Chapter III

MATERIALS & METHODS
3.1 Plant Material and Growth Conditions

Plants of *C. borivilianum* were grown under controlled conditions in a growth chamber with day/night temperature of 27 ± 1°C, for 16 h photoperiod (flux density of 200 μmol m⁻² sec⁻¹) at Panjab University, Chandigarh (India) [latitude: 30°44'14N; longitude 76°47'14E; altitude 350 m above mean sea level]. Leaves were harvested during the period of active growth (May-June, average temperature 38°C), snap frozen in liquid nitrogen and later transferred to a -80°C freezer until further processing.

3.2 Biochemical Reagents and Equipments

The sources of various enzymes, chemicals and kits used in the present work are tabulated in Appendix I. Preparation of various solutions is described in Appendix II. The instruments and equipments used during the course of the work are mentioned in Appendix III.

3.3 RNA Isolation

3.3.1 Inactivation of contaminating RNases

All the glasswares and plasticwares were dipped overnight in DEPC-treated deionized water for overnight at 37°C. Thereafter, these were autoclaved (1.05 kg/cm²) for 20 min in order to eliminate all possible RNase contamination. Glassware, pestle and mortars were baked at 250°C for 4 h. Gel running apparatus was cleaned with 0.5% SDS solution, washed with DEPC-treated autoclaved water, treated with 3% H₂O₂ for 1 h and finally rinsed thoroughly with DEPC treated autoclaved water. All the solutions were prepared in DEPC-treated water and then autoclaved or prepared in autoclaved DEPC treated water as per the requirements. All the experiments were carried out wearing powder free nitrile gloves to avoid RNase contamination.

3.3.2. Isolation of total RNA

RNA was isolated using the method developed by Ghawana et al., (2011) (patent filed in US PTO).

**Procedure:**

1. One hundred mg of leaf tissue was taken and ground to make fine powder in liquid N₂ using a pre-chilled pestle and mortar.
2. Added Solution I (2 ml) and ground further to make fine powder of the frozen
material. Continued grinding prepared a homogenous mixture to ensure close contact of the tissue ingredients and the reagents that would help in instantaneous denaturation of proteins. Allowed the mixture to thaw completely with intermittent grinding.

3. Added Solution II (800 μl) and mixed by grinding.

4. Transferred the contents to two 2 ml micro-centrifuge tubes and left undisturbed for 5 min at room temperature.

5. Added chloroform (200 μl) to each tube, vortexed briefly and left undisturbed for 10 min at room temperature. Then the tubes were centrifuged at 13,000 rpm for 10 min at 4°C.

6. Transferred upper aqueous phase into fresh tubes (avoided contamination with interphase).

7. Added 0.6 volume of isopropanol. Vortexed briefly and left undisturbed for 10 min at room temperature.

8. Centrifuged at 13,000 rpm for 10 min at 4°C. Decanted off the supernatant.

9. Washed RNA pellet with 70% ethanol (in DEPC treated water) by centrifuging at 13,000 rpm.

10. Ethanol was aspirated and samples were air dried.

11. Dissolved the pellet in 20-30 μl of DEPC water.

3.3.3 Denaturing Agarose Gel Electrophoresis to Check RNA Integrity

The quality of RNA was determined by denaturing agarose gel electrophoresis (Sambrook et al., 1989). RNA (2 μg) was run on a 1% FAA gel (Appendix II) to check the integrity of RNA by monitoring distinct 28S and 18S rRNA bands. RNA was mixed with RNA loading dye (Appendix II) and denatured by incubating the samples at 65 °C for 10 min followed by chilling on ice for 5 min. Denatured samples were loaded onto 1% FAA gel and electrophoresed at 72 V in 1× FAA gel running buffer. The gel was viewed on a UV trans-illuminator and captured on Molecular Imager Gel Doc™ XR+ Imaging System (Biorad, USA).

3.3.4 Quantification of RNA

Recovery and purity of RNA was determined by spectrophotometric analysis. RNA was diluted with DEPC water and the absorbance was recorded at 260 nm and 280 nm on U-2900 Spectrophotometer (Hitachi, Japan). A260 /A280 ratio was
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calculated to check the purity of RNA samples, which should be more than 1.8. The following formulae were used to calculate the concentration and the yield of RNA:

\[
\text{Concentration of RNA (µg/ml)} = \frac{A_{260} \times 40 \times \text{dilution factor}}{}
\]

\[
\text{Total yield (µg/ml)} = \text{Concentration} \times \text{Volume of stock RNA sample (ml)}
\]

3.4 Cloning of Early Genes Involved in Saponin Biosynthesis

SqS and SE genes associated with saponin biosynthesis pathway in C. borivilianum were cloned using degenerate primers as follows.

3.4.1 Cloning of Partial cDNA Sequences

3.4.1.1 Primer Designing

Degenerate primer sequences for SqS and SE were taken from Kribi et al., 1997 and Uchida et al., 2007 respectively. Sequences of the primers are mentioned in Table 3.1.

3.4.1.2 Preparation of cDNA from total RNA

3.4.1.2.1 Removal of Genomic DNA Contamination

Contaminating DNA from RNA was removed by DNase I digestion (Amplification grade, Invitrogen, USA).

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (1µg)</td>
<td>2.0</td>
</tr>
<tr>
<td>10X DNase I buffer</td>
<td>1.0</td>
</tr>
<tr>
<td>DNase I, Amp Grade (1U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>RNase free ADW</td>
<td>6.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>11.0</strong></td>
</tr>
</tbody>
</table>

Contents were mixed well and incubated at 25°C for 15 min. Then added 1µl of 25 mM EDTA solution and heated the above reaction mixture at 65°C for 10 min to inactivate the DNase I. The tube was placed on ice for 2 min.

