4.1 Introduction

CCoAOMT gene expression was for the first time reported in *Zinnia* and found to be closely associated with lignification (Ye et al., 1994). After this finding, the emphasis shifted towards determining whether this expression pattern was common to other plants. Localization of CCoAOMT at the protein and mRNA levels confirmed CCoAOMT gene expression in lignifying tissues in herbaceous plants like tobacco, tomato, soybean (Ye, 1997), alfalfa (Kersey et al., 1999; Inoue et al., 1998), and in woody plants like forsythia (Ye, 1997) and poplar (Chen et al., 2000; Zhong et al., 2000). CCoAOMT gene expression and localization was shown to be essentially restricted to the tracheary elements, xylary fibres, phloem fibers and xylem ray parenchyma cells. In tobacco after tobacco mosaic virus infection, two members of the CCoAOMT gene family are constitutively expressed in various plant organs and tissue, whereas two other are preferentially expressed in flower organs (Martz et al., 1998). Three distinct classes of CCoAOMT have been characterized in tobacco and levels of expression varied with the stages of stem development and in accordance with their involvement in the synthesis of guaiacyl units of lignin (Maury et al., 1999). In quaking aspen seasonal expression of CCoAOMT gene was analyzed in developing secondary xylem. CCoAOMT activity and expression was maximum during the middle of the growing season (Meng and Campbell, 1998). CCoAOMT gene expression and activity has also been studied in pine. Promoter of pine CCoAOMT localized GUS activity to secondary xylem suggesting the expression of CCoAOMT gene in secondary xylem and a close correlation with xylem lignification (Li et al., 1999).

4.2 Material and Methods

4.2.1 Cloning CCoAOMT cDNA in Expression vector

The pET-30b(+) vector (Novagen, USA) was used for expression of CCoAOMT gene in *E.coli* BL 21 (DE3). This vector carries a N-terminal His•Tag®/thrombin/S•Tag™/enterokinase configuration plus an optional C-terminal His•Tag sequence. This vector has unique restriction sites for cloning ease (Fig. 4.1). The cloned gene is expressed under the T7 RNA polymerase promoter.
Fig. 4.1: Vector diagram of expression vector pET 30 a,b and c(+). The maps for pET-30b(+) and pET-30c(+) are the same as pET-30a(+) (shown) with the following exceptions: pET-30b(+) is a 5421 bp plasmid; subtract 1 bp from each site beyond BamHI at 198. pET-30c(+) is a 5423 bp plasmid; add 1 bp to each site beyond BamHI at 198. The sequence of pET 30b(+) is highlighted in yellow.

The CCoAOMT1 gene specific, MF4 forward and the MR4 reverse primer sequences (Chapter 3; section 3.3.6) were modified to include NdeI restriction site at the 5’end of the
MF4 forward primer and Xho I site at the 5’ end of the MR4 reverse primer. The TGA stop codon was deleted from the MR4 reverse primer. This ensured that the His•Tag from the cloning vector was in frame at the C-terminus of the expressed protein. The modified primers were designated as PetF [5’ (d) CAT ATG GCG GAT CAG AAT CAA AGC GAA G 3’] and PetR [5’ (d)CTC GAG GCT GAT CCT ACG GCA GAG AGT GAT 3’] were used to reamplify the CCoAO MT1 cDNA gene clone. The reamplified gene was cloned in the Nde I and Xho I sites of the pET-30b(+) vector under the control of the T7 promoter.

4.2.2 Protein expression and purification
Protein was expressed in the host E. coli BL21 (DE3) cell line and purified as described earlier (Chapter 2; section 2.8). Purity of protein was checked on 10% SDS PAGE (Chapter 2; section 2.9).

4.2.3 Primary and Secondary Antibodies
Purified CCoAOMT protein was used for raising rabbit immune-serum. Primary Polyclonal antibodies were purified from the immune-serum. Secondary antibodies i.e anti rabbit goat IgG conjugated with alkaline phosphatase was from Merck, USA.

4.2.4 Affinity purification of CCoAOMT IgG from rabbit immune serum
The CCoAOMT protein specific polyclonal IgG were antigen affinity purified from rabbit immune-serum using Affi-gel 15 (BIORAD) (Chapter 2; section 2.10.2)

4.2.5 Histology, histochemical staining and immunocytolocalization
Transverse sections of root, shoot, leaf rachis and inflorescence of 0, 5, 10 and 15 days old seedlings as well as from one and two season old plant were used for histology, immunocytolocalization and histochemical staining (Chapter 2; section 2.11 and 2.13).

