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In silico study of binding motifs in squalene synthase enzyme of secondary metabolic pathway solanaceae family

Sanchita · Garima Singh · Ashok Sharma

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Abstract Solanaceae is an important family with several plants of medicinal importance. These medicinal plants have distinctive pathways for secondary metabolite biosynthesis. In most of the plants, two important compounds, dimethylallyl diphosphate and isopentenyl diphosphate, synthesize isoprenoid or terpenoids. Squalene synthase (SQS) is a key enzyme of the biosynthesis of isoprenoid (farnesyl pyrophosphate (FPP) → squalene). *Withania somnifera* (ashwagandha), an important medicinal plant of family solanaceae produces withanolides. Withanolides are secondary metabolites synthesized through isoprenoid pathway. In this study, 13 SQS protein sequences from the plants of solanacae family and *Arabidopsis thaliana* were analyzed. The conserved domains in corresponding sequences were searched. The multiple sequence alignment of conserved domains revealed the important motifs and identified the residue substitution in each motif. Our result further indicated that residue substitution in motifs might not lead to functional variation, although it may affect the binding affinity of Mg^{++}, FPP and NAD(P)H. In addition, the homology modelling of SQS enzyme of *W. somnifera* was done for the prediction of three-dimensional structure. Molecular docking study of considered substrates with WsSQS was performed and the docked structure were analyzed further. The docked structures showed binding affinity for motif 2 of WsSQS. Our analysis revealed that 29 residues of motif 2 might be important for catalytic/functional activity of SQS enzyme of *W. somnifera*. This study may provide an understanding of metabolic pathways responsible for the production of secondary metabolites. The motifs may play a key role in regulating the pathway towards enhanced production of metabolites.

Keywords Solanacae · Squalene synthase · Sesquiterpenoid and triterpenoid biosynthesis · *Withania somnifera*

Sanchita (✉) · G. Singh · A. Sharma
Biotechnology Division, CSIR-Central Institute of Medicinal and Aromatic Plants, Post Office CIMAP, Lucknow 226015, India
e-mail: 0804sanchita@gmail.com

Introduction

Solanaceae is a diverse family of plants with approximately 90 genera and 3,000–4,000 species [1]. It comprises a number of economically important plants, *Solanum tuberosum, Solanum lycopersicum, Solanum melongena* and *Capsicum annum* are used as food, *Nicotiana tabacum, Nicotiana rustica, Atropa belladonna, Mandragora officinarum, Withania somnifera* and *Datura innoxia* for drugs and *Petunia hybrida, Salpiglossis sinuate* are *Schizanthus pinnatus* as ornamentals [2]. *W. somnifera* (ashwagandha) is known since thousands of years for its pharmacological and medicinal properties. The well described pharmacological activities of *W. somnifera* include physiological and metabolic restoration, anti-arthritic, anti-aging, anti-cancer, cognitive function improvement in geriatric states and recovery from neurodegenerative disorders [3–6]. Medicinal importance of *W. somnifera* is due to the presence of withanolides in the leaves and roots [7]. The therapeutic value of *W. somnifera* is attributed to its secondary metabolite synthesis by the process of secondary metabolism. Secondary metabolic pathway are involved in allelopathic and plant–pathogen interactions and protects plants from herbivores, microorganisms, UV radiation and stress conditions [8]. Withanolides are a large group of steroidal lactones found in Solanaceous plants [9].