3.4.1.2.2 First strand cDNA synthesis

First strand cDNA from DNA-free RNA was synthesized using total RNA in the presence of 0.5 µg oligo (dT)12-18 primer (Invitrogen, USA) and 400 U of Superscript III reverse transcriptase (Invitrogen, USA) following manufacturer’s instructions. Following components were added in a sterile RNase-free 0.2 ml PCR tube:
Heated the above reaction mixture to 65°C in a thermal cycler for 5 min. Cooled the tube on ice for 2 min and then centrifuged briefly. This serves to remove RNA secondary structure. Then, added the following components to the reaction tube:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X First strand buffer</td>
<td>4.0</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2.0</td>
</tr>
<tr>
<td>Superscript III reverse transcriptase (200 U/µl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total</td>
<td>20.0</td>
</tr>
</tbody>
</table>

All the contents were mixed well and incubated at 42°C for 60 min in a hot lid thermal cycler. The reaction was terminated by incubating at 70°C for 15 min. The tubes containing cDNA were stored at -20°C till further use.

### 3.4.1.3 Amplification of cDNA by 26S rRNA gene primers

The 26S rRNA gene primer, (26SF: 5'-CACAATGATAGGAAGAGCCGAC-3'; 26SR: 5'-CAAGGGACGGGCTTGGCAGAATC-3'), designed for highly conserved regions of 26S rRNA genes, were used as internal control primers in order to check whether DNA and cDNA are of amplification grade (Singh et al., 2004).

For amplification, following components were mixed in 0.2 ml PCR tube.

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADW</td>
<td>19.75</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>0.5</td>
</tr>
<tr>
<td>Forward primer (10 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primer (10 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA</td>
<td>1</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/µl; New England Biolabs)</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>
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Added the above components into the PCR tubes, mixed well and PCR was performed (S1000 Thermal Cycler, Bio-Rad, USA) with PCR conditions 3 min at 94°C, 30 cycles (94°C for 30 sec, 52°C for 1 min, 72°C for 2 min).

3.4.1.4 Agarose Gel Electrophoresis

Analytical gel electrophoresis of PCR fragments was performed as per the standard protocols (Sambrook et al., 1989). Agarose gel (1.2% (w/v)) was prepared in 1X TAE, supplemented with the fluorescent intercalating dye EtBr (0.2 μg/ml). To determine the fragment size, DNA markers with known sizes of the fragments were co-electrophoresed. Bands were visualized using GeNei uvitec Ultraviolet radiation torch (254 nm) and photographs were taken using Molecular Imager Gel Doc™ XR+ Imaging System (Biorad, USA).

3.4.1.5 PCR Amplification of the desired genes

PCR was performed with C. borivilianum cDNA using degenerate oligonucleotide primers as described in Table 3.1. For amplification, following components were mixed in 0.2 ml PCR tubes for each gene:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADW</td>
<td>18.75</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>0.5</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>1.0</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>1.0</td>
</tr>
<tr>
<td>cDNA</td>
<td>1.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl; New England Biolabs)</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
</tr>
</tbody>
</table>

Forward and reverse primers for CbSqS and CbSE were added to their respective tubes labelled for each gene. PCR was performed using Thermal Cycler (S1000, Bio-Rad, USA) with conditions described in Table 3.1.
### Table 3.1: Primer sequences and PCR conditions used in the present work for the amplification of genes from *C. borivilianum*

<table>
<thead>
<tr>
<th>Name of the gene</th>
<th>Primer Sequence (Forward primer, F and Reverse Primer, R)</th>
<th>PCR conditions</th>
</tr>
</thead>
</table>
| SqS              | F: 5'-TAYTGAYTGCTGGGTYTGGTNGG-3'  
R: 5'-ATIGCCATIACYTGNGRATNGCRAAA-3'| 94°C, 30 sec; 56°C, 40 sec; 72°C, 1 min (35 cycles) |
| SE               | F: 5'-GTICAGYGIATIGHGARMGIGA-3'  
R: 5'-TGICKCATRTTAAAGCR-3' | 94°C, 30 sec; 48°C, 40 sec; 72°C, 1 min (35 cycles) |

\*R=A/G; M=A/C; W=A/T; Y=C/T; S=C/G; K=G/T; D=A/G/T; H=A/C/T; B=G/C/T; N=A/T/G/C

3.4.1.6 DNA Extraction from agarose gel

DNA fragment of interest was eluted from the agarose gel and extracted using GenElute™ Gel Extraction Kit (Sigma-Aldrich, USA) as per the manufacturer’s instructions:

**Procedure:**

1. The DNA fragment of desired size was excised from agarose gel with a clean, sharp scalpel or razor blade and weighed the gel slice in a pre-weighed microfuge tube.
2. Three gel volumes of 'Gel Solubilization Solution' were then added to the microfuge tube containing gel slice. Incubated the gel mixture at 50-60°C for 10 min and vortexed briefly every 2-3 min during incubation in order to dissolve the gel.
3. One gel volume of 100% isopropanol was added to the dissolved gel and mixed thoroughly.
4. The binding columns were prepared by adding 500 μl of the 'Column Preparation Solution' to each column placed in a 2 ml collection tube and centrifuged for 1 min. Flow-through liquid was discarded.
5. The solubilised gel solution mixture was loaded into the binding column and centrifuged for 1 min. The flow-through liquid was discarded.
6. Added 700 μl of wash solution to the binding column and centrifuged for 1 min.
7. The binding column was placed back into the collection tube and centrifuged once again for 1 min without any additional wash solution.
8. The binding column was transferred to a fresh collection tube. 30 µl of Elution Solution was added to the centre of the membrane and incubated for 1 min. The column was then centrifuged for 1 min for the elution of DNA.

9. DNA collected in collection tube was stored at -20°C for future use.

3.4.1.7 Cloning of Amplified PCR Products

The amplified and purified PCR fragments were cloned in TA cloning vector (pGEM®-T Easy vector system, Promega, USA).

3.4.1.7.1 Characteristic Features of pGEM®-T Easy Vector

The pGEM®-T Easy is a linearized vector with 3' terminal thymidine at both ends (Fig 3.1). The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing re-circularization of the vector and providing a compatible overhang for the PCR products generated by Taq polymerase. Taq polymerase often adds a single deoxyadenosine, in a template-independent fashion at 3’-ends of the amplified fragments.

