transgene dissemination by pollen. Chloroplast transformation vectors require an

 

expression cassette flanked by homologous plastid sequences to drive plastome recombination. Thus, *C. odorata* plastome sequences are a prerequisite. The *rrn16-rrn23* plastome region was selected, cloned, and characterized. When the sequence identity among the *rrn16-rrn23* regions from *C. odorata* and *Nicotiana tabacum* was compared, 3 inDels of 240, 104, and 39 bp were found that might severely affect transformation efficiency. Using this region, a new transformation vector was developed using pUC19 as a backbone by inserting the *rrn16-trnI* and *trnA-rrn23* sequences from *C. odorata* and adding 2 independent expression cassettes into the *trnI-trnA* intergenic region, conferring spectinomycin resistance, the ability to express the *gfp* reporter gene, and a site that can be used to express any other gene of interest.

**Key words:** Chloroplast transformation; Expression vector; Flanking regions; Plastome sequence; Tropical tree biotechnology

## INTRODUCTION

*Cedrela odorata*, *C. fissilis*, *Swietenia macrophylla*, *Khaya senegalensis*, *Melia azedarach*, and *Toona ciliata* are species that belong to the Meliaceae family with relevant economic importance because of the quality of their wood. These species are used in tropical areas to establish commercial plantations; however, their success has been limited by the high prevalence of *Hypsipyla* spp borers (Griffiths, 2001). The use of genetic modifications for improving materials by expressing entomotoxic proteins in some tropical tree species has been reported in previous studies (Nichols et al., 2002); however, ethical and environmental concerns are relevant as forest species are publicly perceived as natural components of the landscape (Gamborg and Sandøe, 2010). Adopting a precautionary approach has been recommended in the development of transgenic trees that are unable to spread genetically modified material. Thus, a transplastomics approach has become one of the most promising alternatives for developing genetically modified varieties. Because plastids are mostly inherited maternally, the amount of transgene content in pollen is extremely low, resulting in improved containment (Svab and Maliga, 2007). Lowering the level of transgenes in the pollen substantially reduces gene flow into wild trees (Brunner et al., 2007). Furthermore, plastid transformation exhibits other advantages, such as high levels of transgene and protein expression, which remains stable because silencing mechanisms do not take place on the plastids, and the possibility of transforming multiple genes (Bock, 2001). However, chloroplast genetic transformation presents technical challenges, most importantly the vector mode-of-insertion. Because of their bacterial origin, chloroplasts primarily resemble the prokaryotic machinery, including homologous recombination processes. Typically, plastid transformation vectors include an expression cassette of interest located at an intergenic region that is flanked by plastid sequences to target transgene insertion at a discrete locus in the host plastome without gene disruption. The selection of flanking regions and intergenic sequences for introducing the gene of interest is a key step in the development of chloroplast transformation vectors because of interspecies sequence diversity, thus limiting the concept of a "universal" chloroplast transformation vector (Verma and Daniell, 2007). Recent reports have indicated a positive effect on transformation

 

efficiency when homologous flanking sequences are used rather than universal vectors containing heterologous *N. tabacum* sequences (Kumar et al., 2004a,b; Scotti et al., 2011). In this study, we focused on the design and *de novo* construction of a chloroplast expression vector suitable for plastid transformation of trees from the Meliaceae family containing homologous *C. odorata* sequences.

