5.1 Introduction

Promoter analysis has been the main tool for studying gene regulation. Many phenylpropanoid pathway genes have been cloned and the activities of their promoters analyzed. Promoter analysis has been done for Phenyl ammonia-lyase (PAL; Bevan et al., 1989; Ohl et al., 1990; Hauffe et al., 1991; Levy et al., 1992), cinnamic acid 4-hydroxylase (C4H; Bell-Lelong et al., 1997), hydroxycinnamate: CoA ligase (4CL; Douglas et al., 1991), caffeic acid O-methyltransferase (COMT; Capellades et al., 1996), caffeoyl CoA O-methyltransferase (CCoAOMT; Grimmig and Matern, 1997; Chen et al., 2000) and hydroxycinnamyl alcohol dehydrogenase (Feuillet et al., 1995). All these gene promoters directed the expression of GUS reporter gene in lignifying tissues. Deletion analyses in the PAL and 4CL gene promoters, identified the cis-acting AC elements as being critical for tissue-specific expression (Hauffe et al., 1991; Levy et al., 1992). These elements are also conserved in the promoters of other phenylpropanoid pathway genes. The Myb protein binds to the AC elements and turns on the expression of a reporter gene driven by the PAL promoter (Sablowski et al., 1994, 1995). Over expression of the two Antirrhinum Myb genes in transgenic tobacco plants down-regulated the expression of certain genes in the phenylpropanoid biosynthetic pathway, resulting in a reduction in lignin accumulation (Tamagnone et al., 1998). The common cis-elements in the promoter regions of a number of known PAL genes, which were also found in 4CL gene, have been reported as box P (box 2: C/TTT/CC/TA/CA/CCA/CAA/CCC/AC/AC), box A (C/ACGTCT/C), and box L (box 1: T/CCT/CC/TACCTACC). These elements play important roles in the regulation of PAL promoter activity (Cramer et al., 1989; Lois et al., 1989; Logemann et al., 1995).

Other cis-elements, such as the FP56 identified in the 4CL promoter, have also been shown to be important for regulation of gene expression in addition to the conserved AC elements (Neustaedter et al., 1999).

In ~5Kb CCoAOMT promoter fragment isolated from parsley (Grimmig and Matern, 1997), presence of three putative cis-regulatory elements, boxes P, A and L, were shown. These elements represent motifs recurring in the promoters of several genes of stress inducible phenylpropanoid pathway. The transient expression assays with sets of 5’- truncated promoter –GUS fusion showed that significant promoter activity was retained in the 354 bp
fragment. Presence of a novel cis-regulatory element (E box) was also shown by in vitro
DNase I footprinting.

Chen et al.(2000) reported the activity of two CCoAOMT promoters from poplar in
regulating gene expression in xylem and differentially in phloem. In xylem, expression was
preferentially observed in vessels and contact rays, whereas no detectable expression was
found in storage rays and fibers. Increased expression levels were reported after fungal
infection, wounding and bending. Upon bending and leaning of stem, it was reported that the
cell specific expression pattern was lost and both genes were expressed in all cell types of
xylem.

In the present study the partial promoters of CCoAOMT1 and CCoAOMT2 genes from L.
leucocephala were analyzed for presence of regulatory elements, using online software
MatInspector 2.2. The two promoters were also analyzed for their ability to drive the
expression of the reporter green fluorescent protein gene (GFP).

5.2 Material and Methods

5.2.1 Promoter analysis

The two partial CCoAOMT1 and CCoAOMT2 gene promoter sequences, designated ProC1
and ProC2 respectively, were analyzed for presence of regulatory elements using online web
based software MatInspector 2.2 available at www.genomatix.de.

