Molecular Studies on Withanolides Biosynthetic Pathway Gene(s) (Squalene Synthase) from *Withania somnifera*

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IN
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BY
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UNDER THE GUIDANCE OF
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CERTIFICATE

This is to certify that the work incorporated into the thesis entitled "**Molecular Studies on Withanolides Biosynthetic Pathway Gene(s) (Squalene Synthase) from *Withania somnifera***" submitted by **Neha Gupta** for the degree of Doctor of Philosophy, was carried out under my supervision at Plant Tissue Culture Division, National Chemical Laboratory, Pune. Materials obtained from other sources have been duly acknowledged in the thesis.

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DECLARATION

I, hereby, declare that the work of the thesis entitled **"Molecular Studies on Withanolides Biosynthetic Pathway Gene(s) (Squalene Synthase) from *Withania somnifera*"** has been carried out at the Plant Tissue Culture Division, National Chemical Laboratory, Pune under the guidance of **Dr. B. 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.

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ABSTRACT

Title: Molecular Studies on Withanolides Biosynthetic Pathway Gene(s) (Squalene Synthase) from *Withania somnifera*

Medicinal plants are in great demand to extract various medicinally important compounds for human health. Herbal medicines are gaining popularity in day-to-day life as 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. *Withania somnifera* is being used for centuries in Ayurvedic system of medicine as anti-tumor, anti-oxidant, anti-inflammatory, anti-aging, antiserotogenic, antistress agent and various other biological activities. These pharmacological properties are mainly attributed to the steroidal lactones present in the plant called as withanolides. Withanolides are synthesized by diverting the metabolite flux away from the isoprenoid pathway by the reductive condensation of farnesyl diphosphate to squalene, through the activity of squalene synthase.

The plant produces relatively small amount of withanolides and large biomass have been used to overcome the shortage to pharmaceutical industry for medicinal formulations. If this continues, it may cause *Withania* spp. to fall in the category of endangered species very soon. In order to overcome this problem, the present study is focused on metabolic engineering of isoprenoid biosynthetic pathway in *W. somnifera* by overexpression of squalene synthase gene. The efficient regeneration and transformation system for *Withania* has been developed which can be used for plant transformation and micropropagation at large scale.

Chapter 1: General Introduction

This chapter gives information on medicinal plant, *W. somnifera* and its secondary metabolites, withanolides, their biosynthesis and wide range of biological activities. The biosynthetic pathway of withanolides reveals a key regulatory enzyme, squalene synthase (SQS), which diverts the carbon flux towards their synthesis. Background of research done on SQS has been dealt in detail. A thorough literature survey of work done on SQS in the area of genetic engineering of isoprenoid pathway with regards to the current status of

research in this area has been presented. Isolation, cloning and protein expression studies on SQS from various plants have been discussed in detail and need of more research on SQS has been elaborated. Finally, the scope of the present study and objectives of the thesis work have been discussed.

Chapter 2: Isolation, cloning and characterization of squalene synthase gene(s) from *Withania somnifera*

This chapter deals with the isolation, cloning and characterization of withanolide biosynthetic pathway gene, SQS, from *W. somnifera*. PCR based approach was used for the isolation of gene. Nucleotide sequencing revealed two different sequences of *SQS* from *W. somnifera*, differing in 6 nucleotides. Rapid amplification of cDNA ends (RACE) was then used to confirm the terminal sequences of the isolated genes and to find out the untranslated regions (UTRs). The full length cDNAs encoding SQSs from *W. somnifera* was designated as *WsSQS1* and *WsSQS2*. *WsSQS1* consisted of 1236 bp (open reading frame) ORF, 53 bp 5' UTR and 257 bp of 3'UTR while *WsSQS2* consisted of 1242 bp ORF**,** 65 bp 5' UTR and 253 bp 3'UTR. Both the genes have been submitted to NCBI GenBank database having accession number GU181386 and GU732820 respectively. The calculated mass of polypeptide WsSQS1 (411 AA) and WsSQS2 (413AA) were observed to be 47.0 and 47.16 kDa, respectively with predicted pI value 8.1. WsSQS1 and WsSQS2 amino acid sequences share 99% similarity and 99% identity with each other. Both the gene showed maximum similarity and identity with *Solanum tuberosum* followed by *Capsicum annum* and *Nicotiana tabacum*. According to conserved domain database 6 highly conserved peptide domains have been identified in WsSQSs. Hydropathy analysis showed one large transmembrane region at C terminal of the polypeptide. Phylogenetic analyses showed that isolated genes are closely related with dicot plant spp., clustered with *Solanum* spp. and *Capsicum* spp.

Chapter 3: Heterologous expression, purification and characterization of the recombinant WsSQS protein(s)

This chapter deals with the protein studies of the isolated genes. The genes were cloned in expression vector (pET 30b +) and overexpressed in *Escherichia coli* (BL21). The

expressed proteins were purified by Ni-NTA affinity and gel filteration chromatography. The activity of the purified proteins were determined by assaying the enzyme activities and analyzing the product squalene by GC-MS. Polyclonal antibodies were raised in rabbit against recombinant WsSQS1, which was then used for immunological studies. ELISA showed maximum WsSQS in root followed by leaf and stem tissue of the plant which was also reflected in western blot analysis. Comparative modeling and docking studies of WsSQS1 was done to predict its 3D structure and interactions with ligands: NADPH and FPP. The overall study showed that generated model was reliable, and interacts with ligands through some specific amino acids present on the catalytic site.

Chapter 4: Tissue specific expression analyses of *WsSQS* under stress conditions

Quantitative Real Time PCR (qRT-PCR) analysis showed that *WsSQS* express differentially in different tissues of the plant such as leaf, stem and root. Relatively higher expression of *WsSQS* was observed in roots followed by leaf and minimum in stem. Same pattern was also observed in WsSQS protein accumulation in these tissues with maximum protein in root, which was almost equal to protein in leaf tissue and minimum in stem. The differential expression of WsSQS was also investigated under stress conditions at two different concentrations of salt (NaCl), salicylic acid and methyl jasmonate. qRT-PCR data showed different levels of increased transcript accumulation of the gene in all cases where methyl jasmonate was found to induce transcript expression at greater extent. There was no co-relation found between the mRNA expression and protein accumulation, however, consistent with the qRT-PCR results, WsSQS protein also increased in presence of different stressors but there was variation in the level of accumulation.

Chapter 5A: Genetic transformation of *W. somnifera* for overexpression of *WsSQS* and molecular analyses of transformed plants

This part of the study covers cloning of *WsSQS* in sense orientation in binary vector, propagation of *W. somnifera in vitro*, plant transformation and analysis of transformed plants. The full length *WsSQS* was introduced in pCAMBIA 1301 binary vector in between the CaMV 35S promoter and Nos terminator, and the recombinant vector was then transformed in *Agrobacterium tumefaciens* GV2260 for plant transformation. *Withania* plants were germinated from seeds *in vitro*, and nodal and apical segments derived from these plantlets were used as explant for plant transformation.

Transformation was achieved by *Agrobacterium* mediated, microprojectile bombardment method as well as the combination of both where the latter gave maximum transformation frequency (8.7%). The concentration of hygromycin B and cefotaxime were optimized. Out of various combinations of growth regulators, MS basal medium supplemented with 0.2 mg/L BAP and 0.1 mg/L kinetin supported maximum and quick shoot elongation and multiplication. The transformants were screened by Gus histochemical assay and finally by molecular analyses by carrying out PCR of hygromycin phosphotransferase (*hpt* II) and *WsSQS* gene.

Chapter 5B: Enhanced withanolide biosynthesis in transformed *W. somnifera* overexpressing *WsSQS* gene

This chapter deals with the analyses of the transformants for increased levels of WsSQS mRNA and protein, and finally estimation of withanolide content of the transformed plants. qRT-PCR analyses of various transformed tissues indicated 2-5 fold increase in *WsSQS* transcripts. The tissue specific protein expression studies revealed 2-3 fold increase in WsSQS, which was further confirmed by enzyme activity from these tissues. These observations were corroborated with the 1.5-2 fold increase in total withanolide content of the transformed tissues, as analyzed by LC-MS. However, in leaf tissue, the levels of Withaferin A and Withanolide A increased significantly up to 4-4.5 fold.

These findings demonstrate metabolic engineering of isoprenoid pathway in *W. somnifera* resulting in enhanced production of withanolides, and also provide understanding of the metabolic networks leading to improvement of the pharmacological content of different medicinally important plants.

ABBREVIATIONS

AMV-RT	Avian Myeloblastosis Virus-Reverse Transcriptase
APS	Ammonium Per Sulphate
BAP	6-Benzylaminopurine
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
DEPC	Diethylpyrocarbonate
DTT	Dithiothritol
DW/ FW	Dry/ Fresh Weight
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme Linked Immuno-Sorbent Assay
ER	Endoplasmic Reticulum
EtBr	Ethidium bromide
GC	Gas Chromatography
HEPES	4-(2-Hydroxy Ethyl)-1-Piperazine Ethane Sulfonic acid
hptII	Hygromycin Phosphotransferase-II
IBA	Indole-3-butyric acid
IPTG	Isopropyl β-D-thiogalactoside
LC	Liquid Chromatography
LD_{50}	Lethal dose 50%
MS	Mass Spectrometry
NBT	Nitro-Blue Tetrazolium
pI	Isoelectric point
pNPP	p-Nitrophenyl Phosphate
PVP	Polyvinylpyrrolidone
qRT-PCR	Quantitative Real Time PCR
SM	Secondary Metabolites
SMQ	Sterile Milli Q
TEMED	Tetramethylethylenediamine
UTR	Untranslated Region
WsSQS	*Withania somnifera* Squalene Synthase
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactoside

CHAPTER 1

INTRODUCTION

1.1 Medicinal Plants

Humans are dependent on plants for their various needs such as food, shelter, clothing, flavors, fragrances and medicines (Cragg and Newman 2005). Plants have formed the basis of traditional medicine system from thousands of years to provide mankind with new remedies. Until the advent of modern medicine, man depended on plants for treating human and livestock diseases. Even today, modern medicinal system and traditional plant therapy are used independently. Natural products and their derivatives represent more than 50% of all the drugs in clinical use worldwide today and higher plants contribute 25% of the total (Farnsworh et al. 1985; Cragg and Newman 2005).

Medicinal and aromatic plants play an important role in the healthcare, especially in developing countries. About 80% of the population of most developing countries still use traditional medicines derived from plants for treating human diseases (De Silva 1997). The Indian systems of medicine 'Ayurveda,' 'Sidha' and 'Unani' entirely, and homeopathy to some extent, depend on plant materials or their derivatives for treating human ailments (Prajapati et al. 2003). Some important medicinal plants are listed in the Table 1.1.

Botanical Name	Common name	Parts Used	Medicinal Use
Withania somnifera	Aswagandha	Whole plant	Restorative tonic, stress, nerves disorder, aphrodiasiac
Bacopa monnieri	Brahmi	Whole plant	Nervous, memory enhancer, mental disorder
Azadirachta indica	Neem	Leaf, fruit	Sedative, analgesic, epilepsy, hypertensive
Ocimum sanctum	Tulsi	Leaf, seed	Cough, cold, bronchitis, expectorant
Vinca rosea	Sada Bahar	Whole plant	Leukemia, hypotensive, antispasmodic, antidot
Cinnamomum zeylanicum	Dalchini	Bark, oil	Bronchitis, asthma, cardiac disorder, fever
Rauvolfia serpentina	Sarpagandha	Root	Hypertension, insomnia
Aloe vera	Ghritakumari	Leaf	Laxative, wound healing, skin burns & care, ulcer
Mesua ferrea	Nagakesara	Bark, leaf and flower	Asthma, skin burning, vomiting, dysentery, piles

Table 1.1: List of few important medicinal plants and their uses

India has the highest proportion of medicinal plants in its existing flora than any country of the world (Shiva 1996). Ayurveda, the oldest medical system in Indian sub-continent, has alone reported approximately 2000 medicinal plant species, followed by Siddha and Unani. About 25% of drugs in modern pharmacopoeia are derived from plants (phytomedicines) and many others are synthetic analogues built on prototype compounds isolated from plants. Upto 60% of the drugs prescribed in Eastern Europe consists of unmodified or slightly altered higher plant products (Lancet 1994). These drugs carry important therapeutic properties including contraceptives, steroids and muscle relaxants for anesthesia and abdominal surgery, quinine and artemisinin against malaria; digitalis derivatives for heart failure; and the anticancer drugs vinblastin, etoposide and taxol. These compounds cannot be synthesized cost effectively, which means that their production requires reliable supplies of plant material (Van Seters 1997).

1.2 Secondary metabolites (SMs)

In plants, as a result of metabolic processes, many different kinds of organic compounds or metabolites are produced. These metabolites are grouped into primary and secondary metabolites. The primary metabolites like chlorophyll, amino acids, nucleotides, carbohydrates and lipids are essential for normal growth and development of plants and are also utilized as food by animals. They play important roles in photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation. However, the SMs 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 low molecular weight compounds which differ from primary metabolites in having a restricted distribution in the plant kingdom. The relationship between primary compounds, intermediary metabolism and the groups of secondary compounds is described in Fig 1.1 (Dörnenburg and Knorr 1996). SMs often found in only one plant species or a taxonomically related group of species, whereas the basic primary metabolites are found throughout the plant kingdom (Taiz and Zeiger 2006).

It was believed that plant SMs have no recognized role in the maintenance of fundamental life processes in plants; however, they do have an important role in the interaction of the

plant with its environment. The production of these compounds is very low (less than 1% dry weight) and it depends greatly on the physiological and developmental stage of the plant. The production of SMs in plants is enhanced by both biotic and abiotic stress conditions. These compounds have complex and unique structures, stored in specific cells and/or organs of the plant and often accumulate in vacuoles (Dixon 2001). Originally, secondary products were seen as end points in metabolism, with a less specific role. Many of the compounds were shown to have an active turnover and now it is accepted that they have a much more defined function in plants (Fig 1.2).

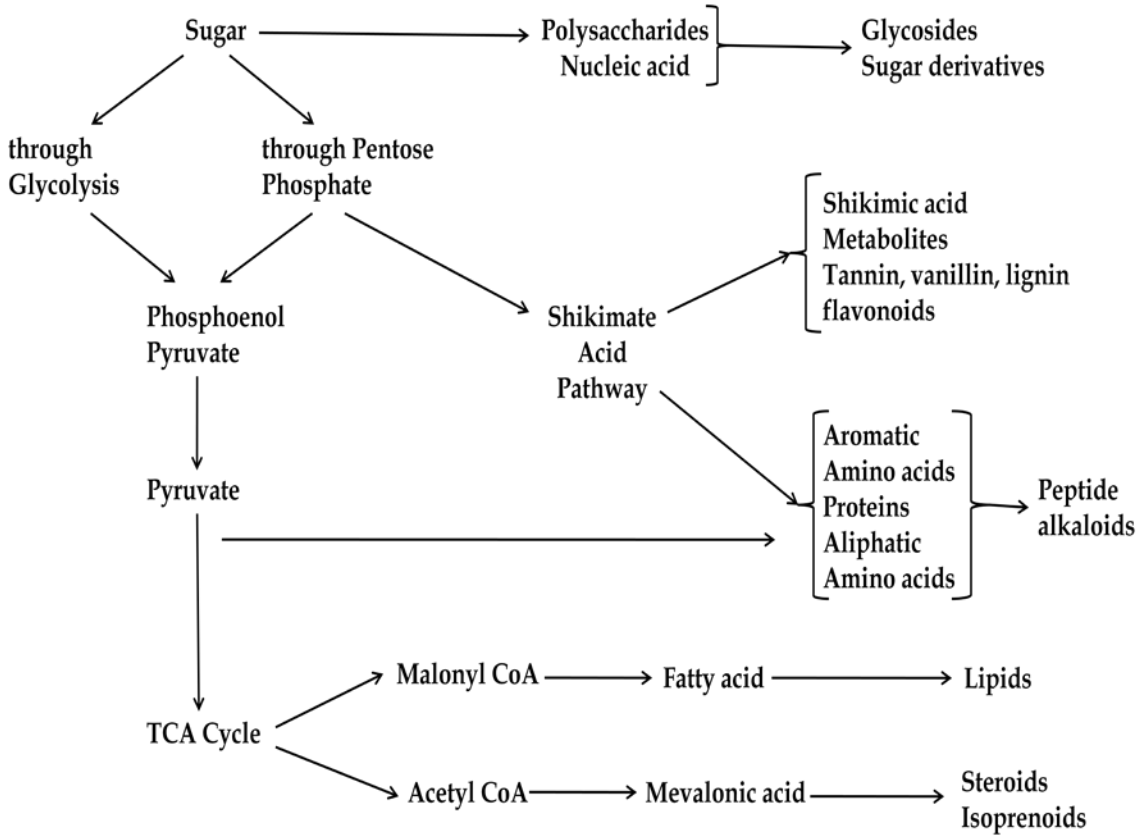


Fig 1.1: Primary and secondary metabolites derived from carbon metabolism

The major SMs 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. SMs are sometimes considered as waste or secretory products of plant metabolism but many of them possess pharmaceutical importance. Mankind has been using SMs for multiple purposes, such as dyes, flavors,

fragrances, stimulants, hallucinogens, insecticides, vertebrate and human poisons as well as therapeutic agents. Such fine chemicals are extracted and purified from plant sources (Verpoorte and Alfermann 2000). The interest in SMs 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 cancer and many chronic diseases (Enfissi et al. 2010).

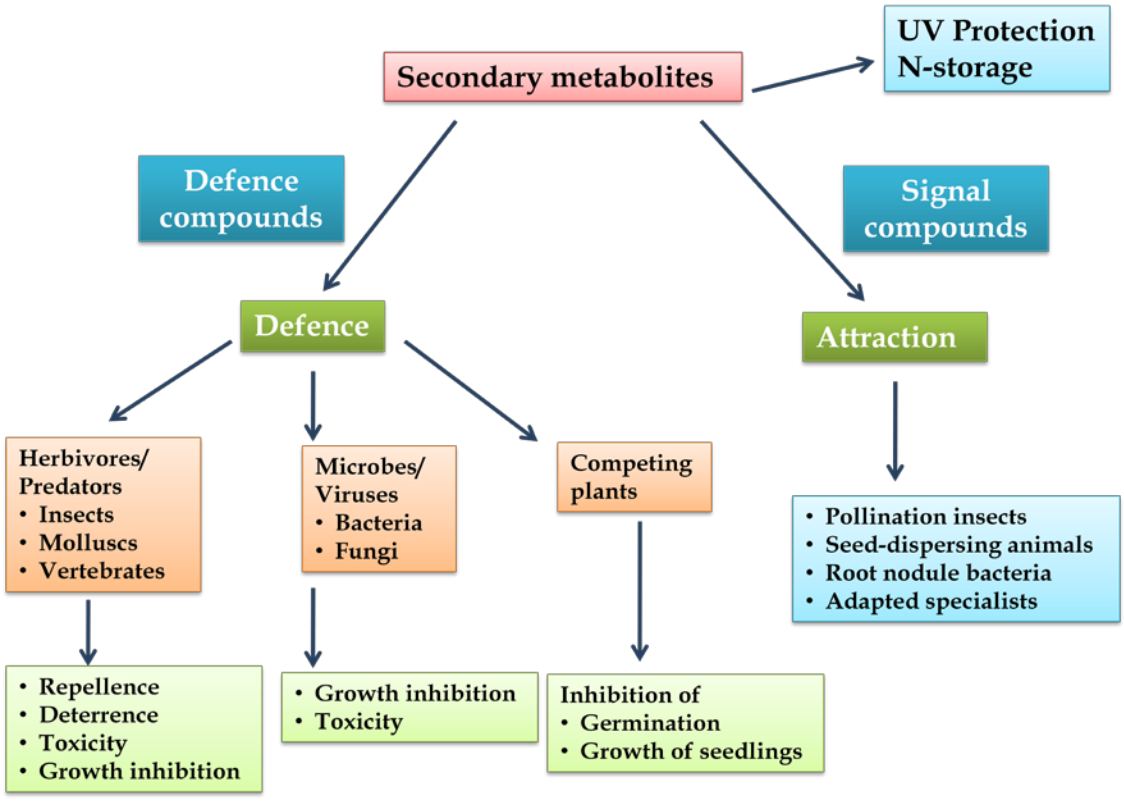


Fig 1.2: Ecological and physiological functions of plant secondary metabolites

1.3 *Withania somnifera*

Withania somnifera L. Dunal (Ashwagandha), member of Solanaceae family, is a well known medicinal plant. It is a perennial branched herbaceous species, commonly used as a domestic remedy for several diseases and described as a herbal tonic and health food in the famous book of Vedas, and is considered as 'Indian Ginseng' in traditional Indian system of healing (Dhuley 2000). *Withania* appears in WHO monographs on Selected Medicinal Plants and an American Herbal Pharmacopoeia monograph (Marderosion 2001). *Withania* is widely claimed to have potent aphrodisiac, sedative, rejuvenative and life prolonging

properties. Several recent reports have demonstrated immuno-modulator and antitumor effect of *W. somnifera* (Agarwal et al. 1999). It is used as a general energy-enhancing tonic known as 'Medharasayana' which means 'to promote learning and a good memory' (Williamson 2002). The plant was traditionally used to promote youthful vigor, endurance, strength, health and increasing the production of vital fluids, muscle fat, blood, lymph and semen. The similarity between these restorative properties and those of ginseng roots has led to Ashwagandha roots being called Indian ginseng (Singh and Kumar 1998).

1.3.1 Classification and botanical description

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Angiosperma
Class	Dicotyledoneae
Subclass	Asteridae
Order	Solanales
Family	Solanaceae
Genus	***Withania***
Species	***somnifera***

The plant has erect branching, usually clothed with minutely stellate hairy tomentum; leaves ovate with dense hair beneath and sparse above. The flowers are greenish or lurid yellow in axillary fascicles, bisexual with long pedicel while the fruits are globose berries enclosed in a persistent calyx carrying seeds (Fig 1.3). The fleshy roots are cylindrical when dry, gradually tapering down with a brownish white surface and pure white inside when broken. Flowering occurs in fall and spring.

1.3.2 Geographical distribution and cultivation

W. somnifera is native to India and cultivated as an annual shrub. The plant is cosmopolitan growing throughout the drier parts and sub-tropical India including Gujarat, Rajasthan, Punjab plains, Himachal Pradesh and Jammu. It is also cultivated in Madhya Pradesh, Andhra Pradesh and Uttar Pradesh (Arun et al. 2007). It is also found in Congo, Spain, Canary Islands, South Africa, Egypt, Morocco, Jordan, Palestine, Pakistan and Afganistan.

W. somnifera grows well in sandy loam or light red soil, having pH 7.5-8.0 with good drainage. It can be cultivated between 600-1200 m altitudes. The crop requires dry season

during its growing period. Temperature between 20 ºC to 35 ºC is most suitable for cultivation. Late winter rains are conducive for the proper development of the plant roots.

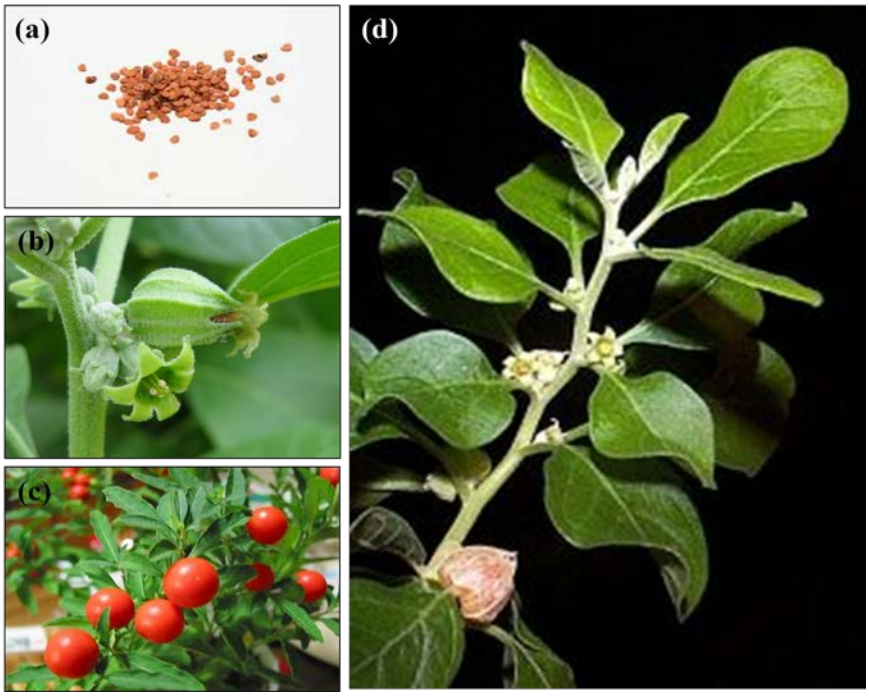


Fig 1.3: *Withania somnifera* **(a)** seeds; **(b)** flowers; **(c)** fruits; and **(d)** plant

1.3.3 Chemical constituents

The chemistry of *Withania* species has been extensively studied and several groups of chemical constituents such as steroidal lactones (withanolides), alkaloids (isopelletierineanaferine), flavonoids, tannin and many more have been identified, extracted and isolated (Atta-ur-Rahman et al. 1991; 1993). At present, more than 12 alkaloids, 40 withanolides and several sitoindosides (a withanolide containing a glucose molecule at carbon 27) have been isolated and reported from aerial parts, roots and berries of *Withania* species (Choudhary et al. 1996). Withanolides are known to be the chemical markers of the plant which is responsible for its pharmacological properties. The detailed description of withanolides is covered in section 1.4 of this chapter.

1.3.4 Therapeutic uses of *W. somnifera*

W. somnifera helps counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature ageing, emaciation, debility and muscle tension.

The various parts of the plant have been reported to possess antiserotogenic and anabolic properties, and have shown beneficial effects in the treatment of arthritis and geriatric problems (Prakash et al. 2001). The plant is known to be adaptogen, aboritifacient, aphrosidiac, astringent, deobstruent, narcotic and sedative (Mirjalili et al. 2009b). *W. somnifera* is also used into dietary supplements with good nutritional properties. The roots are a constituent of over 200 formulations in Ayurvedha, Siddha and Unani medicines, which are used in the treatment of various physiological disorders (Asthana and Raina 1989; Singh and Kumar 1998). A review on the medicinal evaluation of *W. somnifera* describes the potential of all parts of the species to cure many painful and deadly diseases (Kumar and Kushwaha 2006). The roots are used as a nutrient and health restorative medicine in pregnant women and old people. The decoction of the roots boiled with milk and ghee is recommended for curing sterility in women. The roots are also used in constipation, senile debility, rheumatism, general debility, loss of memory, loss of muscular energy and spermatorrhoea (Watt 1972; Mirjalili, et al. 2009b).

The leaves of the plant are bitter in taste and used as an anthelmintic. Bruised leaves and fruits are locally applied to tumors and tubercular glands, carbuncles and ulcers (Kapoor 2001). The plant has antibiotic, anti-viral and anti-amoebic properties. Its fruits and seeds are diuretic, hypnotic and employed in curdling plant milk to prepare vegetable cheese. They are also rich in saponins and can be used as substitutes of soap (Saritha and Naidu 2007). Saponins are important components of a number of herbal medicines and known for a wide range of bioactivities like allelopathic, antimicrobial, anticholesterolemic, adjuvant and haemolytic activities (Madina et al. 2007). The natural wild populations of *W. somnifera* contain a tongue paralyzing constituent in their roots and hence cannot be used for ayurvedic treatments. The improved, cultivated varieties however do not have such an ingredient. The wild and cultivated plants contain the same alkaloids but exhibit different morphologies and therapeutic actions (Kaul 1957).

1.3.4.1 Anti-inflammatory properties

The effectiveness of Ashwagandha in a variety of rheumatologic conditions may be due in part to its anti-inflammatory properties. Rats given powdered root of Ashwagandha orally

one hour before being given injections of Freund's complete adjuvant over a period of three day showed considerable reduction in inflammation (Begum and Sadique 1988).

1.3.4.2 Antitumor properties

The use of *W. somnifera* on animal cell cultures caused decrease in levels of the nuclear factor kappa B, suppression of intercellular tumor necrosis factor and also potentiates apoptotic signaling in cancerous cell lines (Ichikawa et al. 2006). *W. somnifera* has the capacity to fight cancers by reducing the size of tumour (Prakash et al. 2002; Jayaprakasam et al. 2003**).** Antitumor and radio-sensitizing effects of Withaferin were also seen in mouse Ehrlich ascites carcinoma *in vivo* (Sharad et al. 1996).

1.3.4.3 Antistress effect

W. somnifera has traditionally been used to stabilize mood in patients with behavioral disturbances. Research has revealed that the herb produces an anti-depressant and anti-anxiety effect in rodents comparable to the anti-depressant drug imipramine and the anti-anxiety drug lorazepam (Archana and Namasivayam 1999).

1.3.4.4 Antioxidant effect

Sitoindosides VII-X and Withaferin A found in *W. somnifera* are powerful antioxidants which were tested for their antioxidant activity using the major free-radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) in the rat brain frontal cortex and striatum (Dhuley 2000).

1.3.4.5 Immunomodulatory properties

In a study using mice, administration of powdered root extract from Ashwagandha was found to enhance total white blood cell count. In addition, this extract inhibited delayed type hypersensitivity reactions and enhanced phagocytic activity of macrophages when compared to a control group (Davis and Kuttan 2000).

1.3.4.6 Hematopoietic effect

Administration of *W. somnifera* extract was found to significantly reduce leukopenia induced by cyclophosphamide (CTX) treatment in Swiss albino mice. The major activity of *Withania* may be the stimulation of stem cell proliferation (Davis and Kuttan 1998).

1.3.4.7 Effects on nervous system

Total alkaloid extract (ashwagandholine, AG) of *Withania* roots has been studied for its effects on the central nervous system (Malhotra et al. 1965). Effects of sitoindosides VII-X and Withaferin isolated from aqueous methanol extract of roots of cultivated varieties of *Withania* were studied on brain cholinergic, glutamatergic and GABAergic receptors in male Wistar rats (Schliebs et al. 1997).

1.4 Withanolides

1.4.1 Discovery and significance

The first withanolide, "withaferin", was discovered as a constituent of the leaves of *W. somnifera* (Yarden and Lavie 1962). This metabolite represented a novel type of steroids characterized by an α,β-unsaturated lactone linked to C-17 of the sterane skeleton (Lavie et al. 1965a). However, "withaferin" turned out to be 2, 3-dihydro-3-methoxywithaferin A co-occurring with Withaferin A (Fig 1.4) (Lavie et al. 1965b). Stereochemistry of the latter constituent was determined by the same authors (Lavie et al. 1966). The yields of Withaferin A from intact plants of *Withania* spp. (Israel chemotype) are 0.2-0.3% of DW of leaves (Abraham et al. 1968). To date, about 400 withanolides or closely related congeners have been discovered in altogether 58 solanaceous species belonging to 22 genera.

Some of the solanaceous species containing withanolides or closely related derivatives are: *Acnistus, Brachistus, Browallia, Datura, Deprea, Discopodium, Dunalia, Eriolarynx, Exodeconus, Hyoscyamus, Iochroma, Jaborosa, Leucophysalis, Lycium, Nicandra, Physalis, Salpichroa, Solanum, Tubocapsicum, Vassobia, Withania* and *Witheringia.*

Non-solanaceous occurrence of withanolides has been reported in: *Tacca* spp. of the Taccaceae, *Ajuga* spp. of Lamiaceae and some marine organisms.

The early discoveries of withanolides were caused by bioassay-guided fractionation, e.g., Withaferin A, since alcoholic extracts of *A. arborescens* leaves showed significant tumor inhibitory activity (Kupchan et al. 1965). Consequently, many other biological/pharmacological activities of withanolides were discovered: immunomodulating properties, cytotoxic/tumor inhibiting activities, anti-inflammatory effects,

hepatoprotective properties, antifungal and antibacterial activities, antifeedant and insecticidal properties.

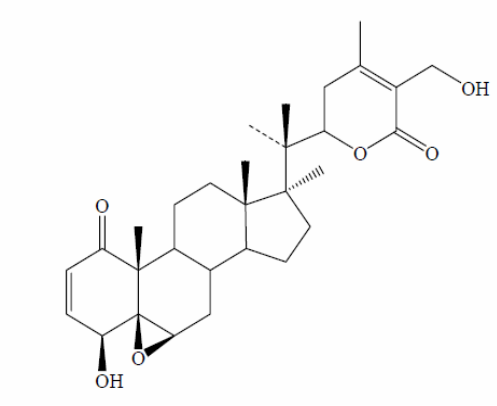


Fig 1.4: Structure of Withaferin A

In addition, Veleiro et al. (2005) have summarized certain recently discovered properties such as antifertility effects or trypanocidal and leishmanicidal activities as well as phytotoxicity (15, 21-cyclowithanolides from *J. bergii*). In particular, the induction of the phase II drug metabolizing enzyme quinone reductase (QR) by withanolides, used to determine their potential cancer chemopreventive property, turned out to be a promising field.

1.4.2 Structure

Withanolides are a group of naturally occurring C_{28} steroidal lactones built on an intact or rearranged ergostane framework, in which C-22 and C-26 are appropriately oxidized to form a six-membered lactone ring (Glotter 1991) (Fig 1.5). "Withanolide" represents the term for 22-hydroxyergostan-26-oic acid-22, 26-olide (Lavie et al. 1965a, b).

Typical substitutions and other modifications for the existing metabolites are the following:

- Oxo group at C-1; instead less frequently a hydroxyl group
- Double bond C-2 → C-3; instead less frequently a hydroxyl group at C-3
- δ-Lactone (26 → 22*O*) often unsaturated (24,25)
- γ-Lactone moiety (26 → 23*O*) instead of δ-lactone, often also unsaturated
- Lactol moiety instead of lactone residue

- High degree of oxidation at many positions of the whole molecule (e.g., oxo groups, hydroxyl groups, epoxide substructures, hemiketals)
- High diversity with regard to such functionalizations with the consequence of oxidative cleavages and/or novel cyclizations

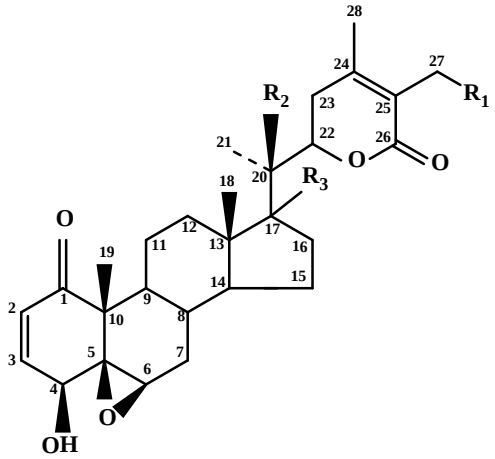


Fig 1.5: Basic skeleton of some withanolides (Alfonso and Kapetanidis 1994)

Withaferin A R_1=OH, R_2=H, R_3=H; Withanolide D R_1=H, R_2=OH, R_3=H; 27-Deoxywithaferin A R_1=H, R_2=H, R_3=H; 14α-OH, R_1=H, R_2=H; 17α-OH, R_1=H, R_2=H, R_3=H; 27-Hydroxywithanolide D R_1=OH, R_2=OH, R_3=H; 14α-OH, R_1=OH, R_2=H, R_3=H; 17α-OH, R_1=OH, R_2=H, R_3=H; Dihydrodeoxywithaferin A 2,3-diH, R_1=H, R_2=H, R_3=H; Dihydrowithaferin A 2,3-diH, R_1=H, R_2=OH, R_3=H; 17-Hydroxywithaferin A, R_1= R_3=OH, R_2=H

1.4.2.1 Metabolites with an unmodified withanolide skeleton

Withanolides with an unmodified β-oriented side chain: This group of withanolides is a voluminous one comprising, e.g., metabolites from the genera *Withania*, *Datura*, *Dunalia* and *Lycium*. A classical example is represented by Withaferin A (Fig 1.4).

Withanolides with an unmodified α-oriented side chain: This group is less frequent though not rare; they occur in the genera *Withania* and *Jaborosa* eg. Withanolide E and the withanolide derivative jaborosalactol N.

1.4.2.2 Metabolites with a modified withanolide skeleton

This group includes:

- Withanolides with an aromatic moiety
- 18-Functionalized withanolides
- 13, 14-Secowithanolides

- 21-Functionalized withanolides
- Withanolides with modifications due to an integration of C-12 into an additional ring system
- Withanolides with unusual hetero atoms (chlorowithanolides, thiowithanolides)
- Withanolide glycosides
- Glycosidic withanolide congeners without lactone cyclization

1.4.3 SMs in *W. somnifera*

Withanolides are the major chemical constituents of *W. somnifera*, mainly localized in leaves, and their concentration usually ranges from 0.001 to 0.5% DW (Bandyopadhyay et al. 2007). Use of *W. somnifera* as a valuable and very popular drug in Ayurvedic medicine (Ray and Gupta 1994) and *Physalis alkekengi* in Chinese medicine are two outstanding examples for an extensive significance of withanolide containing plants in traditional/folk medicine. The properties of withanolides in *W. somnifera* are elaborated in section 1.3.4.

Analysis of *W. somnifera* roots has resulted in the isolation of a new dimeric thiowithanolide, named ashwagandhanolide (Fig 1.6) (Subaraju et al. 2006). A bioassay-guided purification of the methanolic extract of *W. somnifera* fruits yielded withanamides A-I. The structure of these compounds was determined by using serotonin, glucose and long-chain hydroxyl fatty acid moieties (Jayaprakasam et al. 2004). The isolation of nicotine, somniferine, somniferinine, withanine, withananine, pseudowithanine, tropine, pseudotropine, 3α-trigloyloxytropane, choline, cuscohygrine, *dl*-isopelletierine and new alkaloids anaferine and anhygrine has been described earlier (Gupta and Rana 2007). The reported total alkaloid content in the roots of Indian *W. somnifera* varies between 0.13 and 0.31%. In addition to the alkaloids, the roots are reported to contain starch, reducing sugars, hentriacontane, glycosides, dulcitol, withanicil and four types of peroxidases. The leaves are reported to contain five unidentified alkaloids chlorogenic acid, calystegines (nitrogen-containing polyhydroxylated heterocyclic compounds) withanone, condensed tannin and flavonoids (Johri et al. 2005).

1.4.4 Biosynthesis

The biosynthetic pathways of withanolides and other chemical constituents of *W. somnifera* are not fully known, and there is very little information about their biogenetic

aspects (Kirson et al. 1977; Nittala and Lavie 1981; Ray and Gupta 1994). 24-Methylene-cholesterol was proposed as a C_{28} sterol precursor of the withanolides based on corresponding feeding experiments with *W. somnifera* (Lockley et al. 1976). Isolated Withaferin A and Withanolide D showed radioactive incorporation of the supposed precursor whereas labelled 24-(*R, S*)-methyl-cholesterol failed to be incorporated.

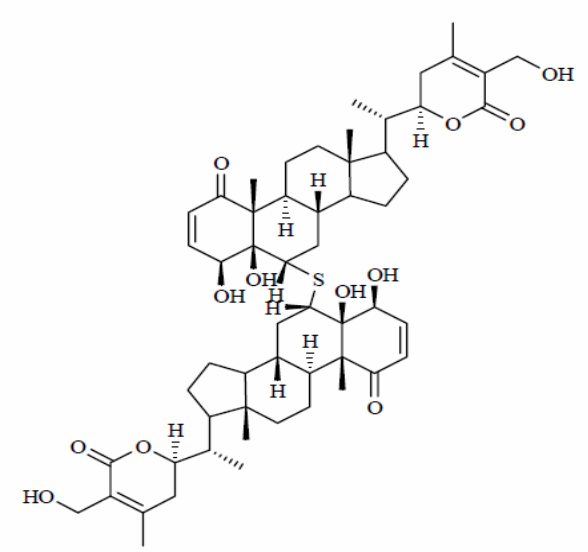


Fig 1.6: Ashwagandhanolide, a new compound isolated from *W. somnifera*

The first step in the biosynthesis of cholesterol is the activation of acetate by its conversion to acetyl Co-enzyme A. Two units of acetylCoA are combined and metabolized to mevalonic acid. Only the *R*-form of mevalonic acid is used by the living system to produce terpenes, while the *S*- form is metabolically inert. The (*R*)-mevalonic acid is converted into isopentenyl pyrophosphate (IPP) through the loss of one carbon atom. IPP then condenses in a head-to-tail manner with its isomer, 3, 3-dimethyl allyl pyrophosphate (DMAPP), to give geranyl pyrophosphate (GPP). A condensation reaction of *trans* GPP with another molecule of IPP yields farnesyl pyrophosphate (FPP). The enzyme squalene synthase catalyses the condensation of two molecules of FPP in a head to head manner in the presence of NADPH to produce squalene. Oxidation of squalene by atmospheric oxygen is catalyzed by NADPH-linked oxide to afford squalene 2, 3-epoxide. The latter undergoes ring closure to form lanosterol which is then converted into a variety of different steroidal triterpenoidal skeletons. The bioconversion of lanosterol to 24-methylenecholesterol is still not fully understood and the latter may be a biosynthetic precursor of steroidal lactones. It

has been proposed that the hydroxylation in C22 and δ-lactonization between C22 and C26 of 24-methylenecholestrol yields withanolides (Fig 1.7). It has also been suggested that the α,β-unsaturated ketone in ring A of common withanolides may be produced through the sequence 20-23 (Manitto 1981; Danishefsky 1980; Velde and Lavie 1981).