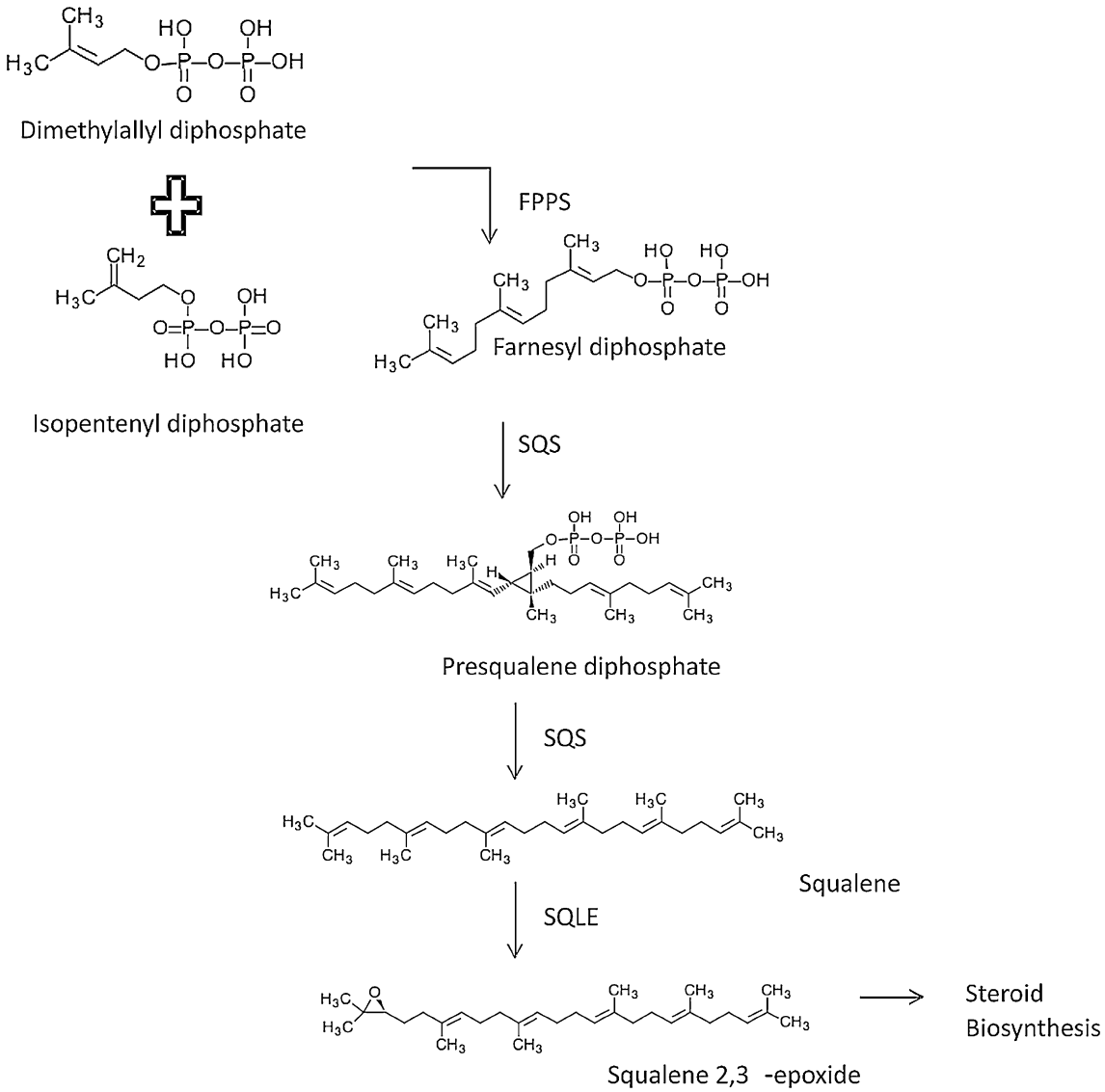

Fig. 1 Schematic representation of steroid biosynthesis

Withanolides are alkaloid in nature and are synthesized through isoprenoid pathway probably via both mevalonate and non-mevalonate pathways. Squalene synthase (SQS) (EC 2.5.1.21) represent the first committed step to diverge the carbon flux from terpenoid biosynthesis towards steroid biosynthesis [10]. The 24-methylene cholesterol is the first branching point towards the biosynthesis of different withanolides through a series of denaturation, hydroxylation, epoxidations, cyclization, chain elongation and glycosylation steps [11]. SQS is a critical branch point enzyme of isoprenoid biosynthesis that is thought to regulate the flux of isoprene intermediates through the sterol pathway. It is a bifunctional enzyme catalyzing the condensation of two molecules of farnesyl pyrophosphate (FPP) in head to head manner to form presqualene diphosphate (PSPP) and then converts the PSPP to squalene in the presence of NAD(P)H and Mg^{2+} (Fig. 1). In addition, the formation of PSPP or a prior conformational change in SQS is the rate limiting step for synthesis of squalene from FPP via PSPP in the presence of NAD(P)H and for synthesis of PSPP in the absence of NAD(P)H [12]. SQS is an endoplasmic reticulum associated enzyme with carboxy terminus anchored in the ER membrane and the amino terminus including active site is exposed in the cytosol [13]. In phytosterol biosynthetic pathway, SQS play an important regulatory role [14]. SQS genes are shown to be highly expressed in *Panax ginseng* [15] and *Eleutherococcus senticosus* [16] to increase the accumulation of phytosterols and triterpenes. In the present work, we did an *in silico* analysis of SQS enzyme in order to find out the conserved motifs playing

role in substrate binding. The 3D structure of *W. somnifera* squalene synthase (WsSQS) is not known. In this study, a 3D model of WsSQS was constructed by a homology modeling procedure based on the crystal structure of the human SQS.

