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CINNAMATE 4-HYDROXYLASE DOWNREGULATION IN TRANSGENIC TOBACCO ALTERS TRANSCRIPT LEVEL OF OTHER PHENYLPROPANOID PATHWAY GENES

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ABSTRACT:

Cinnamate 4-hydroxylase (*C4H*) is the second gene of phenylpropanoid pathway responsible for the production of a variety of compounds including lignin. Transgenic tobacco plants were raised using antisense construct harbouring partial *C4H* gene from *Leucaena leucocephala*. C4H down-regulation in transgenic tobacco had profound effect on the transcript level of *C4H* and other lignin biosynthesis pathway genes investigated. Transgenics had reduced *C4H* transcript level, as low as about 5% of the level in control plants in one of the transgenics. It was interesting to see higher reduction in the transcript level of phenylalanine ammonia lyase (*PAL*), the enzyme preceding C4H in the pathway, than *C4H* itself. Reduction in the transcript level of 4-Coumarate: CoA Ligase (*4CL*), the enzyme that follows C4H in the pathway, was comparable to that of *C4H*. Transcript levels of two other phenylpropanoid pathway genes, Caffeoyl coenzyme A 3-*O*- methyltransferase (*CCoAOMT*) and cinnamyl alcohol dehydrogenase (*CAD*) were also found reduced compared to control. In the transgenic line AS9, a small reduction of about 11% in the transcript level of *C4H* resulted in about 88% reduction in the transcript level of *PAL* and 19-29% reduction in the transcript level of *4CL*, *CCoAOMT* and *CAD*. In our knowledge, this is the first quantitative report of concomitant decrease in the transcript level of five phenylpropanoid pathway genes as a result of down-regulation of one of the pathway genes.

Keywords: Cinnamate 4-hydroxylase (C4H), phenylpropanoid pathway, transcript level, transgenics, quantitative real time PCR

[I] NTRODUCTION

Eclipsed only by cellulose, lignin is the second most abundant biopolymer on earth and represents approximately 30% of the organic carbon [1]. To engineer plants with agronomically desirable lignin related traits, it is necessary to devise strategies to predictably and flexibly reduce lignin content and/or alter its monomer composition. One of the ways to reduce lignin content or alter its composition is to explore the potential of genetic engineering by means of down-regulating key lignin biosynthesis gene(s). Lignin biosynthesis in plants begins with a core group of three reactions termed as general

phenylpropanoid pathway catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) respectively [1–3]. C4H catalyzes the second step of phenylpropanoid pathway and hydroxylates *trans*-cinnamic acid at the *para* position, which is the reaction product of first enzyme of phenylpropanoid pathway, PAL [1–5]. C4H catalyzes slow, irreversible reaction with high substrate specificity and is located at strategically important point as the pathway branches after C4H. Considering the early position of C4H in the pathway, several workers generated transgenic plants down-regulated for C4H in order to reduce lignin content, alter lignin composition or to study the effects of C4H reduction in plants [6–10]. Majority of C4H down-regulation study in plants have revolved around reduction in lignin content, alteration in its monomeric composition, delayed lignification and reduction in C4H activity in transgenics compared to control plants. Although down-regulation of C4H and the resulting effects have been extensively studied in these cases, the relationship between C4H down-regulation and its effect on the transcript level or activity of other phenylpropanoid pathway gene(s) is largely underrepresented and is mostly limited to the study of PAL. In the present paper, we are reporting quantitative changes in the transcript level of *PAL*, *C4H*, *4CL*, caffeoyl coenzyme A 3-*O*- methyltransferase (*CCoAOMT*) and cinnamyl alcohol dehydrogenase (*CAD*) as a result of C4H down-regulation in transgenic tobacco carrying partial *C4H* gene from *Leucaena leucocephala* in antisense orientation. *L. leucocephala* is a pulp yielding leguminous tree species accounting for about $1/4^{th}$ of raw material supplied to pulp and paper industry in India. Removal of lignin from pulp is chemical and energy intensive process which releases toxic pollutants and damages polysaccharide components of wood. Lignin content in *L. leucocephala* is 24-30% [11], which needs to be reduced for better commercial exploitation of this species. The results of the present study related to reduction in lignin content and transcript level of phenylpropanoid pathway genes in tobacco transgenics will provide a basis to achieve similar degree of results in *L. leucocephala*.

