STUDIES ON PEROXIDASES AND THEIR POTENTIAL ROLE IN LIGNIN METABOLISM OF *Leucaena leucocephala.*

A THESIS
SUBMITTED TO THE
UNIVERSITY OF PUNE

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY
ABHILASH O. U.

UNDER THE GUIDANCE OF
DR. B. M. KHAN
HEAD
PLANT TISSUE CULTURE DIVISION

NATIONAL CHEMICAL LABORATORY
PUNE – 411008
INDIA

November, 2009

DEDICATED TO PARENTS AND FAMILY

Contents	Page No.

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Studies on peroxidases and their potential role in lignin metabolism of *Leucaena leucocephala***" submitted by ***Abhilash O. U.*** 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, "**Studies on peroxidases and their potential role in lignin metabolism of *Leucaena leucocephala***" has been carried out at the 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.

(**Abhilash O. U.**)

Date: November, 2009
Place: Plant Tissue Culture Division,
National Chemical Laboratory (NCL),
Pune – 411008, India.

ACKNOWLEDGEMENTS

First and foremost with a deep sense of gratitude and indebtedness, I thank my mentor, ***Dr. B. M. Khan*** *for his invaluable guidance, constant encouragement, constructive criticism, valuable suggestions, help and freedom in finalizing the research and preparation of this manuscript without which the task would have been difficult. My experience with him has inculcated in me qualities that will always remain ingrained in my heart, mind and soul. Thank you Sir for making me a better person, guiding me and improving my silliest and minutest mistakes.*

I want to express my sincere and heartfelt gratitude to ***Dr. S. K. Rawal****, Former Head, Plant Tissue Culture Division for his immense interest and constant guidence during these years. He has taught me the ways of scientific thinking and stimulated discussions by his vast knowledge of molecular biology, plant secondary metabolism and science in general.*

My heartfelt thanks go to ***Dr.B. L. V. Prasad*** *whose expertise in nano-biotechnology has also been essential for this research work; his long experience in physical chemistry and nanotechnology made this research more competitive and meaningful. I thank him for the pleasant collaboration.*

I would extend my sincere thanks to ***Dr. M. S. Patole*** *(NCCS,Pune) &* ***Dr. Ashok Giri*** *(PMB group NCL) for their critical suggestions and guidences during my interim research evaluations. I am grateful to Dr. M.I. Khan, Dr. S. Kendurkar, Dr. D.C. Agrawal and Mrs. M. V. Shirgurkar for providing me the opportunity to share lab facilities.*

To complete any mission successfully in life, it is not just the roadmap that helps, but also the company of good friends which soars you to the heights greater than those reached alone. I have always been blessed with the best of good hearts, to relieve me from the most tiresome moments of life. I am indebted to all my friends throughout my lifetime for their care and kindness.

My special thanks are due to my friend Dr.Vijay Kumar Shanmugham, whose help and valuable suggestions are worth mentioning. My sincere thanks to Dr. Sucheta, Dr. S. Kendurkar and Dr. Neelima for making me feel homely during the course of this work at NCL and in giving perfect family atmosphere and a home away from home.

My special thanks are due to Dr. D. K. Kulkarni, Dr. Subhada Thengane, Dr. M. M. Jana, Mrs. Varsha Parasurami, Mr. R Suryaprasad and all the staff of Plant Tissue Culture division and Biochemical sciences.

I owe many cherished moments to ***Nishi*** *who soothed away all my worries with her bright smile, sweet words and prayers.*

The life of a researcher can only be truly understood by another. Therefore, my past and present colleagues in the Lignin project deserve heartfelt thanks for creating the most enjoyable atmosphere both in and out of the lab.

Many thanks are due to my Seniors Dr. Rohini, Dr.Manish, Dr. Sushim, my lab mates Dr. Noor, Arun, Pallavi, Santosh Jadhav, Ruby, Santosh Gupta, Sumita, Jai, Malini, Azfar, Sameena, Rishi, Somesh and Prashanth for their encouragement and help during the course of this work and cordial atmosphere in the lab.

Special thanks to my project students and trainees ***Akshata, Sandeep, Anupama, Sonia, Kapil, Veera, Anurag, Nazneen, Tripti*** *for the support, best wishes and lighter moments shared together throughout my work period and in making myself more competent.*

I take this opportunity to thank my friends Bhuban, Suresh, Alson, Sajid, Venu, Nagraj, Bhushan, Atul, Anish, Kiran Pawar, Arshad, Sarvesh Soni, Sampa Sarkar, Varsha, Shyla, Prashanth, Amol, Parul and Priyanka. My ***Cricket team*** *Ramanujan, Kannan, Kamu, Abishek, Dillu, Kannan, Ganesh, Nagraj, Sudershan, Kapil, Amit, Naveen, Jay, somesh and* ***TLEP 2008 group*** *for the support, best wishes and lighter moments shared together throughout my work period.*

I owe my deepest gratitude to my best buddies ***Sangeetha, Anu, suganya, Divya*** *and* ***Biji*** *for their constant help, encouragement and for pumping confidence in me during my testing times.*

*Last but not the least, it is difficult to express my gratitude towards my parents and family members, without whose encouragement and unstinted moral support, this endeavor would have never been possible. The main credit of my thesis goes to my dear parents (****Mr. and Mrs.Ushar Raj****) whose unending love, encouragement and sacrifice has made me achieve this milestone today. To my father who has always encouraged me to fulfill my dreams and to my Mother for her perseverance, encouragement and support. To my dearest brothers* ***Arun Raj*** *and* ***Anish Raj*** *their cheerful, loving nature and blessings was also instrumental in the completion of my thesis and obtaining the degree of Philosophy.*

This research has been supported by grant from ***The Council of Scientific and Industrial Research (CSIR****), New Delhi who awarded me Junior and Senior Research Fellowship for this research work. The financial support from CSIR for the fellowship and research grant provided in the network program TLP 001426 is thus gratefully acknowledged. I would like to thank* ***Dr. Sivaram,*** *Director****, National Chemical Laboratory****, for allowing me to submit my work in the form of a thesis.*

Above all these people, behind all my efforts and aspirations, there was one force that enriched my heart with the hope for tomorrow; that often assured me of the invincible power of care, love, and affection; that never deserted me in desperate times and never ceased to enthuse me when life touched its nadir. It is my beloved wife Pyary, who is indeed, the great force behind my perseverance; without whom, nothing would have been possible and nothing is going to be possible in future too!

Last but not least I thank God Almighty for giving me the strength and courage at every step of life.

Abhilash Raj

ABBREVIATIONS

AAS	Atomic Absorption Spectrometer
ALP	Alkaline phosphatase
CA	Coniferyl alcohol
CaMV	Cauliflower mosaic virus
4CL	4 - Coumarate Co A: ligase
AldOMT	5-Hydroxyconiferaldehyde O- methyltransferase
APS	Ammonium per sulphate
BAP	6-benzylaminopurine
BSA	Bovine serum albumin
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
C3H	Coumarate 3- hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CAld5H/ F5H	Coniferaldehyde 5- hydroxylase / Ferulate 5- hydroxylase
CCR	Cinnamoyl coenzyme A reductase
CCoAOMT	Caffeoyl coenzyme A 3-O- methyltransferase
COMT	Caffeate O-methyltransferase
cm	Centimeter
2,4-D	2,4-dichlorophenoxyacetic acid
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol; Cleland's reagent
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FTIR	Fourier Transform Infra Red
g	Grams
G	Guaiacyl
GUS	β-glucuronidase

$g\ L^{-1}$	grams per litre
$HAuCl_4$	Hydrochloroauric acid
h	Hour (s)
hph	hygromycin phosphotransferase gene
HTCG 600	Heat treated carbon-gold at 600^{o}C
2ip	2-isopentenyl adenine
IPTG	Isopropyl-beta-D-thiogalactopyranoside
ITCC	Indian Type Culture Collection
kb/Kbp	Kilobase pairs
kD/kDa	Kilo Daltons
L	Liter
LB	Luria-Bertani
min	minutes
mg	Milligram
mL	Milli liter
mM	Milli molar
MCS	Multiple cloning sites
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
µL	Micro liter
µg	Microgram
NAA	1-Napthyl aceticacid
N	Normality
ng	Nanogram
nm	Nano meter
OD	Optical density
O/N	Overnight
PAL	Phenylalanine ammonia lyase
pCA	p-coumaryl alcohol
pI	Isoelectric point
PEG	Polyethylene glycol

PMSF	Phenylmethanesulphonylfluoride
psi	pounds per square inch
PVPP	Poly vinyl pyro phosphate
pmol	Pico mole
RACE	Rapid amplification of cDNA ends
RNase A/ (H)	Ribonuclease A/(H)
rpm	Rotations per minute
RT	Room temperature
SA	Sinapyl alcohol
SAD	Sinapyl alcohol dehydrogenase
SEM	Scanning electron microscope
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMQ	Sterile milli Q water
SSC	Saline sodium citrate
s	Second
TAE	Tris acetic EDTA buffer
T-DNA	Transfer DNA
TDZ	Thidiazuron
TE	Tris EDTA buffer
TEM	Transmission Electron Microscopy
TEMED	Tetramethylethylenediamine
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
x g	Relative centrifugal force
XRD	X-ray diffractograms
YEP	Yeast extract peptone
°C	Degree centigrade

ABSTRACT

Lignin is a hydrophobic heteropolymer that is synthesized in the secondary cell walls of all vascular plants. In plants, lignin enables upright growth habits, water conduction through the stem and also protects the plants against invading pests and pathogens. From the commercial point of view, lignin hinders the utilisation of the cellulosic cell walls of plants in pulp and paper industry and as forage. Furthermore, the resistance of lignin to microbial degradation enhances persistence of plant biomass in soil leading to decreased biodegradation. From these industrial and agricultural perspectives, it is desirable to develop improved plant varieties with altered lignin content and composition

Biosynthesis of lignin precursors (p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) takes place in cytoplasm through phenylpropanoid pathway, these monomers are transported into the cell wall and oxidised by peroxidases and laccases to phenoxy radicals that couple to form the lignin heteropolymer. The presence of residual lignin results in pulp and paper with inferior performance characteristics and poor brightness stability or yellowing with age. During paper pulping, lignin is removed from wood by chemical and energy intensive processes, which are neither economical nor eco-friendly. Paper industry in India mainly uses bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena* sp. as a source for paper pulp. Although all these plant species are of importance to the paper industry, *Leucaena leucocephala* is exclusively used in India and contribute to around 25% of raw materials to paper and pulp industry.

The present study is aimed at understanding lignin biosynthesis pathway in *Leucaena leucocephala*. The current research gave emphasis on the less well-known polymerisation stage, so as to identify, isolate and characterize lignin biosynthetic peroxidases (class III). Classical peroxidases are monomeric, heme-containing glycoproteins that are found exclusively in the secretory pathways. These enzymes can dehydrogenate cinnamyl alcohols, and many other phenolic substrates, at the expense of H_2O_2.

No study has been done so far in this regard, anywhere on *Leucaena sp.* Most of the lignin biosynthetic enzymes are encoded by several genes, all of which may not participate in lignin biosynthesis. Therefore, in this study, we investigated the expression of identified lignin biosynthetic peroxidases during developmental and stress-induced lignification process, using

real-time RT-PCR and immuno-localization. Also, the responses of peroxidase down-regulated *Leucaena leucocephala* to their wild type plants were evaluated. Over all, the present study may be helpful for development of transgenic *L. leucocephala* plants with desired characters suitable for Indian pulp and paper industry.

The main features of the present thesis are:

- Cloning and characterization of lignin biosynthetic peroxidase (*POX*) gene from *Leucaena leucocephala.*
- Studies on spatial and temporal expression of *POX* gene in *Leucaena leucocephala*.
- Transformation of *Leucaena leucocephala* plant with antisense *POX* construct and its analysis.

Isolation, cloning and characterization of lignin biosynthetic Class III peroxidase gene

This chapter deals with the PCR based approach for fishing out the peroxidase gene from *Leucaena leucocephala*. Primers were designed based on consensus region of various reported sequences of the peroxidase genes from the NCBI GenBank database. A partial peroxidase gene was amplified which showed highest homology to Class III peroxidases. Rapid Amplification of cDNA Ends (RACE) was performed to obtain the 5' and 3' UTR regions. Primers were designed from these UTRs to amplify the full length peroxidase gene. Characterization of the gene encoding lignin biosynthetic Class III peroxidase is embodied in this chapter.

(A) Heterologous expression of lignin biosynthetic Class III peroxidase, its purification and characterization

The cDNA clone of peroxidase was cloned into the expression vector pET-28a(+) and was expressed in *E. coli* BL21 (DE3) strain. The Protein was purified from inclusion bodies using Ni-chelated affinity column and used to raise polyclonal anti-bodies in rabbit. The present study showed that, peroxidase being a highly glycosylated protein need to be expressed in eukaryotic expression system to get the protein expressed in its active form. Hence, cDNA coding for mature peptide of peroxidase was cloned into pPICZα A vector and expressed in *Pichia pastoris* as secretory protein and was purified and characterized.

(B) Spatio-temporal expression and immuno-cytolocalization of peroxidase

This section will describe the expression pattern of lignin biosynthetic Class III peroxidase (*POX*) gene in different plant parts at different intervals of time. The spatial and temporal expression of the peroxidase gene was studied using quantitative PCR *i.e.* Real Time PCR. The 5.8S rRNA was used as the internal standard. Plants of different age *i.e.* seedling of age 5 day, 10 day, 15 day and 20 days were used for temporal expression studies. Plant parts mainly root and shoot only used for preliminary spatial expression studies.

Antibodies raised in rabbit against, lignin biosynthetic Class III peroxidase (POX) protein was used to immuno-cytolocalize the candidate protein in *Leucaena leucocephala*. Transverse sections of root and shoot of 5 day, 10 day, 15 day and 20 days old seedlings were used for immuno-cytolocalization studies.

Transformation of antisense peroxidase gene in *Leucaena leucocephala* and analysis of transformants.

This chapter presents, the first time report of the biological synthesis of a carbon-gold (HTC 600-Au) composite nano particle and its use as microcarrier in biolistic transformation of plant systems. The plant transformation binary vector pCAMBIA 2301 with the reporter gene GUS, harbouring the peroxidase gene in anti-sense orientation was used for the study. Two different strategies namely, particle bombardment and particle bombardment followed by co-cultivation were used and will be described in detail. Evaluation and analysis of transgenic plants, for putative transformants and confirmation of integration of these genes in *Leucaena leucocephala* genome by GUS assay and by molecular techniques like PCR, DNA sequencing and Southern/slot blotting is presented in this chapter.

CHAPTER 1

1. Introduction

Evolution of the land plants and their ability to colonise the earth required a mechanism that allowed the transport of water and nutrients from the roots into the aerial parts. This was achieved by the evolution of water conducting elements, which are hollow, interconnected cells that form a continuum from the roots to the leaves and to the shoot apex. These water conducting cells contain a thick secondary cell wall that mainly consists of parallel chains of cellulose, which are organized through hydrogen bonding to microfibrils and further lamellar structures. In addition to cellulose hemicellulose are also incorporated. To allow water conduction through the cells, the hydrophilic carbohydrates are incrusted with lignin, which makes the cell walls hydrophobic and impermeable to water. Lignin also gives compressive strength to the vascular elements to withstand the negative pressure that is thought to be generated by transpiration and the cohesive movement of water along the vascular elements (Steudle, 2001). Compressive strength is also needed to support the weight of the stem and the crown, especially in trees. Therefore, lignin is indispensable to land plants. In addition to the vascular elements, a few other cell types namely, fibres and sclereids are also lignified in plants (Esau, 1960). Lignified fibres are long cells, in which the lumen is nearly filled with secondary cell wall. Sclereids also contain secondary cell wall but they are smaller in size than fibres. Lignin also functions as a defence barrier against invading pathogens and is formed in response to wounding. On the other hand, lignin hinders utilization of the cellulosic cell walls of plants as forage and its commercial use in pulp and paper industry, and thus has a tremendous economical impact.

The occurrence of lignin in the plant kingdom is restricted to vascular plants. It is found from pteridophytes and higher plants but not from bryophytes, although some Phenylpropanoid metabolites have been detected in mosses (Lewis and Yamamoto, 1990). Majority of lignin is synthesized in trees, in which most of the secondary xylem is lignified. In gymnosperms, the xylem consists of lignifying tracheids, which act as both water conducting and supporting structures, and ray parenchyma performs horizontal transport of nutrients. Ray tracheids are associated with ray parenchyma, which also have a lignified secondary cell wall. In angiosperms, the water conducting cells are the large

vessel elements that contain secondary thickenings, as well as the less abundant tracheids, whereas fibres act as supporting structures.

Lignin biosynthesis is a part of the Phenylpropanoid metabolism, which also includes the diverse flavanoids, coumarins, suberin and lignans. The biosynthetic pathways of these compounds are partly shared, which has complicated the view on lignin biosynthesis. The research on lignin biosynthesis has focused on a few model species, which include both trees and herbaceous plants, example, tobacco (*Nicotiana tabacum* L.). Among the tree species, poplars are commonly used due to the available genomic sequence from black cottonwood (*Populus trichocarpa* Torr. & Gray) (Tuskan *et al*., 2006) and to the relative ease of the generation of transgenic plants and clonal material. Species of *Eucalyptus* and *Pinus* have also been utilized. The herbaceous model plant *Arabidopsis thaliana* (L.) Heynh. is widely used for studies on both secondary cell wall development and lignin biosynthesis (Nieminen *et al*., 2004; Groover, 2005). In addition to the lignified vascular elements, the *Arabidopsis* inflorescence stem contain interfascicular fibres that have thickened secondary cell walls with both G and S lignin (Ehlting *et al*., 2005).

The following sections will describe the existing knowledge on the lignin hetero polymer and its biosynthesis, including the synthesis of the precursors, their transport into apoplast and polymerization to form lignin. The current views on the regulation of the enzymes involved lignification process, their importance and the role of *Leucaena leucocephala* with special reference to Indian paper and pulp industry is discussed.

1.1 Pulp and Paper Industry

In India the annual production of paper is close to 5 million MT. Worldwide annual production of paper has increased more than three fold in the past forty years, amounting to a total production of 120 x 10^6 tons. India's annual soft woods and pulp imports are worth US $ 300 million and the projected annual short fall of paper and paper products is expected to grow to approximately 4 million MT by the year 2010. In addition, the growth of paper and paper products consumption in India is expected to be the highest in Asia during the current decade.

To produce bright and good quality paper, pulp may require a further bleaching treatment to remove lignin residues. The delignification process consumes large quantities of

energy and hazardous chemicals. Reducing the content or changing the quality of lignin in pulp wood species without compromising the mechanical strength of the plant is desirable for paper industry. This would be beneficial, both from the economical as well as environmental point of view. The results could be extrapolated to the forage crops to improve digestibility since the presence of lignin limits the ability of microorganisms to break down the cellulose and hemicellulose in the animal alimentary canal. Thus, it has become imperative to achieve self sufficiency in paper and pulp production, which may be achieved with the development of fast growing trees providing higher biomass with low lignin content per unit of land. Wood, agricultural residues and many other plant materials, which can be used for pulp and paper production, consist largely of lingo-cellulose (a composite of mainly cellulose, hemicellulose and lignin). In the production of paper, pulp (which is mainly cellulose), the hemicellulose and much of the lignin are removed using mechanical or chemical processes or a combination of both.

1.2 Lignin

The term Lignin was introduced in 1819 by *de Candolle* and is derived from the Latin word *lignum*, meaning wood. After cellulose, lignin is most abundant natural (terrestrial) organic polymer and a major constituent of wood. It forms an integral cell wall component of all vascular plants, representing on an average of 25% of the terrestrial plant biomass. Lignin content is higher in softwoods (27-33%) than in hardwood (18-25%) and grasses (17-24%). The highest amounts of lignin (35-40 %) occur in compression wood on the lower part of branches and leaning stems (Fengel and Wegner, 1984; Sarkanen and Ludwig, 1971). Lignin does not occur in algae, lichens or mosses (Nimz and Tutschek, 1977), whereas the “lignins” of bark differ in their structure from typical wood lignins (Zimmermann *et al.*, 1985). The ability to synthesize lignin has been essential in the evolutionary adaptation of plants from an aquatic environment to land and provides crucial structural integrity to the cell wall stiffness and strength of the stem (Chabannes *et al.,* 2001 and Jones *et al*., 2001). Lignin biosynthesis begins in the cytosol with the synthesis of glycosylated monolignols from the amino acid phenylalanine. These first reactions are shared with the phenylpropanoid pathway. The attached glucose renders them water soluble and less toxic. Once transported through the cell membrane to the apoplast, the glucose is removed and the polymerization commences. Lignin is

predominantly synthesized and deposited in the secondary cell wall of specialized cells such as xylem vessels, tracheids and fibers. It is also deposited in minor amounts in the periderm where in association with suberin it provides a protective role against pathogens (Sarkanen and Ludwig, 1971). In addition, lignin waterproofs the cell wall, enabling transport of water and solutes through the vascular system. Though lignin has been studied for more than a century, many aspects of its biosynthesis remain unresolved. The monolignol biosynthetic pathway has been redrawn many times and remains a matter of debate (Dixon *et al.,* 2001; Humphreys and Chapple, 2002). Likewise, the biochemical processes leading to dehydrogenation of the monolignols in the cell wall and their polymerization and deposition are fields of active discussion (Davin and Lewis, 2000; Hatfield and Vermerris, 2001; Lee *et al.,* 1997; Sederoff *et al.,* 1999).

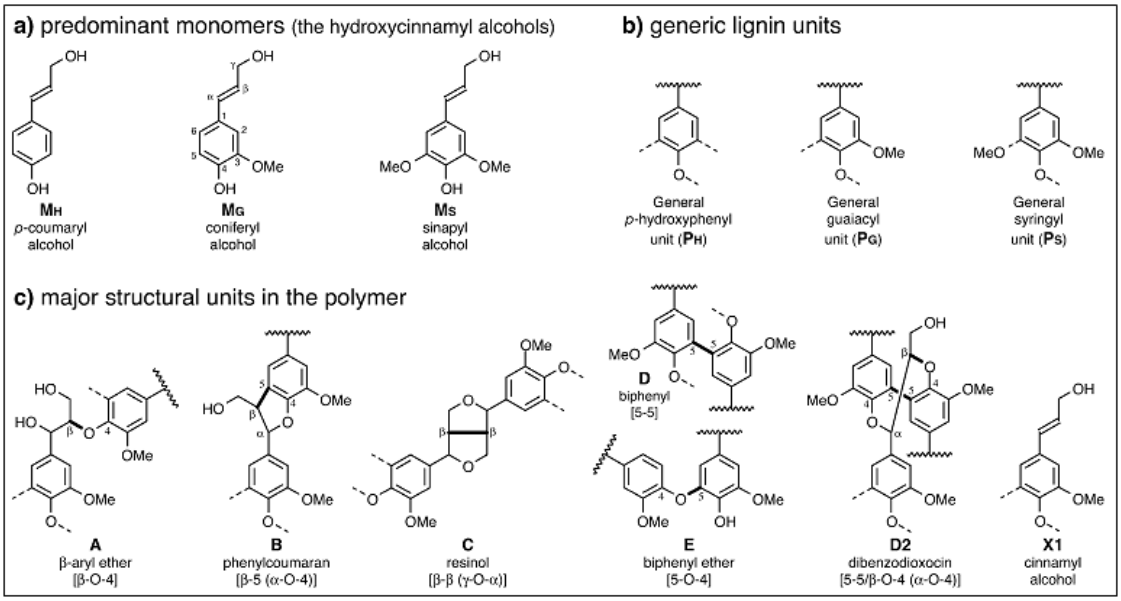


Fig. 1: (a) Primary lignin monomers **M** , the monolignols. (b) Lignin polymer **P** units are denoted based on the methoxyl substitution on the aromatic ring as generic **PH**, **PG** and **PS** units. (c) Major structural units in the polymer; the bolded bonds are the ones formed in the radical coupling reactions. The numbering follows that established by Boerjan *et al*., 2003.

Lignins are complex racemic aromatic heteropolymers synthesized from the dehydrogenative polymerization of monolignols, namely coumaryl, coniferyl and sinapyl alcohol monomers differing in their degree of methoxylation (Freudenberg and Neish, 1968) (Fig. 1a). These monolignols produce, respectively, *p*-hydroxyphenyl (**H)**,

guaiacyl (**G)**, and syringyl (**S)** phenylpropanoid units when incorporated into the lignin polymer (Fig. 1b). The amount and composition of lignins vary among taxa, cell types and individual cell wall layers, and are influenced by developmental and environmental cues (Campbell and Sederoff, 1996).

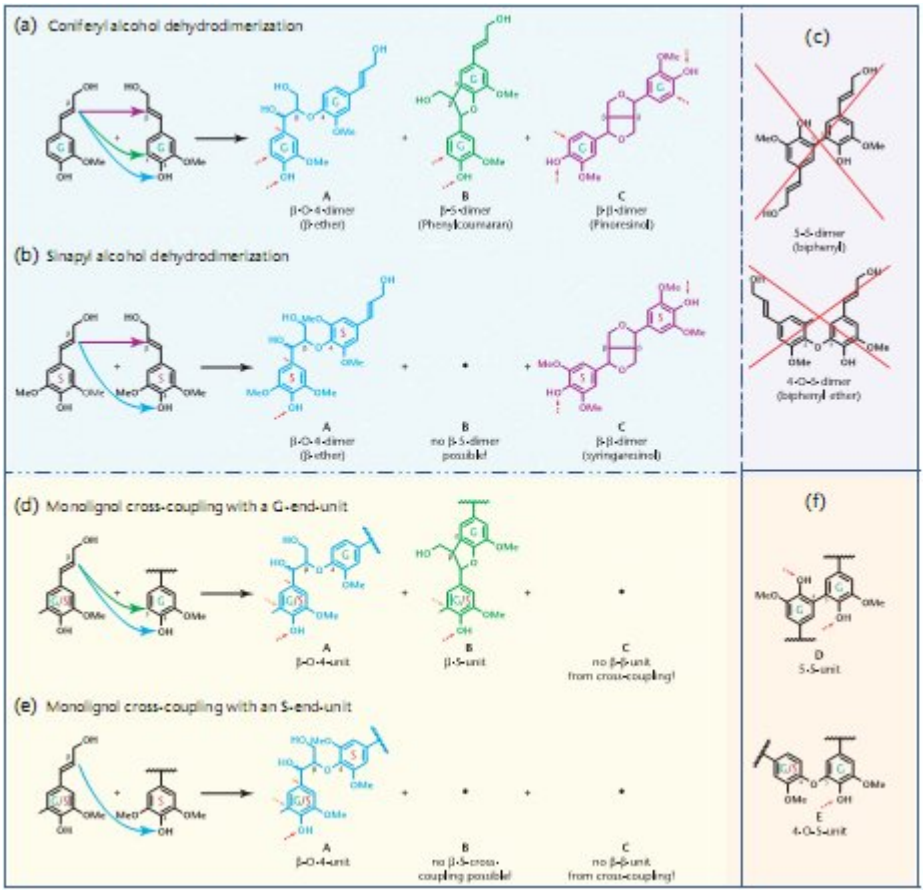


Fig. 2: Lignification differs substantially from dimerization of monolignols. (a) Dehydrodimerization of coniferyl alcohol produces three dehydrodimers in comparable a mounts. The new bond formed by the radical coupling reaction is drawn bolder. (b) Dehydrodimerization of sinapyl alcohol produces only two products. (c) Dehydrodimerization does not produce these structures. (d) Cross-coupling of a hydroxycinnamyl alcohol with a G unit gives only two main products. (e) Cross-coupling of a hydroxycinnamyl alcohol with an S unit leads almost exclusively to β -ether unit s A. When the polymer phenolic end unit is a β ether, β–1-coupling may also occur to a relatively minor extent. (f) Coupling of preformed oligomers is the source of the 5–5- and 4–O–5 units. Red arrows indicate sites at which further radical coupling can occur during lignification; the lighter arrow to the 5-positions in (d) and (e) structures shows where coupling can occur in G units but not in S units (where the 5 -position is occupied by a methoxyl group).

Dicotyledonous angiosperm (hardwood) lignins consist principally of **G** and **S** units and traces of **H** units, whereas gymnosperm (softwood) lignins are composed mostly of **G** units with low levels of **H** units. Lignins from grasses (monocots) incorporate **G** and **S** units at comparable low levels, and more **H** units than dicots (Baucher *et al*., 1998). Lignification is the process by which H, G and S units are linked together via radical coupling reactions (Sarkanen and Ludwig, 1971; Freudenberg and Neish, 1968). The main "end-wise" reaction couples a new monomer (usually a monolignol and usually at its β position) to the growing polymer, giving rise to different structures (Fig. 1c) all of which are β-linked. Coupling between preformed lignin oligomers results in units linked 5–5 and 5–O–4. The coupling of two monolignols is a minor event, with resinol (β–β) units or cinnamyl alcohol end groups as the outcome. Monolignol dimerization and lignin are substantially different processes (Adler, 1977), explaining why lignification produces frequencies of the various units that are different from those produced by dimerization or bulk polymerization *in vitro* (Fig. 2).

1.3 Lignification

Lignification takes place during the growing season. In tree species, the extensive secondary xylem is formed by the vascular cambium, which is an undifferenciated layer of cells, equivalent to the shoot apical meristem (Groover, 2005). It surrounds the stem and produces xylem inwards and the phloem outwards as the cambial cells divide. This division is followed by the differentiation process, including cell expansion and elongation through the turgor driven expansion of the wall and the deposition of new primary cell wall material, mainly pectin, hemicellulose and cellulose. Once the final dimensions of the cell has been reached, the deposition of the secondary cell wall begins, followed closely by lignification. In the final phase, the cells undergo programmed cell death (Plomion *et al*., 2001). In gymnosperm species, *Picea abies* (L.) Karst. (Norway spruce) and *Pinus sylvestris* L. (Scots pine) secondary growth and lignification takes place between late May and August. In deciduous trees, such as *Betula pendula* Roth. (Silver birch) this occurs in a shorter time frame, in june and july (Marjamaa *et al*., 2003),

allowing time for the formation of photosynthesizing new leaves in the spring, and for the recycling of the leaf nutrients towards autumn before the dormant season.

The formation of a thick secondary cell wall is typical for all lignifying cell types. The wall usually contains three layers, S1 to S3, in which the cellulose microfibril (Fig. 3A) angle changes relative to the longitudinal cell axis, resulting in increased tensile strength in multiple dimensions (Donaldson, 2001; Plomion *et al*., 2001). Lignification starts already during cellulose synthesis, beginning from the cell corners and middle lamella and proceeding towards the lumen (Terashima *et al*., 1988). This first stage begins after the start of S1 formation (Fig. 3B).

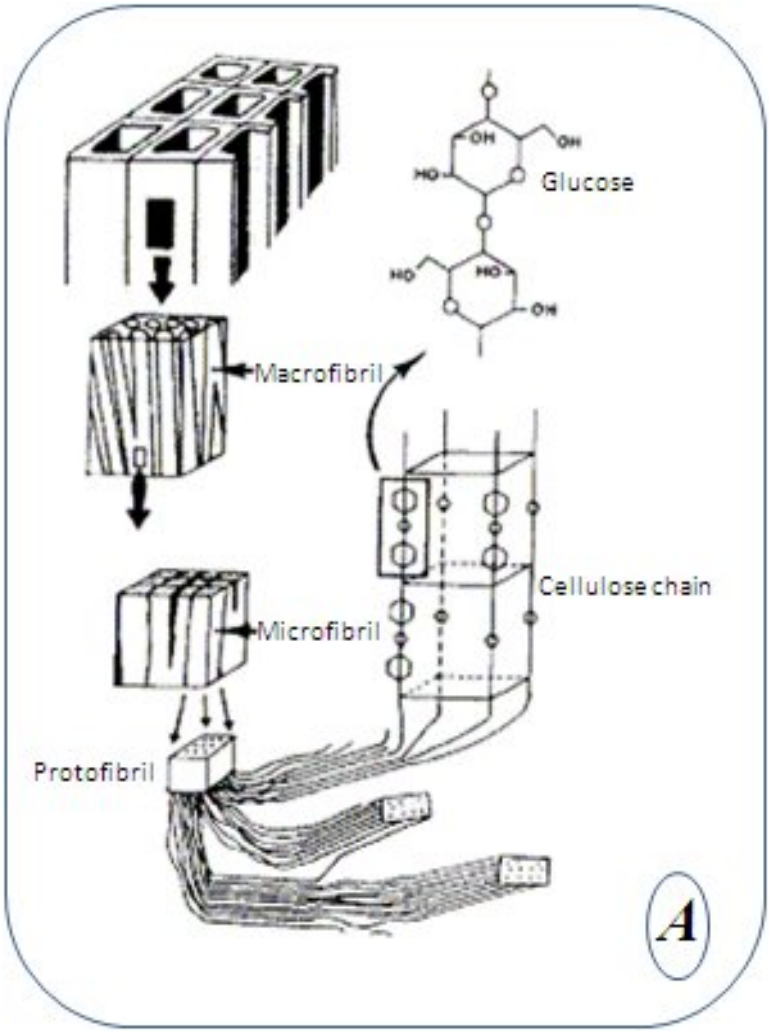


Fig. 3 A: Organization of the cellulose skeletons in the fiber wall (Parham, 1987).

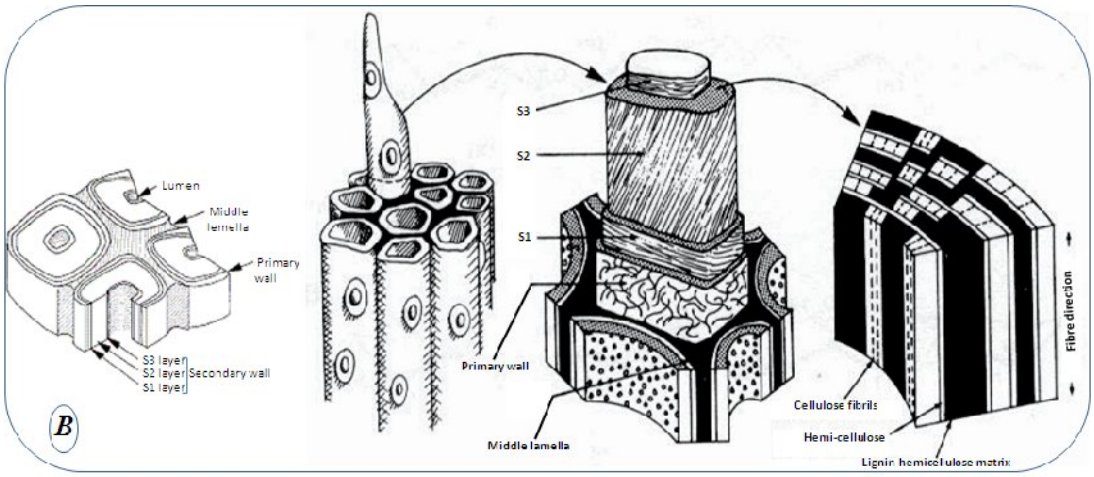


Fig. 3 B: A schematic presentation of wood structure showing adjacent tracheids, diameter of each tracheid is approximately 30 µm (left), wood cell wall layers S1, S2 and S3: secondary cell wall layers, primary wall, middle lamella (middle) and lignin-carbohydrate complex of the secondary cell wall (right). Figure from Kirk and Cullen (1998).

During the second stage, lignin deposition into the S2 layer is started while polysaccharide deposition still continues. The main lignification takes place after the formation of S3 layer (Terashima *et al*., 1988). During lignification, the water-filled pores between the cellulose microfibrils are filled with lignin, which replaces water and is thought to form covalent linkages with the carbohydrates. In the mature wall, the secondary cell wall contains upto 80% of the total lignin because of its greater volume. However, the lignin concentration is highest in the middle lamella, upto 85% of weight (Donaldson, 2001).

Wood

Wood characteristics vary in different types of plant. For instance, conifers (gymnosperms) produce softwood whereas, angiosperms produce hardwoods. Softwoods are mainly composed of three cell types, tracheids (which play a role both in rigidity and conduction), and axial and ray parenchyma cells. Hardwoods are mainly made of fibers, vessels, and axial and ray parenchyma cells. Vessels transport water and solutes through the vascular system while fibers provide rigidity, and ray cells facilitate centripetal nutrition (Higuchi, 1997). Tracheids, vessels and fibers vary in shape and size (Table 1).

The dimensions and chemical composition of the different cell types of wood depend on genetic, developmental and environmental factors (Vallette and de Choudens, 1992).

Softwoods and hardwoods differ in their pulping characteristics. Indeed, the individual cell types within wood differ in their chemical characteristics, reflecting the underlying differences in biochemistry and molecular biology that are only beginning to be appreciated. The three major components of wood cell walls are cellulose, hemicellulose and lignin. Long molecules of cellulose provide the skeleton of the walls. Linear cellulose chains are aligned together in structures known as 'elementary fibrils' or 'protofibrils' that, in turn, associate into more complex structures called microfibrils (Fig. 3A). Microfibrils are highly organized and form distinct fibrillar cell wall layers (Delmer and Amor, 1995). Hemicelluloses and other carbohydrates provide the matrix of the cell wall. Lignin, a heterogenous hydrophobic phenolic polymer, encrusts the other wall components to waterproof and strengthen the wall. In a transverse plane, the parietal structure of wood cells is made of a primary and a secondary wall, the latter consisting of two or three layers, designated S1, S2, and S3 (Fig. 3B).

Table 1: Dimensions of the different cell types of softwoods and hardwoods (Fengel and Wegener, 1984).

Cell Type	Character	Cell Dimensions
Soft wood (***Picea abies***)		
Tracheids	Diameter	20 – 40 µm
	Wall thickness	2.1 – 4.3 µm
	Length	1.7 – 3.7 mm
Hard wood (***Fagus sp.***)		
Fibers	Diameter	15 – 20 µm
	Wall thickness	5 µm
	Length	0.6 – 1.3 mm
Vessels	Diameter	5 – 100 µm
	Wall thickness	1 µm
	Length	0.3 – 0.7 mm

The various cell wall layers differ in chemical composition (Mellerowicz *et al*., 2001). Lignin deposition is one of the final stages of xylem cell differentiation and mainly takes place during secondary thickening of the cell wall (Donaldson, 2001). Lignin deposition proceeds in different phases, each preceded by the deposition of carbohydrates, and starts at the cell corners in the region of the middle lamella and the primary wall when S1 formation has initiated. When the formation of the polysaccharide matrix of the S2 layer is completed, lignification proceeds through the secondary wall. The bulk of lignin is deposited after cellulose and hemicellulose have been deposited in the S3 layer. Generally, lignin concentration is higher in the middle lamella and cell corners than in the S2 secondary wall (Baucher *et al*., 1998; Donaldson, 2001; Saka and Goring, 1985). The three monolignols (S, G and H) are incorporated at different stages of cell wall formation. Typically, H units are deposited first, followed by G units and S units still later in angiosperms (Donaldson, 2001; Terashima *et al*., 1995). Lignin in vessels is generally enriched in G units, whereas lignin in fibers is typically enriched in S units (Saka and Goring, 1985). A large proportion of S units is also found in secondary walls of ray parenchyma (Fergus and Goring, 1970). In gymnosperms, the lignin deposited in compression wood is enriched in H units (Timell, 1986). The difference in timing of monolignol deposition is associated with variations in lignin condensation in the individual cell wall layers, as shown by immunocytochemistry with antibodies raised against pure H, pure G, or mixed GS synthetic lignins (Chabannes *et al*., 2001; Joseleau and Ruel, 1997). Lignin deposition and the relative incorporation of the different monolignols into the polymer, are spatially and temporally regulated. The mechanisms controlling this process are not yet fully resolved but are likely governed by the interplay between the spatio-temporal expression of monolignol biosynthetic genes, the kinetics of monolignol delivery to the cell wall and the chemistry of monolignol coupling to the growing polymer in the complex macromolecular environment of the cell wall.

1.4 Review of the literature

The major source of fiber for paper products comes from the vegetative tissues of vascular plants. Although almost any vascular plant could be used for paper production, the economics of scale require a high fiber yield for paper manufacture. By far, the principal source of paper making fibers is wood from trees, the largest vascular plants

available. The type of wood used can also decide the final characteristics of the pulp. In general, softwood fibers are longer than those from hardwood and have thinner cell walls. The longer fibers of softwood promote inter-fiber bonding and produce papers of greater strength.

The fibrous particles used to make paper are made of cellulose, a primary component of the cell walls of vascular plant tissues. The cellulose fibers must be removed from a chemical matrix like lignin, hemicelluloses, and resins leading to relatively pure fibers. In this context, biotechnological approaches are followed to improve soft wood quality by reducing the content of lignin, the second largest organic component, next to cellulose.

Worldwide annual production of paper has increased more than three fold in the past forty years, amounting to a total production of 120 x 10^6 tons. In India the annual production of paper is close to 5 million MT. India's annual soft woods and pulp imports are worth US $ 300 million and the projected annual short fall of paper and paper products is expected to grow to approximately 4 million MT by the year 2010. In addition, the growth of paper and paper products consumption in India is expected to be the highest in Asia during the current decade. Thus, it has become imperative to achieve self sufficiency in paper and pulp production, which may be achieved with the development of fast growing trees providing higher biomass with low lignin content per unit of land.

On the global scale, the production of trees with improved characteristics is expected to help meet the growing need for wood and wood-related products (FAO, 2005), and hence to diminish the pressure on the domestication of natural forest resources in the future (Boerjan, 2005; Merkle & Nairn, 2005; Nehra *et al.,* 2005). Several conifer and angiosperm tree species have been genetically transformed, with the major focus on traits including wood quality, insect and disease resistance and abiotic stress tolerance (Boerjan, 2005; Merkle & Nairn, 2005; Nehra *et al.,* 2005). Transgenic trees with various genetically modified (GM) traits have also been tested in field conditions, e.g. insect resistant *Populus nigra* (Hu *et al*., 2001; Lin *et al.,* 2006), and *Picea glauca* (Lachance *et al.,* 2007), several herbicide-resistant *Populus hybrids* (Meilan *et al.,* 2002), lignin-modified *Populus tremula* x p*opulus alba* (Pilate *et al.,* 2002), fungal disease resistant

Betula pendula (Pasonen *et al.,* 2004), sterile *B. pendula* (EC, 2007) and sterile *P. tremula* x *P. alba* (Wei *et al.,* 2006). Field testing is generally considered to be important for assessment of the potential environmental effects of genetic modification in organisms such as trees that are characterized by e.g. longevity, wind pollination, and multiple ecological interactions in forest ecosystems. Up to now, the commercialization of GM trees has lagged well behind that of GM crop plants. The area of cultivated, commercialized GM crop plants in 2006 covered 102 million hectares (James, 2006), whereas there are only two commercially cultivated GM trees, i.e. the virus-resistant papaya fruit tree in Hawaii (Ferreira *et al.,* 2002) and insect-resistant poplar cultivations in China (FAO, 2004). The ability to modify lignin as a part of cell wall modification is an important area of research in attempts to improve the utilization of plant biomass as a renewable source for sustainable development (Boerjan, 2005; Chiang, 2006; Higuchi, 2006; Li *et al.,* 2006). The development of trees with improved wood quality through modification of the genes involved in lignin biosynthesis could be important for the improved end use of wood material (Chiang, 2006; Higuchi, 2006). In chemical wood pulping, lignin is the main factor hindering the effective utilisation of cellulose fibres, from which it needs to be separated by costly and pollutant generating processes (Chiang, 2002; Baucher *et al.,* 2003; Boerjan, 2005). Genetic modifications resulting in increased delignification (through a modified lignin content and/or chemical composition) could thus be highly beneficial at both the economical and environmental scale (Baucher *et al.,* 2003; Boerjan, 2005; Chiang, 2006).

1.4.1 Lignin Biosynthesis and its Regulation

For the last two decades, there has been a great deal of interest in cloning and characterization of the genes controlling monolignol biosynthesis in order to understand monolignol biosynthetic pathways in trees and other plants. A number of reviews have been done about the advancements of monolignol biosynthesis pathways (Whetten and Sederoff, 1995; Whetten *et al*., 1998; Humphreys and Chapple, 2002; Boerjan *et al*., 2003). There is enormous variation in lignin content and composition among plant species, tissues, cell types, and even developmental stages and environmental conditions. Data from the studies using different plant materials display many agreements as well as certain disagreements. Thus, it is debatable whether lignin biosynthesis in all plants

follows the exact same pathway or not. To date, most of the genes for monolignol biosynthesis have been identified and characterized in various plant species. A summarized picture of the main and possible monolignol biosynthesis pathways of wood formation in trees is shown in Fig. 5. The genes involved in the pathway, the reaction catalyzed by them and their regulation will be discussed.

Natural variations in lignin content and composition observed between different plants, tissues and cell types (Grand *et al*., 1985; 1983; Monties, 1998; Wu *et al*., 1992; Campbell and Sederoff, 1996; Buxton and Redfearn, 1997; Sederoff *et al*., 1999; Donaldson, 2001) as well as occurrence of natural mutants (Ralph *et al*., 1997; Halpin *et al*., 1998) suggest that human induced changes in lignin content and composition through genetic engineering approaches is a realistic possibility. Attempts have been made to genetically modify plant species with the aim of lignin reduction. Conceptually, genetic engineering of lignin can be accomplished at three levels of control, namely synthesis of monolignols, its transport and polymerization at the site of deposition (Fig. 4) as the overall rate of lignification is regulated not only by the monolignol biosynthesis, but also by the coordinated transport, storage, mobilization and polymerization of monolignol precursors to the cell wall. During the past decade, significant headway has been made in the cloning and subsequent expression of sense and antisense constructs of a number of genes pertaining to monolignol biosynthesis, transport and polymerization (Whetten *et al*. 1998; Whetten and Sederoff 1991; Boudet *et al*. 1995; Boerjan *et al*. 2003; Boudet *et al*. 1997; Baucher *et al*. 2003; Boudet 2000, Sederoff 1999; Grima-Pettenati and Goffner 1999). The transgenics have produced unexpected findings leading to a profound reappraisal of our understanding of the phenylpropanoid “metabolic grid”. The enzymes and their genes involved in lignin biosynthesis and related findings of gene regulation of lignin biosynthesis pathway genes are discussed in the following section.

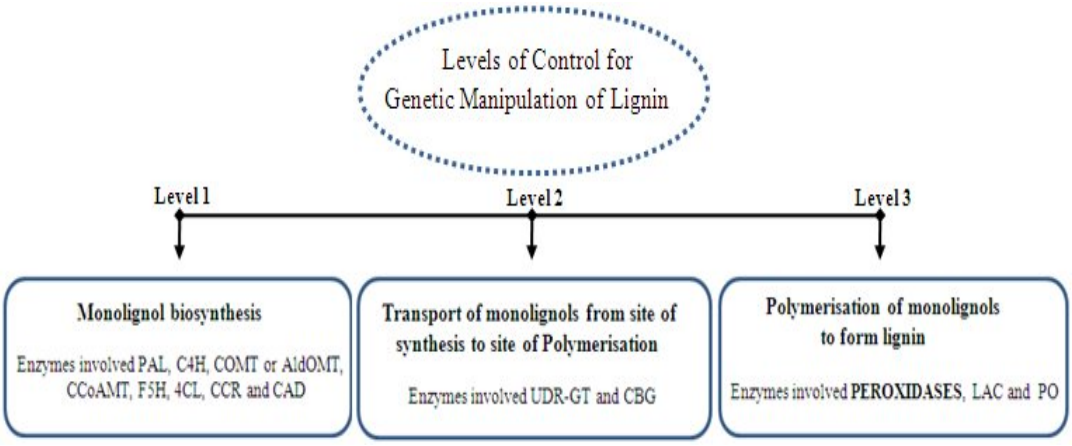


Fig. 4: Levels of control for manipulation of lignin biosynthesis. The control level 1 involves manipulation of genes for PAL, C4H, COMT or AldOMT, CCoAOMT, F5H, 4CL, CCR and CAD, level 2 involves the genes for UDPG-GT and CBG, while level 3 includes POD, LAC and PO

1.4.2 Key enzymes in Lignin Biosynthesis:

Phenylalanine ammonia-lyase (PAL)

Monolignol biosynthesis is considered to start from phenylalanine. The enzyme Phenylalanine ammonia-lyase, PAL, that catalyzes the conversion of phenylalanine to transcinnamic acid, is the initial step towards monolignol biosynthesis and other phenolic secondary plant metabolites. Genes encoding PAL have been studied in *Populus* species (Osakabe *et al*., 1995; Kao *et al*.; 2002), loblolly pine and other plant species (Jones, 1984; Ohl *et al*., 1990; Leyva *et al*., 1992; Bate *et al*., 1994; Hatton *et al*., 1995; Kumar and Ellis, 2001). *PAL* exists as a multiple member gene family and the individual members can be involved in different metabolic pathways as suggested by their expression patterns in association with certain secondary compounds accumulated in specific tissue or developmental stage. The expression studies of two *PAL* genes suggested that one is associated with condensed tannin metabolism and the other with monlignol biosynthesis (Kao *et al*., 2002). In the Arabidopsis genome, four *PAL* genes were identified and were phylogenetically classified into two groups based on sequence similarity (Raes *et al*., 2003). The biochemical activity of all known *PAL*s is verified to specifically catalyze deamination of phenylalanine, but genetic and physiological

function may vary among different *PAL* members. The expression of *PAL* genetic function is controlled by various genetic circuits and signaling pathways.

The *cis*-element structures in *PAL* gene promoters can be part of the molecular circuit that directs a variety of the *PAL* genetic and physiological functions. In some *PAL* promoters, conserved AC *cis*-elements of box P, box A, and box L are identified for regulating the phenylpropanoid genes expression related to monolignol biosynthesis (Cramer *et al*., 1989; Lois *et al*., 1989). In other *PAL* member promoters, the *cis*-elements of H box and G box are found (Cramer *et al*., 1989; Lois *et al*., 1989; Osakabe *et al*., 1995; Leyva *et al*., 1992; Raes *et al*., 2003). Many other *cis*-elements have been identified in members of the *PAL* gene family, however, their function and regulation remains to be studied. *PAL* gene expression has been suppressed by 85% and >98% in transgenic plants with resultant 52% (Sewalt *et al*., 1997) and 70% (Korth *et al*., 2001) reduction in Klason lignin content, respectively. Lignin monomeric composition, determined by pyrolysis GC-MS, was characterized by a lower proportion of G units and a 1.7-fold increase in S/G ratio (Sewalt *et al*., 1997). Because PAL catalyzes the first step of the phenylpropanoid pathway, reduction of its activity results in a wide range of abnormal phenotypes. The transgenic plants were stunted, had curled leaves, and had thinner cell walls in the secondary xylem with less lignin than those of the control (Elkind *et al*., 1990; Bate *et al*., 1994). These plants were also more susceptible to the fungal pathogen *Cercospora nicotianae* (Maher *et al*., 1994). A slight increase in Klasone lignin and dry matter content was observed in the stem of *PAL*-over expressing plants (Howles *et al*., 1996; Korth *et al*., 2001). Over expression of *PAL* did not lead to changes in lignin composition as determined by pyrolysis GC-MS (Sewalt *et al*., 1997), but to a decrease in the amount of S units, yielding a reduction in the S/G ratio when lignin was analyzed by thioacidolysis (Korth *et al*., 2001).

Cinnamate 4-hydroxylase (C4H)

The conversion of cinnamate to *p*-coumarate is catalyzed by C4H. C4H is a cytochrome P450-dependent monooxygenase, belonging to the *CYP73* family. Similar to PAL, C4H is thought to be involved in a number of secondary metabolism pathways in addition to monolignol biosynthesis as *p*-coumarate is an intermediate for biosynthesis of many

secondary compounds (Croteau *et al*., 2000). Multiple *C4H* gene members are identified in many plant species, however, only one *C4H* is known in the Arabidopsis genome (Raes *et al*., 2003). The expression study of two *C4H* members in quaking aspen indicated that one is strongly expressed in developing xylem tissues and the other is more active in leaf and young shoot tissues. In other species, *C4H* gene is expressed in a variety of tissues and the expression is induced by wounding, light, pathogen attacks and other biotic and abiotic stimuli (Bell-Lelong *et al*., 1997; Raes *et al*., 2003). The mechanisms that regulate the genetic function of *C4H* gene and its family members are yet unknown.

In transgenic tobacco plants, C4H activity was altered by expressing the alfalfa class I *C4H* (CYP73A3) (Sewalt *et al*., 1997; Blount *et al*., 2000) or the French bean class II *C4H* (CYP73A15) (Blee *et al*., 2001) genes in sense or antisense orientation (class I and class II *C4H* share approximately 60% similarity). Overexpression of class I *C4H* had no effect on Klasone lignin, nor on the S/G ratio. In contrast, a 76% reduction in total *C4H* activity led to a 63% decrease in Klason lignin and a modification of the lignin monomeric composition. The amount of S units was strongly reduced and S/G decreased by over 90% (Sewalt *et al*., 1997). Similarly, a reduction by 90% of C4H activity by down-regulation of the class II *C4H* resulted in a 27% decreased lignin content and one tobacco transgenic line had a decreased S/G ratio (Blee *et al*., 2001). In transgenic tobacco modified to reduce *C4H* expression, decreased *PAL* activity has also been shown (Blount *et al*., 2000). Regulation may be mediated by pathway intermediates, for example, cinnamic acid may act as a feedback regulator of the phenylpropanoid pathway.

Coumarate 3-hydroxylase (C3H)

Early biochemical evidence suggested that the reaction coumarate to caffeate is catalyzed by a nonspecific phenolase, but that suggestion did not receive much support in other studies (Stafford and Dresler, 1972; Boniwell and Butt, 1986; Kojima and Takeuchi, 1989; Petersen *et al*. 1999). Recently, the gene encoding *p*-coumarate 3-hydroxylase (C3H) was cloned and an alternative pathway proposed based on the enzyme activity of *CYP98A3* gene from Arabidopsis (Schoch *et al*., 2001; Franke *et al*., 2002 a; Nair *et al*., 2002).

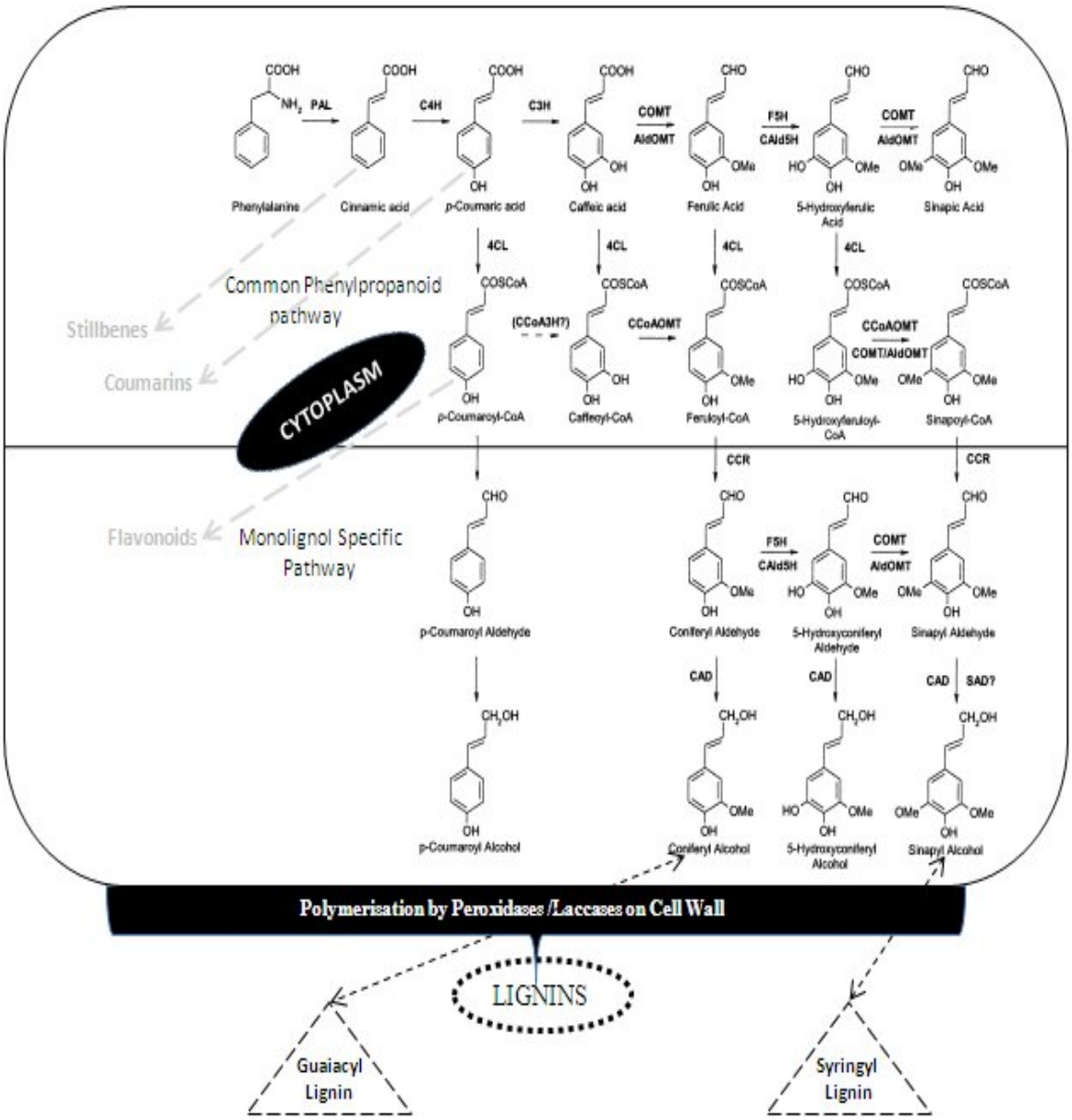


Fig. 5: Biosynthetic pathway for the monolignol precursors of lignin.

PAL*-phenylalanine ammonia-lyase;* ***C4H****-cinnamic acid 4-hydroxylase;* ***C3H****- p-coumarate-3-hydroxylase;* ***CST-****hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase;* ***4CL-*** *4-coumaroyl-CoA-ligase;* ***CCoAOMT-*** *caffeoyl-CoA O-methyltransferase;* ***CCR****-cinnamoyl coenzyme A reductase;* ***CAld5H****-coniferyl aldehyde 5-hydroxylase;* ***AldOMT****-5hydroxyconiferyl aldehyde O-methyltransferase;* ***CAD****-cinnamyl alcohol dehydrogenase;* ***SAD-*** *sinapyl alcohol dehydrogenase*

The proposed alternative suggested that the hydroxylation at the 3-position of the aromatic ring of cinnamic acid does not directly occur on *p*-coumarate, instead, *p*-coumarate is first converted to *p*-coumaroyl CoA ester by 4-cinnamoyl-CoA ligase (4CL), then the CoA ester group of *p*coumaroyl CoA is exchanged by hydroxycinnamoyl-CoA:shikimate hydroxycinnamoyltransferase (CST) to form *p*-coumaroyl shikimic acid which serves as a substrate of *C3H* to produce caffeoyl shikimic acid. Subsequently caffeoyl shikimic acid reverts back to caffeoyl CoA to push metabolism towards the biosynthesis of monolignols. Among tree species, a *CYP98* cDNA was cloned from sweetgum and aspen (Osakabe *et al*., 1999), but the postulated genetic and biochemical functions in monolignol biosynthesis have not been demonstrated for its role in wood formation.

The gene encoding *p*-coumarate 3-hydroxylase (*C3H*) has only recently been cloned by two independent research groups. Using a functional genomics approach, Schoch *et al*. (2001) identified CYP98A3 as a possible candidate for *C3H*. In parallel, by screening *Arabidopsis* mutants under UV light, Franke *et al*. (2002a) isolated the reduced epidermal fluorescence 8 (*ref8*) mutant. By positional cloning, the *REF8* gene was identified as the cytochrome P450 dependent monooxygenase *CYP98A3*. The *ref8* mutant had collapsed xylem vessels, a higher cell wall degradability and a higher susceptibility to fungal colonization (Franke *et al*., 2002b), associated with the accumulation of *p*-coumarate esters instead of sinapoylmalate and with a reduction in lignin content of 60-80%. A range of analyses showed that lignin composition was dramatically altered, being almost entirely madeup of *p*-coumaryl alcohol units (Franke *et al*., 2002b).

Coumarate coenzyme A ligase (4CL)

Genetic and biochemical functions of 4-Coumarate Coenzyme A ligase (*4CL*) genes have been clearly demonstrated in association with monolignol biosynthesis (Lewis and Yamamoto, 1990; Lee *et al*., 1997; Hu *et al*., 1998, 1999; Harding *et al*., 2002). *4CL* genes usually exist as a multi-gene family. Different expression patterns of *4CL* members are found in herbaceous and tree species. Four *4CL* genes were detected in the Arabidopsis genome and the expression of each member was regulated differentially in

tissues and development stages (Raes *et al*., 2003). In aspen trees, two *4CL* genes were cloned and their expression clearly distinct, with one in epidermal and leaf tissue and the other specifically in developing xylem tissue (Hu *et al*., 1998; Harding *et al*., 2002). Furthermore, the enzymatic activities of *4CL* members from aspen, loblolly pine, tobacco, soybean, *Arabidopsis*, and many other species were found to have distinct substrate specificities (Voo *et al*., 1995; Zhang and Chiang, 1997; Hu *et al*., 1998; Lindermayr *et al*., 2003; Schneider *et al*., 2003; Hamberger and Hahlbrock, 2004). Whether the substrate specificity of the *4CL* members relates to different metabolic pathways is unknown. As the *4CL* catalytic kinetics vary among species, it is also likely that the mainstream pathway mediated by *4CL* may not be exactly the same in all plant species or tissues. Nevertheless, monolignol biosynthesis is tightly controlled by *4CL*.

Suppression of *4CL* expression through antisense technology has demonstrated the effectiveness of reducing total lignin content (Lee *et al*., 1997; Hu *et al*., 1999; Li *et al*., 2003). In aspen, suppression of *4CL* expression led to more than 55% lignin reduction in wood. Thus, technology aimed at *4CL* suppression could be applied to plant genetic modification for better fiber production and other utilizations. Transgenic plants with reduced *4CL* activity have been produced in tobacco (Kajita *et al*., 1996, 1997), *Arabidopsis* (Lee *et al*., 1997), and aspen (Hu *et al*., 1999; Li *et al*., 2003). In tobacco, reduction of *4CL* by over 90% resulted in 25% less lignin. In poplar and *Arabidopsis* with a >90% reduced *4CL* activity, lignin content was reduced by 45–50%. In tobacco, the low *4CL* activity was associated with browning of the xylem tissue (Kajita *et al*., 1996). In transgenic aspen down-regulated for *4CL*, Hu *et al*. (1999) also detected an increase in non lignin alkali-extractable wall-bound phenolics (p-coumaric acid, caffeic acid, and sinapic acid), which were not incorporated into the lignin polymer. However, no difference in lignin S/G composition for *Arabidopsis* and tobacco was observed. Discrepancy between the results published by Kajita *et al*. (1997) and Hu *et al*. (1999) is that the transgenic tobacco lines with the most severe reduction in lignin content (25%) were characterized by a collapse of vessel cell walls and reduced growth (Kajita *et al*., 1997), whereas the transgenic poplars with a 45% reduction in lignin content had a normal cell morphology and a higher growth rate than the control (Hu *et al*., 1999). The increased level of hydroxycinnamic acids as non-lignin cell wall constituents has been

suggested to contribute to the cell wall strength in transgenic poplar (Hu *et al*., 1999). Because several 4CL isozymes exist with different cell-specific expression, down-regulation of several or all isozymes simultaneously may perturb metabolite levels other than those involved in lignin, with a secondary effect on growth as a consequence. Antisense inhibition of *4CL* in aspen trees led to a 15% increase in cellulose content. These results suggest that lignin and cellulose deposition are regulated in a compensatory fashion and that a reduced carbon flow toward phenylpropanoid biosynthesis increases the availability of carbon for cellulose biosynthesis (Hu *et al*., 1999; Li *et al*., 2003). A combinatorial down-regulation of *4CL* along with an over expression of *F5H* in xylem has been achieved by co-transformation of two *Agrobacterium* strains in aspen (Li *et al*., 2003). Additive effects of independent transformation were observed, in particular a 52% reduction in lignin content associated with a proportional increase in cellulose and a higher S/G ratio.

Caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT) and Caffeate-*O*-methyl-transferase (COMT) or 5-hydroxyconiferaldehyde-*O*-methyltransferase (AldOMT)

In monolignol biosynthesis, methylation is required at the 3-carbon and at the 5-carbon positions on the aromatic ring of the monolignol unit. The 3-carbon methylation leads to guaicyl unit formation and methylations on the 3-and 5-positions results in a syringyl unit. In early studies, it was postulated that two types of methyltransferases were necessary for methylations (Higuchi, 1997). Mono-functional *O*-methyltransferase to methylate the 3rd position and therefore controlled the G monolignol unit biosynthesis, and the bi-functional *O*-methyltransferase to catalyze both 3-and 5-methylations leading to S monolignol unit biosynthesis. However, molecular studies have revealed that there are two kinds of genes encoding for the enzymes that specifically catalyze the 3-and 5-methylation, respectively (Ye *et al*., 1994; Li *et al*., 1999, 2000; Chen *et al*., 2001). Biochemical evidence from the studies using tree material suggests that the 3-and 5-methylations occur at different biosynthesis stages. The 3-methylation occurs on the CoA ester intermediate while the 5th position is methylated at the aldehyde intermediate (Osakabe *et al*., 1995; Li *et al*., 2000). The two genes are: one encoding a *CCoAOMT* and the other encoding a 5-*O*methyltransferase that preferably methylates 5-hydroxyconiferaldehyde. The 5-*O*methyltransferase was thought to methylate caffeic acid

and was named *COMT* accordingly. However, more recent evidence indicates that the methylation catalyzed by COMT enzyme basically occurs at 5-hydroxyconiferaldehyde, therefore the enzyme was renamed *AldOMT* (Li *et al*., 2000). In addition to these two types of OMTs involved in the monolignol biosynthesis in angiosperms, another OMT (named AEOMT) that can methylate both hydroxycinnamic acids and hydroxycinnamoyl CoA esters was found in the gymnosperm loblolly pine (Li *et al*., 1997). However, identification of *AldOMT* in gymnosperms has not been reported. It appears that *CCoAOMT* gene plays a predominant role in the gymnosperm lignin biosynthesis. In loblolly pine *CCoAOMT* was detected as a single copy and specifically expressed in developing xylem (Li *et al*., 1999). Down-regulation of COMT activity has been achieved using either antisense or sense transgenes in tobacco (Dwivedi *et al*., 1994; Ni *et al*., 1994; Atanassova *et al*., 1995), poplar (Van Doorsselaere *et al*., 1995; Tsai *et al*., 1998; Jouanin *et al*., 2000) and alfalfa (Guo *et al* 2001). In all three species, drastic reductions in the lignin S/G ratio were apparent and an unusual phenolic compound 5-hydroxyconiferyl alcohol (5OHG) was present in the polymer (Atanassova *et al*., 1995; Van Doorsselaere *et al*., 1995; Tsai *et al*., 1998; Lapierre *et al*., 1999; Jouanin *et al*., 2000; Guo *et al*., 2001; Marita *et al*., 2003).

In the lignin of the transgenic poplars described by Jouanin *et al*. (2000), the level of 5OHG units even exceeded that of S units. In COMT suppressed alfalfa, β-β, β-1, and β-5 linkages involving S units, were absent (Guo *et al*., 2001), whereas in *COMT*-down-regulated poplar, free phenolic groups in β-O-4-linked G units were less abundant (Lapierre *et al*., 1999). In *COMT*-suppressed poplar, reduced lignin content has been reported (Jouanin *et al*., 2000) as well as no change in lignin amount in poplar (Van Doorsselaere *et al*., 1995) and aspen (Tsai *et al*., 1998). Reports describing *COMT*-suppression in tobacco also differ on whether lignin content is (Ni *et al*., 1994) or is not (Dwivedi *et al*., 1994; Atanassova *et al*., 1995) reduced. Despite these discrepancies, the data from all of the *COMT*-suppressed tobacco and poplar plants indicate that COMT plays a predominant role in determining the incorporation of S units into the lignin polymer. In alfalfa, a reduction in *COMT* activity affected both the content of G and S units (Guo *et al*., 2001; Marita *et al*., 2003). In accordance with these results, Parvathi *et al*. (2001) found that in alfalfa COMT is also involved in the methylation of caffeoyl

aldehyde. Down regulation of *CCoAOMT* affected the Klason lignin content by 12–50% in transgenic tobacco (Zhong *et al*., 1998; Pincon *et al*., 2001a), alfalfa (Guo *et al*., 2001; Marita *et al*., 2003) and poplar (Meyermans *et al*., 2000; Zhong *et al*., 2000). In tobacco and poplar, the decreased lignin content was due to reduction of both G and S units as determined by pyrolysis GC-MS (Zhong *et al*., 1998, 2000) or thioacidolysis (Meyermans *et al*., 2000). Because, the decrease in G units was more pronounced, the S/G ratio increased (Zhong *et al*., 1998; Meyermans *et al*., 2000). In contrast, the S unit amount was not reduced in transgenic alfalfa (Guo *et al*., 2001) nor transgenic tobacco (Pincon *et al*., 2001a). In contrast to the transgenic poplars, which were not affected in growth or morphology, the transgenic tobacco plants down-regulated for *CCoAOMT* had collapsed vessel walls (probably because of the reduced lignin content) and altered growth and flower development (Pincon *et al*., 2001a). Simultaneous down-regulation of both COMT and CCoAOMT in tobacco (Zhong *et al*., 1998; Pincon *et al*., 2001a) and alfalfa (Guo *et al*., 2001) resulted in combinatorial and/or additive effects. There was a greater reduction in Klasone lignin content in tobacco (Pincon *et al*., 2001a) but not in alfalfa (Guo *et al*., 2001). In both species, the lignin S/G ratio was reduced although in tobacco this was due to decreases in both G and S units (Zhong *et al*., 1998), whereas only S units decreased in alfalfa (Guo *et al*., 2001).

Cinnamoyl coenzyme A reductase (CCR)

The reduction of cinnamoyl CoA esters to cinnamaldehydes is the first metabolic step committed to monolignol formation. This step is catalyzed by CCR. Many studies of CCR activity indicated that five cinnamoyl-CoA esters *viz. p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA, could be used as substrate (Wengenmayer *et al*., 1976; Luderitz and Grisebach, 1981; Sarni *et al*., 1984; Goffner *et al*., 1994). The CCR enzyme purified from *Eucalyptus* xylem tissue was active toward *p*coumaroyl-CoA, feruloyl-CoA, caffeoyl-CoA and sinapoyl-CoA with approximately equal affinity (Goffner *et al*., 1994). Similar to the native protein, the recombinant *Eucalyptus* CCR protein was also demonstrated to be active with the substrates *p*-coumaroyl-CoA, feruloyl-CoA and sinapoyl-CoA (Lacombe *et al*., 1997). Recently, the characterization of aspen CCR recombinant protein indicated that CCR selectively catalyzed the reduction of feruloyl-CoA from the five cinnamoyl CoA esters

(Li *et al*., 2005). When CCR and CCoAOMT were coupled together, the linked reactions constitute the pathways from caffeoyl-CoA ester to coniferaldehyde (Fig. 5). In addition, the results also suggested that the neighboring CCoAOMT and CCR enzymes require different pH environments and compartmentalization *in vivo*.

The *CCR* genes in various species appear as a multiple members family. In the *Populus* genome, there exist 8 CCR-homolog or *CCR*-like gene sequences. Transgenic tobacco (Piquemal *et al*., 1998; Ralph *et al*., 1998; O'Connell *et al*., 2002) and *Arabidopsis* (Goujon *et al*., 2003b) down-regulated for *CCR*, are characterized by an approximate 50% decrease in Klason lignin. The lignin S/G ratio was increased (mainly because of a decrease in the G unit amount) in transgenic tobacco and variable, depending on the growth conditions, in transgenic *Arabidopsis*. A change in the lignin structure was also indicated by the higher amount of alkali-labile material that could be released from the extractive-free lignin polymer of the transgenic lines (O'Connell *et al*., 2002). The transgenic plants with the lowest *CCR* activity and 50% reduced lignin had abnormal phenotypes, such as collapsed vessels, stunted growth, and abnormal leaf development. Important alterations in the fiber cell walls were observed, such as a loosening in the arrangement of the cellulose microfibrils, which resulted in reduced cell wall cohesion (Pincon *et al*., 2001b; Goujon *et al*., 2003b). Also an increased amount of tyramine ferulate (Figure 5), an unsual component of tobacco cell walls that is probably a sink for feruloyl-CoA, was incorporated into the lignin of the CCR-down-regulated tobacco plants (Ralph *et al*., 1998). A *CCR* mutant, designated irregular xylem (*irx4*), has been identified in *Arabidopsis* (Jones *et al*., 2001*)*. Like the *CCR*down-regulated tobacco and *Arabidopsis* described above, this mutant is characterized by a 50% reduced lignin content, collapse of the vessels, and an altered growth and morphology. By crossing transgenic tobacco down-regulated for *COMT* (Atanassova *et al*., 1995) with tobacco down-regulated for *CCR* (Piquemal *et al*., 1998), a simultaneous reduction in *COMT* and *CCR* expression was achieved (Pincon *et al*., 2001b).

Ferulate 5-hydroxylase/ Coniferaldehyde 5-hydroxylase (F5H/CAld5H)

A necessary step to biosynthesize S monolignols is hydroxylation at the 5-position on the aromatic ring of cinnamic intermediates. This reaction was thought to occur using ferulic

acid as the substrate and catalyzed by F5H, which is encoded by a P450 protein gene belonging to *CYP84* family. Although forward genetics evidence demonstrated that *F5H* gene is essential for S-lignin formation in Arabidopsis (Meyer *et al*., 1996), it was unable to identify the intermediate on which the 5-hydroxylation biochemically occurs. Homologous genes have been cloned from a number of tree species. The biochemical function of this P450 gene was first demonstrated by expressing a sweetgum *CYP84* gene in yeast (Osakabe *et al*., 1999). The biochemical data suggest that the *CYP84* protein catalyzes 5-hydroxylation using coniferaldehyde, instead of the postulated ferulic acid, as a substrate to produce 5-hydroxyconiferaldehyde. Thus, *F5H* is actually a *CAld5H*. The 5-hydroxylation of coniferaldehyde was further confirmed with an Arabidopsis *CYP84* recombinant protein (Humphreys *et al*., 1999). According to the biochemical function of this *CYP84* gene, it was suggested that the S-monolignol biosynthesis pathway is branched out from a guaicyl intermediate at coniferaldehyde. Consistent with this view, 5-hydroxyconiferaldehyde is then methylated by *COMT* or *AldOMT* as described above. The genetic function of *CYP84* is also demonstrated through a reverse genetics approach by over expression of the gene, which leads to the intensified S units in lignin (Franke *et al*., 2000; Li *et al*., 2003). Because the lignin with higher percentages of S-unit has a potentially significant value in the pulping economy (Chang and Sarkanen, 1973), over expression of *CAld5H* gene in trees has great potential to produce desirable wood material for fiber production. An *Arabidopsis* mutant deficient in *F5H* (*fah1*) has been described and it produced lignin deficient in S units (Chapple *et al*., 1992) with a consequently increased frequency of phenylcoumaran (β-5) and biphenyl (5-5) linkages (Marita *et al*., 1999). When *Arabidopsis F5H* was overexpressed from the *C4H* promoter in the mutant, a lignin almost entirely composed of S units linked by β-O-4 linkages was produced (Meyer *et al*., 1998; Marita *et al*., 1999). The proportion of S units in the lignin of these plants was the highest ever reported for any plant (Ralph, 1996). Similarly, lignin of tobacco and poplar transformed with the same chimeric gene was enriched in S units (Franke *et al*., 2000). Li *et al*. (2003) overexpressed a sweetgum *F5H* (*Cald5H*) under the control of a xylem-specific promoter (*Pt4CL1P*) in transgenic aspen and reported a 2.5-fold increase in the S/G ratio and no changes in lignin content. An accelerated maturation/lignification of stem secondary xylem cells was noted in these *F5H* over

expressing plants (Li *et al*., 2003). A 25–35% reduction in Klasone lignin content was observed in *F5H*-overexpressing *Arabidopsis* (Marita *et al*., 1999) and tobacco (Franke *et al*., 2000).

Cinnamyl alcohol dehydrogenase (CAD) and Sinapyl alcohol dehydrogenase (SAD)

In gymnosperm wood, coniferyl alcohol is the major monolignol unit while both coniferyl alcohol and sinapyl alcohol are monolignols in angiosperm wood. The last metabolic step forming these monolignols is reduction of coniferaldehyde and sinapaldehyde. CAD is suggested to catalyze multiple cinnamyl alcohol formations from their corresponding cinnamaldehydes (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995; Whetten *et al*., 1998). In loblolly pine, *CAD* is a single copy gene and its mutation leads to abnormal lignin formation in wood (MacKay *et al*., 1997; Lapierre *et al*., 2000). When the *Populus* tree was studied for monolignol biosynthesis in wood-forming tissue, in addition to *CAD*, it was found in aspen that another gene, its sequence similar to but distinct from *CAD*, is also associated with lignin biosynthesis (Li *et al*., 2001). The biochemical characterization of the recombinant protein encoded by this gene indicated that the enzymatic activity has specific affinity toward sinapaldehyde, therefore it was named SAD. Compared with SAD enzyme kinetics, CAD showed a catalytic specificity towards coniferaldehyde instead. The catalytic specificities of the two enzymes have recently been further verified in protein structure analysis (Bomati and Noel, 2005).

Furthermore, it was demonstrated that the expression of *CAD* is associated with G-lignin accumulation while *SAD* was associated with S-lignin formation during xylem differentiation (Li *et al*., 2001). The evidence from molecular, biochemical and cellular characterizations strongly suggest that *CAD* is involved in G-monolignol biosynthesis and *SAD* in S-monolignol biosynthesis in aspen wood formation. However, a recent genetic study using an *Arabidopsis* model system suggests a broad CAD function for both G-and S-lignin biosynthesis in the herbaceous species (Sibout *et al*., 2005). Nevertheless, more evidence connecting the biochemical function to its genetic role may be required in order to completely understand how *CAD* and *SAD* genes play a role in monolignol biosynthesis during wood formation. Transgenic plants with reduced *CAD*

activity have been produced in tobacco (Halpin *et al*., 1994; Hibino *et al*., 1995; Stewart *et al*., 1997; Yahiaoui *et al*., 1998), poplar (Baucher *et al*., 1996) and alfalfa (Baucher *et al*., 1999), whereas *CAD* mutants exist in pine (MacKay *et al*., 1997), maize (Halpin *et al*., 1998) and *Arabidopsis* (Sibout *et al*., 2003). An unusual monomer, dihydroconiferyl alcohol, was shown to be incorporated into the lignin of the pine *CAD* mutant and accounted for 30% of the lignin compared to only 3% in wild-type lignin (Ralph *et al*., 1997). Accordingly, higher amounts of arylpropane-1,3-diol structures, arising from dihydroconiferyl alcohol, have been found in the lignin of the pine *CAD* mutant (Ralph *et al*., 1999b, 2001a). In contrast, no dihydroconiferyl alcohol has been found in the lignin of transgenic angiosperms down-regulated for *CAD*, such as tobacco and poplar (Ralph *et al*., 1998). A higher amount of cinnamaldehydes has been detected in the lignin of *CAD*-down-regulated tobacco (Halpin *et al*., 1994: Ralph *et al*., 1999a, 2001a), poplars (Kim *et al*., 2002), pine *CAD* mutant (Ralph *et al*., 1997) and in the *Arabidopsis Atcad-D* mutant (Sibout *et al*., 2003). However, only a slighty lower Klasone lignin content was measured in the wood of transgenic poplar lines downregulated for *CAD* (Lapierre *et al*., 1999; Pilate *et al*., 2002), in the pine *CAD* mutant (MacKay *et al*., 1997) and in the *Arabidopsis Atcad-D* mutant (Sibout *et al*., 2003). The lignin of plants with low *CAD* activity was more extractable in alkali (Halpin *et al*., 1994; Baucher *et al*., 1996; Bernard-Vailh´e *et al*., 1996; Yahiaoui *et al*., 1998; MacKay *et al*., 1999). The S/G ratio of the lignin of transgenic tobacco (Ralph *et al*., 1998) and transgenic alfalfa (Baucher *et al*., 1999) was reduced, suggesting that in these plants the uncondensed S structures are more affected than their G analogs. These data are in apparent conflict with the recent proposal that *SAD*, and not *CAD*, is involved in S lignin biosynthesis in angiosperms (Li *et al*., 2001).