4.2.6 Semi- and absolute quantification of CCoAOMT1 and CCoAOMT2 gene expression
Total RNA isolated from root, shoot, leaf and inflorescence of 0, 5, 10 and 15 day old seedlings as well as of one and two season old plant (Chapter 2; section 2.4.6) was used for
cDNA first strand synthesis. The cDNA first strands were used as template for semi - and absolute quantification of CCoAOMT transcripts as described earlier (Chapter 2; section 2.4.11).

4.3 Results and Discussion

4.3.1 Expression of CCoAOMT gene in E.coli

The CCoAOMT1 gene clone in pET-30b(+) vector was expressed in E.coli BL21 (DE3) cell line. In view of the fact that the deduced amino acid sequence of CCoAOMT1 and CCoAOMT2 genes is 99% similar and differ only at the amino acids L 46 I and R 133 K (see Chapter 3; Fig. 3.30), and the hydropathy plots (Kite and Dolittle, 1982; Fig. 4.2 a, b) for the two proteins was highly similar and superimposable, protein expressed from only one gene was used for raising antibodies. The hydropathy plots also show similar strong negative peaks indicating possible exposed surface regions of the protein at almost similar amino acid positions (Hopp and Woods, 1981). Because of the similarity of the hydropathy plots and the deduced amino acid sequence of CCoAOMT1 and CCoAOMT2, it was assumed that selective antibodies for the two proteins cannot be raised.

The pET-30b(+) expression vector provides the choice of having the **His•Tag** either at the N or the C terminus of the expressed protein. The CCoAOMT protein is reported to form homodimer in solution (Inoue et al., 1998) and during this homodimerization the N - terminus of the protein is not exposed. Since protein was purified from inclusion bodies and the use of Ni – chelated affinity column was intended, this information was taken into account and the **His•Tag** was provided at the exposed C-terminus of the protein.

The CCoAOMT1 gene reamplified using PetF and PetR primers was cloned in pGEM T Easy vector and sequenced. The gene insert was released with **Nde I** and **Xho I** restriction digestion, purified and cloned in the **Nde I** and **Xho I** sited of the pET-30b(+) vector. The pET-30b(+) vector harboring CCoAOMT1 gene was designated as pETCT7 (Fig. 4.3). The vector pETCT7 was then transformed into E. coli BL21 (DE3) cell line.
Fig. 4.2: Kite and Dolittle plots for the CCoAOMT 1 (a) and CCoAOMT 2 (b). In both the plots window size is 9.
Fig. 4.3: Vector map of pETCT7
The deduced amino acids sequence of the CCoAOMT 1 gene sequence was used to calculate its approximate molecular mass, percent amino acid composition and theoretical pI. The calculated approximate molecular mass of CCoAOMT 1 was 27.5KDa with pI of 5.31. These calculations compare well with other CCoAOMT proteins reported from Zinnia, tobacco, tomato, soybean (Ye, 1997), alfalfa (Inoue et al., 1998), poplar (Zhong et al., 2000). Percent amino acid composition is shown in table 4.1.

Table 4.1: Percentage amino acid composition of CCoAOMT 1

<table>
<thead>
<tr>
<th>Amino Acid Comp.</th>
<th>Aa</th>
<th>no</th>
<th>Mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18</td>
<td>7.38%</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>0.82%</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>17</td>
<td>6.97%</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>18</td>
<td>7.38%</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>2.05%</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>16</td>
<td>6.56%</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>6</td>
<td>2.46%</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>16</td>
<td>6.56%</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>15</td>
<td>6.15%</td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>29</td>
<td>11.89%</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>7</td>
<td>2.87%</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>13</td>
<td>5.33%</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>13</td>
<td>5.33%</td>
<td></td>
</tr>
<tr>
<td>Q</td>
<td>7</td>
<td>2.87%</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>12</td>
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<td></td>
</tr>
<tr>
<td>S</td>
<td>9</td>
<td>3.69%</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>9</td>
<td>3.69%</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>17</td>
<td>6.97%</td>
<td></td>
</tr>
<tr>
<td>W</td>
<td>2</td>
<td>0.82%</td>
<td></td>
</tr>
<tr>
<td>Y</td>
<td>13</td>
<td>5.33%</td>
<td></td>
</tr>
</tbody>
</table>

