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ORIGINAL PAPER

Identification of a UDP-glucose pyrophosphorylase from cotton (*Gossypium hirsutum* L.) involved in cellulose biosynthesis in *Arabidopsis thaliana*

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Abstract UDP-glucose pyrophosphorylase is an important regulatory enzyme for the development of plants and a critical enzyme in synthesis of glycogen. Here, we reported the cloning of a full-length *UGP* cDNA from cotton, named *GhUGP*. Real-time PCR analysis indicated the *GhUGP* expression in root, stem, leaf and flower of cotton, with a higher level in flower and root. The transcription level of *GhUGP* depended on sucrose and light in short time and increased under low temperature, but decreased in O_2 deficiency. Interestingly, the expression of *GhUGP* was significantly up-regulated after ethylene induction in cotton ovules. The over-expression of the *GhUGP* in Arabidopsis showed discrepant phenotype: increase in height and growth rate when compared with control lines. What is more, the transgenic Arabidopsis had increased contents of soluble sugars, starch and cellulose, but not in lignin content. Collectively, these results indicate that cotton UGPase participates in sucrose/polysaccharides metabolism and cell wall biosynthesis and provide theoretical deduction supporting *GhUGP* as a good candidate gene for improving the development of cotton fibers cell.

Keywords UDP-glucose pyrophosphorylase · *GhUGP* · Cotton · *Arabidopsis thaliana* · Cellulose

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Abbreviations

UDP	Uridine diphosphate
UGPase	UDP-glucose pyrophosphorylase
UDPG	Uridine diphosphate glucose
UTP	Uridine triphosphate
Glc-1-P	Glucose 1-phosphate
PPi	Pyrophosphate
SPS	Sucrose phosphate synthase
SS	Sucrose synthase
AGPase	ADP-glucose pyrophosphorylase
ADP	Adenosine diphosphate
SuSy	Sucrose synthase
NADP	Nicotinamide adenine dinucleotide phosphate
HXK	Hexokinase
OKA	Okadaic acid
USPase	UDP-sugar pyrophosphorylase
NB	Nucleotide binding

Introduction

UDP-glucose pyrophosphorylase (UGPase) is an important regulatory enzyme, which could catalyze the glucose-1-phosphate (Glc-1-P) reaction with uridine triphosphate (UTP) to form uridine diphosphate glucose (UDPG) and pyrophosphate (PPi), and catalyze this reaction to the reversible direction. UDPG, the substrate/product of the enzyme, is the key precursor for synthesizing sucrose, mannose, cellulose, cutin, glucoprotein, glycolipid and carbohydrate (Kleczkowski et al. 2004). In source tissues, UGPase mainly participates in the reaction of synthesizing sucrose, by producing UDPG for sucrose phosphate synthase (SPS) or sucrose synthase (SS); in sink tissues, UGPase is involved in sucrose degradation and converts

UDPG into Glc-1-P. The Glc-1-P produced from UGPase reversible reaction can either be catabolized through glycolysis pathway or converted into ADP-glucose (ADPG) by ADP-glucose pyrophosphorylase (AGPase) and finally stored as starch (Kleczkowski 1994).

So far, *UGP* cDNAs have been isolated from various plants, such as potato (Katsube et al. 1990), barley (Eimert et al. 1996), banana (Pua et al. 2000), *Astragalus membranaceus* (Wu et al. 2002), rice (Abe et al. 2002; Chen et al. 2007), melon (Dai et al. 2006), aspen (Meng et al. 2007) and bamboo (Weng et al. 2009). In barley, only one type of *UGP* transcript was found in leaves, embryos and endosperm (Eimert et al. 1996). In potato, two small differences in *UGP* cDNAs have been explained as resulting from allelic polymorphism (Sowokinos et al. 1997). Consequently, UGPase had been thought represented by a single *UGP* gene in plants. However, evidently, there are two highly homologous *UGP* genes belonging to *UGP-A* class and have been found in Arabidopsis, rice, aspen, Japanese pear and slime mold (Kleczkowski et al. 2004; Kiyosumi et al. 2002). Recently, Chang et al. (2005) and Okazaki et al. (2009) reported a distinct *UGP-B* class gene from *Chlamydomonas* and *Arabidopsis*, respectively. The UGP-B proteins have not much or no homology with UGP-A, but show the same catalytic function (Kleczkowski et al. 2010). These suggested that plants generally have two *UGP-A* gene to encode UGP-A protein and a single gene encoding UGP-B protein (Kleczkowski et al. 2010).