## MATERIAL AND METHODS

### Plastid DNA isolation

Five grams *C. odorata* tender leaves were incubated in the dark for 48 h and ground in a mortar with 20 mL STE buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 1 mM EDTA). The resulting homogenate was filtered through sterilized mesh and the filtrate was centrifuged at 300 *g* for 20 min. The supernatant was recovered and centrifuged at 3000 *g* for 20 min. The pellet was resuspended in 20 µL ST buffer [400 mM sucrose, 50 mM Tris, pH 7.8, 0.1% (w/v) seroalbumin] and treated with 5 U DNAse I (Invitrogen, Carlsbad, CA, USA) and incubated for 30 min at 37°C. Next, 50 mL NEFT buffer (1.25 M NaCl, 50 mM Tris-Cl, pH 8.0, 50 mM EDTA, pH 8.0, 50 mM NaF) was added to the mixture. The solution was centrifuged at 3000 *g* for 20 min and the pellet was resuspended in 600 µL TEN buffer [100 mM NaCl, 100 mM Tris, pH 7.2, 50 mM EDTA, pH 8.0, and 0.2% (v/v) 2-mercaptoethanol], followed by incubation on ice for 5 min. Next, 30 mL of 20% (w/v) sodium dodecyl sulfate was added and the solution was mixed at room temperature. Chloroplast DNA (cpDNA) was extracted with phenol, followed by further extraction with phenol-chloroform; the phenol was eliminated through a chloroform wash. cpDNA was precipitated overnight at -70°C with 1 volume isopropanol and 1/10 volume 5 M ammonium acetate. The cpDNA was recovered by centrifugation at 10,000 *g* for 20 min and resuspended in 20 µL distilled water.

### Cloning of flanking regions

To clone the *C. odorata rrn16* to *trnA* region, a PCR reaction was set up using 20 pM-primers CLB1 and CLB2 (Table 1), which carry the restriction enzyme sites *Hin*dIII and *Pst*I/*Not*I, respectively, 20 ng *C. odorata* cpDNA, 10 mM dNTPs, 1X amplification buffer, 2 mM $MgCl_2$, and 0.5 U iProof DNA polymerase (BioRad, Hercules, CA, USA) for a final volume of 50 µL. The thermalcycler program consisted of 1 cycle at 95°C for 4 min, 34 cycles at 95°C for 30 s, 59°C for 1 min, 72°C for 3 min, and a final cycle of 72°C for 15 min. To amplify the *trnI* to *rrn23* region, primers CLB3 and CLB4, carrying the *Sal*I/*Not*I and *Xba*I restriction enzymes sites, respectively, (Table 1) were synthesized. Polymerase chain reactions (PCRs) were performed as described above. To obtain pCBL1 and pCBL3, the resulting amplicons were column-purified and digested at 37°C for 3 h with *Hin*dIII/*Pst*I for *rrn16*-*trnI* and *Sal*I/*Xba*I for *trnA*-*rrn23* and ligated independently into the pUC19 vector, which was previously digested with either *Hin*dIII/*Pst*I, or *Sal*I/*Xba*I. Ligations were performed with a 3:1 insert: vector ratio and 20 U T4 ligase (New England Biolabs, USA) in a final volume of 20 µL. Then, 10 µL each ligation reaction were added to 100 µL competent DH5αF' *Escherichia coli* cells (Invitrogen) for standard thermal shock transformation followed by selection on LB media plates supplemented with 20 mM isopropyl β-D-1-thiogalactopyranoside, 80 µg/mL X-gal,

and 100 µg/L ampicillin. To introduce the *Not*I site into the *trnI-trnA* intergenic region, a *Not*I/*Xba*I fragment carrying the *trnA-rrn23* region from pCBL3 was inserted into pCBL1, which was digested using the same enzymes, and the resulting plasmid was named pCBL4.

## Expression cassette design and synthesis

An expression cassette was designed *in silico* based on previously reported sequences available in the GenBank generating 2 operons. The sequences and accession numbers are as follows: Accession number *Prrn16* 5' untranslated region (UTR) [(AN) EU520587], gene *aadA* (AY895148), *PG10T7* (NC_001604.1), and T7 5'UTR (EU450674), *gfp* gene (AB199889) and *Trps16S* 3'UTR (EU520589). The *Pst*I, *Sph*I, *Not*I, *Xba*I, and *Hin*dIII sites were eliminated from all native sequences to facilitate the cloning strategy. Only a single *Sph*I site was introduced between *PG10T7* and the structural *gfp* gene. The *in silico* design and edition of sequences was performed using CLC Genomics Workbench (CLC Bio, Aarhus, Denmark) and pDRAW32 by AcaClone (http://www.acaclone.com). The expression cassette was chemically synthetized at the BiomatiK Corporation (Cambridge, Ontario, Canada) and was received cloned into a pBMH vector, which was named pBMH-Co.

