Research Article

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Isolation and characterization of Ribulose 1, 5 – bisphosphate carboxylase small subunit gene from *Hevea brasiliensis*

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Abstract

Hevea brasiliensis, the major source of natural rubber is a member of the family *Euphorbiaceae*. In the present study an attempt has been made for the isolation and characterization of ribulose 1, 5 – bisphosphate carboxylase small subunit gene (rbcS) from *Hevea brasiliensis* (clone RRII 105). The RuBisCO gene was PCR amplified from the genomic DNA of *Hevea brasiliensis*, cloned in Topo TA® vector and sequenced. The sequence was compared with the earlier reported cDNA sequence of RuBisCo from *Hevea brasiliensis*. The sequence revealed the presence of a 1.7 kb gene which includes a short sequence of 176 bp corresponding to a signal peptide. Two introns of 1115 bp and 97 bp were present in the genomic sequence. The first intron was seated in the sequence coding for the transit peptide. The exon region of the isolated genomic sequence was found to be identical with the reported cDNA sequence. Signal P prediction results showed a signal peptide probability of 0.991. The existence of signal peptide and the identification of the promoter can be exploited for a number of transgenic applications including targeted transport of various proteins in *Hevea* leaf chloroplasts.

Key words: Hevea brasiliensis, promoter, RuBisCO, signal peptide

Introduction

Hevea brasiliensis has been established as the only commercial source of natural rubber due to its good yield and excellent physical properties of the products developed (Archer and Audley, 1973). The inventions of novel biotechnological tools for gene transfer and the introduction of agronomically desirable traits has added new dimensions to crop improvement programmes in *Hevea*. The ability to obtain specific expression of foreign or native genes in *Hevea* opens up the possibility of improving the crop commercially by genetic manipulation for increased stress tolerance, rubber yield and other agronomic traits. The lack of genetic diversity of this crop makes it highly susceptible to pathogenic attack and other failures.

RuBisCO is the most abundant protein in the photosynthetic organisms (Gutteridge and Keys, 1985). RuBisCO is the bi-functional enzyme playing the key role of catalyzing the carboxylation or the oxygenation of ribulose 1, 5-bisphosphate (RuBp) with carbon dioxide or oxygen in the Calvin cycle. Magnesium ions (Mg^{2+}) are needed for enzymatic activity. In this way non-assimilatory form of atmospheric carbon dioxide is made available to organisms in the assimilatory form of carbohydrates through the ene - diol mechanism. In algae and other higher plants eight large subunits containing the catalytic site, are supplemented by eight small subunits which are thought to play a regulatory role. The large subunit is synthesized in the chloroplast; where as the small subunit is made on cytoplasmic 80 S ribosomes and imported into the chloroplast

RuBisCO is often rate limiting for photosynthesis in plants, and hence it is possible to improve photosynthetic efficiency by modifying RuBisCO genes in plants to increase its catalytic activity and/or decrease the rate of the oxygenation activity (Spreitzer and Salvucci, 2003). Approaches have been initiated to transfer RuBisCO genes with varying specifity from one organism to another for increasing the level of expression of the RuBisCO subunits and to alter RuBisCO genes so as to increase specificity for carbon dioxide or otherwise to increase the rate of carbon fixation (Parry *et al.*, 2003). One particularly interesting avenue is to introduce RuBisCO variants with naturally high specificity values such as the one from the red alga *Galdieria partita* into plants. This would be expected to improve the photosynthetic efficiency of crop plants (Whitney and Andrews, 2001). Important advances in this area include the replacement of the tobacco RuBisCO enzyme with that of the purple photosynthetic bacterium *Rhodospirillum rubrum* (Andrews and

Whitney, 2003). Although the rbcS genes have been cloned in higher plants, the chromosomal loci of individual members of the rbcS genes have not been well examined (Dean *et al.*, 1989).

In the present study, an attempt has been made for the isolation and characterization of ribulose 1, 5 – bisphosphate carboxylase small subunit gene (rbcS) from *Hevea brasiliensis* (clone RRII 105). The genomic sequence can be later used for the characterization of the promoter for the rbcS gene as well as to derive the signal peptides which opens the door for developing transgenic rubber trees with increased leaf specific expression of agronomically important genes.

