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Cloning of chloroplastic rbcL gene region for plastidial vector in *Medicago sativa* L.

Somayeh Khalili Kolikand*, Bahram Baghban Kohnehrouz* and Ashraf Gholizadeh **
**Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, I.R. IRAN*
***Research Institute for Fundamental Sciences, University of Tabriz, Tabriz, I.R. IRAN*

(Corresponding author: Bahram Baghban Kohnehrouz)
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ABSTRACT: Alfalfa (*Medicago sativa* L.) is a valuable source of proteins, vitamins and minerals for animal nutrition. This crop is cultivated worldwide in more than 80 countries with area exceeding 30 million ha. Due to its high wet and dry performance and also storage capability, this plant is fit as host plant for production of recombinant proteins either by transgenic or transplastomic approach. Construction of species specific plastidial vector was targeted in this research for efficiently developing transplastomic alfalfa. First, the flanking regions (rbcL gene region) in plastid genome were selected for integration of cassettes and cloned in a plasmid vector by PCR method. In this job, total DNA of alfalfa seedling leaves was extracted and its quality and quantity were determined by agarose gel electrophoresis. The PCR amplification of the target fragment was carried out by designed specific primer set and the expected size was examined by 8% agarose gel. After extraction of target fragment from gel, it was ligated in pTG19-T vector. Colony PCR and enzymatic confirmation of recombinant plasmids were done and then sequencing was carried out. Finally, the resulted nucleotide data was compared with reported data in nucleotide bank indicating 96% similarity with *Medicago truncatula*.

Keywords: alfalfa, cloning, flanking region, plastidial vector, rbcL

INTRODUCTION

Alfalfa (*Medicago sativa* L.), is the most important forage crop grown in the temperate regions and is cultivated over 30 million hectares worldwide (USDA, 2013). Due to higher amounts of protein, vitamins and minerals in this plant, it is used not only for animal feed but for human as medicine (Yang *et al.*, 2008; Zhengyi *et al.*, 2011). Alfalfa has several characteristics, such as used for silage, hay, green chop, erosion control, honey production, rotational crop and nitrogen soil fixer (Monteiro *et al.*, 2003). More importantly, since alfalfa has high biomass production, this is ideal for genetic manipulation in production of recombinant proteins. Among these technologies, plastid genetic engineering has a number of merits over nuclear transformation in higher plants and algae too (Mata *et al.*, 2010), such as high protein content levels, lack of positional effect, maternal inheritance and gene containment, prokaryotic nature and the feasibility of expressing multiple proteins from polycistronic mRNAs (Bock, 2007; Corriveau and Coleman 1988; Maliga, 2002; Quesada-Vargas *et al*, 2005). Since the accomplishment of genetic transformation of chloroplast from two decades ago (Boyton *et al.*, 1988; Svab *et al.*, 1990), chloroplast genetic transformation offered several advantages in improving agronomic traits of crops (Daniell *et al.* 2005; Grevich and Daniell, 2005; Kumar *et al*, 2004) as well as producing value-added vaccines, antigens and biopharmaceuticals via plant or alga as bioreactors (Daniell *et al*, 2009; Specht *et al*, 2010).

Plastid expression vectors possessed left and right flanking sequences originated from plastid genome each with about 1kb in size (Verma and Daniel, 2007). These regions are used for foreign gene integration into plastid DNA through double homologous recombination events. The site of insertion in the plastid genome is determined by plastid DNA segments which are being used as flanking regions at both sides of selectable marker gene and the gene(s) of interest. Insertion of foreign DNA in intergenic regions of the plastid genome has been reported for 16 sites. Three of the insertion sites, trnV-3'rps12,trnI-trnA and trnfM-trnG, were most commonly used in more plants (Anil *et al*, 2011; Maliga, 2004). The trnV-3'rps12, and trnI-trnA sites are located in the 25 kb inverted repeat (IR) region of plastid DNA and thus a gene inserted into these sites would be rapidly copied into two copies in the IR region. In this study, cloning of the flanking rbcL regions from *Medicago sativa* was targeted for constructing the alfalfa specific plastidial vectors.

MATERIALS AND METHODS

Alfalfa (*Medicago sativa* L.) seeds of synthetic variety of Ghareyonjeh (Developed in Iran, Uni. of Tabriz) were planted in pots. Plants were grown at constant temperature (26ºC) with a 16-h light and 8-h dark cycle for 20 days.

A. DNA extraction

To prepare high quality DNA, the plants were incubated in a dark place for 24 hours for the full breakdown of cellular starch content. Total DNA was extracted from leaf sample at 2-3 leaves stage using CTAB method (Saghaei *et al,* 1984). Quality, quantity and concentration of the extracted DNA were evaluated by 0.8% agarose gel electrophoresis using DNA weight marker (SinaClon).