## pCBL5 construction, sequencing, and sequence analysis

The pCBL4 insert was sequenced at the Clemson University Genomics Institute (Clemson, SC, USA) by the Sanger method using BigDye Terminators and the primers shown in Table 1. BLAST analyses and sequence alignments were performed using the nBLAST algorithm at NCBI and Clustal W/Ω. The expression cassette from pBMH-Co was inserted into the *Not*I site of pCBL4 to obtain pCBL5, and the orientation was confirmed by restriction analysis.

# RESULTS

## Cloning and characterization of the *rrn16-rrn23 C odorata* region

To generate a chloroplast transformation vector suitable for transforming *C. odorata* and other Meliaceae trees, we first isolated and characterized the *rrn16-rrn23* flanking sequences directing homologous recombination. This region was chosen because it is useful for the development of plastid transformation vectors (McNutt et al., 2007). Moreover, this region is close to the replication origins *oriA* and *oriB* responsible for the high copy number cpDNA, thus increasing the probability of reaching homoplasty after a few regeneration steps (Guda et al., 2000). Because the plastome of *C. odorata* was not available, an *in silico* approach was used to design primers for PCR amplification. In an early attempt to obtain amplicons from the *C. odorata rrn16-rrn23* region, "universal chloroplast primers", known as 16S-F and IRB23R (Tsumura et al., 1995; Dhingra and Folta, 2005) (Table 1) based on the *N. tabacum* sequence, were used in PCR reactions with plastid DNA from *C. odorata* and *Tabebuia rosea* (Bignonaceae). An approximately 5700-bp amplicon was obtained using *C. odorata* as a template (Figure 1A); however, we were not able to directly clone this sequence. The *T. rosea* amplicon of similar size was cloned (Peña-Ramírez et al., unpublished results), and a partial 923-bp sequence from the *T. rosea* plastome was determined [GenBank Accession No. (AN): KF741278]. A nucleotide BLAST search of the NCBI databases comparing this *T. rosea*

sequence to other relevant plant species produced 10 hits with maximum score values of 1652-1572 and coverage of 98-99% for the following plant species: *Olea europaea*, *Daucus carota*, *Lactuca sativa*, *Eucalyptus globulus*, *Coffea arabica*, *Jatropha curcas*, *Nicotiana tabacum*, *Morus indica*, *Populus trichocarpa*, and *Populus alba* (Table 2).

**Table 1.** Highest scores for BLAST analysis referencing a *rrn23* fragment from *Tabebuia rosea*. The *rrn16-rrn23* sequences from species most related to *C. odorata* (bold) were used for consensus sequence determination and primer design for *C. odorata* region amplification, cloning, and sequencing.

| Species | Accession No. | Max score | Total score | Coverture | Identity |
|---|---|---|---|---|---|
| ***Olea europaea*** | GU228899.1 | 1652 | 3304 | 99% | 99% |
| *Daucus carota* | DQ898156.1 | 1600 | 3200 | 98% | 98% |
| ***Lactuca sativa*** | DQ383816.1 | 1600 | 3200 | 99% | 98% |
| ***Eucalyptus globulus*** | AY780259.1 | 1600 | 3200 | 99% | 98% |
| ***Coffea arabica*** | EF044213.1 | 1594 | 3189 | 99% | 98% |
| ***Jatropha curcas*** | FJ695500.1 | 1589 | 3178 | 99% | 98% |
| *Nicotiana tabacum* | Z00044.2 | 1589 | 3178 | 99% | 98% |
| ***Morus indica*** | DQ226511.1 | 1583 | 3167 | 99% | 98% |
| ***Populus trichocarpa*** | EF489041.1 | 1572 | 3145 | 99% | 98% |
| ***Populus alba*** | AP008956.1 | 1572 | 3145 | 99% | 98% |

