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Research article

Molecular characterization of *Glycine max* squalene synthase genes in seed phytosterol biosynthesis

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ABSTRACT

The reaction catalyzed by squalene synthase (EC.2.5.1.21) that converts two molecules of farnesyl pyrophosphate to squalene represents a crucial branch point of the isoprenoid pathway in diverting carbon flux towards the biosynthesis of sterols. In the present study two soybean squalene synthase genes, *GmSQS1* and *GmSQS2*, were identified in the soybean genome and functionally characterized for their roles in sterol biosynthesis. Both genes encode a deduced protein of 413 amino acids. Complementation assays showed that the two genes were able to convert yeast sterol auxotrophy *erg9* mutant to sterol prototrophy. Expression of *GmSQS1* and *GmSQS2* was ubiquitous in roots, stem, leaves, flower and young seeds of soybean, however *GmSQS1* transcript was preferential in roots while *GmSQS2* transcript was more in leaves. Their expression was lower in response to dehydration treatments suggesting they might be negative regulators of water stress adaptation. Transgenic *Arabidopsis* plants overexpressing *GmSQS1* driven by either constitutive or seed-specific promoters showed increases in the major end product sterols: campesterol, sitosterol and stigmasterol, which resulted in up to 50% increase in total sterol content in the seeds. The increase in the end product sterols by *GmSQS1* overexpression was at the level achievable by previously reported overexpression of individual or combination of other key enzymes in the sterol pathway. Together the data demonstrate that soybean *SQS* genes play an important role in diverting carbon flux to the biosynthesis of the end product sterols in the seeds.

1. Introduction

Sterols are found primarily in cell membranes of all eukaryotic organisms. In plants, the dominant sterols are 24-alkyl sterols which comprise sitosterol, stigmasterol and campesterol while the other non-methylated C-24 sterols, such as cholesterol, are present in relatively low amounts [1]. Sterols play multiple roles in developmental stages in higher plants [2]. Most of the sterols exist as free sterols and serve as components of the cell membrane. A small amount of sterols, specifically campesterol, are precursors to brassinosteroids, the critical hormones for plant growth and development [2]. Sterols have been reported to have roles in the adaptation of plants to environmental stress. The abundance of free sterols, sitosterol in particular, are associated with the adaptability of potato to low temperature [3] and down-regulation of *Arabidopsis* SQS increased susceptibility to pathogen attack [4].

Sterols are majorly derived from the mevalonate pathway of isoprenoid biosynthesis (Fig. 1). Catalyzing the first committed reaction in sterol biosynthesis [5], squalene synthase (SQS, EC.2.5.1.21) acts in the condensation of two farnesyl pyrophosphate (FPP) molecules to produce squalene which occur in two steps. The first step is the head-to-head condensation of two FPP molecules to form presqualene diphosphate, which is then rearranged and reduced by NADPH to form squalene in the second step [5]. Squalene is further metabolized to synthesize end product sterols including sitosterol, stigmasterol and campesterol. Because FPP is also the substrate to synthesize other non-sterol isoprenoids (Fig. 1), including ubiquinones, sesquiterpenoids and geranylgeranyl diphosphate in plant cells [6,7], regulation of SQS has been considered important for redirecting carbon flux to end product sterol biosynthesis, which was reflected by a number of evidences.

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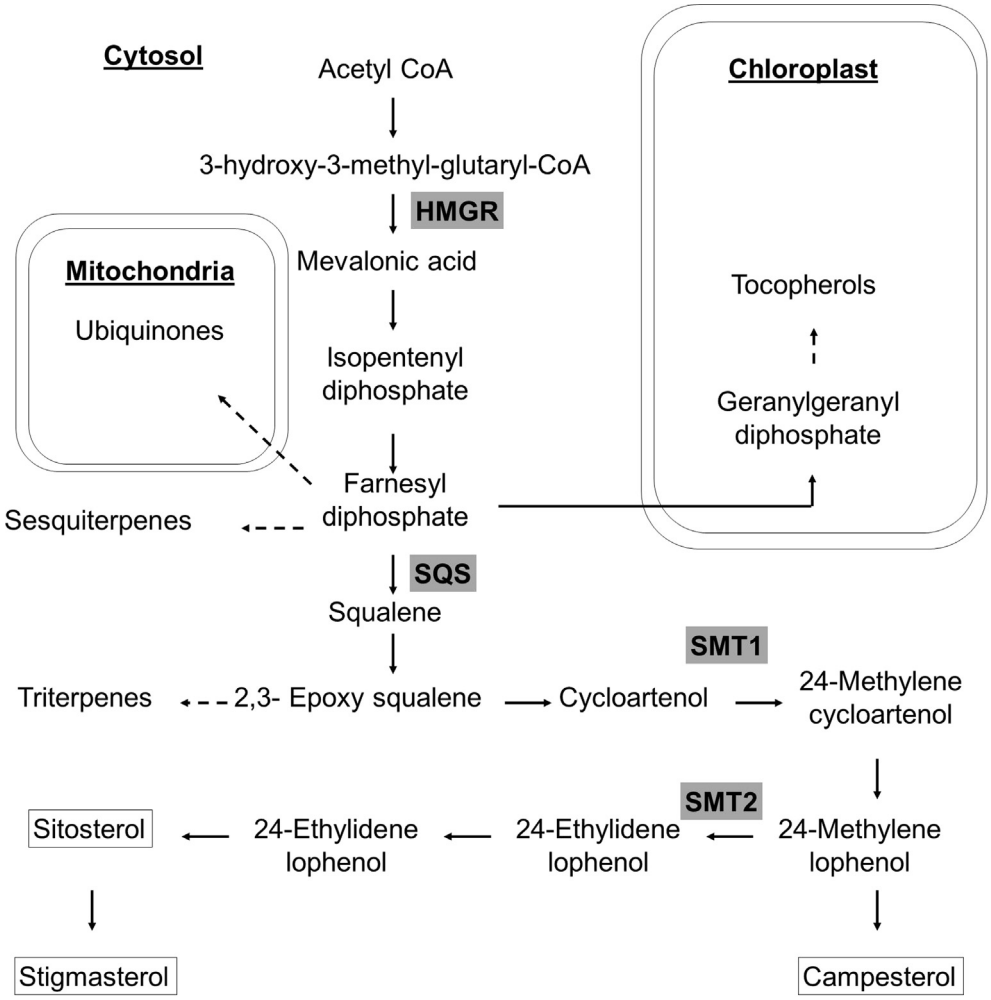

Fig. 1. Major sterol biosynthesis pathway in plants. Shaded boxes represents rate limiting enzymes that have been reported to enhance total sterol biosynthesis in plants. Major sterol products are boxed. Dotted arrow marks represent multiple enzymatic steps.

