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Cloning and transformation of rbc L gene in Solanum melongena L.

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Abstract

Plants would not able to undergo photosynthesis without rubisco, which is a powerful tool in phylogenic analysis, species diversity estimates, varietal identification and population analysis. Transformation is a parasexual method of introducing new genes into an organism. pGEM- T and pGEM –T easy vector, containing multiple cloning region, flanked by recognition sites for the restriction enzyme EcoR1 and Not I, are employed for cloning PCR product and to transform bacterial strain E. coli JM- 109 which is deficient in B- galactosidase activity due to deletion in both genomic and episomal copies of lacZ gene. Amp^r and lacZ gene are used for recombinant selection. On the other hand cloning of the gene for sequence divergence amongst species and genera is also a powerful tool in comparison to direct sequencing of PCR product. The rbcL isolated from two cultivars indigenous (A) & exogenous (B), separately. In both the cultivars the sequence of rbcL gene found more or less similar either cultivars taken from greenhouse (A)or from field (B). after sequencing, rbcL probe may be used for screenable related taxa as well as the taxa which have the low photosynthetic rate, the insertion of rbcL gene through recombinant DNA technology or other recent similar technologies, in higher amount may increase photosynthesis rate. Resulting this the crop may be improved either for qualitative or quantitative traits.

Keywords: Rubisco, Cloning, Transformation, E.coil, pEGM, pEGM® - T Easy vector systems.

INTRODUCTION

In the early1970s recombinant DNA techniques permitted unprecedented levels of detail in gennetic investigation by providing tools for genetic manipulations that were fundamentally different. From those previously available. The primary change was the ability to purify and amplify small, unique

DNA fragments from background of millions of virtually identical fragments. The discovery of restriction enzymes [1,2], the development of cloning vectors [3,4] and the isolation of modification enzymes e.g.; DNA ligases [5], provided many of the necessary tools.

On the other hand,Chloroplast DNA (cp-DNA) has proven to be a useful source of data for phylogenic reconstruction in all taxonomic level [6-9]. Early studies employed restriction- sites variation of cp-DNA where restriction–site maps were produced via–southern hybridization of radioactively labeled cp-DNA fragments [8,9]. Recent investigation have used nucleotide sequences of one or few chloroplast gene (e.g. *rbc*L, *ndhf* and *matk*) for phylogenetic inference [9,10].

Universal occurrence of rubisco in all land plants as prime fixer of Carbon which is one of the most essential elements in the chemistry of life underlines its importance. Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) initiates the process of photosynthesis by combining carbon dioxide with ribulose bisphosphate to form two molecules of 3-phosphoglycerate (PGA). PGA goes through a series of reactions to yield Glucose. Plants would not be able to undergo photosynthesis with out rubisco. Rubisco is composed of eight large and eight small catalytic subunits separately i.e. 477 amino acid residues and 123 amino acid residues. The large subunits are encoded by the chloroplast genome and the small subunits are encoded by the nuclear genome.

The role and importance of rubisco has been known by the scientists continue to investigate experiment. The variation in the nucleotide sequence of the genes coding for the large subunit of rubisco (*rbc*L) which exist in the chloroplast genome has warranted its use as a powerful tool in phylogenetic analysis, species diversity estimates, varietals identification and population analysis. Cloning of the gene for sequence divergence amongst different species and genera is a powerful tool in comparison to direct sequencing of the PCR products. The advantages of cloning are that the gene is there for longer periods of times and fixes the ambiguities of PCR reaction by miss incorporation of bases by *Taq* polymerase as well as resolves the problem of repeated amplification using genomic DNA which is critical in case of fossilized specimens as well as for materials which are exotic.

Brinjal *(Solanum melongena* L.) is one of the most popular vegetable crop in Central, South and Southeast Asia. Varied in different forms, colors and shapes [11,12]. Suggested that brinjal originated in India but has secondary centre of diversity in China. Expectedly, India possesses rich germplasm diversity of *Solanum melongena* and related species..Chloroplasts use the energy of light to oxidize water to give O_2 and generate ATP and NADPH. A series of so called dark reaction in the chloroplast stroma use ATP and NADPH to grab carbon di oxide from the air and build it into molecules, a process called carbon fixation.

