Molecular characterization of Cinnamoyl Co A Reductase (CCR) gene in *Leucaena leucocephala*

A THESIS
SUBMITTED TO THE

UNIVERSITY OF PUNE

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN
BIOTECHNOLOGY

BY

Sameer Srivastava

NATIONAL CHEMICAL LABORATORY
PUNE – 411 008
INDIA

January 2009

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Under the Guidance of
Dr. B. M. Khan
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Dedicated to my family……..

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Molecular Characterization of Cinnamoyl Co A Reductase (CCR) gene in *Leucaena leucocephala*"** submitted by **Sameer Srivastava** 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 **"Molecular Characterization of Cinnamoyl Co A Reductase (CCR) gene in *Leucaena leucocephala*"**, submitted for the Degree of **Doctor of Philosophy** to the University of Pune, has been carried out by me at Plant Tissue Culture Division, National Chemical Laboratory, Pune - 411 008, Maharashtra, India, under the supervision of **Dr. B. M. Khan** (Research Guide). 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.

Sameer Srivastava

(Research Scholar)

Acknowledgements

The work presented in this thesis is a compilation of ideas, challenges, excitement, and also ofcourse the techniques and research carried out during the course of my PhD. I take this opportunity to acknowledge all the people who have contributed in some way or the other to help me get where I am today.

I am deeply grateful to my Research Guide Dr. Bashir Mohammad Khan, who has been a constant source of inspiration, motivation and a mentor in true sense. His invaluable guidance throughout the course of my research has rendered me a thorough researcher. It was a privilege to be a part of his team at NCL.

Special thanks are due to Dr. S. K. Rawal for being an unrelenting source of encouragement and support. His expert advice always gave new strength to face the challenges.

I also wish to acknowledge Dr. S. Sivaram, Director, NCL for providing necessary infrastructure to carry out my research.

Thanks are due to all members of the scientific and supporting staff of the Plant Tissue Culture Division, Biochemical Sciences Division and NCL Library.

My labmates and friends Ruby, Shweta, Pallavi, Kannan, Santosh, Sumita, Santosh Gupta, Sucheta ma'm, Arun, Abhilash, Neha, Priya, Jai, Azfar, Malini, Ulfat and Jay Kumar deserve special thanks, for the cordial atmosphere in the lab.

I am obliged to Nooka Raju and Rajeshri for their expertise in Transformation experiments.

I thank my seniors RC, Rohini, Neelima, Manish and Sushim for their encouragement and help during the course of this work.

I am immensely grateful to my trainees Rishi, Yasir and Rahul for their invaluable help during the crucial period of my work.

I take this opportunity to thank my friends Ashish, Sreekanth, Nagraj, Shasidhar, JP, Sagar, Manje, Elan, Santosh, Chetan, Atul F., Satya, Ambrish, Anish, Aarohi, Nitin, SuryaPrasad, Sachin, Shabab, Shadab, Poonam, Ansary, Asad, Kishore bhaiya, Asutosh, Sunil, Bhuban, Kiran, Shweta, Swapna, Shyla, Sujata, Arshad, Dillu, Venkatesh, Rajendra, Sridharji, Sudhir K, Jitu Pandey, Sarvesh, Kanna, Kamu, Rakesh (and many others whom I might have forgetten to mention here) for their help, support, best wishes and lighter moments shared together through out my work period.

I would like to acknowledge my Goa Biotech friends Ajay, Atul, Akkhu, Viku, Prashant, Priti, Gayatri, Pankaj, Sanjay, Rohtas, Nishant, Abhishek, Sridevi, Chinu, Reeti, Imran, Prashant S, Punnu, Ashutosh, Akanksha, Monku, Vini, and Gautam for support and affection.

Special thanks to Viku for his guidance in heterologous protein expression experiments and Hamid for helping with FTIR experiments.

Sincere thanks to Dr. K. V. Pandhare (FTIR spectroscopy) and Dr. Yogesh Shouche (Real Time PCR) for extending the necessary facilities.

Special mention is a must for Noor (Papa), Ashish (KF), Rishi (Muni), Somesh (Thokia), Mohsin (Mozane), Rodu (Bhokali) and Anubhav (Chaman) for being my extended family during the course of my work. They have given me a perfect family atmosphere and a home away from home.

I would like to gratefully acknowledge the fellowship awarded by the CSIR, India.

I owe my deepest gratitude to my grandparents, parents, brothers and sister who have constantly encouraged me to excel.

I am falling short of words to appreciate the encouragement and efforts put in by my wife Tonima for the completion of this thesis.

Sameer :-)

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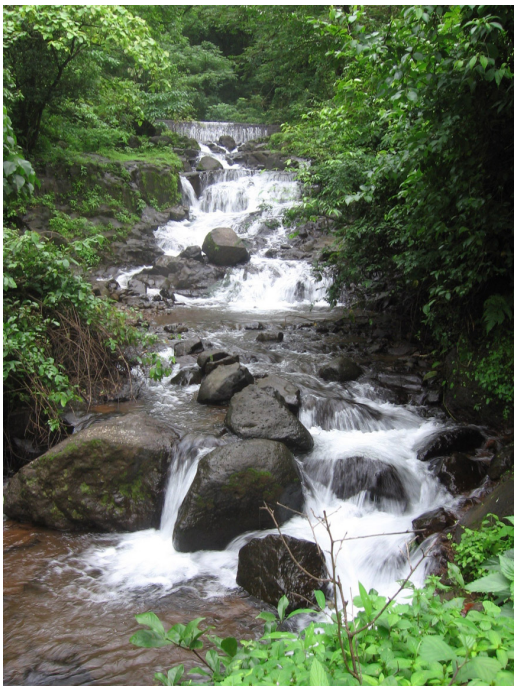
Abbreviations

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

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

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

Introduction



Nature serves the mankind, in one way or the other, in every aspect of life. Trees are reservoirs of many economically and biotechnologically significant products. Wood is one such gift of nature, which has diverse applications for mankind. One of the most well known applications of wood is paper and paper products.

In the past forty years, the world wide annual production of paper has increased more than three folds. Wood, agricultural residues and many other plant materials, which can be used for pulp and paper production, consist largely of 'lignocellulose', which is a composite of mainly cellulose, hemicellulose and lignin.

Lignin, one of the most abundant natural (terrestrial) organic polymers and a major constituent of wood, forms an integral cell wall component of all vascular plants. It represents, on an average, 25% of the terrestrial plant biomass.

In the production of pulp (which is mainly cellulose), the hemicellulose and much of the lignin is removed using alkali. To produce bright paper, pulp may require a further bleaching treatment to remove lignin residues. This delignification process consumes large quantities of energy and hazardous chemicals and is neither economical nor environment friendly. Reduced lignin content or altered quality of lignin in pulp wood species without compromising the mechanical strength of the plant is desirable for paper industry. The alteration of the lignin components has to be achieved in a manner where in the sinapyl alcohol (S unit, less compact lignin) to guaiacyl alcohol (G unit, more compact lignin) ratios are in favour of sinapyl alcohol (higher S/G ratio desirable for paper industry).

Although lignin has been studied for over a century, many aspects of its biosynthesis still remain unresolved. The monolignol biosynthetic pathway has been redrawn many times and yet remains a matter of debate. The enzymes involved in lignin biosynthesis include: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), coumarate 3-hydroxylase (C3H), 4-coumarate CoA ligase (4CL), caffeoyl CoA 3-O- methyltransferase (CCoAOMT), caffeate O-methyltransferase (COMT), ferulate 5-hydroxylase/ coniferaldehyde 5-hydroxylase (F5H/CAld5H), **cinnamoyl CoA reductase (CCR)**,

cinnamyl alcohol dehydrogenase (CAD), sinapyl alcohol dehydrogenase (SAD), UDP-glycosyltransferase (UDP-GT), coniferin β glucosidase (CBG), peroxidases and laccases.

It has been hypothesized that CCR plays a key regulatory role in lignin biosynthesis as the first committed step in the production of monolignols from phenylpropanoid metabolites. Cinnamoyl CoA Reductase (CCR) catalyzes the reduction of hydroxycinnamoyl-CoA thioesters to the corresponding aldehydes. This reaction is considered to be a potential control point that regulates the overall carbon flux towards lignin. Studies have shown that altering the expression of CCR gene (down-regulation and up-regulation) has a direct effect on quality and quantity of lignin produced.

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 about 25% raw material for pulp industry comes from this plant. *Leucaena leucocephala* is native of Central America and belongs to the family Fabaceae. It is one of the most versatile and fast growing tree species.

Lignin biosynthesis genes have not been studied so far in *Leucaena* sp. Study of these genes 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.

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 (Council of Scientific and Industrial Research) is actively engaged in pursuing research on genes involved in lignin biosynthetic pathway in *L. leucocephala*. I had an opportunity to work under the guidance of Dr. B. M. Khan at this institute and the work carried out is presented here.

The present study is the first instance towards isolation, characterization and expression studies of Cinnamoyl-CoA-Reductase (CCR) gene involved in lignin biosynthetic pathway in *L. leucocephala*.

The thesis comprises of four chapters:

- **Chapter One: Lignin and its biosynthesis in nature**
  This chapter deals with the occurrence and chemistry of lignin and various aspects of lignin biosynthesis with special reference to CCR gene.

- **Chapter Two: Isolation, cloning and characterisation of CCR gene from *Leucaena leucocephala***
  This chapter deals with isolation and characterization of c-DNA clones of CCR gene from *L. leucocephala*.

- **Chapter Three:** This Chapter has been sub divided into two sections

  Section 1: Heterologous expression of CCR gene, its purification and characterisation
  This section deals with cloning of cDNA for CCRH2 in expression vector pET 30b+ and its expression in *E. coli* strain BL21 (DE3). It also covers purification and characterisation of the protein.

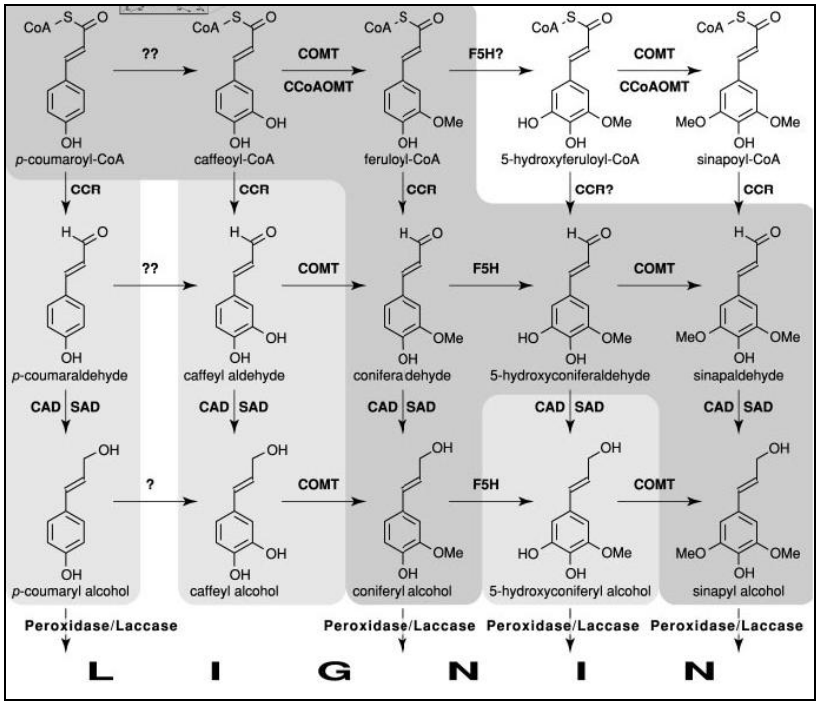
  Section 2: Spatio-temporal expression and Immuno-cytolocalization of CCR gene
  This section covers studies on the spatial and temporal expression of the CCR genes using semi quantitative, Real Time PCR and ELISA based technique. Immuno-cytolocalization of CCR was studied by raising antibodies in rabbit against recombinant CCR protein.

- **Chapter Four: Antisense cloning of CCR gene, transformation of *L. leucocephala* and analysis of transformants**

  This chapter deals with cloning of CCR gene in pCAMBIA 1301 vector in antisense orientation and transformation of *Leucaena* using *Agrobacterium* co-cultivation and particle bombardment. The transformants were analyzed for integration using PCR based methods, SLOT Blot and ELISA analysis.

Chapter One

Lignin and its biosynthesis in nature



1.1 Introduction:

Lignin (from Latin *lignum* meaning wood) is the most abundant natural (terrestrial) organic polymer, next only to cellulose, and a major constituent of wood. It is a vital cell wall component of all vascular plants and represents on an average of 25% of the terrestrial plant biomass. Lignins provide mechanical support and are functional in water transport, and disease resistance in higher terrestrial plants. 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; Jones *et al.,* 2001).

1.2 Occurrence:

Lignin is primarily 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. 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).

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 (Sarkanen and Ludwig, 1971).

1.3 Chemistry of lignin:

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

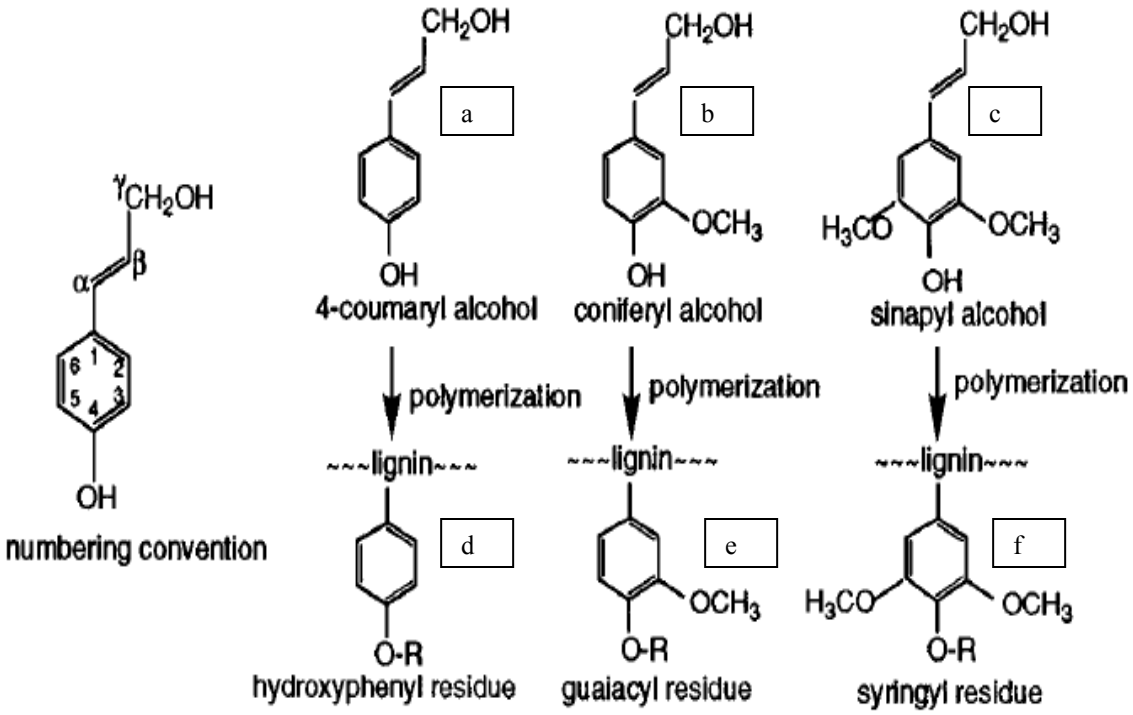
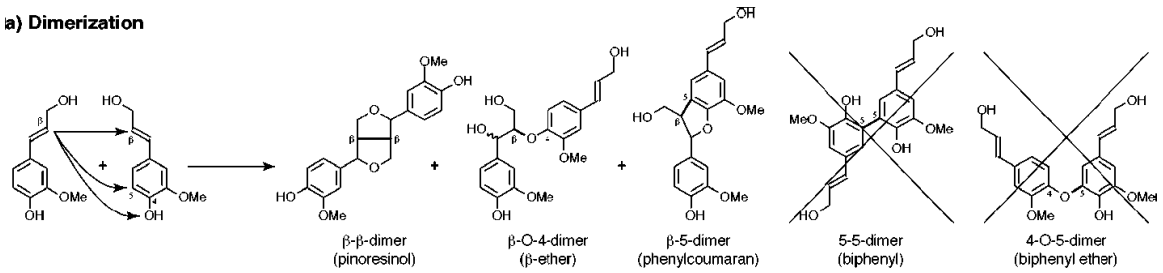
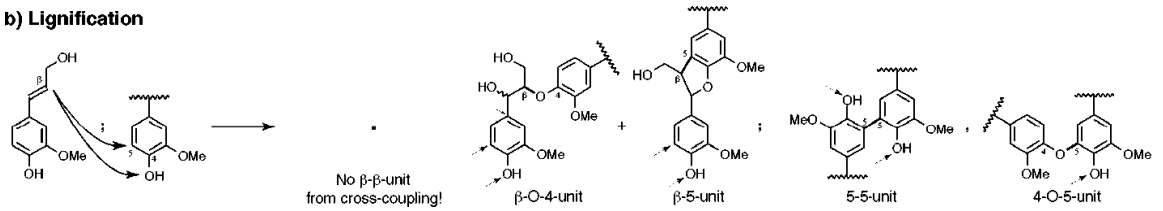


Fig. 1: Monolignols and Lignin units: Monolignols (a) *p*- coumaryl (b) coniferyl, and (c) sinapyl alcohol and Lignin units (d) *p*-Hydroxyphenyl or H unit, (e) Guaiacyl or G unit, and (f) Syringyl or S unit.

These monolignols as such are toxic to the plant cell and hence are converted to respective glycosides, which are transported to the cell wall via cell membrane (Whetten and Sederoff, 1995; Boerjan *et al.*, 2003). In the cell wall the glycosides are again hydrolysed to form Hydroxyphenyl, Guaiacyl and Syringyl units respectively (Fig. 1 d-f). These monomers are linked together via end-wise and radical coupling reactions (Sarkanen and Ludwig, 1971; Freudenberg and Neish, 1968) to produce H, G and S lignin respectively. This process is known as lignification. Monolignol dimerization and lignification 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 a and b).



(*a*) Dimerization of coniferyl alcohol produces only three dimers, in each of which at least one of the coniferyl alcohols is coupled at its β position. The 5–5 and 5–O–4 dimers (crossed out) do not arise in any significant way from monomer dimerization reactions.



(*b*) Cross coupling of coniferyl alcohol with a G unit gives only two main products, explaining why there are more β-ethers formed during lignification than in monolignol dimerization. Coupling of preformed oligomers is the source of most of the 5–5- and 5–O–4 units. Sites of further coupling reactions during lignification are indicated by arrows (Baucher *et al.*, 1998; Boerjan *et al.,* 2003).

Fig. 2: Dimerization and Lignification: Dimerization (a) differs substantially from simple Lignification (b) of monolignols.

As the monolignols differ in their degree of methoxylation, the respective lignins produced are also different in their compactness. Syringyl unit has less number of potential radical forming sites than guaiacyl unit and hence the lignin formed from S unit monomers is less compact than that formed from G unit monomers.

The three monolignols (S, G and H) are incorporated at different stages of cell wall formation. In angiosperms, H units are deposited first, followed by G and S units (Donaldson *et al.*, 2001; Terashima *et al.,* 1993). 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. 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 G-S synthetic lignins (Chabannes *et al.,* 2001). 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).

1.4 Lignins in wood:

Wood lignins are composed of S and/or G units linked by a series of ether and carbon-carbon bonds (Adler, 1977). 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 levels, and more H units than dicots (Baucher *et al.,* 1998).

In angiosperms, lignin is predominately composed of S and G units and total lignin is a combination of S and G lignin in varied proportion. Low Syringyl (S) to Guaiacyl (G) lignin ratio plays a detrimental role in economy and ecology of paper production. As it has been discussed that S lignin is less compact than G lignin, removal of S lignin is economically and ecologically more favorable than G lignin and hence a higher S/G ratio is desired for paper and pulp industry.

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 unit is predominately found in fiber cells.

1.5 Lignin Biosynthesis:

Lignin biosynthesis is a significant part of Phenylpropanoid biosynthetic pathway which itself can be broadly divided into common phenylpropanoid pathway and monolignol specific biosynthetic pathway. Monolignol specific branch of phenylpropanoid pathway leads to the formation of monolignols and subsequently lignin (Fig.4). Phenylpropanoid metabolism comprises a complex series of branching biochemical reactions that provide the plant with a host of important phenolic compounds. Phenylpropanoids have a range of important functions in plants, including as structural components (such as lignin), protectants against biotic and abiotic stresses (antipathogenic phytoalexins, antioxidants and UV-absorbing compounds), pigments (particularly the anthocyanins) and signaling molecules (*e.g.* flavonoid nodulation factors).

The synthesis of lignin represents one of the most energy demanding biosynthetic pathways in plants, requiring large quantities of carbon skeletons. The monolignols that make up the lignin polymer are derived from the sequential action of three key biosynthetic pathways (Lewis & Yamamoto, 1990; Whetten & Sederoff, 1995; Boerjan *et al*, 2003;). Shikimic acid metabolism gives rise to the amino acid phenylalanine, which in turn is used by general phenylpropanoid metabolism to synthesise the hydroxycinnamoyl-CoA-thioesters that are used in the two-step reduction, and phenyl substitution reactions of monolignol biosynthesis (Fig.3 and 4).

The monolignol biosynthesis pathway, functions of its products, and regulation of expression of biosynthesis genes, have been discussed in several recent reviews. Manipulating the phenylpropanoid metabolism holds significant commercial potential. Increasing or reducing the expression of particular genes in transgenic plants raises many possibilities to alter phenylpropanoid biosynthesis both quantitatively and qualitatively. However, making a predictable change may not be always possible as the pathways are complex and alternative routes to the synthesis of particular compounds may be present (Fig.3).

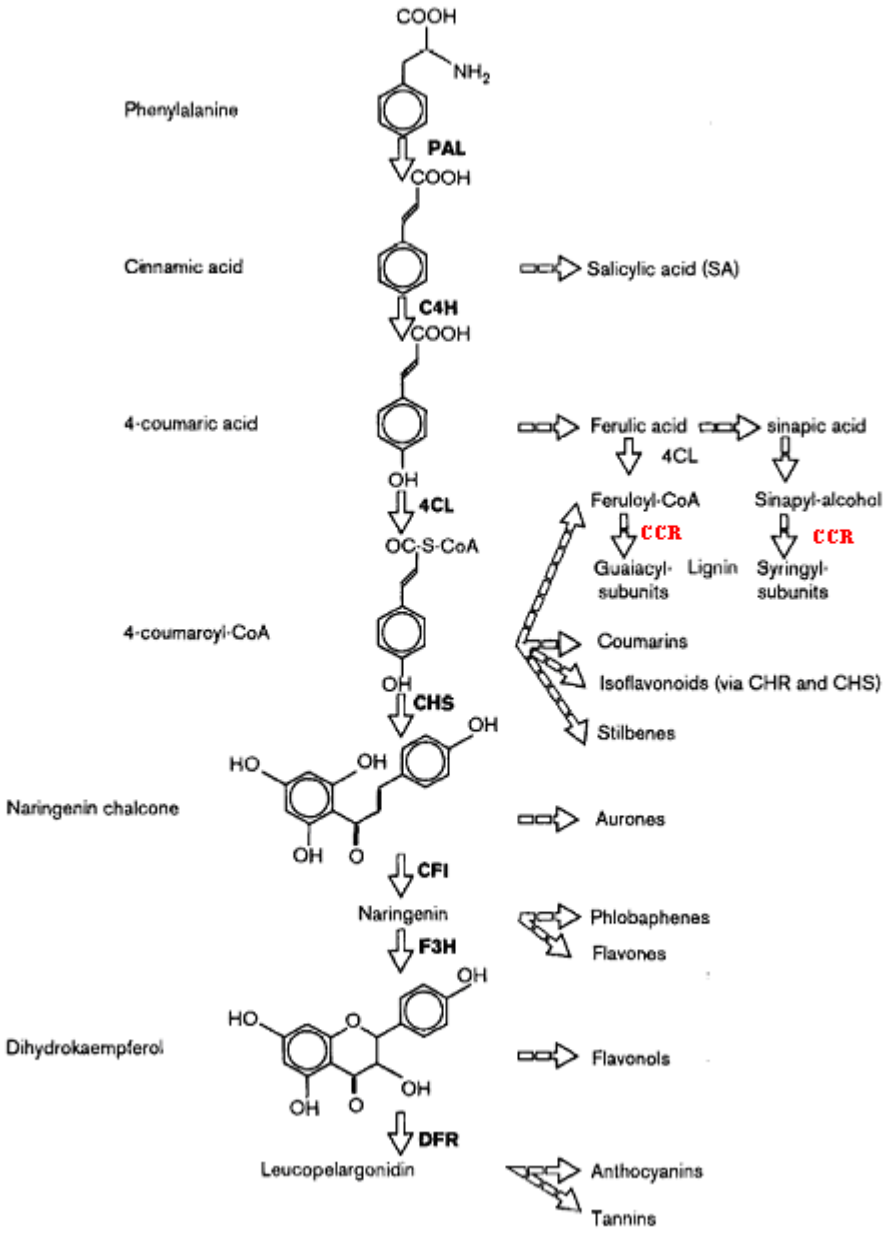


Fig. 3: Schematic view of some branches of phenylpropanoid metabolism (Weisshaar and Jenkins, 1998).

The lignin biosynthetic pathway can be subdivided into four steps starting with
(a) The biosynthesis of monolignols
(b) Transport of monolignols from the site of synthesis to the site of polymerization
(c) Dehydrogenation and
(d) Polymerization of monolignols to give the final product.

1.5.1 Monolignol Biosynthetic pathway:

During the last two decades, there has been a great deal of interest in cloning and characterization of the genes controlling monolignol biosynthesis 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 an enormous variation in lignin content and composition among plant species, tissues, and cell types. Developmental stages of plant and environmental conditions play a significant role in deciding the content and composition of lignin and its deposition. 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.

Several enzymes involved in lignin biosynthesis have been isolated and characterized. Highlights of some of these enzymes are given below.

1.5.1.1 Phenylalanine Ammonia-Lyase (PAL):

Deamination of phenylalanine to cinnamate is catalyzed by the enzyme phenylalanine ammonia-lyase (PAL; EC: 4.3.1.5). This enzyme is one of the most extensively studied enzyme in plant secondary metabolism because of the key role it plays in phenylpropanoid biosynthesis. PAL is found as a tetramer in vascular plants. Monolignol biosynthesis is considered to start from phenylalanine. Multigene families in angiosperms typically encode PAL, with two to 40 different members depending on the species. Genes encoding

different PAL subunits show tissue-specific patterns of expression in severa1 angiosperms (Leyva *et al.,* 1992; Lois *et al.*, 1989).

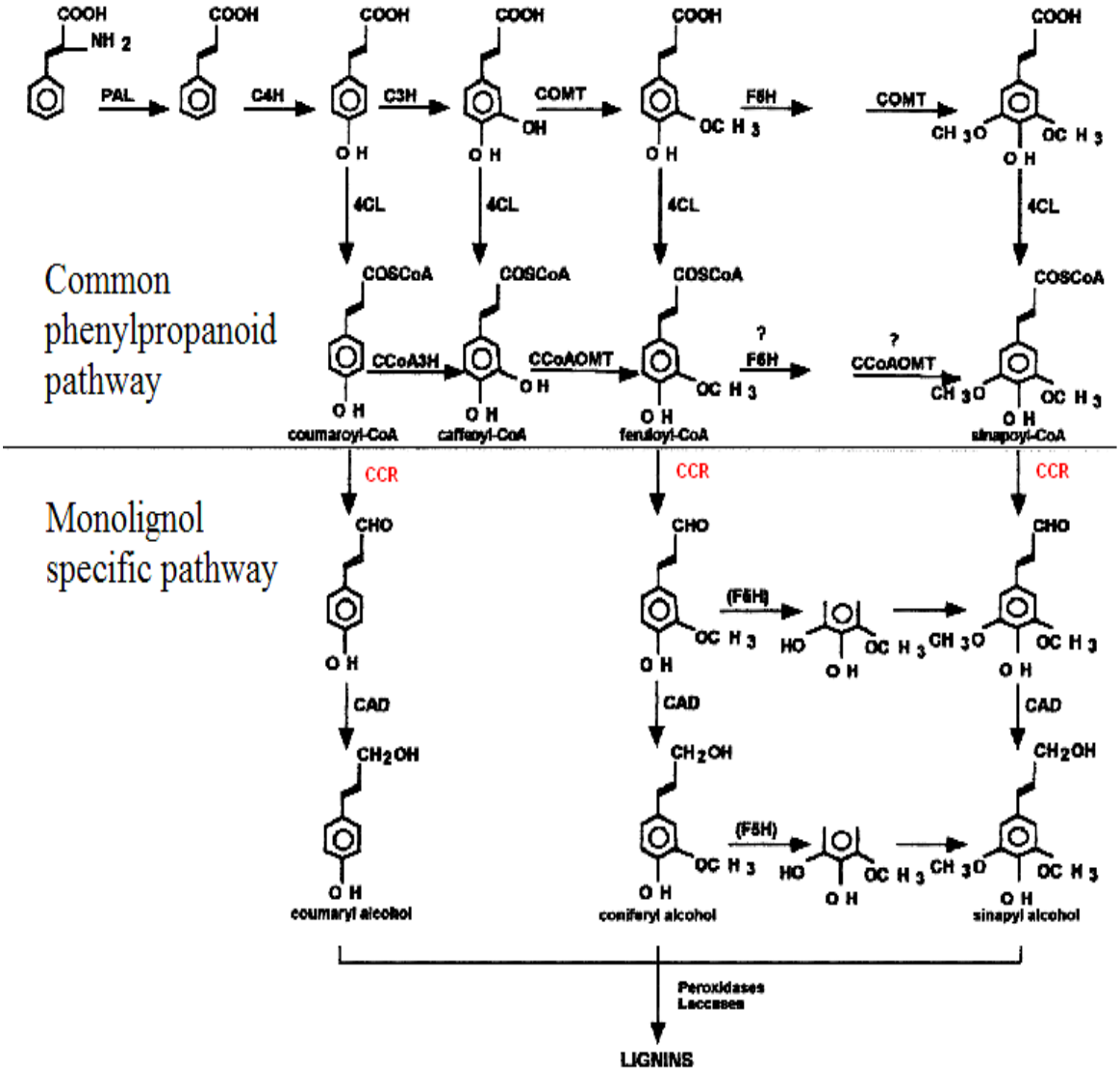


Fig. 4: An overview of the monolignol biosynthesis pathways: PAL, Phenylalanine ammonia-lyase; C4H, Cinnamic acid 4-hydroxylase; C3H, *p*-Coumarate 3-hydroxylase; HCT, Hydroxycinnamoyltransferase; 4CL, 4-Coumaroyl-CoA ligase; *CCoAOMT*, Caffeoyl-CoA O-methyltransferase; *CCR*, Cinnamoyl Coenzyme A reductase; CAld5H, Coniferyl aldehyde 5-hydroxylase; AldOMT, 5- hydroxyconiferyl aldehyde O-methyltransferase; *CAD*, Cinnamyl alcohol dehydrogenase; SAD, Sinapyl alcohol dehydrogenase (Li *et al.,* 2006).

1.5.1.2 Cinnamate 4-Hydroxylase (C4H):

Hydroxylation of cinnamic acid to p-coumaric acid is catalyzed by cinnamate hydroxylase (C4H; EC: 1.14.13.11), a cytochrome P-450-linked monooxygenase belonging to the *CYP73* family. Molecular oxygen is cleaved during this reaction, with one oxygen atom added to the aromatic ring and the other reduced to water. C4H has been purified and characterized to different degrees from severa1 plant species. Analysis of cDNA clones shows that C4H represents a class of cytochrome P-450s distinct from previously characterized P-450 enzymes (Bell-Lelong *et al.*, 1997).

1.5.1.3 Coumarate 3-Hydroxylase (C3H):

Little is known about coumarate 3-hydroxylase, the enzyme that catalyzes the hydroxylation of p-coumarate to form caffeate. Severa1 plant oxidases can carry out hydroxylation of phenolic molecules, and identifying the enzyme that catalyzes this reaction in the synthesis of monolignols has been difficult. Early workers suggested that the reaction is catalyzed by phenolase (C3H; EC: 1.10.3.1), a copper-containing oxidase capable of oxidizing a wide variety of substrates.

1.5.1.4 4-Coumarate Coenzyme A Ligase (4CL):

4-Coumarate CoA ligase (4CL; EC: 6.2.1.12) catalyzes the formation of CoA thioesters of cinnamic acids in the biosynthesis of a wide variety of phenolic derivatives, including benzoic acid, condensed tannins, flavonoids, and the cinnamyl alcohols. 4CL depends strictly on ATP, and the reaction resembles the activation of fatty acids, proceeding through an intermediate acyl adenylate, which reacts with COA to form the thioester. Early studies on 4CL suggested that multiple isoforms might control formation of different phenylpropanoid products. 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). *4CL* genes usually exist as a multi-gene family. Three isoforms of 4CL have been identified with different substrate specificities in stems of poplar and it is hypothesized that expression of different 4CL enzymes could regulate the relative abundance of the different precursors for lignin.

1.5.1.5 Caffeoyl Coenzyme A 3-O- methyltransferase (*CCoAOMT*) and Caffeate O-methyltransferase (COMT) or 5-Hydroxyconiferaldehyde O-methyltransferse (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 result in a syringyl unit. Caffeic acid is methylated to form ferulic acid by caffeic acid -O-methyltransferase (C-OMT; EC: 2.1.1.68), using S-adenosyl methionine as the methyl group donor. This methylation reaction limits the reactivity of the 3-hydroxy group, thus reducing the number of sites on the aromatic ring that can form bonds to other monolignol molecules during polymerization. The same enzyme is also believed to catalyze the methylation of 5-hydroxyferulate to sinapate. An enzyme distinct from C-OMT, caffeoyl-CoA 3-O-methyltransferase (CCoA-OMT; EC: 2.1.1.104), has been identified in connection with the defense response in several dicot plant species (Pakusch *et al.,* 1989), and a cDNA clone has been isolated from parsley cell cultures (Schmitt *et al.,* 1991). Ye *et al.,* (1994) proposed that CCoA-OMT plays a role in methylation of both caffeoyl-COA and 5-hydroxyferuloyl-COA during monolignol biosynthesis.

1.5.1.6 Cinnamoyl Coenzyme A Reductase (CCR):

As discussed above, the biosynthesis of lignin begins with the common phenylpropanoid pathway starting with the deamination of phenylalanine and leading to the cinnamoyl-CoA esters which are the common precursors of a wide array of end-products such as flavonoids, coumarins and many small phenolic molecules, (Hahlbrock and Scheel, 1989; Boerjan *et al.,* 2003) that play key roles during plant development and defense. Cinnamoyl-CoA esters are then channeled into the lignin branch pathway to produce monolignols via two reductive steps using Cinnamoyl CoA Reductase (CCR; EC: 1.2.1.44) and cinnamyl alcohol dehydrogenase (CAD). The first reductive step in lignin biosynthetic pathway is acted upon by CCR and it controls the over-all carbon flux towards lignin (Lacombe *et al.,* 1997). The reduction of cinnamoyl CoA esters to cinnamaldehydes is the first metabolic step committed to monolignol formation.

To meet the increasing demand of high quality wood for paper industry it is essential to provide designer plant species. Studies have shown that transgenic tobacco (Abbott *et al.,* 2002; Chabannes *et al.,* 2001) and *Arabidopsis* (Goujon *et al.,* 2003) down regulated for *CCR* were characterized by an approximate 50% decrease in Klason lignin. The lignin S/G ratio increased (mainly because of a decrease in the G unit amount) in transgenic tobacco and was variable, depending on the growth conditions, in transgenic *Arabidopsis*. Hence, work was initiated with the objective of cloning and characterizing *CCR* cDNA and genomic clones in *Leucaena* and also for its antisense down-regulation.

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

Hydroxylation of ferulate to 5-hydroxyferulate is catalyzed by another cytochrome P-450-linked monooxygenase, ferulate 5-hydroxylase. A genetic approach to the study of F5H function has yielded new tools for the study of this enzyme. A mutation in the *Arabidopsis* gene encoding F5H ***(fah-7)*** has been identified, and mutant plants were shown to lack sinapate-derived residues in lignin (Chapple *et al.,* 1992). It is a necessary step to biosynthesize S monolignols by 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. Genetic evidences have demonstrated that *F5H* gene is essential for S-lignin formation in *Arabidopsis*.

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

The reduction of hydroxycinnamaldehydes to hydroxycinnamyl alcohols is catalyzed by cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195). CAD has been considered to be an indicator of lignin biosynthesis because of its specific role at the end of the monolignol biosynthetic pathway. CAD is also expressed in response to stress, pathogen elicitors, and wounding. CAD is therefore, regulated by both developmental and environmental pathways, much like other well-studied enzymes of phenylpropanoid metabolism. 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/SAD is suggested to catalyze multiple cinnamyl alcohol formations from their corresponding cinnamaldehydes (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995; Whetten *et al.,* 1998).

1.5.2 Transport of monolignols:

After the synthesis, the lignin precursors or monolignols are transported to the cell wall where they are oxidized and polymerized. The monolignols formed are as such insoluble and toxic to the plant cell and hence are converted to their respective glucosides by the action of enzyme UDP-glycosyltransferase. This conversion renders the monolignols, soluble and less toxic to the plant cells. These glucosides can be stored in plant vacuoles, which are transported to the cell wall as the need arises. In gymnosperms and some angiosperms, monolignol 4–O–β-D-glucosides accumulate to high levels in the cambial tissues (Steeves *et al.,* 2001). It has been hypothesized that these monolignol glucosides are storage or transport forms of the monolignols. A uridine diphosphate glucose (UDP-G) coniferyl alcohol glucosyl transferase (Steeves *et al.,* 2001), together with coniferin-beta-glucosidase (CBG), may regulate storage and mobilization of monolignols for lignan and/or lignin biosynthesis (Dharmawardhana *et al.,* 1995).

1.5.3 Dehydrogenation:

After the transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols. The dehydrogenation to monolignol radicals has been attributed to different classes of proteins, such as peroxidases, laccases, polyphenol oxidases, and coniferyl alcohol oxidase. Which of these enzymes or a combination thereof are responsible for the dehydrogenation of the monolignols in plants and whether monolignol oxidation occurs through redox shuttle-mediated oxidation are still unclear (Onnerud *et al.,* 2002).

1.5.4 Polymerization:

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. Based on biochemical and cellular evidence, it has been suggested that laccases and peroxidases may be two types of possible enzymes involved in lignin polymerization (Christensen *et al.,* 1998;). However, convincing genetic evidence to support this suggestion is lacking.

Whether lignin is polymerized from monomer units randomly or in a guided way remains an enigma (Ralph *et al.,* 2004; Davin and Lewis, 2005). A gene encoding a dirigent protein was cloned and the biochemical results suggested that the dirigent protein might play a role in guiding a stereo-specific lignin polymerization (Davin *et al.,* 1997). This hypothesis still remains to be confirmed.

The actual process of lignin polymer formation occurs without the rigid biochemical controls seen in the biosynthesis of the precursor monolignols, giving rise to a unique class of polymers. Lignins are racemic mixtures (Ralph *et al.,* 1999), derived from radical coupling reactions under chemical (but no apparent biochemical) control between phenolic radicals (monolignol radicals) in an essentially combinatorial fashion. The accepted model for lignin polymerization is based on simple chemically controlled combinatorial coupling reactions, which was recently challenged by Davin and Lewis (2000). According to Davin and Lewis group, the macromolecular assembly of lignin is not based on "random coupling" of monolignols. Instead, this group proposed a strong biological control over the outcome of phenoxy radical coupling *in vivo*. The new theory arose from the discovery of a fascinating class of dirigent proteins implicated in lignin biosynthesis (Boerjan *et al.,* 2003); lignans are de-hydrodimers of monolignols and are typically optically active. 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 (Davin and Lewis, 2000).

1.5.4.1 Radical generation and radical coupling:

After their dehydrogenation, the radicals, which are relatively stable owing to electron delocalization that provides single-electron density to the side-chain β position, are coupled. The most important reaction is cross-coupling to the growing polymer to extend the complex three-dimensional lignin network (Fig. 2b). But, such coupling reactions are radical quenching. Each extension of the polymer requires new radicals on each of the two coupling partners. Radicals on the growing lignin polymer are thought to be generated by radical transfer from monolignols or other intermediaries (Takahama and Oniki, 1994).

Similar radical transfer mechanisms can be envisioned between the monolignols and the growing polymer, *i.e*. the monolignols may act as the radical shuttles. When a monolignol radical encounters a polymer radical, it may cross-couple with it, but when the polymer is not electron-deficient, radical transfer may occur and the monolignol will diffuse back to the peroxidase/laccase to be reoxidized. Alternatively, redox shuttles, such as a Mn^{2+}/Mn^{3+} system (Onnerud *et al.,* 2002), may be involved.

1.6 Lignin deposition:

Lignin deposition begins at the cell corners in the region of the middle lamella and the primary wall when S1 (outermost layer of secondary wall) layer formation has initiated. It is one of the final stages of xylem cell differentiation and mainly takes place during secondary thickening of the cell wall (Donaldson *et al.*, 2001). The deposition of lignin proceeds in different phases; each proceeded by the deposition of the polysaccharide matrix in the S2 (middle layer of secondary wall) layer. The bulk of lignin is deposited after cellulose and hemicellulose have been deposited in the S3 (innermost layer of secondary wall) layer. Generally, lignin concentration is higher in the middle lamella and cell corners than in the S2 layer of secondary wall (Baucher *et al.,* 1998; Donaldson *et al.*, 2001; Saka and Goring, 1985).

Given that the middle lamella and the cell corners are rich in Ca^{2+} pectate 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. Also, evidence is available to show that ferulates and diferulates may act as attachment sites for monolignols (Ralph *et al.,* 1995).

1.7 Regulation of Lignin biosynthesis genes:

The potential for phenylpropanoid biosynthesis in a given species is determined by the nature and expression patterns of the genes present in that species. It is well established that there is spatial and temporal regulation of specific genes, and also regulation in response to both endogenous (metabolic and hormonal) and environmental (light, pathogen attack, cold, etc.) stimuli. New information has recently been obtained on the transcription factors that regulate specific genes and the processes that control transcription factor activity. A large body of data has accumulated on the structures and cis-acting elements of several phenylpropanoid biosynthetic genes. The genes used in many of these studies are ***PAL, CHS,*** and dihydroflavonol reductase ***(DFR)***. The promoter analysis has become more and more refined, and extended to other genes and various species.

1.7.1 Transcription factors controlling the genes involved in lignin biosynthesis:

To date, LIM, R2R3-MYB, KNOX and MADS proteins have all emerged as equally plausible regulators of lignin biosynthesis (Kawaoka and Ebinuma, 2001; Weisshaar and Jenkins, 1998). Perhaps different proteins are used in different developmental contexts, or in response to different external or internal cues, such as day length or resource availability, to modulate lignin biosynthesis. In several maize genes (*e.g.* in the BRONZE2 promoter; a Cl-motif and a R-motif) have been identified which are important for co-activation by MYB-like and bHLH (basic region/helix-loop-helix) classes of transcription factors respectively (Faktor *et al.,* 1997*;* Droge-Laser, *et al.*, 1997).

Most of the genes involved in lignin biosynthesis have been identified and their promoter regions have been studied. The AC rich-element Pal-box is an important cis-acting

element. The Pal-box motif, CCA (C/A) (A/T) A (A/C) C (C/T) CC, is a highly conserved sequence that is involved in the expression of phenylpropanoid biosynthetic genes such as *PAL*, *4CL*, *CHS* and *CAD*. The Pal-box motif appears to be similar to the nucleotide sequence bound by MYB transcription factors. The first evidence for the role of MYB proteins in the regulation of genes in the phenylpropanoid pathway came from the study of a flower-specific MYB from Antirrhinum. Overexpression of two Antirrhinum MYB genes in transgenic tobacco down-regulated the expression of some genes in phenylpropanoid biosynthesis. This result suggests that MYB-type transcription factors might be involved in regulating the expression of genes in lignin biosynthesis. However, there are many members of the MYB gene family in a plant genome and the DNA binding specificity of plant MYB proteins varies considerably. The binding-site performance and affinity of MYB proteins is also likely to be strongly influenced by other protein factors that interact with them. Hence, it is difficult to believe that the MYB-type transcription factors alone play a pivotal role in the expression of lignin biosynthetic genes.

Recent analysis of the French bean CHSZS promoter indicated that a 39 base pair region, containing G-box and H-box elements, directed both tissue-specific expression and stress-responsive activation in transgenic tobacco plants. The H-box shares similarity with the Cl-motif and may act as a MYB recognition element. The G-box belongs to a large family of ACGT elements (ACES), which generally are recognised by bZIP-type (basic region/leucine zipper) factors. Taken together, there are three types of elements, which are identified in many of the analyses: ACES or G-box-like elements, bHLH factor binding sites (R-motifs), which are related to ACES, and MYB recognition elements. The combinatorial interaction of these elements, which has been found repeatedly, provides the specificity in the promoter- and stimulus-dependent gene activation process.

1.8 Conclusion:

Natural variations in lignin content and composition observed between different plants, tissues and cell types (Campbell and Sederoff, 1996; Sederoff *et al.,* 1999; Donaldson *et al.*, 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 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 polymerisation (Whetten *et al*., 1998; Boudet *et al*., 1995; Boerjan *et al*., 2003; Baucher *et al*., 2003; Boudet, 2000, Sederoff, 1999).

Significant amount of study has been done on various lignin biosynthesis genes in past ten odd years in various laboratories around the world. Prime objectives were to increase forage value, proper utilization of biomass and increase resistance against pathogens. Lignin biosynthesis genes are also being targeted to produce designer plants using antisense and siRNA technology with low lignin content. Down-regulation of various key genes have been achieved to obtain transgenic plant with low or modified lignin content.

This research work was started with a background of utilization of *Leucaena leucocephala* in paper production in India. Removal of lignin is a major hurdle for obtaining good quality pulp. *L. leucocephala* is extensively used in paper industry in India, therefore as a first step to generate transgenic plant with low lignin content; cDNA clone of *CCR* gene from *L. leucocephala* was isolated and characterized. Heterologous expression and spatio-temporal expression has also been studied in this research work and down-regulation of *CCR* gene using antisense construct has been attempted.

1.9 References:

Adler E. 1977. Lignin chemistry: past present and future. *Wood Science and Technology* **11,** 169–218.

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: Downregulation of three unrelated lignin biosynthetic genes in tobacco. *Plant Physiology* **128,** 844–853.

Baucher M, Halpin C, Petit-Conil M, Boerjan W. 2003. Lignin: genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology* **38,** 305–350.

Baucher M, Monties B, Van Montagu M, Boerjan W. 1998. Biosynthesis and genetic engineering of lignin. *Critical Reviews in Plant Science* **17,** 125–197.

Bell-Lelong DA, Cusumano JC, Meyer K, Chapple C. 1997. Cinnamate-4-hydroxylase expression in *Arabidopsis*. *Plant Physiology* **113,** 729–738.

Boerjan W, Ralph J, Baucher M. 2003. Lignin Biosynthesis. *Annual Reviews in Plant Biology* **54**, 519- 546.

Boudet AM. 2000. Lignins and lignification: selected issues. *Plant Physiology and Biochemistry* **38,** 81-96.

Boudet AM, Lapierre C, Grima-Pettenati J. 1995. Biochemistry and molecular biology of lignification. *New Phytologist* **129,** 203–236.