The pGEM®-T Easy vector is 3018 bp in size and is a high copy number vector, containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide code allows the recombinant clones to be directly identified by colour screening on indicator plates. Various reference points of pGEM®-T Easy vector are shown in Table 3.2.
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Fig 3.1: Partial map and cloning region of pGEM®-T Easy Vector

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3.4.1.7.2 Ligation

The appropriate insert volume was adjusted according to insert:vector molar ratio which was optimized as 3:1 and it was calculated for each reaction using the following equation.

\[
\frac{\text{ng of vector}}{\text{kb size of vector}} \times \frac{\text{kb size of insert} \times \text{insert:vector molar ratio}}{\text{ng of insert}}
\]

The ligation-reaction mix was prepared in a tube according to the following scheme:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Ligation master mix*</td>
<td>5.0</td>
</tr>
<tr>
<td>pGEM®-T Easy vector</td>
<td>1.0</td>
</tr>
<tr>
<td>Eluted PCR product</td>
<td>3.0</td>
</tr>
<tr>
<td>T4 DNA ligase (3 U/μl)</td>
<td>1.0</td>
</tr>
<tr>
<td>Total</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Supplied by the manufacturer

Briefly mixed the ligation-reaction mixture and incubated at 16°C for overnight. Stored the ligation mixture at -20°C till further use.
3.4.1.8 Preparation of DH5α *E. coli* ‘Ultra Competent’ Cells

Competent cells have been treated to enhance their ability to take up the DNA from the surroundings. Competent cells can be prepared either freshly for each use or can be pre-made and stored at -80°C for the transformation experiments. Preparation of the competent cells was performed under sterile conditions (Inoue et al., 1990).

Procedure for Preparation of “ultra competent” cells:-

1. A trace of DH5α cells was removed from the vial with a sterile toothpick or inoculating loop, and streaked on LB agar plate.
2. The plate was incubated overnight at 37°C.
3. A single colony was picked to inoculate 10 ml of LB medium and incubated overnight at 37°C in an orbital shaker (200 rpm).
4. One ml overnight grown culture was added to 100 ml of LB medium and incubated on shaker at 20°C until an OD₆₀₀ of 0.5 was obtained after approximately 24-30 h.
5. The culture was cooled on ice for 10 min, and transferred to a sterile, round-bottom centrifuge tube.
6. The cells were collected by centrifugation at low speed (10 min, 2500g, 4°C).
7. Supernatant was discarded carefully.
8. The cells were resuspended gently (by swirling) in 80 ml of ice cold “TB” and kept on ice for 10 min.
9. The cells were collected by centrifugation (10 min, 2500g, 4°C).
10. Supernatant was discarded carefully and cells were re-suspended carefully in 7 ml of ice cold “TB”.
11. DMSO was then to a final concentration of 7% and placed the culture tube on ice for 10 min.
12. Aliquots of 100 µl were prepared in sterile micro-centrifuge tubes and frozen in liquid nitrogen.
13. Competent cells were stored at -80°C till further use.
3.4.1.8.1 Transformation

1. Ligation mix was transferred into a cold sterile 1.5 ml microcentrifuge tube and kept on ice.
2. Thawed the competent cells on ice.
3. Ligation mix approximately 10 μl was added to the tubes containing the competent cells. This was then mixed well by finger tipping and incubated on ice for 45 min.
4. The tube was then transferred to a 42°C water bath for 2 min and then set the tubes back on ice for 2 min.
5. Added 500 μl of LB medium to the cells and incubated for 2-3 h at 37°C with agitation.
6. X-Gal (80 μl; 20μg/μl) and IPTG (40 μl; 1 M) were evenly spread on LB plates containing 100μg/ml ampicillin and allowed to dry for 30 min.
7. Transformation cultures were plated on above plates and incubated at 37°C overnight.
8. Next day appearance of white/blue colonies confirmed transformation.

3.4.1.9 Colony PCR

The white colonies, possible positive clones, were subjected to colony PCR to verify the presence of insert in the vector using plasmid specific SP6 (5'-GATTTAGGTGACACTATAG -3') and T7 (5'- TAATACGACTCACTATAGGG-3') primers flanking the cloning site. A small portion of transformed bacterial colony was picked with a clean pipette tip and transferred into the colony lysis buffer (Appendix II). The tubes were incubated in boiling water for 10 min. After brief cooling, the cell debris was pelleted and supernatant (colony lysate) was transferred to new microfuge tube. PCR was set up as below:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADW</td>
<td>13.8</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>0.5</td>
</tr>
<tr>
<td>T7 primer (10 μM)</td>
<td>0.5</td>
</tr>
<tr>
<td>SP6 primer (10 μM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Colony lysate</td>
<td>2.0</td>
</tr>
<tr>
<td>Taq DNA polymerase (5 U/μl; New England Biolabs)</td>
<td>0.20</td>
</tr>
<tr>
<td>Total</td>
<td>20.0</td>
</tr>
</tbody>
</table>
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PCR was performed in a thermal cycler (S1000 Thermal Cycler, Bio-Rad, USA) as follows 30 cycles of 94°C for 30 sec, 54°C for 1 min, 72°C for 1 min. PCR products were analyzed on 1.2% agarose gel with EtBr staining and picture was taken on Molecular Imager Gel Doc ™XR Imaging System (Biorad, USA).

3.4.1.10 Isolation of Recombinant Plasmid DNA from E.coli Host Cells

Plasmid DNA was isolated using GenElute™ Plasmid Miniprep Kit (Sigma-aldrich, USA) as follows:

1. Overnight grown recombinant DH5a E.coli culture (5 ml) was centrifuged at 10,000 rpm for 1 min and supernatant was discarded.
2. The cell pellet was resuspended in 200 µl of the resuspension solution (RNase added) by pipetting up and down.
3. Resuspended cells were lysed by adding 200 µl of lysis buffer and contents were mixed immediately by gentle inversion until the mixture became clear and viscous. The mixture was allowed to stay at room temperature for 2 min.
4. Cell debris was precipitated by adding 350 µl of neutralization solution and gently inverted the tube 4-6 times to mix. The mixture was incubated for 5-10 min and centrifuged at 12,000 rpm for 10 min.
5. The binding column was prepared by adding 500 µl of the column preparation solution to each binding column placed in 2 ml collection tubes and centrifuging for 1 min. Flow-through liquid was discarded.
6. The cleared lysate was transferred to the column and centrifuged at 12,000 rpm for 1 min. Flow-through liquid was discarded.
7. 750 µl of wash solution was added to the binding column and centrifuged at 12,000 rpm for 1 min.
8. The binding column was placed back into the collection tube and centrifuged once again for 1 min without any additional wash solution.
9. The binding column was transferred to a fresh collection tube. Elution solution (30 µl) was added at the centre of the membrane of the column and centrifuged at 12,000 rpm for 1 min.
10. The size and quality of DNA was determined on 1% agarose gel electrophoresis.
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3.4.1.11 Sequencing

Isolated plasmids were sent for automated sequencing. The sequencing was performed using big dye terminator v 3.1 cycle sequencing kit on an automated sequencer (ABI 3100 genetic analyser, Applied Biosystems, USA).

