Isolation and characterization of Cinnamyl Alcohol Dehydrogenase gene from *Leucaena leucocephala*

A THESIS
SUBMITTED TO THE
UNIVERSITY OF PUNE

FOR THE DEGREE OF
DOCTORATE OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY
PALLAVI KULKARNI

UNDER THE GUIDANCE OF
DR. B. M. KHAN

PLANT TISSUE CULTURE DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE-411008
INDIA

SEPTEMBER, 2009

DEDICATED TO

MY BELOVED PARENTS...

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Isolation and characterization of Cinnamyl Alcohol Dehydrogenase gene from *Leucaena leucocephala***" submitted by **Pallavi Kulkarni** for the degree of Doctor of Philosophy, was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. B. M. Khan
(Research guide)

Declaration

I hereby declare that the thesis entitled, "**Isolation and characterization of Cinnamyl Alcohol Dehydrogenase gene from *Leucaena leucocephala***" has been carried out at Plant Tissue culture Division, National Chemical Laboratory, Pune, under the guidance of **Dr. Bashir M. Khan**. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other university. I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

(Pallavi Kulkarni)

ACKNOWLEDGEMENTS

I wish to thank my research guide Dr. B. M. Khan for his exceptional guidance, unfailing support & motivation and for his extraordinary kindness and consideration throughout my doctoral studies. He has been a research guide, a mentor, a well-meaning critic, a tough task-master and a kind teacher, all rolled into one amazing person. I consider myself rather fortunate that I got the opportunity to work under him. The experience and learning that I take form here is enormous and will stay with me throughout my life.

I am deeply grateful to Dr. S.K. Rawal, former Head, Plant Tissue Culture Division, for his support and invaluable comments and suggestions.

I wish to thank Dr. D. C. Agrawal and Mrs. M. V. Shirgurkar for allowing me to use Gel Documentation, Particle gun and Microscopy facilities.

I appreciate the kind and timely help extended by the scientific and supporting staff of the Plant Tissue Culture Division and NCL library.

Special thanks are due to Dr. Nooka Raju and Rajeshri for their extensive help in plant transformations. Their efforts have been critical in taking my work towards completion.

The remarkable help and assistance extended by Prashant and Gayatri is highly appreciated.
I am thankful to the Director, NCL for giving me the opportunity to work in this reputed laboratory.

I would like to thank my seniors Lata Mahishi, RC, Rohini, Manish, Sushim, Noor, Sameer, Arun and my lab mates Abhilash, Sumita, Santosh J, Santosh RJ, Prashant, Rishi, Somesh, Kannan, Sameena, Nishi, Ruby, Anagha for their timely help and for making work fun. Thanks to Poonam S, Poonam L, Swapna, Varsha, Sridevi, Ambrish, Bhuban, Sagar, Neha, Sucheta, Neelima, Malini, Ulfat, Jai, Azfar, Shweta, Shashidhar, Chetan, Suryaprasad, Priya, Shreekanth, Kiran and Trupti for maintaining a lively and cordial atmosphere in the lab.

The fellowship support given by CSIR is duly acknowledged.

I would like to thank my parents and brother for their unwavering support throughout my life.
The words are not enough to thank my in-laws for their extraordinary support in every sense, especially during the crucial last phase of my work.

Finally, words would not suffice to express my gratitude towards my husband Vikram for his support throughout this endeavor and in every aspect of my life.

CONTENTS

Abbreviations

AA	Amino acid
AldOMT	5-Hydroxyconiferaldehyde O- methyltransferase
BAP	6- Benzylaminopurine
bp	Base pairs
BSA	Bovine serum albumin
C3H	Coumarate 3- hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CAGR	Compound Annual Growth Rate
CAld5H/ F5H	Coniferaldehyde 5- hydroxylase / Ferulate 5- hydroxylase
CBG	Coniferin β glucosidase
CCoAOMT	Caffeoyl coenzyme A 3-O- methyltransferase
CCR	Cinnamoyl coenzyme A reductase
cDNA	Complementary DNA
Ci/ mmol	Curie per milli mole
CIAP	Calf Intestinal Alkaline Phosphatase
COMT	Caffeate O-methyltransferase
Cps	Counts per second
Da	Dalton
DEPC	Diethylpyrocarbonate
DNA	Deoxyribose nucleic acid
DTT	Dithiothritol
EDTA	Ethylene Diamine Tetra Acetic acid
EDTA	Ethylene diamine tetra acetic acid disodium salt
ELISA	Enzyme linked immuno sorbent assay
F5H/CAld5H	Ferulate 5-hydroxylase/ coniferaldehyde 5-hydroxylase
FTIR	Fourier Transform Infra Red
g /L	grams per litre
g	gram
G	Guaiacyl
gDNA	GenomicDNA

GSP	Gene Specific Primers
h	Hour(s)
IPTG	Isopropyl β-D-thiogalactoside
Kb	Kilobase pairs
KDa	Kilo Daltons
Kg	Kilogram
Km	Michaelis-Menton constant
l	Litre
LD_{50}	Lethal dose 50%
MCS	Multiple cloning sites
mg	milligram
min	Minute(s)
ml	millilitre
mM	millimolar
mRNA	messenger RNA
NAA	1-Napthyl acetic acid
nM	nano molar
nm	nanometer
NUP	Nested Universal Primers
O/N	Overnight
PAL	Phenylalanine ammonia lyase
pg	picogram
pmol	picomole
PMSF	Phenyl methyl sulphonyl fluoride
ppm	Parts per million
RNA	Ribose nucleic acid
rpm	Rotations per minute
RT	Room temperature
s	second(s)
S	Syringyl
SAD	Sinapyl alcohol dehydrogenase
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)

SMQ	Sterile Milli Q
Soln	Solution
sp.	Species
TDZ	Thiadiazuron
U	Units
UDP-GT	UDP-glycosyltransferase
UPM	Universal Primer Mix
UTR	Untranslated Region
UV	Ultraviolet
V	Volt
v/v	volume / volume
Vmax	Maximum velocity
w/v	weight / volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
α	Alpha
β	Beta
λ	Lamda
%	Percentage
°C	degree Celsius
µg	microgram
µg/l	Micrograms per liter
µl	microlitre
µm	micrometer
µM	micromolar
4CL	4-Coumarate coenzyme A ligase

CHAPTER 1

INTRODUCTION

1.1 Paper

Paper has been the most common tool for transfer of knowledge over the generations as it is the basic material used for compilation and dissemination of information, written communication etc. Significance of paper is evident in the numerous ways in which we use it. We write on it, read it, draw on it, copy on it, wrap with it and wipe with it as well. Thus, paper is an integral part of our life. We use over 100 million tons of it per year. Forty percent of our solid waste is paper. The socio-economic importance of paper has its own value in the country's development and it is directly related to the industrial and economic growth of the country. As India is one of the fastest growing economies in the world, paper consumption is bound to increase manifold.

The word paper is derived from the word *papyrus* as in ancient times, the Egyptians made paper from *papyrus*, a member of the sedge family. However the earliest paper makers are the Paper wasps. They remove the exposed fibres of weathered wood and mix them with saliva. Then this mixture is converted into pulp and is spread thinly over their nest where it dries into thin strong paper-like sheet. True paper production was invented by the Chinese in A.D. 105. The first paper mill was established in A.D. 794 at Baghdad, with flax fibre and linen rags as raw materials.

1.2 Paper industry

Paper industry is one of the 35 high-priority industries in India and is presently growing at a rate of 6.3% per annum. Indian paper industry accounts for about 1.6% of the world's production of paper and paperboard. The estimated turnover of the industry is Rs. 25,000 Cr. (USD 5.95 billion) approximately and its contribution to the exchequer is around Rs. 2918 Cr. The per capita consumption of paper in India is 7.2 kg, which is far lower than other emerging economies like China (45 kg) and developed economies like US and EU (Eleventh five year plan 2007-2012, Planning commission, Government of India).

India is the fastest growing market for paper globally and it presents an exciting scenario; paper consumption is poised for a big leap forward in sync with the economic growth and is estimated to touch 13.95 million tons by 2015-16. The futuristic view is

that growth in paper consumption would be in multiples of GDP and hence an increase in consumption by 1 kg per capita would lead to an increase in demand of 1 million tons. As per industry estimates, paper production are likely to grow at a CAGR of 8.4% while paper consumption will grow at a CAGR of 9% till 2012-13. The import of pulp & paper products is likely to show a growing trend.

Several growth triggers are in place to take paper industry in India to a new level. These include: economic growth, increasing literacy rate, increasing government spending on education, population growth, changing demographics, increasing living standards etc.

The country is almost self-sufficient in most varieties of paper and paperboard, and imports are taking place only of certain specialty items such as coated paper, cheque paper, etc. However, the industry has failed to keep pace with the technological advances and is beset with major difficulties such as high production cost, pollution problems, and finished paper quality not conforming to international standards. The growth of the paper industry has not been able to match the growth in the demand for paper as the industry has been acutely short of domestically available wood. The limitation on cutting trees and other forest produce has led it to depend on imported wood and wood-based materials.

A variety of fibrous raw materials, viz. bamboo, hardwoods, agricultural residues, and waste paper are being used for paper-making. In India, the most commonly used raw material is Bamboo, *Casuarina*, *Leucaena* and *Eucalyptus*.

Wood consists of following components:

- **Cellulose** consists of long, straight chains of glucose molecules. It forms the skeleton of the plant wall and has the most desired properties for making paper. These fibres are long, strong and translucent.
- **Hemicelluloses** are short, branched chains of glucose and other sugar molecules. They fill in space in the plant wall. Hemicelluloses are more soluble in water and are thus often removed during the pulping process.
- **Lignin** is a three dimensional phenolic polymer network. It holds the cellulose fibres together and makes them rigid. Chemical pulping and bleaching processes

selectively remove the lignin without significantly degrading the cellulose fibres.

- **Extractives** account for 3(+/-2) % of softwoods. These materials include plant hormones, resin and fatty acids along with other substances that help the tree grow and resist disease and pests

1.3 Process of paper production

The five principal steps in paper production are wood preparation, pulping, bleaching, chemical recovery, and paper-making.

1.3.1 Wood preparation

It involves debarking and cutting wood logs into uniform chips.

1.3.2 Pulping

It can be performed using chemical. mechanical., or combined chemical-mechanical techniques. The most common mechanical pulping technique involves separating the cellulose fibres by pressing logs against wet grindstones or by passing wood chips between counter revolving grooved metal disks (refiners). Lignins and other residues are not removed. This results in a higher yield, but there is more damage to the fibres. In addition, lignin degrades in time. The lower quality fibre limits the use of this process to less expensive grades of paper, such as newsprint.

In chemical pulping, wood chips are cooked in an aqueous solution at high temperature and pressure. Chemical processes dissolve most of the lignin while leaving cellulose fibres relatively undamaged. This process results in high quality paper with a yield of only 40%-60% of the weight of dry wood. The processes principally used in chemical pulping are Kraft and sulfite.

The Kraft process, the most common one, uses a solution of sodium hydroxide and sodium sulfite. The Kraft process produces a darker pulp which requires more bleaching. In the sulfite process the pulping chemical is a mixture of metals (sodium, magnesium, potassium or calcium) or ammonium sulfite or bisulfite. The Kraft process

does less damage to the cellulose fibres than the sulphite process, thereby producing stronger fibres, but the sulfite process makes pulp that is easier to bleach.

Combined chemical and mechanical pulping can produce varying grades of paper depending on the particular process used. These processes include thermo-mechanical., chemical thermo-mechanical., and semi-chemic*al*

Large Indian mills that are predominantly based on forest raw materials use the Kraft process. Agro-based mills use a soda process while newsprint mills use mechanical. chemical., chemi-mechanical and chemi-thermo-mechanical processes (CTMP).

1.3.3 Bleaching

It whitens pulp for the manufacture of writing, printing, and decorative papers. The process alters or removes the lignin attached to the wood fibre. Chemical pulps are bleached through the use of alternating treatments of oxidizing agents and alkali solutions. Bleaching is performed in stages. Early stages remove remaining lignin while final stages brighten the pulp. Pulp is usually washed between stages to remove any soluble organic materi*al* Strong oxidizing agents used for bleaching are elemental chlorine, chlorine dioxide, Sodium hypochlorite, ozone, oxygen and hydrogen peroxide.

Elemental chlorine (Cl_2) is an inexpensive and effective bleaching agent. It adds chlorine atoms to the lignin degradation products, thus producing significant amounts of chlorinated organic materi*al* The concentration of chloride ions in the waste water makes it too corrosive to be re-circulated to the recovery system, so it must be discharged to the effluent treatment system and ultimately in rivers and lakes.

Chlorine dioxide (ClO_2) is a highly selective, more expensive but somewhat less effective chemical that can both delignify and brighten pulp. In this method also chlorinated organic compounds are formed but in less amount as compared to elemental chlorine.

Ozone (O_3) is also a cost-effective bleaching agent. Oxygen (O_2) is an inexpensive, highly effective delignifying agent that is usually used at the beginning of the bleaching process. It has intermediate selectivity.

Sodium hypochlorite (NaOCl) is an inexpensive delignifying agent. However mills are phasing out hypochlorite as it generates large quantities of chloroform when used to bleach pulp.

Hydrogen peroxide (H_2O_2) is mainly used to brighten pulp in the final bleaching stages. Peroxide is often used at the end of a conventional bleaching sequence to prevent the pulp from losing brightness over time.

1.3.4 Environmental concerns

The chemical soup of pulp mill pollutants entering the air, water and land includes ammonia, carbon monoxide, carbon dioxide, carbonyl sulphide, chlorine, chlorine dioxide, chloroform, dioxins, furans, hydrogen chloride, methanol, nitrogen oxides (NO_X), particulate matter, phenols, sulphur oxides, total reduced sulphur compounds, cadmium, mercury, zinc, resin acids, alcohols, terpenes, acetaldehyde, nitrates, fungi *(Aspergillus fumigatus* and *A. versicolor)*, bioaerosols (endotoxins), benzene, chlorinated benzenes and phenolics, guaiacols, other volatile organic compounds, including dichloroacetic acid, methyl ester, 2,5- dichlorothiophane, styrene, toluene and xylenes (Delores Broten and Robyn Budd, 1994).

Pulp mill emissions and effluents are harmful to the environment. Organochlorines, heavy metals and benzene based compounds are especially dangerous because they are persistent. The most deadly organochlorines are not only toxic, but highly bio-accumulative. The dioxin is toxic to some animals in very tiny amounts, and studies indicate that it can occupy the receptor site of a hormone, blocking the hormones' natural function in wildlife and humans. Dioxin causes cancer, immune system suppression and reproductive failure. Chlorine/chlorine dioxide gas causes or worsens lung disease; reacts to form organochlorines which are linked to cancer, hormone problems and reproductive ailments. Sulphur compounds and soot go up the stack, and chlorinated organic compounds like chloroform evaporate every day at pulp and paper mills. Volatile chlorinated organic compounds affect the ozone layer and, at ground level, the air we breathe.

It would be highly beneficial to the paper industry from economic as well as environmental point of view if there is reduction in consumption of large amounts of

chemicals used in pulping and bleaching. For this, raw material with less amount of lignin or with easily extractable lignin must be available. Thus, it is the need of the hour to provide designer plant species with altered lignin content/composition.

1.4 *Leucaena leucocephala*

In India *Leucaena leucocephala* is widely used as raw material (≈ 25%) for pulp and paper industry. Other plants used as raw material are Bamboo, Eucalyptus, *Casuarina* etc.

Figure 1.1 *Leucaena leucocephala* A- Twig; B- Inflorescence; C- Pods; D- Seeds

Taxonomic name: *Leucaena leucocephala* (Lam.) De wit

Synonyms: *Acacia leucocephala* (Lamark) Link 1822, *Leucaena glabrata* Rose 1897, *Leucaena glauca* (L.) Benth. 1842, *Mimosa leucocephala* Lamark 1783.

1.4.1 Classification

Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Rosidae*
Order	*Fabales*
Family	*Fabaceae* – Pea family
Genus	*Leucaena* Benth. – leadtree
Species	*Leucaena leucocephala* (Lam.) de Wit – white leadtree
Sub species	Glabrata (Rose; S. Zarate); Ixtahuacana (Hughes) and Leucocephala (Benth) Var. Peru and Cunningham

1.4.2 Description

Leucaena leucocephala is an American tropical and subtropical tree/shrub with its diversity center located in Mexico. It is a fast growing tree adapted to a variety of soil and climatic conditions. It is arborescent, deciduous, nitrogen fixing species, growing up to 20 m tall.

Leucaena leucocephala is highly invasive and has been reported as a weed in more then 20 countries across all continents except Europe and Antarctica. It grows quickly, and forms dense thickets which crowd out any native vegetation

Leucaena leucocephala is a 'conflict tree' being widely promoted for tropical forage production and reforestation, whilst at the same time it is spreading naturally and is widely reported as a weed. *Leucaena* genus is represented by 22 species. Of these, 6 are intraspecific taxa and 2 are widespread spontaneous hybrids. Most of the species are diploid 2n=52 or 56. However, 4 species are tetraploid 2n = 4X=104 or 112 (Hughes, 1998)

L. leucocephala has three growth forms that are commonly found in tropics (Hughes, 1998). The first and common type is a shrubby free-shading form known as the Hawaiian Leucaena, which is weedy and low yielding. The second growth form is giant type, which is a tall tree of about 20 meters, with large leaves, pod and seeds, and almost without branches. It is native of Central America and Mexico, and produces twice the biomass of the common type. It is suitable for timber, wood product and industrial fuel. The last is Peru type, which are a multi-branched, semi-erect, medium height (about 10 meters) and the most productive form.

1.4.3 Uses

It is used for a variety of purposes, such as firewood, fibre and livestock feed. It is an excellent pulpwood (Hu, 1987). *Leucaena* wood is among the best hardwoods for paper and rayon making. It produces pulp that is high in holocellulose, low in silica, ash, and lignin, is alcohol-benzene soluble and hot water soluble. Pulp yield is 50 to 52%. Its short fibre is suitable for rayon production (Pottinger and Hughes, 1995).

1.5 Lignin

1.5.1 Introduction

The discovery of lignin can be traced back to Augustin P. de Candolle in 1813, when he described a substance ‘‘la lignine’’ that remained in wood following treatment with solvents and mild acid. Later, in 1838, Anselme Payen reported the presence of two products from wood, ‘‘la cellulose’’ and ‘‘l’incrustation ligneuse’’

The term lignin was coined from Latin *lignum* (wood). Lignin binds the cellulose and strengthens the cell wall. It is the second most abundant biopolymer on earth after cellulose. Lignins are absent from algae, fungi and mosses. They apparently first emerged with the appearance of pteridophytes, but became most abundant with the evolution of gymnosperms and angiosperms. Thus the evolution of water conducting cells with lignified cell walls is closely associated with the first wave of structural diversification among land plants (Friedman & Cook, 2000).

1.5.2 Significance

Lignin provides mechanical support for the plant body and also enables water and mineral transport through the xylem under high negative pressure without collapse of tissue (Jones *et al.*, 2001). Lignins provide protection against pathogen attack by providing a significant barrier to pathogen ingress. They also limit the digestibility of plant matter by herbivores, reducing the nutritive value of the plant, and presumably decreasing its desirability as a foodstuff (Moore & Jung, 2001). Lignin has a major influence on the ease of wood pulping during paper making, on the digestibility of forage crops and on post harvest quality of certain vegetables.

1.5.3 Deposition

Lignin is deposited in secondary cell wall of certain differentiating cell types, principally the tracheids and vessel elements of xylem, sclerenchyma, phloem fibres and periderm. Lignin deposition generally occurs when cell growth is completed and the cell wall undergoes secondary thickening. Deposition subsequently begins at sites far removed from the plasma membrane, namely in the cell corners and primary wall/S1 outer layer regions, and then extends into middle lamella and secondary cell wall regions until completed (Lewis, 1999). The bulk of lignin is deposited after cellulose and hemicellulose have been deposited in the S3 layer. Generally lignin concentration is higher in middle lamella and cell corners, than in S2 secondary wall, but because it occupies a larger portion of the wall, the secondary wall has highest lignin content. Secondary walls of vessels generally have higher lignin content than that of fibres.

1.5.4 Chemistry

Chemically lignins are complex racemic aromatic heteropolymers derived mainly from three hydroxycinnamyl alcohol monomers, differing in their degree of methoxylation namely, p-coumaryl, coniferyl and sinapyl alcohols. These monolignols produce p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenyl propanoid units when incorporated into lignin polymer. The composition and amount of lignins vary among taxa, cell types and individual cell wall layers. It is also influenced by developmental

and environmental cues. Although exceptions exist, gymnosperm lignins are composed almost entirely of guaiacyl units with low levels of p-hydroxyphenyl units. Angiosperm lignins consist principally of guaiacyl and syringyl monomers and traces of H units. Lignins from monocots incorporate G and S units at comparable levels and more hydroxyphenyl units than dicots. The proportion of H lignin greatly increases in compression wood which forms in regions under compressive stress.

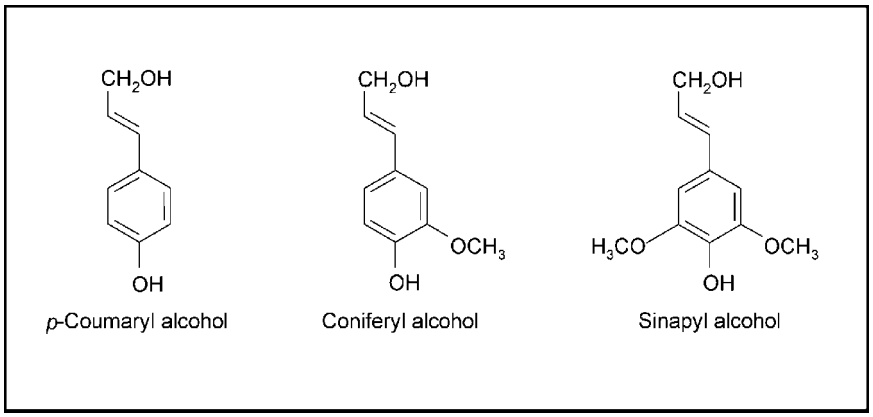


Figure 1.2 Lignin monomers

Three monolignols are incorporated at different stages of cell wall formation. Lignin in vessels is generally enriched in G units whereas fibre lignins are enriched in H units. Secondary walls of ray parenchyma are abundant in S units. The chemical nature of carbohydrate matrix and the orientation of the cellulose microfibrils influence lignin deposition. In the middle lamella and the primary wall, lignin forms spherical structures, whereas in secondary wall, lignin forms lamellae that follow the orientation of microfibrils.

In the process of lignification, monomer units are linked together via radical coupling reactions. The main "end-wise" reaction couples a new monomer (usually a monolignol at its β position) to the growing polymer giving rise to β-linked structures. The most frequent inter-unit linkage is the β-O-4 (β-aryl ether) linkage. It is also one of the most easily cleaved linkages, providing a basis for industrial processes such as chemical pulping, and several analytical methods. The other linkages are condensed β-5, β-β, 5-5, 5-O-4 and β-1 which are more resistant to chemical degradation. The relative abundance of a particular linkage depends largely on the contribution of a particular

monomer. Gymnosperm lignin has relatively more condensed carbon bonds than angiosperm lignins because of abundance of G units where the aromatic C_5 position is free to make ring to ring linkages. These C-C bonds are resistant to chemical degradation, thus making gymnosperm wood harder to pulp than angiosperm wood in chemical pulping processes (Halpin, 2004).

Lignins also contain several monomers other than three monolignols stated above. All lignins contain traces of units from apparently incomplete monolignol biosynthesis and other side reactions that occur during that biosynthesis viz. acetylated monomers, ferulate dehydrodimers (in grasses), dihydroconiferyl alcohol (DHCA), cinnamyl aldehydes etc.

1.5.5 Monolignol Biosynthesis

The phenylpropanoid pathway, which is unique to plants, catalyzes the conversion of phenylalanine to a myriad of phenolic secondary metabolites, including lignins, sinapate esters, stilbenes, and flavonoids. These compounds play diverse roles in essential plant processes including growth, defense, protection from UV light, and reproduction (reviewed in Hahlbrock and Scheel, 1989, Chapple *et al.,* 1994, Koes *et al.,* 1994, Rhodes, 1994, Shirley, 1996). Accordingly, the activities of the various branch pathways are highly regulated. Transcriptional controls play an important role in regulating the overall activity of phenylpropanoid metabolism in response to biotic and abiotic signals (reviewed in Weisshaar and Jenkins, 1998). The phenylpropanoid pathway is located exclusively in the cytoplasm.

The biosynthesis of the monolignols starts with the deamination of phenylalanine and involves successive hydroxylation reactions of the aromatic ring, followed by phenolic *O*-methylation and conversion of the side-chain carboxyl to an alcohol group. The figure 2 shows all possible enzymatic conversions that have been shown by *In vitro* experiments. For most of the enzymes in the pathway, multiple isoforms that are differentially expressed during development and upon environmental cues (Chen C. *et al.,* 2000, Hu W-J. *et al.,* 1998, Lauvergeat V. *et al.,* 2001, Lindermayr C. *et al.,* 2002) exist, that may have different kinetics and substrate preferences (Ehlting J. *et al.,* 1999, Harding S. A. *et al.,* 2002, Zubieta C. *et al.,* 2002). Certain paths in the grid are

therefore expected to be kinetically favored in given cell types or environmental conditions, allowing for metabolic flexibility.

1.5.5.1 Phenylalanine Ammonia-Lyase

Jane Koukol and Eric Conn in 1961 described phenylalanine ammonia lyase (PAL., EC 4.3.1.5), which is now one of the most extensively studied enzymes in plant metabolism. This is the first enzyme in the pathway and catalyses the deamination of phenylalanine to cinnamate. It generally serves as the entry point to phenylpropanoid metabolism, although some species (particularly grasses) are able to metabolize tyrosine into p-coumaric acid via the action of the related enzyme, tyrosine ammonia lyase. The first gene encoding a bona fide PAL was published by Lamb's group in 1985.

PAL is found as a tetramer in vascular plants (Hanson and Havir, 1981; Jones, 1984). Genes encoding different PAL subunits show tissue-specific patterns of expression in severa1 angiosperms (Bevan *et al.,* 1989; Liang *et al.,* 1989; Lois and Hahlbrock, 1992; Shufflebottom *et al.,* 1993). PAL subunits are typically encoded by multigene families in angiosperms, with 2 to 40 different members, depending on the species (reviewed by Wanner *et al.,* 1995). Different PAL isoforms play different roles in the many aspects of phenylpropanoid metabolism.

1.5.5.2 4-Coumarate: Coenzyme A Ligase

The involvement of CoA esters in the biosynthesis of monolignols from hydroxycinnamic acids was first established by Gross & Zenk. In a substrate-versatile manner, 4-coumarate CoA ligases (4CL; EC 6.2.1.12) can convert hydroxycinnamic acids into the corresponding hydroxycinnamoyl CoA esters through the intermediacy of AMP derivatives. The first gene encoding a bona fide 4CL was obtained from parsley in 1987.

4CL depends strictly on ATP, and the reaction resembles the activation of fatty acids, proceeding through an intermediate acyl adenylate, which reacts with CoA to form the thioester. 4CL is a multi gene family and all known 4CL enzymes have fairly broad but differential substrate versatilities for potential hydroxycinnamic acid substrates.

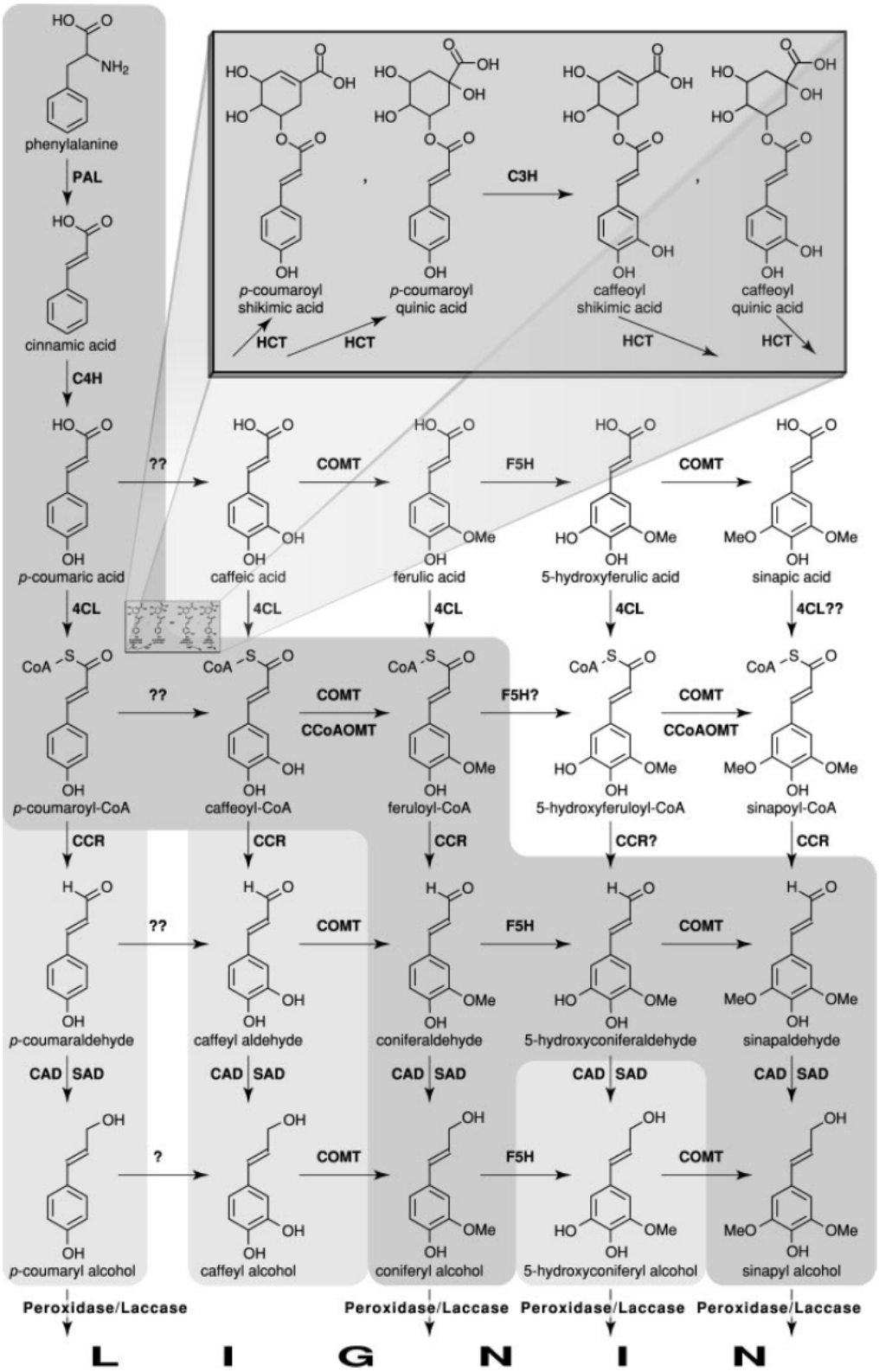


Figure 1.3 Lignin biosynthetic pathways (Boerjan *et al.*, 2003)

1.5.5.3 Cytochrome P450s and hydroxycinnamoyl CoA transferases

The pathway to monolignols involves a series of hydroxylation steps. The first of these is the hydroxylation of cinnamic acid by cinnamate-4-hydroxylase (C4H) to form p-coumaric acid. The second hydroxylation step, catalyzed by p-coumarate-3-hydroxylase (pC3H), introduces a hydroxyl group at C3 on the aromatic ring to give caffeic acid-like derivatives, whereas the third, ferulate-5-hydroxylase (F5H), places a hydroxyl group at C5. While C4H affords entry into the vast majority of the phenylpropanoid pathway derived metabolites (i.e. lignins, suberins, sporopollenins, hydroxycinnamic acids, etc.), pC3H and F5H are largely involved (from a carbon allocation perspective) in formation of the two monolignols coniferyl alcohol and sinapyl alcohol, the major precursors of the lignans/lignins. All of these hydroxylation reactions are cytochrome P450 mediated, with C4H discovered by Russell and Conn, pC3H by Heller and Ku¨hnl and F5H by Grand.

p-coumarate is first converted to *p*coumaroyl- CoA by 4CL, with subsequent conversion to *p*-coumaroyl-shikimate and *p*-coumaroyl-quinate, the substrates for C3H, by *p*-hydroxycinnamoyl-CoA:Dquinate-(CQT) or *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyltransferase (CST). These enzymes, described as reversible enzymes, can convert caffeoyl-shikimate or caffeoyl-quinate (chlorogenic acid) into caffeoyl-CoA, the substrate for CCoAOMT (Schoch G. *et al.,* 2001, Ulbrich B. and Zenk M. H., 1980). Recently, a reversible acyl transferase with both CQT and CST activity, designated HCT, has been purified and the corresponding gene cloned from tobacco (Hoffmann L. *et al.,* 2002) Taken together, these data argue that none of the C3 and C5 substitutions of the aromatic ring take place at the cinnamic acid level in monolignol biosynthesis.

Metabolic flux studies demonstrated that C4H has a rate-limiting (regulatory) role in carbon allocation to the pathway, as does pC3H for entry to the guaiacyl/syringyl lignins. F5H is apparently not involved in determining/controlling carbon allocation to the pathway (Anterola and Lewis 2002, Anterola *et al* 1999, Anterola 2002).

1.5.5.4 Caffeate O-Methyltransferase and Caffeoyl-Coenzyme A O-Methyltransferase

Conversion of caffeic acid into ferulic acid *In vitro* by cell-free extracts from apple cambial tissue was first described by Finkle & Nelson. Caffeic acid is methylated to form ferulic acid by caffeic acid -O-methyltransferase (COMT; EC 2.1.1.68**),** using S-adenosyl methionine as the methyl group donor. COMT is actually specifically involved in the second methylation step of S-lignin deposition *In vivo*. Caffeoyl CoA O-methyltransferases (CCoAOMT/CCOMT; EC 2.1.1.104) catalyzes the conversion of caffeoyl CoA into feruloyl CoA.

1.5.5.5 Cinnamoyl-Coenzyme A Reductase

Reduction of hydroxycinnamoyl-COA thioesters to the corresponding aldehydes is catalyzed by cinnamoyl-COA reductase (CCR; EC 1.2.1.44). CCR was first detected in cell-free extracts of *Salix alba* by Gross *et al* CCR is a type B reductase, CCR gene was first reported in 1997 by Lacombe *et al* Goffner *et al* (1994) hypothesized that CCR plays a key regulatory role in lignin biosynthesis as the first committed step in the production of monolignols from phenylpropanoid metabolites.

1.5.5.6 Cinnamyl Alcohol Dehydrogenase

The reduction of hydroxycinnamaldehydes to hydroxycinnamyl alcohols is catalyzed by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195). CAD has been considered to be an indicator of lignin biosynthesis because of its specific role at the end of the monolignol biosynthetic pathway (Walter *et al.,* 1988). Differences in substrate affinities of CAD enzymes from angiosperms and gymnosperms may play a role in controlling the formation of different types of lignin (Kutsuki *et al.,* 1982).

CAD was first detected by Gross *et al* in cell-free extracts of *S. alba*. The following year it was purified to apparent homogeneity by Mansell *et al* and shown to be a type A reductase, abstracting the 4-pro-R hydrogen of NADPH. The first bona fide clone encoding a *CAD* gene was later described by Knight *et al* using tobacco. The Boudet laboratory later reported a second bona fide *CAD* (so-called *CAD2*) from *Eucalyptus gunnii* using the tobacco cDNA (Knight *et al.,* 1992) as a probe.

Sinapyl Alcohol Dehydrogenase (SAD) obtained from aspen (*Populus tremuloides*) (Li L. *et al.,* 2001) was reported as solely responsible for generation of syringyl lignin in this species. However, re-analysis of these data (Davin *et al.,* 2008, Anterola and Lewis, 2002) robustly questioned those conclusions: Firstly, SAD was substrate-versatile and thus was able to reduce each of the aldehydes *In vitro*. Furthermore, it was catalytically more active overall for all potential substrates than a presumed CAD which was also present; (Anterola and Lewis, 2002) hence, it could not be substrate-specific based on these considerations. Additionally, the complementation of a CAD double mutant (cad-4 cad-5) in Arabidopsis with the *P. tremuloides* SAD gene was unable to restore the massively reduced G and S levels in the lignin of this mutant to that of wild type (WT), thereby suggesting its non-involvement in S-lignin formation.(Sibout *et al.,* 2005). X-ray crystal structure of SAD was also obtained (Bomati and Noel, 2005) however, its substrate binding pocket only contained 2 of the 12 residues present in bona fide CADs (Youn *et al.,* 2006) and it was also much larger, perhaps suggesting an alternative biochemical/physiological role. Taken together, the proposed involvement of SAD in the formation of S-units of lignins is tenuous. In short, the physiological role of the putative SAD is considered to be unknown.

1.5.6 Metabolic Regulation

Based on metabolic flux analysis and transcriptional profiling, (Anterola *et al.,* 1999, 2002) it was also demonstrated that carbon allocation to the phenylpropanoid pathway was determined by factors controlling Phe (Freudenberg K. *et al.,* 1968) availability, and differential control over enzymatic activities of C4H and pC3H. The latter (pC3H), in particular, largely controls carbon allocation to the G/S segments of the monolignol/lignin pathway

Another level of complexity is that pathway intermediates may affect the synthesis or activity of certain enzymes in the pathway. Cinnamic acid inhibits phenylalanine ammonia-lyase (PAL) expression at the transcriptional (Blount J. W. et *al.,* 2000, Mavandad M. et *al.,* 1990) and post-translational level (Bolwell G. P. et *al.,* 1986) and induces the activity of CQT (Lamb C. J., 1977). Accordingly, down-regulation of cinnamate 4-hydroxylase (C4H) in transgenic tobacco reduces PAL activity by feedback modulation (Blount J.W. et *al.,* 2000). Phenylalanine concentrations also have

a profound effect on flux through the pathway. Feeding lignifying *Pinus taeda* cell suspension cultures with phenylalanine increases *p*-coumaryl and coniferyl alcohol synthesis and PAL., 4CL, CCoAOMT, and CCR transcript levels, but only slightly up-regulates those of C4H and C3H (Anterola A. M. *et al.,* 2002). *In vitro* enzymatic experiments have shown that 5-hydroxyconiferaldehyde is a competitive inhibitor of caffeic and 5-hydroxyferulic acid methylation and that coniferaldehyde is a noncompetitive inhibitor of ferulate 5-hydroxylation, corroborating the conclusion that C3 and C5 substitutions in monolignol biosynthesis do not take place at the cinnamic acid level (Li L. et *al.,* 2002, Osakabe K. et *al.,* 1999). In aspen, coniferaldehyde is additionally a competitive inhibitor of the sinapaldehyde reduction by CAD (Li L. et *al.,* 2001). Caffeic acid competitively inhibits the 4CL activation of p-coumaric acid (Harding S. A. et *al.,* 2002) and feedback regulation by hydroxycinnamic acid levels has also been suggested for CCoAOMT (Jin H. *et al.,* 2000). Down-regulation in tobacco induces a strong increase in CCR activity and has a negative effect on the production of CCoAOMT (Pincon G. *et al.,* 2001), indicating additional regulatory mechanisms controlling the flux through the pathway.

1.5.7 Glycosylation, Storage, and Transport

The monolignols p-hydroxycinnamyl alcohol, coniferyl alcohol, and sinapyl alcohol are relatively toxic and unstable compounds. Glycosylation on the phenolic hydroxyl group, a reaction performed by UDP-g1ucose: coniferyl alcohol glucosyltransferase (EC 2.4.1.111), forms the monolignol glucosides p-hydroxycinnamyl alcohol glucoside, coniferin, and syringin respectively. These glucosides accumulate in some species of plants, most notably in conifers. Coniferyl alcohol glucosyl transferase is found in many species of plants, as is coniferin β-glucosidase (EC 3.2.1.126) (Ibrahim and Grisebach, 1976; Hosel *et al.,* 1982). The monolignol glucosides are transported from the vacuole to the cell wall at the appropriate time during xylem differentiation.

1.5.8 Dehydrogenation

After transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols (Christensen J. H. *et al.,* 2000). The dehydrogenation to monolignol radicals has been attributed to different classes of proteins, such as peroxidases, laccases, polyphenol oxidases, and coniferyl alcohol

oxidase. Peroxidases use hydrogen peroxide (H_2O_2) to oxidize their substrates (Christensen J. H. *et al.,* 2000). The high number of peroxidase genes [73 in the Arabidopsis genome (Tognolli M. *et al.,* 2002) is one reason why isozymes that are responsible for monolignol oxidation *In vivo* cannot be identified easily. Laccases (*p*-diphenol: O_2 oxidoreductases) are copper-containing, cell wall–localized glycoproteins that are encoded by multigene families in plants. In contrast to peroxidases, laccases consume O_2 instead of H_2O_2 to oxidize the monolignols. Laccases of a variety of species are expressed in lignifying cells (Bao W. *et al.,* 1993; Driouich A. *et al.,* 1992; Ranocha P. *et al.,* 1999; Sterjiades R. *et al.,* 1992). However, down-regulation of laccase in aspen and yellow poplar did not affect lignin content or composition (Dean J. F. D. *et al.,* 1998; Ranocha P. *et al.,* 2002).

1.5.9 Polymerization

Considerable controversy exists as to whether the polymerization of the monolignols is a random, spontaneous phenomenon (Hatfield and Vermerris, 2001), or whether it is highly ordered (Gang *et al.,* 1999) and perhaps mediated by dirigent proteins such as the one isolated from Forsythia, which is capable of catalyzing the stereoselective coupling of two coniferyl alcohol radicals into lignan pinoresinol (Davin *et al.,* 1997). Involvement of shuttle-mediated oxidation has also been proposed (Onnerud *et al.,* 2002).

1.5.10 Transcriptional regulation

Few regulatory or transcription factor genes have been identified that can promote or inhibit expression of lignin biosynthetic enzymes. Only a small fraction has been characterized. MYB proteins are involved in transcriptional regulation. Conserved motifs (AC elements or PAL boxes) which are common recognition sites for MYB binding are found in the promoters of some lignin biosynthetic genes, including PAL (Lois *et al.,* 1989), C4H (Bell-Lelong *et al.,* 1997), 4CL (Hauffe *et al.,* 1993), CCoAOMT (Chen *et al.,* 2000), CCR (Lacombe *et al.,* 2000) and CAD (Lauvergeat *et al.,* 2002). In some cases, AC elements have been shown to reside within promoter regions that are essential for expression in vascular tissues, particularly xylem, suggesting that these elements may be important in directing tissue specific expression (Hatton *et al.,* 1995; Lacombe *et al.,* 2000; Lauvergeat *et al.,* 2002). Other conserved

elements such as H boxes or G boxes are found in certain lignin biosynthetic genes and are thought to be associated with stress and defense responsiveness. Some lignin biosynthetic genes are induced by wounding, and the cis elements responsible for wounding responsiveness have been localized within their promoters.

A MYB protein from *Pinus taeda* PtMYB has recently been shown to bind to AC elements and to induce expression of certain lignin biosynthetic genes when over-expressed in tobacco (Patzlaff *et al.,* 2003). A PAL-box binding protein, Ntlim 1, with a zinc finger motif and similarity to members of LIM protein family has been isolated from tobacco (Kawaoka and Ebinuma, 2001). Transgenic tobacco containing an antisense Ntlim 1 gene showed a 20% decrease in lignin content and reduced activity of PAL., 4CL and CAD (Kawaoka and Ebinuma, 2001).

1.5.11 Analysis of lignin content, structure and composition

Determining the content, structure, and composition of lignin in plant materials is a challenging problem because of the complexity and heterogeneity of the polymer and its cross links to other wall components. It is not currently possible to fully elucidate lignin structure and composition in any species. Most techniques for lignin characterization rely on initial solublization of a lignin fraction under relatively harsh chemical conditions but the resulting soluble fraction does not represent native lignin quantitatively or qualitatively, in particular, condensed lignin fractions resistant to degradation are absent from such preparations and solublised fractions are often modified during extraction.

Lignin analysis techniques also vary considerably in sensitivity. No single technique is completely reliable or unbiased. Lignin is most frequently quantified by gravimetric techniques after extracting the other, more soluble, wall components (Klason lignin determination) or, alternatively, by extracting the lignin component itself (e.g., with thioglycolic acid or acetyl bromide). Lignin structure and composition can be determined by analysis of its degradation products derived by pyrolysis gas chromatography-mass spectrometry (pyrolysis GC-MS), alkaline nitro-benzene oxidation, thioacidolysis, or derivatization followed by reductive cleavage (DFRC).

Two-dimensional nuclear magnetic resonance (NMR) spectroscopy of isolated lignin fractions has allowed an improved picture of lignin structure. Methods that allow the whole of the polymer to be analyzed *In situ*, such as solid-state NMR or F-TIR (Fourier-transform infrared spectroscopy), are generally not as sensitive as 'wet' chemical methods, and can also be useful when dealing with novel lignins that are hard to extract.

1.6 CAD as a candidate gene

1.6.1 Introduction

Cinnamyl Alcohol Dehydrogenase (CAD) catalyses reduction of hydroxy-cinnamyl aldehydes to respective hydroxy cinnamyl alcohols in the presence of NADPH as the cofactor. CAD is considered to be an indicator of lignin biosynthesis as it is present at the end of monolignol biosynthetic pathway.

Figure 1.4 Crystal structure of CAD (*Arabidopsis thaliana* CAD5) homodimer (Youn *et al.,* 2006)

CADs are dimers and belong to the Zn^{2+}-dependent medium-chain dehydrogenase/reductase (MDR) super family with two zinc ions per subunit (one catalytic and the other structural). Its biochemical mechanism of catalysis was also reported with a hydride abstraction/proton shuttle for substrate reduction initially being possibly considered involving amino acid residues Thr49, His52, and Asp57. Site-directed mutagenesis has since determined Thr49 is essentially only involved.

CAD is synthesized in polysomes and released in cytosol during secondary cell formation. The expression of CAD is regulated by both developmental and

environmental cues. CAD is also expressed in response to stress (Galliano *et al.,* 1993), pathogen elicitors (Campbell and Ellis, 1992), and wounding. CAD preparations from gymnosperms are generally much more active on coniferaldehyde, whereas angiosperm CAD preparations show equal activities with coniferaldehyde and sinapaldehyde (reviewed in Gross, 1985). Isoforms of CAD with markedly different substrate affinities are detected in such species as soybean (Wyrambik and Grisebach, 1975), wheat (M Pillonel *et al.,* 1992), Eucalyptus (Goffner *et al.,* 1992), and Salix (Mansell *et al.,* 1976).

1.6.2 CAD down-regulation studies

In transgenic plants down-regulated for CAD activity, it was found that large reductions in CAD activity only slightly reduced lignin content because the plants were able to circumvent the block in CAD activity by shipping its substrates, the cinnamaldehydes, to the cell wall for polymerization. Also the altered lignin structure did not affect overall plant growth and development.

Interestingly, chemical pulping experiments with wood harvested from 4-year-old, field-grown transgenic poplars down-regulated for CAD have demonstrated that the modifications in lignin structure result in an altered chemical reactivity, which reduces the consumption of chemicals needed to remove lignin from the pulp. The pulp yield was simultaneously enhanced. These data also show that significant improvements in pulping efficiency can be achieved without strong reductions in lignin content.

Transgenic plants with reduced CAD activity have been produced in tobacco (Halpin *et al.,* 1994; Hibino *et al.,* 1995; Stewart *et al.,* 1997; Yahiaaoui *et al.,* 1998), poplar (Baucher *et al.,* 1996), and alfalfa (Baucher *et al.,* 1999) whereas CAD mutant exists in pine (MacKay *et al.,* 1997), maize (Halpin *et al.,* 1998), Arabidopsis (Sibout *et al.,* 2003), and probably sorghum (Pillonel *et al.,* 1991).