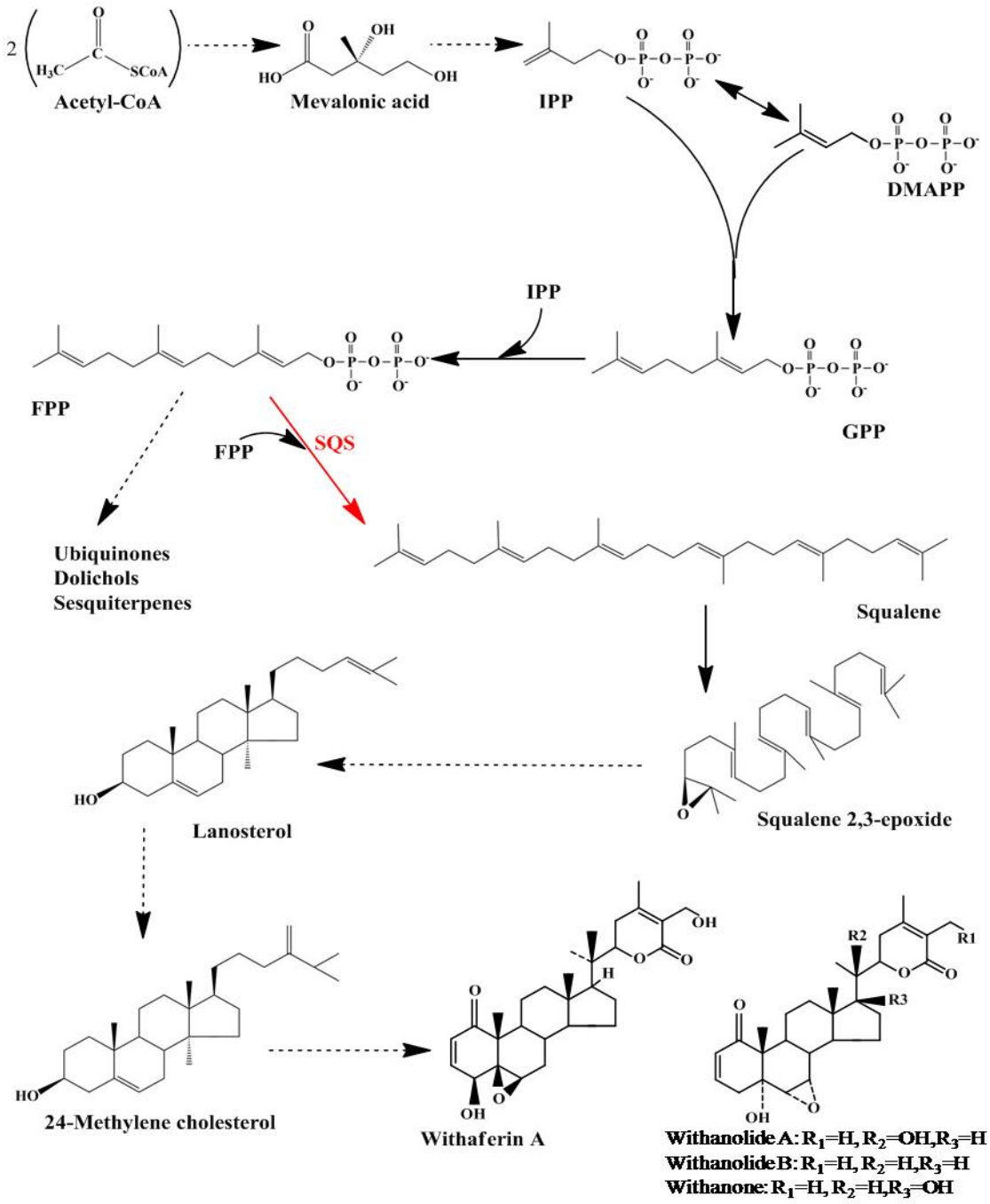


Fig 1.7: Simplified scheme of withanolide biosynthetic pathway. Abbreviations: IPP: Isopentenyl-5-pyrophosphate; DMAPP: Dimethylallyl pyrophosphate; GPP: Geranyl pyrophosphate; FPP:

Farnesyl pyrophosphate; and SQS: Squalene synthase. Dashed arrows show that multiple steps are involved in the reaction

1.5 Biosynthesis of triterpenoids

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

Isoprenoids are synthesized in plants via two pathways, the mevalonic acid (MVA) pathway localized in cytoplasm, and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway that occurs in plastids (Fig 1.8) (Eisenreich et al. 1998; Lichtenthaler 1999; Rohmer 1999). 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 starts 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 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 DMAPP *via* the enzyme IPP isomerase.

The 5-carbon compounds IPP and DMAPP are condensed to form the 10-carbon GPP which serves as the precursor for the synthesis of all monoterpenes. The addition of another IPP unit to GPP yields the 15-carbon farnesyl 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 lies 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.

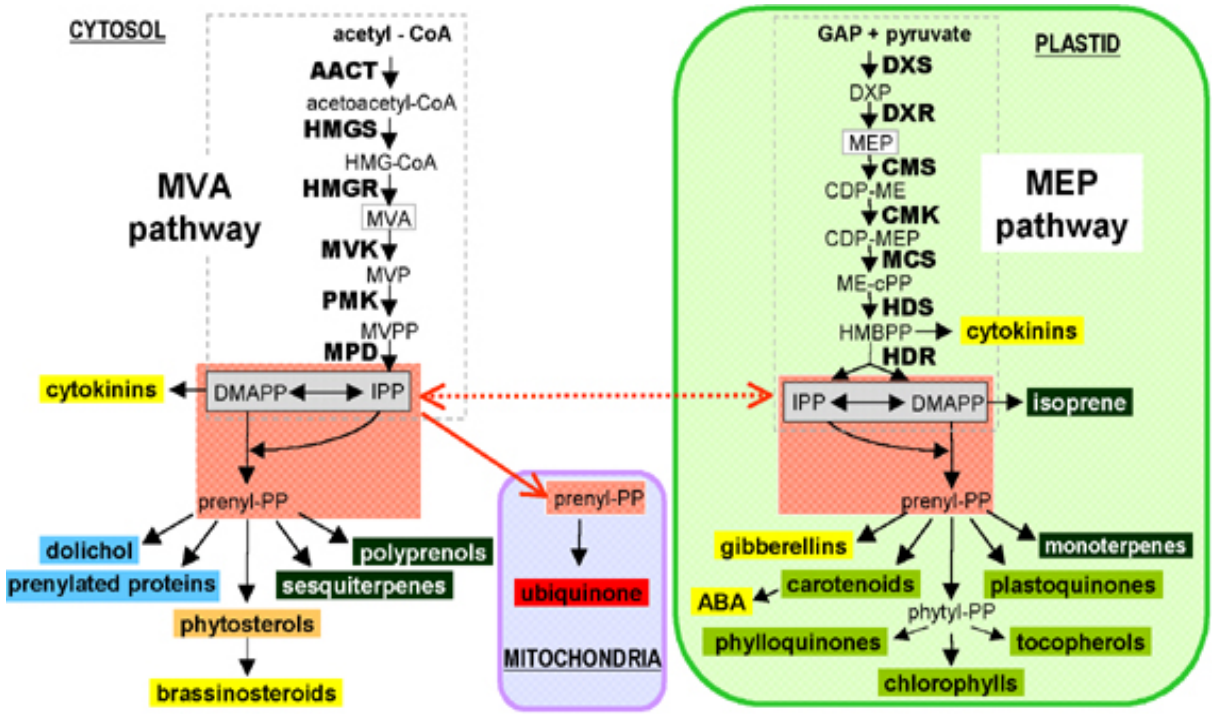


Fig 1.8: Diagrammatic representation of various terpenoids biosynthesis via MVA and MEP pathway. AACT: Acetyl-CoA-Acetly transferase; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl-CoA reductase; MVA: Mevalonic acid; MVK: Mevalonate kinase; MVP: Mevalonate 5-Phosphate; PMK: Phosphomevalonate kinase; MVPP: Mevalonate 5-Pyrophosphate; MPD: Mevalonate 5-Pyrophosphate decarboxylase; 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: 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; HDR: 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase

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 SMs 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. Squalene synthase is one such enzyme which represents the putative branch point in terpenoid biosynthetic pathway capable of diverting carbon flow specifically to the biosynthesis of triterpenes and sterols. It plays a key role and considered as committed step in triterpene biosynthesis, so engineering at this point may lead to manipulation of end products.

1.6 Squalene synthase

Squalene synthase (SQS; EC 2.5.1.21) (farnesyl diphosphate:farnesyl diphosphate farnesyltransferase) is a regulatory branch point enzyme of isoprenoid pathway diverting the carbon pool away from the central pathway towards the biosynthesis of phytosterols and triterpenoids (Abe et al. 1993) (Fig 1.7). SQS catalyses head to head reductive dimerization of two molecules of farnesyl diphosphate (FPP) (1'-1) to form a linear 30 carbon compound, squalene. SQS is also known as a bifunctional monomeric enzyme that catalyzes the reaction in two steps. First, two FPPs are condensed to form stable cyclopropylcarbinyl diphosphate intermediate, presqualene diphosphate (PSPP) (Rilling and Epstein 1969). Second, PSPP is reductively rearranged into squalene in the presence of NADPH and Mg^{2+} (Fig 1.9). The activity of SQS has been localized to the smooth ER with its carboxy terminal portion anchored to the ER membrane, whereas the catalytic site of the enzyme is associated with the amino terminal portion of the protein found on the cytoplasmic face of the ER (Robinson et al. 1993).

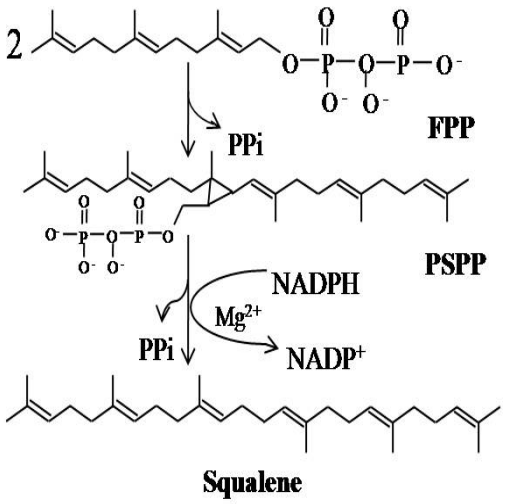


Fig 1.9: Condensation of two FPP molecules to form squalene, catalyzed by squalene synthase

SQS has been reported to play an important regulatory role in the triterpene and steroids biosynthetic pathway. Because of its particular position at the interface between hydrophilic and hydrophobic intermediates, SQS might constitute a major control point for regulating the sterol branch in directing FPP molecules into either sterols or non-sterol isoprenoids in response to changing cellular requirements (Wentzinger et al. 2002). Evidences support that inhibition of the SQS 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 SQS step 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 SQS (Keller 1996) and similar effects have been observed in plants (Fulton et al. 1995). In another study in *Artemisia anuua*, the antisense squalene synthase resulted in increase in production of artemisinin, which is belongs to isoprenoid pathway by diverting the carbon flux away from squalene (Wang et al. 2012). Similar results were obtained in *S. cerevisiae* where the quantity of amorphadiene increased to five folds by the down regulation of squalene synthase while the production of ergosterol decreased due to the decrease in squalene (Paradise et al. 2008).

cDNA clones for SQS 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. 2005), *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).

Several biochemical investigations were carried out to obtain soluble and active recombinant SQS enzyme. SQS amino acid sequence analysis provided insights for engineering more soluble variants. Jennings et al. (1991) cloned the yeast SQS gene and suggested that the enzyme consisted of a large cytosolic domain anchored to the ER by a single C-terminal transmembrane helix. Subsequently, a soluble and fully active version of recombinant yeast SQS 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 *S. 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 *S. 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).

There is only one report of crystal structure of SQS i.e. from human (Pandit et al. 2000). The structure showed that SQS is folded as a single domain, with a large channel in the middle of one face. The active sites of the two half-reactions catalyzed by the enzyme are located in the central channel, which is lined on both sides by conserved aspartate and arginine residues. One end of this channel is exposed to solvent, whereas the other end leads to a completely enclosed pocket surrounded by conserved hydrophobic residues. These observations identified residues that affect substrate binding and activity, suggesting that two molecules of FPP bind at one end of the channel, where the active center of the first half-reaction is located, and then the stable reaction intermediate moves into the deep pocket, where it is sequestered from solvent and the second half-reaction occurs. Five α helices surrounding the active center are structurally homologous to the active core in the

three other isoprenoid biosynthetic enzymes whose crystal structures are known, even though there is no detectable sequence homology (Pandit et al. 2000). The human protein sequence is identical to that of the chimpanzee and shares between 86% and 93% protein identity with other mammals (Do et al. 2009). The sequence of the catalytic site of human squalene synthase is similar to that of another enzyme involved in isoprenoid biosynthesis, phytoene synthase, which catalyzes the reaction of two molecules of geranylgeranyl diphosphate into phytoene (Summers et al. 1993).

1.7 Biotechnology and tissue culture of *W. somnifera*

W. somnifera is in great demand owing to the presence of withanolides responsible for various biological efficacies of the plant. This species propagates easily by seeds but mature and healthy seeds are not always available for germination. The viability period of seeds is very short and their germination is also poor (Vakeswaran & Krishnasamy 2003). The plant does not have the natural ability for regenerative propagation and hence it is difficult to fix the variability generated by sexual recombination (Jayanthi and Sharma 1991). Its commercial cultivation is limited due to relatively long gestation period between planting and harvesting, and the large cloning variation in the withanolide profiles. Moreover, the conventional method cannot meet the increasing demand of pharmaceutical products of the plant. *In vitro* propagation of this useful medicinal plant could provide a means of disease free healthy clones as well as for establishing biotechnological approaches to produce SMs. This technology has sound and extensive potential for rapid multiplication of plants because it is quick and allows continuous production of identical plants. Micropropagation in *Withania* through direct multiple shoot formation has been studied using different explants such as shoot tip (Jayanthi and Sharma 1991; Sen and Sharma 1991), leaf (Sharma et al. 2010; Kulkarni et al. 1996), leaf dics (Abhyankar and Chinchanikar 1996), axillary meristems (Roja et al. 1991), hypocotyls and axillary leaf (Rani and Grover 1999), node (Siddique et al 2004) and internode (Valizadeh and Valizadeh 2009) while indirect regeneration of plant through callus (Baburaj and Gunasekaran 1995; Manickam et al. 2000; Rani et al. 2003) has also been done but it is less preferred due to recalcitrance of callus to differentiation.

In one study, a simple effective protocol was developed for conservation and plant propagation through callus cultures of *W. somnifera* where seed germination percentage

reached a maximum value of 64.3% on ½ MS + 0.25 mg/L GA_3 at third week of culture. Three different basal media compared for seed germination where MS was found to be most effective. Out of 25 combinations of growth regulators evaluated, MS + 1.0 mg/L BA + 1.0 mg/L 2, 4-D found to be best for callus induction and proliferation regardless to explants. Among the four different explants tested, *in vivo* leaf explant was found most suitable for callus induction, proliferation and fresh weight gain. The highest callus induction frequency percentage 86.4% was recorded with *in vivo* leaf explant whereas, 43.4% in *in vitro* leaf explant at day 30 on MS augmented with 1.0 mg/L BA + 1.0 mg/L 2,4-D. Among different growth regulator combinations tested, 2.0 mg/L BA + 1.0 mg/L NAA was the best eliciting a maximum of 82.3% shoot induction with highest shoots/callus. Of three different auxins tested for *in vitro* rooting, IBA was most effective at an optimum concentration of 2.0 mg/L inducing rooting in 83.1% of the *in vitro* derived shoots (Rout et al. 2011).

An efficient method of *in vitro* shoot propagation of six elite accessions of *W. somnifera* was developed. Maximum numbers of shoots in all accessions were achieved from axillary explant on MS medium supplemented with 1 mg/L BAP and 1 mg/L kinetin. The *in vitro* raised shoots of all the accessions could be easily rooted on MS medium supplemented with 2 mg/L IBA (Sabir et al. 2007).

A number of experiments were undertaken with nodal explants of Ashwagandha in an attempt to improve the process of regeneration of multiple shoots *in vitro*. The optimum medium for multiple shoot formation was MS medium with BAP (1.5 mg/L) and IAA (1.5 mg/L). Gibberellic acid (0.15 mg/L) was effective in enhancing the maximum shoot elongation. 72% of *in vitro* raised shoots responded rooting on MS medium fortified with IBA (5.0 mg/L) (Kumar et al. 2011).

Regeneration from leaf explants of *W. somnifera* for mass propagation was studied on MS medium supplemented with kinetin and BAP alone or in combination. Shoot buds were induced from the midrib on the abaxial side in presence of kinetin and BAP (4 μM). Rooting of these shoots was achieved in 0.5 μM of IBA (Joshi and Padhya 2010).

Tissue culture was established from axillary meristems of the *W. somnifera* where multiple shooting was observed in MS medium supplemented with BA, rooting in MS + coconut milk (10%) and callus formation in MS + 2,4-D (2.0 ppm). Callus cultures failed to

synthesize withanolides, but multiple shoot cultures synthesized significant amounts; with a highest concentration in 2.0 mg/L BA (Roja et al. 1991).

Multiple shoot cultures of *W. somnifera* from single shoot tip explants and their potential for the production of Withaferin A and Withanolide D have also been investigated. Shoot tips grown on MS medium supplemented with BA (1 mg/L) induced 10.0 microshoots per explant which accumulated Withaferin A and Withanolide D. Supplementation of MS solid agar medium with 1.0 mg/L BA and 4% sucrose enhanced accumulation of both withanolides. MS liquid medium containing 1.0 mg/L BA and 10% coconut milk favoured a maximum increase in biomass (27 fold), induced microshoots as well as accumulation of Withaferin A (Ray and Jha 2001).

Direct rooting from leaf explants of *W. somnifera* has been achieved on 1/2 MS medium supplemented with 15 g/l sucrose, and different concentrations of growth regulators. The roots were cultured on MS liquid medium for the establishment of root-organ cultures. The concentration of alkaloids increased compared to field grown roots (Wadegaonkar et al. 2006).

Recently, Withanolide A biogeneration in shoot cultures of *W. somnifera* has been reported using nodal segments as explants (Sangwan et al. 2007). The production of Withanolide A in the cultures varied considerably (*ca.* 10-fold, 0.014 to 0.14 mg/g FW) according to the hormone composition of the culture media as well as the genotype of the explant source. Hormonal combination of 1.0 ppm of BAP and 0.5 ppm of kinetin displayed the highest concentration of Withanolide A in the green shoots.

1.8 Hairy root cultures as a source of withanolides

Several studies have been done on investigating the potential of hairy root cultures transformed with *A. rhizogenes* for the production of SMs (Mano et al. 1986; Payne et al., 1987; Shanks and Morgan 1999).

Banerjee *et al*. (1994) carried out hairy root transformation of *W. somnifera* by three different strains of *A. rhizogenes* (A4, LBA 9402 and LBA 9360) and analyzed the specificity and frequency of their withanolide production with special reference to Withaferin A. The best response in terms of transformation ability and growth of the hairy roots was obtained with strain A4, followed by LBA 9402; LBA 9360 failed to induce a

transformation event. The maximum production of Withaferin A was observed in the media and hairy roots of 10-week-old cultures.

Mirjalili *et al*. (2008) showed that the inoculation of leaf sections of *W. coagulans* with *A. tumefaciens* strain C58C1 (pRiA4) induced transformed roots with two morphologies: callus-like roots (CR) with a high capacity to produce withanolides and typical hairy roots (HR) with faster growth capacity and lower withanolide accumulation.

Withanolide production by *in vitro* cultures of *W. somnifera* transformed by *A. rhizogenes* was investigated on MS media either supplemented with BA or 2,4-D, or without any plant growth regulators. Limited production was observed in shoot and callus cultures and no withanolides were detected in hairy roots (Vitali et al. 1996).

Hairy roots cultures of *W. somnifera* transformed with *A. rhizogenes* (LBA 9402) were grown on MS medium containing 3% (w/v) sucrose in the absence of exogenous plant growth regulators where the productivity of Withanolide D in transformed roots was higher than untransformed root cultures (Ray et al. 1996).

Other studies have also been done in *Withania* using *A. rhizogenes* to produce hairy root culture for the increased production of withanolides (Bandyopadhyay et al. 2007; Kumar et al. 2005).

1.9 Molecular engineering for secondary products

Medicinal plants are highly exploited for the production and extraction of SMs, pushing them towards the verge of extinction. The production of plant SMs has, for a long time, been achieved through the field cultivation of medicinal plants. However, plants originating from particular biotypes can be hard to grow outside their local ecosystems. Moreover, the 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 SMs. Initially transgenic plants were used to increase the level of most secondary products. A more analytical approach is now used for the synthesis of secondary products. It includes identification of the enzymes in a secondary pathway and an attempt to modify these enzymes by enhancing gene expression. The problem with this approach is that the concentration of enzymes in secondary product pathways is too low for them to be isolated and purified in sufficient quantities for analysis, which is an essential step prior to

identification of the corresponding gene(s). Recognition of the regulatory genes would be the ideal approach, since these genes are going to control a large part, or the whole, of the pathway. However, many secondary pathways are not linear; rather, they are so complex network of interactions that increasing total flux through a pathway requires the engineering of a number of genes. These regulatory genes would also have to be modified for continuous expression, so that their products are not sensitive to the usual controls such as feedback inhibition (Collin 2001).

Since the low level of production of most secondary product enzymes is a major problem in identifying enzymes and subsequently genes, the methods that are now being used to clone secondary product genes are transposon tagging and differential screening. Using a PCR approach, with differential screening, it is possible to isolate a number of genes related to a pathway. The individual genes must then be heterologously expressed, the protein identified and its function judged by assay or by comparison with other proteins.

1.10 Genetic engineering of isoprenoid pathway in *W. somnifera* using squalene synthase as a target gene

W. somnifera is well known in Ayurveda for its potential use in cure of many diseases and these pharmacological properties are attributed to the presence of various steroidal lactones collectively called as withanolides. Withanolides 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 *Withania* with high withanolide contents using advanced molecular biology tools.

SQS is identified as a potential target for metabolic engineering of the plant SMs biosynthetic pathway as it regulates at a branch point of the pathway diverting the carbon flux towards the withanolide biosynthesis. Previously also, the genes that express the enzymes of isoprenoid pathway have been manipulated to enhance carbon flow into specific downstream products in various plant species (Re et al. 1995; Hey et al. 2006; Chen et al. 2000). 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 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* (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*. Similar study was also performed in *E. senticosus* (Seo et al. 2005) where overexpression of SQS in transformed plants resulted in higher triterpene saponin. Likewise, transgenic roots of *B. falcatum* overexpressing *BfSS1* in the sense orientation resulted in *SQS* mRNA accumulation and enhanced production of both phytosterol and saikosaponins (Kim et al. 2011b). Overexpression of *SQS* in *G. uralensis* led to increased glycyrrhizin content as compared to control hairy roots (Lu et al. 2008). Recently, *SQS* overexpression in *W. somnifera* also resulted in increase in Withanolide A and production of Withaferin A from the callus cell suspension cultures (Grover et al., 2012). In *W. coagulans* also, the overexpression of *SQS* from *A. thaliana* resulted in increased production of phytosterols, withanolides and triterpenoids (Mirjalili et al. 2011).

The detailed description of wide range of therapeutic properties of *W. somnifera* and withanolides, and elaboration of secondary metabolic pathway and their engineering is beyond the scope of this chapter. However, one can refer the reviews for the better understanding about the same (Mirjalili et al. 2009b; Mishra et al 2000). The present study was taken up with the objective to isolate, clone and characterize squalene synthase gene from *W. somnifera* (*WsSQS*). This was followed by the studies for the expression of WsSQS protein in heterologous system, *in-silico* studies of WsSQS (Homology modeling and docking studies) and differential expression studies of WsSQS in normal as well as in stress conditions. Transformation of *W. somnifera* plants with sense construct of *WsSQS* in binary vector was achieved to improve the content of medicinally important compounds of the plant. Efficient regeneration system and stable transformation methods were designed and applied as a prerequisite for development of transgenics.

CHAPTER 2

ISOLATION, CLONING AND CHARACTERIZATION OF SQUALENE SYNTHASE GENE(S) FROM *WITHANIA SOMNIFERA*

2.1 Introduction

W. somnifera is well known since thousands of years for its pharmacological and medicinal properties which are attributed to the characteristic SMs of the plant, called withanolides (Singh et al. 2001; Misra et al. 2005; Matsuda et al. 2001). The biosynthetic pathway of withanolides is still unclear but they are supposed to be derived from cholesterol. Squalene synthase (SQS) is a regulatory branch point enzyme which diverts carbon flux away from the central isoprenoid pathway towards the biosynthesis of withanolides. It is a NADPH dependent alkyl transferase which catalyses head-to-head transfer of a farnesyl group to another FPP to form PSPP and its subsequent conversion to squalene in the presence of NADPH and Mg^{2+}. As studied in engineered yeasts (Paradise et al. 2008; Shimada et al. 1998), down regulation of the squalene synthase in the sterol biosynthetic pathway leads to the accumulation of FPP, which is redirected away from this pathway and towards the synthesis of other commercially important isoprenoids.

As a key enzyme in the regulation of isoprenoid biosynthesis, SQS encoding genes have been cloned and characterized from several organisms including bacteria (Lee and Poulter 2008), yeasts (Jennings et al. 1991; LoGrasso et al. 1993; Kennedy et al. 1999; Zhang et al. 1993), fungi (Zhao et al 2007; Merkulov et al. 2000; Zhao et al. 2010), algae (Okada et al. 2000), protozoa and animals (Bhargava et al. 2010; Inoue et al. 1995; McKenzie et al. 1992; Sealey-Cardona et al. 2007), and human beings (Robinson et al. 1993; Summers et al. 1993; Guan et al. 1995). Plant SQS genes have also been characterized in *Nicotiana tabacum* (Devarenne et al. 2002), *P. ginseng* (Lee et al. 2004; Kim 2011a), *G. glabra* (Hayashi et al. 1999), *Euphorbia tirucalli* (Uchida et al. 2009) and other monocot and dicot plants (Kribii et al. 1997; Yoshioka et al. 1999; Hata et al. 1997; Nakashima et al. 1995, Zhou et al. 2012, Belingheri et al. 1991, Huang et al. 2007, Yun et al. 2003, Kim et al. 2005; Lee et al. 2002; Suzuki et al. 2002; Akamine et al. 2003).

Though SQS plays an imperative function in terpenoid biosynthesis, the role of this gene is still unexplored in *W. somnifera.* Hence, the present chapter is aimed at the molecular studies on squalene synthase from *W. somnifera* with regard to its isolation, cloning and bioinformatic characterization with the help of online bioinformatic tools. There is considerable interest in understanding role of this gene in withanolides biosynthesis which

will facilitate future metabolic pathway engineering for development of elite plant species with improved withanolide contents.

2.2 Materials

Taq polymerase was procured from Bangalore Genei (India), ligases, reverse transcriptase and restriction enzymes were procured from Promega (USA). PCR primers were synthesized from MWG (India). Gel elution kit and RACE kit was purchased from Axygen (USA) and Clontech (USA), respectively.

Withania somnifera (Solanaceae) plant was collected from the Department of Horticulture, National Chemical Laboratory, Pune, Maharashtra.

Refer Appendix-I, II and VI for vectors, bacterial strains and bacterial media, respectively.

2.3 Methods

2.3.1 Total RNA isolation

RNase free lab environment was created and maintained as described by Blumberg (Blumberg 1987). All glassware and plasticware were DEPC (0.1% in water) treated overnight and autoclaved. All materials were dried in a vacuum oven. The pestle and mortar were also treated in DEPC and heated at 300 ºC for about 6 h.

Total RNA was isolated from aerial parts of *W. somnifera* using TRIzol reagent (Sigma, USA). The plant tissue was collected, washed with DEPC water, frozen in liquid nitrogen and crushed to a fine powder. To 100 mg of the fine powder, 1 mL of TRIzol reagent was added and vortexed thoroughly. 300 µL Chloroform: isoamyl alcohol (24:1) was added and vortexed. The tubes were centrifuged at 13,000 g for 15 min at 4 ºC. The supernatant was transferred to 1.5 mL tubes and the chloroform: isoamyl alcohol step was repeated. The aqueous phase was transferred to 1.5 mL tubes and 0.6 volume isopropanol added. It was mixed thoroughly and kept for RNA precipitation for 1 h at room temperature. Total RNA was pelleted out by centrifugation at 13,000 g for 15 min at 4 ºC, washed with 70% ethanol twice and dried in a Speedvac centrifugal concentrator. RNA pellet was dissolved in 40 µL of DEPC treated water, checked on 1% agarose gel and stored at -80 ºC in aliquots. DNA/RNA purity and concentration of nucleic acid was determined by measurement of absorbance at 260 nm on Nanovue (GE Healthcare, Sweden).

2.3.2 Synthesis of cDNA first strand by reverse transcription (RT)

cDNA was synthesized using total RNA by AMV reverse transcriptase using AMV-RT system (Promega) as per the manufacturer's guidelines. RT-PCR was performed in a 20 µL reaction mixture as follows:

RNA (2 µg)	1-6 µL
Oligo (dT_{15})/Random-primers (10 pmol)	0.5 µL
Adjusted with nuclease free water	**10.0 µL**

The primer-template mixture was denatured at 70 ºC for 5 min and snap chilled on ice. A reverse transcription reaction mix was prepared simultaneously on ice as follows:

Nuclease-free water	1.5 µL
5X Reaction Buffer	4.0 µL
$MgCl_2$ (15 mM)	2.0 µL
dNTP Mix (10 mM)	1.0 µL
RNasin® (40U/µL)	0.5 µL
AMV Reverse Transcriptase	1.0 µL
Final volume	**10.0 µL**

The template-primer mixture was added to the above 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 or directly added to amplification reactions.

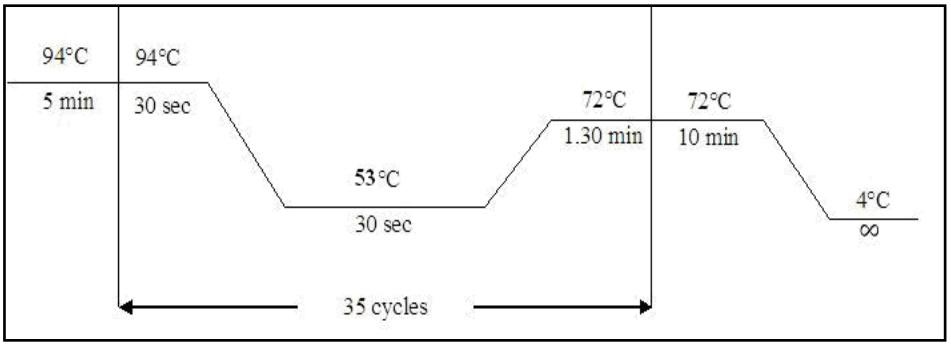
2.3.3 Isolation of partial *SQS* fragment

2.3.3.1 PCR amplification

The nucleotide sequences of *SQS* from various sources available in the NCBI GenBank database were aligned and multiple sets of primers were designed from the conserved regions. The PCR reaction mixture and generalized cycling conditions used were as follows:

Reagents 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:



The amplified PCR product was recovered from agarose gel by using gel elution kit as per manual instructions. The gel eluted fragment was ligated into pGEM-T® Easy vector (Appendix-I) in the molar ratio of vector: insert (3:1) with the help of T4 DNA ligase (1.5 U) and incubated at 16 ºC overnight for ligation.

2.3.3.2 Bacterial transformation

E. coli XL10 Gold (Appendix-II) was grown at 37 ºC with shaking at 200 rpm in LB broth and maintained on LB plates with 1.5% agar. Competent cells were prepared according to Sambrook et al. (1989). Briefly, 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. Overnight grown culture (1%) was used to inoculate 50 mL of SOB medium which was grown at 37 ^{o}C under shaking 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 (Appendix-VI) and kept on ice for 30 min

followed by centrifugation for 10 min at 5,000 rpm at 4 ^{o}C. The pellet obtained was resuspended in 5 mL TB buffer containing 7% DMSO. It was then distributed into aliquots of 100 µL in sterile 1.5 mL eppendorf tubes, frozen in liquid nitrogen and stored at -80 ^{o}C till use.

Competent cells were transformed with ~50 ng DNA/ ligation mixture according to protocol of Sambrook et al. (1989). Transformed cells were grown in LB medium supplemented with appropriate antibiotics (Appendix-VII) used for their selection.

2.3.3.3 Screening of recombinant cells

Single transformed colonies were individually picked from the agar plates with the help of microtip and suspended in 20 µL of sterile miliQ water in 1.5 mL eppendorf tubes. Bacteria were lyzed by heating at 95 ^{o}C for 5 min, and after brief spin, 2 –3 µL of supernatant was used as a template for PCR using T7 and SP6 primers (Appendix-IV). 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**.** Plasmids of the PCR positive clones were isolated according to the alkaline lysis method of Sambrook et al. (1989). These recombinant pGEM-T plasmids were then digested by *Eco*RI restriction enzyme to check the size and insertion of the cloned DNA fragment in the vector. The additions were done as follows:

Sterile MilliQ water	14.5 µL
Plasmid DNA	3.0 µL
Buffer H (10 X)	2.0 µL
*Eco*RI (10 U/ µL)	0.5 µL
Total volume	**20.0 µL**

The reaction mixture was incubated at 37 ^{o}C for 1 h and checked by 1% agarose gel electrophoresis. Plasmids with appropriately sized digestion bands were then sequenced on the ABI Prism 3730 DNA analyzer (Applied Biosystems) at Genomebio Biotech Pvt. Ltd., Pune, Maharashtra, India. The transformed cells harboring the recombinant plasmids with SQS inserts were stored as stock with 20% sterile glycerol, frozen in liquid nitrogen and stored at -80 ^{o}C.

2.3.4 Isolation of full-length *SQS* fragment

The primers SQS F0 and SQS R0 (Appendix-IV) were used for isolating full length *SQS* gene. PCR was performed with Hi-fidelity Platinum-*pfx* DNA polymerase (Invitrogen) by using cDNA as a template. Amplicon was cloned in pGEM-T Easy vector, sequenced and maintained as mentioned in section 2.3.3.3.

2.3.5 Rapid amplification of cDNA ends (RACE)

SMART^{TM} RACE cDNA Amplification Kit was used to perform RACE and the reactions were set up as per the manufacturer's guidelines with minor modifications.

For synthesis of 5' and 3' RACE ready cDNAs, the oligonucleotides provided in the kit were used as follows:

Components	**5' RACE ready cDNA**	**3' RACE ready cDNA**
RNA	1 µg	1 µg
5'/ 3' CDS Primer A	1 µL	1 µL
SMART^{TM} II A oligonucleotide	1 µL	-
Total volume (adjusted with sterile milliQ water)	Adjusted to 5 µL	Adjusted to 5 µL

The components were mixed and incubated for 2 min at 70 ºC. The tubes were cooled on ice for 2 min and rests of the components were added to it as follows:

Components	**Volume**
RNA + oligos	5 µL
5 X First strand buffer	2 µL
DTT (20 mM)	1 µL
dNTPs mix (10 mM)	1 µL
MMLV reverse transcriptase	1 µL
Total volume	**10 µL**

Both 5' and 3' cDNAs were diluted to 50 µL with Tricine EDTA buffer and primary PCR was performed as per kit's manual. Primers used were: UPM, NUP, RACE R,and RACE FI (Appendix-IV), and the conditions used were as:

Temperature	Time	No. of cycles
94 ^{o}C	30 s	5
72 ^{o}C	1 min	
94 ^{o}C	30 s	5
70 ^{o}C	30 s	
72 ^{o}C	1 min	
94 ^{o}C	30 s	30
68 ^{o}C	30 s	
72 ^{o}C	1 min	
72 ^{o}C	10 min	1

Primary PCR product was diluted 50 times with Tricine EDTA buffer and a secondary (nested) PCR was performed using NUP, RACE NR, and RACE NFI (Appendix-IV) primers with conditions:

Temperature	Time	No. of cycles
94 ^{o}C	5 min	1
94 ^{o}C	1 min	35
70 ^{o}C	30 s	
72 ^{o}C	1 min	
72 ^{o}C	7 min	1

RACE PCR was used to isolate full-length *SQS* gene with UTRs which was further cloned in pGEM-T Easy vector and sequenced as described in section 2.3.3.3.

2.3.6 Bioinformatic characterization and phylogenetic tree analysis

Nucleotide and amino acid sequence analysis was done using software pDRAW 32, ClustalX 2.0 and online bioinformatics analysis servers 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 ProtParam (http://web.expasy.org/protparam/). Phylogenetic analysis was conducted in MEGA4.0.2 (Tamura et al. 2007). 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).