Campbell MM, Sederoff RR. 1996. Variation in lignin content and composition.Mechanisms of control and implications for the genetic improvement of plants. *Plant Physiology* **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.

Chabannes M, Ruel K, Yoshinaga A, Chabbert B, Jauneau A, Joseleau J-P, Boudet A-M. 2001. 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 Journal* **28**, 271–82.

Chapple CCS, Vogt T, Ellis BE, Somerville CR. 1992. An Arabidopsis mutant defective in the general phenylpropanoid pathway. *Plant Cell* **4,** 1413–1424.

Christensen JH, Bauw G, Welinder KG, Montagu M, Boerjan W. 1998. Purification and characterization of peroxidases correlated with lignification in poplar xylem. *Plant Physiol*ogy **118,** 125–135.

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 Physiology* **123,** 453–461.

Davin LB, Lewis NG. 2005. Lignin primary structures and dirigent sites. *Current Opinion in Biotechnology* **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.

Dharmawardhana DP, Ellis BE, Carlson JE. 1995. A _-glucosidase from lodgepole pine xylem specific for the lignin precursor coniferin. *Plant Physiology* **107,** 331–339.

Donaldson LA. 2001. Lignification and lignin topochemistry—an ultrastructural view. *Phytochemistry* **57,** 859–873.

Droge-Laser W, Kaiser A, Lindsay WP, Halkier BA, Loake GJ, Doerner P, Dixon RA, Lamb C. 1997. Rapid stimulation of a soybean protein-serine kinase that phosphorylates a novel bZlP DNA-binding protein, G/HBF-1, during the induction of early transcription-dependent defenses. *EMBO Journal* **16**.726-738.

Faktor O, Loake G, Dixon RA, Lamb CJ: 1997. The G-box and H-box in a 39 bp region of a French bean chalcone synthase promoter constitute a tissue-specific regulatory element *Plant Journal* **11**.1105-1113.

Freudenberg K, Neish AC, ed. (1968) Constitution and Biosynthesis of Lignin. Berlin: Springer-Verlag. pp 129

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.

Hahlbrock K, Scheel D. 1989. *Annual Review of Plant Physiology and Plant* **40***,* 347-369.

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 Journal* **14,** 545–553.

Humphreys JM, Chapple C. 2002. Rewriting the lignin roadmap. *Current Opinion in Plant Biology* **5**, 224–229.

Jones L, Ennos AR, Turner SR. 2001. Cloning and characterization of irregular xylem4 (irx4): a severely lignin deficient mutant of Arabidopsis. *Plant Journal* **26,** 205–216.

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

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 Journal* **11,** 429–441.

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

Lewis NG, Yamamoto E. 1990. Lignin: occurrence, biogenesis and biodegradation. *Annual Review of Plant Physiology and Plant Molecular Biology* **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.

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

Nimz HH, Tutschek R. 1977. *Holzforschung* **31,** 101–106.

Onnerud H, Zhang L, Gellerstedt G, Henriksson G. 2002. Polymerization of monolignols by redox shuttle-mediated enzymatic oxidation: a new model in lignin biosynthesis. *Plant Cell* **14,** 1953–1962.

Pakusch A-E, Kneusel RE, Matern U. 1989. S-Adenosyl-lmethionine: trans-caffeoylcoenzyme A 3-O-methyltransferase from elicitor-treated parsley cell suspension cultures. *Archives in Biochemistry and Biophysics* **271,** 488–494.

Ralph J, Grabber JH, Hatfield RD. 1995. Lignin-ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydrate Research* **275,** 167–178.

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 Reviews* **3,** 29–60.

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, Peng J, Lu F, Hatfield RD. 1999. Are lignins optically active? *Journal of Agriculture and Food Chemistry* **47,** 2991–2996.

Saka S, Goring DAI. 1985. Localization of lignins in wood cell walls. In Biosynthesis and Biodegradation of Wood Components, ed. Higuchi T, pp 51–62. Orlando: Academic. pp 679.

Sarkanen KV, Ludwig CH. 1971. Lignins: Occurrence, Formation, Structure, and Reactions. New York: Wiley- Intersci pp 916.

Schmitt D, Pakusch A-E, Matern U. 1991. Molecular cloning, induction, and taxonomic distribution of caffeoyl-CoA 3-O-methyltransferase, an enzyme involved in disease resistance. *Journal of Biology and Chemistry* **266,** 17416–17423.

Sederoff RR, MacKay JJ, Ralph J, Hatfield RD. 1999. Unexpected variation in lignin. *Current Opinion in Plant Biology* **2,**145–152.

Steeves C, Forster 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,** 1085–1093.

Takahama U, Oniki T. 1994. Effects of ascorbate on the oxidation of derivatives of hydroxycinnamic acid and the mechanism of oxidation of sinapic acid by cell wallbound peroxidases. *Plant Cell Physiology* **35,** 593–600.

Terashima N, Fukushima K, He LF, Takabe K.1993. Comprehensive model of the lignified plant cell wall. In Forage Cell Wall Structure and Digestibility, ed. Jung HG, Buxton DR, Hatfield RD, Ralph D pp 247–70. Madison: ASACSSA-SSSA pp 794.

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.

Weisshaar B and Jenkins G. 1998. Phenylpropanoid biosynthesis and its regulation. *Current Opinion in Plant Biology* **1**. 251-257.

Whetten R, Sederoff R. 1995. Lignin biosynthesis. *Plant Cell* **7,** 1001–1013.

Whetten Ross W., John J. MacKay, and Ronald R. Sederoff. 1998. Recent advances in understanding lignin biosynthesis. *Annual Review Plant Physiology and Plant Molecular Biology* **49,** 585–609.

Ye Z-H. 1997. Association of caffeoyl coenzyme A 3-O-methyltransferase expression with lignifying tissues in several dicot plants. *Plant Physiology* **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.

Zimmermann HH, Nimz, E. Seemuller. 1985. *Holzforschung* **39,** 45–49.

Chapter Two

Isolation, cloning and characterisation of CCR gene from *Leucaena leucocephala*

2.1 Introduction:

Lignin biosynthesis has been already discussed in detail in Chapter One of this thesis. The present chapter deals with why *Leucaena leucocephala* and *CCR* from *L. leucocephala* were used as candidates for this study. In its later part isolation, cloning and characterization of *CCR* gene from *L. leucocephala* is described in detail.

Paper industry in India mainly uses bamboo, *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 given industrial unit. In developing countries, like India, the proportionate use of bamboo and hardwood species is in the ratio of 15:85 although all these plant species are of equal importance to the paper industry.

2.1.1 *Leucaena* as a source of pulp:

Leucaena sp. is extensively used in India and about 25% of raw material for pulp and paper industry is contributed by this hard wood tree. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. It will thus be vital to raise plantations with elite materials and/or genetically modified plants that meet the demands of the pulp and the paper industry in economical and sustainable manner.

2.1.2 About *Leucaena leucocephala*:

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, rich in protein and grow in a wide range of soils (Jones, 1979; Hughes, 1998). However, these attributes are limited by the occurrence of anti-nutritive factors in the fodder, such as tannins and mimosine (Jones, 1979; Hegarty *et al.*, 1964; Hammond *et al.*, 1989 a, b).

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

Classification:

Division:	Magnoliophyta
Class:	Magnoliopsida
Sub- class:	Rosidae
Order:	Fabales
Family:	Fabaceae
Sub-family:	Mimosoideae
Tribe:	Mimoseae
Genus:	Leucaena
Species:	leucocephala

Common name: Lead tree, white popinac, subabul.

Sub species: Glabrata (Rose; S. Zarate); Ixtahuacana (Hughes) and Leucocephala (Benth) Var. Peru and Cunningham

Related genera: *Desmanthus*, *Schleinitzia*, *Calliandropsis*, *Neptunia, Alantsilodendron*, *Gagnebina, Dichrostachys* and *Kanaloa*.

2.1.3 Lignin Biosynthsis and *CCR*:

Detailed Lignin biosynthesis has been discussed in Chapter One. In brief, the biosynthesis of lignin begins with the common phenylpropanoid pathway starting with the deamination of phenylalanine and leading to the cinnamoyl-CoA esters. These esters are the common precursors of a wide array of end-products such as flavonoids, coumarins and many small phenolic molecules (Hahlbrock and Scheel, 1989; Boerjan *et al.*, 2003) that play key roles during plant development and defense. Cinnamoyl-CoA esters are then channeled into the lignin branch pathway to produce monolignols via two reductive steps using *CCR* and Cinnamyl Alcohol Dehydrogenase (*CAD*). By the action of *CAD*, monolignols are formed which are toxic to plant cells. They are acted upon by monolignol specific UDP-glucosyltransferase, which adds a glucose moiety to monolignols (Glycosides), and are transported to outside the cell membrane. In the cell wall the glycosides are again hydrolyzed to form Hydroxyphenyl (H), Guaiacyl (G) and Syringyl (S) units respectively. These monomers are linked together via end-wise and radical coupling reactions (Sarkanen and Ludwig, 1971; Freudenberg and Neish, 1968) to produce H, G and S lignin respectively. Here, dehydrogenative polymerization of monolignols takes place to form lignin (Boerjan *et al.*, 2003; Dixon *et al.*, 2001).

The first reductive step in lignin biosynthetic pathway is acted upon by *CCR* and it controls the over-all carbon flux towards lignin (Piquemal *et al.,* 1998). The reduction of cinnamoyl CoA esters to cinnamaldehydes is the first metabolic step committed to monolignol formation (Lacombe *et al.,* 1997). *CCR* is apparently encoded by a single gene per haploid genome in *Eucalyptus* (Boudet *et al.*, 1997), poplar (Leple *et al.*, 1998), ryegrass (Larsen, 2004; McInnes *et al.*, 2002), *Triticum* (Ma, 2007) and tobacco (Piquemal *et al.*, 1998) and by two genes in maize (Pichon M, 1998) and *Arabidopsis* (Lauvergeat *et al.*, 2001). The *CCR* genes in various species appear as a multiple member family. In the *Populus* genome, there exist 8 *CCR*-homolog or *CCR*-like gene sequences (Li, 2005). Several other *CCR* gene sequences have been deposited in the GenBank database, but their functions have still not been demonstrated. It is proposed that all *CCR* enzymes have a similar catalyzing mechanism for converting the CoA ester to aldehyde in monolignol biosynthesis.

2.1.3.1 *CCR* as a candidate gene:

Out of three (H, G and S) monomer units of lignin, S and G lignins are found predominantly 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 and removal of total lignin is easier. Transgenic tobacco (Chabannes *et al.,* 2001; Abbott *et al.*, 2002) and *Arabidopsis* (Lauvergeat *et al.,* 2001; Goujon *et al.*, 2003) down regulated for *CCR* were characterized by an approximately 50% decrease in Klason lignin. The lignin S/G ratio increased (mainly because of a decrease in the G unit amount) in transgenic tobacco and was variable, depending on the growth conditions, in transgenic *Arabidopsis* (Abbott *et al.*, 2002; Goujon *et al.,* 2003 and Chabannes *et al.,* 2001). A change in 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). Transgenic plants with the lowest *CCR* activity and 50% reduced lignin showed abnormal phenotypes, such as important alterations in the fiber cell walls and loosening in the arrangement of the cellulose microfibrils that resulted in reduced cell wall cohesion (Elkind *et al.,* 1990; Pincon a or b *et al.*, 2001).

Based on the above information the work was started with the objective of cloning and characterization of the *Leucaena CCR* gene and its antisense down-regulation. This study is the first instance towards isolation and characterization of lignin biosynthetic pathway gene(s) for the development of transgenic *Leucaena* plants. The study is intended to lay down criteria for the development of the transgenic plants, which would ultimately strive for: (a) reduced lignin content and (b) altered ratio of S/G lignin. These criteria would be met by isolation, cloning and characterization of the selected and key lignin biosynthesis pathway genes *viz.* cinnamyl alcohol dehydrogenase (*CAD*), 4-coumarate-CoA-ligase (4CL), Caffeate-O-methyltransferase (COMT or AldOMT), caffeoyl-CoA-3-O-methyltransferase (CCoAOMT), cinnamoyl-CoA-reductase (*CCR*), UDP glucose-glucosyl transferase (UDPG-GT) and coniferin β-glucosidase (CBG). The above genes of the lignin biosynthesis pathway in the target plant species could then be down regulated by transformations with the gene constructs in sense or antisense orientations under the control of suitable tissue specific promoters. It will, however, be beyond the scope of this

thesis to cover all the genes listed above. This thesis elaborates isolation and characterization of *CCR* gene, its differential expression, and down regulation.

No study was carried out on lignin biosynthetic pathway gene(s) so far in *Leucaena* and the study of Cinnamoyl Co A Reductase will help in better understanding of the pathway in *Leucaena* sp. and its manipulation. This research work deals with isolation, cloning and characterization of *CCR* gene from *Leucaena*. In the present study the genomic and the cDNA gene clones of *CCR* were isolated from *Leucaena leucocephala*. In this report, two ful-length *CCR* cDNAs from *Leucaena* have been isolated and characterized.

2.2 Materials and Methods:

2.2.1 Materials:

Chemicals:

Ampicillin, Kanamycin, Tetracycline, Tris-buffer, IPTG, X-gal, SDS, BSA, EDTA and Ethidium bromide were purchased from Sigma-Aldrich, USA. Agarose, restriction enzymes, T4 DNA ligase, RNase A and lysozyme were obtained from GIBCO-BRL (USA), Promega (USA) and Amersham (UK). Taq DNA polymerase was obtained from Bangalore Genei (India). 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 Kodak (USA). All other chemicals and solvents of analytical grade were purchased from HIMEDIA, Qualigens Fine Chemicals and E-Merck Laboratories, India.

Plasticware:

Sterile disposable filter sterilization units and petri dishes were procured from Laxbro (India). Micro-centrifuge tubes and micropipette tips were procured from Axygen and Tarsons (India).

Glassware:

Glassware used in the experiments such as test tubes, glass bottles, petridishes, Erlenmeyer flasks and pipettes were procured from Borosil (India).

Bacterial strains and plasmids used in the study:

Escherichia coli XL-1 blue (Stratagene, USA)

E. coli MRF-XL-1 (Stratagene, USA)

E. coli XOLR (Stratagene, USA)

E. coli SOLR (Stratagene, USA)

λ ZAP II vector (Stratagene, USA)

pGEM-T Easy Vector Cloning vector (Promega, USA)

Stock solutions:

1) IPTG stock solution 200 mg/mL in sterile distilled water.

2) X-gal stock solution 20 mg/mL in dimethylformamide.

3) IPTG stock solution 1 M in sterile distilled water.

4) Kanamycin 30 mg/mL.

5) Ampicillin 100 mg/mL.

6) Tetracycline 12.5 mg/mL.

Plant material: RACE, Isolation of full-length *CCR* gene:

Xylem tissue of mature healthy plants, growing in NCL campus was harvested as and when required. Outer bark was scraped to expose the xylem tissue. The tissue was scraped and ground using liquid nitrogen and RNA was isolated for normal cDNA preparations (RACE PCR).

2.2.2 Methods:

2.2.2.1 Bacterial culture conditions:

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

2.2.2.2 *E. coli* transformation and selection:

LB medium (50 mL) was inoculated with 1% of the overnight grown *E. coli* culture and allowed to grow till A_{600} was 0.5. The cells were harvested by centrifugation at 5,000 g for 10 min at 4 °C, suspended in 100 mM ice-cold TB Buffer and again centrifuged at 5,000 g for 10 min at 4 °C. Cells were suspended in 4.65 mL of ice-cold TB Buffer and 350 µL DMSO. Aliquots of 100 µL were frozen in liquid nitrogen and stored at –80 °C till further use. The competent *E. coli* cells, thus formed, were transformed according to Sambrook *et al.* (1989). Briefly, DNA (~50 ng in 10 µL or less) was added to 100 µL of competent *E. coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42 °C for 2 min. To each tube 900 µL of LB broth was added and further incubated at 37 °C for 1 h. About 100 µL of the transformed competent cells was spread onto LB agar plates containing appropriate antibiotic, IPTG (4 µL per 90 mm plate) and X-gal (40 µL per 90 mm plate) (Sambrook *et al.*, 1989).

2.2.2.3 Genomic DNA extraction:

Solutions:

Extraction buffer: 100 mM Tris-HCl, pH 8.0; 20 mM Sodium EDTA; 1.4 M NaCl and 2.0% (w/v) CTAB (cetyltrimethylammoniumbromide). Store at 37 °C. Add 0.2% (w/v) β-mercaptoethanol before use.

Other solutions used were Chloroform:Isoamyl alcohol: 24:1 (v/v); autoclaved 5 M NaCl, RNase A (10 mg/mL); 95% ethanol; 75% ethanol; autoclaved TE buffer: 10 mM Tris-HCl and 1 mM EDTA pH 8.0.

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 1g 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 polyvinylpolypyrrolidone (PVPP) added. The tube was gently inverted several times 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: isoamyl-alcohol 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: isoamylalcohol extraction step repeated. To the clear supernatant, 0.5 volume of 5 M NaCl was added and mixed gently. Next, two volumes of cold (-20 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 the DNA pellet washed with cold (4 ºC) 75% ethanol and air-dried. DNA was dissolved in 300 µL of TE buffer.

The DNA solution was treated with 1µL RNase A (10 mg/mL) per 100 µL DNA and incubated at 37 ºC for 30 min. The sample was extracted with chloroform: isoamyl alcohol, re-precipitated and dissolved in TE buffer. Purity of DNA was checked spectro-photometrically by measuring the ratio of OD at 260/280 nm. DNA was stored at 4 ºC.

2.2.2.4 Isolation of plasmid DNA from *E. coli* cells:

Solutions:

Soln. I (TEG Buffer): 25 mM Tris-HCl, pH 8.0; 10 mM EDTA , pH 8.0; 50 mM Glucose

Soln. II: 0.2 N NaOH, 1% SDS (freshly prepared)

Soln. III: 3.0 M Potassium acetate (pH 4.8)

Chloroform, absolute ethanol, 3.0 M Sodium acetate, 70% ethanol, de-ionized water

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 DNA per 1.5 mL culture depending on the host strain and the plasmid vector. An important feature of this protocol was the use of poly-ethyleneglycol (PEG) for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination. The bacterial cultures were grown overnight (O/N) with shaking (200 rpm) at 37 °C in LB broth, with appropriate antibiotic(s). About 3 mL culture was centrifuged for 1 min at 4,000 g to pellet the bacterial cells. The pellet was resuspended in 200 µL of TEG buffer by vigorous pipetting, 300 µL of Soln. II was added, mixed by inversion till the solution becomes clear and incubated on ice for 5 min. The cell lysate was

neutralized by addition of 300 µL of Soln. III, mixed well and incubated on ice for 5 min. The cell debris was removed by centrifuging for 5 min at 12,000 g at room temperature. The supernatant was transferred to a clean tube; RNase A to a final concentration of 20 µg/mL (Sambrook *et al.,* 1989) was added and incubated at 65 ^{o}C for 15 min. To the above solution 400 µL of chloroform was added, mixed for 30 s and centrifuged for 5 min at 12,000 g at 4 ^{o}C. The upper aqueous layer was transferred to a clean tube, 1/10th volume sodium acetate and 0.6 volume iso-propanol was added with mixing and kept at RT for 10 min. The sample was centrifuged at 12,000 g for 15 min at room temperature. The pellet was washed twice with 70% ethanol and dried under vacuum. The dried pellet was dissolved in 100 µL of de-ionized water and 100 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 °C. The supernatant was aspirated carefully, the pellet was washed with 75% ethanol and air-dried. The dried pellet was resuspended in 40 µL de-ionized water and stored at -70 °C.

2.2.2.5 Restriction digestion of DNA:

Plasmid and genomic DNA restriction digestion was set up as per manufacturer's (Promega, USA; NEB, UK; Amersham, USA) recommendations.

2.2.2.6 Extraction and purification of DNA from agarose gel:

The restriction digested DNA or PCR amplified products were run on an agarose gel in 1X TAE buffer (see Appendix). The gel was stained with ethidium bromide (0.5 µg/mL) and viewed using a hand held long wavelength UV illuminator. The fragment of interest were excised from the gel and weighed. A 50-200 mg gel slice was transferred to a 1.5 mL micro-centrifuge tube. Purification of DNA from gel was done according to the manufacturer's protocol (Auprep, Lifetechnologies; Axygen India Pvt. Ltd; SIGMA-ALDRICH, India.).

2.2.2.7 Total RNA Isolation:

Solutions:

TRI Reagent (SIGMA)

Chloroform and Isoamyl Alcohol (24:1); Isopropanol; 75% ethanol in DEPC treated de-ionized water; DEPC treated de-ionized water; 8 M LiCl.

RNase free environment was created and maintained as described below. The glassware 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 TRI reagent. The plant tissue was collected, washed with DEPC treated water, frozen in liquid nitrogen and crushed to a fine powder. TRI reagent (1 mL) was added to 100 mg of the fine powder and mixed thoroughly using a vortex. Chloroform: isoamyl alcohol (300 µL) was added and mixed thoroughly again using vortex. The tubes were centrifuged at 4 ºC at 13,000 g for 10 min. The supernatant was transferred to 1.5 mL microcentrifuge tube and the chloroform: isoamyl alcohol step was repeated. The aqueous phase was transferred to 1.5 mL tubes and 0.6 volume isopropanol was added. It was mixed thoroughly and kept for RNA precipitation for 1 h at 4 °C. Total RNA was pelleted out by centrifugation at 13,000 g for 15 min at 4 ºC. The RNA pellet was washed with 75% ethanol twice and dried in a SpeedVac (Savant, USA) centrifugal concentrator. RNA pellet was dissolved in 100 µL of DEPC treated water. 50 µL of 8 M LiCl was added, mixed and kept at -20 °C overnight. Next day total RNA was pelleted out by centrifugation at 13,000 g for 15 min at 4 ºC, washed with 75% ethanol, dried in SpeedVac and dissolved in 50 µL DEPC treated de-ionized water. Purity of RNA was confirmed by measuring OD at 260/280 nm and also by visualization on 1.5% TAE agarose gel.

2.2.2.8 cDNA first strand synthesis by Reverse Transcription:

Complementary DNA (cDNA) is synthesized from a mature mRNA template in a reaction catalyzed by the enzyme reverse transcriptase. The resulting molecule is a DNA-RNA hybrid and the process is called as cDNA first 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.

In the present study cDNA first strand was synthesized using ImProm-II™ Reverse Transcription System (Promega, USA). The reactions were set up as per the manufacturer's guidelines.

In brief, reverse transcription reactions of up to 1 µg of Total RNA performed in 20 µL reactions comprised of following components of the ImProm-II Reverse Transcription System.
The first strand reaction was set up as follows:

Experimental RNA (1µg)	1- 6 µL
Primer [Oligo $(dT)_{15}$] or Random (10 pmol)	1 µL
Nuclease-Free Water	3 µL
Final volume	10 µL

The tubes were incubated at 70 °C for 5 min and then chilled on ice for 2 min. The tubes were briefly spun in a micro-centrifuge to collect the condensate and maintain the original volume. The tubes were kept on ice until addition of the reverse transcription reaction mix was over. The reverse transcription reaction mix was prepared by combining the following components of the ImProm-II Reverse Transcription System in a sterile 1.5 mL micro-centrifuge on ice.

Nuclease-free water	1.5 µL
ImProm-II. 5X Reaction Buffer	4.0 µL
$MgCl_2$ (15 mM)	2.0 µL
dNTP Mix (10 mM)	1.0 µL
RNasin® Ribonuclease Inhibitor (40U/µL)	0.5 µL
ImProm-II Reverse Transcriptase	1.0 µL
Final volume	10.0 µL

An aliquot of 1.0 µg total RNA and oligo $(dT)_{15}$ or random hexamer primer (10 pmol) mix was added to the above reaction for a final reaction volume of 20 µL per tube. The tube was incubated at 42 °C for 1 h for cDNA first strand synthesis. Reverse transcriptase was thermally inactivated by incubation at 90 °C for 5 min prior to proceeding with PCR amplification.

2.2.2.9 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. This technique has been successfully used for various purposes like fishing out of gene(s) from genomic DNA or from cDNA population, introducing restriction sites of interest in the amplified product for directional cloning, creating sequence mismatch/ deletion/ addition resulting in mutant version of a gene or nucleotide sequence, differentiating between two alleles etc. In the present study applications of PCR were exploited for a few of the above specified applications (Mullis *et al.*, 1992). The PCR reaction mixture and cycling conditions used were as follows:

Reaction mixture:

Sterile de-ionized water	6.7 µL
Template (100 ng/µL)	1.0 µL
Forward primer (7 pmol)	1.0 µL
Reverse primer (7 pmol)	1.0 µL
dNTPs (0.2 mM)	4.0 µL
10 X Taq Buffer A (Mg^{+2} 15 mM)	1.0 µL
Taq Polymerase (1 U/µL)	0.3 µL
Total volume	15.0 µL

PCR cycle conditions:

No. of cycles	Temperature	Time
1	95 ºC	5 min
35	95 ºC 45-65 ºC (annealing temperature was dependent on primer Tm) 72 ºC	1 min 30-45 s 1min 30 s
1	72 ºC	7 min
1	4 ºC	Hold

2.2.2.10 Southern Hybridization, Slot Blot Hybridisation and Random Primer Labeling:

2.2.2.10.1 Southern Hybridization:

Solutions:

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

Depurination solution: 0.25 N HCl

Denaturation solution: 1.5 M NaCl, 5 M NaOH

Neutralization solution: 0.5 M Tris-HCl (pH 7.4); 3 M NaCl

Gel loading dye (6X): 0.25% Bromophenol blue in 40% (w/v) sucrose in water

For Southern hybridization (Southern, 1975) the DNA samples were electrophoresed on an agarose gel in 1X TAE buffer containing 0.5 µg/mL ethidium bromide. The gel was rinsed with de-ionized water (DW) and placed in depurination solution for 15 min. It was then rinsed with de-ionized water and immersed in denaturation solution for 30 min with gentle shaking. The gel was again rinsed with de-ionized 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 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, USA) of the exact gel size was wetted with de-ionized water followed by transfer buffer (20X SSC) and then placed

on top of the gel. A glass rod was rolled over the membrane to remove any trapped air bubbles. One piece of Whatman #3 paper wetted with 20X SSC was placed on the membrane followed by Whatman #3 paper pre-wetted in 2X SSC. On this paper another dry Whatman #3 paper was placed followed by a stack of absorbent paper towels. A glass plate and a ~0.5 kg weight were placed on the top of the paper towels. Transfer of DNA was allowed to proceed for 18 h. The membrane was marked for orientation, removed carefully and washed with 6X SSC. The membrane was air dried and baked for 2 h at 80 °C to immobilize DNA onto the nylon membrane. Hybridization and autoradiography were carried out as is described in the section 2.2.2.10.4.

2.2.2.10.2 Slot Blot Hybridization:

Solutions:

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

3 M NaOH.

For slot blot hybridization DNA samples were diluted according to experimental requirements. The DNA samples were denatured by adding 1/10th volume 3 M NaOH and incubation at 65 ºC for 10 min. An equal volume of 6X SSC was added to the denatured samples. Two layers of Whatman #3 filter paper wetted with sterile de-ionized water and 6X SSC were placed in the Slot Blot apparatus (Hoefer Scientific, USA) followed by Hybond-N^+ membrane (Amersham, USA) 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 appeared dry. The unit was carefully disassembled and the membrane taken out. The membrane was air dried and then baked for 2 h at 80 °C to immobilize DNA. Hybridization and autoradiography were carried out as described in the following section 2.2.2.10.4.

2.2.2.10.3 Random Primer Labeling:

Random primer labeling of the DNA probes was done using the Megaprime DNA labeling kit (Amersham, USA) Reaction (50 µL) was set up as follows:

25 ng DNA (used as probe)	5.0 µL
Primer solution (random hexanucleotides)	5.0 µL

Above mixture was heated in a boiling water bath for 10 min and cooled to room temperature facilitating primer annealing to the DNA. 40 µL of the following reaction mixture was added to above mixture.

10X reaction buffer	5.0 µL
dCTP/dATP	4.0 µL
dGTP	4.0 µL
dTTP	4.0 µL
α-32P-dATP/dCTP Sp. activity 3000 Ci/ mmol	2 to 5.0 µL
Sterile de-ionized water	17 to 20.0 µL
Exonuclease free Klenow fragment (2 U/µL)	1.0 µL
Total volume	50.0 µL

The reaction was carried out at 37 ^{o}C for 60 min and stopped by incubation in a boiling water bath for 10 min and snap chilled on ice.

2.2.2.10.4 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 2.2.2.10.1 and 2.2.2.10.2 above were pre-hybridized at 50 °C 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 50 °C for 14-18 h. The solution was decanted and the membrane washed with low stringency buffer at 55 °C for 15 min followed by a high stringency wash at 55 °C for 15 min. This process was repeated two or three times, till the desired (20– 50 cps) count from membrane is achieved. The blot was checked for 20 to 50 counts per seconds by Geiger Muller counter, wrapped in Saran wrap and exposed to X-ray film at –70 °C in a cassette with intensifying screen. After four days films were developed using developer and fixer solutions described in Appendix.

2.2.2.11 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 °C. After two hours of incubation all SM buffer (refer Appendix) was pooled in one tube and kept at 4 °C for further use (Plaques stable up to 6 months at 4 °C).

2.2.2.11.1 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 $^{\circ}$C for further use.

2.2.2.11.2 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 $^{\circ}$C for 8 h.

2.2.2.11.3 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 $^{\circ}$C for 2h before pre hybridization.

2.2.2.11.4 Preparation of probe, Pre-hybridization and Hybridization:

PCR was used (as described in section 2.2.2.9) to prepare radiolabelled probe. 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 2.2.2.10.4

Necessary care was taken while working with radioactive dATP and dCTP. Lab coat, spectacles, and gloves were used while working.

2.2.2.11.5 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.

2.2.2.11.6 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 1000 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.

2.2.2.12 Rapid Amplification of cDNA Ends (RACE):

Generally using reverse transcription, 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 and amplified by PCR using RACE. In the present study, SMART RACE cDNA Amplification Kit (BD Biosciences, Clontech, USA) was used. The reactions were set up as per the manufacturer's guidelines.

Briefly, SMART technology provides a mechanism for generating full length cDNA's in reverse transcription reactions. This is done by the joint action of the SMART II™ A Oligonucleotide and the PowerScript™ Reverse Transcriptase (RT). PowerScript RT is a variant of MMLV RT, which upon reaching the end of a RNA template exhibits terminal transferase activity by adding 3–5 residues (predominantly dC) to the 3' end of the first strand cDNA. The SMART oligo contains a terminal stretch of G residues that anneal to the dC-rich cDNA tail and serves as an extended template for RT. PowerScript RT switches templates from the mRNA molecule to the SMART oligo, generating a complete cDNA copy of the original RNA with the additional SMART sequence at the end. Following reverse transcription, the first strand cDNA is used directly in 5'- and 3'-RACE PCR reactions. The only requirement for SMART RACE cDNA amplification is 23–28 nucleotides of sequence information in order to design gene specific primers (GSPs) for the 5'- and 3'-RACE reactions.

Using SMART RACE Kit two separate cDNA populations, 5'-RACE cDNA and 3'-RACE cDNA are synthesized. The cDNA for 5'-RACE is synthesized using a modified lock-docking oligo (dT) primer and the SMART II A oligo as described above. The modified oligo (dT) primer termed the 5'-RACE CDS Primer A (5'-CDS), has two degenerate nucleotide positions at the 3' end (Table 2.2.1). These nucleotides position the primer at the start of the A+ tail and thus eliminate the 3' heterogeneity inherent with conventional oligo (dT) priming. Once, RACE cDNAs are prepared, 5'- and 3'-RACE can be performed using gene specific primers. All PCR reactions in the SMART RACE protocol are carried out using the Advantage® 2 Polymerase Mix. The Polymerase Mix is comprised of

TITANIUM™ Taq DNA Polymerase—a nuclease-deficient N-terminal deletion of Taq DNA polymerase plus TaqStart® Antibody to provide automatic hot-start PCR and a minor amount of a proofreading polymerase.

The reactions were set up for 5' and 3' RACE cDNA as below:

2.2.2.12.1 RACE cDNA preparation:

For preparation of 5'-RACE cDNA

RNA sample (1 µg/µL)	5 µL
5'-CDS primer A (12 µM)	1 µL
SMART II A oligo (12 µM)	1 µL
SMQ	3 µL

For preparation of 3'-RACE cDNA

RNA sample (1 µg/µL)	5 µL
3'-CDS primer A (12 µM)	1 µL
SMQ	4 µL

Sterile H_2O was added to a final volume of 10 µL for each of the above reaction. Contents were mixed and the tubes centrifuged briefly. The tubes were incubated at 70 °C for 2 min and cooled on ice for 2 min. The tubes were briefly centrifuged and to each reaction tubes following reagents were added:

5X First-Strand Buffer	4 µL
DTT (20 mM)	1 µL
dNTP Mix (10 mM)	1 µL
$MgCl_2$ (25 mM)	3 µL
PowerScript Reverse Transcriptase (5U/ µL)	1 µL
Total volume	10 µL

The contents of the tube were mixed by gentle pipetting and were briefly centrifuged. The tubes were incubated at 42 ºC for 1.5 h in a hot-lid thermal cycler. The first strand reaction mixture was diluted to 100 µL with Tricine- EDTA buffer (provided with the kit) and heated at 70 ºC for 7 min. The diluted first strands were used for 5' and 3' RACE. The following master mix was prepared according to the number of PCR reactions to be set up:

2.2.2.12.2 Primary and Nested PCR:

Master mix for RACE PCR reaction

PCR-Grade Water	34.5 µL
10X Advantage 2 PCR Buffer	5.0 µL
dNTP Mix (10 mM)	1.0 µL
50X Advantage 2 Polymerase Mix	1.0 µL
Total volume	41.5 µL

The above master mix was used for 5' and 3' RACE PCR.

The reaction was set up as follows, for 5'RACE:

5' RACE cDNA	2.5 µL
UPM (10X)	5.0 µL
GSP1 (10 µM) (Sam r1)	1.0 µL
Master Mix	41.5 µL
Total volume	50.0 µL

The reaction was set up as follows, for 3'RACE:

3' RACE cDNA	2.5 µL
UPM (10X)	5.0 µL
GSP2 (10 µM) (SCHF2)	1.0 µL
Master Mix	41.5 µL
Total volume	50.0 µL

Following PCR cycling conditions were used for amplifying 5' and 3' RACE products:

No. of Cycles	Temperature	Time
5 cycles	94 °C 72 °C	30s 3 min
30 cycles	94 °C 60 °C 72 °C	30 s 30 s 3 min

After the PCR, 7 µL of the reaction mix was loaded on 1.0% agarose gel in 1X TAE buffer and checked for amplification. Nested PCR was run according to cycling conditions mentioned in section 2.2.2.9

Nested PCR reaction mix:

Nested 5' RACE and 3' RACE were performed as separate reactions.

PCR-Grade Water	41 µL
10X Advantage 2 PCR Buffer	5.0 µL
dNTP Mix (10 mM)	1.0 µL
50X Advantage 2 Polymerase Mix	1.0 µL
Forward primer (SCHF2, 3' RACE)	1 µL
Reverse primer (Sam r1, 5' RACE)	1 µL
NUP (Table 1)	1.0 µL
5' and 3' Primary reaction	2.0 µL
Total volume	50 µL

Following is the list of primers (refer Table 2.2.1), which were used for the RACE: Provided with kit

Name	Sequence 5'-3'
SMART II™ A Oligonucleotide	AAGCAGTGGTATCAACGCAGAGTACGCGGG
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAGAGTAC $(T)_{30}$V N
5'-RACE CDS Primer A (5'-CDS)	$(T)_{25}$V N
10X universal Primer A Mix (UPM)	Long: CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT Short: CTAATACGACTCACTATAGGGC
Nested universal Primer A (NUP)	AAGCAGTGGTATCAACGCAGAGT

Table 2.2.1: List of Primers used for the RACE.

2.2.2.13 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.

2.2.2.14 Bioinformatics analysis:

Nucleotide and amino acid sequence analysis was done using software **pDRAW 32** and **ClustalX 1.8.** Primer designing was done by aligning *CCR* sequences in NCBI database using ClustalX. Ll-*CCR* gene sequences were characterized with the Genscan software and homology was verified by database searching at the National Center for Biotechnology Information server using BLAST algorithm (http://www.ncbi.nlm.nih.gov). The deduction of the amino acid sequences, calculation of the theoretical molecular mass and pI, as well as prediction of sub-cellular localization was performed with ExPASy Proteomic tools provided at http://www.expasy.ch/tools/. Global alignment of two nucleotide or amino acid sequences and percentages of identity were calculated using the EMBOSS Pairwise Alignment algorithms (http://www.ebi.ac.uk/emboss/). 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). Online bioinformatics analysis facility available at www.justbio.com, Mat-Inspector (for promoter analysis.) and www.expasy.org was used for detailed analysis.

2.2.2.15 Estimation of *CCR* gene copy number:

Genomic DNA was isolated using Lodhi *et al.* method. Gene copy number was estimated by southern hybridization and Real time PCR analysis. DNA was digested with four restriction enzymes, *Hinc* II*, Nde* I, *Bgl* I and *Sac* I. One of the enzymes, S*ac* I does not cut inside the gene while the other three have one site in the gene. Southern hybridization was done using an ≈800 bp fragment (from 5' of *CCR* coding region and UTR) as a probe at 62 °C hybridization temperature (Sambrook *et al.*, 1989). Gene copy number was also estimated using Taqman based PCR chemistry. Standard dilutions of *CCR* gene cloned in pGEM-T vector was used to make a standard graph The reaction was run in triplicates. Primers and probe were designed from Eurogenetec, Belgium and care was taken to design it from one single exon (Table 2.2.2). Genomic DNA was quantified and dilutions were made according to the dilutions made for standard graph (1.81 pg of DNA per haploid genome of *L. leucocephala*). Number of copies initially present in amount of gDNA used in Taqman PCR was compared with standard graph and copy number was estimated (Freeman *et al.*, 1999).

Name	Sequence 5'-3'
*CCR*2 Taqprobe Modifications 3'- Black Hole Quencher-3 ™ 597.63 5'-Cy5Indocarbocyanin ® 533.63	ACT CCT TCT GCC TCC GGT CGT TAT
*CCR*2F2	TGT TGC ATT AGC CCA TGT TCT TG
*CCR*2R2	GAG GAT CTC GAC CAG TTC TCC

Table 2.2.2: Primers used to determine Gene copy Number using Taqman probe.

2.3 Results:

2.3.1 The presence of the *CCR* gene in *L. leucocephala*:

The presence of Cinnamoyl Co A Reductase (*CCR*) gene in *Leucaena leucocephala* was established by slot blot analysis. Genomic DNA of good integration was isolated from the leaves of *L. leucocephala.*

Slot Blot Hybridization:

The genome size of *L. leucocephala* has ~1.81 pg of DNA per haploid genome. 1.81 pg corresponds to a single representation of *L. leucocephala* genomic DNA, 0.18 µg and 0.36 µg representing the genome $1x10^5$ and $2x10^5$ times respectively was spotted on Hybond N^+ membrane (Amersham, USA). The CCR cDNA gene clone from tobacco (1061 bp; a kind gift from Dr. Claire Halpin, Dundee, UK) was spotted as standard dilutions representing $1x10^5$ and $2x10^5$ copies in duplicates. The blot was probed with the radiolabelled tobacco *CCR* gene. Positive signal obtained under high stringency hybridization conditions was indicative of the presence of the *CCR* gene in *L. leucocephala.* Based on signal intensity it was also inferred that in *L. leucocephala CCR* belonged to a gene family represented possibly by two members (Fig. 2.3.1).

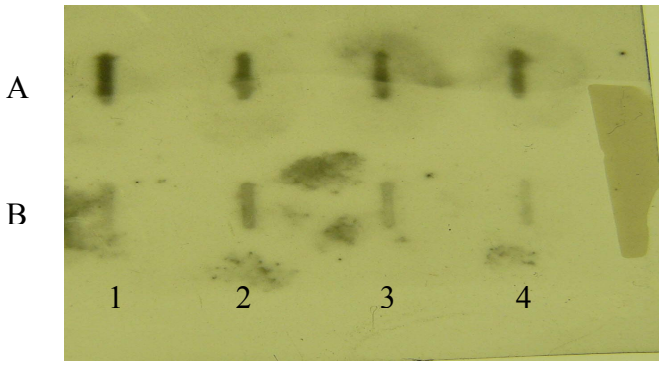


Fig. 2.3.1: Slot Blot Hybridization for *CCR* gene in *L. leucocephala*: Slot Blot of *L. leucocephala* genomic DNA hybridized with radiolabelled tobacco *CCR* gene (U20736): Lane A1, A2 and A3, A4 signals from $2x10^5$ and $1x10^5$copies of genomic DNA representations of *L. leucocephala* in duplicates, respectively. Lane B1, B2 and B3, B4 signals from $2x\ 10^5$ and $1x\ 10^5$ copies of tobacco *CCR* gene clone (each blotted in duplicates, respectively).

2.3.2 Isolation of partial fragment of *CCR* gene:

After establishment of presence of *CCR* gene in *L. leucocephala*, the next step was to isolate and characterize the gene in the same plant. This could be done in three ways

1. PCR based approach
2. cDNA/gDNA library screening
3. Reverse genetics

In the present study, PCR based approach and cDNA/ gDNA library screening were used as a tool for isolation of *CCR* gene from *L. leucocephala*. In preliminary attempts, PCR based method was followed to achieve above objective.

2.3.2.1 Total RNA isolation and cDNA preparation:

RNA was isolated from xylem tissue of *L. leucocephala* (Fig. 2.3.2) and cDNA first strand was prepared using above stated protocol section (2.2.2.8).

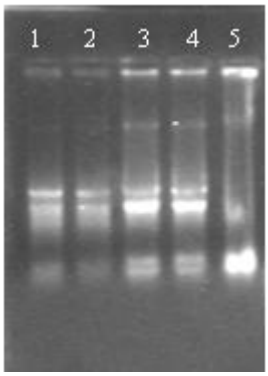


Fig. 2.3.2: Electrophorogram of RNA isolation from *L. leucocephala* run on 1% agarose gel: Lane 1-4 intact RNA, Lane 5 degraded RNA.

2.3.2.2 Primer designing:

All known *CCR* amino acid and cDNA sequences from NCBI GenBank database were aligned using CLUSTAL X software. Few conserved domains were recognized in the aligned sequences. Alignments were also performed using different combinations of all known sequences e.g.

1. Sequences reported from one type of family.
2. All tree species.
3. All full-length sequences reported.

On the basis of above alignments 10-12 pairs of primers were designed. These primers were used to perform PCR using gDNA and cDNA as a template.

2.3.2.3 PCR, Cloning and sequence analysis:

Above sets of primers, in all possible combinations were used in PCR reactions using cDNA and gDNA as a template. Out of all the primer sets only one set gave amplification with estimated size (Table 2.3.1).

S. No	Name	Sequence: 5'- 3'	Tm
1	SCHF2	GATGAGGTGG TTGACGAGTC TT	64 °C
2	Samr1	TTAGCTGAGCCAGTGAGGTACTT	62 °C

Table 2.3.1: Primers used for isolation of partial *CCR* gene fragment.

Using above primers, ~250bp cDNA fragment and ~600 bp gDNA fragment were amplified (Fig. 2.3.3).

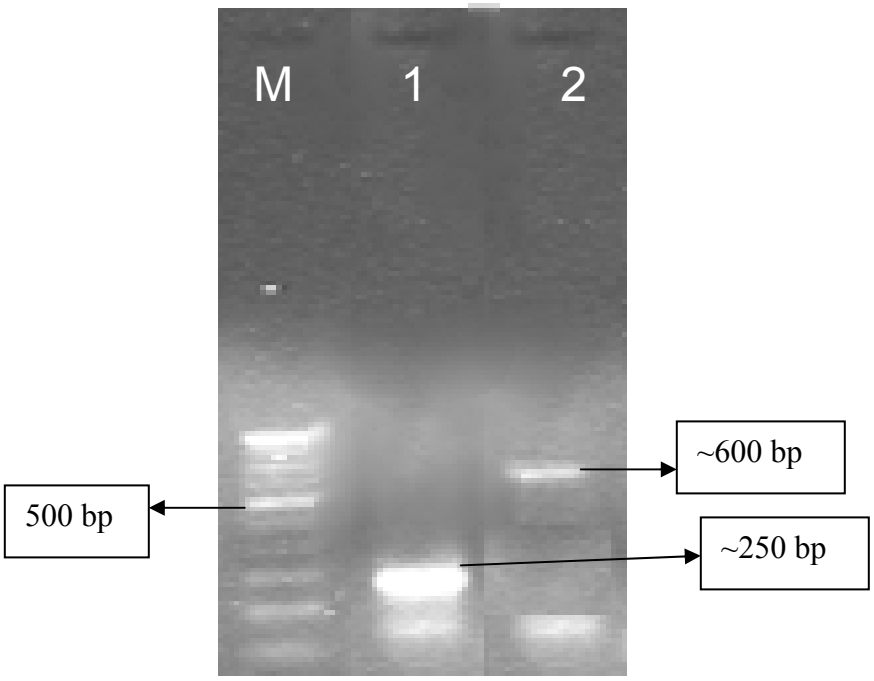


Fig. 2.3.3: PCR amplified fragment of *CCR* gene run on 1% agarose gel: Lane M: 100 bp ladder Bangalore Genei, Lane 1: 250 bp cDNA fragment, Lane 2: 600 bp gDNA fragment.

The above two fragments were cloned in pGEM-T easy vector (Fig. 2.3.4) and sequenced. Sequencing revealed that cDNA fragment is 230 bp and gDNA fragment is 585 bp.