5.2.2 Vector construction

Two primer sets were designed to reamplify the promoters ProC1 and ProC2. The forward
and the reverse primers were modified to introduce EcoR I and BamH I restriction site and
the modified primer sets were designated as ProC1F: 5’ (d) GAA TTC CGA CGG CCC
GGG CTG GTA A 3’ and ProC1R: 5’ (d) GGA TCC TAC TTC TTA TCC TTT TTG
TTT CCT CG 3’ for promoter ProC1 , and ProC2F: 5’ (d) GAA TTC CGA TGG CCC
GGG CTG GTA A 3’ and ProC2R: 5’ (d) GGA TCC ACT TCT TAT CCT TTT TTG
TTT CCT TG 3’ for promoter ProC2. The modified promoters were reamplified, cloned in
pGEM T Easy vector and sequenced for validation. The modified promoter fragments were
separately cloned in EcoR I and BamH I digested pJ4GFP-XB (a gift from Dr Anjan
Banerjee, Iowa, USA; Fig. 5.1 a) and upstream of the GFP gene. The two plasmid constructs
were designated pProG1 and pProG2 (Fig. 5.1 b, c). The pProG1 and pProG2 plasmids were digested with EcoR I and Hind III to release the ProC1/ProC2 promoter-GFP-NOS 3’ cassette. The released cassette was cloned in EcoR I and Hind III digested pCAMBIA 1300 MCS (Fig. 5.2). The two resulting plasmid were designated as pPC1G and pPC2G respectively.

### 5.2.3 Agrobacterium tumefaciens transformation

The pPC1G and pPC2G vectors harboring ProC1 or ProC2 – GFP – NOS 3’ cassette were transformed independently into A. tumefaciens GV2260 (Chapter 2; section 2.3.2).

### 5.2.4 Agrobacterium mediated transformation of tobacco

Tobacco plants were transformed independently using the above A. tumefaciens cultures, harboring the pPC1G or the pPC2G vectors (Chapter 2; section 2.14).

### 5.2.5 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 2.4.3 and 2.4.8).

### 5.2.6 Histology and Fluorescent Microscopy

Transverse sections of root, stem and leaf midrib were cut, dehydrated and rehydrated (Chapter 2; section 2.11). The dechlorophyllled putative transformed plants and transverse sections of leaf mid rib, shoot and root were visualized for green fluorescence and pictures captured (Chapter 2; section 2.12).
Fig. 5.1: Vector maps: (a) pJ4GFP-XB, (b) pProG1 and (c) pProG2.
Fig. 5.2: (a) Vector map of pCAMBIA 1300MCS; (b) ProC1 promoter-GFP-NOS 3' (pPC1G) and ProC2-GFP-NOS 3' (pPC2G) cassettes.
5.3 Results and Discussion

5.3.1 Promoter analysis using MatInspector version 2.2

The nucleotide sequences of the promoters ProC1 and ProC2 were analyzed using online software MatInspector 2.2. The analysis revealed presence of different motifs, which have been earlier reported in different plant promoters and play role in gene regulation (Table 5.1 a, b).

Fourteen and twelve gene regulation related motifs respectively were identified in ProC1 and ProC2. Out of these, eight motifs were of common occurrence in both the promoters. In promoter ProC1 six motifs were identified which were absent in promoter ProC2. Similarly three motifs were seen in ProC2, which were absent in ProC1.

Table 5.1: MatInspector 2.2 analysis of ProC1 (a) and ProC2 (b) showing presence of different motifs involved in gene regulation. Highlighted in bold letters are motifs unique to each of the promoters.

