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DEVELOPMENT OF LOW LIGNIN *EUCALYPTUS CAMALDULENSIS* THROUGH DOWN-REGULATION OF ENDOGENOUS CINNAMOYL COA REDUCTASE (CCR)

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Abstract

Renewable fuel resources can provide an alternative to meet the energy demands and compensate the diminishing resources of fossil fuels. Lignocellulosic biomass from non-crop plants can provide an alternative for the production of biofuels. However, presence of lignin in lignocellulosic biomass is a limiting factor to achieve industrial objectives. Industrial methods for the removal of lignin from lignocellulosic biomass are cumbersome, inefficient and expensive. The objective of this study was to produce lignocellulosic biomass with low lignin contents by down regulation of endogenous Cinnamoyl CoA Reductase (*CCR*) in *Eucalyptus camaldulensis*. Lignin downregulation was accomplished through RNAi. Putative transgenic plants were screened for the presence of transgene through polymerase chain reaction (PCR). *CCR* expression profiling, lignin estimation and effects of lignin down regulation leading to morphological changes were studied in transgenic and control *E. camaldulensis* plants. Real time Reverse transcriptase PCR for expression profiling of endogenous *CCR* gene revealed down regulation of *CCR* RNA transcripts from 72% to 80% in transgenic as compared to control and wild type plants. *CCR* enzyme activity was significantly reduced in transgenic plant as compared to control plant. Lignocellulosic biomass from stem of transgenic plants showed reduction in Klason lignin to 25.5% in transgenic plants as compared to wild type *E. camaldulensis*. Transgenic plants showed ~28% height reduction and ~53% stem girth reduction in comparison to untransformed control plants. Transformation efficiency of 0.13% was observed after successful transformation, regeneration, selection, molecular screening and expression profiling of the transgenic plants. This study confirmed role of *CCR* downregulation leading to lignin reduction in *E. camaldulensis* through RNAi for development of lignocellulosic biomass with low lignin contents.

Key words: *Eucalyptus Camaldulensis*; Low lignin; Cinnamoyl Coa Reductase

Introduction

There has been an increase in energy demand since last few decades (Ding *et al.,* 2021). The energy produced from fossil fuel is one of the major energy resources. Fossil fuel resources are depleting rapidly which has increased fuel prices, unemployment and global economic crisis (Aktar *et al.,* 2021). Hence, there is a dire need to produce efficient, low cost and renewable energy resources. Biofuels are hope for the future as being "renewable energy resource". Low cost and abundance of lignocellulosic biomass makes it a future renewable energy resource for the production of biofuels (Rezania *et al.,* 2020). The fuel derived from living matter is termed as "biofuel" (Voloshin *et al.,* 2016). Processed forms of biofuels are available in solid, liquid, gaseous form which have been further classified in to first-generation, second generation and third generation biofuels. This classification is based upon the type of biomass and technique used for the biofuel production (Jouzani *et al.,* 2020). Most common form of biofuel feed stock is from staple crops like corn, sugarcane etc. The use of food crops, fertile land and water for the production of biofuels are limitations for their production (Podkuiko *et al.,* 2014) due to high food demand with increasing global population. Hence, use of food crops, fertile land and water is undesirable for biofuel production as it creates the dilemma 'food versus fuel'. Therefore, there has been great surge in research interest for the production of biofuels from the lignocellulosic biomass from non-food crops (Sims *et al.,* 2010; Raud *et al.,* 2014: Kikas *et al.,* 2016).

Lignin is a complex heteropolymer present in lignocellulosic biomass (Donaldson *et al.,* 2017). After cellulose and hemicellulose, lignin is the third major constituent of lignocellulosic biomass of hard wood plants. Lignin plays key role in providing physical support, water proofing and resistance against microbial and pest attacks (Hendriks & Zeeman, 2009). The role of lignin in plant growth and development is crucial for providing support to plants in adapting terrestrial harsh habitats. However, presence of lignin determines the recalcitrance of lignocellulosic biomass for its conversion to biofuels (Peter *et al.,* 2007; Oliveira *et al.,* 2020). The coverage and bondage between hemicellulose, cellulose and lignin inhibits enzymatic degradation, hydrolysis, saccharification and availability of fermentable sugars which ultimately affects digestibility of lignocellulosic biomass for biofuel production (Agbor *et al.,* 2011; Zabed *et al.,* 2016; Xie *et al.,* 2020). Biomass having high cellulose as compared to lignin is desirable for the production of high grade biofuels (Raud *et al.,* 2014, Bajpai, 2016).