Plant UGPase plays key roles in carbohydrate metabolism on plant growth and secondary cell wall biosynthesis. Spychalla et al. (1994) and Borovkov et al. (1996) found sucrose content in transgenic potato tuber had a significant decrease along with a 30–50% reduction of UGPase activity. The over-expression of *UGP* from *Acetobacter xylinum* in tobacco and hybrid poplar has shown significant changes in phenotype and carbohydrate content (Coleman et al. 2006, 2007). In rice, *OsUGP1* and *OsUGP2* played critical roles in plant growth and reproduction (Chen et al. 2007; Woo et al. 2008; Mu et al. 2009). Although double mutant Arabidopsis still maintain normal growth and development, the UGPase activity was reduced by 85%, and resulted in a significant decrease in output under field conditions (Meng et al. 2009b). It suggested that UGPase is not a rate-limiting enzyme in carbohydrate synthesis pathway, but still is essential for development of Arabidopsis plant. In contrast, a double knockout mutant analysis of *AtUGP1* and *AtUGP2* demonstrated plant growth defect and male sterility and indicated that *UGP* genes are critical factors for carbohydrate metabolism in both vegetative and reproductive phases (Park et al. 2010). However, not much is known about the effect of *UGP* gene from cotton on plant growth, in this paper, the role of *UGP* gene from cotton will be further studied by transgenic Arabidopsis.

Cotton is one of the most important and widely grown agricultural and industrial crops and cotton textile industries have become central factor to the economic growth of both developed and developing countries. Cotton fibers, the most prevalent natural raw materials, already been became the research focus. Cellulose deposition is the main factor in secondary cell wall biosynthesis, and hence it is essential for improving the quantity and quality of cotton fibers. Cellulose is formed from UDPG which can be synthesized by three enzymes, UGPase, UDP-sugar pyrophosphorylase (USPase) and sucrose synthase (SuSy). To synthesize UDPG, UGPase and USPase use monosaccharide-1-phosphates as substrate (Kotake et al. 2004), whereas SuSy catalyzes sucrose cleavage and provides UDPG directly to cellulose synthase complex associated with the plasmalemma (Amor et al. 1995). Evidently, the expression of *UGP* in poplar has elucidated the role of UGPase in late cell expansion and secondary cell wall formation (Hertzberg et al. 2001; Coleman et al. 2007). These findings support the putative relationship between UGPase and cellulose biosynthesis. UGPase activity has also been positively correlated with the development of cotton fiber cells. Hence, cloning *UGP* gene from cotton and further studying its function might add a new insight to improve the quality of cotton fibers and cellulose biosynthesis. However, the complete cDNA of cotton UGPase, the basic character in the expression of mRNA under different stresses and function have not been reported. In this paper, we successfully cloned a novel *UGP* from cotton, studied on the variation of *UGP* expression in investigated cotton tissues and under different stresses and evaluated its critical role of carbohydrate metabolism when over-expression in Arabidopsis.

Materials and methods

Plant materials and treatments

Cotton seeds (yu 2067) were provided by Cotton Research Institute, Chinese Academy of Agricultural Sciences. Bigplump seeds were selected, delineated with concentrated sulfuric acid, rinsed three times by sterile distilled water, and then put on moist sterile filter paper overnight at 25°C. After seeds germination, they were planted into the mixture of vermiculite and nutrient soil (1:2, w/w) and cultured in growth chamber under the photoperiod of 16 h/8 h with the light intensity of 150 μmol/m^2/s and the day/night temperature of 25°C/22°C.

Arabidopsis thaliana, ecotype Columbia, was provided by the Laboratory of Molecular Biology of China Agricultural University. Seeds were sterilized by 75% ethanol and NaClO solution, and then sowed on MS culture medium

(1× MS salts, 1× MS vitamins, 3% sucrose, 1% agar, pH 5.7). After 4°C vernalization for 3 days, the seeds were cultured in chamber under the photoperiod of 16 h light/8 h dark, at 22 and 18°C, respectively, and maintaining the humidity at 80%. After 10 days, seedlings of *Arabidopsis thaliana* were transplanted into pots with mixture of soil and vermiculite (1:1, w/w) under the same condition.

For sucrose feeding experiments, leaves were detached from intact cotton plants (plants were pre-adapted to dark for 5–6 h) and placed into tubes and fed in different concentrations of sucrose solutions for 12 h. The submerged parts of the leaf petioles were removed from samples before leaves were frozen with liquid nitrogen and stored at −80°C. For light exposure, the leaves were put into the 150 mM sucrose solution and treated in dark and light for 8 h, respectively, saved at −80°C. Under the low temperature, the seedlings were placed in growth chamber at 4°C in normal light intensity and collected at different time intervals. For the hypoxia treatment, seedlings of cotton were immerged into water (flooding) for 1–3 days, sampled at different times and stored at −80°C. For ethylene induction, cotton ovules were aseptically removed from ovaries 24 h after anthesis and floated on the surface of liquid medium according to the method of Beasley and Ting (1973). 0.1 μM ethylene was mixed in the liquid medium and cultured at 37°C, samples were collected at 1, 3, 6, 12 h and stored at −80°C.

