# Chapter 3: Materials and methods

## 3.1. Reagents and equipment

The various chemicals used in the current study were purchased from Sigma–Aldrich (USA), Qiagen (Germany), Takara Bio USA, Inc (USA), Thermo Fisher Scientific (USA), Chromadex (USA), Merck Ltd (India), Himedia (India) and SD Fine Chemical Ltd (India).

The laboratory equipment used in this study was from BioRad Laboratories (USA), Eppendorf (India), Tarsons Products Pvt. Ltd. (India), Merck Millipore (Germany), Thermo Fisher Scientific (USA), New Brunswick Scientific Co., Inc. (USA), Simag (Italy), Lab Companion (South Korea) and Sartorius (Germany) as detailed in Appendix A.

Primers were synthesized from Sigma–Aldrich (India), Integrated DNA Technologies (India) and Imperial Life Sciences Pvt. Ltd (India) as detailed in Annexure III

## 3.2. Investigation of major ginsenosides in rhizomes of *Panax sokpayensis* from Sikkim Himalaya

The major ginsenosides, viz., Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 were investigated using HPLC in the rhizomes of *P. sokpayensis* Shiva K. Sharma & Pandit from Sikkim Himalaya.

### 3.2.1. Plant materials and chemicals

Rhizome samples of *P. sokpayensis* were collected from niche habitat in Sopakha, West Sikkim district (altitude 2447 m; N27° 16′ 17″, E88° 04′ 55″) during the month of August/September (Figure 3.1). After collection, the ages of the plants were determined by counting the annual bud scars along the rhizomes (Anderson et al. 1993). Ten years old rhizome samples were used for the experiment. The standards of major ginsenosides, Rg1, Rg2, Rf, Re, Rd, Rc, Rb1 and Rb2 were purchased from ChromaDex (Irvine, USA). HPLC-grade acetonitrile and methanol were obtained from Merck (Merck, Darmstadt, Germany). Deionized water was obtained from Milli-Q water-purification system (Millipore, Bedford, USA).

### 3.2.2. Preparation of calibration curve

Eight major ginsenoside standards Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 were dissolved in methanol and reconstituted at six different concentrations, viz., 0.05, 0.1, 0.2, 0.3, 0.4 and


**Figure 3.1** Representative pictures of *P. sokpayensis* (A) *P. sokpayensis* in its niche (B) *P. sokpayensis* rhizome.

0.5 mg per ml. All the standards were separated and identified using Dionex UltiMate 3000 BioRS HPLC System (Thermo Scientific, USA) equipped with UV detector. An X-bridge™ amide column (4.6 mm x 150 mm, i.d. 3.5 µm) purchased from Waters Technologies (Ireland Ltd, Ireland) was used for all chromatographic separations. The injection volume was 20 µl and column temperature was set at 60 °C. The gradient program used for the separation of analytes using acetonitrile and Milli Q water combination is shown in table 3.1. The standards were identified and compared on the basis of their retention times and absorbance at 203 nm. A calibration curve of peak area versus concentration (mg per ml) of each analyte was plotted by taking the mean of three readings.

The linearity was evaluated by linear regression analysis calculated by least squares regression method (Table 3.2).

**Table 3.1** Gradient parameters for the ginsenoside analysis

| Time (min) | Flow rate (ml/min) | ACN[$] (%) | Milli Q[#] (%) |
|---|---|---|---|
| start | 1.3 | 100 | 0 |
| 5 | 1.3 | 95 | 5 |
| 15 | 1.3 | 87 | 13 |
| 35 | 1.3 | 80 | 20 |
| 41 | 1.3 | 80 | 20 |

$ - Acetonitrile, # - Deionized milli Q water

### 3.2.3. Sample preparation and extraction

The rhizomes were dried at 25 °C in an innova 42R incubator shaker (New Brunswick Scientific Company, USA) for 7 days and sliced into small pieces. These samples were ground to fine powder using mortar and pestle. Extraction of ginsenosides was performed following the protocol as described by Yang et al. (2013) with certain modifications as described below:

1. Rhizome powder (100 mg) was dissolved in 5 ml of methanol and incubated in an innova 42R incubator shaker (New Brunswick Scientific Company, USA) set at 25 °C and 200 rpm for 24 h.
2. The mixture was centrifuged at 3,500 x g for 5 min at 4 °C in Sorvall Biofuge Primo R centrifuge (Thermo Scientific, USA).
3. The supernatant was filtered using 0.45 µm polyvinylidenedifluoride (PVDF) syringe filter (Himedia, India) and used for HPLC analysis. The samples were extracted in triplicates and 20 µl of each extract was injected for the detection and quantification of ginsenosides.

### 3.2.4. Quantification of ginsenosides in *P. sokpayensis*

The methanol extracts from *P. sokpayensis* were analyzed through HPLC-UV by loading the samples on X-bridge™ amide column. The data were analyzed using Chromeleon software version 6.8. The ginsenosides profile was detected at wavelength of 203 nm and compared the retention times of sample peaks with that of standard peaks. The quantities of detected ginsenosides were calculated from the respective regression equations obtained from the calibration curves prepared from the ginsenoside standards (Table 3.2). Final quantity of each ginsenoside was calculated as a mean of three independent readings and the data were represented as mg per gram dry weight of rhizome.

**Table 3.2** Linearity of calibration curve for eight ginsenosides

| Ginsenoside | Calibration curve[#] | $r^2$ |
|---|---|---|
| Rg2 | Y = 4.7820X – 263.86 | 0.9943 |
| Rg1 | Y = 4.1497X – 361.07 | 0.9992 |
| Rf | Y = 4.2950X – 561.26 | 0.9990 |
| Re | Y = 3.5187X – 744.79 | 0.9989 |
| Rd | Y = 3.6149X – 804.65 | 0.9991 |
| Rc | Y = 2.7799X – 683.79 | 0.9992 |
| Rb2 | Y = 3.2161X – 786.35 | 0.9993 |
| Rb1 | Y = 3.0467X – 724.98 | 0.9993 |

[#]Where X represents an amount of ginsenoside and Y represents peak area.

### 3.2.5. Statistical analysis

Data are presented as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) was employed to determine whether the results had statistical significance ($p<0.05$).

## 3.3. Plant materials for molecular biological studies

Plants of *P. sokpayensis* for cDNA SSH library and other molecular biology experiments were collected with soil from their niche habitat in Sopakha, (altitude 2200 m; N27° 16′ 57″, E88° 05′ 05″), West Sikkim district, and brought to the institute at Tadong, Gangtok (altitude 1230 m asl; N27° 18′ 41″, E88° 35′ 44″), East Sikkim district, Sikkim, India. These plants were maintained in net house conditions at the institute (Figure 3.2). After one year, actively growing 5-years-old plants were selected for further experiments. Plant tissues (leaf, stem and rhizome) were harvested during the active growth period in the month of June (average temperature 18 °C). All the samples were harvested in liquid nitrogen and stored at -80 °C until further use.

## 3.4. Identification of differentially expressed genes using Suppression Subtractive Hybridization (SSH) cDNA libraries

The mRNAs from leaves and rhizomes were used for the construction of cDNA Suppression subtractive hybridization (SSH) library (Diatchenko et al., 1996). Two cDNA SSH libraries, viz., forward and reverse libraries were constructed. In the forward library,

leaf cDNA was used as a tester and rhizome cDNA as a driver. Thus all the genes that were equally expressed in both the tissues were subtracted and the library consisted of expressed sequence tags (ESTs) of only those genes that were either upregulated or expressed only in the leaf tissue. Similarly, the reverse library represented ESTs of genes that were either upregulated or expressed only in the rhizome. The steps involved in the construction of forward and reverse SSH libraries are described below:

### 3.4.1. Isolation of total ribonucleic acid (RNA)

Total RNA was isolated using the protocol as described by (Ghawana et al., 2011). The tissues sampled under section 3.3 were used for this purpose. The isolation was repeated several times for each tissue and then the RNAs were pooled together to get total RNA amount sufficient for purification of 2 µg of mRNA for each tissue.

#### 3.4.1.1. Solutions and reagents

1. Treatment of water – The distilled water used for the isolation of RNA was treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC) following the standard procedure as mentioned in Sambrook and Russell (2001).
2. All the tips, micro-centrifuge tubes, gel trays etc used for RNA isolation were treated with DEPC water (unautoclaved). All the chemicals used for RNA isolation were prepared using DEPC treated autoclaved distilled water (ADW).
3. Solution I (see appendix B).
4. Chloroform.
5. Isopropanol.
6. 70 % ethanol.