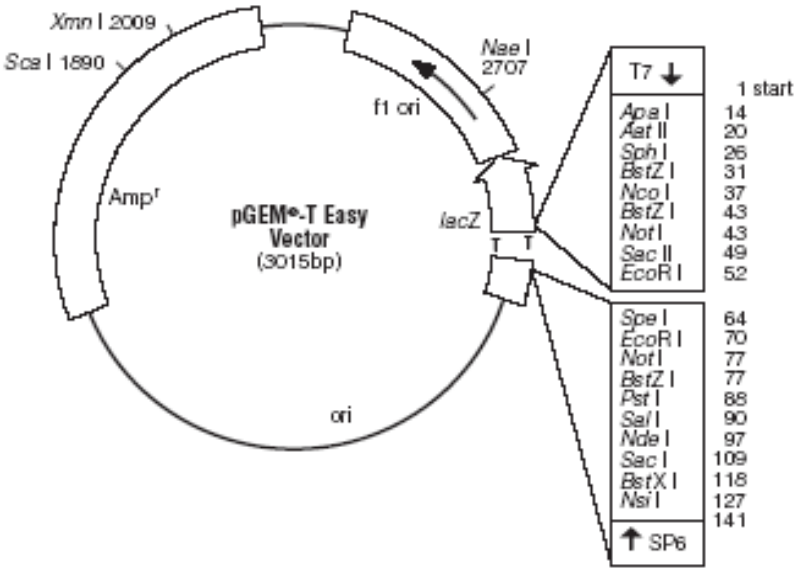


Fig. 2.3.4: Map of pGEM-Teasy vector

2.3.2.4 Sequence analysis of cDNA and gDNA Fragments:

The above cloned fragments of cDNA and gDNA were assigned as p***CCR*230** and p***CCR*585.** BLAST result of both the sequences showed significant similarity with other known *CCR* sequences in database.

p*CCR*585: Genomic DNA nucleotide sequence

```
  1 GATGAGGTGG TTGACGAGTC TTGCTGGAGC AATTTGGATT ATTGCAAGAA
 51 CACAAAGGTA TATACTTCCA ACCTACCCCC TCTCTTCTCA ATCATAAATC
101 CCCTAATATT TTGTTGACAC GTGATCTTGG AGGTCAATCA AGAGACAATA
151 GTTCTAAATT CTATTTTTAG AAGTAATATT ATATGCATAT GTCCCTCCCC
201 AGACTCCATC TGATAGAACC TGGGCTGCCC TAACGCTCAT TTTGAAACTC
251 GAGTAAAAAG GCTGCTCAGT GCATTAGTCT CTCTAGGAAG GATAGATGCA
301 CATGACCTTA TCTTGAGGAT TTGAATCATT GACTTTCATG TCACATATTA
351 GCAATCTTAC TTCTGCGCTA AGCTCATGCT CATGGATACA ATATGAGGGA
401 TGGATTTTGC AGAATTGGTA TTGCTATGGG AAGGCAGTGG CAGAGAAATC
451 AGCATGGGAT GAGGCAAAAG CAAGAGGGGT GGATTTGGTT GTGGTGAATC
501 CAGTTTTGGT GTTGGGACCA TTGCTTCAAA CCACCATGAA TGCAAGCACA
551 ATTCACATCC TCAAGTACCT CACTGGCTCA GCTAA
```

The sequence consists of an incomplete exon from nucleotide 1 to 57, a complete intron from nucleotide 58 to 412 (in red), one partial exon from nucleotide 413 to 585. Spidey hosted on www.ncbi.nlm.nih.gov/tools has been used for gene/exon prediction and to predict the splice sites. The intron splice junctions followed the GT and AG rule and were confirmed through cDNA analysis.

p*CCR*230: cDNA nucleotide sequence

```
  1 GATGAGGTGG TTGACGAGTC TTGCTGGAGC AATTTGGAAT ATTGCAAGAA
 51 CACAAAGAAC TGGTATTGCT ATGGGAAGGC AGTGGCAGAG CAAGCAGCAT
101 GGGATGAGGC AAAAGCAAGA GGGGTGGATT TGGTTGTGGT GAATCCAGTT
151 TTGGTGTTGG GACCATTGCT TCAAACCACC ATGAATGCAA GCACAATTCA
201 CATCCTCAAG TACCTCACTG GCTCAGCTAA
```

Alignment of the spliced gDNA sequence and cDNA sequence showed marginal sequence differences, which may be due to sequencing error. Alignment of the spliced partial gDNA and the cDNA sequence with known *CCR* sequences in NCBI GenBank Databases is shown below, which suggests that the sequence is a partial fragment of *CCR* gene from *L. leucocephala.*

```
pCCR585     ------GATGAGGTGGTTGACGAGTCTTGCTGGAGCAATTTGGATTATTGCAAGAACACA
pCCR230     ------GATGAGGTGGTTGACGAGTCTTGCTGGAGCAATTTGGAATATTGCAAGAACACA
Fragaria    GGTCCCGATGTCGTTGTCGACGAGTCTTGTTGGAGTGACCTCGAGTTTTGCAAGAACACC
Populus     GGCCCAGATGTTGTCATTGATGAATCTTGCTGGAGTGATCTTGAATTCTGCAAGAACACC
Raphanus    GACCCTGAAGCCGTTGTCGACGAAAGTTGTTGGAGTGATCTTGAGTTCTGCAAAAACACT
                  ** *  **  * ** **   *** *****  *  * ** *  ***** *****

pCCR585     AAGAATTGGTATTGCTATGGGAAGGCAGTGGCAGAGAAATCAGCATGGGATGAGGCAAAA
pCCR230     AAGAACTGGTATTGCTATGGGAAGGCAGTGGCAGAGCAAGCAGCATGGGATGAGGCAAAA
Fragaria    AAGAACTGGTACTGCTACGGCAAAGCTGTGGCGGAGCAAGCAGCGTGGGAAGAGGCCAAA
Populus     AAGAATTGGTATTGCTATGGAAAGGCTGTGGCAGAACAAGCTGCATGGGATATGGCTAAG
Raphanus    AAGAATTGGTATTGTTACGGCAAGATGGTGGCGGAGCAAGCGGCGTGGGAGACGGCAGAG
            ***** ***** ** ** ** **    ***** **  ** * ** *****   ***  *

pCCR585     GCAAGAGGGGTGGATTTGGTTGTGGTGAATCCAGTTTTGGTGTTGGGACCATTGCTTCAA
pCCR230     GCAAGAGGGGTGGATTTGGTTGTGGTGAATCCAGTTTTGGTGTTGGGACCATTGCTTCAA
Fragaria    GAGAGAGGAGTGGACTTGGTGGTGGTGAACCCAGTTCTGGTGCTTGGACCACTGCTCCAA
Populus     GAGAAAGGGGTGGACCTAGTGGTGGTTAACCCAGTGCTGGTGCTCGGACCATTGTTCCAG
Raphanus    GAGAAAGGTGTTGACCTGGTGGTTTTGAATCCGGTGCTGGTTCTTGGTCCGCCGTTACAG
            *  * *** ** **  * ** **  * ** ** **  ****  * ** **   * * **
```

```
pCCR585     ACCACCATGAATGCAAGCACAATTCACATCCTCAAGTACCTCACTGGCTCAGCTAA----
pCCR230     ACCACCATGAATGCAAGCACAATTCACATCCTCAAGTACCTCACTGGCTCAGCTAA----
Fragaria    CCAACCATCAACGCCAGCATCATCCACATCCTCAAGTACTTGACTGGCTCGGCCAAGACT
Populus     CCCACTGTCAATGCTAGCATCGTTCACATCCTCAAGTACCTCACCGGCTCAGCCAAGACT
Raphanus    CCGACGATCAACGCCAGTCTCTTCCACGTACTAAAGTATCTAACCGGCTCGGCTAAGACT
             * **  * ** ** **     * *** * ** *****  * ** ***** ** **
```

2.3.3 Screening of genomic DNA Library:

To fish out the full-length gene the second option, screening of genomic DNA library was done, The partial 585 bp genomic DNA fragment was used to screen genomic DNA library in λ phage (ZAP II, Stratagene.). Screening was performed as described in 2.2.2.11. Ten plates with average number of plaques around 5000 were used for primary screening. Few positive plaques were picked and used for secondary and subsequently for tertiary screening (Fig. 2.3.5).

Secondary and tertiary screening was performed at a higher hybridization temperature (60 °C). Good positive signals were obtained. In the tertiary screening, transfection was performed with an aim to obtain isolated plaques. The positive plaques from tertiary screening were cut individually and excision was performed according to the manufacturer's protocol. Excised plaque was further characterized to be a 5 kb fragment. This 5 kb insert was later characterized to be a part of a calcium dependent protein kinase.

Due to major failure of genomic library screening, next step to fish out full-length gene was performed. Information of partial cDNA sequence showing significant similarity with known orthologous *CCR* sequence was exploited to perform RACE. RACE reactions were performed as described in section 2.2.2.12 of this chapter.

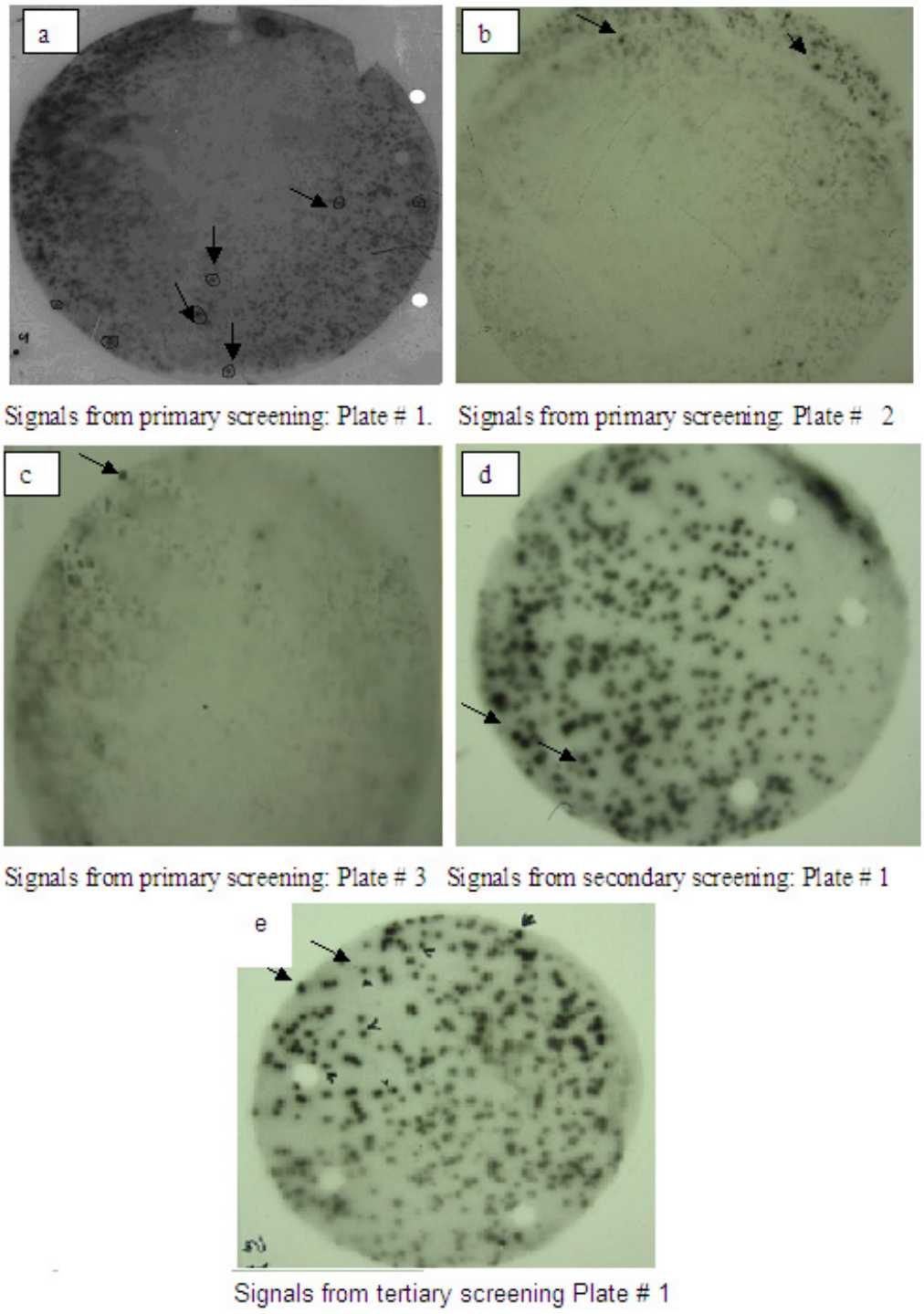


Fig. 2.3.5: Screening of genomic library of *L. leucocephala*: Arrow shows positive signals. A, B and C: Signals from primary screening. D: Signals from secondary screening. E: Signals from tertiary screening.

2.3.4 Rapid Amplification of cDNA Ends (RACE):

2.3.4.1 5' and 3' RACE:

SMART-RACE cDNA amplification kit (Clontech Laboratories Inc., Mountain View, USA) and Total RNA isolated from xylem tissue were used to amplify the 5' and 3' ends of *L. leucocephala CCR* (Ll-*CCR*) cDNA. The 5' and 3' ends of Ll-*CCR* were amplified in two rounds of PCR with the Ll-*CCR* gene-specific primers (Table 2.3.1) and with primers provided with the kit (Table 2.2.1). The first PCR for 5' end amplification of *Leucaena CCR* gene was performed with the Samr1 and UPM primers under following conditions: 3 min at 95 ºC, 35 cycles of 1 min at 95 ºC, 30 s at 45 ºC, 3 min at 72 ºC, and a final extension step for 10 min at 72 ºC. The PCR product of the first round PCR was 40 fold diluted and used as template in the nested PCR amplification with Samr1 and NUP primers at the annealing temperature of 55 ºC and under the same PCR conditions as in the case of first PCR. The first PCR for 3' end amplification of *Leucaena CCR* gene was performed with SCHF2 and UPM primers under following conditions: 3 min at 95 ºC, 35 cycles of 1 min at 95 ºC, 1 min at 58 ºC, 3 min at 72 ºC, and a final extension step for 7 min at 72 ºC. The PCR product of the first round PCR was 40 fold diluted and used as template in the second round of nested PCR with SCHF2 and NUP primers for Ll-*CCR* at the annealing temperatures of 60 ºC (Lacombe *et al.*, 1997; Ma, 2007).

Secondary reaction of 5' and 3' RACE yielded an 850 bp and 825 bp fragments respectively (Fig. 2.3.6). These two fragments were cloned in pGEM-T easy vector and six clones from each 5' and 3' RACE product were sequenced. Two 5' Clones and three different 3' clones were characterized. On sequence validation it was found that two type of 5'UTR and one type of 3' UTR were present. These clones were assigned as pGEM5'A, pGEM5'C, pGEM3' (Genbank Accession Nos. 5'A-EU195226, 5'C-EU195225, 3'-DQ986908).

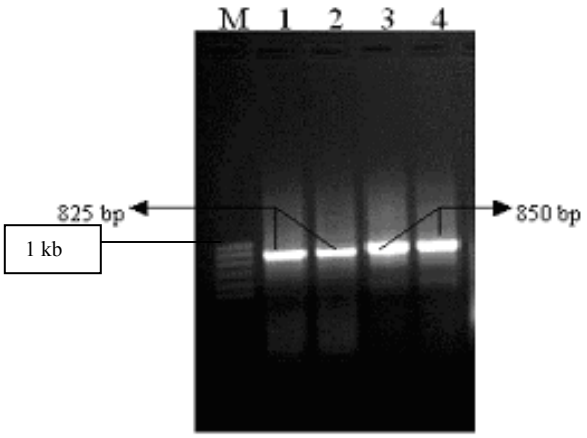


Fig. 2.3.6: 5' and 3' RACE PCR reaction: Lane M: Marker (100 bp ladder, Bangalore Genei, India), Lane 1 and 2: Secondary PCR product of 3' RACE reaction, Lane 3 and 4: Secondary PCR product of 5' RACE reaction.

Analysis of two 5' clones showed that they are 850 (Sam5' A) and 844 bp (Sam 5' C) fragments with coding region starting at 177th and 157th base, respectively. Coding region showed significant similarity to known *CCR* sequence in NCBI GenBank. UTR of 176 and 156 bases was identified in these fragments (Fig. 2.3.7 a, b and c).

2.3.4.2 Sequence analysis of 5' RACE clones:

5' UTR region and Start codon have been highlighted in blue and red color respectively.

Nucleotide sequence of pGEM5'A, 5' RACE product (GenBank Acc. No.EU195226):

```
  1 GGTCAAGCCA GAGATCATCT TCTCCACTCT CCAACTTCTT CCTTCTTTCT CTCTCCTTCT
 61 CACCAACCCC CGCACAGAGA ATTTTCTCAT TTCCCTCTTT TGCTCTATCC TACGCCTCGT
121 TCTGATTCCA AATTCGTCTC TGTTTACAGA TAATCTTATT AACACTACCC ACCACCATGC
181 CTGCTGCCGC CCCAGCCCCC ACCGCCGCTA ACACCACCTC ATCAGGTTCC GGCCAAACCG
241 TCTGCGTCAC AGGCGCCGGT GGCTTCATCG CCTCTTGGAT TGTCAAGCTC TTGCTAGAGA
301 GAGGCTACAC TGTTAGAGGC ACCGTCAGAA ATCCAGATGA TTCTAAGAAC TCTCACTTGA
361 AAGAGTTGGA AGGAGCAGAG GAGAGGCTAA CTCTTCATAA GGTTGATCTT CTTGATCTTG
421 AATCTGTGAA AGCTGTTATC AATGGTTGTG ATGGCATCAT TCACACGGCT TCTCCAGTCA
481 CAGACAACCC CGAAGAGATG GTGGAGCCGG CGGTGAATGG AGCAAAGAAT GTGATCATCG
541 CAGCTGCAGA AGCGAAAGTG AGAAGAGTAG TGTTCACGTC ATCCATTGGA GCCGTCTACA
```

601 TGGACCCCAG CAGGAACATT GATGAGGTGG TTGACGAGTC TTGCTGGAGC AATTTGGAAT
661 ATTGCAAGAA CACAAAGAAC TGGTATTGCT ATGGGAAGGC AGTGGCAGAG CAAGCAGCAT
721 GGGATGAGGC AAAAGCAAGA GGGGTGGATT TGGTTGTGGT GAATCCAGTT TTGGTGTTGG
781 GACCATTGCT TCAATCCACC ATGAATGCAA GCACAATTCA CATCCTCAAG TACCTCACTG
841 GCTCAGCTAA

Nucleotide sequence of pGEM5'C, 5' RACE product (GenBank Acc. No. EU195225):

1 GGAGAAGTCA TCTCTCCACT CTCACTCTTC CTCTCTCTCG TTTCAACCAA CCCCGCACAG
61 AGAATTTCTC ACTCCCTCTT TTGCTCTATC CTACGCTTCG TTCTGATTCC AAATCTTCTC
121 TGTTTACAGA TAGTCTTATT AACACTACCC ACCACCATGC CTGCTGCCGC CCCCGCCGCC
181 GCTAACACCA CCTCATCAGG TTCCGGCCAA ACCGTCTGCG TCACAGGCGC CGGTGGCTTC
241 ATCGCCTCTT GGATTGTCAA GCTCTTGCTA GAGAGAGACT ACACTGTCAG AGGCACCGCC
301 AGAAATCCAG ATGATTCTAA GAACGCACAC TTAAAAGAGT TGGAAGGAGC AGAGGAGAGG
361 CTAACTCTTC ATAAGGTGGA TCTTCTTGAT CTGGAATCTG TGAAAGCTGC TATCAATGGC
421 TGTGATGGCG TCATTCACAC GGCTTCTCCA GTCACAGACA ACCCCGAAGA GATGGTGGAG
481 CCGGCGGTGA ATGGAGCAAA GAATGTGATC ATCGCAGCTG CAGAAGCGAA AGTGAGAAGA
541 GTAGTGTTCA CGTCATCCAT TGGAGCCGTC TACATGGACC CCAGCAGGAA CATTGATGAG
601 GTGGTTGACG AGTCTTGCTG GAGCAATTTG GAATATTGCA AGACCACAAA GAACTGGTAT
661 TGCTATGGGA AGGCAGTGGC AGAGCAAGCA GCATGGGATG AGGCAAAAGC AAGAGGGGTG
721 GATTTGGTTG TGGTGAATCC AGTTTTGGTG TTGGGACCAT TGCTTCAAAC CACCATGAAT
781 GCAAGCACAG TTCACATCCT CAAGTACCTC ACTGGCTCAG CTAA

Deduced amino Acid sequence of coding region of pGEM5'A RACE product:

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

Deduced amino Acid sequence of coding region of pGEM5'C RACE product:

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

Alignment of deduced amino acid sequence of pGEM5'A and pGEM5'C showed few differences at amino acid level too. The sequence alignment showed major difference near the start codon, which strengthens the possibility of two different isoforms of *CCR* gene present in *L. leucocephala* (Fig. 2.3.7 c).

2.3.4.3 Analysis of two 5' UTRs:

Inspection of both the UTRs revealed that there is a possibility of two different UTR, driving two different *CCR* genes. Some motifs were characterized which were already reported in promoters of plant origin. Motif like P$GAGA/GAGABP.01 and P$IBOX/IBOX.01 (IUPAC name for motifs) were characterized only in 5'C UTR and they were missing in 5'A UTR. This analysis supported the information of differential expression of two *CCR* genes, which is already reported for maize and *Arabidopsis* (Table 2.3.2).

P$GAGA/GAGABP.01	(GA)n/(CT)n binding proteins (GBP, soybean; BBR, barley)	20 - 44(5'C)	gaaacgAGAGagaggaagagtgaga
P$MIIG/PALBOXL.01	Cis-acting element conserved in various PAL and 4CL promoters	61 – 75 (5'A) 58 - 72(5'C)	cggggttGGTGaga cggggttGGTGaga
P$MYBS/MYBST1.01	MybSt1 (Myb Solanum tuberosum 1) with a single myb repeat	104 – 120(5'A) 82 - 98(5'C)	tgctctATCCtacgcct tgctctATCCtacgctt
P$IBOX/IBOX.01	I-Box in rbcS genes and other light regulated genes	109 - 125(5'C)	aaacaGAGAagatttgg
P$MIIG/ATMYB84.01	R2R3-type myb-like transcription factor (IIG-type binding site)	166 – 180(5'A) 143 - 157(5'C)	tggtggtgGGTAgtg tggtggtgGGTAgtg

Table 2.3.2: Nucleotide sequence analysis of 5'A and 5'C UTRs.

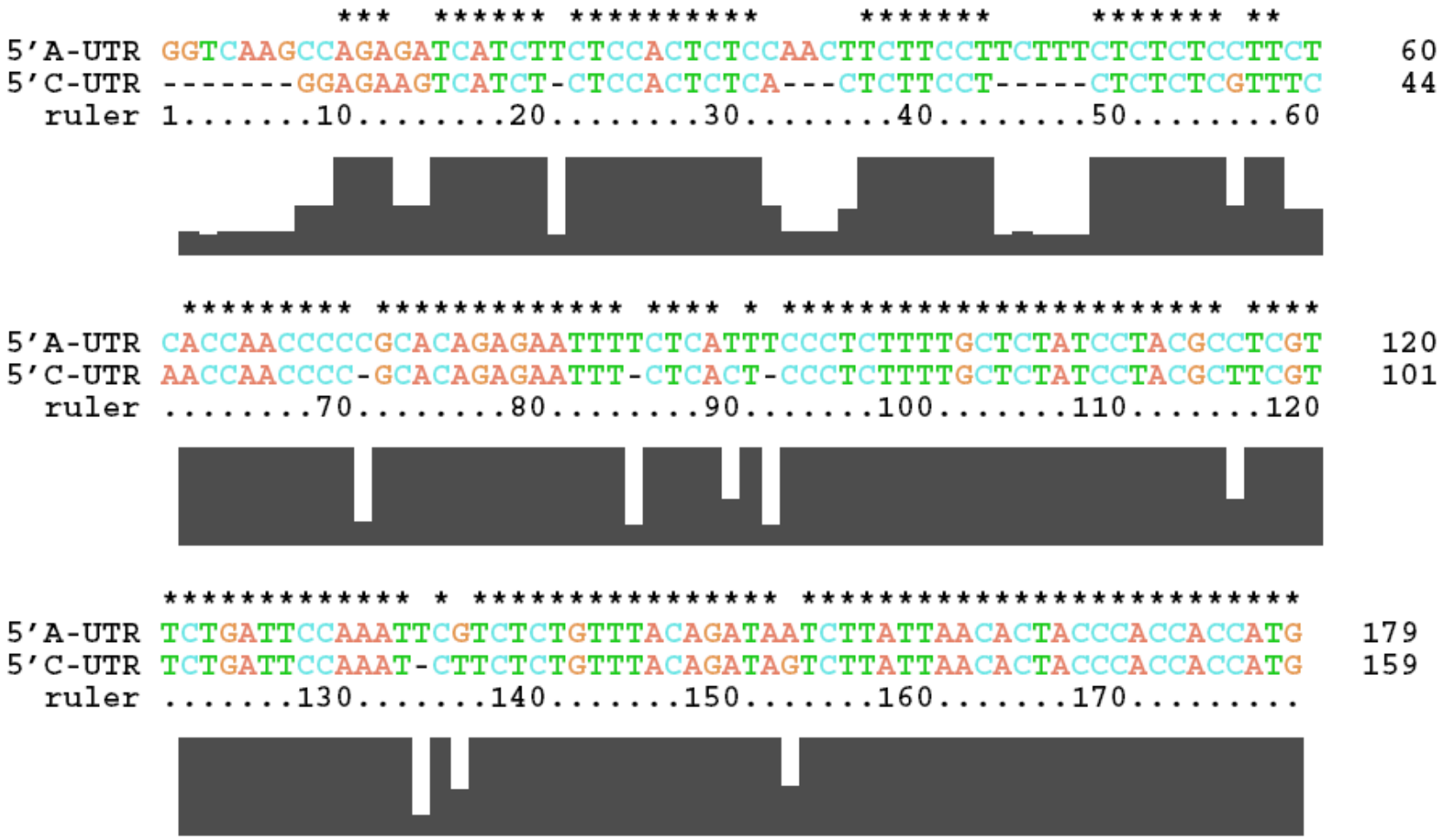


Fig. 2.3.7 a: Alignment of 5'A and 5'C UTR region.

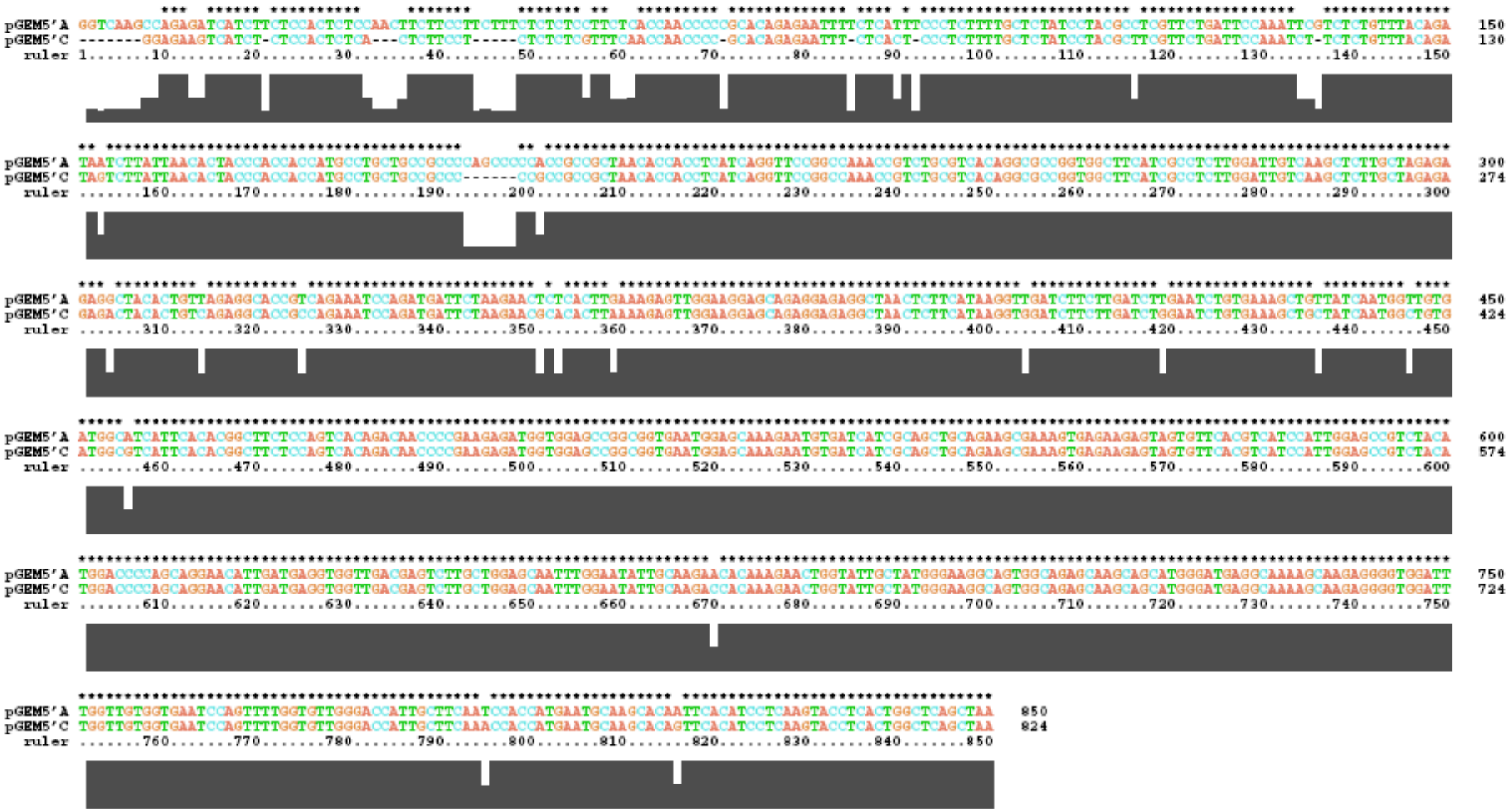


Fig. 2.3.7 b: Alignment of nucletide sequence of pGEM5'-A and pGEM5'-C clone

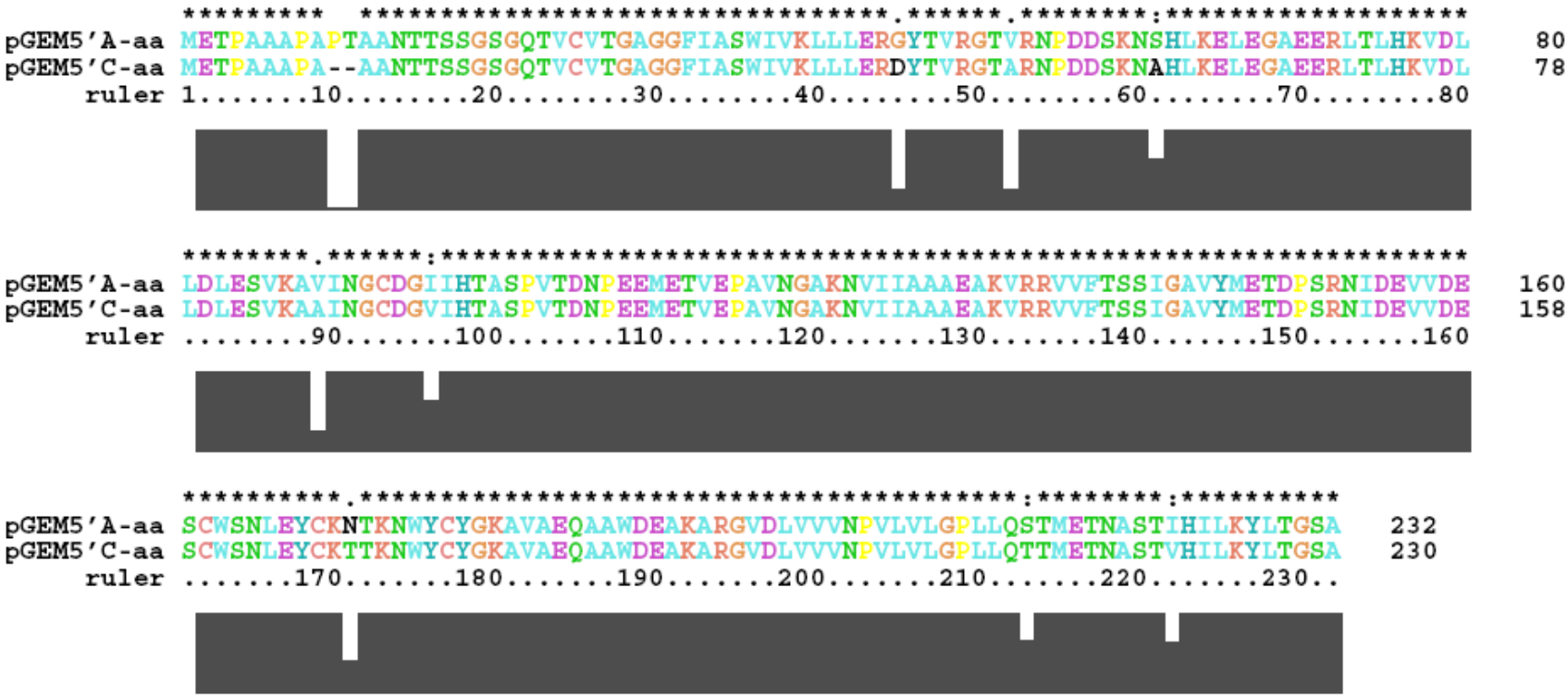


Fig. 2.3.7 c: Alignment of amino acid sequence of coding region of pGEM5'A and pGEM5'C

2.3.4.4 Sequence analysis of 3' clone:

Analysis of 3' clone showed that it is 825 bp fragments with coding region ending at 564th base. Coding region showed significant similarity to known *CCR* sequence in NCBI GenBank. A UTR of 260 bases was identified in this fragment. 3' UTR region is highlighted in red.. This clone was assigned as pGEM3'A

Nucleotide sequence of pGEM3'A clone (GenBank Acc. No. DQ986908):

```
  1 GATGAGGTGG TTGACGAGTC TTGCTGGAGC AATTTGGAAT ATTGCAAGAA
 51 CACAAAGAAC TGGTATTGCT ATGGGAAGGC AGTGGCAGAG CAAGCAGCAT
101 GGGATGAGGC AAAAGCAAGA GGGGTGGATT TGGTTGTGGT GAATCCAGTT
151 TTGGTGTTGG GACCATTGCT TCAAACCACC ATGAATGCAA GCACAATTCA
201 CATCCTCAAG TATCTCACTG GCTCTGCCAA GACCTATGCA AATGCCACTC
251 AGGCCTATGT TCATGTTAAG GATGTTGCAT TAGCCCATGT TCTTGTTTAC
301 GAGACTCCTT CTGCCTCCGG TCGTTATCTA TGTTCCGAGA GTTCTCTCCA
351 CCGTGGAGAA CTGGTCGAGA TCCTCGCCAA ATATTTCCCA GAATACCCAA
401 TTCCTACCAA ATGTTCGGAC GAGAAGAATC CAAGAGCAAA ACCCTACACA
451 TTCTCTAACA AGAGGCTGAA GGATTTAGGA TTAGAGTTTA CACCAGTCCA
501 TCAGTGTCTA TACGACACCG TTAAGAGCCT GCAGGACAAA GGCCATCTTC
551 TCCTTCCCAC CAAGTAACAG AGATCTGTCA GATTAAATCC TAGACACTTT
601 ATTTAGTTAC TACTTCCAAG TATTATTATT ATTAAAGAAG AAGAAGAAGA
651 AGCAGTTTTT AGCAATTTGG TGGCTCCATA TGAAGCTACT AATTTAAGAT
701 AGAATTATGT GAACATGTTG AATATCTTCA TCTGTATCAG ACAATTCCTT
751 GTAACGGTTT TTCTGTGTAA CAGTGGTGAC AATCAATATG TTTGTTTTAA
801 AAAAAAAAAA AAAAAAAAAA AAAAAAA
```

Deduced amino acid sequence of coding region of 3' RACE Product:

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

3' UTR of *CCR* gene:

Putative poly-adenylation site is highlighted (grey).

```
  1 CAGAGATCTG TCAGATTAAA TCCTAGACAC TTTATTTAGT TACTACTTCC
 51 AAGTATTATT ATTATTAAAG AAGAAGAAGA AGAAGCAGTT TTTAGCAATT
101 TGGTGGCTCC ATATGAAGCT ACTAATTTAA GATAGAATTA TGTGAACATG
151 TTGAATATCT TCATCTGTAT CAGACAATTC CTTGTAACGG TTTTTCTGTG
201 TAACAGTGGT GACAATCAAT ATGTTTGTTT TAAAAAAAAA AAAAAAAAAA
251 AAAAAAAAAA
```

2.3.5.1 Amplification of full-length *CCR* gene:

Coding region of RACE products showed significant similarity to known *CCR* sequences in NCBI GenBank. Difference in 5' UTR regions allowed designing two different forward and one reverse primer. Using combination of two different sets of primers, PCR was performed to amplify two different full-length *CCR* genes.

First strand cDNA was prepared from RNA and full-length *CCR* was amplified using cDNA as a template. Amplified fragment was of ~1 kb and was cloned in pGEM-T easy vector and sequenced (Fig. 2.3.8).

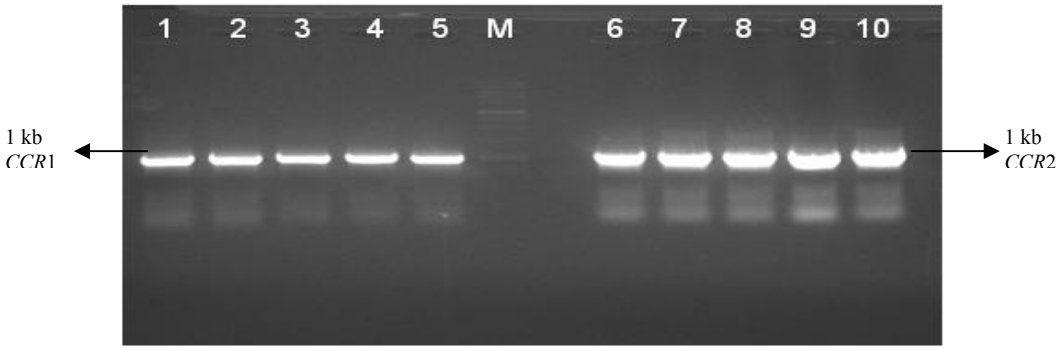


Fig. 2.3.8: Amplification of full-length *CCR* gene: Lane1 to 5: PCR amplification of *CCR*1, Lane M: 1 kb ladder, Lane 5 to 10: PCR amplification of *CCR* 2.

2.3.5.2 Sequence analysis of *CCR* 1 *CCR* 2:

Two *CCR* fragments were characterized and designated as pGEM*CCR*1 of 1011 bases (GenBank Accession No.DQ986907) and pGEM*CCR*2 of 1005 bases (GenBank Accession

No. EU195224) respectively. Deduced Protein sequence showed that *CCR*1 is 336 AA and *CCR*2 is 334 AA long (the amino acid composition is given in Table 2.3.3). They were designated as Ll-*CCR*H1 and Ll-*CCR*H2 respectively (Fig. 2.3.10).

Nucleotide Sequence of Full-length Ll-*CCR*H1 gene:

```
   1 - ATGCCTGCTGCCGCCCCAGCCCCCACCGCCGCTAACACCACCTCATCAGGTTCCGGCCAA - 60
   1 - M  P  A  A  A  P  A  P  T  A  A  N  T  T  S  S  G  S  G  Q   - 20

  61 - ACCGTCTGCGTCACAGGCGCCGGTGGCTTCATCGCCTCTTGGATTGTCAAGCTTTTGCTA - 120
  21 - T  V  C  V  T  G  A  G  G  F  I  A  S  W  I  V  K  L  L  L   - 40

 121 - GAGAGAGGCTACACTGTTAGAGGCACCGTCAGAAATCCAGATGATTCTAAGAACTCTCAC - 180
  41 - E  R  G  Y  T  V  R  G  T  V  R  N  P  D  D  S  K  N  S  H   - 60

 181 - TTGAAAGAGTTGGAAGGAGCAGAGGAGAGGCTAACTCTTCATAAGGTTGATCTTCTTGAT - 240
  61 - L  K  E  L  E  G  A  E  E  R  L  T  L  H  K  V  D  L  L  D   - 80

 241 - CTTGAATCTGTGAAAGCTGTTATCAATGGCTGTGATGGCATCATTCACACGGCTTCTCCA - 300
  81 - L  E  S  V  K  A  V  I  N  G  C  D  G  I  I  H  T  A  S  P   - 100

 301 - GTCACAGACAACCCCGAAGAGATGGTGGAGCCGGCGGTGAATGGAGCAAAGAATGTGATC - 360
 101 - V  T  D  N  P  E  E  M  V  E  P  A  V  N  G  A  K  N  V  I   - 120

 361 - ATCGCAGCTGCAGAAGCGAAAGTGAGAAGAGTAGTGTTCACGTCATCCATTGGAGCCGTC - 420
 121 - I  A  A  A  E  A  K  V  R  R  V  V  F  T  S  S  I  G  A  V   - 140

 421 - TACATGGACCCCAGCAGGAACATTGATGAGGTGGTTGACGAGTCTTGCTGGAGCAATTTG - 480
 141 - Y  M  D  P  S  R  N  I  D  E  V  V  D  E  S  C  W  S  N  L   - 160

 481 - GAATATTGCAAGAACACAAAGAACTGGTATTGCTATGGGAAGGCAGTGGCAGAGCAAGCA - 540
 161 - E  Y  C  K  N  T  K  N  W  Y  C  Y  G  K  A  V  A  E  Q  A   - 180

 541 - GCATGGGATGAGGCAAAAGCAAGAGGGGTGGATTTGGTTGTGGTGAATCCAGTTTTGGTG - 600
 181 - A  W  D  E  A  K  A  R  G  V  D  L  V  V  V  N  P  V  L  V   - 200

 601 - TTGGGACCATTGCTTCAATCCACCATGAATGCAAGCACAATTCACATCCTCAAGTATCTC - 660
 201 - L  G  P  L  L  Q  S  T  M  N  A  S  T  I  H  I  L  K  Y  L   - 220

 661 - ACTGGCTCTGCTAAGACCTATGCAAATGCCACTCAGGCCTATGTTCATGTTAAGGATGTT - 720
 221 - T  G  S  A  K  T  Y  A  N  A  T  Q  A  Y  V  H  V  K  D  V   - 240

 721 - GCATTAGCCCATGTCCTTGTTTACGAGATTCCTTCTGCCTCCGGTCGTTATCTATGTTCC - 780
 241 - A  L  A  H  V  L  V  Y  E  I  P  S  A  S  G  R  Y  L  C  S   - 260

 781 - GAGAGTTCTCTCCACCGTGGAGAACTGGTCGAGATCCTCGCCAAATATTTCCCAGAATAC - 840
 261 - E  S  S  L  H  R  G  E  L  V  E  I  L  A  K  Y  F  P  E  Y   - 280

 841 - CCAATTCCTACGAAATGTTCGGACGAGAAGAATCCAAGAGCAAAAGCCTACACATTCTCT - 900
 281 - P  I  P  T  K  C  S  D  E  K  N  P  R  A  K  A  Y  T  F  S   - 300


 901 - AACAAGAGGCTGAAGGATTTAGGATTAGAGTTTACACCAGTTCATCAGTGTCTATACGAC - 960
 301 - N  K  R  L  K  D  L  G  L  E  F  T  P  V  H  Q  C  L  Y  D   - 320

 961 - ACCGTTAAGAGCCTGCAGGACAAAGGCCATCTTCCTCTTCCCACCAAGTAA          - 1011
 321 - T  V  K  S  L  Q  D  K  G  H  L  P  L  P  T  K  *           - 340
```

Nucleotide Sequence of Full-length Ll-*CCR*H2 gene:

```
   1 - ATGCCTGCTGCCGCCCCCGCCGCCGCTAACACCACCTCATCAGGTTCCGGCCAAACCGTC - 60
    1 - M  P  A  A  A  P  A  A  A  N  T  T  S  S  G  S  G  Q  T  V   - 20

  61 - TGCGTCACAGGCGCCGGTGGCTTCATCGCCTCTTGGATTGTCAAGCTCTTGCTAGAGAGA - 120
  21 - C  V  T  G  A  G  G  F  I  A  S  W  I  V  K  L  L  L  E  R   - 40

 121 - GACTACACTGTCAGAGGCACCGCCAGAAATCCAGATGATTCTAAGAACGCACACTTAAAA - 180
  41 - D  Y  T  V  R  G  T  A  R  N  P  D  D  S  K  N  A  H  L  K   - 60

 181 - GAGTTGGAAGGAGCAGAGGAGAGGCTAACTCTTCATAAGGTGGATCTTCTTGATCTGGAA - 240
  61 - E  L  E  G  A  E  E  R  L  T  L  H  K  V  D  L  L  D  L  E   - 80

 241 - TCTGTGAAAGCTGCTATCAATGGCTGTGATGGCGTCATTCACACGGCTTCTCCAGTCACA - 300
  81 - S  V  K  A  A  I  N  G  C  D  G  V  I  H  T  A  S  P  V  T   - 100

 301 - GACAACCCCGAAGAGATGGTGGAGCCGGCGGTGAATGGAGCAAAGAATGTGATCATCGCA - 360
 101 - D  N  P  E  E  M  V  E  P  A  V  N  G  A  K  N  V  I  I  A   - 120

 361 - GCTGCAGAAGCGAAAGTGAGAAGAGTAGTGTTCACGTCATCCATTGGAGCCGTCTACATG - 420
 121 - A  A  E  A  K  V  R  R  V  V  F  T  S  S  I  G  A  V  Y  M   - 140

 421 - GACCCCAGCAGGAACATTGATGAGGTGGTTGACGAGTCTTGCTGGAGCAATTTGGAATAT - 480
 141 - D  P  S  R  N  I  D  E  V  V  D  E  S  C  W  S  N  L  E  Y   - 160

 481 - TGCAAGACCACAAAGAACTGGTATTGCTATGGGAAGGCAGTGGCAGAGCAAGCAGCATGG - 540
 161 - C  K  T  T  K  N  W  Y  C  Y  G  K  A  V  A  E  Q  A  A  W   - 180

 541 - GATGAGGCAAAAGCAAGAGGGGTGGATTTGGTTGTGGTGAATCCAGTTTTGGTGTTGGGA - 600
 181 - D  E  A  K  A  R  G  V  D  L  V  V  V  N  P  V  L  V  L  G   - 200

 601 - CCATTGCTTCAAACCACCATGAATGCAAGCACAATTCACATCCTCAAGTATCTCACTGGC - 660
 201 - P  L  L  Q  T  T  M  N  A  S  T  I  H  I  L  K  Y  L  T  G   - 220

 661 - TCTGCCAAGACCTATGCAAATGCCACTCAGGCCTATGTTCATGTTAAGGATGTTGCATTA - 720
 221 - S  A  K  T  Y  A  N  A  T  Q  A  Y  V  H  V  K  D  V  A  L   - 240

 721 - GCCCATGTTCTTGTTTACGAGACTCCTTCTGCCTCCGGTCGTTATCTATGTTCCGAGAGT - 780
 241 - A  H  V  L  V  Y  E  T  P  S  A  S  G  R  Y  L  C  S  E  S   - 260

 781 - TCTCTCCACCGTGGAGAACTGGTCGAGATCCTCGCCAAATATTTCCCAGAATACCCAATT - 840
 261 - S  L  H  R  G  E  L  V  E  I  L  A  K  Y  F  P  E  Y  P  I   - 280

 841 - CCTACCAAATGTTCGGACGAGAAGAATCCAAGAGCAAAACCCTACACATTCTCTAACAAG - 900
 281 - P  T  K  C  S  D  E  K  N  P  R  A  K  P  Y  T  F  S  N  K   - 300

 901 - AGGCTGAAGGATTTAGGATTAGAGTTTACACCAGTCCATCAGTGTCTATACGACACCGTT - 960
 301 - R  L  K  D  L  G  L  E  F  T  P  V  H  Q  C  L  Y  D  T  V   - 320

 961 - AAGAGCCTGCAGGACAAAGGCCATCTTCCTCTTCCCACCAAGTAA                - 1005
 321 - K  S  L  Q  D  K  G  H  L  P  L  P  T  K  *                  - 340
```

S. No	Amino acid (Aa)	Number (*CCR*H1)	Number (*CCR*H2)	Percent (*CCR*H1) %	Percent (*CCR*H2) %
1	A	35	37	10.42	11.08
2	C	8	8	2.38%	2.40
3	D	16	17	4.76	5.09
4	E	22	22	6.55	6.59
5	F	5	5	1.49	1.50
6	G	20	19	5.95	5.69
7	H	9	9	2.68	2.69
8	I	14	12	4.17	3.59
9	K	23	23	6.85	6.89
10	L	31	31	9.23	9.28
11	M	4	4	1.19	1.20
12	N	16	15	4.76	4.49
13	P	18	18	5.36	5.39
14	Q	6	6	1.79	1.80
15	R	12	12	3.57	3.59
16	S	24	22	7.14	6.59
17	T	22	24	6.55	7.19
18	V	33	32	9.82	9.58
19	W	4	4	1.19	1.20
20	Y	14	14	4.17	4.19

Table 2.3.3: Amino acid composition of deduced *CCR* sequence.