Cloning of *UGP* cDNA

Total RNA of cotton plants was isolated by the extraction kit (Promega, WI, USA). Poly (A+) was separated with a PolyATract mRNA Isolation System according to the specifications of the supplier (Promega, WI, USA). A cDNA library was prepared by inserting fragments in a λZAP-II vector as the specifications of cDNA Library Construction Kit (Merck, Germany). The library was cultured on the 140-mm diameter plate and obtained about 10^5 plaques of cDNA library. A potato cDNA clone, pTUP (Katsube et al. 1991), which was prepared ^{32}P-dUTP, was used as probe. Screening of positive plaques was carried out by the method of colony in situ hybridization. After the third screening, we obtained three positive plaques. λZAP-cDNA clones were sub-cloned to the pBlueScript II SK (+) through in vivo excision according to the instructions of the kit.

Sequence and phylogenetic analysis

DNA sequencing was performed by Sino Geno Max Corporation (Beijing, China) and analyzed using DNAclub and DNAMAN software. The alignment of cDNA and amino acids was carried out by BLAST in NCBI. Phylogenetic analysis was performed with the mature protein sequence using Clustal X2 and MEGA 4 program.

Real-time PCR

Total RNA was isolated from cotton tissues after different treatments according to the manufacturer's instructions, as mentioned previously. 2-μg total RNA was reverse transcribed into first-strand cDNA with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, CA, USA) according to the specifications. The cDNA diluted five times was used as the template in each well for the real-time PCR analysis. cDNA was amplified by Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) in an ABI7500 thermocycler (Applied Biosystems, CA, USA) as the following conditions: 95°C denaturation for 10 min, followed by 40 cycles of 95°C for 15 s, 57°C for 20 s, 72°C for 30 s. The *GhUGP* gene was detected by the particular primers GhUGP-F (5′-CTTCCAGTGAAGGC AACTTCAG-3′) and GhUGP-R (5′-CCTTAAGGCTAT CAAGCCCAATG-3′), and the length of DNA was 202 bp. The endogenous control was *UBQ7* gene (DQ116441) from cotton, which was 198 bp in length, and can be detected using the sense primer UBQ-F (5′-GAAGGCA TTCCACCTGACCAAC-3′) and antisense primer UBQ-R (5′-CTTGACCTTCTTCTTCTTGTGCTTG-3′). Before proceeding with real-time PCR, we routinely verified that the primers of *GhUGP* and *UBQ7* gene had a similar slop with high correlation coefficients by constructing standard curve ($R^2 = 0.93$, $R^2 = 0.94$, respectively). The threshold cycle (CT) values of the triplicate real-time PCRs were averaged and the fold changes of transcription levels of target gene (*GhUGP*) relative to the reference gene (*UBQ7*) was analyzed by the comparative CT ($2^{-\Delta\Delta CT}$) method, where $\Delta\Delta C_T = (C_T \text{ target} - C_T \text{ reference})_{\text{Sample X}} - (C_T \text{ target} - C_T \text{ reference})_{\text{Sample 1}}$. Sample 1 of *GhUGP* gene was calibrator sample without any treatments, whereas sample X was treated by different stresses. All experiments were repeated three times for cDNA prepared from three batches of plants. Statistical analysis of real-time PCR date and SD (standard deviation) values were performed as previously described by Livak et al. (2001).

Plasmid construction

The ORF of *GhUGP* was amplified with sense primer (5′-*ACAA*AGATCTTATGGAAAAGCTGGAACACCTC-3′) and antisense primer (5′-*AACA*ACTAGTGATGTCTTC GGGGCCATTAAT-3′) (restriction endonuclease *Bgl*II and *Spe*I were underlined). The PCR amplified product was digested by *Bgl*II and *Spe*I, and inserted into a binary

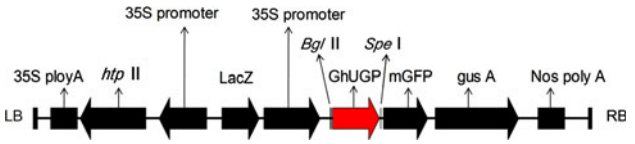

Fig. 1 Outline of the pCAMBIA 1304 transformation vector with GhUGP under the control of CaMV 35S promoter

vector- pCAMBIA 1304 containing a hygromycin phosphotransferase (*hph*) gene, a fusion of *β*-glucuronidase (GUS) and green fluorescent protein (GFP) gene under the control of CaMV35S promoter (Fig. 1). Sequence analysis confirmed the proper insertion of *UGP* gene into the vector.