#### 3.4.1.2. Protocol

1. The tissue (100 mg) was taken and ground in liquid nitrogen using chilled mortar and pestle to a fine powder.
2. The powder was overlaid with 2 ml of Solution I. The mix was further ground intermittently.
3. After the mixture thawed completely, 800 µl of DEPC treated ADW was added and mixed by grinding.
4. The mixture was transferred to two micro-centrifuge tube (2 ml) and left at room temperature (RT) for 10 min.
5. To each tube, 200 µl of chloroform was added and mixed by vortexing briefly and

left at RT for 10 min.

6. The mixture was then centrifuged at 16,600 x g for 10 min at 4 °C and the upper aqueous phase was transferred to a single fresh tube.
7. Isopropanol (0.6 volume) was then added to this solution, vortexed briefly and kept at RT for 10 min.
8. Then the mixture was centrifuged at 16600 x g for 10 min at 4 °C.
9. The supernatant was discarded and the pellet was washed with 70 % ethanol, air dried and dissolved in 20 – 50 µl of DEPC treated ADW.

### 3.4.2. Quantitation and electrophoresis of RNA

The quantitation and purity of RNA was checked using NanoDrop™ 2000c spectrophotometer (Thermo Fisher Scientific, USA) as given in Appendix A.

### 3.4.3. Purification of mRNA from total RNA

The total RNA of each sample was used to purify respective mRNA using Oligotex®


**Figure 3.2** *P. sokpayensis*: (A), (B) plants maintained in net house conditions at the IBSD, Sikkim Centre, (B) 5 years old plants during active growth (D) 5 years old rhizome.

Direct mRNA Mini Kit (Qiagen, Germany) as per manufacturer's protocol as described below:

1. All the reagents were heated at 37 °C in a water bath to dissolve precipitate if present.
2. Total RNA (1 mg) was pipetted into an RNase free micro-centrifuge tube and the volume was made upto 500 µl with DEPC treated ADW.
3. To the above RNA solution, 500 µl of OBB (binding buffer, supplied by manufacturer) and 55 µl of oligotex suspension was added and mixed thoroughly by flicking the tube.
4. The sample was incubated for 3 min at 70 °C in a water bath.
5. The sample was removed from the water bath, and placed at 20 to 30 °C for 10 min.
6. The Oligotex:mRNA complex was then pelleted by centrifugation for 2 min at 14,000 x g. The supernatant was removed carefully by pipetting.
7. The Oligotex:mRNA pellet was dissolved in 400 µl buffer OW1 (wash buffer, supplied by manufacturer) by vortexing and pipetted onto a small spin column placed in a 1.5 ml micro-centrifuge tube. The tube was centrifuged for 1 min at 14,000 x g.
8. The spin column was then transferred to a new RNase-free 1.5 ml micro-centrifuge tube, 400 µl of wash buffer OW2 (supplied by manufacturer) was pipetted on to the column and centrifuged for 1 min at 14,000 x g.
9. The column was transferred to a new RNase-free 1.5 ml micro-centrifuge tube and 100 µl of hot (heated at 70 °C) elution buffer, OEB was transferred onto the column and pipetted up and down 3 or 4 times to resuspend the resin. The column was centrifuged for 1 min at 14,000 x g.
10. The eluted mRNA was stored at -70 °C till further use.

### 3.4.4. Ethanol precipitation of mRNA

The purified mRNA was concentrated using ethanol precipitation method as described below:

1. Glycogen (20 µg) was added to 100 µl of mRNA sample followed by addition of 0.1 volume of 3M sodium acetate (pH 5.2) and 3 volumes of chilled absolute ethanol and incubated overnight at -20 °C.
2. The sample was then centrifuged at 14000 x g at 4 °C for 15 min and the supernatant was discarded.
3. The pellet was then washed with 70 % ethanol and air dried for 30 min.
4. The pellet was dissolved in 10 µl DEPC treated ADW.

5. The mRNA concentration and purity was checked as mentioned under section 3.4.2.

### 3.4.5. Construction of cDNA SSH library

Leaf (forward) and rhizome (reverse) cDNA libraries were constructed using PCR-select$^{TM}$ cDNA SSH kit (Clontech, USA).

#### 3.4.5.1. First strand cDNA synthesis

1. The components for each tester and driver were added as follows:

| Components | Forward library (tester – leaf ), µl | Reverse library (tester – rhizome), µl |
|---|---|---|
| mRNA (2.0 µg) | 2 – 4 | 2 – 4 |
| cDNA synthesis primer (10.0 µM) | 1.0 | 1.0 |
| ADW | X$^{\$}$ | Y$^{\$}$ |
| Total | 5.0 | 5.0 |

$^{\$}$the volume of X and Y depends on the volume of mRNA taken

2. The contents were mixed and spun briefly in a micro-centrifuge and the tubes were incubated in a thermal cycler at 70 °C for 2 min and cooled on ice for 2 min and spun briefly.
3. The following components were added to each of the above mixture:

| Components | Volume (µl) |
|---|---|
| 5X first strand buffer | 2.0 |
| dNTP mix (10 mM) | 1.0 |
| Sterile water | - |
| DTT (20 mM) | 2.0 |
| SMARTScribe Reverse Transcriptase (100 units/µl) | 2.0 |
| Total | 7.0 |

4. The tubes were vortexed gently and spun briefly.
5. The tubes were then incubated at 42 °C for 1.5 h in an incubator.
6. The tubes were kept on ice to terminate first strand synthesis and immediately proceeded for second strand synthesis.

#### 3.4.5.2. Second strand synthesis

The following procedure was followed with each first strand tester and driver for the synthesis of second strands:

1. The following components were added to the first strand reaction tubes:

| Components | Volume (µl) |
|---|---|
| 5X second strand buffer | 16.0 |
| dNTP mix (10 mM) | 1.6 |
| Sterile water | 48.4 |
| 20X second strand enzyme cocktail | 4.0 |
| Total | 70.0 |

2. The components were mixed and spun briefly and incubated at 16 °C for 2 h in a water bath.
3. T4 DNA polymerase (2 µl; 6 U) of was added to the tube and mixed well.
4. The tube was incubated at 16 °C for 30 min in water bath.
5. The reaction was terminated by adding 4 µl of 20X EDTA/glycogen mix (0.2 M EDTA, 1 mg per ml glycogen).
6. Phenol:chloroform:isoamyl alcohol (100 µl; 25:24:1) was added and the contents were mixed by vortexing and centrifuged at 19,000 x g for 10 min at RT.
7. The upper aqueous layer was transferred to a fresh 0.5 ml micro-centrifuge tube. The inter- and lower phases were discarded.
8. To the above aqueous phase, 100 µl of chloroform:isoamyl alcohol (25:24) was added. The contents were mixed by vortexing and centrifuged at 19,000 x g for 10 min at RT.
9. The upper aqueous layer was transferred to a fresh 0.5 ml micro-centrifuge tube. The inter- and lower phases were discarded.
10. To this aqueous layer, 40 µl of 4 M ammonium acetate ($NH_4OAc$) and 300 µl of 95 % ethanol were added.
11. The contents were vortexed thoroughly and centrifuged at 19,000 x g for 20 min at RT.
12. The supernatant was carefully collected and discarded.
13. The pellet was overlaid with 500 µl of 80 % ethanol.
14. The tube was centrifuged at 19,000 x g for 10 min at RT.
15. The supernatant was discarded and the pellet was air dried for about 10 min.
16. The pellet was then dissolved in 50 µl of ADW.
17. From the above solution of cDNA, 6 µl was stored in a fresh micro-centrifuge tube

at -20 °C until after *Rsa* I digestion (for agarose gel electrophoresis) to estimate yield and size range of ds cDNA products synthesized.

### 3.4.5.3. *Rsa I* digestion

Each experimental double stranded (ds) tester and driver cDNA was restriction digested with *Rsa* I to create shorter, blunt ended ds cDNA fragments which are optimal for subtraction and adaptor ligation.