[II] MATERIALS AND METHODS

2.1. Preparation of *C4H* antisense construct

Initial preparation of the antisense construct has been described elsewhere [12]. In short, *L. leucocephala 4CL* (*Ll4CL*) gene was double digested using restriction endonucleases *Kpn*I and *Sac*I, and cloned in to the multiple cloning sites (MCS) of pCAMBIA1300 flanked by CaMV 35S promoter and Nos terminator on either side. The CaMV 35S promoter-antisense *Ll4CL*-Nos terminator cassette from recombinant pCAMBIA1300 was digested using *Eco*RI and *Hin*dIII and cloned in to the MCS of pCAMBIA1301. The recombinant pCAMBIA1301 harbouring antisense *Ll4CL* gene [12] was modified as per the requirement of the present study by replacing the antisense *Ll4CL* gene by partial antisense *L. leucocephala C4H1* (*LlC4H1*) gene. In short, *Sac*I and *Kpn*I sites were introduced in to the forward (C4H-ANTS-F3) and reverse primers (C4H-ANTS-R3), respectively and 438 bp long partial fragment of *LlC4H1* was amplified using a plasmid harbouring *LlC4H1* cDNA clone (NCBI accession no- GU183363) as template. The amplified partial *LlC4H1* fragment was cloned in to pGEM T-Easy vector and digested with *Sac*I and *Kpn*I to recover the digested 'insert'. Antisense *Ll4CL* fragment was removed from the recombinant pCAMBIA1301 [12] by digesting the vector using *Kpn*I and *Sac*I, and the digested partial *LlC4H1* insert was ligated to the digested vector backbone. This modified pCAMBIA1301 containing partial *LlC4H1* gene in antisense orientation (pCAMBIA1301-asC4H) was transferred to *Agrobacterium tumefaciens* strain GV2260 and subsequently used for plant transformation experiments (Fig. 1). Primer

sequences used in the study have been given in Table 1.

2.2. Plant transformation, regeneration and selection for transformants

Tobacco (*Nicotiana tabacum* var. Anand 119) was transformed by pCAMBIA1301-asC4H vector using leaf disc method as described by [13]. Shoot regeneration from leaf disks was achieved on half strength MS semi-solid medium [14] supplemented with 4.4 µM 6-benzylaminopurine (BAP) and 5.37 µM 1-naphthyl acetic acid (NAA). Hygromycin (25 mg/L) served as the selective antibiotic during in vitro culture. Cefotaxime (250 mg/L) was incorporated in the selection medium to check *Agrobacterium* contamination. Rooting was achieved in half strength MS semi-solid medium devoid of growth regulators. Rooted plantlets were transferred to soil, hardened and shifted to green house to reach maturity. Plants emerging from leaf disks gone through the same procedure without co-cultivation with *Agrobacterium* served as control plants. Putative tobacco transformants were selected on the basis of *GUS* assay taking a small portion of leaf from the regenerated shoot of tobacco. Transformants were confirmed on the basis of PCR using *hpt*II specific primers (for primer sequence, see Table 1) and genomic DNA isolated from the putative transformants as template. The amplified fragments were cloned and sequenced.

2.3. RNA isolation and cDNA synthesis

All the analyses have been performed on T_0 transformants. Seven transformants (named AS1, AS4, AS5, AS6, AS8, AS9 and As10) and three control plants were used for RNA isolation and cDNA synthesis. Leaves of transgenic tobacco and control plants, emerging from nodes 8-10 from the first fully opened leaf at the top were harvested and stored at -80 ºC. Petioles from leaves were crushed in liquid nitrogen and RNA was isolated from the tissue using RNA isolation kit (Invitrogen, USA) in duplicate. On column DNaseI treatment was given as per the manufacturer's recommendation and the absence of genomic DNA in isolated RNA was confirmed by PCR. Quality of RNA was checked by gel electrophoresis. RNA was quantified using NanoVue plus (GE healthcare, USA) and first strand cDNA was synthesized using cDNA synthesis kit (SuperScript III Reverse Transcriptase, Invitrogen) taking 1 µg total RNA as template as per the instructions.

2.4. Quantitative real time (Q-RT) PCR analysis

Mx3000P real time PCR machine (Stratagene, USA) was used for all the experiments. Primer sequences specific to tobacco gene sequences were designed by retrieving the specific gene sequences from NCBI database. Genbank accession numbers DQ350352, DQ350353 and DQ350354 were used to design tobacco specific *C4H* primers; AB008199, AB008200, D17467 and EU883670 for tobacco specific *PAL* primers; U50845 and U50846 for tobacco specific *4CL* primers; X62343 and X62344 for tobacco specific *CAD* primers; and U38612, U62734, U62735 and U62736 for tobacco specific *CCoAOMT* primers. Primers were designed so as to amplify all the isoforms of a particular gene. Specificity of primers was determined by PCR and detection of single band after 2% agarose gel fractionation. Primer sequences used for Q-RT PCR are given in Table 1. Suitability of 5.8S and 18S rRNA as internal controls was checked by directly comparing their transcription range [15] on the basis of non-normalized expression level in the cDNA of all the plant petiole samples. Later, cDNA samples were normalized for 5.8S rRNA and the corresponding 18S rRNA C_T values were noted [16].

