CHAPTER - 1

General Introduction
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Indian paper industry holds a share of 2% of the world paper production and is continuously expanding (FAOSTAT 2011). About 660 manufacturing units in India are currently engaged in manufacturing paper, paperboards, newsprints and paper products like tissue paper, paper bag, filter paper, teabag, medical grade coated paper and light weight online coated paper etc. To keep the Indian paper industry rolling, availability of superior quality raw material is must. Paper industry is primarily dependent upon forest-based raw materials with Indian paper industry currently relying on four major species viz. Bamboo, Eucalyptus, Casuarina, and *L. leucocephala*. Among these plants, *L. leucocephala* accounts for about 25% of the raw material (Srivastava et al. 2011). The wood pulp, that is processed to form paper, consists of three major components, cellulose, lignin and hemicellulose. Cellulose is the fibrous component of wood used to make paper, while lignin is responsible for sticking of the cellulose microfibrils together. As a major cell wall polymer, lignin imparts rigidity to the plant cell and renders the cell wall impermeable to water. This impermeability holds key role in water conduction through the xylem vessels to withstand the negative pressure, thought to be generated by transpiration and water conduction along the vascular bundle (Steudle 2001). In addition, lignin acts as a barrier to plant pathogens and prevents lodging, a problem in many agronomically important plants. Other than its critical role in plants’ sustenance, it is seen as a major hindrance for the agro-industrial exploitation of various plant species such as source of pulp for paper industry; and fodder for ruminants. The presence of lignin adversely affects paper quality and is responsible for poor performance characteristics and brightness of paper associated with yellowing of paper with age (Rastogi and Dwivedi 2006). To produce high quality paper, lignin needs to be removed from the pulp involving various chemical and energy intensive processes which in turn release various toxic pollutants and damage the polysaccharide components of wood (Rastogi and Dwivedi 2006). There is currently intense interest to reduce, modify or alter lignin content of *L. leucocephala* to be used as model plant in paper industry or as easily digestible and palatable fodder for animals. Therefore, various biotechnological programs have been undertaken to raise transgenics down-
regulated for key lignin biosynthetic gene(s) in order to tackle this problem. In the current work, the author has undertaken one such step by isolating a lignin biosynthetic pathway gene, Cinnamate 4-Hydroxylase (C4H) from *L. leucocephala* and characterizing it at molecular level to understand its role in lignification in *L. leucocephala*. Downregulation of C4H expression has also been studied in transgenic tobacco and *L. leucocephala* plants and the transgenics have been analysed for parameters like lignin content and the effect of reduction of C4H activity on the transcript abundance of C4H and other phenylpropanoid pathway genes.

1.1 *Leucaena leucocephala*

We need to have high cellulosic pulp yielding tree acclimatized to grow in a range of climatic conditions to serve as a source of pulp for paper industry in India. *L. leucocephala* is one such multiple purpose and one of the most versatile and productive tree legume commonly found in India (Fig. 1.1). This tree species was once known as the ‘miracle tree’ because of its worldwide success of being a perennial and highly nutritious forage tree and a variety of other uses (Shelton and Brewbaker 1994). Besides forage, *L. leucocephala* has been used as a potential source of pulp, firewood, timber, mulch, organic fertilizer, gum, shade and as cover to control soil erosion (Shelton and Brewbaker 1994; Vietmeyer and Cottom 1977). *L. leucocephala* pulp is of high quality and is suitable for use in printing and writing papers owing to its high opacity, good printability and good formation. It has been estimated that the dried leaves of *L. leucocephala* contain 27-34% protein of high nutritional quality (Rastogi and Dwivedi 2006) making it very healthy source of fodder. A large volume of medium-light hardwood for fuel (specific gravity 0.5 – 0.75) with low moisture and high calorific value that produces excellent quality charcoal, low smoke and ash can be produced from this species (Shelton and Brewbaker 1994). Being a leguminous tree, it is also associated with atmospheric nitrogen fixation and has been used in alley cropping in Philippines and Indonesia (Shelton and Brewbaker 1994; Vietmeyer and Cottom 1977). The *Leucaena-Rhizobium* partnership can fix more than 500 kg of nitrogen per hectare of land (Vietmeyer and Cottom 1977). The major limitation in using *L. Leucocephala* as a source of pulp or forage is its extent
of lignification. Despite being a good quality source of pulp, *L. leucocephala* is traditionally high in lignin content (Dutt et al. 2007) and this problem needs to be addressed by raising transgenics with low lignin content.

