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Distinct biochemical activities and heat shock responses of two UDP-glucose sterol glucosyltransferases in cotton

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ABSTRACT

UDP-glucose sterol glucosyltransferase (SGT) are enzymes typically involved in the production of sterol glycosides (SG) in various organisms. However, the biological functions of SGTs in plants remain largely unknown. In the present study, we identified two full-length *GhSGT* genes in cotton and examined their distinct biochemical properties. Using UDP-[U-^{14}C]-glucose and β-sitosterol or total crude membrane sterols as substrates, GhSGT1 and GhSGT2 recombinant proteins were detected with different enzymatic activities for SG production. The addition of Triton (X-100) strongly inhibited the activity of GhSGT1 but caused an eightfold increase in the activity of GhSGT2. The two GhSGTs showed distinct enzyme activities after the addition of NaCl, $MgCl_2$, and $ZnCl_2$, indicating that the two GhSGTs exhibited distinct biochemical properties under various conditions. Furthermore, after heat shock treatment, GhSGT1 showed rapidly enhanced gene expression *in vivo* and low enzyme activity *in vitro*, whereas GhSGT2 maintained extremely low gene expression levels and relatively high enzyme activity. Notably, the *GhSGT2* gene was highly expressed in cotton fibers, and the biochemical properties of GhSGT2 were similar to those of GhCESA in favor for $MgCl_2$ and non-reduction reaction condition. It suggested that GhSGT2 may have important functions in cellulose biosynthesis in cotton fibers, which must be tested in the transgenic plants in the future. Hence, the obtained data provided insights into the biological functions of two different GhSGTs in cotton and in other plants.

1. Introduction

Sterol glycosides (SGs) are ubiquitous in bacteria [1,2], plants, and animals. The sugar moiety in typical SGs is attached to the 3 β-hydroxy group at the C3-atom of a sterol. The structural multiplicity of SGs is due to variations in sugar moieties, sterols, and linkages [3]. Although glucose is the most common sugar moiety in plant SGs, galactose [4], xylose [5], and other carbohydrates are also present [6,7]. The acylation of the sugar moiety occurs in the structure of SGs, and phytosterols differ in terms of the presence of C24, bond positions of the rings, and the side chains [8,9].

SG biosynthesis is catalyzed by UDP-glucose sterol glucosyltransferase (SGT) [10–12]. A UDP-Glc-dependent glucosylceramide from cotton has been proposed to play a role in SG biosynthesis [13]. Although eukaryotic SGTs are members of the UGT family (GT1) [14,15], the SGTs from *Borrelia burgdorferi* and *Heliobacter pylori* belong to GT4 because of different catalytic mechanisms [2,16].

The substrates of SGTs are limited to steroids, such as steroidal alkaloids [17,18], steroidal sapogenin [18], sterols, and their derivatives [12,19]. Steroidal glycoalkaloids or saponins have resistance to insects or microbes [20–23]. The three most common phytosterols are sitosterol, stigmasterol, and campesterol [24]. β-Sitosterol to stigmasterol conversion occurs in plant–pathogen interactions [25]. In addition to free sterol form, phytosterols are found as

Abbreviations: SGT, UDP-glucose sterol glucosyltransferase; SG, sterol glycoside; ASG, acyl sterol glycoside; GT, glucosyltransferase; UGT, UDP-glucosyltransferase; Gh, *Gossypium hirsutum*; CESA, cellulose synthase; PSPG, the putative secondary plant glycosyltransferase.

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conjugates, with the three forms being sterol esters, sterol glycosides, and acylated sterol glycosides. Conversions between free sterols and steryl esters involve in phytosterol homeostasis [26] and the response of *Arabidopsis thaliana* to *Phytophthora infestans* [27]. Sterols and sterylglucosides are enriched in detergent-resistant plasma membrane microdomains (DRMs) in plants [28,29]. The DRM fractions contain more than 70% of the total glucan synthase activities present in original plasma membranes [28]. Three *Arabidopsis* sterol biosynthesis mutants exhibit a deficiency in cellulose [30]. The reports suggest that sterols are important for cellulose synthesis because sterols are the major component of DRMs for maintaining the subcellular location, structural integrity and/or activity of the cellulose synthase [31].

SGs are the primers for cellulose synthesis in cotton fibers, and SGTs are thought to be involved in SG synthesis [32]. However, *Arabidopsis* SGT-double mutants with reduced SG and acyl SG (ASG) contents do not show a phenotype with impaired cellulose biosynthesis [33], suggesting that low levels of SGs may be sufficient for initiating cellulose synthesis [31]. Furthermore, SGs can be used as an acceptor by cellulose synthase, indicating that they do not necessarily mean as a primer for cellulose synthesis *in vivo* [34]. In addition, heat stress can induce membrane sterol glycosylation in various organisms of *Physarum polycephalum* [35,36]. Heat stress can also increase the amount of cholesteryl glucoside in human fibroblasts [37]. Furthermore, SG is a lipid mediator involved in stress-responsive signal transduction [38]. Overexpression of the SGT gene of *Withania somnifera* in *Arabidopsis* can enhance salt tolerance, heat tolerance and cold accumulation ability in transgenic *Arabidopsis* plants [39].