3.4.1.12 Analysis of sequences

Sequence homology search was carried out against a gene sequence database at NCBI (http://www.ncbi.nlm.nih.gov) using BLAST analysis through BLASTN and BLASTX programs (Altschul et al., 1997). The significant scores were obtained in terms of E-value, which represents the number of different alignments with scores equivalent to or better than S (alignment score) that are expected to occur in a database search by chance. The lower E-value indicated the more significant score.

3.4.2 Cloning of Full Length cDNA through Rapid Amplification of cDNA Ends (RACE)

RACE is a method to clone full length cDNAs corresponding to mRNAs (Frohman et al., 1988). RACE protocol is a rapid PCR based method and requires very little amount of RNA. RACE differs from conventional PCR in that it requires knowledge of only a small region of sequence within either the target RNA or a partial clone of cDNA. A set of gene specific primers were used to generate 5' and 3' ends of the gene, separately.

RACE was performed using SMART™ RACE cDNA Amplification Kit (BD, Biosciences, USA), following the manufacturer's instructions. The kit includes recent events in PCR technology that both increase the sensitivity as well as reduce the background of the RACE reactions. SMART (Switching Mechanism At 5' end of RNA Transcript) cDNA synthesis technology is expected to isolate complete 5' sequence (Fig 3.2).

SMART technology eliminates the need for adapter ligation and uses first strand cDNA directly in RACE PCR (Chenchik et al., 1998). It provides a mechanism for generating full length cDNAs in reverse transcription reactions (Zhu et al., 2001). This is made possible by the joint action of the SMART II™ oligonucleotide and the MMLV reverse transcriptase. MMLV-RT has a property of exhibiting terminal transferase activity adding 3-5 residues (predominantly dC) to the 3' end of cDNA. The SMART oligo contains a terminal stretch of G residues that anneal to the dC-rich cDNA tail and serves as an extended template for RT. MMLV-RT switches templates...
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from the mRNA molecule to the SMART oligo, generating a complete cDNA copy of the original RNA with the additional complementary SMART sequence at the end.

Similarly, complete 3’ sequence can be isolated using 3’-CDS primer. This contains an oligo (dT) sequence that anneals to the polyA tail of cDNA and an extended SMART sequence. MMLV-RT generates a complete cDNA copy of the original RNA with the additional complimentary SMART sequence at the end (Fig 3.3).

Occasionally, SMART oligo does random priming of mRNA during cDNA synthesis. However, template switching mechanism creates the cDNA which has SMART sequence at both the ends. Long UPM primer incorporates the inverted repeats using these SMART sequences which lead to the intermolecular hybridization of inverted repeat sequences forming panhandle structures. In such cDNAs no primer binding can occur hence they are not amplified during PCR cycles. This is called as suppression PCR which ensure high specificity in the amplification of target cDNA (Fig 3.4).
First strand cDNA synthesis by Superscript RT Enzyme

Attachment of SMART II A Oligonucleotide at polyC tail

Template extension by Superscript RT Enzyme

PCR cycle with Long Universal Primer

Second strand cDNA synthesis by Taq DNA polymerase

Template synthesis through Gene Specific Primer

PCR cycles with Gene Specific Primer

PCR cycles with Short Universal Primer and Gene Specific Primer

Fig 3.2 Detailed mechanism for 5’RACE reaction
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Fig 3.3 Detailed mechanism for 3' RACE reaction
3.4.2.1 Designing of RACE Primers

Primers were designed such that the amplified 5' and 3' ends overlap each other over a small stretch of nucleotides. Primers were designed using Primer3 software (http://www.frodo.wi.mit.edu). For 3'-RACE, a gene specific primer GSP1 for primary PCR and a nested gene specific, NGSP1 for secondary PCR was designed (Table 3.3). For 5'-RACE a gene specific primer 2, GSP2 for primary PCR and a nested primer, NGSP2 was designed (Table 3.3).

3.4.2.2 Preparation of RACE-Ready cDNA

The SMART RACE Kit includes a protocol for the synthesis of two separate cDNA populations: 5' RACE Ready cDNA and 3'-RACE Ready cDNA
3.4.2.2.1 Preparation of 5' RACE-Ready cDNA

The cDNA for 5'-RACE is synthesized using a modified lock-docking oligo(dT) primer and the SMART II A oligo as described above. The modified oligo(dT) primer, termed the 5'-CDS, has two degenerate nucleotide positions at the 3' end. These nucleotides position the primer at the start of the polyA tail and thus eliminate the 3' heterogeneity inherent with conventional oligo(dT) priming (Borson et al., 1992).

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Sample (1 μg)</td>
<td>1.0 -3.0</td>
</tr>
<tr>
<td>5'-CDS primer (10 μM)</td>
<td>1.0</td>
</tr>
<tr>
<td>SMART II A oligo (10 μM)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Sterile dH₂O (final volume of 5.0 μl) was added for each reaction and the contents were mixed and spun the tubes briefly in a micro-centrifuge. Tubes were incubated at 70°C for 2 min and cooled on ice for 2 min and spun briefly to collect the contents at the bottom. Added the following to reaction tube:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X first-strand buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>DTT (20 mM)</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1.0</td>
</tr>
<tr>
<td>MMLV reverse transcriptase</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mixed well by gentle pipetting and spun briefly to collect the contents at the bottom. Tubes were incubated at 42°C for 1.5 h in an air incubator. First strand reaction product was diluted with 100 μl of Tricine-EDTA buffer and tubes were heated at 72°C for 7 min. Samples were stored at -80°C till further use.

