CHAPTER 4: RESULTS, Part 1
Isolation and cloning of partial CA-PRO promoter

The *Clerodendrum aculeatum* L genomic DNA was digested either with BamHI, BglII or Sou3A restriction enzymes (Fig. 1 and 2-A). The cut ends were partially filled with A and G nucleotides to prevent self-ligation of the digested DNA fragments. These partially filled genomic DNA fragments were ligated with adapter-primers (ADOP-32 and ADOP-27, Fig. 1). The adaptor-primers were designed in such a way so that they can anneal to other DNA and also anneal with the partially filled genomic DNA. The ADOP-32 primer contained sequences of T7 primer and the ADOP-27 primer 3’ end was blocked to prevent the extension during the PCR reaction. The ligated DNA was used as template and the promoter sequences were PCR amplified using T7 and MES-1 primers (Fig. 2-B). The PCR amplified DNA was used as template for the second PCR with T7 and MES-2 primer (Fig. 2-C). A band of approximately 0.35 kb was seen in the Sau3AI digested DNA. The amplified DNA was purified, cloned into pGEM-T vector to create pGEMT-CA-PRO1 vector (Fig. 3). Cloning of the CA promoter was further confirmed using vector specific T7 and SP6 primers and finally by sequencing.

Isolation and Cloning of 5' DNA Fragment Further Upstream to CAP Gene

Similar approach was adopted to isolate about 800 bp length DNA fragment upstream to CAP gene by walking the genomic DNA using primers MES-4, MES5, MES17 and MES19 The complete sequence of the DNA was
Fig. 1. Flow diagram outlining the strategy used to isolate the CA-PRO promoter.
Details given in Materials and Methods. T7, MES-1 and MES-2 are primers used for amplification. MES-1 and MES-2 are gene-specific primers.
Fig. 2. Isolation of CA-PRO promoter.
(A) Genomic DNA was digested partially with Sou3A, BamHI, BglII, and BcII, end-filled and ligated with specific adapter-primer (for details see Materials and Methods). (B) In the Primary PCR amplification the adapter ligated genomic DNA was amplified with T7 and MES-1 gene specific primers. The amplified products were run on 0.8% agarose gel. (C) Secondary amplification by using various dilutions of the Primary PCR products using T7 and second gene specific MES-2 primer.
Fig. 3. PCR Amplification products for colone of CA-PRO 1 promoter first fragment after cloned the promoter in pGEMT vector. Lanes from 1 to 9 and 11 to 20 show the amplification with CAPRO5-1 and CAPRO3-1 primers. Lane (M) contains DNA digested with lambda HindIII used a size marker.
presented in Figure 4. Also the sequence information was deposited in a public data base GenBank under the accession number AF527488.

Computer Aided Sequence Analysis to Identify Cis-Acting Elements

Computer aided sequence analysis (Signal Scan Program) of 5' regulatory sequence reveled the presence of several cis-acting elements such as TTATT (nucleotide position 102), ATAGAA (231), CAAT (316), GATA (320 and 458), GRWAAW (448), TATAAAT (538), AAAG (703). A comparison of these Cis-acting elements with the previously reported Cis-acting elements were present in figure 5. CAAT, GATA and TATA Boxes that are typically present in other promoters were present in CA promoter. Also GT-1 binding site involved in light regulated expression was present. Figure 6 show a partial sequence of CA promoter that contained AATTAA Box and CAAT Box elements.

Primer Extension to Determine the Transcription Start Site of the Promoter

In order to determine the transcription initiation for the CA promoter, a primer extension reaction was carried out and the results are presented in figure 7. It was found that the transcript initiate from two different nucleotides for this promoter. The major transcript initiated from “C” nucleotide, position 170 and the minor transcript initiated from “A” nucleotide, position 150.
Fig. 4. Promoter sequence of CA-PRO. The nucleotide sequence of the CA-PRO promoter is shown above. The sequence shown in lower case represents the portion overlapping with the cDNA clone of CA-SRI. The start codon (ATG) is underlined. The sequence (minus the portion overlapping with the CA-SRI cDNA) was deposited in Genbank with the Accession number (AF527488). The start sites as mapped by primer extension are marked in blue. The putative cis-acting elements present in the CA-PRO promoter are indicated by coloured boxes over the sequence. The cis-acting elements are listed in next fig.
<table>
<thead>
<tr>
<th>Motif</th>
<th>Found in</th>
<th>Site Position</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Box II&quot;</td>
<td>tobacco (N.t.) plastid atpB gene promoter;</td>
<td>231</td>
<td>ATAGAA</td>
<td>Kapoor, and Sugiura (1999)</td>
</tr>
<tr>
<td>CIACADIANLELHC</td>
<td>tomato (L.e.) Lhc gene</td>
<td>126</td>
<td>CAANNNNATC</td>
<td>Piechulla, et.al., (1998)</td>
</tr>
<tr>
<td>DOFCOREZM</td>
<td>required for binding of Dof proteins in maize</td>
<td>703</td>
<td>AAAG</td>
<td>Yanagisawa and Schmidt (1999)</td>
</tr>
<tr>
<td>GATABOX</td>
<td>GATA motif in CaMV 35S promoter</td>
<td>458</td>
<td>GATA</td>
<td>Gilmartin, et.al., (1990)</td>
</tr>
<tr>
<td>GRWAABOX</td>
<td>the promoter of Petunia</td>
<td>320</td>
<td>GATA</td>
<td>Benfey and Chua (1990)</td>
</tr>
<tr>
<td>GTGANTGTG10</td>
<td>promoter of the tabacco (N.t.) late</td>
<td>255</td>
<td>GTGA</td>
<td>Rogers, et.al., (2001)</td>
</tr>
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<td>TATABOX5</td>
<td>5’upstream region of pea</td>
<td>102</td>
<td>TTATTT</td>
<td>Tjaden, et.al., (1995)</td>
</tr>
<tr>
<td>POLASIG2</td>
<td>poly A signal found in rice alpha-amylase</td>
<td>290</td>
<td>AATTAAA</td>
<td>Kumagai, et.al., 1990</td>
</tr>
<tr>
<td>TATABOX2</td>
<td>5’upstream region of pea legA gene</td>
<td>538</td>
<td>TATAAT</td>
<td>Shirsat, et.al., (1989)</td>
</tr>
<tr>
<td>GT1CONSENSUS</td>
<td>GT-1 binding site in many light-regulated genes</td>
<td>448</td>
<td>GRWAAW</td>
<td>Le Gourrierec et.al., (1999)</td>
</tr>
</tbody>
</table>