2.4 Results and discussion

2.4.1 Multiple sequence alignment for primer designing

Nucleotide sequences of *SQS*s [*Lotus japonicus* (AB102688), *Glycine max (*AB007503) and *Capsicum annuum* (AF124842)] available at NCBI GenBank database were aligned using Clustal W program (Fig 2.1).

```
L. japonicus        ATGGGGAGTTTGGGAGCGATTGTGAAGCATCCAGATGATTTGTACCCGCTGCTGAAGCTC 60
G. max              ATGGGGAGTTTGGGAGCGATTCTGAAACATCCAGATGACTTTTACCCTCTGCTGAAGCTG
C. annuum           ATGGGGACTTTGAGAGCGATTTTGAAGAATCCAGATGATTTGTATCCATTGATAAAGCTA
                    ***   *   *     *  *  * *   * ** ** **  *     *  *  * **  *

                                          SQSF1
L. japonicus        AAAATGGCGGCGAGGCACGCCGAGAAGCAGATCCCTTCGGAGCCGCATTGGGGCTTCTGC 120
G. max              AAAATGGCGGCGAGGAACGCCGAGAAGCAGATCCCGCCGGAGCCGCACTGGGCGTTCTGC
C. annuum           AAACTAGCGGCTCGACATGCCGAAAAGCAGATCCCGCCGGAGCCACATTGGGGATTCTGT
                     **    **        * **    ** ***** **     * **  *    *  ** *

                                   SQSF2
L. japonicus        TACTCCATGCTCCACAAGGTTTCTAGAAGCTTCGCCCTCGTCATTCAGCAACTCGACACT 180
G. max              TACACAATGCTCCACAAGGTTTCTCGAAGTTTCGCCCTCGTCATTCAGCAACTCGGCATC
C. annuum           TACTTAATGCTTCAAAAAGTCTCTCGTAGTTTTGCTCTCGTCATTCAACAGCTTCCTGTC
                    **    ** ** ** ** ** ***   ** **     * ** ***** ** **

L. japonicus        GATCTTCGCAACGCCGTTTGCATATTCTACTTGGTTCTTCGAGCTCTGGATACCGTTGAG 240
G. max              GAGCTTCGCAACGCCGTTTGCATATTTTATTTGGTTCTGCGAGCTCTCGACACCGTTGAG
C. annuum           GAGCTTCGTGATGCTGTATGCATATTCTATTTGGTCCTTAGAGCACTTGACACTGTCGAG
                    *  * **  * ** ** **  * ** ** **  * **  * *  ** ** ** ** ***

L. japonicus        GATGATACGAGCATAGCTACAGAAGTAAAGGTCCCCATACTGAAAGCTTTTCACCGTCAC 300
G. max              GATGATACTAGCATAGAAACAGATGTCAAGGTGCCAATACTGATAGCCTTTCACCGTCAT
C. annuum           GATGATACCAGCATTCCCACGGATGTTAAAGTACCTATTCTGATCTCTTTTCATCAGCAT
                    ***** *  **  *       **    **  * ** **  ***     ** **   **

L. japonicus        ATCTATGATCGTGATTGGCACTTCTCATGTGGCACAAAGGAGTACAAAGTTCTCATGGGC 360
G. max              ATTTATGATCGTGATTGGCACTTTTCATGTGGCACAAAGGAGTACAAAGTTCTAATGGAC
C. annuum           GATGATACCAGCATTCCCACGGATGTTAAAGTACCTATTCTGATCTCTTTTCATCAGCAT
                    ** **  **  *   ***** *   * ** **    ** ** ** **    ** **** *

L. japonicus        CAGTTTCATCTTGTTTCAACTGCTTTTCTGGAACTTGCAAAGAACTATCAGGAAGCAATT 420
G. max              CAGTTTCATCATGTTTCAACTGCTTTTCTGGAACTTGGAAAGAACTACCAGGAAGCAATT
C. annuum           CAGTTCCATCATGTCTCAACTGCTTTTCTGGAACTTGGAAAAAATTATCAGCAAGCAATT
                    * ** ** *  ** **    ** *** *    **    *     **  *  * *  **

                                          SQSR1
L. japonicus        GAAGACATTACTGACAGAATGGGTGCTGGAATGGCCAAATTTATTTGCAAGGAGGTAGAA 480
G. max              GAGGACATTACCAAAAGAATGGGTGCTGGAATGGCCAAATTTATTTGCAAGGAGGTAGAA
C. annuum           GAGGATATTACCATGAGGATGGGTGCAGGAATGGCAAAATTTATATGCAAGGAGGTGGAA
                    ** ** ** **    ** ***** ** ** ***** ** ** **  *  * ***** **

                                          SQSF3
L. japonicus        ACAATTGATGACTACGACGAATATTGTCACTATGTGGCAGGACTTGTTGGGCTGGGTTTA 540
G. max              ACAATTGATGACTATGATGAATATTGTCACTATGTGGCAGGACTTGTTGGGCTGGGTTTA
C. annuum           ACAACCGATGATTATGACGAATATTGTCACTACGTAGCTGGGCTTGTTGGGCTAGGATTG
                    *    ** ** ** ** ** ** ** ** ** *  ** ** ** *****  * ** **

L. japonicus        TCAAAGCTTTTCCATGCCTCTGGTAAAGAAAATCTGGCAGCGGATT------CCCTTTCC 600
G. max              TCAAAGCTTTTCCATGCATCTGGTTCAGAAGATCTGGCTCCAGATG------ACCTTTCC
C. annuum           TCAAAACTGTTCCATGCATCTGGGAAAGAAGATCTGGCTTCAGATT------CTCTCTCC
                    **     * **     *  *       **     **    * **          * **

L. japonicus        AATTCAATGGGTTTGTTTCTTCAGAAAACAAACATTATTCGAGATTATCTGGAAGACATA 660
G. max              AATTCAATGGGCTTGTTTCTACAGAAAACAAACATTATTCGAGATTATCTGGAAGACATC
C. annuum           AACTCCATGGGTTTATTTCTTCAGAAAACAAACATCATTAGAGATTATCTGGAAGACATA
                    ** ** *  ** ** ***** ***** ** ** ** **   *** **  * ** ** **

                                          SQSR2
L. japonicus        AACGAGATCCCCAAATCACGCATGTTTTGGCCACGGCAGATCTGGAGTAAATATGTTAAC 720
G. max              AATGAGATCCCCAAGTCACGCATGTTTTGGCCACGACAGATTTGGAGTGAATATGTTAAC
C. annuum           AATGAAGTACCCAAGTGCCGTATGTTTTGGCCCCGTGAGATTTGGAGTAAATATGTTAAC
                    ** **    ** ** *  ** ********** *   * ** ***    ******

L. japonicus        AAACTTGAGGACTTGAAATATGAGGAGAACTCTGTTAAGGCAGTGCAATGTCTAAACGAC 780
G. max              AAACTTGAGGATTTGAAATATGAGGAGAACTCTGTTAAGGCAGTGCAATGCTTAAATGAC
C. annuum           AAGCTTGAGGAGTTAAAGTATGAGGAGAACTCGGTCAAGGCAGTGCAATGTCTTAATGAC
                    ** ** *****  * **  * ** ***    *     *  * ** ** **  * ** **
```

```
L. japonicus        ATGGTCACTAATGCTCTGATGCATGCTGAAGATTGCTTAAAGTACATGTCTGATTTACGA 840
G. max              ATGGTCACTAATGCCCTGATGCATCCTGAAGATTGCTTAAAATACATGGCTGCTTTACGT
C. annuum           ATGGTCACCAATGCTTTGTCACATGTAGAAGATTGTTTGATTTACATGTCCAATTTGCGT
                    *** * ** *****  **   **     * ** **  * *  *   **       *

L. japonicus        GACGATTCTATATTTCGCTTTTGTGCTATTCCCCAGATAATGGCAATTGGAACACTTGCA 900
G. max              GACCCGCCTATATTTCGCTTTTGTGCTATACCCCAGATAATGGCAATTGGAACACTTGCA
C. annuum           GATCCTGCCATCTTTCGATTCTGTGCTATTCCACAGGTCATGGCAATTGGGACTTTAGCT
                    *     * ** **    *  ** ** ** ** *** *  ****  * *  **  * *

L. japonicus        ATATGCTACAACAACGTTGAGGTCTTCAGAGGTGTAGTGAAAATGAGGCGAGGTCTAACT 960
G. max              TTATGCTACAACAACATTGAGGTCTTCAGAGGTGTAGTTAAAATGAGGCGAGGTTTAACT
C. annuum           ATGTGCTATGACAACATTGAAGTCTTCAGAGGAGTGGTTAAAATGAGACGTGGTCTGACA
                    * ** **  * **     * ** ** **  * ** ** *   *  * ** *** * *

L. japonicus        GCCAAAGTGATTGATCGGACAAAGACCATTGCTGATGTCTATGGTGCTTTCTTTGATTTT 1020
G. max              GCCAAAGTGATTGACCGAACAAAGACTATGGCTGATGTCTACGGTGCTTTTTTTGATTTT
C. annuum           GCTAAGGCCATTGACCGGACTAGAACTATGGCTGATGTATATGGTGCTTTTTTTGACTTC
                    ** **    ** **  * ** *  *  **    ** ** **    ** ** *  ** **

                                                        SQSR3
L. japonicus        GCTTCTATGTTGGAGTCCAAGGTTGACAAAAATGATCCCAATGCAACAAAGACATTGAGC 1080
G. max              GCTTCTATGTTGGAGCCCAAGGTTGACAAAAACGATCCCAATGCAACAAAAACATTGAGC
C. annuum           TCTTGTATGCTGAAATCCAAGGTTAATAATAATGATCCAAATGCAACAAAAACTTTGAAG
                     *    *   *     * *****    *   * ***** *****       **  * *

L. japonicus        AGGCTGGTAGCTATACAGAAAACTTGCAGAGAATCTGGACTCCTAAA---TAAAAGGAAA 1240
G. max              AGGCTGGAAGCTATACAGAAAACTTGCAGAGAATCTGGTCTCTTAAG---TAAAAGGAAA
C. annuum           AGGCTTGAAGCAATCCTGAAAACTTGCAGAGACTCGGGAACCTTGAA---TAAAAGGAAA
                    * *           *     ****  *     **     *       ** ** *

L. japonicus        TCTTACATTCTGAGGAAAGAGAACGGATATGGCTCAACACTGATTATCATACTGGTCCTC 1300
G. max              TCTTACATTGTGAATGATGAAAGTGGATATGGCTCAACCATGATTGTCATATTGGTCATC
C. annuum           TCTTACGTAATCAAGAGCGAGCCTACTTACAGTCCAGTTCTGATCTTTGTCATCTTCATC
                    *               *                   *       *     *

L. japonicus        TTGTTTTCCATCATGTTTGC-TTATAGCTCTGCTACCCGCCATAGTAACTAG--------
G. max              ATGGTTTCCATCATTTTTGC-TTATCTGTCTGCTAACCACCATAATAGCTAG--------
C. annuum           ATACTGGCTATTATTCTTGC-ACACCTATCTGGAAACCGCTCTTAG--------------
                     *  *  *  *  *   **    *          *
```

Fig 2.1: Multiple sequence alignment of *SQS* nucleotide sequences of *Lotus japonicus* (AB102688), *Glycine max* (AB007503) and *Capsicum annuum* (AF124842). Highlighted regions were considered for primer synthesis (Red: Forward and Green: Reverse primers)

2.4.2 Total RNA isolation and cDNA synthesis

Total RNA (Fig 2.2) was isolated from aerial part of *W. somnifera* plant and cDNA was synthesized as mentioned in section 2.3.1 & 2.

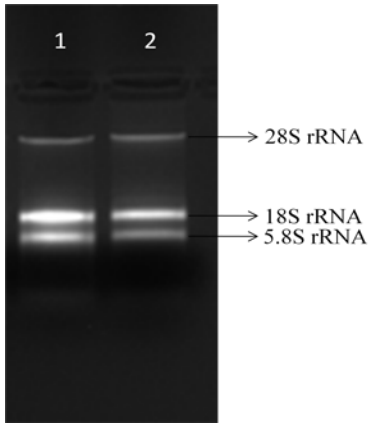


Fig 2.2: Total RNA isolated from aerial tissue of *W. somnifera*, electrophoresed on 1% agarose gel

2.4.3 PCR amplification of partial *SQS* gene

The primer combination of SQSF1-SQSR2 (Appendix-IV) gave an amplification of ~600 bp fragment using cDNA prepared from RNA of *W. somnifera* (Fig 2.4a). The fragment was cloned in pGEM-T Easy vector (Fig 2.3) and confirmed by restriction digestion with *Eco*RI (Fig 2.4b). Sequencing of these plasmids revealed a 601 bp partial fragment (Fig 2.5) showing similarity with *SQS* from different plant sources.

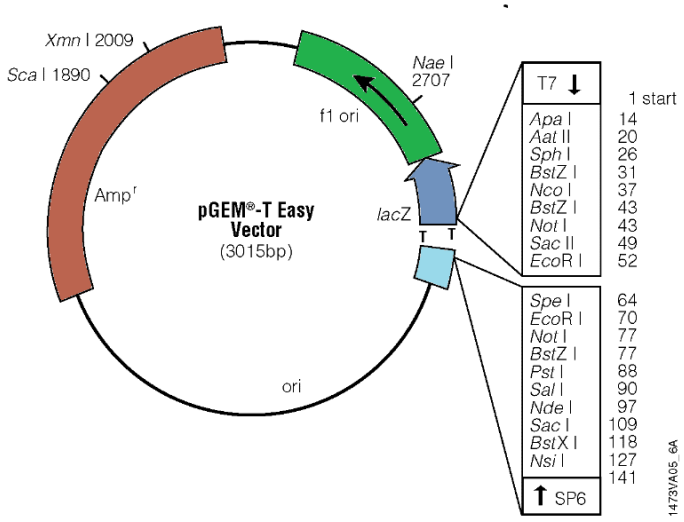


Fig 2.3 Map of pGEM -T® Easy Vector

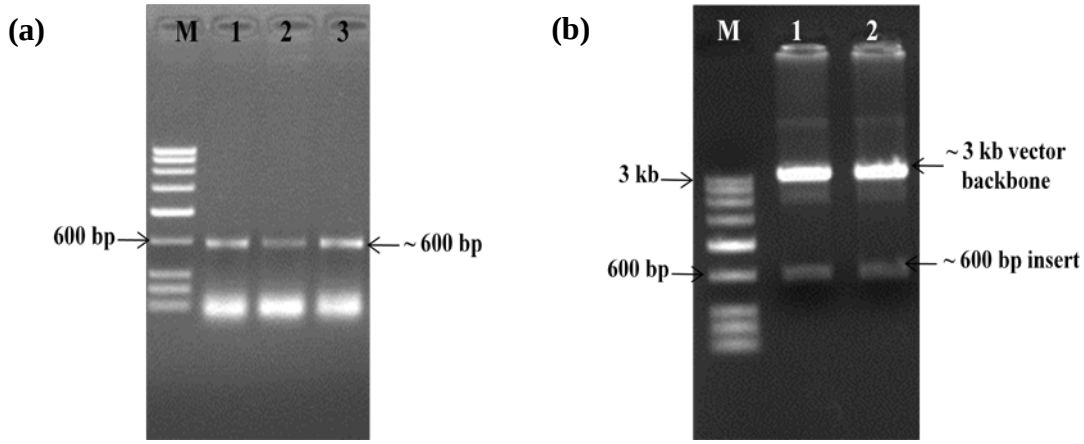


Fig 2.4: (a) PCR amplification product of ~600 bp fragment of *SQS* gene separated on 1% agarose gel. Lane 1, 2 & 3: Amplified product and lane M: Low range DNA ruler; **(b)** Partial clone of *SQS* gene in pGEM-T Easy vector releasing ~600 bp insert when digested with *Eco*RI. Lane 1 & 2: ~600bp insert and lane M: Low range DNA ruler

```
1   AAAGCGGAGA AGCAGATCCC GCCGGAGCCA CATTGGGCCT TCTGTTACTT AATGCTTCAA AAGGTTTCTC GTAGCTTTGC
81   TCTCGTCATT CAACAGCTTC CTGTTGAGCT TCGTGATGCT GTATGCATAT TCTATTTGGT TCTTCGAGCA CTTGACACTG
161 TTGAGGATGA CACCAGCATT CCCGCAGATG TTAAAGTACC TATTCTGATA TCTTTTCATC AGCATGTTTA TGACCGTGAA
241 TGGCACTTTT CATGTGGCAC GAAGGAGTAC AAGGTTCTCA TGGACCAGTT CCATCATGTT TCAACCGCTT TTCTGGAACT
321 TGGGAAACAT TATCAGCAGG CAATTCAGGA TATTACCTTG AGGATGGGTG CAGGAATGGC AAAATTTATA TGCAAGGAGG
401 TGGAAACAAC CGATGATTAT GACGAATATT GTCACTATGT AGCTGGGCTT GTTGGTTTAG GATTGTCAAA ACTGTTTCAT
481 GCCTCTGGGA AGGAAGATCT GGCTCCAGAT TCTCTCTCCA ACTCTATGGG TTTATTTCTT CAGAAAACAA ACATCATCAG
561 AGATTATTTG GAAGACATAA ATGAGGTGCC CAAGTGCCGT ATGTTTTGGC CTCGTGAGAT TT
```

Fig 2.5: Nucleotide sequence of partial *SQS* gene from *W. somnifera*. The sequences in green represent 3' RACE primers and sequences in red represent primers for 5' RACE

2.4.4 PCR amplification of full length cDNA of *SQS*

The partial fragment of SQS showed 93% similarity with *S. tuberosum,* so the primers SQS F0 and SQS R0 (Appendix-IV) were designed from the terminal sequences of *SQS* of *S. tuberosum* to amplify the ORF of *SQS* as a single PCR product. This primer combination gave an amplification of ~1.2 kb fragment (Fig 2.6a) and showed a 1.2 kb fragment upon digestion with *Eco*RI (Fig 2.6b).

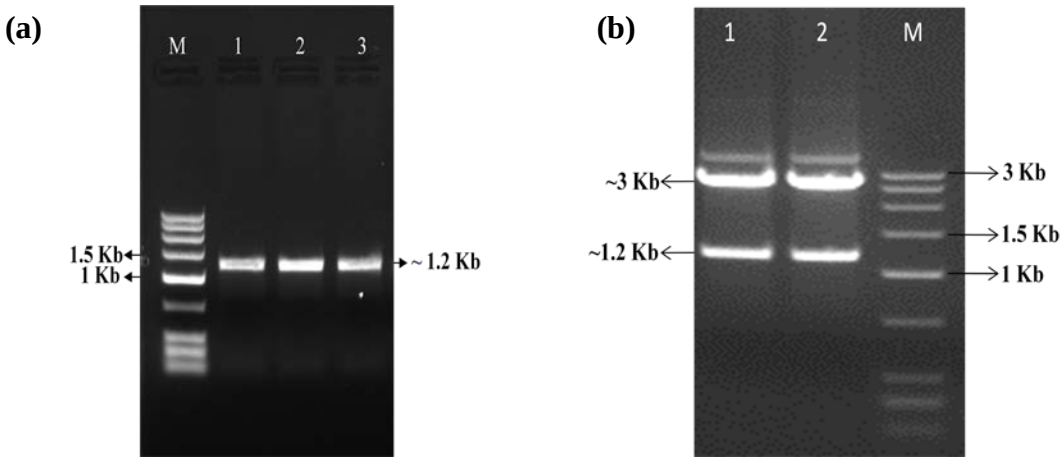


Fig 2.6: (a) PCR amplification of full length *SQS* gene separated. Lane 1, 2 & 3: Amplified product and lane M: Low range DNA ruler; **(b)** EcoRI digestion of recombinant pGEM-T Easy vector. Lane 1 & 2: ~1.2 kb insert and lane M: Low range DNA ruler

Sequencing of 10-12 different clones revealed the presence of two different *SQS* sequences, differing in some nucleotides (Fig 2.7). They were designated as ***WsSQS1*** (1236 bp) and ***WsSQS2*** (1242 bp)**,** and submitted to NCBI GenBank database having accession number GU181386 and GU732820 respectively.

(a)

```
WsSQS1 ATGGGAACATTGAGGGCGATTTTGAAGAATCCAGATGATTTGTATCCATTGATAAAACTAAAGCTTGCGGCTAGAC
WsSQS2 ATGGGAACATTGAGGGCGATTTTGAAGAATCCAGATGATTTGTATCCATTGATAAAACTAAAGCTTGCGGCTAGAC
       **********************************************************************************

WsSQS1 ATGCGGAGAAGCAGATCCCGCCGGAGCCACATTGGGCCTTCTGTTACTTAATGCTTCAAAAGGTTTCTCGTAGCTT
WsSQS2 ATGCGGAGAAGCAGATCCCGCCGGAGCCACATTGGGCCTTCTGTTACTTAATGCTTCAAAAGGTTTCTCGTAGCTT
       **********************************************************************************

WsSQS1 TGCTCTCGTCATTCAACAGCTTCCTGTTGAGCTTCGTGATGCTGTATGCATATTCTATTTGGTTCTTCGAGCACTT
WsSQS2 TGCTCTCGCCATTCAACAGCTTCCTGTTGAGCTTCGTGATGCTGTATGCATATTCTATTTGGTTCTTCGAGCACTT
       ******** **********************************************************************

WsSQS1 GACACTGTTGAGGATGACACCAGCATTCCCGCAGATGTTAAAGTACCTATTCTGATATCTTTTCATCAGCATGTTT
WsSQS2 GACACTGTTGAGGATGACACCAGCATTCCCGCAGATGTTAAAGTACCTATTCTGATATCTTTTCATCAGCATGTTT
       **********************************************************************************

WsSQS1 ATGATCGTGAATGGCACTTTTCAT------GTGGCACGAAGGAGTACAAGGTTCTCATGGACCAGTTCCATCATGT
WsSQS2 ATGATCGTGAATGGCACTTTTCATTGTTAGGTGGCACGAAGGAGTACAAGGTTCTCATGGACCAGTTCCATCATGT
       ************************      ********************************************

WsSQS1 TTCAACCGCTTTTCTGGAACTTGGGAAACATTATCAGCAGGCAATTCAGGATATTACCTTGAGGATGGGTGCAGGA
WsSQS2 TTCAACCGCTTTTCTGGAACTTGGGAAACATTATCAGCAGGCAATTCAGGATATTACCTTGAGGATGGGTGCAGGA
       **********************************************************************************

WsSQS1 ATGGCAAAATTTATATGCAAGGAGGTGGAAACAACCGATGATTATGACGAATATTGTCACTATGTAGCTGGGCTTG
WsSQS2 ATGGCAAAATTTATATGCAAGGAGGTGGAAACAACCGATGATTATGACGAATATTGTCACTATGTAGCTGGGCTTG
       **********************************************************************************

WsSQS1 TTGGTTTAGGATTGTCAAAACTGTTTCATGCCTCTGGGAAGGAAGATCTGGCTCCAGATTCTCTCTCCAACTCTAT
WsSQS2 TTGGTTTAGGATTGTCAAAACTGTTTCATGCCTCTGGGAAGGAAGATCTGGCTCCAGATTCTCTCTCCAACTCTAT
       **********************************************************************************

WsSQS1 GGGTTTATTTCTTCAGAAAACAAACATCATCAGAGATTATTTGGAAGACATAAATGAGGTGCCCAAGTGCCGTATG
WsSQS2 GGGTTTATTTCTTCAGAAAACAAACATCATCAGAGATTATTTGGAAGACATAAATGAGGTGCCCAAGTGCCGTATG
       **********************************************************************************

WsSQS1 TTCTGGCCCCGTGAGATTTGGAGTAAATATGTTAACAATGAGGTGCCCAAGTGCCGTATGTTCTGGCCCCGTGAGA
WsSQS2 TTCTGGCCCCGTGAGATTTGGAGTAAATATGTTAACAATGAGGTGCCCAAGTGCCGTATGTTCTGGCCCCGTGAGA
       **********************************************************************************

WsSQS1 TTTGGAGTAAATATGTTAACAAGCTTGAGGACTTAAAGTATGAGGAGAACTCGGTCAAGGCAGTGCAATGCCTCAAT
WsSQS2 TTTGGAGTAAATATGTTAACAAGCTTGAGGACTTAAAGTATGAGGAGAACTCGGTCAAGGCAGTGCAATGCCTCAAT
       **********************************************************************************

WsSQS1 GACATGGTCACCAATGCTTTGTCACATGTAGAAGATTGTTTGACTTACATGTCCAATTTGCGCGATCCTGCCATCTT
WsSQS2 GACATGGTCACCAATGCTTTGTCACATGTAGAAGATTGTTTGACTTACATGTCCAATTTGCGCGATCCTGCCATCTT
       **********************************************************************************

WsSQS1 TCGATTCTGTGGTATTCCACAGGTCATGGCAATTGGGACATTAGCTATGTGCTACGACAACATTGAAGTCTTCAGAG
WsSQS2 TCGATTCTGTGGTATTCCACAGGTCATGGCAATTGGGACATTAGCTATGTGCTACGACAACATTGAAGTCTTCAGAG
       **********************************************************************************

WsSQS1 GAGTGGTTAAAATGAGGCGTGGTCTGACTGCTAAGGTCATTGACCGGACTAGGACTATGGCAGATGTATATGGTGCT
WsSQS2 GAGTGGTTAAAATGAGGCGTGGTCTGACTGCTAAGGTCATTGACCGGACTAGGACTATGGCAGATGTATATGGTGCT
       **********************************************************************************

WsSQS1 TTTTTTGACTTCTCTTGTATGCTGAAATCCAAGGTTAATAATAATGATCCAAATTCAACTAAAACGTTGAAGAAGCT
WsSQS2 TTTTTTGACTTCTCTTGTATGCTGAAATCCAAGGTTAATAATAATGATCCAAATTCAACTAAAACGTTGAAGAAGCT
       **********************************************************************************

WsSQS1 TGAAGCAATCCTGAAAACTTGCAGAAATTCGGGAATGTTGAATAAAAGGAAGTCTTATGTAATCAGGAGTGAGCCAA
WsSQS2 TGAAGCAATCCTGAAAACTTGCAGAAATTCGGGAATGTTGAATAAAAGGAAGTCTTATGTAATCAGGAGTGAGCCAA
       **********************************************************************************

WsSQS1 ATTACAGTCCAGTTCTGATTATTGTCATCTTCGTCATACTGGCTGTTATTCTTTCACAACTTTCTGGCAACCGATCT
WsSQS2 ATTACAGTCCAGTTCTGATTATTGTCATCTTCGTCATACTGGCTGTTATTCTTTCACAACTTTCTGGCAACCGATCT
       **********************************************************************************

WsSQS1 TAG
WsSQS2 TAG
       ***
```

(b)

```
WsSQS1 MGTLRAILKNPDDLYPLIKLKLAARHAEKQIPPEPHWAFCYLMLQKVSRSFALVIQQLPVELRDAVCIFYLVLRALD
WsSQS2 MGTLRAILKNPDDLYPLIKLKLAARHAEKQIPPEPHWAFCYLMLQKVSRSFALAIQQLPVELRDAVCIFYLVLRALD
       ************************************************** ************************

WsSQS1 TVEDDTSIPADVKVPILISFHQHVYDREWHFS--CGTKEYKVLMDQFHHVSTAFLELGKHYQQAIQDITLRMGAGMA
WsSQS2 TVEDDTSIPADVKVPILISFHQHVYDREWHFSLLGGTKEYKVLMDQFHHVSTAFLELGKHYQQAIQDITLRMGAGMA
       ********************************   ****************************************
```

```
WsSQS1 KFICKEVETTDDYDEYCHYVAGLVGLGLSKLFHASGKEDLAPDSLSNSMGLFLQKTNIIRDYLEDINEVPKCRMFWP
WsSQS2 KFICKEVETTDDYDEYCHYVAGLVGLGLSKLFHASGKEDLAPDSLSNSMGLFLQKTNIIRDYLEDINEVPKCRMFWP
       ************************************************************************

WsSQS1 REIWSKYVNKLEDLKYEENSVKAVQCLNDMVTNALSHVEDCLTYMSNLRDPAIFRFCGIPQVMAIGTLAMCYDNIEV
WsSQS2 REIWSKYVNKLEDLKYEENSVKAVQCLNDMVTNALSHVEDCLTYMSNLRDPAIFRFCGIPQVMAIGTLAMCYDNIEV
       ************************************************************************

WsSQS1 FRGVVKMRRGLTAKVIDRTRTMADVYGAFFDFSCMLKSKVNNNDPNSTKTLKKLEAILKTCRNSGMLNKRKSYVIRS
WsSQS2 FRGVVKMRRGLTAKVIDRTRTMADVYGAFFDFSCMLKSKVNNNDPNSTKTLKKLEAILKTCRNSGMLNKRKSYVIRS
       ************************************************************************

WsSQS1 EPNYSPVLIIVIFVILAVILSQLSGNRS
WsSQS2 EPNYSPVLIIVIFVILAVILSQLSGNRS
       ***************************
```

Fig 2.7: Alignment of **(a)** nucleotide; **(b)** deduced amino acid sequences of WsSQS1 and WsSQS2. The yellow highlighted region in (a) shows the primer sequence used for clone confirmation while the residues in red color indicates the difference in sequences of both the isoforms

2.4.5 Confirmation of *WsSQS* isoforms by PCR

Since the difference in sequences of both the cDNA clones of *WsSQS* was very less, the presence of these isoforms in *W. somnifera* was confirmed by PCR. SQSF0 was used as the forward primer while a reverse primer, NSQSR (Appendix-IV), was designed from the sequences that were different in both the clones (Fig 2.7a). The pGEM-T vector plasmids containing the respective *SQS* clones were used as templates.

The above primer combination gave an amplification of ~350 bp with *WsSQS2* while no amplification was observed with *WsSQS1* (Fig 2.8). This confirmed the presence of two different sequences of *SQS* in plasmids and probably two homologs of *SQS* in *W. somnifera*. Previously, two *SQS* genes were also identified in *A. thaliana* organized in a tandem array (Kribii et al. 1997) and three homologs of SQS have been also recognized in *P. ginseng* (Kim 2011a).

2.4.6 Rapid amplification of cDNA ends (RACE)

RACE was performed to obtain the UTRs of both the *SQS* isoforms and to confirm the terminal sequences of the genes. Based on the sequence information from partial fragment of *WsSQS*, 5' and 3'RACE primers were designed (Fig 2.5).

2.4.6.1 5' RACE PCR

In order to get the 5' regions of *WsSQSs*, 5' RACE PCR was performed as described in section 2.3.5. Approximately 700 bp secondary PCR product (Fig 2.9a) was obtained in

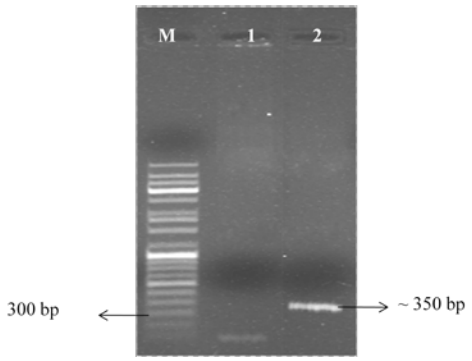


Fig 2.8: PCR for clone confirmation. M: Medium range ladder; Lane 1: PCR product with the plasmid containing the insert *WsSQS1* showing no amplification; Lane 2: PCR product with the plasmid containing the insert *WsSQS2* showing an amplification of ~350 bp

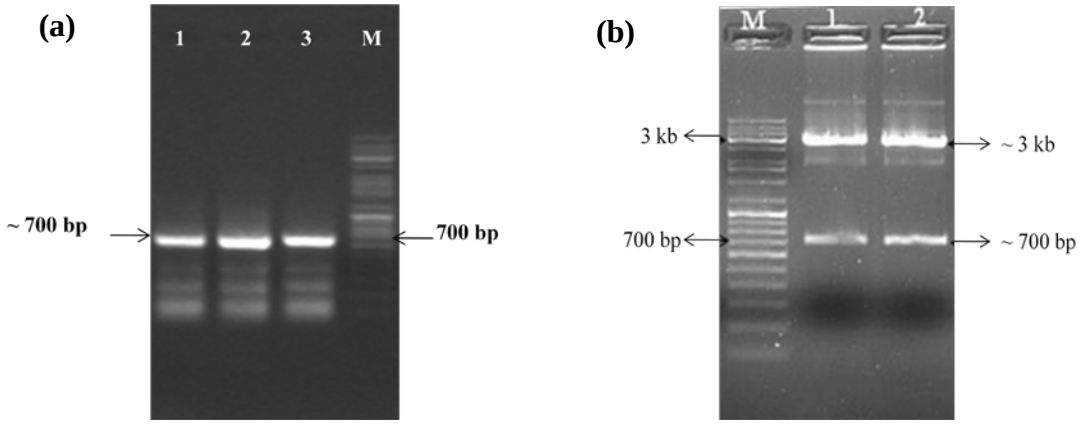


Fig 2.9 **(a)** 5' RACE PCR amplification product of ~700 bp fragment separated on 1% agarose gel. Lane 1, 2 & 3: Amplified product; Lane M: Medium range DNA ruler; **(b)** Restriction digestion with *Eco*RI releasing ~700 bp insert. Lane 1 & 2: vector backbone (~3 kb) and inserts (~700 bp); Lane M- Medium range DNA ruler

secondary PCR which was cloned in pGEM-T Easy vector and confirmed by *Eco*RI digestion (Fig 2.9b). The sequencing analysis of 5' RACE product revealed 53 and 65 bp UTR regions of *WsSQS1* and *WsSQS2* respectively (Fig 2.10).

(a)

```
1   AAGCAGTGGT ATCAACGCAG AGTACATGGG GTACCAAACC TCCGGACGCC AGCCTATTGT CCTCATAACT GGTTGCATGG
81  GAACATTGAG GGCGATTTTG AAGAATCCAG ATGATTTGTA TCCATTGATA AAACTAAAGC TTGCGGCTAG ACATGCGGAG
161 AAGCAGATCC CGCCGGAGCC ACATTGGGCC TTCTGTTACT TAATGCTTCA AAAGGTTTCT CGTAGCTTTG CTCTCGTCAT
241 TCAACAGCTT CCTGTTGAGC TTCGTGATGC TGTATGCATA TTCTATTTGG TTCTTCGAGC ACTTGACACT GTTGAGGATG
321 ACACCAGCAT TCCCGCAGAT GTTAAAGTAC CTATTCTGAT ATCTTTTCAT CAGCATGTTT ATGATCGTGA ATGGCACTTT
401 TCATGTGGCA CGAAGGAGTA CAAGGTTCTC ATGGACCAGT TCCATCATGT TTCAACCGCT TTTCTGGAAC TTGGGAAACA
481 TTATCAGCAG GCAATTCAGG ATATTACCTT GAGGATGGGT GCAGGAATGG CAAAATTTAT ATGCAAGGAG GTGGAAACAA
561 CCGATGATTA TGACGAATAT TGTCACTATG TAGCTGGGCT TGTTGGTTTA GGATTGTCAA AACTGTTTCA TGCCTCTGGG
641 AAGGAAGATC TGGCTCCAGA TTCTC
```

(b)

```
1   AAGCAGTGGT ATCAACGCAG AGTACATGGG AAACGCTCTC CTCTACCAAA CACTCCCACG CCATTCGCTT CACATGCGTT
81  GAAGAAGAAT GGGAACATTG AGGGCGATTT TGAAGAATCC AGATGATTTG TATCCATTGA TAAAACTAAA GCTTGCGGCT
161 AGACATGCGG AGAAGCAGAT CCCGCCGGAG CCACATTGGG CCTTCTGTTA CTTAATGCTT CAAAAGGTTT CTCGTAGCTT
241 TGCTCTCGCC ATTCAACAGC TTCCTGTTGA GCTTCGTGAT GCTGTATGCA TATTCTATTT GGTTCTTCGA GCACTTGACA
321 CTGTTGAGGA TGACACCAGC ATTCCCGCAG ATGTTAAAGT ACCTATTCTG ATATCTTTTC ATCAGCATGT TTATGATCGT
401 GAATGGCACT TTTCATTGTT AGGTGGCACG AAGGAGTACA AGGTTCTCAT GGACCAGTTC CATCATGTTT CAACCGCTTT
481 TCTGGAACTT GGGAAACATT ATCAGCAGGC AATTCAGGAT ATTACCTTGA GGATGGGTGC AGGAATGGCA AAATTTATAT
561 GCAAGGAGGT GGAAACAACC GATGATTATG ACGAATATTG TCACTATGTA GCTGGGCTTG TTGGTTTAGG ATTGTCAAAA
641 CTGTTTCATG CCTCTGGGAA GGAAGATCTG GCTCCAGATT CTC
```

Fig 2.10 Sequence analysis of 5' RACE PCR product of **(a)** *WsSQS1*; **(b)** *WsSQS2*. Underlined- 5' NUP sequence and RACE NR primer; blue: 5' UTR; red: start codon (ATG)

2.4.6.2 3' RACE PCR

To isolate 3' end of the *WsSQSs* from *W. somnifera*, 3' RACE PCR was performed as described in section 2.3.5. Approximately 1.1 kb amplicon (Fig 2.11a) was cloned and checked by *Eco*RI digestion (Fig 2.11b). The sequencing analysis of 3' RACE product revealed 257 and 253 bp UTR regions of *WsSQS1* and *WsSQS2* respectively (Fig 2.12).