A simultaneous down-regulation of *CAD* and *CCR* has been achieved by crossing homozygous transgenic lines in which either *CAD* (Halpin *et al*., 1994) or *CCR* (Piquemal *et al*., 1998) was downregulated (Chabannes *et al*., 2001b). The lignin content was decreased by approximately 50% in tobacco with 32% of wild-type *CCR* activity and 12% of wild-type *CAD* activity. The phenotype of the double transformants was normal with only slight alterations in the vessel shape, showing that, similarly to the results of Zhong *et al*. (1998) and Hu *et al*. (1999), plants can also tolerate important reductions in lignin content. A simultaneous suppression of *COMT* (to 24% of wild-type level), *CCR*

(to18%of wild-type evel), and *CAD* (to 4% of wild-type level) was achieved in tobacco by a single chimeric construct, consisting of partial sense sequences for the three different genes. The transgenic lines were stunted and had characteristics of *COMT*, *CCR*, and *CAD* suppression in lignin; for example, the xylem was red (indicative of *CAD* suppression), contained collapsed vessels (indicative of *CCR* suppression), and had reduced staining for S lignin (indicative of *COMT* suppression) (Abbott *et al*., 2002). In analysis of functional genes involved in monolignol biosynthesis of angiosperm, three genes, *CAld5H*, *AldOMT* and *SAD*, control a line of consecutive metabolic steps and constitute a pathway toward S-monolignol biosynthesis. These three genes have not been known to be present in gymnosperm species that do not synthesize S-lignin. Gymnosperm wood is primarily comprised of tracheid elements, but angiosperm wood contains two types of thickened secondary wall cells, vessel element and fiber cells. Apparently the fiber cell is evolved along with occurrence of angiosperm species. It is known that G-monolignol units are dominant in tracheids and vessels and S units predominate fiber cells. It can be postulated that monolignol biosynthesis pathway evolution may be correlated with cell type specification in the course of plant evolution; however, this hypothesis remains to be verified. It is believed that lignin is polymerized at the outside of the plasma membrane in secondary cell walls. Thus, monolignols that are synthesized inside plasma membrane need to be transported across plasma membranes for polymerization.

1.4.3 Storage and transport of the monolignols into the apoplast

After their synthesis, the monolignols are either stored, putatively in the vacuole, as more hydrophilic and less toxic conjugates, or transported into apoplast (Boerjan *et al*., 2003). Accumulation of monolignol 4-*O*-glucosides (p-coumaryl alcohol glucoside, coniferin and syringin) have been observed in the cambial sap of conifers and some woody angiosperms during cambial activation (Freudenberg and Harkin, 1963; Terazawa *et al*., 1984). Radio tracer experiments have shown that glucoside-derived monolignols can be incorporated into lignin, though unexpectedly partly through conversion into aldehyde first (Terashima *et al*., 1988; Tsuji and Fukushima, 2004). Savidge *et al*., (1998) speculated that glucoside conjugation could prevent a premature lignification during early secondary cell wall development. A role for coniferin as a quickly metabolisable

precursor for defence-related phenolics was also suggested (Bednarek *et al*., 2005). A UDP-glucose: coniferyl alcohol glucosyltransferase activity has been identified from the cambial sap of Norway spruce and pine (Schmid and Grisebach, 1982; Savidge and Forster, 1998). The spruce enzyme preferred coniferyl alcohol, while pine enzyme catalysed the conjugation of both coniferyl alcohol and sinapyl alcohol equally well.

The accumulation of monolignol glucosides could be a tree-specific phenomenon, as reports from herbaceous species are rare. However, three homologous glucosyltransferases that were able to glucosylate both CA and SA, Na the corresponding aldehydes and acids *in vitro,* have been identified in *Arabidopsis* (Lim *et al*., 2001 and 2005). For polymerization into lignin, transport of the monolignols into the apoplast is needed. The transport form, whether glucoside or aglucone, is unknown. However, the identification and cell wall localization of a coniferin- hydrolyzing β-glucosidase in, spruce and pine argues for the glucoside transport (Marcinowski and Grisebach, 1978; Dharmawardhana *et al*., 1995 and 1999; Samuels *et al*., 2002). Two β-glucosidases that hydrolyse monolignol glucosides have also been identified from *Arabidopsis* (Escamilla-Trevino *et al*., 2006). Of these, BGLU45 was specific for coniferin and syringing, while BGLU46 had higher activity towards p-coumaryl alcohol glucoside, and hydrolysed other phenolic glucosides as well (Escamilla-Trevino *et al*., 2006). Their expression pattern coincided with lignifying tissues, supporting a role in lignification. The reported K_m values of β -glucosidases for monolignol glucosides vary from 0.18 to 7 mM (Marcinowski and Grisebach, 1978; Dharmawardhana *et al*., 1995; Escamilla-Trevino *et al*., 2006). The high values suggest that the biological relevance of β-glucosidases might be low; however, if the 10 mM concentrations reported for coniferin in pine cambial sap are generally found, the high K_m values would not be a problem.

Three transport mechanisms have been suggested for monolignol. Firstly, monolignol aglucones could diffuse freely through the membranes (Boija and Johansson, 2006), provided that free monolignols do not reach toxic concentrations. Another possibility is the ATP-binding cassette (ABC) transporters that carry small-molecular-weight compounds across membranes (Yazaki, 2006). Several ABC transporters were coregulated with lignin biosynthetic genes during the development of *Arabidopsis* inflorescence stem (Ehlting *et al*., 2005). Also vesicular secretion has been suggested as a

means for transport. Supporting this, radioactive label derived from phenylalanine and cinnamate was localized to Golgi-associated vesicles in wheat coleoptiles and *Cryptomeria japonica* (L.f) D. Don (Pickett-Heaps, 1968; Takabe *et al*., 1985). In developing contorta pine (*Pinus contorta* Dougl.) tracheids, dark-stained, putatively phenolic vesicles appeared on the trans-Golgi network during secondary cell wall synthesis and early lignification (Samuels *et al*., 2002). As no hemicellulose was localized to the vesicles, they were proposed to be involved in monolignol (glucoside) secretion. Accordingly to this model, a controlled vesicular secretion of glucosides and the consecutive deglucosylation in the cell wall would provide precursors for lignification during cell wall synthesis, whereas the vacuolar rupture during the programmed cell death would release the stored monolignol glucosides from the vacuole for the final stage of lignification (Samuels *et al*., 2002).

1.4.4 Polymerization of monolignols

After transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols (Christesen *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. Lignin is a hydrophobic and optically inactive polymer, which is highly complex and heterogeneous in nature. Lignin polymerization is a radical coupling reaction, where the monolignols are first activated into phenoxy radicals in an enzyme catalysed dehydrogenation reaction. The radicals couple to form dimmers, oligomers and eventually the lignin polymer (Freudenberg, 1968). The radicals are stabilized through resonance structures, which also allow the radical to form a covalent bond with another radical on several locations on the monolignol to form different lignin substructures (Fig. 6). For the polymer to grow, radicals must also be formed on larger molecules, such as monolignol oligomers or polymeric lignin. The reaction is a stepwise addition of monolignols onto the growing polymer, so called ‘end-wise polymerization’ (Sarkanen, 1971). Several possibilities for radical generation on polymeric lignin have been suggested. First, a monolignol radical could withdraw an electron from the polymer, returning to the ground sate for re-oxidation. This was shown to take place between monolignols (Takahama, 1995), and it requires that the monolignol radical has a higher

redox potential than the electron donating lignin substructure. Secondly, a shuttle mechanism involving Mn^{2+}/Mn^{3+} ions could be involved in radical transfer was shown by Onnerud *et al* ., 2002. A poplar peroxidase that oxidises polymeric lignin (Sasaki *et al*., 2004) suggests that a direct enzymatic oxidation may be the predominant mechanism for the generation of radicals on the lignin polymer.

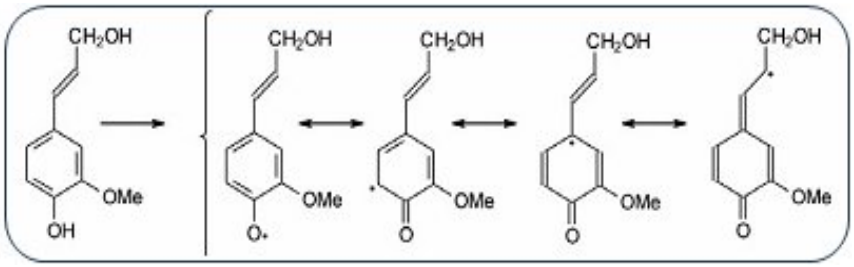


Fig. 6: The resonance structures of a coniferyl alcohol radical

Lignin polymerization at molecular level is a matter of controversy. The simplest model states that the polymerization is a chemical coupling reaction guided by the redox potentials of the radicals and by the chemical environment like, pH and monolignol concentration (Ralph *et al*., 2004). The opposite view emphasizes a strict biochemical/enzymatic control through dirigent like proteins and a template guided polymerization (Guan *et al*., 1997; Gang *et al*., 1999).

Dirigent-like proteins and template polymerization

The new theory arose from the discovery of a class of dirigent proteins implicated in lignan biosynthesis (Davin *et al*., 1997). Dirigent genes have been found by homology in both gymnosperms and angiosperms and they were expressed in the cambial region and the ray parenchyma cells in *Forsythia* (Burlat *et al*., 2001). The first such dirigent protein discovered, guided the dimerization of coniferyl alcohol radicals to produce an optically active lignan, pinoresinol. The corresponding gene was cloned and shown to encode a cell wall localized protein. The finding was extrapolated to lignification, suggesting that such proteins would logically be responsible for specifying the exact structure of the lignin polymer, bringing lignins in line with proteins and polysaccharides that are more carefully biosynthesized (Devin and Lewis, 2000; Lewis, 1999). In mature xylem, dirigent like proteins were immunolocalized to the middle lamella and the S1 layer of the secondary

cell wall (Burlat *et al*., 2001). Dirigent family genes were expressed at a low level in the xylem of western red cedar (*Thuja plicata* D. Don) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) (Kim *et al*., 2002; Ralph *et al*., 2006a). In Sitka spruce shoot, higher expression was found in the shoot tip than in the woody shoot base and only two dirigent gene had a secondary xylem preferred expression pattern (Friedmann *et al*., 2007). The expression of most dirigent-like genes was induced after insect feeding in Sitka spruce bark and xylem, suggesting a preferential role in defence (Ralph *et al*., 2006a). The dirigent proteins are different from lignan forming proteins and to act as monolignol binding sites in the initial phase of lignification to guide the formation of the primary structures of lignin (Gang *et al*., 1999). These primary structures would then be replicated through template polymerization, in which the preformed chain is used as the template (Guan *et al*., 1997; Chen and Sarkanen, 2003). However, no repeating structures have been found in lignin, casting doubt on the template polymerization theory (Ralph *et al*., 2004).

Initiation sites for lignin polymerization

Lignin is first deposited in the middle lamella and the cell corners of the primary wall after the formation of the secondary wall has started. Some factor controls the site of initial lignin polymerization and prevents monolignols from being polymerized next to the plasma membrane directly after the transport into the cell wall. The nature of these nucleation sites is unknown. Ferulates, conjugated to polysaccharides, and their dehydrodimers are well established. Evidence, that ferulates and diferulates may act as attachment sites for monolignols, has been shown (Ralph *et al*., 1995). Given that the middle lamella and the cell corners are rich in Ca^{2+} pectate (Carpita and Gibeut, 1993) and are the first sites to be lignified, Ca^{2+} pectate-bound peroxidases may conceivably play a role in the spatial control of lignin deposition, and changes in Ca^{2+} and H^{+} concentrations may modulate the location of these peroxidases (Carpin *et al*., 2001). The negatively charged pectins are also good binding sites for polyamines (Carpin *et al*., 2001) and, hence, may be suitable sites for H_2O_2 generation by polyamine oxidases (Moller and McPherson, 1998). Pectin-binding peroxidases and polyamine oxidases may act locally in the early stages of lignin deposition both for H_2O_2 generation and oxidation of monolignols, cinnamic acids bound to polysaccharides or polyamines, or aromatic residues on certain proteins, such as glycine-rich proteins (Keller *et al*., 1989). The correct placement of the initiation sites in the cell wall was

suggested to be regulated through the vesicular secretion of pectin and other hemicelluloses and potentially associated initiation factors (Samuels *et al*., 2002).

Enzymes for the formation of monolignol radicals

Oxidative enzymes catalyse the activation of the monolignols into radicals. Several enzymes are being proposed, including peroxidases (Harkin and Obst, 1973), laccases (Sterjiades *et al*., 1992), other phenol oxidases (Savidge and Udagama-Randeniya, 1992) and even cytochrome c oxidase (Koblitz and Koblitz, 1964). Most of these enzymes exist as numerous isoenzymes and generally have broad substrate specificity. Due to this, high affinity to the monolignols or mere temporal and spatial correlation with lignification can only be considered as suggestive for a role in lignin polymerization. Conclusive evidence is only obtained from transgenic plants in which the enzymatic activity has been down-regulated and which show changes in lignin quantity and quality.

1.5 Peroxidases as candidate gene

The plant peroxidase super family contains three classes of peroxidases from plants, fungi and bacteria (Welinder, 1991). The Class I of the super family contains bacterial peroxidases, the yeast cytochrome c peroxidases and intracellular plant ascorbate peroxidases. Class II consists of fungal peroxidases and Class III consists of plant secretory peroxidases. This Class III comprises all the plant peroxidases that are targeted to the endoplasmic reticulum, either for secretion to the cell wall or transport into the vacuole.

Peroxidases are heme-containing oxidoreductases that use H_2O_2 as the ultimate electron acceptor. The natural electron donor molecules in a peroxidase catalysed reaction vary and include, monolignols, hydroxycinnamic acids (Zimmerlin *et al*., 1994), tyrosine residues in extensions (Brownleader *et al*., 1995) and auxin (Hinman and Lang, 1965). In addition to polymerization, peroxidases are also able to produce O_2^{-} and H_2O_2 through an oxidative cycle (Yokota and Yamazaki, 1965) and even produce hydroxyl radicals through a hydroxylic cycle if a suitable reductant is present (Chen and Schopfer, 1999). Hence, in a plant, peroxidase have a plethora of functions, including the regulation of the balance between cell wall growth and cross linking, lignification, suberisation, accumulation of heavy metals, degradation of toxic molecules and production of reactive

oxygen species during wounding or pathogen attack. Peroxidases are also involved in nodulation, mycorrhization and senescence (Passardi *et al*., 2005). The genomic sequences of *Arabidopsis* (Arabidopsis Genome Initiative, 2000) and rice (International Rice Genome Sequencing Project, 2005) contained 73 and 138 peroxidase genes, respectively (Tognolli *et al*., 2002; Welinder *et al*., 2002; Passardi *et al*., 2004).

However, some genes were actively transcribed and 0.85% of all *Arabidopsis* ESTs encoded for Class III peroxidases (Welinder *et al*., 2002). A majority of peroxidases were expressed in roots and several responded to various biotic and abiotic stimuli (Hiraga *et al*., 2000; Welinder *et al*., 2002). Welinder *et al*., 2002 reported that even highly similar (>70%) peroxidase genes were differentially expressed, indicating subfunctionalisation after gene duplication. The amino acid sequences of Class III peroxidases vary, but the size and the general structure is conserved. The classic fold contains several α-helices with two Ca^{2+} ions and a non-covalently bound heme that is mostly buried inside the protein (Schuller *et al*., 1996, Gajhede *et al*., 1997). Based on their isoelectric points (pIs), peroxidases can be divided into acidic, neutral and basic isoforms, but no correlation of the function with the pI of peroxidases has been observed. Isoenzymes are glycosylated to varying degree through asparagines residues, which increases the heterogeneity of peroxidases at the protein level (Veitch, 2004).

Some peroxidases contain a C-terminal extension that is thought to direct the protein into vacuole (Welinder *et al*., 2002). The catalytic cycle involves the reduction of H_2O_2 to water by the electrons from reducing substrates, like monolignols, which are oxidised to radicals ($R^{\cdot}$) **(**$H_2O_2 + 2RH \longrightarrow 2R^{\cdot} + 2H_2O$**)**. The active site of peroxidases contains three amino acid residues important for the catalytic mechanism. The distal His42 (according to horseradish peroxidase C1 (HRP C1)) Arg38 and Pro139, are involved in hydrogen bonding and proton transfers that are prerequisites for electron transfers and O-O cleavage (Henriksen *et al*., 1999). In addition, several hydrophobic residues are important in creating an aromatic binding pocket for the reducing substrate (Henriksen *et al*., 1998; Ostergaard *et al*., 2000). The binding pocket in most peroxidase is, however, large enough to accommodate a variety of reducing substrates, resulting in generally low substrate specificity. This, together with the putative redundancy caused by large number of peroxidase isoenzymes has made it difficult to relate given isoenzymes with specific

physiological processes. Several reports on peroxidase activity or gene expression in lignin-forming tissues have appeared, but only a few isoenzymes or genes have been specifically associated with lignification (Sato *et al*., 1993; Quiroga *et al*., 2000; Christensen *et al*., 2001; Marjamaa *et al*., 2006). Although several reports on the transcriptional regulation of monolignol biosynthesis exist, not much is known for peroxidases. The promoter of the HRP *C2* gene, was shown to contain an AC element that is found in many monolignol biosynthetic genes (Raes *et al*., 2003). The promoter was activated by NtLIM1, an AC element binding transcription factor from tobacco, which was required for both basal and wound-induced expression of the *C2* peroxidase (Kaothein *et al*., 2002). It is likely that the control of the whole lignification process requires a mechanism for the coordinated expression and/or activation of the monolignol biosynthetic genes/enzymes and the radical-forming peroxidases. Data from transgenic plants down-regulated for peroxidase activity has confirmed the role of some peroxidase isoforms in lignin polymerization (Talas-Ogras *et al*., 2001; Blee *et al*., 2003; Li *et al*., 2003b).

Quantitative (up to 50%) and qualitative changes were reported in transgenic plants, but no obvious growth phenotypes, other than larger xylem elements, were found. Antisense expression of the *TP60* peroxidase gene in tobacco resulted in an equal reduction of both G and S units, suggesting the existence of a feedback regulation to decrease the monolignol synthesis and transportation under reduced oxidative capacity in the apoplast (Blee *et al*., 2003). But, no metabolite analysis other than phloroglucinol staining was performed to confirm that the monolignols or their derivatives did not accumulate in xylem. In aspen, down regulation of *PRXA3a* gene reduced the lignin content by 20%. Incorporation of G units into lignin decreased while S units remained at the wild type level (Li *et al*., 2003b). Peroxidases from *Populus alba* (L.) and *Z. elegans* were shown to oxidise SA efficiently (Sasaki *et al*., 2004; Gabaldon *et al*., 2005). The *Populus* peroxidase, CWPO-C was immunolocalized into the middle lamella and cell corners of poplar xylem fibre walls, coinciding partly with the S type lignin (Sasaki *et al*., 2006). Interestingly, CWPO-C was also found in the cytosol of ray parenchyma cells, suggesting that ray parenchyma could provide the fibre middle lamella with CWPO-C (Sasaki *et al*., 2006).

Both anionic and cationic peroxidases have been implicated in lignification based on their affinity for coniferyl alcohol, their location in the cell wall and their expression in lignified tissue (M"ader and F¨ussl, 1982; Lagrimini *et al*., 1987; El Mansouri *et al*., 1999). Nevertheless, no change in lignin content was obvious in transgenic tobacco plants that were deficient in the major anionic peroxidase (Lagrimini *et al*., 1997a). However, transgenic poplar with a 44% reduction in the activity of a stem-specific anionic peroxidase (PRXA3a) had a 21% reduced lignin content and a higher content in β-O-4 linked (uncondensed) structures in lignin (Yahong *et al*., 2001). The over expression of peroxidase genes in transgenic poplar (PXP 3-4; Christensen *et al*., 2001a, 2001b) and in tobacco (spi 2; Elfstrand *et al*., 2002) resulted in 800-fold and 5-fold increased total peroxidase activity, respectively. Only 50% reductions at best in lignin amount have been accomplished, argues for redundancy in peroxidase activities. It is likely that *in vivo* several isoenzymes participate in lignin polymerization. Division of labour between the isoenzymes could also exist, both spatially between different cell wall layers and functionally. Polymeric lignin rarely fits into the active site of a peroxidase; however, CWPO-C was also able to oxidise polymeric lignin (Sasaki *et al*., 2004).

H_2O_2 producing enzymes

Peroxidases require H_2O_2 as the ultimate electron acceptor and H_2O_2 synthesis may be the rate limiting factor for lignin polymerization (Nose *et al*., 1995; Gabaldon *et al*., 2006). It has been suggested that, in addition to the lignifying cells themselves, the non lignifying xylem parenchyma cells could also generate H_2O_2 for their lignifying neighbours (Ros Barcelo, 2005). This could be relevant especially during the later stages of xylem lignification. Several mechanisms and enzymes for H_2O_2 production has been suggested, such as diamine and polyamine oxidases, plasma membrane NADPH oxidase, germin-like oxalate oxidase and even peroxidases themselves. However, only indirect evidence for the role of any of the enzymes in lignification exists, based mainly on co-localization.

Copper-containing diamine oxidases and flavin-containing polyamine oxidases are mainly extracellular enzymes that catalyse the catabolism of putrescine, spermidine and spermine, producing H_2O_2 in the process (Cona *et al*., 2006). Expression of diamine oxidase genes correlated with lignification and peroxidase expression in *Arabidopsis*

seedlings (Moller and McPherson, 1998) and polyamine oxidase activity coincided with peroxidase activity in lignifying tissues in chick pea (*Cicer arietinum* L.) epicotyls and maize mesocotyls (Angelini and Federico, 1989). Germin-like oxalate oxidases can also generate H_2O_2 within the cell wall using oxalate and O_2 as substrates (Lane *et al*., 1993). Deposition of lignin-like material also correlated with increased oxalate oxidase and peroxidase activities in aluminium stressed wheat seedlings (Hossain *et al*., 2005) and three germin-like genes had similar expression patterns with the monolignol biosynthetic genes during *Arabidopsis* inflorescence stem development (Ehlting *et al*., 2005). H_2O_2 can also be produced through plasma membrane NADPH oxidase (Lamb and Dixon, 1997). Several NADPH oxidase-like genes appeared coregulated with the monolignol biosynthetic genes in *Arabidopsis*, further supporting their role in lignification (Ehlting *et al*., 2005). Apoplastic peroxidases can also generate $O_2^{\cdot -}$ from a reaction that uses, NADH as a reductant instead of monolignols (Halliwell, 1978; Mader and Amberg-Fisher, 1982). Other possible reductants for the peroxidase mediated $O_2^{\cdot -}$ production include cysteine and indole-3-acetic acid (Ferrer *et al*., 1990; Bolwell, 1996).

Apparently, regulation of the H_2O_2 concentration in the cell wall is complex process. Several enzyme systems exist for H_2O_2 generation and its ultilisation. But a tight regulation is needed to allow for the controlled expansion of the cells and to restrict lignification only to the cells destined to lignify, while maintaining the ability for massive production of reactive oxygen species during stress or pathogen attack.

Laccases

Laccases belong to a family of multi-copper oxidases and they are copper-containing, cell wall–localized glycoproteins that are encoded by multigene families in plants (Hoegger *et al*., 2006). 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 *et al*., 1993; Driouich *et al*., 1992; Ranocha *et al*., 1999; Sterjiades *et al*., 1992). Plant laccases form monophyletic group within the family (Hoegger *et al*., 2006). Laccases are characterized by a blue colour that results from a specific copper coordination. They contain two catalytic centres where altogether four copper ions are found. Laccases oxidise most *o*- and *p*-phenols and even monophenols into radicals ($R^{\cdot}$), reducing

molecular oxygen into water in the process (Ducros *et al*., 1998).

$$(4RH + O_2 \longrightarrow 2R^{\cdot} + 2H_2O)$$

Plant laccases are thought to have a multiplicity of functions, such as lignin polymerization (Sterjiades *et al*., 1992), wound healing (McCaig *et al*., 2005) and even iron oxidation (Hoopes and Dean, 2004; McCaig *et al*., 2005). In plants laccases exist as multigene families (Ranocha *et al*., 1999; Sato *et al*., 2001). High expression of laccases in developing xylem has been observed in many tree species (Sterky *et al*., 1998; Sato *et al*., 2001). The precise role played by laccases in lignification is not yet understood, but there is correlative evidence that laccase and oxygen participate in the polymerization of monolignols. For instance, when peroxidase is inhibited either in the absence of H_2O_2 or in the presence of H_2O_2 scavengers (catalase and superoxide dismutase), coniferyl alcohol is still oxidized and O_2 consumed in tobacco xylem (McDougall *et al*., 1994).

1.6 *Leucaena leucocephala*

***Leucaena leucocephala* plants at different stages. (a) *Leucaena leucocephala* plant in its bloom, (b) *Leucaena* immature pods and (c) *Leucaena* plants with mature pods (D) immature seeds and (E) mature seeds**

Classification of *Leucaena leucocephala*

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.]

Kingdom	Plantae
Super division	Tracheobionta
Division	Spermatophyta
Sub-division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Rosidae
Order	Fabales
Family	Fabaceae
Genus	*Leucaena*
Sub-species	*leucocephala* (Benth) Var. Peru and Cunningham and *ixtahuacana* (Hughes)
Common name	Lead tree, white popinac

Leucaena is a native of Central America and has been naturalized pan-tropically. Members of the genera are vigorous, drought tolerant, highly palatable, high yielding, and rich in protein and grow in a wide range of soils (Jones, 1979; Hughes, 1998). However, these attributes are limited by the occurrence of antinutritive factors in the fodder, such as tannins and mimosine (Jones, 1979; Hegarty *et al*., 1964b; Hammond *et al*., 1989 a, b). *Leucaena* occupies 2 to 5 million hectares of land worldwide (Brewbaker and Sorenson, 1990).They are recognized as some of the fastest growing and most useful trees in the tropics (NRC, 1984). *Leucaena* 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* is a member of the genus related to the other species within the Mimosoideae sub-family, its subspecies and other related genera.

***Leucaena leucocephala* species growth forms**

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 multibranched, semi-erect, medium height (about 10 meters) and the most productive form.

Fodder production and feed value of *Leucaena leucocephala*.

Leucaena leucocephala produces 6 to 18 tons of forage dry matter per hectare in the wet season and 2 to 3 tons of dry matter per hectare in the dry season. All parts of *Leucaena* are edible to livestock (Shelton, 1995). *Leucaena* leaves contain about 19 to 26 percent crude protein. They are rich in source of carotene and vitamins. The provitamin-A in *Leucaena* is among the highest ever recorded in a plant specimen (Jones, 1994). *Leucaena* is rich in calcium and phosphorus, but deficient in sodium and iodine. The *in-vivo* digestibility is 50 to 70 percent (Norton and Poppi, 1995). The presence of mimosine, a tyrosine analogue, a toxic non-protein amino acid, limits the use of this plant

as forage (Allison *et al*., 1990; Gupta and Atreja, 1999; Tangendjaja and Willis, 1980). It is also efficient in nitrogen fixation, at more than 500 kg/ha/year. During the 1970s and 1980s it was promoted as a "miracle tree" for its multiple uses. It has also been described as a "conflict tree" in that it is both promoted for forage production and spreads like a weed in some places.

Wood

Leucaena wood has a thin bark, which is about 8% dry matter at the age of 5 years. The sapwood is yellow-white, while the heartwood is yellow to reddish brown. Bole wood has a specific gravity of 0.54 at the age of 6 to 8 years. This is similar to the density, tensile, compression, bending and shear strength of oak, ash, birch and sugar maple. It is fine textured and workable. It absorbs preservatives, and can be treated against termites (Pottinger and Huges, 1995). *Leucaena* wood is among the best hardwoods for the paper and rayon making. It produces pulp that is high in holocellulose, low in silica, ash, lignin, alcoholbenzene soluble and hot water soluble. Pulp yield is 50 to 52%. Its short fibre is suitable for rayan production (Pottinger and Hughes, 1995). Wood from giant *Leucaena* has a heating value of 4640 Kcal.kg $^{-1}$ at the age of 2 to 4 years, and 7000 Kcal.kg $^{-1}$ at the age of 8 years, which is equivalent to 70% of the heating value of fossil fuel.

1.7 Why *Leucaena leucocephala* was selected for study

Paper industry in India mainly uses Bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena sp*. as a source for paper pulp. Selection of the species depends upon availability, price and acceptability by any one given industrial unit. In bamboo growing countries, like India, the proportionate use of bamboos and hardwood species is in the ratio of 15:85. Although all these plant species are of importance to the paper industry, *Leucaena sp*. is exclusively used in India and about 25% of raw material for pulp and paper industry comes from this plant. *Leucaena sp.* is a fast growing multipurpose tree adapted to a variety of soils and climatic conditions.

Paper industry is one of the 35 high priority industries in India and currently growing at a rate of 6.3% per annum. The estimated turnover of the industry is Rs. 25,000 Cr. (USD 5.95 billion) approximately. The per capita consumption of paper in India is 7.2 kg, which is far lower than other emerging economies like China (45 ka) and developed

economies like US and EU (Eleventh five year plan 2007-2012, Planning Commission, Government of India). To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. However, as a safeguard for the future no plant should be harvested from areas that may challenge sustainability. It will thus be crucial to raise plantations of the plant species with elite materials and or genetically modified plants that meet the demands of the pulp and the paper industry in economical and sustainable manner. However no study has been done on Lignin Biosynthesis gene(s) so far in *Leucaena sp.* and study of these gene(s) will help in understanding the Lignin Biosynthetic Pathway in *Leucaena sp*. and its manipulation so as to meet the needs of pulp and paper industry.

1.8 Rationale of thesis

Lignin is one of the main structural elements of wood, and in angiosperms it is mainly composed of G (guaiacyl) and S (syringyl) monomers (Baucher *et al*., 1998; Boerjan *et al*., 2003). Considerable scientific interest has been focused on the development of trees with improved wood quality through modification of the genes involved in lignin biosynthesis, it could be important for the improved end use of wood material (Chiang 2006; Higuchi 2006). In chemical wood pulping, lignin is the main factor hindering the effective utilisation of cellulose fibres, from which it needs to be separated by costly and pollutant-generating processes (Chiang, 2002; Baucher *et al*., 2003; Boerjan, 2005). Genetic modifications resulting in increased delignification (through a modified lignin content and/or chemical composition) could thus be highly beneficial at both the economical and environmental scale (Baucher *et al*., 2003; Boerjan, 2005; Chiang, 2006). Lignin biosynthetic, Class III peroxidases (EC 1.11.1.7) are heme-containing glycoproteins that are found exclusively in the secretory pathways. These enzymes can dehydrogenate cinnamyl alcohols, and many other phenolic substrates, at the expense of H_2O_2; there by playing important role in the final step (Fig. 4) of lignin biosynthesis. So far no study has been made regarding the role of peroxidases on lignin biosynthesis in *Leucaena leucocephala*. The present study will give sufficient information regarding the role of peroxidase in lignin biosynthetic pathway; also will help in understanding the temporal and spatial expression of the peroxidase gene. This valuable information can be in turn used for manipulation of lignin content in perennial leguminous tree like

Leucaena leucocephala. Being fast growing tree, which is adapted to wide range of soils and agro-climatic conditions *Leucaena leucocephala* forms 25% of the total raw material for paper, and pulp industry in India. The outcome of this study that is, genetic manipulation of peroxidase to down regulate lignin in *Leucaena* can benefit commercially the paper industries and in turn will be an eco-friendly industrial approach since chemicals used in removing lignin can be reduced drastically.

CHAPTER 2

2. Materials and methods

This chapter includes general laboratory techniques routinely followed in the studies. Other important specific methodologies followed are discussed separately in the respective chapters.

2.1 Plant material

2.1.1 *Leucaena leucocephala*

Studies on *in vitro* plant regeneration were carried out using seeds obtained from field grown *L. leucocephala* cultivar K-636 which is a selection from Hawaii (Bray *et al*., 1998). This cultivar produces erect boles suitable for timber production. Seeds of *L. leucocephala* (K-636) were given scarification using conc. H_2SO_4 for 8-10 min and washed extensively with tap water. The scarified seeds were surface sterilized using 1.5% (v/v) sodium hypochlorite for 10 min followed by five rinses with sterile distilled water. Seeds were soaked overnight in sterile water at 30 ºC on a shaker for imbibing and embryos were isolated from imbibed seeds aseptically using a pair of sharp, sterile forceps and plated with their shoot apex facing up in the center of a 90 mm diameter Petri plate containing regeneration media (½-MS (Murashige and Skoog, 1962) + TDZ (0.5 mg/L)). The medium was solidified using 0.8% agar and the pH of the medium was adjusted to 5.8 prior to autoclaving.

For spatio-temporal studies, the imbibed seeds were transferred to ½-MS basal medium supplemented with 2% sucrose and 1.5% glucose. The medium was solidified using 0.8% agar and the pH of the medium was adjusted to 5.8 prior to autoclaving. The cultured bottles were incubated at 25±2 ^{o}C under 16 h photoperiod with a light intensity 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 the experiments.

For genomic DNA isolation, young leaves were harvested and washed thoroughly with water, dried and then crushed using liquid nitrogen. For RNA isolation, xylem tissues from mature field grown plants were harvested as and when required. Outer bark/phloem was removed and carefully isolated xylem scrapping was used in RNA isolation for cDNA preparations, RACE and RT PCR.

2.1.2 *Nicotiana tabacum*

Tobacco seeds from *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. 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 with a light intensity 24.4 µmol /m^2/s. One month old axenic cultured pant leaves were the raw material for further experiments.

2.2 Glassware

Glassware used in all the experiments were procured from "Borosil", India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), petridishes (85 mm x 15 mm; 55 mm x 15 mm), conical flasks (100, 250 & 500 mL; 1, 2 & 5 L capacity) and pipettes (1, 2, 5, 10 and 25 mL capacity) were used during the course of study.

2.2.1 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 and rinsed with distilled 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 ºC and 15 psi for 1 h.

2.3 Plastic ware

Sterile disposable filter sterilization units (0.22 µM) and petridishes (55 mm and 85 mm diameter) 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.

2.4 Chemicals

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), 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 Invitrogen (USA), 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), Affigel matrices, 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. Substrates for enzyme assays i.e. coniferaldehyde, coniferyl alcohol, sinapyl alcohol, ABTS etc. were also obtained from Sigma-Aldrich (USA).

2.5 Equipments: See appendix, Table 2.1 List of Equipments used.

2.6 Buffers and solutions

2.6.1 Buffers and solutions for DNA electrophoresis: See appendix, Table 2.2 Buffers and Solutions for DNA Electrophoresis.

2.6.2 Buffers and solutions for g DNA isolation, Southern and Slot blot: See appendix, Table 2.3 Buffers and Solutions for g-DNA isolation, Southern and Slot Blot.

2.6.3 Solutions for the transformation and selection of *E. coli.*

E.coli was grown at 37 ^{o}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 suppplemented with appropriate antibiotics. See appendix, Table 2.4 Stock solutions for *E. coli* transformation and selection.

2.6.4 Buffers and solutions for plasmid isolation (Alkaline lysis method): See appendix, Table 2. 5 Buffers and solutions for plasmid isolation (Alkaline lysis method).

2.6.5 Buffers and solutions for gel electrophoresis (PAGE): See appendix, Table 2.6 Buffers and Solutions for Gel Electrophoresis (native/ SDS-PAGE).

2.6.6 Buffers and solutions for protein purification under native conditions: See appendix, Table 2.7 Buffers and solutions for protein purification (based on imidazole conc.)

2.6.7 Buffers and solutions for protein extraction under denaturing conditions: See appendix, Table 2.8 Buffers and solutions for protein extraction under denaturing conditions.

2.6.8 Buffers and solution for the peroxidase (POX) enzyme activity

Normal peroxidase activity was checked using 20 mM Phosphate buffer (K_2HPO_4 and KH_2PO_4 pH 6.0 to7.4). Also, 20 mM Tris-HCl buffer (pH 7.5), 50 mM Na-citrate buffer (pH 5.0) and 20 mM MES buffer (pH 6.0) supplemented with 1 mM $CaCl_2$, 1 mM MnCl2 and $MgCl_2$ were also used in the study.

During purification of peroxidase enzyme guaiacol and syringaldazine were used as the substrates to quickly check the activity. In this assay system, increases in absorbance due to the peroxidase reaction were monitored at 470 and 530 nm for guaiacol and syringaldazine, respectively.

2.6.9 POX enzyme assay

The purified peroxidases (POX) were used for polymerization studies. The reaction mixture (1.0 mL) contained the purified peroxidase, 0.1 mM monolignol, and 50 µM H_20_2 in 40 mM phosphate buffer (pH 6.8). The reaction, initiated by adding H_20_2 to the reaction mixture, was carried out at 30 ^{o}C. At the scheduled reaction time (2 min), recorded the absorbance at 263 and 272 nm for coniferyl alcohol and sinapyl alcohol,

respectively. Differences in absorbance were converted to the amount of consumed monolignols using extinction coefficients of 15100 and 141001/mol.cm for coniferyl and sinapyl alcohol, respectively (Aoyama *et al., 2002*). See appendix, Table 2.9 substrates used for the study.

2.6.10. Buffers and solutions for ELISA/ Immuno-cytolocalization/ GUS assay and for DNA coating on to microcarriers in biolistic transformation: See appendix, Table 2.10 Buffers and Solutions used for ELISA, Immuno-cytolocalization, Lignin staining and DNA coating.

2.6.11 Component of Murasighe and Skoog media/ different inducing media and hormones: See appendix, Table 2.11 Component of Murasighe and Skoog media /different inducing media and hormones.

2.6.12 Different media used for studies: See appendix, Table 2.12 Different media used for bacterial studies.

2.7 Host cells: See appendix, Table 2.13 Bacterial cell lines used.

2.8 Methods

2.8.1 Bacterial culture conditions

E. coli was grown at 37 ºC 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 medium supplemented with appropriate antibiotic(s).

2. 8.2 Bacterial transformation

2.8.2.1 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 ^{o}C in incubator shaker at 200 rpm. Overnight grown culture (500 µL) was added to 50 mL of SOB medium and grown for 2-3 hours at 37 ^{o}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 ^{o}C. The cell pellet was suspended in 5 mL ice-cold TB buffer containing 7% DMSO. This was then dispensed

into aliquots of 100 µl in 1.5 mL eppendorf tubes, frozen in liquid nitrogen and stored at -80 ^{o}C.

2.8.2.2 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. The cells were harvested by centrifugation at 5,000 g for 10 min at 4 ºC, suspended in ice-cold 100 mM $CaCl_2$ and kept on ice for 30 min. Cells were centrifuged, the pellet suspended in 1 mL of ice-cold 100 mM $CaCl_2$ and stored as aliquots of 200 µL at 4 ºC or in -70 ºC after adding 15% glycerol or 7% DMSO.

2.8.2.3 *E. coli* transformation

The competent *E. coli* cells were transformed according to Sambrook *et al.,* (1989). Gently, 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 ºC for 1.5 min (heat shock) and immediately kept back on ice. To each tube 800 µL of LB broth was added and further incubated at 37 ºC for 1 h. Cells were pelleted by centrifugation at 5,000 rpm for 5 min. The supernatant media was discarded and pellet was dissolved in 100 µL of LB broth. The resuspended cells were plated on LB agar medium with appropriate antibiotics, IPTG and X-gal as per need (Sambrook *et al*., 1989).

Solutions	Stock	Final concentration
IPTG	200 mg mL^{-1} in sterile distilled water	40 µg mL^{-1}
X-gal	20 mg mL^{-1} dimethylformamide	40 µg mL^{-1}

2.8.2.4 Preparation of *Agrobacterium tumefaciens* competant cells, transformation and selection

The method adopted for the preparation of *A. tumefaciens* competent cells was essentially as reported by An (1987). A single colony of *A. tumefaciens* (GV2260) was inoculated in

50 mL LB broth containing Rifampicin 150 µg mL^{-1} and incubated at 28 ºC with shaking at 200 rpm till O.D. 0.5 at 600 nm. Cells were centrifuged at 5,000 g for 10 min at 4 ºC and washed twice with ice cold 150 mM $CaCl_2$. The cells were pelleted and resuspended in 1.0 mL of ice cold 20 mM $CaCl_2$. Aliquots of 200 µL were made. For transformation 1.0 µg DNA of the desired binary plasmid vector was added to an 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 ºC. After thawing 1.0 mL LB medium was added and the tubes incubated at 28 ºC for 2 h with gentle shaking. The cells were centrifuged at 4,000 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 ºC for two days to allow the transformed colonies to grow for further processing.

2.8.3 Colony PCR for screening recombinant colonies after bacterial transformation

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 DNA for amplification. A single bacterial colony was picked up from the agar plate containing transformants with the help of microtip and added to 1.5 mL eppendorf PCR tube containing 25 µL of sterile miliQ water. The microtip was agitated in the water to suspend the bacterial cells. Subsequently, 2 –3 µL of this suspension was used in 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 gene specific primers can be used. An additional 5 min denaturation step at 95 ºC before the amplification cycles will aid the bacterial lysis to enhance PCR product amplification success. The resulting PCR products were checked on an agarose gel for the presence of the cloned gene of interest.

2.8.4 Isolation of nucleic acids and Polymerase Chain Reaction (PCR)

2.8.4.1 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. An important feature of this protocol was the use of PEG for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination. The bacterial cultures were grown overnight with shaking (200 rpm) at 37 ºC in LB broth, with appropriate antibiotic(s). About 1.5 to 3 mL culture was centrifuged for 1 min at 7,000 g to pellet the bacterial cells. The pellet was resuspended in 100 µL of GTE buffer (Tab: 2.5) by vigorous pipetting, 200 µL of Soln. II (Tab: 2.5) was added, mixed by inversion till the solution becomes clear, normally for 2-3 min. The cell lysate was neutralized by addition of 150 µL of Soln. III (Tab: 2.5), mixed well and incubated on ice for 5 min. The cell debris was removed by centrifugation for 10 min at 12,000 g at 4 ºC. The supernatant was transferred to another microfuge tube, RNase A was added to a final concentration of 20 µg/mL (Sambrook *et al*., 1989) and incubated at 37 ºC for 20 min. To the above solution 400 µL of chloroform was added, vortexed for 2 min and centrifuged for 5 min at 12, 000 g at room temperature. The upper aqueous layer was transferred to a clean tube, 1/10th volume sodium acetate and one volume absolute ethanol was added with gentle mixing and kept at -20 ºC for 1-2 h. The sample was centrifuged at 12,000 g for 10 min at 4 ºC. The pellet was washed thrice with 70% ethanol and dried under vacuum. The dried pellet was dissolved in 40 µL of deionized water and 40 µL of PEG/NaCl solution (20% PEG 8000 in 2.5 M NaCl) was added. The mixture was incubated on ice for 20 min and the plasmid DNA pelleted out by centrifugation at 12,000 g for 15 min at 4 ºC. The supernatant was aspirated carefully, the pellet washed with 70% ethanol and air-dried. The dried pellet was resuspended in 20 µL deionized water and stored at -20 ºC.

2.8.4.2 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 Soln. I (Tab: 2.5) containing 10 µL Triton X-100 and

resuspended in 300 µL of Soln. I (Tab: 2.5) and 400 µL of Soln II. (Tab: 2.5). The cells were mixed by inverting till the solution turns clear. This was followed by the addition of 400 µL of Soln. III. (Tab: 2.5). 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 ^{o}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 ^{o}C till further use.

2.8.4.3 Isolation of plant genomic DNA

Extraction buffer: 100 mM Tris-HCl and 20 mM sodium EDTA adjust pH to 8.0 with HCl; add NaCl to 1.4 M and 2.0 % (w/v) CTAB (cetyltrimethylammonium bromide). Dissolve CTAB by heating to 60 °C. Store at 37 ºC. Add β-mercaptoethanol to 0.2% before use. Chloroform: isoamyl alcohol 24:1 (v/v), 5 M NaCl, RNAase A (10 mg mL^{-1}), 95% ethanol, 70% ethanol, TE buffer: 10 mM Tris-HCl and 1 mM EDTA, adjust pH to 8.0 and autoclave.

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 1.0 g of ground tissue was extracted with 10 mL extraction buffer. The slurry was poured into a clean, autoclaved 50 mL centrifuge tube and 100 mg insoluble polyvinylpoly-pyrrolidone (PVPP) as well as 20-40 µL of β-mercaptoethanol was also added. The tube was inverted several times but very gently to thoroughly mix the slurry, incubated at 65 ºC for 30 min and then allowed to cool down to room temperature. 12 mL of chloroform: isoamylalcohol mixture was added and the contents mixed by inverting the tube gently till an emulsion formed. The mixture 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 and two volumes of cold (-20 ºC) 95 % ethanol was added

and the sample kept at 4 ºC 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, and DNA pellet washed with cold (4 ºC) 70 % ethanol and air-dried. DNA was dissolved in 400 µL of TE buffer. The DNA solution was treated with 1 µL RNase A (10 mg mL^{-1}) per 100 µL DNA solution and incubated at 37 ºC for 30 min. The sample was extracted with chloroform: isoamyl alcohol to remove RNAase A. DNA was re-precipitated and dissolved in 40-100 µL TE buffer. Purity of DNA was checked spectrophotometrically by measuring the absorbance ratio (A_{260}/A_{280}) and also by visualization on 0.8 % agarose gel with 1X TAE. Genomic DNA was stored at 4 ºC.

2.8.4.4 Restriction digestion of DNA

Plasmid and genomic DNA restriction digestion was set up as per restriction enzymes manufacturer's recommendations.

2.8.4.5 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^{-1}) and viewed using a hand held long wavelength UV illuminator. The fragment of interest was excised from the gel and weighed. A 100 µg gel slice was transferred to a 1.5 mL micro centrifuge tube and 300 µL Buffer DE-A (Axygen^{TM} GEL elution kit, Biosciences, USA) added. The tube was incubated at 70 ºC 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 molten agarose was put into Axyprep column and placed into 2 mL microfuge collection tube. The assembly was centrifuge 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 spin at 12,000 g for 30 s, filtrate was discarded. It was repeated again with wash buffer 2. One min empty spin was given to ensure the complete removal of salt as well as ethanol. 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 4 ºC. This eluted PCR product or any DNA fragments are of good quality and can be visualized on 1% agarose gel by taking an aliquots of 3-4 µL. The eluted DNA /PCR product was stored at -20 ^{o}C and was used for subsequent reactions.

2.8.4.6 Total RNA Isolation

RNase free environment was created and maintained as described by Blumberg (1987). All glass and plasticware were DEPC (0.1% in water) treated 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 13,000 g for 15 min at 4 ºC. The supernatant was transferred to 1.5 mL tubes and the chloroform: isoamyl alcohol step was repeated. The aqueous phase was transferred to 1.5 mL tubes and 0.6 volume isopropanol added. It was mixed thoroughly and kept for RNA precipitation for 1 h at room temperature. Total RNA was pelleted out by centrifugation at 13,000 g for 15 min at 4 ºC. 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 ºC in aliquots. Purity of RNA was confirmed by measuring OD at 260/280 nm and also by visualization on 1.5% TAE agarose gel.

2.8.4.7 mRNA purification

Total RNA was quantified spectrophotometrically as well as with the Bioanalyser system. The amount of RNA was in the range of 1to 3 mg. Appropriate amount of OBB Buffer and Oligotex suspension were added according to manufacturer's instruction. The sample was incubated for 3 min at 70 ºC in a heating block. Sample was removed from the heating block, and placed at 20 – 30 ºC 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 micro-centrifuge tube, appropriate amount of OW2 buffer added to the column and again centrifuged for 1 min at 12,000 g, flow-through was discarded. Spin column was transferred to a new RNase free 1.5 mL micro-centrifuge tube. Appropriate amount of hot (70 ºC) OEB buffer was pipetted onto the column and resuspended by pipetting up and down; spin it down for 1 min at 12,000 g. The Eluted mRNA sample was quantified spectrophotometrically and used for further downstream processes.

2.8.4.8 Spectrophotometric determination of nucleic acids concentration

DNA concentration was determined by measurement of the absorption at 260 nm. 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 1 cm light path quartz cuvette. Absorbance value (A_{260}) should fall between 0.1-1.0 to be accurate. Sample dilution was adjusted accordingly. An absorbance of 1.0 at 260 nm corresponds to 50 µg DNA/ mL.

The Bioanalyser system (Agilent 2100) was also used to check the quantity of the nucleic acids (according to manufacturer's instructions). Some other useful parameters like gel electrophoresis pattern of the samples (Nucleic acids and protein), the integrity of the RNA and DNA, concentration of the individual bands can also be analyzed simultaneously with this system. The basis of the system to perform multiple analysis simultaneously is that, one has to load the samples, standards, suitable ladder and the gel matrix in the different wells of a single microchip provided with the system and then the system primarily performs electrophoretic separation of all the samples loaded through the inbuilt micro-capillaries of the chip and thereafter, analyses the various parameters in one go.

2.8.4.9 cDNA first strand synthesis by reverse transcription

Complementary DNA (cDNA) was 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 mixture was isothermally denatured at 70 ºC 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 ^{o}C for 5 min, the reaction was incubated at 42 ºC for up to 1-1.5 h. The cDNA synthesized was directly added to amplification reactions. The first strand reaction was set up as follows:

Reagent and concentration	**Volume**
Experimental RNA (1 µg)	1.0 µL
Primer (Oligo$(dT)_{15}$ or Random (10 pmol))	1.0 µL
DEPC treated Water	3.0 µL
Final volume	**5.0 µL**

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

Reagent and concentration	Volume
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	**15.0 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 reaction mixure was incubated at 25 ºC for 5 min for primer annealing and then at 42 ºC for 1 h for cDNA first strand synthesis. Reverse transcriptase was thermally inactivated by incubation at 70 ºC for 15 min prior to proceeding with PCR amplification (Chumakov, 1994).

2.8.4.10 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

Reagent and concentration	Volume
Sterile deionized water	6.2 µL
Template (50 ng/ µL)	1.0 µL
Forward primer (8 pmol)	1.0 µL
Reverse primer (8 pmol)	1.0 µL
dNTPs (0.2 mM)	4.0 µL
10 x 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

95 ºC - 5 min

95 ºC - 1 min

45-65 ºC - 45-90 s

35 cycles

(Annealing temperature (Ta) was dependent on melting Temperature of the primer (Tm); Ta = Tm - 5 ºC)

72 ºC - 1.5 min.

1 cycle 72 ºC 10 min

1 cycle 4 ºC hold.

2.8.4.11 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). RACE ready cDNA Kit (Invitrogen, USA) was used to perform

RACE. The reactions were set up as per the manufacturer's guidelines with minor modification. Briefly, the RACE technique is based on oligo-capping and RNA ligase-mediated (RLM) RACE methods (Maruyama and Sugano, 1994; Vollo Ch *et al*., 1994). 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. Application of GeneRacer race ready cDNA Kit is as follows:

- Identifying the 5' and 3' untranslated 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.

GeneRacer RACE Ready cDNA Synthesis

The pre-requisite 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 mix is as follows:

Reagent and concentration	**Volume**
RNA	7.0 µL
10X CIAP Buffer	1.0 µL
RNaseOut™ (40 U/µL)	1.0 µL
CIP (10 U/µL)	1.0 µL
Total Volume	**10.0 µL**

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 50 ºC for 1 h. The reaction volume was made up to 100 µL by adding DEPC water and precipitated by adding 2 µL of 10 mg/mL glycogen, 10 µL of 3 M sodium acetate (pH 5.2) and 220 µL of 95% ethanol. The precipitated sample was washed with 70% ethanol and pellet was air-dried. Air-dried pellet was

dissolved in 7 µL of DEPC water. 5' mRNA cap structure from full- length mRNA was removed by following reaction,

Reagent and concentration	Volume
Dephosphorylated RNA	7.0 µL
10X TAP Buffer	1.0 µL
RNaseOut™ (40 U/µL)	1.0 µL
TAP (0.5 U/µL)	1.0 µL
Total Volume	**10.0 µL**

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 37 ºC for 1 h. The reaction volume was made up to 100 µL by adding DEPC water and precipitated by adding 2 µL of 10 mg/mL glycogen, 10 µL of 3 M sodium acetate (pH 5.2) and 220 µL 95% ethanol. The precipitated sample was washed with 70% ethanol and pellet was air-dried. Air dried pellet was dissolved in 7 µL of DEPC water. After decapping of full length mRNA, the RNA Oligo was ligated to the 5' end of the mRNA. The reaction of ligating the RNA Oligo to decapped mRNA is as follows:

Reagent and concentration	Volume
Decapped RNA	7.0 µL
10 X Ligase Buffer	1.0 µL
10 mM ATP	1.0 µL
RNaseOut™ (40 U/ µL)	1.0 µL
T4 RNA ligase (5 U/µL)	1.0 µL
Total Volume	**11.0 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 ºC for 5 minutes and kept on ice, reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 37 ºC for 1 h. The reaction volume was made up to 100 µL by adding DEPC water and precipitated by adding 2 µL of 10 mg /mL glycogen, 10 µL of 3 M sodium acetate (pH 5.2) and 220 µL 95% ethanol. The precipitated sample was washed with 70% ethanol and pellet was air dried. Air dried pellet was dissolved in 10 µL of DEPC water. The decapped full length mRNA ligated with GeneRacer™ RNA - Oligo was used to prepare cDNA by reverse transcription. 1 µL of the desired primer and 1 µL of dNTP Mix (25 mM each) were added to the ligated RNA and incubated at 65 ºC for 5 min. Following reagents were added to the 11 µL ligated RNA and primer,

Reagent and concentration	**Volume**
5 X RT Buffer	4.0 µL
SuperScript™ III RT (200 U/µL)	1.0 µL
0.1 M DTT	1.0 µL
RNaseOut™ (40 U/µL)	1.0 µL
Sterile water	2.0 µL
Total Volume	**20.0 µL**

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 45 ºC for 1 h. The reaction was inactivated by incubating the reaction mixture at 70 ºC 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 ºC for 20 min. Centrifuged the sample and kept at -20 ºC for further use in PCR.

PCR reaction setup

Reactions were setup to amplify either the 5′ end or the 3′ end of gene of interest. The reaction was set up as given below:

Reagent and concentration	5′ RACE	3′ RACE
GeneRacer 5′ Primer10 µM	3.0 µL	-
Reverse GSP 10 µM	1.0 µL	-
GeneRacer™ 3′ Primer 10 µM	-	3.0 µL
Forward GSP 10 µM	-	1.0 µL
RT Template	1.0 µL	1.0 µL
10X High Fidelity PCR Buffer	5.0 µL	5.0 µL
dNTP Solution (10 mM each)	1.0 µL	1.0 µL
Platinum® Taq DNA Polymerase or High Fidelity, 5 U/ µL	0.5 µL	0.5 µL
$MgSO_4$, 50 mM	2.0 µL	2.0 µL
Sterile Water	36.5 µL	36.5 µL
Total Volume	**50.0 µL**	**50.0 µL**

Cycling conditions

Temperature	Time	Cycles
94 ºC	2 min	1
94 ºC	30 S	5
72 ºC 1 min/ 1 kb	2 min	
94 ºC	30 S	5
70 ºC 1 min/ 1 kb	2 min	
94 ºC	30 S	25
66 ºC	30 S	
72 ºC	2 min	
72 ºC	10 min	1

Nested PCR

Nested PCR was done to increase the specificity and sensitivity of RACE products for the 5′ or 3′ ends of gene. 1 µL of the original amplification reaction was used as template for nested PCR. Reactions were set up as described below:

Reagent	5′ RACE	3′ RACE
Gene Racer 5′Nested Primer10 µM	1.0 µL	-
Reverse Nested GSP 10 µM	1.0 µL	-
Gene Racer 3′ Nested Primer 10 µM	-	1.0 µL
Forward Nested GSP 10 µM	-	1.0 µL
Primary PCR Product	1.0 µL	1.0 µL
10 X High Fidelity PCR Buffer	5.0 µL	5.0 µL
dNTP Solution (10 mM each)	1.0 µL	1.0 µL
Platinum® Taq DNA Polymerase High Fidelity, 5 U/µL	0.5 µL	0.5 µL
$MgSO_4$, 50 mM	2.0 µL	2.0 µL
Sterile Water	38.5 µL	38.5 µL
Total Volume	**50.0 µL**	**50.0 µL**

Following cycling condition was used for the nested PCR reactions,

Temperature	Time	Cycles
94 ºC	2 min	1
94 ºC	30 S	25
66 ºC	2 min	
72 ºC	2 min	
72 ºC	10 min	1

10-20 µL of nested PCR product was analyzed 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.

2.8.5 Quantitative real time PCR (QRT PCR)

Quantitative Real Time PCR is a powerful tool for gene expression analysis and was first demonstrated by Higuchi *et al*., (1992, 1993). QPCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferred 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; Raeymaekers, 2000). QPCR 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 (Higuchi *et al*., 1992, 1993). The QPCR system is based on the detection and quantitation of a fluorescent reporter (Lee *et al*., 1993; Livak *et al*., 1995). There are three main fluorescence-monitoring systems for DNA amplification (Wittwer *et al*., 1997a): (1) hydrolysis probes, (2) hybridizing probes and (3) DNA-binding agents (Wittwer *et al*., 1997b, Vander Velden *et al*., 2003). Most commonly used are the hydrolysis probes, which include TaqMan probes (Heid *et al*., 1996), molecular beacons (Tan *et al*., 2004; Vet and Marras, 2005) and scorpions (Saha *et al*., 2001; Terry *et al*., 2002). They use the 5' exonuclease activity of *Taq* polymerase to measure the amount of target sequences in cDNA samples. The use of fluorescent probe technologies reduces the risk of sample contamination while maintaining convenience, speed and high throughput screening capabilities (Reischl *et al*., 2002).

In the present study the Brilliant® II QPCR Master Mix (Stratagene, USA) was used. This kit supports quantitative amplification and detection with multiplex capability, and shows consistent high performance with various fluorescent detection systems, including molecular beacons and TaqMan® probes. The kit supports PCR amplifications and detection of a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA. The 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.

2.8.5.1 Pre-protocol considerations

Magnesium chloride concentration

Magnesium chloride concentration in the PCR reaction mix affects the specificity of the PCR primers and probe hybridization (Eckert and Kunkel, 1990). The Brilliant® II QPCR Master Mix kit contains $MgCl_2$ at a concentration of 5.5 mM (in the 1 x solution), which is suitable for most targets.

Probe design

Probes are designed in a fashion so as to have a melting temperature 7–10 ºC higher than the annealing temperature of the primers. Lyophilized custom molecular beacon or TaqMan probes are constituted in 5 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA (low TE buffer).

Fluorescence detection

Fluorescence may be detected either in real-time or at the endpoint of cycling using a real-time spectrofluorometric thermal cycler. For endpoint analysis, PCR reactions can be run on any thermal cycler and then analyzed with a fluorescence plate reader that has been designed to accommodate PCR tubes and is optimized for the detection of fluorescent PCR reactions. Readings may be acquired before and after PCR for comparison.

Optimal concentrations for experimental probes and primers

The optimal concentration of the experimental probe 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. In present study TaqMan® probes were used.

TaqMan® probes

The TaqMan probe concentration can be optimized by varying the final concentration from 50 to 300 nM in increments of 50 nM.

PCR primers

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 primer concentration for use with TaqMan probes can be optimized by varying the primer concentration from 100 nM to 900 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

Reference dye

A passive reference dye may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm respectively.

Reference dye dilution

Reference dye is prepared fresh prior to setting up the reactions. All reaction tubes containing reference dye are protected from light. Initial dilutions of the reference dye are made in nuclease-free PCR-grade H_2O and used at a final concentration of 30 to 300 nM depending upon the instrument configuration. In the present studies, 30 nM concentration of reference dye has been used.

Data acquisition with a spectro-fluorometric thermal cycler

Acquisition of real-time data generated by fluorogenic probes was as recommended by the instrument manufacturer. Data collection was either at the annealing step of each cycle (3-step cycling protocol) or the annealing/extension step (2-step cycling protocol).

2.8.5.2 Preparing the RT-qPCR reactions

The reference dye was diluted 1:500 (recommended in kit) using nuclease-free PCR-grade H_2O resulting in a final reference dye concentration of 30 nM in the reaction mixture. Real time PCR model Stratagene Mx 3000P was used in present study. The experimental reactions were prepared by adding the following components in order:

Reagent Mixture

Component	**Volume**
2 X master mix	12.5 µL
Experimental probe (optimized concentration)	X µL
Upstream primer (optimized concentration)	X µL
Downstream primer (optimized concentration)	X µL
Diluted reference dye	0.375 µL
Experimental gDNA, cDNA or plasmid	X µL
Final volume	**25 µL**

2.8.5.3 PCR cycling programs

The reactions were placed in the instrument and a 2 step program was run (recommended for TaqMan reactions).

Two step cycling Protocol,

Temperature	**Time**	**No. of Cycles**
95 ºC	10 min	1
95 ºC	30 S	40
55-57 ºC	30 S	
72 ºC	1 min	

Initial 10 min incubation was to activate the DNA polymerase completely. The temperature cycler was set to detect and report fluorescence during the annealing/extension step of each cycle.

2.8.6 Nucleic acids blotting/hybridization

2.8.6.1 Southern blotting

For Southern hybridization (Southern, 1975) the DNA samples were electrophoresed on an agarose gel in 1X TAE buffer containing 0.5 µg mL^{-1} ethidium bromide. The gel was rinsed with deionized water (D/W) and placed in depurination solution for 15 min. The gel was rinsed with deionized water and immersed in denaturation solution for 30 min with gentle shaking. The gel was again rinsed with deionized water and transferred to neutralization solution for 45 min. The gel was next 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 3MM paper wetted with 20X SSC was placed on the membrane followed by Whatman 3MM paper pre-wetted in 2X SSC. On this paper another dry Whatman 3MM 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 ºC to immobilize DNA onto the nylon membrane. Hybridization and autoradiography were carried out as is described in the following section.

2.8.6.2 Slot blotting (Slot blot hybridisation)

Genomic DNA was extracted from *L. leucocephala* and was quantified; DNA was checked for its quality and digestability using spectrophometer and on an agarose gel respectively. 20 µg of gDNA was digested up to completion with respective restriction enzymes in separate 1.5 mL eppendorf tubes. The restriction enzyme was selected on the

basis of its presence within the gene, such that it cuts the DNA only once. The digested DNA was electrophoretically separated on 0.8% agarose gel.

For slot blot hybridization DNA or RNA samples were diluted according to experimental requirements. The DNA samples were denatured by adding 1/10th volume of 3 M NaOH and incubated at 65 ºC for 10 min. To the denatured sample an equal volume of 6X SSC was added. Two layers of Whatman 3 MM filter paper wetted with sterile deionized water and 6X SSC were placed in the Slot blot apparatus followed by Hybond-N+ membrane (Amersham, UK) treated in the manner as above. The Slot blot unit was assembled and wells washed with 500 µL of 6X SSC by applying vacuum. After washing, samples prepared earlier were applied in the wells and vacuum applied till whole sample volume passed through the well slit and wells appear dry. The unit was carefully disassembled and the membrane taken out. The membrane was air dried and baked for 2 h at 80 ºC to immobilize DNA. Radio-labeling of probe, hybridization and autoradiography were carried out as described in the following sections.

2.8.6.3 Random primer labeling

Random primer labeling of the DNA probes (Feinbeng and Vogelstein, 1983) 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 hexamers (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. This reaction mixture was added to the following reaction mixture.

Component	Volume
10 X reaction buffer (500 mM Tris-HCl, pH 8.0; 100 mM $MgCl_2$; 10 mM DTT; 0.5 mg/ mL acetylated BSA)	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)	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 ºC for 1 h and stopped by incubation at 95 ºC in a dry bath for 10 min and snap chilled on ice.

2.8.7 Pre-hybridization and hybridization

Solutions:

20X SSC: 3 M NaCl; 0.3 M Sodium citrate (pH 7.0)

Hybridization buffer: 1% BSA; 1.0 mM EDTA, pH 8.0; 0.5 M Sodium phosphate, pH 8.0; 7% SDS

Low stringency wash buffer: 2 X SSC, 0.1% SDS

High stringency wash buffer: 0.2 X SSC, 1% SDS

The blots made as in sections 2.8.6.1 and 2.8.6.2 above were pre-hybridized at 62 ºC in 30 mL of hybridization buffer for 6-8 h in a hybridization incubator (Robin Scientific, USA). The buffer was decanted and fresh buffer added with the denatured radiolabelled probe. Hybridization was carried out at 62 ºC for 14-18 h. The solution was discarded and the membrane washed with low stringency buffer at 55-65 ºC for 5 min followed by a high stringency wash at 55-65 ºC for 5 min. The moist blot was wrapped in saran wrap

and placed in the developing / intensifying screen provided with the Typhoon TRIO+ scanner. After 6-8 h of exposure, remove the membrane and scan the screen with Typhoon trio scanner system. One can increase the exposure time if the band intensity is weak. The membrane scanning is done usually in the resolution mode instead of sensitivity mode of the scanner. Good quality of band pictures can be obtained by scanning at 500 micron resolution with the system. This instrument will provide many more options and functions to get exquisite band pictures of the exposed membrane. After scanning the intensifying screen, the signals can easily be erased from it, upon exposure to a white light provided with the system for 10 min and the same screen can be used to develop the other membrane.

2.9 Construction and screening of genomic DNA library

Genomic DNA library was prepared using λ- ZAP II vector according to manufacturer's guidelines (Stratagene). In Brief Genomic DNA was digested with *Sau* 3A restriction enzyme to generate approximately 8 to 10 kb fragments. Preliminary digestions were done to standardize the time and quantity of enzyme required to generate the required fragment of same size. Digested Genomic DNA was run on 0.8 % agarose gel and smear ranging from 8 to 10 kb was purified. Gel purified fragment were blunt ended and *Eco* RI adapters were ligated. These fragments were now ligated into λ-ZAP II vector (pre digested with *Eco* RI, and CIAP treated). The recombinant λ-ZAP II vectors were now packaged with packaging extract according to the manufacturer's instructions. The packaged recombinant λ-ZAP II vector was used to transfect *E.coli* XL1-MRF strain. Twenty to thirty plates of 135 mm were plated according to transfection protocol discussed later.

Plaques appeared after 8 to 10 h. 5 mL of SM buffer was poured in each plate and kept on shaker for two hours at 4 ºC. After two hours of incubation all SM buffer (refer Appendix) was pooled in one tube and kept at 4 ºC for further use (Plaques stable up to 6 months at 4 ºC).

2.9.1 Preparation of cells for Transfection

Plates were poured with NZY bottom agar (refer Appendix) and tetracycline (12.5 µg/ mL of media). A single colony of *E. coli* XL–Blue MRF was inoculated in 5 mL of LB

media and kept on shaker overnight. 500 µL of this culture was taken and inoculated in 50 mL LB media for further sub culturing for 4 h on shaker. 25 mL of culture was taken in autoclaved tubes; these steps were performed in laminar airflow unit to avoid contamination of cells. Cells were centrifuged at 5,000 g for 10 min, supernatant was discarded and pellet was washed gently, twice, with chilled 10 mM $MgSO_4$. Pellet was dissolved in approximately 20 mL of chilled $MgSO_4$. These cells were kept at 4 ºC for further use.

2.9.2 Transfection

500 µL of cultured cells were taken in micro-centrifuge tube and 1 µl of λ Phage representing genomic library of *Leucaena leucocephala,* was added, mixed gently and kept in incubator at 37 ºC for 15 min. In an autoclaved test tube 5 mL of melted top NZY media (containing 0.7 % agarose) and transfected bacterial cells were added and spread on plates having bottom NZY-agar media. These plates were kept in incubator at 37 ºC for 8 h.

2.9.3 Plaques lifting

The Plates with a density of 4000 – 5000 plaques per plate were used for screening. Nylon membrane (Hybond-Amersham) was placed on the plate gently with the help of forceps. It was marked asymmetrically for alignment. The membrane was peeled off the plate gently and kept in denaturing solution (refer Appendix) for 2 min followed by in neutralization solution (refer Appendix) for 5 min. It was then kept in rinsing solution (2X SSC and 0.2 M Tris HCl) for 30 s. The membrane was air-dried for about 2 h and then baked in oven at 80 ^{o}C for 2h before pre hybridization.

2.9.4 Preparation of probe, pre-hybridization and hybridization

PCR was used (as described in section 2.8.4.10) to prepare radiolabelled probe (as described in section **2.8.6.3**). dATP having α-P^{32} (3000 Ci/ mmol) was used as one of the dNTPs replacing normal dATP. Genomic DNA partial clone was used as a probe for screening purpose. After completion of PCR, tubes were kept at 99 °C for 10 min. Tube was snap chilled and used for hybridization.

Pre-hybridization and hybridization was done as discussed in section **2.8.7** Necessary care was taken while working with radioactive dATP. Lab coat, spectacles, and gloves were used while working.

2.9.5 First, second and third round of screening

Plaques with positive signals were cut and resuspended in 200 µl SM buffer with 10 µl chloroform. These plaques represent the λ phage having gene of interest. Plaques were kept as such for 4 h and then centrifuged at 8,000 g for 2 min. Transfection was done with phage collected after first round of screening. Subsequent screening steps i.e. secondary and tertiary screenings were followed as in case of primary screening. Single plaque was amplified and kept for further experiments.

2.9.6 Single clone excision

For single clone excisions, following components were mixed in a Falcon 50 mL polypropylene tube:

200 µL of XL1-Blue MRF cells (A_{600} = 1.0)

250 µL of phage stock (containing >1 × 10^5 phage particles)

1 µL of the ExAssist helper phage (supplied with Kit)

The mixture was incubated at 37 °C for 15 min to allow the phage to attach to the cells. 3 mL of LB broth was added with supplements in the tube and was incubated for 2.5–3 h at 37 °C with shaking. Tube was heated at 65–70 °C for 20 min to lyse the λ phage particles and the cells. It was centrifuged at 1,000 g for 15 min for pelleting the cell debris. Supernatant was saved in another sterile tube and transformation was done in freshly grown *E. coli* SOLR cells according to manufacturer's instructions. Plasmid was isolated from colonies, which appeared on LB agar (Kanamycin 30 µg/mL) plates and analysed.

2.10 Expression and purification of recombinant Protein

Heterologous expression and activity of lignin biosynthetic peroxidase (*POX*) gene was attempted in *E.coli* (BL21) host cell. The transformants were grown at 37 ºC in Luria – Bertani medium containing kanamycin (50 µg/mL). 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. 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 the addition of isopropyl β-D-thiogalactopyranoside (IPTG), and the culture was grown for 4 to 6 h at 37 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4 °C. Pellets were resuspended in 6.25 mL lysis buffer. Cells were disrupted by sonication for 5 mins 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 ^{o}C for ½ h. It was centrifuged at 10,000 rpm for 10 minute and supernatant was saved as lysate and pellet was resuspended in 2 mL sonication buffer. Suspension was again sonicated for 1 min at 70 amplitude to disrupt the inclusion bodies and the disrupted inclusion bodies were dissolved in 3 mL of dispersion buffer and an aliquot of 20 µL checked on SDS PAGE (Chapter2: section 2. 10.3 and table: 2.6) to check heterologous expression of *POX* gene.

2.10.1 Protein isolation from inclusion body

Cells were isolated by centrifugation and resuspended in 100 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 20% glycerol, 1.5 mM DTT, 1 mM PMSF and 0.4% Triton. Lysozyme (0.5 mg/mL) was added, and the suspension was incubated at 37 ºC. Bacterial cells were further lysed by sonication on ice with an MSE soniprep cell disrupter by using 20 pulses for 10 s with 20 s-cooling intervals. Cell debris was removed by centrifugation and the pellet was resuspended in sonication buffer which contain 100 mM Tris (pH 8.0) and 50 mM glycine. Cells were sonicated once again for 5-6 pulses 10 s on and 20 second cooling.

2.10.2 Affinity purification of recombinant protein Using Ni^{+} NTA beads

The recombinant protein, among several other bacterial proteins is loaded on affinity matrix column such as Ni-agarose. This affinity matrix contains bound metal ion nickel, to which the poly histidine-tag binds with micro molar affinity. The matrix is then washed with buffer to remove unbound proteins. This can be achieved either by

increasing the imidazole concentration in buffer or by lowering the pH of the washing and elution buffer. The column(s) 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 was passed through the column followed by addition of soluble protein in dispersion buffer. The column was kept at 4 ºC for 1 h for binding of recombinant protein to Ni^+ beads. Flow through was collected in different tubes after 1 h and column was washed with two bed volume of washing buffer (chapter 2: section 2.6.6). 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. (Chapter 2: section 2.6.6, Tab 2.7). 6x His-tagged protein was eluted in 4 aliquots of elution buffer, 0.5 mL each. Protein elution was monitored by measuring the absorbance at 280 nm of collected fractions. The eluted protein was separated and analyzed by SDS-PAGE.

2. 10.3 Polyacrylamide gel electrophoresis (PAGE)

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

2. 10.3.1 Preparation of the separating gel

A vertical slab gel (Hoeffer Scientific, U.S.A.) was assembled using 1.0 mm spacers. In a side armed vacuum flask, 10% separating gel solution was made according to Table 2.6, 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. 10.3.2 Preparation of the stacking gel

Stacking gel solution was prepared according to Table 2.6, excluding 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. 10.3.3 Preparation of the sample

Equal parts of the protein sample and the loading buffer were mixed in a microcentrifuge tube and kept in a boiling water bath for 3-5 min for SDS-PAGE. Then the samples were centrifuged at 10,000 g for 10 min, supernatant collected and used for electrophoresis. Gel was run at room temperature at constant ampere 20 mA or 80 V. The protein samples for native PAGE were mixed with native dye and gel was run at 80 V at low temperature ranging from 4-10 ºC.

2. 10.3.4 Loading and running the polyacrylamide Gel

Once well, have been formed in the gel, flushed it 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 70-80 units. The run was stopped when the dye reached the bottom of the polyacrylamide gel.

2. 10.3.5 Silver staining of the gel

Protein separated on SDS or native PAGE, was transferred to the fixer solution for 1 h. This was followed by 2 x 20 min washes in 50% ethanol. The gel was transferred to sodium thiosulfate solution for 1 min and rinsed thrice with deionized H_2O (20 s each). The gel was silver stained (HCHO was added to the solution just before use) for 20 min with intermittent shaking in dark. The gel was then rinsed thrice with deionized H_2O (20 s each) and transferred to the developer till the bands developed. The gel was washed with deionized H_2O and stored in fixer.

2.11 Raising polyclonal antibody against POX in rabbit

The purified recombinant *Leucaena* POX protein was used for raising polyclonal antibodies in rabbit (New Zealand White).

2.11.1 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.

2.11.2 Determination of titre of antibodies and ELISA

Buffers

1X Phosphate buffer saline (PBS): 8 gm NaCl; 1.44 g Na_2HPO_4; 0.24 g KCl; 0.200 g K_2HPO_4 in 1 L De-ionized water.

Wash Buffer (PBST): 1X PBS, 0.05% Tween 20 and 0.1% BSA

Antibody diluting buffer: 1X PBS and 0.25% BSA

Blocking reagent: 1% BSA in PBS

Substrate diluting buffer: 200 mM Tris-HCl, pH 9.5 and 10 mM $MgCl_2$

All reagents were prepared in sterile milliQ water.

ELISA 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 PBST, 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, 1:10000, 1:20000, 1:30000, 1:40000, 1:60000, 1:80000 and 1:100000. All dilutions were in triplicates and plate was kept at 37 °C for 2 h again. Plate was washed as described earlier with PBST 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 PBST, 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.

2.11.3 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% and 50% ethanol and 0.5X SSC for 2 min. The rehydrated sections were soaked in two changes of 1X PBS 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 ºC using 75 µL of diluted antibody (1 µg m/L) 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 ºC 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 containing 10% polyvinyl alcohol) to the color development buffer (100 mM Tris, pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$) treated slides. In present study we have got the ready to use BCIP/NBT solution therefore the volume from this solution was added sufficiently to submerge the sections in the watch glass. The slides were placed in humidified (color development buffer) chamber at RT in dark for 45 min. Upon color development, 10 mM EDTA was used to stop the reaction, rinsed with water, air dried and cover slip-mounted using glycerol and then it was observed under microscope, and microscopic image captured.

2.11.4 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.

2.11.5 ELISA of peroxidase (POX) protein in different tissues of *Leucaena leucocephala*

Fresh tissues were collected, frozen in liquid nitrogen and crushed to a fine powder. Crude protein was extracted with 2 mL of protein extraction buffer. Total Protein was quantified using Bradford reagent. Equal amount of protein was coated on 96 well micro titer plates. Antigen (recombinant protein) was diluted in PBS to an optimal concentration and coated on 96 well micro-titer plate (100 µL/ well). Plate was incubated for 2 h at room temperature or over night at 4 ºC and washed twice with PBS 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, primary antibody (Anti rabbit IgG) was added and incubated for 1 h at room temperature. The unbound primary antibody was washed twice with PBS and secondary antibody conjugated with alkaline phosphatase was added and incubated for 2 h at room temperature. Enzyme specific substrate BCIP/ NBT or PNPP was added and incubated for 45 min for the color development and the absorbance was measured at 405 nm.

2.12 Plant genetic engineering

2.12.1 *Agrobacterium* mediated tobacco transformation

The tobacco regeneration and transformation protocol is modified from Horch *et al*., (1985). *Agrobacterium tumefaciens* strain GV2260 harboring the binary plasmid vector to be transformed in tobacco was inoculated in 5mL YEP media containing rifampicin (250 mg L^{-1}) and kanamycin (50 mg L^{-1}). The culture was allowed to grow overnight at 28 ºC with shaking at 200 rpm. Next day 1 mL inoculum from this tube was taken and

added to 50 mL YEP flask containing appropriate antibiotics. The culture was incubated at 28 ºC 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 ºC. 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 kanamycin (7.09 µM) and cefotaxime (200 µM). The cultures were incubated at 25±2 ºC with 18/6 h photoperiod in diffused light (24.4 µmol/m^2 s^{-1}). Leaf discs were sub-cultured 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.

2.12.2 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 scarification with concentrated sulphuric acid (7 min) and 1.5% sodium hypochlorite (10 min), were used to isolate embryos. Embryo axes were excised from the seeds and inoculated on regeneration medium [1/2-MS + TDZ (0.5 mg/ L)]. The embryos were then used for transformation. LD_{50} for Kanamycin was estimated in previous experiments.

The transformation was carried out by two methods:

1) Particle bombardment

2) Particle bombardment followed by co-cultivation

2.12.2.1 Particle bombardment

Calculated amount (approximately 500 µg) of gold particle of 1µm size and HTC 600-Au were used for bombardment. The gold particles taken in microcentrifuge tubes were washed with sterile water and supernatant was decanted after centrifuging 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 at 13,000 g for 10 min. Again particles were suspended in 500 µL sterile water and were pelleted down after centrifuging at 13,000 g for 10 min. DNA (1.0 µg plasmid) was dissolved in 100 µL of TE buffer. DNA was added to the prepared gold particle and mixed 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. It was then given a brief spin to settle down the sample at the bottom. The sample 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. Helium gas pressure (1100 psi) was used to bombard the particle. Microcarrier travel distance was adjusted to 3 or 6 cm. The embryo axes were arranged in the centre of the petridishes and bombarded twice at 4 h interval with gold particles coated with DNA (plasmid/cassette). The explants were incubated in dark for 72 h after the bombardment.

2.12.2.2 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* (GV2260) harboring pCAMBIA2301 containing *POX* gene in anti-sense orientation. Cultured *Agrobacterium* 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 immerging them into the bacterial suspension. The agro infected embryo axes were then transferred onto the regeneration medium [1/2-MS + TDZ (0.5 mg/L)] with or without 0.1 mM acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Sigma-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 [1/2- MS + TDZ (0.5 mg/ L)].

2.13 GUS histochemical assay

The GUS staining solution was prepared by taking 1 mM X-Gluc (5-bromo, 4chloro, 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 calli. The GUS assay was carried out on 10% of the randomly selected calli 48 h after co-cultivation (transient GUS assay) as well as, 21 days after co-cultivation. Embryo axes were immersed in GUS assay solution and incubated at 28 ºC O/N. *GUS* gene expression was observed and photographed by using an Axioplan 4 microscope from Carl Zeiss.

2.14 FTIR analysis

FTIR was done using Spectrum One, machine from Perkin Elmer, USA. Growing seedlings in normal condition were considered for this experiment to monitor any change in quality of lignin produced at different point of time. Spectrum One (Perkin Elmer, USA) is capable of analyzing powder as well as liquid samples. Powdered lignin samples were analysed according to the manufacturer's recommendation.

Sample preparation

Developing seedlings (root and stem) were harvested at different time intervals and crushed to a fine powder in a mortar-pestle using liquid nitrogen. Powder was transferred to a glass crucible and kept at 80 °C for two days to remove moisture. After drying it was again mixed to homogenize and passed through a filter to get uniform size particle. Sample (0.85 mg) was mixed with dried potassium bromide (85 mg). Mixture was made to a fine powder and pellet was made using KBr press (pellet making machine) and dye (10 mm mould for pellet). In brief, fine powder of sample and KBr was put in assembled dye apparatus and the dye was kept on KBr press machine. 10-ton pressure was applied

for 5 min and dye apparatus was dismantled to get KBr pellet. Pellet was used to take reading in Spectrum One FTIR instrument. A pellet of KBr and KBr + commercial lignin (Sigma, Germany) was also made for baseline correction and standard lignin respectively.

2.15 Lignin Estimation

A modified method from Luo (2008) was used for estimating lignin in transformed plants. The plant material is ground finely in a mortar and pestle and then dried in oven at 55 ^{o}C. The pre-weighed dried tissue is extracted continuously with acetone:water (10:1) for 24 h at 55 ^{o}C. the solvent was discarded after a spin at 10,000 rpm for 5 min. H_2SO_4 (1 mL) was added and kept at 25 ^{o}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 through 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 weighed after drying. The final weight of crucible was substracted from initial weight to give the final weight of residue. For determining acid insoluble lignin, the percentage weight of residue was calculated. Acid soluble lignin was calculated according to the given formula.

$$\text{Acid soluble lignin } (\%) = \frac{100 \times \mathbf{A}_{205} \times \text{Vol. of filtrate (mL)} \times \text{dilution factor}}{110 \times \text{Initial dry powder weight (mg)}}$$

Total lignin = Acid soluble lignin + Acid insoluble lignin.

