"ISOLATION, CLONING AND CHARACTERIZATION OF LIGNIN BIOSYNTHESIS PATHWAY GENE(S) 4-COUMARATE Co A LIGASE (4CL) FROM *Leucaena leucocephala*"

A THESIS
SUBMITTED TO THE
UNIVERSITY OF PUNE

FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

BY
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UNDER THE GUIDANCE OF
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NOVEMBER, 2008

DEDICATED TO MY PARENTS

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Isolation, Cloning and Characterization of Lignin Biosynthesis Pathway Gene(s) 4-Coumarate Co A Ligase (4CL) from *Leucaena leucocephala***" submitted by ***Sushim kumar Gupta*** for the degree of Doctor of Philosophy, was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. B. M. Khan
(Research guide)

DECLARATION

I hereby declare that the thesis entitled, "**Isolation, Cloning and Characterization of Lignin Biosynthesis Pathway Gene(s) 4-Coumarate Co A Ligase (4CL) from *Leucaena leucocephala***" has been carried out at the Plant Tissue Culture Division, National Chemical Laboratory, Pune, under the guidance of **Dr. Bashir M. Khan**. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University. I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

(**Sushim Kumar Gupta**)

Date: November, 2008
Place: Plant Tissue Culture Division,
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Sushim

ABBREVIATIONS

4CL	4 - Coumarate Co A: ligase
AldOMT	5-Hydroxyconiferaldehyde O- methyltransferase
APS	Ammonium per sulphate
BSA	Bovine serum albumin
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
C3H	Coumarate 3- hydroxylase
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CAld5H/ F5H	Coniferaldehyde 5- hydroxylase / Ferulate 5- hydroxylase
CCR	Cinnamoyl coenzyme A reductase
CCoAOMT	Caffeoyl coenzyme A 3-O- methyltransferase
cm	Centimeter
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol; Cleland's reagent
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
g	Grams
L	Liter
h	Hour
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb/Kbp	Kilobase pairs
kD/kDa	Kilo Daltons
mg	Milligram
mL	Milli liter
mM	Milli molar
mRNA	Messenger ribonucleic acid

µL	Micro liter
µg	Microgram
N	Normality
ng	Nanogram
nm	Nano meter
OD	Optical density
O/N	Overnight
PEG	Polyethylene glycol
PMSF	Phenylmethanesulphonylfluoride
PVPP	Poly vinyl pyro phosphate
pmol	Pico mole
RACE	Random amplified complementary deoxyribonucleic acid end
RNase A/ (H)	Ribonuclease A/(H)
rpm	Rotations per minute
SAD	Sinapyl alcohol dehydrogenase
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMQ	Sterile milli Q water
SSC	Saline sodium citrate
s	Second
TAE	Tris acetic EDTA buffer
TE	Tris EDTA buffer
TEMED	Tetramethylethylenediamine
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
x g	Relative centrifugal force
°C	Degree centigrade
GUS	β-glucuronidase

ABSTRACT

Leucaena sp. is a fast growing multipurpose tree adapted to a variety of soils and climatic conditions. *Leucaena* is recognized as most useful trees in the tropics. Paper industry in India mainly uses bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena sp*. as a source for paper pulp. This hard wood *Leucaena sp*. is exclusively used in India and about 25% of raw material for pulp and paper industry comes from this plant. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. The present study was aimed at understanding lignin biosynthesis pathway in *Leucaena leucocephala*. No study has been done so far in this regard anywhere on *Leucaena sp.*

4CL (4-Coumarate CoA Ligase, EC (6.2.1.1.2)) is one of the important key enzymes of general phenyl propanoid metabolism, which provides the precursor molecules for a large variety of important plant secondary products such as lignin, flavonoid etc. It catalyzes the activation of 4-coumarate and various other cinnamic acid derivatives to the corresponding thiol esters in two-step reaction via an adenylate intermediate. Thio reaction represents the last step in a short series of biochemical conversions, known as phenyl propanoid metabolism leading from phenylalanine to the activated cinnamic acid derivatives. These derivatives are precursors for the biosynthesis of a large variety of plant secondary metabolites. This gene was chosen as the target gene for the study if we could down regulate this 4CL enzyme then there would be scarcity of precursor molecules for lignin biosynthesis *i.e*. total lignin content of plant will get altered. Thus it may be helpful for development of transgenic *L. leucocephala* plants with desired characters suitable for Indian pulp and paper industry. The main features of the present thesis are:

- Cloning and characterization of 4-coumarate CoA ligase (4CL) gene from *Leucaena leucocephala.*
- Spatial and temporal expression of 4CL in *Leucaena leucocephala*.
- Transformation of *Leucaena leucocephala* plant with antisense 4CL construct and its analysis.

Cloning and Characterization of 4-coumarate CoA ligase (4CL) Gene from *Leucaena leucocephala*

A PCR based approach to fish out the c-DNA clones of 4CL genes from *Leucaena leucocephala* was followed. Primers were designed on the basis of consensus regions of various reported 4CLs from the NCBI Gen-Bank database. A partial 4CL gene sequence was amplified and its sequence utilized to design gene specific primers. Rapid amplification of cDNA ends (RACE) was performed to fish out the full length cDNA clones of 4CL. Characterization of the two genes encoding Ll4CL1 and Ll4CL2 was done.

Spatial and Temporal Expression of 4CL in *Leucaena leucocephala*

4CL was immunolocalized in xylem and fibers proving its presence at the sites of active lignification. The spatial and temporal expression of the 4CL gene was studied using semi quantitative and absolute quantification ***i.e.*** Real Time PCR. Primers were designed so as to distinguish between two genes. The 5.8S RNA was used as the internal standard. For absolute quantification Taqman probes were used. Plants of different age ***i.e.*** seedling of age 1 day, 5 day, 10 day and 15 day; one season and two seasons old plants were used for temporal expression studies. Different plant part ***i.e.*** root, shoot, leaf and inflorescence were used for spatial expression studies.

Transformation of *Leucaena leucocephala* plant with Antisense 4 CL construct and its Analysis

Leaves of *Nicotiana tabacum* var. Anand 119 and embryo axes excised form germinated seeds of *Leucaena leucocephala* cultivar K-636 were used as explant for transformation studies. Explants were transformed by three different methods i.e. particle bombardment, particle bombardment followed by co-cultivation and agro-infusion method. The integration of genes was confirmed by PCR with gene specific primers. The sequences of PCR products of putative transformants with Hygromycin, 35S promoter *and* 35Spromoter and a part of Ll4Cl1 genes showed complete homology with the sequences of the corresponding genes in the plasmids. Slot blot analysis of the transformed plants showed strong positive signals confirming the integration of the genes.

Chapter 1

1. General introduction

This chapter includes a thorough literature survey of work done in the area of Lignin genetic engineering with regards to the current status of research in this area. A special emphasis on the techniques and strategies used in study of the genes involved in the lignin biosynthesis pathway is dealt with. Role of a key enzyme, importance and role of *L. leucocephala* with special reference to Indian paper and pulp industry is discussed. Potential of biotechnology in improving the pulp qualitatively and quantitatively will be discussed.

1.1. Biotechnological approaches to forest trees

On the global scale, the production of trees with improved characteristics is expected to help meet the growing need for wood and wood-related products (FAO, 2005), and hence to diminish the pressure on the domestication of natural forest resources in the future (Boerjan, 2005; Merkle & Nairn, 2005; Nehra *et al.,* 2005). Several conifer and angiosperm tree species have been genetically transformed, with the major focus on traits including wood quality, insect and disease resistance and abiotic stress tolerance (Boerjan, 2005; Merkle & Nairn, 2005; Nehra *et al.,* 2005). Transgenic trees with various genetically modified (GM) traits have also been tested in field conditions, e.g. insectresistant *Populus nigra* (Hu *et al*., 2001; Lin *et al.,* 2006), and *Picea glauca* (Lachance *et al.,* 2007), several herbicide-resistant *Populus hybrids* (Meilan *et al.,* 2002), lignin-modified *Populus tremula* x p*opulus alba* (Pilate *et al.,* 2002), fungal disease resistant *Betula pendula* (Pasonen *et al.,* 2004), sterile *B. pendula* (EC, 2007) and sterile *P. tremula* x *P. alba* (Wei *et al.,* 2006). Field testing is generally considered to be important for assessment of the potential environmental effects of genetic modification in organisms such as trees that are characterized by e.g. longevity, wind pollination, and multiple ecological interactions in forest ecosystems. Up to now, the commercialization of GM trees has lagged well behind that of GM crop plants. The area of cultivated, commercialized GM crop plants in 2006 covered 102 million hectares (James, 2006), whereas there are only two commercially cultivated GM trees, i.e. the virus-resistant papaya fruit tree in Hawaii (Ferreira *et al.,* 2002) and insect-resistant poplar cultivations in China (FAO, 2004). The

ability to modify lignin as a part of cell wall modification is an important area of research in attempts to improve the utilization of plant biomass as a renewable source for sustainable development (Boerjan, 2005; Chiang, 2006; Higuchi, 2006; Li *et al.,* 2006). The development of trees with improved wood quality through modification of the genes involved in lignin biosynthesis could be important for the improved end use of wood material (Chiang, 2006; Higuchi, 2006). In chemical wood pulping, lignin is the main factor hindering the effective utilisation of cellulose fibres, from which it needs to be separated by costly and pollutant-generating processes (Chiang, 2002; Baucher *et al.,* 2003; Boerjan, 2005). Genetic modifications resulting in increased delignification (through a modified lignin content and/or chemical composition) could thus be highly beneficial at both the economical and environmental scale (Baucher *et al.,* 2003; Boerjan, 2005; Chiang, 2006).

1.2. Lignin

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

through the cell membrane to the apoplast, the glucose is removed and the polymerisation commences.

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

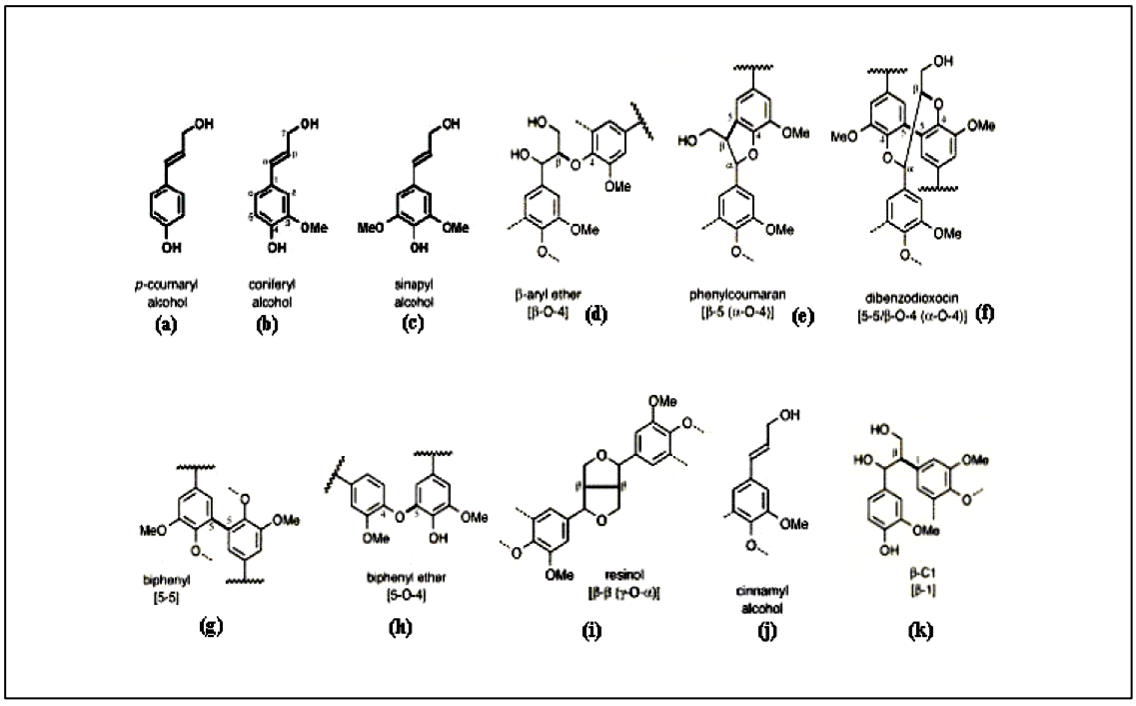


Fig. 1.1: Monolignols *p*- coumaryl (a), coniferyl (b) and sinapyl alcohol (c). Different structures formed due to "end wise" coupling (d-f). Coupling between preformed lignin oligomers results in units linked 5–5 and 5–O–4 (g, h). Coupling of two monolignols form resinol β – β units (i) or cinnamyl alcohol (j) end groups (Boerjan *et al*., 2003).

Lignins are complex racemic aromatic heteropolymers synthesized by the dehydrogenative polymerization of monolignols, namely coumaryl, coniferyl and sinapyl alcohol monomers differing in their degree of methoxylation (Freudenberg and Neish, 1968) (Fig. 1 a-c). These monolignols produce, respectively, *p*-hydroxyphenyl (**H)**, guaiacyl (**G)**, and syringyl (**S)** phenylpropanoid units when incorporated into the lignin polymer. 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). 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.

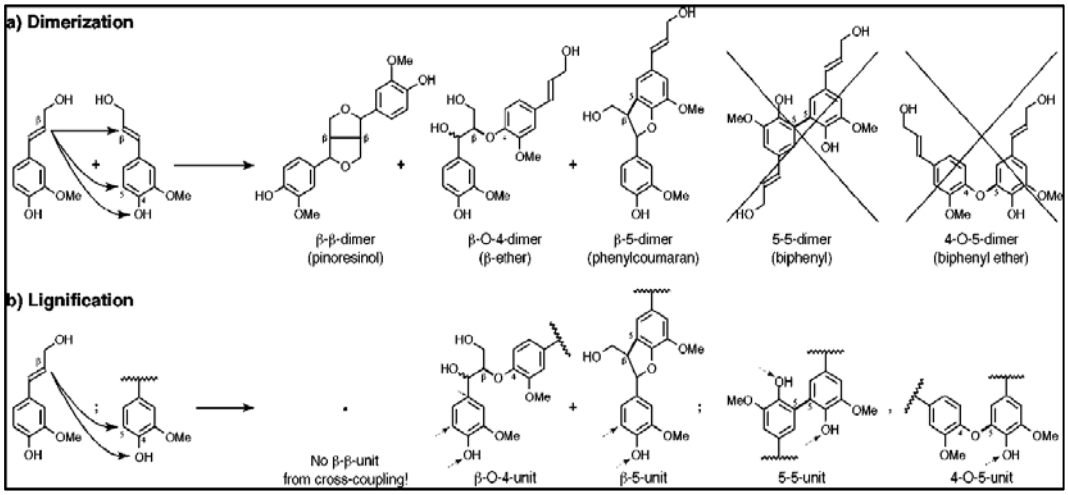


Fig. 5.2: Lignification differs substantially from simple dimerization of monolignols. (*a*) Dimerization of coniferyl alcohol produces only three dimers, in each of which at least one of the coniferyl alcohols is coupled at its β position. The 5–5 and 5–O–4 dimers (crossed out) do not arise in any significant way from monomer dimerization reactions. The new bond formed by the radical coupling reaction is noted in bold. (*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 (Boerjan *et al*., 2003).

(Baucher *et al.,*1998). Lignification is the process by which H, G and S units are linked together via radical coupling reactions (Sarkanen and Ludwig, 1971). The main "end-wise" reaction couples a new monomer (usually a monolignol and usually at its β position) to the growing polymer, giving rise to different structures (Fig. 1 d-f) all of which are β linked. Coupling between preformed lignin oligomers results in units linked 5–5 and 5–O–4 (Fig. 1 g, h). The coupling of two monolignols is a minor event, with resinol (β – β) units (Fig. 1 i) or cinnamyl alcohol end groups (Fig. 1 j) as the outcome. Monolignol dimerization and lignin are substantially different processes (Adler, 1997), explaining why lignification produces frequencies of the various units that are different from those produced by dimerization or bulk polymerization *in vitro* (Fig. 2).

Lignins can serve the following purposes:

1) Binder 2) Dispersant 3) Emulsifier 4) Sequestrant

1) Lignin as a Binder:

Lignosulfonates are a very effective and economical adhesive, acting as a binding agent or "glue" in pellets or compressed materials. Lignosulfonates used on unpaved roads reduce environmental concerns from airborne dust particles and stabilize the road surface. This binding ability makes it a useful component of Coal briquettes, Ceramics, Carbon black, Fertilizers & herbicides, Dust suppressants, Animal feed pellets, Fiberglass insulation, Linoleum paste, Soil stabilizers, Plywood and particle board.

2) Lignin as a Dispersant:

Lignosulfonate prevents the clumping and settling of undissolved particles in suspensions. By attaching to the particle surface, it keeps the particle from being attracted to other particles and reduces the amount of water needed to use the product effectively. The dispersing property makes lignosulfonate useful in Cement mixes, Clay, ceramics, dyes, pigments, oil drilling mud's, leather tanning, concrete admixtures, gypsum board pesticides and insecticides

3) Lignin as an Emulsifier:

Lignosulfonate stabilizes emulsions of immiscible liquids, such as oil and water, making them highly resistant to breaking. Lignosulfonates are at work as emulsifiers in Asphalt emulsions, Pesticide, Wax emulsions, Pigments and dyes.

4) Lignin as a Sequestrant:

Lignosulfonates can tie up metal ions, preventing them from reacting with other compounds and becoming insoluble. Metal ions sequestered with lignosulfonates stay dissolved in solution, keeping them available to plants and preventing scaly deposits in water systems. As a result, they are used in Micronutrient systems, Cleaning compounds, Water treatments for boilers and cooling systems,

1.3. Lignin Biosynthesis and its Regulation

Phenylpropanoid metabolism comprises a complex series of branching biochemical reactions, which provide the plant with a host of important phenolic compounds. The general phenylpropanoid pathway leads from phenylalanine to coumaroyl-CoA and is initiated by the enzyme phenylalanine ammonia-lyase (PAL; Figure 3). Phenylpropanoid metabolism branches to give rise to literally thousands of compounds, many of which are specific to particular plant species. An important branch leads to the production of flavonoids, including flavonols, anthocyanins and tannins. Phenylpropanoid 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 signalling molecules (e.g. flavonoid nodulation factors). Recent studies indicate that flavonols are required for male fertility and, more specifically, pollen tube growth in maize, petunia and tobacco. The pathways, the functions of their products, and the regulation of expression of biosynthesis genes have been discussed in several recent reviews (Dixon *et al.,* 2001; Chapple, 1992; Boerjan *et al.,* 2003; Lee *et al.,* 1996). There is significant commercial potential in the manipulation aspects of phenylpropanoid metabolism (Lee *et al.,* 1997). The ability to increase or reduce the expression of particular genes in transgenic plants raises many possibilities to

alter phenylpropanoid biosynthesis both quantitatively and qualitatively. Making predictable changes, however, may be problematic because the pathways are complex and alternative routes to the synthesis of particular compounds may be present. For the last two decades, there has been a great deal of interest in cloning and characterization of the genes controlling monolignol biosynthesis in order to understand monolignol biosynthetic pathways in trees and other plants. A number of reviews have been done about the advancements of monolignol biosynthesis pathways (Whetten and Sederoff, 1995; Whetten *et al.,* 1998; Humphreys and Chapple, 2002; Boerjan *et al.,* 2003). There is enormous variation in lignin content and composition among plant species, tissues, cell types, and even developmental stages and environmental conditions play a role. Data from the studies using different plant materials display many agreements as well as certain disagreements. Thus, it is debatable whether lignin biosynthesis in all plants follows the exact same pathway or not.

To date, most of the genes for monolignol biosynthesis have been identified and characterized in various plant species. A summarized picture of the main and possible monolignol biosynthesis pathways of wood formation in trees is shown in Fig.5. The genes involved in the pathway, the reactions catalyzed by them and their regulation will be discussed. Natural variations in lignin content and composition observed between different plants, tissues and cell types (Campbell and Sederoff, 1996; Buxton and Redfearn, 1997; Sederoff *et al*., 1999; Donaldson, 2001) as well as occurrence of natural mutants (Kuc and Nelson, 1964; 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.

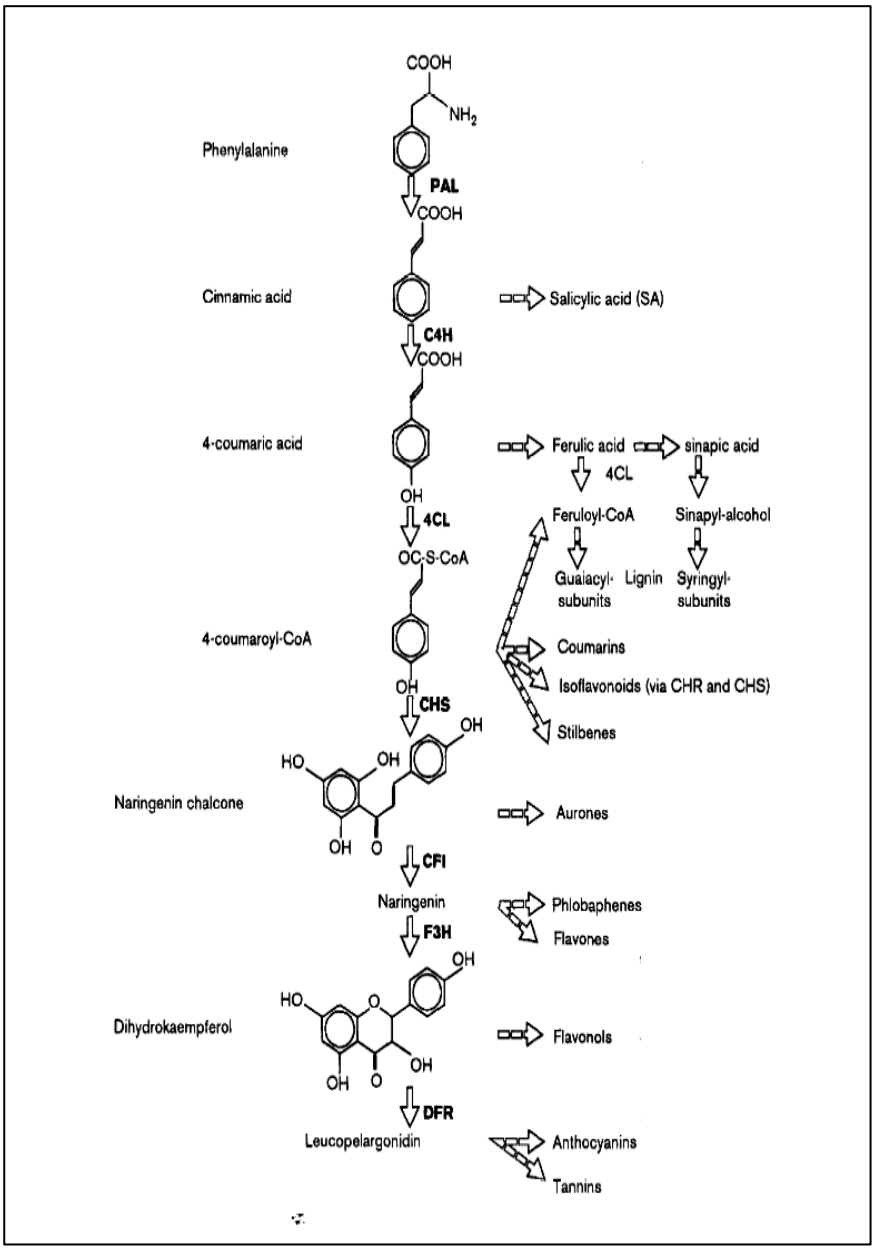


Fig.1.3: Schematic view of some branches of phenoil propanoid pathway. Solid arrow indicates enzymic reactions with the respective enzyme indicated to right. PAL, Phenylalanine ammonia-lyase, C4H cinnamate 4-hydroxylase; 4CL, Coumarate coenzyme A ligase; CHS, chalcone synthase; CFI, chalcone flavanone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol reductase; CHR, chalcone reductase. Broken arrow indicated metabolic branches towards several classes of phenylpropanoids, or several subsequent enzymatic steps. In some cases the enzymes indicated are also involved in other reactions, not shown.

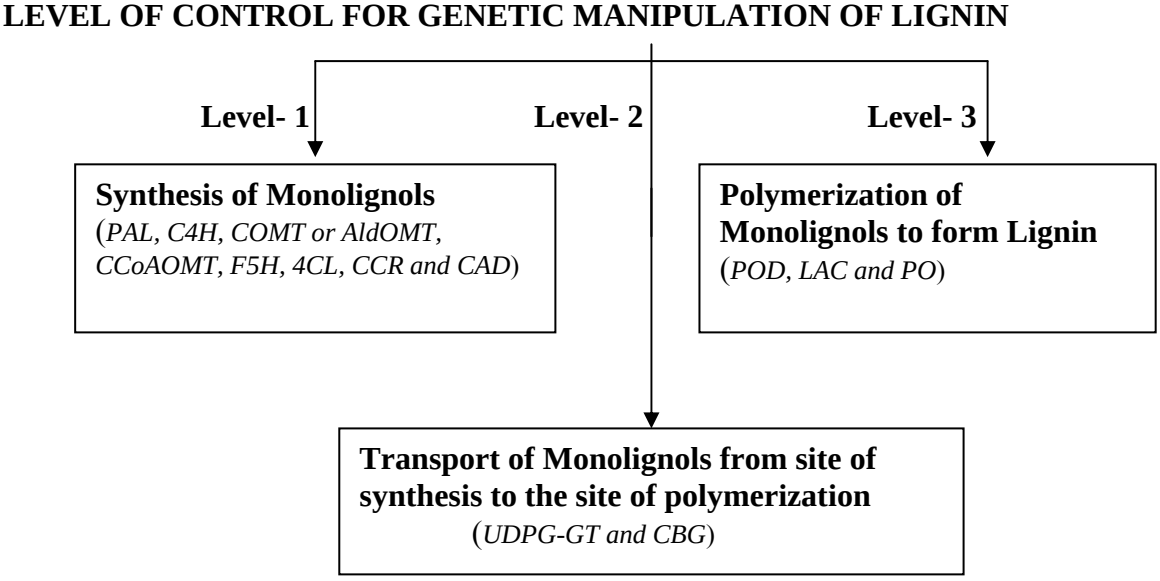


Fig. 1.4: Levels of control for manipulation of lignin biosynthesis. The control level 1 involves manipulation of genes for PAL, C4H, COMT or AldOMT, CCoAOMT, F5H, 4CL, CCR and CAD, level 2 involves the genes for UDPG-GT and CBG, while level 3 includes POD, LAC and PO.

Attempts have been made to genetically modify plant species with the aim of lignin reduction. Conceptually, genetic engineering of lignin can be accomplished at three levels of control, namely synthesis of monolignols, its transport and polymerization at the site of deposition (Fig.4) as the overall rate of lignification is regulated not only by the monolignol biosynthesis, but also by the coordinated transport, storage, mobilization and polymerization of monolignol precursors to the cell wall. During the past decade, significant headway has been made in the cloning and subsequent expression of sense and antisense constructs of a number of genes pertaining to monolignol biosynthesis, transport and polymerisation (Whetten *et al*., 1998; Whetten and Sederoff, 1995; Boudet *et al*., 1995; Boerjan *et al*., 2003; Baucher *et al*., 2003; Boudet, 2000; Sederoff, 1999; Grima-Pettenati and Goffner, 1999; 2000). The transgenics have produced unexpected findings leading to a profound reappraisal of our understanding of the phenylpropanoid "metabolic grid". The enzymes and their genes involved in lignin biosynthesis and related findings of gene regulation of lignin biosynthesis pathway genes are discussed in the following section (1.4).

1.4. Key enzymes in Lignin Biosynthesis:

1.4.1. Phenylalanine ammonia-lyase (PAL)

Monolignol biosynthesis is considered to start from phenylalanine. The enzyme Phenylalanine ammonia-lyase, PAL, that catalyzes the conversion of phenylalanine to transcinnamic acid, is the initial step towards monolignol biosynthesis and other phenolic secondary plant metabolites. Genes encoding PAL have been studied in *Populus* species (Osakabe *et al*., 1995; Kao *et al.,* 2002) and other plant species (Jones, 1984; Ohl *et al.,* 1990; Leyva *et al.,* 1992; Kumar and Ellis, 2001). *PAL* exists as a multiple member gene family and the individual members can be involved in different metabolic pathways as suggested by their expression patterns in association with certain secondary compounds accumulated in specific tissue or developmental stage. The expression studies of two *PAL* genes suggested that one is associated with condensed tannin metabolism and the other with monolignol biosynthesis (Kao *et al.,* 2002).

PAL gene expression has been suppressed by 85% and >98% in transgenic plants with resultant 52% (Sewalt *et al*., 1997) and 70% (Korth *et al.,* 2001) reduction in Klason lignin content, respectively. Lignin monomeric composition, determined by pyrolysis GC-MS, was characterized by a lower proportion of G units and a 1.7-fold increase in S/G ratio (Sewalt *et al.,* 1997). Because PAL catalyzes the first step of the phenylpropanoid pathway, reduction of its activity results in a wide range of abnormal phenotypes. The transgenic plants were stunted curled leaves, and with thinner cell walls in the secondary xylem with less lignin than those of the control. These plants were also more susceptible to the fungal pathogen *Cercospora* nicotianae (Maher *et al.,* 1994). A slight increase in Klasone lignin and dry matter content was observed in the stem of *PAL*-over expressing plants (Korth *et al.,* 2001). Over expression of *PAL* did not lead to changes in lignin composition as determined by pyrolysis GC-MS (Sewalt *et al.,* 1997), but to a decrease in the amount of S units, yielding a reduction in the S/G ratio when lignin was analyzed by thioacidolysis (Korth *et al*., 2001).

1.4.2. Cinnamate 4-hydroxylase (C4H)

The conversion of cinnamate to *p*-coumarate is catalyzed by C4H. C4H is a cytochrome P450- dependent monooxygenase, belonging to the *CYP73* family. Similar to PAL, C4H is thought to be involved in a number of secondary metabolism pathways in addition to monolignol biosynthesis as *p*-coumarate is an intermediate for biosynthesis of many secondary compounds (Croteau *et al.,* 2000). Multiple *C4H* gene members are identified in many plant species, however, only one *C4H* is known in the Arabidopsis genome (Raes *et al.,* 2003). The expression study of two *C4H* members in quaking aspen indicated that one is strongly expressed in developing xylem tissues and the other is more active in leaf and young shoot tissues. In other species, *C4H* gene is expressed in a variety of tissues and the expression is induced by wounding, light, pathogen attacks and other biotic and abiotic stimuli (Raes *et al.,* 2003). The mechanisms that regulate the genetic function of *C4H* gene and its family members are yet unknown.

In transgenic tobacco plants, C4H activity was altered by expressing the alfalfa class I *C4H* (CYP73A3) (Sewalt *et al.,* 1997) or the French bean class II *C4H* (CYP73A15) (Blee *et al.,* 2001) genes in sense or antisense orientation (class I and class II *C4H* share approximately 60% similarity). Over expression of class I *C4H* had no effect on Klason lignin, nor on the S/G ratio. In contrast, a 76% reduction in total *C4H* activity led to a 63% decrease in Klason lignin and a modification of the lignin monomeric composition. The amount of S units was strongly reduced and S/G decreased by over 90% (Sewalt *et al.,* 1997). Similarly, a reduction of 90% in C4H activity by down-regulation of the class II *C4H* resulted in a 27% decreased lignin content and one tobacco transgenic line had a decreased S/G ratio (Blee *et al*., 2001).

In transgenic tobacco modified to reduce *C4H* expression, decreased *PAL* activity has also been shown (Blount *et al*., 2000). Regulation may be mediated by pathway intermediates, for example, cinnamic acid may act as a feedback regulator of the phenylpropanoid pathway.

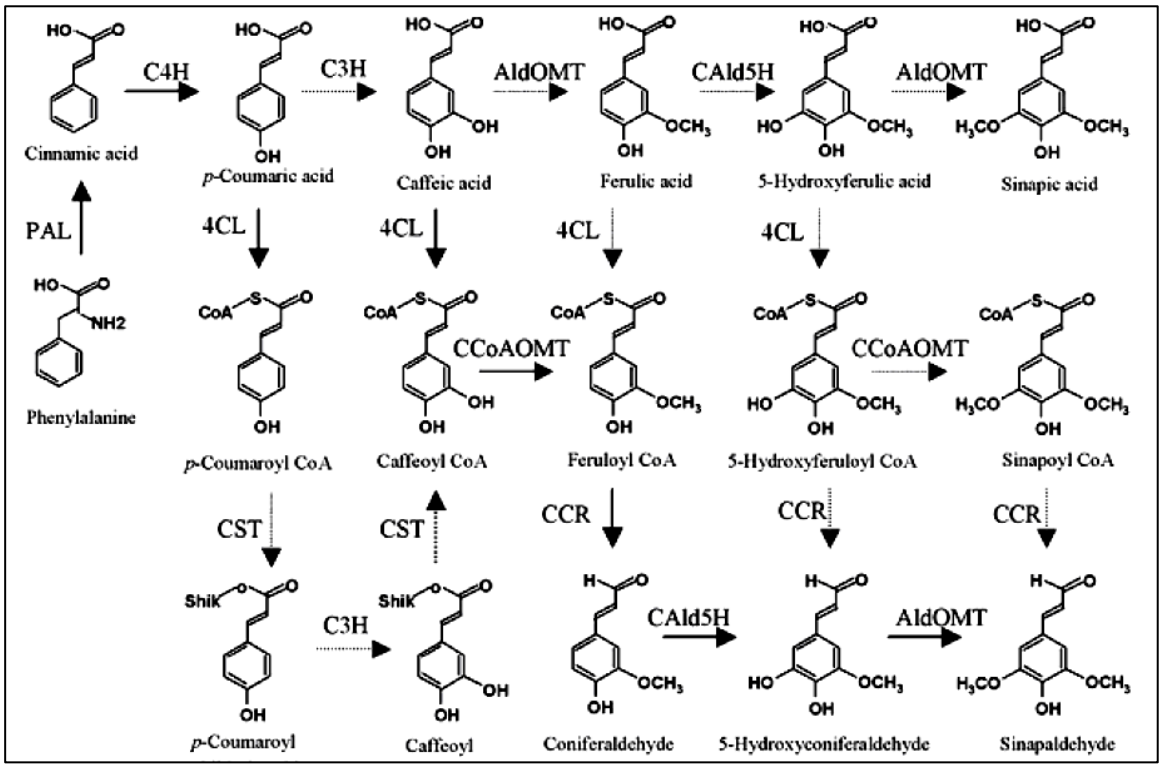


Fig. 1.5: An overview of the monolignol biosynthesis pathways. The main pathways are indicated in solid line arrows and possible pathways in dotted line arrows. *PAL*, phenylalanine ammonia-lyase; *C4H*, cinnamic acid 4-hydroxylase; *C3H*, *p*-coumarate 3-hydroxylase; *CST*, hydroxycinnamoyl- CoA:shikimate hydroxycinnamoyltransferase; *4CL*, 4-coumarate-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).

1.4.3. Coumarate 3-hydroxylase (C3H)

Early biochemical evidence suggested that the reaction, coumarate to caffeate, is catalyzed by a nonspecific phenolase, but that suggestion did not receive much support in other studies (Kojima and Takeuchi, 1989; Petersen *et al*., 1999). Recently, the gene encoding *p*-coumarate 3-hydroxylase (C3H) was cloned and an alternative pathway was proposed based on the enzyme activity of *CYP98A3* gene from Arabidopsis (Schoch *et al*., 2001; Franke *et al*., 2002a). The proposed alternative suggested that the hydroxylation at the 3- position of the aromatic ring of cinnamic acid does not directly occur on *p*-coumarate, instead, *p*-coumarate is first converted to *p*-coumaroyl CoA ester by 4- coumaroyl-CoA ligase (4CL),

then the CoA ester group of *p*-coumaroyl CoA is exchanged by hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase (CST) to form *p*-coumaroyl shikimic acid which serves as a substrate of *C3H* to produce caffeoyl shikimic acid. Subsequently caffeoyl shikimic acid reverts back to caffeoyl CoA to push metabolism towards the biosynthesis of monolignols. Among tree species, a *CYP98* cDNA was cloned from sweetgum and aspen (Osakabe *et al*., 1999), but the postulated genetic and biochemical functions in monolignol biosynthesis have not been demonstrated for its role in wood formation. The gene encoding *p*-coumarate 3-hydroxylase (*C3H*) has only recently been cloned by two independent research groups. Using a functional genomics approach, Schoch *et al*. (2001) identified CYP98A3 as a possible candidate for *C3H*. In parallel, by screening *Arabidopsis* mutants under UV light, Franke *et al*. (2002a) isolated the reduced epidermal fluorescence (*ref8*) mutant. By positional cloning, the *REF8* gene was identified as the cytochrome P450- dependent monooxygenase *CYP98A3*. The *ref8* mutant had collapsed xylem vessels, a higher cell wall degradability and a higher susceptibility to fungal colonization (Franke *et al*., 2002b), associated with the accumulation of *p*-coumarate esters instead of sinapoylmalate and with a reduction in lignin content of 60-80%. A range of analyses showed that lignin composition was dramatically altered, being almost entirely madeup of *p*-coumaryl alcohol units (Franke *et al*., 2002b).

1.4.4. 4-coumarate coenzyme A ligase (4CL)

Genetic and biochemical functions of 4-Coumarate Coenzyme A ligase (*4CL*) genes have been clearly demonstrated in association with monolignol biosynthesis (Lewis and Yamamoto, 1990; Lee *et al*., 1997; Hu *et al*., 1999; Harding *et al*., 2002). *4CL* genes usually exist as a multi-gene family. Four *At4CLs* have been identified in *Arabidopsis*, of which *At4CL1* and *At4CL2* are associated with lignin biosynthesis (Soltani *et al*., 2006). Different expression patterns of *4CL* members are found in herbaceous and tree species. Four *4CL* genes were detected in the Arabidopsis genome and the expression of each member was regulated differentially in tissues and development stages (Raes *et al*., 2003). In aspen trees, two *4CL* genes were cloned and their expression was clearly distinct, with one in epidermal and leaf tissue and the other specifically in developing xylem tissue

(Harding *et al*., 2002). Furthermore, the enzymatic activities of *4CL* members from aspen, loblolly pine, tobacco, soybean, Arabidopsis, and many other species were found to have distinct substrate specificities (Voo *et al*., 1995; Zhang and Chiang, 1997; Lindermayr *et al*., 2003; Hamberger and Hahlbrock, 2004). Whether the substrate specificity of the *4CL* members relates to different metabolic pathways is unknown. As the *4CL* catalytic kinetics vary among species, it is also likely that the mainstream pathway mediated by *4CL* may not be exactly the same in all plant species or tissues. Nevertheless, monolignol biosynthesis is tightly controlled by *4CL*. Suppression of *4CL* expression through antisense technology has demonstrated the effectiveness of reducing total lignin content (Lee *et al*., 1997; Hu *et al*., 1999; Li *et al*., 2003). In aspen, suppression of *4CL* expression led to more than 55% lignin reduction in wood. Thus, technology aimed at *4CL* suppression could be applied to plant genetic modification for better fiber production and other utilizations.

Transgenic plants with reduced *4CL* activity have been produced in tobacco (Kajita *et al*., 1996, 1997), *Arabidopsis* (Lee *et al*., 1997), and aspen (Hu *et al*., 1999; Li *et al*., 2003). In tobacco, reduction of *4CL* by over 90% resulted in 25% less lignin. In poplar and *Arabidopsis* with a >90% reduced *4CL* activity, lignin content was reduced by 45–50%. In tobacco, the low *4CL* activity was associated with browning of the xylem tissue (Kajita *et al*., 1996). In transgenic aspen down-regulated for *4CL* (Hu *et al*., 1999) also detected an increase in non lignin alkali-extractable wall-bound phenolics (p-coumaric acid, caffeic acid, and sinapic acid), which were not incorporated into the lignin polymer. However, no difference in lignin S/G composition for *Arabidopsis* and tobacco was observed. Discrepancy between the results published by Kajita *et al*. (1997) and Hu *et al*. (1999) is that the transgenic tobacco lines with the most severe reduction in lignin content (25%) were characterized by a collapse of vessel cell walls and reduced growth (Kajita *et al*., 1997), whereas the transgenic poplars with a 45% reduction in lignin content had a normal cell morphology and a higher growth rate than the control (Hu *et al*., 1999). The increased level of hydroxycinnamic acids as non-lignin cell wall constituents has been suggested to contribute to the cell wall strength in transgenic poplar (Hu *et al*., 1999). Because several 4CL isozymes

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

1.4.5. Caffeoyl coenzyme A 3-O- methyltransferase (CCoAOMT) and Caffeate Omethyltransferase(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 results in a syringyl unit. In early studies, it was postulated that two types of methyltransferases were necessary for methylations (Higuchi, 1990). Mono-functional *O*-methyltransferase to methylate the 3- position and therefore controlled the G monolignol unit biosynthesis, and the bi-functional *O*-methyltransferase to catalyze both 3- and 5-methylations leading to S monolignol unit biosynthesis. However, molecular studies have revealed that there are two kinds of genes encoding for the enzymes that specifically catalyze the 3- and 5-methylation, respectively (Ye *et al*., 1994; Li *et al*., 1999, 2000; Chen *et al*., 2001). Biochemical evidence from the studies, using tree material, suggests that the 3- and 5-methylations occur at different biosynthesis stages. The 3-methylation occurs on the CoA ester intermediate while the 5- position is methylated at the aldehyde intermediate (Osakabe *et al*., 1995; Li *et al*., 2000). The two genes are: one encoding a *CCoAOMT* and the other encoding a 5-*O*methyltransferase that preferably methylates 5 hydroxyconiferaldehyde. The 5-

*O*methyltransferase was thought to methylate caffeic acid and was named *COMT* accordingly.

Down-regulation of COMT activity has been achieved using either antisense or sense transgenes in tobacco (Dwivedi *et al*., 1994; Ni *et al*., 1994; Atanassova *et al*., 1995), poplar (Jouanin *et al*., 2000) and alfalfa (Guo *et al*., 2001). In all three species, drastic reductions in the lignin S/G ratio were apparent and an unusual phenolic compound 5-hydroxyconiferyl alcohol (5OHG) was present in the polymer (Atanassova *et al*., 1995; Tsai *et al*., 1998; Lapierre *et al*., 1999; Jouanin *et al*., 2000; Guo *et al*., 2001; Marita *et al*., 2003). In the lignin of the transgenic poplars described by Jouanin *et al*. (2000), the level of 5OHG units even exceeded that of S units. In *COMT* suppressed poplar, reduced lignin content has been reported (Jouanin *et al*., 2000) as well as no change in lignin amount in aspen (Tsai *et al*., 1998). Reports describing *COMT*-suppression in tobacco also differ on whether lignin content is (Ni *et al*., 1994) or is not (Dwivedi *et al*., 1994; Atanassova *et al*., 1995) reduced. Despite these discrepancies, the data from all of the *COMT*-suppressed tobacco and poplar plants indicate that COMT plays a predominant role in determining the incorporation of S units into the lignin polymer. In alfalfa, a reduction in *COMT* activity affected both the content of G and S units (Guo *et al*., 2001; Marita *et al*., 2003). In accordance with these results, Parvathi *et al*. (2001) found that in alfalfa COMT is also involved in the methylation of caffeoyl aldehyde. Down regulation of *CCoAOMT* affected the Klason lignin content by 12–50% in transgenic tobacco (Pincon *et al*., 2001a), alfalfa (Guo *et al*., 2001; Marita *et al*., 2003) and poplar (Zhong *et al*., 2000). In tobacco and poplar, the decreased lignin content was due to reduction of both G and S units as determined by pyrolysis GC-MS (Zhong *et al*., 2000) and thioacidolysis. Simultaneous down-regulation of both COMT and CCoAOMT in tobacco (Zhong *et al*., 1998; Pincon *et al*., 2001a) and alfalfa (Guo *et al*., 2001) resulted in combinatorial and/or additive effects. There was a greater reduction in Klasone lignin content in tobacco (Pincon *et al*., 2001a) but not in alfalfa (Guo *et al*., 2001). In both species, the lignin S/G ratio was reduced although in tobacco this was due to decreases in both G and S units, whereas only S units decreased in alfalfa (Guo *et al*., 2001).

1.4.6. Cinnamoyl coenzyme A reductase (CCR)

The reduction of cinnamoyl CoA esters to cinnamaldehydes is the first metabolic step committed to monolignol formation. This step is catalyzed by CCR. Many studies of CCR activity indicated that five cinnamoyl-CoA esters *viz. p*-coumaroyl- CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA and sinapoyl-CoA, could be used as substrate (Luderitz and Grisebach, 1981; Goffner *et al*., 1994). The CCR enzyme purified from *Eucalyptus* xylem tissue was active toward *p*-coumaroyl- CoA, feruloyl-CoA, caffeoyl-CoA and sinapoyl-CoA with approximately equal affinity (Goffner *et al*., 1994). Similar to the native protein, the recombinant *Eucalyptus* CCR protein was also demonstrated to be active with the substrates *p*-coumaroyl-CoA, feruloyl- CoA and sinapoyl-CoA (Lacombe *et al*., 1997). Recently, the characterization of aspen CCR recombinant protein indicated that CCR selectively catalyzed the reduction of feruloyl-CoA from the five-cinnamoyl CoA esters (Li *et al*., 2005). When CCR and CCoAOMT were coupled together, the linked reactions constitute the pathways from caffeoyl-CoA ester to coniferaldehyde (Fig. 5). In addition, the results also suggested that the neighboring CCoAOMT and CCR enzymes require different pH environments and compartmentalization *in vivo*. The *CCR* genes in various species appear as a multiple members' family. In the *Populus* genome, there exist 8 CCR-homolog or *CCR*-like gene sequences.

Transgenic tobacco (Ralph *et al*., 1998; O'Connell *et al*., 2002) and *Arabidopsis* (Goujon *et al*., 2003) down regulated for *CCR*, are characterized by an approximate 50% decrease in Klason lignin. The lignin S/G ratio was increased (mainly because of a decrease in the G unit amount) in transgenic tobacco and variable, depending on the growth conditions, in transgenic *Arabidopsis*. A change in the lignin structure was also indicated by the higher amount of alkali-labile material that could be released from the extractive-free lignin polymer of the transgenic lines (O'Connell *et al*., 2002). The transgenic plants with the lowest *CCR* activity and 50% reduced lignin had abnormal phenotypes, such as collapsed vessels, stunted growth, and abnormal leaf development. Important alterations in the fiber cell walls were observed, such as a loosening in the arrangement of the cellulose microfibrils that resulted in reduced cell wall

cohesion (Pincon *et al*., 2001b; Goujon *et al*., 2003; Leple *et al.,* 2007). Also an increased amount of tyramine ferulate (Figure 5), an unsual component of tobacco cell walls that is probably a sink for feruloyl-CoA, was incorporated into the lignin of the CCR-down-regulated tobacco plants (Ralph *et al*., 1998). A *CCR* mutant, designated irregular xylem (*irx4*), has been identified in *Arabidopsis* (Jones *et al*., 2001*).* Like the *CCR*down- regulated tobacco and *Arabidopsis* described above, this mutant is characterized by 50% reduced lignin content, collapse of the vessels, and an altered growth and morphology. By crossing transgenic tobacco down-regulated for *COMT* (Atanassova *et al*., 1995) with tobacco down-regulated for *CCR,* a simultaneous reduction in *COMT* and *CCR* expression was achieved (Pincon *et al*., 2001b).

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

A necessary step to biosynthesize S monolignols is hydroxylation at the 5-position on the aromatic ring of cinnamic intermediates. This reaction occurs using ferulic acid as the substrate and catalyzed by F5H, which is encoded by a P450 protein gene belonging to *CYP84* family. Although forward genetics evidence demonstrated that *F5H* gene is essential for S-lignin formation in Arabidopsis (Meyer *et al*., 1996), it was unable to identify the intermediate on which the 5-hydroxylation biochemically occurs. Homologous genes have been cloned from a number of tree species. The biochemical function of this P450 gene was first demonstrated by expressing a sweetgum *CYP84* gene in yeast (Osakabe *et al*., 1999). The biochemical data suggests that the *CYP84* protein catalyzes 5-hydroxylation using coniferaldehyde, instead of the postulated ferulic acid, as a substrate to produce 5- hydroxyconiferaldehyde. Thus, *F5H* is actually a *CAld5H*. The 5-hydroxylation of coniferaldehyde was further confirmed with an Arabidopsis *CYP84* recombinant protein (Humphreys *et al*., 1999).

According to the biochemical function of this *CYP84* gene, it was suggested that the S-monolignol biosynthesis pathway is branched out from a guaicyl intermediate at coniferaldehyde. Consistent with this view, 5-hydroxyconiferaldehyde is then methylated by *COMT* or *AldOMT* as described above. The genetic function of *CYP84* is also demonstrated through a reverse

genetics approach by over expression of the gene, which leads to the intensified S units in lignin (Franke *et al*., 2000; Li *et al*., 2003). Because the lignin with higher percentages of S-unit has a potentially significant value in the pulping economy (Chang and Sarkanen, 1973), over expression of *CAld5H* gene in trees has great potential to produce desirable wood material for fiber production. An *Arabidopsis* mutant deficient in *F5H* (*fah1*) has been described and it produced lignin deficient in S units (Chapple *et al*., 1992) with a consequently increased frequency of phenylcoumaran (β-5) and biphenyl (5-5) linkages (Marita *et al*., 1999). When *Arabidopsis F5H* was overexpressed from the *C4H* promoter in the mutant, a lignin almost entirely composed of S units linked by β-O-4 linkages was produced (Meyer *et al*., 1998; Marita *et al*., 1999). The proportion of S units in the lignin of these plants was the highest ever reported for any plant (Ralph, 1996). Similarly, lignin of tobacco and poplar transformed with the same chimeric gene was enriched in S units (Franke *et al*., 2000). Li *et al*. (2003) overexpressed a sweetgum *F5H* (*Cald5H*) under the control of a xylem-specific promoter (*Pt4CL1P*) in transgenic aspen and reported a 2.5-fold increase in the S/G ratio and no changes in lignin content. An accelerated maturation/lignification of stem secondary xylem cells was noted in these *F5H* over expressing plants (Li *et al*., 2003). A 25–35% reduction in Klasone lignin content was observed in *F5H*-overexpressing *Arabidopsis* (Marita *et al*., 1999) and tobacco (Franke *et al*., 2000).

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

The last metabolic step forming the monolignols is reduction of coniferaldehyde and sinapaldehyde. CAD is suggested to catalyze multiple cinnamyl alcohol formations from their corresponding cinnamaldehydes (Lewis and Yamamoto, 1990; Whetten and Sederoff, 1995). In loblolly pine, *CAD* is a single copy gene and its mutation leads to abnormal lignin formation in wood (MacKay *et al*., 1997; Lapierre *et al*., 2000). When the *Populus* tree was studied for monolignol biosynthesis in wood-forming tissue, in addition to *CAD*, it was found in aspen that another gene, its sequence similar to but distinct from *CAD*, is also associated with lignin biosynthesis (Li *et al*., 2001). The biochemical characterization of the

recombinant protein encoded by this gene indicated that, the enzymatic activity has specific affinity toward sinapaldehyde, therefore it was named SAD. Compared with SAD enzyme kinetics, CAD showed a catalytic specificity towards coniferaldehyde instead. The catalytic specificities of the two enzymes have recently been further verified in protein structure analysis (Bomati and Noel, 2005). Furthermore, it was demonstrated that the expression of *CAD* is associated with G-lignin accumulation while *SAD* was associated with S-lignin formation during xylem differentiation (Li *et al*., 2001). The evidence from molecular, biochemical and cellular characterizations strongly suggest that *CAD* is involved in G monolignol biosynthesis and *SAD* in S-monolignol biosynthesis in aspen wood formation. However, a recent genetic study using an Arabidopsis model system suggests a broad CAD function for both G- and S-lignin biosynthesis in the herbaceous species (Sibout *et al*., 2005). So far, nine Arabidopsis CAD members have been annotated as the bona fide CAD homologs, and their biochemical properties have been well studied (Kim *et al*., 2004). AtCADC and AtCADD of the family have been studied in null mutants and double mutants (Sibout *et al*., 2003, 2005) and proven to be the primary CAD genes in Arabidopsis lignification. Tobias and Chow (2005) have recently shown that there are 12 CAD family members in rice based on bioinformatics criteria.

Transgenic plants with reduced *CAD* activity have been produced in tobacco (Yahiaoui *et al*., 1998), poplar (Baucher *et al*., 1996) and alfalfa (Baucher *et al*., 1999), whereas *CAD* mutants exist in pine (MacKay *et al*., 1997), maize (Halpin *et al*., 1998) and *Arabidopsis* (Sibout *et al*., 2003). An unusual monomer, dihydroconiferyl alcohol, was shown to be incorporated into the lignin of the pine *CAD* mutant and accounted for 30% of the lignin compared to only 3% in wild-type lignin (Ralph *et al*., 1997). The S/G ratio of the lignin of transgenic tobacco (Ralph *et al*., 1998) and transgenic alfalfa (Baucher *et al*., 1999) was reduced, suggesting that in these plants the uncondensed S structures are more affected than their G analogs. These data are in apparent conflict with the recent proposal that *SAD*, and not *CAD*, is involved in S lignin biosynthesis in angiosperms (Li *et al*., 2001). A simultaneous down-regulation of *CAD* and *CCR* has been achieved by crossing homozygous transgenic lines in which either *CAD* (Halpin *et al*.,

1994) or *CCR* (Piquemal *et al*., 1998) was down regulated (Chabannes *et al*., 2001b). The lignin content was decreased by approximately 50% in tobacco with 32% of wild-type *CCR* activity and 12% of wild-type *CAD* activity. The phenotype of the double transformants was normal with only slight alterations in the vessel shape, showing that, similarly to the results of Hu *et al*., (1999), plants can also tolerate important reductions in lignin content.

A simultaneous suppression of *COMT* (to 24% of wild-type level), *CCR* (to18 % of wild-type 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).

1.5. Transport of monolignols

After monolignol's synthesis, the lignin precursors are transported to the cell wall where they are oxidized and polymerized. 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 and that a uridine diphosphate glucose (UDPG), coniferyl alcohol glucosyl transferase (Steeves *et al*., 2001), together with coniferin-beta-glucosidase (CBG), may regulate storage and mobilization of monolignols for lignan or lignin biosynthesis (Dharmawardhana *et al*., 1995; Samuels *et al*., 2002). Whether these glucosides are transported via Golgi-derived vesicles or through direct plasma membrane pumping by ABC transporters is still unknown (Samuels *et al*., 2002). According to early studies with radiolabeled monolignol precursors, lignification of the cell wall has been hypothesized to proceed after cell death (Pickett-Heaps, 1968). These findings are now supported by experiments with the zinnia cell system showing that lignification of tracheary elements that have undergone programmed

cell death still progresses by supply of monolignols from the surrounding xylem parenchyma cells (Hosokawa *et al*., 2001).

