SCIENCE CHINA
Life Sciences

THEMATIC ISSUE: Cotton genome plus
• RESEARCH PAPER •

February 2016 Vol.59 No.2: 213–218
doi: 10.1007/s11427-016-5004-y

Antisense expression of *Gossypium barbadense UGD6* in *Arabidopsis thaliana* significantly alters cell wall composition

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Received May 14, 2015; accepted July 20, 2015; published online January 22, 2016

Uridine diphosphate-glucose dehydrogenase (UGD, EC1.1.1.22 oxidizes UDP-Glc (UDP-D-glucose) to UDP-GlcA (UDP-D-glucuronate), a critical precursor of cell wall polysaccharides. *GbUGD6* from *Gossypium barbadense* is more highly expressed late in the elongation of cotton fibers (15 d post-anthesis (DPA)) and during the stage of secondary cell wall thickening (30 DPA). Subcellular localization analysis in onion epidermis revealed that fluorescently labeled GbUGD6 protein was distributed throughout the cell membrane, as well as the nucleus and vacuoles. Examination of UGD function in *Arabidopsis* revealed that the antisense *GbUGD6* lines had shorter roots, deferred blossoming, compared to wild-type plants. Activities of associated enzymes were also affected by UGD reduction, and biochemical analysis of cell wall samples showed an increase in cellulose levels and a decrease in UGP-GlcA contents. The results of the present study as well as previous studies on UGD support the conclusion that UGD plays a major role in synthesizing polysaccharides synthesis in the cell wall.

UDP-glucose dehydrogenase, UDP-GlcA, *Gossypium*, fiber

Citation: Han, J., Pan, Y., Wang, X., Zhang, Y., and Ma, Z. (2016). Antisense expression of *Gossypium barbadense UGD6* in *Arabidopsis thaliana* significantly alters cell wall composition. Sci China Life Sci 59, 213−218. doi: 10.1007/s11427-016-5004-y

INTRODUCTION

Uridine diphosphate-glucuronic acid (UDP-GlcA) is an essential precursor of eukaryotic hemicelluloses and pectin matrix polysaccharides (Diet et al., 2006; Karkonen et al., 2005; Malinova et al., 2014; Seifert, 2004) UDP-galacturonic acid (a pectin precursor) and UDP-xylose (precursor of hemicellulose) are formed through catalysis of UDP-GlcA by UDP-d-glucuronic acid 4-epimerase (GAE) and UDP-glucuronic acid decarboxylase (UXS), respectively (Clough and Bent, 1998; Zablackis et al., 1995).

Synthesis of UDP-GlcA involves two pathways: nucleotide sugar oxidation (Kuhn et al., 2010) and myo-inositol oxidation (MIO) (Karkonen et al., 2005); however, it is unclear which is more important. Both utilize the substrate D-glucose-6-P. The nucleotide sugar oxidation (NSO) pathway utilizes three enzymes: phosphoglucomutase (PGM), UDP glucose pyrophosphorylase (UGP), and UDP-glucose dehydrogenase (UGD). The first two catalyze reversible reactions that form UDP-glucose (UDP-Glc), and as a key enzyme, UGD then catalyzes an irreversible reaction that converts UDP-Glc to UDP-GlcA (Diaz-De-Leon et al., 1993). The UGD enzyme has important functions in tobacco (*Nicotiana tabacum*) and *Zea mays*, and its differential expression has been described in *Arabidopsis thaliana*, poplar (*Populus*), soybean (*Glycine max*), and *Phaseolus vulgaris* (Bindschedler et al., 2005; Johansson et al., 2002; Karkonen et al., 2005; Klinghammer and Tenhaken, 2007; Robertson et al., 1996; Tenhaken and Thulke, 1996). In those cases, UGD expression is higher in

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young tissues and is also induced by biotic stress. In particular, down-regulation of UGD-A reduces pentose contents in the cell walls (Klinghammer and Tenhaken, 2007), which suggests that UGD has a critical role in the biosynthesis of UDP-GlcA and precursors of other cell wall polysaccharides.