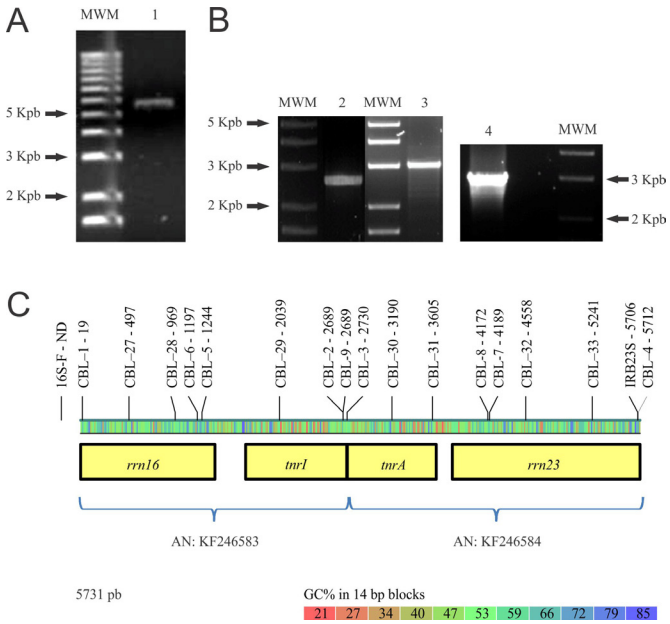


**Figure 1.** PCR analysis of *Cedrela odorata rrn16-rrn23* region and localization of primers. **A.** Agarose gel electrophoresis. *Lane 1* was loaded with the amplification product (~5700 bp) amplified using the 16S-F/IRB23S primers and corresponding to the *C. odorata* chloroplast region used as flankers for transgene insertion. **B.** *Lane 2* was loaded with the PCR product of the amplification of region *rrn16-trnI* using the CLB-1/CLB-2 primers. *Lane 3* was loaded with the PCR product of the amplification of region *trnA-rrn23* using the CLB-3/CLB-4 primers. *Lane 4* was loaded with the PCR product of the amplification of region 3' end *rrn16* to 5' end *rrn23* using the CLB-6/CLB-7 primers. Lane molecular weight markers in A) and B) corresponds to a 1 kbp ladder. **C.** Graphic representation of the *rrn16-rrn23* region showing the name and position of the primers used in this study. The position of the 16S-F primer was upstream of the sequence at a non-determined position (ND). The horizontal bar indicates the GC% content according to the reference bar shown at the bottom. Braces below sequences indicate the accession numbers for regions *rrn16-trnI* and *trnA-rrn23*.

**Table 2.** Primers employed in this study. Name, sequence, orientation, and target gene is shown. Letters in bold italics in the sequence of primers CBL-1 to CBL-4 correspond to nucleotides introducing new restriction sites. All primers reported here, except 16S-F (Shinozaki et al., 1986) and IRB23S (Dhingra and Folta, 2005), were designed by the authors.

| Name | Sequence | Orientation | Restriction sites added | Target |
|---|---|---|---|---|
| 16S-F | ACGGGTGAGTAACGCGTAAG | FW | None | *rrn16S* |
| IRB23S | CGCTACCTTAGGACCGTTATAGTTAC | RV | None | *rrn23S* |
| CBL-1 | ***AAAGCTT***AGAACCTGCCTGCCCTTGGGAGG | FW | *Hin*dIII | *rrn16S* |
| CBL-2 | ***CTGCAGGCGGCCGC***TGGGCCATCCTGGACTTG | RV | *Pst*I/*Not*I | *trnI* |
| CBL-3 | ***GTCGACGCGGCCGC***GGGGATATGCTCAGTTGGTAGAGC | FW | *Sal*I/*Not*I | *trnA* |
| CBL-4 | ***TCTAGA***CGCTACCTTAGGACCGTTAT | RV | *Xba*I | *rrn23S* |
| CBL-5 | CCAGCTCCCATAGTGTGAC | RV | None | *rrn16S* |
| CBL-6 | ATCGCTAGTAATCGCCGGTC | FW | None | *rrn16S* |
| CBL-7 | GGTCCTTGCTGATTCACAC | RV | None | *rrn23S* |
| CBL-8 | CTCTGACCCGAGTAGCATG | FW | None | *rrn23S* |
| CBL-9 | GGGCGAGGTCTCTGGTTC | FW | None | *trnI* |
| CBL-10 | GCCGACTACCTTGGTGATC | RV | None | *aadA* |
| CBL-27 | TTCCCTCTGCCCCTACCG | RV | None | *rrn16S* |
| CBL-28 | ACCCTCGTGCTTAGTTGCCA | FW | None | *rrn16S* |
| CBL-29 | GAGCACAGGTTTAGGTTCGG | FW | None | *trnI* |
| CBL-30 | TTCCACTTATTGAGCAGGGT | RV | None | *trnA* |
| CBL-31 | GGATGTCAGCGGTTCGAGT | FW | None | *trnA* |
| CBL-32 | CATTTCACCCCTAACCACAA | RV | None | *rrn23S* |
| CBL-33 | GAGGGACGGAGGAGGCTA | FW | None | *rrn23S* |