Materials and Methods

Retrieval of SQS protein sequences and motif identification

The protein sequences of SQS (FASTA format) of eight plants of Solanaceae family and *Arabidopsis thaliana* were retrieved from NCBI. The conserved domain database (CDD) [17] was used to find the conserved domains in the protein sequences of SQS of different plants. MOTIF search tool (http://www.genome.jp/tools/motif/) was used for the identification of motifs using PROSITE database. The known structures of FPP, Mg^{2+} and NAD(P)H were retrieve from Pubchem database of NCBI.

Multiple sequence alignment and phylogenetic analysis of SQS domain

The MegAlign module of Lasergene, DNASTAR, Inc., Madison, WI, USA [18] was used for multiple sequence alignment of obtained domains of SQS using CLUSTAL-W. The phylogenetic analysis was performed through MEGA 5.05 [19]. A neighbour-joining method was applied on SQS domain sequences using clustering metric.

Structure prediction and molecular docking study

Secondary structure prediction was done using SOPMA [20]. Homology modeling was performed using Modeller 9.13 [21]. Molecular docking study of SQS protein was performed using Autodock 4 [22]. Identification and visualization of SQS binding motifs was done through Pymol viewer [23].

Results

Sequence analysis of SQS

Protein sequences of SQS enzyme of *A. thaliana*, *C. annum*, *D. inoxia*, *N. tabacum*, *S. lycopersicum*, *S. tuberosum*, *S. chacoense* and *W. somnifera* were obtained from NCBI. Multiple numbers of SQS sequences has been reported from some plant species (Table 1). The length of amino acids varied between 411 and 413 in all the considered sequences.

Table 1 Number of SQS sequences present in plants species

Sr. no.	Plants	Number of protein sequences
1.	*Arabidopsis thaliana*	3
2.	*Capsicum annum*	1
3.	*Datura inoxia*	1
4.	*Nicotiana tabacum*	3
5.	*Solanum lycopersicum*	1
6.	*S. tuberosum*	1
7.	*S. chacoense*	1
8.	*Withania somnifera*	2

Conserved domain analysis

The conserved domains were identified in each corresponding sequence through CDD. The amino acid length of domains was found to be varying from species to species. From all the identified domains, two of *A. thaliana* and one of *W. somnifera* showed longest 285 residues whereas *N. tabacum* had smallest domain of length 248 residues. All the identified domains were considered further for multiple sequence alignment to analyze the conserved motifs (Fig. 2).

Motif analysis

MSA analysis of SQS domain resulted in two conserved motifs (Fig. 2). All protein sequence of SQS reported two conserved motifs viz., motif 1 "YCHYVAGLVGLGLSKL" which was 16 residue long and motif 2 "MGLFLQKTNIIRDYLEDINEIPKSRMFWP" which was 29 residue long. In positions of motifs lied between 170 and 186 for motif 1 and 205 and 234 for motif 2. Only in the case of *N. tabacum* the length of motif 2 varied. These motif sequences were previously reported in other plants with several residues substitution [22, 23]. viz., motif1 "YCHYVAGLVGLGLSKL" and motif2 "MGLFLQKTNIIRDYLEDINEIPKSRMFWP" which were 16 and 29 residues long, respectively (Fig. 2). These conserved motifs were present in all protein sequences considered for the study. In protein sequence of *W. somnifera*, the positions of motifs lied between 168 to 183 for motif1 and 201 to 229 for motif 2. These motif sequences were reported earlier in other plants also with several residual substitutions [24, 25]. In this study, motif 2 of *W. somnifera* showed one residual substitution from I to V.