<table>
<thead>
<tr>
<th>S. No</th>
<th>IUPAC</th>
<th>Position</th>
<th>Sequence Capitals: core sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P$FAM267/P$STAAAGSTKST1</td>
<td>15 - 21</td>
<td>GGTAAAG</td>
</tr>
<tr>
<td>2</td>
<td>P$FAM010/P$WBOXHVISO1</td>
<td>23 - 37</td>
<td>CATGACCTTTATTTATC</td>
</tr>
<tr>
<td>3</td>
<td>P$FAM012/P$IBOXCORE</td>
<td>44 - 50</td>
<td>GATAATT</td>
</tr>
<tr>
<td>4</td>
<td>P$FAM272/P$SV40COREENHAN</td>
<td>57 - 64</td>
<td>GTGGATTG</td>
</tr>
<tr>
<td>5</td>
<td>P$FAM004/P$PALBOXLPC</td>
<td>111 - 121</td>
<td>CCTCAACCAACC</td>
</tr>
<tr>
<td>6</td>
<td>P$FAM003/P$MYBPLANT</td>
<td>114 - 124</td>
<td>CACCAACCACA</td>
</tr>
<tr>
<td>7</td>
<td>P$FAM171/P$BOXLCOREDCPAL</td>
<td>115 - 121</td>
<td>ACCAACC</td>
</tr>
<tr>
<td>8</td>
<td>P$FAM263/P$DPBF COREDCDC3</td>
<td>122 - 128</td>
<td>ACACCCCG</td>
</tr>
<tr>
<td>9</td>
<td>P$FAM270/P$RAV1AAT</td>
<td>143 - 147</td>
<td>CAACA</td>
</tr>
<tr>
<td>10</td>
<td>P$FAM241/P$STATABOX2</td>
<td>203 - 209</td>
<td>TATAAAT</td>
</tr>
<tr>
<td>11</td>
<td>P$FAM061/P$AGCBOXNPGLB</td>
<td>216 - 222</td>
<td>AGCCGCC</td>
</tr>
<tr>
<td>12</td>
<td>P$FAM305/P$ANAERO1CONSENSUS</td>
<td>293 - 299</td>
<td>AAACAAA</td>
</tr>
<tr>
<td>13</td>
<td>P$FAM014/P$MYBST1</td>
<td>302 - 308</td>
<td>AGGATAA</td>
</tr>
<tr>
<td>14</td>
<td>P$FAM012/P$IBOXCORENT</td>
<td>304 - 310</td>
<td>GATAAGA</td>
</tr>
</tbody>
</table>

(a)
5.3.1.1 Common Regulatory Motifs

The eight common motifs present in the ProC1 and ProC2 are described below:

<table>
<thead>
<tr>
<th>S. No</th>
<th>IUPAC Name</th>
<th>Position</th>
<th>Sequence capitals: core sequence</th>
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<td>1</td>
<td>P$FAM107/P$CGACGOSAMY3</td>
<td>1 - 5</td>
<td>CGACG</td>
</tr>
<tr>
<td>2</td>
<td>P$FAM267/P$TAAAGSTKST1</td>
<td>15 - 21</td>
<td>GGTAAAG</td>
</tr>
<tr>
<td>3</td>
<td>P$FAM010/P$WBOXHVISO1</td>
<td>23 - 37</td>
<td>CGTGACTTATTATTC</td>
</tr>
<tr>
<td>4</td>
<td>P$FAM243/P$TATABOX4</td>
<td>38 - 44</td>
<td>TATAAA</td>
</tr>
<tr>
<td>5</td>
<td>P$FAM267/P$TAAAGSTKST1</td>
<td>40 - 46</td>
<td>TATAAAG</td>
</tr>
<tr>
<td>6</td>
<td>P$FAM272/P$SV40COREENHAN</td>
<td>57 - 64</td>
<td>GTGGATTG</td>
</tr>
<tr>
<td>7</td>
<td>P$FAM302/P$SITEIIATCYTC</td>
<td>85 - 95</td>
<td>TGGGCCACGT</td>
</tr>
<tr>
<td>8</td>
<td>P$FAM003/P$MYBPLANT</td>
<td>145 - 155</td>
<td>AACCAACCACT</td>
</tr>
<tr>
<td>9</td>
<td>P$FAM171/P$BOXLCOREDCPAL</td>
<td>146 - 152</td>
<td>ACCAACC</td>
</tr>
<tr>
<td>10</td>
<td>P$FAM305/P$ANAERO1CONSENSUS</td>
<td>299 - 305</td>
<td>AAACAAAA</td>
</tr>
<tr>
<td>11</td>
<td>P$FAM014/P$MYBST1</td>
<td>307 - 313</td>
<td>AGGATAAA</td>
</tr>
<tr>
<td>12</td>
<td>P$FAM012/P$IBOXCORENT</td>
<td>309 - 315</td>
<td>GATAAGA</td>
</tr>
</tbody>
</table>

