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Cloning of rbcL region from *Cicer arietinum* L. for species specific plastidial vector

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ABSTRACT: Chickpea is a good source of protein and carbohydrate among crop plants. There are some agronomic and economically important traits could be improved by transgenic and transplastomic research in this field crop. Despite the high yield potential of chickpea (over 4000 Kg.ha^{-1}) transgenic breeding is going to develop new cultivars against biotic and abiotic stresses as well as modern industrial usages. To do this by transplastomic approach chickpea specific plastidial vector is an essential need which is pursued in this work. The nucleotide sequence of chickpea organellar genome of plastid was used for determining the rbcL regional to design specific primer set using on line Primer blast software. Total genomic DNA was extracted from seedling leaves and used for PCR reaction as template. Expected length of amplified rbcL fragment was confirmed by agarose gel electrophoresis. The fragment extracted from agarose gel (0.8 %) using the gel purification and extraction kits and used for the T/A cloning. White blue screening used for recovering recombinant colonies followed by colony PCR, restriction analysis, sequencing and bioinformatic studies. Obtained results showed the hight similarity of cicer with trifolium by 95% and low similarity with vicia by 81%.

Keywords: chickpea, *Cicer arietinum* L, cloning, plastidial vector, rbcL

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is one of the oldest and most widely consumed legumes in the world due to relatively high protein content and wide adaptability as a food grain. It is the second most widely grown legume in the world (FAO, 2008).

Its protein quality is better than other legumes such as pigeon pea, black gram and green gram. Chickpea has significant amounts of all the essential amino acids except sulfur containing types, which can be complemented by adding cereals to daily diet. Chickpea has several potential health benefits and in combination with other pulses and cereals, it could have beneficial effects on some of the important human diseases like cardiovascular, type 2 diabetes, digestive diseases and some cancers. Overall, chickpea is an important pulse crop with a diverse array of potential nutritional and health benefits (Kaur and Singh, 2005).

There is a growing demand for chickpea due to its nutritional value. In the semi-arid tropics chickpea is an important component of the diets of those individuals who cannot afford animal proteins or those who are vegetarian by choice. Chickpea is a good source of carbohydrates and protein, together constituting about 80% of the total dry seed mass in comparison to other pulses. Chickpea is cholesterol free and a good source of dietary fiber, vitamins and minerals. Chickpea is grown in many parts of the world and yields a total of about 9.8 M t from an area of 11.1 M ha (FAO STAT 2009). Among the major chickpea producer countries, India, Pakistan, Turkey and Iran, has the most growing areas (Anonymous 2011). Despite the high yield potential of chickpea of over 4000 kg ha^{-1} (Singh 1987; Singh 1990), the actual yields are significantly lower which is considered to be due to biotic and abiotic stresses (Singh 1993). The protein quality of chickpea is better than other legumes like pigeon pea, black gram and green gram (Kaur and Singh, 2005). According to the size, shape and color of the seeds, two types of chickpea are usually acknowledged. Kabuli chickpea is large seeded with salmon white testa, is grown mainly in the Mediterranean area, central Asia and America and Desi chickpea is small seeded with a light brown testa, is cultivated mostly in India and east Africa (Rincon *et al.,* 1998). Chloroplast genetic engineering has got a number of merits over nuclear transformation in higher plants (Mata *et al.* 2010).

These advantages are targeted gene integration, high level heterologous protein expression and the feasibility of expressing multiple proteins from polycistronic mRNAs (Maliga, 2002; Bock, 2007; Kittiwongwattana *et al.,* 2007). It is also important that in the majority of flowering plants including major crops, inheritance of the plastid genome is through the maternal parent (Corriveau and Coleman, 1988), and transmission of plastids through pollen is very rare (Ruf *et al.,* 2007; Svab and Maliga, 2007). Since the accomplishment of genetic transformation of chloroplast from 25 years ago (Boyton *et al.,* 1988., Svab *et al.,* 1990), several reports are published in producing value-added vaccines, antigens and biopharmaceuticals via plant or alga as bioreactors (Daniell *et al.* 2005, 2009; Specht *et al.* 2010). The most prominent is that plastid transgene expression can be remarkably high and the desired recombinant protein may represent up to 70% of leaf total soluble protein (Daniell *et al.,* 2009; Oey *et al.,* 2009a; Ruhlman *et al.,* 2010).The plastid transformation vectors are developed through restriction and ligation of excised chloroplast DNA fragment using plasmid DNA in such a manner that it flanks the foreign genes so that the integration of gene(s) of interest and selectable marker gene is inserted at a precise and predetermined location in the plastome through homologous recombination. For this purpose, the plastid expression vectors usually possessed left and right flanking sequences each with about 1-2 kb in size from the plastid genome. Insertion of foreign DNA in intergenic regions of the plastid genome had been accomplished at 16 sites (Maliga, 2004). In the present study, we cloned the rbcL region to use it as flanking region for plastidial vectors in *Cicer arietinum.*

MATERIALS AND METHODS

A. Plant material

Kabuli chickpea seeds were planted in pots and were grown up at 26ºC temperature with a 16L/8D photoperiod for 15 days.