In comparison, the MIO pathway involves five enzymatic steps that are catalyzed consecutively by myo-inositol phosphate synthase (MIPS; EC 5.5.1.4), myo-inositol monophosphatase (IMP; EC 3.1.3.25) and inositol oxygenase (MIOX; EC 1.13.99.1), glucuronokinase (EC 2.7.1.43), and UDP-glucuronic acid pyrophosphatase (EC 2.7.7.44). MIOX is the key enzyme in that pathway.

As valuable crops worldwide, various types of cotton (*Gossypium* spp.) have highly desirable fiber characteristics that are determined by matrix polysaccharides cross-linked by cellulose, hemicelluloses, and pectin. To improve their quality and yield, however, researchers must understand the mechanism by which those polysaccharides are formed. We previously used cDNA-AFLP technology to isolate a UGD fragment from *G. barbadense* (Pan, 2007). In the study described here, we cloned a full-length *GbUGD6* gene that is preferentially expressed during the stages of late cotton-fiber elongation and secondary cell wall thickening. We then examined its functioning in *Arabidopsis thaliana,* and found that antisense expression of *GbUGD6* changed the functioning of the above pathways, suggesting that the gene modulates the biosynthesis of cotton fibers.

RESULTS

Features of GbUGD6 cDNA

The reconstituted cDNA of *GbUGD6* was 1,912 bp long, including a 1,443-bp open reading frame (ORF), a 168-bp 5′-untranslated region (UTR), and a 301-bp 3′-UTR. The ORF was determined to encode a peptide of 480 amino acid residues (Figure S1A in Supporting Information), and the deduced amino acid sequence was highly homologous to *A. thaliana* UGD catalytic subunits and differed from a previously cloned UGD (Figure S1 in Supporting Information) (Pang et al., 2010).

Subcellular location of GFP-fused GbUGD6

Although green fluorescent protein (GFP) signals from the empty vector control were detected in the cytomembrane and nucleus, pCamE- GbUGD6::GFP expression was observed in the cell membrane system, including the plasma membrane, nucleus, and vacuoles (Figure 1).

mRNA expression profile for GbUGD6 in cotton fibers

Results of our quantitative RT-PCR revealed similar *GbUGD6* expression profiles for *G. hirsutum* (“CCRI 8”) and *G. barbadense* (“Pima90-53”). In particular, transcripts accumulated sharply at 15 DPA, and then maintained a high level until 30 DPA (Figure S2 in Supporting Information). These patterns indicated that *GbUGD6* expressed dramaticlly elongation of cotton fibers and during the stage of secondary cell wall thickening.

Phenotypic alterations in transgenic *Arabidopsis*

Phenotypic differences were obvious for growth habit and morphology between wild-type (WT) plants and antisense transgenic lines. After 7 d of development, roots of *Arabidopsis* plants from antisense transgenic lines were shorter than those of the WT (Figure 2). The WT plants began