**IUPAC Name: P$TAAAGSTKST1**
Position in Promoter ProC1: 15 - 21 bp.
Position in Promoter ProC2: 15 - 21 and 40 – 46 bp.
This motif belongs to family P$FAM267 and its IUPAC ambiguity code is TAAAG. This TAAAG motif is found in promoter of *Solanum tuberosum* KST1 gene, which encodes a K+ influx channel. It is the target site for trans-acting StDof1 (DNA binding with one finger) protein controlling guard cell-specific gene expression (Plesch et al., 2001).

**IUPAC Name: P$WBOXHVISO1**
Position in Promoter ProC1: 23 – 37 bp.
Position in Promoter ProC2: 23 – 37 bp.
This motif belongs to family P$FAM010 and its IUPAC ambiguity code is TGACT. A novel WRKY transcription factor SUSIBA2, involved in sugar signaling in barley by binding to the
sugar-responsive elements of the iso1 promoter, bind to W-box element in barley iso1 (encoding isoamylase1) promoter (Sun et al., 2003).

**IUPAC Name: P$SV40COREENHAN**

Position in Promoter ProC1: 57 – 64 bp.
Position in Promoter ProC2: 57 – 64 bp.
This motif belongs to family PSFAM272 and its IUPAC ambiguity code is GTGGWWHG.
This sequence shows similarity to SV40 core enhancer sequence found in rbcS genes (Weiher et al., 1983; Green et al., 1987; Donald and Cashmore, 1990).

**IUPAC Name: P$MYBPLANT**

Position in Promoter ProC1: 114 – 124 bp.
This motif belongs to family PSFAM003 and its IUPAC ambiguity code is MACCWAMC.
This element is plant MYB binding site and consensus sequence is related to box P in promoters of phenylpropanoid biosynthetic genes such as PAL, CHS, CHI, DFR, CL, Bz1. The AmMYB308 and AmMYB330 transcription factors from *Antirrhinum majus* have been shown to regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco (Tamagnone et al., 1998; Sablowski et al., 1994).

**IUPAC Name: P$BOXLCOREDCPAL**

Position in Promoter ProC1: 115 – 121 bp.
Position in Promoter ProC2: 146 – 152 bp.
This motif belongs to family PSFAM171 and its IUPAC ambiguity code is ACCWWCC.
This element is consensus of the putative core sequence of box –L- like sequence in carrot (*Daucus carota*) PAL1 promoter region. *In vitro* it has been shown that DCMYB1 binds to these sequences (Maeda et al., 2005).

**IUPAC Name: P$ANAERO1CONSENSUS**

Position in Promoter ProC1: 293 – 299 bp.
This motif belongs to family: P$FAM305 and its IUPAC ambiguity code is AAACAAA. It is one of 16 motifs found in silico in promoters of 13 anaerobic genes involved in the fermentative pathway (anaerobic set 1) (Mohanty et al., 2005).

**IUPAC Name: P$MYBST1**
Position in Promoter ProC1: 302 – 308 bp.
This motif belongs to family P$FAM014 and its IUPAC ambiguity code is GGATA. It is core motif of MybSt1 (a potato MYB homolog) binding site. The MybSt1 cDNA clone was isolated by using CaMV 35S promoter domain A as a probe. The Myb motif of the MybSt1 protein is distinct from the other plant Myb DNA binding domain described so far (Baranowskij et al., 1994).

**IUPAC Name: P$IBOXCORENT**
Position in Promoter ProC1: 304 – 310 bp.
This motif belongs to family P$FAM012 and its IUPAC ambiguity code is GATAAGR. It is "I-box core motif" in the CAMs (conserved DNA modular arrays) and associated with light-responsive promoter regions (Martinez et al., 2002).