Increased levels of cinnamaldehydes have been detected in the lignin of CAD antisense tobacco (Halpin *et al.,* 1994; Ralph *et al.,* 1998, 2001) and poplar (Kim *et al.,* 2002), as well as in the pine (Ralph *et al.,* 1997), Arabidopsis (Sibout *et al.,* 2003) and maize (Marita *et al.,* 2003) CAD mutants. In tobacco, poplar, and Arabidopsis, increases in both coniferaldehyde and sinapaldehyde were identified (Halpin *et al.,* 1994; Kim *et*

al., 2002; Ralph *et al.,* 2001; Sibout *et al.,* 2003). In CAD antisense poplar and in the maize *bm*1 mutant (Halpin *et al.,* 1998; Lapierre *et al.,* 1999), the proportion of S and G units in lignin thioacidolysis products is not altered, whereas in CAD antisense tobacco and alfalfa (Baucher *et al.,* 1999; Halpin *et al.,* 1994), the S:G ratio is reduced. This suggests that in CAD-deficient plants the deposition of S lignin units is equally or more affected than that of G lignin units.

A striking characteristic of transgenic plants down-regulated for CAD is the red or brownish colour of xylem tissues, initially observed in maize brown midrib (*bm*) mutants. This colour has been attributed to the incorporation of cinnamaldehydes in the polymer because synthetic dehydrogenation polymers (DHPs) of coniferyl alcohol and coniferaldehyde also from a red polymer (Higuchi *et al.,* 1994).

The unusual monomer, dihydroconiferyl alcohol, is apparently also incorporated into the lignin of the pine CAD mutant, where it accounts for 30% of the polymer compared to only 3% in wild type plants (Ralph *et al.,* 1997). Altered structure in lignin polymer of CAD-suppressed plants is also indicated by its increased extractability in alkali (Baucher *et al.,* 1996; Bernard-Vailhe *et al.,* 1996; Halpin *et al.,* 1994; MacKay *et al.,* 1999; Yahiaaoui *et al.,* 1998).

Greenhouse-grown transgenic tobacco and poplar with down-regulated CAD activity have been subjected to chemical pulping analysis (Baucher *et al.,* 1996; Jouanin *et al.,* 2000; Lapierre *et al.,* 1999; O'Connell *et al.,* 2002). In both species, the changes to lignin structure in CAD suppressed plants resulted in a greater ease of pulping by the chemical Kraft process, and the Kappa number, a measure of residual lignin in the pulp after cooking, was reduced compared to that of wild-type plants. Subsequent bleaching of the pulp was also easier and there were no detrimental changes to other pulp properties. Wood from pine CAD mutant has also been subjected to Kraft process, but in this case no enhanced delignification was evident (MacKay *et al.,* 1999). In transgenic poplar and tobacco, the lignin was enriched in free phenolic groups in both S and G units (Lapierre *et al.,* 1999 and O'Connell *et al.,* 2002). The increase in free phenolic groups may be important in altering the solubility of lignin which in turn has implications for the ease with which wood from these plants can be pulped (Lapierre *et al.,* 1999 and O'Connell *et al.,* 2002).

1.7 Rationale of the thesis

All these data indicate that CAD is one of the most suited genes for genetic manipulation of lignin biosynthetic pathway, as the down-regulation of CAD gene does not significantly affect the lignin content, rather it increases the lignin extractability. Increased lignin extractability is highly desirable for pulp and paper industry as it reduces the amount of harsh and expensive chemicals used for lignin removal and also reduces the pollutant load on environment. Leucaena is widely used raw material in pulp and paper industry in India. However no study has been done so far on Lignin Biosynthesis gene(s) and on the CAD gene in particular, in *Leucaena sp.* The study of these genes will help in understanding the lignin biosynthetic pathway and its manipulation so as to meet the needs of pulp and paper industry. The present work deals with the study of CAD gene and its down-regulation in *Leucaena leucocephala.*

CHAPTER 2

MATERIALS & METHODS

Materials

2.1 *Leucaena leucocephala*

Leucaena leucocephala cultivar K-636 was used for the studies and it was a selection from Hawaii (Bray *et. al*. 1998). The cultivar produces erect boles suitable for timber production.

For regeneration and transformation experiments, embryo axis of *Leucaena leucocephala* was used as the ex-plant. Seeds of *L. leucocephala* were treated with concentrated H_2SO_4 for 2-3 min and then washed extensively with tap water. The scarified seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 min followed by five rinses with sterile deionized water. The seeds were soaked in sterile water for two days for imbibing. Embryo was excised from imbibed seeds and was transferred to ½ MS basal medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 1.5% glucose. One day old excised embryos were used for the transformation experiments.

For spatio-temporal studies, the imbibed seeds were transferred to ½ MS basal medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 1.5% glucose for germination. The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. The culture bottles were incubated at 25±2 ºC under a 16 h photoperiod, 70% relative humidity, with a light intensity of 24.4 µmol/m^2/s. The day of inoculation was considered as day zero. Root, shoot and leaves were harvested from 5, 10, 15 and 20 day old seedlings and used for further experiments.

For genomic DNA isolation, young *Leucaena* leaves were harvested and washed thoroughly with water, dried and then crushed with liquid nitrogen.

For RNA isolation, xylem tissue of mature healthy plants was harvested as and when required. Outer bark was scraped to expose the xylem tissue. The tissue was scraped and ground using liquid nitrogen and RNA was isolated for normal cDNA preparations, RACE and RT PCR.

2.2 *Medicago sativa*

Medicago sativa was obtained from Agriculture College, Pune. Genomic DNA was isolated from young leaves of *Medicago*.

2.3 *Nicotiana tabaccum*

Tobacco seeds (*Nicotiana tabacum* var. Anand 119) were germinated on wet sterile paper towel. Germinated seeds were transferred to ½ MS basal medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 1.5% glucose for germination. The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. The culture bottles were incubated at 25±2 ºC and 70% relative humidity under 16 h photoperiod. One month old axenic cultured plant leaves were used as the explant for further transformation experiments.

2.4 Bacterial strains and plasmids used

Strain or Plasmid	Important features (reference or source)
Escherichia coli XL-1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´proAB lacIqZ_M15 Tn10 (Tet^r)] (Stratagene, USA)
E. coli BL 21(DE3)	F^- ompT gal dcm lon hsdSB(r_B^- m_B^-) _(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) (Invitrogen, USA)
Agrobacterium tumefaciens GV2260	C58, Rif^r, pGV2260 (pTiB6S3_T-DNA), $Carb^r$, Octopine type
pGEM-T Easy Vector	Cloning vector (Promega, USA)
pCAMBIA 1301	Binary cloning plant transformation vector (CAMBIA)
pET30b(+)	Expression vector, Novagen (USA)

2.5 Glassware

Glassware used in all the experiments were procured from “Borosil”, India. Test tubes, glass bottles, petridishes, conical flasks, beakers and pipettes were used during the course of study.

Preparation of Glassware

Glassware used for all the experiments were cleaned by boiling in a saturated solution of Sodium bicarbonate for 1 h followed by repeated washing in tap water. Thereafter, it was immersed in 30% HNO_3 solution for 30 min followed by repeated washing in tap water. Washed glassware were thereafter dried at room temperature. Test tubes and flasks were plugged with absorbent cotton (Mamta Surgical Cotton Industries Ltd., Rajasthan, India). Autoclaving of the glassware and above items was done at 121 °C and 15 psi for 1 h.

2.6 Plastic ware

Sterile disposable filter sterilization units (0.22 µm) and petridishes were procured from "Laxbro", India. Microfuge tubes (1.5 ml and 2 ml capacity), microtips (10, 200 and 1000 µl capacity) and PCR tubes (0.2 ml and 0.5 ml capacity) were obtained from "Tarsons" and "Axygen", India.

2.7 Chemicals

Acetosyringone, Tris, IPTG, X-gal., SDS, BSA, EDTA, PMSF, PIPES, DTT, PVPP, APS, Imidazole, TES buffer, Urea and Ethidium bromide were purchased from Sigma-Aldrich (USA) and Bio-world (USA). Agarose, restriction enzymes, T4 DNA ligase, RNase A and lysozyme were obtained from NEB (USA), Promega (USA), Bioenzymes (USA) and Amersham (UK). Different kits were purchased from BD CLONETECH (JAPAN), Invitrogen (USA), Promega (USA), and Sigma-Aldrich (USA). *Taq* DNA polymerase was obtained from Sigma-Aldrich (USA) and Bangalore Genei (India). Plasmid vectors, pGEM-T Easy Vector and pET30b (+) were purchased from Promega (USA) and Novagen (USA) respectively. Megaprime labeling kit and Hybond-N^+ membrane were obtained from Amersham (UK). [α-^{32}P]-dATP and [α-^{32}P] –dCTP were obtained from Bhabha Atomic Research Centre (BARC), India. X-ray films were obtained from Konica (Japan) or Kodak (USA). Agarose A (Sigma), gold particles were purchased from BioRad (USA). All other chemicals and solvents of analytical grade were purchased from HiMedia, Qualigens Fine Chemicals and E-Merck Laboratories, India. All chemicals used in the tissue culture study were of analytical

grade (AR) and were obtained from "Qualigens", "S.D. Fine Chemicals" or "HiMedia", India. The Sucrose, glucose and agar-agar were obtained from "Hi- Media". Bacto-Agar for microbial work was obtained from "DIFCO" laboratories, USA.

2.8 Different nutrient media used

Name	Ingredients	Preparation and storage
Luria Bertani Broth (LB)	1% Bactotryptone 0.5%Yeast extract 1% NaCl	pH adjusted to 7.0 with NaOH, Store at room temperature or at +4 °C
SOB media	2% Bactotryptone 0.5% Yeast extract 10 mM NaCl 10 mM $MgCl_2.6H_2O$ 2 mM KCl	pH adjusted to 6.8 with NaOH, Store at room temperature or at +4 °C
YEP	1% Bactotryptone 1% Yeast extract 0.1% Glucose	pH adjusted to 7.0 with NaOH, Store at room temperature or at +4 °C

Methods

2.9 Bacterial culture conditions

E. coli was grown at 37 °C with shaking at 200 rpm in Luria Bertani (LB) broth/ SOB broth and maintained on LB/ SOB plates with 1.5% agar (Sambrook *et al*., 1989). For plasmid DNA preparation recombinant *E. coli* was grown in LB media supplemented with appropriate antibiotic.

2.10 Bacterial transformation

2.10.1 *E. coli* transformation

2.10.1.1 Preparation of competent cells using TB Buffer

A single colony of *E. coli* XL1 Blue was inoculated in 5 ml of LB medium containing tetracycline (12.5 mg/l.) and grown overnight at 37 °C in incubator shaker at 200 rpm. 500 µl of the overnight grown culture was added to 50 ml of SOB medium and grown for 2-3 hours at 37 °C in incubator shaker at 200 rpm. Cells were kept on ice for 10 min

and harvested by centrifugation at 5,000 rpm for 10 min at 4 °C. The cell pellet was suspended in 15 ml ice-cold TB-buffer (Table 2.3) and kept on ice for 15 min and centrifuged at 5,000 g for 10 min at 4 °C. The pellet was resuspended in 5 ml of ice cold TB-buffer containing 7% DMSO. This was then dispensed in 100 µl aliquots to 1.5 ml eppendorf tubes, frozen in liquid Nitrogen and stored at -80 °C.

2.10.1.2 Preparation of competent cells using $CaCl_2$

A single colony of *E. coli* XL1 Blue was inoculated in 5 ml of LB medium containing tetracycline (12.5 mg/l). LB medium (50 ml) was inoculated with 1% of the overnight grown *E. coli* culture and allowed to grow till 0.5 O.D. at 600 nm at 37 °C. The cells were harvested by centrifugation at 5,000 g for 10 min at 4 °C, suspended in 100 mM ice-cold $CaCl_2$ and kept on ice for 30 min. Cells were centrifuged, the pellet suspended in 1ml of 100 mM ice-cold $CaCl_2$ and stored as aliquots of 200 µl at 4 °C.

2.10.1.3 *E. coli* transformation

The competent *E. coli* cells, thus formed, were transformed according to Sambrook et al. (1989). Briefly, DNA (~50 ng in 10 µl or less) was added to the (200 µl) competent *E. coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42 °C for 2 min. To each tube 800 µl of LB broth was added and further incubated at 37 °C for 1 h. About 100 µl of the transformed competent cells were spread onto LB plates containing appropriate antibiotics, IPTG and X-gal as per need (Sambrook et al., 1989).

2.10.2 *Agrobacterium tumefaciens* transformation and selection

2.10.2.1 Preparation of competent cells for *Agrobacterium*

The method adopted for the preparation of *A. tumefaciens* competent cells was essentially as reported by Annes (1987). A single colony of *A. tumefaciens* (GV2260) was inoculated in 50 ml LB broth containing Rifampicin 150 µg / ml and incubated at 28 °C with shaking at 200 rpm till O.D. was 0.5 at 600 nm. Cells were centrifuged at 5000 g for 10 min at 4 °C and washed twice with ice-cold 150 mM $CaCl_2$. The cells were pelleted and resuspended in 1ml of ice-cold 20 mM $CaCl_2$. Aliquots of 200 µl were made for further use.

2.10.2.2 *A. tumefaciens* transformation and selection

For transformation 1µg DNA of the desired binary plasmid vector was added to a 200 µl aliquot of the competent *A. tumefaciens* cells and incubated on ice for 30 min. The cells were then snap frozen in liquid nitrogen and allowed to thaw at 37 °C. After thawing 1 ml LB medium was added and the tubes incubated at 28 °C for 2 h with gentle shaking. The cells were centrifuged at 4000 g for 5 min and 100 µl supernatant was retained. The cells were resuspended in the 100 µl supernatant and plated on LB agar medium with appropriate antibiotic(s). The plates were incubated at 28 °C for two days to allow the transformed colonies to grow for further processing.

2.11 PCR Colony Screening

This method bypasses DNA purification, and relies on the selectivity of PCR amplification to determine whether a bacterial colony of interest does indeed contain the desired DNA. Simply adding a small portion of a bacterial colony to a PCR master mix will introduce enough templates of DNA for amplification. A single bacterial colony was added to 1.5 ml eppendorf PCR tube containing 25 µl of water. The microtip was agitated in the water to remove the colony. Subsequently, the 2 –3 µl of this suspension was put into 15 µl of PCR reaction. The remaining components were added to the PCR reaction and subjected to normal cycling parameters for the particular primers. If insert orientation, as well as presence, needs to be determined, utilization of a forward vector-specific primer and a reverse insert-specific primer, or vice versa, allows such determination. If only the presence of the insert needs to be determined, then two insert-specific primers can be used. An additional 5 min denaturation step at 95 °C before the amplification cycles will aid in lysing the bacteria to enhance PCR product amplification success. The resulting PCR products were checked on an agarose gel for the presence of the predicted band.

2.12 Isolation of Nucleic Acids

2.12.1 Isolation of plasmid DNA from *E. coli* cells

The alkaline lysis method of Sambrook *et al*., (1989) was improvised upon so that 12-24 samples could be processed conveniently for plasmid DNA extraction within 3 h,

with yields of 5-30 µg per 1.5 ml culture depending on the host strain and the plasmid vector. The bacterial cultures were grown overnight (O/N) with shaking (200 rpm) at 37 °C in LB broth, with appropriate antibiotic(s). About 1.5 to 3 ml culture was centrifuged for 1 min at 4000 g to pellet the bacterial cells. The pellet was resuspended in 100 µl of TEG buffer (Table 2.4) by vigorous pipetting, 200 µl of Solution II (Table 2.4) was added, mixed by inversion till the solution becomes clear and incubated on ice for 5 min. The cell lysate was neutralized by addition of 150 µl of Solution III (Table 2.4), mixed well and incubated on ice for 5 min. The cell debris was removed by centrifuging for 5 min at 12000 g at room temperature. The supernatant was transferred to a clean tube; RNase A to a final concentration of 20 µg/ml (Sambrook et al., 1989) was added and incubated at 37 °C for 20 min. To the above solution 400 µl of chloroform was added, mixed for 30 s and centrifuged for 5 min at 12000 g at 4 °C. The upper aqueous layer was transferred to a clean tube, 1/10th volume sodium acetate (3 M) and one volume absolute ethanol was added with mixing and kept at -20 °C for 1-2 h. The sample was centrifuged at 12000 g for 10 min at room temperature. The pellet was washed thrice with 70% ethanol and dried under vacuum. The dried pellet was resuspended in 20 µl deionized water and stored at -20 °C.

2.12.2 Isolation of plasmid DNA from *Agrobacterium* cells

Overnight grown culture of *A. tumefaciens* was centrifuged at 4,000 g for 10 min. The cells were washed with 500 µl of Solution I (Table 2.4) containing 10 µl Triton X 100 and resuspended in 300 µl of Solution I (Table 2.4) and 400 µl of Solution II (Table 2.4). The cells were mixed by inverting till the solution turns clear. This was followed by the addition of 400 µl of Solution III (Table 2.4). The suspension was mixed by inverting the tubes several times and centrifuged at 12,000 g for 10 min. Clear supernatant was transferred to fresh 1.5 ml tubes and 2 µl of RNase A (10 mg/ ml) added. The tube was incubated at 37 °C for 30 min and then extracted with 400 µl of Chloroform:isoamyl alcohol. For phase separation the tubes were centrifuged at 12,000 g for 10 min. The upper aqueous phase was transferred to a 1.5 ml tube and 600 µl of isopropanol added. It was mixed thoroughly and the plasmid DNA pelleted by centrifugation at 12,000 g for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, dissolved in 40 µl deionized sterile water and stored at -20 °C till further use.

2.12.3 Isolation of plant Genomic DNA

Genomic DNA was isolated by using the protocol of Lodhi et al. (1994). Fresh young leaves were collected, frozen in liquid nitrogen and crushed to a fine powder. About 1 g of ground tissue was extracted with 10 ml extraction buffer (Table 2.2). The slurry was poured into a clean autoclaved 50 ml centrifuge tube and 100 mg insoluble polyvinylpolypyrrolidone (PVPP) added. The tube was inverted several times to thoroughly mix the slurry, incubated at 60 °C for 30 min and then allowed to cool down to room temperature. 12 ml of chloroform: isoamyl alcohol mix was added and the contents mixed by inverting the tube gently till an emulsion formed. The mix was then centrifuged at 6,000 g for 15 min at room temperature. Supernatant was carefully collected in a fresh tube and chloroform: isoamyl alcohol (24:1) extraction step repeated. To the clear supernatant 0.5 volume of 5 M NaCl was added and mixed gently. Next, two volumes of cold (-20 °C) 95% ethanol was added and the sample kept at 4 °C until DNA strands appeared. The tube was centrifuged at 3,000 g for 3 min and then at 5,000 g for next 3 min. The supernatant was poured off, the DNA pellet washed with cold (4 °C) 70% ethanol and air-dried. DNA was dissolved in 400 µl of TE buffer (Table 2.2). The DNA solution was treated with 1 µl RNase A (10 mg/ml) per 100 µl DNA and incubated at 37 °C for 30 min. The sample was extracted with chloroform: isoamyl alcohol to remove RNAase A. DNA was reprecipitated and dissolved in 40 µl TE buffer. Purity of DNA was checked spectrophotometrically by measuring the absorbance ratio at 260/280 nm. DNA was stored at 4 °C.

2.13 Restriction digestion and ligation of DNA

Plasmid and genomic DNA restriction digestion and the DNA ligation reactions were set up as per manufacturer's recommendations.

2.14 Extraction and purification of DNA from agarose gels

The restriction digested DNA or PCR amplified products were run on an agarose gel in 1X TAE buffer. The gel was stained with ethidium bromide (0.5 µg/ml) and viewed using a hand held long wavelength UV illuminator. The fragments of interest were excised from the gel and weighed. A 100 µg gel slice was transferred to a 1.5 ml

microcentrifuge tube and 300 µl Buffer DE-A (Axygen™ GEL elution kit, Biosciences, USA) was added. The tube was incubated at 70 °C for 5 to 10 min with intermittent mixing until the gel slice was completely dissolved. The gel mixture was cooled down to room temperature and 150 µl of Buffer DE-B was added. The above solubilized agarose was put into Axyprep column and placed into 2 ml microfuge tube. The assembly was centrifuged at 12,000 g for 1 min and filtrate was discarded. 500 µl of wash buffer 1 (provided by Axygen) was added and centrifuged at 12,000 g for 30 s, filtrate was discarded. 700 µl of wash buffer 2 was added and spun at 12,000 g for 30 s, filtrate was discarded. It was repeated again with buffer 2. 1 min empty spin was given to ensure the complete removal of salt. Axyprep column was transferred into a fresh 1.5 ml microfuge tube and 25-30 µl of elution buffer was added to the centre of the membrane and kept it as such for 1 min at room temperature. Centrifuge at 12000 g for 1 min. The eluted DNA was stored at -20 °C. This eluted PCR product was further used for further steps.

2.15 Total RNA isolation

RNase free environment was created and maintained as described by Blumberg (1987). All glass and plastic ware were treated with DEPC (0.1% in water) overnight and autoclaved. The pestle and mortar were also DEPC treated and then baked at 300 ºC for 6 h. All materials were dried in a vacuum oven. Total RNA from different plant tissues was isolated using TRIzol reagent. The plant tissue was collected, washed with DEPC treated water, frozen in liquid nitrogen and crushed to a fine powder. To 100 mg of the fine powder 1 ml TRIzol reagent was added and mixed thoroughly using a vortimix. Chloroform:isoamyl alcohol (300 µl) was added and mixed thoroughly using vortimix. The tubes were centrifuged at 4 °C at 13,000 g for 15 min. The supernatant was transferred to 1.5 ml tubes and the chloroform:isoamyl alcohol step repeated. The aqueous phase was transferred to 1.5 ml tubes and half volume isopropanol added. It was mixed thoroughly and kept for RNA precipitation for 1 h. Total RNA was pelleted out by centrifugation at 13,000 g for 15 min at 4 °C. The RNA pellet was washed with 70% ethanol twice and dried in a SpeedVac centrifugal concentrator. RNA pellet was dissolved in 40 µl of DEPC treated water and stored at -80 °C in aliquots. Purity of

RNA was confirmed by measuring OD at 260/280 nm and also by visualization on 1.5% TAE Agarose gel.

2.15.1 mRNA Purification

Total RNA was quantified spectrophotometrically. QiaGen mRNA purification kit was used for mRNA purification. The amount of RNA was in the range of 1 mg to 3 mg. Appropriate amount of OBB Buffer and Oligotex suspension were added according to manual instruction. The sample was incubated for 3 min at 70 °C in a heating block. Sample was removed from the heating block, and placed at 20 °C to 30 °C for 10 min. Oligotex: mRNA complex was pelleted down by centrifugation for 2 min at maximum speed (14,000–18,000 g), and carefully removed the supernatant by pipetting. Oligotex:mRNA pellet was resuspended in the appropriate amount of OW2 buffer by vortexing and pipetted onto a small spin column placed in a 1.5 ml microcentrifuge tube and centrifuged for 1 min at maximum speed. Spin column was transferred to a new RNase-free 1.5 ml microcentrifuge tube, appropriate amount of OW2 buffer added to the column and again centrifuged for 1 min at maximum speed, flow-through was discarded. Spin column was transferred to a new RNase free 1.5 ml microcentrifuge tube. Appropriate amount of hot (70 °C) OEB buffer was pipetted onto the column and resuspended by pipetting up and down; spin it down for 1 min at maximum speed. The eluted mRNA sample was quantified spectrophotometrically and used for further downstream processes.

2.15.2 cDNA first strand synthesis by Reverse Transcription

Complementary DNA (cDNA) is synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase. The resulting molecule is a DNA-RNA hybrid and the process is called as cDNA 1st strand synthesis. For DNA double strand synthesis this hybrid molecule is digested with RNase H (specific for degrading RNA strand in a DNA- RNA hybrid), DNA second strand is synthesized using DNA polymerase I (Kimmel and Berger, 1987). In the present study cDNA first strand was synthesized using ImPromII™ Reverse Transcription System (Promega, USA). The reactions were set up as per the manufacturer's guidelines. Briefly, reverse transcription reactions of up to 1 µg of total RNA performed in 20 µl reactions

comprised of components of the ImPromII Reverse Transcription System. Experimental RNA was combined with the oligo $(dT)_{15}$ primer. The primer/template mix was isothermally denatured at 70 °C for 5 min and snap chilled on ice. A reverse transcription reaction mix was assembled on ice to contain nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor RNasin®. As a final step, the template-primer combination was added to the reaction mix on ice. Following an initial annealing at 25 °C for 5 min, the reaction was incubated at 42 °C for up to 1 h. The cDNA synthesized was directly added to amplification reactions. The first strand reaction was set up as follows:

Experimental RNA (1µg)	1 µl
Primer [Oligo $(dT)_{15}$ or Random (10 pmol)	1 µl
DEPC treated Water	3 µl
Final volume	**5 µl**

The tubes were incubated at 70 °C for 5 min and then chilled in ice-water for 5 min. Tubes were briefly spun in a microcentrifuge to collect the condensate and maintain the original volume. The tubes were kept closed and on ice until addition of the reverse transcription reaction mix. The reverse transcription reaction mix was prepared by combining the following components of the ImProm-II Reverse Transcription System in a sterile 1.5 ml microcentrifuge tube on ice.

ImProm-II. 5X Reaction Buffer	4.0 µl
$MgCl_2$ (15 mM)	2.0 µl
dNTP Mix (7.5 mM)	1.0 µl
RNasin® Ribonuclease Inhibitor (40 U/µl)	0.5 µl
ImProm-II Reverse Transcriptase	1.0 µl
Nuclease-free water	6.5 µl
Final volume	**20.0 µl**

An aliquot of 1.0 µg total RNA and oligo $(dT)_{15}$ or Random hexamer primer (10 pmol) mix was added to the above reaction for a final reaction volume of 20 µl per tube. The

tube was incubated at 25 °C for 5 min for primer annealing and then at 42 °C for 1 h for cDNA first strand synthesis. Reverse transcriptase was thermally inactivated by incubation at 70 °C for 15 min prior to proceeding with PCR amplification (Chumakov, 1994).

2.16 Spectrophotometric determination of nucleic acid concentration

DNA concentration was determined by measurement of the absorption at 260 nm. A Lambda 25 Perkin Elmer photometer was used to determine the concentration of 1:50 diluted RNA or DNA samples in a volume of 1ml in a 10 mm light path Quartz cuvette. Absorbance readings (A_{260}) should fall between 0.1 and 1.0 to be accurate. Sample dilution was adjusted accordingly. An absorbance of 1.0 at 260 nm corresponds to 50 µg DNA/ ml.

2.17 Polymerase Chain Reaction (PCR)

PCR is a powerful technique to amplify a desired nucleotide sequence using sequence specific primers. This amplification may be either of and from a single template or of a template from a mixture of templates (Saiki *et al.,* 1985; Mullis and Faloona, 1987; Saiki *et al.,* 1988; Mullis, 1990; Arnheim and Erlich, 1992). This technique has been successfully used for various purposes like fishing out of gene(s) from genomic DNA or from cDNA population (Todd *et al.,* 1987), introducing restriction sites of interest in the amplified product for directional cloning (Scharf *et al.,* 1986), creating sequence mismatch/ deletion/ addition resulting in mutant version of a gene or nucleotide sequence (Goodenow *et al.,* 1989), differentiating between two alleles (Kwok *et al.,* 1990) etc. In the present study applications of PCR were exploited for a few of the above specified applications. The PCR reaction mixture and cycling conditions used were as follows:

Reaction mixture

Sterile deionized water	6.2 µl
Template (50 ng/µl)	1.0 µl
Forward primer (7 pmol)	1.0 µl

Reverse primer (7 pmol)	1.0 µl
dNTPs (0.2 mM)	4.0 µl
10X Buffer (Mg^{+2} 1.5 mM)	1.5 µl
Taq DNA Polymerase (1 U/µl)	0.3 µl
Total volume	**15.0** µl

PCR cycle conditions

1 cycle- 95 °C 5 min

35 cycles- 95 °C 1 min / 45-65 °C 30-45 s (annealing temperature was dependent on primer Tm) / 72 °C 1 min 30 s

1 cycle- 72 °C 10 min

1 cycle- 4 °C hold

2.18 Rapid amplification of cDNA ends (RACE)

Generally, using reverse transcription PCR, either partial cDNA fragments (both 5' and 3' ends missing) or cDNA with full 5' end missing are amplified from total cDNA. If a partial cDNA sequence is known, unknown sequences to the 5' and 3' of the known sequence can be reverse transcribed from RNA, amplified by PCR using RACE (Frohmman *et al.,* 1988). BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clonetech) and RACE Ready cDNA Kit (Invitrogen, USA) was used for 3' and 5' RACE respectively. The reactions were set up as per the manufacturer's guidelines. The RACE technique is based on oligo-capping and RNA ligase-mediated (RLM) RACE methods (Maruyama and Sugano, 1994; Volloch *et al.*, 1994).

Application of RACE is as follows:

- Identifying the 5' and 3' un-translated regions of genes
- Studying heterogeneous transcriptional start sites
- Characterizing promoter regions
- Obtaining the complete cDNA sequence of a gene
- Amplifying the full-length cDNA for downstream cloning and expression

2.18.1 GeneRacer RACE Ready cDNA method

The GeneRacer method involves selectively ligating an RNA oligonucleotide (GeneRacer RNA Oligo) to the full-length 5' ends of decapped mRNA using T4 RNA ligase.

The prerequisite to begin the RACE was to have the good quality RNA or mRNA. 1-5 µg total RNA or 50- 250 ng purified mRNA in DEPC water was used for the RACE reaction**.** Dephosphorylation reaction of 10 µl was set in 1.5 ml sterile micro centrifuge tube. The reaction is as follows.

Reagent	**Volume**
RNA	2 µl
10X CIAP Buffer	1 µl
RNaseOut™ (40 U/µl)	1 µl
CIP (10 U/µl)	1 µl
DEPC water	5 µl
Total Volume	**10 µl**

7 µl of dephosphorylated, decapped RNA was added to the tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 µg). RNA Oligo was resuspended by pipetting up and down several times. Mixture was incubated at 65 °C for 5 minutes and kept on ice.

Reagent	**Volume**
10X Ligase Buffer	1 µl
10 mM ATP	1 µl
RNaseOut™ (40 U/ µl)	1 µl
T4 RNA ligase (5 U/µl)	1 µl
Total Volume	**11 µl**

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 45 °C for 1 h. The reaction was inactivated by incubating the reaction mixture at 70 °C for 15 min. 1 µl of RNase-H (2 U) was added

to reaction mixture to chew up the DNA / RNA hybrid by incubation at 37 °C for 20 min. Centrifuged the sample and kept at -20 °C for further use in PCR.

Primers

GeneRacer. 5' Primer 5' –GCA CGA GGA CAC UGA CAU GGA CUG A-3'

5' Nested Primer 5'-GGA CAC TGA CAT GGA CTG AAG GAG TA-3'

CAD 8L2 (GSP) 5'-ACC ACC TCA TGC CCA GGA ACC ATG GGA T-3'

2.18.1.1 PCR Setup

Reactions were setup to amplify the 5′ end of gene of interest. The reaction was set up as given in table below:

Reagent	**5′ RACE**
GeneRacer 5′ Primer10 µM	3 µl
Reverse GSP 10 µM	1 µl
RT Template	1.0 µl
10X High Fidelity PCR Buffer	5.0 µl
dNTP Solution (10 mM each)	1.0 µl
Platinum® Taq DNA Polymerase High Fidelity, 5 U/µl	0.5 µl
$MgSO_4$, 50 mM	2.0 µl
Sterile Water	36.5 µl
Total Volume	**50.0 µl**

2.18.1.2 Cycling

Temperature	Time	Cycles
94 °C	2 min	1
94 °C	30 s	5
72 °C 1 min/1 kb	2 min	
94 °C	30 s	5
70 °C 1 min/1 kb	2 min	
94 °C	30 s	25
66 °C	30 s	
72 °C	2 min	
72 °C	10 min	1

Following program was used for the nested PCR reactions.

Temperature	Time	Cycles
94 °C	2 min	1
94 °C	30 s	25
66 °C	2 min	
72 °C	2 min	
72 °C	10 min	1

10-20 µl of nested PCR product was analysed on a 1% agarose/ethidium bromide gel. Nested PCR products were shorter by the number of bases between the original primers and the nested primers. The largest product was representing the most full-length message. The band(s) were excised, cloned and sequenced.

2.18.2 BD SMART RACE method

First-strand synthesis is primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The BD SMART II A Oligonucleotide anneals to the tail of the cDNA and serves as an

extended template for BD PowerScript RT. Following reverse transcription, the first-strand cDNA is used directly in 5'- and 3'-RACE PCR reactions.

Primers

3'-RACE CDS Primer A (3'-CDS)-

5'–AAGCAGTGGTATCAACGCAGAGTAC(T)30V N–3'

(N = A, C, G, or T; V = A, G, or C)

10X Universal Primer A Mix (UPM)

Long (0.4 µM):

5'CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT3'

Short (2 µM): 5'–CTAATACGACTCACTATAGGGC–3'

Nested Universal Primer A

(NUP; 10 µM) 5'–AAGCAGTGGTATCAACGCAGAGT–3'

GSP 2 5'–CAACGTGACA GTGATAAGCT CGTCG–3'

2.18.2.1 Reaction

For preparation of 3'-RACE-Ready cDNA, following components were combined in 0.5-ml microcentrifuge tube

Reagent	Volume
RNA sample	1–3 µl
3'-CDS primer A	1 µl

Sterile H_2O was added to a final volume of 5 µl. The contents were mixed and the tubes were spun briefly in a microcentrifuge. The tubes were incubated at 70 °C for 2 min. The tubes were then cooled on ice for 2 min and again spun briefly to collect the contents at the bottom.

The following components were added to tube (already containing 5 µl earlier reaction)

Reagent	Volume
5X First-Strand Buffer	2 µl
DTT (20 mM)	1 µl
dNTP Mix (10 mM)	1 µl

BD PowerScript Reverse Transcriptase	1 µl
Total final volume	10 µl

The contents were mixed gently by pipetting and the tubes were given a brief spin and were then incubated at 42 °C for 1.5 h in an air incubator or a hot-lid thermal cycler. The first-strand reaction product was diluted with Tricine-EDTA Buffer: The tubes were heated at 72 °C for 7 min and the samples were stored at –20 °C.

3'-RACE

Reagent	**Volume**
3'-RACE-Ready cDNA	2.5 µl
UPM (10X)	5 µl
GSP2 (10 µM)	1 µl
Master Mix	41.5 µl
Final volume	**50 µl**

2.18.2.2 Cycling conditions

Temperature	**Time**	**Cycles**
94 °C	30 s	25
68 °C	30 s	
72 °C	3 min	

Following program was used for the nested PCR reactions.

Temperature	**Time**	**Cycles**
94 °C	2 min	1
94 °C	30 s	25
65 °C	2 min	
72 °C	2 min	
72 °C	10 min	1

2.19 Quantitative Real Time PCR (Q-PCR):

Quantitative Real Time PCR is a powerful tool for gene expression analysis and was first demonstrated by Higuchi *et al.* (1992, 1993). Q-PCR quantifies the initial amount

of the template most specifically and is a preferable alternative to other forms of quantitative reverse transcriptase PCR that detect the amount of final amplified product at the end point (Freeman *et al.,* 1999). Q-PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (*i.e.*, in real time) as opposed to the endpoint detection. In the present study the SYBR Brilliant® II QPCR Master Mix (Stratagene, USA) was used. This kit supports quantitative amplification and detection systems. The kit supports PCR amplifications and detection of a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA. The SYBR Brilliant II QPCR master mix includes SureStart® *Taq* DNA polymerase, a modified version of *Taq2000*™ DNA polymerase with hot start capability. A passive reference dye (an optional reaction component) is provided so as to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms. The fluorescent dye SYBR Green I in the master mix enables the analysis of many different targets without having to synthesize target-specific labeled probes.

Real time PCR can be divided into four major phases: the linear ground phase, early exponential phase, log-linear (also known as exponential) phase, and plateau phase. During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. The cycle at which this occurs is known as Ct in ABI Prism® literature (Applied Biosystems, Foster City, CA, USA) or crossing point (CP) in LightCycler® literature (Roche Applied Science, Indianapolis, IN, USA). This value is representative of the starting copy number in the original template and is used to calculate experimental results. During the log-linear phase, PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction conditions. Finally, the plateau stage is reached when reaction components become limited and the fluorescence intensity is no longer useful for data calculation. In general., lower Ct value indicates higher initial copies.

2.19.1 Q-PCR considerations:

The optimal concentration of the upstream and downstream PCR primers is determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The best concentrations of the upstream and downstream primers are not always of equal molarity. In this study, 40 nM was considered optimum. Reaction was standardized in such a way that there was no primer dimer formation. Acquisition of real-time data generated by SYBR Green 1 was done as recommended by the instrument manufacturer. Data collection was either at the annealing step (3- step cycling protocol) or extension step of each cycle.

Magnesium chloride concentration in the PCR reaction mix affects the specificity of the PCR primers and probe hybridization. The SYBR Green Brilliant® II QPCR Master Mix kit contains $MgCl_2$ at a concentration of 5.5 mM (in the 1X solution), which is suitable for most targets.

2.19.2 Preparing the reactions:

Real time PCR model Mx 3000P (Stratagene, USA) was used in the present study. The experimental reactions were prepared by adding the following components in order.

2.19.2.1 Reaction Mixture:

Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µl (including experimental DNA)

Reagent	**Volume**
2X master mix	12.5 µl
Upstream primer (optimized concentration)	1 µl
Downstream primer (optimized concentration)	1 µl
Total	**25 µl**

The reactions were mixed without creating bubbles and 1 µl of experimental genomic DNA, cDNA or plasmid DNA was added to each experimental reaction. Reaction was mixed gently and briefly spun.

2.19.4 Real-time quantitative PCR for *CAD* gene:

Total RNA was extracted individually from leaves, roots and shoots at different time intervals from developing seedlings of *L. leucocephala.* One µg of total RNA was used for making cDNA using ImProm cDNA synthesis kit (Promega, Madison, USA). Brilliant SYBRGreen QPCR kit (Stratagene, USA) and Stratagene Mx3000P Real time Machine were used for all reactions. The primer sequences that were designed for *Leucaena CAD* gene and 5.8 S rRNA are given in Table 2.0. Optimal numbers of PCR cycles within the linear range of amplification for each gene were determined in preliminary experiments. RT-PCR reactions were performed under the following conditions: 2 min at 50 °C, followed by 10 min at 94 °C, 37 cycles (for *CAD*) or 35 cycles (for 5.8 S rRNA) of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C. The reaction was run in triplicates and repeated twice. It was ensured that equal quantity of RNA template was used for each reaction (Pfaffl 2001; Freeman *et al*., 1999; Edyta Zdunek-Zastocka 2008).

Primer	Sequence 5' – 3'	Tm in °C
CAD F1	TGC CAT TCC GAT CTG CAT CAG	62
CAD R1	TTA AAC TTG GTC ACA TTC GAA CCC	68
CTF	CTAAACGACTCTCGGCAAC	58
CTR	TTCAAAGACTCGATGGTTCAC	60

Table 2.0 Primer sequences designated for *Leucaena CAD* gene and 5.8 S rRNA

2.20 Nucleic acid blotting & hybridization

2.20.1 Southern Blotting

For Southern hybridization (Southern 1975) the DNA samples were electrophoresed on an 0.8% agarose gel in 1X TAE (Table 2.1) buffer containing 0.5 µg / ml ethidium bromide. The gel was rinsed with deionized water (DW) and placed in depurination

solution (Table 2.2) for 15 min. The gel was rinsed with deionized water and immersed in denaturation solution (Table 2.2) for 30 min with gentle shaking. The gel was again rinsed with deionized water and transferred to neutralization solution (Table 2.2) for 45 min. The gel was then set up for capillary transfer of DNA to solid membrane support.

A tray was filled with the transfer buffer (20X SSC). A platform was made and covered with a wick, made from 2 sheets of Whatman 3 mm filter paper saturated with transfer buffer and the gel was placed on it. It was surrounded with Saran Wrap to prevent the transfer buffer from being absorbed directly by the paper towels stacked above the membrane. A sheet of Hybond-N^+ membrane (Amersham, UK) of the exact gel size was wetted with deionized water followed by transfer buffer (20X SSC) and then placed on top of the gel. A glass rod was rolled over the membrane to remove any trapped air bubbles. One piece of Whatman 3 mm paper wetted with 20X SSC was placed on the membrane followed by Whatman 3 mm paper pre-wetted in 2X SSC. On this paper another dry Whatman 3 mm paper was placed followed by a stack of absorbent paper towels. A glass plate and a ~0.5 kg weight were placed on the top of the paper towels. Transfer of DNA was allowed to proceed for 18 h. The membrane was marked for orientation, removed carefully and washed with 6X SSC. The membrane was air dried and baked for 2 h at 80 °C to immobilize DNA onto the nylon membrane. Hybridization and autoradiography were carried out as described in the following section.

2.20.2 Dot Blot Hybridization

For dot blot hybridization DNA samples were diluted according to experimental requirements. The DNA samples were denatured by adding $1/10^{th}$ volume 3M NaOH and incubation at 65 °C for 10 min. The genomic DNA of equal quantity was applied on the Hybond-N+ membrane (Amersham, UK) in small volumes. The membrane was air dried and then baked for 2 h at 80 °C to immobilize DNA. Hybridization and autoradiography were carried out as described in the following section 2.20.4.

2.20.3 Random primer labeling

Random primer labeling of the DNA probes (Feinbeng and Vogelstein 1983, 1984) was done using the Megaprime DNA labeling kit (Amersham, UK). Reaction (50 µl) was set up as follows:

Component	**Volume**
25 ng DNA (used as probe)	5.0 µl
Primer solution (Random hexanucleotides) (3.5 A_{260} U)	5.0 µl
Final Volume	**10.0 µl**

Above mixture was heated in a boiling water bath for 10 min and cooled to room temperature facilitating primer annealing to the DNA.

Component	**Volume**
10 X reaction buffer	5.0 µl
dATP (0.5 mM)	4.0 µl
dGTP (0.5 mM)	4.0 µl
dTTP (0.5 mM)	4.0 µl
(α-^{32}P)-dCTP (Sp. activity 3000 Ci mmol^{-1})	5.0 µl
Sterile deionized water	16.0 µl
Exonuclease free Klenow fragment (2 U/µl)	2.0 µl
Total volume	**50.0 µl**

The reaction was carried out at 37 °C for 45 min and stopped by incubation in a boiling water bath for 10 min and snap chilled on ice.

2.20.4 Hybridization

The blots made as in sections 2.11.1 and 2.11.2 above were pre-hybridized at 57 °C in 30 ml of hybridization buffer (Table 2.2) for 6-8 h in a hybridization incubator (Robin Scientific, USA). The buffer was decanted and fresh buffer added with the denatured radio-labeled probe. Hybridization was carried out at 60 °C for 14-18 h. The solution was decanted and the membrane washed with low stringency buffer at 55-65 °C for 15 min followed by a high stringency wash at 60 °C for 15 min. The moist blot was

wrapped in Saran wrap and exposed to X-ray film at -70 °C in a cassette with intensifying screen.

2.21 Expression and purification of recombinant protein

2.21.1 Expression of recombinant protein

A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 µg/ml kanamycin) was used to inoculate 5 ml liquid LB medium containing the same concentrations of the antibiotic. Culture was grown overnight with shaking at 225 rpm at 37 °C. 1 ml aliquot of each culture was used to inoculate 100 ml liquid cultures containing 50 µg/ml kanamycin. Once the cultures reached A_{600} 0.4 - 0.5, recombinant protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG), and the culture was grown for 4–6 h at 37 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. Pellets were resuspended in 6.25 ml lysis buffer (Table 2.6). Cells were disrupted by sonication for 5 min at 70 amplitude on a Sonifer Cell Disruptor. $MgSO_4$ of final concentration of 10 mM and lysozyme of final concentration 100 µg/ml was added to the disrupted cell and kept at 37 °C for ½ h. It was centrifuged at 10000 rpm for 10 min and supernatant was saved as lysate and pellet was resuspended in 2 ml sonication buffer (Table 2.6). Suspension was again sonicated for 1 min at 70 amplitude to disrupt the inclusion bodies and this disrupted inclusion bodies were dissolved in 3 ml of dispersion buffer (Table 2.6) and an aliquot of 20 µl checked on SDS PAGE (Table 2.5) to check the expression.

2.21.2 Affinity purification of recombinant protein using Ni^+ NTA beads

The recombinant protein, among several other bacterial proteins was loaded on affinity matrix column such as Ni-agarose. This affinity matrix contains bound metal ion nickel, to which the polyhistidine-tag binds with micro molar affinity. The matrix is then washed with buffer to remove unbound proteins. The washing efficiency may be improved by the addition of 20 mM imidazole and histidine-tagged proteins are then usually eluted with 150-300 mM imidazole. The purity and amount of protein is assessed by SDS-PAGE**.** The column(s) and buffers were equilibrated to room

temperature. The Ni^{+} beads column was equilibrated with binding buffer (Table 2.7) for 30 min and then three bed volume of binding buffer was passed through the column followed by addition of soluble protein in dispersion buffer. The column was kept at 4 °C for 1 h for binding of recombinant protein to Ni^{+} beads. Flow through was collected in different tube after 1 h and column was washed with two bed volume of washing buffer (Table 2.7). The 6X His-tagged bound protein was eluted in 4 aliquots of elution buffer (Table 2.7) 0.5 ml each. Protein elution was monitored by measuring the absorbance at 280 nm of collected fractions. The eluted protein was analyzed by SDS-PAGE.

2.21.3 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE system is the widely used electrophoresis system for protein separations (Laemmeli 1970). The resolution in a Laemmli gel is excellent because the treated peptides are stacked in a stacking gel before entering the separating gel.

2.21.3.1 Preparation of the separating gel

A vertical slab gel (Hoeffer Scientific, U.S.A.) was assembled using 1.0 mm spacers. In a sidearmed vacuum flask, 10% separating gel solution was made according to Table. 2.5 except for the addition of ammonium persulfate and TEMED. The solution was degassed by applying vacuum while stirring on a magnetic stirrer. TEMED and ammonium persulfate were added, and mixed gently without generating bubbles. The solution was pipetted into the gel cassette leaving 1.5 cm from the top unfilled. The gel solution was overlaid with water saturated n-butanol to remove trapped air bubbles and to layer evenly across the entire surface. When a sharp liquid-gel inter-surface was observed after the gel polymerization, the slab was tilted to pour off the overlay.

2.21.3.2 Preparation of the stacking gel

Stacking gel solution was prepared according to Table. 2.5, leaving out ammonium per sulfate and TEMED. As in the separating gel, this solution was degassed. TEMED and ammonium per sulfate were added, mixed and overlaid on the separating gel. A comb was inserted taking care not to trap air bubbles beneath the comb teeth. The gel was left to polymerize.

2.21.3.3 Preparation of the sample

Equal parts of the protein sample and the loading buffer (Table 2.5) were mixed in a microcentrifuge tube and kept in a boiling water bath for 90 s. The samples were centrifuged at 10,000 g for 10 min, supernatant collected and used for electrophoresis.

2.21.3.4 Loading and running the gel

The comb was removed from the gel, the wells were flushed with distilled water and drained off completely. The wells were filled with tank buffer and the samples under laid using a syringe. Lower and upper buffer chambers were filled with tank buffer. Voltage was set between 70 and 80 volts. The run was stopped when the dye reached the bottom of the gel.

2.21.3.5 Coomassie blue staining of the gel:

Coomassie blue staining solution: 45 ml Methanol, 10 ml acetic acid, 45 ml de-ionized water and 0.25% Coomassie blue (R 250)

After running the gel it was transferred directly to a tray containing Coomassie blue staining solution and was kept for three to four hours at room temperature on rocker. Staining solution was poured off and de-staining solution (Table 2.5) was poured in. De-staining step was repeated two-three times till clear bands appeared.

2.22 Raising polyclonal Antibody against CAD in Rabbit

The purified CAD protein was used for raising polyclonal antibodies in rabbit (New Zealand White).

2.22.1 Pre-treatment of serum:

Rabbit immune serum (stored at -70 ºC), containing polyclonal antibodies against the antigen, was thawed overnight at 4 ºC. The Serum was kept at 55 °C for one hour and the immune serum was centrifuged at 12,000 g for 15 min at 4 ºC. Aliquots according to the requirements were made and stored at -70 ºC. Prior to use again antiserum was thawed overnight at 4 ºC.