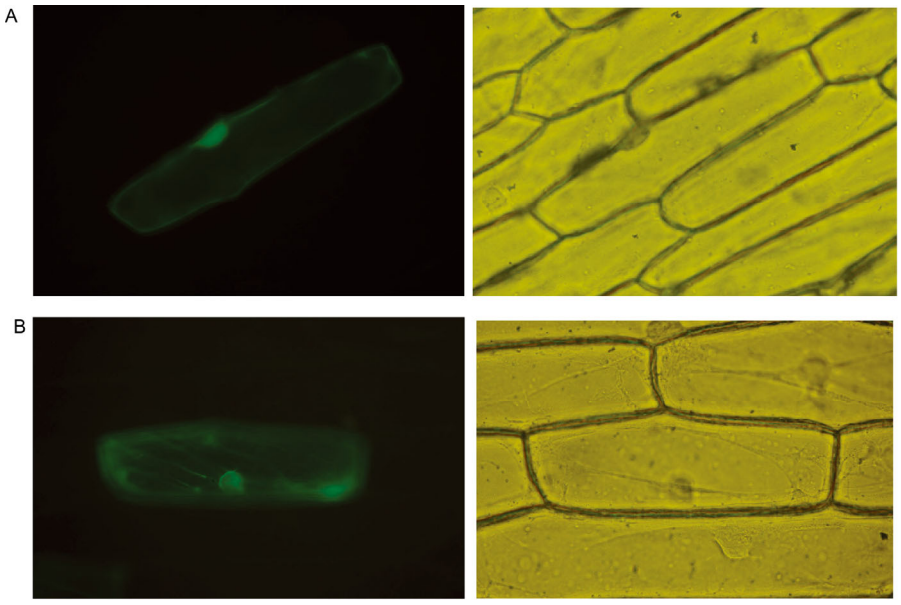

Figure 1 Subcellular localization of GbUGD6 fused with GFP in onion cells. A, Images of GFP in cells under confocal microscope (magnify 20×) (left, green fluorescence image; right, bright field image). B, Images of pCamE-UGD6::GFP expression vector in onion epidermal cells (magnify 20×) (left, green fluorescence image; right, bright field image).

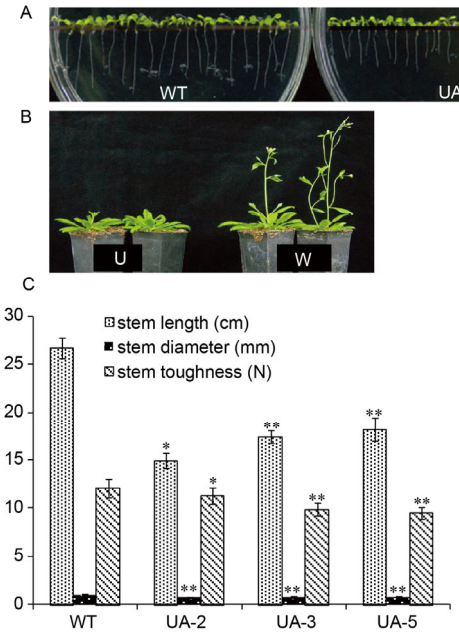

Figure 2 Phenotypes for wild-type (WT) and T3 transgenic (UA, GbUGD6-antisense) plants of *Arabidopsis*. A, Seedling roots from one week old seedlings. B, Shoot growth from 6-week-old seedlings. C, Phenotypic characters for wild-type (WT) and T3 transgenic (UA, GbUGD6-antisense) plants of *Arabidopsis*. **, values are significantly different at $P<0.01$.

blooming after only 35 d in the light chamber, while the antisense transgenic lines remained in their vegetative phase.

After 42 d of culturing, floral stems from the antisense transgenic lines were 37% shorter, had diameters that were 28% smaller, and had toughness values that were 15% lower when compared with WT plants

GbUGD6 enzymatic activities and qRT-PCR results for selected fiber-related genes

qRT-PCR profiles for *GbUGD6* showed that transcript levels wereabout 50% lower in the antisense transgenic lines than in the WT (Figure 3A). Our assays indicated that endogenous UGD activity was 30% to 50% lower in the antisense transgenic lines than in the WT (Figure 3B). The transgenic lines had higher cellulose contents but lower levels of UGP-GlcA than the WT (Figure 3C). For the NSO pathway genes *AtUGP1*, *AtUGP2*, and *AtPGM2,* expression was greatly reduced in the antisense transgenic lines. However, the MIO pathway genes *AtMIOX4*, *AtMIPS1*, and *AtIMP* exhibited increased expression in the antisense transgenic plants. The UGD downstream gene *AtUXS* was decreased, while *AtGAE* was increased (Figure 3D). These findings suggested that *GbUGD6* has a role in regulating