**5.3.1.2 Different Regulatory Motifs**
The motifs which are present only in individual ProC1 and ProC2 promoter are as follows:

**Promoter ProC1**
**IUPAC Name: P$IBOXCORE**
Position: 44 – 50 bp
This motif belongs to family P$FAM012 and its IUPAC ambiguity code is GATAA. I-box is conserved sequence found upstream of light-regulated genes of both monocots and dicots (Terzaghi and Cashmore, 1995).
IUPAC Name: P$PALBOXLPC
Position: 111 - 121
This motif belongs to family P$FAM004 and its IUPAC ambiguity code is YCYYACCCWACC. This element is ‘Box L’ which is one of three putative cis-acting elements (boxes P, A, and L) of phenylalanine ammonia lyase genes in parsley (*Petroselinum crispum*). None of these elements (boxes P, A, and L) alone, or the promoter region containing all of them together, conferred elicitor or light responsiveness. These elements appear to be necessary but not sufficient for elicitor or light-mediated PAL gene activation (Logemann et al., 1995).

IUPAC Name: P$DPBFCOREDCDC3
Position: 122 - 128
This motif belongs to family P$FAM263 and its IUPAC ambiguity code is ACACNNG. It is a novel class of bZIP (basic leucine zipper) transcription factors, DPBF-1 and 2 (Dc3 promoter-binding factor-1 and 2), binding core sequence found in the carrot Dc3 gene promoter. Dc3 expression is normally embryo-specific, and also can be induced by ABA. The *Arabidopsis* abscisic acid response gene ABI5 encodes a bZIP transcription DE factor. The abi5 mutant has pleiotropic defects in ABA response. The ABI5 DE regulates a subset of late embryogenesis-abundant genes. GIA1 (growth-insensitivity to ABA) is identical to ABI5 (Kim et al., 1997; Finkelstein et al., 2000; Lopez-Molina and Chua, 2000).

IUPAC Name: P$RAV1AAT
Position: 143 – 147 bp.
This motif belongs to family P$FAM270 and its IUPAC ambiguity code is CAACA. It is binding consensus sequence of *Arabidopsis* transcription factor, RAV1. RAV1 specifically binds to DNA with bipartite sequence motifs of RAV1-A (CAACA) and RAV1-B (CACCTG). RAV1 protein contain AP2-like and B3-like domains which recognize the CAACA and CACCTG motifs, respectively. The expression level of RAV1 was relatively high in rosette leaves and roots (Kagaya et al., 1999).
IUPAC Name: P$TATABOX2
This motif belongs to family P$FAM241 and its IUPAC ambiguity code is TATAAAT. It is commonly known as "TATA box". This TATA box is found in the 5' upstream region of pea legA gene (Shirsat et al., 1989), sporamin A of sweet potato and in beta-phaseolin promoter (Grace et al., 2004). The sequence and spacing of TATA box elements are critical for accurate initiation (Grace et al., 2004).

IUPAC Name: P$AGCBOXNPGLB
Position: 216 – 222 bp.
This motif belongs to family P$FAM061 and its IUPAC ambiguity code is AGCCGCC. This element, "AGC box", is repeated twice in a 61 bp enhancer element in tobacco class I beta-1,3-glucanase (GLB) gene. "GCC-box" which is a binding sequence of Arabidopsis AtERFs (ethylene response factors). AtERF1, 2 and 5 functions as activators of GCC box-dependent transcription whereas AtERF3 and 4 acts as repressors. AtERF proteins are stress signal-response factors and are found in the promoter of tobacco Osmotin-like protein (OLP) genes. It is EREBP2 (ethylene-responsive element binding proteins) binding site and is conserved in most PR-protein genes (Hart et al., 1993; Sato et al., 1996; Fujimoto et al., 2000; Ohme et al., 2000).

Promoter ProC2
IUPAC Name: P$CGACGOSAMY3
Position: 1 – 5 bp.
This motif belongs to family P$FAM107 and its IUPAC ambiguity code is CGACG. "CGACG element" has been reported from the GC-rich regions of the rice Amy3D and Amy3E amylase genes, but not in Amy3E gene (Hwang et al., 1998). This element may function as a coupling element for the G box element.
IUPAC Name: **P$TATABOX4**  
Position: 38 – 44 bp.  
This motif belongs to family P$FAM243 and its IUPAC ambiguity code is TATATAA. It is commonly known as "TATA box". This TATA box is found in the 5'upstream region of sweet potato sporamin A gene and in beta-phaseolin promoter (Grace et al., 2004). Sequence and spacing of TATA box elements are critical for accurate initiation (Grace et al., 2004).