2.23 Determination of titre of antibodies and ELISA:

2.23.1 Determination of titre of antibodies

ELISA (Enzyme-Linked Immunosorbent Assay) was performed to determine the titre of first, second and third bleed of rabbit serum. Equal quantity of antigen *i.e.* 100 ng was coated in triplicates on ELISA plates and kept overnight at 4 °C. Next morning, the plate was washed with 250 µl of PBS-T, three times for 5 min and 300 µl of blocking reagent was added. The plate was wrapped in aluminium foil and kept at 37 °C for 2 h. ELISA plate was washed again as described earlier and challenged to different dilution of serum such as, 1:50, 1:100, 1:200,……. 1:6553600, 1:13107200 and 1:26214401. All dilutions were in triplicates and plate was kept at 37 °C for 2 h again. Plate was washed as described earlier with PBS-T buffer and secondary antibodies (anti goat IgG against rabbit IgG) tagged with alkaline phosphatase was added to a dilution of 1:20000. Plate was incubated at 37 °C for two more hours. After 2 hours of incubation plate was washed with 250 µl of PBS-T, three times for 5 min and 100 µl of 1 mg/ml substrate (*p*- Nitro phenyl phosphate) was added and incubated for 45 min. Reaction was stopped by adding 10 mM EDTA. Once the antibody titre was determined then a fixed dilution of antibodies was used for rest of the experiments.

2.23.2 ELISA of CAD in different tissues of *L. leucocephala*

Fresh tissues of *L. leucocephala* were collected, frozen in liquid nitrogen and crushed to a fine powder. Crude protein was extracted with 2 ml of protein extraction buffer (2.24.2). Total Protein was quantified using Bradford reagent. Equal amount of protein (25 µg) was coated on 96 well micro titer plates. Antigen (crude extract) was diluted in 50 mM sodium carbonate buffer to an optimal concentration and coated on 96 well microtiter plate (100 µl/well). Plate was incubated for 2 h at room temperature or over night at 4 °C and washed twice with PBS-T after incubation. Non-specific sites were blocked with blocking buffer 300 µl/well and incubated for 1h at room temperature. After washing out the unbound blocking agent with PBS-T, primary antibody (Anti rabbit IgG) was added and incubated for 1 h at room temperature. The unbound primary antibody was washed twice with PBS-T and secondary antibody conjugated with alkaline phosphatase was added and incubated for 2 h at room temperature. Enzyme

specific substrate pNPP was added at a concentration of 1mg/ml and incubated until the color develops and the colorimetric reaction was measured at 405 nm.

2.24 Enzyme Assay

2.24.1 Recombinant CAD protein extraction and purification

2.24.1.1 Recombinanat CAD protein extraction

A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 µg/ml kanamycin) was used to inoculate 5 ml liquid LB medium containing the same concentrations of the antibiotic. Culture was grown overnight with shaking at 225 rpm at 37 °C. 1 ml aliquot of each culture was grown in 100 ml liquid cultures containing 50 µg/ml kanamycin and 1 mM $ZnCl_2$ until they reached A_{600} 0.4-0.5. The cells were then cooled to 15 °C and then the recombinant protein expression was induced by the addition of 1mM isopropyl β-D-thiogalactopyranoside (IPTG), and the culture was grown for 16 h at 15 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. Pellets were resuspended in 6.25 ml lysis buffer (Table 2.8). Lysozyme was added to a final concentration of 1mg/ml and the cells were incubated at 4 °C until lysis occurred. The cells were then sonicated four times for 5 s, cooling on ice between each sonication. Cell debris was then pelleted by centrifugation at 10000 rpm for 10 minute at 4 °C. The supernatant was loaded on affinity matrix column such as Ni-agarose.

2.24.1.2 Recombinanat CAD protein purification

The affinity matrix contains bound metal ion nickel, to which the polyhistidine tag binds with micro molar affinity. The matrix is then washed with buffer to remove unbound proteins. The washing efficiency may be improved by the addition of 20 mM imidazole and histidine-tagged proteins are then usually eluted with 150-300 mM imidazole. The purity and amount of protein is assessed by SDS-PAGE**.** The column and buffers were equilibrated to room temperature. The Ni^+ beads column was equilibrated with binding buffer for 30 min and then three bed volume of binding buffer (Table 2.8) was passed through the column followed by addition of supernatant. The column was kept at 4 °C for 1 h for binding of recombinant protein to Ni^+ beads. Flow

through was collected in different tube after 1 h and column was washed with two bed volume of washing buffer (Table 2.8). The 6X His-tagged bound protein was eluted in 4 aliquots of elution buffer (Table 2.8) 0.5 ml each. The eluent 2 and 3 were checked for enzyme activity.

2.24.2 Plant protein extraction

The method given by Sato *et al.* (1997) was followed for plant crude protein extraction. Plant tissue was crushed to a fine powder in liquid nitrogen. Tissue was extracted with the grinding buffer (Table 2.9) for 10 min at 4 ºC. The homogenized tissue was transferred to eppendorf tubes and centrifuged at 12,000 g for 10 min. The clear extract was transferred to another tube. The extract was subjected to 80% ammonium sulfate precipitation. The precipitate was dissolved in dialysis buffer and was dialysed against the dialysis buffer (Table 2.9) O/N. The dialysed extract was used for CAD enzyme activity studies.

2.24.3 CAD enzyme assay

CAD enzyme activity was measured according to the method of Wyrambik (1975). Coniferaldehyde was used as substrate for the reaction. The reaction mixture contained 1.156 µM substrate, 40 µM NADP, and 200-500 µM enzyme in 0.2 M Potassium phosphate buffer (pH 6.5). Reaction without substrate was taken as blank. For each reaction, decrease in absorbance at 340 nm was monitored for 3 min. Readings were taken every 30 seconds. Each reaction was run in triplicate. The readings were plotted against time and the slope was calculated. The activity was calculated and expressed as nKat/mg of protein.

2.25 Histology and Immuno-cytolocalization:

Free hand transverse sections were fixed overnight under vacuum in freshly prepared cold 4% buffered formaldehyde (4% paraformaldehyde in 1X PBS). The sections were dehydrated by passages through increasing ethanol: water series (30%, 50%, 70%, 85%, 95% and 100% ethanol) for 30 min each. This was followed by passages through tertiary butanol: ethanol series (25:75, 50:50, 75:25, and 100:0). The sections were rehydrated by treating with 70%, 50% ethanol and 0.5X SSC (Table 2.10) for 2 min.

The rehydrated sections were soaked in two changes of 1X PBS (Table 2.10) for 10 min each. Next, the sections were washed in 1X PBS containing 0.1% BSA for 5 min and subjected to 30 min of blocking with 10% BSA at room temperature in a humidified chamber. Post blocking washes included three washes of 15 min each with 1X PBS containing 0.1% BSA. Primary antibody incubation was carried out overnight in a humidified chamber at 4 °C using 75 µl of diluted antibody (1µg/ml) in 1 X PBS containing 0.1% BSA. Negative controls included either the use of pre-immune serum or the omission of both antibody and pre-immune serum. Following the primary antibody incubation, the sections were washed thrice for 15 min each in 1X PBS containing 0.1% BSA. A secondary antibody, 0.2% Anti-rabbit-IgG-goat alkaline phosphate conjugate antibody (diluted in 1X PBS with 0.1% BSA), was added to the tissue sections at this stage and incubated at 37 °C in a humidified chamber for 2 h in dark. Post secondary antibody washes were carried out at room temperature using 1X PBS with 0.1% BSA. Color was developed in dark by addition of 60 µl of color development reagent (20 µl BCIP/NBT mix in color development buffer (Table 2.10) containing 10% polyvinyl alcohol) to the color development buffer treated slides. The slides were placed in humidified chamber at RT in dark. Upon color development, 10 mM EDTA was used to stop the reaction, rinsed with water, air-dried, cover slip-mounted using glycerol and observed under microscope, and microscopic image captured.

2.25.1 Phloroglucinol staining

Free hand transverse sections were prepared for histochemistry as described above. Phloroglucinol staining was done by covering pretreated slide with phloroglucinol for half an hour and then concentrated HCl was added (Harris *et. al*., 1980; Speer *et. al*., 1987). Lignified tissue takes red color. A cover slip mounted on the specimen using glycerol was sealed to the slide using nail enamel. The slides were viewed under a light microscope and pictures captured.

2.26 *Agrobacterium* mediated tobacco transformation

The tobacco regeneration and transformation protocol is modified from Horch *et al*. (1985). *Agrobacterium tumefaciens* strain GV2260 harbouring the binary plasmid

vector to be transformed in tobacco was inoculated in 5 ml YEP medium containing rifampicin (250 mg/l) and kanamycin (50 mg/l). The culture was allowed to grow overnight at 28 °C with shaking at 200 rpm. Next day 1ml inoculum from this tube was taken and added to 50 ml YEP flask containing appropriate antibiotics. The culture was incubated at 28 °C for 24 h with shaking at 200 rpm. Fresh leaves were taken from axenic cultures of *Nicotiana tabacum* var. Anand 119 and ~6 mm diameter disc were punched out. The leaf discs were inoculated on MS medium agar plates containing 4.4 µM BAP and 5.37 µM NAA (MSBN) and kept for 48 h in dark. *A. tumefaciens* GV2260 cells grown for 24 h were centrifuged for 10 min at 5,000 g. Cells were suspended in 20 ml of 50 mM $MgSO_4$ and 200 µM Acetosyringone and allowed to stand for 10 min with intermittent shaking. The tobacco leaf discs from axenic tobacco cultures were co-cultivated in this suspension for 10 min. with intermittent shaking. The leaf discs were then transferred to MSBN plates without any antibiotics. The adaxial side of the leaf disc was in contact with the medium. The plates were incubated in dark at 28 °C. After two days the leaf discs were harvested, washed with liquid MSBN and dried on sterile filter paper. Up to 10 leaf discs per plate were transferred to MSBN agar plates containing Hygromycin (7.09 µM) and Cefotaxime (200 µM). The cultures were incubated at 28 °C with 18/6 h photoperiod in diffused light (60-80) mE/mS.). The leaf discs were subcultured every week till elongated shoots were excisable. Elongated shoots were excised and transferred to root induction medium. The rooted plants were hardened and further analyzed.

2.27 Genetic Transformation of *Leucaena leucocephala*

One day old embryo axes without cotyledons were used as explants for transformation. Seeds of *Leucaena* imbibed in distilled water after the treatment with conc. sulphuric acid (7 min) and mercuric chloride (0.1% for 10 min), were used as source of embryo axes. Embryo axes excised from the seeds and inoculated on regeneration medium (½ MS+ TDZ (0.5 mg/l)). The embryos were then used for transformation.

The transformation was carried out by three methods:

1) Particle bombardment

2) Particle bombardment followed by co-cultivation

3) Agro-infusion method

2.27.1 Particle Bombardment

Calculated amount (approximately 500 µg) of gold particle of 1µm size was used for bombardment. The gold particles taken in microcentrifuge tubes were washed with sterile water and supernatant was decanted after centrifuging it at 13,000 g for 10 min. The washing of particles was repeated three times with 70% (freshly prepared) ethanol and supernatant was decanted after centrifuging it at 13,000 g for 10 min. Again particles were dissolved in 500 µl sterile water and pelleted down after centrifuging it at 13,000 g for 10 min. 1 µg of DNA (plasmid/cassette) was dissolved in 100 µl of Xho buffer. DNA was added to the prepared gold particle and mixed it by vortexing. 100 µl of 0.1 M spermidine was added to it and mixed by vortexing. 100 µg of PEG 3000 was added and mixed by vortexing followed by addition of 100 µl of 2.5 M $CaCl_2$ and incubated in ice bath for 10 min, gave brief spin to settle down sample at the bottom. It was resuspended in absolute ethanol and sonicated briefly to resuspend the particle and was used for bombardment. Particle bombardment was done using BioRad PDS 1000/ He system. 1100 psi He gas pressure was used to bombard the particle at the distance of 6 cm. Microcarrier travel distance was adjusted to 6 cm. The embryo axes were arranged in the centre of the petridishes and bombarded with gold particles coated with DNA (plasmid/cassette). The explants were incubated in dark for 72 h after the bombardment.

2.27.2 Particle bombardment followed by co-cultivation

The embryo axes were first bombarded with gold particles coated with DNA (plasmid/cassette) as mentioned above followed by co-cultivation with *Agrobacterium* (GV2230) harboring pCAMBIA1301 containing *CAD* gene in anti-sense orientation. Cultured bacterial cells were pelleted by centrifugation at 5,000 g for 5 min and resuspended in the YEM medium at a density of 3-5 × 10^9 cells/ml, considering the optical density of bacterial culture at 600 nm. The selected embryo axes after bombardment were co-cultured with *Agrobacterium* for one hour by immersing them into the bacterial suspension. The agro infected embryo axes were then transferred onto the regeneration medium (½ MS+ TDZ (0.5 mg/l)) with or without 0.1 mM acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Aldrich Chemical Co.) and

co-cultivated in the dark at 28 ± 2 °C for 3 days. After co-cultivation, the embryo axes were washed thoroughly with cefotaxime 250 mg/l in sterile distilled water and transferred onto the regeneration medium (½ MS+ TDZ (0.5 mg/l)).

2.27.3 Agro-infusion method

In this method, the *Agrobacterium* was introduced forcibly with a sterile needle to the meristematic region of the explants. After transformation (Particle bombardment / Particle bombardment followed by co-cultivation /Agro-infusion), embryo axes were cultured on regeneration medium (½ MS+ TDZ (0.5 mg/l)) without selection for one week. Then the explant was shifted to selection medium containing hygromycin (10 mg/l) for 3 weeks followed by selection on hygromycin 15 mg/l for another 3 weeks. The survived ex-plants on hygromycin (15 mg/l) were shifted to ½- MS without hygromycin selection. Cytokinin 2ip (2-isopentenyl adenine at 0.5 mg/l) was used in the medium to have better elongation of transformed shoots.

2.28 GUS histochemical assay

The GUS staining solution was prepared by taking 1 mM X-Gluc (5-bromo, 4-chloro, 3-indolyl-β-D-glucuronide: Cyclohexylammonium salt (X-GlcA) from a 20 mM stock made in dimethylformamide, 100 mM sodium dihydrogen phosphate dihydrate and 0.5% Tween-20. The pH of the solution was adjusted to 7.0 with 1N NaOH. The histochemical GUS assay was performed as described by Jefferson (1987) to monitor *GUS* gene expression in putative transgenic embryo axis. The GUS assay was carried out on 10% of the randomly selected embryo axis immediately after co-cultivation as well as 21 days after co-cultivation. Embryo axes were immersed in GUS assay solution and incubated at 37 °C overnight. *GUS* gene expression was observed and photographed by using Axioplan2 microscope from Carl Zeiss.

2.29 Lignin estimation

A modified method from Luo (2008) was used for estimating lignin in tranformant plants. The plant material is ground finely in a mortar and pestle and then dried in oven at 55 °C. The preweighed dried tissue is extracted continuously with acetone:water (10:1) for 24 h at 55 °C. The solvent was discarded after a spin at 10,000 rpm for 5 min.

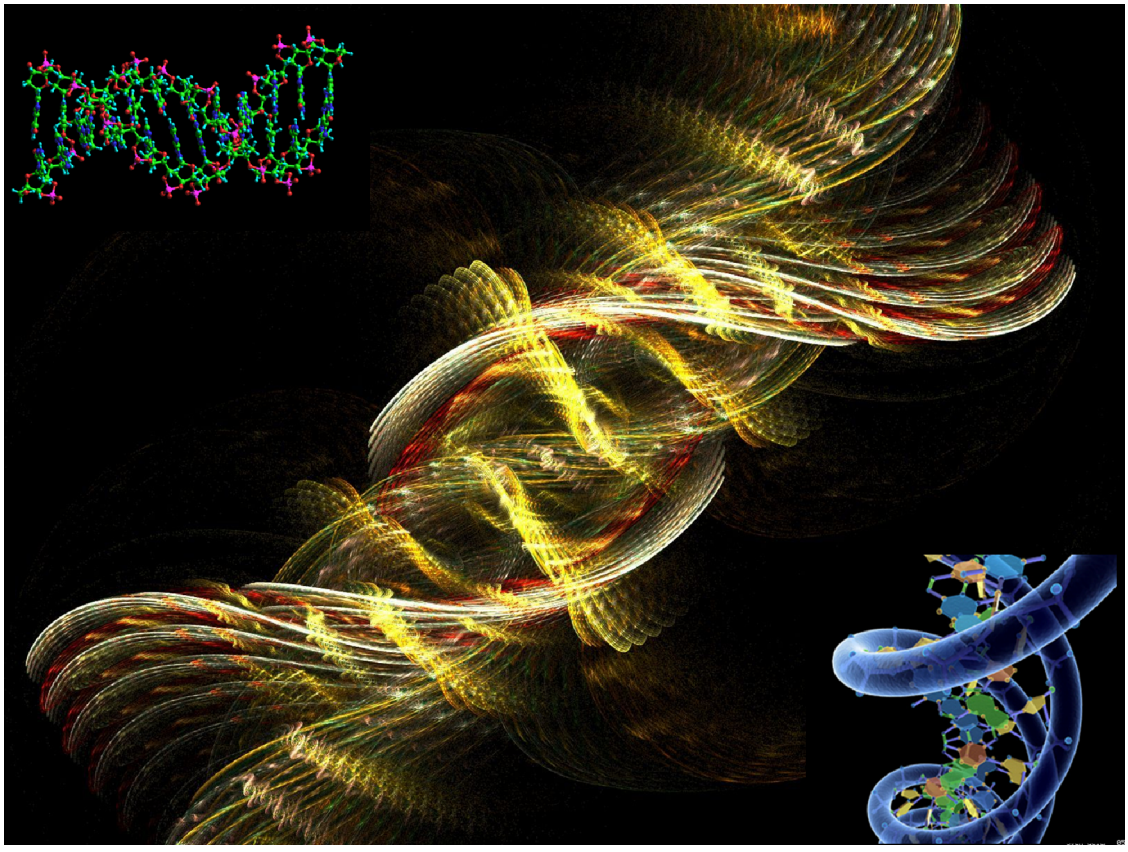
1 ml of 72% H_2SO_4 was added and kept at 25 °C in shaking condition for 3 h. After 3 h the sample was diluted upto 3% H_2SO_4. The samples were autoclaved for 1 h. After cooling to RT the samples were filtered with Whatman paper no.3. The volume of filtrate was measured and after appropriate dilution, absorbance of the sample was taken at 205 nm. The residue was washed with water 2-3 times. The residue was then transferred to a pre-weighed crucible. The residue was allowed to dry for 1-2 days at 80 °C. The crucible was weighed after drying. The final weight of crucible was subtracted from initial weight to give the final weight of residue. For determining acid insoluble lignin the percent weight of residue was calculated. Acid soluble lignin was calculated according to the given formula

$$\text{Acid soluble lignin} = \frac{100 \times A205 \times \text{Vol.of filtrate} \times \text{dilution factor}}{110 \times \text{initial dry powder weight}}$$

Total lignin = Acid soluble lignin + Acid insoluble lignin

CHAPTER 3

ISOLATION, CLONING & CHARACTERISATION OF CINNAMYL ALCOHOL DEHYDROGENASE (CAD) GENE



3.1 Introduction

Isolation of a gene is the pre-requisite for its thorough study or characterization. To isolate any gene of interest generally there are two major approaches as follows :-

1. Genomic/cDNA library screening- It is the classical method of gene isolation in which genomic/cDNA libraries are screened with homologous or heterologous probe. After 2-3 rounds of screenings, the plaques showing strong positive signals are excised and the DNA fragment sequenced.
2. PCR – It is one of the most popular approaches of gene isolation because of its simplicity and rapidity. Forward and reverse primers are designed on the basis of available sequences in the database. PCR is performed using these primers and genomic/cDNA as template. The amplicon is sequenced to confirm its identity.

In the present study, PCR based approach was followed to fish out the *CAD* gene from *Leucaena leucocephala.*

3.2 Materials and methods

3.2.1 Genomic DNA extraction

Genomic DNA was extracted from *L. leucocephala* and *Medicago sativa* using method given by Lodhi *et al*. (section 2.12.3).

3.2.2 Isolation of *CAD* from *Medicago sativa*

Primers were synthesized from the *Medicago CAD* available in NCBI GenBank database which were used to perform PCR using *Medicago* genomic DNA as template. The amplicon thus obtained was sequenced to confirm its identity. This gene fragment was used as heterologous probe in southern hybridization for establishing CAD gene in *L. leucocephala.*

3.2.3 Southern hybridization

Southern hybridization was done (section 2.20.1) to establish the presence of *CAD* gene and determine its copy number in *L. leucocephala*.

3.2.4 RNA isolation and cDNA first strand synthesis

Total RNA was isolated from *L. leucocephala* xylem tissue of stem (section 2.15). mRNA was purified from total RNA using oligotex dT resins (section 2.15.1). cDNA Reverse Transcription (RT) 1st strand was synthesized (section 2.15.2).

3.2.5 Polymerase Chain Reaction

PCR amplification was done using cDNA 1st strand or genomic DNA as template (section 2.17). Amplified PCR products were eluted from Agarose gel (section 2.14). The eluted PCR products were used for ligation into suitable vector.

3.2.6 Transformation and selection

The ligation mixture was used for transformation into suitable host cell line. Putative transformants were selected using Blue-white screening by adding X-gal and IPTG. Colony PCR (section 2.11) was done to screen the putative transformants. Plasmid was isolated from clones showing positive colony PCR results. The plasmid was restriction digested to check for the insert size and the insert was further sequenced to confirm its identity.

3.2.7 Bioinformatic analysis

Nucleotide and amino acid sequence analysis was done using software pDRAW 32 and online bioinformatic analysis facility available at www.justbio.com, www.expasy.org and www.ncbi.nlm.nih.gov. Phylogenetic analyses were conducted in MEGA4 (Tamura, 2007).

3.2.8 Rapid Amplification of cDNA Ends (RACE)

RACE (section 2.18) was done to fish out full length gene with its 5' and 3' UTRs.

3.3 Results and Discussion

3.3.1 Establishing presence of *CAD* gene in *L. leucocephala*

Prior to isolation of *CAD* gene from *L. leucocephala*, it is necessary to confirm its presence in the plant. It was done with the help of southern hybridization using *Medicago sativa CAD* gene fragment as the heterologous probe.

Using the primers from *Medicago sativa* CAD sequence and *Medicago* genomic DNA, a 800 base pair fragment was amplified. This fragment was cloned in pGEM-T Easy vector and sequenced to confirm its identity and was further used as probe in southern hybridization.

3.3.1.1 Southern hybridization

Good quality high molecular weight genomic DNA (Fig. 3.1) was isolated from young leaves of *L. leucocephala*. The genomic DNA was quantified spectophotometrically and about 10 µg DNA was used for restriction digestion reactions.

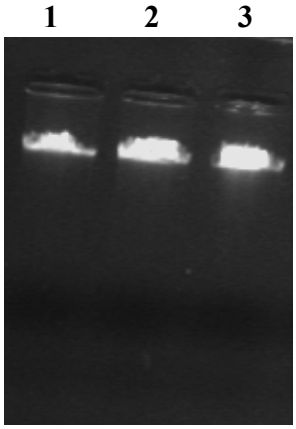


Figure 3.1 Lane 1, 2, 3 *L. leucocephala* genomic DNA

The genomic DNA was restriction digested with *Eco* RI and *Hind* III (Fig. 3.2 A). The digested DNA was electrophoresed on 0.8% agarose gel in 1X TAE buffer. The 800 bp *Medicago* CAD gene fragment was used as heterologous probe for hybridization.

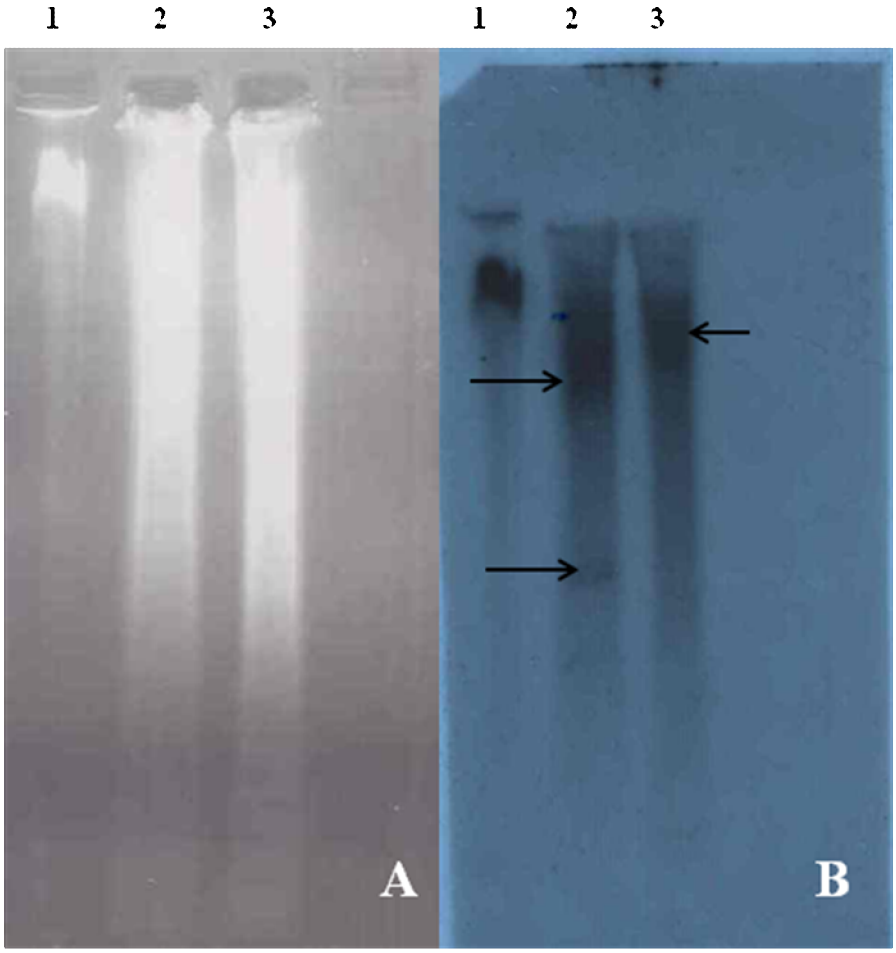


Figure 3.2 Southern hybridization of *L. leucocephala*. **A.** Agarose gel showing *L. leucocephala* genomic DNA digested with *Eco* RI (Lane 2) and *Hind* III (Lane 3) and uncut genomic DNA (Lane 1). **B-** X-RAY film showing regions hybridized with probe

Southern hybridization with *Medicago CAD* gene revealed that from each of the restriction digested DNA samples multiple bands hybridized to the *Medicago CAD* gene clone (Fig. 3.2 B). The hybridization signals from low to very high molecular weight DNA fragments indicated possible multiple gene copies in the *L. leucocephala* genome. Low molecular weight signals from *Eco* RI and *Hind* III digests suggested the presence of these restriction sites within the gene(s). It has been obvious from southern hybridization that *CAD* gene in *L. leucocephala* exist as multi gene as reported earlier in other plants like *Medicago sativa, Saccharum officinarum* and *Fragaria* X *Ananassa* (Brill *et. al*., 1999; Selman-Housein *et. al*., 1999; Blanco-Portales *et. al*., 2002).

3.3.2 Isolation of partial *CAD* gene from *L. leucocephala*

In order to obtain the *CAD* gene from *Leucaena*, primers were designed from the region showing maximum homology in nucleotide sequences of *CAD* gene from the Fabaceae members.

3.3.2.1 RNA extraction from *L. leucocephala*

Total RNA (Fig. 3.3) was extracted from xylem tissue of *L. leucocephala* stem and cDNA 1st strand was prepared from the mRNA.

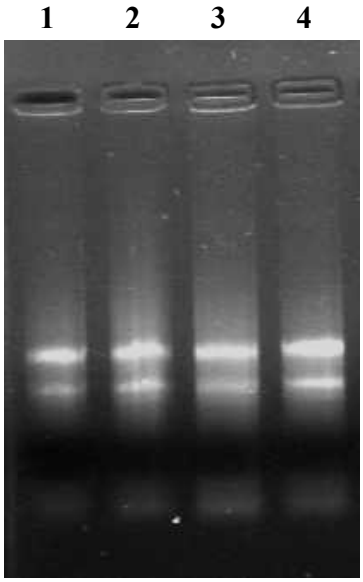
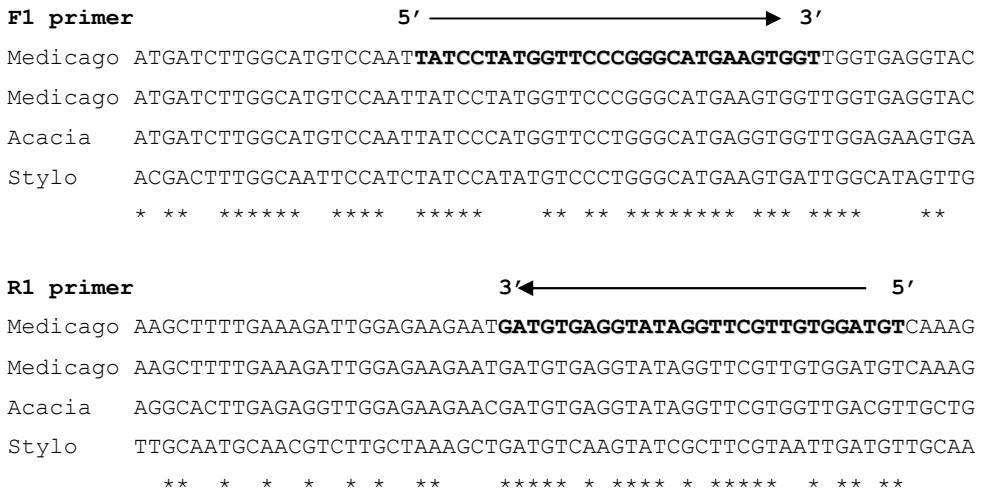


Figure 3.3 All the lanes showing total RNA from *L. leucocephala*

3.3.2.2 Primer designing

CAD sequences of Fabaceae members available at NCBI GenBank database were aligned using Clustal W software. Forward and reverse primers were designed from regions showing highest homology.



Forward primer CAD F1-5' TAT CCT ATG GTT CCC GGG CAT GAA GTG GT 3'

Reverse primer CAD R1-5' ACA TCC ACA ACG AAC CTA TAC CTC ACA TC 3'

3.3.2.3 Amplification and cloning

Primers CAD F1 and CAD R1 were used to amplify 863 bp fragment (CADc1) (Fig. 3.4) using cDNA 1st strand as template. The fragment was cloned in pGEM-T Easy vector (Fig. 3.5) and sequenced. The clone was designated as LlCADc1.The sequence was submitted in NCBI GenBank data base with Accession number DQ 361031.

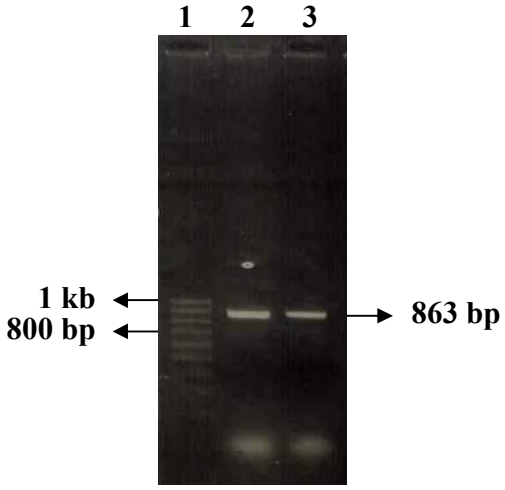


Figure 3.4 Lane 1-DNA molecular weight marker, Lane 2 & 3- 863 bp amplicon

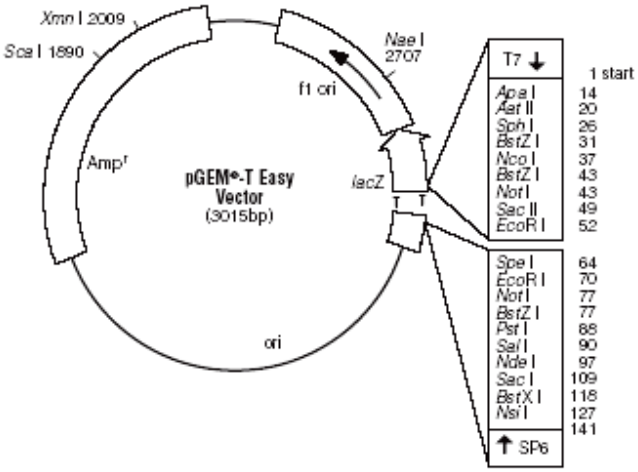


Figure 3.5 Map of pGEM-T Easy vector

3.3.2.4 Sequence analysis of LlCADc1

Nucleotide sequence of LlCADc1

```
  1 TATCCTATGG TTCCCGGGCA TGAAGTGGTC GGAGAGGTGA TCGAGGTGGG
 51 TTCGAATGTG ACCAAGTTTA AGGTCGGAGA AGTAGTCGGA GCAGGACTCA
101 TCGTTGGGAG CTGCCGGAAT TGCAGGGCAT GCAAATCTGA TATTGAGCAA
151 TATTGCGCCA AGAAGATCTG GAATTGCAAC GATGTTTACA CCGATGGAAA
201 ACCCACCCAA GGTGGCTTCG CTGAAACCAT GGTCGTCAAT CAAAATTTCG
251 TTGTGAAGAT ACCAGAGGGG ATGTCTCCGG AACAAGTGGC TCCACTGTTG
301 TGCGCCGGCG TGACGGTGTA CAGTCCACTG TCACACTTTG GACTGAAAGA
351 GAGTGGGCTA AGAGGAGGAA TATTAGGGCT TGGAGGAGTG GGACACATGG
401 GCGTGAAGAT AGCCAAAGCC ATGGGACACA ACGTGACAGT GATAAGCTCG
451 TCGGAGAAGA AGAAGCAGGA GGCTCTGGAA CACCTCGGAG CAGACGATTA
501 TGTGGTTAGC TCAGACGAAA CTCAGATGCA GAAGATTGCT GATTCACTTG
551 ATTATATCAT CGATACGGTG CCAGTGGGTC ACCCTCTTGA GCCTTATCTT
601 TCTCTGCTCA AAGTTGATGG CAAGTTGATC TTAATGGGTG TTATCAACAC
651 TCCTCTGTAA TTCGTCAGCC CCATGGTCAT GCTCGGGAGG AAGACGATAA
701 CGGGAAGCTT CATTGGGAGC ATAAAGGAGA CGGAAGAGAT GTTGGGGTTC
751 TGGAAAGAGA AGGGGCTGAG TTCAATGATA GAGGTTGTGA AGATGGATTA
801 CATCAACAAA GCCTTCGAGA GGTTGGAGAA GAACGATGTG AGGTATAGGT
851 TCGTTGTGGA TGT
```

BLAST analysis of the 863 bp fragment nucleotide sequence revealed it to be a *CAD* gene with 92% homology with *Acacia auriculiformis* x *Acacia mangium* cinnamyl alcohol dehydrogenase mRNA. Thus a partial CAD gene fragment from *L. leucocephala* was obtained.

Percentage homology with other Fabaceae *CAD* genes in NCBI GenBank database

Plant	Nucleotide	Amino acid
Acacia auriculiformis x Acacia mangium	92 %	94 %
Medicago sativa	80 %	84 %

3.3.3 Isolation of full length *CAD* gene from *L. leucocephala*

3.3.3.1 Primer designing

Clone LlCADc1 showed highest homology with *Acacia mangium* CAD gene nucleotide sequence. Since *Acacia* is from the same family Fabaceae and same sub-family Mimosoideae as *Leucaena*, highest homology between the *CAD* sequences from both the plants is expected. Therefore, to isolate full length *CAD* gene from *L. leucocephala*, forward primer was designed from the initiation codon of *Acacia CAD* sequence (Acc. No. AY769938) and similarly reverse primer was designed upto termination codon of *Acacia CAD* sequence.

CAD AF 5' ATG GGA AGC ATT GAA GGA GAA AGA AC 3'

CAD AR 5' TCA CTG ATG ATC ATC AAG TTT GCT GCC 3'

3.3.3.2 Amplification and cloning

A 1080 bp fragment was obtained when PCR was carried out using CAD AF and CAD AR primers with cDNA 1st strand template from *L. leucocephala*. The amplicon (CADc2) was cloned in pGEM-T Easy vector and sequenced. The clone was designated as Ll CADc2 (Fig. 3.6).

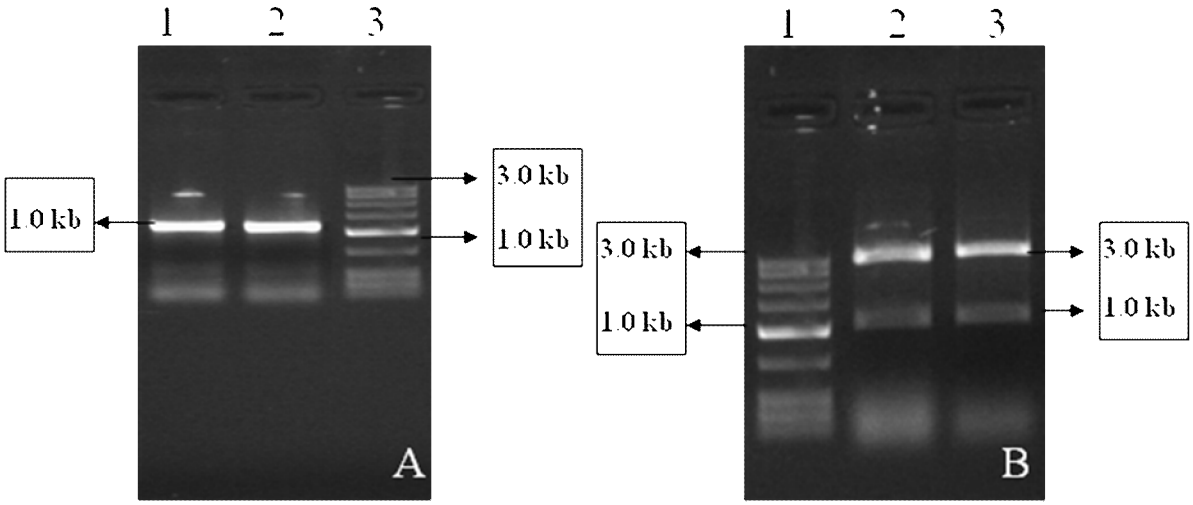


Figure 3.6; A: Lane 1 & 2- 1.0 kb amplicon, Lane 3- DNA molecular weight marker. **B**: Lane 1- DNA molecular weight marker, Lane 2 & 3- *Eco* RI digestion of pGEM-T Easy vector with cloned CADc2 fragment

3.3.3.3 Sequence analysis of LlCADc2

Nucleotide sequence of LlCADc2

```
   1 ATGGGAAGCA TTGAAGGAGA AAGAACAACG GTTGGTTGGG CTGCTCGAGA
  51 CCCTTCTGGG ACTCTCTCTC CCTACACTTT CAATCTCAGG AACACGGGAC
 101 CTGATGATGT GTATATCAAG GTTCACTACT GTGGAATATG CCATTCCGAT
 151 CTGCATCAGA TTAAGAATGA TCTTGGCATG TCCAATTATC CCATGGTTCC
 201 TGGGCATGAG GTGGTCGGAG AGGTGATCGA GGTGGGTTCG AATGTGACCA
 251 AGTTTAAGGC CGGAGAAGTA GTCGGAGCAG GACTCATCGT TGGGAGCTGC
 301 CGGAATTGCA GGGCATGCAA ATCTGATATT GAGCAATATT GCGCCAAGAA
 351 GATCTGGAAT TGCAACGATG TTTACACCGA TGGAAAACCC ACCCAAGGTG
 401 GCTTCGCTGA AACCATGGTC GTCAATCAAA ATTTCGTTGT GAAGATACCA
 451 GAGGGGATGT CTCCGGAACA AGTGGCTCCA CTGTTGTGCG CCGGCGTGAC
 501 GGTGTACAGT CCACTGTCAC ACTTTGGACT GAAAGAGAGT GGGCTAAGAG
 551 GAGGAATATT AGGGCTTGGA GGAGTGGGAC ACATGGGCGT GAAGATAGCC
 601 AAAGCCATGG GACACAACGT GACAGTGATA AGCTCGTCGG AGAAGAAGAA
 651 GCAGGAGGCT CTGGAACACC TCGGAGCAGA CGATTATGTG GTTAGCTCAG
 701 ACGAAACTCA GATGCAGAAG ATTGCTGATT CACTTGATTA TATCATCGAT
 751 ACGGTGCCAG TGGGTCACCC TCTTGAGCCT TATCTTTCTC TGCTCAAAGT
 801 TGATGGCAAG CTGATCTTAA TGGGTGTTAT CAACACTCCT CTGCAATTCG
 851 TCAGCCCCAT GGTCATGCTC GGGAGGAAGA CGATAACGGG AAGCTTCATT
 901 GGGAGCATAA AGGAGACGGA AGAGATGTTG GGGTTCTGGA AAGAGAAGGG
 951 GCTGAGTTCA ATGATAGAGG TTGTGAAGAT GGATTACATC AACAAAGCCT
1001 TCGAGAGGTT GGAGAAGAAC GATGTCAGAT ATAGGTTCGT TGTTGACGTT
1051 GCCGGCAGCA AACTTGATGA TCATCAGTGA
```

The underlined nucleotides are the primers CAD AF and CAD AR

Deduced amino acid sequence of LlCADc2

5'3' Frame 1

```
   1 - ATGGGAAGCATTGAAGGAGAAAGAACAACGGTTGGTTGGGCTGCTCGAGACCCTTCTGGG - 60
   1 - M  G  S  I  E  G  E  R  T  T  V  G  W  A  A  R  D  P  S  G   - 20

  61 - ACTCTCTCTCCCTACACTTTCAATCTCAGGAACACGGGACCTGATGATGTGTATATCAAG - 120
  21 - T  L  S  P  Y  T  F  N  L  R  N  T  G  P  D  D  V  Y  I  K   - 40

 121 - GTTCACTACTGTGGAATATGCCATTCCGATCTGCATCAGATTAAGAATGATCTTGGCATG - 180
  41 - V  H  Y  C  G  I  C  H  S  D  L  H  Q  I  K  N  D  L  G  M   - 60

 181 - TCCAATTATCCCATGGTTCCTGGGCATGAGGTGGTCGGAGAGGTGATCGAGGTGGGTTCG - 240
  61 - S  N  Y  P  M  V  P  G  H  E  V  V  G  E  V  I  E  V  G  S   - 80

 241 - AATGTGACCAAGTTTAAGGCCGGAGAAGTAGTCGGAGCAGGACTCATCGTTGGGAGCTGC - 300
  81 - N  V  T  K  F  K  A  G  E  V  V  G  A  G  L  I  V  G  S  C   - 100

 301 - CGGAATTGCAGGGCATGCAAATCTGATATTGAGCAATATTGCGCCAAGAAGATCTGGAAT - 360
 101 - R  N  C  R  A  C  K  S  D  I  E  Q  Y  C  A  K  K  I  W  N   - 120

 361 - TGCAACGATGTTTACACCGATGGAAAACCCACCCAAGGTGGCTTCGCTGAAACCATGGTC - 420
 121 - C  N  D  V  Y  T  D  G  K  P  T  Q  G  G  F  A  E  T  M  V   - 140

 421 - GTCAATCAAAATTTCGTTGTGAAGATACCAGAGGGGATGTCTCCGGAACAAGTGGCTCCA - 480
 141 - V  N  Q  N  F  V  V  K  I  P  E  G  M  S  P  E  Q  V  A  P   - 160

 481 - CTGTTGTGCGCCGGCGTGACGGTGTACAGTCCACTGTCACACTTTGGACTGAAAGAGAGT - 540
 161 - L  L  C  A  G  V  T  V  Y  S  P  L  S  H  F  G  L  K  E  S   - 180

 541 - GGGCTAAGAGGAGGAATATTAGGGCTTGGAGGAGTGGGACACATGGGCGTGAAGATAGCC - 600
 181 - G  L  R  G  G  I  L  G  L  G  G  V  G  H  M  G  V  K  I  A   - 200

 601 - AAAGCCATGGGACACAACGTGACAGTGATAAGCTCGTCGGAGAAGAAGAAGCAGGAGGCT - 660
 201 - K  A  M  G  H  N  V  T  V  I  S  S  S  E  K  K  K  Q  E  A   - 220

 661 - CTGGAACACCTCGGAGCAGACGATTATGTGGTTAGCTCAGACGAAACTCAGATGCAGAAG - 720
 221 - L  E  H  L  G  A  D  D  Y  V  V  S  S  D  E  T  Q  M  Q  K   - 240

 721 - ATTGCTGATTCACTTGATTATATCATCGATACGGTGCCAGTGGGTCACCCTCTTGAGCCT - 780
 241 - I  A  D  S  L  D  Y  I  I  D  T  V  P  V  G  H  P  L  E  P   - 260

 781 - TATCTTTCTCTGCTCAAAGTTGATGGCAAGCTGATCTTAATGGGTGTTATCAACACTCCT - 840
 261 - Y  L  S  L  L  K  V  D  G  K  L  I  L  M  G  V  I  N  T  P   - 280

 841 - CTGCAATTCGTCAGCCCCATGGTCATGCTCGGGAGGAAGACGATAACGGGAAGCTTCATT - 900
 281 - L  Q  F  V  S  P  M  V  M  L  G  R  K  T  I  T  G  S  F  I   - 300

 901 - GGGAGCATAAAGGAGACGGAAGAGATGTTGGGGTTCTGGAAAGAGAAGGGGCTGAGTTCA - 960
 301 - G  S  I  K  E  T  E  E  M  L  G  F  W  K  E  K  G  L  S  S   - 320

 961 - ATGATAGAGGTTGTGAAGATGGATTACATCAACAAAGCCTTCGAGAGGTTGGAGAAGAAC - 1020
 321 - M  I  E  V  V  K  M  D  Y  I  N  K  A  F  E  R  L  E  K  N   - 340

1021 - GATGTCAGATATAGGTTCGTTGTTGACGTTGCCGGCAGCAAACTTGATGATCATCAGTGA - 1080
 341 - D  V  R  Y  R  F  V  V  D  V  A  G  S  K  L  D  D  H  Q  *   - 360
```

Blast analysis revealed CADc2 to be a full length *CAD* gene starting from the initiation codon (Italics) up to the termination codon (Italics). The sequence was submitted in NCBI GenBank data base with Accession number DQ914843.