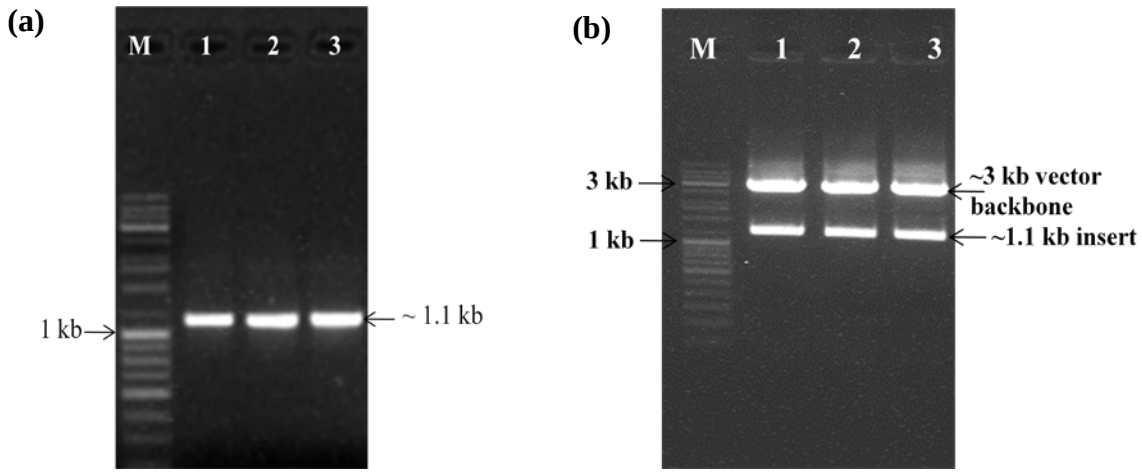


Fig 2.11 (**a)** 3' RACE PCR amplification product of ~1.1 kb fragment separated on 1% agarose gel. Lane 1, 2 & 3: Amplified product; Lane M: Medium range DNA ruler; **(b)** Restriction digestion with *Eco*RI releasing ~1.1 kb inserts. Lane 1, 2 & 3: vector backbone (~3 kb) and inserts (~1.1 kb); Lane M- Medium range DNA ruler

(a)

```
1 GACGAATATT GTCACTATGT AGCTGGGCTT GTTGGTTTAG ACTGTTTCAT GCCTCTGGGA AGGAAGATCT GGCTCCAGAT
81 TCTCTCTCCA ACTCTATGGG TTTATTTCTT CAGAAAACAA ACATCATCAG AGATTATTTG GAAGACATAA ATGAGGTGCC
161 CAAGTGCCGT ATGTTCTGGC CCCGTGAGAT TTGGAGTAAA TATGTTAACA AGCTTGAGGA CTTAAAGTAT GAGGAGAACT
241 CGGTCAAGGC AGTGCAATGC CTCAATGACA TGGTCACCAA TGCTTTGTCA CATGTAGAAG ATTGTTTGAC TTACATGTCC
321 AATTTGCGCG ATCCTGCCAT CTTTCGATTC TGTGGTATTC CACAGGTCAT GGCAATTGGG ACATTAGCTA TGTGCTACGA
```

401 CAACATTGAA GTCTTCAGAG GAGTGGTTAA AATGAGGCGT GGTCTGACTG CTAAGGTCAT TGACCGGACT AGGACTATGG
481 CAGATGTATA TGGTGCTTTT TTTGACTTCT CTTGTATGCT GAAATCCAAG GTTAATAATA ATGATCCAAA TTCAACTAAA
561 ACGTTGAAGA AGCTTGAAGC AATCCTGAAA ACTTGCAGAA ATTCGGGAAT GTTGAATAAA AGGAAGTCTT ATGTAATCAG
641 GAGTGAGCCA AATTACAGTC CAGTTCTGAT TATTGTCATC TTCGTCATAC TGGCTGTTAT TCTTTCACAA CTTTCTGGCA
721 ACCGATCTTA GAGACGTTAC CGTGCCATCG TAGCTTACGG ATCTCAAGGG ATTAGCTAGC TTAGCTAGGG CTAGCTTCGA
801 TCGACTAACG TGGTACGATT ACGTAGGCTA GCCTTTAGCT AGGGCTAGCT TTACGTTAGC TTTATGAGCT ACGTTTACGT
881 AGGCTAGCTT AGCTAAACCC GTACTTGTTT TTTACGTATT CGATAAAGCA ACGTATTCGA TCGATCACGT AGCAATAAAA
961 ACAGTCAGTG ACTTGTAGGT ACCCTGTCAA AAAAAAAAAA AAAAAAAAAA AAAAACTCTG CGTTGATACC AGTCGCTT

(b)

1 GACGAATATT GTCACTATGT AGCTGGGCTT GTTGGTTTAG GATTGTCAAA ACTGTTTCAT GCCTCTGGGA AGGAAGATCT
81 GGCTCCAGAT TCTCTCTCCA ACTCTATGGG TTTATTTCTT CAGAAAACAA ACATCATCAG AGATTATTTG GAAGACATAA
161 ATGAGGTGCC CAAGTGCCGT ATGTTCTGGC CCCGTGAGAT TTGGAGTAAA TATGTTAACA AGCTTGAGGA CTTAAAGTAT
241 GAGGAGAACT CGGTCAAGGC AGTGCAATGC CTCAATGACA TGGTCACCAA TGCTTTGTCA CATGTAGAAG ATTGTTTGAC
321 TTACATGTCC AATTTGCGCG ATCCTGCCAT CTTTCGATTC TGTGGTATTC CACAGGTCAT GGCAATTGGG ACATTAGCTA
401 TGTGCTACGA CAACATTGAA GTCTTCAGAG GAGTGGTTAA AATGAGGCGT GGTCTGACTG CTAAGGTCAT TGACCGGACT
481 AGGACTATGG CAGATGTATA TGGTGCTTTT TTTGACTTCT CTTGTATGCT GAAATCCAAG GTTAATAATA ATGATCCAAA
561 TTCAACTAAA ACGTTGAAGA AGCTTGAAGC AATCCTGAAA ACTTGCAGAA ATTCGGGAAT GTTGAATAAA AGGAAGTCTT
641 ATGTAATCAG GAGTGAGCCA AATTACAGTC CAGTTCTGAT TATTGTCATC TTCGTCATAC TGGCTGTTAT TCTTTCACAA
721 CTTTCTGGCA ACCGATCTTA GACCATCTTT TGGGCTATAA AAAAGAAGTC TGGTCAAGGG AGACAGCACA AGCTTTAGGT
801 CAATTATGTG ATCACTGCAA ATTGTAATGT TTGTATTCAT TAAGTGAGAT AATTGCACGT TCAACCGAAC CAGAGATTAG
881 AAAGCCTGTT TTTGGTAGTT TGTTGGGTAT GTACTTGTTT GCAGGGTGCT ACGGAAAGCA AATTCCAATC GTAATAAAAG
961 TCACCGTAAG GCTTAATACA CATTCCATTT TGCCAAAAAA AAAAAAAAAA AAAAAAAAAC TCTGCGTTGA TACCAGTCGC
1041 TT

Fig 2.12 Sequence analysis of 3' RACE PCR product of **(a)** *WsSQS1*; **(b)** *WsSQS2*. Underlined-RACE NF1 primer and 3'' NUP sequence; blue: 3' UTR; red: stop codon (TAG); blue underlined: putative polyadenylation site

WsSQS1: 53 bp 5' UTR + 1236 bp ORF + 257 bp of 3'UTR, 411 AA

WsSQS2: 65 bp 5' UTR + 1242 bp long ORF + 253 bp 3'UTR, 413 AA

2.4.7 Amino acid analysis

WsSQS1 and WsSQS2 amino acid sequences share 99% similarity and 99% identity with each other. One more SQS was reported from *W. somnifera* (Bhat et al. 2012) which was found to be 94.6% identical and 97.3% similar to WsSQS1, while 93.7% identical and 96.6% similar to WsSQS2. WsSQSs are also similar to the SQS from other plants. WsSQS1 and WsSQS2 show 97.6% and 96.6% similarity and 94.6% and 93.7% identity with *C. annuum*; 98.1% and 97.1% similarity and 94.4% and 93.5% identity with *S. tuberosum*; 97.3% and 96.4% similarity and 93.2% and 92.3% identity with *N. tabacum*; 91.5% and 90.6% similarity and 88.3% and 87.4% identity with *Datura inoxia*; 84.8% and 83.9% similarity and 70.1% and 69.3% identity with *A. thaliana* respectively. Two SQS

proteins are known from *A. thaliana* which share 79 % identity and 88 % similarity (Kribii et al. 1997). Different parameters of both the WsSQSs are mentioned in Table 2.1

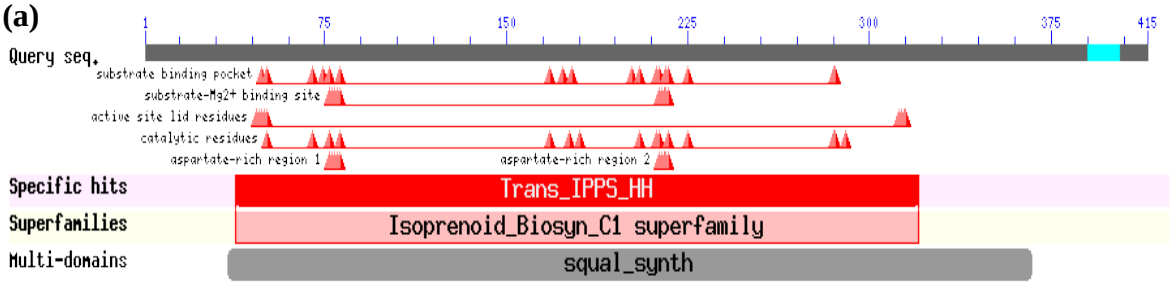
Parameters	**WsSQS1**	**WsSQS2**
Total amino acids	411	413
Molecular weight (Mw)	47.0 kDa	47.16 kDa
Theoretical Isoelectric point (pI)	8.15	8.18
Instability index	42.71	42.06
Atomic formula	$C_{2113}H_{3342}N_{560}O_{597}S_{27}$	$C_{2122}H_{3358}N_{562}O_{599}S_{26}$
Positively charged amino acids (Arg + Lys)	50	50
Negatively charged amino acids (Asp + Glu)	47	47
Aliphatic index	97.23	98.18

Table 2.1: Table indicating various parameters of WsSQS1 and WsSQS2 computed by ExPASy tool: Protparam (http://web.expasy.org/protparam/)

2.4.8 Multiple sequence alignment of WsSQSs with other plants and CDD search

WsSQSs show specific hits with trans-isoprenyl diphosphate synthases and belongs to superfamily of isoprenoid biosynthsis class 1 enzyme (Fig 2.13a). According to conserved domain database 6 highly conserved peptide domains have been identified in WsSQSs (Fig 2.13b) which was exactly similar to the domains found in SQS from *S. tuberosum* (Yoshioka et al. 1999), *S. lycopersicum* (ACY25092.1), *C. annuum* (Lee et al. 2002), *D. inoxia* (AAY22201.1), *N. tabacum* (Devarenne et al. 2002) and *D. kaki* (ACN69082.1). While these domains of WsSQSs were found to be almost similar with the SQS from *S. pombe* (Robinson et al. 1993), *Taxus cuspidata* (Huang et al. 2007) and *G. lucidum* (Zhao et al. 2007), although there are differences in some amino acid residues of these domains. The amino acid residues in these domains are also conserved in the WsSQS polypeptides. These domains are situated between 40 to 340 amino acid residues. Amino acids at the carboxy terminal region of the protein exhibit least sequence identity because carboxy terminal is very hydrophobic and it is considered to anchor the protein in the ER membrane. Domain I (highlighted in yellow) is substrate binding pocket or chemical binding site which consists of about 18 amino acids distributed throughout the polypeptide. Domain II (highlighted in blue) is substrate–Mg^{2+} binding site which consists of 10 amino acids divided in two patches of 5 each in which domain V and VI (aspartate rich region 1 and 2 respectively) are embedded. Domain III (highlighted in green) is active site which is composed of about 9 amino acid residues. Domain IV (residues in red) is catalytic domain of about 14 amino acids distributed throughout the polypeptide. The catalytic site is

(a)



(b)

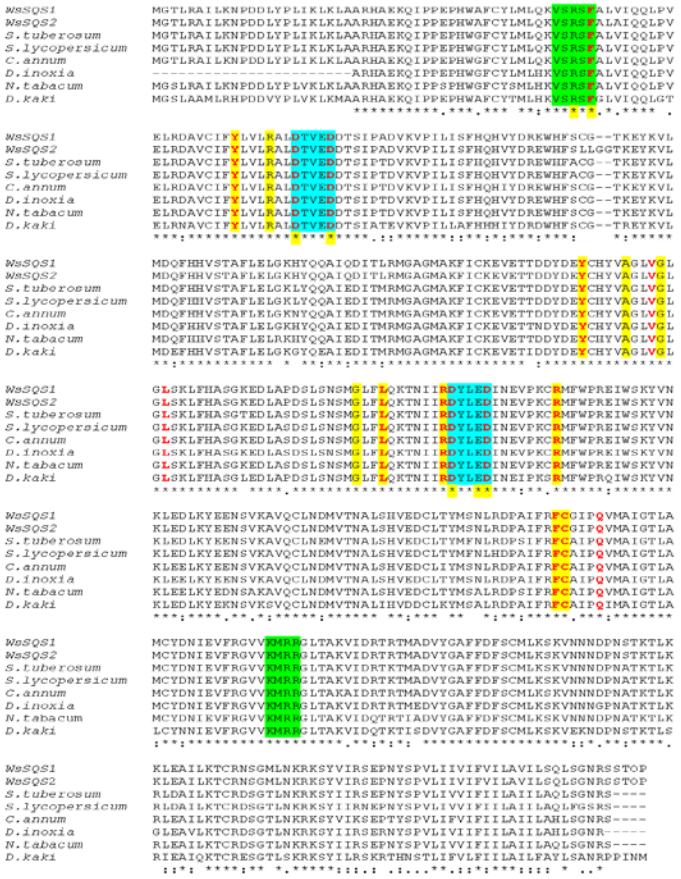


Fig 2.13: (a) CDD search on NCBI; **(b)** ClustalW alignment of WsSQSs with other plant SQS sequences. Different motifs and catalytic residues are highlighted with their respective colors

composed of the large central cavity formed by antiparallel alpha helices with two aspartate rich regions (DXXXD) on opposite walls (Chen et al. 1994; Pandit et al. 2000; Reipen et al. 1995; Marrero et al. 1992). These residues are considered to play role in binding of prenyl phosphates by binding Mg^{2+} ions. Extremely high amino acid conservation is observed within the domains that have been implicated with the substrate binding and catalytic activities. Amino acid residues in the C-terminal region exhibited a

low level of sequence identity among all SQS proteins. This region is very hydrophobic in all SQS enzymes and may function as an anchor in the ER membrane.

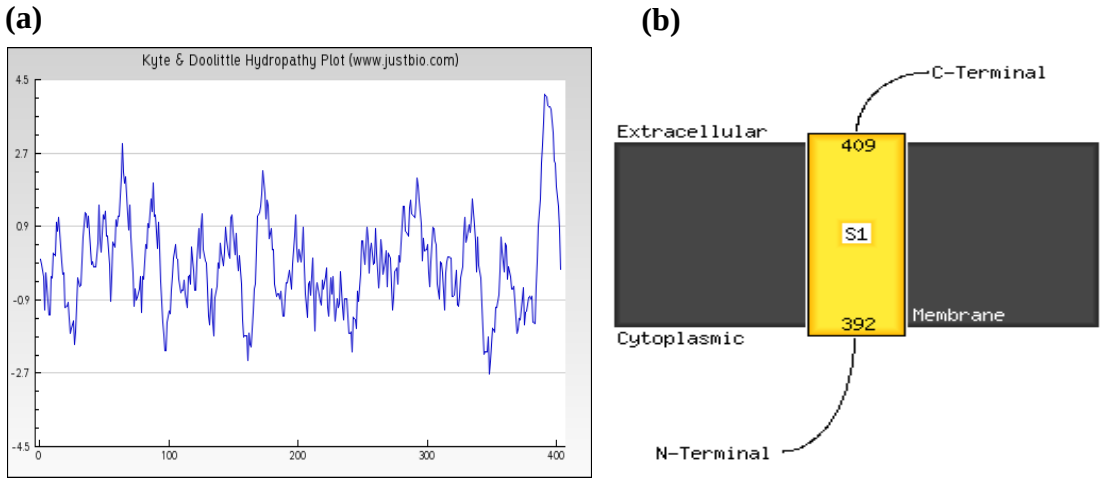


Fig 2.14: (a) Kyte-Doolittle Hydropathy plot, **(b)** Transmembrane domain analysis by Psipred

2.4.9 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 WsSQSs 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 WsSQSs, one large transmembrane region is supposed to be present at C terminal and a small region at N terminal (Fig 2.14a). Only WsSQS2 was taken into account for this study due to the high similarity in both the sequences.

Presence of transmembrane domains was also predicted on Psipred server (http://bioinf.cs.ucl.ac.uk/psipred). The amino acid residues from 392 to 409 were found to be anchored in membrane (Fig 2.14b).

2.4.10 Phylogenetic tree analysis

A phylogenetic tree was constructed by using known SQS sequences from broad range of organisms including dicots, monocots, mammals, fungi and bacteria. Phylogenetic tree was

divided in 4 clusters. Cluster I and II were composed of dicots, in which WsSQSs were closely related to the *Solanum spp.* and *C. annuum*. All mammalian enzymes were grouped in cluster III and cluster IV consisted of all fungal SQS enzymes (Fig 2.15). Phylogenetic analysis suggested that WsSQSs has evolved from a single ancestral gene.

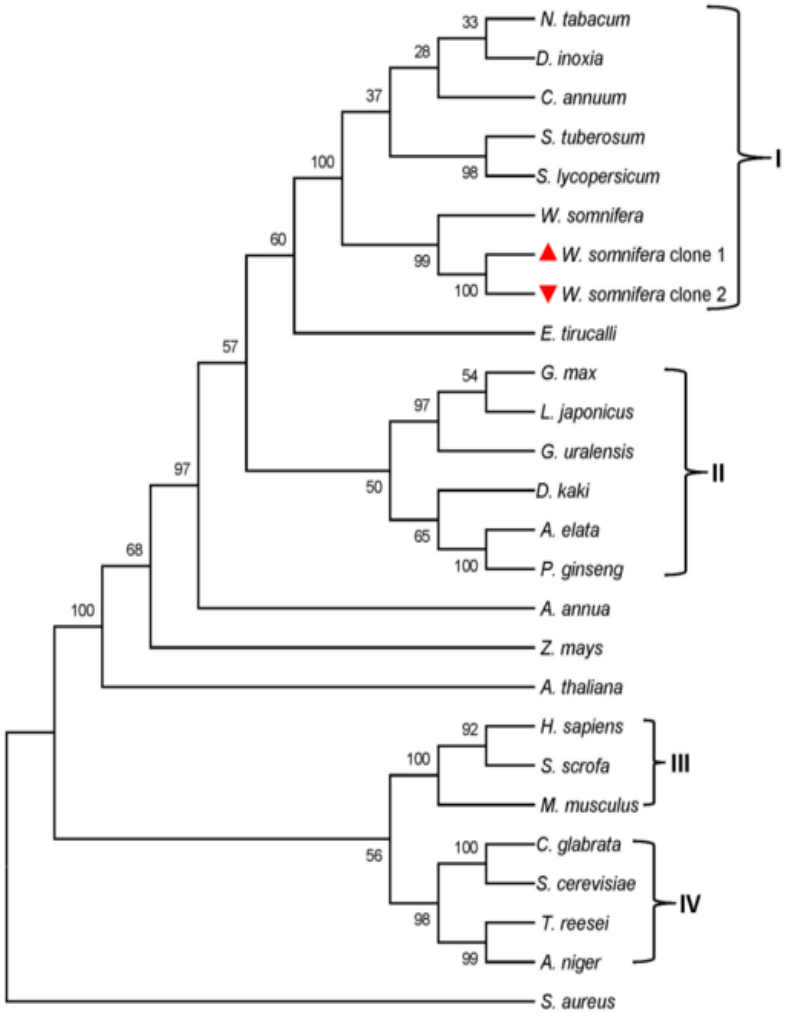


Fig 2.15: Phylogenetic relationship of WsSQSs with other SQSs. The evolutionary tree was formed using the ClustalX2 program and MEGA 4.0.2 software with neighbor-joining method. Accessions numbers. are as follows: *N. tabacum* SQS (AAB08578), *D. inoxia* SQS (AAY22201), *C. annuum* SQS (AAD20626), *S. tuberosum* SQS (BAA82093), *S. lycopersicum* SQS (ACY25092), *W. somnifera* SQS (ADC95435), ***W. somnifera* SQS clone 1 (GU181386), *W. somnifera* SQS clone 2 (GU732820),** *E. tirucalli* SQS **(**BAH23428), *G. max* SQS (BAA22559), *L. japonicus* SQS (BAC56854), *G. uralensis* SQS (ACS66750), *D. kaki* SQS (ACN69082), *A. elata* SQS (ADC32654), *P. ginseng* SQS (ACV88718), *A. annua* SQS (AAR20329), *Z. mays* SQS (NP_001104839), *A. thaliana* SQS (AAB61927), *H. sapiens* SQS (NP_004453), *S. scrofa* SQS (NP_001161120), *M. musculus* SQS (NP_034321), *C. glabrata* SQS (BAB12207), *S. cerevisiae* SQS (ACD03847), *T. reesei* SQS (EGR47283), *A. niger* SQS (XP_001395275) and *S. aureus* SQS (YP_001317747)

2.5 Conclusions

Though the plant, *W. somnifera* has been well characterized in terms of pharmaceutical activities as well as phytochemical profiles, not much is known about the genes responsible for biosynthesis of these compounds. To gain new insights into the role of *SQS* in the isoprenoid biosynthetic pathway in higher plants, we isolated cDNA coding *W. somnifera SQS*. This is the first attempt of cloning and characterization of this gene from *W. somnifera*. Two isoforms of *WsSQS* (*WsSQS1 and WsSQS2*) have been identified sharing 99% identity and 99% sequence homology with each other in BLAST analysis. RACE PCR was used to identify 5' and 3'UTRs of both the genes and have been submitted to NCBI database. CDD search revealed 6 conserved peptide domains in WsSQS. Phylogenetic analysis showed WsSQSs are clustered in dicots group and are closely related to *Solanum* spp. and *C. annuum*.

CHAPTER 3

HETEROLOGOUS EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE RECOMBINANT WsSQS PROTEIN(S)

3.1 Introduction

Triterpenoids (C_{30} isoprenoids) are a diverse class of naturally occurring organic products and include various sterols, steroidal sapogenins, steroidal alkaloids and steroidal lactones. Withanolides are one of the most widely studied steroidal lactones which are commonly found in plants of Solanaceae. The enzyme SQS plays an important role in biosynthesis of withanolides by catalyzing a reductive condensation of two FPP molecules into C_{30} 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) leading to the biosynthesis of withanolides in *W. somnifera*. The role of SQS has been described in detail in Chapter 1.

Being a regulatory enzyme, SQS encoding genes have been cloned and characterized from several organisms and their functional characterization has also been studied. The protein purification and *in vitro* studies have proved this enzyme to be transmembrane being anchored in ER membrane (Zhang et al. 1993; Kim et al. 2005). SQS amino acid sequence analysis provided insights for engineering more soluble variants (Jennings et al. 1991). Jennings et al. cloned the yeast *SQS* gene and suggested that the enzyme consisted of a large cytosolic domain anchored to the ER by a single C terminal transmembrane helix (Jennings et al. 1991). Subsequently, a soluble and fully active version of recombinant yeast SQS was constructed by deletion of a C terminal hydrophobic region from the enzyme (Zhang et al. 1993; LoGrasso et al. 1993). Similar C terminal hydrophobic domains were also found in the *S. 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 *S. 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).

In Chapter 2 we described isolation, cloning and characterization of genes encoding SQS from *W. somnifera*. This chapter deals with their heterologous expression in *E. coli*, their purification and characterization using various bioinformatics tools.

3.2 Materials

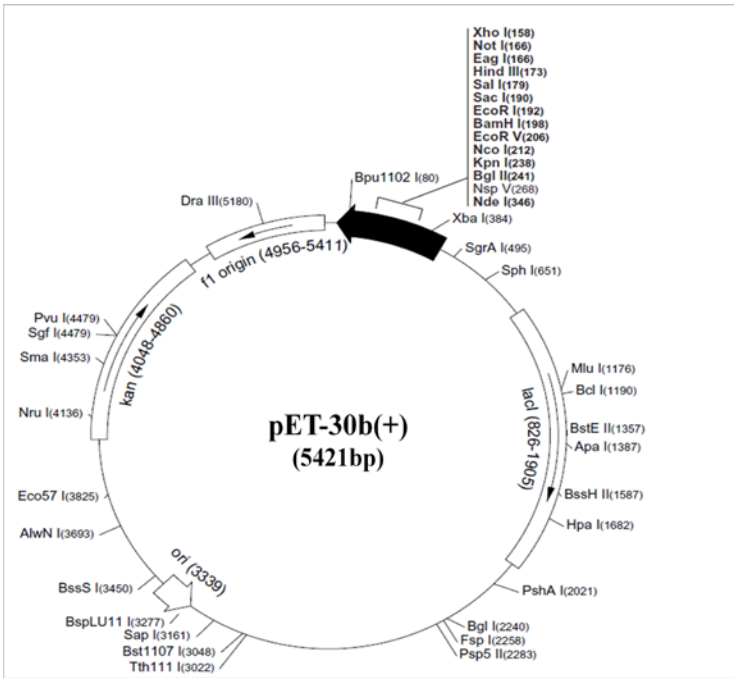
FPP, NADPH and authentic squalene were purchased from Sigma. HEPES were procured from Bioworld (USA). GC vials were ordered from Merck and glass syringes were purchased from Hamilton (Switzerland).

Refer Appendix-IX, X and XI for various buffers and other reagents.

3.3 Methods

3.3.1 Directional cloning of full length *WsSQSs* in expression vector pET-30 b (+)

The ORFs of *WsSQSs* were amplified by PCR with primer set FSQSP-RSQSP (Appendix-IV) having appropriate restriction sites (*Nde*I and *Xho*I), the amplicons were cloned in pGEM-T Easy vector and the inserts were excised from pGEM-T plasmids using the same restriction enzymes (Fig 3.2a) and directionally cloned into pET-30b (+) vector (Fig 3.1) already double digested with *Nde*I and *Xho*I enzymes (Fig 3.2b).



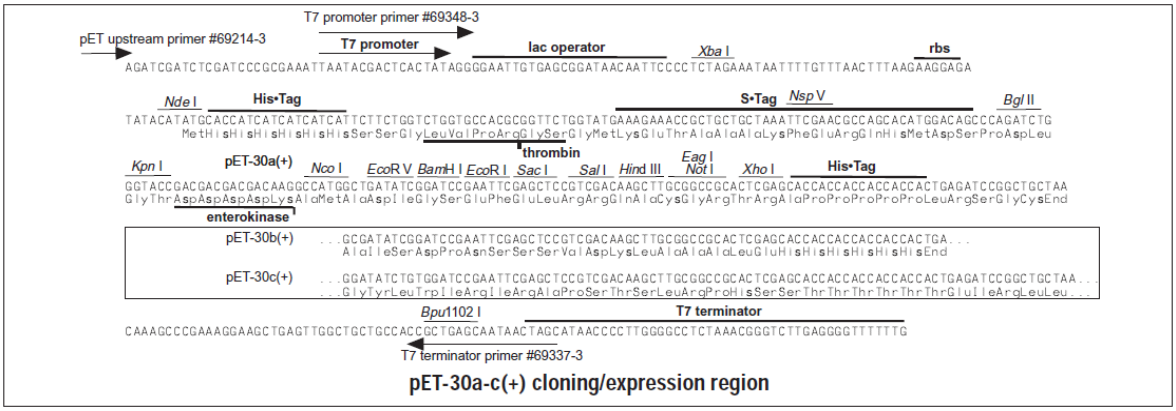
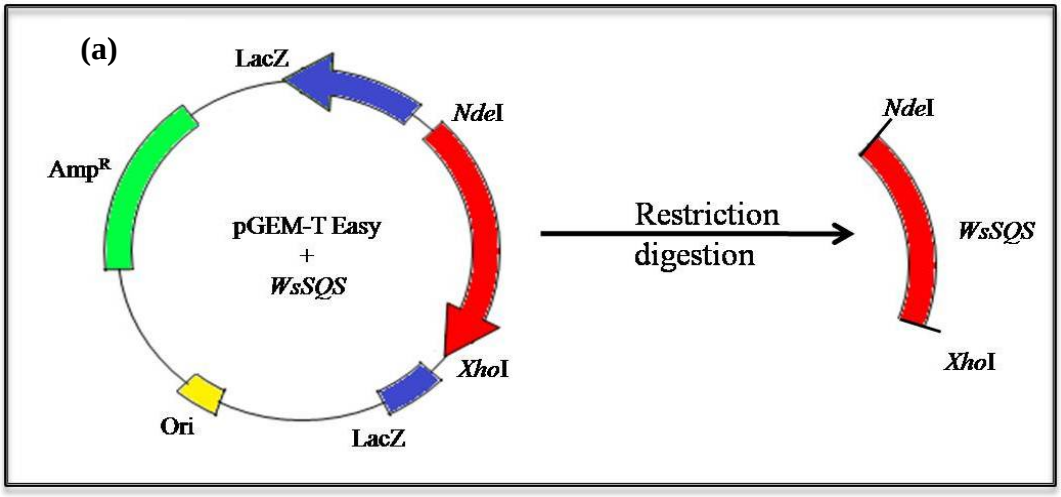


Fig 3.1: Vector map of pET 30b (+)



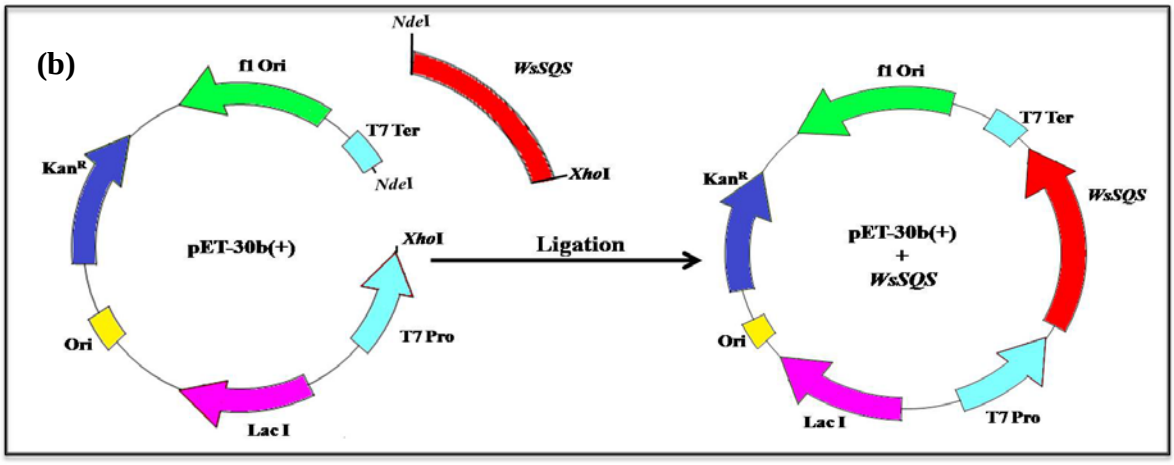


Fig 3.2: Strategy used for directional cloning of *WsSQS* gene in pET-30b (+) vector. **(a)** Double digestion of pGEM-T Easy vector to release *WsSQS* fragment with sticky ends; **(b)** Ligation of the *WsSQS* fragment in pET-30 b (+) vector already digested with the same restriction sites to generate expression construct for over-expression of recombinant protein

The ligation mixtures were used to transform *E. coli* XL-10 cells and plasmids were isolated and confirmed by double digestion. The plasmids were then mobilized into the expression cell line *E. coli* BL21 (DE3) strain. Colony PCR with primers from T7 promoter and T7 terminator (Appendix-IV) was done to screen the recombinant pET-30b (+) clones. Integration of genes in pET-30b (+) were confirmed by digestion with respective restriction enzymes. All clones were also confirmed by sequencing.

3.3.2 Directional cloning of truncated *WsSQSs*

To make the recombinant enzymes soluble and to avoid problems regarding their expression, C terminal truncated WsSQSs were generated using the forward primer FSQSP that harbored the *Nde*I restriction site and a reverse primer T-RSQSP (Appendix-IV) that contained a *Xho*I restriction site. The reverse primer was located 24 amino acids upstream from the native stop codon in the full length *WsSQS*. The amplicon was cloned in pET-30b (+) and finally the constructs, pET-30b (+)-*WsSQS*1 and pET-30b (+)-*WsSQS*2, were transformed in *E. coli* strain BL21 (DE3) for protein expression.

3.3.3 Heterologous expression in *E. coli*

Heterologous expression of *WsSQS* genes was attempted in *E. coli* BL21 (DE3) host cells. The transformants were grown at 37 ºC in LB 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 concentration of the antibiotic. The culture was grown overnight with shaking at 200 rpm at 37 °C and 1% of this culture was used to inoculate 100 mL liquid LB containing 50 µg/mL kanamycin. Once the culture reached A_{600} 0.5 - 0.6 (log phase), recombinant protein expression was induced by the addition of 1 mM 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 and cells disruption was done by sonicating cells at 75% amplitude at 4 ^{o}C for 5-7 min with pulse of 30 s on/off. Lysozyme (100 µg/mL) was added to the disrupted cells and kept at 37 ^{o}C for 20 min. It was centrifuged at 10,000 rpm for 10 min at 4 ^{o}C 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 dissolved in 3 mL of dispersion buffer and an aliquot of 20 µL was checked on SDS-PAGE to check heterologous expression of the proteins.

3.3.4 Optimization of recombinant protein expression

The level of expression and solubility of recombinant proteins by transformed cells showing promising expression were optimized for IPTG concentration, incubation temperature and duration of incubation after induction. After achieving proper bacterial growth in secondary culture, expression of protein was induced by adding IPTG. Both IPTG concentration and incubation temperature were kept low initially for increasing solubility of proteins while incubation duration was increased to compensate the decreased growth rate of the cells. The IPTG concentration tried were from 0.1 mM to 1.2 mM, incubation temperature from 15 to 37 ^{o}C and incubation time duration from 2 to 16 h.

3.3.5 Purification of recombinant protein using Ni^{+}-NTA affinity and gel filteration chromatography

The proteins were expressed as a His-tag fusion protein and purified by Metal Chelate (Ni^{+}-NTA) Affinity Chromatography. The Ni^{+} beads column was equilibrated with binding buffer for 30 min and then by passing three bed volume of binding buffer through the column. The recombinant protein, among several other bacterial soluble proteins, was then loaded on affinity matrix column and kept for binding to the Ni^{+} beads for 1 h at 4 ºC on dancing shaker. After 1 h, beads were allowed to settle down followed by washing of the matrix with 6-8 bed volume of wash buffer, until OD_{280} reaches to baseline to remove unbound proteins. The recombinant proteins were eluted with 250 mM imidazole and were monitored by measuring A_{280} of collected fractions. The protein was eluted in aliquots of 1 mL each and protein content in different fractions was estimated using Bradford assay (Bradford 1976). A standard graph of BSA was made for determination of protein concentration in unknown samples.

The Ni^{+}-NTA purified fractions were pooled, concentrated (Amicon Ultra, Merck) and further purified by gel filteration chromatography on Akta Explorer (GE Healthcare). Standard proteins of known sizes were run on gel filteration column (Superdex 75 pg) and size of unknown protein was determined by comparing with the standard proteins.

Collected fractions were concentrated and desalted on column (HiLoad Sephadex desalting 16/60, GE Healthcare) and protein content was estimated by Bradford method.

3.3.6 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.

Preparation of the polymerizing gel

A vertical slab gel was assembled using 1.5 mm spacers, 10% separating gel solution was prepared (Appendix-X) and mixed gently without generating bubbles. The solution was pipetted into the gel assembly leaving the space of about 1.5 cm from the top unfilled. The gel solution was overlaid with water to remove trapped air bubbles and to check for any leakage. When a sharp liquid-gel inter-surface was observed after the gel polymerization, the slab was tilted to pour off the water overlay. Simultaneously, stacking gel solution was prepared (Appendix-X) and, TEMED and ammonium per sulfate were added to the solution just before pouring it over the separating gel. A comb was inserted taking care not to trap air bubbles beneath the comb teeth. The gel was left to polymerize.

Loading of protein samples and running polyacrylamide gel

Equal concentration of the protein samples were mixed with gel loading dye in a microcentrifuge tube and kept at 97 ºC for 5 min for SDS-PAGE followed with a brief spin. These samples were then directly used for loading onto the protein gel. Once solidified, the gel cassette was transferred to the tank connected to the power pack. The samples were loaded in the wells using a syringe along with the protein molecular weight ladder (Appendix-III). Lower and upper buffer chambers were filled with tank buffer and the circuit was closed to start run. Gel was run at room temperature at constant current of 20 mA or voltage of 80 V. The run was stopped when the dye reached the bottom of the polyacrylamide gel.

3.3.7 Coomassie blue staining

After completion of run, the gel was transferred directly to a gel staining box containing Coomassie blue staining solution (Appendix-X) 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.

3.3.8 Silver staining

Proteins separated on SDS PAGE gel were transferred to the fixer solution I for 1 h. This was followed by two washes in fixer solution II for 20 min each. The gel was then transferred to sensitizing solution for 1 min and rinsed thrice with deionized H_2O (1 min 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 H_2O (1 min each) and transferred to the developer till the bands appeared. The gel was washed with deionized H_2O and stored in fixer.

3.3.9 WsSQS enzyme assay and product identification

Assay for WsSQS activity is based on the conversion of FPP to squalene. The reaction was carried out with 200 µg purified protein, 200 µM FPP, 1mM NADPH and the total reaction volume was made to 400 µl with buffer (1 mg/mL BSA, 1% Tween, 1% β-mercaptoethanol, 10 mM $MgCl_2$ and 20 mM HEPES buffer). The reaction mixture was incubated at 30 ºC for 2 h and then extracted using NaCl and TBME solvent thrice. It was then concentrated using N_2 bubbles to 100 µl and 1 µl of it was loaded to GC column with a splitless injection. The product formation after the reaction was analyzed by a GC-MS (Agilent 5975C mass selective detector interfaced with an Agilent 7890A gas chromatograph) fitted with a β-cyclodextrine column (30 m X 0.32 mm X 0.25 µm, J & W Scientific). The injector temperature was 200 ºC with a total run of 40 min.

3.3.10 Raising polyclonal antibodies against purified WsSQS in rabbit

For further studies, only WsSQS2 was considered because of high similarity of both at the nucleotide and amino acid level, and will be named as WsSQS hereafter. The purified recombinant WsSQS protein was used for raising polyclonal antibodies in rabbit (New Zealand White). Three bleeds of serum were collected. Rabbit immune serum (stored at -80 ºC), containing polyclonal antibodies against the antigen, was thawed overnight at 4 ºC followed by incubation at 55 °C for 1 h and 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.

ELISA was performed to determine the titer 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 followed by washings of the plate with 250 µL of PBST (thrice) for 5 min and 300 µL of blocking reagent was added and incubated at 37 °C for 2 h. ELISA plate was again washed and challenged to different dilutions such as, 1:500, 1:1000, 1:5000, 1:10000, 1:20000, 1:50000 and 1:100000 of serum and pre-immune serum (100 µL) in separate wells. The plate was kept at 37 °C for 2 h again followed with washes as described earlier with PBST buffer. 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, pNPP, 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 titer was determined, ELISA was performed with different concentrations of recombinant purified protein and standard graph was plotted between protein concentrations *versus* OD_{405}. Protein concentration of unknown samples was determined from the standard curve.

3.3.11 WsSQS protein determination in different tissues of *W. somnifera* by ELISA

Fresh *W. somnifera* tissues (500 mg each of leaf, stem and root) were collected, frozen in liquid nitrogen and crushed to a fine powder. Crude protein was extracted with 1 mL of crude protein extraction buffer (Appendix-XI) and supernatant was collected after centrifugation. Total Protein was quantified in supernatant using Bradford assay. Equal quantity of plant protein was coated on 96 well microtiter plate in triplicates as an antigen. The volume of each protein loaded was made to 100 µL by dilution with PBS and ELISA was performed as mentioned in section 3.3.10. Non-specific sites were blocked with blocking buffer (300 µL/ well), 1:5000 dilution of Anti-SQS antibody was used and the absorbance was measured at 405 nm.

3.3.12 Western blotting

Protein blot analysis was performed for analyzing the plant native SQS protein from the crude protein extracts. Total soluble protein was extracted from different parts of the plant (leaf, stem and root) in appropriate crude protein extraction buffer and 50 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). After transfer, the 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 WsSQS) for 1 h. The membrane 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. Protein bands were detected with ready to use BCIP/NBT solution (Calbiochem) (Harlow and Lane 1988).

3.3.13 *In-silico* characterization of WsSQS

3.3.13.1 Comparative modeling

Protein sequence of WsSQS was generated from the nucleotide sequence of *WsSQS* gene using ExPasy tools. Secondary structure elements were predicted using the PSIPRED server. BLASTp search by using the query sequence against Protein Data Bank (PDB) were performed to identify close homologous crystal structures available in the database. The coordinates of crystal structure of template were used to build initial models of WsSQS. Finally 3D models were generated using MODELLER 9v9 software. The quality of the selected models was assessed by DOPE (Discrete Optimized Protein Energy) method.

3.3.13.2 Refinement and validation of the model

The models generated were improved by molecular Dynamics and Equilibration methods using Chemistry at HARvard Macromolecular Mechanics (CHARM27) force field for lipids, proteins and nucleotides to remove bad contacts in the side chains. The final model was validated by the Ramachandran Plot, PROCHECK, ERRAT, Verify3D and ProSA programs. Finally, the resultant energy minimized protein model was used for the active site identification and for docking analysis with the substrate. Diagrammatic representations of the structures were generated using PyMOL 1.3 software.

3.3.13.3 Substrate docking

The structure data files (sdf) of the ligands were obtained from the PubChem database. Docking studies were carried out with NADPH and FPP. Protein-ligand docking simulations were conducted using AutoDock Vina tool to prepare the systems for calculations. For each ligand, around 100 docking runs with default parameters were performed treating protein as rigid and the ligand as flexible molecules. The results were visualized using PyMOL software, wherein all the conformations of each of the ligand were found to be within the cavity of protein indicating that the docking run was free from errors. The conformational clusters with lowest binding energy (Ea) for each ligand were considered for further studies.

3.4 Results and discussion

3.4.1 Directional cloning of full length *WsSQS* gene in pET-30b (+)

The *WsSQS* genes, amplified using primers FSQSP-RSQSP, were cloned in pGEM-T Easy vector and then restricted digested, ligated into pET-30b (+) vector flanked with *Nde*I and *Xho*I restriction sites and transformed into *E. coli* XL10 competent cells (as described in section 3.3.1). *E. coli* colonies having recombinant plasmids were screened by colony PCR using T7 promoter and T7 terminator primers (Fig 3.3a). Plasmids isolated from PCR positive clones were digested with *Nde*I and *Xho*I to confirm the integration of *WsSQS* gene fragments in pET-30b (+) vector (Fig 3.3b). Integration of the *WsSQS* gene along with restriction sites was further confirmed by sequencing. The sequence was translated using proteomic tools available at http://web.expasy.org/translate/ and was checked for in frame translation up to 6x HIS tag.

3.4.2 Recombinant WsSQSs protein expression

E. coli BL21 (DE3) cells transformed with recombinant pET-30b (+) plasmids having *WsSQS* genes were screened for overexpression of protein. Different conditions for optimization of protein expression were tried like IPTG concentrations, induction duration and temperature but all the protein was found concentrated in inclusion bodies and no protein was appearing in soluble fraction. Since it is known from the literature (Lee et al. 2002; Jennings et al. 1991; Zhang et al. 1993) and bioinformatic study (Section 2.4.10), SQS is a transmembrane protein anchored in endoplasmic reticulum through its

hydrophobic patch of 24 amino acid at carboxy terminus, efforts were taken to obtain truncated gene for protein expression.