1. The following reagents were added in each of the two 1.5 ml micro-centrifuge tubes:

| Components | Volume, µl |
|---|---|
| ds cDNA | 43.5 |
| 10X *Rsa* I restriction buffer | 5.0 |
| *Rsa* I (10 units/µl) | 1.5 |
| Total | 50.0 |

2. The contents were mixed by vortexing, centrifuged briefly and then incubated at 37 °C for 1.5 h in a thermal cycler.
3. EDTA/Glycogen mix (20X, 2.5 µl) was added to terminate the reaction.
4. From the above digested mixture, 5 µl was set aside to analyze the efficiency of *Rsa* I digestion.
5. To the remaining digested mixture, 50 µl of phenol:chloroform:isoamyl alcohol (25:24:1) was added.
6. The contents were mixed by vortexing and centrifuged at 19,000 x g for 10 min at RT to separate the phases.
7. The top aqueous was carefully transferred to a fresh 0.5 ml micro-centrifuge tube.
8. To the above aqueous layer, 50 µl of chloroform:isoamyl alcohol (24:1) was added.
9. The steps 6 and 7 were repeated.
10. To the aqueous phase, 25 µl of 4 M $NH_4OAc$ and 187.5 µl of 95 % ethanol were added.
11. Step 6 was repeated.
12. The supernatant was discarded and the pellet was gently overlaid with 200 µl of 80 % ethanol and centrifuged at 19,000 x g for 5 min at RT.
13. The supernatant was carefully removed and the pellet was air dried at for 5 – 10 min.
14. The air dried pellet was then dissolved in 5.5 µl sterile water and stored at -20 °C. This would serve as an experimental driver cDNA.

15. The *Rsa* I digested cDNA from step 3 was checked using 1 % agarose gel containing 0.05 µg per ml ethidium bromide (EtBr).

### 3.4.5.4. Adaptor ligation

Adaptor was ligated to each experimental tester cDNA, viz., leaf and rhizome cDNA in a separate reaction. For forward library, leaf cDNA digested with *Rsa* I was used as tester and the adaptors are ligated to it to produce subtracted cDNA population with sequences specific to leaf. Similarly, for reverse library the adaptors were ligated to *Rsa* I digested rhizome cDNA to generate a subtracted cDNA population with sequences specific to rhizome. The schematic diagrams of adaptor ligations for forward and reverse libraries are shown in figure 3.3. Two separate adaptor ligation reactions, one each for forward and reverse subtraction were performed. For each subtraction, respective tester cDNA (leaf cDNA labelled tester 1 in case of forward subtraction and rhizome cDNA labelled tester 2 in case of reverse subtraction) was aliquoted into two separate tubes: one aliquot was ligated with adaptor 1 and the second one was ligated with adaptor 2R. Portions from each tube were combined so that the cDNA is ligated with both adaptors (unsubtracted tester control 1 – c and 2 – c). Each unsubtracted tester control cDNA served as positive control for ligation and as a negative control for subtraction. The procedure for adaptor ligation is described below:

1. Each experimental tester cDNA (1.0 µl) was diluted with 5.0 µl of sterile water.
2. The ligation master mix was prepared by combining the following reagents in a 0.5 ml micro-centrifuge tube:

| Components | Volume, µl |
|---|---|
| ADW | 3.0 |
| 5X ligation buffer | 2.0 |
| T4 DNA Ligase (400 units/µl) | 1.0 |
| Total | 6.0 |

3. For each experimental tester cDNA, the reagents are combined in a 0.5 ml micro-centrifuge tube in an order shown below:

| Components | Tester 1 – 1[$] | Tester 1 – 2[$] |
|---|---|---|
| Diluted tester cDNA | 2.0 µl | 2.0 µl |
| Adaptor 1 | 2.0 µl | - |
| Adaptor 2R | - | 2.0 µl |
| Ligation master mix | 6.0 µl | 6.0 µl |
| Total | 10.0 µl | 10.0 µl |

[$]Similar set up was used for reverse subtraction in which the tester was rhizome cDNA

4. Tester 1 – 1 and tester 1 – 2 (2 µl each) were mixed in a fresh centrifuge tube and kept aside to be used as unsubtracted tester control 1 – c (Figure 3.3 A). Similarly, unsubtracted control 2 – c is prepared for reverse library using 2 µl of tester 2 – 1 and 2 µl of tester 2 – 2 (Figure 3.3 B).
5. The tubes containing ligation mixtures were given short spin in a centrifuge to mix the contents and incubated at 16 °C overnight.
6. EDTA/Glycogen mix (1 µl) was added to stop the reaction.
7. The samples were heated at 72 °C for 5 min to inactivate the ligase. The tubes were centrifuged briefly.
8. Unsubtracted control (1 µl) from each library (1 – c in case of forward library and 2 – c in case of reverse library) was taken separately and diluted to 1 ml each with ADW to be used for PCR confirmation.
9. The ligation efficiency test was performed using 1 µl each from the ligation reaction as per the manufacturer's protocol.
10. The samples are stored at -20 °C.

**3.4.5.5. First hybridization**

After ligation efficiency test, first hybridization was carried out to equalize and enrich differentially expressed sequences. An excess of driver was added to each tube/sample of the tester. For example, in forward library (leaf cDNA/cDNA 1/tester 1) to each adaptor ligated cDNA sample (tester 1 – 1 ligated with adaptor 1 and tester 1 – 2 ligated with adaptor 2R), an excess of rhizome cDNA (cDNA2/tester 2) was added, heat denatured and allowed to anneal at 68 °C. This process equalizes the concentration of high and low abundance sequences and at the same time enriches differentially expressed sequences in the tester. The first hybridization was carried out as described below:

1. Hybridization buffer (4X) was kept at RT for 20 min to warm up the solution in order to dissolve any pellet if present. The reagents were combined as given below:

| Components | Hybridization sample 1 (tester 1 – 1[$]) | Hybridization sample 2 (tester 1 – 2[$]) |
|---|---|---|
| *Rsa*I digested driver cDNA (rhizome cDNA) | 1.5 µl | 1.5 µl |
| Adaptor I ligated tester 1 – 1 | 1.5 µl | - |
| Adaptor 2R ligated tester 1 – 2 | - | 1.5 µl |
| 4X hybridization buffer | 1.0 µl | 1.0 µl |
| Final volume | 4.0 µl | 4.0 µl |

[$] Similar set up was used for tester 2 – 1 and 2 – 2

2. The samples were mixed by brief centrifugation and incubated at 98 °C for 1.5 min in a thermal cycler.
3. The samples were then incubated at 68 °C for 8 h.

**3.4.5.6. Second hybridization**

In this step, two hybridization samples are mixed and freshly denatured driver cDNA is added to further enrich the differentially expressed sequences. A new type of hybrid cDNA is formed. Each strand of this type of cDNA contains different adaptor at the 5′ end which helps in the exponential amplification of these hybrid sequences during PCR enrichment. The following procedure was followed for each of the experimental tester cDNA:

1. The following reagents were added in a 0.5 ml micro-centrifuge tube:

| Components | Volume (µl) |
|---|---|
| *Rsa* I digested driver cDNA | 1.0 |
| 4X hybridization buffer | 1.0 |
| ADW | 2.0 |
| Total | 4.0 |

2. From the above mixture, 1 µl was taken in a micro-centrifuge tube and overlaid with one drop of mineral oil.
3. The sample was incubated at 98 °C for 1.5 min in a thermal cycler.
4. Freshly denatured driver cDNA was then mixed with hybridization samples 1 and 2.
5. The sample was mixed by brief centrifugation and then incubated overnight at 68 °C.

6. Dilution buffer (200 µl) was added and mixed by pipetting. The sample was then heated at 68 °C for 7 min in a thermal cycler.
7. The sample was stored at -20 °C until further use.