Total: 244 100.00%
The CCoAOMT protein with **His•Tag** at its C-terminus was purified from *E.coli* culture lysate and inclusion bodies by using Ni- chelated column (Pierce, USA). The protein purity was checked by 10% SDS PAGE. The Ni- column purified CCoAOMT protein was homogenous and free of other contaminating protein. Total denatured protein profile, before and after induction, from cell lysate of *E.coli* BL21 (DE3) harbouring pETCT7 plasmid was compared. No appreciable difference was observed in the level of protein expression pre- and post –IPTG induction. The Ni-chelated affinity column purified CCoAOMT protein was seen to be of ~ 29KDa molecular mass (Fig. 4.4).

Cloning of alfalfa and tobacco CCoAOMT cDNA in expression vectors, its expression in *E.coli* BL21 (DE3) cells and purification through metal ion (Ni$^{2+}$ and Co$^{2+}$) chelated affinity columns and agarose-glutathione matrix have been reported. The purified protein was then used for raising polyclonal antibodies (Gowri et al., 1991; Inoue et al., 1998; Maury et al., 1999) and crystal formation (Ferrer et al., 2005).

**Fig. 4.4:** SDS PAGE (10%) of *E. coli* BL21 (pETCT7) induced lysate (lanes 1, 7), uninduced lysate (lane 2, 6), protein from inclusion bodies ~29Kda (lane 3), protein size markers BSA (66 KDa) and Carbonic anhydrase (29KDa) (lane 4), Ni column purified protein (lane 5).
4.3.2 Purification of polyclonal antibodies

CCoAOMT protein purified from recombinant *E. coli* inclusion bodies (since the yield was better than from lysate) was used to raise rabbit immune serum. The immune serum was centrifuged at 12,000 x g for 15 min and the clear supernatant heated for 1h at 55ºC to deactivate the compliment system. Heat denatured immune serum was again centrifuged and the supernatant transferred to fresh tubes. EDTA was added to a final concentration of 10mM and Themersol to a final concentration of 0.02%. The immune serum was aliquoted, frozen in liquid nitrogen and stored at -70ºC till further use.

The CCoAOMT specific polyclonal IgG was antigen affinity purified using Affi-Gel 15 (BioRad). The IgG was checked for purity on 10% SDS PAGE. As is obvious from Fig. 4.5, antigen affinity purified IgG show characteristic 44KDa heavy chain and the 22KDa light chain peptides. The recovered IgG was also free of other contaminating serum proteins.

![Fig. 4.5: 10% SDS PAGE of the affinity purified CCoAOMT specific IgG. BSA (lane 1), eluted fractions of affinity purified IgG (lane 2- 12), crude serum (lane 13) and serum flow through (lane 14-15).](image-url)
4.3.3 Histology

*Leucaena leucocephala* seeds were scarified using concentrated \( \text{H}_2\text{SO}_4 \). The seeds were washed thoroughly with running tap water and imbibed for 12 h (in dark). Imbibed seeds were transferred to pots (1:1 coco peat and sand mixture) for germination. Transverse sections from the shoot, root, rachis and inflorescence were prepared. Seedlings at 0 day (when the seedling fully emerged from seed coat), 5, 10 and 15 days old, and one and two seasons old plants were used for analysis.

Anatomy of *L. leucocephala* resembles the anatomy of a typical dicot plant (Esau, 1977). The sequential and progressive development of vascular tissue was evident in the transverse sections of different plant parts of 0, 5, 10 and 15 days old seedlings, and in one and two season old plants. Phloroglucinol-HCl staining of the transverse section stained lignified xylem, secondary xylem and phloem fiber in brown red (Figure 4.6 a, b; Left panel). Development of vascular tissue, particularly xylem tissue, was visible in phloroglucinol-HCl stained transverse sections at different growth stage of seedlings. Increase in number of differentiating xylem cells as well as their stain intensity indicated progression of lignification. In older tissue well developed and lignified secondary growth and development of fibers was observed. When the sections were visualized under polarized light (Fig. 4.2 a, b; Central panel), the xylem tissue which was bright reddish yellow in 0 day plant, progressively changed to bright and deep orange red in 5 and 10 days old seedlings and then to light pinkish red in 15 days old seedlings, and one season and two season old plants. The phloem fibers, however, were of bright reddish yellow initially up to 15 day but appeared orange red and pink red in I and II season old plant. Since, polarizing light produces different hues depending on the chemical bond configurations the change in color may be as a consequence of the change in cell wall chemical composition with age. As the plant matures, change in the color of lignified tissue might also be as a consequence of change in ratio or the type of lignin.