**Table 4. Cis-acting elements found in the CA-PRO promoter.** The putative cis-acting elements found in the CA-PRO promoter along with their positions are listed above. These elements were identified using the Signal Scan Program at PLACE (http://www.dna.affrc.go.jp).
Fig. 5A. Autoradiogram showing the sequencing reactions with the T7 primer for second fragment of CA-PRO promoter. The sequence indicates some of the cis-acting elements found in the CA-PRO promoter along with their positions. The sequence of loading was ATGC (from the left to right).
Fig. 5B. Autoradiogram showing the sequence of CA-SR1 gene promoter and coding region-junction. Also shown was the comparison between the UTR of previously published sequence (Acc. No. X96583) and the sequence determined in the present study. Note the high degree of divergence between these two UTR regions which is an indirect evidence for the existence of at least a second CA-SR1 gene in Clerodendrum aculeatum.
Fig. 6. Characterisation of the CA-PRO promoter. Primer extension analysis predicts two sites for initiation of the CA-SIR transcript. The primer extension product was run on a sequencing gel along with the sequencing reaction for the CA-PRO promoter. The major transcript initiates from C, 170 nucleotides upstream of the translation initiation site (ATG) while the minor transcript starts from A, 150 nucleotides upstream of ATG. The start sites are indicated by arrows. The letters A, T, G & C on the top of the lane indicate the base at which the DNA synthesis was terminated of primer extension products.
Fig. 7. Structure of plasmid pBI121. The construct carry left and right borders (LB, RB) of the transferred DNA that demarcates the sequences that are incorporated into the plant genomic DNA via Agrobacterium-mediated transformation. The GUS gene coding region lies downstream of the CaMV 35S promoter in pBI121 and is followed by the nopaline synthase (NOS) terminator. In pBI121, the 35S promoter is replaced by a different fragment of CA-PRO promoter (CAPRO1, CAPRO1+2, CAPRO1+3). Restriction endonuclease cleavage site are indicated.
Deletion Analysis of CA Promoter

In order to identify the functional domain of CA promoter, three different constructs that differed in the promoter length were generated using pBI121 plant transformation vector (Fig. 8). The CA promoter containing 300 bp (CA-PRO 1), 500 bp (CA-PRO 1+2) and 850 bp (CA-PRO 1+3) were cloned as HindIII and BamHI fragments in the same sites to drive the uidA (GUS) reporter gene in pBI121 vector. Cloning of CA promoter into pBI121 was confirmed by PCR (Fig. 8) and restriction analysis (data not shown). When PCR amplified with CA promoter specific internal primers (CA-PR05-1 and CA-PR03-1), all the 13 colonies tested were found to be positive and a band of 350 bp was observed. For the comparison, CA promoter present in pGEMT was used as a template (Fig. 9, lane P). Similar approach was followed to clone 500 bp and 850 bp fragments of CA promoter into pBI 121 vector.

Transformation of pBI121-CA-PRO1, pBI121-CA-PRO 1+2 and pBI121-CA-PRO 1+3 Vectors and Regeneration of Stable Tobacco Transgenic Plants

The A. tumefaciens Ti- plasmid based binary vectors pBI121 harbouring the nptII gene and a gus gene under CA promoter was used to transform N. tabaccum by co-cultivation (Fig. 10A). Several transformed plants resistant to kanamycin (100 – 200 µg/ml) were obtained following transformation. The DNA transfer events were studied by monitoring the transgenes associated with T-DNA (uidA and nptII) among the kanamycin resistant plants.
Fig. 8. PCR Amplification products for colone of CA-PRO 1 promoter first fragment after cloned the promoter in pBI121 vector for Agrobactrium transformation. Lanes from 1 to 13 show the amplification with CAPRO5-1 and CAPRO3-1 primers. Lane (P) Positive colone. Lane (M) contains DNA digested with lambda HindIII used size marker.
Tobacco explants after incubating the wounded plant tissue with *Agrobacterium* carrying the pBI121-CA-PRO Plasmid.

