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Isolation and characterization of a green tissue-specific promoter from pigeonpea [*Cajanus cajan* (L.) Millsp.]

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Expression of *rbcS* genes encoding small subunit of rubisco, most abundant protein in green tissue, is regulated by at least three parameters—tissue type, light conditions and stage of development. One of the green tissue-specific promoters of *rbcS* gene family was isolated from pigeonpea by PCR. Expression of *uidA* gene encoding β-glucuronidase in the transgenic tobacco plants under the control of pigeonpea *rbcS* promoter, clearly showed that this promoter was as strong as pea *rbcS3A* promoter characterized earlier. Study of the sequence similarity with pea *rbcS3A* promoter, especially the region (boxes I and III) that is required for *rbcS3A* expression, showed more than 50% divergence. In contrast, pigeonpea promoter sequence isolated in the present study was more similar to that of spinach and rice *rbcS* promoters.

Keywords: Light regulation, *rbcS* Genes, Tissue-specific promoter, Transgenic tobacco, *uidA* Expression.

Most abundant protein that increases in concentration during greening of the plant tissues is the chloroplast stromal enzyme ribulose 1,5-bisphosphate-carboxylase/oxygenase (Rubisco). This enzyme complex, which accounts for >50% of soluble protein in plants, is composed of eight copies each of two non-identical subunits. The large subunit (rbcL) is encoded in the chloroplast; whereas small subunit (rbcS) is encoded in the nucleus by a small multigene family. The expression of the *rbcS* genes is regulated by at least three parameters—tissue type, light conditions and stage of development[1,2]. The *rbcS3A* gene transcript levels are found to be the highest in leaves, lower in stems and below the levels of detection in roots[3,4]. The gene transfer experiments carried out by Fluhr *et al.*[4] have demonstrated that a 280 base pair enhancer like element in the upstream region at positions –330 to –50 relative to the transcriptional start site can confer both light induction and organ specificity. Tissue specific expression of chloramphenicol acetyl transferase (*cat*) gene under the control of *rbcS* gene promoter of pea in tobacco[5] and transgenic expression of two marker genes under the control of an *Arabidopsis rbcS* promoter have already been reported[6]. Isolation of tissue-specific promoters from pigeonpea is an important endeavour because of the necessity of expressing foreign genes in a tissue-specific manner. Normally constitutive expression of foreign genes is avoided when expressing them for specific purposes. In the present study, one of the promoters of *rbcS* gene was isolated and characterized in pigeonpea.

Plant DNA extraction and purification—Total plant genomic DNA was isolated by CTAB method following the protocol given by Doyle and Doyle[7]. DNA was treated with RNase A (50 μg/ml) and purified to remove proteins and RNase A by extracting with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Spectrophotometric measurements of A_{260} and A_{280} were undertaken to determine the concentration and purity of DNA.

Polymerase chain reaction—The primers were designed from the nucleotide sequence of pea *rbcS3A* promoter[8] by using Lasergene programme (DNASTAR Inc, USA). The primer sequences were—

5' GGAATT CCG ATC CAA AAG CTT GGA C 3′ (Forward primer; *Eco*RI); and 5' GCTCTAGA GCC ATT TTT CTC ACT T 3' (Reverse primer; *Xba*I)

Restriction sites for *Eco*RI and *Xba*I were added to forward and reverse primers to facilitate cloning of the amplified product in a desired cloning/binary vector.

PCR was carried out in a thermal cycler (Biometra T-gradient) with *Taq* polymerase (MBI Fermentas) in 50 µl reaction volume. The cycle conditions were—initial denaturation –94°C for 3 min; cycle denaturation –94°C for 1 min; annealing –57.2°C for 1 min; extension –72°C for 2 min; and final extension –72° C for 5 min.

Cloning of promoter sequence—PCR product was purified and ligated in pGEM-T easy vector (Promega, USA). Finally, the *rbcS* pigeonpea promoter was cloned into the binary vector pBI101 (Clontech, USA), and the cloning was confirmed by restriction analysis. Similar method was followed to isolate and clone pea *rbcS3A* promoter in a binary vector pBI101. The recombinant binary vectors carrying pigeonpea *rbcS* promoter and pea *rbcS3A* promoter were transformed into *A. tumefaciens* strain EHA-105 containing the helper plasmid pEHA105 by freeze-thaw transformation method[9,10].