Materials and Methods

DNA isolation

The plant material used in the present study was collected from the germplasm nursery of Rubber Research Institute of India. Genomic DNA was isolated from the young, uninfected leaves of *Hevea clone* RRII 105 following the modified CTAB protocol of Doyle *et al.* (1990). The isolated DNA was checked for quality via agarose gel electrophoresis

PCR amplification of RuBisCO small subunit gene from the *Hevea* clone RRII 105

For the amplification of rbcS gene, primers were designed based on a earlier reported cDNA sequence (Chye *et al.*, 2004; Genbank accession no: M60274). The nucleotide sequence of the designed primers were as follows:

Forward primer: 5' ATG GCT TCA TCT ATG CTTT C 3'

Reverse primer: 5' TTA TTC AGC GCC TTT AGG CTT GTA 3'

The PCR was carried out in 20 µl reactions, which contained 1 X buffer having 1.5 mM $MgCl_2$ (pH 8.3), 100 µM of each dNTP's, 0.5 unit of *Taq* DNA polymerase (M/ S Bangalore Genei, India), 20 ng of template DNA (genomic DNA from leaf) and 250 nM of primers in a thermal-cycler (Perkin Elmer 480). The PCR conditions were; initial denaturation at 94°C for 3 minutes followed by 36 cycles with denaturation at 94°C for 1 minute, annealing temperature of 60°C for 1 minute and an extension at 72°C for 1 minute. The final extension was carried out at 72°C for 10 minutes. Amplified products were separated in a 1 % agarose gel.

Cloning and Sequence Characterization of the rbcS gene

The amplified rbcS fragment was eluted out using the DNA gel band purification kit (M/S Amersham Pharmacia Biotech, USA) following the manufacturer's instructions. The amplified product after purification was used directly for cloning. Cloning of the amplified genomic DNA was carried out using TOPO TA® cloning kit for sequencing (M/S Invitrogen Life Technologies, USA) following the manufacturer's protocol. The cloned gene was transformed into the chemically competent *E. coli* (DH5a) cells supplied along with the kit. The transformed cells were plated on the LB agar plates containing 50 µg/ ml ampicillin and the positive colonies were selected.

Confirmation of transformation

A few colonies were selected after 16 hours of incubation and colony PCR was done to identify positive clones. A tip touch of individual colonies were used as template. The initial denaturation temperature was increased to 96° C for 10 minutes. Plasmids were isolated from positive clones using Perfect prep Plasmid ı Kit (M/S Eppendorf, USA). The confirmation of transformation was done by amplification of the insert from the plasmids obtained. About 2 ng of plasmid DNA was used as template in a PCR reaction of 30 cycles with the same primer used to amplify the rbcS gene. Amplification was checked through 1.5 % agarose gel electrophoresis.

Characterization of the DNA sequence for rbcS gene

The recombinant plasmids obtained were sequenced at M/S Macrogen, Korea. Sequence was aligned using the CLUSTAL W software at the EMBL EBI site (Thompson *et al.*, 1994) with the reported cDNA sequence of rbcS sequence (Accession no: M60274). BLAST analysis (Altschul *et al.*, 1997) was also performed to compare the obtained sequences with the reported sequence from other species.

Results and Discussion

The genomic DNA isolated from the leaves (Fig. 1) was used for the isolation of the gene through PCR amplification using the designed gene specific primers PCR amplified product had a molecular weight of approximately 1.7 kb (Fig. 2). The amplified product was cloned in TOPO TA vector (M/S Invitrogen life technologies, USA). Positive transformants were identified through colony PCR (Fig. 3). The isolated plasmids from the positive transformants (Fig. 4) were sequenced to characterize the gene insert.

The sequence analysis showed that the isolated gene fragment is having 1762 base pairs with two introns

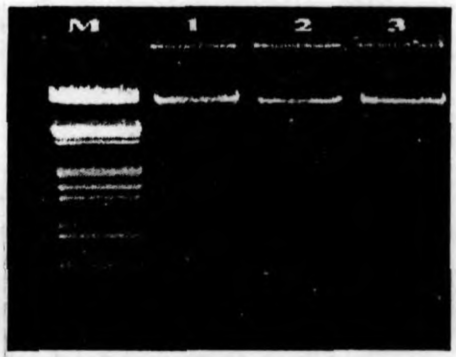

Fig. 1. DNA Isolation from RR III105

(183 – 1298bp, 1432 – 1529 bp) (Fig. 5). The genomic sequence has been registered in NCBI with the accession no: EU449763. The sequence comparison using CLUSTAL W of the isolated sequence with the reported cDNA sequence of rbcS gene (NCBI accession No: M60274) showed 100 % sequence homology in the 549 basepair exon region with (Fig. 6). The gene codes for a protein of 183 amino acids. BLAST analysis revealed sequence homology with rbcS mRNA sequence (M60274) from *Hevea brasiliensis* (100%)and from other species like *Manihot esculenta* (88%), *Oryza sativa* (79%) etc. Bioinformatic tools like 'Signal P' was used for the prediction of signal peptides present in the sequence. Signal P prediction results showed a signal peptide probability of 0.991 (Fig. 7). The existence of transit peptide sequence (176bs) is located between 27 – 203 nucleotides within the isolated genomic sequence.