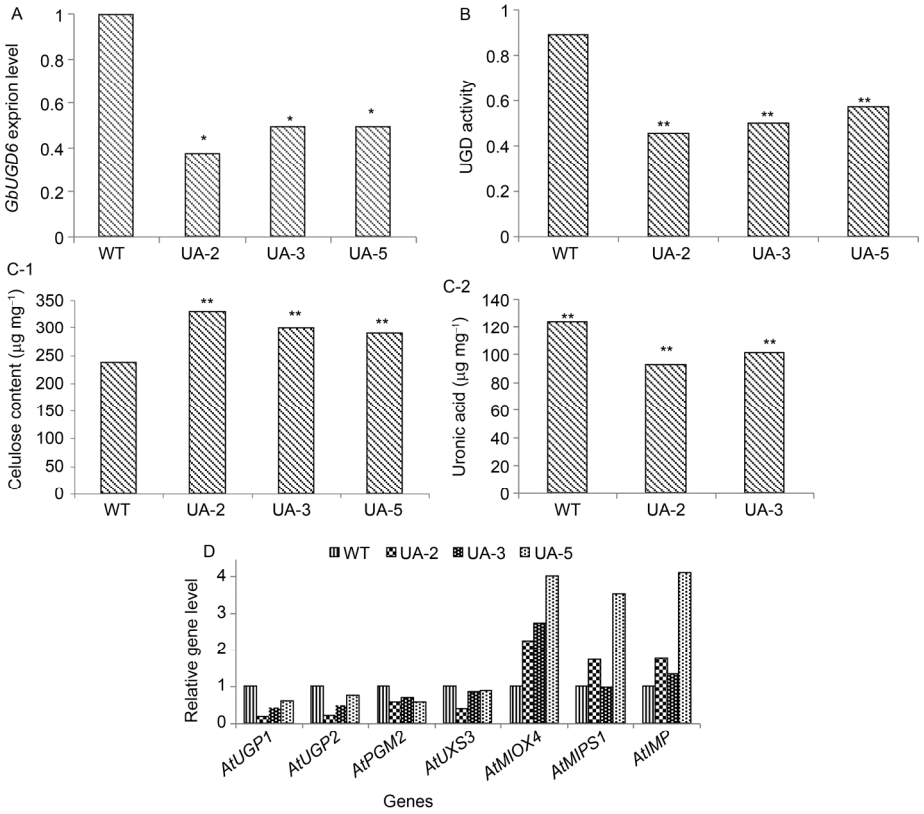

Figure 3 Characterization of antisense *Arabidopsis* plants with lower *UGD6* expression**.** A, Results from real-time PCR analysis showing relative expression level for GbUGD6 in wild-type (WT) and GbUGD6-antisense (UA) plants of *Arabidopsis*. **, significantly different at $P<0.01$. B, Analysis of endogenous UGD activity in wild-type (WT) and GbUGD6-antisense (UA) plants of *Arabidopsis*. **, significantly different at $P<0.01$. C, Contents of cellulose (C-1) and UDP-GlcA (C-2) in cell walls from wild-type (WT) and GbUGD6-antisense (UA) plants of *Arabidopsis*. D, Results from real-time PCR analysis for transcripts of genes related to genes sugar metabolism in wild-type (WT) and GbUGD6-antisense (UA) plants of *Arabidopsis*. **, significantly different at $P<0.01$.

the synthesis of polysaccharides.

DISCUSSION

UGD is the key enzyme for generating UDP-GlcA, the primary precursor of hemicelluloses and pectin polysaccharide in plant cell walls. Its functions have been investigated in many plants, including soybean, poplar, tobacco, and *Arabidopsis*. In the current study, we examined the UGD from *Gossypium barbadense* and confirmed that the enzyme participates in pathways that are involved in polysaccharide synthesis, as has been found for UGDs from other species.

Expression of *GbUGD6* was detected in several stages of cotton fiber development, especially during the formation of primary and secondary cell walls. Our observations support previous reports that have implicated *UGD1* in cotton fiber elongation (Pang et al., 2010) and clearly demonstrate that *GbUGD6* is involved in the pathways of polysaccharide synthesis in cotton. The functions of *GbUGD6* were further verified using antisense transgenic *Arabidopsis* plants. We noted that plants from *GbUGD6* antisense transgenic lines had defective growth, which is in agreement with earlier studies that have shown that an *Arabidopsis* mutant (*ugd2,3*) that lacks two of four UGD isoforms has a strong dwarfed phenotype and severe root defects (Reboul et al., 2011). The results from our expression analysis and examination of cell wall components also provide evidence that UGDs with different plant origins can have similar effects on phenotype.