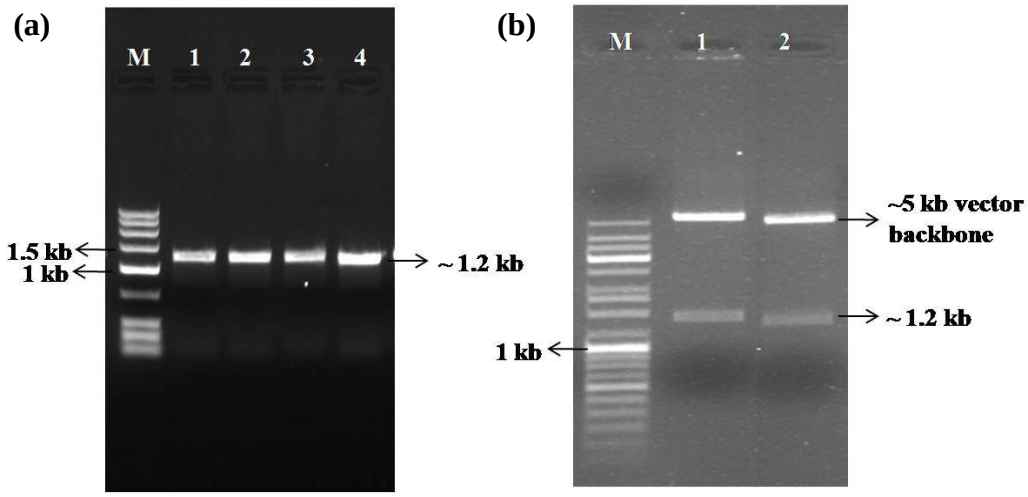


Fig 3.3: (a) Colony PCR showing ~1.2 kb *WsSQS* gene in recombinant colonies; Lane M-Low range DNA ruler; Lane 1, 2, 3 & 4: positive clones; **(b)** Restriction analysis of recombinant pET-30b (+) with *Nde*I and *Xho*I; Lane M: Low range DNA ruler; Lane 1 & 2: recombinant clones releasing ~1.2 kb WsSQS gene and ~5 kb vector backbone

3.4.3 Directional cloning of truncated *WsSQS* genes in pET-30b (+)

To amplify truncated genes, new reverse primer (T-RSQSP) was designed leaving last 72 nucleotides and having *Xho*I restriction site, which was again cloned in pGEM-T Easy vector, restriction digested with the same enzymes, ligated into pET-30b (+), checked by colony PCR and sequencing, and finally transformed in *E. coli* BL21 (DE3) cells for protein expression. The truncated gene was checked by its translation into protein using ExPASy translate tool (Fig 3.4).

MGTLRAILKNPDDLYPLIKLKLAARHAEKQIPPEPHWAFCYLMLQKVSRSFALVIQQLPVELRDAVCIFYLVLRALDTVEDD
TSIPADVKVPILISFHQHVYDREWHFSCGTKEYKVLMDQFHHVSTAFLELGKHYQQAIQDITLRMGAGMAKFICKEVETT
DDYDEYCHYVAGLVGLGLSKLFHASGKEDLAPDSLSNSMGLFLQKTNIIRDYLEDINEVPKCRMFWPREIWSKYVNKLED
LKYEENSVKAVQCLNDMVTNALSHVEDCLTYMSNLRDPAIFRFCGIPQVMAIGTLAMCYDNIEVFRGVVKMRRGLTAKVI
DRTRTMADVYGAFFDFSCMLKSKVNNNDPNSTKTLKKLEAILKTCRNSGMLNKRKSYVIRSEPNY**SPVLIIVIFVILAVILS**
QLSGNRS

Fig 3.4: Amino acid sequence of full length WsSQS1 protein where red residues show the truncated portion at the C terminus

3.4.4 Heterologous expression and purification of truncated recombinant WsSQS

E. coli BL 21 (DE3) cells transformed with recombinant pET-30b (+) plasmids having truncated *WsSQS* genes were screened for over-expression of protein. An approximately 43

kDa overexpressed protein was observed on 10% SDS-PAGE (Fig 3.5a). The *E. coli* (BL21) containing control pET-30b (+) plasmid did not show corresponding overexpressed band. Conditions were optimized for various parameters including IPTG conc., temperature and induction duration. It was found that recombinant cells grown at 30 ^{o}C for 2 and 4 h in the presence of 0.5 and 1 mM IPTG expressed recombinant protein in good amount in soluble fractions as compared to other conditions. Although, some protein was still going in inclusion bodies which was difficult to recover, we got good amount of soluble protein in lysate (Fig 3.5b). For further protein studies, the induction conditions chosen were: temperature of 30 ^{o}C at IPTG concentration of 1 mM for 2 h.

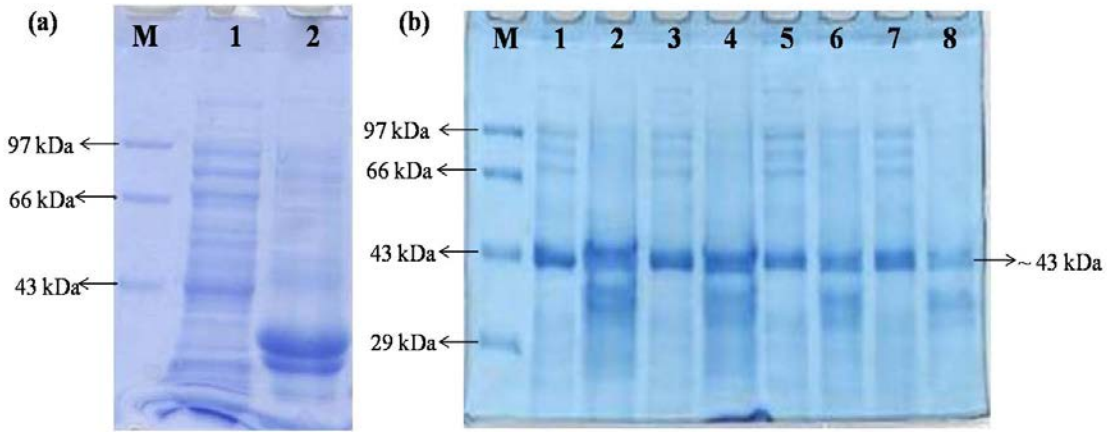


Fig 3.5: 10% SDS-PAGE Coomassie Blue staining **(a)** Gel Showing protein from lysate under induced and uninduced conditions. M: Molecular weight ladder; Lane1: Lysate from induced *E. coli* transformed with recombinant pET-30b (+) plasmid; Lane2: Lysate from uninduced *E. coli*; **(b)** Recombinant WsSQS1 overexpression under different induction conditions. M: Molecular weight ladder; Lane 1 & 2: lysate & IB at 1 mM IPTG induction for 2 h, respectively; Lane 3 & 4: lysate and IB at 1 mM IPTG for 4 h, respectively; Lane 5 & 6: lysate & IB at 0.5 mM IPTG induction for 2 h, respectively; and, Lane 7 & 8: lysate and IB at 0.5 mM IPTG for 4 h, respectively. Same expression profile was obtained for WsSQS2

Lysate obtained from above optimized conditions was then used to purify protein by Ni^{+}-NTA affinity chromatography. The peak of expected protein was obtained by elution with 250 mM imidazole elution buffer (Fig 3.6a), the protein fractions were collected, run on SDS-PAGE and silver stained which showed presence of ~ 43 kDa purified protein bands and there was also small contaminating protein present (Fig 3.6b). To further purify WsSQS, the Ni^{+}-NTA purified fractions were pooled, concentrated and subjected to Superdex 75 pg gel filteration chromatography. The proteins were separated according to their size and the protein of expected size was estimated by running different protein standards on the column (Fig 3.6c). The purified fractions were run on SDS-PAGE and silver stained which

revealed the presence of single bands of ~ 43 kDa without any impurity (Fig 3.6d). The purified protein fractions were pooled, concentrated, desalted and further used for protein and enzyme assays.

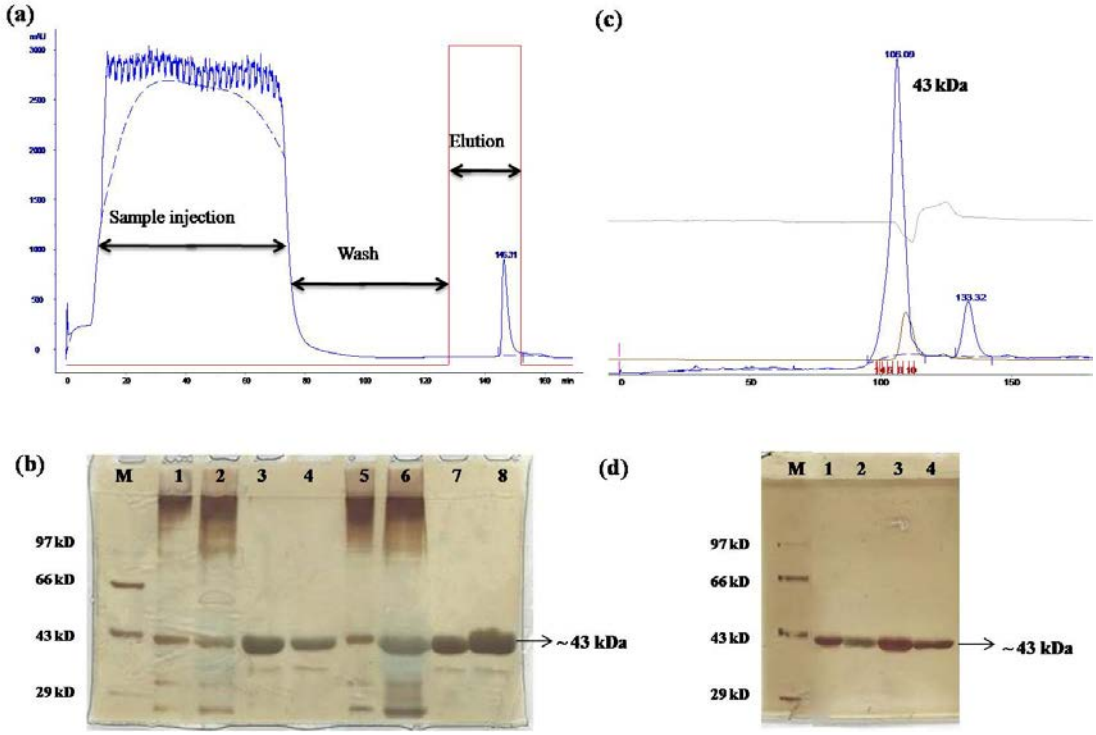


Fig 3.6: Protein purification of WsSQS. **(a)** Ni^{+}-NTA affinity chromatogram showing peak of WsSQS; **(b)** SDS-PAGE of WsSQS subjected to Ni^{+}-NTA affinity chromatography. M: Molecular weight ladder; Lane 1, 2 & 5, 6: lysate and IB from WsSQS1 and WsSQS2, respectively; Lane 3, 4 & 7, 8: Ni^{+}-NTA purified fractions of WsSQS1 and WsSQS2, respectively; **(c)** Gel filteration chromatogram of WsSQS1 showing peak of ~ 43 kDa and a small peak of contaminating protein obtained just after the elution of WsSQS; and **(d)** Silver stained SDS-PAGE after gel filteration of WsSQS. M: Molecular weight ladder; Lane 1-4: gel filteration purified WsSQS fraction having no contaminating protein bands

3.4.5 WsSQS enzyme assay and product identification

The WsSQS enzyme assay was performed as mentioned in section 3.3.9, and the product was extracted and analyzed by GC-MS. The authentic squalene was run to obtain gas chromatogram and mass spectrum of squalene (Fig 3.7a & b). The product in the reaction mixture was identified by comparing retention time and mass fragmentation pattern of the authentic squalene (Fig 3.7c & d). The reactions catalyzed by both WsSQS1 and WsSQS2 showed the good resolved peak for squalene while there was no peak observed in control reactions without enzyme or substrate. The reactions were also set in absence of NADPH

which showed no peak for squalene, thus indicating that NADPH is necessary for carrying out the reaction (Fig 3.7e).

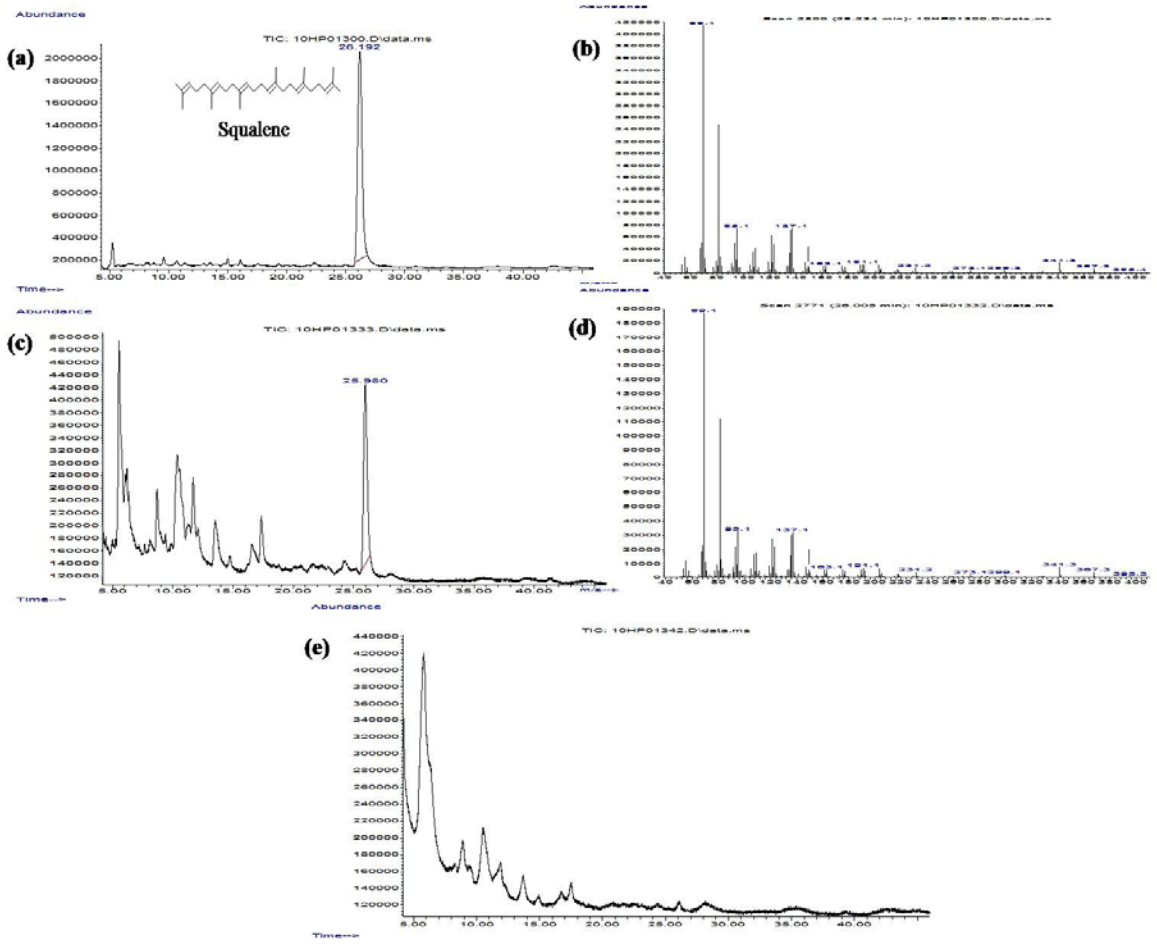


Fig 3.7: GC-MS detection of squalene in WsSQS assay. **(a)** gas chromatogram of authentic squalene, **(b)** mass spectrum of authentic squalene, **(c)** gas chromatogram of reaction mixture of purified WsSQS, **(d)** its mass spectrum; and **(e)** gas chromatogram of control reaction in absence of NADPH

3.4.6 Raising polyclonal antibodies against purified WsSQS in rabbit

Approximately 300 µg of purified protein was used for first injection in New Zealand rabbit to raise antibodies against WsSQS 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 WsSQS protein. Titer of 1st bleed was ~1:10000 (Fig 3.8a) and the dilution of 1:5000 was used for further experiments. For exact quantification of SQS in total plant tissue protein, ELISA was performed with different concentrations of purified WsSQS and a standard graph was plotted between WsSQS concentrations and

OD_{405} (Fig 3.8b). The R^2 value of the graph was 0.99 and the concentration of WsSQS (x) in unknown sample was calculated from the equation y = a + b * x, where y = OD_{405} of the unknown sample, a = the intercept and b = slope of the curve.

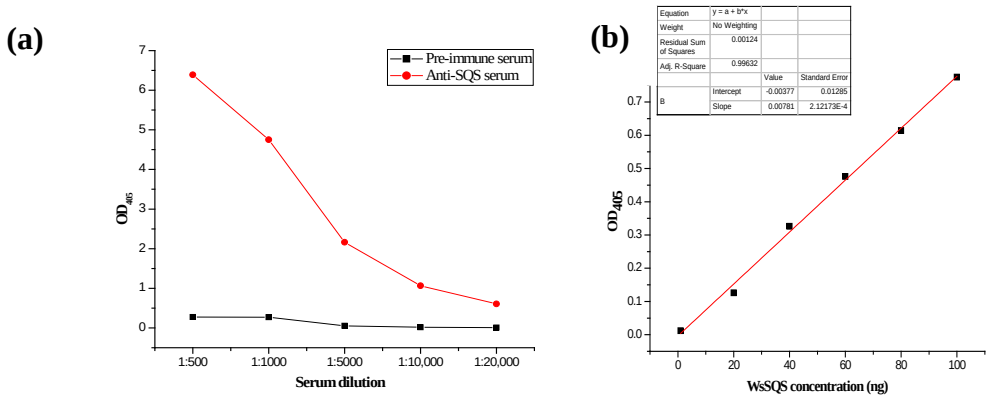


Fig 3.8: Immunological studies of WsSQS. **(a)** Titer of Anti-WsSQS polyclonal antibody; and **(b)** a standard graph between WsSQS concentrations and OD_{405}

3.4.7 WsSQS analysis in different tissues of *W. somnifera* by ELISA and Western blot

WsSQS quantitative analysis in leaf, stem and root of *W. somnifera* was done by using the determined titer of above polyclonal antibodies raised against purified recombinant WsSQS protein. Total protein from different tissues was extracted and 20 µg protein from each tissue was coated on 96 well microtiter plates. Among all three types of tissues, maximum expression of WsSQS protein was observed in root (1.4 µg/g FW) which was almost equal to leaf (1.3 µg/g FW) and least was obtained in stem tissue (0.5 µg/g FW) (Fig 3.9a).

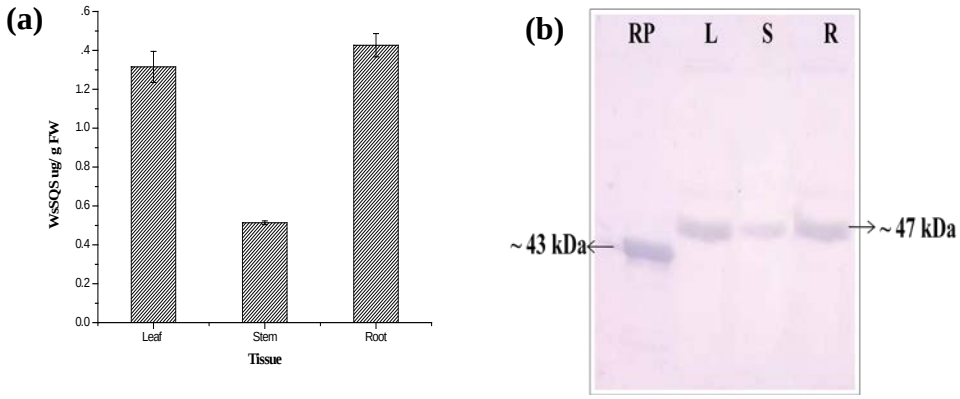


Fig 3.9: WsSQS protein expression analysis in different tissues of *W. somnifera* by **(a)** ELISA, and **(b)** Western blot (RP: recombinant truncated protein, L: leaf, S: stem and R: root)

Total soluble protein extracted from *W. somnifera* (50 µg from each tissue) was separated on 10% SDS-PAGE. Purified truncated WsSQS protein was also used as a positive control and size marker. These bands correspond to the molecular weight of ~ 47 kDa (molecular weight of untruncated SQS) and was found slightly above the band of positive control (~43 kDa) (Fig 3.9b). The band of root protein was found to be slightly intense followed by leaf and stem indicating the level of expressed SQS in these tissues.

3.4.8 Prediction of WsSQS secondary structure by PSIPRED

The secondary structure of WsSQS was predicted by the PSIPRED Protein Structure Prediction Server (PSIPRED v3.0). The structure showed 21 α-helices and no β-sheets (Fig 3.10).

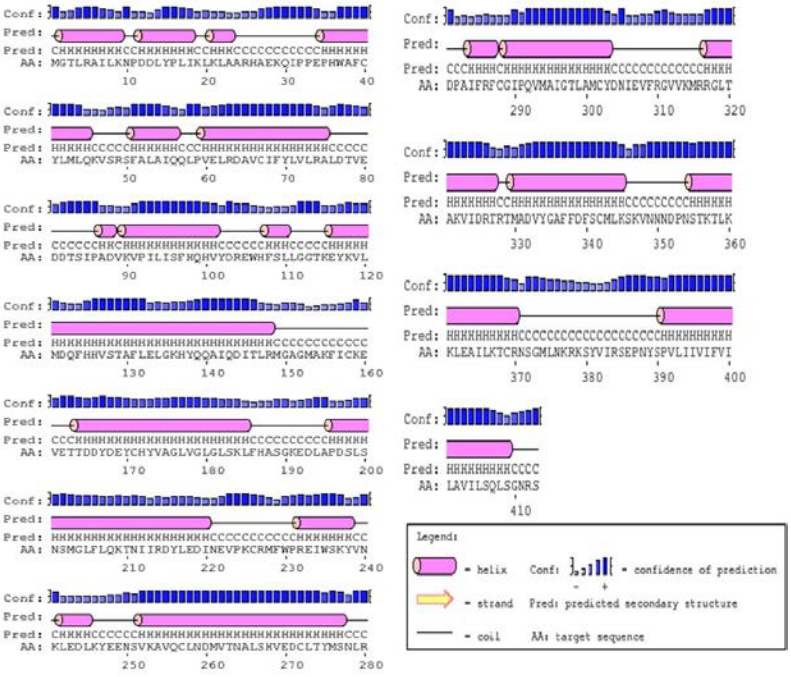


Fig 3.10: Secondary structure of WsSQS from *W. somnifera*

3.4.9 Homology modeling of WsSQS

Squalene synthase from *W. somnifera* (GU181386) 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 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 WsSQS (Fig 3.11a). Total 50 models were generated and their discrete optimized potential energy (DOPE) was calculated, and the model having lowest DOPE score was considered as the best model of WsSQS. Fig 3.11b 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 and was found to be 0.268 Å.

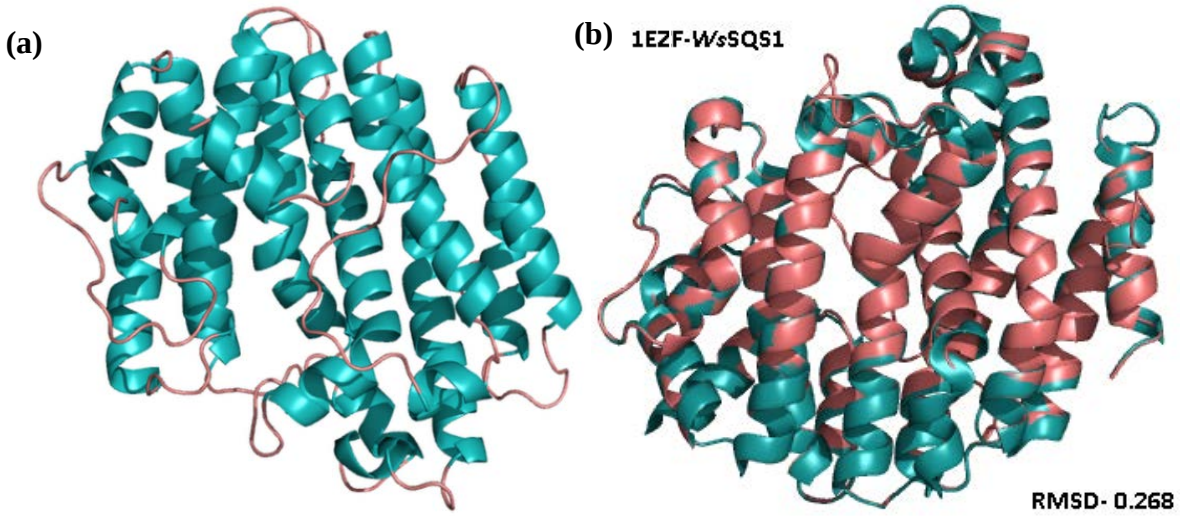


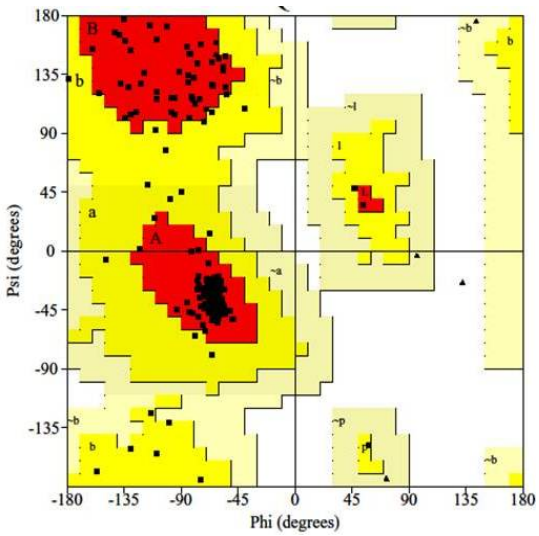
Fig 3.11 (a) 3D structure of WsSQS showing α-helices and coils; **(b)** Superimposition of modeled WsSQS (blue) with template 1EZF (red)

3.4.10 Validation of modeled WsSQS

The model was energy-minimized and then validated using different tools available on SAVEs server. Based upon these analyses the model was further refined using the loop refinement scripts of Modeller until satisfactory model was generated. PROCHECK was used to prepare the Ramachandran plot of final refined model of WsSQS (Fig 3.12). The results indicate that the protein model is reliable with more than 90% residues in favoured region and none in disallowed regions of the plot.

Further, ERRAT analysis of the model showed no regions of the structure that can be rejected at the 95% confidence level (Fig 3.13). The overall quality factor of the model was 97.22, expressed as the percentage of the protein for which the calculated error value

falling below the 95% rejection limit. Good high resolution structures generally produce values around 95% or higher. The Verify 3D score was 96.4, indicating that generated model was reliable. The Z score of the model (-8.5) is located within the space of protein related to X-ray (Fig 3.14) This value is nearly close to that of the template 1EZF which suggests that the obtained model is reliable.



Residues in most favored regions [A, B, L]	281	91.5%
Residues in additional allowed regions [a, b, 1, p]	26	8.5%
Residues in generously allowed regions [~a, ~b, ~1, ~p]	0	0.0%
Residues in disallowed regions	0	0.0%
Number of non-glycine and non-proline residues	**307**	**100.0%**
Number of end residues (excl. Gly and Pro)	1	
Number of glycine residues (shown as triangles)	15	
Number of proline residues	9	
Total number of residues	**332**	

Fig 3.12: Ramachandran plot of modeled WsSQS

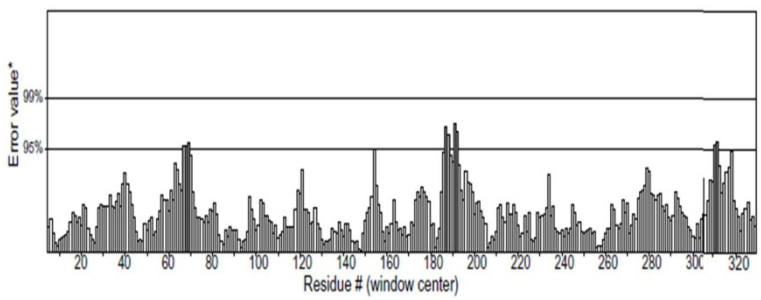


Fig 3.13: ERRAT plot

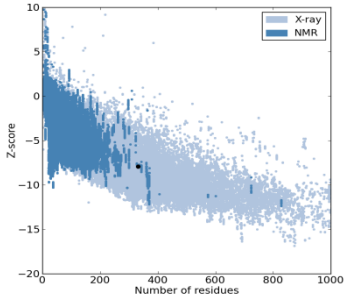


Fig 3.14: Z-Score Plot

3.4.11 Overall structure of WsSQS

WsSQS folded as a single domain having a large channel in the middle of one face. Multiple sequence alignment of WsSQS with other homologous SQS and also with template was performed using ClustalW 2.0 to locate the conserved residues. The conserved feature in the structures of WsSQS and other Class I isoprenoid biosynthetic enzymes is an α-helical core surrounding a central cavity, with hydrophobic residues at one end, and more hydrophilic at the other. A "flap" like structure covers one end of the central channel formed by residues VSRSF (47-51) while the other end is exposed to the solvent with two conserved aspartate-rich motifs DTVED (77-81) and DYLED (213-217) (Fig 3.15). These residues along with those lining the central channel are highly conserved in different squalene synthases as well as other plant and bacterial phytoene synthases (Ashby and Edwards 1990; Robinson et al. 1993). It is highly imperative that due to similar functions of these enzymes, this highly conserved central channel may harbor the active sites for the two half-reactions.

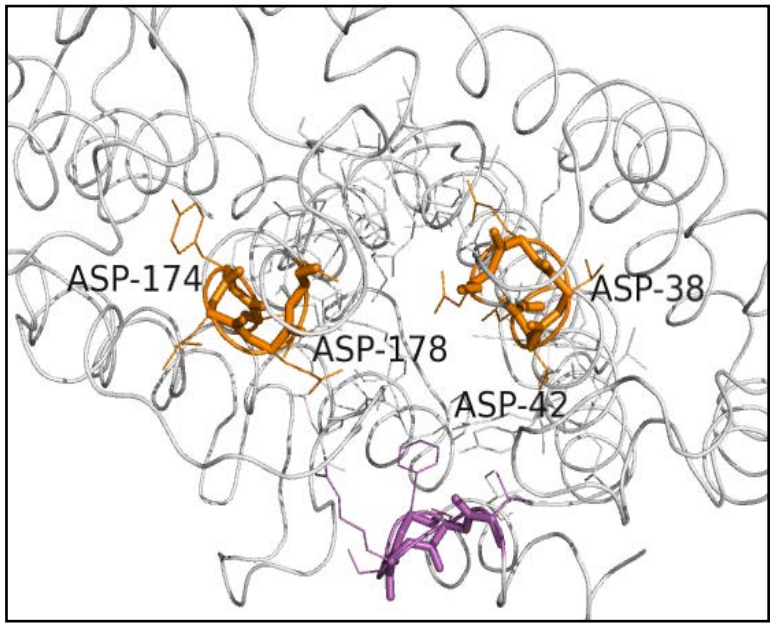


Fig 3.15: Conserved regions on WsSQS. The highly conserved aspartates which are a part of two DDXXD motifs at one end of the central channel, expected to be involved in binding the diphosphate moiety of the substrates via Mg^{2+} ions are indicated in orange color. The "Flap" is shown in magenta color with the conserved Tyr residue at the other end of the central channel. Note that the residue number mismatches with the ones mentioned in text are due to deletion of 39 residue transmembrane domain of the model

3.4.12 Active-site geometry

Substrates FPP and NADPH were docked with modeled WsSQS in order to understand the active-site morphology of the enzyme. The 3D structure of the substrates was taken from Pubchem server of NCBI. The X, Y, Z dimensions of the grid were set to -10.96, 32.68 & 26.63 Å, with grid points separated by 40 Å. The amino acid residues of WsSQS protein playing active role in the interaction with ligands FPP (Fig 3.16a) and NADPH (Fig 3.16b) are shown. NADPH binding site pocket consisted of S48, Y70, R74, D77, V172, Q206, N209, Q290, and R315. The substrate binding site of WsSQS is made up of 3 residues D77, Q206 and N209. Residues corresponding to the active site in WsSQS are highly conserved among the SQSs from various plant species and also with template 1EZF. Docking conformations of FPP and NADPH with WsSQS obtained from AutoDock Vina showed binding energies -7.8 and -9.5 Kcal/mole for FPP and NADPH, respectively. The key intermolecular interactions between WsSQS and substrates were identified from docking studies and are discussed below.

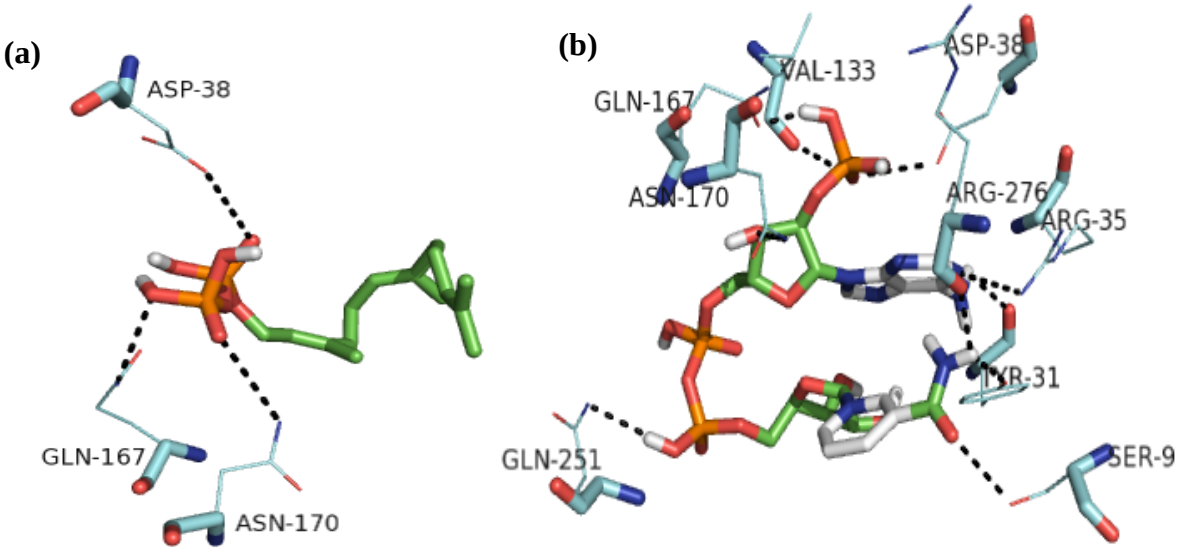


Fig 3.16: Stick-line diagram showing interaction of WsSQS with **(a)** FPP; and **(b)** NADPH. Black dotted lines shows H-bonding. Note that the residue number mismatches with the ones mentioned in text are due to deletion of 39 residue transmembrane domain of the model

3.4.13 Catalytic reaction

In the first half-reaction, the condensation of two molecules of FPP forms a cyclopropyl containing intermediate, PSPP. Class I isoprenoid biosynthetic enzymes contain a DD*XX*D sequence motif that binds the diphosphate moiety of the substrates via Mg^{2+} ions,

facilitating phosphate release. Structural superposition of WsSQS on Human SQS shows two conserved sequence motifs (D77-D81 and D213-D217) (Fig 3.15) which are on opposite sides of the central cleft. It is likely that these motifs bind the diphosphates of two FPP molecules with the isoprene tails of both FPP molecules extending toward the hydrophobic end of the channel. The 30 C intermediate PSPP can be accommodated in a large hydrophobic cavity present on the same side of the cleft along with DTVED motif suggesting the prenyl acceptor binding on this side of the cleft. Also, Q216 and N209 located adjacent to DYLED domain have their side chains extended into the cleft near the side chains of the conserved aspartate residues, suggesting their role in stabilizing the diphosphate leaving group by forming electrostatic or hydrogen bonding interactions.

The product of the first half-reaction is the stable intermediate, PSPP, which is rearranged and reduced by NADPH to form squalene in a second reaction. An important part of the SQS mechanism includes effective shielding of the higly reactive carbocation formed from premature solvent quenching. Human SQS crystal structure shows a cavity accommodating the carbocationic intermediates likely to be defined by two pockets forming the upper end of the central cleft; formed by the amino acids constituting part of the “flap” corresponding to the residues VSRSF of WsSQS (Fig 3.15). The residues lining these pockets are predominantly hydrophobic and completely conserved in all known SQS sequences (Pandit et al. 2000).

3.5 Conclusions

The truncated version of the WsSQS was obtained to get more protein in soluble fraction and purified recombinant protein was obtained after Ni^{+}–NTA affinity chromatography. GC–MS analysis confirmed that the recombinant SQS proteins could catalyze the formation of squalene from FPP and NADPH is essential requirement of the reaction. The purified WsSQS was used to raise polyclonal antibody which was used to determine the levels of WsSQS in different tissues of *W. somnifera* which revealed maximum protein in root followed by leaf and stem. Secondary structure prediction showed the presence of 21 α-helices and no β-sheets in the structure and 3D structure was validated by PROCHECK, ERRAT and ProSA, and the structure was found to be reliable. The WsSQS model was docked with FPP and NADPH to understand the role of amino acids involved in interactions with the ligands.

CHAPTER 4

TISSUE SPECIFIC EXPRESSION ANALYSES OF *WsSQS* UNDER STRESS CONDITIONS

4.1 Introduction

W. somnifera synthesize wide range of SMs called withanolides which are well known for their biological efficacies, but at the same time, these compounds possess various other properties of plant benefit and are highly important for plant survival in natural environment. SMs are responsible for defense, signaling, flavor, fragrance, hormonal, antibiotic, insecticidal and pharmaceutical properties of the plant. When the plants are stimulated by a wound and/or pathogen in the environment, they respond to stress through the synthesis of defensive SMs and other defense-related proteins. The activation of defense responses is associated with the increased expression of a large number of genes involved in rapid and localized cell death, the production of pathogenesis-related proteins, and the accumulation of antimicrobial compounds (Stoessl 1980; Subba and Strange 1994; VanEtten et al. 1994). The synthesis of withanolides is discussed in detail in Chapter 1 (section 1.4.4) and the role of squalene synthase is well explained. Thus, in this chapter the relative expression of *WsSQS* transcript in different parts of the plant (leaf, stem and root) were studied by qRT-PCR in the presence of stressors: salt (NaCl), salicylic acid (SA) and methyl jasmonate (MJ). Later on, the WsSQS protein expression level was also analyzed in these samples using ELISA.

4.2 Materials

Tissue culture raised *W. somnifera* plants of same age were used for differential expression studies.

Brilliant II™ SYBR® Green Q-PCR master mix was purchased from Stratagene, Agilent Technology, USA. BCIP/NBT solution was procured from Bioworld, USA.

4.3 Methods

4.3.1 Stress treatment of plant tissues

Tissue culture raised *W. somnifera* plants were transferred to half strength MS (1/2 MS) liquid medium having different stressors separately. Root, stem and leaf tissues were harvested separately at time interval of 2 h, 6 h and 12 h. Two different concentrations of NaCl (100 & 200 mM), salicylic acid (50 & 100 μM) and methyl jasmonate (250 & 500 μM) were taken into consideration.

4.3.2 *WsSQS* transcript expression analysis by qRT-PCR

4.3.2.1 Total RNA extraction and cDNA synthesis

Total RNA was isolated from the stress treated tissues and first strand cDNA was synthesized, primed with an oligo $(dT)_{15}$ primer, according to the protocol described in Chapter 2; section 2.3.1 & 2.

4.3.2.2 Quantitative real time PCR

qRT-PCR is an established technique for quantifying mRNA in biological samples. Advantages of this procedure over conventional methods for measuring RNA include its sensitivity, large dynamic range and the potential for high throughput as well as accurate quantification. Furthermore, many of the key proteins are found in such low abundance that qRT-PCR quantification of their mRNAs represents the only technique sensitive enough to measure reliably their expression *in vivo* (Huggett et al. 2005). qRT-PCR is a powerful tool for gene expression analysis and was first demonstrated by Higuchi et al. (1992, 1993). qRT-PCR quantitates the initial amount of the template most specifically, sensitively and reproducibly, and 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; 1993).

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, 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.

4.3.2.3 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 done 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.

4.3.2.4 qRT-PCR reactions

The cDNA first strands were used as templates for quantification of WsSQS gene transcripts. Brilliant II™ SYBR® Green Q-PCR master mix was used in the reactions which contains 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. Ubiquitin was used as a housekeeping gene and its amplification was achieved by using UBC primer mix (Eurogentec, Belgium) while

gene specific amplification was achieved by using RT-SQSF – RT-SQSR primers (Appendix-IV) designed from the sequence of *WsSQS*.

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:

Reagents	**Volume**
PCR grade water	10.5 µL
Primer Mix (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 SYBR Green mastermix 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 as:

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

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 ($2^{-\Delta\Delta Ct}$) in terms of mean fold expression (Pfaffl 2001).

4.3.2.5 Relative quantification methods

Relative (comparative) quantification relates to the PCR signal of the target transcript in a treatment group to that of another such as untreated control.