3.4.2.2.2 Preparation of 3' RACE-Ready cDNA

The 3'-RACE cDNA is synthesized using a traditional reverse transcription procedure, but with a special oligo(dT) primer. This 3'-RACE CDS Primer A (3'-CDS) includes the lock-docking nucleotide positions and has a flanking portion of the SMART sequence at its 5' end.
Sterile dH₂O (final volume of 5 µl) was added for each reaction and the contents were mixed and spun the tubes briefly in a micro-centrifuge. Tubes were incubated at 70°C for 2 min, cooled on ice for 2 min and spun briefly to collect the contents at the bottom. Added the following to a reaction tube:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X first-strand buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>DTT (20 mM)</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1.0</td>
</tr>
<tr>
<td>MMLV reverse transcriptase</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Mixed well by gentle pipetting and spun briefly to collect the contents at the bottom. Tubes were incubated at 42°C for 1.5 h in an air incubator. First strand reaction product was diluted with 100 µl of Tricine-EDTA buffer and tubes were heated at 72°C for 7 min. Samples were stored at -80°C till further use.

### 3.4.2.3 RACE PCR

Incorporation of SMART sequence into 5'- and 3'-RACE-ready cDNA populations, enables priming both the RACE PCR reactions using UPM and NUP that recognizes the SMART sequence in conjunction with distinct GSPs. Master mix was prepared for all of the PCR reactions plus one extra reaction to ensure sufficient volume. For each 25 µl reaction, the following reagents were mixed:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADW</td>
<td>18.25</td>
</tr>
<tr>
<td>10X Advantage 2 PCR buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1.0</td>
</tr>
<tr>
<td>UPM (10 µM)*</td>
<td>0.5</td>
</tr>
<tr>
<td>50X Advantage 2 Polymerase Mix*</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>22.5</td>
</tr>
</tbody>
</table>
Mixed well and spun briefly in a micro-centrifuge. Following components were added in order in a 0.2 ml PCR tubes.

<table>
<thead>
<tr>
<th>Contents</th>
<th>3' RACE components (µl)</th>
<th>5' RACE Components (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' RACE ready cDNA</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>5' RACE ready cDNA</td>
<td>-</td>
<td>1.0</td>
</tr>
<tr>
<td>GSP1 (10 mM)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>GSP2 (10 mM)</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Master mix</td>
<td>22.5</td>
<td>22.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
<td><strong>25</strong></td>
</tr>
</tbody>
</table>

PCR was performed in a thermal cycler (Gene Amp® PCR System 9700, Applied Biosystems, USA) using the program mentioned in Table 3.3. Primary PCR product was checked on a 1.2% agarose gel along with appropriate DNA size markers.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>RACE Primer</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SqS</td>
<td>GSP1- 5'-GGGCTGGTGGGCTAGGATTGTCTAA-3'</td>
<td>Primary PCR and Nested PCR</td>
</tr>
<tr>
<td></td>
<td>NGSP1- 5'-GTATGTTTTTGGCCTCAGCATCTG-3'</td>
<td>94°C, 30 sec; 68°C, 35 sec; 72°C, 2 min (35 cycles)</td>
</tr>
<tr>
<td></td>
<td>GSP2- 5'-TTACATTATTAGCACAAGGCTATGTCC-3'</td>
<td>94°C, 30 sec; 68°C, 35 sec; 72°C, 2 min (35 cycles)</td>
</tr>
<tr>
<td></td>
<td>NGSP2- 5'-CTGTCAGCCATCTTCACTGAAACCC-3'</td>
<td>94°C, 30 sec; 68°C, 35 sec; 72°C, 2 min (35 cycles)</td>
</tr>
<tr>
<td>SE</td>
<td>GSP1- 5'-TCTGTTTCTACAAATGCGAGAG-3'</td>
<td>Primary PCR and Nested PCR</td>
</tr>
<tr>
<td></td>
<td>NGSP1- 5'-TGTCCAGTGGGAGGAAACAGTA-3'</td>
<td>94°C, 30 sec; 68°C, 35 sec; 72°C, 2 min (35 cycles)</td>
</tr>
<tr>
<td></td>
<td>GSP2- 5'-CCAAACAAACAAACAGGGGACA-3'</td>
<td>94°C, 30 sec; 68°C, 35 sec; 72°C, 2 min (35 cycles)</td>
</tr>
<tr>
<td></td>
<td>NGSP2- 5'-CAGATTGAGGGAACCATCGCAT-3'</td>
<td>94°C, 30 sec; 68°C, 35 sec; 72°C, 2 min (35 cycles)</td>
</tr>
</tbody>
</table>

Table 3.3 Primer sequences and PCR conditions used in RACE reactions

"GSP1, gene specific primer for 3'-RACE; NGSP1, nested gene specific primer for 3'-RACE; GSP2, gene specific primer for 5'-RACE; NGSP2, nested gene specific primer for 5'-RACE"
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3.4.2.4 “Nested” RACE PCR

The primary PCR product (1 μl) was diluted into 99 μl of ADW. PCR reaction was set up as follows:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADW</td>
<td>17.25</td>
</tr>
<tr>
<td>10X Advantage 2 PCR buffer*</td>
<td>2.5</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>1.0</td>
</tr>
<tr>
<td>NUP (10 μM)*</td>
<td>0.5</td>
</tr>
<tr>
<td>NGSP (10 μM)</td>
<td>1.0</td>
</tr>
<tr>
<td>Diluted primary PCR product</td>
<td>2.5</td>
</tr>
<tr>
<td>50X Advantage 2 Polymerase Mix*</td>
<td>0.25</td>
</tr>
<tr>
<td>Total</td>
<td>25.0</td>
</tr>
</tbody>
</table>

*Supplied by the manufacturer

PCR was performed in a thermal cycler (S1000 Thermal Cycler, Biorad USA) using the following program described in Table 3.3.

Cloning, sequencing and analysis of RACE products was carried out essentially as described in section 3.4.1. After BLAST analysis, overlapping regions of sequences of 5’ and 3’ ends of genes were aligned to make a complete gene sequence.

3.5 Glycerol Stock Preparation

For long term storage of the cloned cDNA of all the genes, the bacterial colony with the insert was grown in LB medium supplemented with ampicillin (100 μg/ml) for overnight at 37 °C. Equal volume of overnight culture and autoclaved glycerol were mixed in Cryovial® tubes, frozen in liquid nitrogen and stored at -80 °C.