GbUGD6 is critical for function of the NSO and MIO pathways, and consequently, we observed dwarfing and reduced expression of *AtUGP* and *AtPGM2* in our antisense transgenic lines. Similar to the dwarfism observed in our transgenic lines, a decrease in PGM activity can negatively influence plant growth rates and carbohydrate partitioning, and overexpression of *G. hirsutum* or *Larix gmelinii* UGPs in *Arabidopsis* has been shown to enhance vegetative growth and cellulose biosynthesis (Li et al., 2014; Malinova et al., 2014; Wang et al., 2011).

Furthermore, lines, expression of *AtMIOX*, *AtMIPS*, and *AtIMP* was up-regulated in the MIO pathway, in accord with previous studies on *MIOX* induction (Reboul et al., 2011). Our findings demonstrate that *GbUGD6* expression is able to alter the MIO pathway and reduce the amount of UDP-GlcA that can be synthesized from UGD. When that pathway is limited, then NSO may become the key pathway for forming UDP-GlcA. This is consistent with a previous report by Reboul et al. (Reboul et al., 2011). As the common precursor for synthesizing cell wall polysaccharides, such as pectin and hemicellulose, reduced levels of UDP-GlcAwould result in a decrease in cellulose contents in transgenic seedlings.

In *Arabidopsis* mutants, down-regulation of *GbUGD6* led to a decline in the expression of *AtUXS*, a gene downstream of *UGD*. Microarray analysis has shown that transcripts of *UGD1* and *UXS* are significantly reduced during the elongation stage in *G. hirsutum* Li2 fibers (Naoumkina et al., 2013). Moreover, *UGP*, *UXS*, and *UGD* are down-regulated during that stage in the *fl* mutant of the same species (Padmalatha et al., 2012).

In summary, our results indicate that the cotton gene, *GbUGD6*, is involved in polysaccharide and cell wall biosynthesis. Therefore, this gene may represent a valuable target for schemes to improve the cellular development of cotton fibers. Whole genome sequencing of cotton provides a chance for fiber development research (Cao, 2015). So the UGD function will be more and more clear.

MATERIALS AND METHODS

Plant materials

Plants of *Gossypium hirsutum* "CCRI 8" and *G. barbadense* "Pima90-53" were grown in an experimental field in Baoding, China. Ovules and epidermal fibers were collected at 0, 5, 10, 15, 20, 25, 30, 35, and 40 DPA, respectively, and were immediately immersed in liquid nitrogen.

Leaf, stem and root tissues *Arabidopsis* ("Columbia" ecotype, or "Col-O") seedlings were sampled from light chamber-grown (16 h light period, 23°C; 8 h dark period, 22°C; 70% relative humidity) and placed in liquid nitrogen.

Total RNAs were isolated from the ovules and fibers of both cotton cultivars with RNAplant Kits (TIANGEN, China), while RNA was isolated from *Arabidopsis* tissues with a TransZol Kit (TransGen Biotech, China). Purified RNA was treated with DNase I (Promega, USA), according to the manufacturer's instructions. Then, cDNA was synthesized using an M-MLV Kit (TaKaRa, China).

Subcellular localization of GhUGD and GFP fusion proteins expressed in onion epidermal cells

In order to test the subcellular localization of *GbUGD6,* the coding region of *GbUGD6* was inserted into the pCamE: GFP expression vector to generate a pCamE-GbUGD6:: GFP fusion protein with green fluorescent protein (GFP). The correctly sized recombinant pCamE-GbUGD6::GFP plasmid and a control pCamE-GFP plasmid were transferred into living onion epidermal cells with a gene gun (PDS-1000/He Particle Delivery System; Bio-Rad, USA), according to the manufacturer's instructions. The transformed cells were incubated on Murashige and Skoog (MS) agar plates in the dark at 25°C for 48 h before being observed under a fluorescence microscope (BX51; Olympus, Japan) to detect GFP signals.