1.6. Dehydrogenation

After transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols (Christensen *et al*., 2000). The dehydrogenation to monolignol radicals has been attributed to different classes of proteins, such as peroxidases, laccases, polyphenol oxidases, and coniferyl alcohol oxidase. Which of these enzymes or a combination thereof are responsible for the dehydrogenation of the monolignols in planta and whether monolignol oxidation occurs through redox shuttle-mediated oxidation are still unclear (Onnerud *et al*., 2002). Although peroxidases are believed to catalyze the final condensation of cinnamyl alcohols in the formation of lignin, no definitive proof has been presented yet for the involvement of any specific peroxidase isozyme *in vivo*, mainly because of the high number of genes that encode peroxidases (Tognolli *et al*., 2002) and the typically low substrate specificities of these enzymes. Peroxidases use hydrogen peroxide (H_2O_2) to oxidize their substrates (Christesen *et al*., 2000). How H_2O_2 is generated in the cell wall is still a matter of debate. Evidence is emerging for a role for an NADPH oxidase in lignifying tissues, which would supply H_2O_2 for monolignol oxidation (Ogawa *et al*., 1997; Ros-Barcela *et al*., 2002).

Five divergent laccase genes have been cloned and characterized from poplar (Ranocha *et al*., 1999, 2000). Both anionic and cationic peroxidases have been implicated in lignification based on their affinity for coniferyl alcohol, their location in the cell wall and their expression in lignified tissue (Lagrimini *et al*., 1987). Nevertheless, no change in lignin content was obvious in transgenic tobacco plants that were deficient in the major anionic peroxidase (Lagrimini *et al*., 1997a). However, transgenic poplar with a 44% reduction in the activity of a stem-specific anionic peroxidase (PRXA3a) had a 21% reduced lignin content and a higher content in β-O-4 linked (uncondensed) structures in lignin (Yahong *et al*., 2001).

The over expression of peroxidase genes in transgenic poplar (PXP 3-4; Christensen *et al*., 2001a, 2001b) and in tobacco (Elfstrand *et al*., 2002) resulted in 800-fold and 5-fold increased total peroxidase activity, respectively. No effects of the genetic modification on the overall phenotype, the Klasone lignin content, or the recovery yield of G and S units were identified. Transgenic *Liriodendron* (Dean *et al*., 1998) and poplar (Ranocha *et al*., 2000, 2002) downregulated in laccase had neither altered phenotype nor any change in lignin amount or S/G composition. However, in transgenic poplar down-regulated for one of the laccase genes (*lac3*), the walls of xylem cells were irregular in contour when compared with the control and had adhesion defects either at the primary cell wall of adjacent cells or within the secondary cell wall of a given cell (Ranocha *et al*., 2002).

1.7. Polymerization

1.7.1. Radical generation and radical coupling

After their dehydrogenation, the radicals, which are relatively unstable owing to electron delocalization that provides single-electron density to the side-chain position, are coupled. The most important reaction is cross-coupling to the growing polymer to extend the complex three-dimensional lignin network (Fig. 3). 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 polymers 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.

1.7.2. Polymerization Process

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 (Ralph *et al*., 1999), deriving from radical coupling reactions under chemical (but no apparent biochemical control) between phenolic radicals in an essentially combinatorial fashion. The accepted model for lignin polymerization based on simple chemically controlled combinatorial coupling reactions, was recently challenged by Devin and Lewis (2000). It was proposed that the macromolecular assembly of lignin is not based on "random coupling" of monolignols. The new theory arose from the discovery of a class of dirigent proteins implicated in lignan biosynthesis (Davin *et al*., 1997). The first such dirigent protein discovered guided the dimerization of coniferyl alcohol radicals to produce an optically active lignan, pinoresinol. The corresponding gene was cloned and shown to encode a cell wall–localized protein. The finding was extrapolated to lignification, suggesting that such proteins would logically be responsible for specifying the exact structure of the lignin polymer, bringing lignins in line with proteins and polysaccharides that are more carefully biosynthesized (Devin and Lewis, 2000; Lewis, 1999).

1.7.3. Nucleation sites

Lignin is first deposited in the middle lamella and the cell corners of the primary wall after the formation of the secondary wall has started, nucleation sites, from which the lignin polymers can grow. The nature of these nucleation sites is unknown. Ferulates, conjugated to polysaccharides, and their dehydrodimers are well established. Evidence that ferulates and diferulates may act as attachment sites for monolignols (Ralph *et al*., 1995). Given that the middle lamella and the cell corners are rich in Ca^{2+} pectate (Carpita and Gibeut, 1993) and are the first sites to be lignified, Ca^{2+} pectate-bound peroxidases may conceivably play a role in the spatial control of lignin deposition, and changes in Ca^{2+} and H^{+} concentrations may modulate the location of these peroxidases (Carpin *et al*., 2001). The negatively charged pectins are also good binding sites for polyamines (Carpin *et al*., 2001) and, hence, may be suitable sites for H_2O_2 generation by polyamine oxidases (Moller and McPherson, 1998). Pectin-binding peroxidases

and polyamine oxidases may act locally in the early stages of lignin deposition both for H_2O_2 generation and oxidation of monolignols, cinnamic acids bound to polysaccharides or polyamines, or aromatic residues on certain proteins, such as glycine-rich proteins (Keller *et al*., 1989).

As described above, many different transgenic plants and a few mutants are now available with altered lignin content, altered lignin composition/ structure, or both. Whether or not these changes in cell wall biochemistry could have advantages in industrial operations, such as pulping, can only be determined experimentally. A few such experiments have been performed. To put the results into context are described the different methods and parameters important in the production of pulp and paper before reviewing the impact that specific genetic engineering can have on pulping properties.

1.8. Pulp and Paper Industry

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

To produce bright and good quality paper, pulp may require a further bleaching treatment to remove lignin residues. The delignification process consumes large quantities of energy and hazardous chemicals. Reducing the content or changing the quality of lignin in pulp wood species without compromising the mechanical strength of the plant is desirable for paper industry. This would be beneficial, both from the economical as well as environmental point of view. The results could be extrapolated to the forage crops to improve digestibility since the presence of lignin limits the ability of microorganisms to break down the cellulose and hemicellulose in the animal alimentary canal. Thus, it has become imperative to

achieve self sufficiency in paper and pulp production, which may be achieved with the development of fast growing trees providing higher biomass with low lignin content per unit of land. Wood, agricultural residues and many other plant materials, which can be used for pulp and paper production, consist largely of lignocellulose (a composite of mainly cellulose, hemicellulose and lignin). In the production of paper, pulp (which is mainly cellulose), the hemicellulose and much of the lignin are removed using mechanical or chemical processes or a combination of both.

1.9. Aims of the thesis

Lignin is one of the main structural elements of wood, and in angiosperms it is mainly composed of G (guaiacyl) and S (syringyl) monomers (Baucher *et al*., 1998; Boerjan *et al*., 2003). Considerable scientific interest has been focused on the development of trees with improved wood quality through modification of the genes involved in lignin biosynthesis, could be important for the improved end use of wood material (Chiang 2006; Higuchi 2006). In chemical wood pulping, lignin is the main factor hindering the effective utilisation of cellulose fibres, from which it needs to be separated by costly and pollutant-generating processes (Chiang, 2002; Baucher *et al*., 2003; Boerjan, 2005). Genetic modifications resulting in increased delignification (through a modified lignin content and/or chemical composition) could thus be highly beneficial at both the economical and environmental scale (Baucher *et al*., 2003; Boerjan, 2005; Chiang, 2006). 4CL (4-Coumarate Co A ligase, EC (6.2.1.1.2)) is one of the important key enzymes of general phenyl propanoid metabolism, which provides the precursors molecules for lignin biosynthetic pathway. The Study of 4CL gene and its role in lignin synthesis is of great importance (1.6.4). The present study was done with the aim to isolate, clone and characterized 4CL gene and its role in lignin biosynthesis. Furthermore, development of *Leucaena leucocephala* transgenic lines with anti 4CL and analysis of these transgenic lines for down regulation of 4CL and other wood characters.

1.10. *Leucaena sp.*

Leucaena is a native of Central America and has been naturalized pan-tropically. Members of the genera are vigorous, drought tolerant, highly palatable, high yielding, and rich in protein and grow in a wide range of soils (Jones, 1979; Hughes, 1998). However, these attributes are limited by the occurrence of anti-nutritive factors in the fodder, such as tannins and mimosine (Jones, 1979; Hegarty *et al*., 1964b; Hammond *et al*., 1989 a, b). *Leucaena* occupies 2 to 5 million hectares of land worldwide (Brewbaker and Sorenson, 1990). They are recognized as some of the fastest growing and most useful trees in the tropics (NRC, 1984). *Leucaena* is represented by 22 species. Of these, 6 are intraspecific taxa and 2 are widespread spontaneous hybrids. Most of the species are diploid 2n=52 or 56. However, 4 species are tetraploid 2n = 4X=104 or 112 (Hughes, 1998). *L. leucocephala* is a member of the genus related to the other species within the Mimosoideae sub-family, its subspecies and other related genera.

Fig. 1.6: *Leucaena leucocephala* plants at different stages. (a) *Leucaena leucocephala* plants in its full bloom, (b) *Leucaena* plants with immature pods, (c) *Leucaena* plants with mature pods and flowers.

Classification:

Classification of *Leucaena leucocephala*

Kingdom	Plantae
Super division	Tracheobionta
Division	Spermatophyta
Subdivision	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Fabales
Family	Fabaceae
Genus	*Leucaena*
Common name	Lead tree, white popinac
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*

1.10.1. *Leucaena leucocephala* species growth forms

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

1.10.2. Fodder production and feed value of *Leucaena leucocephala.*

Leucaena leucocephala produces 6 to 18 tons of forage dry matter per hectare in the wet season and 2 to 3 tons of dry matter per hectare in the dry season. All

parts of *Leucaena* are edible to livestock (Shelton, 1995). *Leucaena* leaves contain about 19 to 26 percent crude protein. They are rich in source of carotene and vitamins. The provitamin-A in *Leucaena* is among the highest ever recorded in a plant specimen (Jones, 1994). *Leucaena* is rich in calcium and phosphorus, but deficient in sodium and iodine. The *in- vivo* digestibility is 50 to 70 percent (Norton and Poppi, 1995). The presence of mimosine, a tyrosine analogue, a toxic non-protein amino acid, limits the use of this plant as forage (Allison *et al*., 1990; Gupta and Atreja, 1999; Tangendjaja and Willis, 1980). It is also efficient in nitrogen fixation, at more than 500 kg/ha/year. During the 1970s and 1980s it was promoted as a "miracle tree" for its multiple uses. It has also been described as a "conflict tree" in that it is both promoted for forage production and spreads like a weed in some places.

1.10.3. Wood

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

1.11. Why *Leucaena leucocephala* was selected for study

Paper industry in India mainly uses bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena sp*. as a source for paper pulp. Selection of the species depends upon availability, price and acceptability by any one given industrial unit. In bamboo growing countries, like India, the proportionate use of bamboos and hardwood

species is in the ratio of 15:85. Although all these plant species are of importance to the paper industry, *Leucaena sp*. is exclusively used in India and about 25% of raw material for pulp and paper industry comes from this plant. *Leucaena sp.* is a fast growing multipurpose tree adapted to a variety of soils and climatic conditions. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. However, as a safeguard for the future no plant should be harvested from areas that may challenge sustainability. It will thus be crucial to raise plantations of the plant species with elite materials and or genetically modified plants that meet the demands of the pulp and the paper industry in economical and sustainable manner. However no study has been done on Lignin Biosynthesis gene(s) so far in *Leucaena sp.* and study of these gene(s) will help in understanding the Lignin Biosynthetic Pathway in *Leucaena sp*. and its manipulation so as to meet the needs of pulp and paper industry.

Chapter 2

2. Materials and methods

This chapter deals with the general laboratory techniques routinely followed during the course of work. Other important specific methodologies followed will be discussed separately in the respective chapters.

2.1. Plant Material

2.1.1. *Leucaena leucocephala*

Studies on in vitro plant regeneration were carried out using seeds obtained from field grown *L. leucocephala* cultivar K-636. Seeds of *L. leucocephala* (K-636) were treated with conc. H_2SO_4 for 2 – 3 min and then washed extensively with tap water. The scarified seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 10 min followed by five rinses with sterile deionized water. The seeds were soaked in sterile water for two days and then transferred to ½ x MS basal medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 1.5% glucose for germination. The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. The culture bottles were incubated at 25± 2^0C and 60% relative humidity under 16 h photoperiod. One month old axenic cultured pants were the raw material for further experiments.

2.1.2. *Nicotiana tabacum*

Tobacco seeds *Nicotiana tabacum* var. Anand 119 were germinated on wet sterile paper towel. Germinated seeds were transferred to ½x MS basal medium (Murashige and Skoog, 1962) supplemented with 2% sucrose and 1.5% glucose for germination. The medium was solidified with 0.8% agar. The pH of the medium was adjusted to 5.8 prior to autoclaving. The culture bottles were incubated at 25± 2 ^{0}C and 60% relative humidity under 16 h photoperiod. One month old axenic cultured pant leaves were the raw material for further experiments.

2.2. Glassware

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

2.2.1. Preparation of Glassware

Glassware used for all the experiments were cleaned by boiling in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Thereafter, it was immersed in 30% HNO_3 solution for 30 min followed by repeated washing in tap water. Washed glassware were thereafter dried at room temperature. Test tubes and flasks were plugged with absorbent cotton (Mamta Surgical Cotton Industries Ltd., Rajastan, India). Autoclaving of the glassware and above items was done at 121 ^{0}C and 15 psi for 1 h.

2.3. Plasticware

Sterile disposable filter sterilization units (0.22 μm) and petridishes (55 mm and 85 mm diameter) were procured from "Laxbro", India. Eppendorf tubes (1.5 mL and 2 mL capacity), microtips (10, 200 and 1000 μL capacity) and PCR tubes (0.2 mL and 0.5 mL capacity) were obtained from "Tarsons" and "Axygen", India.

2.4. Chemicals

Acetosyringone, Tris, IPTG, X-gal, SDS, BSA, EDTA, PMSF, PIPES, DTT, PVPP, APS, Imidazole, TES buffer, Urea and Ethidium bromide were purchased from Sigma-Aldrich (USA), Bio-world (USA). Agarose, restriction enzymes, T4 DNA ligase, RNase A and lysozyme were obtained from NEB (USA), Promega (USA), Bioenzymes(USA) and Amersham (UK). Different kits were purchased from BD CLONETECH (JAPAN). Invitrogen (USA), Promga (USA), and Sigma-Aldrich (USA). Taq DNA polymerase was obtained from Sigma-Aldrich (USA) and Bangalore Genei (India). Plasmid vectors Pichia vector, pGEM-T Easy Vector and

pET30b (+) were purchased from Invitrogen (USA), Promega (USA) and Novagen (USA) respectively. Megaprime labeling kit and Hybond-N+ membrane were obtained from Amersham (UK). [α-32P]-dATP and [α-32P] –dCTP were obtained from Bhabha Atomic Research Centre (BARC), India. X-ray films were obtained from Konica (Japan) or Kodak (USA). Agarose A (Sigma), Affi-gel matrices, gold particles were purchased from BioRad (USA). All other chemicals and solvents of analytical grade were purchased from HiMedia, Qualigens Fine Chemicals and E-Merck Laboratories, India. All chemicals used in the tissue culture study were of analytical grade (AR) and were obtained from "Qualigens", "S.D. Fine Chemicals" or "HiMedia", India. The Sucrose, glucose and agar-agar were obtained from "Hi-Media". Bacto-Agar for microbial work was obtained from "DIFCO" laboratories, USA.

2.5. Equipment

Tab 2.1: Equipment

S.No.	Equipment	Make
1	Balances	Contech/ Sartorious
2	Water bath	Julabo/
3	Dry Bath	Eppendorf/BGenei
4	Incubator	New Brunswick
5	Centrifuge	Sorvall/Haereus/eppendorf/Sigma
6	Gel Documentation system	Bio-Rad
7	Thermo Cycler PCR machine	MJResearch/Stratagene
8	Spectrophotometer	Applied Biosystem
9	Power pack	Bio-Rad
10	Agarose Gel Electrophoresis Units	Bangalore Genei/ Bio-Rad
11	protein Gel Electrophoresis Units	Hoeffer Scientific/ BioRad
12	Speed Vac concentrater	Savant
13	pH-Meter	Global
14	Water purification system	Millipore Unit (Milli RO/ Milli Q)
15	Microwave oven	Bilbol
16	Fridge/0 C/Deep freezer	Vestfrost/Leonard/Godrej
17	Magnetic rotator	REMI
18	Laminar Air Flow	
19	Particle bombardment system	Bio-Rad
20	ELISA Plate Reader	Amersham

2.6. Buffers and solutions

2.6.1. Buffers and Solutions for DNA Electrophoresis

Tab. 2.2: Buffers and Solutions for DNA Electrophoresis

Name	Ingredients	Preparation and Storage
50x TAE	2 M Tris 0.05 M EDTA	pH was Adjusted to 8.0 with glacial acetic acid and stored at RT
TBE buffer	90 mM Tris 90 mM Boric acid 2 mM EDTA	RT
DNA loading buffer	0.25 g Xylencyanol 0.25 g Bromophenol blue 0.25 g Ficoll 400 1.46 g EDTA make up the volume to 100ml with H_2O	The solutions were filtered sterilized using .22µ filter and stored at RT

2.6.2. Buffers and Solutions for genomic DNA isolation, Southern and Slot Blot

Tab. 2.3: Buffers and Solutions for g-DNA isolation, Southern and Slot Blot

Name	Ingredients	Preparation and Storage
Extraction buffer	100 mM Tris-HCl (pH 8.0) 20 mM Na EDTA (pH 8.0) 1.4 M NaCl 2.0% (w/v) CTAB Add ßmercaptoethanol 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

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

Tab. 2.4: Solutions for the Transformation and Selection of *E. coli*

Name	Ingredients	Preparation and Storage
IPTG solution	200 mg/mL in SMQ	Sterile filtration and Storage at -20 ^{0}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^{0}C
Ampicillin	100 mg/mL in SMQ	Sterile filtration and Storage at -20 ^{0}C
Tetracycline	12.5 mg/mL in 70% ethanol	Sterile filtration and Storage at -20 ^{0}C
Kanamycine	50 mg/mL in SMQ	Sterile filtration and Storage at -20 ^{0}C
Riffamycine	50 mg/mL in DMSO	Sterile filtration and Storage at -20 ^{0}C
Hygromycin	25 mg/mL in SMQ	Sterile filtration and Storage at -20 ^{0}C

2.6.4. Buffers and Solutions for Plasmid Isolation (Alkaline lysis)

Tab. 2.5: Buffers and Solutions for Plasmid isolation (Alkaline lysis)

Name	Ingredients	Preparation and Storage
Solution I (TEG)	25 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM Glucose.	Stored at 4 ^{0}C
Solution II	0.2 N NaOH, 1% SDS	Freshly Prepared
Solution III	3 M Potassium acetate (pH 4.8)	Stored at 4 ^{0}C
RNase A	10 mg/mL	Stored at -20 ^{0}C
Other solutions	Chloroform, Absolute ethanol, 3.0 M Sodium acetate 70% ethanol Deionized water	

2.6.5. Buffers and Solutions for Gel Electrophoresis (SDS-PAGE)

Tab. 2.6: Buffers and Solutions for Gel Electrophoresis (SDS-PAGE)

Name	Ingredients	Preparation and Storage
Monomer solution	29.2% acrylamide 0.8% bisacrylamide in water	Stored at 4 ^{0}C in the dark
Stacking gel	Distilled water 3.4 mL 1 M Tris-HCl (pH 6.8) 0.63 mL Acrylamide/bis 30% 0.83 mL 10% (w/v) SDS 0.05 mL 10% (w/v) APS 0.05 mL TEMED 5 µL	

Separating gel (12%)	Distilled Water 3.3 mL 1.5 M Tris-HCl (pH 8.8) 2.5 mL Acrylamide/Bis 30% 4.0 mL 10% (w/v) SDS 0.1 mL 10% (w/v) APS 0.1 mL TEMED 4 µL	
2x Protein loading buffer	Distilled Water 2.7 mL 0.5 M Tris-HCl (pH 6.8) 1.0 mL Glycerin 2.0 mL 10% (w/v) SDS 3.3 mL β-Mercaptoethanol 0.5 mL 0.5% (w/v) Bromophenol blue 0.5 mL	Store at 4 ^{0}C
10x SDS-electrode buffer	Tris base 15 g Glycine 72 g SDS 5 g Water up to 500 mL	Store at 4 ^{0}C, dilute 1:10 before use
Staining solution	Coomassie-blue R 250 25 mL Methanol 100 mL Acetic acid 20 mL Water up to 200 mL	
Coomassie blue	Coomassie-blue R 250 0.5 g Water up to 50 mL	Dissolve the dye in water and filtrate
Destaining solution	Methanol 30 mL Acetic acid 20 mL Distilled water up to 200 mL	
Silver staining Fixing solution	30% ethanol (150 mL) 10% acetic acid (50 mL) Distilled water up to 500 mL	Store at room temperature
Sensitizing solution	30% ethanol (150 mL) 0.5 M sodium acetate 0.2% $Na_2S_2O_3$ Distilled water up to 500 mL	Store at room temperature
Silver solution	0.2% silver nitrate (0.6 g) 0.01 % formaldehyde (30 µL) Distilled water up to (300 mL)	Prepare fresh
Developing solution	6 % Na_2CO_3 (18 g) 0.02 % formaldehyde (60 µL) Distilled water up to 300 mL	Prepare fresh
Stop solution	1.5% Na_2EDTA (4.5 g) Distilled water up to 300 ml	Store at room temperature

2.6.6. Buffers and Solutions for Protein Extraction

Tab 2.7: Buffers and Solutions for Protein Extraction

Name	Ingredient	Preparation and storage
Lysis buffer	50 mM Tris-HCl (pH 8.) 5 mM EDTA 100 mM NaCl 0.5% Triton-X100 0.7 mM DTT 0.1 mM PMSF (Freshly added). 10 mM Mg SO_4 Lysozyme100µg/mL (Added freshly).	Stored at 4 ^{0}C
Sonication buffer	100 mMTris HCl (pH8.0) 50 mM Glycine	Stored at 4 ^{0}C
Dispersion buffer:	100 mM TrisCl(pH8.0) 50 mM Glycine 8 M Urea	Stored at 4 ^{0}C

2.6.7. Buffers and Solutions for Protein Purification

Tab 2.8: Buffers and Solutions for Protein Purification

Name	Ingredients	Preparation and Storage
Binding buffer	35 mM Tris 150 mM NaCl 10 mM imidazole 5% glycerol (pH 7.2)	Adjust pH by adding concentrated HCl and Store at 4 ^{0}C
Wash buffer	50 mM Tris 300 mM NaCl 25 mM imidazole 10% glycerol (pH 6.8)	Adjust pH by adding concentrated HCl and Store at 4 ^{0}C
Elution buffer	50 mM Tris 300 mM NaCl 200 mM imidazole 10% glycerol (pH 6.8)	Adjust pH by adding concentrated HCl and Store at 4 ^{0}C

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

Tab 2.9: Buffers and Solutions for ELISA/Immunocytolocalization/GUS Assay/ particle coating.

Name	Ingredients	Preparation and Storage
Crude protein extraction buffer	100mM Tris (pH 7.4) 2% PVPP 2% PEG 6000 10 mM PMSF 5 mM DTT	Stored at RT
PBS T	1.44 g Na_2HPO_4 0.24 g KH_2PO_4 0.2 g KCl 8 g NaCl 0.05% v/v Tween-20	Stored at RT
Antibody dilution Buffer	PBS with 0.25% BSA	store at 4 ^{0}C
Subustrate buffer	200 mM TrisCl pH 9.5 0.5 mM $MgCl_2$	Store at 4 ^{0}C
1X PBS	10 mM NaH_2PO_4- Na_2HPO4 Buffer (pH 7.2) 130 mM NaCl	Adjust pH to pH 7.2 and Stored at 4 ^{0}C
0.5 X SSC	75 mM NaCl 7.5 mM Na Citrate	Adjust pH to pH 7.0 and Stored at 4 ^{0}C
Color development buffer	100 mM Tris (pH 9.5) 150 mM NaCl, 50 mM $MgCl_2$	Stored at 4 ^{0}C
BCIP/NBT mix	0.577 mM BCIP 0.122 mM NBT	Stored at 4 ^{0}C In Dark
Stop Solution	10mM EDTA	Stored at RT
Other reagents used	Polyvinyl alcohol, Ethanol, Tertiary butanol, Paraffin, Xylene, Glycerol	

X-GluC	1 mM X-Gluc (5-bromo, 4-chloro, 3-indolyl-β-D-glucuronide: Cyclohexylammonium salt (X-GlcA) in dimethylformamide 100 mM sodium dihydrogen phosphate dihydrate 05% Tween-20	Adjust pH to 7.0 and Stored at 4 ^{0}C
Phloroglucinol	2% Phloroglucinol in 95% Ethanol	Stored at 4 ^{0}C
Xho buffer	150 mM Nacl 10 mM Tris Cl pH 8.0	Stored at -20 ^{0}C
Spermidine	0.1 M Spermidine in H_2O	Stored at -20 ^{0}C
$CaCl_2$	2.5 M $CaCl_2$	Stored at -20 ^{0}C

2.6.9. Component of murasighe and Skoog Media/ Different inducing media and hormones

Tab 2.10: Component of murasighe and Skoog Media / Different inducing media and hormones

Name	Ingredients	Preparation and Storage
Major component	20.61 mM NH_4NO_3 18.75 mM KNO_3 2.99 mM $CaCl_2.2H_2O$ 1.5 mM $MgSO_4.7H_2O$ 1.24 mM KH_2PO_4	Stored At 4 ^{0}C
Minor Components	0.147 mM $MnSO_4$ 5.3 x 10^{-2} mM $ZnSO_4$ 1.56 x 10^{-4} mM $CuSO_4$ 1.05 x 10^{-4} mM $CoCl_2.6H_2O$ 4.99 x 10^{-3} mM KI 0.1mM H_3BO_4 1.03 x 10^{-3} mM $Na_2Mo_4.2H_2O$	Stored At 4 ^{0}C
Vitamins	5.55 x10^{-2} mM Myoionsitol 4.06 x 10^{-3} mM Nicotinic acid 2.43 x 10^{-3}mM Pyridoxine HCl 2.96 x 10^{-4} mM Thymine HCl 2.66 x 10^{-2} mM Glycine	Stored At 4 ^{0}C
Iron	0.1mM $FeSO_4.7H_20$ 0.1mM Na_2EDTA	Stored At 4 ^{0}C
BAP	1.776 mM BAP (dissolve in NaOH and make up the volume by adding ethanol)	Stored At 4 ^{0}C
NAA	2.148 mM NAA (Dissolve in H_2O)	Stored At 4 ^{0}C
Shoot Induction Medium	Major (40x)-25mL Minor (100x)-10mL Iron (100x)-10mL Vitamins (200x)-5mL	Stored At 4 ^{0}C

	BAP (4.4 µM))-2.5mL NAA (5.37 µM)-250µL Glucose-1.5% Sucrose-2.0% pH-5.6 to 5.8 Agar-0.8% (make up to volume 1L)	
Resuspension Medium	Major (40x) 25mL Minor (100x) 10mL Iron (100x) 10mL Vitamins (200x) 5mL BAP (4.4µM) 2.5mL NAA (5.37µM) 250µL Glucose 1.5% Sucrose 2.0% pH-5.6 to 5.8 Acetosyringone-200µM $MgSO_4$ 40mM (make up to volume 1L)	Stored At 4 ^{0}C
Root Induction Medium	Major(40x) 25mL Minor(100x) 10mL Iron (100x) 10mL Vitamins (200x) 5mL NAA(5.37 µM) 250µL Glucose 1.5% Sucrose 2.0% pH-5.6 to 5.8 Agar 0.8% (make up to volume 1L)	Stored At 4 ^{0}C
Selection medium	7.09 µM hygromycin 200/250 µM cefotaxime	

2.7. Host Cells

Tab 2.11: Host Cells

E.coli	**Genotype**
DH 5α	*F' _80_lacZ_M15 end A1 hsdR17 (rk-mk+) supE44 thi-1_-gyrA96 relA1 _(lacZYA-argFV169) deoR*
JM 109	*e14-(McrA-) recA1 endA gyrA96 th-1 hsdR17(rk-mk+) supE44 relA1 _(lac-proAB) [F' traD36 proAB lacqZ _M15*
XL1 Blue	*RecA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)
TOPO 10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (StrR) endA1 nupG*
BL 21	*F-, ompT hsdSB (rB – mB -) gal dcm (DE3) pLysS (CamR)*

2.6.10. Different Media used for studies

Tab. 2.11: Different Media used for studies

Name	Ingredients	Preparation and storage
Luria Bertani Broth (LB)	1% Bactotryptone 0.5%Yeast extract 1% NaCl	pH adjusted to 7.0 with NaOH, Store at room temperature or at $+4^0$ C
SOB media	2% Bactotryptone 0.5% Yeast extract 10 mM NaCl 10 mM $MgCl_2.6H_2O$ 2 mM KCl	pH adjusted to 6.8 with NaOH, Store at room temperature or at $+4^0$ 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% Bactotrypton 1% Yeast extract 0.1% Glucose	pH adjusted to 7.0 with NaOH, Store at room temperature or at $+4^0$ C

2.7. Methods

2.7.1. Bacterial culture conditions

E. coli was grown at 37 ^{0}C with shaking at 200 rpm in Luria Bertani (LB) broth/ SOB broth and maintained on LB/ SOB plates with 1.5% agar (Sambrook et al., 1989). For plasmid DNA preparation recombinant *E. coli* was grown in LB media supplemented with appropriate antibiotic.

2.8. Bacterial transformation

2.8.1. Preparation of competent cells using TB Buffer

A single colony of *E.coli* XL1 Blue was inoculated in 5 mL of LB medium containing tetracycline (12.5mg / L.) and grown overnight at 37 ^{0}C in incubator shaker at 200 RPM. 500 µL of the overnight grown culture was added to 50 mL of SOB medium and grown for 2-3 hours at 37 ^{0}C in incubator shaker at 200 RPM. Cells were kept on ice for 10 min and harvested by centrifugation at 5,000 RPM for 10 minutes at 4 ^{0}C. The cell pellet was suspended in 15 mL ice-cold TB-buffer and kept on ice for 15 min

and centrifuged at 5,000 g for 10min at 4 ^{0}C. The pellet was resuspended in 5 mL of ice cold TB-buffer containing 7% DMSO. This was then dispensed in 100 µL aliquots to 1.5 mL eppendorf tubes, frozen in liquid Nitrogen and stored at -80 ^{0}C.

2.8.2. Preparation of competent cells using $CaCl_2$

A single colony of *E.coli* XL1 Blue was inoculated in 5 ml of LB medium containing tetracycline (12.5mg / L). LB medium (50 mL) was inoculated with 1% of the overnight grown *E. coli* culture and allowed to grow till 0.5 O.D. at 600nm. The cells were harvested by centrifugation at 5,000 g for 10 min at 4 ^{0}C, suspended in 100 mM ice-cold $CaCl_2$ and kept on ice for 30 min. Cells were centrifuged, the pellet suspended in 1mL of 100 mM ice-cold $CaCl_2$ and stored as aliquots of 200 µL at 4 ^{0}C.

2.8.3. Preparation of competent cells for *Agrobacterium*

The method adopted for the preparation of *A. tumefaciens* competent cells was essentially as reported by Annes (1987). A single colony of *A. tumefaciens* (GV2260) was inoculated in 50 mL LB broth containing Rifampicin 150 µg / mL and incubated at 28 ^{0}C with shaking at 200 rpm till O.D. was 0.5 at 600nm. Cells were centrifuged at 5000 g for 10 min at 4 ^{0}C and washed twice with ice-cold 150 mM $CaCl_2$. The cells were pelleted and resuspended in 1mL of ice-cold 20 mM $CaCl_2$. Aliquots of 200 µL were made for further use.

2.8.4. *E. coli* transformation

The competent *E. coli* cells, thus formed, were transformed according to Sambrook et al. (1989). Briefly, DNA (~50 ng in 10 µL or less) was added to the (200 µL) competent *E. coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42 ^{0}C for 2 min. To each tube 800 µL of LB broth was added and further incubated at 37 ^{0}C for 1 h. About 100 µL of the transformed competent cells were spread onto LB plates containing appropriate antibiotics, IPTG and X-gal as per need (Sambrook et al., 1989).

2.8.5. *A. tumefaciens* transformation and selection

For transformation 1μg DNA of the desired binary plasmid vector was added to an 200μL 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 ^{0}C. After thawing 1mL LB medium was added and the tubes incubated at 28^0 C for 2 h with gentle shaking. The cells were centrifuged at 4000 g for 5 min and 100 μL supernatant was retained. The cells were resuspended in the 100 μL supernatant and plated on LB agar medium with appropriate antibiotic(s). The plates were incubated at 28 ^{0}C for two days to allow the transformed colonies to grow for further processing.

2.9. PCR Colony Screening

This method bypasses DNA purification, and relies on the selectivity of PCR amplification to determine whether a bacterial colony of interest does indeed contain the desired DNA. Simply adding a small portion of a bacterial colony to a PCR master mix will introduce enough templates DNA for amplification. A single bacterial colony was added to 1.5 mL eppendorf PCR tube containing 25 μL of water. The microtip was agitated in the water to remove the colony. Subsequently, the 2 –3 μL of this suspension was put into 15 μL of PCR reaction. The remaining components were added to the PCR reaction and subjected to normal cycling parameters for the particular primers. If insert orientation, as well as presence, needs to be determined, utilization of a forward vector-specific primer and a reverse insert-specific primer, or vice versa, allows such determination. If only the presence of the insert needs to be determined, then two insert-specific primers can be used. An additional 5 min denaturation step at 95 ^{0}C before the amplification cycles will aid in lysing the bacteria to enhance PCR product amplification success. The resulting PCR products were checked on an agarose gel for the presence of the predicted band.

2.10. Isolation of Nucleic Acids

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

The alkaline lysis method of Sambrook *et al*., (1989) was improvised upon so that 12-24 samples could be processed conveniently for plasmid DNA extraction within 3 h, with yields of 5-30 µg per 1.5 mL culture depending on the host strain and the plasmid vector. An important feature of this protocol was the use of PEG for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination. The bacterial cultures were grown overnight (O/N) with shaking (200 rpm) at 37 ^{0}C in LB broth, with appropriate antibiotic(s). About 1.5 to 3 mL culture was centrifuged for 1 min at 4000 g to pellet the bacterial cells. The pellet was resuspended in 100 µL of TEG buffer (Tab: 2.5) by vigorous pipetting, 200 µL of Soln. II (Tab: 2.5) was added, mixed by inversion till the solution becomes clear and incubated on ice for 5 min. The cell lysate was neutralized by addition of 150 µL of Soln. III (Tab: 2.5), mixed well and incubated on ice for 5 min. The cell debris was removed by centrifuging for 5 min at 12000 g at room temperature. The supernatant was transferred to a clean tube, RNase A to a final concentration of 20 µg/ mL (Sambrook et al*.,* 1989) was added and incubated at 37 ^{0}C for 20 min. To the above solution 400 µL of chloroform was added, mixed for 30 s and centrifuged for 5 min at 12000 g at 4 ^{0}C. The upper aqueous layer was transferred to a clean tube, 1/10th volume sodium acetate and one volume absolute ethanol was added with mixing and kept at -20 ^{0}C for 1-2 h. The sample was centrifuged at 12000 g for 10 min at room temperature. The pellet was washed thrice with 70% ethanol and dried under vacuum. The dried pellet was dissolved in 40 µL of deionized water and 40 µL of PEG/NaCl solution (20% PEG 8000 in 2.5 M NaCl) was added. The mixture was incubated on ice for 20 min and the plasmid DNA pelleted out by centrifugation at 12000 g for 15 min at 4 ^{0}C. The supernatant was aspirated carefully, the pellet washed with 70% ethanol and air-dried. The dried pellet was resuspended in 20 µL deionized water and stored at -20 ^{0}C.

2.10.2. Isolation of plasmid DNA from *Agrobacterium* cells

Overnight grown culture of *A. tumefaciens* was centrifuged at 4,000 g for 10 min. The cells were washed with 500 µL of Sol I (Tab: 2.5) containing 10 µL Triton X 100 and resuspended in 300 µL of Soln I (Tab: 2.5) and 400 µL of Soln II. (Tab: 2.5). The cells were mixed by inverting till the solution turns clear. This was followed by the addition of 400 µL of Soln III. (Tab: 2.5). The suspension was mixed by inverting the tubes several times and centrifuged at 12,000 g for 10 min. Clear supernatant was transferred to fresh 1.5 mL tubes and 2 µL of RNase A (10 mg/ mL) added. The tube was incubated at 37 ^{0}C for 30 min and then extracted with 400 µL of Chloroform: isoamyl alcohol. For phase separation the tubes were centrifuged at 12,000 g for 10 min. The upper aqueous phase was transferred to a 1.5 mL tube and 600 µL of isopropanol added. It was mixed thoroughly and the plasmid DNA pelleted by centrifugation at 12,000 g for 10 min. The DNA pellet was washed with 70% ethanol, air-dried, dissolved in 40 µL deionized sterile water and stored at -20 ^{0}C till further use.

2.10.3. Isolation of plant Genomic DNA

Genomic DNA was isolated by using the protocol of Lodhi et al. (1994). Fresh young leaves were collected, frozen in liquid nitrogen and crushed to a fine powder. About 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 inverted several times to thoroughly mix the slurry, incubated at 60 ^{0}C for 30 min and then allowed to cool down to room temperature. 12 mL of chloroform: isoamylalcohol mix was added and the contents mixed by inverting the tube gently till an emulsion formed. The mix was then centrifuged at 6,000 g for 15 min at room temperature. Supernatant was carefully collected in a fresh tube and chloroform: isoamylalcohol (24:1) extraction step repeated. To the clear supernatant 0.5 volume of 5 M NaCl was added and mixed gently. Next two volumes of cold (-20 ^{0}C) 95% ethanol was added and the sample kept at 4 ^{0}C until DNA strands appeared. The tube was centrifuged at 3,000 g for 3

min and then at 5,000 g for next 3 min. The supernatant was poured off, the DNA pellet washed with cold (4 ^{0}C) 70% ethanol and air-dried. DNA was dissolved in 400 µL of TE buffer. The DNA solution was treated with 1 µL RNase A (10 mg/ mL) per 100 µL DNA and incubated at 37 ^{0}C for 30 min. The sample was extracted with chloroform: isoamyl alcohol to remove RNAase A. DNA was reprecipitated and dissolved in 40 µL TE buffer. Purity of DNA was checked spectrophotometrically by measuring the absorbance ratio at 260/280nm. DNA was stored at 4 ^{0}C.

2.10.4. Restriction digestion of DNA

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

2.10.5. Extraction and purification of DNA from agarose gels

The restriction digested DNA or PCR amplified products were run on an agarose gel in 1X TAE buffer.The gel was stained with ethidium bromide (0.5 µg mL^{-1}) and viewed using a hand held long wavelength UV illuminator. The fragment of interest were excised from the gel and weighed. A 100 µg gel slice was transferred to a 1.5 mL microcentrifuge tube and 300 µL Buffer DE-A (Axygen^{TM} GEL elution kit, Biosciences, USA) added. The tube was incubated at 70 ^{0}C for 5 to 10 min with intermittent mixing until the gel slice was completely dissolved. The gel mixture was cooled down to room temperature and 150 µL of Buffer DE-B was added. The above solubilized agarose was put into Axyprep column and placed into 2ml microfuge tube. The assembly was centrifuge at 12,000 g for 1 min and filtrate was discarded. 500 µL of wash buffer 1 (provided by Axygen) was addd and centrifuged at 12,000 g for 30s, filtrate was discarded. 700 µL of wash buffer 2 was added and spin at 12,000 g for 30 s, filtrate was discarded. It was repeated again with buffer 2. 1 min empty spin was given to ensure the complete removal of salt. Axyprep column was transferred into a fresh 1.5 mL microfuge tube and 25-30 µL of elution buffer was added t to the centre of the membrane and kept it as such for 1 min at room

temperature. Centrifuge at 12000 g for 1 min. The eluted DNA was stored at 20 ^{0}C. This eluted PCR product was further used for further steps.

2.10.6. Total RNA Isolation

RNase free environment was created and maintained as described by Blumberg (1987). All glass and plastic ware was DEPC (0.1% in water) treated overnight and autoclaved. The pestle and mortar were also DEPC treated and then baked at 300 ºC for 6 h. All materials were dried in a vacuum oven. Total RNA from different plant tissues was isolated using TRIzol reagent. The plant tissue was collected, washed with DEPC treated water, frozen in liquid nitrogen and crushed to a fine powder. To 100 mg of the fine powder 1 mL Trizol reagent was added and mixed thoroughly using a vortimix. Chloroform: isoamyl alcohol (300 µL) was added and mixed thoroughly using vortimix. The tubes were centrifuged at 4 ^{0}C at 13,000 g for 15 min. The supernatant was transferred to 1.5 mL tubes and the chloroform: isoamyl alcohol step repeated. The aqueous phase was transferred to 1.5 mL tubes and half volume isopropanol added. It was mixed thoroughly and kept for RNA precipitation for 1 h. Total RNA was pelleted out by centrifugation at 13,000 g for 15 min at 4 ^{0}C. The RNA pellet was washed with 70% ethanol twice and dried in a SpeedVac centrifugal concentrator. RNA pellet was dissolved in 40 µL of DEPC treated water and stored at -80 ^{0}C in aliquots. Purity of RNA was confirmed by measuring OD at 260/280 nm and also by visualization on 1.5% TAE Agarose gel.

2.10.7. mRNA Purification

Total RNA was quantified spectrophotometrically. The amount of RNA was in the range of 1 mg to 3 mg. Appropriate amount of OBB Buffer and Oligotex Suspension were added according to manual instruction. The sample was incubated for 3 min at 70 ^{0}C in a heating block. Sample was removed from the heating block, and place at 20 ^{0}C to 30 ^{0}C for 10 min. Oligotex: mRNA complex was pelleted down by centrifugation for 2 min at maximum speed (14,000–18,000 *g*), and carefully removed the supernatant by pipetting. Oligotex:mRNA pellet was resuspended in the

appropriate amount of OW2 buffer by vortexing and pipetted onto a small spin column placed in a 1.5 mL microcentrifuge tube and centrifuged for 1 min at maximum speed. Spin column was transferred to a new RNase-free 1.5 mL microcentrifuge tube, appropriate amount of OW2 buffer added to the column and again centrifuged for 1 min at maximum speed, flow-through was discarded. Spin column was transferred to a new RNase free 1.5 mL microcentrifuge tube. Appropriate amount of hot (70 ^{0}C) OEB buffer was pipetted onto the column and resuspended by pipetting up and down, spin it down for 1 min at maximum speed. The Eluted mRNA sample was quantified spectrophotometrically and used for further downstream processes.

2. 10. 8. Spectrophotometric determination of concentration

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

2.10.9. cDNA first strand synthesis by Reverse Transcription

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

Experimental RNA was combined with the oligo $(dT)_{15}$ primer. The primer/template mix was isothermally denatured at 70 ^{0}C for 5 min and snaps chilled on ice. A reverse transcription reaction mix was assembled on ice to contain nuclease-free water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor RNasin®. As a final step, the template-primer combination was added to the reaction mix on ice. Following an initial annealing at 25 ^{0}C for 5 min, the reaction was incubated at 42 ^{0}C for up to 1 h. The cDNA synthesized was directly added to amplification reactions. The first strand reaction was set up as follows:

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

The tubes were incubated at 70 ^{0}C for 5 min and then chilled in ice-water for 5 min. Tubes were briefly spun in a microcentrifuge to collect the condensate and maintain the original volume. The tubes were kept closed and on ice until addition of the reverse transcription reaction mix. The reverse transcription reaction mix was prepared by combining the following components of the ImProm-II Reverse Transcription System in a sterile 1.5 mL microcentrifuge tube on ice.

ImProm-II. 5X Reaction Buffer	4.0µL
MgCl2 (15 mM)	2.0 µL
dNTP Mix (7.5 mM)	1.0 µL
RNasin® Ribonuclease Inhibitor (40U μl^{-1})	0.5 µL
ImProm-II Reverse Transcriptase	1.0 µL
Nuclease-free water	6.5 µL
Final volume	**15.0 µL**

An aliquot of 1.0 µg total RNA and oligo $(dT)_{15}$ or Random hexamer primer (10 pmol) mix was added to the above reaction for a final reaction volume of 20 µL per tube. The tube was incubated at 25 ^{0}C for 5 min for primer annealing and then at 42 ^{0}C for 1 h for cDNA first strand synthesis. Reverse transcriptase was thermally inactivated by incubation at 70 ^{0}C for 15 min prior to proceeding with PCR amplification (Chumakov, 1994).

2.10.10. Polymerase Chain Reaction (PCR)

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

Reaction mixture

Sterile deionized water	6.2 µL
Template (50ng μL^{-1})	1.0 µL
Forward primer (6 pmol)	1.0 µL
Reverse primer (6 pmol)	1.0 µL
dNTPs (0.2 mM)	4.0 µL
10 x Buffer (Mg^{+2} 1.5 mM)	1.5 µL
Taq DNA Polymerase (1U μL^{-1})	0.3 µL
Total volume	**15.0** µL

PCR cycle conditions

1 cycle 95 ^{0}C 5 min

35 cycles 95 ^{0}C 1 min / 45-65 ^{0}C 30-45 s (annealing temperature was dependent on primer Tm) / 72 ^{0}C 1 min 30 s

1 cycle 72 ^{0}C 10 min

1 cycle 4 ^{0}C hold.

2.10.11. Rapid amplification of cDNA ends (RACE)

Generally, using reverse transcription PCR, either partial cDNA fragments (both 5' and 3' ends missing) or cDNA with full 5'end missing are amplified from total cDNA. If a partial cDNA sequence is known, unknown sequences to the 5' and 3' of the known sequence can be reverse transcribed from RNA, amplified by PCR using RACE (Frohmman *et al.,* 1988). RACE Ready cDNA Kit (Invitrogen, USA) was used for RACE. The reactions were set up as per the manufacturer's guidelines. Briefly the RACE technique is based on oligo-capping and RNA ligase-mediated (RLM) RACE methods (Maruyama and Sugano, 1994; Volloch *et al.*, 1994). The GeneRacer method involves selectively ligating an RNA oligonucleotide (GeneRacer RNA Oligo) to the full-length 5' ends of decapped mRNA using T4 RNA ligase. Application of GeneRacer race ready cDNA Kit is as follows:

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

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

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

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 50 ^{0}C for 1 h. The reaction volume was madeup to 100 µL by adding DEPC water and precipitated by adding 2 µL of 10 mg/mL glycogen, 10 µL of 10 µL 3 M sodium acetate (pH 5.2) and 220 µL 95% ethanol. The precipitated sample was washed with 70% ethanol and pellet was air-dried. Air-dried pellet was dissolved in 7 µL of DEPC water. 5' mRNA cap structure from full- length mRNA was removed by following reaction:

Reagent	Volume
Dephosphorylated RNA	7 µL
10X TAP Buffer	1 µL
RNaseOut™ (40 U/µL)	1 µL
TAP (0.5 U/µL)	1 µL
Total Volume	**10 µL**

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 37 ^{0}C for 1 h. The reaction volume was makeup to 100 µL by adding DEPC water and precipitated by adding 2 µL of 10 mg/mL glycogen, 10 µL of 3 M sodium acetate (pH 5.2) and 220 µL 95% ethanol. The precipitated sample was washed with 70% ethanol and pellet was air-dried. Air-dried pellet was dissolved in 7 µL of DEPC water. After decapping of full-length mRNA the RNA Oligo was ligated to the 5' end of mRNA. The reaction of ligating the RNA Oligo to decapped mRNA is as follows:-

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

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

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 37 ^{0}C for 1 h. The reaction volume was made up to 100 µL by adding DEPC water and precipitated by adding 2 µL of 10 µL /mL glycogen, 10 µL of 3 M sodium acetate (pH 5.2) and 220 µL 95% ethanol. The precipitated sample was washed with 70% ethanol and pellet was air-dried. Air-dried pellet was dissolved in 10 µL of DEPC water. After ligation of GeneRacer™ RNA Oligo to decapped, full-length mRNA, and the mixture was ready to reverse transcribe the mRNA into cDNA. 1 µL of the desired primer and 1 µL of dNTP Mix (25 mM each) was added to ther ligated RNA and incubated at 65 ^{0}C for 5 min. Following reagents were added to the 11 µl ligated RNA and primer:

Reagent	**Volume**
5 X RT Buffer	4 µL
SuperScript™ III RT (200 U/µL)	1 µL
0.1 M DTT	1 µL
RNaseOut™ (40 U/µL)	1 µL
Sterile water	2 µL
Total Volume	**20 µL**

The reaction mixture was mixed gently by pipetting, followed by brief spin to collect the liquid at the bottom and incubated at 45 ^{0}C for 1 h. The reaction was inactivated by incubating the reaction mixture at 70 ^{0}C for 15 min. 1 µL of RNase H (2 U) was added to reaction mixture to chew up the DNA / RNA hybrid by incubation at 37 ^{0}C for 20 min. Centrifuged the sample and kept at -20 ^{0}C for further use in PCR.

PCR Setup

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

Reagent	5′ RACE	3′ RACE
GeneRacer 5′ Primer10 µM	3 µL	-
Reverse GSP 10 µM	1 µL	-
GeneRacer™ 3′ Primer 10 µ M	-	3 µL
Forward GSP 10 µM	-	1.0 µL
RT Template	1.0 µL	1.0 µL
10X High Fidelity PCR Buffer	5.0 µL	5.0 µL
dNTP Solution (10 mM each)	1.0 µL	1.0 µL
Platinum® Taq DNA Polymerase High Fidelity, 5U/ µL	0.5 µL	0.5 µL
MgSO4, 50 mM	2.0 µL	2.0 µL
Sterile Water	36.5 µL	36.5 µL
Total Volume	**50.0 µL**	**50.0 µL**

Cycling

Temperature	Time	Cycles
94 ^{0}C	2 min	1
94 ^{0}C	30 S	5
72 ^{0}C 1 min/ 1 kb	2 min	
94 ^{0}C	30 S	5
70 ^{0}C 1 min/ 1 kb	2 min	
94 ^{0}C	30 S	25
66 ^{0}C	30 S	
72 ^{0}C	2 min	
72 ^{0}C	10 min	1

Nested PCR:

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

Reagent	5′ RACE	3′ RACE
Gene Racer 5′Nested Primer10 µM	1 µL	-
Reverse Nested GSP 10 µM	1 µL	-
Gene Racer 3′ Nested Primer 10 µM	-	1 µL
Forward Nested GSP 10 µM	-	1.0 µL
Primary PCR Product	1.0 µL	1.0 µL
10 X High Fidelity PCR Buffer	5.0 µL	5.0 µL
dNTP Solution (10 mM each)	1.0 µL	1.0 µL
Platinum® Taq DNA Polymerase High Fidelity, 5U/µL	0.5 µL	0.5 µL
$MgSO_4$, 50 mM	2.0 µL	2.0 µL
Sterile Water	38.5 µL	38.5 µL
Total Volume	**50.0 µL**	**50.0 µL**

Following program was used for the nested PCR reactions.

Cycling

Temperature	Time	Cycles
94 ^{0}C	2 min	1
94 ^{0}C	30 S	25
66 ^{0}C	2 min	
72 ^{0}C	2 min	
72 ^{0}C	10 min	1

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

2.10.12. Quantitative real time PCR (QRT PCR)

Quantitative Real Time PCR is a powerful tool for gene expression analysis and was first demonstrated by Higuchi *et al*., (1992, 1993). QPCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a 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; Raeymaekers, 2000). QPCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (*i.e.*, in real time) as opposed to the endpoint detection (Higuchi *et al.,* 1992, 1993). The QPCR system is based on the detection and quantitation of a fluorescent reporter (Lee *et al.,* 1993; Livak *et al.,* 1995). There are three main fluorescence-monitoring systems for DNA amplification (Wittwer *et al.,* 1997a): (1) hydrolysis probes, (2) hybridizing probes and (3) DNA-binding agents (Wittwer *et al.,* 1997b, Vander Velden *et al.,* 2003). Most commonly used are the hydrolysis probes, which include TaqMan probes (Heid *et al.,* 1996), molecular beacons (Tan *et al.,* 2004; Vet and Marras, 2005) and scorpions (Saha *et al.,* 2001; Terry *et al.,* 2002). They use the 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples. The

use of fluorescent probe technologies reduces the risk of sample contamination while maintaining convenience, speed and high throughput screening capabilities (Reischl *et al.,* 2002).

In the present study the Brilliant® II QPCR Master Mix (Stratagene, USA) was used. This kit supports quantitative amplification and detection with multiplex capability, and shows consistent high performance with various fluorescent detection systems, including molecular beacons and TaqMan® probes. The kit supports PCR amplifications and detection of a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA. The Brilliant II QPCR master mix includes SureStart® *Taq* DNA polymerase, a modified version of *Taq2000*™ DNA polymerase with hot start capability. A passive reference dye (an optional reaction component) is provided so as to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms.

2. 10.12.1. Pre-protocol considerations

Magnesium Chloride Concentration

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

Probe Design

Probes are designed in a fashion so as to have a melting temperature 7–10 ^{0}C higher than the annealing temperature of the primers. Lyophilized custom molecular beacon or TaqMan probes are constituted in 5 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA (low TE buffer).

Fluorescence Detection

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

Optimal Concentrations for Experimental Probes and Primers Probes

The optimal concentration of the experimental probe is determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. In present study TaqMan® probes were used.

TaqMan® Probes

The TaqMan probe concentration can be optimized by varying the final concentration from 100 to 500 nM in increments of 100 nM.

PCR Primers

The optimal concentration of the upstream and downstream PCR primers is determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The primer concentration for use with TaqMan probes can be optimized by varying the primer concentration from 50 nM to 600 nM. The best concentrations of the upstream and downstream primers are not always of equal molarity.

Reference Dye

A passive reference dye may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are

normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm respectively.

Reference Dye Dilution

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

Data acquisition with a spectrofluorometric thermal cycler

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

2.10.12.2. Preparing the Reactions

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

Reagent Mixture

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

2.10.12.3. PCR Cycling Programs

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

Two-step Cycling Protocol

Temperature	Time	No. of Cycles
95 ^{0}C	10 min	1
95 ^{0}C	30 S	40
60 ^{0}C	30 S	
72 ^{0}C	1 min	

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

2.11 Nucleic acids blotting/hybridization

2.11.1 Southern Blotting

For Southern hybridization (Southern, 1975) the DNA samples were electrophoresed on an agarose gel in 1X TAE buffer containing 0.5 µg / mL ethidium bromide. The gel was rinsed with deionized water (DW) and placed in depurination solution for 15 min. The gel was rinsed with deionized water and immersed in denaturation solution for 30 min with gentle shaking. The gel was again rinsed with deionized water and transferred to neutralization solution for 45 min. The gel was then set up for capillary transfer of DNA to solid membrane support.