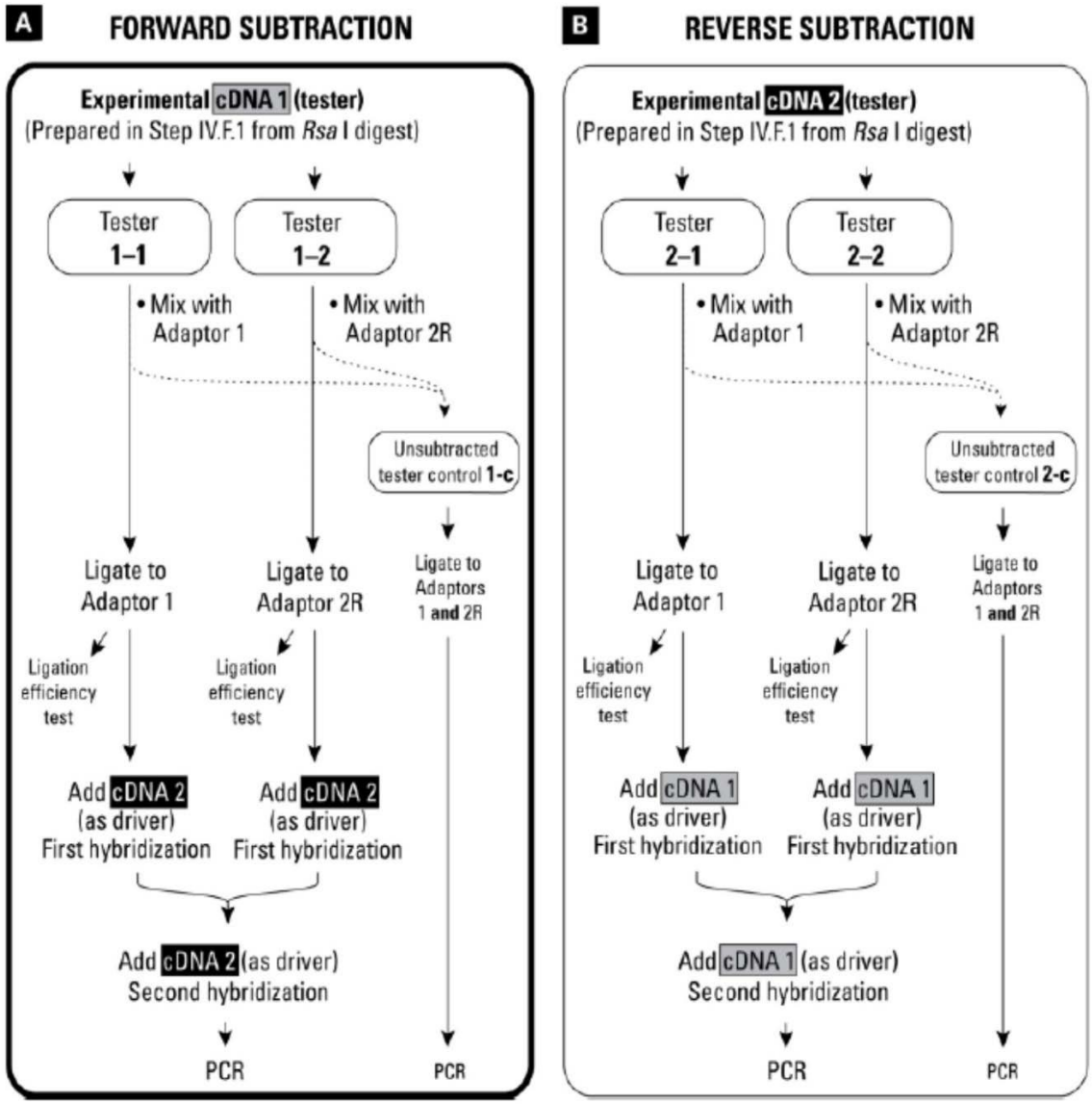


**Figure 3.3** Preparation of adaptor ligated tester cDNAs for hybridization and PCR: (A) Forward subtraction, (B) Reverse subtraction (www.clontech.com). cDNA 1 – *Rsa* I digested leaf cDNA, cDNA 2 – *Rsa* I digested rhizome cDNA.

### 3.4.5.7. PCR amplification

This process involves two rounds of PCR (primary and secondary PCR) to selectively enrich differentially expressed sequences. PCRs were performed for each experimental cDNA:

Forward library – (i) forward subtracted cDNA and (ii) unsubtracted tester control, 1 – c

Reverse library – (ii) reverse subtracted cDNA and (ii) unsubtracted tester control, 2 – c

The PCRs were carried out as follows:

1. The PCR master mix was made for the required number of reactions plus one additional reaction using the following components:

| Components | Volume (µl) |
|---|---|
| Sterile water | 19.5 |
| 10X PCR buffer | 2.5 |
| dNTP mix (10 mM) | 0.5 |
| PCR primer 1 (10 µM) | 1.0 |
| 50X Advantage cDNA Polymerase mix | 0.5 |
| Total | 24.0 |

2. The contents were mixed well by vortexing and brief centrifugation. 24 µl each of master mix was added to 1 µl of diluted forward subtracted cDNA and unsubtracted tester control, 1 – c. Similarly, 24 µl each of master mix was added to 1 µl of diluted reverse subtracted cDNA and unsubtracted tester control, 2 – c.
3. The reaction mixture was incubated at 75 °C for 5 min in a thermal cycler to extend the adaptors.
4. Thermal cycling was commenced immediately in a thermocycler (Eppendorf Mastercycler pro S, USA) using the following parameters: 94 °C for 25 s followed by 27 cycles of 94 °C for 10 s, 66 °C for 30 s and 72 °C for 1.5 min.
5. From each tube, 8 µl PCR product was analyzed on a 2.0 % agarose gel containing 0.05 µg per ml EtBr and run on 1X TAE buffer.
6. Each primary PCR product was diluted 10 times (3 µl PCR product in 27 µl of ADW), mixed properly by tapping and brief centrifugation and kept aside on ice to be used for secondary PCR.
7. The PCR master mix was made for the required number of reactions plus one additional reaction using the following components:

| Components | Volume, µl |
|---|---|
| ADW | 18.5 |
| 10X PCR buffer | 2.5 |
| dNTP mix (10 mM) | 0.5 |
| Nested PCR primer 1 (10 µM) | 1.0 |
| Nested PCR primer 2R (10 µM) | 1.0 |
| 50X advantage cDNA Polymerase mix | 0.5 |
| Total | 24.0 |

8. The contents were mixed well by vortexing and brief centrifugation. 24 µl each of master mix was added to 1 µl of diluted primary PCR product of each sample and control belonging to forward and reverse subtraction. The sample was overlaid with one drop of mineral oil.
9. The thermal cycling was commenced immediately with the following parameters: 12 cycles of 94 °C for 10 s, 68 °C for 30 s and 72 °C for 1.5 min.
10. From each tube, 8 µl was analyzed on a 2.0 % agarose gel containing 0.05 µg per ml EtBr and run on 1X TAE buffer.
11. The remaining mixture was stored at -20 °C until required.

### 3.4.6. Cloning of cDNA products

The PCR product now contains enriched ESTs belonging to differentially expressed genes. The ligation of these differentially expressed sequences from both forward and reverse libraries, transformation of the competent cells with the recombinant constructs, selection of the transformants, colony PCR and plasmid isolation were done as mentioned below:

#### 3.4.6.1. Ligation

The amplified fragment was ligated into pCR2.1 vector (Figure 3.4) supplied with TA cloning kit (Invitrogen, USA). The components of the reaction are as follows:

| Reagents | Volume (µl) |
|---|---|
| 10X ligation buffer | 1.0 |
| pCR 2.1 vector (25 ng/µl) | 2.0 |
| PCR product (X ng) | Y |
| T4 DNA ligase (5 units/µl) | 1.0 |
| Sterile water | Make up the volume to 10.0 µl |
| Total | 10.0 |

X – amount of PCR product to be ligated in a 1:1 (vector:insert) molar ratio and is given by the following formula: X ng PCR product = [(Y bp PCR product) (50 ng PCR 2.1 vector)]/(size in bp of pCR2.1 vector-3929 bp); Y – depends on the amount of PCR product, X. .

The ligation mixture was incubated at 16 °C for 16 h in a circulatory water bath.

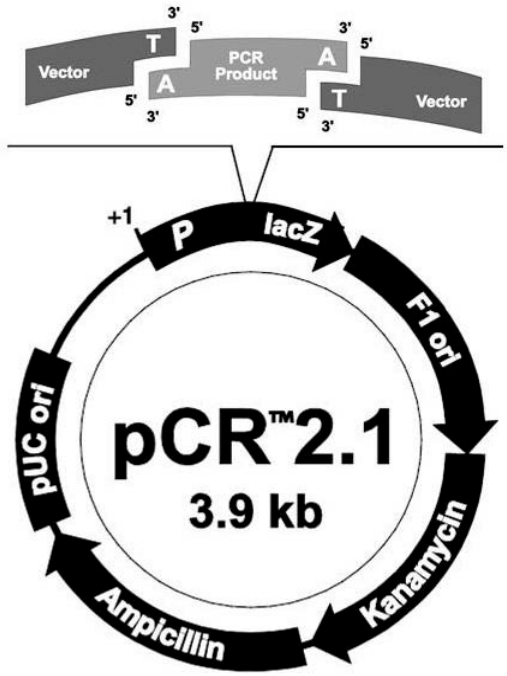


**Figure 3.4** Graphical map of pCR2.1 vector showing cloning site. (Reproduced from Invitrogen, Thermo Fisher Scientific, USA).