SG may be specific only for cellulose biosynthesis in cotton fibers because mature fibers contain more than 95% of cellulose [40]. This assumption was tested by investigating the biochemical activity of SGTs for SG production. In the present study, we initially identified two distinct *SGT* genes in cotton and then characterized their enzymatic activities *in vitro* with various biochemical conditions. The response of SGT to heat shock was also detected to understand the potential role of SG in cotton fibers.

2. Materials and methods

2.1. Plant materials and heat shock treatment

Upland cotton plants (*Gossypium hirsutum*, Huamian99) were grown in the fields of Huazhong Agricultural University. Cotton fibers at various stages were collected every 5 days from 4 days post-anthesis (DPA) to 24 DPA and immediately frozen in liquid nitrogen. The leaves, stems, and roots of cotton were also harvested from the field. The cotton seedlings were grown in a growth chamber and subjected to heat shock treatment. The cotyledons of seedlings were cut after heat shock after 0, 20, 40, and 60 min at 42 °C and immediately ground to powder under liquid nitrogen and stored until use.

2.2. Cloning of SGT coding sequences

The 3′ ends of *GhSGT1* and *GhSGT2* genes were isolated using rapid amplification of cDNA ends (3′RACE) technique, as previously described by Frohman et al. [41]. The 5′ cDNA regions were cloned by 5′RACE using the 5′-Full RACE kit (TaKaRa). Based on sequence information from both ends, full-length cDNAs of the two *GhSGT*s were identified. Primers for the two *GhSGT* genes in cotton are listed in Supplemental Table S1. The coding sequence of *GhSGT1* was isolated by 3′RACE kit (TaKaRa) using primers (4F, 4R). The coding sequence of *GhSGT2* was obtained using primers (6F and 8R) designed by 3′-end and 5′-end sequences.

2.3. Subcellular localization of GFP-GhSGT fusion protein

The coding regions of the two GhSGTs were amplified and subsequently cloned into the pEGAD vector. Recombinant and empty vectors were separately transformed into onion epidermal cells by particle bombardment. Culture and plasmolysis of the transformed onion epidermal cells were conducted using a previously described method [43]. Cells were observed using a fluorescence microscope (Olympus BX61, Japan) and photographed using a RETIGA-4000DC camera (RETIGA, Canada).

2.4. Real-time PCR

Total RNA was extracted from *G. hirsutum* fibers and leaves by employing the modified method [42]. First-strand cDNA was obtained using M-MLV Reverse Transcriptase (Promega). Real-time PCR was carried out on a Bio-rad MyCycler thermal cycler with the SYBR premix ExTaq (TaKaRa) according to the manufacturer's instructions. *UBQ7* was used as the internal control. All primers used are listed in Table S1. The experiments were carried out in biological triplicate.

2.5. Heterologous expression in Escherichia coli and GhSGT protein purification

The ORFs of two *GhSGT* genes were amplified using primer pairs (9F, 9R; 10F, 10R). The amplified ORFs of 1.8 kb GhSGT1 and 1.9 kb GhSGT2 were cloned in T vector (TaKaRa) and verified. The ORFs were then constructed into pGEX-6P-3 and transformed into *E. coli* Rosetta (DE3). Transformants were cultured at 37 °C in LB liquid medium containing 100 μg mL^{-1} ampicillin until the OD_{600} readings fell within the range of 0.4 to 0.6. Isopropyll-thio β-D-galactoside was added to the final concentration of 1 mM in GhSGT expression cultures. The cells were harvested and resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0. containing 2.5 mM DTT, 1 mM EDTA, and PMSF). The suspension was sonicated, centrifuged (12,000 × *g*, 15 min), filtered through the membrane with 22 μm bore diameters, and incubated with 1 mL of 50% glutathione Sepharose 4B (Amersham Bioscience). Fusion proteins were eluted in lysis buffer containing 10 mM reduced glutathione. The fusion proteins were concentrated using Centrifugal Filters (Amicon Ultra-0.5, Ultracel-50 Membrane, 50 kDa) for SGT activity assay.

2.6. Total crude sterol extraction

Cotton 24 DPA fibers were ground to fine powder in liquid nitrogen. The powder was added to the MOPS buffer (pH 7.5) and stirred as homogenate. The homogenate was centrifuged at 3000 × *g* for 10 min at 4 °C. The supernatant was obtained by filtering with a micro cloth and centrifugation at 100,000 × *g* for 30 min. The pellet was homogenized with the solvents (chloroform/methanol/water; 1/2/0.8, v/v/v), centrifuged at 12,000 × *g*, and added with water to form two phases. The bottom phase was obtained and evaporated to remove chloroform, and the residue was used as total sterols in the membrane.