Phylogenetic analysis of SQS

Phylogenetic analysis was performed to see the evolutionary relationship between *A. thaliana* and other plants of solanacae family. It was observed that *W. somnifera* SQS domain sequence were more closely related to other solanaceous plants as compared to *A. thaliana* (Fig. 3). The

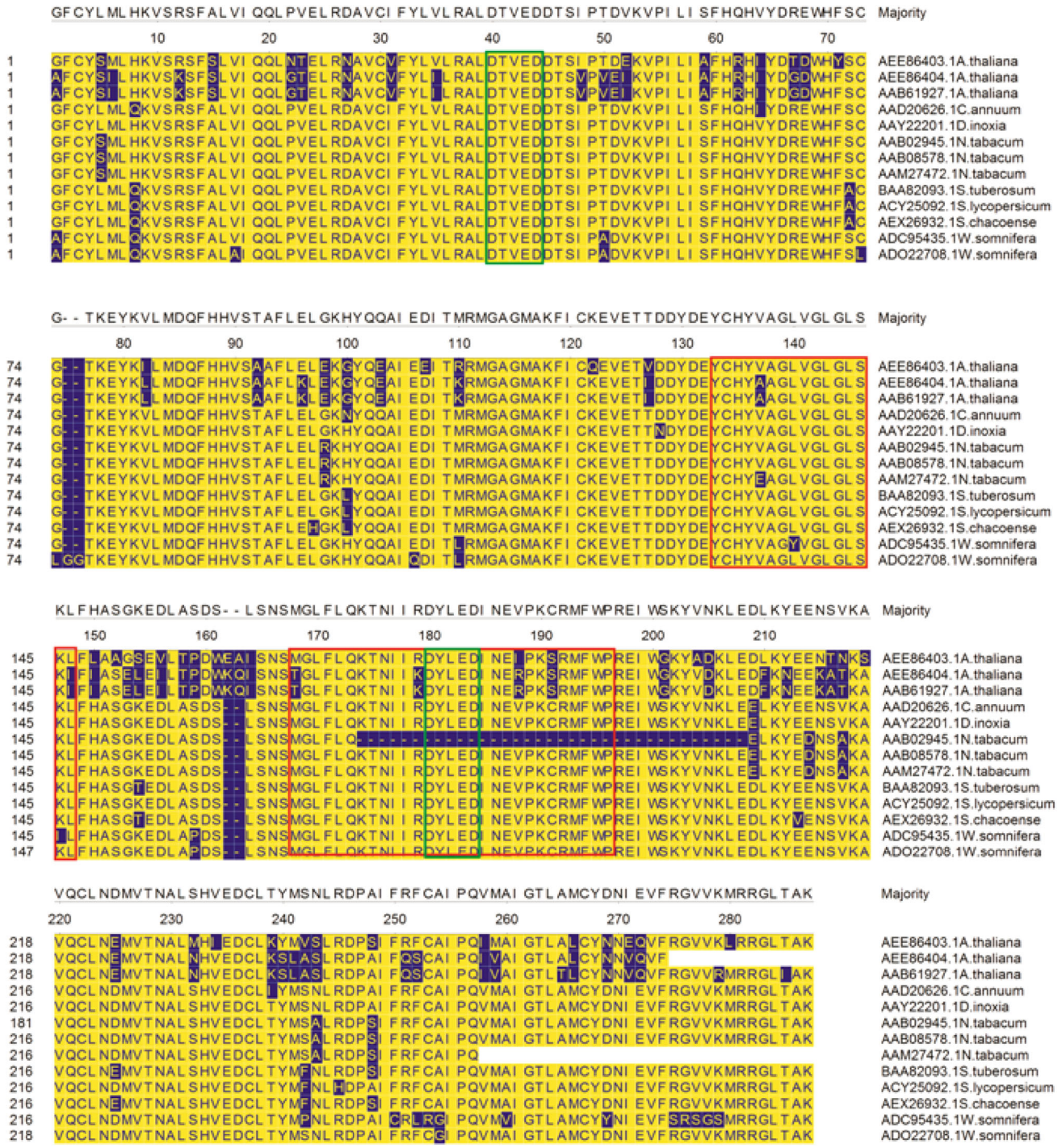

Fig. 2 Multiple sequence alignment of domain sequences of squalene synthase. *Shade with solid bright yellow* residues indicates that they match with the consensus exactly. *Shade with solid bright cobalt* residues indicate that they differ from the consensus. The putative squalene synthase binding motifs were indicated in the shape. Alignments were performed with ClustalW method. (Color figure online)

results indicated that SQS were derived from one ancestor gene which further developed into different branches. The phylogenetic tree demonstrated that SQS have a relationship with each plant.