4.3.4 Immunocytolocalization

In the present study CCoAOMT was immunolocalized in the transverse sections of root, shoot, rachis and inflorescence of 0, 5, 10, 15 days old seedling as well as from one and two season old plant. The affinity purified CCoAOMT specific IgG and anti rabbit goat IgG
conjugated with alkaline phophatase were used as primary and secondary antibodies respectively.

Results revealed CCoAOMT enzyme expression in differentiating xylem tissue and phloem fibers (Figure 4.6 a, b; Right panel). The CCoAOMT presence was detected as early as in 0 day old seedling. In transverse sections of 0 day seedling blue black precipitate was visible even in the potential phloem fiber regions. This observation was confirmed when these areas differentiated into fiber as seedlings grew older. Comparison of the sections where CCoAOMT was immunolocalized, with phloroglucinol-HCl stained sections confirmed the presence of CCoAOMT at the locations where lignin was stained. Hence, suggesting that CCoAOMT activity was confined to cell undergoing lignification.

No CCoAOMT protein was immunolocalized in cortex and phloem tissue (except in phloem fibers) showing absence of CCoAOMT activity in these regions. No CCoAOMT was detected in pith cells either.

As in the present study, CCoAOMT protein immunolocalized in xylem and phloem fiber and its active participation in lignin biosynthesis has been reported in many plants like Zinnia (Ye and Varner, 1995; Ye et al., 1997), alfalfa (Kersey et al., 1999), poplar (Chen et al., 2000; Zhong et al., 2000; Ye et al., 2001), tobacco and forsythia (Ye et al., 2001).

### 4.3.5 Semiquantitative and Quantitative Real Time PCR

The immunocytolocalization of CCoAOMT in different parts of *L. leucocephala* plants at different growth stages showed that CCoAOMT activity was localized to xylem tissue and phloem fibers. This study, however, could not elucidate or discriminate the involvement of CCoAOMT 1 and CCoAOMT 2 in lignification during different stages of plant development. The differential expression of CCoAOMT1 and CCoAOMT2 genes during lignification process was assayed by semiquantitative and quantitative real time PCR (QPCR). TaqMan probe based chemistry was used during the study.
The nucleotide sequence alignment of the CCoAOMT1 and CCoAOMT2 cDNA clones reveals many positions where the nucleotide sequences differed. One of these differences in nucleotide sequence was used to design and synthesize gene specific primers to selectively amplify CCoAOMT1 and/or CCoAOMT2 transcripts from a cDNA population (Figs. 4.7 and 4.8 a, b). TaqMan probes were also designed and synthesized.

![Nucleotide sequence alignment](image.png)

**Fig. 4.7:** Nucleotide sequence alignment (nucleotide position 232 to 416) of CCoAOMT 1 (DQ431233) and CCoAOMT 2 (DQ431234) genes. Nucleotide sequences highlighted in bold are regions from where forward primer, probe and reverse primers were designed.

- **C1expF** 5' (d) AAGAATACCAGACGAGGGGCAATCTTGAAACATGCTCCTCTTTAAGCTTATGCTTAAGAATACC
- **C2expF** 5' (d) AAGAACACGAGGGGCAATCTTGAAACATGCTCCTCTTTAAGCTTATGCTTAAGAACACC
- **C1expR** 5' (d) GCCCTCTCTGAACTCAATTTTGTG
- **C2expR** 5' (d) GCCCTCTCTTCTGAACTCAATTTTGTG

**Taq1** – 5'-/6-FAM/TACTCCCTGCTTGCCACATGCTTCTGCTCTCTTCAAA/3'
**Taq2** – 5'-/Cy5/TACTCCCTGCTTGCCACTGCTTCTGCTCTCTTCAAA/3'

**Fig. 4.8:** (a) C1expF and C1expR are gene specific forward and reverse primer sequences for CCoAOMT 1. C2expF and C2expR are gene specific forward and reverse primer sequences for CCoAOMT 2. (b) Taq1 and Taq2 are TaqMan probes for the CCoAOMT 1 and CCoAOMT 2 genes respectively.
Total RNA was isolated from shoot, root, leaf and inflorescence of 0, 5, 10, 15 day old seedling, and one and two season old *L. leucocephala* plants. An aliquot of total RNA normalized for uniform amplification of the 5.8S rRNA was used for synthesis of cDNA first strand which was used as template for semiquantitative PCR.