Cloning and transformation

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Cloning refers to making an exact copy of an original form. The root of the word comes from the Greek word for 'twin' or 'breaking in two' – *klon* in biology, the term usually refers to asexual reproduction (like bacteria).

In biotechnology, the term often refers to 'cloned genes' - making multiple, identical copies of a gene (or protein) in bacteria or animal cells. In 1996, cloning took on a new meaning: a new individual grown from a single somatic cell and genetically identical to it. This technique is also called somatic cell nuclear transfer.

Transformation is a parasexual method of introducing new genes into an organism. These transgenes may give the new functions organism. Transgenic organisms may be selected by the ability to grow on a new food source, a metabolic poison (antibiotic), or by a change in rate of growth or in morphology, such as change in color or shape that may be seen by eye. Here *E. coli* gives new traits by transformation with a plasmid DNA carrying a functional gene. One trait is ampicillin resistance and the other trait is a new color due to the inactivation of the β-galactosidase gene. The transformed cells seem white whereas non-transformed cells seem blue. This property is utilized in their screening. The resistance is conferred to the bacterium by transforming it with a plasmid carrying the gene for lactamase. Lactamase is an enzyme that cleaves and destroys chemical structures with lactam rings, such as penicillin and ampicillin.

Recombinant DNA technology is important for learning about other related technologies, such as gene therapy, genetic engineering of organisms, and sequencing genomes. Gene therapy can be used to treat certain genetic conditions by introducing virus vectors that carry corrected copies of faulty genes into the cells of a host organism. Genes from different organisms that improve taste and nutritional value or provide resistance to particular types of disease can be used to genetically engineer food crops. With genome sequencing, fragments of chromosomal DNA must be inserted into different cloning vectors to generate fragments of an appropriate size for sequencing.

Cloning of PCR products

"Vector" is an agent that can carry a DNA fragment in to a host cell. If it is used for reproducing the DNA fragment , it is called a "cloning vector. It is used expressing certain gene in the DNA fragment called an expression vector. The pGEM®-T and pGEM®-T Easy vectors systems are convenient systems for the cloning of PCR products (Fig. 1 and 2). pGEM®-T Easy vector offer all of the advantages of the pGEM®-T vector systems with the added convenience of recognition sites for EcoR I and Not I flanking the insertion site. Thus several options for removal of the desired insert DNA with a single restriction digestion are provided.

Screenig on indicator plate

Both the pGEM®-T and pGEM®-T Easy Vectors contain multiple restriction sites within the multiple cloning regions. The pGEM®-T Easy Vector multiple cloning region is flanked by recognition sites for the restriction enzymes EcoR I, BstZ I and Not I, thus providing three single-enzyme digestions for release of the insert. The pGEM®-T Vector cloning region is flanked by recognition sites for the enzyme BstZ I. Alternatively, a double-digestion may be used to release the insert from either vector.

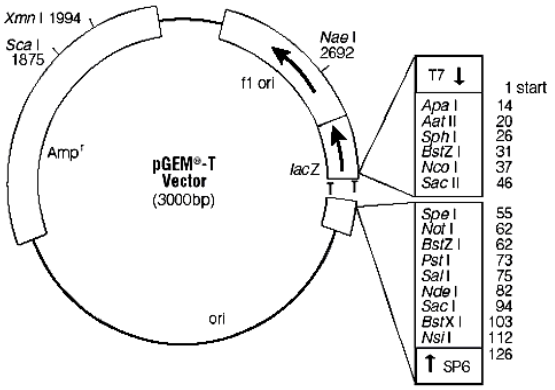

Fig 1. pGEM®-T vector circle map and sequence reference points.