IUPAC Name: **P$SITEIIATCYTC**  
Position: 85 – 95 bp.  
This motif belongs to family P$FAM302 and its IUPAC ambiguity code is TGGGCY. This element, "Site II element", is found in the promoter regions of cytochrome genes (Cytc-1, Cytc-2) in *Arabidopsis* and is located between -147 and -156 from the translational start sites (Welchen et al., 2005).

### 5.3.2 Promoter ProC1 and ProC2 directed GFP gene expression in Tobacco

The two isolated promoters ProC1 (313 bp) and ProC2 (319 bp) were relatively small compared to earlier reported promoters sequences for different phenylpropanoid pathway genes (Bevan et al., 1989; Ohl et al., 1990; Hauffe et al., 1991; Capellades et al., 1996; Mizutani et al., 1997; Bell-Lelong et al., 1997). Grimmig and Matern (1997) reported ~5.0 Kb promoter from parsley CCoAOMT gene. Two CCoAOMT promoters of 2.0 Kb and 1.4 Kb have been reported from poplar (Chen et al., 2000). The two promoters ProC1 and ProC2 were used to construct vectors pPC1G and pPC2G where the GFP gene was placed under their control (section 5.2.2 above).

### 5.3.2.1 Tobacco transformation

Tobacco (*N. tabaccum* var. Anand 119) leaf discs were transformed separately with *A. tumefaciens* cultures harboring the pPC1G or the pPC2G vectors. Shoots regenerated *in vitro* under selection pressure from the cut surface of the leaf disc after 2 weeks (Fig. 5.3). The sets of putative transformants were designated as CProG1 and CProG2 depending on the promoter used.
The regenerants were allowed to grow for 12 weeks and then shifted to root induction medium. Roots were initiated within 2 weeks of shifting (Fig. 5.4). The transformed plants were hardened and transferred to pots (Fig. 5.5 a, b).

5.3.2.2 GFP visualization in transformed tobacco plants

The two transformation events, CProG1 and CProG2 were visualized at 395nm using NightSea GFP flash light (NightSea, USA). The dechlorophylled putative transgenic tobacco plants, (regenerated in vitro) showed green fluorescence, when illuminated at 395nm (Fig. 5.6 a, b). No fluorescence was seen in untransformed control tobacco plants (Fig. 5 c). This was first indication of the integration and expression of the GFP gene under the control of both the promoters ProC1 and ProC2. As compared to other reported CCoAOMT promoters, the ProC1 and ProC2 were small and are possibly partial promoter sequences. Grimmig and Matern (1997) have shown that a 5’ truncated 354 bp fragment of CCoAOMT promoter from parsley was sufficient to drive the expression of GUS gene. The truncated ProC1 and ProC2 also are sufficient to drive GFP gene expression respectively.

5.3.2.3 Integration of promoters ProC1, ProC2 and GFP in tobacco genome

Genomic DNA isolated from untransformed control tobacco and the two transformation events CProG1 and CProG2 was used as template for PCR based amplification of the GFP gene using gene specific forward primer GF: 5’ (d) ATG GTG AGC AAG GGC GAG GAG CTG TTC A 3’ and the reverse primer GR: 5’ (d) TTA GAT CTC TTG TAC AGCTCG TCC ATG CCG TG 3’. A fragment of ~700bp was amplified from the genomic DNA templates of CProG1 and CProG2. There was no amplification from the untransformed tobacco plant (Figure 5.7 a). The amplicons were isolated, cloned in pGEM-T Easy vector and sequenced. The nucleotide sequence of the amplicons was confirmed to be that of the GFP gene. PCR amplifications from the genomic DNA of the transformation events CProG1 and CProG2 with forward primer Pro1F and Pro2F designed from ProC1 and ProC2 promoter sequences respectively and the GFP gene reverse primer GR, amplified an ~1Kb band (Fig. 5.7 b). The amplicons were cloned in pGEM-T Easy vector and sequenced. The sequence data matched the promoter plus the GFP gene sequences. These results indicate the
integration of the CCoAOMT promoters ProC1 and ProC2 together with the GFP gene in the tobacco genome.