Reactions were performed in triplicates in 25 µL reaction volume containing 12.5 µL of mesa green qPCR master mix plus for SYBR assay (low rox, Eurogentec), experimentally determined amount of primers (optimized after performing primer titration according to the manufacturer's recommendation) and normalized

cDNA dilution as template. PCR parameters were 95 ºC for 5 min, followed by 40 cycles of 95 ºC for 10 s, 55 ºC for 30 s, 72 ºC for 1 min and after completion of run, melt curve analysis to ensure that only a specific product and no primer dimers are formed. C_T values of *C4H*, *PAL*, *4CL*, *CAD* and *CCoAOMT* were geometrically averaged form the three control plants. Relative transcript level of *C4H*, *PAL*, *4CL*, *CAD* and *CCoAOMT* in transgenics compared to control were determined using the model proposed by Pfaffl (2001) [17] and expressed as the percentage transcript level of control plants.

2.5. Lignin estimation from tobacco stem

Lignin was estimated from five transformants (AS1, AS4, AS5, AS9 and AS10) and three control plants. Mature tobacco mid stem material between nodes 8-10 from the first fully opened leaf on top was used for lignin estimation. Lignin was estimated in duplicates using the Klason method [18] with slight modification. Tissues were chopped and air dried. Air dried tissues were ground to fine powder, oven dried and about 200 mg of accurately weighed tissue powder was continuously extracted in acetone:water (10:1 v/v) for 48 hours at 55 ºC. Cell wall residue (CWR) was obtained by drying the tissue powder to constant weight at 105 ºC. For control plants lignin estimation, oven dried tissue powder from three independent control plants were mixed in equal proportion (wt/wt) and lignin estimated in duplicate. Acid hydrolysis of CWR (72 % H_2SO_4 for 3 h at 25 ºC in stirring condition) was followed by dilution of the hydrolyzed CWR to 3% H_2SO_4 using molecular biology grade water and autoclaving for one hour. After cooling down, residue was filtered, washed with hot water to remove acid and dried to constant weight at 105 ºC to get acid insoluble or Klason lignin (KL), which was expressed as percentage of original sample. The filtrate after 50 times dilution was used for spectrophotometric determination of acid soluble lignin (ASL) at 205 nm [18]. Total lignin was calculated as the sum of ASL and KL.

[III] RESULTS

3.1. Sequence identity between *C4H* from *Leucaena* and *C4H* from tobacco

Three highly similar isoforms of *C4H* are reported from tobacco (NCBI accession numbers DQ350352, DQ350354 and DQ350353 respectively). The partial *LlC4H1* used to down-regulate C4H activity in transgenic tobacco shares 70%-71% identity with tobacco *C4H* sequences available in NCBI database. Apart from these three isoforms, two additional wound inducible *C4H* sequences from tobacco are also reported (NCBI accession numbers AF368379 and AF368378). These two sequences share more than 50% identity with the partial *L. leucocephala C4H* sequence.

3.2. Difference in phenotype between control and C4H antisense plants

In vitro grown plants showed no clear difference between control and transgenic plants. However, differences started appearing a few weeks after the plants were kept for hardening or shifted to green house. Transgenics displayed varied phenotypes, but as a general rule their growth was stunted compared to control plants. Some antisense lines needed support during growth and had narrower, curled and wrinkled leaves (Fig. 2). Severely down-regulated plants' stems were weak and fragile at the top. Flowering and senescence was also delayed in transgenics.

3.3. Phloroglucinol staining of stem sections of control and transgenic plants

Fresh stem sections from tobacco plants (which had been cut for lignin estimation) were taken from the plants and used for acidic phloroglucinol staining [19], which stains lignin typical pink. Staining of 2-3 mm thick stem sections from control and transgenic line AS1 clearly shows that while staining in control plant is uniform and dark pink, staining in AS1 is non-uniform and light pink. Gaps in staining can be clearly seen in the section (Fig. 3B and 3D). Phloroglucinol staining of free hand stem sections at higher

magnification (10X and 40X) further delineates the differences in staining. Under 10X magnification, it can be clearly seen that while some part of xylem tissue is stained; other part is left unstained/lightly stained (Fig. 3F). Focusing the lightly stained portion of xylem tissue of AS1 at 40X magnification reveals that some selected tissues have taken very light stain and most of the xylem tissue is unstained (Fig. 3H).

3.4. Quantitative real time (Q-RT) PCR analysis

Transcription range (for definition see, Radonic et al. 2004, [15]) for 5.8S rRNA and 18S rRNA in non-normalized cDNA of all the samples (control plants as well as transgenics) come out to be 3.31 and 3.57 respectively. Hence, 5.8S rRNA was used as a preferred internal control and the cDNA samples were normalized against 5.8S rRNA. The expression ratio of 18S rRNA relative to 5.8S rRNA between all the samples after normalization against 5.8S rRNA (calculated using the formula suggested by Pfaffl (2001) [17]) varied between 0.804 to 1.137. Nevertheless, this variation does not seem to be significant in the transgenics considering the large reduction in the transcript level of genes used in this study in transgenics.

