Chapter 5
Chapter 5 Transformation studies of Leucaena

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 cocultivation 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 overexpression of the genes but, as originally observed through the introduction of chalcone synthase transgene into petunia (Napoli et al., 1990), the sense strategy may also lead to silencing (down-regulation) of both the endogene and the transgene due to co-suppression (i.e. post-transcriptional gene silencing, PTGS). The molecular mechanism of the gene silencing was unclear for long time until the discovery of RNA interference (RNAi) (Yu & Kumar, 2003; Matthew, 2004; Chen, 2005; Bonnet et al., 2006; Zhang et al., 2006). In the RNAi silencing process, the transgene gives rise to long double-stranded (ds) RNA molecules, which are enzymatically cleaved into very small pieces of RNA (21 nt), referred to as small interfering RNAs (siRNAs). siRNAs are then incorporated in an RNA
silencing system (RISC: RNA induced silencing complex) which is able to recognize, bind and induce cleavage or translation repression of complementary mRNAs (Bonnet et al., 2006; Zhang et al., 2006). The RNAi technique is currently being applied for the efficient production of down-regulated or 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-Orczyk, 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.
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### 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 weak absorption), which results from an internal p-hydroxybenzylideneimidazolinine fluorophore generated by an autocatalytic cyclization and oxidation of a ser-gly sequence at aminoacid residues. The other advantage of gfp as a reporter gene is that no exogenously supplied substrate/cofactors are needed for its fluorescence emission at 508 nm.

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

### 5.1.5. 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 thioacidolysis, 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
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promoter, Pt4CL1IP. 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 Li4CL1 gene was transferred into model plant system Nicotiana tabacum 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 explant 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 L14CL1 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 L14CL1 gene has internal site for *KpnI* and *SacI*, thus L14CL1 was double digested using these two enzymes. The released fragment was eluted from agarose gel and was cloned in *KpnI* and *SacI* 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 shortcomings, 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. *EcoRI* and *HindIII* 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 *EcoRI* and *HindIII* enzymes. The digested cassette was eluted from agarose gel and cloned in *EcoRI* and *HindIII* digested pCAMBIA1301 vector (Fig. 5.2).

(a) **L14CL1 gene**

![Diagram of L14CL1 gene with restriction sites](image-url)
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(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
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(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 Li4CL1 gene and Nos terminator, (c) Constructed vector map of pCAMBIA1301.

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

Fig 5.3: *Kpn*I/*Sac*I digested 1.0 kb fragment of Li4CL1 gene from pCAMBIA1301 vector. Lane M: 1 Kb Ladder, Lane-1& 2: *Kpn* I/ *Sac* I digested 1.1 Kb cloned fragment of Li4CL1 gene. Lane 3: *Kpn*I digested constructed vector, Lane 4: *Sac*I digested constructed vector.
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5.2.4. *Agrobacterium tumefaciens* transformation

*A. tumefaciens* GV2260 was transformed with the pCAMBIA1301 vectors harboring 5’ 35SPro-AntiLI4CL1- 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* cultures, harboring the 5’ 35SPro-AntiLI4CL1- 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-AntiLI4CL1- 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
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ccentration from 2 to 40mg/L. LD₅₀ 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, LD₅₀ 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
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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 antiL14CL1 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 plant (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 L14CL1 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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(Cont.)
5.3.3.1. 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 hygromycin 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 antiLi4CL1 with 35S forward and 35S reverse primer of 35S promoter.

\[
\begin{align*}
35S F & 5' \text{ACAGTCTCAGAAGACCAAAGGGCT} 3' \\
35S R & 5' \text{AGTGGGATTGTGCGTCATCCCTA} 3'
\end{align*}
\]

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 anti-Ll4CL1 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).

35S F 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.

![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 35S 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).](image)

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

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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 (Chapter 2: 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).
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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).
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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