Pakistan has total geographical area of 79.61 million hectare out of which approximately 21 million hectare is cultivable. Water logging (30%) and salinity (21%) of the agricultural land is one the major problem for Pakistan. Water logging and salinity have affected major part of agricultural lands in Punjab and Sindh (Qureshi, 2016; Ashraf *et al.,* 2020). *E. camaldulensis* can be planted on these waste lands for the production of biomass. Most importantly *Eucalyptus* plantation can also protect cultivable agricultural land for biomass production (Subhani *et al.,* 2000; Singh & Dhillon, 2020).

E. camaldulensis belongs to family Myrtaceae and is native to Australia (Bown, 1995; Younoussa *et al.,* 2020). *E. camaldulensis* is often referred as "river red gum. *E. camaldulensis* is a perennial ever green tree with single stem and can attain height of up to 30-45 meters (Bren & Gibbs, 1986; Keatley *et al.,* 2021). This *Eucalyptus* specie has fast growth, biomass yield and short rotation period. It has capability to grow in variety of environments including saline and water logged soils making it an ideal candidate for the good source of biofuel (Girijashankar, 2011; Singh & Dhillon, 2020).

Cinnamoyl CoA Reductase (*CCR*) is involved in final steps of lignin biosynthesis pathway as it confers the conversion of p-coumaroyl CoA and feruloyl CoA to p-coumaraldehyde and coniferylaldehyde respectively. This reduction step of CoA esters is the first step in monolignol formation which cannot be completed without *CCR*. *CCR* is considered crucial and can act as the key limiting enzyme in lignin biosynthesis (Ralph *et al.,* 1998; Goujon *et al.,* 2003). *CCR* down regulation has been proved a useful technique for the downregulation of lignin in plants (Van der Rest *et al.,* 2006; Leplé *et al.,* 2007; Jackson *et al.,* 2008; Wadenbäck *et al.,* 2008; Prashant *et al.,* 2011, Ponniah *et al.,* 2017). Down regulation of *CCR* in *E. camaldulensis* was an interesting prospect for development of a non-food plant with low lignin contents having potential to grow on non-cultivable lands in Pakistan. We have studied down regulation of *CCR* in *E. camaldulensis* for down regulation of lignin contents.

Material and Methods

Transformation of *EuCCR* pSB219 into *Agrobacterium* strain LBA 4404: *EuCCR* sense-antisense construct (Fig. 1) was developed and cloned in plant expression vector pSB219 at National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad. The construct was provided in *E. coli* strain dh10α. Plant expression vector pSB219 harboring *EuCCR* sense-antisense construct was isolated from *E. coli* dh10α and transformed into electro competent *Agrobacterium tumefaciens* strain LBA4404 and selected on LB medium 50mg/L Spectinomycin and 50mg/L Rifampicin. Colony Polymerase Chain Reaction (PCR) analysis of selected *A. tumefaciens* colony was conducted for the presence of target *CCR* gene using *CCR* primers set given in Table 1.

Table 1. Primers used for molecular screening.

Oligo name		Sequence 5´-3´
CCR	*CCR*-F	GCAAGGCAGTGGCGAAGA
	CCR-R	CAGGCTGAAGTCCAGCTGCCAGAAAC
Bar	*bar*-F	TGCACCATCGTCAACCACTACATCGAG
	bar-R	GTGTACAAAGGGCAGGGA

Plant material, transformation and tissue culture: *E. camaldulensis* seeds were obtained from Ayub Agricultural Research Institute, Faisalabad. These seeds were used in all transformation experiments. Seed sterilization, transformation and tissue culture methods were used already reported by Ahad *et al.,* 2014 and Ahad *et al.,* 2018.

Optimization of selection medium for putative transgenic plants: Phosphinothricin acetyl transferase (*pat*) resistance gene (*bar* gene) was used as selection marker to select putative transgenic plants. Shoot induction medium supplemented with Basta/glufocinate from 1-8mg/L was used for the optimization of selection medium. Shoot induction medium supplemented with stringent Basta concentration was used to select putative transgenic shoots after 4 weeks of inoculation on selection medium.

Acclimatization and hardening of putative transformants: Rooted putative transgenic and untransformed control plantlets were transferred to the pots containing 50% peat moss+ 25% perlite + 25% vermiculite mixture under light condition. Plantlets were kept under high humidity which was gradually reduced to acclimatize plants. Acclimatized plants were shifted to soil containing pots under glass house condition for hardening. In mild weather at 25-30°C all transgenic plants were shifted to net house condition.

Molecular screening of putative transgenic plants: Molecular screening of the hardened putative transgenic shoots was performed to confirm the integration of the construct through polymerase chain reaction (PCR) using phosphinothricin acetyl transferase resistance gene (*bar gene)* primers as a target. Plant DNA was isolated from 100mg of freshly collected *E. camaldulensis* leave by using Murray and Thompson, 1980 CTAB method. The confirmation of putative transformants was conducted by amplification of target fragment of *bar* gene using primer sequence described in Table 1.

