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Genetic Engineering of Phenylpropanoid Pathway in *Leucaena leucocephala*

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1. Introduction

Trees are reservoirs of many economically and biotechnologically significant products. Wood, one such gifts of nature, consists of lignin, hemicelluloses, and cellulose. Biochemistry of lignin, which being one of the most abundant biopolymers on earth, has been studied extensively, partly due to the significance, and interest of such knowledge from industrial point of view (Boerjan et al., 2003). Lignin has far reaching impacts on agriculture, industry and the environment, making phenylpropanoid metabolism, a major route for synthesis of lignin in plants, a globally important part of plant chemistry.

Besides its critical role in normal plant health and development, high levels of lignin are problematic in the agro-industrial exploitation of various plant species. It is considered an undesirable component in paper manufacture due to the cost, energy consumption, and pollutant generating processes required for its removal (Baucher et al., 2003; Boerjan, 2005; Chiang, 2002). Thus, making it essential to provide designer plant species with altered lignin content, and hence, to diminish the pressure on the domestication of natural forest resources in the future. Considerable scientific interest has been focused on the development of trees with improved wood quality through modification of different genes involved in lignin biosynthesis, which could be important for the improved end use of wood material (Chiang, 2006; Higuchi, 2006). *Leucaena leucocephala*, one of the most versatile fast growing commercially important trees for paper and pulp industry in India, contributes nearly a quarter of the total raw material. The wider use of this tree species in the pulp industry is due to its high rate of biomass production and ability to adapt to a variety of soils and climatic conditions. Every step towards the development of this tree variety in terms of increased biomass and reduced lignin content would be of great help to pulp and paper industry as it will decrease cost and release of hazardous chemicals during the production of paper pulp.

This chapter will briefly cover, the chemistry of lignin deposition in plants, role of different monolignol biosynthesis pathway genes, followed by studies concentrated on genetic

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engineering of phenylpropanoid pathway in *Leucaena leucocephala* as tool for altering its lignin composition, thereby its application in pulp and paper industries.

2. Lignin: Occurrence, structure and function

Lignin (from Latin *lignum* meaning wood), is one of the most abundant natural organic polymer next only to cellulose (Boerjan et al., 2003). It is a vital cell wall component of all vascular plants and represents on an average of 25 % of the terrestrial biomass. It plays crucial role in structural integrity of cell wall & stiffness and strength of stem (Chabannes et al., 2001; Jones et al., 2001). Lignin is primarily synthesized and deposited in the secondary cell wall of specialized cells such as xylem vessels, tracheids and fibers. It is also deposited in minor amounts in the periderm where association with suberin provides a protective role against pathogens. In addition, lignin waterproofs the cell wall; enabling transport of water and solutes through the vascular system (Sarkanen & Ludwig, 1971). Lignins are complex racemic aromatic heteropolymers synthesized from the dehydrogenative polymerization of monolignols, namely coumaryl, coniferyl and sinapyl alcohol monomers differing in their degree of methoxylation (Freudenberg & Neish, 1968). These monolignols produce respectively, p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units when incorporated into the lignin polymer (Fig. 1). The amount and composition of lignins vary among taxa, cell types and individual cell wall layer, and are influenced by developmental and environmental cues (Campbell & Sederoff, 1996). Lignin content is higher in softwoods (27-33%) than in hardwood (18-25%) and grasses (17-24%). The highest amounts of lignin (35-40%) occur in compression wood on the lower part of branches and leaning stems (Sarkanen & Ludwig, 1971).

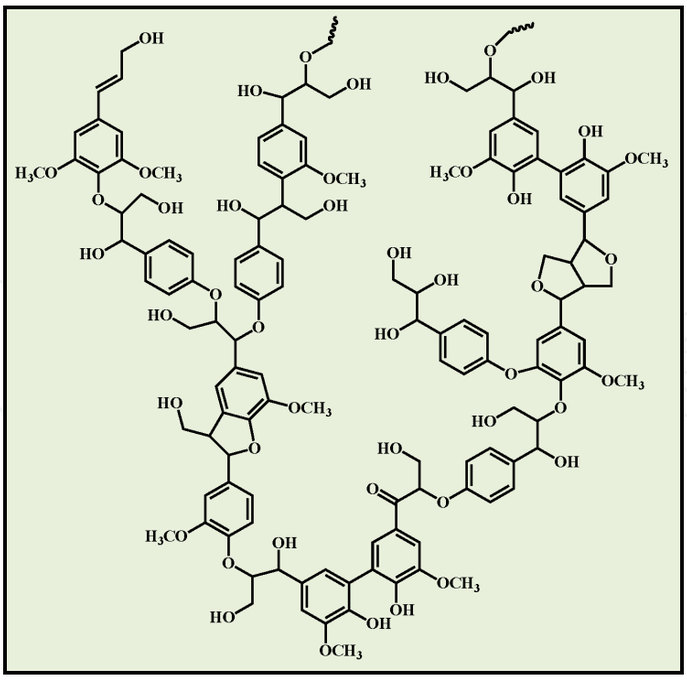


Fig. 1. Structure of a lignin oligomer. These structures consist of phenylpropanoid units H, G and S. A number of such oligomers cross-polymerizes to form a complex structure of lignin

2.1 Lignin biosynthesis

Although lignin has been studied for over a century, many aspects of its biosynthesis still remain unresolved. The monolignol biosynthetic pathway has been redrawn many times, yet still remains a matter of debate. During the last two decades, significant headway has been made in isolating and characterizing a number of genes pertaining to monolignol biosynthesis from different plants. Several reviews on the advancements of monolignol biosynthesis pathways are also available (Boerjan et al., 2003; Humphreys & Chapple, 2002; Whetten & Sederoff, 1995; Whetten et al., 1998).

In plants, lignins are synthesized by the polymerization of monolignols, namely p-coumaryl, coniferyl and sinapyl alcohol monomers differing in their degree of methoxylation (Higuchi, 1985; Sederoff & Chang, 1991) via phenylpropanoid biosynthetic pathway (Gross, 1985). This pathway comprises a complex series of branching biochemical reactions responsible for synthesis of a variety of products like lignin, flavonoids and hydroxycinnamic acid conjugates. Many intermediates and end products of this pathway play important role in plant such as phytoalexins, antiherbivory compounds, antioxidants, ultra violet (UV) protectants, pigments and aroma compounds. Finally, the diverse functions of lignin and related products in resistance to biotic and abiotic stresses make this pathway vital to the health and survival of plants.

The synthesis of lignin represents one of the most energy demanding biosynthetic pathways in plants, requiring large quantities of carbon skeletons. Deposition of lignin in plants proceeds via the following steps:

1. The biosynthesis of monolignols
2. Transport of monolignols from the site of synthesis to the site of polymerization
3. Dehydrogenation & Polymerization of monolignols.

2.1.1 Biosynthesis of monolignols

The biosynthesis of monolignols proceeds through the phenylpropanoid pathway starting with deamination of phenylalanine to produce cinnamic acid and involves successive hydroxylation reactions of the aromatic ring, followed by phenolic *o*-methylation and conversion of the side chain carboxyl to an alcohol group (Fig. 2). Immense amount of work has been done in characterizing the monolignol biosynthesis pathway in past two decades. It is a complex pathway comprising of enzymes with functions like methyltransferase, hydroxylase, reductase and dehydrogenase. Some of the important enzymes involved in monolignol biosynthesis have been discussed below.