Real time PCR efficiency for every primer pair was > 85%. The transcript level of *C4H* and other genes in transgenics relative to control plants has been shown in Fig. 4. Careful study of the results suggests that *C4H* down-regulation has deleterious effect on the transcript level of *C4H* in transgenics. The minimum *C4H* transcript level was observed in AS1 with only 4.9±0.6% of the transcript level observed in control, whereas maximum transcript level was observed in AS9 (88.6±2.2% relative to control). *C4H* transcript level in AS4, AS5, AS6, AS8 and AS10 were found to be 17.1±1.8%, 23.2±0.2%, 15.5±0.5%, 20.4±1.2% and 16±1.2%, respectively. Transcript level of all the genes of phenylpropanoid pathway studied in transgenics, showed reduced transcript level compared to control. It is interesting to see that *C4H* down-regulation in tobacco resulted in higher reduction in the transcript level of *PAL*, the enzyme preceding *C4H* in the pathway, than *C4H* itself. In AS1, compared to *C4H* transcript level of about 4.9±0.6% of its level in control plants, *PAL* transcript level decreased to a much lower level, 0.5±0.1% of its level in control plants. Maximum *PAL* transcript level among transgenics was observed in AS9 (11.8±1.2% of control), which had only slight decrease in *C4H* transcript level (88.6±2.2% relative to control). Another general phenylpropanoid pathway gene, *4CL* had comparable reduction in transcript level with that of *C4H*. *4CL* transcript level in transgenics varied between 7.2±0.8 to 77.6±2.3%. In all transgenics, percent reduction in *4CL* transcript level is slightly higher than *C4H*, except in AS9, where *4CL* level (77.6±2.5% of control) is below *C4H* level. *CAD* and *CCoAOMT* transcript levels had also reduced in transgenics. Minimum *CAD* and *CCoAOMT* transcript level was observed in AS1 (11.5±0.3% and 6.0±0.7% respectively), whereas maximum *CAD* and *CCoAOMT* transcript level was observed in AS9 (70.8±2.3% and 81.1±2.4% respectively). Among all the genes, lowest transcript level observed was that of *PAL* in all the transgenics.

3.5. Lignin estimation from stem tissues of control and transgenic tobacco

Compared to control plants, both ASL and KL had decreased in stem tissues of all the transgenics (Table 2). Transgenics having higher reduction in *C4H* transcript level had greater reduction in Klason lignin than acid soluble lignin, for example AS1 had 41.3% reduction in KL and 3.4% reduction in ASL (37.4% reduction in total lignin). Another transgenic, AS9 having marginal decrease in *C4H* transcript level had 3.6% decrease in KL and 15.4% decrease in ASL (4.8% in total lignin). AS4 and AS10, which had respectively about 17% and 16% *C4H* transcript level relative to control, had 24.6% and 29.8% reduction in total lignin level, respectively.

[Table-1].

Name of the primer	**Sequence of the primer (5' → 3')**
C4H-ANTS-F3	GAGCTCATGGATCTCCTACTCCTGGAG
C4H-ANTS-R3	GGTACCTGTATTGCTGAACCACCTTGT
hptII-F	GCGTCGGTTTCCACTATCGG
hptII-R	TCTCGGAGGGCGAAGAATCT
Tob-*C4H*-F	GTGGCAAGTGTAATTGAGGATGTG
Tob-*C4H*-R	GCCAATCTACTCCTTTCACCATTC
Tob-*PAL*-F	CGGTGGATTTTTCGAGTTGCAGCC
Tob-*PAL*-R	TGAGCCGCCTTCACATAAGAGCT
Tob-*4CL*-F	GGTTACACACTGGCGACATTGG
Tob-*4CL*-R	GGAACTTCTCCTGCTTGCTCATC
Tob-*CAD*-F	GGGAGTGAAAATAGCAAAGG
Tob-*CAD*-R	GCCAACAGGGACAGTATC
Tob-*CCoAOMT*-F	ACACCCTATGGAATGGATCAG
Tob-*CCoAOMT*-R	GCCTTGTTGAGTTCCAATACG

Table: 1. Primer sequences used in the present study. Restriction site overhang in the primers (*Sac*I and *Kpn*I in the forward and reverse primers, respectively) used to amplify partial *L. leucocephala C4H* gene are underlined

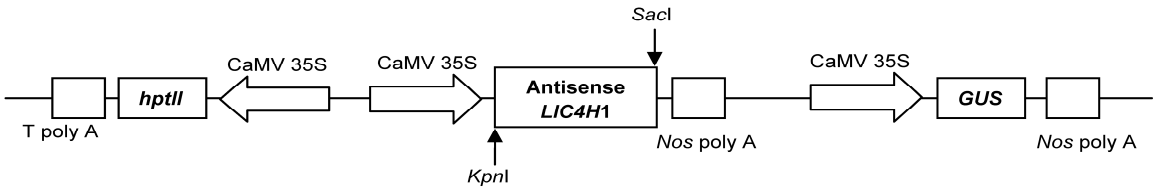

Fig. 1 Antisense *LlC4H* construct (pCAMBIA1301-asC4H) used in the present study. CaMV 35S: Cauliflower mosaic virus 35S rRNA promoter, *hpt* II: Hygromycin phosphotransferase gene, T poly A: CaMV 35S rRNA terminator sequence, Antisense *LlC4H1* sequence: partial *Leucaena leucocephala C4H* gene in antisense orientation, *Nos* poly A: nopaline synthase gene terminator sequence, GUS: *β*-glucuronidase gene