<table>
<thead>
<tr>
<th>pCam.1301.hyg</th>
<th>Transgenic plant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTATTCTTTTCGCTGCCGACGACGGCTGGGCGTCTGTTTCCACTATCGGCGAGTACTT</strong></td>
<td><strong>------------------------AGGTTGCGGGCGCTGTGTT--CAGTATCTGGGAGTACTT</strong></td>
</tr>
<tr>
<td>*** ******* ****  *******************</td>
<td>*** ******* ****  *******************</td>
</tr>
<tr>
<td><strong>TACACAGCCATCGGCAGACGGCCGCTCGGATGCTGCTGCTGCTGCT</strong></td>
<td><strong>TACACAGCCATCGGCAGACGGCCGCTCGGATGCTGCTGCTGCTGCT</strong></td>
</tr>
<tr>
<td>************************************************************</td>
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<tr>
<td><strong>CCCAGCTCCGAGATCGGGCGGATCTGCGCTCACTGGGCGACGCACTGACGGTGTCGTCCATC</strong></td>
<td><strong>CCCAGCTCCGAGATCGGGCGGATCTGCGCTCACTGGGCGACGCACTGACGGTGTCGTCCATC</strong></td>
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<tr>
<td><strong>GATCGCATCCATAGCCTCCGCGACCGGTTGTAGAACAGCGGGCAGTTCGGTTTCAGGCAG</strong></td>
<td><strong>GATCGCATCCATAGCCTCCGCGACCGGTTGTAGAACAGCGGGCAGTTCGGTTTCAGGCAG</strong></td>
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<tr>
<td><strong>CACAGCTCATCGAGAGCCTGCGCGACGGACGCACTGACGGTGTCGTCCATC</strong></td>
<td><strong>CACAGCTCATCGAGAGCCTGCGCGACGGACGCACTGACGGTGTCGTCCATC</strong></td>
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<td><strong>GATCGCATCAGCACTGGGCAATGGGCGGAACCCGCTCGTCTGGCTAAAGATCGGCCGCAGC</strong></td>
<td><strong>GATCGCATCAGCACTGGGCAATGGGCGGAACCCGCTCGTCTGGCTAAAGATCGGCCGCAGC</strong></td>
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<tr>
<td><strong>CCAGTGATACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCATGTAGTGTATTG</strong></td>
<td><strong>CCAGTGATACACATGGGGATCAGCAATCGCGCATATGAAATCACGCCATGTAGTGTATTG</strong></td>
</tr>
<tr>
<td>************************************************************</td>
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</tr>
<tr>
<td><strong>ACCAGTATACACTATCGGCCAATCGCCGCATATGAAATACCGCCATATGAGTATGAT</strong></td>
<td><strong>ACCAGTATACACTATCGGCCAATCGCCGCATATGAAATACCGCCATATGAGTATGAT</strong></td>
</tr>
<tr>
<td>************************************************************</td>
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<tr>
<td><strong>GATCGCATCAGCACTGGGCAATGGGCGGAACCCGCTCGTCTGGCTAAAGATCGGCCGCAGC</strong></td>
<td><strong>GATCGCATCAGCACTGGGCAATGGGCGGAACCCGCTCGTCTGGCTAAAGATCGGCCGCAGC</strong></td>
</tr>
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<td>************************************************************</td>
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</tr>
</tbody>
</table>

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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.

\[
\begin{align*}
35SF & \ 5' \ ACAGTCTCAGAAGACCAAAGGGCT \ 3' \\
35SR & \ 5' \ AGTGGGATTGTGCCGTCATCCCTTA \ 3'
\end{align*}
\]

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

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.
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CLUSTAL W (1.8) multiple sequence alignment

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 LI4CL1 gene in antisense orientation in transgenic Leucaena genome

PCR amplifications from the genomic DNA of the transformation events antiLI4CL1 with forward primer of 35S promoter and forward primer from the mid region of LI4CL1 gene sequences (this forward primer from LI4CL1 gene was used as reverse primer as the LI4CL1 gene was in antisense orientation).

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 LI4CL1 gene. The nucleotide sequences were aligning with the 35S
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promoter of pCAMBIA1301 using CLUSTAL W (1.8) multiple sequence alignment and it showed almost 100% similarity with 35S promoter and a part of L14CL1 gene (Fig 5.20).

Fig 5.19: PCR amplification of 1.2 Kb 35S promoter and a part of L14CL1 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: Positive control using pCAMBIA vector, Lane N: non transformed plant, Lane Nt: No template control, Lane M: 1 Kb ladder.

CLUSTAL W (1.8) multiple sequence alignment

| anti4CLinpCAMBIA1301 | ACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAAC |
| pCAMamp135SF&Lec5F   | ACAGTCTCAGAAGACCAAAGGGCTATTGAGACTTTTCAACAAAGGGTAATATCGGGAAAC |
|                       | ***************************************************************** |
| anti4CLinpCAMBIA1301 | CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAA |
| pCAMamp135SF&Lec5F   | CTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTCATCAAAAGGACAGTAGAAAAGGAA |
|                       | ***************************************************************** |
| anti4CLinpCAMBIA1301 | GGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT |
| pCAMamp135SF&Lec5F   | GGTGGCACCTACAAATGCCATCATTGCGATAAAGGAAAGGCTATCGTTCAAGATGCCTCT |
|                       | ***************************************************************** |
| anti4CLinpCAMBIA1301 | GTTCCAACCACGTCTTCAAAAGCAGTGGGATAGTGATATCCTCCACTGACGTAAGGGAT |
| pCAMamp135SF&Lec5F   | GTTCCAACCACGTCTTCAAAAGCAGTGGGATAGTGATATCCTCCACTGACGTAAGGGAT |
|                       | ***************************************************************** |

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anti4CLinpCAMBIA1301
pCAMampI35SFLec5F

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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

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

- Approximately 1.0 kb fragment of Li4CL1 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.