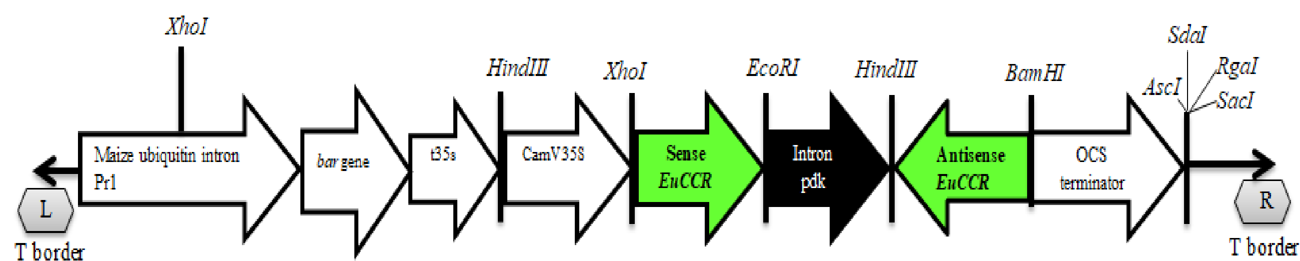

Fig. 1. A schematic diagram of pSB219 T-DNA region. *Eucalyptus CCR* in sense-antisense orientation under *CamV35S* promoter and Octopine synthase (*OCS*) terminator and *bar* gene under *t35S* terminator. Restriction enzyme specific restriction sites are represented by the enzyme names between Right and Left border of T-DNA.

Expression analysis by Reverse transcriptase Real time RT-PCR: Total RNA from control, transgenic and wild type *E. camaldulensis* plants was isolated using Thermo Scientific® GeneJET® Plant RNA purification kit. RNA quantity was measured using NanoDrop®. cDNA was prepared using oligo dT primers at 42°C for 60 minutes followed by reaction termination 70°C for 10min.

Primer against *Eucalyptus* Cinnamoyl CoA reductase (*CCR*) and ribosomal 18s primers (Unnikrishnan *et al.,* 2011) were designed by using pBLAST at NCBI with 200bp amplicon size and manufactured from Gene Link™ Hawthorne. Primer set sequence for *CCR* and 18s rRNA are given in Table 2.

Table 2. Primers used for expression profiling.

Oligo name		Sequence 5´-3´
CCR	CCR-RT-F	CTTGGACCGCTCCTTCAG
	CCR-RT-R	CACTCTCGTCGCACAAATAG
18s rRNA	Eu18srRNAF	ATGCCCTTAGATGTTCTGGG
	Eu18srRNAR	GTGTACAAAGGGCAGGGA

***CCR* activity assay:** Assay mixtures were prepared using total protein extract from untransformed control and transgenic plant. Total protein extract was obtained through sonication in the presence of Triton X-100 and 10 µg total protein was used for *CCR* assay. *CCR* assay was performed according to the procedure describe by Wengenmayer *et al.,* 1976. 50µL reaction was carried out in a 1.5mL microfuge tube at 30°C. The reaction mixture consisted of 0.1 M potassium phosphate buffer pH 6.25, 0.1 mM of NADPH, 10µg of purified protein sample and 70µM feruloyl CoA. The reaction was started by the addition of substrate feruloyl CoA, the absorbance was taken at 366 nm between 0-20 min with equal interval of 5 minutes.

Estimation of lignin content: Wood from main stem of 6 year old transgenic and control was collected by cutting the stem approximately 2 inch from the bottom while wild type *E. camaldulensis* mature branch was used as sample for lignin estimation. Extraction of samples was performed using a Soxhlet apparatus as described by Sluiter *et al.,* 2005. Total lignin from the extractive free biomass was estimated by using Sluiter *et al.,* 2008 method. Lignin was estimated as acid insoluble lignin (AIL) and acid soluble lignin (ASL).

Total lignin was estimated on extractive free basis as follow:

$$\%Lignin_{ext\ free} = \%\ AIL + \%\ ASL$$

Study of physical parameter of transgenic and control plants: Effects of *CCR* down regulation in transgenic and untransformed control *E. camaldulensis* plants were studied by observation and recording of stem length, width and change in coloration of wood biomass.

Results

Transformation of pSB219 *EuCCR* into Agrobacterium strain LBA 4404: Selected agrobacterium colonies were subjected to colony PCR for the presence of transgene using *CCR* gene target primers and amplified PCR product was visualized on 1% Agarose gel (Fig. 2). A PCR product of 336bp pertaining to *CCR* was observed after staining and visualization of 1% Agarose gel.