A tray was filled with the transfer buffer (20 X SSC). A platform was made and covered with a wick, made from 2 sheets of Whatman 3MM filter paper saturated with transfer buffer and the gel was placed on it. It was surrounded with Saran Wrap to prevent the transfer buffer from being absorbed directly by the paper towels stacked above the membrane. A sheet of Hybond-N+ membrane (Amersham, UK) of

the exact gel size was wetted with deionized water followed by transfer buffer (20 X SSC) and then placed on top of the gel. A glass rod was rolled over the membrane to remove any trapped air bubbles. One piece of Whatman 3 MM paper wetted with 20 X SSC was placed on the membrane followed by Whatman 3 MM paper pre-wetted in 2 X SSC. On this paper another dry Whatman 3 MM paper was placed followed by a stack of absorbent paper towels. A glass plate and a ~0.5 kg weight were placed on the top of the paper towels. Transfer of DNA was allowed to proceed for 18 h. The membrane was marked for orientation, removed carefully and washed with 6X SSC. The membrane was air dried and baked for 2h at 80 ^{0}C to immobilize DNA onto the nylon membrane. Hybridization and autoradiography were carried out as is described in the following section.

2.11.2 Slot Blot Hybridization

For slot blot hybridization DNA or RNA samples were diluted according to experimental requirements. The DNA samples were denatured by adding 1/10th volume 3M NaOH and incubation at 65 ^{0}C for 10 min. To the denatured sample an equal volume of 6 X SSC was added. Two layers of Whatman 3 MM filter paper wetted with sterile deionized water and 6 X SSC were placed in the Slot Blot apparatus followed by Hybond-N+ membrane (Amersham, UK) treated in the manner as above. The Slot Blot unit was assembled and wells washed with 500 µL of 6 X SSC by applying vacuum. After washing, samples prepared earlier were applied in the wells and vacuum applied till whole sample volume passed through the well slit and wells appear dry. The unit was carefully disassembled and the membrane taken out. The membrane was air dried and then baked for 2 h at 80 ^{0}C to immobilize DNA. Hybridization and autoradiography were carried out as described in the following section 2.11.3 & 2.12.

2.11.3 Random primer labeling

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

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

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

Component	**Volume**
10 X reaction buffer (500 mM Tris-HCl, pH 8.0; 100 mM $MgCl_2$; 10 mM DTT; 0.5 mg/ mL acetylated BSA)	5.0 µL
dATP (0.5 mM)	4.0 µL
dGTP (0.5 mM)	4.0 µL
dTTP (0.5 mM)	4.0 µL
(α-^{32}P)-dCTP (Sp. activity 3000 Ci $mmol^{-1}$)	5.0 µL
Sterile deionized water	16.0 µL
Exonuclease free Klenow fragment (2 U μL^{-1})	2.0 µL
Total volume	**50.0 µL**

The reaction was carried out at 37 ^{0}C for 45 min and stopped by incubation in a boiling water bath for 10 min and snap chilled on ice.

2.12. Hybridization

The blots made as in sections 2.11.1 and 2.11.2 above were prehybridized at 45 ^{0}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 ^{0}C for 14-18 h. The solution was decanted and the membrane washed with low stringency buffer at 55-65 ^{0}C for 15 min followed by a high stringency wash at 55 ^{0}C for 15 min. The moist blot was wrapped in Saran wrap and exposed to X-ray film at –70 ^{0}C in a cassette with intensifying screen.

2.13. Expression and purification of recombinant protein

2.13.1. Expression of recombinant protein

A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 µg / ml kanamycine) was used to inoculate 5 ml liquid LB medium containing the same concentrations of the antibiotic. Culture was grown overnight with shaking at 225 rpm at 37 °C. One 1 ml aliquot of each culture was used to inoculate 100 ml liquid cultures containing 50 µg/ml kanamycine. Once the cultures reached OD_{600} 0.4 - 0.5, recombinant protein expression was induced by the addition of isopropyl ß-D-thiogalactopyranoside (IPTG), and the culture was grown for 4–6 h at 37 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. Pellets were resuspended in 6.25 ml lysis buffer. Cells were disrupted by sonication for 5 mins at 70 amplitude on a Sonifer Cell Disruptor. $MgSO_4$ of final concentration of 10 mM and lysozyme of final concentration 100 µg/ml was added to the disrupted cell and kept at 37^{0}C for ½ h. It was centrifuged at 10000 rpm for 10 minute and supernatant was saved as lysate and pellet was resuspended in 2 mL sonication buffer. Suspension was again sonicated for 1 min at 70 amplitude to disrupt the inclusion bodies and the this disrupted inclusion bodies were dissolved in 3 mL of dispersion buffer and an aliquot of 20 µL checked on SDS PAGE to check the expression (Chapter2: section 2.6.6 and table: 2.7).

2.13.2. Affinity purification of recombinant protein using Ni^+ NTA beads

The recombinant protein, among several other bacterial proteins is loaded on affinity matrix column such as Ni-agarose. This affinity matrix contains bound metal ion nickel, to which the polyhistidine-tag binds with micro molar affinity. The matrix is then washed with buffer to remove unbound proteins. The washing efficiency may be improved by the addition of 20mM imidazole and histidine-tagged proteins are then usually eluted with 150-300 mM imidazole. The purity and amount of protein is assessed by SDS-PAGE**.** The column(s) and buffers were equilibrated to room temperature. The Ni^+ beads column was equilibrated with binding buffer for 30 minutes and then three bed volume of binding buffer was passed through the column followed by addition of soluble protein in dispersion buffer (hapter 2: section 2.13.1). the column was kept at 4 ^{0}C for 1 h for binding of recombinant protein to Ni^+ beads. Flow through was collected in different tube afrter 1 h and column was washed with two bed volume of washing buffer. The 6xHis-tagged bind protein was eluted in 4 aliquots of elution buffer 0.5 mL each. Protein elution was monitored by measuring the absorbance at 280 nm of collected fractions. The eluted protein was analyzed by SDS-PAGE.

2.13.3. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

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

2.13.4. Preparation of the separating gel

A vertical slab gel (Hoeffer Scientific, U.S.A.) was assembled using 1.0 mm spacers. In a sidearmed vacuum flask, 10% separating gel solution was made according to Table. 2.6 except for the addition of ammonium persulfate and TEMED. The solution was degassed by applying vacuum while stirring on a magnetic stirrer. TEMED and ammonium persulfate were added, and mixed gently without generating bubbles. The

solution was pipetted into the gel cassette leaving 1.5 cm from the top unfilled. The gel solution was overlaid with watersaturated n-butanol to remove trapped air bubbles and to layer evenly across the entire surface. When a sharp liquid-gel inter-surface was observed after the gel polymerization, the slab was tilted to pour off the overlay.

2.13.5. Preparation of the stacking gel

Stacking gel solution was prepared according to Table. 2.6 leaving out ammonium per sulfate and TEMED. As in the separating gel, this solution was degassed. TEMED and ammonium per sulfate were added, mixed and overlaid on the separating gel. A comb was inserted taking care not to trap air bubbles beneath the comb teeth. The gel was left to polymerize.

2.13.6. Preparation of the sample

Equal parts of the protein sample (as described in section 2.8 above) and the loading buffer were mixed in a microcentrifuge tube and kept in a boiling water bath for 90 s. The samples were centrifuged at 10,000 g for 10 min, supernatant collected and used for electrophoresis.

2.13.7. Loading and running the gel

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

2.13.8.. Silver staining of the gel

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 deionized H_2O (20 s each). The gel was silver stained (HCHO was added to the solution just before use) for 20 min with

intermittent shaking in dark. The gel was then rinsed thrice with deionized H_2O (20 s each) and transferred to the developer till the bands developed. The gel was washed with deionized H_2O and stored in the fixer.

2.14 Raising Polyclonal Antibody against 4CL in Rabbit and IgG affinity purification

The purified 4CL protein was used for raising polyclonal antibodies in rabbit (New Zealand White).

2.15 Histology and Immunocytolocalization:

Free hand transverse sections were fixed overnight under vacuum in freshly prepared cold 4% buffered formaldehyde (4% paraformaldehyde in 1X PBS). The sections were dehydrated by passages through increasing ethanol: water series (30%, 50%, 70%, 85%, 95% and 100% ethanol) for 30 min each. This was followed by passages through tertiary butanol: ethanol series (25:75, 50:50, 75:25, and 100:0). The sections were rehydrated by treating with 70% , 50% ethanol and 0.5X SSC for 2 min. The rehydrated sections were soaked in two changes of 1X PBS for 10 min each. Next, the sections were washed in 1X PBS containing 0.1% BSA for 5 min and subjected to 30 min of blocking with 10% BSA at room temperature in a humidified chamber. Post blocking washes included three washes of 15 min each with 1X PBS containing 0.1% BSA. Primary antibody incubation was carried out overnight in a humidified chamber at 4 ^{0}C using 75 µL of diluted antibody (1µg mL^{-1}) in 1 X PBS containing 0.1% BSA. Negative controls included either the use of pre-immune serum or the omission of both antibody and pre-immune serum. Following the primary antibody incubation, the sections were washed thrice for 15 min each in 1X PBS containing 0.1% BSA. A secondary antibody, 0.2% Anti-rabbit-IgG-goat alkaline phosphate conjugate antibody (diluted in 1X PBS with 0.1% BSA), was added to the tissue sections at this stage and incubated at 37 ^{0}C in a humidified chamber for 2 h in dark. Post secondary antibody washes were carried out at room temperature using 1X PBS with 0.1% BSA. Color was developed in dark by addition of 60 µL of color

development reagent (20 µL BCIP/NBT mix in color development buffer containing 10% polyvinyl alcohol) to the color development buffer (100 mM Tris, pH 9.5, 150 mM NaCl, 50 mM $MgCl_2$) treated slides. The slides were placed in humidified (color development buffer) chamber at RT in dark. Upon color development, 10 mM EDTA was used to stop the reaction, rinsed with water, air-dried, cover slip-mounted using glycerol and observed under microscope, and microscopic image captured.

2.16 Phloroglucinol staining

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

2.17 *Agrobacterium* mediated tobacco transformation

The tobacco regeneration and transformation protocol is modified from Horch et al. (1985). *Agrobacterium tumefaciens* strain GV2260 harbouring the binary plasmid vector to be transformed in tobacco was inoculated in 5 mL YEP media containing rifampicin (250 mg/ L) and kanamycin (50 mg/ L). The culture was allowed to grow overnight at 28 ^{0}C with shaking at 200 rpm. Next day 1mL inoculum from this tube was taken and added to 50 mL YEP flask containing appropriate antibiotics. The culture was incubated at 28 ^{0}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 ^{0}C. After two days the leaf discs were harvested, washed with liquid MSBN and dried on sterile filter paper. Up to 10 leaf discs per plate were transferred to MSBN agar plates containing Hygromycin (7.09 μM) and Cefotaxime (200 μM). The cultures were incubated at 28 ^{0}C with 18/6 hours photoperiod in diffused light (60-80) mE/ mS.). The leaf discs were subcultured every week till elongated shoots were excisable. Elongated shoots were excised and transferred to root induction medium. The rooted plants were hardened and further analyzed.

2.18 Genetic Transformation of *Leucaena leucocephala*

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

The transformation was carried out by three methods:

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

2.18.1 Particle Bombardment

Calculated amount (approximately 500 μg) of gold particle of 1μm size was used for bombardment. The gold particles taken in microcentrifuge tubes were washed with sterile water and supernatant was decanted after centrifuging it at 13,000g for 10 min. The washing of particles was repeated three times with 70% (freshly prepared) ethanol and supernatant was decanted after centrifuging it at 13,000g for 10 min. Again particles were dissolved in 500 μL sterile water and were pelleted down after

centrifuging it at 13,000g for 10 min. 1µg of DNA (plasmid/cassette) was dissolved in 100 µL of Xho buffer. DNA was added to the prepared gold particle and mixed it by vertexing. 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, gave brief spin to settle down sample at the bottom. It was resuspended in absolute ethanol and sonicated briefly to resuspend the particle and was used for bombardment. Particle bombardment was done using BioRad PDS 1000/ He system. 1100 psi He gas pressure was used to bombard the particle at the distance of 6 cm. Microcarrier travel distance was adjusted to 6 cm. The embryo axes were arranged in the centre of the petridishes and bombarded with gold particles coated with DNA (plasmid/cassette). The explants were incubated in dark for 72 h after the bombardment.

2.18.2 Particle bombardment followed by co-cultivation

The embryo axes were first bombarded with gold particles coated with DNA (plasmid/cassette) as mentioned above followed by co-cultivation with *Agrobacterium* (GV2230) harboring pCAMBIA1301 containing 4CLgene in anti-sense orientation. Cultured bacterial cells were pelleted by centrifugation at 5,000g for 5 minutes and resuspended in the YEM medium at a density of 3-5 $\times 10^9$ cells/ mL, considering the optical density of bacterial culture at 600 nm. The selected embryo axes after bombardment were co-cultured with *Agrobacterium* for one hour by immerging them into the bacterial suspension. The agro infected embryo axes were then transferred onto the regeneration medium (1/2 MS+ TDZ (0.5 mg/ L)) with or without 0.1 mM acetosyringone (3', 5'-dimethoxy-4'-hydroxy-acetophenone; Aldrich Chemical Co.) and co-cultivated in the dark at 28 ± 2 ^{0}C for 3 days. After co-cultivation, the embryo axes were washed thoroughly with cefotaxime 250 mg/ L in sterile distilled water and transferred onto the regeneration medium (1/2 MS+ TDZ (0.5 mg/ L)).

2.18.3 Agro-infusion method

In this Agro-infusion method The *Agrobacterium* was introduced forcibly with a sterile needle to the meristematic region of the explants. After transformation (Particle bombardment / Particle bombardment followed by co-cultivation /Agro-infusion), embryo axes were cultured on regeneration medium 1/2 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 MS without hygromycin selection. Cytokinin 2ip (2-isopentenyl adenine at 0.5 mg/ L) was used in the medium to have better elongation of transformed shoots.

2.19. GUS histochemical assay

The GUS staining solution was prepared by taking 1 mM X-Gluc (5-bromo, 4-chloro, 3-indolyl-β-D-glucuronide: Cyclohexylammonium salt (X-GlcA) from a 20 mM stock made in dimethylformamide, 100 mM sodium dihydrogen phosphate dihydrate and 0.5% Tween-20. The pH of the solution was adjusted to 7.0 with 1N NaOH. The histochemical GUS assay was performed as described by Jefferson (1987) to monitor *GUS* gene expression in putative transgenic calli. The GUS assay was carried out on 10% of the randomly selected calli immediately after co-cultivation as well as 21 days after co-cultivation. Embryo axes were immersed in GUS assay solution and incubated at 37 ^{0}C overnight. *GUS* gene expression was observed and photographed by using a Axioplan2 microscope from Carl Zeiss.

2.20 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. Total Protein was quantified using Bradford reagent. Equal amount of protein was coated on 96 well micro titer plates. Antigen (crude extract) was diluted in PBS to an optimal

concentration and coated on 96 well microtiter plate (100 µL/ well). Plate was incubated for 2 h at room temperature or over night at 4 ^{0}C and washed twice with PBS after incubation. Non-specific sites were blocked with blocking buffer 300 µL/ well and incubated for 1h at room temperature. After washing out the unbound blocking agent with PBS, primary antibody (Anti rabbit IgG) was added and incubated for 1 h at room temperature. The unbound primary antibody was washed twice with PBS and secondary antibody conjugated with alkaline phosphatase was added and incubated for 2 h at room temperature. Enzyme specific substrate BCIP/ NBT was added at a concentration of and incubated until the color develops and the colorimetric reaction was measured at 405 nm.

Chapter 3

3. Isolation, Cloning and Characterization of 4-Coumarate Co A Ligase (4CL) Gene(s)

This chapter deals with different strategies followed to fish out the full-length c-DNA clones of 4CL gene(s) from *Leucaena leucocephala* and their analysis.

3.1. Introduction

In higher plants, 4CL is typically encoded by a gene family consisting of two to four members. In some species, such as *Arabidopsis* (Ehlting *et al.,* 1999), aspen (Hu *et al.,* 1998), soybean (Uhlmann and Ebel, 1993), potato (Becker-André *et al.,* 1991), parsley (Douglas *et al.,* 1987, Lozoya *et al.,* 1988), and tobacco (Lee and Douglas 1996), specific 4CL genes appear to be associated with the formation of unique phenylpropanoid end-products. The *Arabidopsis* 4CL gene family consists of four members, At4CL1, At4CL2, At4CL3 and At4CL5 (Cukovic *et al.,* 2001; Ehlting *et al.,* 1999; Schneider *et al.,* 2003; Hamberger and Hahlbrock, 2004). In soybean, the two discrete 4CL genes characterized, Gm4CL14 and Gm4CL16, have been proposed to correspond to two distinct 4CL isoforms identified in this species, consistent with the idea that discrete 4CL genes can encode function-specific isoforms (Knobloch and Hahlbrock, 1977, Uhlmann and Ebel, 1993). In aspen, the Pt4CL1 and Pt4CL2 gene products are both structurally and functionally distinct, and the two genes are expressed in a compartmentalized manner (Hu *et al.,* 1998).

3.1.1. Reaction mechanism catalyzed by 4CL

4-Coumarate: coenzyme A ligase (4CL [EC 6.2.1.12]) also called 4- coumaryl: coenzyme A synthetase and *p*-hydroxycinnamic acid: coenzyme A ligase) plays a pivotal role in the general pathway of phenylpropanoid metabolism by providing activated coenzyme A (Co-A) esters of hydroxycinnamic acids (4-coumaric acid and its methoxylated derivatives; (Ranjeva *et al.,* 1976, Lee *et al.,* 1996), 4CL activates hydroxycinnamates by esterification with coenzyme A. The overall ATP and Mg^{2+} dependent reaction (Fig: 3.1) can be divided into the following two steps.

The first step is the formation of the adenylate intermediate. ATP apparently binds first to the enzyme followed by the substrate with pyrophosphate being released while the 4-hydroxycinnamoyl-adenylate intermediate still remains bound. Subsequent binding of coenzyme A (CoA) is followed by release of the 4-hydroxycinnamoyl-CoA thiol ester and AMP (Knobloch, and Hahlbrock., 1975; Becker-Andre *et al.,* 1991). The phenylpropanoid enzyme 4-coumarate: coenzyme A ligase (4CL) is considered necessary to activate the hydroxycinnamic acids for biosynthesis of the coniferyl and sinapyl alcohols subsequently polymerized into lignin**.**

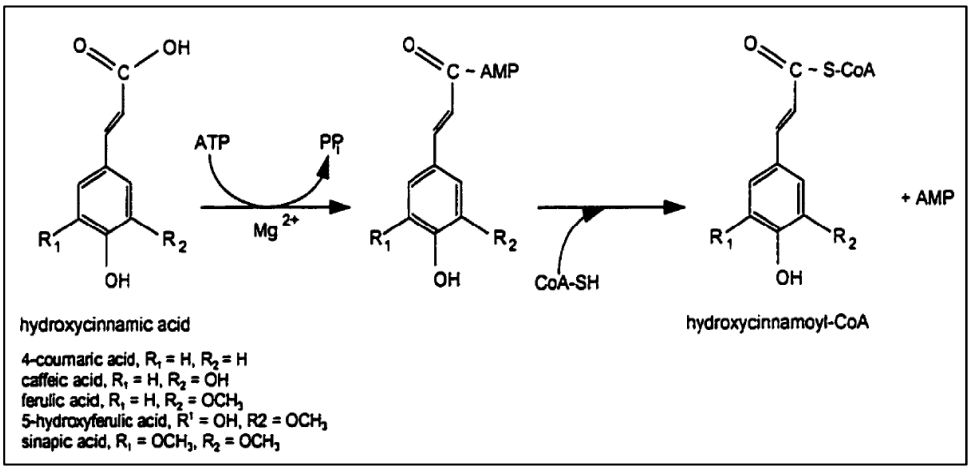


Fig. 3.1: Reaction catalyzed by 4-coumarate: CoA ligase

3.2. Materials and methods

3.2.1. Genomic DNA extraction

Genomic DNA was extracted from young and disease free leaves of *L. leucocephala* (Chapter 2; section 2.10.3).

3.2.2. Slot Blot, Southern hybridization and random primer labeling

Slot blot, Southern blot and random primer labeling were done as described earlier (Chapter 2; sections 2.11.1, 2.11.2, 2.11.3 and 2.11.4 respectively).

3.2.3. RNA isolation and RT^{1st} strand cDNA synthesis

Total RNA was isolated from the xylem tissue of the stem portion of *L. leucocephala (*Chapter 2; section 2.10.6*).* mRNA was purified from total RNA using oligotex dT resins(Chapter 2; section 2.10.7). cDNA RT^{1st} strand was synthesized as described in (Chapter 2; section 2.10.8).

3.2.4. PCR amplification and cloning

PCR amplification was done using RT 1st strand as template (Chapter 2; section 2.10.8). Amplified PCR products were eluted from Agarose gel (Chapter 2; section 2.10.5). The eluted PCR products were used for ligation (Chapter 2; section 2, 10).

3.2.5. Transformation and selection

Transformation (Chapter 2; section 2.9) was done with ligated mixture and putative transformants were selected using Blue/White screening by adding IPTG/X-GAL (Chapter 2; section 2.9.4). Colony PCR was done to screen the positive clones (Chapter 2; section 2.9.6). Plasmids were isolated (Chapter 2; section 2.10.1) from the colony PCR positive clones (Chapter 2; section 2.9.6), confirmed by restriction digestion (Chapter 2; section 2.10.4) and further by sequencing.

3.2.6. Bioinformatics analysis of nucleotide sequences

Nucleotide and amino acid sequence analysis was done using software pDRAW 32 and online bioinformatics analysis facility available at www.justbio.com, www.expasy.org and www.ncbi.nlm.nih.gov.

3.2.7. RACE (Rapid amplification of cDNA ends)

RACE (Chapter 2; section 2.10.11) was done to fish out full length gene with its 5’ and 3’ UTRs.

3.3. Results and Discussion

3.3.1. Establishing the presence of the 4CL gene in *L. leucocephala*

The presence of 4-coumarate Co A ligase (4CL) gene in *Leucaena leucocephala* was established by slot blot analysis and Southern hybridization. High molecular weight good quality genomic DNA was isolated from the tender leaves of *L. leucocephala* (Fig. 3.2).

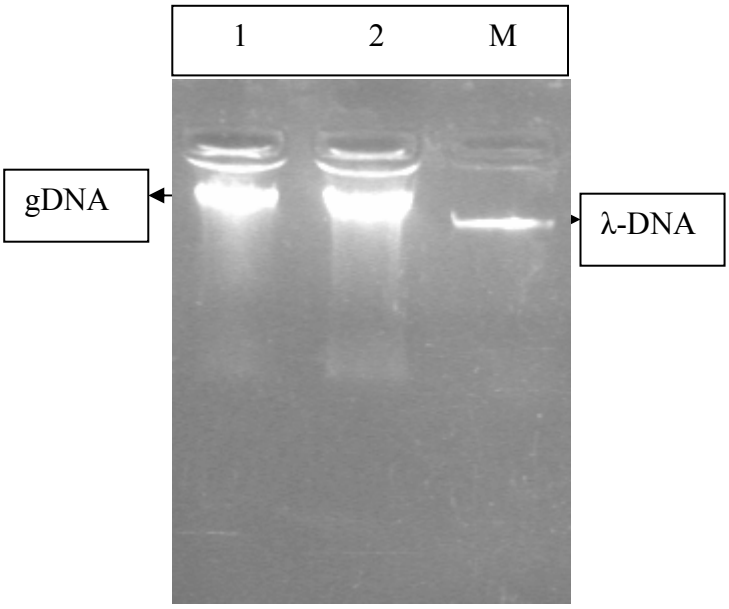


Fig. 3.2: g-DNA isolated from *Leucaena leucocephala* by CTAB method.
Lane M: λ DNA, Lane 1& 2: gDNA of *Leucaena leucocephala*.

3.3.2. Slot blot hybridization

The genome size of *L. leucocephala* is 1.18 x 10^9 bp and 1.2 pg of the genomic DNA correspond to a single representation of the genome. *L. leucocephala* genomic DNA, 1.2µg representing the genome one million times was spotted on Hybond N^+ membrane. The 4CL cDNA gene clone from *Nicotiana* (980 bp; NCBI Gen-Bank (Accession number U40085) was amplified, cloned and isolated was also spotted. The *Nicotiana* DNA 1.1pg, representing one million copies of the gene clone was spotted in triplicate to represent these gene copies once (1.1pg), twice (2.2pg) three (3.3pg) times and four (4.4pg) times. The blot was probed with the radiolabelled *Nicotiana* 4CL gene. Positive signal obtained under high stringency hybridization conditions was indicative of the presence of the 4CL

gene in *L. leucocephala.* Based on signal intensity it was also inferred that in *L. leucocephala 4*CL belonged to a gene family represented possibly by six members (Fig 3.3).

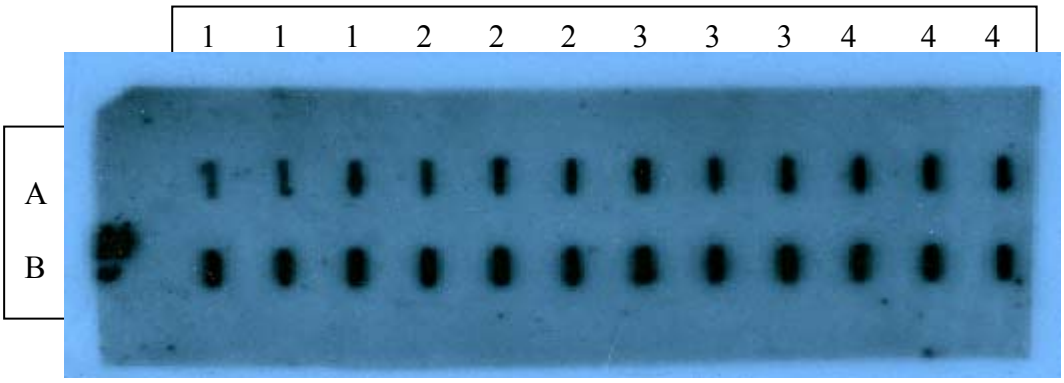
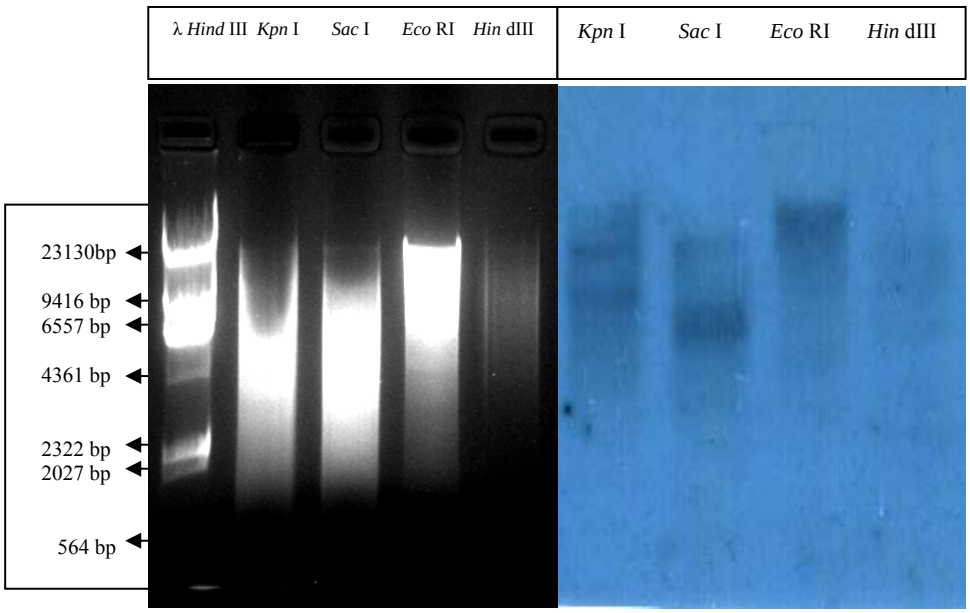


Fig. 3.3: Slot Blot of *L. leucocephala* genomic DNA hybridized with radiolabelled *Nicotiana* 4CL gene (U40485): (A) signals from 10^6 x 1, 2, 3 and 4 copies of *Nicotiana* 4CL gene clone (each blotted in triplicate), (B) signals from 10^6 genomic DNA representations of *L. leucocephala*.



(a) (b)

Fig 3.4: Southern Hybridization of *L. leucocephala*. (a) 0.7% agarose gel showing *L. leucocephala* genomic DNA digestion with *Kpn* I, *Sac* I, *EcoR* I, and *Hind* III. λphage DNA *Hind* III digest used as DNA size marker; (b) Southern hybridization of (a) with *Nicotiana* 4CL gene.

3.3.3. Southern hybridization

To further validate the results from slot blot experiment and to understand the distribution of the 4CL gene in the *L. leucocephala* genome, Southern hybridization was performed. A 10µg of *L. leucocephala* genomic DNA was restriction digested with *Kpn* I, *Sac I, Eco* RI and *Hind* III. The digested genomic DNA was electrophoresed on 0.7% agarose gel in 1X TAE buffer (Fig. 3.4 a). Southern hybridization with *Nicotiana* 4CL gene (U40485) revealed that from each of the restriction digested DNA samples multiple bands hybridized to the *Nicotiana* 4CL gene clone (Fig. 3.4 b). The hybridization signals from very low to very high molecular weight DNA fragments indicated possible multiple gene copies in the *L. leucocephala* genome. Low molecular weight signals from *Eco* RI and *Hind* III digests suggested the presence of these restriction sites within the gene(s). It has been obvious from southern hybridization that 4CL gene in *L. leucocephala* exist as multi gene as reported earlier in other plants like four *At4CLs* have been identified in *Arabidopsis* (Soltani *et al*., 2006) two copies in pine (Zhong and Chiang, 2000) and two copies in tobacco (Lee *et al.,* 1998) etc.

3.3.4. Isolation of the 4-Coumarate CoA ligase (4CL) gene

There are different approaches to isolate gene like gDNA library screening, cDNA library screening and direct PCR based approach (RACE). We approached to isolate 4CL gene using PCR based method *i.e.* through RACE. The prerequisite for RACE reaction is to have a partial known sequence of the gene to be isolated. So the first thing is to isolate a partial cDNA of gene.

3.3.4.1. Designing of oligonucleotide primer PCR (DOP-PCR)

The following 4CL sequences publically accessible from the National Center for Biotechnology Information were aligned to generate a 4CL nucleotide consensus sequence. The alignment of these nucleotide sequences indicated that large stretches of the nucleotides are highly conserved and thus could be used as primer targets. The consensus regions were used to design primers. Selected nucleotide sequences of 4CL genes available from NCBI Gen-Bank database were aligned using multiple sequence alignment program Clustal W 1.8 (www.bioedit.com).

Six sequences with high degree of sequence similarity were selected for the design and synthesis of primers from highly conserved region (Fig. 3.5).

3.3.4.2. Multiple sequence alignment for forward primer

```
Juglans/ AJ278455      5' ---CAGGGATACGGAATGACAGAGGCAGG 3'
Populus/ AF008184      5' ---CAGGGATACGGAATGACAGAGGCAGG 3'
Betula/ AY792353       5' ---CAGGGATACGGAATGACAGAGGCAGG 3'
Amorpha/ AF435968      5' ---CAGGGATACGGAATGACTGAGGCAGG 3'
Glycine/ X69954        5' GGCCAGGGATACGGAATGACTGAGGCAGG 3'
Allium/ AY541033       5' GGCCAGGGTTACGGAATGACTGAAGCAGG 3'
                             ***** *********** ** *****
```

Designed Forward Primer

Fab4clF 5'GGCCAGGGATACGGAATGACTGAGGCAGG 3'

3.3.4.3. Multiple sequence alignment for reverse primer

```
Nicotiana/ U50845           5' ATCAAATACAAAGGATTTCAAGTGGCTCC--- 3'
Solanum/ AF150686           5' ATCAAATACAAAGGATTTCAAGTGGCTCC--- 3'
Glycine/ X69954             5' ATCAAATACAAAGGATTTCAAGTGGCTCCAGC 3'
Amorpha/ AF435968           5' ATCAAATACAAAGGATTTCAGGTGGCTCC--- 3'
Alrabidopsis/ AY376728      5' ATCAAGTACAAAGGATTTCAAGTGGCTCCAGC 3'
Brassica/ AF207572          5' ATCAAGTACAAAGGTTTTCAAGTGGCTCC--- 3'
                               ***** ******** ***** ********
```

Designed Reverse Primer

Fab4clR 5' GCTGGAGCCACTTGAAATCCTTTGTATTTGAT 3'

Fig. 3.5: Clustal W 1.8 multiple sequence alignment of the 4CL gene sequences: *Juglans* (AJ278455), *Populus* (AF008184), *Betula* (AY792353), *Amorpha* (AF435968), *Glycine* (X69954), *Allium* (AY541033), *Nicotiana* (U50845), *Solanum* (AF150686), *Arabidopsis* (AY376728) and *Brassica* (AF207572). The region selected and is conserved for designing primers are in bold letters.Only the 5' and the 3' regions of the gene coding sequences are shown.

3.3.4.4. Amplification and analysis of 362 base pair fragment

Total RNA was extracted from xylem tissue of *L. leucocephala* to obtain a rich population of lignin biosynthetic pathway gene transcripts. RNA (Fig 3.6) was extracted using TRIzol reagent (Chapter 2, section section 2.10.6). RT^{1st} strand

cDNA was synthesized using the ImProm-II Reverse Transcription System (Promega, USA), brief protocol is described in Chapter 2, section section 2.10.8.

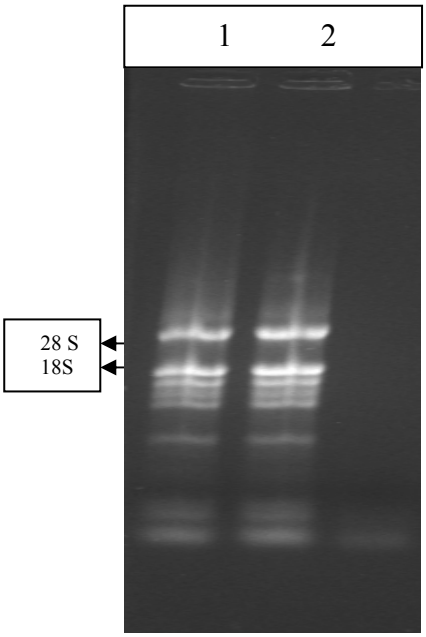


Fig 3.6: Lane 1 & 2 Total RNA isolated from xylem tissue of *Leucaena leucocephala*.

PCR was setup with the cDNA first strand as template using Fab4CLF as forward and Fab4CLR as reverse primers. A DNA fragment of 0.36 Kb was amplified (Fig. 3.6 a). The amplicon was gel eluted and cloned in pGEM-T Easy Vector (Promega, USA). Plasmid DNA were isolated and digested with *Eco*R I from three recombinant. The digestion pattern revealed presence of inserts (Fig. 3.6 b). The clone was designated as SUSH4CL362 as sequenced. The nucleotide sequence of SUSH4CL362 cDNA clone was searched for sequence similarity using BLAST in NCBI Gen-Bank database and SUSH4CL362 cDNA clone showed maximum sequence similarity with other reported plant 4CL3 mRNA sequences from other plants (72% with *Glycine* max 4CL 3: AF002258, 79% with *Rubus idaeus* 4CL3: AF239685 and 77% with *Arabidopsis thaliana* 4CL3 : AF106088). The above nucleotide sequences alignged using ClustalW 1.8 with

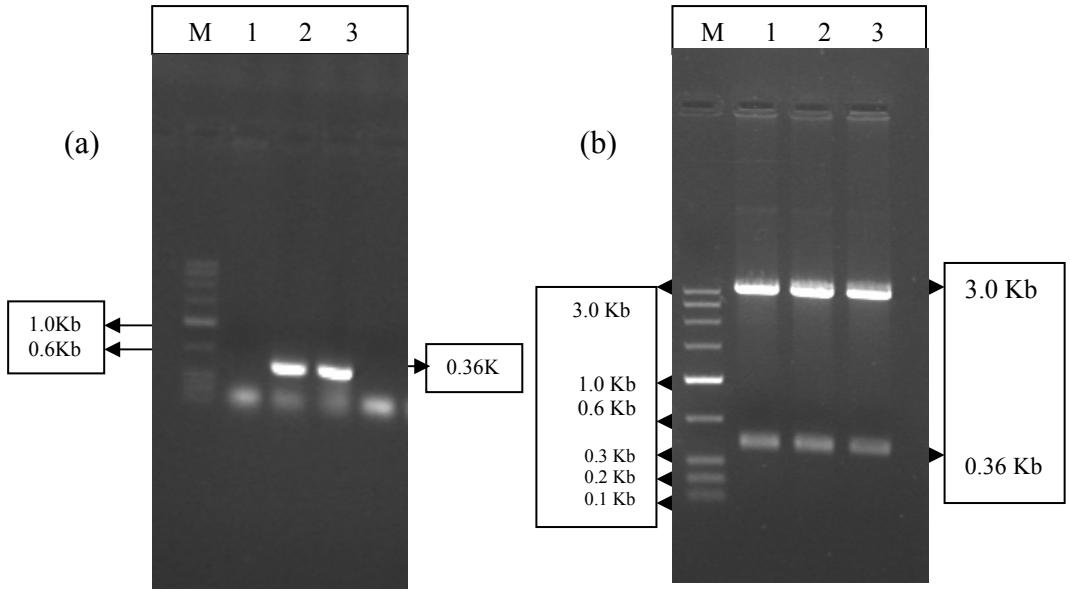


Fig 3.7: (a) PCR amplified from cDNA of *L. leucocephala* using Fab4CLF and Fab4CLR primers, Lane M: Low range Ruler, Lane 2 & 3: PCR amplified 0.36 Kb fragment and Lane 4: no template control. (b): cDNA clone of *L. leucocephala* with Fab4CLF and Fab4CLR primers, Lane M: Low range ruler, Lane 1, 2 & 3: *Eco* RI digested 0.36 Kb cloned fragment in pGEMT easy vector.

Dicot (*Glycine*: AF002258), Gymnosperm (*Pinus*: U12013) and Monocot (Rice: X52623) and this SUSH4CL362 sequence showing 79%, 65% and 69% sequence similarity, respectively**.** This sequence was searched for the amino acid homology using BLAST in NCBI database. It shows 70-85% homology with the other reported 4CL amino acid sequences. This amino acid sequence contains the conserved catalytic domain (GEICEIRG), which is present in all the other 4 - coumarate Co A ligase (4CL) gene. The central cystein residue plays an important role in catalytic activity. The SUSH4CL362 gene sequence was submitted in NCBI Gen-Bank database and allotted accession number for this gene is DQ267975.

Nucleotide sequences of SUSH4CL362:

```
   1 GGCCAGGGAT ACGGAATGAC TGAGGCAGGG CCAGTGTTGT CAATGTGTTT
  51 GGGTTTTGCA GAAGAAGCAT TCCCAACAAA GTCAGGATCA TGTGGCACAG
 101 TCGTCAGAAA CGCAGGACTC AAAGTCGTTG ACCCTGAAAC TGGTCGCTCT
 151 CTTAGCTATA ACCAGCCTGG TGAGATTTGC ATCCGAGGCC ACCAAATCAT
 201 GAAAGGGTAT CTGAATGATG AGAACGCGAC AGCAACAACT ATAGACGCAG
 251 AGGGATGGCT TCACACAGGG GATATTGGGT ACATAGATGA CGACGAGGAG
 301 TTATTCATAG TGGACAGAGT TAAGGAGCTC ATCAAATACA AAGGATTTCA
 351 AGTGGCTCCA GC
```

Fig. 3.8: Nucleotide sequence of SUSH4CL362. Forward and reverse primers are indicated by underlined sequences.

3.3.5. 4CL1 specific primers

The SUSH4CL362 was showing maximum homology with the member of fabaceae family. Therefore further primers were designed from the members of fabaceae 4CL genes reported from NCBI Gen-BanK data. The 4CL gene sequences available in the NCBI database of fabaceae Family were aligned using CLUSTAL W 1.8. The alignment of these nucleotide sequences indicated that large stretches of the nucleotides are highly conserved and this could be used as primer targets. These consensus regions were used to design primers.

3.3.5.1. Multiple sequence alignment of member of Fabaceae for forward primers

```
Amorpha/AF435968   5' CCAATCTCCAAACACCTTCCCCTTCACTCTTACTGCTTTGAGA 3'
Glycine/AF002259   5' TACATCCCCAAACATATGCCCCTCCACTCTTACTGCTTCGAGA 3'
Glycine/AF279267   5' CCGATCCCCACACATCTCCCATTGTACTCTTACTGCTTCCAAA 3'
                       *** *** ***  * **  *  ************  * *

      Gly F------- --5' CCAAACACCTCCCTCTCCACTCTTACTGCTT 3'
```

Fig 3.9: Clustal W 1.8 multiple sequence alignment of the 4CL gene sequences of fabaceae members: *Amorpha* (AF435968), *Glycine* (AF002259) and *Glycine* (AF279267). The conserved region selected for designing primers is in bold letters.

3.3.5.2. Amplification and analysis of 1.3 Kb fragment

The primer combination **GlyF** and **Fab4clR** was used for amplification using RT 1st strand cDNA as template. A fragment of approximately 1.3 kb was (Sush4CL1300, Fig. 3.10 a) amplified using **GlyF** and **Fab4clR** primers. The amplicon was gel eluted and cloned in pGEM-T easy vector (Promega, USA). Recombinant plasmid DNA were isolated and digested with *Eco*R I. The digestion pattern revealed presence of inserts (Fig. 3.10 b). The clone was designated as SUSH4CL1300 (Fig. 3.11). SUSH4CL1300 sequence was used for searching sequence similarity with the sequences available in the gene data base (NCBI) using BLAST and it showed 65-75% homology with the other reported 4CL gene sequences. SUSH4CL1300 was aligned using ClustalW 1.8 with of with Dicot (*Glycine max* 4CL1: AF279267) Gymnosperm (*Pinus, Acc*. No.U12013) and Monocot (Rice, X52623) and this SUSH4CL1300 sequences were showing 72% sequence similarity with *Glycine* , 68% sequence similarity with *Pinus* and 66% sequence similarity with rice 4CL genes respectively**.** The SUSH4CL1300 gene sequence was submitted in NCBI Gen-Bank database and allotted accession number for this gene is DQ986905.

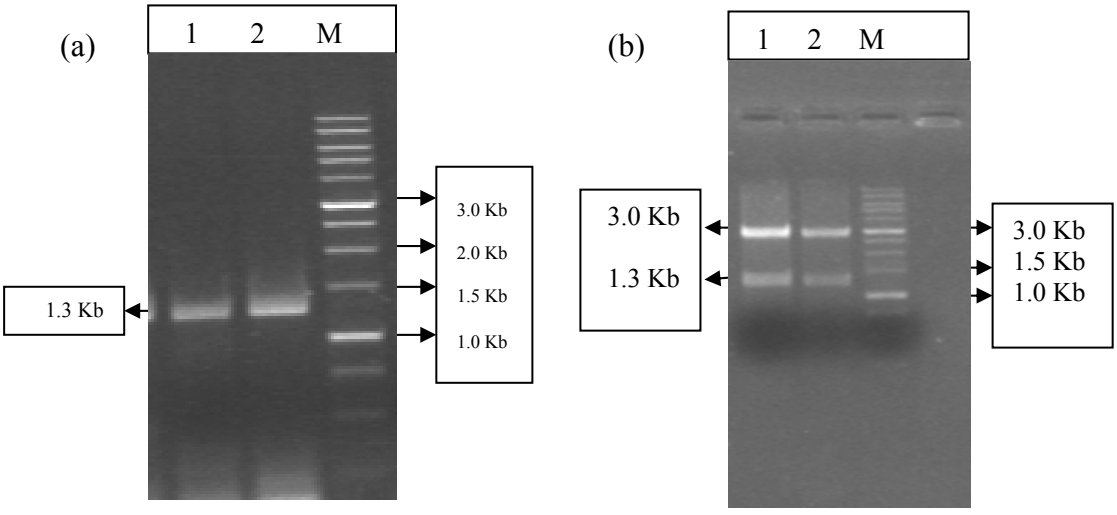


Fig 3.10: cDNA amplification and restriction digestion of 1.3 kb fragment using GlyF & Fab4CLR primers. (a) Lane M:1 Kb Ladder, Lane-1 & 2 : PCR amplified 1.3 Kb fragment (b) Lane M: 1 Kb Ladder Lane-1 & 2 *EcoRI* digested 1.3 Kb cloned in pGEMT Easy vector.

Nucleotide sequences of SUSH4CL1300:

```
   1 CCAAACACCT CCCTCTCCAC TCTTACTGCT TCGAGAACCT ATCTCAGGTC
  51 AAAGATCGAC CCTGCCTCAT CAACGGCGAC ACCGGCGAGA CCTTTACCTA
 101 TGCCGATGTT GAGCTCACCG CCCGCCGTGT CGCCGCCGGC CTCACCAAAC
 151 TTGGTATCCG ACAAGGTGAC GTCATCATGC TCGTGCTCCG GAACTGTCCC
 201 CAATTCGCTC TGGCCTTCCT GGGAGCCTCC TTCGCCGGCG CCGTGGTCAC
 251 CACGGCCAAC CCCTTCTTCA CTCCCGCCGA GCTGGCGAAA CAAGCCACCT
 301 CGTCCAAGTC AAAGCTGATC ATAACGCATG CGGCTTTCGT TGAGAAAATC
 351 AAGGATTTCG CGGATACCAA TGGCGTTTCA CTGATGCTCA TTGACTCTAC
 401 TTTTCCAGAA AAGGAAGGTA TTTCACATTT TTCTCTACTC ACCAAAGCTG
 451 ACGAAACTGA TACGCCGGCC GTCAAGATCA GCCCTGACGA CATTGTGGCA
 501 CTGCCGTACT CCTCCGGAAC CTCCGGCGTT CCCAAGGGCG TGATGCTAAC
 551 ACACAAAAAT CTGGTAACTT CCGTGGCTCA GCTCGTCGAC GGCGAAAACC
 601 CGAACCAGTA CATCACCAGC GACGACGTAC ATATCTGCGT TCTTCCAATG
 651 TTCCATATCT ATGCGCTTAA CCCCATTTTG CTCTGCGGTA TCCGAGCCGG
 701 AGCAGCCATC CTGACCATGA GCAAGTACGA CATCACCACG TTGTTGAAGA
 751 TGATTGAGAC TTACAAGGTG ACAATGGCGT CGTTCGTGCC TCCGATTCTA
 801 TTAAACATCG TGAAGAGTGA GAAAGTTGAT CGGCACGACC TGTCCTCCAT
 851 AAGGATGATC GTTACCGGAG CAGCGCCGGT GAGCGGGGAG CTGGAACAAG
 901 CCCTGAGAGC TAAGATTCCT CACGCCATAC TTGGACAGGG ATATGGGATG
 951 ACGGAGGGTG GTGCTCTGTC GATAAGCCTG TCCTTCGCGA AGGAGCCTGT
1001 GGAGATGAAA TCAGGCGCGT GCGGGAGCGT GATAAGGAAT GCAGAGATGA
1051 AGATCGTGGA CATTGAAACG GGAGCTTCGC TTCCAAGGAA CAGAGCCGGT
1101 GAAATCTGCA TTAGAGGAAA TCAAGTTATG AAAGGGTACC TAAACGATTC
1151 GGAGGCGACC AAAACGACGA TAGATGAAGA AGGATGGCTT CACTCGGGGG
1201 ACATCGGATA CATAGACGAC GACGAGGAGG TGTTCGTTGT GGATCGTTTG
1251 AAGGAGATCA TCAAATACAA AGGATTTCAA GTGGCTCCAG C
```

Fig. 3.11: Nucleotide sequence of: SUSH4CL1300. GlyF forward and Fab4CLR reverse primers are indicated in italics and are underlined.

3.3.6. Rapid amplification of cDNA ends (RACE)

If a partial cDNA sequence is known, unknown sequences to the 5' and 3' of the known sequence can be reverse transcribed from RNA, amplified by PCR using RACE. SUSH4CL1300 sequences was showing maximum similarity with the reported 4CL1 gene in NCBI data base. This 4CL1 gene has a pivotal role in lignin biosynthetic pathway. Hence, the 4CL1 gene specific primers were designed from SUSH4CL1300 nucleotide sequences to perform RACE to fish out

the full-length gene. The RACE reaction was performed as describe in the chapter 2 (Section: 2.10.11).

3.3.6.1. 3' Rapid amplification of cDNA ends (3'RACE)

Gene Specific forward primers 4CLRCFI and 4CLRCFII were designed from the known sequence of SUSH4CL1300. These primers 4CLRCFI and 4CLRCFII were used as gene specific reverse and nested reverse primer respectively.

4CLRCFI 5'GGGATGACGGAGGGTGGTGCTCTGTCGA 3'
4CLRCFII 5' GCTTCCACACGCCATACTTGGACAGGGA 3'

RACE reaction (Section 2.10.11) was done using total RNA (3.0 μg) isolated from xylem tissue of *L. leucocephala* as described (Section 2.10.7). 3' Race was done using RACE ready cDNA as template and primary PCR was done using 4CLRCFI and 3'GRP (3' gene racer primer provided with the kit), approximately 900 bp long fragments was amplified. This primary PCR product was diluted to 100 times and used as template to perform secondary PCR for 3'RACE using 4CLRCFII and 3'NGRP (3' nested gene racer primer provided with the kit) primers, approximately 900 bp fragment was amplified (Fig: 3.12) cloned inpGEMT Easy vector (Fig: 3.13) and sequenced. The sequencing data revealed that the 4CL gene ends with TAA not with TGA as reported in so many genes. The length of 3'UTR is 238 bp and it ends with 20 poly A tails (Fig: 3.14).

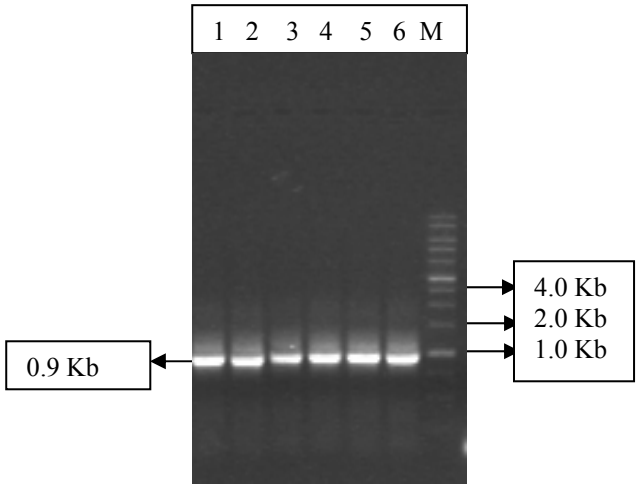


Fig 3.12 PCR amplified 0.9kb 3'RACE product from RACE ready cDNA using 4CLRCFII and 3'NGRP (3' nested gene racer primer) primers. Lane M: 1 Kb Ladder, Lane1, 2, 3, 4, 5 & 6: PCR amplified 0.9 Kb fragment.

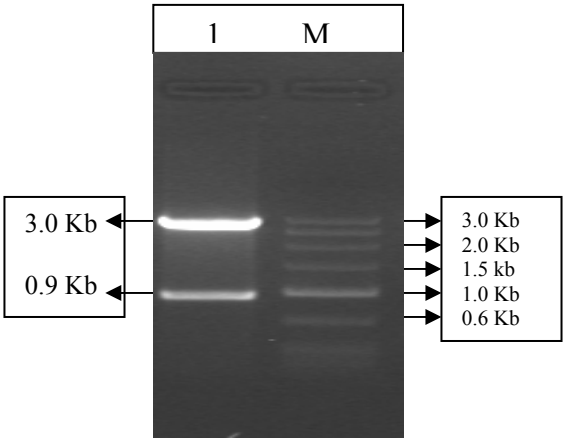


Fig 3.13: cDNA clone of 3'RACE from *L. leucocephala* using 4CLRCFII and 3'NGRP primers. Lane M: low range ruler (B.Genei), Lane 1: *EcoRI* digested 0.9 kb cloned fragment in T vector

1 AGGGATATGG GATGACGGAG GGTGGTCCTC TGTCGATAAG CTTGTCGTTC
51 GCGAAGGAGC CGGTGGAGAT GAAATCAGGC GCGTGCGGGA GCGTGATAAG
101 AAACGCAGAG ATGAAGATTG TTGACATTGA GACGGGAGCT TCGCTTCCAA
151 GAAACAGGGC TGGTGAAATC TGCATTAGAG GAAATCAAGT TATGAAAGGG
201 TACCTAAACG ACCCGGAGGC GACGAAAACG ACGATAGACG AAGAAGGATG
251 GCTTCACACG GGGGACATCG GACACGTAGA CGACGACGAC GAGGTGTTCG
301 TTGTGGATCG TTTGAAGGAG ATCATCAAAT ACAAAGGTTT CCAAGTGGCT
351 CCTGCTGAGC TGGAGGCTCT TCTCATCTCT CACCCTTTCA TTTCTGATGC
401 TGCCGTTGTT CCGATGAAAG ATGAGGCTGC GGGGGAGCTT CCGGTGGCAT
451 TTGTGGTTAG GTCAAATGGT TTTAAGATCT CTGAGGATGA CATCAAGCTT
501 TTCATTTCAC AACAGGTGGT GTATTACAAG AGAATCCACA AGGTTATTTT
551 CACAGACACT ATTCCTAAAG CAGTTTCTGG CAAAATTTTA CGAAAGGATT
601 TAAAAGCAAG GCTTGCATCA GATTTGGGCA ATTAA*TTAAT TAGCCTAATC*
651 CATTTGCGAA CTAATTAAGG GTTAAAACAA GTCAACTAAT TAATTACGTG
701 TATGAATTTT TAATACATTA TTATTAGTGG TTGGTTTATA TATATGTTTT
751 TTGTTTTCAT TTGGTTTTGT ATACGAGTAT GTTGCTATAA ATGCAATTGG
801 ATCTTGTGTC TTGTAAGTAC TTCAAATTAT ATATTGTATC CCATCCGTGG
851 TTCAAAAAAA AAAAAAAAAA AAA

Fig 3.14: Nucleotide sequence of cDNA clone of 3'RACE of *L. leucocephala* using 4CLRCFII and 3'NGRP (3' nested gene racer primer). The sequenced in bold represent the sequence in reading frame till stop, sequence in italics represent the 3'UTR region.

3.3.6.2. 5' Rapid amplification of cDNA ends (5' RACE).

The primer 4CLRCRI was designed from the known sequence of SUSH4CL1300. The primer Fab4CLR and 4CLRCRI was used as reverse and reverse nested primer for 5' RACE respectively.

Fab4CLR 5' CGATCCACAACGAACACCTCCTCGTCGTCGTCT 3'
4CLRCRI 5' GAGCCTCCAGCTCAGCAGGAGCCACT 3'

5' RACE was done using RACE ready cDNA as template and primary PCR was done using Fab4CLR and 5'GRP (5' gene racer primer provided with the kit) primers, approximately 1400 bp long fragment was amplified. This primary PCR product was diluted to 100 times and used as template to perform secondary PCR for 5'RACE using 4CLRCRI and 5'NGRP (5' nested gene racer primer provided with the kit) primers, approximately 1400 bp fragment was amplified (3.15) and cloned in pGEMT Easy vector (3.16). The sequencing data revealed that the 4CL gene starts with ATG and the length of 5'UTR is 68 bp (Fig: 3.17).