2.3.5.3: *In-silico* Restriction digestion analysis of Ll-*CCR*Hs:

Restriction digestion was performed using online software NebCutter. The restriction pattern suggested that out of frequently used hexa-cutters, *Hinc* II*, Pvu* II*, Taq* I*, Stu* I*, Acc* I and *Bgl* I cut both the sequence but *Hind* III exclusively cuts only Ll-*CCR*H1 and this restriction site was absent in Ll-*CCR*H2 (Fig. 2.3.9). pGEM*CCR*H1 and pGEM*CCR*H2 were double digested with *Eco* RI and *Hind* III and run on 1% agarose (Fig. 2.3.9 C).

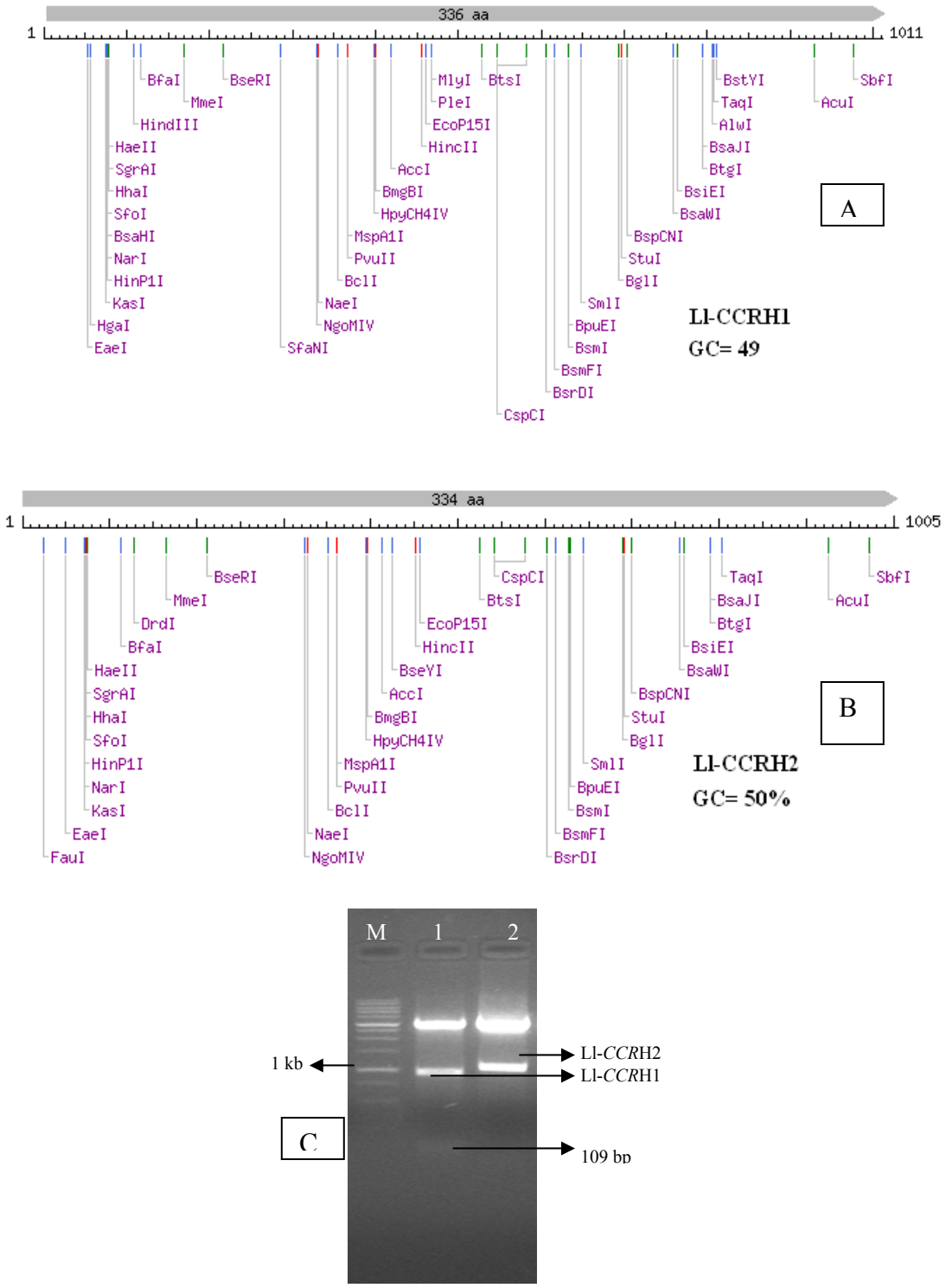


Fig. 2.3.9: Restriction digestion analysis: A and B: *In-silico* restriction analysis of Ll-*CCR*H1 and 2; C: Restriction digestion of Ll-*CCR*H1 and 2 by *Hind* III, Lane M: Marker, Lane 1: Ll-*CCR*H1 and lane 2: Ll-*CCR*H2.

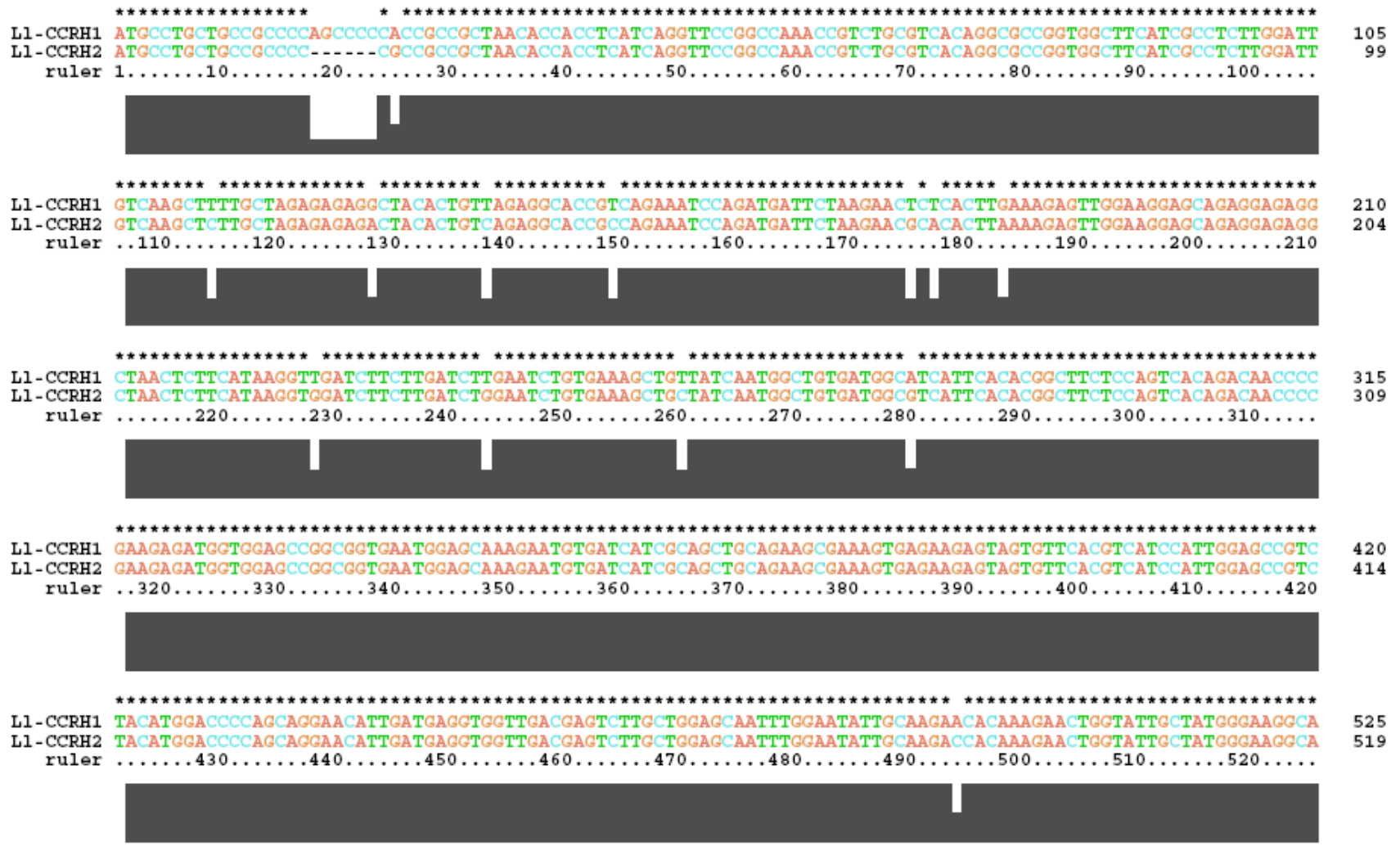


Fig. 2.3.10: Alignment of Ll-*CCR*H1 and Ll-*CCR*H2 nucleotide sequence:

Continued to next page

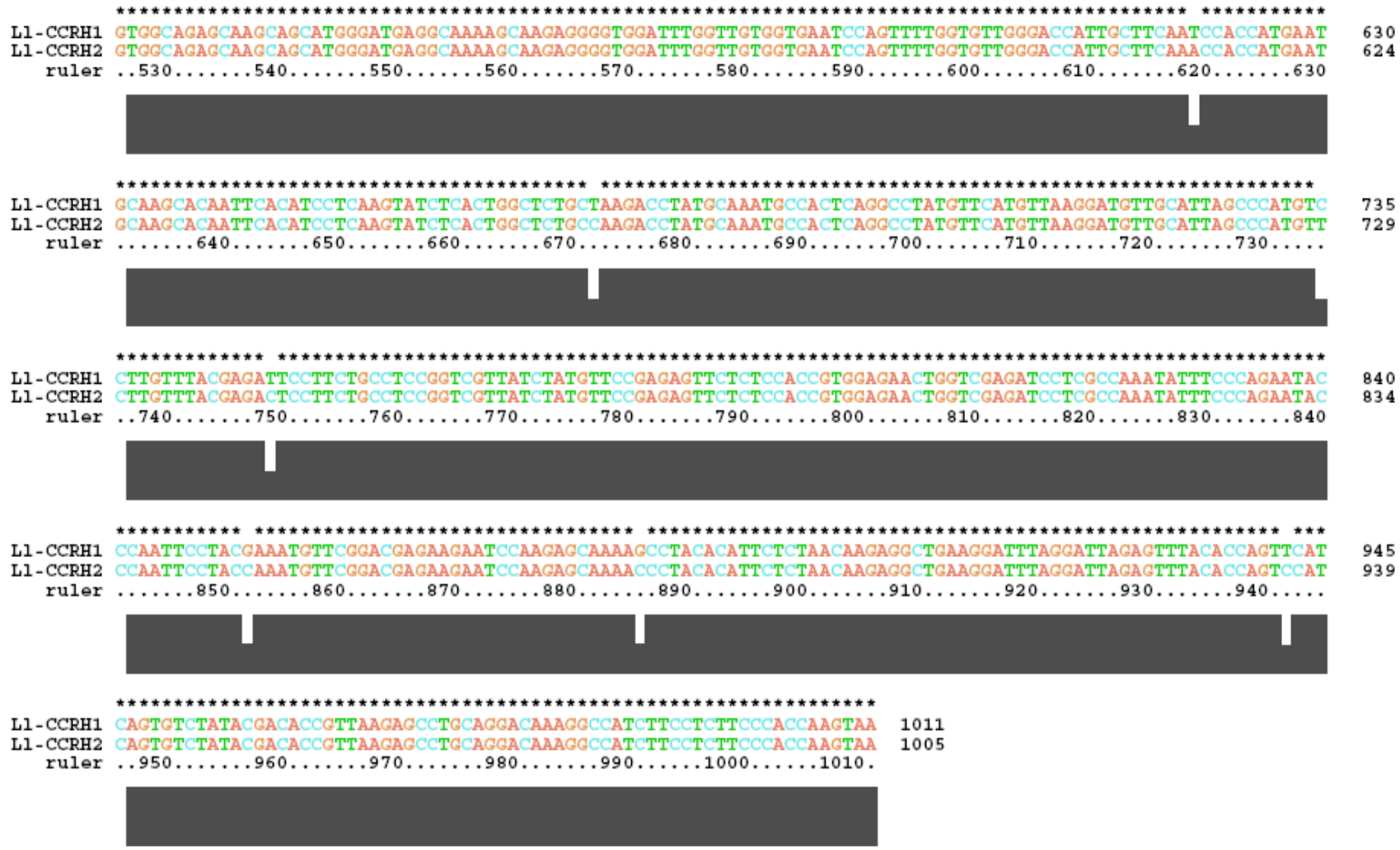


Fig. 2.3.10: Alignment of Ll-*CCR*H1 and Ll-*CCR*H2 nucleotide sequence.

2.3.5.4 Bioinformatic analysis of deduced *CCR* proteins:

The *L. leucocephala CCR* cDNAs were conceptually translated and protein sequence was analyzed. The predicted molecular weight and pI for Ll-*CCR*H1and Ll-*CCR*H2 were estimated to be 36.5 kD /6.32 and 36.3 kD / 6.16, respectively and identified as cytosolic proteins (www.expasy.org/tools; Fig. 2.3.11). When comparative primary structure analysis of the deduced Ll-*CCR*s with orthologous *CCR* sequences was performed using Pfam and rps-blast search, putative domains such as 3-beta-hydroxysteroid dehydrogenase/isomerase (3Beta_HSD), NADH-flavin reductase, and NAD dependent epimerase/dehydratase for binding cofactors were observed in both the Ll-*CCR* amino acid sequences (Lacombe *et al.*, 1997; McInnes *et al.*, 2002; Larsen, 2004). Apart from these domains, it contains conserved domains for various types of reductase and dehydrogenase activities (McInnes *et al.*, 2002; Lacombe *et al.*, 1997). This data classifies the protein as a member of oxido-reductase family (Fig. 2.3.12a). Ll-*CCR*s were aligned with other *CCR* sequences and found to be similar to dihydro-kaempeferol reductase from *Arabidopsis*, which also possesses the above mentioned conserved domains indicating that these domains are conserved evolutionarily in plant systems. Site for NADP binding (marked with solid blue arrow) and exon-intron junction was identified (McInnes *et al.*, 2002) and a signature sequence of NWYCYGK (marked by thin red arrow) was also observed (Fig. 2.3.12b) (Larsen, 2003: Larsen, 2004). Hydropathic plot was plotted with a window size of 6 and hydrophobic regions were characterized by positive values. The hydrophilic regions were characterized as negative values and they correspond to exposed part of globular protein, which may be putative antigenic epitope (Fig. 2.3.13). As both the genes showed very little to no difference, they were designated as two isoforms of *CCR* gene in *Leucaena* genome. Considering the identical molecular weight, isoelectric point, conserved domains and homology between two genes only Ll-*CCR*H2 gene was over-expressed and characterized further.

Deduced amino acid sequence Ll-*CCR*H 1 Gene:

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

Deduced amino acid sequence Ll-*CCR*H 2 Gene:

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

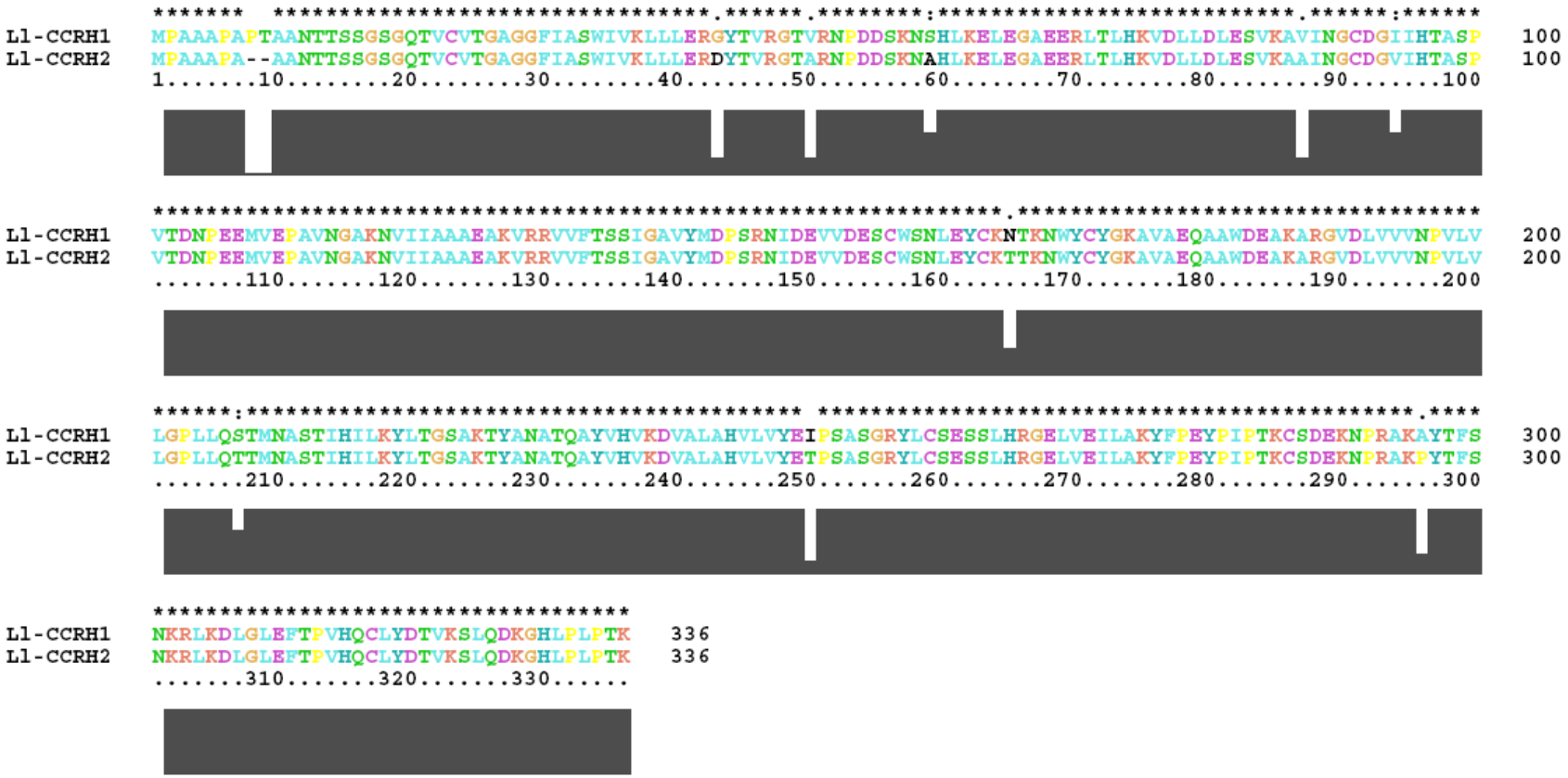


Fig. 2.3.11: Alignment of Ll-*CCR*H1 and Ll-*CCR*H2 amino acid sequence.

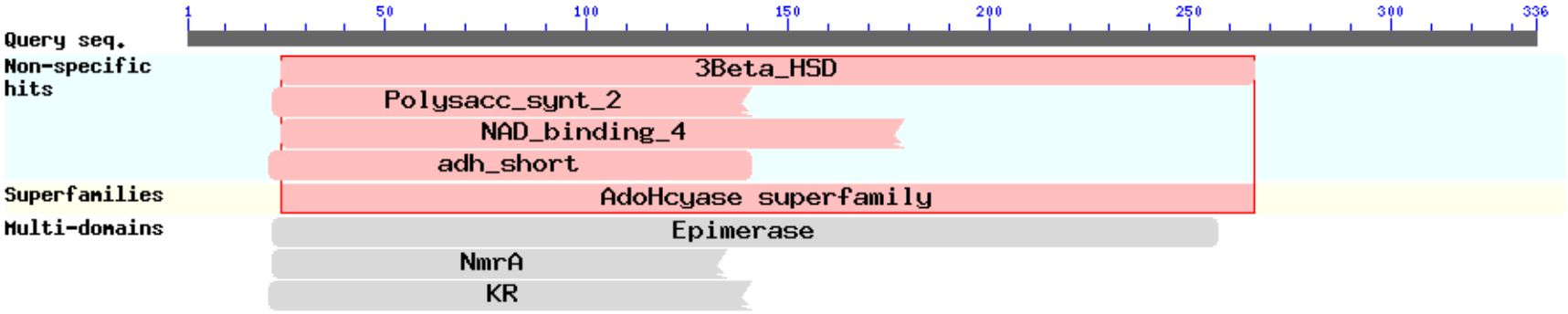


Fig. 2.3.12 a: Putative conserved domains in Ll-*CCR*H1.

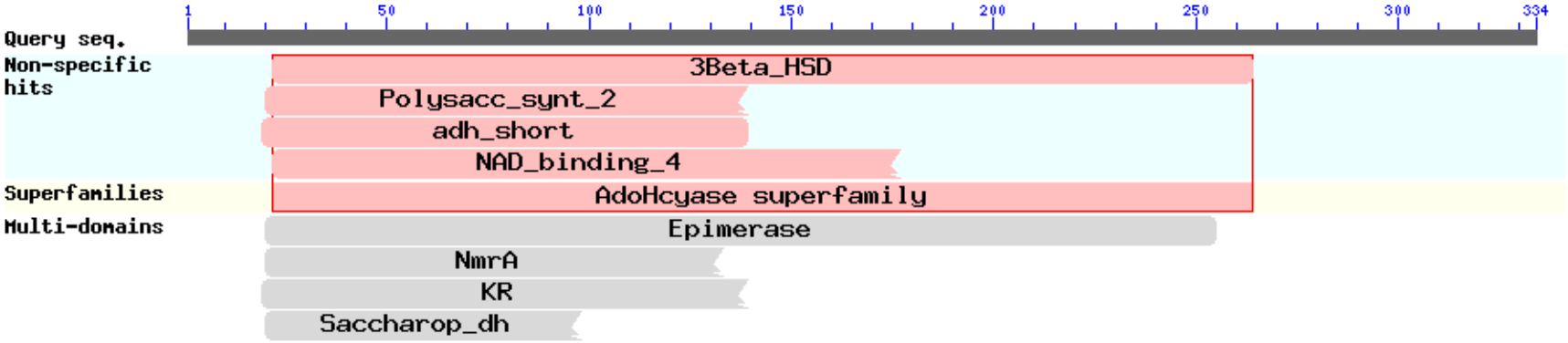


Fig. 2.3.12 a: Putative conserved domains Ll-*CCR*H2.

3Beta_HSD: 3- Beta-hydroxysteroid; Polysacc_synt_2: Polysaccharide synthatase; NAD_binding_4: NAD binding domain; Adh_short: Aldehyde dehydrogenase; AdoHcyase superfamily; Epimerase; NmrA: NmrA like protein; KR: KR like protein

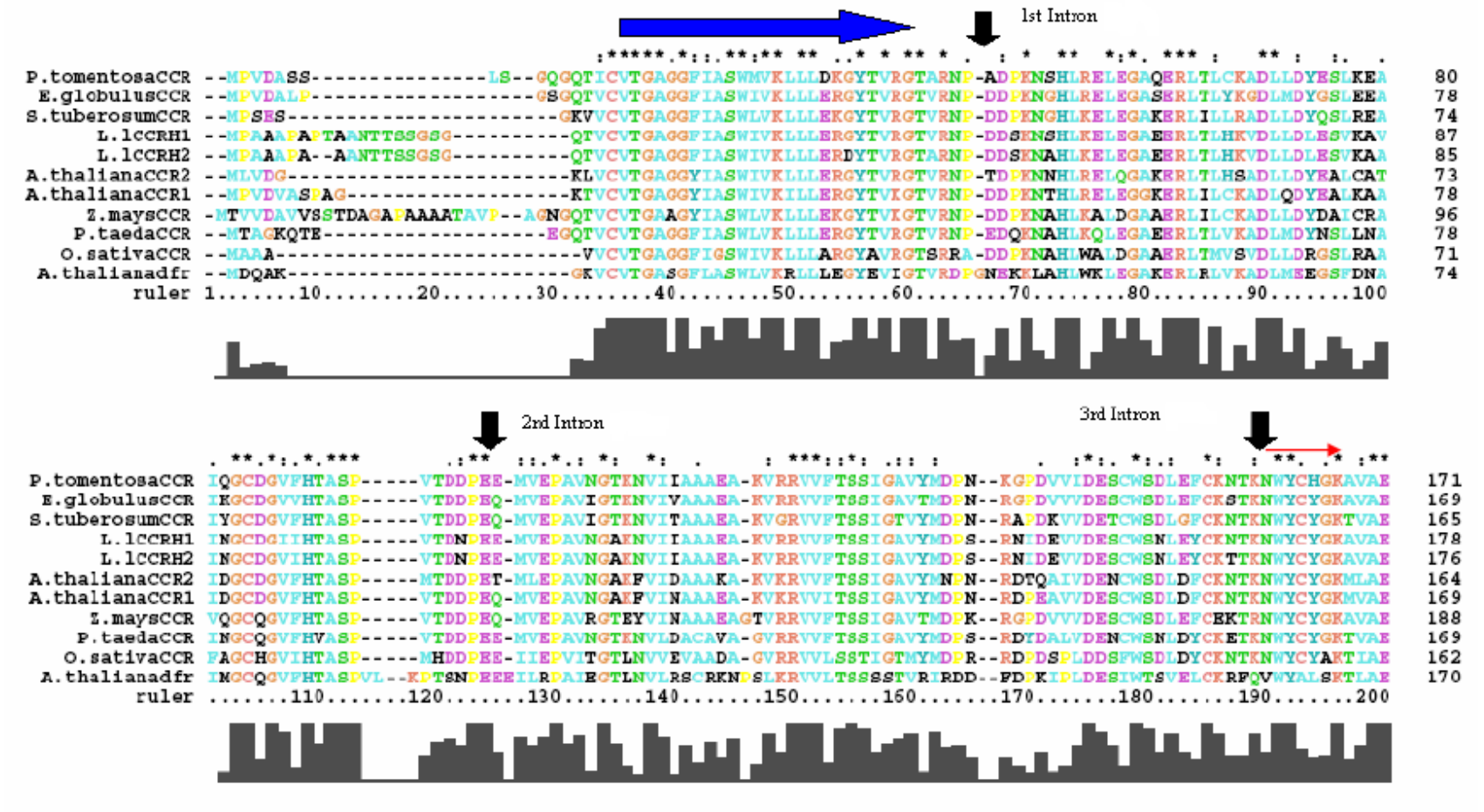


Fig. 2.3.12 b: Alignment of Ll-*CCR*s with orthologous *CCR* sequences from different plant system:

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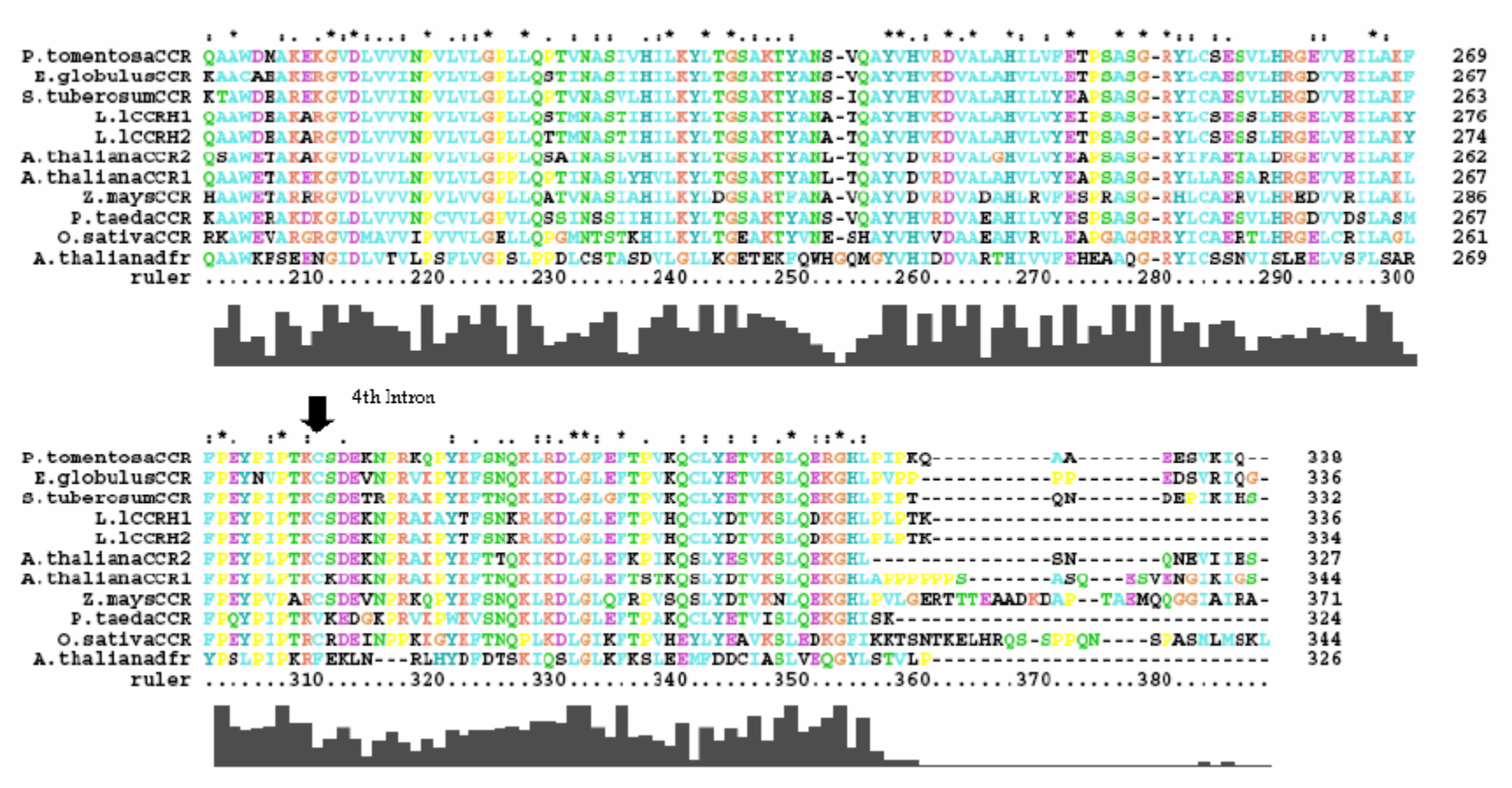


Fig. 2.3.12 b: Alignment of Ll-*CCR*s with orthologous *CCR* sequences from different plant system: Putative NADP binding site, marked in solid blue arrow, Thin red arrow marks *CCR* signature sequence and solid black arrow denotes Exon-Intron junction.

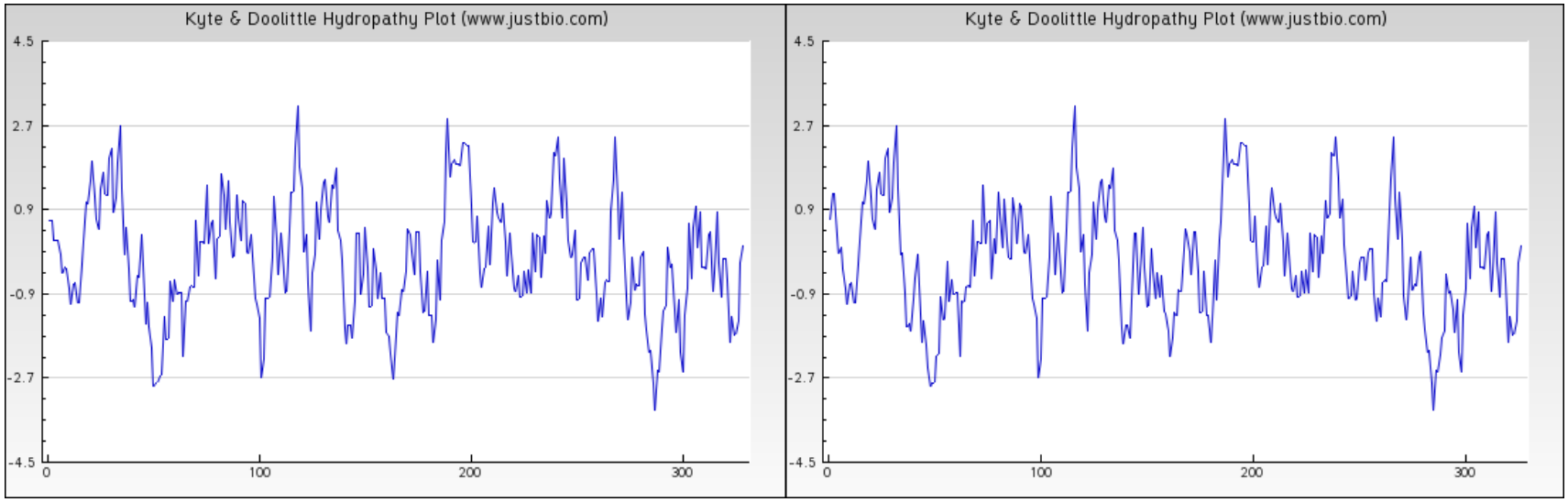


Fig. 2.3.13: Hydropathic plot. Window size of 6 suggested a good value for finding putative surface-exposed regions (Hydrophilic region characterized by positive values). Both the *CCR*s showed exactly similar putative antigenic epitopes (www.justbio.com).

2.3.5.5 Phylogenetic analysis of *CCR*s:

The closest *in silico* match for Ll-*CCR*H1 and Ll-*CCR*H2 proteins was *Eucalyptus globulus* and *Populus tomentosa,* which showed 76-77% identity and 84-86% similarity at the amino acid level respectively. Ll-*CCR* protein sequences share overall 54-66% identities with *CCR* of monocotyledonous plants and 77-65% identities with *CCR* of dicotyledonous plants, respectively. *CCR* sequence also showed 38 and 39% identities with dihydro-flavanoid reductases from *Arabidopsis thaliana* and *Zea mays* respectively and only 40% identities with *Acacia mangium x Acacia auriculiformi*. Ll-*CCR*H1 and Ll-*CCR*H2 bears 70% identity to AT-*CCR*1 and around 68% identity to AT-*CCR*2. Interestingly, they showed 70% identity with *Pinus taeda*. To investigate the evolutionary relationship between Ll-*CCR*s and *CCR*s from other plants, a phylogenetic tree (Neighbor-Joining) was constructed based on the deduced amino acids sequence. *Z. mays*-Dihydroflavanol-reductase (Zm-DFR) and *A. thaliana* Dihydrokempferol-reductase (At-DKFR) were taken as an out-group for analyzing evolutionary significance and *CCR* protein sequences from other plant species were used to deduce the similarity index. The tree can be divided into three clusters. The first cluster comprises of monocotyledonous plant species, which can be divided in two groups. The first group comprises of *CCR*s from *Lolium perene, Zea mays-CCR1, Hordeum vulgare* and *Sacharrum officinarum* and other group consist of *Zea mays-CCR*2 and *Oryza sativa-CCR*. The second cluster comprises of dicotyledonous plant species, which again were further, divided in two groups. First group comprises of *P. tomentosa, E. Globulus, Solanum tuberosum, Codonopsis lanceolata, Lycopersicon esculentum* and *Fragaria x ananassa*; whereas the second group comprises of *A. thaliana CCR*1*, A. thaliana CCR*2 and *L. leucocephala CCR*H1 and *CCR*H2. The third cluster comprises of *Oryza sativa and Z. mays-CCR2. Acacia mangium x Acacia auriculiformi* (*A. mangium*). *A. mangium*, *O. sativa* and *Z. mays-CCR*2 are found to be least evolved *CCR* genes in their respective families. At-DKFR and Zm-DFR are treated as out-group. Phylogenetic analysis clearly demonstrated that Ll-*CCR*Hs are closely related to *CCR*s of dicots than monocots and is distantly related to gymnosperm (*P. taeda*) and DFRs (Fig. 2.3.14).

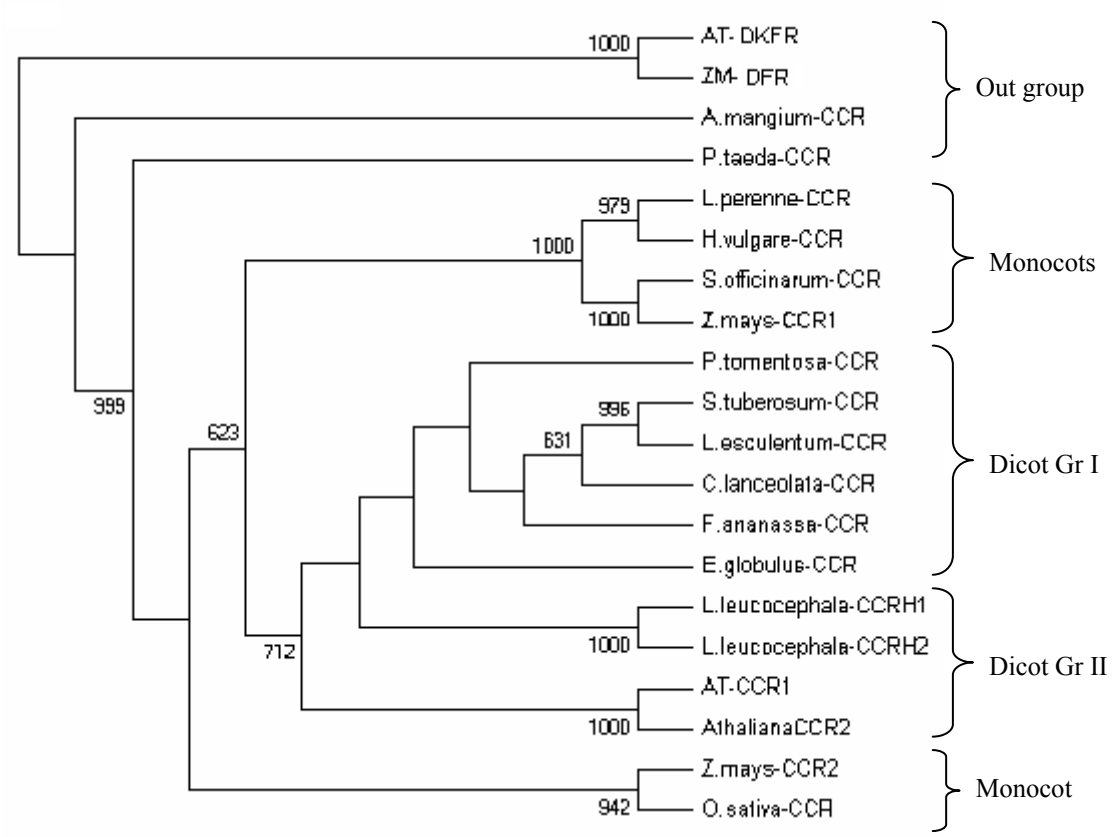


Fig. 2.3.14: Phylogenetic tree made using the Neighbor-Joining method: Numbers at nodes represent the bootstrap values. The evolutionary history was inferred using the Neighbor-Joining method. *Z. mays* Dihydroflavanol-reductase and *A. thaliana* Dihydrokaempferol-reductase were taken as an out-group for analyzing evolutionary significance. *CCR* protein sequence from other plant species were used to deduce the similarity index. Monocot group consist of *Lolium perene* (AAG09817), *Zea mays-CCR1* (CAA66707), *Hordeum vulgare* (AAN71760) and *Sacharrum officinarum* (CAA13176) and other group consist of *Zea mays-CCR*2 (AAO42621) and *Oryza sativa-CCR* (BAF08006). Dicot group comprises of *P. tomentosa* (AAR83344), *E. Globulus* (AAT74878), *Solanum tuberosum* (AAN71761), *Codonopsis lanceolata* (BAE48787), *Lycopersicon esculentum* (AAY41879), *Fragaria x ananassa* (AAP46143), *A. thaliana CCR*1 (AAG48822), *A. thaliana CCR*2 (AAG53687) and *L. leucocephala CCR*H1 (ABL01801) and *CCR*H2 (ACB45309). *Acacia mangium x Acacia auriculiformi* (*A. mangium*- AAY86360), *O. sativa* and *Z. mays-CCR*2 are found to be least evolved *CCR* genes in their respective families. At-DKFR (ABF74722) and Zm-DFR (AAK52955) are treated as out-group. Phylogenetic analysis clearly demonstrated that Ll-*CCR*Hs are closely related to *CCR*s of dicots than monocots and is distantly related to gymnosperm (*P. taeda*-AAL47684) and DFRs. GenBank accession numbers given in brackets.

2.3.6.1 Gene copy number-Southern Hybridization:

To further validate the results from slot blot experiment and to understand the distribution of the *CCR* gene in the *L. leucocephala* genome, Southern hybridization was performed. A 25 µg aliquot of *L. leucocephala* genomic DNA was restriction digested individually with the restriction enzymes *Nde* I, *Hinc* II, *Sac* I and *Bgl* I, which are predicted not to cut (*Sac* I) or cut once (*Nde* I, *Bgl* I and *Hinc* II) within the sequence. As shown in Fig. 2.3.15, four bands were detected in the *Hinc* II and *Nd*e I digest, while two were recorded in the *Sac* I digest. *Bgl* I showed only three distinct bands, which may be due to the reason that fourth band, may be a very small and of low intensity, which is not seen in blot. These results suggest that Ll-*CCR*H2 is present as a two-copy number gene. Southern hybridization was done using a part of coding region and part of 5' UTR. An approximately 800 bp fragment was used as a probe for hybridization at 62 °C. Banding pattern in Southern hybridization suggested that at least two copy of *CCR* gene is present in *L. leucocephala* genome.

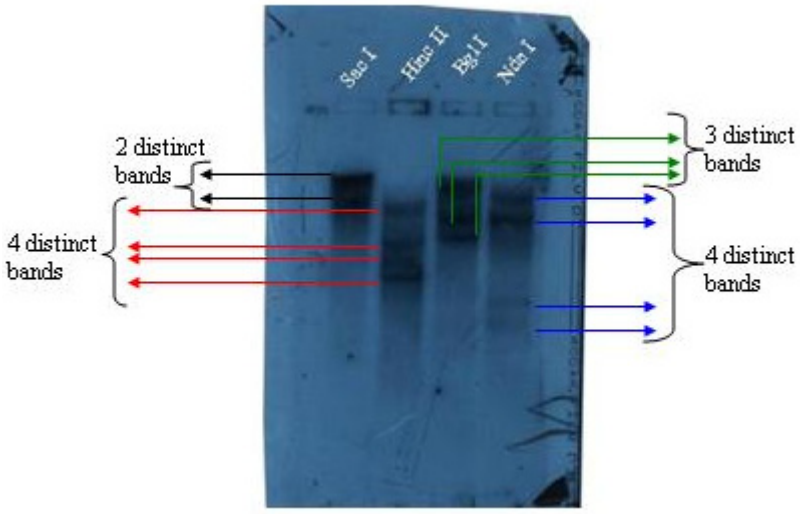


Fig. 2.3.15: Southern hybridization analysis: Four distinct band are seen in *Hinc* II and *Nde* I lane, two bands in *Sac* I lane, and three bands in *Bgl* I lane.

2.3.6.2 Gene copy number -Real time PCR

Copy number was also estimated using Real-Time PCR analysis. Briliant II PCR master mix (Stratagene, USA) was used for the reactions. Dilution of *CCR* gene in pGEMT vector ranging from 1 x 10^5 to 7 x 10^5 copies was treated as standard. Known amount of *Leucaena* genomic DNA was used in each reaction, which was extrapolated to estimate copies of *CCR* gene present in *Leucaena* genome. The gene copy number in *Leucaena leucocephala* was estimated to be two in numbers (Fig. 2.3.16).

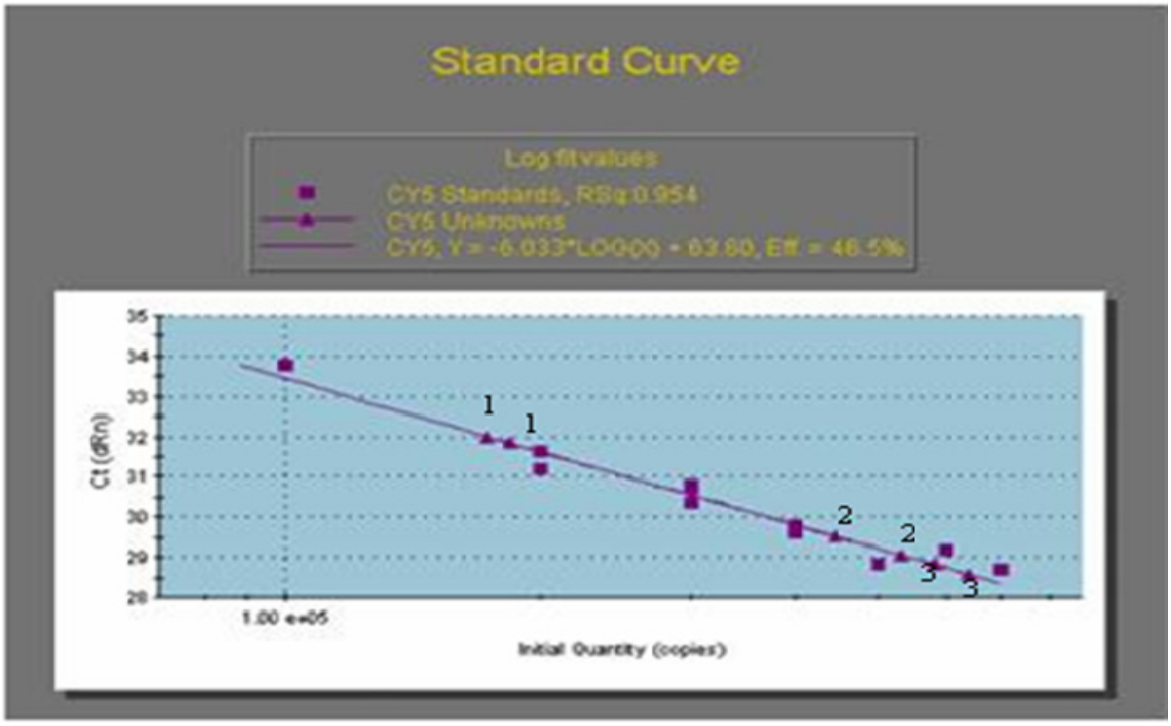


Fig. 2.3.16: Estimation of gene copy number using Real-Time PCR: Solid triangle shows (1) 1 x 10^5, (2) 2 x 10^5 and (3) 3 x 10^5 copies of Leucaena gDNA in duplicates. Solid square represents 1 x 10^5 to 7 x 10^5 copies of *CCR* gene in pGEMT easy vector (Standard graph).

2.4 Discussion:

CCR is one of the key genes involved in lignin biosynthesis. Hydroxycinnamoyl-CoA esters of general phenyl propanoid pathway, when acted upon by *CCR*, become destined to form respective monolignols. *CCR* activity is found to be generally low in plants so it is hypothesized that it may play a crucial role as a rate limiting step in regulation of lignin biosynthesis (Ma, 2007). *CCR* gene was first reported from *E. gunnii* (Piquemal *et al.*, 1997). *CCR* gene has also been characterized from maize, ryegrass, *Arabidopsis* and several other plants (Pichon *et al.*, 1998; McInnes *et al.*, 2002; Lauvergeat *et al.*, 2001). Two cDNAs have been identified to encode *CCR* gene in maize (Pichon *et al.,*1998), ryegrass (McInnes *et al.*, 2002) and *Arabidopsis* (Lauvergeat *et al.*, 2001) and their chemical properties have been analysed. Down regulated tobacco plants with anti-sense *CCR* gene showed a strong decrease in lignin content with an alteration in development. These plants when crossed with homozygous *CAD* gene down regulated lines, showed hybrid with reduced lignin content without affecting the plant development (Chabannes *et al.,* 2001). The above information suggests that there is a need for a detailed study of *CCR* gene. Apart from *Euclyptus*, poplar and Norway spruce, only few *CCR* genes have been cloned and characterized from tree species. *Leucaena* is a tree species which is extensively used in paper and pulp industry in India. To achieve transgenic *L. leucocephala* down regulated with *CCR* gene with reduced/altered lignin content which can be utilized properly in paper and pulp industry, it was neccessary to isolate and characterise *CCR* gene from it.