CHAPTER 3

3.1 Introduction

Lignin biosynthesis has been already discussed in detail in Chapter two of this thesis. The present chapter deals in detail regarding isolation, cloning and characterization of lignin biosynthetic peroxidase (*POX*) gene from *L. leucocephala*.

There is considerable interest in the biotechnological modification of (woody) plant lignin content and composition, for reasons which are essentially threefold. One is to delineate the mechanism(s) of lignin assembly in different cell types (Lewis *et al*., 1999), whereas the other two include manipulating vascular plant biosynthetic pathways for improving the efficiency of pulp and paper making for quality, economic and environmental reasons and/or obtaining more readily digestible animal feed (Boudet, 1998; O'Connell *et al*., 1998; Baucher *et al*., 2000; Anterola and Lewis, 2002a, b). In this regard, next to cellulose, lignin is nature's second most abundant biopolymer in vascular plants.

3.1.1 Peroxidase (*POX*) as a candidate gene

Peroxidases are ubiquitous enzymes that catalyze oxidation of cellular components in the presence of H_2O_2. Most higher plants contain a number of peroxidase isozymes, which can be classified into two (anionic and cationic) or three (anionic, neutral, and cationic) subgroups according to their isoelectrophoretic mobilities, and these isozymes exist in cytosol, chroloplast, vacuole and cell wall (Lagrimini *et al*., 1987; Asada, 1992). Their physiological roles have been extensively investigated, and it has been demonstrated that they catalyze a variety of important reactions, such as indole-3-acetic acid catabolism (Hinman and Lang, 1965), suberization of cell wall (Christensen *et al*., 1998), detoxification of H_2O_2 (Asada, 1992) and lignin biosynthesis (Espelie, 1986).

The peroxidase superfamily contains three main structural groups described as I, II and III. In terms of catalysis, all three groups can undergo a catalytic cycle involving oxidation of the heme group by hydrogen peroxide followed by reduction by various phenolic compounds [including lignin monomers thereby generating the corresponding free-radical species (Dunford, 1991; Anterola and Lewis, 2002)]. However, only the class I (ascorbate peroxidases) and class III forms are found in plants, with most plant species containing a large number of isoforms of the latter. However, members of the family

show large variations in pI and extent of glycosylation and substrate specificity; thus appears to be dependent upon residues at the active site and substrate access channel. The substrate preference for p-coumaryl and coniferyl alcohols over sinapyl alcohol has been proposed as the basis for a peroxidase involved in lignification (Ostergaard *et al*., 2000).

There have been very few substantiated reports of the biochemical consequences on lignin and cell wall assembly, as regards manipulation of processes controlling either monomer transport to lignin initiation sites or in the subsequent oxidative polymerization stages involving peroxidases. These are necessary to define further the strategies for global optimization of plant fibre production, and for further dissection of the lignin assembly process.

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:-

- 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.
- 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, both the methods were attempted to fish out the *POX* gene from *Leucaena leucocephala.*

3.2 Materials and methods

3.2.1 Genomic DNA extraction

Genomic DNA was extracted from *L. leucocephala* using the method given by Lodhi *et al*. (Chapter 2, Section 2.8.4.3).

3.2.2 Restriction digestion of DNA

Plasmid and genomic DNA restriction digestions were set up as per manufacturer's (Promega, USA; NEB, UK; Amersham, USA) recommendations (Chapter 2, Section 2.8.4.4).

3.2.3 Bacterial strains and plasmids used in the study

Escherichia coli XL-1 blue (Stratagene, USA)
E. coli -XL1-Blue MRF (Stratagene, USA)
E. coli SOLR (Stratagene, USA)
λ ZAP II vector (Stratagene, USA)
pGEM-T Easy Vector Cloning vector (Promega, USA)

3.2.4 Construction and screening of genomic DNA library

Leucaena leucocephala genomic DNA library was constructed and screening was performed as mentioned in Chapter 2, Section 2.9.

3.2.5 Southern hybridization

Southern hybridization was done (Chapter2, Section 2.8.6.1) to establish the presence of *POX* gene and determine its copy number in *L. leucocephala*.

3.2.6 RNA isolation and cDNA first strand synthesis

Total RNA was isolated from *L. leucocephala* xylem tissue of stem (Chapter2, Section 2.8.4.6); mRNA was purified from total RNA using oligotex dT resins (Chapter2, Section 2.8.4.7) and cDNA Reverse Transcription (RT) 1st strand was synthesized (Chapter2, Section 2.8.4.9).

3.2.7 Polymerase Chain Reaction

PCR amplification was done using cDNA 1st strand or genomic DNA as template (Chapter2, Section 2.8.4.10). Amplified PCR products were eluted from Agarose gel (Chapter2, Section 2.8.4.5). The eluted PCR products were used for ligation into suitable vector.

3.2.8 Transformation and selection

The ligation mixture was used for transformation of *E.coli* XL1-Blue, host cell line. Putative transformants were selected using Blue-white screening by adding X-gal and IPTG. Colony PCR (Chapter2, Section 2.8.3) 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.

3.2.9 Bioinformatic analysis

The *POX* gene/nucleotide sequences available in the NCBI gene data bank were aligned and multiple sets of primers were designed from the conserved regions. Several sets of degenerate primers were also designed based on the conserved domains of the POX peptide sequences.

Nucleotide and amino acid sequence analysis was done using software pDRAW 32, ClustalX 1.8 and online bioinformatics analysis facility available at www.justbio.com, www.expasy.org and www.ncbi.nlm.nih.gov. Multiple alignments of the amino acid sequences were carried out with the Clustal W1.8 program (http://www.ebi.ac.uk/clustalw/). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura, 2007).

3.2.10 Rapid Amplification of cDNA Ends (RACE)

RACE (Chapter2, Section 2.8.4.11) was done to fish out full length gene with its 5' and 3' UTRs.

3.2.11 Sequencing

DNA sequencing was performed with the ABI Prism Big- Dye Terminator Cycle Sequencing Kit on the ABI Prism 3730 DNA analyzer (Applied Biosystems) at GenomeBio Biotech Pvt. Ltd., Pune, Maharashtra, India.

3.2.12 Estimation of *POX* gene copy number

Genomic DNA was isolated from *L. leucocephala* using Lodhi *et al.* (1994) method. Gene copy number was estimated by Southern hybridization. DNA was digested with six restriction enzymes, *Hae* III*, Eco* RI, *EcoR* V, *Sma* I, *Dra* I and *Pme* I. Four of these enzymes, *Eco* RI, *Hae* III, *Dra* I and *Pme* I do not cut inside the gene while the other two have one site in the gene. Southern hybridization was done using an approximately ~500 bp fragment (from 5' region of *POX* coding region and UTR which hybridizes only with one fragment of *EcoR* V and *Sma* I digest) as probe at 62 °C hybridization temperature (Sambrook *et al.*, 1989).

3.3 Results and discussion

3.3.1 PCR based method for *POX* gene isolation

3.3.1.1 Multiple sequence alignment of amino acid sequences of reported peroxidase (POX) proteins from Fabaceae

POX protein sequences of Fabaceae members (*Medicago sativa* and *Stylosanthes humilis*) available at NCBI GenBank database were aligned using Clustal W (1.8) multiple sequence alignment software. Forward and reverse degenerate primers were designed from regions showing highest homology. The conserved regions selected for primer designing have been shown high-lighted yellow and green in Fig 3.1. In the figure (Fig 3.1), the conserved region, whose primers successfully enabled gene amplification is high-lighted yellow. Forward primers were designed from the regions VHFHDCFVQ & VSCADILALAA) and reverse primers were designed from VSCADILALAA **&** LNTTYLQTLRTICPNGGPG as shown in figure 3.1.

```
>ALFPXDCMedicagosativa       MNSSFNLTLAALCCVVVVLG-G-LPFSSNAQLDNSFYRDTCPNVHSIVRE
>MSRNAPE1AMedicagosativa     ---MLGLSATAFCCMVFVLI-GGVPFS-NAQLDPSFYNSTCSNLDSIVRG
>SSNPEROXIBStylosanthes      -MRWFHVSVCNVVVVVAMLILGALPFSSHAQLDPSFYKNTCANVHSIVRG
>MSRNAPE1CMedicagosativa     ---MNSL-ATSMWCVVLLVVLGGLPFSSDAQLSPTFYSKTCPTVSSIVSN
>MSRNAPE1BMedicago           ---MNSLRAVAIALCCIVVVLGGLPFSSNAQLDPSFYRNTCPNVSSIVRE
                                   :    .     ::  * :*** .***. :** .**..: ***

>ALFPXDCMedicagosativa       VLRNVSKTDPRILASLMRVHFHDCFVQGCDASILLNTTSTITSEQTAFGN
>MSRNAPE1AMedicagosativa     VLTNVSQSDPRMLGSLIRLHFHDCFVQGCDASILLNDTATIVSEQSAPPN
>SSNPEROXIBStylosanthes      VVRNVSKSDRRMLGSLMRLHFHDCFVQGCDGSILLNSTSSIVSEQGAAPN
>MSRNAPE1CMedicagosativa     VLTNVSKTDPRMLASLVRLHFHDCFVLGCDASVLLNNTATIVSEQQAFPN
>MSRNAPE1BMedicago           VIRSVSKKDPRMLASLVRLHFHDCFVQGCDASVLLNKTDTVVSEQDAFPN
                             *: .**:.* *:*.**:*:********:***.*:*** * ::.*** *  *

>ALFPXDCMedicagosativa       NNSIRGLDVVNQIKTAVENACPNTVSCADILALAAEISSVLAHGPDWKVP
>MSRNAPE1AMedicagosativa     NNSIRGLDVINQIKTAVENACPNTVSCADILALSAEISSDLANGPTWQVP
>SSNPEROXIBStylosanthes      NNSIRGLDVVNQIKTAVENACPGVVSCADILALAAEISSVLAGGPNWKVP
>MSRNAPE1CMedicagosativa     NNSLRGLDVVNQIKLAVEVPCPNTVSCADILALAAQASSVLAQGPSWTVP
>MSRNAPE1BMedicago           RNSLRGLDVVNQIKTAVEKACPNTVSCADILALSAELSSTLADGPDWKVP
                             .**:*****:**** *** .**..*********:*: ** ** ** * **

>ALFPXDCMedicagosativa       LGRRDSLTANLTLANENLPSPAFNLSELKKNFDRQGLDTTDLVALSGAHT
>MSRNAPE1AMedicagosativa     LGRRDSLTANNSLAAQNLPAPTFNLTRLKSNFDNQNLSTTDLVALSGGHT
>SSNPEROXIBStylosanthes      LGRRDSLTANLTLANQKLPAPTFNLTQLISTFGNQTLNITDLVALSGAHT
>MSRNAPE1CMedicagosativa     LGRRDGLTANRTLANQNLPAPFNSLDQLKAAFTAQGLNTTDLVALSGAHT
>MSRNAPE1BMedicago           LGRRDGLTANQLLANQNLPAPFNTTDQLKAAFAAQGLDTTDLVALSGAHT
                             *****.****  ** ::**:*  .  .*   *  * *. ********.**

>ALFPXDCMedicagosativa       IGRGQCRFFVDRLYNFSNTGNPDPTLNTTYLQTLRTICPNGGPGSTLTDL
>MSRNAPE1AMedicagosativa     IGRGQCRFFVDRLYNFSNTGNPDSTLNTTYLQTLQAICPNGGPGTNLTDL
>SSNPEROXIBStylosanthes      IGRAQCRFFSSRLYNFSSSGNPDPSLNTTYLQTLRSIFPNGGPGNTLTNL
>MSRNAPE1CMedicagosativa     FGRAHCAQFVSRLYNFSSTGSPDPTLNTTYLQQLRTICPNGGPGTNLTNF
>MSRNAPE1BMedicago           FGRAHCSLFVSRLYNFSGTGSPDPTLNTTYLQQLRTICPNGGPGTNLTNF
                             :**.:*  * .******.:*.**.:******* *::* ******..**::

>ALFPXDCMedicagosativa       DPTTPDTFDSAYYSNLRIQKGLFESDQVLASTSGADTIAIVNSFNNNQTL
>MSRNAPE1AMedicagosativa     DPTTPDTFDSNYYSNLQVGKGLFQSDQELFSRNGSDTISIVNSFANNQTL
>SSNPEROXIBStylosanthes      DLTTPDTFDNKYFSNLQSLNGLLQSDQELFSTSGASSVSIVYSFSSNHTL
>MSRNAPE1CMedicagosativa     DPTTPDKFDKNYYSNLQVKKGLLQSDQELFSTSGADTISIVNKFSTDQNA
>MSRNAPE1BMedicago           DPTTPDKFDKNYYSNLQVKKGLLQSDQELFSTSGSDTISIVNKFATDQKA
                             * ****.**. *:***:  :**::*** * * .*:.:::** .* .::.

>ALFPXDCMedicagosativa       FFEAFKASMIKMSKIKVLTGSQGEIRKQC---NFVNGNS-GLATKVIRE-
>MSRNAPE1AMedicagosativa     FFENFVASMIKMGNIGVLTGSQGEIRTQC---NAVNGNSSGLATVVTKE-
>SSNPEROXIBStylosanthes      FFEAFKASMIKMGNIGVLTGSLGEIQNSV---S----------------
>MSRNAPE1CMedicagosativa     FFESFKAAMIKMGNIGVLTGTKGEIRKQCNFVNFVNSNSAELDLATIASI
>MSRNAPE1BMedicago           FFESFRAAMIKMGNIGVLTGNQGEIRKQC---NFVNSKSAELGLINVAS-
                             *** * *:****.:* ****. ***:..    .

>ALFPXDCMedicagosativa       --SSEDGIVSSYS
>MSRNAPE1AMedicagosativa     --SSEDGMASSFS
>SSNPEROXIBStylosanthes      -------------
>MSRNAPE1CMedicagosativa     VESLEDGIASVIS
>MSRNAPE1BMedicago           ADSSEEGMVSSMS
```

Fig 3.1: Multiple sequence alignment of peroxidase (POX) amino acid sequences of Fabaceae members *Medicago sativa* and *Stylosanthes humilis*. Highlighted are conserved regions considered for primer synthesis.

3.3.1.2 Multiple sequence alignment of nucleotide sequences of reported Peroxidase (*POX*) gene(s) from Fabaceae

POX gene nucleotide sequences of Fabaceae members (*Medicago sativa* and *Stylosanthes humilis*) available at NCBI GenBank database were aligned using Clustal W (1.8) multiple sequence alignment software. Forward and reverse primers were designed from regions showing highest homology. The conserved regions selected for primer designing has been shown high-lighted (yellow & green (**Fig 3.2**)); the conserved region, whose primers successfully enabled gene amplification is high-lighted yellow (**Fig 3.2**).

```
>SSNPEROXIBStylosanthes      ATGAGGTGGTTTC---ATGTAAGTGTGTGTAATGTTGTGGT-TG-TTGTG
>MSRNAPE1AMedicagosativa     ------ATGCTTG---GTCTAAGTGCA-ACAGCTTTTTGCTGTA-TGGTG
>MSRNAPE1CMedicagosativa     ATGAACTCCTTA------GCAACTTCT-AT-GTGGTGTGTTGTGCTTTTA
>MSRNAPE1BMedicago           ATGAACTCCCTTC---GTGCTGTAGCA-ATAGCTTTGTGCTGTA-TTGTG
>ALFPXDCMedicagosativa       ATGAACTCTTCTTTCAATCTCACATTG-GCAGCTTTGTGCTGTG-TAGTG
                                                          * ** * *  *  *

>SSNPEROXIBStylosanthes      GCTATGCTCATTCTTGGAGCACTTCCATTTTCCTCACATGCACAGTTAGA
>MSRNAPE1AMedicagosativa     TTTGTGCTAATTGGAGGAGTACCC------TTTTCAAATGCACAACTAGA
>MSRNAPE1CMedicagosativa     GTTGTGCT---TGGAGGACTACCC---TTTTCCTCAGATGCACAACTTAG
>MSRNAPE1BMedicago           GTTGTGCT---TGGAGGGTTACCC---TTCTCTTCAAATGCGCAACTTGA
>ALFPXDCMedicagosativa       GTTGTGCT---TGGAGGGTTACCC---TTCTCTTCAAATGCACAACTTGA
                               * ****   *   **   **        *  *** **** **  *

>SSNPEROXIBStylosanthes      TCCATCATTTTACAAGAACACTTGTGCCAATGTGCATTCCATTGTTAGAG
>MSRNAPE1AMedicagosativa     TCCTTCATTTTACAACAGTACATGTTCTAATCTTGATTCAATCGTACGTG
>MSRNAPE1CMedicagosativa     TCCCACTTTTTACAGCAAAACGTGTCCAACTGTTAGTTCCATTGTTAGCA
>MSRNAPE1BMedicago           TCCATCCTTTTACAGGAACACTTGTCCAAATGTTAGTTCCATTGTTCGTG
>ALFPXDCMedicagosativa       TAATTCCTTCTATAGGGACACCTGTCCTAATGTTCATTCCATTGTTCGTG
                             *    * ** ** *     ** *** * * * *   *** ** **  *

                                                    Amino acid sequence LHFDCFV
>SSNPEROXIBStylosanthes      GAGTTGTAAGAAACGTTTCAAAATCTGATCGAAGAATGCTTGGTAGTCTC
>MSRNAPE1AMedicagosativa     GTGTGCTCACAAATGTTTCACAATCTGATCCCAGAATGCTTGGTAGTCTC
>MSRNAPE1CMedicagosativa     ATGTCTTAACAAACGTTTCTAAGACAGATCCTCGCATGCTTGCTAGTCTC
>MSRNAPE1BMedicago           AAGTCATAAGGAGTGTTTCTAAGAAAGATCCTCGTATGCTTGCTAGTCTT
>ALFPXDCMedicagosativa       AAGTACTAAGGAATGTTTCTAAGACAGATCCTCGTATACTTGCTAGTTTG
                               **  * *  *  *****  *    ****   * ** **** **** *

>SSNPEROXIBStylosanthes      ATGAGGCTCCACTTTCATGATTGTTTTGTTCAAGGTTGTGATGGATCGAT
>MSRNAPE1AMedicagosativa     ATCAGGCTACATTTTCATGACTGTTTTGTTCAAGGTTGCGATGCCTCGAT
>MSRNAPE1CMedicagosativa     GTCAGGCTTCACTTTCATGACTGTTTTGTTCTGGGATGTGATGCCTCAGT
>MSRNAPE1BMedicago           GTCAGGCTTCACTTTCATGACTGTTTTGTTCAAGGTTGTGATGCATCAGT
>ALFPXDCMedicagosativa       ATGAGAGTTCACTTCCATGATTGCTTTGTTCAAGGTTGTGATGCATCAAT
                              * **  * ** ** *****  ** ******  ** ** ****  ** *

>SSNPEROXIBStylosanthes      TTTGTTGAATAGCACAAGTAGCATAGTGAGTGAACAAGGTGCAGCTCCAA
>MSRNAPE1AMedicagosativa     TTTGCTGAACGATACGGCTACAATAGTGAGCGAGCAAAGTGCACCACCAA
>MSRNAPE1CMedicagosativa     TTTGCTGAACAATACTGCTACAATCGTAAGCGAACAACAAGCTTTTCCAA
>MSRNAPE1BMedicago           TTTACTAAACAAAACTGATACCGTTGTGAGTGAACAAGATGCTTTTCCAA
>ALFPXDCMedicagosativa       CTTGTTGAATACCACTTCGACGATTACGAGTGAGCAAACAGCTTTTGGAA
                              **  * **    **    *   *    ** ** ***   **      **

>SSNPEROXIBStylosanthes      ATAACAACTCAATAAGAGGCTTAGATGTTGTGAATCAGATCAAAACAGCT
>MSRNAPE1AMedicagosativa     ATAACAACTCCATAAGAGGTTTGGATGTGATAAACCAGATCAAAACAGCG
>MSRNAPE1CMedicagosativa     ATAACAACTCTCTAAGAGGTTTGGATGTTGTGAATCAGATCAAACTGGCT
>MSRNAPE1BMedicago           ACAGAAACTCATTAAGAGGTTTGGATGTTGTGAATCAAATCAAAACAGCT
>ALFPXDCMedicagosativa       ATAACAACTCAATAAGAGGTTTGGATGTTGTGAATCAAATCAAAACAGCA
                             * *  *****  ******* ** *****  * ** ** ******   **
```

Amino acid sequence VSCADILAA

```
>SSNPEROXIBStylosanthes      GTAGAAAATGCTTGTCCTGGTGTTGTCTCTTGTGCTGATATTCTTGCACT
>MSRNAPE1AMedicagosativa     GTGGAAAATGCTTGTCCTAACACAGTTTCTTGTGCTGATATTCTTGCTCT
>MSRNAPE1CMedicagosativa     GTAGAAGTGCCTTGTCCTAACACAGTTTCTTGTGCTGATATTCTTGCACT
>MSRNAPE1BMedicago           GTGGAAAAGGCTTGTCCTAACACAGTTTCTTGTGCTGATATTCTTGCTCT
>ALFPXDCMedicagosativa       GTTGAAAATGCTTGTCCTAACACTGTTTCTTGTGCTGATATTCTTGCACT
                             ** ***    *******     ** ******************** ** **

>SSNPEROXIBStylosanthes      TGCAGCTGAGATTTCATCTGTTTTGGCTGGAGGTCCTAATTGGAAAGTTC
>MSRNAPE1AMedicagosativa     TTCTGCTGAAATATCATCTGATCTGGCAAATGGTCCTACTTGGCAAGTTC
>MSRNAPE1CMedicagosativa     TGCTGCTCAAGCATCCTCTGTTCTGGCACAAGGTCCTAGTTGGACGGTTC
>MSRNAPE1BMedicago           TTCTGCTGAATTATCATCTACACTGGCAGATGGTCCTGACTGGAAGGTTC
>ALFPXDCMedicagosativa       TGCTGCTGAAATTTCCTCTGTTCTGGCTCATGGTCCTGATTGGAAAGTGC
                             * * *** *    ** ***    ****    ******   ***   ** *

>SSNPEROXIBStylosanthes      CATTAGGGAGAAGAGATAGTTTAACAGCAAACTTAACCCTTGCTAATCAG
>MSRNAPE1AMedicagosativa     CATTAGGAAGAAGGGATAGTTTGACAGCAAATAATTCCCTTGCAGCTCAA
>MSRNAPE1CMedicagosativa     CTTTAGGAAGAAGGGATGGTTTAACCGCAAACCGAACACTTGCAAATCAA
>MSRNAPE1BMedicago           CTTTAGGAAGAAGAGATGGTTTAACGGCAAACCAGTTACTTGCTAATCAA
>ALFPXDCMedicagosativa       CATTAGGAAGAAGAGATAGTTTAACAGCAAATTTAACCCTTGCTAATGAA
                             * ***** ***** *** **** ** *****       *****   * *

>SSNPEROXIBStylosanthes      AAACTTCCAGCTCCTACCTTCAATCTTACTCAACTCATCTCTACTTTTGG
>MSRNAPE1AMedicagosativa     AATCTTCCTGCCCCCACTTTCAACCTTACTCGACTAAAATCTAACTTTGA
>MSRNAPE1CMedicagosativa     AATCTTCCGGCTCCATTCAATTCCTTGGATCAACTTAAAGCTGCATTTAC
>MSRNAPE1BMedicago           AATCTTCCAGCTCCTTTCAATACTACTGATCAACTTAAAGCTGCATTTGC
>ALFPXDCMedicagosativa       AATCTTCCTAGTCCCGCTTTCAACCTTAGTGAACTGAAAAAAAACTTTGA
                             ** *****    **               *  *** *       ***

 >SSNPEROXIBStylosanthes      TAATCAAACCCTCAACATAACTGATCTAGTTGCACTCTCAGGTGCTCATA
>MSRNAPE1AMedicagosativa     TAATCAAAACCTCAGTACTACTGATCTAGTTGCACTCTCAGGTGGCCATA
>MSRNAPE1CMedicagosativa     TGCTCAAGGCCTCAATACTACTGATCTAGTTGCACTCTCGGGTGCTCATA
>MSRNAPE1BMedicago           TGCTCAAGGTCTCGATACTACTGATCTGGTTGCACTCTCCGGTGCTCATA
>ALFPXDCMedicagosativa       TAGACAAGGTCTGGACACTACAGATCTAGTTGCACTTTCAGGTGCTCATA
                             *   ***   **    *  ** ***** ******** ** ****  ****

>SSNPEROXIBStylosanthes      CAATTGGAAGAGCTCAATGCAGATTTTTCAGCAGCAGATTATACAATTTC
>MSRNAPE1AMedicagosativa     CAATTGGAAGAGGTCAATGCAGATTTTTCGTTGATCGATTATACAATTTC
>MSRNAPE1CMedicagosativa     CATTTGGAAGAGCTCATTGCGCACAATTTGTTAGTCGATTGTACAACTTC
>MSRNAPE1BMedicago           CATTTGGAAGAGCTCATTGCTCTTTATTTGTTAGCCGATTGTACAACTTC
>ALFPXDCMedicagosativa       CAATTGGTAGAGGTCAATGCAGATTCTTCGTTGATCGATTATACAATTTC
                             ** **** **** *** ***      **        **** ***** ***

>SSNPEROXIBStylosanthes      AGCAGCAGTGGAAACCCTGATCCAAGTTTGAACACAACCTACCTACAAAC
>MSRNAPE1AMedicagosativa     AGCAACACTGGAAACCCCGATTCAACTCTTAACACGACCTATTTACAAAC
>MSRNAPE1CMedicagosativa     AGCAGTACTGGAAGTCCCGATCCAACTCTTAACACAACTTACTTACAACA
>MSRNAPE1BMedicago           AGCGGTACGGGAAGTCCCGATCCAACTCTTAACACAACTTACTTACAACA
>ALFPXDCMedicagosativa       AGCAATACTGGAAATCCCGATCCAACTCTCAACACAACCTACTTACAAAC
                             ***   *  ****  ** *** *** * * ***** ** **  *****
```

Amino acid sequence CPNGGPG

```
>SSNPEROXIBStylosanthes      ACTTAGATCAATTTTCCCCAATGGTGGACCAGGGAATACCCTCACCAATT
>MSRNAPE1AMedicagosativa     ATTGCAAGCAATATGTCCCAATGGTGGACCTGGTACAAACCTAACCGATT
>MSRNAPE1CMedicagosativa     ACTGCGCACAATATGTCCCAATGGTGGACCTGGCACAAACCTTACCAATT
>MSRNAPE1BMedicago           ATTGCGCACAATATGTCCCAATGGTGGACCTGGCACGAACCTTACCAATT
>ALFPXDCMedicagosativa       ATTGAGAACAATATGTCCCAATGGTGGACCTGGTAGCACCCTCACTGATT
                             * *     ****    ********************  ***  **   ***

>SSNPEROXIBStylosanthes      TGGACCTAACAACACCTGACACATTTGACAACAAATACTTCTCCAATCTT
>MSRNAPE1AMedicagosativa     TGGACCCAACCACACCAGATACATTTGACTCCAACTACTACTCCAATCTC
>MSRNAPE1CMedicagosativa     TCGATCCAACGACTCCTGATAAATTTGACAAGAACTATTACTCCAATCTT
>MSRNAPE1BMedicago           TCGATCCAACGACTCCTGATAAATTTGACAAGAACTATTACTCTAATCTT
>ALFPXDCMedicagosativa       TGGATCCAACCACTCCAGATACATTCGACTCGGCCTACTATTCAAACCTA
                             * ** * *** ** ** ** * *** ***      ** *  ** ** **

>SSNPEROXIBStylosanthes      CAATCTCTGAATGGATTGCTTCAGAGCGATCAAGAGCTTTTCTCCACAAG
>MSRNAPE1AMedicagosativa     CAAGTTGGAAAGGGCTTGTTTCAGAGTGACCAAGAGCTTTTTCCAGAAA
>MSRNAPE1CMedicagosativa     CAAGTGAAAAAGGGTTTGCTCCAAAGTGATCAAGAGTTGTTCTCAACTTC
>MSRNAPE1BMedicago           CAAGTGAAAAAAGGTTTGCTTCAAAGTGATCAAGAGTTGTTCTCAACATC
>ALFPXDCMedicagosativa       AGGATTCAAAAAGGCTTGTTTGAGAGTGACCAAGTATTGGCTTCCACAAG
                                      ** ** *** *  * ** ** ****   *    ** *
```

```
>SSNPEROXIBStylosanthes       TGGTGCATCCTCGGTTAGCATTGTCTACAGTTTCAGCAGTAACCATACTC
>MSRNAPE1AMedicagosativa      TGGTTCTGACACTATTTCTATTGTCAATAGTTTCGCCAATAATCAAACTC
>MSRNAPE1CMedicagosativa      TGGTGCAGATACCATTAGCATTGTCAACAAATTCAGCACCGATCAAAATG
>MSRNAPE1BMedicago            TGGTTCAGATACCATTAGCATTGTCAACAAATTCGCAACCGATCAAAAAG
>ALFPXDCMedicagosativa        TGGCGCGGACACGATTGCCATTGTCAATAGTTTCAACAACAACCAAACTC
                              ***  *     *  **   ****** * *  ***   *   * ** *

>SSNPEROXIBStylosanthes       TCTTCTTTGAGGCATTTAAGGCTTCAATGATTAAGATGGGTAACATTGGA
>MSRNAPE1AMedicagosativa      TCTTCTTTGAAAATTTTGTAGCCTCAATGATAAAAATGGGTAATATTGGA
>MSRNAPE1CMedicagosativa      CTTTCTTTGAGAGCTTTAAGGCTGCAATGATTAAAATGGGCAATATTGGT
>MSRNAPE1BMedicago            CTTTTTTTGAGAGCTTTAGGGCTGCTATGATCAAAATGGGAAATATTGGT
>ALFPXDCMedicagosativa        TTTTCTTTGAAGCTTTTAAGGCCTCAATGATTAAAATGAGTAAAATTAAG
                                ** *****    ***   **  * ***** ** *** * ** ***

>SSNPEROXIBStylosanthes       GTTCTAACTGGCTCTCTAGGAGAAATTCAGAACTCAGTGTAACTTTGT--
>MSRNAPE1AMedicagosativa      GTTTTAACTGGATCTCAAGGTGAAATT-AGAACACAGTGTAATGCTGT--
>MSRNAPE1CMedicagosativa      GTGCTAACAGGGACAAAAGGAGAGATT-AGAAAACAATGCAACTTTGTGA
>MSRNAPE1BMedicago            GTGTTAACCGGGAACCAAGGAGAGATT-AGAAAACAATGCAACTTTGT--
>ALFPXDCMedicagosativa        GTGTTAACTGGATCTCAAGGTGAAATT-AGAAAACAATGTAACTTTGT--
                              **  **** **      *** ** *** ****  ** ** **   ***


>SSNPEROXIBStylosanthes       -------TAATGCCAACTCTTC------TGGATTGCCTACTTTTGCTACT
>MSRNAPE1AMedicagosativa      -------GAATGGGAATTCTTC------TGGATTGGCTACTGTAGTCACC
>MSRNAPE1CMedicagosativa      ACTTTGTGAACTCAAATTCTGCAGAACTAGATTTAGCCACCATAGCATCC
>MSRNAPE1BMedicago            -------TAATTCAAAATCAGCAGAACTTGGTCTTATCAATGTTGCCTC-
>ALFPXDCMedicagosativa        -------TAATGGAAATTCTG---------GTCTTGCCACTAAAGTCATC
                                     **    ** **             *    *     *

>SSNPEROXIBStylosanthes       AGAGTAGAAGGAGAAGGAGGTGTTACTATTACTTCAGTCTAAAAAGGTTA
>MSRNAPE1AMedicagosativa      AAA---GAATCATCAGAAGATGGAATGGCTAGCTCATTCTAA--------
>MSRNAPE1CMedicagosativa      ATAGTAGAATCATTAGAGGATGGTATTGCTAGTGTAATATAA--------
>MSRNAPE1BMedicago            --AGCAGATTCATCTGAGGAGGGTATGGTTAGCTCAATGTAA--------
>ALFPXDCMedicagosativa        ---AGAGAGTCGTCAGAGGATGGTATTGTTAGCTCATACTAA--------
                                   **       *  *  *  *    **    *   ***
```

Figure 3.2: Multiple sequence alignment of peroxidase (*POX*) nucleotide sequences of Fabaceae members *Medicago sativa* and *Stylosanthes humilis.* Highlighted are conserved regions considered for primer synthesis.

The nucleotide sequences of the *POX* gene from Fabaceae were aligned using Clustal W 1.8 program. The nucleotide sequences corresponding to conserved regions (highlightened in Fig 3.1 and Fig 3.2) were selected to design forward and reverse primers. Though the amino acid seems conserved in the selected regions (Figure 3.1), their nucleotide counter parts donot show much homology (Figure 3.2). In an attempt to design an unambiguous primer, the codon usage table for *L. leucocephala* was used and the nucleotide bases were modified at the wobble positions.

3.3.1.3 Forward and reverse primers

Forward (Raj3F): 5’ ATG CTT GGT AGT CTC ATG AGG CTC CAC TTT CAT GA 3’

Reverse (Super3R): 5’ AGC AGC AAG TGC AAG AAT ATC AGC ACA AGA 3’

3.3.1.4 Genomic DNA extraction

Genomic DNA was isolated (**Fig 3.3**) by using the protocol of Lodhi *et al.,* (1994) as mentioned in Chapter 2, Section 2.8.4.3. The concentration of the gDNA was measured by spectrophotometer as mentioned in Chapter 2, Section 2.8.4.8. PCR was performed using 100 ng of good quality of gDNA as the template. All possible primer combinations and cycling parameters were tried. None of the reactions gave a positive result.

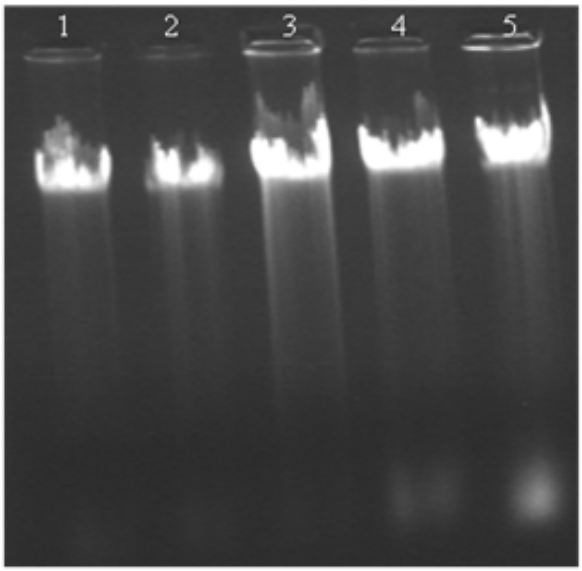


Figure 3.3: *Leucaena leucocephala* genomic DNA (lanes 1 to 5) resolved on 0.7% agarose gel.

3.3.1.5 Isolation of total RNA from *L. leucocephala*, its cDNA synthesis and PCR

RNase free environment was created and maintained as described, the glassware and plasticware were treated overnight with DEPC (0.1% in water) and autoclaved. The pestle and mortar were also DEPC treated and then baked at 300 ºC for 6 h.

Total RNA (**Fig.3.4**) was isolated from xylem tissue of field grown plants or from *in vitro* grown seedlings of *L. leucocephala* (as mentioned in Chapter 2, Section 2.8.4.6) and purified mRNA (as mentioned in Chapter 2, Section 2.8.4.7) was used for cDNA 1st strand synthesis (as mentioned in Chapter 2, Section 2.8.4.9).

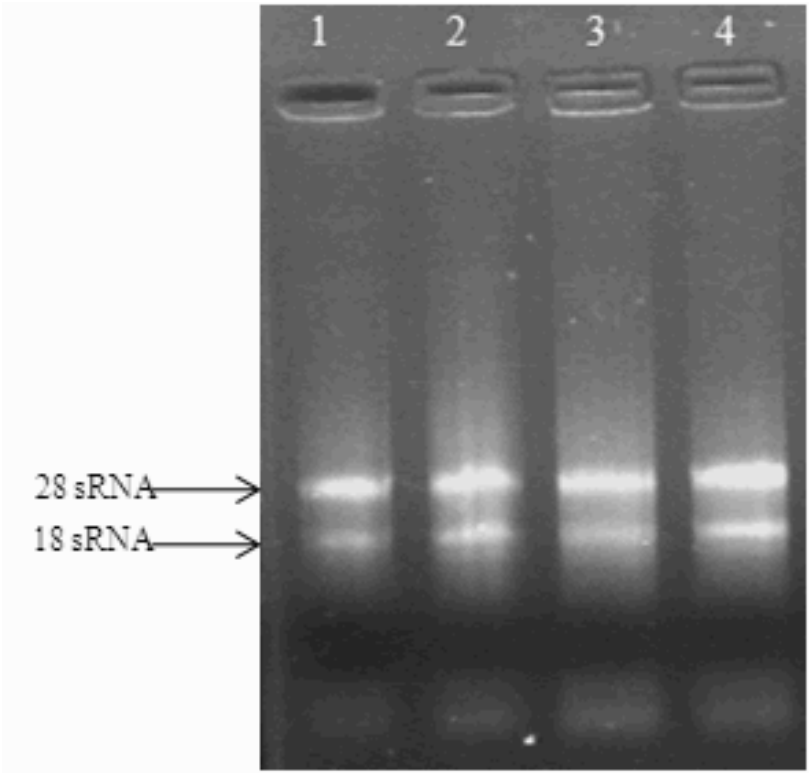


Fig 3.4: Total RNA isolated from xylem tissue (lanes 1 to 4) of *L. leucocephala*, resolved on 1% denaturing gel. Arrows indicates both 28s RNA and 18s RNA bands.

3.3.1.6 PCR amplification of partial cDNA fragments of *POX* gene from *L. leucocephala*

Primers Raj3F and Super3R (mentioned in section 3.3.1.3) were used to amplify 222 bp fragment of *POX* gene, using cDNA 1st strand as the template (Fig 3.5A). The fragment was cloned in pGEM-T Easy vector; confirmed by restriction analysis (Fig 3.5B) and sequencing (Fig 3.6). This partial clone will be referred as LlPOXA in the subsequent discussions. The LlPOXA showed maximum homology (>80 to 90%) with the Fabaceae members *Medicago sativa*, *Stylosanthes humulis*.

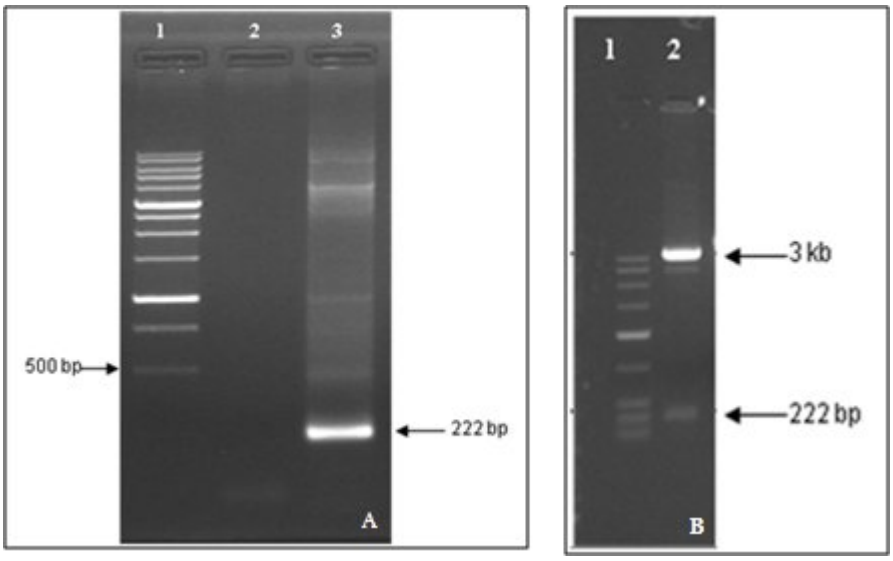


Fig 3.5A: PCR amplification product of 222 bp fragment (designated as LlPOXA**)** separated on 1.5% agarose gel. Lane 1- marker, lane 2 non-template control and lane 3 amplified product. **Fig 3.5B** LIPOXA releasing 222 bp insert when digested with *Eco* RI, lane 1- marker, lane 2- cloned 222 bp insert in LIPOXA

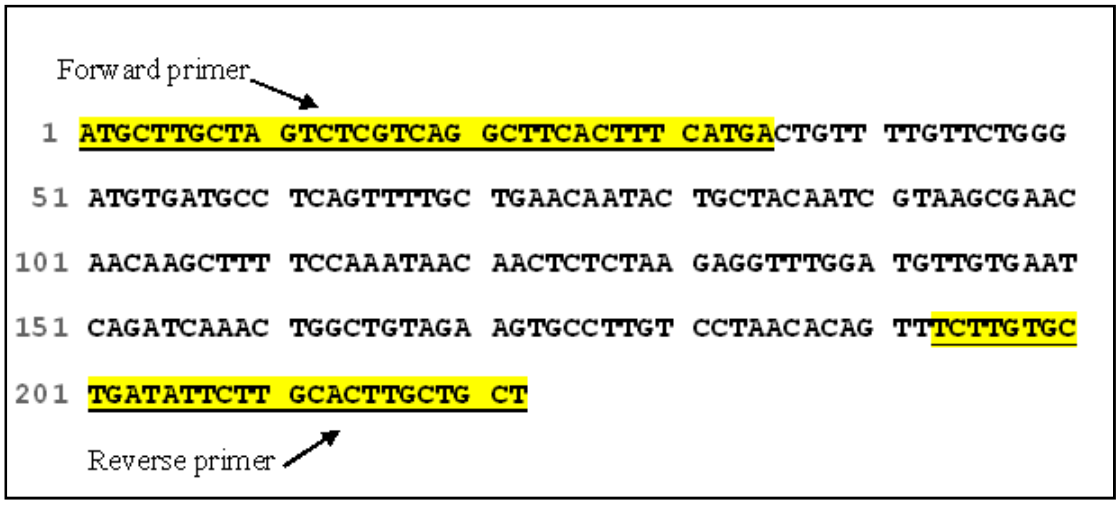


Fig 3.6: Nucleotide sequence of partial peroxidase clone LlPOXA, highlighted regions show primer sequences.

3.3.2 Slot Blot Hybridization

The genome size of *L. leucocephala* has ~1.81 pg of DNA per haploid genome. 1.8 pg corresponds to a single representation of *L. leucocephala* genomic DNA, 0.18 µg representing the genome $1x10^5$ times (Fig 3.7 A1 to A12) was spotted on Hybond N^+ membrane (Amersham, USA). The POX cDNA clone LlPOXA (222 bp; Section 3.3.1.6) was spotted as standard dilutions representing $1x10^5$, $2x10^5$, $3 x10^5$, $4 x10^5$, $5 x10^5$ and 6

x10^5 copies in duplicates. The blot was probed with the radio-labelled LlPOXA gene. Positive signal obtained under high stringency hybridization conditions was indicative of the presence of the *POX* gene in *L. leucocephala.* Based on signal intensity it was also inferred that in *L. leucocephala POX* belonged to a gene family represented possibly by 3 to 4 members (Fig 3.7).

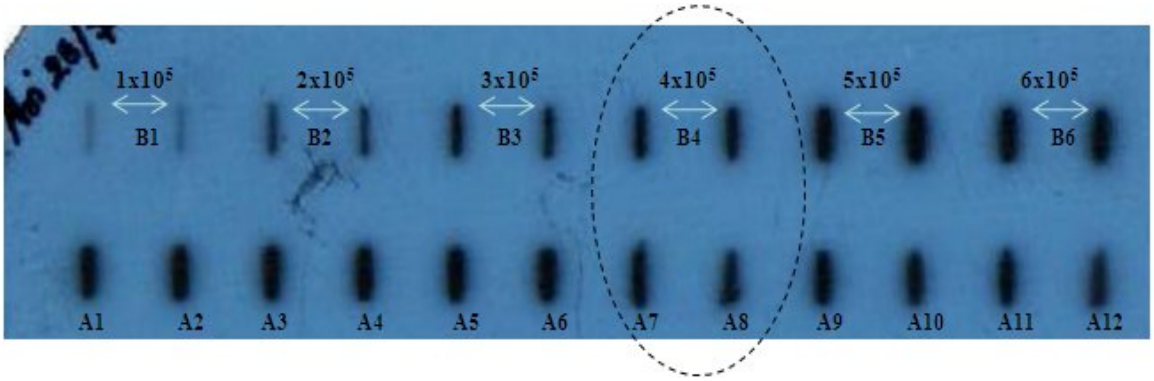


Fig. 3.7: Slot Blot Hybridization for *POX* gene in *L. leucocephala*: Slot Blot of *L. leucocephala* genomic DNA hybridized with radio-labelled LlPOXA. Lanes A1 to A12 represents genomic DNA of *L. leucocephala* once (1x10^5). Lane B1, B2, B3, B4, B5 and B6 signals from 1x10^5, 2x10^5, 3 x10^5, 4 x10^5, 5 x10^5 and 6 x10^5 copies in duplicates of LlPOXA gene. From the figure we can possibly infer that *POX* gene may occur in 3 to 4 copies

3.3.3 Gene copy number by Southern Hybridization

To further validate the results from slot blot experiment and to understand the distribution of the *POX* gene in the *L. leucocephala* genome, Southern hybridization was performed (Chapter 2, Section 2.8.6). A 25 µg aliquot of *L. leucocephala* genomic DNA was restriction digested individually with six restriction enzymes, *Dra* I, *Eco* RI, *EcoR* V, *Hae* III*, Sma* I, and *Pme* I (Fig 3.8A). Four enzymes, *Dra* I, *Eco* RI, *Hae* III and *Pme* I do not cut inside the gene while the other two have one site within the gene. As shown in Fig 3.8B, four bands were detected in the *Dra* I, *Eco* RI, *Hae* III and *Pme* I digests, while the other two doesn't show any distinct bands, may be due to the reason that bands may be very small and of low intensity, which is not seen in blot. Southern hybridization was done using a part of coding region and part of 5' UTR (Section 3.3.5.1). Post hybridization membrane was exposed on intensifying screen. The screen was scanned for signal development using a very robust and much sensitive Typhoon TRIO+ scanner. An

approximately 500 bp fragment was used as a probe for hybridization at 62 °C. Banding pattern in Southern hybridization suggested that at least 4 copy of *POX* gene is present in *L. leucocephala* genome, as four bands are observed in wells digested with *Dra* I, *Eco* RI, *Hae* III and *Pme* I and subjected to Southern hybridization (Fig 3.8B).

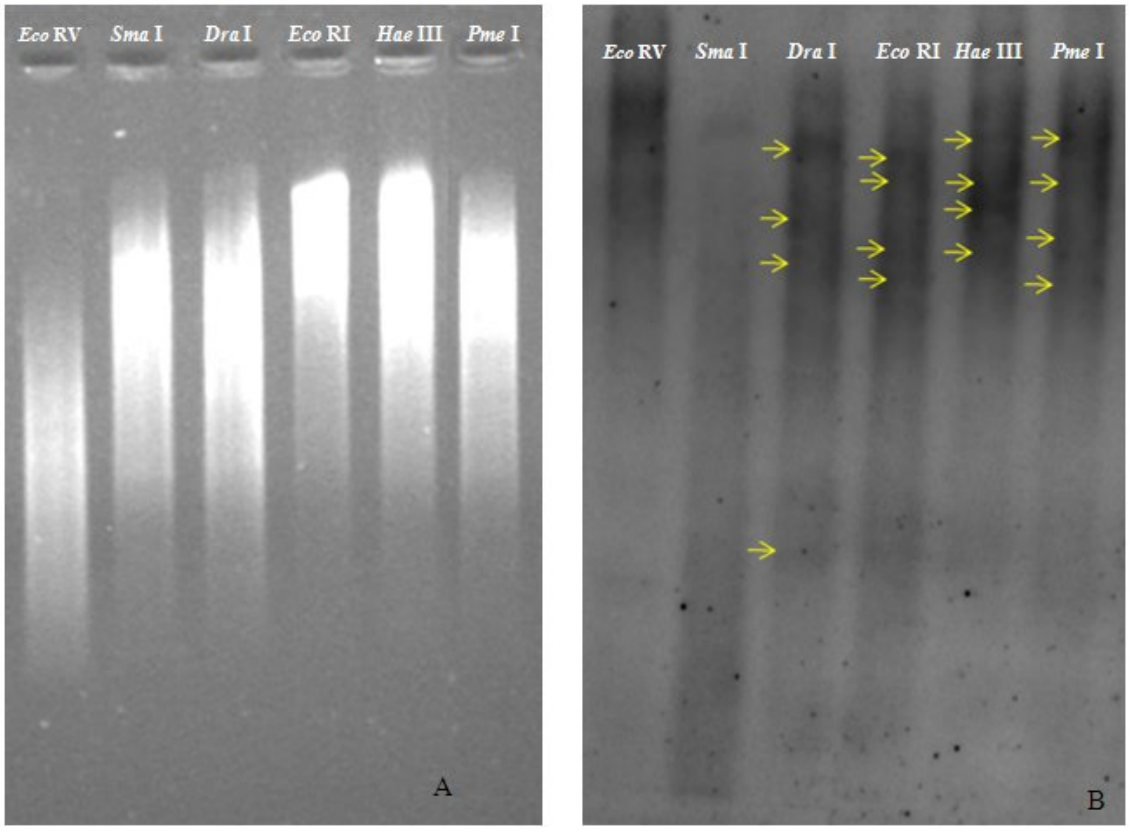


Figure 3.8: A. 0.7% agarose gel showing *L. leucocephala* gDNA digested with *EcoR* V (lane 1), *Sma* I (lane 2), *Dra* I (lane 3), *Eco* RI (lane 4), *Hae* III (lane 5), and *Pme* I (lane 6). **B**. Southern hybridized signals of the above mentioned gel scanned using Typhoon TRIO+ scanner. Arrows indicate hybridization signals with respective enzymes.

3.3.4 Screening of genomic DNA Library:

To fish out the full-length genomic clone of *POX* gene, the next option (first PCR amplification using gDNA as template, mentioned in section 3.3.1.4) was, screening of *Leucaena* genomic DNA library. The partial 222 bp cDNA fragment was used to screen *Leucaena* genomic DNA library prepared in λ phage (ZAP II, Stratagene) as mentioned in section 3.2.4. The average size of gDNA fragments cloned was standardizing by time-course reaction (Fig. 3.9) and fragments of size between 8 to 10 kb were selected for

making the library as mentioned in section 3.2.4. Screening was performed on 10 plates with an average of 5000 plaques as described in section 3.2.4.3 (plaques lifting), section 3.2.4.4 (preparation of probe, pre-hybridization and hybridization), section 3.2.4.5 (first, second and third round of screening, (Fig. 3.10)) and section 3.2.4.6 (single clone excision). Secondary and tertiary screening was performed at a higher hybridization temperature (62 °C). The positive plaques from tertiary screening were cut individually and excision was performed according to the manufacturer's protocol. After sequencing it was confirmed that, none of the plaques contains peroxidase, the gene of interest.

Next step was to fish out full-length gene, using the available information of partial cDNA sequence of LlPOXA (section 3.3.1.6), showing significant similarity with known POX sequences. Hence, decided to proceed with RACE reactions.

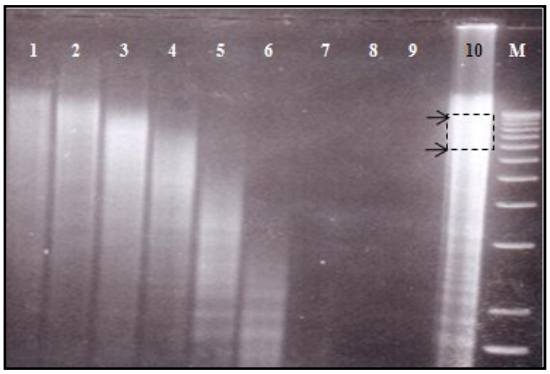


Fig 3.9: Time course reaction of *L. leucocephala* gDNA with Sau 3AI enzyme. Lanes 1 to 10 DNA samples at different time intervals of digestion. Arrows indicate the region of DNA taken for library construction.

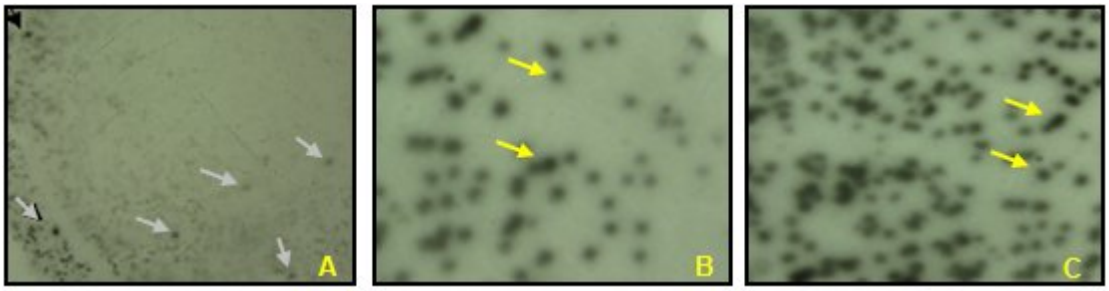


Fig 3.10: A representation of primary (A), secondary (B) and tertiary (C) round of screening of plaques from the gDNA library prepared in λ ZAP II vector. Arrows indicate plaques showing signals after southern hybridization.

3.3.5 Rapid Amplification of cDNA Ends (RACE)

Primers Raj3F and Super3R (mentioned in section 3.3.1.3) used to amplify LlPOXA fragment (222 bp; Section 3.3.1.6) using cDNA 1st strand as template was used for RACE reactions.

Raj3F →

```
  1 ATGCTTGCTA GTCTCGTCAG GCTTCACTTT CATGACTGTT TTGTTCTGGG
 51 ATGTGATGCC TCAGTTTTGC TGAACAATAC TGCTACAATC GTAAGCGAAC
101 AACAAGCTTT TCCAAATAAC AACTCTCTAA GAGGTTTGGA TGTTGTGAAT
151 CAGATCAAAC TGGCTGTAGA AGTGCCTTGT CCTAACACAG TTTCTTGTGC
201 TGATATTCTT GCACTTGCTG CT
```

Super3R ←

3.3.5.1 5’ RACE reaction

5’ Rapid amplification of cDNA ends was performed using gene specific reverse primer ((Super3R) 5’ AGC AGC AAG TGC AAG AAT ATC AGC ACA AGA 3’) and 5’Generacer forward primer provided with the kit, as described in Chapter 2, Section 2.8.4.11. In this reaction 5’ RACE ready cDNA was used as the template. The above RACER product was diluted (1:50) and secondary PCR was done using Super3R and a nested 5’ GeneRacer primer provided with the kit. Agarose gel electrophoresis analysis revealed an amplification product of approximately 500 bp as depicted in figure 3.11A. This amplicon was cloned in pGEMT-Easy vector (Promega, USA) and the resulting construct was confirmed by *Eco*RI restriction analysis (Figure 3.11B) and by sequencing.

The analysis of the sequenced product revealed the exact size of the amplicon to be 477 bp. The 5’ GeneRacer nested primer and gene specific reverse primer sequences are underlined (Fig 3.12). The 5’ UTR region is shown highlighted in yellow (67 bp) and the start codon (ATG) is highlighted blue (Fig 3.12). The partial 5’ coding sequence of *POX* gene obtained from 5’ RACE reaction is 384 bp.

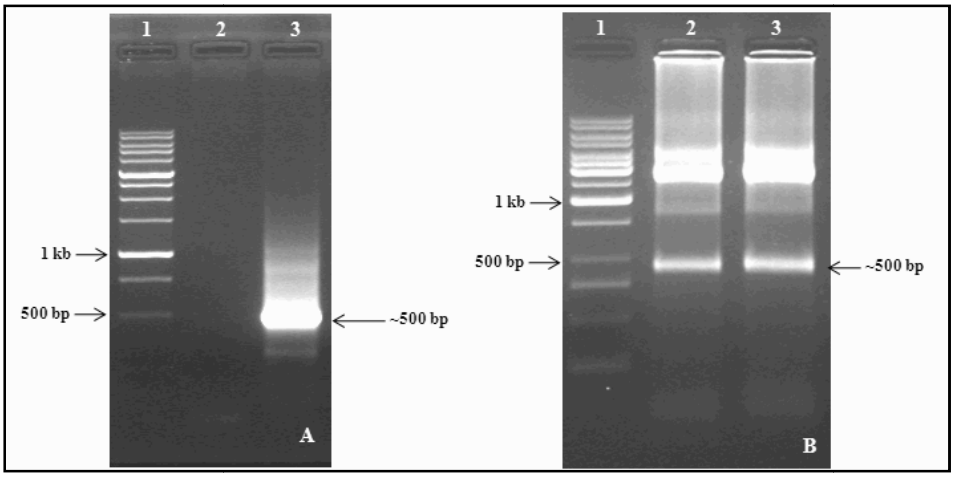


Fig 3.11: A 1% agarose gel showing primary 5'RACE product. Lane 1- marker, lane 2- non-template control and lane 3 ~500 bp 5'RACE product. **B** *EcoR*I digested recombinant pGEMT-Easy vector releasing ~500 bp fragment in lanes 2 & 3, lane 1- marker.

(5'NUP)

```
  1 GGACACTGAC ATGGACTGAA GGAGTAGAAA AATTCTTCAT CTTCTTTCTT
 51 CTCTCTTATC CTTTATTGTT GTTCTCAGTT TCAAGCTCCA GCTATGGCTT
101 CCCTTACCCA TTTCTTCTTA CTTGCATTGT CTGTTCTTTC TCTCTTTGCT
151 TCTTCTTCCA ATGCTCAGCT CTCTCCCAAC TTCTACGCCA GATCTTGCCC
201 TAACGTCCGG GCCATTGTTC GCAACACAAT GAGGCAAGCT CTCGCCCGAG
251 AAGCCCGCCT TGGCGCCTCC ATCCTCCGCC TCCTCTTCCA CGACTGCTTC
301 GTCAATGGTT GTGACGCCGG AATCCTCCTA GACGACACCG CAAGCTTCAC
351 CGGAGAGAAA AACGCCGGCC CCAACCAGTC TGCAAGAGGC TATGAAGTGA
401 TTGATGCTAT CAAAACCAAC GTGGAAGCTG CCTGCAGGGG CACTGTGTCT
451 TGTGCTGATA TTCTTGCACT TGCTGCT
```

Reverse primer (Super3R)

Fig 3.12: Sequence analysis of 5' RACE product of *POX* gene: Primers (5' NUP & Super3R) shown underlined, 5' UTR region shown highlighted in yellow, start codon (ATG) highlighted blue and rest is 5' coding region of POX gene 384 bp.

3.3.5.1.1 Deduced amino acids of 5' RACE coding region

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

3.3.5.2 3' RACE reaction

To isolate the 3' sequence of the *POX* gene, 3' RACE reaction was performed (as described in Chapter 2, Section 2.8.4.11) using a forward gene specific primer ((Raj3F) 5' ATG CTT GGT AGT CTC ATG AGG CTC CAC TTT CAT GA 3') designed from LlPOXA (section 3.3.1.6) and a reverse 3'GeneRacer primer provided with the kit. 3' RACE ready cDNA was used as the template. The RACE reaction resulted in an amplified product of approximately 1.2 Kb; which was further re-amplified using Raj3F and 3' nested universal primer (3'NUP- is provided with kit). The resultant PCR product (Fig 3.13A) was cloned into pGEMT-Easy vector (Promega, USA) and the recombinant construct was confirmed by *Eco* RI restriction analysis (Fig 3.13B) and by sequencing.

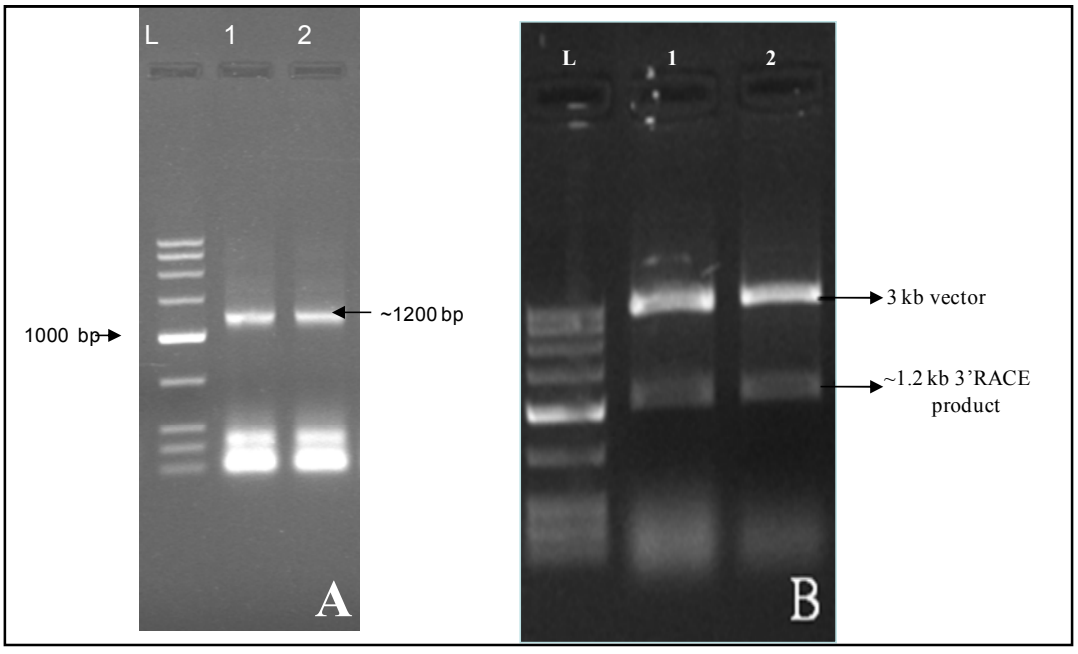


Fig 3.13: A 1% agarose gel showing 3'RACE product. Lane L- marker, lane 1 & 2 is ~1.2 kb 3'RACE product. **B** Lane L- marker, *Eco* RI digested recombinant pGEMT-Easy vector releasing ~ 1.2 kb fragment in lanes 2 & 3.

The analysis of the sequenced 3'RACE product revealed 297 bp 3' UTR region, which is shown highlighted in blue (Fig 3.14) and the stop codon (TGA) is highlighted pink. The primary cleavage site selection in pre- mRNA (CP Joshi, 1987) is highlighted yellow. The poly-A tail of mRNA is shown underlined and the 3'NUP is shown in red letters (Fig 3.12).

```
  1 GGTGTTACCC AGCTAGGGGG ACCCTCATGG GCAGTACCAC TTGGCCGGAG
 51 GGATGCGAGA ACGGCGAGCC AGAGCAAAGC CAACAGCGAG ATCCCGGGGC
101 CGTCATCGGA CCTATCGACT CTGATCTCCA TGTTCGCCGC GAAAGGGCTG
151 ACAGCCCGGC AGATGACGGT GCTGTCGGGC GCTCACACCA TAGGTCAGGG
201 ACAGTGCAAC TTCTTCAGGA ACCGAATCTA CAACGAGAAC AACATTGACC
251 CAAGTTTTGC AGCCACGAGA AGGGCCACTT GCCCTAGAAC TGGTGGGGAC
301 ATCAACTTGG CCCCACTTGA CTTTACCCCC AGCAGGTTCG ACAACACTTA
351 CTACAAGGAC CTTGTAAATC GACGTGGCCT GTTCCATTCG GACCAAGTTC
401 TCTTCAATGG TGGCTCTCAA GATGCTATAG TTAGGGCATA TAGCACCAAC
451 AGCGTCCTCT TCTTTGGGGA CTTTGCTTCT GCAATGGTCA AGGTGAGCAG
501 TATCACTCCC CTCACTGGGT CCCAGGGAGA GATCCGAAAG AATTGTAGGG
551 TTGTCAACTG ATTAGTTCCT CCGTGATTTT TGTTTTTTGT TTTTAATTTT
601 TGGTCATAAA CAGCGATCAA ATAATCAAGG GTTGTATGGG TTGGGGGGGT
651 GTTAATTAGT TTAATCGGTT GTAATTATTA TAGACAAGGT TTTACTATTA
701 GTCATAAGTT GACTAAATGG ATCTTGGATA TATGTATTTG TATGTATGCA
751 TGACACGGCT CATCAAGTGT TGTGAGCTGT ATGTGTCTTT TTTTAAGCAA
801 CGAAATGATT CCAAGTGTTT GCTTGTTTAT GAAAAAAAAA AAAAAAAAAA
851 AAAAACACTG TCATGCCGTT ACGTAGCG
```

Fig 3.14: Sequence analysis of 3' RACE product of *POX* gene: The 3'UTR is represented by 297 bp and it extends from stop codon till end of poly-A tail. Stop codon (TGA) is highlighted in pink, poly-A tail is underlined, the primary cleavage site in pre- mRNA (CP Joshi, 1987) is highlighted in yellow and 3, NUP is represented by red letters

3.3.5.2.1 Deduced amino acids of 5' RACE coding region

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

3.3.5.3 Isolation of Full-length coding region of *POX* gene from cDNA of *L. leucocephala.*

The full length open reading frame (ORF) of peroxidase (*POX*) gene was achieved by designing a forward primer from the start codon in 5'RACE product and a reverse primer from 3'RACE product, leaving the stop codon. cDNA from *L. leucocephala* xylem tissue was used as the template. The primers designed were PeroxidaseF (5' ATG GCT TCC CTT ACC CAT TTC TTC 3') and PeroxidaseR (5' GTT GAC AAC CCT ACA ATT CTT TCG 3'). PCR was performed as mentioned in chapter 2, section 2.8.4.10 and an amplicon of 948 bp was obtained as expected with the full length *POX* gene (Fig 3.15A). The amplicon was excised from the agarose gel and purified using Axygen^{TM} GEL elution kit as mentioned in Chapter2, Section 2.8.4.5. The full length PCR product was cloned into pGEMT-Easy vector (Promega, USA) and was mobilized into *E. coli* XL1-Blue. Plasmids were isolated from a few of the white recombinant colonies which survived on LB ampicillin plates containing X-gal – IPTG and were digested with *Eco* RI to confirm the presence of cloned insert (Fig 3.15B).

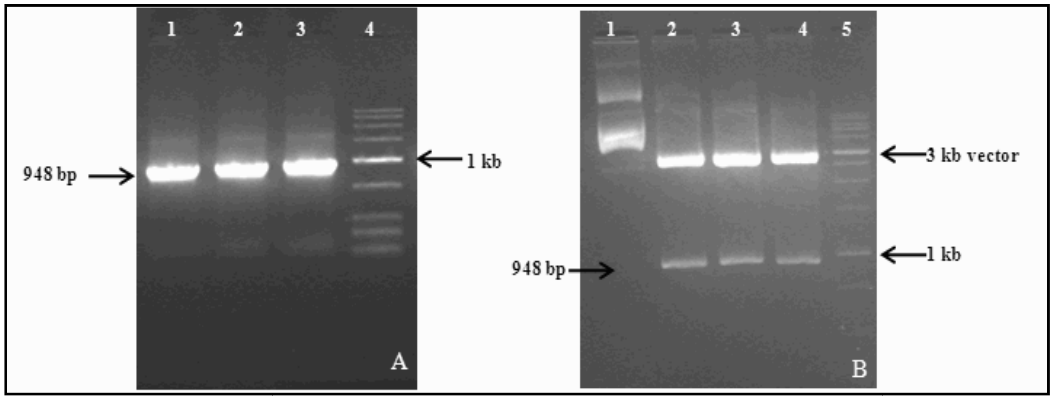


Fig 3.15: A 1% Agarose gel showing full length *POX* gene PCR amplified from cDNA in lanes 1 to 3 (948 bp) and lane 4 is marker. **B** Restriction analysis of recombinant clones releasing 948 bp insert (lanes 2 to 4), lane1 uncut recombinant vector and lane 5 is marker.

A few recombinant pGEMT-Easy vectors carrying *POX* gene were sequenced and confirmed for cloned full length *POX* gene. The sequence analysis revealed existence of two different genes of *POX* gene in randomly sequenced recombinant clones. A few more fresh clones/colonies were sequenced with a view to fish out new clones of *POX* and with

a view to reconfirm the presence of two full length clones in the amplified product (Fig. 3.15A).

3.3.5.4 Characterization of *POX* cDNA sequence

The PCR amplification of full length gene resulted in amplification of two cDNA clones. These two full length cDNA sequences of *POX* genes were submitted to NCBI GenBank database under accession no. EU 649680.1 (LBPox) and GU 143879 (LlLBPOX).

The *Leucaena POX* gene (both EU 649680.1 (LBPox) and GU 143879 (LlLBPOX)) sequence contains a 948 bp open-reading frame, which codes for a protein of 316 amino acids with a molecular weight 34.7 kDa. The putative initiation codon ATG is flanked by a G at + 4, in keeping with the nucleotide commonly found flanking the initiator methionine in plants (Lutcke *et al.,* 1987). The sequence analysis shows that RACE amplification has been well extended downstream to the stop codon (TGA) and has yielded a 297 bp 3'UTR. Upstream to poly-A tail an ubiquitous polyadenylation (poly-A) signal sequence AATAA was present in the 3'UTR (Fig 3.14) which is present in most eukaryotes and forms a complex with U4 SnRNP for primary cleavage site selection in pre- mRNA (CP Joshi, 1987).