2.1.1.1 Phenylalanine ammonia-lyase (PAL)

The enzyme phenylalanine ammonia-lyase (PAL; EC: 4.3.1.5) 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 (Kao et al. 2002; Osakabe et al., 1995), loblolly pine and other plant species (Bate et al., 1994; Hatton et al., 1995; Jones, 1984; Kumar & Ellis, 2001; Leyva et al., 1992; Ohl et al., 1990). *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

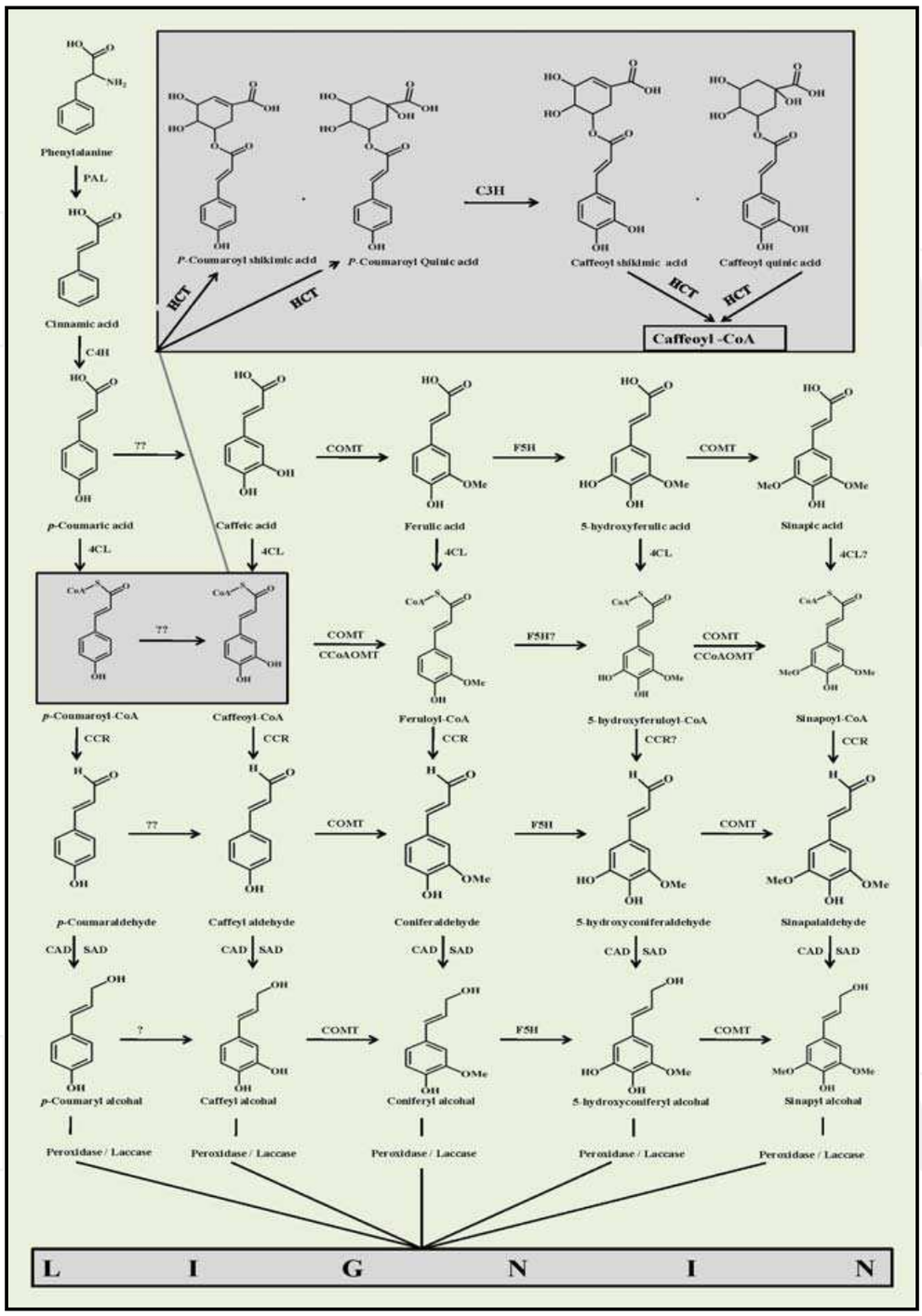


Fig. 2. An overview of monolignol biosynthesis pathway: PAL, Phenylalanine ammonia-lyase; C4H, Cinnamic acid 4-hydroxylase; C3H, p-Coumarate 3-hydroxylase; HCT, Hydroxycinnamoyltransferase; 4CL, 4-Coumarate-CoA ligase; CCoAOMT, Caffeoyl-CoA O-methyltransferase; CCR, Cinnamoyl CoA reductase; CAld5H, Coniferyl aldehyde 5-hydroxylase; AldOMT, 5- hydroxyconiferyl aldehyde O-methyltransferase; CAD, Cinnamyl alcohol dehydrogenase; SAD, Sinapyl alcohol dehydrogenase

developmental stage. The biochemical activity of all known PALs is verified as specific deamination of phenylalanine, but genetic and physiological function may vary among different *PAL* members. The expression of *PAL* genetic function is controlled by various genetic circuits and signaling pathways.

2.1.1.2 Cinnamate 4-Hydroxylase (C4H)

C4H (EC: 1.14.13.11) constitutes the CYP73 family of the large group of Cyt P450 monooxygenases. It catalyzes the 4-hydroxylation of *trans*-cinnamate, the central step in the generation of phenylalanine-derived substrates for the many branches of phenylpropanoid metabolism. The first and the last enzymes of this short sequence of closely related reactions, termed the general phenylpropanoid metabolism, are PAL and 4CL, respectively. A second metabolic link couples C4H to the membrane-localized Cyt P450 Reductase (CPR). The expression patterns of all three C4H-linked enzymes, PAL, 4CL, and CPR, and of the corresponding mRNAs have been analyzed in cell-suspension cultures and various intact tissues of parsley (Logemann et al., 1995) and *Arabidopsis* (Mizutani and Ohta, 1997). A reduction in PAL levels leads to an increase in the S/G ratio, whereas reduced C4H activity leads to a decrease in the S/G ratio. These observations support the existence of some sort of metabolic channeling between the enzymes of the central phenylpropanoid pathway and those of monolignol biosynthesis and also provide a basis for the development of new strategies for modified or reduced lignin content.

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 (Shanfa et al., 2006). 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 & abiotic stimuli (Bell-Lelong et al., 1997; Raes et al., 2003). The mechanisms that regulate the genetic function of *C4H* gene and its family members are yet unknown.