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

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$

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

Means,

Δ Ct $_{sample}$ = Ct $_{sample}$ – Ct $_{endogenous\ control}$ (Ubiquitin gene or any other housekeeping gene)

Δ Ct $_{reference}$ = Ct $_{reference}$ – 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 Δ Ct varies with template dilution. If the plot of cDNA dilution versus Δ Ct 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.

4.3.3 WsSQS protein expression studies by ELISA

Protein was isolated from 0.5 g of different tissues of *W. somnifera* and ELISA was performed with total protein according to the protocol described in section 3.3.11.

4.4 Results and discussion

4.4.1 Tissue specific expression analysis of WsSQS

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. It was found that *WsSQS* expressed in all tissues but the level of expression was different. It was highest in root followed by leaf and minimum in stem tissue (Fig. 4.1a**).** Root and leaf tissue accumulated 4 and 2 fold higher *WsSQS* mRNA than stem tissue, respectively. Similar pattern was observed for WsSQS protein in different plant tissues with maximum expression in root which was almost equal to leaf and lowest in stem (Fig. 4.1b).

The similar results with highest SQS transcript accumulation in root tissue were also observed in other plants including *L. japonicus* (Akamine et al. 2003), *M. truncatula* (Suzuki et al. 2002), *A. thaliana* (Kribii et al. 1997) and *T. cuspidata* (Huang et al. 2007). While some different observations were seen in *E. tirucalli* where *SQS* transcript accumulated in almost equal amounts in the stem and the leaf with a stalk, while a lower amount was detected in the root (Uchida et al. 2009), unlikel tobacco, where SQS was

found to be localized predominantly in shoot apical meristem (Devarenne et al. 2002). In *P. 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).

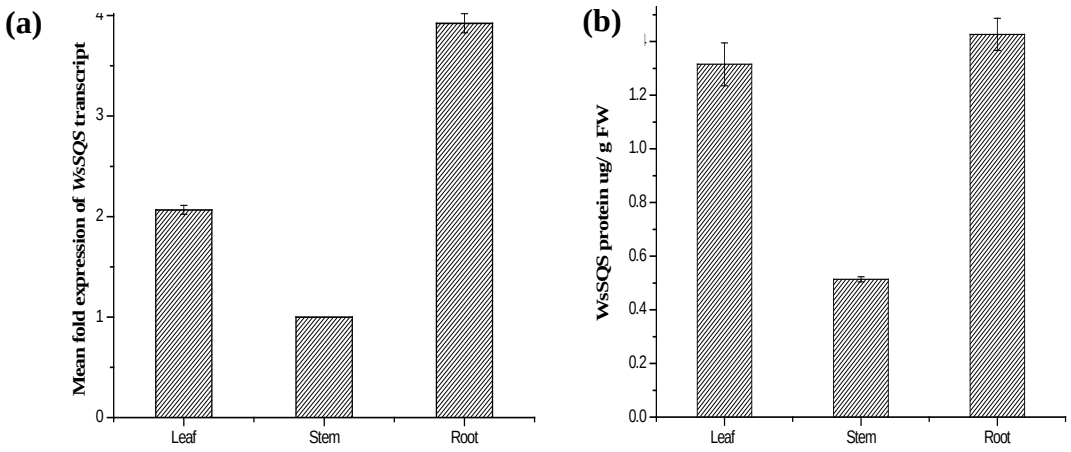


Fig. 4.1: Relative accumulation of (a) *WsSQS* transcript in terms of mean fold expression in different tissues; (b) WsSQS protein as determined by ELISA. All values are plotted with standard error of mean of triplicate analysis

4.4.2 Differential expression analysis of WsSQS under salt stress

Salt stress negatively affects many physiological processes in plants. It can lead to changes in development, growth and productivity, and severe salt stress may also threaten survival. The two different concentrations of salt stress were selected so as to study the maximum effect of this stress on plant tissues for the defined period of time.

4.4.2.1 NaCl (100 mM)

Here we report the expression analysis of *WsSQS* transcripts (Fig. 4.2a) and WsSQS protein (Fig. 4.2b) in the presence of 100 mM NaCl. There was no co-relation between transcript expression and protein accumulation. qRT-PCR revealed the variation in level of transcript expression in all the tissue with time. ELISA showed no significant difference in protein expression in leaf and stem while the expression increased to 4 times in root.

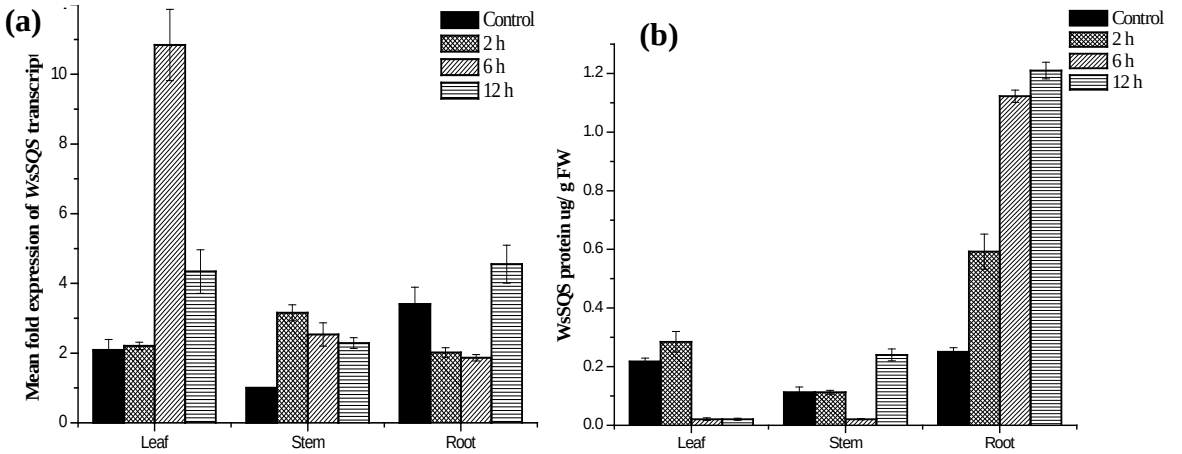


Fig. 4.2: Relative accumulation of **(a)** *WsSQS* transcript in terms of mean fold expression; **(b)** WsSQS protein in µg/g FW of tissue under salt (NaCl 100 mM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis

4.4.2.1 NaCl (200 mM)

qRT-PCR showed that at this concentration of salt, transcript first increases in leaf and then decreases with time while in stem the level of expression increases with time. Root tissue showed decrease in expression in 2 and 12 h while there was no significant increase at 6 h also (Fig. 4.3a).

ELISA showed completely different pattern of protein expression with maximum protein in root tissue esp. at 6 h. The expression in leaf and stem increased at 2 h followed by decrease at 6 h while it again increased at 12 h (Fig. 4.3b).

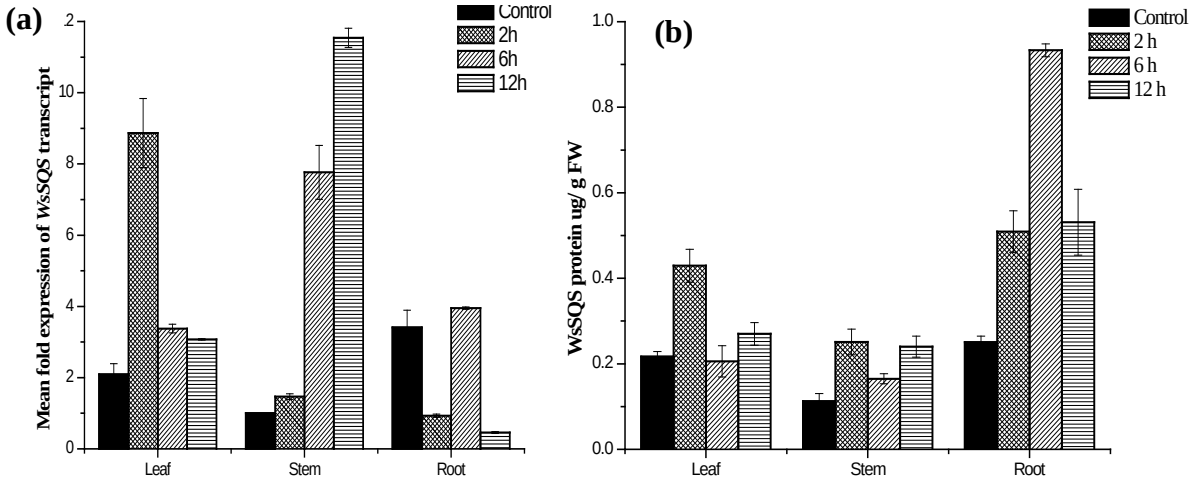


Fig. 4.3: Relative accumulation of **(a)** *WsSQS* transcript in terms of mean fold expression; **(b)** WsSQS protein in µg/g FW of tissue under salt (NaCl 200 mM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.4.3 Differential expression analysis of WsSQS under salicylic acid

Salicylic acid have been identified as key signaling phytohormone regulating a complex network of signal transduction pathways responsible for induced plant defense against biotic and abiotic stresses (Smith et al. 2009; Zhao 2004; Shah 2009; Misra et al. 2010a). These responses lead to the induction of various SMs like isoprenoids as a defence mechanism (Ament et al. 2006; Martin et al. 2002; Misra et al. 2010b). Salicylic acid is very effective inducer for gene expression while its higher amount leads to death of the plant, so its concentrations for the present study were selected to study the maximum effect on *WsSQS* expression without any damage to the plant.

4.4.3.1 Salicylic acid (50 µM)

The *WsSQS* transcript increased in all the tissues esp. in 6 h sample of leaf and stem. The expression was maximum in 2 h root which was about 8 fold greater than control root (Fig. 4.4a). The protein expression pattern was similar to the transcript except for root tissue where protein quantity increased with time (Fig. 4.4b).

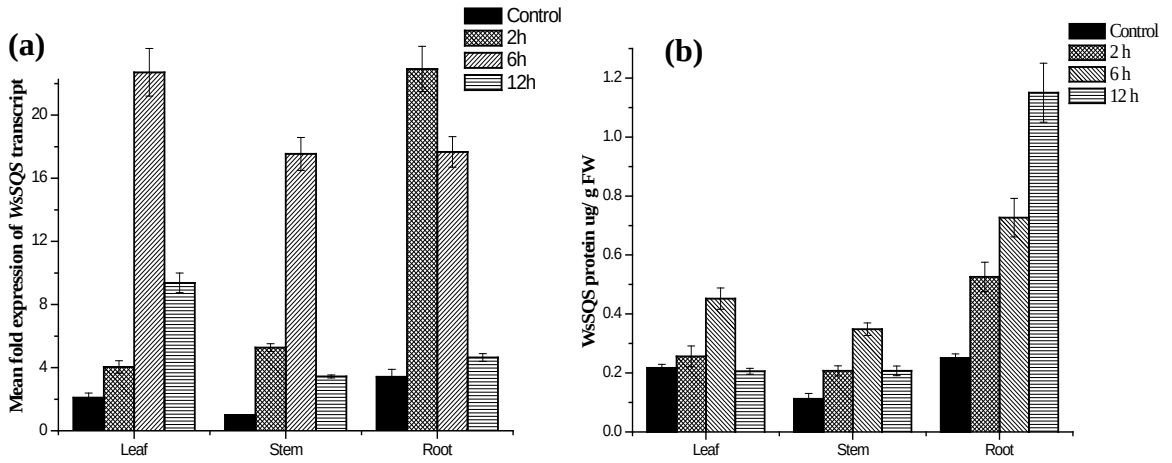


Fig. 4.4: Relative accumulation of **(a)** *WsSQS* transcript in terms of mean fold expression; **(b)** WsSQS protein in µg/g FW of tissue under salicylic acid (50 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.4.3.2 Salicylic acid (100 µM)

The mRNA expression was observed to increase with time on exposing the plant tissues with 100 µM salicylic acid till 6 h, after which the expression decreased except for root tissue where expression decreased with time. Maximum expression was shown by 6 h stem

sample where mRNA increased up to 14 fold (Fig 4.5a). ELISA revealed somewhat different pattern of protein expression where 2 h leaf and stem showed higher expression rather than 6 h, and protein expression increased in root with time (Fig 4.5b).

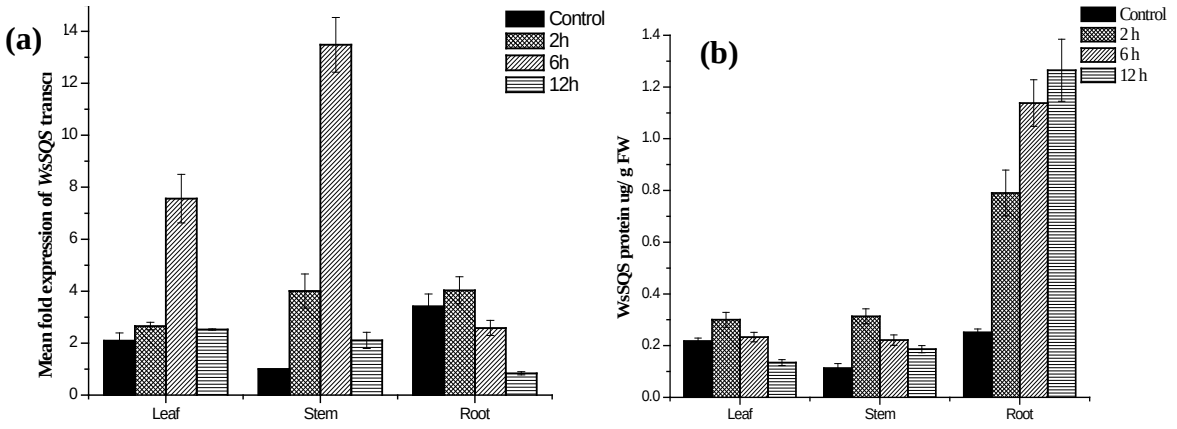


Fig. 4.5: Relative accumulation of **(a)** *WsSQS* transcript in terms of mean fold expression; **(b)** WsSQS protein in µg/g FW of tissue under salicylic acid (100 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis

4.4.4 Differential expression analysis of WsSQS under methyl jasmonate

Methyl jasmonate is an organic volatile compound used in plant defense and also affects diverse developmental plant pathways (Cheong et al. 2003). Plants produce jasmonic acid and methyl jasmonate in response to many biotic and abiotic stresses (in particular, herbivory and wounding), which build up in the damaged parts of the plant. The methyl jasmonate can be used to signal the original plant's defense systems which causes the overexpression of many unknown genes of the pathways leading to enhanced SMs of the plant.

4.4.4.1 Methyl jasmonate (250 µM)

The mRNA expression increased with time in leaf tissue, was found maximum in 12 h stem and was maintained at lowest in root (Fig 4.6a). The pattern was reflected in protein where 12 h tissue samples showed maximum expression, increased with time in root (Fig 4.6b).

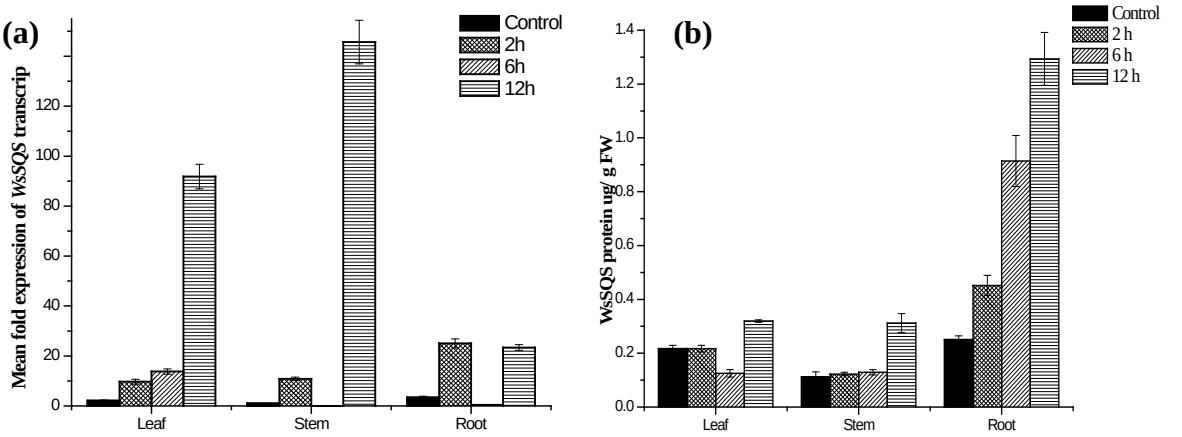


Fig. 4.6: Relative accumulation of **(a)** *WsSQS* transcript in terms of mean fold expression; **(b)** WsSQS protein in µg/g FW of tissue under methyl jasmonate (250 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

4.4.4.2 Methyl jasmonate (500 µM)

The transcript expression increased with time in leaf and stem till 6 h, after which it decreased. While in root tissue, the expression increased with time. Highest expression was observed in 6 h stem tissue which was about 100 fold greater than the respective control (Fig 4.7a). The protein expression was observed to be higher in 12 h of all the three tissues with highest in root (Fig 4.7b).

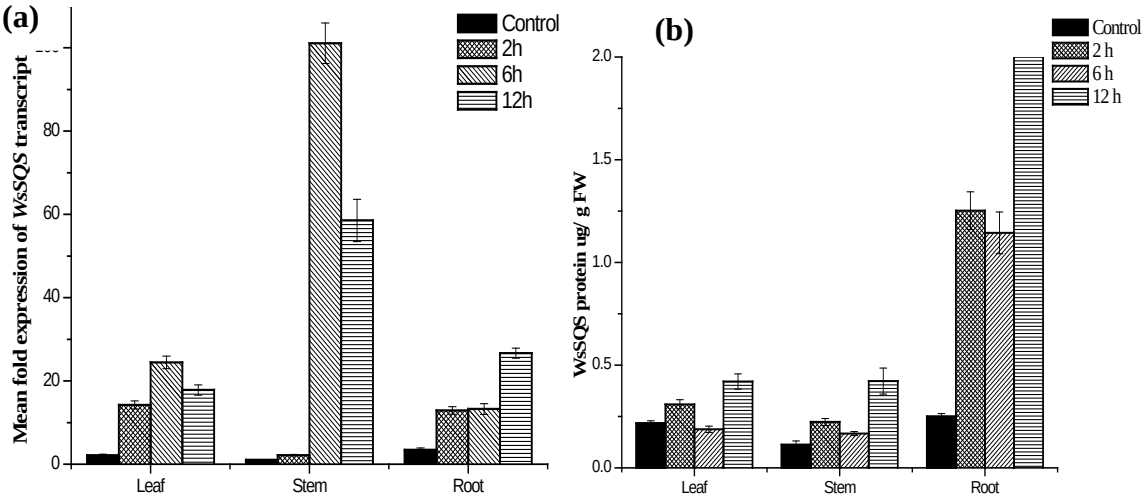


Fig. 4.7: Relative accumulation of **(a)** *WsSQS* transcript in terms of mean fold expression; **(b)** WsSQS protein in µg/g FW of tissue under methyl jasmonate (500 µM) stress with respect to control plant at different time intervals. All values are plotted with standard error of mean of triplicate analysis.

Gupta at al. (2011) performed similar studies in *W. somnifera* to understand the effect of salicylic acid, methyl jasmonate and mechanical wounding on expression of farnesyl

diphosphate synthase gene where all these treatments led to the enhanced expression of WsFPPS. The increase of gene expression was observed within 6 h of salicylic acid treatment, which reached to its maximum at 12 h post treatment and declined thereafter, and a biphasic pattern of expression was observed in case of MJ treatment.

4.5 Conclusions

The transcript and protein expression of WsSQS was analyzed in different plant tissues and our observations suggest that WsSQS expression is responsive to NaCl, salicylic acid and methyl jasmonate treatments. Differential expression patterns of WsSQS observed with different elicitors propose that *SQS* gene expression is tightly regulated to the kind of the defense signals applied. The noted response of rapid and steady accumulation *WsSQS* transcripts and protein in response to these stressors might reflect onset of a perpetual defense against the threat (e.g. herbivory etc.). Altogether, differential expression of *WsSQS* with various defense signal suggests that expression of this gene is differentially regulated recognizing each effectors signal individually. Our aim was to study responsiveness of *WsSQS* to different elicitors where enhanced expression of WsSQS may modulate withanolide content of the plant.

CHAPTER 5A

GENETIC TRANSFORMATION OF *W. SOMNIFERA* FOR OVEREXPRESSION OF *WsSQS* AND MOLECULAR ANALYSES OF TRANSFORMED PLANTS

5A.1 Introduction

W. somnifera is an important medicinal plant, well known for its use in Ayurveda and all the pharmaceutical properties of the plant are accredited to the characteristic SMs of the plant, called withanolides (Matsuda et al. 2001; Singh et al. 2001; Misra et al. 2005). Withanolides are mainly recognized for their antitumor, antioxidant, antistress and many other properties. The plant has been extensively exploited for the extraction of these SMs for their use in medicinal formulations, which may lead to the extinction of *W. somnifera* in near future. So people have already started looking for the better alternative for improvement of withanolide content of *W. somnifera*. Various conventional tissue culture techniques and chemical synthesis of withanolides were attempted (Kovganko and Kashkan 1997; Gamoh et al. 1984; Jana et al. 2011) which failed to produce withanolides in sufficient quantities (Yu et al. 1974). There are few reports on plant cell and hairy root cultures developed for the production of the important metabolites from *Withania* extracts (Murthy et al. 2008; Roja et al. 1991), although withanolide production by *in vitro* cultures is still far from the levels required for economical exploitation. However, this limitation can be overcome by using genetic engineering as a tool to manipulate crucial steps of metabolic network to increase the yield of withanolides. Development of an efficient genetic transformation protocol is of great significance for manipulating SMs biosynthetic pathway. In this regard, an integrated protocol was designed to increase transformation efficiency of *W. somnifera* which was further utilized for the introduction of an important gene that can divert the metabolic flux towards the synthesis of compounds of interest. In the present chapter, three methods are described to transform *W. somnifera* tissues for the overexpression of squalene synthase. In the later part of the chapter, the transformants were analyzed by biochemical Gus assay and molecular analyses to ensure the integration of introduced gene into the host genome.

5A.2 Materials

Seeds of *W. somnifera* were procured from Vindhya herbals, Bhopal, Madhya Pradesh, India to raise tissue culture plants.

Plant genomic DNA isolation kit was purchased from Hipura, Himedia, India.

Refer Appendix-XII for plant tissue culture media.

5A.3 Methods

5A.3.1 Cloning of *WsSQS* in binary vector pCAMBIA 1301

WsSQS was cloned in sense orientation in a modified plant transformation binary vector pCAMBIA 1301 (Appendix-I). Primers (SnsSQSF & SnsSQSR; Appendix-IV) were designed having sites for restriction enzymes *Kpn*I and *Sac*I and gene was amplified by PCR using pGEM-T Easy vector harboring full length *WsSQS* as a template. The amplified product was ligated into pGEM-T Easy vector, transformed into *E. coli* XL10 followed by plasmid isolation, restriction digestion and sequencing. *WsSQS* was then released from pGEM-T Easy vector by double digestion and finally ligated into pCAMBIA 1301 (digested with same restriction enzymes) and transformed into *E. coli*. Plasmids were isolated and used for transformation of *Agrobacterium tumefaciens* GV2260 (Appendix-II) for plant transformation.

5A.3.2 *A. tumefaciens* competent cell preparation and transformation

A single colony of *A. tumefaciens* (GV2260) was inoculated in 50 mL YEB broth (Appendix-VI) containing rifampicin (50 µg/mL) and incubated at 28 ºC with shaking at 200 rpm till A_{600} reached 0.5-0.7 followed by centrifugation at 5000 g for 10 min at 4 ºC and washings 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 of pCAMBIA 1301 binary vector having *WsSQS* sense construct 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 which 1 mL YEB medium was added and the tubes incubated at 28 ºC for 2 h with gentle shaking. The cells were centrifuged at 4000 g for 5 min and 100 µL supernatant was retained in the tube with the pellet. The cells were resuspended and plated on YEB 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.

5A.3.3 Initiation and propagation of *W. somnifera* tissue culture

W. somnifera seeds were washed with sterile distilled water and then rinsed with 1% teepol for 1 min followed by sterile distilled water washings in laminar air flow cabinet. Seeds were then treated with 0.1% mercuric chloride for 5 min, washed thoroughly with sterile

distilled water to remove traces of $HgCl_2$, inoculated on germination medium (Appendix-XII) and incubated in dark for 15 days to obtain germination. The germinated seeds were then transferred to 1/2 MS liquid media for further development of the seedlings.

For rapid multiplication of *W. somnifera*, *in vitro* grown healthy and young seedlings were used as a source of explant. The apical and nodal explants of one cm length containing a single node along with a small portion of petiole were excised and micropropagated by inoculating on proliferation medium (Appendix-XII) supplemented with appropriate growth regulators (kinetin and BAP) to check the effect of different concentrations of growth hormone on shoot elongation. The axillary meristem proliferation and shoot elongation was obtained within 4-6 weeks. The elongated shoots were subcultured after every 5-6 weeks. For rooting, the developed shoots were transferred to rooting medium (Appendix-XII) to develop into complete plants. All cultures were incubated under 60 µmol m^{-2} s^{-1} light intensity at 26 ± 2 ºC for 16 h photoperiod.

5A.3.4 Hygromycin and Cefotaxime sensitivity assay

Explants of same age and size were taken from *in vitro* grown shoots and ten explants in three replicates were inoculated aseptically on proliferation medium for each concentration of hygromycin B (0, 5, 10, 15, 20, 25 and 30 mg/L). The growth of the explants was observed for a month to check their sensitivity towards hygromycin B and determine LD_{50} for hygromycin B.

Cefotaxime, in stipulated concentrations, is an antibiotic which inhibits excess growth of *Agrobacterium* in plants. To check the sensitivity of the *A. tumefaciens* GV2260 towards cefotaxime, disc diffusion method was carried out for the concentrations ranging from 100 – 300 mg/L.

5A.3.5 Genetic transformation of *W. somnifera*

The genetic transformation in *W. somnifera* was achieved by following three methods:

5A.3.5.1 *A. tumefaciens* mediated transformation

Disease free, healthy young apical and nodal segments from *in vitro* grown shoots were used as an explant for transformation studies. The explants were precultured for 2 days on proliferation medium. *A. tumefaciens* was inoculated in YEB minimal medium containing

50 mg/L rifampicin and 50 mg/L kanamycin and incubated at 28 ºC for overnight with constant agitation of 175 rpm. This fresh bacterial culture (A_{600} 0.62) was harvested by centrifugation at 5000 rpm for 10 min and pellet was resuspended in liquid MS medium to obtain a density of 10^8 cells/mL. The preconditioned explants were immersed in freshly prepared bacterial suspension for 15 min. After blotting away the excess of bacterial culture, the explants were transferred to proliferation medium. Co-cultivation was carried out for 48 h in dark.

5A.3.5.2 Microprojectile bombardment

The alkaline lysis method of Sambrook et al. (1989) was improvised to isolate pCAMBIA 1301 plasmid from *E. coli* XL10 cells with yield of 5-30 µg plasmids per 1.5 mL of culture. Gold microparticles (10 mg, 1 µm diameter) were suspended in 500 µl sterile milli Q water by vigorous vortexing for 10 min followed by washing with freshly prepared 70 % ethanol. The microparticles were soaked in ethanol for 15 min and washed three times with water. For coating, 10 µg plasmid DNA was dissolved in 100 µl buffer (5M NaCl, 2M Tris-HCl, pH 8.0) and added to gold microparticles. Spermidine (100 µl, 0.1M), PEG (100 µg, MW 1300-1600) and calcium chloride (100 µl, 2.5M) were added to this solution and incubated for 10 min on ice for precipitation followed by centrifugation for 1-2 min at 12000 rpm. Supernatant was discarded; pellet was washed with absolute ethanol and re-suspended in 100 µl absolute ethanol. Vortexing and brief spin after each step was done to ensure uniform particle distribution and minimize agglomeration.

Since this was the first attempt of microprojectile bombardment in *W. somnifera*, some of the parameters influencing transformation were optimized:

i. **Preculture duration of the explants**: The explants were precultured on proliferation medium for 0 h, 48 h and 96 h.

ii. **Medium support to the explant**: The explants were placed as a monolayer on petri plates with wet filter paper, solidified media and no media during bombardment.

iii. **He gas pressure**: BioRad PDS 1000/He device was used for bombardment which utilizes a burst of He gas as the accelerating force. The explants were bombarded with the plasmid coated gold particles at the He gas pressure of 900, 1100, 1300 and 1500 psi.

iv. **Target distance**: The explants were bombarded at the distance of 3, 6 and 9 cm from the stopping screen

The microprojectile bombardment was performed according to the protocol described by Sanford et al. (1993). After bombardment, the cultures were maintained on proliferation medium for ten days.

5A.3.5.3 Microprojectile bombardment followed by agroinfection

The precultured explants were bombarded using above optimized protocol and immediately co-cultivated with *A. tumefaciens* for 15 min. The cultures were kept in dark for 48 h at 26 ± 2 ºC.

5A.3.6 Selection of putative transformed shoots

After 48 h, the co-cultivated explants and bombarded plus co-cultivated explants were washed in sterile distilled water containing cefotaxime to remove most of the bacteria, blot dried and transferred to proliferation medium supplemented with cefotaxime under the same culture conditions for ten days. Shoot development was observed from the explants transformed with all the three methods used which were then shifted to selection medium. The cultures were constantly maintained on selection pressure and after a month, the concentration of cefotaxime was reduced to half and removed completely later on. The green shoots growing on this selection medium were subcultured and necrotic shoots (if any appeared) were removed.

5A.3.7 Histochemical Gus Assay

The putative transformants were checked for transient expression of *gus* gene after 7 days of transformation. For confirming expression in the subcultured tissues maintained on hygromycin B, Gus assay was done periodically to detect the β–Glucuronidase activity (Jefferson et al. 1987). The tissues were incubated overnight at 37 °C in sodium dihydrogen phosphate buffer (100 mM, pH 7.0) containing 1 mM X-Gluc and 0.5 % tween-20. Stained material was cleared in 70% ethanol for 3-4 h to remove chlorophyll. Non transformed leaves were also included as a control assay against background staining. The tissues were observed under stereomicroscope and photo-documented.

5A.3.8 Molecular characterization of transgenic events

The presence of integrated DNA into the genome of Gus-positive and hygromycin B-resistant transformants was confirmed finally by PCR using primers specific to the *hpt* II and *WsSQS* gene (Appendix-IV). For *WsSQS* specific PCR, the forward primer was designed from the sequence of CaMV 35S promoter (GeneBank GQ336528.1) and reverse primer was designed from the 3' terminal sequence of *WsSQS* (SnsSQSR). Total genomic DNA was extracted from young leaves of putative transformed shoots and tissue culture raised non transformed control plant of the same age by using plant DNA extraction kit**.** Plasmid pCAMBIA 1301 was used as a positive control. The components of PCR reaction mixture were: 15 ng template DNA, 80 µM dNTPs, *hpt* II/*WsSQS* specific forward and reverse primers (0.66 pmol each), 1 U of *Taq* DNA polymerase and 1X reaction buffer, in a total volume of 15 µl. The PCR reaction for both the genes was carried out under the following conditions, initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C (1 min for *hpt* II/1.6 min for *WsSQS*), and a final extension cycle of 72 °C for 5 min. For checking bacterial contamination in transformed plants, PCR amplification of above extracted DNA was carried out using 16S rRNA primer pair 16srRNA-F - 16srRNA-R (Appendix-IV) (Aggarwal et al. 2011) specific to bacterial 16S rRNA. Bacterial genomic DNA was used as positive control. Transformation efficiency was calculated as the percent explants showing positive result on the basis of *hpt* II and *WsSQS* PCR.

5A.4 Results and discussion

Although, regeneration has been reported frequently in *W. somnifera*, transformation studies were not carried out in any one of them. Genetic transformation of *W. somnifera* for increased production of withanolides have been confined to hairy root using *A. rhizogenes* (Banerjee et al. 1994; Kumar et al. 2005; Bandyopadhyay et al. 2007; Murthy et al. 2008) and none of these cases resulted in stable and successful regeneration of plants from the transgenic tissue. As far our knowledge goes, there are only few reports on *A. tumefaciens*-mediated transformation in *W. somnifera.* In one study, the wild type strain of *A. tumefaciens* resulted in the formation of shooty teratomas (Ray and Jha, 1999) while

another report by Pandey et al. (2010) demonstrated very low transformation efficiency using leaf explants from green house grown plants.

5A.4.1 Cloning of *WsSQS* in binary vector pCAMBIA 1301

Full length *WsSQS* was cloned in sense orientation in binary vector pCAMBIA 1301 with restriction sites *Kpn*I and *Sac*I. Its transformation was checked by restriction digestion of the plasmid isolated from transformed *E. coli* cells (Fig 5A.1) and the clone in pCAMBIA was designated as pCAM-WsSQS. Whole pCAMBIA constructs with target gene in sense orientation was used for transformation. The *WsSQS* open reading frame was positioned between CaMV 35S promoter and Nos terminator within the T-DNA region of the vector using the site specific restriction enzymes to ensure high levels of gene expression. The CaMV 35S promoter was chosen, a well-characterized strong constitutive promoter, to study the expression pattern of the candidate gene in different tissues of the plant. T-DNA regions also contained a *gus* reporter gene and a selectable marker *hpt* II gene imparting resistance to hygromycin B under the control of the constitutive CaMV 35S promoter (Fig 5A.2).

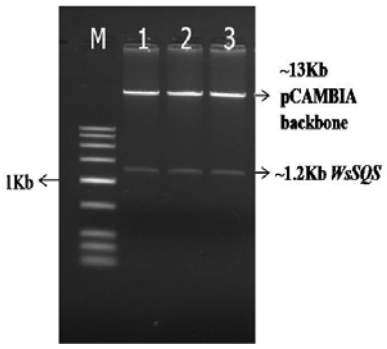


Fig 5A.1: Restriction analysis of recombinant pCAMBIA1301 with *Kpn*I and *Sac*I. Lane 1, 2 & 3: recombinant pCAM-WsSQS releasing ~1.2 kb *WsSQS* gene and vector backbone; Lane M- Low range DNA ruler

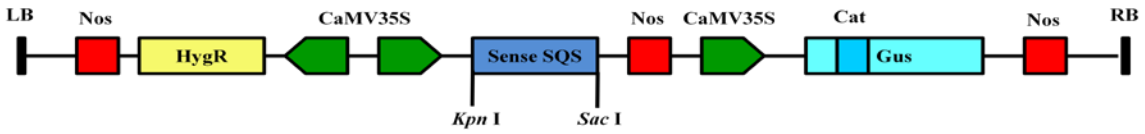


Fig 5A.2: Map of T-DNA region of modified pCAMBIA 1301 vector construct with target genes used for plant transformation. CaMV 35S: Cauliflower mosaic virus 35S rRNA promoter; Nos*:* Nopaline synthase terminator; *Hpt* II: Hygromycin phosphotransferase; *WsSQS*: *W. somnifera* squalene synthase; *Gus*: β–Glucuronidase reporter gene; Cat: Catalase intron; LB: left border; and RB: right border of T-DNA

5A.4.2 *In vitro* propagation of *W. somnifera*

The seeds were inoculated on germination media where radical emergence was observed after 15 days which grew upto 3-5 cm within 4 weeks after inoculations (Fig 5A.3a & b). They were transferred on ½ MS liquid medium onto the filter paper support to develop into complete seedlings of up to 9-10 cm in 6 weeks (Fig 5A.3c).

The apical and nodal segments obtained from these seedlings were inoculated on MS media supplemented with growth regulators (kinetin and BAP). The growth was observed in almost all combinations of kinetin and BAP but the rate of multiplication and proliferation varied significantly. The media containing kinetin (0.1 mg/L) + BAP (0.2 mg/L) supported maximum and quick shoot elongation and multiplication (Table 5A.1). This media was named as proliferation media and was used consistently after that for further subculturing and transformation studies. The sprouting of the apical and nodal meristems was observed within a week after inoculation in proliferation media and shoot elongation was achieved in 1 month with optimum number of shoots. The regenerated shoots were separated and transferred to rooting media which supported maximum root development *in vitro*.

Growth regulator (mg/L)		**No. of shoots**	**Shoot length**
Kinetin	**BAP**		
0.1	0.1	3 ± 0.04	7 ± 1.12
0.1	0.2	10 ± 0.45	12 ± 2.3
0.1	0.3	7 ± 0.54	10 ± 3.2
0.2	0.1	8 ± 1.11	4 ± 0.7
0.2	0.2	5 ± 0.32	8 ± 0.57
0.2	0.3	4 ± 0.34	5 ± 1.23

Table 5A.1: Effect of growth hormones on shoot elongation and multiplication from apical and nodal meristematic explants of *W. somnifera*, determined after 1 month of inoculation into the media. Values are the mean ± SD of three experiments

5A.4.3 Hygromycin and cefotaxime sensitivity assay

The range of hygromycin B concentration was tested to study the lethal dose and standardize its concentration for the selection of transformed explants. Hygromycin B at 10 mg/L was found to be optimum for selection of explants (Fig 5A.4). The explants growing on lower concentrations of hygromycin B showed luxuriant growth while the explants turned completely necrotic above 10 mg/L. In soyabean, Olhoft et al. (2003) examined the suppressive effects of hygromycin B on cotyledonary nodes and found that at 10 mg/L

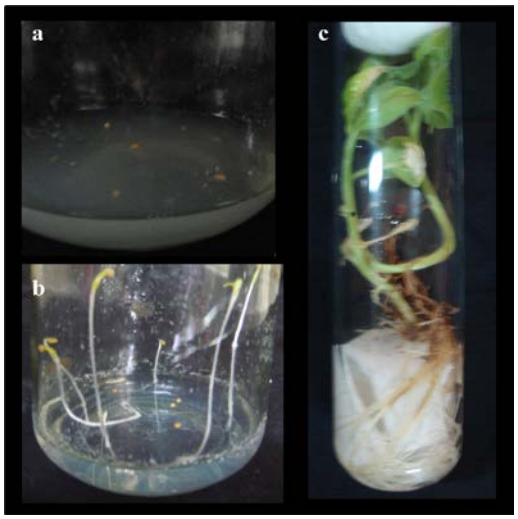


Fig 5A.3: Tissue culture of *W. somnifera*. **(a)** *W. somnifera* seeds inoculated in germination medium; **(b)** germination achieved after 15 days; **(c)** fully grown plant after 6 weeks

hygromycin B, significant portions of non-transformed callus tissue had senescenced and hygromycin B-sensitive shoots were completely necrotic without affecting the survival of the transformed tissues. Some crops appear to react or are hypersensitive to *Agrobacterium* inoculation by forming necrotic barrier. Overgrowth of *Agrobacterium* in explants after co cultivation may lead to necrosis of the plant material. So it is necessary to inhibit the excess growth of *Agrobacterium* to ensure survival of the explants. Cefotaxime sensitivity of *Agrobacterium* was studied by disc diffusion method with different concentrations (0, 100, 150, 200, 250 and 300 mg/L) of cefotaxime. Largest zone of inhibition was observed at 250 mg/L concentration of cefotaxime against *A. tumefaciens* GV 2260. No zone of inhibition was obtained in control (Table 5A.2).