3.3.5.4.1 Characterization of *POX* cDNA sequence EU649680.1 (LBPox)

3.3.5.4.1a Nucleotide sequence of EU649680.1 (LBPox)

```
   1 ATGGCTTCCC TTACCCATTT CTTCTTACTT GCATTGTCTG TTCTTTCTCT
  51 CTTTGCTTCT TCTTCCAATG CTCAGCTCTC TCCCAACTTC TACGCCAGAT
 101 CTTGCCCTAA CGTCCGGGCC ATTGTTCGCA ACACAATGAG GCAAGCTCTC
 151 GCCCGAGAAG CCCGCCTTGG CGCCTCCATC CTCCGCCTCT TCTTCCACGA
 201 CTGCTTCGTC AATGGTTGTG ACGCCGGAAT CCTCCTAGAC GACACCGCAA
 251 GCTTCACCGG AGAGAAAAAC GCCGGCCCCA ACCAGTCTGC AAGAGGCTAT
 301 GAAGTGATTG ATGCTATCAA AACCAACGTG GAAGCTGCCT GCAGGGGCAC
 351 TGTGTCGTGT GCTGATATCT TGGCACTTGC GGCACAAGAG GGTGTTACCC
 401 AGCTTGGAGG ACCTCATGGG CAGTACCACT TGGCCCGGAG GGACGCGAGA
 451 ACGGCGAGCC AGAGCAAAGC CAACAGCGAG ATCCCGGGGC CGTCATCGGA
 501 ACTCTCAACT CTGATCTCCA TGTTCGCCGC AAAAGGGCTG AACGCCCGGG
 551 AGATGACGGT GCTGTCCGGC GCGCACAGCA TAGGTCAGGG ACAGTGCAAC
 601 TTCTTCAGGA ACCGAATCTA CAACGAGAAC AACATTGACC CAAGTTTTGC
 651 AGCCACGAGA AGGGCCACTT GCCCTAGAAC TGGTGGGGGC ATCAACTTGG
 701 CCCCACTTGA CTTTACCCCC AACAGGTTTG ACAACACTTA CTACAAGGAC
 751 CTTGTAAATC GACGTGGCCT GTTCCATTCG GACCAAGTTT TTTTCAATGG
 801 TGGCTCTCAA GATGCTATAG TTAGGGCATA TAGCACCAAC AGTGTCCTTT
```

```
851 TTTTTGGGGA CTTTGCTTTT GCAATGGTCA AGATGAGCAG TATCACTCCC
901 CTCACTGGGT CCCAGGGAGA GATCCGAAAG GATTGTAGGG TTGTCAACTG
951 A
```

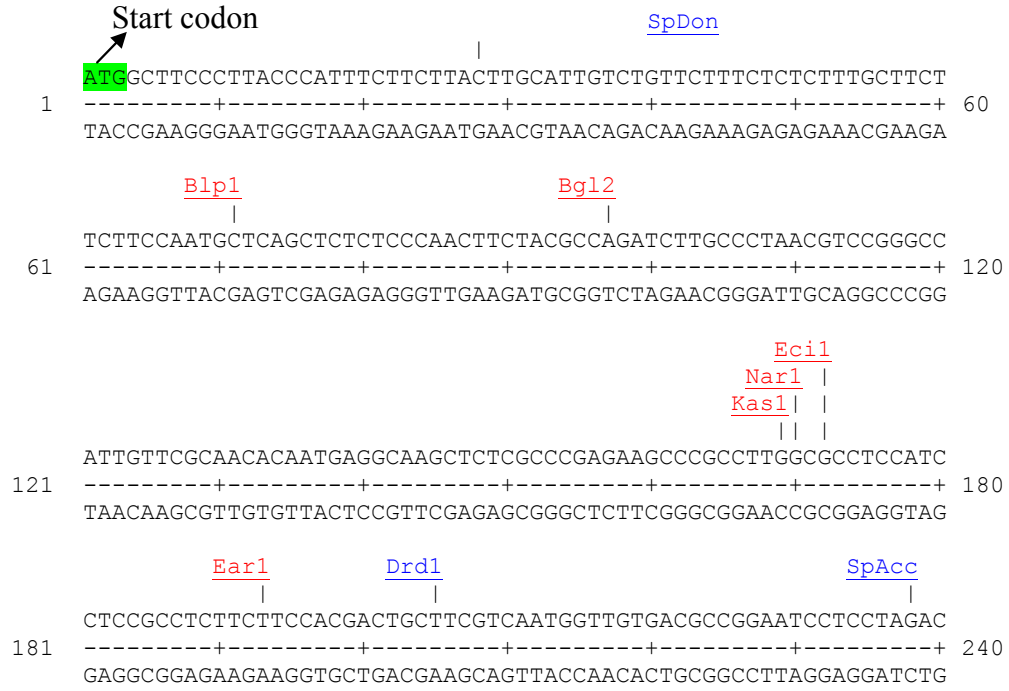
3.3.5.4.1b Deduced amino acid sequence of EU649680.1 (LBPox)

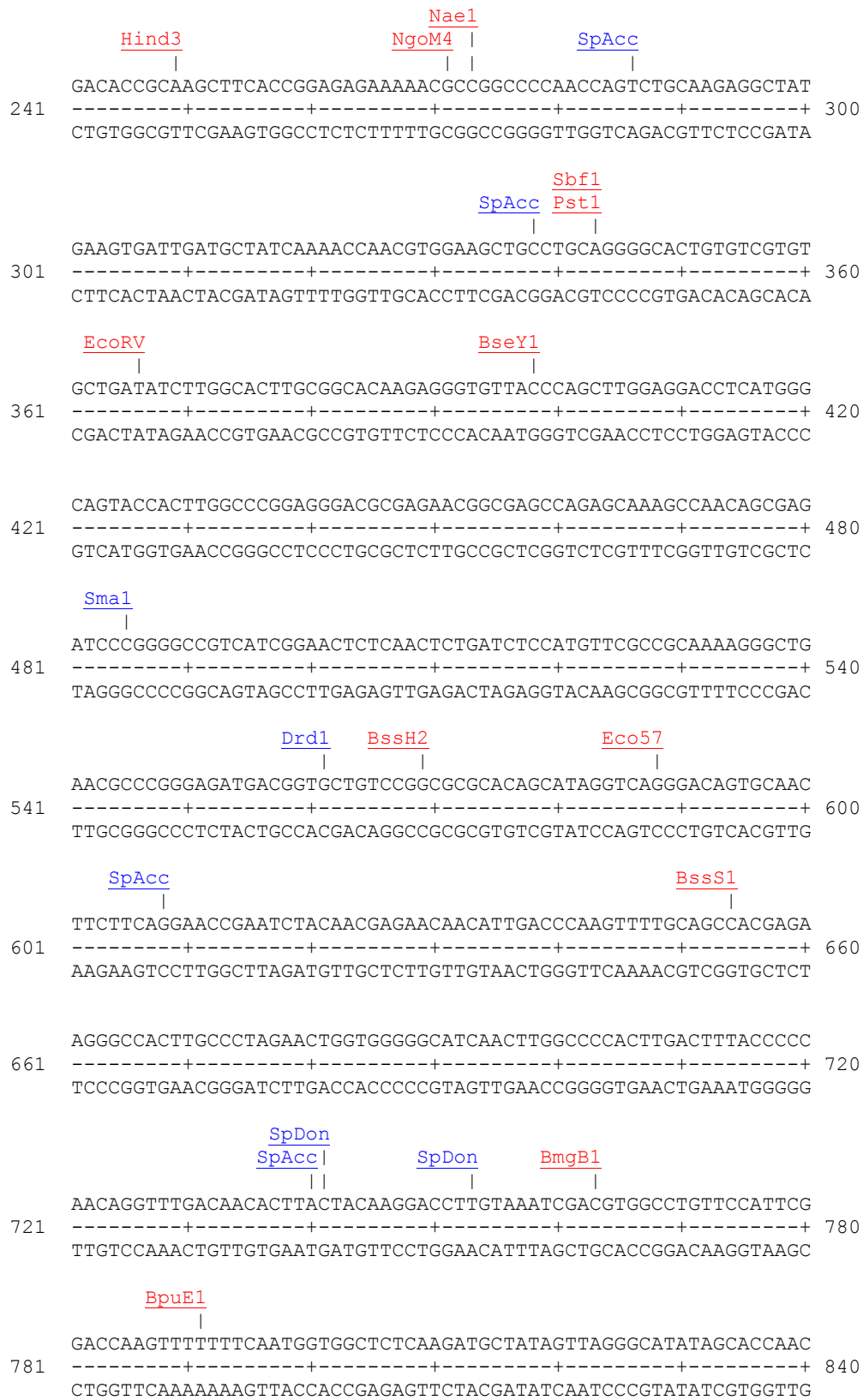
M A S L T H F F L L A L S V L S L F A S S S N A Q L S P N F Y A R S C P N V R A I V R N
T M R Q A L A R E A R L G A S I L R L F F H D C F V N G C D A G I L L D D T A S F T G
E K N A G P N Q S A R G Y E V I D A I K T N V E A A C R G T V S C A D I L A L A A Q E
G V T Q L G G P H G Q Y H L A R R D A R T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

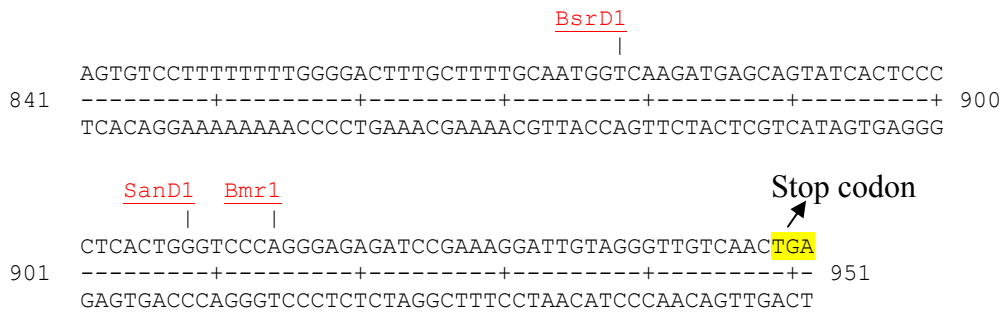
3.3.5.4.1c Restriction map of peroxidase gene - EU649680.1 (LBPox)

Restriction map of *POX* gene was created by using an online bioinformatic tool, Webmap DNA. Map created at http://pga.mgh.harvard.edu/web_apps/web_map/start. Editor at http://pga.mgh.harvard.edu/web_apps/dna_utilities.html

Sites protected by *E. coli* methylation are not shown in this map




Nae1
Hind3
NgoM4
SpAcc
GACACCGCAAGCTTCACCGGAGAGAAAAACGCCGGCCCCAACCAGTCTGCAAGAGGCTAT
241
CTGTGGCGTTCGAAGTGGCCTCTCTTTTTGCGGCCGGGGTTGGTCAGACGTTCTCCGATA
300
Sbf1
SpAcc
Pst1
GAAGTGATTGATGCTATCAAAACCAACGTGGAAGCTGCCTGCAGGGGCACTGTGTCGTGT
301
CTTCACTAACTACGATAGTTTTGGTTGCACCTTCGACGGACGTCCCCGTGACACAGCACA
360
EcoRV
BseY1
GCTGATATCTTGGCACTTGCGGCACAAGAGGGTGTTACCCAGCTTGGAGGACCTCATGGG
361
CGACTATAGAACCGTGAACGCCGTGTTCTCCCACAATGGGTCGAACCTCCTGGAGTACCC
420
CAGTACCACTTGGCCCGGAGGGACGCGAGAACGGCGAGCCAGAGCAAAGCCAACAGCGAG
421
GTCATGGTGAACCGGGCCTCCCTGCGCTCTTGCCGCTCGGTCTCGTTTCGGTTGTCGCTC
480
Sma1
ATCCCGGGGCCGTCATCGGAACTCTCAACTCTGATCTCCATGTTCGCCGCAAAAGGGCTG
481
TAGGGCCCCGGCAGTAGCCTTGAGAGTTGAGACTAGAGGTACAAGCGGCGTTTTCCCGAC
540
Drd1
BssH2
Eco57
AACGCCCGGGAGATGACGGTGCTGTCCGGCGCGCACAGCATAGGTCAGGGACAGTGCAAC
541
TTGCGGGCCCTCTACTGCCACGACAGGCCGCGCGTGTCGTATCCAGTCCCTGTCACGTTG
600
SpAcc
BssS1
TTCTTCAGGAACCGAATCTACAACGAGAACAACATTGACCCAAGTTTTGCAGCCACGAGA
601
AAGAAGTCCTTGGCTTAGATGTTGCTCTTGTTGTAACTGGGTTCAAAACGTCGGTGCTCT
660
AGGGCCACTTGCCCTAGAACTGGTGGGGGCATCAACTTGGCCCCACTTGACTTTACCCCC
661
TCCCGGTGAACGGGATCTTGACCACCCCCGTAGTTGAACCGGGGTGAACTGAAATGGGGG
720
SpDon
SpAcc
SpDon
BmgB1
AACAGGTTTGACAACACTTACTACAAGGACCTTGTAAATCGACGTGGCCTGTTCCATTCG
721
TTGTCCAAACTGTTGTGAATGATGTTCCTGGAACATTTAGCTGCACCGGACAAGGTAAGC
780
BpuE1
GACCAAGTTTTTTTCAATGGTGGCTCTCAAGATGCTATAGTTAGGGCATATAGCACCAAC
781
CTGGTTCAAAAAAAGTTACCACCGAGAGTTCTACGATATCAATCCCGTATATCGTGGTTG
840



3.3.5.4.2 Characterization of *POX* cDNA sequence GU143879 (LlLBPOX)

3.3.5.4.2a Nucleotide sequence of GU143879 (LlLBPOX)

```
   1 ATGGCTTCCC TTACCCATTT CTTCTTACTT GCAGTGTCTC TTCTTTCTCT
  51 CTTTGCCTCT TCTTCCAATG CTCAGCTCTC TCCCAAATTC TACTCCAAAT
 101 CTTGCCCTAA TCTCCAGACT ATTGTTCGCA ACACAACGAG GCAGGCTCTC
 151 GCCAGAGAGG CCCGCCTTGG CGCCTCCATC CCCCGCCTCT TCTTTCACGA
 201 CTGCTTCGTC AATGGTTGCG ACGCCGGAAT CCTCCTAGAC GACACCGCAA
 251 GCTTCACCGG AGAGAAAAAC GCCGGCCCCA ACCAGTCTGC AAGAGGCTAT
 301 GAAGTGATTG ATGCTATCAA AACCAACGTG GAAGCTGCCT GCAGGGGCAC
 351 TGCGTCATGT GCTGATATCT TGGCACTTGC GGCACAAGAG GGTGTTACCC
 401 AGCTAGGGGG ACCCTCATGG GCAGTACCAC TTGGCCGGAG GGATGCGAGA
 451 ACGGCGAGCC AGAGCAAAGC CAACAGCGAG ATCCCGGGGC CGTCATCGGA
 501 CCTATCGACT CTGATCTCCA TGTTCGCCGC GAAAGGGCTG ACAGCCCGGC
 551 AGATGACGGT GCTGTCGGGC GCTCACACCA TAGGTCAGGG ACAGTGCAAC
 601 TTCTTCAGGA ACCGAATCTA CAACGAGAAC AACATTGACC CAAGTTTTGC
 651 AGCCACGAGA AGGGCCACTT GCCCTAGAAC TGGTGGGGAC ATCAACTTGG
 701 CCCCACTTGA CTTTACCCCC AGCAGGTTCG ACAACACTTA CTACAAGGAC
 751 CTTGTAAATC GACGTGGCCT GTTCCATTCG GACCAAGTTC TCTTCAATGG
 801 TGGCTCTCAA GATGCTATAG TTAGGGCATA TAGCACCAAC AGCGTCCTCT
 851 TCTTTGGGGA CTTTGCTTCT GCAATGGTCA AGGTGAGCAG TATCACTCCC
 901 CTCACTGGGT CCCAGGGAGA GATCCGAAAG AATTGTAGGG TTGTCAACTG
 951 A
```

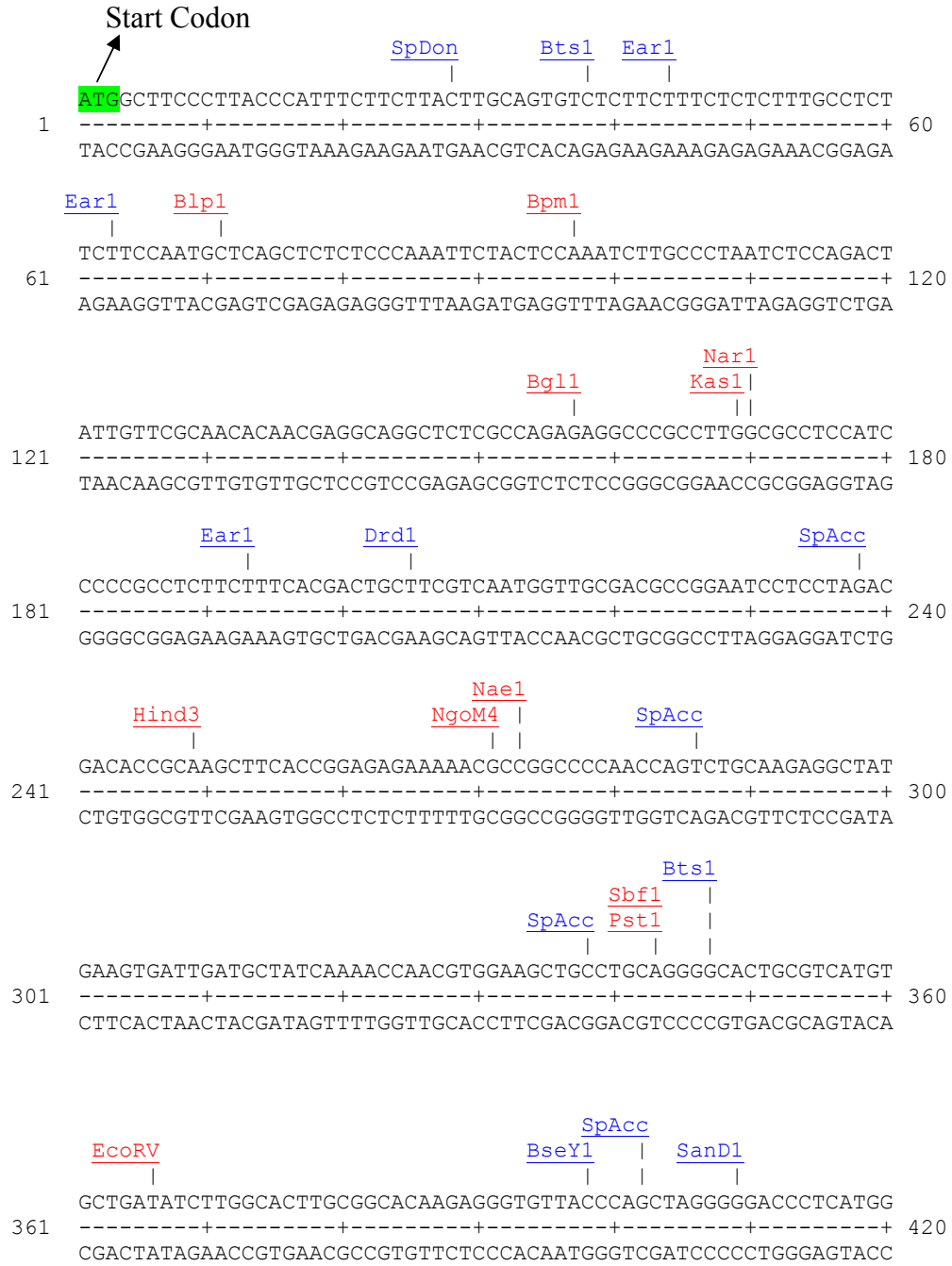
3.3.5.4.2b Deduced amino acid sequence of GU143879 (LlLBPOX)

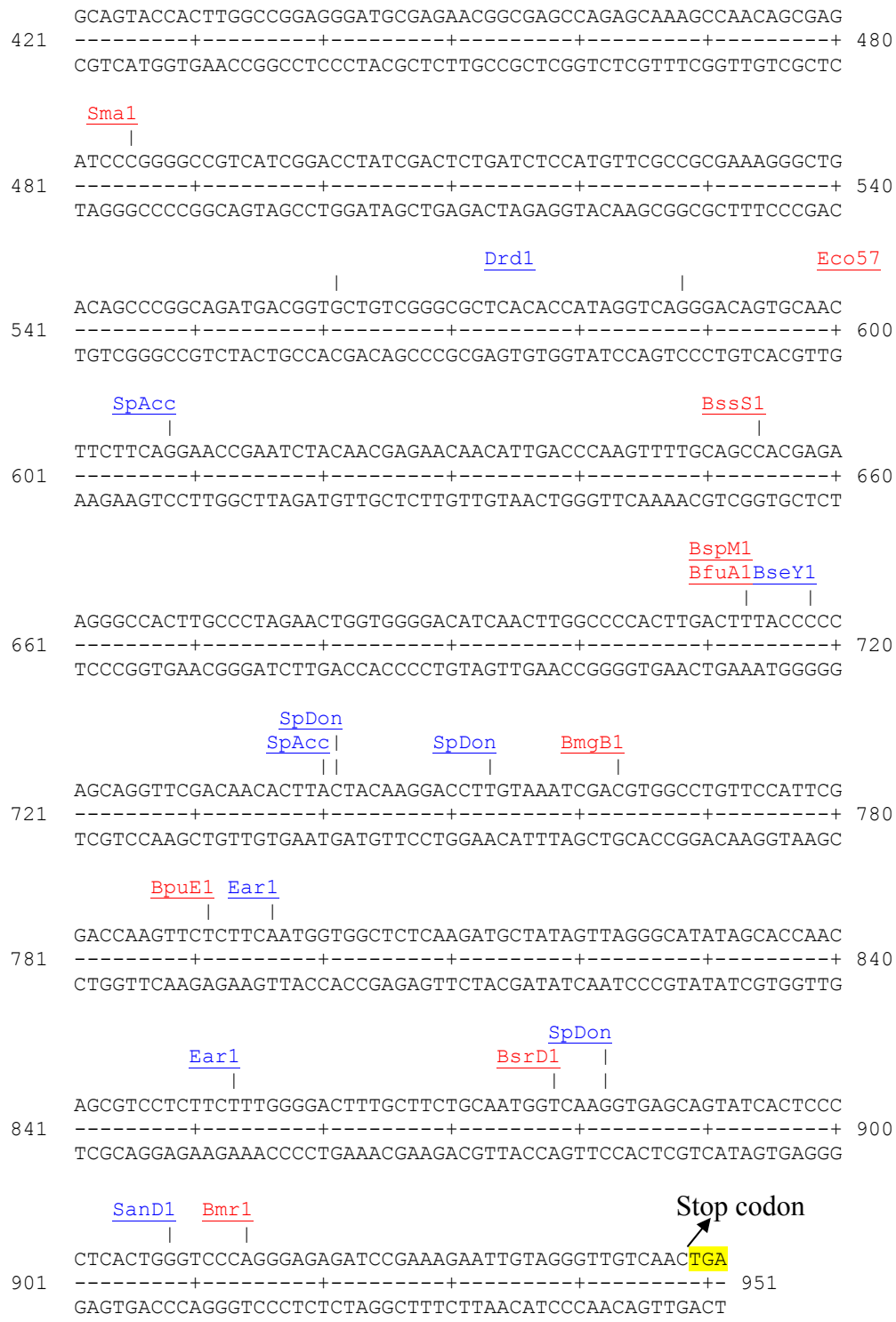
M A S L T H F F L L A V S L L S L F A S S S N A Q L S P K F Y S K S C P N L Q T I V R N
T T R Q A L A R E A R L G A S I P R L F F H D C F V N G C D A G I L L D D T A S F T G E
K N A G P N Q S A R G Y E V I D A I K T N V E A A C R G T A S C A D I L A L A A Q E G
V T Q L G G P S W A V P L G R R D A R T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

3.3.5.4.2c Restriction map of peroxidase gene - GU143879 (LlLBPOX)

Restriction map of *POX* gene was created by using an online bioinformatic tool, Webmap DNA. Map created at http://pga.mgh.harvard.edu/web_apps/web_map/start. Editor at http://pga.mgh.harvard.edu/web_apps/dna_utilities.html

Sites protected by *E. coli* methylation are not shown in this map




GCAGTACCACTTGGCCGGAGGGATGCGAGAACGGCGAGCCAGAGCAAAGCCAACAGCGAG
421 480
CGTCATGGTGAACCGGCCTCCCTACGCTCTTGCCGCTCGGTCTCGTTTCGGTTGTCGCTC
Sma1
ATCCCGGGGCCGTCATCGGACCTATCGACTCTGATCTCCATGTTCGCCGCGAAAGGGCTG
481 540
TAGGGCCCCGGCAGTAGCCTGGATAGCTGAGACTAGAGGTACAAGCGGCGCTTTCCCGAC
Drd1
Eco57
ACAGCCCGGCAGATGACGGTGCTGTCGGGCGCTCACACCATAGGTCAGGGACAGTGCAAC
541 600
TGTCGGGCCGTCTACTGCCACGACAGCCCGCGAGTGTGGTATCCAGTCCCTGTCACGTTG
SpAcc
BssS1
TTCTTCAGGAACCGAATCTACAACGAGAACAACATTGACCCAAGTTTTGCAGCCACGAGA
601 660
AAGAAGTCCTTGGCTTAGATGTTGCTCTTGTTGTAACTGGGTTCAAAACGTCGGTGCTCT
BspM1
BfuA1
BseY1
AGGGCCACTTGCCCTAGAACTGGTGGGGACATCAACTTGGCCCCACTTGACTTTACCCCC
661 720
TCCCGGTGAACGGGATCTTGACCACCCCTGTAGTTGAACCGGGGTGAACTGAAATGGGGG
SpDon
SpAcc
SpDon
BmgB1
AGCAGGTTCGACAACACTTACTACAAGGACCTTGTAAATCGACGTGGCCTGTTCCATTCG
721 780
TCGTCCAAGCTGTTGTGAATGATGTTCCTGGAACATTTAGCTGCACCGGACAAGGTAAGC
BpuE1
Ear1
GACCAAGTTCTCTTCAATGGTGGCTCTCAAGATGCTATAGTTAGGGCATATAGCACCAAC
781 840
CTGGTTCAAGAGAAGTTACCACCGAGAGTTCTACGATATCAATCCCGTATATCGTGGTTG
SpDon
Ear1
BsrD1
AGCGTCCTCTTCTTTGGGGACTTTGCTTCTGCAATGGTCAAGGTGAGCAGTATCACTCCC
841 900
TCGCAGGAGAAGAAACCCCTGAAACGAAGACGTTACCAGTTCCACTCGTCATAGTGAGGG
SanD1
Bmr1
Stop codon
CTCACTGGGTCCCAGGGAGAGATCCGAAAGAATTGTAGGGTTGTCAACTGA
901 951
GAGTGACCCAGGGTCCCTCTCTAGGCTTTCTTAACATCCCAACAGTTGACT

3.3.5.4.3 CLUSTAL W (1.8) multiple sequence alignment of nucleotide sequences of EU649680.1 (LBPox) and GU143879 (LlLBPOX)

The start codon, ATG is mentioned in red letters, stop codon TGA is shown highlighted in pink and the nucleotide mismatch between the two sequences is shown highlighted in green.

```
EU649680.1_LBPox_       ATGGCTTCCCTTACCCATTTCTTCTTACTTGCATTGTCTGTTCTTTCTCTCTTTGCTTCT
GU143879_LlLBPOX_       ATGGCTTCCCTTACCCATTTCTTCTTACTTGCAGTGTCTCTTCTTTCTCTCTTTGCCTCT
                        ***********************************  *****  ***************  ***

EU649680.1_LBPox_       TCTTCCAATGCTCAGCTCTCTCCCAACTTCTACGCCAGATCTTGCCCTAACGTCCGGGCC
GU143879_LlLBPOX_       TCTTCCAATGCTCAGCTCTCTCCCAAATTCTACTCCAAATCTTGCCCTAATCTCCAGACT
                        *************************** ******  ***  ************  ***  * *

EU649680.1_LBPox_       ATTGTTCGCAACACAATGAGGCAAGCTCTCGCCCGAGAAGCCCGCCTTGGCGCCTCCATC
GU143879_LlLBPOX_       ATTGTTCGCAACACAACGAGGCAGGCTCTCGCCAGAGAGGCCCGCCTTGGCGCCTCCATC
                        ***************** ******  *********  ****  **********************


EU649680.1_LBPox_       CTCCGCCTCTTCTTCCACGACTGCTTCGTCAATGGTTGTGACGCCGGAATCCTCCTAGAC
GU143879_LlLBPOX_       CCCCGCCTCTTCTTTCACGACTGCTTCGTCAATGGTTGCGACGCCGGAATCCTCCTAGAC
                        * ************  ************************  **********************

EU649680.1_LBPox_       GACACCGCAAGCTTCACCGGAGAGAAAAACGCCGGCCCCAACCAGTCTGCAAGAGGCTAT
GU143879_LlLBPOX_       GACACCGCAAGCTTCACCGGAGAGAAAAACGCCGGCCCCAACCAGTCTGCAAGAGGCTAT
                        ************************************************************

EU649680.1_LBPox_       GAAGTGATTGATGCTATCAAAACCAACGTGGAAGCTGCCTGCAGGGGCACTGTGTCGTGT
GU143879_LlLBPOX_       GAAGTGATTGATGCTATCAAAACCAACGTGGAAGCTGCCTGCAGGGGCACTGCGTCATGT
                        ************************************************** *** ***

EU649680.1_LBPox_       GCTGATATCTTGGCACTTGCGGCACAAGAGGGTGTTACCCAGCTTGGAGGACC-TCATGG
GU143879_LlLBPOX_       GCTGATATCTTGGCACTTGCGGCACAAGAGGGTGTTACCCAGCTAGGGGGACCCTCATGG
                        ******************************************** ** ***** ******

EU649680.1_LBPox_       GCAGTACCACTTGGCCCGGAGGGACGCGAGAACGGCGAGCCAGAGCAAAGCCAACAGCGA
GU143879_LlLBPOX_       GCAGTACCACTTGGCC-GGAGGGATGCGAGAACGGCGAGCCAGAGCAAAGCCAACAGCGA
                        *************** ******* ***********************************

EU649680.1_LBPox_       GATCCCGGGGCCGTCATCGGAACTCTCAACTCTGATCTCCATGTTCGCCGCAAAAGGGCT
GU143879_LlLBPOX_       GATCCCGGGGCCGTCATCGGACCTATCGACTCTGATCTCCATGTTCGCCGCGAAAGGGCT
                        ******************** ** ** ********************** ********

EU649680.1_LBPox_       GAACGCCCGGGAGATGACGGTGCTGTCCGGCGCGCACAGCATAGGTCAGGGACAGTGCAA
GU143879_LlLBPOX_       GACAGCCCGGCAGATGACGGTGCTGTCGGGCGCTCACACCATAGGTCAGGGACAGTGCAA
                        **  ****** *************** ***** **** ********************

EU649680.1_LBPox_       CTTCTTCAGGAACCGAATCTACAACGAGAACAACATTGACCCAAGTTTTGCAGCCACGAG
GU143879_LlLBPOX_       CTTCTTCAGGAACCGAATCTACAACGAGAACAACATTGACCCAAGTTTTGCAGCCACGAG
                        ************************************************************

EU649680.1_LBPox_       AAGGGCCACTTGCCCTAGAACTGGTGGGGGCATCAACTTGGCCCCACTTGACTTTACCCC
GU143879_LlLBPOX_       AAGGGCCACTTGCCCTAGAACTGGTGGGGACATCAACTTGGCCCCACTTGACTTTACCCC
                        **************************** ****************************

EU649680.1_LBPox_       CAACAGGTTTGACAACACTTACTACAAGGACCTTGTAAATCGACGTGGCCTGTTCCATTC
GU143879_LlLBPOX_       CAGCAGGTTCGACAACACTTACTACAAGGACCTTGTAAATCGACGTGGCCTGTTCCATTC
                        ** ****** ************************************************

EU649680.1_LBPox_       GGACCAAGTTTTTTTTCAATGGTGGCTCTCAAGATGCTATAGTTAGGGCATATAGCACCAA
GU143879_LlLBPOX_       GGACCAAGTTCTCTTCAATGGTGGCTCTCAAGATGCTATAGTTAGGGCATATAGCACCAA
                        ********** * ********************************************
```

```
EU649680.1_LBPox_       CAGTGTCCTTTTTTTTGGGGACTTTGCTTTTGCAATGGTCAAGATGAGCAGTATCACTCC
GU143879_LlLBPOX_       CAGCGTCCTCTTCTTTGGGGACTTTGCTTCTGCAATGGTCAAGGTGAGCAGTATCACTCC
                        *** ***** ** ********************** ********** ***********

EU649680.1_LBPox_       CCTCACTGGGTCCCAGGGAGAGATCCGAAAGGATTGTAGGGTTGTCAACTGA
GU143879_LlLBPOX_       CCTCACTGGGTCCCAGGGAGAGATCCGAAAGAATTGTAGGGTTGTCAACTGA
                        ******************************** *********************
```

3.3.5.4.4 CLUSTAL W (1.8) Multiple sequence alignment of amino acid sequences of EU649680.1 (LBPox) and GU143879 (LlLBPOX)

```
EU649680.1_LBPox_       MASLTHFFLLALSVLSLFASSSNAQLSPNFYARSCPNVRAIVRNTMRQALAREARLGASI
GU143879_LlLBPOX_       MASLTHFFLLAVSLLSLFASSSNAQLSPKFYSKSCPNLQTIVRNTTRQALAREARLGASI
                        ***********:*:**************:**::****:::*****  **************

EU649680.1_LBPox_       LRLFFHDCFVNGCDAGILLDDTASFTGEKNAGPNQSARGYEVIDAIKTNVEAACRGTVSC
GU143879_LlLBPOX_       PRLFFHDCFVNGCDAGILLDDTASFTGEKNAGPNQSARGYEVIDAIKTNVEAACRGTASC
                         ************************************************************.**

EU649680.1_LBPox_       ADILALAAQEGVTQLGGPHGQYHLARRDARTASQSKANSEIPGPSSELSTLISMFAAKGL
GU143879_LlLBPOX_       ADILALAAQEGVTQLGGPSWAVPLGRRDARTASQSKANSEIPGPSSDLSTLISMFAAKGL
                        ******************     *.*********************:**************

EU649680.1_LBPox_       NAREMTVLSGAHSIGQGQCNFFRNRIYNENNIDPSFAATRRATCPRTGGGINLAPLDFTP
GU143879_LlLBPOX_       TARQMTVLSGAHTIGQGQCNFFRNRIYNENNIDPSFAATRRATCPRTGGDINLAPLDFTP
                        .**:********:************************************.**********

EU649680.1_LBPox_       NRFDNTYYKDLVNRRGLFHSDQVFFNGGSQDAIVRAYSTNSVLFFGDFAFAMVKMSSITP
GU143879_LlLBPOX_       SRFDNTYYKDLVNRRGLFHSDQVLFNGGSQDAIVRAYSTNSVLFFGDFASAMVKVSSITP
                        .**********************:************************ ****:*****

EU649680.1_LBPox_       LTGSQGEIRKDCRVVN
GU143879_LlLBPOX_       LTGSQGEIRKNCRVVN
                        **********:*****
```

Both the peroxidases show good level of sequence homology at amino acid levels as given above; the mismatches are highlighted in green. From the multiple sequence alignment, it is clear that both the peroxidases (EU649680.1 and GU143879) have a sequence similarity of 95% both in amino acid (95.25% similarity) as well as at nucleotide level (95.17% similarity).

3.3.5.4.5 Amino acid compositions and theoretical pI and molecular weight of isolated peroxidase genes of *L. leucocephala*.

The deduced amino acid sequences of EU649680.1 (LBPox) and GU143879 (LlLBPOX) gene show the coding region consists of 316 amino acids, with theoretical molecular weight of 34205.6 Daltons for EU649680.1 and 33943.8 for GU143879. They have theoretical pI values of 8.96 and 9.08 respectively (http://www.expasy.ch/cgi-bin/protparam). The amino acid composition of EU649680.1 (LBPox) is given in table 3.1 and for GU143879 (LlLBPOX) is given in table 3.2. In EU649680.1 (LBPox) the

total number of negatively charged residues (Asp + Glu) equals 25 and positively charged residues (Arg + Lys) equal 31. Similarly, total number of negatively charged residues (Asp + Glu) and positively charged residues (Arg + Lys) in GU143879 (LlLBPOX) is 24 and 31 respectively. The empirical formula of EU649680.1 (LBPox) is $C_{1493}H_{2349}N_{441}O_{455}S_{14}$ and of GU143879 (LlLBPOX) is $C_{1482}H_{2347}N_{433}O_{458}S_{12}$.

Table 3.1 Amino acid composition of EU649680.1 (LBPox)

Amino acids	Total No.	Percentage	Amino acids	Total No.	Percentage
Ala (A)	37	11.7	Leu (L)	27	8.5
Arg (R)	24	7.6	Lys (K)	7	2.2
Asn (N)	22	7.0	Met (M)	6	1.9
Asp (D)	15	4.7	Phe (F)	21	6.6
Cys (C)	8	2.5	Pro (P)	11	3.5
Gln (Q)	12	3.8	Ser (S)	29	9.2
Glu (E)	10	3.2	Thr (T)	18	5.7
Gly (G)	25	7.9	Trp (W)	0	0
His (H)	6	1.9	Tyr (Y)	7	2.2
Ile (I)	15	4.7	Val (V)	16	5.1

Table 3.2 Amino acid composition of GU143879 (LlLBPOX)

Amino acids	Total No.	Percentage	Amino acids	Total No.	Percentage
Ala (A)	36	11.4	Leu (L)	28	8.9
Arg (R)	22	7.0	Lys (K)	9	2.8
Asn (N)	20	6.3	Met (M)	4	1.3
Asp (D)	16	5.1	Phe (F)	19	6.0
Cys (C)	8	2.5	Pro (P)	13	4.1
Gln (Q)	13	4.1	Ser (S)	32	10.1
Glu (E)	8	2.5	Thr (T)	22	7.0
Gly (G)	24	7.6	Trp (W)	1	0.3
His (H)	4	1.3	Tyr (Y)	6	1.9
Ile (I)	15	4.7	Val (V)	16	5.1

3.3.5.4.6 Hydropathy index of the peroxidase amino acids

The hydropathy index of an amino acid is a number representing the hydrophobic or hydrophilic properties of its side-chain (Jack Kyte and Russell Doolittle, 1982). The larger the number is, the more hydrophobic the amino acid. The most hydrophobic amino

acids are isoleucine (4.5) and valine (4.2). The most hydrophilic ones are arginine (-4.5) and lysine (-3.9). This is very important in protein structure; hydrophobic amino acids tend to be internal (with regard to the protein's native 3- dimensional structure) while hydrophilic amino acids are more commonly found towards the protein surface. The amino acid sequences of EU649680.1 (LBPox) and GU143879 (LlLBPOX) were analyzed using Kyte-Doolittle Hydropathy plot at http://gcat.davidson.edu/rakarnik/kd.cgi (Fig 3.16A & Fig 3.16B respectively) window size 9. When the window size is **9**, strong negative peaks indicate possible surface regions of globular proteins.

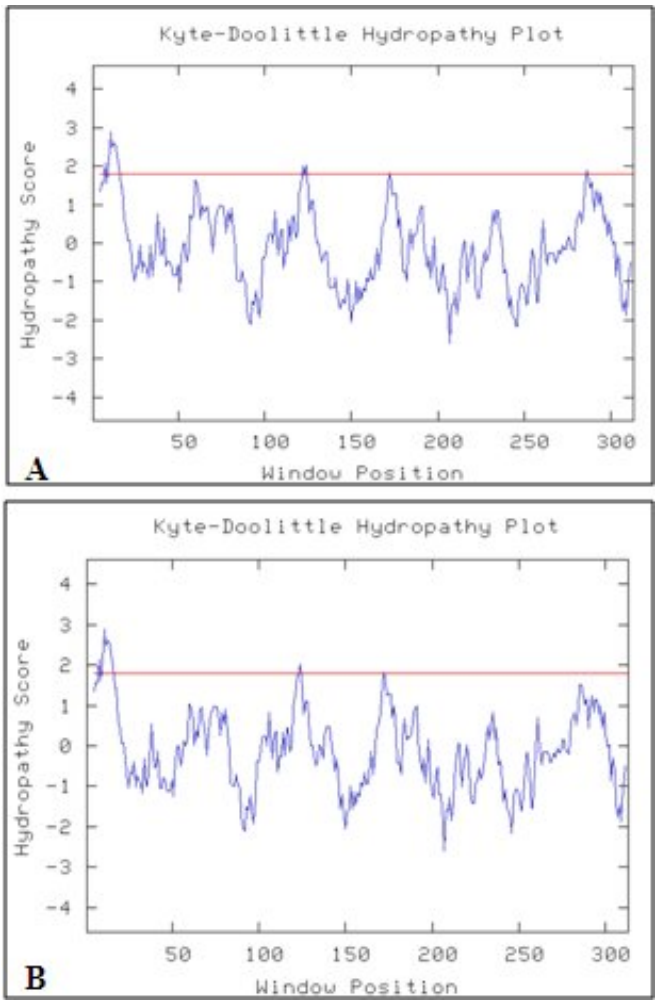


Fig. 3.16: Hydropathic plot. A Kyte-Doolittle Hydropathy for EU649680.1 (LBPox) and **B** Kyte-Doolittle Hydropathy for GU143879 (LlLBPOX). Window size of 9 suggested a good value for finding putative surface-exposed regions. When the window size is **9**, strong negative peaks indicate possible surface regions of globular proteins. Both the *POXs* showed almost similar pattern in (Kyte-Doolittle plot).

The GC% and codon usage of both, the cDNA clones (EU649680.1 (LBPox) and GU143879 (LlLBPOX)) were calculated using online software (www.justbio.com). The GC content of the cDNA clone, EU649680.1 (LBPox) is 54.61% and for GU143879 (LlLBPOX) is 54.22%. The codon usage for *POX* cDNA clone, EU649680.1 (LBPox) is given in Table 3.3 and codon usage of GU143879 (LlLBPOX) is given in table 3.4. It is expressed as % of total codons. Standard genetic codes were used for determining codon usage.

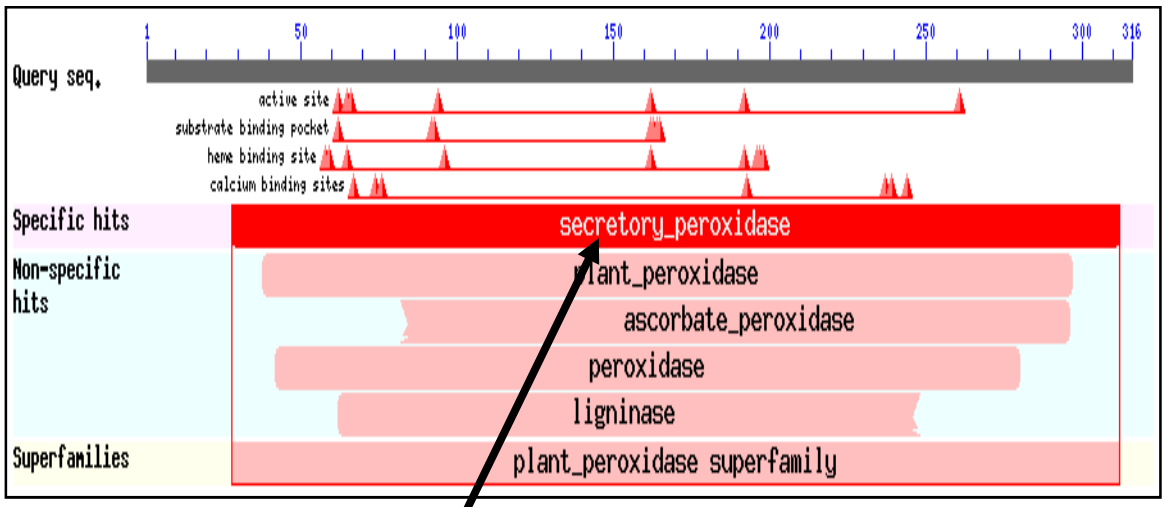
Table 3.3 Codon usage of *POX* gene EU649680.1 (LBPox)

Codon	Mean	Codon	Mean	Codon	Mean	Codon	Mean
UUU (F)	2.52	UCU (S)	0.63	UAU (Y)	0.63	UGU (C)	0.95
UUC (F)	2.84	UCC (S)	1.26	UAC (Y)	1.26	UGC (C)	1.26
UUA (L)	0.00	UCA (S)	0.63	UAA (*)	0.00	UGA (*)	0.00
UUG (L)	0.95	UCG (S)	0.95	UAG (*)	0.00	UGG (W)	0.00
CUU (L)	1.89	CCU (P)	0.63	CAU (H)	0.63	CGU (R)	0.32
CUC (L)	1.89	CCC (P)	0.95	CAC (H)	0.95	CGC (R)	0.63
CUA (L)	0.32	CCA (P)	0.63	CAA (Q)	1.26	CGA (R)	1.26
CUG (L)	1.26	CCG (P)	0.63	CAG (Q)	2.21	CGG (R)	0.63
AUU (I)	0.63	ACU (T)	2.21	AAU (N)	0.95	AGU (S)	0.95
AUC (I)	3.15	ACC (T)	1.89	AAC (N)	4.73	AGC (S)	2.21
AUA (I)	0.63	ACA (T)	0.00	AAA (K)	1.26	AGA (R)	1.26
AUG (M)	1.58	ACG (T)	0.95	AAG (K)	0.95	AGG (R)	2.52
GUU (V)	1.26	GCU (A)	1.89	GAU (D)	1.26	GGU (G)	1.58
GUC (V)	1.26	GCC (A)	4.10	GAC (D)	3.47	GGC (G)	2.52
GUA (V)	0.32	GCA (A)	2.52	GAA (E)	1.26	GGA (G)	1.89
GUG (V)	1.26	GCG (A)	1.26	GAG (E)	1.89	GGG (G)	1.89

Table 3.4 Codon usage of *POX* gene EU649680.1 (LBPox)

Codon	Mean	Codon	Mean	Codon	Mean	Codon	Mean
UUU (F)	1.89	UCU (S)	2.84	UAU (Y)	0.63	UGU (C)	0.63
UUC (F)	4.10	UCC (S)	1.89	UAC (Y)	1.26	UGC (C)	1.89
UUA (L)	0.32	UCA (S)	0.95	UAA (*)	0.00	UGA (*)	0.00
UUG (L)	0.63	UCG (S)	1.26	UAG (*)	0.00	UGG (W)	0.32
CUU (L)	2.84	CCU (P)	0.63	CAU (H)	0.63	CGU (R)	0.32
CUC (L)	2.84	CCC (P)	1.89	CAC (H)	0.63	CGC (R)	0.95
CUA (L)	0.95	CCA (P)	0.95	CAA (Q)	0.95	CGA (R)	0.95
CUG (L)	1.26	CCG (P)	0.63	CAG (Q)	3.15	CGG (R)	0.63
AUU (I)	0.95	ACU (T)	2.52	AAU (N)	1.89	AGU (S)	0.63
AUC (I)	3.15	ACC (T)	2.52	AAC (N)	4.42	AGC (S)	2.52
AUA (I)	0.63	ACA (T)	0.63	AAA (K)	1.89	AGA (R)	1.58
AUG (M)	1.26	ACG (T)	1.26	AAG (K)	0.95	AGG (R)	2.52
GUU (V)	1.58	GCU (A)	2.84	GAU (D)	1.26	GGU (G)	1.58
GUC (V)	1.26	GCC (A)	4.10	GAC (D)	3.79	GGC (G)	2.52
GUA (V)	0.63	GCA (A)	2.84	GAA (E)	0.63	GGA (G)	1.58
GUG (V)	1.58	GCG (A)	1.58	GAG (E)	1.89	GGG (G)	1.89

3.3.5.5 Analysis of amino acid sequence of the *L. leucocephala* peroxidase genes, EU649680.1 and GU143879 for their conserved domain



Specific hits with Secreatory peroxidases

The amino acid sequences of EU649680.1 (LBPox) and GU143879 (LlLBPOX) when analysed for their sequence similarity, it matched with secretory peroxidases. Horseradish peroxidase and related secretory peroxidases belong to Class III of the plant heme-dependent peroxidase superfamily. All members of the superfamily share a heme prosthetic group and catalyze a multistep oxidative reaction involving hydrogen peroxide as the electron acceptor. Class III peroxidases are found in the extracellular space or in the vacuole in plants, where they have been implicated in hydrogen peroxide detoxification, auxin catabolism and lignin biosynthesis and stress response. Class III peroxidases contain four conserved disulphide bridges and two conserved calcium binding sites.

3.3.5.6 Phylogenetic analysis of *POX* gene (EU649680.1) with other reported peroxidases

Phylogenetic analysis was done using 19 reported protein sequences of POX, which were retrieved from the GenBank database. Among the two genes of POX, only EU649680.1 was used for phylogenetic analysis, as they are almost similar at nucleotide as well as amino acid level (>95%). Multiple alignments of the predicted protein coding sequences

were performed using Clustal X. The phylogenetic tree was constructed by neighbor-joining methods using Mega 4.0 software program. It is clear from the phylogenetic tree that, EU649680.1 is evolutionarily most similar with *Pisum sativum*, then to other peroxidases (Fig. 3.17).

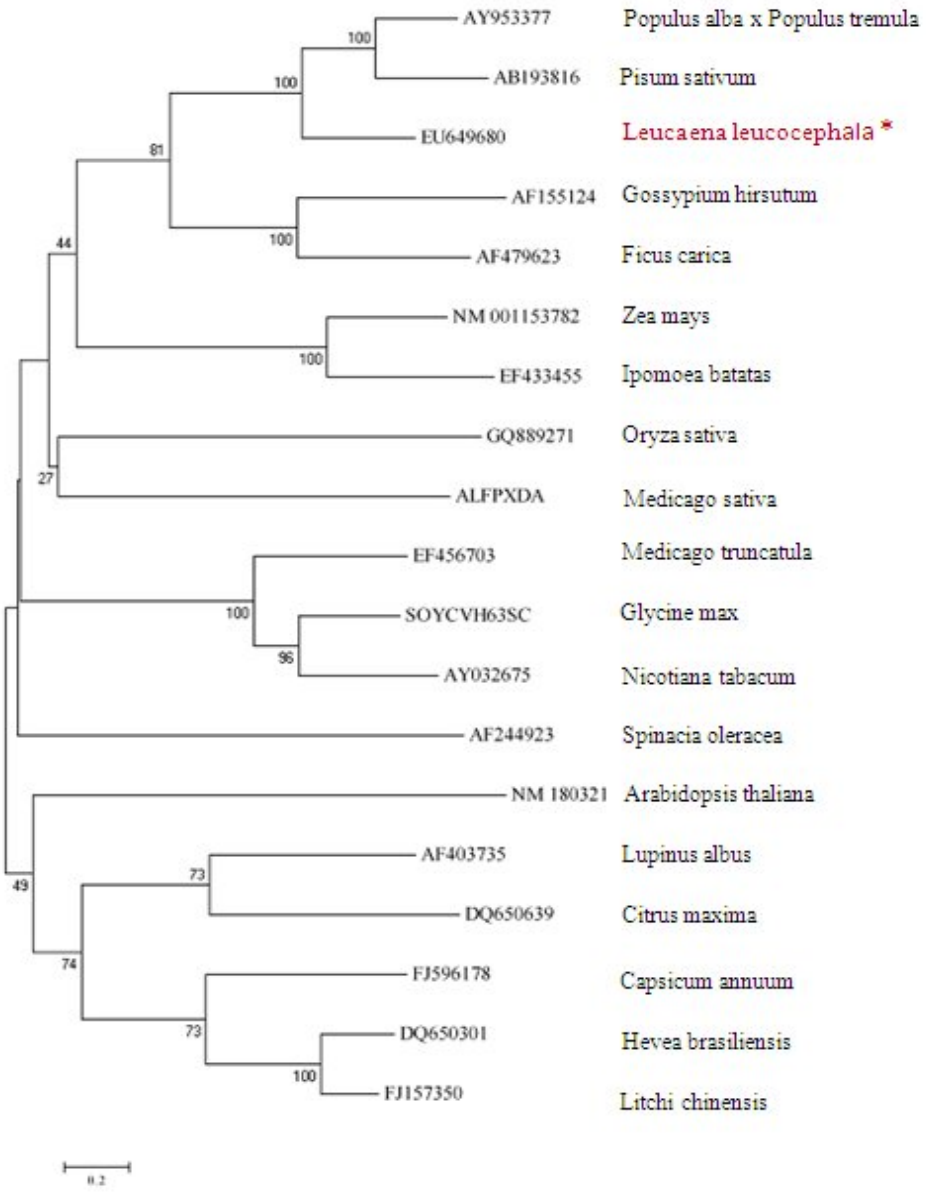


Fig. 3.17: Phylogenetic tree using 19 selected plant peroxidase proteins, constructed by neighbor-joining methods using Mega 4.0 software program, after alignment of amino acids sequences by Clustal X. The numbers shown at each node represents, the support values (percent) obtained by bootstrap analysis (1000 replicates). The tree shows the relatedness between amino acid sequences of EU649680.1, to the other plant peroxidases. The closest homologues of *Leucaena* POX protein is POX of *Pisum sativum* (AB193816).

3.3.5.7 Analysis of deduced amino acid sequences of EU649680.1 (LBPox) and GU143879 (LlLBPOX)

The SignalP 3.0 program predicted that in both the peroxidases, upto the 24th amino acid residues from the first methionine, which are shaded in yellow (Fig. 3.18) is the signal peptide region. Both the peroxidases possess several highly homologous regions including the peroxidase active site (Fig. 3.18, shaded in *pink*) and the ligand of heme (Fig. 3.18, shaded in *light blue*). Concerning tyrosine residue, which is reported to be involved in binding of proteins to lignin and its related compound (McDougall, 1993; McDougall *et al*., 1996), both the peroxidases showed a higher tyrosine content (shaded green; 7 tyrosine residues in EU649680.1 and 6 tyrosine residues in GU143879). Among them, six tyrosine residues were conserved in both the peroxidases.

Both *POX* homologs EU649680.1 (LBPox) and GU143879 (LlLBPOX) exhibited similar property. Hence, for all further study, only one of the *POX* homologs *i.e* EU649680.1 was characterized.

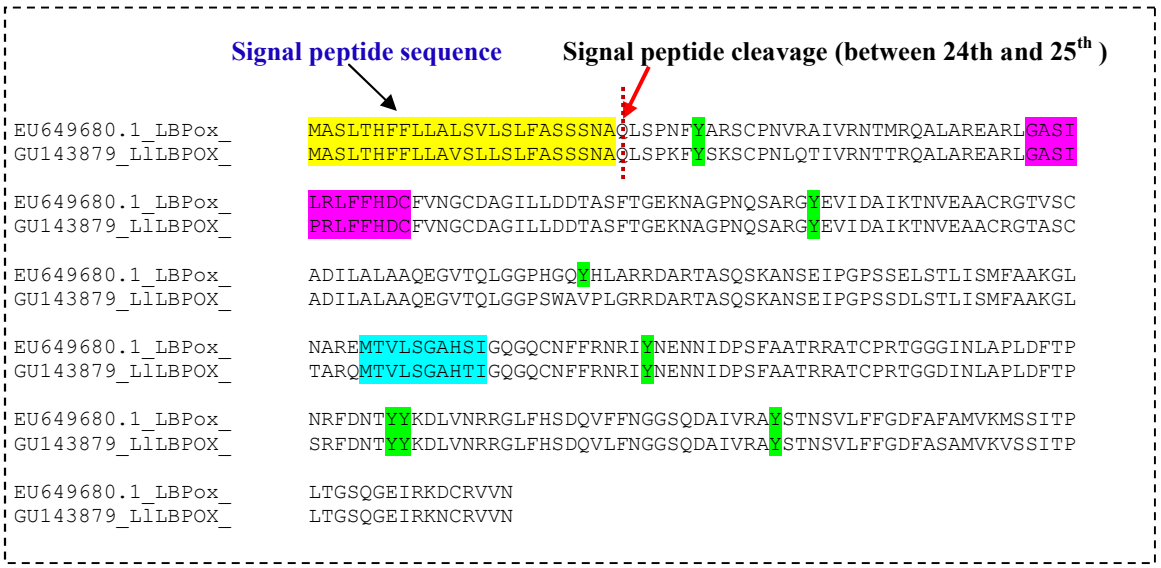


Fig. 3.18: Deduced amino acid sequences of peroxidases EU649680.1 (LBPox) and GU143879 (LlLBPOX): The regions shaded in *yellow, pink,* and *blue* are signal peptide, peroxidase active site, and ligand of heme, respectively.

3.4 Discussions

Peroxidase is one among the several genes involved in lignin biosynthesis (Chapter 1, Section 1.5). Both anionic and cationic peroxidases have been implicated in lignification based on their affinity for coniferyl alcohol, their location in the cell wall and their expression in lignified tissue (M"ader and F¨ussl, 1982; Lagrimini *et al*., 1987; El Mansouri *et al*., 1999). Functional identification of various suspected lignin-specific peroxidase isoenzymes had been previously attempted by modification of the expression of particular anionic peroxidases in tobacco (Lagrimini *et al*., 1987, 1997a,b; Lagrimini,1996) and poplar (Christensen *et al*., 1998; Baucher *et al*., 2000) these manipulations however, had no substantial effect on lignin deposition of biotechnological significance.

In this study, two cDNAs encoding *POX* gene(s) have been isolated which were designated as LBPox (Accession No. EU649680.1) and LlLBPOX (Accession No. GU143879) as mentioned in section 3.3.5.4. They have among themselves 95% homology, both at nucleotide as well as amino acid levels. Phylogenetic analysis of EU649680.1 grouped it among other peroxidases from dicots (Fig. 3.15). Gene copy number of these *POX* was estimated to be four in *L. leucocephala*. Considering the analysis of LBPox (Accession No. EU649680.1) and LlLBPOX (Accession No. GU143879) genes it can be suggested that both belongs to Class III peroxidases of plant species. As both *POX* homologs (EU649680.1 (LBPox) and GU143879 (LlLBPOX)) exhibited similar property, for all further study only one of the *POX* homologs *i.e* EU649680.1 was characterized.

3.5 Conclusion

PCR based RACE approach was used to fish out the *POX* gene. Two *POX* cDNA clones were isolated, LBPox (Accession No. EU649680.1) and LlLBPOX (Accession No. GU143879). They both showed 95% sequence homology with each other. Putative polyadenylation sites and the poly A tails were identified in the 3' UTRs. The full length cDNA clone of both the peroxidases were of 948 bp. BLAST analysis revealed about 60-75% identity with other *POX*s in the database. Banding pattern in Southern hybridization suggested that at least 4 copy of *POX* gene is present in *L. leucocephala* genome.

Analysis of deduced amino acid sequences of both the POX revealed highly homologous regions including the peroxidase active site and the ligand of heme binding. Phylogenetic analysis of deduced amino acid sequence was done using 19 protein sequences of POX using the neighbor joining method. The results showed that isolated POXs are evolutionarily most similar to *Pisum sativum,* than other dicots.

CHAPTER 4A

4.1 Introduction

In the past decade, cloning and characterization of genes involved in lignin biosynthesis and modification of lignin content and composition in plants have provided new insights into the lignin biosynthesis pathway. The enzyme kinetics of a few tree species (Lacombe *et al.*, 1997; Leple *et al.*, 1998, 2007) has been studied, however, the biochemical data of lignin biosynthetic enzymes in monocot plants is still limited (Baucher *et al.*, 2003; Boerjan *et al.*, 2003). Furthermore, the evidence for physiological functions of lignin biosynthesis at gene levels is very scarce.

Peroxidases are ubiquitous enzymes that catalyze oxidation of cellular components in the presence of H_2O_2. Most higher plants contain a number of peroxidase isozymes, which can be classified into two (anionic and cationic) or three (anionic, neutral, and cationic) subgroups according to their isoelectrophoretic mobilities, and these isozymes exist in cytosol, chroloplast, vacuole and cell wall (Lagrimini *et al*., 1987, Asada, 1992). Their physiological roles have been extensively investigated, and it has been demonstrated that they catalyze a variety of important reactions, such as indole-3-acetic acid catabolism (Hinnman and Lang, 1965), lignin biosynthesis (Grisebach and Luderitz, 1981, Espelie *et al*., 1986), suberization of cell wall (Christensen *et al*., 1998 and detoxification of H_2O_2 (Asada, 1992). These peroxidases are soluble in buffers containing detergents or high concentration of salt, whereas peroxidases that cannot be extracted using these buffers are also found in various plants (Goldberg *et al*., 1986). Although the latter peroxidases have been shown to be covalently bound to cell walls, almost nothing is known about their physiological importance, their structural characteristics, or the mechanism of covalent bond formation.

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 possible

with its functional expression in heterologous host cells. Heterologous expression of plant genes provides a new technique for determining gene-product function.

In the previous chapter, two cDNAs encoding *POX* gene were characterized. *In silico* studies of these two sequences revealed that both the genes have approximately equal molecular weight, isoelectric point and hydropathicity. Therefore, one of the cDNA encoding *POX* was chosen for over-expression and further studies.

4.2 Materials and methods

4.2.1 Materials

Glassware and plasticware: As discussed in Chapter 2, Section 2.2 and Section 2.3

Chemicals: As discussed in Chapter, Section 2.4

***Escherichia coli*, *Pichia pastoris* strains and plasmids used in the study**

Escherichia coli XL-1 Blue (Stratagene, USA)
E. coli BL 21(DE3 (Invitrogen, USA)
pGEM-T Easy Cloning vector (Promega, USA)
pET28a (+) Expression vector (Novagen, USA)
X-33 Mut^+ (*Pichia pastoris*) (Invitrogen, USA)
GS-115 His^-, Mut^+ (*Pichia pastoris*) (Invitrogen, USA)and
pPICZα A (Zeocine resistant vector for *Pichia* expression system. Invitrogen, USA)

Stock solutions: As discussed in Chapter 2, Section 2.6.3

4.2.2 Methods

4.2.2.1 Bacterial culture conditions: As discussed in Chapter 2, Section 2.8.1

4.2.2.2 Bacterial cells transformation: *E. coli* transformation and selection was done as discussed in Chapter 2, Section 2.8.2.3

4.2.2.3 Isolation of plasmid DNA from *E. coli*: As described in Chapter 2, Section 2.8.4.1

4.2.2.4 Restriction digestion of DNA: As described in Chapter 2, Section 2.8.4.4

4.2.2.5 Extraction and purification of DNA from agarose gels: As described in Chapter 2, Section 2.8.4.5

4.2.2.6 Polymerase Chain Reaction (PCR): As described in Chapter 2, Section 2.8.4.10

4.2.2.7 Colony PCR method: As described in Chapter 2, Section 2.8.3

4.2.2.8 Cloning of *POX* in pET-28a(+)

POX gene was cloned in pGEM-T Easy vector by incorporating the restriction sites *Nde* I and *Xho* I in the primers; POX F with *Nde* I and POX R with *Xho* I respectively. High fidelity *Taq pol* (*Pfx* Invitrogen) was used to amplify *POX* gene using the above set of primers from the cDNA clone, sequenced and maintained in pGEM-T Easy vector as the template. The above recombinant plasmid DNA was diluted 100 times and 1 µL was used as the template and PCR was performed (Chapter 2, Section 2.8.4.10). A 1 kb band was amplified (exactly 948 bp coding region of *POX* gene + *Nde* I and *Xho* I sites added into primer sequences). The band was excised from gel, purified, ligated in pGEM-T Easy vector and transformed in *E.coli* XL1 MRF cells. Clones with *POX* gene with *Nde* I and *Xho* I restriction sites were screened by colony PCR and a few colonies were inoculated in 5 mL LB (Ampicilin 100 µg/mL) tubes. Isolated individual plasmids were restriction digested with *Nde* I and *Xho* I enzymes to confirm the integration of *POX* gene.

PCR cycling condition:

Temperature	Time	No. of cycles
95 ºC	5 min	1
95 ºC 60 ºC 72 ºC	40 s 30 s 1.2 min	35
72 ºC	5 min	1
4 ºC	hold	1

The recombinant *POX* clones with *Nde* I and *Xho* I restriction sites were directionally cloned in pET-28a(+) vector (Fig 4A.1). Colony PCR (Chapter 2 Section 2.8.3) was done

to screen the recombinant pET-28a(+) clones. Integration of *POX* gene in pET-28a(+) was confirmed by digestion with *Nde* I and *Not* I (Fig 4A.2).

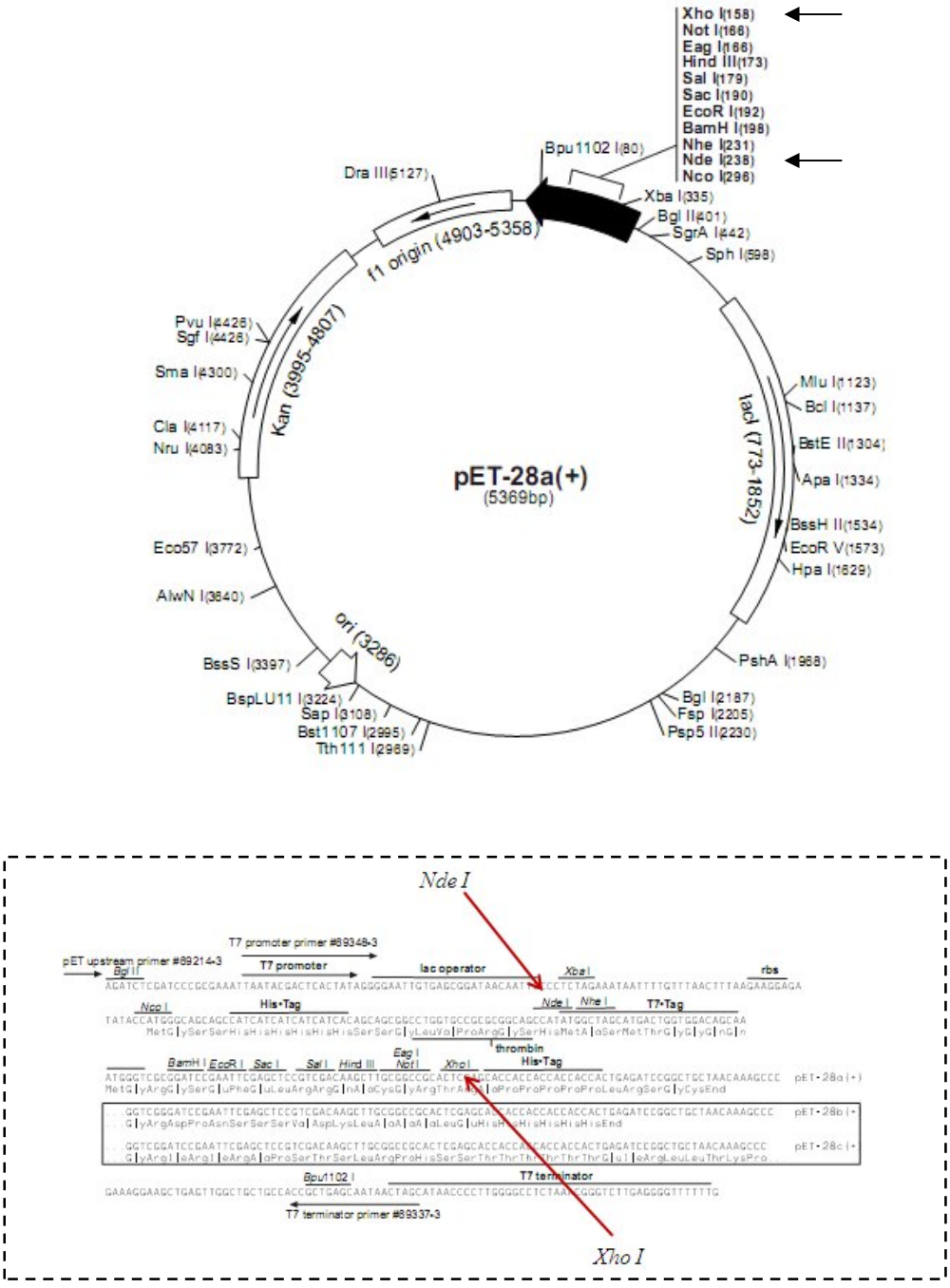


Fig 4A.1: Vector map of pET-28a (+) and the lower panel shows MCS of the vector and arrows indicates the sites where *POX* gene is cloned in frame with the promoter.

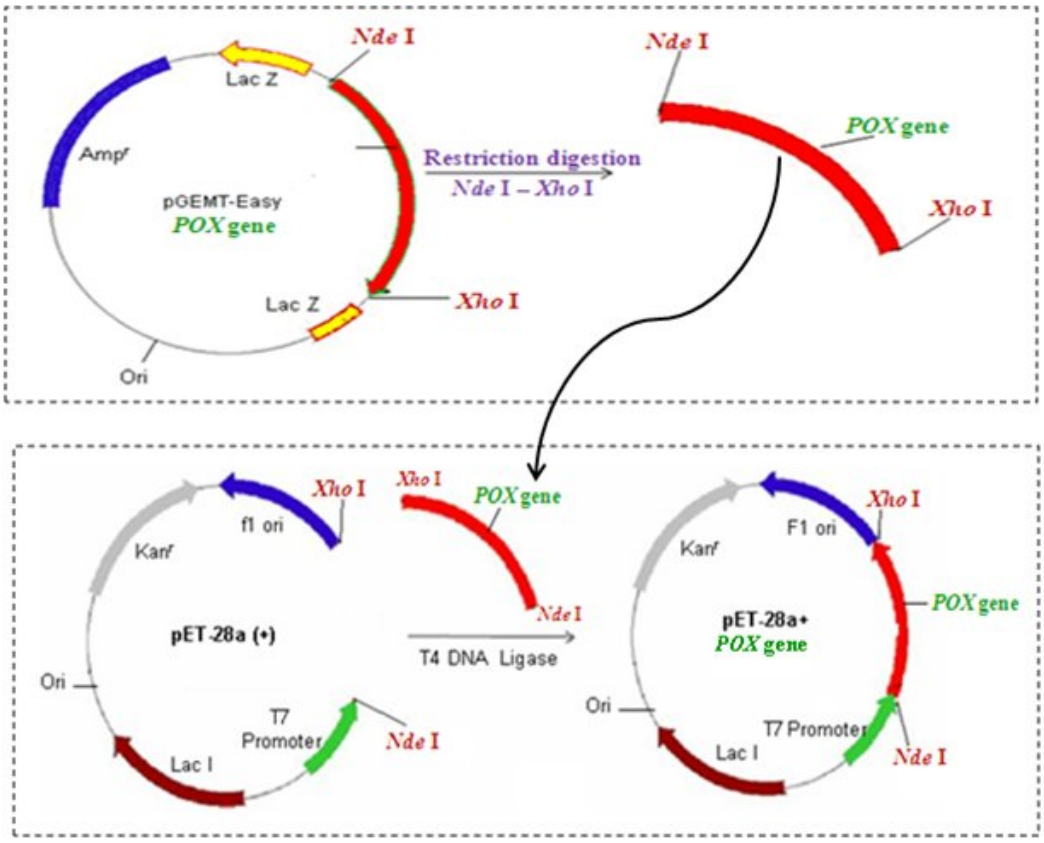


Fig 4A.2: Strategy used for directional cloning of *POX* gene in pET-28a (+) vector

4.2.2.9 Recombinant POX protein expression and its purification from inclusion bodies

4.2.2.9.1 Recombinant protein expression in *E. coli* (BL21)

A single bacterial (BL21) colony carrying recombinant pET-28a(+) with *POX* gene, from freshly streaked plates (grown on LB agar medium containing 50 µg / mL kanamycin) were 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. One mL aliquot of over-night grown culture was used to inoculate 100 mL LB broth containing 50 µg / mL kanamycin. Once the cultures reached OD_{600} 0.4 to 0.5, recombinant protein expression was induced by the addition of isopropyl -β-D-thiogalactopyranoside (IPTG), and the culture was grown for 4 to 6 h at 37 °C with shaking at 150 rpm. Recombinant protein extraction was done according to the protocol described in Section 2.10.

Likewise, four positive recombinant pET-28a(+) clones were screened for POX protein over-expression on 10% SDS PAGE (Chapter 2, Section 2.10.3). The clone showing maximum over-expression was chosen for further studies.

4.2.2.9.2 Purification of recombinant protein

His-tagged recombinant 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 POX protein carrying a 6xHis-tag was conducted using Ni NTA Agarose beads (Qiagen) (Chapter 2, Section 2.10.2). Purity of protein was checked on 10% SDS-PAGE (Chapter 2, Section 2.10.3).

4.2.2.9.3 Raising polyclonal antibody against purified POX protein in rabbit

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

4.2.2.9.4 Pre-treatment of serum: As described in Chapter 2, Section 2.11.1

4.2.2.9.5 Determination of titre of antibodies

ELISA was performed to determine the titre of first, second and third bleed of rabbit serum (Chapter2, Section 2.11.2). Once the antibody titre was determined then, a fixed dilution of antibody was used for rest of the experiments.

4.2.2.9.6 Standardization of time for protein expression in soluble form

The information obtained from above section 4.2.2.9 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 recombinant pET-28a(+) plasmid carrying *POX* gene. Induction with 0.1 to 1.0 mM IPTG was done and the cells were grown till A_{600}

reached 0.5. The flasks of 50 mL were used for analysis at different temperatures 37 °C, 25 °C, 22 °C and 15 °C for 4 h, 6 h, 10 h and 16 h after induction. One flask as an uninduced control sample was used before inducing with IPTG. The soluble fraction of cell lysate was analysed on SDS-PAGE.

4.2.2.9.7 Protein estimation

Protein estimation was done using Bradford assay (1976). A standard graph was made for BSA and concentration of unknown sample was determined by plotting standard graph (See appendix).

4.2.2.9.8 POX enzyme assay and kinetics

The purified peroxidases (POX) were attempted for polymerization studies. The reaction mixture (1.0 mL) contained the purified peroxidase, 0.1 mM monolignol, and 50 µM H_20_2 in 40 mM phosphate buffer (pH 6.8). The reaction, initiated by adding H_20_2 to the reaction mixture, was carried out at 30 ^oC. At the scheduled reaction time (2 min), recorded the absorbance at 263 and 272 nm for coniferyl alcohol and sinapyl alcohol, respectively. Differences in absorbance were converted to the amount of consumed monolignols using extinction coefficients of 15100 and 14100 $M^{-1}.cm^{-1}$ for coniferyl and sinapyl alcohol, respectively (Aoyama *et al., 2002*). See appendix, Table 2.9 substrates used for the study

4.3 Results and discussions

4.3.1 Cloning of *L. leucocephala POX* gene in pET-28a (+)

POX gene was cloned in pET-28a(+) expression system to get the gene expressed in its active form and for its characterization.

4.3.1.1 Incorporation of restriction sites

The *POX* gene cloned in pGEM-T Easy vector was amplified using gene specific primers ***POX Forward*** and ***POX Reverse*** to incorporate the *Nde* I site at the 5' end and *Xho* I site at 3' end of *POX* gene.

POX Forward - 5' CAT ATG GCT TCC CTT ACC CAT TTC TTC TTA C 3'

POX Reverse - 5' CTC GAG GTT GAC AAC CCT ACA ATT CTT TCG 3'

Approximately, 1 kb (948 bp *POX* + *Nde* I and *Xho* I restriction sites) band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in *E.coli* XL1 Blue cells. Clones with *POX* gene with *Nde* I and *Xho* I restriction sites were screened by inoculating a few colonies in 5 mL LB (Ampicilin 100 µg/mL) tubes. Isolated individual plasmids were restriction digested with *Nde* I and *Xho* I enzymes to confirm the integration of *POX* gene.

4.3.1.2 Directional cloning of *L. leucocephala POX* gene in pET-28a (+)

The above clone in pGEM-T Easy vector was restriction digested with *Nde* I and *Xho* I restriction enzymes and gene of interest was purified. pET-28a(+) vector DNA was also digested with same restriction enzymes and purified. *POX* gene was directionally cloned in purified restriction digested pET-28a(+) vector. Ligation mixture was transformed into *E.coli* XL1 competent cells and plated on LB-agar plate (Kanamycin 50 µg/mL). Colonies of recombinant plasmids were screened by colony PCR (Fig 4A.3), with reaction cycles as shown in table below. Plasmids were isolated from PCR positive clones and were digested with *Nde* I and *Xho* I to confirm the integration of *POX* gene fragment in pET-28a(+) vector (Fig 4A.4).

Temperature	Time	No. of cycles
95 ºC	5 min	1
95 ºC 60 ºC 72 ºC	40 s 30 s 1min	35
72 ºC	5 min	1
4 ºC	hold	1

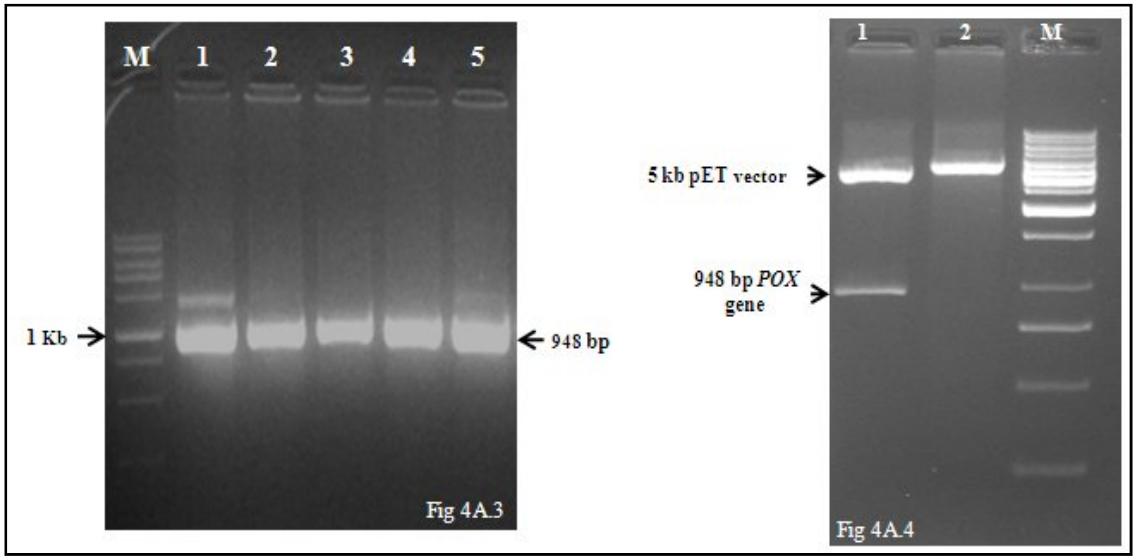


Fig 4A.3: Colony PCR showing 948 bp *POX* gene containing recombinant colones; Lane M- marker, lane 1, 2, 3, 4 and 5 are positive clones. **Fig 4A.4** Restriction analysis of recombinant pET-28a (+): lane 1 recombinant vector releasing 948 bp *POX* gene and 5 kb vector backbone, lane 2 linearised recombinant vector with *Nde* I restriction enzyme.

4.3.2 Recombinant POX protein expression and its purification from inclusion bodies

4.3.2.1 Recombinant POX protein expression and purification

E.coli BL 21 (DE3) cells transformed with recombinant pET-28a(+) plasmids were screened for over-expression. A few positive recombinant clones were screened for recombinant POX protein over-expression. An approximately 36 kD protein was found to express in all clones analysed on 10% SDS-PAGE (Fig 4A.5a) with varying levels of expression and purified using Ni-NTA Agarose beads (4A.5b).

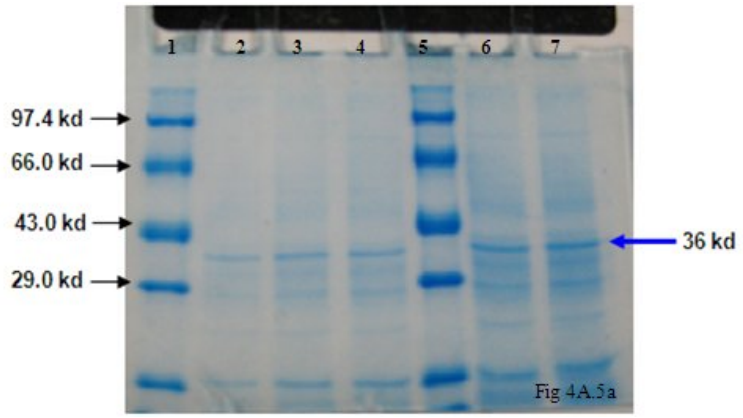


Fig 4A.5a: 10% SDS-PAGE; Coomassie (R 250) stained: Lane 1 & 5 Protein Molecular weight marker. Lanes 2, 3, 4, 6 & 7 POX protein expressed in *E. coli* BL 21 (DE3) carrying recombinant pET-28a(+)

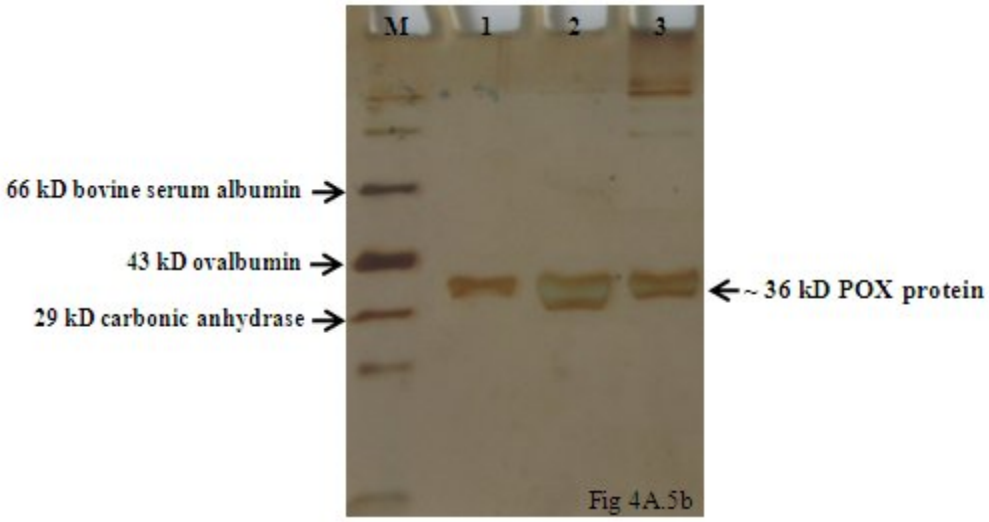


Fig 4A.5b: 10% SDS PAGE; silver stained: Lane M Protein Molecular weight marker. Lanes 1 & 2 purified POX using Ni-NTA Agarose beads, lane3 POX protein from inclusion bodies.

Purified POX protein was dialysed against 10 mM PBS buffer supplemented with 1 mM DTT and 0.1 mM PMSF overnight with two changes of fresh buffer. Dialysed POX protein was concentrated, quantified by Bradford assay (Bradford reagent, 1976) and checked for its activity. Under all the experimental conditions followed using different substrates, the purified protein in its native form failed to produce active protein. Since peroxidases are highly glycosylated proteins and glycosylation is absent in bacterial system we assume lack of glycosylation as the probable reason behind this expressed

POX protein not to give activity. This purified POX protein was used for raising polyclonal antibodies in rabbit.

4.3.3 Raising polyclonal antibodies in rabbit

300 μg of purified POX protein was used for first injection in New Zealand rabbits 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. The titre of third and fourth bleed is almost the same. The 3rd bleed serum dilution of 1:10000 was used for further experiments.

4.3.4 Directional cloning of *POX* gene in *Pichia pastoris* expression system

The association of peroxidase and glycans has been known for a long time, Van Huystee and Chibbar 1987, Olden *et a*l., 1985. Overall, the glycans are important for the activity and stability of cPrx75 and for proteins in general (Faye *et al*., 1989). HRP contains seven glycans (Harthill and Ashford, 1992) would suggest that they have some special functions. To date a minimum of 250 to 300 enzymes are involved in the glycosylation processes (Eijnden and Joziasse, 1993) which requires a great amount of metabolic activity and thus the glycans probably have a purpose. Biochemical compounds without a role are usually eliminated in the evolutionary process. That would hold particularly true for glycans.

With this knowledge from the previous reports on peroxidases and assuming our lignin biosynthetic *POX* gene also to be a highly glycosylated protein, it was decided to clone and express *POX* gene in eukaryotic expression system to get the protein in its active form. Eukaryotic system shows glycoslation, a major factor involved in post-translational modification, a phenomenon which is not observed in prokaryotic system. So, to successfully obtain the above POX protein in its active form, its expression in *Pichia pastoris* (Invitrogen, USA) system was attempted over *E. coli* where *POX* gene was not getting expressed in its active protein.

As a eukaryote, Pichia pastoris has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and post-translational modification, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*.

Many of the techniques developed for *Saccharomyces* may be applied to *Pichia pastoris*. These include:

. Transformation by complementation

. Gene disruption

. Gene replacement

4.3.4.1 Signal peptide prediction using Signal-P bioinformatics tool

The signal peptide was predicted using Signal-P bioinformatics tool (www. cbs.dtu.dk/services/signalp/) and the mature protein was deduced by considering the output of the prediction. The output data (Fig. 4A.6) suggests the presence of a signal peptide of 25 amino-acids at N-terminal. The cleavage site was predicted between 24th and 30th amino acids (Fig 4A.6)

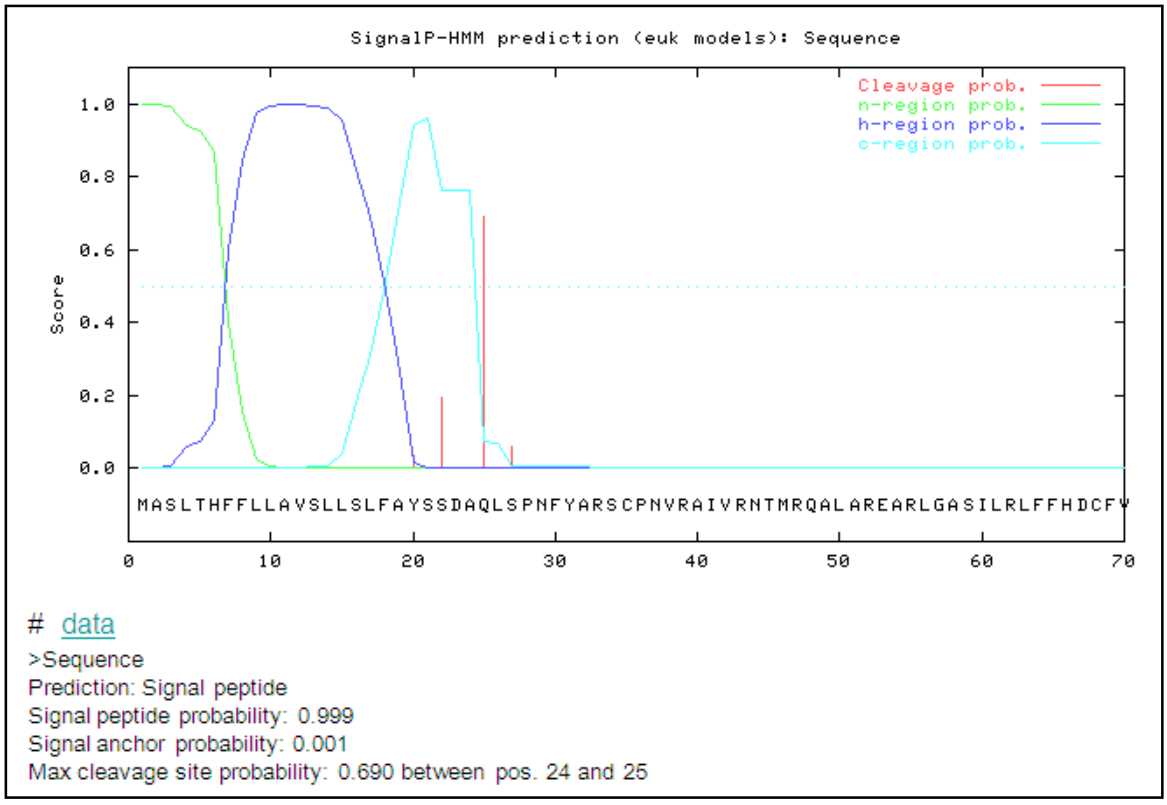


Fig 4A.6: Picture showing the **POX** signal peptide predicted by Signal–P tools. The most probable signal peptide cleavage site has been shown by red vertical line on x-axis.

The *POX* gene cloned in pGEM-T Easy vector was amplified using gene specific primers for *Pichia pastoris* namely, ***POX Picforward*** and ***POX Picreverse*** to incorporate the *EcoR* I site at the 5' end and *Not* I site at 3' end of *POX* gene to express mature peptide. All PCR reactions were performed using high fidelity *taq* DNA polymerase (Invitrogen, USA).

POX Picforward – 5' GAA TTC GCC CGA GAA GCC CGC CTT GGC 3'

POX Picreverse – 5' GCG GCC GC GTT GAC AAC CCT ACA ATT CTT TCG 3'

Approximately, 900 bp (858 bp *POX* gene mature peptide coding region + *EcoR* I and *Not* I restriction sites) band was amplified. The band was cut, purified, ligated in pGEM-T Easy vector and transformed in *E.coli* XL1 Blue cells. Clones with *POX* gene with *EcoR* I and *Not* I restriction sites were screened by colony PCR and were further confirmed by inoculating a few PCR positive colonies in 5 mL LB (Ampicilin 100 µg/mL) tubes. Isolated individual plasmids were restriction digested with *EcoR* I and *Not* I enzymes to confirm the integration of *POX* gene.

4.3.4.2 Directional Cloning of *POX* gene in pPICZα vector

If protein is normally secreted, glycosylated, or directed to an intracellular organelle, it is recommended to use pPICZα vectors. pPICZα A, B, and C are 3.6 kb vectors used to express and secrete recombinant proteins in *Pichia pastoris*. Recombinant proteins are expressed as fusions to an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal. In the present study the nucleotide sequence coding for mature peptide for *POX* gene was amplified (using primers *POX Picforward* and *POX Picreverse*) and cloned directionally into pPICZα A vector (Fig 4A.7). The *POX* gene cloned in pPICZα A was confirmed by PCR and restriction analysis (Fig 4A.8).

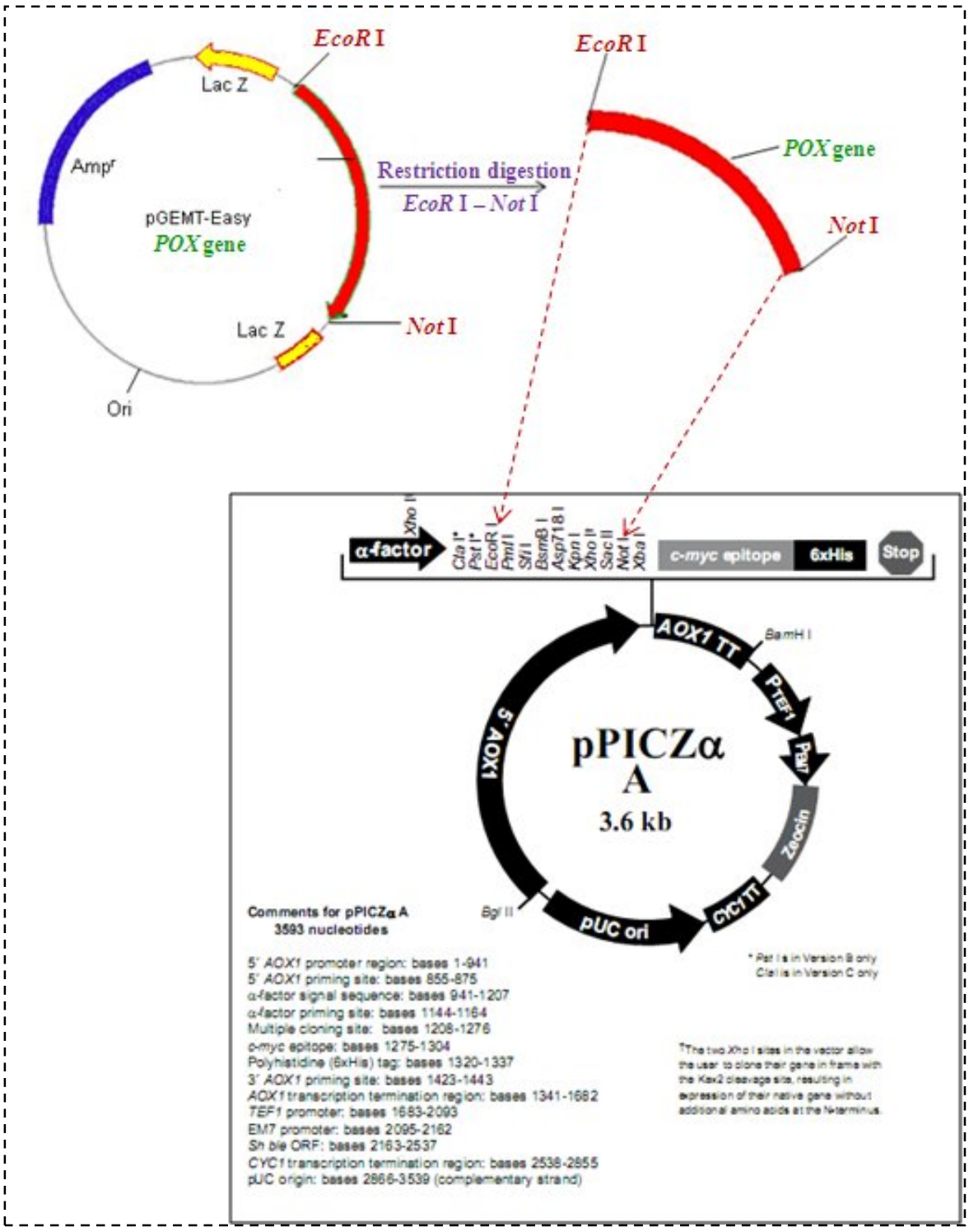


Fig 4A.7: Strategy used for directional cloning of *POX* gene in pPICZα A vector between *EcoR* I and *Not* I sites

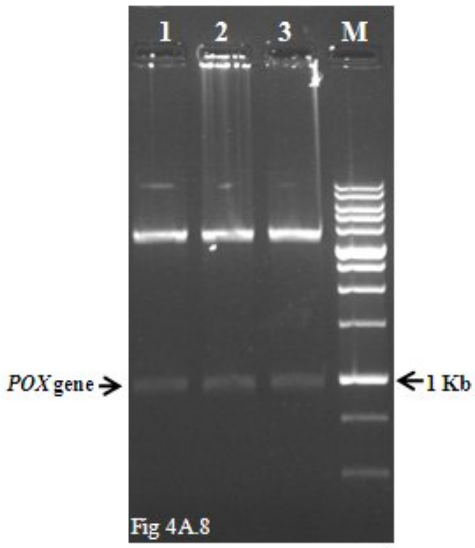


Fig 4A.8: Restriction analysis of recombinant pPICZα A releasing *POX* gene using *EcoR* I and *Not* I restriction enzymes in lanes 1, 2 & 3. Lane 4 is marker

4.3.4.3 Transformation of *Pichia* strain X-33 with recombinant pPICZα A vector

4.3.4.3.1 Preparation of DNA for transformation

The recombinant pPICZα A carrying *POX* gene was linearised using *BstX* I restriction enzyme for *Pichia pastoris* transformation.