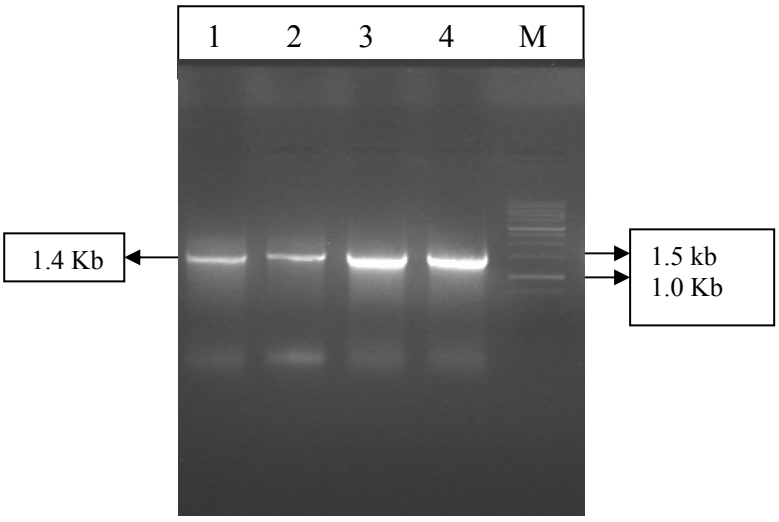


Fig.3.15: PCR amplified 1.4kb 5'RACE product from RACE ready cDNA using 4CLRCRI and 5'NGRP (5' nested gene racer primer) Lane M: 1 Kb Ladder, Lane1, 2, 3, & 4: PCR amplified 1.4 Kb fragment.

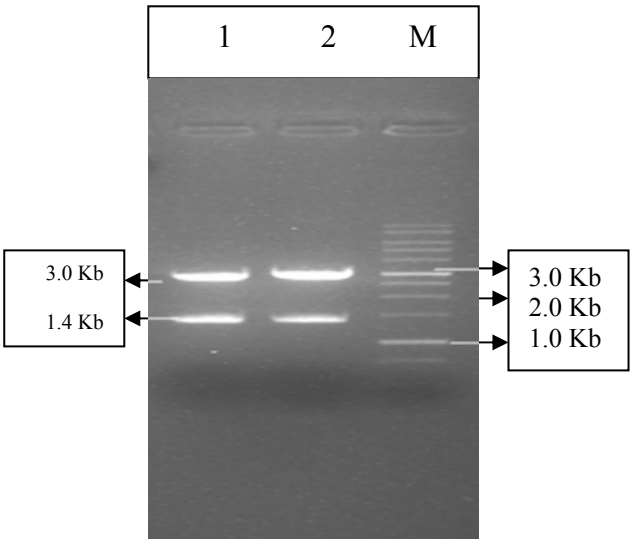


Fig 3.16: cDNA clone of 5'RACE from *L. leucocephala* using 4CLRCRI and 5'NGRP (5' nested gene racer primer) primers, Lane M: 1 Kb Ladder, Lane 1 and 2: *EcoRI* digested 1.4 Kb cloned fragment in pGEMT Easy vector.

Nucleotide sequences of 5'RACE:

```
   1 GAAAACTTCA TTTCTCTCTC CCTTTCTTTC TCTTTGTCTG TTCAACAAAC
  51 TGAATAAAGC AATTCTCAAT GGAAACTCCT TCGATGGAAT TCATCTTCCG
 101 ATCGAAACTC CCCGATATTT ACATCCCAGA CCACCTCCCA CTTCACTCCT
 151 ACGTCTTCGA GAACCTATCT CAGGTCAAAG ATCGACCCTG CCTCATCGAC
 201 GGCGACACTG GCGAGACCCT TACCTATGCC GACGTCGAGC TCACCGCCCG
 251 CCGTGTTGCC GCCGGCCTCA CCAAACTCGG CATCCAACAA GGTGACGTCA
 301 TCATGCTCGT GCTCCGTAAC TGTCCCCAGT TCGCTCTGGC CTTCCTGGGA
 351 GCCTCCTTCG CCGGCGCCGC AGTCACCACT GCCAACCCCT TGTCCACTCC
 401 AGCCGAGCTG GCGAAACAAG CCACCGCGTC CAAGTCGAAA CTGATCATAA
 451 CGCAAGCGGC TTTCGTTGAG AAAATCAAGG ATTTCGCTGA CAAACGTGGC
 501 GTTTCTTTGA TGTGCATTGA TTCTACTTTC CCAGAAACGG AAGGTATTTC
 551 ACATTTTTCT TTACTCACCC AAGCCGACGA AGCTTGTATG CCGGCCGTCA
 601 AGATCAGCCC CGACGACGTT GTGGCACTGC CGTATTCCTC CGGCACCTCC
 651 GGCTTTCCCA AGGGCGTGAT GCTAACGCAC AAGAATCTGG TGACCTCCGT
 701 GGCTCAGCTC GTCGACGGCG AAAACCCGAA CCAGTACACC ACTAGCGACG
 751 ACGTACATAT CTGCGTTCTT CCAATGTTCC ATATCTATGC GCTGAACTCC
 801 ATTTTGCTCT GCTGCATCCG AGCCGGAGCC GCCATTCTGA CGATGGGTAA
```

```
 851 GTACGACATC GCCACGTTGT TGAAGATGAT CAAGACTTAC AAGGTGACAA
 901 TGGCGTCGTT TGTGCCTCCG ATCCTATTAA ACATCGTGAA GAGTGAGGAA
 951 GTTGACCGGC ACGACCTGTC GTCCATAAGG ACGATCGTCA CCGGAGCAGC
1001 GCCGGTGAGC GTGGAGCTGG AACAAGCCCT GAGGGCTAAG CTTCCACACG
1051 CCATACTTGG ACAGGGATAT GGGATGACGG AGGGTGGTCC TCTGTCGATA
1101 AGCTTGTCGT TCGCGAAGGA GCCGGTGGAG ATGAAATCAG GCGCGTGCGG
1151 GAGCGTGATA AGAAACGCAG AGATGAAGAT TGTTGACATT GAGACGGGAG
1201 CTTCGCTTCC AAGAAACAGG GCTGGTGAAA TCTGCATTAG AGGAAATCAA
1251 GTTATGAAAG GGTACCTAAA CGACCCGGAG GCGACGAAAA CGACGATAGA
1301 CGAAGAAGGA TGGCTTCACA CGGGGGACAT CGGACACGTA GACGACGACG
1351 ACGAGGTGTT CGTTGTGGAT CGTTTGAAGG AGATCATC
```

Fig 3.17: Nucleotide sequences of cDNA clone of 5'RACE of *L. leucocephala* using 4CLRCRI and 5'NGRP primers. The sequence in bold represent the sequence in reading frame till reverse gene specific primer (4CLRCRI), sequence in italics represent the 5'UTR region.

Both 3' RACE and 5' RACE sequences were aligned together and the common region was identified. Both the sequence were arranged to make full length 4Cl gene along with 5' and 3' UTR. The exact length of coding region of full length 4CL gene are 1629 nucleotide long (Fig. 3.18).

Nucleotide sequences of Ll4CL1:

```
   1 GAAAACTTCA TTTCTCTCTC CCTTTCTTTC TCTTTGTCTG TTCAACAAAC
  51 TGAATAAAGC AATTCTCAAT GGAAACTCCT TCGATGGAAT TCATCTTCCG
 101 ATCGAAACTC CCCGATATTT ACATCCCAGA CCACCTCCCA CTTCACTCCT
 151 ACGTCTTCGA GAACCTATCT CAGGTCAAAG ATCGACCCTG CCTCATCGAC
 201 GGCGACACTG GCGAGACCCT TACCTATGCC GACGTCGAGC TCACCGCCCG
 251 CCGTGTTGCC GCCGGCCTCA CCAAACTCGG CATCCAACAA GGTGACGTCA
 301 TCATGCTCGT GCTCCGTAAC TGTCCCCAGT TCGCTCTGGC CTTCCTGGGA
 351 GCCTCCTTCG CCGGCGCCGC AGTCACCACT GCCAACCCCT TGTCCACTCC
 401 AGCCGAGCTG GCGAAACAAG CCACCGCGTC CAAGTCGAAA CTGATCATAA
 451 CGCAAGCGGC TTTCGTTGAG AAAATCAAGG ATTTCGCTGA CAAACGTGGC
 501 GTTTCTTTGA TGTGCATTGA TTCTACTTTC CCAGAAACGG AAGGTATTTC
 551 ACATTTTTCT TTACTCACCC AAGCCGACGA AGCTTGTATG CCGGCCGTCA
 601 AGATCAGCCC CGACGACGTT GTGGCACTGC CGTATTCCTC CGGCACCTCC
 651 GGCTTTCCCA AGGGCGTGAT GCTAACGCAC AAGAATCTGG TGACCTCCGT
 701 GGCTCAGCTC GTCGACGGCG AAAACCCGAA CCAGTACACC ACTAGCGACG
```

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   751 ACGTACATAT CTGCGTTCTT CCAATGTTCC ATATCTATGC GCTGAACTCC
   801 ATTTTGCTCT GCTGCATCCG AGCCGGAGCC GCCATTCTGA CGATGGGTAA
   851 GTACGACATC GCCACGTTGT TGAAGATGAT CAAGACTTAC AAGGTGACAA
   901 TGGCGTCGTT TGTGCCTCCG ATCCTATTAA ACATCGTGAA GAGTGAGGAA
   951 GTTGACCGGC ACGACCTGTC GTCCATAAGG ACGATCGTCA CCGGAGCAGC
  1001 GCCGGTGAGC GTGGAGCTGG AACAAGCCCT GAGGGCTAAG CTTCCACACG
  1051 CCATACTTGG ACAGGGATAT GGGATGACGG AGGGTGGTCC TCTGTCGATA
  1101 AGCTTGTCGT TCGCGAAGGA GCCGGTGGAG ATGAAATCAG GCGCGTGCGG
  1151 GAGCGTGATA AGAAACGCAG AGATGAAGAT TGTTGACATT GAGACGGGAG
  1201 CTTCGCTTCC AAGAAACAGG GCTGGTGAAA TCTGCATTAG AGGAAATCAA
  1251 GTTATGAAAG GGTACCTAAA CGACCCGGAG GCGACGAAAA CGACGATAGA
  1301 CGAAGAAGGA TGGCTTCACA CGGGGGACAT CGGACACGTA GACGACGACG
  1351 ACGAGGTGTT CGTTGTGGAT CGTTTGAAGG AGATCATCAA ATACAAAGGT
  1401 TTCCAAGTGG CTCCTGCTGA GCTGGAGGCT CTTCTCATCT CTCACCCTTT
  1451 CATTTCTGAT GCTGCCGTTG TTCCGATGAA AGATGAGGCT GCGGGGGAGC
  1501 TTCCGGTGGC ATTTGTGGTT AGGTCAAATG GTTTTAAGAT CTCTGAGGAT
  1551 GACATCAAGC TTTTCATTTC ACAACAGGTG GTGTATTACA AGAGAATCCA
  1601 CAAGGTTATT TTCACAGACA CTATTCCTAA AGCAGTTTCT GGCAAAATTT
  1651 TACGAAAGGA TTTAAAAGCA AGGCTTGCAT CAGATTTGGG CAATTAATTA
  1701 ATTAGCCTAA TCCATTTGCG AACTAATTAA GGGTTAAAAC AAGTCAACTA
  1751 ATTAATTACG TGTATGAATT TTTAATACAT TATTATTAGT GGTTGGTTTA
  1801 TATATATGTT TTTTGTTTTC ATTTGGTTTT GTATACGAGT ATGTTGCTAT
  1851 AAATGCAATT GGATCTTGTG TCTTGTAAGT ACTTCAAATT ATATATTGTA
  1901 TCCCATCCGT GGTTCAAAAA AAAAAAAAAA AAAAA
```

Fig 3.18: Nucleotide sequence of 4CL with 5' and 3' UTRs. Both the 5' (Green) and 3' (yellow) UTRs are shaded bold capitals, 'Start (Pink) and stop (Blue) codons are shaded bold capitals. Polyadenylation signal is underlined and shaded bold capitals. The common regions are in italics.

3.3.7. Analysis of Ll4CL1 gene

Gene specific forward and reverse primers were designed from the start and stop codon of 5'and 3'RACE sequences respectively and designated as Ll4CLF and Ll4CLR.

Ll4CLF 5' ATGGAAACTCCTTCGATGGAATTCA 3'
Ll4CLR 5' TTAATTGCCCAAATCTGATGCAAGC 3'

These gene specific forward and reverse primers were used to amplify full length gene using RT^{1st} strand cDNA as template. A fragment of approximately 1.6 kb was amplified (Fig: 3.19).This PCR amplified 1.6 Kb fragment was cloned in pGEM T Easy vector (Fig: 3.20). The clone was designated as Ll4CL1. The sequencing data revealed that the sequence shows exactly the same sequences as were in 5' and 3' RACE. The Ll4CL1 was submitted in NCBI Gen-Bank database and allotted accession number for this gene is FJ205490.

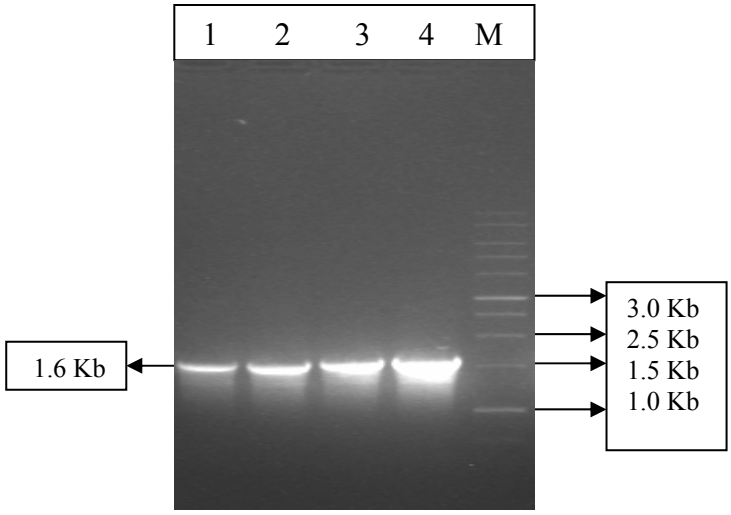

Fig 3.19: PCR amplified 1.6 kb fragment from cDNA of *L. leucocephala* using Ll4CLF and Ll4CLR primers, Lane M: 1 Kb Ladder, Lane1, 2, 3 & 4: PCR amplified 1.6 kb fragment.

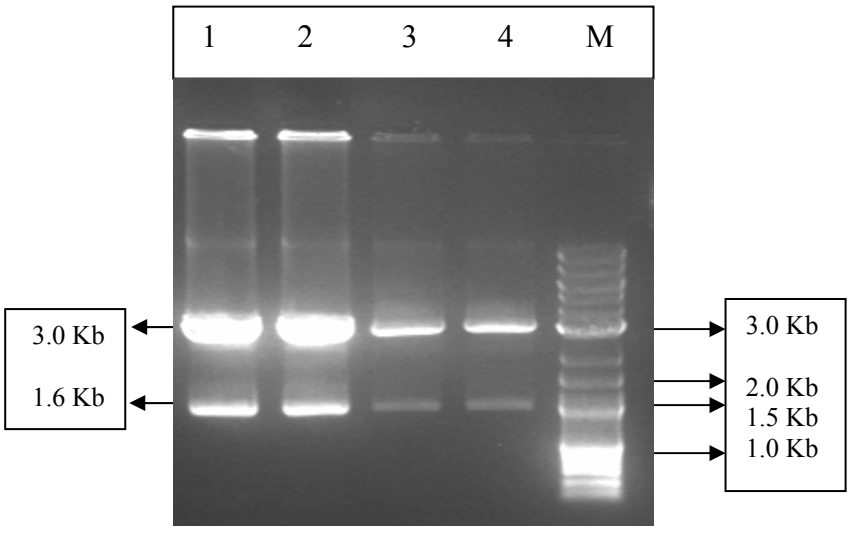

Fig 3.20: cDNA clone of 4CL from *L. leucocephala* using Ll4CLF and Ll4CLR primers, Lane M: 1 Kb Ladder, Lane 1, 2, 3 and 4: *EcoRI* digested 1.6 Kb cloned fragment in pGEM T Easy vector.

The deduced amino acid sequences of Ll4CL1 gene shows that, the coding region consist of 542 amino acids, with molecular weight 58 kd, theoratical Pi 5.56 and empirical formula $C_{2632}H_{4230}N_{688}O_{787}S_{24}$. Deduced amino acid sequences of Ll4CL1 gene show the presence of AMP binding superfamily domain. It shows the presence of two conserved motifs (Allina *et al.,* 1998; Becker-Andre *et al.,* 1991; Ehlting *et al.,* 1999; Hu *et al.,* 1998; Uhlmann and Ebel, 1993). This first motif is AMP binding box I conserved motif. The box I (AMP binding) conserved motif (LPYSSGTSGFPK) of Ll4CL1 is present at amino acid residues 187 – 198, this putative AMP binding box I motif is not only almost absolutely conserved in all 4CL gene , but highly similar motifs are also found in luciferases, acetyl CoA synthetases. The second conserved domain Box II domain is GEICIRG which is at amino acid residues 386-392, this putative domain is absolutely conserved in all 4CL genes and its central cystein residue has been suggested to be directly involved in catalysis (fig. 3.21). The total 9 cystein residues are present which are proposed to be involved in catalytic activity (Bhushan *et al*., 2006; Ehlting *et al*., 2001; Kumar and Ellis, 2003).

Deduced amino acids sequences of Ll4CL1:

```
  1 METPSMEFIF  RSKLPDIYIP  DHLPLHSYVF  ENLSQVKDRP  CLIDGDTGET
 51 LTYADVELTA  RRVAAGLTKL GIQQGDVIML  VLRNCPQFAL  AFLGASFAGA
101 AVTTANPLST  PAELAKQATA  SKSKLIITQA  AFVEKIKDFA  DKRGVSLMCI
151 DSTFPETEGI  SHFSLLTQAD  EACMPAVKIS  PDDVVALPYS  SGTSGFPKGV
201 MLTHKNLVTS VAQLVDGENP  NQYTTSDDVH  ICVLPMFHIY  ALNSILLCCI
251 RAGAAILTMG KYDIATLLKM  IKTYKVTMAS  FVPPILLNIV  KSEEVDRHDL
301 SSIRTIVTGA  APVSVELEQA  LRAKLPHAIL  GQGYGMTEGG PLSISLSFAK
351 EPVEMKSGAC GSVIRNAEMK  IVDIETGASL  PRNRAGEICI  RGNQVMKGYL
401 NDPEATKTTI  DEEGWLHTGD  IGHVDDDDEV  FVVDRLKEII  KYKGFQVAPA
451 LEALLISHPF  ISDAAVVPMK  DEAAGELPVA FVVRSNGFKI  SEDDIKLFIE
501 SQQVVYYKRI  HKVIFTDTIP  KAVSGKILRK  DLKARLASDL  GN
```

Fig 3.21: Deduced amino acid sequences of Ll4CL1 gene. The Box1 AMP binding domains is shaded in green and other highly conserved catalytic domain Box II is shaded in yellow.

This enzyme belongs to the family of ligases, specifically those forming carbon-sulfur bonds as acid-thiol ligases. The systematic name of this enzyme class is 4-coumarate Co A ligase (AMP-forming). Other names in common use include 4-coumaroyl-Co A synthetase, p-coumaroyl CoA ligase, 4-coumaryl coenzyme A synthetase, p-coumaryl Co A synthetase, p-coumaryl-CoA ligase, feruloyl CoA ligase, hydroxycinnamoyl CoA synthetase, 4-coumarate:coenzyme A ligase, caffeolyl coenzyme A synthetase, p-hydroxycinnamoyl coenzyme A synthetase, feruloyl coenzyme A synthetase, sinapoyl coenzyme A synthetase, 4-coumaryl-CoA synthetase, hydroxycinnamate:CoA ligase and p-hydroxycinnamic acid:CoA ligase. The deduced amino acid sequences of the Ll4CL1 BLAST in the NCBI database and it was found that it has conserved AMP binding domain. This domain present in all the 4CL proteins reported from different plants was located here too (Fig. 3.22).

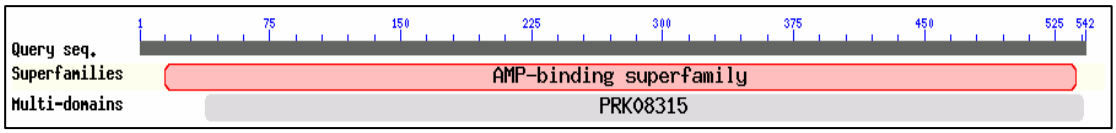

Fig 3.22: Image showing AMP binding superfamily present in 4CL gene (FJ205490).

The nucleotide sequences of Ll4CL1 (FJ205490) cDNA clone and their respective deduced amino acids sequences were searched for sequence similarity in NCBI Gen-Bank database using BLAST and ClustalW 1.8 and it showed sequence similarity with other reported plant 4CL cDNA clones. The nucleotide sequence alignment of the Ll4CL1 gene with representative member of the dicots *e.g. Glycine* 4CL1: AF279267, the monocots *e.g. Zea mays* 4CL: AY566301 and the gymnosperms *e.g. Pinus taeda*: U12012 showed 73%, 68% and 65% sequence similarity respectively (Fig 3.23).

Nucleotide sequence alignment using Clustal W 1.8.

```
Glycine4CL1/AF279267      --------------------------------------------------
Leucaena4CL1/FJ205490     --------------------------------------------------
Pinustaeda4CL1/U12012     ------------------------------------------ATGGC
Zeamays4CL/AY566301       ATGGGTTCCGTAGACGCGGCGATCGCGGTGCCGGTGCCGGCGGCGGAGGA
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Glycine4CL1/AF279267     -ATGGCACCTTCTCCACAAGAAATCATCTTCCGATCCCCACTCCCCGATA
Leucaena4CL1/FJ205490    -ATGGAAACTCCTTCGATGGAATTCATCTTCCGATCGAAACTCCCCGATA
Pinustaeda4CL1/U12012    CAACGGAATCAAGAAGGTCGAGCATCTGTACAGATCGAAGCTTCCCGATA
Zeamays4CL/AY566301      GAAGGCGGTGGAGGAGAAGGCGATGGTGTTCCGGTCCAAGCTTCCCGACA
                          *  *             *      * * * * **    ** ***** *

Glycine4CL1/AF279267     TTCCGATCCCCACACATCTCCCATTGTACTCTTACTGCTTCCAAAACTTG
Leucaena4CL1/FJ205490    TTTACATCCCAGACCACCTCCCACTTCACTCCTACGTCTTCGAGAACCTA
Pinustaeda4CL1/U12012    TCGAGATCTCCGACCATCTGCCTCTTCATTCGTATTGCTTTGAGAGAGTA
Zeamays4CL/AY566301      TCGAGATCGACAGCAGCATGGCGCTGCACACCTACTGCTTCGGGAAGATG
                         *    ***          *  *  *  *  * **   ***    *   *

Glycine4CL1/AF279267     TCACAGTTCCATGACCGTCCATGCCTCATCGACGGCGACACCGGCGAGAC
Leucaena4CL1/FJ205490    TCTCAGGTCAAAGATCGACCCTGCCTCATCGACGGCGACACTGGCGAGAC
Pinustaeda4CL1/U12012    GCGGAATTCGCAGACAGACCCTGTCTGATCGATGGGGCGACAGACAGAAC
Zeamays4CL/AY566301      GGCGAGGTGGCGGAGCGGGCGTGCCTGATCGACGGGCTGACGGGCGCGTC
                             *  *    **  *  * ** ** ***** **    ** * *    *

Glycine4CL1/AF279267     CCTCACCTACGCCGACGTCGACCTCGCTGCTCGCCGCATCGCCTCCGGCC
Leucaena4CL1/FJ205490    CCTTACCTATGCCGACGTCGAGCTCACCGCCCGCCGTGTTGCCGCCGGCC
Pinustaeda4CL1/U12012    TTATTGCTTTTCAGAGGTGGAACTAATTTCTCGCAAGGTCGCTGCCGGTC
Zeamays4CL/AY566301      GTACACGTACGCGGAGGTGGAGTCCCTGTCCCGGCGCGCCGC-GTCGGGG
                                *   * ** ** **        * **       **   ***

Glycine4CL1/AF279267     TCCACAAAATCGGCATCCGCCA-GGGTGACGTCATCATGCTCGTCCTACG
Leucaena4CL1/FJ205490    TCACCAAACTCGGCATCCAACA-AGGTGACGTCATCATGCTCGTGCTCCG
Pinustaeda4CL1/U12012    TGGCGAAGCTCGGGTTGCAGCAGGGGCAG-GTTGTCATGCTTCTCCTTCC
Zeamays4CL/AY566301      CTGCGCGCCATGGGGGTGGGCAAGGGCGACGTGGTGATGAGCCTGCTCCG
                                    **      **  **    **  * ***    * ** *

Glycine4CL1/AF279267     CAACTGCCCGCAGTTCGCCCTCGCCTTCCTCGGCGCCACCCACCGTGGCG
Leucaena4CL1/FJ205490    TAACTGTCCCCAGTTCGCTCTGGCCTTCCTGGGAGCCTCCTTCGCCGGCG
Pinustaeda4CL1/U12012    GAATTGCATCGAATTTGCGTTTGTGTTCATGGGGGCCTCTGTCCGGGGCG
Zeamays4CL/AY566301      CAACTGCCCCGAGTTCGCCTTCACCTTCCTGGGCGCCGCCCGCCTGGGCG
                          ** **     * ** **  *    *** * ** *** *   *   ****

Glycine4CL1/AF279267     CCGTCGTCACCACAGCCAACCCCTTCTACACGCCGGCGGAGCTTGCGAAG
Leucaena4CL1/FJ205490    CCGCAGTCACCACTGCCAACCCCTTGTCCACTCCAGCCGAGCTGGCGAAA
Pinustaeda4CL1/U12012    CCATTGTGACCACGGCCAATCCTTTCTACAAGCCGGGCGAGATCGCCAAA
Zeamays4CL/AY566301      CCGCCACCACCACGGCCAACCCGTTCTACACCCCGCACGAGGTGCACCGC
                         **      ***** ***** ** ** * **  **    *** *

Glycine4CL1/AF279267     CAAGCGACGGCCACGAAAACCAGGCTCGTCATAACGCAATCCGCGTACGT
Leucaena4CL1/FJ205490    CAAGCCACCGCGTCCAAGTCGAAACTGATCATAACGCAAGCGGCTTTCGT
Pinustaeda4CL1/U12012    CAGGCCAAGGCCGCAGGCGCGCGCATCATAGTTACCCTGGCAGCTTATGT
Zeamays4CL/AY566301      CAGGCGGAGGCGGCCGGCGCCCGGCTCATCGTGACCGAGGCCTGCGCCGT
                         ** **    **  *     *     *  *  * **     *       **
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Glycine4CL1/AF279267     AGAGAAAATCAAGAGTTTCGCGGACAGCAGCAGCGATGTCATGGTGATGT
Leucaena4CL1/FJ205490    TGAGAAAATCAAGGATTTCGCTGACAAACGTGGCGTT-TCTT--TGATGT
Pinustaeda4CL1/U12012    TGAGAAACTGGCCGA-TCTGCAGAGCCACGATGTGCT--CGT--CA-TCA
Zeamays4CL/AY566301      GGAGAAGGTGCGGGAGTTCGCGGCGGAGCGGGGCA-T--CCC--CG-TGG
                          *****  *       *  ** *      *  *   *  *       *

Glycine4CL1/AF279267     GCATTGATGATGATTTTTCTTATGAAAACGACGGCGTTTTGCATTTCTCA
Leucaena4CL1/FJ205490    GCATTGATTCTACTTTCCCA---GAAACGGAAGGTATTTCACATTTTTCT
Pinustaeda4CL1/U12012    CAATCGATGATGCTCCCAAG---------GAAGGTTGCCAACATATTTCC
Zeamays4CL/AY566301      TCACCGTCGACGGGCGCTTC---------GACGGCTGCGTGGAGTTCGCC
                           *  *                      ** **       *  *  *

Glycine4CL1/AF279267     ACGCTCAGTAACGCCGACGAAACGGAAGCCCCTGCCGTTAAGATTAACCC
Leucaena4CL1/FJ205490    TTACTCACCCAAGCCGACGAAGCTTGTATGCCGGCCGTCAAGATCAGCCC
Pinustaeda4CL1/U12012    GTTCTGACCGAAGCCGACGAAACCCAATGCCCGGCCGTGAAAATCCACCC
Zeamays4CL/AY566301      GAGCTGATCGCGGCCGA-GGAGCTGGAGGCT--GACGCCGACATCCACCC
                            ** *     ***** * * *          * **   * **   ***

Glycine4CL1/AF279267     TGACGAGCTCGTTGCGCTTCCGTTTTCTTCTGGCACGTCTGGGCTCCCCA
Leucaena4CL1/FJ205490    CGACGACGTTGTGGCACTGCCGTATTCCTCCGGCACCTCCGGCTTTCCCA
Pinustaeda4CL1/U12012    GGACGATGTCGTGGCGTTGCCCTATTCTTCCGGAACCACGGGGCTCCCCA
Zeamays4CL/AY566301      CGACGACGTCGTCGCGCTGCCNTACTCCTCCGGCACCACCGGGCTGCCCA
                          *****  * ** **  * ** *  ** ** ** **  * **  * ****

Glycine4CL1/AF279267     AGGGCGTTATGTTATCGCATAAAAACTTGGTCACCACGATAGCGCAGTTA
Leucaena4CL1/FJ205490    AGGGCGTGATGCTAACGCACAAGAATCTGGTGACCTCCGTGGCTCAGCTC
Pinustaeda4CL1/U12012    AGGGCGTGATGTTAACGCACAAAGGCCTGGTGTCCAGCGTTGCCCAGCAG
Zeamays4CL/AY566301      AGGGCGTCATGCTCACCCACCGCAGCCTCATCACCAGCGTCGCGCAGCAG
                         ******* *** *  * **       *  *  **    * ** ***

Glycine4CL1/AF279267      GTTGACGGCGAAAACCCGCACCAATACACTCACAGCGAGGATGTGCTACT
Leucaena4CL1/FJ205490     GTCGACGGCGAAAACCCGAACCAGTACACCACTAGCGACGACGTACATAT
Pinustaeda4CL1/U12012     GTCGATGGTGAAAATCCCAATCTGTATTTCCATTCCGATGACGTGATACT
Zeamays4CL/AY566301       GTTGATGGCGAGAACCCGAACCTGTACTTCCGCAAGGACGACGTGGTGCT
                          ** ** ** ** ** **  * *  **          ** ** **     *

Glycine4CL1/AF279267      CTGTGTGTTGCCTATGTTTCATATCTATGCGCTCAATTCCATTTTGCTCT
Leucaena4CL1/FJ205490     CTGCGTTCTTCCAATGTTCCATATCTATGCGCTGAACTCCATTTTGCTCT
Pinustaeda4CL1/U12012     CTGTGTCTTGCCTCTTTTCCACATCTATTCTCTCAATTCGGTTCTCCTCT
Zeamays4CL/AY566301       GTGCCTGCTGCCGCTGTTCCACATCTACTCGCTGAACTCGGTGCTGCTGG
                           **  *  * **  * ** ** *****  * ** ** **  *  * **

Glycine4CL1/AF279267      GCGGGATTCGTTCCGGTGCGGCCGTGCTTATTTTGCAGAAGTTTGAGATC
Leucaena4CL1/FJ205490     GCTGCATCCGAGCCGGAGCCGCCATTCTGACGATGGGTAAGTACGACATC
Pinustaeda4CL1/U12012     GCGCGCTCAGAGCCGGGGCTGCGACCCTGATTATGCAGAAATTCAACCTC
Zeamays4CL/AY566301       CCGGCCTGCGCGCGGGCTCCACCATCGTGATCATGCGCAAGTTCGACCTG
                           *    *  *  * **  *  *    * *   **   ** *   *  *
```

```
Glycine4CL1/AF279267      ACTACTCTGTTGGAGCTCATCGAGAAGTACAAGGTGACGGTTGCGTCGTT
Leucaena4CL1/FJ205490     GCCACGTTGTTGAAGATGATCAAGACTTACAAGGTGACAATGGCGTCGTT
Pinustaeda4CL1/U12012     ACGACCTGTCTGGAGCTGATTCAGAAATACAAGGTTACCGTTGCCCCAAT
Zeamays4CL/AY566301       GGCGCGCTGGTTGACCTGGTGCGCAGGTACGTGATCACCATCGCGCCCTT
                              *     *  *  *  *    *  ***  * * **  * **  *  *

Glycine4CL1/AF279267      TGTGCCGCCCATCGTTTTGGCGTTGGTTAAGAGCGGAGAGACTCATCGCT
Leucaena4CL1/FJ205490     TGTGCCTCCGATCCTATTAAACATCGTGAAGAGTGAGGAAGTTGACCGGC
Pinustaeda4CL1/U12012     TGTGCCTCCAATTGTCCTGGACATCACAAAGAGCCCCATCGTTTCCCAGT
Zeamays4CL/AY566301       CGTGCCGCCCATCGTGGTGGAGATCGCCAAGAGCCCCCGCGTGACCGCCG
                            ***** ** **  *  *     *    *****

Glycine4CL1/AF279267      ACGACCTGTCGTCTATTCGCGCTGTGGTCACCGGCGCGGCACCCTTAGGA
Leucaena4CL1/FJ205490     ACGACCTGTCGTCCATAAGGACGATCGTCACCGGAGCAGCGCCGGTGAGC
Pinustaeda4CL1/U12012     ACGATGTCTCGTCCGTCCGGATAATCATGTCCGGCGCTGCGCCTCTCGGG
Zeamays4CL/AY566301       GCGACCTCGCGTCCATCCGCATGGTCATGTCCGGCGCCGCGCCCATGGGC
                           ***  *  ****  *  *     *  *  **** ** ** **  *  *

Glycine4CL1/AF279267      GGGGAACTTCAAGAAGCCGTTAAGGCTAGGCTACCACACGCTACTTTTGG
Leucaena4CL1/FJ205490     GTGGAGCTGGAACAAGCCCTGAGGGCTAAGCTTCCACACGCCATACTTGG
Pinustaeda4CL1/U12012     AAGGAACTCGAAGATGCCCTCAGAGAGCGTTTTCCCAAGGCCATTTTCGG
Zeamays4CL/AY566301       AAGGAGCTCCAGGACGCCTTCATGGCCAAGATCCCCAATGCCGTGCTCGG
                            *** **  *  * *** * *  *      * **  * **     * **

Glycine4CL1/AF279267      ACAGGGATATGGGATGACAGAAGCAGGACC---ACTTGCCATTAGCATGG
Leucaena4CL1/FJ205490     ACAGGGATATGGGATGACGGAGGGTGGTCC---TCTGTCGATAAGCTTGT
Pinustaeda4CL1/U12012     GCAGGGCTACGGCATGACAGAAGCAGGCCCGGTGCTGGCAATGAACCTAG
Zeamays4CL/AY566301       GCAGGGGTACGGGATGACGGAGGCAGGCCCCGTGCTGGCGATGTGCCTGG
                           ***** ** ** ***** ** *  ** **    **  * **   * *

Glycine4CL1/AF279267      CATTTGCAAAAGTACCCTCTAAGATTAAACCAGGTGCATGCGGAACCGTT
Leucaena4CL1/FJ205490     CGTTCGCGAAGGAGCCGGTGGAGATGAAATCAGGCGCGTGCGGGAGCGTG
Pinustaeda4CL1/U12012     CCTTCGCAAAGAATCCTTTCCCCGTCAAATCTGGCTCCTGCGGAACAGTC
Zeamays4CL/AY566301       CCTTCGCCAAGGAGCCGTACCCGGTCAAGTCCGGGTCGTGCGGCACCGTG
                          * ** ** **    **       * **  * **  * ***** *  **

Glycine4CL1/AF279267      GTGAGAAACGCCGAGATGAAAATCGTGGATACAGAAACGGGTGATTCACT
Leucaena4CL1/FJ205490     ATAAGAAACGCAGAGATGAAGATTGTTGACATTGAGACGGGAGCTTCGCT
Pinustaeda4CL1/U12012     GTCCGGAACGCTCAAATAAAGATCCTCGATACAGAAACTGGCGAATCTCT
Zeamays4CL/AY566301       GTGCGGAACGCGGAGCTGAAGATCGTCGACCCCGACACCGGCGCCGCCCT
                           *  * *****  *  * ** **  * **    ** ** ** *   * **

Glycine4CL1/AF279267      TCCAAGAAACAAACACGGTGAAATTTGCATAATAGGCACAAAGGTCATGA
Leucaena4CL1/FJ205490     TCCAAGAAACAGGGCTGGTGAAATCTGCATTAGAGGAAATCAAGTTATGA
Pinustaeda4CL1/U12012     CCCGCACAATCAAGCCGGCGAAATCTGCATCCGCGGACCCGAAATAATGA
Zeamays4CL/AY566301       CGGCCGGAACCAGCCCGGCGAGATCTGCATCCGCGGGGAGCAGATCATGA
                                 **       ** ** ** *****    **     *  * ****
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```
Glycine4CL1/AF279267      AAGGATATCTAAATGACCCAGAGGCTACAGAGAGAACTGTAGACAAAGAA
Leucaena4CL1/FJ205490     AAGGGTACCTAAACGACCCGGAGGCGACGAAAACGACGATAGACGAAGAA
Pinustaeda4CL1/U12012     AAGGATATATTAACGACCCGGAATCCACGGCCGCTACAATCGATGAAGAA
Zeamays4CL/AY566301       AAGGTTACCTGAACGACCCCGAGTCGACGAAGAACACCATCGACCAGGAC
                          **** **  * ** ***** **  * **       **  * **  * **

Glycine4CL1/AF279267      GGATGGTTACACACAGGAGATATTGGTTTCATTGATGATGATGATGAACT
Leucaena4CL1/FJ205490     GGATGGCTTCACACGGGGGACATCGGACACGTAGACGACGACGACGAGGT
Pinustaeda4CL1/U12012     GGCTGGCTCCACACAGGCGACGTCGAATACATTGACGATGACGAAGAAAT
Zeamays4CL/AY566301       GGCTGGCTGCACACCGGCGACATCGGCTACGTGGACGACGACGACGAGAT
                          ** *** * ***** ** **  * *    * * ** ** ** ** **  *

Glycine4CL1/AF279267      CTTCATTGTTGATCGGTTAAAGGAATTGATCAAATACAAAGGATTCCAAG
Leucaena4CL1/FJ205490     GTTCGTTGTGGATCGTTTGAAGGAGATCATCAAATACAAAGGTTTCCAAG
Pinustaeda4CL1/U12012     CTTCATAGTCGACAGAGTAAAGGAGATTATCAAATATAAGGGCTTCCAGG
Zeamays4CL/AY566301       CTTCATCGTCGACAGGCTCAAGGAGATCATCAAGTACAAGGGCTTCCAGG
                           *** * ** **  *  * *****  * ***** ** ** ** ***** *

Glycine4CL1/AF279267      TGGCTCCTGCTGAGCTTGAAGCATTGTTGATTGCCCACCCAAACATTTCT
Leucaena4CL1/FJ205490     TGGCTCCTGCTGAGCTGGAGGCTCTTCTCATCTCTCACCCTTTCATTTCT
Pinustaeda4CL1/U12012     TGGCTCCTGCTGAGCTGGAAGCTTTACTTGTGGCTCATCCGTCAATCGCT
Zeamays4CL/AY566301       TGCCGCCGGCGGAGCTGGAGGCGCTCCTCATCACGCACCCGGAGATCAAG
                          ** * ** ** ***** ** **  *  *  *  * ** **    **

Glycine4CL1/AF279267      GATGCTGCCGTTGTAGGCATGAAAGATGAAGCTGCAGGGGAAATTCCAGT
Leucaena4CL1/FJ205490     GATGCTGCCGTTGTTCCGATGAAAGATGAGGCTGCGGGGGAGCTTCCGGT
Pinustaeda4CL1/U12012     GACGCAGCAGTCGTTCCTCAAAAGCACGAGGAGGCGGGCGAGGTTCCGGT
Zeamays4CL/AY566301       GACGCCGCCGTCGTCTCAATGAACGACGACCTTGCTGGTGAAATCCCGGT
                          ** ** ** ** **       **  * **    ** ** **  * ** **

Glycine4CL1/AF279267      TGCATTTGTTGTAAGGTCAAATGGTTCTGAGATAGCCGAGGATGAAATCA
Leucaena4CL1/FJ205490     GGCATTTGTGGTTAGGTCAAATGGTTTTAAGATCTCTGAGGATGACATCA
Pinustaeda4CL1/U12012     GGCCTTCGTGGTGAAGTCG------TCGGAAATCAGCGAGCAGGAAATCA
Zeamays4CL/AY566301       CGCCTTCATCGTGCGGACCGAAGGTTCTCAAGTCACCGAGGATGAGATCA
                           ** **  * **   * *       *   *  *    *** * ** ****

 Glycine4CL1/AF279267     AGAAATACATTTCACAACAGGTGGTTTTTTACAAGAGAATATGTAGAGTT
 Leucaena4CL1/FJ205490    AGCTTTTCATTTCACAACAGGTGGTGTATTACAAGAGAATCCACAAGGTT
 Pinustaeda4CL1/U12012    AGGAGTTCGTGGCAAAGCAGGTGATTTTCTACAAGAAAATACACAGAGTT
 Zeamays4CL/AY566301      AGCAATTCGTCGCCAAGGAGGTGGTTTTCTACAAGAAGATCCACAAGGTC
                          **   * * *  *  *  ***** * *  *******  **    *  **

Glycine4CL1/AF279267     TTCTTCACGGACTCTATTCCTAAAGCACCCTCAGGCAAAATTCTGCGAAA
Leucaena4CL1/FJ205490    ATTTTCACAGACACTATTCCTAAAGCAGTTTCTGGCAAAATTTTACGAAA
Pinustaeda4CL1/U12012    TACTTTGTGGATGCGATTCCTAAGTCGCCGTCCGGCAAGATTCTGAGAAA
Zeamays4CL/AY566301      TTCTTCACCGAATCCATCCCCAAGAACCCGTCGGGCAAGATCCTGAGGAA
                            **    **  * ** ** **       ** ***** **  *  * **
```

```
Glycine4CL1/AF279267        GGTATTAACTGCAAGACTTAACGAAGGTTTGGTGGTGGCCAATTAG
Leucaena4CL1/FJ205490       GGATTTAAAAGCAAGGCTTGCATCAGATTTGG--GCAATTAA----
Pinustaeda4CL1/U12012       GGATTTGAGAAGCAGACTGGCAGCAAAATGA---------------
Zeamays4CL/AY566301         GGACTTGAGAGCCAGGCTCGCCGCCGG-TGTTCACTGA--------
                            **  ** *     ** **          *
```

Fig. 3.23: Nucleotide sequence alignment of the coding sequences of Ll4CL1 of *L. leucocephala* (FJ205490), *Glycine* 4CL1 (AF279267), *Zea mays* 4CL (AY566301) and *Pinus taeda* 4CL1(U12012) genes using Clustal W 1.8.

The cDNA deduced amino acid sequences were searched for sequence similarity in the Gen-Bank database (NCBI) using BLAST. The deduced amino acid sequences of Ll4CL1 were aligned with representative member of the dicots *e.g.* Glycine 4CL1 AF279267, the monocots *e.g. Zea mays* 4CL AY566301and the gymnosperms *e.g. Pinus taeda* U12012 showed 85%, 80% and 79% sequence similarity respectively (Fig. 3.24) using clustal W multiple sequence alignment software.

Amino acid sequence alignment using Clustal W 1.8

```
Pinustaeda4CL1/U12012        --------------MANGIKKVEHLYRSKLPDIEISDHLPLHSYCFERV
Zeamays4CL/AY566301         MGSVDAAIAVPVPAAEEKAVEEKAMVFRSKLPDIEIDSSMALHTYCFGKM
Glycine4CL1/AF279267         ----------------MAPSPQEIIFRSPLPDIPIPTHLPLYSYCFQNL
Leucaena4CL1/FJ205490        ----------------METPSMEFIFRSKLPDIYIPDHLPLHSYVFENL
                                                ::** **** *   :.*::* * .:

Pinustaeda4CL1/U12012       AEFADRPCLIDGATDRTYCFSEVELISRKVAAGLAKLGLQQGQVVMLLLP
Zeamays4CL/AY566301         GEVAERACLIDGLTGASYTYAEVESLSRRAASGLRAMGVGKGDVVMSLLR
Glycine4CL1/AF279267        SQFHDRPCLIDGDTGETLTYADVDLAARRIASGLHKIGIRQGDVIMLVLR
Leucaena4CL1/FJ205490       SQVKDRPCLIDGDTGETLTYADVELTARRVAAGLTKLGIQQGDVIMLVLR
                            .:. :*.***** *. :  :::*:  :*: *:**  :*: :*:*:* :*

Pinustaeda4CL1/U12012       NCIEFAFVFMGASVRGAIVTTANPFYKPGEIAKQAKAAGARIIVTLAAYV
Zeamays4CL/AY566301         NCPEFAFTFLGAARLGAATTTANPFYTPHEVHRQAEAAGARLIVTEACAV
Glycine4CL1/AF279267        NCPQFALAFLGATHRGAVVTTANPFYTPAELAKQATATKTRLVITQSAYV
Leucaena4CL1/FJ205490       NCPQFALAFLGASFAGAAVTTANPLSTPAELAKQATASKSKLIITQAAFV
                            ** :**:.*:**:  ** .*****: .* *: :** *: :::::* :. *

Pinustaeda4CL1/U12012       EKLADLQSHDV-LVITIDDAPKE----GCQHISVLTEADETQCPAVKIHP
Zeamays4CL/AY566301         EKVREFAAERG-IPVVTVDGRFD----GCVEFAELIAAEELEA-DADIHP
Glycine4CL1/AF279267        EKIKSFADSSSDVMVMCIDDDFSYENDGVLHFSTLSNADETEAPAVKINP
Leucaena4CL1/FJ205490       EKIKDFADKRG-VSLMCIDSTFP-ETEGISHFSLLTQADEACMPAVKISP
                            **: .:      : :   *       *  .:: *  *:*     ..* *
```

```
Pinustaeda4CL1/U12012     DDVVALPYSSGTTGLPKGVMLTHKGLVSSVAQQVDGENPNLYFHSDDVIL
Zeamays4CL/AY566301       DDVVALPYSSGTTGLPKGVMLTHRSLITSVAQQVDGENPNLYFRKDDVVL
Glycine4CL1/AF279267      DELVALPFSSGTSGLPKGVMLSHKNLVTTIAQLVDGENPHQYTHSEDVLL
Leucaena4CL1/FJ205490     DDVVALPYSSGTSGFPKGVMLTHKNLVTSVAQLVDGENPNQYTTSDDVHI
                          *::****:****:*:******:*:.*::::** ******: *  .:** :

Pinustaeda4CL1/U12012     CVLPLFHIYSLNSVLLCALRAGAATLIMQKFNLTTCLELIQKYKVTVAPI
Zeamays4CL/AY566301       CLLPLFHIYSLNSVLLAGLRAGSTIVIMRKFDLGALVDLVRRYVITIAPF
Glycine4CL1/AF279267      CVLPMFHIYALNSILLCGIRSGAAVLILQKFEITTLLELIEKYKVTVASF
Leucaena4CL1/FJ205490     CVLPMFHIYALNSILLCCIRAGAAILTMGKYDIATLLKMIKTYKVTMASF
                          *:**:****:***:**. :*:*:: : : *::: : :.::. * :*:*.:

Pinustaeda4CL1/U12012     VPPIVLDITKSPIVSQYDVSSVRIIMSGAAPLGKELEDALRERFPKAIFG
Zeamays4CL/AY566301       VPPIVVEIAKSPRVTAGDLASIRMVMSGAAPMGKELQDAFMAKIPNAVLG
Glycine4CL1/AF279267      VPPIVLALVKSGETHRYDLSSIRAVVTGAAPLGGELQEAVKARLPHATFG
Leucaena4CL1/FJ205490     VPPILLNIVKSEEVDRHDLSSIRTIVTGAAPVSVELEQALRAKLPHAILG
                          ****:: :.**  .   *::*:* :::****:. **::*.  ::*:* :*

Pinustaeda4CL1/U12012     QGYGMTEAGPVLAMNLAFAKNPFPVKSGSCGTVVRNAQIKILDTETGESL
Zeamays4CL/AY566301       QGYGMTEAGPVLAMCLAFAKEPYPVKSGSCGTVVRNAELKIVDPDTGAAL
Glycine4CL1/AF279267      QGYGMTEAGP-LAISMAFAKVPSKIKPGACGTVVRNAEMKIVDTETGDSL
Leucaena4CL1/FJ205490     QGYGMTEGGP-LSISLSFAKEPVEMKSGACGSVIRNAEMKIVDIETGASL
                          *******.** *:: ::*** *  :*.*:**:*:***::**:* :** :*

Pinustaeda4CL1/U12012     PHNQAGEICIRGPEIMKGYINDPESTAATIDEEGWLHTGDVEYIDDDEEI
Zeamays4CL/AY566301       GRNQPGEICIRGEQIMKGYLNDPESTKNTIDQDGWLHTGDIGYVDDDDEI
Glycine4CL1/AF279267      PRNKHGEICIIGTKVMKGYLNDPEATERTVDKEGWLHTGDIGFIDDDDEL
Leucaena4CL1/FJ205490     PRNRAGEICIRGNQVMKGYLNDPEATKTTIDEEGWLHTGDIGHVDDDDEV
                           :*: ***** * ::****:****:*  *:*::*******: .:***:*:

Pinustaeda4CL1/U12012     FIVDRVKEIIKYKGFQVAPAELEALLVAHPSIADAAVVPQKHEEAGEVPV
Zeamays4CL/AY566301       FIVDRLKEIIKYKGFQVPPAELEALLITHPEIKDAAVVSMNDDLAGEIPV
Glycine4CL1/AF279267      FIVDRLKELIKYKGFQVAPAELEALLIAHPNISDAAVVGMKDEAAGEIPV
Leucaena4CL1/FJ205490     FVVDRLKEIIKYKGFQVAPAELEALLISHPFISDAAVVPMKDEAAGELPV
                          *:***:**:********.********::** * *****  :.: ***:**

Pinustaeda4CL1/U12012     AFVVKS--SEISEQEIKEFVAKQVIFYKKIHRVYFVDAIPKSPSGKILRK
Zeamays4CL/AY566301       AFIVRTEGSQVTEDEIKQFVAKEVVFYKKIHKVFFTESIPKNPSGKILRK
Glycine4CL1/AF279267      AFVVRSNGSEIAEDEIKKYISQQVVFYKRICRVFFTDSIPKAPSGKILRK
Leucaena4CL1/FJ205490     AFVVRSNGFKISEDDIKLFISQQVVYYKRIHKVIFTDTIPKAVSGKILRK
                          **:*::   :::*::** :::::*::**:* :* *.::***  *******
```