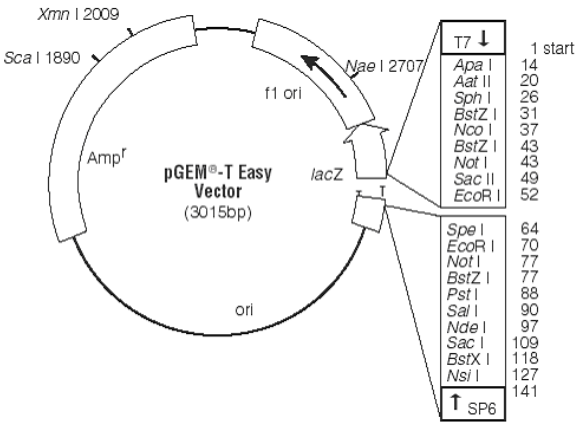

Fig 2. pGEM®-T Easy vector circle map and sequence reference points.

Transformation of E. coli

Bacterial Strain *E. coli* JM109 is a useful host for transformation of pGEM®-T vectors and for production of single-stranded DNA from M13 or phagemid vectors. The strain grows well and is transformed efficiently by a variety of methods. Because JM109 is recA– and lacks the *E. coli* K restriction system, undesirable restriction of cloned DNA and recombination with host chromosomal DNA are prevented. The endonuclease A– mutation leads to an improved yield and quality of isolated plasmid DNA.

JM109 is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the lacZ gene. The deletion in the episomal (F´ factor) copy of the lacZ gene (lacZΔM15) can be complemented by addition of a functional α-peptide encoded by a pGEM®-Z or pGEM®-Zf vector. If complementation does not occur, bacterial colonies are white. To maintain the F´, JM109 should be grown on minimal (M-9) media supplemented with 1mM thiamine.

MATERIALS AND METHODS

Genomic DNA isolation

The Young and juvenile leaves of two brinjal cultivars i.e. indigenous (A and exogenous (B)) were obtained from the green house as well as field of Delhi state respectively, NBPGR, Pusa campus, New Delhi in a brown paper bag and brought to the laboratory for DNA extraction. The leaves were carefully washed with distilled water, wiped in clean filter papers and allowed to air dry. The

required amount of sample leaf tissue was taken and the rest was sealed in another plastic bag and stored in cold place at 4°C. The samples were carefully labeled according to the plant from which the sample was taken. Plant DNA was isolated following the CTAB method [13]. The quantity of DNA isolated was improved by RNAase treatment with phenol chloroform and isoamyl alcohol and further desolved in TE buffer and the quantity of the purified DNA was estimated using DyNa QUANT Fluorimeter and DNA estimates were 475 ng /ul for sample A and 635 ng /ul for sample B .Universal primers were used for amplification for rbcLgene. The sequence of primer used as follows.

F-5'ATGTCACCACAAACAGAAACTAAAGC 3'

R-5' CTTCAACAAGCAGCAGCTAGTTCAGGACTCC 3'

Plasmid isolation and restriction of recombinent plasmid

The plasmid DNA was isolated from host *E.coli* JM 109 cells by the alkaline lysis miniprep method.The recombinant plasmid isolated was restricted with Eco RI' which flank the insert in case of pGEM-T easy vector.

Sequencing of the insert DNA (rbc L gene)

This data as produced above is utilized for sequencing of the insert DNA segment i.e. *rbc* L gene. The sequencing of the insert DNA cloned in the plasmid vector was done manually by Sanger's dideoxy method [14], using reader TM DNA sequencing kit from MBT fermentar. The primers used for sequencing were universal primer (T7, SP6, M13/pUC forward and reverse primer).

The isolation of large subunit of Rubisco (*rbc* L) gene, gene cloning and transformation in *E. coli* was manually done in the laboratory of "National Research Centre on DNA Fingerprinting" NBPGR, Pusa Campus, New delhi and the sequencing of the insert (i.e. *rbc* L gene) is presented in the paper carried out by a leading multinational company "GENEI, Bangalore"

RESULTS AND DISCUSSION

Genomic DNA extraction

Isolation of DNA from fresh leaf tissues were tried using CTAB method outlined in materials and methods and isolated DNA was checked on 0.8% agarose for purity assessment.

Quality assessment

The quality of DNA isolated was checked using 0.7 per cent agarose gel which showed that the DNA isolated from the above method was intact. DNA having more than 10kb can be resolved easily using 0.7 per cent agarose gel. The DNA was checked on and the extracted DNA was found to have no smears and there was no hanging of DNA in the wells. After checking the purity the dilution of 5 ng per µl were made for each of the samples (Fig. 3).