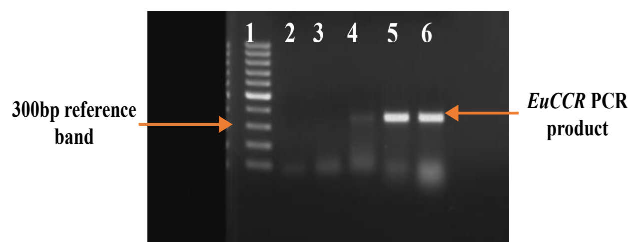

Fig. 2. Confirmation of transgene through colony PCR in *Agrobacterium* strain LBA4404 targeting *CCR* gene in construct. Colony PCR amplified product was resolved on 1% Agarose gel supplemented with Ethidium bromide and gel imaging was performed using UVP GelDoc-IT® Imager after resolving the gel. Lane 1. 100bp Marker. 2. No-template negative control. 3. Non-target template negative controls. 4. Positive control. 5 & 6 *CCR* gene PCR product of selected *Agrobacterium* colonies.

Optimization of selection medium: Regenerating untransformed control shoots were incubated on shoot induction medium supplemented with 1-8 mg/L Basta® for 4 weeks with sub culturing after every 2 weeks. 8mg/L of Basta was found to be efficient for selection of putative transgenic plants for 1 month of culturing on selection medium. 8 mg/L Basta concentration gave maximum necrosis to control shoots. These results suggested the use of 8 mg/L of Basta for selection of putative transgenic shoots. Whereas, 1mg/L Basta had no effect and 2-4mg/L Basta had mild to moderate effects on regenerating control shoots on selection medium (Fig. 3).

Selection of putative transgenic shoots: Transgenic shoot were selected by using 8mg/L Basta in SIM for four weeks with sub culturing after every 2 weeks. Healthy growing shoots were observed after one month on selection medium while non-transformed regenerated shoots showed necrosis (Fig. 4).

Acclimatization and hardening of putative transgenic and control plants: Acclimatization and hardening procedures were found to be effective for the transfer of transformed and untransformed control plants from *In vivo* to net house conditions (Fig. 5A and 5B).

Molecular screening of putative transgenic plants: Genomic DNA from putative transgenic plants PCR showed amplification of 500bp *bar* gene amplicon on 1% Agarose gel. Genomic DNA from control plants served negative control and plasmid DNA with the target construct served as positive control in PCR reaction (Fig. 6).

***CCR* Expression analysis of transgenic plants:** Real time RT PCR was performed by isolating total RNA from transgenic, wild type and control plants. Wild type and control plants showed high relative expression of *EuCCR* gene as compared to transgenic plants. The high relative expression of *EuCCR* in control and wild type plants confirmed successful expression of sense-antisense *EuCCR* cassette in transgenic plants leading to down regulation of endogenous *CCR* mRNA (Fig. 7).

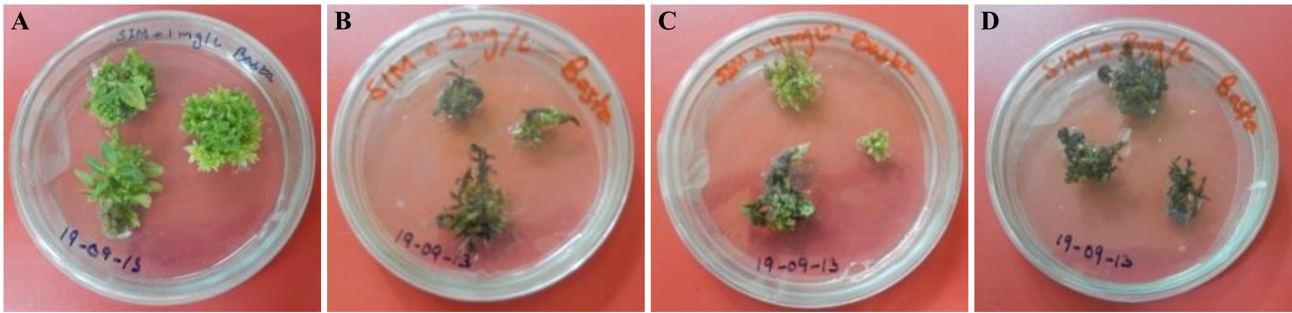

Fig. 3. Optimization of Basta selection medium.
Optimization of selection medium was performed by transferring *E. camaldulensis* untransformed control regenerated shoots to Shoot Induction Medium (SIM) supplemented with 2g/L gelzan and 1-8 mg/L Basta under light condition at 40.5 µmolm-2s-1 in tissue culture room. **A)** Regenerated shoots on SIM with 1mg/L Basta showed no growth restriction and necrosis **B-C)** Regenerated explants on SIM with 2 and 4 mg/L Basta respectively **(D)** Regenerated explants on SIM with 8mg/L Basta showing necrosis in untransformed control explants this concentration of Basta was found to control growth of untransformed tissues on the selection medium after 1 month of culturing.