B. Designing the primers

Primers were designed according to the sequences of Medicago truncatula chloroplast genome available in nucleotide data bank (accession number: AC093544.8). This species is accounted for a close relative of alfalfa. Due to lack of information on Medicago sativa's chloroplast genome, truncatula's was downloaded from databank and used for designing the specific primers of (F: GAGCTCAATTTTCAAAGTCAACCCAGT' and R: AAGCTTAAACGGTCTCTCCAAC GCAT) by the online software of Primer-Blast. The cleavage sites of HindIII and sacI enzymes were embedded in the 5' end of the forward and reverse primers to ensure the cloning procedure.

C. PCR amplification and bacterial transformation

Total genomic DNA of alfalfa was used as a template for amplification of target fragment in concentration of 5ng.µL^{-1}. The PCR program consisted of an initial denaturing at 94°C for 5 min, continued by 35 cycles of 94°C for 60s, 59°C for 30s and 72°C for 60s, with a final extension step at 72°C for 2 min. About 1400bp target amplificant was eluted using extraction kit from agarose gel and used at concentration of 38ng.µL-1 in ligation reaction with pTG19-T vector at 4°C for 24 hours (Fig. 1).

In this work, amplificants were checked on 0.8% agarose gel and purified by gel extraction kit (Bioneer Co, South Korea). After reconfirmation and quantifying the eluted DNA, it was used in 1:3 molar ratios (Vector to Insert) for ligation reaction with pTG-19 vector by T4 DNA ligase.

Bacterial transformation was carried out with 5µL of ligation reaction using *E. coli* DH5' competent cells by Heat Shock method. Transformation mixture were grown overnight at 37°C in LB agar plates containing Ampicillin, X-gal and IPTG. Bacteria that transformed with vectors containing recombinant plasmid DNA produced white colonies and false bacterial cells without recombinant plasmids (i.e. only the vector) grew only blue colonies. The colony PCR and gel electrophoresis were used to confirm presence of target fragment in the white colonies. Single positive white colonies were selected and inoculated to 10 mL LB/ampicillin (100µg.mL^{-1}) broth medium for plasmid extraction. Plasmid extraction from overnight culture was performed using Plasmid extraction Kit (Bioneer, South Korea). Restriction enzyme (Fermentas Co.) digestion was performed to verify the presence of insert in plasmids. The final confirmation of DNA target sequence was done commercially using automated sequencing (Bioneer, South Korea).

RESULTS AND DISCUSSION

Successfully amplification of target fragment by truncatula based primers in alfalfa showed the first clue for nucleotide similarity between these two species at primer levels only. But comparison of full length sequence of rbcL fragment was attempted by cloning of this region and revealing its nucleotide sequence (Nucleotide sequences not shown).

Results of bacterial transformation showed that overnight incubation on of transformed mixture on LB agar containing 100 ?g.mL-1 ampicillin and IPTG/X-gal plate leads to growing white and blue colonies (Fig. 1). Blue colonies were originated from either small ligated fragments present in eluted fraction or any fails in vector supplied by company. This kind of problems is seen some times in the white clones too. For this reason from each transformation reaction, 3-5 white colonies were randomly chosen and subjected to direct colony PCR analysis.

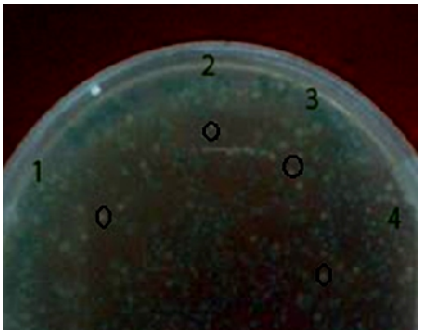

Fig. 1. White blue screening for cloning rbcL region in *E. coli*-DH5α on the LB agar/amp containing IPTG and X-gal. Colonies were grown overnight at 37°C.

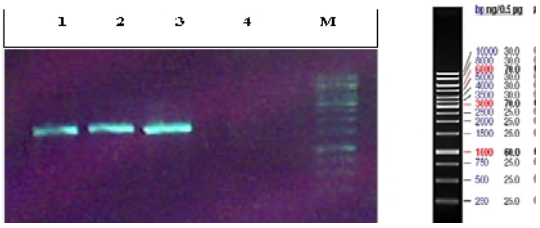

Fig. 2. Colony PCR for rbcL region. 1, 2 and 3)PCR confirmation of white colonies using specific primer gives the band size ~1400 bp, M) 1 Kb DNA Ladder (Fermentas); 4) Negative control.

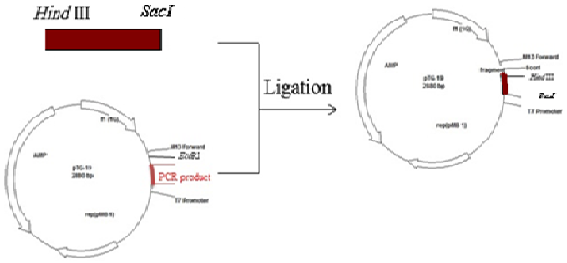

Fig. 3. Ligation reaction. using heat shock method. The pTG19-T is a linear-type vector which takes a circular form after ligation.