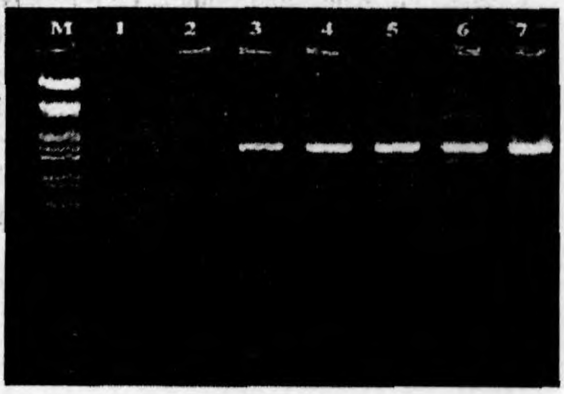

Fig. 2. PCR amplification of RuBisCo small subunit
M-marker
Lanes 1&2-primer controls

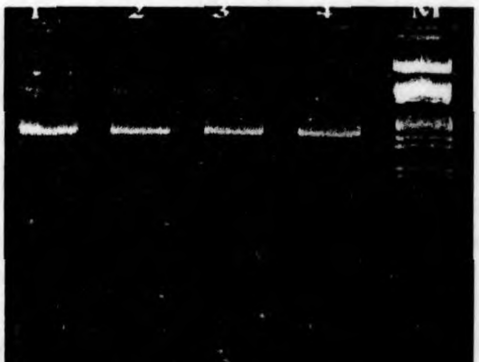

Fig. 3. Colony PCR of trasformed colonies
M-Marker

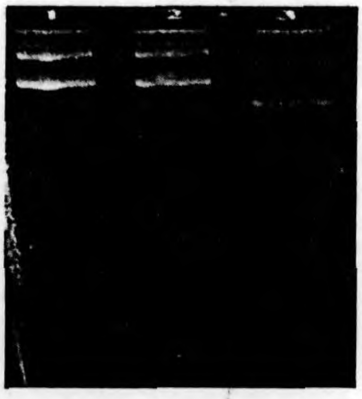

Fig. 4. Plasmid 1&2 : Plasmid osolation from transrerred colonies
Lnaes 1&2:Plasmid osolated from the positive transformed cononies
Lane 3 : Control plasmid

Different proteins synthesized in the cytoplasm find out their target to corresponding organelle by means of a transit peptide present in the N- terminal end. This principle is widely used in transgenic technologies (Gupta *et al.*, 1993). The transit peptide aids in the translocation of the protein to the respective organellles. The role of these nuclear-encoded small subunits in RuBisCO structure and function is not yet fully understood. It is assumed that the small subunits may have originated during evolution to support large-subunit