1.1.1 Botanical description of *Leucaena leucocephala*

*L. leucocephala*, previously known as *L. glauca*, is a perennial tree or shrub belonging to fabaceae family, that grows up to heights of 7-18 m (Shelton and Brewbaker 1994). Other than *L. leucocephala*, there are at least 14 other species recognized in the genus. Majority of these species are diploid, but *L. leucocephala* is a self-pollinating tetraploid (2n=4x=104) species (Shelton and Brewbaker 1994). *L. leucocephala* is the most common species of *Leucaena* genus and hence is sometimes referred to as ‘*Leucaena*’ only (Vietmeyer and

![Fig. 1.1: *Leucaena leucocephala*](image-url)
Cottom 1977). The author has followed this convention and hence in this thesis, ‘Leucaena’ written anywhere in the text refers to *Leucaena leucocephala* only and not to other species of the genus *Leucaena*.

### 1.1.2 Botanical classification of *Leucaena leucocephala*

Kingdom: Viridiplantae  
Division: Streptophyta  
Class: Magnoliopsida  
Order: Fabales  
Family: Fabaceae  
Genus: *Leucaena*  
Species: *leucocephala*

### 1.2 Pulp and paper industry statistics

The world’s paper and paperboard production decreased from 392 million tonnes in 2008 to 377 million tonnes in 2009 (FAOSTAT 2011) whereas the demand for paper is growing day by day. In India, annually, 6.8 million tonnes (mt) of wood is consumed to produce 1.9 mt of pulp, where nearly 80% of wood is procured from agro-forestry sources and rest 20% from government sources (Kulkarni 2008). To maintain the supply of raw materials to the industry, the industry relies on input from social and farm forestry. *Leucaena* accounts for approximately 1/4th of the raw material supplied to the industry and thus plays crucial role to maintain the demand-supply chain. Quality wise, pulp generated from *Leucaena* is rich in holocellulose and low in ash, silica, hot water solubles and alcohol-benzene solubles, which is comparable to other fast growing hardwoods used in paper industry (Dutt et al. 2007). *Leucaena* wood contains about 60% holocellulose and the pulp yield varies between 48.5 – 51.5% depending upon the age of the tree (Dutt et al. 2007).

India, despite featuring among the top 15 paper producing countries of the world, the per capita consumption of paper in India is among the lowest and is poised at about 4.5 kg per year, far below the South Asian and global average of 10.89 and 53.07 kg per year, respectively. But the demand for paper is expected to increase particularly because of the upsurge of the
disposable income of the expanding middle income group. So far, the growth of paper industry in India has matched the growth of GDP and on an average stands between 6-7% over the last few years. India is the fastest growing market for paper globally and the paper production is estimated to touch 13.95 mt by 2015-16 (http://www.ipma.co.in/paper_industry_overview.asp). As per the estimates from paper industry, paper production is expected to grow at a Compounded Annual Growth Rate (CAGR) of 8.4% while paper consumption will grow at a CAGR of 9% till 2012-13. The import of pulp & paper products is hence, likely to show a growing trend.

1.3 The pulping process (Pulp production)
Pulping is the process to reduce wood to a fibrous mat by separating the wood component cellulose from lignin and hemicellulose. Pulping can be divided broadly into three steps.

Step 1: Initial processing
Initial processing can be done either through (a) chemical, (b) mechanical or (c) semi-mechanical (combination of both).

a) Chemical method involves cooking wood in a digester at elevated pressure with a solution of chemical that dissolves lignin and leave behind cellulose. This process is hazardous releasing air pollutants including formaldehyde, methanol, acetaldehyde and methyl ethyl ketones.

b) Mechanical method physically separates the fibres by pressing the wood against a grinder. This is energy demanding and produces weak and opaque product that discolours easily after exposure to light.

c) Semi-mechanical pulping involves both chemical and mechanical pulping methods. Wood chips are first partially cooked with chemicals and then is subjected to mechanical pulping.