Plant transformation—Fresh leaves from *in vitro* cultured tobacco plants were taken and cut into small pieces (2×2 mm) with a sterile scalpel. MS medium supplemented with BAP (1.0 mg/l) and NAA (0.1 mg/l) was used for pre-culturing and co-cultivation of explants. The explants were infected with overnight grown *Agrobacterium* carrying *uidA* gene under the control of pea *rbcS3A* and pigeonpea *rbcS* promoters, independently. The explants were cultured on selection medium containing antibiotics cefotaxime (500 mg/l) and kanamycin (300 mg/l). The explants were subcultured on fresh medium every 15 days. Plantlets were transferred to the rooting medium when they attained 5-8 cm height.

Molecular and biochemical analysis

PCR analysis—DNAs isolated from the leaves of putative transgenic plants were taken and PCR was performed using *rbcS3A* primers. The plasmids carrying pea and pigeonpea *rbcS* promoter sequences were taken as positive controls and non-transgenic tobacco DNA as negative control.

GUS assay—Leaves from PCR positive samples were taken and the transient and stable expression of *uidA* gene were detected histochemically[11]. The staining solution was kept at *p*H 7 to avoid expression of endogenous GUS-like activity[12]. Stained materials were washed with ethanol (70%) before visual evaluation.

Sequence analysis: The nucleotide sequence analysis of pigeonpea *rbcS3A* promoter was carried out by automated DNA sequencer (DNA SeqC, Amersham Biosciences MegaBACE 1000). The sequences of pea and pigeonpea *rbcS3A* promoters were compared using Lasergene program (DNASTAR Inc, USA) and BLAST for sequence homology.

A 419 bp length *rbcS* promoter (Fig. 1) from pigeonpea was isolated and cloned in pGEM T-easy vector (Fig. 2). The nucleotide sequence of pigeonpea promoter was compared with that of pea, maize, *Lemna,* spinach, rice and rape *rbcS* promoters by using Lasergene software. From the sequence analysis it was observed that pigeonpea *rbcS* promoter sequence shared more similarity with the sequences of rice and spinach *rbcS* promoters. The pigeonpea *rbcS* promoter showed only 18.7% similarity with maize promoter[13], 23.4% with rape, 23.4% with *Lemna*, and 23% with pea *rbcS* promoters. According to Kuhlemeier *et al.*[8] two highly conserved sequences (Box I and Box II) around –150 bp related to the transcriptional start site are required for *rbcS3A* expression and sequences at both 5′ and 3′ of –170 can direct light regulated and organ specific expression. Sequence upstream of –170 is dispensable only in the mature leaves of a green plant. Gene transfer experiments carried out by Fluhr *et al.*[4] have demonstrated that a 280 bp enhancer like element in the upstream region (at –330 to –50 related to the transcription start site), could confer both light induction and organ specificity. From the nucleotide sequence clustering by using

Lasergene software, the regions of Box I and Box II of pigeonpea *rbcS* promoter were found around –160 and –149 (related to the transcriptional start site) respectively. The sequence of Box I in pigeonpea promoter (TTCAGAA) at the position –160 related to the transcriptional start site differed in three nucleotides CAG with that of pea promoter sequence (TTTCAAA), which is also

-419 -369
AAGCTTGGACAGATCATGGCAAGCAAAGGTGACTACCATAGAAGAGTCAAA

-317
GGACATGACTTTATTGACCCTTGCTACTCTCTTTGGAAAGCTAAGAGAGCATG

-267
AGAAAAAGCTACATATATTTGAGGAGAATGAACAACAAGATAAAAAGGGCA

-217
AGGGAGTGTCATTAAGAGCCTTGAAATCAGTCAAAGGCAAAGAAGTGCATG

-165
AAGAATCAAGCTTCGAAGATTCTGAAACAGAGAACTTTAACTTGCTAGTGAA

Box I Box II Box III -113
GAAGTTCAGAAAATTCTTAAGAAAGAAGAGGAATTCCCCCTACAAGTTCAAC

-61
AAGAAGACTAACAAGAAAGAGAAAGCAAGTACATCCTCCTACAATTGTTTG

-10
AGTGTGGGAAACCAGGACACATAAAAGATGAATGTCCAAACCTCTTAAAGA

-1
AGCAACAAT AGGAAAAGAAAGTGAGAAAAATG

Fig. 1—Nucleotide sequence of pigeonpea *rbcS* promoter.