Molecular modeling and structure validation

The amino acid sequence of the target protein WsSQS was obtained from NCBI (GenBank: ADC95435.1) with 411

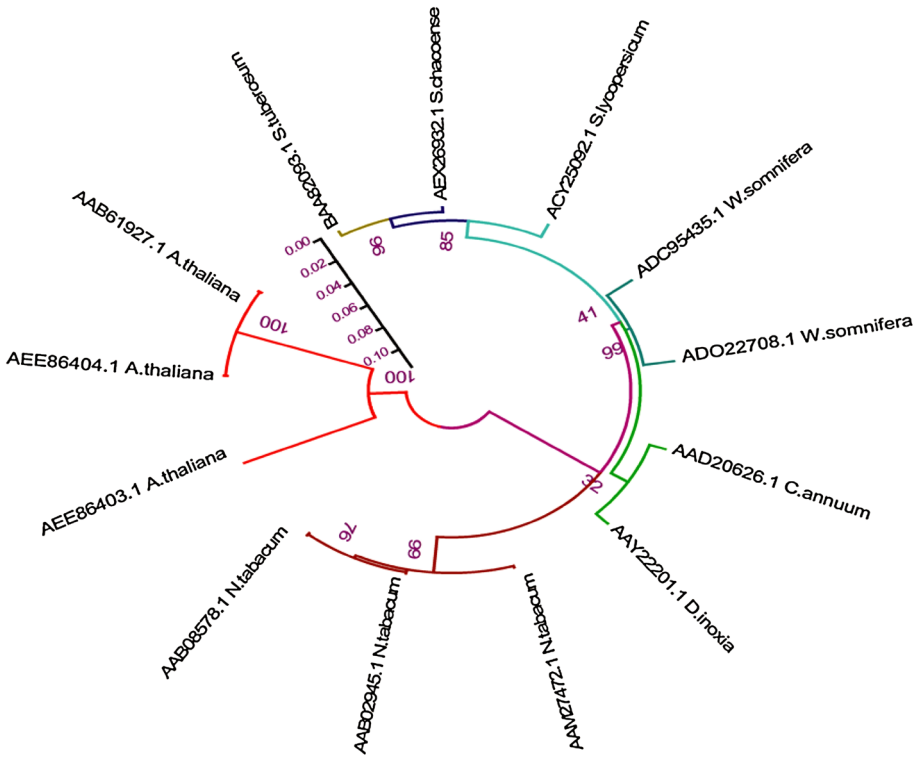

Fig. 3 A neighbour-joining tree showing the evolutionary relationship of SQS conserved domains. Neighbour joining phylogenetic tree constructed from the binding domain sequences of *C. annum*, *D. innoxia*, *N. tabacum*, *S. lycopersicum*, *S. chacoense*, *S. tuberosum*, *W. somnifera* and *A. thaliana* using MEGA 5.05, number above the branches indicate bootstrap values. The percentage of 5,000 bootstrap replicates was given at each node

residues. The analysis of SOPMA for secondary structure of WsSQS was performed and the result revealed that the peptide of WsSQS has 73.14 % of alpha helices, 5.30 % of extended strand, 3.53 % and 18.02 % of beta turns and random coils, respectively. The three dimensional structure of this protein is not available in any structural database. For deducing the structure through homology modeling approach, human SQS (PDB id 1EZF) was considered as template protein. The quantitative comparison between WsSQS and human SQS was calculated using Root Mean Square Deviation (RMSD) value. The RMSD value was 0.864 Å, which was identified through superimposition suggesting good similarity. WsSQS showed 47.44 % identity against human SQS enzyme. The predicted structure model was further validated using Ramachandran plot. The modeled structure was found to fulfill the criteria of proper distribution of residues in different regions. Seventy seven and 15.6 % of the residues were present in favoured and allowed regions, respectively (Fig. 4). The analyzed motifs are represented in the modeled protein (Fig. 5).