B. DNA extraction

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

C. Designing the primers

The specific primers (F: GAGCTCAATTTTCAAAGTCAACCCAGT-3' and R:AAGCTTAAA CGGTCTCTCCAACGCAT) were designed according to genome of plastid from *Cicer arietinum* (accession number: EU 835853.1) by the online software of Primer-Blast. The cleavage sites of HindIII and sacI were embedded in the 5' end of the forward and reverse primers to ensure the further sub cloning procedure.

D. PCR amplification and Bacterial Transformation

Total genomic DNA was used as a template for amplification of target fragment in the concentration of 5 ng/µl. The PCR program consisted of an in 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. The 1235 bps target amplificant was eluted from agarose gel using gel extraction kit (Bioneer, South Korea) and used at concentration of 38ng/µl in ligation reaction with pTG19-T vector by T4 DNA ligase at molar ratio of 1:3 vector to insert at 4°C for 24 hours. The recombinant colonies were verified by colony PCR, restriction digestion and sequencing at the end.

Plasmid transformation into DH5 alpha *E. coli* competent cells was done using heat shock method. To do this, the bacteria were freshly grown overnight at 37°Cin LB agar plates containing ampicillin, x-gal, and IPTG. Cells transformed with pTG vector containing recombinant DNA will produce white colonies; cells transformed with non recombinant plasmids (i.e. only the vector) grow into blue colonies. To perform a colony PCR, small piece of a single white colony was used as template DNA containing target and the PCR product was electrophoresed in 0.8 % agarose gel. After the first confirmation step, single white colonies were inoculated into 10 mL LB/ampicillin (100µg mL^{-1}) broth medium. The overnight broth culture was harvested by centrifuged at 130,000 rpm for 2 min and supernatant was discarded. Plasmid extraction was performed using Bioneer Plasmid Kit (Bioneer, South Korea). Restriction enzyme digestion was performed to determine the presence of the insert and final confirmation of rbcL fragment was carried out using automated sequencing facilities (Bioneer, South Korea).

RESULTS AND DISCUSSION

After overnight incubation of transformed competent bacterial cells on the LB agar containing 100 µg°mL^{-1} ampicillin, the white and blue colonies were appeared on IPTG/X-gal agar plate (Fig. 1). Blue colonies were the false and lack inserts and may either get in framed filled of lacZα by any artifact PCR product or company product fails while white colonies contained almost the right insert.

As a control in transformation process, competent *Escherichia coli* DH5α without insert was spread on ampicillin agar plate containing 100µg mL^{-1} and showed no colony growth after overnight incubation. For each transformation reaction, 5 white colonies were randomly chosen from the LB agar plate and subjected to PCR-colony analysis. Coloneis screening of rbcL gene region from *Cicer arietinum* by PCR using specific primer gives the band size ~1235 bp. Restriction analysis of recombinant plasmids was done with restriction enzyme EcoRI (Fig. 2). Obtained sequencing results were analyzed using bioinformatic software (Fig. 5).

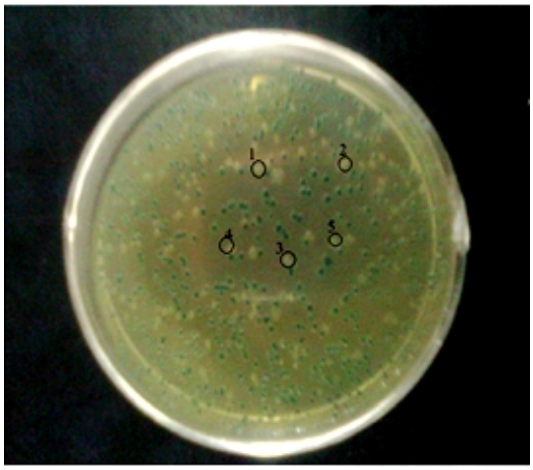

Fig. 1. White-blue screening of bacterial tranformants on the LB agar/amp. Overnight growth of *Escherichia coli* DH5alpha colonies after transformation with pTG-19 plasmid vector at 37°C.

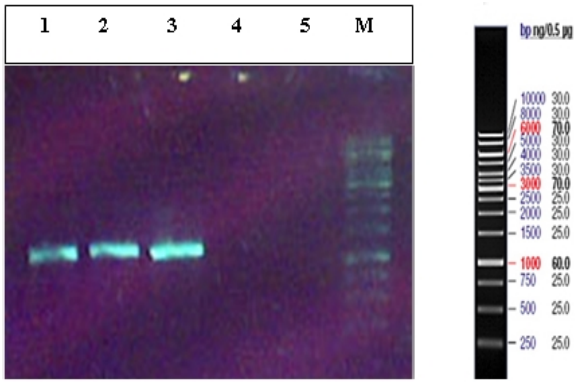

Fig. 2. Colony PCR for white colonies. Screening of white clones by PCR using specific primer for fragment size of ~1235bps. M, 1 Kb DNA Ladder (Fermentas).