4.3.4.3.2 Preparation of *Pichia* competent cells

Pichia pastoris cell line X-33 was used for competent cell preparation. Invitrogen's *Pichia* EasyComp kit was used for chemical synthesis of competent *Pichia* cells, which is an alternative to electroporation and a rapid, convenient method for transformation. All reagents and solutions and cell lines were procured from Invitrogen, USA and strictly manufacturer's (Invitrogen, USA) protocol was followed.

4.3.4.3.3 *Pichia* transformation with *POX* gene construct

Pichia EasyComp transformation protocol was used strictly following manufacturer's instructions (Invitrogen, USA)

4.3.4.4 PCR analysis of *Pichia* transformants for gene integration

Genomic DNA was isolated from *Pichia* clones which survived on the selection antibiotic, zeocine. Amplification of the gene of interest was carried out in putative

recombinant clones using 5' *AOX1* primer paired with the 3' *AOX1* primer provided with the kit (Invitrogen, USA). Recombinant X-33 Mut$^+$ integrants should give two bands; one will correspond to the size of cloned gene of interest (Fig 4A.9) and the other to the *AOX1* gene (approximately 2.2 kb).

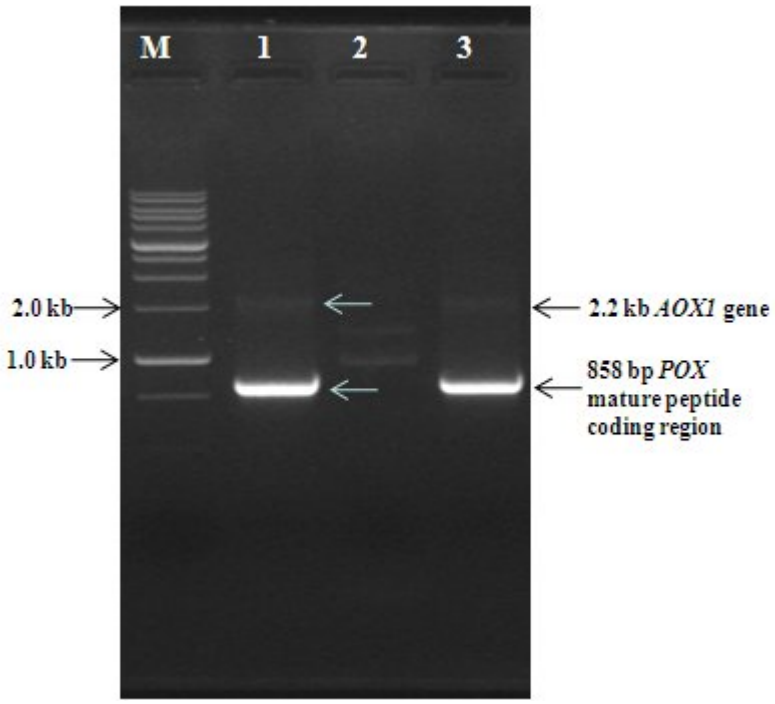


Fig 4A.9: PCR analysis of recombinant X-33 clones using *AOX1* primers. Lane M- marker, lanes 1 & 3 positive clones and lane 2 negative

4.3.4.5 Recombinant POX protein expression and its purification

Randomly selected, six recombinant *Pichia* (strain X-33) clones and a control X-33 cell line, used for preparing competent cells was subjected to expression studies strictly following manufacturer's (Invitrogen, USA) protocol. Extra-cellular broth (2-3 mL) was collected at 0 h, 3h, 6h, 12 h, 18 h, 24 h, 48 h and 72 h (methanol was added every 24 h to enhance induction as per manufacturer's recommendation) after inoculation and checked for peroxidase activity using guaiacol 100 mM as substrate. It was noticed that from 12 h onwards detectable amount of peroxidase activity was observed in the broth and comparatively good activity was noticed between 18 and 24 h (Section 4.3.5). The extra-cellular broth was then subjected to ammonium sulphate precipitation and the fractions (0-40%; 40-60% and 60-80%) were subjected to dialysis against 20 mM

phosphate buffer pH 7.4 and was concentrated and checked on SDS-PAGE. No noticeable bands were observed when stained using Coomassie blue-R250, due to low protein expression levels. The same fraction was run on native PAGE and stained using 20 mM Guaiacol and H_2O_2, where the fraction obtained between 60-80% showed a band for peroxidase activity. The band was around 58 to 66 kD over the expected 32 kD expected for the cloned 858 bp mature peptide coding region. This increase in size of the protein may be due to extensive glycosylation in eukaryotic (*Pichia*) expression system. Average yield of POX protein obtained per liter of broth was around 0.9- 1.2 mg, which was further purified using anion exchange chromatography by mounting Q Sepharose ™ Fast Flow column on to AKTAexplorer FPLC system (GE Health care). In FPLC system, the POX protein was dialysed against 20 mM phosphate buffer; same buffer was used to equilibriate and bind the protein; elution was done as continuous gradient using 1M NaCl in 20 mM phosphate buffer (Fig 4A.10).

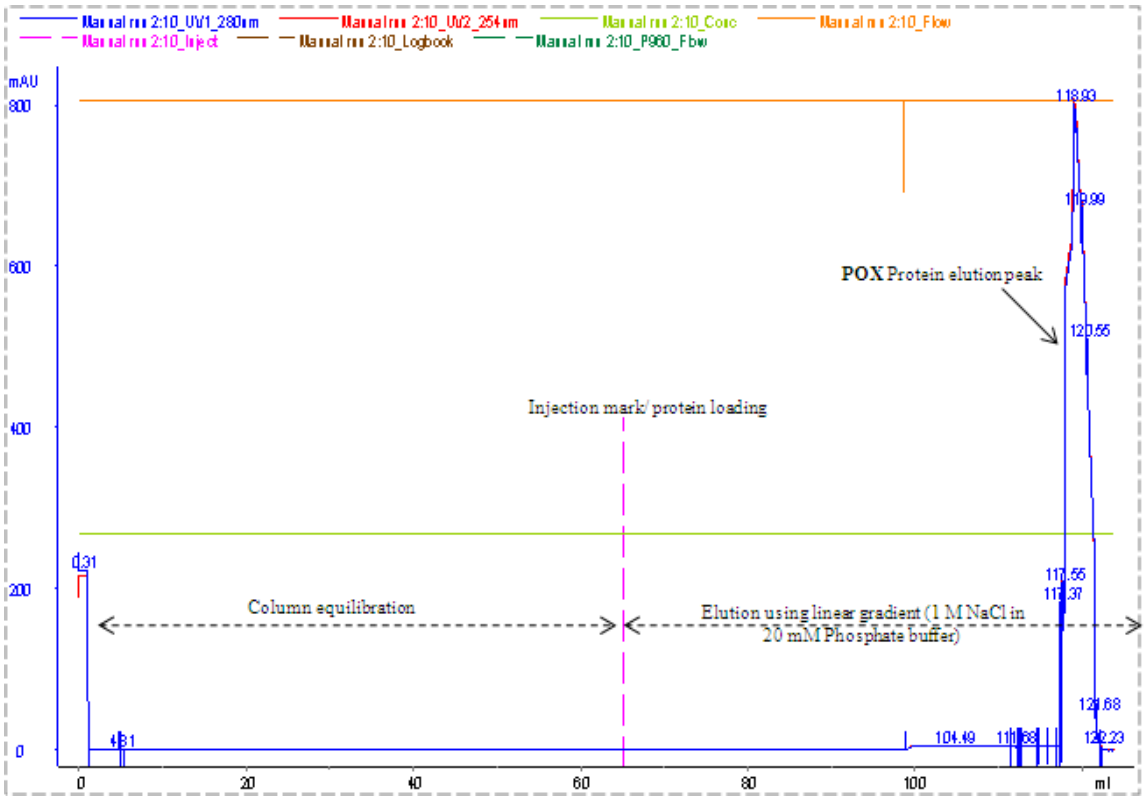


Fig 4A.10: Purification of recombinant protein expressed in *Pischia* expression system using Q Sepharose ™ Fast Flow column mounted on to AKTAexplorer FPLC system

4.3.4.6 Western blot analysis

The recombinant protein expressed in Chapter 4, Section 4.3.2 was used to raise polyclonal antibody against POX protein in rabbit, Chapter 4, Section 4.3.3 (Bangalore Genei, Bangalore, India). The above raised anti-POX polyclonal antibody obtained from 3rd bleed serum was diluted 1:10000 times and was used for western blot experiments. Secondary antibody *i.e.* goat anti-rabbit IgG conjugated with alkaline phosphatase was purchased from Bangalore Genei, Bangalore, India.

iBlot Gel Transfer System was used to transfer the protein from SDS-PAGE gels or from slot blot membranes by strictly following manufacturer's (Invitrogen, USA) instructions. The PVDF membrane containing transferred protein samples were processed as per standard procedure, blocking, treatment with primary antibody (anti-POX antibody Chapter 4, Section 4.3.3), washing followed by treatment with secondary antibody which is ALP conjugated, followed by washing and colour development using BCIP/NBT as substrate. The samples subjected for western analysis includes recombinant proteins expressed in *Pichia pastoris* expression system and crude protein from different tissues of *Leucaena leucocephala* seedlings. The recombinant proteins were slot blotted and subjected to western analysis (Fig 4A.11a), whereas, the plant extracts were run on 10% SDS-PAGE and blotted on to PVDF membrane (Fig 4A.11b).

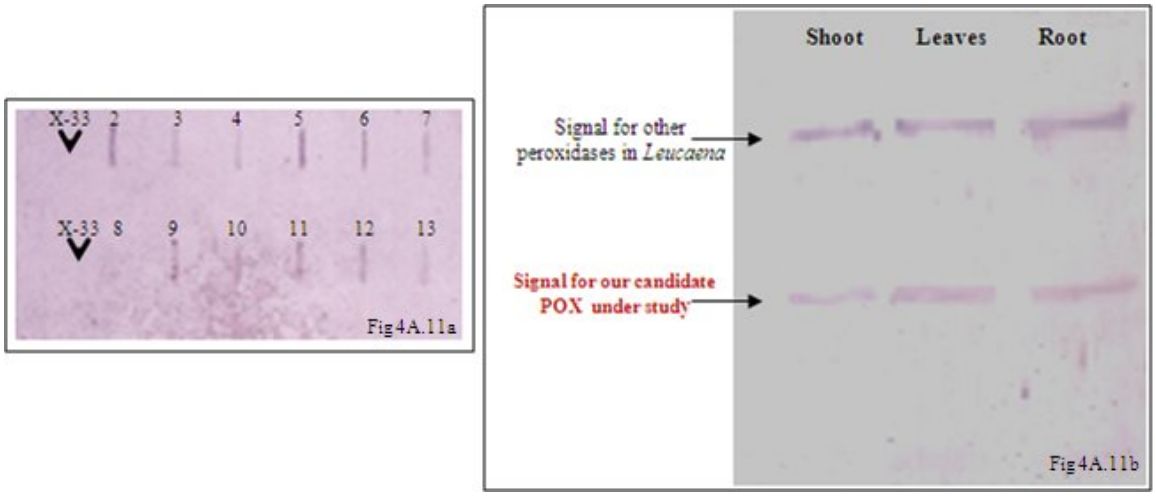


Fig 4A.11a: Western blot analysis of putative *Pichia* clones; lane mentioned as X-33 is control non-recombinant cell line, clone 8 showed no POX expression, rest all showed positive signal for cloned POX gene product. **Fig 4A.11 b:** Western analysis of *Leucaena* plant extracts from stem, leaves and root tissues

4.3.4.7 Guaiacol staining of peroxidase

In western blot analysis, it was revealed that the anti-POX antibody gave signals at two places on the blot when total *Leucaena* crude protein from shoot, leaf and root were challenged with the antibody. To study the above western result that is, whether there exist two peroxidase populations in *Leucaena leucocephala* and to know which one among those two population of *POX* is our isolated and characterized *POX* gene, guaiacol staining was performed using crude protein extracts from shoot, leaf and root samples along with the protein expressed in *Pichia pastoris*. From the guaiacol stained native PAGE gel (Fig 4A. 12) profile it is clear that there exists two major peroxidase populations based on their molecular weight (Fig 4A. 12 lanes 1. 2 & 3) and the *Pichia pastoris* expressed recombinant POX protein matched with the low molecular weight peroxidase population noticed in *Leucaena leucocephala* (Fig 4A. 12 lane 4). In lane 4, the recombinant protein expressed in *Pichia,* we can see the POX protein in varying level/extend of glycosylation in the form of a trailing band unlike clear-cut bands noticed in lanes 2 & 3 (Fig 4A. 12).

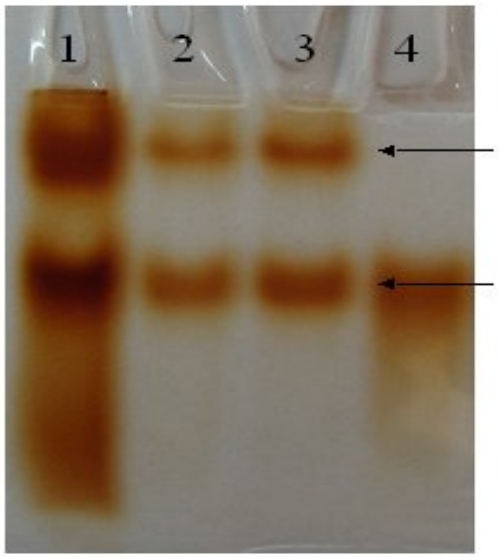


Fig 4A.12: Peroxidase stained using guaiacol on native PAGE: Lane 1 crude protein extract from shoot, lane 2 and 3 crude protein extracts from leaves and root respectively. Lane 4 recombinant POX expressed in *Pichia.*

The heterologous expression of POX in *Pichia pastoris* was checked using guaiacol as substrate. Extra cellular broth was collected from recombinant clones at 0 h, 3h, 6h, 12 h, 18 h, 24 h, 48 h and 72 h after inoculation and checked for peroxidase activity using guaiacol on native PAGE. It was noticed that from 12 h onwards detectable amount of peroxidase activity was observed and comparatively good activity was noticed between 18 and 24 h (Fig. 4A.13).

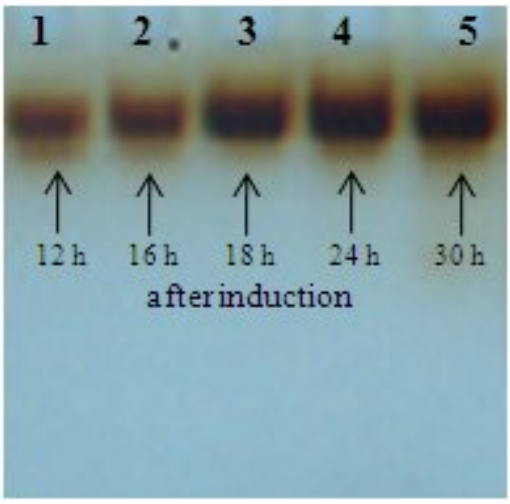


Fig 4A.13: Guaicol staining of recombinant POX expression in Pichia at different time intervals on native 12% gel showing gradual increse in POX expression with best expression at 24 h after induction

4.3.5 Peroxidase enzyme assay

The activity of peroxidase fractions during purification was routinely monitored by oxidation of guaiacol. The assay contained 20 mM potassium phosphate buffer pH 7.0, 13.3 mM guaiacol, and 100 µM H_2O_2. The reaction was initiated by addition of peroxidase (2 µL) and the color development was monitored over a period of 2 min at 470 nm. For assays purified peroxidase was used at 10 nM concentration.

The substrates mainly tested were coniferyl alcohol and sinapyl alcohol the lignin monomers found in plants. The objective was to check whether the isolated and expressed lignin bisynthetic peroxidase can perform the proposed role, that is, polmerisation of lignin monomers or the monolignols. Substrate specificity of peroxidase was monitored with the following substrates coniferyl alcohol (A_{263} nm), sinapyl alcohol

(A_{272} nm) and guaiacol (A_{470} nm). The incubation medium contained 20 mM potassium phosphate buffer, pH 7.4, and 1mM H_2O_2. Reactions were monitored over a 2-min time course at the appropriate wavelength mentioned above. For coniferyl alcohol and sinapyl alcohol the differences in absorbance were converted to the amount of consumed monolignols using extinction coefficients of 15100 and 14100 $M^{-1}.cm^{-1}$ respectively. The apparent K_m and V_{max} for coniferyl alcohol, sinapyl alcohol and guaiacol are mentioned in the table below.

	Substrate	**K_m**	**V_{max} (nKat/mg protein)**
1	Coniferyl alcohol	3.1 ± 0.37	2780 ± 28.7
2	Sinapyl alcohol	5.2 ± 0.8	741.2 ± 17
3	Guaiacol	23.2 ± 1.9	2073 ± 35

From the preliminary studies, it is revealed that among the three substrates (coniferyl alcohol, sinapyl alcohol and guaiacol) tested coniferyl alcohol is the preferred substrate over sinapyl alcohol and guaiacol. The order of affinity for the above substrates is in the order coniferyl alcohol > sinapyl alcohol > guaiacol. When compared with sinapyl alcohol and guaiacol, enzyme has more affinity to sinapyl alcohol but the catalytic rate is low as per the V_{max} values. Further enzyme characterization and studies using different substrates are in progress; but at this point we can infer that the *POX* gene isolated from *L. leucocephala* expressed in *Pichia* expression system in its active form have affinity to monolignols and plays a role in lignification process.

4.3.6 Discussion

Class III lignin biosynthetic peroxidase (*POX*) isolated from *L. leucocephala* was over-expressed using pET-28a(+) expression system (Novagen, USA). The pET vectors carry N-terminal His•Tag®/thrombin/S•Tag™/enterokinase configuration plus an optional C-terminal His•Tag sequence. Unique sites (*Nde* I and *Xho* I) in pET-28a(+) vector (Fig. 4A.1) was selected to clone *POX* gene inframe with the promoter to get the protein expressed along with the His tags. The 6X His affinity tag facilitates binding of POX protein to Ni-NTA agarose beads and thereby its purification. Since the expressed POX

protein was not giving activity under all experimental conditions tried, the purified protein was used to raise polyclonal antobodies in rabbit (New Zealand White) and these antibodies were used for futher studies like western, ELISA and immuno-cytolocalization. Since peroxidases are highly glycosylated proteins, the nucleotides coding for mature POX protein was cloned in pPICZα A (using Signal-P bioinformatics tool) and was expressed as secreated protein in *Pichia pastories* strain X-33. The expressed protein was purified using anion exchange matrix (Q Sepharose^{TM} Fast Flow column) mounted on to AKTAexplorer FPLC system (GE Health care). The purified protein was obtained in its active glycosylated form and was confirmed by western analysis. This POX protein was partially characterized and kinetic studies revealed based on its low K_m value that, expressed POX enzyme have more affinity to coniferyl alcohol (K_m3.1 ± 0.37) than sinaply alcohol (K_m5.2 ± 0.8). Since the isolated, cloned and expressed *L. leucocephala POX* gene is able to polymerise monolignols, it is confirmed beyond doubt that they have definite role in lignin biosynthesis in *L. leucocephala*. Further enzyme characterization and analysis of the polymerised compounds is in progress.

4.3.7 Conclusion

The peroxidase gene (*POX*) isolated from *L. leucocephala* was directionally cloned in pET-28a(+) expression system. Recombinant POX protein was standardized for over-expression and purified from inclusion bodies; it was used to raise antibodies in rabbit (New Zealand white). The candidate peroxidase protein was detected in *L. leucocephala* by Western blotting. The gene coding for mature peptide of POX was cloned in eukaryotic expression system of *Pichia pastoris* and was expressed as glycosylated protein in its active form. POX enzyme activity for substrates like coniferyl alcohol, sinaply alcohol and guaiacol was also standardized from purified POX protein expressed in *Pichia*. The functional expression of recombinant POX protein confirms that the cDNA isolated from *L. leucocephala* encodes for lignin biosynthetic peroxidase enzyme.

CHAPTER 4B

4.4 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 in woody angiosperms or tree species.

Previously studies were done to identify the monolignol biosynthetic genes in lignifying Sotaka spruce shoots (Friedmann *et al*., 2007) and in different tissues of maize (Guillaumie *et al*., 2007b) using micro/macroarray hybridisations. Appearance of several peroxidase isoforms has been correlated with tracheary element differentiation and lignifications (Fukuda and Komamine, 1982, Pesquet et al., 2005, Sato et al., 2006). Highly similar peroxidases from *Arabidopsis* were temporally and spatially linked with lignifications (Sato et al., 2006). In the present study, we used reverse transcription followed by polymerase chain reaction (real-time RT-PCR), since the method is very sensitive and specific when differentiation between highly similar transcripts is needed (Huggett *et al*., 2005).

The reports on temporal expression pattern of *POX* gene(s) involved in monolignol polymerisation in woody angiosperms is very limited. In the present study, expression of the identified lignin biosynthetic *POX* gene was performed in more detail in *L. leucocephala* to verify the expression patterns and to explore their roles under specific developmental conditions. Studying different aspects of *POX* like determining the protein expression pattern in stem and root tissues by ELISA, transcript analysis using RT-PCR and immuno-cytolocalization, especially with respect to different developmental stages can provide sufficient knowledge for genetic engineering of tree species, in context of lignin biosynthesis.

4.5 Materials and methods

4.5.1 Plant material

Seeds of *L. leucocephala* (K-636) were treated as described in Chapter 2: section 2.1.1. 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 (Fig 4B.1a, Fig 4B.2a, Fig 4B.3a, Fig 4B.4a and Fig 4B.5a). For 10, 15 and 20 day seedlings root, shoot and leaves were harvested separately and were used for Real-Time PCR analysis, ELISA and immuno-cytolocalization studies.

4.5.2 Primary antibodies for anti POX protein

Purified POX protein was used to raise antibodies in rabbit as discussed in Chapter two, Section 2.10 and 2.11.

4.5.3 Secondary antibody

Alkaline phosphatase tagged goat anti- rabbit IgG antibodies were purchased from Merck.

4.6 Methods

4.6.1 Total RNA extraction and cDNA synthesis

Total RNA was isolated from experimental germinated seedlings according to the TRI reagent (Sigma, Germany) or hot phenol method at different time intervals. First strand cDNA synthesis, primed with an oligo $(dT)_{15}$ primer, was performed with Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) according to the manufacturer's protocol (Promega Corp., Madison, USA).

4.6.2 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 SYBRBrilliant 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.

4.6.2.1 Q-PCR considerations: See chapter 2: section 2.8.5.1

4.6.2.2 Preparing the RT-qPCR reactions: See chapter 2: section 2.8.5.2

4.6.2.3 Real-time quantitative PCR for *POX* gene

Total RNA was extracted individually from roots and shoots at different time intervals from developing seedling 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 POX* gene and 5.8S rRNA are given in Table 4B.1. Optimal numbers of PCR cycles within the linear range of amplification for each gene were determined in preliminary experiments. RT-PCR reactions were performed following conditions mentioned in Chapter 2: section 2.8.5.3 with annealing temperature of 55 ^{o}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).

Table 4B.1 Primer sequences designated for *Leucaena POX* gene and 5.8S rRNA

Primer	Sequence 5' – 3'	T_m in °C
Abi POX F1	CTGTGTCTTGTGCTGATATCTTGG	64
Abi POX R1	GATCTCGCTGTTGGCTTTGC	62
Ctrl5.8S F	CTAAACGACTCTCGGCAAC	58
Ctrl5.8S R	TTCAAAGACTCGATGGTTCAC	60

4.7 Enzyme extraction from developing seedlings and ELISA Analysis

0, 5, 10, 15, 20 and 30 day old stem and root samples were harvested and homogenized in buffer (100 mM Tris-HCl pH.7.5, 2% PVPP, 2% PEG 4000, DTT 5 mM and PMSF 1 mM). Leaf tissue was not considered for this experiment. Homogenized tissues were centrifuged at 12000 g at 4 °C and supernatant was collected in fresh microfuge tube. All supernatants were quantified using Bradford assay (Bradford reagent, BioRad, USA). Total protein used per well for ELISA was 25 μg. All samples were coated in triplicates. Primary antibody was used at a dilution of 1/10000 and secondary antibody was anti-rabbit goat IgG tagged with alkaline phosphatase at a dilution of 1/20000. PNPP (*p*-nitro phenyl phosphate) was used as a substrate for all reactions at a concentration of 1 mg/mL. A standard graph was made using 1 ng to 32 ng of purified recombinant POX protein. Experiment was repeated twice for reproducibility.

4.8 Immuno-cytolocalization of POX protein in *Leucaena leucocephala*

Solutions: See Appendix: Table 2.10

Protocol: See Chapter 2: Section 2.11.3

4.9 Histochemical staining of lignin

Solutions: Phloroglucinol 2% in 95% Ethanol

Free hand transverse sections were prepared for histochemistry as described in Chapter 2: Section 2.11.3. Phloroglucinol staining was done by covering pretreated slide with phloroglucinol for half an hour and then concentrated HCl was added. Lignified tissue takes up 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 (Axioplan 2, Zeiss).

4.10 FTIR analysis

FTIR was done using Spectrum One, machine from Perkin Elmer, USA. Growing seedlings in normal condition were considered for this experiment to monitor any change in quality of lignin produced at different point of time. Spectrum One (Perkin Elmer, USA) is capable of analyzing powder as well as liquid samples. Powdered lignin samples were analysed according to the manufacturer's recommendation.

4.10.1 Sample preparation: See Chapter 2: Section 2.14.

4.10.2 FTIR Scanning

FTIR scanning was done ten times for each sample and values were recorded in percent transmission. All values were corrected for baseline KBr scan.

4.11 Results and discussions

Expression studies were done using developing seedlings of *L. leucocephala.* Mx 3000P (Stratagene, USA) was used for expression studies. Spectrum One (Perkin Elmer, USA) was used for FTIR analysis.

4.11.1 Spatio-Temporal expression pattern of *POX* gene, Real-time PCR

Temporal expression of *POX* was carried out by isolating total RNA from 5, 10, 15 and 20 day old developing seedlings. Seedlings were subjected to treatments (1% mannitol, 2% mannitol, 100 mM NaCl and 200 mM NaCl) under light and dark conditions, along with control not subjected to any stress.

4.11.1.1 Real Time PCR analysis for spatio-temporal *POX* expression pattern in developing shoots and roots of *L. leucocephala* grown under light and dark (Fig.4B.1a)

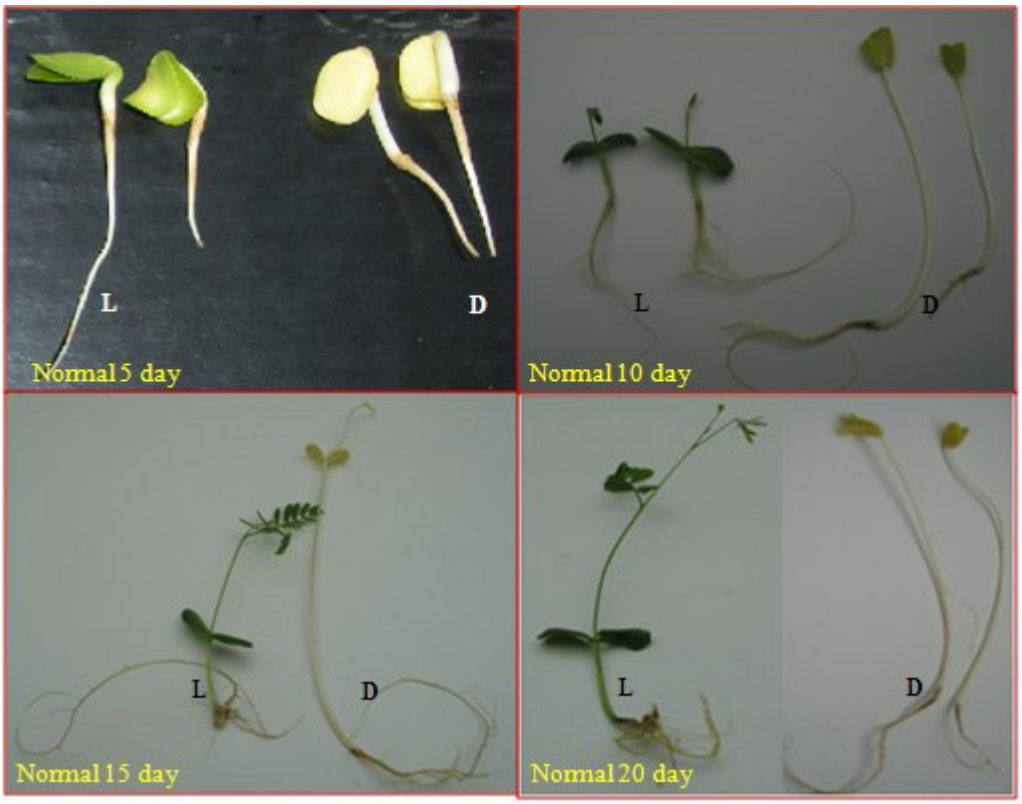


Fig 4B.1a: *Leucaena leucocephala* seedlings of 5day, 10 day, 15 day and 20 day grown under light and dark conditions. "L"-stands for light grown, "D"- stands for dark grown plants

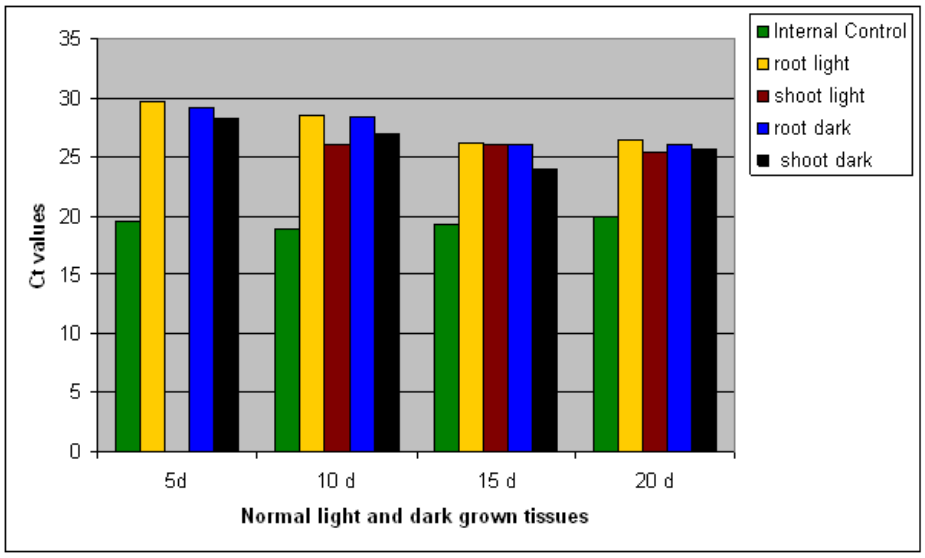


Fig 4B.1b: Relative expression of POX transcript in terms of Ct value in different plant tissues of different age *L. leucocephala* seedlings under light and dark conditions. On X-axis is tissue (shoot and stem) with age of seedling. On Y-axis is quantity in terms of Ct value. The numbers 5d, 10d, 15d and 20d represents age of seedling in days

Based on the Ct values, the expression patterns in different tissue types of different age groups were analyzed. The mean Ct value was calculated for three replicates of each treatment along with the internal control (5.8S rRNA) gene. The mean Ct values obtained from all the samples along with the standard deviation were used for analysis. It was assumed that the reference gene (5.8S rRNA) doesnot vary in copy number or expression level in the samples of study which is evident by the very little fluctuations in the Ct values obtained from RT-PCR amplification plots.

For normal light and dark grown plants the highest expression of *POX* was observed in 15 day dark grown shoot (Fig 4B.1b), followed by 20 day light grown shoot and 15 day light grown shoot. Lowest expression was noticed in 5 day light and dark grown root (Fig 4B.1b). The $2^{-\Delta\Delta Ct}$ method is a convenient way to analyze the relative changes in gene expression from real time quantitative PCR experiments (Pfaffl MW. 2001), as $2^{-\Delta\Delta Ct}$ actually reflects the fold expression. Based on $2^{-\Delta\Delta Ct}$ values, the highest expression obtained for *POX* transcript was for 15 day dark grown shoot; which showed 18.25 fold expression in comparison with the lowest expression in 5 day old root (Fig 4B.1b).

4.11.1.2 Real Time PCR analysis for spatio-temporal *POX* expression pattern in developing shoots and roots of *L. leucocephala* grown under light and dark (Fig 4B.2a**) with 1% mannitol as drought stress.**

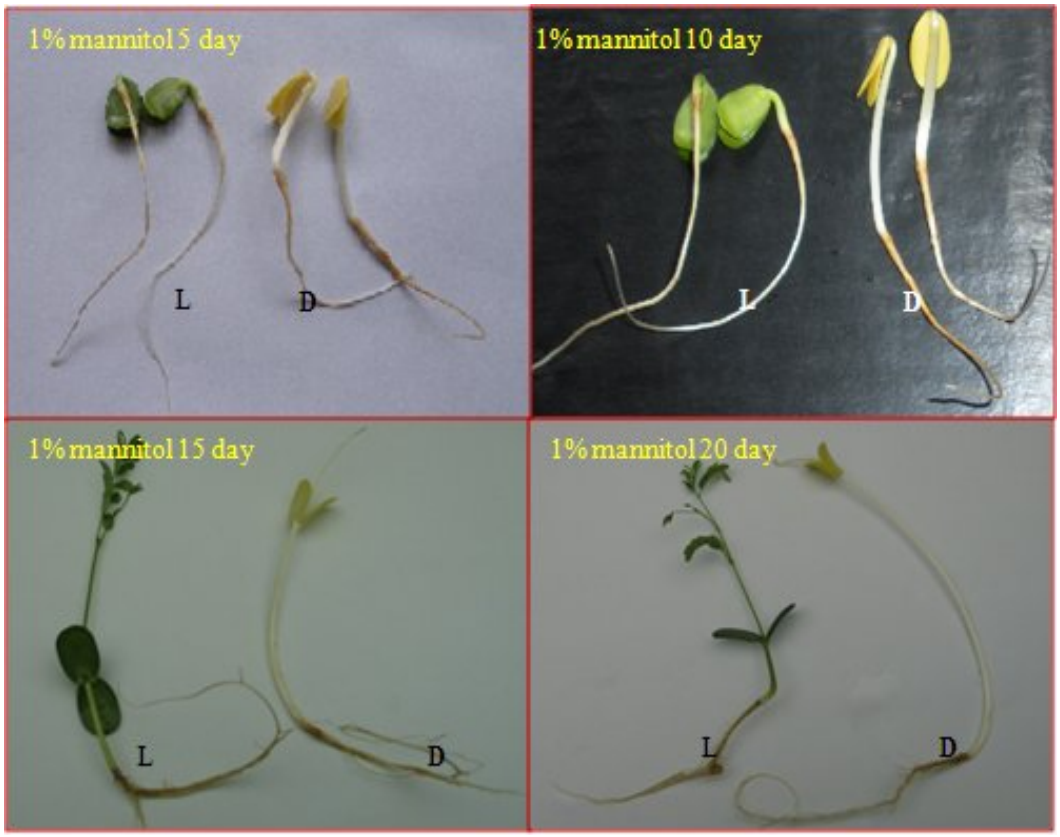


Fig 4B.2a: *Leucaena leucocephala* seedlings of 5day, 10 day, 15 day and 20 day grown under light and dark conditions with 1% mannitol as stress to induce drought. "L"-stands for light grown, "D"- stands for dark grown plants

For 1% mannitol light and dark grown plants the highest expression of *POX* was observed in 20 day dark grown shoot (Fig 4B.2b), followed by 20 day light grown shoot and 10 day dark grown shoot. Lowest expression was noticed in 5 day dark grown root (Fig 4B.2b). Based on $2^{-\Delta\Delta Ct}$ values, the highest expression obtained for *POX* transcript was for 20 day dark grown shoot; which showed 36.25 fold expression in comparison with least expressed 5 day old root (Fig 4B.2b).

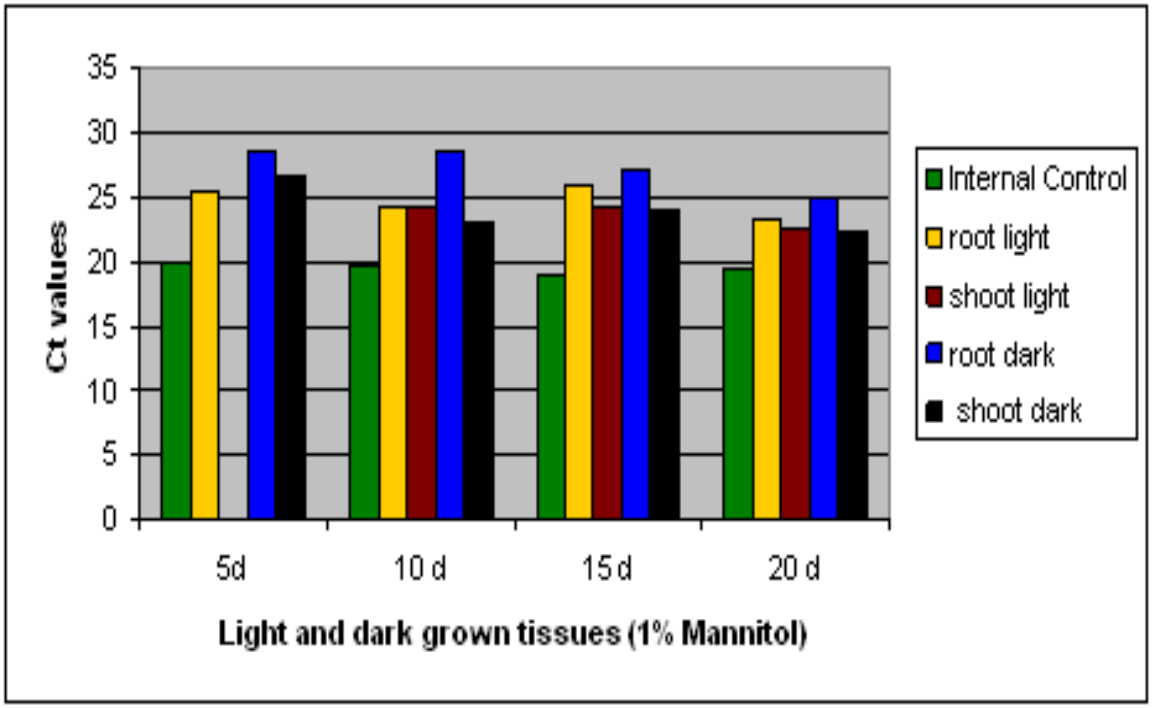


Fig 4B.2b: Relative expression of POX transcript in terms of Ct value in different plant tissues of different age *L. leucocephala* seedlings under light and dark conditions with 1% mannitol stress. On X-axis is tissue (root and stem) with age of seedling. On Y-axis is quantity in terms of Ct value. The numbers 5d, 10d, 15d and 20d represents age of seedling in days

4.11.1.3 Real Time PCR analysis for spatio-temporal *POX* expression pattern in developing shoots and roots of *L. leucocephala* grown under light and dark (Fig 4B.3a) with 2% mannitol as drought stress.

For 2% mannitol light and dark grown plants the highest expression of *POX* was observed in 20 day light grown shoot (Fig 4B.3b), followed by 20 day dark grown shoot and 20 day light grown root. Lowest expression was noticed in 5 day dark grown root (Fig 4B.3b). Based on $2^{-\Delta\Delta Ct}$ values, the highest expression obtained for *POX* transcript was for 20 day light grown shoot; which showed 14.32 fold expression in comparison with least expressed 5 day old dark grown root (Fig 4B.3b).

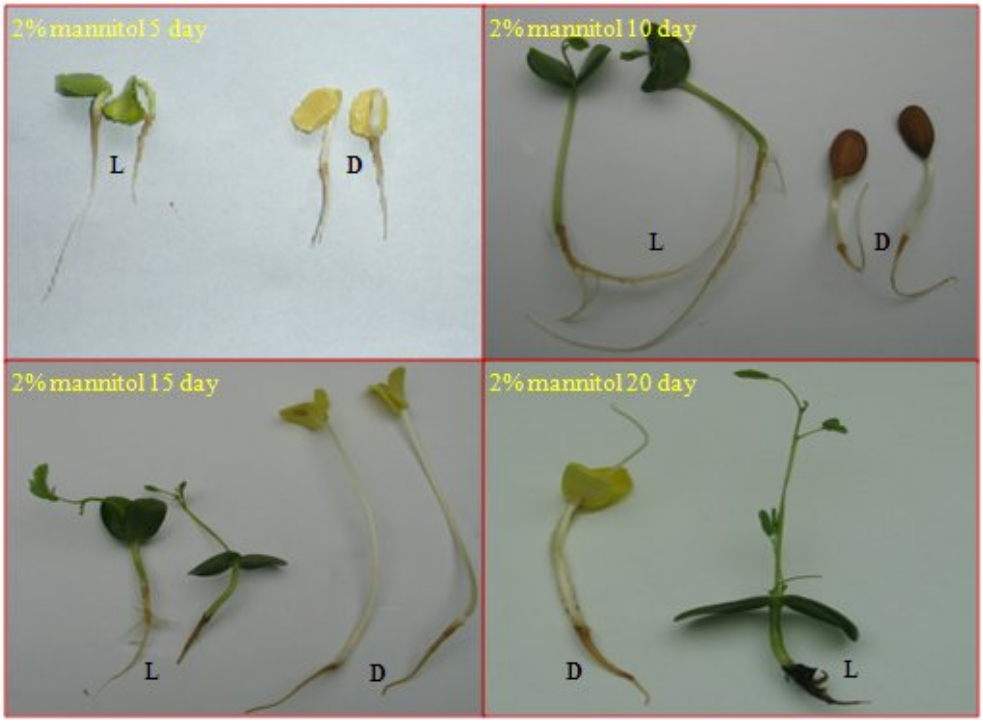


Fig 4B.3a: *Leucaena leucocephala* seedlings of 5day, 10 day, 15 day and 20 day grown under light and dark conditions with 2% mannitol as stress to induce drought. "L"-stands for light grown, "D"- stands for dark grown plants

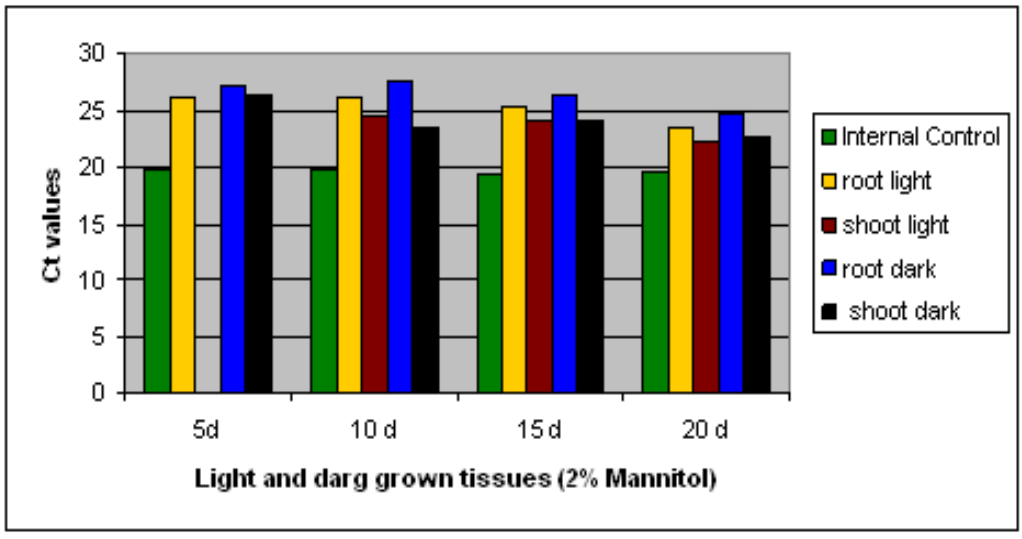


Fig 4B.3b: Relative expression of POX transcript in terms of Ct value in different plant tissues of different age *L. leucocephala* seedlings under light and dark conditions with 2% mannitol stress. On X-axis is tissue (root and stem) with age of seedling. On Y-axis is quantity in terms of Ct value. The numbers 5d, 10d, 15d and 20d represents age of seedling in days

4.11.1.4 Real Time PCR analysis for spatio-temporal *POX* expression pattern in developing shoots and root of *L. leucocephala* grown under light and dark (Fig 4B.4a**) with 100 mM NaCl as salt stress.**

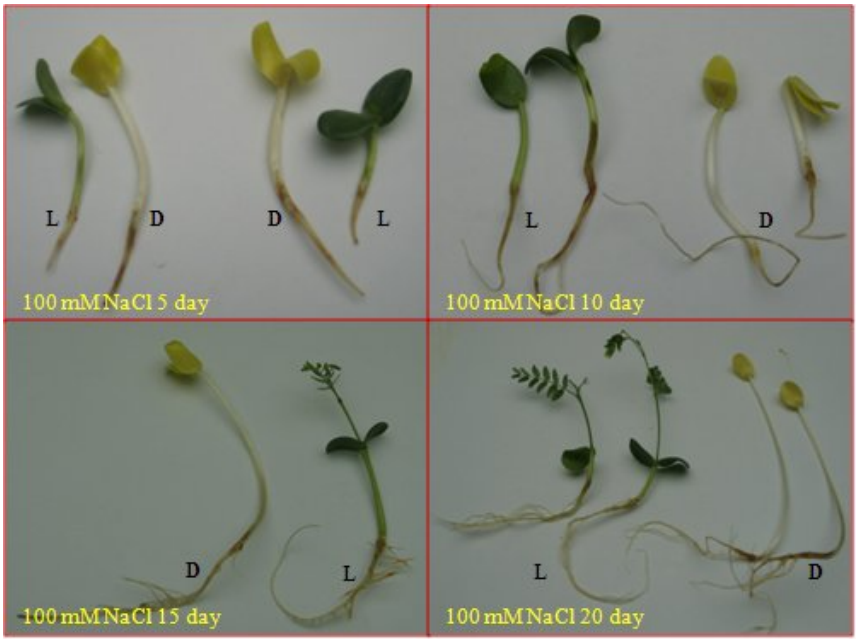


Fig 4B.4a: *Leucaena leucocephala* seedlings of 5day, 10 day, 15 day and 20 day grown under light and dark conditions with 100 mM NaCl as salt stress. "L"-stands for light grown, "D"- stands for dark grown plants

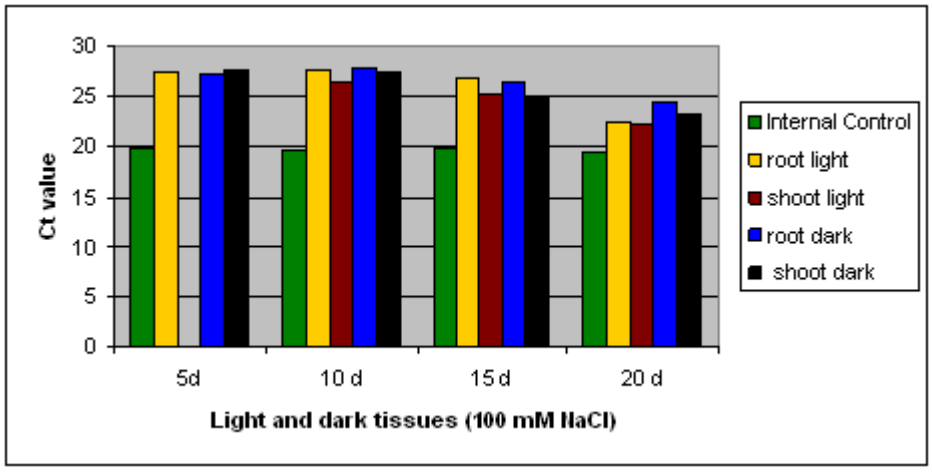


Fig 4B.4b: Relative expression of POX transcript in terms of Ct value in different plant tissues of different age *L. leucocephala* seedlings under light and dark conditions with 100 mM NaCl stress. On X-axis is tissue (root and stem) with age of seedling. On Y-axis is quantity in terms of Ct value. The numbers 5d, 10d, 15d and 20d represents age of seedling in days

For 100 mM NaCl, light and dark grown plants the highest expression of *POX* was observed in 20 day light grown shoot (Fig 4B.4b), followed by 20 day light grown root and 20 day dark grown shoot. Lowest expression was noticed in 10 day dark grown root (Fig 4B.4b). Based on $2^{-\Delta\Delta Ct}$ values, the highest expression obtained for *POX* was for 20 day light grown shoot; which showed 23.89 fold expression in comparison with least expressed 10 day old dark grown root (Fig 4B.4b).

4.11.1.5 Real Time PCR analysis for spatio-temporal *POX* expression pattern in developing shoots and root of *L. leucocephala* grown under light and dark (Fig 4B.5a**) with 200 mM NaCl as salt stress.**

Fig 4B.5a: *Leucaena leucocephala* seedlings of 5day, 10 day, 15 day and 20 day grown under light and dark conditions with 200 mM NaCl as salt stress. "L"-stands for light grown, "D"- stands for dark grown plants

For 200 mM NaCl, light and dark grown plants the highest expression of *POX* was observed in 20 day light grown shoot and root (Fig 4B.5b), followed by 20 day dark grown shoot. Lowest expression was noticed in 10 day light grown root (Fig 4B.5b). Based on $2^{-\Delta\Delta Ct}$ values, the highest expression obtained for *POX* transcript was for 20 day

light grown shoot; which showed 18.7 fold expression when compared with the lowest expressed 10 day old dark grown root (Fig 4B.5b).

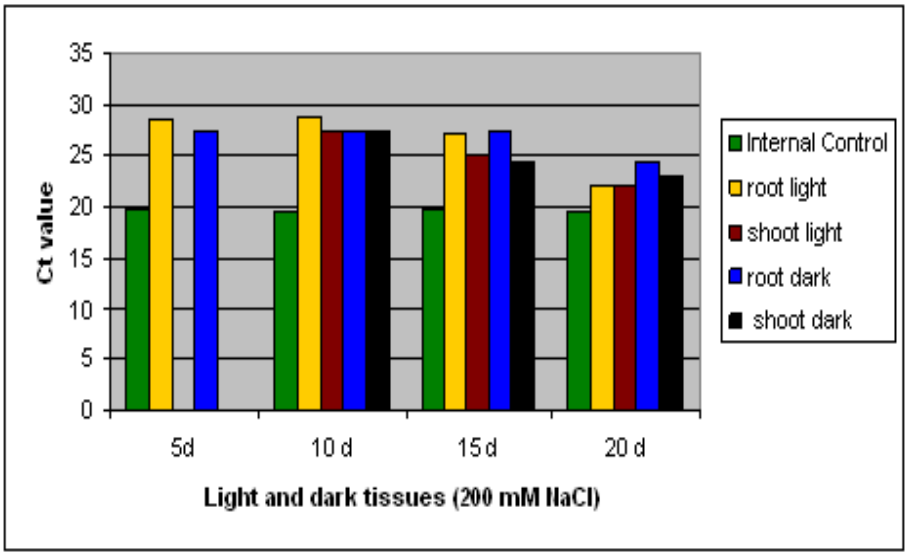


Fig 4B.5b: Relative expression of POX transcript in terms of Ct value in different plant tissues of different age *L. leucocephala* seedlings under light and dark conditions with 200 mM NaCl stress. On X-axis is tissue (root and stem) with age of seedling. On Y-axis is quantity in terms of Ct value. The numbers 5d, 10d, 15d and 20d represents age of seedling in days

4.11.2 Temporal expression pattern of *POX in Leucaena* seedlings, ELISA analysis

A standard graph for ELISA analysis was made using serial dilution of purified POX protein, which revealed that an OD_{405} of 0.0256 ± 0.0088 corresponds to 1 ng of purified POX protein (Fig 4B.6). Graph was made using Graph Pad Prism software.

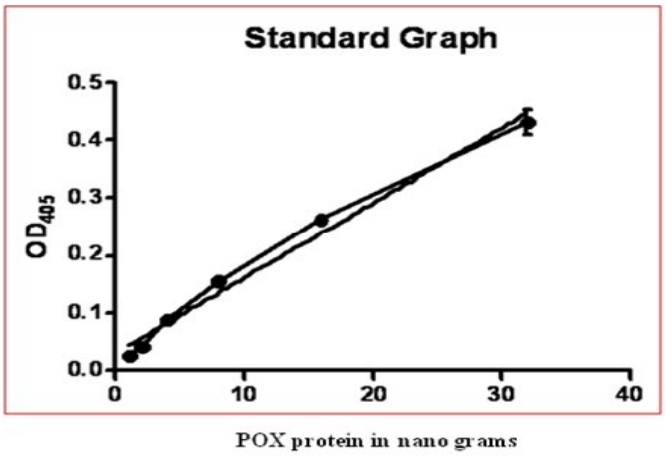


Fig 4B.6: Standard graph: An OD_{405} of 0.0372 ± 0.0068 represents 1 ng of extractable POX protein

In case of root samples, 15 day old root (R 15) (Fig 4B.7) showed maximum amount of extractable POX protein, followed by detectable amounts of proteins in 5 day old root (R 5), 20 day (R20), 10 day (R 10), 30 day (R 30) and zero day ((R 0) - corresponds to radical axis after scarification and imbibitions on the day of inoculation).

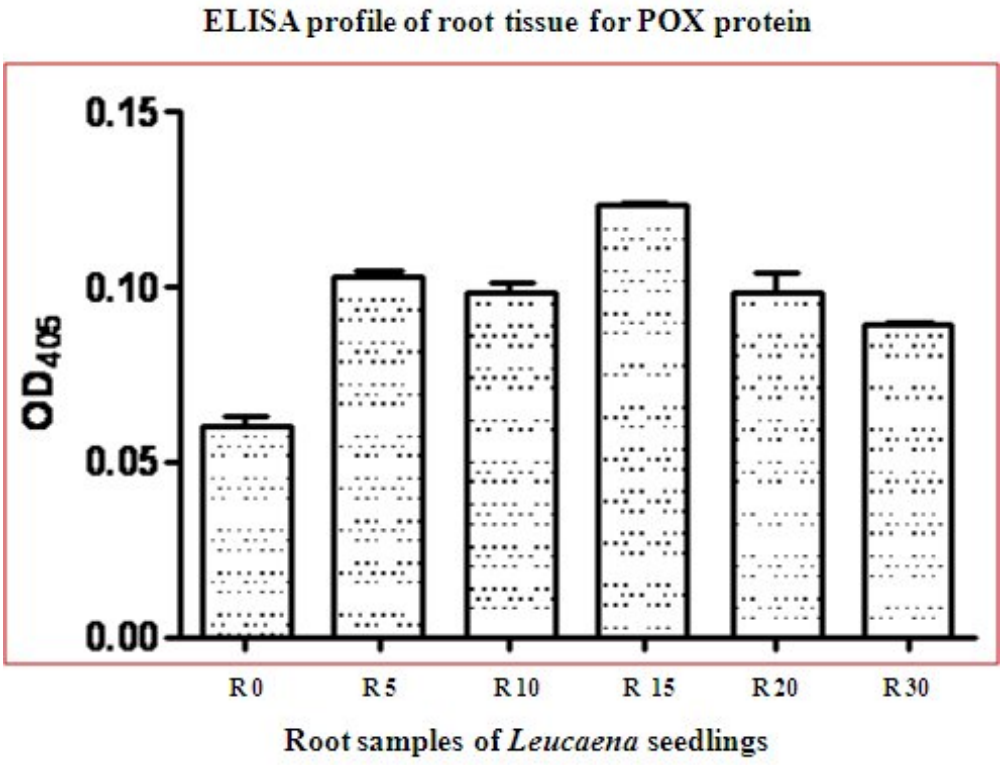


Fig 4B.7: ELISA profile for temporal expression of extracted POX protein in growing seedlings of *L. leucocephala*: Values on y-axis represent O.D at 405 nm in ELISA assay using PNPP (*p*-Nitro phenyl phosphate) as substrate. It can be directly correlated with amount of extractable POX protein in the tissue. R0, R 5, R 10, R 15, R 20, R 30 are 0-30 day old developing root tissues. All values are plotted with standard deviation taken into account.

In case of shoots/stem samples, expression of extractable POX protein was highest in case of S0 (0 day stem sample) and lowest in S 5 (Stem 5 day old) and S10 (10 day old stem) followed by S 20 (20 day old stem) and S 30 (30 day old stem) Fig 4B.8.

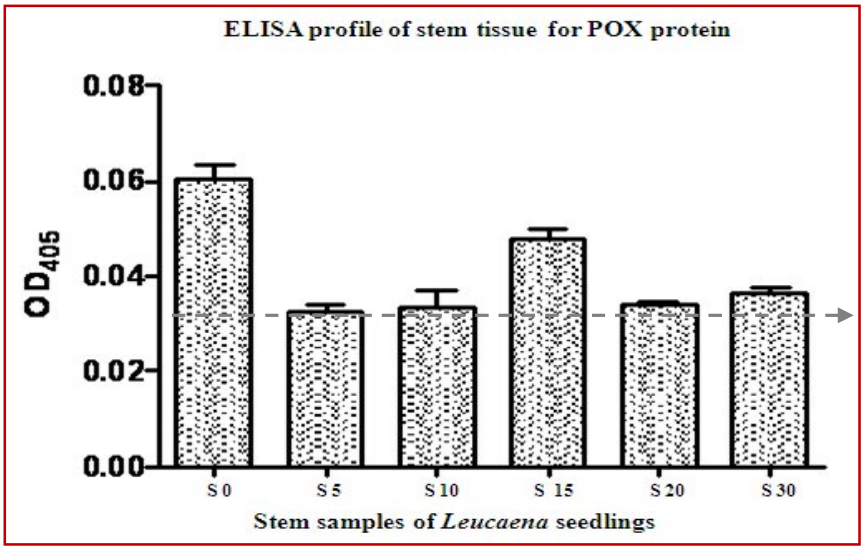


Fig 4B.8: ELISA profile for temporal expression of extracted POX protein in growing seedlings of *L. leucocephala*: Values on y-axis represent O.D at 405 nm in ELISA assay using pNPP (*p*-Nitro phenyl phosphate) as substrate. It can be directly correlated with amount of extractable POX protein in the tissue. S0, S 5, S 10, S 15, S 20, and S 30 are 0-30 day old developing stem tissues. All values are plotted with standard deviation taken into account.

4.11.3 Immuno-cytolocalization of POX in developing seedlings of *Leucaena leucocephala*

POX 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 POX protein near the sites of lignification (Fig 4B.9A, 4B.9B, 4B.9C and 4B.9D). Comparison of the sections where POX was immuno-localized, was done with lignin staining using phloroglucinol-HCl; the stained sections of 5 day and 15 day old root and stem samples at low and high magnification clearly shows lignification at the sites were POX was immuno-localized.

In root and stem as the xylem tissue develops, the expression of POX protein is also found increasing; correspondingly the extent of lignification was also found increasing in the order 5 day to 20 day stage, very well reflected by immunolocalization of POX protein and lignin staining by phloroglucinol-HCl shown in Fig 4B.9A to Fig 4B.9D. In transverse sections of root and stem xylem and phloem fibers shows presence of POX protein and lignin (stained tissues), which is clearly absent in pith tissue and cortex.

Images under higher magnification clearly show presence of POX protein and corresponding lignin deposition in cell wall of xylem tissue.

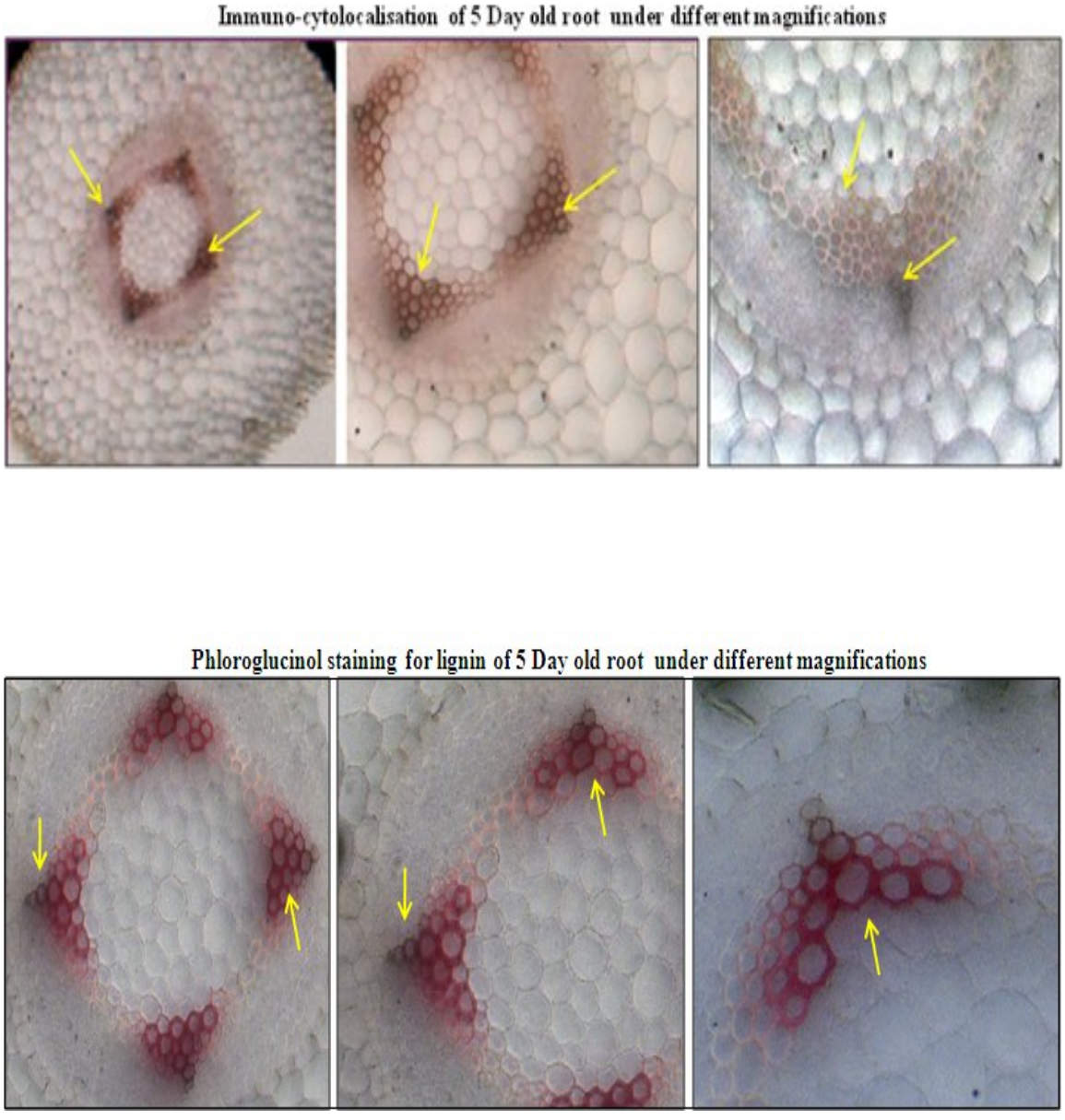


Fig 4B.9A: Immuno-cytolocalization and Phloroglucinol stained sections of POX protein in 5 day old root of *L. leucocephala*: Arrows denotes regions of POX localization and corresponding lignifications

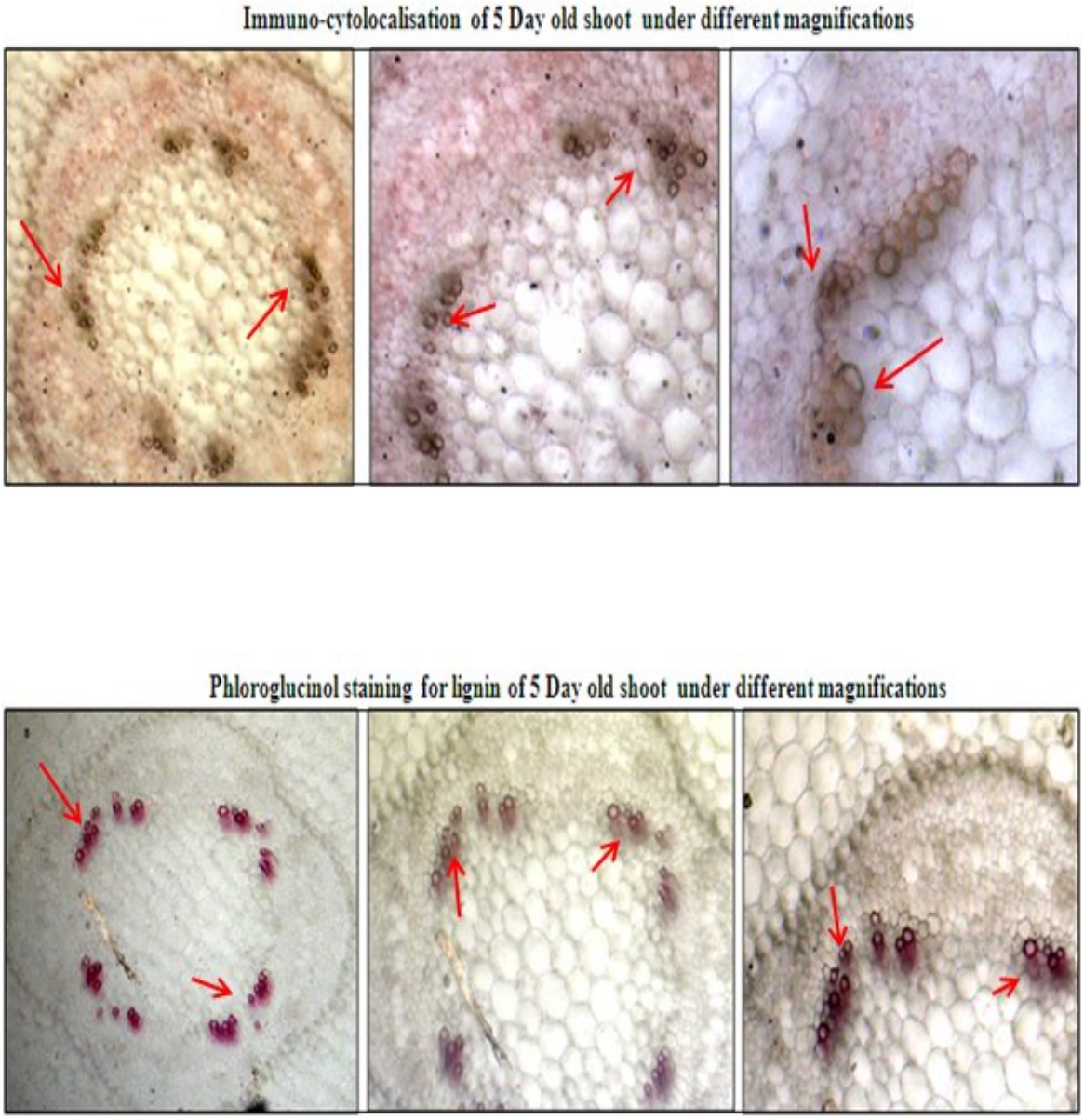


Fig 4B.9B: Immuno-cytolocalization and Phloroglucinol stained sections of POX protein in 5 day old stem of *L. leucocephala*: Arrows denotes regions of POX localization and corresponding lignifications

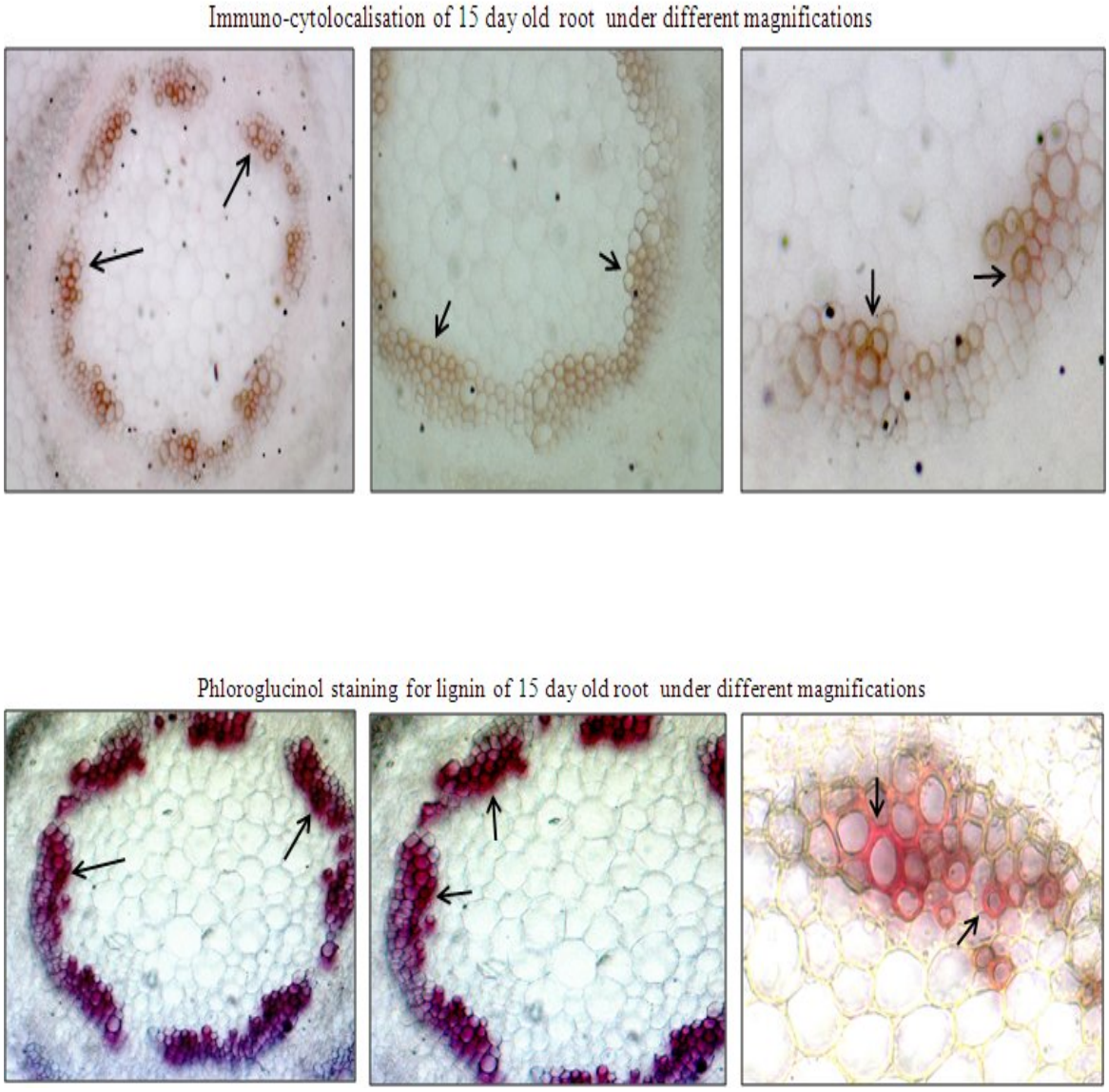


Fig 4B.9C: Immuno-cytolocalization and Phloroglucinol stained sections of POX protein in 15 day old root of *L. leucocephala*: Arrows denotes regions of POX localization and corresponding lignifications

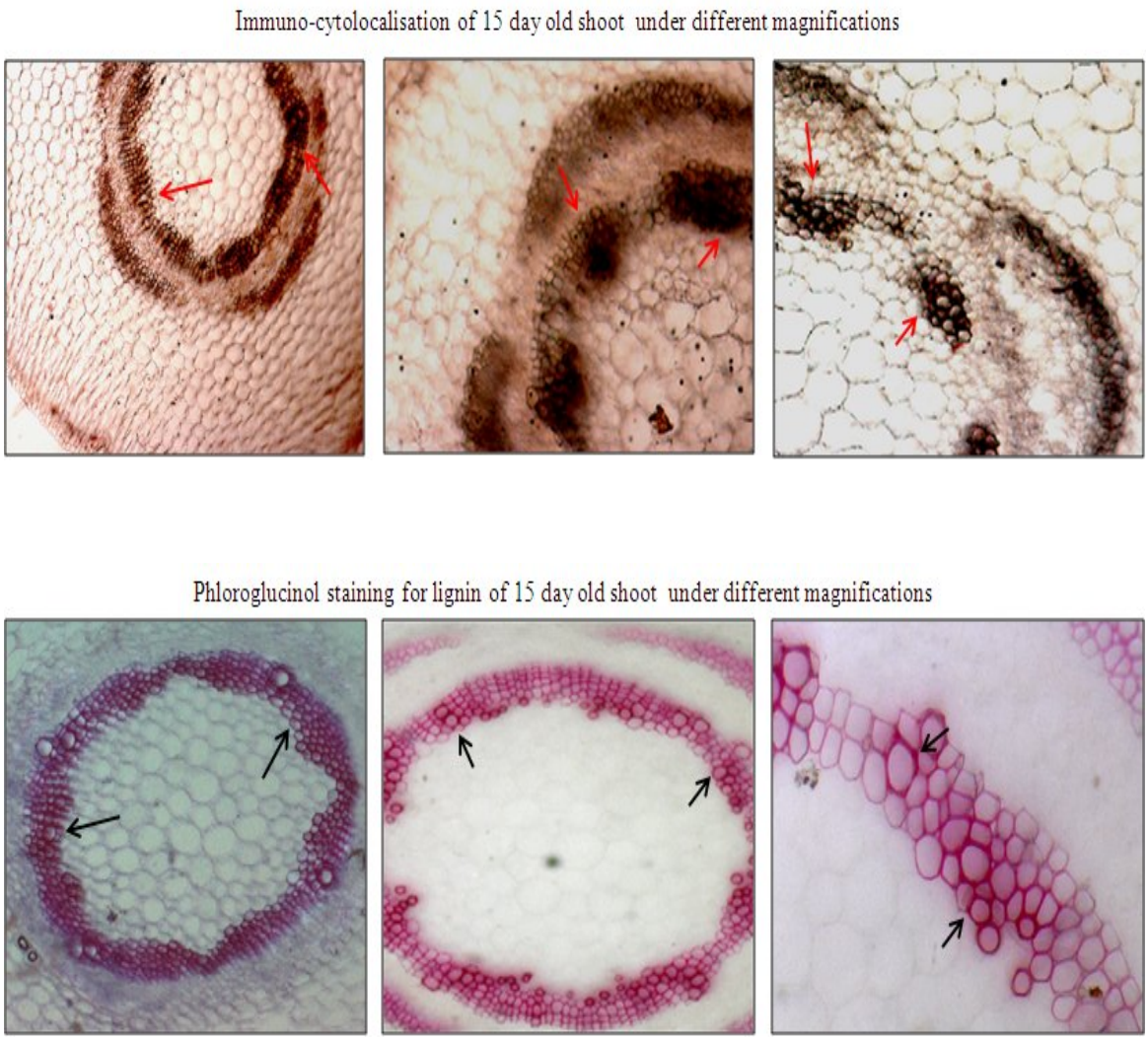


Fig 4B.9D: Immuno-cytolocalization and Phloroglucinol stained sections of POX protein in 15 day old stem of *L. leucocephala*: Arrows denotes regions of POX localization and corresponding lignification

4.11.4 FTIR analysis

FTIR analyses of 10 day and 15 day old root and stem of developing seedlings were done. FTIR analysis revealed that there is no qualitative difference in lignin composition of 10 & 15 day old developing stem (Fig 4B.10A) and 10 & 15 day old developing root (Fig 4B.10B). FTIR scan of commercial lignin was also performed to characterize signature peaks of Lignin (Fig 4B.10A and B). Peaks with wavenumber 1040 cm^{-1} denote dialkyl ether linkage (between cinnamyl alcohol subunits), peak 1270 cm^{-1} corresponds to C-O stretching, aromatic (methoxy), peak 1595 cm^{-1} is aromatic ring with C=O stretching and peak 1510 cm^{-1} represents aromatic ring with C-O stretching (MacKay *et al.,* 1997).

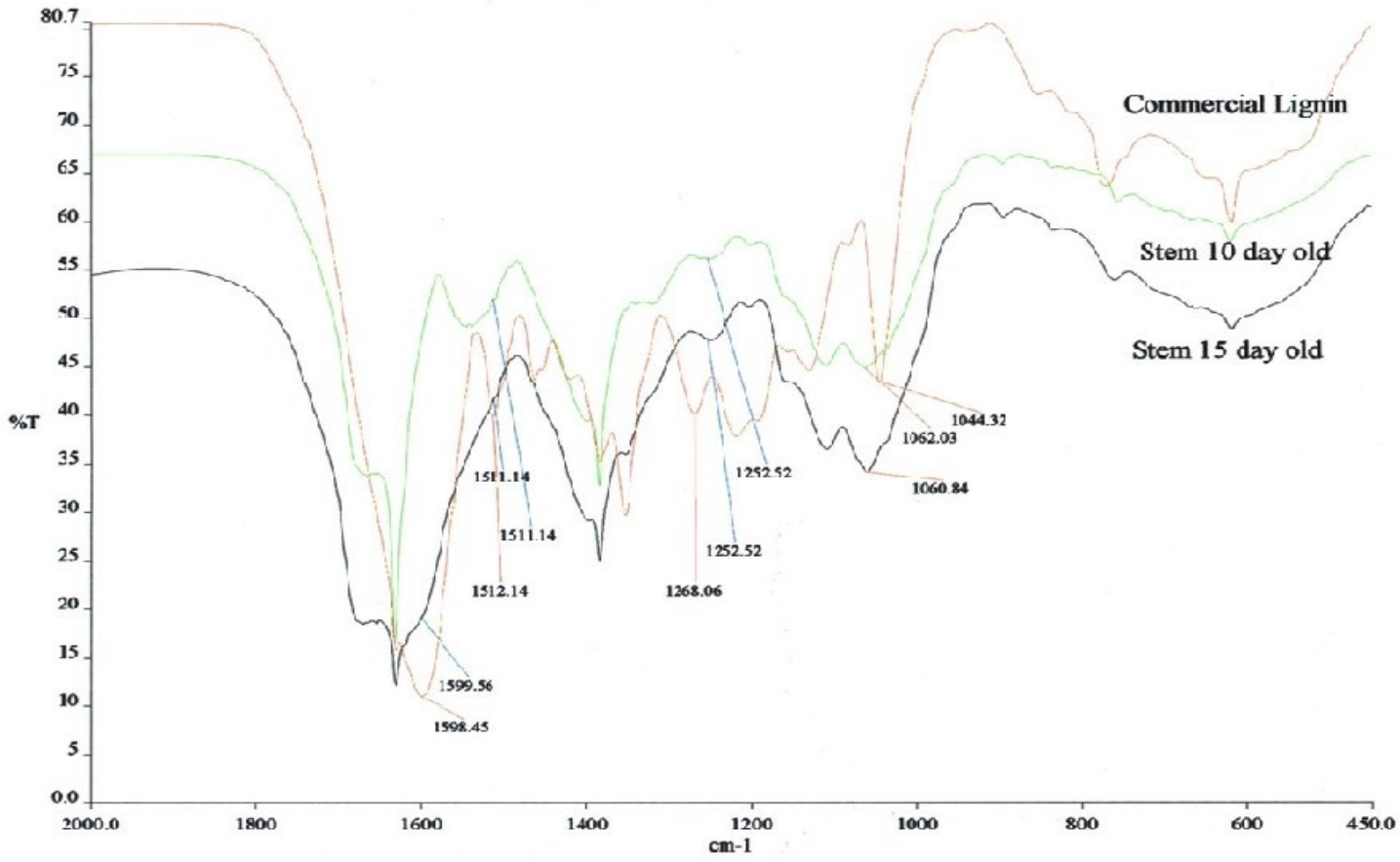


Fig 4B.10A: FTIR analysis of 10 & 15 day old developing stem: Peaks with wave number in the range of 1595 to 1599 cm^{-1}, 1040 to 1062 cm^{-1}, 1250 to 1270 cm^{-1} and 1510 to 1512 cm^{-1} are characterized to be signature peaks of monolignols and lignin. Signature peaks of commercial lignin are 1044 cm^{-1}, 1268 cm^{-1}, 1512 cm^{-1} and 1598 cm^{-1}.