Percentage homology of LlCADc2 with other *CAD* genes in NCBI GenBank database

plant	Nucleotide	Amino acid
Acacia auriculiformis X *Acacia mangium*	92%	95%
Medicago sativa	79%	83%
Populus tomentosa	77%	81%
Gossypium hirsutum	75%	83%

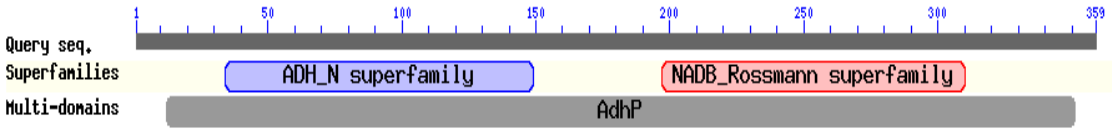


Figure 3.7: Putative conserved domains showing CADc2 to be a member of Zn binding alcohol dehydrogenase family

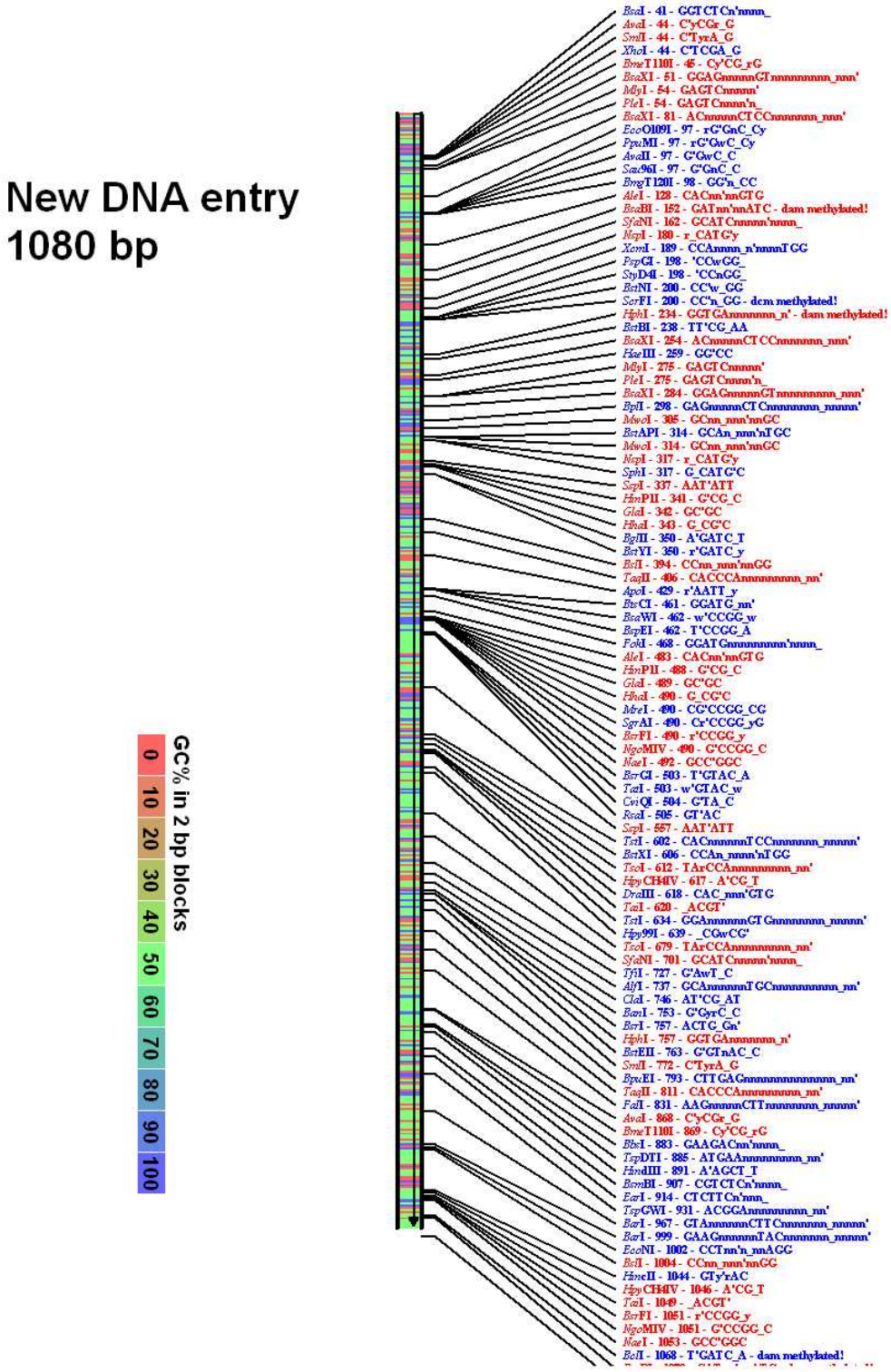


Figure 3.8: *In silico* restriction analysis of CADc2

The deduced amino acid sequence is of 359 amino acids. The nucleotide sequence shows significant similarity with other CAD nucleotide sequences in database, highest homology is with *Acacia* (92%). BLAST analysis of the amino acid sequence shows the conserved domain of zinc binding alcohol dehydrogenase family. In silco restriction analysis is shown in Figure 3.8. This full length clone LlCADc2 was used for further studies like heterologous expression and antisense cloning. Since the primers used for amplification of CADc2 were designed from *Acacia* sequence, some mismatches were expected in the primer region of CADc2 fragment. So, in order to obtain the genuine full length *CAD* gene and to obtain the untranslated regions of the gene, Rapid Amplification of cDNA Ends (RACE) was performed.

3.3.4 Rapid Amplification of cDNA Ends (RACE)

RACE is an important tool to obtain the UTRs (Un-Translated Regions) of a particular gene and is also useful to obtain full length gene when a partial fragment is known.

3.3.4.1 Primer designing and RACE reaction

For RACE, gene specific primers were designed from CADc2 fragment.

For 5' RACE the following primer was designed.
CAD 8L2 5' ACC ACC TCA TGC CCA GGA ACC ATG GGA T 3'

For 3' RACE following primer was designed.
CAD LF 5' CAACGTGACAGTGATAAGCTCGTCG 3'

3' RACE

BD SMART-RACE cDNA amplification kit (Clontech Laboratories Inc., Mountain View, USA) and total RNA isolated from xylem tissue were used to amplify the 3' end of *L. leucocephala CAD* cDNA. The 3' end of *CAD* was amplified in two rounds of PCR with the *CAD* gene-specific primers and with primers provided with the kit (Section 2.18.2). The first PCR for 3' end amplification of *Leucaena CAD* gene was performed with the CAD LF and UPM primers under following conditions: 3 min at 95 ºC, 35 cycles of 1 min at 95 ºC, 30 s at 45 ºC, 3 min at 72 ºC, and a final extension step

for 10 min at 72 ºC. The PCR product of the first round PCR was 40 fold diluted and used as template in the nested PCR amplification with CAD LF and NUP primers at the annealing temperature of 60 ºC and under the same PCR conditions as in the case of first PCR (Lacombe *et al.,* 1997; Ma, 2007).

5' RACE

GeneRacer RACE kit (Invitrogen) and total RNA isolated from xylem tissue were used to amplify the 5' end of *L. leucocephala CAD* cDNA. The 5' end of CAD was amplified in two rounds of PCR with the CAD gene-specific primers and with primers provided with the kit (Section 2.18.1). The first PCR for 5' end amplification of *Leucaena CAD* gene was performed with the CAD 8L2 and GeneRacer 5' primers under following conditions: 3 min at 95 ºC, 35 cycles of 1 min at 95 ºC, 30 s at 45 ºC, 3 min at 72 ºC, and a final extension step for 10 min at 72 ºC. The PCR product of the first round PCR was 40 fold diluted and used as template in the nested PCR amplification with CAD 8L2 and 5' nested primers at the annealing temperature of 60 ºC and under the same PCR conditions as in the case of first PCR.

3.3.4.2 Results of RACE reaction

Secondary reaction of 5' and 3' RACE yielded a 318 bp and 638 bp fragments respectively. These two fragments were cloned in pGEM-T Easy vector and four clones from each 5' and 3' RACE products were sequenced. Two different 5' clones and three different 3' clones were characterized. On sequence validation it was found that two type of 5' UTR and three types of 3' UTR were obtained. The 5' clones were assigned names 5'CADA and 5'CADB and the 3' clones were assigned names as 3'CADA, 3'CADB and 3'CADC.

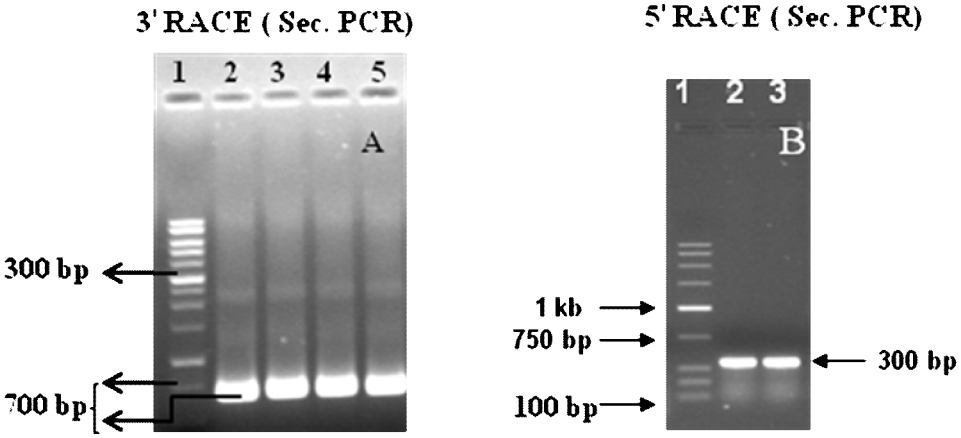


Figure 3.9 A: Lane 1- DNA molecular weight Marker, Lane 2-5 secondary 3' PCR

B: Lane 1- DNA molecular weight Marker, Lanes 2,3- Secondary 5' PCR

Nucleotide sequences and deduced amino acid sequences of 5' RACE products 5'CADA and 5'CADB

5'CADA

```
  1 GGACACTGAC ATGGACTGAA GGAGTAGAAA ATTCTTTCTT CTTCCCTCTC
 51 TCTTTGTTTG CCTTTTTTCC CTCTGTGGAA TTCTCTCTTT CCCTCTTGAG
101 AAAACTATGG GTAGCGTCGA AGGAGAAAGA ACAACGGTTG GTTGGGCTGC
151 TCGAGACCCT TCTGGGACTC TCTCTCCCTA CACTTTCAAT CTCAGGAACA
201 CGGGACCTGA TGATGTGTAT ATCAAGGTTC ACTACTGTGG AATATGCCAT
251 TCCGATCTGC ATCAGATTAA GAATGATCTT GGCATGTCCA ATTATCCCAT
301 GGTTCCTGGC ATGAGGTGGT
```

5'-3' Frame 2

D T D **M** D **X** R S R K F F L L P S L F V C L F S L C G I L S F P L E K T **M** G S V E
G E R T T V G W A A R D P S G T L S P Y T F N L R N T G P D D V Y I K V H Y
C G I C H S D L H Q I K N D L G **M** S N Y P **M** V P G **M** R W

5'CADB

```
  1 GGACACTGAC ATGGACTGAA GGAGTAGAAA ATTCTTTCTT CTTCTCTCTC
 51 TTTCTTTGTC TTTTTCCCCT TTGTTGATTT CTCTCTCTCC CTCTGAGAAA
101 ACTATGGGCA GCGTCGAAGG AGAAAGAACA ACAGTTGGTT GGGCTGCTCG
151 AGACCCTTCT GGGATTCTCT CCCCCTACAC CTTCAATCTC AGGAACACGG
201 GACCTGATGA TGTGTACATC AAGGTTCACT ACTGTGGAAT CTGCCATTCC
251 GATCTGCATC AGATTAAGAA TGATCTTGGC ATGTCCAATT ATCCCATGGT
301 TCCTGGGCAT GAGGTGGT
```

5'-3' Frame 2

D T D **M** D **X** R S R K F F L L L S L S L S F S P L L I S L S P S E K T **M** G S V E G
E R T T V G W A A R D P S G I L S P Y T F N L R N T G P D D V Y I K V H Y C G
I C H S D L H Q I K N D L G **M** S N Y P **M** V P G H E V

CLUSTAL W (1.8) multiple sequence alignment 5'CADA and 5'CADB

```
5'CADA          GGACACTGACATGGACTGAAGGAGTAGAAAATTCTTTCTTCTTC--TCTCTCTTTCTTTG
5'CADB          GGACACTGACATGGACTGAAGGAGTAGAAAATTCTTTCTTCTTCCCTCTCTCTTTGTTTG
                ****************************************  ********* ****

5'CADA          TCTTTTTCCCCTTTGTTGATTTCTCTCTCTCCCTCT-GAGAAAACTATG
5'CADB          CCTTTTTTCCCTCTGTGGAATTCTCTCTTTCCCTCTTGAGAAAACTATG
                ****** **** *** ** ******** ******* ************
```

Nucleotide sequences and deduced amino acid sequences of 3' RACE products 3'CADA, 3'CADB and 3'CADC

3'CADA

```
  1 CAACGTGACA GTGATAAGCT CGTCGGAGAA GAAGAAGCAG GAGGCTCTGG
 51 AACACCTCGG AGCAGACGAT TATGTGGTTA GCTCAGACGA AACTCAGATG
101 CAGAAGATTG CTGATTCACT TGATTATATC ATCGATACGG TGCCAGTGGG
151 TCACCCTCTT GAGCCTTATC TTTCTCTGCT CAAAGTTGAT GGCAAGTTGA
201 TCTTAATGGG TGTTATCAAC ACTCCTCTGC AATTCGTCAG CCCCATGGTC
251 ATGCTCGGGA GGAAGACGAT AACGGGAAGC TTCATTGGGA GCATAAAGGA
301 GACGGAAGAG ATGTTGGGGT TCTGGAAAGA GAAGGGGCTG AGTTCAATGA
351 TAGAGGTTGT GAAGATGGAT TACATCAACA AAGCCTTCGA GAGGTTGGAG
401 AAGAACGATG TCAGATATAG GTTCGTTGTT GACGTTGCCG GCAGCAAACT
451 TGATCAATGA AATGGAACAC GCATGTGTCA TCTTATAATT TCTTAAATAA
501 CCTTCTTCTG CAAAACTTAT GAATACACCT GGGAGTTGTT TGTTGTGTGT
551 TACGTTGTCG AATATTAAAT GTGATCTGTT TTCAGGGAAA AAAAAAAAAA
601 AAAAAAAAAA AAAGTACTCT GCGTTGATAC CACTGCTT
```

5'-3' Frame 2

N V T V I S S S E K K K Q E A L E H L G A D D Y V V S S D E T Q **M** Q K I A D S L D Y
I I D T V P V G H P L E P Y L S L L K V D G K L I L **M** G V I N T P L Q F V S P **M** V **M** L
G R K T I T G S F I G S I K E T E E **M** L G F W K E K G L S S **M** I E V V K **M** D Y I N K
A F E R L E K N D V R Y R F V V D V A G S K L D Q **X** N G T R **M** C H L I I S **X** I T F F
C K T Y E Y T W E L F V V C Y V V E Y **X** **M** **X** S V F R E K K K K K K K K V L C V D
T T A

3'CADB

```
  1 CAACGTGACA GTGATAAGCT CGTCGGAGAA GAAGCGGGAG GCTCTGGAAC
 51 ACCTCGGAGC AGACGATTAT GTGGTTAGCT CAGACGAAAC TCAGATGCAG
101 AAGATTGCTG ATTCACTTGA TTATATCATC GATACGGTGC CAGTGGGTCA
151 CCCTCTTGAG CCTTATCTTT CTCTGCTCAA AGTTGATGGC AAGTTGATCT
201 TAATGGGTGT TATCAACACT CCTCTGCAAT TCGTCAGCCC CATGGTCATG
251 CTCGGGAGGA AGACGATAAC GGGAAGCTTC ATTGGGAGCA TAAAGGAGAC
301 GGAAGAGATG TTGGGGTTCT GGAAAGAGAA GGGGCTGAGT TCAATGATAG
351 AGGTTGTGAA GATGGATTAC ATCAACAAAG CCTTTGAGAG GTTGGAGAAG
401 AACGATGTCA GATATAGGTT CGTTGTTGAC GTTGCCGGCA GCAAACTTCA
451 ATGAAATGAA ACACGCATGT TTCATCTTAT AATTCTTAAA TAACCTTCTT
501 CTGCAAAACT TATGAATATG AATACACCCG GGCGTTGTTT GTTGTGTTAC
551 GTTGTCCAAT ATTAAATGTG ATCTGTTTTC AGCGAAAAAA AAAAAAAAAA
601 AAAAAAAAAA AGTACTCTGC GTTGATACCA CTGCTT
```

5'-3' Frame 2

N V T V I S S S E K K R E A L E H L G A D D Y V V S S D E T Q **M** Q K I A D S L D Y I I
D T V P V G H P L E P Y L S L L K V D G K L I L **M** G V I N T P L Q F V S P **M** V **M** L G
R K T I T G S F I G S I K E T E E **M** L G F W K E K G L S S **M** I E V V K **M** D Y I N K A F
E R L E K N D V R Y R F V V D V A G S K L Q **X** N E T R **M** F H L I I L K **X** P S S A K L
M N **M** N T P G R C L L C Y V V Q Y **X** **M** **X** S V F S E K K K K K K K K S T L R **X** Y H
C

3'CADC

```
  1 CAACGTGACA GTGATAAGCT CGTCGGAGAA GAAGAAGCGG GAGGCTCTGG
 51 AACACCTCGG AGCAGACGAT TATGTGGTTA GCTCAGACGA AACTCAGATG
101 CAGAAGATTG CTGATTCACT TGATTATATC ATCGATACGG TGCCAGTGGG
151 TCACCCTCTT GAGCCGTATC TTTCTCTGCT CAAAGTTGAT GGCAAGTTGA
201 TCTTAATGGG TGTTATCAAC ACTCCTCTGC AATTCGTCAG CCCCATGGTC
251 ATGCTCGGGA GGAAGGCGAT AACGGGAAGC TTCATAGGGA GCATAAAGGA
301 GACGGAAGAG ATGTTGGAGT TCTGGAAAGA GAAGGGGCTG AGTTCAATGA
351 TAGAGGTTGT GAAGATGGAT TACATCAACA AAGCCTTTGA GAGGTTGGAG
401 AAGAACGATG TCAGATATAG GTTCGTTGTT GACGTTGCCG GCAGCAAACT
451 TCAATGAAAT GAAACACACA TGTTTCATCT TATAATTCTT AAATAACCTT
501 CTTCTGCAAA ACTTATGAAT ATGAATACAC CCGGGCGTTG TTTGTTGTGT
551 CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA GTACTCTGCG TTGATACCAC
601 TGCTT
```

5'3' Frame 2

N V T V I S S S E K K K R E A L E H L G A D D Y V V S S D E T Q **M** Q K I A D S L D Y
I I D T V P V G H P L E P Y L S L L K V D G K L I L **M** G V I N T P L Q F V S P **M** V **M** L
G R K A I T G S F I G S I K E T E E **M** L E F W K E K G L S S **M** I E V V K **M** D Y I N K
A F E R L E K N D V R Y R F V V D V A G S K L Q X N E T H **M** F H L I I L K X P S S A
K L **M** N **M** N T P G R C L L C Q K K K K K K K K K V L C V D T T A

CLUSTAL W (1.8) multiple sequence alignment 3'CADA, 3'CADB and 3'CADC

```
3'CADA          TGAAATGAAACACGCATGTTTCATCTTATAATT-CTTAAATAACCTTCTTCTGCAAAACT
3'CADB          TGAAATGAAACACACATGTTTCATCTTATAATT-CTTAAATAACCTTCTTCTGCAAAACT
3'CADC          TGAAATGGAACACGCATGTGTCATCTTATAATTTCTTAAATAACCTTCTTCTGCAAAACT
                ******* ***** ***** ************* *************************

3'CADA          TATGAATATGAATACACCCGGGCGTTGTTTGTTGTGTTACGTTGTCCAATATTAAATGTG
3'CADB          TATGAATATGAATACACCCGGGCGTTGTTTGTTGTGTCA--------------------
3'CADC          TATGAATA------CACCTGGGAGTTGTTTGTTGTGTGTTACGTTGTCGAATATTAAATG
                ********      **** *** **************

3'CADA          ATCTGT-TTTCAGCGAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACC
3'CADB          --------------AAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACC
3'CADC          TGATCTGTTTTCAGGGAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGATACC
                          ******************************************

3'CADA          ACTGCTT
3'CADB          ACTGCTT
3'CADC          ACTGCTT
                *******
```

Four clones were randomly selected for sequencing, after 5' RACE reaction. Upon sequence analysis, two different clones were obtained viz. 5'CADB (273 bp) and 5'CADA (276 bp). Clustal W (1.8) multiple alignment of these two sequences shows total 10 base difference. In clone 5'CADA an *Eco* RI site (underline) is present which is not observed in clone 5'CADB. The initation codon is indicated in blue and the differences are highlited in red.

Upon 3' UTR sequence analysis, three different clones were obtained viz. 3'CADA, 3'CADB and 3'CADC. These sequences were submitted in NCBI GenBank database with Accession numbers as following, 3'CADA-Acc. No.EF017700, 3'CADB-Acc. No. EF017701 and 3'CADC-Acc. No. EF017702. On Clustal W (1.8) multiple alignment of these sequences, many differences in the sequences were found. 3'CADC cPAL 14 is the shortest (98 bp), 3'CADB cPAL 13 is the longest (133 bp) and 3'CADA cPAL 12 is of intermediate (130 bp) length. The differences in the sequences are indicated in red. The putative polyadenylation sites are shown in brown and the termination codon is in blue. The differences in three UTRs might play a role in differential expression of CAD genes since CAD is known to be regulated by both developmental and environmental cues (Whetten and Sederoff 1995).

3.3.5 Isolation of full length cDNA and genomic CAD genes

3.3.5.1 Primer designing and PCR

Primers were designed using 5' UTR and 3' UTR sequences to obtain full length *CAD* cDNA and genomic clone.

PALF5-2 5'- TCC CTC TGT GGA ATT CTC TCT -3'

PAL R3-15 5'- TAT TCA TAA GTT TTG CAG AAG AAG GT -3'

3.3.5.2 Amplification and cloning

PCR was performed using these primers and Genomic DNA & cDNA as template. A 1178 bp amplicon from cDNA and a 1621 bp amplicon from genomic DNA were obtained. These amplicons were cloned in pGEM-T Easy vector and sequenced. The cDNA clone was designated as LlCADc3 (1074 bp from ATG to TGA) and the genomic clone was designated as LlCADg1. These sequences were submitted in NCBI GenBank database with accession numbers LlCADc3-EU870436 and LlCADg1-EU870437.

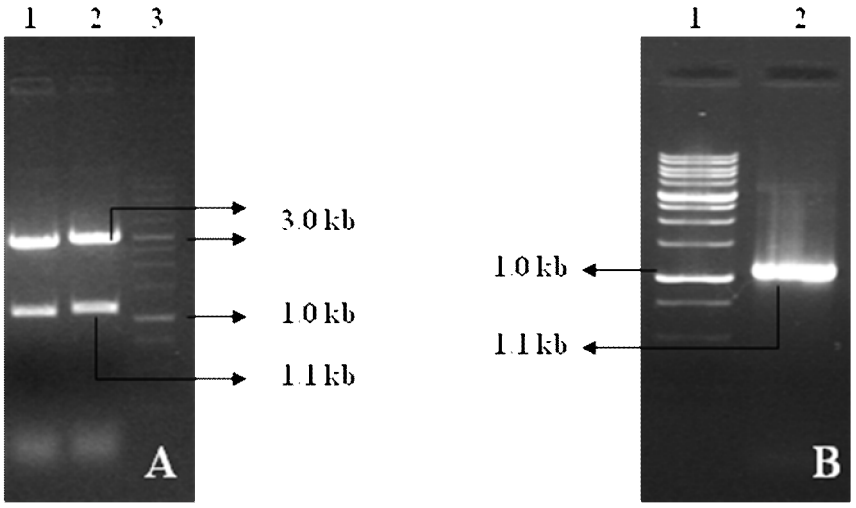


Figure 3.10; A: Lane 1 & 2- *Eco* RI digestion of pGEM-T EASY vector with cloned CADc3, Lane 3- DNA molecular weight marker **B**: Lane 1 DNA molecular weight marker, Lane 2- 1.1 kb amplicon.

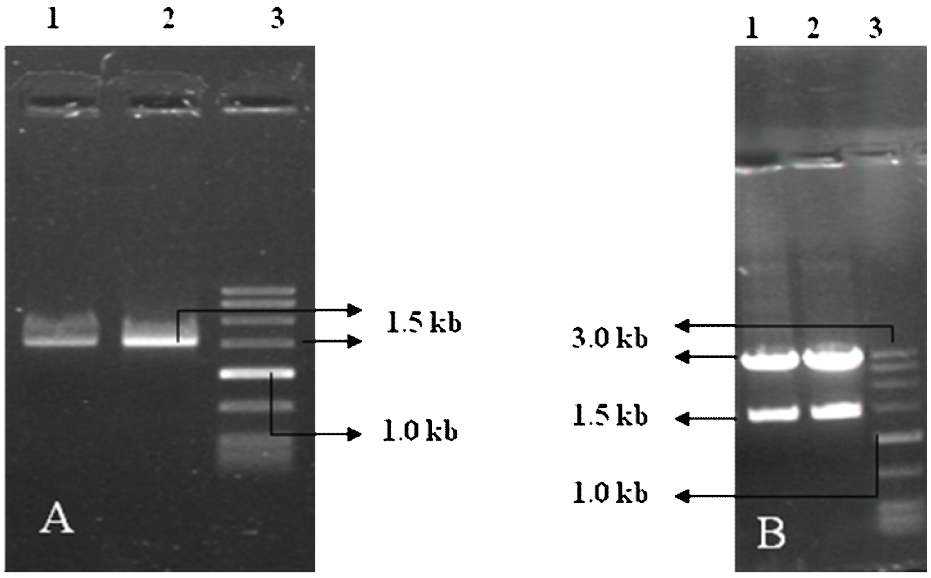


Figure 3.11; A: Lane 1& 2- 1.5 kb amplicon, Lane 3- DNA molecular weight marker.

B: Lane 1& 2- *Eco* RI digestion of pGEM-T Easy vector with cloned CADg1

3.3.5.3 Sequence analysis of LlCADc3

Nucleotide sequence of LlCADc3

```
   1 ATGGGTAGCG TCGAAGGAGA AAGAACAACG GTTGGTTGGG CTGCTCGAGA
  51 CCCTTCTGGG ACTCTCTCTC CCTACACTTT CAATCTCAGG AACACGGGAC
 101 CTGATGATGT GTATATCAAG GTTCACTACT GTGGAATATG CCATTCCGAT
 151 CTGCATCAGA TTAAGAATGA TCTTGGCATG TCCAATTATC CCATGGTTCC
 201 TGGGCATGAG GTGGTCGGAG AGGTGATCGA GGTGGGTTCG AATGTGACCA
 251 AGTTTAAGGT CGGAGAAGTA GTCGGAGCAG GACTCATCGT TGGGAGCTGC
 301 CGGAATTGCA GGGCATGCAA ATCTGATATT GAGCAATATT GCGCCAAGAA
 351 GATCTGGAAT TGCAACGATG TTTACACCGA TGGAAAACCC ACCCAAGGTG
 401 GCTTCGCTGA AACCATGGTC GTCAATCAAA ATTTCGTTGT GAAGATACCA
 451 GAGGGGATGT CTCCGGAACA AGTGGCTCCA CTGTTGTGCG CCGGCGTGAC
 501 GGTGTACAGT CCACTGTCAC ACTTTGGACT GAAAGAGAGT GGGCTAAGAG
 551 GAGGAATATT AGGGCTTGGA GGAGTGGGAC ACATGGGCGT GAAGATAGCC
 601 AAAGCCATGG GACACAACGT GACAGTGATA AGCTCGTCGG AGAAGAAGAA
 651 GCAGGAGGCT CTGGAACACC TCGGAGCAGA CGATTATGTG GTTAGCTCAG
 701 ACGAAACTCA GATGCAGAAG ATTGCTGATT CACTTGATTA TATCATCGAT
 751 ACGGTGCCAG TGGGTCACCC TCTTGAGCCT TATCTTTCTC TGCTCAAAGT
 801 TGATGGCAAG TTGATCTTAA TGGGTGTTAT CAACACTCCT CTGCAATTCG
 851 TCAGCCCCAT GGTCATGCTC GGGAGGAAGA CGATAACGGG AAGCTTCATT
 901 GGGAGCATAA AGGAGACGGA AGAGATGTTG GGGTTCTGGA AAGAGAAGGG
 951 GCTGAGTTCA ATGATAGAGG TTGTGAAGAT GGATTACATC AACAAAGCCT
1001 TCGAGAGGTT GGAGAAGAAC GATGTCAGAT ATAGGTTCGT TGTTGACGTT
1051 GCCGGCAGCA AACTTGATCA ATGA
```

Initation and termination codons are underlied

Percentage homology of LlCADc3 with other *CAD* genes in NCBI GenBank database

Plant	Nucleotide	Amino acid
Acacia auriculiformis x Acacia mangium	92%	95%
Medicago sativa	79%	83%
Populus tomentosa	77%	81%
Gossypium hirsutum	75%	83%

Codon usage analysis of LICADc3

%GC- 49.22

codon	mean	codon	mean	codon	mean	codon	mean
UUU (F)	0.56	**UCU** (S)	0.84	**UAU** (Y)	1.68	**UGU** (C)	0.00
UUC (F)	1.96	**UCC** (S)	0.28	**UAC** (Y)	0.84	**UGC** (C)	1.68
UUA (L)	0.56	**UCA** (S)	1.12	**UAA** (*)	0.00	**UGA** (*)	0.00
UUG (L)	1.12	**UCG** (S)	0.84	**UAG** (*)	0.00	**UGG** (W)	0.56
CUU (L)	1.40	**CCU** (P)	1.12	**CAU** (H)	0.28	**CGU** (R)	0.00
CUC (L)	1.12	**CCC** (P)	0.84	**CAC** (H)	1.40	**CGC** (R)	0.00
CUA (L)	0.28	**CCA** (P)	1.12	**CAA** (Q)	1.68	**CGA** (R)	0.00
CUG (L)	1.96	**CCG** (P)	0.28	**CAG** (Q)	0.84	**CGG** (R)	0.28
AUU (I)	0.84	**ACU** (T)	0.56	**AAU** (N)	1.68	**AGU** (S)	0.84
AUC (I)	2.23	**ACC** (T)	1.12	**AAC** (N)	1.40	**AGC** (S)	1.96
AUA (I)	1.96	**ACA** (T)	0.28	**AAA** (K)	2.23	**AGA** (R)	0.56
AUG (M)	3.63	**ACG** (T)	1.40	**AAG** (K)	4.47	**AGG** (R)	1.12
GUU (V)	3.07	**GCU** (A)	1.12	**GAU** (D)	3.07	**GGU** (G)	1.12
GUC (V)	2.23	**GCC** (A)	1.68	**GAC** (D)	0.84	**GGC** (G)	1.40
GUA (V)	0.28	**GCA** (A)	0.84	**GAA** (E)	1.68	**GGA** (G)	3.91
GUG (V)	4.47	**GCG** (A)	0.00	**GAG** (E)	4.19	**GGG** (G)	2.51

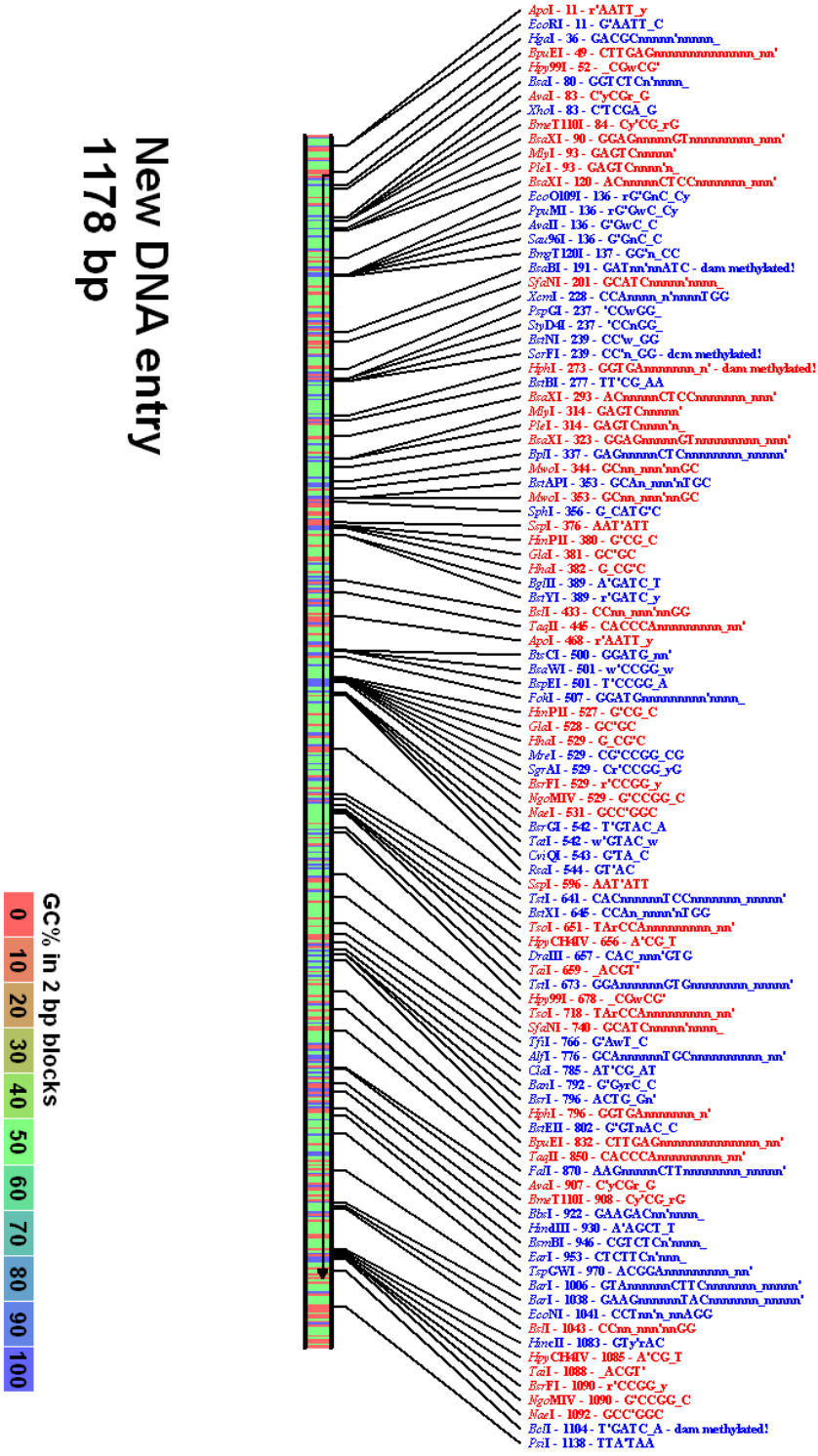


Figure 3.12: *In silico* restriction analysis of LlCADc3

The nucleotide sequence of LlCADc3 is of 1074 bp. It shows 75-92% homology at nucleotide level and 83-95% at amino acid level with other CAD sequences in database. The percentage GC of LlCADc3 is 49.22%. In silico restriction analysis is shown in figure 3.12

CLUSTAL W (1.8) multiple sequence alignment of LlCADc2 and LlCADc3

```
LlCADc2         ATGGGTAGCGTCGAAGGAGAAAGAACAACGGTTGGTTGGGCTGCTCGAGACCCTTCTGGG
LlCADc3         ATGGGAAGCATTGAAGGAGAAAGAACAACGGTTGGTTGGGCTGCTCGAGACCCTTCTGGG
                ***** *** * **********************************************

LlCADc2         ACTCTCTCTCCCTACACTTTCAATCTCAGGAACACGGGACCTGATGATGTGTATATCAAG
LlCADc3         ACTCTCTCTCCCTACACTTTCAATCTCAGGAACACGGGACCTGATGATGTGTATATCAAG
                ************************************************************

LlCADc2         GTTCACTACTGTGGAATATGCCATTCCGATCTGCATCAGATTAAGAATGATCTTGGCATG
LlCADc3         GTTCACTACTGTGGAATATGCCATTCCGATCTGCATCAGATTAAGAATGATCTTGGCATG
                ************************************************************

LlCADc2         TCCAATTATCCCATGGTTCCTGGGCATGAGGTGGTCGGAGAGGTGATCGAGGTGGGTTCG
LlCADc3         TCCAATTATCCCATGGTTCCTGGGCATGAGGTGGTCGGAGAGGTGATCGAGGTGGGTTCG
                ************************************************************

LlCADc2         AATGTGACCAAGTTTAAGGTCGGAGAAGTAGTCGGAGCAGGACTCATCGTTGGGAGCTGC
LlCADc3         AATGTGACCAAGTTTAAGGCCGGAGAAGTAGTCGGAGCAGGACTCATCGTTGGGAGCTGC
                ******************************************************

LlCADc2         CGGAATTGCAGGGCATGCAAATCTGATATTGAGCAATATTGCGCCAAGAAGATCTGGAAT
LlCADc3         CGGAATTGCAGGGCATGCAAATCTGATATTGAGCAATATTGCGCCAAGAAGATCTGGAAT
                ************************************************************

LlCADc2         TGCAACGATGTTTACACCGATGGAAAACCCACCCAAGGTGGCTTCGCTGAAACCATGGTC
LlCADc3         TGCAACGATGTTTACACCGATGGAAAACCCACCCAAGGTGGCTTCGCTGAAACCATGGTC
                ************************************************************

LlCADc2         GTCAATCAAAATTTCGTTGTGAAGATACCAGAGGGGATGTCTCCGGAACAAGTGGCTCCA
LlCADc3         GTCAATCAAAATTTCGTTGTGAAGATACCAGAGGGGATGTCTCCGGAACAAGTGGCTCCA
                ************************************************************

LlCADc2         CTGTTGTGCGCCGGCGTGACGGTGTACAGTCCACTGTCACACTTTGGACTGAAAGAGAGT
LlCADc3         CTGTTGTGCGCCGGCGTGACGGTGTACAGTCCACTGTCACACTTTGGACTGAAAGAGAGT
                ************************************************************

LlCADc2         GGGCTAAGAGGAGGAATATTAGGGCTTGGAGGAGTGGGACACATGGGCGTGAAGATAGCC
LlCADc3         GGGCTAAGAGGAGGAATATTAGGGCTTGGAGGAGTGGGACACATGGGCGTGAAGATAGCC
                ************************************************************

LlCADc2         AAAGCCATGGGACACAACGTGACAGTGATAAGCTCGTCGGAGAAGAAGAAGCAGGAGGCT
LlCADc3         AAAGCCATGGGACACAACGTGACAGTGATAAGCTCGTCGGAGAAGAAGAAGCAGGAGGCT
                ************************************************************

LlCADc2         CTGGAACACCTCGGAGCAGACGATTATGTGGTTAGCTCAGACGAAACTCAGATGCAGAAG
LlCADc3         CTGGAACACCTCGGAGCAGACGATTATGTGGTTAGCTCAGACGAAACTCAGATGCAGAAG
                ************************************************************

LlCADc2         ATTGCTGATTCACTTGATTATATCATCGATACGGTGCCAGTGGGTCACCCTCTTGAGCCT
LlCADc3         ATTGCTGATTCACTTGATTATATCATCGATACGGTGCCAGTGGGTCACCCTCTTGAGCCT
                ************************************************************

LlCADc2         TATCTTTCTCTGCTCAAAGTTGATGGCAAGTTGATCTTAATGGGTGTTATCAACACTCCT
LlCADc3         TATCTTTCTCTGCTCAAAGTTGATGGCAAGCTGATCTTAATGGGTGTTATCAACACTCCT
                ******************************************************
```

```
LlCADc2         CTGCAATTCGTCAGCCCCATGGTCATGCTCGGGAGGAAGACGATAACGGGAAGCTTCATT
LlCADc3         CTGCAATTCGTCAGCCCCATGGTCATGCTCGGGAGGAAGACGATAACGGGAAGCTTCATT
                ************************************************************

LlCADc2         GGGAGCATAAAGGAGACGGAAGAGATGTTGGGGTTCTGGAAAGAGAAGGGGCTGAGTTCA
LlCADc3         GGGAGCATAAAGGAGACGGAAGAGATGTTGGGGTTCTGGAAAGAGAAGGGGCTGAGTTCA
                ************************************************************

LlCADc2         ATGATAGAGGTTGTGAAGATGGATTACATCAACAAAGCCTTCGAGAGGTTGGAGAAGAAC
LlCADc3         ATGATAGAGGTTGTGAAGATGGATTACATCAACAAAGCCTTCGAGAGGTTGGAGAAGAAC
                ************************************************************

LlCADc2         GATGTCAGATATAGGTTCGTTGTTGACGTTGCCGGCAGCAAACTTGATCAATGA------
LlCADc3         GATGTCAGATATAGGTTCGTTGTTGACGTTGCCGGCAGCAAACTTGATGATCATCAGTGA
                ********************************************** *
```

CLUSTAL W (1.8) multiple sequence alignment

```
LlCADc2         MGSIEGERTTVGWAARDPSGTLSPYTFNLRNTGPDDVYIKVHYCGICHSDLHQIKNDLGM
LlCADc3         MGSVEGERTTVGWAARDPSGTLSPYTFNLRNTGPDDVYIKVHYCGICHSDLHQIKNDLGM
                ***:********************************************************

LlCADc2         SNYPMVPGHEVVGEVIEVGSNVTKFKAGEVVGAGLIVGSCRNCRACKSDIEQYCAKKIWN
LlCADc3         SNYPMVPGHEVVGEVIEVGSNVTKFKVGEVVGAGLIVGSCRNCRACKSDIEQYCAKKIWN
                **************************.*****************************

LlCADc2         CNDVYTDGKPTQGGFAETMVVNQNFVVKIPEGMSPEQVAPLLCAGVTVYSPLSHFGLKES
LlCADc3         CNDVYTDGKPTQGGFAETMVVNQNFVVKIPEGMSPEQVAPLLCAGVTVYSPLSHFGLKES
                ************************************************************

LlCADc2         GLRGGILGLGGVGHMGVKIAKAMGHNVTVISSSEKKKQEALEHLGADDYVVSSDETQMQK
LlCADc3         GLRGGILGLGGVGHMGVKIAKAMGHNVTVISSSEKKKQEALEHLGADDYVVSSDETQMQK
                ************************************************************

LlCADc2         IADSLDYIIDTVPVGHPLEPYLSLLKVDGKLILMGVINTPLQFVSPMVMLGRKTITGSFI
LlCADc3         IADSLDYIIDTVPVGHPLEPYLSLLKVDGKLILMGVINTPLQFVSPMVMLGRKTITGSFI
                ************************************************************

LlCADc2         GSIKETEEMLGFWKEKGLSSMIEVVKMDYINKAFERLEKNDVRYRFVVDVAGSKLDDHQ
LlCADc3         GSIKETEEMLGFWKEKGLSSMIEVVKMDYINKAFERLEKNDVRYRFVVDVAGSKLDQX-
                ************************************************************:
```

The nucleotide and amino acid sequences of LlCADc2 and LlCADc3 were aligned using Clustal W multiple alignment tool. The alignment showed that there are differences in the sequences that correspond to the primer region of LlCADc2 (shown in bold). These differences were expected as the primers (CAD AF and CAD AR) for LlCADc2 were designed from *Acacia CAD* nucleotide sequences. There are two more differences in nucleotide sequence and one in corresponding amino acid sequence in the region not corresponding to the primer (shown as underlined). Thus the alignment results show that there are no major differences in LlCADc2 and LlCADc3 and both the clones can be treated as one.

LlCADc3 Deduced amino acid sequence

```
   1 - ATGGGTAGCGTCGAAGGAGAAAGAACAACGGTTGGTTGGGCTGCTCGAGACCCTTCTGGG - 60
   1 - M  G  S  V  E  G  E  R  T  T  V  G  W  A  A  R  D  P  S  G   - 20

  61 - ACTCTCTCTCCCTACACTTTCAATCTCAGGAACACGGGACCTGATGATGTGTATATCAAG - 120
  21 - T  L  S  P  Y  T  F  N  L  R  N  T  G  P  D  D  V  Y  I  K   - 40

 121 - GTTCACTACTGTGGAATATGCCATTCCGATCTGCATCAGATTAAGAATGATCTTGGCATG - 180
  41 - V  H  Y  C  G  I  C  H  S  D  L  H  Q  I  K  N  D  L  G  M   - 60

 181 - TCCAATTATCCCATGGTTCCTGGGCATGAGGTGGTCGGAGAGGTGATCGAGGTGGGTTCG - 240
  61 - S  N  Y  P  M  V  P  G  H  E  V  V  G  E  V  I  E  V  G  S   - 80

 241 - AATGTGACCAAGTTTAAGGTCGGAGAAGTAGTCGGAGCAGGACTCATCGTTGGGAGCTGC - 300
  81 - N  V  T  K  F  K  V  G  E  V  V  G  A  G  L  I  V  G  S  C   - 100

 301 - CGGAATTGCAGGGCATGCAAATCTGATATTGAGCAATATTGCGCCAAGAAGATCTGGAAT - 360
 101 - R  N  C  R  A  C  K  S  D  I  E  Q  Y  C  A  K  K  I  W  N   - 120

 361 - TGCAACGATGTTTACACCGATGGAAAACCCACCCAAGGTGGCTTCGCTGAAACCATGGTC - 420
 121 - C  N  D  V  Y  T  D  G  K  P  T  Q  G  G  F  A  E  T  M  V   - 140

 421 - GTCAATCAAAATTTCGTTGTGAAGATACCAGAGGGGATGTCTCCGGAACAAGTGGCTCCA - 480
 141 - V  N  Q  N  F  V  V  K  I  P  E  G  M  S  P  E  Q  V  A  P   - 160

 481 - CTGTTGTGCGCCGGCGTGACGGTGTACAGTCCACTGTCACACTTTGGACTGAAAGAGAGT - 540
 161 - L  L  C  A  G  V  T  V  Y  S  P  L  S  H  F  G  L  K  E  S   - 180

 541 - GGGCTAAGAGGAGGAATATTAGGGCTTGGAGGAGTGGGACACATGGGCGTGAAGATAGCC - 600
 181 - G  L  R  G  G  I  L  G  L  G  G  V  G  H  M  G  V  K  I  A   - 200

 601 - AAAGCCATGGGACACAACGTGACAGTGATAAGCTCGTCGGAGAAGAAGAAGCAGGAGGCT - 660
 201 - K  A  M  G  H  N  V  T  V  I  S  S  S  E  K  K  K  Q  E  A   - 220

 661 - CTGGAACACCTCGGAGCAGACGATTATGTGGTTAGCTCAGACGAAACTCAGATGCAGAAG - 720
 221 - L  E  H  L  G  A  D  D  Y  V  V  S  S  D  E  T  Q  M  Q  K   - 240

 721 - ATTGCTGATTCACTTGATTATATCATCGATACGGTGCCAGTGGGTCACCCTCTTGAGCCT - 780
 241 - I  A  D  S  L  D  Y  I  I  D  T  V  P  V  G  H  P  L  E  P   - 260

 781 - TATCTTTCTCTGCTCAAAGTTGATGGCAAGTTGATCTTAATGGGTGTTATCAACACTCCT - 840
 261 - Y  L  S  L  L  K  V  D  G  K  L  I  L  M  G  V  I  N  T  P   - 280

 841 - CTGCAATTCGTCAGCCCCATGGTCATGCTCGGGAGGAAGACGATAACGGGAAGCTTCATT - 900
 281 - L  Q  F  V  S  P  M  V  M  L  G  R  K  T  I  T  G  S  F  I   - 300

 901 - GGGAGCATAAAGGAGACGGAAGAGATGTTGGGGTTCTGGAAAGAGAAGGGGCTGAGTTCA - 960
 301 - G  S  I  K  E  T  E  E  M  L  G  F  W  K  E  K  G  L  S  S   - 320

 961 - ATGATAGAGGTTGTGAAGATGGATTACATCAACAAAGCCTTCGAGAGGTTGGAGAAGAAC - 1020
 321 - M  I  E  V  V  K  M  D  Y  I  N  K  A  F  E  R  L  E  K  N   - 340

1021 - GATGTCAGATATAGGTTCGTTGTTGACGTTGCCGGCAGCAAACTTGATCAATGA - 1074
 341 - D  V  R  Y  R  F  V  V  D  V  A  G  S  K  L  D  Q  *  X     - 360
```

The deduced amino acid sequences of LlCADc3 gene shows that, the coding region consist of 357 amino acids, with molecular weight 38872.7 daltons, theoretical pI 5.89 and empirical formula $C_{1721}H_{2740}N_{458}O_{520}S_{22}$. BLAST analysis revealed the presence of conserved domain for alcohol dehydrogenase and the zinc binding domains. The amino acid residues indicated in red show the NADPH binding domain, the underlined residues are responsible for zinc binding. Residues in green are the zinc structural motifs, while the residues in pink are the zinc catalytic centers. The residue shown in blue is the one specific for NADP binding.

