"Isolation, Cloning and Characterization of Terpenoids Biosynthesis Pathway gene(s) from *Bacopa monniera*"

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SUBMITTED TO THE
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FOR THE DEGREE OF
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IN
BIOTECHNOLOGY

BY
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UNDER THE GUIDANCE OF
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INDIA
MAY, 2012

Dedicated to my Family

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Isolation, Cloning and Characterization of Terpenoids Biosynthesis Pathway gene(s) from *Bacopa monniera*"** submitted by **Rishi Kishore Vishwakarma** for the degree of Doctor of Philosophy, was carried out under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. B. M. Khan
(Research guide)

DECLARATION

I hereby declare that the thesis entitled **"Isolation, Cloning and Characterization of Terpenoids Biosynthesis Pathway gene(s) from *Bacopa monniera*"** has been carried out at Plant Tissue culture Division, National Chemical Laboratory, Pune, under the guidance of **Dr. Bashir M. Khan**. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other university. I further declare that the materials obtained from other sources have been duly acknowledged in the thesis.

(Rishi Kishore Vishwakarma)

Date: May, 2012

Place: Plant Tissue Culture Division,

National Chemical Laboratory (NCL),

Pune – 411008, India.

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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-oxidosquale, 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 pI 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 *hpt*II 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-amyrin 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.

ABBREVIATIONS

AA	Amino acid
AMV-RT	Avian Myeloblastosis Virus-Reverse Transcriptase
BAP	6-Benzylaminopurine
BM	*Bacopa monniera*
BSA	Bovine serum albumin
cDNA	Complementary DNA
DEPC	Diethylpyrocarbonate
DTT	Dithiothritol
EDTA	Ethylene diamine tetra acetic acid disodium salt
ELISA	Enzyme linked immuno sorbent assay
*hpt*II	Hygromycin phosphotransferase II
IAA	Indole-3-acetic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LD_{50}	Lethal dose 50%
MEP Pathway	2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway
MS	Murashige and Skoog
MVA pathway	Mevalonate pathway
OD	Optical density
ORF	Open Reading Frame
pI	Isoelectric point
PMSF	Phenyl methyl sulphonyl fluoride
RACE	Rapid amplification of cDNA ends
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMQ	Sterile Milli Q
SOD	Superoxide dismutase
TAE	Tris acetic EDTA buffer
TEMED	Tetramethylethylenediamine
UTR	Untranslated Region
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indolylß-D-galactoside

CHAPTER: 1

INTRODUCTION

Chapter 1. Introduction

1.1 Primary and secondary metabolites and its biosynthesis in plants

Plants are a great source of secondary metabolites used as pharmaceuticals. The primary metabolites like sugar, fatty acids and amino acids are needed for normal growth and development of plants and also utilized as food by animals. The secondary metabolites such as alkaloids, flavonoids, terpenoids, glycosides etc. are biosynthetically derived from primary metabolites and represents chemical adaptations in response to environmental stresses, microorganisms, insects and higher herbivorous predators. They are sometimes considered as waste or secretory products of plant metabolism and have pharmaceutical importance.

The biosynthesis of primary metabolites in plants starts with photosynthesis to form carbohydrates and secondary metabolites are ultimately derived from carbohydrate metabolism. The primary and secondary metabolites derived from carbon metabolism are shown in **Fig. 1**. The interrelationship of biosynthetic pathways leading to formation of secondary metabolites is shown in **Fig. 2**. The major secondary metabolite produced by plants can be divided into three main groups: phenolic compounds; terpenoids/ isoprenoids; nitrogen or sulfur containing compounds such as the alkaloids and glucosinolates, respectively. The three major classes of secondary metabolites are produced from pathways of different primary metabolites, including glycolysis, the TCA cycle, aliphatic amino acids, pentose phosphate pathway, shikimate pathway and notably the aromatic amino acids (Aharoni and Galili, 2011). Mankind has been using secondary metabolites for multiple purposes, such as dyes, flavors, fragrances, stimulants, hallucigenes, insecticides, vertebrate and human poisons as well as therapeutic agents. The interest in secondary metabolites has increased in recent years since many investigations with respect to human nutrition pointed out that modest, long-term intake of certain metabolites will have a major impact on preventing incidences of cancers and many chronic diseases (Enfissi et al., 2010). The tight link between metabolic fluxes of primary metabolites and the accumulation of secondary metabolites renders the engineering of the latter compounds quite complex as it demands the consideration of the entire metabolic network in order to redirect primary

metabolites resources into secondary metabolites without interfering with plant fitness (Aharoni and Galili, 2011).

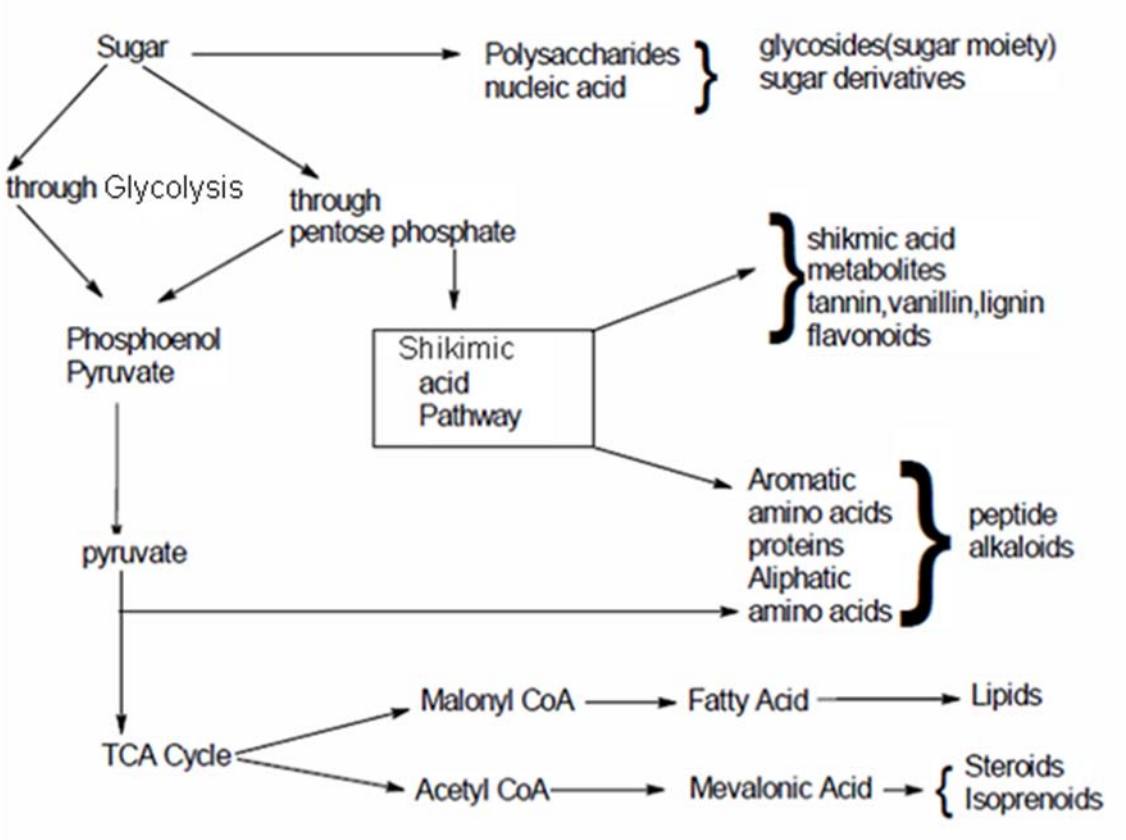


Fig. 1: Primary and secondary metabolites derived from carbon metabolism

Major plant secondary metabolites are synthesized via isoprenoid pathway, phenylpropanoid pathway and shikimic acid pathway and on the basis of biosynthetic pathway three large secondary metabolite families are generally considered: phenolics, terpenes and steroids, and alkaloids. These secondary metabolites are useful to protect plants against herbivores (insects and vertebrates), mammals, bacteria, fungi, viruses and even other competing plants. In addition, some plants use secondary metabolites to attract pollinators and seed dispersers, as signals for communication between plants and symbiotic microorganisms or for protection against UV light and other physical stress (Wink, 2003; 2008).

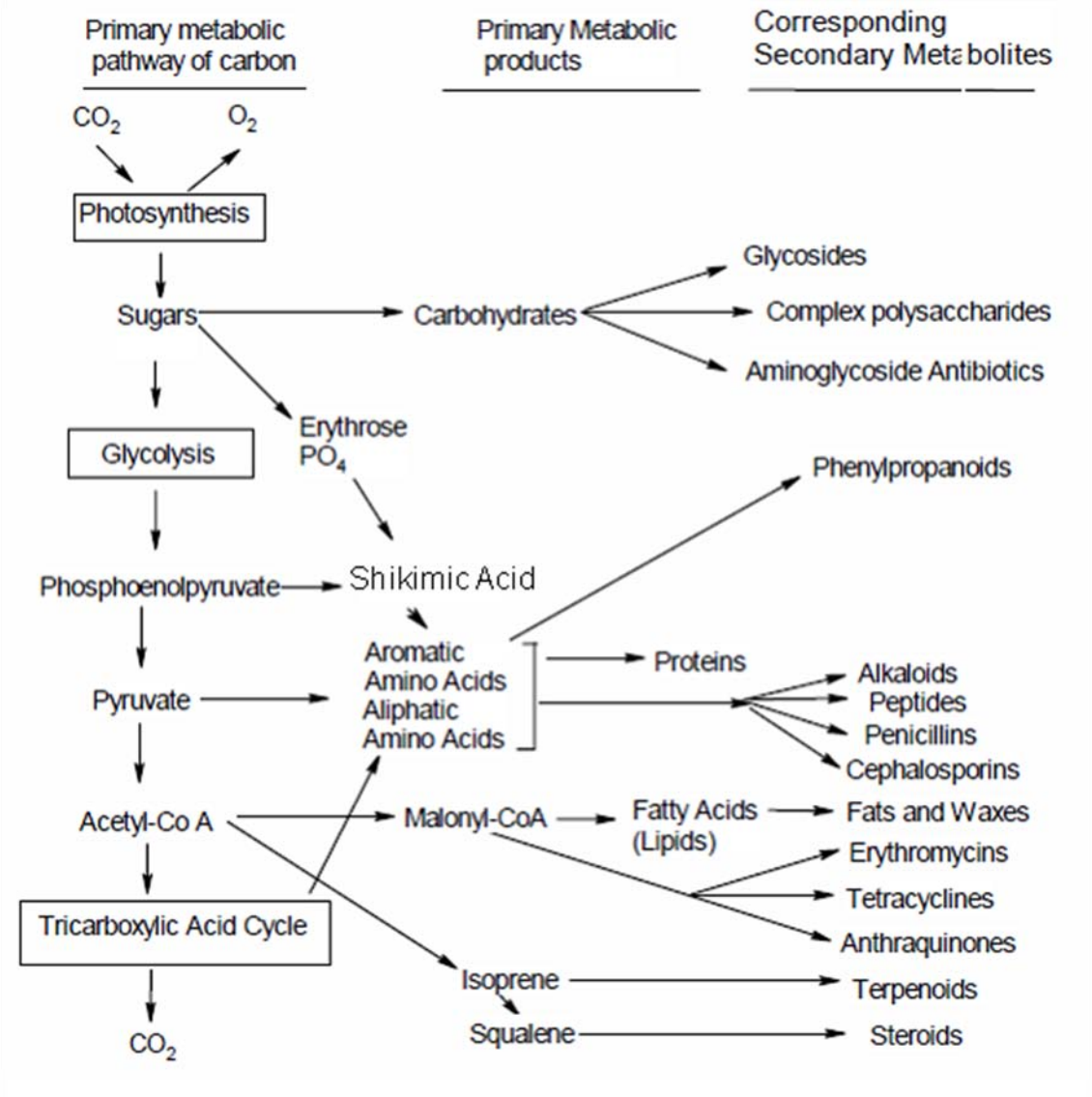


Fig. 2: Interrelationship of biosynthetic pathway leading to secondary constituents in plants

Nature and its huge biodiversity harbour an endless source of compounds containing unique chemical structures. In pharmaceutical industry, more than 25% molecules used are of natural plant origin (Payne et al., 1991). The genuine molecule can be isolated in large quantities from many plants, but the chemical is synthesized as an acetyl-derivative in order to lower secondary effects. Chemical synthesis apart, the production of plant secondary metabolites has, for a long time, been achieved through the field cultivation of medicinal plants. However, plants originating from particular biotopes can be hard to grow outside their local ecosystems. It also happens that common plants do not withstand large field cultures due to pathogen sensitiveness and

environmental conditions. This has led scientists and biotechnologists to consider plant cell, tissue and organ cultures as an alternative way to produce the corresponding secondary metabolites.

1.2 Medicinal plants

Plants synthesize and conserve a variety of biochemical products, many of which are extractable and used as raw material for various scientific investigations. Many secondary metabolites from plant are commercially important and are used as pharmaceutical compounds. Over the past few years, the medicinal plants have regained a wide recognition due to a growing faith in herbal medicine in view of its lesser side effects compared to allopathic medicine and the necessity of meeting the requirements of medicine for an increasing human population. However, a continuous supply of the source material often becomes difficult due to the factors like environmental changes, cultural practices, diverse geographical distribution, labor cost, and selection of the superior plant stock and over exploitation by pharmaceutical industry (Joy et al., 2001).

It has been estimated that in developed countries such as United States, plant drugs constitute as much as 25% of the total drugs, while in fast developing countries such as China and India, the contribution is as much as 80%. Thus, the economic importance of medicinal plants is much more in countries such as India than to rest of the world. These countries provide two third of the plants used in modern system of medicine and the health care system of rural population depend on indigenous systems of medicine (Joy et al., 2001). In India, of the 17,000 species of higher plants, 7500 are known for medicinal uses (Shiva, 1996). This proportion of medicinal plants is the highest proportion of plants known for their medical purposes in any country of the world for the existing flora of that respective country. Ayurveda, the oldest medical system in Indian sub-continent, has alone reported approximately 2000 medicinal plant species, followed by Siddha and Unani. The Charak Samhita, an age-old written document on herbal therapy, reports on the production of 340 herbal drugs and their indigenous uses. Most of these continue to be gathered from wild plants to meet the demand of the medical profession. Thus, despite the rich heritage of knowledge on the use of plant drugs, little attention had been paid to grow them as field crop in the

country till the latter part of the nineteenth century. Approximately 25% of drugs are derived from plants, and many others are synthetic analogues built on prototype compounds isolated from plant species in modern pharmacopoeia (Rao et al., 2004).

The northern part of India harbors a great diversity of medicinal plants because of the majestic Himalayan range. About 8000 species of angiosperms, 44 species of gymnosperms and 600 species of pteridophytes have been reported in the Indian Himalaya (Singh and Hajra, 1996); of these 1748 species are known as medicinal plants (Samant et al., 1998). The maximum medicinal plants (1717 species) have been reported around the 1800 m elevation range. On the regional scale, the maximum species of medicinal plants have been reported from Uttaranchal (Kala, 2002), followed by Sikkim and North Bengal (Samant et al., 1998). The trans-Himalaya sustains about 337 species of medicinal plants (Kala, 2002), which is low compared to other areas of the Himalaya due to the distinct geography and ecological marginal conditions (Kala, 2002).

The remedial properties of drugs are due to the presence of complex chemical substances of varied composition (present as secondary plant metabolites) in one or more parts of these plants. These plant metabolites according to their composition are grouped as alkaloids, glycosides, corticosteroids, essential oils, etc. The alkaloids form the largest group, which includes morphine and codein (Poppy), strychnine and brucine (nux vomica), quinine (cinchona), ergotamine (ergot), hypocampus (beeladona), scopolamine (datura), emetine (ipecac), cocaine (coco), ephedrine (ephedra), reserpine (*Rauwolfia*), caffeine (tea dust), aconitine (aconite), vascine (vasaca), santonin (*Aremisia*), lobelin (*Lobelia*) and a large number of others. Glycosides form another important group represented by digoxin (foxglove), stropanthin (*Strophanthus*), glycyrrhizin (liquorice), barbolin (aloe), sennocide (senna), etc. Corticosteroids have come into prominence recently and diosgenin (*Dioscorea*), solasodin (*Solanum* sp.), etc. now command a large world demand. Some essential oils such as those of valerian kutch and peppermint also possess medicating properties and are used in pharmaceutical industry. However, it should be stated in all fairness that our knowledge of the genetic and physiological make-up of most of the medicinal plants is poor and

we know still less about the biosynthetic pathways leading to the formation of active constituents for which these crops are valued.

1.3 *Bacopa monniera*

The plant under study was *Bacopa monniera* (commonly known as Bramhi). Bramhi is widely used in ayurvedic medicines since long time as a powerful brain tonic. The compounds responsible for the memory enhancing effects of *Bacopa monnieri* are triterpenoid saponins called as Bacosides.

1.3.1 Botanical description

Bacopa monniera, a member of the Scrophulariaceae family (figwort or snapdragon family) is a small herb with light purple flowers. It grows in wet and sandy areas. The plant is prostrate or creeping, tender, annual herb, rooting at the nodes with numerous ascending branches (**Fig. 3**). Leaves are simple, opposite, sessile, entire, fleshy, and obscurely veined. Flowers are pale blue to whitish, axillary, solitary, on long slender pedicles; fruits are ovoid, acute, 2-celled, 2- valved capsules, tipped with style base; seeds minute, numerous. Flowers and fruits appear in summer. Dispersal and propagation is by seeds and stem fragments. Crushed leaves have a distinctive 'lemon' scent. These plants grow in grasslands occurring in aquatic sites, sand and wet soil occupying in the edges of freshwater or brackish pools and streams and lake beds. The plant is distributed in the major part of the plains of India, Pakistan, Afghanistan, Nepal, Sri Lanka, Africa and Australia. *B. monniera* is commercially cultivated for medicinal purpose and the annual production is around 40,000–50,000 kg per hectare (Russo and Borrelli, 2005). The whole plant is medicinally useful (Bone, 1996).

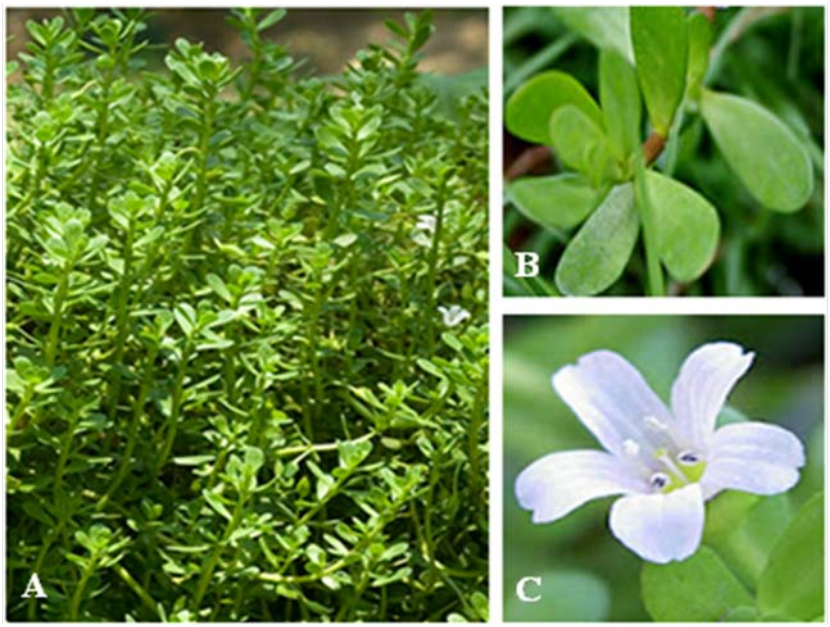


Fig. 3: A. Complete *Bacopa* plant **B.** Leaves **C.** Flower

1.3.2 Classification

- Kingdom: Plantae
- Phylum: Magnoliophyta
- Class: Magnoliopsida
- Order: Scrophulariales
- Family: Scrophulariaceae
- Genus: *Bacopa*
- Species: *monniera*

The herb has been described in Ayurvedic texts since around 800 BC and recorded as a treatment for the range of mental disorders in the 'Charak Samhita' (Singh and Dhawan, 1997). In a priority list of the most important medicinal plants, evaluated on the basis of their medicinal importance, commercial value and potential for further research and development, *B. monniera* was placed second according to a sector study by the Export- Import Bank of India. According to an estimate, the annual requirement of the plant was projected to be about 12,700 tonnes of dry material, valued at approximately Rs 15 billion.

1.3.3 Uses of *B. monniera*

B. monniera often called "Brahmi"or "The thinking person's herb," is widely used in traditional Indian Ayurvedic System of medicine as a potent nervine tonic to enhance memory function and improve intellectual and cognitive functions (Rastogi et al., 1994). It is also used in treatment of asthama, leprosy, hoarseness, renal disease, water retention and blood cleaning (Singh and Dhawan, 1982).

B. monniera has been used in Ayurvedic formulations for conditions ranging from catarrhal complaints, gastrointestinal disturbances due to excessive tobacco use, habitual abortions and high blood sugar due to anxiety disorders, hysteria, epilepsy etc. (Chopra et al., 1956; Nadkarni, 1976). In certain parts of India, Brahmi is believed to be an aphrodisiac. In Srilanka, under the name of Loonooweela, Brahmi is prescribed for fevers. In the Phillipines, it is used as a diuretic (Uphof, 1968). It has a bitter taste. Traditionally, the fleshy leaves and stems were made into paste or pressed for juice extraction.

In recent years, "Memory Plus", a product that contains the standardized extract of bacosides from brahmi, has been marketed in India. Under the definition of herbal drugs in the guidelines of herbal medicines issued by the World Health Organization (WHO) in 1991, an herbal product that has been used traditionally without demonstrable harm does not require specific regulatory action unless new evidence demands a revised risk/ benefit analysis. Subsequently, several formulations containing *B. monniera* extracts standardized for bacoside content have appeared in the global market place.

The evaluation of BacoMind^{TM} in healthy adult volunteers revealed that at the given oral dose of 300 mg once a day for first 15 days and 450 mg once a day for next 15 days, the herbal supplement was found to be safe and tolerable. Though minor gastrointestinal side effects were reported in 3 out of 23 volunteers, the general physical, systemic, hematological, biochemical and electrocardiographic parameters were within the normal limits (Pravina et al., 2007). It was also reported that BacoMind^{TM} can be used as a natural dietary supplement for supporting optimal health and mental functioning and is safe for oral consumption. It also has the capacity to be used as an anti-oxidant and an anti-carcinogenic agent particularly against those

carcinogens that mediate their effects via formation of oxygen free radicals (Deb et al., 2008).

Therefore *B. monniera*, ayurvedic medicines have been used for traditional treatment of a number of disorders, particularly those involving anxiety, intellect and poor memory (Singh and Dhawan, 1997), epilepsy, insanity and retardation (Mathura et al., 2002), to counteract the effects of mental stress and neurosis and revitalization of sensory organs (Sivarajan and Balachandran, 1994). The well-known nootropic plant has been reported to possess sedative (Malhotra and Das, 1959), cardiotonic (Mathura et al., 2002), cognitive enhancer (Nathan et al., 2001; Roodenrys et al., 2002), broncho-vasodilator (Channa et al., 2003), hepatoprotective (Sumathy and Nongbri, 2008; Sumathy et al., 2001), antidepressant (Sairam et al., 2002), calcium antagonistic (Dar and Channa, 1999), smooth muscle relaxant (Dar and Channa, 1997), neuropharmacological (Russo and Borrelli, 2005) cell stabilizing (Samiulla et al., 2001) and antiulcer (Sairam et al., 2001) properties. The said plant also showed antistress effect, via modulation of Hsp 70 expression, superoxide dismutase and cytochrome p450 inhibitory activity in rat brain (Chowdhuri et al., 2002) and antioxidant activity (Tripathi et al., 1996). *B. monniera* is reported to play a protective role on morphine-induced brain mitochondrial enzyme status in rats (Sumathy et al., 2002). It is also active against leishmaniasis (Sinha et al., 2002).

Anbarasi et al. (2005a; 2005b) have reported the protective effects of bacoside A on cigarette smoking-induced brain damage. The role of bacoside A against chronic cigarette smoking-induced hsp70 expression and apoptosis in rat brain cerebral cortex. Apoptosis was monitored by DNA fragmentation and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining, and further confirmed by electron microscopy (Anbarasi et al., 2006).

The studies have shown that it improves the rate of learning in a brightness discrimination task and a conditional avoidance task, that it improves retention, as demonstrated by savings in relearning, and that it attenuates amnesia induced by immobilization, electroconvulsive shock and scopolamine (Singh and Dhawan, 1997). This latter finding involved administration of the extracted bacosides A and B, and suggests that they influence cholinergic systems.

Recently, the study have shown that, the stem extract of *Bacopa monniera* was corrosion inhibitor of aluminium in 0.5 M NaOH solution and weight loss measurements. The results revealed that *Bacopa* stem extract was an effective inhibitor, and the inhibition efficiencies obtained from polarization and weight loss experiments were in good agreement (Singh et al., 2012).

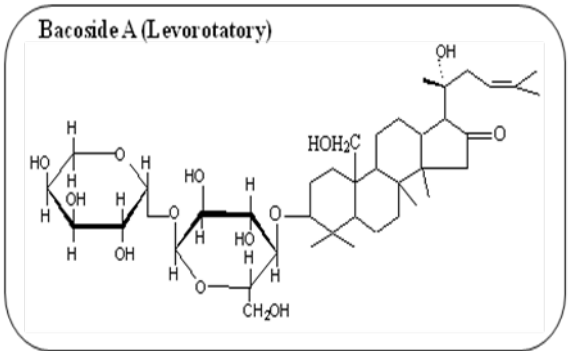
1.3.4 Medicinally active constituents

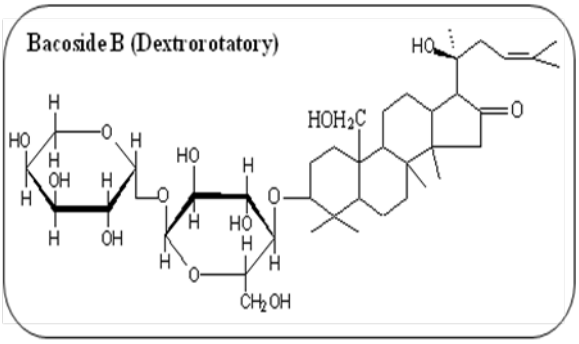
In view of the importance of this plant in the indigenous system of medicine, systematic chemical examinations of the plant have been carried out by several laboratories. Detailed investigations were first documented in 1931, when Bose and Bose reported the isolation of the alkaloid ‘‘brahmine’’ from *Bacopa monniera*. Later, other alkaloids like nicotine and herpestine have also been reported (Chopra et al., 1956). Several glycosides such as asiaticoside and thanakunicide, flavonoids such as apigenin and luteonin, phytochemicals such as betulinic acid, wogonin, oroxindin, betulic acid, stigmastarol, beta-sitosterol, as well as brahamoside, brahminoside, brahmic acid, isobrahmic acid, vallerine, pectic acid, fatty acids, tannin, volatile oil, ascorbic acid, thanakunic acid and asiatic acid (Mathew et al., 2010) have also been reported. The isolation of D-mannitol, and a saponin, hersaponin and potassium salts by (Sastri et al., 1959) provided further details of the chemical components of *B. monniera*.

The major chemical entity shown to be responsible for the memory-facilitating action of Bacopa monniera, bacoside A, was assigned as 3-(α-L-arabinopyranosyl)-O-β-D-glucopyranoside-10, 20-dihydroxy-16-keto-dammar-24-ene (Chatterji et al., 1965). Bacoside A usually co-occurs with bacoside B, the latter differing only in optical rotation and probably an artifact produced during the process of isolating bacoside A (Rastogi, 1990) as shown in **Fig. 4**. The chemical composition of bacosides, contained in the polar fraction, has been established on the basis of chemical and physical degradation studies. On acid hydrolysis, bacosides yield a mixture of aglycones, bacogenin A1, A2, A3 (Kulshreshtha and Rastogi, 1973), which are artifacts, and two genuine sapogenins, jujubogenin and pseudojujubogenin (Rastogi et al., 1994).

Another bacogenin, A4 was identified as ebelin lactone pseudojujubogenin (Rastogi et al., 1994). Successively, a minor saponin bacoside A1 was isolated and characterized as 3-O-[α-L-arabinofuranoyl(1-3)-β-L-arabinopyranosyl] jujubogenin (Garai et al., 1996a; Rastogi et al., 1994). Garai et al. (1996b) isolated from *B. monniera* three new dammarane-type triterpenoid saponins of biological interest, bacopasaponins A, B and C, identified as 3-O-α-L-arabinopyranosyl-20-O-α-L-arabinopyranosyl-jujubogenin, 3-O-[α-L-arabinofuranosyl(1-2)α-L-arabinopyranosyl] pseudojujubogenin and 3-O-[β-D-glucopyranosyl(1-3) {α-L-arabinofuranosyl(1-2)}α-Larabinopyranosyl] pseudojujubogenin by spectroscopic and chemical transformation methods. Successively, the same authors (Garai et al., 1996a) isolated a new dammarane-type pseudojujubogenin glycoside, bacopasaponin D, defined as 3-O-[α-L-arabinofuranosyl(1-2)β-D glucopyranosyl] pseudojujubogenin by spectroscopic and chemical transformation methods. It is noteworthy that dammarane-type triterpenoid saponins are major constituents of a number of reputed herbal drugs, including ginseng. Although jujubogenin glycosides have been isolated from several reputed medicinal plants (i.e. Rhamnaceae and Scrophulariaceae), pseudojubogenin glycosides have only been reported in this Indian herbal drug.

In addition, Hou et al. (2002) have isolated a new saponin, 3-O-[α-1-arabinofuranosyl-(1-2)]-α-L-arabinopyranosyl jujubogenin, named bacopasaponin G, a new matsutaka alcohol derivative, (3R)-1-octan-3yl-(6-O-sulfonyl)-β-D-glucopyranoside, a new phenylethanoid glycoside,3,4-dihydroxyphenylethyl alcohol (2-O-feruloyl)-β-D-glucopyranoside, and a new glycoside, phenylethyl alcohol [5-O-p-hydroxybenzoyl-β-D-apiofuranosyl-(1-2)]-β-D-glucopyranoside (Hou et al., 2002). Two new constituents from *Bacopa,* bacopaside N1 and bacopaside N2 were also reported (Sivaramakrishna et al., 2005).





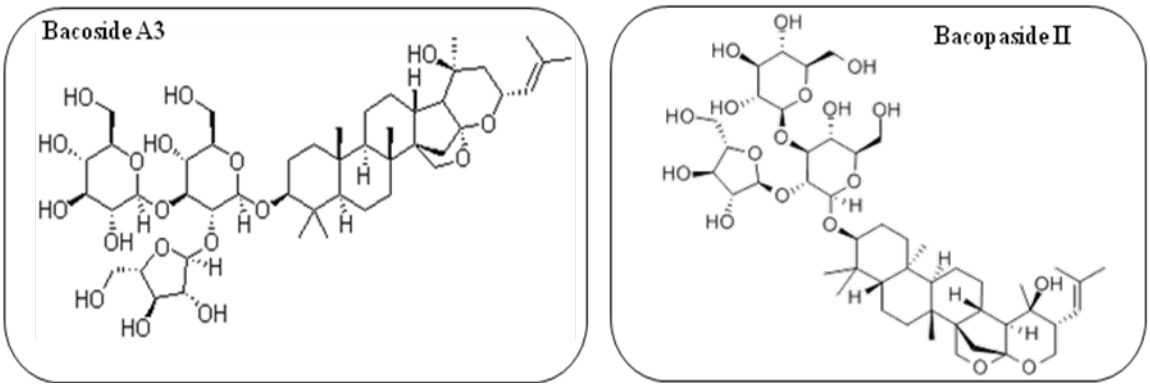


Fig. 4: Chemical structure of different bacosides from *B. monniera*

1.3.5 Mechanism of action

The therapeutic potential of brahmi in treatment or prevention of neurological diseases is due to its ability to reduce NO-induced cellular alterations (Russo et al., 2003a). It was also reported that brahmi extract rich in saponins, is able to induce a dose-related increase in superoxide dismutase (SOD), catalase (CAT) and glutathione peroxide (GSH-Px) activities in rat frontal cortex, striatum and hippocampus (Bhattacharya et al., 2000).

The triterpenoid saponins and their bacosides are responsible for *Bacopa's* ability to enhance nerve impulse transmission. The bacosides aid in repair of damaged neurons by enhancing kinase activity, neuronal synthesis, and restoration of synaptic activity, and ultimately nerve impulse transmission and boosting the synthesis of new proteins in the brain (Singh and Dhawan, 1997).

Animal research has shown that *Bacopa* extracts modulate the expression of certain enzymes involved in generation and scavenging of reactive oxygen species in the brain (Chowdhuri et al., 2002). *In vitro* research has also shown that *Bacopa* exerts a protective effect against DNA damage in astrocytes and human fibroblasts (Russo et al., 2003a; Russo et al., 2003b). *Bacopa* has a relaxant effect on pulmonary arteries, aorta, trachea, and ileal and bronchial tissue, possibly mediated by inhibition of calcium- ion influx into cell membranes (Channa et al., 2003). *Bacopa* appears to stabilize mast cells *in vitro*, (Samiulla et al., 2001) and possesses anti-inflammatory activity via inhibition of prostaglandin synthesis and lysosomal membrane stabilization

(Jain et al., 1994). *In vitro* research suggests an anticancer effect for *Bacopa* extracts, possibly due to inhibition of DNA replication in cancer cell lines (Elangovan et al., 1995).

In a recent study, the neuroprotective role of *Bacopa* extract was investigated in hippocampus of temporal lobe of epileptic rat. The study concluded that the extract potentiate the therapeutic effect by reversing the alterations in glutamate receptor binding and NMDA R1 gene expression that occur during epilepsy (Paulose et al., 2008).

1.3.6 Clinical highlights

Bacopa monniera has been studied clinically for its effect on various diseases and disorders. Some clinical studies are summarized in **Table 1**.

Table1: Some clinical studies of *B. monniera*

Disease/ disorders	**Treatment/dosages**	**Effects**	**References**
Cognitive effect	300 mg/day (standardized to 55% combined bacoside A & B)	No significant effect on various measures of memory performance found with acute administration of *Bacopa*.	(Nathan et al., 2001)
Anxiety and depression	12 g/ day	Decreased anxiety, Improvement in concentration and memory span, No side effects.	(Singh and Singh, 1980)
Epilepsy	High dose (close to 50% of LD50)	Anticonvulsant activity was observed	(Martis et al., 1992)
Bronchitis and Asthma	0.1-0.7 mg/mL ethanolic extract	Vasorelaxant action, inhibited hypotension and bradycardia.	(Dar and Channa, 1997)
Gastrointesti -nal disorder	5 g/ Day	Direct spasmolytic activity on intestinal smooth muscle via inhibition of calcium influx across cell membrane channels.	(Dar and Channa, 1999)
Cardiovascu -lar effects	100 mg/mL	Vasodilatory effect on calcium chloride induced contraction of tissues.	(Rashid et al., 1990)
Hypothyroid -ism	High dose (200-400 mg/ day)	Stimulate synthesis or release of T4 not T3	(Kar et al., 2002)

Cancer		Cytotoxic activity for sarcoma-180 cells	(Elangovan et al., 1995)
Drug toxicity (morphine)	1 mg	Significantly reduced the naloxone-induced withdrawal effect	(Sumathi et al., 2002)

1.3.7 Biotechnology and tissue culture studies on *B. monniera*

In an effort to study the genetic diversity in the germplasm of *B. monniera*, 24 accessions from different geographical regions of India and one introduction from Malaysia maintained at the Central Institute of Medicinal and Aromatic Plants, Lucknow, India, were analyzed for random amplified polymorphic DNA variation. The similarity between accessions was found to be in the range of 0.8-1.0, which is indicative of a narrow genetic base and a low to medium level of polymorphism. The low level of genetic variation was attributed to the interplay between sexual and vegetative reproduction and similarity of local environments in the habitats of the plant (Darokar et al., 2001).

The plant has very high morphogenic potential, because of which explants from it respond very readily to treatment with plant growth regulators. Several protocols have been reported for the rapid multiplication of shoots and shoot cultures, and for the micropropagation of the plant (Rajni et al., 2004). In most of the tissue culture studies, a very good response was obtained by 6-benzyl amino purine in producing adventitious shoots from different explants (Singh and Dhawan, 1982; 1994) and somatic embryogenesis (Tiwari et al., 1998), which led to the development of successful protocols for the mass propagation of the plant (Tiwari et al., 2001).

As *B. monniera* has a high morphogenic potential, the explants readily regenerated shoot buds in medium containing small amounts of cytokinins like 6-benzyl amino purine or kinetin (Shriavstava and Rajni, 1999). With a high regenerative potential, the stem and leaf explants showed a tendency to regenerate shoots and/ or roots even in media that are known to support callus initiation and growth (e.g., media supplemented with 2,4-dichlorophenoxyacetic acid), while the callusing response of the explants was very poor. The best response for shoot regeneration from both stem and leaf explants was obtained with 2 µM 6-benzyl amino purine in Murashige and Skoog (MS) media gelled with 0.2% Gelrite, where profuse induction of multiple shoot buds

was obtained in 9 days of incubation. Furthermore, after 3 weeks of shoot culture growth, when the shoots were harvested and explants were transferred into fresh medium, it continued to expand exponentially and regenerate new shoot buds. In addition, the leaf explants from shoot cultures proved to be superior explant material compared to the leaf explants from field-grown plants. The shoot cultures performed better when grown on medium gelled with Gelrite (0.2%) rather than with agar (0.7%). The phytochemical profile of the regenerated shoots was found to be similar to that of field-grown plants, as revealed by TLC analysis (Shrivastava and Rajni, 1999).

Recently several efficient regeneration systems for *B. monniera* have been reported. For high efficient shoot regeneration from leaf and internode explants a two stage culture procedure has been developed. In the first medium containing 1.5 mg/L TDZ and 0.5 mg/L NAA, adventitious shoot buds were obtained and then subcultured on second medium supplemented with BAP (Antony Ceasar et al., 2010). Approximately 90% response was recorded in case of leaf explants grown on MS supplemented with BAP (17.8 µM) and IAA (2.28 µM) (Mahender et al., 2012). In another report, explants on MS supplemented with BAP (2.5 mg/L and IAA (0.01 mg/L), maximum no. of shoots/explants was obtained (Jain et al., 2012).

In an interesting study, *B. monniera* was shown to respond to the antibiotic trimethoprim and the fungicide bavistin, with bavistin inducing a good number of shoots from internodal explants (Tiwari et al., 2012). However, the growth of the regenerated shoots was stunted and for further optimum shoot growth it required the supplementation of plant growth regulators. This calls for further exploration of the potential of bavistin in shoot regeneration, especially since the induction response itself was not gradual and was a concentration-dependent response, causing a sudden surge of induction at 300 mg/L in internode and explants (Tiwari et al., 2012).

Cell suspension cultures were established from leaf explants of *in vitro* plants, and two cell lines were identified that showed a 5- to 6-fold increase in fresh and dry weight in 40 days of culture in MS medium supplemented with 1 mg/L α-naphthalene acetic acid and 0.5 mg/L kinetin. In these two cell lines, bacosides A and B were detected from the 10^{th} day of culture, and they progressively accumulated up to the 40^{th} day. Bacoside A content was found to be higher than bacoside B all through the culture

period, with a maximum of 1% dry weight basis (dwb) in 40-day cultures. Bacoside B accumulated up to 0.25- 0.37% dwb in 40-day cultures (Rahman et al., 2002).

Ali and coworkers (Ali et al., 1998) used *B. monniera* grown *in vitro* as a model system for studying the effects of cadmium on plants, towards an effort to establish metal-tolerant plants, since cadmium is a widespread pollutant. It was found that the cultures could become acclimatized to cadmium through gradual exposure to increased concentrations of cadmium (Ali et al., 2000). Cadmium caused a reduction in photosynthetic rate, stomatal conductance, and internal carbon dioxide concentration, and supplementation with zinc improved these parameters under cadmium stress. There was a gradual increase in the accumulation of cadmium with an increase in concentration and duration of treatment, with maximum accumulation in root (Ali et al., 2000). Cadmium was shown to accumulate in the cell wall. Cadmium induced an increase in total protein content in the cultures (up to 50 µM cadmium), and caused the accumulation of proline (Ali et al., 2000; 2001). At higher concentrations (200 µM) cadmium caused oxidative damage with increased lipid peroxidation, decreased chlorophyll and protein content, and a concomitant induction in the activities of SOD, ascorbate peroxidase, and guicol peroxidase, perhaps to combat the oxidative stress caused by cadmium. However, there was significant reduction in catalase activity (Mishra et al., 2007).

In a study of the adaptation of *B. monniera* regenerants to salinity stress *in vitro*, the proline content of the plants was found to increase six-fold compared to the control, while photosynthetic rate, fresh mass, and root length of the regenerates decreased (Ali et al., 2000).

Tetraploid plants were generated by treating nodal explants from *in vitro*-grown plants with colchicine followed by culture in MS medium supplemented with 0.25 mg/L 6-benzyl amino purine. The tetraploid plants had larger leaves and flowers, but its growth was slow. The tetraploid was named as a new variety, Ali INTA-JICA (Escandon et al., 2006).

1.4 Biosynthesis of triterpenoids

Triterpenoids are the isoprenoid compounds, represent highly diverse group of natural products with many functions in plant primary and secondary metabolism. Many isoprenoids have important applications in areas such as human health and nutrition, and much effort has been directed towards their biosynthesis pathway studies and improvement of yield of such compounds through genetic engineering.

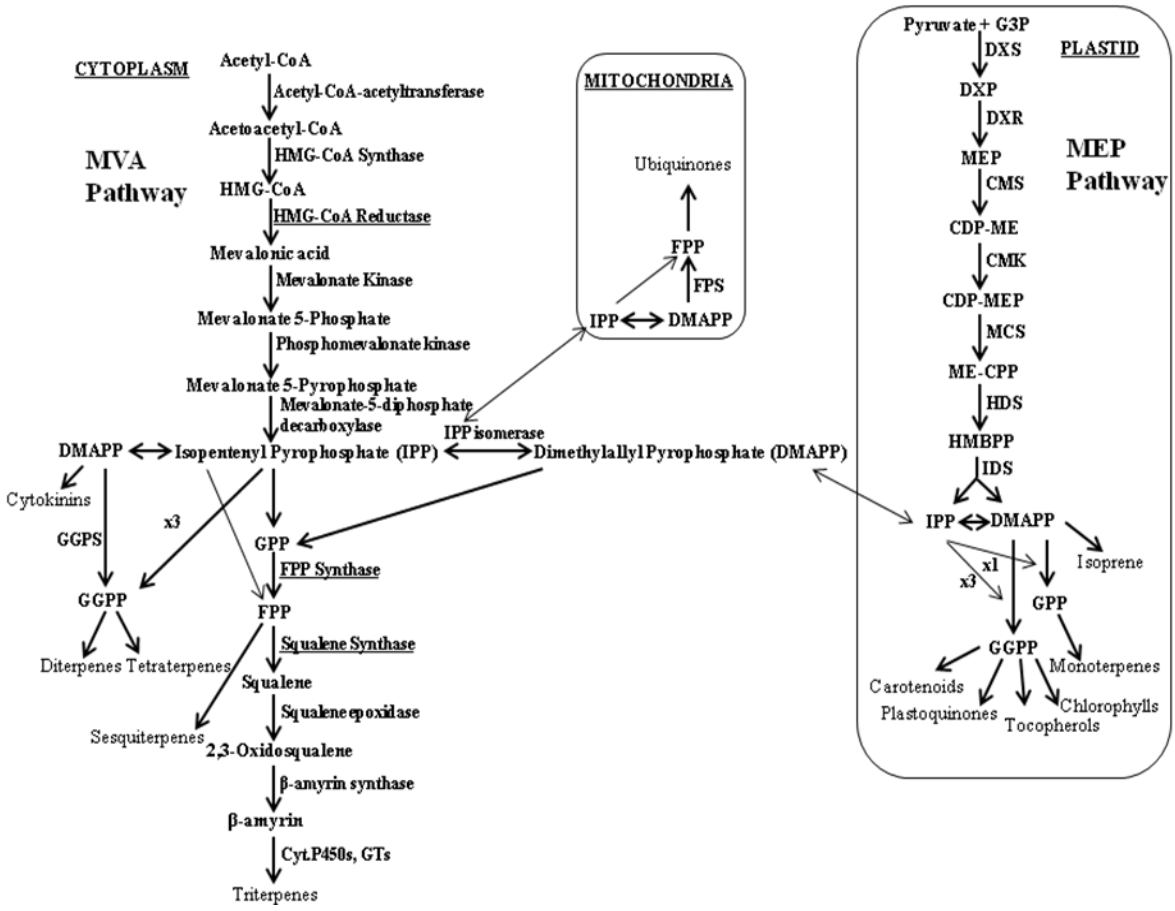


Fig. 5: Diagrammatic representation of various terpenoid biosynthesis via MVA and MEP pathway. GPP-Geranyl pyrophosphate; FPP-Farnesyl pyrophosphate; GGPP-Geranylgeranyl pyrophosphate; GGPS- Geranylgeranyl pyrophosphate synthase; DXS-1-deoxy-D-xylulose 5-phosphate synthase; DXP- 1-Deoxy-D-xylulose 5-phosphate; DXR- 1-Deoxy-D-xylulose 5-phosphate reductase; MEP- 2-C-methylerythritol 4-phosphate; CMS- 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; CDP-ME-4-Diphosphocytidyl-2-*C*-methylerythritol; CMK- 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase; CDP-MEP- 4-Diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; MCS- 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; ME-CPP-2-C-Methyl-D-erythritol-2,4-cyclopyrophosphate; HDS- 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase; HMBPP- **(**E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate.

Isoprenoids are synthesized in plants via two pathways, the mevalonic (MVA) pathway localized in cytoplasm, and the MEP pathway that occurs in plastids (**Fig. 5**.) (Eisenreich et al., 1998; Lichtenthaler, 1999; Rohmer, 1999). Isopentenyl diphosphate (IPP) is the central intermediate in the biosynthesis of isoprenoids and leads to formation of variety of natural products (Lange et al., 2000).

The MVA-pathway starts with a Claisen-condensation of two acetate units followed by an aldol-addition of a third, catalyzed by thiolase and the HMG-CoA synthase, to give 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). After reduction of the HMG-CoA by the membrane bound HMG-CoA reductase, the resulting mevalonic acid is transformed to IPP by two sequential phosphorylations and a co-elimination of the tertiary phosphate and the carboxyl group (Spurgeon and Porter, 1981). MEP pathway starting with condensation of pyruvate and glyceraldehydephosphate to 1-deoxy-D-xylulose-5-phosphate (DOX) (Rohmer, 1999; Rohmer et al., 1993). The latter is rearranged and reduced to the key intermediate 2-C-methyl-D-erythritol 4-phosphate (MEP) (Takahashi et al., 1998). Transformation of MEP to IPP proceeds via reductive elimination of a cyclic diphosphate intermediate (Rohdich et al., 2000). IPP may be isomerized to dimethylallyl pyrophosphate (DMAPP) *via* the enzyme isopentenyl pyrophosphate isomerase.

The five-carbon compounds IPP and DMAPP are condensed to form the 10-carbon geranyl pyrophosphate (GPP). GPP serves as the precursor for the synthesis of all monoterpenes. The addition of another IPP unit to GPP yields the 15-carbon farnesyl pyrophosphate (FPP). The enzyme FPP synthase catalyzes the synthesis of both GPP and FPP in mammals, whereas in plants a separate GPP synthase has been identified. FPP sits at the branch-point between sterol and longer-chain nonsterol synthesis. The enzyme squalene synthase catalyzes the head-to-head condensation of two FPP molecules to form the sterol and triterpene precursor squalene. Subsequent cyclization steps lead to sterol and triterpene synthesis. Plants also use FPP as a substrate for sesquiterpene synthesis. Geranylgeranyl pyrophosphate (GGPP) synthase catalyzes the addition of IPP to FPP to form the 20-carbon product GGPP. In plants, GGPP serves as the precursor for carotenoids, diterpenes, and chlorophylls.

Triterpenoid saponins are synthesised by cyclization of 2, 3-oxidosqualene to give primarily oleanane (β-amyrin) or dammarane triterpenoid skeletons. The triterpenoid backbone then undergoes various modifications (oxidation, substitution and glycosylation), mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases and other enzymes. Still very little is known about the enzymes and biochemical pathways involved in saponin biosynthesis. The genetic machinery required for the elaboration of this important family of plant secondary metabolites is as yet largely uncharacterized, despite the considerable commercial interest in this important group of natural products (Haralampidis et al., 2002).

The terpenoid biosynthetic pathway is regulated by several enzymes. HMG Co-A reductase, which catalyzes the formation of mevalonate from HMG-CoA, is the first committed step in mevalonate pathway for isoprenoid biosynthesis in plants. Farnesyl pyrophosphate synthase catalyzes the formation of 15 carbon compound FPP, which is the major substrate used by cytosolic and mitochondrial branches of the isoprenoid pathway. Squalene synthase represents the putative branch point in the terpenoid biosynthetic pathway capable of diverting carbon flow specifically to the biosynthesis of triterpenes and sterols. These three points in isoprenoid biosynthetic pathway play key role and considered as committed steps in triterpene biosynthesis, so alteration at these points may lead to development of plants rich in such compounds.

1.4.1 HMG-CoA reductase

The HMG-CoA reductase (HMGR EC.1.1.1.34) plays a key role in the regulation of the isoprenoid biosynthesis in plants and animals (Bach et al., 1999). It catalyzes the conversion of HMG-CoA to mevalonic acid (**Fig. 6**), which is the rate limiting enzyme in isoprenoid biosynthesis via MVA pathway (Buhaescu and Izzedine, 2007). Mevalonate is the general precursor of various identified isoprenoid compounds in plants. HMGR has been examined in a wide variety of plant species at the biochemical and molecular levels. The plant HMGR is encoded by a gene family and regulated by light, growth regulators, wounding and treatment with elicitors (Maldonado-Mendoza et al., 1997; Park et al., 1992b). Loss of function of HMGR1 in *Arabidopsis* leaded to dwarfism, early senescence and male sterility, and reduce sterol levels, suggested that HMGR plays a critical role in isoprene biosynthesis (Suzuki et al., 2004).

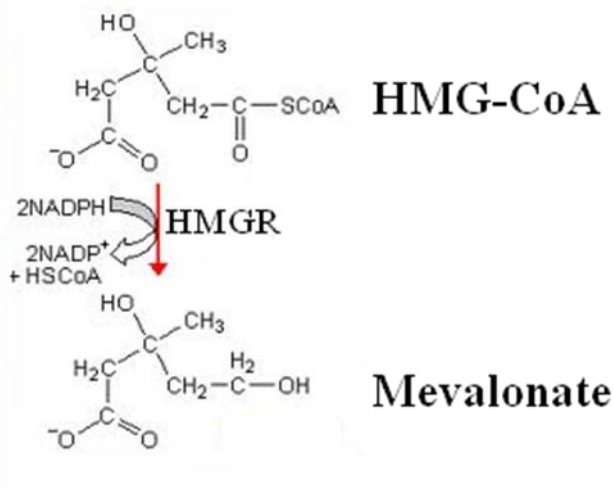


Fig. 6: Conversion of HMG-CoA to mevalonate catalyzed by HMG-CoA reductase

Genes encoding HMGRs have been isolated and characterized from many plant species including *Hevea* (Chye et al., 1991; Chye et al., 1992), *Salvia miltiorrhiza* (Liao et al., 2009), *Arabidopsis* (Enjuto et al., 1994; Enjuto et al., 1995), *Catharanthus roseus* (Maldonado-Mendoza et al., 1992), melon (Kato-Emori et al., 2001), tomato (Park et al., 1992a), potato (Korth et al., 1997), mulberry (Jain et al., 2000), *Michelia chapensis* (Cao et al., 2011) and *Euphorbia pekinensis* (Cao et al., 2010). Chye et al. (1992) reported that independent isoprenoid pathways do occur and the pathway for rubber biosynthesis in *Hevea* is distinct from the pathway(s) leading to the biosynthesis of other isoprenoid compounds in plants. Recently, Sando et al. (2008) reported that biosynthesis of natural rubber takes place biochemically by the mevalonate (MVA) pathway in *Hevea* (Sando et al., 2008). Unlike animals, which have single-copy *hmgr* genes (Gertler et al., 1988), plant *hmgr* usually occurs in small gene families and the number of genes encoding HMGR in plants varies depending on the species. Although HMGR proteins share high sequence identity, the expression patterns of individual family members are generally distinct (Stermer et al., 1994). HMGR is encoded by at least two and/or even larger multigene families in plants. The presence of multiple genes is consistent with the hypothesis that different isoforms of HMGR are involved in separate sub cellular pathways for isoprenoid biosynthesis.

1.4.2 Farnesyl pyrophosphate synthase (FPS)

FPP synthase (FPS; EC 2.5.1.1/EC.2.5.1.10) catalyzes the sequential head-to-tail coupling of dimethylallyl pyrophosphate (DMAPP) with two molecules of isopentenyl pyrophosphate (IPP), producing FPP (**Fig. 7**). FPS belongs to the family of short-chain prenyltransferases that also includes GPP synthase (GPPS) (Ogura and Koyama, 1998) and the GPPS catalyzes a head-to-tail of IPP and DMAPP to form GPP (Ogura and Koyama, 1998). These enzymes function at the branch points of isoprenoid metabolism and can thus play a regulatory role in controlling IPP flux into different terpenoid families (Gershenzon and Croteau, 1993).

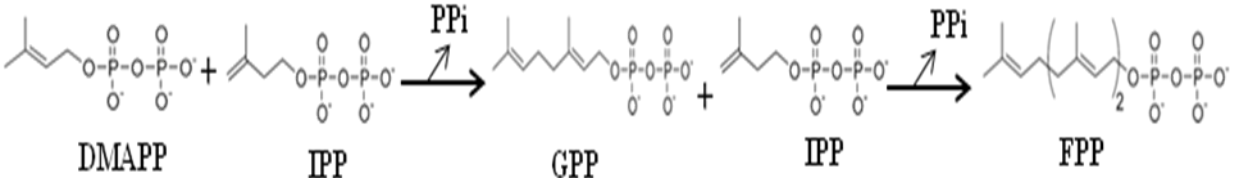


Fig. 7: FPP synthase catalyzed reaction to form FPP

The gene encoding FPS have been isolated from many plant species, such as *Artemisia annua* (Matsushita et al., 1996), *Hevea brasiliensis* (Adiwilaga and Kush, 1996), rice (Sanmiya et al., 1997), *Arabidopsis thaliana* (Delourme et al., 1994), *Lupinus albus* (Attucci et al., 1995), *Centella asiatica* (Kim et al., 2005b) and *Euphorbia pekinensis* (Cao et al., 2012). Two FPS genes that encode isozymes FPS1L (mitochondrial), FPS1S and FPS2 (both cytosolic) were reported from *A. thaliana* and they showed that simultaneous knockout of both FPS genes is lethal for *Arabidopsis*, and embryo development is arrested at the pre-globular stage, demonstrating that FPP-derived isoprenoid metabolism is essential (Closa et al., 2010). Tissue-specific and developmental analyses of the mRNA levels of FPP synthase from *Chimonanthus praecox* (CpFPPS) and volatile sesquiterpenoids levels in flowers revealed that the FPS may play a regulatory role in floral volatile sesquiterpenoids biosynthesis (Xiang et al., 2010). In tomato, *FPS* gene showed biphasic transcript accumulation pattern during fruit development. As fruits mature, the abundance of *FPS* transcript decreased slightly with a minimum at the mature green stage. During the ripening process, *FPS* mRNA amount increased again, but to a lesser extent compared with young fruits (Gaffe et al.,

2000). Cao et al. (2012) functionally characterized FPS from *Euphorbia* in ergoesterol auxotroph yeast strain and showed that accumulation pattern of the total triterpenoid contents depend not only on the effect of *FPS* gene, but also the effect of some other genes.

1.4.3 Squalene synthase

Squalene synthase (SQS) (farnesyl diphosphate:farnesyl diphosphate farnesyltransferase, EC 2.5.1.21) is a common downstream enzyme of both MVA and MEP pathways and catalyses the first enzymatic step committing carbon pool away from the central isoprenoid pathway towards the biosynthesis of phytosterols, brassinosteroids and triterpenoids (Abe et al., 1993). Squalene synthase catalyses the condensation of two molecules of farnesyl diphosphate to form a linear 30 carbon compound squalene (**Fig. 8**) and this activity has been localized to the smooth endoplasmic reticulum with its carboxy-terminal portion anchored to the ER membrane, whereas the catalytic site of the enzyme is associated with the aminoterminal portion of the protein found on the cytoplasmic face of the ER (Robinson et al., 1993). SQS has been reported to play an important regulatory role in the triterpene and steroids biosynthetic pathway.

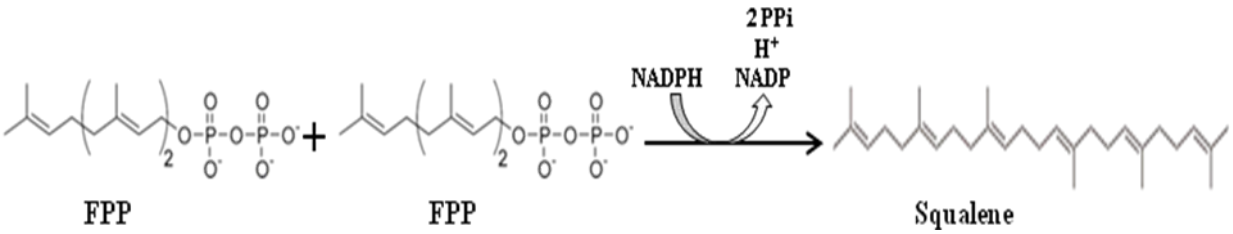


Fig. 8: Condensation of two FPP molecules to form squalene, catalyzed by squalene synthase

cDNA clones for squalene synthase have been isolated and characterized from various plant species, such as rice, maize, soybean (Hata et al., 1997), tobacco (Devarenne et al., 1998; Hanley et al., 1996), *Arabidopsis thaliana* (Kribii et al., 1997; Nakashima et al., 1995), *Panax ginseng* (Kim et al., 2011a), *Diospyros kaki* (Zhou et al., 2012), *Centella asiatica* (Kim et al., 2005a), *Lotus japonicus* (Akamine et al., 2003), *Capsicum annum* (Lee et al., 2002), *Glycyrrhiza glabra* (Hayashi et al., 1999), and

other plants, as well as mammals (Inoue et al., 1995; Robinson et al., 1993) and yeast (Jennings et al., 1991; Merkulov et al., 2000; Robinson et al., 1993).

Several biochemical investigations were carried out to obtain soluble and active recombinant SQS enzyme. Squalene synthase amino acid sequence analysis provided insights for engineering more soluble variants. Jennings et al. (1991) cloned the yeast squalene synthase gene and suggested that the enzyme consisted of a large cytosolic domain anchored to the endoplasmic reticulum by a single C-terminal transmembrane helix. Subsequently, a soluble and fully active version of recombinant yeast squalene synthase was constructed by deletion of a C-terminal hydrophobic region from the enzyme (LoGrasso et al., 1993; Yoshioka et al., 1999). Similar C-terminal hydrophobic domains were also found in the *Schizosaccharomyces pombe* (Robinson et al., 1993) and *Homo sapiens* proteins (Jiang et al., 1993). Membrane bound SQS enzyme has been purified to homogeneity from microsomal membranes of *Saccharomyces cerevisiae* (Sasiak and Rilling, 1988) and in a truncated soluble form from rat liver (McKenzie et al., 1992). In plants, the enzyme has been solubilized and partially purified from daffodil microsomal membranes (Belingheri et al., 1991) and from tobacco cell-suspension cultures (Hanley and Chappell, 1992). C-terminal truncation was also carried out in *C. annuum* by removing last 24 amino acids and fully active SQS protein was purified from recombinant *E. coli* (Lee et al., 2002).

Squalene synthase is commonly described as an incipient and crucial branch point enzyme away from the main isoprenoids biosynthetic pathway and a potential regulatory point that controls carbon flux into sterols and triterpenes biosynthesis (Huang et al., 2007). Lots of evidence support that inhibition of the squalene synthase enzyme is a potential means of redirecting FPP away from the sterol biosynthesis, towards the synthesis of other commercially interesting isoprenoids. The disruption of sterol biosynthesis at squalene synthase leads a remarkable accumulation of FPP in an erg9 mutant strain of *Saccharomyces cerevisiae* (Song, 2003). Besides FPP, the increase of IPP and GGPP was also observed when the rat liver cells were treated with zaragozic acid A, a potent inhibitor of squalene synthase (Keller, 1996) and similar effects have been observed in plants (Fulton et al., 1995).

1.5 Regulation of plant terpenoid biosynthesis

Environmental factors including biotic and abiotic stimuli, carbon-nutrition balance, genotype and ontogenesis usually control and regulate the biosynthesis of secondary metabolites in plants (Kliebenstein, 2004; Laitinen et al., 2005; Lerdau and Coley, 2002; Lila, 2006). The regulation of plant terpenoid biosynthesis is complicated and in general shows two broad categories: the spatial and the temporal (Dudareva et al., 2004). Formation of volatile compounds is spatially regulated. Several investigations have shown that terpenoid volatiles are often synthesized *de novo* in some special physical structures, such as the oil glands (Gershenzon et al., 2000) or resin ducts (Miller et al., 2005; Phillips and Croteau, 1999), where large amounts of terpenoids accumulate. The monoterpenes and sesquiterpenes emitted as volatiles contribute to indirect defenses against herbivores, whereas diterpenoids and some of the sesquiterpenoids provide direct protection through formation of phytoalexins (Phillips and Croteau, 1999). Elucidation of biosynthetic pathways and regulatory mechanism of plant terpenoids, and dissecting the role of plant secondary metabolites in an ecosystem will provide a novel insight into the sustainable development of agriculture and the environment (Cheng et al., 2007).

Many aspects of plant development and metabolism are light regulated. The rhythmic and developmental regulation of volatile terpenoid emission from different species has been reported (Aharoni et al., 2003; Dudareva et al., 2003; Lu et al., 2002). In *Artemisia annua*, the emission of β-pinene fluctuates with the day-night rhythm and is generally higher in the day than in the night (Lu et al., 2002). In snapdragon flowers, emissions of monoterpene compounds follow a diurnal rhythm that is controlled by a circadian clock (Kolosova et al., 2001). In *Arabidopsis*, nearly all terpenoids detected in the flower headspace show a clear diurnal emission pattern, whereas other non-terpenoid components do not show such a rhythm (Aharoni et al., 2003). Terpenoid volatile emissions from *Picea sitchensis* saplings on treatment with methyl jasmonate (MeJA) also showed a diurnal rhythm pattern (Miller et al., 2005).

Aside from rhythmic regulation, many terpenoids are specifically induced in response to various elicitors or herbivorous damage. This defense-related formation of terpenoids has been studied in many species (Rodriguez-Saona et al., 2003; Van Poecke

et al., 2001). In poplar, several terpenoids were induced and emitted from the forest tent caterpillar infested local and systemic leaves (Arimura et al., 2004a). In *Lotus japonicus*, two-spotted spider mites induced the emission of (E)-β- ocimene and the accumulation of (E)-β-ocimene synthase gene transcripts (Arimura et al., 2004b). In recent years, great progress has been made in elucidating plant terpenoid biosynthetic pathways at the gene and enzyme levels. However, regulation of terpenoid metabolism in context of regulatory factors controlling pathway enzyme genes of secondary metabolism requires further investigations.

1.6 Genetic engineering of terpenoid biosynthesis pathway in plants using HMG-CoA reductase, FPP synthase and squalene synthase as target genes

Genes that express the enzymes of the MVA, MEP, and downstream pathways have been manipulated to enhance carbon flow into specific downstream products in various plant species. The genetic manipulations suggested that, in most cases, synthesis of the upstream isoprenoid precursors IPP and DMAPP should be maximized to boost production of downstream metabolites (Kumar et al., 2012). The rate-limiting step in the MVA pathway, 3-hydroxy 3-methylglutaryl-coenzyme A reductase (HMGR) has been the target of genetic manipulations in several studies. Overexpression of heterologous HMGR resulted in increased levels of total sterols in tomato (Enfissi et al., 2005) and tobacco (Chappell et al., 1995; Harker et al., 2003; Schaller et al., 1995). Many other isoprenoid products have been successfully enhanced, including monoterpenes and sesquiterpenes (Davidovich-Rikanati et al., 2007).

Farnesyl diphosphate synthase (FPS) plays a vital role in organ development in plants. However, FPS has not previously been identified as a key regulatory enzyme in triterpene biosynthesis. To elucidate the functions of FPS in triterpene biosynthesis, *C. asiatica* was transformed with a construct harboring *Panax ginseng* FPS (PgFPS) encoding cDNA coupled to the cauliflower mosaic virus 35S promoter. Significant increase in sterol and triterpene content was observed, which suggested that FPS plays an important role in phytosterol and triterpene biosynthesis (Kim et al., 2010). Squalene synthase functions as a key regulator in channeling the carbon flux into both the primary and secondary metabolite branches. The regulatory role of SQS in triterpene sterol biosynthesis has been demonstrated in various plants (Vogeli and Chappell 1988;

Devarenne et al., 2002; Devarenne et al., 1998; Wentzinger et al., 2002). There is also a positive correlation between the expression levels of SQS and the amount of triterpenes produced in *Ganoderma lucidum* and *P. ginseng* (Lee et al., 2004; Zhao et al., 2007). Lee et al. (2004) demonstrated the role of the squalene synthase (PgSS1) gene in the biosynthesis of phytosterols and triterpenoids in *P. ginseng*. (Kim et al., 2011b) demonstrated that enhanced triterpenoid and sterol content in *Bupleurum falcatum* over-expressed squalene synthase gene. Some examples of transgenic plants over-expressing HMGR, FPS and SQS, and their phenotypic alterations are summarized in **Table 2**.

Table 2: Highlights of secondary metabolite content in transgenic plants over-expressing terpenoid biosynthesis pathway genes, HMGR, FPS and SQS.

Genes	**Over-expression in Plant**	**Effects on secondary metabolite content**	**References**
HMGR	*Arabidopsis*	Although very high levels of HMG CoA reductase mRNA were expressed in these transgenic plants, only modest increases in enzyme activity and isoprenoids content was observed.	(Re et al., 1995)
HMGR (*Hevea brasiliensis*)	Tobacco	Transgenic lines showed an increase in the level of total sterols up to 6-fold	(Schaller et al., 1995)
Truncated HMGR (*Hevea brasiliensis*)	Tobacco	Total seed sterol content 2.4 fold increased. 4-desmethylsterols were increased by 2.2-fold Enhanced seed HMGR activities by 11-fold	(Harker et al., 2003)
HMGR (*A. thaliana*)	Tobacco	Levels of seed phytosterols were up to 2.44-fold higher in plants transformed. Levels of phytosterols in leaves were unchanged in transformed plants Phytosterol content elevated up to 2.4 fold	(Hey et al., 2006) (Enfissi et al., 2005)

HMGR (*Catharanthus roseus*)	*Artemisia annua*	22.5% artemisnin content increased as compared to wild type 7.65 fold higher artemisnin content than control plant.	(Aquil et al., 2009) (Alam and Abdin, 2011)
FPS (*Gossypium arboretum*)	*Artemisia annua*	Artemisnin content 2-3 fold higher than control plant.	(Chen et al., 2000)
FPS	*Arabidopsis thaliana*	FPS activity increased 8-12 folds as compared to wild type. Reduced the level of endogenous zeatin-type cytokinins in leaves, ultimately resulting in their phenotypic alteration. Sterol content similar to control plant.	(Masferrer et al., 2002)
FPS (*Saccharomyces cerevisiae*)	Tobacco	Total FPP specific activity increased 12-fold compared to control and has been correlated to a clear increase of both sterol and carotenoid biosynthesis	(Daudonnet et al., 1997)
FPS (*P. ginseng*)	*Centella asiatica*	Madecassoside and asiaticoside was transiently increased by 1-1.5 fold as compared to control. Total sterol contents increase 3 fold as compared to control. Squalene content 1.1-1.5 fold increased as compared to control.	(Kim et al., 2010)
SQS	*Panax ginseng*	Enhanced phytosterols and triterpene ginsenoside contents	(Lee et al., 2004)
SQS	*Eleutheroccus senticosus*	Increased phytosterols and triterpenoid contents.	(Seo et al., 2005)
SQS (*A. thaliana*)	*Withania coagulans*	Increased phytosterols, withanolides and triterpenoid contents.	(Mirjalili et al., 2011)
SQS	*Bupleurum falcatum*	Enhanced triterpenoid and sterol contents	(Kim et al., 2011b)

All plant isoprenoids are derived from the common precursor isopentenyl diphosphate (IPP), which is synthesized by two different pathways: the mevalonic acid (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. IPP is then used in condensation reactions to produce geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP). These compounds are the key intermediates for the synthesis of a wide range of end products derived from the isoprenoid pathway. There is scarce information about biosynthetic pathway involved in terpenoid saponins. The understanding of genetic machinery required for the elaboration of this important family of plant secondary metabolites is as yet not clearly characterized. This is likely to be due in part to the complexity of the molecules and the lack of pathway intermediates for biochemical studies (Haralampidis et al., 2002).

B. monniera is an important herb in Ayurvedic medicine for the improvement of intelligence, memory and revitalization of sensory organs and the medicinal property of this plant attributed due to presence of triterpenoid saponins collectively called as bacosides. Bacosides are present in very low quantity in the plant and the extraction procedure requires huge biomass leading to environmental imbalance and accounting this plant as an endangered species. To alleviate these issues, there is urgent need to develop efficient methods for plant propagation and develop elite species of *Bacopa* with high bacoside contents using advanced molecular biology tools.

The present study was taken up with the objective to isolate, clone and characterize terpenoid biosynthetic pathway gene (s) from *B. monniera*. Transformation of *B. monniera* plants with sense constructs in binary vector to achieve improved medicinally important compound content. Efficient regeneration system and stable transformation method are prerequisite for development of transgenic. The other objectives were to study the expression of protein in heterologous system, *in-silico* studies of proteins (Homology modeling and docking studies) and differential expression pattern study of terpenoid biosynthetic pathway genes in the plant in normal as well as in stress conditions.

CHAPTER: 2

MATERIALS AND METHODS

Chapter 2. Materials and methods

This chapter deals with all the routine chemicals used during the research and their resources. It also deals with general laboratory methods and techniques used during experiments. Other important specific methodologies followed and chemicals used are discussed separately in the respective chapters.

2.1 Materials and equipments used in the study

2.1.1 Glasswares and their preparation

Glassware used for the experiments were purchased from Borosil, India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), conical flasks (250, 500, 1000, 2000 and 5000 mL capacity), measuring cylinders (10, 50, 100, 500, 1000 and 2000 mL), beakers (250, 500, 1000 and 2000 mL), petriplates and funnels etc were used during course of study. All the glasswares were cleaned by boiling in a saturated solution of sodium bicarbonate for 1 h followed by repeated washing in tap water. Thereafter, it was immersed in 30% HNO_3 solution for 30 min followed by repeated washing in tap water and rinsed with distilled water. Washed glassware was thereafter dried at room temperature. Autoclaving of the glassware was done when mandatory at 121 ºC and 15 psi for 1 h.

2.1.2 Plasticwares

Sterile petridishes (55 mm and 85 mm diameter) were procured from “Axygen” and “Tarsons”, India. Disposable filter sterilization units (0.22 μM) were purchased from Millipore (USA). Microfuge tubes (1.5 mL and 2 mL capacity), microtips (10, 200, 1000 and 5000 μL capacity) and PCR tubes (0.2 mL capacity, flat cap) were obtained from “Tarsons” and “Axygen”, India. EIA/RIA medium binding flat bottom, 96 well plates were procured from “Costar” USA.

2.1.3 Chemicals

All chemicals were purchased from Sigma-Aldrich (USA), Bioworld (USA), Merck, SD fine chemicals, Hi-Media,. Restriction enzymes, T4 DNA ligase, RNase A and lysozyme were obtained from NEB (USA), Promega (USA), and Amersham (UK). Different kits were purchased from BD Clonetech (Japan), Invitrogen (USA), Promega

(USA), Sigma-Aldrich (USA), Amersham, Vibantis and Hi-Media. Taq DNA polymerase was obtained from Sigma-Aldrich, Invitrogen and Bangalore GeNei (India). All solvents of analytical as well as HPLC grade were purchased from Qualigens, India. MS media and growth regulators used for plant tissue culture were purchased from Hi-Media and Sigma. Standards for HPLC were obtained from Sigma-Aldrich (USA) and Chromadex (USA). Different types of matrix for protein purifications were purchased from Qiagen (USA) and GE Healthcare (Sweden).

