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ISOLATION OF CINNAMYL ALCOHOL DEHYDROGENASE (CAD) GENE FROM *SHOREA LEPROSULA* (DIPTEROCARPACEAE) USING DEGENERATE OLIGONUCLEOTIDE PRIMER (DOP)-PCR: PRELIMINARY RESULTS

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Lignin, an aromatic polymer found in the cell walls of all vascular plants, provides structural support to the stem and contributes to plant defenses. Considerable research into lignin biosynthesis during the last few years has increased our understanding of lignin formation and deposition, and led to the identification and characterisation of a number of enzymes involved such as o-methyltransferase, peroxidase, phenylalanine ammonialyase and cinnamyl alcohol dehydrogenase (Whetten & Sederoff 1995). Among these, cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) has recently become the focus of a number of molecular analyses of lignification. CAD has been purified from *Nicotiana tabacum* (Halpin *et al.* 1992), *Eucalyptus gunnii* (Goffner *et al.* 1992), *Pinus taeda* (O'Malley *et al.* 1992) and *Aralia cordata* (Hibino *et al.* 1993b); cDNA clones have also been isolated for CAD from *N. tabacum* (Knight *et al.* 1992), *P. taeda* (O'Malley *et al.* 1992), *E. gunnii* (Grima-Pettenati *et al.* 1993) and *A. cordata* (Hibino *et al.* 1993a). In tobacco, a sequence encoding CAD was introduced in the antisense orientation, and a reduction in the level of CAD mRNA in the transgenic tobacco plants resulted in the production of a modified lignin composed of fewer cinnamyl alcohol monomers and more cinnamyl aldehyde monomers than normal lignin (Halpin *et al.* 1994, Hibino *et al.* 1995, Yahiaoui *et al.* 1998). This result provides strong evidence that CAD participates in the reduction of cinnamyl aldehyde in the biosynthesis pathway to lignin *in vivo*. Recent transgenic approaches in poplar have also proved that it is possible to alter lignin composition and structure to achieve better pulpability without severely affecting other vital functions such as water conduction, defense response, or simply the mechanical support the tree needs to remain standing (Baucher *et al.* 1996).

Shorea leprosula (Dipterocarpaceae), or meranti tembaga, a common timber tree of Southeast Asia, grows on well-drained or swampy sites on deep clay soil in lowland and hill dipterocarp forests below 700 m altitude (Symington 1943, Ashton 1982). It is one of the fastest growing dipterocarp species, with an average annual diameter increment of about 1.2 cm. However, production of genetically improved planting materials through conventional breeding programmes would take a long time as timber production can be achieved only after 35–40 y. With the establishment of gene banks containing cDNA clones for specific genes (e.g. lignin biosynthesis genes that determine the quality of timber), it is hoped that it would provide a good source of gene probe that can be used for early selection and advanced breeding. In addition, fully sequenced lignin biosynthesis genes can also be constructed for genetic transformation studies to alter the quality of the timber.

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Construction of good quality genomic or cDNA libraries for the purpose of gene isolation and characterisation is time consuming and expensive. With the recent advancement of polymerase chain reaction (PCR) techniques, isolation and characterizations of a specific gene can also be done using degenerate primers, which are much simpler and cost-effective than using genomic or cDNA libraries. This paper describes the preliminary results on the isolation of CAD gene from *S. leprosula* using degenerate oligonucleotide primer (DOP)-PCR.

Tissues (developing xylem and woody stem) of *S. leprosula* sapling were collected from the Forest Research Institute Malaysia (FRIM). Total RNA was extracted using the method described by Schultz *et al.* (1994) and further used to synthesize first-strand cDNA with RT-PCR method with poly(T) adaptor primer (5'-ggCCACgCgTCgACTAgTACT [17]-3') and Super Script II RNase H Reverse Transcriptase (GIBCO BRL). The first-strand cDNA obtained served as the template for the DOP-PCR amplification of partial CAD fragments. Two sense (5'-TAYCCIATggTICCNggNCAYgARg-3' [sense1] and 5'-gAYggIAARCCNACNCARggNgg-3' [sense2] and one antisense (5'-IATIACNgTNACRTgRTgNCC-3') degenerate primers corresponding to the peptides conserved in *E. gunnii* (Grima-Pettenati *et al.* 1993), *P. taeda* (O'Malley *et al.* 1992), *N. tabacum* (Halpin *et al.* 1992) and *A. cordata* (Hibino *et al.* 1993b) and an universal amplification primer (UAP) (5'-CUACUACUACUAggCCACgCgTCgACTAgTAC-3') corresponding to poly(T) adaptor primer were used to isolate the partial CAD. A schematic representation of the annealing sites for degenerate primers and UAP primer is given in Figure 1.