2.1.1.3 Coumarate 3-hydroxylase (C3H)

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

2.1.1.4 p-hydroxycinnamoyl-CoA: quinate shikimate p-hydroxycinnamoyl- transferase (HCT)

The enzyme *p*-coumarate 3-hydroxylase (C3H) converts *p*-coumaric acid into caffeic acid and has been shown to be a cytochrome p450-depenedent monooxygenase. It is interesting to note that enzymatic assays have demonstrated that the shikimate and quinate esters of *p*-coumaric acid are the preferred substrates for C3H over *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 are good substrates (Franke et al., 2002; Nair et al., 2002; Schoch et al., 2001). *p*-Coumarate is first converted to *p*-coumaroyl-CoA by 4CL, with subsequent conversion to *p*-coumaroyl-shikimate and *p*-coumaroyl-quinate, the substrates for C3H, by *p*-hydroxycinnamoyl-CoA:quinate-(CQT) or *p*-hydroxycinnamoyl-CoA:shikimate *p*-hydroxycinnamoyltransferase (CST) (Schoch et al., 2001). These enzymes, described as reversible enzymes, can convert caffeoyl-shikimate or caffeoyl-quinate (chlorogenic acid) into caffeoyl-CoA, the substrate for CCoAOMT. A reversible acyltransferase with both CQT and CST activity, designated HCT, has been purified and the corresponding gene cloned from tobacco (Hoffmann et al., 2002). Silencing of *HCT* through RNA interference (RNAi) lead to reduction in lignin, hyper accumulation of flavonoids and growth inhibition in *Arabidopsis* (Chapple, 2010; Hoffmann et al., 2004, 2007)

2.1.1.5 Coumarate Coenzyme-A ligase (4CL)

Genetic and biochemical functions of 4-Coumarate Coenzyme A ligase (4CL; EC: 6.2.1.12) genes have been clearly demonstrated in association with monolignol biosynthesis (Lewis and Yamamoto, 1990; Lee et al., 1997; Hu et al., 1998, 1999; Harding et al., 2002). *4CL* genes usually exist as a multi-gene family. 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 were clearly distinct, with one in epidermal & leaf tissue and the other specifically in developing xylem tissue (Harding et al., 2002; Hu et al., 1998). 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 (Hu et al., 1998; Lindermayr et al., 2003; Voo et al., 1995). 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*.

2.1.1.6 O-methyltransferases (O-MT)

S-adenosyl-L-methionine methyltransferases are key enzymes in the phenylpropanoid, flavanoid and many other metabolic pathways in plants. The enzymes Caffeate 3-O methyltransferase (COMT; EC: 2.1.1.68) and Caffeoyl CoA 3-O methyltransferase (CCoAOMT; EC: 2.1.1.104) control the degree of methoxylation in lignin precursors *i.e. p*- hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units of lignin (Higuchi, 1990; Ralph et al., 1998; Boerjan et al., 2003). The methylation reactions at the C3 and C5 hydroxyl functions of the lignin precursors were thought to occur mainly at the cinnamic acid level via a bi-functional COMT. However, the association of *CCoAOMT* expression with lignification (Pakusch et al., 1991; Ye et al., 1994; Ye & Varner, 1995; Ye, 1997; Martz et al., 1998; Chen et al., 2000) and the observation that down-regulation of *COMT* preferentially affected the amount of S units suggested the existence of an alternative pathway for the methylation of the lignin precursors at the hydroxycinnamoyl CoA level and specific O-methyltransferase *i.e.* CCoAOMT converts caffeoyl CoA into feruloyl CoA and 5-hydroxyferuloyl CoA into sinapyl CoA (Martz et al., 1998).

2.1.1.7 Cinnamoyl CoA reductase (CCR)

The reduction of cinnamoyl CoA esters to cinnamaldehydes is the first metabolic step committed to monolignol formation (Lacombe & Hawkins, 1997). This first reductive step in

lignin biosynthetic pathway is performed by Cinnamoyl CoA reductase (EC: 1.2.1.44) and it controls the over-all carbon flux towards lignin. CCR activity is found to be generally low in plants so it is hypothesized that it may play a crucial role as a rate limiting step in regulation of lignin biosynthesis (Ma & Tian, 2005). CCR is apparently encoded by a single gene per haploid genome in *Eucalyptus* (Lacombe & Hawkins, 1997), poplar (Leple et al., 1998), ryegrass (Larsen, 2004; McInnes et al., 2002), *Triticum* (Ma, 2007) and tobacco (Piquemal & Lapierre, 1998) and by two genes in maize (Pichon, Courbou et al., 1998), and *Arabidopsis* (Lauvergeat & Lacomme, 2001). The *CCR* genes in various species appear as a multiple member family. In the *Populus* genome, there exist 8 *CCR*-homolog or *CCR*-like gene sequences (Li & Cheng, 2005). *Triticum* (Ma & Tian, 2005; Ma, 2007), maize (Pichon & Courbou, 1998), switchgrass (Escamilla-Trevino & Shen, 2010), *Medicago* (Zhou & Jackson, 2010) and *Arabidopsis* (Lauvergeat & Lacomme, 2001) have been shown to possess two or more than two isoforms (*CCR1* and *CCR2*) which are involved in mutually exclusive or redundant functions like, constitutive lignifications and defense. Several other *CCR* gene sequences have been deposited in the GenBank database, but their functions have still not been demonstrated. It is proposed that all CCR enzymes have a similar catalyzing mechanism for converting the CoA esters to aldehydes in monolignol biosynthesis.

2.1.1.8 Coniferaldehyde 5-hydroxylase (CAld5H)

CAld5H enzyme like C4H belongs to cytochrome P450 monoxygenase family. The hydroxylation reaction in the biosynthesis of S-unit (syringyl) was first considered to occur at the ferulate level (Grand, 1984), and hence, the enzyme was called Ferulate 5-hydroxylase (F5H). However, studies later have revealed that F5H can also function at later steps in the pathway, mainly at the coniferyl aldehyde or coniferyl alcohol level (Humphreys et al., 1999; Li et al., 2000). This enzyme was therefore alternatively renamed coniferaldehyde- 5-hydroxylase (CAld5H) (Osakabe et al., 1999). F5H/CAld5H is unusual in that it is a multifunctional plant P450 with three physiologically relevant substrates. The *K*m for the substrates such as coniferaldehyde, coniferyl alcohol and for the ferulic acid are 1 µM, 3 µM and 1000 µM respectively. This study demonstrates that the coniferaldehyde is the most preferred substrate for the enzymes (Humphrey et al., 1999). Considerable evidence is now available that shows that in angiosperm trees, the syringyl monolignol pathway branches out from guaiacyl pathway through coniferaldehyde and is regulated in sequence by three genes encoding coniferaldehyde 5-hydroxylase (*CAld5H*), 5-hydroxyconiferaldehyde *O*-methyltransferase (*COMT*) and sinapyl alcohol dehydrogenase (*SAD*).

2.1.1.9 Cinnamyl/Sinapyl alcohol dehydrogenases (CAD/SAD)

In gymnosperm wood, coniferyl alcohol is the major monolignol units while both coniferyl and sinapyl alcohols are present in angiosperm wood. CAD (E.C: 1.1.1.195), depicts a class of NADPH dependent oxidoreductase, suggested to catalyze multiple cinnamyl alcohol formations from their corresponding cinnamaldehydes (Lewis & Yamamoto, 1990; Whetten & Sederoff, 1995; Whetten et al., 1998). This reduction of aldehydes to corresponding alcohols has been considered to be an indicator of lignin biosynthesis because of its specific role at the end of the monolignol biosynthesis pathway (Baucher et al., 1996). 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 been further verified in protein structure analysis (Bomati & 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.