2.1.4 Vectors and bacterial strains

Vector	**Source**	**Features**
pGEM-T Easy	Promega	T/A cloning vector
pET-30(b+)	Novagen	Expression vector
Modified pCAMBIA1301	CAMBIA University Australia	Binary vector
Bacterial strain	**Source**	**Genotype**
E. coli XL-10 gold	Stratagene	*Tet^r Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F´ proAB lacI^{q}ZΔM15 Tn10 (Tet^r) Amy Cam^r].*
XL1-Blue MRF´ strain	Stratagene	*Δ(mcrA)183 Δ(mcrCB -hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96relA1 lac [F´ proAB lacI^{q}ZΔM15 Tn10 (Tet^r)]*
E. coli BL21 (DE3)	Stratagene	*E. coli B F$^-$ompT hsdS(rB$^-$mB$^-$) dcm$^+$Tet^r gal λ(DE3) endA Hte*
Agrobacterium tumefaciens (GV2260)		*C58, Rifr, pGV2260 (pTiB6S3_T-DNA), Carbr, Octopine type*

2.1.5 Equipments used during study

S. No	Equipment	Make
1	Centrifuge	Sorvall/Haereus/Eppendorf
2	Gel Documentation system	Bio-Rad
3	Thermo Cycler PCR machine	Bio-Rad
4	Real time PCR	Stratagene, USA
5	Spectrophotometer	Perkin Elmer
6	Protein Gel Electrophoresis Units	Hoeffer Scientific/GE Healthcare
7	Speed Vac concentrator	Eppendorf
8	Water purification system	TKA (Milli RO/Milli Q)
9	Bioanalyser agilent 2100	Agilent Technology
10	Typhoon Trio + Scanner	GE Healthcare (Sweden)
11	HPLC	Perkin Elmer, USA
12	ELISA Plate Reader	BIO-RAD (USA)
13	iBlot Gel Transfer System	Invitrogen, USA
14	NanoVue (Nanodrop)	GE Healthcare

2.2 Plant material

2.2.1 *Bacopa monniera*

Bacopa monniera (Linn.) Pennel (*Scrophulariaceae*) was collected from the Department of Horticulture, National Chemical Laboratory, Pune.

For genomic DNA and RNA isolation young *Bacopa* shoots were harvested, washed thoroughly with water, blotted and then crushed with liquid nitrogen. For Real-time PCR experiments, *B. monniera* plants were maintained as potted plant in the garden under moderate sunlight. They were watered at regular intervals. Shoots were collected from these healthy and disease free plants and were used for initiation of the cultures. Shoots from these *in vitro* grown plants were used for qRT-PCR experiments.

2.2.2 Surface sterilization

The shoots were cleaned under tap water and treated with savlon (10% v/v) for one minute followed by thorough washing with sterile distilled water for 2-3 times. They were treated with 70% alcohol in sterile flask for few seconds in laminar air flow cabinet. Alcohol was quickly decanted and shoots were washed thoroughly with sterile distilled water. After that washed shoots were treated with 0.01% $HgCl_2$ solution for 30 s with frequent shaking and rinsed with sterile distilled water several times to remove all the traces of $HgCl_2$. Explants were prepared by dissecting the shoots into pieces with 1-2 axillary buds.

2.2.3 Media used

All media were based on Murashige and Skoog basal medium (Murashige and Skoog, 1962). The pH of the media was adjusted to 5.8 prior to autoclaving (121 ^oC and 15 psi for 20 min). Initiation medium was MS basal medium supplemented with 0.5 mg/L 6-benzyl amino purine and 3% sucrose, solidified with 0.8% agar. Proliferation medium was plain MS medium with 3% sucrose.

2.2.4 Inoculation and incubation

The surface sterilized explants were inoculated on initiation medium under aseptic conditions. Cultures were incubated at the temperature 25±1°C under 16 h photoperiod at 11.7 µmol/m^2/sec light intensity/8 h dark cycles for about 20 days. The proliferating multiple adventitious shoots were separated and re-inoculated on proliferation medium for further growth.

2.2.5 Stress treatment

In vitro proliferated shoots were used for study of different stress treatments like salt, methyl jasmonate, salicylic acid, yeast extract, chlorogenic acid and cold by transferring them to MS basal liquid medium supplemented with 200 mM NaCl, 500 µM methyl jasmonate, 200 µM salicylic acid, 0.5 mg/L Chlorogenic acid and 0.1% Yeast extract separately. The cultures were further incubated under the same conditions as described earlier. Sample tissue was collected from different time intervals for RNA isolation. In case of cold (4 °C) treatment, cultures were reinoculated without any supplement in MS basal liquid medium and kept at 4 ^oC. Tissues were harvested from

cold treated plants at different time interval and RNA was isolated and used for qRT-PCR analysis.

2.3 Buffers and solutions

2.3.1 Buffers and solutions for agarose gel electrophoresis

Name	Components	Preparation and Storage
50 X TAE	2 M Tris Acetic acid (242 g Tris-base and 57.1 mL Glacial acetic acid for 1L) 0.05 M EDTA	pH was adjusted to 8.0 and stored at room temperature.
10 X TBE buffer	890 mM Tris base 890 mM Boric acid 20 mM EDTA (pH-8.0)	Room temperature.
DNA loading buffer	0.25% (w/v) Xylencyanol 0.25% w/v) Bromophenol blue 40% Sucrose 40 mM EDTA (pH 8.0)	The solutions were filter sterilized using 0.22 micron (µ) filter and stored at 2-8 ^{o}C

2.3.2 Different buffers and media used for bacterial studies

Name	Components	Preparation and Storage
Luria Bertani Broth (LB)	1% Tryptone 0.5% Yeast extract 1% NaCl	pH adjusted to 7.0 with NaOH, store at room temperature or at 4 ºC after autoclaving
SOB media	2% Bactotryptone 0.5% Yeast extract 10 mM NaCl 10 mM $MgCl_2.6H_2O$ 2 mM KCl	pH adjusted to 6.8 with NaOH, store at room temperature or at 4 ºC after autoclaving
TB buffer	10 mM PIPES 15 mM $CaCl_2$ 250 mM KCl	pH was adjusted 6.8 with KOH. $MnCl_2$ was added to final concentration of 55 mM and filter sterilized. Store at -20 ^{o}C
YEB	Beef Extract (0.5%) Yeast Extract (0.1%) Peptone (0.5%) Sucrose (0.5%) Magnesium Sulfate (2.0 mM)	pH adjusted to 7.0 with NaOH, store at room temperature or at 4 ºC after autoclaving

2.3.3 Stock solutions for bacterial and plant transformation and selection

Name	Components	Preparation and Storage
IPTG solution	200 mg/mL in SMQ	Sterile filtration and storage at -20 ºC
X-Gal (5-bromo-4chloro -3-indolyl-β-D-galactoside)	20 mg/mL in N,N'-Dimethyl formamide (DMF)	Light sensitive, covered & store at -20 ºC
Ampicillin	100 mg/mL in SMQ	Filter sterilization and storage at -20 ºC
Tetracycline	12.5 mg/mL in 70% ethanol	Filter sterilization and storage at -20 ºC
Kanamycin	50 mg/mL in SMQ	Filter sterilization and storage at -20 ºC
Hygromycin	10 mg/mL in SMQ	Filter sterilization and storage at -20 ºC
Cefotaxime	250 mg/mL in SMQ	Filter sterilization and storage at -20 ºC
Rifampicin	100 mg/mL in ethanol	Filter sterilization and storage at -20 ºC

2.3.4 Buffers and solutions for plasmid DNA isolation (Alkaline lysis method)

Name	Components	Preparation and Storage
Solution I (GTE Buffer)	50 mM Glucose, 25 mM Tris-HCl (pH 8.0) 10 mM EDTA (pH 8.0)	Autoclave for 10 min and Store at 4 ºC
Solution II	0.2 N NaOH 1% SDS	Freshly prepared
Solution III	3M Potassium acetate (pH-5.5)	Store at 4 ºC
RNase A	10 mg/mL	Store at -20 ºC
Other solutions or Reagents	Chloroform, Absolute ethanol, 3.0 M Sodium acetate, 70% ethanol & Deionized sterile water	Room temperature

2.3.5 Buffers and solutions for protein extraction and purification

Name	Components	Preparation and Storage
Lysis buffer	50 mM Tris-HCl (pH 8.0) 1 mM EDTA 150 mM NaCl 0.5% TritonX-100 0.7 mM DTT 0.1 mM PMSF (Freshly added) 10 mM $MgSO_4$ Lysozyme 100 µg/mL (Added freshly)	Store at 4 ºC
Sonication buffer (for inclusion bodies)	100 mM Tris HCl (pH8.0) 50 mM Glycine	Store at 4 ºC
Dispersion buffer (for inclusion bodies)	100 mM Tris-HCl (pH8.0) 50 mM Glycine & 8 M Urea /6 M GdnHCl	Store at 4 ºC
Binding buffer	50 mM Tris (pH 8.0) 300 mM NaCl 20 mM imidazole	Adjust pH by adding concentrated HCl and store at 4 ºC
Wash buffer	50 mM Tris (pH 8.0) 300 mM NaCl 30 mM imidazole	Adjust pH by adding concentrated HCl and store at 4 ºC
Elution buffer	50 mM Tris (pH 8.0) 300 mM NaCl 250 mM imidazole	Adjust pH by adding concentrated HCl and store at 4 ºC

2.3.6 Buffers and solutions for protein gel electrophoresis (SDS-PAGE) and staining

Name	Components	Preparation and Storage
Monomer solution	29.2% acrylamide 0.8% bis-acrylamide in water	Store at 4 ºC (in darkness)
Stacking gel	Distilled water 2.76 mL 1 M Tris-HCl (pH 6.8) 0.50 mL Acrylamide/bis 30% 0.65 mL 10% SDS 0.04 mL 10% (w/v) APS 0.04 mL TEMED 4 .0 µL	Freshly prepared
Separating gel (10%)	Distilled water 3.162 mL 1.5 M Tris-HCl (pH 8.8) 2.0 mL Acrylamide/Bis 30% 2.67 mL 10% SDS (SDS-PAGE) 0.08 mL 10% (w/v) APS 0.08 mL TEMED 8.0 µL	Freshly prepared

2x Protein loading buffer	Distilled water 2.7 mL 0.5 M Tris-HCl (pH 6.8) 1.0 mL Glycerol 2.0 mL 10% SDS(SDS-PAGE) 3.3 mL β-Mercaptoethanol 0.5 mL 0.5% Bromophenol blue 0.5 mL	Store at 4 ºC
10x SDS-electrode buffer	Tris base 15.1 g Glycine 94.0 g SDS 00.5 g Adjust pH-8.3 *And make-up the volume up to 500 mL.*	Store at 4 ºC Dilute 1:10 before use
Staining solution (Coomassie)	Coomassie-blue R 250, 0.25 g in Methanol, 40 mL and Acetic acid, 10 mL *Make-up volume up to 100 mL*	Store at RT (in brown bottle)
Destaining solution	Methanol, 40 mL and Acetic acid, 10 mL *Make- up volume up to 100 mL*	Freshly prepared
Silver staining Fixer solution I	40% Methanol (40 mL) and 10% acetic acid (10 mL) *Make-up volume up to 100 mL*	Store at RT
Silver staining Fixer solution II	50% Ethanol	Freshly prepared
Sensitizing solution	0.2% $Na_2S_2O_3$	Store at RT
Silver solution	0.2% silver nitrate (0.6 g) 0.01 % formaldehyde (225 µL) *Make-up volume up to 300 mL*	Prepared freshly in darkness
Developing solution	6% Na_2CO_3 (18 g) 0.02% formaldehyde (450 µL) *Make-up volume up to 300 mL*	Prepared freshly
Stop solution	1.5% Na_2EDTA (4.5 g) *Make-up volume up to 300 mL*	Store at RT

2.3.7 Buffers and solutions used for ELISA and Western blotting

Name	**Components**	**Preparation and Storage**
Crude protein extraction buffer	100 mM Tris-HCl pH.7.5, 1% PVPP, 2% PEG 4000, 5 mM DTT and 1 mM PMSF	Store at room temperature
PBST	1.44 g Na_2HPO_4 0.24 g KH_2PO_4 0.2 g KCl 8 g NaCl 0.05% v/v Tween-20 *Make-up volume 1000 mL with water*	Store at room temperature

Antibody dilution Buffer	PBS with 0.25% BSA	Store at room temperature
Substrate buffer	200 mM Tris-HCl pH 9.5 0.5 mM $MgCl_2$	Store at room temperature
1X PBS	10 mM NaH_2PO_4- Na_2HPO Buffer (pH-7.2) 130 mM NaCl	Adjust pH to pH 7.2 and stored at 4 ºC
0.5 X SSC	75 mM NaCl 7.5 mM Na-Citrate	Adjust pH to pH 7.0 and stored at 4 ºC
Color development buffer	100 mM Tris (pH 9.5) 150 mM NaCl 50 mM $MgCl_2$	Store at 4 ºC
BCIP/NBT mix	0.577 mM BCIP 0.122 mM NBT	Store at 4 ºC in dark
Stop Solution	10 mM EDTA	Store at room temperature

2.3.8 DNA ladders and protein molecular weight markers used

All DNA rulers and protein molecular weight markers were purchased from Banglore GeNei (India).

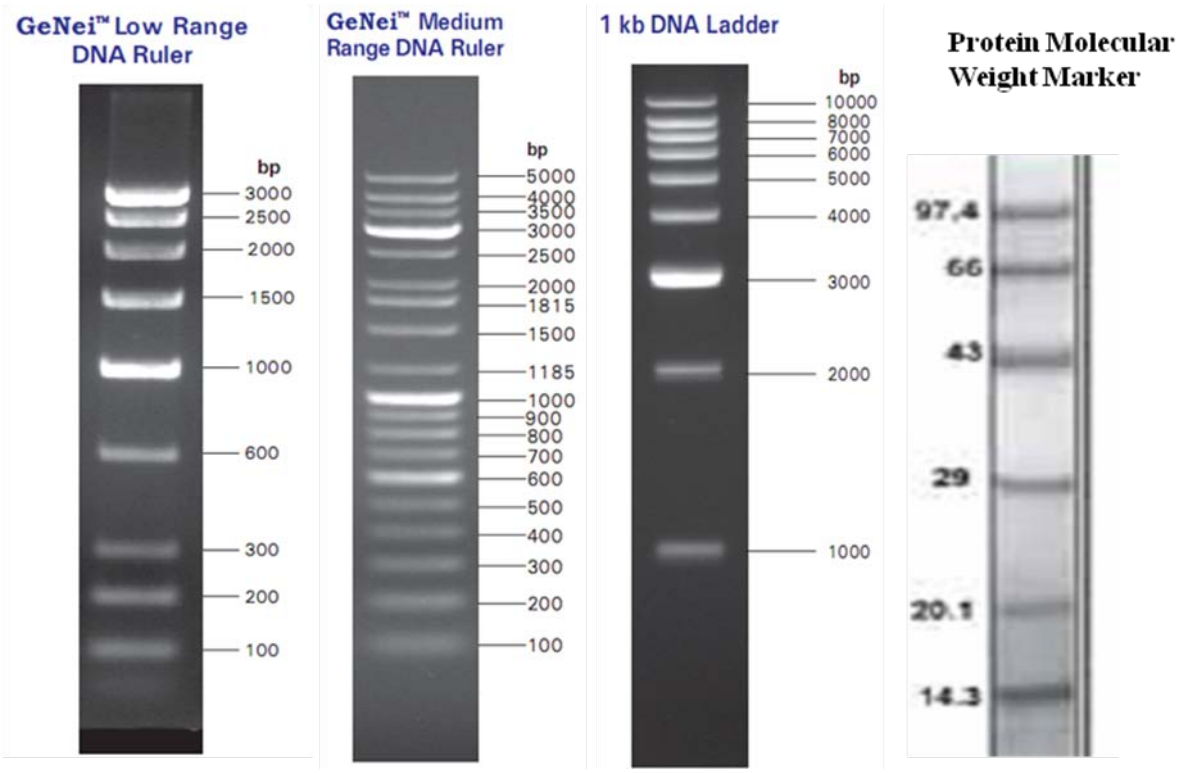


Fig. 2.1: Map of different DNA rulers and protein molecular weight marker.

2.4 Methods

2.4.1 Bacterial culture conditions

E. coli was grown at 37 ºC with shaking at 200 rpm in Luria Bertani (LB) broth/SOB broth and maintained on LB/SOB plates with 1.5% agar (Sambrook et al., 1989). Recombinant *E. coli* was grown in LB medium supplemented with appropriate antibiotic(s) used for plasmid DNA preparation. *Agrobacterium tumefaciens* (GV2260) was grown at 28 ^{o}C with shaking at 280 rpm in YEB medium with appropriate antibiotic(s).

2.4.2 Bacterial transformation

2.4.2.1 Preparation of competent cells using TB buffer

A single colony of *E. coli* XL10 Gold was inoculated in 5 mL of LB medium containing tetracycline (12.5 mg/L) and grown overnight at 37 ^{o}C in incubator shaker at 200 rpm. Overnight grown culture (500 µL) was added to 50 mL of SOB medium and grown for 2 h at 37 ^{o}C in incubator shaker at 200 rpm (until OD_{600} reaches 0.5-0.7). Cells were kept on ice for 10 min and harvested by centrifugation at 5,000 rpm for 10 min at 4 ^{o}C. The cell pellet was suspended in 10 mL ice-cold TB buffer and kept on ice for 30 min. Centrifuged for 10 min at 5,000 rpm and 4 ^{o}C. Pellet was resuspended in 5 mL TB buffer containing 7% DMSO. This was then dispensed into aliquots of 100 µL in sterile 1.5 mL eppendorf tubes, frozen in liquid nitrogen and stored at -80 ^{o}C.

2.4.2.2 Bacterial transformation

The competent *E. coli* cells were transformed according to Sambrook et al. (1989). Competent cells were thawed on ice and DNA (~ 50 ng /or ligation mixtures) was added gently to the (100 µL) competent *E. coli* cells, mixed and kept on ice for 30 min. The cells were then incubated at 42 ºC for 2 min (heat shock) and immediately kept back on ice. LB broth (800 µL) was added and further incubated at 37 ºC for 1 h in shaker incubator. Cells were pelleted by centrifugation at 5,000 rpm for 10 min. The supernatant media was discarded and pellet was dissolved in 100 µL of LB broth. The resuspended cells were plated on LB agar medium with appropriate antibiotics, IPTG and X-gal as per need (Sambrook et al., 1989).

2.4.3 Colony PCR for screening recombinant colonies after bacterial transformation

This method bypasses DNA purification, and relies on the selectivity of PCR amplification to determine whether a bacterial colony of interest does indeed contain the desired DNA. Simply adding a small portion of a bacterial colony to a PCR master mix will introduce enough templates DNA for amplification. A single bacterial colony was picked up from the agar plate containing transformants with the help of microtip and added to 1.5 mL eppendorf tube containing 20 µL of sterile miliQ water. The microtip was agitated in the water to suspend the bacterial cells. Bacterial lysis was done by placing the suspension on dry bath at temperature 95 ^{o}C for 5 min. After brief spin 2 –3 µL of supernatant was used in 15 µL of PCR reaction. The remaining components were added to the PCR reaction and subjected to normal cycling parameters for the particular primers. The resulting PCR products were checked on 1 % agarose gel for the presence of the cloned gene of interest amplicon**.**

2.4.4 Cryopreservation of bacterial culture

Bacterial culture harboring plasmid with target gene was preserved for further use in glycerol at ultra low temperature. In a microfuge tube (1.5 mL) culture with 20% sterile glycerol were mixed thoroughly by pipeting and frozen in liquid nitrogen and stored at -80 ^{o}C. The stocks were revived periodically (~6 months) and fresh stocks were prepared.

2.4.5 Isolation of nucleic acids and Polymerase Chain Reaction (PCR)

2.4.5.1 Isolation of plasmid DNA from *E. coli* cells

The alkaline lysis method of Sambrook et al. (1989) was improvised upon so that 12-24 samples could be processed conveniently for plasmid DNA extraction within 3 h, with yields of 500 µg per 1.5 mL culture depending on the host strain and the plasmid vector. An important feature of this protocol was the use of PEG for purification, which resulted in precipitation of high quality super-coiled plasmid DNA free of contamination. The bacterial cultures were grown overnight with shaking (200 rpm) at 37 ºC in LB broth, with appropriate antibiotic(s). About 2.0 mL culture was centrifuged for 10 min at 7000 rpm to pellet the bacterial cells. The pellet was resuspended in 200 µL of GTE buffer (solution I) by vigorous pipetting, 400 µL of solution II was added,

mixed by inversion till the solution becomes clear, normally for 2-3 min. The cell lysate was neutralized by addition of 300 µL of solution III, mixed well and incubated on ice for 5 min. The cell debris was removed by centrifugation for 10 min at 10,000 rpm at 4 ºC. The supernatant was transferred to another microfuge tube, RNase A was added to a final concentration of 20 µg/mL (Sambrook *et al*., 1989) and incubated at 55 ºC for 20 min. To the above solution, 400 µL of chloroform was added, vortexed for 2 min and centrifuged for 10 min at 10, 000 rpm at room temperature. The upper aqueous layer was transferred to a clean tube, 0.1 volume of sodium acetate and 0.6 volume of propanol was added with gentle mixing and kept at room temperature for at least 30 min. The sample was then centrifuged at 13,000 rpm for 15 min at 4 ºC. The pellet was washed twice with 70% ethanol and dried under vacuum. The dried pellet was dissolved in 40 µL of nuclease free water and 40 µL of PEG/NaCl solution (20% PEG 8000 in 2.5 M NaCl) was added. The mixture was incubated on ice for 20 min and the plasmid DNA pelleted out by centrifugation at 13,000 rpm for 15 min at 4 ºC. The supernatant was aspirated carefully, the pellet washed with 70% ethanol and air dried. The dried pellet was resuspended in 20 µL of nuclease free water and stored at -20 ºC.

2.4.5.2 Isolation of plant genomic DNA

Plant genomic DNA isolation kit (Himedia and Banglore GeNei, India) was used for DNA isolation and manufacture's protocol was followed.

2.4.5.3 Restriction digestion of DNA

Plasmid and genomic DNA restriction digestion was set up as per restriction enzymes manufacturer's recommendations**.**

2.4.5.4 Extraction and purification of DNA from agarose gels

DNA/PCR product was recovered from agarose gel by using Gel elution kit (Invitrogen/Sigma) as per manual instructions. The eluted DNA /PCR product was stored at -20 ^{o}C and was used for subsequent reactions.

2.4.5.5 Total RNA isolation

RNase free environment was created and maintained. All glass and plasticware were DEPC (0.1% in water) treated overnight and autoclaved. The pestle and mortar were

also DEPC treated and then baked at 300 ºC for 6 h. All materials were dried in a vacuum oven. Total RNA from different plant tissues was isolated using TRIzol reagent (Sigma) and Plant RNA isolation kit (Sigma, Invitrogen) as per manual instructions. Isolated total RNA samples were treated with DNase with DNase I Digest kit (Sigma, USA) according to manual instructions and checked on 1% agaorse gel and stored at -80 ^{o}C.

2.4.5.6 Spectrophotometric determination of nucleic acids concentration

DNA/RNA concentration was determined by measurement of the absorption at 260 nm. Nanodrop was used to determine the concentration of 1:50 diluted RNA or DNA samples. Absorbance value (A_{260}) should fall between 0.1-1.0 to be accurate. Sample dilution was adjusted accordingly. An absorbance of 1.0 at 260 nm corresponds to 50 µg DNA/ mL (40 µg/mL in case of RNA).

The Bioanalyser system 2100 (Agilent) was also used to check the quantity of the nucleic acids (according to manufacturer's instructions). Some other useful parameters like gel electrophoresis pattern of the samples (nucleic acids and protein), the integrity of the RNA and DNA, concentration of the individual bands can also be analyzed simultaneously with this system. The basis of the system to perform multiple analysis simultaneously is that, one has to load the samples, standards, suitable ladder and the gel matrix in the different wells of a single microchip provided with the system and then the system primarily performs electrophoretic separation of all the samples loaded through the inbuilt micro-capillaries of the chip and thereafter, analyses the various parameters in one go within 30 min.

2.4.5.7 Synthesis of cDNA first strand by reverse transcription

Complementary DNA (cDNA) was synthesized using total RNA in a reaction catalyzed by the enzyme reverse transcriptase. The reactions were set up as per the manufacturer's guidelines. Reverse-Transcription PCR (RT-PCR) was performed on 1 µg of total RNA using AMV reverse transcription system (Promega, USA) with oligo (dT) primers in a 20 µL reaction volume. Experimental RNA was combined with the oligo $(dT)_{15}$ primer. The primer/template mixture was isothermally denatured at 70 ºC for 5 min and snap chilled on ice. A reverse transcription reaction mix was assembled on ice to contain nuclease-free water, reaction buffer, reverse transcriptase, magnesium

chloride, dNTPs and ribonuclease inhibitor RNasin®. As a final step, the template-primer combination was added to the reaction mix on ice and reaction was incubated at 42 ºC for up to 1-1.5 h. The reaction was terminated by enzyme denaturation at 70 ^{o}C for 15 min. The cDNA synthesized was diluted as per requirement and stored at -20 ^{o}C for further use and directly added to amplification reactions.

2.4.5.8 Polymerase Chain Reaction (PCR)

PCR is a powerful technique to amplify a desired nucleotide sequence using sequence specific primers. This amplification may be either of and from a single template or of a template from a mixture of templates (Arnheim and Erlich, 1992; Mullis, 1990; Mullis and Faloona, 1987; Saiki et al., 1985; Saiki et al., 1988). This technique has been successfully used for various purposes like fishing out of gene(s) from genomic DNA or from cDNA population, introducing restriction sites of interest in the amplified product for directional cloning (Scharf et al., 1986), creating sequence mismatch/ deletion/ addition resulting in mutant version of a gene or nucleotide sequence, differentiating between two alleles (Kwok et al., 1990) etc. In the present study applications of PCR were exploited for a few of the above specified applications. The PCR reaction mixture and generalized cycling conditions used were as follows:

Reaction mixture

Reagent and concentration	Volume
Sterile deionized water	9.5 µL
Template (100 ng/ µL)	1.0 µL
Forward primer (10 pmol/ µL)	1.0 µL
Reverse primer (10 pmol/ µL)	1.0 µL
dNTP mix (0.2 mM)	5.0 µL
10 X Buffer (Mg^{2+} 1.5 mM)	2.0 µL
Taq DNA Polymerase (1 U/µL)	0.5 µL
Total volume	20.0 µL

PCR cycle conditions

1 cycle of 94 ºC - 5 min

35 cycles of 94 ºC – 30 s; 50 $^{o}C^{\#}$- 30 s; 72 ºC - 1 min*

1 cycle 72 ºC 10 min (Final extension) and

4 ºC holds.

#- Annealing temperature depends on T_m of primers

* Extension time 1min/kb

The PCR conditions used to amplify various gene fragments have been mentioned in the respective sections. In this study PCR was used to isolate genes for 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), farnesyl pyrophosphate synthase (FPS) and squalene synthase (SQS) from *B. monniera* and designated as ***BmHMGR***, ***BmFPS*** and ***BmSQS*** respectively.

2.4.5.9 Rapid amplification of cDNA ends (RACE)

Generally, using reverse transcription PCR, either partial cDNA fragments (both 5' and 3' ends missing) or cDNA with full 5'end missing are amplified from total cDNA. If a partial cDNA sequence is known, unknown sequences to the 5' and 3' of the known sequence can be reverse transcribed from RNA, amplified by PCR using RACE (Frohmman *et al*., 1988). RACE ready cDNA Kit (Invitrogen, USA) was used to perform RACE. The reactions were set up as per the manufacturer's guidelines (GeneRacer Race kit, Invitrogen) with minor modifications. RACE PCR was used to isolate full-length genes *BmHMGR*, *BmFPS* and *BmSQS*. RACE PCR products were cloned in pGEM-T Easy vector and sequenced.

2.4.5.10 Full-length genes ORF amplification by PCR

The sequence information of 5'and 3' RACE PCR product clones were used for designing of primers from start and stop codon to obtain full-length of the genes. PCR was performed with Hi-fidelity *pfx Taq* DNA polymerase (Invitrogen) by using cDNA as a template. Full-length amplicons of all three genes (BmHMGR, BmFPS and BmSQS) were cloned in pGEM-T Easy vector and sequenced.

2.4.6 Quantitative real time PCR (qRT-PCR)

Quantitative real time PCR is a powerful tool for gene expression analysis and was first demonstrated by Higuchi et al. (1992, 1993). qPCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and is a preferred alternative to other forms of quantitative reverse transcriptase PCR that detect the amount of final amplified product at the end-point (Freeman et al., 1999; Raeymaekers, 2000). qPCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e., in real time) as opposed to the endpoint detection (Higuchi et al., 1992; Higuchi et al., 1993). In the present study the SYBR Brilliant® II QPCR Master Mix (Stratagene, USA) was used. This kit supports quantitative amplification and detection systems. The kit supports PCR amplifications and detection of a variety of DNA targets, including genomic DNA, plasmid DNA, and cDNA. The SYBR Brilliant II QPCR master mix includes SureStart® *Taq* DNA polymerase, a modified version of *Taq2000*™ DNA polymerase with hot start capability. A passive reference dye (an optional reaction component) is provided so as to control the final dye concentration, increasing the flexibility of the reagents for use with different platforms. The fluorescent dye SYBR Green I in the master mix enables the analysis of many different targets without having to synthesize target-specific labeled probes.

Real time PCR can be divided into four major phases: the linear ground phase, early exponential phase, log-linear (also known as exponential) phase, and plateau phase. During the linear ground phase (usually the first 10–15 cycles), PCR is just beginning, and fluorescence emission at each cycle has not yet risen above background. Baseline fluorescence is calculated at this time. At the early exponential phase, the amount of fluorescence has reached a threshold where it is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. The cycle at which this occurs is known as Ct in ABI Prism® literature (Applied Biosystems, Foster City, CA, USA) or crossing point (CP) in LightCycler® literature (Roche Applied Science, Indianapolis, IN, USA). This value is representative of the starting copy number in the original template and is used to calculate experimental results. During the log-linear phase, PCR reaches its optimal amplification period with the PCR product doubling after every cycle in ideal reaction conditions. Finally, the plateau

stage is reached when reaction components become limited and the fluorescence intensity is no longer useful for data calculation. In general, lower Ct value indicates higher initial copies.

2.4.6.1 qRT-PCR considerations

The optimal concentration of the upstream and downstream PCR primers is determined empirically. The optimal concentration is the lowest concentration that results in the lowest Ct and an adequate fluorescence for a given target concentration. The best concentrations of the upstream and downstream primers are not always of equal molarity. In this study, 120 nM was considered optimum. Reaction was standardized in such a way that there was no primer dimer formation. Acquisition of real-time data generated by SYBR Green was done as recommended by the instrument manufacturer. Data collection was either at the annealing step (3- step cycling protocol) or extension step of each cycle. Magnesium chloride concentration in the PCR reaction mix affects the specificity of the PCR primers and probe hybridization. The SYBR Green Brilliant® II QPCR Master Mix kit contains $MgCl_2$ at a concentration of 5.5 mM (in the 1X solution), which is suitable for most targets.

A passive reference dye may be added to compensate for non-PCR related variations in fluorescence. Fluorescence from the passive reference dye does not change during the course of the PCR reaction but provides a stable baseline to which samples are normalized. In this way, the reference dye compensates for changes in fluorescence between wells caused by slight volume differences in reaction tubes. The excitation and emission wavelengths of the reference dye are 584 nm and 612 nm respectively. The SYBR Green Brilliant® II QPCR Master Mix kit contains reference dye which is suitable for most targets.

2.4.6.2 Preparing the reactions and PCR cycling parameters

Real time PCR model Mx 3000P (Stratagene, USA) was used in the present study. The experimental reactions were prepared by adding the following components in order. Nuclease-free PCR-grade H_2O to adjust the final volume to 25 µL (including experimental cDNA)

Reagent	**Volume**
PCR grade water	10.5 µL
Primer Mix(optimized concentration, 120 nM)	1.0 µL
cDNA	1.0 µL
Total	12.5 µL

The reactions were mixed without creating bubbles and 12.5 µL of 2 X SYBER Green master mixes was added to each experimental reaction. Reaction was mixed gently and briefly spun. The reactions were placed in the instrument and a 2 step program was run. Two step cycling Protocol:

Temperature	Time	No. of cycles
95 ºC	10 min	1
95 ºC	30 s	40
*55 ºC	30 s	
72 ºC	30 s	

*Depending upon primer's T_m

Initial 10 min incubation was to activate the DNA polymerase completely. The temperature cycler was set to detect and report fluorescence during the annealing/extension step of each cycle. Amplicon dissociation curve was also recorded at the end of the PCR cycles. All reactions were run in triplicate and repeated twice. Relative expression of gene was analyzed using comparative Ct method ($2^{-\Delta\Delta Ct}$).

2.4.7 Expression and purification of recombinant protein

2.4.7.1 Directional cloning of genes in expression vector (pET 30 b+)

After restriction analysis appropriate restriction sites were introduced by PCR in all three gene ORFs and cloned in pGEM-T Easy vector. Inserts were excised from pGEM-T Easy clone plasmids and ligated in pET 30b (+) vector flanked with same restriction sites. Ligation mixtures were transformed in *E. coli* XL-10 and plasmids were isolated. Plasmids were mobilized in expression cell strain *E. coli* BL21 (DE3).

2.4.7.2 Heterologous expression in *E. coli*

Heterologous expression of *BmHMGR*, *BmFPS* and *BmSQS* genes was attempted in *E. coli* (BL21 DE3) host cell. The transformants were grown at 37 ºC in Luria–Bertani medium containing kanamycin (50 µg/mL). A single isolated bacterial colony from freshly streaked plates (grown on LB agar medium containing 50 µg/mL kanamycin) was used to inoculate 5 mL liquid LB medium containing the same concentrations of the antibiotic. Culture was grown overnight with shaking at 200 rpm at 37 °C. One mL aliquot of each culture was used to inoculate 100 mL liquid LB containing 50 µg/mL kanamycin. Once the cultures reached A_{600} 0.4 - 0.6, recombinant protein expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG), and the culture was grown for 4 to 6 h at 37 °C with shaking at 200 rpm. Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4 °C. Pellets were resuspended in 6.25 mL lysis buffer. Cells were disrupted by sonication for 5 mins at 70% amplitude on an ultrasonic liquid processor, XL 2000 model (MISONIX). $MgSO_4$, final concentration of 10 mM and lysozyme, final concentration 100 µg/mL was added to the disrupted cells and kept at 37 ^oC for 20 min. It was centrifuged at 10,000 rpm for 10 min at 4 ^oC and supernatant was saved as lysate and pellet was resuspended in 2 mL sonication buffer. Suspension was again sonicated for 1 min at 70% amplitude to disrupt the inclusion bodies and the disrupted inclusion bodies were dissolved in 3 mL of dispersion buffer and an aliquot of 20 µL checked on SDS PAGE to check heterologous expression of genes.

2.4.7.3 Optimization of *BmHMGR, BmFPS* and *BmSQS* expression in *E. coli*

Level of expression and solubility of recombinant proteins was optimized for different IPTG concentration (for induction), temperature (to grow the culture after induction) and duration of induction. Both IPTG concentration and temperature were reduced to increase solublization of proteins. Optimized conditions for all the genes have been mentioned in respective sections in later chapter.

2.4.7.4 Affinity purification of recombinant protein using Ni^+- NTA agarose beads

The recombinant protein, among several other bacterial proteins is loaded on affinity matrix column such as Ni^+-agarose. This affinity matrix contains bound metal ion nickel, to which the poly histidine-tag binds with micro molar affinity. The matrix is

then washed with buffer to remove unbound proteins. This can be achieved either by increasing the imidazole concentration in buffer or by lowering the pH of the washing and elution buffer. The column(s) and buffers were equilibrated to room temperature. The Ni^{+} beads column was equilibrated with binding buffer for 30 min and then three bed volume of binding buffer was passed through the column followed by addition of soluble protein. The column was kept at 4 ºC for 1 h for binding of recombinant protein to Ni^{+} beads on dancing shaker. After 1 h, beads were allowed to settle down and flow through was collected. Column was washed with 6-8 bed volume of washing buffer, until OD_{280} reaches to baseline. The washing stringency may be increase by increasing imidazole concentration and histidine-tagged proteins are usually eluted with 150-300 mM imidazole. The 6x His-tagged protein was eluted in 4 aliquots of elution buffer, 1 mL each. Protein elution was monitored by measuring the absorbance at 280 nm of collected fractions. The eluted protein was separated and analyzed by SDS-PAGE. The gel was stained with silver stain and protein concentration was determined by Bradford (Bio-Rad) method using BSA as the standard.

2.4.8 Polyacrylamide gel electrophoresis (PAGE)

PAGE system is the widely used electrophoresis system for protein separations (Laemmeli, 1970). The resolution in a Laemmeli gel is excellent because the treated peptides are stacked in a stacking gel before entering the separating gel.

2.4.8.1 Preparation of the separating gel

A vertical slab gel (GE Healthcare, Sweden) was assembled using 1.5 mm spacers. In a side armed vacuum flask, 10% separating gel solution was made as mentioned in section 2.3.6, except for the addition of ammonium persulfate and TEMED. The solution was degassed by applying vacuum while stirring on a magnetic stirrer. TEMED and ammonium persulfate were added, and mixed gently without generating bubbles. The solution was pipetted into the gel cassette leaving 1.5 cm from the top unfilled. The gel solution was overlaid with water saturated n-butanol to remove trapped air bubbles and to layer evenly across the entire surface. When a sharp liquid-gel inter-surface was observed after the gel polymerization, the slab was tilted to pour off the overlay.

2.4.8.2 Preparation of the stacking gel

Stacking gel solution was prepared as given in section 2.3.6, excluding ammonium per sulfate and TEMED. As in the separating gel, this solution was degassed. TEMED and ammonium per sulfate were added, mixed and overlaid on the separating gel. A comb was inserted taking care not to trap air bubbles beneath the comb teeth. The gel was left to polymerize.

2.4.8.3 Preparation of the sample

Equal parts of the protein sample and the loading buffer were mixed in a microcentrifuge tube and kept in a boiling water bath for 3-5 min for SDS-PAGE. Then the samples were centrifuged at 10,000 rpm for 2 min, supernatant collected and used for electrophoresis. Gel was run at room temperature at constant ampere 20 mA or 80 V. The protein samples for native PAGE were mixed with native dye and gel was run at 80 V at low temperature ranging from 4-10 ºC.

2.4.8.4 Loading of samples and running the polyacrylamide gel

Once well, have been formed in the gel, flushed it with distilled water and drained off completely. The wells were filled with tank buffer and the samples under laid using a syringe. Lower and upper buffer chambers were filled with tank buffer. Voltage was set between 70-80 units. The run was stopped when the dye reached the bottom of the polyacrylamide gel.

2.4.8.5 Coomassie blue staining of the gel

Coomassie blue staining solution: 45 mL Methanol, 10 mL acetic acid, 45 mL de-ionized water and 0.25% Coomassie blue (R 250). After running the gel it was transferred directly to a gel staining box (Tarsons, India) containing Coomassie blue staining solution and was kept for 2-3 h at room temperature on dancing shaker. Staining solution was poured off and de-staining solution was poured in. De-staining step was repeated two-three times till clear bands appeared.

2.4.8.6 Silver staining of the gel

Protein separated on SDS or native PAGE, was transferred to the fixer solution for 1 h. This was followed by 2 x 20 min washes in 50% ethanol. The gel was transferred to

sodium thiosulfate solution for 1 min and rinsed thrice with deionized H2O (20 s each). The gel was silver stained (HCHO was added to the solution just before use) for 20 min with intermittent shaking in dark. The gel was then rinsed thrice with deionized H2O (20 s each) and transferred to the developer till the bands developed. The gel was washed with deionized H2O and stored in fixer.

2.4.9 Raising polyclonal antibody against BmFPS and BmSQS in rabbit

The purified recombinant BmFPS and BmSQS proteins were used for raising polyclonal antibodies in rabbit (New Zealand White).

2.4.9.1 Pre-treatment of serum

Rabbit immune serum (stored at -80 ºC), containing polyclonal antibodies against the antigen, was thawed overnight at 4 ºC. The Serum was kept at 55 °C for 1h and the immune serum was centrifuged at 12,000 rpm for 15 min at 4 ºC. Aliquots (according to the requirements) were made and stored at -80 ºC. Prior to use, antiserum was thawed again overnight at 4 ºC.

2.4.9.2 Determination of titre of antibodies and ELISA

Buffers

1X Phosphate buffer saline (PBS): 8 gm NaCl; 1.44 g Na_2HPO_4; 0.24 g KCl; 0.200 g K_2HPO_4 in 1 L De-ionized water.

Wash Buffer (PBST): 1X PBS, 0.05% Tween 20 and 0.1% BSA

Antibody diluting buffer: 1X PBS and 0.25% BSA

Blocking reagent: 1% BSA in PBS

Substrate diluting buffer: 200 mM Tris-HCl, pH 9.5 and 0.5 mM $MgCl_2$

All reagents were prepared in sterile milliQ water.

ELISA was performed to determine the titre of first, second and third bleed of rabbit serum. Equal quantity of antigen (100 ng) was coated in triplicates on ELISA plates and kept overnight at 4 °C. Next morning, the plate was washed with 250 µL of PBST, three times for 5 min and 300 µL of blocking reagent was added. The plate was wrapped in aluminium foil and kept at 37 °C for 2 h. ELISA plate was washed again as described earlier and challenged to different dilution such as, 1:1000, 1:10000, 1:20000, 1:30000, 1:40000, 1:60000, and 1:100000 of serum and pre-immune serum

(100 µL) in separate wells. All dilutions were in triplicates and plate was kept at 37 °C for 2 h again. Plate was washed as described earlier with PBST buffer and secondary antibodies (goat anti-rabbit IgG-ALP conjugate) tagged with alkaline phosphatase was added to a dilution of 1:20000 (100 µL). After 2 h of incubation at 37 ^{o}C plate was washed thrice with 250 µL of PBST for 5 min and 100 µL of 1 mg/mL substrate (*p*-Nitro phenyl phosphate) was added and incubated for 45 min in dark at room temperature. Reaction was stopped by adding 10 mM EDTA and absorbance was taken at 405 nm. Once the antibody titre was determined then a fixed dilution of antibodies was used for rest of the experiments.

2.4.10 ELISA of BmFPS and BmSQS proteins in different tissues of *B. monniera*

Fresh *B. monniera* tissues (stem, leaf and root) were collected, frozen in liquid nitrogen and crushed to a fine powder. Crude protein was extracted with 2 mL of protein extraction buffer (Bicarbonate buffer). Total Protein was quantified using Bradford reagent. Equal amount of protein was coated on 96 well microtiter plates. Antigen (recombinant protein) was diluted in PBS to an optimal concentration and coated on 96 well micro-titer plate (100 µL/ well). Plate was incubated for 2 h at 37 ^{o}C or over night at 4 ºC and washed thrice with PBST after incubation. Non-specific sites were blocked with blocking buffer 300 µL/ well and incubated for 1h at 37 ^{o}C. After washing thrice with PBST, primary antibody (Anti rabbit IgG against BmFPS and BmSQS, 1:5000 dultion) was added (100 µL) and incubated for 1 h at 37 ^{o}C. The unbound primary antibody was washed thrice with PBST and secondary antibody (Goat anti-rabbit IgG-ALP conjugate, 1:20000) conjugated with alkaline phosphatase was added (100 µL) and incubated for 2 h at 37 ^{o}C. Enzyme specific substrate PNPP (1 mg/mL, 100 µL) was added and incubated for 45 min for the color development in dark and the absorbance was measured at 405 nm.

2.4.11 Protein blots (Western) analysis of BmFPS and BmSQS

Protein blot analysis was performed for analyzing the plant native FPS and SQS protein from the crude protein extracts of shoots. Total soluble protein was extracted in appropriate buffer (100 mM Tris-HCl, pH.8.0, 1% PVPP, 2% PEG 4000, DTT 5 mM and PMSF 1 mM) and 100 µg of total protein was separated on 10% SDS-PAGE. After complete separation protein was electro-transferred on to PVDF membrane as per

manual guidelines (iBlot gel transfer system, Invitrogen). WesternBreeze kit (Invitrogen) was used for further processing of blots as per instructions. In brief, blot was placed in 10 mL of blocking solution and incubated at room temperature for 30 min on rotatary shaker. The membrane was rinsed twice with 20 mL of sterile milliQ water and incubated with 10 mL of primary antibody solution (1:5000 dilutions, antibody against BmFPS and BmSQS) for 1 h. The membranes was washed thrice with 20 mL of antibody wash buffer followed by two washes with 20 mL of SMQ and incubated in 10 mL of secondary antibody solution for 30 min. The membrane washed again thrice with antibody wash buffer and rinsed with 20 mL of water twice. Signals were detected with ready to use BCIP/NBT solution (Calbiochem).

2.4.12 Multiple shoots regeneration and genetic transformation of *B. monniera*

2.4.12.1 Multiple shoots regeneration

The disease free, healthy young nodal segments of 5-6 cm of *B. monniera* were collected from green house at National Chemical Laboratory, Pune. They were washed under running tap water for 30 min in order to remove the external contaminants and treated with 10% v/v savlon for one min followed by washing with sterile distilled water for 2-3 times. They were treated with 70% v/v ethanol for few seconds in laminar air flow cabinet. Ethanol was quickly decanted and segments were washed thoroughly with sterile distilled water and then treated with 0.01% w/v mercuric chloride ($HgCl_2$) solution for one min followed by thorough rinsing with sterile distilled water for 3-4 times. Explants were prepared by cutting nodal segments into pieces of 1-2 cm length containing 2-3 nodes and inoculated on initiation medium and kept for 2-3 weeks. The cultures were then maintained by regular sub culturing on proliferation medium after every 30-35 days. Leaves produced from these shoots were used as explants for regeneration experiments by placing 2-3 longitudinal cuts on the leaf lamina. After 20 days, multiple shoots regenerated from leaf surface were transferred to proliferation medium for root induction. All media were based on MS basal medium (Murashige and Skoog, 1962), Initiation medium was composed of MS basal medium containing 0.5 mg/L BAP supplemented with 30 g/L sucrose, solidified with 8 g/L agar, and 20 ml of this medium was distributed in each of the culture tubes of 60 ml capacity, plugged with nonabsorbent cotton. Proliferation medium (PM) was composed of MS basal

medium supplemented with 30 g/L sucrose and solidified with 8 g/L agar. Media used for regeneration experiments using leaves as explants were composed of MS basal medium containing (1-2 mg/L) BAP and (0-0.2 mg/L) IAA with 30 g/L sucrose and 8 g/L agar and poured in sterile petriplates. All cultures were incubated under 16 h photoperiod, 60 μmol m^{-2} s^{-1} light intensity at 25 ±1 ºC.

2.4.12.2 Histological studies

The leaf explants (control and experimental) incubated on shoot regeneration medium (SRM) composed of MS basal medium containing 2 mg/L BAP and 0.2 mg/L IAA, supplemented with 30 g/L sucrose, solidified with 8 g/L agar, were collected at regular interval from the day of inoculation till the visible growth of the new shoot buds. The leaf samples were fixed in 5: 5: 90 v/v ratio of formalin/glacial acetic acid/ethanol solution. Fixed tissues were dehydrated through an ethanol-xylene series (3:1, 1:1, and 1:3 v/v). Tissues then were infiltrated with paraffin wax (Melting point 58-60˚C) initially at room temperature for 24 h followed by hot infiltration by keeping the samples in wax at 65 ^{o}C in hot air oven for 48-60 h till xylene was replaced by wax completely in the tissues. Paraffin blocks were made by embedding the tissues in paraffin wax and allowing solidifying. Sections of 15 μm thickness (Leica RM 2155 microtome, USA) were cut and stained with 1% w/v safranin and 1% w/v light green prior to microscopic observation. Observations were taken under Axioplan microscope (Carl Zeiss, USA). The regenerating leaf explants were observed under APO 11 microscopes (Carl Zeiss). The digital photomicrography was done using Axiocam MRC5 system for both the microscopes.

2.4.12.3 Plasmid vector and *Agrobacterium tumefaciens* preparation

Agrobacterium tumefaciens, strain GV2260 harboring binary vector pCAMBIA1301with gene of interest (BmHMGR, BmFPS and BmSQS) was used for genetic transformation. This vector contains a reporter gene namely β–Glucuronidase (*gus*) and selectable marker gene, hygromycin phosphotransferase (*hpt*II), imparting resistance to hygromycin B under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter to confirm transgenic expression. The transformed *A. tumefaciens* was inoculated in YEB medium containing 50 mg/L rifampicin and 50 mg/L kanamycin and incubated at 28 ^{o}C for overnight with constant agitation at 200

rpm. The culture having A_{600} of 0.5-1.0 was used for the genetic transformation of leaf explants of *B. monniera*.

2.4.12.4 *Agrobacterium* mediated transformation and selection

Leaf explants were aseptically excised from the micro shoots. 2-3 longitudinal cuts were made on leaf lamina and co-cultivated with overnight grown culture of *A. tumefaciens* strain GV2260 harboring binary vector pCAMBIA1301 with gene of interests, for 15 min. Explants were subsequently blotted on a sterile Whatman filter paper to remove excess of the bacterial suspension and transferred to SRM. Co-cultivation was carried out for two days under same culture conditions as described above.

After 2 days of co-cultivation period with *Agrobacterium*, explants were washed with sterile distilled water containing 200 mg/L cefotaxime to remove most of the bacteria, blotted dry on sterile Whatman filter paper and cultured on selection medium SRM supplemented with 200 mg/L cefotaxime and 10 mg/L hygromycin B for 10-15 days under the same culture conditions. After 4 weeks on selection medium, hygromycin resistant plants were transferred to proliferation medium.

2.4.13 Analysis of transgenics

2.4.13.1 PCR with *hpt*II primers

Total genomic DNA was extracted from young leaves of transformed plant and non transformed control plant by using plant DNA extraction kit (Himedia, Hipura Plant Genomic Purification. Putative transformants were screened by PCR for the presence of *hpt*II gene. The *hpt*II gene forward and reverse primer sequences were 5´ TCCTGCAAGCTCCGGATGCCTC-3´ and 5´-CGTGCACAGGGTGTCACGTTGC-3´. The components of PCR reaction mixture were: template DNA, 0.2 mM of dNTPs, *hpt*II gene specific forward and reverse primers (500 nM each), 1 U of *Taq* DNA polymerase and 1X reaction buffer, in a total volume of 20 μL. The PCR reaction was carried out in following conditions, initially denatured at 94 ^{o}C for 5 min, followed by 35 cycles at 94 ^{o}C for 30 s, 55 ^{o}C for 30 s, 72 ^{o}C for 1 min 20 s, and a final extension cycle of 72 ^{o}C for 5 min. The PCR amplified product for *hpt*II gene was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

2.4.13.2 GUS histochemical assay

Histochemical assay was performed on the hygromycin resistant leaves of putative transgenic plants to detect the β–Glucuronidase activity. GUS activity was analyzed histochemically as described in Jefferson et al. (1987). For the histochemical detection of the *gus* reporter gene, hygromycin resistant leaves were incubated overnight at 37 ^{o}C, in sodium dihydrogen phosphate buffer (100 mM, pH 7) containing the X-Gluc (1 mM) and Tween-20 (05 %). Stained material was cleared in 70 % (v/v) ethanol for 3-4 h to remove chlorophyll. Non transformed leaves were also included as a control assay against background staining.

2.4.14 Bacosides estimation (HPLC)

Analysis of the plant extracts were done by using reversed-phase HPLC (Perkin Elmer series 200) on C18 column (Supelco Analytical, 5µm, 15 cm x 4.6 mm). The dried coarsely powdered plant material was extracted with HPLC grade methanol for 48 h. The extract was filtered through Whatman (No. 1) filter paper and dried under vacuum. The dried extract was dissolved in DMSO and used for HPLC analysis. Detection was done at 205 nm. Mobile phase consisted of sterile milliQ water (SMQ) with 0.1% TFA and was programmed as follows for 40 min run: Flow rate - 1mL/min; 10% acetonitrile for 5.0 min, 30% acetonitrile for 15.0 min and 35% acetonitrile for 20.0 min.

CHAPTER: 3

ISOLATION, CLONING AND CHARACTERIZATION OF TERPENOIDS BIOSYNTHETIC PATHWAY GENE (S)

Chapter 3. Isolation, Cloning and Characterization of Terpenoids Biosynthetic pathway gene (s)

This chapter deals with different strategies used for isolation of full-length cDNA clones of terpenoid biosynthetic pathway genes, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), farnesyl diphosphate synthase (FPS) and squalene synthase (SQS) from *B. monniera* and characterization of isolated genes.

3.1 Introduction

Plants synthesize various kinds of secondary metabolites like terpenoids, carotenoids, flavonoids, phytosterols, saponins, phytoestrogens, glucosinolates and much more. Plant secondary metabolites can boost the immune system, protect the body from free radicals, and kill pathogenic germs. In plants several important classes of terpenoid compounds are synthesized via the mevalonate pathway. The terpenoids sometimes referred to as isoprenoids represent the largest group of biologically active metabolites with at least 30000 chemical compounds. Plant isoprenoids are essential for the numerous physiological and developmental processes in plants and many plant isoprenoids are of considerable commercial interest as pharmaceuticals, flavors and fragrances or food colorants. Understanding the regulation of isoprenoid synthesis is therefore of immense scientific and commercial interest. In today's world medicinal plants are very important, as they contribute to about 80% in global economy in the form of plant or plant extracts (Dhyani and Kala, 2005).

As discussed in chapter one, *B. monniera* is an important drug in Ayurvedic medicine system for the improvement of intelligence, memory and revitalization of sensory organs. The active medicinal principle compounds of this plant are **terpenoid saponins** collectively known as bacosides and different types of alkaloids. Biosynthesis of these compounds takes place via isoprenoid pathway. Some important genes such as HMG-CoA reductase, FPP synthase, squalene synthase etc., play an important role in biosynthesis of secondary metabolites and considered as key steps. The details of these genes have been already discussed in chapter one.

The present chapter deals in detail regarding isolation, cloning and characterization of three key steps genes namely HMG-CoA reductase, FPP synthase and squalene synthase from *B. monniera*. There is considerable interest in understanding role of these

genes in bacosides biosynthesis which will facilitate future metabolic pathway engineering for development of elite plant species with improved bacoside contents.

Isolation of a gene is the pre-requisite for its thorough study or characterization. To isolate any gene of interest generally there are two major approaches as follows:

1. Genomic/cDNA library screening- It is the classical method of gene isolation in which genomic/cDNA libraries are screened with homologous or heterologous probe. After 2-3 rounds of screenings, the plaques showing strong positive signals are excised and the DNA fragment sequenced.
2. PCR- It is one of the most popular approaches for gene isolation because of its simplicity and rapidity. Forward and reverse primers are designed on the basis of available sequences in the database. PCR is performed using these primers and genomic/cDNA as template. The amplicon is sequenced to confirm its identity.

In the present study, PCR based approach was followed to fish out the terpenoid biosynthetic pathway gene(s) from *B. monniera*.

3.2 Materials and methods

3.2.1 Bacterial strains and plasmids used in the study

Escherichia coli XL-10 Gold (Stratagene, USA)

pGEM-T Easy Vector Cloning vector (Promega, USA)

3.2.2 RNA isolation and cDNA first strand synthesis

Total RNA was isolated from *B. monniera* leaves and 1 µg of total RNA was used for first-strand cDNA synthesis using oligo $(dT)_{15}$ primer and AMV-RT (as mentioned in Chapter 2). cDNA was stored at -20 ^{o}C and further used for PCR reactions.

3.2.3 Polymerase Chain Reaction (PCR)

PCR amplification was done using cDNA 1st strand as template. Amplified PCR products were eluted from agarose gel and used for ligation into T/A cloning vector pGEM-T Easy. The PCR program is as follows:1 cycle of 94 ^{o}C for 5 min; 35 cycles of 94 ^{o}C 30 s, 50-58 ^{o}C for 30 s, 72 ^{o}C for 1 min 30 s and a final extension of 72 ^{o}C for 7 min.

3.2.4 Transformation and selection

The ligation mixture was used for transformation of *E. coli* XL10 GOLD, host cell strain. Putative transformants were selected using antibiotic ampicillin and Blue-white screening by adding X-gal and IPTG. Colony PCR of white colonies with primers T7 (5'-TAATACGACTCACTATAGGG-3') and SP6 (5'-ATTTAGGTGACACTATAGAA-3') was done to screen the putative transformants. Plasmids were isolated from clones showing positive colony PCR results and subjected to restriction digestion. The plasmids with expected insert release were further sequenced to confirm its identity with reported sequences available at NCBI GenBank database.

3.2.5 Bioinformatic and phylogenetic tree analysis

The nucleotide sequences available in the NCBI GenBank database were aligned and multiple sets of primers were designed from the conserved regions. Nucleotide and amino acid sequence analysis was done using software pDRAW 32, ClustalX 2.0 and online bioinformatics analysis facility available at www.justbio.com, www.expasy.org and www.ncbi.nlm.nih.gov. Various physical and chemical parameters of proteins were predicted by using ExPasy tool ProtParam (http://web.expasy.org/protparam/). Multiple sequence alignments of the amino acid sequences were carried out with the ClustalW 2.0 program (http://www.ebi.ac.uk/clustalw/). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analysis was conducted in MEGA4.0.2 (Tamura et al., 2007).

3.2.6 Rapid amplification of cDNA ends (RACE)

RACE was done to isolate the full-length genes with its 5' and 3' UTRs. The PCR conditions are as follows:

Primary PCR

Temperature	Time	Cycles
94 ^{o}C	3 min	1 cycle
94 ^{o}C	30 s	10 cycles
70 ^{o}C	*1 min 30 s	
94 ^{o}C	30 s	25 cycles
$^{\#}$60 ^{o}C	30 s	
72 ^{o}C	*1 min 30 s	
72 ^{o}C	10 min	1 cycle

Annealing temperature may vary according to T_m of primers. *1 min/kb DNA extension.

Secondary (Nested) PCR

Temperature	Time	Cycles
94 ^{o}C	5 min	1 cycle
94 ^{o}C	30 s	35 cycles
65 ^{o}C	30 s	
72 ^{o}C	*1 min 30 s	
72 ^{o}C	10 min	1 cycle

*1 min/kb DNA extension.

3.2.7 Sequencing

DNA sequencing was performed with the ABI Prism Big-Dye Terminator Cycle Sequencing Kit on the ABI Prism 3730 DNA analyzer (Applied Biosystems) at GenomeBio Biotech Pvt. Ltd., Pune, Maharashtra, India.

3.3 Results and discussion

3.3.1 Total RNA isolation and cDNA synthesis

Total RNA was isolated from aerial part of *B. monniera* plant and cDNA synthesis was done using protocol as mentioned in materials and methods chapter.

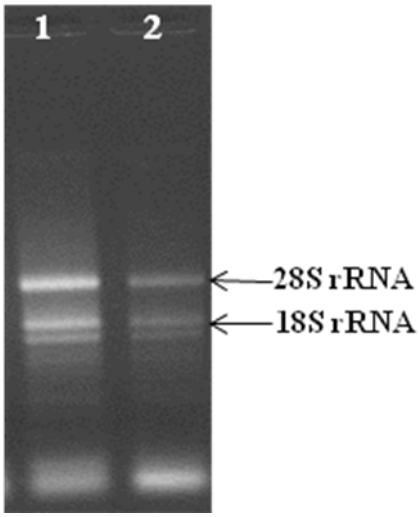


Fig. 3.1: Total RNA isolated from aerial tissue (Lanes 1 & 2) of *B. monniera*, electrophoresed on 1% agarose gel.

3.4 PCR based approach for the isolation of terpenoid biosynthetic pathway gene(s)

3.4.1 HMG-CoA reductase: Its isolation, cloning and characterization

3.4.1.1 Multiple sequence alignment of HMG-CoA reductase nucleotide sequences from different plants

HMG-CoA reductase gene nucleotide sequences available at NCBI GenBank database were aligned using Clustal W program. Different set of primers were designed from conserved regions, as shown in figure in red and green colours (**Fig. 3.2**).

```
S.miltiorrhiza      ATGACGACGAAAATGGAGAAAGAGGCGGCGGCCAAG---------------------- 36
A.majus             ATGATGAGGATGAGGATGGAAGGG-CAGCGGACGAGGAAGGGCGATTCTGATTCTGATTC 59
                    **** ** **:.* *.:*.***.* *.****.*.**

                                                                    HMG F1
S.miltiorrhiza      ----------------GCCTCGGACGCACTCCCCCTCCCTCTCTACCTCACCAACGGAGT 80
A.majus             GGGCGTGATTATTAAAGCATCGGACGCCCTGCCTCTTCCGCTGTACCTGACCAACGGCGT 119
                              **.********.** ** ** ** ** ***** ********.**

                                                                    HMG F2
S.miltiorrhiza      CTTCTTCACCCTCTTCTTCTCCGTCGTCTACTTTCTCCTCCTCCGCTGGCGTGAGAAGAT 140
A.majus             GTTCTTCACTCTCTTCTTCTCGGTGGTGTATTATTTGCTCCTCCGATGGCGAGAGAAGAT 179
                     ******** ********** ** ** ** *:* * ********.*****:********
```

```
S.miltiorrhiza      CCGCAATTCCGTCCCCCTCCACGTCGTCACCCTCTCCGAGATCTCCGCCATCGTCGTCTT 200
A.majus             CCGAAACTCCACCCCCTCTCCATGTCGTCACCCTCTCCGAATTAGCCGCCATCCTCACCTT 239
                    ***.** ***. *** ***** *****************.:*. ******** **. ***

S.miltiorrhiza      CGTCGCCTCCTTCATCTATCTCCTCGGCTTCTTCGGCGTCACCTTCGTGCAGTCCCTCGT 260
A.majus             CCTCGCCTCCTTCATCTATCTCCTTGGCTTCTTTGGCATCGGCTTTGTTCAGTCCCTTAT 299
                    * ********************** ******** ***.**. *** ** ******** .*

S.miltiorrhiza      CATCCCCCG--CTCCCCCAGCG------------ACGAGATTTTGGACGACGACGACGA 305
A.majus             CATTCCCCGAGCTTCCCATTTGGATTTAGATTTGGACTTGGATTTGGATGAAGGGGAAGA 359
                    *** *****  ** ***.:  *            ** :*.:****** **.*. **.**

S.miltiorrhiza      GATCATCG-------ACGACTTGATG-------------------CTCAAGGAGGACTC 338
A.majus             ATTCAAAATTAGAAAACAAGTTGTTGTTGATGATGATCGCCTCCTGCTCAAGGAGGACTC 419
                    .:***:..       **.* ***:**                   ************

S.miltiorrhiza      CCGCGCCGCCCCCTGCAGCGCCGCCGCA----------------CCCTGCAAGATG---- 378
A.majus             ACGTACCTCCCCCTACGCTGCTGCTGCTGCTGCTGCTGCTCCCCAAATCTGATGATAA 479
                    .** .** ******.*.  ** ** **:               **.:. .:****

S.miltiorrhiza      -------TTACCCGCTGAGCCGCTCCCGGAGGAAGACGAGGAGATCGTCAAGGCCGTGGT 431
A.majus             GATTCCTATTCCCCCTTTGGTGTTGGACGATGAAGACGAGGCGTTTGTGAGGTCCGTGGT 539
                           :*:*** ** :*  * *  . ** **********.*:* ** *.* *******

S.miltiorrhiza      GGAGGGGAAGATCCCTTCCTACGCCCTGGAGTCCAAGCTGGGAGACTGCCGCCGCGCCGC 491
A.majus             TGAGGGCAAAATCCCCTCATACGCCCTCGAATCCAAGCTTGGCGATTGCCACCGTGCCGC 599
                     ***** **.***** **.******** **.******** **.** ****.*** *****

S.miltiorrhiza      CTTTGTCCGCCGCGAGGCCCTGCAGCGCACCACCGGAAAGTCCCTGGAGGGGCTTCCCCT 551
A.majus             TTCCATACGCCGTGAGGCCCTGCAGCGCATTACAGGAAAGTCCTTGGCTGGAATGCCCCT 659
                     *  .*.***** ***************  **.********* ***. **..* *****

S.miltiorrhiza      TGAAGGGTTCAACTACGAGGCCATACTGGGGCAGTGCTGCGAGATGCCGGTCGGGTACGT 611
A.majus             CGAGGGCTTCGACTACGAGGCCATACTCGGGCAGTGCTGCGAGATGCCCGTGGGGTACGT 719
                     **.** ***.**************** ******************** ** ********
```

HMGR1

```
S.miltiorrhiza      GCAGATTCCGGTGGGCATCGCCGGGCCGTTGTTGCTGGACGGGGTCGAGTATTCGGTCCC 671
A.majus             GCAGATCCTCGTGGGCATTGCGGGCCCTCTCCTTCTCGACGGCNGGGAGTACTCCGTCCC 779
                    ****** *  ******** ** ** **  *  * ** ***** .  ***** ** *****

S.miltiorrhiza      GATGGCGACGACGGAGGGGTGCCTGGTGGCGAGCACAAACAGAGGGTGCAAGGCCATCAT 731
A.majus             GATGGCCACCACAGAGGGGTTGCTTTGTGCCAGCACCAACAGGGGATGCAAAGCCATATA 839
                    ****** ** **.*******  **    ** *****.*****.**.*****.*****.::

S.miltiorrhiza      GGCGTCGGGAGGCGCGAGCAGCGCCGTCCACCGGGACGGAATGACGCGCGCCCCCGTCGT 791
A.majus             CGCCTGCGGAGGGGCCACCAGCGTCCTCCTCAGAGACGGAATGACCAGGGCTCCCGTTGT 899
                     ** *  ***** ** * ***** * ***:*.*.*********** .* ** ***** **

S.miltiorrhiza      CAGATTCGGGTCGGCGAAGCGGGCCGTGGAGCTGAAGCTCTTCCTAGAGGATCCTCTCAA 851
A.majus             TAGGTTCGCCACCGCCAAGCGGGCTGCTGACTTGAAGTTCTTCCTCGAGGACCCTCTCAA 959
                     **.****  :* ** ******** *  **  ***** *******.***** ********

S.miltiorrhiza      CTTCGAGTCCCTCTCCCTAGTGTTCAACAGCTCGAGCCGCTTTGGGAGGCTGCAGAAGAT 911
A.majus             CTTCGACACGTTGTCCCTTGTCTTCAACAGTTCCAGCCGATTCGCCAGGCTCCAGACCAT 1019
                    ****** :*  * *****:** ******** ** *****.** *  ***** ****. **
```

HMG F3

```
S.miltiorrhiza      CAAGTGCGCCATCGCGGGCAAGAATCTGTACATGAGGTTCACCTGCAGCACGGGCGACGC 971
A.majus             CAAGTGCGCTGTTGCTGGCAAGAATCTCTATATCAGGTTCTCCTCGAGCACGGGGATGC 1079
                    ********* .* ** ********** ** ** ******:***  ******** ** **

S.miltiorrhiza      CATGGGCATGAACATGGTCTCTAAGGGCGTGCAGAACGTGTTGGACTTCCTCGTCAACCA 1031
A.majus             CATGGACACTGACATGGTCTCAAAGGGGGTGCAGAACGTCATGGATTTCCTAACCAACGA 1139
                    *****.**  .*********:***** ********** :**** *****.. **** *

S.miltiorrhiza      GTTCCCCGACATGGACGTGCTCGGCATCTCCGGAAACTACTGCTCCGACAAGAAGCCCGC 1091
A.majus             GTTCCCTGACATGGATGTCATTGGCATCTCTGGAAACTATTGTTCCGACAAGAAACCAGC 1199
                    ****** ******** **  .* ******** ******** ** **********.**.**

S.miltiorrhiza      CGCGGTCAATTGGATCGAAGGGCGGGGCAAGTCCGTGGTGTGCGAGGCCGTCATCAAAGA 1151
A.majus             TGCGGTGAACTGGATTGAAGGGCGGGGCAAGTCAGTGGTGTGTGAGGCAATCATTAAGGA 1259
                     ***** ** ***** *****************.******** *****..**** **.**

S.miltiorrhiza      GGAGGTGGTGCACAAGGTGCTCAAAACTAACGTCGCCGCCCTCGTGGAGCTCAACATGCT 1211
A.majus             AGAGGTTGTGAACAAGGTACTCAAGACTAGCGTTGCTTCTCTGGTGGAGCTCAACATGCT 1319
                    .***** ***.********.*****.****.*** **  * ** ****************

S.miltiorrhiza      CAAGAACCTCACCGGCTCCGCCATCGCCGGAGCCCTCGGGGGCTTCAACGCCCACGCCAG 1271
A.majus             CAAGAACCTCACTGGTTCAGCCATGGCTGGAGCTTTGGGTGGCTTCAATGCTCATGCCAG 1379
                    ************ ** **.***** ** *****  * ** ******** ** ** *****

S.miltiorrhiza      CAACATCGTCTCCGCCGTCTACATCGCCACGGGCCAGGACCCCGCACAGAACGTGGAGAG 1331
A.majus             CAATATTGTCTCCGCCATCTTCATCGCTACGGGTCAGGATCCCGCACAAAATGTCGAGAG 1439
                    *** ** *********.***:****** ***** ***** ********.** ** *****

S.miltiorrhiza      CTCGCACTGCATCACCATGATGGAAGCCGTCAACGGCGGCAAGGACCTCCACGTGTCAGT 1391
A.majus             TTCTCACTGCATCACCATGATGGAAGCCGTGAATGACGGAAGGGACCTTCACATCTCTGT 1499
                     ** ************************** ** *.***.*.****** ***.* **:**

S.miltiorrhiza      GAGCATGCCGTCCATCGAGGTCGGCACCGTCGGAGGCGGCACTCAGCTGGCGTCGCAGTC 1451
A.majus             CACAATGCCTTCCATTGAGGTTGGTACTGTCGGAGGTGGGACTCAACTGCCCTCTCAATC 1559
                     * .***** ***** ***** ** ** ******** ** *****.*** * ** **.**
```

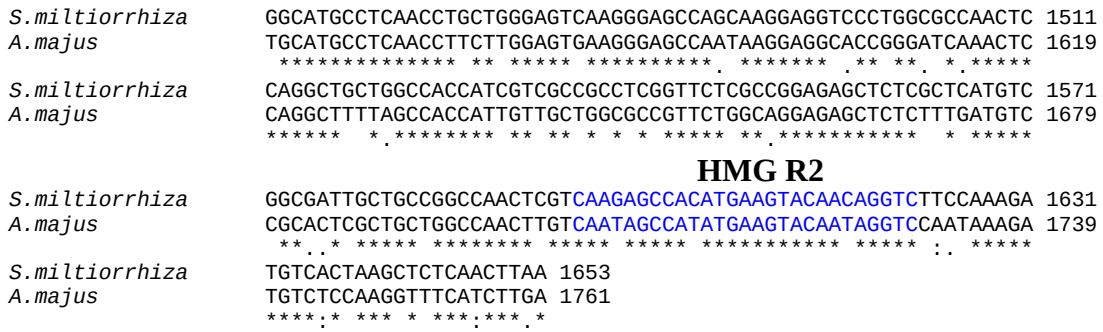


Fig. 3.2: Multiple sequence alignment of HMGR nucleotide sequences of *Salvia miltiorrhiza* (Acc. No. FJ747636) and *Antirrhinum majus* (Acc. No. EF666139) Highlighted regions are considered for primer synthesis (Red colored-Forward and Blue colored- Reverse primers).

Table 3.1: Primers used for isolation of partial HMGR cDNA

Gene Name	**Forward and Reverse primers (5'→3')**	
HMG CoA-Reductase (HMGR)	HMG F1	CTCTCTACCTCACCAACGG(A/C)GT(C/G)TTC
	HMG F2	CG(A/C)TGGCGAGAGAAGATCCGAAAC
	HMG F3	GGCGACGCCATGGGCATGAACATG
	HMG R1	TCTGTGGTGGCCATCGGGAC
	HMG R2	GACCTATTGTACTTCATATGGCTATTG

3.4.1.2 PCR amplification of partial cDNA of *HMGR* gene from *B. monniera*

Two primer sets i.e. HMG F2-HMGR1 and HMG F3-HMG R2 (as mentioned in table 3.1) gave amplifications of ~600 bp fragments of *HMGR* gene using cDNA 1st strand as a template (**Fig. 3.3 A & B**). The fragments were cloned in pGEM-T Easy vector (**Fig. 3.4**) and confirmed by restriction analysis (**Fig. 3.5A & B**) and sequencing (**Fig. 3.6A & B**). These partial clones were named as HMG F2/R1 and HMG F3/R2 respectively.