Fig. 2 Difference in morphology between leaf from a control plant (a), and leaf from an *LlC4H1* antisense transformed line(b). Leaf from transformed line is wrinkled (shown by arrow) and narrower than the leaf from control plant

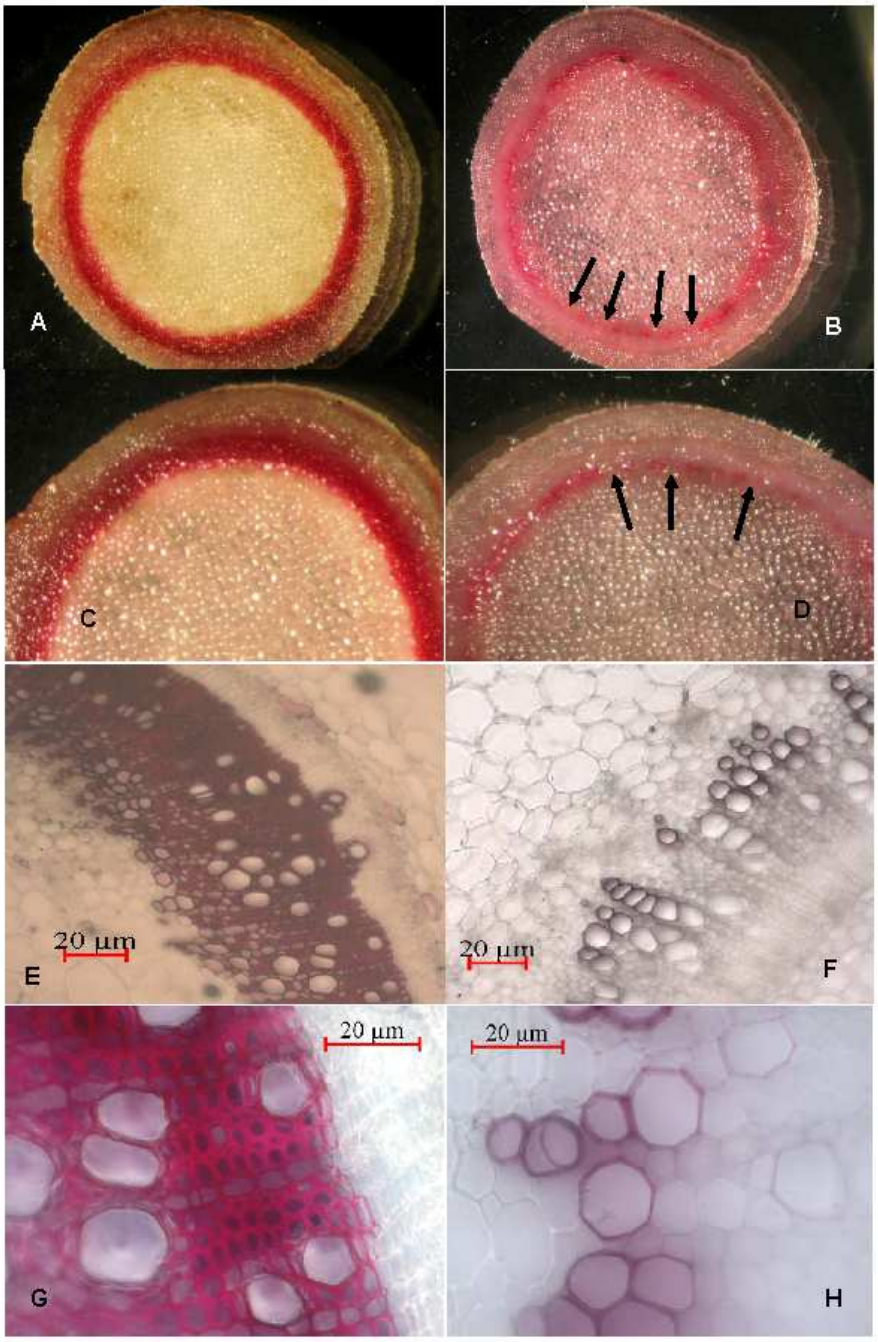

Fig. 3 Phloroglucinol staining of stem sections from control and a C4H down-regulated transgenic line (AS1). Phloroglucinol staining of 2-3 mm thick stem section of a control plant (a), transgenic line (b), closer view of 2-3 mm thick stem section of a control plant showing intense and continuous staining (c), closer view of 2-3 mm thick stem section of AS1 showing discontinuous staining with gaps clearly visible indicated by arrow (d), free hand section of control stem under 10X magnification (e), free hand section of AS1 stem under 10X magnification (f), free hand section of control stem under 40X magnification (g) and free hand section of AS1 stem under 40X magnification (h)