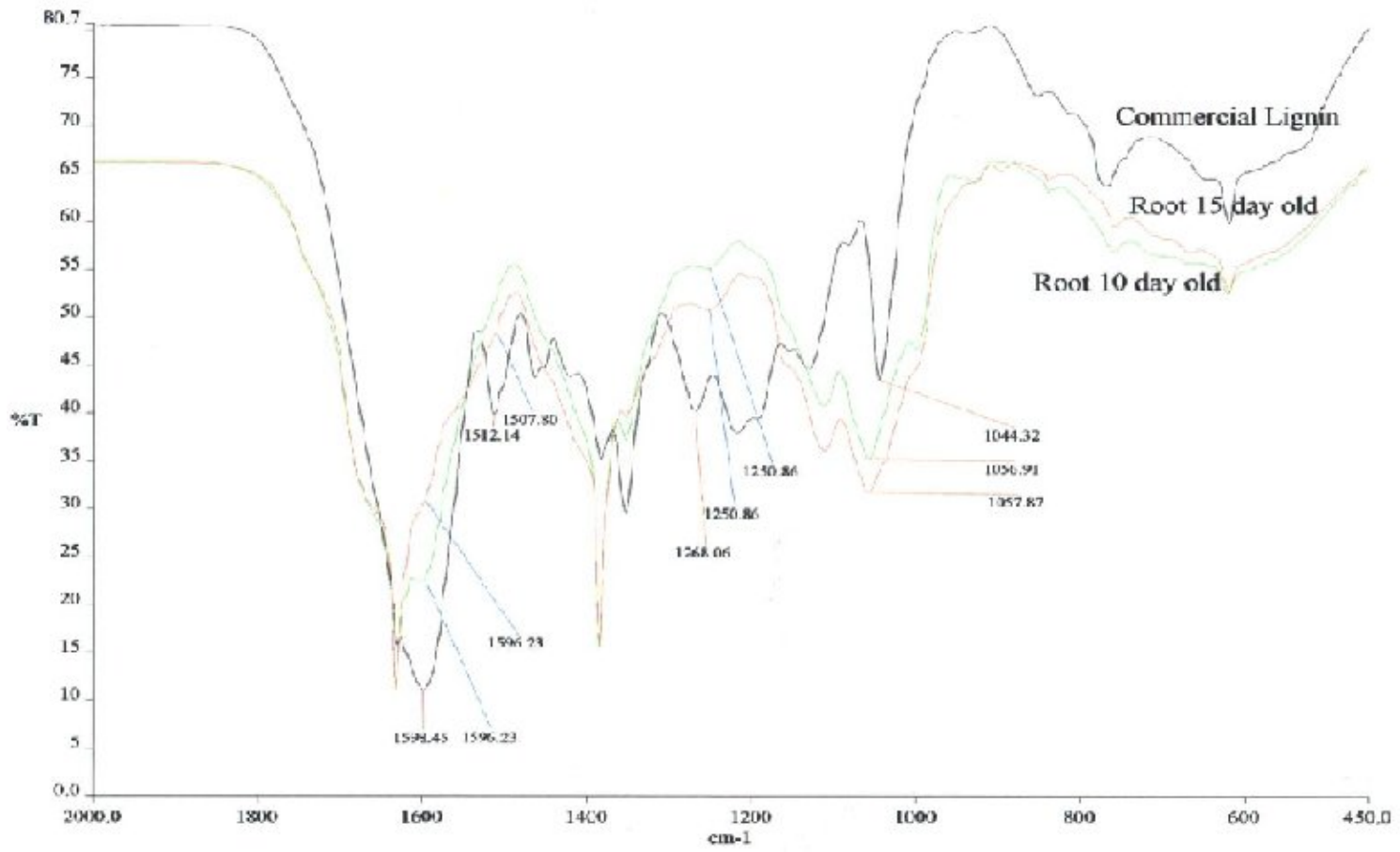


Fig 4B.10B: FTIR analysis of 10 & 15 day old developing root: Peaks with wave number in the range of 1595 to 1599 cm^{-1}, 1040 to 1057 cm^{-1}, 1250 to 1270 cm^{-1} and 1507 to 1512 cm^{-1} are characterized to be signature peaks of monolignols and lignin. Signature peaks of commercial lignin are1044 cm^{-1}, 1268 cm^{-1}, 1512 cm^{-1} and 1598 cm^{-1}

4.12 Discussion

In this report, spatio-temporal expression of the lignin biosynthetic *POX* gene in *Leucaena leucocephala* has been investigated in stem and root for the first time. The expression pattern of *POX* was monitored from 5to 20 day of growing seedlings using real-time PCR to throw some light on the role of *POX* gene in plant development (Section 4.11.1). As the plant grows from seedling, the level of expression of *POX* gene also increases and this can be directly correlated to the developing xylem tissue inside the vascular bundle. Reports have shown that *POX* gene is expressed mostly in tissues undergoing active lignifications and in case of *Leucaena POX* activity increases from 0-20 day (stem and root) as lignification is also in progress at this time. Taking cognizance of the above data of spatio-temporal expression profile, it can be suggested that *POX* is involved in constitutive lignification in developing tissues of developing seedling.

The expression of *POX* transcripts during stem and root development was found paralleled with extractable POX enzyme quantity using ELISA based technique. It was also found that the increase of *POX* gene expression is not in proportion to the increase in Klason lignin (data not shown). This can be explained as follows: mRNA levels and quantity of protein just reflect a time-point value when measured in plant tissues, while Klason lignin is accumulated progressively. Once lignin is deposited in plant tissues, it cannot be converted or degraded. For this reason, it is often seen that the higher expression activity of lignin biosynthetic gene just leads to a small increase in lignin content (Ma, 2007). The almost exclusive expression of our candidate *POX* in stem and root tissues suggests that it is involved in lignification in developing tissues. To date, however, there is little information at the molecular level as to how the regulations of lignin synthesis affects stem and root development. Hence this information could be helpful in predicting the role of lignin in stem and root development in future.

Regarding the expression of the *POX* gene, it was revealed from the study conducted on 0 day to 20 day old seedlings that, the level of expression generally increased with the age of the seedlings both in light and dark grown plants (without any other stress; section 4.11.1.1). The highest expression was noticed in 15 day old dark grown shoot and lowest

was in 5 day old dark grown root. That is, when represented in terms of fold expression the 15 day old dark grown shoot showed 18.25 fold expression over 5 day old dark grown root.

When mannitol was used to induce water-stress in the experimental seedlings, it was found that the expression of *POX* gene getting increased. Initially, with 1% mannitol (section 4.11.1.2) the 20 day old dark grown shoot showed 36.25 fold expression over the least expressed 5 day old dark grown root. When stress was increased using 2% mannitol (section 4.11.1.3), the 20 day old light grown shoot showed highest level of expression, followed by 20 day dark grown shoot. The 20 day old light grown shoot showed 14.32 fold expression, over the least expressed 5 day old dark grown root.

Similarly, when NaCl was used to induce salt stress, it was observed that with 100 mM NaCl as stress (section 4.11.1.4), the 20 day old light grown shoot showed highest expression of 23.89 fold expression over the lowest expressed 10 day old dark grown root. When stress was increased using 200 mM NaCl (section 4.11.1.5) the result observed was 20 day old light grown shoot showed 18.7 fold expression as compared with the lowest expressed 10 day old dark grown root

FTIR is a technique, which is mostly used to characterize chemical molecules. Peaks registered in FTIR scanning correspond to some special character of molecule like functional group, double bond, C-H bond, C-O bond *etc*. Peaks generated in lignin samples are also characterized by presence of aromatic group, O-H group, double bond, *etc*. This information was utilized to analyse quality of lignin in developing seedlings of *L. leucocephala.* It was found that quality of lignin did not change in 10 day and 15 day root and stem samples. Most peaks such as 1595 cm^{-1}, 1512 cm^{-1}, 1270 cm^{-1} and 1040 cm^{-1} were common in all samples which suggest that similar quality of lignin is present in 10 and 15 day developing root and stem sample.

4.13 Conclusion

Spatio-temporal expression profile was investigated using real-time PCR analysis. It was found that *POX* gene transcript was expressed in developing stem and root specifically in xylem tissue. Extractable POX protein from developing seedlings were harvested at same point of time as in case of real-time PCR experiment, which also revealed the same pattern of *POX* gene expression. POX protein was immuno-cytolocalized in tissues which are undergoing lignification. Taking cognizance of above expression pattern and localization of POX protein near lignifying tissue it can be stated that cDNA isolated and characterized in Chapter Three of this thesis have definite role in lignin biosynthesis in *Leucaena leucocephala*.

CHAPTER 5

5. Transformation of *Leucaena leucocephala* with peroxidase gene (*LlPOX*) and its analysis

This chapter includes the different strategies used to genetically modify the plant systems. The plant transformation vector pCAMBIA1301 and 2301 harbouring the *L. leucocephala* peroxidase gene (*LlPOX*) in antisense orientation along with the reporter gene/scorable marker *GUS* were used for the study. Synthesis of a composite carbon-gold nano particles and its efficient use as micro-carriers is also dealt in detail in this section. This composite carbon-gold nano particles denoted as HTC 600-Au (heat treated carbon gold at 600 °C) having gold nanoparticles embedded in sharp graphite like carbonaceous supports showed DNA delivery to model plant *Nicotiana tabacum* that was further taken to monocot *Oryza sativa* and a hardy dicot tree species *Leucaena leucocephala*. Two different strategies *i.e.* particle bombardment and particle bombardment followed by co-cultivation with *Agrobacterium* is described in detail in this chapter. Evaluation and analysis of putative transgenic plants and confirmation of integration of these genes in *L. leucocephala* genome by GUS assay and by molecular techniques (like PCR, DNA sequencing and slot blot) is performed. Further these transgenics were subjected to ELISA and immuno-cytolocalization studies. This present study shows nano gold immobilized on sharp edge carbon, which can be synthesized with minimal energy through biological approach**,** proved to be a better plant transformation abiotic carrier over the micron gold used in classical biolistic/gene-gun approach.

5.1 Introduction (general aspects of genetic transformation of trees)

Worldwide annual production of paper has increased more than three fold in the past forty years, amounting to a total production of 330 million tonnes (FAO, Forest report 2003, http://maps.grida.no/go/graphic/paperandpaperboardproduction). In India, the annual production of paper is close to 5 million tonnes. India's annual soft wood and pulp imports are worth US $ 300 million and the projected annual short fall of paper and paper products is expected to grow to approximately 4 million tonnes by the year 2010. Besides, the growth of paper and paper product consumption in India is expected to be the highest in Asia during the current decade. Thus, it has become essential to be self sufficient in paper and pulp production, which may be achieved with the development of

fast growing trees that provide higher biomass with low lignin content per unit of land. As discussed in Chapter Two; paper industry in India mainly uses bamboo, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena* sp. as a source of raw material for paper and pulp production. Though all these species are important to the paper industry, *Leucaena* sp. is extensively used in India because of its easy adaptation to a wide range of agro-climatic conditions and high rate of biomass production and hence about 25% of raw material comes from this genus.

The undesirable component for paper and pulp industry is lignin. In *Leucaena* sp. its content ranges from 17 to 30% of total biomass. Lignins, second to cellulose in abundance, are conventionally considered to be polymers of three alcohol monomers, or monolignols: *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) monomers differing in their degree of methoxylation (Boerjan *et al.,* 2003; Freudenberg and Neish, 1968; Baucher *et al.,* 2003 and Lewis *et al.*, 1990). Each type of precursor may form several types of bonds with other precursors in forming a lignin polymer (Whetten *et al.*, 1998). Lignin is closely linked to cellulose and hemicelluloses. In plants, lignin is mainly deposited in the walls of tracheary elements, sclerenchyma, phloem fibres, and periderm. This imparts firmness and structural support to the wall and assists in the transport of water and nutrients within xylem tissue by decreasing the permeability of the cell wall (Baucher *et al.*, 2003; Humphreys *et al.,* 2002; Lewis *et al*., 1990; Hahlbrock and Scheel, 1989).

Out of the three monomer (H, G and S) units of lignin; S and G lignins are found largely in angiosperms. A higher S/G ratio is desirable for paper industry, as S lignin is less compact (higher degree of methoxylation) than G lignin. Higher S/G ratio facilitates the removal of total lignin in pulp making process.

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 have included both sense and antisense strategies (referring to the orientation of the introduced gene into the plant genome) (Strauss *et al.,* 1995; Baucher *et al.,* 1998) and RNAi technology (Merkle & Nairn, 2005). In the antisense

strategy, duplex formation between the antisense transgene and the endogenous gene transcripts is proposed to induce the degradation of duplexes and correspondingly, lead to suppressed gene expression (Strauss *et al.,* 1995). The sense strategy was originally targeted for overexpression of the genes but, as originally observed through the introduction of chalcone synthase transgene into petunia (Napoli *et al.,* 1990), the sense strategy may also lead to silencing (down-regulation) of both the endogene and the transgene due to co-suppression (i.e. post-transcriptional gene silencing, PTGS). The molecular mechanism of the gene silencing was unclear for long time until the discovery of RNA interference (RNAi) (Yu & Kumar, 2003: Matthew, 2004; Chen, 2005; Bonnet *et al.,* 2006; Zhang *et al.,* 2006). In the RNAi silencing process, the transgene gives rise to long double-stranded (ds) RNA molecules, which are enzymatically cleaved into very small pieces of RNA (21 nt), referred to as small interfering RNAs (siRNAs). siRNAs are then incorporated in an RNA silencing system (RISC: RNA induced silencing complex) which is able to recognize, bind and induce cleavage or translation repression of complementary mRNAs (Bonnet *et al.,* 2006; Zhang *et al.,* 2006). The RNAi technique is currently being applied for the efficient production of down-regulated or knockout plants (Wesley *et al.,* 2001), e.g. in genetic transformation of *Betula pendula* for achieving sterility (Lannenpaa, 2005).

5.1.1 Nanogold loaded sharp edged carbon bullets (HTC 600-Au), as Gene carrier for plant genetic engineering

We present our material, basically with the sharp sp^2 carbon to make the injection. This sharp matrix supports gold nanoparticles giving the platform for the DNA to bind and provide minimum density for the carrier, to gain threshold velocity in the gene gun to pierce the hard cell wall in plant systems. The particles were prepared by inert heating of the fungus that produces intracellular gold nanoparticles, which also have no application otherwise. Intracellular synthesis of nanoparticles was first reported by Beveridge *et al*., followed by Klaus *et al.,* 1999 in bacteria. Subsequently, screening and documentation of several eukaryotic fungal genera especially *Verticellium sp.* and actinomycetes for intracellular nanoparticle synthesis were done extensively by the groups at National Chemical Laboratory, India. The extracellular synthesis of nanoparticles with *Fusarium*

oxysporum and *Trichothecium sp.* was also well studied with various combinations of metal ions to produce different metal and metal oxide nanoparticles. In this context, the extracellular synthesis masked further research on intracellular nanoparticles as finding an application for the intracellular synthesis where nanoparticles are entangled in cellular matrix looked very difficult.

We envisaged that, simple heat-treatment under inert conditions would convert biomatrix into a carbonaceous support with embedded nanoparticles. The obtained product was grained and used as potential carrier for gene delivery. Since the material formed by heating the intracellular nanoparticles get sand-witched and clamped well in the carbon mask, it can withstand the shattering in the DNA coating procedure. Otherwise, the inert carbon cannot be bonded to the gold to withstand sonication and high pressure involved in the DNA coating and the impact of gene delivery process respectively.

Further advantage of these particles are, first higher gold surface area to gold used, hence more gene cargo can be handled. Since the gold is known to support high DNA packing density that will give better transformation efficiency and low nuclease degradation. Second, graphite like carbon with the sharp edge can place the gene piercing the hard plant cell wall and nuclear membrane into the chromosomal site. The gene gun plant transformation creates hard puncture that requires the young embryo or the callus to be placed in the dark for the DNA to get integrated and for the wound to get healed. Where our composite nano material shows complementary effect with carbon, which is sharp and biocompatible leading to minimal injury and rapid wound healing to individual cells and thereby, leading to higher survival rates of the bombarded cells.

5.1.2 *Agrobacterium* mediated plant transformation

The natural ability of the soil microorganism *Agrobacterium* to transform plants is exploited in the *Agrobacterium*-mediated transformation method. During the infection process, a specific segment of the plasmid vector, T-DNA, is transferred from the bacterium to the host plant cells and integrates into the nuclear genome.

5.1.3 Biology and life cycle of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens is a gram negative soil inhabiting bacteria that causes, crown gall disease in a wide range of dicotyledonous plants, especially in members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses. The strain, biovar 3, causes crown gall of grapevine. Although, this disease reduces the marketability of nursery stock, it usually does not cause serious damage to older plants. *Agrobacterium* infection was first described by Smith and Townsend in 1907. The bacterium transfers part of its DNA to the plant, and this DNA integrates into the plant's genome, causing the production of tumors and associated changes in plant metabolism. The unique mode of action of *A. tumefaciens* has enabled this bacterium to be used as a tool in plant transformation. Desired genes, such as insecticidal or fungicidal toxin genes or herbicide-resistance genes, can be engineered into the bacterial T-DNA and thereby inserted into a plant. The use of *Agrobacterium* allows entirely new genes to be engineered into crop plants. *Agrobacterium*-mediated gene transfer is known to be a method of choice for the production of transgenic plants with a low copy number of introduced genes (Hiei *et al.,* 1997).

5.1.4 Infection process

Agrobacterium tumefaciens infects the plants through wounds, either naturally occurring or caused by transplanting of seedlings and nursery stock. In natural conditions, the motile cells of *A. tumefaciens* are attracted to wound sites by chemotaxis. This is partly a response to the release of sugars and other common root components. Strains that contain the Ti plasmid respond more strongly, because they recognise wound phenolic compounds like acetosyringone even at very low concentrations (10^{-7}M). Acetosyringone plays a further role in the infection process by activating the virulence genes (*Vir* genes) on the Ti plasmid at higher concentrations (10^{-5} to 10^{-4} M). These genes coordinate the infection process. It is important to note that only a small part of the plasmid (T-DNA) enters the plant and the rest of the plasmid remains in the bacterium to serve further roles. When integrated into the plant genome, the genes on the T-DNA code for auxins, cytokinins and synthesis and release of novel plant metabolites (opines and agrocinopines). These plant hormones upset the normal balance of cell division leading

to the production of galls. Opines are unique aminoacid derivatives and the agrocinopines are unique phosphorylated sugar derivatives. All these compounds can be used by the bacterium as the sole carbon and energy source.

5.1.5 Markers for plant transformation

5.1.5.1 Selectable markers

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. Another selectable marker gene, phosphomanoisomerase(pmi), which catalyzes mannose-6phosphate to fructose-6-phosphate, an intermediate of glycolysis that positively supports growth of transformed cells, is also recently being used. Mannose absorbed by the plant cells converts into mannose-6- phosphate, an inhibitor of glycolysis, inhibits growth and development of non-transformed cells. Transformed cells having PMI gene can utilize mannose as a carbon source.

5.1.5.2 Screenable markers

The oncogenes of *Agrobacterium* are replaced by reporter/screenable marker genes like *ß-glucuronidase* gene (*gus*) (Baribault *et al.,* 1990), *luciferase (luc)*gene for analyzing gene expression. Since the first demonstration of the *green fluorescent protein (gfp)* gene from jellyfish *Aequorea victiria* as a marker gene (Chalfie *et al.,* 1994), *gfp* has attracted increasing interest and is considered advantageous over other visual marker genes. Unlike other reporter proteins, GFP expression can be monitored in living cells and tissues in a non-destructive manner. This gene has been used as a visible reporter gene in genetic transformation of both monocots and dicots (Haseloff *et al*., 1997; Reichel *et al*., 1996; Kaeppler and Carlson, 2000). The fluorescence emission of GFP only requires the excitation of living cells by UV or blue light (390 nm strong absorption and 470 nm weak absorption), which results from an internal p-hydroxybenzylideneimidazolinine fluorophore generated by an autocatalytic cyclization and oxidation of a ser-gly sequence at aminoacid residues. The other advantage of *gfp* as a reporter gene is that no

exogenously supplied substrate/ cofactors are needed for its fluorescence emission at 508 nm.

Red fluorescent protein marker (DsRed2, a mutant form of DsRed from *Discosoma* sp.) was first used as a visual reporter gene for transient expression and stable transformation of soybean (Nishizawa *et al*., 2006). DsRed2 fluorescence can be monitored with any fluorescence stereomicroscope equipped with a filter set for excitation at 530–560 nm and emission at 590–650 nm.

5.1.6 Genetic transformation of plants with peroxidase (*LlPOX*) gene(s)

Numerous reports on peroxidase activity or gene expression in lignin forming tissues have been reported, but only a few isoenzymes or genes have been specifically associated with lignification (Sato *et al*.,1993; Quiroga *et al*., 2000; Christensen *et al*., 2001; Marjamaa *et al*., 2006). Although several reports on the transcriptional regulation of monolignol biosynthesis exists, not much is known for peroxidases. It is likely that the control of the whole lignification process requires a mechanism for the co-ordinated expression and/or activation of the monolignol biosynthetic genes/enzymes and the radical forming peroxidases. This was supported by the apparent co-regulation of five peroxidase genes with the 'lignification tool box' genes during *Arabdopsis* inflorescence stem development (Ehlting *et al*., 2005).

Data from transgenic plants down regulated for peroxidase activity has confirmed the role of some peroxidase isoforms in lignin polymerization in tobacco and *Populus sieboldii* (Miq.)X *Populus grandidentata* (Michx.) (Talas-Ogras *et al*., 2001; Blee *et al*., 2003; Li *et al*., 2003b). Both quantitative (up to 50% reduction) and qualitative changes were reported, but no obvious growth phenotypes, other than larger xylem elements were found. Antisense expression of the TP60 peroxidase gene in tobacco resulted in an equal reduction of both G and S units, suggesting the existence of a feed-back regulation to decrease the monolignol synthesis and transportation under reduced oxidative capacity in the apoplast (Blee *et al*., 2003). However, no metabolite analysis other than phloroglucinol staining was performed to confirm that the monolignols or their derivatives did not accumulate in xylem.

In aspen, down-regulation of the PRXA3a gene reduced the lignin content by 20%. Incorporation of G units into lignin decreased while S units remained at the wild type level (Li *et al*., 2003b). This is not surprising, as most peroxidases are likely to be inefficient in SA oxidation due to structural constraints (Ostergaard *et al*., 2000 ; Nielsen *et al*., 2001). However, peroxidases from, e.g., *Populus alba (L.) and Z.elegans* were shown to oxidize SA efficiently (Sasaki *et al*., 2004; Gabaldon *et al*., 2005). The Populus peroxidase, CWPO-C, was immunolocalized into the middle lamella and cell corners of poplar xylem fiber walls, coinciding partly with S type lignin. Interestingly, CWPO-C was also found in the cytosol of ray parenchyma cells, suggesting that ray parenchyma could provide the fiber middle lamella with CWPO-C (Sasaki *et al*.,2006).

Polymeric lignin rarely fits into the active site of peroxidase; however, CWPO-C was also able to oxidize polymeric lignin (Sasaki *et al*.,2004). In fungal lignin peroxidase from *Phanerochaete chrysosporium,* oxidation of polymeric lignin was shown to take place on the protein surface via long–range electron transfer (Johjima *et al*., 1999). It will be intresting to see if a similar mechanism is functional in secretory plant peroxidases as well. The fact that only 50% reductions at best in lignin amount have been accomplished, argues for redundancy in peroxidase activities. It is likely that *in vivo* several isoenzymes participate in lignin polymerization. Division of labour between the isoenzymes could also exists, both spatially between different cell wall layers and functionally, for example in the radical generation on the free monolignols versus the polymer.

In the present study, the efficacy of our synthesized HTC 600-Au and the conventional micron gold to transfer antisense constructs of the Peroxidase (*LlPOX*) gene efficiently into the monocot system of *Oryza sativa,* dicot model plant *Nicotiana tabacum* and woody leguminous tree *Leucaena leucocephala* were analysed. Transfromants were analyzed using GUS assay, PCR and slot blot.

5.2 Materials and methods

Agar, maltose, glucose, yeast extract, peptone, 0.1% sodium hypochlorite, MS medium (Murashige and Skoog, 1962), were procured from Himedia chemicals. Hydrochloroauric acid ($HAuCl_4$), spermidine and calcium chloride were purchased from Sigma chemicals and used as received. Polyethylene glycol was purchased from Fluka Biochemica. Ethanol (Merck) and reagents required for polymerase chain reaction (PCR) was purchased from Bangalore Genei, India. Deionised Milli-Q® water was used wherever necessary. Gene constructs used were carrying; Class III lignin biosynthetic peroxidase isolated from *Leucaena leucocephala* cloned in anti-sense orientation flanked by CaMV35S promoter and nos terminator in pCAMBIA 1301 and 2301. Isolation and purification of plasmid was carried using Wizard® Plus Minipreps DNA Purification Systems (Promega, Madison, USA) following the manufacturer's instructions.

5.2.1 Instrumental details

The X-ray diffractograms (XRD) were recorded on a PAN analytical Xpert pro machine using a CuKα source at operating conditions of 40 mA and 30 kV at a scan rate of 4 degrees / min. TEM samples of carbon supported HTC 600-Au °C were prepared by placing drops of their aqueous dispersions over amorphous carbon coated copper grids and allowing the solvent to air dry. Transmission Electron Microscopy (TEM) images were recorded using a Technai G^2 F-30 model operated at an accelerating voltage of 300 kV and JEM 2100 instrument operated at 200 kV. Raman spectra were measured in the back scattering configuration using a 514.5-nm Ar laser excitation. The scattered light was analyzed in a Jobin-Yvon HR460 single-grating spectrometer equipped with a charge-coupled array detector and a holographic notch filter (Kaiser Optical Systems, Inc., Ann Arbor, MI). To avoid laser damage to the sample the experiments were conducted at low laser powers (2 W/cm^2). The gold concentrations were measured with a Chemito - Atomic Absorption Spectrometer (AAS) 201 with a gold hollow cathode lamp.

The tissue culture grown explants were transformed using Biolistic-PDS 1000/He system (Bio-Rad Laboratories, Hercules, USA). The eppendorf's Bio Photometer was used for plasmid DNA quantification and to check its purity. Bio-Rad C1000™ Thermal Cycler

was used for PCR based molecular analysis of putative transgenic plants. Typhoon TRIO+ phospho imager was used for scanning Southern blots.

5.2.2 Synthesis and characterization of HTC 600-Au

The fungus isolated from a foundry-polluted area was identified and registered at Indian Type Culture Collection (ITCC) as *Aspergillus ochraceus* no. 6102. This fungus was already known to grow in metal ion rich concenteration. In a typical reaction the isolated fungal spores were inoculated in 500 mL Erlenmeyer flask containing 200 mL of MGYP broth. The spores were allowed to germinate and produce hyphal biomass for three days in a shaker (200 rpm) at 37 °C. It produces 60 g biomass on wet weight basis that was harvested and three rounds of washing (1000 rpm 15 min) were done with autoclaved MilliQ® water under sterile condition. The biomass was then resuspended in 200 mL of 10^{-3} M $HAuCl_4$ followed by incubation in a shaker (200 rpm) for two days at 37 °C. The product biomass was washed with MilliQ® water and heat treated (600 °C for 6 h) in a tubular furnace under nitrogen flow. It gave 400 mg product that was ground finely and characterized. The gold concentration in this sample was analyzed using AAS and found to be ~2.5 wt%. This is denoted as HTC 600-Au (heat treated carbon gold at 600 °C).

While testing DNA transformation efficiency, along with HTC 600-Au, three more controls were used. The first of such control used was the classical 1 μm gold particles. Next control was HTC 600 (heat treated carbon at 600 °C) prepared by calcinations of the pure biomass without challenging it with gold ion. This material wasn't discussed further in text since it was not showing appreciable carrier results. Final control is the extracellular nanoparticles (synthesized by making 10^{-3} M $HAuCl_4$ in 200 mL extracellular extract collected by incubating the fungus in sterilized distilled water).

To investigate how efficiently this nano gold immobilized sharp edged carbon can genetically modify plant species, studies were conducted on the model plant *Nicotiana tabacum*, rice (*Oryza sativa*), which forms staple food for one third of world population especially in East and South-East Asia (Tang *et al.,* 2001) and a perennial leguminous tree *Leucaena leucocephala* a member of family Fabaceae, which forms 25% of raw material for paper and pulp industry, where in genetic transformation is being attempted with a view to down regulate lignin biosynthesis.

5.2.3 Standardization of plasmid DNA to HTC 600-AU for plant transformation.

To prove this composite material can function as a better DNA delivery agent, the above synthesized carbon-gold nano particles were coated with recombinant binary vector with normal procedure specified for the micron sized gold particles (Bio-Rad laboratories, Hercules, USA). The 1 μm gold particles are very well used as DNA carriers in biolistic approach of plant transformation. Standardized proportion followed in lab with 1μm gold is, 200 μg of microcarrier per bombardment and 600 ng DNA, which resulted on an average 25±7 GUS expressing foci in the immature embryos of *Leucaena leucocephala*. To find at which minimal ratio of DNA to HTC 600-Au exceeds the classical micron carrier in GUS efficiency, HTC 600-Au was kept constant (1mg) with varying the amount of DNA used for coating (200ng, 400ng, 600ng, 800ng, 1000ng and 2000ng). Finally, the DNA coated HTC 600-Au was suspended in absolute ethanol and stored at – 20 ^{o}C prior to use. The transformation events were carried out using Biolistic-PDS 1000/He System after checking the particles adhesion strength using the agarose gel electrophoresis.

5.2.4 Explant

Tobacco **(***N. tabacum* var. Anand 119**)** plants were grown *in vitro* on basal Murashige and Skoog medium (MS medium, 1962) in absence of any plant growth regulators. Fresh leaves from two months old plants were used as explants and were cut into pieces of about 5 mm^2 and inoculated in MS basal medium (pH 5.8) with 1.4 % agar, supplemented with 2% sucrose, 0.8% BA and NAA for callus induction under darkness at 25±2 ^{o}C in a growth incubator.

In case of rice, manually dehusked seeds were surface sterilized with 70% ethanol for 3 min followed by 1.5% (v/v) sodium hypochlorite for 10-15 min and washed 5-7 times with sterile distilled water. Then, the sterilized seeds were plated onto MS medium containing 2.5 mg/L 2,4-D (Callus induction medium) and incubated in dark at 25±2 ^{o}C for 3 weeks.

With a view to show the potential of our material to deliver gene of interest into hardy woody tree species, we selected *L. leucocephala* (Lead tree, white popinac, subabul) a perennial leguminous tree species of commercial importance for our studies. The seeds

were taken out from the pods and were surface-sterilised with 1.5% (v/v) sodium hypochlorite solution for 10-15 min, followed by 4 washes with sterile water. The immature embryos were isolated asceptically using a pair of sharp, sterile forceps and plated with their shoot apex facing up in the centre of a 90 mm diameter Petri plate containing regeneration media (1/2-MS + TDZ (0.5 mg/ L).

5.2.5 *Agrobacterium* strain and plasmids

Agrobacterium tumefaciens strain GV2260 was used. The strain carried recombinant pCAMBIA1301 binary vector, harboring partial sequence of peroxidase (*LlPOX*) gene in antisense orientation under the control of CaMV35S constitutive promoter. This construct was used for rice transformation. This vector has hygromycin *(hpt)* and kanamycin (*npt II*) resistance genes as selection markers for plant and bacterial systems respectively. GV2260 strain carrying recombinant pCAMBIA2301 binary vector, harboring partial Peroxidase (*LlPOX*) gene in antisense orientation and plant selection marker kanamycin was used for *Nicotiana* and *Leucaena* transformation systems.

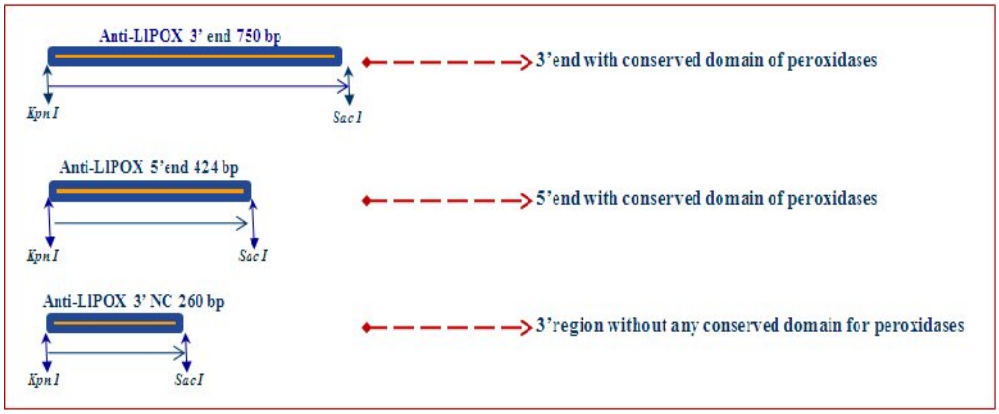
5.2.6 Construction of recombinant binary vector

Three fragments of *Leucaena* peroxidase (*LlPOX*) gene (750 bp, 424 bp and 260 bp), to be cloned in anti-sense orientation was amplified using PCR with restriction sites for *Kpn*I and *Sac*I, cloned (in pGEM-T vector (Promega, USA.)), sequenced and confirmed and they were double digested using these two enzymes (Fig. 5.1a). The released fragments were eluted from agarose gel and cloned in *Kpn*I and *Sac*I digested pCAMBIA 1300 MCS (Fig. 5.1b). The right and left hand T-border of pCAMBIA1300 vector harbours the hygromycin gene (selectable marker) and multiple cloning sites. This vector does not have any reporter gene thus the transformants using this vector cannot be analyzed by reporter gene. To avoid these short comings, pCAMBIA 1301 was used for *Oryza* transformation system and pCAMBIA 2301 vector was used for *Nicotiana* and *Leucaena* transformation systems. The right and left hand T-border of pCAMBIA1301 and pCAMBIA2301 vector harbours the hygromycin gene and kanamycin respectively as plant selectable markers, multiple cloning sites and GUS reporter gene.

The MCS of pCAMBIA1301 and pCAMBIA2301 does not have any promoter and terminator to drive and stop the gene respectively. *Eco*RI and *Hin*dIII restriction sites are present flanking MCS of both pCAMBIA vectors and same sites are also there in pCAMBIA1300 just before the 35S promoter and after the nos terminator, thus the recombinant pCAMBIA1300 was double digested using *Eco*RI and *Hind*III enzymes. The digested cassette **(**Fig. 5.2a) was eluted from agarose gel and cloned in *Eco*RI and *Hind*III digested pCAMBIA1301 vector (Fig. 5.2b) and pCAMBIA2301 vector (Fig. 5.2c).

The constructed vector was transferred in to *E.coli* for multiplication and the integration of gene into the vector was further confirmed by *Kpn* I and *Sac* I digestion of the constructed vector (Fig: 5.3A, 5.3B, 5.3C).

5.1a *LIPOX gene(s)* with KpnI-SacI sites



5.1b Vector map of pCAMBIA 1300

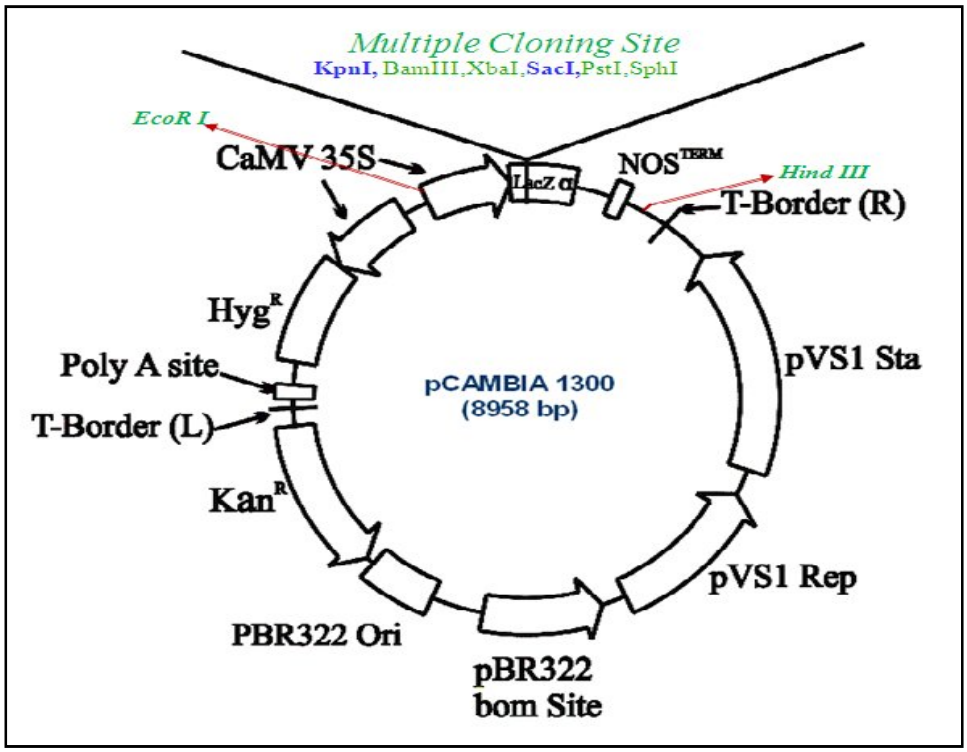


Fig:5.1: Topographical representation of (a) *LIPOX gene(s)* with KpnI-SacI restriction sites, (b) MCS of pCAMBIA1300 along with 35S promoter and Nos terminator flanked by EcoRI and HindIII restriction sites.

Fig. 5.2a Anti-sense *LIPOX* gene(s) Cassette in pCAMBIA1300 flanked by EcoR I - Hind III

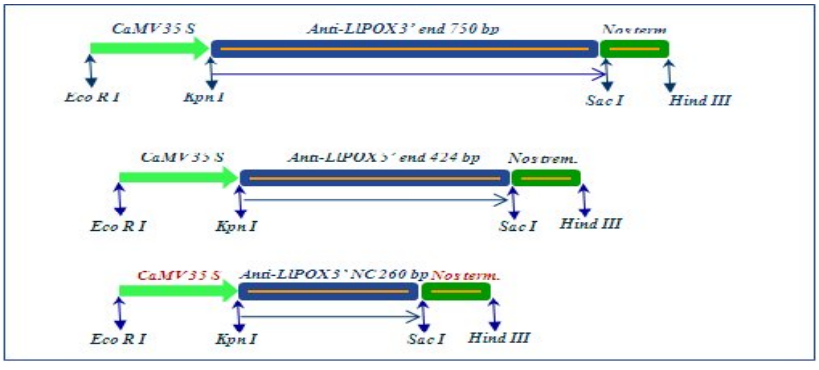


Fig. 5.2b Constructed vector map of pCAMBIA1301 recombinant binary vectors

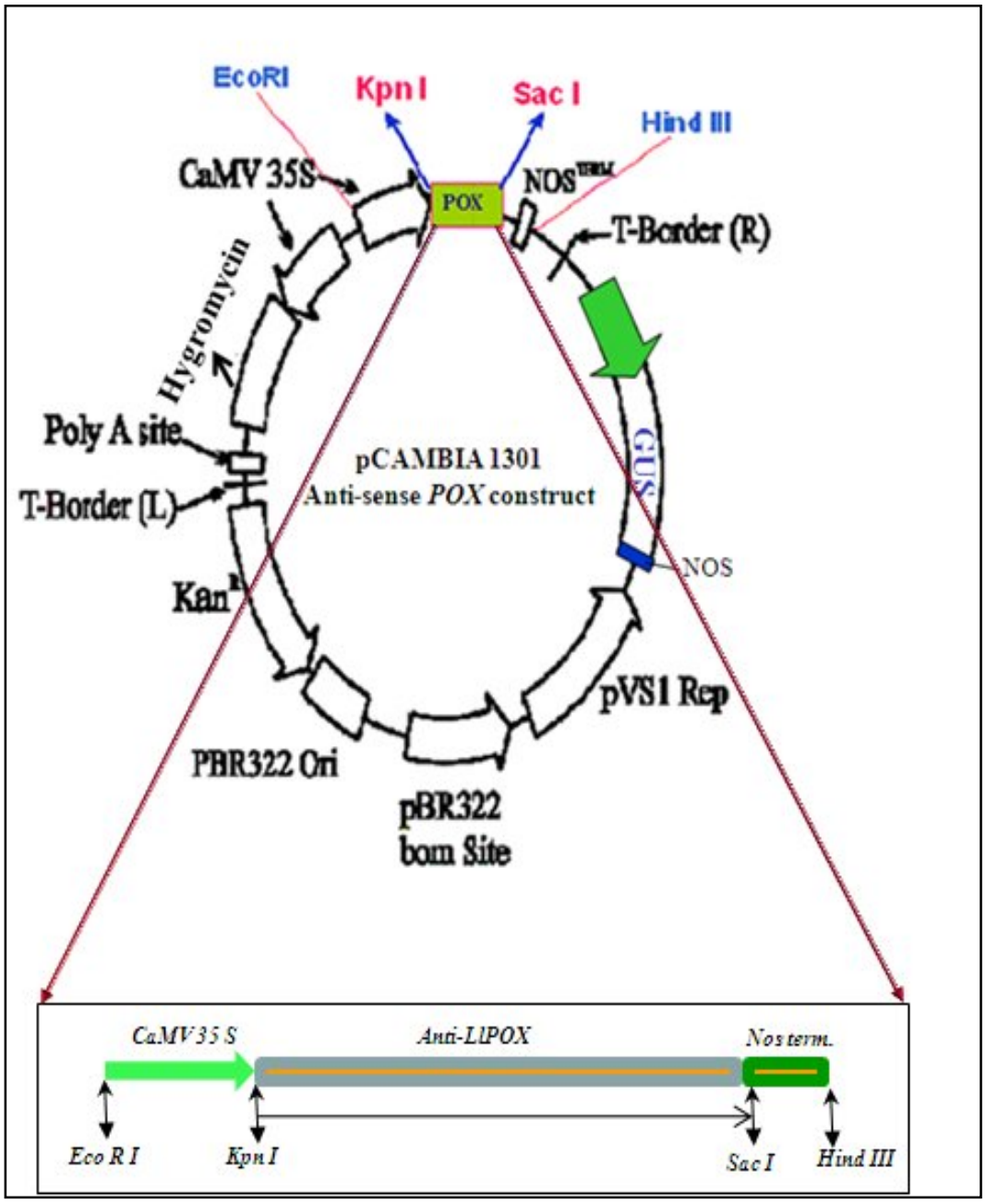


Fig. 5.2c Constructed vector map of pCAMBIA2301 recombinant binary vector

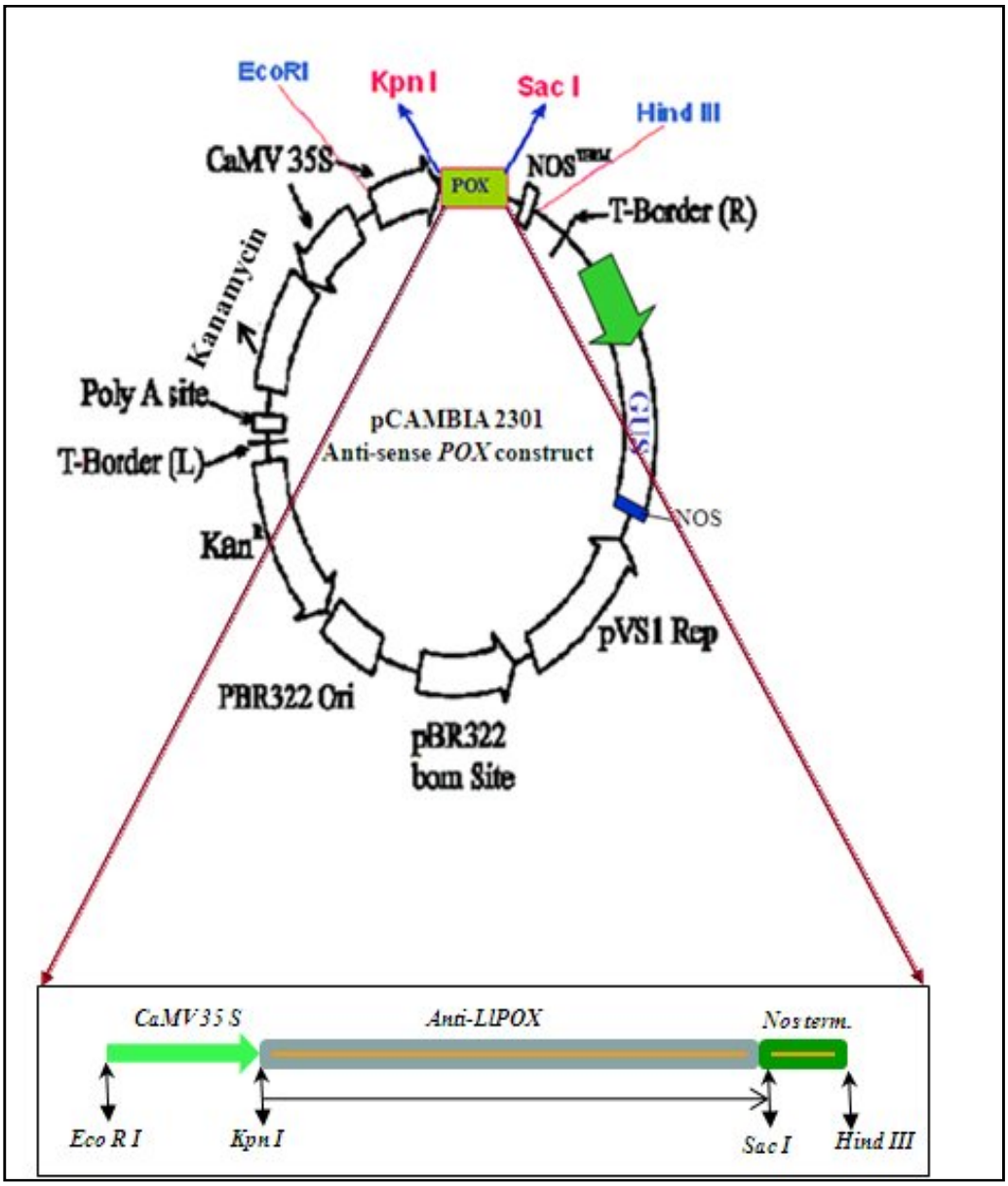


Fig 5.2: Topographical representation of (a) Anti-sense *LlPOX* gene(s) Cassette with flanking EcoR I - Hind III restriction sites (b) Recombinant pCAMBIA1301 binary vector harbouring 35S promoter, Partial anti-sense *LlPOX* gene(s) and Nos terminator, (c) Constructed Recombinant pCAMBIA2301 binary vector harbouring 35S promoter, Partial anti-sense *LlPOX* gene(s) and Nos terminator.

```
  1 CACGACTGCT TCGTCAATGG TTGTGACGCC GGAATCCTCC TAGACGACAC
 51 CGCAAGCTTC ACCGGAGAGA AAAACGCCGG CCCCAACCAG TCGGCAAGGG
101 GCTATGAAGT GATTGATGCC ATCAAAACCA ACGTGGAAGC AGCCTGCAAC
151 GGCACTGTGT CCTGTGCTGA TATCTTGGCA CTCGCAGCAC AAGAGGGTGT
201 TACCCAGCTA GGGGGACCCT CATGGGCAGT ACCACTTGGC CGGAGGGATG
251 CGAGAACGGC GAGCCAGAGC AAAGCCAACA GCGAGATCCC GGGGCCGTCA
301 TCGGACCTAT CGACTCTGAT CTCCATGTTC GCCGCGAAAG GGCTGACAGC
351 CCGGCAGATG ACGGTGCTGT CGGGCGCGCA CACCATAGGT CAGGGACAGT
401 GCAACTTCTT CAGGAACCAA ATCTACAACG AGAACAACAT CGATCCAAGT
451 TTTGCAACCA CGAGAAGGGC CACTTGCCCT AGAACTGGCG GGGACACCAA
501 CTTGGCCCCA CTTGACCCCA CCCCAAACAA GTTTGATAAC ACTTACTATC
551 AGGACCTTGT TGTCCGACGT GGCCTCTTCC ATTCGGACCA AGAGCTCTTC
601 AATGGTGGCT CTCAAGATGC TCTGGTTAGG ACTTATAGTT CCAATAGTGC
651 CCTCTTCTTT AGGGATTTTG CTTCTGCCAT GGTCAAGGTA AGCAGTATCA
701 CTCCCCTCAC GAGGTCCCAG GGAGAGATCC GAAAGAATTG TAGGGTTGTC
```

HDCFVNGCDAGILLDDTASFTGEKNAGPNQSARGYEVIDA
IKTNVEAACNGTVSCADILALAAQEGVTQLGGPSWAVPLG
RRDARTASQSKANSEIPGPSSDLSTLISMFAAKGLTARQMT
VLSGAHTIGQGQCNFFRNQIYNENNIDPSFATTRRATCPRT
GGDTNLAPLDPTPNKFDNTYYQDLVVRRGLFHSDQELFNG
GSQDALVRTYSSNSALFFRDFASAMVKVSSITPLTRSQGEI
RKNCRVV

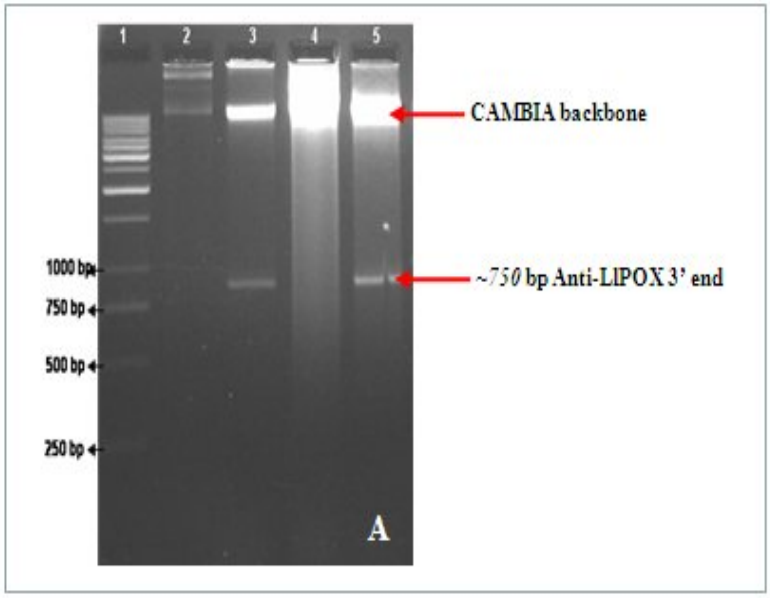


Fig: 5.3A: Restriction analysis of recombinant anti-sense pCAMBIA vector releasing 750 bp 3'end of *LlPOX* gene carrying conserved domains underlined in the sequence above. Lane 1-ladder, lane 2 & 4 uncut recombinant binary vector, lane 3 & 5 recombinant binary vector digested with *Kpn* I & *Sac* I

```
  1 ATGGCTTCCC TTACCCATTT CTTCTTACTT GCAGTGTCTC TTCTTTCTCT
 51 CTTTGCCTAT TCTTCCGATG CTCAGCTCTC TCCCAACTTC TACGCCAGAT
101 CTTGCCCTAA CGTCCGGGCC ATTGTTCGCA ACACAATGAG GCAAGCTCTC
151 GCCCGAGAAG CCCGCCTTGG CGCCTCCATC CTCCGCCTCT TTTTCCACGA
201 CTGCTTCGTC AATGGTTGTG ACGCCGGAAT CCTCCTAGAC GACACCGCAA
251 GCTTCACCGG AGAGAAAAAC GCCGGCCCCA ACCAGTCGGC AAGGGGCTAT
301 GAAGTGATTG ATGCCATCAA AACCAACGTG GAAGCAGCCT GCAACGGCAC
351 TGTGTCCTGT GCTGATATCT TGGCACTCGC AGCACAAGAG GGTGTTACCC
401 AGCTAGGGGG ACCCTCATGG GCAG
```

MASLTHFFLLAVSLLSLFAYSSDAQLSPNFYARSCPNVRAIV
RNTMRQALAREARLGASILRLFF**HDCFVN**GCDAGILLDDTA
SFTGEKNAGPNQSARGYEVIDAIKTNVEAACNGT**VSCADIL**
ALAAQEGVTQLGGPSWA

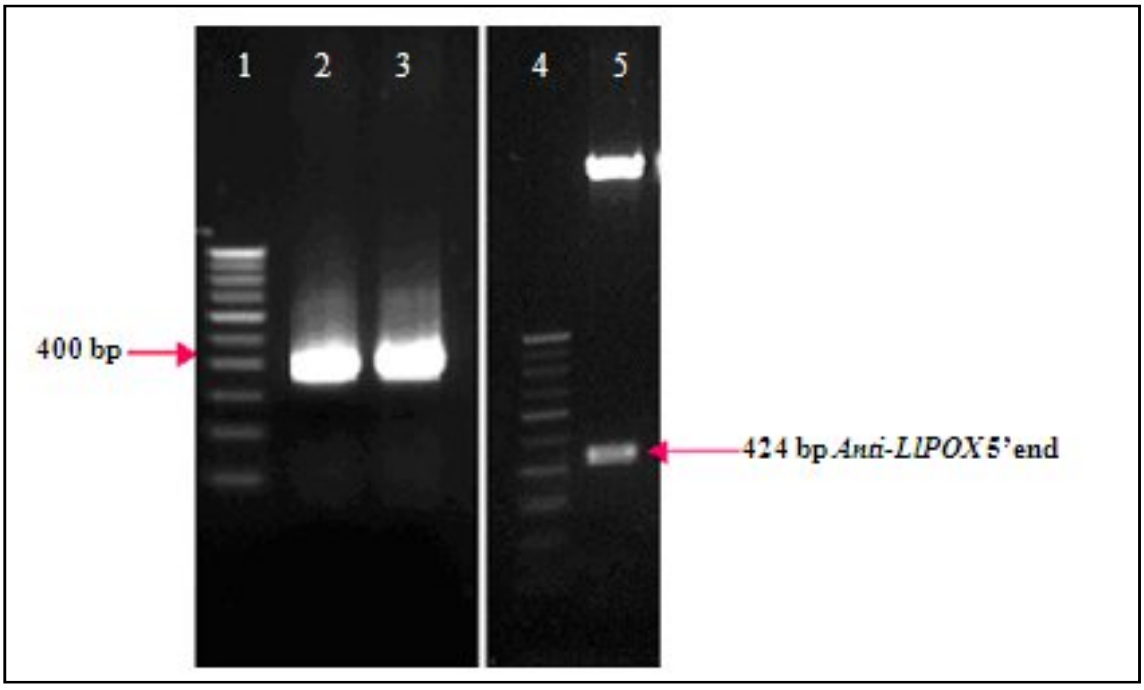


Fig: 5.3B: Restriction analysis of recombinant anti-sense pCAMBIA vector releasing 424 bp 5' ends of *LlPOX* gene carrying conserved domains underlined in the sequence above. Lane 1 & 4 ladder, lane 2&3 PCR amplified product, Lane 5 recombinant clone digested with *Kpn* I & *Sac* I releasing 424 bp 5'conserved region of peroxidase gene

```
  1 AGAACTGGCG GGGACACCAA CTTGGCCCCA CTTGACCCCA CCCCAAACAA
 51 GTTTGATAAC ACTTACTATC AGGACCTTGT TGTCCGACGT GGCCTCTTCC
101 ATTCGGACCA AGAGCTCTTC AATGGTGGCT CTCAAGATGC TCTGGTTAGG
151 ACTTATAGTT CCAATAGTGC CCTCTTCTTT AGGGATTTTG CTTCTGCCAT
201 GGTCAAGGTA AGCAGTATCA CTCCCCTCAC GAGGTCCCAG GGAGAGATCC
251 GAAAGAATTG TAGGGTTGTC

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

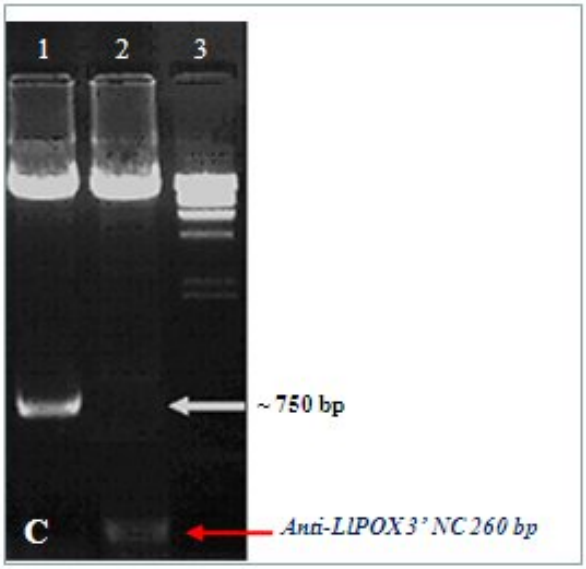


Fig: 5.3C: Restriction analysis of recombinant anti-sense pCAMBIA vector releasing 260 bp 3'end of *LlPOX* gene without any conserved domains of *POX* gene. Lane 1 is 3' 750 bp antisense clone in binary vector, lane 2 restriction analysis of recombinant clone with *Kpn* I & *Sac* I releasing 260 bp 3'non conserved region of *LlPOX* gene

5.2.7 *Agrobacterium tumefaciens* transformation

A. tumefaciens GV2260 was transformed with the recombinant pCAMBIA1301 and pCAMBIA 2301 vectors harboring antisense *LlPOX* gene cassette (Chapter 2, Section 2.8.2.4).

5.2.8 Biolistic transformation using PDS-1000/He Biolistic Particle Delivery System

The selected embryogenic calli derived from leaf explants of *Nicotiana tabacum*, were bombarded twice after arranging at the centre of a 90 mm diameter petri-plate using rupture discs with 900 pounds per square inch (psi) specification with 25 inches of Hg vaccum at an interval of 4 hours. After two days of incubation under darkness the bombarded calli were shifted to differentiating media containing basal MS medium, B5 vitamins, 1 mg/L BAP, 0.1 mg/L NAA, 30 g/L sucrose, 4 g/L phytagel for a week and later transferred to the same medium containing plant selection marker kanamycin 100 mg/L concentration.

In case of *Oryza* 50 friable embryogenic calli were counted and placed at the centre of a 90 mm diameter petri plate with callus induction medium containing osmotica (MS medium + 2.5 mg/L 2,4-D supplemented with 36.4 g/L mannitol and 36.4 g/L sorbitol), 4 hours prior to the first shooting using PDS-1000/He Biolistic Particle Delivery System. The explants were bombarded twice using rupture discs with 900 pounds psi specification with 25 inches of Hg vaccum at an interval of 4 hours. After forty-eight hours of the second bombardment, the calli were directly transferred on to MS callus induction medium containing 50 mg/L hygromicin B, and incubated at 25±2 ^{o} C for 15-18 days. Actively proliferating calli were subcultured onto a fresh selection medium thrice at 15-18 days interval.

The proliferating embryogenic calli after 3 rounds of selection on hygromycin B were transferred onto an MS regeneration medium (MS medium supplemented with BAP and NAA (Sigma, USA) 3 mg/L and 0.5 mg/L respectively). The calli were cultured at 16 hours light (24.4 µmol/m^2/s) and 8 hours dark at 25±2 ^{o}C till shoots established. Emerging shoots were transferred to MS rooting medium (1/2-MS basal salts, MS vitamins and 15 g/L sucrose) containing 30 mg/L hygromycin B.

Similarly, *Leucaena* embryos were bombarded with PDS-1000/He Biolistic Particle Delivery System twice using rupture discs 900 psi with 25 inches of Hg vaccum at an interval of 4 hours and kept under darkness for two days. After growing the embryos on the above regeneration media without selection for one week, were subjected to three rounds of selections in the same regeneration medium containing Kanamycin 250 mg/ L,

at an interval of 15 days. The plants, which survived three rounds of selection on Kanamycin 250 mg/ L, were shifted to ½-MS with Cytokinin, 2ip (2-isopentenyl adenine) 0.5 mg/ L to enhance elongation of transformed shoots.

In all the above cases, the bombarded explants were subjected to transient GUS assay 48 hrs after second bombardment and the putative transgenic plants, which survived three rounds of selection were analysed for the gene integration into the plant genome.

5.2.9 Particle bombardment followed by co-cultivation

The *Leucaena* embryo axes were first bombarded with microcarriers coated with recombinant pCAMBIA vectors as mentioned above followed by co-cultivation with *Agrobacterium* (GV2260) harboring respective recombinant CAMBIA vectors containing *POX* gene in anti-sense orientation. Cultured *Agrobacterium* 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 respective regeneration medium with or without 0.1 mM acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Sigma-Aldrich Chemical Co.) and co-cultivated in the dark at 25 ± 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.

5.2.10 Immuno-cytolocalization of *POX* protein in *L. leucocephala* transformants

Solutions

1X PBS: 10 mM NaH_2PO_4; Na_2HPO_4, pH 7.2; 130 mM NaCl
0.5X SSC: 75 mM NaCl; 7.5 mM Sodium citrate, pH 7.0
Color development buffer: 100 mM Tris-HCl, pH 9.5; 150 mM NaCl, 50 mM $MgCl_2$
BCIP/NBT mix: 0.577 mM BCIP; 0.122 mM NBT (Merck, USA)
10 mM EDTA; Ethanol; Tertiary butanol; Paraffin; Xylene and Glycerol.

Stem of developing control seedlings and putative transformants were used for the study. Free hand transverse sections were fixed overnight, under vacuum, in freshly prepared cold 4% buffered formaldehyde (4% formaldehyde in 1X PBS). The sections were dehydrated through increasing ethanol: water series. This was followed by tertiary butanol: ethanol series (Lacombe *et al.*, 1997). The sections were rehydrated by treating with 70% and 50% ethanol and 0.5X SSC for 2 min. The rehydrated sections were subjected to 1 h of blocking with 10% BSA at room temperature in a humidified chamber. Primary antibody (antibodies raised against purified *LlPOX* protein in rabbit) incubation was carried out overnight in a humidified chamber at 4 ºC using 1:5000 antibody dilution in 1 X PBS containing 0.1% BSA. Negative controls included the use of pre-immune serum. Following the primary antibody incubation, the sections were washed thrice, for 15 min each, in 1X PBS containing 0.1% BSA. Alkaline phosphate (ALP) conjugated anti-rabbit goat IgG in 1:10000 dilution (diluted in 1X PBS with 0.1% BSA), was added to the tissue sections at this stage and incubated at 37 ºC in a humidified chamber for 2 h in dark. Post secondary antibody washes were carried out at RT using 1X PBS with 0.1% BSA. Color was developed in dark by addition of color development reagent (one part BCIP/NBT mixed with two parts color development buffer) to treated sections. The sections were placed in humidified chamber at RT, in dark, for 45 min. Upon color development, 10 mM EDTA was used to stop the reaction. Cover slip was mounted using glycerol and observed under the microscope, and microscopic image captured (Axioplan 2, Zeiss).

5.2.11 Histochemical staining of lignin

Solutions

Phloroglucinol 2% in 95% Ethanol

Free hand transverse sections were prepared for histochemical analysis as described above. Phloroglucinol staining was done by covering pre-treated sections with phloroglucinol for half an hour and then concentrated HCl was added. Lignified tissue takes up red color. A cover slip mounted on the specimen using glycerol and the slide was sealed using nail enamel. The slides were viewed under a light microscope and pictures captured (Axioplan 2, Zeiss).

5.2.12 ELISA (Enzyme-Linked Immunosorbent Assay)

Fresh tissues were collected from control as well as putative transgenic plants, frozen in liquid nitrogen and crushed to a fine powder. Crude protein was extracted with 2 mL of protein extraction buffer (100 mM Tris-HCl, pH.7.5; 2% PVPP; 2% PEG 4000; DTT 5 mM and PMSF 1 mM). Total Protein was quantified using Bradford reagent. Equal amount of protein was coated on 96 well micro titre plates. ELISA protocol was performed as discussed in Chapter 2, Section 2.11.5.

5.2.13 Lignin estimation

Randomly selected *L. leucocephala* transformants showing positive signals (PCR, ELISA and Slot blot) for transformation events were chosen for lignin estimation studies. Acid soluble and acid insoluble lignin was determined to give the total lignin content. Protocol for lignin estimation is given in Chapter2, Section 2.15.

5.3 Results and discussions

5.3.1 Synthesis and characterization of nano particles used as micro-carriers

In this chapter, a composite carbon supported gold nanopartilces, as an alternative material (microcarrier) for gene delivery using the gene gun developed by Sanford and co-workers has been presented. While the carbon support is expected to be having the required traits such as inertness, better piercing capacity, the embedded gold nanopartilces provide the best support to DNA. This is the first report where such composite material has been tried for gene delivery.

The first step towards the preparation of this material involves, the incubation of fungal biomass with gold ions leading to the development of ruby red colour to the biomass indicating the reduction of gold ion to Au nanoparticles inside the cellular matrix (Fig.5.6A and B). SEM image of calcined and uncalcined fungal hyphae shown in fig C and D.

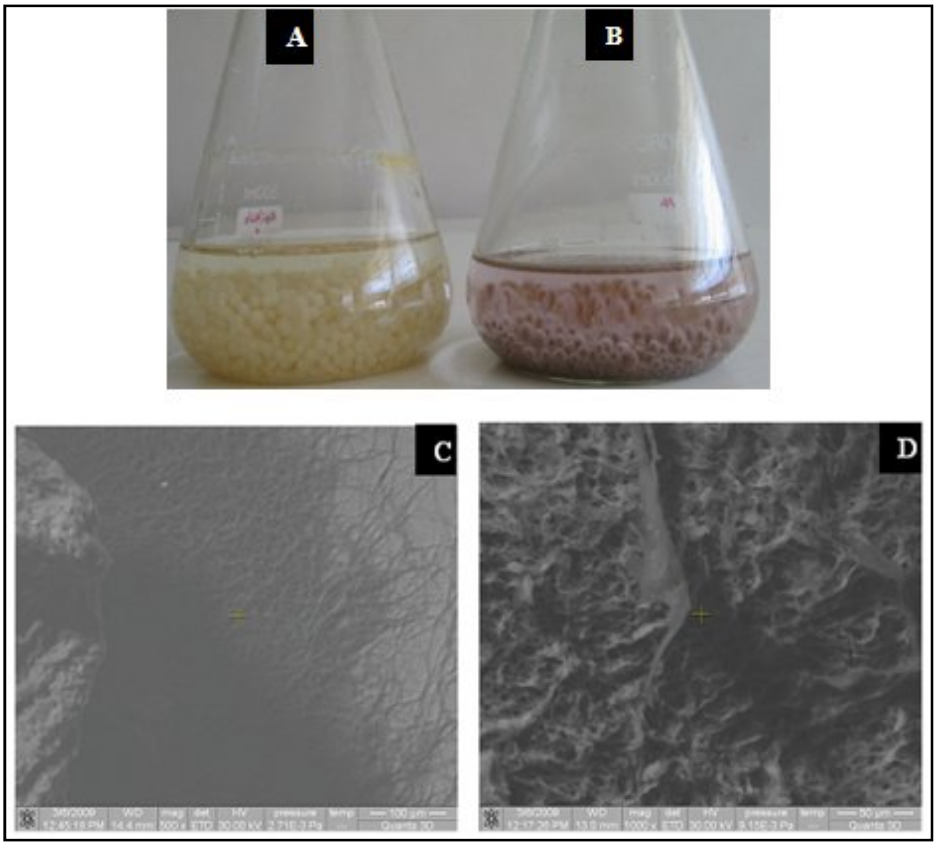


Fig: 5.6. A: Biomass before the incubation with the gold ions. B: Biomass after the incubation with the gold ions. C: SEM image of uncalcined fungus micro hyphal thread: D: SEM image Calcined biomass before grinding.

The XRD signal of the biomass dried at room temperature and grained to powder (Fig. 5.7A curve 1) and calcined in the inert atmosphere at 600 ^{o}C (Fig. 5.7A curve 2) were recorded. The XRD patterns of the as prepared sample (curve 1) showed a number of Bragg reflections at *d* values ≈ 2.36, 2.04, 1.45 and 1.23 Å characteristic of the fcc gold. This clearly shows that the nanoparticles are formed just by the treatment of gold ions with the biomass.

The peaks in the HTC 600-Au (curve 2) were sharper than the as prepared sample indicating an improvement in the crystallinity upon heating. The Raman signals of the HTC 600-Au are plotted in Fig. 5.7C. The curves show features characteristic of disordered carbon with peaks at ~1590 cm^{-1} and ~1350 cm^{-1} that are designated as G and D peak respectively. The data was deconvoluted and fitted to two Gaussian peaks. From the intensity of the peak at 1365 and 1590 cm^{-1} the graphite in-plane domain size (L_a)

value was determined to be 1.35 nm in HTC 600-Au (B. L. V. Prasad *et al*., 2000), as compared to 0.90 nm in the pure carbon sample without gold nanoparticles. The increase in L_a of HTC 600-Au (Fig. 5.7C) could be explained on the basis of previous works, where presence of metal impurities were shown to facilitate graphitization (H.Murayama and Maeda, 1990). However the inplane size of 1.35 nm in HTC 600-Au still suggested the presence of an amorphous carbon matrix.

The SEM/TEM (Fig.5.8) analysis for the HTC 600-Au was in-line with the above XRD and Raman analysis. The SEM image in fig.5.8A shows a sharp particle made up of many flat sheet like material. This figure was selected to highlight the sharp tips/edges such materials could possess. The TEM image (Fig.5.8B) at lower magnification showed the presence of 50 nm Au nanoparticles embedded in the carbon matrix with sharp edges. Lack of any long range ordered structure confirms the poor development of the graphite like carbon. At higher magnification in the Fig.5.8C apart from the few 50 nm particles, plenty of 5 nm particles spread throughout the carbon sheet is observed. Still closer view of the carbon in the Fig.5.8D shows wavy edge, showing bundle of sheet arrangement with one sheet getting protruded to form a sharp edge. The HRTEM image (inset Fig.5.8D) of one of the isolated particle shows a "d" spacing of 2.4 Å that matches with the lattice spacing 2.36 Å of gold plane.

To compare the DNA binding capacity of the HTC 600-Au with commercial micro gold, agarose gel electrophoresis was performed (Fig.5.9). From the gel picture it is evident that, the HTC 600-Au coated with the DNA coupled with adhesive coating (lane 5) showed significant difference in the distance it had migrated compared to the free plasmid (lane 1). The behavior of the DNA of HTC 600-Au is infact comparable to that of micro gold (lane 3). Without adopting coating procedures, physically mixed plasmid and HTC 600-Au was loaded on lane 4, which have shown free movement of the plasmid negating the argument that the particle chocking of the well would have lead to the slow movement in the DNA bound with HTC 600-Au (in lane 5). The standardization of the plasmid DNA to the HTC 600-Au was reproducibly carried out in the dicot tree *L. leucocephala*. The study (Fig.5.10) revealed that, only 200 ng of plasmid is enough to match the results of the 600 ng classical abiotic micron gold carriers used in plant transformation. Also it was found that on increasing the DNA concentration per unit of

HTC 600-Au still higher levels of GUS expressing foci can be achieved, which can be optimized and very well used for other plant transformation systems.

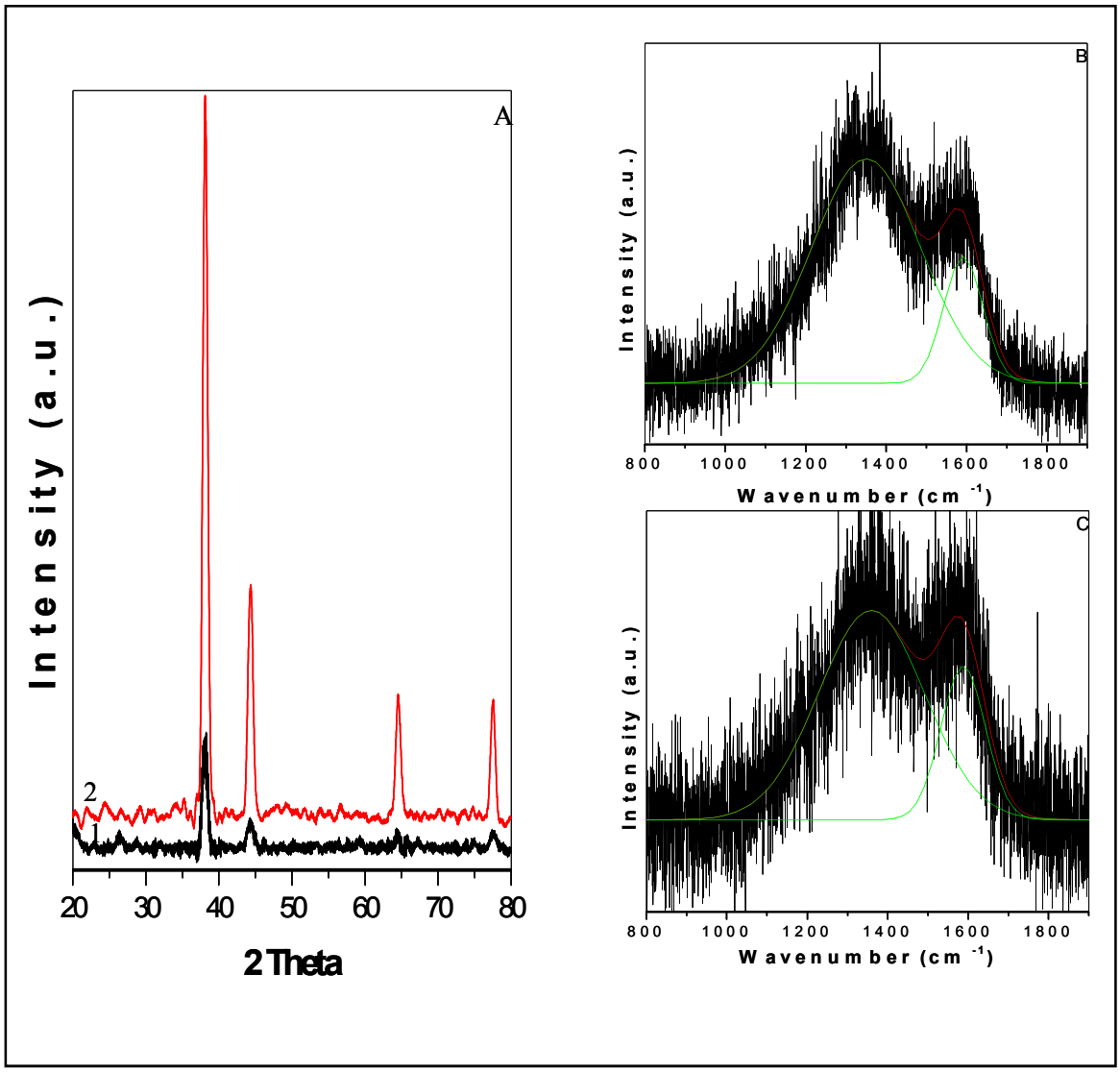


Fig. 5.7 A: XRD spectrum. Curve - 1: Uncalcined biomass incubated with 1 X 10^{-3} M $HAuCl_4$; Curve- 2: Heat treated carbon gold at 600 °C **(HTC 600-Au)**; **B**: Raman spectrum of heat treated carbon gold at 600 °C **(HTC 600-Au)**; **C**: Raman spectrum of biomass.

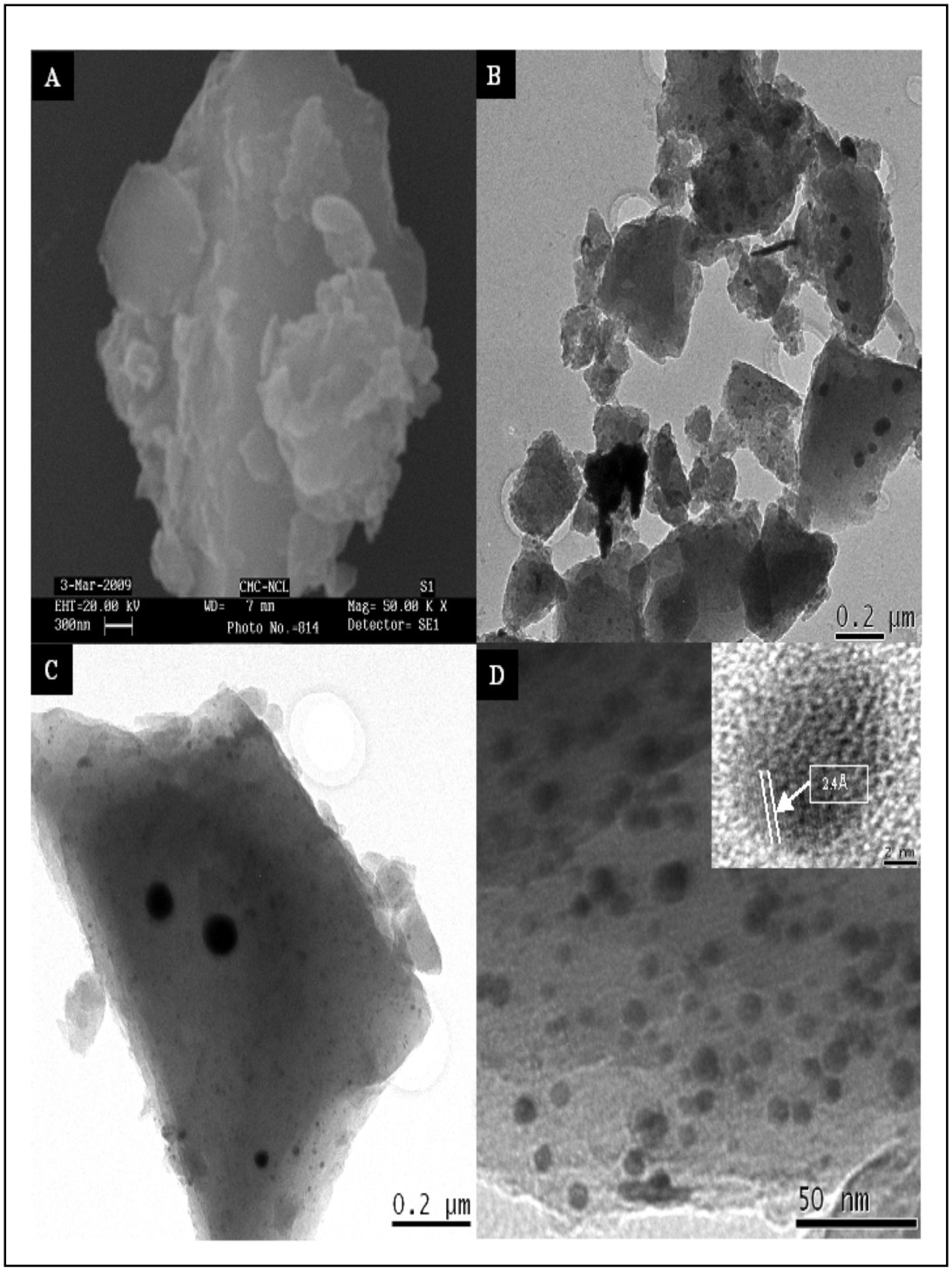


Fig. 5.8: (A) SEM image of heat treated carbon gold at 600 °C **(HTC 600-Au)**; (B, C and D) TEM image of heat treated carbon gold at 600 °C **(HTC 600-Au)** note the presence small gold particles plane embedded in carbon matrix as revealed by the TEM images in C and D**;** Inset D HRTEM image of the gold nanoparticle showing the "*d*" spacing corresponding to 111 phase of gold.

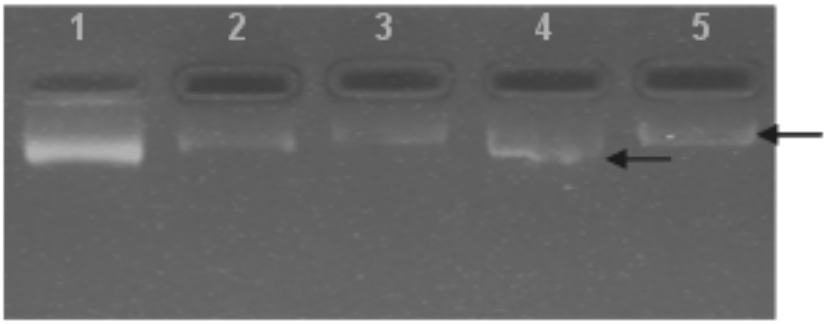


Fig. 5.9: Gel showing plasmid adhesion to the **HTC 600-Au**. Well 1, plasmid; Well 2, gold micro particles mixed with plasmid; Well 3, gold micro particles adhesively coated with plasmid; Well 4, **HTC 600-Au** particles physically mixed with plasmid; Well 5, **HTC 600-Au** particles adhesively coated with plasmid.

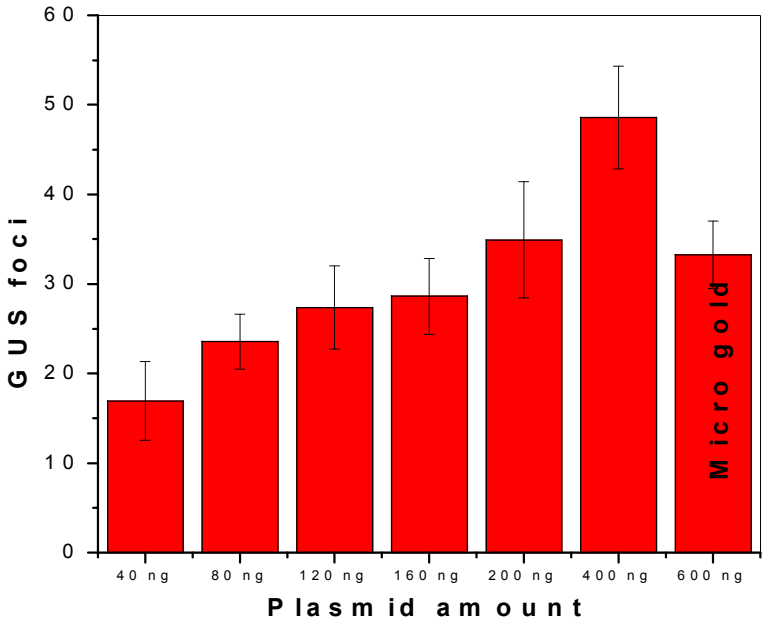


Fig.5.10: Standardization of different concentrations of plasmid on 1 mg heat treated carbon gold at 600 °C **(HTC600-Au)** to match the number of GUS foci as obtained with specifications for commercial micro gold (Explant used is *Leucaena leucocephala*).

5.3.2 Antibiotic sensitivity

In a preliminary study conducted to find out the optimum concentration of cefotaxime for the control of *Agrobacterium* contamination after co-cultivation, it was observed that cefotaxime at a minimum concentration of 250 mg/L could control the growth of *Agrobacterium* completely. Cell density of the *Agrobacterium* strain carrying **5' 35SPro-*Anti-LlPOX*-NOS 3'** plotted against time showed a typical growth with lag phase upto 4 h followed by log phase upto 16 h with intense cell division. After this, the curve became stationary and later started declining indicating mortality of the bacterium as per the growth curve, *Agrobacterium* culture during the log phase (4–16 h old) was used for particle bombardment followed by co-cultivation studies.

In kanamycin free treatment, freshly excised embryo axes from *Leucaena* seeds showed normal proliferation, growth and germination (Fig: 5.11a). There was a gradual increase in necrosis of the embryo axis with the increase in kanamycin concentration from 50 to 400 mg/L. LD_{50} for kanamycin was observed at a concentration 250 mg/L showing necrosis and death of the 50% of the inoculated embryos(Fig: 5.11b). Explants showing callusing, germination and further proliferation became brownish and necrotic at later stages in most of the kanamycin treatments. In case of tobacco, LD_{50} for kanamycin was observed at a concentration 100 mg/L showing necrosis and death of the 50% of the inoculated tobacco leaves. For rice seed as explants LD_{50} for hygromycin was observed at a concentration 50 mg/L.

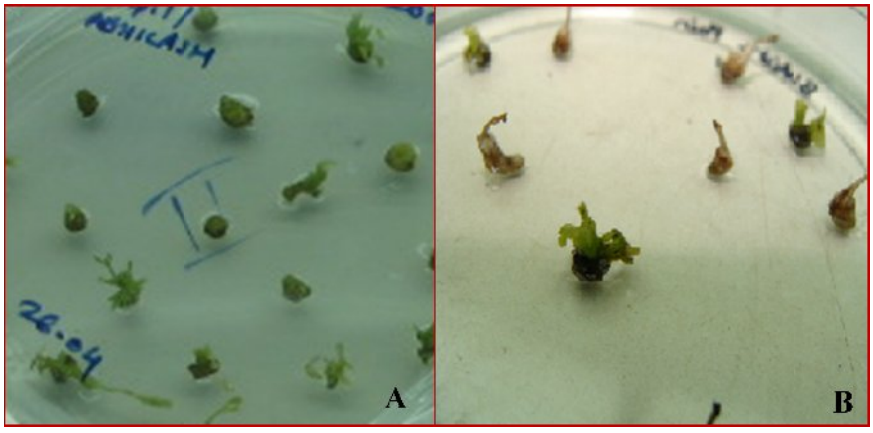


Fig: 5.11: Antibiotic sensitivity of embryo axes of *L. leucocephala*. (a) Embryo axes cultured in kanamycin (0 mg/L) (b) embryo axes cultured in kanamycin (250 mg /L).

5.3.3 GUS histochemical assay

GUS histochemical assay (**Chapter 2; section 2.19**) was performed 48 hours after the second bombardment on embryo axes of *L. leucocephala* and on calli of both *Oryza* and *Nicotiana*. Transient GUS expressing foci (Fig: 5.4B, 5.4D; 5.5C, 5.5D & 5.6a B, 5.6a C) were observed in almost all the explants subjected to bombardment.

5.3.3.1 Genomic DNA extraction and polymerase chain reaction

Genomic DNA was extracted from putative transgenic plant leaves and PCR reactions performed as described earlier (**Chapter 2; section 2.10.3 and 2.10.12.3**).

5.3.4 Genetic Engineering of plant systems (*Oryza*, *Nicotiana* and *Leucaena*)

Using gene gun system, a recombinant binary vector was efficiently introduced into calli (tobacco (Fig.5.4) and rice (Fig. 5.5)) and the immature embryos of *Leucaena* (Fig.5.6a). Transient GUS expressing foci were observed in almost all the explants subjected to bombardment. Studies proved a distance of 1/4" as optimal between rupture disc and macrocarrier to get reproducible results when experiments were carried out in triplicates in case of *Nicotiana, Oryza* and *Leucaena*. While the target distance (the distance between stopping screen and target explants), found optimal for tobacco and rice was 6 cm (level 2) and for *Leucaena* it was 3 cm (level 1). With above standardized conditions, tobacco on an average showed 30±11 GUS foci with HTC 600-Au as the carrier, whereas 1.0 µm gold particles yielded 27±9 foci. An average of 40±13 and 24±17 GUS spots were noticed in rice when HTC 600-Au and 1.0 µm gold particles were used as carriers respectively. *Leucaena* showed higher levels of transient GUS expression on an average with HTC 600-Au (35±11), in comparison to the conventional gold micron carriers (25±7). It is important to reiterate that all these results were obtained with 200 ng of plasmid on HTC 600-Au as compared to 600 ng of plasmid on conventional micron sized gold. Similarly the gold required for transformation in HTC 600-Au form was ¼ times less than what is used in micron sized particles.

The putative transgenic tobacco plants, which showed GUS expression, were subjected for polymerase chain reaction (PCR) as recommended by Sambrook *et al.,* (1989) using 100 ng of genomic DNA in a 25 µL reaction mixture from putative transgenic plants,

10X PCR buffer (50 mM Tris-HCl pH 8.8, 50 mM KCl and 1.5 mM $MgCl_2$), 200 µM of each dNTPs, 1µL of each primer (containing 8 pico moles forward and reverse each) and 1 unit of *Taq* DNA polymerase. Using GUS primers (Forward 5'GAA CCG ACG ACT CGT CCG TCC TG 3'and Reverse5' TGC TTT TTC TTG CCG TAA TCA CC 3') 490 bp internal fragment of GUS gene was amplified (Fig. 5.12A) and the transformation event was further confirmed by sequencing (Fig. 5.13).

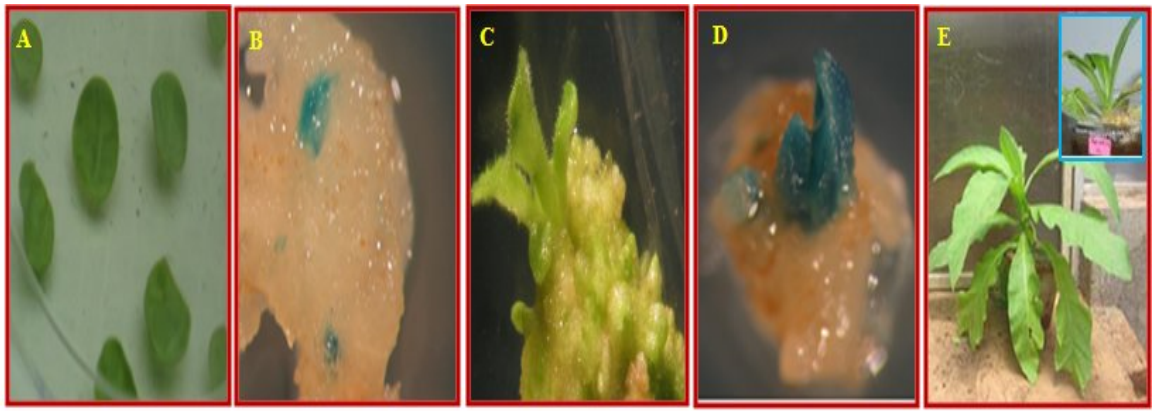


Fig: 5.4: Biolistic transformation in Tobacco. **A**.Tobacco leaf explants, **B**. transient GUS expression in calli, **C**. Shoot induction from callus, **D**. Stable GUS expression on calli derived shoot, **E**. Hardened putative transgenic plants which survived three rounds of kanamycin selection.

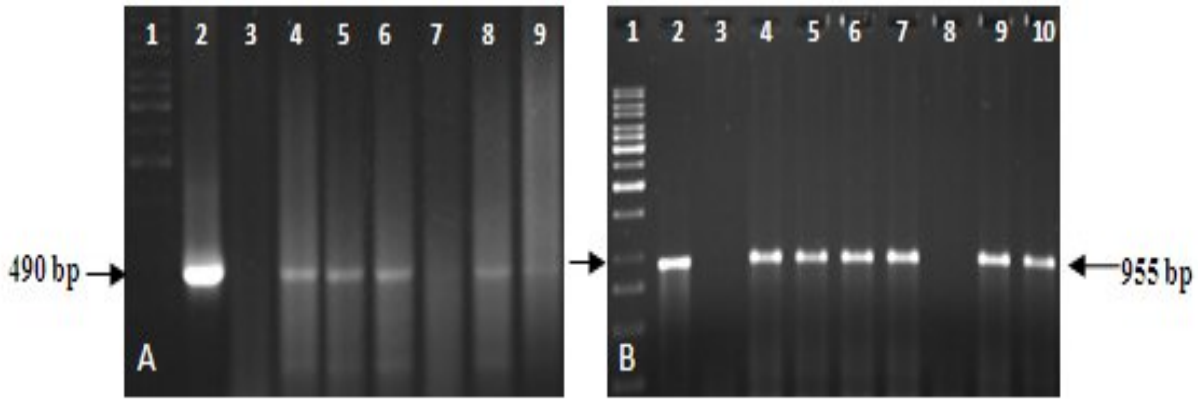


Fig. 5.12: PCR analysis of putative transgenic tobacco (**A**) and rice (**B**) plants. **A**. Lane1 DNA marker, lane 2 positive control showing a 490 bp internal fragment of GUS gene, lane 3 untransformed control plant, lane 4,5,6,8 & 9 shows positive for GUS, lane 7 untransformed plant**. B**. Lane 1 DNA marker, lane 2 positive control showing a 955 bp internal fragment of *hph* gene, lane 3 untransformed control plant, lane 4,5,6,7,9 & 10 shows positive for *hph* gene, lane 8 untransformed plant.