2.7. SGT activity assay in vitro

Enzyme activities of the two GhSGTs were measured using 20 mM Tris–HCl buffer, 0.23 mM β-sitosterol (aliquoted from an ethanolic stock solution) or total crude sterols extracted from cotton fibers as described above, 1.3 μM UDP-[U-^{14}C]-glucose (specific activity 302 mCi mmol^{-1}), and enzyme extracts. The reaction was performed at 27 and 37 °C, respectively, for 30 min, and then the

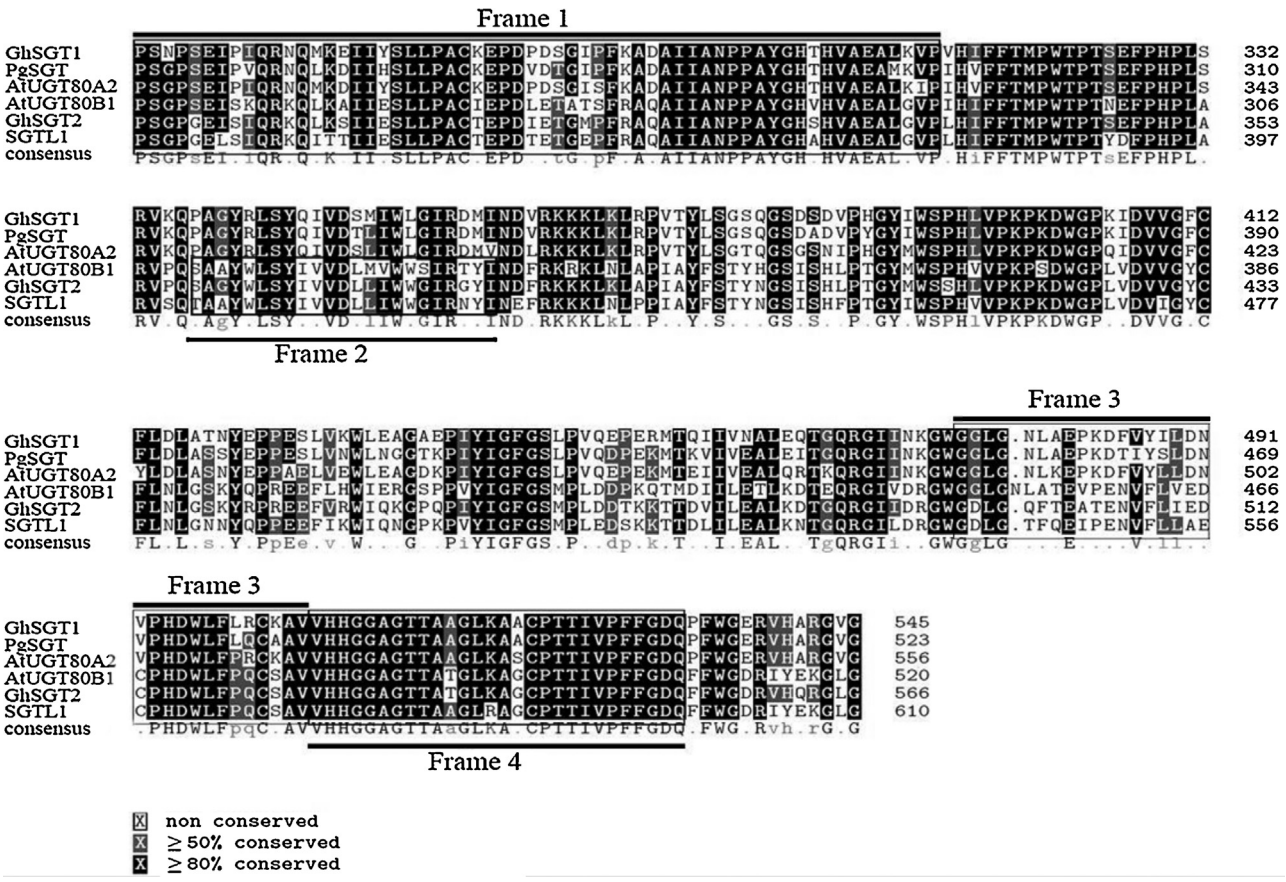

Fig. 1. Comparison of the conserved domains of SGT proteins in cotton (GhSGT1, JN004107; GhSGT2, JN004108) and other plants. Sequences of SGTs in *Arabidopsis thaliana* (AtUGT80A2/At1g43620; AtUGT80B1/At3G07020), *Panax ginseng* (PgSGT, AB071962), and *Withania somnifera* (SGTL1, DQ356887) obtained from the NCBI database; Putative transmembrane domains shown in Frame 2, PSBD domain in Frame 1, UGT prosite motif in Frame 4; Frame 3 indicates the additional stretch from the UGT prosite motif.

reaction SG product was extracted using ethyl acetate. Radioactivity in the ethyl acetate extract was detected with liquid scintillation counter (Wallac). All experiments were carried out in biological triplicate. Furthermore, the extracted SG product was concentrated and separated by silica gel plate using chloroform/methanol (85/15, v/v). The radioactivity on the silica gel plate was detected by photographic film. The radioactivity bands were scraped from the plate, extracted with chloroform/methanol (2/1, v/v), and dried in air. The residues were hydrolyzed with 2 M trifluoroacetic acid at 121 °C in autoclave (15 psi) for 1 h and dried under vacuum at 38 °C. Methoxylamine hydrochloride dissolved in pyridine (50 μL) was added under nitrogen and incubated at 30 °C for 90 min. N-methyl-N-(trimethylsilyl) trifluoroacetamide (50 μL) was added under nitrogen and incubated at 37 °C for 120 min. The derivative products were then dried under nitrogen and re-dissolved in 2 mL *n*-hexane for GC/MS analysis. GC/MS analytical conditions: Restek Rxi-5 ms, 30 m × 0.25 mm ID × 0.25 μm df column. Carrier gas: He. Injection method: Split. Injection port: 250 °C, Interface: 250 °C. Injection volume: 1.0 μL. GC temperature program: 155 °C (held for 10 min) and then rise to 300 °C at the rate of 50 °C/min for 15 min. Ion source temperature: 200 °C, ACQ Mode: SIM. The mass spectrometer was operated in the EI mode with ionization energy of 70 eV. Mass spectra were acquired with full scans based on the temperature program from 50 to 500 *m*/*z* in 0.45 s.