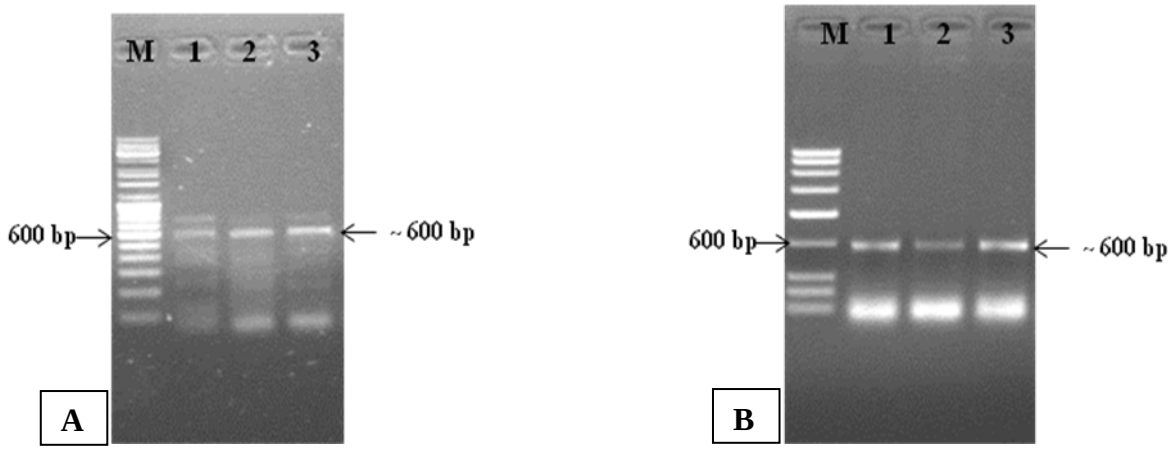


Fig. 3.3: A. PCR amplification product of ~600bp (HMG F2/R1) fragment of HMGR gene separated on 1% agarose gel. Lane 1-3: Amplified product and lane M- Medium range DNA ruler. **B.** PCR Amplicon of ~600bp (HMG F3/R2) of HMGR gene on 1% agarose gel. Lane 1- 3 amplified product and lane M- Low range DNA ruler.

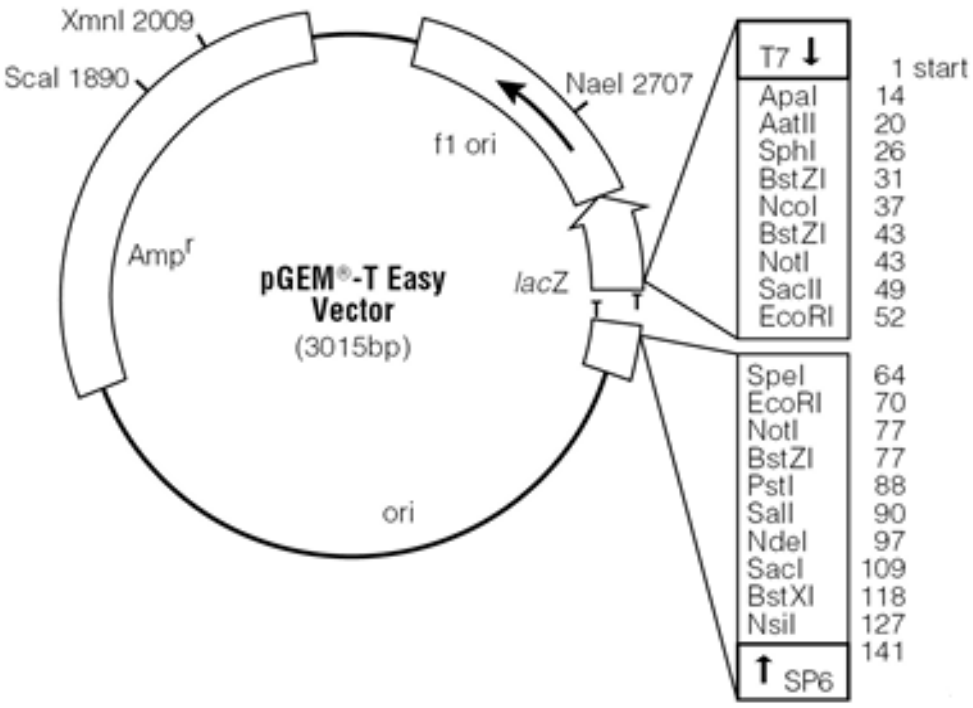


Fig. 3.4: Map of pGEM-T Easy vector.

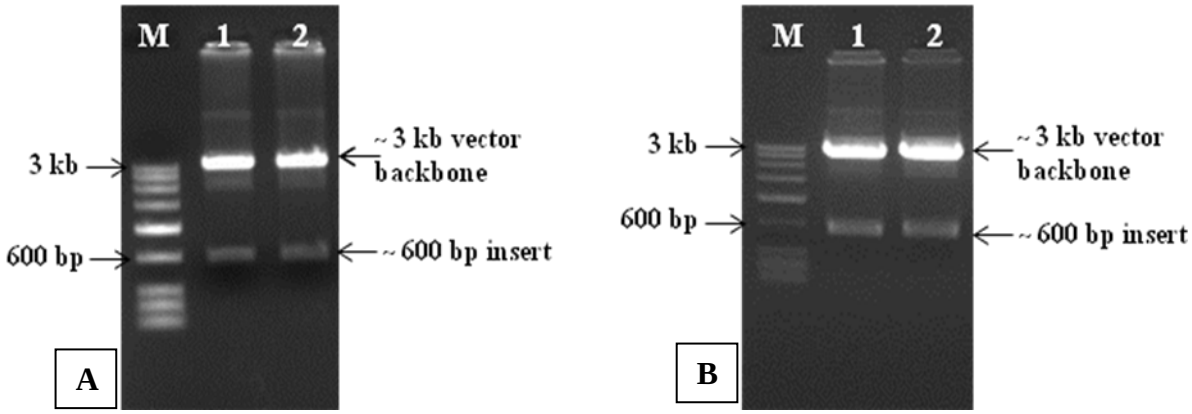


Fig. 3.5: A. Partial clone of HMGR (HMG F2/R1) gene in pGEM-T Easy vector releasing ~600 bp insert when digested with *Eco*RI, lane 1& 2: cloned ~600bp insert. **B.** Partial clone of HMGR (HMG F3/R2) gene digested with *Eco*R1, lane 1 & 2- Digested product, lane M- Low range DNA ruler.

After confirmation by restriction digestion, 6-8 plasmids from both clones were sequenced. Sequencing result revealed that fragment HMG F2/R1 and HMG F3/R2 were 598 bp and 657 bp respectively (**Fig. 3.6**).

A. HMG F2/R1

```
  1 CGATGGCGTG AGAAGATCAG AAACTCCACC CCCCTCCACG TCGTCACCCT
 51 TTCTGAAATC GCCGCCATCG TCACCTTCGT TGCCTCCTTT ATTTATCTCA
101 TTGGTTTCTT TGGCGTTGGA TTTGTTCAAT CCCTAATCAT TCCCCGTGCT
151 TCCCACGACG ATATCCTTGG GGAAGATGAC GAAATTGTTG ATGGAGATCT
201 AGATCGCCAG ATGCTGAAGG AGGATTCCCG CTCCGCCCCT TGCGCCGCCG
251 CGCTTCCATC CGTGGATTCT GGCAAGCTTG CTTCTCCCAG ATCCAAGGTC
301 CACGGCGGTG ATCTAATCGT CACGCCTCCG GCGTCGGAGG AAGATGAGGA
351 GATAATCAAA TCGGTGGTGG AGGGCAAGAC TCCTTCTTAT TCACTCGAAT
401 CGAAGCTTGG GGATTGCCGT CGCGCCGCCG CCATCCGCCG TGAGGCTTTG
451 CAGCGTACGA CCGGAAAATC CCTTGACGGG CTTCCCCTCG ATGGTTTTAA
501 CTACGATTCA ATATTGGGTC AGTGCTGCGA GATGCCGGTG GGATACGTCC
551 AGATTCCGGT GGGCATCGCC GGCCCTCTGT CCCGATGGCC ACCACAGA
```

B. HMG F3/R2

```
  1 GGCGACGCCA TGGGCATGAA CATGGTCTCA AAGGGAGTAC AGAACGTCAT
 51 GGATTTCCTC AACAAAGAGT TCCCCGACAT GGACGTCATC GGAATTTCAG
101 GCAACTACTG CTCCGACAAG AAACCGGCGG CGGTGAACTG GATTGAAGGC
151 AGGGGAAAGT CAGTTGTGTG TGAAGCCATA ATCAAAGAAG ATGTGGTCAA
201 GAAAGTCCTC AAGACCGATG TTGCTTCATT GGTTGAGCTC AACATGCTTA
251 AGAACCTCAC CGGCTCCGCC ATGGCCGGAG CTCTGGGTGG TTTCAACGCT
301 CACGCCAGCA ATATAGTCTC TGCCGTCTAC ATCGCCACTG GACAAGATCC
351 CGCACAGAAC GTCGAGAGCT CTCACTGTAT CACAATGATG GAAGCCGTCA
401 ATGATGGGAA GGATCTTCAT ATCTCTGTCA CTATGCCTTC CATTGAGATC
451 GGGACAGTCG GAGGTGGGAC CCAATTGGCG TCTCAGTCGG CATGCCTAAA
501 CCTGCTTGGA GTGAAGGGAG CCAGTAAAGA TGCTCCAGGG TCGAACTCCC
```

551 GGCAGCTGGC CACCATTGTT GCCGGGGCGG TTCTCGCCGG AGAGCTGTCT
601 CTGATGTCGG CTTTGGCCGC AGGACAACTG CAATAGCCAT ATGAAGTACA
651 ATAGGTC

Fig. 3.6: A & B. Nucleotide sequences of partial *HMGR* gene from *B. monniera*. Primers used for 5' (HMG F2/R1 sequence) & 3' (HMG F3/R2) RACE are underlined.

BLAST analysis of both the partial sequences showed significant similarity with reported *HMGR* nucleotide sequences at NCBI GenBank database.

3.4.1.3 Rapid amplification of cDNA ends (RACE)

RACE is an important tool to obtain the UTRs (Un-Translated Regions) of a particular gene and is also useful to obtain full-length gene. The limitation of RACE PCR is partial sequence of the gene must be known to design gene specific primers.

Primers for 5' and 3' RACE

Based on the sequence of partial HMGR gene fragments, primers for 5' RACE and 3'RACE were designed from HMGF2/R1 and HMG F3/R2 clone sequences respectively (Underlined in **Fig. 3.6 A & B**).

RACE HMG R 5'-GAGCGGGAATCCTCCTTCAGCATC-3'
RACE HMG NestedR 5'-CTAGATCTCCATCAACAATTTCGTC-3'

Primers supplied with kit:

5'GeneRacer primer (5'GRP) 5'-CGACTGGAGCACGAGGACACTGA-3'
5'GeneRacer nested primer (5'NGRP) 5'-GGACACTGACATGGACTGAAGGAGTA-3'

RACE HMG F 5'- CTCACTGTATCACAATGATGGAAGCC-3'
RACE HMG NestedF 5'- GGGAAGGATCTTCATATCTCTGTCAC-3'

Primers supplied with kit:

3'GeneRacer primer (3'GRP) 5'-GCTGTCAACGATACGCTACGTAACG-3'
3'GeneRacer nested primer (3'NGRP) 5'-CGCTACGTAACGGCATGACAGTG-3'

3.4.1.3.1 5' RACE PCR

In order to get the 5' region of *HMGR* gene, 5' Rapid amplification of cDNA ends (RACE) PCR was performed as per manual instructions. Primary PCR was done with RACE HMG R & 5'GRP primers and 5'RACE ready cDNA as a template. The

primary PCR product was diluted (1:50) and used as a template for secondary PCR with primers RACE HMG NestedR and 5' NGRP. Approximately 550 bp secondary PCR product (**Fig. 3.7**) was cloned in pGEM-T Easy vector and sequenced.

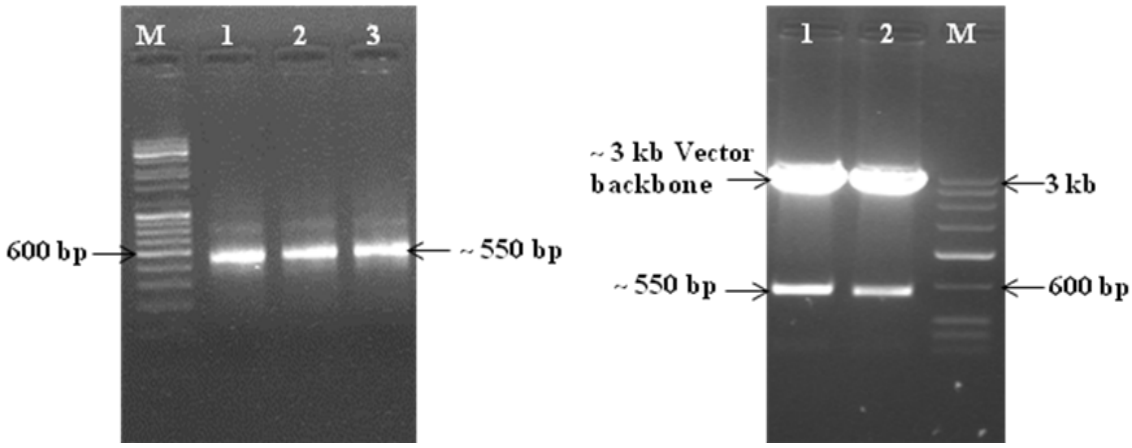


Fig: 3.7: A. 1 % agarose gel showing 5' RACE product of *HMGR* gene. Lane M- Medium range DNA ruler, lane 1-3- ~550 bp 5' RACE product. **B.** Restriction digestion with *Eco*RI. Lane 1 & 2- Showing vector backbone (~3 kb) and release of insert (~550 bp), Lane M- Low range DNA ruler.

The analysis of the sequenced 5' RACE product of *HMGR* gene revealed 132 bp 5' UTR regions, which is shown highlighted in red and the start codon (ATG) is highlighted in green (**Fig. 3.8**).

```
  1 GGACACTGAC ATGGACTGAA GGAGTAGAAA ATCCAAATCC ACTTTCCAAT
 51 TCTAACACAT TTCTGCAACA TATTCCTCCA ATATCATATC AGAAACTACA
101 AATCGACCTT AATTTTTCCC CTTTCTCCCG ATCTGCCGTG GCCGGGAAAA
151 AGGCGGCCAT GGAGGCTAAG GGACGATCCA TGAAATTGAA AGGCGGTGCC
201 CTTGATCGGA AGCTTCAACG CAAATCCTCC GATTCCGATC AGCCCAAGGC
251 TTCCGACGCA CTTCCCCTTC CATTAGCCTT CACCAACGCC GTCTTCATCA
301 CCCTTTTCTT CTCCGTCGTG TATTATCTCC TCCGCCGTTG GCGTGAGAAG
351 ATCAGAAACT CCACCCCCCT CCACGTCGTC ACCCTTTCTG AAATCGCCGC
401 CATCGTCACC TTCGTTGCCT CCTTTATTTA TCTCATTGGT TTCTTTGGCG
451 TTGGATTTGT TCAATCCCTA ATCATTCCCC GTGCTTCCCA CGACGATATC
501 CTTGGGGAAG ATGACGAAAT TGTTGATGGA GATCTAG
```

Fig. 3.8: Sequence analysis of 5' RACE PCR product of *HMGR* gene. Underlined- 5' NGRP sequence; 5' UTR (132 bp) highlighted with red colour; Start codon (ATG) highlighted with green and underlined.

3.4.1.3.2 3’ RACE PCR

To isolate 3’ end of the *HMGR* gene from *Bacopa*, 3’ RACE PCR was performed. Primary PCR was done using 3’ RACE ready cDNA and primers RACE HMG F & 3’GRP. Diluted (1:50) primary PCR product was used as a template for secondary PCR with RACE HMG Nested F and 3’ NGRP primers. Approximately 600 bp amplicon (**Fig. 3.9**) was cloned and sequenced.

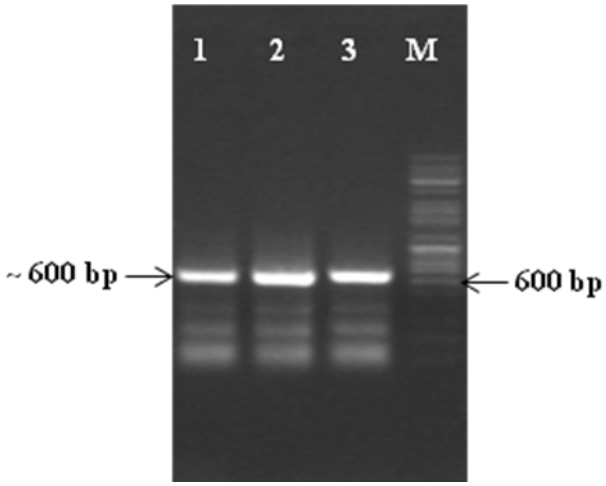


Fig. 3.9: 1 % agarose gel showing 3’ RACE product of *HMGR* gene. Lane M- Medium range DNA ruler, lane 1-3- ~600 bp 3’ RACE product.

Sequence analysis of 3’ RACE clone showed stop codon (TAA), 3’ UTR (224 bp), putative polyadenylation site and poly ‘A’ tail (**Fig. 3.10**).

```
  1 GGGAAGGATC TTCATATCTC TGTCACTATG CCTTCGATTG AGGTTGGTAC
 51 AGTCGGAGGT GGGACCCAAT TGGCGTCTCA GTCGGCATGC CTAAACCTGC
101 TTGGAGTGAA GGGAGCCAGT AAAGATGCTC CAGGGTCGAA CTCCCGGCAG
151 CTGGCCACCA TTGTTGCCGG GGCGGTTCTC GCCGGAGAGC TGTCTCTGAT
201 GTCGGCTTTG GCCGCAGGAC AACTGGTGAA GAGCCATATG AAATACAATA
251 GGTCCAGTAA GGATGTCTCC AAGGTTTCGT CTCAATAAGT TTCTTTTTCC
301 TTTATTTTAT TTTATTTTAT TTTTGTTGGT TCGTTTATTT TACTCCGGCT
351 AGGAACAACC GATTCCCGCC GGCGAAGAAA CGGAATAGGT AAATACTTGT
401 TTGAAACAAC AGTGGTGGTA CCAAAGCCAT GTAAATGTAA AAGGGATGTT
451 CTCTATTTAG TTCTTTTTAT TATGTTGGTA ATTTATGGCA ATAAAATACC
501 TTCATTTTTA TCAAAAAAAA AAAAAAAAAC ACTGTCATGC CGTTACGTAG
551 CG
```

Fig. 3.10: Sequence analysis of 3’ RACE PCR product of HMGR gene. Underlined-Black (RACE HMG Nestd F) and blue (3’ NGRP); Stop codon TAA (Red underlined); 3’ UTR (224 bp) highlighted with green colour; Putative polyadenylation site (AATAAA) orange underlined.

3.4.1.4 Amplification of full-length *HMGR* cDNA from *B. monniera*

On the basis of 5' and 3' RACE sequence information, primers were designed from start and stop codon to amplify the ORF of HMGR gene as a single PCR product.

HMG Full F 5'- ATGGAGGCTAAGGGACGATCCATG-3'
HMG Full R 5'- TTATTGAGACGAAACCTTGGAGACATC-3'

PCR was performed with primers HMG Full F/R and cDNA as a template with high fidelity *Taq* DNA polymerase. Approximately 1.7 kb amplicon was cloned in pGEM-T Easy vector and confirmed by restriction digestion and sequencing (**Fig. 3.11 A & B**).

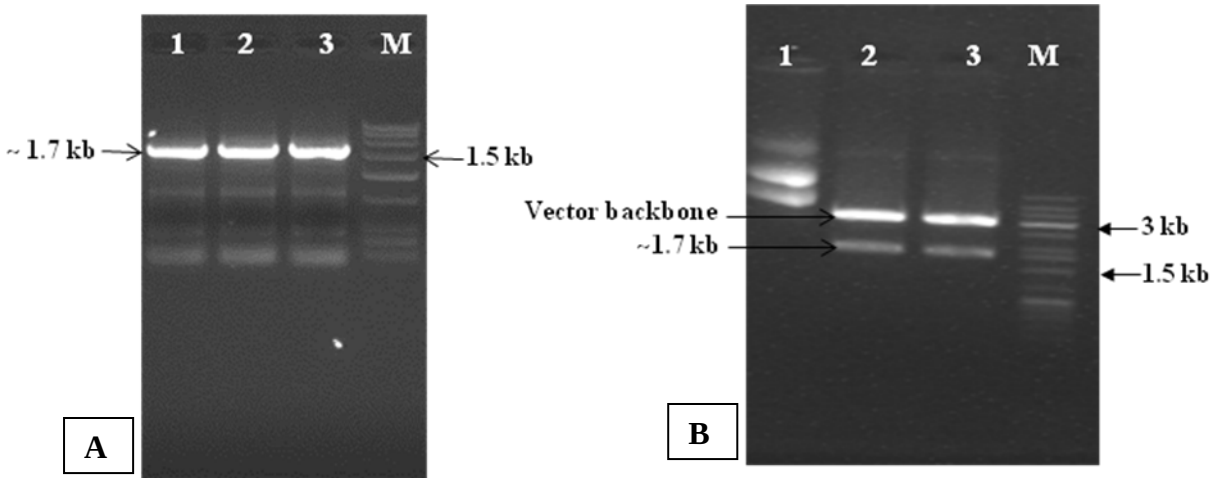


Fig. 3.11: A. 1% agarose gel showing 1.7 kb amplification (Lane 1-3), Lane M- Low range DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1- Undigested plasmid, Lane 2 & 3- Showing vector backbone (~3 kb) and release of insert (~1.7 kb), Lane M- Medium range DNA ruler

Sequence analysis of 6-8 clones showed the exact size of 1770 bp and two types of clones probably isoforms of the *HMGR* gene. Both isoforms were designated as *BmHMGR1* and *BmHMGR6* and submitted to NCBI GenBank data base having accession number HM222606 and HM222607 respectively (**Fig. 3.12**).

Nucleotide Sequence (BmHMGR1; Accession no.:HM222606)

```
  1 ATGGAGGCTA AGGGACGATC CATGAAATTG AAAGGCGGTG CCCTTGATCG
 51 GAAGCTTCAA CGCAAATCCT CCGATTCCGA TCAGCCCAAG GCTTCCGACG
101 CACTTCCCCT TCCATTAGCC TTCACCAACG CCGTCTTCAT CACCCTTTTC
151 TTCTCCGTCG TGTATTATCT CCTCCGCCGT TGGCGTGAGA AGATCAGAAA
201 CTCCACCCCC CTCCACGTCG TCACCCTTTC TGAAATCGCC GCCATCGTCA
251 CCTTCGTTGC CTCCTTTATT TATCTCATTG GTTTCTTTGG CGTTGGATTT
```

```
 301 GTTCAATCCC TAATCATTCC CCGTGCTTCC CACGACGATA TCCTTGGGGA
 351 AGATGACGAA ATTGTTGATG GAGATCTAGA TCGCCAGATG CTGAAGGAGG
 401 ATTCCCGCTC CGCCCCTTGC GCCGCCGCGC TTCCATCCGT GGATTCTGGC
 451 AAGCTTGCTT CTCCCAGATC CAAGGTCCAC GGCGGTGATC TAATCGTCAC
 501 GCCTCCGGCG TCGGAGGAAG ATGAGGAGAT AATCAAATCG GTGGTGGAGG
 551 GCAAGACTCC TTCTTATTCA CTCGAATCGA AGCTTGGGGA TTGCCGTCGC
 601 GCCGCCGCCA TCCGCCGTGA GGCTTTGCAG CGTACGACCG GAAAATCCCT
 651 TGACGGGCTT CCCCTCGATG GTTTTAACTA CGATTCAATA TTGGGTCAGT
 701 GCTGCGAGAT GCCGGTGGGA TACGTCCAGA TTCCGGTGGG CATCGCCGGC
 751 CCTCTGTTGT TGAACGGAAA GGAATACTCT GTTCCAATGG CCACCACAGA
 801 GGGGTGCCTG GTCGCAAGCA CAAACAGGGG ATGCAAAGCC ATCTTCGCCT
 851 CCGGCGGCGC CTCCAGCGTC ATACTCAAGG ATGGTATGAC CAGAGCTCCC
 901 GTCGTAAGAT TTGGTACAGC CAAACGTGCC GCCGAGCTCA AATTCTTCCT
 951 GGAGGACCCT CTCAATTTCG AACCCCTTTC CCTCATTTTC AACAGTTCCA
1001 GCCGATTCGG AAGGCTGCAG AGCATCAAAT GCGCCGTCGC CGGAAAGAAT
1051 CTGTACATGA GATTCACTTG CAGCACAGGG GATGCCATGG GGATGAACAT
1101 GGTCTCAAAG GGAGTACAGA ACGTCATGGA TTTTCTCAAC AAAGAGTTCC
1151 CCGACATGGA CGTCATCGGA ATCTCAGGCA ACTACTGCTC CGACAAGAAA
1201 CCAGCGGCAG TGAACTGGAT TGAAGGCACG GGAAAGTCAG TTGTGTGTGA
1251 AGCCATAATC AAAGAAGATG TGGTCAAGAA AGTCCTCAAG ACCGATGTTG
1301 CTTCATTGGT TGAGCTCAAC ATGCTTAAGA ACCTCACCGG CTCCGCCATG
1351 GCCGGAGCTC TGGGTGGTTT CAACGCTCAC GCCAGCAATA TAGTCTCTGC
1401 CGTCTACATC GCCACTGGAC AAGATCCCGC ACAGAACGTC GAGAGCTCTC
1451 ACTGTATCAC AATGATGGAA GCCGTCAATG ATGGGAAGGA TCTTCATATC
1501 TCTGTCACTA TGCCTTCCAT TGAGATCGGG ACAGTCGGAG GTGGGACCCA
1551 ATTGGCGTCT CAGTCGGCAT GCCTAAACCT GCTTGGAGTG AAGGGAGCCA
1601 GTAAAGATGC TCCAGGGTCG AACTCCCGGC AGCTGGCCAC CATTGTTGCG
1651 GGGGCGGTTC TCGCCGGAGA GCTGTCTCTG ATGTCGGCTT TGGCCGCAGG
1701 ACAACTGGTG AAGAGCCATA TGAAATACAA TAGGTCCAGT AAGGATGTCT
1751 CCAAGGTTGC GTCTCAA
```

TAA

Deduced amino acid sequence

```
MEAKGRSMKLKGGALDRKLQRKSSDSDQPKASDALPLPLAFTNAVFITLFFSVVYYLLRR
WREKIRNSTPLHVVTLSEIAAIVTFVASFIYLIGFFGVGFVQSLIIPRASHDDILGEDDE
IVDGDLDRQMLKEDSRSAPCAAALPSVDSGKLASPRSKVHGGDLIVTPPASEEDEEIIKS
VVEGKTPSYSLESKLGDCRRAAAIRREALQRTTGKSLDGLPLDGFNYDSILGQCCEMPVG
YVQIPVGIAGPLLLNGKEYSVPMATTEGCLVASTNRGCKAIFASGGASSVILKDGMTRAP
VVRFGTAKRAAELKFFLEDPLNFEPLSLIFNSSSRFGRLQSIKCAVAGKNLYMRFTCSTG
DAMGMNMVSKGVQNVMDFLNKEFPDMDVIGISGNYCSDKKPAAVNWIEGTGKSVVCEAII
KEDVVKKVLKTDVASLVELNMLKNLTGSAMAGALGGFNAHASNIVSAVYIATGQDPAQNV
ESSHCITMMEAVNDGKDLHISVTMPSIEIGTVGGGTQLASQSACLNLLGVKGASKDAPGS
NSRQLATIVAGAVLAGELSLMSALAAGQLVKSHMKYNRSSKDVSKVASQ
```

Nucleotide Sequence (BmHMGR6; Accession no.:HM222607)

```
   1 ATGGAGGCTA AGGGACGATC CATGAAATTG AAAGGCGGTG CCCTTGATCG
  51 GAAGCCTCAA CGCAAATCCT CCGATTCCGA TCAGCCCAAG GCTTCCGACG
 101 CACTTCCCCT TCCATTAGCC TTCACCAACG CCGTCTTCAT CACCCTTTTC
 151 TTCTCCGTCG TGTATTATCT CCTCCGCCGT TGGCGTGAGA AGATCAGAAA
 201 CTCCACCCCC CTCCACGTCG TCACCCTTTC TGAAATCGCC GCCATCGTCA
 251 CCTTCGTTGC CTCCTTTATT TATCTCATTG GTTTCTTTGG CGTTGGATTT
 301 GTTCAATCCC TAATCATTCC CCGTGCTTCC CACGACGATA TCCTTGGGGA
 351 AGATGACGAA ATTGTTGATG GAGATCTAGA TCACCAGATG CTGAAGGAGG
 401 ATTCCCGCTC CGCCCCTTGC GCCGCCGCGC TTCCATCCGT GGATTCTGGC
```

```
 451 AAGCTTGCTT CTCCCAGATC CAAGGTCCAC GGCGGTGATC TAATCGTCAC
 501 GCCTTCGGTG TCGGAGGAAG ATGAGGAGAT AATCAAATCG GTGGTGGAGG
 551 GCAAGACTCC TTCTTATTCA CTCGAATCGA AGCTTGGGGA TTGCCGCCGC
 601 GCCGCCGCCA TCCGCCGTGA GGCTTTGCAG CGTACGACCG GAAAATCCCT
 651 TGACGGGCTT CCCCTCGATG GTTTTAACTA CGATTCAATA TTGGGTCAGT
 701 GCTGCGAGAT GCCGGTGGGA TACGTCCAGA TTCCGGTGGG CATCGCCGGA
 751 CCTCTGTTGT TGAACGGAAA GGAATTCTCT GTTCCAATGG CCACCACAAA
 801 GGGGTGCCTG GTCGCAAGCA CAAACAGGGG ATGCAAAGCC ATCTTCGCCT
 851 CCGGCGGCGC CTCCAGCGTC ATACTCAAGG ATGGTATGAC CAGAGCTCCC
 901 GTCGTAAGAT TTGGTACAGC CAAACGTGCC GCCGAGCTCA AATTCTTCCT
 951 GGAGGACCCT CTCAATTTCG AATCCCTTTC CCTCATTTTC AACAGTTCCA
1001 GCCGATTCGG AAGGCTGCAG AGCATCAAAT GCGCCGTCGC CGGAAAGAAT
1051 CTGTACATGA GATTCACTTG CAGCACAGGG GATGCCATGG GGATGAACAT
1101 GGTCTCAAAG GGAGTACAGA ACGTCATGGA TTTTCTCAAC AAAGAGTTCC
1151 CCGACATGGA CATCATCGGA ATCTCAGGCA ACTACTGCTC CGACAAGAAA
1201 CCAGCGGCAG TGAACTGGAT TGAAGGCAGG GGAAAGTCAG TTGTGTGTGA
1251 AGCCATAATC AAAGAAGATG TGGTCAAGAA AGTCCTCAAG ACCGATGTTG
1301 CTTCATTGGT TGAGCTCAAC ATGCTTAAGA ACCTCACCGG CTCCGCCATG
1351 GCCGGAGCTC TGGGTGGTTT CAACGCTCAC GCCAGCAGTA TAGTCTCTGC
1401 CGTCTACATC GCCACTGGAC AAGATCCCGC ACAGAACGTC GAGAGCTCTC
1451 ACTGTATCAC AATGATGGAA GCCGTCAATG ATGGGAAGGA TCTTCATATC
1501 TCTGTCACTA TGCCTTCCAT TGAGGTCGGG ACAGTCGGAG GTGGGACCCA
1551 ATTGGCGTCT CAGTCGGCAT GCCTAAACCT GCTTGGAGTG AAGGGAGCCA
1601 GTAAAGATGC TCCAGGGTCG AACTCCCGGC AGCTGGCCAC CATTGTTGCC
1651 GGGGCGGTTC TCGCCGGAGA GCTGTCTCTG ATGTCGGCTT TGGCCGCAGG
1701 ACAACTGGTG AAGAGCCATA TGAAATACAA TAGGTCCAGT AAGGATGTCT
1751 CCAAGGTTGC GTCTCAATAA
```

Deduced amino acid sequence

```
MEAKGRSMKLKGGALDRKPQRKSSDSDQPKASDALPLPLAFTNAVFITLFFSVVYYLLRR
WREKIRNSTPLHVVTLSEIAAIVTFVASFIYLIGFFGVGFVQSLIIPRASHDDILGEDDE
IVDGDLDHQMLKEDSRSAPCAAALPSVDSGKLASPRSKVHGGDLIVTPSVSEEDEEIIKS
VVEGKTPSYSLESKLGDCRRAAAIRREALQRTTGKSLDGLPLDGFNYDSILGQCCEMPVG
YVQIPVGIAGPLLLNGKEFSVPMATTKGCLVASTNRGCKAIFASGGASSVILKDGMTRAP
VVRFGTAKRAAELKFFLEDPLNFESLSLIFNSSSRFGRLQSIKCAVAGKNLYMRFTCSTG
DAMGMNMVSKGVQNVMDFLNKEFPDMDIIGISGNYCSDKKPAAVNWIEGRGKSVVCEAII
KEDVVKKVLKTDVASLVELNMLKNLTGSAMAGALGGFNAHASSIVSAVYIATGQDPAQNV
ESSHCITMMEAVNDGKDLHISVTMPSIEVGTVGGGTQLASQSACLNLLGVKGASKDAPGS
NSRQLATIVAGAVLAGELSLMSALAAGQLVKSHMKYNRSSKDVSKVASQ
```

Fig. 3.12: Nucleotide and deduced amino acid sequences of BmHMGR1 and BmHMGR6**.**

3.4.1.5 Characterization of BmHMGR1 and BmHMGR6

The full-length cDNA sequences having ORF of 1770 bp (including stop codon), encode polypeptide of 589 amino acid residues. The presence of internal *Eco*RI restriction site was observed in *BmHMGR6* (**Fig. 3.12**, Blue underlined). Deduced amino acid sequences show identity and similarity of 98.1% and 99% respectively with each other. Differences are highlighted with green colour in alignment (**Fig. 3.13**).

```
BmHMGR1         MEAKGRSMKLKGGALDRKLQRKSSDSDQPKASDALPLPLAFTNAVFITLFFSVVYYLLRR 60
BmHMGR6         MEAKGRSMKLKGGALDRKPQRKSSDSDQPKASDALPLPLAFTNAVFITLFFSVVYYLLRR 60
                ****************** ****************************************

BmHMGR1         WREKIRNSTPLHVVTLSEIAAIVTFVASFIYLIGFFGVGFVQSLIIPRASHDDILGEDDE 120
BmHMGR6         WREKIRNSTPLHVVTLSEIAAIVTFVASFIYLIGFFGVGFVQSLIIPRASHDDILGEDDE 120
                ************************************************************

BmHMGR1         IVDGDLDRQMLKEDSRSAPCAAALPSVDSGKLASPRSKVHGGDLIVTPPASEEDEEIIKS 180
BmHMGR6         IVDGDLDHQMLKEDSRSAPCAAALPSVDSGKLASPRSKVHGGDLIVTPSVSEEDEEIIKS 180
                *******:****************************************..**********

BmHMGR1         VVEGKTPSYSLESKLGDCRRAAAIRREALQRTTGKSLDGLPLDGFNYDSILGQCCEMPVG 240
BmHMGR6         VVEGKTPSYSLESKLGDCRRAAAIRREALQRTTGKSLDGLPLDGFNYDSILGQCCEMPVG 240
                ************************************************************

BmHMGR1         YVQIPVGIAGPLLLNGKEYSVPMATTEGCLVASTNRGCKAIFASGGASSVILKDGMTRAP 300
BmHMGR6         YVQIPVGIAGPLLLNGKEFSVPMATTKGCLVASTNRGCKAIFASGGASSVILKDGMTRAP 300
                ****************.********:**********************************

BmHMGR1         VVRFGTAKRAAELKFFLEDPLNFEPLSLIFNSSSRFGRLQSIKCAVAGKNLYMRFTCSTG 360
BmHMGR6         VVRFGTAKRAAELKFFLEDPLNFESLSLIFNSSSRFGRLQSIKCAVAGKNLYMRFTCSTG 360
                ************************.***********************************

BmHMGR1         DAMGMNMVSKGVQNVMDFLNKEFPDMDVIGISGNYCSDKKPAAVNWIEGTGKSVVCEAII 420
BmHMGR6         DAMGMNMVSKGVQNVMDFLNKEFPDMDIIGISGNYCSDKKPAAVNWIEGRGKSVVCEAII 420
                *************************:**********************************

BmHMGR1         KEDVVKKVLKTDVASLVELNMLKNLTGSAMAGALGGFNAHASNIVSAVYIATGQDPAQNV 480
BmHMGR6         KEDVVKKVLKTDVASLVELNMLKNLTGSAMAGALGGFNAHASSIVSAVYIATGQDPAQNV 480
                ****************************************.*********************

BmHMGR1         ESSHCITMMEAVNDGKDLHISVTMPSIEIGTVGGGTQLASQSACLNLLGVKGASKDAPGS 540
BmHMGR6         ESSHCITMMEAVNDGKDLHISVTMPSIEVGTVGGGTQLASQSACLNLLGVKGASKDAPGS 540
                ****************************:*****************************

BmHMGR1         NSRQLATIVAGAVLAGELSLMSALAAGQLVKSHMKYNRSSKDVSKVASQ 589
BmHMGR6         NSRQLATIVAGAVLAGELSLMSALAAGQLVKSHMKYNRSSKDVSKVASQ 589
                ************************************************
```

Fig. 3.13: Deduced amino acid sequence alignment of BmHMGR1 and BmHMGR6. Differences are highlighted with green.

3.4.1.5.1 Multiple sequence alignment of BmHMGRs with other plant HMGRs and CDD search

Multiple sequence alignment showed substrate binding motifs (highlighted in green), NAD (P) H binding motifs (Red highlighted) and different catalytic residues (Yellow highlighted). Conserved domain database search on NCBI server showed specific hits with HMG-CoA_reductase_classI and super family of HMG-CoA_reductase (**Fig. 3.14 A & B**).

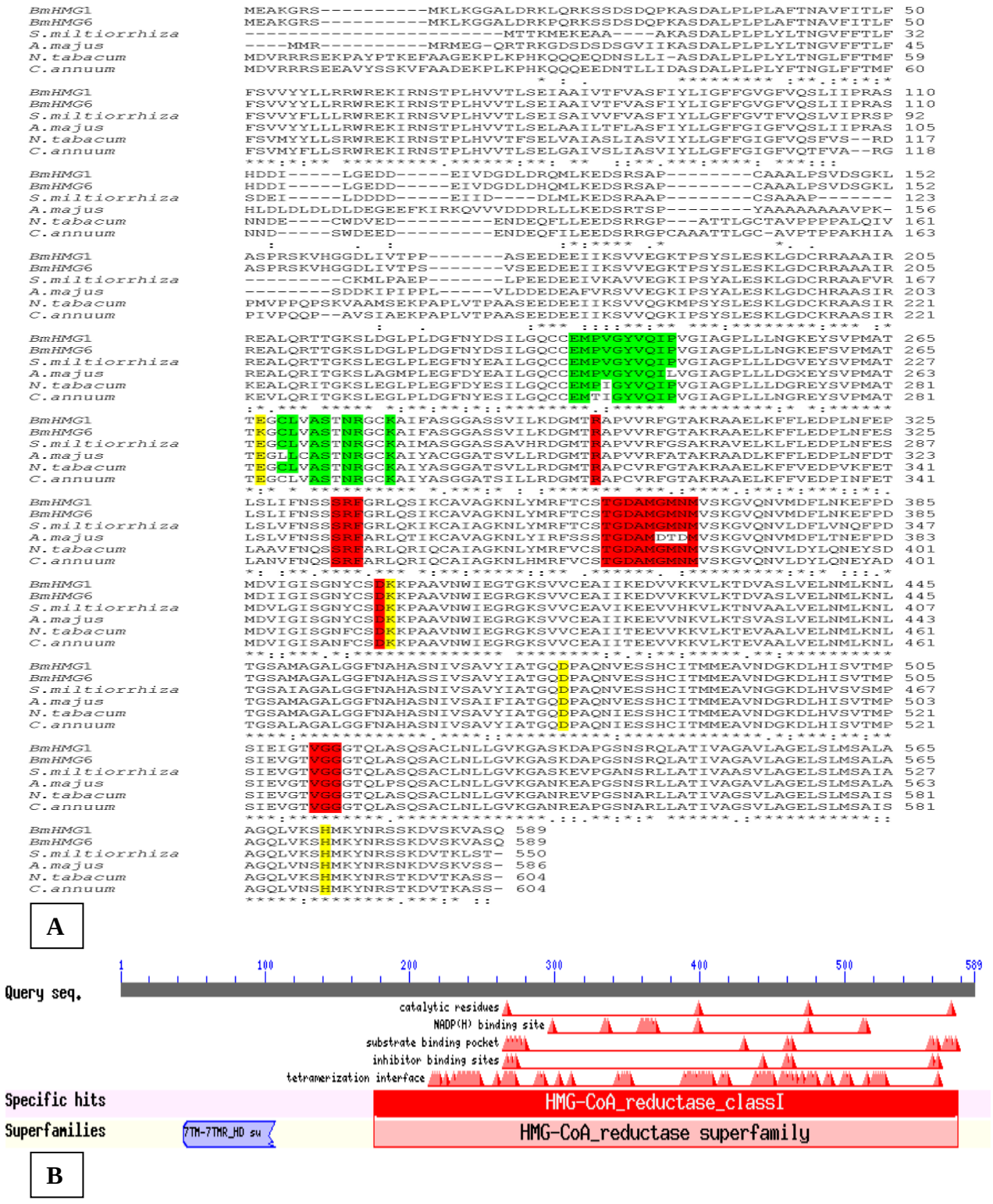


Fig. 3.14: A. ClustalW alignment of BmHMGRs with other plant HMGR amino acid sequences. Different motifs and catalytic residues are highlighted with different colours. **B.** CDD search on NCBI server.

3.4.1.5.2 Amino acid sequence analysis

The deduced amino acid sequence of BmHMGR shared 86.8%, 85.0%, 83.7% and 82.4% similarity and identities of 77.8%, 76.6%, 72.8% and 71.7% with *Salvia miltiorrhiza*, *A. majus*, *N. tabaccum* and *C. annuum* respectively. The calculated molecular mass and predicted pI value of BmHMGRs were 62.63 kDa and 8.33 respectively. The instability index was computed to be 37.11, indicates that protein is stable and atomic formula of BmHMGR was $C_{2749}H_{4462}N_{758}O_{843}S_{31}$. The total number of positively (Arg+Lys) and negatively (Asp+Glu) charged amino acids were 64 and 60 respectively.

3.4.1.5.3 Hydropathy plot and transmembrane domain analysis

The hydropathy index of an amino acid is a number representing the hydrophobic or hydrophilic properties of its side-chain (Kyte and Doolittle, 1982). Each amino acid is given a hydrophobicity score between -4.5 and 4.5. A score of 4.5 is the most hydrophobic and a score of -4.5 is the most hydrophilic. The amino acid sequences of BmHMGRs were analyzed using Kyte-Doolittle Hydropathy plot of JustBio hosted tool (http://www.justbio.com/index.php?page=plots) at window size 9. Strong negative peaks indicate possible surface regions of globular proteins and score greater than 1.8 indicate possible transmembrane regions. In BmHMGRs two transmembrane regions at N-terminal and one at C-terminal were observed (**Fig. 3.15 A**). Here, only one isoform (BmHMGR6) was taken an account because both sequences are 99% similar.

Presence of transmembrane domains was also predicted on TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/). Three domains (I- 39-58 amino acid; II- 79-101 amino acid & III- 545-566 amino acid residues) were found (**Fig. 3.15 B**).

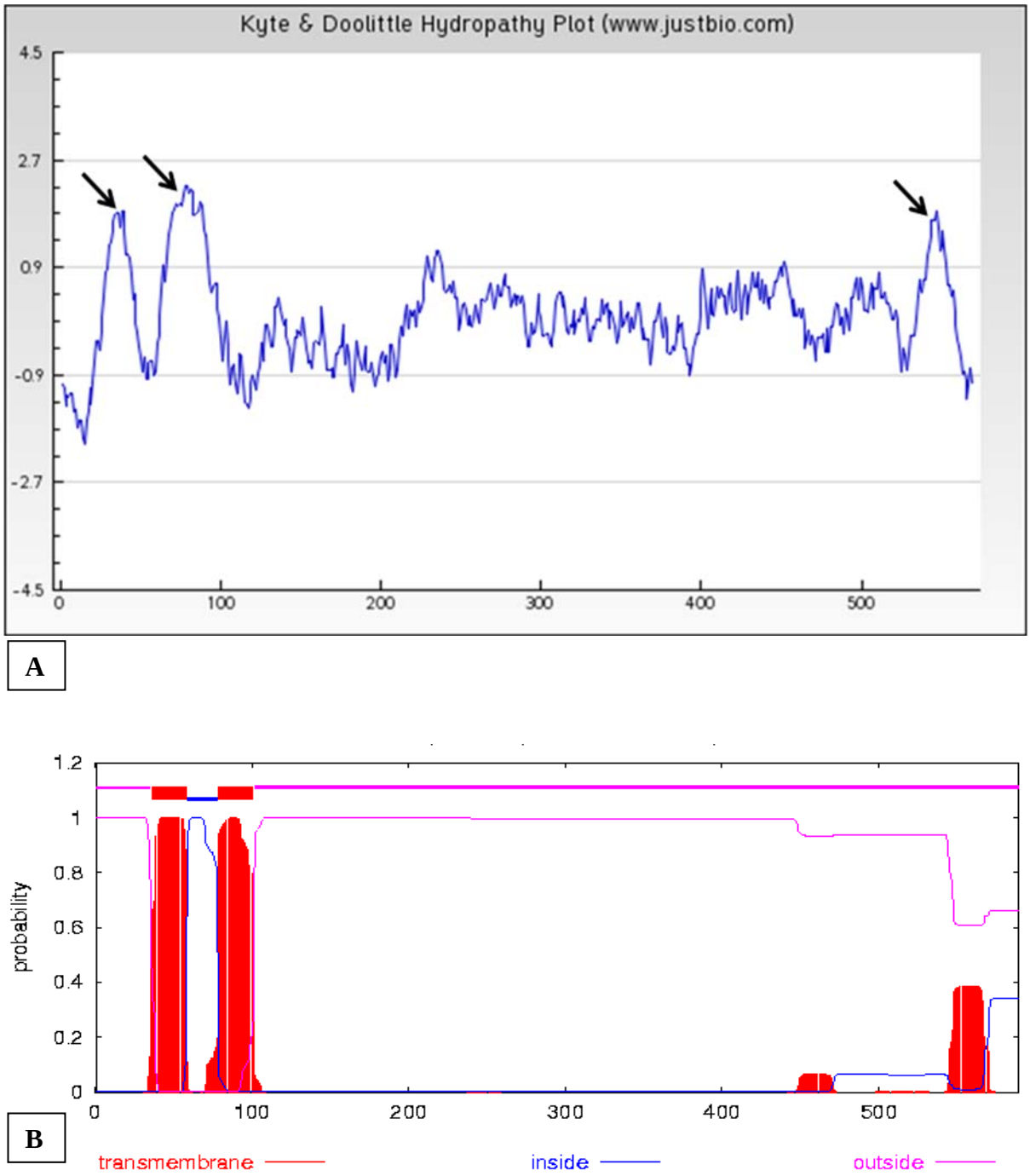


Fig. 3.15: A. Kyte-Doolittle Hydropathy plot **B.** Transmembrane domains (Domain I 39-58 AA; Domain II 79-101 AA; Domain III-545-566 AA).

3.4.1.5.4 Phylogenetic tree analysis

A phylogenetic tree was constructed by using known HMGR sequences from broad range of organisms including dicots, monocots, gymnosperms and bacteria. The dicot HMGRs were clustered into one group, in which BmHMGR was closely related to the *S. miltiorrhiza* and *A. majus* (**Fig. 3.16**).

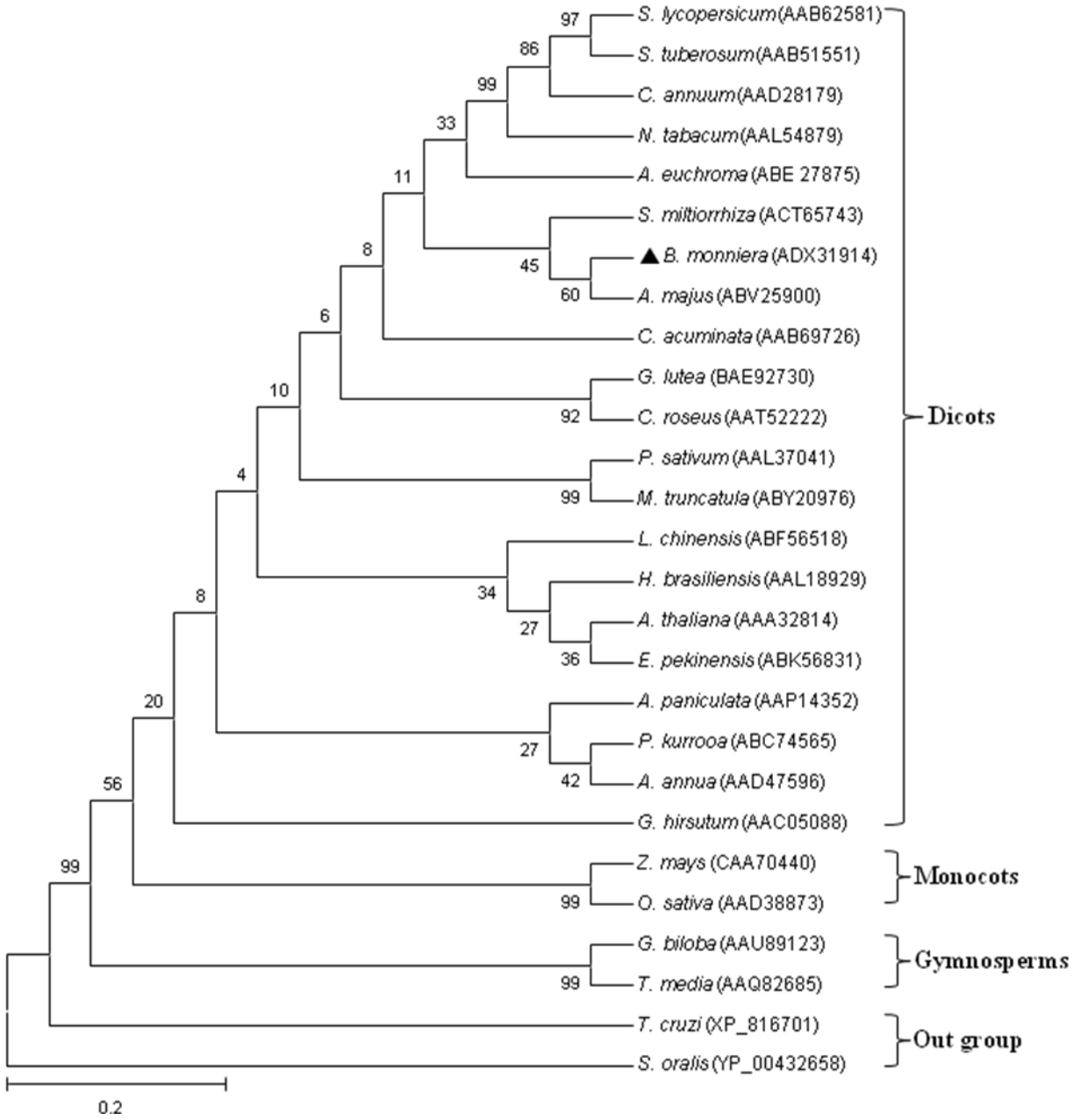


Fig. 3.16: Phylogenetic Relationship of BmHMGR with other HMGRs. The evolutionary tree was formed using the ClustalX2 program and MEGA 4.0.2 software with neighbor-joining method. Accessions Nos. are given in brackets.

3.4.2 Farnesyl pyrophosphate (FPP) synthase: Its isolation, cloning and characterization

3.4.2.1 Multiple sequence alignment for designing of primers

Nucleotide sequences of FPP synthase available at NCBI GenBank database were aligned using Clustal W program. Different set of primers were designed from conserved regions. Forward and reverse primers are highlighted with red and blue respectively (**Fig. 3.17**).

```
L.albus         ATGG---CAGATCT--------------------AAGGTCAACTTTCTTGAATGTGTAT 36
G.lutea         ATGG---CAAATCTGAACGGAACTACATCCGATCTGAGAACTACATTCTTGCAAGTCTAT 57
A.thaliana      ATGGAGACCGATCT--------------------CAAGTCAACCTTTCTCAACGTTTAT 39
                ****   *..****                     *.:*:** **  * .* ** ***
L.albus         TCGGTTCTGAAATCAGAGCTCCTTCATGACCCAGCTTTTGAATTCTCTCCTGATTCTCGT 96
G.lutea         TCCGTACTCAAATCTGAGCTTTTGAATGACCTTGCTTTCGAGTGGTCTCCTGATTCTCGT 117
A.thaliana      TCTGTTCTCAAGTCTGACCTTCTTCATGACCCTTCCTTCGAATTCACCAATGAATCTCGT 99
                ** **:** **.**:** **  * .****** : * ** **.*  :* ..***:******
                                                FPS F1
L.albus         CAATGGCTCGACCGGATGCTGGACTACAATGTGCCTGGAGGAAAGCTAAACCGTGGACTG 156
G.lutea         CAATGGGTCGAACGGATGCTGGATTACAATGTTCCTGGAGGAAAGCTCAACAGAGGCCTT 177
A.thaliana      CTCTGGGTTGATCGGATGCTGGACTACAATGTACGTGGAGGGAAACTCAATCGGGGTCTC 159
                *:.*** * ** *********** ******** * ******.**.**.** .* ** **
L.albus         TCAGTTATTGACAGCTACAGATTGTTGAAAGATGGACATGAATTAAACGATGATGAAATT 216
G.lutea         TCTGTCATTGATAGCTATGAACAGCTAAAAGAAGGAAAGGAGTTAACAAAGGATGAGGTC 237
A.thaliana      TCTGTTGTTGACAGTTTCAAACTTTTGAAGCAAGGCAATGATTTGACTGAGCAAGAGGTC 219
                **:** .**** ** *: ..* :  *.**. *:**..* ** **.*. .*  *:**..*
                                                FPS F2
L.albus         TTTCTTGCTAGTGCTCTTGGTTGGTGTATTGAATGGCTTCAGGCATATTTCCTTGTTCTT 276
G.lutea         TTTCTTGCAAGTGCTCTAGGCTGGTGTATTGAATGGCTCCAGGCATACTTTCTTGTCCTT 297
A.thaliana      TTCCTCTCTTGTGCTCTCGGTTGGTGCATTGAATGGCTCCAAGCTTATTTCCTTGTGCTT 279
                ** **  *::******* ** ***** *********** **.**:** ** ***** ***
                FPS F3
L.albus         GATGACATTATGGATAACTCCCACACACGGCGTGGTCAGCCATGTTGGTTCAGAGTACCC 336
G.lutea         GATGACATTATGGATGGCTCTCACACTAGGCGTAGCCAACCTTGTTGGTTCAGATTGCCA 357
A.thaliana      GATGACATTATGGATAACTCTGTCACCCGCCGTGGTCAACCTTGCTGGTTCAGAGTTCCT 339
                ***************..***  :*** .* ***.* **.**:** ********* * **
L.albus         AAGGTTGGAATGATTGCAGCAAATGATGGGGTGCTGCTACGGAACCATATTCCTCGTATC 396
G.lutea         AAGGTTGGAATGATAGCTGTAAATGACGGTGTTCTCCTTCGCAACCACATACCCAGAATT 417
A.thaliana      CAGGTTGGTATGGTTGCCATCAATGATGGGATTCTACTTCGCAATCACATCCACAGGATT 399
                .*******:***.*:** . .***** ** .* ** **:** ** ** ** *. .* **
L.albus         CTTAAGAAACACTTCAGGGGAAAACCTTATTATGCTGATCTTCTTGATCTGTTTAATGAG 456
G.lutea         CTCAAGAAGCACTTTAGAGAAAAGCCTTACTATGTAGACCTATTGGACCTTTTCAACGAG 477
A.thaliana      CTCAAAAAGCATTTCCGTGATAAGCCTTACTATGTTGACCTTGTTGATTTGTTTAATGAG 459
                ** **.**.** ** .* *.:**.***** **** :** **: * **  * ** ** ***
L.albus         GTTGAGTTTCAAACTGCTTCAGGGCAGATGATAGATCTGATCACTACACTGGAAGGAGAA 516
G.lutea         GTTGAGTTCCAGACTGCATCCGGACAGATGATAGATTTGATTACTACTCTTGAAGGAGAA 537
A.thaliana      GTTGAGTTGCAAACAGCTTGTGGCCAGATGATAGATTTGATCACCACCTTTGAAGGCGAA 519
                ******** **.**:**:*  ** ************ **** ** **  * *****.***
L.albus         AAAGACCTGTCTAAATACACATTATCACTACACCGCCGTATTGTTCAGTACAAGACTGCC 576
G.lutea         AAGGATCTATCAAAATATTCCTTGTCTCTACACCGGAGGATTGTTCAGTACAAAACTGCG 597
A.thaliana      AAGGATTTGTCCAAGTACTCATTGTCAATCCACCGTCGTATTGTCCAGCACAAAACGGCT 579
                **.**  *.** **.** :*.**.**:.*.***** .* ***** *** **** .** **
L.albus         TATTATTCGTTTTACCTCCCAGTCGCATGTGCATTACTCATGGTGGGTGAGAATCTTGAT 636
G.lutea         TACTACTCGTTTTACCTTCCTGTTGCATGTGCATTGCTTATGGCTGGTGAAAATTTGGAC 657
A.thaliana      TATTACTCATTTTATCTCCCTGTTGCTTGTGCGTTGCTTATGGCGGGCGAAAATTTGGAA 639
                ** ** **.***** ** **:** **:*****.**.** ****  ** **.*** * **
                                                                FPS R1
L.albus         AACCATATTGACGTGAAAAACATTCTTGTTGATATGGGAACGTACTTTCAAGTACAGGAT 696
G.lutea         AATTATGTAGATGTTAAGAACGTTCTTATTGACATGGGAATATACTTCCAAGTTCAGGAT 717
A.thaliana      AACCATATTGACGTGAAAAATGTTCTTGTTGACATGGGAATCTACTTCCAAGTGCAGGAT 699
                **  **.*:** ** **.**  .*****.**** *******  ***** ***** ******
L.albus         GATTATTTGGATTGCTTTGGTGCTCCTGAAACAATTGGAAAGATAGGTACAGATATTGAA 756
G.lutea         GACTATTTGGACTGCTTTGGTGAACCAGAGAAGATTGGAAAGATTGGAACCGATATTGAA 777
```

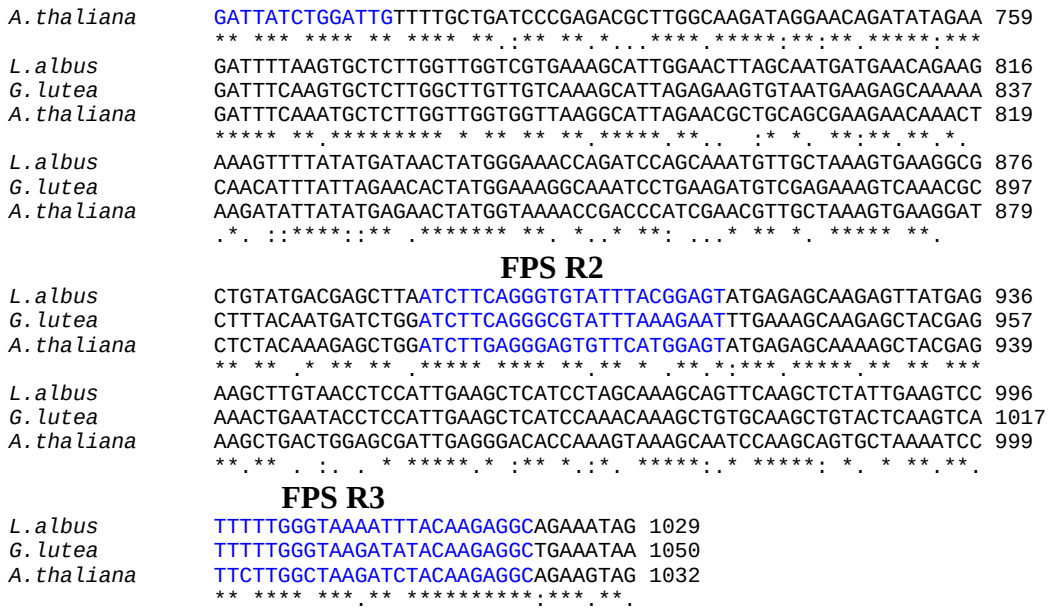


Fig. 3.17: Multiple sequence alignment of FPS nucleotide sequences of *Lupinus albus* (Acc. No. LAU15777), *Gentiana lutea* (AB017371) and *Arabidopsis thaliana* (Acc. No. X75789). Forward (Red) and reverse (Blue) primer sequences are highlighted.

Table 3.2: List of primers used for isolation of BmFPS partial gene.

Gene Name	**Forward and Reverse primers (5'→3')**	
Farnesyl pyrophosphate synthase (FPS)	FPSBM F1	GACTACAATGTACG(T/C)GGAGG
	FPSBM F2	CTCGGTTGGTGCATTGAATGGCT
	FPSBM F3	CTTGTGCTTGATGACATTATGGA
	FPSBM R1	CAATCCAGATAATCATCCTGCACTTG
	FPSBM R2	ACTCCATGAACACTCCCTCAAGAT
	FPSBM R3	GCCTCTTGTAGATCTTAGCCAAGAA

3.4.2.2 Amplification of partial cDNA fragment of *FPS* gene from *B. monniera* by PCR

Approximately 900 bp amplicon was obtained in PCR with primer combination (FPSBM F1and FPSBM R3) and cDNA as a template (**Fig. 3.18 A**). This amplicon was cloned in pGEM-T Easy vector and insert release was confirmed by restriction digestion with *Eco*RI (**Fig. 3.18 B**). Sequence analysis and restriction digestion with *Eco*RI showed presence of *Eco*RI restriction site inside the gene (**Fig. 3.19**). BLAST analysis showed more than 80% homology with reported FPS sequences at NCBI. Blast

search of deduced amino acid sequence predicted the presence of putative conserved domains specific to Isoprenoid_Biosyn_C1 super family. This confirmed that isolated partial sequence was FPP synthase from *B. monniera*.

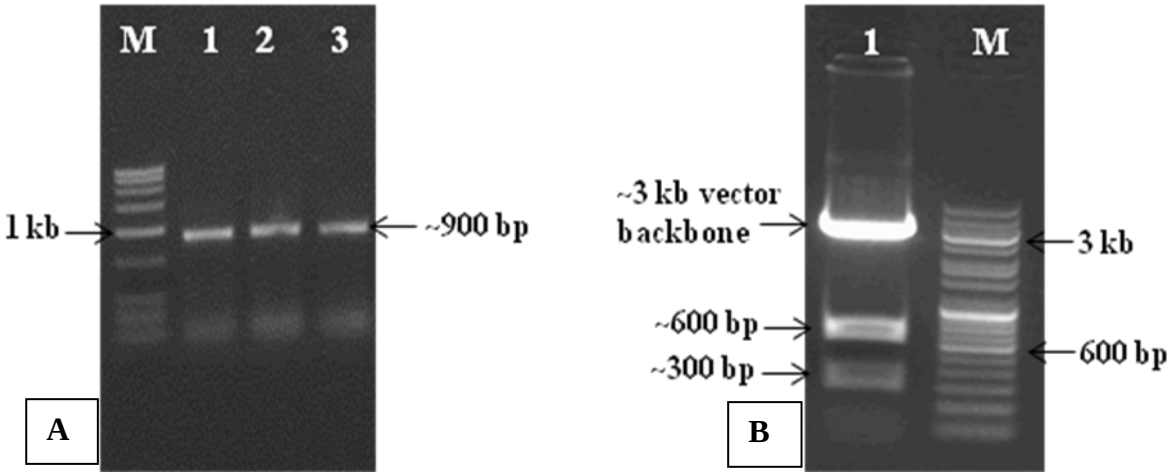


Fig. 3.18: A. 1% agarose gel showing amplification of ~ 900 bp (Lane1-3), Lane M- Low range DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1- Showing vector backbone (~3 kb) and release of inserts (~600 bp and 300 bp), Lane M- Medium range DNA ruler

Partial BmFPS nucleotide sequence

```
  1 GACTACAATG TGCGCGGAGG GAAGTTAAAC CGAGGTCTGT CAGTAATTGA
 51 TAGCTATAAG TTACTAAAAG AAGGAAAGGA CCTTACTGAA GAAGAAGTGT
101 TTCTTGCTAG TGCTCTCGGC TGGTGTATCG AATGGCTTCA GGCATATTTT
151 CTTGTCCTTG ATGATATTAT GGATAATTCT CACACTCGAC GTGGTCAACC
201 TTGTTGGTTT AGGGTCCCCA AGGTTGGTAT GATCGCGGTC AATGATGGAA
251 TCATCCTTCG GAATCACATA CCTAGAATTC TCAAGAAGCA CTTCAGAAAC
301 AAACCTTACT ATGTGGATTT GGTGGATTTA TTCAATGAGG TGGAGTTTCA
351 AACTGCTTCA GGACAGATGA TAGATTTAAT TACCACTATT GAAGGAGAAA
401 AGGATTTGTC AAAATACTCA TTGCCACTTC ACCGCCGCAT TGTTCAGTAT
451 AAGACTGCCT ACTACTCATT TTACCTTCCA GTTGCATGTG CACTGCTCAT
501 GGCAGGCGAA AACTTAGACA ACCATGTAAA TGTGAAGGAT GTCCTTATTG
551 ACATGGGAAT CTATTTCCAA GTACAGGATG ACTACCTAGA TTGCTTTGGC
601 GAGCCTGAGA AGATTGGAAA GATTGGAACT GATATTGAAG ATTTCAAGTG
651 TTCCTGGCTG GTTGTGCAAG CCCTGGAACA CTGCAACGAA GAGCAGAAGA
701 AAATTTTATT CGAGCACTAC GGGAAGCCAG ATCCTGCCGA CGTTGCCAAG
751 ATCAAAGCCC TTTATAATGA GATTAATCTC CAATCTATAT TTACCGATTA
801 TGAAAGCAAG AGCTACGAGA AATTGAACAG CTCTATTGAA TCTCATCCAA
851 GCAAAGCTGT GCAAGCAGTG CTCAAGTCTT TCTTGGCTAA GATCTACAAG
901 AGA
```

Fig. 3.19: Nucleotide sequence of partial BmFPS. Presence of *Eco*RI site is highlighted. Primers used for 5' RACE (Red, Reverse and Nested reverse) and 3' RACE (Blue, Forward and Nested forward) are underlined.

3.4.2.3 RACE PCR (5' and 3') of BmFPS

To obtain the 5' and 3' end of the gene two steps RACE PCR was done. Gene Specific Primers were designed from above partial sequence of BmFPS (**Fig. 3.19**, Underlined).

3.4.2.3.1 5' RACE

Primers

RACE FPSBM R 5'- GATCATACCAACCTTGGGGACCCT-3' (For primary PCR)

RACE FPSBM NR 5'- GCCATTCGATACACCAGCCGAG-3' (For Nested PCR)

The primary PCR product with RACEFPSBM R/ 5'GRP was diluted (1:50) and used as a template for secondary PCR. Nested PCR with primers RACEFPS NR and 5' NGRP yielded approximately 350 bp fragment (**Fig. 3.20 A**). It was cloned in pGEM-T Easy vector, confirmed by restriction digestion (**Fig. 3.20 B**) and sequenced.

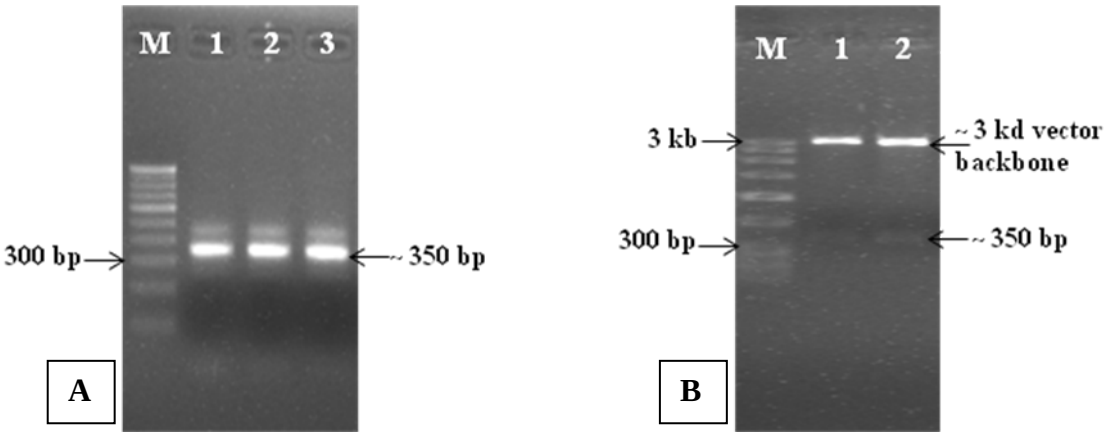


Fig. 3.20: A. 1.5% agarose gel showing ~ 350 bp amplification (Lane1-3), Lane M- 100bp DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1and 2 Showing vector backbone (~3 kb) and of inserts (~ 350 bp), Lane M- Low range DNA ruler.

Sequence analysis of 5'RACE clone revealed start codon (green), 9 bp 5'UTR (Red) and 5' NGRP sequens is underlined (**Fig. 3.21**).

```
  1 GGACACTGAC ATGGACTGAA GGAGTAGAAA AAGAGATGGC GAATCATAAT
 51 GGACAGACGA CGGATCTGAG GGAGGCATTT CTAGGTGTCT ACTCTGTCCT
101 TAAATCTGAG CTGTTGAACG ACCCAGCTTT TGAATGGACC GACGCATCTC
151 GCGAATGGGT CGAGCGGATG CTGGACTACA ATGTGCCTGG AGGGAAGTTA
201 AACCGAGGTC TGTCAGTAAT TGATAGCTAT AAGTTACTAA AAGAAGGAAA
251 GGACCTTACT GAAGAAGAAG TGTTTCTTGC TAGTGCTCTC GGCTGGTGTA
301 TCGAATGGC
```

Fig. 3.21: Nucleotide sequence of 5' RACE clone. Start codon (ATG, underlined) in green and 5' UTR in red. 5' NGRP black underlined.

3.4.2.3.2 3' RACE

Primers

RACE FPSBM F 5'- GTGCAAGCCCTGGAACACTGCAA-3'
RACE FPSBM NF 5'- ATCCTGCCGACGTTGCCAAGATC-3'

To isolate 3' end and UTR of the gene two steps RACE PCR was done. Nested PCR with RACEFPSBM NF/ 3'NGRP yielded an amplicon of ~400 bp (**Fig. 3.22 A**) which was cloned in pGEM-T Easy vector, confirmed by restriction digestion (**Fig.3. 22 B**) and sequencing.

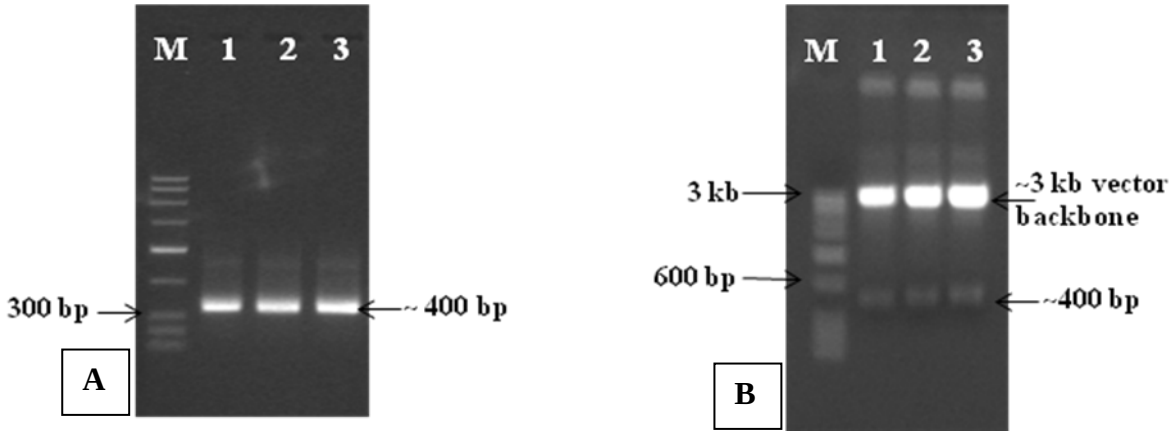


Fig. 3.22: A. 1% agarose gel showing amplification of ~ 400 bp 3' RACE PCR product. **B.** Restriction digestion with *Eco*RI. Lane 1- Showing vector backbone (~3 kb) and release of insert (~400 bp), Lane M- Low range DNA ruler

Sequencing of 3'RACE clone showed stop codon (TAA, red) 3' UTR (204 bp), putative polyadenylation site (Orange) and poly A tail (**Fig. 3.23**).

```
  1 ATCCTGCCGA CGTGCCAAGA TCAAAGCCCT TTATAATGAG ATTAATCTCC
 51 AATCTATATT TACCGATTAT GAAAGCAAGA GCTTCGAGAA ATTGAACAGC
101 TCTATTGAAT CTCATCCAAG CAAAGCTGTG CAAGCAGTGC TCAAGTCTTT
151 CTTGGGTAAG ATATACAAGA GGCAGAAGTA AGTCCAATAC ATAGGCCCTT
201 GTTTGGAGAC GGAGATATTG TGGATAACTT GAAAGTTTAA AACCACATAT
251 TTTCCTGATT CATATTTGTA TTCTAGTTGG GGTGGAAAAA TAATAGCCCC
301 CATTATCTTT TTTGTATTAG TCAATGTTGT TTTTCTAATT GTAATATTCA
351 CAGTCTCCAA GATTATGATC TTGTTCTCGG ACTTATGAAA AAAAAAAAAA
401 AAACACTGTC ATGCCGTTAC GTAGCG
```

Fig. 3.23: Nucleotide sequence of 3' RACE clone. Stop codon (TAA) in red, 3'UTR in green and polyadenylation site in orange underlined. Primer 3' NGRP is underlined.