The PCR reaction mixtures contained 100 pM each of the paired primer (sense1-antisense; sense1-UAP; sense2-UAP and sense2-antisense), approximately 5 ng of first-strand cDNA, 0.2 mM dNTP, and 1 U of Ex *Taq* DNA polymerase (Takara). They were incubated in a thermocycler (Perkin-Elmer Gene Amp PCR System 2400) for 5 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C and finally a 5 min incubation at 72 °C. Figure 2 shows the products of first PCR using the above combinations of paired primers. A clear and distinct PCR product (approximately 210 bp) was produced only with the paired primer of sense2 and antisense (lane 4).

In order to confirm that the products of first PCR were those of the partial sequence of CAD gene, the products of first PCR were further used as the templates for the second nested PCR with sense2 and antisense as the paired primer. As shown in Figure 3, a PCR fragment of approximately 210 bp was obtained. This fragment was extracted from the gel using Geneclean III kit (Bio101), sub-cloned into pGEM-3Z vector (Promega) and transformed into competent *Escherichia coli*. The transformed *E. coli* was multiplied on agar plate and screening of the transformed *E. coli* colonies using colony PCR showed that 37 colonies were positive with the insert of approximately 210 bp (Figure 4). From these, 10 colonies were selected and sequenced using the chain-termination method of Sanger *et al.* (1977) with ABI PRISM BigDye sequencing kit and ABI PRISM 377 Genetic Analyser (Perkin-Elmer). The nucleotide sequences were analysed with the aid of the GENETYX-MAC program (Software Development Co. Ltd., Tokyo). Comparisons of the sequences using BLAST e-mail server at the National Center for Biotechnology Information (NCBI) showed that the partial sequence had high homology to

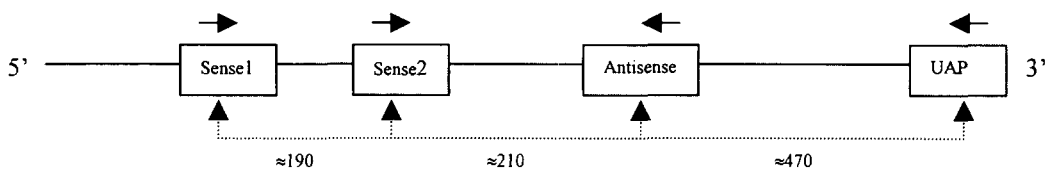

Figure 1. Schematic representation of the annealing sites for degenerate primers and UAP primer

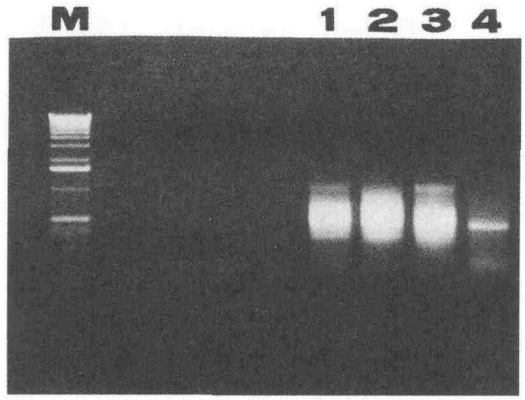

Figure 2. Products of first PCR using the following combination of primers: lane 1–sense1 and antisense; lane 2–sense2 and UAP; lane 3–sense2 and UAP; lane 4–sense2 and antisense. M = marker 4.

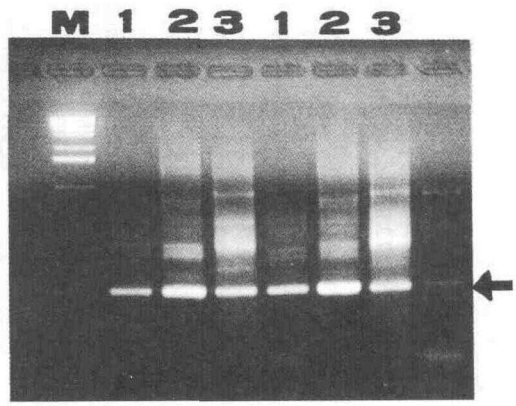

Figure 3. Products of second PCR (nested PCR) using sense2 and antisense as the paired primer. The templates used are: lane 1–first PCR product of sense1 and antisense; lane 2–first PCR product of sense1 and UAP; lane 3–first PCR product of sense2 and UAP. Arrow indicates potential CAD gene fragment (approximately 210 bp). M = marker 4.