Cholesterol level of human fibroblasts was found to be modulated by SQS activity [8,9]. Changes in the activity of SQS in the yeast *Saccharomyces cerevisiae* sterol-auxotrophic mutants of upstream genes were associated with the treatment of exogenous sterol [10]. In plants, there is an association of SQS activity and the partitioning of FPP between sterol and sesquiterpenoid biosyntheses, which compete for this prenyl diphosphate [11–13]. Lowering sterol levels resulted from treatments with SQS inhibitor were found to trigger increased activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) an upstream enzyme in the pathway [14]. Overexpression of squalene synthase was reported to increase the major phytosterols up to 200% in *Panax ginseng*, *Eleutherococcus senticosus* and *Euphorbia tirucalli* [15–17] while down-regulation of SQS reduced the level of stigmasterol in tobacco [4].

Soybean is the world's leading oilseed crop and provides the largest source of processed vegetable oil. Sterol content in soybean crude oil is about 300 mg/100 g [1] and present mainly as free sterols [1]. Consumption of plant sterols is considered beneficial to human health due to their proposed inhibitory effects on lung, stomach, ovarian and breast cancers, and their promotion of anti-oxidant enzyme activities thereby reducing oxidative stress [18]. Phytosterols also inhibit and lower cholesterol absorption in human and consequently reduce cardiovascular risk [19]. With the given benefits, improving sterol content in the soybean oil could increase the soybean nutritional values. In this study, we aimed to investigate the function of two soybean SQS genes, *GmSQS1* and *GmSQS2*, in the regulation of end product sterol biosynthesis. *GmSQS1* (GenBank accession number AB007503) was previously cloned from soybean (Hata, 1997, unpublished); however, its function in seed sterol biosynthesis has not yet been investigated. *GmSQS1* and *GmSQS2* encoded two highly identical proteins and they were able to convert yeast *erg9* mutant, which is defective in SQS, to ergosterol autotrophic. Transgenic *Arabidopsis* plants over-expressing *GmSQS1*, driven by either constitutive or seed-specific promoter, were able to enhance the production of phytosterols in the seeds. Use of seed-specific expression of soybean SQS can potentially avoid negative effect on drought adaptation that may be associated with the elevated overexpression of SQS in the vegetative tissues.

2. Results

2.1. Identification and sequence information of soybean squalene synthases

Using known GmSQS1 protein (NCBI accession BAA22559) sequence as a query, Phytozome TBLASTN searches against soybean genome showed two distinct high scored hits of 1.8e−39 and 9.5e−32 in chromosome 12 and 11, respectively. Other BLAST hits landed on non-genic regions or resulted in substantially low scores.

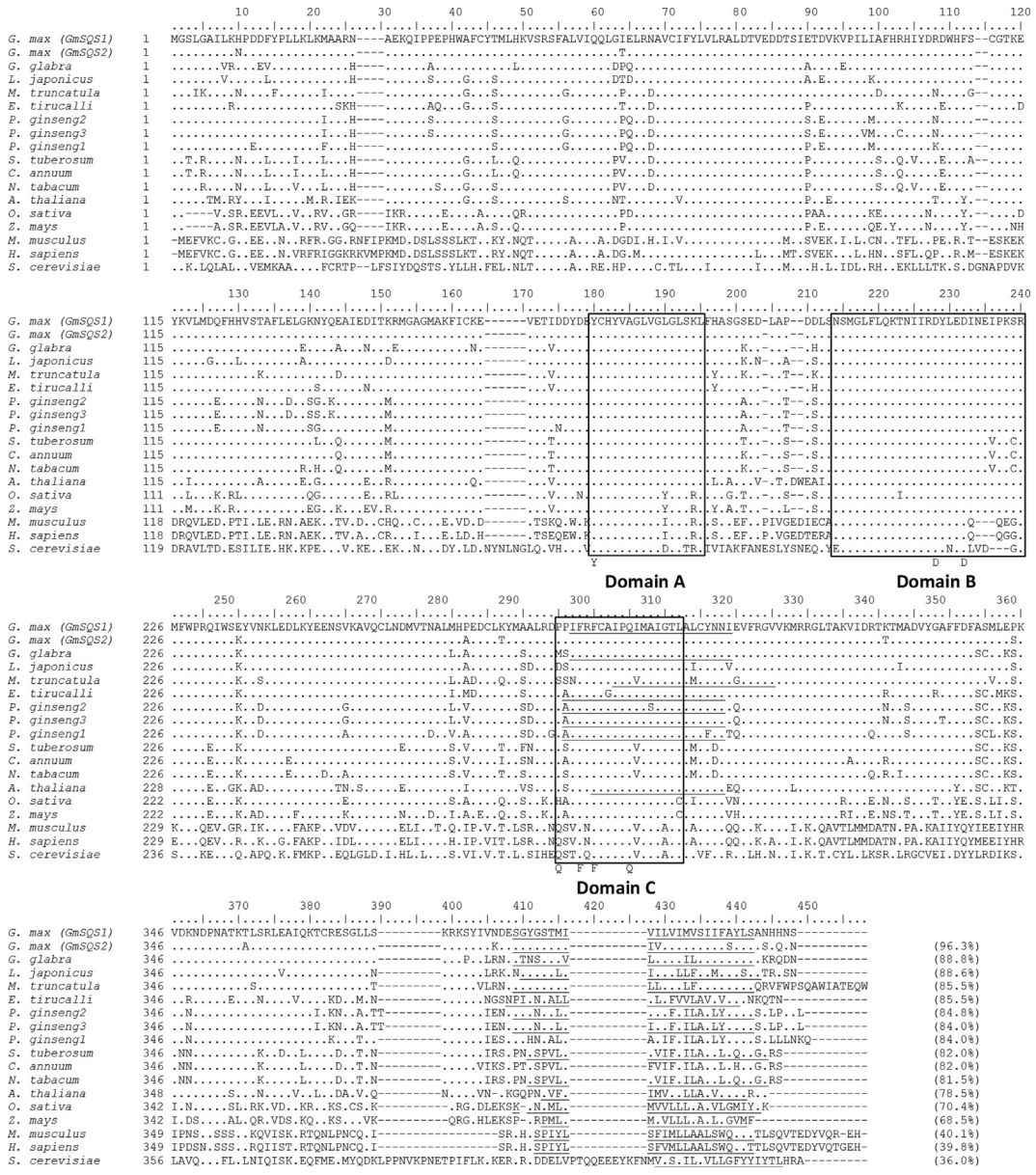

Fig. 2. Alignment of soybean SQS amino acid sequences with those of other organisms. Dots represent identical amino acids to soybean GmSQS1 and dashes represent gaps in aligned sequences. The three conserved catalytic domains are marked, and specific amino acids that are required for SQS functions [38] are written in the bottom lines. Predicted transmembrane domains were underlined based on prediction using TMHMM software [59] and a previous prediction [60]. The accession numbers that were retrieved from NCBI database are listed in Materials and methods.