K_m and V_{max} assay was performed in 50 μL reaction buffer with 0.23 mM β-sitosterol. Afterward, a series of concentrations of UDP-[U-^{14}C]-glucose (0.16, 0.32, and 0.48 μM) was added to the reactions. Reactions without added enzymes were used as controls. K_m and V_{max} were determined by Hanes–Woolf mapping based on the concentrations of UDP-[U-^{14}C]-glucose ([S]) and enzymatic reaction rates (V). In the same manner, K_m and V_{max} of β-sitosterol were determined.

3. Results

3.1. Identification of two GhSGT genes

Two UDP-glucose sterol glucosyltransferase genes identified as GhSGT1 and GhSGT2 were submitted to GenBank (Accession Nos. JN004107 and JN004108). GhSGT1 and GhSGT2 encoded the protein, with 625 and 660 amino acids, respectively. Alignment between GhSGTs and the known SGTs was performed using AlignX (Fig. 1). The alignment indicated the presence of conserved putative sterol-binding domains and UGT prosite motifs in all SGT proteins. The UGT prosite motif, a PROSITE glycosyltransferase signature sequence, displayed the signature sequence of glycosyltransferase for secondary metabolites with additional 15 N-terminal amino acids to the PROSITE consensus. The 15 amino acids were defined as the putative secondary plant glycosyltransferase (PSPG) motif. The PSPG sequence composition of SGTs differs significantly from the incorporation of additional residues within the PSPG motif [44]. In addition to the difference in the PSPG motif, the multi-introns in the genome presented characteristics of the SGT gene family. A total of 13 and 14 introns were embedded in the *GhSGT1* and *GhSGT2* genomes, respectively. The major difference between the two genes is the presence of the transmembrane domain in GhSGT2. To test bioinformatics prediction, we performed a subcellular location experiment by observing GFP-GhSGT fusion transient expression in onion epidermal cells. The results showed that GFP and GFP-GhSGT1 signals were distributed throughout the

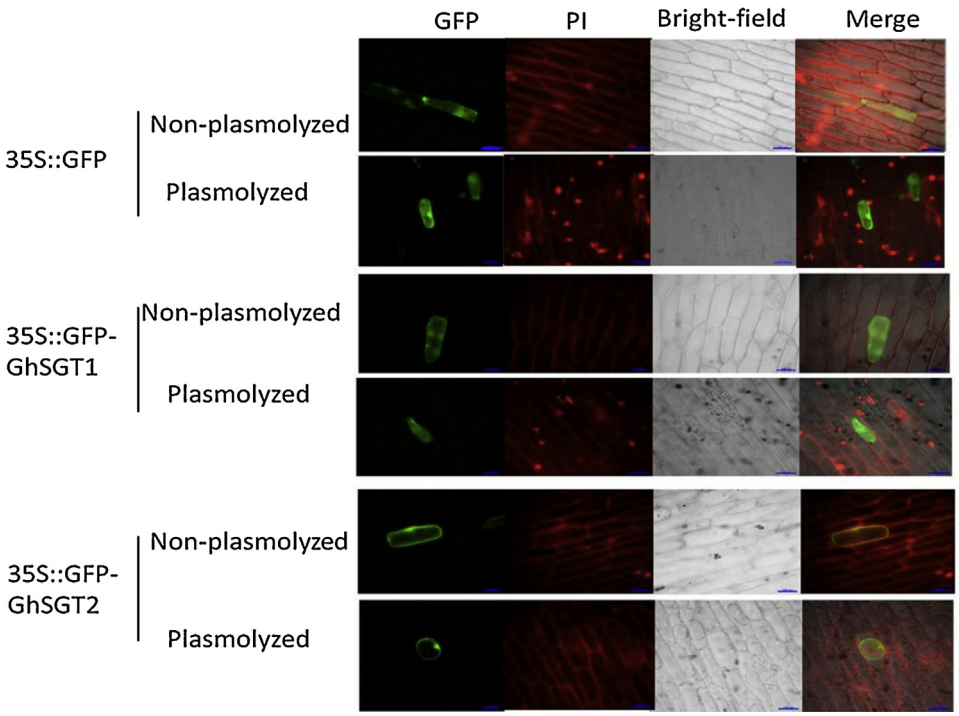

Fig. 2. Subcellular localization of GhSGT fused to GFP on onion epidermal cells. 35S::GFP indicated as empty control. Cells were observed by GFP fluorescence of the GhSGT protein and red propidium iodide fluorescence. Bars represent 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intracellular area of the cells. GFP-GhSGT2 signals were observed in the intracellular area, but stronger GFP-GhSGT2 signals occurred around the plasma membrane. After plasmolysis, the GFP-GhSGT1 signal was much brighter in the protoplast, whereas GFP-GhSGT2 imagine was more concentrated in the plasma membrane (Fig. 2). This finding suggests that GhSGT2 mainly participates in catalyzing glucosylation of membrane sterols.