```
Pinustaeda4CL1/U12012      DLRSRLAAK-----
Zeamays4CL/AY566301        DLRARLAAGVH---
Glycine4CL1/AF279267       VLTARLNEGLVVAN
Leucaena4CL1/FJ205490      DLKARLASDLGN--
                                  :**
```

Fig. 3.24: Deduced amino acid sequence alignment of the coding sequences of *L. leucocephala* (FJ205490), *Glycine* 4CL1 (AF279267), *Zea mays* 4CL (AY566301) and *Pinus taeda* 4CL1(U12012) genes using Clustal W 1.8. The conserved amino acid residues or conservative exchanges are marked by asterisks and dots, respectively, in the bottom line. Five conserved cystein residue shown in the green shade. The two conserved domain box I AMP binding domain and box II GEICIRG shown in green shade.

The GC% and codon usage of the cDNA clones was calculated using online software (**www.justbio.com**). The GC content of Ll4CL1 cDNA clones was 51.58%. The codon usage for Ll4CL1 cDNA clones is given in Table 3.1 and it is expressed as % of total codons. Standard genetic codes were used for the purpose.

Tab 3.1: Codon usage of 4CL1 gene *Leucaena leucocephala.*

codon	mean	codon	mean	codon	mean	codon	mean
UUU (F)	0.94	UCU (S)	1.32	UAU (Y)	0.75	UGU (C)	0.38
UUC (F)	2.44	UCC (S)	1.69	UAC (Y)	1.13	UGC (C)	1.13
UUA (L)	0.75	UCA (S)	0.94	UAA (*)	0.00	UGA (*)	0.00
UUG (L)	1.50	UCG (S)	1.13	UAG (*)	0.00	UGG (W)	0.19
CUU (L)	1.69	CCU (P)	0.94	CAU (H)	0.56	CGU (R)	0.56
CUC (L)	1.13	CCC (P)	0.75	CAC (H)	1.32	CGC (R)	0.00
CUA (L)	0.56	CCA (P)	0.94	CAA (Q)	1.32	CGA (R)	0.38
CUG (L)	2.44	CCG (P)	1.69	CAG (Q)	0.94	CGG (R)	0.19
AUU (I)	2.26	ACU (T)	1.13	AAU (N)	0.75	AGU (S)	0.19
AUC (I)	3.57	ACC (T)	1.32	AAC (N)	1.69	AGC (S)	0.94
AUA (I)	1.13	ACA (T)	0.38	AAA (K)	2.44	AGA (R)	0.75
AUG (M)	2.44	ACG (T)	2.26	AAG (K)	3.57	AGG (R)	0.94
GUU (V)	2.44	GCU (A)	2.44	GAU (D)	1.50	GGU (G)	1.32
GUC (V)	0.75	GCC (A)	3.01	GAC (D)	3.76	GGC (G)	1.69
GUA (V)	0.38	GCA (A)	1.50	GAA (E)	1.69	GGA (G)	1.69
GUG (V)	3.57	GCG (A)	1.88	GAG (E)	3.20	GGG (G)	0.94

3.3.7.1. Restriction analysis of Ll4CL1

Restriction analysis of Ll4CL1 was done using bioinformatics software pDRAW32 (Fig. 3.25). Analysis was limited to enzyme site cutting not more than four times in the sequence. Major restriction sites are present at following position. *Eco* RI at 19th , *Sac* I at 173rd, *Fat* I at 233rd , *Nar* I at 296th , *Eag* I at 524th, *Sal* I at 643rd, *Nru* I 1045th, *Kpn* I 1197th and *Dra* I at 1595th positions respectively.

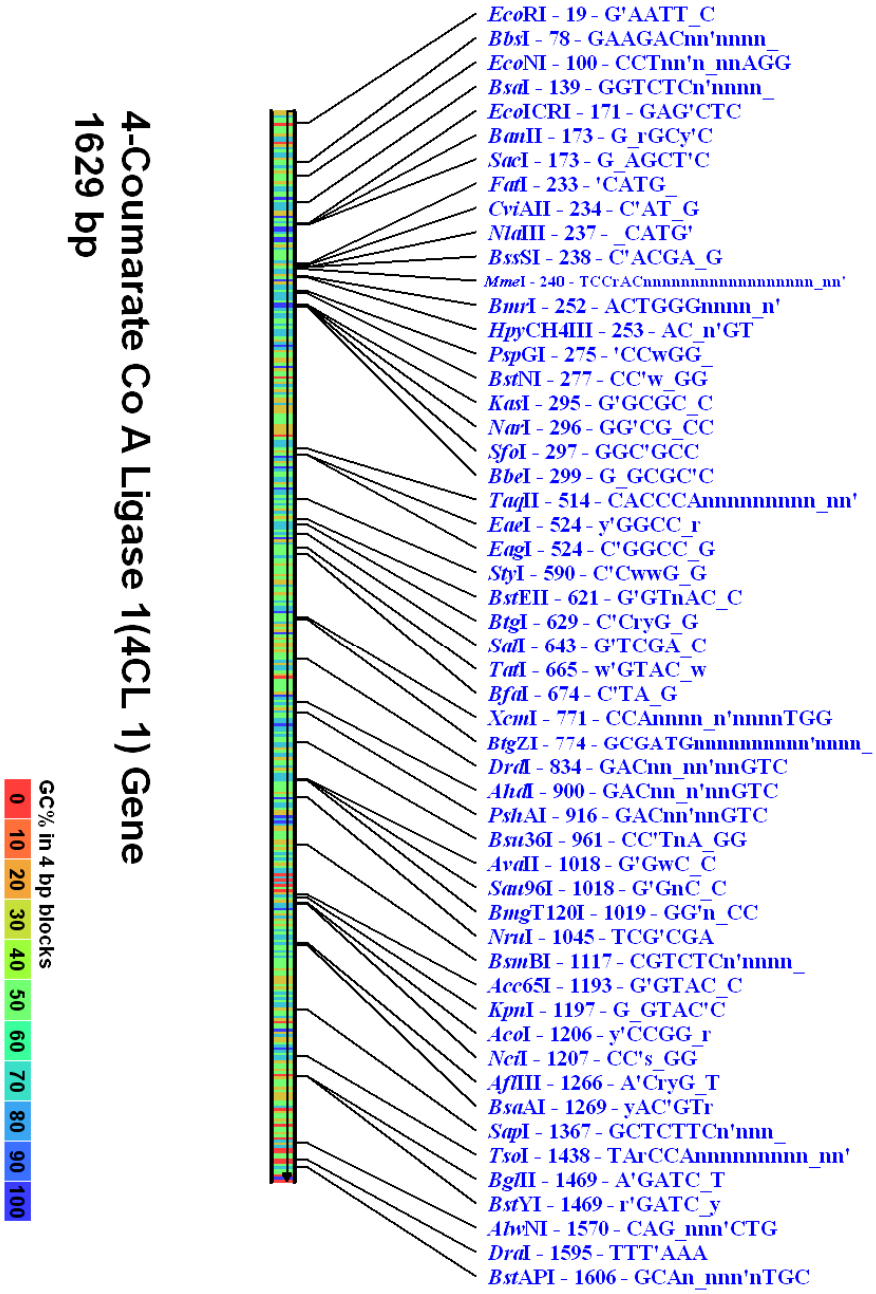


Fig 3.25: Restriction map of Ll4CL1 (FJ205490) gene using pDRAW32.

3.3.8. Isolation and analysis of Ll4CL2 gene

GlyF forward primer was designed from the conserved 4CL nucleotide sequences (Chapter 3, section 3.4.3) of fabaceae and there was a possibility that this primer may be useful to amplify other isoenzymes of 4CL family. It has been already reported that this gene belongs to multi gene family. 3'RACE was done using GlyF and 3'GRP as forward and reverse primer respectively. A fragment of approximately 1.8 kb (Ll4CL2) was amplified using these primers (Fig 3.26). The amplicon was gel eluted and cloned in pGEM-T Easy vector (Promega, USA).

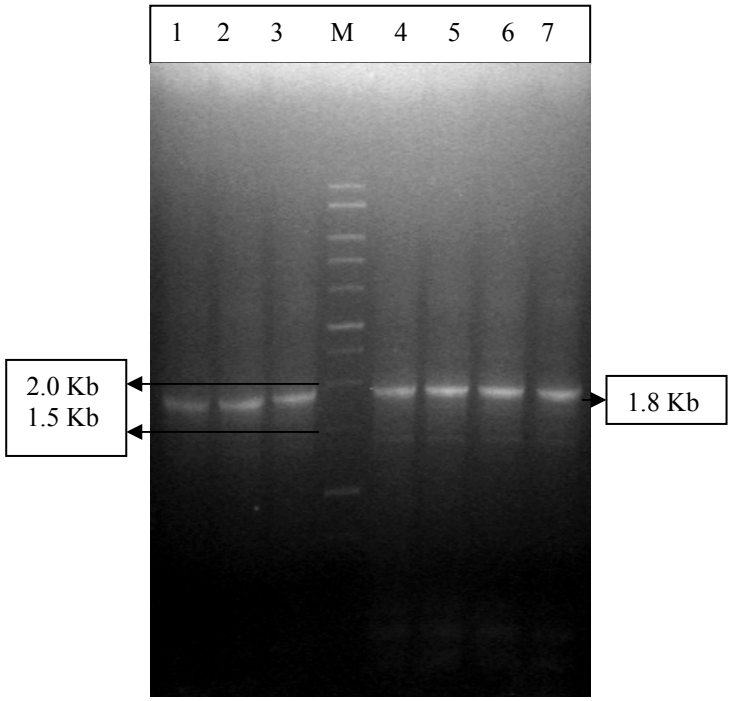


Fig 3.26: PCR amplified from cDNA of *L. leucocephala* using GlyF and 3'GRP primers, Lane M: 1 Kb Ladder, Lane1, 2, 3, 4, 5, 6 & 7: 1.8 Kb PCR amplified fragment.

Plasmid DNA were isolated from three recombinant and digested with *Eco*R I. The digestion pattern revealed presence of inserts (Fig. 3.27). All the three clones were sequenced, out of three clones, two clones were the part of same gene (Ll4CL1), but the third clone sequence was entirely different and it was designated as Ll4CL2. Ll4CL2 was searched for sequence similarity with the sequences available in the gene data base (NCBI) and the sequences showed maximum homology with the reported plant 4CL2 gene in NCBI data base. The

sequencing data revealed that the Ll4CL2 gene also ends with TAA not with TGA as reported in so many genes. The length of 3'UTR is 271 bp and it ends with 25 poly A tails (Fig: 3.28). The designated Ll4CL2 gene is the partial gene of 1560 bp and it codes 519 amino acids. The Ll4CL2 was submitted in NCBI Gen-Bank database and allotted accession number for this Ll4CL2 gene is FJ205491.

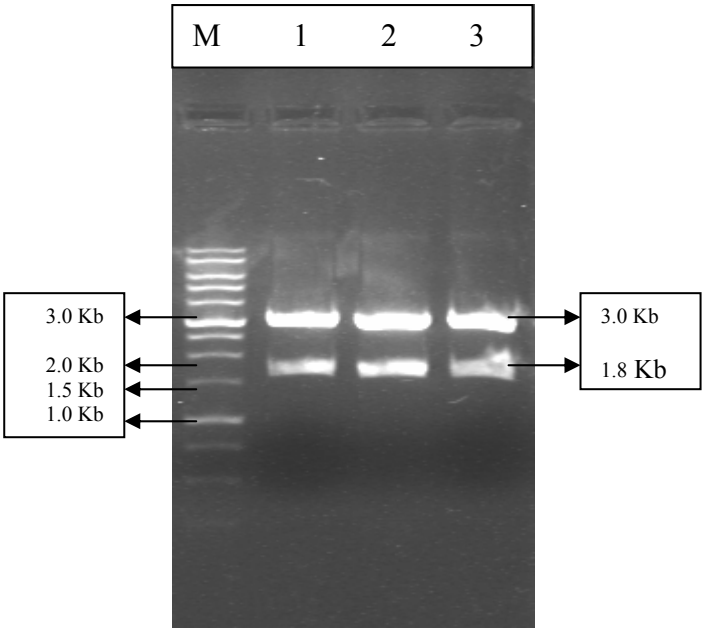


Fig 3.27: cDNA clone of *L. leucocephala* amplified using GlyF and 3'GRP primers, Lane M: 1 Kb Ladder, Lane-1, 2 & 3: *Eco*RI digested 1.8 Kb cloned fragment in pGEM-T Easy vector.

Nucleotide sequences of Ll4CL2:

```
   1 CCCAAACACC TCCCTCTCCA TTCTTACTGC TTCGAGAATC TCTCCGAATT
  51 CGGCTCCCGT CCTTGCCTGA TCAATGCCCC CACCGGCGAA ATCTATACTT
 101 ACTACGACGT CGAGCTCACC TCCCGGCGTG TCGCCTCCGG ATTGAACAAA
 151 TTCGGCGTCG GACAGGGTGA CGTCATCATG GTCCTCCTTT CCAATTCCCC
 201 TGAATTCGTC TTCTCCTTCC TCGGCGCCTC CTTCCGCGGC GCCTTGACAA
 251 CCGCCGCGAA TCCCTTCTTC ACCGCCGCCG AGATTTCCAA ACAATTCAAA
 301 GCCTCCAACG CGAAAATCCT CATAACTCAA TCGGCCTACT ACGAGAAAGT
 351 GAAGGACCTC GACGTGAAGC TAATCTTCGT CGATTCTCCA CCGGACGGAC
 401 ACTCTCATTT CTCGGAGCTA TCTCAAGCCG ACGAGAACGA CATGCCCGAG
 451 GTCAAAATCA AACCGGACGA CGTCGTCGCA TTGCCGTATT CCTCCGGCAC
 501 CACCGGGTTA CCAAAAGGCG TGATGCTCAC TCACAAGGGA TTACTGACGA
```

551 **GCATAGCCCA ACAAGTCGAC GGAGAGAATC CGAACCTGTA TTTCCACCAT**
601 **GAAGATGTGA TTCTCTGTGT CCTTCCCCTG TTCCATATCT ACTCGTTGAA**
651 **CTCTGTTTTG CTGTGTGGAT TGAGGGCCAA GGCGGCGATC TTGTTGATGC**
701 **CCAAGTTTGA AATCAATGCG TTGTTAGGCT TGATACAGAA ACACAAGGTG**
751 **TCGATAGCTC CGGTGGTGCC GCCGATCGTG CTTGCCGTGT CAAAGTCGGC**
801 **GGATATCGAT AAGTACGACC TGTCGTCGAT CAGAGTATTC AAATCCGGTG**
851 **GGGCTCCTCT GGGTAAAGAG CTTGAAGACT CTGTGAGAGC CAAGTTCCCT**
901 **AAGGCCAGAC TTGGTCAGGG ATATGGGATG ACGGAGGCAG GACCGGTGTT**
951 **GACAATGAGC TTAGCATTTG CAAAGGAACC CATGGGAGTG AAGGCAGGAG**
1001 **CATGTGGAAC AGTAGTTAGA AATGCCGAGA TGAAGATTGT AGATCCTGAG**
1051 **ACGTCTGAAT CTCTTCCCCG TAACCGCCCG GGCGAGATCT GCATTAGAGG**
1101 **CGATCAAATT ATGAAAGGGT ATTTAAATGA TCCGGAGGCC ACGAAGAGAA**
1151 **CAATCGACGA GGAGGGGTGG TTGCATACGG GAGACATAGG CTACATCGAT**
1201 **GACGACGATG AGCTGTTCAT CGTTGATAGG CTGAAGGAGC TCATCAAGTT**
1251 **CAAGGCCTTC CAGGTGGCCC CCGCTGAACT TGAGGCCCTC CTTCTCACTC**
1301 **ATCCTCACAT ATCCGATGCC GCCGTGGTCC CAATGAAGGA TGAATCTGCT**
1351 **GGAGAGGTGC CCGTTGCCTT TGTTGTCAGA TCCAACGGTC ACACTCAAAC**
1401 **CACCGAGGAT GACATCAAAC GGTTTGTCTC CAAACAGGTG GTGTTTTACA**
1451 **AGAGAATAAG CAGAGTGTTC TTCATTGATG CGATTCCAAA ATCACCCTCA**
1501 **GGCAAGATAT TGAGAAAGGA CCTCAGAGCA AAACTAGCTG CTGGTGTTGC**
1551 **TGCTAATTAA** *AATTGAAGCT TCTAATGTAT AATTACATTC CTACCCTTCA*
1601 *ACTTCTCCTC CTCATATCGG CAAAGGCTTT CATAGATTTG AAGATCATAT*
1651 *ATGGATTTCA TCTTTATAGC TATCCATCAT ACTTCATATC TGTGTGATGA*
1701 *TAGTATTGTA TTTTTATAAT TTTTTTTTAT CACTTTGTTC CAAAGAAAGG*
1751 *ACTATATTGT ATTTCCCACC CTTTCAAATA ATATATATTG ATTTTATATT*
1801 *TGTCCAAAAA AAAAAAAAAA AAAAAAAAAA A*

Fig 3.28: Nucleotide sequnces of Ll4CL2 cDNA clone. The sequences in bold represent the sequence in reading frame till stop, sequence in italics represent the 3'UTR region.

The deduced amino acid sequences of Ll4CL2 gene show that total number of amino acid are 519, molecular weight is 57.0 kd, theoratical Pi of 6.27 and empirical formula $C_{2561}H_{4062}N_{672}O_{748}S_{16}$ it shows the presence of two conserved domains (Uhlmann and Ebel, 1993). Deduced amino acid sequences of Ll4CL2 gene show the presence of AMP binding superfamily domain. This AMP binding superfamily domain have box I (AMP binding) conserved motif. The box I (AMP binding) conserved motif of Ll4CL2 LPYSSGTTGLPK is present at amino acid

residues 161 – 172, this putative AMP binding domain is not only almost absolutely conserved in all 4CL gene , but highly similar motifs are also found in luciferases, acetyl CoA synthetases. The second conserved domain Box II motif is GEICIRG at amino acid residues 361-367, this putative domain is absolutely conserved in all 4CL genes and its central cystein residue has been suggested to be directly involved in catalysis (fig. 3.29). The total 9 cystein residues are present which are proposed to be involved in catalytic activity (Bhushan *et al*., 2006; Ehlting *et al*., 2001; Kumar and Ellis, 2003).

Deduced amino acid sequence of Ll4CL2 gene:

```
  1 PKHLPLHSYC  FENLSEFGSR  PCLINAPTGE IYTYYDVELT SRRVASGLNK
 51 FGVGQGDVIM  VLLSNSPEFV  FSFLGASFRG ALTTAANPFF TAAEISKQFK
101 ASNAKILITQ  SAYYEKVKDL  DVKLIFVDSP PDGHSHFSEL SQADENDMPE
151 VKIKPDDVVA  LPYSSGTTGL  PKGVMLTHKG LLTSIAQQVD GENPNLYFHH
201 EDVILCVLPL  FHIYSLNSVL  LCGLRAKAAI LLMPKFEINA LLGLIQKHKV
251 SIAPVVPPIV  LAVSKSADID  KYDLSSIRVF KSGGAPLGKE LEDSVRAKFP
301 KARLGQGYGM  TEAGPVLTMS  LAFAKEPMGV KAGACGTVVR NAEMKIVDPE
351 TSESLPRNRP  GEICIRGDQI  MKGYLNDPEA TKRTIDEEGW LHTGDIGYID
401 DDDELFIVDR  LKELIKFKAF  QVAPAELEAL LLTHPHISDA AVVPMKDESA
451 GEVPVAFVVR  SNGHTQTTED  DIKRFVSKQV VFYKRISRVF FIDAIPKSPS
501 GKILRKDLRA  KLAAGVAAN
```

Fig 3.29: Deduced amino acid sequences of Ll4CL2 gene. The AMP binding domains is shaded in green and other highly conserved catalytic domain is shaded in yellow. The entire cystein residue is shaded in pink.

The nucleotide sequences of Ll4CL2 (FJ205491) cDNA clone and their respective deduced amino acids sequences were searched for sequence similarity in NCBI Gen-Bank database using BLAST and Clustal W 1.8 and it showed sequence similarity with other reported plant 4CL cDNA clones. The nucleotide sequence alignment of the Ll4CL2 gene sequences with representative member of the dicots *e.g. Glycine* 4CL2: AF002259, the monocots *e.g Oriza* 4CL: NM_001064787 and the gymnosperms e.g *Pinus taeda* 4CL2: U12013 (Fig. 3.30) showed 73%, 72% and 65% sequence similarity, respectively.

CLUSTAL W (1.8) multiple sequence alignment:

```
4CL2/Leucaena/FJ205491    --------------------------------------------------
4CL2/Glycine/AF002259     --------------------ATGGCTGATGATGGAAGCAGGAGGGAATT
4CL/Oriza/NM_001064787    ATGGGGTCCATGGCGGCGGCGGCGGAGGCGGCGCAGGAGGAGGAGACGGT
4CL2/Pinus/PTU12013       --------------------ATGGCCAACGGAATCAAGAAGGTCGAGCA

4CL2/Leucaena/FJ205491    ---------------------------------CCCAAACACCTCCCTC
4CL2/Glycine/AF002259     GATATTCAGGTCGAAGCTTCCGGATATCTACATCCCCAAACATATGCCCC
4CL/Oriza/NM_001064787    GGTGTTCCGGTCCAAGCTCCCCGACATCGAGATCCCCAGCCACCTCACCC
4CL2/Pinus/PTU12013       TCTGTACAGATCGAAGCTTCCCGATATCGAGATCTCCGACCATCTGCCTC
                                                      **   **  *  * *

4CL2/Leucaena/FJ205491    TCCATTCTTACTGCTTCGAGAATCTCTCCGAATTCGGCTCCCGTCCTTGC
4CL2/Glycine/AF002259     TCCACTCTTACTGCTTCGAGAATTTGAGAGAGTGTGGTTCACGCCCCTGC
4CL/Oriza/NM_001064787    TGCAGGCCTACTGCTTCGAGAAGCTTCCGGAGGTGGCCGCCCGCCCCTGC
4CL2/Pinus/PTU12013       TTCATTCGTATTGCTTTGAGAGAGTAGCGGAATTCGCAGACAGACCCTGT
                          * **  * ** ***** ****   *    **    *      * ** **

4CL2/Leucaena/FJ205491    CTGATCAATGCCCCCACCGGCGAAATCTATACTTACTACGACGTCGAGCT
4CL2/Glycine/AF002259     CTGATCAATGCCCCCACGGGAGACGTCTACAGCTACCACGAGGTGGACAG
4CL/Oriza/NM_001064787    CTCATCGACGGGCAGACCGGGGCGGTGTACAGCTACGGCGAGGTGGAGGA
4CL2/Pinus/PTU12013       CTGATCGATGGGGCGACAGACAGAACTTATTGCTTTTCAGAGGTGGAACT
                          ** *** * *     ** *        **    *     ** ** **

4CL2/Leucaena/FJ205491    CACCTCCCGGCGTGTCGCCTCCGGATTGAACAAATTCGGCGTCGGACAGG
4CL2/Glycine/AF002259     CACCGCCAGAAAGGTGGCGAGGGGGCTGAAGAAAGAGGGCGTGGAACAGG
4CL/Oriza/NM_001064787    GCTCTCCCGGCGCGCGGCGGCGGGGCTGCGGCGGCTCGGGGTGGGGAAGG
4CL2/Pinus/PTU12013       GATTTCTCGCAAGGTCGCTGCCGGTCTGGCGAAGCTCGGGTTGCAGCAGG
                               *  *    *  **    **  **         **  *     ***

4CL2/Leucaena/FJ205491    GTGACGTCATCATGGTCCTCCTTTCCAATTCCCCTGAATTCGTCTTCTCC
4CL2/Glycine/AF002259     GCCAGGTCATCATGATCCTCCTCCCCAATTGCCCCGAATTCGTCTTCTCG
4CL/Oriza/NM_001064787    GCGACGTGGTGATGAGCCTCCTCCGCAACTGCCCCGAGTTCGCCTTCACC
4CL2/Pinus/PTU12013       GGCAGGTTGTCATGCTTCTCCTTCCGAATTGCATCGAATTTGCGTTTGTG
                          *  * **  * ***   *****    ** * *   ** ** *  **

4CL2/Leucaena/FJ205491    TTCCTCGGCGCCTCCTTCCGCGGCGCCTTGACAACCGCCGCGAATCCCTT
4CL2/Glycine/AF002259     TTTCTTGGCGCATCCCACCGCGGTGCCATGGCCACTGCCGCCAACCCTTT
4CL/Oriza/NM_001064787    TTCCTCGGCGCCGCCAGGCTGGGCGCGGCCACCACCACCGCCAACCCGTT
4CL2/Pinus/PTU12013       TTCATGGGGGCCTCTGTCCGGGGCGCCATTGTGACCACGGCCAATCCTTT
                          **  * ** **  *    *  ** **       **  * ** ** ** **

4CL2/Leucaena/FJ205491    CTTCACCGCCGCCGAGATTTCCAAACAATTCAAAGCCTCCAACGCGAAAA
4CL2/Glycine/AF002259     CTTCACCCCCGCCGAGATTGCAAAGCAAGCCCATGCCTCCAATGCCAAGC
4CL/Oriza/NM_001064787    CTACACCCCGCACGAGATCCACCGCCAGGCGTCGGCGGCGGGCGCCAGGG
4CL2/Pinus/PTU12013       CTACAAGCCGGGCGAGATCGCCAAACAGGCCAAGGCCGCGGGCGCGCGCA
                          ** **   *   ******       **       **  *    **
```

```
4CL2/Leucaena/FJ205491    TCCTCATAACTCAATCGGCCTACTACGAGAAAGTGAAGGACCTC---GAC
4CL2/Glycine/AF002259     TCCTCATCACCCAGGCCTCTTACTACGACAAAGTCAAGGACCTCCGCGAC
4CL/Oriza/NM_001064787    TGATCGTGACCGAGGCGTGCGCGGTGGAGAAGGTGCGCGGGTTC---GCC
4CL2/Pinus/PTU12013       TCATAGTTACCCTGGCAGCTTATGTTGAGAAACTGGCCGATCTGCAGAGC
                          *  *  * **     *          ** **  *    *   *      *

4CL2/Leucaena/FJ205491    GTGAAGCTAATCTTCGTCGATTCT-----CCACCGGACGGA---------
4CL2/Glycine/AF002259     ATCAAGCTCGTATTCGTCGACTCTTGTCCCCCCCATACAGAGGAGAAGCA
4CL/Oriza/NM_001064787    GCCGACCGCGGCATCCCCGTGGTCG--C-CGTCGACGGGGACTTCGACGG
4CL2/Pinus/PTU12013       CACGATGTGCTCGTCATCACAATCGATGATGCTCCCAAGGAA------GG
                              *        **  *                     **

 4CL2/Leucaena/FJ205491    -CACTCTCATTTCTCGGAGCTATCTCAAGCCGACGAGAACGAC-ATGCC-
4CL2/Glycine/AF002259     GCATCTCCATTTCTCACATCTGTGTGAGGATAACGGTGACGCTGATGTTG
4CL/Oriza/NM_001064787    CTGCGTCGGGTTCGGGGAGGCGATGCTGGACGCGTCCATCGAGCCGCTCG
4CL2/Pinus/PTU12013       TTGCCAACATATTTCCGTTCTGACCGAAGCCGACGAAACCCAA--TGCC-
                                     *               *         *

4CL2/Leucaena/FJ205491    ----CGAGGTCAAAATCAAACCGGACGACGTCGTCGCATTGCCGTATTCC
4CL2/Glycine/AF002259     ATGTTGATGTTGATATCAAGCCCGATGACGTGGTTGCATTGCCTTATTCT
4CL/Oriza/NM_001064787    ACGCCGACGAGGAGGTCCACCCCGACGACGTCGTCGCCCTCCCTTACTCC
4CL2/Pinus/PTU12013       ---CGGCCGTGAAAATCCACCCGGACGATGTCGTGGCGTTGCCCTATTCT
                               *  *   *  ** * ** ** ** ** ** **  * ** ** **

4CL2/Leucaena/FJ205491    TCCGGCACCACCGGGTTACCAAAAGGCGTGATGCTCACTCACAAGGGATT
4CL2/Glycine/AF002259     TCGGGAACAACTGGTCTTCCCAAGGGAGTGATGCTGAGCCACAAGGGGCT
4CL/Oriza/NM_001064787    TCCGGCACCACCGGCTTGCCCAAGGGCGTCATGCTCACCCACCGCAGCCT
4CL2/Pinus/PTU12013       TCCGGAACCACGGGGCTCCCCAAGGGCGTGATGTTAACGCACAAAGGCCT
                          ** ** ** ** **  * ** ** ** ** *** * *  ***    *  *

4CL2/Leucaena/FJ205491    ACTGACGAGCATAGCCCAACAAGTCGACGGAGAGAATCCGAACCTGTATT
4CL2/Glycine/AF002259     GGTGACCAGCATTGCTCAGCAGGTTGACGGTGATAATCCCAACCTCTATT
4CL/Oriza/NM_001064787    CGTCACCAGCGTCGCGCAGCAGGTGGACGGGGAGAACCCGAACCTGTACT
4CL2/Pinus/PTU12013       GGTGTCCAGCGTTGCCCAGCAGGTCGATGGTGAAAATCCCAATCTGTATT
                            *  * *** * ** ** ** ** ** ** ** ** ** ** ** ** *

4CL2/Leucaena/FJ205491    TCCACCATGAAGATGTGATTCTCTGTGTCCTTCCCCTGTTCCATATCTAC
4CL2/Glycine/AF002259     ACCACTGCCACGACACCATCCTCTGCGTGCTTCCCCTCTTTCACATTTAC
4CL/Oriza/NM_001064787    TCAGGAGGGAGGACGTGGTGCTGTGCTTGCTGCCGCTGTTCCACATCTAC
4CL2/Pinus/PTU12013       TCCATTCCGATGACGTGATACTCTGTGTCTTGCCTCTTTTCCACATCTAT
                           *       * **     * ** **  *  * ** ** ** ** ** **

4CL2/Leucaena/FJ205491    TCGTTGAACTCTGTTTTGCTGTGTGGATTGAGGGCCAAGGCGGCGATCTT
4CL2/Glycine/AF002259     TCTCTCAACTCCGTTCTTCTCTGTGGCTTGCGTGCCAAGGCTACCATTCT
4CL/Oriza/NM_001064787    TCGCTCAACTCCGTGCTGCTGGCGGGGCTGCGAGCCGGGTCGGCGATCGT
4CL2/Pinus/PTU12013       TCTCTCAATTCGGTTCTCCTCTGCGCGCTCAGAGCCGGGGCTGCGACCCT
                          **  * ** ** **  * **    *   *  * ***  * *  * *   *
```

```
4CL2/Leucaena/FJ205491    GTTGATGCCCAAGTTTGAAATCAATGCGTTGTTAGGCTTGATACAGAAAC
4CL2/Glycine/AF002259     CCTCATGCCCAAGTTCGATATTAACTCCTTGCTTGCTCTCATTCACAAGC
4CL/Oriza/NM_001064787    GATCATGCGCAAGTTCGACCTGGGCGCGCTGGTGGACCTGACGCGGAGGC
4CL2/Pinus/PTU12013       GATTATGCAGAAATTCAACCTCACGACCTGTCTGGAGCTGATTCAGAAAT
                            * ****  ** **  *  *     *     * *   * *  *  *

4CL2/Leucaena/FJ205491    ACAAGGTGTCGATAGCTCCGGTGGTGCCGCCGATCGTGCTTGCCGTGTCA
4CL2/Glycine/AF002259     ACAAAGTCACTATTGCCCCTGTCGTCCCTCCCATTGTTCTCGCCATTTCC
4CL/Oriza/NM_001064787    ACGGCGTCACCGTCGCGCCGTTCGTGCCGCCCATCGTGGTGGAGATCGCC
4CL2/Pinus/PTU12013       ACAAGGTTACCGTTGCCCCAATTGTGCCTCCAATTGTCCTGGACATCACA
                          **   **  *  * ** **  * ** ** ** ** **  * *   *  *

4CL2/Leucaena/FJ205491    AAGTCGGCGGATATCGATAAGTACGACCTGTCGTCGATCAGAGTATTCAA
4CL2/Glycine/AF002259     AAATCACCCGATCTCCACAAGTACGACCTGTCTTCCATCAGAGTCTTGAA
4CL/Oriza/NM_001064787    AAGAGCCCCCGCGTCACCGCCGACGACCTCGCCTCCATCCGCATGGTTAT
4CL2/Pinus/PTU12013       AAGAGCCCCATCGTTTCCCAGTACGATGTCTCGTCCGTCCGGATAATCAT
                          **     *     *        ****  *  * **  ** *  *  * *

4CL2/Leucaena/FJ205491    ATCCGGTGGGGCTCCTCTGGGTAAAGAGCTTGAAGACTCTGTGAGAGCCA
4CL2/Glycine/AF002259     GTCCGGGGGAGCCCCTCTGGGTAAGGAACTCGAAGACACTCTCAGAGCTA
4CL/Oriza/NM_001064787    GTCCGGCGCCGCCCCCATGGGGAAGGACCTCCAGGACGCCTTCATGGCCA
4CL2/Pinus/PTU12013       GTCCGGCGCTGCGCCTCTCGGGAAGGAACTCGAAGATGCCCTCAGAGAGC
                           ***** *  ** **  * ** ** ** **  * **  *  * *  *

4CL2/Leucaena/FJ205491    AGTTCCCTAAGGCCAGACTTGGTCAGGGATATGGGATGACGGAGGCAGGA
4CL2/Glycine/AF002259     AATTCCCCAACGCCAAACTTGGCCAGGGATACGGAATGACTGAGGCAGGG
4CL/Oriza/NM_001064787    AGATCCCCAACGCCGTCCTGGGACAGGGGTATGGGATGACTGAGGCTGGA
4CL2/Pinus/PTU12013       GTTTTCCCAAGGCCATCTTCGGGCAGGGCTACGGCATGACAGAAGCAGGC
                            * ** ** ***    * ** ***** ** ** ***** ** ** **

4CL2/Leucaena/FJ205491    CCGGTGTTGACAATGAGCTTAGCATTTGCAAAGGAACCCATGGGAGTGAA
4CL2/Glycine/AF002259     CCTGTGCTAACAATGTCTTTAGCTTTTGCTAAAGAACCGATAGACGTGAA
4CL/Oriza/NM_001064787    CCTGTGCTGGCCATGTGTCTGGCCTTCGCAAAGGAGCCATTCGAGGTCAA
4CL2/Pinus/PTU12013       CCGGTGCTGGCAATGAACCTAGCCTTCGCAAAGAATCCTTTCCCCGTCAA
                          ** *** *  * ***    * ** ** ** **  * **  *    ** **

4CL2/Leucaena/FJ205491    GGCAGGAGCATGTGGAACAGTAGTTAGAAATGCCGAGATGAAGATTGTAG
4CL2/Glycine/AF002259     ACCAGGTGCATGTGGAACCGTTGTAAGAAATGCAGAGATGAAGATTGTCG
4CL/Oriza/NM_001064787    GTCCGGCTCGTGCGGGACAGTCGTCAGAAACGCGGAGCTGAAGATCGTTG
4CL2/Pinus/PTU12013       ATCTGGCTCCTGCGGAACAGTCGTCCGGAACGCTCAAATAAAGATCCTCG
                            * **  * ** ** ** ** **  * ** **  *  * *****  * *

4CL2/Leucaena/FJ205491    ATCCTGAGACGTCTGAATCTCTTCCCCGTAACCGCCCGGGCGAGATCTGC
4CL2/Glycine/AF002259     ATCCTGAAACCGGTCATTCTTTACCCAGAAACCAATCCGGTGAAATTTGC
4CL/Oriza/NM_001064787    ACCCTGACACCGGCGCCACCCTTGGCCGGAACCAGTCCGGGGAGATTTGC
4CL2/Pinus/PTU12013       ATACAGAAACTGGCGAATCTCTCCCGCACAATCAAGCCGGCGAAATCTGC
                          *  * ** **        *  *       ** *   * ** ** ** ***
```

```
4CL2/Leucaena/FJ205491    ATTAGAGGCGATCAAATTATGAAAGGGTATTTAAATGATCCGGAGGCCAC
4CL2/Glycine/AF002259     ATAAGAGGCGACCAGATTATGAAAGGTTATCTAAATGATGGAGAGGCTAC
4CL/Oriza/NM_001064787    ATCCGGGGAGAACAAATCATGAAAGGTTATCTGAATGACCCGGAGTCCAC
4CL2/Pinus/PTU12013       ATCCGCGGACCCGAAATAATGAAAGGATATATTAACGACCCGGAATCCAC
                          **  * **     * ** ******** *** * ** **    **  * **

4CL2/Leucaena/FJ205491    GAAGAGAACAATCGACGAGGAGGGGTGGTTGCATACGGGAGACATAGGCT
4CL2/Glycine/AF002259     AGAGAGAACCATAGACAAAGATGGTTGGTTGCACACAGGTGACATCGGTT
4CL/Oriza/NM_001064787    GAAGAACACCATCGACAAGGGCGGTTGGCTGCACACAGGAGACATTGGTT
4CL2/Pinus/PTU12013       GGCCGCTACAATCGATGAAGAAGGCTGGCTTCACACAGGCGACGTCGGGT
                                 ** ** **  * *  ** *** * ** ** ** *** * ** *

4CL2/Leucaena/FJ205491    ACATCGATGACGACGATGAGCTGTTCATCGTTGATAGGCTGAAGGAGCTC
4CL2/Glycine/AF002259     ACATCGACGATGACGATGAGTTATTCATCGTTGACAGGCTCAAGGAATTG
4CL/Oriza/NM_001064787    ATGTTGACGACGACGACGAGATTTTCATTGTTGACCGGCTCAAGGAAATA
4CL2/Pinus/PTU12013       ACATTGACGATGACGAAGAAATCTTCATAGTCGACAGAGTAAAGGAGATT
                          *  * ** ** ***** **  * ***** ** **  *  * *****  *

4CL2/Leucaena/FJ205491    ATCAAGTTCAAGGCCTTCCAGGTGGCCCCCGCTGAACTTGAGGCCCTCCT
4CL2/Glycine/AF002259     ATCAAATACAAAGGATTTCAAGTGGCTCCAGCTGAACTTGAAGCCCTTCT
4CL/Oriza/NM_001064787    ATAAAATACAAGGGGTTCCAAGTACCTCCTGCGGAACTTGAAGCTCTTCT
4CL2/Pinus/PTU12013       ATCAAATATAAGGGCTTCCAGGTGGCTCCTGCTGAGCTGGAAGCTTTACT
                          ** ** *  ** *  ** ** **  * ** ** ** ** ** **  * **

4CL2/Leucaena/FJ205491    TCTCACTCATCCTCACATATCCGATGCCGCCGTGGTCCCAATGAAGGATG
4CL2/Glycine/AF002259     TCTCACTCATCCTAAGATCTCTGATGCTGCTGTTGTTCCAATGAAGGATG
4CL/Oriza/NM_001064787    CATCACACACCCTGATATCAAGGATGCTGCCGTTGTACCGATGATAGACG
4CL2/Pinus/PTU12013       TGTGGCTCATCCGTCAATTGCTGACGCAGCAGTCGTTCCTCAAAAGCACG
                            *  * ** **    **    ** ** ** ** ** **    *   * *

4CL2/Leucaena/FJ205491    AATCTGCTGGAGAGGTGCCCGTTGCCTTTGTTGT-CAGATCCAACGGTCA
4CL2/Glycine/AF002259     AAGCCGCGGGAGAGGTACCTGTTGCATTTGTGGT-CATATCAAATGGTTA
4CL/Oriza/NM_001064787    AAATTGCGGGTGAAGTGCCGGTTGCATTCATTGTACGGATTGAAGGAT--
4CL2/Pinus/PTU12013       AGGAGGCGGGCGAGGTTCCGGTGGCGTTCGTGGT------GAAGTCGT--
                          *    ** ** ** ** ** ** ** **  * **        *    *

4CL2/Leucaena/FJ205491    CACTCAAACCACCGAGGATGACATCAAACGGTTTGTCTCCAAACAGGTGG
4CL2/Glycine/AF002259     TACCGACACAACCGAGGATGAAATTAAGCAGTTTATCTCCAAACAGGTGG
4CL/Oriza/NM_001064787      CTGCAATCAGCGAGAATGAGATCAAGCAATTTGTGGCAAAGGAGGTTG
4CL2/Pinus/PTU12013         CGGAAATCAGCGAGCAGGAAATCAAGGAGTTCGTGGCAAAGCAGGTGA
                            *    *  * **** * ** ** **    **  *  * **  ****

4CL2/Leucaena/FJ205491    TGTTTTACAAGAGAATAAGCAGAGTGTTCTTCATTGATGCGATTCCAAAA
4CL2/Glycine/AF002259     TGTTTTACAAAAGAATAAACCGAGTATTCTTCATTGATGCAATTCCCAAG
4CL/Oriza/NM_001064787    TTTTCTACAAGAGGCTCAACAAAGTTTTCTTCGCGGATTCAATTCCGAAG
4CL2/Pinus/PTU12013       TTTTCTACAAGAAAATACACAGAGTTTACTTTGTGGATGCGATTCCTAAG
                          * ** ***** *   *   *  *** * ***    *** * ***** **
```

```
4CL2/Leucaena/FJ205491    TCACCCTCAGGCAAGATATTGAGAAAGGACCTCAGAGCAAAACTAGCTGC
4CL2/Glycine/AF002259     TCACCGTCAGGCAAAATCTTGCGCAAGGATCTAAGAGCAAAGATAGCGGC
4CL/Oriza/NM_001064787    AGTCCTTCTGGCAAGATTCTCAGGAAGGACCTCAGAGCAAAGCTTGCAGC
4CL2/Pinus/PTU12013       TCGCCGTCCGGCAAGATTCTGAGAAAGGATTTGAGAAGCAGACTGGCAGC
                             ** ** ***** **  *  * *****  * ***   *   * ** **
```

Fig. 3.30: Nucleotide sequence alignment of the coding sequences of Ll4CL2 of *L. leucocephala* (FJ205491) with *Glycine* 4CL2 (AF002259), *Oriza* 4CL (NM_001064787) and *Pinus taeda* 4CL2(U12013) genes using Clustal W 1.8.

The Ll4CL2 cDNA deduced amino acid sequences searched for sequence similarity in the Gen-Bank database (NCBI) using BLAST. The amino acid sequence alignment of the Ll4CL2 gene sequences with representative member of the dicots *e.g.* Glycine 4CL2 AF002259, the monocots *e.g Oriza* 4CL NM_001064787 and the gymnosperms e.g *Pinus taeda* U12013 showed 88%, 81% and 82% sequence similarity, respectively.(Fig 3.31)

CLUSTAL W (1.8) multiple sequence alignment:

```
4CL2/Leucaena/FJ205491      -------------------------PKHLPLHSYCFENLSEFGSRPC
4CL2/Glycine/AF002259       ---MADDGSRRE--LIFRSKLPDIYIPKHMPLHSYCFENLRECGSRPC
4CL2/Pinus/PTU12013         ---MANGIKKVE--HLYRSKLPDIEISDHLPLHSYCFERVAEFADRPC
4CL/Oriza/NM_001064787    MGSMAAAAEAAQEEETVVFRSKLPDIEIPSHLTLQAYCFEKLPEVAARPC
                                                  ..*:.*::****.: * . ***

4CL2/Leucaena/FJ205491    LINAPTGEIYTYYDVELTSRRVASGLNKFGVGQGDVIMVLLSNSPEFVFS
4CL2/Glycine/AF002259     LINAPTGDVYSYHEVDSTARKVARGLKKEGVEQGQVIMILLPNCPEFVFS
4CL2/Pinus/PTU12013       LIDGATDRTYCFSEVELISRKVAAGLAKLGLQQGQVVMLLLPNCIEFAFV
4CL/Oriza/NM_001064787    LIDGQTGAVYSYGEVEELSRRAAAGLRRLGVGKGDVVMSLLRNCPEFAFT
                          **:. *.  * : :*:  :*:.* ** : *: :*:*:* ** *. **.*

4CL2/Leucaena/FJ205491    FLGASFRGALTTAANPFFTAAEISKQFKASNAKILITQSAYYEKVKDL--
4CL2/Glycine/AF002259     FLGASHRGAMATAANPFFTPAEIAKQAHASNAKLLITQASYYDKVKDLR-
4CL2/Pinus/PTU12013       FMGASVRGAIVTTANPFYKPGEIAKQAKAAGARIIVTLAAYVEKLADLQS
4CL/Oriza/NM_001064787    FLGAARLGAATTTANPFYTPHEIHRQASAAGARVIVTEACAVEKVRGFAA
                          *:**:  ** .*:****:.. ** :*  *:.*::::* :.  :*: .:

4CL2/Leucaena/FJ205491    --DVKLIFVDSPP-----DGHSHFSELS--QADENDMPEVKIKPDDVVAL
4CL2/Glycine/AF002259     --DIKLVFVDSCPPHTEEKQHLHFSHLCEDNGDADVDVDVDIKPDDVVAL
4CL2/Pinus/PTU12013       H-DVLVITIDDAP----KEGCQHISVLT--EADETQCPAVKIHPDDVVAL
4CL/Oriza/NM_001064787    DRGIPVVAVDGDF-----DGCVGFGEAMLDASIEPLDADEEVHPDDVVAL
                            .: :: :*.       .    :.      .        .::*******
```

```
4CL2/Leucaena/FJ205491    PYSSGTTGLPKGVMLTHKGLLTSIAQQVDGENPNLYFHHEDVILCVLPLF
4CL2/Glycine/AF002259     PYSSGTTGLPKGVMLSHKGLVTSIAQQVDGDNPNLYYHCHDTILCVLPLF
4CL2/Pinus/PTU12013       PYSSGTTGLPKGVMLTHKGLVSSVAQQVDGENPNLYFHSDDVILCVLPLF
4CL/Oriza/NM_001064787    PYSSGTTGLPKGVMLTHRSLVTSVAQQVDGENPNLYFRREDVVLCLLPLF
                          ***************:*:.*::*:******:*****:: .*.:**:****

4CL2/Leucaena/FJ205491    HIYSLNSVLLCGLRAKAAILLMPKFEINALLGLIQKHKVSIAPVVPPIVL
4CL2/Glycine/AF002259     HIYSLNSVLLCGLRAKATILLMPKFDINSLLALIHKHKVTIAPVVPPIVL
4CL2/Pinus/PTU12013       HIYSLNSVLLCALRAGAATLIMQKFNLTTCLELIQKYKVTVAPIVPPIVL
4CL/Oriza/NM_001064787    HIYSLNSVLLAGLRAGSAIVIMRKFDLGALVDLTRRHGVTVAPFVPPIVV
                          **********..*** :: ::* **:: : : * ::: *::**.*****:

4CL2/Leucaena/FJ205491    AVSKSADIDKYDLSSIRVFKSGGAPLGKELEDSVRAKFPKARLGQGYGMT
4CL2/Glycine/AF002259     AISKSPDLHKYDLSSIRVLKSGGAPLGKELEDTLRAKFPNAKLGQGYGMT
4CL2/Pinus/PTU12013       DITKSPIVSQYDVSSVRIIMSGAAPLGKELEDALRERFPKAIFGQGYGMT
4CL/Oriza/NM_001064787    EIAKSPRVTADDLASIRMVMSGAAPMGKDLQDAFMAKIPNAVLGQGYGMT
                           ::**. :   *::*:*:. **.**:**:*:*:.  ::*:* :*******

4CL2/Leucaena/FJ205491    EAGPVLTMSLAFAKEPMGVKAGACGTVVRNAEMKIVDPETSESLPRNRPG
4CL2/Glycine/AF002259     EAGPVLTMSLAFAKEPIDVKPGACGTVVRNAEMKIVDPETGHSLPRNQSG
4CL2/Pinus/PTU12013       EAGPVLAMNLAFAKNPFPVKSGSCGTVVRNAQIKILDTETGESLPHNQAG
4CL/Oriza/NM_001064787    EAGPVLAMCLAFAKEPFEVKSGSCGTVVRNAELKIVDPDTGATLGRNQSG
                          ******:* *****:*: **.*:********:**:*.:*. :* :*:.*

4CL2/Leucaena/FJ205491    EICIRGDQIMKGYLNDPEATKRTIDEEGWLHTGDIGYIDDDDELFIVDRL
4CL2/Glycine/AF002259     EICIRGDQIMKGYLNDGEATERTIDKDGWLHTGDIGYIDDDDELFIVDRL
4CL2/Pinus/PTU12013       EICIRGPEIMKGYINDPESTAATIDEEGWLHTGDVGYIDDDEEIFIVDRV
4CL/Oriza/NM_001064787    EICIRGEQIMKGYLNDPESTKNTIDKGGWLHTGDIGYVDDDDEIFIVDRL
                          ****** :*****:** *:*  ***: *******:**:***:*:*****:

4CL2/Leucaena/FJ205491    KELIKFKAFQVAPAELEALLLTHPHISDAAVVPMKDESAGEVPVAFVVRS
4CL2/Glycine/AF002259     KELIKYKGFQVAPAELEALLLTHPKISDAAVVPMKDEAAGEVPVAFVVIS
4CL2/Pinus/PTU12013       KEIIKYKGFQVAPAELEALLVAHPSIADAAVVPQKHEEAGEVPVAFVVKS
4CL/Oriza/NM_001064787    KEIIKYKGFQVPPAELEALLITHPDIKDAAVVPMIDEIAGEVPVAFIVRI
                          **:**:*.***.********::** * ******  .* ********:*

4CL2/Leucaena/FJ205491    NGHTQTTEDDIKRFVSKQVVFYKRISRVFFIDAIPKSPSGKILRKDLRAK
4CL2/Glycine/AF002259     NGYTDTTEDEIKQFISKQVVFYKRINRVFFIDAIPKSPSGKILRKDLRAK
4CL2/Pinus/PTU12013       ---SEISEQEIKEFVAKQVIFYKKIHRVYFVDAIPKSPSGKILRKDLRSR
4CL/Oriza/NM_001064787    EG-SAISENEIKQFVAKEVVFYKRLNKVFFADSIPKSPSGKILRKDLRAK
                                :*::**.*::*:*:***:: :*:* *:***************::
```

```
4CL2/Leucaena/FJ205491      LAAGVAAN-------
4CL2/Glycine/AF002259       IAASVPK--------
4CL2/Pinus/PTU12013         LAAK-----------
4CL/Oriza/NM_001064787      LAAGIPTNDNTQLKS
                            :**
```

Fig. 3.31: Deduced amino acid sequence alignment of the coding sequences of Ll4CL2 of *L. leucocephala* (FJ205490), *Glycine* 4CL2 (AF002259), *Oriza* 4CL (NM_001064787) and *Pinus taeda* 4CL2 (U12013) genes using Clustal W 1.8. The amino acid residues are numbered in the left margin, and conserved amino acid residues or conservative exchanges are marked by asterisks and dots, respectively, in the bottom line. Five conserved cystein residue shown in the green shade. The two conserved domain box I AMP binding domain and box II GEICIRG shown in blue shade.

The GC% and codon usage of the Ll4CL2 cDNA clones was calculated using online software (www.justbio.com). The GC content of Ll4CL2 cDNA clones was 51.23 %. The codon usage for Ll4CL2 cDNA clones is given in Table 3.2 and it is expressed as % of total codons. Standard genetic codes were used for the purpose.

Tab 3.2: Codon usage of Li4CL2 gene *Leucaena leucocephala.*

codon	mean	codon	mean	codon	mean	Codon	mean
UUU (F)	0.99	UCU (S)	1.59	UAU (Y)	0.80	UGU (C)	0.60
UUC (F)	3.58	UCC (S)	2.39	UAC (Y)	1.19	UGC (C)	0.20
UUA (L)	0.99	UCA (S)	0.60	UAA (*)	0.00	UGA (*)	0.00
UUG (L)	2.39	UCG (S)	1.39	UAG (*)	0.00	UGG (W)	0.20
CUU (L)	1.59	CCU (P)	0.99	CAU (H)	0.99	CGU (R)	0.20
CUC (L)	1.99	CCC (P)	1.79	CAC (H)	1.19	CGC (R)	0.40
CUA (L)	0.60	CCA (P)	0.80	CAA (Q)	1.39	CGA (R)	0.00
CUG (L)	1.59	CCG (P)	1.99	CAG (Q)	0.80	CGG (R)	0.20
AUU (I)	1.39	ACU (T)	0.80	AAU (N)	1.39	AGU (S)	0.00
AUC (I)	2.98	ACC (T)	1.19	AAC (N)	1.19	AGC (S)	0.60
AUA (I)	1.59	ACA (T)	0.80	AAA (K)	2.98	AGA (R)	2.19
AUG (M)	1.99	ACG (T)	0.99	AAG (K)	4.17	AGG (R)	0.40
GUU (V)	1.19	GCU (A)	1.59	GAU (D)	2.78	GGU (G)	0.99
GUC (V)	2.19	GCC (A)	4.17	GAC (D)	3.18	GGC (G)	1.79
GUA (V)	0.60	GCA (A)	1.39	GAA (E)	1.59	GGA (G)	2.19
GUG (V)	3.58	GCG (A)	1.39	GAG (E)	3.78	GGG (G)	0.99

3.3.8.1. Restriction analysis of Ll4CL2 gene

Restriction analysis of Ll4CL2 was done using bioinformatics software pDRAW32 (Fig. 3.32). Analysis was limited to enzyme site cutting not more than four times in the sequence. Major restriction sites are present at following position. *Bcl* I at 68th, *Sac* I at 237th, *Sal* I at 565th, *Pvu* I at 776th, *Eco* RV at 804th, *Nco* I at 980th, *Xma* I at 1977th, *Sma* I at 1079th, *Bgl* II at 1085th at, *Dra* I at 1124th and *Stu* I at 1255th positions respectively.

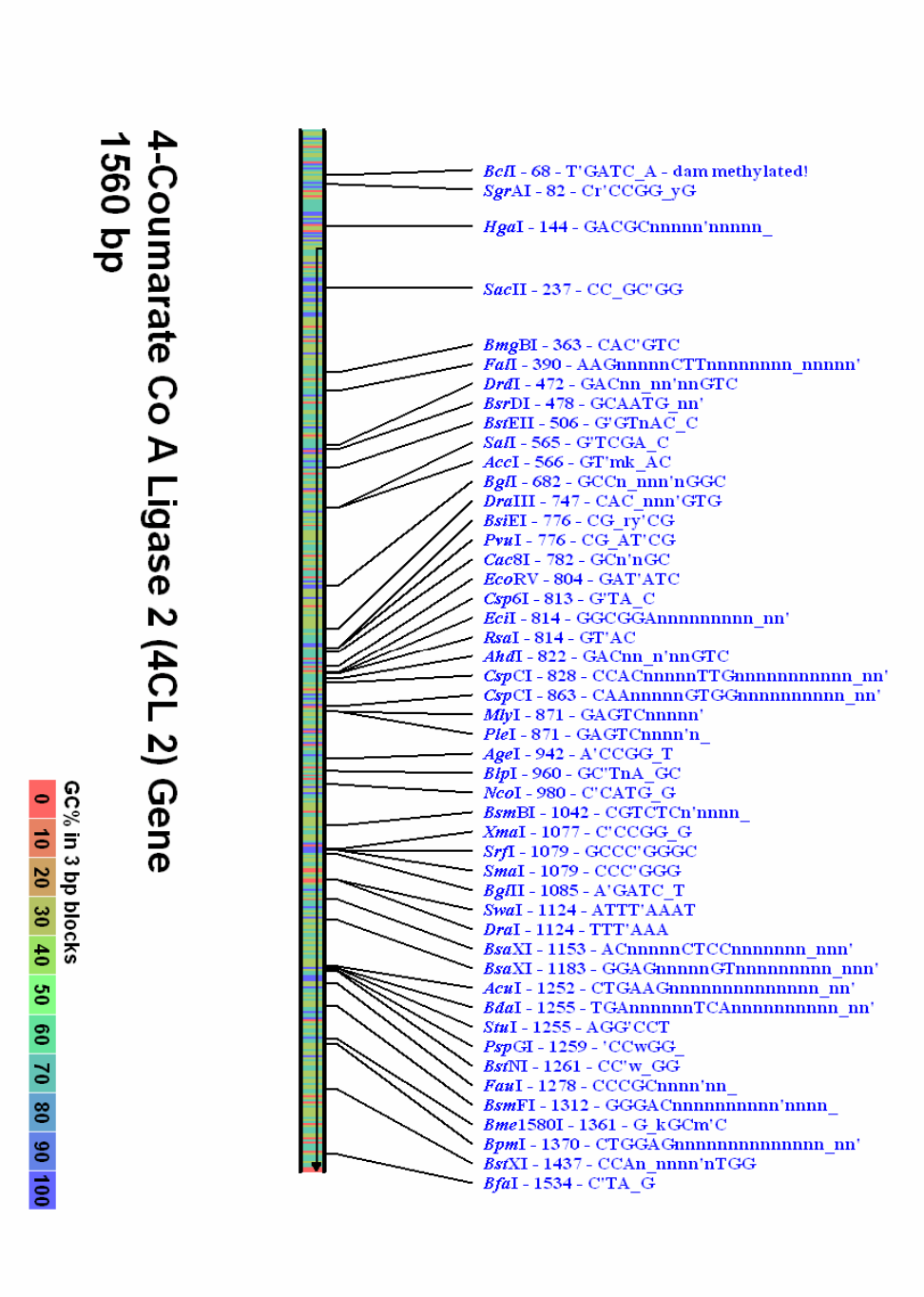


Fig 3.32: Restriction map of Ll4CL2 (FJ205491) gene using pDRAW32.

3.3.9. Distributions of 4CL genes

To validate the distribution of the Ll4CL1 and Ll4CL2 genes in *L. leucocephala* genome, Southern hybridization was performed. 10µg of *L. leucocephala* genomic DNA was restriction digested with *Hin* dIII, *Kpn* I, *Sac I,* and double digested with *KpnI* / *SacI* in duplicates. The digested genomic DNA, *Hind* III, *Kpn* I, *Sac* I and *Kpn* I/*Sac* I) was run in duplicates on 0.7% agarose gel in 1X TAE buffer. (Fig. 3.33 a) and transferred on Nylon N^+ membrane. The nylon N^+ membrane was cut from the middle and the half of nylon N^+ membrane was Southern hybridized with Ll4CL1 gene (FJ205490) and another half of nylon N^+ membrane was hybridized with Ll4CL2 gene (FJ205491). Southern hybridization revealed that from each of the restriction digested DNA samples multiple bands hybridized to the Ll4CL1 (Fig. 3.33 b) and Ll4CL2 gene clone (Fig. 3.33 c). The hybridization signals from very low to very high molecular weight DNA fragments indicated possible multiple gene copies in the *L. leucocephala* genome. Low molecular weight signals of Ll4CL1 from *Hind* III, *kpn*I , *Sac*I and *kpn*I / *Sac*I digests suggested the presence of these restriction sites within the gene(s). As from restriction analysis of this gene it is clear that there is only single site for *kpn*I, *Sac*I and no site for *Hind*III thus there is a possibility that these sites are present in multiples in introns. Ll4Cl2 hybridized membrane also show low molecular weight signals but very less in number as compared to signals from Ll4CL1. Low molecular weight signals of Ll4CL2 from *Hind* III digests suggested the presence of these restriction sites in introns. There is no low molecular weight signal with *kpn*I and one low molecular weight signal with *Sac*I suggested that there are two *Sac*I sites present in introns. This result was further affirmed by same signal with KpnI/SacI double digested genomic DNA.

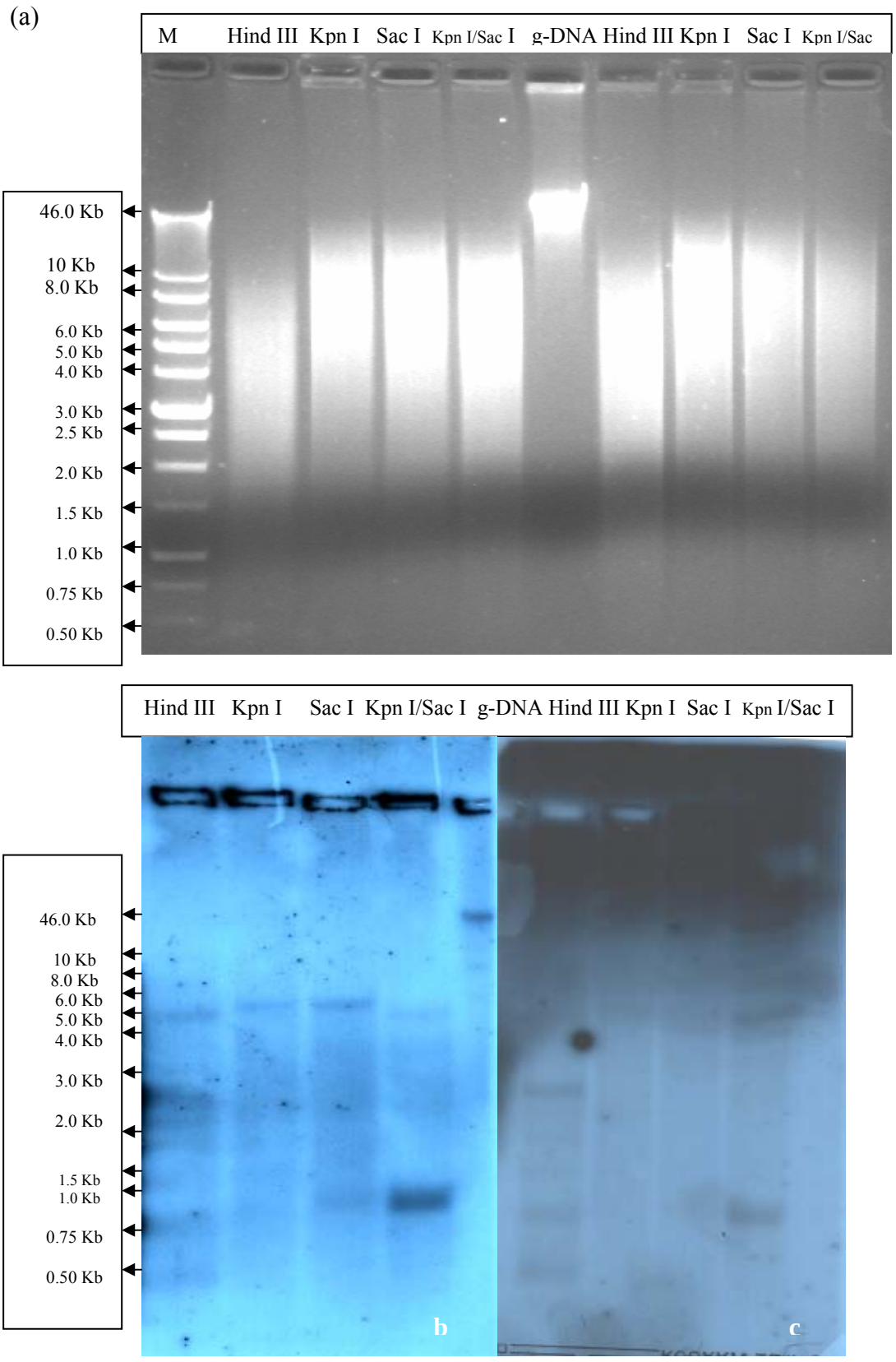


Fig 3.33: (a) 0.7% agarose gel showing *L. leucocephala* genomic DNA digestion with *Hind* III, *Kpn* I, *Sac* I and *Kpn* I / *Sac* I.Lane M λ phage DNA and 1 Kb DNA ladder ; (b) Southern hybridization of left half part of (a) with *Leucaena* Ll4CL1 gene ; (c) Southern hybridization of right half part of (a) with *Leucaena* Ll4CL2 gene.

The nucleotide sequences of Ll4CL1 (FJ205490) and Ll4CL2 (FJ205491) cDNA clones were aligned together using Clustal W 1.8(Fig 3.34) and both the sequences showed 68% sequence similarity.

CLUSTAL W (1.8) multiple sequence alignment:

```
Ll4CL1          ATGGAAACTCCTTCGATGGAATTCATCTTCCGATCGAAACTCCCCGATATTTACATCCCA
Ll4CL2          ----------------------------------------------------------CCCA
                                                                          ****

Ll4CL1          GACCACCTCCCACTTCACTCCTACGTCTTCGAGAACCTATCTCAGGTCAAAGATCGACCC
Ll4CL2          AAC-ACCTCCCTCTCCATTCTTACTGCTTCGAGAATCTCTCCGAATTCGGCTCCCGTCCT
                 ** ******* ** ** ** ***  ********* ** **  *  **      ** **

Ll4CL1          TGCCTCATCGACGGCGACACTGGCGAGACCCTTACCTATGCCGACGTCGAGCTCACCGCC
Ll4CL2          TGCCTGATCAATGCCCCCACCGGCGAAATCTATACTTACTACGACGTCGAGCTCACCTCC
                ***** *** * * *  *** ***** * *  *** **   *************** **

Ll4CL1          CGCCGTGTTGCCGCCGGCCTCACCAAACTCGGCATCCAACAAGGTGACGTCATCATGCTC
Ll4CL2          CGGCGTGTCGCCTCCGGATTGAACAAATTCGGCGTCGGACAGGGTGACGTCATCATGGTC
                ** ***** *** ****  * * **** ***** **  *** *************** **

Ll4CL1          GTGCTCCGTAACTGTCCCCAGTTCGCTCTGGCCTTCCTGGGAGCCTCCTTCGCCGGCGCC
Ll4CL2          CTCCTTTCCAATTCCCCTGAATTCGTCTTCTCCTTCCTCGGCGCCTCCTTCCGCGGCGCC
                 * **    ** *  **  * ****   *  ******* ** *********  *******

Ll4CL1          GCAGTCACCACTGCCAACCCCTTGTCCACTCCAGCCGAGCTGGCGAAACAAGCCACCGCG
Ll4CL2          TTGACAACCGCCGCGAATCCCTTCTTCACCGCCGCCGAGATTTCCAAACAATTCAAAGCC
                      *** * ** ** ***** * ***  * ****** *  * ******  **  **

Ll4CL1          TCCAAGTCGAAACTGATCATAACGCAAGCGGCTTTCGTTGAGAAAATCAAGGATTTCGCT
Ll4CL2          TCCAACGCGAAAATCCTCATAACTCAATCGGCCTACTACGAGAAAGTGAAGGACCTCG--
                *****  ***** *  ******* *** **** * *   ****** * *****  ***

Ll4CL1          GACAAACGTGGCGTTTCTTTGATGTGCATTGATTCTACTTTCCCAGAAACGGAAGGTATT
Ll4CL2          -----ACGTGAAG-----CTAATCTTCGTCGATTCTCC---------ACCGGACGGACAC
                     *****  *      * ** * * * ****** *         * **** **


Ll4CL1          TCACATTTTTCTTTACTCACCCAAGCCGACGAAGCTTGTATGCCGGCCGTCAAGATCAGC
Ll4CL2          TCTCATTTCTCGGAGCTATCTCAAGCCGACGAGAACGACATGCCCGAGGTCAAAATCAAA
                ** ***** **    **  * ***********       ***** *  ***** ****

Ll4CL1          CCCGACGACGTTGTGGCACTGCCGTATTCCTCCGGCACCTCCGGCTTTCCCAAGGGCGTG
Ll4CL2          CCGGACGACGTCGTCGCATTGCCGTATTCCTCCGGCACCACCGGGTTACCAAAAGGCGTG
                ** ******** ** *** ******************** **** ** ** ** ******
```

```
Ll4CL1          ATGCTAACGCACAAGAATCTGGTGACCTCCGTGGCTCAGCTCGTCGACGGCGAAAACCCG
Ll4CL2          ATGCTCACTCACAAGGGATTACTGACGAGCATAGCCCAACAAGTCGACGGAGAGAATCCG
                ***** ** ******    *  ****   * * ** ** *  ******** ** ** ***

Ll4CL1          AACCAGTACACCACTAGCGACGACGTACATATCTGCGTTCTTCCAATGTTCCATATCTAT
Ll4CL2          AACCTGTATTTCCACCATGAAGATGTGATTCTCTGTGTCCTTCCCCTGTTCCATATCTAC
                **** ***   *      ** ** **   * **** ** *****  **************

Ll4CL1          GCGCTGAACTCCATTTTGCTCTGCTGCATCCGAGCCGGAGCCGCCATTCTGACGATGGGT
Ll4CL2          TCGTTGAACTCTGTTTTGCTGTGTGGATTGAGGGCCAAGGCGGCGATCTTGTTGATGCCC
                 ** *******  ******* **  *  *  * ***   ** ** **  **  ****

Ll4CL1          AAGTACGACATCGCCACGTTGTTGAAGATGATCAAGACTTACAAGGTGACAATGGCGTCG
Ll4CL2          AAGTTTGAAATCAATGCGTTGTTAGGCTTGATACAGAAACACAAGGTGTCGATAGCTCCG
                ****  ** ***    *******     ****  ***   ******** * ** **  **

Ll4CL1          TTTGTGCCTCCGATCCTATTAAACATCGTGAAGAGTGAGGAAGTTGACCGGCACGACCTG
Ll4CL2          GTGGTGCCGCCGATCGTGCTTGCCGTGTCAAAGTCGGCGGATATCGATAAGTACGACCTG
                 * ***** ****** *  *   * *    ***   * ***  * **   * ********

Ll4CL1          TCGTCCATAAGGACGATCGTCACCGGAGCAGCGCCGGTGAGCGTGGAGCTGGAACAAGCC
Ll4CL2          TCGTCGATCAGAGTATTCAAATCCGGTGGGGCTCCTCTGGGTAAAGAGCTTGAAGACTCT
                ***** ** **     **    **** *  ** **  ** *    ***** *** *  *

Ll4CL1          CTGAGGGCTAAGCTTCCACACGCCATACTTGGACAGGGATATGGGATGACGGAGGGTGGT
Ll4CL2          GTGAGAGCCAAGTTCCCTAAGGCCAGACTTGGTCAGGGATATGGGATGACGGAGGCAGGA
                 **** ** *** * **  * **** ****** **********************  **

Ll4CL1          CCTCTGTCGATAA---GCTTGTCGTTCGCGAAGGAGCCGGTGGAGATGAAATCAGGCGCG
Ll4CL2          CCGGTGTTGACAATGAGCTTAGCATTTGCAAAGGAACCCATGGGAGTGAAGGCAGGAGCA
                **  *** ** **   ****  * ** ** ***** **  ***   ****  **** **

Ll4CL1          TGCGGGAGCGTGATAAGAAACGCAGAGATGAAGATTGTTGACATTGAGACGGGAGCTTCG
Ll4CL2          TGTGGAACAGTAGTTAGAAATGCCGAGATGAAGATTGTAGATCCTGAGACGTCTGAATCT
                ** ** *  **  * ***** ** ************** **   *******   *  **

Ll4CL1          CTTCCAAGAAACAGGGCTGGTGAAATCTGCATTAGAGGAAATCAAGTTATGAAAGGGTAC
Ll4CL2          CTTCCCCGTAACCGCCCGGGCGAGATCTGCATTAGAGGCGATCAAATTATGAAAGGGTAT
                *****  * *** *  * ** ** **************  ***** *************

Ll4CL1          CTAAACGACCCGGAGGCGACGAAAACGACGATAGACGAAGAAGGATGGCTTCACACGGGG
Ll4CL2          TTAAATGATCCGGAGGCCACGAAGAGAACAATCGACGAGGAGGGGTGGTTGCATACGGGA
                 **** ** ******** ***** *  ** ** ***** ** ** *** * ** *****

Ll4CL1          GACATCGGACACGTAGACGACGACGACGAGGTGTTCGTTGTGGATCGTTTGAAGGAGATC
Ll4CL2          GACATAGGCTACATCGATGACGACGATGAGCTGTTCATCGTTGATAGGCTGAAGGAGCTC
                ***** **  ** * ** ******** *** ***** * ** *** *  ******** **
```

```
Ll4CL1          ATCAAATACAAAGGTTTCCAAGTGGCTCCTGCTGAGCTGGAGGCTCTTCTCATCTCTCAC
Ll4CL2          ATCAAGTTCAAGGCCTTCCAGGTGGCCCCCGCTGAACTTGAGGCCCTCCTTCTCACTCAT
                ***** * *** *  ***** ***** ** ***** ** ***** ** **  ** ****

Ll4CL1          CCTTTCATTTCTGATGCTGCCGTTGTTCCGATGAAAGATGAGGCTGCGGGGGAGCTTCCG
Ll4CL2          CCTCACATATCCGATGCCGCCGTGGTCCCAATGAAGGATGAATCTGCTGGAGAGGTGCCC
                ***  *** ** ***** ***** ** ** ***** *****  **** ** *** * **

Ll4CL1          GTGGCATTTGTGGTTAGGTCAAATGGTTTTA---AGATCTCTGAGGATGACATCAAGCTT
Ll4CL2          GTTGCCTTTGTTGTCAGATCCAACGGTCACACTCAAACCACCGAGGATGACATCAAACGG
                ** ** ***** ** ** ** ** ***   *   * * * * ************** *

Ll4CL1          TTCATTTCACAACAGGTGGTGTATTACAAGAGAATCCACAAGGTTATTTTCACAGACACT
Ll4CL2          TTTGTCTCCAAACAGGTGGTGTTTTACAAGAGAATAAGCAGAGTGTTCTTCATTGATGCG
                **  * **  ************ ************   **  **  * ****  **  *

Ll4CL1          ATTCCTAAAGCAGTTTCTGGCAAAATTTTACGAAAGGATTTAAAAGCAAGGCTTGCATCA
Ll4CL2          ATTCCAAAATCACCCTCAGGCAAGATATTGAGAAAGGACCTCAGAGCAAAACTAGCTGCT
                ***** *** **   ** ***** ** **  *******  * * *****  ** **  *

Ll4CL1          GAT-TTG--GGCAATTAA
Ll4CL2          GGTGTTGCTGCTAATTAA
                    * ***  *  ******
```

Fig. 3.34: Nucleotide sequence alignment of the coding sequences of Ll4CL1 of *L. leucocephala* (FJ205490) and coding sequences of Ll4CL2 *L. leucocephala* (FJ205491), genes using Clustal W 1.8.

The deduced amino acid sequence of the Ll4CL1 gene with Ll4CL2 gene (Fig. 3.35) showed 66% ((347/521)) identities and 81% (424/521) positives. The AMP-binding domain sequences of Ll4CL1 and LL4CL2 showed 84% identities and 100% positives sequences. The second conserved domain is GEICIRG; this putative domain is absolutely conserved in both the genes.

CLUSTAL W (1.8) multiple sequence alignment:

```
Ll4CL1(FJ295490)    METPSMEFIFRSKLPDIYIPDHLPLHSYVFENLSQVKDRPCLIDGDTGETLTYADVELTA
Ll4CL2(FJ205491)    -------------------PKHLPLHSYCFENLSEFGSRPCLINAPTGEIYTYYDVELTS
                                      *.******* *****:. .*****:. ***  ** *****:

Ll4CL1(FJ295490)    RRVAAGLTKLGIQQGDVIMLVLRNCPQFALAFLGASFAGAAVTTANPLSTPAELAKQATA
Ll4CL2(FJ205491)    RRVASGLNKFGVGQGDVIMVLLSNSPEFVFSFLGASFRGALTTAANPFFTAAEISKQFKA
                    ****:**.*:*: ******::* *.*:*.::****** ** .*:***: *.**::** .*

Ll4CL1(FJ295490)    SKSKLIITQAAFVEKIKDFADKRGVSLMCIDSTFPETEGISHFSLLTQADEACMPAVKIS
Ll4CL2(FJ205491)    SNAKILITQSAYYEKVKDLD----VKLIFVDS---PPDGHSHFSELSQADENDMPEVKIK
                    *::*::***:*: **:**:     *.*: :**    .:* **** *:****  ** ***.
```

```
Ll4CL1(FJ295490)      PDDVVALPYSSGTSGFPKGVMLTHKNLVTSVAQLVDGENPNQYTTSDDVHICVLPMFHIY
Ll4CL2(FJ205491)      PDDVVALPYSSGTTGLPKGVMLTHKGLLTSIAQQVDGENPNLYFHHEDVILCVLPLFHIY
                      *************:*:*********.*:**:** ******* *   :** :****:****

Ll4CL1(FJ295490)      ALNSILLCCIRAGAAILTMGKYDIATLLKMIKTYKVTMASFVPPILLNIVKSEEVDRHDL
Ll4CL2(FJ205491)      SLNSVLLCGLRAKAAILLMPKFEINALLGLIQKHKVSIAPVVPPIVLAVSKSADIDKYDL
                      :***:*** :** **** * *::* :** :*:.:**::*..****:* : ** ::*::**

Ll4CL1(FJ295490)      SSIRTIVTGAAPVSVELEQALRAKLPHAILGQGYGMTEGGP-LSISLSFAKEPVEMKSGA
Ll4CL2(FJ205491)      SSIRVFKSGGAPLGKELEDSVRAKFPKARLGQGYGMTEAGPVLTMSLAFAKEPMGVKAGA
                      ****.: :*.**:. ***:::***:*:* *********.** *::**:*****: :*:**

Ll4CL1(FJ295490)      CGSVIRNAEMKIVDIETGASLPRNRAGEICIRGNQVMKGYLNDPEATKTTIDEEGWLHTG
Ll4CL2(FJ205491)      CGTVVRNAEMKIVDPETSESLPRNRPGEICIRGDQIMKGYLNDPEATKRTIDEEGWLHTG
                      **:*:********* **. ******.*******:*:************ **********

Ll4CL1(FJ295490)      DIGHVDDDDEVFVVDRLKEIIKYKGFQVAPAELEALLISHPFISDAAVVPMKDEAAGELP
Ll4CL2(FJ205491)      DIGYIDDDDELFIVDRLKELIKFKAFQVAPAELEALLLTHPHISDAAVVPMKDESAGEVP
                      ***::*****:*:******:**:*.************::**.************:***:*

Ll4CL1(FJ295490)      VAFVVRSNG-FKISEDDIKLFISQQVVYYKRIHKVIFTDTIPKAVSGKILRKDLKARLAS
Ll4CL2(FJ205491)      VAFVVRSNGHTQTTEDDIKRFVSKQVVFYKRISRVFFIDAIPKSPSGKILRKDLRAKLAA
                      *********  : :***** *:*:***:**** :*:* *:***: *********:*:**:

Ll4CL1(FJ295490)       DLGN-
Ll4CL2(FJ205491)      GVAAN
```

Fig. 3.35: Deduced amino acid sequence alignment of Ll4CL1 (FJ205490) and (FJ205491) using Clustal W 1.8.the shaded region show the presence of both the catalytic domain, Single shaded sequences are conserved cystein residue in both genes.

3.3.7.4 Phylogenetic analysis of Ll4CLgene

Phylogenetic analysis was done using 41 protein sequences of 4CLs, which were retrieved from the Gen-Bank database. Multiple alignments of the predicted protein coding sequences were performed using Clustal W. The phylogenetic tree was subsequently analyzed and displayed by Neighbor joining mega software. Bootstrap values were calculated from 1000 replications for statistical analysis. The phylogenetic analysis was calculated from *Streptomyces coelicolor*, 4CL Cinnamate:CoAligase, an enzyme of unclear physiological significance which was not included in a distinct clade containing bona fide 4CL in phylogenetic analysis (Kaneko *et al.* 2003). The phylogenetic reconstruction of known plant 4CLs had revealed the evolution of two major classes (Cukovic et al. 2001). (Fig.

3.36). Two distinct phylogentic classes were distinguished as reported previously, and Ll4CL1 was associated with class II clustering with *Glycine*, *Arabidopsis, Populus* and *Rubus*, whereas Ll4CL2 showed a closer relationship to class I enzymes, *i.e. Amorpha, Betula.* .The constructed Phylogentic tree (Fig 3.36) shows Ll4CL1 showed high similarity with that of the GM4CL1 (*Glycine*). Ll4CL1 and GM4CL1 belong to the family fabaceae, While Ll4CL2 placed close to AF4CL. The Ll4CL2 shows high similarity with Af4CL (*Amorpha*) which also belongs to the same family fabaceae (Endler *et al.,* 2008).

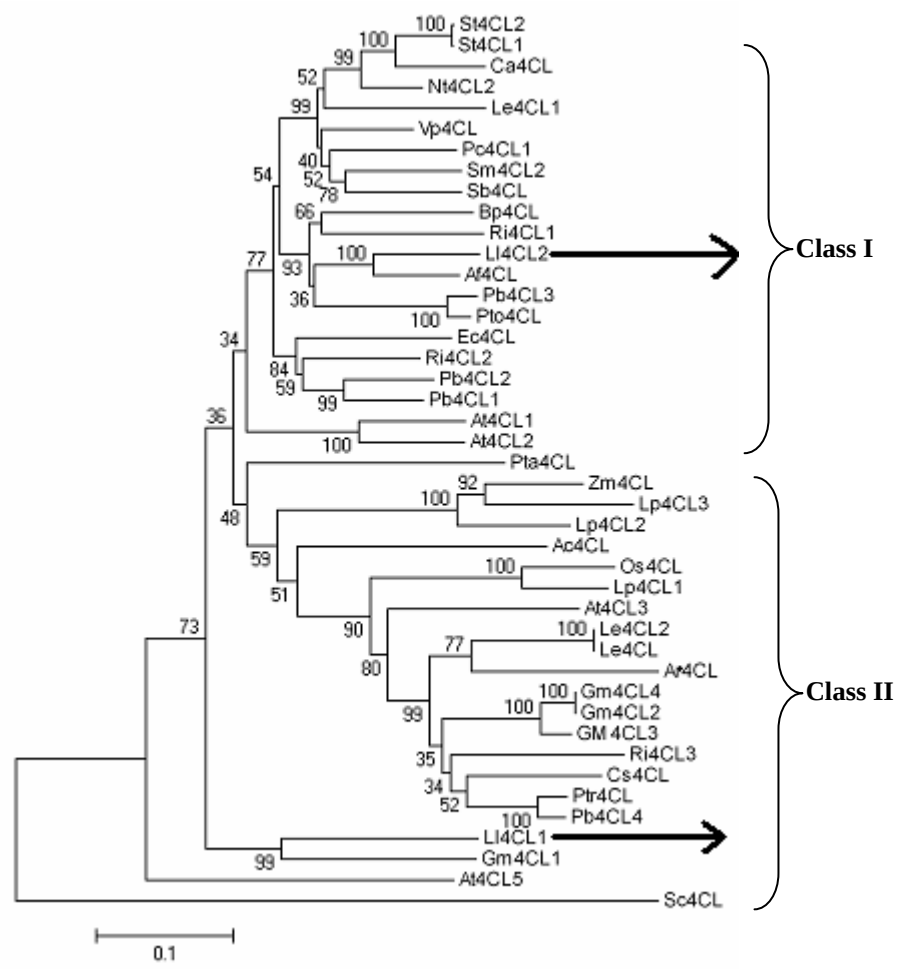


Fig. 3.36: Phylogenetic tree constructed of Ll4CL1, Ll4CL2 and 41 heterologous 4CL polypeptide sequences. The phylogenetic analysis was calculated using Streptomyces coelicolor 4CL as the outgroup, and the Ll4CL sequences are marked by arrows. At4CL1 (*Arabidopsis thaliana*, NP_175579); At4CL2 (*Arabidopsis thaliana*, NP_188761); At4CL3 (*Arabidopsis thaliana*; NP_176686); AT4CL4 (*Arabidopsis thaliana*, AAM_19949), Os4CL (*Oryza sativa* , NP_001047819); At4CL5 (*Arabidopsis thaliana*, NP_188760), GM_4CL3 (*Glycine max*, AAC97389), Gm4CL1 (*Glycine max*, AAL98709), Gm4CL4 (*Glycine max*, CAC36095); Gm4CL2 (*Glycine max*, P31687); Ptr4CL (*Populus tremuloides*, AAC24504); Pb4CL4 (*Populus balsamifera*, AAK58909); Pb4CL2 (*Populus balsamifera*, AAC39365); Pb4CL3 (*Populus balsamifera*, AAK58908); Pb4CL1 (*Populus balsamifera*, AAC39366); Le4CL (*Lithospermum erythrorhizon* BAA0836); Ri4CL3 (*Rubus idaeus*, AAF91308); Cs4CL (*Camellia sinensis*, ABA40922); Ar4CL (*Agastache rugosa*, AAT02218); Lp4CL1 (*Lolium perenne*, AAF37732); Pta4CL (*Pinus taeda*, AAA92669); Bp4CL (*Betula platyphylla*, AAV65114); Sm4CL2 (*Salvia miltiorrhiza*, AAP68991); Ri4CL2 (*Rubus idaeus*, AAF91309); Pc4CL1 (*Petroselinum crispum*, P14913); Ac4CL (*Allium cepa*, AAS48417); Ri4CL1 (*Rubus idaeus*, AAF91310); Nt4CL2 (*Nicotiana tabacum*, O24146); Ec4CL (*Eucalyptus camaldulensis*, AAZ79469); Ca4CL (*Capsicum annuum*, AAG43823); St4CL2 (*Solanum tuberosum*, P31685); St4CL1 (*Solanum tuberosum*, P31684); Af4CL (*Amorpha fruticosa*, AAL35216); Le4CL (*Lithospermum erythrorhizon*, BAA08366); Sb4CL (*Scutellaria baicalensis*, BAD90936); Zm4CL (*Zea mays*, AAS67644); Vp4CL (*Vanilla planifolia*, O24540); Pto4CL (*Populus tomentosa*, AAY84731); Lp4CL2 (*Lolium perenne*, AAF37733); Lp4CL3 (*Lolium perenne*, AAF37734); Ll4CL1 (*Leucaena leucocephala*, FJ205490) and Ll4CL2 (*Leucaena leucocephala*, FJ205491) and c4CL (*Streptomyces coelicolor*, CAB95894)

3.4. Conclusion

- 4-coumarate: CoA ligase (4CL) in L. leucocephala is a gene family of possibly 6 members.
- A 0.36 kb fragment was amplified using primers designed from the consensus 4CL gene sequences present in the NCBI Gen-Bank data base. This fragment was cloned and sequenced. Sequencing data revealed that this fragment showed maximum homology with the reported 4CL3 genes (72% with *Glycine max* 4CL 3: AF002258, 79% with *Rubus idaeus* 4CL3: AF239685 and 77% with *Arabidopsis thaliana* 4CL3: AF106088). This sequence was submitted to the NCBI Gen-Bank database and the allotted accession number for this 0.36 kb fragment is DQ267975.
- A 1.30 kb fragment was amplified using primers designed from the consensus 4CL1 gene sequences of member of fabaceae family present in the NCBI Gen-Bank database. This fragment was cloned and sequenced. Sequencing data revealed that this fragment showed maximum homology with the reported 4CL1 genes (72% with *Glycine max* 4CL1: AF279267, 67% with *Rubus idaeus* 4CL1: AF239687 and 67% with *Scutellaria baicalensis* 4CL1: AB166767). This sequence was submitted to the NCBI Gen-Bank database and the allotted accession number for this 1.30 kb fragment is DQ986905.
- Full-length 4CL1 gene was isolated using RACE. 5' and 3' RACE reactions were performed separately and both 5' RACE and 3' RACE sequences were aligned and the common region was identified. Both the sequence were arranged to make full-length 4Cl gene along with 5' and 3' UTR. The total length of Ll4CL1 along with 5' and 3' UTR was 1935 bp. The exact length of coding region of full length, 5'UTR and 3'UTR are 1629, 68 and 238 nucleotides respectively. The full length gene was designated as Ll4CL1.
- Primers were designed from the initiation codon ATG and the end of coding region of the 4CL full length gene. This gene was amplified from RT^{1st} strand, cloned and sequenced. Sequencing data revealed that this

fragment showed maximum homology with the reported 4CL1 genes (73% with *Glycine max* 4CL1: AF279267, 69% with *Rubus ideus* 4CL1: AF239687 and 68% with *Scutellaria baicalensis* 4CL1: AB166767). This sequence along with 5' and 3' UTRs was submitted to the NCBI GenBank database and the allotted accession number for this fragment is FJ205490.

- A fragment of 1.80 kb was amplified using forward primer designed from the consensus region of member of fabaceae family and reverse primer from RACE kit. The amplified fragment was cloned and sequenced. Sequencing data revealed that this fragment showed maximum homology with the reported 4CL2 genes (76% with *Amorpha fruticosa* 4CL: AF435968, 73% with *Glycine max* 4CL2: AF002259, 72% with *Rubus idaeus* 4CL2: AF239686 and 72% with *Ruta graveolens* 4CL2: EU196764). This sequence along with 3' UTRs was submitted to the NCBI Gen-Bank database and the allotted accession number for this fragment is FJ205491. This gene was designated as Ll4CL2. The exact length of coding region and 3'UTR of Ll4CL2 are 1560 and 271 nucleotides respectively.
- Two cDNA clones Ll4CL1 and Ll4CL2, showed 68% nucleotide and 66% deduced amino acid sequence similarity with each other. Nucleotide sequence similarity with 4CL cDNA gene clones from other plants was 65-75%.
- Deduced amino acid sequences of Ll4CL1 and Ll4CL2 genes show the presence of AMP binding superfamily domain 415 to 519 amino acid. This AMP binding superfamily domain have box I (AMP binding) conserved motif. This box I sequences are slightly different in Ll4CL1 (LPYSSGTSGFPKG) and Ll4CL2 (LPYSSGTTGLPKG). The box I sequences showed 84% identities and 100% positive sequences. The second conserved domain is box II *i.e.* GEICIRG which are absolutely conserved in all reported 4CL gene.
- Southern hybridization was done to analyze the distribution of both the genes in the *L. leucocephala* genome and it was concluded that both the genes were differently located in the genome.

- Phylogenetic analysis was done using 41 protein sequences of 4CLs, which were retrieved from the Gen-Bank database. Multiple alignments of the predicted protein coding sequences were performed using Clustal W. The phylogenetic tree was subsequently analyzed and displayed by Neighbor joining mega software. Bootstrap values were calculated from 1000 replications for statistical analysis. The phylogenetic analysis was calculated using *Streptomyces coelicolor* 4CL as the out-group. Ll4CL1 showed high similarity with that of the *Glycine* and Ll4CL2 shows high similarity with *Amorpha*. Two distinct phylogentic classes were distinguished and Ll4CL1 was associated with class II clustering with *Glycine*, *Arabidopsis, Populus* and *Rubus*, whereas Ll4CL2 showed a closer relationship to class I enzymes, *i.e. Amorpha, Betula.*

Chapter 4

4. A. Cloning and Heterologous Expression of 4CL Gene and Immuno- cytolocalization

This chapter includes the heterologous expression of 4CL gene in *E. coli* BL21 (DE3) and purification of recombinant protein from inclusion bodies using Ni-chelated affinity column. Purification of polyclonal IgG raised against recombinant 4CL protein using agarose A affinity matrix. Immunolocalization of the transverse sections of root and shoot of 0, 5, 10, 15 and 30 days old seedling as well as from one and two season old plant was done using purified IgG. Phloroglucinol-HCL staining of the transverse sections of root and shoot of 0, 5, 10, 15 and 30 days old seedling as well as from one and two season old plant was done to study the extent of lignification.

4.1. Introduction

4-coumarate Co A ligase (4CL) protein was for the first time isolated from soybean (Knobloch and Hahlbrock, 1975), later recombinant 4CL protein was expressed from different plants and found to be closely associated with lignification in tobacco (Kajita, *et al*., 1996), pine (Zhang and Chiang, 1997), poplar (Douglas *et al.,* 1998), *Rubus idaeus (Ellis and Kumar, 2003) soybean* (Lindermayr, *et al*., 2002), *Salvia (*Liu *et al*., 2006). After this finding, the emphasis shifted towards determining whether this expression pattern was common to other plants. Localization of 4CL at the protein and mRNA levels confirmed 4CL gene expression in lignifying tissues in herbaceous plants like tobacco (kajita,1996) , alfalfa (Kersey, *et al.*, 1999; Inoue, *et al.*, 1998), and in woody plants like forsythia (Ye, 1997) and poplar (Chen *et al.*, 2000; Zhong *et al.*, 2000).

4.1.1. Expression of 4CL gene and protein purification

To maximize expression, the cloned gene must be transcribed and translated as efficiently as possible. This is feasible due to the construction of expression vectors, i.e. modified plasmids with useful features which can be propagated and controlled in special hosts (expression systems). Usually, vectors for cloning and

expressing target DNA are derived from medium-copy plasmids such as pET. *E.coli* expression systems should meet several criteria including:

(I) Minimal basal expression of the gene to be expressed under repressed conditions,

(II) Fast and uncomplicated induction of a wide variety of genes to a high level of expression,

(III) Easy cloning and DNA manipulation features.

The most common expression system is the T7 expression system derived from bacteriophage T7. The T7 expression system is based on the use of the T7 bacteriophage promoter and RNA polymerase. The T7 RNA polymerase is useful for synthesizing selectively large amounts of RNA because the T7 RNA polymerase recognizes only the T7 promoter and not the *E.coli* promoters. Conversely, the *E.coli* RNA polymerase does not recognize the T7 promoter. The T7 RNA polymerase is able to transcribe genes five times faster than the *E.coli* RNA polymerase. The gene encoding the T7 RNA polymerase was inserted into the chromosome of the bacteria used for over expression. Expression of the target gene is induced by addition of IPTG (isopropyl-ß-D-thiogalactopyranoside) to a growing culture.

4.2. Material and Methods

4.2.1. Cloning 4CL cDNA in expression vector

The pET-30b (+) vector (Novagen, USA) was used for expression of Ll4CL1 gene in *E.coli* BL 21 (DE3). This vector carries a N-terminal His•Tag®/thrombin/Ser•Tag™/ enterokinase configuration plus an optional C-terminal His•Tag sequence. This vector has unique restriction sites for cloning (Fig. 4.1). The cloned gene is expressed under the T7 RNA polymerase promoter.

4.2.2. Construction of pET expression vector

The Ll4CL1 gene specific Ll4CLF forward and the Ll4CLR reverse primer sequences (Chapter 3; section 3.3.6) were modified by incorporating *Nde*I restriction site at the 5'end of the Ll4CLF primer and *Xho*I restriction site at the 5' end of the Ll4CLR primer excluding the stop codon from the end.

Pet4CLF 5′ ***CATATG***GAAACTCCTTCGATGGAATTCA 3′
Pet4CLR 5′ ***CTCGAG***ATTGCCCAAATCTGATGCAAGC 3′

High fidelity *Taq pol* (*Pfx* Invitrogen) was used to amplify Ll4CL1 full length gene using Pet4CLF and Pet4CLR primers using RT[1st] strand cDNA as template. The amplified fragment was gel eluted and cloned in pGEMT easy vector after tailing it with dATP. This cloning strategy is topographically represented in Fig 4.2.

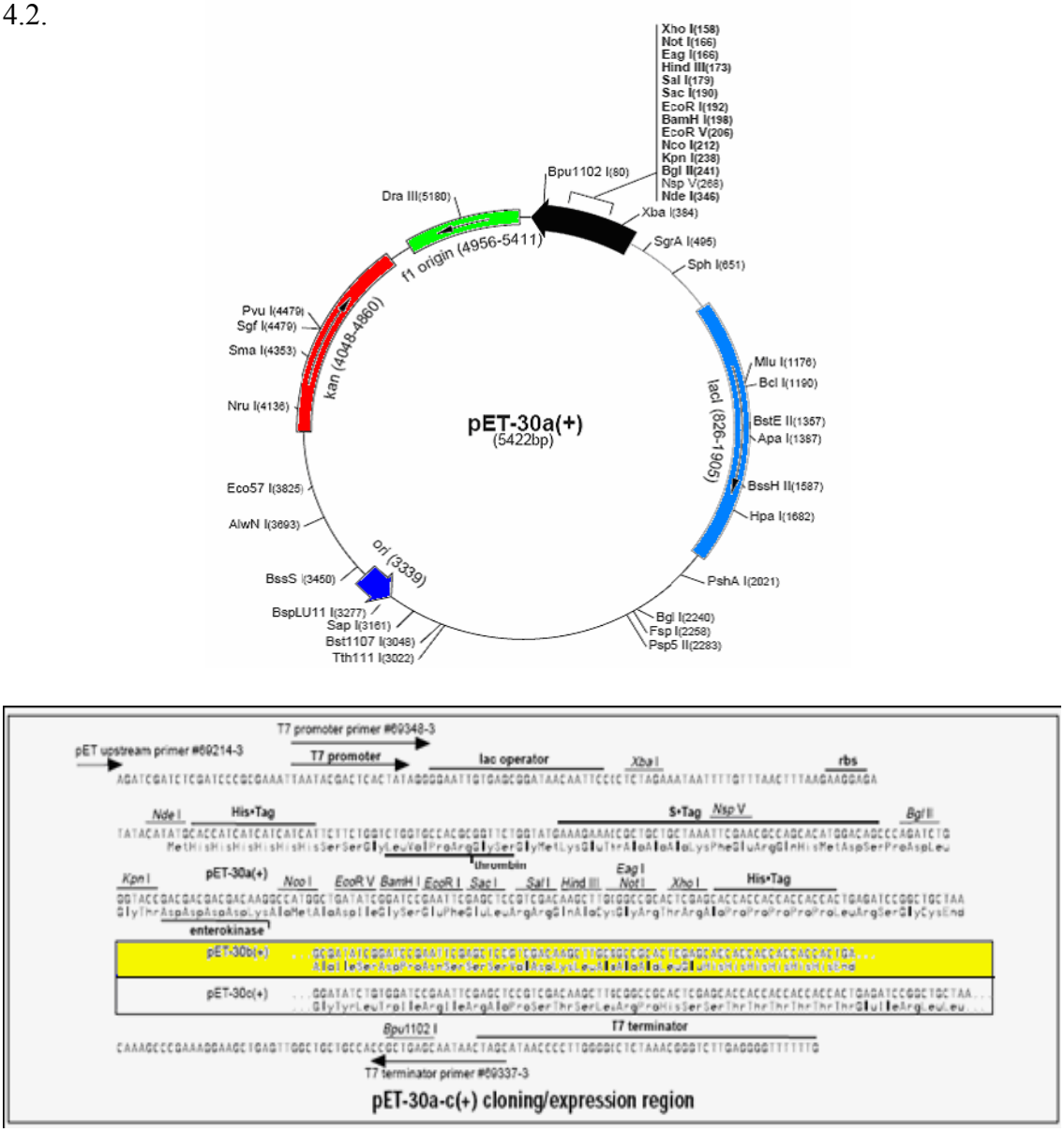


Fig. 4.1: Vector diagram of expression vector pET 30 a,b and c(+).The maps for pET 30b(+) and pET-30c(+) are the same as pET-30a(+) (shown) with the following exceptions: pET-30b(+) is a 5421 bp plasmid; subtract 1 bp from each site beyond BamH I at 198. PET-30c (+) is a 5423 bp plasmid; add 1 bp to each site beyond BamH I at 198. The sequence of pET 30b (+) is highlighted in yellow.

Directional cloning of the 4CL gene fragment into the suitable expression vector:

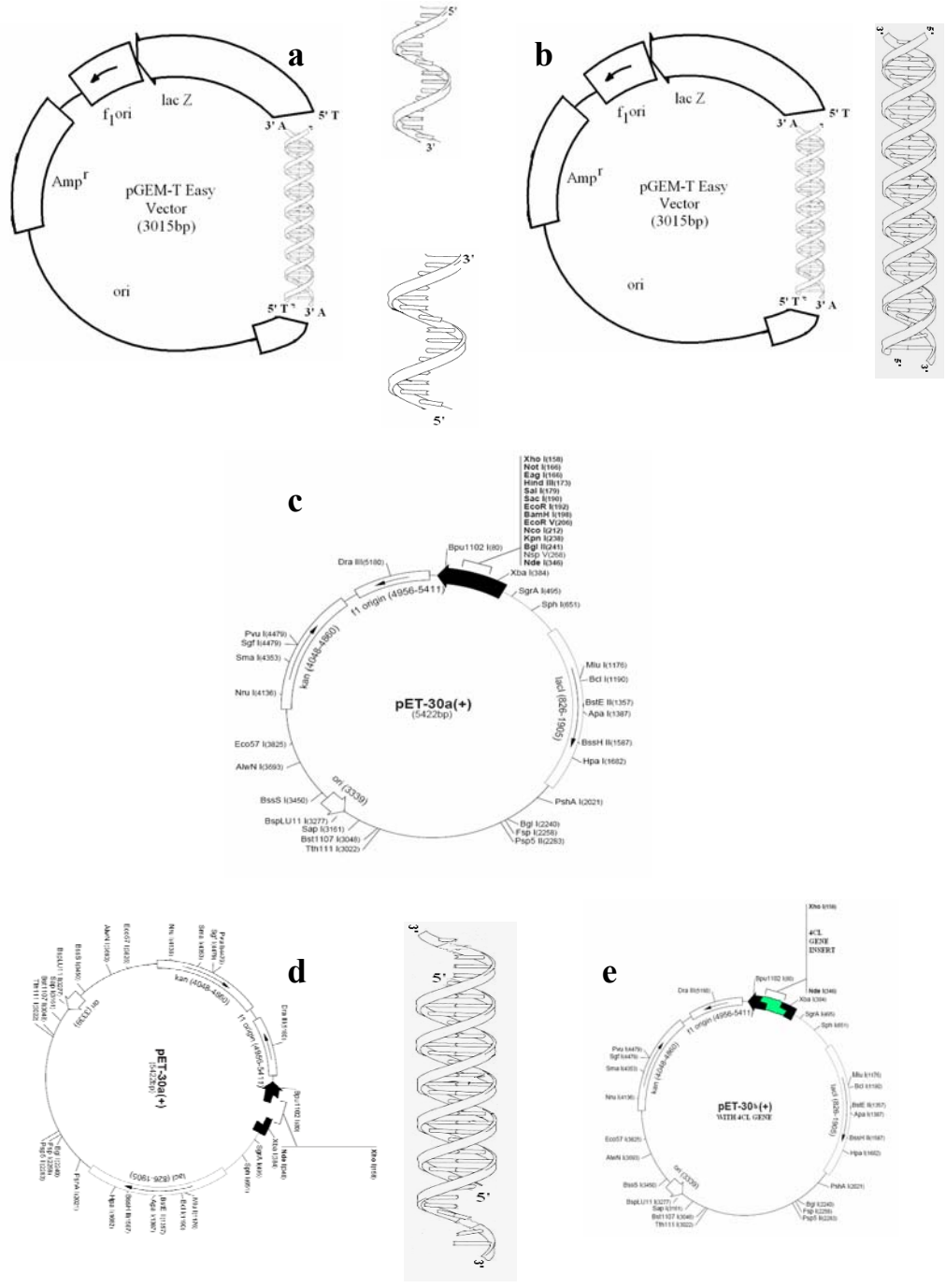


Fig 4.2: Topographical representation of directional cloning:(a) Amplification of gene using designed primers with restriction sites, (b) Digestion of amplified genes ei from clone or from amplicon, (c) Intact pET vector, (d) Directional cloning of insert in digested pET vector, (e) Insert cloned in pET vector.

Digestions of DNA insert and vector pET -30b (+)

1 µg purified DNA insert in pGEMT easy	: 3.0 µl
Or pET 30b (+) vector	
10 x restrictions Buffer	: 3.0 µl
10 U/µl *Nde*I	: 0.5 µl
20 U/µl *Xho*I	: 0.5 µl
H_2O up to	: 25.0 µl
Total Volume	**: 30.0 µl**

The reaction was incubated at 37 °C for 2 h.

Ligation of insert (pETLl4CL1) in pET -30b (+) vector

1 µg pET -30b (+) vector	: 3.0 µl
300 ng purified DNA insert (pETLl4CL1)	: 1.0 µl
2 x ligation Buffer	: 5.0 µl
3 U/µl *Ligase*	: 1.0 µl
Total Volume	**: 10.0 µl**

The reaction was incubated at 16 °C for 18 h.

The cloned fragment was sequentially digested with *Xho*I followed by *Nde*I instead of double digestion although the restriction buffer was common for both the enzymes. This was done because the MCS (Multiple Cloning Site) of pGEMT had a restriction site *Nde*I. The digested fragment (pETLl4CL1) was gel eluted and checked on 1% agarose gel for integrity and cloned in pre digested pET 30b (+) vector containing a histidine-tag. The expression vector was transferred into an *E.coli* BL 21 (DE3) strain, the growth of bacterial cells and the induction of expression with IPTG were preformed.

4.2.3. Confirmation of insert

After isolation, the plasmids were analyzed for the presence of an insert into the multiple cloning sites (MCS). This was done by sequence analysis of the insert across the MCS using T_5 forward and reverse primers (Invitrogen) which flank the multiple cloning site of pET 30b (+) vector.

4.2.4. Protein expression and purification

4.2.4.1. Growth and IPTG treatment of transformed *E.coli* BL21

A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 µg / mL kanamycine) was used to inoculate 5 mL liquid LB medium containing the same concentrations of the antibiotic. Culture was grown overnight with shaking at 225 rpm at 37 °C. One 1 ml aliquot of each culture was used to inoculate 100 ml liquid cultures containing 50 µg / mL kanamycine. Once the cultures reached OD_{600} 0.4 - 0.5, recombinant protein expression was induced by the addition of isopropyl -ß-D-thiogalactopyranoside (IPTG), and the culture was grown for 4–6 h at 37 °C with shaking at 150 rpm. Cells were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. Pellets were resuspended in lysis buffer. Cells were disrupted by sonication on a Sonifer Cell Disruptor on ice.

4.2.4.2. Preservation of bacteria

Bacterial cultures containing plasmids with target insert were stored in glycerol. In an Eppendorf tube, 800 µL of bacterial culture were mixed with 200 µL of glycerol (sterilized by autoclaving). The mixture was vortexed to ensure that the glycerol is dispersed. The tube was stored at -80 °C for long-term storage. To recover the bacteria, the frozen surface of the culture was scraped with a sterile inoculating needle and the bacteria adhered to the needle were immediately streaked on the surface of a LB agar plate containing the appropriate antibiotic. The plates were incubated overnight at 37 °C.

4.2.4.3. Expression and purification of recombinant proteins

Many natural proteins have metal binding sites which can be used for purification. The concept of this type of purification tool is rather simple. A gel bead is covalently modified to display a chelator group for binding a heavy metal ion like Ni^{2+}. Affinity chromatography is viewed as a group-selective tool for purifying the metal-binding class of proteins. His-tagged recombinant protein can be purified by Metal Chelate Affinity Chromatography. The initial stage of His-tagged protein purification is based on the remarkable selectivity and high affinity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an

affinity tag of six consecutive histidine residues, the 6xHis-tag. NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal-chelating purification systems that have only three sites available for interaction with metal ions. The extra chelation site prevents nickel ion leaching, providing a greater binding capacity and high-purity protein preparations. Isolation and purification of proteins were conducted using Ni NTA Agarose beads (Qiagen). Fusion proteins were purified as described earlier (Chapter 2; section 2.13.1 and 2.13.2). Purity of protein was checked on 10 % SDS PAGE (Chapter 2; section 2.13.3).

4.2.5. Primary and secondary antibodies

Purified 4CL protein was used for raising rabbit immune-serum. Primary Polyclonal antibodies were purified from the immune-serum. Secondary antibodies *i.e.* anti rabbit goat IgG conjugated with alkaline phosphatase were from Merck, USA.

4.2.6. Histology, histochemical staining and immunocytolocalization

Transverse sections of root and shoot of 0, 5, 10 15 and 30 days old *Leucaena leucocephala* seedlings were used for histology, immunocytolocalization and histochemical staining (Chapter 2; section 2.15 and 2.16). Immunoassays are immunological methods that make use of the binding specificity of an antibody to measure either the antigen or the antibody. To quantitate the reaction, either the antigen or the antibody is labeled. In an enzyme-linked immunosorbent assay (ELISA), the label is an enzyme with high turnover number, such as horseradish peroxidase, alkaline phosphatase, or β-galactosidase. In most applications of ELISA, one of the components is adsorbed passively to a solid phase, most often plastic in the form of 96-well microtiter plates.

4.2.7. Semi- and absolute quantification of 4CL gene expression

Total RNA isolated from root, shoot, leaf and inflorescence of 0, 5, 10 and 15 day old *Leucaena leucocephala* seedlings as well as of one and two seasons old plant (Chapter 2; section 2.10.6) was used for cDNA first strand synthesis. The cDNA first strands were used as template for semi and absolute quantification of 4CL transcripts as described earlier (Chapter 2; section 2.10.9).

4.3. Results and discussion

4.3.1. Expression of 4CL gene in *E.coli*

The Ll4CL1 gene was cloned in pET 30b (+) vector and expressed in *E.coli* BL21 (DE3) cell line. The Ll4CL1 gene reamplified using pETLl4CLF and pETLl4CLR primers (Chapter 4, section 4.2.2.) was cloned in pGEM T Easy vector and sequenced. The gene insert was released with *Nde* I and *Xho* I restriction digestion, purified and cloned in the *Nde*I and *Xho*I sited of the pET-30b (+) vector. The directionally cloned insert Ll4CL1 was released from pET vector with *Nde* I and *Xho* I restriction digestion (Fig: 4.4) and integration of Ll4CL1 gene in frame was further confirmed by sequencing using t5 forward and reverse primers. The pET 30b (+) vector harboring Ll4CL1 gene was designated as pETLl4CL1 (Fig: 4.5). The vector pETLl4CL1 was transformed into *E. coli* BL21 (DE3) cell line for expression. The deduced amino acids sequence of the Ll4CL1 gene sequence was used to calculate its approximate molecular mass, percent amino acid composition and theoretical pI. The calculated approximate molecular mass of Ll4CL1 was 58.00KDa with pI of 5.56. These Calculations compare well with other 4CL proteins reported from tobacco (Kajita *et al*., 1996), pine (Zhang and Chiang, 1997), poplar (Douglas *et al.,* 1998), *Rubus idaeus* (Ellis and Kumar, 2003) *soybean* (Lindermayr *et al*., 2002), *Salvia* (Liu, *et al*., 2006). Percent amino acid composition is shown in table 4.1. The deduced amino acid sequence (Total number of negatively charged residues (Asp + Glu): 66) and the hydropathy plots (Kyte and Doolittle, 1982; Fig. 4.3) for the 4CL proteins also show strong negative peaks indicating possible exposed surface regions of the protein (Hopp and Woods, 1981) as reported in other 4CL gene tobacco (Kajita *et al*., 1996), pine (Zhang and Chiang, 1997), poplar (Douglas *et al.,* 1998), *Rubus idaeus*(Ellis and Kumar, 2003) soybean (Lindermayr, *et al*., 2002), *Salvia (*Liu, *et al*., 2006)).

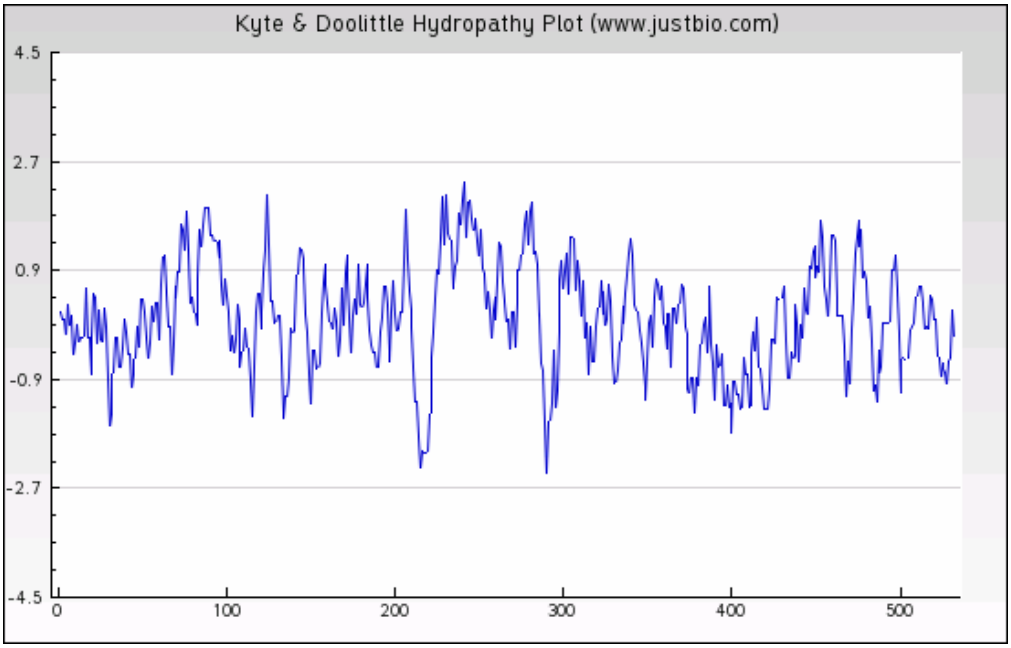


Fig 4.3: Kyte and Doolittle plots for the Ll4CL1 deduced amino acids in window size 9.

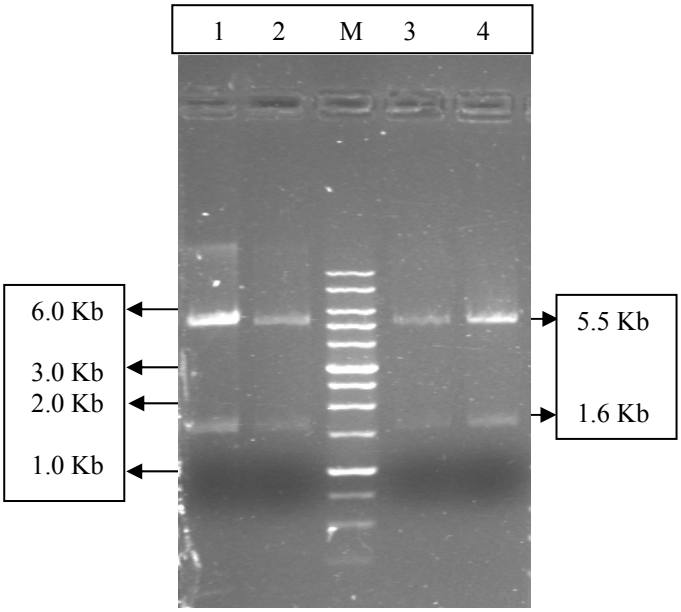


Fig 4.4: Agarose gel picture of *Nde I / Xho I* digested 1.6 Kb Ll4CL1 directionally cloned fragment in pET vector. Lane M: 1 Kb Ladder, Lane-1, 2, 3 & 4 Nde *I / Xho I* digested 1.6 Kb fragment.

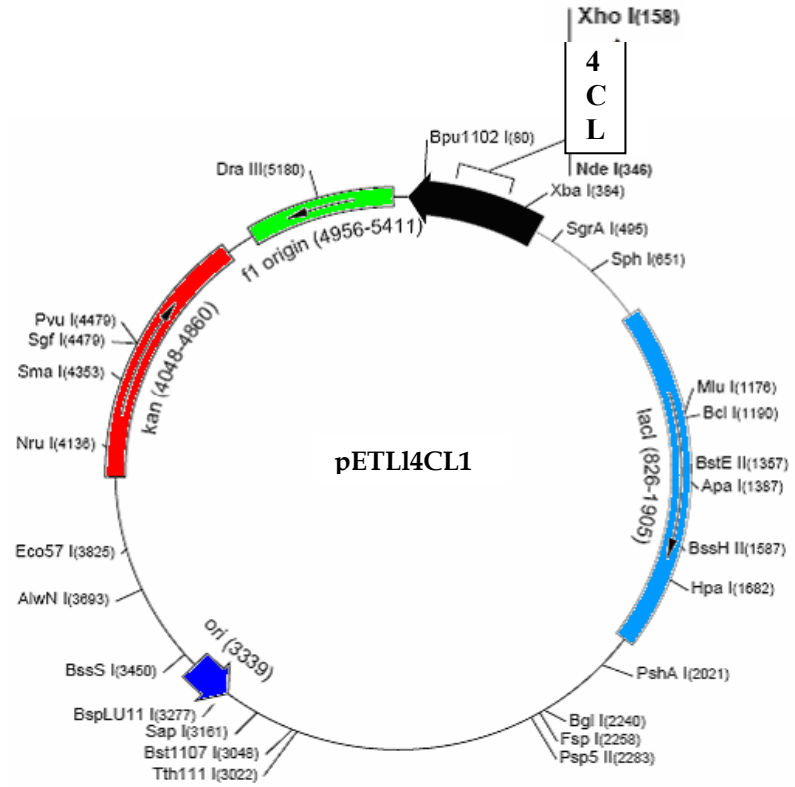


Fig 4.5: Vector map of pETLl4CL1.

The 4CL protein was expressed in BL21 (DE3) cell line of *E. coliI* (Fig: 4.6). The expressed protein with **His•Tag** at its C-terminus was purified from *E.coli* inclusion bodies by using Ni^{+} chelated column (Qiagen, Germany). The protein purity was checked by 12 % SDS PAGE (Fig: 4.7). The Ni^{+} column purified 4CL protein was homogenous and free of other contaminating protein. The Ni-chelated affinity column purified 4CL protein was seen to be of ~ 58 KDa molecular mass (Fig: 4.7). Cloning of alfalfa and tobacco 4CL cDNA in expression vectors, its expression in *E coli* BL21 (DE3) cells and purification through metal ion (Ni^{+2} and Co^{+2}) chelated affinity columns and agarose-glutathione matrix have been reported. The purified protein was then used for raising polyclonal antibodies (Inoue *et al.*, 1998).

Table 4.1: Percentage amino acid composition of 4CL

Amino Acid Comp.		
Aa	**No**	**Mol%**
Ala (A)	**51**	**9.4%**
Arg (R)	**19**	**3.5%**
Asn (N)	**14**	**2.6%**
Asp (D)	**35**	**6.5%**
Cys (C)	**9**	**1.7%**
Gln (Q)	**15**	**2.8%**
Glu (E)	**31**	**5.7%**
Gly (G)	**35**	**6.5%**
His (H)	**12**	**2.2%**
Ile (I)	**43**	**7.9%**
Leu (L)	**52**	**9.6%**
Lys (K)	**35**	**6.5%**
Met (M)	**15**	**2.8%**
Phe (F)	**21**	**3.9%**
Pro (P)	**28**	**5.2%**
Ser (S)	**37**	**6.8%**
Thr (T)	**33**	**6.1%**
Trp (W)	**1**	**0.2%**
Tyr (Y)	**13**	**2.4%**
Val (V)	**43**	**7.9%**
Total:	**542**	**100.00%**

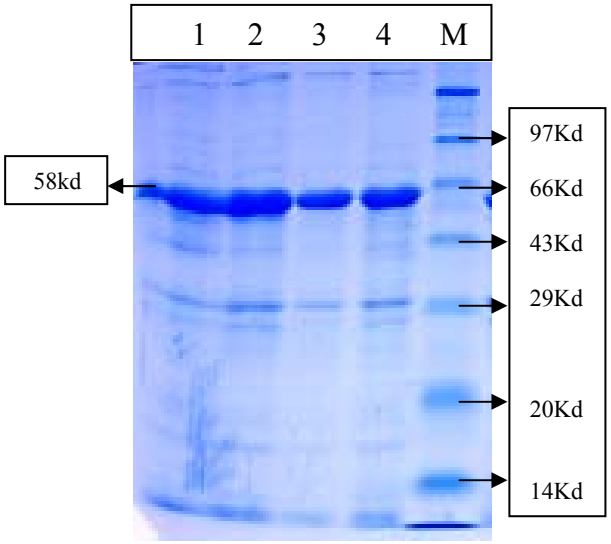


Fig. 4.6: SDS PAGE (12%) of *E. coli* BL21 (pETLl4CL1) induced 4CL protein from inclusion bodies. Lane 1, 2, 3 and 4: ~58Kda induced 4CL protein from inclusion bodies. Lane M: Low molecular weight protein size markers (Bangalore genie)

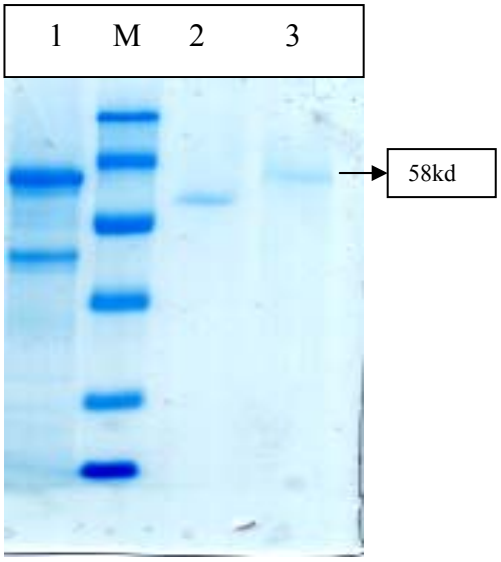


Fig. 4.7: SDS PAGE (12%) of Ni^{+} beads purified 4CL protein from inclusion bodies.Lane 1: ~58Kda induced 4CL protein from inclusion bodies. Lane 3: Ni^{+} beads purified ~58Kda 4CL protein. Lane M: Low molecular weight protein size markers (Bangalore genie)

4.3.2. Purification of polyclonal antibodies

4CL protein purified from recombinant *E. coli* inclusion bodies was used to raise rabbit immune serum. The immune serum was centrifuged at 12,000 g for 15 min

and the clear supernatant heated for 1 h at 55 ºC to deactivate the compliment system. Heat denatured immune serum was again centrifuged and the supernatant transferred to fresh tubes. EDTA was added to a final concentration of 10mM and Thimersol to a final concentration of 0.02 %. The immune serum was aliquoted, frozen in liquid nitrogen and stored at -70 ºC till further use.

The polyclonal IgG was raised against the expressed and purified 4CL protein. The total IgG was purified using agaroseA (Sigma). The IgG was checked for purity on 12 % SDS PAGE. As is obvious from Fig. 4.8, affinity purified IgG show characteristic 44 KDa heavy chain and the 22 KDa light chain peptides. The recovered IgG was also free of other contaminating serum proteins.

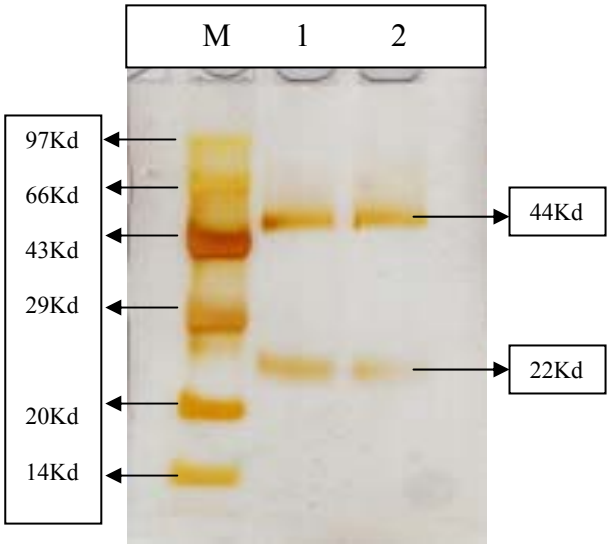


Fig 4.8: 12 % SDS PAGE (Sliver stained) of the purified IgG. Lane 1 and 2: purified IgG heavy chain of 44Kd and Light chain of 22Kd. Lane M: Low molecular weight protein size markers (Bangalore genie).

4.3.3. Histology

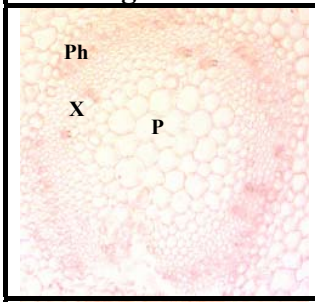
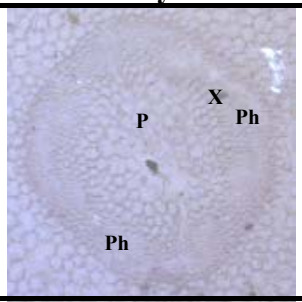
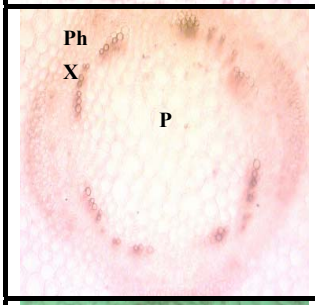
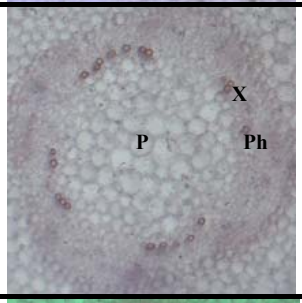
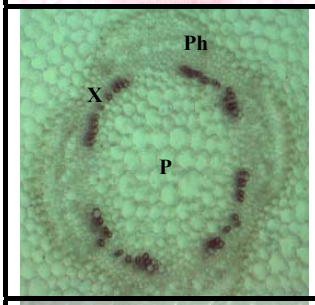
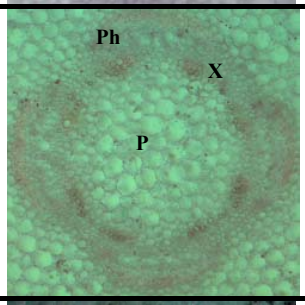
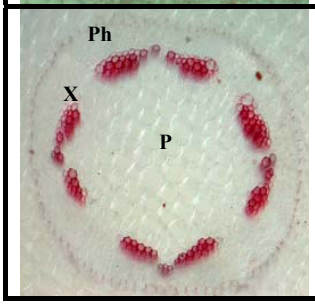
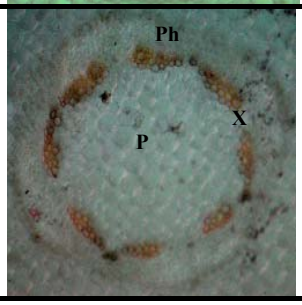
Hand cut transverse sections from the shoot and root of seedlings at 0 day (when the seedling fully emerged from seed coat), 5, 8, 10, 15 and 30 days were used for analysis.

Anatomy of *L. leucocephala* resembles the anatomy of a typical dicot plant (Esau, 1977). The sequential and progressive development of vascular tissue was evident in the transverse sections of different plant parts of 5, 8, 10, 15 and 30 days' old seedlings. Phloroglucinol-HCl staining of the transverse section stained lignified xylem, secondary xylem and phloem fiber in brown red (Figure 4.9 a, b; Left panel). Development of vascular tissue, particularly xylem tissue, was visible in phloroglucinol-HCl stained transverse sections at different growth stage of seedlings. Increase in number of differentiating xylem cells as well as their stain intensity indicated progression of lignification. In older tissue well developed and lignified secondary growth and development of fibers was observed.

4.3.4. Immunocytolocalization

In the present study 4CL was immunolocalized in the transverse sections of shoots and roots of 0, 5, 8, 10, 15 and 30 days old seedling. The purified IgG raised against 4CL protein and anti rabbit goat IgG conjugated with alkaline phophatase were used as primary and secondary antibodies respectively. Results revealed 4CL enzyme expression in differentiating xylem tissue and phloem fibers (Figure 4.9 a, b; Right panel). The presence of 4CL was detected as early as in 0 day old seedling. In transverse sections of 0 day seedling blue black precipitate was visible even in the potential phloem fiber regions. This observation was confirmed when these areas differentiated into fiber as seedlings grew older. Comparison of the sections where 4CL was immunolocalized, with phloroglucinol-HCl stained sections showed that the presence of 4CL was just not restricted at the locations where lignin was stained in the beginning of the development (0 and 5 days sections, Fig. 4.9 a and b), but also in other nearby tissue. At later stage of the development expression of 4CL activity was confined to cell undergoing lignification. No 4CL protein was immunolocalized in cortex and phloem tissue (except in phloem fibers) showing absence of 4CL activity in these regions. No 4CL was detected in pith cells either. As in the present study, 4CL protein immunolocalized in xylem and phloem fiber and its active participation in lignin

biosynthesis has been reported in many plants like Populus (Li *et al.*, 2003), *Zinnia* (Ye and Varner, 1995; Ye *et al.*, 1997), alfalfa (Kersey *et al.*, 1999), poplar (Chen et al., 2000; Zhong et al., 2000; Ye et al., 2001), tobacco (Kajita et al., 1997) and forsythia (Ye *et al.*, 2001).

Left Panel Phloroglucinol stained	**Right panel Immunocytolocalized**	
		**S0**
		**S5**
		**S8**
		**S10**

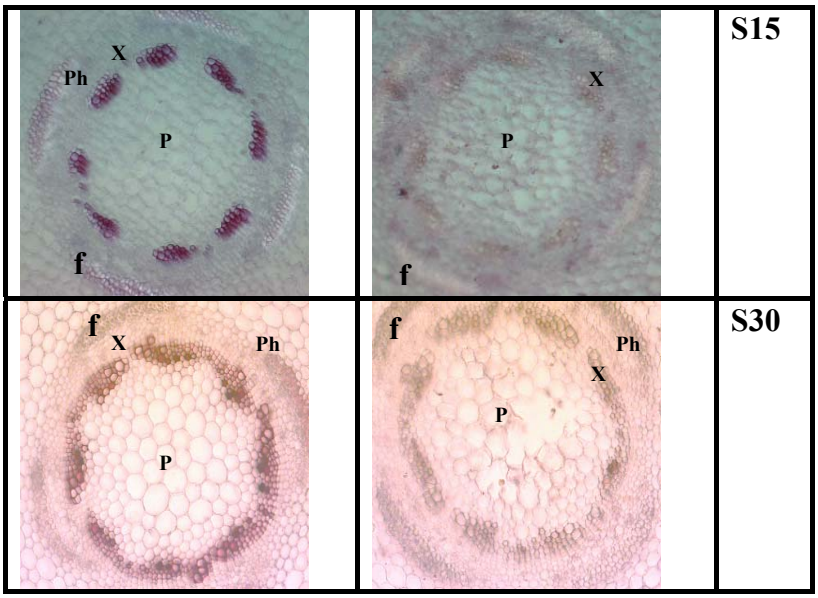
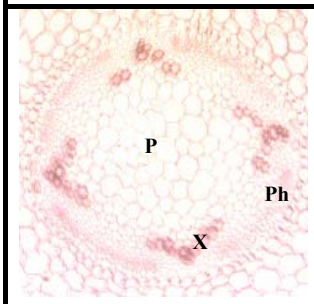
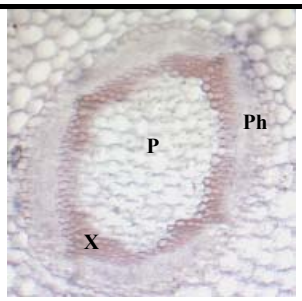


Fig. 4.9 a: Transverse sections of shoot and root of *L. leucocephala*. Left panel are phloroglucinol-HCl stained sections visualized under normal Light and Right panel shows immunocytolocalization of 4CL (20 X magnification). S0 - 0 day seedling shoots (when seedling fully emerges from the seed coat.), S5 - 5 days old seedling shoots, S10 - 10 days old seedling shoots, S15 - 15 days old seedling shoots, S30 - 30 days old seedling shoots. (X- Xylem, Ph- Phloem, f- Pholem fibers and P- Pith).

Left Panel **Phloroglucinol stained**	**Right panel** **Immunocytolocalized**	
		**R5**

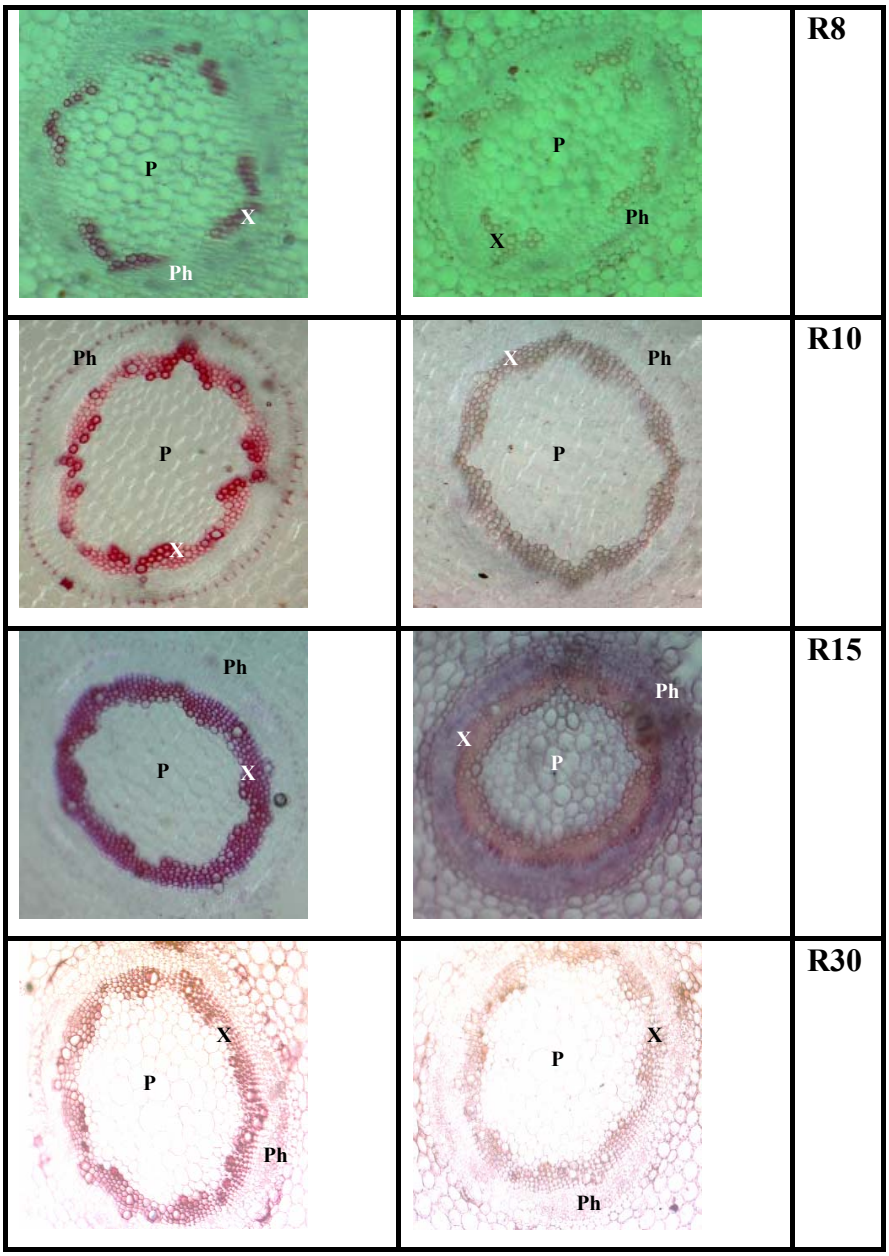


Fig 4.9 b: Transverse sections of root of *L. leucocephala.* Left panel phloroglucinol-HCl stained sections visualized under normal light and Right panel sections immunocytolocalization of 4CL (20 X magnification). R0 - 0 day old seedling root (when seedling fully emerges from the seed coat), R5 - 5 days old seedling root, R10 -10 days old seedling root, R15 -15 days old seedling root, R30 -30 days old seedling root. (X- Xylem, Ph- Phloem and P- Pith).

4. B. Spatial and Temporal Expression of 4-CL Gene in Different Tissues of *Leucaena leucocephala*

4.3.5. Semiquantitative and quantitative real time PCR

The immunocytolocalization of 4CL in different parts of *L. leucocephala* plants at different growth stages showed that 4CL activity was localized to xylem tissue and phloem fibers. The differential expression of 4CL genes during lignification process was assayed by semiquantitative and quantitative real time PCR (QPCR). TaqMan probe based chemistry was used during the study. The nucleotide sequence from highly diverse region of Ll4CL1 was taken when compared to Ll4CL2. the diverse region nucleotide sequence was used to design and synthesize gene specific primers to selectively amplify Ll4CL1 transcripts from a cDNA population (Figs. 4.10 and 4.11). TaqMan probes were also designed and synthesized (Eurogene, Belgium).

ACGTACATATCTGCGTTCTTCCAATGTTCCATATCTATGC*GCTGAACTCCATTTTGC*

sushtaqFF

***TCTG*CTGCATCCG*AGCCGGAGCCGCCATTCTGACGAT*GGGTAAGTACGACATCGCCA**

sushtaqprobe

CGTTGTTGAAGATGATCAA*GACTTACAAGGTGACAATGGCG*TCGTTTGTGCCTCCGA

sushtaqR

TCCTATTAAACATCGTGAAGAGTGAGGAA

Fig. 4.10: Nucleotide sequence (nucleotide position 751 to 950) of Ll4CL1 (FJ205490) gene. Nucleotide sequences highlighted in italics and underlined are regions from where forward primer, probe and reverse primers were designed.

sushtaqF 5′GCTGAACTCCATTTTGCTCTG 3′

sushtaqR 5′CGCCATTGTCACCTTGTAAGTC 3′

sushtaqprobe 5′-6-FAM/AGCCGGAGCCGCCATTCTGACGAT/BHQ-2-3′

Fig. 4.11: sushtaqF and sushtaqR is gene specific forward and reverse primer sequences for Ll4CL1 gene, sushtaqprobe is the TaqMan probe for the 4CL gene.

Total RNA was isolated from shoot, root, leaf and inflorescence of 0, 5, 10, 15 day old seedling, and one and two season old *L. leucocephala* plants. An aliquot of total RNA was used for synthesis of cDNA first strand which was used as template for semiquantitative PCR. 5.8 SrRNA primers were used to normalize and uniform amplification of the RT^{1st} strand cDNA which was used as template for semiquantitative PCR. The primers sushtaqF and sushtaqR were used for semiquantitative PCR of Ll4CL1 transcripts. The amplification obtained from cDNAs of different plant tissues at different growth stages is shown in Fig. 4.12.

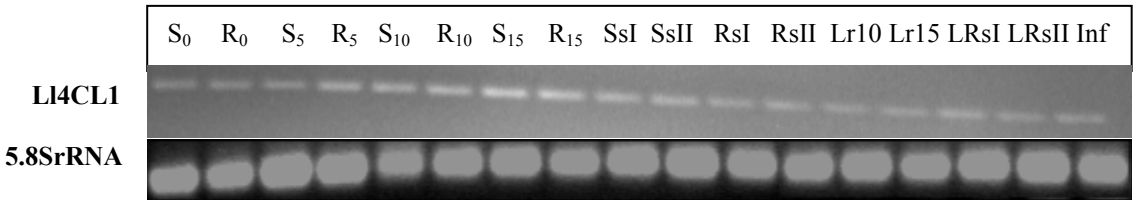


Fig. 4.12: Semiquantitative PCR for Ll4CL1. S, R, Ss, Rs, Lr, LRs and Inf stands for shoot, root, shoot season I, root season I, leaf rachis, leaf rachis season I and inflorescence respectively. The numbers 0, 5, 10, 15 represents age of seedling in days. I and II represents season one and two. 5.8S rRNA was used as internal control.

The Ct values obtained for Ll4CL1 gene are given in table 4.2. The relative expression of the gene in terms of Ct value is shown in Fig. 4.13. The Ct value is inversely proportional to the level of expression. Low gene expression of Ll4CL1 was observed in 15-day shoots, root of one season old plant and leaf rachis of 10-day-old seedling. The expression increased to its maximum level by day 10 in shoots, 5 and 15 day in root. There after the gene expression was more or less similar in all the plants. Similar expression was seen in one-season and two season old plants shoots, however in two season old plants the gene expression levels was slightly more in root. Expression of Ll4CL1 was little higher in the roots of 0 day old seedlings than the shoot of same day old seedlings. Expression level was again higher in 5-day-old roots the than that of shoot of 5day old

seedling. The gene expression of Ll4CL1 was higher in 10- day- old shoot as compared with the root of 10-day-old seedlings but again level of expression was more in root of 15-day-old seedlings as compared to the shoots of 15-day-old seedlings. Almost similar expression was seen in roots from one season and two season old plants. Expression was slightly higher in the leaf rachis of 15-day-old seedlings compared to the leaf rachis of 10-day-old seedlings. The level of expression was very low in shoot of 0 day, shoot of 15 days, I season old root, II season old root, Leaf Rachis 10 day, Leaf Rachis 15 day, Leaf Rachis I season, Leaf Rachis I season and Inflorescence**.**

Table 4.2: Ct values for the Ll4Cl1 gene of different plant tissue at different growth stages:

S.No	Tissue type and Plant age	Ct value
1	Shoot 0 day (S_0)	39.17
2	Shoot 5 day (S_5)	37.30
3	Shoot 10 day (S_{10})	35.72
4	Shoot 15 day (S_{15})	41.60
5	Shoot I season (SsI)	37.46
6	Shoot II season (SsII)	37.39
7	Root 0 day (R_0)	38.77
8	Root 5 day (R_5)	35.92
9	Root 10 day (R_{10})	38.16
10	Root 15 day (R_{15})	34.95
11	Root I season (RsI)	42.03
12	Root II season (RsII)	38.92
13	Leaf Rachis 10 day (LR_{10})	42.35
14	Leaf Rachis 15 day (LR_{15})	40.50
15	Leaf Rachis I season (LRsI)	41.35
16	Leaf Rachis II season (LRsII)	41.03
17	Inflorescence (Inf)	40.71

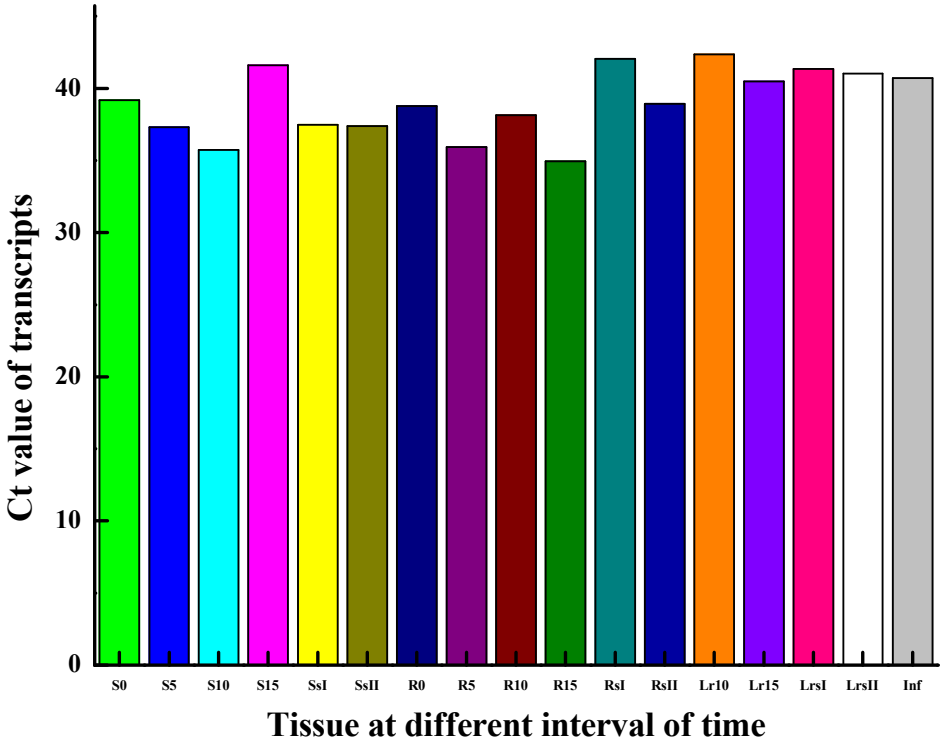


Fig. 4.13: Relative expression of Ll4CL1 in terms of Ct value in different plant tissues of different age plants. On X-axis is tissue type with age of seedling or plant. On Y-axis is quantity in terms of Ct value. S, R, LR, LRs and Inf stands for shoot, root and leaf rachis, leaf rachis with season and inflorescence respectively. The numbers 0, 5, 10, 15 represents age of seedling in days. I and II represents seasons one and two respectively.

4.3.6. Developmental expression of 4CL protein in *L. leucocephala* by ELISA:

25µg of total soluble protein (as estimated by using BSA standard curve) in 100µl of all samples were coated in microtiter ELISA plate and incubated at 37°C for 2hrs. Two more controls were included in the experiment, one was extraction buffer coated directly on wells (buffer control) and the other was standard purified 4Cl protein. The plates were processed as per the protocol mentioned (Chapter 2, section 2.21). The absorbance at 405nm of all unknown plant protein samples were extrapolated on the standard 4Cl curve and concentration of the 4Cl protein in plant protein samples were recorded(Tab: 4.3).

Table 4.3: Calculated Ll4CL1 protein concentration in different plant tissue at different growth stages:

S.No.	Plant Samples	Amount of 4CL protein (ng)	% of 4CL protein
1	Shoot 0 day (S_0)	6.98	0.028
2	Shoot 5 day (S_5)	9.27	0.037
3	Shoot 8 day (S_{10})	5.51	0.022
4	Shoot 12 day (S_{12})	6.53	0.026
5	Shoot 15 day (S_{15})	8.60	0.034
6	Root 5 day (R_5)	4.54	0.018
7	Root 8 day (R_8)	8.03	0.032
8	Root 12 day (R_{12})	5.54	0.022
9	Root 15 day (R_{15})	10.43	0.042

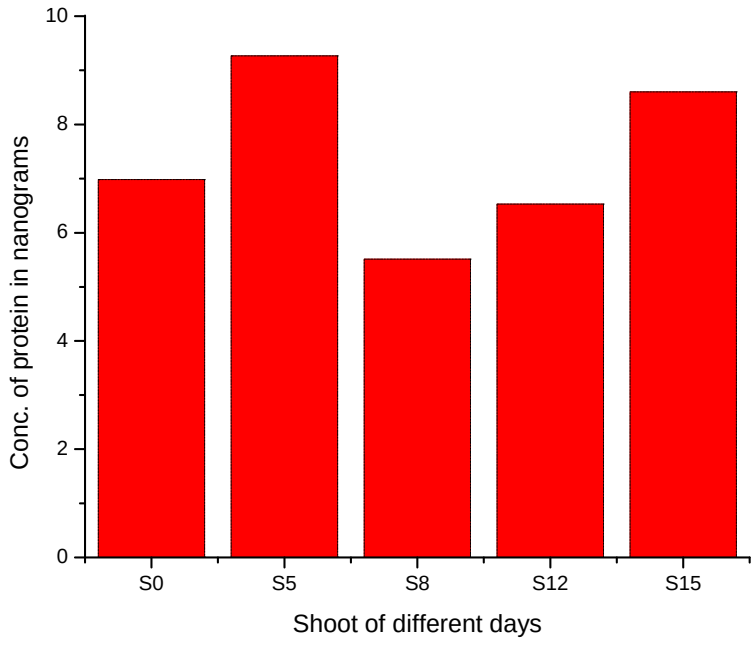


Fig 4.14: Relative expression of 4CL in terms of concentration in shoots tissues of different age plants. X-axis is tissue type with age of seedling or plant. Y-axis is 4CL protein quantity in terms of nanograms. S stands for shoot. The numbers 0, 5, 8, 12, and 15 represents age of seedling in days.

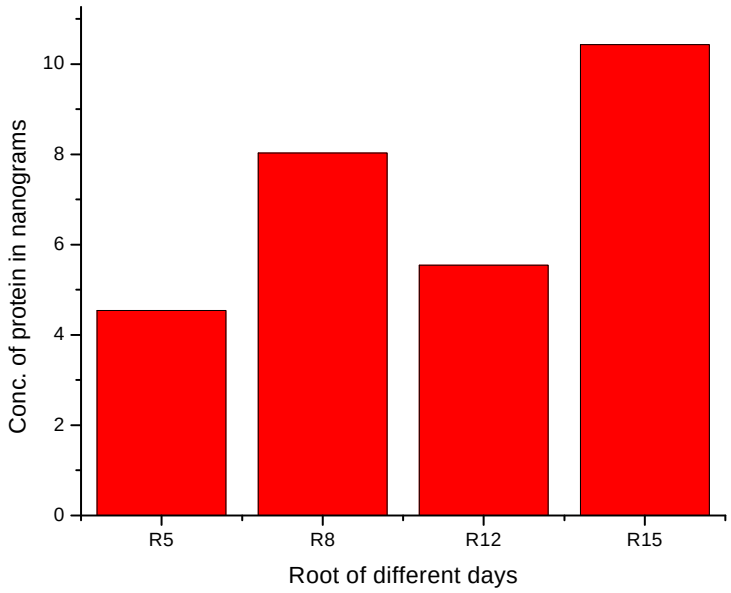


Fig 4.15: Relative expression of 4CL in terms of concentration in root tissues of different age plants. X-axis is tissue type with age of seedling or plant. Y-axis is 4CL protein quantity in terms of nanograms. R stands for root. The numbers 0, 5, 8, 12, and 15 represents age of seedling in days.

The calculated 4CL protein concentration in different plant tissue at different growth stages is given in table 4.3. The relative expression of the 4CL protein in terms of concentration (ng) is shown in Fig. 4.14 and 4.15. The expression of 4CL protein in shoot at different days varies as it is clear from the table 4.3 and fig 4.14 that the expression of 4CL protein was maximum in 5th day followed by 15th day and minimum expression was observed in 8th day. There was gradual increase in the expression after 8th day. The expression of 4CL protein in root at different days varies as it is clear from the table 4.3 and fig. 4.15 that the expression was maximum in 15th day followed by root of 10th day. Minimum expression was found in the root of 5th day. The data could not be correlated with the real time data, the probable reason would be that the 4CL gene exists in multi gene family and the polyclonal antibody generated from total protein not from some Ll4CL1 specific epitope so this polyclonal antibody may binds to other 4CL isoenzymes. Thus data could not be correlated with the real time data.

4.4. Conclusions

- Primers were designed from the start and the end of Ll4CL1 with restriction sites compatible to clone in MCS of pET 30 b expression vector.
- Ll4CL1 was amplified using designed primers and cloned in pET 30b vector.
- Ll4CL1 gene was expressed in *E. coli* BL21 (DE3) and 58.00KDa protein purified from inclusion bodies using Ni-NTA affinity column.
- Polyclonal antibodies were raised against purified recombinant 4CL protein in rabbit.
- Polyclonal IgG were purified using Agarose A affinity matrix.
- Transverse sections of different plant parts at different age stained with phloroglucinol-HCL show increase in number of differentiating xylem cells as well as their stain intensity indicating progression of lignification with age.
- 4CL immunolocalized in xylem and fibers suggesting its presence at the sites of extensive lignifications along with other tissues.
- Semiquantitative and QPCR results showed that the Ll4CL1 gene expression was differed in spatio-temporal manner.
- ELISA results also showed that the 4CL protein expressed differentially in spatio-temporal manner.

Chapter 5

5. Transformation of *Leucaena leucocephala* with 4CL Gene and its Analysis

This chapter includes the different strategies used to transform the plant. The plant transformation vector pCAMBIA1301 (harbouring the 4CL gene in antisense orientation and genes for *GUS* (marker gene)) were used for the study. Three different strategies i.e. *Agrobacterium* mediated, particle bombardment and co-cultivation is described in detail in this chapter. Evaluation and analysis of putative transformants and confirmation of integration of these genes in *L. leucocephala* genome by GUS assay and by molecular techniques like PCR, DNA sequencing and Slot blot.

5.1. Introduction (General aspects of genetic transformation of trees)

The genetic transformation protocols based on *Agrobacterium*-mediated and/or direct gene transfers by biolistic bombardment have been successfully applied for numerous woody angiosperm species (Merkle & Nairn, 2005), including *Populus* and *Betula.* The introduction of transgenes have included both sense and antisense strategies (referring to the orientation of the introduced gene into the plant genome) (Strauss *et al.,* 1995; Baucher *et al.,* 1998) and RNAi technology (Merkle & Nairn, 2005). In the antisense strategy, duplex formation between the antisense transgene and the endogenous gene transcripts is proposed to induce the degradation of duplexes and, correspondingly, lead to suppressed gene expression (Strauss *et al.,* 1995). The sense strategy was originally targeted for over-expression of the genes but, as originally observed through the introduction of chalcone synthase transgene into petunia (Napoli *et al.,* 1990), the sense strategy may also lead to silencing (down-regulation) of both the endogene and the transgene due to co-suppression (i.e. post-transcriptional gene silencing, PTGS). The molecular mechanism of the gene silencing was unclear for long time until the discovery of RNA interference (RNAi) (Yu & Kumar, 2003: Matthew, 2004; Chen, 2005; Bonnet *et al.,* 2006; Zhang *et al.,* 2006). In the RNAi silencing process, the transgene gives rise to long double-stranded (ds) RNA molecules, which are enzymatically cleaved into very small pieces of RNA (21 nt), referred to as small interfering RNAs (siRNAs). siRNAs are then incorporated in an RNA

silencing system (RISC: RNA induced silencing complex) which is able to recognize, bind and induce cleavage or translation repression of complementary mRNAs (Bonnet *et al.,* 2006; Zhang *et al.,* 2006). The RNAi technique is currently being applied for the efficient production of down-regulated or knock-out plants (Wesley *et al.,* 2001), e.g. in genetic transformation of *Betula pendula* for achieving sterility (Lannenpaa, 2005).

Plants are genetically engineered by introducing gene(s) into plant cells that are growing *in vitro* or *ex vitro*. The development of transgenic plants is based on the stable insertion of foreign DNA into the plant genome, regeneration of these transformants to produce the whole plant and expression of the introduced gene(s). *Agrobacterium*-mediated transformation has provided a reliable means of producing transgenics in a wide variety of plant species that can be cultured and regenerated *in vitro*. Recently, some plants such as *Arabidopsis thaliana* have also been transformed by *Agrobacterium*-mediated transformation by dipping the young buds of flowers of *ex vitro* grown plants (Rakoczy-Trojanowska, 2002). This method is known as infiltration or, in general, *in planta* transformation. Other methods of gene-transfer systems include particle bombardment, electroporation and membrane permeabilization using chemicals. Of these, particle bombardment has proved to be successful with plants that are less sensitive to *Agrobacterium* infection, such as cereals and legumes (Walden & Wingender , 1995). However, recently, *Agrobacterium*-mediated transformation has become the method of choice for these plants (Nadolska-Orcyzyk, Orczyk & Przetakiewicz, 2000). The development and optimization of several regeneration protocols, efficient vector constructs and availability of defined selectable marker genes and different methods of transformation have resulted in the production of transgenic plants in more than 100 plant species (Babu *et al.*, 2003; Wimmer, 2003). These transgenic plants include many important crops, fruits and forest plants. The plant transformation technology is not only used to improve plants but also a versatile platform for studying gene function in plants. Plant genetic transformation technology has a great potential in increasing productivity through enhancing resistance to diseases, pests and environmental stresses and by qualitative changes such as chemical composition of the plants. Plants can also be used for high

volume production of pharmaceuticals, nutraceuticals and other beneficial chemicals. Transgenic plants might be used as drug delivery devices, with vaccines being synthesised in plants (Hansen & Wright, 1999). Many plant species previously considered to be recalcitrant to transformation, with advances in tissue culture combined with improvements in transformation technology, have now been transformed.

5.1.1. *Agrobacterium* mediated plant transformation

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

5.1.2. Biology and life cycle of *Agrobacterium tumefaciens*

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

5.1.3. Infection process

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

These plant hormones upset the normal balance of cell division leading to the production of galls. Opines are unique aminoacid derivatives and the agrocinopines are unique phosphorylated sugar derivatives. All these compounds can be used by the bacterium as the sole carbon and energy source.

5.1.4. Markers for Plant Transformation

5.1.4.1. Selectable markers

Genes conferring resistance to antibiotics like *neomycin phosphotransferase* II (nptII) (Baribault *et al.,* 1989), *hygromycin phosphotransferase (hpt)* (Le Gall *et al.,* 1994), *phosphinothricin acetyl transferase / bialaphos resistance (pat/bar*) (Perl *et al.,* 1996) are being used to select transgenic cells. Another selectable marker gene, phosphomanoisomerase(pmi), which catalyzes mannose-6-phosphate to fructose-6-phosphate, an intermediate of glycolysis that positively supports growth of transformed cells, is also recently being used. Mannose absorbed by the plant cells converts into mannose-6- phosphate, an inhibitor of glycolysis, inhibits growth and development of nontransformed cells. Transformed cells having PMI gene can utilize mannose as a carbon source.

5.1.4.2. Screenable markers

The oncogenes of *Agrobacterium* are replaced by reporter / screenable marker genes like *β-glucuronidase* gene (*gus*) (Baribault *et al.,* 1990), *luciferase (luc)* gene for analyzing gene expression. Since the first demonstration of the *green fluorescent protein (gfp)* gene from jellyfish *Aequorea victiria* as a marker gene (Chalfie *et al.,* 1994), *gfp* has attracted increasing interest and is considered advantageous over other visual marker genes. Unlike other reporter proteins, GFP expression can be monitored in living cells and tissues in a non-destructive manner. This gene has been used as a visible reporter gene in genetic transformation of both monocots and dicots (Haseloff *et al*., 1997; Reichel *et al*., 1996; Kaeppler and Carlson, 2000). The fluorescence emission of GFP only requires the excitation of living cells by UV or blue light (390 nm strong absorption and 470 nm week absorption), which results from an internal p-hydroxybenzylideneimidazolinine fluorophore generated by an autocatalytic cyclization and oxidation of a ser-gly sequence at aminoacid residues. The other advantage of *gfp* as a reporter gene is that no exogenously supplied substrate/ cofactors are needed for its fluorescence emission at 508 nm.

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

5.1.5. Genetic Transformation of Plants with 4CL Gene(s)

Genetic and biochemical functions of 4-Coumarate Coenzyme A ligase (*4CL*) genes have been clearly demonstrated in association with monolignol biosynthesis (Lewis and Yamamoto, 1990; Lee *et al.,* 1997; Hu *et al.,* 1998, 1999; Harding *et al.,* 2002). As it provides precursor molecule for lignin biosynthesis pathway thus directly regulate the total carbon flow towards the pathway to regulate the total monolignols biosynthesis. Genetic manipulation of 4CL could be a promising strategy for reducing lignin content to improve wood-pulp production efficiency. Some of the most drastic changes in lignin quantity have been seen in transgenic

trees with modified expression of the 4CL (4-coumarate: coenzyme A ligase). Transgenic plants with reduced 4CL activity have been produced in tobacco (Kajita *et al*., 1996, 1997), *Arabidopsis* (Lee *et al*., 1997), and aspen (Hu *et al*., 1999; Li *et al*., 2003). In tobacco, reduction of 4CL by over 90% resulted in 25% less lignin. In poplar and *Arabidopsis* with a >90% reduced 4CL activity, lignin content was reduced by 45–50%. In tobacco, the low 4CL activity was associated with browning of the xylem tissue (Kajita *et al*., 1996). The monomeric composition of lignin was altered and characterized by a 3-fold increase in the amount of *p*-hydroxybenzaldehyde and an 80% and a 67% decrease in the amount of syringaldehyde (Syr) and vanillin (Van), respectively, resulting in a 40% reduction in the Syr/Van ratio (Kajita *et al*., 1997). The amount of the ester- and ether-linked *p*-coumaric, ferulic, and sinapic acids increased dramatically in the brown xylem tissue (as determined by alkaline hydrolysis of the cell walls followed by gas chromatography and NMR of milled wood lignin). In contrast, in transgenic *Arabidopsis,* the Sy/V (Sy is the sum of syringaldehyde and syringic acid and V is the sum of vanillin and vanillic acid) ratio was increased because of a 40% reduction in the amount of V units, suggesting that 4CL is required for the synthesis of G, but not S units, as postulated (Lee *et al*., 1997; and Hu *et al*., 1998). In transgenic aspen down-regulated for 4CL, Hu *et al*. (1999) also detected an increase in nonlignin alkali-extractable wall-bound phenolics (*p*-coumaric acid, caffeic acid, and sinapic acid), and showed by NMR that these acids were not incorporated into the lignin polymer. However, they did not detect any difference in lignin S/G composition using thioacidolysisi, in contrast to the data obtained for *Arabidopsis* and tobacco. Another discrepancy between the results published by Kajita *et al*., (1997) and Hu *et al*., (1999) is that the transgenic tobacco lines with the most severe reduction in lignin content (25%) were characterized by a collapse of vessel cell walls and reduced growth (Kajita *et al*., 1997), whereas the transgenic poplars with a 45% reduction in lignin content had a normal cell morphology and a higher growth rate than the control (Hu *et al*., 1999). However, the increased growth was probably due to pleiotropic effects caused by the constitutive down-regulation of 4CL governed by the CaMV35S promoter, because it was not observed in the transgenic aspen reported by Li *et al*., (2003), in which the antisense *Pt4CL* was under the control of an aspen xylem-specific

promoter, *Pt4CL1P*. The increased level of hydroxycinnamic acids as non-lignin cell wall constituents has been suggested to contribute to the cell wall strength in transgenic poplar (Hu *et al*., 1999). Because several 4CL isozymes exist with different cell-specific expression, down-regulation of several or all isozymes simultaneously may perturb metabolite levels other than those involved in lignin, with a secondary effect on growth as a consequence. Interestingly, antisense inhibition of 4CL in aspen trees led to a 15% increase in cellulose content. These results suggest that lignin and cellulose deposition are regulated in a compensatory fashion and that a reduced carbon flow toward phenylpropanoid biosynthesis increases the availability of carbon for cellulose biosynthesis (Hu *et al*., 1999; Li *et al*., 2003). A combinatorial down-regulation of 4CL along with an overexpression of F5H in xylem has been achieved by co-transformation of two *Agrobacterium* strains in aspen (Li *et al*., 2003). Additive effects of independent transformation were observed, in particular a 52% reduction in lignin content associated with a proportional increase in cellulose and a higher S/G ratio. These results show that stacking transgenes allows several beneficial traits to be improved in a single transformation step (Halpin & Boerjan, 2003).

In the present study antisense construct of the partial fragment of Ll4CL1 gene was transferred in to model plant system *Nicotiana tobacum* and *Leucaena leucocephala.* Transfromants were analyzed using PCR, GUS assay and slot blot.

5.2. Materials and methods

5.2.1. Explant

Leaf discs of Tobacco (*N. tabaccum* var. Anand 119) were used as the ex plant for the *Agrobacterium*-mediated transformation. Seeds of *Leucaena,* imbibed in distilled water after the treatment with conc. sulphuric acid (7 min) and mercuric chloride (0.1 % for 10 min), were used as source of embryo axes. Embryo axes excised from the seeds and inoculated on regeneration medium (half strength MS+ TDZ (0.5 mg/L)) were used as the target material for the *Agrobacterium*-mediated transformation and particle bombardment mediated transformation.

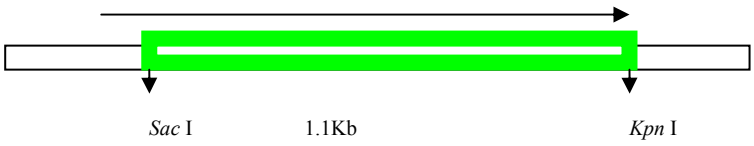
5.2.2. *Agrobacterium* strain and plasmids

Agrobacterium tumefaciens strain GV2260 was used. The strain carried plasmid pCAMBIA1301, a binary vector harboring partial Ll4CL1 gene in antisense orientation under the control of a constitutive promoter CaMV35S and a plant and bacterial selectable marker gene '*hygromycin phosphotransferase (hpt)*' responsible for hygromycin resistance in T-DNA region.

5.2.3. Construction of the vector

The Ll4CL1 gene has internal site for *Kpn*I and *Sac*I, thus Ll4CL1 was double digested using these two enzymes. The released fragment was eluted from agarose gel and was cloned in *Kpn*I and *Sac*I digested pCAMBIA 1300 MCS (Fig. 5.1). The right and left hand T-border of pCAMBIA1300 vector harbours the hygromycin gene (selectable marker) and multiple cloning sites. This vector does not have any reporter gene thus the transformants using this vector can not be analyzed by reporter gene. Selection of the transformants can not be done at the beginning of the transformation and failure of the experiment would be noticed at later stage. To avoid these short comings, pCAMBIA1301 vector was used for transformation. The right and left hand T-border of pCAMBIA1301 vector harbours the hygromycin gene (selectable marker), multiple cloning sites and GUS (reporter gene) with axon and introns. The MCS of pCAMBIA1301 does not have any promoter and terminator to drive and stop the gene respectively. *Eco*RI and *Hin*dIII restriction site are present either site of MCS of pCAMBIA1301 and same sites are also there in pCAMBIA1300 just before the 35S promoter and after the nos terminator, thus pCAMBIA1300 was double digested using *Eco*RI and *Hind*III enzymes. The digested cassette was eluted from agarose gel and cloned in *Eco*RI and *Hind*III digested pCAMBIA1301 vector (Fig. 5.2).

(a) Ll4CL1 gene



(b) MCS of pCAMBIA 1300

(c) Vector map of pCAMBIA 1300

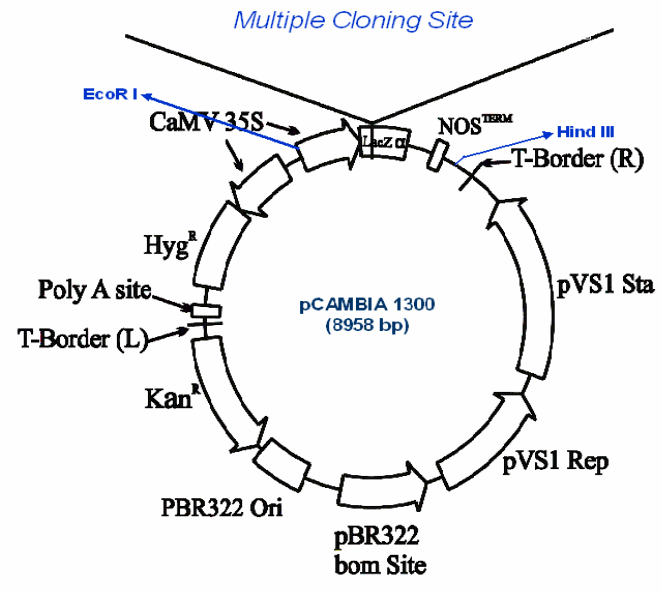
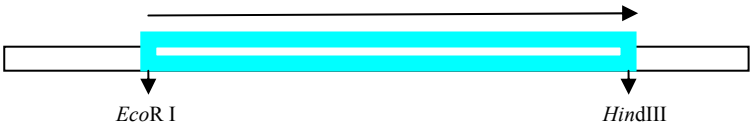
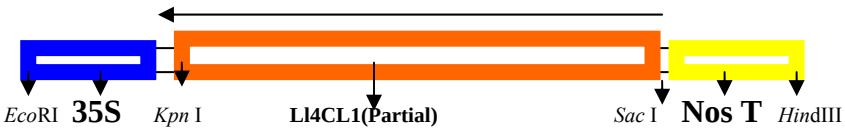


Fig:5.1 Topographical representation of (a) Full length Ll4CL1 gene with restriction sites, (b) MCS of pCAMBIA1300 along with 35S promoter and Nos terminator, (c) Vector map of pCAMBIA 1300**.**

(a) MCS of pCAMBIA1301



(b) Cassette in pCAMBIA1300



(c) Constructed vector map of pCAMBIA1301

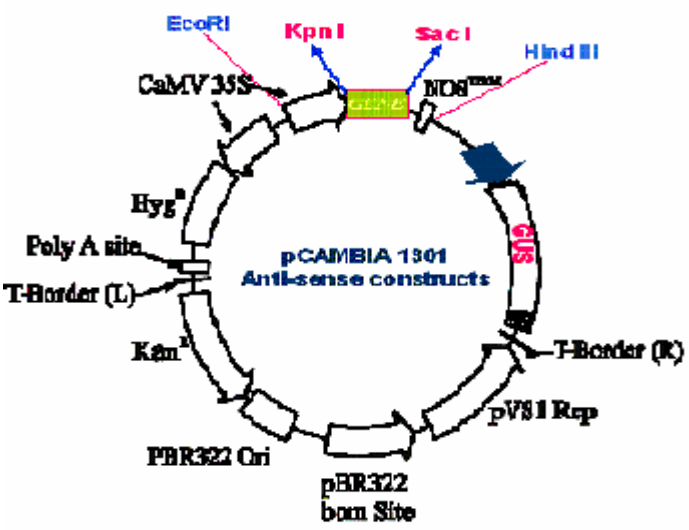


Fig 5.2: Topographical representation of (a) MCS of pCAMBIA1301 without 35S promoter and Nos terminator (b) Cassette of pCAMBIA1300 harbouring 35S promoter, Partial antisense Ll4CL1 gene and Nos terminator, (c) Constructed vector map of pCAMBIA1301.

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

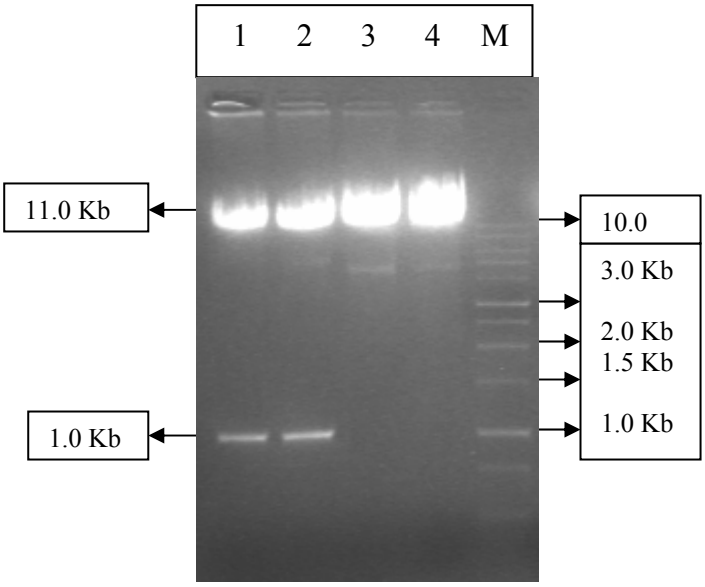


Fig: 5.3: *Kpn*I/*Sac*I digested 1.0 kb fragment of Ll4CL1 gene from pCAMBIA1301 vector. Lane M: 1 Kb Ladder, Lane-1& 2: *Kpn I/ Sac I* digested 1.1 Kb cloned fragment of Ll4CL1 gene. Lane 3: *Kpn*I digested constructed vector, Lane 4: *Sac*I digested constructed vector.

5.2.4. *Agrobacterium tumefaciens* transformation

A. tumefaciens GV2260 was transformed with the pCAMBIA1301 vectors harboring 5' 35SPro-AntiLl4CL1- NOS 3' cassette (Chapter 2; section 2.9.5).

5.2.5. *Agrobacterium* mediated transformation of tobacco

Tobacco plants were transformed independently using the above *A. tumefaciens c*ultures, harboring the 5' 35SPro-AntiLl4CL1- NOS 3' cassette vectors (Chapter 2; section 2.17).

5.2.6. Genomic DNA extraction and polymerase chain reaction

Genomic DNA was extracted from plant leaves and PCR reactions set up as described earlier (Chapter 2; section 2.10.3 and 2.10.12.3).

5.2.7. GUS histochemical assay

GUS histochemical assay (Chapter 2; section 2.19) was performed on bombarded embryo axes of *L. leucocephala*, leaves of putative transformants selected on hygromycin and *Agrobacterium* mediated transformed *Nicotiana* leaves.

5.3. Results and discussion

In a preliminary study conducted to find out the optimum concentration of cefotaxime for the control of *Agrobacterium* contamination after co-cultivation, it was observed that cefotaxime at a minimum concentration of 250 mg/l could control the growth of *Agrobacterium* completely. Cell density of the *Agrobacterium* strain carrying 5' 35SPro-AntiLl4CL1- NOS 3' plotted against time showed a typical growth with lag phase up to 4 h followed by log phase up to 16 h with intense cell division. After this, the curve became stationary and later started declining indicating mortality of the bacterium as per the growth curve, *Agrobacterium* culture during the log phase (4–16 h old) was used for transformation studies.

5.3.1. Antibiotic sensitivity

In hygromycin free treatment, freshly excised embryo axes from *Leucaena* seeds showed normal proliferation, growth and germination (Fig: 5.4 a). There was a gradual increase in necrosis of the embryo axis with the increase in hygromycin

concentration from 2 to 40mg/L. LD_{50} for hygromycin was observed at a concentration 10 mg/L showing necrosis and death of the 50% of the inoculated embryos(Fig: 5.4 b). Complete necrosis and mortality (100%) was observed at a minimum concentration of 15 mg/L. Explants showing callusing, germination and further proliferation became brownish and necrotic at later stages in most of the hygromycin treatments.

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

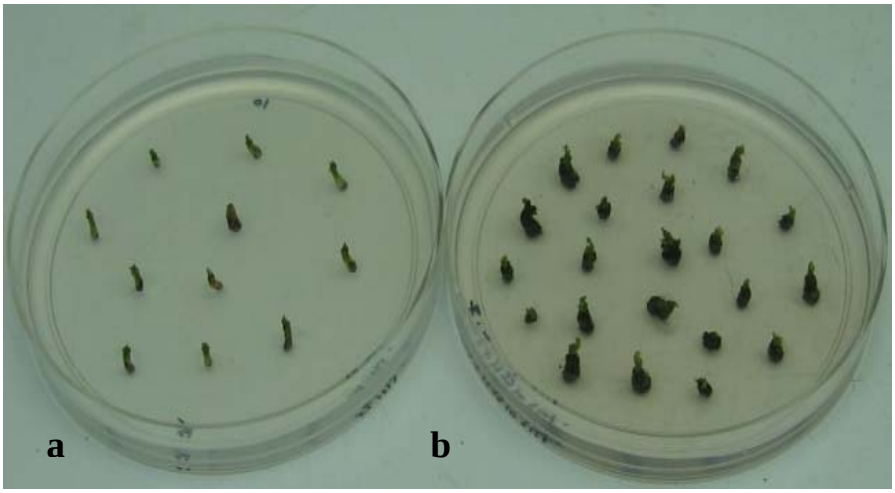


Fig: 5.4. Antibiotic sensitivity of embryo axes of *L. leucocephala*. (a) Embryo axes cultured in hygromycin (0 mg/l) (b) embryo axes cultured in hygromycin (10 µg /ml).

5.3.2. *Tobacco* transformation

Tobacco (*N. tabaccum* var. Anand 119) leaf discs were transformed separately with *A. tumefaciens* cultures harboring the partial Ll4CL1 gene in antisense orientation along with 35S promoter and Nos terminator in pCAMBIA1301 vector. Shoots induction started after 2 weeks under selection pressure (Hygromycin 3 mg/L) from the cut surface of the leaf disc and shoot (Fig. 5.5 a). Proliferation of induced shoot started and noticed after 4 week (Fig. 5.5 b,c). The regenerants were allowed to grow for 12 weeks and then shifted to root induction

medium. Roots were initiated within 2 weeks of shifting (Fig. 5.5 d). The transformed plants were kept for hardening with continuous supply of light for four weeks (Fig. 5.5 e) and then transferred to pots (Fig. 5.5 f).

5.3.3. Integration of antiLl4CL1 gene, and GUS in tobacco genome

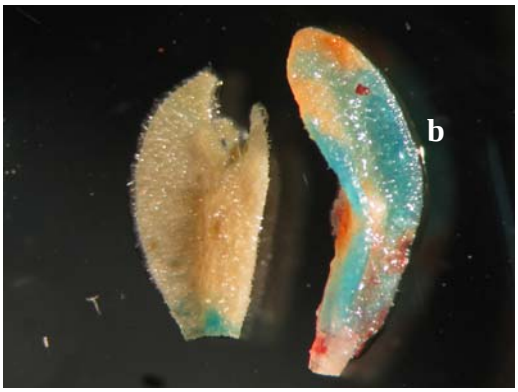
The putative transformed plants were further analyzed for integration of the gene. The first step to analyze the plants was the GUS assay, GUS assay was performed using tender leaves taken from the non transformed and transformed plant. Non transformed plants were taken as control plant. Blue color (Fig 5.6, b) was observed in most of the transformed leaves of the tobacco plant and no color was observed in non-transformed leaves (Fig 5.6, a).The transformed plant were compared with the non transformed plant after 8 week of transfer into the green house and following differences were observed.

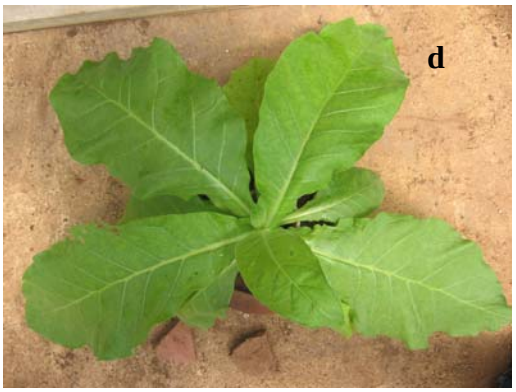
- Transformed plants were more clustered (Fig 5.6 d) as compared to normal tobacco palnt (Fig 5.6 c).
- The leaves were wider (17cm) and lengthier (40cm) in transformed plant (Fig 5.6 f) as compared to non transformed plant (9cm & 30cm Fig 5.6 e).
- Stunted growth was observed in transformed plant when compared with non transformed plant (Fig 5.6 i). Internodal distance was 1/3rd in transformed plant (Fig 5.5 h) as compared to non transformed plant (Fig 5.6 g).

5.3.3.1. Analysis of integration of gene in transgenic tobacco through PCR

The right and left hand border of pCAMBIA1301 harbours 35 S promoter, a part of Ll4CL1 gene in antisense orientation, hygromycin gene and GUS gene. If the tobacco plants were transformed then these genes would have been integrated somewhere in to the tobacco genome. To authenticate the transformed tobacco plant we exploited this integration of different genes in the tobacco genome through PCR.

Fig 5.5: Putative transformed tobacco shoots regenerated *in vitro* on selection medium. Shoot bud induction after 2 weeks (a), proliferation of shoot bud (b), shoots after 8 weeks (c), shoots transferred to root inducing media (d), regenerated plant kept for hardening (e) and transferred plant in green house (f).





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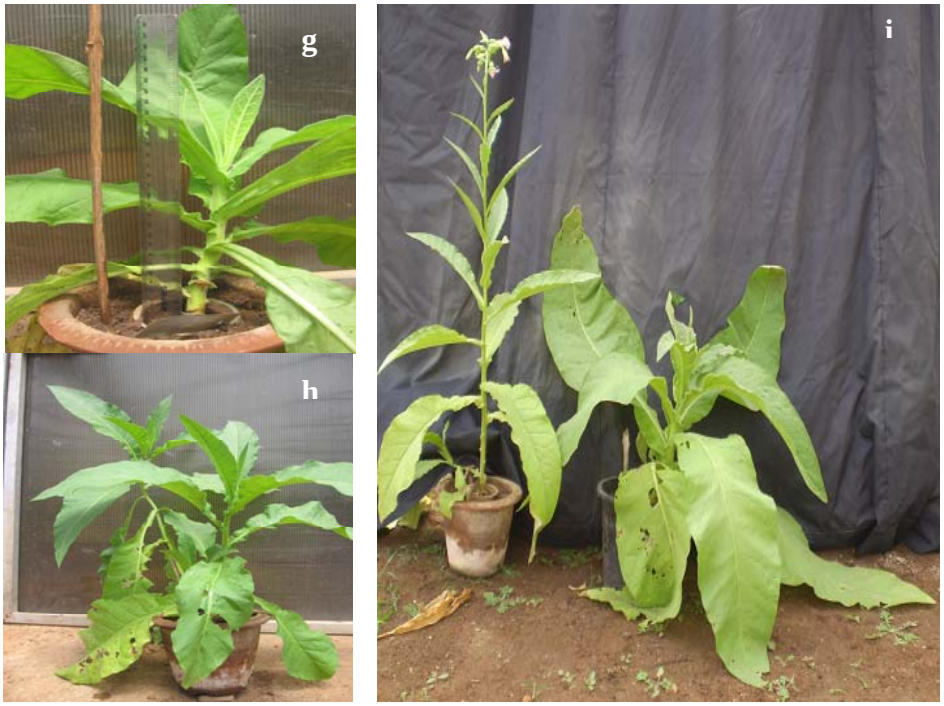

Fig 5.6: Comparision of transgenic tobacco plant with control tobacco plant. GUS assay of non transformed leaves (a) and transformed leaves (b), view of non transformed(c) and transformed (d) plant from top, leaf of non transformed (e) and transformed (f), non transformed (g) and transformed (h) tobacco plant. Normal grown (i) non transformed (Tall plant) and transformed plant (stunted growth).

5.3.3.1.1. Integration of hygromycin gene in transgenic tobacco genome

Genomic DNA was isolated from nontransformed control *Nicotiana* plant and from several transformation events of antiLL4CL1 tobacco plants. Approximately 50ng gDNA was used as template for PCR based amplification of the hygromycin gene using gene specific forward (HygAF) and reverse (HygAR) primers.

Forward primer HygAF 5'ATTTGTGTACGCCCGACAGT 3'
Reverse primer HygAR 5'GGCGAAGAATCTCGTGCTTTC 3'

A fragment of ~800bp was amplified using genomic DNA as templates. There was no amplification from the nontransformed tobacco plant (Figure 5.7). The amplicons were gel eluted, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequence of the amplicons was confirmed to be same as that of the hygromycin gene.

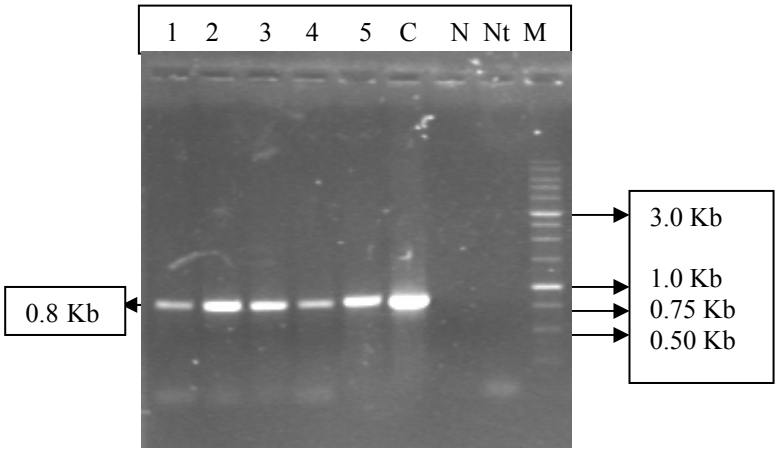

Fig 5.7: PCR amplification of hygromycin gene from transformed tobacco plant. Lane 1, 2, 3, 4 & 5 PCR amplified 0.8 Kb hygromycing gene from transgenic plant (*Tobacco*). Lane C Positive control using constructed pCAMBIA1301 vector. Lane N non transformed plant. Lane Nt No template control. M 1 Kb ladder

5.3.3.1.2. Integration of 35S promoter region in transgenic tobacco genome

PCR amplifications from the genomic DNA of the transformation events antiLl4CL1 with 35S forward and 35S reverse primer of 35S promoter.

35SF 5′ ACAGTCTCAGAAGACCAAAGGGCT 3′
35SR 5′ AGTGGGATTGTGCGTCATCCCTTA 3′

A fragment of ~ 0.30 kb was amplified from the genomic DNA used as templates. There was no amplification from the untransformed tobacco plant (Figure 5.8). The amplicons were gel eluted, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequence of the amplicons was confirmed to be that of 35S promoter.

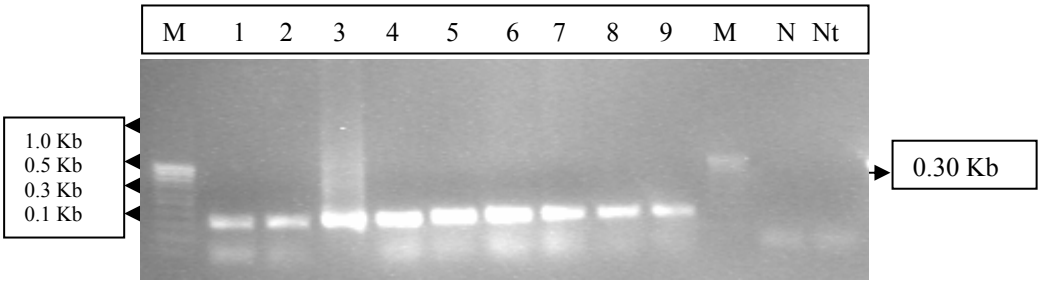

Fig 5.8: PCR amplification of 0.30 Kb of 35S promoter using 35SF and 35SR from transgenic tobacco plant. Lane 1 – 9: PCR amplified 0.30 Kb 35S promoter region from transgenic Plant (tobacco), Lane M: 100 bp ladder, Lane N: non transformed plant, Lane Nt: No template control.

5.3.3.1.3. Integration of Ll4CL1 gene in antisense orientation in transgenic tobacco genome

PCR amplifications from the genomic DNA of the transformation events antiLl4CL1 with forward primer of 35S promoter and forward primer from the mid region of Ll4CL1 gene sequences (this forward primer from LL4CL1 gene was used as reverse primer as the Ll4CL1 gene was in antisense orientation).

35SF 5' ACAGTCTCAGAAGACCAAAGGGCT 3'
Lec5F 5' GGATTTCGCTGACAAACGTGG 3'

A fragment of ~ 1.2 kb was amplified from the genomic DNA used as templates. There was no amplification from the non transformed (N) tobacco plant (Figure 5.9). The amplicons were gel eluted, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequence of the amplicons was confirmed to be that of 35S promoter and a part of Ll4CL1 gene.

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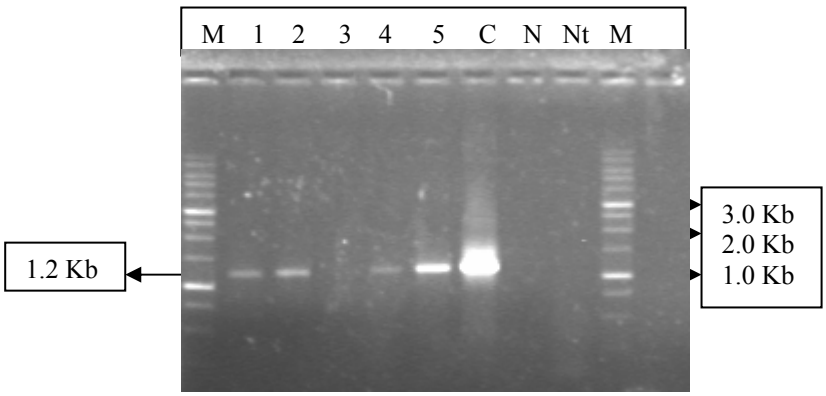

Fig 5.9: PCR amplification of 1.2 Kb 35S promoter and a part of Ll4CL1 gene from transgenic plant (*Tobacco*). Lane 1, 2, 3, 4 & 5: PCR amplified 1.2 Kb 35S promoter and a part of Ll4CL1 gene from transgenic plant (tobacco), Lane C: Positive control using constructed pCAMBIA1301 vector, Lane N: Non transformed plant, Lane Nt: No template control, Lane M: 1 Kb ladder.

5.3.3.2. Analysis of integration of gene in transgenic tobacco through slot blot

The right and left hand border of pCAMBIA1301 harbours 35 S promoters, a part of Ll4CL1 gene in antisense orientation, hygromycin gene and GUS gene. Thus except Ll4CL1 gene other nucleotides could be used as probe to analyze the transgenic plant. Slot blot was done using various transgenic events of tobacco

and a non transformed plant (Fig 5.10). Blot was hybridized using hygromycin gene. Very profound signals were observed in four transgenic events out of seven and very low signals were observed in rest of blotted transgenic gDNA. No signal was observed in non transformant tobacco plant. The difference in the signal intensity in different transgenic events may be due to the presence of different copy numbers of genes.

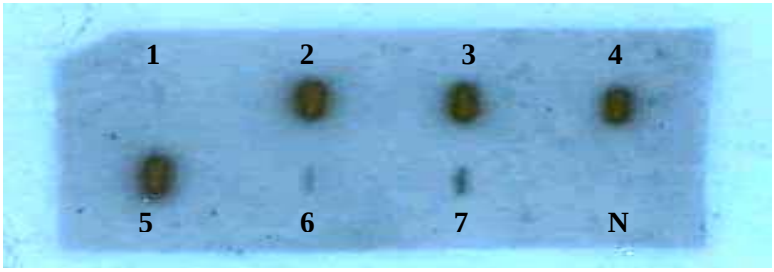

Fig 5.10: Slot blots analysis of transgenic *Tobacco.* Lane 1-7: +ve signal of gDNA of transgenic tobacco blotted on membrane. Lane N: No signal of gDNA of non transgenic tobacco.

5.3.4. Transformation of *Leucaena leucocephala*

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

The transformation was carried out by three methods:

1) Particle bombardment

2) Particle bombardment followed by co-cultivation

3) Agro-infusion method

The method was described in detail in chapter 2, section 2.18.

5.3.4.1. Selection of transformed plant on hygromycin

The non transformed and transformed embryo axes (Particle bombardment / Particle bombardment followed by co-cultivation /Agro-infusion) were kept on plane 1/2 MS + TDZ (0.5 mg/L) regeneration medium for one week. Then the

embryo axes were shifted to selection medium containing Hygromycin (10 mg/L) for 3 weeks. Transformed embryo axes were survived on selection medium (Hygromycin (10 mg/L)) while non transformed embryo axes turn black on selection medium (Fig 5.11). The survived embryo axes on hygromycin (10 mg/L) were further selected on hygromycin 15 mg/L for another 3 weeks. The survived explants on hygromycin (15 mg/L) were shifted to half 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.

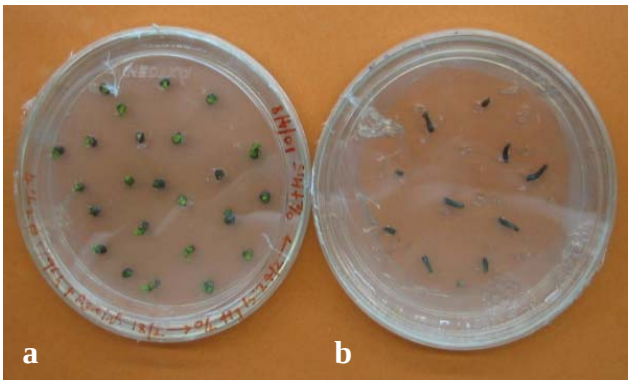

Fig 5.11: Antibiotic sensitivity of embryo axes of *L. leucocephala*. (a) Transformed embryo axes cultured in hygromycin (10 µg/mL) (b) Non transformed embryo axes cultured in hygromycin (10 µg/mL).

The putative transformed plants were further analyzed using molecular tools to confirm the integration of the gene. The first step to analyze the plant was the GUS assay. It was performed using transformed embryo axes and non transformed embryo axes. Blue color (Fig 5.12: a, b & c) spots were observed in most of the transformed embryo axes of *Leucaena* and no color was observed in non-transformed embryo axes. The transformed embryo axes were selected on hygromycin 10mg/L after one week of transformation. Necrosis was observed just after ten day of transfer and the embryo axes started sprouting (Fig 5.12 d). The sprouted embryo axes were transferred to further selection on hygromycin (15mg/L) after three weeks (Fig 5.12, e). The survived explants on hygromycin (15 mg/L) were further elongated till two weeks (Fig 5.12, f) and then further branching started (Fig 5.12, g). The survived explants on hygromycin (15 mg/L) were further shifted to half 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 (Fig 5.12, h & i). The plants were kept in Cytokinin 2IP medium for 6-8 week and then transferred to root inducing media (Chapter2: section 2.6.9, Fig 5.13, a). The elongated plants were transfer for hardening (Fig 5.13, b) and later transferred to pots (Fig 5.14).

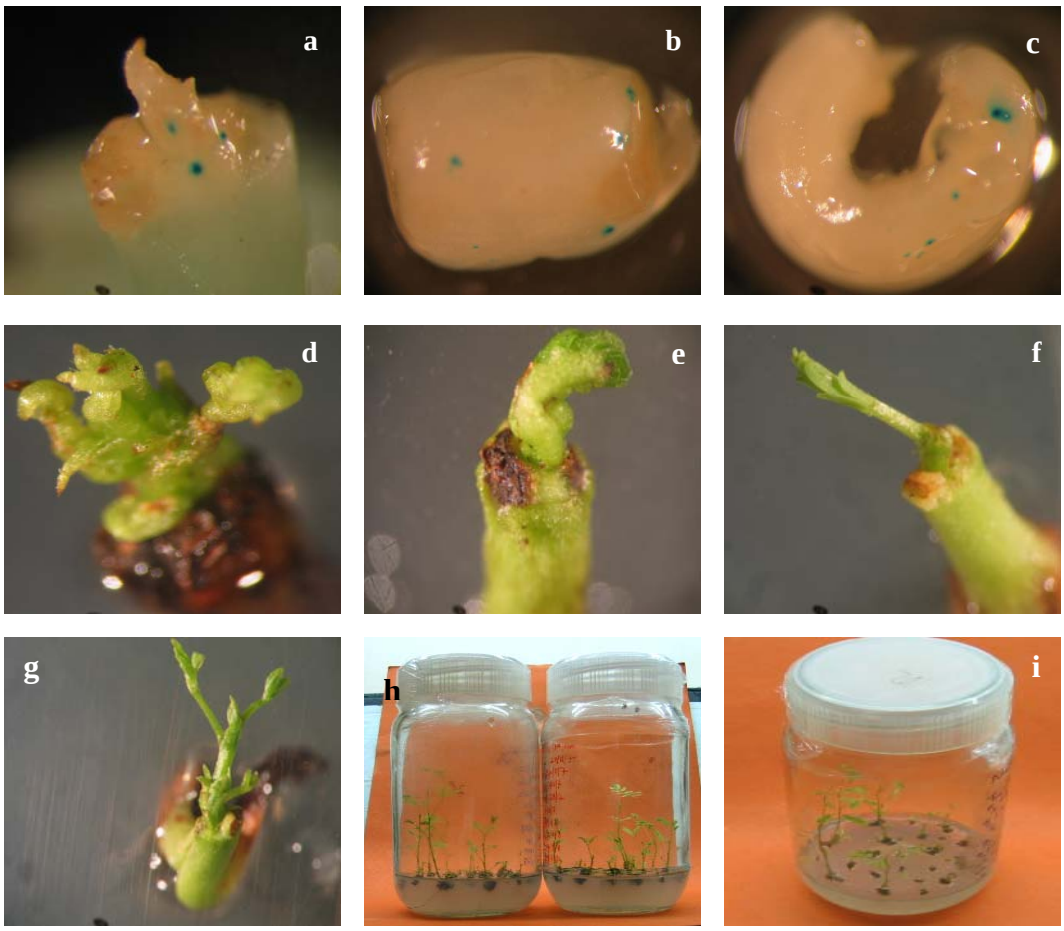

Fig 5.12: Putative transformed *Leucaena* shoots regenerated *in vitro* on selection medium. Transient GUS expression on bombarded embryo axes (a,b and c), Transformed embryo axes on selection medium after two weeks (d), five weeks (e),eight weeks (f), twelve weeks (g), regenerated plant on nutrient media (h & i).

Fig 5.13: a) regenerated plant on root inducing media and b) Hardening of transformed and regenerated *Leucaena* plant.

Fig 5.14: Transformed *Leucaena* plant in pots (a & b).

5.3.5. Integration of antiLl4CL1 gene and GUS in *Leucaena* genome

The right and left hand border of pCAMBIA1301 harbours 35 S promoters, a part of Ll4CL1 gene in antisense orientation, hygromycin and GUS gene. So if the plant were transformed then these genes would have been integrated somewhere in the *Leucaena* genome. To authenticate the transformed *Leucaena* plant we exploited this integration of different gene in to the *Leucaena* genome through PCR.

5.3.5.1. Integration of hygromycin gene in transgenic *Leucaena* genome

Genomic DNA was isolated from nontransformed control *Leucaena* plant and from the several transformation events of antiLL4CL1 plants. Approximately 50ng gDNA was used as template for PCR based amplification of the hygromycin gene using gene specific forward (HygAF) and reverse (HygAR) primers.

Forward primer HygAF 5'ATTTGTGTACGCCCGACAGT 3'
Reverse primer HygAR 5'-GGCGAAGAATCTCGTGCTTTC-3'

A fragment of ~800bp was amplified from the genomic DNA used as templates. There was no amplification from the non transformed control *Leucaena* plant (Figure 5.15). The amplicons were gel eluted, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequences were aligning with the hygromycin gene sequences of pCAMBIA1301 using CLUSTAL W (1.8) multiple sequence alignment and it shows almost 100% match with hygromycin gene (Fig 5.16).

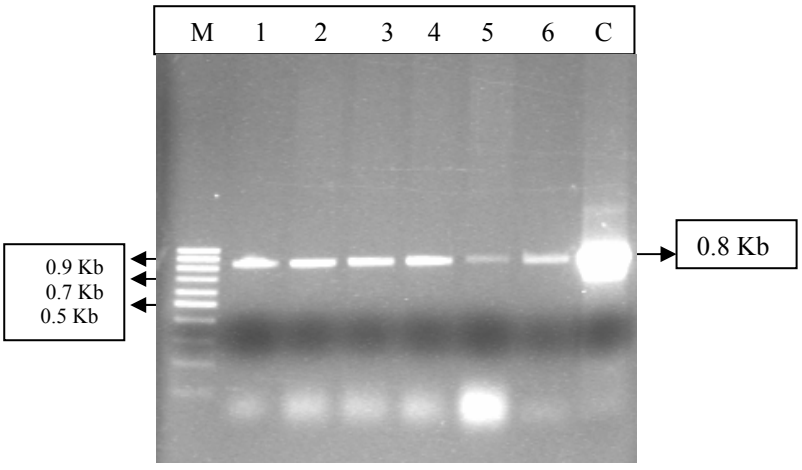

Fig 5.15: PCR amplification of hygromycin gene from transformed *Leucaena* plant. Lane 1, 2, 3, 4, 5 & 6: PCR amplified 0.8 Kb hygromycin gene from transgenic plant (*Leucaena*), Lane C: Positive control using pCAMBIA vector, Lane M: 100 bp ladder.

CLUSTAL W (1.8) multiple sequence alignment