2.1.2 Transport of monolignols

After the synthesis, the lignin precursors or monolignols are transported to the cell wall where they are oxidized and polymerized. The monolignols formed are insoluble and toxic to the plant cell and hence are converted to their respective glucosides by the action of UDP-glycosyltransferases (UDP-GT). This conversion renders the monolignols, soluble and less toxic to the plant cells, which can be stored in plant vacuoles, and transported to the cell wall as the need arises. It has been hypothesized that these monolignol glucosides are storage or transport forms of the monolignols (Steeves et al., 2001).

2.1.3 Dehydrogenation and polymerization

After transport of the monolignols to the cell wall, lignin is formed through dehydrogenative polymerization of the monolignols (Christensen et al., 2000). The dehydrogenation of monolignol radicals has been attributed to different class of enzymes, such as peroxidases (POX), laccases (LAC), polyphenol oxidases, and coniferyl alcohol oxidase. Lignin is a hydrophobic and optically inactive polymer, which is highly complex and heterogeneous in nature. Lignin polymerization is a radical coupling reaction, where the monolignols are first activated into phenoxy radicals in an enzyme catalyzed dehydrogenation reaction. These radicals couple to form dimers, oligomers and eventually the lignin polymer (Freudenberg, 1968). Peroxidases are heme-containing oxidoreductases that use H_2O_2 as the ultimate electron acceptor. The natural electron donor molecules in a peroxidase catalyzed reaction vary and include, monolignols, hydroxycinnamic acids (Zimmerlin et al., 1994), tyrosine residues in extensions (Brownleader et al., 1995) and auxin (Hinman & Lang, 1965). Several reports on peroxidase activity or gene expression in lignin-forming tissues have appeared, but only a few isoenzymes or genes have been specifically associated with lignification (Christensen et al., 2001; Marjamaa et al., 2006; Quiroga et al., 2000; Sato et al., 1993).

2.2 Regulation of monolignol biosynthesis

Developmental program of lignification associated with certain types of plant cells, such as xylem and fibers, require coordinated regulation of different lignin biosynthesis genes, as well as with genes controlling other aspects of plant growth and development. Different transcription factors such as R2R3-MYB, KNOX, MADS, LIM have been found to be regulating lignin biosynthesis in many plants, although the understanding of the molecular mechanism of pathway regulation is still limiting (Campbell & Rogers, 2004; Zhou et al., 2006, 2008). Lignification can be modified in a more efficient and precise way by

understanding the regulation of these pathways via altering the expression of relevant transcription factors.

3. Lignin as barrier for paper production

From an agro-economical point of view, lignin is considered to have a negative impact because it affects the paper manufacture and limits digestibility of forage crops. High quantity and low Syringyl (S) to Guaiacyl (G) lignin ratio plays a detrimental role in economy and ecology of paper production. Every unit increase in S/G ratio decreases the cost of paper production by two and half times. Both lignin content and composition are known to have impact on pulp & paper because residual lignin in the wood fibers causes a discoloration and a low brightness level of the pulp (Chaing et al., 1988). Consequently, for the production of high quality paper, lignin has to be removed from cellulose during the pulping process without damaging the polysaccharide component of wood. During chemical (Kraft) pulping, a large amount of Sodium hydroxide (NaOH) and Sodium sulfide (Na_2S) are required to extract lignin from the pulp (Axegard et al., 1992). Subsequently, the residual lignin is further removed with bleaching agents, such as Chlorine dioxide (ClO_2), Hydrogen peroxide (H_2O_2), Sodium hypochlorite(NaOCl), Oxygen(O_2), or Ozone (O_3) (Axegard et al.,1992; Biermann, 1993; Christensen et al., 2000).

These lignin extraction & bleaching procedures can partly degrade cellulose and consequently, reduce pulp quality and paper strength. Lignin extraction consumes large quantities of chemicals and energy leading to poor environmental image for these industries (Biermann, 1996; Higuchi, 1985; Odendahl, 1994). For this reason, engineering of plants with cell wall structures that are more susceptible to the krafting, and thus, more amenable to hydrolysis, or are sufficiently altered so as to shunt the above processes is an attractive approach to improve pulping efficiency and potentially alleviate some of the negative environmental impacts of the paper making industry. Apart from the great deal of work in the lignin field for improving the pulping process, many examples can be found based on research aimed at altering the lignin content for improving bio-fuel production (Chen, 2006; Chen & Dixion, 2007; Davision, 2006; Franke et al., 2002), as well as for improving forage crop digestibility (Table 1).

4. Genetic engineering of phenylpropanoid biosynthetic pathway

Despite the extensive literature on genetic modifications of lignin biosynthesis in a variety of plants, only a few studies have reported the impact of modified lignin content and composition on pulping and bleaching processes. Nevertheless, significant progress has been made in this field, as summarized in Table 1.

Emerging genetic engineering strategies *in planta* including manipulation of lignin biosynthesis at regulatory level, controlling monolignol polymerization enzymes, and modifications of lignin polymer structure, together with exploration of lignin degrading enzymes from other organisms provide us the necessary tools for producing designer plant species with reduced/altered lignin traits, so as to meet the needs of paper, livestock industries, etc. However, traditional genetic engineering strategies such as upregulation and downregulation of monolignol biosynthetic genes are still applied and have been successful in facilitating lignin decomposition by altering both lignin content and composition. One

such example based on genetic modifications in *Leucaena* is described here, providing insights into the reactions, and regulation of genes involved in lignin biosynthesis, and its impact on determining lignin quality for paper industries.

Plant	Gene	Lignin Content	Lignin Composition	Phenotype	References
Medicago	CAD	Reduced	S/G Ratio↓	Forage Digestibility↑	Baucher et al., 1999
	HCT	"	-	"	Shadle et al., 2007
	C3H/ HCT	"	H/G↑	"	Ziebell et al., 2010
	CP 450 enzymes	"	-	"	Reddy et al., 2005
	COMT	"	-	Cellulose content↑	Marita et al., 2003
	CCoAOMT	"	-	Forage Digestibility↑	Guo et al., 2001
Nicotiana	PAL/CCoAOMT	"	S/G Ratio↑	'	Sewalt et al., 1997
	CCR	"	S/G Ratio↑	Pulping Efficiency↑	O'Connell et al., 2002
	CAD	Unchanged	Aldehyde units↑	"	Halpin et al., 1994
	CAD/CCoAOMT	"	S/G Ratio↓	Forage Digestibility↑	Vailhe et al., 1998
	POX	Reduced	S/G Ratio↑	Saccharification↑	Kavousi et al., 2010
Arabidopsis	CCR	"	-	Forage Digestibility↑	Goujon et al., 2003
Alfalfa	C4H	"	S/G Ratio↓	"	Reddy et al., 2005
	HCT	"	High H	"	Shadle et al., 2007
	C3H	"	"	"	Reddy et al., 2005
	CCoAOMT	"	S/G Ratio↑	"	Guo et al., 2001a, b
	Cald5H	Unchanged	S/G Ratio↓	Unchanged	Reddy et al., 2005
	CAD	"	"	Forage Digestibility↑	Baucher et al., 1999
Populus	COMT	Reduced	"	Pulping Efficiency↓	Jouanin et al., 2000
	CCoAOMT	"	S/G Ratio↑	Pulping Efficiency↑	Petit, Conil et al., 1999
	CAD	Unchanged	Aldehyde units↑ free phenolics ↑	"	Lapierre et al., 2004
Picea	4CL	Reduced	S/G Ratio↓	Cellulose content↑	Voelker et al., 2010
Festuca	CCR	"	H/G Ratio↓	Pulping Efficiency↑	Wadenback et al., 2007
Maize	CAD	"	-	Forage Digestibility↑	Chen et al., 2003
Linum	COMT	"	S/G Ratio↓	"	He et al., 2003
	CCoAOMT	"	"	-	Day et al., 2009