The glucosyltransferase containing the PSPG motif can catalyze the glucosylation of flavonoids, flavonols, anthocyanins, terponoids, and sterols [45]. Accordingly, we conducted a phylogenetic comparison between GhSGTs and other SGTs (Fig. S1). The data indicated that two GhSGTs belonged to type A enzymes that mainly catalyze sterols other than steroids referred to as steroidal alkaloids and sapogenins, because it has been reported that AtSGT (Z83833, AtUGT80A2) and SGTL1 in type A can glucosylate sterols [19], whereas SaGT6, SaGT4A, and SaGT4R utilize steroidal saponins as substrates [18] and StSGT is able to produce steroidal alkaloid glucosides [17].

3.2. GhSGT activity assay in vitro

SGT genes have been reported in plants, but only a single SGT enzyme activity was found in the following three plant species: *A. thaliana* (Gene code: Z83833) [19], *W. somnifera* (DQ356887) [19], and *Avena sativa* (Z83832) [12]. In the present study, two *GhSGT* genes were overexpressed in *E. coli*, and their corresponding recombinant GST fusion proteins were purified for the enzyme activity assay (Fig. 3A). As GhSGT1, GhSGT2 and GST tag proteins are respectively predicted with the molecular mass at 68.7 kDa, 73.7 kDa and 26.9 kDa, the GST-fusion GhSGT1 and GhSGT2 proteins were detected at their corresponding locations at 95.6 kDa and 100.6 kDa as shown in Fig. 3A. In addition, the recombinant GhSGTs were also verified by incising GST tag (data not shown).

For measurements of enzyme activity of the two GhSGTs, β-sitosterol obtained from a commercial company was used as a common substrate, and the total crude sterol extracted from the plasma membrane of 24 DPA cotton fibers was applied as a natural substrate [32]. The results showed that the two GhSGT enzymes could catalyze UDP-glucose and β-sitosterol or total crude sterols for SG production (Table 1; Figs. 3B and S3). The enzyme activity of GhSGT2 for the total crude sterol substrate was 54.8% higher than that of GhSGT1. However, no significant differences were observed between the two GhSGT enzymes in terms of β-sitosterol substrate. Enzyme kinetics (V_{max}/K_m) data indicated that GhSGT2 exhibited higher activity than GhSGT1 although β-sitosterol was used as the substrate (Table 2).

3.3. GhSGT activity regulation by solvents and metal ions

Based on the distinct activities of the two GhSGTs, we detected the effects of Triton (X-100) on the GhSGT enzyme activity *in vitro* using UDP-glucose and β-sitosterol substrates. The activity of GhSGT1 was strongly inhibited by Triton, whereas that of GhSGT2 was remarkably enhanced to a maximum activity under 8.3 μM Triton (Fig. 4A). The data suggest that the two GhSGTs may bind the sterol substrate differently. The activity of GhSGT2 was slightly reduced upon the addition of β-mercaptoethanol, whereas that of GhSGT1 was enhanced with the addition of 1.4 μM β-mercaptoethanol (Fig. 4B). However, the activities of both GhSGTs were largely inhibited by the addition of 2.8 μM β-mercaptoethanol. The results also indicated that GhSGT1 had higher activity than GhSGT2 at the reduction state when β-mercaptoethanol was used as the classic reduction agent.

Given that enzyme activity can be affected by various metal ions in plants, the activities of the two GhSGTs were also examined *in vitro* using the salts NaCl, $MgCl_2$, and $ZnCl_2$. The activities of both GhSGTs were significantly inhibited after treatment with two $ZnCl_2$ concentrations (2.5 and 5.0 μM) (Fig. 4C). The addition of 2.5 μM $MgCl_2$ increased the activities of both GhSGTs, although the activity GhSGT2 was almost twofold higher than that of GhSGT1 (Fig. 4D). Notably, the two GhSGT enzymes showed opposite responses to 5.0 μM NaCl treatment (Fig. 4E). Thus, GhSGT1 and GhSGT2 have