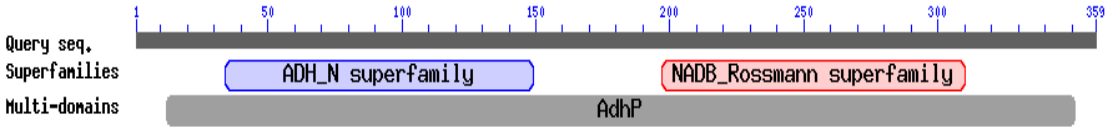


Figure 3.13: Putative conserved domains showing CADc3 to be a member of Zn binding alcohol dehydrogenase family

Amino acid composition of LlCADc3

Ala	(A)	15	4.2%
Arg	(R)	10	2.8%
Asn	(N)	14	3.9%
Asp	(D)	19	5.3%
Cys	(C)	8	2.2%
Gln	(Q)	10	2.8%
Glu	(E)	23	6.4%
Gly	(G)	39	10.9%
His	(H)	9	2.5%
Ile	(I)	21	5.9%
Leu	(L)	27	7.6%
Lys	(K)	26	7.3%
Met	(M)	14	3.9%
Phe	(F)	10	2.8%
Pro	(P)	15	4.2%
Ser	(S)	25	7.0%
Thr	(T)	17	4.8%
Trp	(W)	3	0.8%
Tyr	(Y)	12	3.4%
Val	(V)	40	11.2%
Pyl	(O)	0	0.0%
Sec	(U)	0	0.0%

Phylogenetic analysis of CAD gene

Phylogenetic analysis was done using 18 protein sequences of CAD, which were retrieved from the GenBank database. The protein sequences used for analysis were representatives from each group, namely fabaceae (dicots), other dicots, monocots, gymnosperms and *Saccharomyces cervesiae* alcohol dehydrogenase and horse liver alcohol dehydrogenase were take as out groups. Multiple alignments of the predicted protein coding sequences were performed using Clustal X. The phylogenetic tree was subsequently analyzed and displayed by Neighbor joining mega software. The tree can be divided in three major groups: first consisting of dicots and others of monocots and gymnosperms. Within dicot the fabaceae members form a small group which indicates that they are the most evolutionarily related. It is clear from the tree that LlCADc3 is evolutionarily most similar to fabaceae followed by other dicots, monocots and gymnosperms. *Saccharomyces* alcohol dehydrogenase is more closely related to the CADs than the horse liver alcohol dehydrogenase

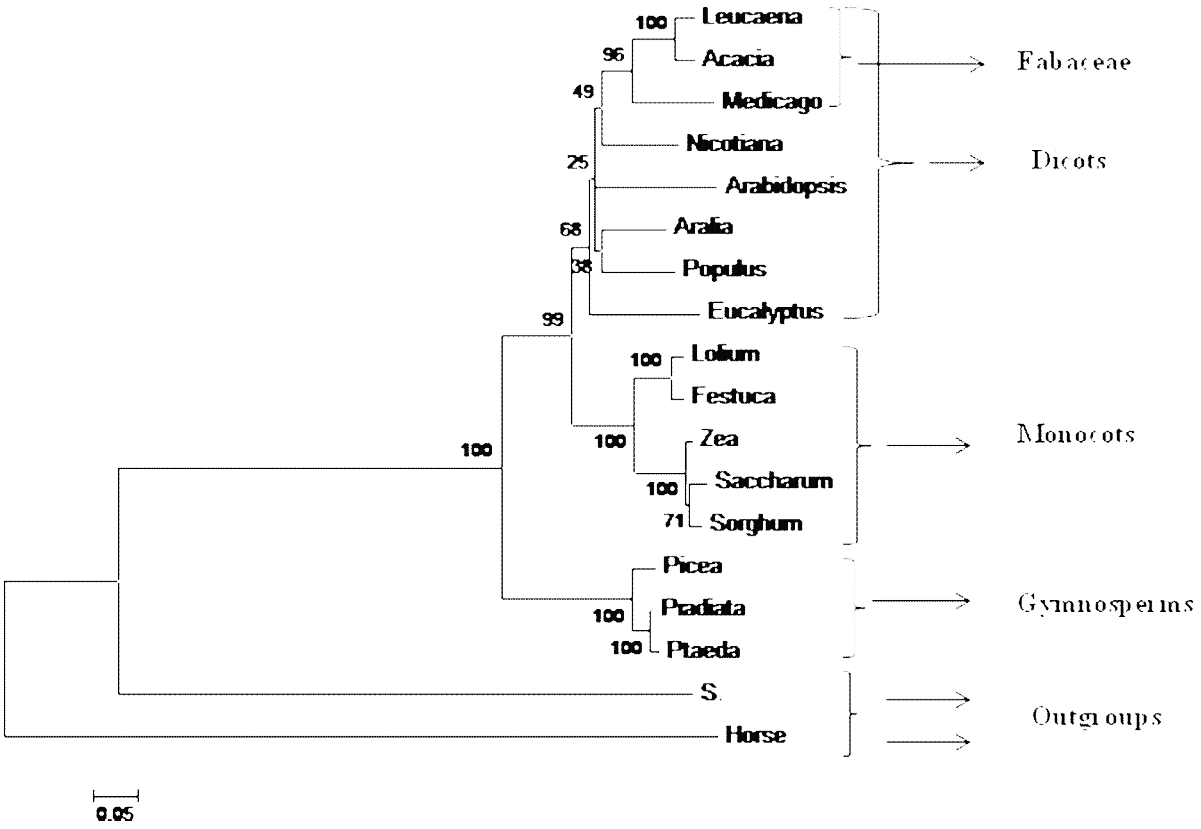


Figure 3.14 Phylogenetic analysis of LlCADc3

Phylogenetic tree made using Neighbour-Joning method (Figure 3.14). The values at the node indicate the bootstrap values. The evolutionary history of CAD was inferred using the neighbor-joining method. Saccharomyces cervisiae alcohol dehydrogenase (M38457) shown in the figure as "S" and horse liver alcohol dehydrogenase (M64865) were used as out groups. CAD protein sequence from other plant species were used to deduce the similarity index. The Fabaceae CAD protein sequences were from *Acacia auriculiformis* X *Acacia mangium* (EU275981) and *Medicago sativa* (AF083332). The other dicot group included *Nicotiana tabacum* (X62343), *Arabidopsis thaliana* (NM112832), *Aralia cordata* (D13991), *Populus tomentosa* (EU760897) and *Eucalyptus globulus* (AF038561). The monocot group consists of *Lolium perenne* (AF010290), *Festuca arundinace*a (AF188294), *Zea mays* (Y13733), *Saccharum officinarum* (AJ231135) and *Sorghum bicolor* (AB288109). The gymnosperm group consists of *Picea abies* (AJ868574), *Pinus radiata* (U62394) and *Pinus taeda* (Z37991). GenBank accession numbers are given in brackets.

3.3.5.4 Sequence analysis of LlCADg1

Nucleotide sequence of LlCADg1

```
   1 GTTTGCCTTT TTTCCCTCTG TGGAATTCTC TCTTTCCCTC TTGAGAAAAC
  51 TATGGGTAGC GTCGAAGGAG AAAGAACAAC GGTTGGTTGG GCTGCTCGAG
 101 ACCCTTCTGG GACTCTCTCT CCCTACACTT TCAATCTCAG GTACTTAATT
 151 AATTACTTTC ATTAAAAAAA ATCTCTTTTT TATACGTTTC GGTTCACCAT
 201 GTTTTAACTC CTGAAATTGA CCGCTTTCAG GAACACGGGA CCTGATGATG
 251 TGTATATCAA GGTTCACTAC TGTGGAATAT GCCATTCCGA TCTGCATCAG
 301 ATTAAGAATG ATCTTGGCAT GTCCAATTAT CCCATGGTTC CTGGGTAATT
 351 AATGAAACAA CCGACTACAA AACCCATTGA ATTATCCTCT GCTTTGTGGC
 401 TGAGAGCTGA TGATGAGTTA TGTTATGATG TGTATAGGCA TGAGGTGGTC
 451 GGAGAGGTGA TCGAGGTGGG TTCGAATGTG ACCAAGTTTA AGGTCGGAGA
 501 AGTAGTCGGA GCAGGACTCA TCGTTGGGAG CTGCCGGAAT TGCAGGGCAT
 551 GCAAATCTGA TATTGAGCAA TATTGCGCCA AGAAGATCTG GAATTGCAAC
 601 GATGTTTACA CCGATGGAAA ACCCACCCAA GGTGGCTTCG CTGAAACCAT
 651 GGTCGTCAAT CAAAAGTAAT CAACAGTACT CCCTGCTTTA TAATTCTTTA
 701 AAAAGTTCCT TCAGAATCCC ACTCCAAAGT TTTATGCTTT ATGCTCTTCA
 751 AAGATTCCTT CAAAACTTCA GTACTTAGCT GTGTTTTCTT GGTTTTTCAG
 801 TTTCGTTGTG AAGATACCAG AGGGGATGTC TCCGGAACAA GTGGCTCCAC
 851 TGTTGTGCGC CGGCGTGACG GTGTACAGTC CACTGTCACA CTTTGGACTG
 901 AAAGAGAGTG GGCTAAGAGG AGGAATATTA GGGCTTGGAG GAGTGGGACA
 951 CATGGGCGTG AAGATAGCCA AAGCCATGGG ACACAACGTG ACAGTGATAA
1001 GCTCGTCGGA GAAGAAGAAG CAGGAGGCTC TGGAACACCT CGGAGCAGAC
1051 GATTATGTGG TTAGCTCAGA CGAAACTCAG ATGCAGAAGA TTGCTGATTC
1101 ACTTGATTAT ATCATCGATA CGGTGCCAGT GGGTCACCCT CTTGTGCCTT
1151 ATCTTTCTCT GCTCAAAGTT GATGGCAAGT TGATCTTAAT GGGTGTTATC
1201 AACACTCCTC TGCAATTCGT CAGCCCCATG GTCATGCTCG GTGCGCTCTC
1251 TCTCTCTCTC TCTGTGTTAT CAGCATCTTT TGTCTTCAAA TTGTAACTTA
1301 ATTATTAATG ATGTGTCCGG CGGCAGGGAG GAAGACGATA ACGGGAAGCT
1351 TCATTGGGAG CATAAAGGAG ACGGAAGAGA TGTTGGGGTT CTGGGAAGAG
1401 AAGGGGCTGA GTTCAATGAT AGAGGTTGTG AAGATGGATT ACATCAACAA
1451 AGCCTTCGAG AGGTTGGAGA AGAACGATGT CAGATATAGG TTCGTTGTTG
1501 ACGTTGCCGG CAGCAAACTT GATCAATGAA ATGGAACACG CATGTGTCAT
1551 CTTATAATTT CTTAAATAAC CTTCTTCTGC AAAACTTATG AATACACCTG
1601 GGAGTTGTTT GTTGTGTGTT A
```

Exon-intron analysis

The genomic clone of *CAD*, LlCADg1 is 1621 bp long with the initiation codon at 52nd position and the termination codon at1527th position. The intiation and termination codons are highlighted. The open reading frame (ORF) of LlCADg1 is of 1478 bp. LlCADg1 consists of four introns and five exons. The introns are shown in italics in the nucleotide sequence and the exon-intron junctions are underlined. The first intron is 90 bp in length (Nt.141-230), second intron is 93 bp in length (Nt.345-437), third intron is 135 bp in length (Nt.666-800) and the fourth intron is 86 bp in length (Nt.1241-1326).

3.3.6 Distribution of *CAD* gene- Southern hybridization

To further validate the results from southern hybridization done with heterologous probe and to understand the distribution of the *CAD* gene in the *L. leucocephala* genome, southern hybridization was performed. A 10 µg aliquot of *L. leucocephala* genomic DNA was restriction digested individually with the restriction enzymes *Eco* RI, *Xba* I and *Kpn* I, which are predicted not to cut (*Xba* I and *Kpn* I) or cut once (*Eco* RI) within the sequence. The site *Eco* RI does not cut within the fragment used as probe. As shown in Fig. 3.15, two bands were detected in each *Eco* RI, *Xba* I and *Kpn* I digest. These results suggest that LlCADc3 is present as a two-copy number gene. An approximately 800 bp fragment from the coding region was used as a probe for hybridization at 62 °C. Banding pattern in Southern hybridization suggested that at least two copy of *CAD* gene is present in *L. leucocephala* genome.

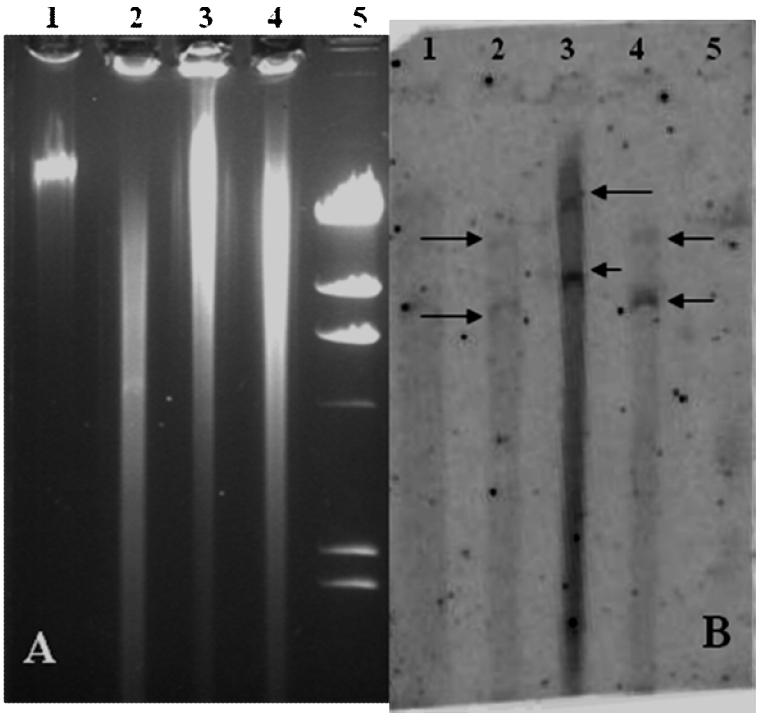


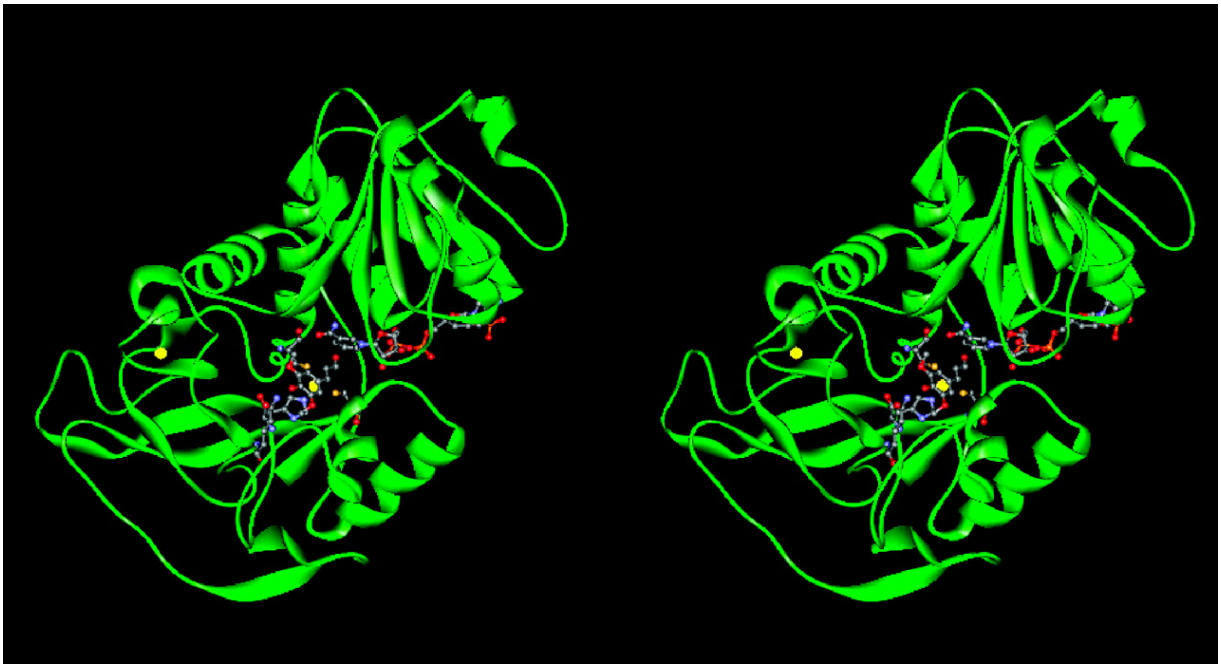
Figure 3.15 A: Agarose gel showing *L. leucocephala* genomic DNA digested with *Eco* RI (Lane 2), *Xba* I (Lane 3), *Kpn* I (Lane 4), Lambda *Hind* III digestion (Lane 5) and uncut genomic DNA (Lane 1). **B-** Phosphor-imager scan, arrows showing regions hybridized with probe

3.4. Conclusions

- PCR based approach was used to fish out the *CAD* gene. Three similar CAD cDNA clones were isolated namely LlCADc1, LlCADc2 and LlCADc3.
- LlCADc1 is the partial cDNA clone, obtained by using primers designed from fabaceae members. The accession number for this clone is DQ 361031.
- LlCADc1 showed maximum homology (92%) with *CAD* from *Acacia auriculiformis* X *Acacia mangium*. So, primers were designed from initiation codon and upto termination codon of *Acacia*. Using these primers LlCADc2 was obtained and its accession number is DQ914843.
- RACE reaction yielded three different 3' UTRs and two different 5' UTRs which may play a role in differential regulation of *CAD*. Putative polyadenylation sites and the polyA tails were identified in the 3' UTRs.
- Primers were designed from 5' and 3' UTRs and were used to get full length *CAD* cDNA and genomic clones.
- The full length cDNA clone LlCADc3 is of 1074 bp with accession number EU870436. BLAST analysis revealed about 70-92% identity with other *CAD*s in the database. There were no major differences in the LlCADc2 and LlCADc3 sequences except the primer regions of LlCADc2 and two other differences, so both the sequences can be treated as one.
- Analysis of deduced amino acid sequence of LlCADc3 showed the conserved domains for NADPH binding and zinc binding domains. BLAST analysis showed it to be a member of zinc binding alcohol dehydrogenase family.
- Phylogenetic analysis of LlCADc3 deduced amino acid sequence was done using 18 protein sequences of CAD using the neighbor joining method. The results show that LlCADc3 is evolutionarily most similar to fabaceae followed by other dicots, monocots and gymnosperms. *Saccharomyces* alcohol dehydrogenase is more closely related to the *CAD*s than the horse liver alcohol dehydrogenase.
- The genomic clone LlCADg1 is of 1621 bp with four introns and five exons.
- *CAD* in *L. leucocephala* is a gene family of possibly 2 members.

CHAPTER 4(A)

HETEROLOGOUS EXPRESSION OF *LEUCAENA LEUCOCEPHALA* CAD GENE, ITS PURIFICATION & CHARACTERIZATION



A. Heterologous expression of *Leucaena leucocephala CAD* gene, its purification and characterization

4.1 Introduction

Analysis of gene function is of central importance for the understanding of physiological processes. Expression of genes in heterologous organisms has allowed the isolation of many important genes (e.g. for nutrient uptake and transport) and has contributed a lot to the functional analysis of the gene products. Heterologous expression systems are powerful tools for isolating new genes and for characterizing proteins from all organisms.

An efficient way to clone and simultaneously prove the function of a gene is functional expression in heterologous host cells. Heterologous expression of plant genes provides a new technique for determining gene-product function.

Cinnamyl alcohol dehydrogenase catalyses the conversion of p-hydroxy-cinnamaldehydes to the corresponding alcohols and is considered a key enzyme in lignin biosynthesis (Poratles *et al.,* 2005). The enzyme kinetics of few tree species (Lacombe *et al.*, 1997; Leple *et al.*, 1998) has been studied. Hence functional expression of CAD gene in heterologous system will be of immense help in understanding the characteristics and kinetics of the CAD enzyme.

The pET 30b(+) vector from Novagen was used as the heterologous expression system. The pET system is the powerful system for cloning and expression of recombinant proteins in *E. coli*. Target genes are cloned in pET plasmids under control of strong bacteriophage T7 transcription and (optionally) translation signals; expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 RNA polymerase is so selective and active that, when fully induced, almost all of the cell's resources are converted to target gene expression; the desired product can comprise more than 50% of the total cell protein within few hours after induction.

Purification of the recombinant protein is done under fully denaturing conditions using His-Tag fusion proteins and His-Bind immobilized metal affinity chromatography.

In the previous chapter isolation, cloning and characterization of full length *CAD* gene was done. Clone LlCADc2 was used for heterologous expression analysis.

4.2 Materials and methods

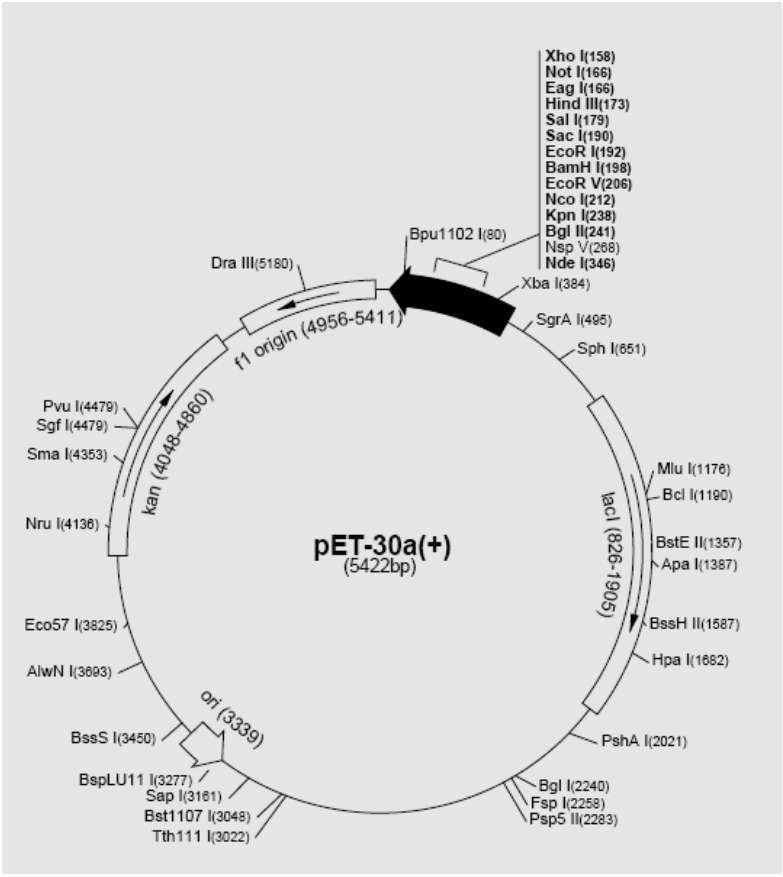
4.2.1 Cloning of CADc2 in pET 30b (+) vector

CADc2 fragment from clone LlCADc2 was cloned in pGEM-T Easy vector by incorporating the restriction sites *Nde* I and *Sal* I in the primers CAD AF and CAD AR respectively. High fidelity *Taq pol* (*Pfx* Invitrogen) was used to amplify CADc2 using AF pET (CAD AF with *Nde* I) and AR pET (CAD AR with *Sal* I) primers using LlCADc2 plasmid as template. Plasmid LlCADc2 was diluted 100 times and 1 µl was used as a template. PCR was performed as described in section 2.17. A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in *E.coli* XL1 MRF cells. Clones with CADc2 fragment with *Nde* I and *Sal* I restriction sites were screened by inoculating few colonies in 5 ml LB (Ampicillin 100 µg/ml) tubes. Isolated individual plasmids were restriction digested with *Nde* I and *Sal* I enzymes to confirm the integration of CADc2 insert.

PCR cycling condition:

No. of cycles	Temperature	Time
1	95 ºC	5 min
35	95 ºC 60 ºC 72 ºC	1 min 30 s 1min
1	72 ºC	5 min
1	4 ºC	hold

The CADc2 fragment with the *Nde* I and *Sal* I restriction sites was directionally cloned in pET 30b(+) vector (Figure 4.1 & 4.2). Colony PCR was done to screen the recombinant pET 30b(+) clones. Integration of CADc2 fragment in pET30b (+) was confirmed by digestion with *Nde* I and *Sal* I.



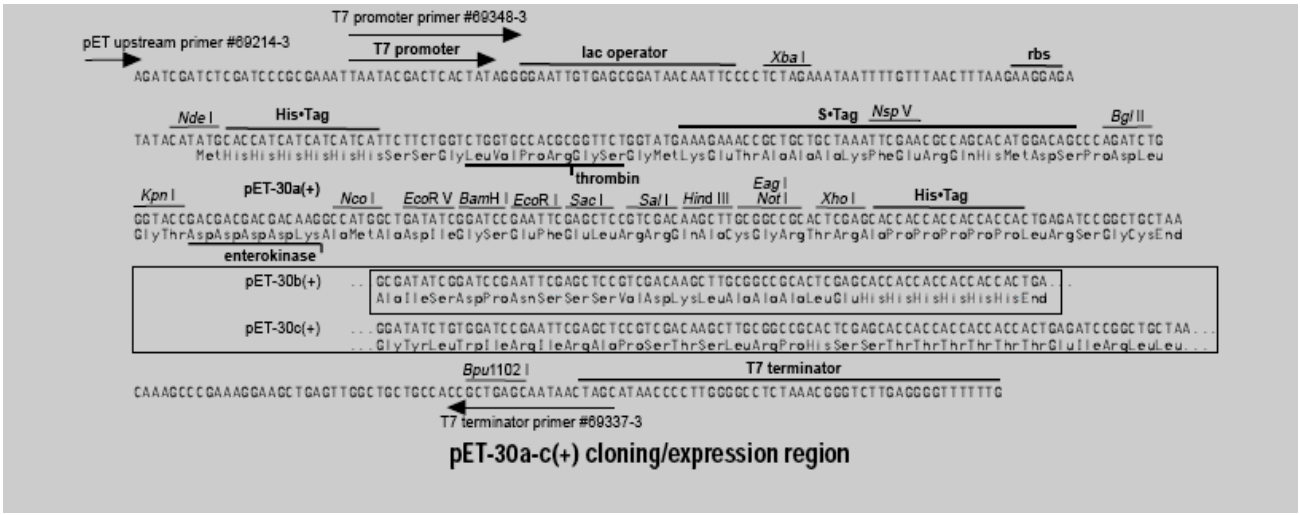


Figure 4.1 Vector map of pET 30. pET 30b (+) is shown in box.

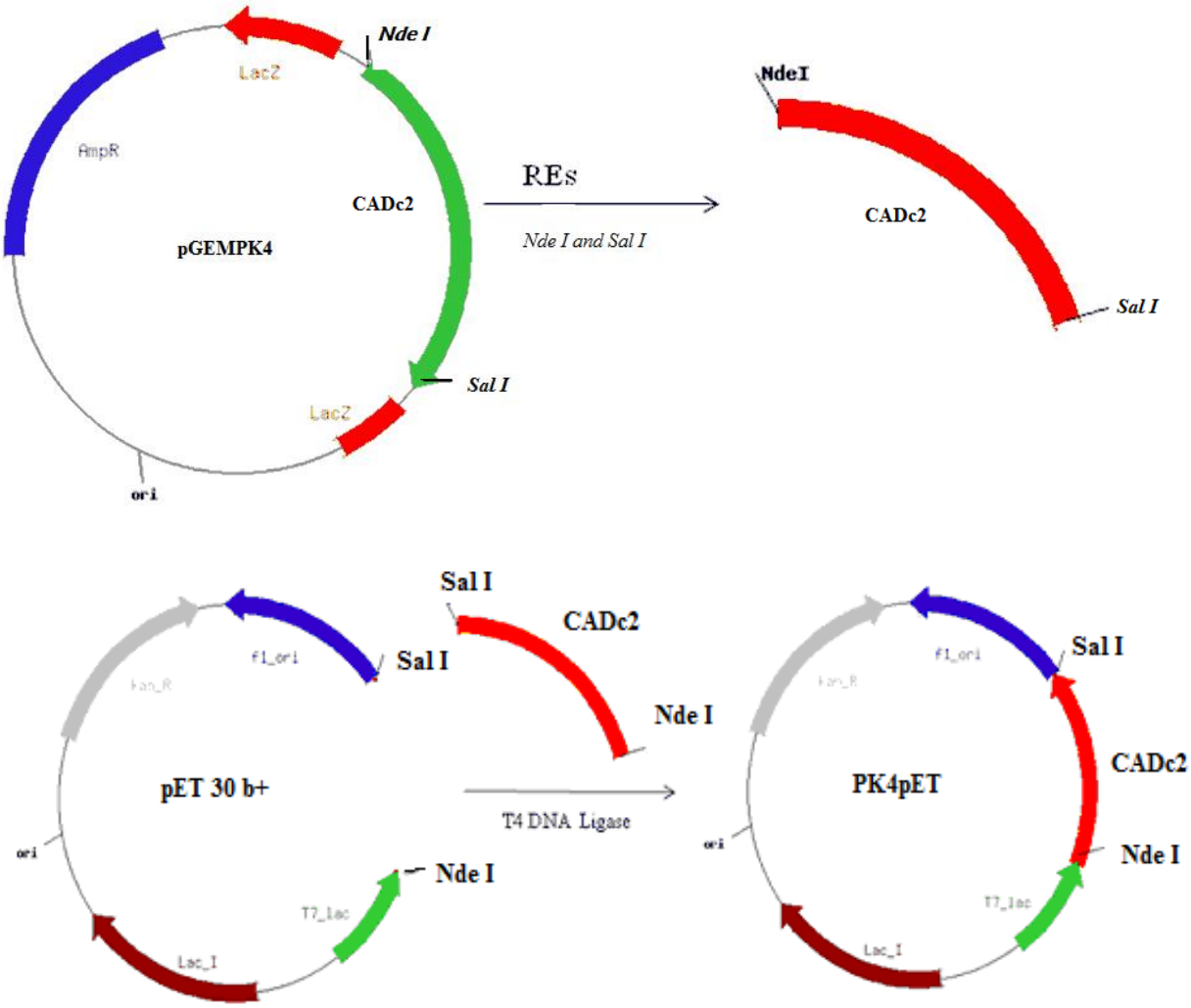


Figure 4.2: Strategy for directional cloning of CADc2 in pET30b(+) vector.

4.2.2 Recombinant CAD protein expression and purification from inclusion bodies

4.2.2.1 Recombinant CAD protein expression

A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 µg/ml kanamycin) was used to inoculate 5 ml liquid LB medium containing the same concentration of the antibiotic. Culture was grown overnight with shaking at 225 rpm at 37 °C. One ml aliquot of each culture was used to inoculate 100 ml liquid cultures containing 50 µg/ml kanamycin. Once the cultures reached OD_{600} 0.4 - 0.5, recombinant protein expression was induced by addition of isopropyl -ß-D-thiogalactopyranoside (IPTG), and the culture was grown for 4–6 h at 37 °C with

shaking at 150 rpm. Recombinant protein extraction was done according to the protocol described in Section 2.21.1.

Four positive recombinant pET 30b(+) clones were screened for CAD protein over-expression on 10% SDS PAGE (Section 2.21.3). The clone showing maximum over-expression was chosen for further studies.

4.2.2.2 Purification of recombinant CAD protein

His-tagged recombinant CAD protein was purified by metal chelate affinity chromatography. The initial stage of His-tagged protein purification is based on the remarkable selectivity and high affinity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues, the 6xHis-tag NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that have only three sites available for interaction with metal ions. The extra chelation site prevents nickel ion leaching, providing a greater binding capacity and high-purity protein preparations. Purification of recombinant CAD protein carrying a 6xHis-tag was conducted using Ni NTA Agarose beads (Qiagen) (Section 2.21.2). Purity of protein was checked on 10% SDS PAGE (Section 2.21.3).

4.2.3 Raising polyclonal antibody against purified CAD protein in Rabbit

The purified CAD protein was used for raising polyclonal antibodies in New Zealand White rabbit.

4.2.3.1 Pre-treatment of serum:

Rabbit immune serum (stored at -70 ºC), containing polyclonal antibodies against the CAD antigen, was thawed overnight at 4 ºC. The Serum was kept at 55 °C for one hour and the immune serum was centrifuged at 12,000 g for 15 min at 4 ºC. Aliquots according to the requirements were made and stored at -70 ºC. The antiserum aliquots were thawed overnight again at 4 ºC prior to use.

4.2.3.2 Determination of titre of antibodies

ELISA was performed to determine the titre of first, second, third and fourth bleed of rabbit serum (Section 2.23.1). Once the antibody titre was determined then a fixed dilution of antibodies was used for rest of the experiments.

4.2.4 Extraction and purification of recombinant CAD protein in soluble form

The recombinant CAD protein was extracted in soluble form for the CAD enzyme activity assay. The extraction and purification was done according to the protocol mentioned in section 2.24.1.

4.2.4.1 Standardization of time for protein expression in soluble form

The information obtained from above experiment was utilized for this experiment. Four flasks with 50 ml LB broth (kanamycin 30 µg/ml) were inoculated with O/N grown culture of *E. coli* BL21 harboring PK4pET recombinant plasmid. Induction with 1.0 mM IPTG was done and the cells were grown till A_{600} reached 0.5. The cultures were grown at different temperatures (20-37 °C) and different durations (4 to 6 h) for optimization of maximum expression of recombinant CAD protein in soluble form. One flask as an uninduced control sample was used before inducing with IPTG. The soluble fraction of cell lysate was analysed on 10% SDS-PAGE.

4.2.4.2 Purification of recombinant CAD protein in soluble form

The extracted recombinant CAD protein was purified from the crude cell lysate using the Ni NTA Agarose beads (Qiagen) under non-denaturing conditions (Section 2.24.1.2).

4.2.5 CAD enzyme assay

The activity of the purified recombinant CAD enzyme was assayed according to the method given by Wyrambik (1975). The protocol for enzyme assay is given in section 2.24.3. The activity was calculated according to the formula given below

$$\text{Activity (U/ml)} = \frac{\Delta A340/\text{min} \times 1000}{6.22 \times 150}$$

Where, 1000 is the reaction volume in µl, 150 is the volume of enzyme in µl and 6.22 is the extinction coefficient of NADPH. The activity is expressed as units/ml. The protein concentration is measured using the Bradford method. The specific activity is expressed as nKat/mg (1 Unit = 16.67 nkatals)

4.3 Results and discussion

4.3.1 Cloning of CADc2 in pET 30b(+)

4.3.1.1 Incorporation of restriction sites and PCR

The CADc2 gene specific primers CAD AF and CAD AR were modified to incorporate the *Nde* I site at the 5' end and *Sal* I site at 3' end of CADc2.

AF pET – 5' ATA TAT T**CAT AT G**GG AAG CAT TGA A 3'

AR pET - 5' ACG C**GT CGA C**CT GAT GAT CAT CAA GTT TGC TG 3'

A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in *E. coli* XL1 Blue cells. Clones with CADc2 fragment with *Nde* I and *Sal* I restriction sites were screened by inoculating few colonies in 5 ml LB (Ampicillin 100 µg/ml) tubes. Isolated individual plasmids were restriction digested with *Nde* I and *Sal* I enzymes to confirm the integration of CADc2 insert.

4.3.1.2 Directional cloning of CADc2 fragment in pET 30b(+) vector

The above clone in pGEM-T Easy vector was designated as pGEMPK4. This clone was restriction digested with *Nde* I and *Sal* I restriction enzymes and 1 kb fragment was purified. pET 30b(+) vector DNA was also digested with same restriction enzymes and purified. A 1 kb fragment from pGEMPK4 was directionally cloned in purified restriction digested pET 30b (+) vector. Ligation mixture was transformed in *E. coli* XL1 competent cells and plated on LB-agar plate (kanamycin 50µg/ml). Colonies for recombinant plasmids were screened by colony PCR method (Figure 4.3). Plasmid was isolated from clones showing 1 kb band in colony PCR reactions. The plasmid was digested with *Nde* I and *Sal* I to confirm the integration of pGEMPK4 fragment in pET 30b (+) vector.

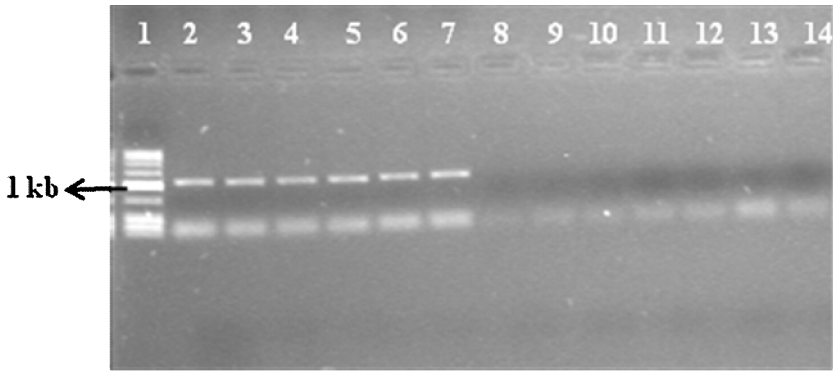


Figure 4.3 PCR of colonies screened. Lane 1- DNA molecular weight marker. Lane 2-7 positive clones.

The recombinant pET 30b(+) plasmids harboring CADc2 gene was confirmed after sequencing. The sequence was translated using proteomic tools available on www.expasy.ch and was checked for in frame translation up to HIS tag. Above recombinant plasmids were mobilized in *E. coli* BL21 strain for over-expression.

4.3.2 Recombinant CAD protein expression and purification from inclusion bodies

4.3.2.1 Recombinant CAD protein expression

E. coli BL21(DE3) cells transformed with recombinant pET 30b(+) plasmids were screened for over-expression. Four positive recombinant clones were screened for recombinant CAD protein over-expression. The clones were designated as PK1pET, PK2pET, PK3pET and PK4pET. A ~40 kD protein was expressed in all clones as analysed on 10% SDS PAGE. Maximum expression was found in clone PK4pET, which was then used for all experiments (Figure 4.4).

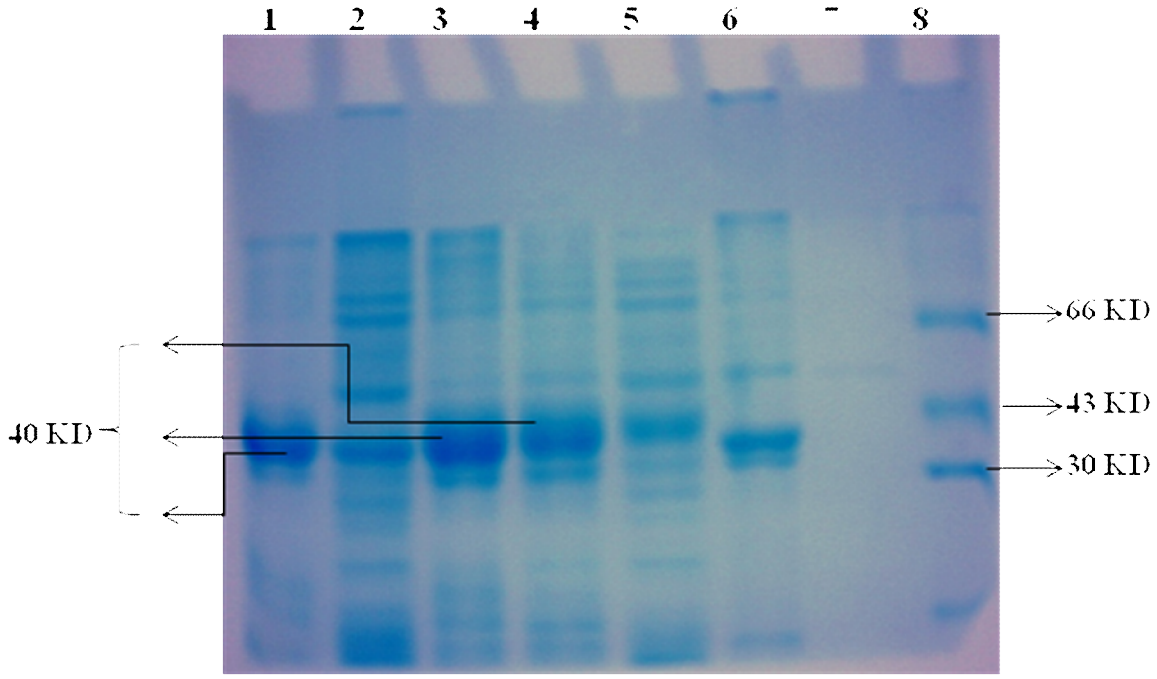


Figure 4.4 10% SDS PAGE**:** Lane 1, 3, 4- Inclusion bodies from clones PK1pET, PK4pET and PK2PET respectively. Lane 2, 5- Cell lysate from clones PK4pET and PK2pET respectively. Lane 8- Protein molecular weight marker.

4.3.2.2 Purification of recombinant protein

Large-scale production and purification was done using the protocol described in section 2.21.1 and 2.21.2. The purified protein was approximately 40 kD in size and was eluted at 200 mM imidazole concentration. It was found that four hours of post-induction at 37 °C was enough to achieve the purification of CAD protein from inclusion bodies (Figure 4.5).

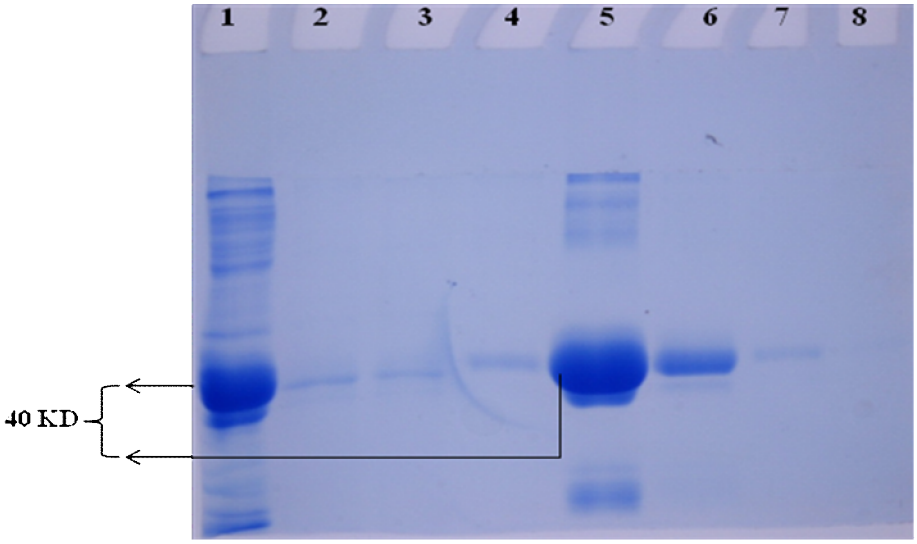


Figure 4.5 10% SDS PAGE: Lane 1-Inclusion bodies. Lane 2- Flow through. Lane 3-Wash 1. Lane 4-8- Eluent 1, 2, 3, 4 and 5 respectively.

Purified CAD protein was dialysed against 1X PBS buffer supplemented with 1 mM DTT and 0.1 mM PMSF overnight with two changes of fresh buffer. Dialysed CAD protein was concentrated, quantified by Bradford assay (Bradford reagent, Promega, USA) and given for raising antibodies in New Zealand rabbit.

4.3.3 Raising antibodies in rabbit:

300 µg of purified protein was used for first injection in New Zealand rabbit to raise antibodies. Same amount of protein was used for booster doses. Antibody titer of first, second, third and fourth bleed was determined by ELISA. Titer of third bleed is ~1:153600 (Figure 4.6). The titre of third and fourth bleed is almost the same. 3rd bleed serum dilution of 1:10000 was used for further experiments.

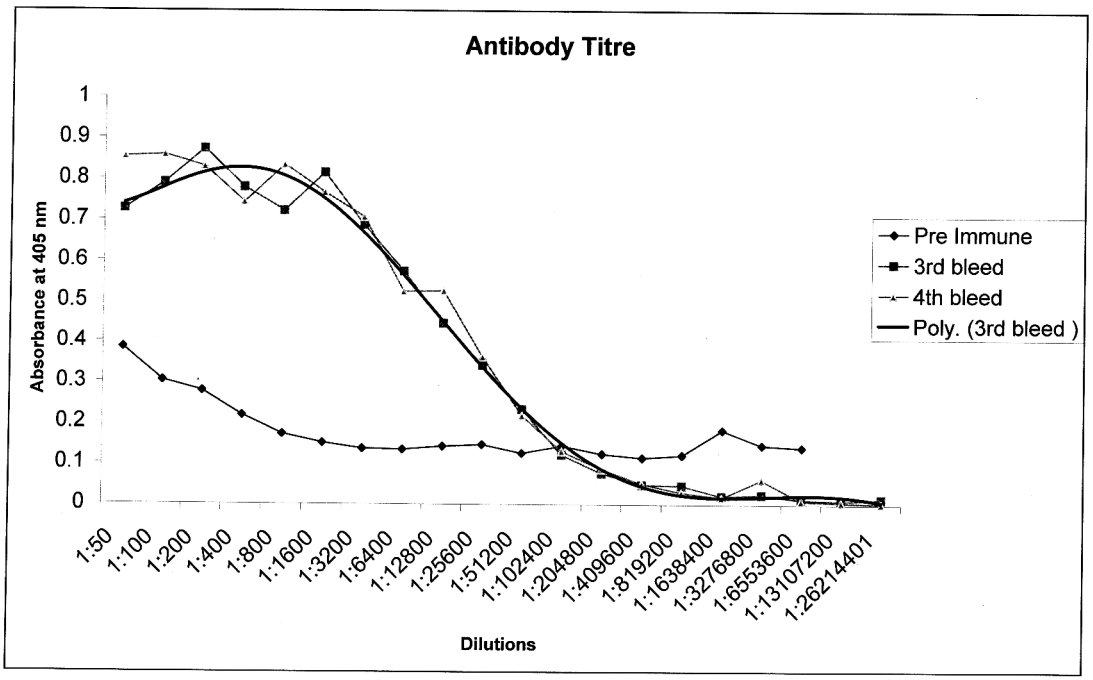


Figure 4.6 Graph showing antibody titre of third and fourth bleed.

4.3.4 Extraction and purification of recombinant CAD protein in soluble form

PK4pET and PK1pET clones were used for extraction of recombinant protein in soluble form to test the enzyme activity. Temperature and time required after induction was standardized for maximum CAD protein expression in soluble form that is in cell lysate. PK4pET showed the maximum CAD expression in soluble form. The optimum parameters were as follows, after initial growth at 37 ^{o}C, the cells were induced with 1mM IPTG. Before induction cells were cooled to 15 ^{o}C and were grown for 16 h at 15 ^{o}C after induction (Figure 4.7).

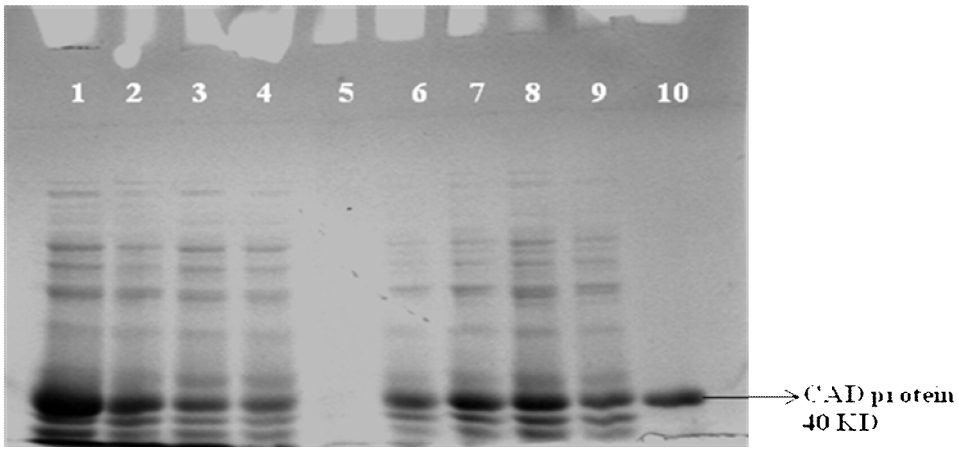


Figure 4.7 Analysis of recombinant CAD protein expressed under temperature and duration on 10% SDS PAGE: Lane1, 3, 6 and 8- PK4pET cell lysate at 15 °C for 16 h, 20 °C for 16 h, 20 °C for 6 h and 37 °C for 4 h respectively. Lane 2, 4, 7 and 9- PK1pET cell lysate at 15 °C for 16 h, 20 °C for 16 h, 20 °C for 6 h and 37 °C for 4 h respectively. Lane 10- purified CAD protein for comparison.