The primers C1expF and C1expR, C2expF and C2expR were used for, semiquantitative PCR of CCoAOMT1 and CCoAOMT2 transcripts respectively. The amplification obtained from cDNAs of different plant tissues at different growth stages is shown in Fig. 4.9. The results revealed that both CCoAOMT 1 and CCoAOMT 2 were expressed simultaneously, but the levels of expression differed with the tissue type and plant age.

Low gene expression of CCoAOMT1 and CCoAOMT2 was observed in 0 day shoots. The expression increased to its maximum level by day 5. There after the gene expression declined by days 10 and 15. Low expression was seen in one season old plants also, however, the CCoAOMT2 gene expression was marginally higher than CCoAOMT1. In the two season old plants the gene expression levels increased again and here too the CCoAOMT2 gene expression was substantially higher than the expression level of CCoAOMT1.

In the roots of 0 day old seedlings both CCoAOMT1 and CCoAOMT2 genes were expressed, CCoAOMT2 relatively more than CCoAOMT1. In 5 day old seedling roots the expression levels of both the genes declined. The gene expression of CCoAOMT1 and CCoAOMT2 increased again by days 10 and 15. The expression level at day 10 in the roots was slightly higher for the CCoAOMT2 as compared to CCoAOMT1. This trend, however, reversed by day 15, when CCoAOMT1 expression was higher than CCoAOMT2. Negligible expression was seen in roots from one season and two season old plants.

Expression of CCoAOMT1 and CCoAOMT2 gene in the leaf tissue was seen in the leaves from 15 day old seedlings. Here too the expression level of CCoAOMT2 gene seemed to be marginally better than that of CCoAOMT1. No gene expression was seen in leaves of one season and two season old plants. No gene expression was seen in the inflorescence.

From the above study it was also obvious that both the CCoAOMT genes expressed or shut down in tandem.
The CCoAOMT 1 and CCoAOMT 2 specific sets of primers with TaqMan probes were used for quantitative real time PCR. Absolute quantification method was used for the purpose. In this method a standard curve was prepared using cDNA clone of the gene to be quantified. Serial dilutions were used for real time PCR reactions and a Ct value for each dilution plotted against diluted gene clone quantity. Once Ct values for experiments were obtained, the expression levels of the target gene in the sample were determined using the standard curve.

The cDNA clones of CCoAOMT 1 (DQ431233) and CCoAOMT 2 (DQ431234) were used for standard curve preparation for absolute quantification of the two gene transcripts. CCoAOMT 1 and CCoAOMT 2 concentrations used were in nanogram (ng) quantities. The cDNA first strand synthesized using total RNA quantities, earlier normalized for uniform amplification of the 5.8S rRNA isolated from shoot, root, leaf and inflorescence of 0, 5, 10, 15 day old seedling, and one and two season old L. leucocephala plants was used as template and reactions were carried out in ABI PRISM 7700 instrument.

