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Proceedings of 24th Kerala Science Congress, 29-31 January 2012, RRII, Kottayam, pp. 226-227

Characterization of cinnamyl alcohol dehydrogenase gene involved in lignification for improving timber quality in *Hevea brasiliensis*

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INTRODUCTION

Wood quality relies on secondary xylem formation, and more particularly on lignin deposition in secondary cell walls. Lignin is a phenylpropanoid derivative and heteropolymer of three monolignols: p-coumaryl, coniferyl, and sinapyl alcohols. Deposition of lignin reinforces plant cell walls, providing rigidity, impermeability to water, and protection against pathogens. The potential of rubber wood as timber for various industrial uses has been well established. But major limitations preventing utilization of rubber wood are the presence of unlignified or partially lignified tension wood fibres and high susceptibility to biological deterioration. Significant information on individual genes involved in the phenylpropanoid pathway was obtained by altering the expression of the genes and their consequences on lignin content and composition. Cinnamyl alcohol dehydrogenase (CAD) (EC 1.1.1.195) is found to be one of the key enzymes involved in lignification, which catalyzes the final step in lignin precursor synthesis reducing the cinnamyl aldehydes to the corresponding alcohols. Therefore, cloning and functional characterization of full-length CAD gene was carried out as initial steps towards improving wood quality through enhanced lignin biosynthesis in rubber.

MATERIALS AND METHODS

Cloning and characterization of CAD gene

Reverse transcription polymerase chain reaction (RT-PCR) technique was adopted to amplify the gene from the bark specific RNA pool derived from the popular *Hevea brasiliensis* clone RRII 105. An RT-PCR product of 734 bp was amplified with a degenerated primer-pair (CADL: 5′-GGTYCCYGGRCATGAAGTG-3′ and CADR: 5′-CATYTCCTCTGTYTCCTTCA-3′) based on the conserved regions of the CAD gene sequences from other plant species existing in the NCBI GenBank. The fragment was gel-purified, cloned and sequenced. Nucleotide sequences of the clones were subjected to homology search with the GenBank sequences. Maximum sequence homology (85%) was detected with *Populus deltoids* CAD gene. Cloning of cDNA ends of CAD gene from rubber was carried out using RACE technique. Two clones each of the 5′ and 3′ RACE products were sequenced in both directions and full-length gene sequence was deduced. Based on the sequence data generated by 5′ and 3′ RACE, a primer-pair was designed and synthesized (CADFF: 5′-CCAATCCAAAACTTCCCCCTCT-3′ and CADFR: 5′-GGGCCAAGTTGGTATCTTTCATTG-3′) to clone a full-length cDNA of *CAD*, designated as *HbCAD* from bark mRNA. The identity of the clones was inferred by homology search. A phylogenetic relationship was established for *HbCAD* retrieving the CAD cDNA sequences of different tree species and well-studied herbaceous plants. A long PCR was attempted to

amplify the genomic sequence of CAD gene from RRII 105. Amplified genomic fragment was cloned successfully and sequenced. Southern hybridization was performed with the partial CAD cDNA as probe to obtain approximate gene copy number.

Expression analysis of HbCAD

Expression of *HbCAD* in various tissues of the clones belonging to *H. brasiliensis* as well as in different species of *Hevea* was analyzed. Relative quantification of CAD expression in the bark tissues of *H. brasiliensis, H. benthamania* and *H. spruciana* was performed using real-time PCR.

Developing bacterial expression cassette

The *HbCAD* cDNA fragment was sub-cloned into an expression vector (pRSET-C) and subsequently transformed into *E. coli* BL21(DE3) pLysS cells to express the recombinant protein under the control of a T7 promoter.

RESULTS AND CONCLUSIONS

A full-length cDNA (*HbCAD*) of 1413 bp was cloned successfully from bark mRNA of rubber. Sequence of the full-length cDNA revealed 1074 bp long open reading frame (ORF) including the translation initiation codon ATG and stop codon TGA. The cDNA sequence was found to encode a protein of 357 amino acid residues having maximum homology with the CAD of *Citrus sinensis* (E value: 7e-150) followed by *Populus tricocarpa* (E value: 2e-146). At the carboxy-terminal region, a motif 'Ser-Lys-Lue' was identified, which appeared to be the signal for translocation of CAD into peroxisome like other CAD. Along with the coding sequences of *HbCAD* cDNA, 74 bp 5' untranslated region (5'UTR) at the upstream and 296 bp 3'UTR at the down-stream, which contained polyadenylation signal, was identified. Phylogenetically *HbCAD* grouped closely with citrus, *Ricinus* and *Vitis*, whereas the same was distantly related with CAD of two tree species, *Populus* and *Eucalyptus*, and maximum divergence was noticed with the latter, *Eucalyptus*. Southern hybridization with full-length cDNA probe of *HbCAD* clearly indicated the presence of minimum two forms of the respective genes in rubber.

Full-length genomic clone was also generated. Nucleotide sequence information showed that the *HbCAD* gene is ~ 2091 bp in size and the coding sequence was interrupted with the presence of four introns.

Semi-quantitative RT-PCR was performed for expression analysis of *Hb-CAD*. Expression of *CAD* gene was comparatively higher in leaf than in bark tissues of RRII 105. CAD activity was also found to be highly variable in bark tissues of different *Hevea* species. In RRII 105 (*H. brasiliensis*), CAD expression was higher than other two species, *H. benthamiana* and *H. spruceana*. Similar trend was also noticed with quantitative gene expression analysis using real-time PCR.

A bacterial expression cassette for the CAD gene controlling lignin biosynthesis in rubber was developed to generate recombinant protein in *E. coli* under the control of a T7 promoter. About 49 kD protein (fusion protein) band was noticed on SDS-PAGE within an hour of induction of the transformed *E. coli* cells with 1 mM IPTG.

Cloning and characterization of *HbCAD* is a significant step for enhancing the lignin content in rubber wood through cisgenic approaches. Bacterial expression cassette for *HbCAD* will facilitate developing *Agrobacterium*--based transformation system initially for the model plant like *Arabidopsis* and then finally for the commercially cultivated clones of rubber. *HbCAD* could also be used as a potential marker for lignin content/ timber quality once a linear relationship is established between gene expression and its lignin content, which may fasten the identification of potential timber clones among both popular and wild relatives of *Hevea brasiliensis*.