The recombinant CAD protein from cell lysate was purified using the Ni-NTA agarose beads under non denaturing conditions. The protein was eluted in the second fraction at 250 mM imidazole concentration.

4.3.5 CAD Enzyme assay

The activity of the recombinant CAD enzyme was assayed using the three substrates namely coniferaldehyde, cinnamaldehyde and sinapaldehyde. 1.156 µM of substrate was taken for the assay. The second eluent fraction was used as the CAD enzyme. The quantity of substrate, enzyme and NADPH was same for all the assay reactions. For each reaction decrease in absorbance at 340 nm was monitored for 3 min (Figure 4.8). The specific activity was expressed as nKat/mg of protein (Table 4.1). The results show that CAD specific activity is maximum for cinnamaldehyde. The results can be further validated by enzyme kinetic studies. Even so this data gives preliminary idea about substrate preference of *Leucaena* CAD enzyme.

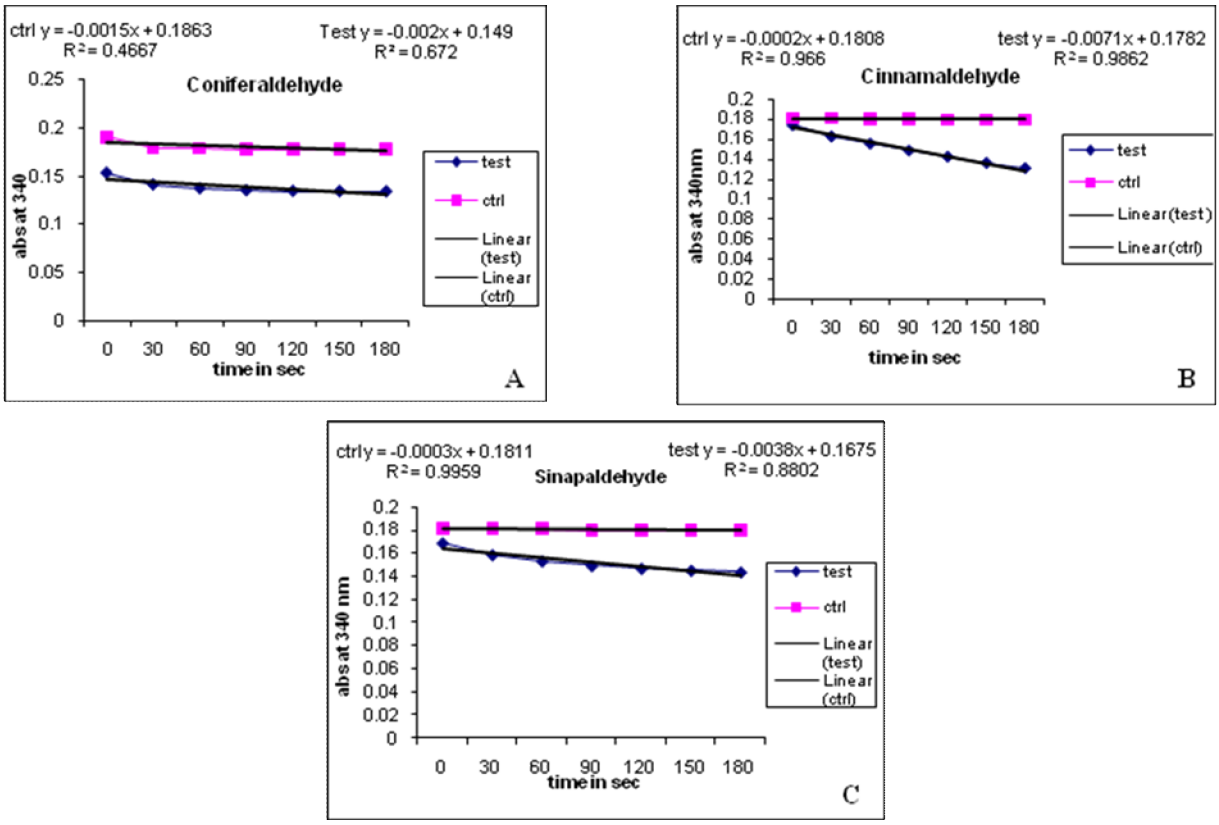


Figure 4.8 Graphs showing activity of CAD enzyme with coniferaldehyde (A), cinnamaldehyde (B) and sinapaldehyde (C).

	Substrate	Specific Activity (nKat/mg)
1	Coniferaldehyde	4.052330913
2	Sinapaldehyde	12.89378018
3	Cinnamaldehyde	25.41916664

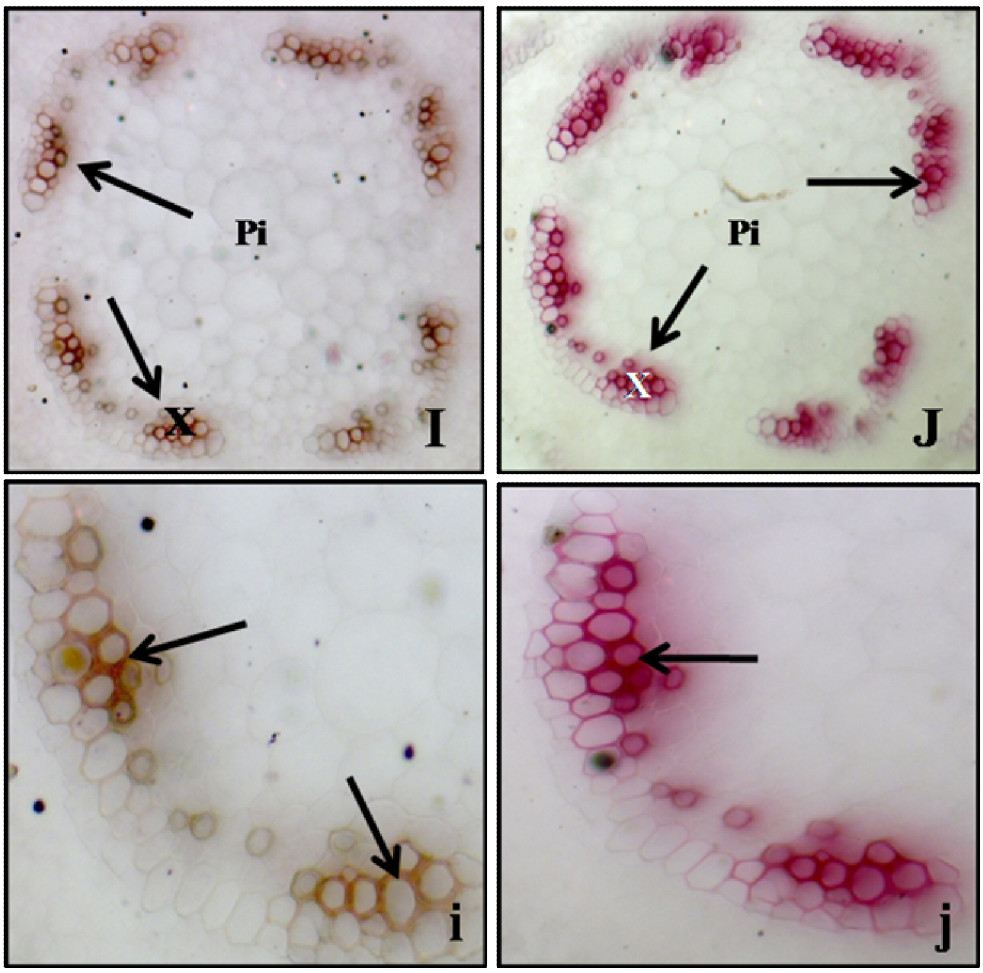
Table 4.1 Specific activities of recombinant CAD enzyme for coniferaldehyde, sinapaldehyde and cinnamaldehdyde substrates.

4.4 Conclusions

- The CADc2 fragment isolated from *L. leucocephala* was directionally cloned in pET 30b(+) expression system.
- Recombinant CAD protein was standardized for over-expression and purified from inclusion bodies. A 40 kD purified protein, from inclusion bodies, was used to raise polyclonal antibodies in New Zealand rabbit.
- CAD enzyme activity for substrates like cinnamaldehyde, coniferaldehyde and sinapaldehyde, was determined using purified CAD protein extracted in soluble form. Peliminary data show that recombinant CAD enzyme has maximum specific activity with cinnamaldehyde.
- The functional expression of recombinant CAD protein confirms that the cDNA isolated from *L. leucocephala* encodes for cinnamyl alcohol dehydrogenase protein.

CHAPTER 4(B)

SPATIO-TEMPORAL EXPRESSION & IMMUNO-CYTOLOCALIZATION OF CAD GENE



B Spatio-temporal expression and immuno-cytolocalisation of CAD gene

4.5 Introduction

Although the roles of most genes of the monolignol biosynthesis pathway in determining lignin amount and composition have been elucidated, our knowledge is still scarce on how monolignol biosynthesis integrates into wider plant metabolism and how plant metabolism responds to changes in the expression of individual monolignol biosynthesis genes. With the advent of genomic tools that enable unbiased transcriptome and metabolome wide analyses, such interactions can now be elucidated. Lignin deposition imparts rigidity and structural support to the cell wall. However, the data of lignin biosynthesis in relation to stem development at gene/transcript level is still limited. A knowledge of localization of enzymes involved in lignifications is essential to better understand the dynamics of lignin biosynthesis in cell, as well as regulation of lignifications in woody plants (Takabe *et al*., 2001). Studying different aspects of CAD like determining the protein amount by ELISA, assaying the activity of CAD, transcript analysis using RT-PCR and immuno-cytolocalization, especially with respect to developmental stages can help us in further studies regarding CAD gen,e from transcript to actual lignin synthesis stage. Such study will form a platform, very useful for transgenic analyses in future.

4.6 Materials and methods

4.6.1 Plant material

Seeds of *L. leucocephala* were treated as described in section 2.1. The imbibed seeds were transferred to ½ MS basal medium. The day of inoculation was considered as the zero day. Seedlings were harvested at 5, 10, 15 and 20 days respectively (Figure 4.9). For 10, 15 and 20 day seedlings root, shoot and leaves were harvested separately and were used for Real-Time PCR analysis, enzyme assay, ELISA and immuno-cytolocalisation studies.

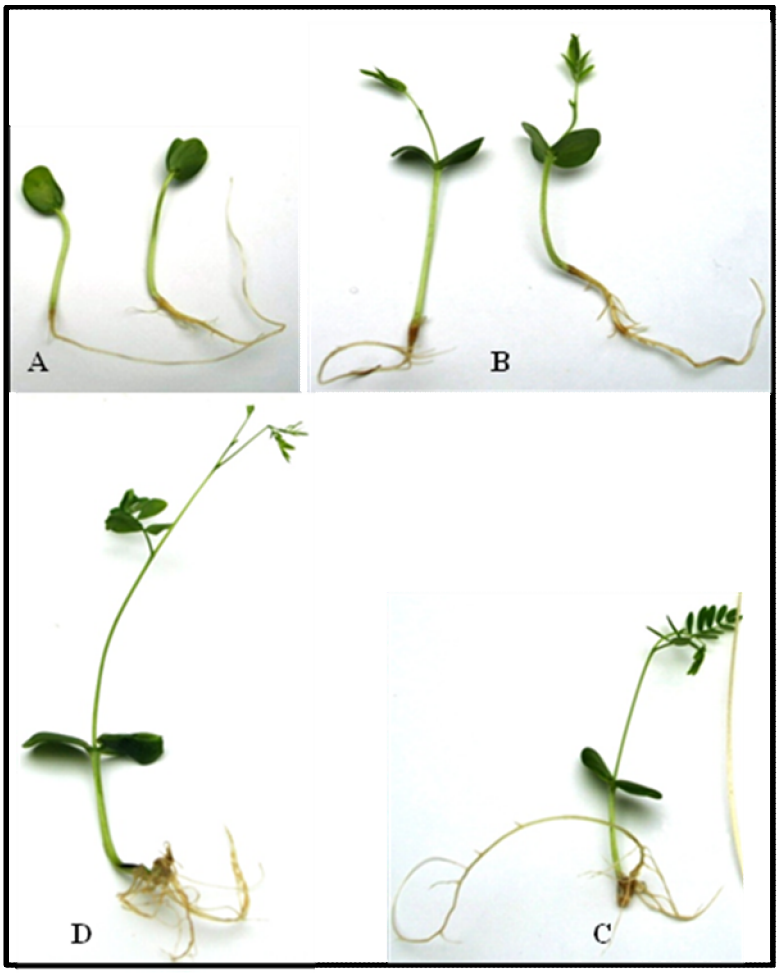


Figure 4.9 *L. leucocephala* seedlings at different stages after germination. A- 5 days. B- 10 days. C- 15 days. D- 20 days.

4.6.2 Quantitative real-time PCR analysis for *CAD* gene

Spatial and temporal expression studies of CAD gene was done using the quantitative real-time PCR analysis. Total RNA was isolated from the root, shoot and leaves of 5, 10, 15 and 20 days old seedlings. One µg of total RNA was used for making cDNA using ImProm cDNA synthesis kit (Promega, Madison, USA). Brilliant SYBRGreen QPCR kit (Stratagene, USA) and Stratagene Mx3000P real time machine were used for all reactions. 5.8 SrRNA was used as the internal control for normalization of expression. The sequence of primers designed for CAdc2 gene and 5.8 SrRNA used for the reactions is given in section 2.19.4, Table 2.0. Real time PCR reactions were performed as described in section 2.19.4.

4.6.3 CAD enzyme assay and ELISA

CAD enzyme was extracted from root, shoot and leaves of 5, 10, 15 and 20 days old *L. leucocephala* seedlings according to the protocol described in section 2.24.2. The purified plant tissue extract was used for enzyme assay after dialysis. CAD enzyme assay was done using coniferaldehyde as substrate (Section 2.24.3). Root, shoot and leaf extracts of 5, 10, 15 and 20 days old seedlings were used for the ELISA analysis (Section 2.23.2). A standard was prepared using the heterologously expressed CAD protein. 0.1 to 1 ng protein was used to make a standard graph. Same sample was used in both CAD assay and ELISA studies. Plant protein was quantified using Bradford method. 25 µg total plant protein was coated on ELISA plate. 1:10000 dilution of primary (anti-CAD) antibody was used. The detection antibody was goat anti rabbit IgG conjugated with alkaline phosphatase. PNPP was used as substrate for alkaline phosphatase. Readings were taken at 405 nm. All the reactions were done in triplicates and the experiment was performed twice.

4.6.4 Immuno-cytolocalisation of CAD in *L. leucocephala* tissues

Free hand transverse sections of root and shoot of 5, 10, 15 and 20 days old seedlings were used for the immuno-cytolocalisation to study the tissue expression pattern of CAD protein in normal growth conditions. Antibodies raised against CADc2 were used for the study. The protocol for immuno-cytolocalisation is described in section 2.25. The same sections were also used for phloroglucinol staining which selectively stains the lignin (Section 2.25.1) to study the correlation between CAD protein detection in tissue sections with lignin deposition.

4.7 Results and discussion

4.7.1 Quantitative real-time PCR analysis of *CAD* gene

Spatial and temporal pattern of *CAD* gene expression during different growth stages was studied using the real-time PCR technique. Root, shoot and leaves of 5, 10, 15 and 20 days old seedlings were used for the study. The relative expression of the gene in terms of Ct value is shown in figure 4.10. The Ct value is inversely proportional to the level of expression.

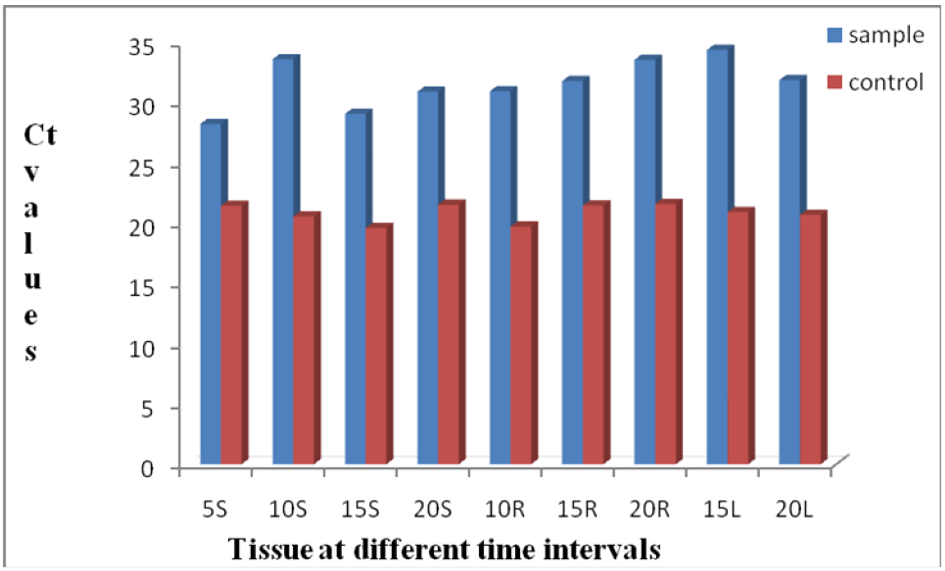


Figure 4.10 Relative expression of LlCADc2 in terms of Ct value in different plant tissues of different age *L. leucocephala* seedlings. On X-axis is tissue type with age of seedling. On Y-axis is quantity in terms of Ct value. S, R, and L stands for shoot, root and leaf respectively. The numbers 5, 10, 15 and 20 represents age of seedling in days.

CAD transcript expression in shoot tissue, is maximum on 5th day, followed by a decrease on 10th day and increase again on 15th day and a marginal decrease on 20th day. In root tissue the transcript level of CAD is maximum on 10th day and increases consecutively on 15th and 20th day. In leaf tissue CAD expression increases from 15th day to 20th day.

4.7.2 CAD enzyme assay and ELISA

4.7.2.1 CAD enzyme assay

CAD enzyme activity was assayed in root, shoot and leaf tissue of 5, 10, 15 and 20 days old seedlings of *L leucocephala.* Coniferaldehyde was used as the substrate for the assay reactions.

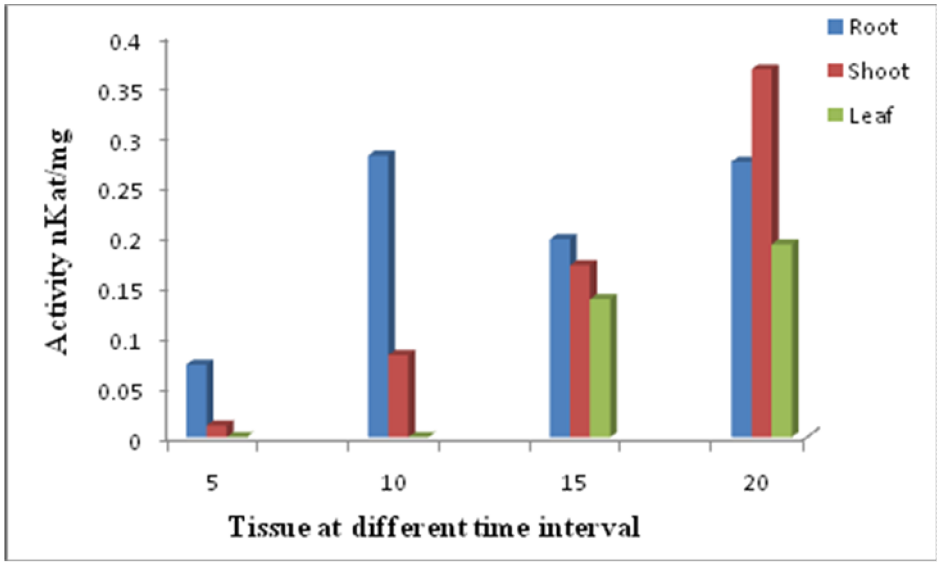


Figure 4.11 CAD activity profiles of *L. leucocephala* tissues at different age of seedlings (5, 10, 15, 20 days). X-axis represents age of seedlings in days; Y-axis represents activity of CAD enzyme.

In case of root tissue CAD activity is highest on 10th day and is lowest on 5th day. The activity in shoot tissue increases gradually from 5th day to 20th day. For leaf tissue the activity level increases from 15th to 20th day (Figure 4.11).

4.7.2.2 Developmental expression of CAD protein in *L. leucocephala* by ELISA

A standard graph for ELISA analysis was made using serial dilution of purified CAD protein (Figure 4.12).

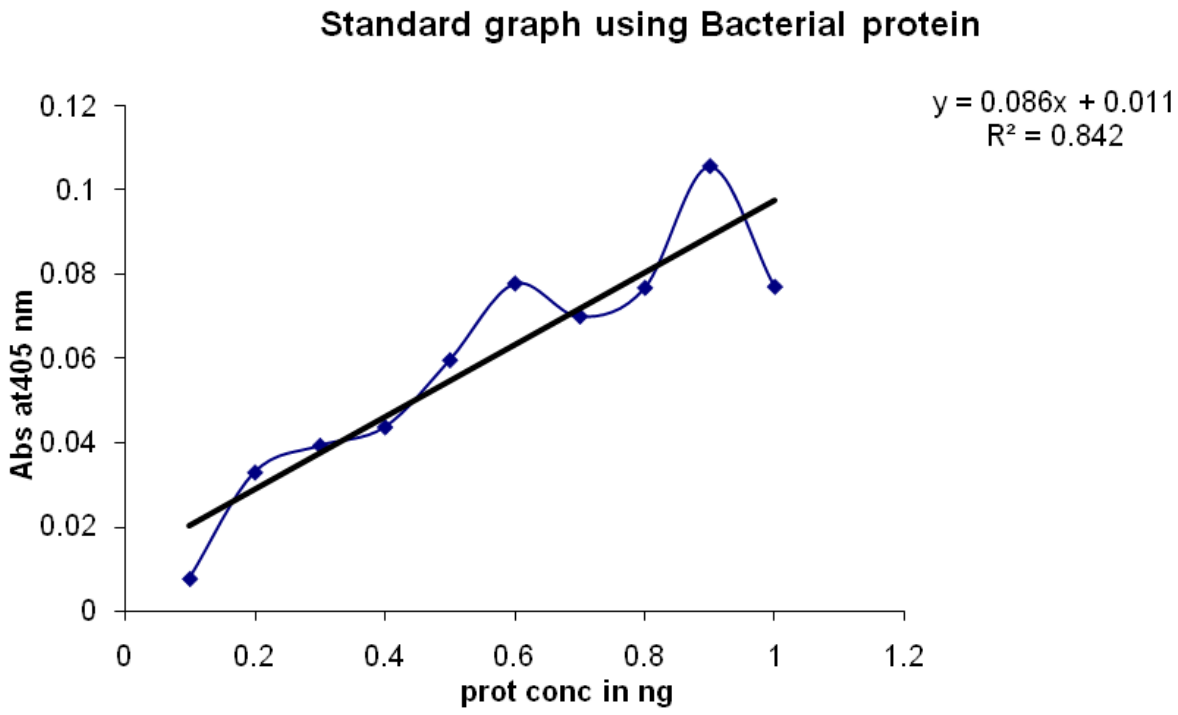


Fig 4.12 Standard graph using recombinant CAD protein

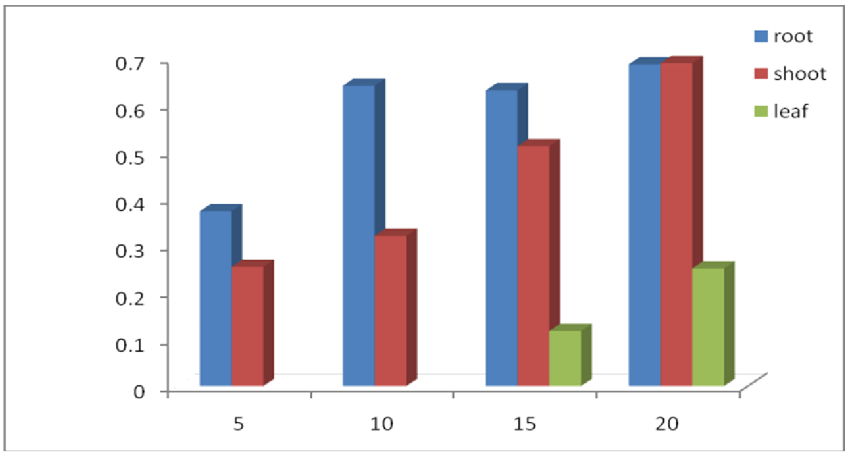


Figure 4.13 ELISA profiles of *L leucocephala* tissues at different age of seedlings (5, 10, 15, 20 days). X-axis represents age of seedlings in days; Y-axis represents protein concentration.

In case of root tissue amount of extractable CAD protein is highest on 20th day and is lowest on 5th day. There is increase in amount of protein from 5th to 20th day with a

slight decrease on 15th day. The extractable protein in shoot tissue increases gradually from 5th day to 20th day. For leaf tissue the protein level increases from 15th to 20th day. The amount of protein in root and shoot is same on 20th day. Although the amount of protein in leaf tissue increases from 15th to 20th day, it is low as compared to root and shoot tissue (Figure 4.13).

4.7.3 Immuno-cytolocalization of CAD in *L. leucocephala* tissues

CAD protein was immuno-cytolocalized in tissues undergoing active lignification *i.e* vascular bundle and xylem fibres. The deposition of blue-black to brownish precipitate after incubating with BCIP/NBT mix confirms the presence of CAD protein near the sites of lignification. Comparison of the sections where CAD was immunolocalized, was done with phloroglucinol-HCl stained sections of 5, 10, 15 and 20 days old root and stem samples at low and high magnification.

In root and stem, as the xylem tissue develops, the expression of CAD protein and therefore synthesis of lignin also increases from 5 day to 20 day stage, very well reflected by immunolocalization of CAD protein and lignin staining by phloroglucinol-HCl shown in Figure 4.14 to Figure 4.17. In transverse sections of root and stem (low magnification view) only tissue like xylem and phloem fibres shows presence of CAD protein and lignin, clearly absent in pith tissue and cortex. Xylem ring formation completes at about 15th day stage of development. Images under higher magnification clearly show presence of CAD protein and corresponding lignin deposition in cell wall of xylem tissue. Immunolocalization studies in strawberry and poplar have similarly shown localization of CAD in developing xylem tissues (Takbe *et al*., 2001; Portales *et al*., 2002).

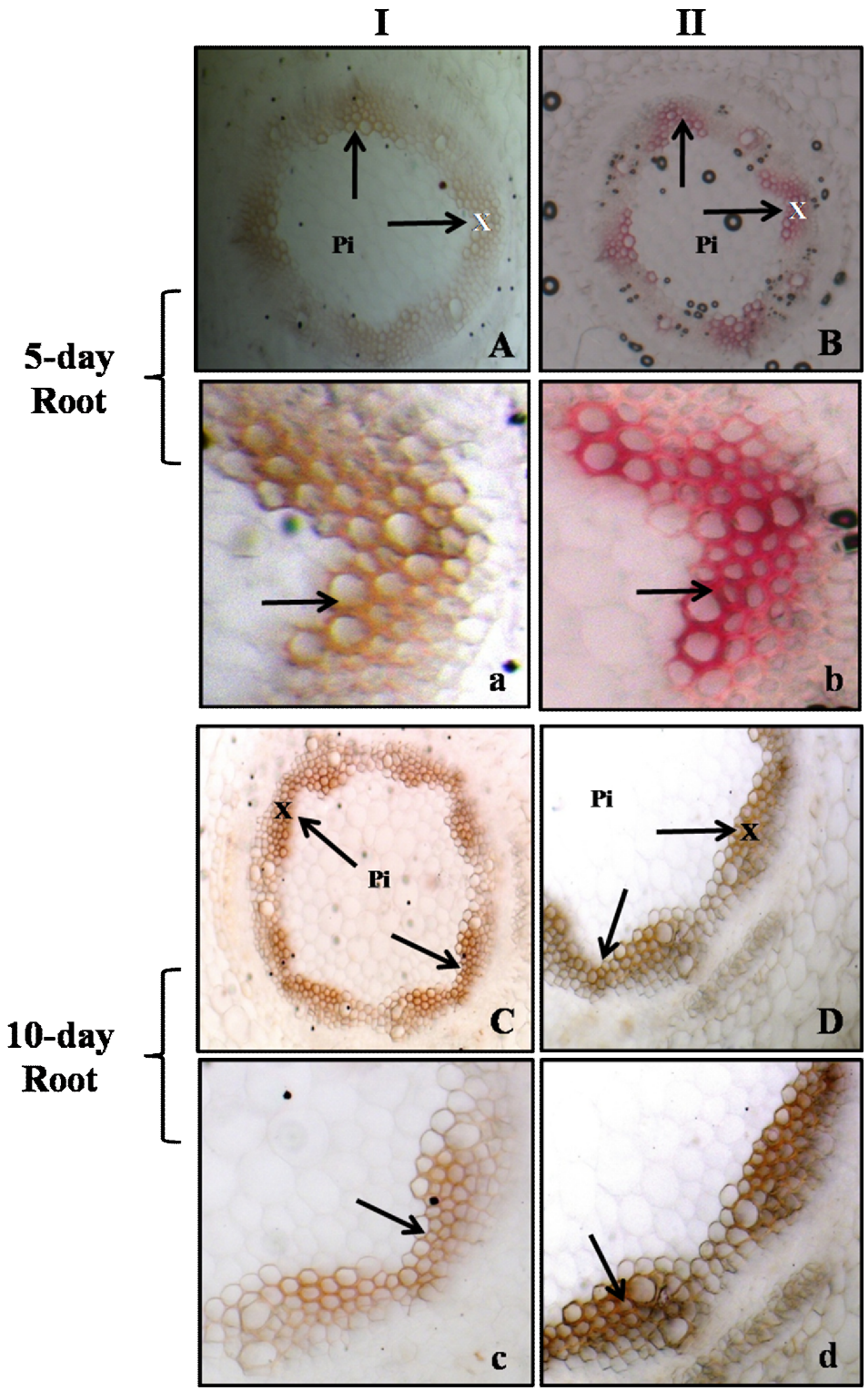


Figure 4.14 Immunolocalization of CAD protein (I) & phloroglucinol staining of lignin (II) in TS of root of *Leucaena*.. Low magnification- A, B, C, D; Higher magnification- a, b, c, d. X- xylem; Pi- pith; arrows indicate CAD protein (I) & lignin deposition (II).

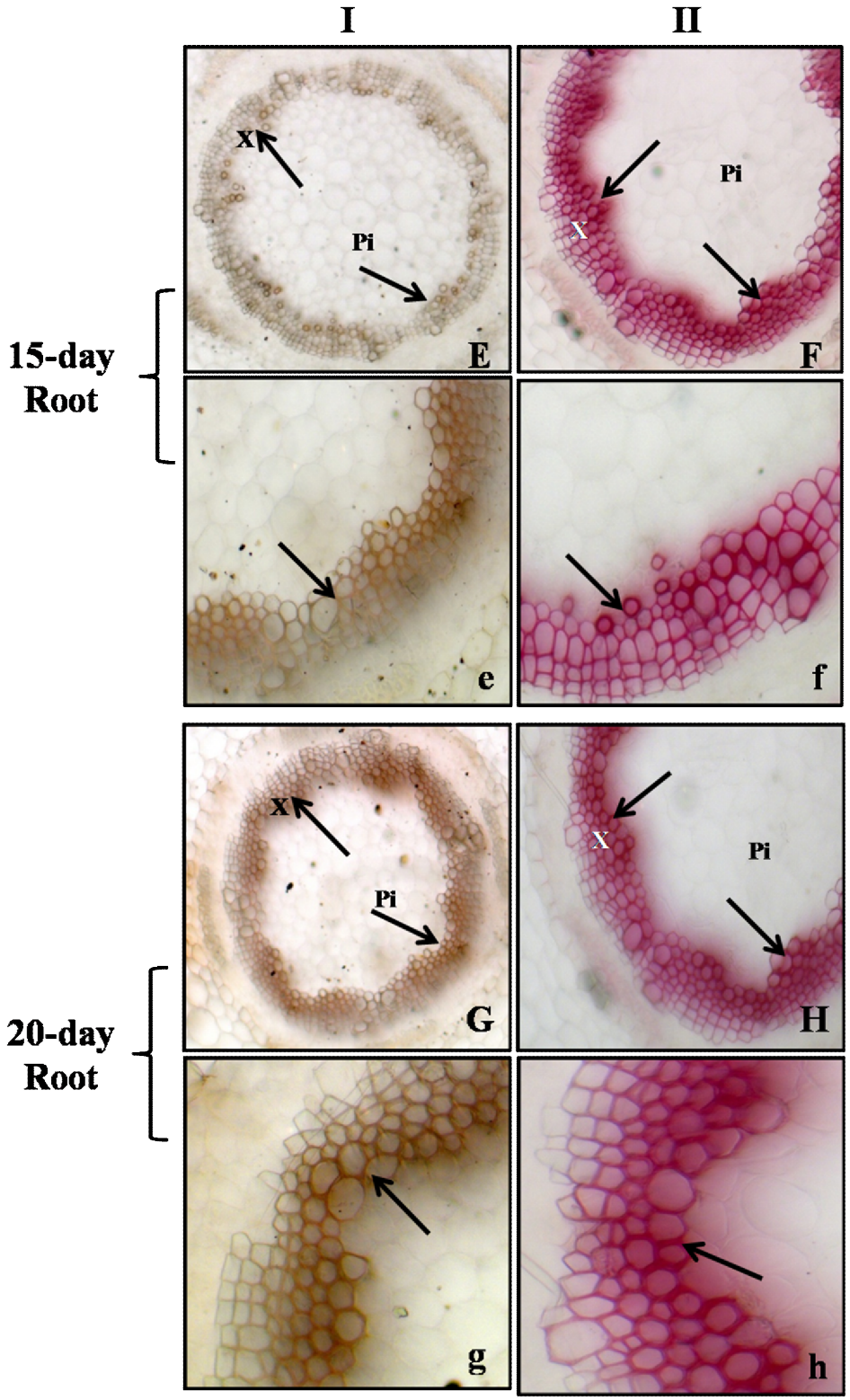


Figure 4.15 Immunolocalization of CAD protein (I) & phloroglucinol staining of lignin (II) in TS of root of *Leucaena*.. Low magnification- E, F, G, H; Higher magnification- e, f, g, h. X- xylem; Pi- pith; arrows indicate CAD protein (I) & lignin deposition (II).

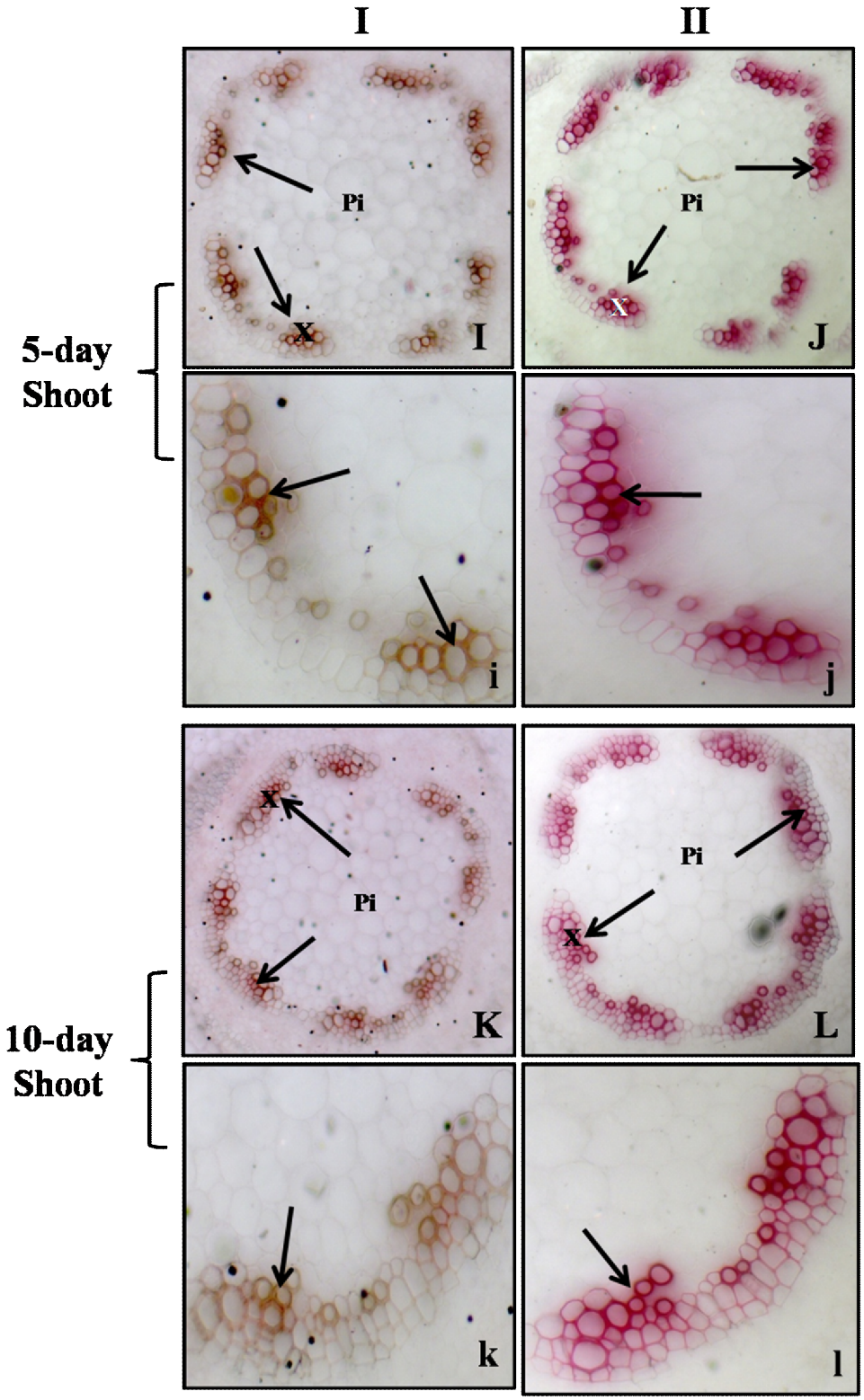


Figure 4.16 Immunolocalization of CAD protein (I) & phloroglucinol staining of lignin (II) in TS of root of *Leucaena*.. Low magnification- I, J, K, L; Higher magnification- i, j, k, l. X- xylem; Pi- pith; arrows indicate CAD protein (I) & lignin deposition (II).

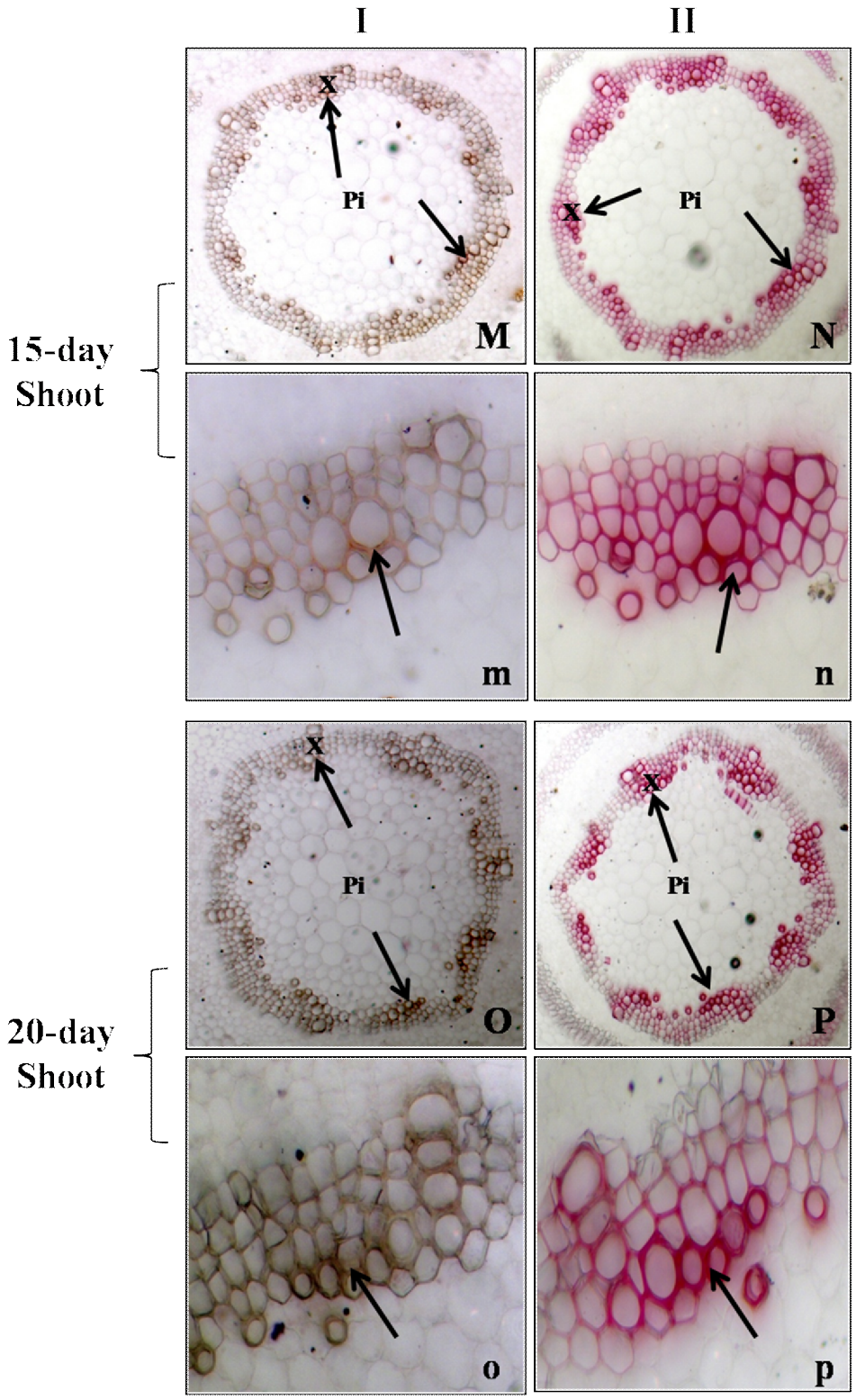


Figure 4.17 Immunolocalization of CAD protein (I) & phloroglucinol staining of lignin (II) in TS of root of *Leucaena*.. Low magnification- M, N, O, P; Higher magnification- m, n, o, p; X- xylem; Pi- pith; arrows indicate CAD protein (I) & lignin deposition (II).

4.8 Conclusions

- Root, shoot and leaf tissue of *L. leucocephala* seedlings were harvested at different time points in development i.e at age 5, 10, 15 and 20 days after germination. These tissue samples were used for real-time PCR, ELISA, CAD assay and immuno-cytolocalization studies.

- Real-time PCR analysis showed differential expression at transcript level with maximum expression in 5 day shoot, 10 day root and in 20 day leaf tissue.

- The results of CAD enzyme assay and ELISA suggested a positive correlation between the amount of CAD enzyme and its activity.

- In root tissue the amount of CAD protein increased from 5 day to 10 day, followed by a decrease on 15th day and a marginal increase on 20th day. Shoot tissue showed a gradual increase in CAD protein from 5th day to 10th day. Amount of CAD protein increased from 15th day to 20th day in case of leaf tissue. Same pattern was observed in case of CAD enzyme assay for root, shoot and leaf tissue.

- Transverse sections of immuno-cytolocalized and phloroglucinol-HCl stained root and stem at different time points of development show that the expression of CAD protein increases as the plant matures from 5th day to 20th day with corresponding increase in lignification. CAD protein expression and corresponding lignin deposition is confined to the cell walls of developing xylem tissue and phloem fibres and is absent in pith and cortex tissue.

- Correlation coefficient was used to analyse the relationship between CAD protein activity vis-à-vis its amount, for root, stem and leaf tissue. Correlation coefficients for root, stem and leaf tissue were 0.9457, 0.9838 and 1.0 respectively, showing positive correlation between the amount of CAD protein and CAD activity.

CHAPTER 5

TRANSFORMATION OF ANTISENSE CAD GENE IN *LEUCAENA LEUCOCEPHALA* & ANALYSIS OF TRANSFORMANTS

5.1 Introduction

5.1.1 Plant genetic transformation

Plant genetic transformation technology has a great potential in increasing productivity through enhancing resistance to diseases, pests and environmental stresses and by qualitative changes such as chemical composition of the plants. Plants can also be used for high volume production of pharmaceuticals, nutraceuticals and other beneficial chemicals. Transgenic plants might be used as drug delivery devices, with vaccines being synthesized in plants (Hansen & Wright, 1999).

Plants are genetically engineered by introducing gene(s) into plant cells that are growing *in vitro* or *in planta*. The development of transgenic plants is based on the stable insertion of foreign DNA into the plant genome, regeneration of these transformants to produce the whole plant and expression of the introduced gene(s). *Agrobacterium*-mediated transformation has provided a reliable means of producing transgenics in a wide variety of plant species that can be cultured and regenerated *in vitro.*

The genetic transformation protocols based on *Agrobacterium*-mediated and/or direct gene transfers by biolistic bombardment have been successfully applied for numerous woody angiosperm species (Merkle & Nairn, 2005), including *Populus* and *Betula.* The introduction of transgenes has included both sense and antisense strategies (Strauss *et al.,* 1995; Baucher *et al.,* 1998) and RNAi technology (Merkle & Nairn, 2005).

Genes conferring resistance to antibiotics like *neomycin phosphotransferase* II (nptII) (Baribault *et al.,* 1989), *hygromycin phosphotransferase (hpt)* (Le Gall *et al.,* 1994), *phosphinothricin acetyl transferase / bialaphos resistance (pat/bar*) (Perl *et al.,* 1996) are being used to select transgenic cells. The oncogenes of *Agrobacterium* are replaced by reporter / screenable marker genes like *β-glucuronidase* gene (*gus*) (Baribault *et al.,* 1990), GFP gene and *luciferase (luc)* gene for analyzing gene expression.

The development and optimization of several regeneration protocols, efficient vector constructs and availability of defined selectable marker genes and different methods of transformation have resulted in the production of transgenic plants in more than 100 plant species (Babu *et al.*, 2003; Wimmer, 2003).

5.1.2 Genetic manipulation of CAD in *Leucaena leucocephala*

The reduction of hydroxycinnamaldehydes to hydroxycinnamyl alcohols is catalyzed by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195). CAD has been considered to be an indicator of lignin biosynthesis because of its specific role at the end of the monolignol biosynthetic pathway (Walter *et al*., 1988). Previous works have shown that in transgenic plants down regulated for CAD activity, large reductions in CAD activity only slightly reduced lignin content because the plants were able to circumvent the block in CAD activity by shipping its substrates, the cinnamaldehydes, to the cell wall for polymerization. Also the altered lignin structure did not affect overall plant growth and development. Green house grown transgenic tobacco and poplar with down-regulated CAD activity have been subjected to chemical pulping analysis (Baucher *et al*., 1996; Jouanin *et al*., 2000; Lapierre *et al*., 1999; O'Connell *et al*., 2002). In both species the changes to lignin structure in CAD suppressed plants resulted in a greater ease of pulping by the chemical Kraft process, and the Kappa number, a measure of residual lignin in the pulp after cooking, was reduced compared to that of wild-type plants. Subsequent bleaching of the pulp was also easier and there were no detrimental changes to other pulp properties.

Genetic manipulation of CAD in *Leucaena leucocephala* could be a promising strategy for altering lignin content/composition to improve wood-pulp production efficiency.

5.2 Materials and methods

5.2.1 Explants

Nicotiana tabacum – Leaf discs from one month old axenic cultured plant leaves were used as the ex-plant for transformation experiments (Section 2.3).

Leucaena leucocephala- Embryo axes of *L. leucocephala* were used as explants for transformation and regeneration experiments (Section 2.1).

5.2.2 *Agrobacterium* strain and plasmids

Agrobacterium tumefaciens strain GV2260 was used. The strain carried plasmid pCAMBIA1301, a binary vector harboring CADc2 gene in antisense orientation under the control of a constitutive promoter CaMV35S and a plant selectable marker gene '*hygromycin phosphotransferase (hpt)*' responsible for hygromycin resistance in T-DNA region and kanamycin resistance gene as bacterial & transformant selectable marker gene.