Molecular docking

Energy minimization was done through 707 steps of steepest descent using the Gromacs 4.5.5 software. The energy minimized model was used for the protein–ligand docking analysis through Autodock 4. All of the ligand molecules FPP, Mg^{2+} and NAD(P)H were docked with WsSQS, respectively. The global structure with lowest energy was chosen for computing intermolecular binding energies and visualized through Pymol. FPP was found to bind with two residues LYS^{223} and VAL^{221} ^{263} ^{261} of the motif 2 (Fig. 6a). Out of ten, confirmation 8 with minimum binding energy (−2.41 kcal/mol) has shown the interaction. Mg^{2+} showed interaction with the region DYLED of motif 2. The interacting residues were ASP^{213} and GLU^{216} (Fig. 6b) whereas the binding energy was −9.42 kcal/mol. NAD(P)H bind with VAL^{221} and LYS^{157}, VAL^{221} is belonged to motif 2 (Fig. 6c). The confirmation 4 of NAD(P)H have shown least binding energy (−3.32 kcal/mol) with the protein.

Discussion

The analysis of multiple sequence alignment of domains was resulted in percentage homology among *A. thaliana* and members of solanaceae family. The highest identity in SQS domain sequences of *A. thaliana* was analyzed with both *N. tabacum* and *S. tuberosum* (82.0%) and lowest identity observed with *W. somnifera* (73.5%) (Fig. 7). Our results revealed that SQS gene family has very conserved domain among plants, indicating that the importance of this enzyme in development and metabolic pathways in plants. Phylogenetic analysis of SQS sequences revealed that throughout evolution, plants of solanaceae family were closely related to

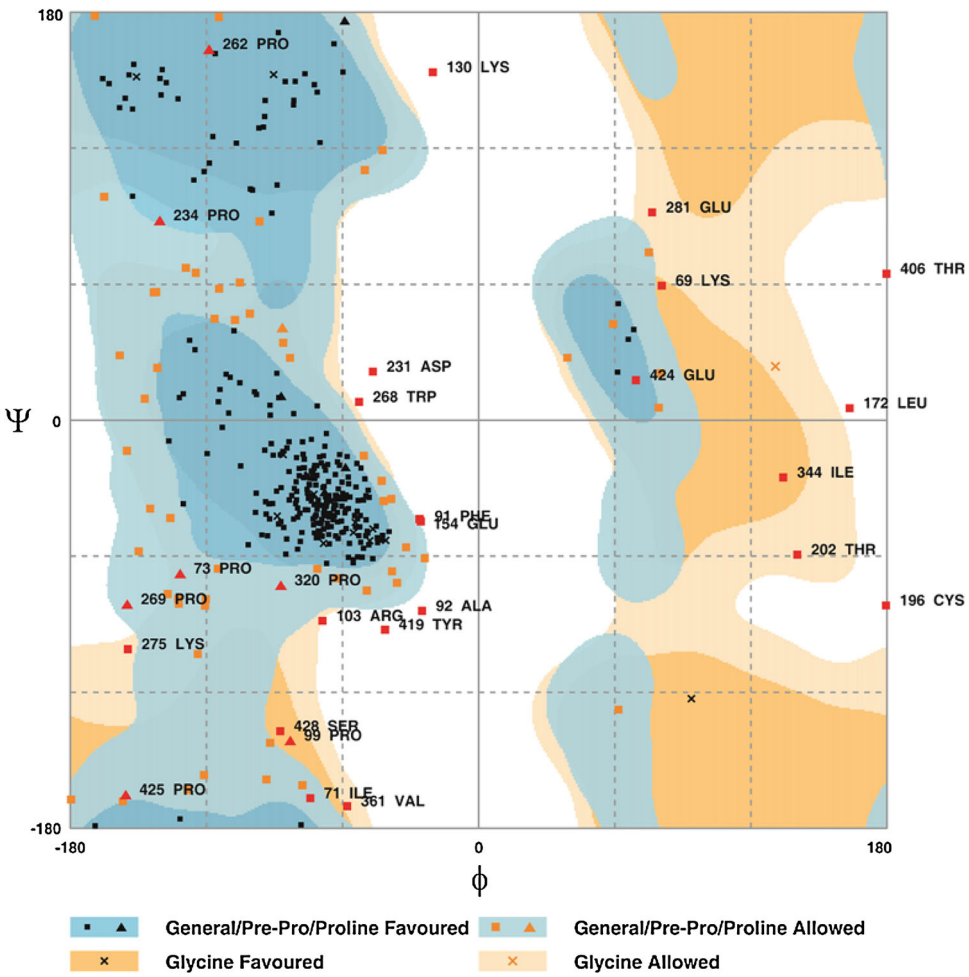

Fig. 4 Ramachandran plot of WsSQS generated by Rampage. *Dark blue* and *dark orange* are favoured regions, *light blue* and *light orange* are allowed regions. (Color figure online)