3.6 Leaf Transcriptome Sequencing using Next Generation Sequencing Technology

3.6.1 cDNA library preparation

Deep sequencing of *C. borivillanum* leaf tissue transcriptome was performed using the HiSeq 2000 Sequencing Platform (Illumina, USA). DNA free Total RNA was extracted followed by mRNA enrichment using miRNA Easy kit
from Qiagen (Germany). Procedure for RNA purification and mRNA enrichment is as follows:

3.6.1.1 Purification of RNA and Enrichment of mRNA

1. Preheat the RNase free microfuge tubes containing extraction buffer at 65°C well in advance in a water bath.

2. Ground 2 g of tissue to a fine powder in liquid nitrogen using a mortar and pestle. Do not let the powder thaw at any time.

3. Using a clean and cold spatula scooped the ground tissue in the preheated extraction buffer tubes. Mixed gently by inverting several times. Incubated at 65°C for 10-15 min and freeze in liquid nitrogen.

4. The juice of the tissue and the extraction buffer was kept at -80°C for a minimum of 2 h.

5. The tubes were thawed (2-4 per sample) at 37°C in a water bath for 5-10 min. The tubes were then vortexed to emulsify all the ground material.

6. The tubes were then centrifuged at 13,000 rpm at 4°C for 20 min and transferred the clear supernatant to a clean tube.

7. The supernatant obtained was again centrifuged at 13,000 rpm at 4°C for 15 min to remove any carryover debris. Transferred the clear supernatant to clean tubes.

8. Added equal volume of chloroform to the supernatant and vortexed thoroughly (for at least one min) and incubated on ice for 5 min.

9. Centrifuged at 13,000 rpm for 20 min at 4°C and transferred the supernatant to a new tube.

10. To the above supernatant, added 1.5 volumes of 100% ethanol and mixed thoroughly by pipetting up and down several times. Continued to next step without delay.

11. Upto 700 μl of the above sample (including any precipitate that was formed) was pipette into an RNeasy mini spin column in a 2 ml collection tube (supplied by the manufacturer). Centrifuged at 10,000 rpm for 15 sec at room temperature. Discarded the flow through.

12. Repeated the above step with the remainder of the sample.
13. Alcohol (350 μl) added RWT buffer was added into the RNeasy mini spin column and centrifuged for 15 sec at 10,000 rpm. Discarded the flow through.

14. Added 10 μl of DNase I stock (Qiagen, Germany) solution to 70 μl of RDD buffer. Mixed by gently inverting the tube.

15. The above prepared DNase I incubation mix (80 μl) was added to the RNeasy spin column membrane and incubated at 20-30°C for 15 min.

16. RWT buffer (350 μl) was added into the RNeasy spin column and centrifuged for 15 sec at 10,000 rpm. Discarded the flow through.

17. Added 500 μl of alcohol added RPE buffer into the RNeasy spin column and centrifuged for 15 sec at 10,000 rpm. Discarded the flow through.

18. RPE buffer (500 μl) was again added to the RNeasy spin column. Centrifuged for 2 min at 10,000 rpm to dry the RNeasy spin column membrane.

19. Placed the RNeasy spin column into a new 2 ml collection tube and centrifuged at full speed for 1min.

20. Transferred the RNeasy spin column to a new 1.5 ml collection tube (supplied by the manufacturer). Pipetted 30-50 μl RNase free water directly onto the RNeasy spin column membrane. Closed the lid gently and centrifuged for 1 min at 10,000 rpm to elute the RNA.

Quality and quantity of RNA was determined using spectrophotometer on Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Thereafter, the RNA samples were sent to the Microarray and Genomic Core Facility, Huntsman cancer institute, University of Utah, Salt Lake City, USA, for preparation of the cDNA library and deep sequencing. Various steps involved in library preparation and sequencing are shown in Fig. 3.5.
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0.1-4 μg Total RNA

Purify and 
Fragment 
mRNA

First strand 
cDNA 
Synthesis

Second strand 
cDNA 
Synthesis

Ligate 
Adapters

Adenylate 3’ 
ends

Repair ends

PCR 
Amplification

Validate 
library

Fig 3.5: Flowchart for High Throughput cDNA synthesis

Then the single-end cDNA library was prepared in accordance with Illumina’s protocols with an insert size of 150 bp and sequenced for 50 bp. The library was sequenced on Illumina HISEQ 2000 platform.

3.6.2 Bioinformatics analysis

Bioinformatics analysis was performed in collaboration with the group of Dr. S. Ramachandran, Scientist, CSIR-IGIB, New Delhi, India. Data of deep sequencing will be made available at Short Read Archive (SRA) database which is maintained by NCBI once it is published. Its temporary submission ID SUB175401 and Biosample accession number is SAMN02045525.

3.6.3 De novo assembly of the sequences and clustering

All the assemblies were performed on Red Hat based SGI workstation with 48 cores, 2.27GHz Intel Xeon processor with 50GB random access memory. We used SOAPdenovo (version 1.04; http://soap.genomics.org.cn/soapdenovo.html) which applies de Bruijn graph algorithm for de novo assembly of high quality (HQ) sequence reads to generate a non-redundant set of transcripts. The cleaned reads were first split into different ‘k-mers’, for assembly in order to produce contigs, using the de Bruijn graph. k-mer size of 23 achieved the best balance between the number of contigs produced, coverage and average sequence length obtained.

In order to reduce sequence redundancy, similar sequences, the clustering process was supplemented with another tool, TGICL, which is based on pairwise sequence similarity and then assembly by individual clusters in order to retrieve better results (Schuhr et al., 2003). The resulting output was then passed to CAP3 assembler for multiple alignment and consensus building. The TGICL and CAP3
assembly of Illumina data was performed using default parameters. The resulting singletons and consensus contigs were merged to get the final list of assembled transcripts.