5.2.3 Multiple shoots regeneration of *L. leucocephala*

Multiple shoot regeneration from embryo axis as explants was optimized in our lab. Briefly, *L. leucocephala* seeds were treated as described in section 2.1. Embryo axis was excised from the imbibed seeds after 24 hours of incubation after the process of imbibing. The embryo axes were then transferred to ½ MS medium + 0.5 mg/l TDZ for 15 days. The explants were sub-cultured on same medium until (~1 month) 4-5 multiple shoots appeared on each embryo axis. After that, embryo axes were transferred on ½ MS medium where it was sub cultured after 15 days regularly until shoots developed up to 4-5 cm of height. The elongated shoots were transferred to rooting media ½ MS + 0.2 mg/l NAA and sub-cultured regularly at the interval of 15 days. Rooted shoots were then transferred to soil in plastic cups containing sand + soil 1:1 proportion covered with plastic bag. After 1 month /sufficient growth of plants, they were transferred to green house.

5.2.4 Determination of LD_{50} for hygromycin

LD_{50} of hygromycin for *L. leucocephala* and tobacco were determined earlier in our lab. In hygromycin free treatment, freshly excised embryo axes from *Leucaena* seeds showed normal proliferation, growth and germination. There was a gradual increase in necrosis of the embryo axis with the increase in hygromycin concentration from 2 to 40 mg/l. LD_{50} for hygromycin was observed at a concentration 10 mg/l showing necrosis and death of the 50% of the inoculated embryos. Complete necrosis and mortality (100%) was observed at a minimum concentration of 15 mg/l. Explants showing callusing, germination and further proliferation became brownish and necrotic at later stages in most of the hygromycin treatments.

In case of tobacco, LD_{50} for hygromycin was observed at a concentration 3 mg/l showing necrosis and death of the 50% of the inoculated tobacco leaves. Complete necrosis and mortality (100%) was observed for tobacco at a minimum concentration of 5 mg/l.

5.2.5 *A. tumefaciens* transformation and selection

Competent cell preparation for *A. tumefaciens* was done according to the protocol mentioned in section 2.10.2.1. Protocol for *A. tumefaciens* transformation and selection is given section 2.10.2.2.

5.2.6 *Agrobacterium* mediated tobacco transformation

The protocol is described in detail in section 2.26

5.2.7 Genetic transformation of *L. leucocephala*

Transformation of *L. leucocephala* was done using embryo axis as ex-plants. The transformation was done by three methods: particle bombardment, particle bombardment followed by co-cultivation and Agro-infusion method. All the protocols are described in section 2.27. After transformation events the embryo axes were shifted to ½ MS media with hygromycin (10 mg/l) and TDZ (0.5 mg/l). After 15 days the embryo axis were shifted to ½ MS media with hygromycin (15 mg/l) and TDZ (0.5 mg/l). After 15 days the axes were transferred to ½ MS with regular sub-culturing after every 15 days. No significant growth was observed for 3-4 months. Therefore 2 ip was

added to the media at a concentration of 0.5 mg/ml. Still no growth was observed. Concentration of 2ip was increased to 1 mg/ml. Shoot induction was observed at 1 mg/ml of 2ip concentration. Shoots were allowed to grow on the same media till shoots were 4-5 cm long. The shoots were then transferred on rooting media (1/2 MS + 0.2 mg/l NAA). After rooting the shoots were transferred to plastic cups containing sand + soil in 1:1 proportion for hardening. The hardened plantlets were shifted to green house after 1 month or sufficient growth.

5.2.8 Construction of pCAMBIA 1301 binary vector with CADc2 antisense gene

CADc2 fragment from clone LlCADc2 was cloned in pGEM-T Easy vector by incorporating the restriction sites *Sac* I and *Kpn* I in the primers CAD AF and CAD AR respectively. High fidelity *Taq pol* (*Pfx* Invitrogen) was used to amplify CADc2 using CAMF (CAD AF with *Sac* I) and CAMR (CAD AR with *Kpn* I) primers using LlCADc2 plasmid template. Plasmid LlCADc2 was diluted 100 times and 1 µl was used as a template. PCR was performed as described in section 2.17. A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in *E. coli* XL1 MRF cells. Clones with CADc2 fragment with *Kpn* I and *Sac* I restriction sites were screened by inoculating few colonies in 5 ml LB (Ampicillin 100 µg/ml) tubes. Isolated individual plasmids were restriction digested with *Kpn* I and *Sac* I enzymes to confirm the integration of CADc2 insert.

PCR cycling condition:

No. of cycles	Temperature	Time
1	95 ºC	5 min
35	95 ºC 60 ºC 72 ºC	1 min 30 s 1min
1	72 ºC	5 min
1	4 ºC	hold

The CADc2 fragment with the *Kpn* I and *Sac* I restriction sites were directionally cloned in pCAMBIA 1301 vector (Figure 5.1). Colony PCR was done to screen the recombinant pCAMBIA 1301 clones. Integration of CADc2 fragment in pCAMBIA 1301 was confirmed by digestion with *Kpn* I and *Sac* I.

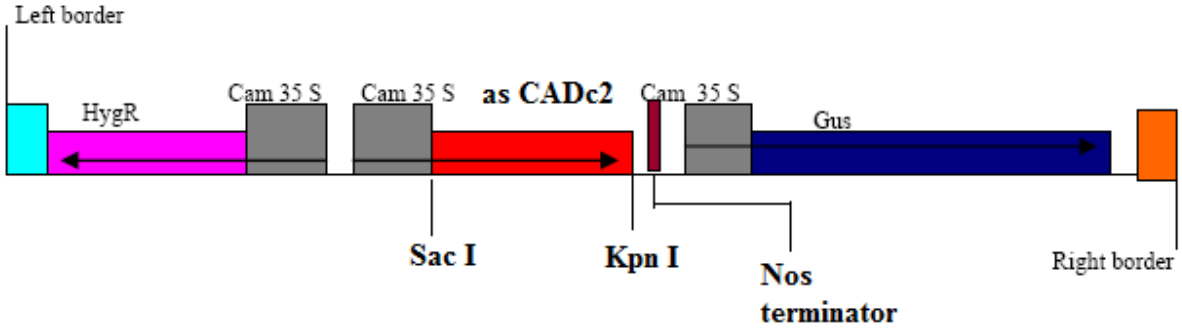


Figure 5.1 Antisense cassette for CADc2 gene (asCADc2) in pCAMBIA 1301

5.2.9 GUS histochemical assay

GUS histochemical assay (Section 2.28) was performed on bombarded embryo axes of *L. leucocephala*, leaves of putative transformants selected on hygromycin and *Agrobacterium* mediated transformed *Nicotiana* leaves.

5.2.10 DNA extraction from transgenic lines

DNA was isolated using a modified protocol of Lodhi *et al*., 1994. In short, one or two leaflets (*Leucaena*) and a piece of leaf (tobacco) were ground in 20–30 µl CTAB extraction buffer (Table 2.2) using glass pestle in a microfuge tube. 500 µl of extraction buffer was added and PVPP (polyvinyl polypyrolidone) was added to a final concentration of 0.2% and mixed gently. The tube was kept at 65 °C for 20 min with intermittent shaking. RNAse was added and kept for 10 more min at 65 °C. Tube was cooled and 400 µl of Chloroform: Isoamyl alcohol (24:1) was added and mixed gently. Tube was centrifuged at 6000 g at 4 °C for 10 min and supernatant was transferred to a fresh microfuge tube. Isopropanol (0.6 volume) was added and kept at room temperature for precipitation. Tube was again centrifuged at 6000 g at 4 °C for 10 min. to pellet DNA. DNA was washed with 70% cold ethanol; air dried and dissolved in 40 µl SMQ water.

5.2.11 PCR amplification

Putative transformants were confirmed by PCR. Hygromycin gene specific primer (Hyg A forward and reverse, Hyg B forward and reverse) and 35S promoter specific primers and genomic DNA from putative transformants was used as template for PCR reactions. The PCR reaction was performed as mentioned in section 2.17.

5.2.12 Dot blot analysis

Putative tobacco transformants confirmed by PCR were used for dot blot analysis. Four tobacco plants were taken for analysis along with non-transformed plant as control. Hygromycin gene fragment was used as probe for hybridization at 60 °C. The protocol followed for dot blot analysis is given in section 2.20.2.

5.2.13 CAD enzyme assay

Tobacco transformants which were showing positive signals in dot blot analysis were used for estimating the CAD enzyme activity which was compared to enzyme activity of non-transformed plants.

5.2.14 Lignin estimation

Tobacco transformants showing positive signals in dot blot analysis were chosen for lignin estimation studies. Acid soluble and acid insoluble lignin was determined to give the total lignin content. The protocol for lignin estimation is given in section 2.29.

5.3 Results and discussion

Multiple shoot regeneration protocol and LD_{50} of hygromycin for *L. leucocephala* and tobacco were standardized in our lab. LD_{50} for hygromycin was determined to be 10 mg/l for *Leucaena* and 3mg/l for tobacco.

5.3.1 Construction of pCAMBIA 1301 vector with antisense CADc2 gene

5.3.1.1 Incorporation of restriction sites and PCR

The CADc2 gene specific primers CAD AF and CAD AR were modified to incorporate the *Sac* I site at the 5' end and *Kpnl* I site at 3' end of CADc2.

CAMF- 5' GGT ACC ATG GGA AGC A TT GAA GGA GAA AGA AC 3'
CAMR- 5' GAG CTC TCA CTG ATG ATC ATC AAG TTT GCT GCC 3'
A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in *E.coli* XL1 Blue cells. Clones with CADc2 fragment with *Kpn* I and *Sac* I restriction sites were screened by inoculating few colonies in 5 ml LB (Ampicillin 100 µg/ml) tubes. Isolated individual plasmids were restriction digested with *Kpn* I and *Sac* I enzymes to confirm the integration of CADc2 insert.

5.3.1.2 Directional cloning of CADc2 fragment in pCAMBIA1301

The above clone in pGEM-T Easy vector was designated as pCAMCADc2. This clone was restriction digested with *Kpn* I and *Sac* I restriction enzymes and 1 kb fragment was purified. Prior to this, pCAMBIA 1301 was modified in our lab by cloning a cassette of CAM35S promoter and NOS terminator within *Eco* RI and *Hind* III site of pCAMBIA1301. This cassette also contained a *Kpn* I (near CAM 35S) and *Sac* I (near NOS terminator) restriction sites. The purified *asCADc2* fragment was directionally cloned in reverse orientation in *Kpn* I and *Sac* I site of the cassette (Figure 5.3). Ligation mixture was transformed in *E. coli* XL1 competent cells and plated on LB-KAN (Kanamycin 50 µg/ml) plate. Colonies for recombinant plasmids were screened by colony PCR method. The PCR positive clones were confirmed by digestion with *Kpn* I and *Sac* I. The recombinant pCAMBIA vector was sequenced to further validate

and was designated as pCAMasCADc2. The plasmid pCAMasCAD2 was isolated in bulk and was used in two ways:

1. Coating of gold particle for particle bombardment and

2. Mobilizing in *Agrobacterium* for co-cultivation.

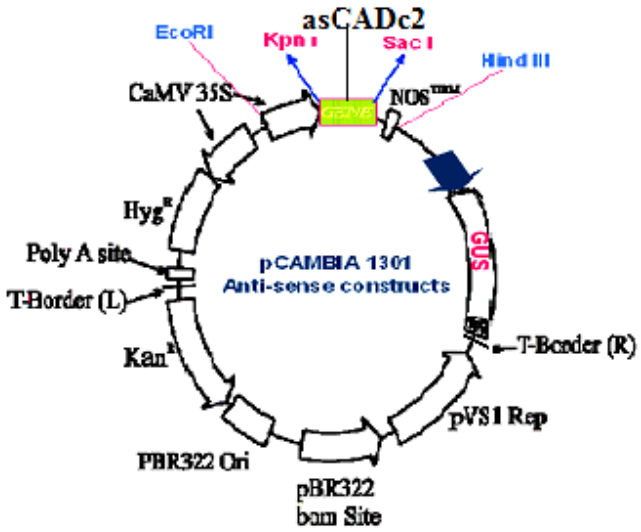


Figure 5.3 CADc2 gene in antisense orientation in pCAMBIA1301

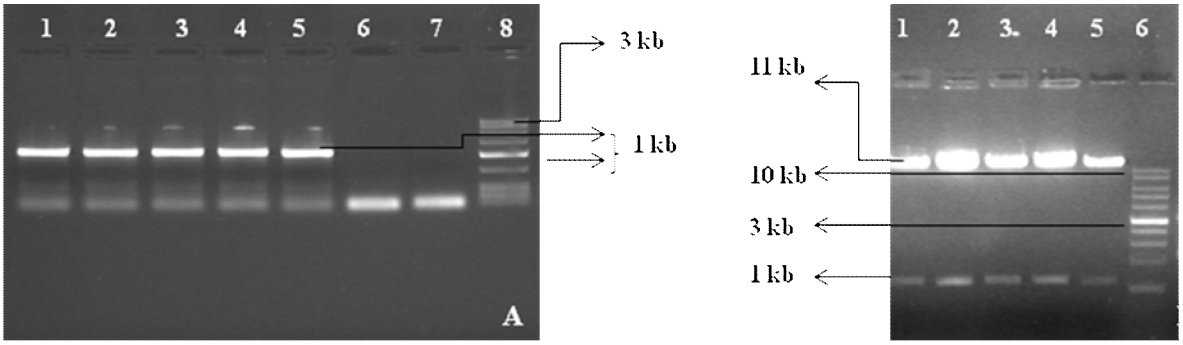


Figure 5.3 A- Lane 1-7 PCR of colonies screened. Lane 1-5 positive clones. Lane 8- DNA molecular weight marker. **B** Lane 1-6 Digestions of PCR positive clones with *Kpn* I and *Sac* I

5.3.2 Mobilizing pCAMasCADc2 in *Agrobacterium tumefaciens* GV2260

Plasmid of pCAMasCADc2 was mobilized in competent *Agrobacterium tumefaceins* cells according to the protocol discussed in section 2.10.2.

5.3.3 Transformation of tobacco with pCAMasCADc2

Tobacco was used as a standard system for transformation. The protocol for tobacco transformation is given in section 2.26. Putative transformants were selected on hygromycin (5 mg/l) supplemented ½ MS medium. Transient GUS assay was done on putative tranformants. Blue colouration was observed in tobacco leaves after GUS assay (Figure 5.4-A). Plants surviving on hygromycin media were then shifted to hygromycin free media (Figure 5.4 C) to allow further growth. Putative transformed plants were shifted to green house (Figure 5.4 E & F) after hardening in plastic cups (Figure 5.4 D).

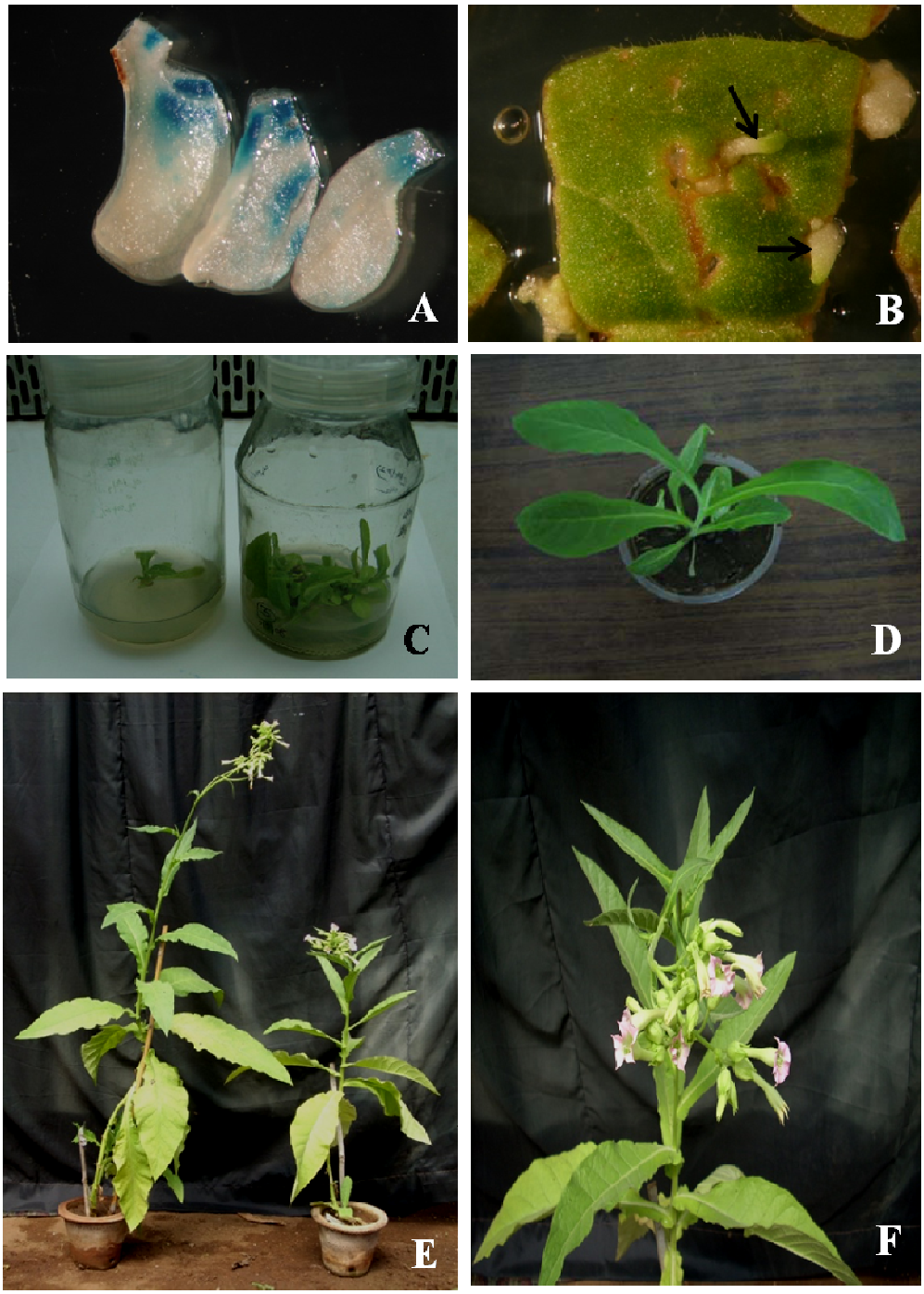


Figure 5.4 Tobacco transformation with pCAMasCADc2
A- transient GUS assay of leaf after 48 h of transformation; B- multiple shoot formation from putative transformed leaf disc; C- plantlets growing on hygromycin-free media; D- plant kept for hardening; E1- Non-transformed, E2- Transformed; F- mature plants in green house

5.3.4 Analysis of putative tobacco transformants

The putative transformed plants were confirmed by PCR and dot blot analysis. The confirmed plants were further analysed for CAD enzyme activity and lignin estimation as compared to non-transformed plants.

5.3.4.1 PCR analysis of putative transformants

Genomic DNA was isolated from putatively transformed plants according to protocol given in section 5.2.10. PCR was performed using diluted genomic DNA as template and hygromycin gene specific primers. PCR analysis was done with seven plants including one non-transformed.

Hygromycin primer set B:

HygBF- 5' GTC GAC CTA TTT CTT TGC CCT CGG AC 3'

HygBR- 5' GGA TCC CCT GAC CTA TTG CAT CTC CC 3'

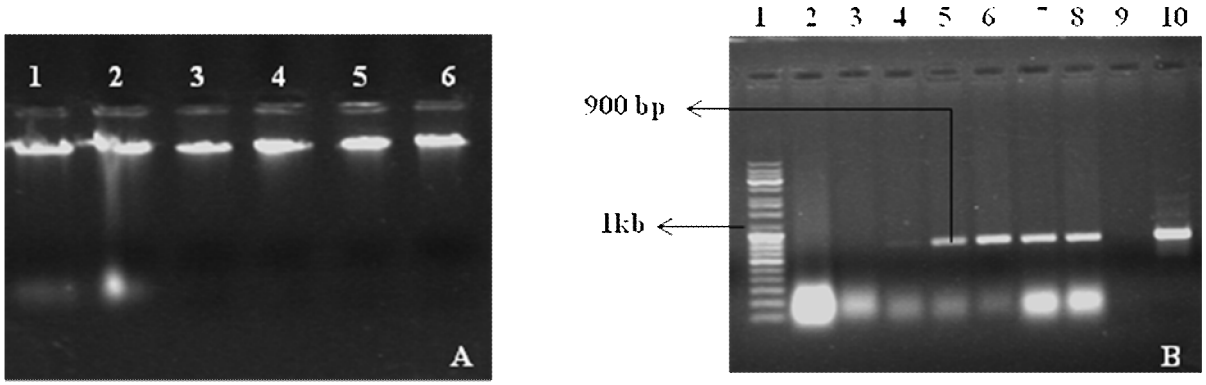


Figure 5.5 A-Lane 1-6 Genomic DNA of putatively transformed tobacco plants. **B**-Lane 1- DNA molecular weight marker. Lane 2- non-transformed. Lane 3-8 putatively transformed plants. Lane 9- NTC. Lane 10- positive control.

5.3.4.2 Dot blot analysis of putative transformants

Plants showing positive results in PCR were taken for dot blot analysis. The plants were designated as 2T, 3T, 6T and 9T. Dot blot analysis was done according to the protocol given in section 2.20.2. Hygromycin gene fragment was used as the probe.

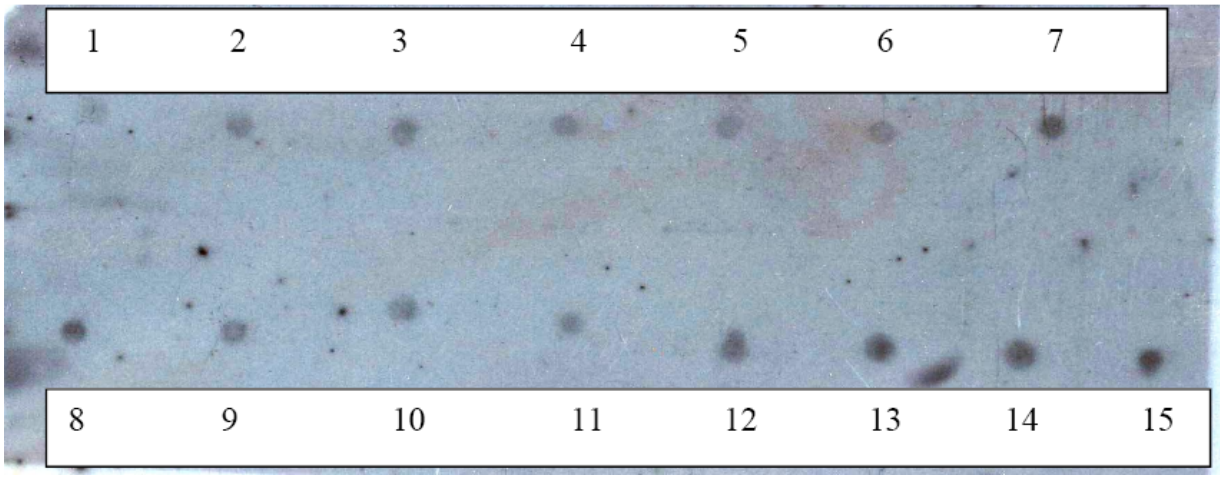


Figure 5.6 X-ray film showing results of dot blot analysis. 1, 2, 3- Non-transformed tobacco. 4, 5, 6- 2T. 7, 8, 9- 3T. 10, 11, 12- 6T. 13, 14,15- 9T.

Genomic DNA was applied in triplicates on the membrane for hybridization. Plants 3T and 9T show strong positive signals as compared to 2T and 6T. Non transformed plants show very weak signals. Plants 3T and 9T were used for further analysis.

5.3.4.3 CAD enzyme assay of transformants

CAD activity assay was done with 3T, 9T and non-transformed plants using cinnamaldehyde as substrate. CAD specific activity was found to be reduced in 3T by ~ 13% while slight increase was observed in 9T (Table 5.1).

Plant	Specific Activity nkat/mg
Non-transformed	0.04655
3T	0.04035
9T	0.04682

Table 5.1 Specific activity of CAD for transformed tobacco plants

5.3.4.4 Lignin estimation in transformants

Lignin estimation was done for 3T and 9T along with non-transformed plant.

Plant	Acid soluble lignin	Acid insoluble lignin (%)	Total lignin
Non-transformed	4.990	26	30.99
3T	2.631	28	30.631
9T	3.838	32	30.87

Table 5.2 Lignin estimation of tobacco transformed plants

It is observed that acid soluble lignin has reduced in both the transformed plants even as acid insoluble lignin has increased. It was observed in various plants downregulated for CAD that lignin content has only slighltly decreased or has remained unchanged. In poplar activity was reduced to 30% with slight decrease in lignin content and increase in S units (Lapierre *et al*., 1999). In antisense tobacco plants CAD activity reductions have been obtained upto 7-56%, lignin content has remained unchanged and decrease in S uits have been observed (Halpin *et al*., 1994, Vailhe *et al*., 1998, Yahiaoui *et al*., 1998). The results obtained in this experiment with tobacco, total lignin shows decrease in both 3T and 9T (Table 5.2). Thus a reduction in total lignin content was observed in 3T (~2%) and 9T (~1%) transformed plants. From the result of lignin estimation it seems that there is increase in G lignin in transformed plants and S lignin is reduced. Therefore S/G ratio is changed in transformed plants as compared to non-transformed (Matsushita *et al*., 2004). These results need to be further validated by doing more analysis of lignin composition.

5.3.5 Transformation of *L. leucocephala* with pCAMasCADc2

Embryo axes of *Leucaena* were used as explant for transformation experiment. Transient GUS assay was done after 48 hours of transformation experiment. The embryo axes showed positive GUS reaction (Figure 5.7 A). After selection on Hygromycin (15 mg/ml) supplemented media (Figure 5.7 B & C), plants were transferred to Hygromycin free media. Out of 354 plants which survived on hygromycin supplemented media, 69 plants showed elongation (Figure 5.7 D). The plantlets were then shifted to rooting media (Figure 5.7 E) (Table 5.3). Survived plants had very weak stem and stunted growth (Figure 5.7 F). Leaves were normal in size

when compared to non-transformed plant. Putative transformed plants when attained stem height of approximately 5-6 cm were transferred to greenhouse (Figure 5.8). Growth of putative transformed plants was very slow and stunted when compared to non-transformed plant.

Gene	**Transformation method**	**No. of embryo axes used**	**No. of explants selected on hygromycin (15 mg/ml)**	**No. of shoots elongated**	**No. of shoots tested for PCR**	**No. of PCR positive samples**
asCAD	Particle bombardment	438	208	42	11	7
asCAD	Particle bombardment & Co-cultivation	173	114	11	2	2
asCAD	Agro-infusion	147	32	16	6	6
Total		758	354	69	19	15

Table 5.3 Details of *Leucaena* embryo axes used for transformation studies and PCR positive

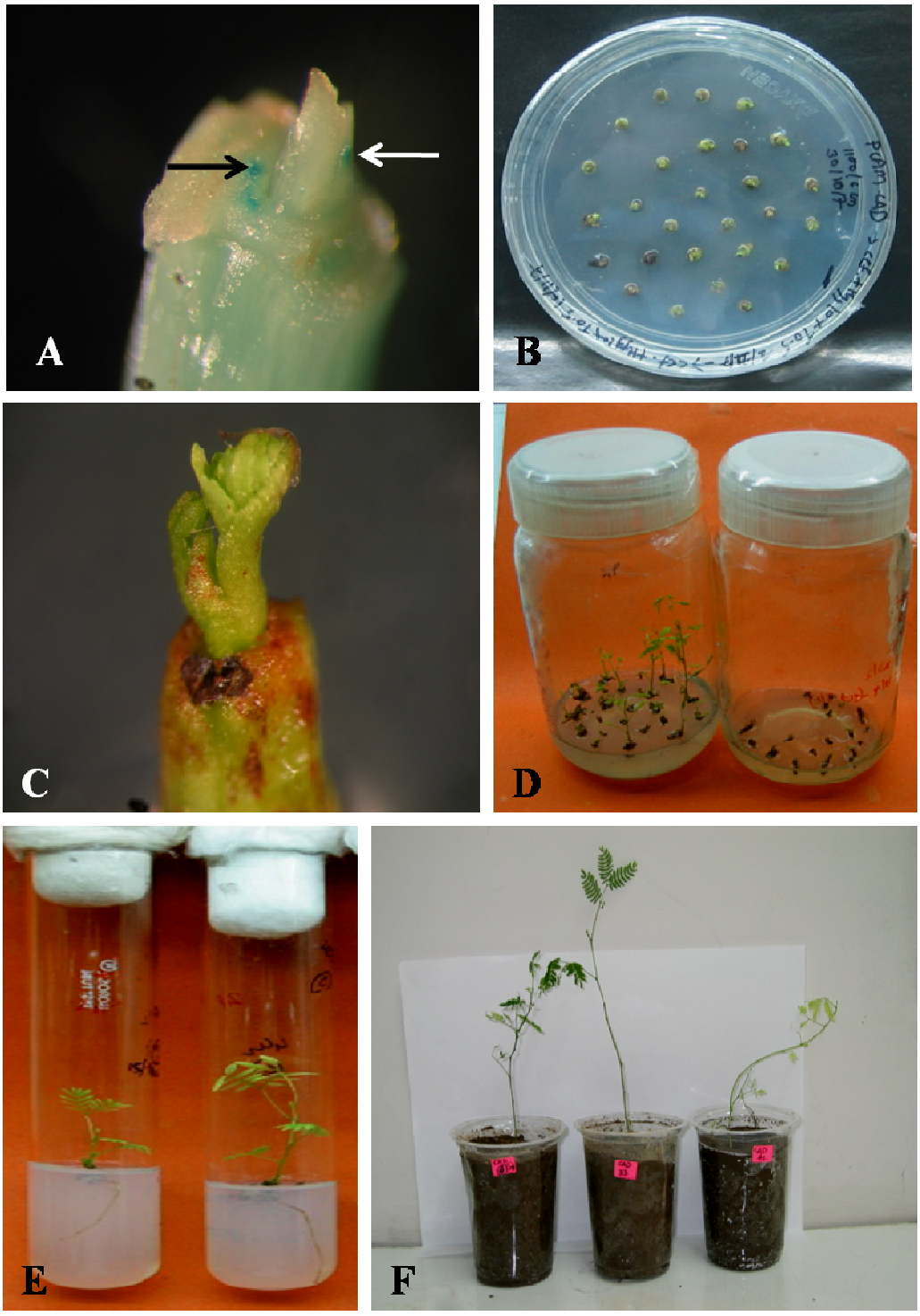


Figure 5.7 Transformation of *L. leucocephala* with pCAMasCADc2
A- Transient GUS assay of embryo axis after 48 h of transformation; B, C- putative transformants growing on medium with hygromycin; D- shoot induction with 2iP; E- root induction with NAA; F- hardening of plantlets

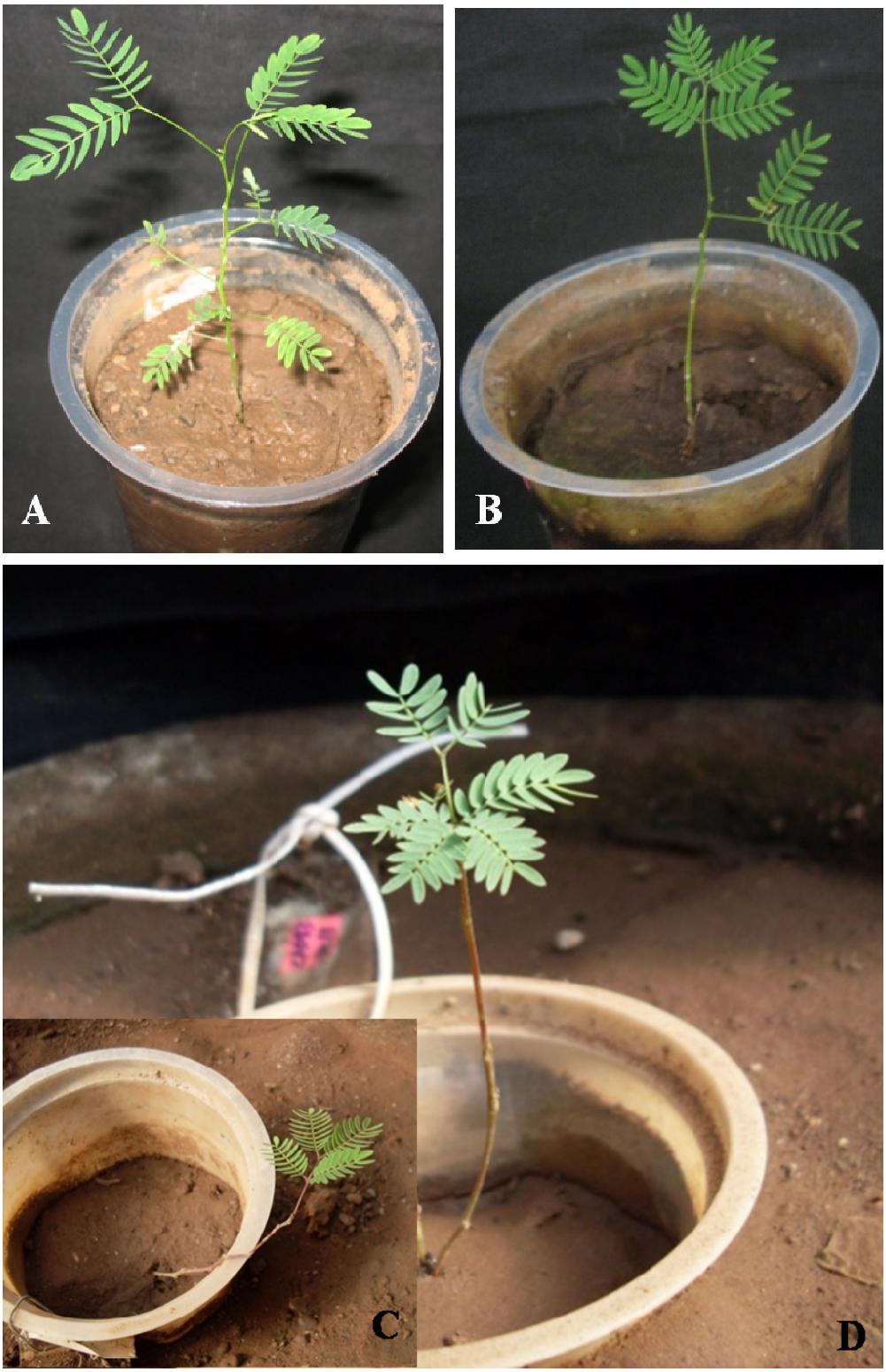


Figure 5.8 Hardening of transformed *L. leucocephala* plants in green house

5.3.6 Analysis of putative *L. leucocephala* transformants

PCR analysis of putative *Leucaena* transformant was done with hygromycin gene specific primers. About 15 putative *Leucanea* transformants show positive PCR results.

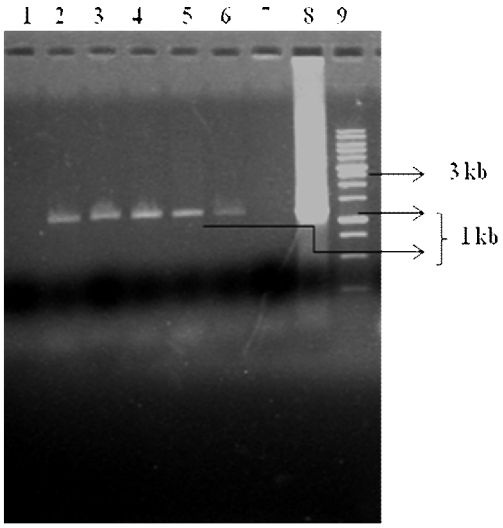


Figure 5.9 PCR of putative *Leucaena* transformants. Lane 1- non-transformed. Lane 2-6 putative transformants. Lane 7- NTC. Lane 8- positive control. Lane-9 DNA molecular weight marker

The growth of *Leucanea* plants was very slow and stunted. There was not enough tissue available for further analysis of *Leucanea* plants due to slow growth. The very stunted growth indicated a possible alteration in lignin content and/or composition which needs to be investigated further once the plants grow sufficiently.

5.4 Conclusions

- Approximately 1.0 kb fragment of CADc2 was cloned in antisense orientation in pCAMBIA1301.
- *Agrobacterium tumefaciens* strain GV2260 was transformed with the antisense CADc2 in pCAMBIA1301.
- *Agrobacterium tumefaciens* strain GV2260 was used to transform tobacco. Transient GUS assay was done to analyze transgenic events.
- Tobacco transgenic events were confirmed by PCR using hygromycin gene specific primer. Integration of gene was further confirmed by dot blot using hygromycin gene fragment as a probe.
- CAD enzyme assay and lignin estimation was done with transformed tobacco plants. CAD activity was found to be reduced by ≈ 13% in one of the two analyzed plants, while the other recorded a slight increase in activity.
- Total lignin was reduced slightly for both the analyzed plants when compared to the non-transformed plant.
- Embryo axes of *Leucaena* were transformed by three different methods viz. particle bombardment, particle bombardment followed by co-cultivation and agro-infusion method. Bombarded embryo axes were analyzed for transient GUS expression.
- Survived putative transformed plants of *Leucaena* were further grown on elongation media followed by root induction media.
- Putative transformed embryo axes of *Leucaena* surviving on the selection medium developed into plantlets which were kept for hardening and then transferred to green house for further growth.
- *Leucaena* transgenic events were further confirmed by PCR using hygromycin gene specific primer.
- Due to very slow and stunted growth of putatively transformed *Leucaena* plants further analysis could not be carried out.

SUMMARY

Summary

Isolation and characterisation of Cinnamyl Alcohol Dehydrogenase gene from *Leucaena leucocephala*

Paper is the integral part of our life. Significance of paper is evident in the numerous ways we use it. Raw material for the pulp and paper industry viz. wood, agricultural residues etc. consists largely of lignocelluloses. Pulping involves delignification of wood and further, bleaching of pulp is necessary for the brightness of paper. These processes consume large quantities of energy and hazardous chemicals. Thus it is the need of the hour to provide designer plant species with reduced/altered lignin characteristics without compromising the mechanical strength of plant.

Lignin, an integral cell wall component of plants, is a phenolic heteropolymer of monolignols namely coumaryl, coniferyl and sinapyl alcohols. It confers rigidity strength, resistance to pathogen attack and water impermeability to cells.

Leucaena leucocephala is one of the important sources of raw material for paper industry. No study has been done on lignin biosynthesis genes so far in *Leucaena* sp.. Study of these genes will help in understanding the lignin biosynthetic pathway in *Leucaena* sp. and its manipulation to meet the needs of pulp and paper industry.

Cinnamyl Alcohol Dehydrogenase (CAD) catalyses the last step in lignin biosynthetic pathway. It converts hydroxycinnamyl aldehydes to respective alcohols. Previous works have shown that down regulation of CAD gene have produced plants with altered lignin composition with higher extractability during pulping.

The thesis deals with

1. **Isolation, cloning and characterization of *CAD* gene.**

PCR based approach was used to fish out the partial 863 bp *CAD* gene (LlCADc1). Bioinformatic analysis of the partial cDNA clone LlCADc1 showed highest homology with *Acacia CAD* sequence. Primers designed from *Acacia* nucleotide *CAD* sequence were used to amplify the full length *CAD* gene (LlCADc2). Primers were designed from LlCADc2 to obtain the 5' and 3' UTRs by RACE reaction. Three different 3'

clones and two different 5' clones were obtained from the RACE reaction which may play a regulatory role for *CAD* gene expression. Full length cDNA and genomic clones were obtained from the primers designed from UTRs. The full length CAD cDNA clone (LlCADc3) was 1074 bp. The sequence of LlCADc3 showed significant homology with other *CAD* nucleotide and amino acid sequences available in database. *In*-silico restriction analysis and codon usage analysis was done for LlCADc3. The deudced amino acid sequence showed the conserved domains for NADPH binding and Zn binding. Phylogenetic analysis of LlCADc3 deduced amino acid sequence show that it is evolutionarily most related to fabaceae members followed by other dicots, followed by monocots and then gymnosperms. The genomic clone LlCADg1 is of 1478 bp with four introns and five exons. *CAD* in *L. leucocephala* is a gene family of possibly 2 members.

2. Heterologus expression of *Leucaena leucocephala* CAD gene, its purification and characterisation

Ll CADc2 was directionally cloned in pET 30b(+) vector. Over-expression in *E. coli*. BL21 (DE3) was standardized and the recombinant CAD protein was isolated from inclusion bodies. The protein was further purified with Nickel NTA column and characterised. The 40 kD purified protein, from inclusion bodies, was used to raise antibodies in New Zealand rabbit. CAD enzyme activity for substrates like cinnamaldehyde, coniferaldehyde and sinapaldehyde, was also standardized from purified CAD protein extracted in soluble form. Recombinant CAD enzyme showed maximum specific activity with cinnamaldehyde. The functional expression of recombinant CAD protein confirms that the cDNA isolated from *L. leucocephala* encodes for cinnamyl alcohol dehydrogenase protein.

3. Spatio-Temporal expression and Immuno-cytolocalisation of CAD gene

Root, shoot and leaf tissue of *L. leucocephala* seedlings were harvested at different time points in development i.e at age 5, 10, 15 and 20 days after germination. These tissue samples were used for Real-time PCR, ELISA, CAD assay and immuno-cytolocalization studies. Real-time PCR analysis showed differential expression at transcript level with maximum expression in 5 day shoot, 10 day root and in 20 day leaf

tissue. The results of CAD enzyme assay and ELISA suggested a positive correlation between the amount of CAD enzyme and its activity. In root tissue the amount of CAD protein increased from 5 day to 10 day, followed by a decrease on 15th day and a marginal increase on 20th day. Shoot tissue showed a gradual increase in CAD protein from 5th day to 10th day. Amount of CAD protein increased from 15th day to 20th day in case of leaf tissue. Same pattern was observed in case of CAD enzyme assay for root, shoot and leaf tissue.

Anti CAD antibodies were used to immunolocalise CAD protein in the transverse sections of root and shoot of developing seedlings (5-20 day). The same tissue sections were used for phloroglucinol staining which selectively stains lignin. The immunolocalised and phloroglucinol stained tissue sections were photographed, which showed that CAD protein was present in tissues undergoing active lignification.

4. Transformation of antisense CAD gene in *Leucaena leucocephala* and analysis of transfromants.

LlCADc2 was cloned in antisense orientation in pCAMBIA 1301 and was transformed in *L. leucocephala* as well as Tobacco using *Agrobacterium* (Agro-infusion method), particle bombardment and particle bombardment followed by co-cultivation methods. Putative transformants were selected on hygromycin media and analysed for integration. Transient GUS assay was done to analyse the transgenic events. Survived putative transformed plants were further grown on elongation media followed by root induction media. The plantlets were transferred to green house after hardening.

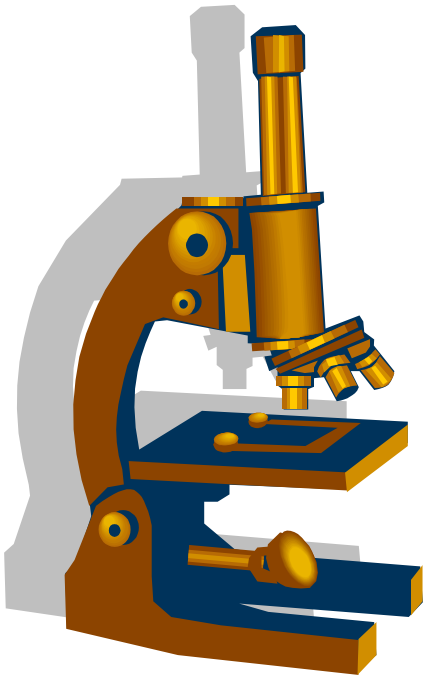
Putative tobacco transformants were confirmed by PCR using hygromycin gene specific primer and 35S promoter specific primer. Integration of gene was further confirmed by slot blot using hygromycin gene fragment as probe. Tobacco transformants showing positive signals in slot blot were analysed for lignin content. Total lignin content had remained unchanged or slightly increased as compared to non-transformed but the acid soluble lignin showed decrease.

Putative *L. leucocephala* transformants were confirmed by PCR using hygromycin gene specific primer and 35S promoter specific primers. Transformed *Leucaena* plants were too stunted and slow growing to provide enough tissue for further analysis.

Conclusion

This work, in future may contribute transgenic *L. leucocephala* plants with altered lignin composition/content having higher lignin extractability, making the paper & pulp industry more economic and eco-friendly.