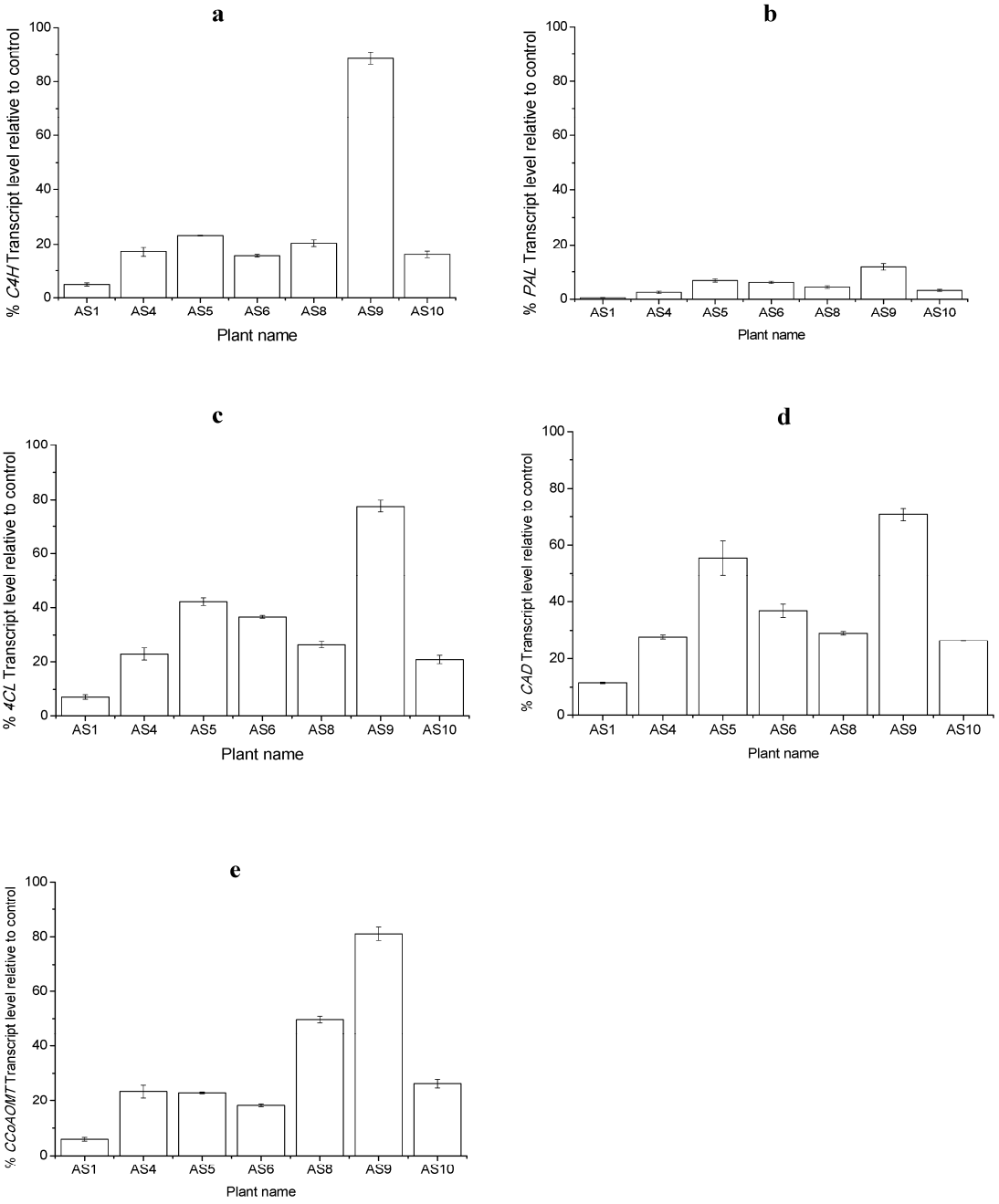

Fig. 4 Transcript level of different phenylpropanoid genes in transgenics relative to control plants and lignin estimation from them. The transcript levels of individual genes from three control plants were averaged and assumed to be 100% and the respective transcript level for the transformants were calculated. C4H transcript level in transgenics relative to control plants (a), PAL transcript level in transgenics relative to control plants (b), 4CL transcript level in transgenics relative to control plants (c), CAD transcript level in transgenics relative to control plants (d), CCoAOMT transcript level in transgenics relative to control plants (e)

[Table-2].