Table 1. Genetic engineering of different lignin biosynthetic genes, and their effects on lignin contents and composition

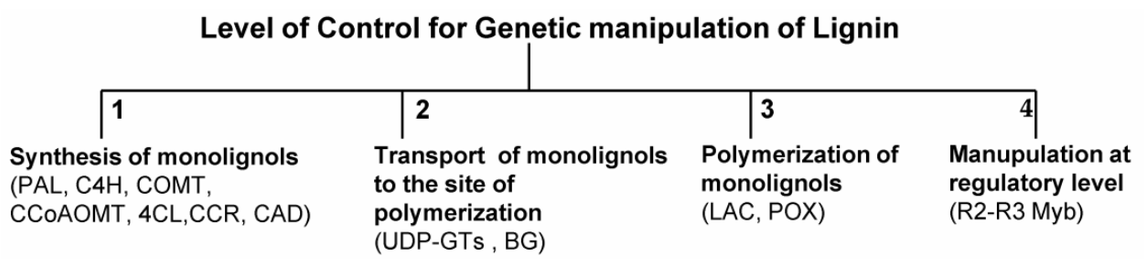


Fig. 3. Levels of control for manipulation of lignin biosynthesis. The control level 1 involves manipulation of lignin biosynthetic genes; level 2 and 3 involves manipulation of genes involved in transport and polymerization of monolignols; while level 4 deals with manipulations of different regulatory factors

4.1 *Leucaena leucocephala* as a source of pulp

A great deal of knowledge on the molecular biology and regulation of phenylpropanoid biosynthesis has been derived from investigations in plants such as *Arabidopsis*, Alfalfa etc.

While these models will continue to serve as platforms for studying lignifications, a number of other plant species, e.g., *Leucaena leucocephala* have recently been selected for such studies. The paper industry in India mainly uses bamboos, *Eucalyptus* sp., *Casuarina* sp. and *Leuacaena* sp. as a source for paper pulp. Selection of the species depends upon availability, price and acceptability by any given industrial unit. However *Leucaena* sp. is extensively used in India and nearly a quarter of raw materials for paper and pulp industry comes from this plant (Srivastava et al., 2011). *Leucaena* is also valued as an excellent source of nutritious forage.



(a) (b)

Fig. 4. (a) *Leucaena leucocephala* cv. K-636 growing at NCL premises, (b) A transgenic plant cultivated in our green-house. (Photographs courtesy of Shakeel Abbassi, NCL)

It has been estimated that dried leaves of *Leucaena* contain nearly 28-35% of protein content of high nutritional quality. Besides this, *Leuceana* is also an excellent source of firewood, industrial fuel, organic fertilizer, timber and gum (Cottom et al., 1977). A native of Central America, *Leucaena* has been naturalized pan-tropically, with members of its genera being vigorous, drought tolerant, highly palatable, high yielding & rich in protein and grow in wide range of soils (Hughes, 1998; Jones, 1979). However, these attributes are limited by the occurrence of anti-nutritive factors in the fodder, such as tannins and mimosine (Hammond et al., 1989; Hegarty et al., 1964; Jones, 1979).

4.2 Phenylpropanoid biosynthesis genes from *Leucaena leucocephala*

Different genes involved in phenylpropanoid biosynthesis were studied in detail from *Leucaena*. Table 2 summarizes the details of such genes isolated from *Leucaena* along with their GenBank accession numbers.

4.3 Regeneration system of *Leucaena leucocephala*

Genetic transformation of plants needs reproducible robust regeneration system. Regeneration of complete plants through tissue culture has made it possible to introduce foreign genes in to plant genome and recover transgenic plants. The limited success rate for

Name of Genes	Accession Nos.	cDNA, kb	Amino acid no.	Mm, kDa	Annotations
PAL	JN540043*	--	--	--	*Ll-PAL*
C4H	GU183363	1.836	505	57.94	*LlC4H1*
	HQ191221	1.761	505	57.93	*LlC4H2*
	HQ191222	1.760	505	57.99	*LlC4H3*
4CL	FJ205490	1.935	542	58.87	*4CL 1*
	FJ205491*	1.831	519	56.74	*4CL2*
CCoAOMT	DQ431234	0.735	244	*27.55*	*CCoAOMT2*
	DQ431233	0.735	244	*27.57*	*CCoAOMT1*
CCR	EU195224	1.005	334	36.30	*Ll-CCRH2*
	DQ986907	1.011	336	36.52	*Ll-CCRH1*
CAld5H	EU041752	1.826	511	57.42	*Ll-Cald5H*
CAD	EU870436	1.178	357	38.87	*Ll-CAD*
Lignin POX	GU143879	0.951	316	33.94	*POX1*
	GU143878	0.951	316	34.03	*POX2*
	EU649680	0.951	316	34.20	*Ll-POX*
Glucosylhydrolase	EU328158	1.524	507	57.57	*Ll-GH Family 1*
R2R3 MYB factor	GU901209	0.911	235	26.50	*Ll_R2R3_MYB2_SSM*
Cellulose Synthase(CesA)	FJ871987	3.391	1075	123.30	*Ll-7CesA*
	GQ267555	3.368	1073	119.97	*Ll-8CesA*

