Summary

Leucaena sp. is a fast growing multipurpose tree adapted to a variety of soils and climatic conditions. Leucaena is recognized as most useful trees in the tropics. Paper industry in India mainly uses bamboos, Eucalyptus sp., Casuarina sp. and Leucaena sp. as a source for paper pulp. This hard wood Leucaena sp. is exclusively used in India and about 25% of raw material for pulp and paper industry comes from this plant. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. The present study was aimed at understanding lignin biosynthesis pathway in Leucaena leucocephala. No study has been done so far in this regard anywhere on Leucaena sp.

4CL (4-Coumarate CoA Ligase, EC (6.2.1.1.2)) is one of the important key enzymes of general phenyl propanoid metabolism, which provides the precursors molecule for a large variety of important plant secondary products such as lignin, flavonoid etc. It catalyzes the activation of 4-coumarate and various other cinnamic acid derivatives to the corresponding thiol esters in two-step reaction via an adenylate intermediate. Thio reaction represents the last step in a short series of biochemical conversions, known as phenyl propanoid metabolism leading from phenylalanine to the activated cinnamic acid derivatives. These derivatives are precursors for the biosynthesis of a large variety of plant secondary metabolites. This gene was chosen as the target gene for the study if we could down regulate this 4CL enzyme then there would be scarcity of precursor molecules for lignin biosynthesis i.e. total lignin content of plant will get altered. Thus it may be helpful for development of transgenic L. leucocephala plants with desired characters suitable for Indian pulp and paper industry. The findings of present study are as follows:

4CL in L. leucocephala is gene family of possibly 6 members. Full length cDNA fragment of 1935 bp was isolated cloned and sequenced. This sequence was submitted to the NCBI Gen-Bank database and the allotted accession number for this fragment is FJ205490. The full-length gene was designated as Ll4CL1. The
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exact length of coding region of full length, 5' UTR and 3' UTR are 1629, 68 and 238 nucleotides respectively. Another 1831 bp long cDNA fragment was isolated, cloned and sequenced. This sequence was submitted to the NCBI Gen-Bank database and the allotted accession number for this fragment is FJ205491. This partial gene with 3' UTR was designated as Ll4CL2. The exact length of coding region and 3' UTR of Ll4CL2 are 1560 and 271 nucleotides respectively. Ll4CL1 and Ll4CL2 show 66% nucleotide and 68% deduced amino acid sequence similarity with each other. Nucleotide sequence similarity with 4CL cDNA gene clones from other plants was 65-75%.

Deduced amino acid sequences of Ll4CL1 and Ll4CL2 genes show the presence of AMP binding superfamily domain of 415 to 519 amino acid. This AMP binding superfamily domain have box I conserved motif. This box I sequences are slightly different in Ll4CL1 (LPYSSGTSGFPKG) and Ll4CL2 (LPYSSGTGTGLPKG). The box I sequences showed 84% identities and 100% positive sequences. The second conserved domain is box II motif i.e GEICIRG which are absolutely conserved in all reported 4CL and its central cystein residue play important role in catalytic activity. Phylogenetic analysis of 4CL genes group the Ll4CL1 and Ll4CL2 genes from *L. leucocephala* with 4CLs of other Fabaceae members.

The Ll4CL1 gene was expressed in *E. coli* BL21 (DE3) and protein purified from inclusion bodies using Ni-chelated affinity column. Polyclonal antibodies were raised against purified recombinant 4CL protein in rabbit. Polyclonal IgG were purified using agarose A affinity matrix. Transverse sections of different plant parts of different age stained with phloroglucinol-HCL show increase in number of differentiating xylem cells as well as their stain intensity indicated progression of lignification with age. 4CL was immunolocalized in xylem and fibers suggesting its presence at the sites of extensive lignification.

The semiquantitative and QPCR results showed that the Ll4CL1 gene was expressed differentially in a spatio-temporal manner in *Leucaena leucocephala*.  

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The ELISA results did not coincide with the results obtained in QPCR; the probable reason is that the polyclonal antibody raised against the Ll4CL1 expressed protein would not be specific only for 4CL1 protein, as this gene belongs to multifamily gene. The raised polyclonal antibody was not from the Ll4CL1 specific epitope and it might be binding to other 4CL isoenzymes present in the plant.

Leaves of *Nicotiana tabacum* var. Anand 119 and embryo axes excised from germinated seeds of *Leucaena leucocephala* cultivar K-636 were used as explant for transformation studies. Explants were transformed by three different methods i.e. particle bombardment, particle bombardment followed by co-cultivation and agro-infusion method. The integration of genes was confirmed by PCR with gene specific primers. The sequences of PCR products of putative transformants with Hygromycin, 35S promoter and 35S promoter and a part of Ll4Cl1 genes showed complete homology with the sequences of the corresponding genes in the plasmids. Slot blot analysis of the transformed plants showed strong positive signals confirming the integration of the genes.