The TBLASTN search was repeated using *Arabidopsis* SQS1 protein (NCBI accession AF004560) as query and resulted in similar results with scores of 2.4e−31 and 1.8e−25, respectively. *GmSQS1* has three mature mRNA as a result of alternative splicing with the only difference is in the 3′UTR region; two mRNAs have 14 exons each while the third one has 13 exons. The second gene was named *GmSQS2* because it had been not reported elsewhere. GmSQS2 protein is highly identical to GmSQS1 with 96% similarity. Mature *GmSQS2* mRNA also has three alternative sequences with 14 exons each and the difference is also detected in the 3′UTR, which does not affect the coding sequences of *GmSQS2*. All the introns are recognized as starting with GT and terminating with AG dinucleotides, universal among eukaryotes [37]. Sequencing the two genes revealed that they have 100% similarity to the predicted mRNA sequences from the Phytozome database and code for the two polypeptides of 413 amino acids.

Multiple alignment and phylogenetic trees of characterized SQS proteins from plants, animals and fungus showed that the soybean SQS proteins are highly similar (>84%) to the SQS from *Glycyrrhiza glabra*, *Medicago truncatula*, *Lotus japonicus*, *E. tirucalli* and *P. ginseng* (Figs. 2 and 3a). The soybean SQS sequences, however, are quite distinct to *Arabidopsis* (78%) and mammalian SQS sequences (40% similarity). Typical mammalian SQS proteins contain three conserved domains, namely A, B, and C, with distinct characterized functions for catalysis and binding [38], comprising of amino acids essential for SQS function. Y168 of section A is presumably involved in the first step of catalysis and Asp213 and 217 of section B might be important for activity and substrate binding via the magnesium salt bridge. Phe285 and 283 of section C might be involved in the second step of catalysis. Sequence alignment showed that both soybean SQS proteins have all these essential catalytic domains and amino acids, except for glutamine 280 which was replaced by

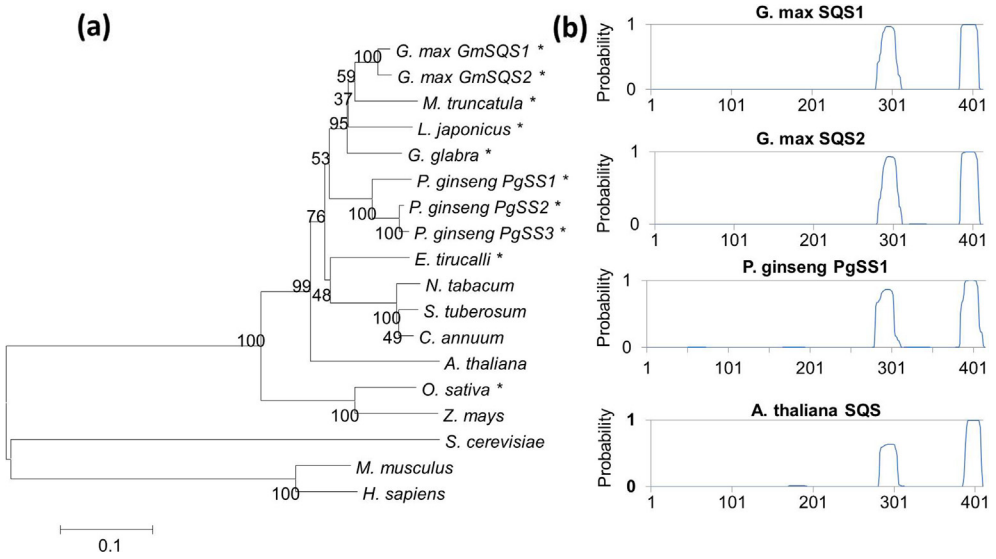

Fig. 3. Phylogenetic tree and transmembrane structure of soybean squalene synthases. (a) Phylogenetic tree of SQS amino acid sequences of soybean and other organisms. The tree was constructed by molecular evolutionary genetics analysis (MEGA) software, version 5.0 [31], using Neighbor-joining method for SQS sequences described in Materials and methods. The deduced proteins with an asterisk mark indicating that the corresponding sequence has two transmembrane domains, which is predicted by TMHMM software [59]. (b) Predicted transmembrane domains of *G. max*, *P. ginseng* and *A. thaliana* SQS.

proline. These data suggest that these two proteins might have conserved function in the biosynthesis of squalene.

SQS proteins are membrane bound enzymes anchored to the endoplasmic reticulum by their highly hydrophobic transmembrane domain located in their C-terminals [39]. This region is poorly conserved among SQS proteins (Fig. 2). Scanning transmembrane protein topology using TMHHM software (Krogh et al., 2001) revealed an additional transmembrane domain close to the domain C (from Ile282 to Ile304) of both soybean SQS proteins (Fig. 3b). This second transmembrane domain was also reported in SQS from closely related sequences of *G. glabra*, *M. truncatula*, *L. japonicas*, *E. tirucalli* and *P. ginseng* [25]. In contrast, *Arabidopsis* [40] and mammalian SQS proteins do not have this additional transmembrane domain (Fig. 3a and b).

2.2. Complementation assay of GmSQS1 and GmSQS2 in *S. cerevisiae*

The function of two soybean SQS in sterol biosynthesis was investigated using the yeast heterologous system. The gene constructs included two intact soybean SQS genes and the chimeric gene cassettes of which the 72 amino acids at the C-terminal of soybean SQS were replaced by the 112 C-terminal residues of the *Schizosaccharomyces pombe* SQS (ERG9), following the design previously reported in *Arabidopsis* [40,41]. This replacement of the C-terminal was used in the present study because it has been shown to be required for the function of *Arabidopsis* SQS1 to channel squalene through the sterol pathway in yeast [41]. The yeast native (*ERG9*), two soybean SQS and the chimeric gene cassettes were overexpressed in the yeast strain 2C1, which has a mutation in the *ERG9* gene that blocks ergosterol biosynthesis, to investigate the SQS activity of two soybean SQS proteins. Fig. 4 shows that the intact genes from soybean, but not the chimeric gene constructs, were able to rescue the mutant. Recovery of the mutant using both soybean SQSs, however, was slow and could not be detected until 8 days of plate culture in the medium lacking ergosterol. In contrast, the WT strain FY167A and the mutant recovered with yeast *ERG9* driven by the same promoter were growing very fast and forming visible colonies after 2–3 days. After seven days, growing patches of yeast strains overexpressing the native soybean SQSs were clearly observed while there were no colonies of the strains overexpressing the fusion gene constructs in the plates lacking ergosterol. The data demonstrate that both soybean SQS could partially restore the yeast mutant *erg9*, suggesting their activity in squalene biosynthesis.