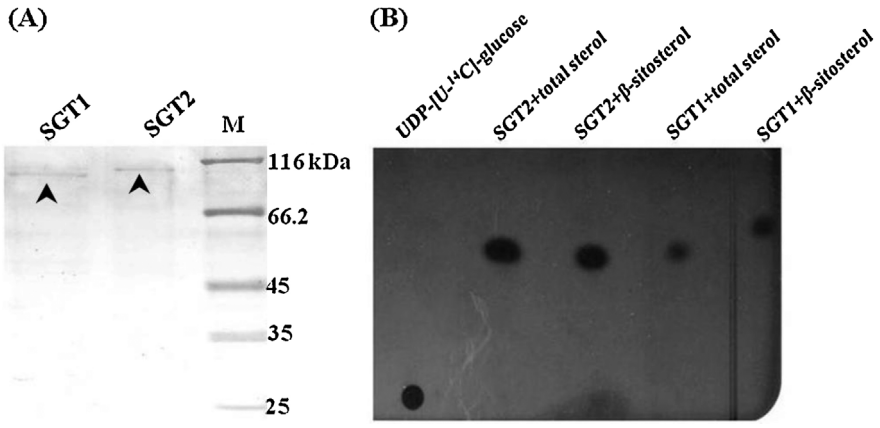

Fig. 3. Detection of two GhSGT activities *in vitro*. (A) SDS-PAGE separation for two recombinant GST-fusion GhSGT proteins. Arrows indicate their corresponding proteins; (B) Analysis of the GhSGT-synthesized product by thin-layer chromatography. Lane 1: UDP-[U-^{14}C]-glucose; lane 2: GhSGT2-synthesized product using total sterol as substrate; lane 3: GhSGT2-synthesized product using pure β-sitosterol as substrate; lane 4: GhSGT1-synthesized product using total sterol; lane 5: GhSGT1-synthesized product using pure β-sitosterol. The band in lane 5 shifted slightly higher than the bands in the other lanes because of faster separation of solvents ran in lane 5.

distinct enzyme activities under various biochemical reaction conditions.

3.4. GhSGT expression and response to heat shock

Cotton fibers have been used as materials in model experiments of cellulose biosynthesis studies, in which SG was reported to have a function [32]. In the present work, the expression levels of the two *GhSGT* genes remarkably increased during the development of cotton fibers (Fig. 5A). This finding is consistent with cellulose biosynthesis in cotton fibers (Fig. S2). Both *GhSGT* genes showed maximum expression levels 24 days after flowering when cellulose synthesis velocity reached the peak [32]. Compared with *GhSGT2*, the expression level of *GhSGT1* was considerably lower in fibers and leaf tissues but slightly higher in root and stem tissues. The obtained results are consistent with SG accumulation and cellulose production in cotton fibers.

The expression of the *SGT* gene in *W. somnifera* (*SGTL1*) is dynamically regulated by heat shock [19]. Thus, we compared the expression levels of the two *GhSGT* genes in cotton seedlings during the course of heat shock treatment at 42 °C. The expression levels of both *GhSGT1* and *GhSGT2* genes initially increased and reached maximum levels after 20 and 40 mins, respectively (Fig. 5B and C). Notably, the expression level of *GhSGT1* was almost 10-fold higher than that of *GhSGT2*. Furthermore, the activities of the two purified recombinant GhSGTs were examined *in vitro* under different heat shock temperatures (Fig. 5D). A significant difference in response patterns to heat shock was found between the two GhSGT enzymes. Moreover, the heat shock stability of GhSGT2 was higher than that of GhSGT1.

4. Discussion

SGs are typical glucolipids found in various organisms [3]. SGs reportedly have primary functions in cellulose biosynthesis in cotton fibers, but their SGTs have not been well characterized [32]. A previous study has reported that the *Arabidopsis* double-SGT mutant does not exhibit considerable cellulose reduction but still contains small amounts of SGs [33]. These results suggest that the double-SGT-mutant may retain weak enzyme activity for the minor

Table 1
Two cotton UDP-glucose sterol glucosyltransferase activities *in vitro*. Reactions were conducted as described in Section 2. Ethyl acetate was used to extract the reaction product, and the cpm value of the extract was converted to mole of incorporated glucoses calculated according to the following equation: 1 mCi = 2,220,000 cpm, and 1 mmol = 302 mCi. All cpm values were calculated after subtraction from the control (background). The concentration of recombinant protein was determined by Bradford method. *All experiments were performed in biological duplicates as means ± SD (n = 3).

Substrate	GhSGT1		GhSGT2	
	Cpm incorporated (10^3 Cpm mg^{-1} protein)	Glucose incorporated (pmol mg^{-1} protein)	Cpm incorporated (10^3 Cpm mg^{-1} protein)	Glucose incorporated (pmol mg^{-1} protein)
β-Sitosterol	47.8 ± 1.9[a]	71.2 ± 2.8	51.6 ± 0.7 (7.6%)	77.0 ± 1.0 (7.8%)[b]
Total sterol	95.8 ± 0.8	143 ± 1	168 ± 2 (47.2%)	251 ± 4 (54.8%)

[a] Means ± SD (n = 3).
[b] GhSGT2 value minus GhSGT1 divided by average of GhSGT2 and GhSGT1.

Table 2
Kinetic comparison of cotton recombinant UDP-glucose sterol glucosyltransferases *in vitro*. K_m and V_{max} assay was described in Section 2, and all experiments were performed in biological duplicates.