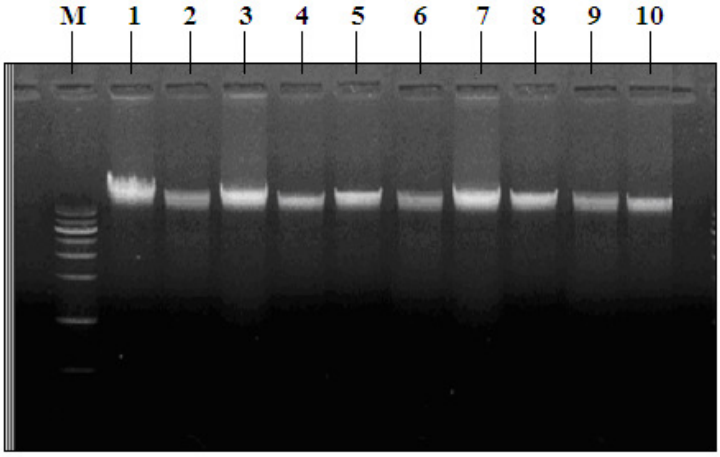

Fig 3. Quality assessment of purified Genomic DNA on gel. (M- 1000bpMarker, Lane- 1-10-Purified genomic DNAs).

Amplification

An approximately 1550bp segment of double stranded DNA contaminating the complete coding sequence for the chloroplast gene *rbc*L was amplified using the Taq polymerase-mediated polymerase chain reaction (PCR). Two synthetic nucleotides were designed for use as amplification primers. The 5' primer is based on the first 26 nucleotide positions of the *rbc* L coding sequence and is two fold degenerate at position 18 to account for the only difference between the maize and tobacco sequences in this region. The 3'is based on a 24bp sequences that contains part of a stem-loop structure beginning 103bp beyond the coding sequences termination for *rbc*L in tobacco.

Reaction parameters

It is important that optimal concentration of PCR mixture is required to produce informative RAPD fingerprints. The reaction volume of each sample consists of DNA template (25 ng), $MgCl_2$ (1.5 mM), dNTP's (100-150 µM), primers (5 picomols), Taq polymerase 3 unit in 1X assay buffer. These concentrations per reaction were found to be optimum for obtaining intense, clear and reproducible banding pattern in brinjal.

Gel purification of PCR fragment, ligation and transformation

PCR amplified rbcL gene is gel purified (Fig. 4). The pGEM®-

Tvectors are prepared by cutting the pGEM® -5Zf(+) and pGEM®-T easy vectors , respectively, with Eco R V and adding a 3'- terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of PCR product. Adenylation at 5' end of GEL purified PCR fragment is simultaneously employed to improve the possibility of lygation. Competent bacterial cells (E. coli stran JM 109) are transformed with the pGEM®-T vector containing rbcL gene via heat shock treatment.

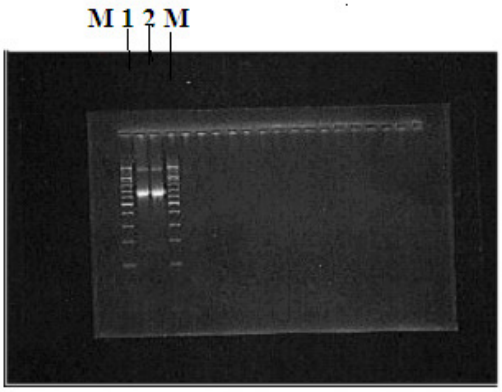

Fig 4. PCR Amplified and gel purified *rbc* L gene. (M- 200bp Marker (O' Range Ruler), Lane- 1 & 2 gels purified *rbc* L gene (~1400bp).

Screening of transformants

Successful cloning of an insert into the pGEM®-T and pGEM®-T Easy Vectors interrupts the coding sequence of β-galactosidase, recombinant clones can usually be identified by color screening on indicator LB plates (Fig. 5). However, the characteristics of PCR products cloned into the pGEM®-T and pGEM®-T Easy vectors can significantly affect the ratio of blue: white colonies obtained following transformation of competent cells. Clones that contain PCR products, in most cases, produce white colonies, but blue colonies can result from PCR fragments that are cloned in-frame with the lacZ gene. Such fragments are usually a multiple of 3 base pairs long (including the 3´-A overhangs), and do not contain in-frame stop codons. There have been reports of DNA fragments of up to 2kb that have been cloned in-frame and have produced blue colonies.