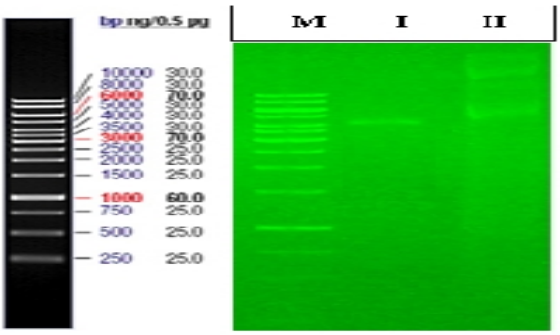

Fig. 4. Enzymatic confirmation of recombinant plasmid on electrophoresis. I) Digested fragment by single cut *Eco*RI gives band size of about 4300bp, II) Uncut recombinant plasmid DNA, M) DNA size marker (Fermentas).

Colonial screening of rbcL region by PCR using specific primer gives the band size ~1400 bp that was accorded with rbcL regional size in truncatula, too (Fig. 2). Before sequencing and for further confirmation of amplificant, extracted plasmids were digested with restriction enzyme EcoRI (Fig. 4).

Final confirmation of cloned DNA sequence was done using automated sequencing (Reseach Biolabs, Bioneer Co, South Korea). Sequencing results were analyzed using bioinformatics software (Fig. 5).

Pair-blast analysis of obtained sequence with species of truncatula's total genome showed not only 96% similarity with organellar plastid genome but also with nuclear chromosomes of 6 and 7. This could be happened by exchanges of genetic material among plastids and nuclei during symbiotic evolution (Gould, *et al*, 2008). The pair-blast of these data with other relative species also showed good amounts of similarities at 85 to 95 percents (Table 1).

Since the plastid transformation offers a good platform of foreign gene expression in higher plants, alfalfa could be the good candidate due to high level of protein production capacities from one hand and industrial/medicinal uses from other hand (Huan-Huan *et al*, 2009; Shaochen, 2011). By this work, we did the first step in plastid transformation by cloning of rbcL region of *Medicago* as flanking regions in alfalfa plastid vector. Till now, tobacco plastidial expression vectors were often used for alfalfa transplastomics (Shaochen *et al*, 2011). Since the cultivated varieties are synthetic worldwide (Flajoulot *et al*, 2005) some differences might be among nucleotide sequences of these variety kinds (Ruhlman *et al*, 2010; Smith, 1989) which could potentially interfere with efficiency of homologous recombination. For this reason using of species specific vector with 100 percent similarity with plastid genome guaranties the exact occurrence of homologous recombination. Here we did not decided on targeting inverted repeat (IRs) regions, because in legumes like alfalfa one of IR regions has been lost during their evolution and the expression potential of this region is remarkable than others (Saski *et al.* 2005).

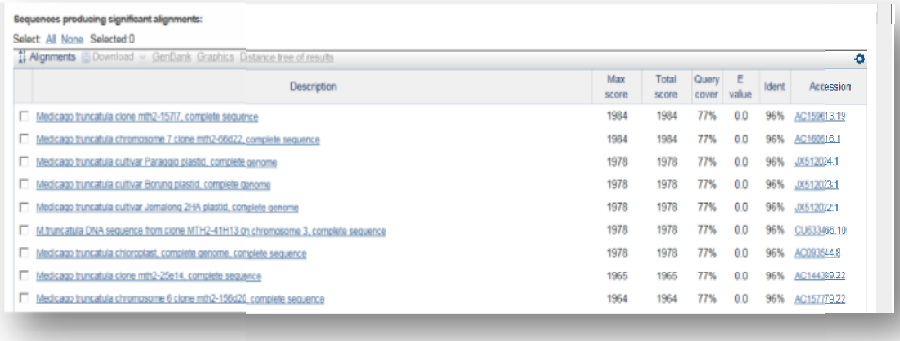

Fig. 5. Result of blastn showed 96% with *Medicago truncatula* chloroplast genome.

Table 1. The results of pair Blast of *Medicago sativa* with some other species

	TrbcL	CrbcL	LrbcL	PrbcL	GrbcL	FbcL	RrbcL
MrbcL	92	91	91	91	86	87	87
TrbcL		92	99	91	88	85	86
CrbcL			92	89	88	85	86
LrbcL				88	87	86	86
PrbcL					98	93	94
GrbcL						94	95

TrbcL (*Trifolium glanduliferum*), KJ788285.1; CrbcL (*Cicer arietinum*), EU835853.1 Lrbcl (*Lathyrus palustris*), HM029366.1; Prbcl (*Pisum sativum*), HM029370.1; Grbcl (*Glycine max*), DQ317523.1; Rrbcl (*Phaseolus vulgaris*), AC254328.1; Rrbcl (*Rosa odorata*) KF753637.1; Frbcl (*Fragaria vesca*), JQ396171.1

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