Substrates	SGT1			SGT2		
	K_m (μM)	V_{max} (pmol mg^{-1} min^{-1})	V_{max}/K_m	K_m (μM)	V_{max} (pmol mg^{-1} min^{-1})	V_{max}/K_m
UDP-glucose	1.12	2.57	2.30	0.29	1.21	4.17
β-Sitosterol	15.1	0.56	0.04	12.6	0.90	0.07

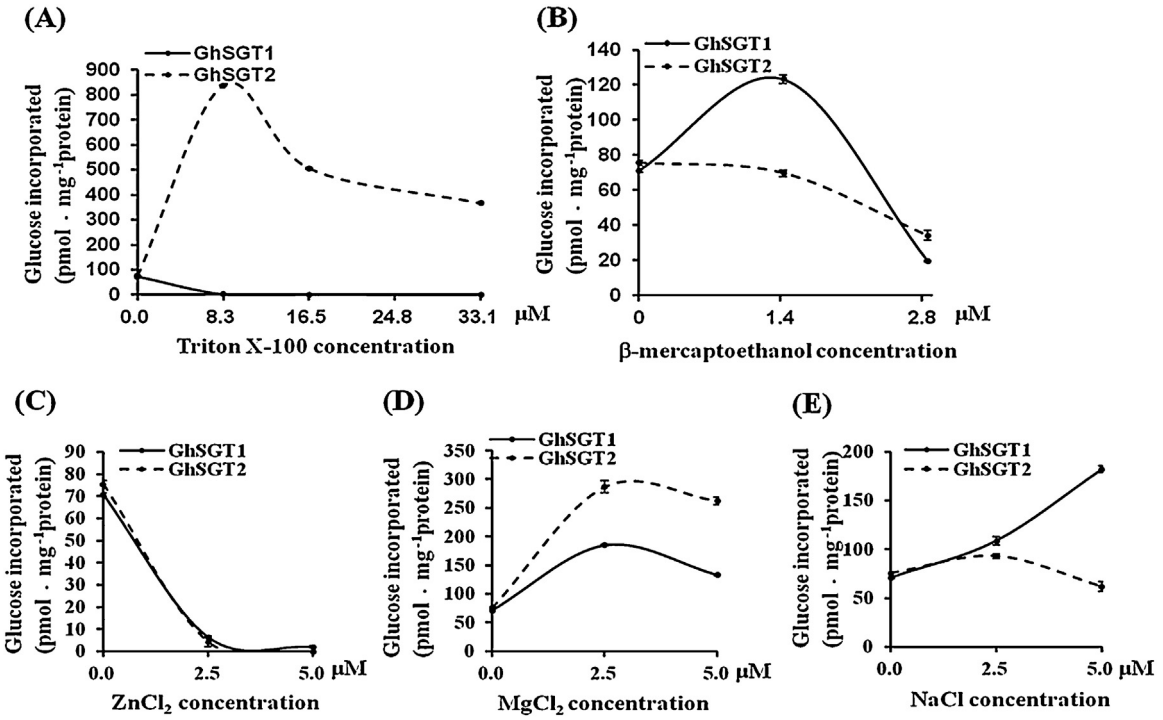

Fig. 4. Detection of two recombinant GhSGT activities *in vitro* supplemented with solvents and metal ions. (A) Triton X-100; (B) β-mercaptoethanol; (C) $ZnCl_2$; (D) $MgCl_2$; (E) NaCl. Each bar represents the mean of three biological duplicates with standard error.

SGs that are sufficient primers for cellulose synthesis in *Arabidopsis*. Thus, characterization of the biochemical properties of SGTs can provide valuable information on the potential functions of SGTs in plants.

In this study, we identified two full-length GhSGT genes in cotton and compared their biochemical activities. Protein-conserved domain analysis and phylogenetic comparison indicated that the two GhSGTs have distinct activities for different sources of sterols as substrates. Due to the transmembrane domain, GhSGT2 may mainly use plasma membrane sterols, whereas GhSGT1 could utilize cytosolic membrane sterols. The above findings were confirmed through the recombinant GhSGT assay *in vitro* using