3.4.2.4 Amplification of full-length cDNA of FPS

Primers were designed from start and stop codon to amplify full-length ORF of BmFPS as a single PCR product on the basis of 5' and 3' RACE sequences.

FPS Full F 5'- ATGGCGAATCATAATGGACAGAC-3'
FPS Full R 5'- TTACTTCTGCCTCTTGTATATCTTACC-3'

PCR was performed with above full-length primers and cDNA as a template using high fidelity *Taq* DNA polymerase. Approximately 1.1 kb amplicon (**Fig. 3.24 A**) was cloned in pGEM-T Easy vector and confirmed by restriction digestion (**Fig. 3.24 B**) and sequencing.

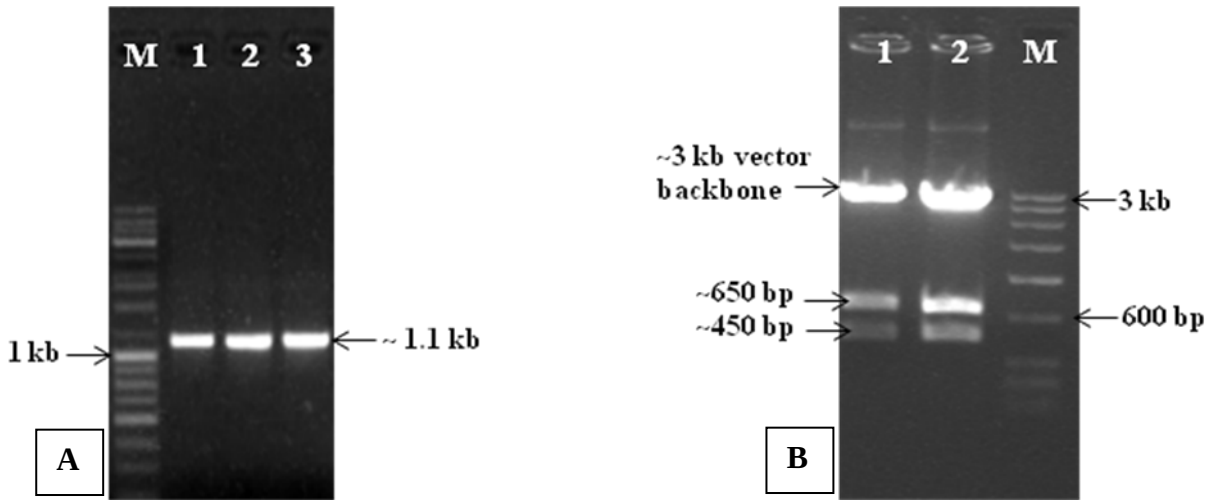


Fig. 3.24: A. 1% agarose gel showing ~ 1.1 kb amplification band (Lane1-3), Lane M-Medium range DNA ruler **B.** Gel showing restriction digestion with *Eco*RI. Lane 1&2-Showing vector backbone (~3 kb) and release of inserts (~650 bp and 450 bp), Lane M-Low range DNA ruler.

Sequence analysis of 6-8 clones showed the exact size of 1050 bp (**Fig. 3.25**) and full-length ORF with 5' and 3' UTRs was submitted to NCBI GenBank data base having accession number **GU385740**.

Nucleotide sequence of ORF (BmFPS)

```
  1 ATGGCGAATC ATAATGGACA GACGACGGAT CTGAGGGAGG CATTTCTAGG
 51 TGTCTACTCT GTCCTTAAAT CTGAGCTGTT GAACGACCCA GCTTTTGAAT
101 GGACCGACGC ATCTCGCGAA TGGGTCGAGC GGATGCTGGA CTACAATGTG
151 CCTGGAGGGA AGTTAAACCG AGGTCTGTCA GTAATTGATA GCTATAAGTT
201 ACTAAAAGAA GGAAAGGACC TTACTGAAGA AGAAGTGTTT CTTGCTAGTG
251 CTCTCGGCTG GTGTATCGAA TGGCTTCAGG CATATTCTCT TGTCCTTGAT
301 GATATTATGG ATAATTCTCA CACTCGACGT GGTCAACCTT GTTGGTTTAG
351 GGTCCCCAAG GTTGGTATGA TCGCGGTCAA TGATGGAATC ATCCTTCGGA
```

```
  401 ATCACATACC TAGAATTCTC AAGAAGCACT TCAGAAACAA ACCTTACTAT
  451 GTGGATTTGG TGGATTTATT CAATGAGGTG GAGTTTCAAA CTGCTTCAGG
  501 ACAGATGATA GATTTAATTA CCACTATTGA AGGAGAAAAG GATTTGTCAA
  551 AATACTCGTT GCCACTTCAC CGCCGCATTG TTCAGTATAA GACTGCCTAC
  601 TACTCATTTT ACCTTCCAGT TGCATGTGCA CTGCTCATGG CGGGCGAAAA
  651 CTTAGACAAC CATGTAAATG TGAAGGATGT CCTTATTGAC ATGGGAATCT
  701 ATTTCCAAGT ACAGGATGAC TACCTAGATT GCTTTGGCGA GCCTGAGAAG
  751 ATTGGAAAGA TTGGAACTGA TATTGAAGAT TTCAAGTGTT CCTGGCTGGT
  801 TGTGAAAGCC CTGGAACACT GCAACGAAGA GCAGAAGAAA ATTTTATTCG
  851 AGCACTACGG GAAGCCAGAT CCTGCTGACG TGGCCAAGAT CAAAGCCCTT
  901 TATAATGAGA TTAATCTCCA ATCTATATTT ACCGATTATG AAAGCAAGAG
  951 CTACGAGAAA TTGAACAGCT CTATTGAATC TCATCCAAGC AAATCTGTGC
 1001 AGGCAGTGCT CAAGTCTTTC TTGGGTAAGA TATACAAGAG GCAGAAGTAA
```

Deduced amino acid sequence

```
MANHNGQTTDLREAFLGVYSVLKSELLNDPAFEWTDASREWVERMLDYNVPGGKLNRGLS
VIDSYKLLKEGKDLTEEEVFLASALGWCIEWLQAYFLVLDDIMDNSHTRRGQPCWFRVPK
VGMIAVNDGIILRNHIPRILKKHFRNKPYYVDLVDLFNEVEFQTASGQMIDLITTIEGEK
DLSKYSLPLHRRIVQYKTAYYSFYLPVACALLMAGENLDNHVNVKDVLIDMGIYFQVQDD
YLDCFGEPEKIGKIGTDIEDFKCSWLVVKALEHCNEEQKKILFEHYGKPDPADVAKIKAL
YNEINLQSIFTDYESKSYEKLNSSIESHPSKSVQAVLKSFLGKIYKRQK
```

Fig. 3.25: Nucleotide and deduced amino acid sequence of full-length BmFPS

3.4.2.5 Characterization of BmFPS

The full-length cDNA sequence having ORF of 1050 bp (including stop codon), encoding a polypeptide of 349 amino acid residues.

3.4.2.5.1 Protein sequence analysis and its identity with other plant FPS

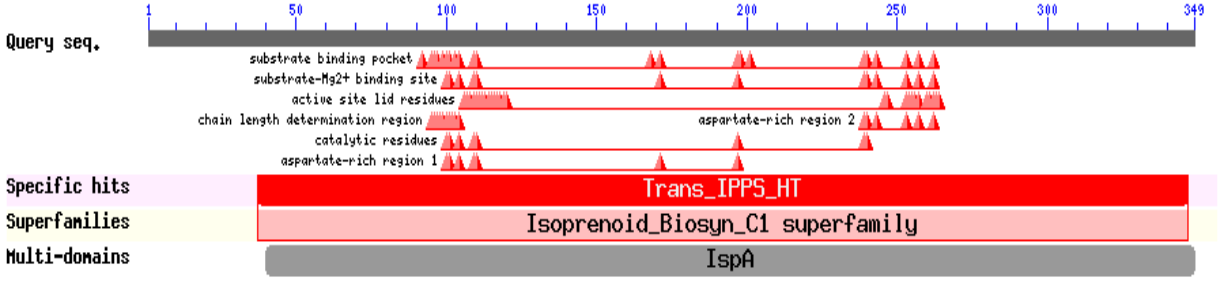
The calculated molecular mass and predicted pI value of BmFPS were 40.26 kDa and 5.57 respectively. The total number of positively (Arg+Lys) and negatively (Asp+Glu) charged amino acids were 42 and 51 respectively. The instability index was computed to be 36.06, indicates that protein is stable and atomic formula of BmFPS was $C_{1825}H_{2824}N_{470}O_{531}S_{13}$. The aliphatic index is 93.27 which indicate thermo stability of proteins. The deduced amino acid sequence of BmFPS shared 95.7, 95.1, 92.3, 90.5 & 90.3% similarity and 90.8, 90.8, 85.1, 82.2 & 81.7% identities with *M. piperita*, *S. miltiorrhiza*, *C. roseus*, *H. brasiliensis* and *P. ginseng* respectively.

3.4.2.5.2 Multiple sequence alignment of BmFPS with other plant FPS and CDD search

Multiple sequence alignment of BmFPS with other plant FPSs showed substrate binding domains (red colored), Mg^{2+} binding sites (Green colored) and active site lid residues (Indigo colored, underlined) (**Fig. 3.26 A**). Active site lid residues shield highly reactive carbocationic intermediates from solvents. The importance of domain I and II (Red colored **Fig. 3.26 A**) in catalysis has been demonstrated by mutagenesis studies. Mutation of aspartate and arginine in region I and, to a lesser extent, of the first aspartate and lysine in region II, reduced the FPP synthase activity (Joly and Edwards, 1993; Marrero et al., 1992; Song and Poulter, 1994). NCBI conserved domain database (CDD) search showed specific hits with Trans_IPPS_HT and super family of Isoprenoid_Biosyn_C1. Different domains and catalytic motifs are also observed (**Fig. 3.26 B**).

```
M.piperita          MANLNGAASDLRKTFLGVYSTLKSELLNDPAFEWTDGSRQWVERMLDYNVPGGKLNRGLS 60
S.miltiorrhiza      MANLNGESADLRATFLGVYSVLKSELLNDPAFEWTDGSRQWVERMLDYNVPGGKLNRGLS 60
B.monniera          MANHNGQTTDLREAFLGVYSVLKSELLNDPAFEWTDASREWVERMLDYNVPGGKLNRGLS 60
P.ginseng           -------MSDLKTRFLEVYSVLKSELLNDPAFEFTDDSRQWVERMLDYNVPGGKLNRGLS 53
C.roseus            ----MANTSDLRTSFLKVYEILKSQLLADPAFEWTEDSRQWVDRMLDYNVPGGKLNRGLS 56
H.brasiliensis      -------MADLKSTFLKVYSVLKQELLEDPAFEWTPDSRQWVERMLDYNVPGGKLNRGLS 53
                           :**:  ** **. **.:** *****:*  **:**:*****************

M.piperita          VIDSYQLLKEGKDLTDDEVFLASALGWCIEWLQAYFLVLDDIMDNSHTRRGQPCWFKVPK 120
S.miltiorrhiza      VIDSYKLLKGGKDLTDDEVFLASALGWCVEWLQAYFLVLDDIMDNSHTRRGQPCWFRVPK 120
B.monniera          VIDSYKLLKEGKDLTEEEVFLASALGWCIEWLQAYFLVLDDIMDNSHTRRGQPCWFRVPK 120
P.ginseng           VIDSYKLLKEGKELSDDEIFLSSALGWCIEWLQAYFLVLDDIMDSSHTRRGQPCWFRLPK 113
C.roseus            VIDSYKLLREGNEPTEDEVFQASALGWCIGWLQAYFLVLDDIMDGSHTRRGQPCWFRVPK 116
H.brasiliensis      VIDSYKLLKEGQELTEEEIFLASALGWCIEWLQAYFLVLDDIMDSSHTRRGQPCWFRVPK 113
                    *****:**: *:: :::*:* :******:**************.***********::**
                                                 I
M.piperita          VGMIAINDGIILRNHIPRILKKHFRSKPYYVDLLDLFNEVEFQTASGQMIDLITTIEGEK 180
S.miltiorrhiza      VGMIAINDGIILRNHIPRILKKHFRTKPYYVDLLDLFNEVEFQTASGQMIDLITTIEGEK 180
B.monniera          VGMIAVNDGIILRNHIPRILKKHFRNKPYYVDLVDLFNEVEFQTASGQMIDLITTIEGEK 180
P.ginseng           VGMIAVNDGILLRNHIPRILKKHFRQKPYYVDLLDLFNEVEFQTASGQMIDLITTLVGEK 173
C.roseus            VGMIAVNDGVLLRNHIPRILKRHFREKPYYVDLLDLFNEVEFQTASGQMIDLITTLEGEK 176
H.brasiliensis      VGLIAANDGILLRNHIPRILKKHFRGKAYYVDLLDLFNEVEFQTASGQIIDLITTLEGEK 173
                    **:** ***::**********:*** *.*****:**************:******: ***

M.piperita          DLSKYSLPLHRRIVQYKTAYYSFYLPVACALLMAGENLENHPTVKDVLIDMGIYFQVQDD 240
S.miltiorrhiza      DLSKYSLPLHRRIVQYKTAYYSFYLPVACALLMAGEDLEKHPTVKDVLINMGIYFQVQDD 240
B.monniera          DLSKYSLPLHRRIVQYKTAYYSFYLPVACALLMAGENLDNHVNVKDVLIDMGIYFQVQDD 240
P.ginseng           DLSKYSLPIHRRIVQYKTAYYSFYLPVACALLMSGEDLEKHTNVKDILIEMGTYFQVQDD 233
C.roseus            DLSKYSLSLHRRIVQYKTAYYSFYLPVACALLMAGENLDNHVDVKNILIDMGIYFQVQDD 236
H.brasiliensis      DLSKYTLSLHRRIVQYKTAYYSFYLPVACALLIAGENLDNHIVVKDILVQMGIYFQVQDD 233
                    *****:*.:************************::**:*::*  **::*::** *******

M.piperita          YLDCFGEPEKIGKIGTDIEDFKCSWLVVKALELCNEEQKKTLFEHYGKENPADVAKIKAL 300
S.miltiorrhiza      YLDCFGEPEKIGKIGTDIEDFKCSWLVVKALELCNEEQKKTLFEHYGKEDPADVAKIKVL 300
B.monniera          YLDCFGEPEKIGKIGTDIEDFKCSWLVVKALEHCNEEQKKILFEHYGKPDPADVAKIKAL 300
P.ginseng           YLDCFGAPEVIGKIGTDIEDFKCSWLVVKALELSNEEQKKFLHENYGKDDPASVAKVKEL 293
C.roseus            YLDCFGEPEKIGKIGTDIEDFKCSWLVVKALERCNEEQKKILLEHYGKADPDDVAKVKAL 296
H.brasiliensis      YLDCFGDPETIGKIGTDIEDFECSWLVVKALELCNEEQKKVLYEHYGKADPASVAKVKVL 293
                    ****** ** ***********:********** .****** * *:*** :* .***:* *

M.piperita          YNDINLQGMFADFESKSYEKITSSIEAHPSKSVQAVLKSFLGKIYKRQK 349
S.miltiorrhiza      YNEINLQGVFAEFESKSYEKLNSSIEAHPSKSVQAVLKSFLGKIYKRQK 349
B.monniera          YNEINLQSIFTDYESKSYEKLNSSIESHPSKSVQAVLKSFLGKIYKRQK 349
P.ginseng           YNTLKLQDVFAEYESKSYDKLIKFIEAHPSQAVQAVLKSFLGKIYKRQK 342
C.roseus            YNNLDLQGVFEEYERKSYEKLTSSIEAHPSKAVQAVLKSFLAKIYKRQK 345
H.brasiliensis      YNELKLQGVFTEYENESYKKLATSIEAHPSKPVQAVLKSFLAKIYKRQK 342
                    ** :.**.:* ::* :**.*: . **:***:.*********.*******
```

A



B

Fig. 3.26: A. ClustalW alignment of BmFPS with other plant FPSs showing different domains and catalytic motifs in different color. **B.** CDD search results on NCBI server.

3.4.2.5.3 Hydropathy plot analysis of BmFPS

The amino acid sequence of BmFPS was analyzed using Kyte-Doolittle Hydropathy plot of JustBio hosted tool (http://www.justbio.com/index.php?page=plots) at window size 9. Plot analysis revealed even distribution of hydrophilic and hydrophobic residues (**Fig. 3.27**).

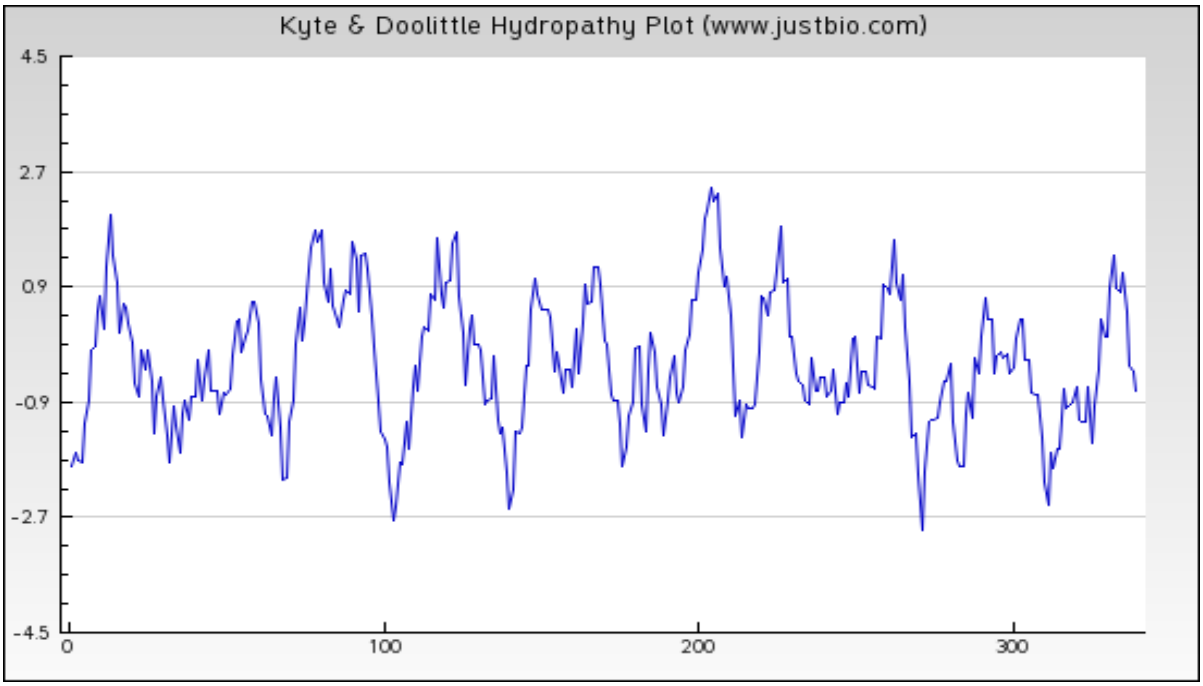


Fig. 3.27: Kyte-Doolittle Hydropathy plot

3.4.2.5.4 Phylogenetic tree analysis

A phylogenetic tree was constructed by using known FPS sequences from broad range of organisms including dicots, monocots, gymnosperms, yeasts and bacteria. The dicot FPSs were clustered in to one group, in which BmFPS was grouped with *S. miltiorrhiza* and *M. peperita* and *C. roseus* (**Fig. 3.28**).

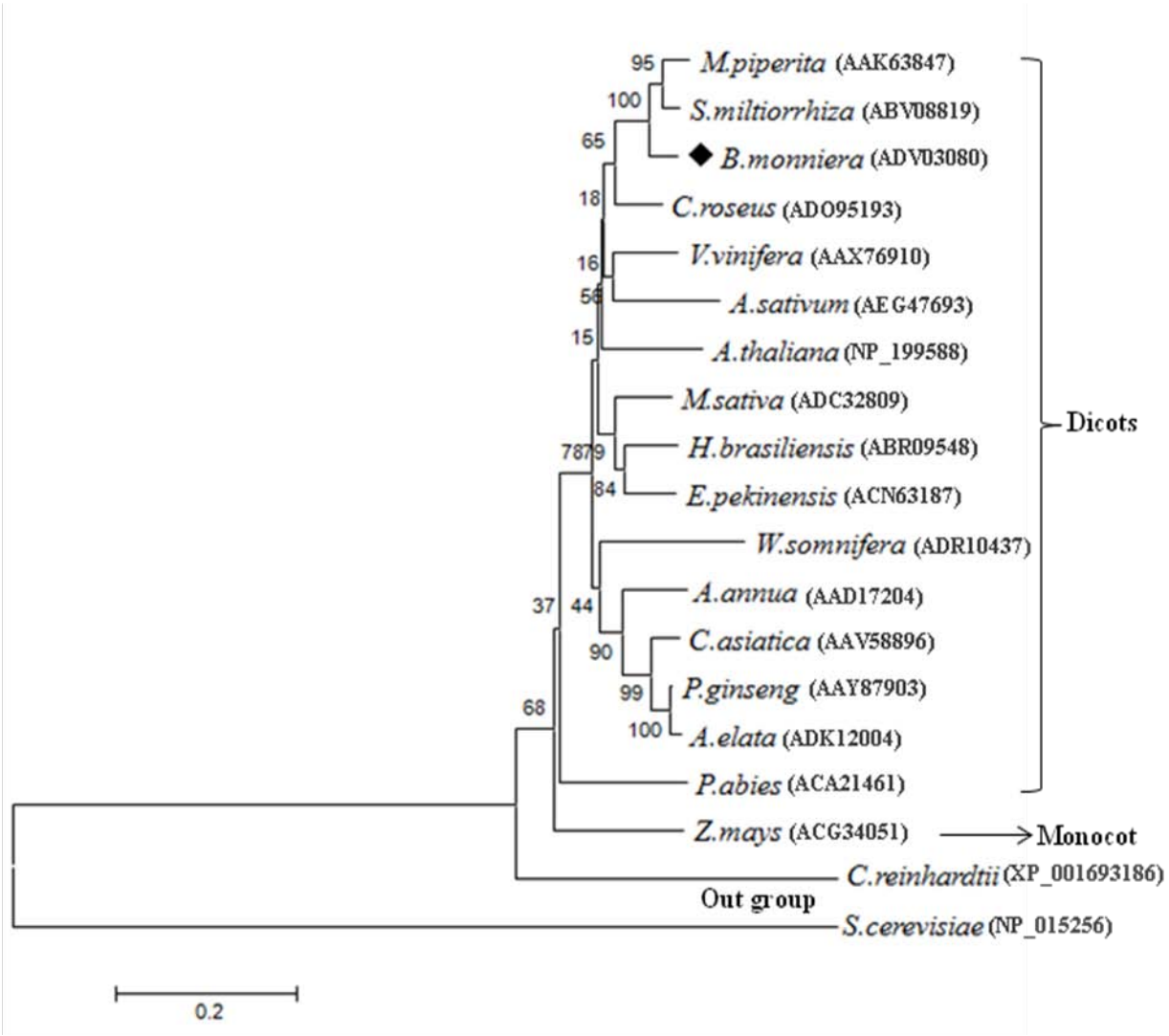


Fig. 3.28: Phylogenetic tree analysis of BmFPS with other FPS amino acid sequences. The tree was formed using MEGA 4.0.2 software with neighbor-joining method. Protein accession nos. is given in brackets.

3.4.3 Squalene synthase (SQS): Its isolation, cloning and characterization from *B. monniera*

3.4.3.1 Multiple sequence alignment for designing of primers

Nucleotide sequences of squalene synthase available at NCBI GenBank database were aligned using Clustal W program. Different set of primers were designed from conserved regions. Forward and reverse primers are highlighted with red and blue respectively (**Fig. 3.29**).

```
L.japonicus     ATGGGGAGTTTGGGAGCGATTGTGAAGCATCCAGATGATTTGTACCCGCTGCTGAAGCTC 60
C.asiatica      ATGGGAAGTTTAGGGGCGATTCTGAAGCATCCGGACGATTTCTATCCGTTGTTGAAGCTG 60
A.thaliana      ATGGGAAGCTTGAGTACGATTTTGAGACATCCGGATGAGTTATATCCGCTCTTGAAGTTG 60
                ***** ** **  *  ***** ***  ***** ** ** ** ** *** *  ***** *

L.japonicus     AAAATGGCGGCGAGGCACGCCGAGAAGCAGATCCCTTCGGAGCCGCATTGGGGCTTCTGC 120
C.asiatica      AAAATGGCGGCGAGGCATGCTGAGAAACAGATCCCGCCGGAGCCGCACTGGGCATTTTGT 120
A.thaliana      AAACTTGCGATTACGAAAGCTCAGAAGCAGATTCCACTTGAGCCACACTTAGCTTTCTGT 120
                *** * ***   * * * **  **** ***** **    ***** ** *  *  ** **

                SQS F1
L.japonicus     TACTCCATGCTCCACAAGGTTTCTAGAAGCTTCGCCCTCGTCATTCAGCAACTCGACACT 180
C.asiatica      TACTCTATGCTTCATAAGGTTTCTAGAAGTTTCGGTCTCGTCATTCAACAGCTCGGTCCG 180
A.thaliana      TATTCAATTCTTCACAAAGTTTCTAAAAGCTTTTCTCTTGTTATTCAACAACTTGGCACC 180
                ** ** ** ** ** ** ******* *** **    ** ** ***** ** ** *   *

                                                              SQS F2
L.japonicus     GATCTTCGCAACGCCGTTTGCATATTCTACTTGGTTCTTCGAGCTCTGGATACCGTTGAG 240
C.asiatica      CAGCTTCGCGATGCTGTATGCATATTTTATTTAGTTCTTCGAGCTCTTGACACTGTTGAG 240
A.thaliana      GAGCTTCGTAACGCCGTGTGTGTGTTCTACTTGATTCTCCGAGCTCTTGATACTGTTGAG 240
                 * *****  * ** ** **  * ** ** **  **** ******** ** ** ******

L.japonicus     GATGATACGAGCATAGCTACAGAAGTAAAGGTCCCCATACTGAAAGCTTTTCACCGTCAC 300
C.asiatica      GATGACACTAGCATATCTACAGAGGTTAAAGTACCAATATTGAAGGCTTTTCATCGTCAC 300
A.thaliana      GATGACACAAGCGTACCAGTGGAGATCAAAGTTCCAATTCTGATAGCTTTCCACCGTCAT 300
                ***** ** *** ** *    **  * ** ** ** **  ***  ***** ** *****

L.japonicus     ATCTATGATCGTGATTGGCACTTCTCATGTGGCACAAAGGAGTACAAAGTTCTCATGGGC 360
C.asiatica      ATATATGATAATAACTGGCACTTTTCATGTGGTACAAAGGAATACAAAATTCTCATGGAC 360
A.thaliana      ATATACGATGGTGACTGGCATTTTTCATGTGGTACAAAAGAGTATAAACTTCTAATGGAC 360
                ** ** ***  * * ***** ** ******** ***** ** ** *** **** **** *

L.japonicus     CAGTTTCATCTTGTTTCAACTGCTTTTCTGGAACTTGCAAAGAACTATCAGGAAGCAATT 420
C.asiatica      GAGTTCCATCATGTTTCTAATGCTTTTCTGGAGCTCGGAAGCGGTTACAAGGAGGCAATA 420
A.thaliana      CAATTTCACCATGTTTCTGCAGCTTTTCTGAAACTTGAAAAAGGGTATCAAGAGGCTATT 420
                 * ** ** * ******    ********* * ** * **     **  * ** ** **

                                                        SQS R1
L.japonicus     GAAGACATTACTGACAGAATGGGTGCTGGAATGGCCAAATTTATTTGCAAGGAGGTAGAA 480
C.asiatica      GAAGATATTACCATGAGAATGGGTGCAGGAATGGCAAAATTTATATGCAAGGAGGTAGAG 480
A.thaliana      GAAGATATAACTAAAAGAATGGGTGCAGGAATGGCCAAGTTCATTTGCAAGGAGGTAGAA 480
                ***** ** **    *********** ******** ** ** ** **************

L.japonicus     ACAATTGATGACTACGACGAATATTGTCACTATGTGGCAGGACTTGTTGGGCTGGGTTTA 540
C.asiatica      ACAATTGACGATTATGATGAATATTGTCACTATGTGGCAGGTCTGGTTGGGTTAGGGTTG 540
A.thaliana      ACAATTGATGACTATGATGAATACTGCCATTATGCTGCAGGACTTGTTGGTTTAGGTTTG 540
                ******** ** ** ** ***** ** ** ****  ***** ** *****  * ** **

L.japonicus     TCAAAGCTTTTCCATGCCTCTGGTAAAGAAAAT-CTGGCAGCGGATTCCC------TTTC 593
C.asiatica      TCAAAGCTCTTCCATGCCTCTGGGGCTGAAGAT-TTGGCTTCAGACTCTC------TGTC 593
A.thaliana      TCAAAAATCTTCATTGCTTC-GGAATTAGAAATACTGACTCCAGATTGGAAGCAGATTTC 599
                *****  * ***  *** ** **      * **  ** *  * ** *         * **

L.japonicus     CAATTCAATGGGTTTGTTTCTTCAGAAAACAAACATTATTCGAGATTATCTGGAAGACAT 653
C.asiatica      TAATTCAATGGGTTTATTTCTCCAGAAGACAAACATCATTCGAGATTATCTGGAGGACAT 653
A.thaliana      AAATTCTACAGGTTTATTTCTGCAGAAAACAAACATTATCAAAGATTATCTTGAAGACAT 659
                 ***** *  ***** ***** ***** ******** **   ********* ** *****

L.japonicus     AAACGAGATCCCCAAATCACGCATGTTTTGGCCACGGCAGATCTGGAGTAAATATGTTAA 713
C.asiatica      AAATGAGATACCCAAGTCACGGATGTTTTGGCCACGGAAAATTTGGAATAAATATGTCAA 713
A.thaliana      TAATGAGAGACCAAAGTCGCGCATGTTTTGGCCTCGTGAGATTTGGGGAAAATATGTTGA 719
                 ** ****  ** ** ** ** ********** **  * ** ***   ********  *
```

```
L.japonicus      CAAACTTGAGGACTTGAAATATGAGGAGAACTCTGTTAAGGCAGTGCAATGTCTAAACGA 773
C.asiatica       CAAACTTGAGGACTTAAAATATGAGGAGAATTCGGTCAAGGCAGTGCAGTGCCTCAACGA 773
A.thaliana       CAAACTTGAGGACTTCAAAAATGAGGAGAAAGCTACAAAAGCAGTGCAGTGTTTGAATGA 779
                 *************** *** **********  *    ** ******** **  * ** **

L.japonicus      CATGGTCACTAATGCTCTGATGCATGCTGAAGATTGCTTAAAGTACATGTCTGATTTACG 833
C.asiatica       CATGGTCACGAATGCTTTGCTGCATGTTGAAGATTGCCTGAAATACATGTCTGATTTGCG 833
A.thaliana       AATGGTCACTAATGCATTGAATCATGTTGAAGATTGTTTGAAATCCTTGGCTTCACTGCG 839
                  ******** *****  **   **** *********  * ** * * ** **    * **

                                                    SQS R2
L.japonicus      AGACGATTCTATATTTCGCTTTTGTGCTATTCCCCAGATAATGGCAATTGGAACACTTGC 893
C.asiatica       AGATCCTGCTATCTTCCGGTTTTGTGCAATTCCACAGATTATGGCTATTGGAACTCTTGC 893
A.thaliana       TGATCCTGCAATATTTCAGTCTTGCGCCATCCCTCAGATCGTGGCGATTGGAACACTTGC 899
                  **   * * ** ** *  * *** ** ** ** *****  **** ******** *****

L.japonicus      AATATGCTACAACAACGTTGAGGTCTTCAGAGGTGTAGTGAAAATGAGGCGAGGTCTAAC 953
C.asiatica       TTTATGTTACAACAATCTTCAAGTCTTCAGAGGGGTAGTGAAAATGAGACGCGGTCTTAC 953
A.thaliana       GTTATGCTATAACAATGTACAAGTGTTTAGAGGTGTCGTGAGATTGAGACGAGGTCTAAT 959
                   **** ** *****  *  * ** ** ***** ** **** * **** ** ***** *

L.japonicus      TGCCAAAGTGATTGATCGGACAAAGACCATTGCTGATGTCTATGGTGCTTTCTTTGATTT 1013
C.asiatica       TGCTAAAGTTATTGACCGAACAAATAAAATGTCAGATGTATATGGTGCTTTCTACGATTT 1013
A.thaliana       AGCTAAAGTCATTGATCGCACAAAGACAATGGATGATGTCTACGGTGCGTTCTATGATTT 1019
                  ** ***** ***** ** ***** *  **    ***** ** ***** ****  *****

L.japonicus      TGCTTCTATGTTGGAGTCCAAGGTTGACAAAAATGATCCCAATGCAACAAAGACATTGAG 1073
C.asiatica       TTCTTGTATGTTGAAGACCAAGGTCGACAACAATGATCCCAATGCTACAAAAACTTTGAG 1073
A.thaliana       TTCTTGCATGCTACAAACAAAGGTTGACAATAACGATCCAAATGCTATGAAAACATTAAA 1079
                 * ***  *** *  *  * ***** ***** ** ***** ***** *  ** ** ** *

L.japonicus      CAGGCTGGTAGCTATACAGAAAACTTGCAGAGAATCTGGACT-CCTAAA---TAAAAGGA 1129
C.asiatica       CAGGCTAGAAGCAATTCAGAAGAAATGCAAGGAGTCAGGAGT-CATAACACCCAACAGGA 1132
A.thaliana       CCGACTCGAAACCATCAAGAAATTTTGCAAAGAAAATGGAGGACTTCA----CAAAAGAA 1135
                 * * ** * * * **  ****    ****  **    ***   * * *     ** ** *

L.japonicus      AATCTTACATTCTGAGGAAAGAGAACGGATATGGCTCAACACTGATTATCATACTGGTCC 1189
C.asiatica       AATCTTACGTACTTGAGAATGACTCGGGATACAACTTAGTCCTGATTGCCATCCTCTTCA 1192
A.thaliana       AATCTTATGT---TAACGATGA-----AACACAATCCAA----GGCTATC------TTTG 1177
                 *******  *       * **      * *      *    *  *  *       *

L.japonicus      TCTTGTTTTCCATCATGTTTGCTTATAGCTCTGCTACCCGCCAT--AGTAACTAG--- 1242
C.asiatica       TTATACTAGC--TCTTGTTTATGCATACCTATCTTCCAATCTATCGAATAACCGATGA 1248
A.thaliana       TTGTA---------ATGTTTGT---------------------------------- 1190
               *  *         *****
```

Fig. 3.29: Multiple sequence alignment of SQS nucleotide sequences of *Lotus japonicus* (Acc. No. AB102688), *Centella asiatica* (AY787628) and *Arabidopsis thaliana* (Acc. No. NM_119631). Forward and reverse primer sequences are highlighted.

Table 3.3: Primers used for isolation of partial BmSQS gene

Gene Name	**Forward and Reverse primers (5' → 3')**	
Squalene synthase (SQS)	SQS F1	TACTCCATGCTCCACAAGGTTTCT
	SQS F2	GCTCTGGATACCGTTGAGGATGATAC
	SQS R1	TCAATTGTTTCTACCTCCTTGCA
	SQS R2	GTTCCAATAGCCATAATCTGTGGAATTG

3.4.3.2 Amplification of partial cDNA fragment of *SQS* gene from *B. monniera* by PCR

Approximately 700 bp amplicon was obtained in PCR with primer combination (SQS F2 and SQS R2) and cDNA as a template (**Fig. 3.30 A**). This amplicon was cloned in pGEM-T Easy vector and insert release was confirmed by restriction digestion with *Eco*RI (**Fig. 3.30 B**) and sequenced. Sequencing result showed nucleotides of 669 bp (**Fig.3.31**). BLAST analysis showed more than 80% homology with reported *SQS* sequences at NCBI. Blast search of deduced amino acid sequence predicted the presence of putative conserved domains specific to Isoprenoid_Biosyn_C1 super family. This confirmed that isolated partial sequence was squalene synthase from *B. monniera*.

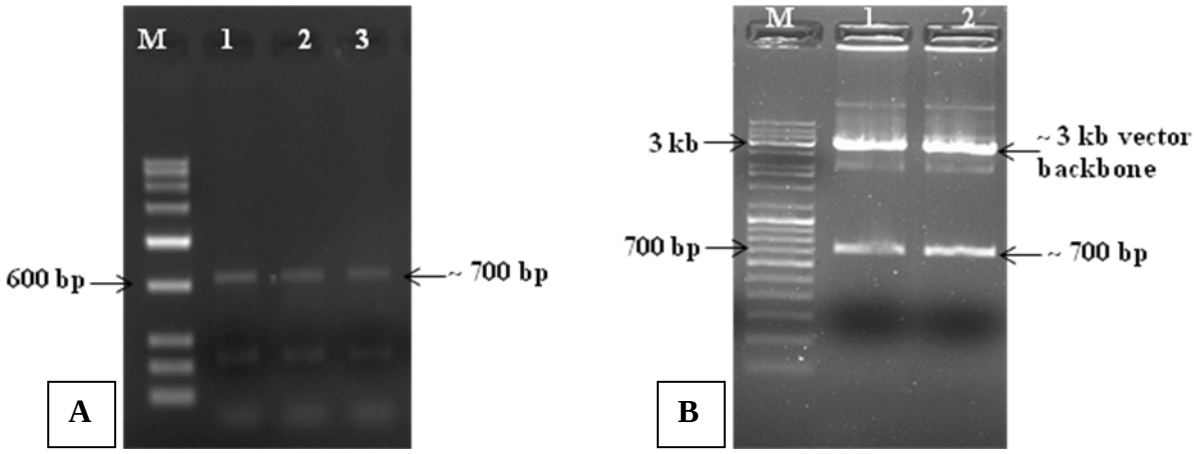


Fig. 3.30: A. 1% agarose gel showing amplification of ~ 700 bp (Lane1-3) of partial BmSQS, Lane M- Low range DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1 & 2- Showing vector backbone (~3 kb) and release of inserts (~700 bp), Lane M- Medium range DNA ruler.

Partial BmSQSS nucleotide sequence

```
  1 GCTCTGGATA CCGTTGAGGA TGATACGAGC ATATCTGCAG AGGTCAAAGT
 51 GCCTATTCTG AAGGCTTTCC ATCGTCACAT ATATGGCCAT GAATGGCATT
101 TTGCATGTGG TACGAAAGAC TATAAAGTTC TCATGGACGA GTTCCATCAT
151 GTTTCAACCG CCTTTCTGGA GCTTGGAAGT GGTTATCAGG AGGCAATTGA
201 GGATATTACC ATGAGAATGG GTGCTGGAAT GGCCAAATTT ATTTGCAAGG
251 AGGTAGAAAC TGTTGATGAT TATGATGAAT ATTGCCATTA TGTTGCTGGA
301 CTAGTTGGAT TAGGGTTGTC AAAGCTGTTC CATGCCTCTG GAAAGGAGGA
351 TCTGGCTTCC GATTCACTTT CGAACTCAAT GGGTTTATTC CTTCAGAAAA
401 CAAATATAAT AAGAGACTAT TTGGAGGACA TTAATGAGAT ACCAAAATCG
451 CGTATGTTTT GGCCTCGTCA AATTTGGAGC AAATATGTTA ACAAACTTGA
501 GGACTTGAAA TATGAGGAAA ACTCTGTTAA GGCTGTGCAG TGTCTGAATG
```

```
551 ACATGGTAAC AAATGCTTTA ATACATGTCG AAGAATTGCA TCAAATACAT
601 GTCTGCATTG CGGGATCCAG CGATTCTTTC GTTTTTTGTT GCAATTCCAC
651 AGATTATGGC TATTGGAAC
```

Fig. 3.31: Nucleotide sequences of partial BmSQS. Primers for RACE are underlined. Red coloured for 5' RACE and blue coloured for 3' RACE.

3.4.3.3 RACE PCR (5' and 3') of BmSQS

To obtain the 5' and 3' end of the gene, two steps RACE PCR was done. Gene Specific Primers were designed from above partial sequence of BmSQS (**Fig. 3.31**, Underlined).

3.4.3.3.1 5' RACE

RaceSQS R 5'-CATCAACAGTTTCTACCTCCTTGC-3'
RaceSQS NR 5'-CAGCACCCATTCTCATGGTAATA-3'

Nested PCR with primers RaceSQS NR and 5' NGRP yielded approximately 500 bp fragment (**Fig. 3.32 A**). It was cloned in pGEM-T Easy vector, confirmed by restriction digestion (**Fig. 3.32 B**) and sequenced.

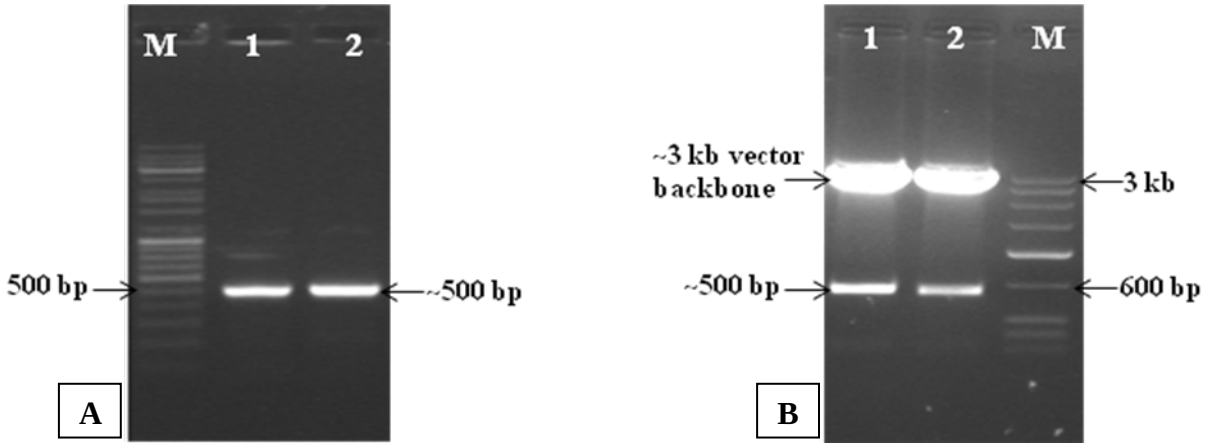


Fig. 3.32: A.1% agarose gel showing amplification of ~ 500 bp (Lane1 & 2) of 5'RACE PCR product, Lane M- Medium range DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1 & 2- Showing vector backbone (~3 kb) and release of inserts (~500 bp), Lane M- Low range DNA ruler.

Sequence analysis of 5' RACE clone revealed 5' UTR of 62 bp (Red) and start codon (ATG, Green underlined) (**Fig. 3.33**). BLAST analysis of sequence showed significant similarity with reported SQS from other plants.

```
  1 GGACACTGAC ATGGACTGAA GGAGTAGAAA AAAATTCATA TTTTCTTCGA
 51 TTCATAAAAA ATTCGAGGCC CAGCGAAAAC GAGAAGGAAT GGGTAGTTTG
101 CGTGCGATTT TGAGGCATCC GGATGATTTA TATCCGATGG TGAAGCTGAA
151 GTTGGCCGTT CGGAATGCGG AGAAGCAGAT CCCACCGGAG CCGCACTGGG
201 GATTCTGCTA TAAGATGCTT CATAAAGTTT CTAGAAGTTT CGCTCTCGTT
251 ATCCAGCAGC TCGATACCGA TCTCCGTGAT GCTGTATGTA TTTTCTATTT
301 GGTTCTTCGG GCACTCGACA CTGTTGAGGA TGATACAAGC ATATCTGCAG
351 AGGTCAAGGT GCCTATTCTG ATGGCTTTCC ACCGTCACAT ATATGACCGC
401 GAATGGCATT TCGCATGTGG TACGAAAGAC TATAAAGTTC TCATGGATGA
451 GTTCCATCAT GTTTCAACTT CCTTTCTGGA GCTTGGAAGT GGTTATCAGG
501 AGGCAATTGA GGATATTACC TTGAGGATGG GTGCTG
```

Fig. 3.33: Nucleotide sequence of *BmSQS* 5' RACE PCR clone. Start codon (ATG) green underlined and 5'UTR (Red). Primer, 5' NGRP is underlined.

3.4.3.3.2 3'RACE

RaceSQS F 5'- GCAGTGTCTGAATGACATGGTAAC-3'
RaceSQS NF 5'- GCATCAAATACATGTCTGCATTGC-3'

Approximately 750 bp amplicon was obtained in nested PCR with RaceSQS NF and 3' NGRP (**Fig. 3.34 A**). This was then cloned in pGEM-T Easy vector and confirmed by restriction digestion (**Fig. 3.34 B**) and sequencing.

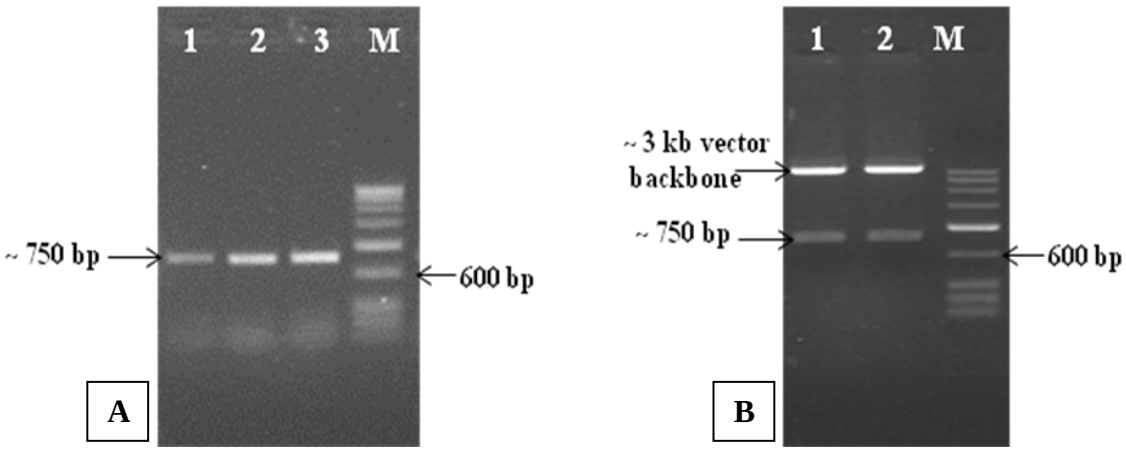


Fig. 3.34: A. 1% agarose gel showing amplification of ~ 750 bp (Lane1-3) of 3'RACE PCR product, Lane M- Low range DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1 & 2- Showing vector backbone (~3 kb) and release of inserts (~750 bp), Lane M- Low range DNA ruler.

Sequence analysis of 3' RACE clone showed stop codon (TGA, red coloured), 3' UTR of 263 bp (Green coloured) and putative polyadenylation site (Orange coloured, underline) followed by poly A tail (**Fig. 3.35**).

```
  1 GCATCAAATA CATGTCTGCA TTGGGAGATC CAGCGATCTT TCGTTTTTGT
 51 GCAATTCCTC AGGTCATGGC AATTGGGACA TTGGCTTTAT GCTACAACAA
101 CATTCAAGTT TTTAGAGGTG TTGTTAAAAT GAGACGTGGG CTCACAGCTA
151 AAGTTATCGA TCGTACCAAA TCAATGGCCG ATGTTTATGG AGCCTTTTAT
201 GATTTTTCTC GTATGCTAAA ATCTAAGGTT GAGGACAGTG ATCCTAATGC
251 TAGAAAGACC AAGGACAGGT TAGAAGCAAT CTTGAAGATT TGTAGGGAGT
301 CTGGAACACT AAACCAAAGG AAATCGTACA TTATTCAGAG CAAGCCCCAA
351 AATTATGCCA CGTCGATTGT CATCATCTTC ATAATAATAG CTATTCTGTT
401 AGCATACTTG TCTGCAACTC GGCCCACTCA CATGTGAATG GTTTTTTGAT
451 GGAAACCTGA AGTGACTGTC CAGTTGTTGG AACTTATTAA TGGACTCAGC
501 GAAATGTTGA TACTTTTTGT TCTTTAAGGA GTGGCCCGTA TGTACCAATT
551 TGTATTAATT ACTCTAGGCA AATAACAGTG GTTAGTTTTT AGTTTGTATT
601 GCACCTAGCC TTTTATTTTG TATATCTCGC TTGGGCATGT ATGTTATACC
651 AAATCAGGAT ATGTTGTAAA GCCAAATAAT GTCTAGGTTT TGTATCTCTT
701 AAAAAAAAAA AAAAAACACT GTCATGCCGT TACGTAGCG
```

Fig. 3.35: Nucleotide sequence of 3' RACE clone of BmSQS. Stop codon (Red), 3' UTR (Green) and polyadenylation site (orange underlined) are shown. Primer 3'NGRP is underlined.

3.4.3.4 Amplification of full-length BmFPS ORF

Primers were designed from start and stop codon to amplify full-length ORF of BmSQS as a single PCR product on the basis of 5' and 3' RACE sequences.

SQS Full F 5'- ATGGGTAGTTTGCGTGCG-3'
SQS Full R 5'- TCACATGTGAGTGGGCCGAG-3'

PCR was performed with above primers SQS Full F/R and cDNA as a template using high fidelity *Taq* DNA polymerase. Approximately 1.2 kb amplicon (**Fig. 3.36 A**) was cloned in pGEM-T Easy vector and confirmed by restriction digestion (**Fig. 3.36 B**) and sequencing.

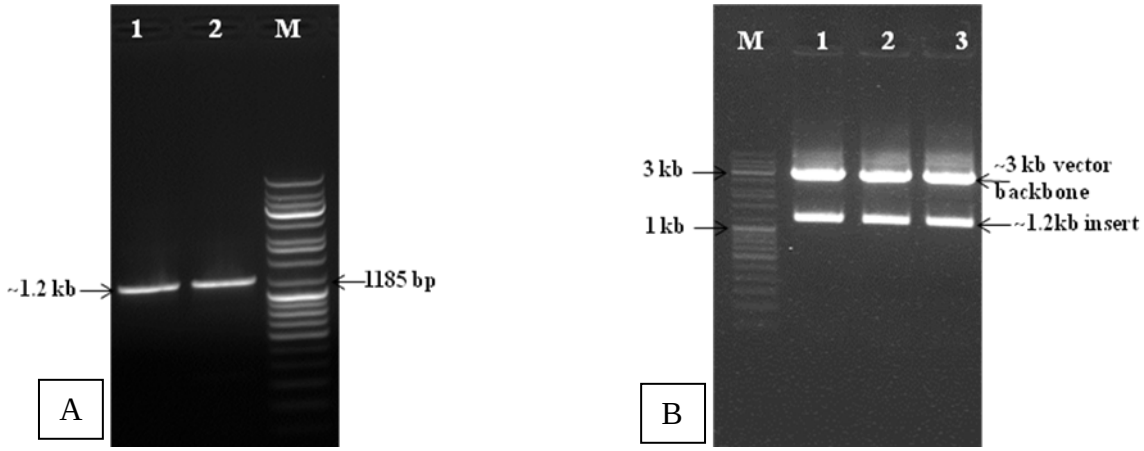


Fig. 3.36: A. 1% agarose gel showing amplification of ~ 1.2 kb (Lane1&2) of full-length *BmSQS* PCR product, Lane M- Medium range DNA ruler. **B.** Restriction digestion with *Eco*RI. Lane 1-3 - Showing vector backbone (~3 kb) and release of inserts (~1.2 kb), Lane M- Medium range DNA ruler.

Sequence analysis of 8-10 clones showed the exact size of 1245 bp (**Fig. 3.37**) and full-length ORF with 5' and 3' UTRs was submitted to NCBI GenBank data base having accession number **GU734711**.

```
   1 ATGGGTAGTT TGCGTGCGAT TTTGAAGCAT CCGGATGATT TGTATCCGAT
  51 GGTGAAGCTG AAGTTGGCCG TTCGGAATGC GGAGAAGCAG ATCCCGCCGG
 101 AGCCGCACTG GGGATTCTGC TACACGATGC TTCATAAAGT TTCCAGAAGT
 151 TTCGCTCTCG TTATTCAGCA GCTCGATACC GATCTCCGTG ATGCTGTACG
 201 TATTTTCTAT TTGGTTCTTC GAGCACTTGA CACTGTTGAG GACGATACAA
 251 GCATATCTGC AGAGGTCAAA GTGCCTATTC TGAAGGCTTT CCATCGACAC
 301 ATATATGACC GTGAATGGCA TTTTGCATGT GGTACGAAAG ACTATAAAGT
 351 TCTCATGGAC GAGTTCCATC ATGTTTCAAC TGCCTTTCTG GAGCTTGGAA
 401 GTGGTTATCA GGAGGCAATT GAGGATATTA CCATGAGAAT GGGTGCTGGA
 451 ATGGCCAAAT TTATTTGCAA GGAGGTAGAA ACTGTTGATG ATTATGATGA
 501 ATATTGCCAT TATGTTGCTG GACTAGTTGG ATTAGGGTTG TCAAAGCTGT
 551 TCCATGCCTC TGGAAAGGAG GATCTGGCTT CCGATTCACT TTCTAACTCA
 601 ATGGGTTTAT TCCTTCAGAA AACAAATATA ATAAGGGACT ATTTGGAGGA
 651 CATTAATGAG ATACCAAAAT CGCGTATGTT TTGGCCTCGT CAAATTTGGA
 701 GCAAATATGT TAACAAACTT GAGGACCTGA AATATGAGGA AAACTCTGTT
 751 AAGGCTGTGC AGTGTCTGAA TGACATGGTA ACAAATGCTT TAATGCATGT
 801 CGAAGATTGC ATCAAATACA TGTCTGCATT GGGAGATCCA GCGATCTTTC
 851 GTTTTTGTGC AATTCCTCAG GTCATGGCAA TTGGGACATT GGCTTTATGC
 901 TACAACAACA TTCAAGTTTT TAGAGGTGTT GTTAAAATGA GACGTGGGCT
 951 CACAGCTAAA GTTATCGATC GTACCAAATC AATGGCCGAT GTTTATGGAG
1001 CCTTTTATGA TTTTTCTCGT ATGCTAAAAT CTAAGGTTGA GGACAGTGAT
1051 CCTAATGCTA GAAAGACCAA GGACAGGTTA GAAGCAATCT TGAAGATTTG
1101 TAGGGAGTCT GGAACACTAA ACCAAAGGAA ATCGTACATT ATTCAGAGCA
1151 AGCCCCAAAA TTATGCCACG TCGATTGTCA TCATCTTCAT AATAATAGCT
1201 ATTCTGTTAG CATACTTGTC TGCAACTCGG CCCACTCACA TGTGA
```

Deduced amino acid sequence

```
MGSLRAILKHPDDLYPMVKLKLAVRNAEKQIPPEPHWGFCYTMLHKVSRSFALVIQQLDTDLRDAVRIFYLVLR
ALDTVEDDTSISAEVKVPILKAFHRHIYDREWHFACGTKDYKVLMDEFHHVSTAFLELGSGYQEAIEDITMRMG
AGMAKFICKEVETVDDYDEYCHYVAGLVGLGLSKLFHASGKEDLASDSLSNSMGLFLQKTNIIRDYLEDINEIP
KSRMFWPRQIWSKYVNKLEDLKYEENSVKAVQCLNDMVTNALMHVEDCIKYMSALGDPAIFRFCAIPQVMAIGT
LALCYNNIQVFRGVVKMRRGLTAKVIDRTKSMADVYGAFYDFSRMLKSKVEDSDPNARKTKDRLEAILKICRES
GTLNQRKSYIIQSKPQNYATSIVIIFIIIAILLAYLSATRPTHM
```

Fig. 3.37: Nucleotide and deduced amino acid sequence of full-length BmSQS.

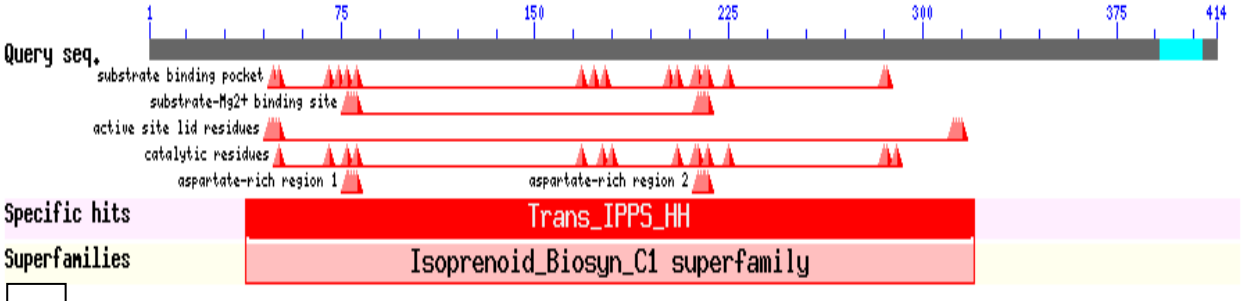
3.4.3.5 Characterization of BmSQS

3.4.3.5.1 Protein sequence analysis of BmSQS

The deduced amino acid sequence of BmSQS shared 84.3%, 83.6%, 82.9% and 78.9% identities with *Salvia miltiorrhiza*, *Diospyros kaki*, *Capsicum annuum* and *Artemisia annua* respectively. The calculated molecular mass and predicted pI value of BmSQS were 47.37 kDa and 7.97 respectively. The total number of positively and negatively charged anino acids was 53 and 51 respectively. The computed instability index and aliphatic index were 36.77 and 95.17 respectively, indicates thermo stability of the BmSQS. According to NCBI conserved domain search, BmSQS showed specific hits with "Trans_IPPS_HH" and super family Ispprenoid_Biosyn_C1. Six (I-VI, **Fig. 3.38 B**) highly conserved SQS domains (Important for the catalytic activity of squalene synthase) were found after multiple alignment of BmSQS deduced amino acid sequence with other SQS proteins. Three domains (III, IV and V) showed highly conserved consensus sequence with other SQS proteins, whereas domains I and II are much less conserved (**Fig. 3.38 A**). Domain II showed highly conserved aspartate rich motif among the other SQS enzymes ((Devarenne et al., 1998). Domain IV was also aspartate rich motif and these motifs were proposed to coordinate/facilitate FPP binding through a magnesium ion requirement (Huang et al., 2007). Even though domain VI exhibits a low level of sequence homology with other SQS in alignment, the domain in the carboxy terminus of the protein contained highly hydrophobic residues, very similar to other SQS sequences and consistent with the putative endoplasmic reticulum membrane anchoring function.

```
B.monniera          MGSLRAILKHPDDLYPMVKLKLAVRNAEKQIPPEPHWGFCYTMLHKVSRSFALVIQQLDT 60
S.miltiorrhiza      MGSLRAILRHPDDLYPLLKLKYAARNAEKQIPPEPHWGFCYTMLHKVSRSFGLVIQQLDT 60
S.lycopersicum      MGTLRAILKNPDDLYPLIKLKLAARHAEKQIPPEPHWGFCYLMLQKVSRSFALVIQQLPV 60
C.asiatica          MGSLGAILKHPDDFYPLLKLKMAARHAEKQIPPEPHWAFCYSMLHKVSRSFGLVIQQLGP 60
                    **:* ***::***:**::*** *.*:**********.*** **:******.******

B.monniera          DLRDAVRIFYLVLRALDTVEDDTSISAEVKVPILKAFHRHIYDREWHFACGTKDYKVLMD 120
S.miltiorrhiza      DLRDAICIFYLVLRALDTVEDDTSIATELKVPILMAFHRHIFDREWHFSCGTKEYRVLMD 120
S.lycopersicum      ELRDAVCIFYLVLRALDTVEDDTSIPTDVKVPILISFHQHVYDREWHFACGTKEYKVLMD 120
C.asiatica          QLRDAVCIFYLVLRALDTVEDDTSISTEVKVPILKAFHRHIYDNNWHFSCGTKEYKILMD 120
                    :****: ***************.:::***** :**:*::*.:***:****:*::***
                         I              II
B.monniera          EFHHVSTAFLELGSGYQEAIEDITMRMGAGMAKFICKEVETVDDYDEYCHYVAGLVGLGL 180
S.miltiorrhiza      QFHHVSTAFLELAPGYQDAIEDITMRMGAGMAKFICKEVETTNDYDEYCHYVAGLVGLGL 180
S.lycopersicum      QFHHVSTAFLELGKLYQQAIEDITMRMGAGMAKFICKEVETTDDYDEYCHYVAGLVGLGL 180
C.asiatica          EFHHVSNAFLELGSGYKEAIEDITMRMGAGMAKFICKEVETIDDYDEYCHYVAGLVGLGL 180
                    :*****.*****.  *::********************** :*****************
                                                                    III
B.monniera          SKLFHASGKEDLASDSLSNSMGLFLQKTNIIRDYLEDINEIPKSRMFWPRQIWSKYVNKL 240
S.miltiorrhiza      SKLFHASGKEDLATDAMSNSMGLFLQKTNIIRDYLEDINEIPKSRMFWPRQIWSKYVNKL 240
S.lycopersicum      SKLFHASGKEDLASDSLSNSMGLFLQKTNIIRDYLEDINEVPKCRMFWPREIWSKYVNKL 240
C.asiatica          SKLFHASGAEDLASDSLSNSMGLFLQKTNIIRDYLEDINEIPKSRMFWPRKIWNKYVNKL 240
                    ******** ****:*::**********************:**.******:**.******
                                                  IV
B.monniera          EDLKYEENSVKAVQCLNDMVTNALMHVEDCIKYMSALGDPAIFRFCAIPQVMAIGTLALC 300
S.miltiorrhiza      EDLKSQENSVKAVQCLNDMVTNALVHVEDCLKYMSDLRDPAIFRFCAIPQIMAIGTLALC 300
S.lycopersicum      EDLKYEENSVKAVQCLNDMVTNALSHVEDCLTYMFNLHDPAIFRFCAIPQVMAIGTLAMC 300
C.asiatica          EDLKYEENSVKAVQCLNDMVTNALLHVEDCLKYMSDLRDPAIFRFCAIPQIMAIGTLALC 300
                    **** :****************** *****:.**  * ***********:*******:*
                                                                  V
B.monniera          YNNIQVFRGVVKMRRGLTAKVIDRTKSMADVYGAFYDFSRMLKSKVEDSDPNARKTKDRL 360
S.miltiorrhiza      YDNIEVFRGVVKMRRGLTAKIIDRTKTMFDVYGAFYDFSRMLKFKVDDKDPNAGTTKDRL 360
S.lycopersicum      YDNIEVFRGVVKMRRGLTAKVIDRTKTMADVYGAFFDFSCMLKSKVNNNDPNATKTLKRL 360
C.asiatica          YNNLQVFRGVVKMRRGLTAKVIDRTNKMSDVYGAFYDFSCMLKTKVDNNDPNATKTLSRL 360
                    *:*::***************:****:.* ******:*** *** **::.**** .* .**

B.monniera          EAILKICRESGTLN-QRKSYIIQSKPQ-NYATSIVIIFIIIAILLAYLSATRPTHM 414
S.miltiorrhiza      DSILKICRDSGTLN-DRKSYIIESGPQYNAAIMVAIFFIILAILQAYFAPKRAV-- 413
S.lycopersicum      DAILKTCRDSGTLN-KRKSYIIRNEPN-YSPVLIVVIFIILAIILAQLFGSRS--- 411
C.asiatica          EAIQKKCKESGVITPNRKSYVLENDSG-YNLVLIAILFIILALVYAYLSSNLSNNR 415
                    ::* * *::**.:. .****::.. .      :.::****:*:: * :  . .
                                                    VI
```

A



B

Fig. 3.38:A. ClustalW alignment of BmSQS with other plant SQSs showing different domains (I-VI) and catalytic motifs in different color. **B.** CDD search results on NCBI server.

3.4.3.5.2 Hydropathy plot analysis of BmSQS

The amino acid sequences of BmSQS was analyzed using Kyte-Doolittle Hydropathy plot of JustBio hosted tool (http://www.justbio.com/index.php?page=plots) at window size 9. Plot analysis revealed even distribution of hydrophilic and hydrophobic residues (**Fig. 3.39**). Presence of two transmembrane domains was found in BmSQS on TMHMM bioinformatics program (**Fig.3.40**).

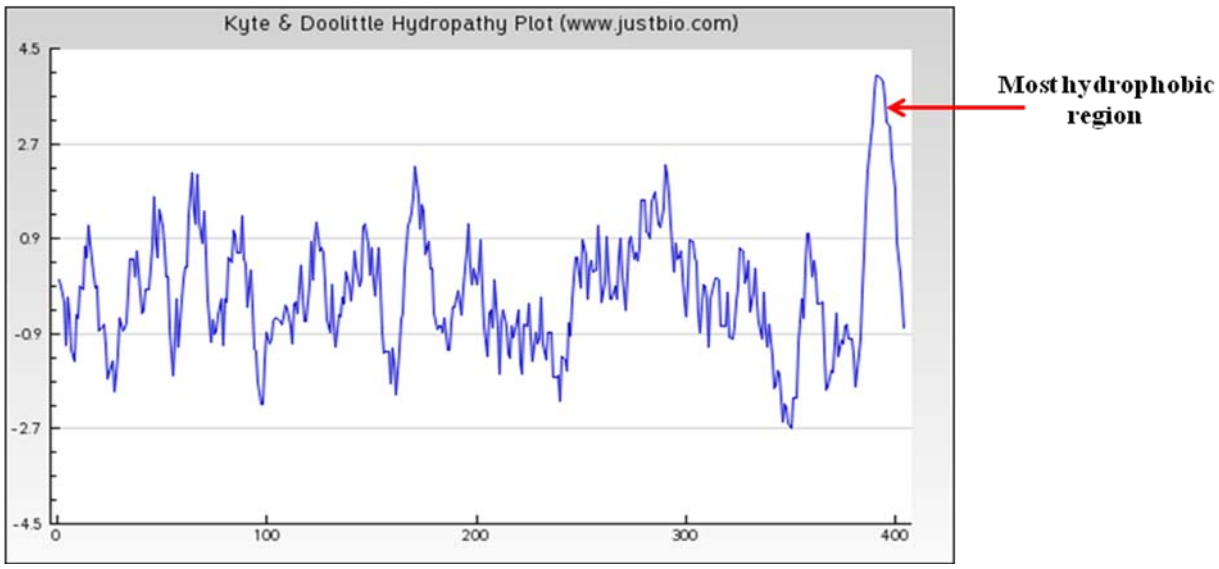


Fig. 3.39: Kyte-Doolittle Hydropathy plot. Arrow indicates C-terminus highly hydrophobic region.

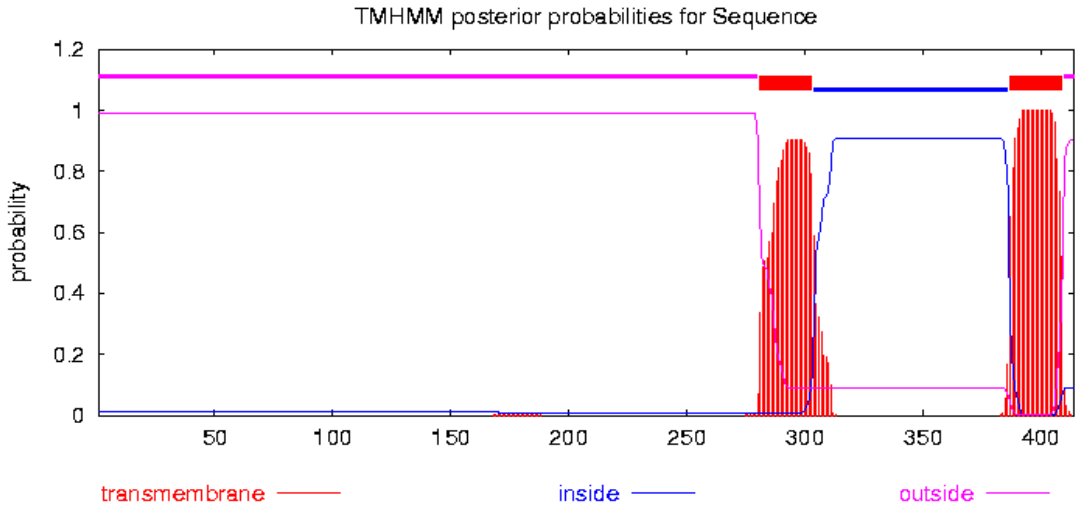


Fig. 3.40: TMHMM analysis of BmSQS. Trans membrane domain I (281-303 AA), domain II (387-409 AA).

3.4.3.5.3 Phylogenetic tree analysis

A phylogenetic tree was constructed by Neighbor-Joining method with MEGA 4.0.2 program from the ClustalX2 alignment of the BmSQS with number of SQSs from different organisms which were retrieved from the NCBI GenBank database. Phylogenetic analysis demonstrated that BmSQS had closer relationship with dicots SQS (Cluster I) including *S. miltiorrhiza*, *D. kaki*, *Lotus japonicas* and *A. annua* (**Fig. 3.41**).