In this study, two cDNAs encoding *CCR* gene have been isolated which were designated as Ll-*CCR*H1 and Ll-*CCR*H2 (Fig. 2.3.10). Ll-*CCR*Hs have almost identical NADP binding motif found in other *CCR* sequences in NCBI GenBank database. Signature amino acid sequence from reported orthologous *CCR*s i.e. NWYCYGK was also present (Fig. 2.3.12**;** Fig. 2.3.13). Conserved domains of various other epimerase, reductase and dehydrogenase were also characterized on analysis of multiple sequence alignment of Ll-*CCR*Hs with other orthologous CCR protein sequences (Lacombe *et al.*, 1997; McInnes *et al.*, 2002). Phylogenetic analysis of Ll- *CCR* grouped it among other *CCR*s from dicots

(Fig. 2.3.15). As both *CCR* homologs exhibited similar property; for further study one of the *CCR* homologs *i.e* Ll-*CCR*H2 was characterized.

Studies have shown that there are eight *CCR* like sequences present in *Arabidopsis* (Anterola and Lewis, 2002) and poplar (Li *et al.*, 2005) genome. In the same way; another key gene in lignin biosynthesis *i.e* Cinnamyl alcohol dehydrogenase (*CAD*) has also been found to be present as different isoforms. Three different *CAD*2 isoforms were isolated from *Euclyptus gunnii*. Different *CAD*2 isoforms exhibited different affinities for different substrates which suggested that a modulation of monolignol pattern could be regulated through differential *CAD* activities (Hawkins and Boudet, 1994; Boudet, 2000). This could be also true in case of *L. leucocephala* as two different cDNAs encoding for *CCR* were characterized with 97% identities and showed very little difference between them (Fig. 2.3.10 and Fig. 2.3.11). According to earlier studies of *ZM*-*CCR*2 and *AT*-*CCR*2, it has been found that these isoforms mainly express in case of pathogen infection and/or wound or stress conditions but in present study of *L. leucocephala* all experiments were performed under normal conditions (no known stress given) hence both isoforms, Ll-*CCR*H1 and Ll-*CCR*H2 are hypothesized to be involved exclusively during constitutive lignification.

Gene copy number was estimated to be two in *L. leucocephala*. Considering the analysis of Ll-*CCR*H2 and Ll-*CCR*H1 gene it can be suggested that it belongs to *CCR* I gene of other plant species. The possibility of one type of *CCR* gene (as two isoforms) in *Leucaena* genome also can not be ruled out as in case of *Eg*-*CCR* (Piquemal *et al.*, 1997) which is present as one copy in number and differentially expresses in normal and stressed conditions.

2.5 Conclusion:

Deduced amino acid sequence of cDNA from *Leucaena leucocephala* contains consensus sequences for NADP-binding site, *CCR* signature sequence (NWYCYGK). Other conserved domains responsible for dehydrogenase and reductase activity of known *CCR* genes were also present in above two cDNAs. All the above data classifies *CCR* to be a member of family oxido-reductase. The phylogenetic analysis of deduced amino acid sequence shows that it is having a close resemblance with other known *CCR* genes from dicots. The gene is present as two copies in the *Leucaena* genome. Considering the molecular mass of the deduced Ll-*CCR* protein and predicted involvement in constitutive lignification, it can be concluded that two cDNAs isolated from *Leucaena* encodes for cinnamoyl-CoA reductase (Ll-*CCR*).

2.6 References:

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. Downregulation of three unrelated lignin biosynthetic genes in tobacco. *Plant Physiology* **128,** 844–853.

Boerjan W, Ralph J, Baucher M. 2003. Lignin Biosynthesis. *Annual Reviews in Plant Biology* **54**, 519- 546.

Burge CB, Karlin S. 1998. Finding the genes in genomic DNA. *Current Opinion in Structural Biology* **8,** 346-354.

Burge CB. 1998. Modeling dependencies in pre-mRNA splicing signals. In: Salzberg S, Searls D, Kasif S, eds. *Computational Methods in Molecular Biology*, Amsterdam: Elsevier Science, 127-163.

Campbell WH, Gowri G. 1990. Codon usage in higher plants, green algae, and cyanobacteria. *Plant Physiology* **92**, 1–11.

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM. 2001. Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous downregulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant Journal* **28,** 257–270.

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.

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. *Proceedings of National Academy of Science* **87,** 9057–9061.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**, 783-791.

Freeman WM, Walker SJ, Vrana KE. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* **26** (1), 112-125.

Freudenberg K, Neish AC, ed.1968. Constitution and Biosynthesis of Lignin. Berlin: Springer-Verlag. pp 129.

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.

Hahlbrock K, Scheel D.1989. *Annual Review of Plant Physiology and Plant Molecular Biology* **40***,* 347-369.

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-DHPdegrading bacteria. *American Journal of Veterinary Research* **50,** 2176.

Hammond AC, Allison MJ, Williams MJ. 1989b. Persistence of DHP-degrading bacteria between growing seasons in subtropical Florida. *Leucaena Research Report* **10,** 66.

Hawkins SW, Boudet AM. 1994. Purification and characterization of cinnamyl alcohol dehydrogenase isoforms from the periderm of *Eucalyptus gunnii*. *Plant Physiology* **104,** 75–84.

Hegarty MP, Court RD, Thorne PM. 1964. The determination of mimosine and 3, 4-DHP in biological material. *Australian Journal of Agricultral Research* **15,** 168.

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. *Current Opinion in Plant Biology* **5**, 224–229.

Jones RJ. 1979. The value of *Leucaena leucocephala* as feed for ruminants in the tropics World Animal review **32,** 10.

Kyte J, Doolittle RF. 1982. A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.

Lacombe E, Hawkins S, Doorsselaers JV, Piquemal J, Goffner D, Poeydomege O, Boudet AM, Grima-Pettenati J. 1997. Cinnamoyl-CoA reductase, the first committed

enzyme of lignin branch biosynthetic pathway: cloning, expression, and phylogenetic relationships. *The Plant Journal* **11**, 429–441.

Larsen K. 2004. Cloning and characterization of a ryegrass (Lolium perenne) gene encoding cinnamoyl-CoA reductase (*CCR*). *Plant Science* **166**, 569–581.

Lauvergeat V, Lacomme C, Lacombe E, Lasserre E , Roby D, Grima-Pettenati J. 2001. Two cinnamoyl-CoA reductase (*CCR*) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry* **57**, 1187–1195.

Li L, Cheng X, Lu S, Nakatsubo T, Umezawa T, Chiang VL. 2005. Clarification of Cinnamoyl Co-Enzyme reductase Catalysis in Monolignol Biosynthesis of Aspen. *Plant and Cell Physiology* **46**, 1073-1082.

Lodhi MA, Ye GN, Norman FW, Bruce IR. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Molecular Biology Reporter* **12**, 6-13.

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.

Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH. 2007. A conserved domain database for interactive domain family analysis. *Nucleic Acids Research* **35**, 237–240.

McInnes R, Lidgett A, Lynch D, Huxley H, Jones E, Mahoney N, Spanganberg G. 2002. Isolation and characterization of cinnamoyl-CoA reductase gene from perennial ryegrass (Lolium perenne L.). *Journal of Plant Physiology* **159**, 415–422.

Mullis K, **Faloona F**, **Scharf S**, **Saiki R**, **Horn G**, **Erlich H.** 1992. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Biotechnology* **24**, 17-27.

O'Connell A, Holt1 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 Research* **11,** 495–503.

Pichon M, Courbon I, Bechert M, Boudet AM, Grima-Pettenati J. 1998. Cloning and characterization of two maize cDNAs encoding cinnamoyl-CoA reducatase (*CCR*) and differential expression of the corresponding genes. *Plant Molecular Biology* **38**, 671–676.

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-Omethyltransferase 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 Physiology* **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, Kmyton A O' Connell, Schuch W, G-Pettenatti J, Boudet AM. 1998. Characterization of a cinnamoyl-CoA reductase that is associated with stem development in *E. gunni*. *The plant journal* **13,** 71-83.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**, 406-425.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, New York: *Cold Spring Harbor Laboratory Press*.

Sarkanen KV, Ludwig CH. 1971. Lignins: Occurrence, Formation, Structure, and Reactions. New York: Wiley- Intersci pp 916.

Steppler HA and Ramachandran PK. 1987**.** Agroforestry a decade of development**.** International Council for Research in Agroforestry, 289-323.

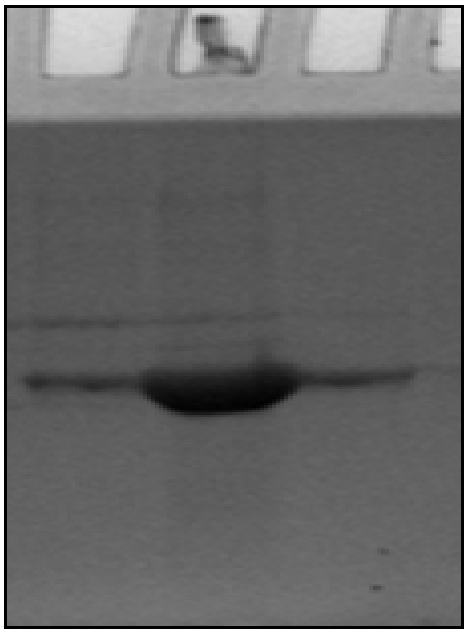
Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

Zdunek-Zastocka E. 2008. Molecular cloning, characterization and expression analysis of three aldehyde oxidase genes from Pisum sativum L. *Plant Physiology and Biochemistry* **46,** 19-28.

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.

Chapter Three

Section 1:
Heterologous expression of CCR gene, its purification and characterization



3.1 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 majority of this work has been done in dicotyledonous angiosperms such as trees and forage crops. The enzyme kinetics of few tree species (Lacombe *et al.*, 1997; Leple *et al.*, 1998) 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.

CCR is one of the key genes involved in lignin biosynthesis. Hydroxycinnamoyl-CoA esters of general phenyl propanoid pathway, when acted upon by CCR, become destined to form respective monolignols. CCR activity is generally low in plants indicating that it may play a crucial role as a rate limiting step in regulation of lignin biosynthesis. As the first committed step of the lignin branch pathway, CCR may be considered as a potential control point regulating the overall carbon flux towards lignins. CCR has been biochemically characterized to a lesser extent than CAD. It has been purified and partially characterized from *Forsythia* and soybean cultures (Gross and Kreiten, 1975; Luderitz and Grisebach, 1981; Wegenmayer *et al.,* 1976), spruce cambial sap (Luderitz and Grisebach, 1981), poplar xylem (Sarni *et al.,* 1984) and *Eucalyptus gunnii* xylem (Goffner *et al.,* 1994). First cloning of a cDNA encoding *CCR* was achieved by screening a differentiating xylem cDNA library with degenerate oligonucleotides designed from internal peptide sequences obtained from purified *Eucalyptus CCR*. The identity of the clone has been unambiguously proven through expression of a functional recombinant enzyme in *E. coli* (Lacombe *et al.,* 1997).

In the previous chapter, two cDNA encoding *CCR* 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 *CCRH2* was chosen to overexpress in *E. coli* BL21 strain.

3.2 Materials and Methods:

3.2.1 Materials:

Chemicals:

Kanamycin, Ampicillin, Tetracycline, Tris HCl, IPTG, X-gal, SDS, BSA, EDTA and Ethidium bromide were purchased from Sigma-Aldrich, USA. Agarose, restriction enzymes, T4 DNA ligase, RNase A and lysozyme were obtained from GIBCO-BRL (USA), Promega (USA) and Amersham (USA). Taq DNA polymerase was obtained from Bangalore Genei (India). Hifi platinum Taq DNA polymerase was procured from Invitrogen (USA). Nickle-NTA Agarose was obtained from Qiagen, USA. All other chemicals and solvents of analytical grade were purchased from HIMEDIA, Qualigens Fine Chemicals and E-Merck Laboratories, India. Pipette tips and micro centrifuge tubes were purchased from Axygen (USA).

Glassware and plasticware:

As discussed in chapter two section 2.2.1

Bacterial 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)

pET30b (+) Expression vector (Novagen, USA)

Stock solutions:

1) IPTG stock solution 200 mg/mL in sterile distilled water (4 μL per 90 mm plate).

2) X-gal stock solution 20 mg/mL in Di-methylformamide (40 μL per 90 mm plate).

3) IPTG stock solution 1 M in sterile distilled water (0.5 – 1.0 mM for induction).

4) Kanamycin 30 mg/mL

5) Ampicillin 100 mg/mL

6) Tetracycline 12.5 mg/mL

3.2.2 Methods:

3.2.2.1 Bacterial culture conditions:

As discussed in Chapter Two section 2.2.2.1

3.2.2.2 Bacterial cells transformation:

E. coli transformation and selection was done as discussed in Chapter Two section 2.2.2.2

3.2.2.3 Genomic DNA extraction:

As described in Chapter Two section 2.2.2.3

3.2.2.4 Isolation of plasmid DNA from *E. coli*:

As described in Chapter Two section 2.2.2.4

3.2.2.5 Restriction digestion of DNA:

As described in Chapter Two section 2.2.2.5

3.2.2.6 Extraction and purification of DNA from agarose gels:

As described in Chapter Two section 2.2.2.6

3.2.2.7 Polymerase Chain Reaction (PCR):

As described in Chapter Two section 2.2.2.9

3.2.2.8 Colony PCR method:

Colony was picked from the master plate and re-suspended in 20 µL SMQ and was re-streaked on a replicate LB-Amp plate. The plate was kept at 37 °C for O/N incubation. The re-suspended colony was boiled at 100 °C for 10 min and centrifuged at 10,000 g for 2 min. From this, 2 µL supernatant was used as a template in PCR. PCR cycling parameters as discussed in detail in section 2.2.2.9 were used**.** The positive colonies were inoculated in LB media with appropriate antibiotics and plasmid was isolated. Isolated plasmids were digested with appropriate restriction enzymes to check for positive clones.

3.2.2.9 Purification of protein from inclusion bodies and cell lysate:

Solutions:

Lysis Buffer: 50 mM Tris-HCl, pH 8.0; 5 mM EDTA; Triton X 100 1%; 0.1 mM PMSF; 1 mM DTT; 10 mM $MgCl_2$ (added later)

Lysozyme 10 mg/mL

Sonication Buffer: 100 mM Tris-HCl, pH 8.0; 50 mM Glycine

Dispersion buffer: 100 mM Tris-HCl, pH 8.0; 50 mM Glycine; 8 M Urea

E. coli cells (BL21 DE3) harboring recombinant pET 30 b+ vector were grown O/N in 5 mL LB Broth at 37 ºC on a shaker (200 rpm). Secondary culture (100 mL LB broth) was inoculated by 1% primary innoculum. When A_{600} was 0.3 to 0.5, cells were induced with IPTG (0.5 mM) and allowed to grow for 6 h. Cells were harvested by centrifuging at 6000 g for 10 min at 4 °C and suspended in 6.25 mL lysis buffer. The suspension was sonicated for 5 min with pulses of 5 s on and 5 s off at 70% amplitude in Branson 450 sonicator. $MgCl_2$ (final concentration 10 mM) and lysozyme (final concentration 0.1 mg/mL) were added after sonication and kept at 37 ºC for 20 min. The suspension was centrifuged at 10,000 g for 10 min. Supernatant was saved and pellet was resuspended in 2.5 mL sonication buffer and sonicated for 30 s. The suspension was dispersed in 2.5 mL dispersion buffer. Protein dissolved in the dispersion buffer was stored at -20 ºC. Protein sample was then analyzed on 10% SDS PAGE (Li, 2001).

3.2.2.9.1 Purification of protein from inclusion bodies

Polyhistidine-tags are often used for affinity purification of polyhistidine-tagged recombinant proteins that are expressed in *Escherichia coli* or other prokaryotic expression systems (Hengen, 1995). 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 polyhistidine-tag binds with micromolar affinity. The matrix is then washed with buffer to remove unbound proteins. The washing efficiency may be improved by the addition of 20 mM imidazole and histidine-tagged proteins are then usually eluted with 150-300 mM imidazole. The purity and amount of protein is assessed by SDS-PAGE**.** In present study Ni-NTA Agarose resin from QIAGEN was used.

Buffers used for purification of protein from inclusion bodies:

Binding Buffer: 10 mM Tris-HCl, pH 8.0; 100 mM Na_2HPO_4; 8 M Urea

Wash Buffer: 10 mM Tris-HCl, pH 8.0; 100 mM Na_2HPO_4; 20 mM Imidazole; 8M Urea

Elution Buffer: 10 mM Tris-HCl, pH 8.0; 100 mM Na_2HPO_4; 250 mM Imidazole; 8 M Urea

Nickel Chelated Columns: Contains nickel (Ni^{+2}) chelated tetradentate Nitriloacetic acid (NTA) covalently immobilized to 4% beaded agarose; stored in 0.02% sodium azide.

The resin comes in 50% ethanol; therefore it was washed with sterile milliQ water and equilibrated to room temperature. Resin after washing was poured in the column and was equilibrated with binding buffer. A 100 mL culture of *E. coli* (A_{600} = 1.5-3.0) expressing the recombinant protein was processed as described in 3.2.2.9. Supernatant was saved as lysate. The Inclusion bodies were suspended in 2.5 mL sonication buffer and subsequently dispersed in 2.5 mL of dispersion buffer to a homogenous suspension and kept at room temperature for 10 min, with gentle shaking. The suspension was centrifuged at 15000 g for 10 min to discard cell debris and supernatant was loaded directly to Ni-NTA agarose column, equilibrated with binding buffer. The column with 6X HIS tagged protein was allowed to bind at 4 °C for one hour. After 1 h column was allowed to stand and flow through was collected in a separate tube. Column was washed with two bed volumes of Wash Buffer and eluted in four 1 mL aliquots with Elution Buffer. Measuring the absorbance at 280 nm monitored the protein elution. The eluted protein was analyzed by SDS-PAGE.

After protein was completely eluted, the gel bed was washed with 2-5 mL of Wash Buffer and stored in binding buffer, the column capped and stored upright at 4 °C. Microbial growth during long-term storage can be prevented by using Wash Buffer containing sodium azide 0.01-0.02%.

3.2.2.9.2 Purification of protein from cell lysate:

Cell lysate obtained in section 3.2.2.9 was utilized to purify protein in native form. Resins and buffers were used as described in above section. Buffers used in purification of protein from cell lysate are given below.

Buffers used for purification of protein from cell lysate:

Binding buffer: 50 mM Na_2HPO_4, pH. 8.0; 300 mM NaCl; 10 mM Imidazole

Wash Buffer: 50 mM Na_2HPO_4, pH. 8.0; 300 mM NaCl; 50 mM Imidazole

Elution Buffer: 50 mM Na_2HPO_4, pH. 8.0; 300 mM NaCl; 250 mM Imidazole

Purification was performed as described in above section but all the protocols were performed at 4 °C to rescue the purified protein in active form.

3.2.2.10 Sodium dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE):

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

Buffers:

Monomer solution: 29.2% Acrylamide and 0.8% Bis-acrylamide in water and was stored at 4 °C in the dark.

Running Gel Buffer: 1.5 M Tris-HCl (pH 8.8)

Stacking Gel Buffer: 0.5 M Tris-HCl (pH 6.8)

10% SDS (w/v) in water

Initiator (APS) 10% Ammonium persulfate (make fresh)

10X Tank Buffer: 0.25 M Tris-HCl; 1.92 M glycine; pH 8.3

Water saturated n-Butanol: 5 mL water in 50 mL n-butanol

5X Loading Buffer: 0.125 M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol (v/v); 0.1% (w/v) Bromophenol blue; 10% β-mercaptoethanol (v/v)

3.2.2.10.1 Preparation of the separating gel:

A vertical slab gel (Hoefer 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 3.1 except for the addition of ammonium persulfate and TEMED. Ammonium persulfate and TEMED 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.

Solution	**Seperating gel for 10 mL**
Gel Buffer (1.5 M Tris-HCl, pH 8.8)	2.5 mL
Monomer Solution	3.33 mL
Distilled water	3.96 mL
APS (10%)	100.0 µL
SDS (10%)	100.0 µL
TEMED	10.0 µL

Table 3.1 Composition of 10% SDS - Polyacrylamide separating gel

3.2.2.10.2 Preparation of the stacking gel:

Stacking gel solution was prepared according to Table 3.2. TEMED and ammonium persulfate were added last and mixed. The mixture was 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.

Solution	Stacking gel for 4 mL
Gel Buffer (0.5 M Tris-HCl, pH 6.8)	1.0 mL
Monomer Solution	0.65 mL
Distilled water	2.25 mL
APS (10%)	40 μL
SDS (10%)	40 μL
TEMED	4 μL

Table 3.2 Composition of SDS- Polyacrylamide, Stacking Gel.

3.2.2.10.3 Preparation of the sample:

Four parts of the protein sample and one part loading buffer were mixed in a micro-centrifuge tube and kept in a boiling water bath for 5 min. The samples were centrifuged at 10,000 g for 10 min, supernatant collected and used for electrophoresis.

3.2.2.10.4 Loading and running the gel:

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

3.2.2.10.5 Coomassie blue staining of the gel:

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

De-staining solution: 45 mL Methanol, 10 mL acetic acid and 45 mL de-ionized water.

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

3.2.2.10.6 Silver staining of the gel:

Solutions:

Fixer: 40% (v/v) methanol, 12% (v/v) Acetic acid in de-ionized water

Thiosulfate solution: 20 mg of $Na_2S_2O_3$ in 100 mL deionized H_2O

Silver Stain: 200 mg $AgNO_3$, 75 µL HCHO in 100 mL deionized H_2O

Developer: 6 g of Na_2CO_3, 50 µL HCHO, 100 µL $Na_2S_2O_3$ (4 mg/mL stock) in 100 mL de-ionized H_2O

After running the gel, it 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 thiosulfate solution for 1 min and rinsed thrice with de-ionized 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 de-ionized H_2O (20 s each) and transferred to the developer till the bands developed. The gel was washed with de-ionized H_2O and stored in the fixer.

3.2.2.11 Raising polyclonal antibody against purified CCR protein in Rabbit:

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

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.

3.2.2.12 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.

3.2.2.13 CCR enzyme assay and kinetics:

CCR enzyme activity was measured according to the method of Luderitz and Grisebach (1981). Coniferaldehyde and sinapaldehyde were used as substrate for reverse reaction. The reaction mixture contained 1nM to 30 nM substrate, 200 µM NADP, 150 µM Coenzyme A and enzyme in 0.2 M Tris-HCl (pH 7.8). For each reaction, increase in absorbance at 366 nm was monitored for 10 mins. Each reation was run in triplicate. A non-induced *E. coli* extract was used as a control. Data was analysed using Graph Pad Prism 5 software programme to determine K_m values.

3.2.2.14 Protein estimation:

Protein estimation was done using Bradford assay (1976). A standard graph was made for BSA (1 μg to 7 μg) and concentration of unknown sample was determined by plotting on standard graph.

3.3 Results:

3.3.1 Incorporation of Restriction sites:

Two primers were designed to incorporate restriction (*Nde* I and *Hind* III) sites to the ends of *CCRH2* gene. Cloned *CCRH2* fragment in pGEMT vector was used as template to amplify *CCRH2* gene with incorporated restriction site.

Primers:

CCRIIpetF 5' **CAT ATG** CCTGCT GCC GCC CCA GCC 3'

CCRIIpetR 5' **AAG CTT** CTT GGT GGG AAG AGG AAG A 3'

Plasmid pGEMCCRH2 was diluted 100 times and 1 μL was used as a template. PCR was performed as described in section 2.2.2.9.

PCR cycling condition:

No. of cycles	Temperature	Time
1	95 ºC	5 min
35	95 ºC 55 ºC 72 ºC	1 min 30 s 1min
1	72 ºC	7 min
1	4 ºC	hold

A 1 kb band was amplified. The band was cut, purified, ligated in pGEM-T easy vector and transformed in *E.coli* XL1 MRF cells. Clones with *CCRH2* fragment with *Nde* I and *Hind* III restriction sites were screened by inoculating few colonies in 5 mL LB (Ampicilin 100 μg/mL) tubes. Isolated individual plasmids were restriction digested with *Nde* I and *Hind* III enzymes to confirm the integration of *CCRH2* insert.

3.3.2 Directional cloning of *CCRH2* in pET30b+

The above clone in pGEM-T easy vector was designated as pGEMCCRH2. This clone was restriction digested with *Nde* I and *Hind* III restriction enzymes and 1 kb fragment was purified. pET 30 b+ vector DNA was also digested with same restriction enzymes and purified (Fig. 3.3.1). A 1 kb fragment from pGEMCCRH2 was directionally cloned in purified restriction digested pET 30b+ vector (Fig. 3.3.2). Ligation mixture was transformed in *E.coli* XL1 competent cells and plated on LB-agar plate (Kanamycin 50µg/mL). Colonies for recombinant plasmids were screened by colony PCR method.

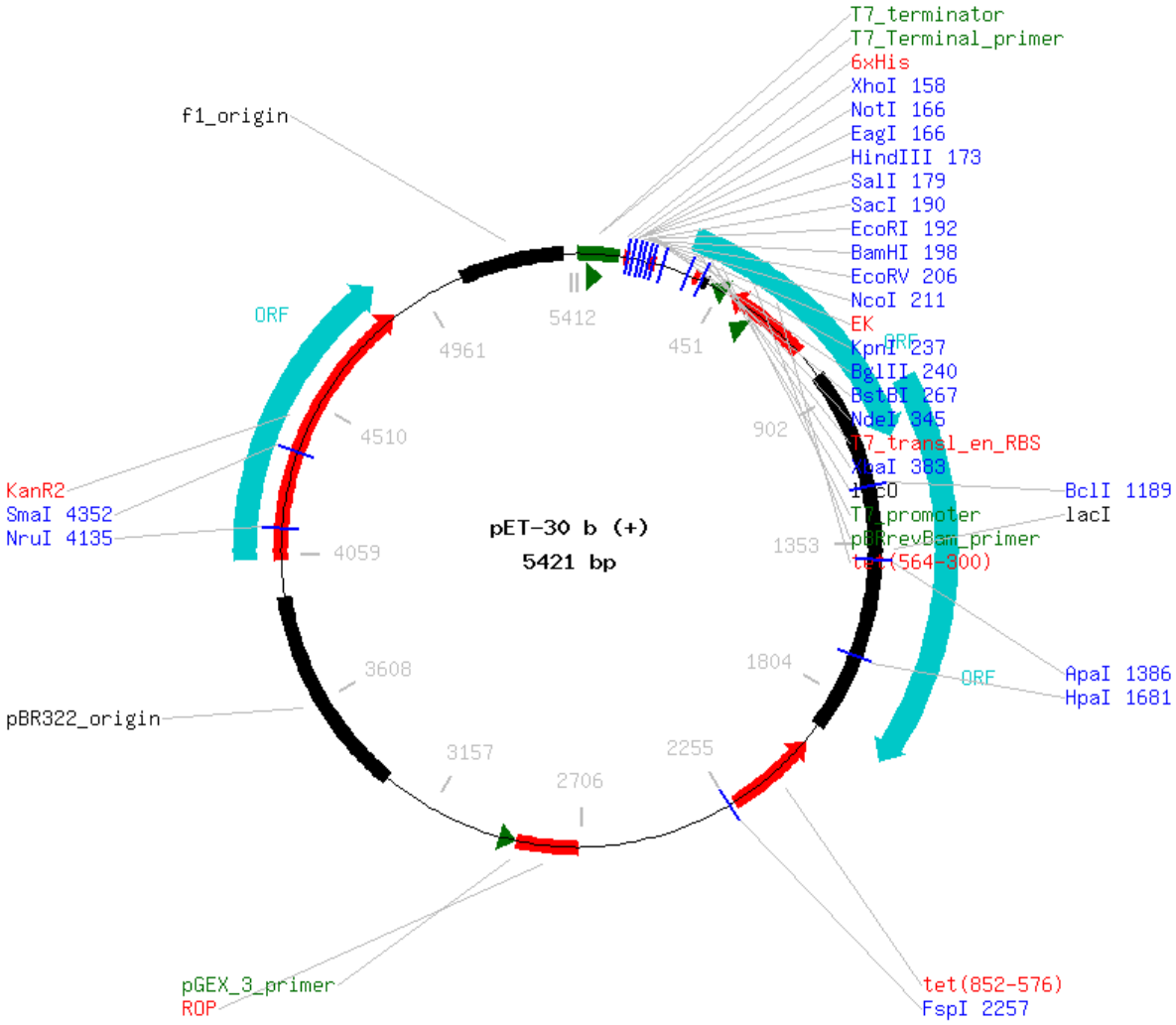


Fig. 3.3.1: Map of pET 30 b+

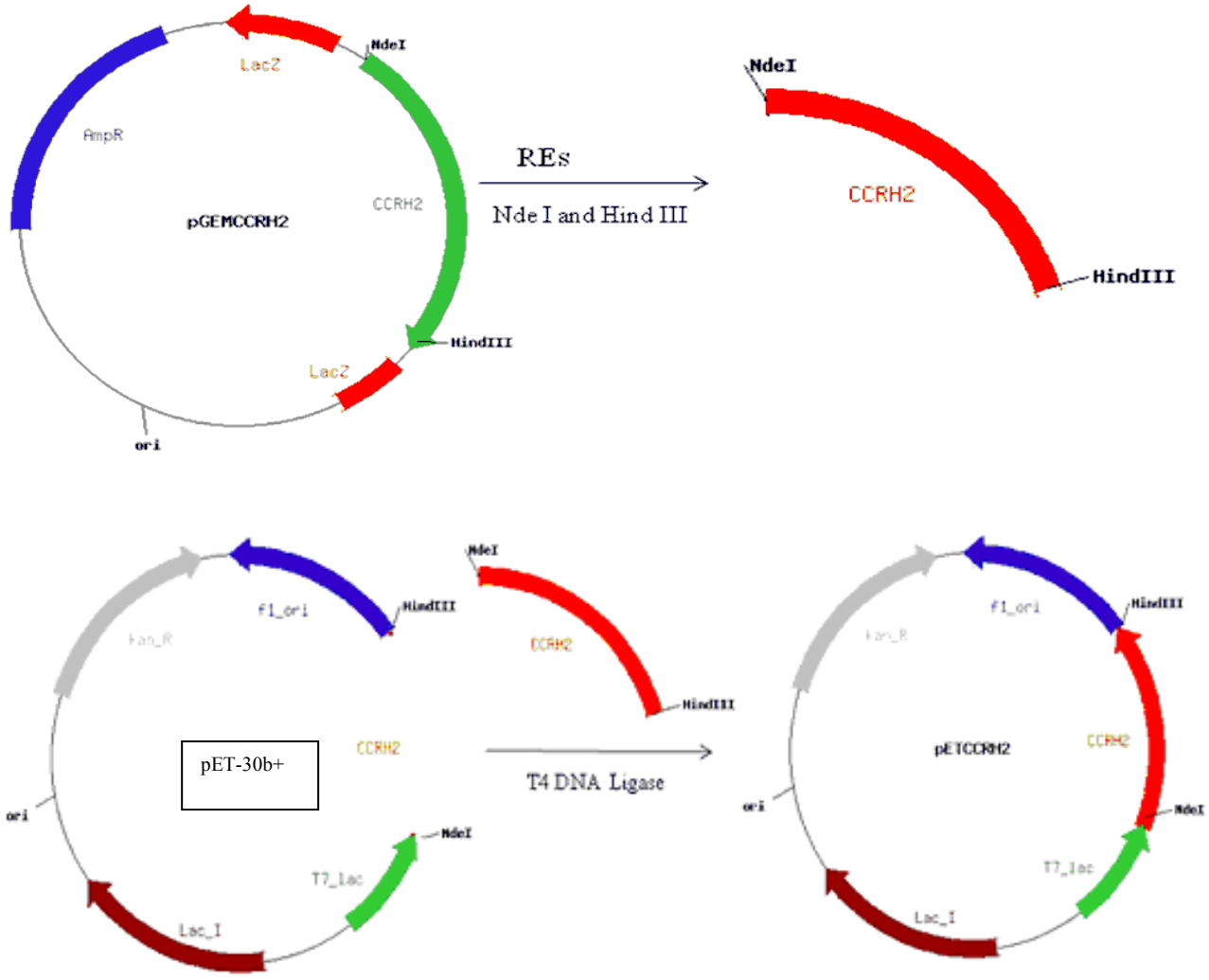


Fig. 3.3.2: Strategy for directional cloning of CCRH2 in pET30 b+ vector

3.3.2.1 Colony PCR:

Colony PCR of around 90 putative clones was performed and out of them four clones gave positive 1 kb band (Fig. 3.3.3).

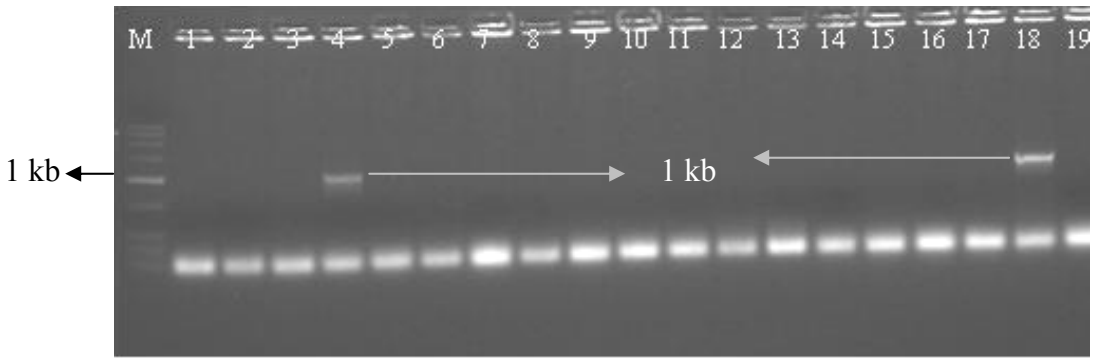


Fig. 3.3.3: Colony PCR of 19 colonies: Lane M: 1 kb marker (Bangalore Genei, India), Lane 1 to 19 PCR of nineteen colonies. Lane 4 and 18 showing positive 1 kb fragment. Bands showed by arrow are 1 kb amplified fragments.

Positive colonies were inoculated in 5 mL LB (ampicilin 100 µg/mL) tubes and plasmids were isolated. Recombinant plasmids were checked by restriction digestion and sequence validated. Four recombinant plasmids were identified. They were designated as pETCCR24, pETCCR38, pETCCR42 and pETCCR54. The recombinant pET 30b+ plasmids harboring *CCRH2* gene was confirmed after sequencing. The sequence was translated using proteomic tools available on www.expasy.ch and was checked for in frame translation upto HIS tag. Above recombinant plasmids were mobilized in *E. coli* BL21 strain for over-expression.

3.3.3 Standardization of protein expression in *E. coli* BL21:

a) Screening for over-expressing recombinant CCRII protein:

E.coli BL 21 (DE3) cells transformed with recombinant pET 30b+ plasmid were screened for over-expression. The *E.coli* BL21 cells harboring recombinant plasmid were grown O/N in 5 mL LB (Kanamycin 30 µg/mL) tubes. The above primary culture was re-innoculated in fresh 5 mL tube containing LB (Kanamycin 30 µg/mL). As the A_{600} reached 0.5, it was induced with 1 mM IPTG solution and kept at 37 °C for 6 h. An uninduced control was taken out before inducing with IPTG. After 6 h of induction, samples were centrifuged at 10000 g for 2 min and re-suspended in 200 µL 2X SDS-Gel loading buffer. The solution was boiled for 5 min, centrifuged at 10000 g for 2 min and loaded onto a 10%

SDS- PAGE (as described earlier). The gel was run at 100 V for 2 h and stained in Coomassie blue (R 250) staining solution. A ~38 kD protein was found to express in all clones (Fig. 3.3.4). Clone pETCCR42 was used for all experiments.

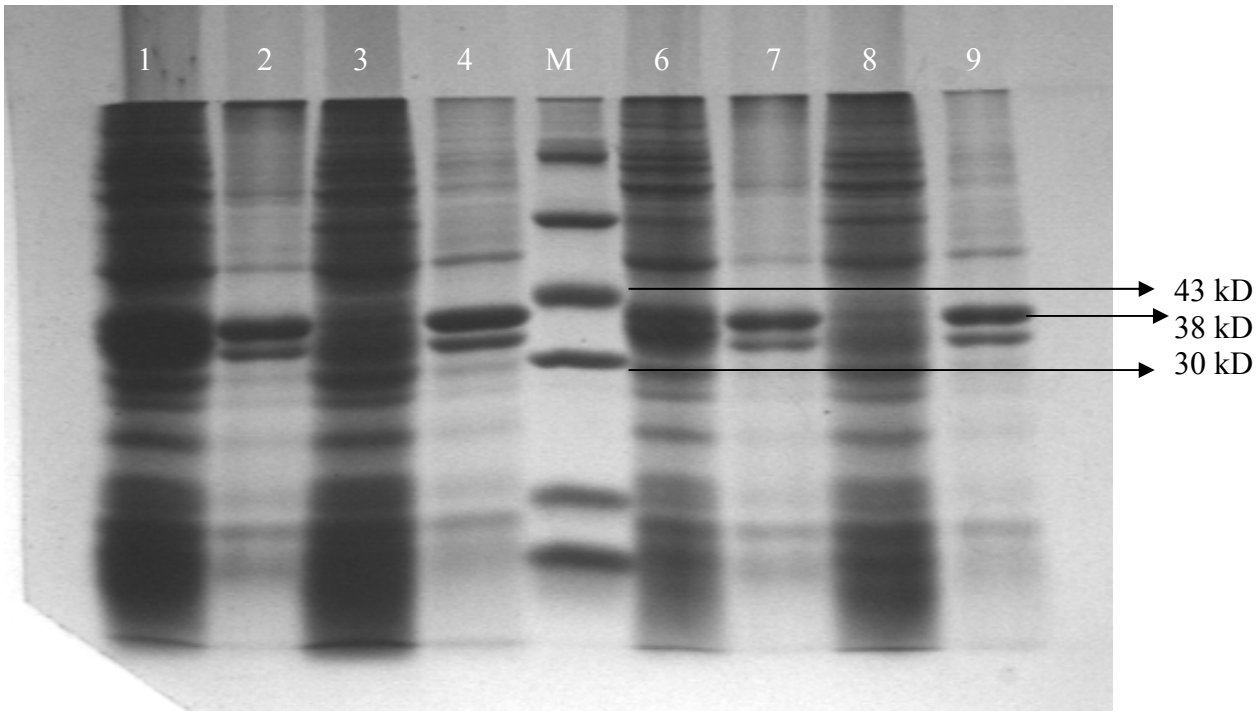


Fig. 3.3.4: Over-expression of *CCRH2* in all positive clones: Lane 1, 3, 6 and 8 are uninduced control, Lane 2, 4, 7 and 9 are induced over-expressed CCRH2 protein (clone 24, 38, 42 and 54 respectively), Lane M Molecular weight marker (Bangalore Genei, India).

b) Standardization of IPTG concentration needed for induction:

The recombinant clone with best expression was utilized for this study. *E.coli* BL 21 (DE3) cells transformed with recombinant plasmid pETCCR42 were grown as described in above section and 0.1 mM, 0.5 mM and 1 mM IPTG concentration were used for induction. Analysis of result showed that 1 mM IPTG concentration was found to be less effective for protein expression and other two tested concentration gave more or less the same result (Fig. 3.3.5). 0.5 mM IPTG concentration was utilized for other experiments.

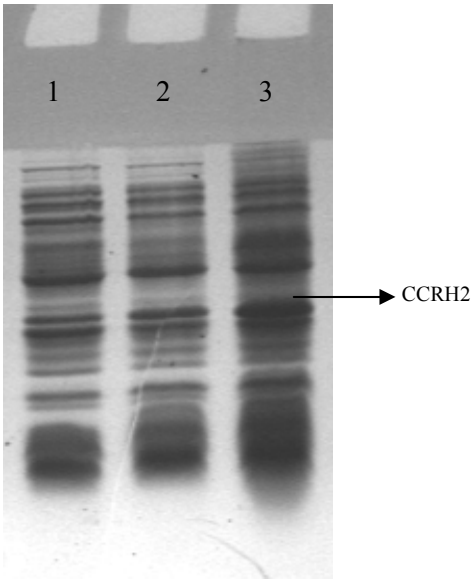


Fig. 3.3.5: Inclusion bodies of over-expressed CCR protein at different IPTG concentration: Equal quantity of total protein was loaded in each well. Lane 1: 0.1 mM IPTG, Lane 2: 1 mM IPTG and Lane 3: 0.5 mM IPTG.

c) Standardization of time for protein expression in soluble form:

The information obtained from above experiment was utilized for this experiment. Six flasks with 50 mL LB broth (Kanamycin 30 μg/mL) were inoculated with O/N grown culture of *E.coli* BL21 harboring pETCCR42 recombinant plasmid. Induction with 0.5 mM IPTG was done when A_{600} reached 0.5 at 28 °C. One flask of 50 mL was used for analysis at 2 h, 4 h, 6 h and 16 h after induction. One flask as an uninduced control sample was used before inducing with IPTG. The SDS-PAGE analysis indicated that the soluble fraction of the cell lysate and pellet fraction of recombinant bacteria contained an over expressed protein with an apparent molecular mass of approximately 38 kDa. SDS-PAGE analysis of protein lysate obtained from harvesting cultures at different time showed that amount of protein in lysate was maximum in 6 h harvested culture (Fig. 3.3.6).

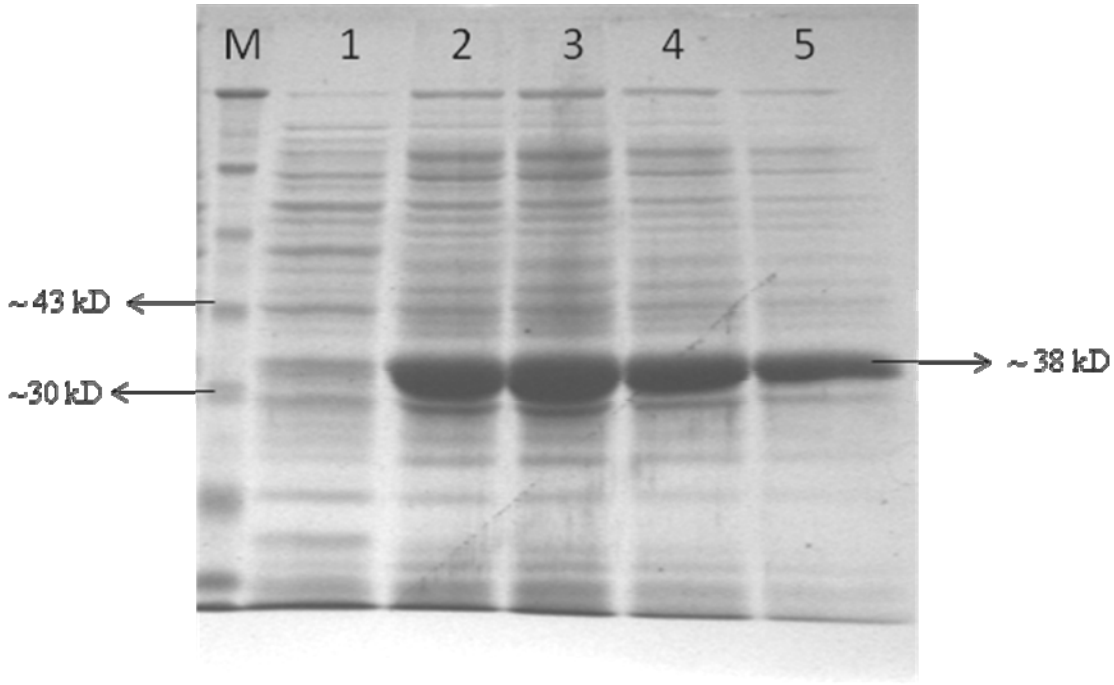


Fig. 3.3.6: Over-expression of CCRH2 protein in soluble form harvesting at different times after induction at 28 °C: Lane M: Molecular weight marker (Bangalore Genei, India), Lane 1: Un-induced lysate, Lane 2: 16 h induced lysate, Lane 3: 6 h induced lysate, Lane 4: 4 h lysate, Lane 5: 2 h lysate.

Since the purification of recombinant protein in soluble form was not achieved in good amount large-scale production and purification to raise antibodies was done from inclusion bodies as described in section 3.2.2.9 of this chapter.

3.3.4 Purification of protein:

Large-scale production and purification was done using the protocol described in section 3.2.2.9. The purified protein was approximately 38 kD in size and was eluted at 200 mM Imidazole concentration. It was found that four hours of post-induction at 37 °C was enough to achieve the purification of CCR protein from inclusion bodies (Fig. 3.3.7). Purified CCRH2 protein was dialysed against 1X PBS buffer supplemented with 1 mM DTT and 0.1 mM PMSF overnight with two changes of fresh buffer. Dialysed CCRH2 protein was concentrated, quantified by Bradford assay (Bradford reagent, Promega, USA) and given for raising antibodies in New Zealand rabbit.

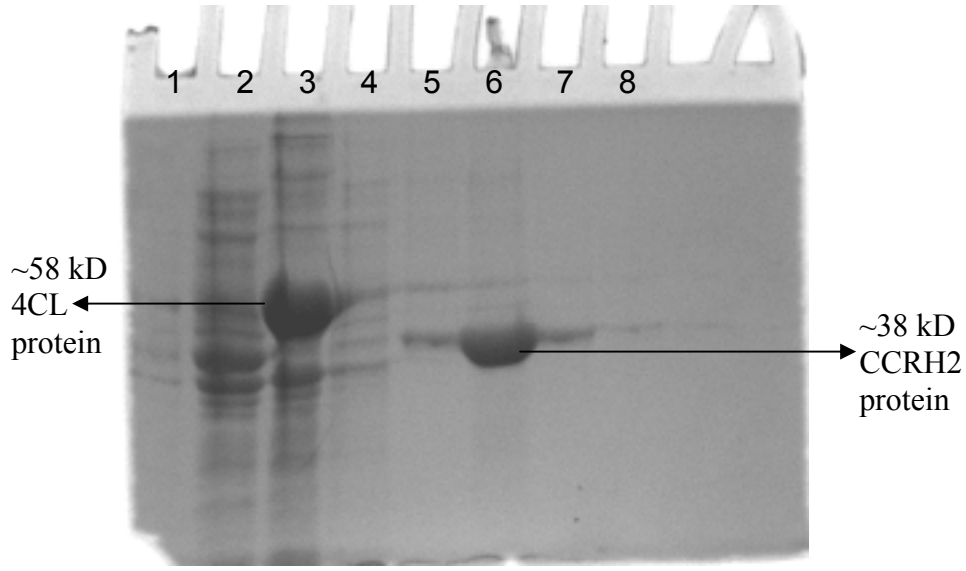


Fig. 3.3.7: Purification of over-expressed CCR protein from Inclusion bodies: 12% SDS-PAGE: Lane 2: Crude protein, Lane 3: Over-expressed 4CL protein 58 kDa (characterized in lab), Lane 4: Wash, Lane 5: Elution 1, Lane 6: Elution 2, Lane 7: Elution 3, Lane 8: Elution 4.