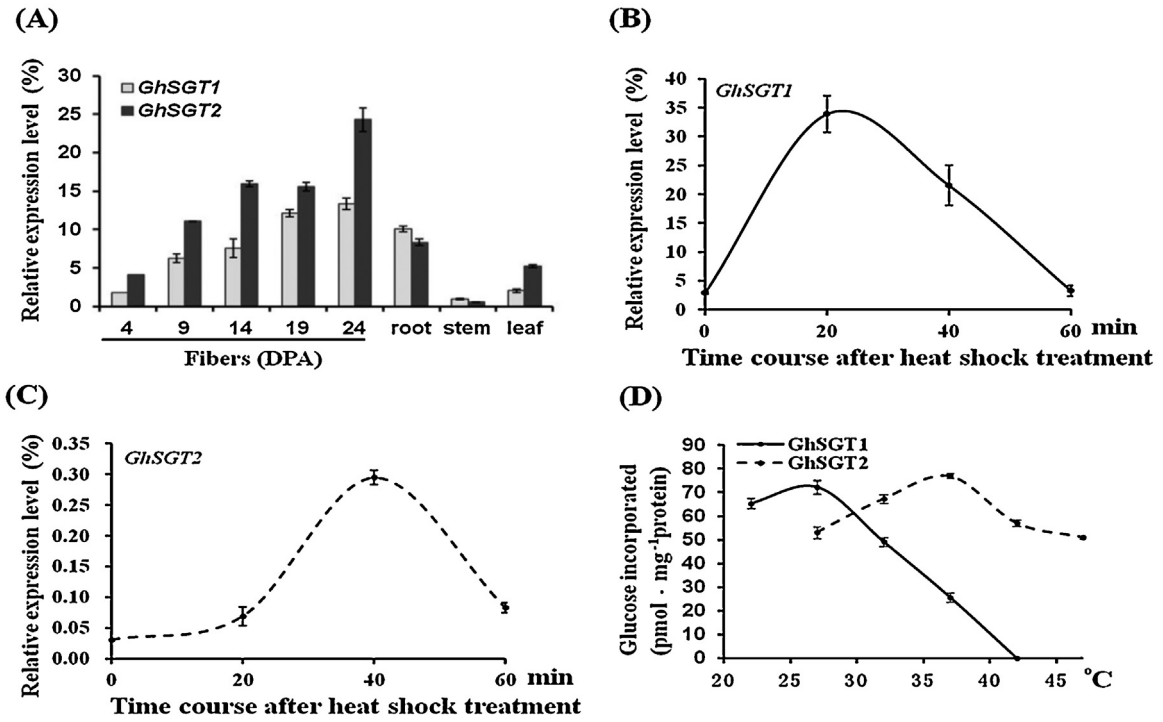

Fig. 5. Two *GhSGT* gene expression levels and GhSGT enzyme responses for heat shock. (A) *GhSGT1* and *GhSGT2* transcript levels during cotton fiber growth and development in three tissues (root, stem, and leaf); (B and C) *GhSGT1* and *GhSGT2* expression responses during the time course of heat shock; (D) effects of different temperatures on two GhSGT recombinant enzyme activities *in vitro*. Each bar represents the mean of three biological duplicates with standard error.

β-sitosterol and total crude membrane-bound sterols of cotton fibers as substrates. Therefore, GhSGT2 has considerably higher biochemical activity than GhSGT1 for total crude membrane-bound sterols (Table 1). Triton X-100, an excellent detergent for membrane protein solubility, strongly enhanced the activity of GhSGT2 but drastically inhibited that of GhSGT1 (Fig. 4A).

Although GhSGT1 and GhSGT2 have been determined with high glucosylation activities for sitosterol-glucoside products in cotton fibers [32], little is known about their ability for other types of sterol substrates. However, since AtUGT80A2 and SGTL1 could glucosylate other sterol substrates [19], it is likely that GhSGT1 and GhSGT2 have the activities similar to AtUGT80A2 and SGTL1 respectively, due to their sharing same conserved domains (Fig. 1). Hence, it remains to test two GhSGT activities for other sterol substrates in the future.

Metal ions are co-factors for high enzyme activity in plants. For example, $MgCl_2$ is added for cellulose (CESA) and callose synthase activity *in vitro* in cotton fibers [32]. In this study, GhSGT2 showed higher activity than GhSGT1 after the addition of $MgCl_2$. By contrast, GhSGT1 exhibited an enhanced activity after the addition of NaCl (Fig. 4D and E). As these two ions (Na^+, Mg^{++}) could play different physiological and chemical roles in plants [46,47], the data were in support of our finding that the two GhSGTs have distinct biochemical properties. In addition, it has been reported that the two *Arabidopsis* AtSGT mutants (Atugt80A2 and ugt80B1) and their double-AtSGT mutant (Atugt80A2, B1) can display a different root growth under various temperatures and a changed neutral sugar level of plant tissues [33].

Notably, GhSGT2 and GhCESA showed high activities *in vitro* after the addition of $MgCl_2$ [32] and were favored under non-reduction reaction conditions (Fig. 4B). In addition, *GhSGT2* displayed higher expression levels than *GhSGT1* during the development of cotton fibers (Fig. 5A). The results suggested that GhSGT2 may have a critical function in cellulose biosynthesis. Nevertheless, we did not find any significant correlation between *GhSGT2* transcript levels and *GhCesAs* expressions during cotton fibers development (data not shown). As SG has been proposed as a primer for initiation of cellulose biosynthesis and it could also be recycled in use by "Korrigan" reaction [32], we presumed that small amount of SG may be enough for cellulose biosynthesis. Hence, *GhSGT* transcript levels should not be correlated with *GhCesAs* expressions. Furthermore, we assumed the other type sterol-glucosides or sterol-like-glucosides may also join as primers for cellulose biosynthesis, which could explain why the double-AtSGT-mutant did not result in cellulose reduction [33]. However, all these hypotheses must be verified by genetic silencing or over-expressing of *GhSGT* genes in cotton fibers and other plants in future studies.

SGT exhibited a dynamic response to heat shock in plants, and a distinct response pattern between the two GhSGTs was observed. The rapid increase in the gene expression of *GhSGT1* under heat shock conditions (Fig. 5B and C) may be caused by its relatively low activity at high temperatures (Fig. 5D). With respect to the stability of GhSGT2 even at high temperatures, its gene expression was extremely low and was considerably later enhanced under heat shock compared with *GhSGT1*. Overall, this study demonstrated that the two GhSGTs have distinct biochemical activities and different response dynamics for heat shock in cotton.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2013.12.013.

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