Place infected tissue on medium containing kanamycin.

Transformed cells divide and grow; these are transferred to medium to allow shoots to develop.

Shoots are placed on another medium to promote root development, so that small plantlets are produced.

**Fig. 9A.** *Agrobacterium* method to transfer DNA from the bacterium into plants.

Modified Ti plasmid in *Agrobacterium tumefaciens*, pBI121-CA-PRO construct and how do we go about producing transgenic tobacco plants.
Plants are transferred from agar medium to soil.

Plants are transferred to soil in greenhouse.

Fig. 9B. Agrobacterium method to transfer DNA from the bacterium into plants. Modified Ti plasmid in Agrobacterium tumefaciens, pBI121-CA-PRO construct and how do we go about producing transgenic tobacco plants.
A total of 50 kanamycin resistant plants from two independent transformation experiments were selected for each promoter construct. Various stages of plantlets development on selection medium was presented in figure 10-A and 10-B. It can be seen from figure 10-A that the kanamycin resistant calli developed from only the cut surface of the tobacco leaf tissue, an indication of transformation using *Agrobacterium*. Transgenic plants were screened by PCR (Fig. 10-A and GUS assay and finally confirmed by Southern hybridization (Fig. 10-B). A band of the expected size (at approximately 2.1 kb) was detected in all transgenic lines (1-13), in a PCR reaction using CA-PRO5-2 and GUS3 primer combination and the genomic DNA from transgenic plants as a template.

Southern blot analysis of genomic DNA digested with HindIII and BamHI and probed with CA promoter or *uidA* coding region further confirmed the transgenic nature of the regenerated tobacco plants (Fig 10-B). The intensity of the hybridized signal was variable, indicating multiple insertions.

Northern blot analysis confirmed the expression of GUS under CA-PRO promoter (Fig.10-C). A transcript of 1.8 kb can be seen in all GUS positive plants.

**Progeny Analysis for Stable Inheritance of NptII and Uida Genes**

The primary transgenic plants (T0 generation) were grown to maturity in the greenhouse to set the seed. Morphologically, transformed plants were
Fig.10. (A) Confirmation of the transgenic tobacco plants transformed with PBI121- CA-PRO using one primer from promoter (CA-PRO5-2) that anneal to promoter and GUS 3 primer. (B) Southern blot analysis of the genomic DNA isolated from the transgenic tobacco plant hybridizes with CA-PRO promoter probe. The DNA was digested with HindIII and BamHI. (C) Northern blot analysis to confirm the expression of GUS gene under CA-PRO 1+3 promoter. The lambda DNA digested with HindIII was used as a marker.
indistinguishable from the control-untransformed plants. These plants grew normally and set seeds. Seeds obtained from individual plants were tested for the segregation of \textit{nptII} gene and for the expression of \textit{uidA} (GUS) gene among the progeny plants. When germinated on kanamycin (100 $\mu$g/ml) containing MS medium in petri dishes, the \textit{nptII} gene containing (green) and absent (white) seedlings segregated in 3:1 Mendelian ratio (Fig. 11). A histochemical assay to detect GUS expression in the green seedlings revealed the presence of blue colour in all the seedlings tested indicating the stable inheritance of \textit{nptII} and \textit{uidA} genes among the progeny plants. Further the expression pattern revealed that the GUS expressed in all the tissues of the plants although the intensity was highest in leaf tissue (Fig. 12-A). Inheritance of GUS among the progeny was further tested using GUS5 and GUS3 internal primers in a PCR reaction. A band corresponding to the size of \textit{uidA} coding region (1.85 kb) was observed in all the seedlings tested (Fig. 12-B). Quantitative analysis revealed that the GUS expression under CA-PRO3+1 promoter ranged from 3-48 nmols/min. per mg protein with an average GUS activity was 70% - 80% equivalent to the average expression of GUS under 35S promoter.
Fig. 11. Selection of CA-PRO promoter transgenic T-1 seedlings on kanamycin containing MS-basal medium. Seeds of CA-PRO transgenics were surface sterilized by washing with 1% bleach solution and then by 70% ethanol and plated on 50 μg/ml kanamycin containing MS-basal medium. Note that seedlings from transformed plants segregated in (3 green : 1 white).
Fig. 12. (A) Detection of transgene in CA-PRO-1 and CA-PRO 1+2 plants using GUS staining and genomic PCR. Transgenic plant treated seedling were stained with x-glu that turned to blue in color, indicating the expression of GUS gene under CA-PRO promoter. (B) Genomic DNA was isolated from GUS positive transgenic plants and tested for the presence of GUS gene using GUS5 and GUS3 primers in a PCR reactions. Note the presence of 1.8kb band corresponding to the uidA gene.