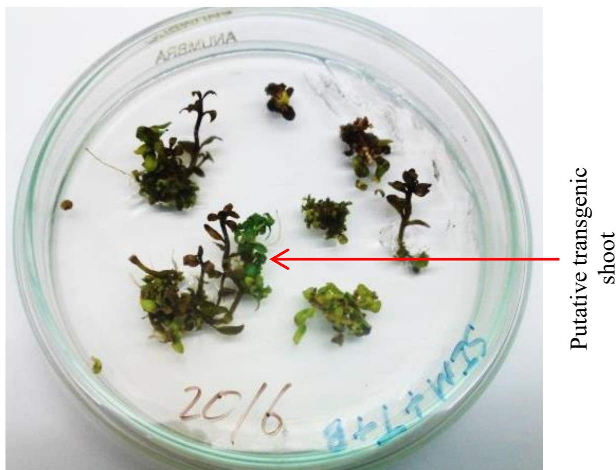

Fig. 4. Selection of transgenic shoot on selection medium
E. camaldulensis transformed regenerated explants were transferred to Shoot Induction Medium (SIM) supplemented with 2g/L gelzan, 400mg/L timentin and 8mg/L Basta under light condition at 40.5 µmolm-2s-1 and 25°C temperature in tissue culture room. Putative transgenic shoots showed growth on Basta selection medium after 1 month of culturing meanwhile all the untransformed tissues showed necrosis. These putative transgenic shoots were separated, transferred to shoot elongation medium (SEM) and subsequently subjected to rooting.

Fig. 5. Hardening of *E. camaldulensis* putative transgenic and untransformed control plants
Acclimatized putative transgenic plants were subjected to hardening by transferring to soil in clay pots in glass house and net house condition. Plants were given 1x Hoagland (Hoagland, 1920) solution for growth. After 2-4weeks in glass house plants were transferred to net house during fall, winter and spring season. **A & B)** Hardening of putative transgenic and untransformed control plant under glass house and net house conditions.

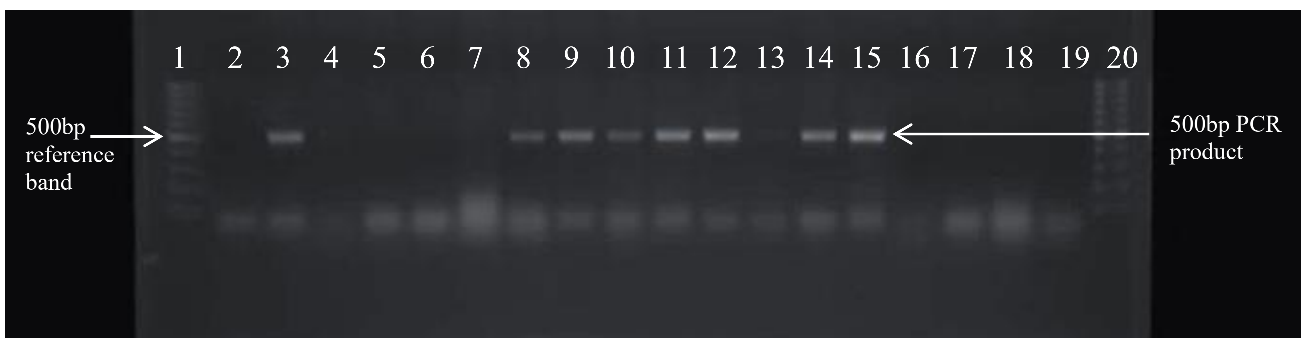

Fig. 6. Molecular screening of putative transgenic plants
Genomic DNA from control plants served as negative control while plasmid with *bar* gene served as positive control. Amplified PCR product was resolved on 1%Agarose gel supplemented with Ethidium bromide for 45 minutes at 100volts gel image was taken using UVP GelDoc-IT® Imager. **Lane 1**. 100bp marker. **Lane 2**. Non-template negative control. **Lane 3**. Positive control. **Lane 4-7**. Untransformed control plants controls. **Lane 8-19**. Putative transgenic plants **Lane 20**. 100bp Marker. A 500bp amplified PCR product was observed in transformed putative transgenic plants related to successful transformation of transgene.