Table 2. Phenylpropanoid biosynthesis genes from *Leucaena*: C4H: Cinnamate 4-Hydroxylase; 4CL: 4 Coumarate CoA Ligase; CCoAOMT: Caffeoyl CoA O-Methyl Transferase; COMT: Caffeate *O*-Methyl Transferase; CCR: Cinnamoyl CoA Reductase; CAld5H: Coniferaldehyde 5-Hydroxylase; CAD: Cinnamyl Alcohol Dehydrogenase; POX: Peroxidase; *Partial sequence

regeneration of leguminous trees has been reported in few research works. The complete *in vitro* plantlet regeneration of *Leucaena* (Cultivar, K-67) from lateral bud explants has been optimized with the maximum shoot multiplication rate of 22 shoots per explants. These regenerated plantlets were transplanted *ex vitro* with 80% survival rates (Goyal et al., 1985). In addition to the regeneration from lateral bud explants, an alternative (both direct and callus mediated/indirect) plantlet regeneration system has been successfully demonstrated with 100% regeneration frequency using cotyledon explants from 3-4 days old plants (Cultivar, K-636). It is interesting to note that the plantlets regenerated from cotyledonary explants rooted without any requirements of growth regulators on basal media (Saafi et al., 2002). Addition of Thidiazuron (TDZ) to the shoot induction medium has substantially improved the number of *in vitro* shoots per explants as compared to the basal shoot induction medium with N^6-Benzyladenine (BA). Liquid pulse treatment of the induced shoots with TDZ resulted in the improvement in the subsequent rooting. The plantlets regenerated in this manner showed more than 90% survival rate *ex vitro* when grown in coco-peat mixture (Shaik et al., 2009). In order to improve overall *in vitro* plantlet regeneration efficiency, attempts to propagate elite (cultivar K-8, K-636) and wild type varieties of *Leucaena* were made by supplementing the basal shoot induction media with puterscin. It has been observed that putriscine (9.3 μM) significantly enhanced the number of regenerated shoots from hypocotyls explants when compared to the induction medium containg only BA (22.2 μM). The incidence of yellowing and leaf abscission was successfully

abridged by addition of glutamine (685 µM) or adenine (540 µM) which indirectly added *ex vitro* survival of the plants. All the regenerated plantlets from hypocotyls explants exhibited 100% *in vitro* rooting and were subsequently transplanted *ex vitro* (Sirisha et al., 2008). *In vitro* regeneration system for some other cultivars of *Leucaena* (K-8, K-29, K-68 and K-850) from mature trees derived nodal explants as well as seedlings derived cotyledonary node explants have also been reported, where cultivar K-29 gave the best response *in vitro*. Indirect (through callus phase) somatic embryogenesis of cv. K-29 using 40.28 µM NAA and 12.24 µM were also established. These somatic embryos were further matured in full strength medium (Rastogi et al., 2008).

Considering all above discussed reports, it can be suggested that our reports (Shaik et al., 2009) of improved method also have produced a consistent regeneration system for *Leucaena* which will be beneficial for the mass propagation and genetic transformation of *Leucaena* species. Lignin content in wood pulp adversely influences the quality of paper produced. In *Leucaena* which is an important paper pulp wood crop, it becomes important to identify and multiply elite clones having naturally low lignin content. In addition to this approach there is a need to develop transgenic plants with altered or reduced lignin content for its efficient and eco-friendly removal from pulp. The above mentioned multiple regeneration pathways are an excellent tool to introducing foreign genes. Out of all these methods shoot regeneration from cotyledonary node explants are more responsive to multiple shoot induction (Hussain et al., 2007). Genetic transformation procedures particularly particle bombardment which is considered as most effective means of gene delivery can be applied to the transformation of these shoot meristems. Cotyledonary explant derived multiple shoots form most suitable tissue for genetic transformation due to their higher regeneration frequency. Therefore, our recent study dealt with multiple shoot induction from the cotyledonary nodes of *Leucaena* in response to cytokinins, thidiazuron (TDZ) and N6-benzyladenine (BA) supplemented in half strength MS (½-MS) medium and also their effect on *in vitro* rooting of the regenerated shoots (Fig. 5). The addition of cytokinins to the medium was found essential for multiple shoot induction. *Leucaena* cotyledonary nodes carried a high potential for rapid multiple shoot regeneration on medium containing lower concentrations of TDZ (0.05 or 0.23 µM) (Shaik et al., 2009). As multiple shoots originated from the mass of closely placed shoot initials of axillary meristems (Fig. 5), this system could be efficiently used for particle bombardment mediated transformation. This efficient and high frequency *in vitro* regeneration system is highly reproducible and can be used for mass propagation and genetic transformation of *Leucaena*.

4.4 Genetic transformation of *Leucaena*

The genetic transformation protocols based on *Agrobacterium*-mediated and/or direct gene transfers by biolistic bombardment have been successfully applied to 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). Regeneration system for *Leucaena* has already been established in our previous works (Shaik et al., 2009; Sirisha et al., 2008). To exploit this to produce transgenic

Leucaena plants for reduced/altered lignin content, various phenylpropanoid pathway genes (C4H, 4CL, CCoAoMT, CCR, CAld5H, CAD and POX) were cloned and used for transformation experiments.

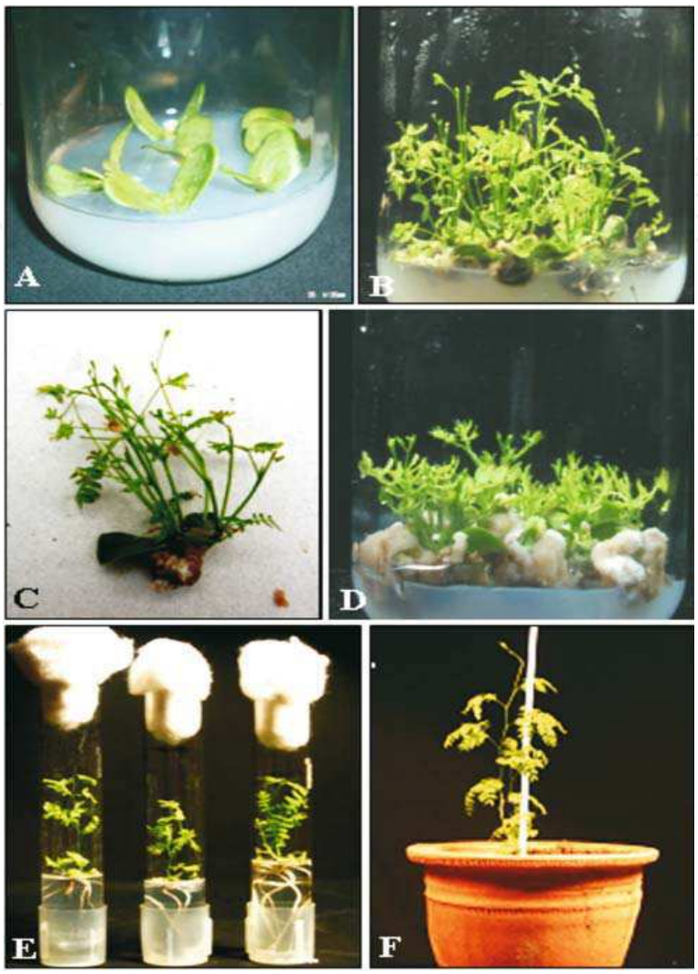


Fig. 5. *In vitro* shoot regeneration in *L. leucocephala* (cultivar K-636). A: Cotyledonary nodes, B-C: Multiple shoot induction in cotyledonary nodes on ½-MS + TDZ (0.23 µM), D: Shortened and fasciated shoots of *Leucaena* on ½-MS + TDZ (0.45 µM), E: Rooted shoots of *Leucaena* on ½-MS + NAA (0.54 µM) and F: Hardened *in vitro* propagated plant of *L. leucocephala* in Sand: soil mixture. (Shaik et al., 2009)

One day old embryo axes without cotyledons were used as explants for transformation. It was carried out by three methods: 1) Particle bombardment; 2) Particle bombardment followed by co-cultivation and 3) Agro-infusion method. The transformation efficiencies with various gene constructs are summarized in Table 3. It can be observed that maximum efficiency (100%) was noted with CAD using particle bombardment followed by co-cultivation as a means of transformation. However, CCR with the same procedure gave only 10% efficiency. In general it was concluded that a combination of particle bombardment method followed by co-cultivation was most effective in transforming the shoot meristems of *Leucaena*.