Step 2: Washing the pulp
After pulping, dissolved lignin and chemicals are removed from pulp by passing pulp through a series of washers and screens at high temperature, again generating hazardous exhausts and air pollutants. The chemical recovery process to recover used chemicals from the effluents also causes severe air pollution.
Step 3: Bleaching the pulp

After washing, pulp is bleached to remove colour associated with remaining residual lignin in the pulp. This is achieved by using either elemental chlorine and hypochlorite; or oxygen and peroxide and washing the pulp with several volumes of water releasing polluted waste water stream and vents releasing air pollutants. Depending upon the bleaching method used, the effluents may contain chlorinated organic compounds and toxic chemicals like dioxins, furans and chlorinated organic compounds and vents discard chloroform, methanol, formaldehyde and methyl ethyl ketone. Many a times, these chemicals pass untreated, through the treatment plants and contaminate water reservoirs like river and ocean.

1.4 Lignin and its biosynthesis

Eclipsed only by cellulose, lignin is the second most abundant biopolymer on earth and represents approximately 30% of the organic carbon (Boerjan et al. 2003). It is a complex racemic aromatic heteropolymer composed of three 4-hydroxycinnamoyl alcohol units differing in their degree of methoxylation in different combinations (Vanholme et al. 2010; Boerjan et al. 2003; Vogt 2010). The hydroxycinnamoyl alcohol units of lignin are termed monolignols (coniferyl, sinapyl and p-coumaryl alcohols) and they constitute the main building block of lignin (Fig. 1.2). These monolignols, when incorporated into the lignin polymer are called guaiacyl (G), syringyl (S) and p-hydroxyphenyl (H) units (Boerjan et al. 2003; Vanholme et al. 2010; Goujon et al. 2003). The amount and composition of lignin vary among different taxa, cell type, individual cell wall layers and is dependent on environmental and developmental factors (Achyuthan et al. 2010; Boerjan et al. 2003). Hardwood (dicotyledonous angiosperm) lignin primarily consists of G and S units with only traces of H units whereas softwood (gymnosperm) lignin consists mostly of G with low level of H units (Achyuthan et al. 2010; Boerjan et al. 2003; Vanholme et al. 2010). G monolignol units are methylated on 3-hydroxy position of the aromatic ring and are derived from caffeic acid (and/or its related aldehyde and alcohol), whereas S monolignol units are methylated on both 3-hydroxy and 5-hydroxy positions and are derived from sinapic acid (and/or its related aldehyde and alcohol) (see Fig. 1.2).
The relative abundance of S and G units in wood plays important role in paper and pulp industry with higher S units favourable than G units. S units in lignin are linked via relatively labile ether bonds which are more easily hydrolyzed than the linkage between G units. On the other hand, majority of G units are linked via more stable (and stubborn) biphenyl and other carbon-carbon linkages difficult to hydrolyze (Eckardt 2002). As a result, higher G content in wood requires more expensive and hazardous chemicals as well as vigorous treatment methods. High variation in the S/G ratio has been observed in between species, within a single species, within individual plant and cell type as a result of genetic, developmental and environmental cues (Eckardt 2002). Even a fractional increase in S/G ratio associated with overall decrease in lignin content has the potential to significantly improve pulping process and bring down the use of environmentally hazardous chemicals to a large extent.

Lignin deposition mainly takes place during secondary thickening of the cell wall in the final stage of xylem cell differentiation (Boerjan et al. 2003). The secondary cell wall consists of three layers: the outer (S1), middle (S2) and the inner (S3) (Fig. 1.3). Lignin deposition takes place in different phases in these layers each preceded by deposition of carbohydrates starting at cell corner of middle lamella and the primary wall when S1 formation has started (Boerjan et al. 2003). (For detailed phenylpropanoid pathway, see Fig. 1.4)
1.4.1 General phenylpropanoid pathway enzymes

Lignin biosynthesis in plants begins with a core group of three reactions termed as general phenylpropanoid pathway catalyzed by Phenylalanine ammonia lyase (PAL), Cinnamate 4-Hydroxylase (C4H) and 4-Coumarate: CoA Ligase (4CL) respectively (Boerjan et al. 2003; Vogt 2010; Hahlbrock and Scheel 1989). The first step towards formation of monolignols commences with the deamination of phenylalanine catalyzed by PAL. Subsequent reactions involve hydroxylation of the aromatic ring followed by phenolic O-methylation and sequential reduction of the side chain carboxyl group to an alcohol group. PAL, being the first enzyme of phenylpropanoid pathway, plays the role of a connecting link between primary and secondary metabolism by directing the carbon flow from Shikimate pathway to phenylpropanoid pathway (Boerjan et al. 2003; Boudet 2007; Hahlbrock and Scheel 1989).