The sequences corresponding to the *rrn16-rrn23* plastid region of these plants (except *D. carota* and *N. tabacum*) were aligned and a 5946-bp consensus was obtained. Based on the consensus sequence, a series of flanking primers was designed to independently clone the *rrn16-trnI* and *trnA-rrn23 C. odorata* plastome regions, as well as internal primers for sequencing (Table 1). The *rrn16-trnI* region of *C. odorata* plastome was amplified from *C. odorata* cpDNA using the CBL-1/CBL-2 primers producing an approximately 2700-bp PCR product (Figure 1B), which was approximately 250 bp longer than expected (based on the *N. tabacum* sequence). To verify the identity of this amplicon, reamplification reactions using internal primers CBL-1/CBL-5 and CBL-2/CBL-6 were conducted. The CLB-1/CLB5 amplicon showed the expected size (~1200 bp), whereas the CBL-2/CBL-6 amplicon was approximately 250 bp larger than the expected amplicon (~1500 bp) (data not shown), indicating the presence of inDels in *C. odorata*. Independently, the *trnA-rrn23* region amplified using the CLB-3/CLB-4 primer pair and *C. odorata* cpDNA as template, resulted in an approximately 3000 bp PCR product, which was similar in size to the *N. tabacum trnA-rrn23* region. Both the *C. odorata rrn16-trnI* and *trnA-rrn23* regions were assembled *in vitro* to regenerate the *rrn16-rrn23* region and was used as a template for PCR in the presence of the CBL6/CBL7 primers. A resulting approximately 3000-bp amplicon of the expected size was obtained spanning from the 3' of *rrn16* to the 5' of the *rrn23* genes (Figure 1C). The assembled *rrn16-rrn23* region was fully sequenced and was used for nBLAST analysis in the NCBI databases, and the identity and orientation of the *rrn16-rrn23* region was confirmed. The best annealing scores matched to chloroplast sequences of *Mangifera indica*, with an identity of 99% and coverage of 100%, and *Theobroma cacao*, showing 99% for both identity and coverage. Other economically important species, such as *Citrus sinensis*, also showed high scores (Table 3). Moreover, alignment of the *rrn16-rrn23* sequences from of *C. odorata* and *N. tabacum* showed important differences. The most relevant differences were 3 regions of 240, 104, and 39 bp in the *C. odorata* plastome that were absent in *N. tabacum* and 2 regions of 64 and 5 bp in *N. tobacco* that were missing in *C. odorata* (Figure 2), representing 3% divergence and 93% coverage.

**Table 3.** nBLAST results for the *Cedrela odorata rrn16-rrn23* region. The species with higher identity from standard alignment are shown. *Nicotiana tabacum* values were obtained independently from a Clustal Ω alignment annealing with *C. odorata* sequence. ND: Non-determined.

| Species | Accession No. | Max score | Coverture | Identity |
|---|---|---|---|---|
| *Mangifera indica* | EF205595.2 | 5446 | 100% | 99% |
| *Rhodoleia championi* | EF207455.1 | 5330 | 100% | 98% |
| *Hamamelis japonica* | EF207445.1 | 5330 | 100% | 98% |
| *Liquidambar styraciflua* | EF207449.1 | 5284 | 100% | 98% |
| *Daphniphyllum* sp | EF207444.1 | 5284 | 100% | 98% |
| *Pterostemon rotundifolius* | EF207454.1 | 5280 | 97% | 99% |
| *Ribes americanum* | EF207456.1 | 5262 | 96% | 98% |
| *Heuchera micrantha* | EF207446.1 | 5256 | 100% | 98% |
| *Cercidiphyllum japonicum* | EF207443.1 | 5245 | 98% | 98% |
| *Kalanchoe daigremontiana* | EF207448.1 | 5186 | 100% | 97% |
| *Ceratophyllum demersum* | AM712908.1 | 4946 | 100% | 96% |
| *Citrus sinensis* | DQ864733.1 | 4900 | 99% | 99% |
| *Theobroma cacao* | HQ244500.2 | 4687 | 99% | 99% |
| *Nicotiana tabacum* | Z00044.2 | ND | 93% | 97% |