3.3.5 Raising antibodies in rabbit:

300 µg of purified protein was used for first injection in New Zealand rabbit to raise antibodies. Same amount of protein was used for booster doses. Titer of first bleed and second bleed was determined to be 1:5000 and 1:50000 respectively. Third booster dose of 60 µg purified protein resulted in increase of titre to ~1:100000 (Fig. 3.3.8). 3rd bleed serum dilution of 1:10000 for ELISA of developing seedlings, 1:5000 for ELISA of transgenic line and 1:5000 for immuno-cytolocalization was used.

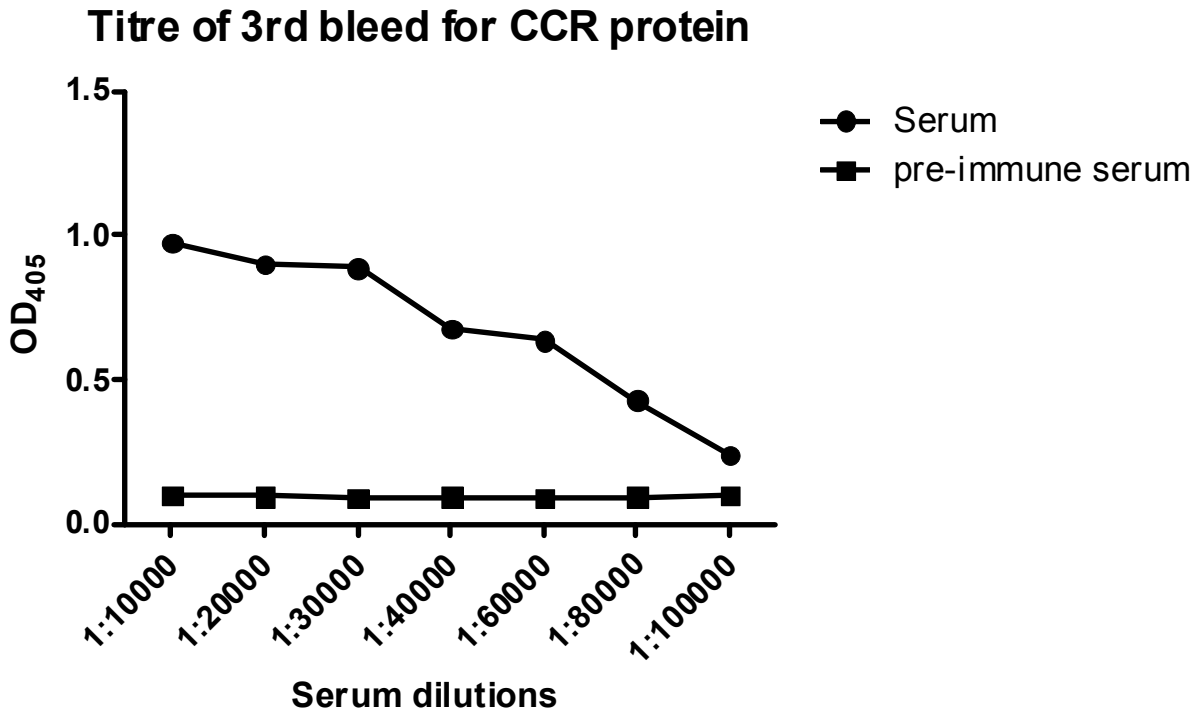


Fig. 3.3.8: Determination of titre of 3rd bleed serum: Solid circles represent OD_{405} of serum dilution; Solid square represents OD_{405} of pre-immune serum.

3.3.6 CCR enzyme assay:

Substrates for CCR enzyme assay *i.e* Feruloyl CoA, Sinapoyl CoA, Coumaryl CoA *etc.* were commercially unavailable and therefore reverse reaction was followed as described by Luderitz and Grisebach (1981). Coniferaldehyde and sinapaldehyde were used as substrate for reverse reaction. Lineweaver- Burk plot revealed that K_m of sinapaldehyde was nearly three times lower than coniferaldehyde which suggests that sinapaldehyde is more preferred substrate (Fig. 3.3.9, Fig. 3.3.10, and Table 3.3).

Substrate	K_m (nM)
Sinapaldehyde	0.264
Coniferaldehyde	0.767

Table 3.3: K_m values for Cinnamoyl Co A reductase from crude lysate: Values are too low because non-homogenous enzyme was used to establish preliminary substrate preference.

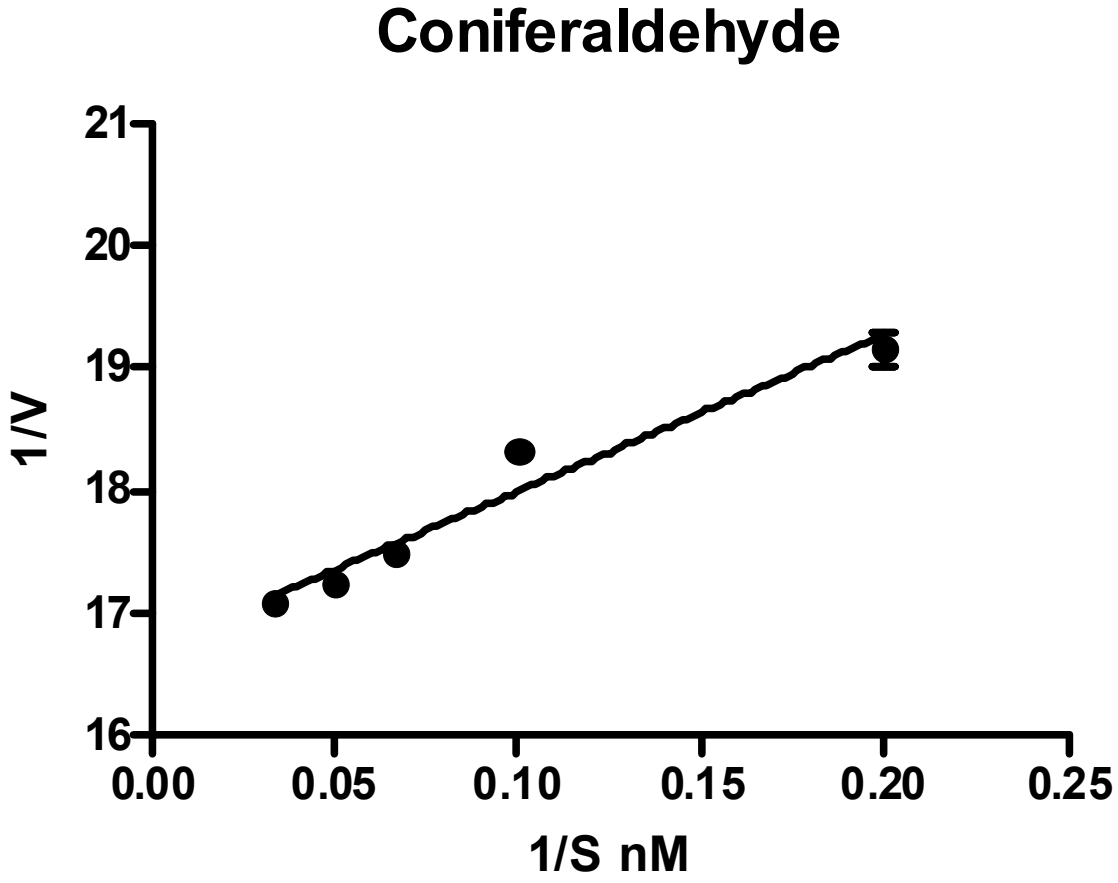


Fig. 3.3.9: Lineweaver- Burk plot: Best-fit values for Coniferaldehyde

	Coniferaldehyde
Y-intercept when X=0.0, 1/Vmax	16.71 ± 0.09874
X-intercept when Y=0.0, 1/Km	-1.303

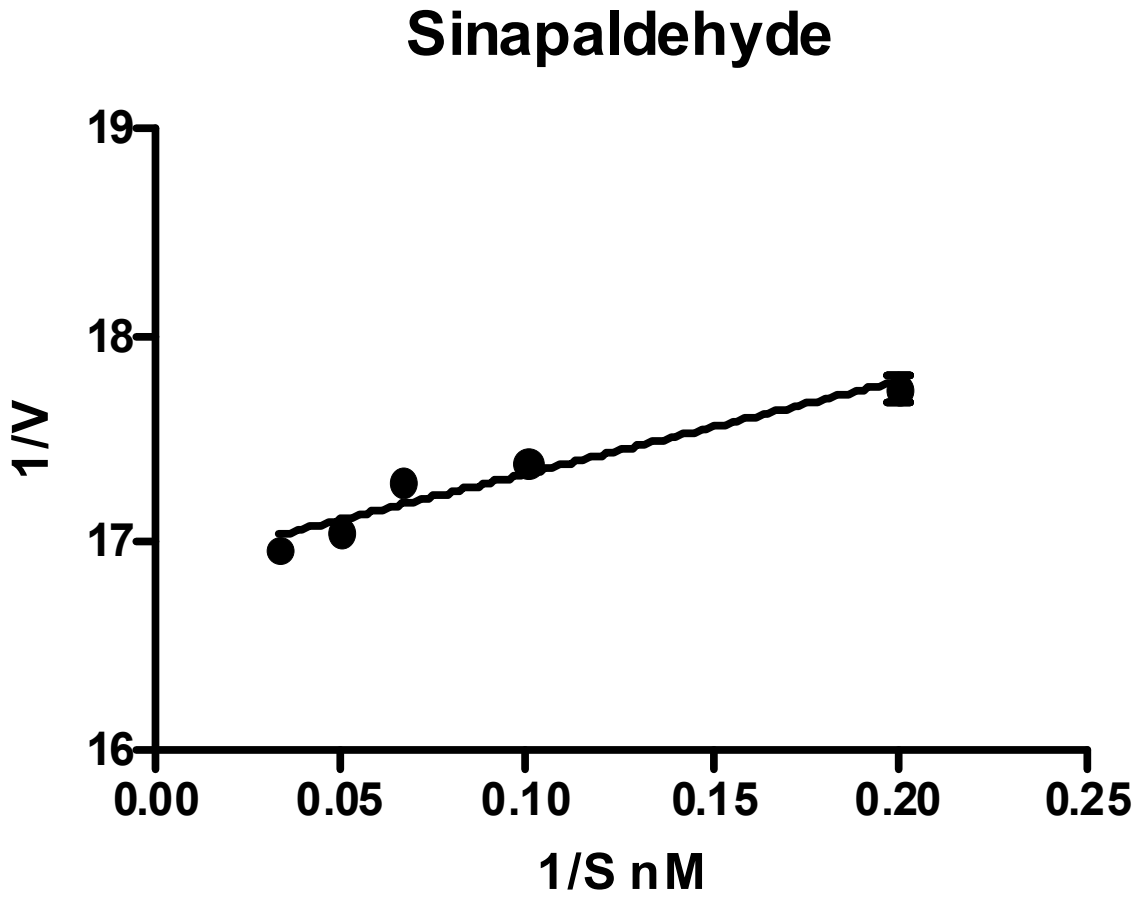


Fig. 3.3.10: Lineweaver- Burk plot: Best-fit values for Sinapaldehyde

	Sinapaldehyde
Y-intercept when X=0.0, 1/Vmax	16.89 ± 0.05045
X-intercept when Y=0.0, 1/Km	-3.783

3.4 Discussion:

Ll-CCRH2 was over-expressed using pET 30b+ expression system (Novagen, USA). The pET 30b+ vectors carry an N-terminal His•Tag®/thrombin/S•Tag™/enterokinase configuration plus an optional C-terminal His•Tag sequence. Unique sites (*Nde I* and *Hind III*) in pET 30b+ vetor (Fig. 3.3.1) allowed us to incorporate sites which were not present inside the *Ll-CCRH2* gene sequence, which ultimately lead to an over-expressed protein with C terminus HIS tag. Incorporation of C- terminus HIS tag enabled us a one step purification of over-expressed LlCCRH2 protein. The 6X His affinity tag at C terminus facilitates binding to Ni-NTA. It is poorly immunogenic, and at pH 8.0 the tag is small, uncharged, and therefore does not generally affect secretion, compartmentalization, or folding of the fusion protein within the cell. In most cases, the 6X His tag does not interfere with the structure or function of the purified protein as demonstrated for a wide variety of proteins, including enzymes. A further advantage of the 6X His tag is that it allows the immobilization of the protein on metal-chelating surfaces such as Ni-NTA. Ni-NTA agarose is composed of Ni-NTA coupled to Sepharose® CL-6B and offers high binding capacity and minimal nonspecific binding. The high surface concentration of the NTA ligand is sufficient for the binding of approximately 5–10 mg of 6X His-tagged protein per mL of resin. Ni-NTA agarose is very stable and easy to handle and antibodies were raised against recombinant CCRH2 protein. Purification of Ll-CCRH2 protein was achieved using above technology from inclusion bodies to homogenity and antibodies were raised in New Zealand rabbit. A total of 300 µg of purified protein was used for first immunization. Two booster dose of 300 µg and one last booster dose of 60 µg was given before third bleed. Titre of third bleed was estimated to be 1:100000. Antibodies were utilised to immuno-cytolocalize CCR protein in transverse section of *L. leucocephala* and ELISA of 0-15 days stem and root and 8 week and 12 week stem and root (described in Chapter Three, Section 2).

CCR enzyme activity was standardized as described by Luderitz and Greisbach (1981). Active CCRH2 protein (crude lysate) was used to check substrate specificity. Purification of Ll-CCRH2 protein from lysate was attempted. But due to loss of activity, purified soluble protein was not utilized for establishing substrate specificity. Determination of K_m

values of sinapaldehyde and coniferaldehyde by cinnamoyl Co A reductase was done using crude lysate, which revealed that Sinapaldehyde is more favored substrate over coniferladehyde (as K_m of coniferaldehyde is three times higher than that of sinapaldehyde). Values given in Table 3.3 represents K_m values of sinapaldehyde and coniferaldehyde. V_{max} and K_m recorded were too low which may be due to reason that enzyme was not purified to homogenity. V_{max} and K_m values may be refined by using purified Ll-CCRH2 protein in further experiments. It is well known that the prefered reaction for cinnamoyl Co A reductase is conversion of feruloyl Co A to coniferaldehyde (Lacombe, *et al.*, 1997; Lauvergeate, *et al.,* 2001; Pichon, *et al.,* 1998). But in case of reverse reaction, preference of sinapaldehyde over coniferaldehyde suggests that, it may have a regulatory role by channelling sinapaldehyde and coniferaldehyde to produce S and G lignin in varied amount (Hawkins and Boudet, 1994).

3.5 Conclusion:

The cDNA isolated from *L. leucocephala* was directionally cloned in pET 30b+ expression system. Ll-CCRH2 protein was standardized for over-expression and purified from inclusion bodies. A 38 kD purified protein, from inclusion bodies, was used to raise antibodies in New Zealand rabbit. CCR enzyme activity for reverse reaction, for substrates like coniferaldehyde and sinapaldehyde, was also standardized. Characterization of cDNA from *L. leucocephala* as Cinnamoyl Co A reductase is described in Chapter Two using various softwares but utilization of sinapaldehyde and coniferaldehyde confirms that the cDNA isolated from *L. leucocephala* encodes for cinnamoyl Co A reductase protein.

3.6 References:

Baucher M, Halpin C, Petit-Conil M, Boerjan W. 2003. Lignin: genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology* **38,** 305–350.

Boerjan W, Ralph J, Baucher M. 2003. Lignin Biosynthesis. *Annual Review in Plant Biology* **54**, 519- 546.

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.

Goffner D, Campbell MM, Campargue C, Clastre M, Borderies C, Boudet A, and Boudet A M. 1994. Purification and Characterization of Cinnamoyl-Coenzyme A:NADP Oxidoreductase in *Eucalyptus gunnii.* Plant Physiology **106.** 625-632.

Gross GG and Kreiten W. 1975. Reduction of Co enzyme A Thioesters of Cinnamic acids with an enzyme preparation from lignifying tissue of *forsythia. FEBS letters* **54** (2). 259-262.

Hawkins SW, Boudet AM. 1994. Purification and characterization of cinnamyl alcohol dehydrogenase isoforms from the periderm of *Eucalyptus gunnii*. *Plant Physiology* **104,** 75–84.

Hengen P. 1995. Purification of His-Tag fusion proteins from *Escherichia coli*. *Trends in Biochemical Science* **20**(7), 285-286.

Lacombe E, Hawkins S, Doorsselaers JV, Piquemal J, Goffner D, Poeydomege O, Boudet AM, Grima-Pettenati J. 1997. Cinnamoyl-CoA reductase, the first committed enzyme of lignin branch biosynthetic pathway: cloning, expression, and phylogenetic relationships. *The Plant Journal* **11**, 429–441.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lauvergeat V, Lacomme C, Lacombe E, Lasserre E , Roby D, Grima-Pettenati J. 2001. Two cinnamoyl-CoA reductase (*CCR*) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry* **57**, 1187–1195.

Li P. 2001. Purification of inclusion bodies and refolding of protein. Basic strong lab protocol. *Immunogenetics* **53**, 279-287.

Lodhi MA, Ye GN, Norman FW, Bruce IR. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Molecular Biology Reporter* **12**, 6-13.

Luderitz T and Grisebach H. 1981. Enzymic Synthesis of Lignin Precursors Comparison of Cinnamoyl-CoA Reductase and Cinnamyl Alcohol: NADP *i*- Dehydrogenase from Spruce *(Picea abies* L.) and Soybean (*GZycine max* L.). *European Journal of Biochemistry* **119**, 115- 124.

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.

Pichon M, Courbon I, Bechert M, Boudet AM, Grima-Pettenati J. 1998. Cloning and characterization of two maize cDNAs encoding cinnamoyl-CoA reducatase (*CCR*) and differential expression of the corresponding genes. *Plant Molecular Biology* **38**, 671–676.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, New York: *Cold Spring Harbor Laboratory Press*.

Sarni F, Grand G, Boudet AM. 1984. Purification and properties of cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase from poplar stems. *European Journal of Biochemistry* **139,** 259–265.

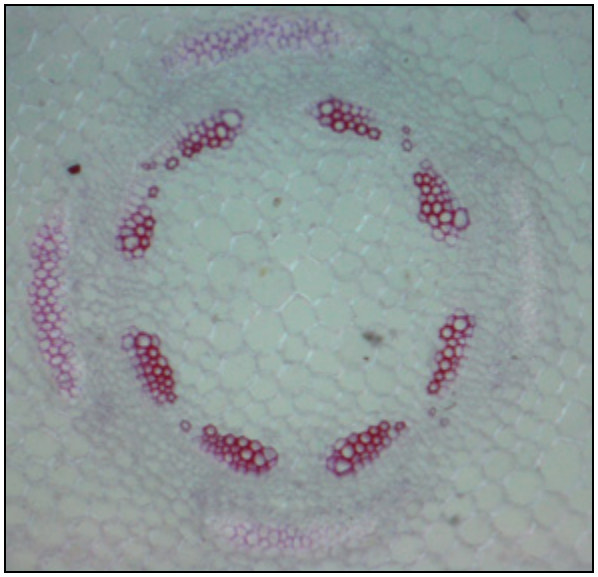
Studier FW, Moffatt BA. 1986. *Journal of Molecular Biology* **189**, 113.

Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. 1990. *Methods in Enzymology* **185**, 60–89.

Wengenmayer H, Ebel J, and 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). European journal of Biochemistry* **65**. 529-536.

Chapter Three

Section 2:
Spatio-temporal expression and Immuno-cytolocalization of CCR gene



3.7 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. The organisation and regulation of phenylpropanoid genes, particularly the first and last step of the ‘‘general phenylpropanoid’’ pathway, catalysed by phenylalanine ammonia-lyase (PAL) and cinnamate-CoA ligase (4CL), have been extensively studied. In contrast to the considerable amount of data accumulated on the regulation of these genes, little is known about the steps devoted more specifically to the lignin monomer biosynthesis.

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. Spatial pattern of *CCR* expression such as *Eg-CCR* (Piquemal *et al*., 1997), *Zm-CCR1* & *Zm-CCR2* (Pichon *et al*., 1998) and *At-CCR1* & *At-CCR2* (Lauvergeat *et al*., 2001) have been reported. The expression of *Eg-CCR* and *Zm-CCR1* is generally associated with tissues undergoing active lignification, including stem and root tissues. The expression of *At-CCR1* was detected in stem and leaf tissues, but it was not reported whether or not this gene was expressed in root tissues.

Temporal pattern of *CCR* gene expression of very few tree species such as *Eucalyptus* (Lacombe *et al.,* 1997) and poplar (Leple. *et al*., 2001) have been reported in detail. In previous section; heterologous expression, purification and characterization has been discussed which suggests that the cDNA isolated from *L. leucocephala* encodes for cinnamoyl Co A reductase. In this section, spatio-temporal expression has been studied in developing seedlings of *L. leucocephala.* Expression pattern of isolated *CCR* gene will help us to understand and predict the involvement of this gene in lignification. To date, however, there is little information at the molecular level as to how the regulation of lignin synthesis affects stem and root development. Hence, the information of expression pattern

of *CCR* gene could be helpful in predicting the role of lignin biosynthesis in stem and root development in future.

Isolation of two cDNA clones has been discussed in Chapter Two and it has been proposed that both the *Ll-CCR*s are isoforms of cinnamoyl Co A reductase, which is involved in lignification. As both *CCR*s had similar binding domains, isoelectric point, molecular weight and hydropathicity only one of the *CCR*s i.e *Ll-CCRH2* was used for spatio-temporal expression analysis.

3.8 Materials and methods:

3.8.1 Materials:

All the chemicals and solvents of analytical grade were purchased from HIMEDIA, Qualigens. Fine chemicals were procured from Sigma-aldrich, USA and Merck Laboratories, USA. Pipette tips and micro centrifuge tubes were purchased from Axygen (India). Medium binding, flat bottom ELISA plates were procured from Costar, USA. Brilliant SYBR Green kit was purchased from Stratagene, USA. BCIP/NBT mix was procured from Merck, USA.

3.8.1.1 Plant material: ELISA and RT PCR experiments

Seeds of *L. leucocephala* were treated with concentrated sulphuric acid for 5 min and then washed thoroughly with sterile distilled water. This was followed by surface sterilization using 0.1% mercuric chloride and washing with sterile distilled water. The seeds were then kept in moist culture bottles, under shaking conditions, in dark. After two days of germination, the seedlings were transferred to bottles/tubes containing half-strength MS media (Murashige and Skoog, 1962). The day of inoculation was considered as zero days. Root, shoots and leaves were harvested from 0, 5, 10, 15 days, 8 week and 12 week old plants. Experiments were conducted in a growth chamber with temperatures of 25±2 ºC under a 16 h photoperiod, 70% relative humidity, with a light intensity of 24.4 μmol/m^2/s.

3.8.1.2 Primary antibodies for anti CCRH2 protein:

Purified Ll-CCRH2 protein was used to raise antibodies in rabbit as discussed in Chapter Three, Section 1.

3.8.1.3 Secondary antibody:

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

3.8.2 Methods:

3.8.2.1 Total RNA extraction and cDNA synthesis:

Total RNA was isolated from experimental germinated seeds 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).

3.8.2.2 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.

3.8.2.2.1 Q-PCR considerations:

The optimal concentration of the upstream and downstream PCR primers is determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The best concentrations of the upstream and downstream primers are not always of equal molarity. In this study, 100 nM was considered optimum. Reaction was standardized in such a way that there was no primer dimer formation.

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 – 300 nM depending upon the instrument configuration. In this study 30 nM concentration was used.

Acquisition of real-time data generated by SYBR Green 1 was done as recommended by the instrument manufacturer. Data collection was either at the annealing step (3- step cycling protocol) or extension step of each cycle.

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

3.8.2.2.2 Preparing the reactions:

The final reference dye concentration of 30 nM was used in the reaction mixture. Real time PCR model Mx 3000P (Stratagene, USA) was used in the present study. The experimental reactions were prepared by adding the following components in order.

Reaction Mixture:

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

2X master mix	12.5 µL
Upstream primer (optimized concentration)	1 µL
Downstream primer (optimized concentration)	1 µL
Diluted reference dye	1 µL
Total	25 µL

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

3.8.2.2.3 Real-time quantitative PCR for *Ll-CCRH2* gene:

Total RNA was extracted individually from leaves, 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 CCRH2* gene and 5.8S rRNA are given in Table 3.4. Optimal numbers of PCR cycles within the linear range of amplification for each gene were determined in preliminary experiments. RT-PCR reactions were performed under the following conditions: 2 min at 50 °C, followed by 10 min at 94 °C, 42 cycles (for *Ll-CCR*) or 30 cycles (for 5.8S RNA) of 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C. The reaction was run in duplicates and repeated twice. It was ensured that equal quantity of RNA template was used for each reaction (Pfaffl, 2001; Freeman *et al*., 1999; Edyta Zdunek-Zastocka, 2008).

Primer	**Sequence 5' – 3'**	**Tm in °C**
SamF1	CTAACACCACCTCATCAGGTT	62
CCRintrev	GTTCTTAGAATCATCTGGATTTCTG	68
CTF	CTAAACGACTCTCGGCAAC	58
CTR	TTCAAAGACTCGATGGTTCAC	60

Table 3.4: Primer sequences designated for *Leucaena CCRH2* gene and 5.8S rRNA

3.8.2.2.4 Enzyme extraction from developing seedlings and ELISA Analysis:

0, 5, 12, 15 day, 8 week and 12 week 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, as expression level of *CCR* gene was very low (Q-PCR data). 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-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 Ll-CCRH2 protein. Experiment was repeated two times.

3.8.2.2.5 Immuno-cytolocalization of CCR protein in *L. leucocephala*:

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 seedlings grown in light in ½ strength MS were used to represent normal growing condition. Leaves were not considered for this experiment as quantity of lignin was estimated to be very low (ELISA). 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 Ll-CCRH2 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. Anti-rabbit-IgG-goat alkaline phosphatase conjugated antibody of 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 mix in 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).

3.8.2.2.6 Histochemical staining of lignin:

Solutions:

Phloroglucinol 2% in 95% Ethanol

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. 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).

3.8.2.2.7 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.

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.

3.9 Results:

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.

3.9.1 Spatio-Temporal expression pattern of *Ll-CCR*, Real-time PCR:

Temporal expression of *CCR* was carried out by isolating total RNA from 0, 5, 10 and 15 day developing seedlings and also 8 week and 12 week old plants. QRT-PCR results showed a gradual increase in *CCR* expression level in roots up to 15 days (Fig. 3.9.1) but decreased drastically in 8 week and 12 week old plants. Expression was maximum in 15 day old root samples.

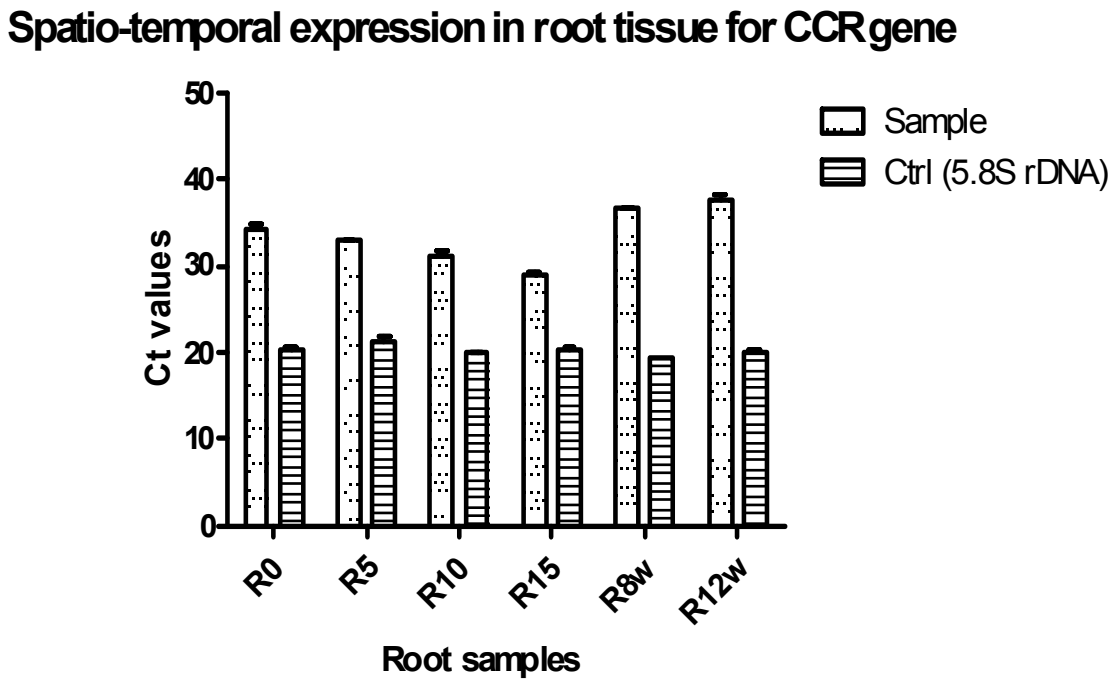


Fig. 3.9.1: Real Time PCR analysis for spatio-temporal *CCR* expression pattern in developing root of *L. leucocephala*: R0, R5, R10, R15, R8w, R12w are 0-15 day root, 8 week and 12 week old developing root tissue. Lower Ct value denotes higher amount of expression. A total of 1 µg RNA was used for QRT-PCR analysis. All values are plotted with standard deviation taken into account.

Stem exhibited detectable levels of expression on day 0 as compared to 5 day old plants but expression was maximum on the 10^{th} day. Detectable amounts of *CCR* expression were also observed in 8 week and 12 week old stem samples (Fig. 3.9.2).

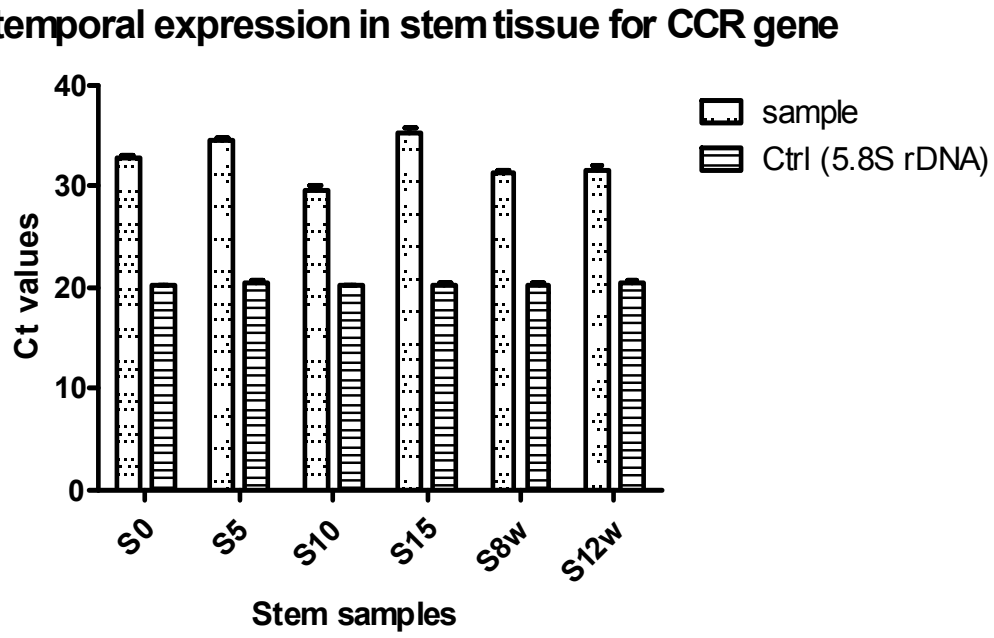


Fig. 3.9.2: Real Time PCR analysis for spatio-temporal *CCR* expression pattern in developing root of *L. leucocephala*: S0, S5, S10, S15, S8w, S12w are 0-15 day stem, 8 week and 12 week old stem of developing seedlings. Lower Ct value denotes higher amount of expression. A total of 1 µg RNA was used for QRT-PCR analysis. All values are plotted with standard deviation taken into account.

Very little to undetectable level of expression was noticed in leaf. *CCR* gene expression was highest in case of 15 day old developing leaf (Fig. 3.9.3). On comparing expression profile of leaf samples to root and stem, it was found that the expression is very low in case of leaf. Taking this data into account leaf was not used for estimating extractable CCR protein in developing seedling.

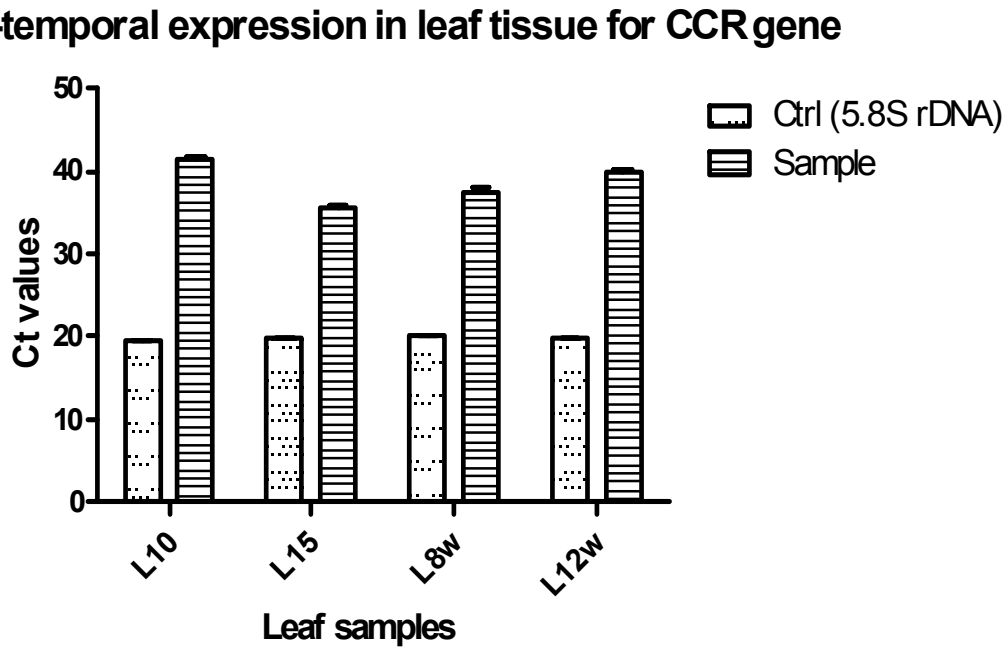


Fig. 3.9.3: Real Time PCR analysis for spatio-temporal *CCR* expression pattern in developing leaf of *L. leucocephala*: L10, L15, L8w and L12w are 10 day, 15 day, 8 week and 12 week old leaf tissue. A total of 1 µg RNA was used for QRT-PCR analysis. All values are plotted with standard deviation taken into account.

Low transcript level of *CCR* gene was observed in leaf, 8 week and 12 week old roots and stem tissue (Fig. 3.9.1; 3.9.2 and 3.9.3). The expression pattern of *Ll-CCRH2* was similar to that of previously recorded organ-specific activity patterns of *CCR*s isolated from other species (Piquemal *et al*., 1998; Ma, 2007; Lauvergeat *et al*., 2001; Pichon *et al*., 1998; McInnes *et al*., 2002).

3.9.2 Temporal expression pattern of *Ll-CCR*, ELISA analysis:

A standard graph for ELISA analysis was made using serial dilution of purified CCRH2 protein, which revealed that an OD_{405} of 0.0256 ± 0.0088 corresponds to 1 ng of purified CCR protein (Fig. 3.9.4). Graph was made using Graph Pad Prism software.

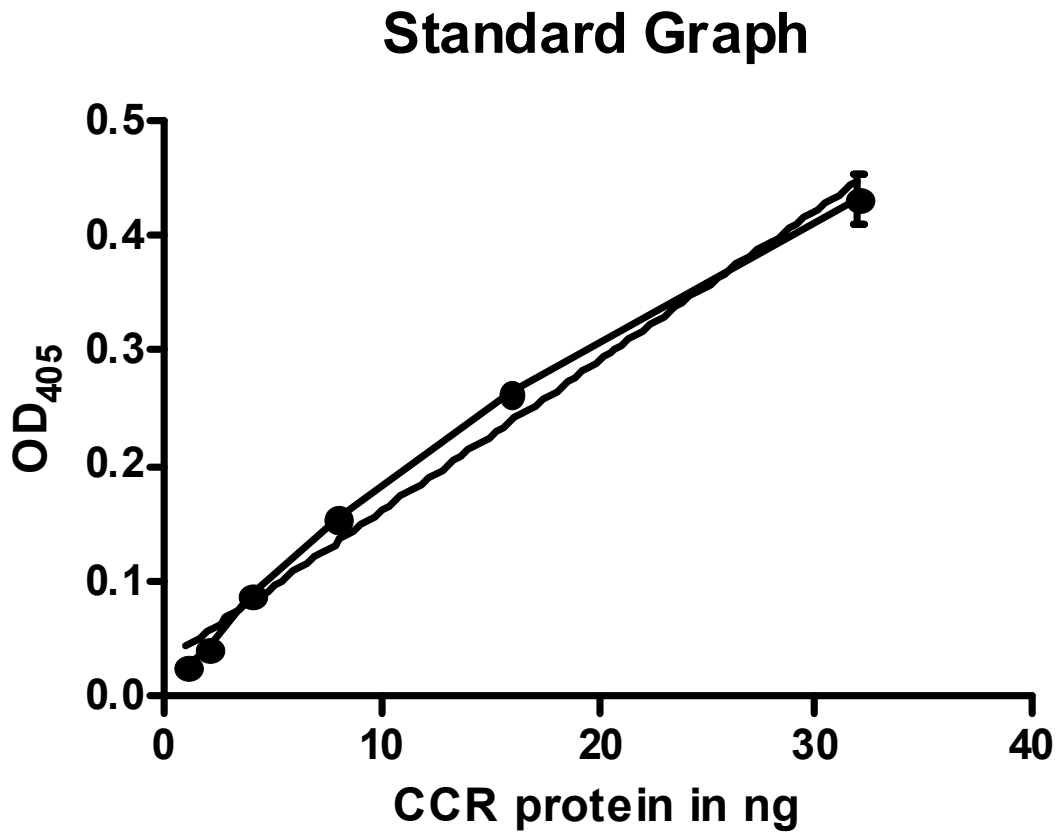


Fig. 3.9.4: Standard graph: An OD_{405} of 0.0256 ± 0.0088 represents 1 ng of extractable CCR protein.

R15 (15 day old root) showed maximum amount of extractable CCR protein and R8w (8 week old root) and R12w (12 week old root) had detectable amount of CCR protein (Fig. 3.9.5).

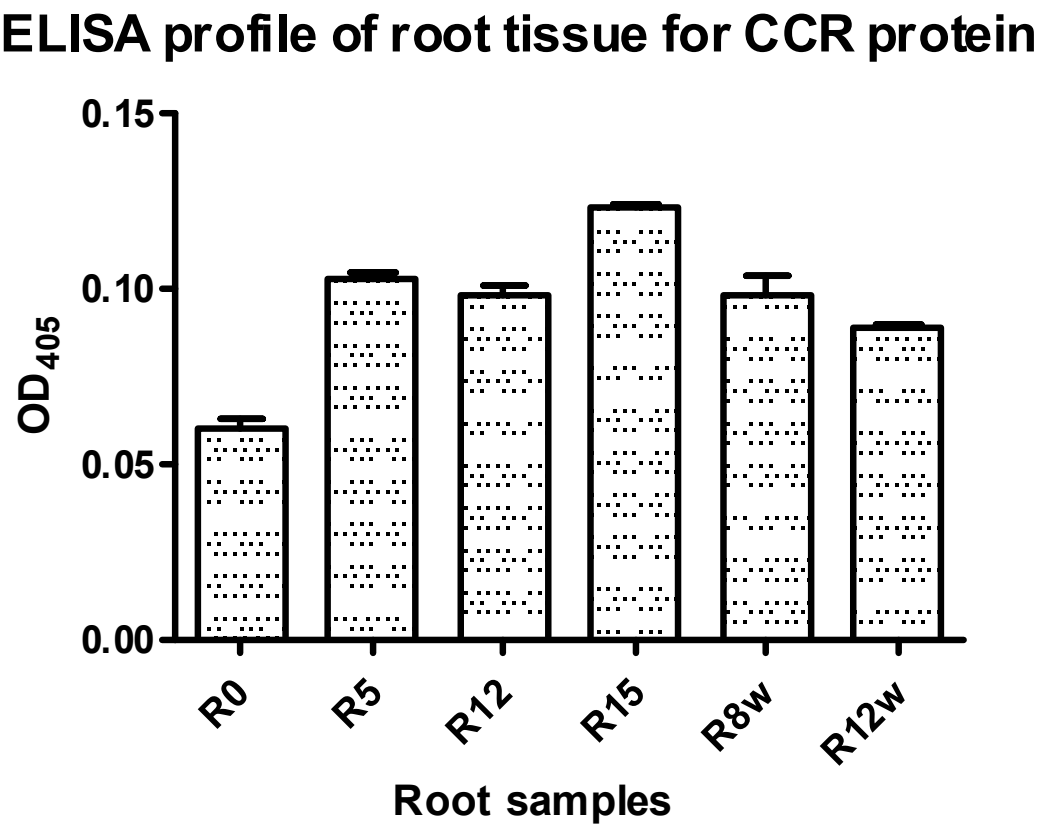


Fig. 3.9.5: ELISA profile for temporal expression of extracted CCR protein in growing seedlings of *L. leucocephala*: Values on y-axis represent O.D at 405 nm in ELISA assay using pNPP (*p*-Nitro phenyl phosphate) as substrate. It can be directly correlated with amount of extractable CCR protein in the tissue. R0, R5, R10, R15, R8w, R12w are 0-15 day root, 8 week and 12 week old developing root tissue. All values are plotted with standard deviation taken into account.

Expression of extractable CCR protein was highest in case of S0 (0 day stem sample) and lowest in S8w (Stem 8 week old) and S12w (12 week old stem), which was almost equal to S5 (5 day old stem) and S12 (12 day old stem) as shown in Fig. 3.9.6.

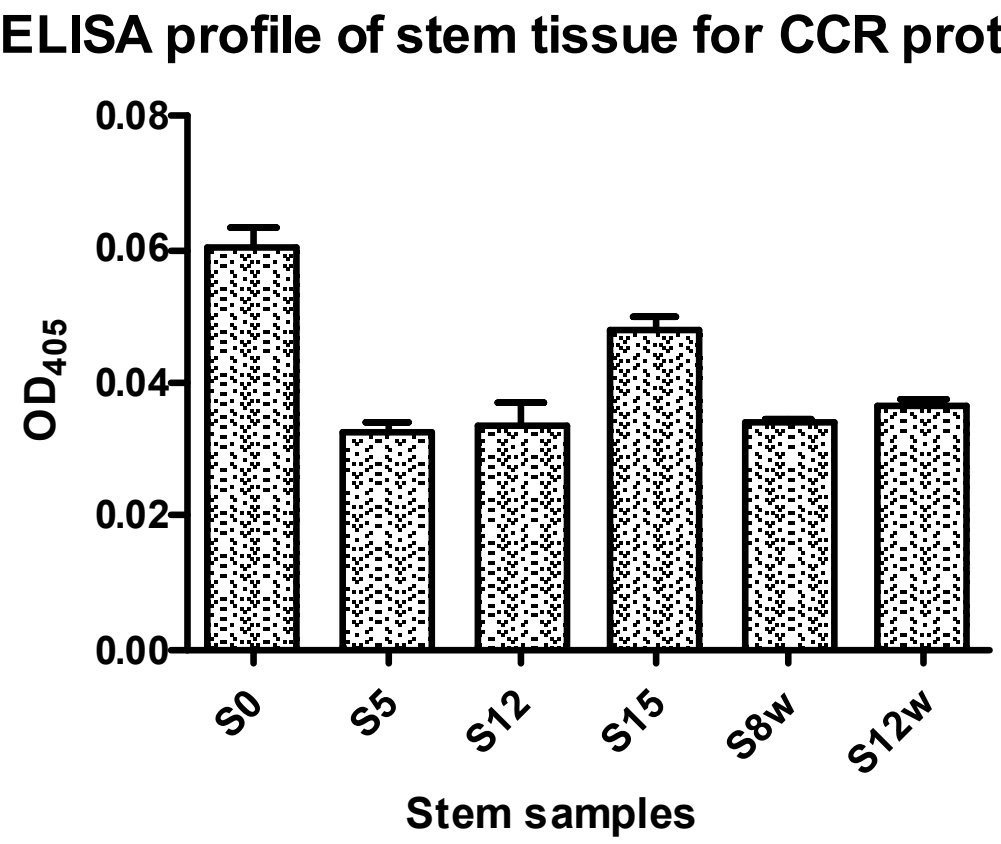


Fig. 3.9.6: ELISA profile for temporal expression of extracted CCR protein in growing seedlings of *L. leucocephala*: Values on y-axis represent O.D at 405 nm in ELISA assay using pNPP (*p*-Nitro phenyl phosphate) as substrate. It can be directly correlated with amount of extractable CCR protein in the tissue. S10, S15, S8w, S12w are 0-15 day stem, 8 week and 12 week old stem of developing seedlings. All values are plotted with standard deviation taken into account.

3.9.3 Immuno-cytolocalization:

CCR protein was immuno-cytolocalized in tissues undergoing active lignification *i.e* vascular bundle and xylem fibres. The deposition of blue-black to purple precipitate after incubating with BCIP/NBT mix confirms the presence of CCR protein near the sites of

lignification (Fig.3.9.7 A and B). 10 day and 15 day old root and stem samples were used for immuno-cytolocalization.

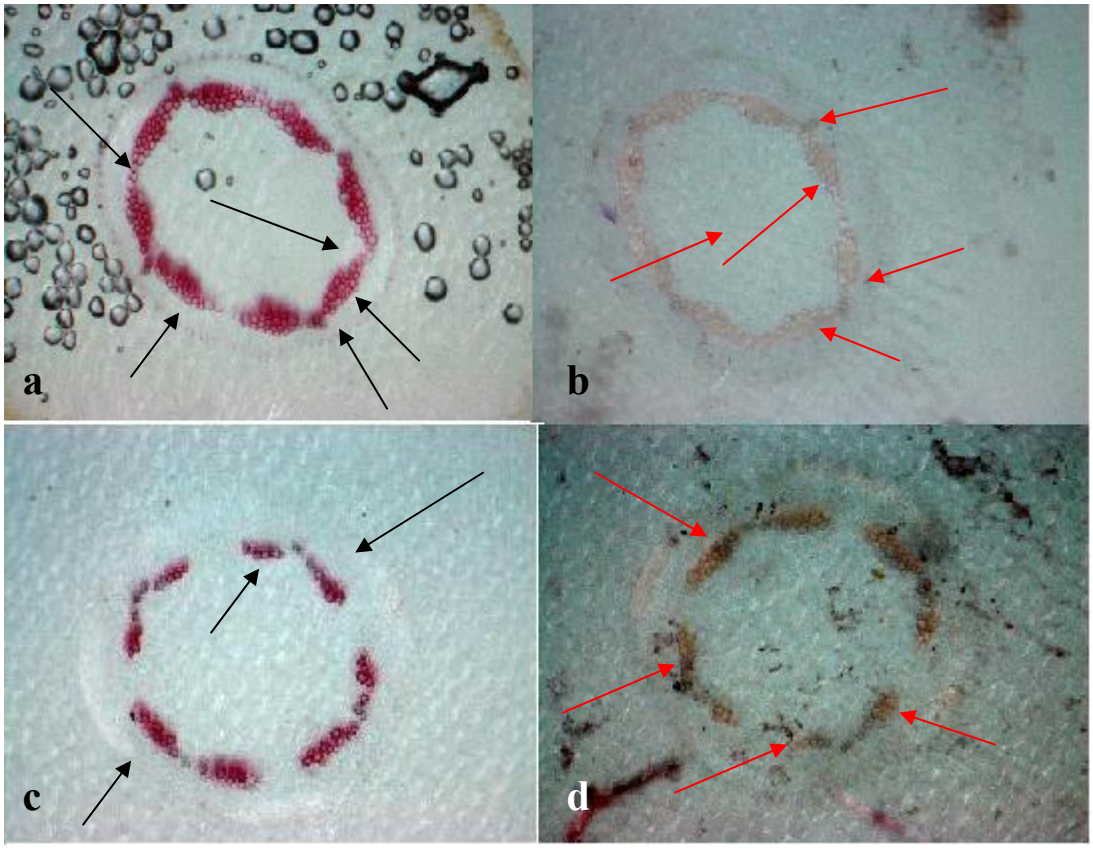


Fig. 3.9.7A: Immuno-cytolocalization of CCR protein in 10 day old growing seedlings of *L. leucocephala*: Panel **a** and **c**: phloroglucinol stained 10 day old stem and root sections of *L. leucocephala*, respectively. Panel **b** and **d**: immuno-cytolocalized 10 day old stem and root sections of *L. leucocephala*, respectively. Black arrow (panel a and c) denotes lignified xylem tissue and red arrow (panel b and d) denotes immuno-cytolocalized CCR protein in stem and root section of *L. leucocephala*, respectively.