Plant name	KL (%)	ASL (%)	Total lignin (%)	% Decrease in total lignin
AS1	11.92±0.18	2.26±0.02	14.18±0.2	37.4
AS4	15.01±0.22	2.06±0.04	17.07±0.26	24.6
AS5	17.23±0.12	2.3±0.03	19.53±0.15	13.7
AS9	19.56±0.24	1.98±0.02	21.54±0.26	4.8
AS10	13.62±0.36	2.28±0.04	15.9±0.4	29.8
C	20.3±0.37	2.34±0.03	22.64±0.4	-

Table: 2. Lignin content in mature stem of control and transgenic lines. AS designations represent transgenic lines; and C represents control plants. KL represents Klason lignin and ASL represents acid soluble lignin. Total lignin equals KL+ASL

[IV] DISCUSSION

Heterologous down-regulation of C4H in tobacco was effectively brought about by using *L. leucocephala C4H* nucleotide sequence in antisense orientation, because *L. leucocephala* and tobacco C4Hs shared more than 70% sequence identity at nucleotide level. In fact, C4H might be one of the most conserved gene families in plants as there is no clear distinction between monocots and dicots based on phylogenetic analysis of C4H [20]. *L. leucocephala C4H* sequence does not share significant similarity with any other known gene sequence so far in tobacco. Thus, it may be expected that down-regulation of C4H in tobacco using *L. leucocephala C4H* will not directly influence the transcript level of any other transcript in tobacco.

C4H downregulation in tobacco affected the normal growth of transgenics compared to control. Most transgenics lines grew to nearly three fourth the height of control plants and had thinner and weaker stem. Severely down-regulated transgenics needed support during maintenance and their leaves were curled and wrinkled. The phenotypic differences observed in transgenics appear to be directly related to the reduced lignin content and altered lignin composition in tissues as a result of reduced C4H activity [6], [8], [10]. The possibility of some phenotypic irregularities in severely down-regulated transgenics arising out of reduction in low molecular weight phenolics derived from phenylpropanoid pathway end products, such as dehydroconiferyl glucoside with proven involvement in cytokinin mediated cell division [21] cannot be excluded. Although, a few transgenic lines did not show any visible morphological difference from the control plants. Differential phenotypic results in transgenics may be explained on the basis of site of integration of t-DNA, which varies randomly in different transgenics and influences the transgene activity in both a qualitative and a quantitative manner [22]. In general, transformed lines had delayed flowering compared to control. It is not known whether it is linked to reduced lignin content and/or altered monomer composition, depletion of some vital compounds or accumulation of some novel compounds. Arabidopsis plants mutant for C4H accumulated a novel hydroxycinnamic ester, cinnamoylmalate not found in wild type plants and were dwarf and male sterile.

Phloroglucinol staining of stem sections of control plants stained the xylem vessels typical pink, whereas xylem vessels in transgenics took little stain of slightly brown colour. This variation in staining gave indication that the composition of lignin had changed in transgenics. Pink colour during phloroglucinol staining is indicative of aldehyde end groups, such as coniferaldehyde end groups in guaiacyl lignins [23]. Change of this colour to brown suggested a possible increase

in the guaiacyl units in lignin in transgenics. Tobacco transgenics carrying an alfalfa (*Medicago sativa*) *C4H* transgene in the antisense orientation resulted in reduced level of Klason lignin and decreased S/G ratio as determined by pyrolysis gas chromatography/mass spectrometry [10].

In the present study, *C4H* transcript level as low as 4.9±0.6% has been recorded from AS1 as compared to control. Bjurhager et al. (2010) [6] reported that in two transgenic hybrid aspen trees down-regulated for homologous *C4H* transgene had *C4H* transcript level in the range of 23.5 ± 15% and 12.5 ± 4% respectively. Given that P450 enzymes catalyze slow reactions (low K_{cat}) [24], a reduction of this level has the potential to abnormally bring down the overall activity of C4H from the transgenic plants leading to accumulation of its substrate, cinnamic acid. Inhibition of *PAL* transcript level and activity accompanied with the synthesis of a proteinaceous PAL inhibitor was recorded in potato tubers when cinnamic acid was exogenously supplied to the tubers [25]. Blount et al. (2000) established that cinnamic acid plays the role of a negative modulator of PAL activity and proposed that the accumulation of cinnamic acid beyond a certain level in the cell is perhaps sensed by the cell machinery to withhold *PAL* transcription and as a result, transgenic tobacco down-regulated for C4H had similar level of PAL activity as that of C4H. Blount et al. (2000) [8] also reported that C4H activity was not reduced in plants genetically engineered for reduced PAL activity. Also, progeny arising as a result of cross between a tobacco line over-expressing bean PAL transgene and a C4H antisense transgenic line had reduced PAL activity than progeny of PAL over-expresser. These results suggest that reduction in C4H activity in plants have strong inhibitory effect on *PAL* transcription and translation.

