ABSTRACT

Medicinal plants are in great demand to extract various medicinally important compounds for human welfare. Herbal medicines are gaining popularity in day-to-day life because they are easily available and have little or no side effects. Terpenoids constitute the largest family of natural products and have a wide range of pharmacological applications. *Bacopa monniera*, has been used for centuries in Ayurvedic system of medicine as brain tonic, memory enhancer, anti-anxiety, cardiotonic, anticancer, anti-inflammatory, analgesic and anticonvulsant agent. These pharmacological properties are mainly attributed to the triterpenoid saponins present in the extract of the plant. Triterpenoid saponins are synthesized starting from isoprenoid pathway through farnesyl pyrophosphate by cyclization of 2, 3-oxidosqualene, leading to formation of triterpenoid skeletons.

The plant produces relatively small amount of bacosides and large biomass have been used to overcome the shortage to pharmaceutical industry. It may lead to *Bacopa* plant in the category of endangered species. In order to meet the challenge, the present study is focused on metabolic engineering of terpenoid biosynthetic pathway and development of efficient multiple shoots regeneration system for *Bacopa*, which can be used for plant transformation and micro propagation at large scale.

As a first step for pathway engineering, the preliminary part of study involved isolation, cloning and characterization of different terpenoid biosynthetic pathway genes including 3-hydroxy 3-methylglutaryl-coenzyme A reductase (HMGR), farnesyl pyrophosphate synthase (FPS) and squalene synthase (SQS) from *B. monniera*. PCR based approach was used for isolation of genes. The full-length cDNA encoding HMGR, FPS and SQS were isolated by rapid amplification of cDNA ends (RACE) from *Bacopa* and designated as BmHMGR, BmFPS and BmSQS respectively. The full-length cDNAs of BmHMGR was 2143 bp containing 1770 bp open reading frame (ORF) including stop codon (132 bp 5’ UTR, 224 bp 3’ UTR and remaining poly ‘A’ tail), encoding a polypeptide of 589 amino acids. The calculated molecular mass and predicted pl values were 62.63 kDa and 8.33 respectively. After sequence analysis of 6-8 clones, two types of HMGRs were identified designated as BmHMGR1 (Accession no. HM222606) and BmHMGR6 (Accession no. HM222607) sharing 98.1% identities with each other. BmFPS full-length cDNA was 1279 bp (Accession no. GU385740)
containing 1050 bp ORF (09 bp 5’ UTR, 204 bp 3’ UTR and remaining poly ‘A’ tail) encoding polypeptide of 349 amino acids with calculated molecular mass of 40.26 kDa and pI value of 5.57. Full-length cDNA of BmSQS was 1586 bp containing 1245 bp ORF (62 bp 5’ UTR, 263 bp 3’ UTR and remaining poly ‘A’ tail) and encoding for 47.37 kDa polypeptide of 414 amino acid residues. Phylogenetic analyses showed isolated genes were closely related with dicot plant species, clustered with *Salvia miltiorrhiza*.

The second major part of the study was heterologous expression, purification and characterization of protein and differential expression study of genes in normal as well as in presence of different stressors. All three genes were cloned in expression vector (pET 30b+) and overexpressed in *E. coli* (BL21). BmFPS and BmSQS were purified by affinity chromatography and polyclonal antibodies were raised against recombinant proteins, which have been used for western blot analysis and ELISA. Comparative modeling and docking studies of BmHMGR, BmFPS and BmSQS were done to predict 3D structures and interactions with ligands. The overall study showed that generated models are reliable and showed interaction and specificity with ligands on the basis of binding energy. qRT-PCR analysis showed that all three genes are differentially expressed in all tissue including stem, leaf root and floral parts. Relatively higher expression of BmHMGR was in roots, whereas BmFPS and BmSQS accumulate more in leaves and petals respectively. The transcript accumulation of BmFPS and BmSQS in stem, leaf and root tissues were coherent with protein expression, confirmed by ELISA. The differential expression of genes was investigated under normal and stress conditions (Methyl jasmonate, salt, cold, salicylic acid, chlorogenic acid and yeast extract). qRT-PCR data showed more or less higher transcript accumulation of all three genes and methyl jasmonate was found to induce transcripts of genes at greater extent. In case of salt stress, BmHMGR mRNA expression in roots declined to large extent.

The final part of the study covered cloning of all three genes in sense orientation in plant transformation binary vector pCAMBIA1301, multiple shoots regeneration of *B. monniera*, plant transformation and analysis of transgenic. Leaf explants with injured lamina was used for regeneration. Out of various combinations of growth regulators, MS basal medium with 2 mg/L 6-benzylaminopurine (BAP) and 0.2 mg/L
indole-3-acetic acid (IAA) was found to yield maximum frequency of shoot regeneration (98.33%). Transformation efficiency was 80-81.48 %, accounted on the basis of PCR with hptII gene specific primers. qRT-PCR analysis showed 2- 4 fold, 3.7- 5.4 fold and 1.4- 4.08 fold higher mRNA expression in transgenic lines over expressing BmHMGR, BmFPS and BmSQS respectively as compared to control non transformed plants. HMGR-transgenic plants exhibited increased transcript accumulation of other downstream genes FPS, SQS and β-amyрин synthase (BAS) which were up to 39, 92 and 16 fold higher respectively as compared to control. FPS and SQS-transgenic plants exhibited slight increase in HMGR transcript, whereas FPS-transgenic plants showed high transcript accumulation of SQS (up to 38 fold) and BAS (up to 18 fold) as compared to control. SQS-transgenic showed very high transcript accumulation (up to 94 fold) of BAS. HPLC analysis of BmFPS transgenic plants showed 2.7– 6.2 fold bacopasaponin C and 3.7– 5.9 fold higher bacoside A as compared to control plants.

This study is the first time report on genetic manipulation of *B. monniera* and the overall results indicate that this study will definitely help in understanding the bacoside biosynthetic pathway and involvement of genes and finally developing designer plants to improve bacoside yield.