Fig. 5.7: (a) PCR amplification of GFP from genomic DNA of control plant (lane 2), CProG1 (lane 3), CProG2 (lane 4). DNA size marker (lane 1). (b) PCR amplification of Promoter plus GFP from genomic DNA of control plant (lane 2), CProG1 (lane 3), CProG2 (lane 4). DNA size marker (lane 1).

5.3.2.4 Visualization of GFP in transverse sections of leaf midrib, stem, and root of CProG1 and CProG2 transgenic tobacco plants

Transverse sections of leaf midrib, stem and root of transgenic tobacco plants, CProG1 and CProG2, were cut. The sections were dehydrated and rehydrated by passing them through alcohol: water series. The sections were mounted in glycerol, visualized at 395nm under fluorescence microscope and pictures captured. In the transverse sections of leaf midrib and stem, green fluorescence was observed, however, the fluorescence was limited to the xylem tissue. Green fluorescence was also observed in occasional epidermal cells (Fig. 5.8 a-d).
The pattern of GFP gene expression in the roots was, however, different. Fluorescence was not limited to the xylem tissue. Fluorescence was also seen on the inner side of the epiblema cell layer and in the pericycle ring (Fig. 5.8 e, f). This observation is in agreement with the involvement of phenylpropanoid pathway genes during suberin biosynthesis. The aromatic moiety of suberin is synthesized via the general phenylpropanoid pathway with its key enzyme PAL (Kolattukudy, 1981). There was no apparent difference in the level or pattern of GFP expression as driven by the ProC1 and ProC2 promoter as was obvious by the extent of fluorescence of GFP in the two transformation events.

The xylem specific expression of GFP gene under control of the two promoters, ProC1 and ProC2, proved that the two truncated promoters were sufficient to drive the expression of a gene (GFP gene in the present case). Similar observation was made by Grimmig and Matern (1997) using truncated CCoAOMT promoter. Moreover, it was evident from the expression pattern of the GFP gene, that the gene regulation elements directing xylem specific gene expression were present in truncated promoters. These results are in confirmation with the CCoAOMT immunocytolocalization results where CCoAOMT protein was shown to be localized in the xylem tissue (Chapter 4; section 4.3.4). Since both ProC1 and ProC2 promoters from CCoAOMT1 and CCoAOMT2 drive GFP gene expression in the xylem tissue, the observation from QPCR that the CCoAOMT1 and CCoAOMT2 are expressed in tandem is substantiated here. However, the stomata guard cell specific expression of GFP was not observed though the TAAAG motif, responsible for it, was present in both the CPro1 and CPro2 promoters. Green fluorescence observed in tissue other than xylem especially in roots, suggests presence of other unidentified elements regulating CCoAOMT1 and CCoAOMT2 expression in roots.

The above results from promoter ProC1 and ProC2 were similar to earlier findings (Grimmig and Matern, 1997 and Chen et al., 2000) of CCoAOMT being selectively expressed in xylem and fibers. Negligible expression in phloem was also in agreement.

5.4 Conclusions

- Analysis of the two CCoAOMT1 (ProC1) and CCoAOMT2 (ProC2) promoter nucleotide sequences revealed presence of different cis-regulatory elements involved in CCoAOMT gene regulation.
Tobacco plants transformed with cassettes where promoters ProC1 and ProC2 drive GFP gene were recovered. GFP was visualized in xylem and fibers in leaf mid rib, shoot and root.

In root GFP was visualized in tissues other than xylem suggesting involvement of other regulatory elements in the two promoters.

5.5 References

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