Fig. 1.3: Lignin, pectin, cellulose and hemicellulose and their occurrence within the boundary of plasma membrane and middle lamella (source: Achyuthan et al. (2010) Molecules 15, 8641-8688)
Scheel 1989; Vanholme et al. 2010; Vogt 2010). The pathway branches after the action of C4H (second step of the pathway) and leads to a variety of compounds including lignin, hydroxycinnamic acid conjugates and flavonoides. Many intermediates and end products of this pathway play important role in plants as phytoalexins, antioxidants, UV ray protectants, pigments, aromatic compounds and antiherbivory compounds (Boudet 2007; Naoumkina et al. 2010). The last step of general phenylpropanoid pathway is catalyzed by 4CL, which commonly exists in plants as a small gene family with distinct roles (Boudet 2007; Vogt 2010). A particular 4CL isoform may direct the flux towards lignin biosynthesis while the other isoform towards other phenolics such as flavonoides with different level of activity in different tissues (Boudet 2007).

1.4.2 Monolignol biosynthesis committed enzymes
Cinnamoyl-CoA-reductase (CCR) and cinnamyl / sinapyl alcohol dehydrogenase (CAD / SAD) belong to the group of monolignol biosynthesis committed enzymes (Boudet 2007; Boudet 2000; Grima-pettenati and Goffner 1999; Vanholme et al. 2008). CCR as the first enzyme committed to monolignol biosynthesis channels phenylpropanoid metabolites into lignin biosynthesis. There may be more than one CCR gene per haploid genome, but in that case only one CCR isoform appears to be regulating lignin and other(s) seem to be related to the production of other phenolic compounds whose accumulation may lead to resistance (Boudet 2000). Another monolignol specific multifunctional enzyme, CAD, catalyzes NADPH dependent reduction of cinnamyl aldehydes to the corresponding cinnamyl alcohol, the last step of monolignol biosynthesis before oxidative polymerization in the cell wall (Boudet 2000; Grima-pettenati and Goffner 1999; Sibout et al. 2005). A CAD homolog from aspen was reported with very high specificity towards reducing sinapaldehyde than the usual CAD substrate and hence was named SAD (Boerjan et al. 2003; Boudet 2007).

1.4.3 Monolignol modification specific phenylpropanoid enzymes
Enzymes of this group control lignin monomeric composition and comprise of methylating enzymes, i.e. caffeic acid-\(O\)-methyltransferase (COMT), caffeoyl-CoA \(O\)-methyltransferase (CCoAOMT); hydroxylating enzymes coumarate-3-
hydroxylase (C3H) and ferulate-5-hydroxylase (F5H) and a newly discovered enzyme HCT (p-hydroxycinnamoyl-CoA:D-quinate; CQT or p-hydroxycinnamoyl-CoA:shikimate p-hydroxycinnamoyltransferase; CST, collectively called HCT) (Boerjan et al. 2003; Boudet 2000; Grim a-pettenati and Goffner 1999). These enzymes are likely candidates in regulating the types of monolignols incorporated into polymeric lignin. C3H was long thought to hydroxylate coumaric acid at C₃ position of the aromatic ring to form caffeic acid, but later it was found that the preferred substrates of C3H are shikimate and quinate esters of coumaric acid and not the conventionally thought p-coumaric acid, p-coumaroyl-CoA, p-coumaraldehyde, p-coumaryl alcohol, nor the 1–O-glucose ester and the 4–O-glucoside of p-coumaric acid (Boerjan et al. 2003; Vanholme et al. 2010). F5H is thought to hydroxylate ferulic acid, coniferaldehyde, coniferyl alcohol and possibly feruloyl-CoA at C₅ of the aromatic ring to form 5-hydroxyferulic acid, 5-hydroxyconiferaldehyde, 5-hydroxyconiferyl alcohol and 5-hydroxyferuloyl-CoA respectively. Whereas, HCT converts p-coumaroyl-CoA to p-coumaroyl shikimic acid / quinic acid (both are substrates of C3H; C3H converts them to caffeoyl shikimic acid / quinic acid) and caffeoyl shikimic acid / quinic acid to caffeoyl-CoA.