Fig. 5. Genomic sequence of RuBisCO small subunit gene from the clone RRII 105

Start codon
Exons
Introns
Nucleotide sequence for the Transit peptide
Stop codon

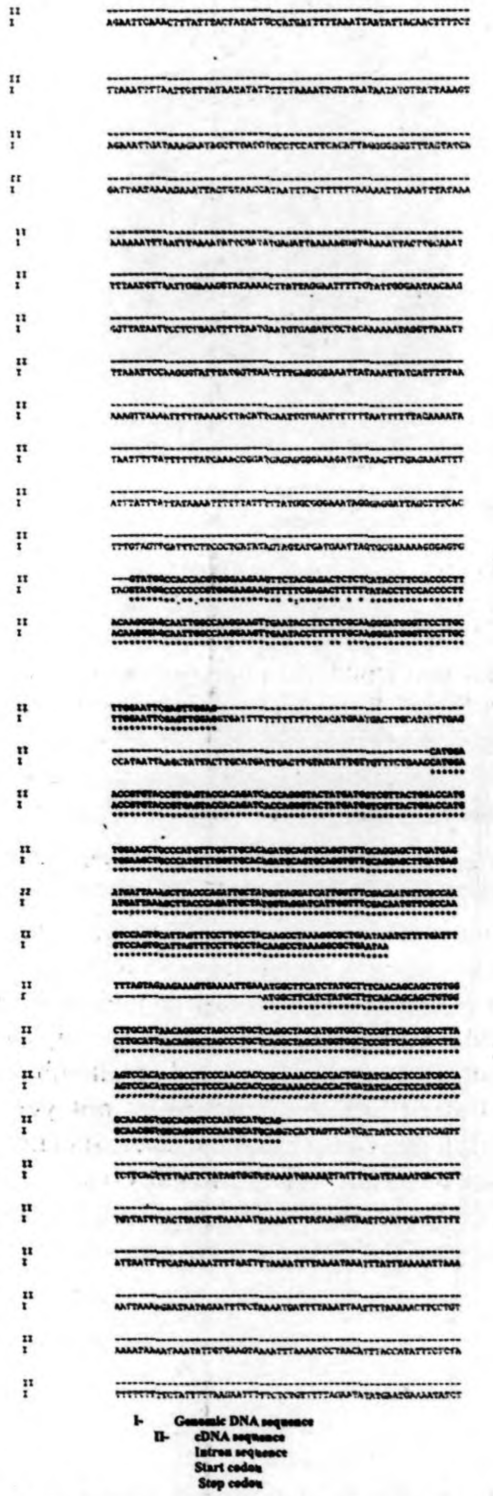

Fig. 6. CLUSTAL W multiple sequence alignment of RuBisCO small subunit gene (rbcS) from the clone RRII 105 with that of thereported cDNA sequence (M60274)

active sites, but the extensive divergence of structures among prokaryotes, algae, and higher plants seems to indicate that small subunits have more-specialized functions. Further, plants and green algae contain families of differentially expressed small subunits, raising the possibility that these subunits may regulate

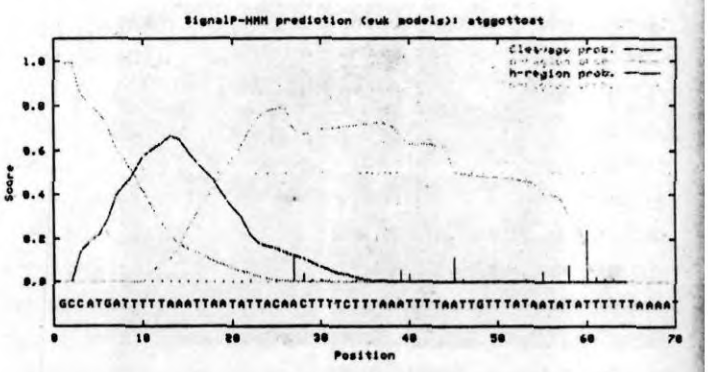

Fig. 7. Signal peptide prediction
>atggcttcat
Prediction: Signal peptide
Signal peptide probability: 0.991
Signal anchor probability: 0.002
Max cleavage site probability: 0.238 between pos. 59 and 60

the structure or function of RuBisCO. Studies of interspecific hybrid enzymes have indicated that small subunits are required for maximal catalysis and in seve cases contribute to CO_2/O_2 specificity. Although geneuc engineering of small-subunit remains difficult in higher plants, directed mutagenesis of cyanobacterial and green-algal genes has identified specific structural regions that influence catalytic efficiency and CO_2/O_2 specificity. It is thus apparent that small subunits will need to be taken into account and strategies developed for creating better RuBisCO enzyme. As any other nuclear encoded chloroplast protein rbcS is also synthesised on cytosolic ribosomes, usually as precursors with a transient N-terminal extension called a transit peptide and imported into chloroplasts.

Some features common to transit peptides found in *Hevea* rbcS transit peptide include the abundance of the hydroxylated aminoacids serine and threonine (30.5%); the presence of small hydrophobic aminoacids alanine and valine (22 %) and the paucity of aci aminoacids (only a single aspartic acid residue at position 14) (Chye *et al.*, 1991).