Gene	Method	No. of embryo axis used	No. of explants survived	No. of shoots elongated	Avg. shoot length*	No. of shoots used for DNA extraction	No. PCR +ve samples	Transformation efficiency (%)#
CCR	*P B*	67	59	30	3.67	8	4	50
CCR	*PB + CC*	107	94	40	2.97	20	2	10
CCR	*A I*	43	6	6	2.07	6	1	16.66
4CL1	*P B*	231	205	154	3.72	154	92	59.74
4CL1	*PB + CC*	109	83	47	3.41	47	38	80.85
4CL1	*A I*	60	22	04	1.17	4	2	50
CAD	*P B*	438	208	42	3.06	11	7	63.63
CAD	*PB + CC*	173	114	11	2.49	2	2	100
CAD	*A I*	147	32	16	1.68	6	6	100
CAld5H	*P B*	74	54	25	3.47	7	2	28.57
CAld5H	*PB* + CC	99	74	31	2.79	14	11	78.57
CAld5H	*A I*	34	5	4	2.00	2	-	-
C4H	*P B*	-	-	-	-	-	-	-
C4H	*PB* + CC	615	61	11	3.5	10	6	60
C4H	*A I*	-	-	-	-	-	-	-
POX	*P B*	58	47	44	1.36	23	17	73.91
POX	*PB* + CC	79	64	59	1.38	31	27	87.09
POX	*A I*	32	23	19	1.15	9	5	55.56

Table 3. Transformation efficiency of *Leucaena* downregulated with phenylpropanoid pathway genes using different methods. PB, Particle Bombardment; CC, Co-Cultivation; AI, Agro Infusion. *Measured after 3 rounds on selection, #Calculated as per the number of plants screened

4.4.1 Peroxidase (*LIPOX*): A case study

Numerous reports on peroxidase activity in lignin forming tissues have been reported, but only a few isoenzymes have been specifically associated with lignification (Sato et al.,1993; Quiroga et al., 2000; Christensen et al., 2001; Marjamaa et al., 2006). It is likely that the control of the whole lignification process requires a mechanism for the co-ordinated expression and/or activation of the monolignol biosynthetic genes/enzymes and the radical forming peroxidases. Data from transgenic plants down regulated for peroxidase activity has confirmed the role of some POX isoforms in lignin polymerization in tobacco and *Populus sieboldii* (Miq.)X *Populus grandidentata* (Michx.) (Talas-Ogras et al., 2001; Blee et al., 2003; Li et al., 2003b). Both quantitative (up to 50% reduction) and qualitative changes were reported, but no obvious growth phenotypes, other than larger xylem elements were found.

Recombinant pCAMBIA1301 binary vector harboring partial sequence of *LlPOX* in antisense orientation was used to transform the embryos isolated from aseptically germinated seeds of *Leucaena* (Fig. 6). Two fragments (one from conserved region and another from non-conserved region) of *LlPOX* cloned in anti-sense orientation were used for transformation. The *Leucaena* embryo axes were bombarded with microcarriers coated with recombinant pCAMBIA vectors using PDS-1000/He Biolistic Particle Delivery System. After growing the embryos on regeneration media without selection for one week, these embryos were subjected to three rounds of selections. The plants, which survived were shifted to ½-MS with Cytokinin, 2-ip (2-isopentenyl adenine) 0.5 mg/ L to enhance elongation of transformed shoots. In all the above cases, the bombarded explants were subjected to transient GUS assay 48 hrs after second bombardment and the putative transgenic plants, which survived three rounds of selection, were analyzed for the gene integration into the plant genome.

As an alternative strategy, *Leucaena* embryo axes were also transformed by a combination of particle bombardment followed by co-cultivation with *Agrobacterium* (GV2260) harboring

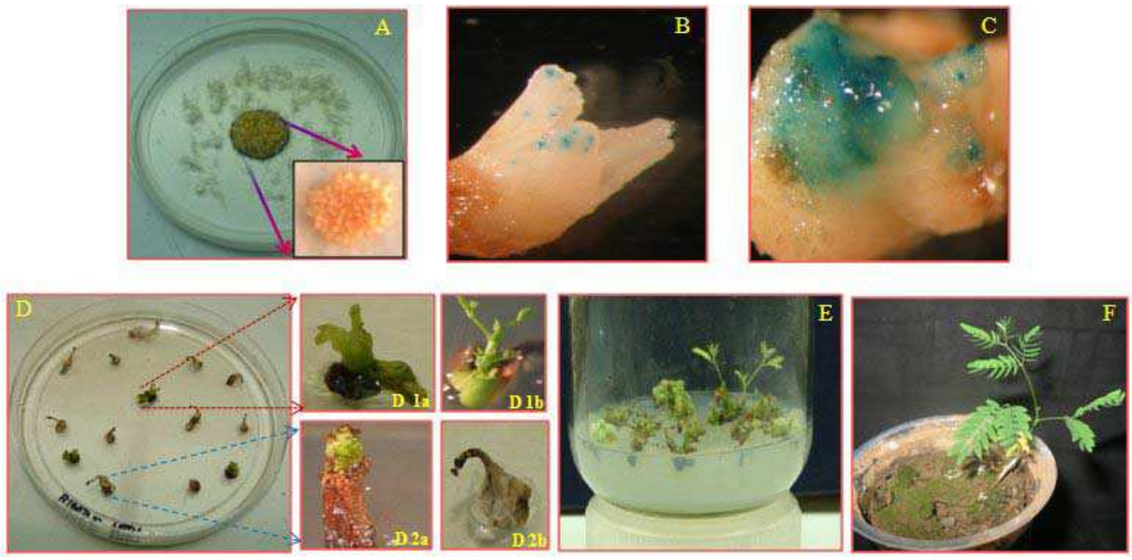


Fig. 6. Genetic transformations in *Leucaena leucocephala* by particle bombardment method using *Anti-LlPOX* construct. A: Embryos arranged for bombardment, B-C: Transient GUS expression, D. Bombarded embryos on selection, D1a and D1b. Regenerating embryos on selection, D2a and D2b. Necrosis and dying of untransformed embryos on selection, E-F: Regeneration and hardening of putative transformants



Fig. 7. a. Untransformed plant not subjected to genetic transformation, b. putative transformants using construct Anti-*LlPOX (NC)* of *POX* gene and c. putative transformants using constructs *Anti-LlPOX (C)* of *POX* gene. NC-Non conserved; C-Conserved

respective recombinant pCAMBIA vectors. After particle bombardment, the embryo axes were then transferred onto the respective regeneration medium, co-cultivated in the dark at 25 ± 2 °C for 3 days. After co-cultivation, the embryo axes were washed thoroughly with Cefotaxime 250 mg/ L in sterile distilled water and transferred onto the regeneration medium. Higher levels of transient GUS expression confirmed the transformation efficiency. The present study was performed using, two different antisense constructs of *Leucaena* peroxidase gene. We observed severe stunted or retarded growth in plants when transformed using constructs having conserved domain. These plants were found to grow barely up to 0.5 cm, soon followed by the death of the apical meristem and rise of a fresh axillary bud from its axis, which again dies and this process was found to be repeating. As a

result, the plant attained a height of 2.5 cm on an average and even failed to produce roots when transferred to rooting medium. When non-conserved *AntiPOX* construct was used in *Leucaena* transformation, normal regeneration was noticed but the plants were thin and slow growing compared to the untransformed control plants. Comparative growth pattern of *Leucaena* are shown in Fig. 7.