2.3. Expression patterns of soybean squalene synthases

The expression of two soybean SQS genes in roots, stem, leaves at the V1 growth stage, flowers at the R2 (reproductive stage 2), and young seeds at the R5 stage of W82 was investigated using quantitative real-time PCR (qRT-PCR). Fig. 5a shows that both *GmSQS1* and *GmSQS2* were ubiquitously expressed in the various tissues studied with slightly differential expression patterns for the two genes. *GmSQS1* expression was strongest in roots with 1.5-2 times higher than the level found in other tissues while *GmSQS2* expression was strongest in leaves. Expression of these two genes was also verified with two mRNA probes using 61K microarray data from Genevestigator [42] which showed ubiquitous expression of the two genes (data not shown). The expression of two soybean SQS was similar to the ubiquitous accumulation of *SQS* transcripts reported in *Arabidopsis thaliana* [41], *M. truncatula* [30] and *L. japonicus* [23], suggesting their roles in various cell types in SQ synthesis.

A number of previous studies in plants revealed that sterol signaling was associated with tolerance to dehydration and manipulation of SQS expression altered stress adaptation. In *Arabidopsis*, overexpression of an *Arabidopsis* HDSTART, a lipid/sterol-binding domain transcription factor [43], improved drought tolerance [44]. Recently, SQS was reported to have negative effects on drought adaptation in rice when constitutive silencing of rice squalene synthase using RNA interference approach resulted in improved water-deficit tolerance [45]. Given the negative effects of SQS on stress adaption of plants, we investigated the expression of soybean SQS genes under various stress treatments. Fig. 5b shows that *GmSQS1* and *GmSQS2* were down regulated under severe dehydration at 5 h (a stem water potential of −28.6 bar was measured using a pressure chamber) and 24 h of exposure. Under

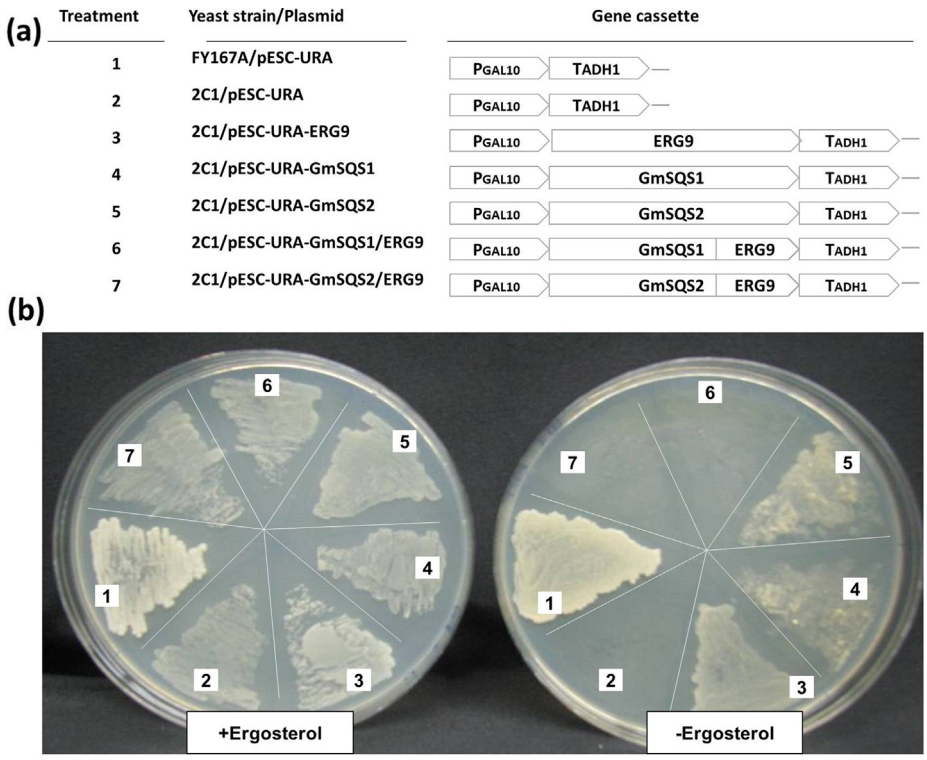

Fig. 4. Complementation assays of soybean SQS1 and SQS2 on *erg9* yeast mutant. (a) Schematic representation of the expression cassettes for *GmSQS1* and *GmSQS2* in yeast under the control of GAL10 promoter (P_{GAL10}). The fusion cassettes (5&6) were constructed by fusing the DNA coding sequences of N-terminals from soybean SQS to C-terminals from yeast strain *S. pombe*. The *S. cerevisiae* 2C1 strain had a mutation in *ERG9* lacking ergosterol biosynthesis and could not grow in the absence of ergosterol. (b) 8-day growth of sterol autotrophic strain FY167A transformed with pESC-URA (1, positive control), and the erg mutant 2C1 transformed with pESC-URA (2, negative control), pESC-URA-ERG9 (3), pESC-URA-GmSQS1 (4), pESC-URA-GmSQS2 (5), pESC-URA-GmSQS1/ERG9 (6) and pESC-URA-GmSQS2/ERG9 (7).

ABA, NaCl, and cold treatments, however, the expression of both genes was upregulated (compared to mock, H_2O treatment). These data, thus, provide an additional evidence that soybean SQS may exhibit a negative role in water deficit adaptation in an ABA-independent pathway.

2.4. Overexpression of GmSQS1 increased end product sterols in Arabidopsis seeds

To verify the function of soybean SQS in sterol biosynthesis, we used *Arabidopsis* to overexpress *GmSQS1* because this plant model has been used successfully for the assessment of the key soybean enzymes in the sterol pathways in our previous study [36]. In this experiment, we used two different promoters to overexpress *GmSQS*1. The constitutive Cauliflower Mosaic Virus 35S promoter regulates transcription in all tissues of host plants [46] and the soybean seed-specific beta-conglycinin 1 promoter [47] was reported to be differentially active during seed maturation from mid to late stages. The use of these two promoters to drive expression of *HMGR* and sterol methyltransferase 1 (*SMT1*) genes in the sterol pathway showed a significant increase in the level of end sterol products in previous studies [36,48]. In addition, we would like to understand effectiveness of the two promoters with the aim to increase phytosterols, but avoid potential negative effect on plant performance under water-deficit condition [45] which might result from the constitutive expression of SQS in the vegetative tissues. We observed that under greenhouse growing conditions, no significant difference in growth, plant structure and maturity was noticeable between the transgenic plants overexpressing *GmSQS1* under both promoters and WT plants (data not shown).