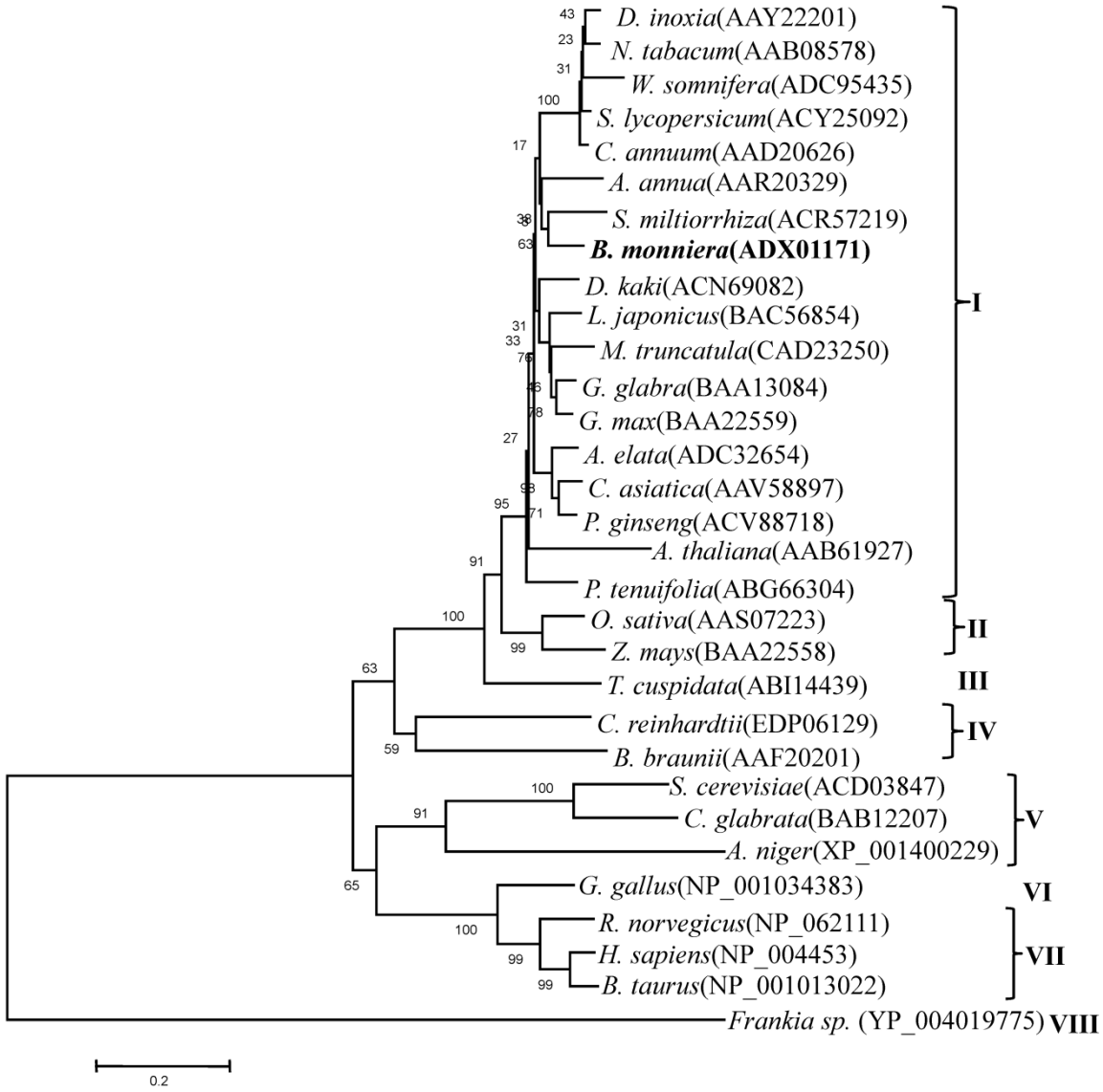


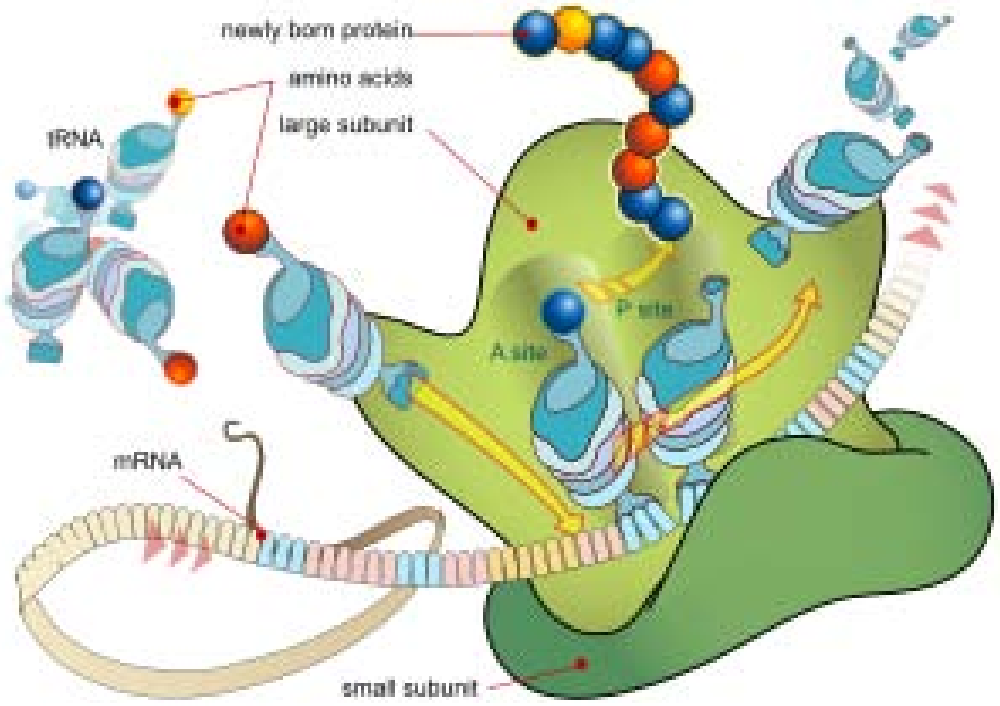
Fig. 3.41: Phylogenetic tree analysis of BmSQS. Cluster I- Dicots, II- Monocots, III- Gymnosperm, IV- Algae, V-Yeasts, VI- Bird, VII- Mammals, VIII- Bacterium. Tree was formed by MEGA 4.0.2 using neighbor-joining method.

3.5 Conclusions

- Three isoprenoids biosynthetic pathway genes encoding HMG-CoA reductase (*BmHMGR*), FPP synthase (*BmFPS*) and Squalene synthase (*BmSQS*) have been isolated from *B. monniera* by PCR based approach.
- Two isoforms of BmHMGR (BmHMGR1 and BmHMGR6) have been identified sharing 98% identity with each other and shared more than 73% sequence homology in BLAST analysis.
- BmFPS and BmSQS showed more than 80% sequence homology with other reported sequences at NCBI.
- RACE PCR was used to identify 5' and 3'UTRs of all three genes.
- Analysis of deduced amino acid of BmHMGR showed presence of different domains involved in catalytic activities. Presence of two transmembrane domains at N- terminus and one at C-terminus were predicted.
- In case of BmFPS presence of two domains (domain I & II) involved in substrate binding, Mg2+ binding sites and active site lid residues shield highly reactive carbocationic intermediates from solvents, were predicted.
- Hydropathy plot analysis of BmSQS showed presence of highly hydrophobic region at C-terminus and two transmembrane domains at C-terminus were also detected.
- Phylogenetic analysis revealed, isolated genes are closely related with other dicot plant species and *B. monniera* genes are close to *S. miltiorrhiza*.

CHAPTER: 4(A)

HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF TERPENOID BIOSYNTHETIC PATHWAY GENE(S)



Chapter 4(A). Heterologous expression, purification and characterization of terpenoid biosynthetic pathway gene(s)

This section of chapter 4 describes the heterologous expression of *B. monniera* HMG-CoA reductase, FPP synthase and squalene synthase genes in *E. coli*, their purification and *in-silico* characterization. This chapter will also describe the specific materials and methods used in the experiments.

4.1 Introduction

Triterpenoids, including steroids, are a highly diverse group of secondary metabolites widely distributed in plants (Vincken et al., 2007). Biosynthesis of terpenoids takes place via isoprenoid pathway starting with acetyl-CoA. Isopentenyl pyrophosphate (IPP) is C5 compound which serves as central metabolite leading to formation of various terpenoids and steroids. In plants IPP is synthesized via MVA an MEP pathway. The details of pathway are described in chapter 1. Several genes of isoprenoid pathway from different sources have been characterized. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonate and has been considered as the first key step in the MVA pathway in plants (Chappell et al., 1995; Ha et al., 2003). Farnesyl pyrophosphate synthase catalyzes the sequential head-to-tail condensations of IPP, first with DMAPP and then with the resulting C10 compound GPP to produce the C15 compound FPP. FPP is located at the first multiple branch point in the terpenoid biosynthetic pathway, suggesting that FPP biosynthesis is tightly regulated (Ding et al., 2008). The enzyme squalene synthase catalyzes a reductive condensation of two FPP molecules into C30 compound squalene. It is commonly described as a crucial branch point enzyme away from the main isoprenoids biosynthetic pathway and the potential regulatory point that controls carbon flux into triterpenes and sterol biosynthesis (Huang et al., 2007). Since these three points are considered as a regulatory point of isoprenoid pathway, the study of these enzymes will facilitate the understanding of their role in triterpenes biosynthesis in *B. monniera*.

In previous chapter (Chapter 3) we described isolation, cloning and characterization of genes encoding HMG-CoA reductase, FPP synthase and squalene synthase from *B. monniera*. This chapter deals with heterologous expression of these

genes in *E. coli*, their purification and characterization using various bioinformatics tools.

4.2 Materials and methods

4.2.1 Stock solutions

IPTG (1M) in sterile milliQ water and filter sterilized

Kanamycin (50 mg/mL) in sterile milliQ water and filter sterilized

4.2.2 Plasmid vectors and host strains

Escherichia coli XL-10 Gold (Stratagene, USA)

E. coli, BL 21 (DE3) (Novagen, USA)

PGEM-T Easy T/A Cloning vector (Promega, USA)

pET30b (+) Expression vector (Novagen, USA).

4.2.3 Methods

Bacterial culture conditions, transformation and selection, plasmid isolation, restriction digestion etc. are already described in chapter 2.

4.2.3.1 Cloning of *BmHMGR*, *BmFPS* and *BmSQS* genes in expression vector pET 30b (+)

ORFs of all three genes were cloned in pGEM-T Easy vector by incorporating appropriate restriction sites in the primers. Since both isoforms of BmHMGRs (BmHMGR 1 and BmHMGR 6) shares 98.1% identity, only BmHMGR 6 was used for expression studies.

Table 4A.1: Primers used for expression studies in *E. coli*.

Gene name	Primer name	Sequence (5' → 3')
BmHMGR	pETHMG F	**GGTACC**ATGGAGGCTAAGGGAC (*Kpn*I)
	pETHMG R	**CTCGAG**TTGAGACGCAACCTTG (*XhoI*)
BmFPS	pETFPS F	**CATATG**GCGAATCATAATGGACA (*Nde*I)
	pETFPS R	**CTCGAG**CTTCTGCCTCTTGTATA (*Xho*I)
BmSQS	pETSQS F	**CATATG**GGTAGTTTGCGTGCG (*Nde*I)
	pETSQS R	**CTC GAG** CGT GGC ATA ATT TTG G (*Xho*I)

In case of *BmHMGR* gene, *Kpn* I restriction site was added to the forward primer and *Xho* I restriction site was added to the reverse primer (namely pETHMG F and pETHMG R) whereas *Nde* I restriction site was added to the forward primer and *Xho*I restriction site was added to the reverse primer of the BmFPS and BmSQS genes (namely pETFPS F & pETFPS R and pETSQS F & pETSQS R). In case of BmSQS, 24 amino acids from C-terminus (most hydrophobic region) were truncated to increase the solubility. High fidelity *Taq* DNA polymerase (*Pfx*, Invitrogen) was used to amplify the genes using the above set of primers and cDNA clone maintained in pGEM-T Easy vector as template. The amplified products with restriction sites were further cloned in pGEM-T Easy vector and transformed in *E. coli* XL10 Gold cells. Clones were screened by colony PCR and a few colonies were subjected to plasmid isolation. Isolated individual plasmids of all three genes were restriction digested with respective restriction enzymes to confirm the integration of the genes.

PCR cycling conditions

Temperature	**Time**	**Cycles**
94 ºC	5 min	1 cycle
94 ºC	30 s	35 cycles
*53 ºC	30 s	
72 ºC	#1 min 30 s	
72 ºC	10 min	1 cycle

*In case of BmHMGR 53 ^{o}C; for BmFPS and BmFPS and BmSQS 58 ^{o}C.

In case of BmHMGR 2 min; for BmFPS and BmSQS 1 min 30 s.

The recombinant BmHMGR, BmFPS and BmSQS clones with respective restriction sites were directionally cloned in pET-30b (+) vector (**Fig. 4A.1**). Colony PCR with T7 promoter (5'-TAATACGACTCACTATAGGG-3') and T7 terminator (5'-GCTAGTTATTGCTCAGCGG-3') was done to screen the recombinant pET 30b (+) clones. Integration of genes in pET 30b (+) were confirmed by digestion with respective restriction enzymes (**Fig. 4A.2**). All clones were also confirmed by sequencing.

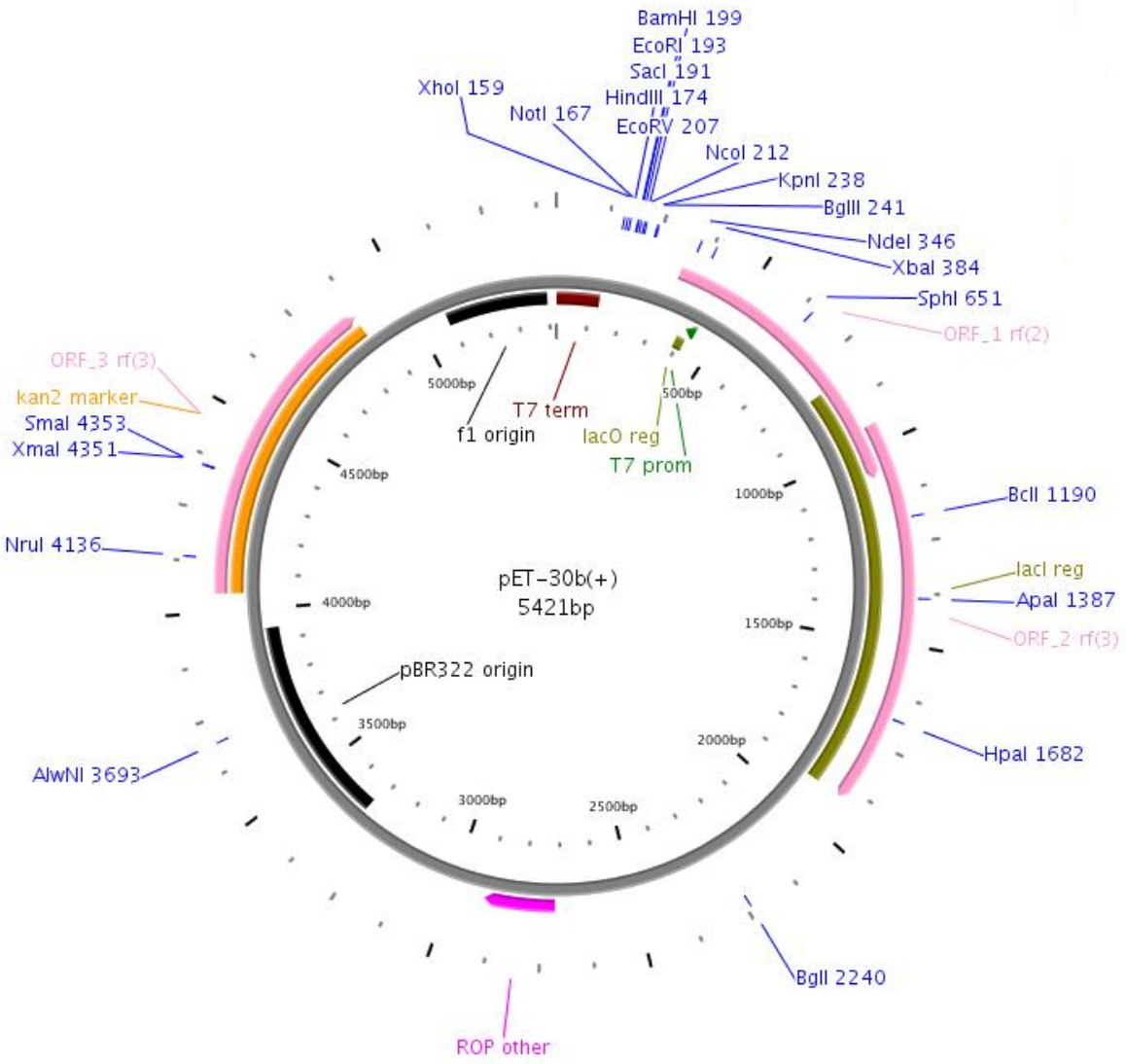
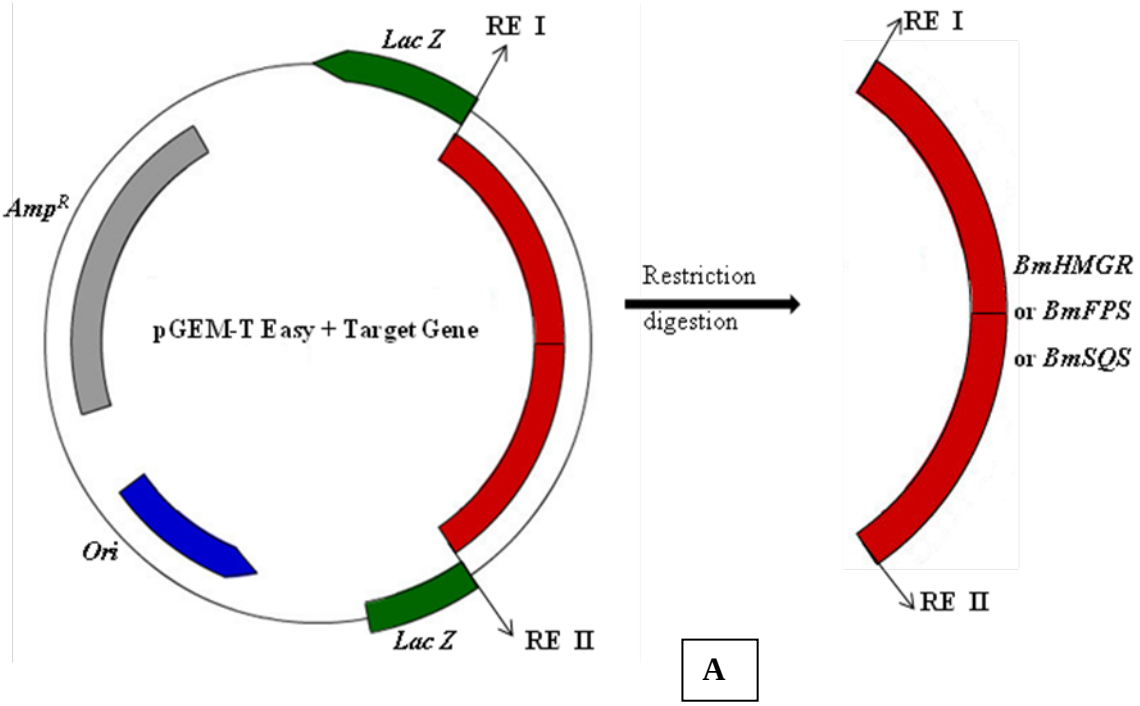
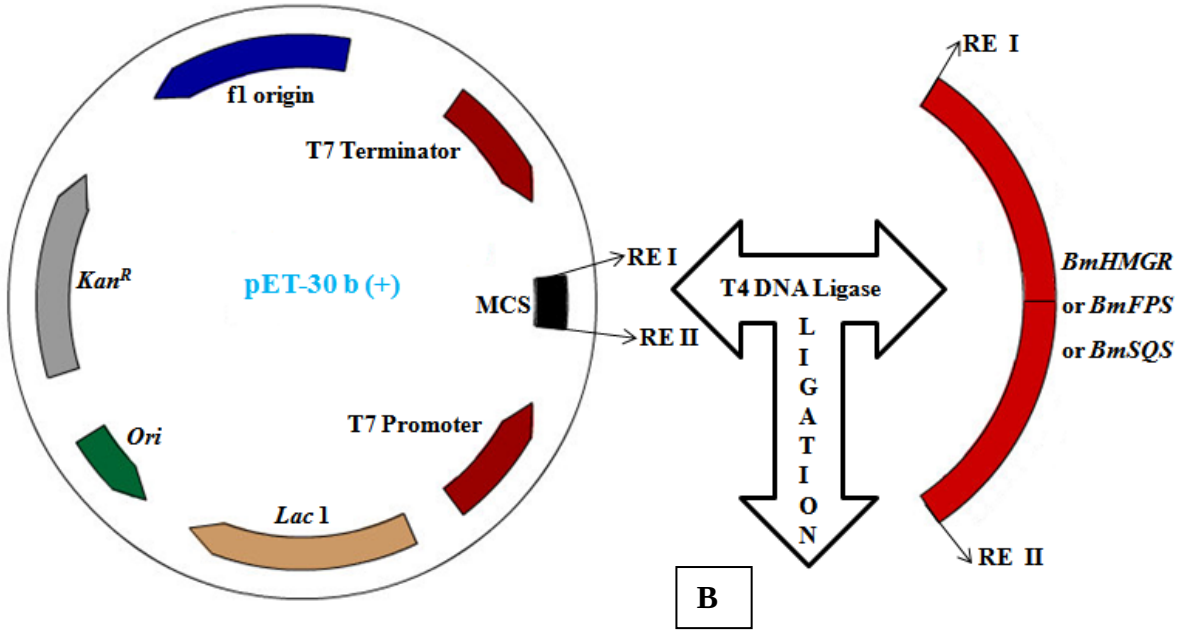


Fig. 4A.1: Vector map of pET 30b (+).


Lac Z
RE I
Amp^R
pGEM-T Easy + Target Gene
Ori
Lac Z
RE II
Restriction
digestion
RE I
BmHMGR
or BmFPS
or BmSQS
RE II
A


f1 origin
T7 Terminator
Kan^R
pET-30 b (+)
MCS
RE I
RE II
Ori
T7 Promoter
Lac I
T4 DNA Ligase
LIGATION
RE I
BmHMGR
or BmFPS
or BmSQS
RE II
B

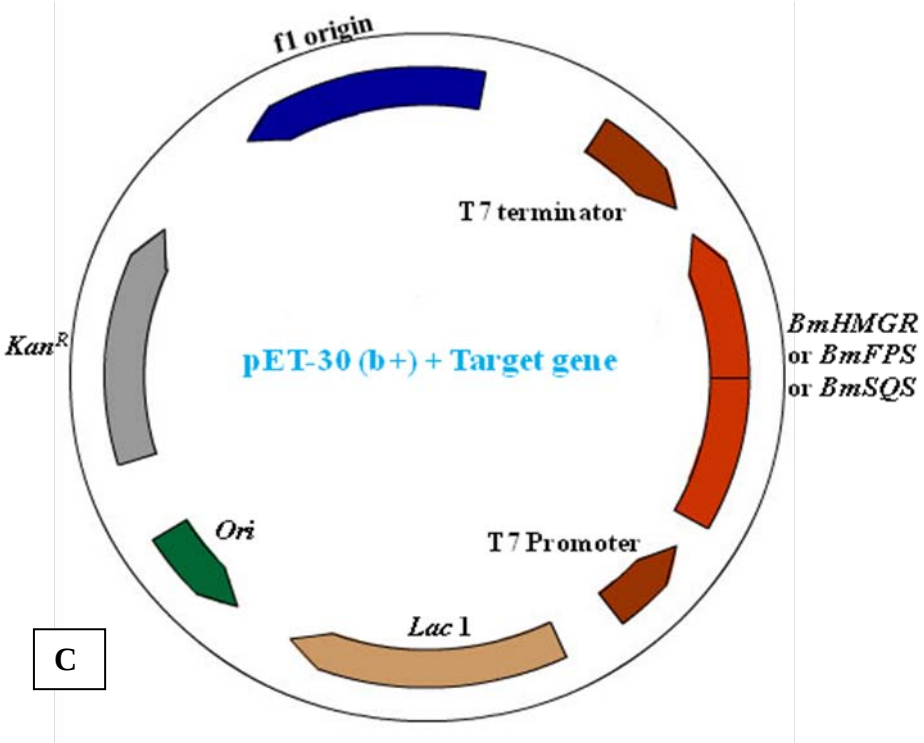


Fig. 4A.2: Strategy used for directional cloning of genes in pET-30b (+) vector. **A.** Target gene in pGEM-T Easy vector and double digested fragment with sticky ends. **B.** Ligation in pET 30 b (+) flanked with same restriction sites. **C.** Expression construct for over-expression of recombinant protein.

4.2.3.2 Recombinant protein expression and its purification

4.2.3.2.1 Preliminary screening of recombinant protein expression in *E. coli* (BL21)

Recombinant pET 30b (+) plasmids harboring *BmHMGR*, *BmFPS* and *BmSQS* were transformed into *E. coli* strain BL21 (DE3). A single bacterial (BL21) colony carrying recombinant pET 30b (+) with respective genes, from freshly streaked plates (grown on LB agar medium containing 50 µg/mL kanamycin) was used to inoculate 5 mL liquid LB medium containing the same concentrations of the antibiotic. Culture was grown overnight with shaking at 200 rpm at 37 °C. One mL aliquot of over-night grown culture was used to inoculate 100 mL LB broth containing 50 µg/mL kanamycin. Once the cultures reached OD_{600} 0.4 to 0.6, recombinant protein expression was induced by the addition of isopropyl β-D thiogalactopyranoside (IPTG), and the culture was grown for 4 to 6 h at 37 °C with shaking at 200 rpm. Recombinant protein extraction was done

according to the protocol described in chapter 2 and over-expression of protein was checked on 10 % SDS-PAGE.

4.2.3.2.2 Optimization of recombinant protein expression

Transformed cells showing promising expression of respective genes were optimized for IPTG concentration, incubation temperature and duration of incubation after induction. After initial screening, cells were grown in 5 mL of LB broth containing kanamycin (50 µg/mL) and grown for overnight as a primary culture. Fresh 100 mL of LB broth containing same concentration of kanamycin was inoculated with 1% of primary culture. Once the cultures reached OD_{600} 0.4 to 0.6, expression of protein was induced by adding IPTG. Both IPTG concentration and incubation temperature were reduced for solubility of proteins and incubation duration was increased to compensate the decreased growth rate of the cells.

4.2.3.2.3 Purification of recombinant proteins

All proteins were expressed as a His-tag fusion protein and purified by Metal Chelate Affinity Chromatography. The initial stage of His-tagged protein purification is based on the remarkable selectivity and high affinity of patented Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of six consecutive histidine residues, the 6xHis-tag. NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal chelating purification systems that have only three sites available for interaction with metal ions. The extra chelation site prevents nickel ion leaching, providing a greater binding capacity and high-purity protein preparations. Purification of recombinant proteins carrying a 6xHis-tag was conducted using Ni-NTA Agarose beads (mentioned in chapter 2). Purity of protein was checked on 10% SDS-PAGE.

4.2.3.2.4 Protein estimation

Protein estimation was done using Bradford assay (Bradford, 1976). A standard graph was made for BSA and concentration of unknown sample was determined by using standard graph.

4.2.3.3 Raising of polyclonal antibodies against purified BmFPS and BmSQS protein in rabbit

The purified BmFPS and BmSQS proteins were used for raising polyclonal antibodies in New Zealand White rabbit. ELISA was performed to determine the titre of first, second and third bleed of rabbit serum. Once the antibody titre was determined then, a fixed dilution of antibody was used for rest of the experiments.

4.2.3.4 Western blot analysis and ELISA of BmFPS and BmSQS with total plant protein

Total soluble protein (TSP) from shoots of *Bacopa* plant was used for western blot analysis. Methodologies are already discussed in chapter 2. For ELISA detection of BmFPS and BmSQS, TSP was extracted from stem, leaves and roots and equal quantities were coated on 96 well microtitre plates (details are discussed in chapter 2).

4.2.3.5 Prediction of secondary and tertiary structure of BmHMGR, BmFPS and BmSQS (Comparative modeling)

The protein sequences of BmHMGR, BmFPS and BmSQS was generated from the nucleotide sequence of the respective genes. BLASTp and a PSI-BLAST search by using the query sequence against the Brookhaven Protein Data Bank were performed to identify close homologs crystal structures which are available. The target and template sequences were aligned using T-coffee server, with default alignment parameter option. The alignment was edited using structural information. Secondary structure analysis was carried out in the PSIPRED server (http://bioinf.cs.ucl.ac.uk/psipred/). 3D models were built using MODELLER 9v10 version. Out of 50 models generated, the model with the lowest DOPE scores was taken as the final model.

4.2.3.6 Refinement and validation of homology model

The protein model was imported to maestro window (Schrödinger, Inc.). The protein was energy minimized using the protein preparation wizard (shipped by Schrödinger) by applying OPLS_2005 force field by application of the autoref.pl script. Progressively weaker restraints (tethering force constants 3, 1, 0.3, 0.1) were applied to non hydrogen atoms only. This refinement procedure is recommended by Schrödinger (technical notes for version 1.8), because Glide uses the full OPLS-AA force field at an

intermediate docking stage and is claimed to be more sensitive towards geometric details than other docking tools. Minimizations were performed until the average root mean square deviation of the non-hydrogen atoms reached 0.3 Å.

The final model was validated by the PROCHECK, ERRAT, Verify3D and ProSA programs. Superimposition and calculation of the root mean square deviations (RMSD) between model and template, was made by Chimera software using the Carbon alpha fitting method. Diagrammatic representations of the structures were generated using PyMOL 1.3 software.

4.2.3.7 Docking studies

The structure data files (sdf) of the ligands were obtained from the PubChem database. Protein–ligand docking simulations were conducted using Glide module of Schrödinger, Inc. (Friesner et al., 2004; Halgren et al., 2004; Krovat et al., 2005). It performs grid-based ligand docking with energetics and searches for favorable interactions between one or more typically small ligand molecules and a typically larger receptor molecule, usually a protein. Schrödinger recommends the performance of test calculations with different scaling factors for the receptor and ligand atom vander Waals radii, because steric repulsive interactions might otherwise be overemphasized, leading to rejection of overall correct binding modes of active compounds. After ensuring that protein and ligands are in correct form for docking, the receptor-grid files were generated using grid-receptor generation program. To soften the potential for nonpolar parts of the receptor, we scaled van der Waals radii of receptor atoms by 1.00 with partial atomic charge 0.25. A grid box dimension was generated at the centroid of the active site and the sizes of ligands to be docked were selected from the workspace. The ligands were docked with the active site using the "xtra precision" Glide algorithm. Glide generates conformations internally and passes these through a series of filters. The first places the ligand center at various grid positions of a 1 Å grid and rotates it around the three Euler angles. At this stage, crude score values and geometric filters weed out unlikely binding modes. The next filter stage involves a grid-based force field evaluation and refinement of docking solutions including torsional and rigid-body movements of the ligand. The OPLS-AA force field was used for this purpose. A small number of surviving docking solutions can then be

subjected to a Monte Carlo procedure to minimize the energy score. The final energy evaluation is done with Glide score (*GScore*) and a single best pose is generated as the output for a particular ligand. The interactions of the ligands on the proteins were visualized and the figures were created by using PyMOL 1.3.

4.3 Results and discussion

4.3.1 HMG-CoA reductase: Heterologous expression and characterization

4.3.1.1 Cloning of *BmHMGR* gene in pET 30b (+) vector

4.3.1.1.1 Incorporation of restriction sites

The *BmHMGR* gene cloned in pGEM-T Easy vector was amplified using gene specific primers pETHMGF and pETHMGR to incorporate the *Kpn*I site at the 5' end and *Xho*I site at 3' end of the gene. Approximately, 1.7 kb (1764 bp *BmHMGR* + *Kpn* I and *Xho* I restriction sites) band was amplified and cloned into pGEM-T Easy vector.

4.3.1.1.2 Directional cloning of *BmHMGR* gene in pET 30b (+)

The above clone of *BmHMGR* gene in pGEM-T Easy vector was restriction digested with *Kpn*I and *Xho*I restriction enzymes and it was ligated in pET 30b (+) vector DNA flanked with same restriction sites. Ligation mixture was transformed into *E. coli* XL10 competent cells and plated on LB-agar plate (kanamycin 50 μg/mL). *E. coli* colonies having recombinant plasmids were screened by colony PCR with T7 promoter and terminator primers (**Fig. 4A.3 A**). Plasmids were isolated from PCR positive clones and were digested with *Kpn* I and *Xho* I to confirm the integration of *BmHMGR* gene fragment in pET 30b (+) vector (**Fig. 4A.3 B**).

Integration of the *BmHMGR* gene along with restriction sites was further confirmed by sequencing. The sequence was translated using proteomic tools available at www.expasy.ch and was checked for in frame translation up to 6x HIS tag.

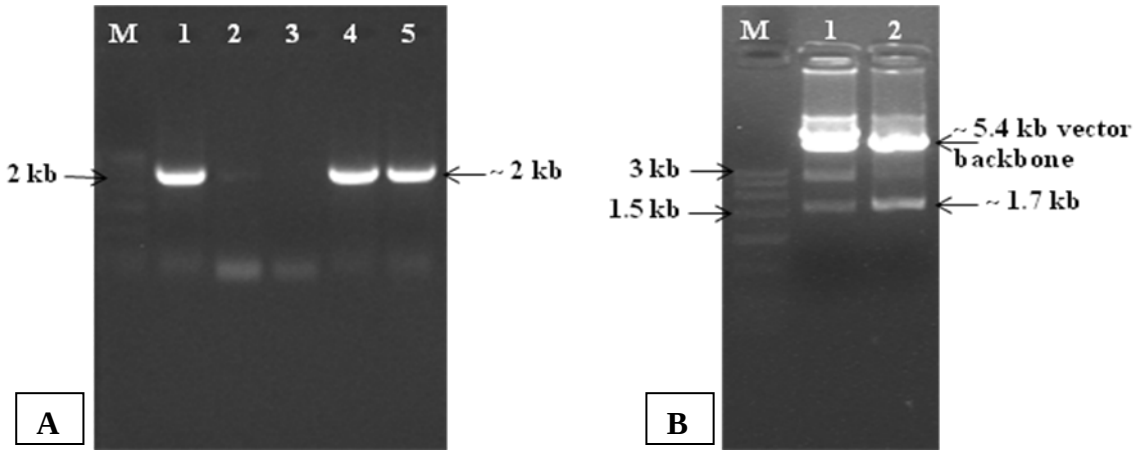


Fig. 4A.3: A. Colony PCR showing ~2.0 kb *BmHMGR* gene (additional amplification is due to presence of sequences between T7 promoter and terminator) in recombinant colonies; Lane M- Low range DNA ruler, lane 1, 4 & 5 are positive clones. **B.** Restriction analysis of recombinant pET 30b (+) with *Kpn*I and *Xho*I: lane 1 & 2 recombinant vector releasing ~1.7 kb *BmHMGR* gene and ~5.4 kb vector backbone, lane M- Low range DNA ruler.

4.3.1.2 Recombinant BmHMGR protein expression and purification

E. coli BL 21 (DE3) cells transformed with recombinant pET-30b (+) plasmids having *BmHMGR* gene were screened for over-expression of protein. An approximately 68 kDa (~4 kDa additional due to presence of vector sequence) over-expressed protein was observed on 10% SDS-PAGE. The *E. coli* (BL21) containing control pET30 b+ plasmid did not show corresponding overexpressed band (**Fig. 4A.4 A**). Conditions were optimized for various parameters including IPTG conc., temperature and induction duration. It was found that recombinant cells grown at 18 ^{o}C for 12 h in the presence of 0.3 and 0.5 mM IPTG expressed recombinant protein in good amount in soluble fractions. But under these conditions protein was still more in insoluble fraction/inclusion bodies (**Fig. 4A.4 B**). For further studies conditions were chosen as, temperature 18 ^{o}C, IPTG 0.3 mM and duration 12 h.

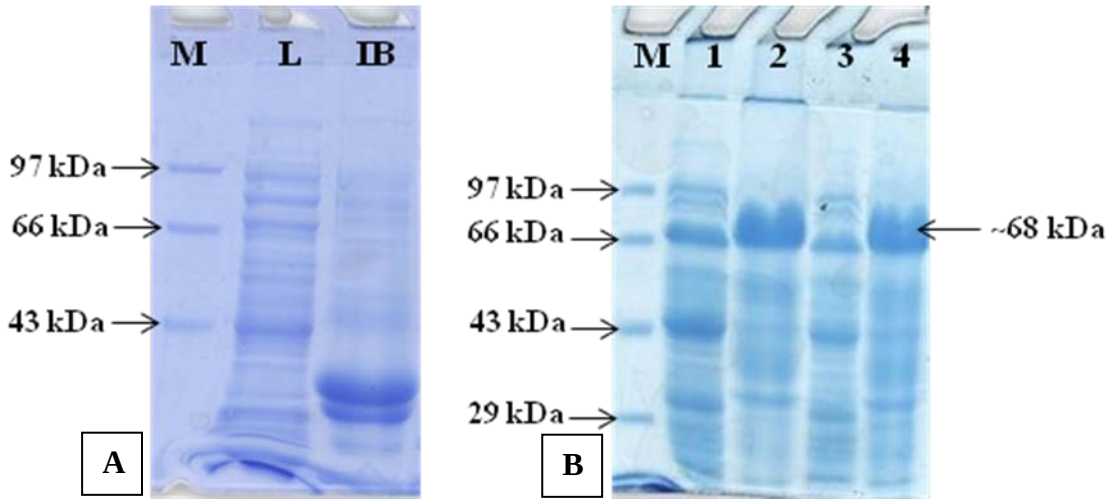


Fig. 4A.4: 10% SDS-PAGE Coomassie Blue staining **A.** Showing Induced control (*E. coli* BL21 (DE3) transformed with pET 30b (+) plasmid) protein expression. Lysate (L) and inclusion bodies (IB), M- molecular weight marker. **B.** Recombinant BmHMGR over-expression. Lane 1 & 2 lysate & IB at 0.3 mM IPTG induction respectively; Lane 3 & 4 lysate and IB at 0.5 mM IPTG induction respectively.

Purification of recombinant BmHMGR was tried with various chromatographic techniques including affinity (Ni-NTA), ion-exchange, ammonium sulphate precipitation and hydrophobic interaction (HIC), but unfortunately we did not get any success. So, some other strategies need to be used for purification, and then biochemical characterization of BmHMGR protein can be done.

4.3.1.3 *In-silico* characterization of BmHMGR

4.3.1.3.1 Secondary structure prediction (PSIPRED)

The secondary structure of BmHMGR was predicted by the PSIPRED Protein Structure Prediction Server (PSIPRED v3.0). The structure showed 14 α-helices and 14 β-sheets (**Fig. 4A.5**).

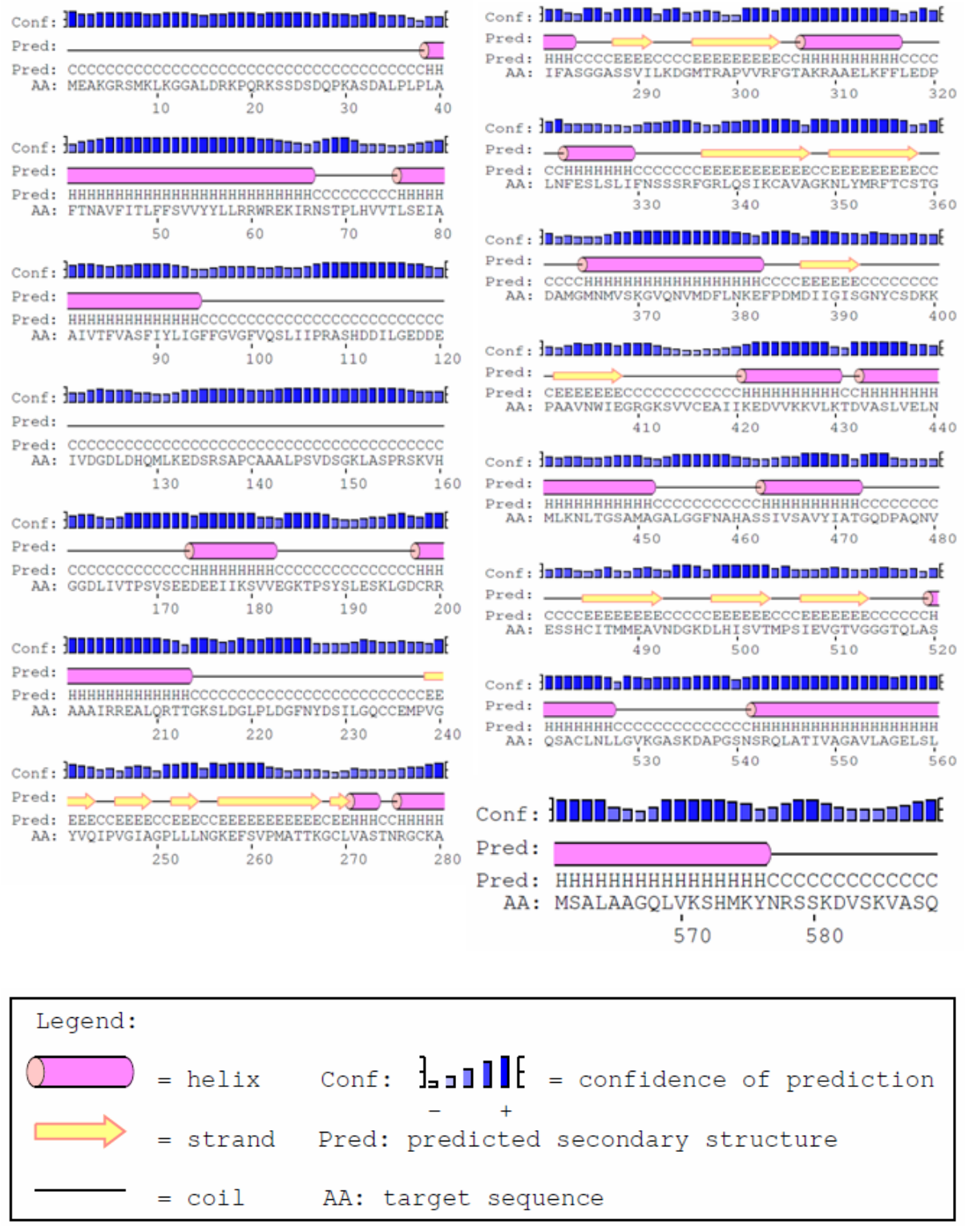


Fig. 4A.5: Predicted Secondary structure of BmHMGR.

4.3.1.3.2 3D structure prediction (Homology modeling)

The HMG-CoA reductase from *B. monniera* BmHMGR6 (NCBI Accession No. ADX31914.1) was used for homology modeling. The Protein-BLAST algorithm (blastp) against the Protein Data Bank was used to carry out the sequence homology search. The sequence and 3D structure of template proteins were extracted from the PDB database. High resolution crystal structures of homologous proteins as a template were considered for homology modeling. The crystal structure of human HMG-CoA reductase (PDB ID- 1DQ8_A; Resolution 2.1Å) with 59% sequence identity was considered as a best hit (E value: 1e-169) and used as a template to generate comparative 3D model of BmHMGR (**Fig. 4A.6 A**) by MODELLR 9v10 (Eswar et al., 2006). Out of 588 residues, 406 (175^{th} -579^{th} AA) residues were modeled in the structure. At N-terminus 174 AA residues and 10 AA residues at C-terminus unmodeled due to presence of hydrophobic regions, not having homology with template and constitute the transmembrane. The crystal structure of the template (1DQ8) from *Homo sapiens* is a tetramer; for activity of protein dimer formation is required, so we generated the model of BmHMGR with homodimer (**Fig. 4A.6 A**). A total 50 models were generated and their discrete optimized potential energy (DOPE) was calculated using model-single.top script. The model no. 21 (BmnHMG.B999900021.pdb) having lowest DOPE score (-92366.95312) was considered as the best model of BmHMGR. The RMSD of alpha carbon (Cα) of the homology model was calculated by structural superimposition of model with templates on Chimera and was found to be 0.232 Å. The low RMSD value of the homology model was a clue for a model of acceptable quality. **Figure 4A.6 B** shows the superimposition of the dimmer target protein model with 1DQ8 (Chain A & B). As expected, the overall conformation of the model is very similar to the template; the yellow is the homology model with the completed closure of the flap domain. PyMOL (DeLano, 2002)) software was used to visualize the model and find out the maximum number of helices, turns and sheets in the protein.

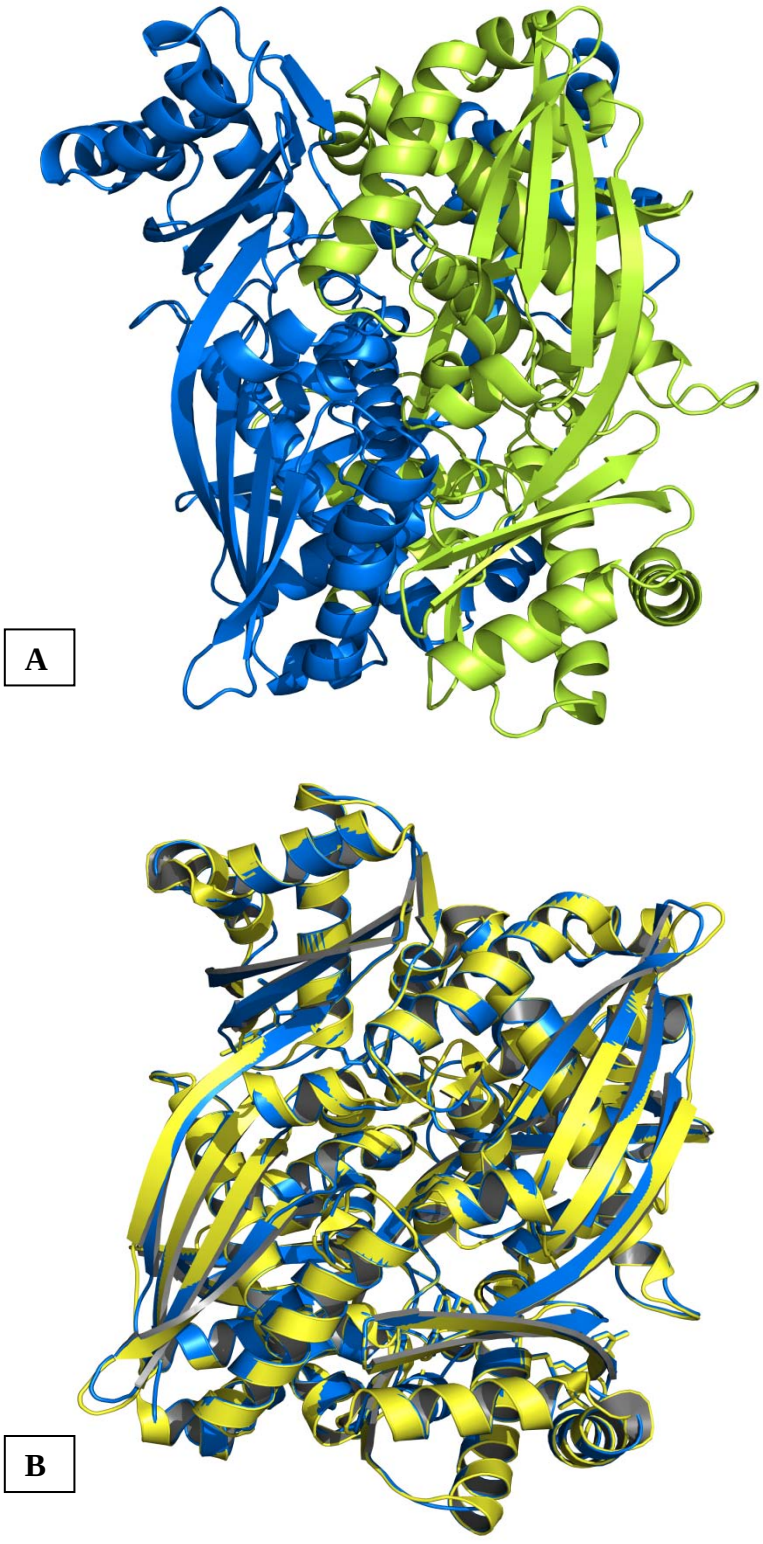


Fig. 4A.6: A. 3D structure of Dimer BmHMGR. **B.** Superimposition of modeled dimer BmHMGR (Yellow) with template 1DQ8_A (Blue).

4.3.1.4 Validation of protein structure model

4.3.1.4.1 Ramachandran plot (PROCHECK)

PROCHECK analysis of the modeled protein showed that 92.7% of the residues were found in the most favoured region, 7.3% in additional allowed region and 0.0 % in generously allowed region and disallowed region of the Ramachandran plot (**Fig. 4A.7**). These results indicate that the protein model is reliable. The good quality model would be expected to have more than 90% residues in most favoured regions.

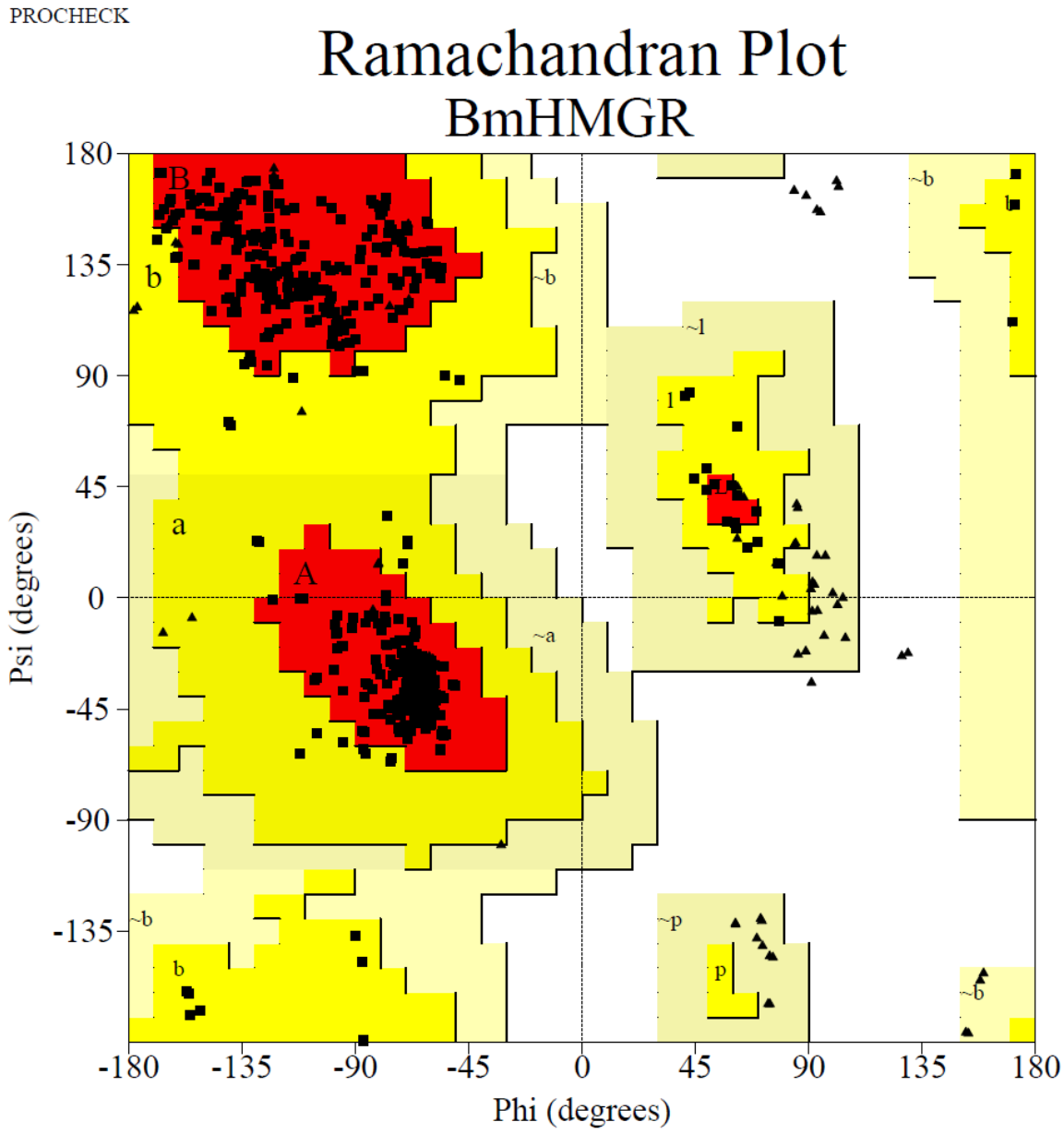


Fig. 4A.7: Ramachandran map of BmHMGR.

The model with the least number of residues in the disallowed region was selected for further studies. The plot statistics are shown in Table 4A.2.

Table 4A.2: Ramachandran plot statistics (BmHMGR).

Plot statistics	No. of residues	%
Most favoured regions [A, B, L]	647	92.7
Additional allowed regions [a, b, l, p]	51	7.3
Generously allowed regions [~a, ~b, ~l, ~p]	0	0.0
Disallowed regions	0	0.0
Non-glycine and non-proline residues	---------- 696	--------- 100.0
End-residues (excl. Gly and Pro)	6	
Glycine residues	82	
Proline residues	28	
Total number of residues	812	

4.3.1.4.2 ERRAT and Verify 3D analysis

ERRAT is a program for verifying protein structures determined by crystallography. Error values are plotted as a function of the position of a sliding 9-residue window. The error function is based on the statistics of non-bonded atom-atom interactions in the reported structure. On the error axis, two lines are drawn to indicate the confidence with which it is possible to reject regions that exceed that error value. Regions of the structure that can be rejected at the 95% confidence level are gray and regions that can be rejected at the 99% level are shown in dark black (**Fig. 4A.8**). The overall quality factor of modeled BmHMGR was 79.302, expressed as the percentage of the protein for which the calculated error value falls below the 95% rejection limit. The Verify 3D score was 86.93%.

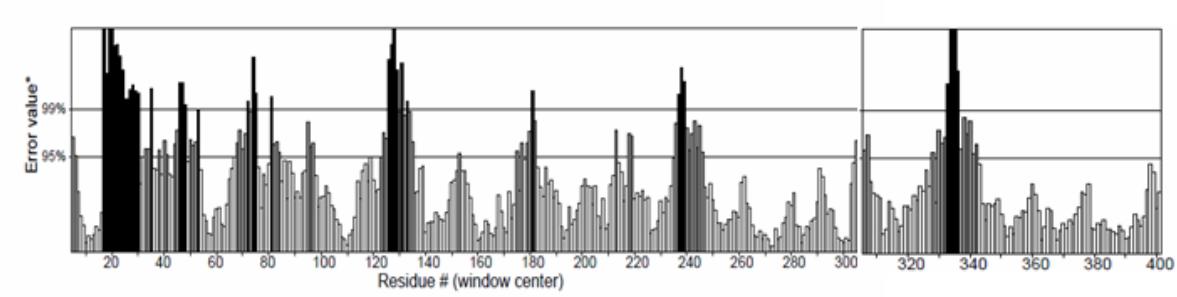


Fig. 4A.8: Overall quality factor determination of BmHMGR (79.302) through ERRAT.

4.3.1.4.3 Zplot analysis (ProSA)

The Z score of the protein is representing the overall quality and measures the deviation of the total energy of the protein structure. The Z score of the protein is displayed in this plot with a dark black point (**Fig. 4A.9**). The structures determined from different sources (NMR, X-ray) are distinguished by different colors (NMR in dark blue and X-ray in light blue). In this plot the Z score value of the obtained model of BmHMGR (−10.25) is located within the space of protein related to X-ray. This value is nearly close to the value of the template 1DQA (−8.81) which suggests that the obtained model is reliable and close to experimentally determined structure. The Z-Score of modeled protein is within the acceptable range (−10 to 10, negative Z-scores are good and it depends on the length of the protein).

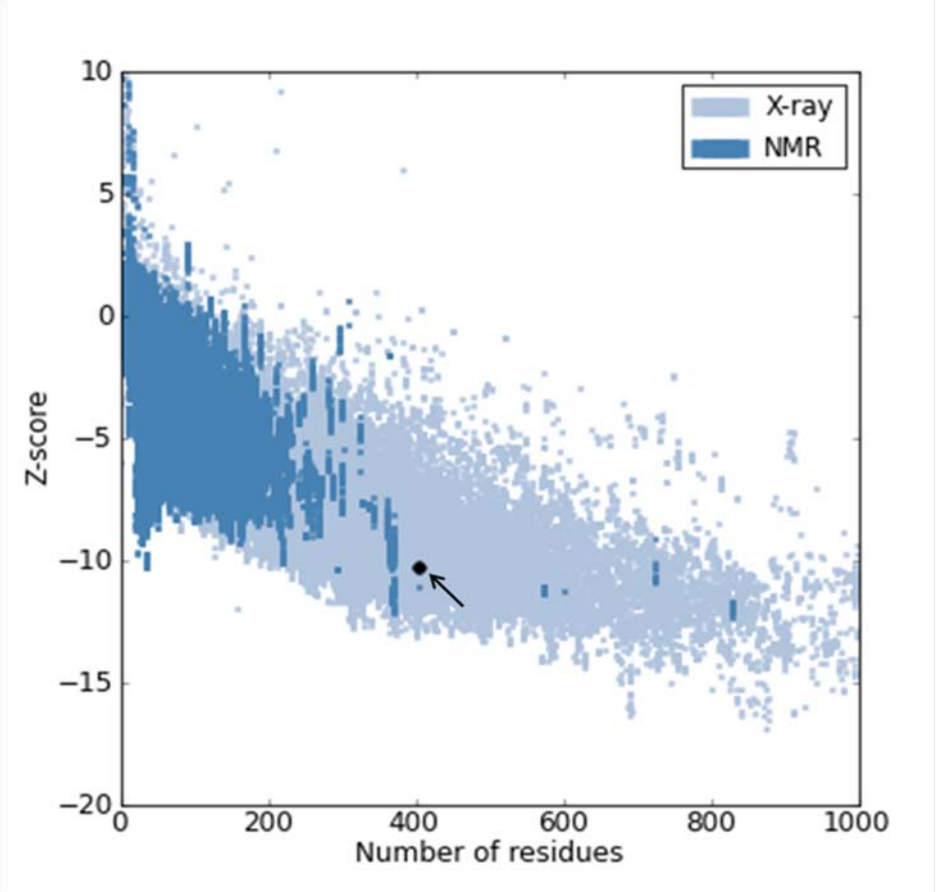


Fig. 4A.9: Z-Score Plot shows that Z score value of BmHMGR (dark blue region represent protein determined by NMR and light blue represents protein determined by X ray). The black dot represents Z-score (indicated by arrow) of the model BmHMGR.

4.3.1.5 Interaction of HMG-CoA and NADPH with BmHMGR (Docking studies)

The 3D structure of substrate (ligand) HMG-CoA and NADPH was taken from pubchem server of NCBI. Default parameters were used except number of runs. Total 1000 docking runs were performed, treating the protein as rigid and the ligand as flexible. Ligands with lower binding energy were considered for further investigations. The X, Y, Z dimensions of the grid for HMG-CoA were set to -31.1308, 13.8801 and 29.6623 Å respectively and for NADPH X, Y, Z dimensions were -27.0795, 7.5787 and 12.4212 Å respectively with grid points separated by 20 Å. The interactions of the ligands on the proteins were visualized and the figures were created by using PyMOL 1.3. The amino acid residues of BmHMGR protein played an active role in the interaction with HMG-CoA (**Fig. 4A.10 A & B**) and NADPH (**Fig. 4A.11 A & B**) ligands shown in Table 4A.3.

Table 4A.3: Residues involved in interaction of HMG-CoA and NADPH with BmHMGR.

Substr-ate	**Glide G-score**	E_{model} **(kJ/mol)**	**No. of H bonds**	**Bond lengths**	**Amino acid residues involved in interactions (BmHMGR)**	**Amino acid residues involved in interactions (Human HMGR)** (Istvan et.al., 2000)
HMG-CoA	-7.60	-79.875	9	2.0-3.4 Å	Gln568(A), Tyr576(A), Tyr189(B); Asn275(A); Lys430(A); Met237(B)	Lys-691, 735; Asp-690, 767; Gln-770; Glu-559; Leu-853; Asn-567; Tyr-479; Ser-565, His-866
NADPH	-7.623	-107.27	14	2.6-3.5 Å	Asn366(A), Asn577(B), Asp361(A), Asp475(A), Arg298(A), Arg335(A), Arg578(B), Met367(A), Gly364(A), Ser369(A), Ser579(B)	Asp-653, 767; Phe-628; Val-805; Ser-626; Gly-656; Arg-590 627,871; Met-655,657,659; Asn-658, 870; Glu-559

(A) and (B) - Chain A and B of homodimer BmHMGR.

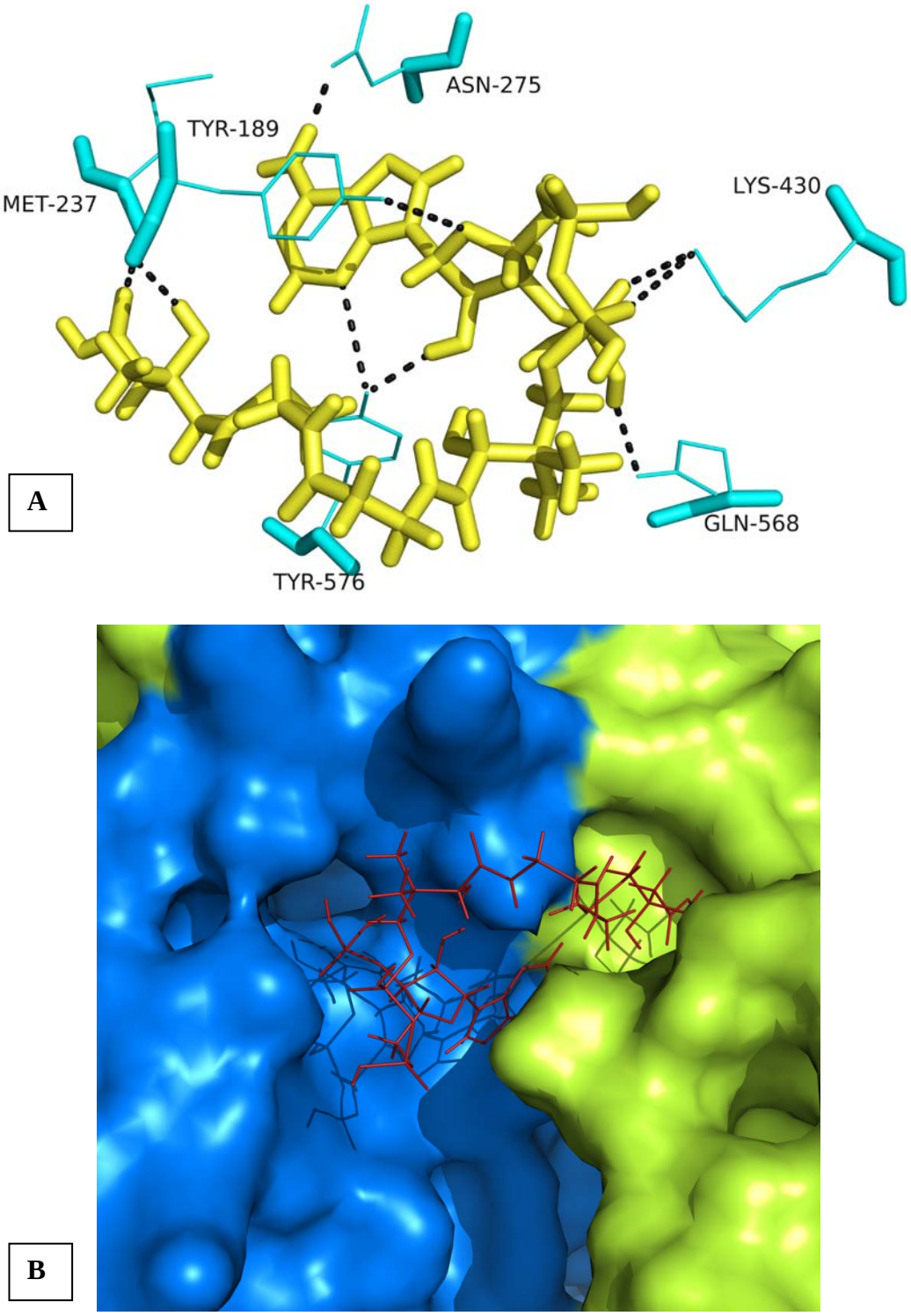
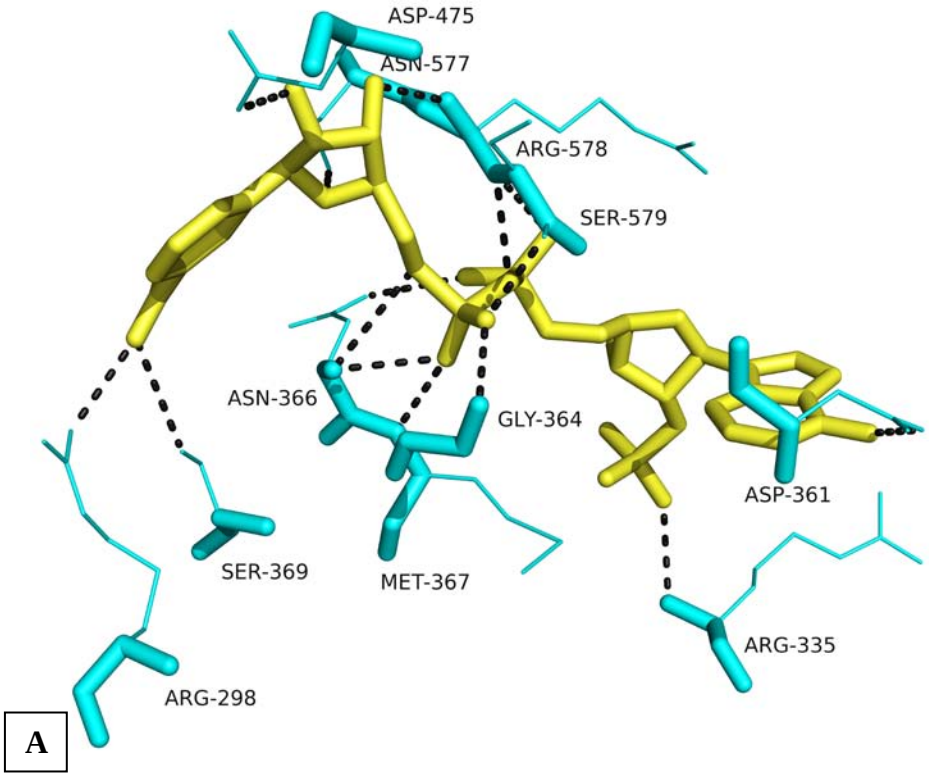


Fig. 4A.10: Interaction of HMG-CoA with BmHMGR. **A.** Stick-line diagram showing residues involved in HMG-CoA (Yellow) binding. Black dotted lines H-bonding. **B.** Surface view showing substrate HMG-CoA (Red) in substrate binding pocket.



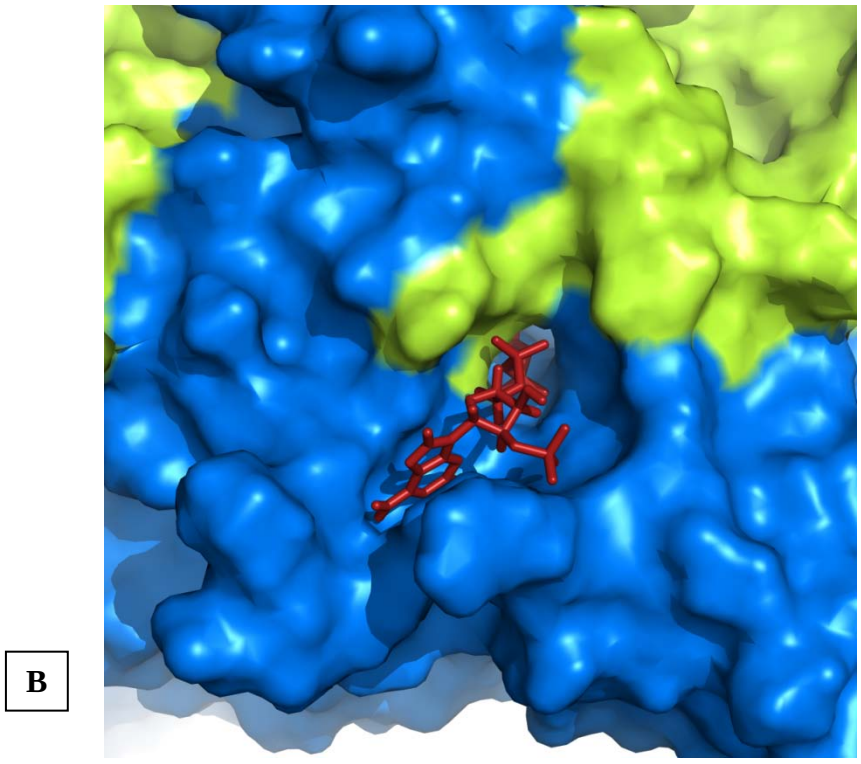
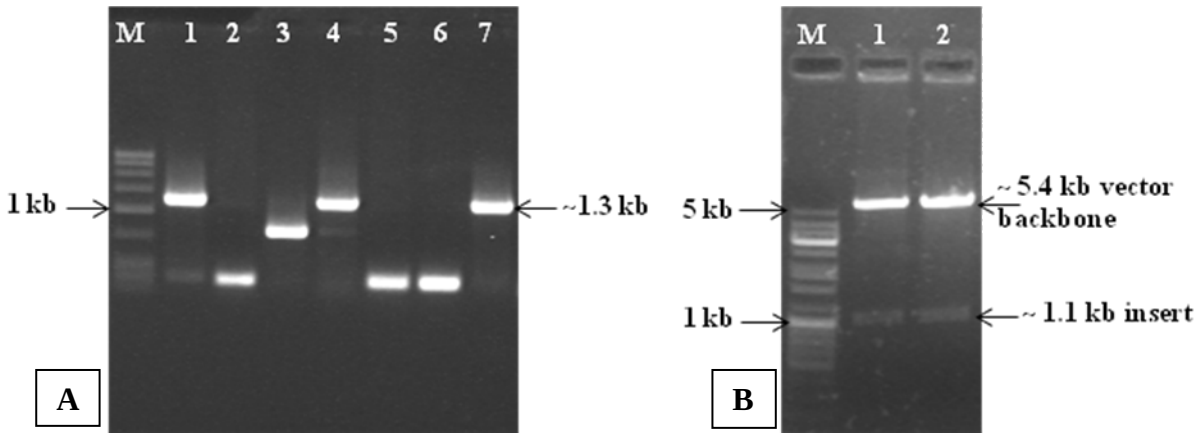


Fig. 4A.11: Interaction of NADPH with BmHMGR. **A.** Stick-line view showing residues involved in NADPH (Yellow) binding. Black dotted lines H-bonding. **B.** Surface view showing substrate NADPH (Red) in substrate binding pocket.

4.3.2 FPP synthase: Heterologous expression, purification and characterization

4.3.2.1 Incorporation of restriction sites and directional cloning of *BmFPS* gene in pET 30b (+)

The *BmFPS* gene already cloned in pGEM-T Easy vector was amplified using gene specific primers pETFPSF and pETFPSR to incorporate the *Nde*I site at the 5' end and *Xho*I site at 3' end of the gene. Approximately, 1.1 kb (1047 bp *BmFPS* ORF + *Nde*I and *Xho*I restriction sites) amplicon was cloned in pGEM-T Easy vector. The above clone of *BmFPS* gene in pGEM-T Easy vector was restriction digested with *Nde*I and *Xho*I restriction enzymes and ~1.1 kb fragment was ligated in pET-30b (+) vector DNA flanked with same restriction sites. Ligation mixture was transformed into *E. coli* XL10 competent cells and plated on LB-agar plate (kanamycin 50 µg/mL). *E. coli* colonies having recombinant plasmids were screened by colony PCR with T7 promoter and terminator primers (**Fig. 4A.12 A**). Plasmids were isolated from colony PCR positive clones were digested with *Nde*I and *Xho*I to confirm the integration of *BmFPS* gene fragment in pET-30b (+) vector (**Fig. 4A.12 B**). Further sequencing was done for confirmation of integration of the *BmFPS* gene along with restriction sites. The sequence was translated using proteomic tools for checking in frame translation up to 6x HIS tag.



4A.12: A. Colony PCR showing ~1.3 kb *BmFPS* gene amplification with T7 promoter and terminator primers. Lane M- Low range DNA ruler, lane 1, 4 & 7 are positive clones. **B.** Restriction digestion of recombinant pET30b (+) with *Nde*I and *Xho*I: lane 1 & 2 recombinant vector releasing ~1.1 kb *BmFPS* gene and ~5.4 kb vector backbone, lane M- Medium range DNA ruler.

4.3.2.2 Heterologous expression and purification of recombinant BmFPS protein

E. coli BL 21 (DE3) cells transformed with recombinant pET30b (+) plasmids harboring *BmFPS* gene were screened for over-expression of protein. An approximately 40 kDa overexpressed protein was observed on 10% SDS-PAGE. Conditions were optimized for various parameters including IPTG conc., temperature and induction duration. It was found that when cells were grown at 28 ^{o}C in presence of 0.5 mM IPTG concentration 0.5 mM induction for 5 h, considerable amount of protein was expressed in soluble fraction (**Fig. 4A.13 A)**. Recombinant BmFPS was purified from lysate using Ni-NTA agrose beads and single band of ~ 40 kDa was observed on SDS-PAGE after silver staining (**Fig. 4A.13 B**).

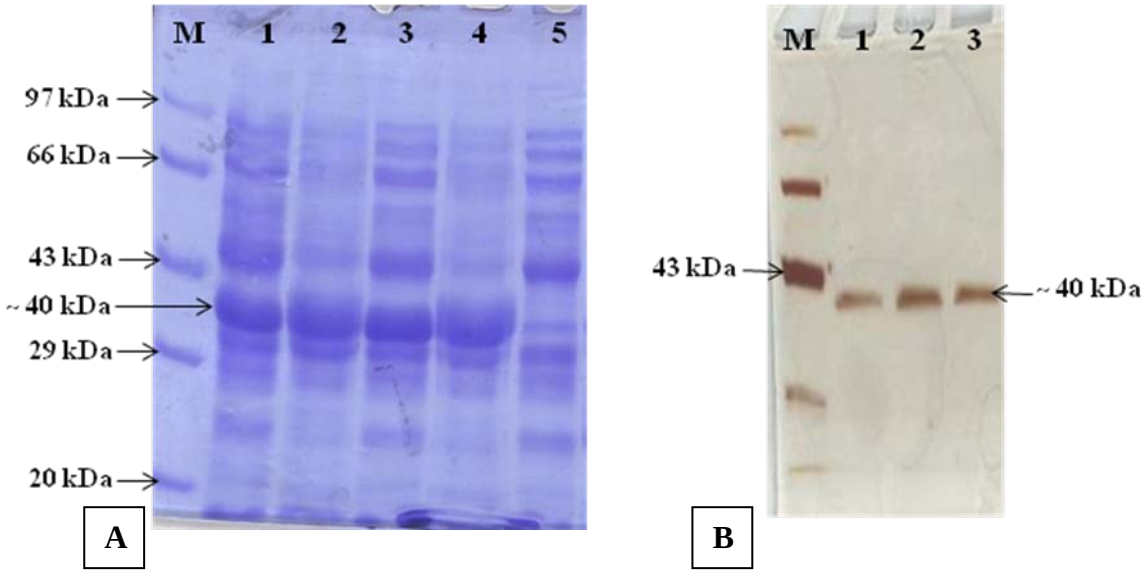


Fig. 4A.13: 10% SDS-PAGE gel. **A.** Optimization of recombinant protein expressin, lane 1 & 2 lysate and IB at 0.3 mM IPTG respectively; lane 3 & 4 lysate and IB at 0.5 mM IPTG; lane 5-uninduced protein; Lane M- Protein molecular weight marker. **B.** Purified recombinant BmFPS protein (Silver stained gel).