Bowler *et al.* (1991), reported a chimaeric gene construct, where signal sequence of mitochondrial MnSOD replaced with chloroplastic signal sequence of RuBisCO small subunit protein and the resultant transgenic plants were found to be resistant to oxidative damage induced by light dependent methyl viologen and ozone. Studies on the functional characterization of sequence motifs in *Arabidopsis* suggested that the transit peptides contain multiple motiffs and that some of them act in concert or synergistically (Lee *et al.*, 2006). Secondary or tertiary structural features of precursor proteins are also important for protein import, along with the transit peptide for the import of protein in the chloroplasts (Lubben *et al.*, 1989). This principle could be better exploited to over express various stress related genes in *Hevea* leaf chloroplasts. The isolation of the rbcS gene from *Hevea* can also be utilized to further characterize the 5' regulatory sequence of the gene. Isolation of the regulatory elements provide a way to express transgenic proteins in *Hevea* leaf using a combination of rbcS gene promoter and the gene of interest. This can be exploited for the over expression of proteins like PR proteins (eg. Chitinase and b-1, 3-Glucanase) which play a major role in the defense mechanism of the host plant against the foliar fungal pathogens. Since most of the PR proteins reported from *Hevea* are potent allergens (Allenius *et al.*, 2002), the over expression of these genes in the latex has serious allergic implications. The leaf specific promoter of RuBisCO can be utilized for the leaf specific over expression of these defense genes in the leaf without causing any harm.

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References

Alenius, H., Turjanmaa, K. and Palosuo, T. 2002. The Natural rubber latex allergy. *Occup Environ Med.* **59**: 419–424.

Altschul, S. F., Thomas, L. M., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402.

Andrews, T. J. and Whitney, S. M. 2003. Manipulating ribulose bisphosphate carboxylase/oxygenase in the chloroplasts of higher plants. *Archives of Biochemistry and Biophysics* **414** (2): 159-169.

Archer, B. L., Audley, B. G. M., Sweeney, G. P. and Hong, T.C. 1969. Studies on composition of latex serum and 'bottom fraction' particles. *J. Rubber Res. Inst. Malaya* **21**: 560 – 569.

Bowler, C., Slooten, L., Vandenbranden, S., De Rycke, R., Botterman, J., Sybesma, C., Van Montagu, M. and Inze, D. 1991. Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO Journal* **10**: 1723-1732.

Chye, M., Sian tan, Chio- Tee tan, Anil Kush and nam – Hai Chua. 1991. Nucleotide sequence of a cDNA clone encoding the precursor of ribulose – 1, 5- bisphosphate carboxylase small subunit from *Hevea brasiliensis* (rubber tree). *Plant Mol Biol* **16**: 1077 – 1078.

Dean, C., Pichersky, E. and Dunsmuir, P. 1989. Structure, evolution and regulation of *rbcS* genes in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**: 415-439.

Doyle, J. J. and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. *BRL Focus* **12**: 13–15.

Gupta, A. S., Heinon, J.L., Holady, A. S., Burke, J. J. and Allen, R. D. 1993. Increased resistance to oxidative stress in transgenic plants that over express chloroplastic Cu/ Zn superoxide dismutase. *PNAS* **90**: 1629-1633.

Gutteridge, S. and Keys, A.J. 1985. The significance of ribulose 1,5-bisphosphate carboxylase in determining the effects of the environment on photosynthesis and photorespiration. In: *Photosynthetic Mechanisms and the Environment* Barber J. Baker, N.R. (Eds.). Elsevier, Amsterdam, PP. 259–285.

Kush, A., Goyvaerts, E., Chye, M. L. and Chua, N. H. 1990. Laticifer-specific gene expression in *Hevea brasiliensis* (rubber tree). *PNAS* **87**: 1787 - 1790.

Lee, D. W., Lee, S., Lee, G., Lee, K. H., Kim, S., Cheong, G. and Hwang, I. 2006. Functional Characterization of Sequence Motifs in the Transit Peptide of ***Arabidopsis*** **Small** Subunit of Rubisco. *Plant Physiology* **140**: 466-483.

Lubben. H. T., Gatenby, A. A., Ahlquist, P. and Keegstra, K. Jan. 1989. Chloroplast import characteristics of chimeric proteins. *Plant Mol Biol* **12**(1): 13-18.

Parry, M. A., Andralojc, P. J., Mitchell, R. A., Madgwick, P. J. and Keys, A. J. 2003. Manipulation of Rubisco: the amount, activity, function and regulation. *Journal of Experimental Botany* **54**: 1321–33.

Spreitzer, R. J. and Salvucci, M. E. 2003. Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. *Annual Review of Plant Biology* **53**: 449–75.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673-4680.

Whitney, S. M. and Andrews, T. J. 2001. Plastome-encoded bacterial ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) supports photosynthesis and growth in tobacco. *PNAS* **98** (25): 14738-14743.