We initially analyzed the composition of sterols in T2 seed bulks for the events with single insertion locus of *GmSQS1*. In this seed generation, 75% of the seeds were expected to be transgenic based on 3:1 segregation. We found that majority of the transgenic events under both constitutive and seed-specific promoters had increased total phytosterol levels from 15 to 58% (Fig. 6a). Measurements were repeated using homozygous seeds at T3 generation for three transgenic events of the two transgenic constructs and the results were consistent with those from the T2 generation. The average amount of sterols (mg/g dry seed weight) in transgenic seeds from three biological repeats ranged from 3.3 to 3.8 mg per gram of dry seeds while WT seeds had 2.5 mg g^{-1} (Fig. 6b). Compared to WT seeds, transgenic lines displayed increased percentages in total end product sterol (sitosterol, stigmasterol and campesterol) contents ranging from 32% to 52%. Two transgenic lines, S_08 and S_11, (under seed-specific promoter) contained the highest amount of end products with around 50% higher than that of WT, followed by 35_01 and 35_08 (under 35S promoter) with around 40% higher than WT.

The change of major phytosterol compositions was monitored in seeds of seven independent homozygous transgenic lines at the T3 generation. Sitosterol composition accounted for around 70–80% in total of end product sterols, followed by campesterol with 15–20%, and stigmasterol from 2% to 10% in all seven transgenic lines using seeds from three independent plants (Fig. 6b). Sitosterol composition in transgenic lines increased significantly from 24% to 46% compared to WT seed. Campesterol had an increase ranging from 23% to 50%. The highest increase was observed in stigmasterol content from 4- to 5-fold compared to non-transgenic controls. Overexpression of *GmSQS1* in *Arabidopsis* did not result in any significant changes in the ratio of campesterol: sitosterol with the

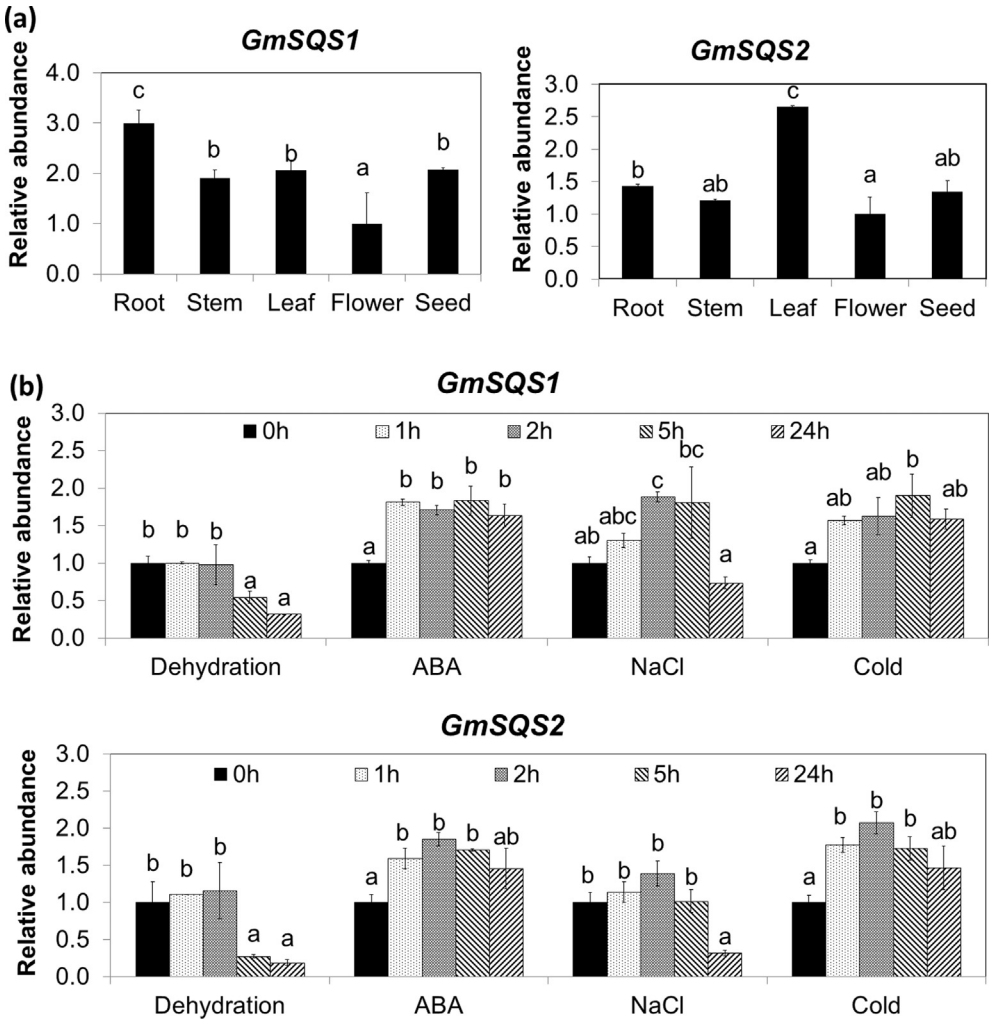

Fig. 5. Expression of soybean *SQS* genes at major developmental stages and under abiotic stresses. (a) Tissue-specific expression. Tissues were collected from soybean W82 growing in greenhouse conditions. Roots, stem and leaves were collected at V1 growth stages, flowers at the R2, and seeds at the early R5 growth stages. (b) Response to abiotic stress treatments. Plants were exposed to stress conditions at the V1 growth stage. Soybean ubiquitin was used as the reference gene for the calculation of relative expression of SQS. Error bars are standard errors of means (SEM) from three independent plants. Different letters indicate significant differences (Duncan test, $P < 0.05$) between means.

ratio ranging from 0.20 to 0.25, compared to 0.22 of the wild-type control.

3. Discussion

Using the current soybean genome database containing large numbers of ESTs coding about 98% of the known proteins (www.phytozome.net), we were able to identify two SQS genes with deduced amino acid sequences of 96% similarity. Multiple alignments (Fig. 2) showed that the two proteins are significantly aligned to the previously characterized proteins from *G. glabra*, *L. japonicas*, *M. truncatula*, *E. tirucalli* and *P. ginseng* while lesser homology was seen to *A. thaliana* and some monocotyledons (*Oryza sativa* and *Zea mays*). Significantly low alignment was observed for animal and yeast SQS which were only about 40% homologous to the soybean sequences. Both GmSQS proteins have the conserved domains which have been functionally characterized in mammals [38]. The essential amino acids whose deletion or replacement by other amino acids demolished or weakened SQS function in various organisms [23,38,40] were present in both soybean SQS, except for Gln280 which is replaced by Pro280. This replacement, however, was also found in functional *Arabidopsis* SQS1 [40] and in all three *P. ginseng* SQSs [25], suggesting that the change from Glutamine to Proline do not alter SQS function in plant cells.