4.3.2.3 Raising antibodies in rabbit against recombinant BmFPS protein

Approximately 300 µg of purified protein (dialyzed in 1X PBS buffer) was used for first injection in New Zealand rabbit to raise antibodies against BmFPS and the same amount of protein was used for 2nd and 3rd booster doses. Antibody titer of first and second bleed was determined by ELISA by using purified recombinant BmFPS protein. Titer of 2nd bleed is ~1:70000 (**Fig. 4A.14**). Second bleed serum dilution of 1:10000 was used for further experiments.

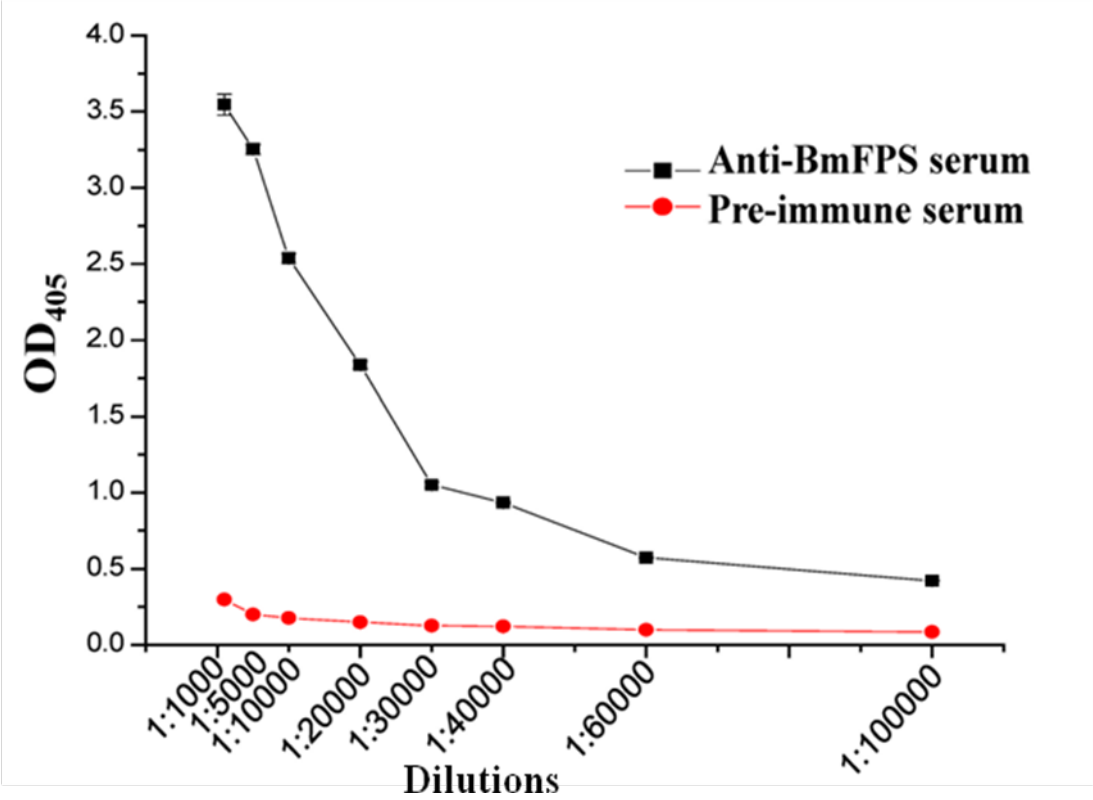


Fig. 4A.14: Titre of Anti-BmFPS polyclonal antibody.

4.3.2.4 Western blot analysis

Total soluble protein from *B. monniera* shoots was extracted in buffer (100 mM Tris-Cl pH-8.0; 5 mM DTT; 1% PVPP and 1 mM PMSF) and 100 µg of protein was separated on 10% SDS-PAGE. Recombinant BmFPS protein was also used as a positive control. After complete separation protein was electro transferred on PVDF membrane using iBlot system (Invitrogen) as per manual instructions. The anti-BmFPS polyclonal antibody obtained from 2nd bleed serum was diluted 1:10000 times used for western blot experiments. Secondary antibody i.e. goat anti-rabbit IgG conjugated with alkaline phosphatase was diluted 1:20000 before use. Color development was done using BCIP/NBT as a substrate. In Western blot, in spite of using polyclonal antibody raised against BmFPS protein, single band was detected in the crude plant extract of *B. monniera.* This band corresponds to the same molecular weight (~40 kDa) as that of recombinant BmFPS (**Fig. 4A.15**).

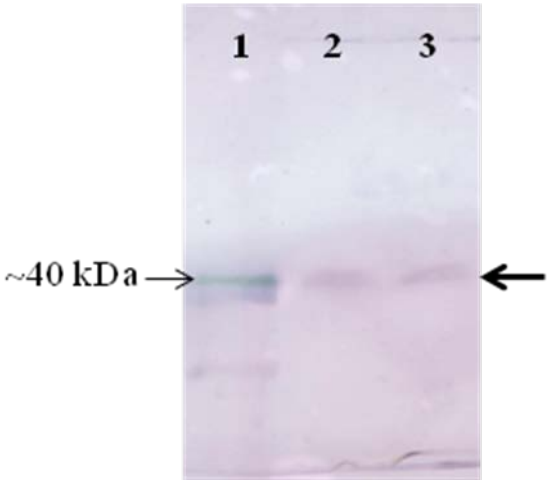


Fig. 4A.15: Western blot analysis of BmFPS. Lane 1- Recombinant protein; Lane 2 & 3- Total plant protein.

4.3.2.5 ELISA analysis of BmFPS in different tissues

BmFPS protein quantitative analysis in stem, leaves and roots was done using above polyclonal antibodies raised against purified recombinant BmFPS protein. Total protein from different tissues was extracted in bicarbonate buffer and equal quantity (30 µg) of protein was coated on 96 well micro titre plate. Among all three types of tissues maximum expression of FPS protein was observed in leaves followed by stem and roots (**Fig. 4A.16**).

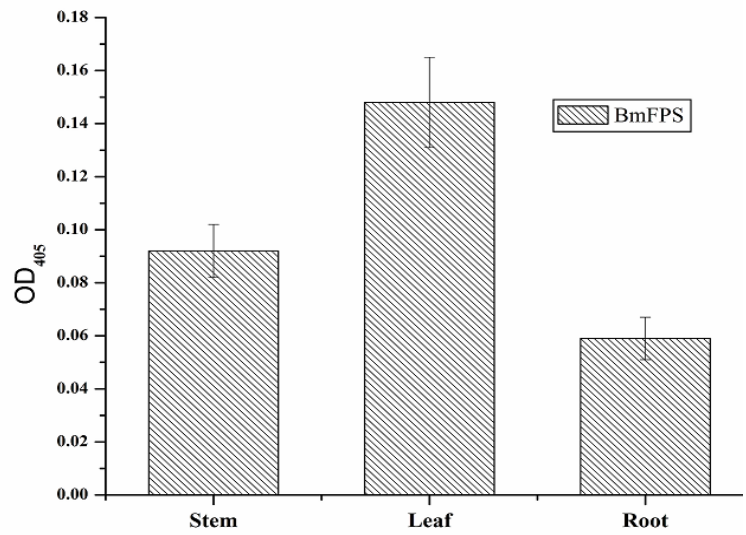


Fig. 4A.16: BmFPS protein expression analysis in different tissues by ELISA.

4.3.2.6 Bioinformatics analysis of BmFPS

4.3.2.6.1 Prediction of secondary structure (PSIPRED)

The secondary structure of BmFPS was predicted by the PSIPRED Protein Structure Prediction Server (PSIPRED v3.0). The structure showed only 14 α-helices and 15 coils. There are no β-sheets (**Fig. 4A.17**).

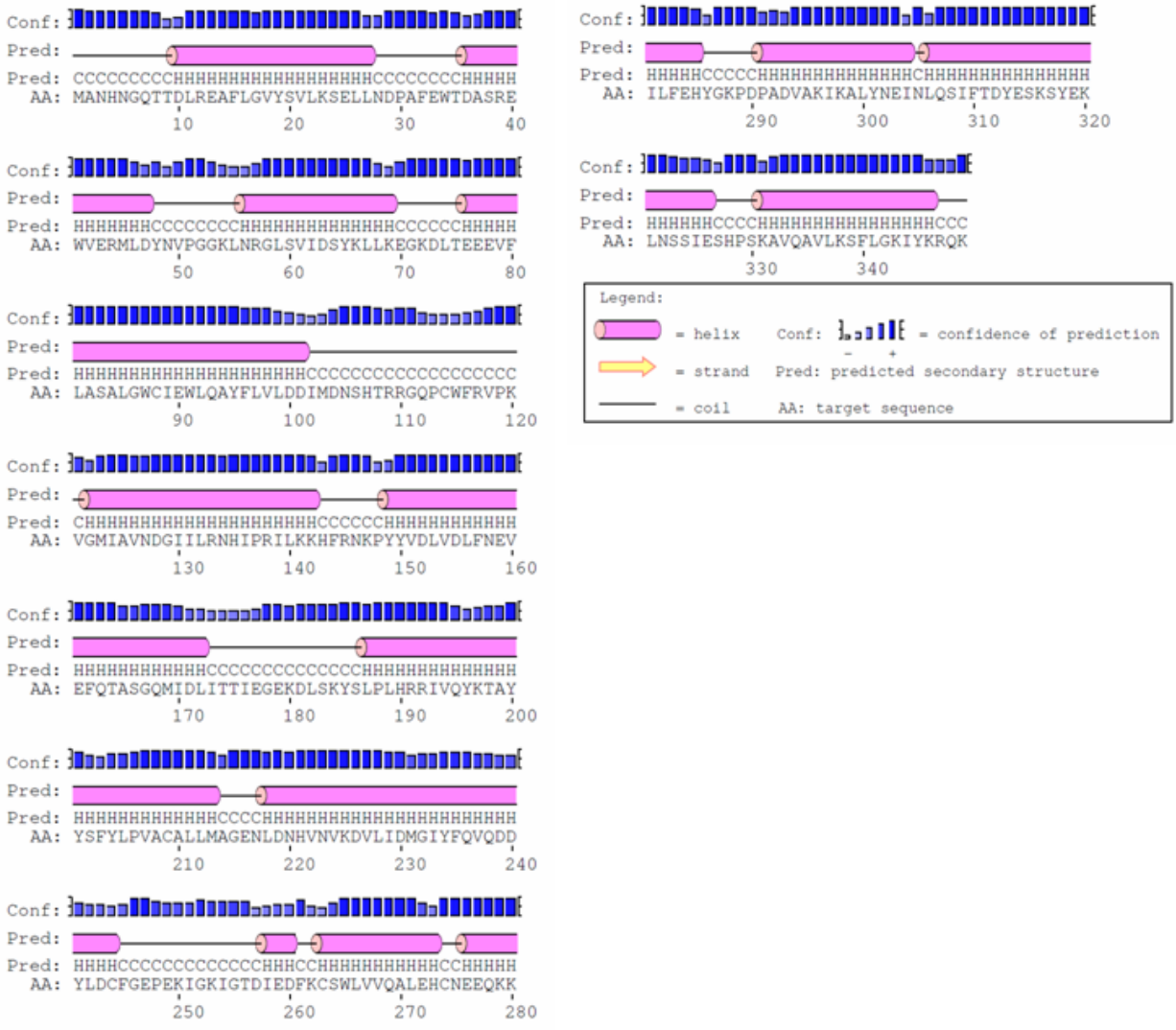


Fig. 4A.17: Secondary structure of BmFPS.

4.3.2.6.2 Homology modeling (3D structure prediction)

The FPP synthase from *B. monniera* (NCBI Accession No. ADV03080.1) was used for homology modeling. The Protein-BLAST algorithm (blastp) against the Protein Data Bank was used to carry out the sequence homology search. High resolution crystal structures of homologous proteins as template were considered for homology modeling. The crystal structure of *Gallus gallus* FPP synthase (PDB ID- 1UBY; Resolution 2.4 Å) with a 47 % sequence identity was considered as a best hit (E value: 4e-105) and was used as a template to generate comparative 3D model of BmFPS (**Fig. 4A.18 A**) by MODELLR 9v10. A total 50 models were generated and their discrete optimized potential energy (DOPE) was calculated using model-single.top script. The model no. 14 (BmPFS.B999900014.pdb) having lowest DOPE score (-44021.3125) was considered as the best model of BmFPS. **Figure 4A.18 B** shows the superposition of the target protein model with 1UBY. The RMSD of alpha carbon (Cα) of the homology model was calculated by structural superimposition of model with templates on Chimera and was found to be 0.953 Å. The overall conformation of the model is very similar to the template; the lemon yellow is the homology model with the completed closure of the flap domain. The low RMSD value of the homology model was a clue for a model of suitable quality. This refined model was then used further for docking studies.

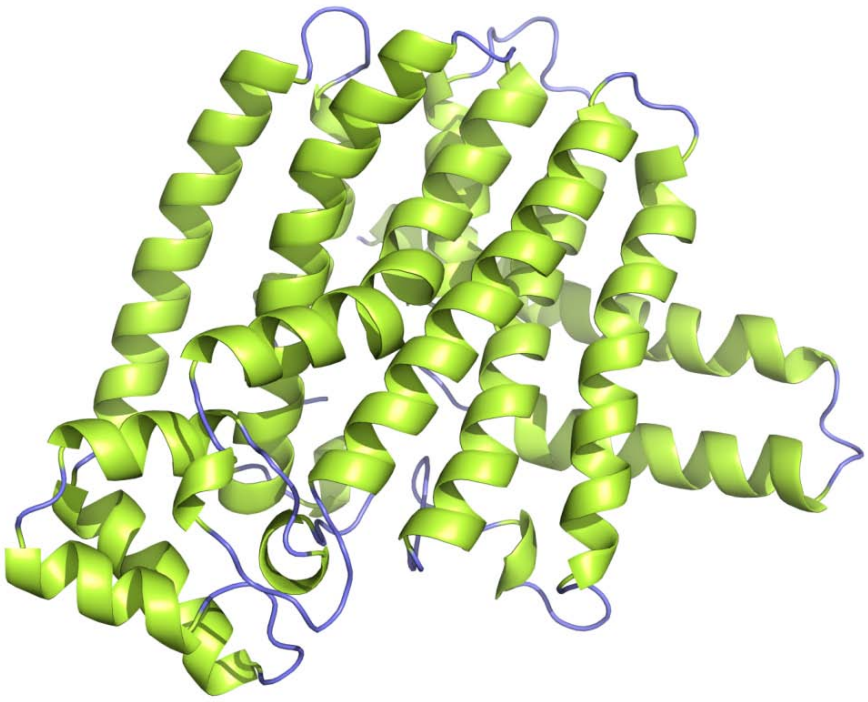


Fig. 4A.18: A.3D structure of BmFPS.

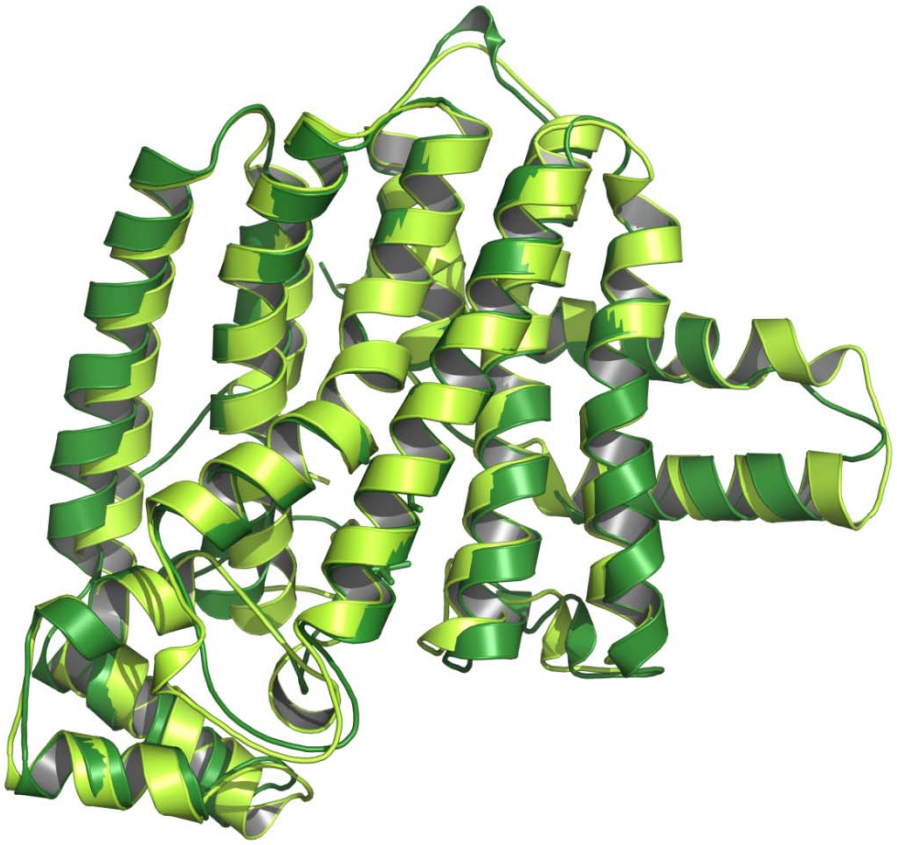


Fig. 4A.18: B. Superimposition of modeled BmFPS (lemon yellow) and template 1UBY (green).

4.3.2.7 Validation of protein structure model

4.3.2.7.1 Ramachandran plot (PROCHECK)

PROCHECK analysis of the modeled protein showed that 95.3 % of the residues were found in the most favoured region, 4.7 % in additional allowed region and 0.0 % in generously allowed and disallowed region of the Ramachandran plot (**Fig. 4A.19**). These results indicate that the protein model is reliable.

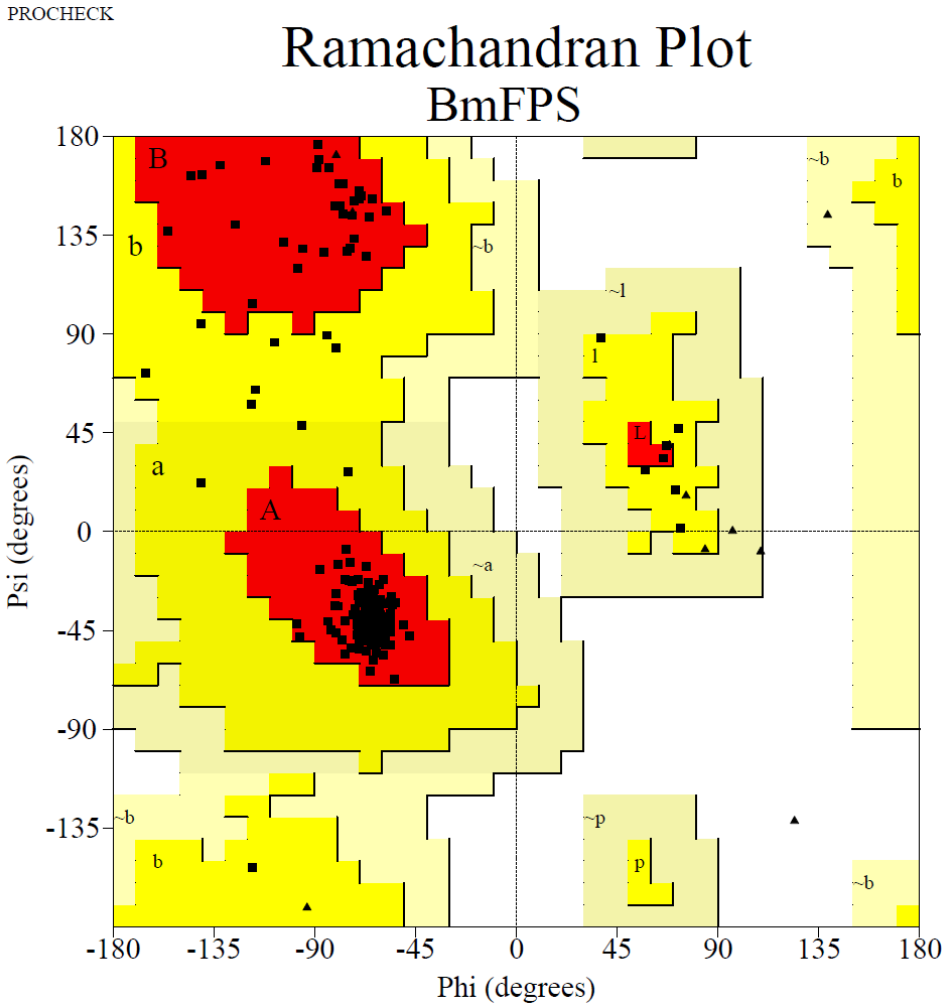


Fig. 4A.19: Ramachandran plot of BmFPS.

The model with the least number of residues in the disallowed region was selected for further studies. The plot statistics are shown in Table 4A.4.

Table 4A.4: Ramachandran plot statistics (BmFPS).

Plot statistics	No. of residues	%
Most favoured regions [A, B, L]	301	95.3
Additional allowed regions [a, b, l, p]	15	4.7
Generously allowed regions [~a, ~b, ~l, ~p]	0	0.0
Disallowed regions	0	0.0
Non-glycine and non-proline residues	---------- 316	--------- 100.0
End-residues (excl. Gly and Pro)	2	

Glycine residues	19	
Proline residues	12	
Total number of residues	349	

4.3.2.7.2 ERRAT and Verify 3D analysis

On the error axis, two lines are drawn to indicate the confidence with which it is possible to reject regions that exceed that error value. Regions of the structure that can be rejected at the 95% confidence level are gray and regions that can be rejected at the 99% level are shown in dark black (**Fig. 4A.20**). The overall quality factor of modeled BmFPS was 81.121, expressed as the percentage of the protein for which the calculated error value falls below the 95% rejection limit. The Verify 3D score was 87.43%.

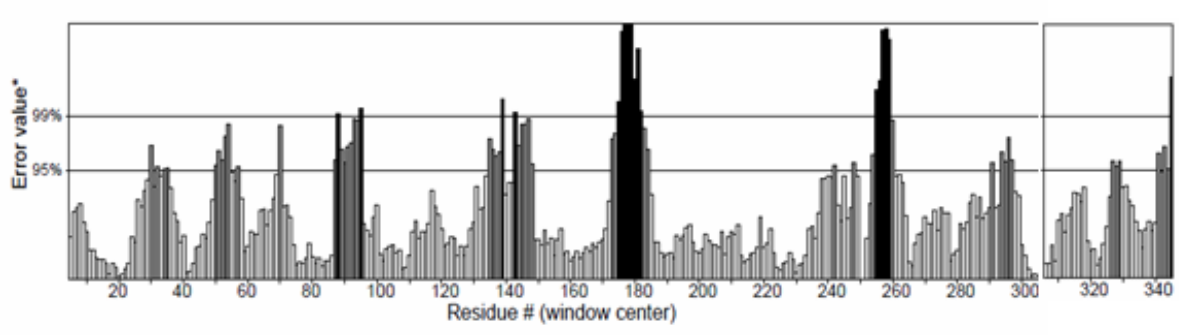


Fig: 4A.20: ERRAT analysis of modeled BmFPS.

4.3.2.7.3 Zplot analysis (ProSA)

The Z score of the protein is displayed in the plot with a dark black point (**Fig.4A.21**). In this plot the Z score value of the obtained model of BmFPS (−9.66) is located within the space of protein related to X-ray. This value is very close to the value of the template 1UBY (−10.72) which suggests that the obtained model is reliable.

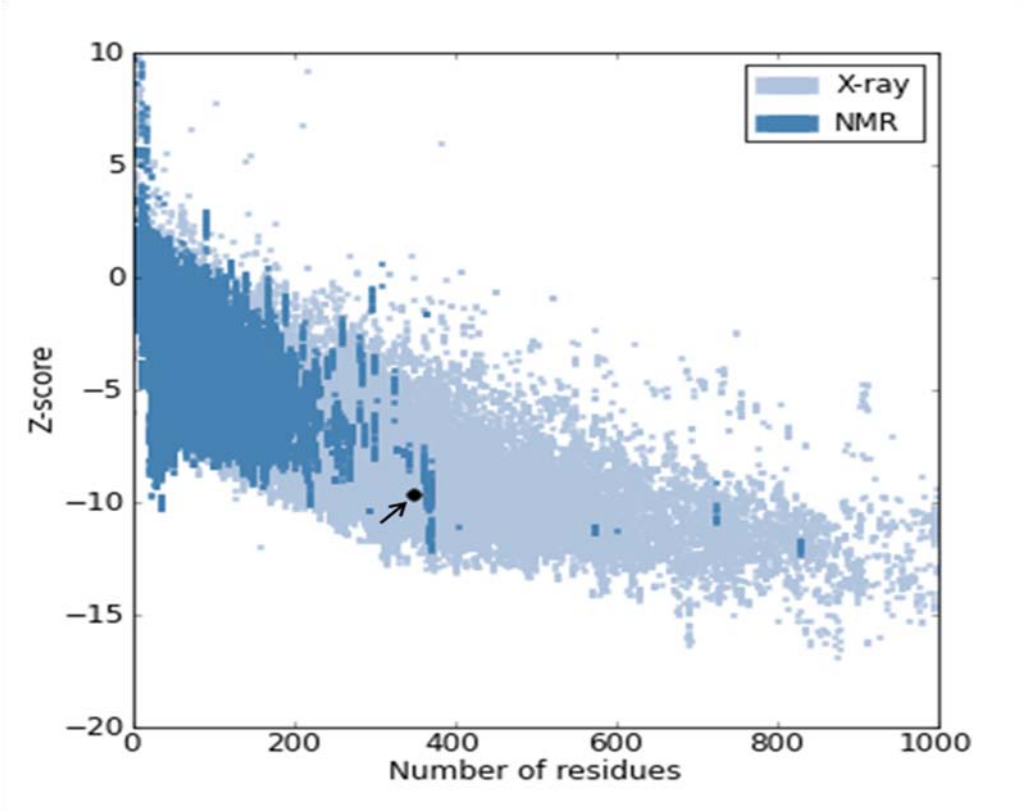


Fig: 4A.21: Z-Score Plot shows that Z score value of BmFPS. The black dot represents Z-score (indicated by arrow) of the model BmFPS.

4.3.2.8 Docking studies of IPP, DMAPP and GPP with BmFPS

The 3D structure of substrates (ligands) IPP, DMAPP and GPP were taken from pubchem server of NCBI. The X, Y, Z dimensions of the grid for IPP, DMAPP and GPP were set to 6.9303, 29.821 and 106.8649 Å respectively with grid points separated by 20 Å.

The amino acid residues of BmFPS protein played an active role in the interaction with IPP (**Fig. 4A.22**), DMAPP (**Fig. 4A.23**) and GPP (**Fig. 4A.24**) compounds are shown in Table 4A.5.

Table 4A.5: Residues involved in interaction of IPP, DMAPP and GPP with BmFPS

Subst -rate	**Glide G-score**	***Emodel* (kJ/mol)**	**No. of H bonds**	**Bond lengths**	**Amino acid residues involved in interactions (BmFPS)**	**Amino acids residues involved in interactions (*Gallus gallus* FPS)** (Tarshis et al., 1996)
IPP	-2.764	-74.978	7	1.7-3.5 Å	Asp-100, 104, 171, 257 and 260	----
DMAPP	-5.509	-90.976	7	2.0-3.4 Å	Asp-100, 104, 171; Arg-109; Lys-197	Asp-117, 118, 121, 188; Lys-214; Arg-126
GPP	-5.390	-98.420	7	1.7-3.5 Å	Asp-100, 104, 109, 171; Arg-109; Lys-197, 262	Asp-117, 118, 121, 188; Lys-214, 271; Arg-126

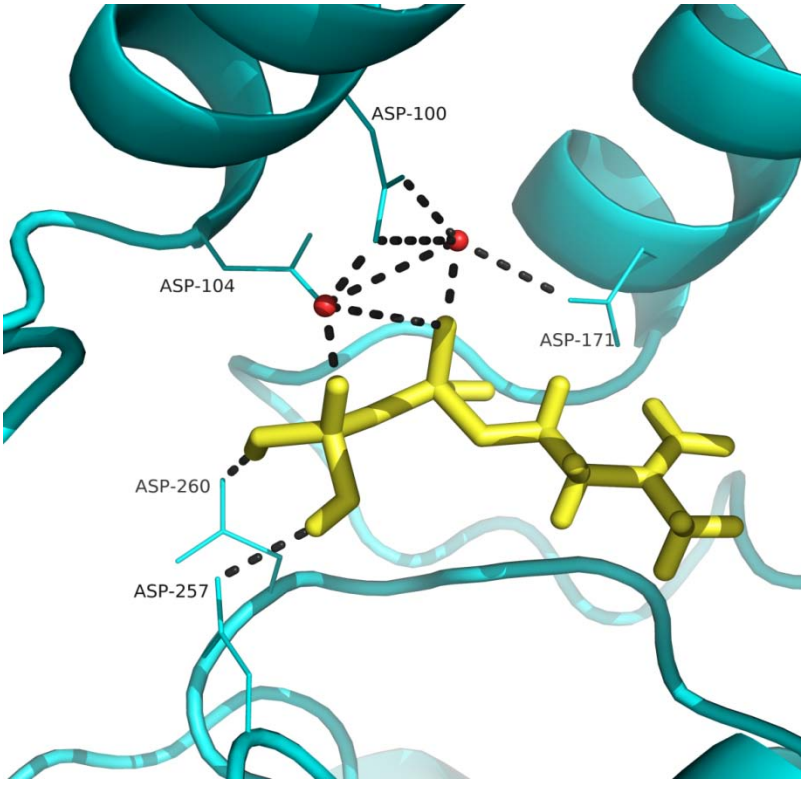


Fig. 4A.22: Ribbon diagram showing interaction of BmFPS with substrate isopentenyl pyrophosphate (Yellow). Mg^{2+} atoms are in red.

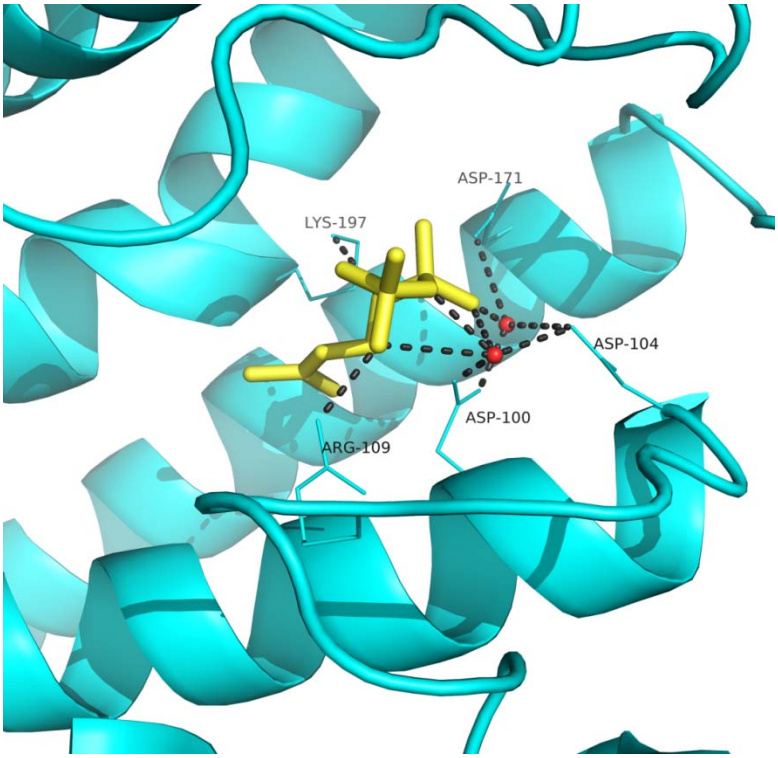


Fig. 4A.23: Ribbon diagram showing interaction of DMAPP (Yellow) with BmFPS.

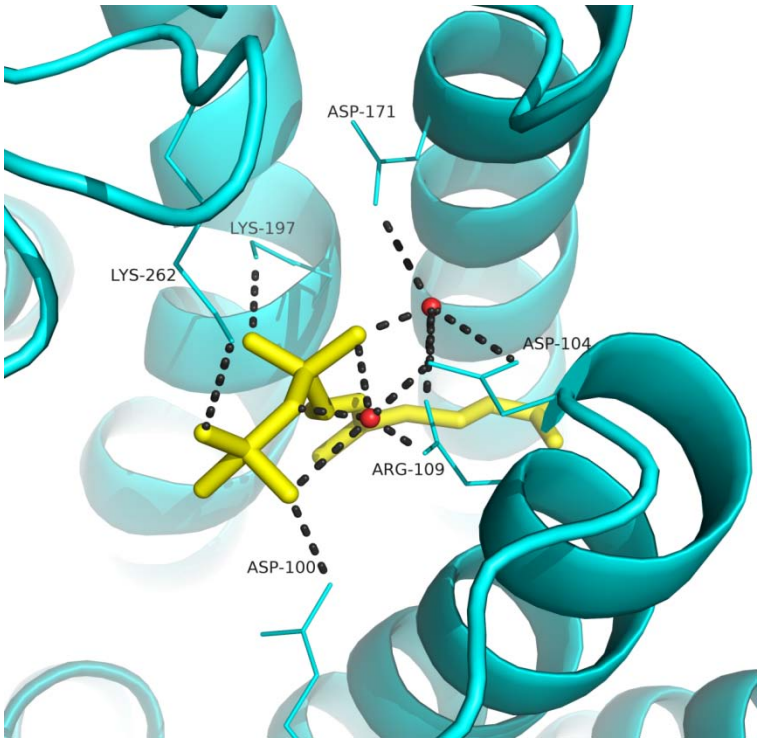


Fig. 4A.24: Ribbon diagram showing interaction GPP (Yellow) with BmFPS. Mg^{2+} atoms are in red and black dotted lines showing H-bonding.

4.3.3 Squalene synthase: Heterologous expression, purification and characterization

4.3.3.1 Directional cloning of BmSQS in expression vector (pET 30 b+)

To resolve the solubility issue due to presence of highly hydrophobic region at C-terminus of protein (Mentioned in chapter 3), C-terminal truncated BmSQS was generated by PCR using the forward primer that harbored the *NdeI* restriction site and a reverse primer that contained an *XhoI* restriction site. The reverse primer was located 75 bp (24 amino acids+ stop codon) upstream from the native stop codon in the full-length *BmSQS*. The truncated *BmSQS* was released from pGEM-T Easy vector and ligated into vector pET30 b (+) and transformed into *E. coli* XL 10. Recombinant clones were confirmed by colony PCR, restriction digestion and sequencing (**Fig. 4A.25 A & B**). After sequence confirmation (ORF in frame with 6x His tag) pET-30 b (+) containing BmFPS was transformed in *E. coli* BL21 (DE3).

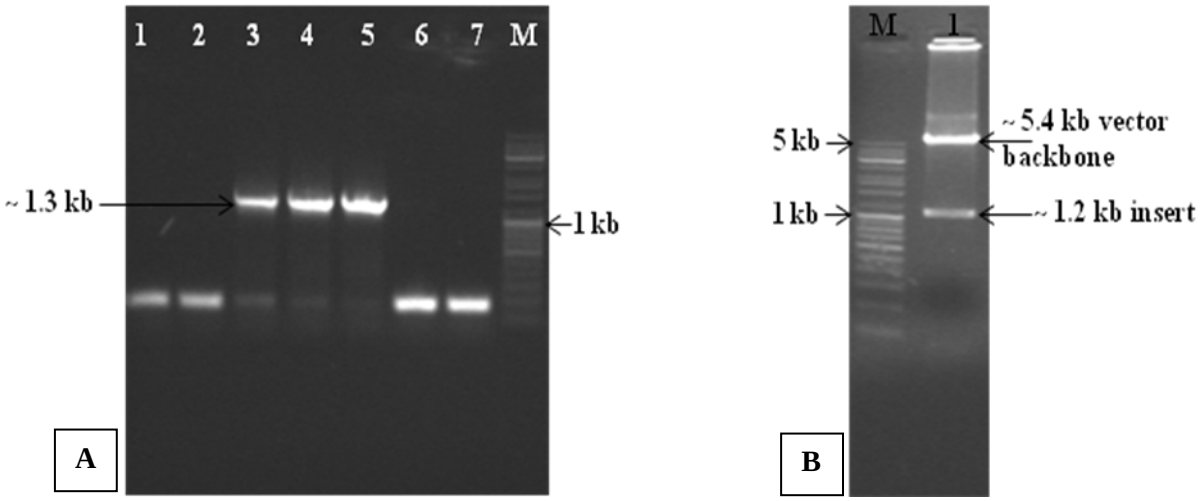


Fig. 4A.25: A. Colony PCR showing ~1.3 kb *BmSQS* gene amplification with T7 promoter and terminator primers. Lane M- Medium range DNA ruler, lane 3, 4 & 5 are positive clones. **B.** Restriction digestion of recombinant pET-30b (+) with *Nde*I and *Xho*I: lane 1- recombinant vector releasing ~1.2 kb *BmSQS* gene and ~5.4 kb vector backbone, lane M- Medium range DNA ruler.

4.3.3.2 Heterologous expression and purification of recombinant BmSQS protein

As mentioned in section 4.3.2.2, similarly heterologous expression of BmSQS was done in *E. coli*. First expression was screened (37 ^{o}C, 1 mM IPTG, 6 h; **Fig. 4A.26 A**, Induced and uninduced protein) and optimized conditions were: 28 ^{o}C, 0.5 mM IPTG and 4 h. An approximately 43 kDa over expressed protein on 10% SDS-PAGE gel was detected in Coomassie staining. Recombinant BmSQS protein was purified by using Ni-NTA agaroge beads (**Fig. 4A.26 B**).

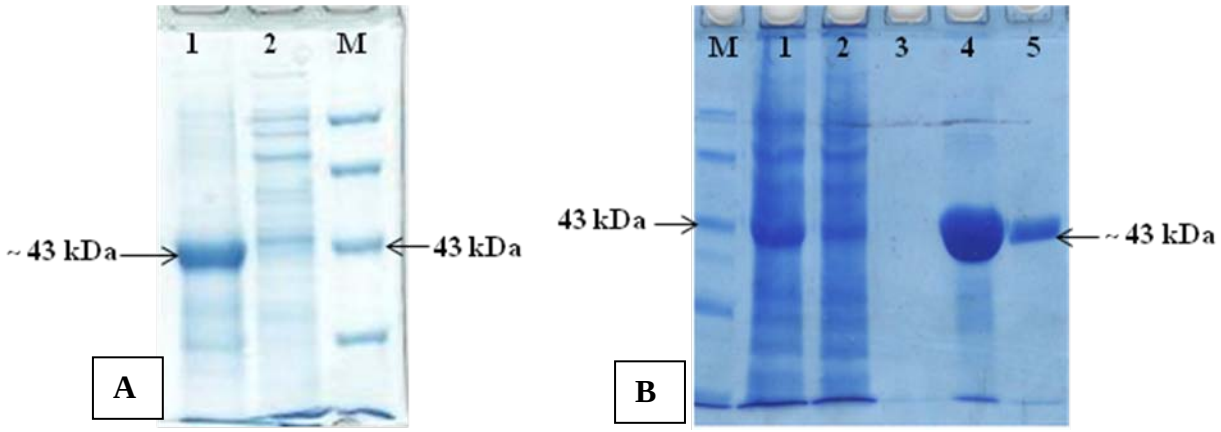


Fig. 4A.26: 10% SDS-PAGE gel. **A.** lane 1- Induced over-expressed protein; lane 2- Un-induced protein and lane M- Protein molecular weight marker. **B.** Lane 1. Lysate; Lane 2- Flow through; Lane 3, 4 and 5- Elution fractions (1, 2 and 3 respectively).

4.3.3.3 Raising antibodies in rabbit against recombinant BmSQS protein

Polyclonal antibodies against truncated BmSQS were raised in rabbit using purified protein. Antibody titer of first and second bleed was determined by ELISA by using purified recombinant BmSQS protein. Titer of 2nd bleed was ~1:60000 (**Fig. 4A.27**). Second bleed serum dilution of 1:5000 was used for further experiments

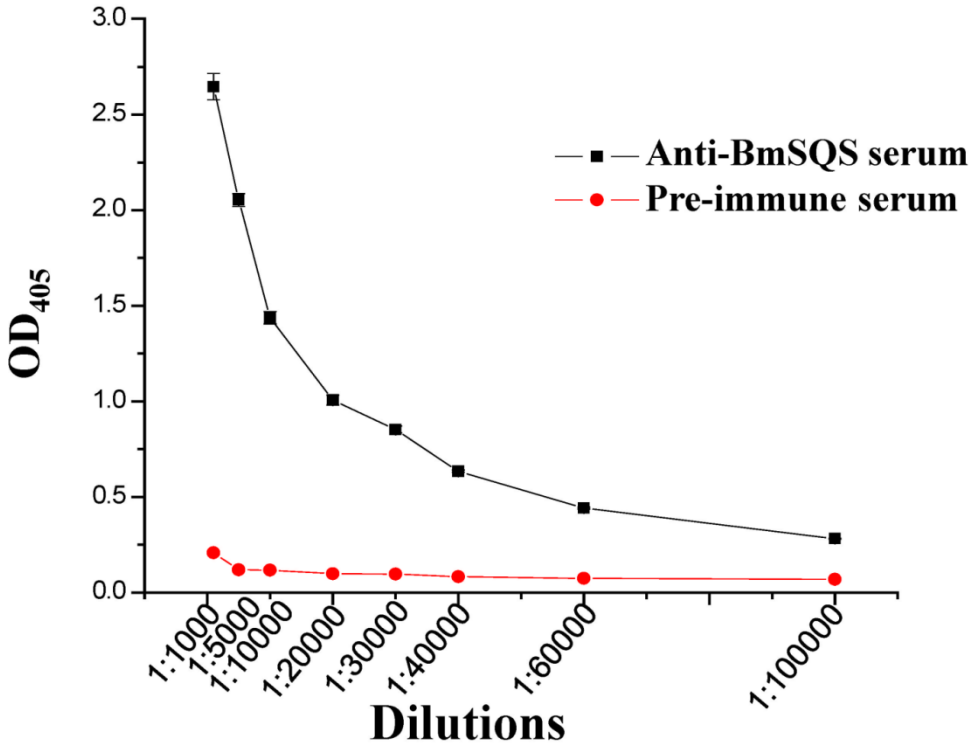


Fig. 4A.27: Titre of Anti-BmFPS polyclonal antibody.

4.3.3.4 Western blot analysis

Total soluble protein from *B. monniera* shoots was extracted and 100 µg of protein was separated on 10% SDS-PAGE and protocol followed as mentioned in section 4.3.2.4. The anti-BmSQS polyclonal antibody obtained from 2nd bleed serum was diluted 1:5000 times and used for Western blot experiments. In Western blot faint band was detected on blot in the crude plant extract of *B. monniera* corresponding to ~ 47 kDa above the truncated recombinant BmSQS positive control (~43 kDa) **(Fig. 4A.28**).

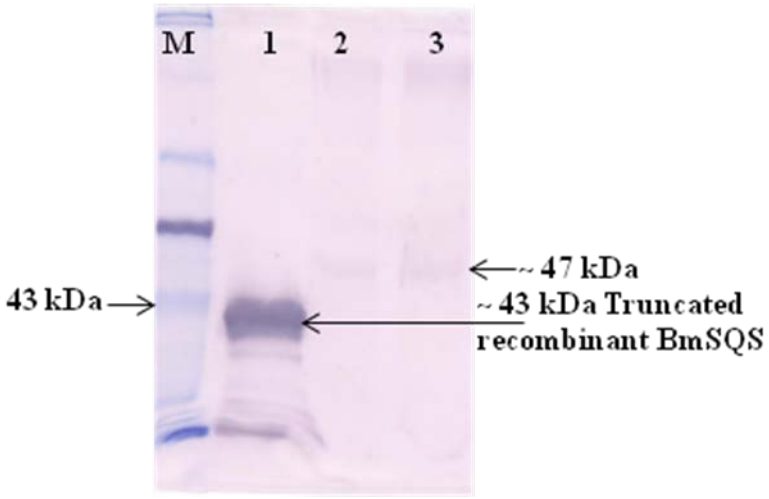


Fig. 4A.28: Western blot analysis of BmSQS protein. Lane 1- Recombinant truncated BmSQS; Lane 2 & 3- total plant protein; Lane M- Prestained protein molecular weight marker.

4.3.3.5 ELISA analysis of BmSQS

Total protein from different tissues was extracted in bicarbonate buffer and equal quantity (30 µg) of protein was coated on 96 well micro titre plate. Among all three types of tissues maximum expression of SQS protein was observed in leaves followed by stem and roots (**Fig.4A.29**). Expression pattern of BmSQS was more/less similar to the BmFPS expression in different tissues taken in consideration.

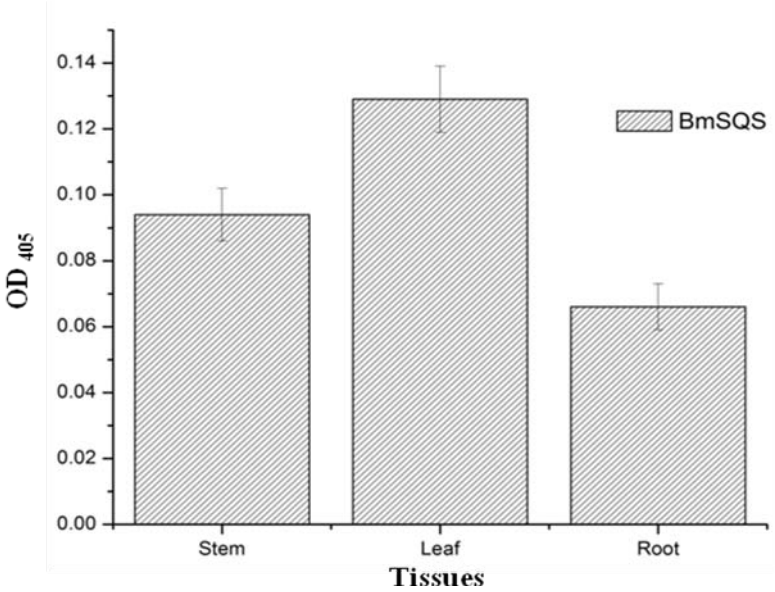


Fig. 4A.29: BmSQS protein expression analysis in different tissues by ELISA.

4.3.4 *In-silico* studies of BmSQS

4.3.4.1 Secondary structure prediction

The secondary structure of BmSQS was predicted by the PSIPRED Protein Structure Prediction Server (PSIPRED v3.0). The structure showed only 18 α-helices and 21 coils. There are no β-sheets (**Fig. 4A.30**).

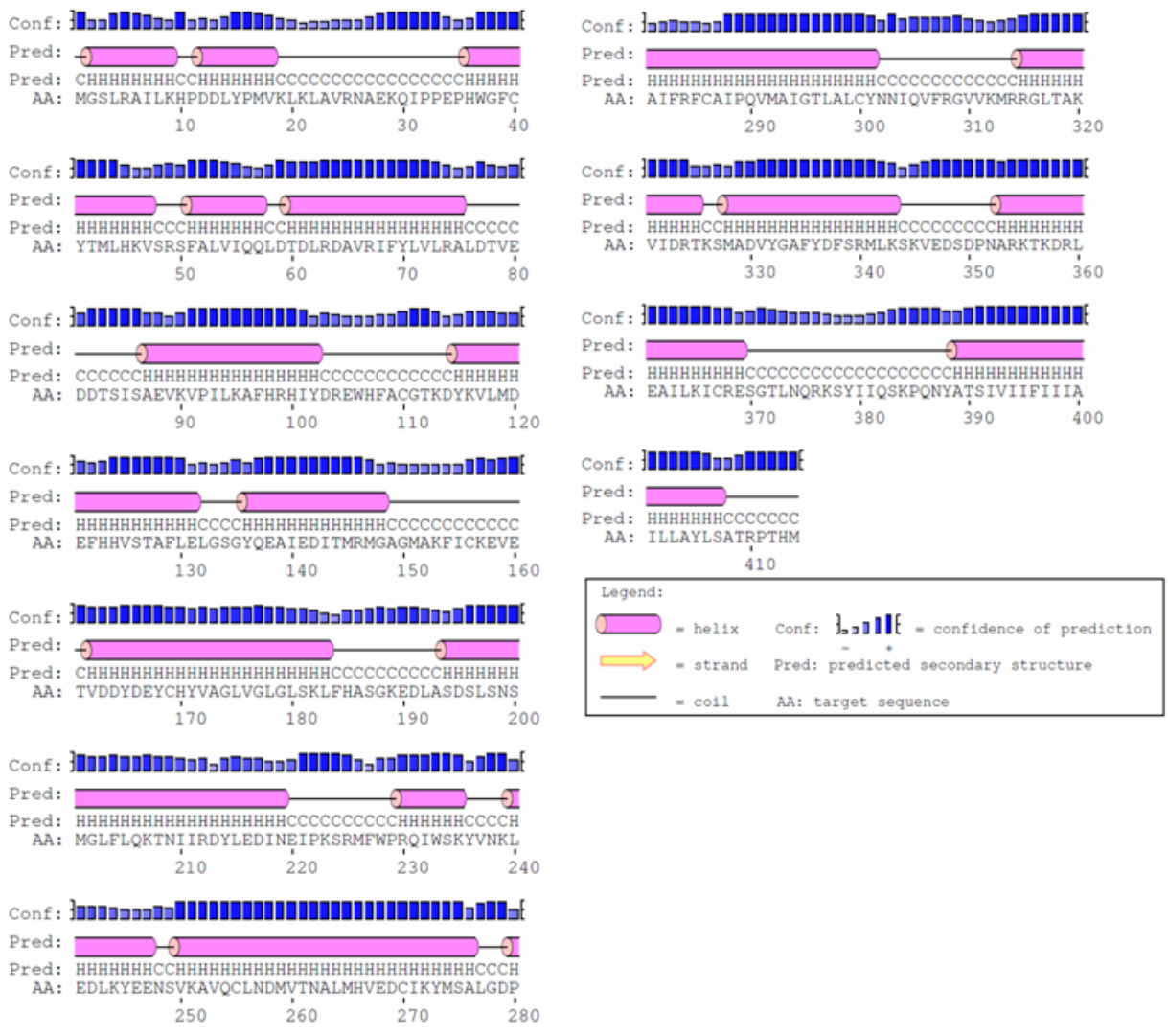


Fig. 4A.30: Secondary structure of BmSQS from *B. monniera*.

4.3.4.2 Homology modeling (Prediction of 3D structure)

Squalene synthase from *B. monniera* (NCBI Accession No. ADX01171.1) was used for homology modeling. The Protein-BLAST algorithm (blastp) against the Protein Data Bank was used to carry out the sequence homology search. The known crystal structures of homologous proteins as template were considered for homology modeling. The crystal structure of *Homo sapiens* squalene synthase (PDB ID- 1EZF; Resolution 2.15 Å) with a 50 % sequence identity was considered as a best hit (E value: 1e-109) and was used as a template to generate comparative 3D model of BmSQS (**Fig. 4A.31 A**). Out of 414 residues, 324 (40^{th} -363^{rd} AA) residues were modeled in the structure. At N-terminus, 39 AA residues and 51 AA residues at C-terminus were unmodeled due to presence of hydrophobic regions. A total 50 models were generated and their discrete optimized potential energy (DOPE) was calculated using model-single.top script. The model no. 12 (BmSQS.B999900012.pdb) having lowest DOPE score (-42431.23828) was considered as the best model of BmSQS. **Figure 4A.31 B** shows the superimposition of the target protein model with 1EZF. The overall conformation of the model is very similar to the template; the blue is the homology model with the completed closure of the flap domain. The RMSD of alpha carbon (Cα) of the homology model was calculated by structural superimposition of model with templates on Chimera and was found to be 0.767 Å.

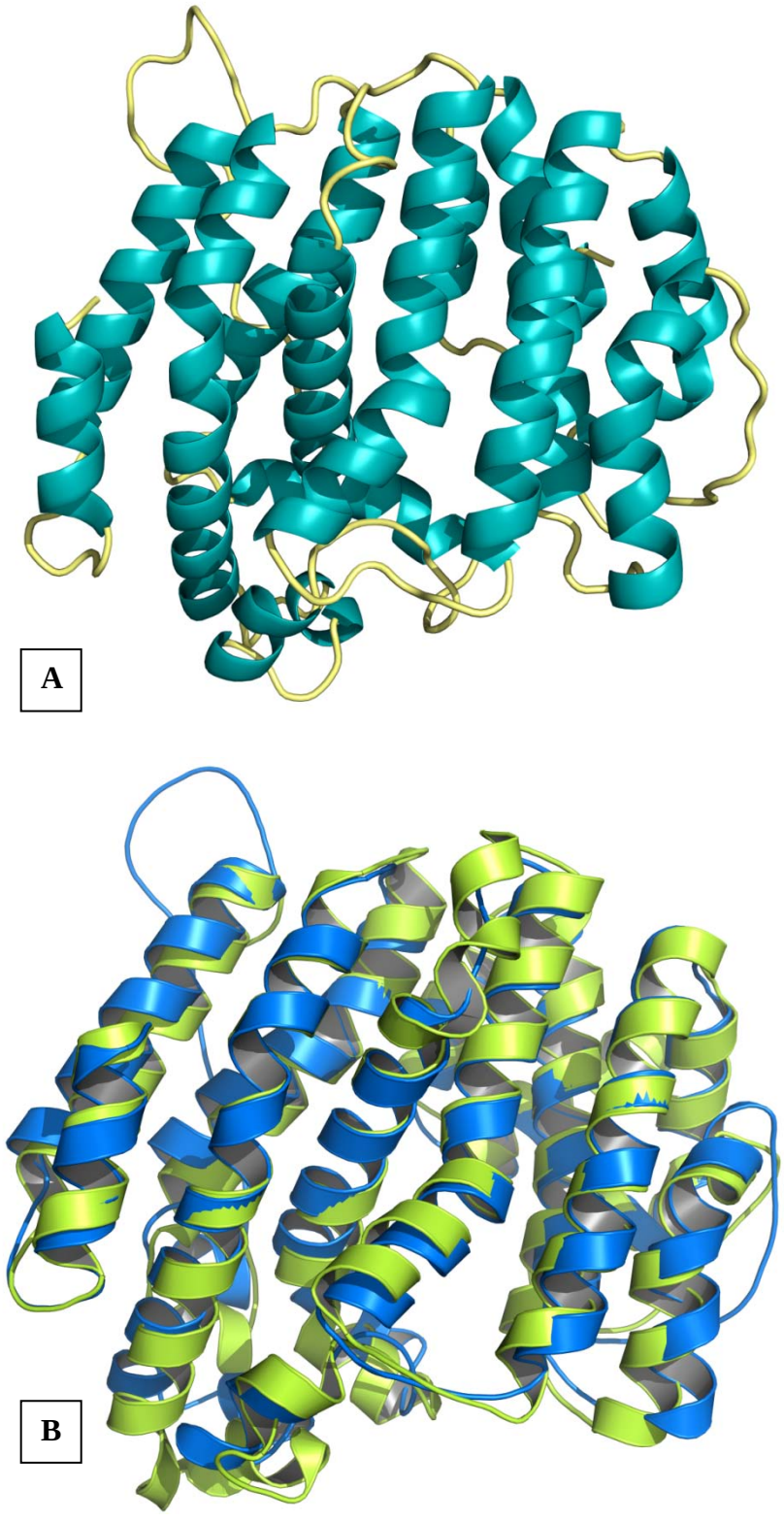


Fig. 4A.31: A. 3D structure of BmSQS **B.** Superimposition of modeled BmSQS (blue) with template 1EZF (lemon yellow).

4.3.4.3 Validation of modeled BmSQS

4.3.4.3.1 Ramachandran plot

PROCHECK analysis of the modeled protein showed that 93.4% of the residues were found in the most favoured region, 6.3% in additional allowed region and 0.3% in generously allowed region and 0.0% disallowed region of the Ramachandran plot (**Fig. 4A.32**). The above results indicate that the protein model is reliable. The plot statistics are shown in Table 4A.6.

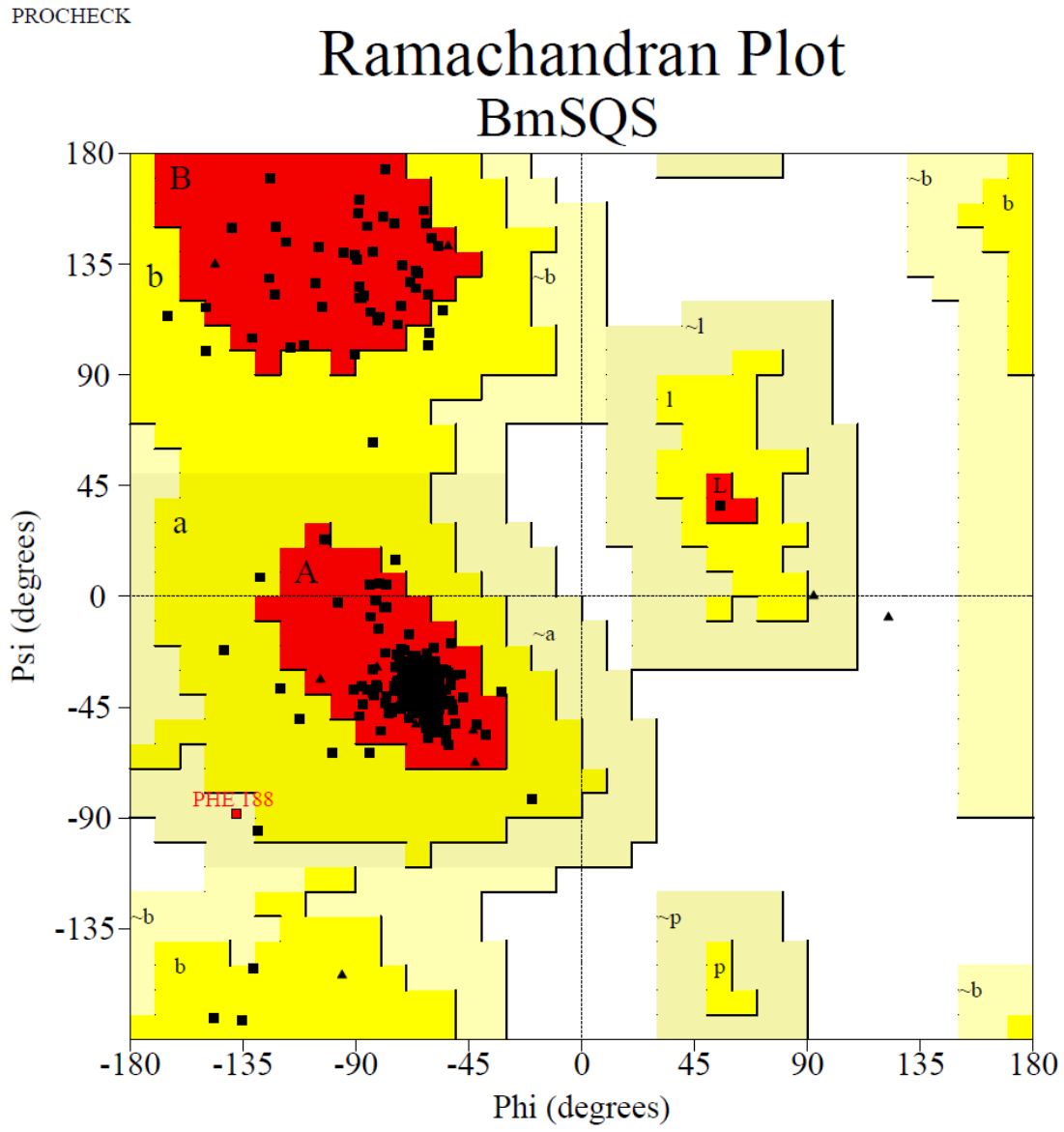


Fig. 4.A 32: Ramachandran plots of modeled BmSQS.

Table 4A.6: Ramachandran plot statistics (BmSQS).

Plot statistics	No. of residues	%
Most favoured regions [A, B, L]	281	93.4
Additional allowed regions [a, b, l, p]	19	6.3
Generously allowed regions [~a, ~b, ~l, ~p]	1	0.3
Disallowed regions	0	0.0
Non-glycine and non-proline residues	---------- 301	---------- 100.0
End-residues (excl. Gly and Pro)	2	
Glycine residues	15	
Proline residues	6	
Total number of residues	---------- 324	

4.3.4.3.2 ERRAT and Verify 3D analysis

Regions of the structure that can be rejected at the 95% confidence level are gray and regions that can be rejected at the 99% level are shown in dark black (**Fig. 4A.33**). The overall quality factor of modeled BmSQS was 85.852, expressed as the percentage of the protein for which the calculated error value falls below the 95% rejection limit. The Verify 3D score was 92.92%, indicating that generated model was reliable.

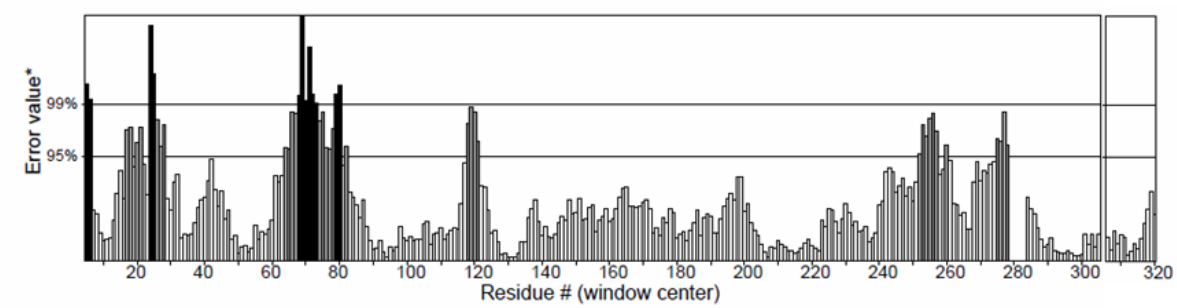


Fig. 4A.33: ERRAT plot.

4.3.4.3.3 Zplot analysis (ProSA)

The Z score of the protein is displayed in the plot with a dark black point (**Fig. 4A.34**). In this plot the Z score value of the obtained model of BmFPS (-8.5) is located within the space of protein related to X-ray. This value is nearly close to the value of the template 1EZF (-9.57) which suggests that the obtained model is reliable.

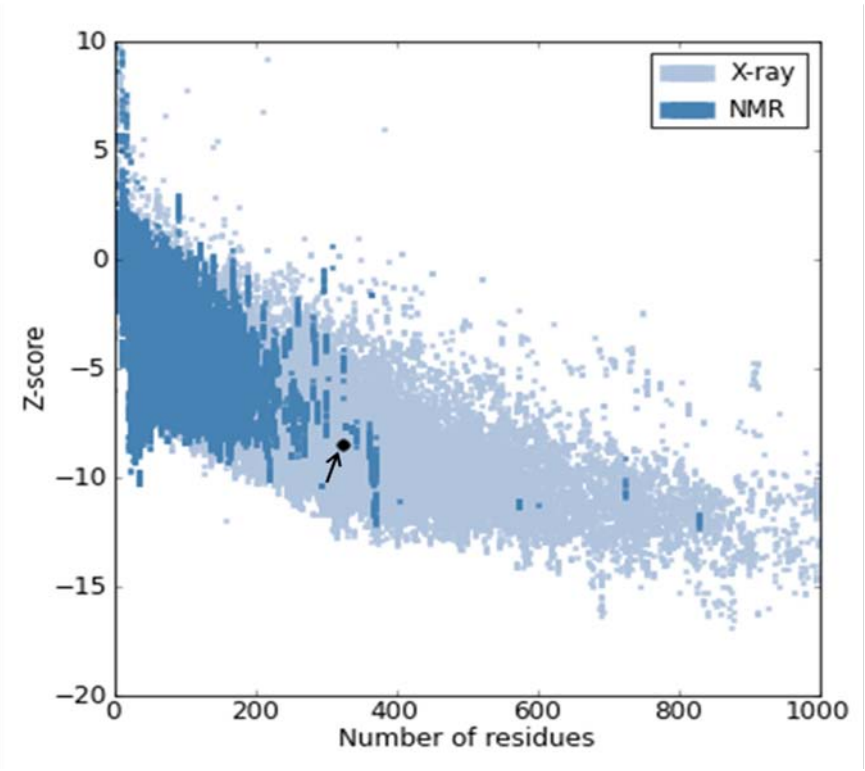


Fig. 4A.34: Z-Score Plot shows that Z score value of BmSQS. The black dot represents Z-score (indicated by arrow) of the model BmSQS.

4.3.4.4 Interaction of BmSQS with ligands (Docking studies)

The 3D structure ligands FPP and NADPH were taken from pubchem server of NCBI. The X, Y, Z dimensions of the grid were set to -27.244, 79.969 & 59.306 for FPP and for NADPH -25.536, 64.700 & 60.621 Å respectively with grid points separated by 30 Å.

The amino acid residues of BmSQS protein played an active role in the interaction with ligands FPP (**Fig. 4A. 35**) and NADPH (**Fig. 4A.36**) are shown in Table 4A.7.

Table 4A.7: Residues involved in interaction of FPP and NADPH with BmFSQS.

Ligands	**Glide G-score**	***Emodel* (kJ/mol)**	**No. of H-bonds**	**Bond lengths**	**Amino acid residues involved in interactions (BmSQS)**	**Amino acid residues involved in interactions (Human SQS)** (Pandit J. et al. 2000)
FPP	-5.421	-72.295	5	1.7-2.4 Å	Arg-48; Asp-76, 212, Tyr-69	Asp-80, 84, 117, 121, 219,223; Arg-228; Tyr-171
NADPH	-9.832	-119.308	16	1.9-3.3 Å	Arg-48, 73; Asp-80, 216, Glu-79, 215, 219; Tyr-69, 167; Ser-47, Asn-208	Phe-54, 288; Cys-289; Pro-292; Val-179; Leu-183, 211; Tyr-73;

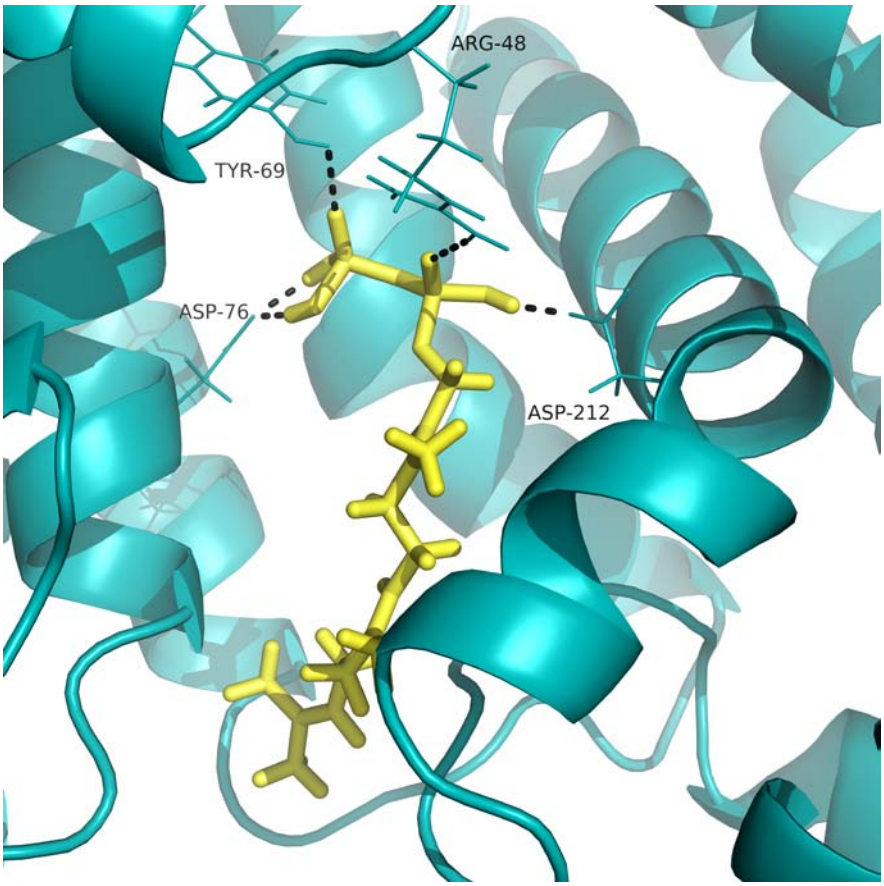


Fig. 4A.35: Ribbon diagram showing interaction of FPP (Yellow) with BmSQS.

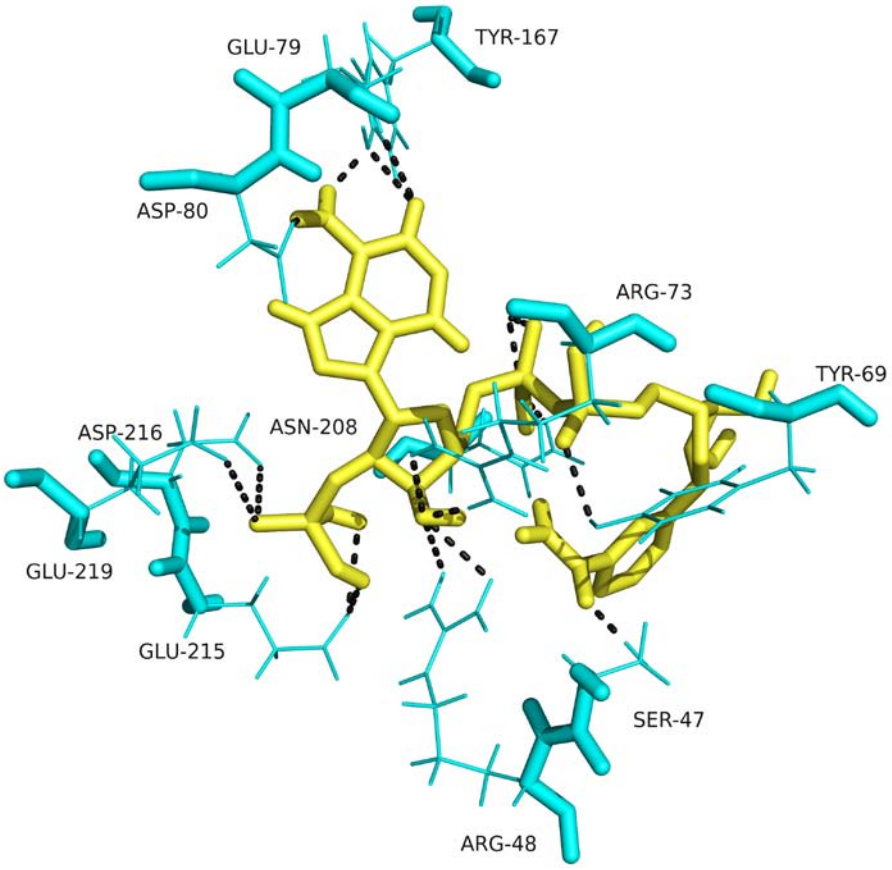


Fig. 4A.36: Stick-line diagram showing interaction of NADPH (Yellow) with BmSQS. Black dotted lines showing H-bonding.

4.4 Conclusions

- The BmHMGR, BmFPS and BmSQS were directionally cloned in pET 30 b (+) expression system and transformed in *E. coli* BL21 (DE3) for over-expression.
- Conditions were optimized for maximum expression of proteins in soluble form and recombinant proteins (BmFPS and BmSQS) were purified by Ni^{+}-NTA affinity chromatography.
- Polyclonal antibodies against purified BmFPS and BmSQS were raised in rabbit, used for ELISA and Western blot analysis.
- Titre of polyclonal antibodies BmFPS and BmSQS were checked which were 1:70000 and 1:60000 respectively.

- ELISA analysis showed that BmFPS and BmSQS expressed more in leaf followed by stem and root.
- Comparative modeling and docking studies of BmHMGR, BmFPS and BmSQS was done to predict 3D structure and interactions with ligands.
- 3D structures were validated by PROCHECK, ERRAT, Verify 3D and ProSA and it was found that all three structures are reliable.
- Secondary structure prediction was done using PSIPRED server. BmHMGR has 14 α-helices and 14 β-sheets, whereas BmFPS and BmSQS have only 14 and 18 α-helices respectively and no β-sheets.
- Interactions of HMG-CoA and NADPH with BmHMGR by docking showed residues Gln568(A); Tyr576(A); Tyr189(B); Asn275(A); Lys430(A); Met237(A) and Asn366(A); Asn475(A); Arg335(A); Arg298(A); Arg578(B); Asp361(A); Asp475(B); Met367(A); Gly364(A); Ser369(A); Ser579(B) respectively are involved.
- Docking study of BmFPS with IPP showed interaction with Asp-100, 104, 171, 257 and 260; with DMAPP Asp-100, 104, 171; Arg-109 and Lys-197; and with GPP Asp-100, 104, 109, 171; Arg-109, Lys-197, 262. It was found that Asp-100 and 104 is common residue interacting with all the three ligands.
- Docking of FPP and NADPH with BmSQS showed residues, Arg-48; Asp-76, 212, Tyr-69 and Arg-48, 73; Asp-80, 216, Glu-79, 215, 219; Tyr-69, 167; Ser-47, Asn-208 interact respectively. It was found that Arg-48 and Tyr-69 are common residues interacting with both ligands.