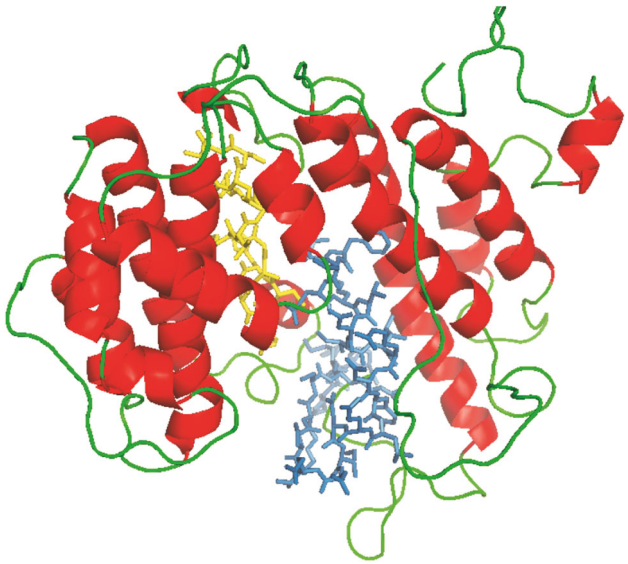

Fig. 5 The modelled 3D structure of WsSQS. Motif 1 and 2 are shown in *yellow* and *blue* colours, respectively. (Color figure online)

each other as compared to *A. thaliana*, which showed phylogenetic divergence (Fig. 3). The alignment of domain sequences showed residue substitution at different positions that may be responsible for the divergence among plants. In MSA analysis, residue substitution occurred in different positions of domains. At positions 15, 27, 31, 59 and 62 sequence of *A. thaliana* showed serine (S), asparagine (N), valine (V), alanine (A) and arginine (R) residues, respectively while plants of solanaceae family showed alanine (A), aspartate (D), isoleucine (I), serine (S), glutamine (Q). The distribution of residue substitution validates the pattern of evolutionary relationship between *A. thaliana* and solanaceous plants. The motif analysis was performed in order to find the pattern of conserveness and resulted into identification of two conserved motifs. These motifs were considered for being binding sites for the ligands [26]. In our findings, in case of *W. somnifera*, the analyzed conserved motifs showed their role in binding of different ligands. In motif 1, transition mutation occurred at position 174 nonpolar and aliphatic amino acid valine was replaced by

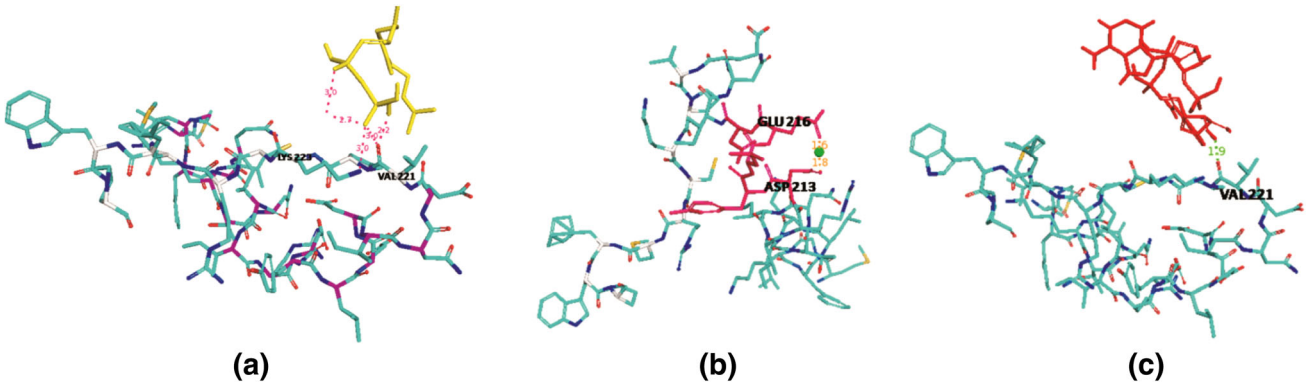

Fig. 6 **a** FPP (*green*) showing hydrogen binding with motif 1 residues. **b** Mg++ (*green* sphere) showing hydrogen binding with two residues (*red*) of motif 1. **c** NAD(P)H (*red*) showing hydrogen binding with motif 1 residue. (Color figure online)