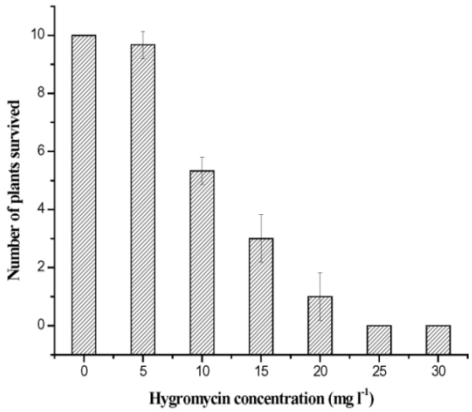


Fig 5A.4: Graph depicting hygromycin B sensitivity assay. LD_{50} of hygromycin B for *W. somnifera* was found to be 10 mg/L. Data and standard error was calculated using 10 plants in 3 replicates

Concentration of cefotaxime (mg/L)	Zone of inhibition (cm)
0	0.0
100	1.8
150	1.9
200	2.7
250	3.0
300	2.3

Table 5A.2: Table representing diameter of zone of inhibition showing effect of different concentrations of cefotaxime on *Agrobacterium tumefaciens* GV2260

5A.4.4 Transformation and selection of putative transformants

The explants, after transformation with the above mentioned three methods, were transferred to selection medium after 10 days where shoot elongation and multiplication was observed in putative transformants (Fig 5A.5b), whereas the escapes and control explants died within 15 days. Following the identification of individual transgenic shoots, they were elongated and multiplied through axillary shoot proliferation (Fig 5A.5c). Modified MS medium supplemented with hormonal combination of 0.1 mg/L kinetin and 0.2 mg/L BAP (proliferation medium) supported maximum transformed shoot proliferation. After 5-6 weeks, elongated shoots selected by random sampling were then transferred to rooting medium containing 2 mg/L IBA (Fig 5A.5d). The rooted transformed plants (Fig 5A.5e) were shifted to the plastic pots containing autoclaved sand and soil in ratio of 1:1 (Fig 5A.5f), hardened for two weeks and then transferred to green house for further acclimatization (Fig 5A.5g). Overall 70% of transferred plants survived in green house. The plants were normal in appearance with no phenotypic aberrations.

5A.4.5 Factors affecting microprojectile bombardment

Different parameters such as preculture duration of the explants, medium support to the explant during bombardment, He gas pressure and target distance have been found to influence the efficiency of microprojectile bombardment method.

i. **Preculture duration of the explants**: Higher Gus expression was observed in the explants precultured on proliferation medium for 48h (Fig 5A.6a). This is in agreement with the proposed concept that during preconditioning the explants undergo a physiological and developmental shift to enter morphogenic competency and when the T-DNA is inserted following this short period, the recipient cells

have already entered the regeneration pathway (Ainsley et al. 2001). Targets should be receptive to transformation and healthy enough so as to withstand the stress of bombardment (Sanford et al. 1993). Young actively dividing cells are best material for transformation, so the explants were set for preculturing of 48 h before transformation. In sugarcane leaf explants, it was found that planar leaf segments bombarded immediately after isolation gave a lower number of Gus expression units as compared to which had been precultured on a MS based medium supplemented with sucrose and 2,4-D for 3 days (Gallo-Meagher and Irvine 1993).

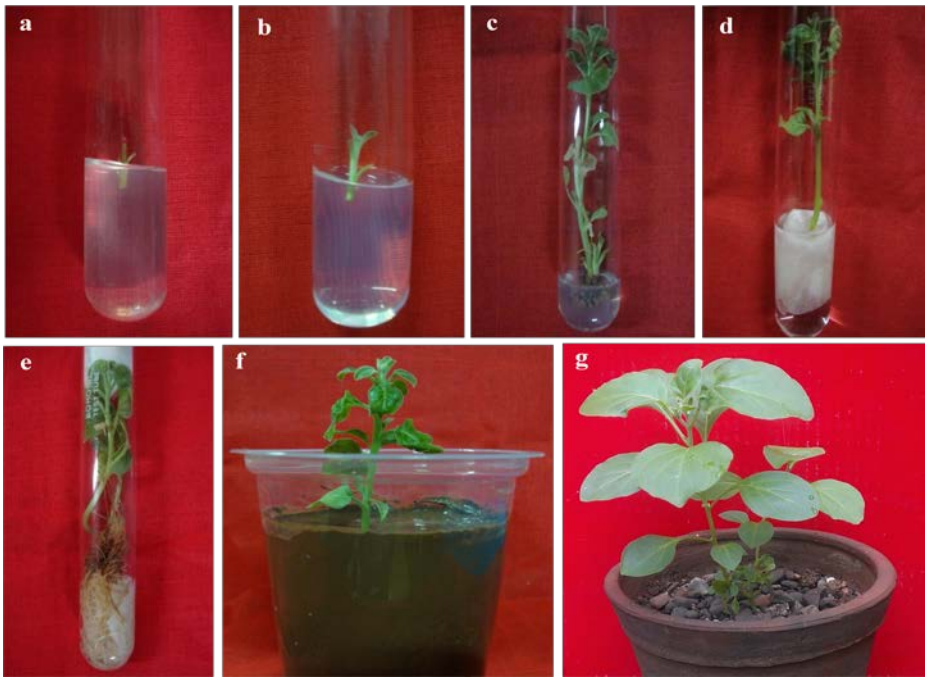


Fig 5A.5: Genetic transformation in *W. somnifera*. **(a)** Nodal explant in proliferation media after two days of transformation; **(b)** Explant in selection media after 10 days of transformation; **(c)** Shoot elongation and proliferation after 5 weeks; **(d)** Plant in rooting media; **(e)** Rooted plantlet; **(f)** Plant transferred in pot; and **(g)** Successfully hardened transgenic plant in green house

ii. **Medium support to the explant**: The explants require support during bombardment to resist the shock waves generated as a result of accelerated particles. Semisolid media has proved to provide the best support while the explants bombarded in an empty plate showed necrosis immediately after bombardment due to the cellular damage caused by high velocity of particles. Wet filter paper did not provide that level of support to the explants thus reducing the transformation frequency (Fig 5A.6b).

iii. **He gas pressure**: Initially no significant difference was found in Gus expression for different He gas pressure but in long term, more Gus expression was obtained at 1100 psi pressure (Fig 5A.6c). He gas pressure accelerates the particles to enter the plant tissues and hence variation in this accelerating force alters the speed of the particles and the degree of penetration (Southgate et al. 1995). He gas pressure of 1100 psi was found to be optimum for microprojectile bombardment. Lower pressure is inadequate for most applications but higher pressure do not necessarily result in significant higher transformation rates and may sometimes cause cell damage. Bombardment of callus tissues of bermudagrass with 1,550 psi He gas pressure resulted in successful transformation (Li and Qu 2004).

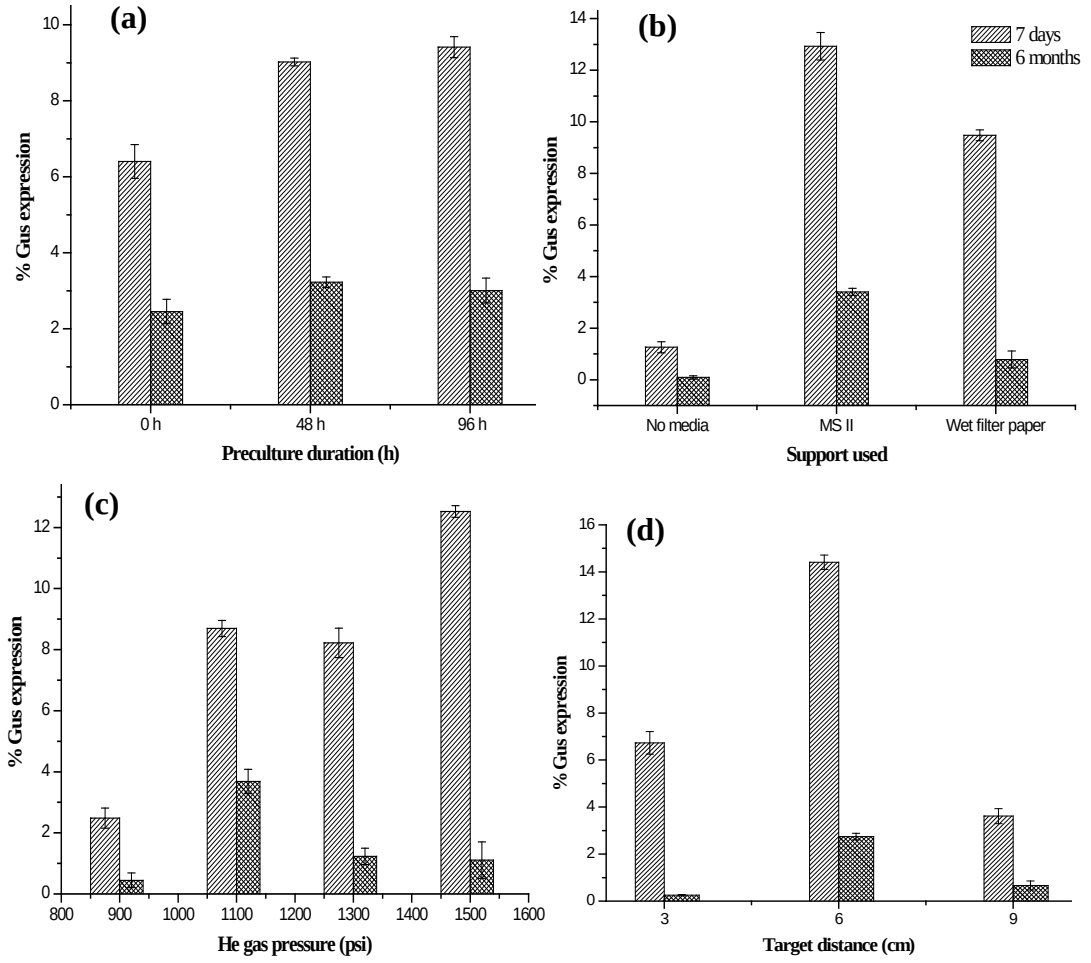


Fig 5A.6: Graphs showing histochemical Gus assay versus factors affecting microprojectile bombardment of *W. somnifera* **(a)** preculture duration of explants; **(b)** media used as a support during bombardment; **(c)** He gas pressure; and **(d)** target distance

iv. **Target distance**: The explants bombarded at 3 cm target distance did not survive for too long due to tissue damage but 6 cm distance maintained adequate impact velocity and showed good Gus expression whereas the low frequency was observed at 9 cm distance (Fig 5A.6d). The distance of the target from the microprojectile stopping screen is critical and affects the velocity of the particles as they enter the plant tissue as well as the distribution of particles in the tissue (Southgate et al. 1995). As the distance increases, the power required to achieve optimum gene delivery decreases, thus decreasing transformation frequency (Hunold et al. 1994; Klein et al. 1988). Taylor et al. (1993) reported visible damage to cells when the latter were placed closer to the stopping plate due to the cells being bombarded by large aggregates of gold particles.

5A.4.6 Histochemical Gus Assay

Transient expression of *gus* gene, histochemically visualized, showed uniform and intense blue staining in different parts of the putative transformed plant used for the assay while no coloration was seen in control plant. The tissues taken from the subcultured plants periodically and after 6 months of transformation (Fig 5A.7) also produced blue coloration, however, the Gus activity decreased considerably (Table 5A.3) due to the escapes with every subculture.

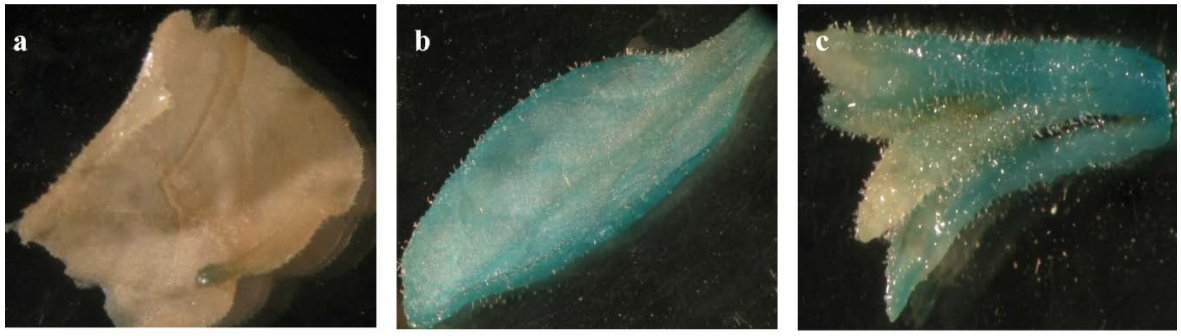


Fig 5A.7: Histochemical Gus assay of *W. somnifera*. **(a)** Control leaf; **(b)** Leaf of transformed plant; and **(c)** Apical meristem of transformed plant

5A.4.7 Molecular analysis of putative transformants

The PCR amplification of DNA extracted from Gus-positive transformants after 6 months using *hpt* II and *WsSQS* specific primers showed ~ 600 bp (Fig 5A.8a) and ~ 1.6 kb (Fig 5A.8b) bands respectively while controls did not show any amplification. Overall transformation efficiencies calculated for *A. tumefaciens*-mediated, microprojectile

bombardment and microprojectile bombardment followed by agroinfection were 3.86%, 3.62% and 8.71% respectively (Table 5A.3). 16S rRNA PCR did not show any amplification, indicating the complete abolition of *A. tumefaciens* contamination from these tissues while positive control (bacterial DNA) showed an amplification of ~1.5 kb 16S rRNA fragment.

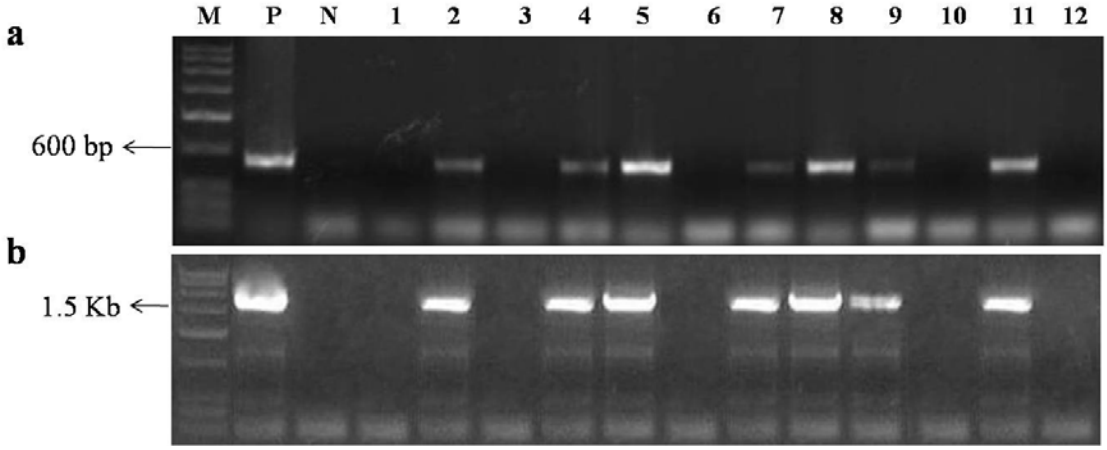


Fig 5A.8: Molecular analyses of different transformed lines. **(a)** *hpt* II specific PCR showing ~ 600 bp amplified products; and **(b)** *WsSQS* specific PCR showing ~ 1.6 bp amplified products. M: Low range molecular weight ladder; P: Positive control (plasmid pCAMBIA 1301); N: Negative control (untransformed plant); and 1-12: randomly selected putative transformed lines

Method of transformation	**Histochemical Gus assay after 7 days (Mean ± SE)**	**Histochemical Gus assay after 6 months (Mean ± SE)**	**Transformation efficiency based on *hpt* II and *gus* PCR**
A. tumefaciens-mediated transformation	5.66 ± 0.47	3.33 ± 1.24	3.86 %
Microprojectile bombardment	6.66 ± 0.47	3.33 ± 0.47	3.62 %
Microprojectile bombardment followed by agroinfection	13.00 ± 0.81	7.66 ± 0.47	8.71 %

Table 5A.3: Transformation efficiencies of three transformation methods used for genetic transformation of *W. somnifera* for overexpression of *WsSQS*. Values are the mean of three replicates each consisting of thirty explants and standard error (SE) of the mean is shown

An ideal transformation method should be simple, economical, quick and should provide selection without producing non-transformed or chimeric plants (Olhoft et al. 2003). *A. tumefaciens* mediated transformation is one of the frequently applied technique for plant transformation but it is very genotype dependent and produces chimeric plants (Cho et al. 1997). This could be possibly one of the reasons for very low transformation efficiency in

earlier reports on *W. somnifera* transformation. Microprojectile system of DNA delivery is another commonly used technique for plant genetic transformation which has no biological constraints and host limitations (Lai et al. 2011). Moreover, it is a simple technique and DNA introduced in this manner can be stably integrated into the plant genome (Bidney et al. 1992). In *Withania, A. tumefaciens*-mediated transformations have not resulted in very satisfactory outcome and there is no published report on microprojectile system in this plant till date. Improving transformation efficiency remains the most important factor in plant transgene technology. The present study deals with comparison of three transformation methods in *W. somnifera.* The transformation efficiency of microprojectile bombardment followed by agroinfection (8.71%) was found to be significantly higher as compared to that obtained by *A. tumefaciens*-mediated (3.86%) and microprojectile bombardment alone (3.62%) (Table 5A.3). The difference in transformation efficiencies of the three approaches used signifies that for the efficient production of transgenic *W. somnifera* plants, the combination of both *A. tumefaciens*-mediated and microprojectile bombardment method offers considerable advantages over these methods used independently. The possible explanation for this increased efficiency may be due to the induction of wound response by microprojectile bombardment used prior to agroinfection (Bidney et al. 1992). The wounds and cut surfaces generated by the impact of high velocity particles bombarded on the explant produce sites where *Agrobacterium* can easily penetrate and cause effective transformation. The same methodology has also been used in some of the plant systems where it is considered superior over these methods used independently. Zimmerman and Scorza (1996) experimented with *N. tabaccum* where two different explants were bombarded with gold particles in one experiment and in another; bombardment was followed by agroinfection and the later resulted in stable transgenic plants. The comparative analysis of transformation techniques was also studied using two different types of explants, tobacco leaves and sunflower meristems where Bidney et al. (1992) observed the increase in transformation frequency in tobacco when the explants were bombarded with plasmid prior to *Agrobacterium* treatment while increased Gus expression was noticed in case of sunflower.

The recovery of large number of transformed plants can also be attributed to the efficient axillary proliferation method employed. Proliferation medium supplemented with

hormonal combination of kinetin (0.1 mg/L) and BAP (0.2 mg/L) supported maximum transformed shoot proliferation. The transformed shoots were rooted in rooting medium containing IBA at a concentration of 2 mg/L. The transformed plants were successfully hardened. The co-cultivation time of 15 min was found suitable for effective transformation as also reported by Pandey et al. (2010).

Here, the apical and nodal segments from in vitro raised plants were used as explants. In very few studies, such kind of target material has been used for gene introduction as it is a group of very well organized tissues, so there are more chances of escapes and chimeras (Domínguez et al. 2004). To verify this, the proliferating transgenic shoots were continuously maintained on hygromycin B at the concentration of 10 mg/L and the Gus assay was performed periodically. The decline in number of transformants as indicated in Table 5A.3 was probably due to the chimera formation. However, the nodal explants have been considered ideal for maintaining cultivar integrity. The use of organized meristematic tissues has the benefit of rapid shoot elongation and proliferation with a minimum period in culture, thus reducing the chances of somaclonal variation (Southgate et al. 1995). Rapid proliferation system is advantageous to provide large number of genetically identical explants for genetic transformation studies.

5A.5 Conclusions

WsSQS was cloned in pCAMBIA 1301 binary vector and was directed under the control of constitutive CaMV 35S promoter. This construct was used to transform apical and nodal meristems of *W. somnifera*. The transformants were recovered under selection pressure and were scrutinized by Gus biochemical assay and molecular analyses. Microprojectile bombardment assisted agroinfection proved to be the better transformation technique with 8.71 % transformation efficiency. The combination of both the methods found to be helpful in recovering transgenic plants at a reasonably high frequency which might lead to genetic improvement of this plant by introduction of desired gene. Therefore, it would be advantageous to combine both the techniques into an integrated protocol for genetic transformation of *W. somnifera.*

CHAPTER 5B

ENHANCED WITHANOLIDE BIOSYNTHESIS IN TRANSFORMED *W. SOMNIFERA* OVEREXPRESSING *WsSQS* GENE

5B.1 Introduction

Genetic engineering can be used as one of the approach to manipulate the biosynthetic pathway of the SMs in order to increase their production. The first committed step which diverts the carbon flux away from the central isoprenoid pathway towards withanolide biosynthesis is the formation of squalene from farnesyl pyrophosphate; catalyzed by a 47 kDa membrane associated enzyme, squalene synthase (Abe et al. 1993). The substrate for this enzyme originates from isoprenoid biosynthetic pathway and can be channeled by metabolic engineering towards squalene accumulation which is the first precursor of triterpenoids. SQS catalyse head to head condensation of two FPP molecules (1'-1) to form PSPP and then converts it to squalene in the presence of NADPH and Mg^{2+}. Squalene oxidizes in presence of NADPH-linked oxide to afford squalene 2, 3-epoxide subsequently cyclizing into lanosterol which acts as a backbone structure for variety of different steroidal triterpenoids (Mirjalili et al. 2009b). The complete biosynthetic pathway of withanolides is still unclear but they are supposed to be derived from 24-methylenecholesterol (Lockley et al. 1976). Since SQS is regulatory branch point enzyme, it has attracted considerable interest as a possible genetic engineering target to promote SMs biosynthesis in plants. Many approaches have been investigated to understand the regulatory role of SQS in sterol biosynthesis using SQS mutants (Karst and Lacroute 1977; Tozawa et al. 1999), fungal elicitors (Devarenne et al. 1998; Threlfall and Whitehead 1988; Vogeli and Chappell 1988) and specific inhibitors of SQS (Baxter et al. 1992; Bergstrom et al. 1993; Wentzinger et al. 2002). The effect of overexpression of *SQS* on accumulation of SMs were studied in *P. ginseng* (Lee et al. 2004) and *E. senticosus* (Seo et al. 2005), and similar study was also performed in *G. uralensis via* Ri-mediated transformation (Lu et al. 2008). Transgenic *B. falcatum* over expressing squalene synthase 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. 2011b). With this objective in mind, the previous chapter explained the transformation of *W. somnifera* with *A. tumefaciens* harboring the *WsSQS* gene in pCAMBIA binary vector. The transformants were screened on selection medium and biochemical assay and finally confirmed by molecular analyses. In the present chapter, the

significance of overexpressing *WsSQS* transcript and protein in different tissues of *W. somnifera* resulting in considerable increase in withanolide content in all the tissues have been demonstrated.

5B.2 Materials

HPLC grade solvents and authentic squalene were purchased from Fischer Scientific (USA) and Sigma (USA), respectively.

External standards of different withanolides were procured from Chromadex (USA).

Refer Appendix-VII and XI for antibiotics and buffers, respectively.

5B.3 Methods

5B.3.1 qRT-PCR analysis of transformants

Total RNA was isolated from different tissues (leaf, stem and root) of transformed lines and control plant according to the protocol described in Chapter 2 (section 2.3.1) and treated with DNase using DNase I Digest kit (Sigma, USA). Total RNA (2 µg) was reverse transcribed into cDNA using AMV reverse transcription system (Chapter 2; section 2.3.2). For normalization of the relative expression data, ubiquitin gene was employed as an internal standard (UBC primer mix, Eurogentec, Belgium). To quantify the *WsSQS* transcripts, first-strand DNA was PCR amplified using gene-specific primers: RT-SQSF and RT-SQSR (Appendix-IV) synthesized from Ws*SQS*. All qRT-PCR reactions were performed as shown in Chapter 4 (section 4.3.2.4) and the data was analyzed by comparative Ct method (Pfaffl 2001).

5B.3.2 Indirect ELISA and western blot of WsSQS protein

Fresh tissues (500 mg each) of transformed and wild-type plants were ground in liquid nitrogen and resuspended in 1 ml of crude protein extraction buffer (Appendix-XI). The supernatant was collected after centrifugation and the total protein quantity was estimated by Bradford assay (Bradford 1976), using BSA as standard. ELISA was performed with 20 ug of total protein as described in Chapter3; section 3.3.10. The concentration of the WsSQS protein present in total protein extracted from wild-type and transformed plants were analyzed in each case of three replications from the standard curve.

Total crude protein (50 µg) from transformed and wild-type tissues was electrophoresed on 10% SDS-PAGE and electro-transferred on to PVDF membrane, with recombinant truncated WsSQS as a size marker. Blot was treated as explained in Chapter3; section 3.3.12 and developed using BCIP/NBT solution.

5B.3.3 WsSQS enzyme acitivity

The microsomal protein fractions were prepared for WsSQS activity measurements from different tissues of the transformed and control plants (Vögeli and Chappell 1988). Essentially, 1 g of frozen tissue was homogenized in 10 mL of crude protein extraction buffer (Appendix-XI). The homogenates were filtered through 40 µm mesh and centrifuged at 10,000 *g* for 25 min at 4 ºC. The supernatant was again centrifuged at 100,000 g for 60 min to obtain the microsomal pellet. The pellet was resuspended in 200 µL of 100 mM Tris-HCl (pH 8.0), 1.5 mM DTT and 20% glycerol and protein concentration was determined by Bradford method.

Assay for WsSQS enzyme was carried out with 10 µg microsomal protein according to the method described previously (Chapter 3; section 3.3.9). The enzyme activity was determined fluorimetrically by measuring NADPH depletion during the reaction on an LS 55 spectrofluorimeter (Perkin Elmer, USA). The assay mixture was excited at 340 nm and emission was recorded in the range 400-500 nm with characteristic maxima around 460 nm corresponding to NADPH fluorescence. Excitation and emission slits were kept at 7.5 and 2.5 nm, respectively, with a scan speed of 100 nm min^{-1}. The reaction was carried out at 30 ºC for 1 h and averaged fluorescence of 5 accumulated scans were recorded at regular time intervals. A standard curve was prepared by plotting fluorescence of commercially available NADPH (dissolved in 50 mM Tris-HCl; pH 8.0) at 460 nm against concentration. In order to validate the enzyme reactions, squalene formed in each reaction was checked on GC-MS. Replicates of the above mentioned reactions, after 2 h of incubation, were extracted using TBME, and concentrated to 100 µL by bubbling dry nitrogen. The concentrate (1 µL) was injected on GC-MS fitted with a capillary column HP-5 (25 m X 0.25 mm, film thickness 0.33 µm 5% methylpolysiloxane cross-linked capillary column, Hewlett-Packard, USA) with a split ratio of 10:1. The injector temperature was set at 290 ºC with helium as the carrier gas (10 mL min^{-1}). The oven temperature was programmed from 150 ºC to 250 ºC at 10 ºC min^{-1} and from 250 ºC to 310 ºC at the rate of 5 ºC min^{-1},

and maintained at final temperature for 5 min. The chromatogram obtained was compared with the authentic squalene (Sigma, USA) for its retention time and mass fragmentation pattern. For quantification of squalene formed in the reactions, the standard curve of squalene was prepared by injecting the different known concentrations of authentic squalene and plotting them against the peak area. The squalene content in the reactions was then calculated from standard curve, and expressed as nmol/mg protein.

5B.3.4 Withanolides extraction and LC-MS analysis

Dried tissues (100 mg each of leaf, stem and root) were separately crushed to fine powder and percolated thrice with 5 ml methanol for 1 h under shaking conditions at room temperature. The extracts of each tissue were pooled, filtered and concentrated under reduced pressure at 45 ºC. This was followed by thorough washes with double volume of n-hexane. The methanolic fraction was dried completely and further partitioned twice with water:chloroform (1:1). The chloroform fractions were pooled, concentrated and finally dissolved in 150 µl methanol. The samples were filtered and subjected to liquid chromatography.

LC-MS of the samples (5 µl each) was performed on Waters Acquity UPLC system (Milford, MA, USA) with an Acquity UPLC® BEH C18 column (2.1 x 100 mm, 1.7 µm) attached to a positive ion elecrospray ionization-mass spectrometer (Waters) for the identification and quantification of withanolides in *W. somnifera* extracts. Separations were achieved using a binary gradient elution of water (solvent A) and acetonitrile (solvent B) with the following program carried out at 25 ºC: 10% B for 2 min; 45% B for 8 min; 75% B for 10 min; and 95% B for 5 min, at a flow-rate of 0.4 mL/min, with a total run time of 25 min. External standards of different withanolides were used to construct calibrated graph of peak area *versus* withanolide concentration, being linear over 10 measurements at different concentrations.

5B. 4 Results and discussion

5B.4.1 Transcript analysis of transformed events

The accumulation of *WsSQS* mRNAs in wild-type and transformed tissues was analyzed by qRT-PCR where ubiquitin gene was used as an internal control to normalize the expression of *WsSQS*. Wild-type tissues were studied for their transcript level where

maximum expression of *WsSQS* was observed in root followed by leaf and stem (Fig 5B.1). Root and leaf tissue accumulated 4 and 2 fold higher *WsSQS* mRNA than stem tissue, respectively. Of all the transformed lines analyzed by qRT-PCR, three best performing lines (T20, T58 and T79) are shown where the mRNA levels increased up to 2-5 fold in the tissues (Fig 5B.1). Similar results were obtained with *E. senticosus* (Seo et al. 2005) and *P. ginseng* (Lee et al. 2004), both overexpressing squalene synthase of *P. ginseng* (*PgSS1*) which resulted in accumulation of *PgSS1* mRNA in the leaves and adventitious roots of the transgenic plants, respectively. This was further observed in *B. falcatum*, where squalene synthase (*BfSS1*) mRNA expression was enhanced in sense transgenic roots but suppressed in antisense roots as compared to non-transgenic roots (Kim et al. 2011b). In the present study, almost 3.5 and 2.5 fold increase in *WsSQS* mRNA was observed in leaf and root tissue of transformed lines, respectively. Although, stem tissue showed maximum fold increase (about 5 fold), the maximum expression of *WsSQS* was observed in root than rest of the tissues (Fig 5B.1). There was variability in levels of expression between different transformed lines where T79 showed maximum overall expression. These results demonstrate that *WsSQS*, catalyzing the regulatory step leading to withanolide biosynthesis, was up-regulated in all the transformed tissues.

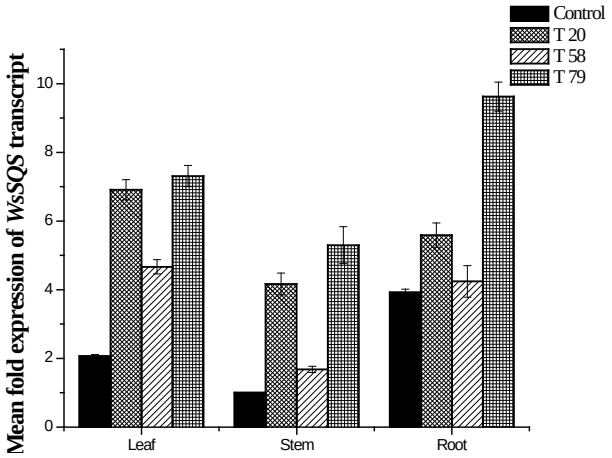


Fig 5B.1: Tissue specific *WsSQS* transcript analysis in transformed *W. somnifera* lines by qRT-PCR. T20, T58 and T79 are three best performing tranformed events. Values are the means of three replicate measurements and error bars show the standard error of the mean

5B.4.2 WsSQS protein levels in transformed tissues

Indirect ELISA was performed to investigate the relative increase of WsSQS protein levels in transformed tissues. The antisera dilution 1:5000 was found to contain optimum antibody titer for carrying out the immunological studies (Chapter 3; section 3.4.6). The wild tissues showed almost equal expression of WsSQS in root and leaf, which was 2 fold greater than stem tissue. It was found completely different from the transcript expression pattern in wild tissues where root showed highest mRNA levels followed by leaf and stem.

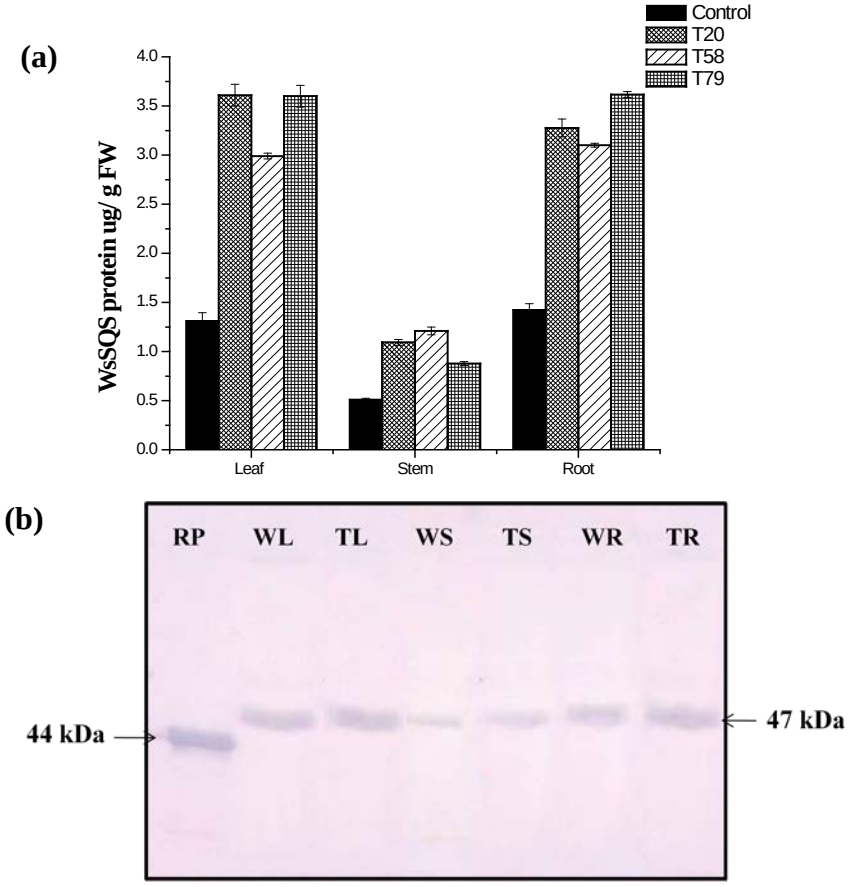


Fig 5B.2: WsSQS protein expression analyses. **(a)** WsSQS protein quantification in total soluble protein extracted from different tissues of wild-type and transformed lines by ELISA, determined from the standard curve plotted between purified recombinant WsSQS protein concentration and absorbance at 405 nm; **(b)** Western blot analysis of different tissues of T79 and wild-type plant. RP: recombinant truncated WsSQS protein as size marker; WL: wild-type leaf; TL: Transformed leaf; WS: Wild-type stem; TS: Transformed stem; WR: Wild-type root; and TR: Transformed root

There was no direct co-relation between mRNA expression and protein accumulation patterns, however, consistent with the transcript expression results, ELISA showed increased protein levels in all the transformed tissues, with maximum and similar accumulation in leaf and root. The stem showed minimum level of WsSQS protein

accumulation. Absolute quantification of protein was carried out by plotting the standard curve for different concentrations of recombinant purified WsSQS and A_{405} obtained spectrophotometrically. In transformed leaf, WsSQS increased to 3.6 µg/g FW as compared to its control (1.3 µg/g FW), in transformed stem it increased to 1.2 µg/g FW as compared to control stem (0.51 µg/g FW) and in root tissue, it increased to 3.6 µg/g FW as compared to its control (1.42 µg/g FW) (Fig 5B.2a).

The overexpression of WsSQS protein in transformed tissues was also witnessed by western blotting (Fig 5B.2b). The recombinant truncated WsSQS protein was run along with other samples to estimate the size of plant SQS. Different tissues of T79 were analyzed against wild-type where clear protein bands were detected corresponding to the expected size. The transformed tissues were found to contain slightly higher SQS than their respective wild-type as co-related with the intensity of the immune-precipitated protein bands.

5B.4.3 WsSQS enzyme activity in transformed tissues

SQS is a membrane bound enzyme anchored to the endoplasmic reticulum through its hydrophobic amino acids at the carboxy terminal (Gupta et al. 2012). For the enzyme activity, microsomal fractions were prepared from wild-type and transformed tissues to find out whether the newly introduced gene is producing active enzyme which is contributing to withanolide production. Quantification of WsSQS enzyme activity was done flourimetrically by calculating the amount of NADPH consumed in the reaction for the conversion of FPP into squalene catalyzed by WsSQS from the standard curve of NADPH. The maximum increase in enzyme activity (pKat/g FW) was found to be 3.3 fold in transformed root tissue followed by 2.7 fold increase in transformed leaf and 2.1 fold in transformed stem tissue as compared to their respective wild-type tissues (Table 5B.1). The increase in enzyme activity indicated that WsSQS enzyme overexpressed in transformed lines.

Likewise, in a previous study from microsomal fractions of *E. senticosus* somatic embryos and plants, the enzyme activity was 1.5 to 3 fold higher in transgenic embryos than in wild-type embryos, while the transgenic plants showed 1.2 to 3 fold higher activity than wild-type plants (Seo et al. 2005). Similar results were also obtained with *P. ginseng* (Lee et al. 2004) and *B. falcatum* (Kim et al. 2011b).

Sample	WsSQS activity (pKat/g FW)	WsSQS activity (pKat/mg protein)	Squalene (µmol/mg protein)
Control Leaf	0.72 ± 0.09	36 ± 2.1	54 ± 3.4
Transformed Leaf	1.98 ± 0.12	54.7 ± 2.5	82 ± 6.7
Control Stem	0.39 ± 0.01	10 ± 0.41	15 ± 8.9
Transformed Stem	0.83 ± 0.02	14.4 ± 0.86	23 ± 1.2
Control Root	0.82 ± 0.02	41 ± 1.5	29 ± 1.2
Transformed Root	2.78 ± 0.32	139.4 ± 10.6	97 ± 3.4

Table 5B.1: Summary of WsSQS activity and squalene formed in the enzyme reactions catalyzed by WsSQS isolated from different tissues of control and transformed plants. The enzymes activity was calculated in pKat/mg protein which was validated by quantifying squalene (µmol/mg protein) formed in each reaction. The fold expression of WsSQS/g FW of tissue was compared. SE of three independent experiments are shown

The replicates of the enzyme reactions were processed for the formation of squalene to validate the results of enzyme activity. The squalene formed in each reaction was confirmed by comparing with the gas chromatogram of the authentic squalene (Fig 5B.3a) and quantified from a standard curve (Fig 5B.3b). The squalene content in transformed tissues was found to be higher than their respective wild-type tissue (Fig 5B.4). The results obtained clearly indicated the increased amount of squalene in the reactions catalyzed by enzyme isolated from transformed lines (Table 5B.1). Therefore, it appears likely that squalene is accumulated as the reaction product is the consequence of the increased expression of *WsSQS*.