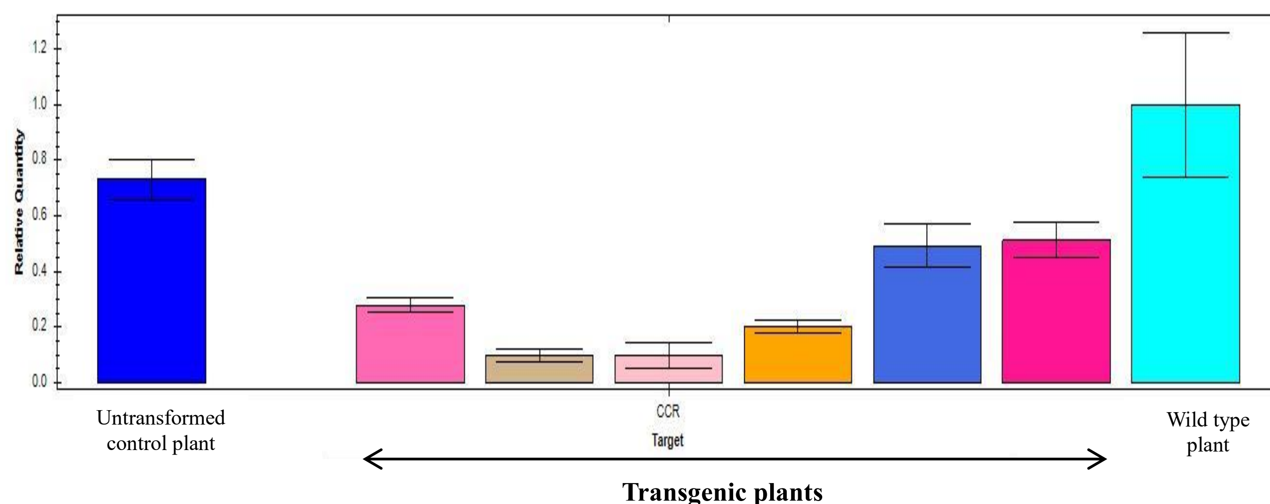

Fig. 7. Real time qPCR bar chart showing relative expression of *CCR* gene in transgenic, control and wildtype *E. camaldulensis* plants. High level *CCR* expression was observed in control and wildtype plants whereas transgenic plants expressed *CCR* at lower levels confirming successful expression of *EuCCR* sense-antisense cassette in transgenic plants.

CCR activity assay: The absorbance change for the extract of untransformed control plant corresponded to 58 nkat whereas no significant change in absorbance was detected in the case of the extract of transgenic plant demonstrating the down-regulation of *CCR* in transgenic plant described in Table 3 and Fig. 8.

Table 3. *CCR* activity assay absorbance taken at 366 nm for control and transgenic protein extracts using feruloyl-CoA as substrate.

Time (minutes)	Untransformed control plant	Transgenic plant
0	2.073	2.101
5	1.969	2.048
10	1.861	2.047
15	1.751	2.053
20	1.645	2.044

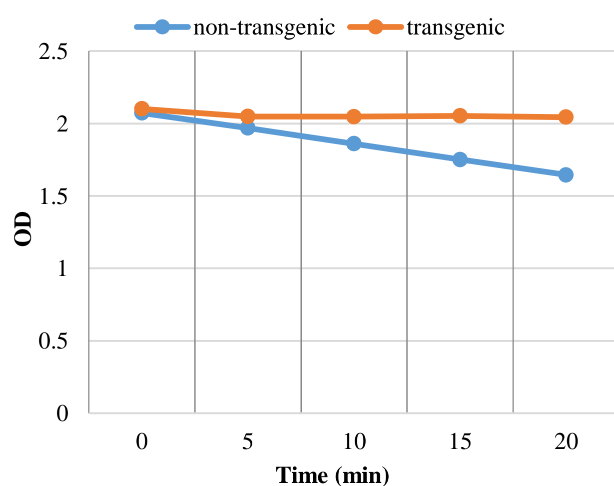

Fig. 8. *CCR* activity comparison of non-transgnic control and transgenic plant for the conversion of feruloyl CoA to coniferaldehyde leading to decrease in absorbance in non-transgnic control plant while feruloyl CoA concentration changed to negligible range confirming the CCR activity down regulation in transgenic plant.

Estimation of lignin contents: Estimation of lignin contents of transgenic and control plants was done using modified method of Sluiter *et. al.,* 2008 results of the lignin estimation in wild type, control and transgenic plants are given in Table 4.

Study of physical parameters and biomass: Stem length and diameter of all of the PCR confirmed transgenic and control plants were measured. A drastic reduction in stem length and diameter was observed as in all of the transgenic plants exhibiting low *CCR* expression which was confirmed through real-time PCR (Table 5 & Fig. 9). Twenty mesh ground biomass from stem of control and transgenic plants showed bleaching effects in transgenic plants (Fig. 10).