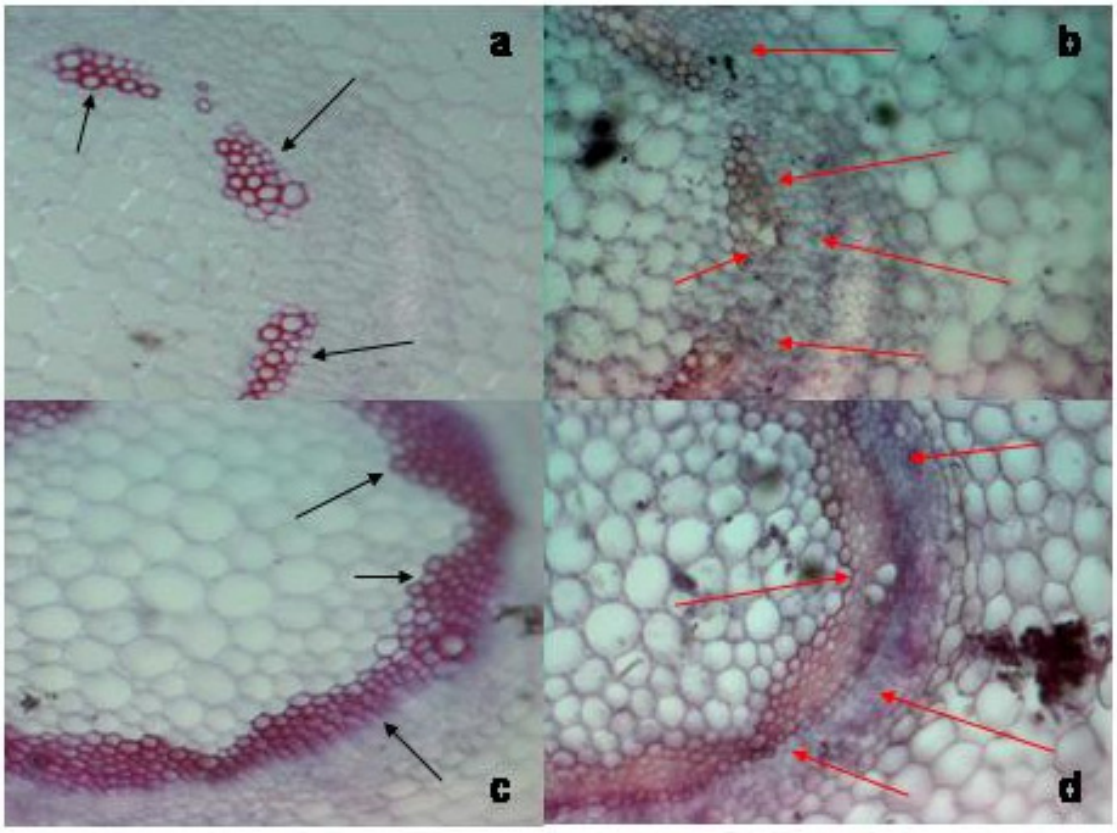


Fig. 3.9.7B: Immuno-cytolocalization of CCR protein in 15 day old growing seedlings of *L. leucocephala*: Panel **a** and **c**: phloroglucinol stained 15 day old stem and root sections of *L. leucocephala,* respectively.. Panel **b** and **d**: immuno-cytolocalized 15 day old stem and root sections of *L. leucocephala,* respectively. Black arrow (panel a and c) denotes lignified xylem tissue and red arrow (panel b and d) denotes immuno-cytolocalized CCR protein in stem and root section of *L. leucocephala*, respectively.

3.9.4 FTIR analysis:

FTIR analysis of 10 day and 15 day old root and stem of developing seedlings was done. FTIR analysis revealed that there is no qualitative difference in lignin composition of 10 & 15 day old developing stem (Fig. 3.9.8 A) and 10 & 15 day old developing root (Fig. 3.9.8 B). FTIR scan of commercial lignin was also performed to characterize signature peaks of Lignin (Fig. 3.9.8 A 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).

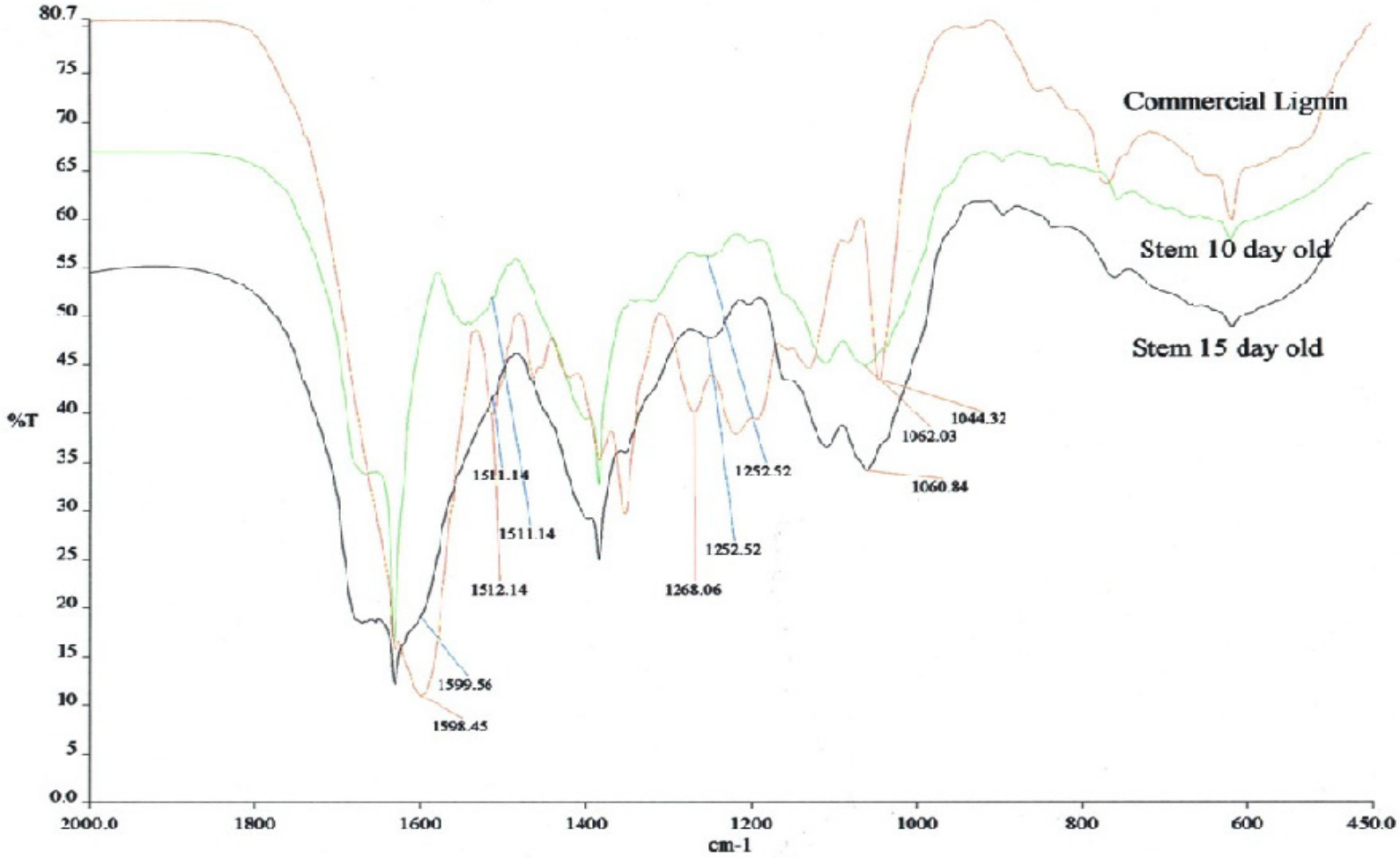


Fig. 3.9.8A: FTIR analysis of 10 & 15 day old developing stem: Peaks with wave number in the range of 1595 to 1599 cm^{-1}, 1040 to 1062 cm^{-1}, 1250 to 1270 cm^{-1} and 1510 to 1512 cm^{-1} are characterized to be signature peaks of monolignols and lignin. Signature peaks of commercial lignin are 1044 cm^{-1}, 1268 cm^{-1}, 1512 cm^{-1} and 1598 cm^{-1}.

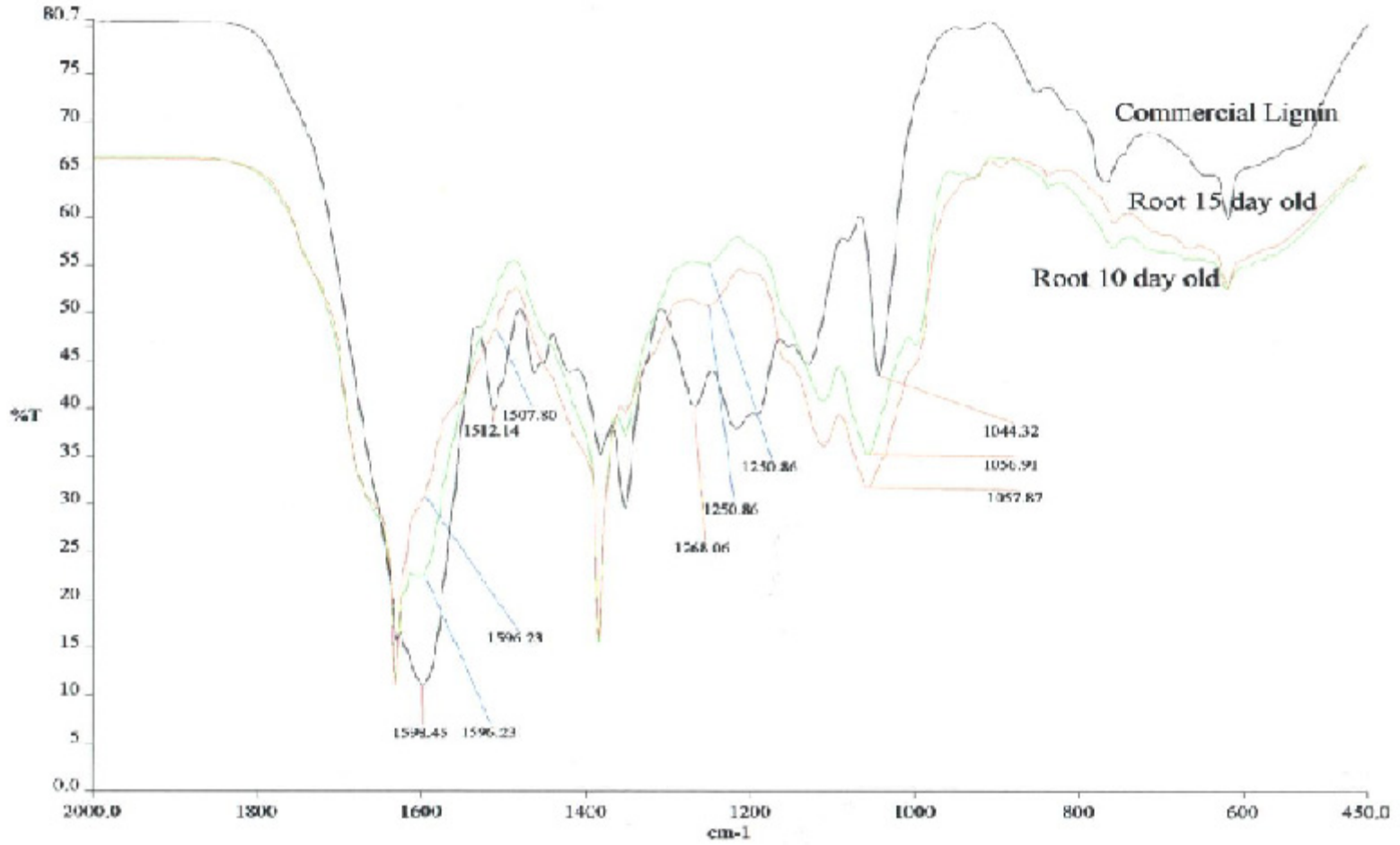


Fig. 3.9.8B: FTIR analysis of 10 & 15 day old developing root: Peaks with wave number in the range of 1595 to 1599 cm^{-1}, 1040 to 1057 cm^{-1}, 1250 to 1270 cm^{-1} and 1507 to 1512 cm^{-1} are characterized to be signature peaks of monolignols and lignin. Signature peaks of commercial lignin are 1044 cm^{-1}, 1268 cm^{-1}, 1512 cm^{-1} and 1598 cm^{-1}.

3.10 Discussion:

In this report, spatio-temporal expression of the *Ll-CCR* gene has been investigated in stem, root and leaf development for the first time. The expression pattern of *Ll-CCRH2* was monitored from 0-15 day of growing seedlings and also in mature plants (8 weeks and 12 weeks) using real-time PCR to throw some light on the role of *CCR* gene in plant development (Fig.3.9.1; 3.9.2; 3.9.3). The expression of *CCR* gene in root and stem was approximately the same and they followed similar pattern. Level of *CCR* transcript was found to increase till day 15 in case of root and 10 day in case of stem. Leaf tissue showed very low to negligible expression. Above data clearly states that the expression of *CCR* gene is mostly in developing parts (stem and root) of plant where lignification is in progress. As the plant grows from seedling, the level of expression of *CCR* gene also increases and this can be directly correlated to the developing xylem tissue inside the vascular bundle. Reports have shown that *CCR* gene is present mostly in tissues undergoing active lignifications and in case of *Leucaena CCR* activity increases from 0-15 day (stem and root) as lignification is also in progress at this time. But when the tissue matures, the *CCR* activity decreases and maintains a low level for normal physiological processes. Taking cognizance of the above data of spatio-temporal expression profile, it can be suggested that *Ll-CCRH2* is involved in constitutive lignification in developing tissues of developing seedling.

The expression of *Ll-CCRH2* transcripts during stem and root development was paralleled with extractable CCR enzyme quantity using ELISA based technique (Fig.3.9.5 and 3.9.6). Again the result supported the real-time PCR data. It was also found that the increase of *Ll-CCRH2* gene expression is not in proportion to the increase in Klason lignin (data not shown, very little to no increase between 10 day old to 15 day old seedling). 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). Therefore, a gradual increase in level of *CCR* transcript in root and in stem does not correlate with amount of lignin estimated for same period of time *i.e*. 10 and 15 day old seedlings. However, ELISA data for 10 and 15 day of stem and root showed direct

relation with amount of Klason lignin for same period of time as extractable CCR protein represents active CCR protein at that point of time.

This behavior of *CCR* gene in case of *Leucaena* was almost same as *CCR* I isoform from Wheat (Ma, 2007), *Arabidopsis* (Lauvergeat *et al.*, 2001) and *Eucalyptus* (Piquemal *et al*., 1998) which are proved to be involved in constitutive lignification. The almost exclusive expression of *Ll-CCRH2* 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 regulation 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.

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 suggests that similar quality of lignin is present in 10 and 15 day developing root and stem sample.

3.11 Conclusion:

Spatio-temporal expression profile was investigated using real-time PCR analysis. It was found that *Ll-CCRH2* gene transcript was expressed in developing stem and root specifically in xylem tissue. Increase in expression level was found from 0-15 days in root and 0-10 days in stem and then decreased gradually in 8 week and 12 week old developing seedlings. Extractable CCR protein from developing seedlings was harvested at same point of time as in case of real-time PCR experiment, which also revealed the same pattern of *CCR* gene expression. This data was comparable to *CCR I* gene expression pattern of studied plant systems such as wheat (Ma, 2007), *Arabidopsis* (Lauvergeat *et al.*, 2001) and *Eucalyptus* (Piquemal *et al*., 1998).

CCR protein was immuno-cytolocalized in tissues which are undergoing lignification.

Taking cognizance of above expression pattern and localization of CCR protein near lignifying tissue it can be stated that cDNA isolated and characterized in Chapter Two of this thesis is *cinnamoyl Co A reductase* gene of *L. leucocephala*.

3.12 References:

Freeman WM, Walker SJ, Vrana KE. 1999. Quantitative RT-PCR: pitfalls and potential. *Biotechniques* **26** (1), 112-125.

Lacombe E, Hawkins S, Doorsselaers JV, Piquemal J, Goffner D, Poeydomege O, Boudet AM, Grima-Pettenati J. 1997. Cinnamoyl-CoA reductase, the first committed enzyme of lignin branch biosynthetic pathway: cloning, expression, and phylogenetic relationships. *The Plant Journal* **11**, 429–441.

Lauvergeat V, Lacomme C, Lacombe E, Lasserre E, Roby D, Grima-Pettenati J. 2001. Two cinnamoyl-CoA reductase (*CCR*) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. *Phytochemistry* **57**, 1187–1195.

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 J, O'malley DM, Presnell T, Booker F, Campbell MM, Whetten RS, and Sederoff RR. 1997. Inheritance, gene expression, and lignin characterization in a mutant pine deficient in cinnamyl alcohol dehydrogenase. *PNAS* **94,** 8255–8260.

McInnes R, Lidgett A, Lynch D, Huxley H, Jones E, Mahoney N, Spanganberg G. 2002. Isolation and characterization of cinnamoyl-CoA reductase gene from perennial ryegrass (Lolium perenne L.). *Journal of Plant Physiology* **159**, 415–422.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology* **15**(3), 473-497.

Pfaffl MW. 2001. A new mathematical model for relative quantification real-time RT-PCR. *Nucleic acid research* **29** (9), 2002-2007.

Pichon M, Courbon I, Bechert M, Boudet AM, Grima-Pettenati J. 1998. Cloning and characterization of two maize cDNAs encoding cinnamoyl-CoA reducatase (*CCR*) and differential expression of the corresponding genes. *Plant Molecular Biology* **38**, 671–676.

Piquemal J, Lapierre C, Kmyton A O' Connell, Schuch W, G-Pettenatti J, Boudet AM. 1998. Characterization of a cinnamoyl-CoA reductase that is associated with stem development in *E. gunni*. *The plant journal* **13,** 71-83.

Zdunek-Zastocka E. 2008. Molecular cloning, characterization and expression analysis of three aldehyde oxidase genes from Pisum sativum L. *Plant Physiology and Biochemistry* **46,** 19-28.

Chapter Four

Antisense cloning of CCR gene, transformation of *L. leucocephala* and analysis of transformants

4.1 Introduction:

World wide annual production of paper has increased more than three fold in the past forty years, amounting to a total production of 330 Million Tones (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 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.

In different studies it has been found that transgenic tobacco (Elkind *et al.,* 1990; Chabannes *et al.,* 2001; Pincon *et al.,* 2001; Abbott *et al*., 2002) and *Arabidopsis* (Goujon *et al*., 2003) down regulated for *CCR* gene were characterized by an approximately 50% decrease in Klason lignin. The S/G ratio increased (mainly because of a decrease in the G unit amount) in transgenic tobacco and was variable, depending on the growth conditions, in transgenic *Arabidopsis* (Abbott *et al*., 2002; Goujon *et al.,* 2003 and Chabannes *et al.,* 2001). *CCR*-down regulated lines in tobacco were characterized by changes at the level of detoxification and carbohydrate metabolism. In addition, the transcript and metabolite data also suggested photo-oxidative stress and increased photorespiration (Dauwe *et al*., 2007). A change in lignin structure was also indicated by the higher amount of alkali labile material that could be released from the extractive-free lignin polymer of transgenic lines (O'connell *et al*., 2002). Transgenic tobacco plants with the lowest *CCR* activity and 50% reduced lignin showed abnormal phenotypes, such as important alterations in the fiber cell walls and loosening in the arrangement of the cellulose microfibrils that resulted in reduced cell wall cohesion (Elkind *et al.,* 1990; Pincon *et al*., 2001; O' connell *et al*., 2002). Among tree species very few plants systems have been studied for down regulation of *CCR* gene. In case of Norway spruce the antisense *CCR* plants had a normal phenotype but smaller stem widths compared to the transformed control plants. The transcript abundance of the sense *CCR* gene was reduced up to 35% relative to the transformed control and the corresponding reduction in lignin content was up to 8% (Wadenback *et al*., 2008). Recent studies indicated that down regulation of *CCR* gene in transgenic poplar (*Populus tremula* x *Populus alba*) was associated with up to 50% reduced lignin content and the reduced levels of lignin and hemicellulose were associated with an increased proportion of cellulose (Leple *et al*., 2007).

Also an increased amount of tyramine ferulate, 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.,*2008). 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 50% reduced lignin content, collapse of the vessels, and an altered growth and morphology. By crossing transgenic tobacco down-regulated for *COMT* with tobacco down-regulated for *CCR* (Piquemal *et al*., 1998), a simultaneous reduction in *COMT* and *CCR* expression was achieved (Pincon *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; Chabannes *et al*., 2001) or *CCR* (Piquemal *et al*., 1998) was downregulated (Chabannes *et al*., 2001). A simultaneous suppression of *COMT* (to 24% of wild-type level), *CCR* (to18% of wild-type level), 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).

Lignin biosynthesis genes especially *CCR* is being targeted to produce designer plants using antisense and siRNA technology with low lignin content. Down-regulation of various key genes has been achieved to obtain transgenic plants with low or modified lignin content. Hence, work was initiated with the objective of cloning and characterizing *CCR* cDNA in *Leucaena* and also for its antisense down-regulation. Significant amount of study has been done on various lignin biosynthesis genes in past ten odd years in various laboratories, around the world. Prime objectives were to increase forage value, proper utilization of biomass and to increase resistance against pathogens. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species.

In previous chapters isolation, cloning, functional expression and spatio-temporal expression pattern of *CCR* gene has been discussed in detail. It has been proposed that the two cDNAs isolated (*Ll-CCRH1* and *Ll-CCRH2*) from *L. leucocephala* are cinnamoyl Co A reductase isoforms, which catalyses the conversion of hydroxycinnamoyl CoA esters to their corresponding hydroxy-cinnamaldehyde. Enzyme kinetic studies have also revealed that the over-expressed protein in *E.coli* utilizes substrates like coniferaldehyde and sinapaldehyde, which again suggests that the cDNAs isolated from *L. leucocephala*, encodes for cinnamoyl Co A reductase.

Spatio-temporal expression profile also suggests that the cDNA isolated, encodes for cinnamoyl CoA reductase; since RT-PCR, ELISA and immuno-cytolocalization studies showed the presence of cDNA transcript and protein to be present in tissues undergoing lignifications. This also proves that the cDNAs isolated from *Leucaena* sp. are the main isoforms of *CCR* gene, which are responsible for constitutive lignification in *Leucaena* sp.

Above inference can be fortified if *CCR* down regulated *Leucaena* plants are found to produce low lignin and this can be achieved by transforming *Leucaena* plant using *Agrobacterium* infusion or particle bombardment method to obtain transgenic *Leucaena* plant. Dual purpose will be served by down-regulation of *CCR* gene in *L. leucocephala*: a transgenic could be obtained with low and altered lignin content, which can be utilized in paper and pulp industry and secondly, it will be proved that cDNA isolated from *Leucaena* encodes for isoform of *CCR* gene, which is involved in constitutive lignification.

In this Chapter, attempt to down-regulate *L. leucocephala* for *CCR* gene is described. Preliminary analysis of transformants was also done to confirm the integration of anti-sense *CCR* gene in *Leucaena* genome. Lignin estimation of these transgenic lines will be done in near future to join links between *CCR* gene down regulation and quantity and quality of lignin produced in transgenics.

4.2 Materials and Methods:

4.2.1 Materials:

Kanamycin, Hygromycin, Rifampicin and Acetosyringone was purchased from Sigma-Aldrich, USA. All other chemicals and solvents of analytical grade were purchased from HIMEDIA, Qualigens Fine Chemicals and E-Merck Laboratories, India. Pipette tips and micro centrifuge tubes were purchased from Axygen, India and Tarson, India. NEBlot kit (NEB, UK) was used for biotinylated probe preparation. Phototope star kit from NEB (UK) was used for detection of chemiluminescence. X-ray films were procured from Kodak, USA.

Glassware and plasticware: As discussed in Chapter Two.

Bacterial strain: *Agrobacterium tumefaciens* GV2260

Plasmid: pCAMBIA 1301 Binary plant transformation vector (CAMBIA)

Plant Material: Transformation

Tobacco explant:

Fresh leaves were taken from axenic cultures of *Nicotiana tabacum* var. Anand 119 and ~ 6 mm diameter disc were punched out.

***Leucaena leucocephala* explant:**

Axenic cultured embryo axes of *L. leucocephala* were taken as explant for transformation experiments.

Plant Material: DNA extraction and analysis of transformants:

Tobacco and *Leucaena* were selected on Hygromycin (LD_{50} determined in earlier experiments) selective media. These putative transformants were used for analysis.

4.2.2 Methods:

4.2.2.1 Bacterial culture conditions and transformation:

E. coli was transformed and maintained as discussed in Chapter 2 section 2.2.2.1 and 2.2.2.2. *Agrobacterium* was grown in YEP media (refer Appendix) containing appropriate antibiotic at 28 °C for 24 h with shaking at 200 rpm.

4.2.2.2 *A. tumefaciens* transformation and selection:

A single colony of *A. tumefaciens* (GV2260) was inoculated in 50 mL LB broth containing Rifampicin 150 µg/mL and incubated at 28 ºC with shaking at 200 rpm till A_{600} was 0.5. Cells were centrifuged at 5000 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 mL of ice cold 20 mM $CaCl_2$. Aliquots of 200 µL were made.

For transformation, 1 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, 1mL 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.

4.2.2.3 Determination of LD_{50} for Hygromycin and Multiple shoot regeneration of *Leucaena leucocephala*:

Seven day old axenic seedlings were used as a source for the embryo axes explants. Multiple shoot regeneration from embryo axes as explant was optimised in our lab. LD_{50} for Hygromycin in *L. leucocephala* was optimised in earlier experiments (15 mg/L). In brief, embryo axes explants were tested in presence of either TDZ or BAP in the medium. All tested concentrations induced multiple shoots from the cultured explants. The number of axillary shoots per explant increased from 4.08 ± 0.36 to 6.80 ± 0.40, with the increase of TDZ concentration from 0.045 μM to 0.227 μM. The number of shoots also increased with increasing concentration of BAP; but the number of shoots was less than those obtained with TDZ. NAA (0.54 μM) in the medium was adequate both for the shoot elongation as well as robust root regeneration. On this medium the shoots elongated and also developed roots within 10 days of transfer. The rooted plantlets were successfully acclimatized and out planted. TDZ concentration of 0.135 μM was found to be the best for multiple shoot induction and rooting of individual shoot. Hence, for all other experiments same concentration of TDZ was used. Same protocol was used for transgenic lines.

4.2.2.4 *Agrobacterium* mediated tobacco transformation:

The tobacco regeneration and transformation protocol is modified from Horsch *et al*. (1985). *Agrobacterium tumefaciens* strain GV2260 harbouring the binary plasmid vector to be transformed in tobacco was inoculated in 5 mL YEP media containing rifampicin (250 mg/ L) and kanamycin (50 mg/ L). The culture was allowed to grow O/N at 28 °C with shaking at 200 rpm. Next day, 1mL inoculum from this tube was added to 50 mL YEP flask containing appropriate antibiotics. The culture was incubated at 28 °C for 24 hours with shaking at 200 rpm. Fresh leaves were taken from axenic cultures of *Nicotiana tabacum* var. Anand 119 and ~ 6 mm diameter disc were punched out. The leaf discs were inoculated on MS medium agar plates containing 4.4 μM BAP and 5.37 μM NAA (MSBN) and kept for 48 h in dark. *A. tumefaciens* GV2260 cells grown for 24 h were centrifuged for 10 min at 5,000 g. Cells were

suspended in 20 mL of 50 mM $MgSO_4$ and 200 µM Acetosyringone and allowed to stand for 10 min with intermittent shaking. The tobacco leaf discs from axenic tobacco cultures were co-cultivated in this suspension for 10 min with intermittent shaking. The leaf discs were then transferred to MSBN plates without any antibiotics. The adaxial side of the leaf disc was in contact with the medium. The plates were incubated in dark at 28 °C. After two days the leaf discs were harvested, washed with liquid MSBN and dried on sterile filter paper. Up to 10 leaf discs per plate were transferred to MSBN agar plates containing Hygromycin (7.09 µM) and cefotaxime (200 µM). LD_{50} for every antibiotic was estimated in previous experiments. The cultures were incubated at 28 °C with 18/6 h photoperiod in diffused light (24.4 µmol/m^2/s). The leaf discs were subcultured every week till elongated shoots were excisable. Elongated shoots were excised and transferred to root induction medium. The rooted plants were hardened and further analyzed.

4.2.2.5 Genetic transformation of *Leucaena leucocephala*:

One day old embryo axes without cotyledons were used as explants for transformation. Seeds of *Leucaena* imbibed in distilled water after the treatment with concentrated sulphuric acid (7 min) and mercuric chloride (0.1 % for 10 min), were used as source of embryo axes. Embryo axes were excised from the seeds and inoculated on regeneration medium [1/2 strength MS + TDZ (0.5 mg/ L)]. The embryos were then used for transformation. LD_{50} for Hygromycin was estimated in previous experiments.

The transformation was carried out by three methods:

1) Particle bombardment
2) Particle bombardment followed by co-cultivation
3) Agro-infusion method

4.2.2.5.1 Particle bombardment:

Calculated amount (approximately 500 µg) of gold particle of 1µm size was used for bombardment. The gold particles taken in microcentrifuge tubes were washed with sterile water and supernatant was decanted after centrifuging 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. 1.0 µg of DNA (plasmid/cassette) 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 minutes. 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 6 cm. The embryo axes were arranged in the centre of the petridishes and bombarded with gold particles coated with DNA (plasmid/cassette). The explants were incubated in dark for 72 h after the bombardment.

4.2.2.5.2 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 pCAMBIA1301 containing *CCR* 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 strength 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 strength MS + TDZ (0.5 mg/ L)].

4.2.2.5.3 Agro-infusion method:

In this method, the *Agrobacterium* was introduced forcibly with a sterile needle to the meristematic region of the explants. After transformation, embryo axes were cultured on regeneration medium [1/2 strength MS + TDZ (0.5 mg/l)] without selection for one week. Then the axes were shifted to selection medium containing Hygromycin (10 mg/

L) for 3 weeks followed by selection on Hygromycin 15 mg/ L for another 3 weeks. The survived explants on Hygromycin (15 mg/ L) were shifted to 1/2 strength MS without Hygromycin selection. Cytokinin 2ip (2-isopentenyl adenine; 0.5 mg/ L) was used in the medium to have better elongation of transformed shoots.

4.2.2.6 DNA extraction from transgenic lines:

DNA was isolated using a modified protocol of Lodhi, *et al*., 1994. In short, one or two leaflets (*Leucaena*) and a piece of leaf (tobacco) were ground in 20 – 30 µL CTAB extraction buffer (Chapter 2, section 2.2.2.3) using glass pestle in a microfuge tube. 500 µL of extraction buffer was added and PVPP (polyvinyl polypyrolidone) was added to a final concentration of 0.2% and mixed gently. The tube was kept at 65 °C for 20 min with intermittent shaking. RNAse was added and kept for 10 more min at 65 °C. Tube was cooled and 400 µL of Chloroform: Isoamyl alcohol (24:1) was added and mixed gently. Tube was centrifuged at 6000 g at 4 °C for 10 min and supernatant was transferred to a fresh microfuge tube. Isopropanol (0.6 volume) was added and kept at room temperature for precipitation. Tube was again centrifuged at 6000 g at 4 °C for 10 mins to pellet DNA. DNA was washed with 70% cold ethanol, air dried and dissolved in 40 µL SMQ water.

4.2.2.7 ELISA (Enzyme-Linked Immunosorbent Assay):

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 (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 section 3.8.2.2.4.

4.2.2.8 Restriction digestion:

As discussed in Chapter Two, section 2.2.2.5.

4.2.2.9 Extraction and purification of DNA from agarose gels:

As discussed in Chapter Two, section 2.2.2.6.

4.2.2.10 PCR amplification, cloning and bacterial transformation:

As discussed in Chapter Two, section 2.2.2.9 and 2.2.2.2.

4.2.2.11 Slot blot analysis:

Slot blot analysis was done as discussed in section 2.2.2.10.2. Probe preparation and detection was done using NEBlot and Phototope star detection kit from NEB, UK according to manufacturer's recommendation.

4.3 Results:

4.3.1 Introduction of desired restriction site for antisense cloning:

For cloning *CCR* gene in antisense orientation, primers were designed with *Sac* I (forward primer) and *Kpn* I (reverse primer) restriction sites.

ASCCRF (*Sac* I) 5' GAG CTC CCT GCT GCC GCC CCA GCC 3'

ASCCRR (*Kpn* I) 5' GGT ACC CTT GGT GGG AAG AGG AAG A 3'

Plasmid pGEMCCRH2 was diluted 100 times and 1 µL was used as a template. PCR was performed as described in section 2.2.2.9:

PCR cycling condition:

No. of cycles	Temperature	Time
1	95 ºC	5 min
35	95 ºC 55 ºC 72 ºC	1 min 30 s 1min
1	72 ºC	7 min
1	4 ºC	Hold

A 1.0 kb band (antisenseCCRH2: *asCCRH2*) was amplified. Band was cut, purified, ligated in pGEM-T easy vector and transformed in *E.coli* XL1 MRF cells. Clones with *asCCRH2* fragment with *Kpn* I and *Sac* I restriction sites were screened by inoculating few colonies in 5 mL LB (Ampicilin 100 µg/mL). Isolated individual plasmids were restriction digested to confirm the integration of *asCCRH2* insert and sequenced.

4.3.2 Directional cloning of *CCRH2* in pCAMBIA 1301 MCS:

The above clone in pGEM-T easy vector with *Sac* I and *Kpn* I restriction site was designated as pKpnSacCCRH2. The clone with restriction sites was digested with *Kpn* I and *Sac* I restriction enzymes. A one kb restriction digested fragment (*asCCRH2*) was purified. Prior to this, pCAMBIA 1301 was modified in our lab by cloning a cassette of CAM 35 S promoter and NOS terminator within *Eco* RI and *Hind* III site of pCAMBIA 1301. This cassette also contained a *Kpn* I (near CAM 35 S) and *Sac* I (near NOS terminator) restriction sites. The purified *asCCRH2* fragment was directionally cloned in reverse orientation in *Kpn* I and *Sac* I site of the cassette (Fig. 4.3.1). Ligation mixture was transformed in *E.coli* XL1 competent cells and plated on LB-KAN (Kanamycin 50 µg/mL) plate. Colonies for recombinant plasmids were screened by colony PCR method (Fig. 4.3.2).

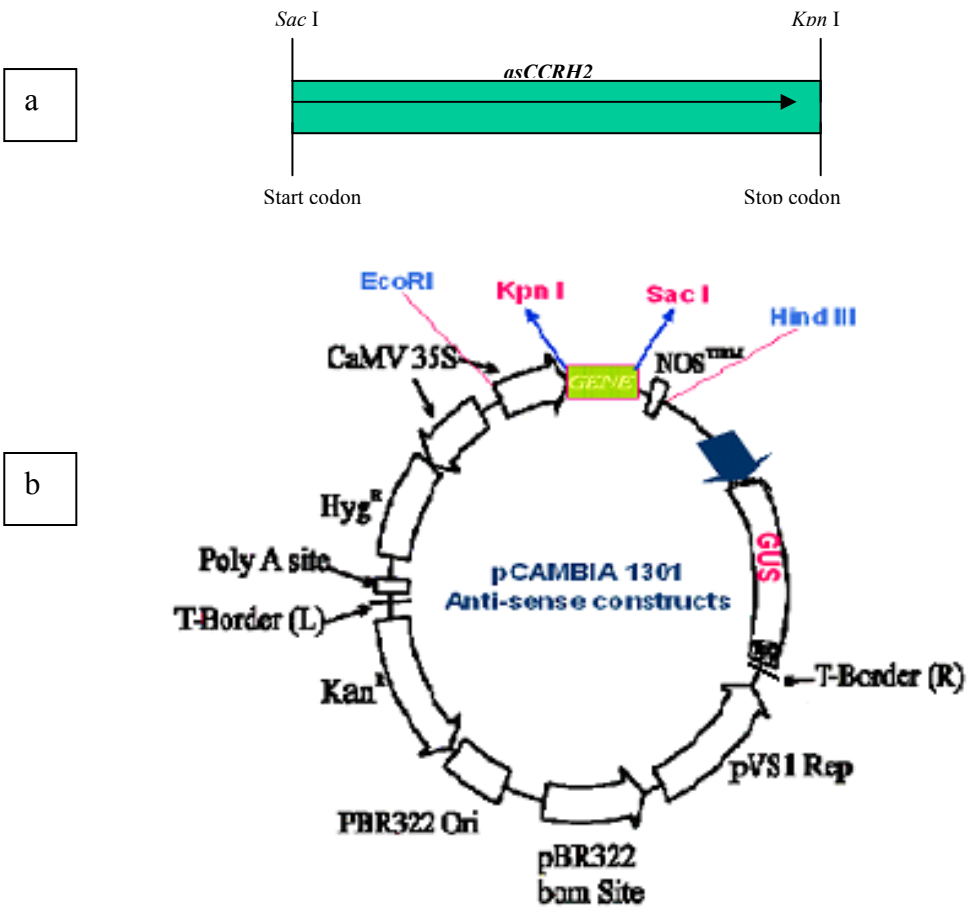


Fig 4.3.1: *CCRH2* gene in antisense orientation in pCAMBIA 1301: a: *asCCRH2* fragment; b: pCAMasCCRH2.

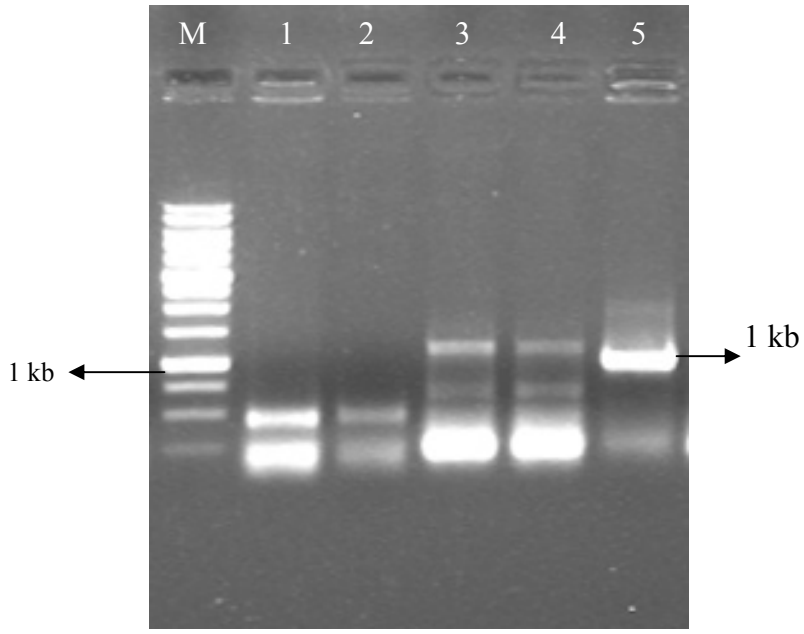


Fig. 4.3.2: Colony PCR: Lane 1 to 5 PCR of colonies screened. Lane M: 1 kb ladder (Bioenzymes, USA). Lane 5: Positive clones.

Positive clones were inoculated in 5 mL LB (Kanamycin 30 µg/mL). Plasmid was isolated and recombinant plasmids were confirmed by restriction digestion to release a 1 kb fragment from pCAMBIA 1301 (Fig. 4.3.3). The recombinant pCAMBIA vector was sequenced to further validate and was designated as pCAMasCCRH2. Cassette is given in Fig. 4.3.4. The plasmid pCAMasCCRH2 was isolated in bulk and was used in two ways:

1. Coating of gold particle for particle bombardment and
2. Mobilising in *Agrobacterium* for co-cultivation.

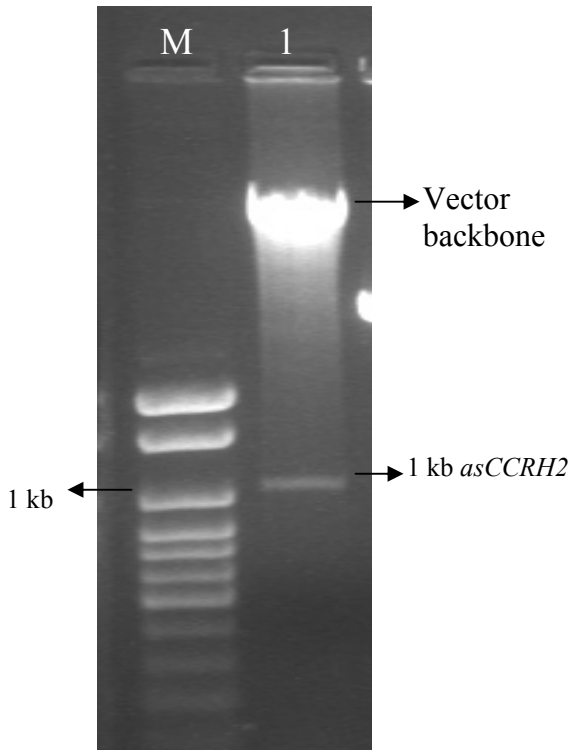


Fig. 4.3.3: Restriction digestion of pCAMasCCRH2 with *Sac* I and *Kpn* I restriction enzymes: Lane M – Marker, Lane 1- 1 kb insert of *asCCRH2*.

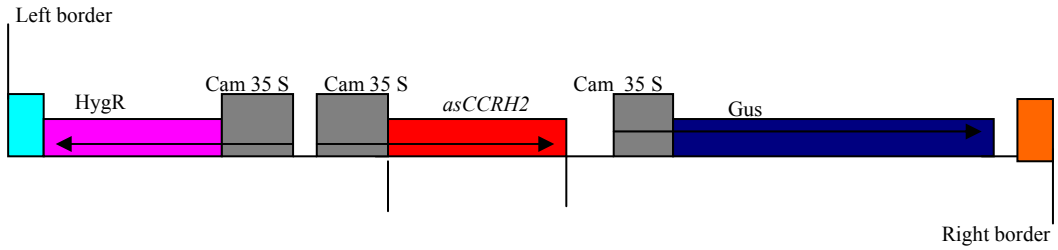


Fig. 4.3.4: Antisense cassette for *CCRH2* gene in pCAMBIA1301.

4.3.3 Mobilizing pCAMasCCRH2 in *Agrobacterium tumefaciens* GV2260:

Plasmid was mobilized in competent *Agrobacterium* according to the protocol discussed in section 4.2.2.2 of this chapter.

4.3.4 Transformation of Tobacco:

Leaf disc of Tobacco was used as explant for transformation experiment. Tobacco was used as a standard system for transformation (Fig. 4.3.5). After selection on Hygromycin (5 mg/mL) supplemented media, plants were transferred to Hygromycin free media. They were transferred to plastic cups with autoclaved soil and kept in

culture room for few days before transferring to green house. Survived plants were like normal plants.

4.3.5 Analysis of Tobacco transformants:

Tobacco was used as a standard transformation system. Transformation of tobacco was done according to the protocol given by Horsch, *et al*., 1985. Approximately 6 plants survived before transferring to plastic cups. Primary screening for putative transformants was done for integration of Hygromycin phosphotransferase and *asCCRH2* gene by following methods.

1. PCR based screening using Hygromycin phosphotransferase gene specific primers.
2. Slot blot of putative PCR positives obtained from Hygromycin phosphotransferase gene primers.
3. ELISA of extracted CCR protein in transgenic lines of Tobacco.

4.3.5.1 PCR based screening using Hygromycin phosphotransferase gene specific primers:

DNA was isolated from plants, which survived on Hygromycin supplemented media. PCR was performed to amplify Hygromycin phosphotransferase gene from putative transgenic lines using Hygromycin phosphotransferase gene specific primers.

Hygromycin primer set B:
HygBF 5'-GTCGACCTATTTCTTTGCCCTCGGAC-3'
HygBR 5'-GGATCCCCTGACCTATTGCATCTCCC-3'

A band of ~900 bps was amplified in putative transformants (Fig. 4.3.6). Two putative transformants of tobacco were characterized to be Hygromycin phosphotransferase gene positive. Further validation of these plants was done using Slot blot analysis.

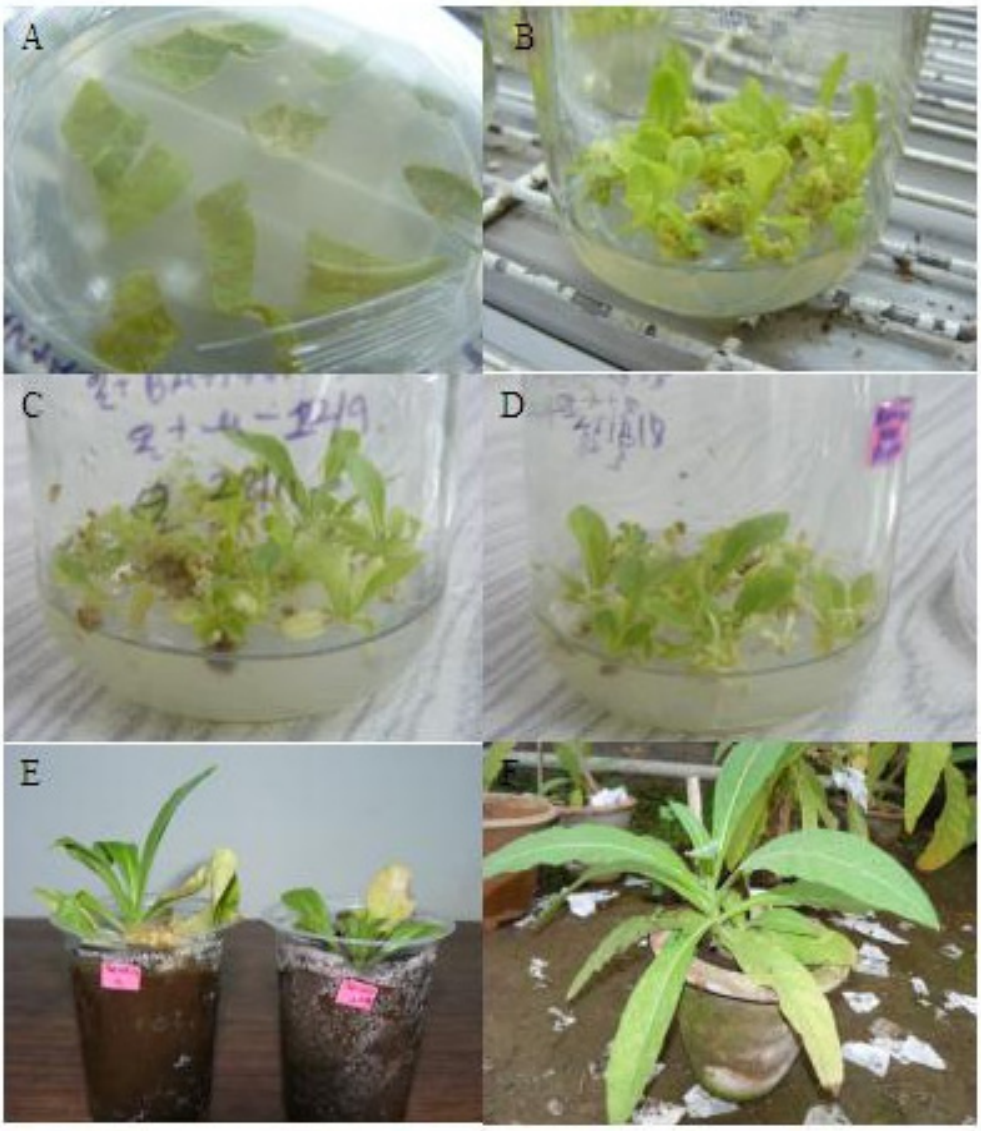


Fig. 4.3.5: Transformation of Tobacco: A: Leaf disc as an explants; B and C: selection on Hygromycin supplemented medium; D: On Hygromycin free medium; E: Transferred in plastic cups; F: hardened in green house.