It was interesting to note that *PAL* transcript level in transgenic tobacco decreased to a much lower fraction of the control plants; the extent of reduction being even greater than *C4H*. The highest *PAL* transcript level in any transgenic was about 12% of control plants, as recorded in AS9. In all the transgenics, there was correspondingly higher reduction in the transcript level of *PAL* than in the transcript level of *C4H*. The transcript level of *PAL* was about six times higher than the transcript level of *C4H* in control plants under the green house condition (calculated on the basis of difference in C_T value of *C4H* and *PAL* from normalized cDNA samples). Down-regulation of C4H in transgenics shifted the ratio to between 0.7- 2.5, i.e. number of mRNA transcripts of *PAL* was in between 0.7 to 2.5 times the number of transcripts of *C4H* in transgenics. Association of C4H and PAL is well documented. It has been successfully demonstrated that C4H was co-regulated with PAL in response to co-induction under various conditions in Parsley. In fact, the association of C4H was stronger with PAL than the redox partner of C4H, NADPH:cytochrome P450 oxidoreductase (CPR) [26]. PAL and C4H activities concomitantly increase in potato tuber when they are illuminated with light [25]. Physical association in the form of a multi-enzyme complex formed of a particular isoform of PAL and C4H in transgenic tobacco is also reported [27]. Formation of multi-enzyme complex does not let PAL generated cinnamic acid to diffuse to the cytosol and thus forms a sort of channelling between PAL and C4H. PAL and C4H are closely associated, nevertheless, it is surprising that down-regulation of C4H reduced *PAL* transcript to a greater level than the reduction of *C4H* transcripts.

Reduction in the transcript level is not limited to only PAL. C4H downregulation also led to the decrease in the transcript level of other phenylpropanoid pathway genes used in the present study. Since as per our best knowledge, there are no previous quantitative reports of reduction of transcript levels of these enzymes, especially *CAD* and *CCoAOMT* in C4H

transgenics, this study holds its own importance. Q-RT PCR analysis suggested that *C4H* and *4CL* transcripts are almost equally abundant in control plants. Individual transgenics also showed similar *4CL* and *C4H* transcript level. Study of transcript level of *4CL* and *C4H* in AS9 shows that even a slight downregulation of *C4H* in transgenics can reduce *4CL* transcript level. In control plants, *CAD* transcript level was about 2.5 times higher than *C4H* transcript level, whereas *CCoAOMT* transcript level was 0.53 times (i.e. transcript level lesser than *C4H*) of *C4H* transcript level. Both, *CAD* and *CCoAOMT* transcript level decreased as a result of reducing C4H activity in transgenic tobacco. In AS9, a small reduction in *C4H* level reduced *CAD* and *CCoAOMT* level to 70.8±2.3% and 81.1±2.4% of their control level, respectively. Co-reduction in the transcript level of different genes in transgenics as a result of reduction in C4H level may be attributed to the limited supply of substrates. The limiting substrates were perhaps sensed by the cell machinery to check the transcription of other genes of the pathway. In general, reduction in *CCoAOMT* was greater than the reduction in *CAD* level except in AS8. Since the transcript levels of these genes have decreased in *C4H* transgenics, it may be assumed that C4H catalyzes a rate limiting step at least in part, at an early point in the phenylpropanoid pathway. Anterola et al. (2002) [28] showed that *Pinus taeda* cell suspension culture were when fed with saturating level of phenylalanine and kept in phenylpropanoid induction medium, induced the transcription level of *PAL*, *4CL*, *COMT*, *CCR* and *CAD*, but did not induce transcription level of *C4H* and *C3H*. This showed that transcript levels of *C4H* and *C3H* are not dependent upon the metabolic requirement of the cells and may represent controlling point in the phenylpropanoid pathway. The present study further supports the hypothesis of Anterola et al. (2002) [28], because reduction in *C4H* transcript level resulted in reduction in the transcript level of *PAL*, *4CL*, *CCoAOMT* and *CAD*.

As expected, lignin content of the transgenics analyzed had decreased compared to the control plants. In AS9, displaying only partial decrease in *C4H* transcript level had 4.8% lesser total lignin content primarily due to major decrease in the acid soluble lignin and slight decrease in Klason lignin. Contrary to it, in AS10, which had only 16±1.2% *C4H* transcript level compared to control, had 30% lesser total lignin, mainly due to decrease in Klason lignin than acid soluble lignin. Maximum reduction (37.4%) in total lignin content was observed in transgenic line AS1 which also observed maximum reduction in the transcript level of all the genes investigated. Although our data did not provide information regarding the composition of lignin monomers as a result of reduction of flux into phenylpropanoid pathway, differential reduction in the later pathway enzymes and differential phloroglucinol staining pattern suggest that the monomer ratio might have changed in transgenics. Cellulose levels from the transgenics were not determined, hence it cannot be said whether reduction in lignin content in transgenics resulted in higher cellulose content or not. Transgenic tobacco down-regulated for another phenylpropanoid pathway gene, cinnamoyl CoA reductase (*CCR*) isolated from *L. leucocephala* also resulted in reduced lignin content, although on the basis of phloroglucinol staining the authors predicted a reduction in aldehyde units and increase in S/G ratio [29]. In short, to conclude, downregulation of C4H activity in transgenic tobacco resulted in reduced lignin content and transcript level of some phenylpropanoid pathway genes. Hence, transgenic *L. leucocephala* trees down-regulated for C4H may qualify as a feasible alternate for high lignin containing plant species which is currently being used as raw material for paper and pulp industry.

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