CHAPTER: 4(B)

TISSUE SPECIFIC EXPRESSION ANALYSES OF THE PATHWAY GENE(S) IN NORMAL AND STRESS CONDITIONS

4 (B) Tissue specific expression analyses of the pathway gene(s) in normal and stress conditions

4.5 Introduction

Terpenoids are synthesized from C5 units, isopentenyl pyrophosphate (IPP). IPP is supplied from MVA pathway and MEP pathway. The MVA pathway uses seven enzymatic reactions to convert acetyl-CoA to IPP and DMAPP. Detailed biosynthetic pathway is already described in chapter 1. Although differential expression pattern of several genes of isoprenoid pathway from different plants have been studied, in *Bacopa* there is no report till date.

In the present study tissue specific expression analysis of three isoprenoid pathway genes from *B. minniera*, *BmHMGR*, *BmFPS* and *BmSQS* were carried out. Expression pattern in response of different abiotic stresses and a biotic stress yeast extract at different time interval were studied.

4.6 Materials and methods

4.6.1 Plant material and stress conditions

Expression analysis of *BmHMGR*, *BmFPS* and *BmSQS* transcripts was done in field grown *Bacopa* plant in green house at flowering stage in different tissues including stem, leaves, roots and floral part.

For stress treatments *B. monniera* shoots were treated as described in Chapter 2. The explants were transferred to MS basal medium for about 20 days till proliferating multiple adventitious shoots were separated and re-inoculated on proliferation medium for further growth. Proliferated shoots were then used for stress treatments. *In vitro* grown complete rooted plants were shifted to liquid MS media for 2 days. Then plants were transferred to fresh liquid medium having different stressors separately. Root, stem and leaves were harvested separately after time interval of 2 h, 4 h, 8 h and 12 h in case of methyl jasmonate (500 µM); 4 h, 8 h and 12 h in case of salt (200 mM), cold (4-7 ^{o}C), salicylic acid (200 µM) and yeast extract (0.1%). In case of chlorogenic acid (0.5 mg/L) root, stem and leaves were harvested at time interval of 4 h, 8 h, 12 h, 24 h and 48 h.

4.6.2 RNA isolation and cDNA synthesis

Total RNA was isolated from different tissues of experimental *in-vitro* plants according to the RNA isolation kit (Sigma) and DNase I treatment was given as per manual instructions. First strand cDNA synthesis, primed with an oligo $(dT)_{15}$ primer, was performed with Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) according to the manufacturer's protocol (Promega Corp., Madison, USA).

4.6.3 Quantitative real time PCR (qRT-PCR)

The cDNA first strands were used as template for qRT-PCR analysis of *BmHMGR*, *BmFPS* and *BmSQS* transcripts. Brilliant II™ SYBR® Green QPCR master mix with low ROX (Stratagene, USA) was used for real-time PCR. The qRT-PCR considerations, preparation of reaction mixtures and PCR cycling parameters were described in chapter 2.

4.6.4 Relative transcript quantification (Comparative Ct method)

Relative (comparative) quantification relates the PCR signal of the target transcript in a treatment group to that of another such as untreated control or sample with lowest expression level at a time, considered as a basal expression.

The comparative Ct method is also known as the $2^{-\Delta\Delta Ct}$ method, where

$\Delta\Delta C_t = \Delta$ Ct, sample - Δ Ct, reference/baseline.

Here, ΔCt, $_{sample}$ is the Ct value for any sample normalized to the endogenous housekeeping gene and ΔCt, $_{reference/baseline}$ is the Ct value for the calibrator also normalized to the endogenous housekeeping gene.

Means,

Δ Ct, $_{sample}$ = Ct, $_{sample}$ – Ct, $_{endogenous\ control\ (5.8S\ rRNA\ gene\ or\ any\ other\ housekeeping\ gene)}$

Δ Ct, $_{reference}$ = Ct, $_{reference/\ normal/\ untreated\ sample/baseline}$ – Ct, $_{endogenous\ control.}$

For the $\Delta\Delta$Ct calculation to be valid, the amplification efficiencies of the target and the endogenous reference must be approximately equal. This can be established by looking at how ΔCt varies with template dilution. If the plot of cDNA dilution versus ΔC_t is close to zero, it implies that the efficiencies of the target and housekeeping genes are

very similar. If a housekeeping gene cannot be found whose amplification efficiency is similar to the target, then the standard curve method is preferred.

4.6.5 qRT-PCR analysis of *BmHMGR, BmFPS* and *BmSQS*

Total RNA was extracted individually from roots, stem and leaves from *in-vitro* grown culture of *B. monniera* and also from the plants under stress. Total RNA (1 µg) was used for making cDNA and Brilliant SYBRGreen QPCR kit (Stratagene, USA) and Stratagene Mx3000P real time machine were used for all reactions. Optimal numbers of PCR cycles within the linear range of amplification for each gene were determined in preliminary experiments. qRT-PCR reactions were performed following conditions mentioned in Chapter 2 with annealing temperature of 55 ^{o}C. The reaction was run in triplicates and repeated twice. It was ensured that equal quantity of cDNA template was used for each reaction (Freeman et al., 1999; Pfaffl, 2001). All the reactions were normalized using 5.8S rRNA gene specific primers.

Table 4B.1: Primers used for qRT-PCR analysis.

Genes name	**Primers name**	**Sequence (5’ → 3’)**
BmHMGR	qHMG F	TGGGTGGTTTCAACGCTCAC
	qHMG R	CATTGACGGCTTCCATCATTGTG
BmFPS	qFPS F	TGTCCTTAAATCTGAGCTGTTGAAC
	qFPS R	TGACAGACCTCGGTTTAACTTCC
BmSQS	qSQS F	TTGCGAGATCCAGCGATCTTTCGT
	qSQS R	TAGCTGTGAGCCCACGTCTCATTT
5.8 S rRNA	CT F	CTA AACGACTCTCGGCAAC
	CT R	TTCAAAGACTCGATGGTTCAC

4.7 Results and discussion

Differential expression studies were done using field grown as well as in *vitro* seedlings in different stress conditions of *B. monniera.* Stratagene Mx 3000P real time machine and SYBR Green chemistry were used for expression studies.

4.7.1 Tissue specific expression analysis of *BmHMGR, BmFPS* and *BmSQS*

Quantitative real time PCR was performed using primers as mentioned above and cDNA as a template. Ct value is inversely proportional to the level of expression. The relative expression of the all three genes in terms of mean fold expression ($2^{-\Delta\Delta Ct}$) was determined. It was found that *BmHMGR* expressed in all tissues tested. It was expressed more in roots as compared to stem, leaf and floral parts (**Fig. 4B.1**). Minimum expression was observed in sepals and it was considered as basal expression. In case of *Euphorbia pekinensis EpHMGR* expression pattern was same, expressed highest in roots, followed by stem and leaves (Cao et al., 2010) which is coherent with our result. In *Salvia miltiorrhiza*, SmHMGR2 expressed more in leaves followed by stems and roots (Dai et al., 2011), whereas in other report SmHMGR expression was highest in root, followed by stem and leaf (Liao et al., 2009).

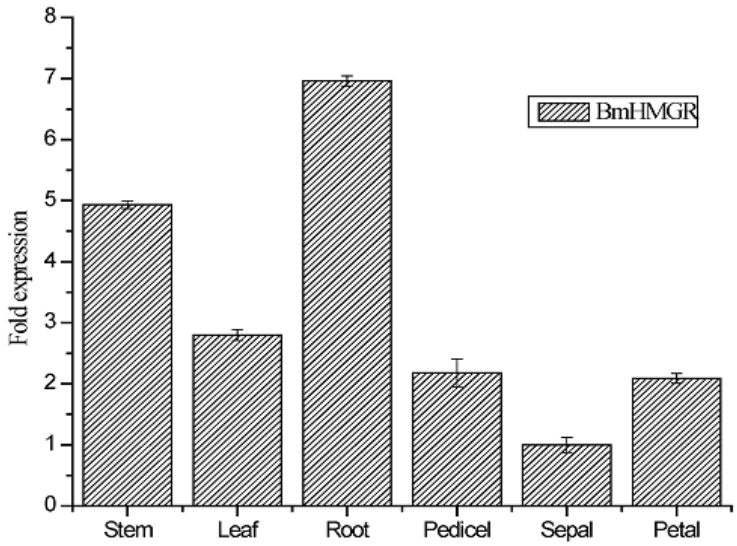


Fig. 4B.1: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene in different tissues. All values are plotted with standard error of mean of triplicate analysis.

BmFPS expression was observed in all tested tissues, and it was about 5.5 fold higher in leaves as compared to petals where basal expression was observed (**Fig. 4B.2**). It was reported that, *Chimonanthus praecox* FPP synthase expressed highest in leaves and petals (Xiang et al., 2010). In case of *BmSQS* it was found that, it expressed more in petals followed by leaf, stem and pedicel (**Fig. 4B.3**). In *Panax ginseng* expression pattern of three squalene synthases (PgSS1, PgSS2 and PgSS3) was reported. PgSS1 and PgSS2 transcripts accumulated more in various organs such as leaves, root and petioles whereas PgSS2 mRNA accumulated preferentially in the leaf only (Kim et al., 2011a). Huang et al. (2007) reported that squalene synthase transcript in *Taxus cuspidata* accumulate higher in roots followed by stem and leaves.

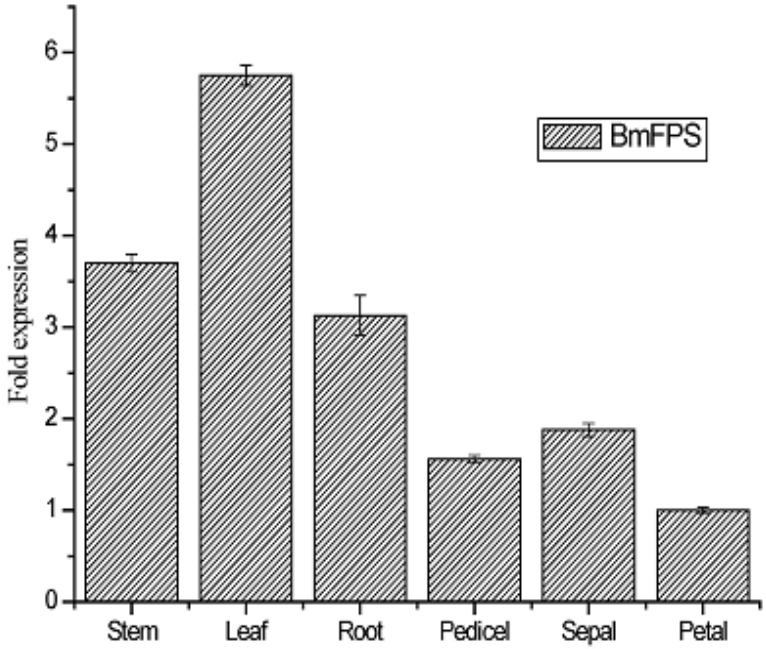


Fig. 4B.2: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene in different tissues. All values are plotted with standard error of mean of triplicate analysis.

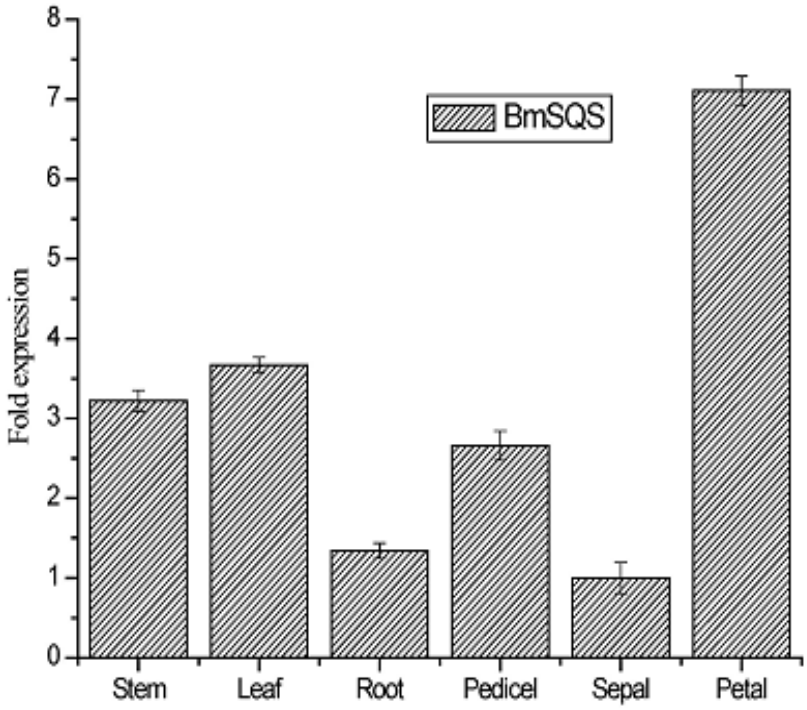


Fig. 4B.3: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene in different tissues. All values are plotted with standard error of mean of triplicate analysis.

4.7.2 Differential expression analysis of *BmHMGR*, *BmFPS* and *BmSQS* under different stress conditions

4.7.2.1 Methyl jasmonate (500 µM)

Methyl jasmonate is an organic compound used in plant defense and induces gene expression of various pathways. Differential expression pattern of *BmHMGR*, *BmFPS* and *BmSQS* in presence of methyl jasmonate are shown in Figures (**Figs. 4B.4**, **4B.5** and **4B.6** respectively). Different tissues showed varied level of overall increased expression as compared to control for all three genes.

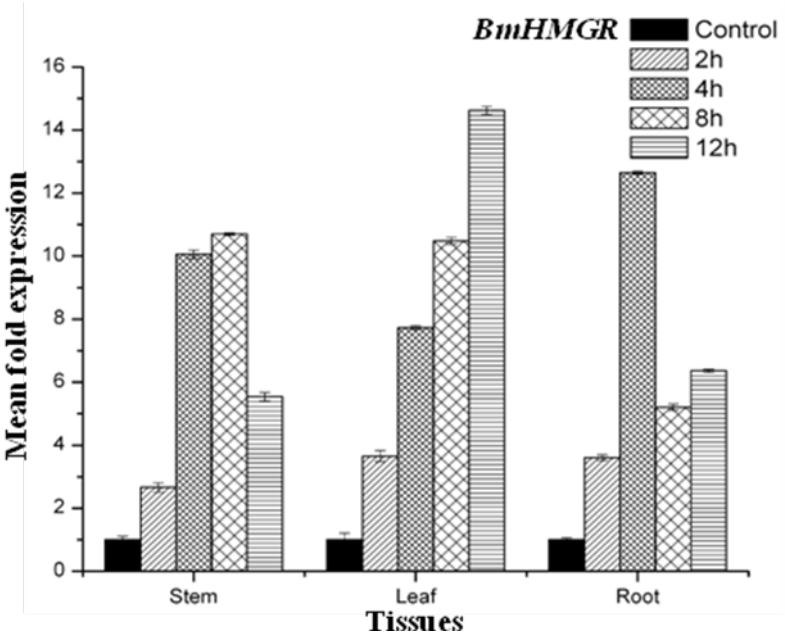


Fig. 4B.4: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene under methyl jasmonate (500 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

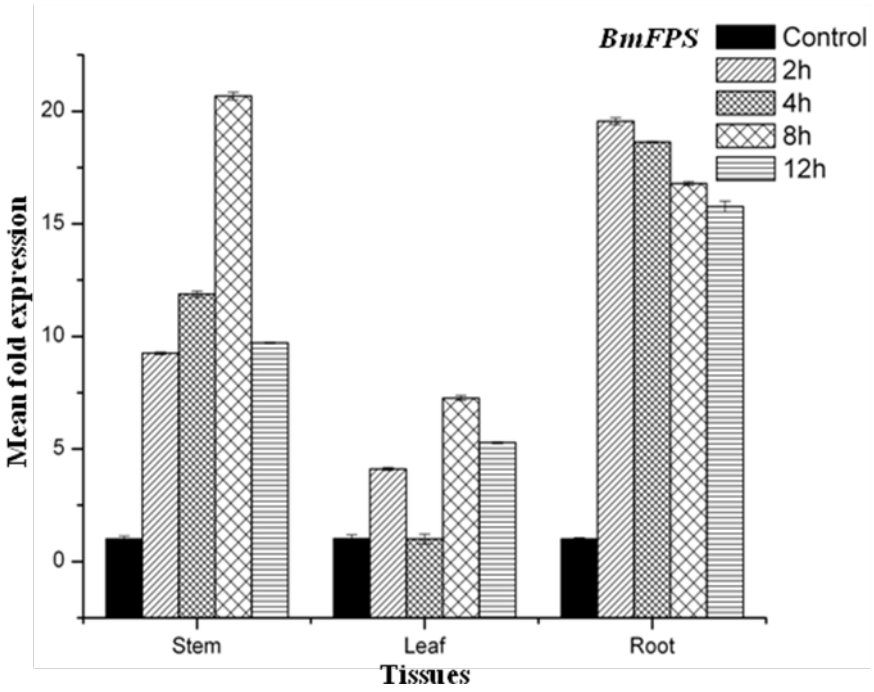


Fig. 4B.5: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene under methyl jasmonate (500 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

Note:

The fold expressions of *BmHMGR*, *BmFPS* and *BmSQS* in presence of different stress conditions were compared independently in individual tissues at different time interval. Although expression levels in stems, leaves and roots were varied, here comparison among tissues was not considered.

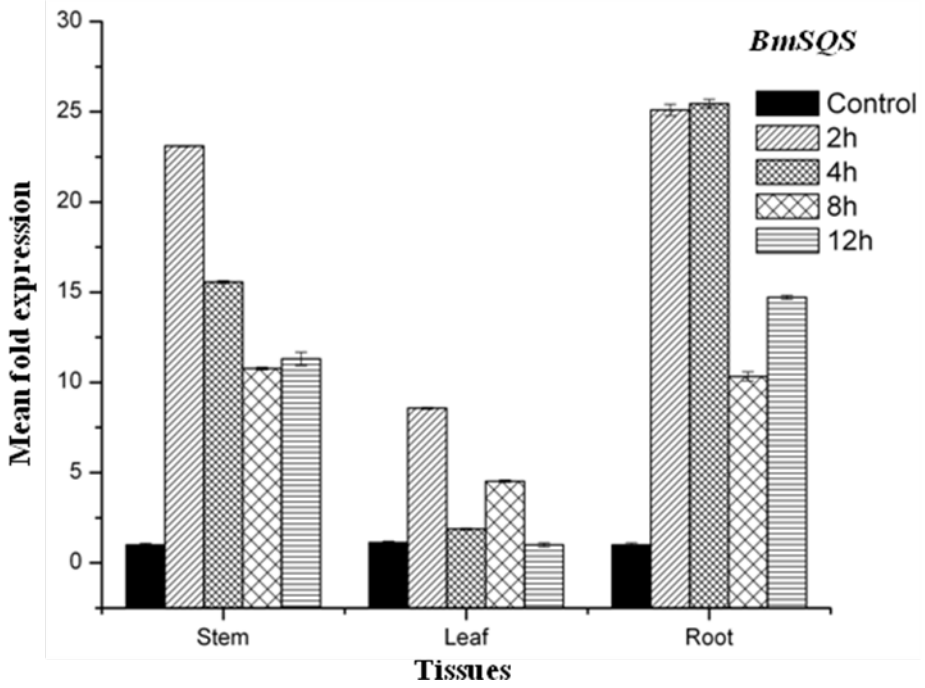


Fig. 4.B.6: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene under methyl jasmonate (500 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.7.2.2 Cold (4- 5 ^{o}C)

Low temperature affects water and nutrient uptake, membrane fluidity and protein and nucleic acid conformation, and it drastically influences cellular metabolism either directly by reducing the rates of biochemical reactions or indirectly through gene expression reprogramming. Expression analysis of *BmHMGR*, *BmFPS* and *BmSQS* under cold stress were showed differential pattern in different tissues at different time intervals (**Figs. 4B.7**, **4B.8** and **4B.9** respectively)

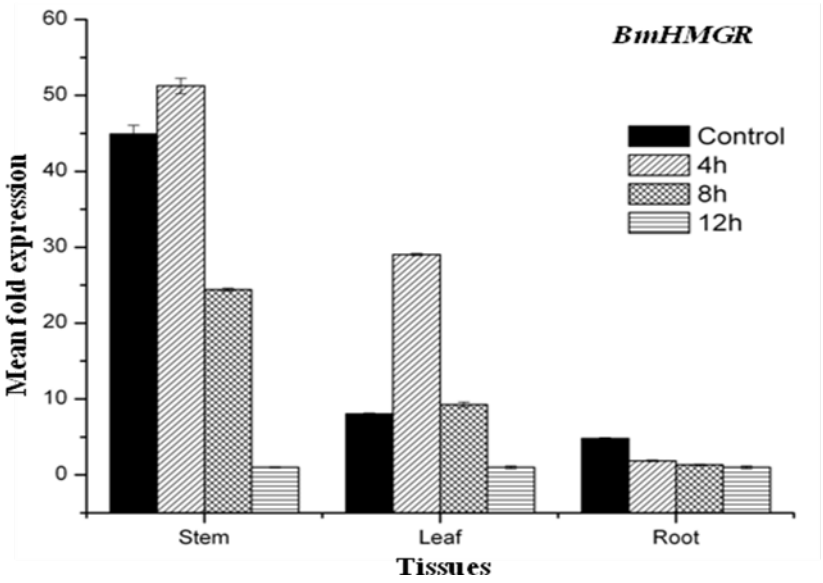


Fig. 4B.7: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene under cold (4-5 ^{o}C) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

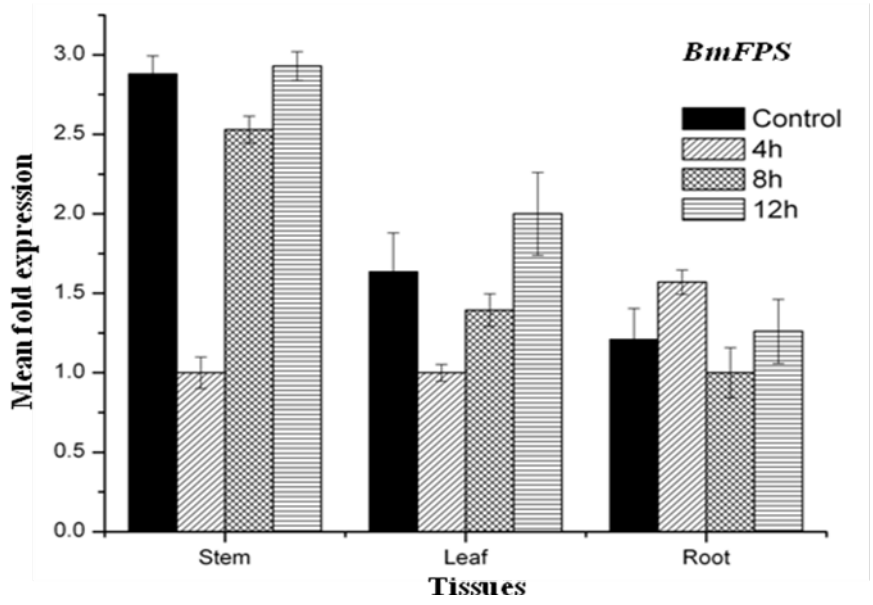


Fig. 4B.8: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene under cold (4-5 ^{o}C) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

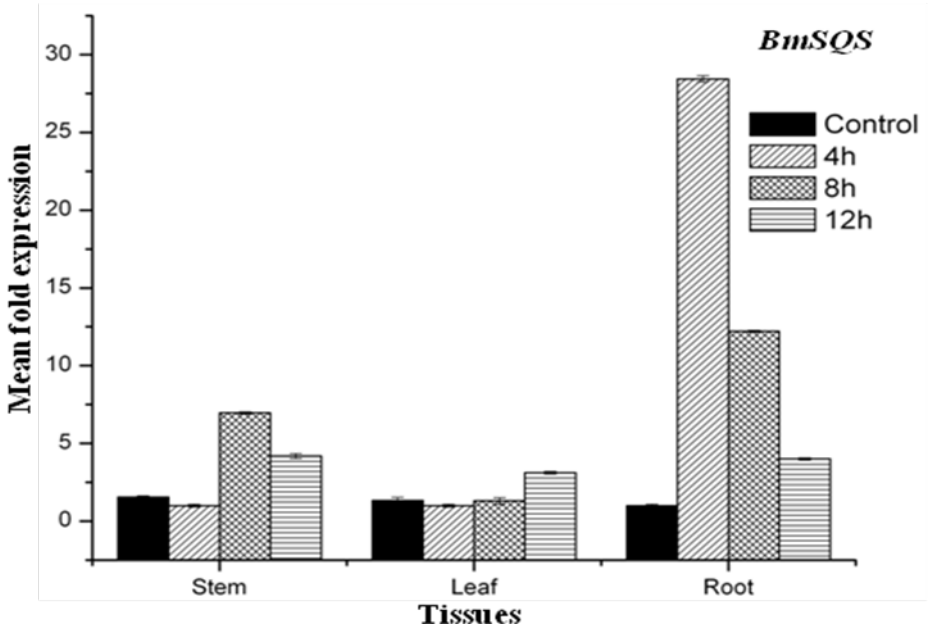


Fig. 4B.9: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene under cold (4-5 ^{o}C) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.7.2.3 Chlorogenic acid (0.5 mg/L)

Chlorogenic acid is a common polyphenol in higher plants. It was reported that the presence of exogenous chlorogenic acid in the medium induced root hair formation. Root hairs increase the efficiency of water absorption and nutrients uptake. Here, we studied the effect of chlorogenic acid on expression pattern of three terpenoid biosynthetic pathway genes *BmHMGR*, *BmFPS* and *BmSQS* at different time interval (**Figs. 4B.10**, **4B.11** and **4B.12** respectively)

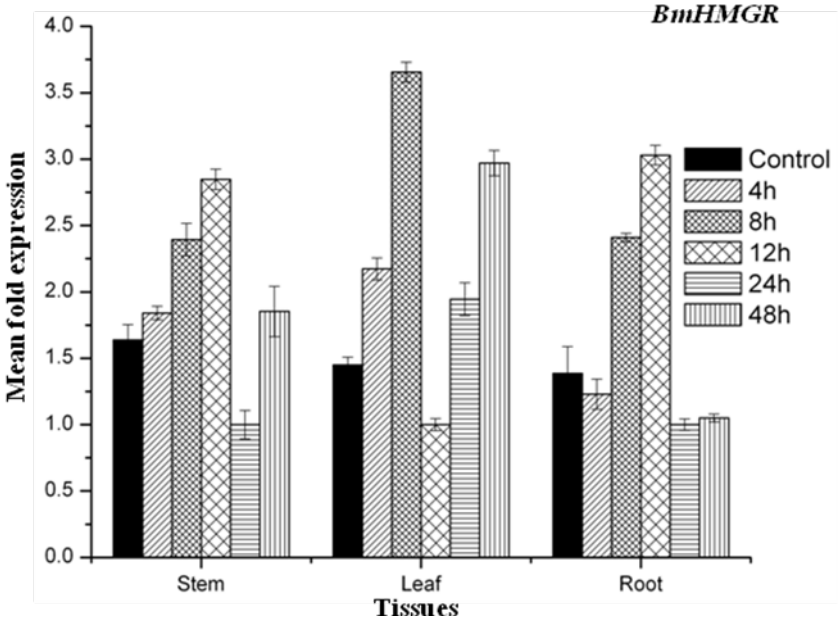


Fig. 4B.10: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene under effect of phenolic compound chlorogenic acid (0.5mg/L) with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

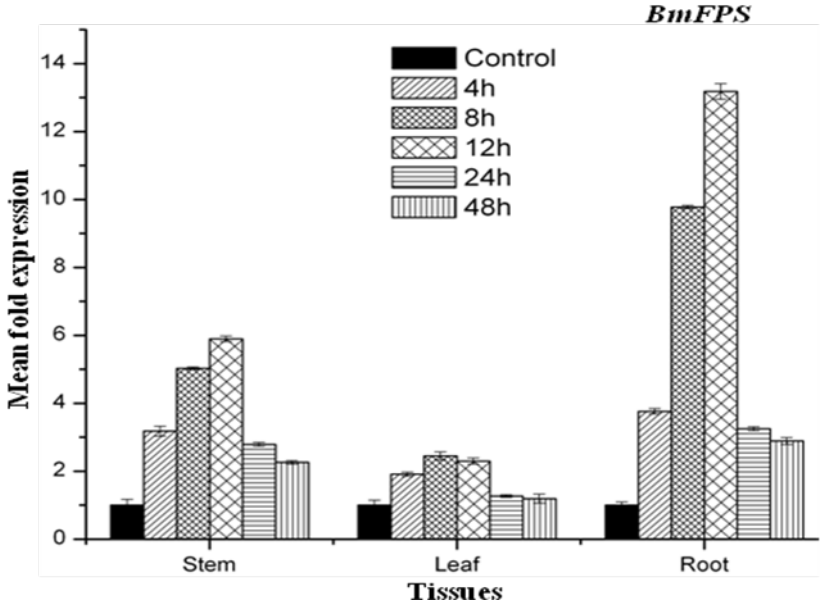


Fig. 4B.11: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene under effect of phenolic compound chlorogenic acid (0.5mg/L) with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

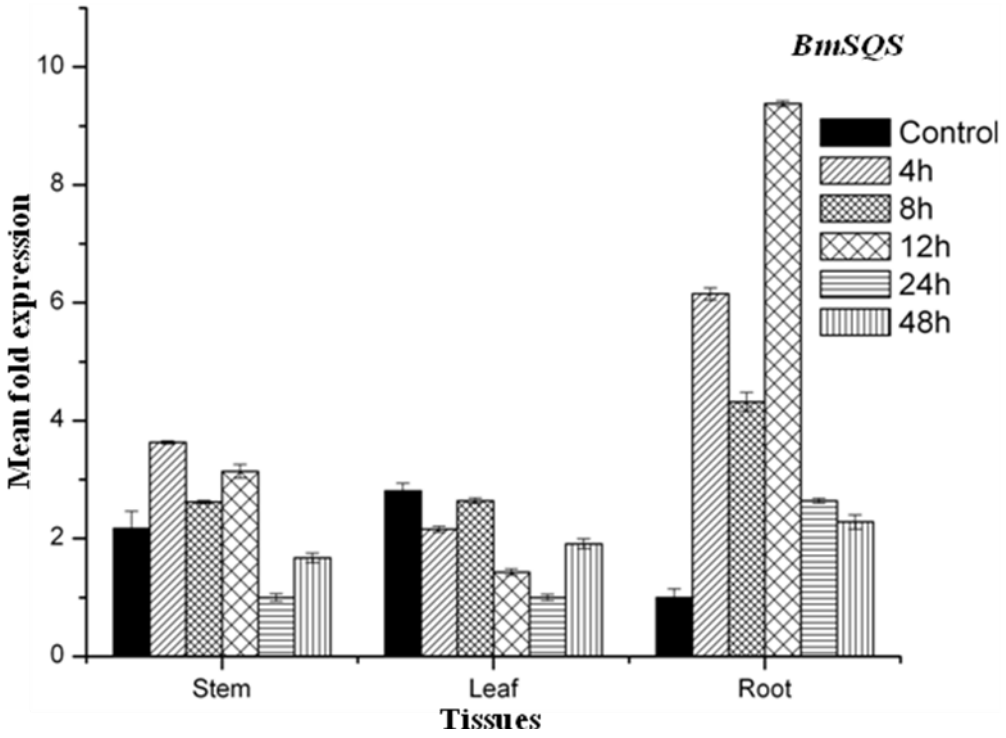


Fig. 4B.12: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene under effect of phenolic compound chlorogenic acid (0.5mg/L) with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.7.2.4 Salt stress (200 mM NaCl)

Salt stress negatively affects many physiological processes in plants. It can lead to changes in development, growth, and productivity and severe stress may threaten survival. Here we report the expression analysis of *BmHMGR*, *BmFPS* and *BmSQS* transcripts in the presence of 200 mM NaCl (**Figs. 4B.13**, **4B.14** and **4B.15** respectively).

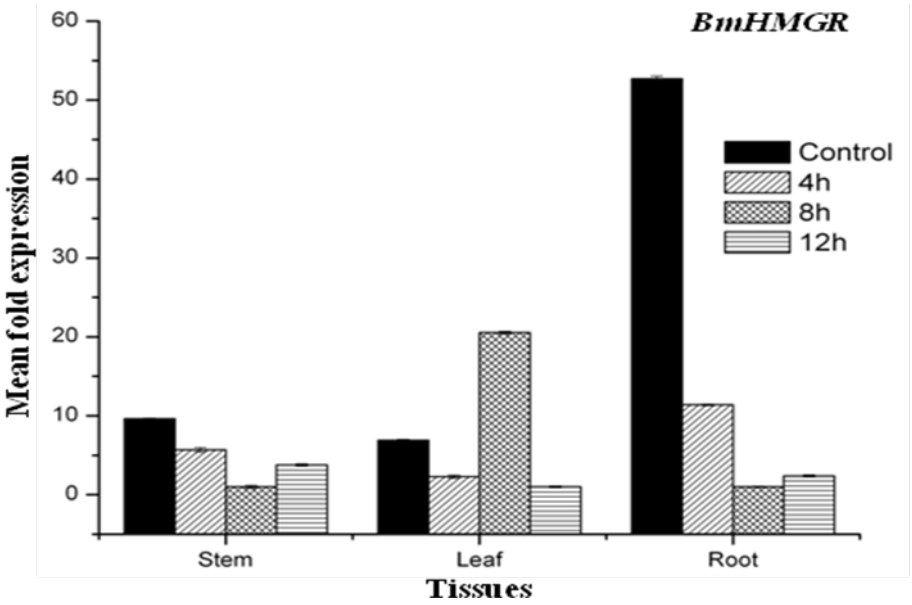


Fig. 4B.13: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene under salt (200 mM NaCl) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

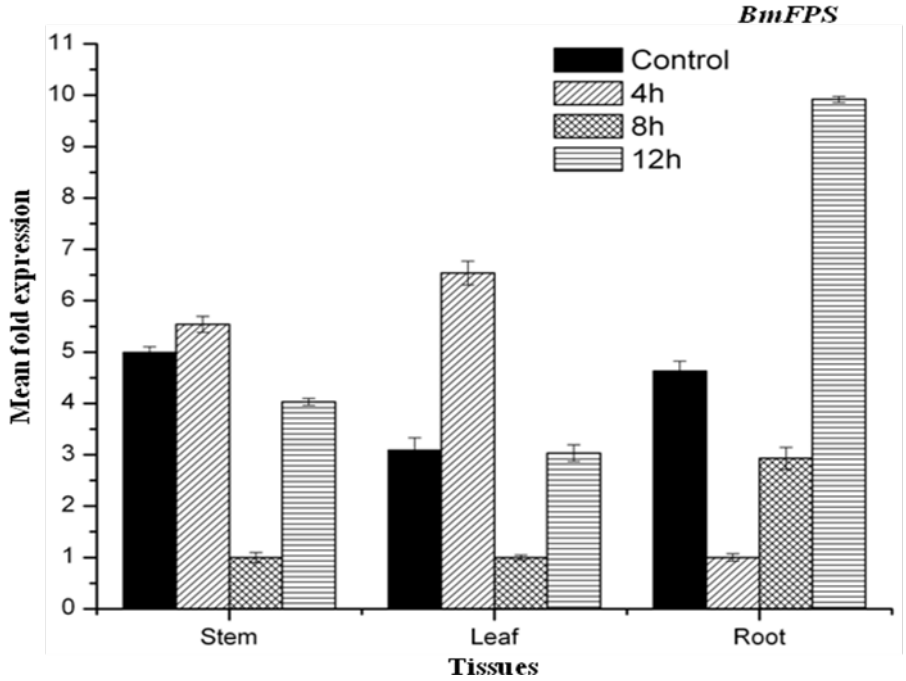


Fig. 4B.14: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene under salt (NaCl 200 mM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

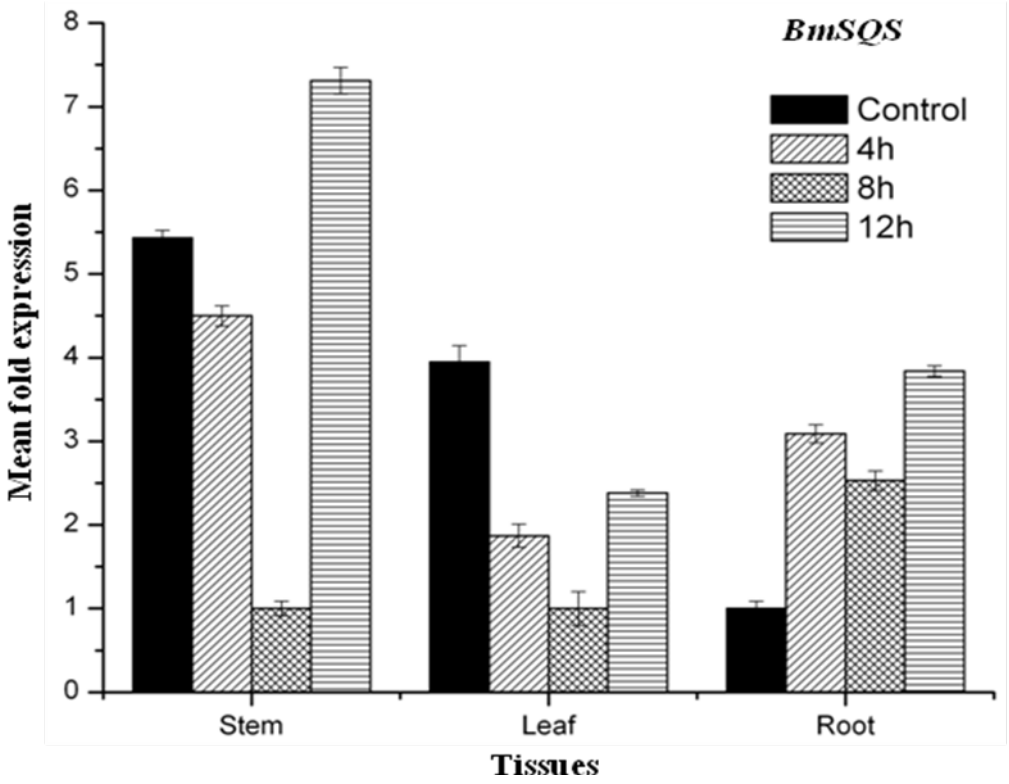


Fig. 4B.15: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene under salt (NaCl 200 mM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.7.2.5 Salicylic acid (200 µM)

Salicylic acid and methyl jasmonate have been identified as key signaling plant hormones regulating a complex network of signal transduction pathways responsible for induced plant defense against biotic and abiotic stresses (Smith et al., 2009{Zhao, 2004 #2377; Zhao et al., 2004). These responses lead to the induction of various secondary metabolites as a defence mechanism like isoprenoids (Ament et al., 2006; Martin et al., 2002). Here, effect of salicylic acid on expression of three isoprenoid pathway genes was investigated. It was found that *BmHMGR* expression induced maximally at 8 h induction and decline in 12 h. But overall expression was maintained higher in stem and leaf as compared to control, whereas decline at 12 h in case of root (**Fig. 4B.16**). Similarly in case of *BmFPS* and *BmSQS* at 8 h induction transcript accumulation was higher and decline after 12 h as compared to control (**Figs.4B.17** and **4B.18**).

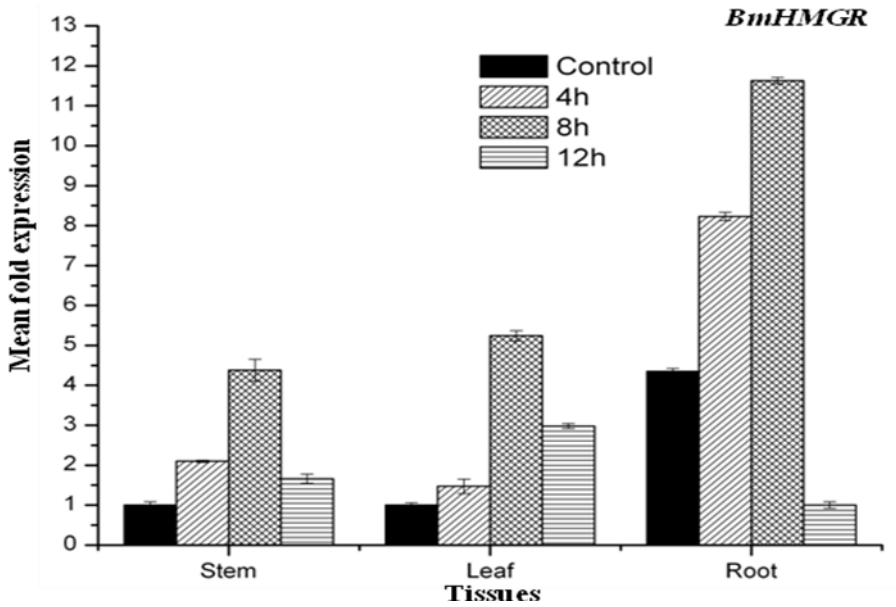


Fig. 4B.16: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene under salicylic acid (200 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

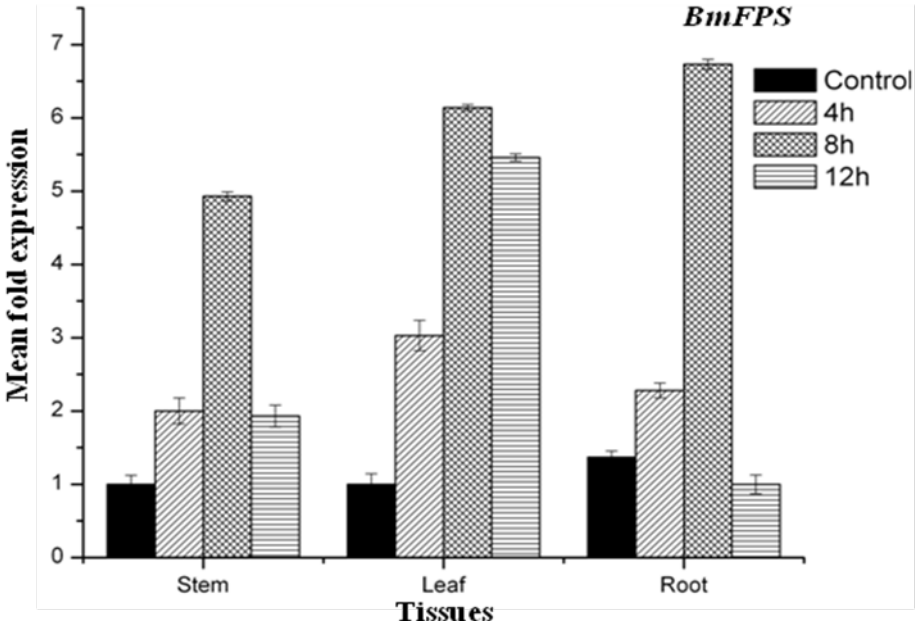


Fig. 4B.17: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene under salicylic acid (200 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

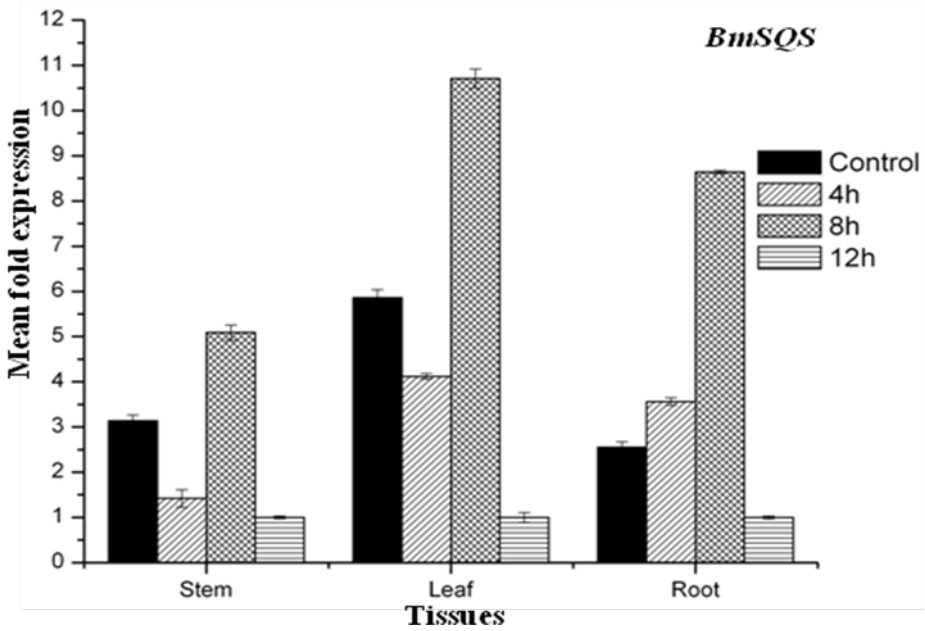


Fig. 4B.18: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene under salicylic acid (200 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.7.2.6 Yeast extract (0.1 %)

It has been reported that yeast extract in the medium resulted in an increased secondary metabolite content and alkaloids. So, it might be affecting biosynthetic pathway by altering the gene expression. As yeast extract is composed of a variety of compounds, apart from amino acids, vitamins, and minerals, it is also possible that the elicitation effects might be due to other components like metal ions, but still unclear. Here, effect of yeast extract on expression of isoprenoid biosynthetic pathway gene transcripts of *BmHMGR*, *BmFPS* and *BmSQS* in *B. monniera* was studied. It was found that *BmHMGR* transcript expression was about 2.5 fold more at 8 h incubation in roots only, whereas there is no significant effect of expression in leaf and stem tissues (**Fig. 4B.19**). The transcript accumulation of *BmFPS* was highest at 12 h incubation in case of stem and root whereas in leaf it was highest at 4 h incubation (**Fig. 4B.20**). In case of *BmSQS* expression was higher at 8 h in stem and root whereas in leaf expression was higher at 12 h incubation (**Fig. 4B.21**).

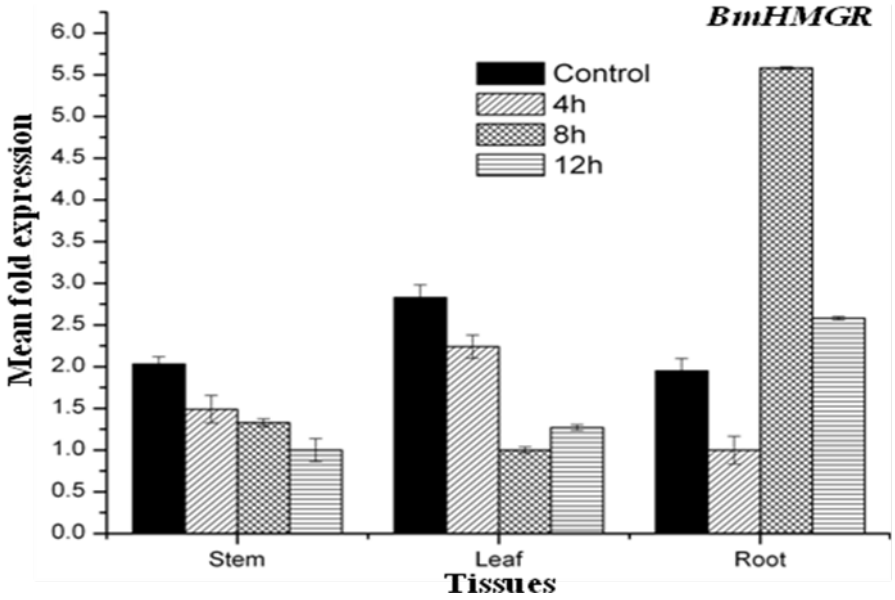


Fig. 4B.19: Relative transcript accumulation in terms of mean fold expression of *BmHMGR* gene under yeast extracts (0.1 %) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

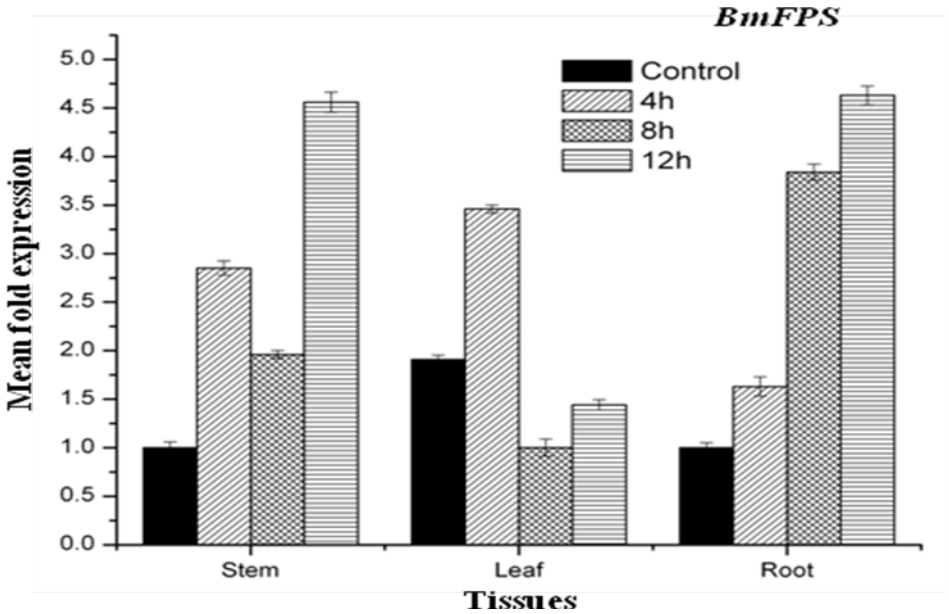


Fig. 4B.20: Relative transcript accumulation in terms of mean fold expression of *BmFPS* gene under yeast extracts (0.1 %) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

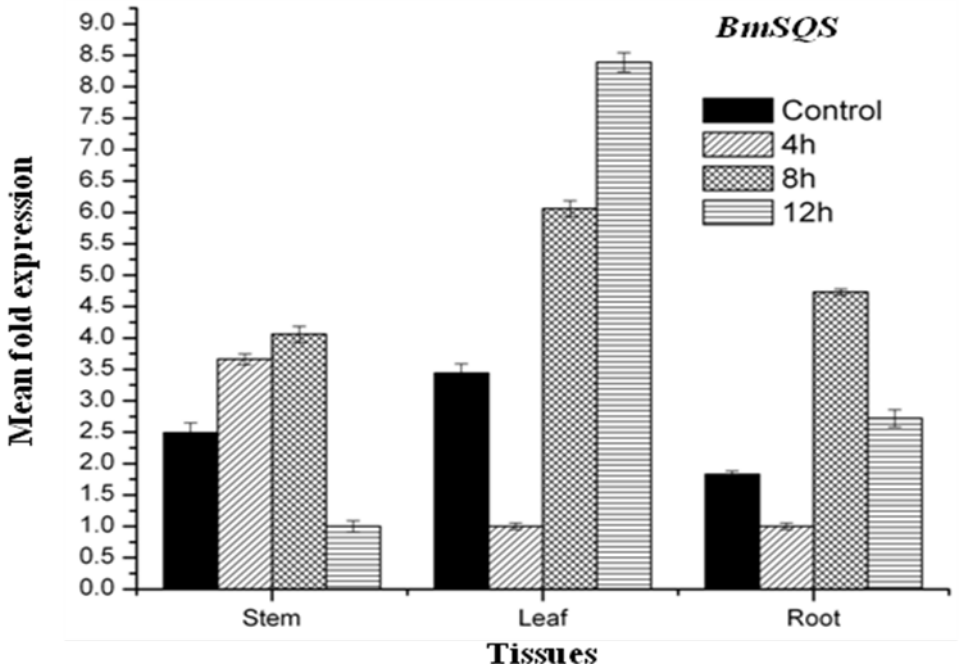


Fig. 4B.21: Relative transcript accumulation in terms of mean fold expression of *BmSQS* gene under yeast extracts (0.1 %) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.8 Conclusions

- Stem, leaf, root and floral parts including pedicel, sepal and petals were harvested and used for gene expression analysis by qRT-PCR.
- *BmHMGR*, *BmFPS* and *BmSQS* were expressed in all tissues tested, but the level of expression was varied. *BmHMGR* expressed more in root, whereas *BmFPS* and *BmSQS* showed higher expression in leaf and petal respectively.
- Root, stem and leaf tissue of control plant as well as the plants which were grown under different stresses like salt, cold, methyl jasmonate, salicylic acid and yeast extract were harvested at different time interval and used for differential gene expression analysis.
- Methyl jasmonate induces approximately 10 fold, 15 fold and 12 fold higher expression of *BmHMGR* in stem, leaf and root at 8 h, 12 h and 4 h respectively.

In case of *BmFPS* it was approximately 20 fold, 6 fold and 18 fold higher expressions in stem, leaf and root at 8 h, 8 h and 2 h respectively. *BmSQS* transcript accumulates 23 fold, 8.5 fold and 25.4 fold higher in stem, leaf and root at 2 h, 2 h and 4 h respectively. Overall expression of all three genes maintained higher as compared to control.

- Cold stress induces *BmHMGR* expression 6 fold higher in leaf 4 h as compared to control, whereas no significant pattern of expression of *BmFPS* was observed. *BmSQS* transcript accumulates 28 fold higher in root at 4 h.

- In case of chlorogenic acid induction *BmHMGR* expression was slightly higher. *BmFPS* expresses 6 fold and 13 fold higher in stem and root at 12 h, 12 h respectively. *BmSQS* transcript in root was 9 fold higher than control at 12 h.

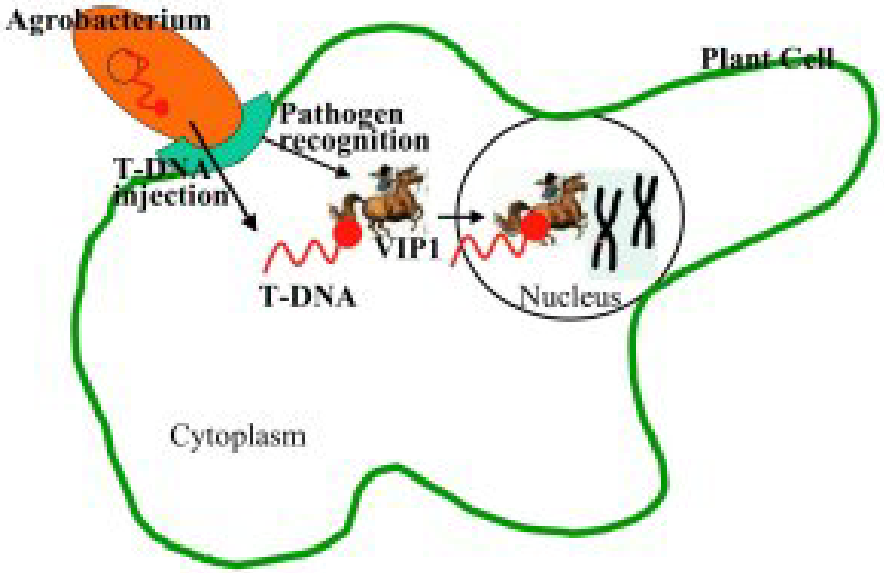
- Salt (NaCl) induces *BmHMGR* expression approximately 19 fold higher at 8 h in leaf whereas drastically decreased in root. *BmFPS* expresses approximately 2.5 fold higher in leaf and root at 4 h and 12 h respectively. A little increase in *BmSQS* expression was observed in stems and roots only at 12 h.

- Salicylic acid induces 4 fold, 5 fold and 11 fold higher expression of *BmHMGR* in stem, leaf and root respectively at 8 h. *BmFPS* and *BmSQS* showed 2- 5 fold higher expression at 8 h.

- In the presence of yeast extract *BmHMGR* showed 3 fold high expressions in root only, whereas *BmFPS* and *BmSQS* showed 1.5- 4.5 fold higher expression.

CHAPTER: 5

OVEREXPRESSION OF TERPENOID BIOSYNTHETIC PATHWAY GENE(S) IN *BACOPA* PLANT AND ANALYSIS OF TRANSGENIC PLANTS



Chapter 5. Overexpression of terpenoid biosynthetic pathway gene(s) in *Bacopa* plant and analysis of transgenic plants

This chapter describes the efficient multiple shoots regeneration and genetic transformation of *Bacopa monniera* for over-expression of HMG-CoA reductase (BmHMGR), FPP synthase (BmFPS) and squalene synthase (BmSQS). This chapter also deals with the analysis of transgenics and specific materials and methods used during the course of this chapter.

5.1 Introduction

Plants are significant source of medicinal compounds and play a key role in world human health (Constabel, 1990; Kala, 2005). In almost all regions and cultures of the world, from ancient times till today, plants have been used as medicines (Kala, 2006). Today, medicinal plants are important to the global economy, as approximately 80% of traditional medicine preparations involve the use of plants or plant extracts (Viera and Skorupa, 1993; Dhyani and Kala, 2005). The increasing demand for herbal medicines in recent years due to their fewer side effects in comparison to synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of in vitro systems for the production of medicinal plants and their extracts (Banerjee and Shrivastava, 2008).

Medicinal compounds in plants are present in very low quantity and extraction and purification of such compounds from plants are multistep process, further resulted in very low yield. Terpenoids are probably the most widespread group of natural products, used by pharmaceutical industry. So, metabolic engineering of terpenoid biosynthetic pathway in plants is a fascinating research topic today. One approach to engineer levels of terpenoids in plants is to alter the availability of precursors. Engineering the terpenoid precursor pool might be insufficient for increasing levels of target terpenoids and simultaneous engineering of downstream genes might be required (Lücker et al., 2007). This could be achieved by altering expression of multiple genes in the pathway or by the use of transcription factors that activate/repress multiple steps in a pathway (Broun, 2004; Capell and Christou, 2004). Various laboratories around the

world are involved in genetic engineering of terpenoid precursors by altering MVA pathway and MEP pathway genes. The rate limiting step in the cytosolic mevalonate pathway has been suggested to be the 3-hydroxymethyl-3-methylglutaryl-CoA reductase (HMGR) and the consequences of over expression of the corresponding gene were studied in several metabolic engineering experiments (Chappell et al., 1995; Harker et al., 2003; Schaller et al., 1995). Total sterol levels were increased three to ten times by constitutive over expression of HMGR in tobacco, but other terpenoids including sesquiterpenes were not altered (Chappell et al., 1995). Overexpression and down-regulation of 1-deoxy-D-xylulose 5-phosphate (DXP) synthase in *Arabidopsis* altered the levels of various isoprenoids, including chlorophylls, tocopherols, carotenoids, abscisic acid and gibberellins (Estevez et al., 2001). Over expression of the gene encoding deoxyxylulose reductoisomerase (DXR) catalyzing the next reaction downstream of DXP synthase in the MEP pathway resulted in a 50% increase of total yield of monoterpenes produced by peppermint (Mahmoud and Croteau, 2001).

When FPP synthase was over expressed in *Arabidopsis*, sterol levels remained unaltered, but the levels of sesquiterpenes were not analyzed (Masferrer et al., 2002). In another plant species, *Artemisia annua*, over expression of FPP synthase resulted in an increase of the antimalarial compound artemisinin (Chen et al., 2000; Han et al., 2006). Transgenic *Bupleurum falcatum* over expressing squalene synthase (BfSS1) resulted in enhanced production of phytosterol and saikosaponins in roots. It also showed increased mRNA accumulation of downstream genes such as squalene epoxidase and cycloartenol synthase but unexpectedly decreased the mRNA levels of β-amyrin synthase (Kim et al., 2011). An enhancement of phytosterol and saponin accumulation by SQS gene over expression has been reported in some species, including *P. ginseng* (Lee et al., 2004), *Eleutherococcus senticosus* (Seo et al., 2005).

Pharmaceutically important compounds from medicinal herb *B. monniera* are mainly triterpenoid saponins collectively called as bacosides, and they are present in very low amount. So, there is urgent need to be developing designer *Bacopa* plants with altered triterpenoid contents. Hence, this work was initiated with the objective of cloning and characterization of terpenoid biosynthetic pathway genes from *Bacopa* and their over expression *in-planta* including HMG-CoA reductase, FPP synthase and

squalene synthase. There is no previous report on genetic manipulation in this plant, so this study will provide insight about genetic machinery and key role of genes involved in bacoside biosynthesis.

5.2 Materials and methods

5.2.1 Materials

Antibiotics: Kanamycin, Hygromycin, Rifampicin

Bacterial strain: *E. coli* (XL10 Gold); *Agrobacterium tumefaciens* GV2260

Plasmid vectors: pGEM-T easy; pCAMBIA 1301 modified plant transformation vector (CAMBIA).

Plant material, culture media and culture conditions: Already discussed in chapter 2.

5.2.2 Methods

5.2.2.1 Cloning of *BmHMGR*, *BmFPS* and *BmSQS* in binary vector pCAMBIA 1301

All the three genes were cloned in sense orientation in modified plant transformation binary vector pCAMBIA 1301. Restriction sites (*Kpn*I in forward primer and *Sac*I in reverse primer) were introduced in genes by PCR. Full-length of the genes already cloned in pGEM-T Easy vector were used as template. The primers used for the cloning is mentioned in Table 5.1.

Table 5.1: Primers used for cloning of BmHMGR, BmFPS and BmSQS in binary vector pCAMBIA1301.

Genes	**Primers name**	**Primer sequences(5'→3')**
BmHMGR	SenseHMG F	**GGTACC**ATGGAGGCTAAGGGAC (*Kpn*I)
	SenseHMG R	**GAGCTC**TTATTGAGACGCAACC (*Sac*I)
BmFPS	SenseFPS F	**GGTACC**ATGGCGAATCATAATGGA
	SenseFPS R	**GAGCTC**TTACTTCTGCCTCTTG
BmSQS	SenseSQS F	**GGTACC**ATGGGTAGTT TGCGTG
	SenseSQS R	**GAGCTC**TCACATGTGAGTGGGC

Amplified product with restriction sites were gel purified and ligated in to pGEM-T Easy vector. After transformation in *E. coli* XL10, plasmids were isolated from positive clones. Further clones were confirmed by restriction digestion and sequencing. Inserts released by double digestion from pGEM-T Easy clones were ligated into binary vector pCAMBIA 1301 (digested with same restriction enzymes) and transformed into *E. coli*. Plasmids were isolated and transformed in *Agrobacterium tumefaciens* for plant transformation.

5.2.2.2 *A. tumefaciens* transformation and selection

A single colony of *A. tumefaciens* (GV2260) was inoculated in 50 mL YEB broth containing Rifampicin 150 μg/mL and incubated at 28ºC with shaking at 200 rpm till A_{600} was 0.5-0.7. Cells were centrifuged at 5000 g for 10 min at 4 ºC and washed twice with ice cold 150 mM $CaCl_2$. The cells were pelleted and resuspended in 1 mL of ice cold 20 mM $CaCl_2$. Aliquots of 200 μL were made.

For transformation, 1 μg DNA of the desired binary plasmid vector was added to an aliquot of the competent *A. tumefaciens* cells and incubated on ice for 30 min. The cells were then snap frozen in liquid nitrogen and allowed to thaw at 37 ºC. After thawing, 1 mL LB medium was added and the tubes incubated at 28 ºC for 2 h with gentle shaking. The cells were centrifuged at 4,000 g for 5 min and 100 μL supernatant was retained. The cells were resuspended in the 100 μL supernatant and plated on LB agar medium with appropriate antibiotic(s). The plates were incubated at 28 ^{o}C for 2 days in dark to allow the transformed colonies to grow for further processing.

5.2.2.3 Multiple shoots regeneration, histological studies and determination of LD50 of hygromycin B for *B. monniera*

The methods of multiple shoots regeneration and histological studies have already been described in chapter 2. Hygromycin sensitivity test (LD50) for *Bacopa* was carried out. Leaf explants were inoculated on shoot regeneration medium containing different concentrations of hygromycin (5, 10, 15 and 20 mg/L). The plants were left for one month under 16 h photoperiod, 60 μmol m-^{2} s^{-1} light intensity and 25 ±1 ºC.

5.2.2.4 *Agrobacterium* mediated *Bacopa* plant transformation and selection

As described in Chapter 2.

5.2.2.5 DNA extraction from transgenic events and PCR with *hpt*II primers

DNA was isolated from leaf tissues by using DNA extraction kit (Himedia, India) as per manual instructions. Detailed methods are described in chapter 2.

Primers used for PCR

HygBF 5'-GTCGACCTATTTCTTTGCCCTCGGAC-3'

HygBR 5'-GGATCCCCTGACCTATTGCATCTCCC-3' For Primary PCR

HygB NF 5'-TCCTGCAAGCTCCGGATGCCTC-3'
HygB NR 5'-CGTGCACAGGGTGTCACGTTGC-3, Used for nested PCR

5.2.2.6 Histochemical GUS assay

As described in Chapter 2.

5.2.2.7 RNA isolation, cDNA synthesis and qRT-PCR analysis

As mentioned in Chapter 2.

Table 5.2: Primers used for differential expression studies of genes in transgenic lines of *Bacopa* plant by qRT-PCR.

Genes	Primers name	Primers sequence (5'→3')
BmHMGR	qHMG F	TGGGTGGTTTCAACGCTCAC
	qHMG R	CATTGACGGCTTCCATCATTGTG
BmFPS	qFPS F	TGTCCTTAAATCTGAGCTGTTGAAC
	qFPS R	TGACAGACCTCGGTTTAACTTCC
BmSQS	qSQS F	TTGCGAGATCCAGCGATCTTTCGT
	qSQS R	TAGCTGTGAGCCCACGTCTCATTT
*BmBAS**	qBAS F	GCATGTGGAATGCACTGCTTCTGT
	qBAS R	TGCCTTCGCCACGGAGATTTCTAT
5.8 S rRNA	CT F	CTA AACGACTCTCGGCAAC
	CT R	TTCAAAGACTCGATGGTTCAC

***BmBAS**- β-amyrin synthase from *B. monniera*.

5.2.2.8 ELISA (Enzyme-Linked Immunosorbent Assay)

As mentioned in Chapter 2. Total soluble proteins were extracted from leaf tissues in bicarbonate buffer (0.2 M sodium carbonate/bicarbonate, pH 9.6) and equal quantity was coated on 96 well microtitre plates, and remaining procedure followed as in chapter 2.

5.2.2.9 Bacosides content analysis (HPLC)

Bacosides content of transgenic events were analyzed by HPLC as mentioned in Chapter 2. In brief, plant samples were extracted in methanol, concentrated and vacuum dried. Dried extracts were dissolved in DMSO and used for HPLC analysis. The column used was analytical C18 (5 um, 15 cm x 4.6 mm) and detection was done at 205 nm. Mobile phase consisted of sterile milliQ water with 0.1% TFA and was programmed for 40 min as, flow rate 1mL/min, 10% acetonitrile for 5 min, 30% acetonitrile for 15 min and 35% acetonitrile for 20 min.

5.3 Results and discussion

5.3.1 Cloning of BmHMGR, BmFPS and BmSQS in binary vector pCAMBIA 1301

The full-length sequence of all three genes was separately cloned in sense orientation in binary vector pCAMBIA 1301 with restriction sites *Kpn*I and *Sac*I. First they were transformed in *E. coli*. Then, plasmids were isolated from positive clones and insert release was confirmed by restriction digestion (**Figs. 5.1 A, B & C**) and the clones in pCAMBIA 1301 were designated as pCAM-BmHMGR, pCAM-BmFPS and pCAM-BmSQS. Whole pCAMBIA constructs with target gene in sense orientation under the control of constitutive CaMV35S promoter were used for transformation (**Fig. 5.2**). Competent cells of *A. tumefaciens* (GV2260) were prepared and transformed with 1 µg of plasmid DNA construct. *Agrobacterium* harboring expression cassette was further used for co-cultivation with *Bacopa* explants.

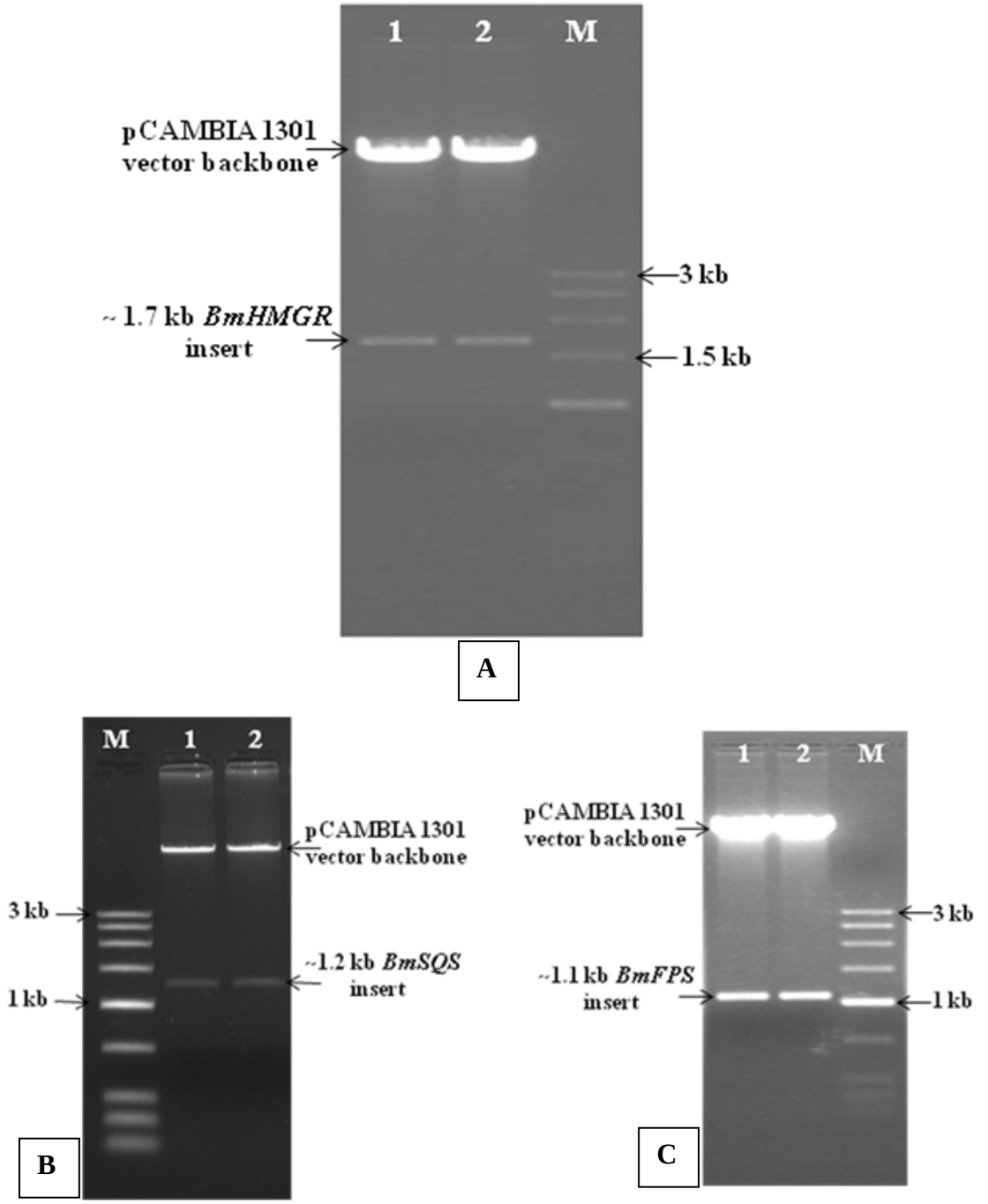


Fig. 5.1: Restriction analysis of recombinant pCAMBIA1301 with *Kpn*I and *Sac*I **A.** lane 1 & 2 recombinant pCAM-BmHMGR releasing ~1.7 kb *BmHMGR* gene and vector backbone, lane M- Low range DNA ruler **B.** lane 1 & 2 recombinant pCAM-BmSQS releasing ~ 1.2 kb *BmSQS* insert and vector backbone. Lane, M- Low range DNA ruler. **C.** lane 1 & 2 recombinant pCAM-BmFPS releasing ~ 1.1 kb *BmFPS* insert and vector backbone. Lane, M- Low range DNA ruler.