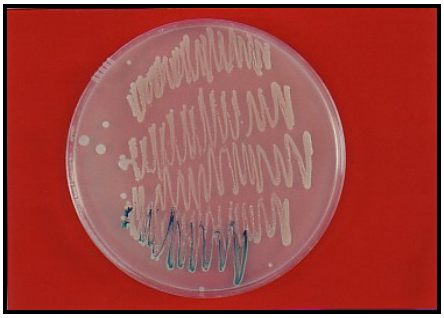

Fig 5. Streaking of *E. coli* cells. (White appeared-transformants, blue appeared –non transformants).

Isolation of plasmid DNA

E. coli cells were cultured in 2XYT medium, though L B broth may also be used for culture of these cells but the 2XYT medium provide good yield of cells. *E. coli* cells were harvested and plasmid DNA was isolated by the alkaline lysis minipreparation method (Fig. 6).

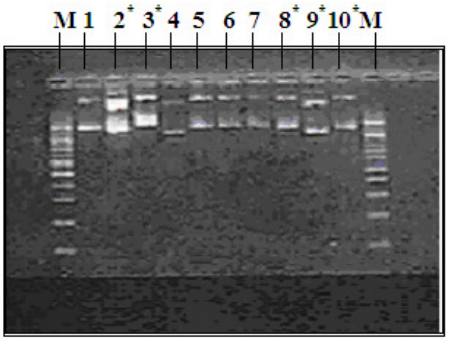

Fig 6. Isolated plasmids DNA run on gel electrophoresis. M - 200bp Marker (O' Range Ruler), Lane 1-10- Plasmid DNA (~3kb, appeared in high intensity, while insert~1400bp with low intensity). (Samples designated with esterisk are negative plasmid without insert, rest are positive with

insert)

Quantification of Plasmid DNA

Quality of the purified DNA was using DyNa QUANT fluorimeter, which is based on Beer –Lbambert's law as described in the following table.1.

Table 1. Quantification of different plasmid DNA Samples calibrated by DyNA QUANT fluorimeter

Plasmid DNA Samples	Mean of three replicates(ng/ml)
A-1	93.33
A-2	70.00
A-3	124.66
A-4	65.33
A-5	132.67
B-1	50.33
B-2	170.00
B-3	112.67
B-4	87.00
B-5	95.67

Restriction digestion andagarose gel electrophoresis

The recombinant plasmid isolated was restricted with Eco R I and the insert were checked on 1.2 % agarose gel. 50bp and 100bp ladder was loaded to check the band of plasmid and insert. The insert gene and plasmid DNA is distinguished separately on the gel as the insert gene is ~1400bp in length and the plasmid is around 3kb in length (Fig. 7).

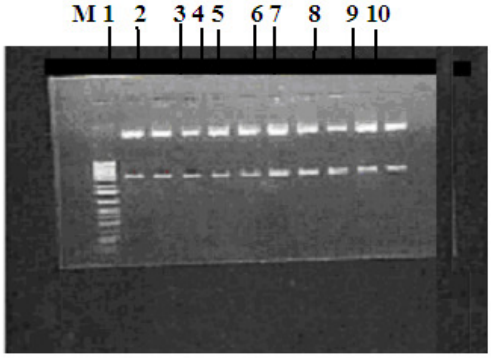

Fig 7. Restriction check of plasmid DNA with *Eco* R I. M – 100bp Marker, Lane 1-10- Plasmid DNA (~3kb, appeared in high intensity, while insert~1400bp with low intensity).

The sequencing of *rbc* L gene isolated from one indigenous and one exotic collection of *Solanum melongena* was manually carried out in "GENEI, Bangalore" as mentioned in the materials and methods and the data of sequencing is hereby presented in the table 2 and 3.