3.6.4 Functional annotation and Classification

All the assembled unigenes (consensus and singletons) longer than 100 bp were annotated by assigning them putative gene descriptions and Gene Ontology (GO) terms on the basis of sequence similarity with previously identified genes annotated with similar details. The unigenes were subjected to BLASTX search against non-redundant protein database. The hits with E-value $<1.0e^{-5}$ were extracted and considered to be significant. Further, the *C. borivilianum* transcripts that did not show any significant hit were searched using BLASTN tool against the non-redundant database. Functional categorization by GO terms (http://www.geneontology.org) (Ashburner et al., 2000) and Enzyme Commission (EC) database was carried out based on Anno8r tool (http://www.nematodes.org/bioinformatics/annot8r/) with E-value threshold of 1.0e^{-05}.

The GOSlim terms for molecular function, biological process, and cellular component categories associated with the best BLASTX hit with non-redundant database were assigned to the corresponding *C. borivilianum* transcripts.

3.6.5 Read mapping onto *C. borivilianum* transcripts

The expression level of each assembled transcript was measured through Reads Per Kilobase per Million mapped reads (RPKM) values. All the reads were mapped onto the non-redundant set of transcripts to quantify the abundance of transcripts assembled. SeqMap (Jiang and Wong, 2008) was used for read mapping and rSeq (Mortazavi et al., 2008) was then applied for RPKM based expression measurement. Assembled sequences were used as the reference sequence to map back short reads and to measure RPKM for all assembled transcripts as suggested by Mortazavi et al., (2008) and Jiang and Wong, (2008). For RPKM measurement, filtered reads were first mapped back to various assembled transcripts, total mapped reads were then estimated, uniquely mapped reads assigned to each assembled transcript, with maximum two mismatches allowed. Based on the above mentioned dissimilar sequence clustering, having homologous sequences in database, for each such cluster, the longest sequence was considered as the representative sequence for the unique gene it represented. The associated GO terms and IDs were parsed...
for each of such sequence and their corresponding RPKM values for different genes assembled was then mentioned.

3.6.6 GC content analysis and SSRs identification

GC content analysis was done using in-house R scripts. A Perl script known as bMicroSATellite (MISA, http://pgrc.ipk-gatersleben.de/misa/) was used to identify microsatellites in the unigenes. The repeats of mono-nucleotide more than 10 times, di-nucleotides repeats more than 6 times, tri-, tetra-, penta- and hexa-nucleotide repeats more than 5 times were considered as search criteria in MISA script.

3.6.7 Identification of Transcription Factor families

For the identification of transcription factor families represented in C. borivilianum transcriptome, the C. borivilianum transcripts were searched against all the transcription factor protein sequences at Plant transcription factor database (PlntTFDB; http://plntfdb.bio.uni-potsdam.de/v3.0/downloads.php) using BLASTX with an E-value cut-off of 1.0e-05.

3.7 Bioinformatics Analysis of the Genes Involved in Saponin Biosynthesis

3.7.1 Sequence Analysis

Similarity search for sequences was conducted using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/). The nucleotide sequence obtained was translated using Translate tool (http://www.expasy.ch/tools/dna.html) Deduced amino acid sequence was used to analyze protein families and domains using Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/). Program at ExPASy server namely ProtParam (http://www.expasy.ch/tools/protparam.html) (Gasteiger et al., 2005) was used to calculate the pl and MW (http://ca.expasy.org/) of the deduced amino acid sequence. Other properties of CbSqS amino acid sequence were estimated using SPLIT v.4.0 (http://split.pmfst.hr/split/4/) and TMHMM (http://www.cbs.dtu.dk/services/) programs. Conseq services (http://conseq.tau.ac.il/, (Ashkenazy et al., 2010) helped in locating the structurally and functionally important regions in the deduced amino acid sequence. Secondary structures of deduced amino acids sequences were predicted using SOPMA program (http://npsa-pbil.ibcp.fr/) and POLYVIEW program (http://sable.cchmc.org/).

3.7.2 Multiple Sequence Alignment and Phylogenetic analysis
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Multiple sequence alignment and pairwise comparison of deduced gene amino acid sequence and other known gene sequences retrieved from GenBank were carried out with MultAlin program (http://multalin.toulouse.inra.fr/multalin/) using default parameters. MEGA 5.05 Beta 2 software (Tamura et al., 2007) was used to create a Neighbour Joining Tree to show the evolutionary relationship.

3.7.3 Three dimensional structure prediction of *CbSqS*, *CbSE* and *CbCAS*

Three-dimensional structure of *CbSqS*, *CbSE* and *CbCAS* was predicted using I-TASSER (http://zhang.bioinformatics.ku.edu/l-TASSER) (Zhang, 2008) software. Ligand binding site was predicted using 3D Ligand Site (http://www.sbg.bio.ic.ac.uk/3dligandsite/). Superimposition analysis of the 3D models of *CbSqS* and its template human SQS protein (PDB ID code 1EZFB) was done using 3-Dimensional Structural Superposition (3 d-SS) service (http://cluster.physics.iisc.ernet.in/3dss/severalinput.html). Conserved amino acids at the protein surface were determined using ConSurf (http://consurf.tau.ac.il/overview.html).

3.8 Gene Expression Analysis

3.8.1 Designing of Gene Specific Primers (GSPs) for Expression Analysis

A set of GSPs were designed for each gene for semi-quantitative RT-PCR studies (Table 3.4). PCR was performed as described in section 3.4.1.4 except that GSPs were used for RT-PCR. Annealing temperatures were optimized for appropriate amplification for each gene (Table 3.4). Amplicons were run on 1.5% agarose gel, excised along with agarose gel, purified from the gel, cloned, and sequenced to confirm that these were part of their respective gene.

3.8.2 Standardization of Annealing Temperature for GSPs

Optimum annealing temperature was selected for GSPs and PCR was set up as mentioned in section 3.4.1.4 using a thermal cycler (S1000 Thermal Cycler, Bio-Rad, USA).
3.8.3 Standardization of Cycling Parameters

Cycle number was optimized to amplify the products within the exponential phase. A PCR tube was taken out after 15, 20, 25 and 30 cycles. Expression analysis was carried out by semi-quantitative RT-PCR as described in following section.