Complementation assay was performed to confirm the functionality of the two soybean SQS genes using yeast mutant in sterol biosynthetic pathway. Although growing slowly in the media lacking ergosterol, the yeast mutants overexpressing *GmSQS1* and *GmSQS2* were recovered, indicating that the two SQS proteins were able to function to convert yeast *erg9* mutant cells to ergosterol prototrophy. These data indicate that both soybean SQS proteins have SQS function and can channel SQ to downstream pathways in yeast heterologous systems. The recovery of the yeast mutant, however, did not occur in the chimeric fusion protein constructs that had the C-terminals of soybean protein replaced by the corresponding yeast sequence. This is in contrast to human and *Arabidopsis* SQSs which were not able to rescue *erg9* yeast mutant [29,41], even though the two enzymes were catalytically active and able to synthesize squalene in yeast cells. Functional complementation on *erg9* yeast mutant using these SQSs can only achieved when replacing the C-terminal of the enzymes with the corresponding sequence from yeast [39,41]. Our current study provides a finding similar to a recent report on *P. ginseng* in which all three PgSQSs were able to convert yeast *erg9* mutant cells to ergosterol

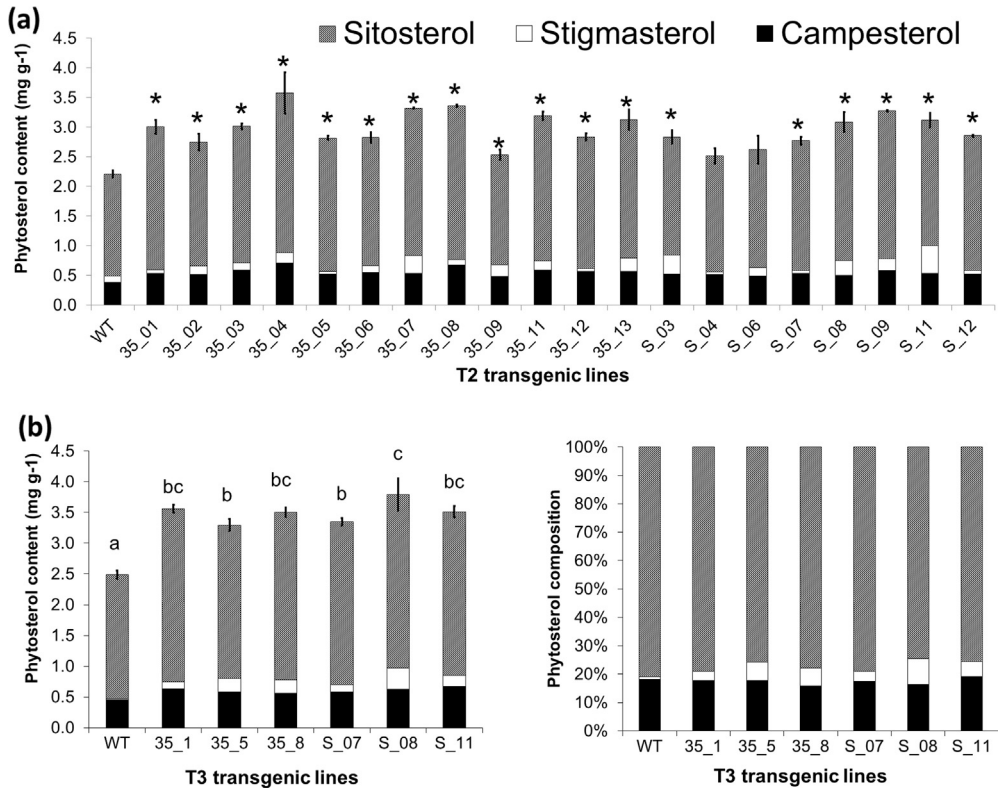

Fig. 6. End product sterols in transgenic *Arabidopsis* seeds overexpressing *GmSQS1*. Transgenic lines were under control of constitutive 35S (35_) and seed-specific beta-conglycinin 1 (S_) promoters. (a) Phytosterol content in T2 transgenic seeds. (b) Seed sterol contents and compositions of T3 homozygous transgenic lines with a single insertion locus. Error bars are standard errors of the means (SEM) from three seed samples from each transgenic event. In (a), student's *T*-test was used to compare end product sterol content between the transgenic and WT lines and significance was denoted using an asterisk mark. In (b), Duncan's multiple range test was used to compare sterol levels of seed-specific and constitutive overexpression of GmSQS.

prototrophy in spite of sequence divergence to yeast [25]. These data are in agreement with the result of sequence analysis from Fig. 3a, which shows that soybean SQS sequences are relatively similar to *P. ginseng* SQSs but distant from *Arabidopsis* and human proteins. In addition, Fig. 3b shows that soybean and *P. ginseng* have a second transmembrane scanning domain that may have been able to anchor into the yeast membrane and channel SQ to the yeast sterol pathways which was suggested in a recent report in *P. ginseng* [25]. The chimeric proteins with replacement of the C-terminal by the corresponding terminal from *S. pombe*, however, could not recover the yeast mutant. A possible conflict in the structure of the C-terminal might have changed the protein conformation, ultimately resulting in losing SQS function the in yeast cells. The inability to recover the yeast *erg9* mutant by the chimeric SQS proteins, however, will require further investigations.

A number of enzymes have been identified to control limiting steps in the biosynthesis of end product sterol biosynthesis in plant cells (Fig. 1). The 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the first committed step in the isoprenoid pathway towards sterol biosynthesis [49,50]. Acting downstream of HMGR, SMT1 which catalyzes the transmethylation of the Δ^{24}-double bond in the cycloartenol to produce 24-methylene cycloartenol [51] which is also an important step in diverting carbon flux to downstream sterol biosynthesis [52,53]. A second class of SMT, SMT2 catalyzes the S-adenosylmethionine-dependent transfer of methyl groups to the C-24(28)-methylene lophenol substrate. SMT2 directs carbon flux towards the synthesis of C-24 ethyl sterols [54], which are the major components of plant cell membranes [1]. Overexpression of individuals or combination of *Arabidopsis HMGR* and soybean *SMT1* and *SMT2* increase significantly end product sterols (sitosterol, stigmasterol and campesterol) in *Arabidopsis* [36] and in soybean [55]. In the present study, transgenic *Arabidopsis* seeds overexpressing *GmSQS1* stably increased the total phytosterols over 50% in the two examined generations which further confirmed the SQS functions in sterol biosynthesis. The increase was comparable to the levels achieved by expression of either individuals or combinations of *Arabidopsis HMGR* and soybean *SMT1* and *SMT2* [36] suggesting that soybean SQS1 is also a rate limiting enzyme in the isoprenoid–sterol biosynthesis pathway.