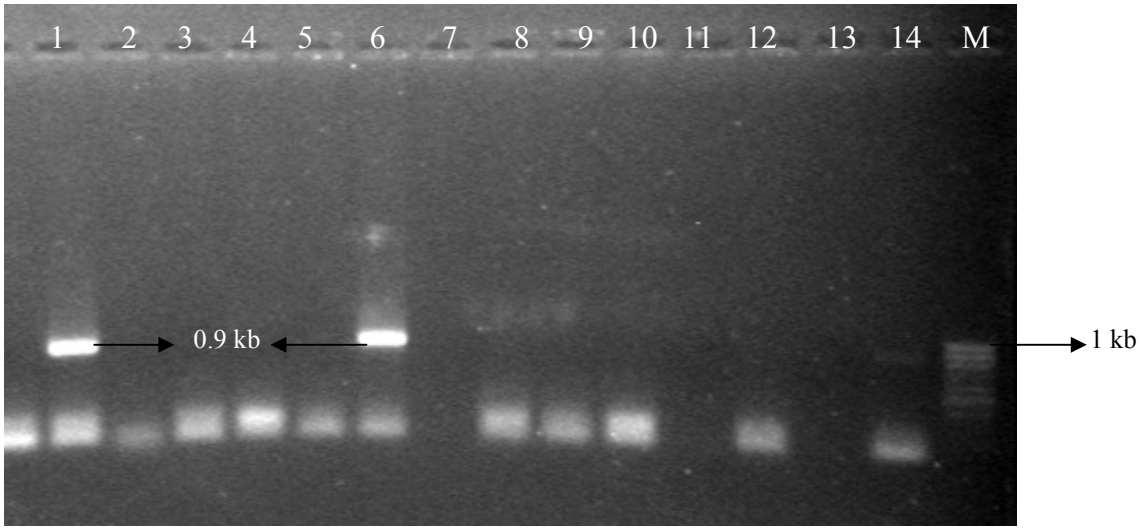


Fig. 4.3.6: PCR positive transgenic lines of tobacco: PCR amplification of integrated Hygromycin phosphotransferase gene. Lane 1 and 6, PCR positive tobacco transformed plants 3 and 4 respectively.

4.3.5.2 Slot blot analysis using Hygromycin phosphotransferase gene as a probe: Slot blot was done using ~900bp Hygromycin phosphotransferase gene amplicon as a probe. NEBlot kit (NEB, UK) was used for biotinylated probe preparation. Phototope star kit from NEB (UK) was used for detection of chemiluminescence. Hybridization was done at 60 °C. Non-transformed tobacco was used as control (Fig. 4.3.7).

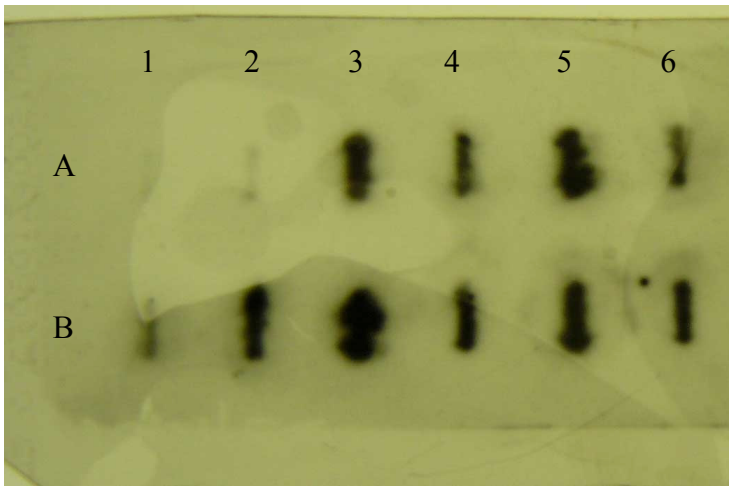


Fig. 4.3.7: Slot blot of putative tobacco transformants: Lane A1 and A2 tobacco, non-transformed. Lane A3, A4: tobacco 3, Lane A5, A6: tobacco 5, Lane B1, B2: tobacco 2, Lane B3, B4: tobacco 4, Lane B5, B6: Tobacco6.

Putative transformant showed positive band in slot blot hybridisation. This indicates the integration of Hygromycin phosphotransferase gene in genome of tobacco. These plants were further analysed by ELISA based experiment.

4.3.5.3 ELISA analysis:

Four transformants of tobacco were tested for amount of extractable CCR protein. ELISA was performed from extractable CCR protein from putative transformants as described in section 3.8.2.2.4. Total of 10 μg protein was coated in each well in triplicates. Non-transformed tobacco was used as a control. ELISA was done for putative tobacco transformants that gave good result in PCR and slot blot along with other transgenic lines. Primary antibodies and secondary antibodies were used at a dilution of 1:5000 and 1:10000 respectively (Fig. 4.3.8).

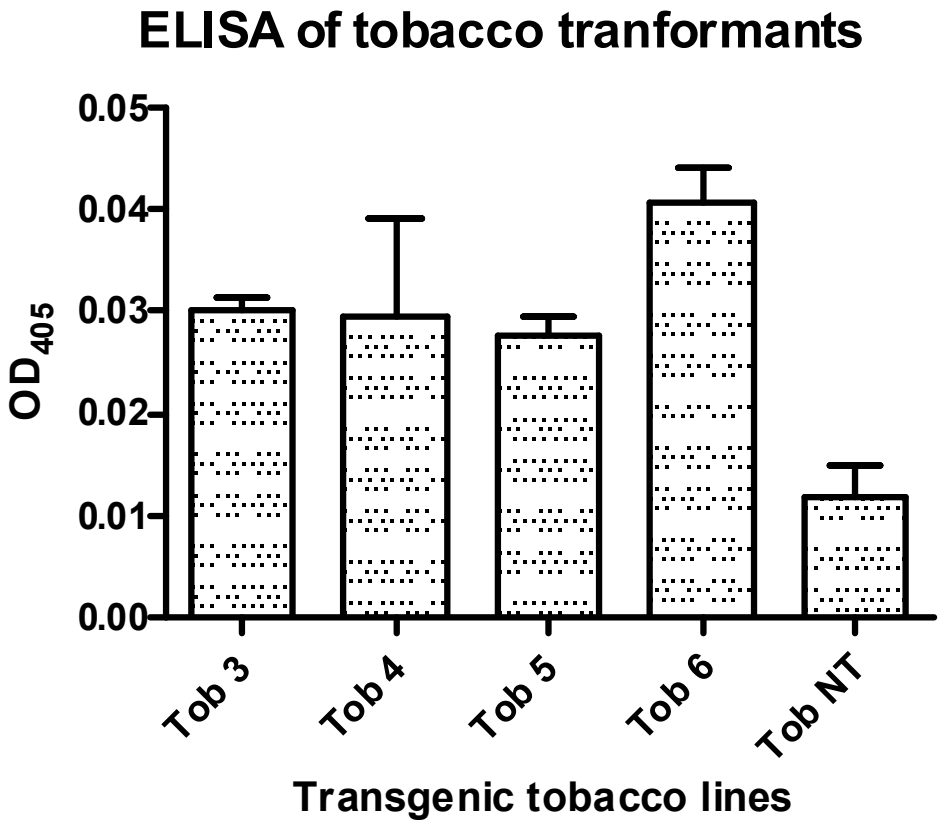


Fig. 4.3.8: ELISA of extractable CCR protein from transgenic line of Tobacco: Tob 3, Tob 4, Tob 5, Tob 6 are Tobacco 3, Tobacco 4, Tobacco 5, Tobacco 6 putative tobacco transformants. Tob NT is tobacco non-transformant.

Putative transformant of tobacco did not give satisfactory result. Extractable CCR protein was lower in case of non-transformed tobacco plant than putative transformed tobacco plant. This result suggests that the putative transformed plants did not show down regulation of *CCRH2*.

4.3.6 Transformation of *Leucaena*:

Embryo axes of *Leucaena* were used as explant for transformation experiment. After selection on Hygromycin (15 mg/mL) supplemented media, plants were transferred to Hygromycin free media. They were transferred to plastic cups with autoclaved soil and kept in culture room for few days before transferring to green house. Out of 159 plants which survived on Hygromycin supplemented media, 76 plants showed elongation on rooting media (Table 4.1). Survived plants were like normal plants with weak stem and stunted growth (Fig. 4.3.9). Leaves were normal in size when compared to non-transformed plant. Putative transformed plants when attained stem height of approximately 5-6 cm were transferred to greenhouse. Growth of putative transformed plants was observed to be less when compared to non-transformed plant.

4.3.7 Analysis of *Leucaena* transformants:

Primary screening was performed to check the integration of Hygromycin phosphotransferase gene and *asCCRH2* gene. Out of 76 putative transformants, DNA of 34 plants was isolated which were approximately of same height (5 to 6 cm). Other plants were very weak and they needed approximately six more months to grow for these analysis. As these plants were very small in size, ELISA was performed for few selected putative transformants. Primary screening for putative transformants was done for integration of Hygromycin phosphotransferase gene and *asCCRH2* gene by following methods.

1. PCR based screening using Hygromycin phosphotransferase gene specific primers.
2. Slot blot of putative PCR positives obtained from Hygromycin phosphotransferase gene primers and
3. ELISA of extracted CCR protein in transgenic lines of *Leucaena.*

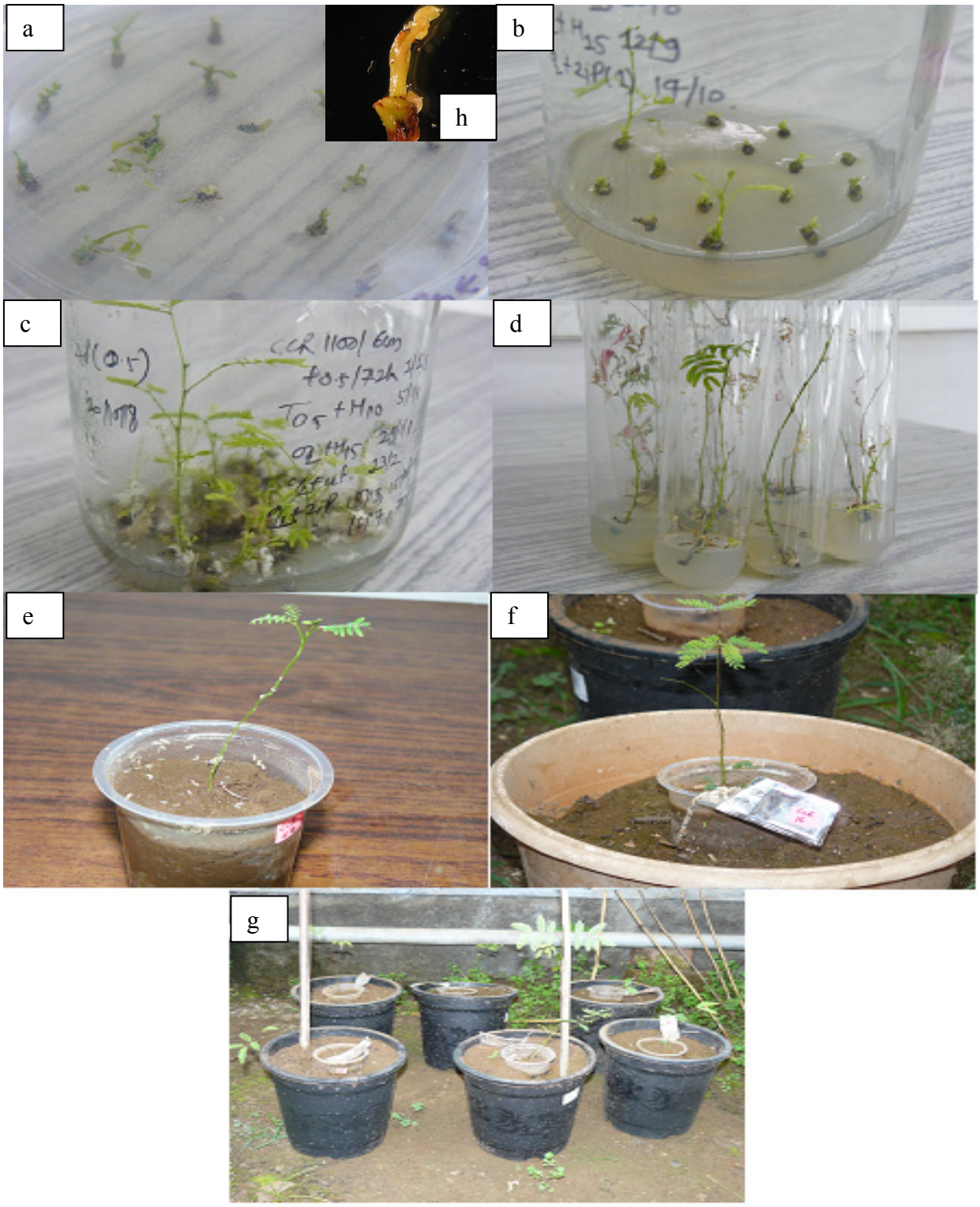


Fig. 4.3.9: Transformation of *Leucaena*: a, b and c: On Hygromycin selection media; d: On Hygromycin free media; e, f and g: Hardening process; h: explant.

4.3.7.1 PCR based screening of *Leucaena* transformants:

PCR was performed to amplify Hygromycin phosphotransferase gene from transgenic lines using Hygromycin phosphotransferase gene specific primers.

Hygromycin primer set B:
HygBF 5'-GTCGACCTATTTCTTTGCCCTCGGAC-3'
HygBR 5'-GGATCCCCTGACCTATTGCATCTCCC-3'

A band of ~900 bp was amplified in transformants (Fig. 4.3.10 and Fig. 4.3.11). Seven putative transformants of *Leucaena* were characterized to be Hygromycin phosphotransferase gene positive. A total of 34 plants were used for this experiment.

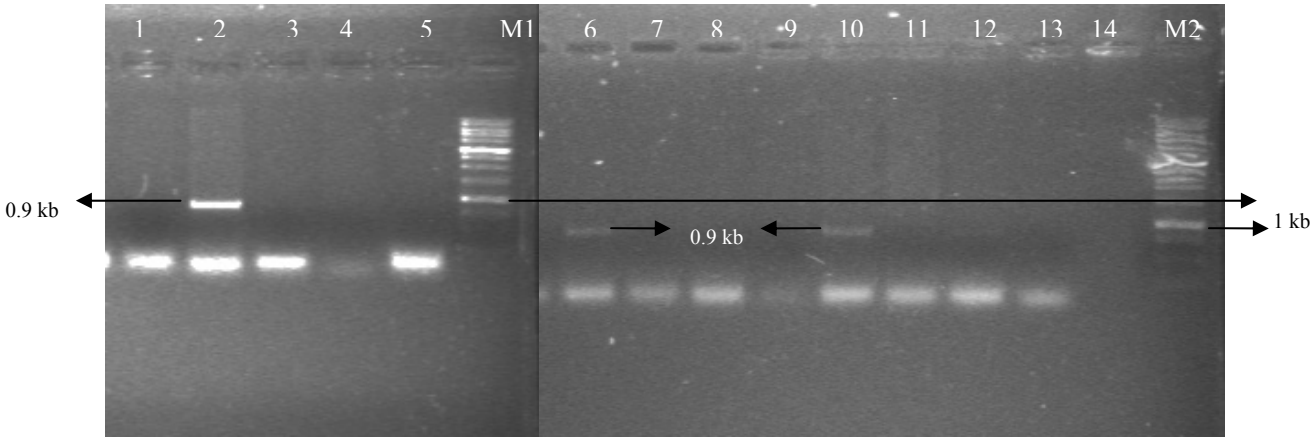


Fig. 4.3.10: PCR positive transgenic lines of *Leucaena*: PCR amplification of integrated Hygromycin gene in *Leucaena*. Lane 2, Lane 6 and Lane 10 are PCR positive ~900 bp fragment. Lane M1 and Lane M2 is DNA ladder (Bangalore Genei, India).

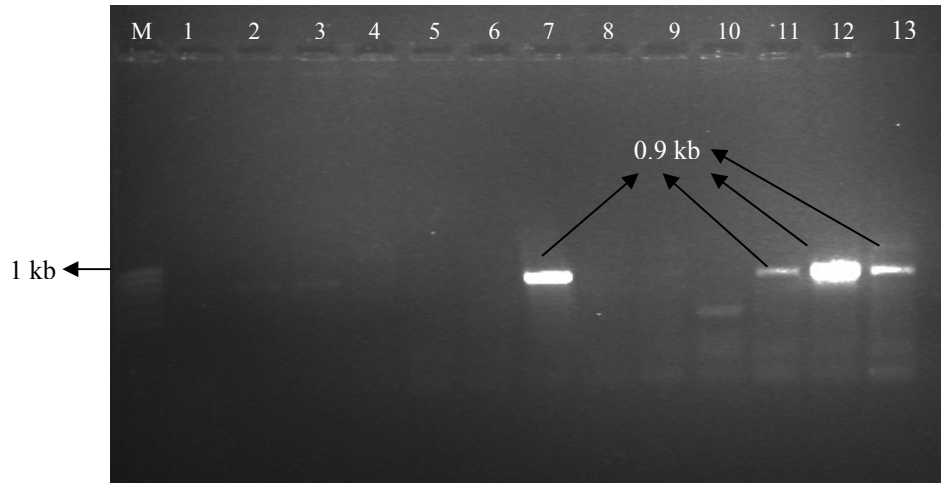


Fig. 4.3.11: PCR positive transgenic lines of *Leucaena*: Lane 7, 11, 12 and 13 are PCR positive (~900 bp fragment). Lane M: 100 bp ladder (Bangalore Genei, India).

900 bp fragment was cloned in pGEM-T easy vector and sequenced. The sequence showed significant similarity with Hygromycin phosphotransferase gene, which is an evidence of integration of Hygromycin phosphotransferase gene in *Leucaena* genome.

Out of 34 putative transgenic lines, seven plants showed PCR positive for Hygromycin phosphotransferase gene. These transformed plants were designated as Leu 14a, Leu 38, Leu 42, Leu 44, Leu 60, Leu 68 and Leu 30. Further analysis of transformants to confirm the integration of Hygromycin phosphotransferase gene was done using slot blot hybridisation.

4.3.7.2 Slot blot analysis using Hygromycin phosphotransferase gene as a probe:
Slot blot was done using slot blot apparatus from Hoefer scientific, USA. DNA from five different transformed plants along with non-transformed control plant was used for slot blot analysis. ~900 bp purified fragment of Hygromycin phosphotransferase gene was used as a probe. Pre-hybridization and hybridisation was performed at 60 °C (Fig. 4.3.12).

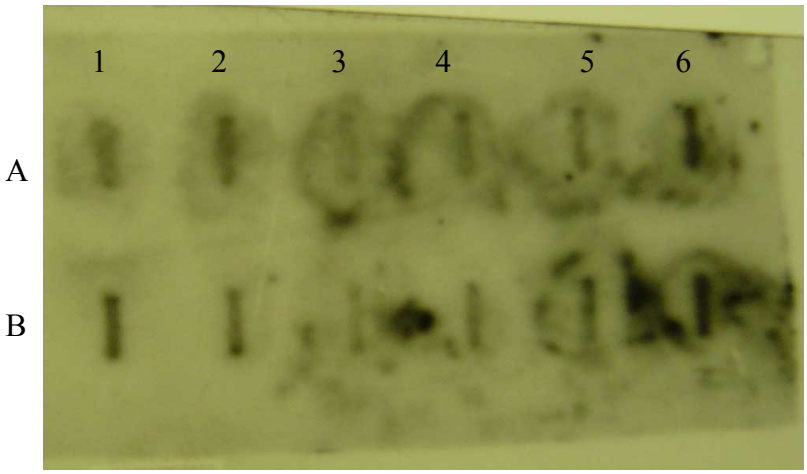


Fig. 4.3.12: Slot blot of putative *Leucaena* transformants: Lane A1 and B1: Leu 14a; Lane A2, B2: Leu 38; Lane A3, B3: Leu non-transformed; Lane A4, B4: Leu 60; Lane A5, B5: Leu 44; Lane A6, B6: Leu 68.

4.3.7.3 ELISA analysis:

Six transgenic lines of *Leucaena* were tested for amount of extractable CCR protein. ELISA was performed from putative transformants as described in section 3.8.2.2.4. Total of 10 µg protein was coated in each well in triplicates. Non-transformed tobacco was used as a control. ELISA was done for putative tobacco transformants that gave good result in PCR and slot blot along with other transgenic lines. Primary antibodies and secondary antibodies were used at a dilution of 1:5000 and 1:10000 respectively.

ELISA analysis of putative transformants showed a decrease in extractable CCR protein than non-transformed *Leucaena*, which suggests lowering of *CCR* activity in putative transformants. This data indicates the integration of *asCCRH2* gene in *Leucaena* genome (Fig. 4.3.12).

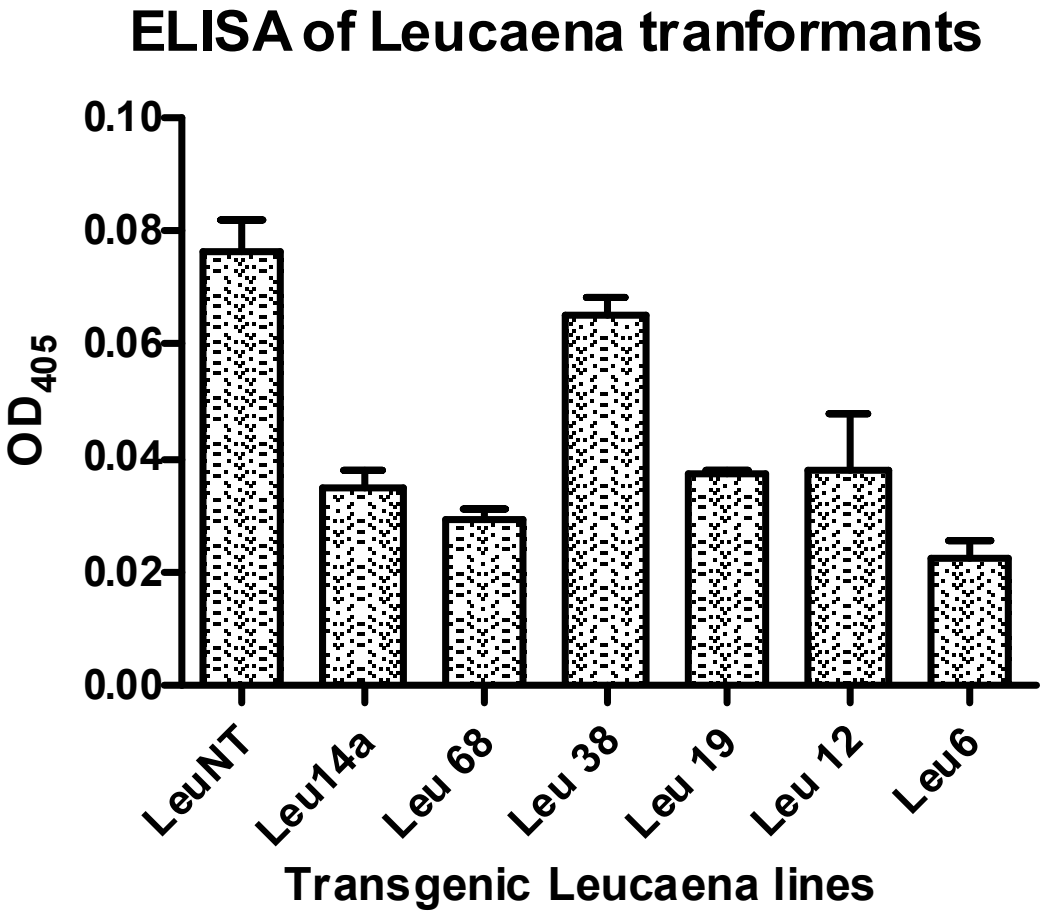


Fig. 4.3.12: ELISA of extractable CCR protein from transgenic line of *Leucaena*: Leu 14a, Leu 68, Leu 38, Leu 19, Leu 12, Leu 6 and Leu NT are *Leucaena* 14a, 68, 38, 19, 12, 6 and non-transformed transgenic lines of *Leucaena* respectively.

To conclude, Tobacco 3 and 4 were found to be positive for Hygromycin phosphotransferase gene (PCR method and Slot blot hybridisation.). Unfortunately, ELISA for extractable CCR protein did not show any convincing result. In case of *Leucaena*: putative transformants 14a, 68, 38, 44, 60, 42 and 30 showed PCR positive and Slot blot positive for Hygromycin phosphotransferase gene integration. ELISA of extractable CCR protein showed that 14a, 68, 38, 19, 12, and 6 have lower level of CCR protein expression as compared to the non-transformed plants, which was according to our expectations.

Table 4.1: Details of the number of *Leucaena* embryo axes used for transformation studies and the PCR positives

Gene	Method used	Number of embryo axes used	Number of explants survived on selection (Hygromycin 15 mg/L) medium	Number of shoots elongated	Average Shoot length (cm)	Number of shoots used for DNA extraction	Number of samples shown PCR positive	Probable Transformation efficiency (%)
asCCR	Particle bombardment	67	59	30	3.67	8	4	5.97
asCCR	Particle bombardment +co-cultivation	107	94	40	2.97	20	2	> 2
asCCR	Agro infusion	43	6	6	2.07	6	1	> 3
	Total	**217**	**159 (73.3%)**	**76**	**-**	**34**	**7 (20.5%)**	**3.22**

4.4 Discussion:

The research work presented in this thesis was started with a backdrop of paper and pulp production in India. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. Lignin biosynthesis genes are also being targeted to produce designer plants using antisense and siRNA technology with low lignin content. Down-regulation of various key genes has been achieved to obtain transgenic plant with low or modified lignin content. Hence, work was initiated with the objective of cloning and characterizing *CCR* cDNA clones in *Leucaena* and also for its down-regulation to raise transgenic lines for proper utilization in paper industry.

Transformation protocol for *Leucaena* and Tobacco was standardized. Transformants were analysed using PCR, Slot blot and ELISA based experiments. PCR and Slot blot based approach was used for primary screening of transformants. Two transgenic lines in tobacco and seven transgenic lines in *Leucaena* were screened in primary experiments.

ELISA of tobacco transformants showed unexpected result, which may be due to the reason that integration of both the genes (Hygromycin phosphotransferase and *asCCRH2*) is purely a chance event. Every transformation event behaves as an individual event and has to be different from other events *i.e.* two transformation events for the same gene need not behave identically. It is not necessary to get a plant positive for both the genes. Plant may be positive for only Hygromycin phosphotransferase gene integration, only *asCCR* gene integration or both. As evident in case of tobacco, two putative transformants showed integration of Hygromycin phosphotransferase gene (PCR and Slot blot) but none showed integration of *asCCR* gene (higher extractable CCR protein in transformed than non-transformed plants). Putative transformants of *Leucaena* showed integration for Hygromycin phosphotransferase gene as well as *asCCR* gene (higher extractable CCR protein in non-transformed than transformed plants).

Further analysis is required to confirm the integration of *CCR* gene in antisense orientation to achieve transgenic lines down regulated for *CCR* gene. Transgenic lines of *Leucaena* are too small and weak to carry on further analysis to confirm the integration of

Hygromycin phosphotransferase and *asCCR* genes. It will take at least six months to nurture them in greenhouse, so that further analysis, such as Southern hybridisation (number of copies integrated), lignin estimation (qualitative and quantitative) could be done to establish *Leucaena* transgenic lines down regulated for *CCR* gene.

4.5 Conclusion:

Primary screening of transformants was done using PCR, Slot blot hybridisation and ELISA based techniques. Seven transgenic lines of *Leucaena* and two transgenic lines of tobacco have been established which will be further analysed for GUS enzyme assay, abundance of CCR transcript using real-time PCR and lignin estimation (quantity and quality) to establish the transgenic lines of *Leucaena* and tobacco down regulated for *CCR* gene. Low/altered lignin producing transgenic lines of *Leucaena* will be utilized for paper and pulp industry in future.

4.6 References:

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: Downregulation of three unrelated lignin biosynthetic genes in tobacco. *Plant Physiology* **128,** 844–853.

Baucher M, Halpin C, Petit-Conil M, Boerjan W. 2003. Lignin: Genetic Engineering and Impact on Pulping. *Critical Reviews in Biochemistry and Molecular Biology* **38,** 305-350.

Boerjan W, Ralph J, Baucher M. 2003. Lignin Biosynthesis. *Annual Review in Plant Biology* **54**, 519- 546.

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM. 2001. Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous

downregulation of cinnamoyl CoA reductase (*CCR*) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *Plant Journal* **28**, 257–270.

Dauwe R, Morreel1 K, Goeminne G, Gielen B, Rohde A, Beeumen JV, Ralph J, Boudet A-M, Kopka J, Rochange SF, Halpin C, Messens E, Boerjan.W. 2007. Molecular phenotyping of lignin-modified tobacco reveals associated changes in cell-wall metabolism, primary metabolism, stress metabolism and photorespiration. *The Plant Journal* **52,** 263–285.

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. *Proceedings of National Academy Science* U S A. **87**(22), 9057–9061.

Freudenberg K, Neish AC, ed. (1968) Constitution and Biosynthesis of Lignin. Berlin: Springer-Verlag. pp 129.

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 At*CCR*1 gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. *Planta* **217**, 218–228.

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

Halpin C, Knight ME, Foxon GA, Campbell M M, Boudet AM, Boon JJ, Chabbert B, Tollier MT and Schuch W. 1994. Manipulation of lignin quality by down-regulation of cinnamyl alcohol dehydrogenase. *Plant Journal* **6**, 339–350.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG and Fraley RT. 1985. A simple and general method for transferring genes into plants. *Science* **227**, 1229–1231.

Humphreys JM, Chapple C. 2002. Rewriting the lignin roadmap. *Current Opinion in Plant Biology* **5**, 224–229.

Jones L, Ennos AR, Turner SR. 2001. Cloning and characterization of irregular xylem4 (irx4): a severely lignin deficient mutant of Arabidopsis. *Plant Journal* **26,** 205–216.

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, Yamamoto E. 1990. Lignin: occurrence, biogenesis and biodegradation. *Annual Review of Plant Physiology and Plant Molecular Biology* **41,** 455–496.

Lodhi MA, Ye GN, Norman FW, Bruce IR. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. *Plant Molecular Biology Reporter* **12**, 6-13.

O'Connell A, Holt1 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 Research* **11,** 495–503.

Pincon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau J-P, Boudet AM, and Legrand M. 2001. Simultaneous Down-Regulation of Caffeic/5-Hydroxy Ferulic Acid-*O*-Methyltransferase I and Cinnamoyl- Coenzyme A Reductase in the Progeny from a Cross between Tobacco Lines Homozygous for Each Transgene. Consequences for Plant Development and Lignin Synthesis, *Plant Physiology* **126**, 145–155.

Piquemal J, Lapierre C, Kmyton A O' Connell, Schuch W, G-Pettenatti J, Boudet AM. 1998. Characterization of a cinnamoyl-CoA reductase that is associated with stem development in *E. gunni*. *The plant journal* **13,** 71-83.

Ralph J, Kim H, Lu F, Grabber JH, Leplé JC, Berrio-Sierra J, Derikvand MM, Jouanin L, Boerjan W, Lapierre C. 2008. Identification of the structure and origin of a thioacidolysis marker compound for ferulic acid incorporation into angiosperm lignins (and an indicator for cinnamoyl CoA reductase deficiency). *Plant Journal* **53**(2), 368-379.

Wadenback J, Sara-von A, Egertsdotter U , Walter MH, Grima-Pettenati J, Goffner D, Gellerstedt G , Gullion T, Clapham D. 2008. Lignin biosynthesis in transgenic Norway spruce plants harboring an antisense construct for cinnamoyl CoA reductase (*CCR*). *Transgenic Research* **17**, 379–392.

Whetten RW, MacKay JJ and Sederoff RR. 1998. Recent advances in understanding lignin biosynthesis. *Annual Review Plant Physiology and Plant Molecular Biology* **49,** 585–609.

Summary

Leucaena leucocephala (subabul) is extensively used in paper industry in India. Removal of lignin is a major hurdle for obtaining good quality pulp, therefore, as a first step to generate transgenic plants with low lignin content, cDNAs of *CCR* gene from *L. leucocephala* were isolated and characterized. The two cDNAs encoding *CCR* were designated as *Ll-CCRH1* and *Ll-CCRH2*. The sequence analysis revealed an Open Reading Frame (ORF) of *CCRH1* to be 1011 and *CCRH2* to be 1005 bp. The predicted molecular weight and pI for *Ll-CCRH1*and *Ll-CCRH2* were estimated to be 36.5 kD /6.32 and 36.3 kD / 6.16, respectively and identified as cytosolic proteins (www.expasy.org/tools). When comparative primary structure analysis of the deduced *Ll-CCR*s with orthologous *CCR* sequences was performed using Pfam and rps-blast search, putative domains such as 3-beta-hydroxysteroid dehydrogenase/isomerase (3Beta_HSD), NADH-flavin reductase, and NAD dependent epimerase/dehydratase for binding cofactors were observed in both the Ll-CCR amino acid sequences. Deduced amino acid sequence showed 76% identity with the *CCR* from *Eucalyptus.* Interestingly it showed 70% homology to *Pinus taeda*. Deduced amino acid sequences of cDNAs from *Leucaena* contain consensus sequences for NADP-binding site, *CCR* signature sequence (NWYCYGK) and other conserved domains responsible for dehydrogenase and reductase activity of known *CCR* genes. All the above information classifies it to be a member of family oxido-reductase. Phylogenetic analysis revealed that *CCRH1* and *CCRH2* sequences are highly homologous to *CCR*s from other dicot plants and are more distantly related to plant dihydro-flavanoid reductase. The haploid genome of *L. leucocephala* contains two copies of *CCRH2* gene. One isoform of *CCR* gene *i.e CCRH2* was over-expressed in *E. coli*, which showed an apparent molecular mass of approximately 38 kDa. Sinapaldehyde was found to be more preferred substrate over coniferaldehyde. The haploid genome of *L. leucocephala* contains two copies of *CCRH2* gene.

Expression of *CCR* gene was monitored in developing seedling of *L. leucocephala*. Root and stem tissue showed good expression of *CCR* gene. *CCR* gene expression increased up to 10 days in case of roots and 15 days in case of stem; but was very low in leaf as revealed by ELISA and RT-PCR studies in 0 to 15 day old seedlings. CCR

protein was immuno-cytolocalised around xylem tissue. FTIR analysis of 15 day and 10 day old developing seedling was done. The result showed that there is no major difference in quality of lignin produced in stem and root tissue.

Considering the molecular mass of the deduced and over-expressed Ll-CCRH2 protein, utilization of sinapaldehyde and coniferaldehyde, developmental and organ-specific gene expression data analysis and immuno-cytolocalization in xylem tissue; the isolated cDNA from *Leucaena leucocephala* can be stated to be involved in constitutive lignification and it can be concluded that the two isoforms code for cinnamoyl-CoA reductase. This report is the first study of spatio-temporal expression profile of *CCR* gene in developing seedling of a tree species. The expression pattern and its presence around lignifying tissue showed that this gene is responsible for constitutive lignifications in *Leucaena leucocephala* and can be used for generating transgenic plants with low lignin content.

To generate transgenic *L. leucocephala*, as a first step, multiple shoot regeneration and transformation protocol was standardized in our lab. Antisense construct of *CCR* gene was made in pCAMBIA 1301 vector and *L. leucocephala* was transformed. Tobacco was used as a standard transformation system. Out of 76 elongated *Leucaena* transformants, randomly 34 plants were selected for analysis. Primary screening of these putative transformants was done using PCR, Slot blot hybridization and ELISA based methods. Seven transgenic lines of *Leucaena* have shown positive result for integration of *asCCR* gene and Hygromycin phosphotransferase gene. Analysis of other transformants is underway.

Future prospects

- Over expressed Ll-CCRH2 protein could be purified in soluble form and substrate specificities may be determined for different substrates of cinnamoyl Co A reductase.
- Attempt could be made to over express both the isoforms in soluble form and substrate specificities may be determined for both proteins. Utilization of different substrates by these two proteins may throw some light on quality of Lignin produced in the plant.
- Promoter region of *CCR* gene could be isolated and can be analysed for its expression using reporter genes like GFP and GUS.
- Putative transformed *Leucaena* plants could be analysed for quality and quantity of lignin being produced.
- Transgenic lines of *Leucaena* most suited for paper industry could be utilized for paper production.

Appendix

Buffers and Solutions for DNA Electrophoresis:

Name	Ingredients	Preparation and Storage
50X TAE	2 M Tris-HCl 0.05 M EDTA	pH was adjusted to 8.0 with glacial acetic acid and store at RT
5X TBE buffer	90 mM Tris-HCl 90 mM Boric acid 2 mM EDTA	RT
DNA loading buffer	0.25 g Xylencyanol 0.25 g Bromophenol blue 0.25 g Ficoll 400 1.46 g EDTA make up the volume to 100ml with H_2O	The solutions were filter sterilize using 0.22µ filter and store at RT

Buffers and Solutions for genomic DNA isolation, Southern and Slot Blot:

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

Solutions for the Transformation and Selection of *E. coli*:

Name	Ingredients	Preparation and Storage
IPTG solution	200 mg/mL in SMQ	Filter sterilise and store at -20 °C
X-Gal	20 mg/mL 5-bromo-4-chloro-3-indolyl-D-galactoside in N,N'-Dimethyl formamide (DMF)	Wrap in aluminum Foil and store at -20 °C
Ampicillin	100 mg/mL in SMQ	Filter sterilise and store at -20 °C
Tetracycline	12.5 mg/mL in 70% ethanol	Filter sterilise and store at -20 °C
Kanamycine	50 mg/mL in SMQ	Filter sterilise and store at -20 °C
Rifampicine	50 mg/mL in DMSO	Filter sterilise and store at -20 °C
Hygromycin	25 mg/mL in SMQ	Filter sterilise and store at -20 °C

Buffers and Solutions for Plasmid Isolation (Alkaline lysis):

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

Buffers and Solutions for Gel Electrophoresis (SDS-PAGE):

Name	Ingredients	Preparation and Storage
Monomer solution	29.2% acrylamide 0.8% bisacrylamide in water	Store at 4 °C, in the dark
Stacking gel	Distilled water 3.4 mL 1 M Tris-HCl (pH 6.8) 0.63 mL Acrylamide/bis 30% 0.83 mL 10% (w/v) SDS 0.05 mL 10% (w/v) APS 0.05 mL TEMED 5 µL	Freshly Prepared
Separating gel (12%)	Distilled Water 3.3 mL 1.5 M Tris-HCl (pH 8.8) 2.5 mL	Freshly Prepared

	Acrylamide/Bis 30% 4.0 mL 10% (w/v) SDS 0.1 mL 10% (w/v) APS 0.1 mL TEMED 4 µL	
2X Protein loading buffer	Distilled Water 2.7 mL 0.5 M Tris-HCl (pH 6.8) 1.0 mL Glycerine 2.0 mL 10% (w/v) SDS 3.3 mL β-Mercaptoethanol 0.5 mL 0.5% (w/v) Bromophenol blue 0.5 mL	Store at 4 °C
10X SDS-electrode buffer	Tris base 15 g Glycine 72 g SDS 5 g Water up to 500 mL	Store at 4 °C, dilute 1:10 before use
Staining solution	Coomassie-blue R 250 0.25 g Methanol 45 mL Acetic acid 10 mL Water 45 mL	Freshly Prepared
Destaining solution	Methanol 40 mL Acetic acid 10 mL Distilled water 50 mL	Freshly Prepared

Buffers and Solutions for Protein Extraction:

Name	**Ingredient**	**Preparation and Storage**
Lysis buffer	50 mM Tris-HCl (pH 8.0) 5 mM EDTA 100 mM NaCl 0.5% Triton-X100 0.7 mM DTT 0.1 mM PMSF (Freshly added). 10 mM Mg SO_4 Lysozyme100µg/mL (Added freshly).	Store at 4 °C
Sonication buffer	100 mMTris HCl (pH 8.0) 50 mM Glycine	Store at 4 °C
Dispersion buffer:	100 mM Tris-HCl(pH 8.0) 50 mM Glycine 8 M Urea /6M GuCl	Store at 4 °C

Buffers and Solutions for Protein Purification under native conditions:

Name	**Ingredients**	**Preparation and Storage**
Binding	50 mM Tris	Adjust pH by adding

buffer	300 mM NaCl 10 mM imidazole	concentrated HCl and store at 4 °C
Wash buffer	50 mM Tris 300 mM NaCl 25 mM imidazole	Adjust pH by adding concentrated HCl and store at 4 °C
Elution buffer	50 mM Tris 300 mM NaCl 200 mM imidazole	Adjust pH by adding concentrated HCl and store at 4 °C

Host Cells:

E.coli	**Genotype**
DH 5α	*F' _80_lacZ_M15 end A1 hsdR17 (rk-mk+) supE44 thi-1_-gyrA96 relA1 _(lacZYA-argFV169) deoR*
XL1 Blue	*RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)
BL 21	*F-, ompT hsdSB (rB – mB -) gal dcm (DE3) pLysS (CamR)*
SOLR	14–(McrA–) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ uvrC umuC::*Tn*5* (Kanr) *lac gyrA96 relA1 thi-1 endA1* λ R [F′ *proAB lacI*qZ Δ *M15*] Su– (nonsuppressing)

Different Media used for studies:

Name	**Ingredients**	**Preparation and Storage**
Luria Bertani Broth (LB)	1% Tryptone 0.5%Yeast extract 1% NaCl	pH adjusted to 7.0 with NaOH, store at room temperature or at +4° C
SOB media	2% Tryptone 0.5% Yeast extract 10 mM NaCl 10 mM $MgCl_2.6H_2O$ 2 mM KCl	pH adjusted to 6.8 with NaOH, store at room temperature or at +4° C
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% Tryptone 1% Yeast extract 0.1% Glucose	pH adjusted to 7.0 with NaOH, store at room temperature or at +4° C
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° C
SM Buffer	0.58% NaCl 0.2% $MgSO_4$. 7 H_2O 50 mM Tris-HCl 0.2% Gelatin	

List of publications

Abstracts published:

1. **S. Srivastava**, M. Arha, S. K. Gupta, N. M. Shaik, A. K. Yadav, P. Kulkarni, O. U. Abhilash, 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.

2. M. Arha, S. K. Gupta, N. M. Shaik**, S. Srivastava**, A. K. Yadav, P. Kulkarni, O. U. Abhilash, B. M. Khan and S. K. Rawal (2006). *Metabolic engineering of Leucaena leucocephala for eco-friendly paper and pulp industry.* In Abstract volume of National Science Day, NCL, Pune, India.

3. M. Arha, S. K. Gupta, N. M. Shaik**, S. Srivastava**, A. K. Yadav, P. Kulkarni, O. U.Abhilash, B. M. Khan and S. K. Rawal (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.

4. 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.

Research papers published/under review/under preparation:

1. N.Sridevi, **Sameer Srivastava**, Bashir Mohammad Khan, Asmita Prabhune (2008). Characterization of the smallest dimeric bile salt hydrolase from a thermophile *Brevibacillus sp.* ***Extremophiles*** (article in press).

2. **Sameer Srivastava**, R. K. Gupta, M. Arha, S. K. Gupta, N. M. Shaik**,** S. Prashant, A. K. Yadav, P. Kulkarni, O. U. Abhilash, R. J. Santosh, R. K. Vishwakarma, V. L. Sirisha, S. K. Rawal, P. B. Kavi Kishor, B. M. Khan (2008). *Molecular cloning, characterization and expression analysis of Cinnamoyl-Co A Reductase (CCR) gene in developing seedlings of Leucaena leucocephala, a paper and pulp yielding tree species* (under review in Journal of Experimental Botany).

3. **Sameer Srivastava**, R. K.Vishwakarma, Manash, B. M. Khan (2009). *Two Cinnamoyl-Co A Reductases (CCR) from Leucaena leucucophala: Homology modelling and docking studies* (Manuscript under preparation).

4. **Sameer Srivastava**, R. K. Vishwakarma, Yasir Arafat and B. M. Khan (2009). *Stress induced structural changes in lignin with respect to Cinnamoyl-Co A*

Reductase (CCR)expression in developing seedlings of Leucaena leucocephala, a paper and pulp yielding tree species (Manuscript under preparation).

5. 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.

6. N. M. Shaik, M. Arha, S. K. Gupta, **S. Srivastava**, A. K. Yadav, P. S. Kulkarni, O. U. Abhilash, B. M. Khan and S. K. Rawal (2008). *High Frequency Regeneration of Leucaena Leucocephala - A Leguminous Pulpwood Tree Species* (under review).

7. N. M. Shaik, **S. Srivastava**, R. Tatkare, M. Arha, S. K. Gupta, A. K. Yadav, P. S. Kulkarni, O. U. Abhilash, N. Akula, B. M. Khan and S. K. Rawal (2008). *Genetic transformation of Leucaena leucocephala with anti-sense constructs of Lignin biosynthesis pathway genes* (Manuscript under preparation).

8. N. M. Shaik, M. Arha, S. K. Gupta, **S. Srivastava**, A. K. Yadav, P. S. Kulkarni, O. U. Abhilash, B. M. Khan and S. K. Rawal (2008). *Molecular cloning and characterization of a Family 1 Glycosyl hydrolase from Leucaena leucocephala* (Manuscript under preparation).

9. M. Arha, S. K. Gupta, N. M. Shaik**, S. Srivastava**, A. K. Yadav, P. Kulkarni, O. U. Abhilash, B. M. Khan and S. K. Rawal (2005). *Isolation, cloning and characterization of Caffeoyl CoA 3-O- methyl transferase (CCoAOMT) from Leucaena leucocephala*. In Proceedings of National Symposium on Plant Biotechnology: New Frontiers, CIMAP, Lucknow, Uttar Pradesh, India.

10. M. Arha, S. K. Gupta, N. M. Shaik, **S. Srivastava**, A. K. Yadav, P. Kulkarni, O. U. Abhilash, B. M. Khan and S. K. Rawal (2005). *High frequency regeneration and transformation of Leucaena leucocephala*. In Proceedings of National Symposium on Plant Biotechnology: New Frontiers, CIMAP, Lucknow, Uttar Pradesh, India.

Books:

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