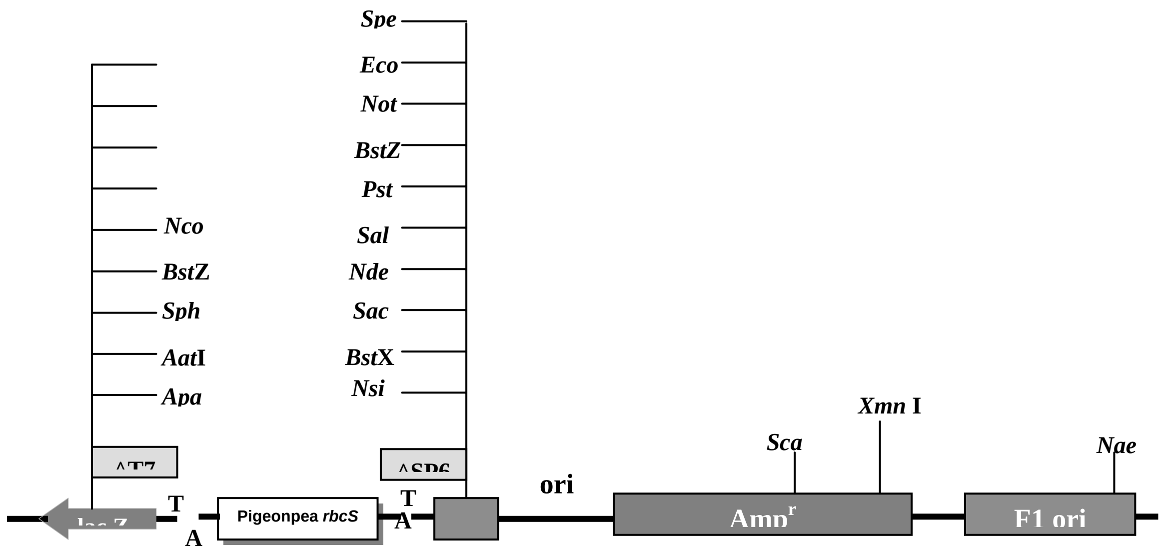

Fig. 2—pGEM T-easy vector carrying pigeonpea *rbcS* promoter.

at –160 related to the transcriptional start site. Similarly, the sequence of Box II in pigeonpea (CTTAAGAAAGAAGA) at the position –149 related to the transcriptional start site differed in 10 nucleotides with the pea promoter sequence (GTGTGGTTAATATG). *In vitro* protein-binding experiments by Green *et al.*[14] on –170 to –50 fragments showed that mutation in either Box II or Box III, abolished expression *in vivo*. The nucleotide sequence of Box III in pigeonpea promoter (ACAAGTTCAACA) at the position –123 related to the transcriptional start site was different in six nucleotides with the sequence of pea promoter (ATCATTTTCACT). Though, there was relatively good amount of divergence between important elements in promoter regions of pea and pigeonpea, the GUS assay showed quite similar results (Fig. 3). It was also observed from the phylogenetic tree generated by Lasergene software (Fig. 4) that the sequence of pigeonpea *rbcS* promoter was more similar to that of rice (Accession. no: AY583764) and spinach (Accession. no: X73236). The pigeonpea *rbcS* promoter was cloned in a binary vector for transformation in tobacco plants by *Agrobacterium* mediated transformation. DNA was isolated from both putative transgenic plants expressing *uidA* gene under the control of pea *rbcS3A* and pigeonpea *rbcS* promoters independently. PCR was performed with the primers that were used to amplify the promoter and resolved by agarose gel electrophoresis. Few leaves from the PCR positive plants were taken for GUS assay. The visual analysis of the leaves incubated with GUS buffer (Fig. 3) clearly showed that the pigeonpea promoter had comparable strength with that of pea *rbcS3A* promoter. Further analysis is needed to find out the effect of sequence divergence in pigeonpea promoter, particularly in the regions of Box I, II and III which are required for *in vitro* expression, and upstream elements of –170 region which are essential for light regulated, organ-specific and stage-specific expression.

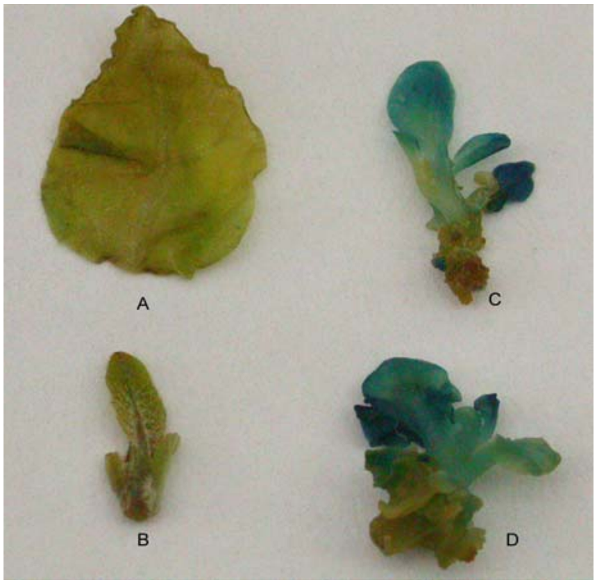

Fig. 3—The glucuronidase assay of transgenic tobacco plants. (A and B)- Control tobacco leaves; (C)- Transgenic tobacco having pea *rbcS* promoter; and (D)- Transgenic tobacco having pigeonpea *rbcS* promoter.