APPENDIX



APPENDIX

Table 2.1 Buffers and Solutions for DNA Electrophoresis

Name	Ingredients	Preparation and Storage
50x TAE	2 M Tris 0.05 M EDTA	pH was Adjusted to 8.0 with glacial acetic acid and stored at RT
TBE buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA	RT
DNA loading buffer	0.25 g Xylencyanol 0.25 g Bromophenol blue 0.25 g Ficoll 400 1.46 g EDTA make up the volume to 100ml with H_2O	The solutions were filter sterilized using 0.22µ filter and stored at RT

Table 2.2 Buffers and Solutions for g-DNA isolation, Southern and Slot Blot

Name	Ingredients	Preparation and Storage
Extraction buffer	100 mM Tris-HCl (pH 8.0) 20 mM Na EDTA (pH 8.0) 1.4 M NaCl 2.0% (w/v) CTAB Add βmercaptoethanol to 0.2 % before use.	RT
TE buffer	10 mM Tris-HCl, pH 8.0 1mM EDTA, pH 8.0	RT
Depurination buffer	0.25 N HCL	Freshly prepared
Denaturation buffer	1.5 M NaCl 1M NaOH	RT
Neutralization buffer	1.5 M NaCl 1.0 M Tris-HCl (pH 7.5)	RT
20X SSC	3 M NaCl 0.3 M Sodium citrate (pH 7.0)	RT
Hybridization buffer	1% BSA 1.0 mM EDTA, pH 8.0 0.5 M Sodium phosphate, pH 7.2 7% SDS	RT
Low stringency wash buffer	6 X SSC 0.1% SDS	RT
Moderate stringency wash buffer	2 X SSC 0.1% SDS	RT

High stringency wash buffer	0.2 X SSC 1% SDS	RT

Table 2.3 Solutions for the Transformation and Selection of *E. coli*

Name	Ingredients	Preparation and Storage
TB buffer	10 mM PIPES 15 mM $CaCl_2$ 250 mM KCl	pH was adjusted 6.8 with KOH . $MnCl_2$ was added to final concentration of 55 mM and filter sterilized
IPTG solution	200 mg/ml in SMQ	Sterile filtration and Storage at -20 °C
X-Gal	20 mg/ml, 5-bromo-4-chloro-3-indolyl-D-galactoside in N,N'-Dimethyl formamide (DMF)	Wrap in aluminum Foil and store at -20 °C
Ampicillin	100 mg/ml in SMQ	Sterile filtration and Storage at -20 °C
Tetracycline	12.5 mg/ml in 70% ethanol	Sterile filtration and Storage at -20 °C
Kanamycin	50 mg/ml in SMQ	Sterile filtration and Storage at -20 °C
Rifampicin	50 mg/ml in DMSO	Sterile filtration and Storage at -20 °C
Hygromycin	25 mg/ml in SMQ	Sterile filtration and Storage at -20 °C

Table 2.4 Buffers and Solutions for Plasmid isolation (Alkaline lysis)

Name	Ingredients	Preparation and Storage
Solution I (TEG)	25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0) 50 mM Glucose	Stored at 4 °C
Solution II	0.2 N NaOH, 1% SDS	Freshly Prepared
Solution III	3 M Potassium acetate (pH 4.8)	Stored at 4 °C
RNase A	10 mg/ml	Stored at -20 °C
Other solutions	Chloroform Absolute ethanol 3.0 M Sodium acetate 70% ethanol Deionized water	

Table 2.5 Buffers and Solutions for Gel Electrophoresis (SDS-PAGE)

Name	Ingredients	Preparation and Storage
Monomer solution	29.2% acrylamide 0.8% bisacrylamide in water	Stored at 4 °C in the dark
Stacking gel	Distilled water-3.4 ml 1 M Tris-HCl (pH 6.8)-0.63 ml Acrylamide/bis 30%-0.83 ml 10% (w/v) SDS-0.05 ml 10% (w/v) APS-0.05 ml TEMED-5 µl	
Separating gel (12%)	Distilled Water 3.3-ml 1.5 M Tris-HCl (pH 8.8)-2.5 ml Acrylamide/Bis 30%-4.0 ml 10% (w/v) SDS-0.1 ml 10% (w/v) APS-0.1 ml TEMED-4 µl	
2x Protein loading buffer	Distilled Water-2.7 ml 0.5 M Tris-HCl (pH 6.8)-1.0 ml Glycerin-2.0 ml 10% (w/v) SDS-3.3 ml β-Mercaptoethanol-0.5ml 0.5% (w/v) Bromophenol blue-0.5 ml	Store at 4 °C
10x SDS-electrode buffer	Tris base-15 g Glycine-72 g SDS-5 g Water up to 500 ml	Store at 4 °C, dilute 1:10 before use
Staining solution	Coomassie-blue R 250-25 ml Methanol-100 ml Acetic acid-20 ml Water up to 200 ml	
Coomassie blue	Coomassie-blue R 250-0.5g Water up to 50 ml	Dissolve the dye in SMQ and filter
Destaining solution	Methanol-30 ml Acetic acid-20 ml Distilled water up to 200 ml	

Table 2.6 Buffers and Solutions for Protein Extraction (Denaturing conditions)

Name	Ingredient	Preparation and storage
Lysis buffer	50 mM Tris-HCl (pH 8) 5 mM EDTA 100 mM NaCl 0.5% Triton-X100 0.7 mM DTT 0.1 mM PMSF (Freshly added). 10 mM $MgSO_4$ Lysozyme 100 µg/ml (Added freshly).	Stored at 4 °C
Sonication buffer	100 mMTris-HCl (pH8.0) 50 mM Glycine	Stored at 4 °C
Dispersion buffer:	100 mM Tris-HCl (pH8.0) 50 mM Glycine 8 M Urea	Stored at 4 °C

Table 2.7 Buffers and Solutions for Protein Purification (Denaturing conditions)

Name	Ingredients	Preparation and Storage
Binding buffer	35 mM Tris 150 mM NaCl 10 mM imidazole 5% glycerol (pH 7.2)	Adjust pH by adding concentrated HCl and Store at 4 °C
Wash buffer	50 mM Tris 300 mM NaCl 25 mM imidazole 10% glycerol (pH 6.8)	Adjust pH by adding concentrated HCl and Store at 4°C
Elution buffer	50 mM Tris 300 mM NaCl 200 mM imidazole 10% glycerol (pH 6.8)	Adjust pH by adding concentrated HCl and Store at 4 °C

Table 2.8 Buffers and Solutions for Protein Extraction and Purification (Non-Denaturing conditions)

Name	Ingredients	Preparation and Storage
Binding buffer	50 mM Na_2HPO_4, pH 8.0 300 mM NaCl 10 mM imidazole	Adjust pH by adding concentrated HCl and Store at 4 °C

Wash buffer	50 mM Na_2HPO_4, pH 8.0 300 mM NaCl 50 mM imidazole	Adjust pH by adding concentrated HCl and Store at 4 °C
Elution buffer	50 mM Na_2HPO_4, pH 8.0 300 mM NaCl 250 mM imidazole	Adjust pH by adding concentrated HCl and Store at 4 °C
Lysis buffer	20 mM Tris-HCl (pH 7.5) 10% glycerol 5mM DTT 1 mM PMSF 1 mM EDTA	Stored at 4 °C

Table 2.9 Buffers and Solutions for Plant Protein Extraction

Name	Ingredients	Preparation and Storage
Grinding buffer	100 mM Tris-HCl (pH 7.5) 10 mM β-Mercaptoethanol	Store at 4 °C
Dialysis buffer	20 mM Tris-HCl (pH 7.5) 10 mM β-Mercaptoethanol 5% Ehtylene glycol	Store at 4 °C

Table 2.10 Buffers and Solutions for ELISA/Immunocytolocalization/GUS Assay/ particle coating.

Name	Ingredients	Preparation and Storage
PBS T	1.44 g Na_2HPO_4 0.24 g KH_2PO_4 0.2 g KCl 8 g NaCl 0.05% v/v Tween-20	Stored at RT
Antibody dilution Buffer	PBS with 0.25% BSA	store at 4 °C
Subustrate buffer	200 mM Tris-HCl, pH 9.5 0.5 mM $MgCl_2$	Store at 4 °C
1X PBS	10 mM NaH_2PO_4-Na_2HPO_4 Buffer (pH 7.2) 130 mM NaCl	Adjust pH to 7.2 and Store at 4 °C
0.5 X SSC	75 mM NaCl 7.5 mM Na Citrate	Adjust pH to pH 7.0 and Stored at 4 °C

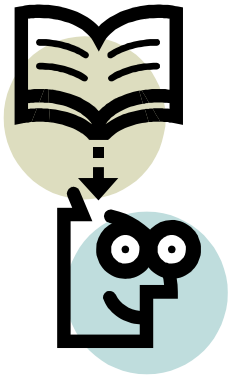
Color development buffer	100 mM Tris-HCl (pH 9.5) 150 mM NaCl, 50 mM $MgCl_2$	Stored at 4 °C
BCIP/NBT mix	0.577 mM BCIP 0.122 mM NBT	Stored at 4 °C In dark
Stop Solution	10mM EDTA	Stored at RT
Other reagents used	Paraformaldehyde, Polyvinyl alcohol, Ethanol, Tertiary butanol, Glycerol	

Table 2.10 Component of Murasighe and Skoog Media / Different inducing media and hormones

Name	**Ingredients**	**Preparation and Storage**
Major component	20.61 mM NH_4NO_3 18.75 mM KNO_3 2.99 mM $CaCl_2.2H_2O$ 1.5 mM $MgSO_4.7H_2O$ 1.24 mM KH_2PO_4	Stored at 4 °C
Minor Components	0.147 mM $MnSO_4$ 5.3 x 10^{-2} mM $ZnSO_4$ 1.56 x 10^{-4} mM $CuSO_4$ 1.05 x 10^{-4} mM $CoCl_2.6H_2O$ 4.99 x 10^{-3} mM KI 0.1mM H_3BO_4 1.03 x 10^{-3} mM $Na_2Mo_4.2H_2O$	Stored at 4 °C
Vitamins	5.55 x10^{-2} mM Myoionsitol 4.06 x 10^{-3} mM Nicotinic acid 2.43 x 10^{-3}mM Pyridoxine HCl 2.96 x 10^{-4} mM Thymine HCl 2.66 x 10^{-2} mM Glycine	Stored at 4 °C
Iron	0.1mM $FeSO_4.7H_20$ 0.1mM Na_2EDTA	Stored at 4 °C
BAP	1.776 mM BAP (dissolve in NaOH and make up the volume by adding ethanol)	Stored at 4 °C
NAA	2.148 mM NAA (Dissolve in H_2O)	Stored at 4 °C
Shoot Induction Medium	Major (40x)-25 ml Minor (100x)-10 ml Iron (100x)-10ml Vitamins (200x)-5 ml BAP (4.4 µM))-2.5 ml NAA (5.37 µM)-250 µl Glucose-1.5% Sucrose-2.0%	Stored at 4 °C

	pH-5.6 to 5.8 Agar-0.8% (make up to volume 1l)	
Resuspension Medium	Major (40x) 25 ml Minor (100x) 10 ml Iron (100x) 10 ml Vitamins (200x) 5 ml BAP (4.4µM) 2.5 ml NAA (5.37µM) 250 µl Glucose 1.5% Sucrose 2.0% pH-5.6 to 5.8 Acetosyringone-200 µM $MgSO_4$ 40 mM (make up to volume 1l)	Stored at 4 °C
Root Induction Medium	Major(40x) 25 ml Minor(100x) 10 ml Iron (100x) 10 ml Vitamins (200x) 5 ml NAA(5.37 µM) 250 µl Glucose 1.5% Sucrose 2.0% pH-5.6 to 5.8 Agar 0.8% (make up to volume 1l)	Stored at 4 °C
Selection medium	7.09 µM hygromycin 200/250 µM cefotaxime	

REFERENCES



Anterola A. M. and Lewis N. G. 2002. Trends in lignin modification: a comprehensive analysis of the effects of genetic manipulations/mutations on lignification and vascular integrity. Phytochemistry. 61: 221–294.

Anterola A. M., Jeon J. H., Davin L. B., Lewis N. G. 2002. Transcriptional control of monolignol biosynthesis in Pinus taeda. Factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. J. Biol. Chem. 277: 18272–80.

Anterola A. M., van Rensburg H., van Heerden P. S., Davin L. B. and Lewis N. G., 1999. Multi-Site modulation of Flux during Monolignol Formation in *Loblolly* Pine (*Pinus taeda*. Biochem. Biophys. Res. Commun. 261: 652–657.

Arnheim and Ehrlich. 1992. Polymerase chain reaction strategy. Annu. Rev. bacteriophage T4. Nature. 227: 680-685.

Babu R.H., Sajeena A., Seetharaman K., Reddy M.S. 2003. Advances in genetically Engineered (transgenic) plants in pest management - an over view. Crop protection. 22: 1071-1086.

Bao W., O'Malley D. M., Whetten R., Sederoff R. R. 1993. A laccase associated with lignification in loblolly pine xylem. Science. 260: 672–74

Baribault T.J., Skene K.G.M., Scott.N.S. 1989. Genetic transformation of grapevine cells. Plant Cell Rep. 8: 137-140.

Baribault T.J., Skene K.G.M., Cain P.A., Scott N.S. 1990. Transgenic grapevines: regeneration of shoots expressing beta-glucuronidase. J. Exp. Bot. 41: 1045-1049.

Baucher M., Monties B., Van Montagu M., Boerjan W. 1998. Biosynthesis and genetic engineering of lignin. Crit. Rev. Plant Sci. 17: 125–97.

Baucher M., Bernard-Vailhe M. A., Chabbert B., Besle J. M., Opsomer C., Van Montagu M. and Botterman J. 1999. Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (Medicago sativa L.) and the effect on lignin composition and digestibility. Plant Molecular Biology. 39: 437–447

Baucher M., Chabbert B., Pilate G., Van Doorsselaere J., Tollier M. T., Petit-Conil M., Cornu D., Monties B., Van Montagu M., Inze D., Jouanin L. and Boerjan W. 1996. Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar. Plant Physiology. 112:1479-1490

Bell-Lelong D. A., Cusumano J. C., Meyer K., Chapple C. 1997. Cinnamate-4-hydroxylase expression in Arabidopsis. Regulation in response to development and the environment. Plant Physiology. 113: 729– 738.

Bernard-Vailhe M. A., Migne C., Cornu A., Maillot M. P., Gernet E., Besle J. M., Atanassova R., Martz F. and Legrand M. 1996. Effect of modificationof the O-methyltransferse activity on cell wall composition, ultra structure and degradability of transgenic tobacco. Journal of the science of food and agriculture. 72: 385-391.

Bernard-Vailhe M. A. *et al*.1998. Effect of down regulation of cinnamyl alcohol dehydrogenase on cell wall composition and on degradability of tobacco stems. Journal of the science of food and agriculture. 76:505-514.

Bevan M., Shufflebottom D., Edwards K., Jefferson R., and Schuch W. 1989. Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO J. 8: 1899-1 906.

Bevan M., Shufflebottom D., Edwards K., Jefferson R., Schuch W. 1989. Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO Journal. 8: 1899–1906.

Blount J. W., Korth K. L., Masoud S. A., Rasmussen S., Lamb C., Dixon R. A. 2000. Altering expression of cinnamic acid 4- hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiol. 122:107–16.

Blumberg D.D. 1987. Creating a ribonuclease-free environment. Meth. Enzymol. 152: 20-24

Boerjan W. et al., 2003. Lignin Biosynthesis. Annu. Rev. Plant Biol. 54: 519-46.

Bolwell G. P., Cramer C. L., Lamb C. J., Schuch W., Dixon R. A. 1986. L-Phenylalanine ammonia-lyase from Phaseolus vulgaris. Modulation of the levels of active enzyme by trans-cinnamic acid. Planta 169:97–107.

Bomati E. K. and Noel J. P. 2005. Structural and Kinetic Basis for Substrate Selectivity in Populus tremuloides Sinapyl Alcohol Dehydrogenase. Plant Cell. 17: 1598–1611.

Brill E. M. et al., 1999 Molecular characterisation and expression of a wound-inducible cDNA encoding a novel cinnamyl-alcohol dehydrogenase enzyme in lucerne (*Medicago sativa* L.) Plant Molecular Biology 41:279-291

Bray R. A. *et al*., 1988. Performance of 14 *Leucaena* lines at five sites in Queensland. Aust. J. exp. Agric. 28: 69-76.

Campbell M. M. and Ellis B.E. 1992. Fungal elicitor-mediated responses in pine cell cultures. 1. lnduction of phenylpropanoid metabolism. Planta. 186: 409-417.

Chapple CCS, *et al.,* 1994. Secondary metabolism in *Arabidopsis*. In: Meyerowitz EM, Somerville CR (eds) Arabidopsis. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp 989–1030

Chen C. Y., Meyermans H., Burggraeve B., De Rycke R. M., Inoue K., De Vleesschauver V., Steenackers M., Van Montagu M. C., Engler G. J. and Boerjan W. A. 2000. Cell specific and conditional expression of caffeioyl-Coenzyme A-3-O-Methyl transferase in poplar. Plant physiology. 123: 853-867.

Chen C., Meyermans H., Burggraeve B., De Rycke R. M., Inoue K., et al. 2000. Cell-specific and conditional expression of caffeoyl-CoA O-methyltransferase in poplar. Plant Physiol. 123:853–67.

Chen C., Meyermans H., Burggraeve B., De Rycke R. M., Inoue K., et al. 2000. Cell-specific and conditional expression of caffeoyl-CoA O-methyltransferase in poplar. Plant Physiol. 123: 853–67.

Christensen J. H., Baucher M., O'Connell A. P., Van Montagu M., Boerjan W. 2000. Control of lignin biosynthesis. In Molecular Biology of Woody Plants, Volume 1, ed. SM Jain, SC Minocha, For. Sci. Dordrecht: Kluwer. 64: 227–67.

Claire H., Holt K., Chojecki J., Oliver D., Chabbert B., Monties B., Edwards K. and Foxon G. A. 1998. Brown-midrib maize (bm1)– a mutation affecting the cinnamyl alcohol dehydrogenase gene. The plant journal. 14(5): 545-553

Claire H., Knight M. E., Foxon G. A., Campbell M. M., Boudet A. M., Boon J. J., Chabbert B., Tollier M. T. and Schuch W. 1994. Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. The plant journal. 6(3): 339-350

Davin L. B., Patten A. M., Jourdes M.and Lewis N. G., 2008. Biomass Recalcitrance. Deconstructing the Plant Cell Wall for Bioenergy.. ed. M. Himmel, Blackwell Publishing, Oxford, UK. pp. 213–305.

Davin L. B., Wang H. B., Crowell A. L., Bedgar D. L., Martin D. M., Sarkanen S., and Lewis N. G. 1997. Stereo-selective bi-molecular phenoxy-radical coupling by an auxiliary (dirigent) protein without an active centre. Science. 275: 362-366.

Davin L. B. *et al*., 2008. Dissection of macromolecular configuration and assembly: Comparison to related biochemical processes in allyl/propenyl phenol and lignin biosynthesis. Nat. Prod. Rep. 25: 1015-1090.

Dean J. F. D., LaFayette P. R., Rugh C., Tristram A. H., Hoopes J. T., et al. 1998. Laccases associate with lignifying vascular tissues. In Lignin and Lignan Biosynthesis (ACS Symp. Series), ed. Lewis N.G., Sarkanen S. Washington, DC: Am. Chem. Soc. 697: 96–108: 436 pp.

Delores Broten and Robyn Budd, 2005. Making paper as if earth matters

Dixon R. A. et al. 2001. The biosynthesis of monolignols: a “metabolic grid”. Or independent pathways to guaicyl and syringyl units? Phytochemistry. 57:1069-1084.

Driouich A., Lain´e A-C., Vian B., Faye L. 1992. Characterization and localization of laccase forms in stem and cell cultures of sycomore. Plant J. 2: 13–24

Edwards K., Cramer C. L., Bolwell G. P., Dixon R. A., Schuch W.and C. J. Lamb. Rapid transient induction of phenylalanine ammonia-lyase mRNA in elicitor-treated bean cells Proc. Natl. Acad. Sci. U. S. A. 1985. 82: 6731– 6735.

Ehlting J., B¨uttner D., Wang Q., Douglas C. J., Somssich I. E., Kombrink E. 1999. Three 4-coumarate:coenzyme A ligases in Arabidopsis thaliana represent two evolutionarily divergent classes in angiosperms. Plant J. 19: 9–20

Eleventh five year plan 2007-2012, Planning commission, Government of India
Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354.

Finkle B. J. and Nelson R. F. 1963. Biochim. Biphys. Acta. 78: 747-749.

Freudenberg K. 1968. Constitution and Biosynthesis of Lignin. ed. K. Freudenberg and A. C. Neish, Springer-Verlag, New York, NY, USA. pp. 45–122.

Friedman W. E., Cook M. E. 2000. The origin and early evolution of tracheids in vascular plants: integration of palaeobotanical and neobotanical data. Philosophical Transactions of the Royal Society of London B Biology Science. 355: 857–868.

Freeman W.M., Walker S.J., Vrana K.E. 1999. Quantitative RT-PCR: pitfalls and potential. Biotechniques 26 (1): 112-125.

Frohman M.A. 1994. On beyond classic RACE (Rapid Amplification of cDNA Ends). PCR Methods Appl. 4: 40-58

Galliano H., Heller W., and Sandermann H. 1993. Ozone induction and purification of spruce cinnamyl alcohol dehydrogenase. Phytochemistry. 32: 557-563.

Gang D. R., Costa M. A., Fujita M., Dinkova-Kostova A. T., Wang H. B., Burlat V., Martin W., Sarakamen S., Davin L. B., and Lewis N. G. 1999. Regio-chemical control of monolignol radical coupling: A new paradigm for lignin and lignan biosynthesis. Chemistry and Biology. 6: 143-151.

Goffner D., Campbell M. M., Campargue C., Clastre M., Borderies O., Boudet A., and Boudet A. M. 1994. Purification and characterization of cinnamoyl-coenzyme ANADP oxidoreductase in Eucalyptus gunnii. Plant Physiol. 106: 625-632.

Goffner D., Joffmy I., Grima-Pettenati J., Halpin C., Knight M.E., Schuch W., and Boudet A.M. 1992. Purification and characterization of isoforms of cinnamyl alcohol dehydrogenase from Eucalyptus xylem. Planta. 188: 48-53.

Goodenow M., Huet T., Saurin W., Kwok S., Sninsky J., Wain-Hobson S. 1989. HIV-1 isolates are rapidly evolving quasi species: evidence for viral mixtures and preferred nucleotide substitutions. J Acquired Immunol Defic. Syndr. 2: 344-352.

Grand. 1984. Ferulic acid 5-hydroxylase: a new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin synthesis FEBS Lett. 169: 7–11.

Grima-Pettenati J., Feuillet C., Goffner D., Borderies G. and Boudet A. M. 1993. Molecular cloning and expression of a Eucalyptus gunnii cDNA clone encoding cinnamyl alcohol Dehydrogenase. Plant Mol. Biol. 21: 1085–1095.

Gross G. 1985. Biosynthesis and metabolism of phenolic acids and monolignols. In Biosynthesis and Biodegradation of Wood Components. Higuchi T., ed (New York: Academic Press). pp. 229-271.

Gross G. G. and W. Kreiten. 1975. Reduction of coenzyme a thioesters of cinnamic acids with an enzyme preparation from lignifying tissue of *Forsythia.* FEBS Lett. 54: 259–262.

Gross G. G. and Zenk M. H. 1966. Z. Naturforsch, B. 21: 683-690.

Gross G. G., Stockigt J., Mansell R. L. and Zenk M. H. 1973. Reduktion aromatischer Sauren zu aldehyden und alkoholen im zellfreien system. FEBS letters. Vol 31: no. 3.

Hahlbrock K. and Scheel D. 1989. Physiology and molecular biology of phenyl propanoid metabolism. Annual review of plant physiology and plant molecular biology. 40: 347-369.

Halpin C. 1994. Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. The plant journal. 6(3): 339-350

Halpin C. *et al.,* 1998. Maize brown midrib (bm1)- amutation affecting the cinnamyl alcohol dehydrogenase gene. The plant journal.6:339-350.

Halpin C. 2004. Investigating and manipulating lignin in the postgenomic era Advances in botanical research. vol. 41: 63-106

Hanson K. R. and Havir E.A. 1981. Phenylalanine ammonia-lyase. In The Biochemistry of Plants: A Comprehensive Treatise, Vol. 7, Secondary Plant Products, E.E. Conn, ed (New York: Academic Press): pp. 577-625.

Hansen G. & Wright M. S. 1999. Recent advances in the transformation of plants. Trends in plant science 4: 226-231.

Harding S. A., Leshkevich J., Chiang V. L., Tsai C-J. 2002. Differential substrate inhibition couples kinetically distinct 4- coumarate: coenzyme A ligases with spatially distinct metabolic roles in quaking aspen. Plant Physiol. 128: 428–38

Hatfield R., and Vermerris W. 2001. Lignin formation in plants. The dilemma of linkage specificity. Plant Physiology 126: 1351–1357.

Hatton D., Sablowski R., Yung M. H., Smith C., Schuch W., Bevan M. 1995. Two classes of cis sequences contribute to tissue-specific expression of a PAL2 promoter in transgenic tobacco. Plant Journal. 7: 859–876.

Hauffe K. D., Lee S. P., Subramaniam R., Douglas C. J. 1993. Combinatorial interactions between positive and negative cis-acting elements control spatial patterns of 4CL-1 expression in transgenic tobacco. Plant Journal. 4: 235–253.

Heller W. and Ku¨ hnl T. 1985. Arch. Biochem. Biophys. 241: 453–460.

Hibino T., Takabe K., Kawazu T., Shibata D., and Higuchi T., 1995. Increase of cinnamaldehydes groups in lignin of transgenic tobacco plants carrying an antisense gene for cinnamyl alcohol dehydrogenase. Bioscience Biotechnology and Biochemistry. 59: 929-931.

Higuchi R., Dollinger G., Walsh P. S., and Griffith R. 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology. 10: 413–417.

Higuchi R., Fockler C., Dollinger G., Watson R. 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology. 11: 1026–1030.

Higuchi T., Ito T., Hibino T. and Shibata T. 1994. Red brown colour of lignified tissues of transgenic plants with antisense CAD gene-wine red lignin from coniferyl aldehydes. Journal of biotechnology. 37: 151-158.

Hoffmann L., Maury S., Martz F., Geoffrey P. and Legrand M. 2002. Purification, cloning and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. J. Biol. Chem. 278:95–103.

Horch R., Fry J., Hoffmann N., Eichholtz P., Rogers R., Fratey T. 1985 A simple method for transferring genes into plants. Science 227: 1229–1231.

Hosel W. *et al*., 1982. Relationship of coniferin-β glucosidase to lignifications in various plant cell suspension cultures. Plant cell tissue organ cult.1:137-148.

Hu Ta Wei. 1987. Use of Nitrogen Fixing Trees for Pulpwood. NFTA, Waimanalo, Hawaii.

Hu W-J., Kawaoka A., Tsai C-J., Lung J., Osakabe K., et al. 1998. Compartmentalized expression of two structurally and functionally distinct 4-coumarate: CoA ligase genes in aspen Populus tremuloides. Proc. Natl. Acad. Sci. USA. 95: 5407–12.

Hughes C. E. 1998. Leucaena: A genetic resources handbook. Tropical Forestry Papers

Ibrahim R., and Grisebach H. 1976. Purification and properties of UDP-glucose: Coniferyl alcohol glucosyltransferase from suspension cultures of Paul's Scarlet rose. Arch. Biochem. Biophys. 176: 700-708.

Jefferson R.A. 1987. Assaying chimeric genes in plants: the genes fusion system. Plant Mol. Biol. Reporter 5: 387-405.

Jin H., Cominelli E., Bailey P., Parr A., Mehrtens F., et al. 2000. Transcriptional repression by AtMYB4 controls production of UV-protecting sunscreens in Arabidopsis. EMBO J. 19:6150–61

Jones D.H. 1984. Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development. Phytochemistry. 23: 1349-1359.

Jones L., Ennos A. R., Turner S. R. 2001. Cloning and characterization of irregular xylem4 (irx4): a severely lignin deficient mutant of Arabidopsis. Plant J. 26: 205–16

Jouanin L., Goujon T., de Nadai V., Martin M. T., Mila I., Vallet C., Pollet B., Yoshinaga A., Chabbert B., Petit-Conil M. and Lapierre C. 2000. lignification in transgenic poplars with extremely reduced caffeic acid O-methyltransferase activity. Plant physiology. 123: 1363-1373

Kawaoka A. and Ebinuma H. 2001. Transcriptional control of lignin biosynthesis by tobacco LIM protein. Phytochemistry. 57: 1149-1157.

Kim H., Ralph J., Lu F. C., Pilate G., Leple J. C., Pollet B. and Lapierre C. 2002. Identification of the Structure and Origin of Thioacidolysis Marker Compounds for Cinnamyl Alcohol Dehydrogenase Deficiency in Angiosperms. The Journal Of Biological Chemistry. Vol. 277, No. 49: 47412–47419.

Kimmel A.R., Berger S.L. 1987. Preparation of cDNA and the generation of cDNA libraries: Overview. Meth Enzymol. 152: 307-316.

Knight M. E., Halpin C. and Schuch W. 1992. Identification and characterization of cDNA clones encoding cinnamyl alcohol dehydrogenase from tobacco Plant Mol. Biol. 19: 793–801.

Koes R. E., Quattrocchio R., Mol JNM (1994) The flavonoid biosynthetic pathway in plants: Function and evolution. BioEssays 16:123–132.

Koukol J. and Conn E. E. 1961. ᴛhe metabolism of aromatic compounds in higher plants. iv. purification and properties of the phenylalanine deaminase of *hordeum vulgare* J. Biol. Chem. 236: 2692–2698.

Kutsuki H., Shimada M. and Higuchi T. 1982. Regulatory role of cinnamyl alcohol dehydrogenase in the formation of guaiacyl and syringyl lignins. Phytochemistry 21: 19-23.

Kwok S., Kellogg D. E., McKinney N., Spasic D., Goda L. 1990 Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. Nucleic Acids Res. 18: 999-1005.

Lacombe E, et al., 1997. Cinnamoyl-CoA reductase, the first committed enzyme of lignin branch biosynthetic pathway: cloning, expression, and phylogenetic relationships. The Plant Journal 11: 429–441.

Lacombe E., Hawkins S., Van Doorsselaere J., Piquemal J., Goffner D., Poeydomenge O.,. Boudet A.M. and Grima- Pettenati J. 1997. Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships Plant J. 11: 429–441.

Lacombe E., Van Doorsselaere J., Boerjan W., Boudet A. M., Grima-Pettenati J. 2000. Characterization of cis-elements required for vascular expression of the cinnamoyl CoA reductase gene and for protein-DNA complex formation. Plant Journal. 23: 663–676.

Laemmli U.K. 1970. Cleavage of structural proteins during the assembly of the head of

Lamb C. J. 1977. trans-cinnamic acid as a mediator of the light-stimulated increase in hydroxycinnamoyl-CoA: quinate hydroxycinnamoyl transferase. FEBS Lett. 75: 37–40.

Lapierre C., Pollet B., Petit-Conil M., Toval G., Romero J., Pilate G., Leple J. C., Boerjan W., Ferret V., de Nadai V. and Jouanin L. 1999. Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. Plant physiology. Vol. 119: 153-163

Lauvergeat V., Lacomme C., Lacombe E., Lasserre E., Roby D., Grima-Pettenati J. 2001. Two cinnamoyl-CoA reductase (CCR) genes from Arabidopsis thaliana are differentially expressed during development and in response to infection with pathogenic bacteria. Phytochemistry 57: 1187–95.

Lauvergeat V., Rech P., Jauneau A., Guez C., Coutos-Thevenot P., Grima-Pettenati J. 2002. The vascular expression pattern directed by the Eucalyptus gunnii cinnamyl alcohol dehydrogenase EgCAD2 promoter is conserved among woody and herbaceous plant species. Plant Molecular Biology. 50: 497–509.

Le Gall O., Torregrosa L., Danglot Y., Candresse T., Bouquet A. 1994. Agrobacterium-mediated genetic transformation of grapevine somatic embryos and regeneration of transgenic plants expressing the coat protein of grapevine chrome mosaic nepovirus, GCMV. Plant Sci. 102: 161-170.

Lewis N.G. 1999. A 20th century roller coaster ride: A short account of lignification. Current opinion in plant biology. 2: 153-162.

Li L., Cheng X. F., Leshkevich J., Umezawa T., Harding S. A., Chiang V. L. 2001. The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell 13: 1567–85

Li L., Popko J. L., Umezawa T., Chiang V. L. 2000. 5-Hydroxyconiferyl aldehydes modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. J. Biol. Chem. 275: 6537–45

Liang X. W., Dron M., Cramer C. L., Dixon R. A and Lamb C. J. 1989. Differential regulation of phenylalanine ammonia-lyase genes during plant development and by environmental cues. Journal of Biological Chemistry. 264: 14486–14492.

Lindermayr C., M¨ollers B., Fliegmann J., Uhlmann A., Lottspeich F., et al. 2002. Divergent members of a soybean (Glycine max L.) 4-coumarate:coenzyme A ligase gene family. Eur. J. Biochem. 269: 1304– 15.

Livak K.J., Flood S.J., Marmaro J., Giusti W., Deetz K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4: 357-362.

Lodhi M. A., Guang-Ning Ye, Norman F. W., Bruce I. R. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, Vitis species and Ampelopsis. Plant Mol. Biol. Reporter. 12: 6-13.

Lois R., and Hahlbrock K. 1992. Differential wound activation of member of the phenylalanine ammonia-lyase and 4toumarate:CoA ligase gene families in various organs of parsley plants. Z. Naturforsch. 47C: 90-94.

Lois R., Dietrich A., Hahlbrock K., Schulz W. 1989. A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive cis-acting elements. EMBO Journal. 8: 1641–1648.

Luo Z. *et al.,* 2008. Accumulation of lignin and involvement of enzymes in bamboo shoot during storage. Eur Food & Res Tech. 226:635-640.

Mac kay J., Presnell T., Jameel H., Taneda H., O' Malley D. and Sederoff R. 1999. Modified lignin and delignification with a CAD deficient loblolly pine. Holzforschung. 53: 403-410.

MacKay John J., O'Malley D. M., Presnell T., Booker F. L., Campbell M. M., Whetten R. W. and Sederoff R. R. 1997. Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. PNAS. vol 94: 8255-8260

Mansell R. L., Gross G. G., Sto¨ckigt J., Franke H. and Zenk M. H. 1974. Phytochemistry. 13: 2427–2435.

Mansell R.B., Babbel G.R., and Zenk M.H. 1976. Multiple forms and specificity of coniferyl alcohol dehydrogenase from cambial regions of higher plants. Phytochemistry. 15: 1849-1853.

Marita J. M., Vermerris W., Ralph J. and Hatfield R. D. 2003. Variations in the cell wall composition of maize brown midrib mutants. Journal of agricultural and food chemistry. 51: 1313-1321

Maruyama K., and Sugano S. 1994. Oligo-Capping: A Simple Method to Replace the Cap Structure of Eukaryotic mRNAs with Oligoribonucleotides. Gene. 138: 171 - 174.

Matsushita M. *et al.,* 2004. Formation and chemical structures of acid-soluble lignin II: reaction of aromatic nuclei model compounds with xylan in the presence of a counterpart for condensation, and behavior of lignin model compounds with guaiacyl and syringyl nuclei in 72% sulfuric acid. J wood sci. 50:136-141.

Mavandad M., Edwards R., Liang X., Lamb C.J., Dixon R.A. 1990. Effects of trans-cinnamic acid on expression of the bean phenylalanine ammonia-lyase gene family. Plant Physiol. 94:671–80.

Merkle SA, Nairn CJ (2005) Hardwood tree biotechnology. In Vitro Cellular and Developmental Biology. Plant 41: 602–619.

Merkle, S.A. & Nairn, C.J. 2005. Hardwood tree biotechnology. In Vitro Cellular and Developmental Biology -Plant 41: 602–619.

Moore K. J., Jung H-J. G. 2001. Lignin and fiber digestion. Journal of Range Management. 54: 420–430.

Mullis K.B. 1990. The unusual origin of the polymerase chain reaction. Scientific American 262: 56–61.

Mullis K.B. and Faloona F. 1987. Specific synthesis of DNA in vitro via a polymerase-cata lyzed chain reaction. Methods Enzymology 155: 335.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant, 15: 473-497.

O'Connell A., Holt K., Piquemal J., Grima-Pettenati J., Boudet A., Pollet B., Lapierre C., Petit-Conil M., Schuch W. and Halpin C. 2002. Improved paper pulp from plants with suppressed cinnamoyl-CoA reductase or cinnamyl alcohol dehydrogenase. Transgenic research. 11: 495-503.

Onnerud H, Zang L., N., Gellerstedt G. and Henriksson G. 2002. Polymerisation of monolignols by redox shuttle-mediated enzymatic oxidation: a new model in lignin biosynthesis. I. Plant Cell. 14: 1953-1962.

Osakabe K., Tsao C. C., Li L., Popko J. L., Umezawa T., et al., 1999. Coniferyl aldehydes 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc. Natl. Acad. Sci. USA. 96: 8955–60

Patzlaff A., Mc Innis S., Courtenay A., Surman C., Newman L. J., Smith C., Bevan M. W., Mansfield S., Whetten R. W., Sederoff R. R., Campbell M. M. 2003. Characterisation of a pine MYB that regulates lignification. Plant Journal. 36: 743–754.

Perl A., Lotan O., Abu-Abied M., Holland D. 1996. Establishment of an *Agrobacterium*-mediated transformation system for grape (Vitis vinifera L.): the role of antioxidants during grape-*Agrobacterium* interactions. Nat. Biotechnol. 14: 624-628.

Pillonel C., Huynziker P., and Binder A. 1992. Multiple forms of the constitutive wheat cinnamyl alcohol ehydrogenase. J. Exp. Bot. 43: 299-305.

Pillonel C., Mulder M., Boon J., Forster B. and Binder A. 1991. Involvement of cinnamyl alcohol dehydrogenase in the control of lignin formation in Sorghum bicolor 1 Monench. Planta. 185: 538-544.

Pincon G., Chabannes M., Lapierre C., Pollet B., Ruel K., et al. 2001. Simultaneous down-regulation of caffeic/ 5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme A reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiol. 126:145–55

Portales R. et al., 2002 Cloning, expression and immunolocalisation pattern of a cinnamyl alcohol dehydrogenase gene from strawberry (FragariaXananassa cv. Chandler). Journal of experimental botany. 53: 1723-1734.

Pottinger A. J., Hughes C. E. 1995. A review of wood quality in Leucaena. In H.M. Shelton, C.M. Piggin & J.L. Brewbaker (Eds.). Leucaena opportunities and limitations. Proceedings of a workshop held in Bogor, Indonesia, January 1994. ACIAR Proceedings Canberra. 57: 98

Raeymaekers L. 2000. Basic principles of quantitative PCR. Mol. Biotechnology. 15: 115-122.

Ralph J., Hatfield R. D., Piquemal J., Yahiaoui N., Pean M., Lapierre C. and Boudet M. 1998. NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamylalcohol dehydrogenase and cinnamoyl-CoA reductase (monolignolygenetic modificationyantisense RNAyconiferyl alcoholyferuloyl-CoA) Proc. Natl. Acad. Sci. USA. Vol. 95: 12803–12808

Ralph J., Lapierre C., Marita J. M., Kim H., Lu F. C., Hatfield R. D., Ralph S., Chapple C., Franke R., Hemm M. R., Van Doorsselaere J., Sederoff R. R., O' Malley D. M., Scott J. T., Mac Kay J. J., Yahiaoui N., Boudet A. M., Pean M., Pilate G., Jouanin L. and Boerjan W. 2001. Elucidation of new structures in lignins of CAD- and COMT-de.cient plants by NMR. Phytochemistry. 57: 993–1003

Ralph J., Mac Kay J. J., Hatfield R. D., O' Malley D. M., Whetten R. W. and Sederoff R. R. 1997. Abnormal Lignin in a Loblolly Pine Mutant. Science Vol. 277.

Ranocha P., Chabannes M., Chamayou S., Danoun S., Jauneau A., et al. 2002. Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. Plant Physiol. 129: 145–55.

Ranocha P., McDougall G., Hawkins S., Sterjiades R., Borderies G., et al. 1999. Biochemical characterization, molecular cloning and expression of laccases—a divergent gene family—in poplar. Eur. J. Biochem. 259: 485–95

Rhodes M.J.C. 1994. Physiological roles for secondary metabolites in plants: Some progress, many outstanding problems. Plant Mol Biol 24: 1–20

Richard S., Eudes A., Pollet B., Goujon T., Mila I., Granier F., Seguin A., Lapierre C. and Jouanin L. 2003. Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in Arabidopsis. Isolation and characterization of the corresponding mutants. Plant Physiology. Vol. 132: 848-860

Russell D. W. and Conn E. E. 1967. Arch. Biochem. Biophys. 122: 256–258.

Sambrook J., Fritsch E. F., Maniatis T. 1989. Molecular cloning; A Laboratory Manual., 2nd ed., New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Scharf S.J., Horn G.T., Erlich H.A. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233: 1076-1078.

Schoch G., Goepfert S., Morant M., Hehn A., Meyer D., et al. 2001. CYP98A3 from Arabidopsis thaliana is a 30-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J. Biol. Chem. 276: 36566–74

Selman-Housein G. et al., 1999 Molecular cloning of cDNAs coding for three sugarcane enzymes involved in lignifications. Plant Science 143: 163–171

Shirley B.W. 1996. Flavonoid biosynthesis: ‘‘New’’ functions for an ‘‘old’’ pathway. Trends Plant Sci 1: 377–382

Shirley B. W. 1999. Evidence for enzyme complexes in the phenylpropanoid and flavonoid Pathways. Physiologia Plantarum. 107: 142–149

Shufflebottom D., Edwards K., Schuch W., and Bevan M. 1993. Transcription of two members of a gene family encoding phenylalanine ammonia-lyase leads to remarkably different cell specificities and induction patterns. Plant J. 3: 835-845.

Sibout R. *et al.,* 2003. Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenase in Arabidopsis. Isolation and characterization of corresponding mutants. Plant physiol. 132: 848-860.

Sibout R., Eudes A., Mouille G., Pollet B., Lapierre C., Jouanin L. and Se´guin A. 2005. cinnamyl alcohol dehydrogenase-C and -D Are the Primary Genes Involved in Lignin Biosynthesis in the Floral Stem of Arabidopsis. Plant Cell. 17: 2059–2076.

Speer E.O. 1987. A method of retaining phloroglucinol proof of lignin. Stain Technol. 62: 279–280.

Sterjiades R., Dean J. F. D., Eriksson K. E. L. 1992. Laccase from sycamore maple (Acer pseudoplatanus) polymerizes monolignols. Plant Physiol. 99: 1162–68

Stewart D., Yahiaoui N., Mc Dougall G. J., Myton K., Marque C., Boudet A. M., Haigh J. 1997. Fourier-transform infrared and Raman spectroscopic evidence for the incorporation of cinnamaldehydes into the lignin of transgenic tobacco (Nicotiana

tabacum L.) plants with reduced expression of cinnamyl alcohol dehydrogenase Planta. 201: 311-318.

Strauss S.H., Rottmann W.H., Brunner,A.M., Sheppard L.A. 1995. Genetic engineering of reproductive sterility in forest trees. Molecular Breeding 1: 5-26.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Takabe K. *et al*., 2001. Immunocytochemical localization of enzymes involved in lignification of cell wall. J. Plant Res. 509-515.

Tognolli M., Penel C., Greppin H., Simon P. 2002. Analysis and expression of the class III peroxidase large gene family in Arabidopsis thaliana. Gene. 288: 129– 38

Ulbrich B. and Zenk M. H. 1980. Partial purification and properties of para-hydroxycinnamoyl- CoA-shikimate-para- hydroxycinnamoyl transferase from higher plants. Phytochemistry. 19:1625–29.

Volloch V., Schweitzer B., and Rits S. 1994. Ligation-Mediated Amplification of RNA from Murine Erythroid Cells Reveals a Novel Class of Beta-Globin mRNA with an Extended 5.-Untranslated Region. Nucleic Acids Research. 22: 2507 - 2511.

Walter M. H., Grima-Pettenati J., Grand C., Boudet A. M. and Lamb C. J. 1988. Cinnamyl-alcohol dehydrogenase, a molecular marker specific for lignin synthesis: cDNA cloning and mRNA induction by fungal elicitor (lignification/plant disease resistance/ transcription). Proc. Natl. Acad. Sci. U. S. A. 85: 5546–5550.

Wanner L. A., Li G., Ware D., Somssich I. E., Davis K. R. 1995. The phenylalanine ammonia-lyase gene family in Arabidopsis thaliana. Plant Molecular Biology. 27: 327–338.

Weisshaar B., Jenkins G.I. 1998. Phenylpropanoid biosynthesis and its regulation. Curr Opin Plant Biol 1: 251–257

Wimmer E.A. 2003. Innovations: applications of insect transgenesis. Nature reviews genetics 4: 225-232.

Whetten R. and Sederoff R. 1995. Lignin biosynthesis. The plant cell. 7: 1001-1013.

Wyrambik D., and Grisebach H. 1975. Purification and properties of isoenzymes of cinnamyl alcohol dehydrogenase from soybean. Eur. J. Biochem. 59: 9-15.

Wyrambik, D., and Grisebach, H. (1975). Purification and properties of isoenzymes of cinnamyl alcohol dehydrogenase from soybean Cell-Suspension Cultures. Eur. J. Biochem. 59, 9-15.

Yahiaoui N., Marque C., Myton K. E., Negrel J., and Boudet A. M. 1998. Impact of different levels of cinnamyl alcohol Dehydrogenase down-regulation on lignins of transgenic tobacco plants. Planta. 204: 8-15

Youn B., Camacho R., Moinuddin S. G. A., Lee C., Davin L. B., Lewis N. G. and Kang C. 2006. Crystal structures and catalytic mechanism of the Arabidopsis cinnamyl alcohol dehydrogenases AtCAD5 and AtCAD4. Org. Biomol. Chem. 41687–1697.

Zubieta C., Kota P., Ferrer J-L., Dixon R. A., Noel J. P. 2002. Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase. Plant Cell. 14: 1265–77

Zubieta C., Kota P., Ferrer J-L., Dixon R. A., Noel J. P. 2002. Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-O-methyltransferase. Plant Cell. 14: 1265–77

PUBLICATIONS

Research papers published/ communicated:

- V L Sirisha, S Prashant, D Ranadheer, P Ramprasad, N M Shaik, Manish Arha, S K Gupta, Sameer Srivastava, A K Yadav, **P S Kulkarni**, O U Abhilash, B M Khan, Shuban K Rawal and P B Kavi Kishor (**2008**) "Direct shoot organogenesis and plant regeneration from hypocotyl explants in selected genotypes of *Leucaena leucocephala*—A leguminous pulpwood tree" .***Indian Journal of Biotechnology*** 7:388-393.
- Sameer Srivastava, D Ranadheer, Manish Arha, S K Gupta, N M Shaik, S Prashant, A K Yadav, **P S Kulkarni**, O U Abhilash, RJ Santosh, Rishi VK, V L Sirisha, Shuban K Rawal., P B Kavi Kishor and B M Khan. (**2008**). 'Molecular cloning, characterization and expression analysis of Cinnamoyl Co A reductase (CCR) gene in developing seedlings of *Leucaena lucocephala*, a paper and pulp yielding tree species". (Communicated).
- N.M.Shaik, M.Arha, S.K.Gupta, S.Srivastava, A.K.Yadav, **P.S.Kulkarni**, OU.Abhilash, B.M.Khan and S.K.Rawal (**2008**) "High Frequency Regeneration of *Leucaena leucocephala* -A Leguminous Pulpwood Tree Species"(Communicated).
- N.M.Shaik, M.Arha, S.K.Gupta, S.Srivastava, A.K.Yadav, **P.S.Kulkarni**, OU.Abhilash,B.M.Khan and S.K. Rawal **(2008)** "Molecular cloning and characterization of a Family 1 Glycosyl hydrolase from *Leucaena leucocephala*" (Manuscript under preparation).
- S.K.Gupta, N.M.Shaik, M.Arha, S.Srivastava, A.K.Yadav, **P.S.Kulkarni**, OU.Abhilash,B.M.Khan and S.K.Rawal. (**2008**). "Molecular cloning, characterization and expression analysis of 4 Coumarate Co A Ligase (4CL) genes from *Leucaena lucocephala"*. (Manuscript under preparation).
- S.K.Gupta**,** N.M.Shaik, R.Tatkare, M.Arha, S.Srivastava, A.K.Yadav, **P.S.Kulkarni**, OU.Abhilash, N.Akula B.M.Khan and S.K. Rawal (**2008**) "Genetic transformation and analysis of *Leucaena leucocephala* with anti-sense constructs of Lignin biosynthesis pathway genes". (Manuscript under preparation)

Books:

- N.M.Shaik, M.Arha, S.K.Gupta, S.Srivastava, A.K.Yadav, **P.S.Kulkarni**, O U.Abhilash, B.M.Khan and S.K.Rawal (**2007**) "Multiple shoot regeneration from the cotyledonary nodes of *Leucaena leucocephala*" in Recent trends in Horticultural Biotechnology I: 337-340 Eds Raghunath Keshavachandran *et al.,* New India Publishing Agency New Delhi.

Abstracts published:

- S. K. Gupta ,M. Arha, N. M. Shaik, S. Srivastava, A. K. Yadav, **P. Kulkarni**, O. U. Abhilash, B. M. Khan and S. K. Rawal (**2005**) 522-527. "High frequency regeneration

and transformation of *Leucaena leucocephala"* in Proceedings of National Symposium on Plant Biotechnology: New Frontiers, CIMAP, Lucknow, Uttar Pradesh, India..

- S. K. Gupta**,** M. Arha, N. M. Shaik, S. Srivastava, A. K. Yadav, **P. Kulkarni**, O. U. Abhilash, B. M. Khan and S. K. Rawal (**2006**). "An approach for genetic modification of *Leucaena leucocephala* for eco-friendly pulp and paper production". In Proceedings of International Symposium on Frontiers of Genetic Engineering and Biotechnology Retrospect and Prospect, Osmania University, Hyderabad, Andhra Pradesh, India.
- M. Arha, S. K. Gupta**,** N. M. Shaik, S. Srivastava, A. K. Yadav, **P. Kulkarni**, O.U. Abhilash, B. M. Khan and S. K. Rawal (**2005**). "Isolation, cloning and characterization of Caffeoyl CoA 3-O- methyl transferase (CCoAOMT) from *Leucaena leucocephala"*. In Proceedings of National Symposium on Plant Biotechnology: New Frontiers, CIMAP, Lucknow, Uttar Pradesh, India.
- N. M. Shaik, M. Arha, S. K. Gupta**,** S. Srivastava, A. K. Yadava, **P. Kulkarni**, O. U.Abhilash, B. M. Khan and S. K. Rawal (**2005)**. "Multiple shoot regeneration from cotyledonary nodes of *Leucaena leucocephala"*. In Proceedings of National Symposium on Biotechnological Interventions for Improvement of Horticultural Crops: Issues and Strategies, Kerala Agriculture University- ICAR, Thrissur, Kerala, India.
- O.U. Abhilash, M.Arha, S. K. Gupta, N. M. Shaik, S. Srivastava, A. K. Yadav, **P. Kulkarni**, B. M. Khan and S. K. Rawal (2006). "Cell wall bound peroxidase from *Leucaena leucocephala*- a candidate enzyme involved in lignin polymerization". In proceedings of International Symposium on Frontiers of Genetic Engineering and Biotechnology Retrospect and Prospect, Osmania University, Hyderabad, Andhra Pradesh, India.