### 3.4.6.2. Transformation

The transformation was carried out in a chemically competent *E. coli* DH5α cells that were prepared using the procedure as described in Appendix A. The transformation was carried out using heat shock. 5 µl of ligated product was mixed with competent cells thawed on slush and mixed by gently tapping. The mixture was then kept on ice for 20 min. The mixture was given a heat shock at 42 °C for 90 s and kept on ice for 2 min. 900 µl of prewarmed SOC medium was added and the cells were grown at 37 °C with 200 RPM shaking in an incubator shaker for 5 h after which the cells were plated for selection through

alpha complementation.

### 3.4.6.3. Selection of transformants

The transformants were selected using blue/white screening system which is based on the principle of α complementation. The procedure is as follows: 300 µl of culture, 40 µl of 100 mM IPTG and 40 µl of 40 mg per ml X gal were spread on LB medium containing 0.1 µg/µl of ampicillin. The plate was incubated at 37 °C overnight in an incubator. The transformants, i.e., cells containing the ligated products form white colonies while the cells containing recircularized vector form blue colonies.

### 3.4.6.4. Colony PCR

To confirm the presence of the desired insert, a colony PCR was performed using M13 forward - 5′ GTAAAACGACGGCCAGTG 3′ and M13 reverse - 5′ GGAAACAGCTATGACCATG 3′ universal primers. Colony lysates were prepared by heat lysis method. White colonies from the blue/white selection plate was streaked on LB ampicillin medium and grown overnight at 37 °C in incubator. A pure single colony from this plate was picked and lysed in 50 µl colony lysis buffer by heating at 80 °C for 10 min in a circulatory water bath. The tube was kept on ice for 2 min and then the cellular debris was pelleted by centrifuging at 10,000 x g for 10 min. The supernatant was transferred to fresh micro-centrifuge tube as colony lysate.

| Components | Volume (µl) |
|---|---|
| ADW | 16.30 |
| 10X buffer | 2.50 |
| dNTP mix (2 mM) | 2.00 |
| M13 forward primer (10 µM) | 1.00 |
| M13 reverse primer (10 µM) | 1.00 |
| Taq polymerase (5units/µl; Qiagen, Germany) | 0.20 |
| Colony lysate | 2.00 |
| Total | 25.00 |

The PCR temperature set up for the colony PCR was: 94 °C – 0 min; 25 x (94 °C – 30 s, 52 °C – 01 min, 72 °C – 02 min). The end product was checked through electrophoresis using 1.2 % agarose gel containing 0.05 µg per ml EtBr.

### 3.4.7. Preparation of glycerol stock and plasmid isolation

After the insert was confirmed through colony PCR, a single colony of transformants was grown in 5 ml LB broth containing 0.1 µg/µl of ampicillin at 37 °C at 200 RPM shaking overnight. A 50 % glycerol stock was prepared by adding 750 µl of the above culture to 750 µl of an autoclaved glycerol. The glycerol stock was snap frozen in liquid nitrogen and then preserved at -80 °C. The remaining culture was used to isolate the recombinant plasmid as detailed in Appendix A.

### 3.4.8. Sequencing of ESTs

The ESTs ligated to plasmids were single pass sequenced using the BigDye terminator (version 3.1) cycle sequencing mix (Applied Biosystems, USA) on an automated DNA sequencer ($3130_{xl}$, Genetic Analyzer, Applied Biosystems, USA) with M13 forward primer.

### 3.4.9. Pre – processing of reads, sequence assembly and functional annotation

The curing of raw sequencing reads and the analysis of high quality ESTs of forward and reverse subtracted libraries were done using bioinformatics softwares as mentioned in table 3.3.

## 3.5. Degenerate primer based amplification of genes

Apart from the three genes, viz., *farnesyl pyrophosphate synthase* (*PsFPS*), *squalene synthase* (*PsSS*) and *dammarenediol synthase* (*PsDS*) cloned through cDNA SSH library based ESTs, partial fragments of few other genes from the ginsenoside biosynthetic pathway were amplified through degenerate primer based approach. The degenerate primers were designed based on the corresponding gene sequences from other plants. Based on these partial fragments obtained through degenerate approach, full length cDNA of *squalene epoxidase* (*PsSE*), *phosphomevalonate kinase* (*PsPMVK*) and *4-diphosphocytidyl-2-C-methyl-D-erythritol kinase* (*PsCMK*) from ginsenoside biosynthetic pathway and *cycloartenol synthase* (*PsCS*) from the phytosterol biosynthetic pathway were amplified. Partial cDNAs of several other genes from mevalonate pathway, non – mevalonate pathway and ginsenoside biosynthetic pathway have also been amplified using the degenerate primers.

The degenerate primers and the PCR conditions used to amplify the partial cDNAs of the desired genes are given in the table 1 (Appendix C).

### 3.5.1. First strand cDNA synthesis

#### 3.5.1.1. Removal of genomic DNA contamination from RNA

The genomic DNA contamination in RNA was removed by treatment with DNase I (Invitrogen, USA). A 1.5 ml micro-centrifuge tube was taken and the following reagents were added:

| Components | Volume (µl) |
|---|---|
| Total RNA (2 µg) | X |
| 10X DNase I buffer | 2.0 |
| DNase I (1 unit/µl) | 2.0 |
| DEPC treated water | Y |
| Total | 20.0 |

X – depends on the concentration of RNA
Y – depends on the volume of X

The mixture was incubated at 25 °C for 15 min in a thermal cycler.

#### 3.5.1.2. Priming

The following reagents were added to the above tube:

| Components | Volume (µl) |
|---|---|
| 25 mM EDTA | 2.0 |
| Oligo $(dT)_{20}$(50 µM) | 2.0 |
| dNTPs (10 mM) | 2.0 |
| Total | 26.0 |

The mixture was incubated at 65 °C for 10 min in a thermal cycler and then kept on ice for 2 min.

#### 3.5.1.3. First strand cDNA synthesis

| Components | Volume (µl) |
|---|---|
| 10 X RT buffer | 4.0 |
| 25 mM $MgCl_2$ | 4.0 |
| 100 mM DTT | 4.0 |
| Superscript® III reverse transcriptase (200 units/µl) | 2.0 |
| Total | 40.0 |

After adding the above reagents to the tube from **3.5.1.2**, the mixture was incubated

at 42 °C for 60 min in a thermal cycler and then at 70 °C for 15 min. The tube was then stored at – 80 °C until required.

### 3.5.2. Polymerase chain reaction (PCR) using degenerate primers

PCR was carried out using a thermocycler. The composition of reagents was as follows:

| Components | Volume (µl) |
|---|---|
| ADW | 19.80 |
| 10X buffer | 2.50 |
| dNTP mix (10 mM) | 0.50 |
| Forward primer (10 µM) | 0.50 |
| Reverse primer (10 µM) | 0.50 |
| cDNA | 1.00 |
| Taq polymerase (5units/µl; Qiagen, Germany) | 0.20 |
| Total | 25.00 |

### 3.5.3. Electrophoresis of PCR products

The PCR products were loaded in 1.2 % agarose gel containing 0.05 µg per ml EtBr and electrophoresed in 1X TAE buffer at 72 volts in a mini – sub cell GT electrophoresis unit (Bio-Rad, USA). The PCR products were mixed with DNA loading dye (Appendix B) before loading into the wells.

### 3.5.4. DNA extraction from gel

The desired DNA amplicon from the agarose gel was extracted using GenElute™ Gel Extraction Kit (Sigma-Aldrich Inc., USA) following the manufacturer's protocol as described in Appendix A.

### 3.5.5. DNA quantitation

The quantitation of the eluted DNA was done as mentioned in Appendix A.

### 3.5.6. Cloning

Cloning of amplicon was done as mentioned under section 3.4.6.

### 3.5.7. Plasmid isolation

Plasmid isolation was done as mentioned under section 3.4.7.

### 3.5.8. Sequencing

Sequencing was done as mentioned under sections 3.4.8.

### 3.5.9. Bioinformatics

Homology search of the amplicon sequence was done using Basic Local Alignment Search Tool (BLAST) (Table 3.3, Altschul et al. 1990).

## 3.6. Rapid Amplification of cDNA ends (RACE)

The full lengths of cDNAs of various genes were obtained by performing RACE using SMARTer$^{TM}$ RACE cDNA Amplification Kit (Clontech, USA). The steps involved are described below:

### 3.6.1. Primer designing for RACE

5′ and 3′ RACE primers were designed using the sequence information of the ESTs/partial sequences obtained through SSH libraries and degenerate primer based approach. Two sets of gene specific primers, one set for primary RACE and the other set for secondary/nested RACE were designed using freely accessible online software Primer3 (Untergasser et al., 2012) (http://bioinfo.ut.ee/primer3-0.4.0/). The primers sequence and Tm are mentioned in Annexure III.