```
GUS-Putativetransformant      TGTCTATGATGATGATGATAGTTACAGAACCGACGACTCGTCCGTCCTGT
GUSfromCAMBIA                 ------------------------CAGAACCGACGACTCGTCCGTCCTGT
                                                       **************************

GUS-Putativetransformant      AGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCA
GUSfromCAMBIA                 AGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCA
                              **************************************************

GUS-Putativetransformant      GTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCG
GUSfromCAMBIA                 GTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCG
                              **************************************************

GUS-Putativetransformant      TTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTT
GUSfromCAMBIA                 TTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTT
                              **************************************************
GUS-Putativetransformant      CGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCG
GUSfromCAMBIA                 CGCCGATGCAGATATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCG
                              **************************************************

GUS-Putativetransformant      AAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTC
GUSfromCAMBIA                 AAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTC
                              **************************************************

GUS-Putativetransformant      GATGCGGTCACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTGAT
GUSfromCAMBIA                 GATGCGGTCACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTGAT
                              **************************************************

GUS-Putativetransformant      GGAGCATCAAGGTGGATATACGCCATTTGAAGCCGATGTCACGCCGTATG
GUSfromCAMBIA                 GGAGCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATG
                              ********* ** ** *********************************

GUS-Putativetransformant      TTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTGTGAACAACGAACTG
GUSfromCAMBIA                 TTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTGTGAACAACGAACTG
                              **************************************************

GUS-Putativetransformant      AACTGGCAGACTATCCCGCCGGGAATGGTGATTAC
GUSfromCAMBIA                 AACTGGCAGACTATCCCGCCGGGAATGGTGATTAC
                              **********************************
```

Fig. 5.13: CLUSTAL W (1.8) multiple sequence alignment of GUS gene, shaded regions shown are part of forward and reverse primers

Similarly, putative transgenic rice plants which survived hygromycin selection were confirmed by PCR analysis under the above mentioned conditions using the hygromycin primers (Forward 5' GAAAAGTTCGACAGCGTCTCC 3'and Reverse 5' GCTGGGGCGTCGGTTTC 3') which amplified ~955 bp internal fragment of *hph* gene (Fig. 5.14).

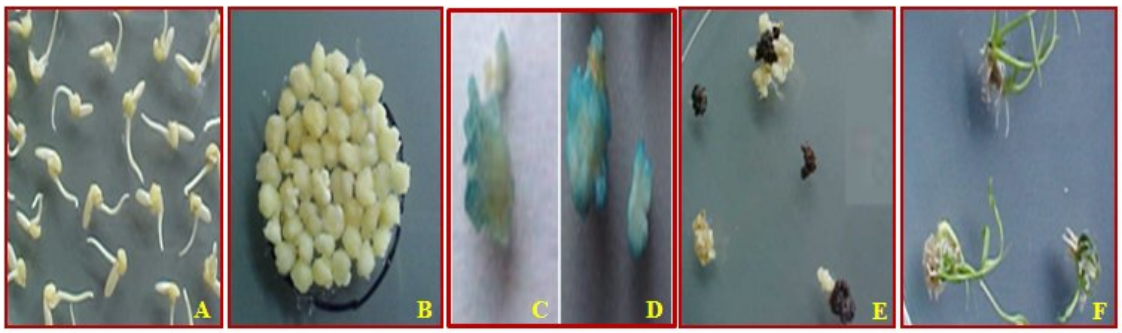


Fig. 5.5: Biolistic transformation in rice. A. Callus induction, B. Calli arranged for bombardment, C. Transient GUS expression on bombarded calli, D. Stable GUS expression in calli which survived three rounds of 50 mg/L hygromycin selection, E. Putative transformed calli on selection media, F. Regeneration.

```
Hygromycingene-Putativetransfo      ----------------ATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCA
Hygromycingene-CAMBIA               GTCGAGAAGTTTCTGATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCA
                                                    **********************************

Hygromycingene-Putativetransfo      GCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGC
Hygromycingene-CAMBIA               GCTCTCGGAGGGCGAAGAATCTCGTGCTTTCAGCTTCGATGTAGGAGGGC
                                    **************************************************

Hygromycingene-Putativetransfo      GTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGAT
Hygromycingene-CAMBIA               GTGGATATGTCCTGCGGGTAAATAGCTGCGCCGATGGTTTCTACAAAGAT
                                    **************************************************

Hygromycingene-Putativetransfo      CGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGT
Hygromycingene-CAMBIA               CGTTATGTTTATCGGCACTTTGCATCGGCCGCGCTCCCGATTCCGGAAGT
                                    **************************************************

Hygromycingene-Putativetransfo      GCTTGACATTGGGGAGTTTAGCGAGAGCCTGACCTATTGCATCTCCCGCC
Hygromycingene-CAMBIA               GCTTGACATTGGGGAGTTTAGCGAGAGCCTGACCTATTGCATCTCCCGCC
                                    **************************************************

Hygromycingene-Putativetransfo      GTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCT
Hygromycingene-CAMBIA               GTGCACAGGGTGTCACGTTGCAAGACCTGCCTGAAACCGAACTGCCCGCT
                                    **************************************************

Hygromycingene-Putativetransfo      GTTCTACAACCGGTCGCGGAGGCTATGGATGCGATCGATGCGCCCGATCT
Hygromycingene-CAMBIA               GTTCTACAACCGGTCGCGGAGGCTATGGATGCGATCGCTGCGGCCGATCT
                                    ********************************** **** *******

Hygromycingene-Putativetransfo      TAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAAT
Hygromycingene-CAMBIA               TAGCCAGACGAGCGGGTTCGGCCCATTCGGACCGCAAGGAATCGGTCAAT
                                    **************************************************

Hygromycingene-Putativetransfo      ACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTAT
Hygromycingene-CAMBIA               ACACTACATGGCGTGATTTCATATGCGCGATTGCTGATCCCCATGTGTAT
                                    **************************************************

Hygromycingene-Putativetransfo      CACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGC
Hygromycingene-CAMBIA               CACTGGCAAACTGTGATGGACGACACCGTCAGTGCGTCCGTCGCGCAGGC
                                    **************************************************

Hygromycingene-Putativetransfo      TCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACC
Hygromycingene-CAMBIA               TCTCGATGAGCTGATGCTTTGGGCCGAGGACTGCCCCGAAGTCCGGCACC
                                    **************************************************

Hygromycingene-Putativetransfo      TCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGC
Hygromycingene-CAMBIA               TCGTGCACGCGGATTTCGGCTCCAACAATGTCCTGACGGACAATGGCCGC
                                    **************************************************
```

```
Hygromycingene-Putativetransfo      ATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATA
Hygromycingene-CAMBIA               ATAACAGCGGTCATTGACTGGAGCGAGGCGATGTTCGGGGATTCCCAATA
                                    **************************************************

Hygromycingene-Putativetransfo      CGAGGTCGCCAACATCTTCTTCTGGAGGCCGTTGTTGGCTTGTATGGAGC
Hygromycingene-CAMBIA               CGAGGTCGCCAACATCTTCTTCTGGAGGCCGTGGTTGGCTTGTATGGAGC
                                    ********************************* ****************

Hygromycingene-Putativetransfo      AGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCA
Hygromycingene-CAMBIA               AGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAGCTTGCAGGATCGCCA
                                    **************************************************

Hygromycingene-Putativetransfo      CGACTCCGGGCGTATATGCTCCGCATTGGTCGTGACCAACTCTATCAGAG
Hygromycingene-CAMBIA               CGACTCCGGGCGTATATGCTCCGCATTGGTCTTGACCAACTCTATCAGAG
                                    ********************************* ****************

Hygromycingene-Putativetransfo      CTTGGTTGACGGCAATTTCGATGATGCAGATTGGGCGCAGGGTCGATGCG
Hygromycingene-CAMBIA               CTTGGTTGACGGCAATTTCGATGATGCAGCTTGGGCGCAGGGTCGATGCG
                                    **************************** *******************

Hygromycingene-Putativetransfo      ACGCAATCGTCCGATCCGGAGCCTGGACTGTCGGGCGTACACAAATCGCC
Hygromycingene-CAMBIA               ACGCAATCGTCCGATCCGGAGCCGGGACTGTCGGGCGTACACAAATCGCC
                                    ********************** *************************



Hygromycingene-Putativetransfo      CGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGA
Hygromycingene-CAMBIA               CGCAGAAGCGCGGCCGTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGA
                                    **************************************************

Hygromycingene-Putativetransfo      TAGTGGAAACCGACGCCCCAGCACTCGTCCGAGG
Hygromycingene-CAMBIA               TAGTGGAAACCGACGCCCCAGCACTCGTCC----
                                    ******************************
```

Fig. 5.14: CLUSTAL W (1.8) multiple sequence alignment of *hph* gene, shaded regions shown are part of forward and reverse primers

5.3.5 Transformation of *Leucaena leucocephala*

In the case of *Leucaena,* as the regeneration time required is more (6 to 7 months). Hence, transformation efficiency with *Leucaena* is not worked out, as plants are in different stages of development and most of them are too small to provide sufficient tissues for the analysis (PCR, ELISA or Slot blot). PCR was performed in plants which survived kanamycin selection using the gene specific for kanamycin (Forward kanamycin 5' GAC CTG TCC GGT GCC CTG 3' & Reverse Kanamycin 5'GCG AAT CGG GAG CGG CGA TAC 3') with a few of the transformants, which amplified expected ~600 bp internal fragment of *kanamycin* gene (Fig. 5.15) and was confirmed by sequencing (Fig. 5.16).

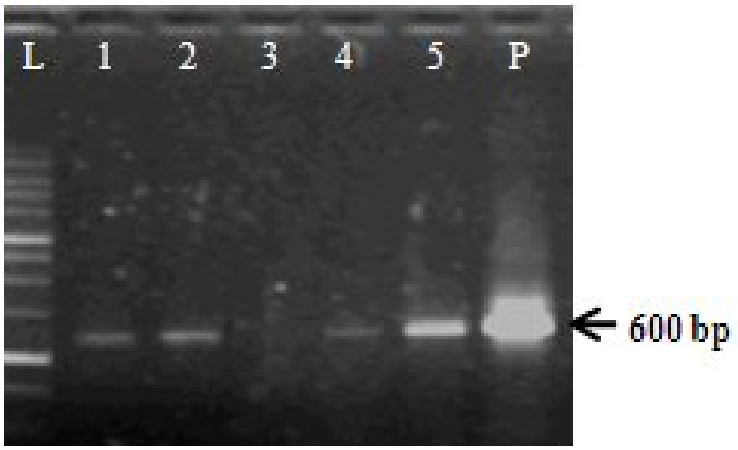


Fig. 5.15: PCR analysis of putative transgenic *Leucaena leucocephala* (Lane1 DNA marker, P- positive control showing a ~600 bp internal fragment of kanamycin gene, lane 1,2,4 & 5 positive for kanamycin, lane 3 untransformed plant.

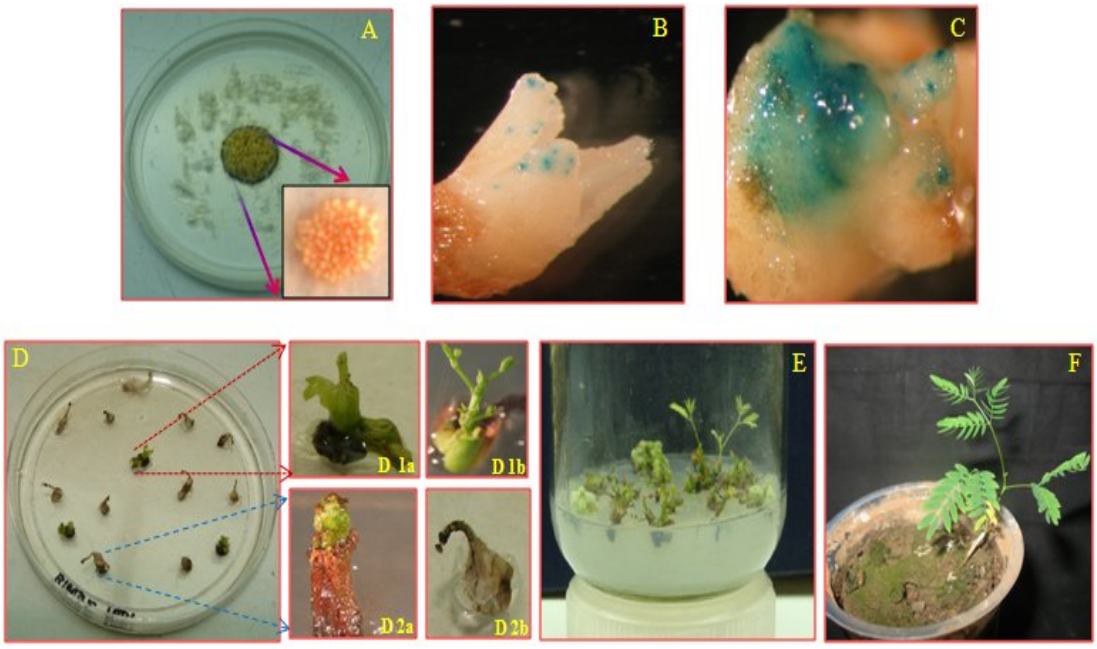


Fig. 5.6a: Biolistic transformation in *Leucaena leucocephala.* A. Embryos arranged for bombardment, B. Transient GUS expression showing fewer and well separated GUS foci using micron gold, C. Transient GUS expression with **HTC 600-Au** showing large number of merged GUS foci, D. Bombarded embryos on 200 mg/L Kanamycin selection, D1a and D1b regenerating embryos on kanamycin selection, D2a and D2b. necrosis and dying of untransformed embryos on kanamycin selection, E and F. Regeneration of putative transgenic plants from bombarded embryos.

```
Kanamycin-putativetransformant      TTGCCAGACCGACCTGTCCGGTGCCCTGAATGAACTCCAGGACGAGGCAG
Kanamycingene-CAMBIA                -----AGACCGACCTGTCCGGTGCCCTGAATGAACTCCAGGACGAGGCAG
                                         ********************************************

Kanamycin-putativetransformant      CGCGGCTATCGTGGCTTGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTC
Kanamycingene-CAMBIA                CGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTC
                                    *************** ********************************

Kanamycin-putativetransformant      GACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCC
Kanamycingene-CAMBIA                GACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCC
                                    **************************************************

Kanamycin-putativetransformant      GGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCA
Kanamycingene-CAMBIA                GGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCA
                                    **************************************************

Kanamycin-putativetransformant      TCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGC
Kanamycingene-CAMBIA                TCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGC
                                    **************************************************

Kanamycin-putativetransformant      CCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGAT
Kanamycingene-CAMBIA                CCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGAT
                                    **************************************************

Kanamycin-putativetransformant      GGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGC
Kanamycingene-CAMBIA                GGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGC
                                    **************************************************

Kanamycin-putativetransformant      TCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGC
Kanamycingene-CAMBIA                TCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGC
                                    **************************************************

Kanamycin-putativetransformant      GAGGATCTCGTCGTGACACATGGCGATGCCTGCTTGCCGAATATCATGGT
Kanamycingene-CAMBIA                GAGGATCTCGTCGTGACACATGGCGATGCCTGCTTGCCGAATATCATGGT
                                    **************************************************

Kanamycin-putativetransformant      GGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGG
Kanamycingene-CAMBIA                GGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGG
                                    **************************************************

Kanamycin-putativetransformant      CGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAG
Kanamycingene-CAMBIA                CGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAG
                                    **************************************************

Kanamycin-putativetransformant      GTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGC
Kanamycingene-CAMBIA                CTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGC
                                     **************************************************