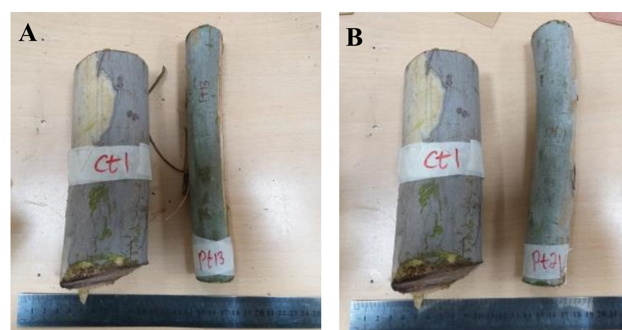

Fig. 9. Stem diameter comparison of control and transgenic plants. Stem from the transgenic and control plants was cut from the 2cm the bottom A: Control plant stem vs transgenic plant Pt13 stem, B: Control plant stem vs transgenic plant Pt 21 stem.

Regeneration and transformation efficiency: Total regeneration and transformation frequency was calculated by calculating total number of explants transformed, regenerated and total PCR confirmed transgenic plants with low CCR expression. The total number of explants successfully regenerated was 1757 with a regeneration frequency of 40.3%. High regeneration rate suggests this regeneration protocol as an efficient method (Table 6) for transgenic and micro-propagation studies. Six transgenic plants expressing reduced amount of *CCR* in real time PCR were selected for further analysis.

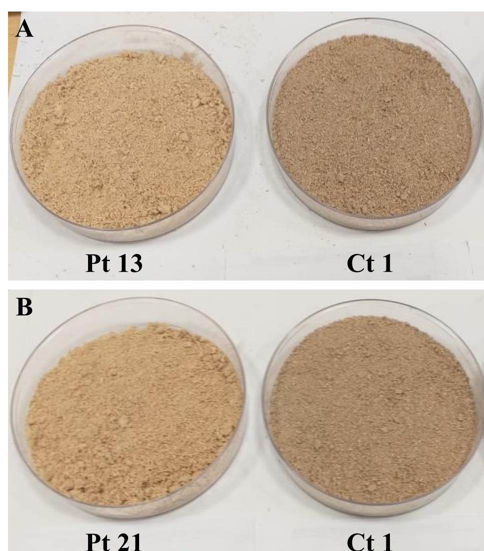

Fig. 10. Comparison of biomass color of control and lignin down regulated transgenic lines.
Air dried biomass was ground to 20 mesh using a knife mill A) Control plant (Ct1) vs transgenic plant Pt13 B) Control plant (Ct1) vs transgenic plant Pt21.

Discussion

Effective selection marker and selection medium can improve separation of transgenic and non-transgenic shoots. Basta® represents the effective non-selective herbicide (Hoerlein, 1994). In this study 1-8mg/L Basta was used to control the growth of non- transgenic shoot, it was observed that 8mg/L Basta controls the growth of non-transgenic shoots of *E. camaldulensis*.

Role of acclimatization is vital for survival of micro propagated and tissue cultured plants. Tissue cultured and micro propagated plants are not familiar to external biotic and a biotic stresses (Mahmood *et al.,* 2021). Acclimatization of laboratory grown plants is a challenge due to high mortality rate due to altered temperature, light, humidity, salts and biotic stresses, hence a dire need of acclimatization method (Deb & Imchen, 2010). *In vitro* grown plants have weak stomatal, root and cuticle functioning due to growth in an excess supply of phytohormones such type of plants are often termed as vitrified plants (Fanourakis *et al.,* 2020). Physiological and anatomical features of *In vitro* grown plants require successful methods for acclimatization of the plants in green house and in fields (Bag *et al.,* 2019). i.e. leaves replacement, changes in leaf tissue morphology, stomatal distribution and morphology, chloroplast and cuticle formation (Preece & Sutter, 1991; Diettrich *et al.,* 1992; Marin *et al.,* 1988; Tichá *et al.,*1999; Hussain *et al.,* 2011). Change in cuticle, epicuticle waxes and stomatal regulation makes plants acclimatized to field conditions by stabilization of water potential (Pospóšilová *et al.,* 1999). In this study *In vitro* plants were acclimatized gradually by shifting from high humidity conditions to low humidity condition after transferring the rooted plantlets to 50% peat moss+ 25% perlite + 25% vermiculite mixtures. Under these condition mortality ≤5% was observed. However, under high humidity condition fungal growth was a problem which was resolved by transferring of the plants to new pots immediately. Acclimatized plants were transferred to soil in pots and kept under glass house conditions maintaining temperature at 25°C during hot summer season (April-October) and transferred to net house condition during winter season (November-March). Hardened plants were observed to give minimum physical symptoms due to less abrupt weather conditions during which they were transferred to net house. Hence, transferring of plants to net house during winter season is recommended as under these temperature acclimatized plants shown no environmental side effects.