In contrast to mammals and yeast, multiple copies of genes encoding SQS have been identified in the plant genomes. Recently, three SQS were identified in *P. ginseng* with ubiquitous expression in various tissues and their functions were confirmed using complementation assays in a yeast mutant in ergosterol biosynthesis. The existence of the two highly similar copies of soybean SQS may have resulted from genome duplication [56] which was also identified by an alignment of the upstream and downstream DNA sequences (data not shown). The expression of both genes was ubiquitous in various tissues (Fig. 5a & b) suggesting functional duplication, or the two genes were co-regulated upon developmental or environmental conditions when high levels of SQS might be required. Squalene and 2,3-oxidosqualene are precursors of plant sterols and triterpenoid saponins [57], and soybean seeds are rich in saponins. Kim *et al.* [25] raised the idea that saponin-rich plants may be associated with plant species having higher SQS copies in their genomes. If that is the case, our data might provide additional evidence to support that hypothesis.

Phytosterols provide benefits to human health by the suppression of cholesterol uptake and inhibition of potential cancers. Genetic engineering of plants for higher phytosterols might be

Table 1
Primers used for PCR cloning and quantitative RT-PCR.

Primer name	Sequence 5′–3′	PCR purpose
GmSQS-EcoRI-F	ATTAGAATTCATGGGAAGTTTGGGAGCG	Common forward primer for GmSQS1 and GmSQS2
GmSQS1-NotI-R	ATTAGCGGCCGCCTAGCTATTATGGTGGTTAGCAGACA	Reverse primer for GmSQS1
GmSQS2-NotI-R	ATTAGCGGCCGCCTAGTTATTTTGGTGGCTAGCAGAC	Reverse primer for GmSQS2
EGR9_972H_ClaI_F	ATCGATATGAGTTTAGCTAACCGCATTGA	Forward primer for *S. pombe* EGR9
EGR9_972H_PacI_R	AAAATTAATTAACTAAAACAAATTAAGC	Reverse primer for *S. pombe* EGR9
linkGm1_1026-Y_1048F	GCTTTTTTTGATTTTGCTTCTATGTTGCATTATAAGAACACTCCTAAAGATCC	Triple-primer PCR to fuse GmSQS1-EGR9
linkGm2_1026-Y_1048F	CTTTCTTTGATTTTGCTTCTATGTTGCATTATAAGAACACTCCTAAAGATCC	Triple-primer PCR to fuse GmSQS2-EGR9
qRT_GmSQS1_F	CCATAGCTGAACAGAAGAAGTCAG	Forward primer for qRT-PCR of GmSQS1
qRT_GmSQS1_R	CGCTGCGATCAAATTTCGATAACC	Reverse primer for qRT-PCR of GmSQS1
qRT_GmSQS2_F	TAGGCGAAGAACGGAAGAAGC	Forward primer for qRT-PCR of GmSQS2
qRT-GmSQS2_R	TTCTGGCTACGATCGAACGAG	Reverse primer for qRT-PCR of GmSQS2
qRT-AtUBI-F	AGAAGAAGACTTACACCAAGCCGA	Forward primer for qRT-PCR of *Arabidopsis* UBI5 gene
qRT-AtUBI-F	CCTCAAACGCTGAACCTTTCC	Reverse primer for qRT-PCR of *Arabidopsis* UBI5 gene

exploited for commercial production [58]. SQS, however, is a negative regulator of drought adaptation in rice [45]. The expression of both SQS genes was lower under water-deficit conditions (Fig. 5b) suggesting they could have negative roles in drought tolerance in soybean. This is consistent with drought stress-related down-regulation of sterol methyltransferase gene expression [36]. Because major soybean production occurs in drought-prone areas, we must consider that overexpression of a gene should not adversely affect drought adaptation. Possible approaches include using a promoter whose inducibility is suppressed by drought stress or excluding the overexpression from vegetative tissues which are known most affected by the stress. Our data showed that overexpressing *GmSQS1* by a seed-specific promoter (beta-conglycinin 1) showed increased phytosterols in *Arabidopsis* to a level comparable to the use of 35S promoter. Using this seed-specific promoter, therefore, can help avoid potential negative effects on the performance of soybean plants under drought conditions, while achieve enhanced phytosterols levels in seeds.

In conclusion, our present study provides evidence that the soybean genome has two functional SQSs regulating an important step in the soybean isoprenoid–sterol pathway. Future studies would target the combination of various characterized important enzymes, including HGMR, SQS, and SMTs, to improve phytosterol levels in crop species like soybean. For genetic engineering approaches to improve phytosterol levels, the use of seed-specific promoters should be considered to prevent possible negative effects of SQS overexpression on stress tolerance in vegetative tissues.

4. Materials and methods

4.1. Soybean plant materials, growth conditions and stress treatments

Soybean cv. Williams 82 (W82) was grown in greenhouse conditions (28/20 °C day/night temperature, 14-h 800 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) day light intensity and 60% humidity). For stress treatment, soybean was maintained at a density of 4 plants per 1-gallon pot, containing a mixture of 1 sand:1 turface. At the V1 (first unfold trifoliolate) growth stage [20], plants were carefully harvested and transferred to stress treatments of dehydration, 100 μM ABA, 250 mM NaCl, and cold (4 °C cold water) for 1, 2, 5, and 24 h of stress. Salt and hormonal solution preparation and stress treatments were performed as previously described [21]. For developmental tissues, flowers (R2 stage) and seeds (R5 stage) were harvested from plants growing in pots containing promix media.

4.2. RNA extraction and cDNA synthesis

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was verified by an absorbance at 260 nm and 280 nm optical density using the NanoDrop ND-1000 UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and by gel electrophoresis (1% agarose). Total RNA was treated with DNase I using the DNA Free Turbo kit (Ambion, TX, USA), according to the manufacturer's instructions, to remove any residual DNA. Complimentary DNA was synthesized from 1 μg of DNase-treated RNA, using either random hexamer oligonucleotides (for quantitative RT-PCR) or oligo(dT) primers (for cloning SQS isoforms), following the manufacturer's instructions (Invitrogen, Carlsbad, CA).

4.3. Identification and sequence analysis of soybean SQS genes

The sequence of GmSQS1 (GenBank accession number AB007503) and AtSQS1 (accession AF004560) proteins were used as queries to blast against the Phytozome database (www.phytozome.net) to retrieve soybean SQS isoforms. The coding sequences (CDS) of the two genes were obtained by PCR using primers listed in Table 1 and cloned into the pGEMT vector for sequencing. *GmSQS1* was amplified from soybean W82 cDNA using the primers GmSQS-EcoRI-F and GmSQS1-NotI-R. Because *GmSQS2* is almost identical to *GmSQS1* at 5′ and 3′ ends of the CDSs, a set of primers were designed for the UTRs to amplify the DNA sequence flanking the CDS of *GmSQS2*, which was subsequently used for a second PCR to amply its CDS using primers GmSQS-EcoRI-F and GmSQS2-NotI-R.