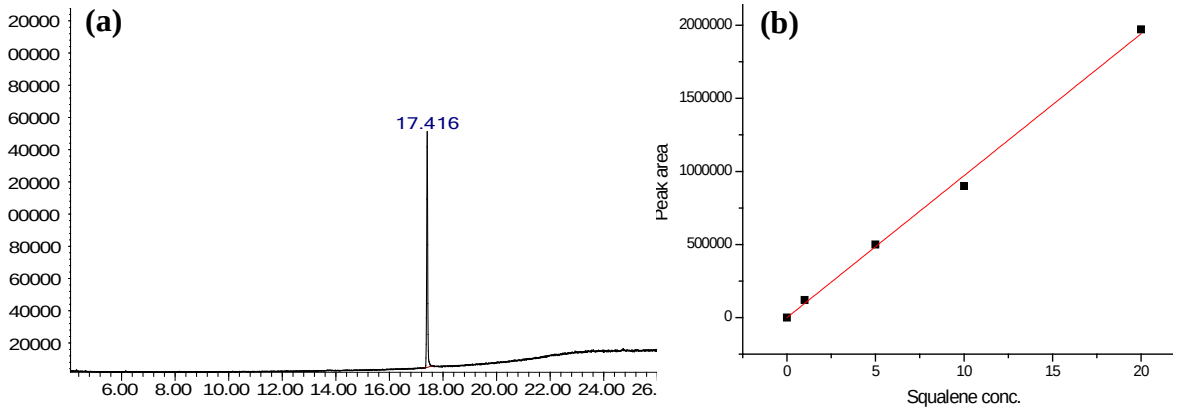


Fig 5B.3: (a) Gas chromatogram of standard squalene (Sigma); **(b)** Standard graph of peak area versus different concentrations of standard squalene loaded on GC column

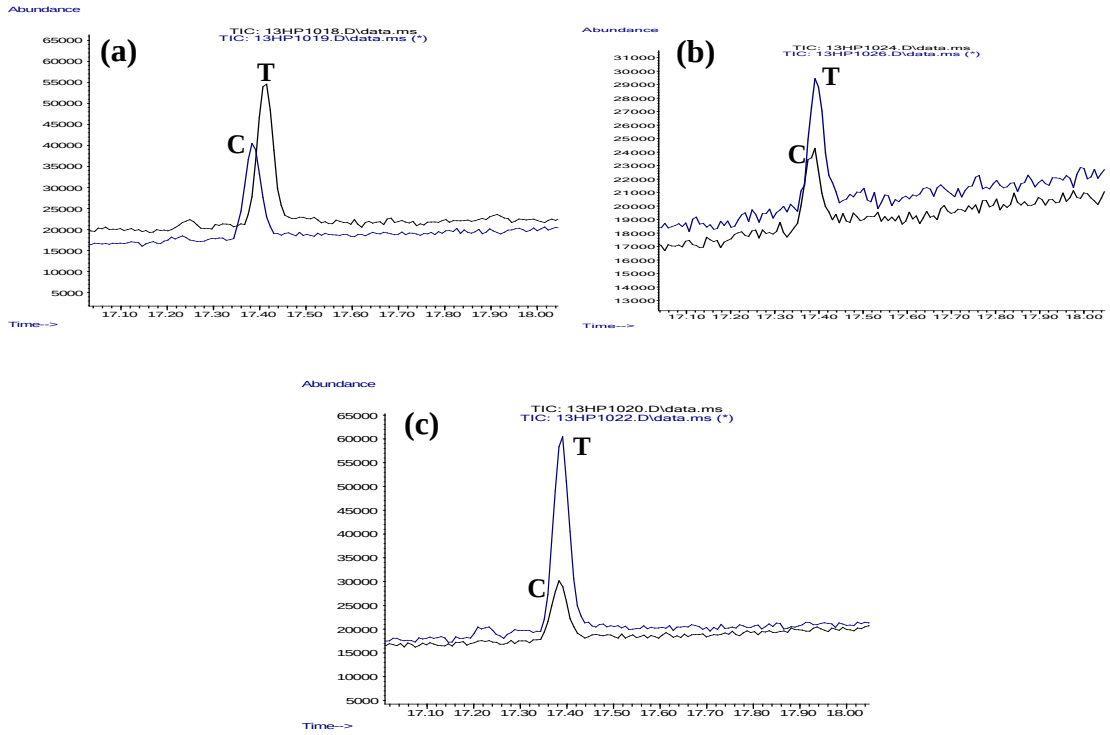


Fig 5B.4: Overlapping gas chromatogram of squalene extracted from reactions of squalene synthase from control and transformed tissues **(a)** leaf; **(b)** stem; and **(c)** root. (C: Control; T: Transformed)

5B.4.4 Tissue specific accumulation of withanolides in transformed *W. somnifera*

The present study offers a simple analytical LC-MS system providing well-resolved and symmetrical peaks required for accurate quantitative analysis of withanolides. This sensitive and selective method allowed identification of four different withanolides in the plant extract which were confirmed by retention time (RT) and mass spectrum of their respective standards facilitating their quantification: Withaferin A (WA; 11.31 min), Withanolide A (Wld A; 12.35 min), Withanolide B (Wld B; 15.00 min) and Withanone (WN; 18.35 min) (Fig 5B.5).

The metabolite profiles indicated variability between the different tissues but their level was found to be higher in all the transformed tissues as compared to wild-types (Fig 5B.6). The overexpression of *WsSQS* in transformed plants resulted in tissue specific accumulation of total withanolides up to 2 fold. The total withanolide content of wild-type leaf, stem and root was 1.84, 2.24 and 2.25 mg/g DW of the tissue, respectively, which

increased to 3.55, 3.37 and 3.98 mg/g DW of their respective transformed tissue (Table 5B.2). The total withanolide increase of about 1.9 fold was achieved in leaf tissue followed by 1.8 fold increase in root and 1.5 fold in stem (Fig 5B.7). The leaf tissue showed maximum fold increase but the overall concentration of total withanolides was observed to be highest in transformed root (Fig 5B.7).

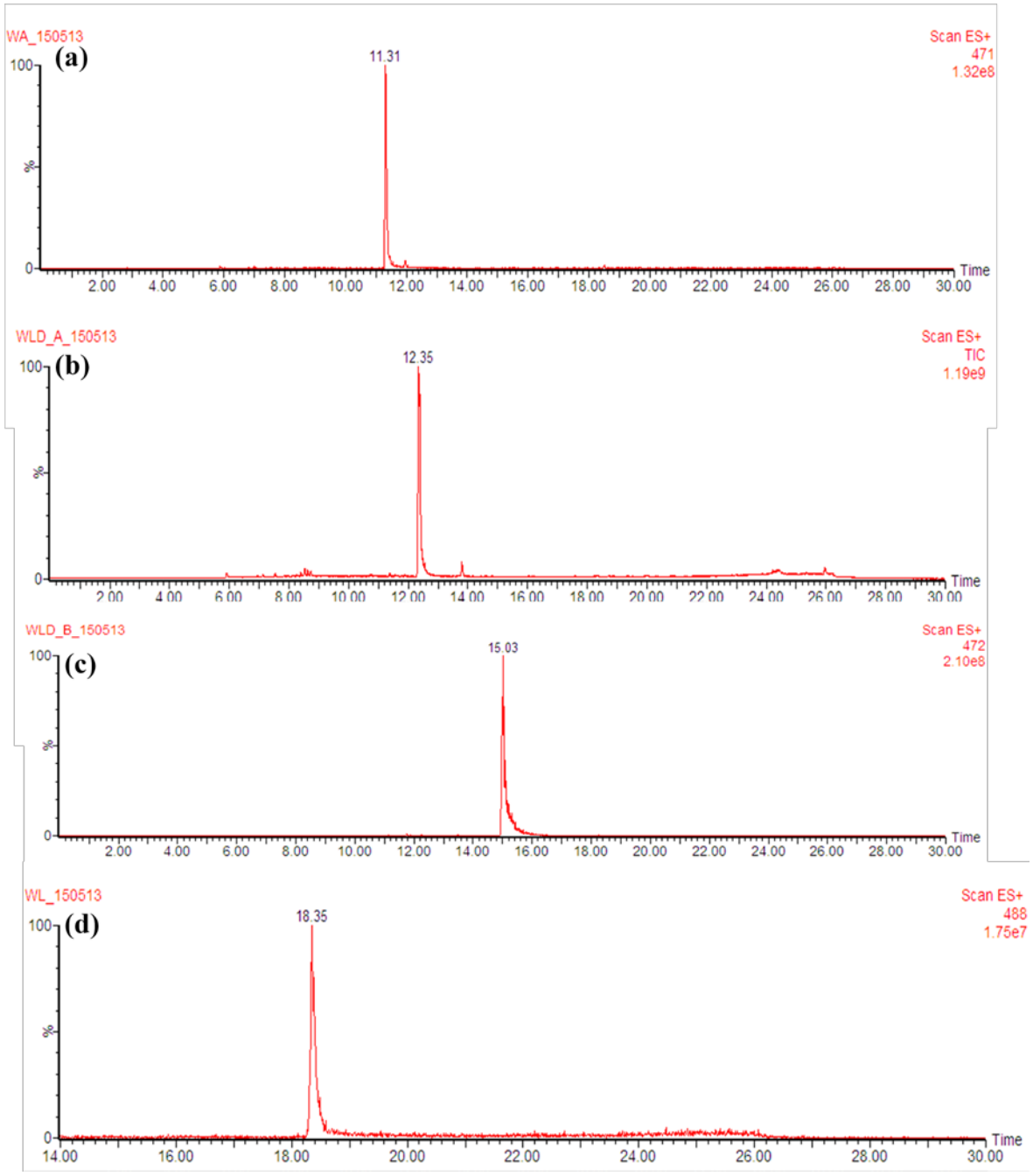


Fig 5B.5: Chromatogram profile of standard withanolides showing their RT **(a)** WA; **(b)** Wld A; **(c)** Wld B; and **(d)** WN

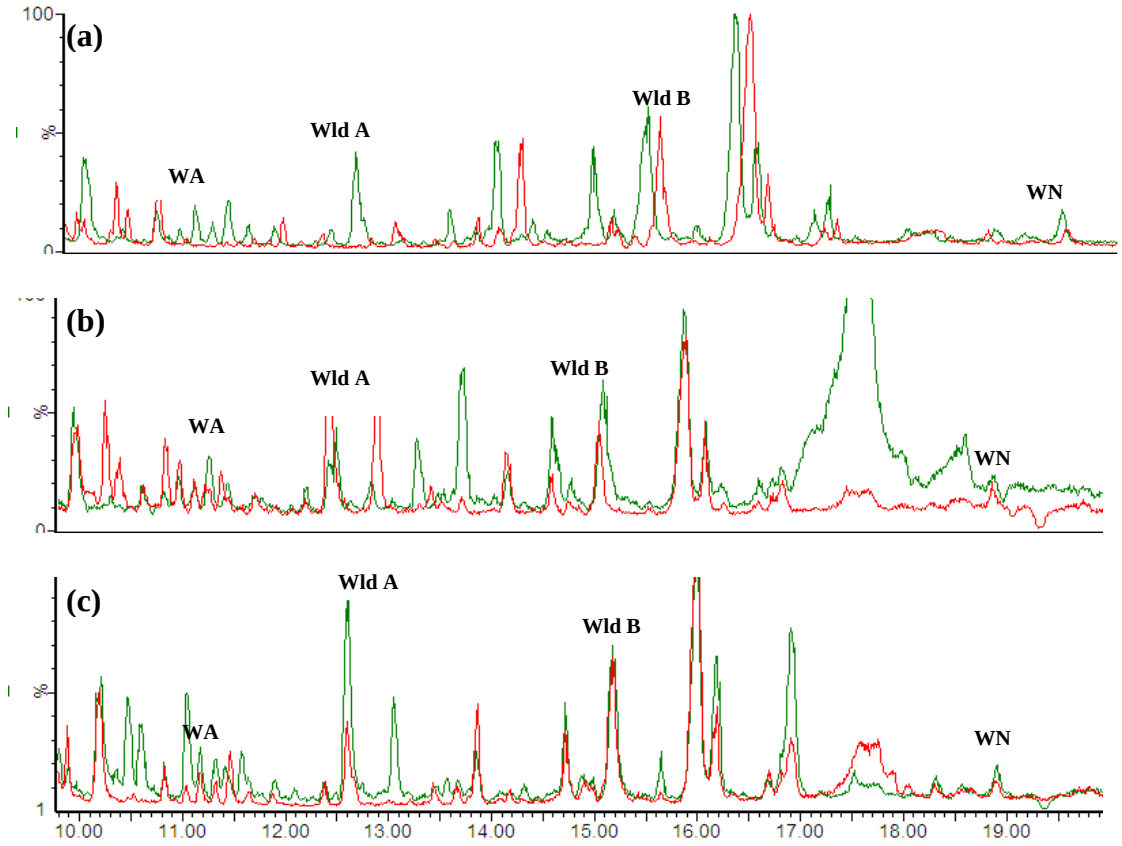


Fig 5B.6: Metabolite profiles of different tissues of wild-type and transformed tissues analyzed by LC-MS **(a)** Leaf; **(b)** Stem; and **(c)** Root. The arrows represent the different withanolides identified in the mixture. (Red: Wild-type; Green: Transformed)

The major change in concentration of withanolides was found in leaf where WA and Wld A increased up to 4 - 4.5 folds while there was negligible change in Wld B and WN. This pattern also reflected in root where WA and Wld A increased up to 2 - 2.5 fold whereas the concentration of Wld B and WN reached only 1.2 and 1.3 fold, respectively. The metabolite profile of stem was completely different from leaf and root where although WA and Wld B increased to about 2 and 1.5 fold, respectively, the quantity of Wld A was found to be almost same, while the concentration of WN increased to 3.7 fold. There are previous reports on tissue specific accumulation of SMs in *W. somnifera* where similar results were obtained in a study accumulating maximum WA and Wld A in root tissue (Manwar et al. 2012), in contrast to another study where the plant was found to accumulate these withanolides more in leaf tissue (Sharma et al. 2007).

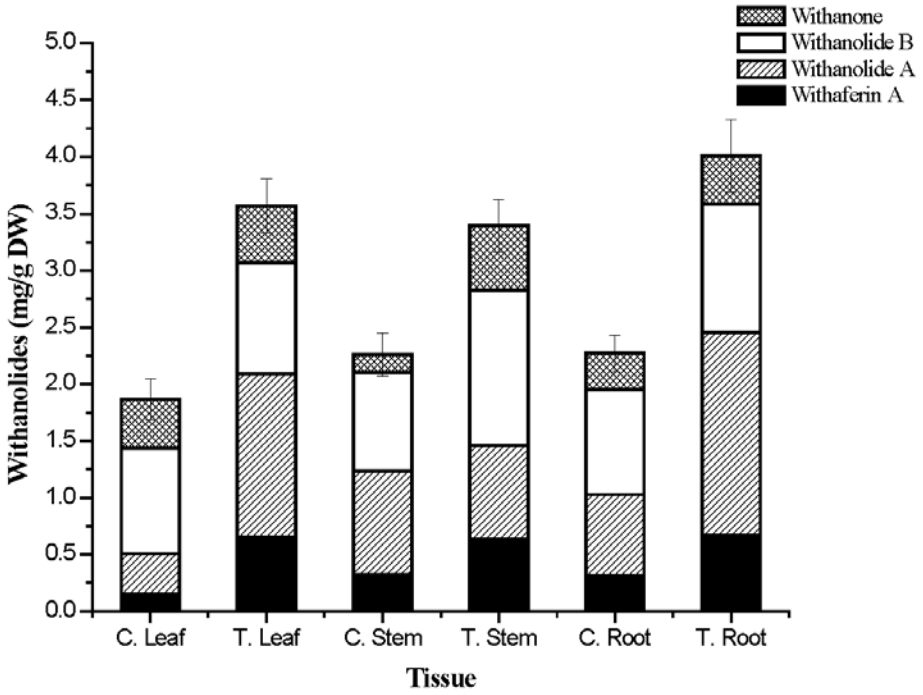


Fig 5B.7: Improved production of withanolides in transformed tissues overexpressing *WsSQS*. Vertical bars indicate the mean values ± SE from three independent experiments. C: control; T: transformed

Withano-lides	RT (min)	Leaf		Stem		Root	
		W	T	W	T	W	T
WA	11.31	0.15 ± 0.01	0.65 ± 0.09	0.32 ± 0.04	0.63 ± 0.07	0.31 ± 0.05	0.66 ± 0.08
Wld A	12.35	0.35 ± 0.03	1.43 ± 0.12	0.91 ± 0.09	0.82 ± 0.08	0.71 ± 0.09	1.78 ± 0.16
Wld B	15.00	0.92 ± 0.1	0.98 ± 0.09	0.86 ± 0.07	1.36 ± 0.18	0.92 ± 0.12	1.12 ± 0.14
WN	18.35	0.42 ± 0.06	0.49 ± 0.08	0.15 ± 0.02	0.56 ± 0.05	0.31 ± 0.04	0.42 ± 0.07
Total	-	**1.84 ± 0.15**	**3.55 ± 0.65**	**2.24 ± 0.32**	**3.37 ± 0.63**	**2.25 ± 0.31**	**3.98 ± 0.66**

Table 5B.2: Quantitative determination of different withanolide content by LC-MS in leaf, stem and root of transformed *W. somnifera* overexpressing *WsSQS*. Values represent the mean of three independent experiments with their SD and expressed as mg/g DW of the respective tissue. WA: Withaferin A: Wld A: Withanolide A: Wld B: Withanolide B; WN: Withanone; W: wild-type; T: Transformed.

There are few reports on plant cell and hairy root cultures initiated for the increased production of metabolites in *Withania*. Murthy et al. (2008) developed the hairy root cultures of *W. somnifera* which produced 2.7 fold more Withanolide A than non-transformed cultured roots. In another study, *W. somnifera* transformation with *A. tumefaciens* resulted in formation of shooty teratomas which accumulated Withaferin A & Withanolide D, and rooty teratomas which accumulated only Withanolide D, but the levels of these withanolides was found to be low (Ray and Jha 1999). In *W. coagulans*

transformed with *A. tumefaciens*, Withanolide A contents was found to be root morphology dependent accumulating higher in callus-like roots than roots displaying typical hairy root morphology but on the contrary, Withaferin A contents were not significantly affected by root morphology (Mirjalili et al. 2009a). Similarly, there are various other hairy root and plant cell culture studies attempted to produce withanolides but none of them resulted in significant levels of the metabolites required for their economical exploitation and hairy root cultures are employed only for the production of few withanolides that are specific to root tissue. Moreover, an important constraint is the commercial utilization of hairy root culture in development and up-scaling of appropriate vessels for the delicate and sensitive hairy roots.

Metabolic engineering of withanolide biosynthetic pathway is an alternative and fascinating approach for their improvement. Squalene synthase operates at a branch point of the pathway regulating the metabolic flux and catalyzes the first committed step leading to the synthesis of different withanolides (Mirjalili et al. 2009b). Thus, manipulating the expression of squalene synthase could be a tool for modifying the SMs of this plant. This study describes the metabolic engineering of isoprenoid pathway by overexpressing *SQS* which resulted in enhanced production of withanolides in *W. somnifera*. Similar results were obtained in the study with *P. ginseng* (Lee et al. 2004) and *E. senticosus* (Seo et al. 2005) where the transformed plants accumulated 1.6-3 fold higher total ginsenoside content in adventitious roots and 2-2.5 times higher triterpene saponin, respectively. Likewise, transgenic roots of *B. falcatum* overexpressing *BfSS1* in the sense orientation resulted in *SQS* mRNA accumulation and enhanced production of both phytosterol and saikosaponins (Kim et al. 2011b). Overexpression of *SQS* in *G. uralensis* led to 2.6 fold increased glycyrrhizin content as compared to control hairy roots (Lu et al. 2008). Recently, *SQS* overexpression in *W. somnifera* resulted in 2-3 fold increase in Withanolide A and production of Withaferin A from the callus cell suspension cultures (Grover et al. 2012). Callus is a chimeric tissue, thus reducing the complete effect of total number of cells actually contributing in withanolide production. The fate of developing cells in callus is also unknown, thus providing no idea about the specific tissue involved in the production of SMs. Besides this, suspension cultures are more prone to contamination, are difficult to maintain and it has also been reported previously that callus cultures are unable

to synthesize withanolides (Roja et al. 1991). The major drawback of callus culture is their tendency to undergo frequent genetic erosions (D'Amato 1977), and therefore, pure clones cannot be maintained. So callus culture could be limited source of metabolites while transformed plant production is suitable option for conservation of germplasm.

5B.5 Conclusions

The present study investigated the enhanced levels of *WsSQS* transcript, WsSQS protein and withanolides in transformed *W. somnifera*, thus establishing significant involvement of squalene synthase in withanolide biosynthesis. Previously, we reported the isolation and cloning of *WsSQS* from *W. somnifera* in Chapter 2, and here we demonstrate the transformation of *W. somnifera* with *WsSQS* through *A. tumefaciens*. The resulting plants were further analyzed which revealed significant increase in WsSQS mRNA and protein in transformed tissues which undoubtedly correlates with increased withanolide content in transformed *W. somnifera* plants. These studies can be used as a tool to obtain greater understanding of the tissue specific regulation of withanolide metabolism, allowing the application of plant metabolic engineering techniques to improve the production of *Withania* bioactive compounds.

SUMMARY

AND

FUTURE PROSPECTS

Withania somnifera is an important plant used in Indian medicine and well known for its wide range of biological efficiencies. The main bioactive compounds of the plant are steroidal lactones called as withanolides. These secondary metabolites of the plant are not only known for their pharmacological activities, but also play physiological roles in plants, such as they act as protectant against pathogen attack. In *Withania* plant, withanolides are present in very low quantity, and large biomass is sacrificed to procure even a small amount of these compounds from plants. As an alternative to increase the yield of such compounds, there is need to develop designer *Withania* 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 squalene synthase (SQS) gene involved in terpenoids biosynthesis from *W. somnifera*. The present investigation also includes development of transformed *Withania* lines overexpressing *SQS* and analyzing its effect on withanolide contents.

SQS was chosen as a target gene for metabolic engineering of isoprenoid pathway because SQS is a key regulatory enzyme of this pathway diverting the metabolic flux towards the synthesis of squalene by catalyzing a reductive dimerization of two farnesyl pyrophosphate (FPP) molecules. Squalene, being a key precursor for the sterol and triterpene biosynthesis, its accumulation controls the channeling of carbon towards the synthesis of withanolides. 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. 2011a), etc. The full-length cDNAs encoding two different *SQSs* were isolated from *W. somnifera* (*WsSQS*) and characterized. Both the cDNAs showed 99% similarity and 99% identity and designated as *WsSQS1* and *WsSQS2*. They showed maximum sequence similarity with *S. tuberosum, C. annum* and *N. tabacum*. The genes were heterologously expressed in *E. coli* and the hydrophobic residues from C terminus (24 amino acids) were truncated for solublization of the protein (Lee et al. 2002). The protein was extracted and purified by affinity and gel filteration chromatography. Antibodies were raised against purified truncated recombinant WsSQS proteins which were then used for ELISA and Western blot analysis. The mRNA expression of WsSQS was tissue specific and it was found that it accumulates more in root than leaf and stem tissue. In *Taxus cuspidata* SQS transcripts

accumulate higher in roots followed by stem and leaves (Huang et al. 2007), on the contrary *P. ginseng* accumulate PgSS1 and PgSS2 transcripts higher in leaf than root and petiole (Kim et al. 2011b).

The transcript and protein expression of WsSQS was analyzed in presence of different stress in different plant tissues of *W. somnifera* by qRT-PCR and ELISA, respectively. It was observed that WsSQS expression increases in presence of NaCl, salicylic acid and methyl jasmonate treatments. This suggests that *SQS* gene expression is tightly regulated to the kind of the defense signals applied.

Efficient shoot regeneration system for *W. somnifera* was established using nodal and apical segments as explants. There are many reports on tissue cultures of *Withania* using various different explants on different kinds of media and using combinations of growth hormones. In majority of the studies, BAP and kinetin have been found to be a good combination for shoot induction and proliferation while IBA is a better hormone for root initiation (Roja et al. 1991; Joshi and Padhya 2010).

Transformation was achieved in *W. somnifera* using *A. tumefaciens* carrying pCAMBIA binary vector having *WsSQS* construct. Microprojectile bombardment method of gene introduction was also used for transformation and the combination of both above techniques was also used. The nodal and apical segments were used as explants for transformation. The putatively transformed plants were analyzed for the introduction of foreign genes by Gus histochemical assay, and molecular PCR of hygromycin phosphotransferase gene and *WsSQS* gene. The combination of microprojectile bombardment and agroinfection gave the transformation frequency of 8.7% which was higher than any of the method used independently. Tranformation has been achieved in *W. somnifera* previously by *A. tumefaciens* using leaf explants from green house but the transformation frequency was turned out to be only 1.1%.

After the preliminary screening of transformed lines by Gus assay and PCR, different tissues were analyzed by qRT-PCR which showed 2-5 fold increase in *WsSQS* transcripts in transformed tissues. The protein levels in the transformed plants were determined by ELISA which resulted in significant increase in WsSQS protein levels in all the three

tissues of the transformed plants. Proteins extracted from one of the best performing transformed plant tissues were then immune-precipitated along with the wild-type tissue proteins for western blot. This showed obvious increase in concentration of WsSQS protein in transformed tissues. This was further confirmed by carrying out the enzyme activity for WsSQS on the basis of NADPH consumption for the conversion of FPP into squalene. The maximum increase in enzyme activity was found to be 3.3 fold in transformed root tissue followed by 2.7 fold increase in transformed leaf and 2.1 fold in transformed stem tissue as compared to their respective wild-type tissues. This was validated by determining the amount of squalene formed in the reactions by GC-MS.

As the final part of the study, the tissue specific accumulation of withanolides was analyzed in the transformed plants by LC-MS. Four different types of withanolides were identified: Withaferin A, Withanolide A, Withanolide B and Withanone. The concentration of each withanolide varied according to the tissue but there was overall increase in concentration of total withanolide content of the transformed plants as compared to control. In leaf tissue, the total withanolide increased to 1.9 fold followed by 1.8 fold increase in root and 1.5 fold in stem. However, the major change was noticed in the concentration of Withaferin A and Withanolide A which increased to 4-4.5 folds in transformed leaf tissue and 2-2.5 folds in root tissue. While transformed stem accumulated 3.7 folds higher Withanone than its wild-type. There was very less increase in the concentration of Withanolide B in all the tissues. In some previous reports, SQS has been shown to play an important role for the improved production of secondary metabolites in plants. *P. ginseng* (Lee et al. 2004) and *E. senticosus* (Seo et al. 2005) transformed with *SQS* resulted in 1.6-3 fold higher total ginsenoside content in adventitious roots and 2-2.5 times higher triterpene saponin, respectively. Similarly, transformed *B. falcatum* overexpressing *SQS* accumulated higher *SQS* mRNA, and both phytosterol and saikosaponins (Kim et al. 2011b). Similar results were obtained with *G. uralensis* which accumulated 2.6 fold increased glycyrrhizin content as compared to control hairy roots (Lu et al. 2008). These data clearly suggests that *SQS* is actively involved in the regulation of withanolide biosynthesis and metabolic engineering of *Withania* for the overexpression of SQS will provide insight about the regulation mechanism of the pathway for further advancement of the process.

Analysis of next generation of transformed plants for the overexpression of squalene synthase and increase in withanolide content would be the next logical step of this work. Since *W. somnifera* is a slow growing shrub and requires specific climatic conditions for their propagation, it was difficult to obtain their seeds. If the next generation of the transformed plants witness the improved production of withanolides as observed in actual transformed lines, this method of plant transformation could establish as a stable means of introduction of genes in *Withania*. Secondly, the expression of SQS is enhanced by various stresses present in the environment, so the present wok can be carried further to analyze the production of withanolides in the stress induced plants, and to study the expression of downstream genes which are involved in the pathway and might be influenced by the overexpression of SQS.

APPENDICES

Appendix-I: Vectors used in the study

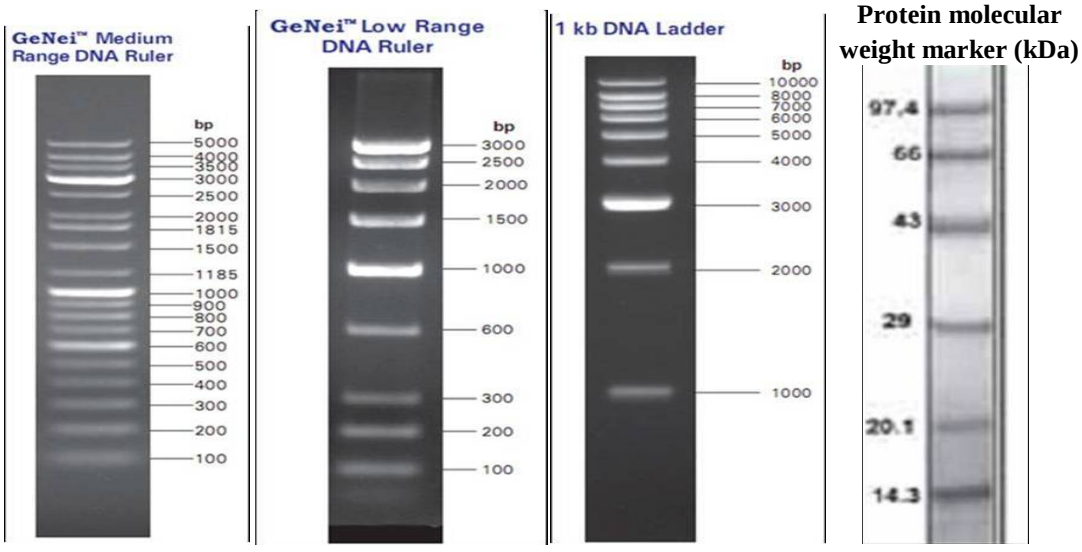
Vector	Source	Features
pGEM-T Easy	Promega	T/A cloning vector
pET-30b (+)	Novagen	Expression vector
Modified pCAMBIA 1301	CAMBIA University Australia	Binary vector

Appendix-II: Bacterial strains used in the study

Bacterial strain	Source	Genotype
E. coli XL-10 gold	Novagen	*Tetr Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacIqZΔM15 Tn10 (Tetr) Amy Camr]*.
E. coli BL21 (DE3)	Stratagene	*E. coli B F-ompT hsdS(rB–mB–) dcm+Tetr gal λ(DE3) endA The*
Agrobacterium tumefaciens (GV2260)		*C58, Rifr, pGV2260 (pTiB6S3_T-DNA), Carbr, Octopine type*

Appendix-III: DNA ladders and protein molecular weight markers used in the study

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



Map of different DNA rulers and protein molecular weight marker

Appendix-IV: Various primers and oligonucleotides used in the study

Primer name	Sequence (5' → 3')	Purpose	Restriction site (underlined)
SQSF1	AAAGCGGAGAAGCAGATCCC	Partial *SQS* amplification	-
SQSR2	AAATCTCACGAGGCCAAAAC AT		-
SQS F0	ATGGGAACATTGAGGGCGATT	Full length *SQS* ampification	-
SQS R0	CTAAGATCGGTTGCCAGAAAG TTGT		-
T7	TAATACGACTCACTATAGGG	Colony PCR with pGEM-T vector	-
SP6	ATTTAGGTGACACTATAGAA		-
NSQSQR	CCTTCGTGCCACCTAACA	Clone confirmation	-
5'-RACE CDS Primer A	$(T)_{25}$ V N	5' RACE ready cDNA synthesis (supplied with kit)	-
SMART II™ A Oligo-nucleotide	AAGCAGTGGTATCAACGCAG AGTACGCGGG		-
3'-RACE CDS Primer A	AAGCAGTGGTATCAACGCAG AGTAC $(T)_{30}$ V N	3' RACE ready cDNA synthesis (supplied with kit)	-
10X universal Primer A Mix (UPM)	Long: CTAATACGACTCACTATAGGG CAAGCAGTGGTATCAACGCAG AGT Short: CTAATACGACTCACTATAGGG C	3' & 5' RACE (supplied with kit)	-
Nested universal Primer (NUP)	AAGCAGTGGTATCAACGCAG AGT		-
RACE R	ACCTCATTTATGTCTTCCAAAT	5' RACE PCR amplification	-
RACE NR (nested)	GAGAATCTGGAGCCAGATCTT CCTTCCCA		-
RACE FI	CTTGGGAAACATTATCAGCAG GC	3' RACE PCR amplification	-
RACE NFI (nested)	GACGAATATTGTCACTATGTA GCTGG		-
FSQSP	<u>CATATG</u>GGAACATTGAGGGCG	Cloning in pET-30b (+)	*Nde*I
RSQSP	<u>CTCGAG</u>AGATCGGTTGCCA		*Xho*I
T7 promoter	TAATACGACTCACTATAGGG	Colony PCR with pET-30b (+) vector	-
T7 terminator	GCTAGTTATTGCTCAGCGG		-
T-RSQSP	<u>CTCGAG</u>GTAATTTGGCTCACT C	Reverse primer for truncation	*Xho*I
SnsSQSF	<u>GGTACC</u>ATGGGAACATTGAGG GCGA	Cloning in pCAMBIA 1301	*Kpn*I
SnsSQSR	<u>GAGCTC</u>CTAAGATCGGTTGCC AG		*Sac*I

HYG-F	TCCTGCAAGCTCCGGATGCCTC	*hpt* II PCR	-
HYG-R	CGTGCACAGGGTGTCACGTTGC		-
GUS-F	AATGGTGATTACCGACGAAAACG	*gus* PCR	-
GUS-R	ATACTCCACATCACCACGCTTG		-
16srRNA-F	AGAGTTTGATCCTGGCTCAG	16srRNA PCR	-
16srRNA-R	ACGGGCGGTGTGTTC		-
CaMV-F	ACAGTCTCAGAAGACCAAAGGGCA	CaMV-WsSQS PCR	-
RT-SQSF	TTTATGATCGTGAATGGCACTTTTC	WsSQS specific qRT-PCR	-
RT-SQSR	AGCGGTTGAAACATGATGGAAC		-

Appendix-V: 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 RT
10 X TBE buffer	890 mM Tris base 890 mM Boric acid 20 mM EDTA (pH-8.0)	RT
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 ºC

Appendix-VI: 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 $MgCl2.6H_2O$ 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 55 mM MnC_{l2}	pH was adjusted 6.8 with KOH. MnCl2 was added adjusting pH and filter sterilized. Store at -20 °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, stored at room temperature or at 4 ºC after autoclaving

Appendix-VII: Stock solutions for bacterial and plant transformation and selection

Name	Stock concentration	Preparation and Storage
IPTG solution	200 mg/ mL in SMQ	Filter sterilized and stored at - 20 ºC
X-Gal	20 mg/ mL in N,N'-Dimethyl formamide (DMF)	Light sensitive, covered & stored at - 20 ºC
Lysozyme	100 mg/ mL in SMQ	Stored at - 20 ºC
Ampicillin	100 mg/ mL in SMQ	Filter sterilized and stored at - 20 ºC
Tetracycline	12.5 mg/ mL in 70% ethanol	Filter sterilized and stored at - 20 ºC
Kanamycin	50 mg/ mL in SMQ	Filter sterilized and stored at - 20 ºC
Hygromycin	10 mg/ mL in SMQ	Filter sterilization and stored at -20 ºC
Cefotaxime	250 mg/ mL in SMQ	Filter sterilized and stored at - 20 ºC
Rifampicin	50 mg/ mL in ethanol	Filter sterilized and stored at -20 ºC

Appendix-VIII: Buffers and solutions for plasmid DNA isolation

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)	Autoclaved for 10 min and Stored at 4 ºC
Solution II	0.2 N NaOH 1% SDS	Freshly prepared
Solution III	3M Potassium acetate (pH-5.5)	Stored at 4 ºC
RNase A	10 mg/ mL	Stored at -20 ºC
Other solutions or Reagents	Chloroform, Absolute ethanol, 3.0 M Sodium acetate, 70% ethanol & Deionized sterile water	RT

Appendix-IX: Buffers and solutions for protein extraction and purification

Name	Components	Preparation and Storage
Lysis buffer	50 mM Tris HCl (pH- 8.0) 300 mM NaCl 0.5% Triton X-100 10% glycerol	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
Denaturing urea buffer	8M Urea 150 mM NaCl	Stored at 4 ºC
Binding buffer	50 mM Tris (pH 8.0) 300 mM NaCl 20 mM imidazole	Adjust pH by adding concentrated HCl and stored at 4 ºC
Wash buffer	50 mM Tris (pH 8.0) 300 mM NaCl 30 mM imidazole	Adjust pH by adding concentrated HCl and stored at 4 ºC
Elutoin buffer	50 mM Tris (pH 8.0) 300 mM NaCl 250 mM imidazole	Adjust pH by adding concentrated HCl and stored at 4 ºC

Appendix-X: Buffers and solutions for SDS-PAGE and staining

Name	Components		Preparation and Storage
Polymer solution	29.2% acrylamide 0.8% bis-acrylamide in water		Stored at 4 ºC (in amber bottle)
Stacking gel	Distilled water 1 M Tris-HCl (pH 6.8) Acrylamide/bis 30% 10% SDS 10% (w/v) APS TEMED	2.76 mL 0.50 mL 0.65 mL 0.04 mL 0.04 mL 4 .0 µL	Freshly prepared
Separating gel (10%)	Distilled water 1.5 M Tris-HCl (pH 8.8) Acrylamide/Bis 30% 10% SDS 10% (w/v) APS TEMED	3.162 mL 2.0 mL 2.67 mL 0.08 mL 0.08 mL 8.0 µL	Freshly prepared
2X Protein loading buffer	Distilled water 0.5 M Tris-HCl (pH 6.8) Glycerol 10% SDS β-Mercaptoethanol 0.5% Bromophenol blue	2.7 mL 1.0 mL 2.0 mL 3.3 mL 0.5 mL 0.5 mL	Stored at 4 ºC
10X SDS-electrode buffer	Tris base Glycine SDS Adjust pH-8.3 *Make-up the volume up to 500 mL.*	15.1 g 94.0 g 00.5 g	Stored at 4 ºC
Staining solution (Coomassie)	Coomassie-blue R 250 Methanol Acetic acid *Make-up volume up to 100 mL*	0.25 g 40 mL 10 mL	Stored at RT (in amber bottle)
Destaining solution	Methanol Acetic acid *Make- up volume up to 100 mL*	40 mL 10 mL	Freshly prepared
Silver staining Fixer solution I	Methanol Acetic acid *Make-up volume up to 100 mL*	40 mL 10 mL	Stored at RT
Silver staining Fixer solution II	50% Ethanol		Freshly prepared
Sensitizing solution	0.2% $Na_2S_2O_3$		Stored 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 dark
Developer	6% Na_2CO_3 (18 g)		Prepared freshly

	0.02% formaldehyde (450 µL) *Make-up volume up to 300 mL*	
Stop solution	1.5% Na_2EDTA (4.5 g) *Make-up volume up to 300 mL*	Stored at RT

Appendix-XI: Buffers and solutions used for ELISA and Western blotting

Name	**Components**	**Preparation and Storage**
Phosphate buffer saline (PBS)	136 mM NaCl 2 mM KCl 8 mM Na_2HPO_4 1 mM KH_2PO_4 pH 7.5	Stored at RT
Crude protein extraction buffer	PBS pH 7.5 1% PVP 5 mM DTT 0.5 M sucrose	Stored at 4 ºC
Wash buffer/ PBST	PBS + 0.05% v/v Tween-20	Stored at 4 ºC
Binding buffer	PBS + 1% BSA	Stored at 4 ºC
Antibody dilution Buffer	PBS + 0.25% BSA	Stored at 4 ºC
Substrate dilution buffer	200 mM Tris-HCl, pH 9.5 0.5 mM $MgCl_2$	Stored at 4 ºC
Substrate (pNPP)	1 mg/ mL in substrate dilution buffer	Freshly prepared
Colour development solution (BCIP/ NBT)	-	Procured from Bioworld Stored at 4 ºC
Stop Solution	10 mM EDTA	Stored at RT

Appendix-XII: Different plant media

Media	**Composition**
Germination media	½ strength MS medium
Proliferation media	MS medium + 0.1 mg/l kinetin + 0.2 mg/l 6-BAP
Rooting media	½ strength MS medium + 2 mg/l IBA
Selection media	Proliferation medium + 10 mg/l hygromycin B

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PATENT

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PUBLICATIONS

Patent:

- **Gupta N**, Patel P, Sharma P, Kendurkar SV, Khan BM. A process of transformation in *Withania somnifera* plants to increase secondary metabolite content. Provisional filing application number: 2824DEL2013; filing date: 25.9.2013.

Research Papers:

- **Gupta N**, Sharma P, Santosh Kumar RJ, Vishwakarma RK, Khan BM (2012) Functional characterization and differential expression studies of squalene synthase from *Withania somnifera*. *Mol Biol Rep* 39:8803–8812.
- Patel P, **Gupta N**, Gaikwad SM, Agrawal D, Khan BM (2013) Leucaena sp. Recombinant cinnamyl alcohol dehydrogenase: purification and characterization. *Int J Biol Macromol* (Accepted DOI: 10.1016/j.ijbiomac.2013.09.005).
- **Gupta N**, Patel P, Sharma P, Kendurkar SV, Thulasiram HV, Khan BM. Enhanced withanolide biosynthesis in *Withania somnifera* overexpressing squalene synthase. (Under review).
- **Gupta N**, Patel P, Kumari U, Kendurkar SV, Khan BM. Microprojectile bombardment assisted agroinfusion of *Withania somnifera* (L.) (Manuscript communicated).
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Posters:

- "Expression profiling of Squalene synthase in *Withania somnifera*". **Gupta N**, Khan BM. Presented at National Chemical Laboratory in Science Day Poster presentation, 2013.
- "Genetic transformation of *Withania somnifera* for improvement of withanolides". **Gupta N**, Mishra S, Kendurkar SV, Khan BM. Presented at National Chemical Laboratory in Science Day Poster presentation, 2012.
- "Role of squalene synthase and squalene epoxidase in secondary metabolite biosynthesis in *Withania somnifera*". **Gupta N**, Sharma P, Abbassi S, Kannan C, Abhilash OU, Khan BM. Presented at National Symposium on "Recent advances in Plant Tissue Culture and Biotechnological Researches in India" at Bikaner, India. Feb 4-6, 2011.