The Ct values obtained for the two genes and their respective quantity determined using standard curve is given in table 4.2. The relative expression of the two genes in terms of ng µL⁻¹ is shown in Fig. 4.10.
Table 4.2: Ct values for the two CCoAOMTs and their respective quantities in ng µL\(^{-1}\).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Tissue type and Plant age</th>
<th>CCoAOMT 1</th>
<th>CCoAOMT 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ct value</td>
<td>Quantity (ng µL(^{-1}))</td>
</tr>
<tr>
<td>1</td>
<td>Shoot 0 day</td>
<td>30.60</td>
<td>6.15 E-4</td>
</tr>
<tr>
<td>2</td>
<td>Shoot 5 day</td>
<td>26.26</td>
<td>1.38 E-2</td>
</tr>
<tr>
<td>3</td>
<td>Shoot 10 day</td>
<td>29.24</td>
<td>1.64 E-3</td>
</tr>
<tr>
<td>4</td>
<td>Shoot 15 day</td>
<td>29.33</td>
<td>1.52 E-3</td>
</tr>
<tr>
<td>5</td>
<td>Shoot I season</td>
<td>31.97</td>
<td>2.29 E-4</td>
</tr>
<tr>
<td>6</td>
<td>Shoot II season</td>
<td>28.96</td>
<td>1.98 E-3</td>
</tr>
<tr>
<td>7</td>
<td>Root 0 day</td>
<td>28.00</td>
<td>3.97 E-3</td>
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<tr>
<td>8</td>
<td>Root 5 day</td>
<td>30.50</td>
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</tr>
<tr>
<td>9</td>
<td>Root 10 day</td>
<td>29.83</td>
<td>1.07 E-3</td>
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<td>10</td>
<td>Root 15 day</td>
<td>29.16</td>
<td>1.72 E-3</td>
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<td>11</td>
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<td>12</td>
<td>Root II season</td>
<td>33.11</td>
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<td>13</td>
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<td>14</td>
<td>Leaf Rachis 15 day</td>
<td>27.98</td>
<td>4.00 E-3</td>
</tr>
<tr>
<td>15</td>
<td>Leaf Rachis I season</td>
<td>36.19</td>
<td>1.11 E-5</td>
</tr>
<tr>
<td>16</td>
<td>Leaf Rachis II season</td>
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<td>17</td>
<td>Inflorescence</td>
<td>36.20</td>
<td>1.10 E-5</td>
</tr>
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</table>
Fig. 4.10: Relative expression of CCoAOMT 1 and CCoAOMT 2 in terms of ng µL\(^{-1}\) in different plant tissues of different age plants. On X axis is tissue type with age of seedling or plant. On Y axis is quantity in ng µL\(^{-1}\). S, R, L and Inf stands for shoot, root and leaf rachis respectively. The numbers 0, 5, 10, 15 represents age of seedling in days. I and II represents season one and two respectively.

QPCR results provided answers to the differential expression of CCoAOMT1 and CCoAOMT2 genes. These experiments also fine tuned and refined the semiquantitative PCR results. The QPCR results revealed that both CCoAOMT1 and CCoAOMT2 gene are expressed throughout the developmental stages of *L. leucocephala*, though the levels of expression differ greatly depending on tissue type and the seedling age. Highest abundance
of the CCoAOMT1 and CCoAOMT2 transcripts was observed in shoots of 5 day old seedling. However, the relative abundance of CCoAOMT1 transcript was more than the CCoAOMT2 transcripts. It was also apparent that with advance in the age of the plants, generally the CCoAOMT2 transcript levels are higher than or equal to the CCoAOMT1 transcript levels. In the root tissues the CCoAOMT1 transcript is of more abundance than the CCoAOMT2 transcript in 0 day old seedlings. The abundance of CCoAOMT1 and CCoAOMT2 is almost negligible in roots from I season and II season old plants. High and equal abundance of CCoAOMT1 and CCoAOMT2 transcript was seen in 15 days old leaves. The abundance of transcripts were negligible in leaves of I season and II season old plants and in inflorescence.

In all the lignifying tissues of different growth stages, the two CCoAOMTs were expressing in tandem. However, no correlation could be drawn between the extent of lignification and CCoAOMT gene expression levels as colour intensity of immunolocalized CCoAOMT was similar in lignifying tissue of different growth stages though QPCR results revealed different levels of transcripts.

4.4 Conclusions

- CCoAOMT gene expressed in *E. coli* BL21 (DE3) and protein purified from inclusion bodies using Ni-chelated affinity column.
- Polyclonal antibodies were raised against purified CCoAOMT protein in rabbit.
- CCoAOMT specific polyclonal IgG purified using Affi-gel 15 affinity matrix.
- Transverse sections of different plant parts of different age stained with phloroglucinol-HCL show increase in number of differentiating xylem cells as well as their stain intensity indicating progression of lignification with age.
- Visualization under polarized light of phloroglucinol-HCL stained tissues showed different colour in different plant parts of different ages, suggesting chemical compositional changes in lignin with tissue age.
- CCoAOMT immunolocalized in xylem and fibers suggesting its presence at the sites of extensive lignification.
- Semiquantitative and QPCR results showed that both the CCoAOMT1 and CCoAOMT2 genes were expressed in tandem.
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