In the both cases either gene isolated from the indigenous collection or from the exotic collection of *Solanum melongena*; the data of sequencing shows that *rbc* L gene is ~ 1400bp in length and also shows more or less similar count of nucleotides i.e. A, C, G, T.

Comparison of these counts of nucleotides in different collections is further utilized in phylogenetic analysis, species diversity estimates, varietals identification and population analysis.

Table 2. Sequencing of *rbc* L gene isolated from indigenous collection of *Solanum melongena.*
Length: 1434 BP, A Count: 396, C Count: 272, G Count: 353, T Count: 413, Others Count: 0 CRC:946139196

Sequence	atgtcaccacaaacagagactaaagcaagtgttggattcaaagctggtgttaaagagtac aaattgacttattatactcctgagtaccaaaccaaggatactgatatattagcagcattc cgagtaactcctcaacctggagttccacctgaagaagcaggggccgcggtagctgccgaa tcttctactggtacatggacaactgtatggaccgatggacttaccagtcttgatcgttac aaagggcgatgctaccgcatcgagcgcgttgttggagaaaaagatcaatatattgcttat gtagcttacccctttagacctttttgaagaaggttccgttaccaatatgtttacttccatt gtaggtaacgtatttgggttcaaagccctgcgcgctctacgtctggargatctgcgaatc cctcctgcttatgttaaaactttccaaggtccgcctcatgggatccaagttgaaagagat aaattgaacaagtatggtcgtcccctgttgggatgtactattaaacctaaattggggtta

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Table 3. Sequencing of *rbc* L gene isolated from exotic collection of *Solanum melongena.*

Length: 1434 BP, A Count: 397, C Count: 272, G Count: 355, T Count: 410, Others Count: 0 CRC:982597193

Sequence	>ABD47065 atgtcaccacaaacagagactaaagcaagtgttggattcaaagctggtgttaaagagtac aaattgacttattatactcctgagtaccaaaccaaggatactgatatattggcagcattc cgagtaactcctcaacctggagttccacctgaagaagcaggggccgcggtagctgccgaa tcttctactggtacatggacaactgtatggaccgatggacttaccagtcttgatcgttac aaagggcgatgctaccgcatcgagcgtgttgttggagaaaaagatcaatatattgcttat gtagcttacccctttagacctttttgaagaaggttccgttaccaatatgcttacttccatt gtaggtaacgtatttgggttcaaagccctgcgcgctctacgtctggaagatctgcgaatc cctgttgcttatgttaaaactttccaaggcccgcctcatgggatccaagttgaaagagat aaattgaacaagtatggtcgtcccctgttgggatgtactattaaacctaaattggggtta tctgcaaaaaactacggtagagctgtttatgaatgtcttcgcggtggacttgattttacc aaagatgatgagaacgtgaactcacaaccatttatgcgttggagagatcgtttcttattt

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gtatggaaagagatcgtatttaattttgcagcaatggacgttttggataagtaa

CONCLUSION

In the present study effort were made largely towards the isolation and cloning of rubisco gene (rbcL) gene taking one indigenous A and other B from brinjal cultivar using the p-GEM T vector. Chloroplast carries genetic information for the larger subunit of ribulosel, 5-biphosphate carboxylase that play a central role in photosynthesis and also 30-40% of the total leaf protein in many plants. The ribulosel, 5-biphosphate carboxylase gene in comparatively most abundantely found protein on earth because of rubisco is a very insufficient catalyst with allowed specific activity (1 mol/min-1 protein) therefore large amount of rubisco are required to support high photosynthetic rate.

Sequencing data of both collection thus generated revealed out that the gene comparises approximately 1400bp and A, C, T, G, count is more or less similar in both collection of brinjal cultivars. Since the photosynthesis rate depend upon the amount of rbcL gene. Thus we can say that the rate of photosynthesis quite similar either cultivar grown in green house or in field. On the other hand rbcL sequence may be utilized for the screening of population, species diversity estimates & vertical identifaction of related taxas. If the related taxas posses low productivity due to low photosynthetic rate, the insertion of rbcL gene in higher amount may increase the photosynthetic rate, therefore, may be utilized in crop improvement.

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