```
pCam.1301.hyg       CTATTTCTTTGCCCTCGGACGAGTGCTGGGGCGTCGGTTTCCACTATCGGCGAGTACTTC
Transgenicplant     --------------------AGGTGGGGGGGCGTGGGTT--CACTATCGGCGAGTACTTC
                                        ***  ******* ****  ******************

pCam.1301.hyg       TACACAGCCATCGGTCCAGACGGCCGCGCTTCTGCGGGCGATTTGTGTACGCCCGACAGT
Transgenicplant     TACACAGCCATCGGTCCAGACGGCCGCGCTTCTGCGGGCGATTTGTGTACGCCCGACAGT
                    ************************************************************

pCam.1301.hyg       CCCGGCTCCGGATCGGACGATTGCGTCGCATCGACCCTGCGCCCAAGCTGCATCATCGAA
Transgenicplant     CCCGGCTCCGGATCGGACGATTGCGTCGCATCGACCCTGCGCCCAAGCTGCATCATCGAA
                    ************************************************************

pCam.1301.hyg       ATTGCCGTCAACCAAGCTCTGATAGAGTTGGTCAAGACCAATGCGGAGCATATACGCCCG
Transgenicplant     ATTGCCGTCAACCAAGCTCTGATAGAGTTGGTCAAGACCAATGCGGAGCATATACGCCCG
                    ************************************************************

pCam.1301.hyg       GAGTCGTGGCGATCCTGCAAGCTCCGGATGCCTCCGCTCGAAGTAGCGCGTCTGCTGCTC
Transgenicplant     GAGTCGTGGCGATCCTGCAAGCTCCGGATGCCTCCGCTCGAAGTAGCGCGTCTGCTGCTC
                    ************************************************************

pCam.1301.hyg       CATACAAGCCAACCACGGCCTCCAGAAGAAGATGTTGGCGACCTCGTATTGGGAATCCCC
Transgenicplant     CATACAAGCCAACCACGGCCTCCAGAAGAAGATGTTGGCGACCTCGTATTGGGAATCCCC
                    ************************************************************

pCam.1301.hyg       GAACATCGCCTCGCTCCAGTCAATGACCGCTGTTATGCGGCCATTGTCCGTCAGGACATT
Transgenicplant     GAACATCGCCTCGCTCCAGTCAATGACCGCTGTTATGCGGCCATTGTCCGTCAGGACATT
                    ************************************************************

pCam.1301.hyg       GTTGGAGCCGAAATCCGCGTGCACGAGGTGCCGGACTTCGGGGCAGTCCTCGGCCCAAAG
Transgenicplant     GTTGGAGCCGAAATCCGCGTGCACGAGGTGCCGGACTTCGGGGCAGTCCTCGGCCCAAAG
                    ************************************************************

pCam.1301.hyg       CATCAGCTCATCGAGAGCCTGCGCGACGGACGCACTGACGGTGTCGTCCATCACAGTTTG
Transgenicplant     CATCAGCTCATCGAGAGCCTGCGCGACGGACGCACTGACGGTGTCGTCCATCACAGTTTG
                    ************************************************************

pCam.1301.hyg       CCAGTGATACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCATGTAGTGTATTG
Transgenicplant     CCAGTGATACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCATGTAGTGTATTG
                    ************************************************************

pCam.1301.hyg       ACCGATTCCTTGCGGTCCGAATGGGCCGAACCCGCTCGTCTGGCTAAGATCGGCCGCAGC
Transgenicplant     ACCGATTCCTTGCGGTCCGAATGGGCCGAACCCGCTCGTCTGGCTAAGATCGGCCGCAGC
                    ************************************************************

pCam.1301.hyg       GATCGCATCCATAGCCTCCGCGACCGGTTGTAGAACAGCGGGCAGTTCGGTTTCAGGCAG
Transgenicplant     GATCGCATCCATAGCCTCCGCGACCGGTTGTAGAACAGCGGGCAGTTCGGTTTCAGGCAG
                    ************************************************************
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pCam.1301.hyg       GTCTTGCAACGTGACACCCTGTGCACGGCGGGAGATGCAATAGGTCAGGCTCTCGCTAAA
Transgenicplant     GTCTTGCAACGTGACACCCTGTGAACGGCGGGAGATGCAATAGG-CAGGGGGGATCCAG-
                    ********************** ******************** ****      * *

pCam.1301.hyg       CTCCCCAATGTCAAGCACTTCCGGAATCGGGAGCGCGGCCGATGCAAAGTGCCGATAAAC
Transgenicplant     ------------------------------------------------------------

pCam.1301.hyg       ATAACGATCTTTGTAGAAACCATCGGCGCAGCTATTTACCCGCAGGACATATCCACGCCC
Transgenicplant     ------------------------------------------------------------
```

Fig 5.16: CLUSTAL W (1.8) multiple sequence alignment of Hygromycin gene shaded region shown are the sequence of forward primer.

5.3.5.2. Integration of 35S promoter region in transgenic *Leucaena* genome

PCR amplifications from the genomic DNA of the transformation events antiLl4CL1 with 35S forward and 35S reverse primer of 35S promoter.

35SF 5′ ACAGTCTCAGAAGACCAAAGGGCT 3′
35SR 5′ AGTGGGATTGTGCGTCATCCCTTA 3′

A fragment of ~ 0.30 kb was amplified from the genomic DNA used as templates. There was no amplification from the untransformed tobacco plant (Figure 5.17). The amplicons were gel eluted, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequence of the amplicons was confirmed to be that of 35S promoter. The nucleotide sequences were aligning with the 35S promoter of pCAMBIA1301 using CLUSTAL W (1.8) multiple sequence alignment and it shows almost 100% similar with 35S promoter sequences (Fig 5.18).

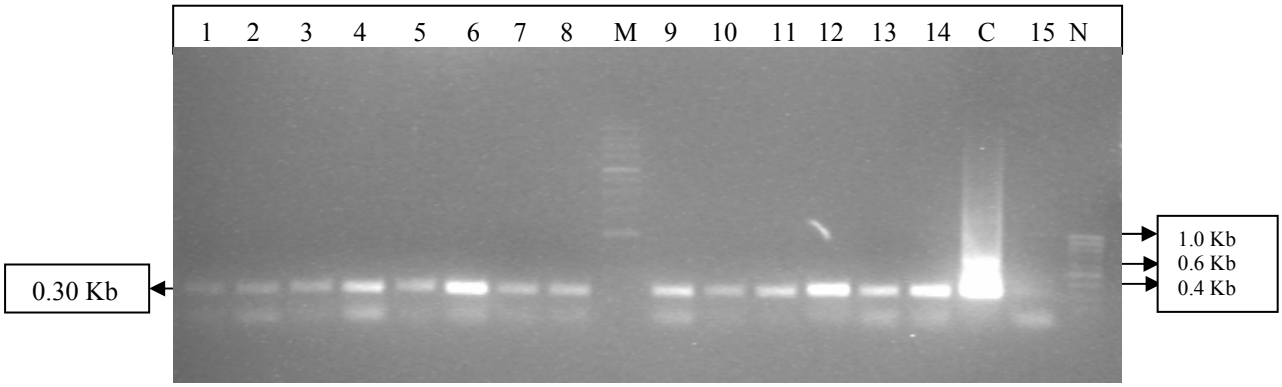

Fig 5.17: PCR amplification of 0.30 Kb of 35S promoter using 35SF and 35SR from transgenic *Leucaena* plant. Lane 1 – 15: PCR amplified 0.30 Kb 35S promoter region from transgenic Plant (*Leucaena*), Lane C: positive control, Lane M: 1 kb ladder, Lane N: 100 bp ladder.

CLUSTAL W (1.8) multiple sequence alignment

```
pCAM130135Sprom     ACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAAC
pCAM35SF&R          ACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAAC
                    ************************************************************

pCAM130135Sprom     CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAA
pCAM35SF&R          CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAA
                    ************************************************************

pCAM130135Sprom     GGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT
pCAM35SF&R          GGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT
                    ************************************************************

pCAM130135Sprom     GCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGAC
pCAM35SF&R          GCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGAC
                    ************************************************************

pCAM130135Sprom     GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGAT
pCAM35SF&R          GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGAT
                    ************************************************************

pCAM130135Sprom     GACGCACAATCCCACT
pCAM35SF&R          GACGCACAATCCCACT
                    ***************
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Fig 5.18: CLUSTAL W (1.8) multiple sequence alignment of 35S promoter region. The shaded regions shown are the region of forward and reverse primer.

5.3.5.3. Integration of Ll4CL1 gene in antisense orientation in transgenic *Leucaena* genome

PCR amplifications from the genomic DNA of the transformation events antiLl4CL1 with forward primer of 35S promoter and forward primer from the mid region of Ll4CL1 gene sequences (this forward primer from LL4CL1 gene was used as reverse primer as the Ll4CL1 gene was in antisense orientation).

35SF 5′ ACAGTCTCAGAAGACCAAAGGGCT 3′
Lec5F 5′ GGATTTCGCTGACAAACGTGG 3′

A fragment of ~ 1.2 kb was amplified from the genomic DNA used as templates. There was no amplification from the untransformed tobacco plant (Figure 5.19). The amplicon were gel eluted, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequence of the amplicons was confirmed to be that of 35S promoter and a part of Ll4CL1 gene. The nucleotide sequences were aligning with the 35S

promoter of pCAMBIA1301 using CLUSTAL W (1.8) multiple sequence alignment and it showed almost 100% similarity with 35S promoter and a part of Ll4CL1 gene (Fig 5.20).

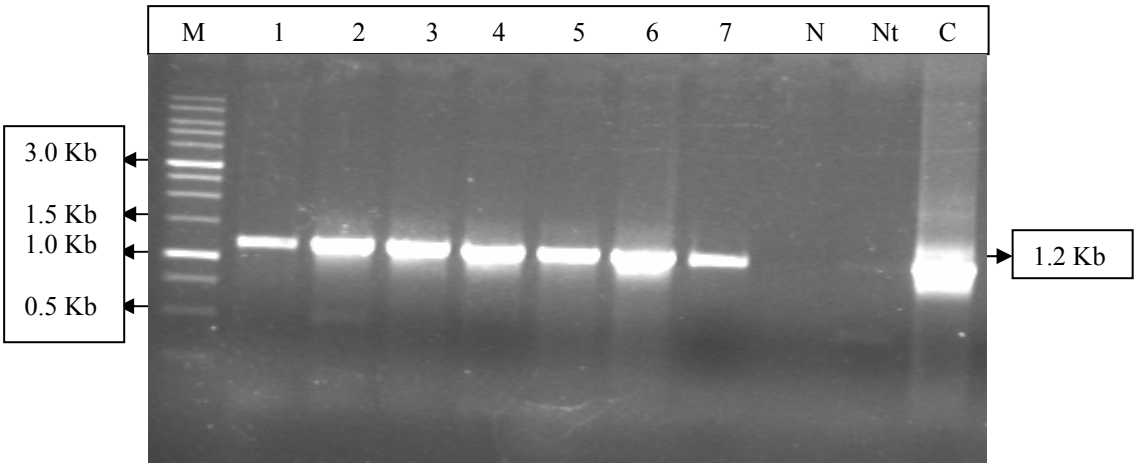

Fig 5.19: PCR amplification of 1.2 Kb 35S promoter and a part of Ll4CL1 gene from transgenic *Leucaena* plant. Lane 1, 2, 3, 4, 5, 6 & 7: PCR amplified 1.2 Kb 35S promoter and a part of 4CL gene from transgenic plant (*Leucaena*), Lane C: Posiive conrol using pCAMBIA vecor, Lane N: non transformed plant, Lane Nt: No template control, Lane M: 1 Kb ladder.

CLUSTAL W (1.8) multiple sequence alignment

```
anti4CLinpCAMBIA1301  ACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAAC
pCAMampli35SF&Lec5F   ACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAAC
                      ************************************************************

anti4CLinpCAMBIA1301  CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAA
pCAMampli35SF&Lec5F   CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAA
                      ************************************************************

anti4CLinpCAMBIA1301  GGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT
pCAMampli35SF&Lec5F   GGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT
                      ************************************************************

anti4CLinpCAMBIA1301  GCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGAC
pCAMampli35SF&Lec5F   GCCGACAGTGGTCCCAAAGATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGAC
                      ************************************************************

anti4CLinpCAMBIA1301  GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGAT
pCAMampli35SF&Lec5F   GTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACTGACGTAAGGGAT
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anti4CLinpCAMBIA1301  GACGCACAATCCCACTATCCTTTCGCAAGACCATCCCTCTATATAAGGAAGTTCATTTCA
pCAMampli35SF&Lec5F   GACGCACAATCCCACTATCCTTTCGCAAGACCATCCCTCTATATAAGGAAGTTCATTTCA
                      ************************************************************

anti4CLinpCAMBIA1301  TTTGGAGAGAACACGGGGGACTCTAGATCTGCAGTCGAGGTACCCTTTCATAACTTGATT
pCAMampli35SF&Lec5F   TTTGGAGAGAACACGGGGGACTCTAGATCTGCAGTCGAGGTACCCTTTCATAACTTGATT
                      ************************************************************

anti4CLinpCAMBIA1301  TCCTCTAATGCAGATTTCACCAGCCCTGTTTCTTGGAAGCGAAGCTCCCGTCTCAATGTC
pCAMampli35SF&Lec5F   TCCTCTAATGCAGATTTCACCAGCCCTGTTTCTTGGAAGCGAAGCTCCCGTCTCAATGTC
                      ************************************************************

anti4CLinpCAMBIA1301  AACAATCTTCATCTCTGCGTTTCTTATCACGCTCCCGCACGCGCCTGATTTCATCTCCAC
pCAMampli35SF&Lec5F   AACAATCTTCATCTCTGCGTTTCTTATCACGCTCCCGCACGCGCCTGATTTCATCTCCAC
                      ************************************************************

anti4CLinpCAMBIA1301  CGGCTCCTTCGCGAACGACAAGCTTATCGACAGAGGACCACCCTCCGTCATCCCATATCC
pCAMampli35SF&Lec5F   CGGCTCCTTCGCGAACGACAAGCTTATCGACAGAGGACCACCCTCCGTCATCCCATATCC
                      ************************************************************

anti4CLinpCAMBIA1301  CTGTCCAAGTATGGCGTGTGGAAGCTTAGCCCTCAGGGCTTGTTCCAGCTCCACGCTCAC
pCAMampli35SF&Lec5F   CTGTCCAAGTATGGCGTGTGGAAGCTTAGCCCTCAGGGCTTGTTCCAGCTCCACGCTCAC
                      ************************************************************

anti4CLinpCAMBIA1301  CGGCGCTGCTCCGGTGACGATCGTCCTTATGGACGACAGGTCGTGCCGGTCAACTTCCTC
pCAMampli35SF&Lec5F   CGGCGCTGCTCCGGTGACGATCGTCCTTATGGACGACAGGTCGTGCCGGTCAACTTCCTC
                      ************************************************************

anti4CLinpCAMBIA1301  ACTCTTCACGATGTTTAATAGGATCGGAGGCACAAACGACGCCATTGTCACCTTGTAAGT
pCAMampli35SF&Lec5F   ACTCTTCACGATGTTTAATAGGATCGGAGGCACAAACGACGCCATTGTCACCTTGTAAGT
                      ************************************************************

anti4CLinpCAMBIA1301  CTTGATCATCTTCAACAACGTGGCGATGTCGTACTTACCCATCGTCAGAATGGCGGCTCC
pCAMampli35SF&Lec5F   CTTGATCATCTTCAACAACGTGGCGATGTCGTACTTACCCATCGTCAGAATGGCGGCTCC
                      ************************************************************

anti4CLinpCAMBIA1301  GGCTCGGATGCAGCAGAGCAAAATGGAGTTCAGCGCATAGATATGGAACATTGGAAGAAC
pCAMampli35SF&Lec5F   GGCTCGGATGCAGCAGAGCAAAATGGAGTTCAGCGCATAGATATGGAACATTGGAAGAAC
                      ************************************************************

anti4CLinpCAMBIA1301  GCAGATATGTACGTCGTCGCTAGTGGTGTACTGGTTCGGGTTTTCGCCGTCGACGAGCTG
pCAMampli35SF&Lec5F   GCAGATATGTACGTCGTCGCTAGTGGTGTACTGGTTCGGGTTTTCGCCGTCGACGAGCTG
                      ************************************************************

anti4CLinpCAMBIA1301  AGCCACGGAGGTCACCAGATTCTTGTGCGTTAGCATCACGCCCTTGGGAAAGCCGGAGGT
pCAMampli35SF&Lec5F   AGCCACGGAGGTCACCAGATTCTTGTGCGTTAGCATCACGCCCTTGGGAAAGCCGGAGGT
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anti4CLinpCAMBIA1301  GCCGGAGGAATACGGCAGTGCCACAACGTCGTCGGGGCTGATCTTGACGGCCGGCATACA
pCAMampli35SF&Lec5F   GCCGGAGGAATACGGCAGTGCCACAACGTCGTCGGGGCTGATCTTGACGGCCGGCATACA
                      ************************************************************

anti4CLinpCAMBIA1301  AGCTTCGTCGGCTTGGGTGAGTAAAGAAAAATGTGAAATACCTTCCGTTTCTGGGAAAGT
pCAMampli35SF&Lec5F   AGCTTCGTCGGCTTGGGTGAGTAAAGAAAAATGTGAAATACCTTCCGTTTCTGGGAAAGT
                      ************************************************************

anti4CLinpCAMBIA1301   AGAATCAATGCACATCAAAGAAACGCCACGTTTGTCAGCGAAATCC
pCAMampli35SF&Lec5F    AGAATCAATGCACATCAAAGAAACGCCACGTTTGTCAGCGAAATCC
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Fig 5.20: CLUSTAL W (1.8) multiple sequence alignment of 35S promoter region. The shaded regions shown are the region of forward and reverse primer

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

The right and left hand border of pCAMBIA1301 harbours 35 S promoters, a part of Ll4CL1 gene in antisense orientation, hygromycin gene and GUS gene. Thus except Ll4CL1 gene other nucleotides could be used as probe to analyze the transgenic plant. Slot blot was done using various transgenic events of *Leucaena* and a non transformed *Leucaena* plant (Fig 5.21). Genomic DNA was isolated from 24 transgenic plants and approximately 200ng of DNA after treatment was blotted on the membrane. Blot was hybridized using hygromycin gene. Very profound signals were observed in all twenty four transgenic events. The positive control and negative control was also blotted and there was no signal on negative control non transformed *Leucaena*.

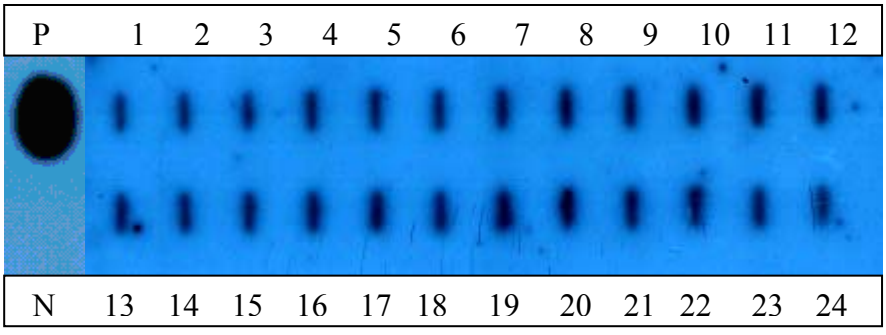

Fig 5.21: Slot blot analysis of transgenic *Leucaena* plant. Lane1-24: positive signal of gDNA of transgenic *Leucaena* blotted on membrane, Lane P: Positive signal with constructed pCAMBIA1301 vector, Lane N: Lane N: no signal with the non transformed *Leucaena* control plant.

5.3.7. Total transformation event

The total 400 transgenic (Tab: 1) events were done with excised embryo axes of *Leucaena*. 77.50% of plants (310) survived on selection medium. 51.25% of plants (205) elongated on selection medium, 66.66% of plants were elongated from the bombarded embryo axes. 46.19% of plants were elongated from the bombarded and followed by co cultivation of embryo axes and 3.33% of transformed plants were elongated from agro-infused embryo axes. The highest transformation efficiency (64.39) was obtained with bombarded plant and lowest efficiency was observed in agro infused (3.33%) embryo axes.

Table 5.1: Details of the number of embryo axes used for transformation studies and the PCR positives

Gene 4CL1	**Method used**	**Number of embryo axes used**	**Number of explants survived on selection (hyg 15 mg/L) medium**	**Number of shoots elongated**	**Av. Shoot length (cm)**	**Range (cm)**	**Number of shoots used for DNA extraction and PCR**	**Number of samples shown PCR positive**	**Transformation efficiency confirmed through PCR (%)**
	Particle bombardment	231	205 (88.75%)	154 (66.66%)	3.72	0.5-12.0	154	92 (39.83%)	59.74%
	Particle bombardment + Co cultivation	109	83 (76.15%)	47 (45.19%)	3.41	1.5-7.0	47	38 (34.86%)	80.85%
	Agro infusion	60	22 (36.67%)	04 (3.33%)	1.17	0.5-5.0	4	2 (3.33%)	50.00%
	Total	**400**	**310 (77.50%)**	**205 (51.25%)**			**205**	**132 (33.00%)**	**64.39%**

5.4. Conclusion

- Approximately 1.0 kb fragment of Ll4CL1 was cloned in antisense orientation in pCAMBIA1301.
- *Agrobacterium tumefaciens* strain GV2260 was transformed with the constructed pCAMBIA1301 vector.
- *Agrobacterium tumefaciens* strain GV2260 was used to transform tobacco. GUS assay were done to analyze transgenic events.
- Embryo axes of *Leucaena* were transformed by three different methods i.e. particle bombardment, particle bombardment followed by co-cultivation and agro-infusion method.
- Bombarded embryo axes were analyzed for transient GUS expression.
- Survived putative transformed plants were further grown on elongation media followed by root induction media.
- Transgenic events were further confirmed by PCR using hygromycin gene specific primer, 35S promoter specific primers and 35S forward and gene specific forward primer.
- Integration of gene was further confirmed by slot blot using hygromycin gene fragment as probe.
- Putative transformed embryo axes of the cultivars K-636 survived on selection medium developed into plantlets, which were kept for hardening and then transferred to green house for further growth.

Summary

Summary

Leucaena sp. is a fast growing multipurpose tree adapted to a variety of soils and climatic conditions. *Leucaena* is recognized as most useful trees in the tropics. Paper industry in India mainly uses bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leucaena sp*. as a source for paper pulp. This hard wood *Leucaena sp*. is exclusively used in India and about 25% of raw material for pulp and paper industry comes from this plant. To meet the increasing demand of high quality wood for paper industry, it is essential to provide designer plant species. The present study was aimed at understanding lignin biosynthesis pathway in *Leucaena leucocephala*. No study has been done so far in this regard anywhere on *Leucaena sp.*

4CL (4-Coumarate CoA Ligase, EC (6.2.1.1.2)) is one of the important key enzymes of general phenyl propanoid metabolism, which provides the precursors molecule for a large variety of important plant secondary products such as lignin, flavonoid etc. It catalyzes the activation of 4-coumarate and various other cinnamic acid derivatives to the corresponding thiol esters in two-step reaction via an adenylate intermediate. Thio reaction represents the last step in a short series of biochemical conversions, known as phenyl propanoid metabolism leading from phenylalanine to the activated cinnamic acid derivatives. These derivatives are precursors for the biosynthesis of a large variety of plant secondary metabolites. This gene was chosen as the target gene for the study if we could down regulate this 4CL enzyme then there would be scarcity of precursor molecules for lignin biosynthesis *i.e.* total lignin content of plant will get altered. Thus it may be helpful for development of transgenic *L. leucocephala* plants with desired characters suitable for Indian pulp and paper industry. The findings of present study are as follows:

4CL in *L. leucocephala* is gene family of possibly 6 members. Full length cDNA fragment of 1935 bp was isolated cloned and sequenced. This sequence was submitted to the NCBI Gen-Bank database and the allotted accession number for this fragment is FJ205490. The full-length gene was designated as Ll4CL1. The

exact length of coding region of full length, 5'UTR and 3'UTR are 1629, 68 and 238 nucleotides respectively. Another 1831 bp long cDNA fragment was isolated, cloned and sequenced. This sequence was submitted to the NCBI Gen-Bank database and the allotted accession number for this fragment is FJ205491. This partial gene with 3'UTR was designated as Ll4CL2. The exact length of coding region and 3'UTR of Ll4CL2 are 1560 and 271 nucleotides respectively. Ll4CL1 and Ll4CL2 show 66% nucleotide and 68% deduced amino acid sequence similarity with each other. Nucleotide sequence similarity with 4CL cDNA gene clones from other plants was 65-75%.

Deduced amino acid sequences of Ll4CL1 and Ll4CL2 genes show the presence of AMP binding superfamily domain of 415 to 519 amino acid. This AMP binding superfamily domain have box I conserved motif. This box I sequences are slightly different in Ll4CL1 (LPYSSGTSGFPKG) and Ll4CL2 (LPYSSGTTGLPKG). The box I sequences showed 84% identities and 100% positive sequences. The second conserved domain is box II motif *i.e* GEICIRG which are absolutely conserved in all reported 4CL and its central cystein residue play important role in catalytic activity. Phylogenetic analysis of 4CL genes group the Ll4CL1 and Ll4CL2 genes from *L. leucocephala* with 4CLs of other Fabaceae members.

The Ll4CL1 gene was expressed in *E. coli* BL21 (DE3) and protein purified from inclusion bodies using Ni-chelated affinity column. Polyclonal antibodies were raised against purified recombinant 4CL protein in rabbit. Polyclonal IgG were purified using agarose A affinity matrix. Transverse sections of different plant parts of different age stained with phloroglucinol-HCL show increase in number of differentiating xylem cells as well as their stain intensity indicated progression of lignification with age. 4CL was immunolocalized in xylem and fibers suggesting its presence at the sites of extensive lignification.

The semiquantitative and QPCR results showed that the Ll4CL1 gene was expressed differentially in a spatio-temporal manner in *Leucaena leucocephala.*

Summary

The ELISA results did not coincide with the results obtained in QPCR; the probable reason is that the polyclonal antibody raised against the Ll4CL1 expressed protein would not be specific only for 4CL1 protein, as this gene belongs to multifamily gene. The raised polyclonal antibody was not from the Ll4CL1 specific epitope and it might be binding to other 4CL isoenzymes present in the plant.

Leaves of *Nicotiana tabacum* var. Anand 119 and embryo axes excised from germinated seeds of *Leucaena leucocephala* cultivar K-636 were used as explant for transformation studies. Explants were transformed by three different methods i.e. particle bombardment, particle bombardment followed by co-cultivation and agro-infusion method. The integration of genes was confirmed by PCR with gene specific primers. The sequences of PCR products of putative transformants with Hygromycin, 35S promoter *and* 35Spromoter and a part of Ll4Cl1 genes showed complete homology with the sequences of the corresponding genes in the plasmids. Slot blot analysis of the transformed plants showed strong positive signals confirming the integration of the genes.

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