The classic historical view of lignin biosynthesis suggested that methylation by COMT occurred only at free acid level; COMT methylating caffeic acid on 3-OH position of the aromatic ring to form ferulic acid (and after the action of F5H on ferulic acid to form 5-hydroxy ferulic acid) and methylating 5-hydroxy ferulic acid at 5-OH position to form sinapic acid. This view was challenged after the finding that COMT is capable of methylating at aldehyde and alcohol levels as well (Boerjan et al. 2003; Boudet 2000; Eckardt 2002). This was further strengthened after the discovery of another class of O-methyltransferase, CCoAOMT. It was successfully proved and is now established that CCoAOMT can methylate CoA esters of 3-hydroxycaffeoyl CoA as well as 5-hydroxyferuloyl CoA (Boudet 2000; Eckardt 2002). The current information on lignin biosynthetic pathway suggests that the pathway can best be described as a metabolic grid with modification of aromatic ring possible at any or all of these levels.
Fig. 1.4: Lignin biosynthetic pathway based on current knowledge (as adapted from Shi et al. (2010) Plant & Cell Physiology 51, (1) 144-163)
1.4.4 Monolignol transport, coupling and polymerization

The monolignols are relatively unstable and toxic to the cell that do not get accumulated in the cytosol but are transported to the cell wall where they are polymerized. Several hypotheses have been put forward to explain the transport mechanism of monolignols to the cell wall but none of them is widely acceptable. The involvement of phenolic glucosides, coniferin and syringin, was proposed to explain the transport of monolignol but it could not be proved (Vanholme et al. 2008). Transport through Golgi body derived vesicles or through exporter proteins to plasma membrane also did not succeed (Vanholme et al. 2010). The picture is still not very clear about the transport of monolignols to the plasma membrane (Boerjan et al. 2003; Vanholme et al. 2010; Vanholme et al. 2008).

After transport of monolignols to the cell wall, they are polymerized through dehydrogenative (oxidative) polymerization which involves oxidative radicalization of phenols followed by combinatorial radical coupling. The dehydrogenation of monolignols has been ascribed to different classes of proteins like, peroxidases, laccases, polyphenol oxidases, and coniferyl alcohol oxidases, but the involvement of any particular protein falling in these categories is very limited because these enzymes belong to large gene families (Vanholme et al. 2010). After dehydrogenation, two monomer radicles may couple to form a dehydro dimer by forming covalent bond between both subunits at their $\beta$ position, resulting in only $\beta-\beta$, $\beta-O-4$ and $\beta-5$ dimers (Vanholme et al. 2010). Since the coupling is in chemical combinatorial fashion, any phenol present in zone of cell wall may enter into the coupling process to the extent as allowed. Thus, polymerization of monolignols follows simple chemical rules and largely depend upon the chemical and physical environment of the site of synthesis as pH, ionic strength, temperature, supply and concentration of individual monolignols, physical proximity of the subunits, hydrogen peroxide and peroxidase concentrations, and the matrix in general (Boerjan et al. 2003; Vanholme et al. 2010; Vanholme et al. 2008). The formed dimer in this fashion again gets dehydrogenated to a phenolic radical and followed by coupling of another monomer radicle. This mode of action of growing the lignin polymer chain is called endwise coupling.
1.5 Cinnamate 4-Hydroxylase (C4H) as target gene

C4H catalyzes the second step of phenylpropanoid pathway and hydroxylates trans-cinnamic acid at the *para* position, which is the reaction product of first enzyme of phenylpropanoid pathway, PAL (Russell 1971; Hahlbrock and Scheel 1989). C4H is a member of the large group of cytochrome P450 monooxygenases (P450) from plants and exclusively constitute the CYP73 family, a typical group A P450 (Chapple 1998). Russell and Conn (1967) provided first evidence of C4H activity from the crude extract of pea seedlings whereas C4H was first purified in active form from Jerusalem artichoke (Gabriac et al. 1991). C4H is one of the most abundant P450 in plants, nevertheless it was a difficult target to purify in active form because of its low concentration, hydrophobicity, instability and membrane bound nature (Chapple 1998; Russell 1971). It took more than 20 years after demonstrating its activity from crude extract, to bring to purity from *J. artichoke* (Gabriac et al. 1991). Following C4H purification from *J. artichoke*, with the aid of peptide sequencing and antibodies raised against the purified C4H, genes corresponding to C4H were isolated from alfalfa, mung bean, *J. artichoke*, Catharathus rosues, hybrid aspen, pea, Arabidopsis (Fahrendorf and Dixon 1993; Frank et al. 1996; Hotze et al. 1995; Kawai et al. 1996; Mizutani et al. 1997; Mizutani et al. 1993a; Teutsch et al. 1993) and the number is still growing. C4H isolated from these plants had high level of identity ranging from 75-80% at nucleotide level to more than 88-90% identity at amino acid level. C4H sequences from plants share many conserved regions and form a cluster of closely related sequences highly similar to each other. Other than this, C4H possess all the characteristics of a typical P450 protein including the light reversible inhibition of enzymatic activity by carbon monoxide (Chapple 1998). C4H exists as multi-family in trees like *Populus tremuloides*, *P. trichocarpa* (Lu et al. 2006), periwinkle (Hotze et al. 1995) and members of fabaceae family plants like pea (Whitbred and Schuler 2000) and alfalfa (Fahrendorf and Dixon 1993). Despite the presence of multiple isoforms of C4H, the precise roles of individual isoforms are not known in every case. Promoter region of C4H genes contain putative cis-acting elements known to occur in other phenylpropanoid pathway genes as well supporting the view that phenylpropanoid genes are subjected to coordinated transcriptional
regulation. Expression pattern of C4H is consistent with the role of this enzyme in lignification. Heterologous expression of C4H in yeast system efficiently converts trans-cinnamic acid to para-coumaric acid.