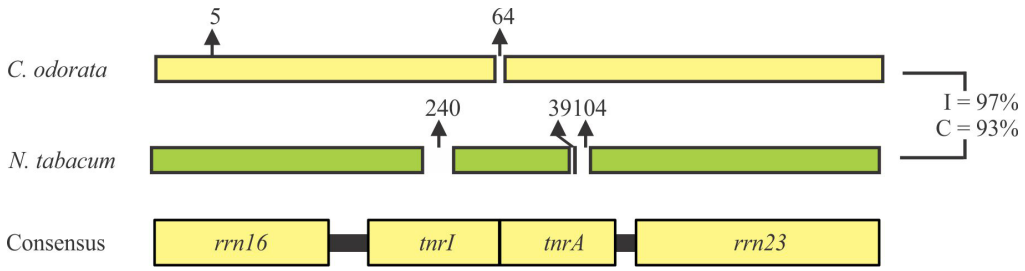


**Figure 2.** Graphic representation of *Cedrela odorata* and *Nicotiana tabacum rrn16-rrn23* sequences. Arrows with numbers represent the number of bp missing on each sequence with respect to the consensus. Clustal Ω annealing produced by identity (I) and coverage (C) values among sequences. Only InDels ≥ 5 bp are shown.

## pCBL4 plasmid assembly

The *rrn16-rrn23* region was cloned following a 2-step strategy. First, the ~2700-bp PCR product obtained from CBL-1/CBL-2 amplification spanning *rrn-trnI* region (AN: KF246583) was cloned into pUC19; yielding pCBL-1 (Figure 3A). In a separate experiment, the ~3000-bp amplicon obtained from CLB3/CLB4 primers corresponding to *trnA-rrn23* region (AN: KF246584) was cloned into pUC19 yielding pCBL3 (Figure 3B). Next, the *Xba*I/*Not*I fragment from the pCBL3 vector (~3000 bp) was cloned into pCBL1, resulting in pCBL4 (Figure 3C).

## Expression cassette design and cloning into pCBL4

An expression cassette was designed *in silico* containing 5' and 3' UTR sequences commonly used to regulate chloroplast expression of structural genes. Thus, the *rrn16* promoter was used to constitutively drive the expression of the *aadA* gene, which encodes an aminoglycoside adenine transferase that confers resistance to spectinomycin (Goldschmidt-Clermont, 1991), followed by the 3' UTR regulatory element *TpsbA* (Dufourmantel et al., 2004). A second expression cassette carrying the *gfp* reporter gene flanked by the *Sph*I and *Pst*I restriction enzymes sites was used to introduce the gene of interest. Gene expression in

this bicistronic operon is regulated by the 5' UTR P*G10T7* (AN: EU450674) (Oey et al., 2009) and the 3' UTR T*rps16* element (Farran et al., 2008). Once assembled *in silico,* codon usage was adapted based on previously reported chloroplast open reading frame sequences from *C. odorata*, eliminating undesired restriction sites for easy molecular manipulations. The resulting expression cassette (2352 bp long) (Figure 3D) was chemically synthesized and cloned into pCBL4 as a *Not*I insert to produce the final *C. odorata* chloroplast transformation vector, named pCBL5 (10,751 bp) (Figure 3E).