CAD sequence of *Nicotiana tabacum, Arabidopsis thaliana, Aralia cordata, Populus deltoides, Eucalyptus gunnii, E. globulus* and *E. botryoides* (Table 1). The details of the partial sequence (204 bp) are shown in Table 2. Based on this partial sequence, a few pairs of specific primers can be easily generated. By using the 5' RACE and 3' RACE reactions, the full-length of CAD gene of *S. leprosula* can be amplified and sequenced.

Table 1. Results of sequence homology search using the BLAST e-mail server at NCBI

Species	Gene	Score (bits)	Homology sequence
Nicotiana tabacum	CAD14	64	Query: 120 - tgccataccatctgggattttcaccacaaacttttgatc - 158 Sbjct: 549 - tgccataccctctggaattttcaccacaaacttttgatc - 511
N. tabacum	CAD19	56	Query: 120 - tgccataccatctgggattttcaccacaaacttttgatc - 158 Sbjct: 553 - tgccataccctctggaattttcaccacaaagttttgatc - 515
Arabidopsis thaliana	CAD	56	Query: 70 - ggactrtacaccgttactccggcgcatagcagcggagccgcctgttccattgccatacca - 129 Sbjct: 2737 - ggactataaaccgttactcccgcgcagagtagcggtgcagcctgctccaccgccattcct - 2678 Query: 130 - tctgggattttcaccacaaac - 150 Sbjct: 2677 - tctggaatcttcaccacaaac - 2657
Aralia cordata	CAD	52	Query: 120 - tgccataccatctgggattttcaccacaaacttttgatcggckacca - 166 Sbjct: 582 - tgccattccatctggtatcttcacaacaaacttttgatcgacgacca - 536
Populus deltoides	CAD	50	Query: 7 - actcctcccagccccagaatgcctcctctgagaccactctgtttcagtccgaagtg - 62 Sbjct: 602 - actcctccaagtcctaaaatccctcctcttagcccactctgtttcagtccaaagtg - 547
Eucalyptus gunnii	CAD	50	Query: 127 - ccatctgggattttcaccacaaac - 150 Sbjct: 566 - ccatctgggattttcaccacaaac - 543
E. globulus	CAD	50	Query: 127 - ccatctgggattttcaccacaaac - 150 Sbjct: 576 - ccatctgggattttcaccacaaac - 553
E. botryoides	CAD	50	Query: 127 - ccatctgggattttcaccacaaac - 150 Sbjct: 1880 - ccatctgggattttcaccacaaac - 1857

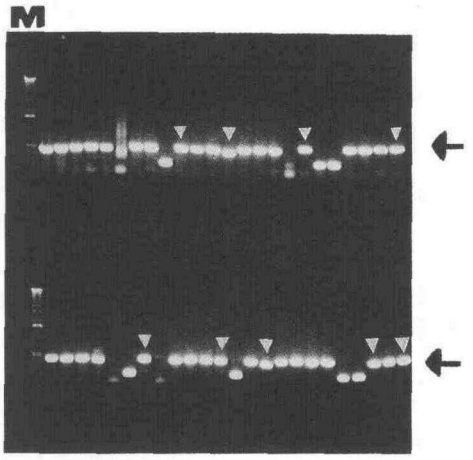

Figure 4. Colony PCR (using For and Rev primers) to screen the potential colonies with partial sequence of CAD construct. Arrows on the right indicate all the potential colonies with approximately 370 bp (210 bp of partial sequence of CAD gene and 160 bp of vector sequence). Pointers inside the plate indicate 10 potential colonies selected for sequencing. M = marker 4.

Table 2. Partial sequence of CAD gene of *Shorea leprosula* (204 bp)

1	:	TGGCCGACTCCTCCCAGCCCCAGAATGCCTCCTCTGAGACCACTCTGTTTCAGTCCGAAG
61	:	TGATGCAGGGGACTRTACACCGTTACTCCGGCGCATAGCAGCGGAGCCGCCTGTTCCATT
121	:	GCCATACCATCTGGGATTTTCACCACAAACTTTTGATCGGCKACCAWRGCRSCGGCRAAS
181	:	CCDCCYTGVGTVGGCTTCCCGTCA

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