C4H is cotranslationally inserted to the endoplasmic reticulum (ER) in a signal recognition particle (SRP) dependent fashion through its helical N-terminal hydrophobic domain and rest of the protein including the heme binding domain faces the cytosol (Chapple 1998). The N terminal transmembrane hydrophobic helix is immediately followed by a proline rich region supposed to destabilize the α helix and produce kink to optimally orient the rest of the sequence with respect to the plasma membrane. C4H also contains heme binding motif near the C- terminus (the conserved sequence is PFGVGRRSCPG) in addition to conserved helices like I helix, K helix, and K’ helix (Durst and Nelson 1995).

C4H catalyzes slow reaction with high substrate specificity (Ehlting et al. 2006) and is located at strategic important point as the pathway branches after C4H. Pinus taeda cell suspension culture when fed with saturating level of phenylalanine did not result in proportional increase in C4H and C3H transcript level as with other phenylpropanoid genes like PAL, 4CL, COMT, CCR and CAD indicating both the P450 genes indeed are rate limiting step in the phenylpropanoid pathway (Anterola et al. 2002). Also, the reaction catalyzed by C4H is exothermic and irreversible (i.e. represent a point of no return) (Ehlting et al. 2006); hence C4H plays a vital role at the very beginning of the phenylpropanoid pathway.

It has been proposed that C4H anchors in the ER membrane and other phenylpropanoid enzymes form multi-enzyme complex by interacting and associating with C4H, although metabolic channelling has only been successfully demonstrated between a specific form of PAL and C4H. This metabolic channelling helps in keeping the local concentration of PAL generated cinnamic acid pool high and does not let it mix with the cytosolic contents (Achnine et al. 2004; Rasmussen et al. 1999). The physical association of membrane anchored C4H, PAL and possibly later
phenylpropanoid pathway enzymes provide indication of effective regulation of phenylpropanoid pathway at the entry level.

In agreement with early position of C4H in the pathway, mutants for C4H exhibit reduced level of phenylpropanoid pathway end products, reduced lignin with altered monomer content with concomitant accumulation of a novel hydroxycinnamic ester, cinnamoymalate not found in wild type plants. Other phenotypic changes observed were dwarfism, male sterility and swelling and branch junctions (Schilmiller et al. 2009). Considering this, several workers generated transgenic plants down-regulated for C4H in order to reduce lignin content in plants to be used as model plants in paper industry (Sewalt et al. 1997) or to be used as forage crops with increased palatability. Sewalt et al. (1997) showed that C4H downregulation in transgenic tobacco reduced C4H activity to up to 20% of wild type and the lines expressing less than 50% C4H activity had significantly reduced lignin level. In another study, Blount et al. (2000) found that transgenic tobacco lines over-expressed for PAL but down-regulated for C4H had reduced PAL activity. This emphasizes the dominant effect of C4H downregulation over PAL over-expression. Rastogi and Dwivedi (2006) generated transgenic L. leucocephala with decreased O-methyl transferase (OMT) and showed that reduction of 55-60% OMT activity in transformants had 13-28% lesser lignin content. Extrapolating this to down-regulation of C4H in L. leucocephala may yield transgenics with significantly reduced lignin level and higher extractability of cellulose from the plants. Keeping this in mind, the present work has been undertaken.