Real-time PCR of GhUGD in cotton fiber cDNA

Real-time quantitative PCR was performed with a Roche LightCycler 1.5 (Germany) in order to compare the *GbUGD6* expression characters of *G. hirsutum* "CCRI 8" and *G. barbadense* "Pima90-53" fibers. Cotton *EF1α*

(GenBank Accession No. DQ174251.1) was amplified as the reference gene and 2×SYBR® Premix Ex Taq™ II (Perfect Real Time; TaKaRa, China) was used. No-template controls were also used for each primer pair. Each sample had three replicates. Data were examined by the $2^{-\Delta\Delta C_T}$ value method.

The PCR mixture contained 1 μL of diluted cDNA, 10 μL of 2×SYBR® Premix Ex Taq™ II (Perfect Real Time; TaKaRa, China), and 20 μmol L^{-1} of each gene-specific primer in a final volume of 20 μL. Conditions for all PCR procedures included 40 cycles of 10 s at 95°C, 10 s at 60°C, and 20 s at 72°C.

Construction of antisense vectors and *Arabidopsis* transformation

To construct our antisense pBI121*p35S*::*AUGD6* expression vector, we amplified the entire coding region of *GbUGD6* via PCR. After ligation into the vector pBI121, the integrated constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 and transferred into WT control plants of Col-O *Arabidopsis* by the floral dip method (Clough and Bent, 1998).

Transgenic plants were selected on a one-half MS medium containing 100 mg L^{-1} kanamycin and were confirmed by PCR. Positive transformants were transferred to soil and allowed to set seed. The T_3 generation of those transgenic plants was used for further investigations.

Real-time PCR analysis and phenotypic examinations of transgenic Arabidopsis plants

Seeds of positive T_3 *Arabidopsis* transgenic lines, along with the WT controls, were germinated on plates containing non-selective 1/2 MS medium. Roots were measured when seedlings were one week old, and phenotypic characteristics, UGD activity, and cellulose and uronic acid content were also recorded after six weeks of development. Cell wall samples were obtained from WT and transgenic *Arabidopsis* whole plants according to the method of Diet et al (Diet et al., 2006). The tissues were homogenized in liquid nitrogen, extracted in a mixture of 70% ethanol and chloroform/methanol (1/1, v/v), and then washed with 100% acetone. The starch was hydrolyzed with α-amylase and DMSO, and the samples were vacuum-dried.

Total cellulose was extracted from the cell walls as described previously (Updegraff, 1969). The levels of UGP-GlcA were quantified by a slightly modified meta-hydroxydiphenyl method (Bindschedler et al., 2005).

The UGD enzyme was extracted from 42-day-old seedlings of *Arabidopsis*. Activity by UGD was assayed as described by Robertson et al. (Robertson et al., 1996).

In addition, the expression of various genes in the NSO and MIO pathways were examined in antisense *Arabidopsis*. RT-PCR was monitored for *AtUXS3* (At5g59290), *AtUGP1* (At5g17310), *AtUGP2* (At3g03250), *AtPGM2* (At1g23190), *AtMIOX4* (At4g262600), *AtMIPS1* (At4g-39800), *AtIMP* (At3g02870), and *AtGAE6* (At3g23820), and *Arabidopsis Ubiquitin5* was amplified as our reference gene. All primers are listed in Table S1.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

Acknowledgements *This research was supported by the Ministry of Agriculture of China (2014ZX08009-003), the Hebei Province Technology Support Program (14962905D), and the Hebei Province Department of Education Fund (Y2012025).*

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SUPPORTING INFORMATION

Figure S1 Nucleotide and deduced amino acid sequences of GbUGD6, with domain underlined and alignment of UGD domain from GbUGD6 with that from other closely related proteins

Figure S2 Patterns of relative expression by *UGD6* over time in stem fibers from *Gossypium hirsutum* cv. CCRI 8 and *G. barbadense* cv. Pima90-53. DPA, days post-anthesis

Table S1 Primers used in RT-PCR analysis of transgenic *Arabidopsis*.

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