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	
1		83.1	82.5	81.6	81.3	81.5	82.0	81.8	82.0	80.9	81.3	77.0	79.5	1
2	19.2		99.6	76.3	75.9	74.5	75.9	76.7	75.9	75.6	75.2	74.8	75.2	2
3	20.0	0.4		76.3	76.0	74.6	76.0	76.7	76.0	75.6	75.3	73.5	75.3	3
4	21.1	28.5	28.5		98.2	96.0	96.5	95.7	96.8	97.5	96.1	92.2	95.4	4
5	21.6	29.1	29.0	1.8		97.2	97.5	96.8	96.8	97.5	96.1	92.6	95.8	5
6	21.4	31.2	31.1	4.1	2.9		100.0	99.5	95.2	95.2	94.4	89.5	93.1	6
7	20.7	29.1	29.0	3.6	2.5	0.0		99.6	95.8	95.8	95.1	90.8	94.0	7
8	20.9	28.0	28.0	4.5	3.2	0.5	0.4		94.9	94.9	94.1	92.1	92.9	8
9	20.7	29.1	29.0	3.3	3.3	5.0	4.4	5.3		98.6	99.3	91.9	94.7	9
10	22.1	29.6	29.5	2.5	2.5	5.0	4.4	5.3	1.4		97.9	92.6	95.4	10
11	21.6	30.2	30.1	4.0	4.0	5.9	5.1	6.2	0.7	2.2		91.2	94.0	11
12	27.5	30.7	32.7	8.2	7.8	11.3	9.8	8.4	8.6	7.8	9.4		94.0	12
13	24.0	30.2	30.1	4.7	4.4	7.2	6.3	7.5	5.5	4.7	6.3	6.3		13
	1	2	3	4	5	6	7	8	9	10	11	12	13	

AEE86403.1A.thaliana
AEE86404.1A.thaliana
AAB61927.1A.thaliana
AAD20626.1C.annuum
AAY22201.1D.inoxia
AAB02945.1N.tabacum
AAB08578.1N.tabacum
AAM27472.1N.tabacum
BAA82093.1S.tuberosum
ACY25092.1S.lycopersicum
AEX26932.1S.chacoense
ADC95435.1W.somnifera
ADO22708.1W.somnifera

Fig. 7 The percentage identity in the domain sequences of SQS enzyme through multiple sequence alignment eight plants of solanacae family and *A. thaliana*

aliphatic amino acid alanine in *A. thaliana* and glutamic acid in *N. tabacum*. Next substitution occurred at position 177 and 185, where aliphatic amino acid leucine is replaced by aromatic amino acid tyrosine and polar positively charged lysine is replaced by aliphatic amino acid isoleucine, respectively in *W. somnifera*. In *A. thaliana* aliphatic amino acid leucine is replaced by aliphatic amino acid isoleucine at position 186. In case of motif 2, the residue substitution occurred only in *A. thaliana.* At positions 205, 216, 225 and 228 the respective substitutions were found (Fig. 2). The modeling of WsSQS showed that protein has spatial architecture that is very similar to human SQS [23, 24]. The structural alignment of WsSQS further showed the superimposition with chain A of human SQS. From molecular docking, motif 2 of WsSQS contained binding sites for cofactor FPP, Mg^{++} and NAD(P)H. The binding of FPP in *P. ginseng* had been earlier reported in DYLED motif [27]. Our results revealed a different residue present in motif 2 for binding with FPP. It was reported earlier that the binding sites for Mg^{++} were aspartate rich regions DYLED and DTVED in SQS [27]. Our results showed that, in *W. somnifera* Mg^{++} binds with two residues of DYLED region of motif 2. The DYLED region is also responsible for the binding of NAD(P)H. Although in human its binding region is reported to be VKIRK [28]. The hydrogen bonds play an important role for structure and function of biological molecules, especially for the enzyme catalysis [27]. Residues of motif 2 showed hydrogen bonds, binding with substrates. LYS^{223} and VAL^{221} were the binding residue for FPP, ASP^{213} and GLU^{216} for Mg^{++} and VAL^{221} were the binding site for NAD(P)H [26, 27]. VAL^{221} of motif 2 plays a key role for substrate interaction that results in secondary metabolite production.

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