LlPOX was immuno-cytolocalized in the transformants generated following the above mentioned protocols. Control and transformed plants of same age group were selected. The control plants showed better growth and bio-metric parameters (height, growth and rooting) over the transformants. POX was immuno-cytolocalized in stem tissues of control untransformed plants (Fig. 8 A, B, C) and putative transformants (Fig. 8 D, E, F), with a view to find whether there exists reduction in peroxidase expression in lignifying tissues (*i.e* vascular bundle and xylem fibres). It was observed that the transformants showed reduced levels of POX near the sites of lignifications. It was also noted that *Leucaena* transformed by *AntiLlPOX* from conserved region resulted in discontinuity in vascular bundle assemblies.

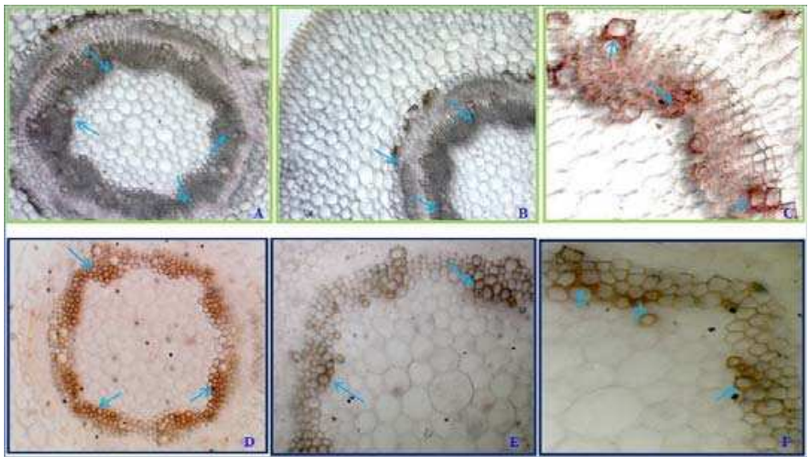


Fig. 8. Immuno-cytolocalization of POX in *Leucaena*. A, B & C stem sections of control plants showing higher levels of POX protein on xylem tissues over the transformed plants D, E & F. Control plants show a well developed vascular bundles (continuous ring) over transformants (discontinuous ring)

Genes Down-regulated	**Morphological Changes**	**Reduction in Lignin content**
4CL	No change	2-7%
CAld5H	No change	Yet to be analyzed
CCR	Stunted growth	4-13%
CAD	Stunted growth	2-8%
C4H	Stunted growth	Yet to be analyzed
POX(NC)	Stunted Growth	4-9%
POX(C)	Stunted and abnormal growth pattern	6-14%

Table 4. Lignin estimation of transgenic *Leucaena* plants. NC-Nonconserved; C-conserved

Likewise, rest of the antisense constructs (*4CL*, *CAld5*, *CCR*, *CAD*, and *C4H*) were successfully utilized for genetic transformation of *Leucaena* and were subsequently

characterized for transformation efficiency and lignin content (Table 4). Plants having antisense construct of *C4H*, *CCR*, *CAD* and *POX* showing stunted growth. But in case of *4CL* transformants no such morphological appearance were observed.

5. Conclusions

Thanks to years of painstaking research in to the chemistry of lignin, it is now seen as a potential target for genetic engineering of plants, mostly aggravated by its industrial and agricultural applications. However, much of our understanding of lignin biochemistry comes from studies of model plants like *Arabidopsis*, Tobacco, Poplar, etc. Furthermore, this technology needs to be transferred to other plant species. *Leucaena,* a multiple utility leguminous tree, is targeted for ongoing research to alter its lignin content due to its importance in paper and pulp industry in India. Keeping this in mind, attempts were made to improve pulp yielding properties by genetically engineering lignin metabolism so as to gratify the demand of such industries. The results presented here highlight the challenges and limitations of lignin down-regulation approaches: it is essential but difficult to find a level of lignin reduction that is sufficient to be advantageous but not so severe as to affect normal growth and development of plants.

These findings may contribute in the development of *Leucaena* with altered lignin composition/content having higher lignin extractability, making the paper & pulp industry more economic and eco-friendly. The multi-purpose benefits of lignin down regulation in this plant can also be extrapolated to improved saccharification efficiency for biofuel production and forage digestibility, apart from enhanced pulping efficiency. Although genetic engineering promises to increase lignin extraction and degradability during the pulping processes, the potential problems associated with these techniques, like increased pathogen susceptibility, phenotypic abnormalities, undesirable metabolic activities, etc. must be addressed before its large scale application. In order to overcome such barriers, significant progress must be made in understanding lignin metabolism, and its effects on different aspects of plant biology.

Nevertheless, the current genetic engineering technology provides the necessary tools for a comprehensive investigation for understanding lignin chemistry, which were hardly possible using classical breeding methods.

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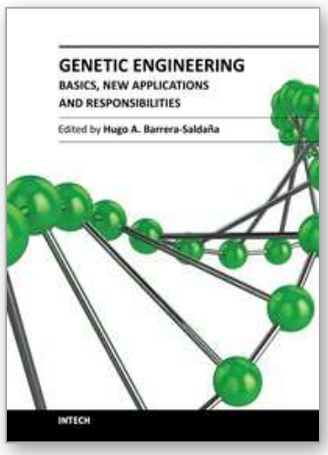
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Genetic Engineering - Basics, New Applications and Responsibilities

Edited by Prof. Hugo A. Barrera-Saldaña

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Leading scientists from different countries around the world contributed valuable essays on the basic applications and safety, as well as the ethical and moral considerations, of the powerful genetic engineering tools now available for modifying the molecules, pathways, and phenotypes of species of agricultural, industrial and even medical importance. After three decades of perfecting such tools, we now see a refined technology, surprisingly unexpected applications, and matured guidelines to avoid unintentional damage to our and other species, as well as the environment, while trying to contribute to solve the biological, medical and technical challenges of society and industry. Chapters on thermo-stabilization of luciferase, engineering of the phenylpropanoid pathway in a species of high demand for the paper industry, more efficient regeneration of transgenic soybean, viral resistant plants, and a novel approach for rapidly screening properties of newly discovered animal growth hormones, illustrate the state-of-the-art science and technology of genetic engineering, but also serve to raise public awareness of the pros and cons that this young scientific discipline has to offer to mankind.

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