### 3.6.2. Preparation of 5′ and 3′ RACE Ready cDNA

The following procedure was used to synthesize RACE Ready first-strand cDNA using 1 µg of total RNA.

1. The following reagents were combined in separate micro-centrifuge tubes as shown below:

| Components | 5′ RACE ready cDNA, µl | 5′ RACE ready cDNA, µl |
|---|---|---|
| RNA sample | 1 µg (1 – 2.75 µl) | 1 µg (1 – 3.75 µl) |
| 5′ CDS primer | 1.00 | - |
| 3′ CDS primer A | - | 1.00 |
| ADW | X* | Y* |
| Total | 3.75 | 4.75 |

* Make up the volume to 3.75 µl and 4.75 µl for 5′ and 3′ RACE ready cDNA respectively

2. The contents were mixed by pipetting and spun briefly in a micro-centrifuge.

3. The tubes were incubated at 72 °C for 3 min and then cooled to 42 °C for 2 min. Then the tubes were spun for 10 s at 14000 x g to collect the contents at the bottom.
4. SMARTer IIA oligo (1 µl) was added to just the 5′ RACE cDNA synthesis reaction.
5. To each tube the following components were added:

| Components | Volume, µl |
|---|---|
| 5X First Strand Buffer | 2.0 µl |
| DTT (20 mM) | 1.0 µl |
| dNTP mix (10 mM) | 1.0 µl |
| Total | 4.0 µl |

6. To the above mixture, 0.25 µl of RNase inhibitor (40 U/µl) and 1.0 µl of SMARTScribe$^{TM}$ Reverse Transcriptase (100 U) was added.
7. The 10 µl mixture was mixed gently by pipetting and spun briefly in a centrifuge.
8. The samples were incubated at 42 °C for 90 min in a hot – lid thermal cycler.
9. The tubes were then heated at 70 °C for 10 min in a thermal cycler.
10. The first strand cDNA was diluted with 100 µl Tricine – EDTA buffer and stored in aliquots of 20 µl at -20 °C.

### 3.6.3. Primary PCR for RACE

1. The PCR components required for primary PCR was added as shown below:

| Components | 5′ RACE, µl | 3′ RACE, µl |
|---|---|---|
| PCR grade water | 34.5 | 34.5 |
| 10X Advantage 2 PCR buffer | 5.0 | 5.0 |
| dNTP mix (10 mM) | 1.0 | 1.0 |
| 10X Universal Primer A Mix (UPM) | 5.0 | 5.0 |
| Gene specific primer* (10 µM) | 1.0 | 1.0 |
| RACE ready cDNA$^{\$}$ | 2.5 | 2.5 |
| 50X Advantage 2 Polymerase mix | 1.0 | 1.0 |
| Total | 50.0 | 50.0 |

*Gene specific primers for 5′ and 3′ RACE are different
$^{\$}$5′ RACE ready cDNA for 5′ RACE and 3′ RACE ready cDNA for 3′ RACE

2. The thermal cycling parameters were as follows: 30 cycles of 94 °C for 10 s, 68 °C for 30 s, 72 °C for 3 min and final extension of 3 min at 72 °C.

### 3.6.4. Secondary (nested) PCR for RACE

1. The components for secondary PCR were added as follows

| Components | 5′ RACE, µl | 3′ RACE, µl |
|---|---|---|
| PCR grade water | 36.0 | 36.0 |
| 10X advantage 2 PCR buffer | 5.0 | 5.0 |
| dNTP mix (10 mM) | 1.0 | 1.0 |
| Nested Universal Primer A (NUP, 10 µM) | 1.0 | 1.0 |
| Nested Gene specific primer* (10 µM) | 1.0 | 1.0 |
| 50X advantage 2 Polymerase mix | 1.0 | 1.0 |
| Diluted primary PCR product[$] | 5.0 | 5.0 |
| Total | 50.0 | 50.0 |

*Nested Gene specific primers for 5′ and 3′ RACE are different, [$] Respective 5′ and 3′ diluted primary PCR product (5 µl of primary PCR product was diluted in 245 µl of ADW)

2. PCR was conducted as mentioned in 3.6.3 (step 2).
3. The primary and secondary PCR products were analyzed on a 1.2 % agarose gel containing 0.05 µg per ml EtBr using appropriate size DNA markers.

The primers used for RACE are given in table 2 in Appendix C.

### 3.6.5. Cloning, sequencing and analysis of RACE products

The agarose gel electrophoresis of RACE amplicons and their extraction from an agarose gel was done as mentioned in section 3.5.3 and 3.5.4. Cloning of these fragments, selection of the transformants, plasmid isolation and sequencing of the inserts were done as mentioned under sections 3.4.6 through 3.4.8. The sequences obtained were analysed using appropriate softwares available at various sites as listed in table 3.3.

## 3.7. Gene expression studies

To validate the qualities of leaf and rhizome subtractive libraries, semi – quantitative RT – PCR and real-time PCR (qRT – PCR) were performed. The total RNA isolation from the leaves, stems and rhizomes and quantitation of their RNAs were carried out as mentioned under section 3.4.1 and 3.4.2, respectively. cDNA synthesis was carried out as mentioned in 3.5.1. List of primers used for the study of expression of various genes using semi – qRT and qRT – PCR is given in table 3, Appendix C.

### 3.7.1. Semi – quantitative reverse transcription PCR (Semi – quantitative RT – PCR)

To validate the quality of the leaf and rhizome subtractive libraries, expression analysis of selected genes was performed by semi-quantitative RT-PCR as described previously (Bhardwaj et al., 2013). Briefly, total RNA was treated with DNase I, (amplification grade, Invitrogen, USA) to remove contaminating genomic DNA. First-strand cDNA was synthesized from 2μg of DNase free RNA using Superscript® III first-strand synthesis system for RT – PCR (Invitrogen, USA) with an oligo (dT) 12-18 primer. PCR was performed with 1 μl of first-strand cDNA and 0.5 μM each of forward and reverse gene specific primers in a final volume of 25 μl. PCR was carried out on a programmable thermocycler using the following cycling conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles at 94 °C for 30 s, 58 °C for 40 s, 72 °C for 1 min, and then a final extension at 72 °C for 7 min. PCR products were separated on 1.2 % agarose gel containing 0.05 μg per ml EtBr and visualized using a gel documentation system (Bio-Rad ChemiDoc™ XRS+ Imaging system, USA). All the primers were designed using bioinformatics software Primer3 (https://www.genscript.com/ssl-bin/app/primer). Expression of *18S rRNA* was used as an internal control (Gao et al., 2014).

### 3.7.2. Quantitative real – time PCR (qRT – PCR)

Differential expression of the all the validated genes was performed using quantitative real-time PCR (qRT-PCR). Relative expression analysis was performed using cDNA synthesized from RNA isolated from leaf and stem and rhizome tissues. Each qRT-PCR reaction was performed with three biological replicates and three technical replicates. The reaction was performed in 10 μl reaction mixture containing diluted cDNA samples as template, 2 X SYBR® Green Master Mix (Applied Biosystems, USA) and 200 nM each of forward and reverse gene specific primers (Table 3, Appendix C). The reactions were performed in StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) using the following programme: initial denaturation at 94 °C for 10 min, followed by 40 cycles of amplification (94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s) and final melt curve analysis was performed. Transcript levels of all the genes were normalized with an internal reference, *18S rRNA* gene. The relative expression ratio of each gene was calculated using comparative Ct value method as described previously (Livak and Schmittgen, 2001). Here, the data represented are relative quantitation (RQ) values of gene expression. Expression is shown after normalization to *18S rRNA* gene. Values were calculated using $\Delta\Delta C_t$ method,

and the error bars represented as $RQ_{MIN}$ and $RQ_{MAX}$.

## 3.8. *In silico* comparative analysis of all the unigenes against available *Panax* transcriptomes:

All the unigenes from both libraries were compared by sequence alignments against *Panax* transcriptome datasets available at NCBI. Information regarding transcriptomes of *P. ginseng*, *P. notoginseng*, *P. quinquefolius* and *P. vietnamensis* was retrieved from NCBI Sequence Read Archive (SRA, http://www.ncbi.nlm.nih.gov/Traces/sra/) (Leinonen et al., 2011). Different transcriptome datasets used for analysis were *P. ginseng* SRX181262 (leaf), SRX181253 (root); *P. notoginseng* SRX017444 (root); *P. quinquefolius* SRX 012184 (root) and *P. vietnamensis* SRX495493 (rhizome). Gene homologues were identified manually against all the unigenes from *P. sokpayensis* using BLASTN analysis (parameter settings: max target sequences 500).