The nucleotide sequence of pigeonpea *rbcS* promoter (419 bp) isolated in the present study exhibited more similarity with those of rice and spinach (Accession no: X73236) *rbcS* promoters than with rape (Accession no: X75334), *Lemna* (Accession no: S45167) and pea sequences. From the clustal analysis it was concluded that pigeonpea *rbcS* promoter differed in ten and six nucleotides in the regions box II and III, respectively, which are found to be essential for *in vitro* expression[14]. GUS histochemical assay clearly indicated that in spite of considerable sequence variation in the boxes II and III, the pigeonpea *rbcS* promoter showed as good an expression as pea *rbcS3A* promoter. Further analysis of pigeonpea *rbcS* promoter sequence and its expression at different wavelengths of light is required to understand various facets of its regulation.

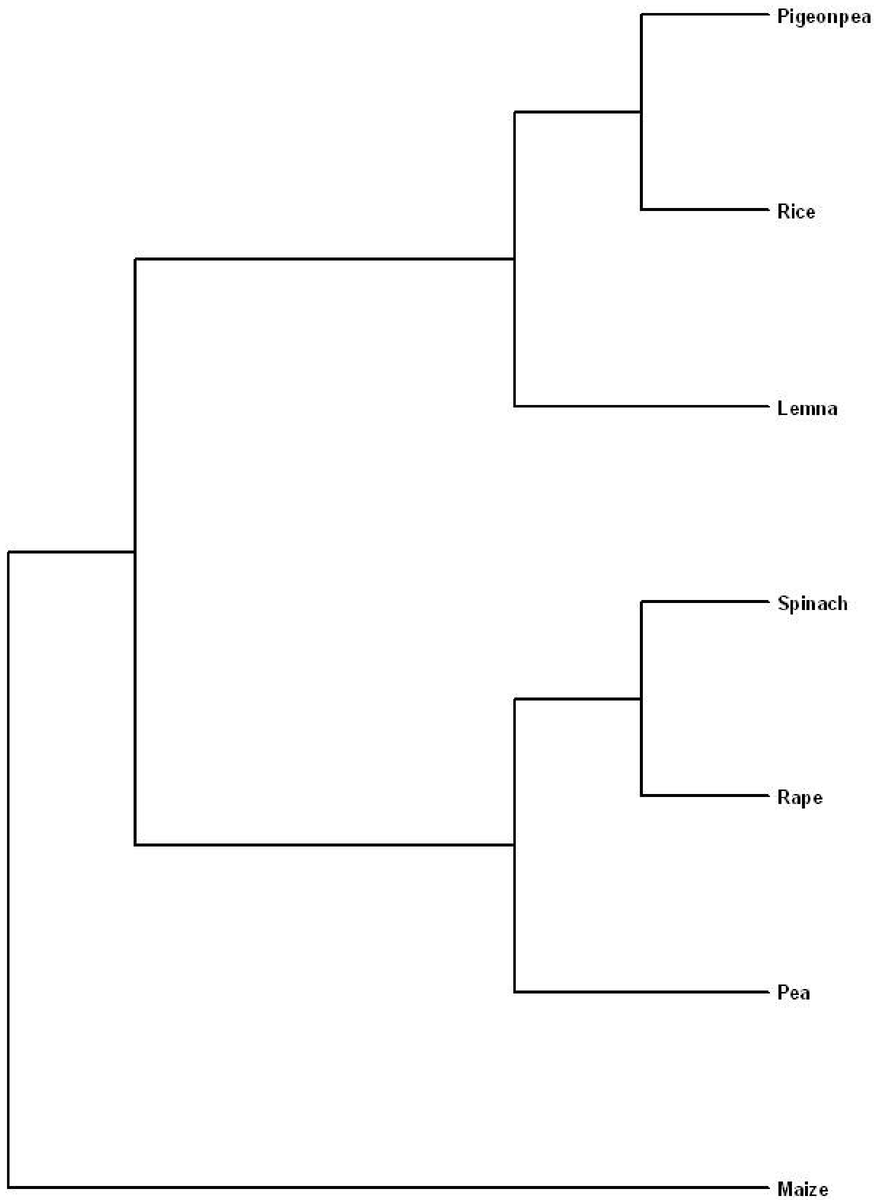

Fig. 4—Phylogenetic tree showing the sequence similarity of pigeonpea *rbcS* promoter with rice, rape, *Lemna*, spinach, pea and maize *rbcS* promoters.

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