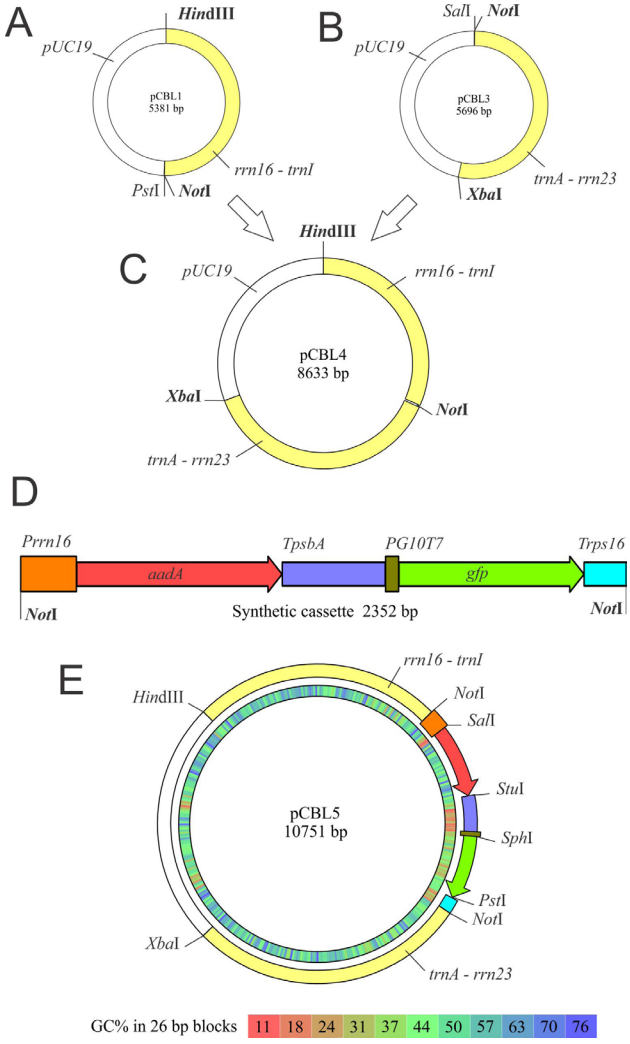


**Figure 3.** Schematic representation of the strategy employed to construct the *Cedrela odorata* chloroplast transformation plasmid (pCBL5). Parental plasmids pCBL1 (**A**), pCBL3 (**B**), and pCBL4 (**C**), harboring *rrn16* to *trnI, trnA-rrn23*, and *rrn16-rrn23* regions from *C. odorata* plastome, respectively. Double expression cassette sequence (**D**) was cloned into the *Not*I site from parental plasmid pCBL4 yielding the pCBL5 transplastomic vector (**E**). In all cases, restriction sites used for subcloning are shown in bold letters. The circle inside pCBL5 indicates the GC% content according to the reference bar shown at the bottom.

## DISCUSSION

We cloned and characterized the *rrn16-rrn23* chloroplast region from *C. odorata* and describe the design and construction of a pUC19-based chloroplast transformation vector named pCBL5 containing a tandem double expression cassette flanked by the plastidic homolog sequences from *C. odorata*, which is useful for directing chloroplast transformation via homologous recombination. The *rrn16-rrn23* sequence showed considerable differences from the *N. tabacum* plastome. Previous reports (Gao et al., 2012; Rigano et al., 2012) suggest that a lack of homology in flanking regions may severely affect the recombination rate if the chloroplast transformations of *C. odorata* and other *Meliaceae* trees used previously reported vectors based on the *N. tabacum* plastome sequence. Recombination efficiency is the first of 2 widely recognized bottlenecks for a successful transplastomic plant regeneration protocol; the regeneration protocol is the second relevant limitation. Specifically, for *C. odorata*, this second bottleneck is significant. Although a reproducible protocol for repetitive somatic embryogenesis has been already reported for *C. odorata* (Peña-Ramírez et al., 2010), as a recalcitrant woody species, the regeneration rate remains low compared to herbaceous model plants, such as tobacco or carrot. Therefore, it is relevant to use homologous sequences flanking expression cassettes for plastidic transformation. This will increase the frequency of homologous recombination and thus reduce the effect of the first bottleneck. In contrast, the high sequence identity between *C. odorata* and other important crops such as *Mangifera indica*, *Theobroma cacao*, and *Citrus sinensis*, suggests that the application of this plastid transformation vector may be extended to these and other valuable tree species to successfully express proteins in the chloroplast as an alternative to existing chloroplast transformation vectors based on *N. tabacum*.

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