PCR confirmed transformants expressing the desired *EuCCR* sense-antisense RNAi transcript were subjected to further confirmation of expression analysis by profiling endogenous *CCR* expression in wild type (Wt), control (Ct) and transgenic plants. From most of the transgenic studies, the expression profiling of transgenic plants gives expression profile of specific insert but in our case the mRNA from endogenous *CCR* was the target. 18s ribosomal RNA primers were designed and used as internal control as suggested by Unnikrishnan *et al.,* 2011. Wild type and control plants showed higher relative expression of *CCR* while lesser expression of *CCR* gene was observed suggesting down regulation of *CCR* expression in transgenic plants from 30%-72%. These results are consistent with many studies which reported down regulation of *CCR* (Van der Rest *et al.,* 2006; Leplé *et al.,* 2007; Wadenbäck *et al.,* 2008; Prashant *et al.,* 2011). However in this study we observed highest down regulation (72%) in transgenic plant as compared to control plants. The down-regulation of *CCR* mRNA expression in transgenic plants was ≤50%-80% as compared to wildtype plants. *CCR* enzyme assay further revealed that control plants had high enzymatic activity against feruloyl CoA. When feruloyl-CoA was used as a substrate, then 0.036 decreases in absorbance at 366 nm corresponds to 100pkat/ml of enzymatic activity. The OD change of 0.02/min corresponds to 58 pkat/ml of enzymatic activity according to Wengenmayer *et al.,* 1976.

Klason lignin in transgenic plants was significantly decreased from 11.9-25.5% as compared to wild type plants. *CCR* expression and down regulation of lignin had no strict correlation as plant expressing 80% *CCR* down regulation showed only 25.5% of total lignin down regulation as compared to wild type plants. Similar results were reported by Wadenbäck *et al.,* 2008 who reported 35% of *CCR* down regulation in Norway spruce but down regulation of lignin was just 8%. The role of the lignin in plant growth in this study was also significant as reported by the study conducted by Wadenbäck *et al.,* 2008 and Prashant *et al.,* 2011 who reported that transgenic plants had attenuated growth i.e. short stem and restricted growth. Prashant *et al.,* 2011 concluded that this restriction in growth in transgenic lines is due to poor lignification of xylem and collapsed vessels. Similar observation were made in the current study that transgenic lines with down regulated lignin contents showed less height (28-67%) and stem girth (53-66%) as compared to control plants.

Table 4. Estimation of lignin contents of wild, control and transgenic *E. camaldulensis*.

Plant type	Percentage of acid soluble lignin (ASL) (A)	Percentage of acid insoluble lignin (AIL) (B)	% Total lignin (C) = A+B	% Ash (D)	Total lignin %-% Ash E = C-D	% Reduction in comparison to wild type F = 100-(E/Ewtx100)	Actual reduction G = Ft-Fct
Wild type (Wt)	3.97%	26.06%	30.03%	1.8%	30.03-1.8 = 28.23%	-	-
Control (Ct)	3.5%	25.2%	28.7%	4.4%	28.7-4.4 = 24.3%	13.92%	-
Transgenic-Pt13	3.84%	20.2%	24.04%	3.1%	24.04-3.1 = 20.94%	25.82%	11.9%
Transgenic-Pt21	3.5%	18.3%	21.8 %	4.7%	21.8-4.7 = 17.1%	39.43%	25.5%

Table 5. Comparison of stem length and width of control and transgenic plants.

Plant	Stem length (inches)	Percentage of stem length reduction as compared to control plants	Stem diameter (inches)	Percentage of stem diameter reduction as compared to control plants
Control	80″	--	2.95″	--
Transgenic-Pt13	57″	28.5%	1.38″	53.2%
Transgenic-Pt21	57″	28.7%	1.38″	53.2%

Table 6. Regeneration and transformation frequency.

Total number of infected explants	Regenerated explants	Regeneration frequency	Total surviving putative transgenic after selection	PCR confirmed transgenic plants	Transgenic plants with low CCR expression	Transformation efficiency
4355	1757	40.3%	40	11	6	0.13%

Transgenic plants exhibiting low lignin contents showed bleaching effect of biomass due to lignin down regulation. This bleaching effect was directly proportional to the lignin down regulation with transgenic plant (Pt13) showing less lignin down regulation as compared to transgenic plant (Pt21) with maximum down regulation of lignin.

The results of the study suggest that lignin playing key role in plant growth and function. Lignin down regulation can be attained to a limited level to conserve biomass production quantity.

Acknowledgements

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