Two types of colonies were observed as white and blue colonies on medium containing IPTG and X-gal.

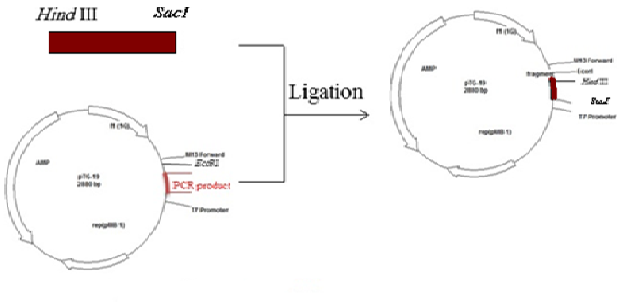

Fig. 3. Schematic presentation of Ligation reaction. The pTG19-T is a linear-type vector which takes a circular form after ligation.

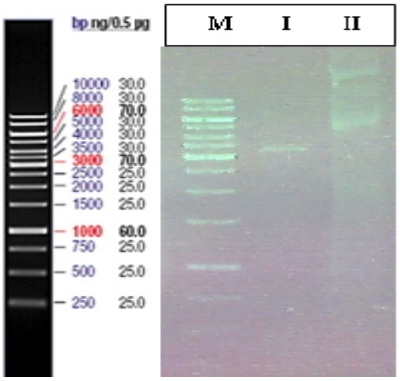

Fig. 4. Restriction analysis of recombinant plasmid. I) Linearized plasmid DNA with *Eco*RI, II) Uncut plasmid. M) DNA size marker 1Kb (Fermentas).

All of analysis done by colony PCR, cutting enzymes and sequencing showed that the rbcL gene region has been successfully cloned from *Cicer arietinum* L. with high similarity to trifolium and low similarity to vicia by 95% and 81% respectively among species of *Cicer arietinum*, (EU835853.1), *Trifolium strictum* (K J788292.1), *Lathyrus palustris* (HM029366.1), *Pisum sativum* (HM029370.1), *Vicia faba* (KF042344.1), *Glycine max* (DQ317523.1), *Eucalyptus umbra* (KC180778.1) and *Fragaria vesca*, (KC507757.1.).

The nutritional importance of chickpea is based on its high protein content of 25.3-28.9% (Hulse, 1991) which is being used more and more as an alternative protein source for the human food and animal feed. Genetic improvement of this crop by selection and hybridization led to introduce high yield cultivars that can grow and provide economic yield under salinity stress conditions to minimize the repercussions of the salinity (Ashraf and McNeilly, 2004).

Since chickpea (*Cicer arietinum* L.) is one of the oldest and most widely consumed legumes in the world with a wide adaptability potential to arid and semi-arid regions, any transgenic improvement could be useful in achieving new goals and aims in this crop. Along with this strategy, transplastomic approach could be attempted based on cloned flanking fragments from chickpea which considered as a frequently reported region from other species (Maliga, 2002; Bock, 2007). The plastid transformation offered a good platform of foreign gene expression in higher plants.

Chloroplast genetic engineering offers several advantages for improving agronomic traits of crops (Daniell *et al.* 2005) as well as producing value-added vaccines, antigens and biopharmaceuticals via plant or alga as bioreactors (Daniell *et al.* 2009; Specht *et al.* 2010). The most prominent is that plastid transgene expression can be remarkably high and the desired recombinant protein may represent up to 70% of leaf protein (Daniell *et al.*, 2009; Oey *et al.*, 2009a; Ruhlman *et al.*, 2010).

Fig. 5. Blastn results showing 96% similarity with *Cicer arietinum.*

Table 1: Pair Blast of *Cicer arietinum* rbcL with some other species.

	LrbcL	**PrbcL**	**VrbcL**	**GrbcL**	**ErbcL**	**CrbcL**
TrbcL	94	94	95	89	92	90
LrbcL		97	97	87	86	88
PrbcL			95	87	85	87
VrbcL				87	85	85
GrbcL					94	84
ErbcL						81

CrbcL(*Cicer arietinum*), EU835853.1; TrbcL (*Trifolium strictum*), K J788292.1; LrbcL (*Lathyrus palustris*), HM029366.1; PrbcL (*Pisum sativum*), HM029370.1; VrbcL (*Vicia faba*), KF042344.1; GrbcL (*Glycine max*) DQ317523.1; ErbcL (*Eucalyptus umbra*), KC180778.1; FrbcL (*Fragaria vesca*) KC507757.1.

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