## 3.9. Heterologous expression of plant protein in *E. coli*

The heterologous expression of protein using open reading frame (ORF) derived from *P. sokpayensis* gene was performed using "The QIAexpressionist$^{TM}$ System" (Qiagen, Germany).

### 3.9.1. The QIAexpressionist system

This system uses *E. coli* strain M15 [pREP4] which permits high level expression of recombinant proteins. M15 strain is derived from *E. coli* K12 and has the phenotype $Nal^S$, $Str^S$, $Rif^S$, $Thi^-$, $Lac^-$, $Ara^+$, $Gal^+$, $Mtl^-$, $F^-$, $RecA^+$, $Uvr^+$ and $Lon^+$. The expression vector consists of QIAexpress pQE vectors that lead to high – level expression of 6xHis – tagged proteins in *E. coli*. pQE plasmids belong to the pDS family of plasmids (Bujard et al., 1987) and were derived from plasmids pDS781/RBSII-DHFRS and pDS56/RBSII (Stuber et al. 1990). The features of these low copy plasmids are as follows:

1. Optimized promoter–operator element with phage T5 promoter that is recognized by the *E. coli* RNA polymerase. It also contains two *lac* operators where *lac* repressor binds to ensure efficient repression of the powerful T5 promoter.
2. Ribosomal binding site II (RBSII), a synthetic ribosomal binding site for high translation rates.
3. 6xHis-tag coding sequence either 5′ or 3′ to the cloning region.

4. Multiple cloning sites with translational stop codons in all reading frames.
5. Two strong transcriptional terminators: T1 from the *rrnB* operon of *E. coli* and *t0* from phage lambda (Schwarz et al. 1978) that prevent read-through transcription and ensure stability of the expression construct.
6. They confer ampicillin resistance at 100 μg per ml due to the presence of β – lactamase gene (*bla*) (Sutcliffe, 1979). The chloramphenicol acetyl transferase gene (*CAT*) present between *t0* and T1 has no promoter and is not normally expressed.
7. ColE1 origin of replication (Sutcliffe, 1979).

The high transcription rate provided by T5 promoter can only be efficiently regulated and repressed by the presence of the *lac* repressor protein at high levels. $M_{15}$ used in this system uses a *lac* repressor to the gene to be expressed. It contains a low-copy plasmid pREP4 with kanamycin resistance gene that confers kanamycin resistance and the *lac* I gene that constitutively expresses the *lac* repressor (Farabaugh, 1978). The pREP4 plasmid contains the p15A replicon and is derived from pACYC. Many copies of pREP4 are present in $M_{15}$ that ensure high level production of *lac* repressors that bind to the operator sequences and tightly regulate the expression of recombinant protein. The pREP4 plasmid is compatible with all plasmids carrying the ColE1 origin of replication, and is maintained in *E. coli* supplied with kanamycin at a concentration of 25 μg per ml. Expression of pQE vector encoded recombinant proteins is rapidly induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) which binds to the *lac* repressor protein and inactivates it. The RNA polymerase of $M_{15}$ can then transcribe the sequences downstream from the promoter once the *lac* repressor is inactivated. The transcripts produced are then translated into the recombinant proteins.

## 3.9.2. Cloning of genes using pQE30 vector

### 3.9.2.1. Preparation of pQE30 vector for cloning

A combination of carefully chosen two restriction enzymes (Thermo Fisher Scientific Inc., USA) was used to linearize pQE30 (Figure 3.5) and create cohesive ends. The restriction sites within the insert to be cloned were taken into consideration while choosing the above enzyme pair. A double digestion reaction in a single tube was carried out according to the protocol described below:

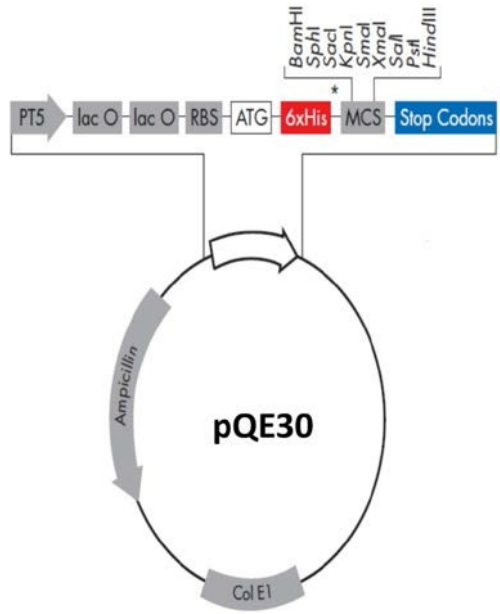


**Figure 3.5** Expression vector pQE30 (www.qiagen.com). PT5 – T5 promoter, *lac* O – *lac* operator, RBS – ribosome binding site, ATG – start codon, 6xHis – His tag sequence, MCS – multiple cloning sites, stop codons – stop codons in all three reading frames, Col E1 – Col E1 origin of replication, Ampicillin – ampicillin resistance gene.

1. The reagents were added as follows

| Components | Volume, µl |
|---|---|
| ADW | 14.0 |
| 10X Fast Digest Green Buffer | 2.0 |
| pQE30 (500 ng/µl) | 2.0 (1 µg) |
| I$^{st}$ Restriction enzyme | 1.0 |
| 2$^{nd}$ Restriction enzyme | 1.0 |
| Total | 20.0 |

2. The mixture was incubated at 37 °C for appropriate time for the respective enzyme as mentioned by manufacturer. For the purpose of cloning of *farnesyl diphosphate synthase* (FPS), pQE30 was double digested with *PaeI* and *PstI* (Thermo Fisher Scientific Inc., USA) and incubated for 10 min at 37 °C.
3. The reaction was stopped by adding 1 µl of 10X TE.
4. The mixture was then electrophoresced in 0.8 % agarose gel containing 0.05 µg per ml EtBr to confirm restriction digestion. Subsequently, gel purification of the processed vector was done as mentioned in section 3.5.4. The eluted vector was quantified as mentioned in section 3.5.5 and stored at – 20 °C until further use.

### 3.9.2.2. Preparation of insert for cloning

#### 3.9.2.2.1. Cloning of open reading frame (ORF)

The open reading frame (ORF) of the concerned gene was amplified using primer set designed from start to stop codon. The cDNA from *P. sokpayensis* rhizome was used as template. The RNA isolation, quantitation of RNA and cDNA synthesis was carried out as mentioned in section 3.4.1, 3.4.2 and 3.5.1 respectively. The composition of reagents for PCR was as mentioned in section 3.5.2 except that the primers were specific primers instead of degenerate primers.

The primer pair used for the amplification of ORFof *FPS* is given in table 4 (Appendix C). The thermal cycling conditions for the amplification of ORF of *FPS* were: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 40 s, 72 °C for 2 min, and then a final extension at 72 °C for 7 min. The PCR products were run in 1 % agarose gel containing 0.05 µg per ml EtBr. DNA extraction of the amplified ORF was done as mentioned in section 3.5.4 and cloning of the ORF and isolation of its recombinant plasmid was done as in section 3.4.6 and 3.4.7, respectively.

#### 3.9.2.2.2. Introduction of cloning sites flankimg ORF

Cloning site flanking each end of the insert was introduced through *in vitro* mutagenesis. For this purpose, different cloning site was introduced in the forward and reverse primer sequence during primer designing. Then PCR was conducted to introduce cloning sites in the insert. The PCR components were added as follows:

| Components | Volume (µl) |
|---|---|
| ADW | 19.80 |
| 10X buffer | 2.50 |
| dNTP mix (10 mM) | 0.50 |
| Forward primer (10 µM) | 0.50 |
| Reverse primer (10 µM) | 0.50 |
| Colony lysate* | 1.00 |
| Taq polymerase (5units/µl; Qiagen, Germany) | 0.20 |
| Total | 25.0 |

*A single colony was lysed in colony lysis buffer as mentioned in section 3.4.6.4

The primer pair used for the introduction of cloning sites flanking the ORF of *FPS* is given in the table 4 (Appendix C). The thermal cycling conditions for the introduction of

cloning sites in the *FPS* ORF were as follows: initial denaturation at 94 °C for 0 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 40 s, 72 °C for 2 min, and then a final extension at 72 °C for 7 min. 2 µl PCR product was run in agarose gel containing 0.05 µg per ml EtBr to confirm the amplification of the desired product. The remaining 23 µl of the PCR product was purified using GenElute PCR cleanup kit (Sigma-Aldrich Inc., USA) as described in Appendix A.