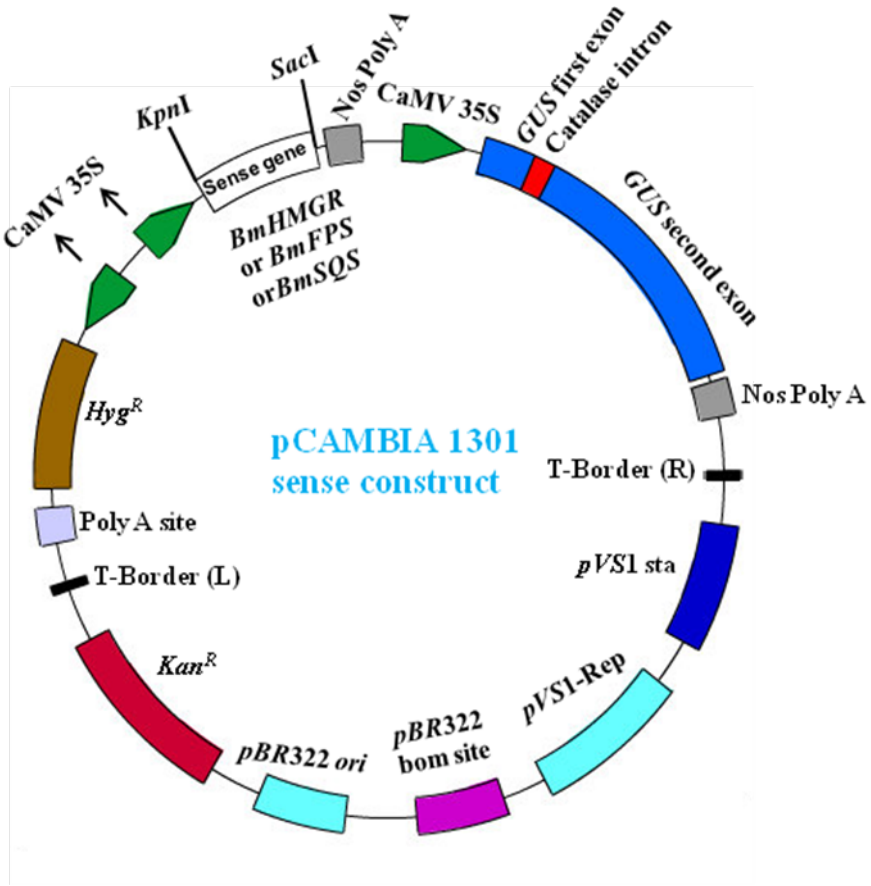


Fig. 5.2: Map of Modified pCAMBIA 1301 vector construct with target gene

5.3.2 Multiple shoots regeneration

The axillary buds of nodal explants incubated on the initiation medium showed shoot formation after 5-6 days of incubation. About 3-4 shoots were formed from each of these explants. These cultures were maintained by regular subculture on proliferation medium (PM).

The leaf explants, obtained from the above mentioned subcultured plants, were inoculated on MS basal medium with various concentrations of 6-benzylaminopurine **(**BAP) and Indole-3-acetic acid (IAA), **s**howed proliferation of multiple shoots after about two weeks of incubation arising especially from the cut regions of leaf lamina (**Fig. 5.3 G**). Excised leaves of *B. monniera* with 2-3 longitudinal cuts on the lamina showed very high frequency induction of shoots as compared to uninjured leaf (control) which showed only shoot formation at petiole (**Fig. 5.3 A,B, F**). After 20 days, multiple shoots produced from leaf surface were transferred to PM which showed root formation within 10 days (**Fig. 5.3 I, J**). **Table 5.3** gives the detailed statistical analysis of the

shoots regeneration frequency per explants incubated on various combinations of these growth regulators. Out of these various combinations, MS basal medium with 2 mg/L BAP and 0.2 mg/L IAA were found to yield maximum frequency of shoot regeneration (98.33%). This medium composition was further used for all the transformation experiments. This result was also found to be consistent with the earlier reports on shoot induction in *B. monniera* where the leaf explants was more responsive than other explants (Shrivastava and Rajani, 1999; Tiwari et al., 1998; Tiwari et al., 2000; Tiwari et al., 2001)

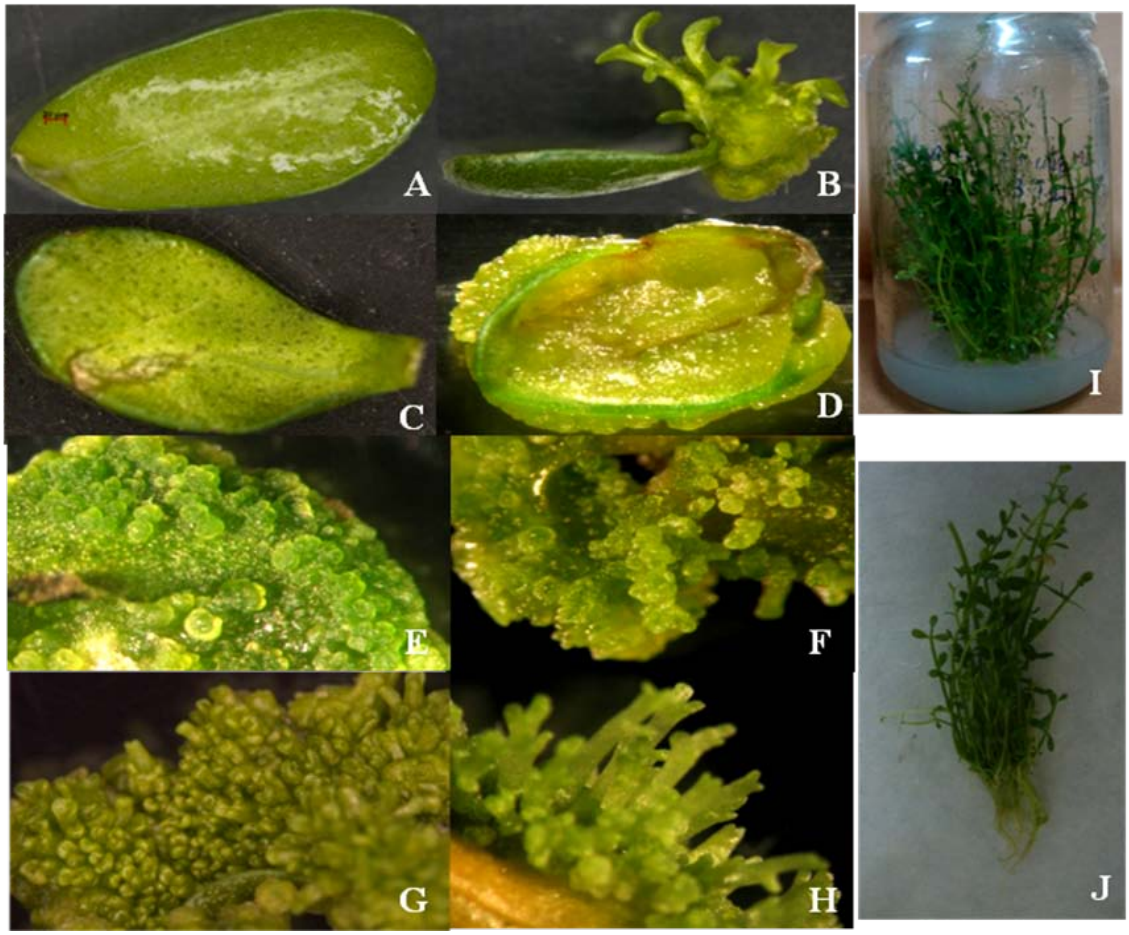


Fig. 5.3: Multiple shoot regeneration on SRM from leaf explants of *B. monniera* **A.** control leaf (without injury). **B.** Control leaf shows shoot formation at petiole. **C.** Leaf with longitudinal cut on the lamina. **D.** Leaf explant after 6 days of incubation. **E.** Leaf explant after 9 days of incubation. **F.** Leaf explant after 12 days of incubation. **G.** Leaf explant after 15 days of incubation, showing multiple shoot formation on surface of leaf. **H.** Leaf explants after 3 weeks incubation. **I, J.** Multiple shoot showed root formation on PM medium.

Table 5.3: Effect of BAP and IAA on shoot formation from leaf explants of *B. monniera*.

Growth regulator (mg/L)		**Shoots regeneration frequency (%)***
BAP	IAA	
1	0.0	6.66 ± 2.8
1	0.05	17.66 ± 2.8
1	0.10	32.67 ± 2.5
1	0.15	45.66 ± 6.0
1	0.20	90.33 ± 0.5
2	0.0	7.67 ± 2.5
2	0.05	20 ± 5
2	0.10	51.66 ± 2.8
2	0.15	71.67± 2.8
2	0.20	98.33 ± 0.5

*Leaf explants were cultured on shoot regeneration medium (SRM) for 2 weeks and then transferred to proliferation medium (PM). Shoot regeneration frequency (%) was recorded after 1 week on PM. Values is the mean ± SD of three experiments.

5.3.3 Histology

Figure 5.4 shows the histology of the leaves from 0 – 15 days of incubation on SRM. Sections of 15 μm thickness (Leica RM 2155 microtome) were cut and stained with 1% w/v safranin and 1% w/v light green prior to microscopic observation. Observations were taken under Axioplan microscope (Carl Zeiss). The regenerating leaf explants were observed under APO 11 microscopes (Carl Zeiss). The digital photomicrography was done using Axiocam MRC5 system for both the microscopes. The emergence of shoot buds from leaf explants was observed on the 6^{th} day of incubation on regeneration medium (**Fig. 5.4**).

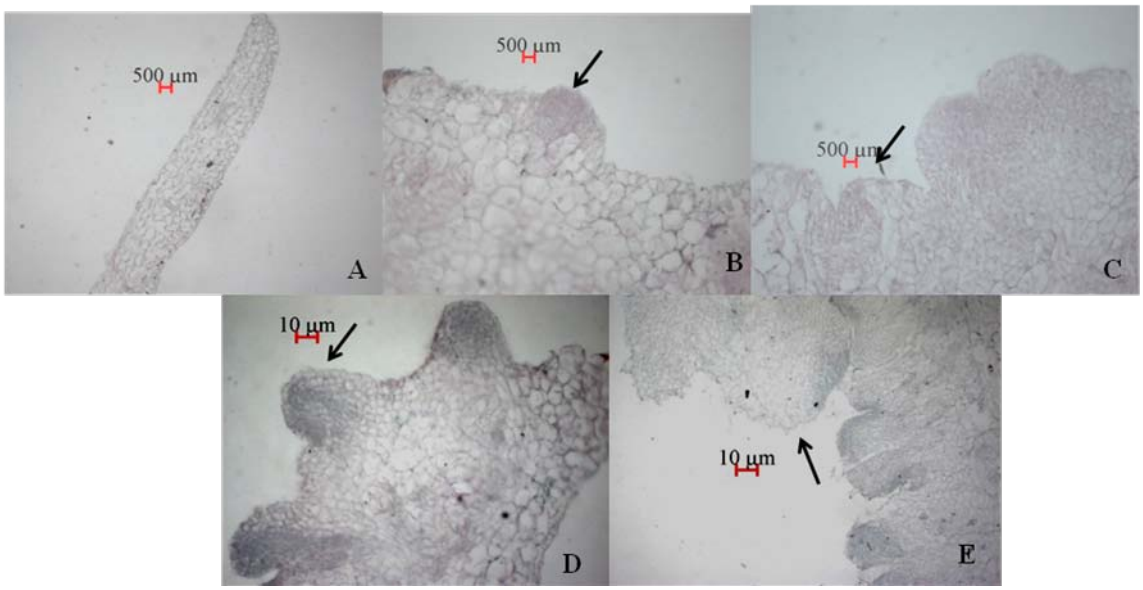


Fig. 5.4: Histological section of leaf explants showing multiple shoot regeneration in various stages of development on SRM. **A.** Control. **B.** After 6 days of incubation. **C.** After 9 days of incubation. **D.** After 12 days of incubation. **E.** After 15 days of incubation.

5.3.4 Transformation of *Bacopa* with sense construct of *BmHMGR*, *BmFPS* and *BmSQS* and selection of transgenic plants

The leaf explants were co-cultivated with *A. tumefaciens* strain GV2260 harboring binary vector pCAMBIA1301 with respective gene construct. After 15 days, the leaf explants showed direct shoot development without an intervening callus phase on SRM selection medium (MS basal medium containing 2 mg/L BAP, 0.2 mg/L IAA, 10 mg/L hygromycin B and 200 mg/L cefotaxime) (**Fig. 5.5 A**). The hygromycin resistant shoots were transferred to fresh SRM selection medium and allowed for further proliferation (**Fig. 5.5 B**). After 15 days, these proliferated shoots were again transferred to PM for rooting. After 10 days, the rooted transformed plants were shifted to the plastic pots containing sterile soil, hardened for a week and then transferred to green house for further acclimatization (**Fig. 5.5 C, D**). Approximately 75% of transferred plants survived in green house. Leaf samples from these plants were used for subsequent transgenic analysis.

The successful genetic transformation of *B. monniera* mediated by *A. tumefaciens* using the *hpt*II gene as a selectable marker was done. A_{600} of 0.5-1 of *A. tumefaciens* harboring gene construct binary vector pCAMBIA 1301 with co-

cultivation time of 15 min was found to be most suitable method for transformation using leaf explants. There are few previous reports of successful *Agrobacterium* mediated transformation of *B. monniera* (Nisha et al., 2003; Ramesh et al., 2011). (Nisha et al., 2003) observed 60% transformation efficiencies using callus while it was found to be 68.8% by (Ramesh et al., 2011) where nodal explants was used for transformation. Mahender et al. (2012) reported 70.6% transformation efficiency by using leaf explants. In this study, we have achieved stable transformation efficiency of 80- 81.48% accounted on basis of *hpt*II gene specific PCR. This reported final transformation efficiency was higher than other medicinal plants; for example, transformation efficiency of 3% was observed in the medicinal plant *Taraxacum platycarpum* using pCAMBIA and pBI121 vector (Tae et al., 2005). (Nanditha et al., 2008) observed an efficiency of 33.33% for *Centella asiatica* using pCAMBIA2301 whereas (Franklin et al., 2007) obtained a transformation frequency of 55% for the *Hypericum perforatum.* Only in few medicinal plants, transformation frequency higher than 78% has been reported. (Khawar et al., 2003) reported 100% transformation efficiency for *Salvia sclarea* and using *Agrobacterium* strain A281 harboring pTiBo542 and pBI121.

Fig. 5.5: Transgenic *B. monniera* plants obtained from *Agrobacterium* mediated transformation. **A.** Hygromycin resistant shoots obtained after 15 days of transformation. **B.** Transgenic plants on selection medium. **C.** Transgenic plants in pot. **D.** Transgenic plants in the green house.

5.3.5 Analysis of transformants

Primary screening for putative all three transformant lines of *Bacopa* over-expressing *BmFPS*, *BmHMGR* and *BmSQS* was done for integration of Hygromycin phosphotransferase (*hpt*II) and target gene by following methods.

- Histochemical GUS assay.
- PCR based screening using Hygromycin phosphotransferase (*hpt*II) gene specific primers.
- Quantitative real-time PCR, and
- ELISA of extracted protein from *BmFPS* and *BmSQS* transgenic lines of *Bacopa*.
- HPLC analysis of BmFPS-transgenic lines.

5.3.5.1 Histochemical GUS Assay

In this experiment the bacterial *gus* gene was used as a reporter gene. Transient expression of *gus* gene was histochemically visualized according to Jefferson *et al*. (1987), that resulted in a uniform and intense blue staining in lamina of the leaf and no GUS activity was visualized in control plant leaf (**Fig. 5.6**). We analyzed total 27, 15 and 21 putative transformants of *B. monniera* over-expressing *BmFPS*, *BmHMGR* and *BmSQS* respectively. The percentages of GUS positives were 60 - 71.42 as shown in **Table 5.4**.

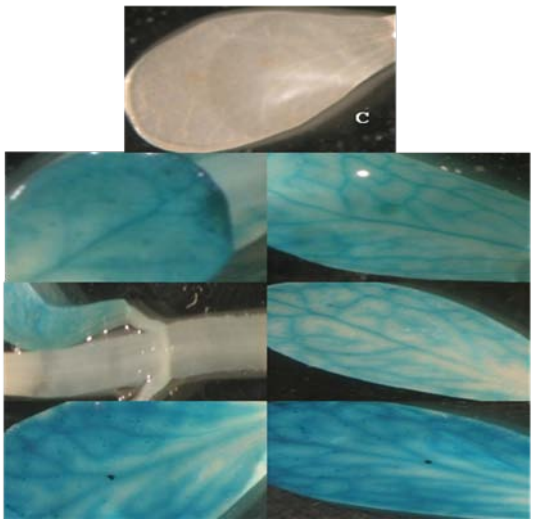


Fig. 5.6: Histochemical GUS assay of *B. monniera*. Leaves of transformed *B. monniera* with sense construct of *BmHMGR*, *BmFPS* and *BmSQS* individually showing varying degrees of expression of *gus* gene along with control leaf (c).

5.3.5.2 Molecular analysis (PCR with *hpt*II primers)

For confirmation of transformation, molecular analysis of transformants was done by PCR which confirmed the incorporation of T-DNA into the plants. Genomic DNA was extracted from putative transformants and PCR was performed using *hpt*II gene specific primers. Total 27 BmFPS, 15 BmHMGR and 21 BmSQS transformants of *B. monniera* were screened for the presence of *hpt*II gene. The PCR amplification of DNA extracted from leaves of putative transformants plants using *hpt*II specific primers (HygB F and Hyb R) was done. Samples showing amplification of approximately 900 bp were further confirmed by nested PCR with primers (HygB NF and HygB NR) and primary PCR product as a template, showed ~500 bp band while control plant did not show any amplification (**Figs. 5.7, 5.8 , 5.9**). Amplification due to bacterial contamination was checked by PCR with 16S rRNA primers, no amplification was observed in plant samples. Overall transformation efficiencies for *Agrobacterium* mediated transformation for all three genes was 80 -81.48% accounted on the basis of *hpt*II gene specific PCR (**Table 5.4**). Some samples of *hpt*II PCR products were also subjected to sequencing for confirmation.

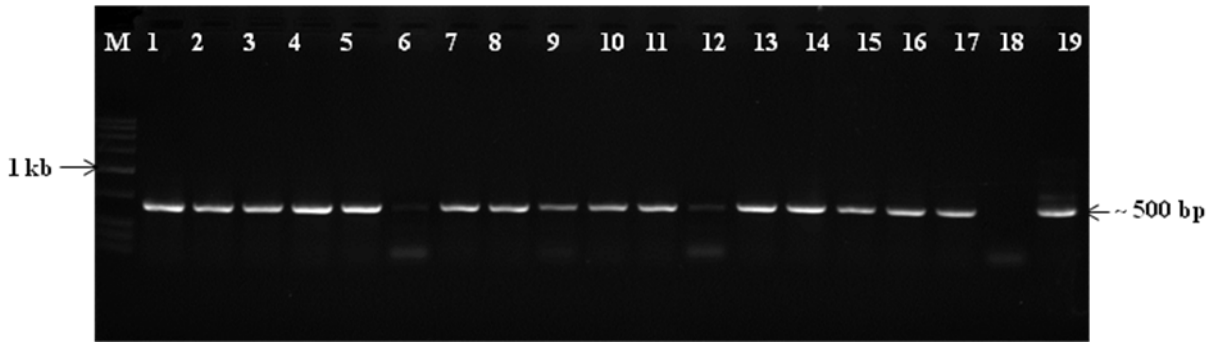


Fig. 5.7: PCR analysis of putative transgenic lines containing sense **BmFPS** construct using *hpt*II gene specific primers and genomic DNA as a template. Lane M, low range DNA ruler; Lane 1-17, putative transformed plants; Lane 18, untransformed plant (negative control); Lane 19, amplification of plasmid pCAMBIA1301 (positive control).

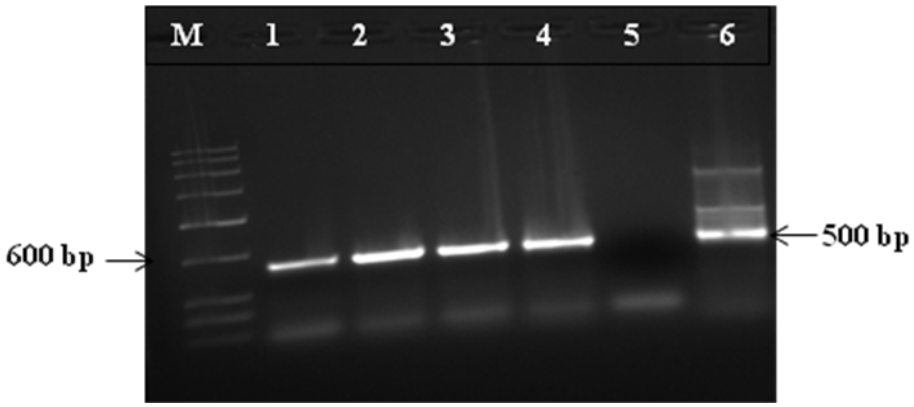


Fig. 5.8: PCR analyses of putative transgenic lines containing sense BmHMGR construct using *hpt*II gene specific primers and genomic DNA as a template. Lane M, low range DNA ruler; Lane 1-4, putative transformed plants; Lane 5, untransformed plant (negative control); Lane 6, amplification of plasmid pCAMBIA1301 (positive control).

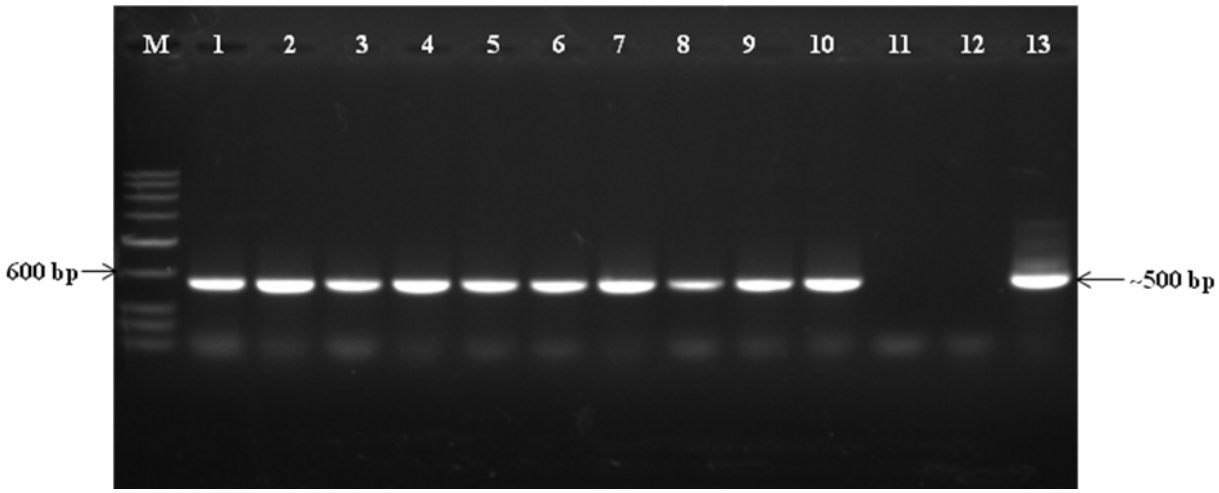


Fig. 5.9: PCR analysis of putative transgenic lines containing sense **BmSQS** construct using *hpt*II gene specific primers and genomic DNA as a template. Lane M, low range DNA ruler; Lane 1-11, putative transformed plants; Lane 12, untransformed plant (negative control); Lane 13, amplification of plasmid pCAMBIA1301 (positive control).

Table 5.4: Transformation efficiencies of *B. monniera* with *A. tumefaciens* GV2260 using different gene constructs (pCAM-BmHMGR, pCAM-BmFPS and pCAM-BmSQS).

Transgenic events	No. of transgenic plants analyzed	No. of transgenic plants *hpt*II gene specific PCR positive	No. of transgenic plants GUS assay positive	Transformation efficiency on the basis of *hpt*II gene specific PCR (%)	GUS activity (%)
BmFPS	27	22	18	81.48	66.66
BmHMGR	15	12	9	80.00	60.00
BmSQS	21	17	15	80.95	71.42

5.3.5.3 qRT-PCR analysis

Total RNA from leaf tissues were isolated from different transgenic events and cDNA was prepared. qRT-PCR was done using cDNA as template and for detection SYBR green chemistry was used. All reactions were normalized by using 5.8S rRNA expression. The data was analyzed by comparative Ct method ($2^{-\Delta\Delta Ct}$) in terms of mean fold expression. Reactions were carried out in triplicates and repeated twice. Four transgenic events of BmHMGR were analyzed, which showed 2 -4 fold higher transcript accumulation as compared to control (**Fig. 5.10**).

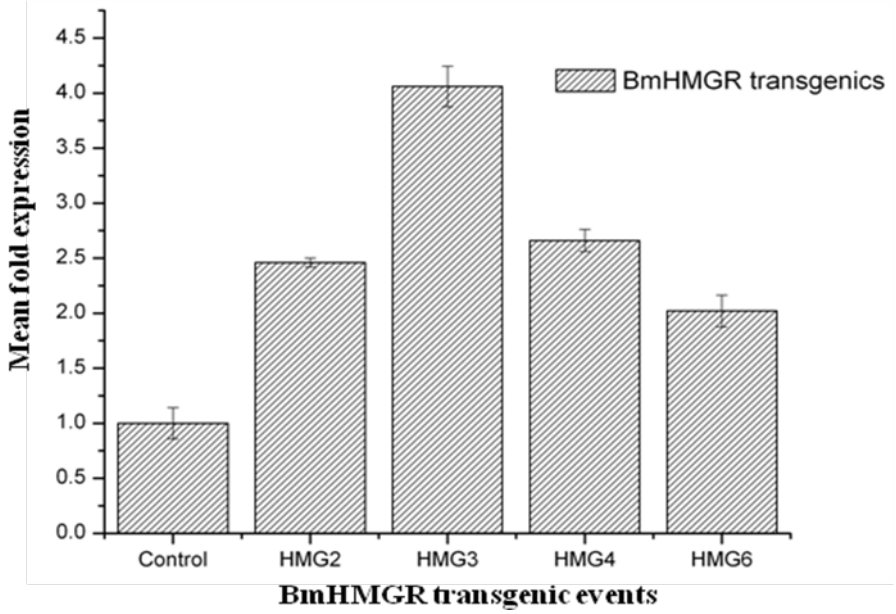


Fig. 5.10: Transcript expression analysis of transgenic events over-expressing *BmHMGR* by qRT-PCR. Standard errors of mean (±SEM) were given for triplicate analysis.

In case of *BmFPS* transgenic events of *B. monniera*, five plants were analyzed. They showed 3.7-5.43 fold higher mRNA expression as compared to control (**Fig. 5.11**). Whereas in BmSQS transgenic events transcript accumulation was 1.4- 4.08 fold higher (six plants) as compared to control (**Fig. 5.12**).

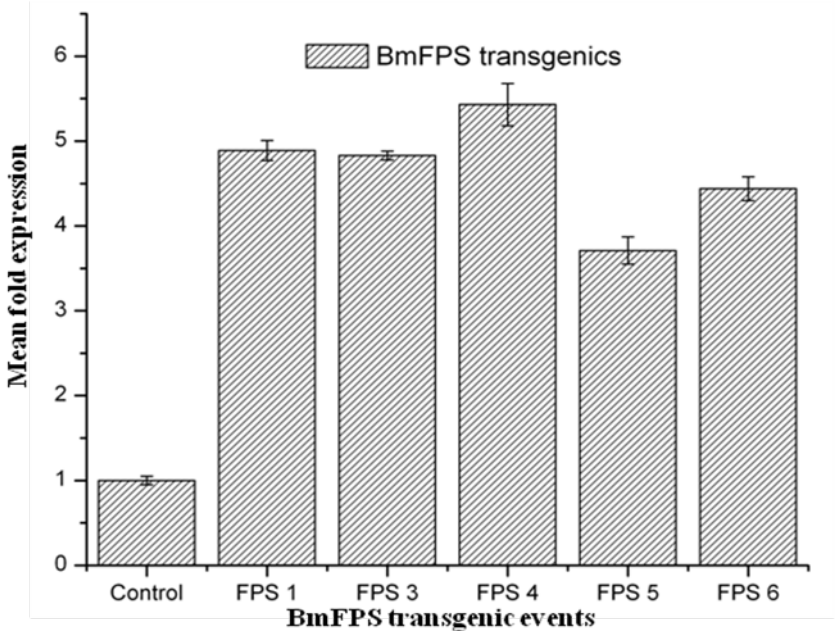


Fig. 5.11: Transcript expression analysis of transgenic events over-expressing *BmFPS* by qRT-PCR. Standard errors of mean (±SEM) were given for triplicate analysis.

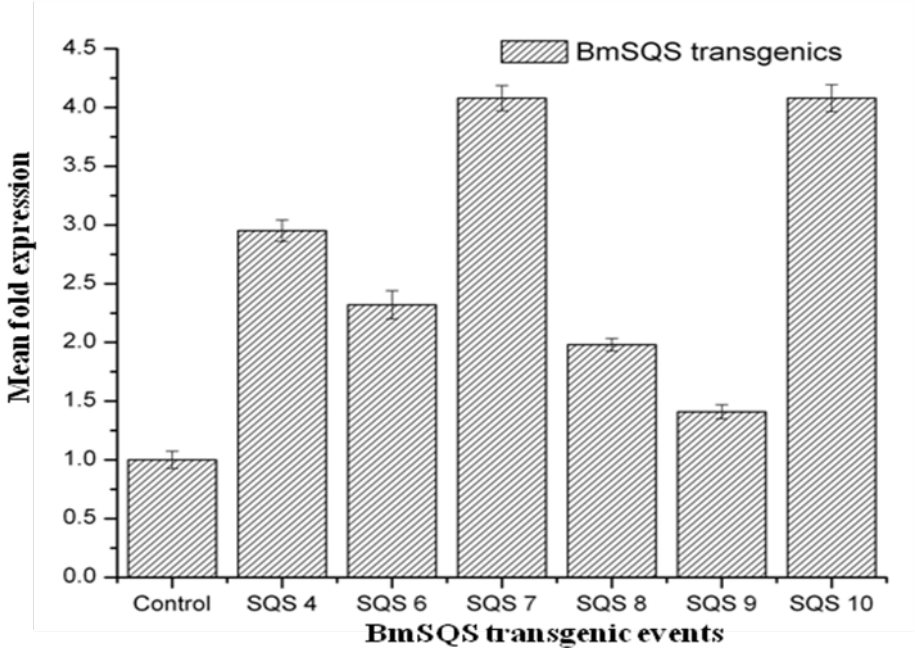


Fig. 5.12: Transcript expression analysis of transgenic events over-expressing *BmSQS* by qRT-PCR. Standard errors of mean (±SEM) were given for triplicate analysis.

5.3.5.4 Transcript analysis of other terpenoid biosynthetic pathway genes in transgenic events (*BmHMGR*, *BmFPS* and *BmSQS*) by qRT-PCR

Quantitative real-time PCR was used to estimate relative steady-state transcript levels of isoprenoid related genes in leaves of the transgenic plants compared to non transgenic controls. HMG-transgenic plants exhibited increased transcript accumulation of FPS, SQS and BAS. It showed FPS transcript accumulation up to 39 fold higher than control, whereas SQS and BAS transcripts were increased up to 92 and 16 fold higher respectively as compared to control (**Fig. 5.13**). 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 (**Fig. 5.14**). SQS-transgenic showed very high transcript accumulation (up to 94 fold) of BAS, whereas FPS and HMGR transcripts are slightly higher than control (**Fig. 5.15**). These data suggests that over-expression of terpenoid biosynthetic pathway genes induce the other genes of the pathway with high effect on genes appeared downstream to gene over-expressed in transgenic plants. In recent study, it has been reported that, potato plant over-expressing HMG-CoA reductase and squalene synthase induced steroidal glycoalkaloid biosynthetic pathway genes. HMGR-transgenic plants exhibited increased transcript accumulation of SQS1, sterol C24-methyltransferase type1 (SMT1), and solanidine glycosyltransferase 2 (SGT2), whereas SQS-transgenic plants, had consistently lower transcript levels of HMGR1 and variable SMT1 and SGT2 transcript abundance among transgenics (Ginzberg et al., 2011) .

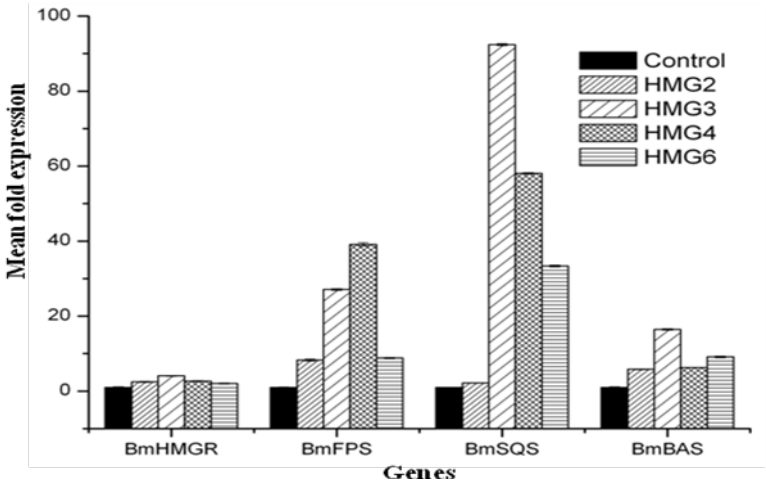


Fig. 5.13: Transcript levels of terpenoid biosynthetic pathway genes (*BmFPS*, *BmSQS* and *BmBAS*) in HMG-transgenic *Bacopa* plants as compared to control non transgenic plants.

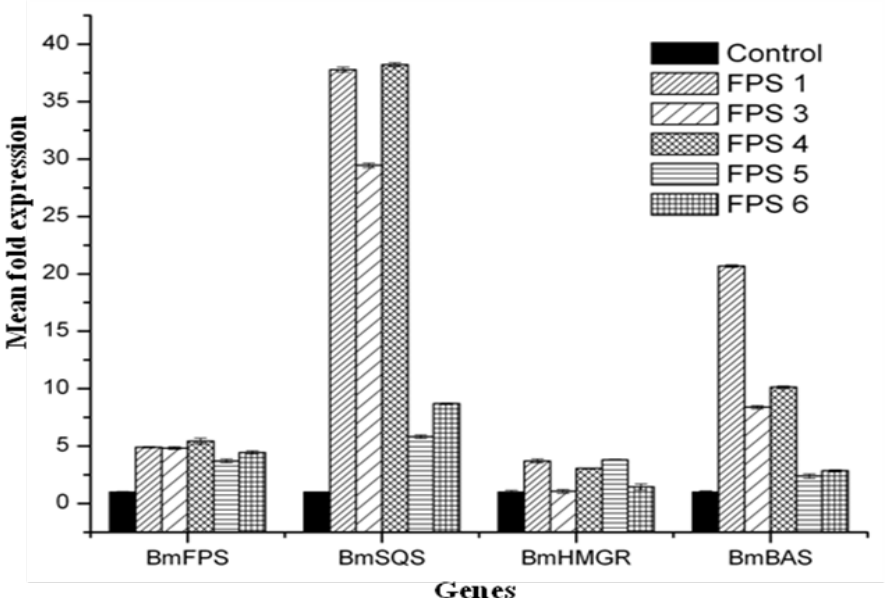


Fig. 5.14: Transcript levels of terpenoid biosynthetic pathway genes (*BmSQS*, *BmHMGR*, and *BmBAS*) in FPS-transgenic *Bacopa* plants as compared to control non transgenic plants.

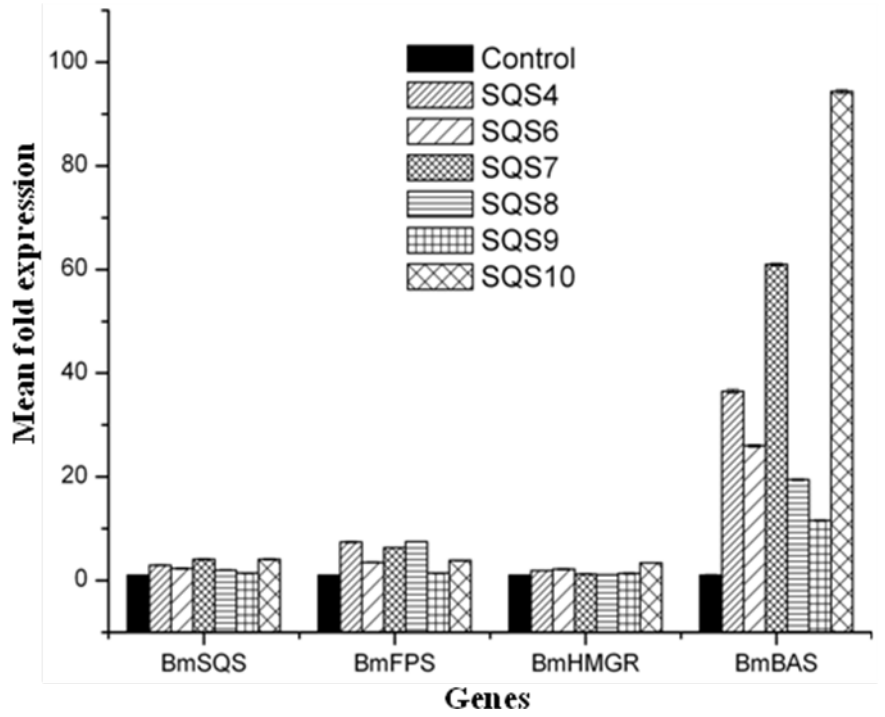


Fig. 5.15: Transcript levels of terpenoid biosynthetic pathway genes (*BmFPS*, *BmHMGR*, and *BmBAS*) in SQS-transgenic *Bacopa* plants as compared to control non transgenic plants.

5.3.5.5 ELISA analysis of *BmFPS* and *BmSQS* transgenic events

Total soluble protein from five BmFPS and six BmSQS transgenic plants were tested for amount of respective protein by ELISA. Total of 25 µg protein was coated in each well of microtitre plate in triplicates. Non-transformed *Bacopa* was used as a control. ELISA was done for putative transformants that gave good result in PCR. Primary antibodies and secondary antibodies were used at a dilution of 1:5000 and 1:20000 respectively. More or less elevated expressions of proteins were observed in transgenic plants as compared to control (**Figs. 5.16**, **5.17**).

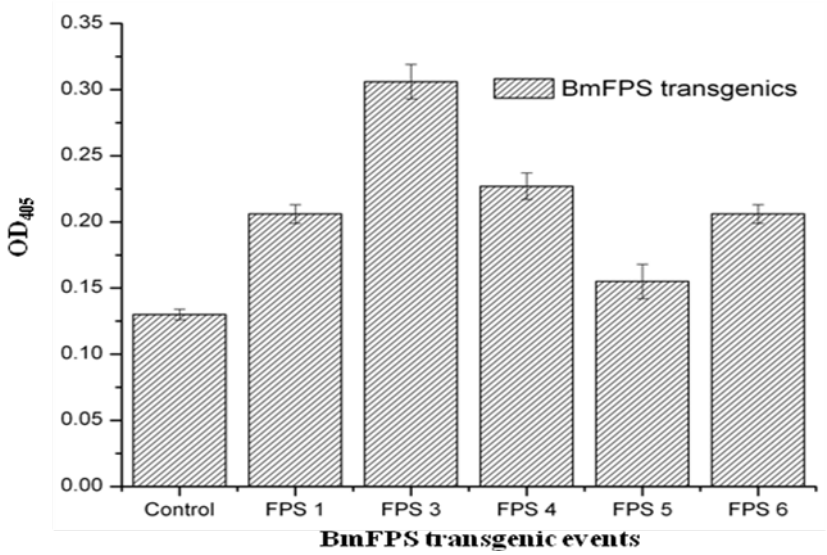


Fig. 5.16: ELISA of extractable FPS protein from transgenic lines of *Bacopa.*

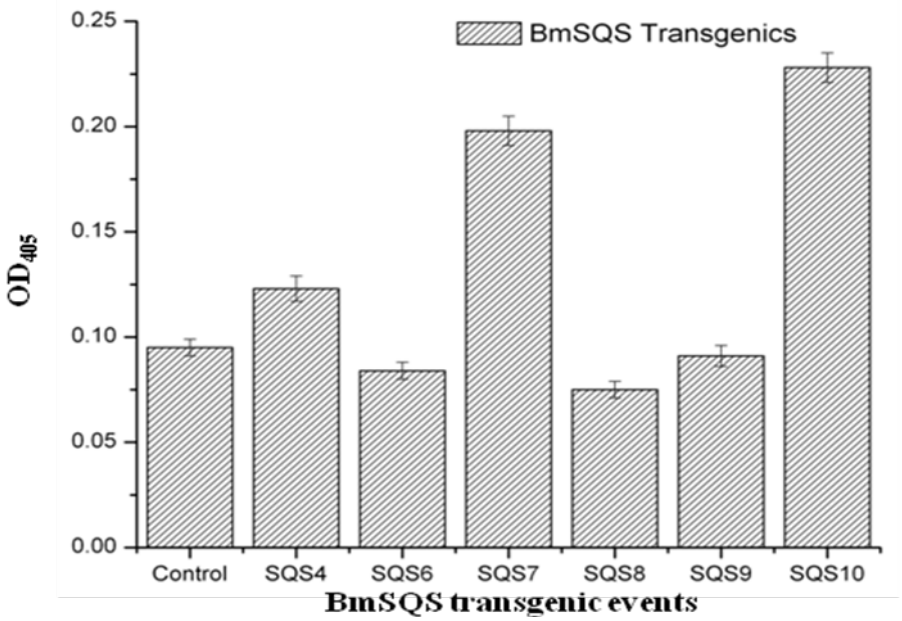


Fig. 5.17: ELISA of extractable SQS protein from transgenic lines of *Bacopa.*

5.3.5.6 HPLC analysis of BmFPS-transgenic lines (Bacoside content analysis)

Our main target was to generate the transgenic lines of *Bacopa* with improved bacosides content. Transgenic lines of *Bacopa* plants overexpressing BmFPS along with nontransformed control plant were subjected to reverse phase HPLC analysis. Here we report bacoside analysis only in BmFPS-transgenic lines, because BmHMGR and BmSQS-transgenic plants growing in green house are small and for HPLC analysis we need to have relatively large plant sample quantity for extraction. Standards (Bacopasaponin C and Bacoside A) were procured from ChromaDex and subjected to HPLC run and detected at 205 nm (**Fig. 5.18**).

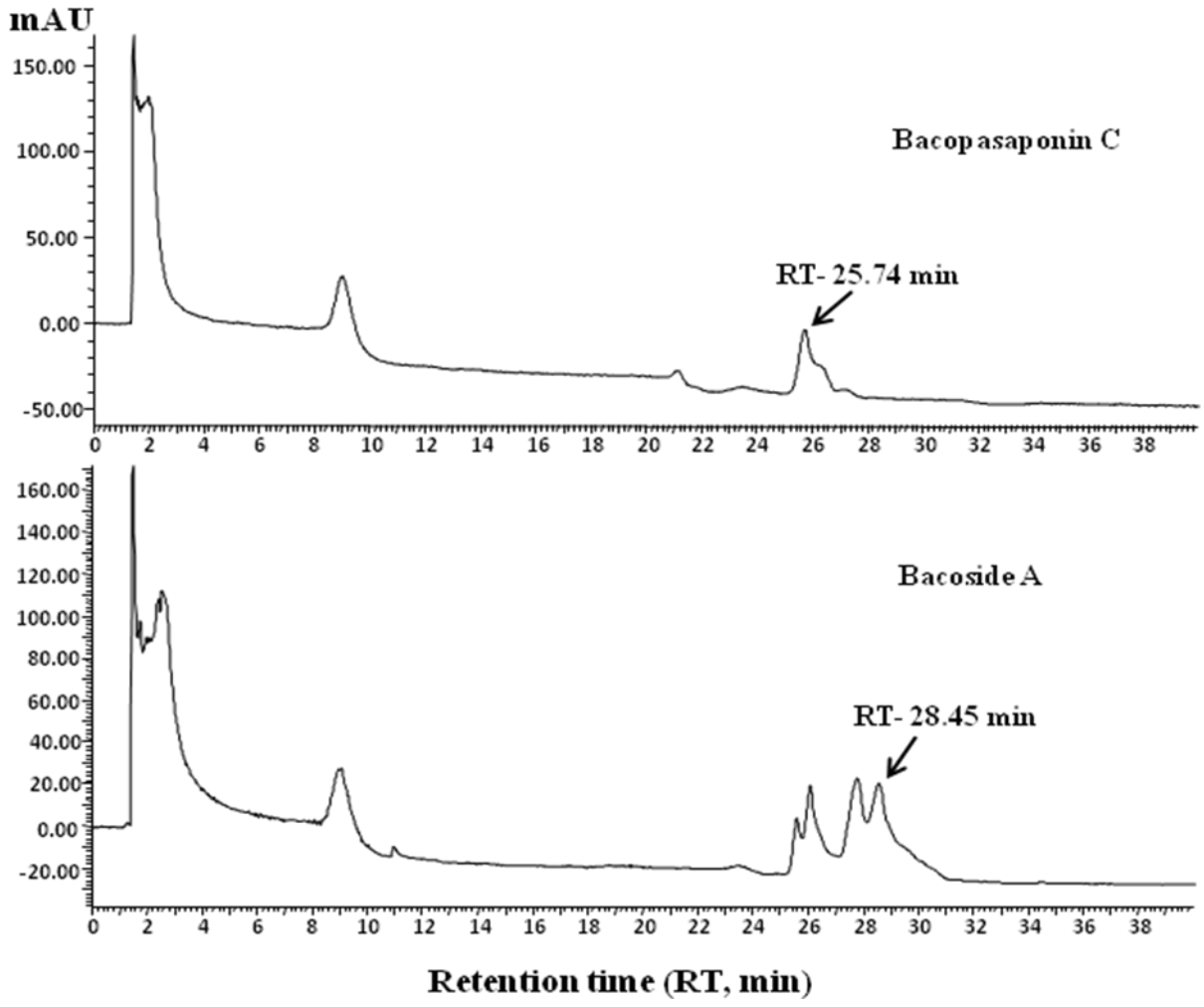


Fig. 5.18: HPLC chromatograms ($A_{205\ nm}$) of standards bacosides. Retention time (RT) of bacopasaponin C is 25.74 min. Bacoside A standard was not pure and 4 peaks were detected at RT 25.57, 26.06, 27.73 and 28.45 mins.

On the basis of standard, two compounds were analyzed in transgenic lines and relative abundance was recorded as compared to control by considering area covered under peaks. Four BmFPS- transgenic lines were analyzed and chromatogram of one plant (FPS 4) along with control is shown in figure (**Fig. 5.19**)

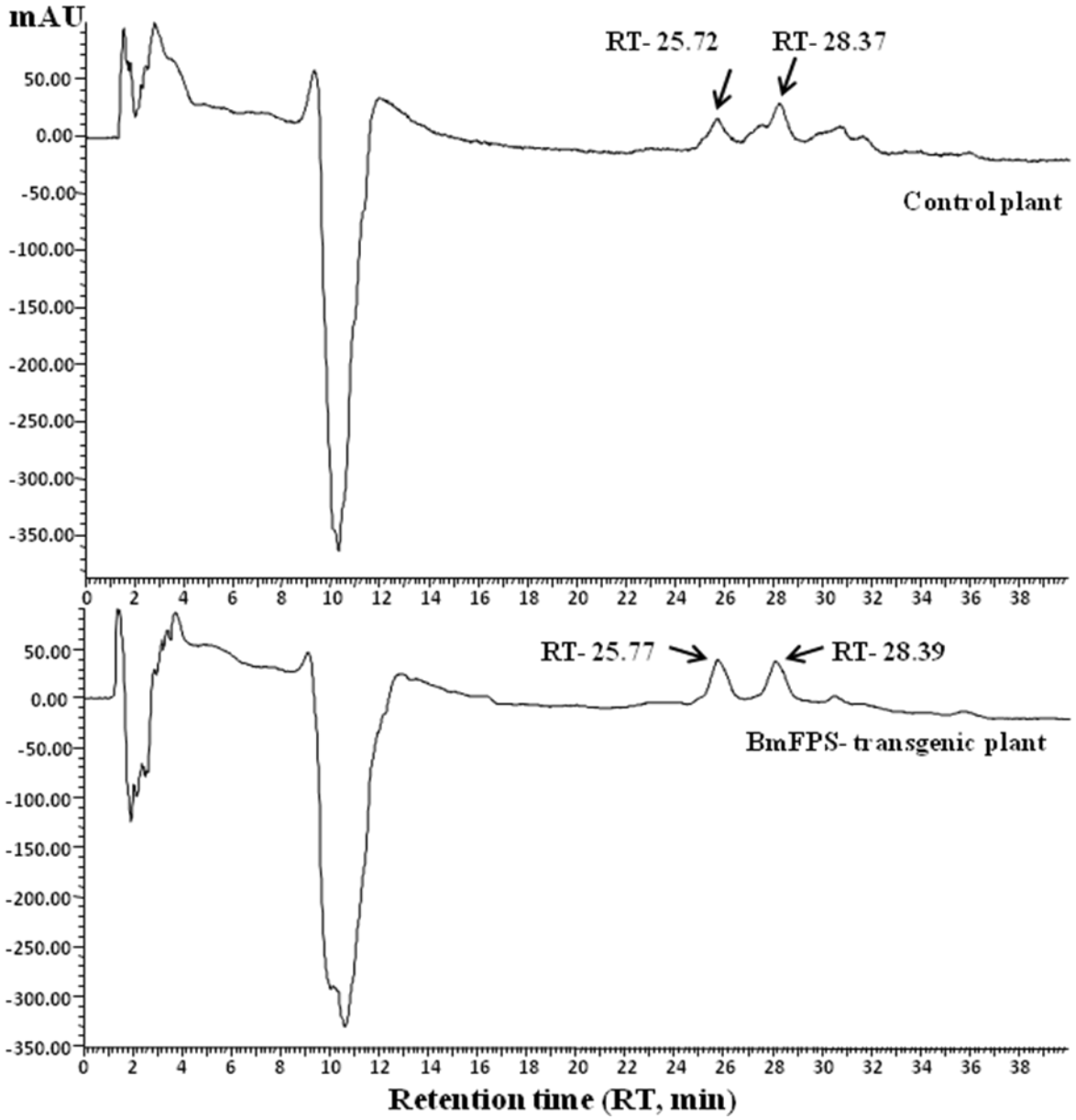


Fig. 5.19: HPLC chromatograms ($A_{205\ nm}$) of extracts from FPS-overexpressing (DC IV) and nontransformed control *Bacopa* plants. The extracts were fractionated by reverse phase HPLC. The peaks at retention time 25.72/25.77 and 28.37/28.39 min correspond to bacopasaponin C and bacoside A respectively.

Equal amount (1 g) of plant samples were extracted in methanol (20 mL) for 24 h, concentrated and vacuum dried at 60 ^{o}C (Deepak *et al*. 2005). Dried extract was dissolved in equal volume (500 µL) of dimethylsulfoxide (DMSO) and injected to HPLC (100 µL). Relative abundance of bacopasaponin C and bacoside A were calculated on the basis of peak area as compare to control nontransformed plants (**Fig. 5.20**). Transgenic lines showed 2.7 – 6.2 fold higher level of bacopasapnin C and 3.7 – 5.9 fold higher bacoside A as compared to control. The levels of triterpenoid contents were coherent with transcript accumulation of BmFPS in transgenic lines. Overexpression of FPP synthase in *A. annua*, resulted 2.5-3.6 fold higher artemisinin content and high correlation was observed in FPS gene expression and artemisinin content (Banyai et al., 2010). In another report 34.4% higher artemisinin content was observed as compared to control, *Artemisia* overexpressing endogenous FPP synthase (Han et al., 2006).

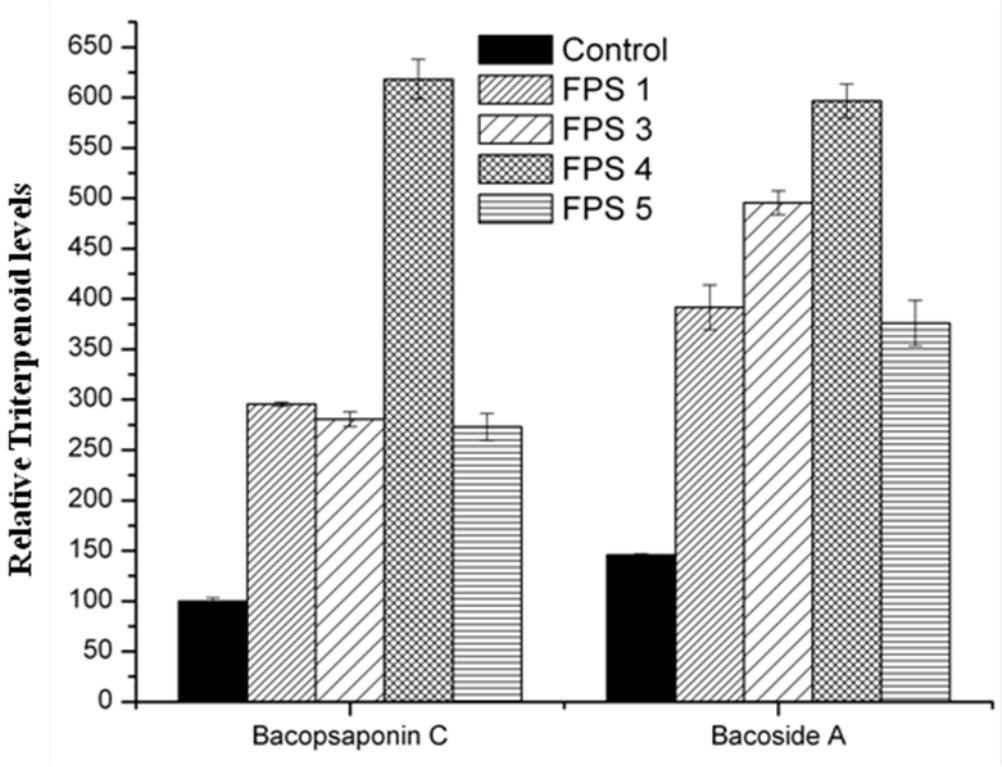


Fig. 5.20: Relative abundance of bacopasaponin C and bacoside A in BmFPS-transgenic lines (FPS 1, 3, 4 and 5).

5.4 Conclusions

- Terpenoid biosynthetic pathway genes from *B. monniera*, BmHMGR, BmFPS and BmSQS, were cloned in sense orientation in plant transformation binary vector pCAMBIA1301.

- Efficient multiple shoots regeneration for *B. monniera* was developed using leaf explants. Approximately 98% shoot regeneration frequency was recorded on basal MS media supplemented with 2 mg/L BAP and 0.2 mg/L IAA.

- Histological study showed the emergence of shoot buds from leaf explants on the 6^{th} day of incubation on regeneration medium.

- *Agrobacterium* mediated transformation of *Bacopa* plant showed 80- 81.48% transformation efficiencies on the basis of PCR with *hpt*II gene specific primers.

- Three transgenic lines of *Bacopa* over-expressing BmHMGR, BmFPS and BmSQS were raised.

- BmHMGR-transgenic events showed 2- 4 fold higher HMGR transcript accumulation as compared to control plants. Three other downstream gene transcript levels in those transgenic plants were investigated. It showed 8.2- 39.1 fold FPS, 2- 92.4 fold SQS and 5.8- 16.4 fold BAS higher expression as compared to control non-transgenic plants.

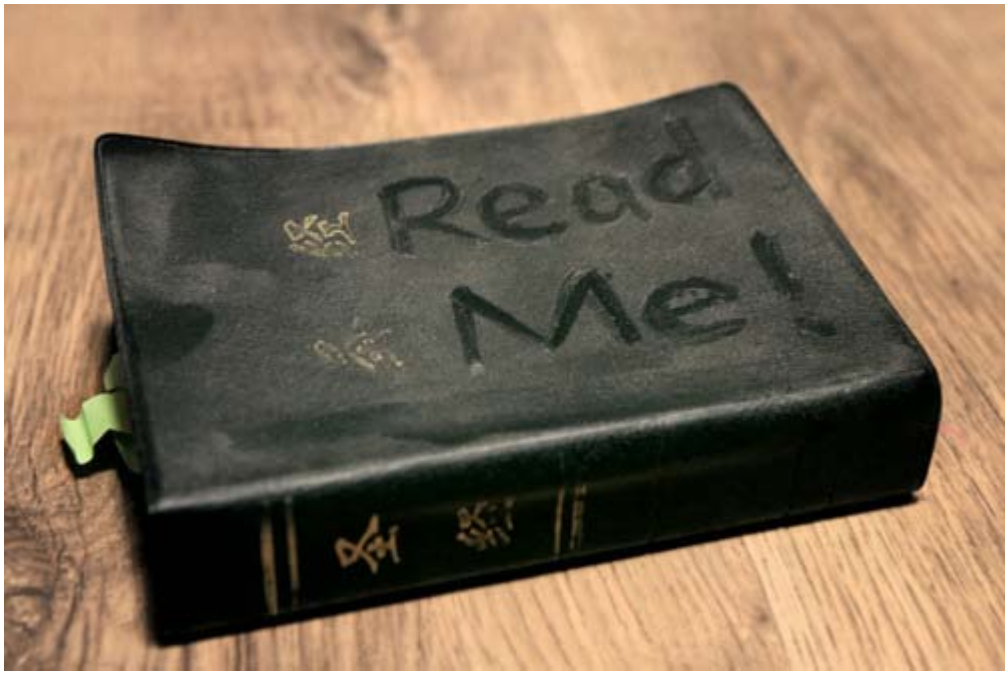
- BmFPS-transgenic lines showed 3.7- 5.4 fold higher FPS mRNA expression as compared to control. ELISA analysis also showed elevated protein expression in transgenic lines. BmHMGR transcript accumulation is slightly high (up to 3.8 fold), whereas BmSQS and BmBAS expression was up to 37.79 and 20.68 fold higher in those transgenics as compared to control.

- BmSQS-transgenic lines showed 1.4 -4.08 fold higher SQS transcript than control plant. ELISA analysis showed elevated protein expression in some of the plants. These transgenic lines were also analyzed for BmHMGR, BmFPS and BmBAS transcript levels. BmHMGR and BmFPS transcripts were slightly

high (up to 3.3 and 7.4 fold respectively), whereas BmBAS transcript accumulation was higher up to 94.35 fold as compared to control plants.

- HPLC analysis of BmFPS transgenic plants showed 2.7 – 6.2 fold bacosaponin C and 3.7 – 5.9 fold higher bacosie A as compared to control nontransformed plants. These data are on the basis of analysis of four BmFPS-transgenic events.

SUMMARY AND FUTURE PROSPECTS



Summary and future prospects

Bacopa monniera is a well-known medicinal herb used as a brain tonic. The main bioactive compounds of the plant are triterpenoid saponins called as bacosides. Triterpene saponins are not only known for pharmacological activities, but also play physiological roles in plants, including as protectant against pathogen attack. In *Bacopa* plant, bacosides are present in very low quantity, as an alternative to increase the yield of such compounds, there is need to develop designer *Bacopa* plants with desired traits by genetic engineering of biosynthetic pathway. The biosynthesis of terpenoids takes place via isoprenoid pathway. In this context the present work was undertaken to isolate and characterize different genes including 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), farnesyl pyrophosphate synthase (FPS) and squalene synthase (SQS) involved in terpenoid biosynthesis from *B. monniera*. It also included development of transgenic *Bacopa* lines overexpressing different isoprenoid biosynthetic pathway genes and their effect on bacoside contents.

The enzyme, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) catalyzes the first committed step of isoprenoid biosynthesis and it has been studied in various medicinal plants including *Eucommia ulmoides* (Jiang et al. 2006), *Catharanthus roseus* (Maldendo-Mendoza et al. 1992), *Salvia miltiorrhiza* (Liao et al. 2009), *Euphorbia pekinnensis* (Cao et al. 2010) and *Arnebia euchroma* (Singh et al. 2010). The HMGR from *B. monniera* (BmHMGR) showed more than 73% sequence identity with reported HMGR from other plants. It contained all the conserved domains including catalytic residues, substrate binding pockets and NADPH binding sites present in HMGR from other plants. BmHMGR was over-expressed in *E. coli* (BL21) and attempts were made for its purification, but unfortunately purification procedures tried, did not work. The 3 D structure of BmHMGR was generated by homology modeling and interactions of residues with different ligands were also investigated by docking studies. Differential expression study showed *BmHMGR* accumulates more in roots as compared to stem, leaf and floral parts and our results are consistent with *Euphorbia* HMGR expression pattern (Cao et al. 2010). In *Salvia*, HMGR expressed more in leaves as compared to stem and roots (Dai et al. 2011). Relative BmHMGR transcript accumulation in response to different stress conditions showed altered expression of mRNA at different time points. BmHMGR expression was induced

highly in presence of methyl jasmonate as compared to other stressors like salt, cold, salicylic acid, chlorogenic acid and yeast extract.

Farnesyl pyrophosphate (FPP), which is catalyzed by farnesyl pyrophosphate synthase (FPS), functions as a substrate in the synthesis of various secondary metabolites, including sesquiterpenoids, phytoalexins, triterpenoids, and phytosterols. FPS has been isolated and characterized from various plant species including *Centella asiatica* (Kim et al. 2005), *Chimonanthus praecox* (Xiang et al. 2010) and *Euphorbia pekinensis* (Cao et al. 2012). The FPS from *B. monniera* (BmFPS) was cloned and characterized. BLAST analysis of BmFPS showed more than 75% identity with reported FPS from other plants. Approximately 40 kDa recombinant protein was purified and polyclonal antibody was raised in rabbit, which was used for Western blot and ELISA analysis. The comparative modeling and docking studies of BmFPS was carried out. The model generated was reliable and docking with ligands showed interactions of amino acid residues with different ligands. qRT-PCR study revealed that BmFPS is differentially expressed, with higher transcript accumulation in leaf as compared to stem, root and floral parts. Xiang et al. (2010) reported that *C. praecox* FPS mRNA expressed highly in leaves and petals. The expression of BmFPS was also induced in presence of different stressors.

Squalene synthase (SQS) catalyzes a reductive dimerization of two farnesyl pyphosphate (FPP) molecules into squalene, a key precursor for the sterol and triterpene biosynthesis. cDNA clones for squalene synthase have been isolated and characterized from various plant species, such as tobacco (Hanley et al. 1996; Devarenne et al. 1998), *Arabidopsis thaliana* (Nakashima et al. 1995; Kribii et al. 1997), *Panax ginseng* (Kim et al. 2011) and *Withania somnifera* (Bhat et al. 2012). The full-length cDNA encoding SQS was isolated from *B. monniera* (BmSQS) and characterized. It showed more than 78% sequence identity with other plants SQS. The hydrophobic residues from C-terminus (24 amino acid) were truncated for solublization of protein in *E. coli* expression (Lee et al. 2002). Antibodies against purified truncated recombinant protein were raised and used for ELISA and Western blot analysis. The mRNA expression of BmSQS was tissue specific and it was found that it accumulates more in petals as compared to leaves and stems. In *Taxus cuspidata* SQS transcripts accumulate higher in roots followed by stem and leaves (Huang et al. 2007), whereas in

P. ginseng PgSS1 and PgSS2 transcripts accumulated more in leaves, roots and petiole (Kim et al. 2011).

Efficient multiple shoots regeneration system for *B. monniera* was established using leaf as explants with shoots regeneration frequency of 98.33%. Recently, Mahender at al. (2012) reported 90% shoots regeneration frequency of *Bacopa* by using same leaf explants. Transgenic lines of *B. monniera*, overexpressing BmHMGR, BmFPS and BmSQS, were achieved and the transformation efficiencies were more than 80% which is higher than other medicinal plants. A 70.76% transformation efficiency was reported in *B. monniera* by using leaf explants (Mahender et al. 2012). Nisha et al. (2003) observed 60% transformation efficiency using callus while it was found to be 68.8% by Ramesh et al. (2011) where nodal explants were used for transformation. Preliminary screening of transgenic lines was carried out by PCR using *hpt*II gene specific primers, GUS assay and qRT-PCR. BmHMGR-transgenic *Bacopa* plants showed 2- 4 fold higher HMGR transcript accumulation as compared to control, whereas BmFPS and BmSQS-transgenic lines showed 3.7-5.4 fold and 1.4- 4.08 fold higher transcript accumulation respectively. These transgenic lines were also analyzed for effect of overexpression of particular gene on expression of other genes of same pathway. BmHMGR-transgeic lines showed 8.2- 39.1 fold BmFPS, 2- 92.4 fold BmSQS and 5.8 – 16.4 fold BmBAS mRNA expression as compared to control. On the other hand BmFPS and BmSQS-transgenic lines showed slight changes in BmHMGR transcripts level whereas BmSQS and BmBAS expression was up to 37.79 and 20.68 fold higher respectively in BmFPS-transgenic lines. In case of BmSQS-transgenic lines the expression level of BmFPS and BmBAS was upto 7.4 and 94.35 fold higher than control plants respectively. In a recent report, potato plants overexpressing HMGR and SQS induced steroidal glycoalkaloid pathway genes expression (Ginzberg et al., 2011). HPLC analysis of BmFPS-transgenic lines showed 2.7- 6.2 fold higher level of bacopasaponin C and 3.7- 5.9 fold higher level of bacoside A as compared to control plants. Overexpression of FPS in *A. annua* resulted in 2.5- 3.6 fold higher artemisinin content and a high correlation was observed in FPS gene expression and artemisinin content (Banyai et al., 2010). These data suggests that the development of transgenic lines of *Bacopa* will provide insight about regulation mechanism of genes involved in bacoside biosynthesis.

The future aspects of this study will include biochemical characterization of BmHMGR, BmFPS and BmSQS and correlation with comparative modeling and docking studies. It will be interesting to know, out of three transgenic lines of *Bacopa* which one contributed more to bacoside biosynthesis. Here we analyzed only few BmFPS-transgenic plants for bacoside estimation because hardening and shifting of transgenic plants of all three lines are continuously going on. BmHMGR and BmSQS-transgenic line analysis work are underway. These data will appear soon as a publication.

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PUBLICATIONS

PUBLICATIONS

Research Papers/ Book Chapter Accepted/ communicated

1. **Vishwakarma R. K**.; Srivastava S.; Singh S.; Khan B. M. (**2012**) Molecular cloning and characterization of two differentially expressed Cellulose synthase gene isoforms in *Leucaena leucocephala*: A pulp yielding tree species. ***Advances in Bioscience and Biotechnology*. 3: 92-100**.

2. Khan B. M.; Rawal S. K.; Arha M.; Gupta S. K.; Srivastava S.; Shaik N. M.; Yadav A. K.; Kulkarni P. S.; Abhilash O.U.; Kumar S.; Omer S.; **Vishwakarma R. K.**; Singh S.; Santosh Kumar R. J.; Sonawane P.; Patel P.; Chinnathambi K.; Abbassi S. (**2012**) Genetic Engineering of Phenylpropanoid Pathway in *Leucaena leucocephala*. ***In: Genetic Engineering- Basics, new applications and responsibilities. InTech. Croatia.* ISBN 978-953-307-671-3, pp 93-120**.

3. **Vishwakarma R. K**.; Ruby; Singh S.; Sonawane P.; Srivastava S.; Santosh Kumar R. J.; Khan B. M. (**2012**) Molecular Cloning, Biochemical Characterization and differential expression studies of Acetyl-CoA C-Acetyltransferase gene (AACT) from Brahmi (*Bacopa monniera*): An Ayurvedic Brain Tonic Herb. ***Molecular Biology Reports* (*Under Revision*; MOLE 4513).**

4. Singh S.; Santosh Kumar R. J.; **Vishwakarma R. K**.; Sonawane P.; Khan B. M. (**2012**) Functional characterization of a flavonoid glycosyltransferase gene from *Withania somnifera* (Ashwagandha). ***Molecular Biology Reports* (*Under Revision*; MOLE 3874)**.

5. Kumari U.; **Vishwakarma R. K**.; Gupta N.; Ruby; Shirgurkar M. V.; Khan B. M. (**2012**) Efficient multiple shoots regeneration of *Bacopa monniera* and its transformation by *Agrobacterium tumefaciens* and particle bombardment. ***In Vitro Cellular & Developmental Biology- Plants* (*Under Revision*, IVPL-D-12-00088)**.

6. Srivastava S.; Gupta R. K.; Arha M.; **Vishwakarma R. K**.; Rawal S. K.; Kavi Kishor P. B.; Khan B. M. **(2011**) Expression analysis of cinnamoyl-CoA reductase (CCR) gene in developing seedlings of Leucaena leucocephala: A pulp yielding tree specie**s. *Plant Physiology and Biochemistry*. 49: 138-145**.

7. Shaik N. M.; Arha M.; Nookaraju A.; Gupta S. K.; Srivastava S.; Yadav A. K.; Kulkarni P. S.; Abhilash O.U.; **Vishwakarma R. K**.; Singh S.; Tatkare R.; Chinnathambi K.; Rawal S. K.; Khan B. M. (**2009**) Improved method of *in vitro* regeneration in *Leucaena leucocephala* – a leguminous pulpwood tree species. ***Physiology and Molecular Biology of Plants*. 15(4): 311-318**.

8. Srivastava S.; **Vishwakarma R. K**.; Arafat Y.; Khan B. M. (**2012**) Abiotic stress induces significant changes in Cinnamoyl CoA Reductase gene expression and lignin deposition in *Leucaena leucocephala*. ***Journal of Plant Research*** (Communicated)

Manuscripts under preparation

1. **Vishwakarma R. K**.; Patel K.; Singh S.; Sonawane P.; Ruby; Santosh Kumar R. J.; Gupta N.; Khan B. M. (**2012**) Squalene Synthase from medicinal herb, *Bacopa monniera*: Its cloning, characterization, comparative modeling and docking studies.

2. **Vishwakarma R. K**.; Kumari U.; Singh S.; Sonawane P.; Ruby.; Ansari Z.; Khan B. M. (**2012**) Terpenoid Biosynthesis Pathway engineering using FPP synthase as a candidate gene: Its cloning, characterization and over-expression in *Bacopa monniera*.

3. **Vishwakarma R. K**.; Kumari U.; Singh S.; Sonawane P.; Ruby.; Khan B. M. (**2012**) Over-expression of HMG CoA Reductase and Squalene Synthase in *Bacopa monniera* and its effects on bacosides content.

4. **Vishwakarma R. K**.; Sonawane P.; Singh S.; Ruby.; Deshmukh N.; Goyal S.; Khan B. M. (**2012**) Stress responsive tissue-specific expression analysis and biochemical characterization of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase gene in *Bacopa monniera* (Brahmi).

5. **Vishwakarma R. K**.; Singh S.; Sonawane P.; Ruby.; Kumari U.; Khan B. M. (**2012**) Molecular Cloning and Characterization of β-amyrin synthase gene from Brahmi (*Bacopa monniera*).

6. **Vishwakarma R. K**.; Singh S.; Sonawane P.; Kumari U.; Ruby.; Ansari Z.; Khan B. M. **(2012**) Alteration in expression of *Bacopa monneira* terpenoid biosynthesis pathway genes by over-expressing of cDNA encoding HMG-CoA reductase, FPP synthase and Squalene synthase.

7. **Vishwakarma R. K.**; Ansari Z.; Abbassi S.; Sonawane P.; Singh S.; Khan B. M. (**2012**) Response of gene encoding Pathogenesis Related Protein (PR1) in *Bacopa monniera*, growing under different stress conditions.

8. Ruby; Santosh Kumar R. J.; **Vishwakarma R. K**.; Khan B. M. (**2012**) Molecular cloning and characterization of genistein 4'-O-glucoside specific glycosyltransferase from *Bacopa monniera*.

Abstracts/ Posters

1. **"Multiple Shoots Regeneration and *Agrobacterium* mediated transformation of *Bacopa monniera*"**. Kumari U.; **Vishwakarma R. K**.; Shirgurkar M. V. and Khan B. M.
   Presented at National Chemical Laboratory in Science Day Celebrations during 27^{th}-28^{th} February **2012**.

2. **"Development of transgenic *Bacopa monniera* plants for altering mevalonate pathway gene(s) with reference to increase bacoside content". Vishwakarma R. K.**; Ruby; Singh S.; Santosh Kumar R. J.; Sonawane P. and Khan B. M.
   Presented in "National symposium on Recent Advances in Plant Tissue Culture and Biotechnological Research in India" at Bikaner, India during 4^{th}-6^{th} February **2011**.

3. "**Metabolic Engineering of Terpenoids Biosynthesis Pathway in *Bacopa monniera*" Vishwakarma R. K**.; Singh S.; Santosh Kumar R. J.; Sonawane P.; Sharma P. and Khan B. M.

   Presented in International symposium on Aromatic and Medicinal Plants (AROMED), at CIMAP Lucknow (CSIR), India during February 21-24 **2010**.

4. **"Over-expression/Up-regulation of Cellulose Synthase (*Ces* A) gene in *Leucaena leucocephala* for improved paper and pulp**" **Vishwakarma R. K**.; Srivastava S.; Shaik N. M.; Gupta S. K.; Singh S.; Rajeshri T.; Omer S.; Kannan C. and Khan B.M.

   Presented in National Symposium at IHBT Palampur (CSIR), India during April 3-4 **2009**.