3.8.4 RT-PCR

Total RNA was isolated from leaf and root tissues of *C. borivilianum* using the methods as described in section 3.3. Isolated RNA was processed further to remove contaminating genomic DNA using DNase I (Invitrogen, USA) and cDNA was synthesized essentially as described in section 3.4.1.2. PCR was carried out using GSPs (Table 3.4) and expression was evaluated at exponential phase of amplification (Kumar et al., 2012). Table 3.4 lists all the PCR parameters for RT-PCR. Expression of 26S rRNA was used as internal control to equalize cDNA quantity in various reactions (Singh et al., 2004). The PCR products were run on 1.5% agarose gel in 1X TAE buffer and photographed on Molecular Imager Gel Doc™ XR⁺ Imaging System (Biorad, USA). IDV of amplicons was calculated by Quantity One software (Biorad, USA). The data was used to calculate the relative change in gene expression.

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Primer Sequence</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SqS</td>
<td>F: 5’-GTGCAGTGCTTGAAAGCAGCAT-3’&lt;br&gt;R: 5’-TG GCCCATGACCTGAGGGAGT-3’</td>
<td>94°C, 30 sec; 60°C, 35 sec; 72°C, 1 min</td>
</tr>
<tr>
<td>SE</td>
<td>F: 5’-TGTTTATCCAAAGAATGCGAGAGA-3’&lt;br&gt;R: 5’-C CAAAACCAACAAAAACAGGACACA-3’</td>
<td>94°C, 30 sec; 68°C, 40 sec; 72°C, 1 min</td>
</tr>
<tr>
<td>CAS</td>
<td>F: 5’-CGAGCCGCTGATGCTGCTGTCG-3’&lt;br&gt;R: 5’-CTCTCTCCCCAACACCACCTGTAG-3’</td>
<td>94°C, 30 sec; 60°C, 40 sec; 72°C, 1 min</td>
</tr>
</tbody>
</table>

Table 3.4: Primer sequences and PCR conditions used for RT-PCR

3.9 Heterologous Expression of *CbSqS* and *CbSE* Protein(s) in *E. coli*

Two cloned genes *CbSqS* and *CbSE* were expressed in *E. coli* M15 strain harbouring pREP4 plasmid using the pQE 30 vector (Qiagen, Germany).
3.9.1 pQE-30 UA Expression Vector

The pQE-30 UA expression vector (Fig 3.6) was used for direct cloning of PCR products. UA-cloning technology exploits the fact that Taq DNA polymerase and other non-proofreading DNA polymerases add a 3’ end A overhangs to PCR products. This allows the direct insertion of such PCR products into the pre-linearized pQE-30 UA vector, which has a U overhang on each 3’ end. This eliminates the need for restriction digestion of the vector or insert, primers with built-in restriction sites, or specially designed adapters, resulting in an efficient and robust cloning.

3.9.2 Cloning of CbSqS and CbSE in Expression Vector

ORFs of CbSqS and CbSE were amplified essentially as described in section 3.4.1 except that in case of CbSqS, in order to amplify the carboxyl terminal, truncated CbSqS cDNA lacking 23 amino acid (389-411 aa) long transmembrane region, was amplified and cloned into pQE-30 UA expression vector (Qiagen, Germany) as described in following sections.

Fig. 3.6: pQE-30 UA expression vector. 'PT5': T5 promoter, 'lac O': lac operator, 'RBS': ribosome binding site, 'ATG': start codon, '6xHis': His tag sequence, 'MCSI/MCSII': multiple cloning sites, 'Stop Codons': stop codons in all three reading frames, 'Col E1': Col E1 origin of replication, 'Ampicillin': Ampicillin resistance gene
3.9.2.1 Ligation

The ligation mix was prepared in a fresh 0.5 ml eppendorf tube according to the following scheme:

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR product (140-210 ng DNA)</td>
<td>4.0</td>
</tr>
<tr>
<td>2X ligation master mix*</td>
<td>5.0</td>
</tr>
<tr>
<td>pQE30 UA vector</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>10.0</strong></td>
</tr>
</tbody>
</table>

* Supplied by the manufacturer

Briefly mixed the ligation-reaction mixture and incubated at 16°C for 16 h before proceeding for transformation in competent *E. coli* cells.

3.9.2.2 Transformation

Competent *E. coli* M15[pREP4] cells were prepared essentially as described in section 3.4.1.8 except that LB agar plate was containing 25 µg/ml kanamycin and 100 µg/ml ampicillin. Transformation in *E. coli* M15[pREP4] competent cells was performed as described in section 3.4.1.8.1 using an aliquot of the 10 µl ligation mix. Colony PCR was performed to confirm the presence of insert following same procedure as mentioned in section 3.4.1.9 except that the vector specific primers UA F(5’-CCCAGAAAAGTGCCACCTG-3’) and UAR (5’-GTTCTGAGGTATTATTGG-3’) flanking the cloning sites were used. Glycerol stocks were prepared for long term storage of cDNA clones.

3.9.3 Protein Expression and Purification

3.9.3.1 Induction of Expression

1. *E. coli* M15 cells harboring plasmid of interest was removed from the glycerol stock vial with a sterile pipette tip and streaked on LB agar plate containing 100 µg/ml ampicillin and 25 µg/ml kanamycin.
2. The plate was incubated overnight at 37°C.
3. A single colony was picked and inoculated in 50 ml of LB medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and was allowed to grow overnight at 37°C.
4. Pre-warmed media (1L with antibiotics) was inoculated with 50 ml of the overnight grown culture and incubated at 37°C with vigorous shaking (~300rpm) until OD$_{600}$ reached 0.5-0.7.
5. Expression was induced by adding IPTG to a final concentration of 1mM.
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6. Cultures were grown for additional 4-5 h. 1 ml sample was collected after regular intervals, cells were pelleted and resuspended in 50 μl of 1X SDS-PAGE sample buffer and frozen until use.

7. Cells were harvested by centrifugation at 4000 × g for 20 min. harvested cells were used for purification of proteins under native conditions.

3.9.3.2 SDS-PAGE Analysis of Expressed Proteins

The cells were resuspended in extraction buffer (Appendix II) and disrupted by sonication. The lysate was centrifuged at 10,000g for 20 min at 4°C, and the supernatant was loaded on 10 % SDS-PAGE (Laemmli, 1970) gel. Protein was run at 25 mA using 1X tris-glycine (containing 0.1% SDS) as running buffer. When the samples reached the resolving gel, current was increased to 35 mA, till the end of the run. The gel was stained with Coomassie Brilliant Blue stain (Hames and Rickwood, 1990).