Kanamycin-putativetransformant      TCCCGATTCGCAGCGC----
Kanamycingene-CAMBIA                TCCCGATTCGCAGCGCATCG
                                    ****************
```

Fig. 5.16: CLUSTAL W (1.8) multiple sequence alignment of kanamycin gene, shaded regions shown are part of forward and reverse primers

It was observed, that in addition to higher levels of transient GUS expression, the transformation efficiency was also on the higher side with HTC 600-Au compared with conventional gold microcarriers (Table 1). This increased transformation efficiency may be due to the crevice formed by the carbon over nanoparticles that help the DNA to be intact without getting lost when they pierce the cells. The average regeneration efficiency

from the three replicas obtained for tobacco was 41.73 and 38.93% with a transformation efficacy of 14.80 and 12.40% for HTC 600-Au and micro gold respectively. In case of rice the regeneration efficiency was 26.42 and 23.25% with a transformation efficacy of 9.33 and 8.42% for HTC 600-Au and micro gold respectively.

Table 1. Average regeneration and transformation efficiency obtained in tobacco and rice using HTC 600-Au and micron gold.

	Tobacco		Rice	
	HTC 600-Au	Micron gold	**HTC 600-Au**	Micron gold
Regeneration efficiency (%)	41.73	38.93	26.42	23.25
Transformation efficiency (%)	14.80	12.40	9.33	8.42

The present study was performed using, three different antisense constructs of *L. leucocephala* peroxidase gene (*LlPOX*) namely, *Anti-LlPOX* 3' end 750 bp, *Anti-LlPOX* 5' end 424 bp and *Anti-LlPOX* 3' NC260 bp (**Fig. 5.1a and Fig. 5.2a).** The constructs, ***Anti-LlPOX 3' end 750 bp*** and ***Anti-LlPOX 5' end 424 bp*** contains the conserved domains present in all the peroxidases; whereas ***Anti-LlPOX 3' NC260 bp*** is particularly designed from 3'region taking care no conserved domain is present in the construct. We observed severe stunted or retarded growth in plants when transformed using constructs having conserved domains (both 5'and 3'constructs). These plants were found growing initially hardly 0.5 cm, soon followed by the dying up of the apical meristem and from its axis a fresh axillary bud grows, which again dies and this process was found repeating; whereby, the plant in-short attained a height of 2.5 cms on an average with stunted appearance and even failed to produced roots when transferred to rooting medium (Fig. 5.17).

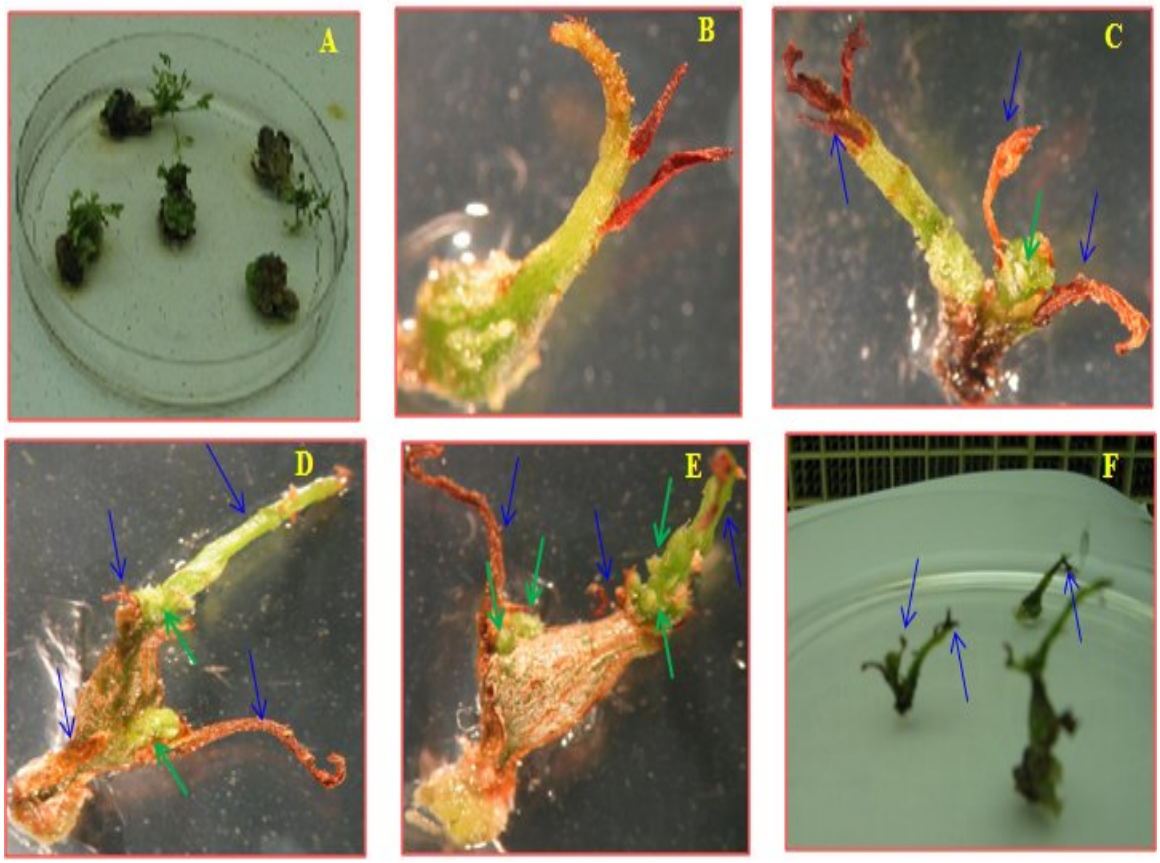


Fig 5.17: Biolistic transformation in *Leucaena leucocephala* using anti-sense constructs ***Anti-LlPOX 3' end 750 bp*** and ***Anti-LlPOX 5' end 424 bp*** of *POX* gene. A, B, C, D, E and F shows stunded growth of plants. The blue arrows indicate dying of growing meristem and green arrows indicate the emerging fresh axillary buds from the axis of dying shoots.

When *Anti-LlPOX 3' NC260 bp* construct was used in *Leucaena* transformation, normal regeneration was noticed but the plants were thin and slow growing compared to the untransformed control plants (Fig. 5.18). The Fig. 5.19 shows a comparative growth pattern of *Leucaena leucocephala* plants both transformed and untransformed.

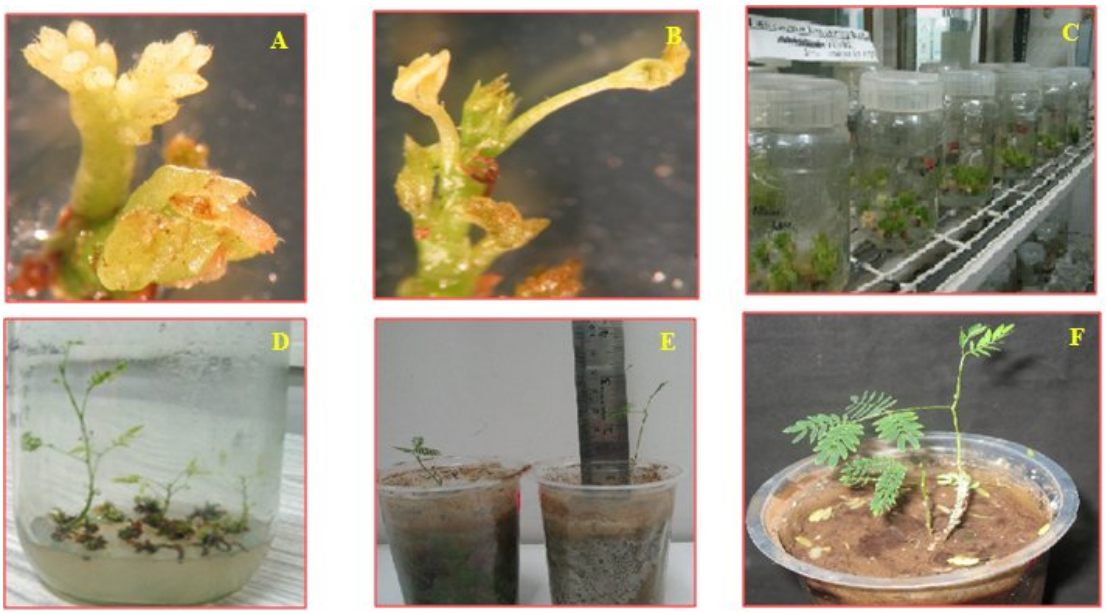


Fig. 5.18: Biolistic transformation in *Leucaena leucocephala* using anti-sense constructs ***Anti-LlPOX 3' NC260 bp.*** A, B, C, D, E & F shows different stages of embryo development after bombardment with *Anti-LlPOX 3' NC260 bp* construct.

Fig. 5.19: a. Untransformed plant not subjected to genetic transformation, **b.** putative transformants using construct ***Anti-LlPOX 3' NC260 bp*** of *POX* gene and **c.** putative transformants using constructs ***Anti-LlPOX 3' end 750 bp*** and ***Anti-LlPOX 5' end 424 bp*** of *POX* gene

5.3.6 Slot blot analysis of integration of gene in transgenic *Leucaena leucocephala*

The right and left hand border of pCAMBIA2301 harbours CaMV35S promoters, a part of *LlPOX gene* in antisense orientation, Kanamycin gene and GUS gene. Thus except *LlPOX* gene other nucleotides could be used as probe to analyze the transgenic plant. Slot blot was done using various transgenic events of *Leucaena* and a non transformed *Leucaena* plant (Fig 5.21). Genomic DNA was isolated from eight PCR positive transgenic plants for kanamycin gene and approximately 200 ng of DNA after treatment was blotted on the membrane. Blot was hybridized using kanamycin gene sequence as the probe. Very profound signals were observed from the putative transgenic lines when the blots were scanned using Typhoon TRIO + Phosho imager. The positive control (**P**) and negative control (**N**) were also blotted and there was no signal on negative control (non-transformed *Leucaena*).

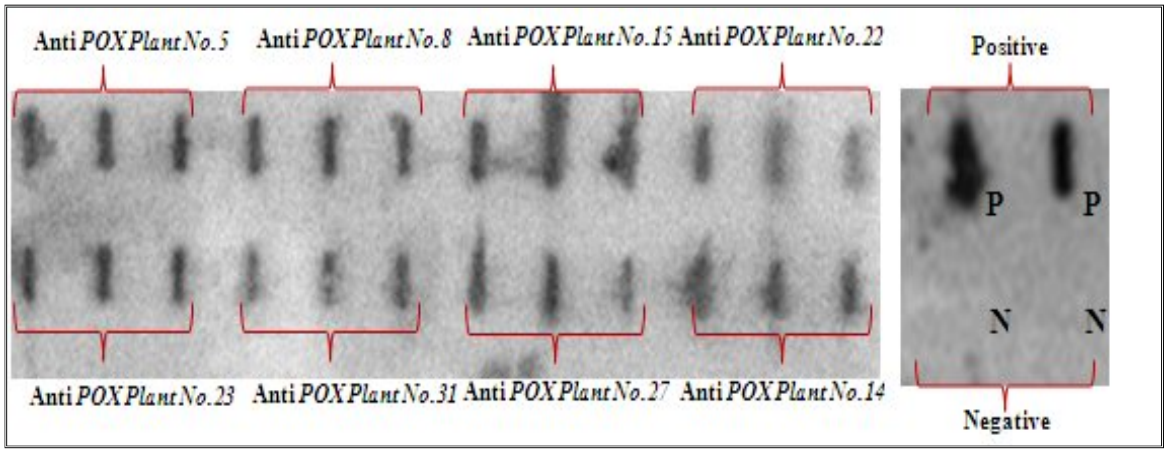


Fig 5.21: Slot blot analysis of transgenic *Leucaena* plants. The experiment was done in triplicates using kanamycin gene as the probe on gDNA from respective transgenic lanes mentioned above, Lanes Positive is, the signal with constructed recombinant pCAMBIA 2301 vector and Lane Negative is, non transformed *Leucaena* control plant showing no signal to kanamycin probe.

5.3.7 Analysis of transgenic plants by ELISA

Eight transgenic lines of *Leucaena* were tested for amount of extractable POX protein and ELISA was performed. A total of 10 μg protein was coated per each well, in triplicates. In this experiment non-transformed *Leucaena* was used as a negative control. ELISA was done for the above eight transformants, which were positive in PCR and slot

blot analysis (Section 5.3.6. (Fig 5.21). Primary antibodies and secondary antibodies were used at a dilution of 1:5000 and 1:10000 respectively.

ELISA analysis revealed a decreased level of extractable peroxidase protein in putative transformants over the non-transformed *Leucaena leucocephala* control plants, which in turn suggests lowering of *POX* activity in transgenic plants. This data indicates the integration of antisense *LlPOX* gene in *Leucaena* genome. ELISA of extractable POX protein showed that *Leucaena* transformants (Anti POX plants 5, 8, 14, 15, 22, 23, 27 and 31) have lower level of POX protein expression as compared to the non-transformed control plants in accordance to our expectations (Fig.5.22). The transgenic *Anti POX plant 8* showed lowest level of POX expression in comparison with other seven transgenic lines, this plant was subjected to immuno-cytolocalization and lignifications studies (Chapter 5; section 5.3.9).

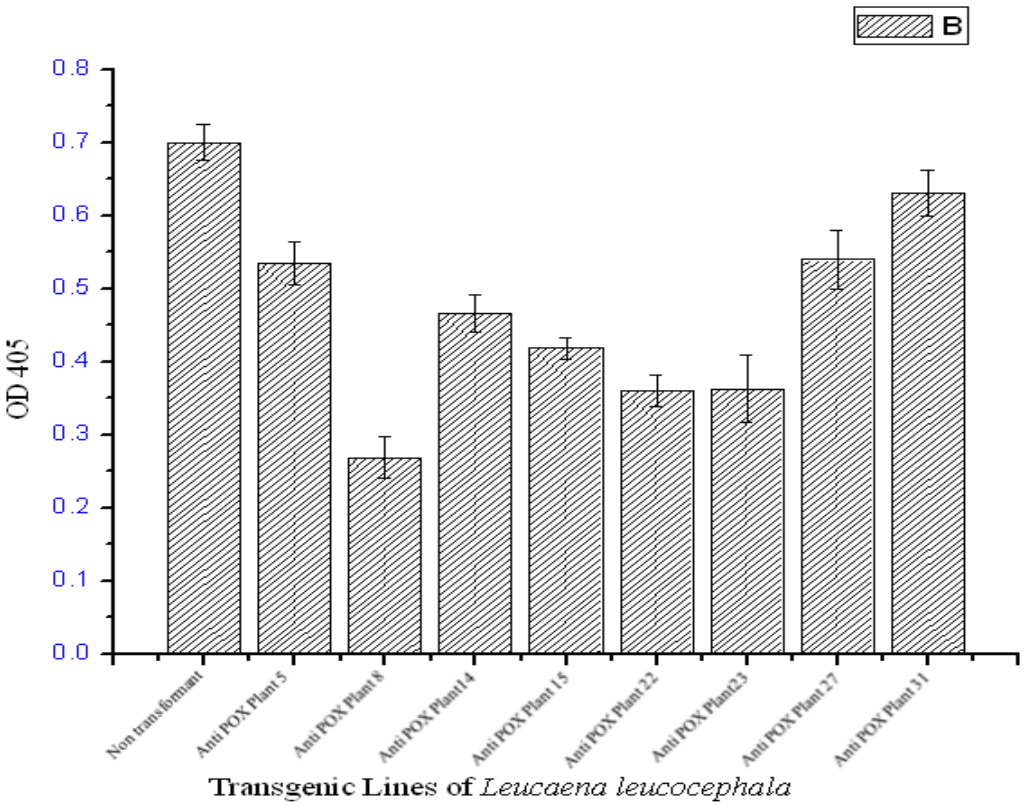


Fig. 5.22: ELISA of extractable POX protein from transgenic lines of *Leucaena leucocephala*. Anti POX plant 5 shows the lowest level of POX protein expression compared to all other transformants.

5.3.8 Comparative SEM analysis of explants surface bombarded with HTC 600-Au and conventional gold micro-carriers

SEM was performed to further confirm and get a better understanding of the HTC 600-Au penetration compared to the classical micro gold particles the SEM images obtained with soft and smooth rice calli are given in Fig 5.23. The Fig A and B shows the HTC 600-Au bombarded on the callus that shows the particles swimming inside the cell media with the surface showing smooth topography. Whereas in the Fig C and D (where the classical micro gold were used) the particles bombarded callus topography is not that smooth. This again indicates that micron sized gold particles causes lots of injury, whereas the carbon supported particles are able to pierce the embryo/ calli without causing much damage.

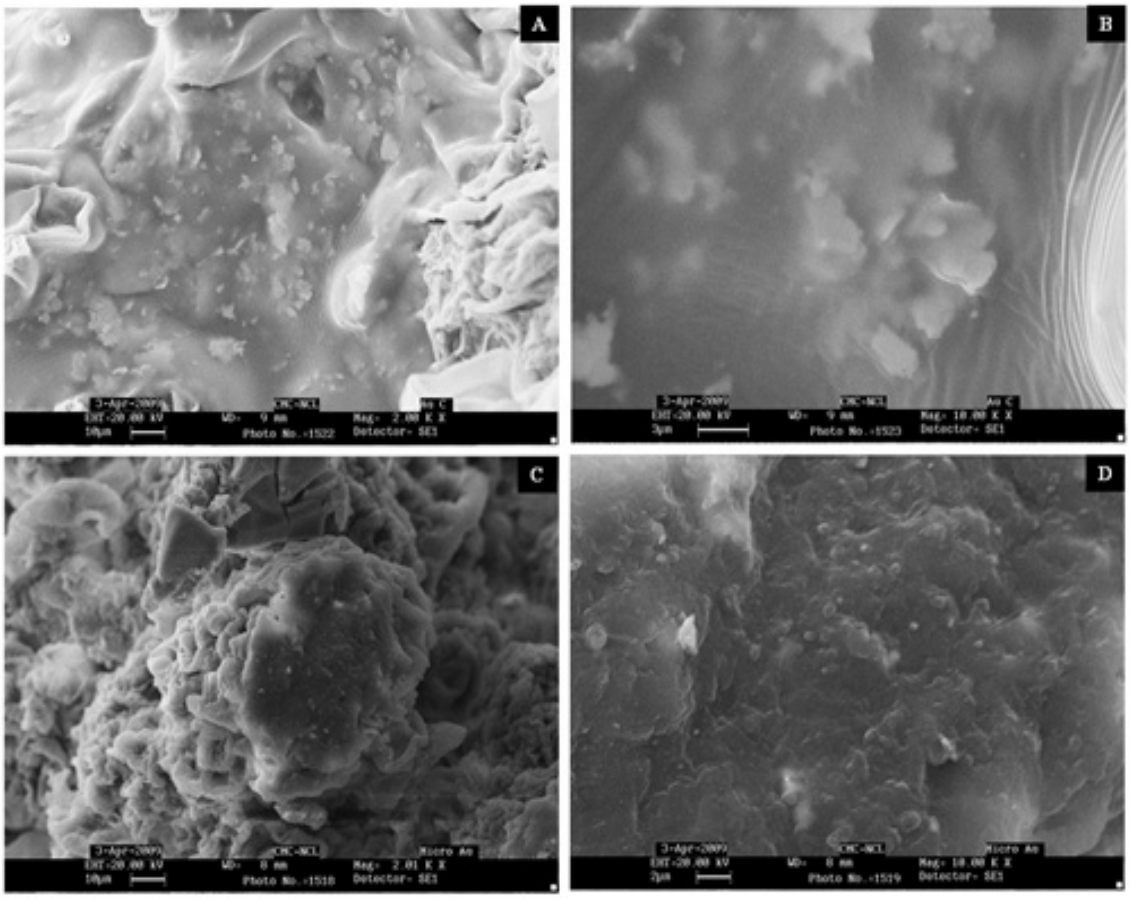


Fig 5.23: SEM image of biolistic bombarded rice callus. A and B Callus bombarded with **HTC 600-Au**; C and D Callus bombarded with micro gold particles.

SEM image of *Leucaena* embryo bombarded with different particles are given in the Fig 5.24. Fig 5.24 A and B shows the location of embryo used for the bombardment with the biolistic gun and the magnified image respectively. The highly convoluted bottom portion was anticipated to be the particle target. Fig 5.24 C and D represents 10 and 3 μm scale images respectively of the convoluted region before the bombardment showing smooth topography. Fig 5.24 E and F shows 10 and 3 μm scale images respectively of the *Leucaena* embryo surface bombarded with the HTC 600-Au particles. In the former plenty of micro particles spread on the surface could be noticed, in the later magnified image the particles were found to be placed inside the embryo slicing the surface. Fig 5.24 G and H shows 10 and 3 μm scale images respectively of the gold micro particle bombarded embryo. Many particles spread on the surface that have not entered the cells could be seen here. Additionally heavy damage is noticed to the embryo compared to the control and the HTC 600-Au bombarded surface. This may be due to the spherical nature of the classical micro gold carrier, which enters into the cell with greater rupture (akin to a cannon shell). The Fig5.24 I and J shows 1 μm and 300 nm scale images respectively of embryo surface bombarded with the extracellularly synthesized gold nanoparticles of ~20-50 nm size (that does not have carbon support) whose TEM image is given in the inset. Most of the particles spread have not penetrated the surface and very few particle injuries are there that showed least transformation output, which was not discussed. This exemplifies the necessity for a sharp carrier and that just spherical nanoparticles could not have resulted in better efficiencies as observed in HTC 600-Au case.

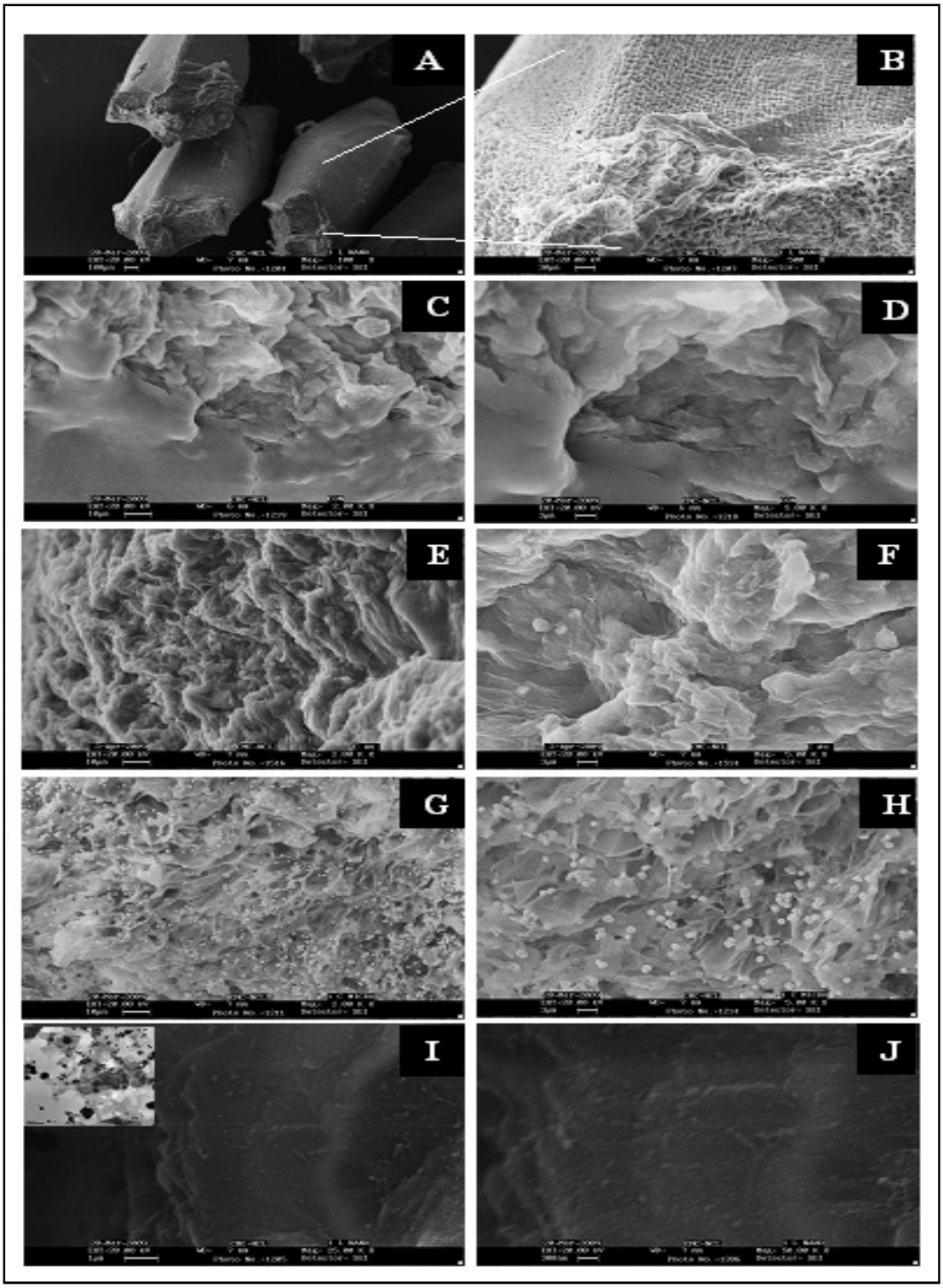


Fig 5.24: SEM image of biolistic bombarded *Leucaena* embryo. A, B, C and D Embryo at 100, 30, 10 and 3 µm scale; E and F Embryo bombarded with **HTC 600-Au** at 10 and 3 µm scale; G and H Embryo bombarded with micro gold particles at 10 and 3 µm scale; I and J embryo without bombardment at1 µm and 300 nm scale.

5.3.9 Immuno-cytolocalization of peroxidase (POX) protein in *L. leucocephala* transformants and lignin staining

For this study, utmost care was taken to select the control plants and transformed plants of same age group. Hence, the control plants are PCR negative (negative for GUS and kanamycin) plants which survived kanamycin selection, this plants showed better growth and bio-metric parameters (height, growth and rooting) over the transformants.

Lignin biosynthetic peroxidase protein was immuno-cytolocalized in stem tissues of control untransformed plants (Fig.5.25 A, B & C) and PCR positive putative transformants (Fig.5.25 D, E & F), with a view to find whether there exists reduction in peroxidase expression in lignifying tissues (*i.e* vascular bundle and xylem fibres). The deposition of blue-black to purple precipitate after incubating with BCIP/NBT mix confirms reduced levels of POX protein near the sites of lignification (Fig.5.25).

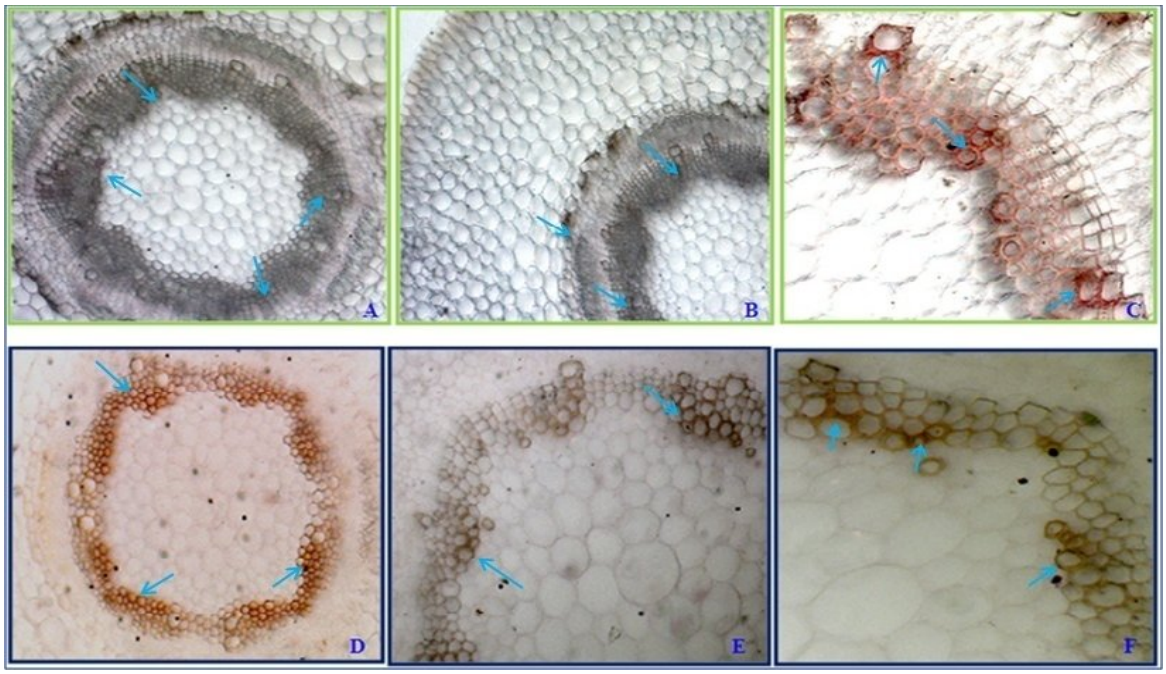


Fig 5.25: Immuno-cytolocalization of lignin biosynthetic peroxidase (POX) protein in *Leucaena leucocephala*. A, B & C stem sections of control plants showing higher levels of POX protein on xylem tissues over the transformed plants D, E & F. Control plants shows a well developed vascular bundles (continuous ring) over transformants (discontinuous ring)

The above sections once analysed for peroxidase were then subjected to staining for lignin using phloroglucinol (Fig.5.26). It is evident to naked eye that the extent of lignification and the level of expression of POX protein is low in putative transformants (Fig.5.26. D, E & F) when compared with the untransformed control plants (Fig.5.26. A, B & C).

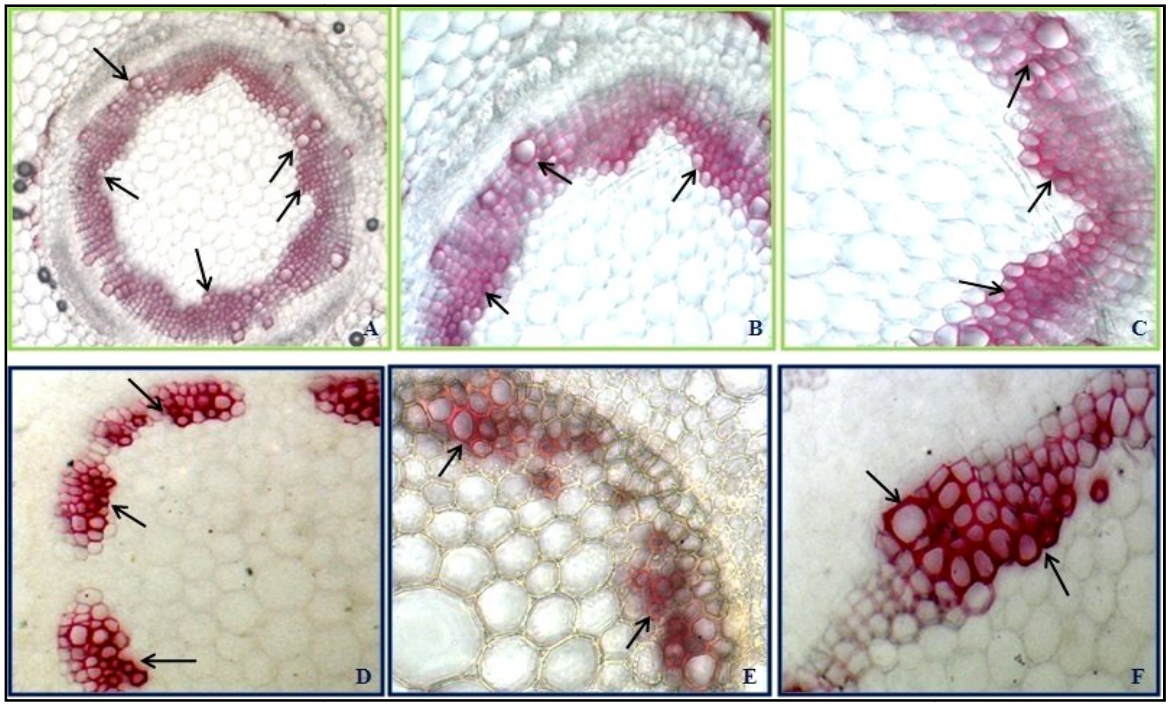


Fig 5.26: Phlorogluicinol staining of vascular bundles of *Leucaena leucocephala* to study extent of lignifications in control and transformed plants. A, B & C stem sections of control plants showing higher levels of lignifications on xylem tissues over the transformed plants D, E & F. Control plants shows a well developed vascular bundles (stained as continuous ring) over transformants (discontinuous ring).

5.3.10 Lignin estimation in transfomants

Lignin estimation was done on three transformed plants *Anti POX plant 5, Anti POX plant 8 and Anti POX plant 31*. Only the shoots were taken for the study, as the root system along with a part of stem was retained for maintaining the transgenic lines.

Table 2. Lignin estimation in transgenic *Leucaena leucocephala*

Plant	Acid soluble lignin (%)	Acid insoluble lignin (%)	Total lignin
Non-transformant	4.2730	32.00	36 (37.273)
Anti POX plant 5	3.838	28.24	32 (32.078)
Anti POX plant 8	2.713	25.73	28 (28.443)
Anti POX plant 31	3.622	30.17	33 (35.792)

Preliminary studies using *Anti-LlPOX 3' NC260 bp*, from non-conserved region of lignin biosynthetic peroxidase revealed that (Table. 2), in all the three transgenic lines analysed for lignin, both acid soluble and acid insoluble lignin content has been reduced compared with control untransformed (Matsushita *et al*., 2004) plants grown under ideal conditions. This inturn, resulted in reduction of total lignin content in the plants. The present study on lignin estimation showed reduction in total lignin content in all transgenics analysed over the control non-transfromed plant.

Further, to validate the results, once sufficient growth has been attained for all the transgenic plants, they will be analysed for G lignin content and S lignin content. Since peroxidases belong to multi gene family and reported (Quiroga *et al*., 2000) to have preferential polymerisation for conifery alcohol and sinapyl alcohol, further studies will give relavent information regarding, whether there exists any preferential down regulation in content of G lignin and S lignin and S/G ratio compared with control plants. Such studies require more amount of tissues for analysis, at present our main focus is to establish this transgenics in field conditions and to see whether they follow Mendelian inheritance for the transgene(s) over generations.

5.3.11 Conclusion

This is the first report of the biological synthesis of a Carbon-gold composite nano particle and its use as microcarrier in biolistic transformation of plant systems. The gene delivery was efficiently tested on a model plant *N. tabacum* that was further extended to monocot *Oryza sativa* and a hardy dicot tree species *L. leucocephala*. These nano gold embedded carbon matrixes reveal good dispersion of the transport material producing more number of GUS foci per unit area, as preferred in gene gun mediated plant transformation. Plant transformation efficacy (*Nicotiana, Oryza*) was found more using HTC 600-Au compared to conventional micron gold carrier. But ofcourse, for the plants analysed till date, HTC 600-Au gave more positive plants over gold carrier. The added advantage of the composite carrier is lower plasmid and gold requirements. SEM study showed least plant cell damage with carbon supported particles that can be related to its increased plant regeneration and transformation efficiency compared with the classical micro gold.

Three different anti-sense constructs of lignin biosynthetic *POX* gene were cloned in pCAMBIA 2301 binary vector and *L. leucocephala* was genetically transformed using particle bombardment and particle bombardment followed by co-cultivation. From the study it was clearly understood that *Leucaena* transformation using antisense *POX* construct from conserved domains of peroxidase genes failed to produce healthy plants. So the strategy to down regulate lignin in dicot plants like *Leucaena* should be by using antisense constructs of *POX* gene from non conserved regions.

Putative transformants of *Leucaena leucocephala* were analyzed by PCR, Slot blot and ELISA techniques; further in these *Leucaena* transformants the reduced levels of POX protein expression and in turn the lignifications was shown by studies like Immuno-cyto localization and lignin staining using phloroglucinol respectively. These transgenic lines were hardened and then transferred to green house for further growth. The transgenics will be further analysed and characterized as low/altered lignin producing transgenic lines of *Leucaena* and will be utilized for paper and pulp industry in future.

SUMMARY

SUMMARY

Evolution of the land plants and their ability to colonise the earth required a mechanism that allowed the transport of water and nutrients from the roots to the aerial parts. To allow water conduction through the cells, the hydrophilic carbohydrates are incrusted with lignin, which makes the cell walls hydrophobic and impermeable to water. Lignin is nature's second most abundant natural biopolymer and a major constituent of wood next to cellulose, it forms an integral cell wall component of all vascular plants and represents, on an average, 25% of the terrestrial plant biomass.

There is considerable interest in the biotechnological modification of (woody) plant lignin content and composition for three reasons; One, to delineate the mechanism(s) of lignin assembly in different cell types, whereas the other two include manipulating vascular plant biosynthetic pathways for improving the efficiency of pulp and paper making for quality, economic and environmental reasons and/or obtaining more readily digestible animal feed.

In the production of pulp (which is mainly cellulose), the hemicellulose and much of the lignin is removed to produce bright paper. This delignification process consumes large quantities of energy and hazardous chemicals and is neither, economical nor environment friendly. Hence, the tree species with reduced lignin content or altered quality of lignin is preferred by pulp and paper industry. Paper industry in India, mainly uses bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena* sp. as a source for pulp. Although all these plant species are of importance to the paper industry, *Leucaena sp.* is extensively used in India and contributes to about 25% raw material for the industry.

Lignin biosynthesis genes have not been studied to great extent in *L. leucocephala* especially, with regards to the enzymes involved in polymerization of monolignols to form the lignin. The reports on temporal and spatial expression pattern of *POX* gene(s) involved in monolignol polymerization is very limited in woody angiosperms. The present study on isolated *POX* gene(s) and their characterization will help in better understanding of their role in lignin biosynthesis in *Leucaena* and hence, genetic engineering could be attempted in this regard to meet the demands commercially raised by the pulp and paper industry.

A Few research groups in India are engaged in raising transgenic *Leucaena* sp. with low or altered lignin content. One of the prominent groups at National Chemical Laboratory (under

Council of Scientific and Industrial Research) is actively engaged in pursuing research on genes involved in lignin biosynthetic pathway in *L. leucocephala*. Myself, being part of this group, had the opportunity to work under the guidance of Dr. B. M. Khan and the work carried out is summarized here.

PCR based, RACE approach was used to fish out two *POX* cDNA clones namely, LBPox (Accession No. EU649680.1) and LlLBPOX (Accession No. GU143879). They both showed 95% sequence homology with each other. The full length cDNA clone of both the peroxidases were of 948 bp. Putative polyadenylation sites and the poly A tails were identified in the 3' UTRs. BLAST analysis revealed about 60 to 75% identity with other reported *POX*s in the database. Banding pattern in Southern hybridization suggested that at least 4 copies of *POX* gene is present in *L. leucocephala* genome. Analysis of deduced amino acid sequences of both the *POX* revealed highly homologous regions including the peroxidase active site and the ligand of heme binding. Phylogenetic analysis of deduced amino acid sequence was done using 19 protein sequences of POX from NCBI database using the neighbor joining method. The results showed that isolated POXs are evolutionarily most similar to *Pisum sativum.* In the present preliminary studies in this thesis, the emphasis is given to only one of the *POX homologs* namely, EU649680.1 and its, cloning, expression and characterization is done in detail.

The peroxidase gene (*POX*) isolated from *L. leucocephala* was directionally cloned in pET-28a(+) expression system. Recombinant POX protein was standardized for its over-expression and purified from inclusion bodies; it was used to raise antibodies in New Zealand white, rabbit. The candidate peroxidase protein was detected in *L. leucocephala* by Western blotting. The gene coding for mature peptide of *POX* was cloned in eukaryotic expression system of *Pichia pastoris* and was expressed as glycosylated protein in its active form. The POX enzyme activity for substrates like coniferyl alcohol, sinaply alcohol and guaiacol was also standardized from purified POX protein expressed in *Pichia*. The functional expression of recombinant POX protein confirms that the cDNA isolated from *L. leucocephala* encodes for lignin biosynthetic peroxidase enzyme.

Spatio-temporal expression profile was investigated using real-time PCR analysis under normal and stress induced (using mannitol and NaCl) conditions. It was found that *POX*

gene transcript was expressed in lignifying tissues of developing stem and root, specifically in vascular bundles or in the xylem. Extractable POX protein from developing seedlings were harvested at same point of time as in case of real-time PCR experiment, which also revealed the same pattern of *POX* gene expression. POX protein was immuno-cytolocalized in tissues which are undergoing lignification. Taking cognizance of above expression pattern and localization of POX protein near lignifying tissue it can be stated that cDNA isolated and characterized in Chapter Three of this thesis have definite role in lignin biosynthesis in *Leucaena leucocephala*.

The present study describes, the first time report of the biological synthesis of a Carbon-gold composite (HTC 600-Au) nano particle and its use as microcarrier in biolistic transformation of plant systems. The gene delivery was efficiently tested on model plant *N. tabaccum* that was further extended to monocot *Oryza sativa* and a hardy dicot tree species *L. leucocephala*. These nano gold embedded carbon matrixes reveal good dispersion of the transport material producing more number of GUS foci per unit area, as preferred in gene gun mediated plant transformation. Plant transformation efficacy (*Nicotiana, Oryza*) was found more using HTC 600-Au compared to conventional micron gold carrier. But ofcourse, for the plants analysed till date, HTC 600-Au gave more positive plants over conventional micron sized gold carrier. The added advantage of the composite carrier is lower plasmid and gold requirements. SEM study showed least plant cell damage with carbon supported particles that can be related to its increased plant regeneration and transformation efficiency compared with the classical micron gold.

Three different anti-sense constructs of lignin biosynthetic *POX* gene were cloned in pCAMBIA 2301 binary vector and *L. leucocephala* was genetically transformed using particle bombardment and particle bombardment followed by co-cultivation. From the study it was clearly understood that *Leucaena* transformation using antisense *POX* construct from conserved domains of peroxidase genes failed to produce healthy plants. So in the strategy using *POX* gene to down regulate lignin, in dicot plants like *Leucaena,* antisense constructs of *POX* gene should be strictly designed from the non conserved regions of the gene.

Putative transformants of *Leucaena leucocephala* were analyzed by PCR, Slot blot and ELISA techniques; further in these *Leucaena* transformants the reduced levels of POX

protein expression and in turn the lignifications was shown by studies like immuno-cyto localization and lignin staining using phloroglucinol respectively. Presently, these transgenic lines were hardened and then transferred to green house for further growth. The transgenics will be further analysed and characterized as low/altered lignin producing transgenic lines of *Leucaena* and will be utilized for paper and pulp industry in future.

FUTURE PROSPECTS

FUTURE PROSPECTS

- The peroxidase (POX) protein which is partially characterized will be studied in depth, including the attempts to isolate more isoforms of the gene from *Leucaena.* All the homologs of the gene will be cloned, expressed and characterized at their molecular and biochemical levels. The crystallization studies will be also attempted with different isoforms.

- Promoter region of *POX* gene(s) could be isolated and can be analysed for its expression using reporter genes like GUS and GFP.

- Putative transformants of *L. leucocephala* will be analysed for the transgene integration in their genome in detail and the plants will be confirmed over generations, to see the genes for which they are transformed follow Mendelian inheritance.

- More number of above characterized transgenic lines of *Leucaena* will be analysed for their performance for desired traits in field and most promising transformants suited for the paper industry could be multiplied for commercialization.

REFERENCES

References

A. Ahmad, P. Mukherjee, S. Senapati, D. Mandal, M. Islam Khan, R. Kumar and M. Sastry Extracellular biosynthesis of silver nanoparticles using the fungus *Fusarium oxysporum Colloids Surf. B*: Biointerfaces*,* 2003, 28, 313-318.

A. Das and G. Nanda, Production of xylanolytic enzymes during growth on pulverized grass by Aspergillus achraceus *Lett Appl Microbiol.,* 1995, 20, 141-144

A. Ahmad, S. Senapati, M. I. Khan, R. Kumar and M.Sastry, Extra/Intracellular Biosynthesis of Gold Nanoparticles by a Novel Alkalotolerant Fungus, Trichothecium sp. *J. Biomed. Nanotechnol.,*1, 2005, 47-53

Abbott JC, Barakate A, Pincon G, Legrand M , Lapierre C, Mila I, Schuch W, Halpin C (2002) Simultaneous suppression of multiple genes by single transgenes. Down-regulation of three unrelated lignin biosynthetic genes in tobacco. Plant Physiol 128: 844–853

Adler E (1977) Lignin chemistry—past present and future. Wood Sci Technol 11: 169–218

Allison MJ, Hammond AC, Jones RJ (1990) Detection of ruminal bacteria that degrade toxic dihydroxypyridine compounds produced from mimosine. Appl Environ Microbiol 56: 590-594

An G (1987) Binary ti vectors for plant transformation and promoter analysis. Meth Enzymol 153: 292-305

Angelini R, Federico R (1989) Histochemical evidence of polyamine oxidation and generation of hydrogen peroxide in the cell wall. J Plant Physiol 135:212-217

Anterola, A.M., Jeon, J.-H., Davin, L.B., Lewis, N.G., 2002a. Transcriptional control of monolignol biosynthesis in Pinus taeda: factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. The Journal of Biological Chemistry 277, 18272–18280.

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

Aoyama W, Sasaki S, Matsumura T. M, Hirai H, Tsutsumu Y and Nishida T. Sinapyl alcohol-specific peroxidase isoenzymes catalyzes the formation of dehydrogenative polymer from sinapyl alcohol. J wood Sci (2002), 497-504

References

Arnheim N, Erlich H (1992) Polymerase Chain Reaction Strategy. Annu Rev Biochem 61:131-156

Asada, K. (1992) Ascorbate peroxidase—a hydrogen peroxide- scavenging enzyme in plants. *Physiol. Plant.* **85,** 235–241

Atanassova R, Favet N, Martz F, Chabbert B, Tollier MT, Monties B, Fritig B, Legrand M (1995) Altered lignin composition in transgenic tobacco expressing O-methyltransferase sequences in sense and antisense orientation. Plant J 8: 465–477

B. L. V. Prasad, H. Sato, T. Enoki, Y. Hishiyama, Y. Kaburagi, A. M. Rao, P. C. Eklund, K. Oshida and M. Endo, Heat-treatment Effect on the nano-sized Graphite p-electron Network During Diamond to Graphite Conversion *Phys. Rev., B,* 2000, 62, 11209-11218

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

Baribault TJ, Skene KGM, Scott.NS (1989) Genetic transformation of grapevine cells. Plant Cell Rep. 8: 137-140.

Baribault TJ, Skene KGM, Cain PA, Scott NS (1990) Transgenic grapevines: regeneration of shoots expressing beta-glucuronidase. J. Exp. Bot. 41: 1045-1049.

Bate NJ, Orr J, Ni W, Meromi A, Nadler-Hassar T, Doerner PW, Dixon RA, Lamb CJ, Elkind Y (1994) Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. Proc Natl Acad Sci USA **91**: 7608–7612

Baucher M, Bernard-Vailhe MA, Chabbert B, Besle J.-M., Opsomer C, Van Montagu M, Botterman J (1999) Down-regulation of cinnamyl alcohol dehydrogenase in transgenic alfalfa (*Medicago sativa* L.) and the impact on lignin composition and digestibility. Plant Mol Biol 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, Boerjan W (1996) Red xylem and higher lignin extractability by down-regulating a cinnamyl alcohol dehydrogenase in poplar (*Populus tremula* x *P. alba*). Plant Physiol 112: 1479–1490

Baucher M, Halpin C, Petit-Conil M, Boerjan W (2003) Lignin: Genetic Engineering and

References

Impact on Pulping. Crit Rev Biochem Mol Biol 38: 305-350

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., van Montagu, M., Boerjan, W., 2000. In: Arencibia, A.D. (Ed), Plant Genetic Engineering: Towards the Third Millennium. Elsevier, Amsterdam, pp. 215–221.

Bednarek P, Schneider B, Svatos A, Oldham NJ, Hahlbrock K (2005) Structural complexity, differential response to infection, and tissue specificity of indolic and phenylpropanoid secondary metabolism in Arabidopsis roots. Plant Physiol 138:1058-1070

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C (1997). Cinnamate-4-hydroxylase expression in *Arabidopsis*. Plant Physiol 113: 729–738

Bernard-Vailhe MA, Cornu A, Robert D, Maillot M-P, Besle J-M (1996) Cell wall degradability of transgenic tobacco stems in relation to their chemical extraction and lignin quality. J Agric Food Chem 44: 1164–1169

Blee K, Choi JW, O'Connell AP, Jupe SC, Schuch W, Lewis NG, Bolwell GP (2001) Antisense and sense expression of cDNA coding for CYP73A15, a class II cinnamate 4-hydroxylase, leads to a delayed and reduced production of lignin in tobacco. Phytochemistry 57: 1159–1166

Blee KA, Choi JW, O'Connell AP, Schuch W, Lewis NG, Bolwell GP (2003) A lignin-specific peroxidase in tobacco whose antisense suppression leads to vascular tissue modification. Phytochemistry 64:163-176

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C, Dixon RA (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: 107–116

Blumberg DD (1987) Creating a ribonuclease-free environment. Meth. Enzymol 152: 20-24

Boerjan W, Ralph J, Baucher M (2003) Lignin Biosynthesis. Annu Rev Plant Biol 54: 519-546

Boerjan, W. 2005. Biotechnology and the domestication of forest trees. Current Opinion in Biotechnology 16: 159–166.

References

Boija E, Johansson G (2006) Interactions between model membranes and lignin-related compounds studied by immobilized liposome chromatography. Biochem Biophys Acta 1758:620-626

Bolwell GP (1996) The origin of oxidativeburst in plants. Biochem Soc Transact 24:438-442

Bomati EK, Noel JP (2005) Structural and kinetic basis for substrate selectivity in Populus tremuloides sinapyl alcohol dehydrogenase. Plant Cell 17: 1598–1611

Boniwell JM, Butt VS (1986) Flavin nucleotide-dependent 3-hydroxylation of 4-hydroxyphenylpropanoid carboxylic acids by particulate preparations from potato tubers. Z Naturforsch 41: 56–60

Bonnet E, Van de Peer Y, Rouzé P (2006) The small RNA world of plants. New Phytologist 171: 451-468.

Boudet AM (2000) Lignins and lignification: selected issues. Plant Physiol Biochem 38: 81-96

Boudet AM, Goffner D, Grima-Pettenati J (1997) In Lignin and Lignification: Recent biochemical and biotechnological advances. C R Acad Sci Paris pp 319, 317-331

Boudet AM, Lapierre C, Grima-Pettenati J (1995) Biochemistry and molecular biology of lignification. New Phytol 129: 203–236

Boudet, A.-M., 1998. A new view of lignification. Trends in Plant Sciences 3, 67–71.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* 72, 248–254.

Brewbaker JL, Sorenson CT (1990) Leucaena: New tree crops from interspecific hybrids. In: Janick. J and Simon J. (Eds). Advances in new crops Timber Press, Oregon. pp 238

Brownleader MD, Ahmed N, Trevan M, Chaplin MF, Dey PM (1995) Purification and partial characterization of tomato extensin peroxidase. Plant Physiol 109:1115-1123

Burlat V, Kwon M, Davin LB, Lewis NG (2001) Dirigent proteins and dirigent sites in lignifying tissues. Phytochemistry 57:883-897

Buxton DR, Redfearn DD (1997) Plant limitation to fiber digestion and utilization. J Nutr 127: S814-S818

References

Campbell MM, Sederoff RR (1996) Variation in lignin content and composition. Mechanisms of control and implications for the genetic improvement of plants. Plant Physiol 110: 3–13

Carpin S, Cr`evecoeur M, de Meyer M, Simon P, Greppin H, Penel C (2001) Identification of a Ca2C-pectate binding site on an apoplastic peroxidase. Plant Cell 13: 511–520

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1– 30

Chabannes M, Ruel K, Yoshinaga A, Chabbert B, Jauneau A, Joseleau J-P, Boudet A-M (2001a) In situ analysis of lignins in transgenic tobacco reveals a differential impact of individual transformations on the spatial patterns of lignin deposition at the cellular and subcellular levels. Plant J 28: 271–82

Chang HM, Sarkanen KV (1973) Species variation in lignin, effect of species on the rate of kraft delignification. Techn Assoc Pulp Pap Ind 56: 132–143.

Chapple CCS, Vogt T, Ellis BE, Somerville CR (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell 4: 1413–1424.

Chen X (2005) microRNA biogenesis and function in plants. FEBS Letters 579: 5923-5931.

Chen F, Kota P, Blount JW, Dixon RA (2001) Chemical syntheses of caffeoyl and 5-OH coniferyl aldehydes and alcohols and determination of lignin O-methyltransferase activities in dicot and monocot species. Phytochemistry 58: 1035–1042

Chen S, Schopfer P (1999) Hydroxyl-radical production in physiological reactions. A novel functions of peroxidase. Eur J Biochem 260:726-735

Chen YR, Sarkanen S (2003) Macro-molecular lignin replication: amechanistic working hyp othesis. Phytochemistry Rev 2:235-255

Christensen JH, Baucher M, O'Connell AP, 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., 64: 227–67. Dordrecht: Kluwer. pp 520 Fengel D and Wegener G, Eds., De Gruyter, Berlin

Christensen JH, Bauw G, Welinder KG, Montagu M, Boerjan W (1998) Purification and characterization of peroxidases correlated with lignification in poplar xylem. Plant

Physiol. 118: 125–135

Christensen JH, Overney S, Rohde A, Ardiles Diaz W, Bauw G, Simon P, Van Montagu M, Boerjan W (2001b) The syringaldazine-oxidizing peroxidase PXP 3-4 from poplar xylem: cDNA isolation, characterization and expression. Plant Mol Biol 47:581–593

Christensen JH, Van Montagu M, Bauw G, Boerjan W (2001a) Xylem peroxidases: purification and altered expression. In Molecular Breeding of Woody Plants, (Progress in Biotechnology Series, Vol. 18), pp 171–176. Morohoshi N and Komamine A, Eds., Elsevier Science, Amsterdam.

Chumakov KM (1994) Reverse transcriptase can inhibit PCR and stimulate primer dimer formation. PCR Methods Appl 4: 62-64

Cona A, Rea G, Angelini R, Federico R, Tavladoraki P (2006) Functions of amine oxidases in plant development and defence. Trends Plant Sci 11:80-88

Cramer CL, Edwards K, Dron M,. Liang X, Dildine SL, Bolwell GP, Dixon RA, Lamb CJ, Schuch W (1989) Phenylalanine ammonialyase gene organization and structure. Plant Mol Biol 12: 367–383

Croteau R, Kutchan TM, Lewis NG (2000) Natural products. In: Biochemistry and Molecular Biology of Plants. pp 1250–1318. Buchana, B. B., Gruissem,W., and Jones, R. L. Eds. American Society of Plant Biologist, Rockville, MD.

David W. Galbraith Silica breaks through in plants Nature Nanotechnology 2, 272- 273

Davin LB, Lewis NG (2000) Dirigent proteins and dirigent sites explain the mystery of specificity of radial precursor coupling in lignan and lignin biosynthesis. Plant Physiol 123: 453–461

Davin LB, Lewis NG (2005) Lignin primary structures and dirigent sites. Curr Opin Biotechnol 16: 407–415

Davin LB, Wang H-B, Crowell AL, Bedgar DL, Martin DM, Sarkanen S, Lewis NG (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275: 362–366

Delmer DP, Amor Y (1995) Cellulose biosynthesis. Plant Cell 7: 987–1000

Dharmawardhana DP, Ellis BE, Carlson JE (1995) A β-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. Plant Physiol 107: 331–39

Dharmawardhana DP, Ellis BE, Carlson JE (1999) cDNA cloning and heterologous

expression of coniferin β- -glucosidase. Plant Mol Biol 40:365-372

Dixon RA, Chen F, Guo D, Parvathi K (2001) The biosynthesis of monolignols: a "metabolic grid," or independent pathways to guaiacyl and syringyl units? Phytochemistry 57: 1069–1084

D'Mello JPF, Devendra C (Eds). Tropical legumes in animal nutrition. CAB International. Wallingford. Oxon, UK. pp 23 NRC (National Research Council) (1984) Nutritional requirements of goats. National Academy Press. Washington DC.

Donaldson L (2001) Lignification and lignin,topochemistry - an ultrastructural view.Phytoch emistry 57:859-873

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

Ducros V, Brzozowski AM, Wilson KS, Brown SH, Ostergaard P, Schneider P, Yaver DS, Pedersen AH, Davies GJ (1998) Crystal structure of the type-2 Cu depleted laccase from Coprinus cinereus at 2.2 Å resolution. Nat Struct Biol 5:310-316

Dunford, H.B.. In: Everse,J., Grisham, M.B. (Eds.), Peroxidases in Chemistry and Biology, Vol.2. CRC Press, Boca Raton, FL, pp.1–24.

Dwivedi UN, Campbell WH, Yu J, Datla RSS, Bugos RC, Chiang VL, Podila GK (1994) Modification of lignin biosynthesis in transgenic *Nicotiana* through expression of an antisense O-methyltransferase gene from *Populus*. Plant Mol Biol 26: 61–71

Eckert KT, Kunkel TA (1990) High fidelity DNA synthesis by the Thermus aquaticus DNApolymerase. Nucleic Acids Res 18: 3739- 3744

Ehlting J, Mattheus N, Aeschliman DS, Li E,Hamberger B, Cullis IF, Zhuang J, Kaneda.M, Mansfield SD, Samuels L, Ritland K,Ellis BE, Bohlmann J, Douglas CJ (2005)Globa l transcript profiling of primary,stems from,Arabidopsis thaliana identifies,candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. Plant J 42:618-640

El Mansouri I, Mercado JA, Santiago-Domenech N, Pliego-Alfaro F, Valpuesta V, Quesada MA (1999) Biochemical and phenotypical characterization of transgenic tomato plants overexpressing a basic peroxidase. Physiol Plant 106: 355–362

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

Elfstrand M, Sitbon F, Lapierre C, Bottin A, von Arnold S (2002) Altered lignin structure and resistance to pathogens in spi 2-expressing tobacco plants. Planta 214: 708–716

Elkind Y, Edwards R, Mavandad M, Hedrick SA, Ribak O, Dixon RA, Lamb CJ (1990) Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene. Proc Natl Acad Sci 87: 9057–9061

Esau K (1960) Anatomy of seed plants. John Wiley & Sons, New York

Escamilla-Trevino LL, Chen W, Card ML, Shih MC, Cheng CL, Poulton JE (2006) Arabidopsis thaliana β- glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides. Phytochemistry 67:1651-166

Espelie, K. E., Franceschi, V. R., and Kolattukudy, P. E. (1986) Academic Press, New York 12. *Plant Physiol.***81,** 487–492

FAO. 2004. [Internet site]. Preliminary review of biotechnology in forestry, including genetic modification. Forest genetic resources working paper FGR/59E. Available at: ftp://ftp.fao. rg/docrep/ fao/008/ae574e/ ae574e00.pdf.

FAO. 2005. [Internet site]. Global forest resources assessment 2005. Progress towards sustainable forest management. FAO forestry paper 147. Available at: ftp://ftp.fao.org/ docrep/fao/008/A0400E/A0400E00.pdf.

Faye, L., Johnson, K.D., Sturm, A., and Chrispeels, M.J. Structure biosynthesis, and function of asparagine-linked glycans on plant glycoproteins. *Physiol. Plant.* 75: 309, 1989.

Feinbeng AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13

Feinbeng AP, Vogelstein B (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal Biochem 137: 266-267

Fengel D, Wegener, G (1984) Lignin. In Wood: Chemistry, Ultrastructure, Reactions pp 132–181

Fergus BJ, Goring DAI (1970) The location of guaiacyl and syringyl lignins in birch xylem

tissue. Holzforschung 24: 113–17

Ferreira SA, Pitz KY, Manshardt R, Zee F, Fitch M., Gonsalves D (2002)Virus coat protein transgenic papaya provides practical control of Papaya ringspot virus in Hawaii. Plant Disease 86: 101-105.

Ferrer MA, Pedre˜no MA, Mu˜noz R, Ros Barcelo A (1990) Oxidation of coniferyl alcohol by cell wall peroxidases at the expense of indole-3-acetic acid and O2. A model for lignification of plant cell walls in the absence of H_2O_2. FEBS Lett 276: 127–130

Food and Agriculture Organization of the United Nations (2001). Pulp and Paper Capacities Survey 2000–2005. FAO, Rome.

Franc¸ Ois Torney, Brian G. Trewyn, Victor S.-Y. Lin and Kan Wang Mesoporous silica nanoparticles deliver DNA and chemicals into plants nature nanotechnology 2 2007

Franke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C (2002b) Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of *Arabidopsis*. Plant J 30: 47–59

Franke R, Humphreys JM, Hemm MR, Denault JW, Ruegger MO, Cusumano JC, Chapple C (2002a) The *Arabidopsis* REF8 gene encodes the 3-hydroxylase of phenylpropanoid metabolism. Plant J 30: 33–45

Franke R, McMichael CM, Meyer K, Shirley AM, Cusumano JC, Chapple C (2000) Modified lignin in tobacco and poplar plants overexpressing the *Arabidopsis* gene encoding ferulate 5-hydroxylase. Plant J 22: 223–234

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: pitfalls and potential. Biotechniques 26: 112-122, 124-125.

Freudenberg K, Neish AC, ed. (1968) Constitution and Biosynthesis of Lignin. Berlin: Springer-Verlag. pp 129

Freudenberg K, Harkin JM (1963) The glucosides of cambial sap of spruce. Phytochemistry 2:189-193

Friedmann M, Ralph SG, Aeschliman D, Zhuang J, Ritland K, Ellis BE, Bohlmann J, Douglas CJ (2007) Microarray gene expression profiling of developmental transitions in Sitka spruce (Picea sitchensis) apical shoots. J Exp Bot 58:593- 614

References

Fukuda H, Komamine A (1980) Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of Zinnia elegans. Plant Physiol 65:57-60

Gabaldón C, López-Serrano M, Pomar F, Merino F, Cuello J, Pedreño MA, Ros Barceló A (2006) Characterization of the last step of lignin biosynthesis in Zinnia elegans suspension cell cultures. FEBS Lett 580:4311-4316

Gabaldón C, López-Serrano M, Pedreño MA, Ros Barceló A (2005) Cloning and molecular characterization of the basic peroxidase isoenzyme from Zinnia elegans, an enzyme involved in lignin biosynthesis. Plant Physiol 139:1138-1154

Gajhede M, Schuller DJ, Henriksen A, SmithAT, Poulos TL (1997) Crystal structure of,hors erad-ish peroxidase C at 2.15 Å resolution. Nature Struct Biol 4:1032-1038

Gang DR, Costa MA, Fujita M, Dinkova- Kostova AT, Wang HB, Burlat V, Martin W, Sarkanen S, Davin LB, Lewis NG (1999) Regiochemical control of monolignol radical coupling: a new paradigm for lignin and lignan biosynthesis. Chem Biol 6:143-151

Goffner D, Campbell MM, Campargue C, Clastre M, Borderies G, Boudet A, Boudet AM (1994) Purification and characterization of cinnamoyl-CoA:NADP oxidoreductase in *Eucalyptus gunnii*. Plant Physiol 106: 625–632

Goldberg, T., Imberty, A., Liberman, M., and Prat, R. (1986) in Molecular and Physiological Aspects of Plant Peroxidases (Greppin, H., Penel, C., and Gaspar, T. ed) pp. 208–220, University of Geneva, Geneva

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

Goujon T, Ferret V, Mila I, Pollet B, Ruel K, Burlat V, Joseleau J-P, Barri`ere Y, Lapierre C, Jouanin L (2003) Down-regulation of the AtCCR1 gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. Planta 217: 218–228.

Grand C, Boudet A, Boudet AM (1983) Isoenzymes of hydroxycinnamate:CoA ligase from poplar stems: properties and tissue distribution. Planta 158: 225–229

Grand C, Parmentier P, Boudet A, Boudet AM (1985) Comparison of lignins and of

enzymes involved in lignification in normal and brown midrib (bm3) mutant corn seedlings. Physiol V´eg 23: 905– 911.

Grima-Pettenati J, Goffner D (1999) Lignin genetic engineering revisited. Plant Sci 145: 51–65.

Groover AT (2005) What genes make a tree a tree? Trends Plant Sci 10:211-214

Guan SY, Mlynar J, Sarkanen S (1997) Dehydrogenative polymerization of coniferyl alcohol on macromolecular lignin templates. Phytochemistry 45:911- 918

Guillaumie S, Pichon M, Martinant JP, Bosio M, Goffner D, Barrière Y (2007a) Differential expression of phenyl- propanoid and related genes in brown- midrib bm1, bm2, bm3 and bm4 young near-isogenic maize plants. Planta 226:235-250

Guillaumie S, San-Clemente H, Deswarte C, Martinez Y, Lapierre C, Murigneux A, Barrière Y, Pichon M, Goffner D (2007b) MAIZEWALL. Database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. Plant Physiol 143:339-363

Guo D, Chen F, Inoue K, Blout JW, Dixon RA (2001) Downregulation of caffeic acid 3-O-methyltransferase and caffeoyl CoA 3-O-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. Plant Cell 13: 73–88

Gupta H.K, Atreja PP (1999) Influence of feeding increasing levels of *Leucaena* leaf meal on the performance of milch goats and metabolism of mimosine and 3, 4-DHP. Anim Feed Sci and Technol 78: 159

H. Murayama and T. Maeda, A novel form of filamentous graphite *Nature,* 1990, 345, 791-793

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.

Halliwell B (1978) Lignin synthesis: the generation of hydrogen peroxide and superoxide by horseradish peroxidase and its stimulation by manganese (II) and phenols. Planta 140:81-88

Halpin C, Holt K, Chojecki J, Oliver D, Chabbert B, Monties B, Edwards K, Barakate A, Foxon GA (1998) Brown-midrib maize (bm1)—a mutation affecting the cinnamyl

alcohol dehydrogenase gene. Plant J 14: 545–553

Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, Chabbert B, Tollier M-T, and Schuch W (1994) Manipulation of lignin quality by downregulation of cinnamyl alcohol dehydrogenase. Plant J 6: 339–350

Hamberger B, Hahlbrock K (2004) The 4-coumarate:CoA ligase gene family in Arabidopsis thaliana comprises one rare, sinapate-activating and three commonly occurring isoenzymes. Proc Natl Acad Sci 101: 2209– 2214

Hammond AC, Allison MJ, Williams MJ (1989b) Persistence of DHP-degrading bacteria between growing seasons in subtropical Florida. Leucaena Research Report. 10: 66

Hammond AC, Allison MJ, Williams MJ, Prine, GM Bates DB (1989a) Prevention of Leucaena toxicosis of cattle in Florida by ruminal inoculation with 3, 4-DHP-degrading bacteria. Am J Vet Res 50: 2176

Harding SA, Leshkevich J, Chiang VL, 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: 428-438

Harkin JM, Obst TR (1973) Lignification in trees: Indication of exclusive peroxidase participation. Science 180:296-297

Harris PJ, Kelderman MR, Kendon MF and McKenzie RJ (1997) Monosaccharide compositions of unlignified cell walls of monocotyledons in relation to the occurrence of wall-bound ferulic acid. Biochem Syst Ecol 25: 167-179

Harthill, J.E. and Ashford, D.A. N-glycosylation of horseradish peroxidase from cell culture. *Biochem. Soc. Trans.* 20: 113s.1992.

Haselhoff J, Siemering KR, Prasher D, Hodge S (1997) Removal of a cryptic introns and subcellular localisation of green fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc. Natl. Acad. Science 94: 2122-2127.

Hatfield R, Vermerris W (2001) Lignin formation in plants. The dilemma of linkage specificity. Plant Physiol 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 J 7: 859–876

Hegarty MP, Court RD, Thorne PM (1964) The determination of mimosine and 3, 4-DHP in

biological material. Aust J Agric Res 15: 168

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6: 986-994

Henriksen A, Schuller DJ, Meno K, Welinder KG, Smith AT, Gajhede M (1998) Structural interactions between horseradish peroxidase C and the substrate benzhydroxamic acid determined by X-ray crystallography. Biochemistry 37:8054-8060

Hibino T, Takabe K, Kawazu T, Shibata D, Higuchi T (1995) Increase of cinnamaldehyde groups in lignin of transgenic tobacco plants carrying an antisense gene for cin9namyl alcohol dehydrogenase. Biosci Biotechnol Biochem 59: 929–931

Hiei Y, Komari T, Kubo T (1997). Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant mol. Biol. 35: 205-208.

Higuchi R, Dollinger G, Walsh PS, 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 (1997) Biochemistry and Molecular Biology of Wood (Springer Series in Wood Science). Springer, Berlin.

Higuchi T (2006) Monolignol biosynthesis and genetic engineering of lignin in trees, a review. Environmental Chemistry Letters 4: 143-146.

Hinman RL, Lang J (1965) Peroxidase catalyzed oxidation of indole3acetic acid. Biochemistry 4:144-158

Hiraga S, Yamamoto K, Ito H, Sasaki K, Matsui H, Honma M, Nagamura Y, Sasaki T, Ohashi Y (2000) Diverse expression,profiles of 21 rice peroxidase genes.FEBS Lett 471:245-250

Hoegger PJ, Kilaru S, James TY, Thacker JR,Kuees U (2006) Phylogenetic comparison,and classification of laccase and related multicopper oxidase protein sequences. FEBS J 273:2308-2326

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

References

Hossain MA, Hossain AKMZ, Kihara T, Koyama H, Hara T (2005) Aluminum-induced lipid peroxidation and lignin deposition are associated with an increase in H2O2 generation in wheat seedlings. Soil Sci Plant Nutr 51:223-230

Howles PA, Sewalt VJH, Paiva NL, Elkind Y, Bate NJ, Lamb C, Dixon R (1996) Over expression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. Plant Physiol 112: 1617–1624

Hu JJ, Tian YC, Han YF, Li L, Zhang BE (2001) Field evaluation of insectresistant transgenic *Populus nigra* trees. Euphytica 121: 123–127.

Hu W-J, Harding SA, Lung J, Popko JL., Ralph J, Stokke DD, Tsai C-J, Chiang, VL (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. Nat Biotechnol 17: 808–812

Hu W-J, Kawaoka A, Tsai C-J, Lung J, Osakabe K, Ebinuma H, Chiang VL (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate:CoA ligase genes in aspen (*Populus tremuloides*). Proc. Natl. Acad. Sci. 95:5407–5412.

Huggett J, Dheda K, Bustin S, Zumla A (2005) Real-time RT-PCR normalisation; strategies and considerations. Genes Immun 6:284

Hughes CE (1998) *Leucaena*: A genetic resources handbook. Tropical Forestry Papers No. 37. Oxford Forestry Institute, Department of Plant Science. Oxford University, UK

Humphreys JM, Chapple C (2002) Rewriting the lignin roadmap. Curr Opin Plant Biol 5: 224–29

Humphreys JM, Hemm MR, Chapple, C (1999) New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. Proc Natl Acad Sci 96: 10045–10050

J. J. Host, J. A. Block, K. Parvin and V. P. Dravid, Effect of annealing on the structure and magnetic properties of graphite encapsulated nickel and cobalt nanocrystals *J. Appl. Phys.,* 1998, 83, 793-801

J.C. Chen, Z.H. Lin and X.X. Ma, *Lett. Appl. Microbiol*, 2003, 37, 105–108 (d) A. Ahmad, S.Senapati, M I. Khan, R. Kumar, R Ramani, V Srinivas and M.Sastry Intracellular

References

Synthesis of Gold nanoparticles by a novel Alkalotolerent Actinomycete, Rhodococcus sp. *Nanotechnology,* 2003, 14, 824-828.

James, C. 2006. [Internet site]. Global status of commercialized biotech/GM crops: 2006. ISAAA Brief No. 35. ISAAA: Ithaca, NY. Available at: http://www.isaaa.org/resources/ publications/briefs/35/.

Jones L, Ennos AR, Turner SR (2001) Cloning and characterization of irregular xylem 4 (irx4): a severely lignin deficient mutant of Arabidopsis. Plant J 26: 205–216

Jones RJ (1979) The value of *Leucaena leucocephala* as feed for ruminants in the tropics World Animal review 32: 10

Jones RJ (1994) Management of anti-nutritive factors with special reference to Leucaena.In: Gutteridge RC, Shelton HM (Eds). Forage tree legumes in tropical agriculture. CAB International. Wallingford, UK. pp 216

Jones, HD (1984) Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development. Phytochemistry 23: 1349–1359

Jordan J. Green, Eugene Chiu, Elizaveta S. Leshchiner, Julie Shi, Robert Langer, and Daniel G. Anderson Electrostatic Ligand Coatings of Nanoparticles Enable Ligand-Specific Gene Delivery to Human Primary Cells 2007 874

Joseleau J-P, Ruel K (1997) Study of lignification by noninvasive techniques in growing maize internodes. An investigation by Fourier transform infrared cross-polarization-magic angle spinning 13C-nuclear magnetic resonance spectroscopy and immunocytochemical transmission electron microscopy. Plant Physiol 114: 1123–1133

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

Kaeppler HF, Menon GK, Skadsen RW, Nuutila AM, Carlson AR (2000) Transgenic oat plants via visual selection of cells expressing green fluorescent protein. Plant Cell Rep. 19: 661-666.

Kajita S, Hishiyama S, Tomimura Y, Katayama Y, Omori S (1997) Structural characterization of modified lignin in transgenic tobacco plants in which the activity

of 4-coumarate: coenzyme A ligase is depressed. Plant Physiol 114: 871– 879

Kajita S, Katayama Y, Omori S (1996) Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate: coenzyme A ligase. Plant Cell Physiol 37: 957–965

Kaothien P, Kawaoka A, Ebinuma H, Yoshida K, Shinmyo A (2002) Ntlim1, a PAL-box binding factor, controls promoter activity of the horse radishwound-inducible peroxidase gene. Plant Mol Biol 49:591-599

KaoYY, Harding SA, Tsai CJ (2002) Differential expression of two distinct phenylalanine ammonia-lyase genes in condensed tannin-accumulating and lignifying cells of quaking aspen. Plant Physiol 130: 796–807

Keller B, Templeton MD, Lamb CJ (1989) Specific localization of a plant cell wall glycine-rich protein in protoxylem cells of the vascular system. Proc Natl Acad Sci USA 86: 1529–1533

Kellogg DE, Rybalkin I, Chen S, Mukhamedova N, Vlasik T, Siebert P, Chenchik A (1994)

Kim H, Ralph J, Lu F, Pilate G, Lepl´e JC (2002) Identification of the structure and origin of thioacidolysis marker compounds or cinnamyl alcohol dehydrogenase deficiency in angiosperms. J Biol Chem 277: 47412–47419

Kimmel AR, Berger SL (1987) Preparation of cDNA and the generation of cDNA libraries:Overview. Meth Enzymol 152: 307-316

Koblitz H, Koblitz D (1964) Participation of cytochrome oxidase in lignification. Nature 204:199-200

Kojima M, Takeuchi W (1989) Detection and characterization of p-coumaric acid hydroxylase in mung bean, *Vigna mungo*, seedlings. J Biochem 105: 265–270

Korth KL, Blount JW, Chen F, Rasmussen S, Lamb C, Dixon RA (2001) Changes in phenylpropanoid metabolites associated with homologydependent silencing of phenylalanine ammonialyase and its somatic reversion in tobacco. Physiol Plant 111: 137–143

Kumar A, Ellis BE (2001) The phenylalanine ammonia-lyase gene family in raspberry. Structure, expression, and evolution. Plant Physiol 127: 230– 239

References

Kwok S, Kellogg DE, 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

Kyte, J. and Doolittle, R. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

Lachance D, Hamel LP, Pelletier F, Valéro J, Bernier-Cardou M, Chapman K, van Frankenhuyzen, K. Séguin, A (2007) Expression of a *Bacillus thuringiensis* cry1Ab gene in transgenic white spruce and its efficacy against the spruce budworm (*Choristoneura fumiferana*). Tree Genetics & Genomes 3: 153-167.

Lacombe E, Hawkins S, Doorsselaere JV, Piquemal J, Goffner D, Poeydomenge O, Boudet, AM (1997) Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: Cloning, expression and phylogenetic relationships. Plant J 11: 429–441

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lagrimini LM, Burkhart W, Moyer M, Rothstein S (1987) Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. Proc Natl Acad Sci USA 84: 7542– 7546

Lagrimini LM, Gingas V, Finger F, Rothstein S, Liu T-TY (1997) Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. Plant Physiol 114: 1187–1196

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251- 275

Lane B, Dunwell J, Ray J, Schmitt M, CumingA (1993) Germin a protein marker of,early pl ant development, is an oxalate oxidase. J Biol Chem 268:12239-12242.

Lannenpaa M (2005) Genetic regulation of birch flower development and potential biotechnological applications. University of Joensuu, PhD dissertations in biology. No 37. 94 p.

Lapierre C, Pollet B, MacKay JJ, Sederoff RR (2000) Lignin structure in a mutant pine deficient in cinnamyl alcohol dehydrogenase. J Agric Food Chem 48: 2326–2331

References

Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leple J-C, Boerjan W, Ferret V, De Nadai V, Jouanin, L (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid Omethyltransferase activity have opposite impact on the efficiency of industrial Kraft pulping. Plant Physiol 119: 153–163

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.

Lee D, Meyer K, Chapple C, Douglas CJ (1997) Antisense suppression of 4-coumarate: coenzyme A ligase activity in *Arabidopsis* leads to altered lignin subunit composition. Plant Cell 9: 985– 998

Lee LG, Connell CR, Bloch W (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res 21: 3761-3766

Leple J-C, Dauwe R, Morreel K, Storme V, Lapierre C, Pollet B, Naumann A, Kang K-Y, Kim H, Ruel K, Andre´e Lefe` bvre, Joseleau J-P, Grima-Pettenati J, De Rycke R, Andersson-Gunneras S, Erban A, Fehrle I, Petit-Conil M, Kopka J, Polle A, Messens E, Bjorn Sundberg,j, Mansfield SD, Ralph J, Pilate G, and Boerjan W. 2007. Downregulation of Cinnamoyl-Coenzyme A Reductase in Poplar: Multiple-Level Phenotyping Reveals Effects on Cell Wall Polymer Metabolism and Structure. *The Plant Cell* 19, 3669–3691.

Lewis NG (1999) A 20th century roller coaster ride: a short account of lignification. Curr Opin Plant Biol 2: 153–162

Lewis NG, Yamamoto E (1990) Lignin: Occurrence, biogenesis, and biodegradation. Annu Rev Plant Physiol Plant Mol Biol 41: 455–496

Leyva A, Liang X, Pintor-Toro JA, Dixon RA, Lamb CJ (1992) cis-Element combinations determine phenylalanine ammonia-lyase gene tissue specific expression patterns. Plant Cell 4: 263–271

Li L, Cheng X, Lu S, Nakatsubo T, Umezawa T, Chiang VL (2005) Clarification of cinnamoyl co-enzyme A reductase catalysis in monolignol biosynthesis of aspen. Plant Cell Physiol 46: 1073–1082

References

Li L, Cheng XF, Leshkevich J, Umezawa T, Harding SA, Chiang VL (2001) The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. Plant Cell 13: 1567–1585

Li L, Lu S, Chiang VL (2006). A genomic and molecular view of wood formation. Critical Reviews in Plant Sciences 25: 215-233.

Li L, Osakabe K, Joshi CP, Chiang VL (1999) Secondary xylem specific expression of caffeoyl-coenzyme A 3-O-methyltransferase plays an important role in the methylation pathway associated with lignin biosynthesis in loblolly pine. Plant Mol Biol 40: 555–565

Li L, Popko JL, Umezawa T, Chiang VL (2000) 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. J Biol Chem 275: 6537–6545

Li L, Popko JL, Zhang X.-H, Osakabe K, Tsai C-J, Joshi C P, Chiang VL (1997) A novel multifunctional O-methyltransferase implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. Proc Natl Acad Sci 94: 5461–5466

Li L, Zhou Y, Cheng X, Sun J, Marita JM, Ralph J, Chiang VL (2003) Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc Natl Acad Sci 100: 4939–4944

Lim EK, Jackson RG, Bowles DJ (2005) Identification and characterisation of Arabidopsis glycosyltransferases capable of glucosylating coniferyl aldehyde and sinapyl aldehyde. FEBS Lett 579:2802- 2806

Lim EK, Li Y, Parr A, Jackson R, Ashford DA, Bowles DJ (2001) Identification of glucosyltransferase genes involved in sinapate metabolism and lignin synthesis in Arabidopsis. J Biol Chem 276:4344- 4349

Lin SZ, Zhang ZY, Zhang Q, Lin YZ (2006) Progress in the study of molecular genetic improvements of Poplar in China. Journal of Integrative Plant Biology 48: 1001-1007.

Lindermayr C, Fliegmann J, Ebel J (2003) Deletion of a single amino acid residue from different 4-coumarate-CoA ligases from soybean results in the generation of new substrate specificities. J Biol Chem 278: 2781–2786

References

Livak KJ, Flood SJ, 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 MA, Guang-Ning Ye, Norman FW, Bruce IR (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, 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 8: 1641–1648

Luderitz T, Grisebach H (1981) Enzyme synthesis of lignin precursors. Comparison of cinnamoyl-CoA reductase and cinnamyl alcohol:NADP+ dehydrogenase from spruce (*Picea abies* L.) and soybean (*Glycine max* L.). Eur J Biochem 119: 115–124

M. Sastry, A. Ahmad, M. I. Khan and R. Kumar, Biosynthesis of metal nanoparticles using fungi and actinomycetes *Curr. Sci.,* 2003, *85*, 162-170.

Ma Q-H. 2007. Characterization of a cinnamoyl-CoA reductase that is associated with stem development in wheat. *Journal of Experimental Botany* 58, 2011–2021

MacKay JJ, O'Malley DM, Presnell T, Booker FL, Campbell MM, Whetten RW, Sederoff, RR (1997) Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. Proc Natl Acad Sci 94: 8255–8260

Mader M, Fussl, R (1982) Role of peroxidase in lignification of tobacco cells. II. Regulation by phenolic compounds. Plant Physiol 70: 1132–1134

Mäder M, Amberg-Fisher V (1982) Role of peroxidase in lignification of tobacco cells. I. Oxidation of nicotinamide adenine dinucleotide and formation of hydrogen peroxide by cell wall peroxidases in Nicotiana tabacum. Plant Physiol 70:1128-1131

Maher EA, Bate NJ, Ni W, Elkind Y, Dixon RA, Lamb CJ (1994) Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proc Natl Acad Sci 91: 7802–7806

References

Marcinowski S, Grisebach H (1978) Enzymology of lignification. Cell wall bound β-glucosidase for coniferin from spruce (Picea abies) seedlings. Eur J Biochem 87:37-44

Marita JM, Ralph J, Hatfield RD, Chapple C (1999) NMR characterization of lignins in Arabidopsis altered in the activity of ferulate 5-hydroxylase. Proc Natl Acad Sci 96: 12328–12332

Marita JM, Ralph J, Hatfield RD, Guo D, Chen F, Dixon RA (2003) Structural and compositional modifications in lignin of transgenic alfalfa down-regulated in caffeic acid 3 O-methyltransferase and caffeoyl coenzyme A 3 O-methyltransferase. Phytochemistry 62: 53–65

Marjamaa K, Hildén K, Kukkola E, Lehtonen,M, Holkeri H, Haapaniemi P, Koutaniemi,S, Teeri TH, Fagerstedt K, Lundell T (2006) Cloning, characterization and localization of three novel class III peroxidases in lignifying xylem of Norway spruce (Picea abies). Plant Mol Biol 61:719-732

Marjamaa K, Lehtonen M, Lundell T, Toikka M, Saranpää P, Fagerstedt KV (2003) Developmental lignification and seasonal variation in β-glucosidase and peroxidase activities in xylem of Scots pine, Norway spruce and silver birch. Tree Physiol 23:977-986

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 compuunds with xylan in 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

Matthew L (2004) RNAi for plant functional genomics. Comparative and Functional Genomics 5: 240-244.

McCaig BC, Meagher RB, Dean JF (2005) Gene structure and molecular analysis of the laccase-like multicopper oxidase (LMCO) gene family in Arabidopsis thaliana. Planta 221:619-636

References

McDougall GJ, Stewart D, Morrison IM (1994) Cell-wall-bound oxidases from tobacco (*Nicotiana tabacum*) xylem participate in lignin formation. Planta 194: 9–14

McDougall, G. J. (1993) *Phytochemistry* **33,** 765–767

McDougall, G. J., Stewart, D., and Morrison, I. M. (1996) *Phytochemistry* **41,** 43–47

Meilan R, Han KH, Ma C, DiFazio SP, Eaton JA, Hoien EA, Stanton BJ, Crockett RP, Taylor ML, James RR, Skinner JS, Jouanin L, Pilate G, Strauss SH (2002). The CP4 transgene provides high levels of tolerance to Roundup® herbicide in fieldgrown hybrid poplars. Canadian Journal of Forest Research 32: 967-976.

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001) Unravelling cell wall formation in the woody dicot stem. Plant Mol Biol 47: 239– 247

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

Meyer K, Cusumano JC, Somerville C, Chapple CCS (1996) Ferulate-5-hydroxylase from Arabidopsis thaliana defines a new family of cytochrome P450-dependent monooxygenases. Proc Natl Acad Sci 93: 6869–6874

Meyer K, Shirley AM, Cusumano JC, Bell-Lelong DA, Chapple C (1998) Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. Proc Natl Acad Sci 95: 6619– 6623

Meyermans H, Morreel K, Lapierre C, Pollet B, De Bruyn A, Busson R, Herdewijn P, Devreese B, Van Beeumen J, Marita JM, Ralph J, Chen C, Burggraeve B, Van Montagu M, Messens E, Boerjan W (2000) Modification in lignin and accumulation of phenolic glucosides in poplar xylem upon down-regulation of caffeoyl-coenzyme A O-methyltransferase, an enzyme involved in lignin biosynthesis. J Biol Chem 275: 36899–36909

Mitchell P (2001) Microfluidics– downsizing large-scale biology. Nat Biotechnol 19: 717–721

Moller SG, McPherson MJ (1998) Developmental expression and biochemical analysis of the Arabidopsis atao1 gene encoding an H_2O_2-generating diamine oxidase. Plant J 13: 781–791

References

Monties B (1998) Novel structures and properties of lignins in relation to their natural and induced variability in ecotypes, mutants and transgenic plants. Polymer Degrad Stabil 59: 53–64

Mullis KB (1990) The unusual origin of the polymerase chain reaction. Sci Am 262: 56–61

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase catalysed reaction. Methods Enzymol 255: 335-350.

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

N. Durán, P. D. Marcato, O. L. Alves, G. I. H. De Souza and E. Esposito, *J Nanobiotechnology*, 2005, 3, 8

Nair RB, Xia Q, Kartha CJ, Kurylo E, Hirji RN, Datla R, Selvaraj G (2002) *Arabidopsis* CYP98A3 mediating aromatic 3-hydroxylation. Developmental regulation of the gene, and expression in yeast. Plant Physiol 130: 210–20

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible cosuppression of homologous genes in trans. The Plant Cell 2: 279289.

NAS [National Academy of Sciences] (1984) *Leucaena*: promising forage and tree crop for the tropics. 2nd ed. Washington, DC: National Academy of Sciences. pp 100

Nehra, N.S., Becwar, M.R., Rottmann, W.H., Pearson, L., Chowdhury, K., Chang, S., Wilde, H.D., Kodrzycki, R.J., Zhang, C., Gause, K.C., Parks, D.W. & Hinchee, M.A.W. 2005. Invited Review: Forest Biotechnology: Innovative Methods, Emerging Opportunities. In Vitro Cellular and Developmental Biology - Plant 41: 701–717.

Ni W, Paiva NL, Dixon RA (1994) Reduced lignin in transgenic plants containing a caffeic acid Omethyltransferase antisense gene. Transgenic Res 3: 120–126

Nieminen K M, Kauppinen L, Helariutta Y (2004) A weed for wood? Arabidopsis as a genetic model for xylem development. Plant Physiol 135:653-659

Nimz HH, Tutschek R (1977) Holzforschung 31: 101–106.

Nishizawa K, Kita Y, Kitayama M, Ishimoto M (2006) A red fluorescent protein, DsRed2, as a visual reporter for transient expression and stable transformation in soybean. Plant Cell Rep. 25: 1355–1361.

References

Norton BW, Poppi DP (1995) Composition and nutritional attributes of pasture legumes. In: D'Mello JPF, Devendra C (Eds). Tropical legumes in animal nutrition. CAB International. Wallingford. Oxon, UK. pp 23.

Nose M, Bernards MA, Furlan M, Zajicek J, Eberhardt TL, Lewis NG (1995)Towards the specification of consecutive steps in macromolecular lignin assembly. Phytochemistry 39:71-79

O. E. Andersson, B. L. V. Prasad, H. Sato, T. Enoki, Y. Hishiyama, Y. Kaburagi, M. Yoshikawa and S. Bandow, *Phys. Rev., B,* 1998, 58, 16387-16395

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

O'Connell, A., Schuch, W., Bolwell, G.P., 1998. Impact of forest biotechnology on the pulp and paper making process in the 21st century. In: Lindsay, K. (Ed.), Transgenic Plant Research. Harwood Academic Publishers, Amsterdam, pp. 175–186.

Ohl S, Hedrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. Plant Cell 2: 837–848

Okayama H, Berg P (1982) High-efficiency cloning of full-length cDNA. Mol Cell Biol 2:161- 170

Olden, K., Bernard, B.A., Humphries, M.J. , Yeo, T.-K., Yeo, K.-T., White, S.L., Newton, S.A., Bauer, H.C., and Parent, J.B. Function of glycoprotein glycans. *TIBS* 10:78, 1985.

Onnerud H, Zhang L, Gellerstedt G, Henriksson G (2002) Polymerization of monolignols by redox shuttle-mediated enzymatic oxidation: a new model in lignin biosynthesis. I Plant Cell 14: 1953–62

Osakabe K, Tsao CC, Li L, Popko JL, Umezawa T, Carraway DT, Smeltzer RH, Joshi CP, Chiang VL (1999) Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. Proc Natl Acad Sci 96: 8955–8960

Osakabe Y, Ohtsubo Y, Kawai S, Katayama Y, Morohoshi N (1995) Structures and tissue-specific expression of genes for phenylalanine ammonialyase from a hybrid aspen. Plant Sci 105: 217–226

References

Ostergaard L, Teilum K, Mirza O, Mattsson O, Petersen M, Welinder KG, Mundy J, Gajhede M, Henriksen A (2000) *Arabidopsis* ATP A2 peroxidase. Expression and high-resolution structure of a plant peroxidase with implications for lignification. Plant Mol Biol 44: 231–243

P. Mukherjee, A. Ahmad, D. Mandal, S. Senapati, S. R. Sainkar, M. I. Khan, R. Parishcha, P. V. Ajaykumar, M. Alam, R. Kumar and M. Sastry, Fungus-Mediated Synthesis of Silver Nanoparticles and Their Immobilization in the Mycelial Matrix: A Novel Biological Approach to Nanoparticle Synthesis *Nano Lett.,*. 2001, 1, 515-519

P. Z. Si, Z. D. Zhang, D. Y. Geng, C. Y. You, X. G. Zhao and W. S. Zhang, Synthesis and Characteristics of Carbon-coated iron and nickel nanocapsules produced by arc discharge in ethanol vapor *Carbon,* 2003, 41, 247-251

Parham RA (1987) Structure, chemistry and physical properties of woody raw materials. In *Pulp and Paper Manufacture, Vol. 1: Properties of Fibrous Raw Materials and Their Preparation for Pulping*, 3rd ed. M.J. Kocurek, and F. Stevens, Eds., Tappi and Canadian Pulp and Paper Association Joint Textbook Committee of the Paper Industry, Montreal.

Parvathi K, Chen F, Guo D, Blount JW, Dixon RA (2001) Substrate preferences of Omethyltransferases in alfalfa suggest new pathways for 3-O-methylation of monolignols. Plant J 25:193–202

Pasonen HL, Seppänen SK, Degefu Y, Rytkönen A, von Weissenberg K, Pappinen A (2004) Field performance of chitinase transgenic silver birches (*Betula pendula*): resistance to fungal diseases. Theoretical and Applied Genetics 109: 562-570.

Passardi F, Cosio C, Penel C, Dunand C(2005) Peroxidases have more functions,than a Swis s army knife. Plant Cell Rep 24:255-265

Passardi F, Longet D, Penel C, Dunand C (2004) The class III peroxidase multigenic family in rice and its evolution in land plants. Phytochemistry 65:1879-1893

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.A

References

Pesquet E, Ranocha P, Legay S, Digonnet C, Barbier O, Pichon M, Goffner D (2005) Novel markers of xylogeneses in Zinnia are differentially regulated by auxin and cytokinin. Plant Physiol 139:1821-1839

Petersen M, Strack D, Matern U (1999) Biosynthesis of phenylpropanoids and related compounds. In: Biochemistry of Plant Secondary Metabolism. Wink, M., Ed., Vol. 2, pp 151–222, Sheffield Academic Press, Sheffield, UK.

Pfaffl MW. (2001). A new mathematical model for relative quantification real-time RT-PCR. *Nucleic acid research* **29 (9):** 2002-2007.

Pickett-Heaps JD (1968) Xylem wall deposition. Radioautographic investigations using lignin precursors. Protoplasma 65: 181–205

Pilate G, Guiney E, Holt K, Petit-Conil M, Lapierre C, Leple J-C, Pollet B, Mila I, Webster EA, Marstorp HG, Hopkins DW, Jouanin L, Boerjan W, Schuch W, Cornu D, Halpin C (2002) Field and pulping performances of transgenic trees with altered lignification. Nature Biotechnol 20: 607–612

Pincon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau J-P, Boudet AM, Legrand M (2001b) 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: 145–155

Pincon G, Maury S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M (2001a) Repression of O-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. Phytochemistry 57: 1167–1176

Piquemal J, Lapierre C, Myton K, O'Connell A, Schuch W, Grima-Pettenati J, Boudet A-M (1998) Down-regulation in cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. Plant J 13: 71–83

Plomion C, Leprovost G, Stokes A (2001) Wood formation in trees. Plant Physiol 127:1513-1523

Pottinger AJ, Hughes CE (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

References

Quiroga M, Guerrero C, Botella MA, Barcelo A, Amaya I, Medina MI, Alonso FJ, De Forchetti SM, Tigier H, Valpuesta V (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiol 122:1119-1127

Raes J, Rohde A, Christensen JH, Peer YV, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in arabidopsis. Plant Physiol 133: 1051–1071

Raeymaekers L (2000) Basic principles of quantitative PCR. Mol Biotechnol 15: 115-122

Ralph J, Grabber JH, Hatfield RD (1995) Lignin-ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydr Res 275: 167–78

Ralph J, MacKay JJ, Hatfield RD, O'Malley DM, Whetten RW, Sederoff RR (1997) Abnormal lignin in a loblolly pine mutant. Science 277: 235–39

Ralph J, Hatfield RD, Piquemal J, Yahiaoui N, Pean M, Lapierre C, Boudet AM (1998) NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamyl alcohol dehydrogenase and cinnamoyl CoA reductase. Proc Natl Acad Sci 95: 12803–12808

Ralph J, Kim H, Peng J, Lu F (1999b) Arylpropane-1, 3-diols in lignins from normal and CAD-deficient pines. Org Lett 1: 323–26

Ralph J, Lapierre C, Lu F, Marita JM, Pilate G (2001) NMR evidence for benzodioxane structures resulting from incorporation of 5-hydroxyconiferyl alcohol into lignins of O-methyltransferase-deficient plants. J Agric Food Chem 49: 86–91

Ralph J, Lapierre C, Marita JM, Kim H, Lu F (2001) Elucidation of new structures in Lignins of CAD-and COMT-deficient plants by NMR. Phytochemistry 57: 993–1003

Ralph J, Lapierre C, Marita JM, Kim H, Lu F, Hatfield RD, Ralph S, Chapple C, Franke R, Hemm MR, Van Doorsselaere J, Sederoff RR, O'Malley DM, Scott JT, Mackay JJ, Yahiaoui N, Boudet A-M, Pean M, Pilate G, Jouanin L, Boerjan W (2001a) Elucidation of new structures in lignins of CAD-and COMT-deficient plants by NMR. Phytochemistry 57: 993–1003

Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Christensen JH, Boerjan W (2004) Lignins: Natural polymers from oxidative

coupling of 4-hydroxyphenylpropanoids. Phytochemistry Rev 3: 29–60

Ralph J, Marita JM, Ralph SA, Hatfield RD, Lu F, Ede RM, Peng J, Quideau S, Helm RF, Grabber JH, Kim H, Jimenez-Monteon G, Zhang Y, Jung H-JG, Landucci LL, MacKay JJ, Sederoff RR, Chapple C, Boudet AM (1999a) Solution-state NMR of lignins. In Advances in Lignocellulosic Characterization. pp 55–108 Argyropoulos DS, Ed.,TAPPI Press, Atlanta, GA.

Ralph J, Peng J, Lu F, Hatfield RD (1999) Are lignins optically active? J Agric Food Chem 47: 2991–2996

Ralph, J (1996) An unusual lignin from kenaf. J Nat Prod 59: 341–342

Ranocha P, McDougall G, Hawkins S, Sterjiades R, Borderies G (1999) Biochemical characterization, molecular cloning and expression of laccases—a divergent gene family—in poplar. Eur J Biochem 259: 485–495

Reichel C, Mathur J, Eckes P, Langenkemper K, Reiss B, Koncz C, Schell J, Maas C (1996) Enhanced green fluorescence by the expression of an *Aequorea victoria* green fluorescent protein mutant in mono- and dicotyledonous plant *cells. Proc. Natl. Acad. Sci. USA. 93: 58885893.*

Reischl U, Wittwer CT, Cockerill F (2002) Rapid Cycle Real-time PCR: Methods and Applications; Microbiology and Food Analysis. New York: Springer-Verlag

Ros Barceló A (2005) Xylem parenchyma cells deliver the H2O2 necessary for lignification in differentiating xylem vessels. Planta 220:747-756

S. Senapati, A. Ahmad, M. I. Khan, M. Sastry and R. Kumar; Extracellular Biosynthesis of Bimetallic Au-Ag Alloy Nanoparticles *Small,* 2005, 1, 517 – 520

Saha BK, Tian B, Bucy RP (2001) Quantitation of HIV-1 by real-time PCR with a unique fluorogenic probe. J Virol Methods 93: 33-42

Saiki R, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350-1354

Saiki RK, Gelfand DH, Stoffel S, Scharf S, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing

phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

Saka S, Goring DAI (1985) Localization of lignins in wood cell walls. In Biosynthesis and Biodegradation ofWood Components, ed. Higuchi T, pp 51–62. Orlando: Academic. pp 679

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

Samuels AL, Rensing K, Douglas CJ, Mansfield S, Dharmawardhana P, Ellis B (2002) Cellular machinery of wood production: differentiation of secondary xylem in *Pinus contorta* var. latifolia. Planta 216:72–82

Sarkanen KV, Ludwig CH (1971) Lignins: Occurrence, Formation, Structure, and Reactions. New York: Wiley-Intersci pp 916

Sarni F, Grand G, Boudet AM (1984) Purification and properties of cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase from poplar stems. Eur J Biochem 139: 259–265

Sasaki S, Nishida T, Tsutsumi Y, Kondo R (2004) Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in β -O-4 linkage. FEBS Lett 562:197-201

Sasaki S, Baba K, Nishida T, Tsutsumi Y, Kondo R (2006) The cationic cell-wall-peroxidase having oxidation ability for polymeric substrate participates in the late stage of lignification of Populus alba (L.) Plant Mol Biol 62:797-807

Sato Y, Demura T, Yamawaki K, Inoue Y, Sato S, Sugiyama M, Fukuda H (2006) Isolation and characterization of a novel peroxidase gene ZPO-C whose expression and function are closely associated with lignification during tracheary element differentiation Plant Cell Physiol 47:493-503.

Sato Y, Bao W, Sederoff R, Whetten R (2001)Molecular cloning and expression of eight laccase cDNAs in loblolly pine (Pinus taeda). J Plant Res 114:147-155

Sato Y, Sugiyama M, Gorecki RJ, Fukuda H, Komamine A (1993) Interrelationship between lignin deposition and the activities of peroxidase isoenzymes in differentiating tracheary elements of Zinnia. Analysis using L-α-aminoxy-β-phenylpropionic acid and 2-aminoindan- 2-phosphonic acid. Planta 189:584-589

References

Savidge R, Förster H (1998) Seasonal activity,of uridine 5'-diphosphoglucose: coniferylalcohol glucosyltransferase in relation to cambial growth and dormancy in conifers. Can J Bot 76:486-493

Savidge RA, Udagama-Randeniya PV (1992) Cell wall-bound coniferyl alcohol oxidase associated with lignification in conifers. Phytochemistry 31:2959-2966

Scharf SJ, Horn GT, Erlich HA (1986) Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science 233: 1076-1078

Schmid G, Grisebach H (1982) Enzymic synthesis of lignin precursors. Purification and properties of UDP-glucose:coniferyl alcohol glucosyltrans-ferase from cambial sap of spruce (Picea abies L.). Eur J Biochem 123:363-370

Schneider K, Hovel K, Witzel K, Hamberger B, Schomburg D, Kombrink E, Stuible H-P (2003) The substrate specificity determining amino acid code of 4-coumarate:CoA ligase. Proc. Natl. Acad. Science 100:8601–8606.

Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichhart D (2001) CYP98A3 from *Arabidopsis thaliana* is a 3 -hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway. J Biol Chem 276: 36566–36574

Schuller DJ, Ban N, Van Huystee RB, McPherson A, Poulos TL (1996) The crystal structure of peanut peroxidase. Structure 4:311-321

Sederoff RR, MacKay JJ, Ralph J, Hatfield RD (1999) Unexpected variation in lignin. Curr Opin Plant Biol 2: 145–152

Sellner LN, Coelen RJ, MacKenzie JS (1992) Reverse transcriptase inhibits *Taq* polymerase activity. Nucleic Acids Res 20: 1487-1490

Sewalt VJH, Ni W, Blount JW, Jung HG, Masoud SA, Howles PA, Lamb C, Dixon RA (1997) Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. Plant Physiol 115: 41– 50

Shelton M (1995) *Leucaena* forage production and quality in South East Queensland. *Leucaena* News. 2: 6 SO-ITFSM-52. New Orleans: USDA Forest Service, Southern Forest Experiment Station. pp 8

References

Sibout R, Eudes A, Mouille G, Pollet B, Lapierre C, Jouanin L, Seguin A (2005) Cinnammyl Alcohol Dehydrogenase C and D are the primary genes involved in lignin biosynthesis in the floral stem of *Arabidopsis.* Plant Cell 17: 2059–2076.

Sibout R, Eudes A, Pollet B, Goujon T, Mila I, Granier F, Seguin A, Lapierre C, Jouanin L (2003) Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenase in *Arabidopsis*. Isolation and characterization of the corresponding mutants. Plant Physiol 132: 848–860

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved method for walking in uncloned genomic DNA. Nucleic Acids Res 23: 1087–1088

Solinas A, Brown LJ, McKeen C, Mellor JM, Nicol J, Thelwell N, Brown T (2001) Duplex Scorpion primers in SNP analysis and FRET applications. Nucleic Acids Res 29 :E96

Speer EO (1987) A method of retaining phloroglucinol proof of lignin. Stain Technol 62:279–280\

Stafford HA, Dresler S (1972) 4-Hydroxycinnamic acid hydroxylase and polyphenolase activities in *Sorghum vulgare*. Plant Physiol 49: 590–595

Steeves C, F¨orster H, Pommer U, Savidge R (2001) Coniferyl alcohol metabolism in conifers. I. Glucosidic turnover of cinnamyl aldehydes by UDPG: coniferyl alcohol glucosyltransferase from pine cambium. Phytochemistry 57:1085–93

Sterjiades R, Dean JFD, Eriksson KEL (1992) Laccase from sycamore maple (*Acer pseudoplatanus*) polymerizes monolignols. Plant Physiol 99:1162–1168

Sterky F, Regan S, Karlsson J, Hertzberg M,Rohde A, Holmberg A, Amini B, Bhalerao.R, L arsson M, Villarroel R, Montagu MV,Sandberg G, Olsson O, Teeri TT, BoerjanW, G ustafsson P, Uhlen M, Sundberg B, Lundeberg J (1998) Gene discovery in the wood-forming tissues of poplar: Analysis of 5,692 expressed sequence tags. Proc Natl Acad Sci 95:13330-13335

Steudle E (2001) The cohesion-tension mechanism and the acquisition of water by plant roots. Annu Rev Plant Physiol Plant Mol Biol 40:19-39

Stewart D, Yahiaoui N, McDougall GJ, Myton K, Marque C, Boudet AM, 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 SH, Rottmann WH, Brunner,AM, Sheppard LA (1995) Genetic engineering of reproductive sterility in forest trees. Molecular Breeding 1: 5-26.

T. Klaus, R.,Joerger, E. Olsson and C. G. Granqvist, Silver-based crystalline , microbially fabricated *Proc*. *Natl. Acad. Sci*. *USA*, 1999, 96, 13611–13614.

Takabe K, Fujita M, Harada H, Saiki H (1985) Autoradiographic investigation of lignification in the cell walls of Cryptomeria (Cryptomeria japonica D. Don). Mokuzai Gakkaishi 31:613-619

Takahama U (1995) Oxidation of hydroxycinnamic acid and hydroxy-cinnamyl alcohol derivatives by laccase and peroxidase. Interactions among p-hydroxyphenyl, guaiacyl and syringyl groups during the oxidation reactions. Physiol Plantarum 93:61-68

Talas-Ogras T, Kazan K, Gözükirmizi N(2001) Decreased peroxidase activity in,transgenic t obacco and its effect on lignification. Biotechnol Lett 23:267-273

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.

Tan W, Wang K, Drake TJ (2004) Molecular beacons. Curr Opin Chem Biol 8: 547-553

Tangendjaja B, Willis RBH (1980) Analysis of mimosine and 3, 4-DHP by High Performance Liquid Chromatography. J Chromatog 202: 317

TaqStart Antibody: Hot start PCR facilitated by a neutralizing monoclonal antibody directed against Taq DNA polymerase. BioTechniques 16: 1134–1137

Terashima N, Fukushima K (1988) Heterogenity in formation of lignin XI: An autoradiographic study of the heterogeneous formation and structure of pine lignin. Wood Sci Technol 22:259-270

Terazawa M, Okuyama H, Miyake M (1984) Isolation of coniferin and syringin from the cambial tissue and inner bark of some angiospermous woods. Mokuzai Gakkaishi 30:409-412

References

Terry CF, Shanahan DJ, Ballam LD, Harris N, McDowell DG, Parkes HC (2002) Real-time detection of genetically modified soya using Lightcycler and ABI 7700 platforms with TaqMan, Scorpion, and SYBR Green I chemistries. J AOAC Int 85: 938-944

Timell TE (1986) CompressionWood in Gymnosperms, Vol. 1: Bibliography, Historical Background, Determination, Structure, Chemistry, Topochemistry, Physical Properties, Origin, and Formation of CompressionWood. Berlin: Springer-Verlag. pp 706

Todd JA, Bell JI, McDevitt HO (1987) HLA-DQ gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. Nature 329: 599-604

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: 129–138

Tsai C-J, Popko JL, Mielke MR, Hu W-J, Podila GK, Chiang VL (1998) Suppression of O-methyltransferase gene by homologous sense transgene in quaking aspen causes red-brownwood phenotypes. Plant Physiol 117: 101–112

Tsuji Y, Fukushima K (2004) Behavior of monolignol glucosides in angiosperms. J Agric Food Chem 52: 7651–7659

Tuskan GA, DiFazio S, Jansson S, BohlmannJ, Grigoriev I, Hellsten U, Putnam N,Ralph S, Rombauts S, Salamov A, Schein J,Sterck L, Aerts A, Bhalerao RR, Bhalerao.RP, Blaudez D, Boerjan W, Brun A,Brunner A, Busov V, Campbell M, Carlson.J, Chalot M, Chapman J, Chen GL, CooperD, Coutinho PM, Couturier J, Covert S,Cronk Q, Cunningham R, Davis J,Degroeve S, Déjardin A, dePamphilis C,Detter J, Dirks B, Dubchak I, Duplessis S,Ehlting J, Ellis B, Gendler K, Goodstein D,Gribskov M, Grimwood J, Groover A,Gunter L, Hamberger B, Heinze B,Helariutta Y, Henrissat B,Holligan D,Holt R, Huang W, Islam-Faridi N, Jones S,Jones-Rhoades M, Jorgensen R, Joshi C,Kangasjärvi J, Karlsson J, Kelleher C,Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leplé JC,Locascio P, Lou Y, Lucas S, Martin F,Montanini B, Napoli C, Nelson DR,Nelson C, Nieminen K, Nilsson O, Pereda.V, Peter G , Philippe R, Pilate G, PoliakovA, Razumovskaya J, Richardson P, Rinaldi.C, Ritland K, Rouzé P, Ryaboy D,Schmutz J, Schrader J, Segerman B, Shin.H, Siddiqui A, Sterky F, Terry A, Tsai CJ,Uberbacher E, Unneberg P, Vahala J, Wall.K, Wessler S, Yang G, Yin T, Douglas C,Marra M, Sandberg G, Van de Peer Y,Rokhsar D (2006) The

genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science 313:1596-1604

V. Bansal, D. Rautaray, A. Ahmad and M. Sastry Biosynthesis of zirconia nanoparticles using the fungus Fusarium oxysporum.*J. Mater. Chem*., 2004, 14, 3303-3333.

V. V. Klechkovskaia, T. A. Otroshko and N. S. Egorov, Proteolytic enzymes produced by *Aspergillus ochraceus* in relation to their plasma coagulating and fibrinolytic activities *Mikrobiologiia,* 1979, 48*,* 820-825

Vallette P, de Choudens C (1992) Le bois, la pate, le papier, 3rd Ed. Centre Technique de l'Industrie des Papiers, Cartons et Celluloses, Grenoble, France.

Van den Eijnden, D.H. and Joziasse, D.H. Enzymes associated with glycosylation. *Curr. Opin. Struct. Biol.* 3: 711, 1993.

Vander Velden VH, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ (2003) Detection of minimal residual disease in hematologic malignancies by realtime quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 17: 1013-1034

Van Doorsselaere J, Baucher M, Chognot E, Chabbert B, Tollier M-T, Petit-Conil M, Leple J-C, Pilate G, Cornu D, Monties B, Van Montagu M, Inze D, Boerjan W, Jouanin, L (1995) A novel lignin in poplar trees with a reduced caffeic acid/5-hydroxyferulic acid O methyltransferase activity. Plant J 8: 855–864

Van Huystee, R.B. and Chibbar, R.N. Peanut peroxidase: a model system for peroxidase analysis *Isozymes: Curr. Top. Biolog. Med. Res.* 13:155, 1987.

Veitch NC (2004) Horseradish peroxidase: a modern view of a classic enzyme. Phytochemistry 65:249-259

Vet JA, Marras SA (2005) Design and optimization of molecular beacon real-time polymerase chain reaction assays. Methods Mol Biol 288: 273-290

Vet JA, Van der Rijt BJ, Blom HJ (2002) Molecular beacons: colorful analysis of nucleic acids. Expert Rev Mol Diagn 2: 77-86

Voo KS, Whetten RW, O'Malley DM, Sederoff RR (1995) 4-coumarate:coenzyme a ligase from loblolly pine xylem. Isolation, characterization, and complementary DNA cloning. Plant Physiol 108: 85–97

References

Wei H, Meilan R, Brunner AM, Skinner JS, Ma C, Gandhi HT, Strauss SH (2006) Field trial detects incomplete barstar attenuation of vegetative cytotoxicity in Populus trees containing a poplar LEAFY promoter::barnase sterility transgene. Molecular Breeding 19: 69-85.

Welinder KG (1991) The plant peroxidase superfamily. In: Lobarzewski J, Greppin H et al (ed) Biochemical, Molecular andPhysiological Aspect of Plant Peroxidases, University of Geneva, Switzerland

Welinder KG, Justesen AF, Kjærsgård IVH,Jensen RB, Rasmussen SK, Jespersen HM,Duro ux L (2002) Structural diversity and transcription of class III peroxidases from Arabidopsis thaliana. Eur J Biochem 269:6063-6081

Wengenmayer H, Ebel J, Grisebach H (1976) Enzymic synthesis of lignin precursors Purification and properties of a cinnamoyl-CoA: NADPH reductase from cell suspension cultures of soybean (*Glycine max*). Eur J Biochem 65: 529–536

Wesley SV, Helliwell CA, Smith NA, Wang M, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, Robinson SP, Gleave AP, Green AG, Waterhouse PM (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. The Plant Journal 27: 581-590.

Whetten R, Sederoff R (1995) Lignin biosynthesis. Plant Cell 7: 1001–1013

Whetten RW, MacKay JJ, Sederoff RR (1998) Recent advances in understanding lignin biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 49: 585–609

Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22: 130-131, 134-138

Wittwer CT, Ririe KM, Andrew RV, David DA, Gundry RA, Balis UJ (1997) The LightCycler: a microvolume multisample fluorimeter with rapid temperature control.Biotechniques 22: 176-81

Won-Kyu Rhim, Jin-Soo Kim, and Jwa-Min Nam. Lipid–Gold-Nanoparticle Hybrid-Based Gene Delivery. Small 2008, 4, 1651–1655

Wu J, Fukazawa K, Ohtani J (1992) Distribution of syringyl and guaiacyl lignins in hardwoods in relation to habitat and porosity. Holzforschung 46: 181-185

Yahiaoui N, Marque C, Myton KE, Negrel J, Boudet AM (1998) Impact of different levels of cinnamyl alcohol dehydrogenase down-regulation on lignins of transgenic tobacco

plants. Planta 204: 8–15

Yahong L, Tsuji Y, Nishikubo N, Kajita S, Morohoshi N (2001) Analysis of transgenic poplar in which the expression of peroxidase gene is suppressed. In Molecular Breeding of Woody Plants, (Progress in Biotechnology Series,Vol. 18). pp. 195–204 Morohoshi N, Komamine A, Eds., Elsevier Science, Amsterdam.

Yazaki K (2006) ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett 580:1183-1191

Ye Z-H (1997) Association of caffeoyl coenzyme A 3-O-methyltransferase expression with lignifying tissues in several dicot plants. Plant Physiol 115: 1341–1350

Ye Z-H, Kneusel RE, Matern U, Varner JE (1994) An alternative methylation pathway in lignin biosynthesis in *Zinnia*. Plant Cell 6: 1427–1439

Ye Z-H, Varner JE (1995) Differential expression of two O-methyltransferases in lignin biosynthesis in *Zinnia elegans*. Plant Physiol 108: 459–467

Yokota K, Yamazaki I (1965) Reaction of peroxidase with reduced nicotinamide-adenine dinucleotide and reduced nicotinamide-adenine dinucleotide phosphate. Biochim Biophys Acta 105:301-312

Yu H, Kumar PP (2003) Post-transcriptional gene silencing in plants by RNA. Plant Cell Reports 22: 167-174.

Zhang B, Pan X, Cobb G.P, Anderson TA (2006) Plant microRNA: a small regulatory molecule with big impact. Developmental Biology 289: 3-16.

Zhang XH, Chiang VL (1997) Molecular cloning of 4-coumarate:coenzyme A ligase in loblolly pine and the roles of this enzyme in the biosynthesis of lignin in compression wood. Plant Physiol 113: 65– 74

Zhong R, Morrison III WH, Himmelsbach DS, Poole II FL, Ye Z-H (2000) Essential role of caffeoyl coenzyme A O-methyltransferase in lignin biosynthesis in woody poplar plants. Plant Physiol 124: 563–577

Zhong R, Morrison WH III, Negrel J, Ye Z-H (1998) Dual methylation pathways in lignin biosynthesis. Plant Cell 10: 2033–2046

Zhu YY, Machleder EM, Chenchik A, Li R & Siebert PM (2001) Reverse transcriptase template switching: A SMART™ approach for full-length cDNA library construction. Biotechniques 30: 892–897

References

Zimmerlin A, Wojtaszek P, Bolwell GP (1994) Synthesis of dehydrogenation polymers of ferulic acid with high specificity by a purified cell-wall peroxidase from French bean (Phaseolus vulgaris L.). Biochem J 299:747-753

Zimmermann HH, Nimz, E. Seemuller (1985) Holzforschung 39: 45–49

Zubieta C, He X-Z, Dixon RA, Noel JP (2001) Structures of two natural product methyltransferases reveal the basis for substrate specificity in plant O-methyltransferases. Nat Struct Biol 8: 271–279

Zubieta C, Kota P, Ferrer J-L, Dixon RA, Noel JP (2002) Structural basis for the modulation of lignin monomer methylation by caffeic acid/5-hydroxyferulic acid 3/5-O methyltransferase. Plant Cell 14: 1265–1277

Zuckerkandl E, Pauling L. 1965. Evolutionary divergence and convergence in proteins. In: Bryson V, Vogel HJ, eds. *Evolving Genes and Proteins*, New York: Academic Press, 97-166.

PUBLICATIONS

PUBLICATIONS

Research papers published/under review/under preparation

- Noor M. Shaik, Manish Arha, Nookaraju A, Sushim. K. Gupta, Sameer Srivastava, Arun K. Yadav, Pallavi S. Kulkarni, **Abhilash O. U.**, Rishi K. Vishwakarma, Somesh Singh, Rajeshri Tatkare, Kannan Chinnathambi, Shuban K. Rawal, Bashir M. Khan (October, 2009). *Improved method of in vitro regeneration in Leucaena leucocephala - a leguminous pulpwood tree species*. Physiology and molecular biology of plants, 15(4).
- 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, Vol 7, pp 388-393.
- **O. U. Abhilash**, P.S.Vijayakumar, B.M. Khan* and B.L.V. Prasad*. Nanogold Loaded Sharp Edged Carbon Bullets as Gene Carrier for Plant Genetic Engineering (2009). Nanoletters. (Communicated).
- **O. U. Abhilash**, S.K.Gupta, M.Arha, N.M.Shaik, S.Srivastava, A.K.Yadav, P.S.Kulkarni, B.M.Khan and S.K.Rawal. (2008). Identification, Molecular characterization and expression analysis of Peroxidase (*POX*) gene(s) from *Leucaena lucocephala.* (Manuscript under preparation).
- **O. U. Abhilash**, S.K.Gupta, M.Arha, N.M.Shaik, S.Srivastava, A.K.Yadav, P.S.Kulkarni, B.M.Khan and S.K.Rawal. (2008). *Molecular modelling and docking studies of lignin biosynthetic ClassIII peroxidase isoforms from Leucaena leucocephala,* (Manuscript under preparation).

Abstract Published

- S. Srivastava, M. Arha, S. K. Gupta, N. M. Shaik, A. K. Yadav, **O. U. Abhilash**, P. Kulkarni, B. M. Khan and S. K. Rawal (2004). *Assessment of Leucaena Biodiversity Using Molecular Marker.* In Abstract volume of National Science Day, NCL, Pune, India.

PUBLICATIONS

- M. Arha, S. K. Gupta, N. M. Shaik, A. K. Yadav, P. Kulkarni, **O. U. Abhilash**, S. Srivastava,B. M. Khan and S. K. Rawal (2006). *Metabolic engineering of Leucaena leucocephala for eco-friendly paper and pulp industry.* In Abstract volume of National Science Day, NCL, Pune, India.
- M. Arha, S. K. Gupta, N. M. Shaik, A. K. Yadav, P. Kulkarni, **O. U. Abhilash**, S. Srivastava, 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 International Symposium on Frontiers of Genetic Engineering and Biotechnology: Retrospect and Prospect, Osmania University, Hyderabad, Andhra Pradesh, India.

Books:

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

Posters Presented:

- **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 polymerisation. In Proceedings of International Symposium on Frontiers of Genetic Engineering and Biotechnology: Retrospect and Prospect, Osmania University, Hyderabad, Andhra Pradesh, India.
- **O. U. Abhilash**, A. K. Yadav, S. K. Gupta, M. Arha, N. M. Shaik, S. Srivastava, , P. Kulkarni, 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.

APPENDIX

APPENDIX

Table 2.1 List of equipment used in present study

S.No.	Equipment	Make
1.	Balances	Contech/ Sartorious
2.	Water bath	Julabo/
3.	Dry Bath	Eppendorf/BGenei
4.	Incubator	New Brunswick
5.	Centrifuge	Sorvall/Haereus/eppendorf/Sigma
6.	Gel Documentation system	Bio-Rad
7.	Thermo Cycler PCR machine/Real Time PCR	MJResearch/Stratagene
8.	Spectrophotometer	Applied Biosystem
9.	Power pack	Bio-Rad
10.	Agarose Gel Electrophoresis Units	Bangalore Genei/ Bio-Rad
11.	Pprotein Gel Electrophoresis Units	Hoeffer Scientific/ BioRad
12.	Speed Vac concentrater	Savant/Eppendorf
13.	pH-Meter	Global
14.	Water purification system	Millipore Unit (Milli RO/ Milli Q)
15.	Microwave oven	Bilbol
16.	Fridge/ Deep freezer	Vestfrost/Leonard/Godrej
17.	Magnetic rotator	REMI
18.	Laminar Air Flow	Microfilt India
19.	Bioanalyser agilent 2100	Agilent Technology
20.	Typhoon Trio + Scanner	GE Healthcare (USA)
21.	FPLC AKTA Explorer	GE Healthcare (USA)
22.	Particle bombardment system	Biolistic-PDS 1000/He system (BioRad Laboratories, Hercules, USA)
23.	ELISA Plate Reader	Amersham (USA)
24.	FTIR machine	Spectrum One (Perkin Elmer,USA)
25.	SEM- Stereoscan 440 model	Leica
26.	TEM G^2 F-30 model	Technai G^2 F-30 model
27.	iBlot Gel Transfer System	Invitrogen

Table 2.2 Buffers and Solutions for DNA Electrophoresis

Name	Components	Preparation and Storage
50x TAE	2 M Tris Acetic acid 0.05 M EDTA	pH was adjusted to 8.0 using glacial acetic acid and stored at room temperature.
TBE buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA	Room temperature.
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 100 ml with H_2O*	The solutions were filter sterilized using 0.22 micron (μ) filter and stored at room temperature.

Table 2.3 Buffers and Solutions for g-DNA isolation, Southern and Slot Blot

Name	Components	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 β-mercaptoethanol to 0.2 % before use.	Room temperature.
Depurination buffer	0.25 N HCL	Freshly prepared.
Denaturation buffer	1.5 M NaCl 0.5 M NaOH	Room temperature.
Neutralization buffer	1.5 M NaCl 1.0 M Tris HCl (pH 7.4)	Room temperature.
20 X SSC	3 M NaCl 0.3 M Sodium citrate (pH 7.0)	Room temperature.
Hybridization buffer	1% BSA 1.0 mM EDTA pH 8.0 0.5 M Sodium phosphate pH 7.2 7% SDS	Room temperature.
Low stringency wash buffer	6 X SSC 0.1% SDS	Room temperature.
Moderate stringency wash buffer	2 X SSC 0.1% SDS	Room temperature.
High stringency wash buffer	0.2 X SSC 1% SDS	Room temperature.

Table 2.4 Stock solutions for *E. coli* transformation and selection

Name	Components	Preparation and Storage
IPTG solution	200 mg/mL in SMQ	Sterile filtration and storage at -20 ºC
X-Gal (***5-bromo-4-chloro-3-indolyl-ß-D-galactoside***)	20 mg/mL in N,N'-Dimethyl formamide (DMF)	Light sensitive, covered & store at -20 ºC
Ampicillin	100 mg/mL in SMQ	Sterile filtration and storage at -20 ºC
Tetracycline	12.5 mg/mL in 70% ethanol	Sterile filtration and storage at -20 ºC
Kanamycin	50 mg/mL in SMQ	Sterile filtration and storage at -20 ºC
Rifampcin	50 mg/mL in DMSO	Sterile filtration and storage at -20 ºC
Hygromycin	25 mg/mL in SMQ	Sterile filtration and storage at -20 ºC

Table 2.5 Buffers and Solutions for plasmid isolation (Alkaline lysis method)

Name	Components	Preparation and Storage
Solution I or Resuspension buffer	50 mM Glucose, 25 mM Tris-HCl (pH 8.0),10 mM EDTA (pH 8.0),	Store at 4 ºC
Solution II or Lysis buffer	0.2 N NaOH and 1% SDS	Freshly prepared
Solution III or Neutralisation buffer	3 M Potassium acetate (pH 4.8)	Store at 4 ºC
RNase A	10 mg/mL	Store at -20 ºC
Other solutions or Reagents	Chloroform, Absolute ethanol, 3.0 M Sodium acetate, 70% ethanol & Deionized sterile water	Room temperature.

Table 2.6 Buffers and Solutions for Gel Electrophoresis (native/ SDS-PAGE)

Name	Components	Preparation and Storage
Monomer solution	29.2% acrylamide 0.8% bis-acrylamide in water	Store at 4 ºC (in darkness)
Stacking gel	Distilled water 03.40 mL 1 M Tris-HCl (pH 6.8) 00.63 mL Acrylamide/bis 30%, 0.83 mL 10% SDS 0.05 mL 10% (w/v) APS 0.05 mL TEMED 04 µL	Freshly prepared
Separating gel (10%)	Distilled water 3.3 mL 1.5 M Tris-HCl (pH 8.8) 2.0 mL Acrylamide/Bis 30%, 4.0 mL 10% SDS (**SDS-PAGE**) 0.1 mL 10% (w/v) APS 0.1 mL TEMED, 07 µL	Freshly prepared
2x Protein loading buffer	Distilled water 2.7 mL 0.5 M Tris-HCl (pH 6.8) 1.0 mL Glycerol, 2.0 mL 10% SDS(**SDS-PAGE**) 3.3 mL β-Mercaptoethanol 0.5 mL 0.5% Bromophenol blue 0.5 mL	Store at 4 ºC
10x SDS-electrode buffer	Tris base 15.1 g Glycine 94.0 g SDS 00.5 g Adjust pH-8.3 ***And make-up the volume up to 500 mL.***	Store at 4 ºC Dilute 1:10 before use
Staining solution	Coomassie-blue R 250, 0.25 g in Methanol, 40 mL Acetic acid, 10 mL ***Make-up to 100 mL***	Store at 4 ºC (in darkness)
Destaining solution	Methanol, 40 mL Acetic acid, 10 mL ***Make- up to 100 mL***	Store at 4 ºC (in darkness)
Silver staining Fixer solution	40% Methanol, (150 mL) 10% acetic acid, (50 mL) ***Make-up to 100 mL***	Store at RT
Sensitizing solution	0.2% $Na_2S_2O_3$	Store at RT
Silver solution	0.2% silver nitrate (0.6 g) 0.01 % formaldehyde (225 µL) ***Make-up to 300 mL***	Prepare fresh in darkness
Developing solution	6% Na_2CO_3 (18 g) 0.02% formaldehyde (150 µL) ***Make-up to 300 mL***	Prepare fresh

Stop solution	1.5% Na_2EDTA (4.5 g) ***Make-up to 300 mL***	Store at RT

Table 2.7 Buffers and solutions for protein purification (based on imidazole conc.)

Name	Components	Preparation and Storage
Binding buffer	50 mM Tris 150 mM NaCl 10 mM imidazole 5% glycerol (pH 8.0)	Adjust pH by adding concentrated HCl and store at 4 ºC
Wash buffer	50 mM Tris 300 mM NaCl 25 mM imidazole 10% glycerol (pH 8.0)	Adjust pH by adding concentrated HCl and store at 4 ºC
Elution buffer	50 mM Tris 300 mM NaCl 200 mM imidazole 10% glycerol (pH 8.0)	Adjust pH by adding concentrated HCl and store at 4 ºC

Table 2.8 Buffers and solutions for protein extraction under denaturing conditions

Name	Components	Preparation and Storage
Lysis buffer	50 mM Tris-HCl (pH 8.0) 5 mM EDTA 100 mM NaCl 0.5% TritonX-100 0.7 mM DTT 0.1 mM PMSF (Freshly added) 10 mM $MgSO_4$ Lysozyme 100 µg/mL (Added freshly)	Store at 4 ºC
Sonication buffer	100 mM Tris HCl (pH8.0) 50 mM Glycine	Store at 4 ºC
Dispersion buffer:	100 mM Tris-HCl (pH8.0) 50 mM Glycine & 8 M Urea /6 M GuHCl	Store at 4 ºC

Table 2.9 substrates used for the study

Substrates	Manufacturer
Coniferyl alcohol	Sigma-Aldrich (USA)
Coniferyl aldehyde	Sigma-Aldrich (USA)
Sinapyl alcohol	Sigma-Aldrich (USA)
Syringaldazine	Sigma-Aldrich (USA)
Sinapyl aldehyde	Sigma-Aldrich (USA)
Guaiacol	Fluka

Table 2.10 Buffers and Solutions used for ELISA, Immunocytolocalization, Lignin staining and DNA coating over micro carriers

Name	Components	Preparation and Storage
Crude protein extraction buffer	20 mM Phosphate buffer (pH 7.4) 2% PVPP 2% PEG 6000 10 mM PMSF	Store at room temperature.
PBST	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	Store at room temperature.
Antibody dilution Buffer	PBS with 0.25% BSA	Store at room temperature.
Substrate buffer	200 mM Tris-HCl pH 9.5 0.5 mM $MgCl_2$	Store at room temperature.
1X PBS	10 mM NaH_2PO_4- Na_2HPO_4 Buffer (pH 7.2) 130 mM NaCl	Adjust pH to pH 7.2 and stored at 4 ºC
0.5 X SSC	75 mM NaCl 7.5 mM Na-Citrate	Adjust pH to pH 7.0 and stored at 4 ºC
Color development buffer	100 mM Tris (pH 9.5) 150 mM NaCl, 50 mM $MgCl_2$	Store at 4 ºC
BCIP/NBT mix	0.577 mM BCIP 0.122 mM NBT	Store at 4 ºC in dark

Stop Solution	10 mM EDTA	Store at room temperature.
Other reagents used	Polyvinyl alcohol, Ethanol, Tertiary butanol, Paraffin, Xylene, Glycerol	Store at room temperature.
X-GluC	2 mM X-Gluc (5-bromo, 4-chloro, 3-indolyl-β-D-glucuronide) in 50 µL DMSO and 50 mM sodium phosphate buffer pH 7.0 containing 0.1 mM potassium ferricyanide and ferrocyanide. Working solution 2.5 mL above stock + 1 mL methanol + 1.5 mL sodium phosphate buffer pH 7.0	Adjust pH to 7.0 and stored at 4 ºC
Phloroglucinol	2% Phloroglucinol in 95% ethanol	Store at 4 ºC
Xho buffer	150 mM Nacl 10 mM Tris-HCl pH 8.0	Store at -20 ºC
Spermidine	0.1 M Spermidine in H_2O	Store at -20 ºC
$CaCl_2$	2.5 M $CaCl_2$	Store at -20 ºC

Table 2.11 Component of Murasighe and Skoog media /Different inducing media and hormones

Name	**Components**	**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	Store at 4 ºC
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 $Na_2Mo_4.2H_2O$	Store at 4 ºC
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	Store at 4 ºC
Iron	0.1 mM $FeSO_4.7H_20$ 0.1 mM Na_2EDTA	Store at 4 ºC
BAP	1.776 mM BAP (*dissolve in NaOH and make up the volume by adding ethanol*)	Store at 4 ºC

NAA	2.148 mM NAA (*Dissolve in H_2O*)	Store at 4 ºC
Callus induction medium (*Nicotiana*)	MS basal medium (pH 5.8) 2% sucrose 0.8% BA and NAA 1.4 % agar	Store at 4 ºC
Shoot Induction medium (*Nicotiana*)	Major (40x)-25 mL Minor (100x)-10 mL Iron (100x)-10 mL Vitamins (200x)-5 mL BAP (4.4 µM))-1 mg/L NAA (5.37 µM)-0.5 mg/L Glucose-1.5% Sucrose-2.0% pH-5.6 to 5.8 Agar-0.8% (*make up to volume 1L*)	Store at 4 ºC
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*)	Store at 4 ºC
Root Induction medium (*Nicotiana*)	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*)	Store at 4 ºC
Callus induction medium (*Oryza sativa*)	MS medium + 2.5 mg/L 2,4-D supplemented with 36.4 g/L mannitol and 36.4 g/L sorbitol	Store at 4 ºC

Shoot Induction medium (*Oryza sativa*)	MS medium supplemented with BAP and NAA (Sigma, USA) 3 mg/L and 0.5 mg/L respectively)	Store at 4 ºC
Root Induction medium (*Oryza sativa*)	1/2- MS basal salts, MS vitamins and 15 g/L sucrose	Store at 4 ºC
Shoot Induction medium (*Leucaena*)	1/2 -basal MS medium + TDZ (0.5 mg/ L)	Store at 4 ºC
Shoot elongation medium (*Leucaena)*	1/2 -MS with Cytokinin, 2ip (2 isopentenyl adenine) 0.5 mg/ L	Store at 4 ºC
Selection medium	50 mg/L hygromycin for *Oryza sativa* 250 mg/L kanamycin for *Nicotiana* and *Leucaena* 200/250 mg/L cefotaxime to control *Agrobacterium*	Store at 4 ºC

Table 2.12 Different media used for studies bacterial growth

Name	Components	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 ºC
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 ºC
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
YEP	1% Bactotrypton 1% Yeast extract 0.1% Glucose	pH adjusted to 7.0 with NaOH, store at RT or at +4 ºC
Terrific Broth	12 g Bactotryptone 24 g Yeast extract 9.4 g K_2HPO_4 2.2 g KH_2PO_4 4 mL Glycerol ***Make up the volume to 1L.***	pH was adjusted to 7.5 and autoclaved.

NZY broth and agar	0.5% NaCl 0.2% $MgSO_4$. 7 H_2O 0.5% Yeast Extract 1.0% Tryptone 1.5% Agar (for NZY agar)	pH adjusted to 7.5 with NaOH, store at room temperature or at +4 ^{o} C
SM Buffer	0.58% NaCl 0.2% $MgSO_4$. 7 H_2O 50 mM Tris-HCl 0.2% Gelatin	Store at room temperature

Table 2.13 Bacterial cell lines used

E.coli	Genotype
DH 5α	F' _80_lacZ_M15 end A1 hsdR17 (rk-mk+) supE44 thi-1_-gyrA96 relA1 _(lacZYA-argFV169) deoR
JM 109	e14-(McrA-) recA1 endA gyrA96 th-1 hsdR17(rk-mk+) supE44 relA1 _(lac-proAB) [F' traD36 proAB lacqZ _M15
XL1 Blue	RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIqZΔM15 Tn10 (Tetr)
TOPO 10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG
BL 21	F-, ompT hsdSB (rB – mB -) gal dcm (DE3) pLysS (CamR)

BRADFORD PROTEIN ASSAY

Principle

The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Both hydrophobic and ionic interactions stabilize the anionic form of the dye, causing a visible color change. The assay is useful since the extinction coefficient of a dye-albumin complex solution is constant over a 10-fold concentration range.

Reagent required

1. Bradford reagent: Dissolve 10 mg Coomassie Brilliant Blue G-250 in 5 mL 95% ethanol, add 10 mL 85% (w/v) phosphoric acid. Dilute to 100 mL when the dye has completely dissolved, and filter through Whatman #1 paper just before use.
2. (Optional) 1 M NaOH (to be used if samples are not readily soluble in the color reagent).

The Bradford reagent should be a light brown in color. Filtration may have to be repeated to rid the reagent of blue components.

ASSAY

1. Warm up the spectrophotometer 15 min. before use.
2. Dilute samples with buffer to an estimated concentration of 20 to 200 micrograms/mL
3. Prepare standards containing a range of 20 to 200 micrograms protein (albumin or gamma globulin are recommended) to a standard volume (generally 1 mL or less).
4. Prepare unknowns to estimated amounts of 20 to 200 micrograms protein per tube, same volume as the unknowns.
5. Add 0.25 mL 1 M NaOH (Optional) to each sample and vortex.
6. Add 5 mL dye reagent and incubate 5 min.
7. Measure the absorbance at 590 nm

ANALYSIS

Prepare a standard curve of absorbance versus micrograms protein (or *vice versa*), and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

REFERENCE

- Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry 72:* 248-254. 1976.