Conserved domain structure and essential amino acids required for the SQS function were identified using alignment of the deduced amino acid sequences of GmSQS1 and GmSQS2 and the characterized SQS proteins from plants, animals, and fungi. The sequences include *A.s thaliana*_BAA06103.1 [22], *L. japonicus*_BAC56854.1 [23], *G. glabra*_BAA13083.1. *Nicotiana tabacum*_AAB08578.1 [24], *P. ginseng* PgSS1_BAD08242.1 [17], PgSS2_GQ468527 and PgSS3_AB115496 [25], *Solanum tuberosum*_BAA82093.1 [26], *E. tirucalli*_ BAH23428.1, *Capsicum annuum*_AAD20626.1, *O. sativa*_BAA22557.1, *Z. mays*_NP_001104839.1, *Mus musculus*_ [27], *S. cerevisiae*_ AAA34597.1 [28], *Homo sapiens*_ AAB33404.1 [29], and *M. truncatula*_XP_003607040.1 [30]. Sequence relationship was analyzed using the ClustalX software (http://www.clustal.org) with settings for a gap open of 10 and a gap extension of 0.2. Phylogenetic tree was created using the molecular evolutionary genetics analysis (MEGA) software version 5.0 [31], with a bootstrap of 1000 replicates.

4.4. Quantitative real-time PCR and expression analysis

Primer sets (Table 1) for qRT-PCR reactions of *GmSQS1* and *GmSQS2* were manually designed based on the alignment of cDNA sequences of the two genes to avoid mis-priming. The primers were verified for specificity using BLAST search against a soybean genomic sequence database (http://www.phytozome.net). The 10-μl qRT-PCR mixture contained 0.2 μM each primer, 5× diluted cDNA template, and 1X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The quantitative RT-PCR reaction was performed using the 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA) with thermal profiles programmed at 50 °C for 2 min, at 95 °C for 10 min and 40 cycles at 95 °C for 15 s, and at 60 °C for 60 s. The RT-PCR data was analyzed using the SDS 2.2.1 software package. The comparative threshold (C_t) method was used to quantify the relative expression of *GmSQS1* and *GmSQS2*, and the data were normalized to the expression levels of ubiquitin (NCBI accession D26092), which was used as an internal reference gene [32]. The relative abundance of mRNA in different tissues was compared by the $2^{-\Delta\Delta Ct}$ method [33].

4.5. Yeast complementation analysis

Coding sequence of *ERG9* (NM_001021271.2) was amplified by PCR from cells of the yeast strain *S. pombe*_972H using primers EGR9_972H_ClaI_F and EGR9_972H_PacI_R (Table 1) and cloned into pGEMT vector (Promega Inc.). Verified sequences of *ERG9*, *GmSQS1* and *GmSQS2* were subsequently cloned into the uracil auxotrophic pESC-URA yeast expression vector (Agilent Technologies), downstream of the GAL10 promoter, to produce pESC-URA-ERG9, pESC-URA-GmSQS1 and pESC-URA-GmSQS2. For fusion constructs, DNA sequences coding the first 342 amino acids of GmSQS1 and GmSQS2 were fused individually with the DNA sequence coding the C-terminal residues 350–460 of the yeast *ERG9* using a triple-primer PCR reaction (GmSQS-EcoRI-F forward, linker and EGR9_972H_PacI_R reverse primers) to produce a fusion protein without an addition of unnecessary residues. The PCR products were digested with *Eco*RI and *Pac*I and cloned into the pESC-URA to produce pESC-URA-GmSQS1/ERG9 and pESC-URA-GmSQS2/ERG9, respectively. All vector constructs were verified by sequencing.

The *S. cerevisiae erg9* mutant strain 2C1 (Mat(α) *erg9*, *His3*, *ura3-1*, *trip1-1*, *leu2*, *aux32*) deficient in SQS activity was used for the complementation assay. All 6 clones: pESC-URA-GmSQS1, pESC-URA-GmSQS2, pESC-URA-ERG9, pESC-URA-GmSQS1/ERG9 and pESC-URA-GmSQS2/ERG9, and the pESC-URA (negative control) were transformed into the 2C1 yeast strain using the Fast™ Yeast Transformation kit (G-Biosciences, St Louis, MO, USA). The transformants were first screened on SD-URA agar medium supplemented with 80 μg ergosterol and verified by PCR. Positive transformants were then screened in SD medium without ergosterol supplementation. pESC-URA was also transformed into a second yeast strain, FY1679-28C: Mat(α), *ura3-52*, *his3Δ200*, *leu2Δ1*, *trip1Δ63* [34] having prototrophic sterol to serve as a positive control.

4.6. Development of transgenic Arabidopsis overexpressing GmSQS1

The soybean seed-specific beta-conglycinin and CaMV35S promoters were used to drive expression of *GmSQS1* in *Arabidopsis*. *GmSQS1* was excised pGEMT vector and subcloned into pBeta-conSoyHyg vector, downstream of the soybean beta-conglycinin promoter and into the pART7 vector, downstream of 35S promoter via *NotI* sites. These two expression cassettes were excised from the subcloning vectors with *Eco*RI and *BamH*I (pART7) and *Asc*I (pBeta-conSoyHyg) and inserted into vector pZY-101-*AscI* to produce binary vectors P35-GmSQS1 and Pseed-GmSQS1, respectively.

A. thaliana ecotype Columbia (Col-gl1) was transformed with the binary vectors containing *GmSQS1* cassettes using the floral dip method [35]. Sterilized seeds were stratified at 4 °C for 3 days to stimulate germination and subsequently placed in a growth chamber (22 °C, 16/8 h day/night). After 2 weeks, the seedlings were transplanted into pots containing promix (SM-2, Premier Sogemix, PA, USA) and maintained in greenhouse (28/22 °C and 16/8 h day/night) until harvested. In all segregating generations, transgenic plants were screened using 25 mg l^{-1} glufosinate ammonium (Crescent chemical company, Islandia, NY, USA) and were further screened by PCR. Homozygous transgenic lines for a single insertion locus were obtained based on segregation ratio at the T2 and T3 generations.

4.7. Sterol analysis

The detailed procedure of phytosterol extraction and analysis using GC-FID was performed as previously described [36]. Briefly, ten milligrams of *Arabidopsis* seeds of the T2 and T3 generations were used for the sterol analysis. Lipids were extracted in chloroform methanol mixture, followed by saponification, solvent extraction with hexane, and subsequent derivatization by silylation, to form trimethylsilyl ether derivatives of sterols, which were used for quantification. GC analysis of sterols was performed with a Finnigan TraceGC Ultra gas chromatograph equipped with a Flame Ionization Detector. Helium was used as the carrier gas at a 1 ml min^{-1} flow rate and a 1:10 split injection ratio. Seeds of three independent plants from each transgenic line were used as three replications. In most cases, ANOVA was used to compare means of transgenic lines and the wild-type control, however, Duncan's multiple range test was also performed when comparing means from lines of the two gene constructs and the WT.

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