The cloning of the *FPS* ORF containing cloning sites and the isolation of its recombinant plasmid was done as mentioned in section 3.4.6 and 3.4.7, respectively.

**3.9.2.2.3. Restriction digestion of the insert to create cohesive ends**

The insert that is to be cloned into the expression vector pQE30 was digested with the same pair of restriction enzymes that was used for digesting pQE30. This creates cohesive ends in the insert that is compatible with the cohesive ends of the vector. The insert was first cloned into pCR2.1 vector, the recombinant plasmid isolated (section 3.4.6 and 3.4.7, respectively) and then this recombinant plasmid was used for the restriction digestion. The double digestion of the insert was performed in a single reaction as follows:

1. The components are added as follows:

| Reagents | Volume (µl) |
|---|---|
| ADW | X |
| 10X Fast Digest Green Buffer | 2.0 |
| pCR2.1 containing insert | Y (1 µg) |
| $I^{st}$ Restriction enzyme | 1.0 |
| $2^{nd}$ Restriction enzyme | 1.0 |
| Total | 20.0 |

Y – depends on the concentration of the recombinant plasmid (pCR2.1 containing insert) and X depends on Y

2. The mixture was incubated at 37 °C for appropriate time for the respective enzyme as mentioned by manufacturer. For the digestion of *farnesyl diphosphate synthase* (*FPS*) insert, pCR2.1 containing *FPS* was double digested with *PaeI* and *PstI* (Thermo Fisher Scientific, USA) and incubated for 10 min at 37 °C.
3. The reaction was stopped by adding 1 µl of 10X TE.
4. The mixture was then electrophoresced in 0.8 % agarose gel containing 0.05 µg per ml EtBr to confirm restriction digestion. Subsequently gel purification of the processed insert was done as mentioned in section 3.5.4. The eluted insert was

quantified as mentioned in section 3.5.5 and stored at – 20 °C until further use.

### 3.9.2.3. Ligation of insert in expression vector pQE30

The ligation of the amplified fragment was done using Rapid ligation kit (Thermo Fisher Scientific, USA). The components of the reaction are as follows:

| Reagents | Volume (µl) |
|---|---|
| Nuclease free water | X |
| 5X Rapid ligation buffer | 4.0 |
| Processed vector (50 ng) | Y |
| Processed insert* | Z |
| Rapid ligase (5U/µl) | 1.0 |
| Total | 20.0 |

*Insert:vector = 3:1; X – depends on the value of Y and Z, Y – depends on the concentration of processed vector, Z – Its value is calculated by using the following formula: Z ng of processed insert = (required ratio) x (insert length in bp/ vector length in bp) x vector mass (ng)

The ligation mixture was incubated at 16 °C for 16 h in a circulatory water bath.

### 3.9.2.4. Transformation in M15 [pREP4] cells

#### 3.9.2.4.1. Preparation of M15 competent cells

The chemically competent cells of M15 [pREP4] were prepared as mentioned in Appendix A.

#### 3.9.2.4.2. Transformation and selection

Transformation was done as in section 3.4.6.2 using M15 [pREP4] chemically competent cells. Selection of the transformants was done by spreading 300 µl of culture on LB medium containing 0.1 µg/µl of ampicillin and 0.025 µg/µl of kanamycin. The plate was incubated at 37 °C overnight in an incubator. The transformants, i.e., cells containing the recombinant vectors grow as white colonies whereas non – transformants fail to grow.

#### 3.9.2.4.3. Confirmation of insert through colony PCR and sequencing

The successful integration of insert into the expression vector pQE30 was confirmed through colony PCR and sequencing. Colony PCR was done using vector specific primers. The reaction set up was done as mentioned under section 3.4.6.4. The plasmids isolated from the positive clones were sequenced to confirm the in – frame cloning of the desired insert.

### 3.9.3. Protein expression

#### 3.9.3.1. Time course analysis of induction of expression of heterologous protein

1. Luria Bertani broth, Miller (10 ml) (Himedia, India) containing 100 µg per ml ampicillin and 25 µg per ml kanamycin in a 50 ml flask was inoculated with a single colony of M15 containing the construct and grown overnight at 37 °C with a shaking at 200 RPM.
2. Next day, 100 ml of pre – warmed Luria Bertani broth, Miller containing 100 µg per ml ampicillin and 25 µg per ml kanamycin was inoculated with 5 ml of overnight culture and grown at 37 °C with a shaking at 200 RPM until the $A_{600}$ of 0.6 was reached.
3. Culture sample (1 ml) was taken immediately before induction and pelleted at 15,000 x g for 1 min. The pellet was resuspended in a 50 µl of 5X sample buffer and frozen at – 20 °C for SDS – PAGE later. This uninduced sample was labelled as $T_0$.
4. The protein expression was induced by adding IPTG to a final concentration of 1 mM.
5. Appropriate volume of culture (cell number should be approximately equal to $T_0$) was collected every hour for 5 hours and procedure mentioned in step 3 was performed. The samples collected after 1 h, 2 h, 3 h, 4 h and 5 h were labelled $T_1$, $T_2$, $T_3$, $T_4$ and $T_5$ respectively.

#### 3.9.3.2. SDS PAGE of heterologously expressed protein

Protein samples in denaturing sample loading buffer were denatured by boiling the solution for 10 min. The samples were kept on ice for 5 min and the crude protein extracts were then separated using 12 % SDS – PAGE at 90 V in a tris – glycine buffer containing 0.1 % SDS in a Bio-RadPROTEAN®II xi cell (Bio-Rad, USA). When the samples reached resolving gel the voltage was increased to 130 V for the rest of the run. The gel was stained using Coomassie Brilliant Blue stain. The molecular weight of the protein of interest was determined using wide range protein marker (Sigma, USA).

## 3.10. Bioinformatics tools used in the current work

The following table lists the bioinformatics tools used for analysis of cDNA libraries, genes and deduced amino acid sequences.

**Table 3.3** Bioinformatics tools used

| Application/task | Bioinformatics tool | Website/reference |
|---|---|---|
| Primer designing | Primer 3, OligoCalc | http://bioinfo.ut.ee/primer3-0.4.0/; http://biotools.nubic.northwestern.edu/OligoCalc (Untergasser et al., 2012) |
| Homology search of nucleotide and deduced amino acid sequences | BLASTN, BLASTX | https://blast.ncbi.nlm.nih.gov/Blast.cgi (Altschul et al., 1990) |
| Homology search of nucleotide sequences against Sequence Read Archive (SRA) | SRA-BLAST | https://www.ncbi.nlm.nih.gov/sra/ (Leinonen et al., 2011) |
| Finding reverse complement of nucleotide sequences | Reverse complement | https://www.bioinformatics.org/sms/rev_comp.html |
| Pairwise and multiple sequence alignment | Clustal W | http://www.genome.jp/tools/clustalw/ (Thompson et al., 1994) |
| Global alignment of two protein sequences to calculate percentage | EMBOSS pairwise alignment algorithm | www.ebi.ac.uk/tools/psa/emboss (Rice et al., 2000) |
| Finding open reading frame/coding sequence | ORF finder | https://www.ncbi.nlm.nih.gov/orffinder/ (Wheeler et al., 2003) |
| *In silico* prediction of amino acid sequences | ExPASy proteomics tool | (www.expasy.org) |
| Secondary structure prediction | SOPMA | www.expasy.org (Geourjon and Deleage 1995) |
| Hydropathicity | Protein plot analysis tool | (www.justbio.com) (Kyte and Doolittle, 1982) |
| Detection of conserved domains | ncbi conserved domain search service | (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer and Bryant, 2004) |
| Presence/absence of transmembrane regions | TMHMM Server v. 2.0 | (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001) |
| Prediction of molecular mass and isoelectric point of deduced polypeptide | ExPASy proteomics tool | (www.expasy.org) |
| Detection of putative poly (A) signal | DNA functional site (DNAFS) miner | (http://dnafsminer.bic.nus.edu.sg) (Liu et al., 2005) |
| Detection of vector contamination in ESTs | VecScreen | https://www.ncbi.nlm.nih.gov/tools/vecscreen |
| Sequence assembly of SSH ESTs | EGassembler | http://www.genome.jp/tools/egassembler/ (Masoudi-Nejad et al., 2006) |
| Functional annotation of SSH ESTs | Blast2go | https://www.blast2go.com (Conesa et al., 2005) |