Plant transformation of Arabidopsis and screening

The constructed vector with *GhUGP* was introduced into *Agrobacterium tumefaciens* strain GV3101 by freeze–thaw method. *Arabidopsis* plants were transformed by the floral dip method via *Agrobacterium*-mediated transformation procedure (Clough and Bent 1998). Transgenic lines were obtained by screening on MS culture medium with hygromycin (25 mg/L) and confirmed by PCR technique. The PCR was performed using vector-specific primers: 1304-F (5′-GAC CCTTCCTCTATATAAG-3′) and 1304-R (5′-GGACAA CTCCATGAAAAG-3′) as the following conditions: 95°C denaturation for 10 min, 33 cycles of 95°C for 1 min, 52°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Chose the stable heritable transgenic Arabidopsis lines (T2) to detect *GhUGP* expression at translation levels.

Carbohydrate analysis

Sucrose, glucose, fructose, and starch were measured in the leaves of 40-day-old Arabidopsis plants exposed 3 h after the beginning of light period. The samples were ground into powder and extracted twice with 80% ethanol at 80°C for 30 min, further washed with 50% ethanol at 80°C for 30 min. After centrifugation (24,000*g*, 30 min, 4°C), sucrose, glucose and fructose in the supernatant were measured, respectively, and enzymatically to determine the reduction of nicotinamide adenine dinucleotide phosphate (NADP) in absorbance at 340 nm after adding successively the coupling enzymes glucose-6-P-dehydrogenase, hexokinase (HXK), phosphoglucose-isomerase and invertase (Sekin 1978). The residual pellets were incubated at 60°C for 3 h with *α*-amylase and amyloglucosidase, and the liberated glucose represented starch content.

Determination of lignin content

Lignin content was determined using modified micro-Klason method (Huntley et al. 2003). The dried 40-day-old transgenic and wild Arabidopsis stems (200 mg) were ground into powder to pass through a 40 mesh screen and extracted with acetone in the Soxhlet apparatus for 6 h. 100 mg of acetone extraction was treated with 5 ml 72% H_2SO_4 for 2 h at the room temperature, and then mixed into 112 mL distilled water and steamed for 1 h. The mixture was filtrated by 40 mesh screen and rinsed with hot water, then dried and weighed. The percentage of lignin equaled leftover divided total weight and multiplied 100%.

Measure of cellulose content

Crystalline cellulose content of Arabidopsis was determined according to the sulfuric acid digestion method (Updegraff 1969) partly modified. A 40-day-old transgenic and wild Arabidopsis stems (200 mg) were ground into powder in liquid nitrogen. The powder was extracted with 70% ethanol for 1 h at 70°C. The remaining ethanol-insoluble residues, which contained the cell wall, were lyophilized and weighed. 100 mg cell wall powder of Arabidopsis and anthrone were hydrolyzed with 67% sulfuric acid (v/v) at 100°C for 16 min. After cooling on ice, the sample was measured using TU-1900 spectrophotometer at A_{650}.

Results

Characterization of *GhUGP* cDNA

The target cDNA was obtained by the method of colony in situ hybridization, named *GhUGP*, logged GU067484 on Genebank, shown in Fig. 2. The *GhUGP* gene was 1,667 bp with an ORF of 1,395 bp, encoding 465 residues protein with a theoretical molecular mass of 51.23 kDa, *pI* 5.5. A polyadenylation signal, aataa, was found at 1,486 and was 183 bp upstream from polyadenylation addition site. The analysis of UGPase functional motif revealed only one putative *N*-glycosylations motifs (N-Q-S) located at amino acids 157–159. The important amino acids in nucleotide-binding loop (NB loop) of cotton UGPase was deduced at 88–92 aa. Several key amino acids were deduced at Try-183, Try-294, Lys-252, Lys-318 and Lys-356, which played a main role in maintaining UGPase catalytic activity and substrate binding(Abe et al. 2002; Meng et al. 2009a). In addition, after analyzing deduced amino acid sequence of cotton UGPase by BLAST in NCBI, we found the UGPase had high identity with *Solanum tuberosum* (87%), *Hordeum vulgare* (84%) and *Arabidopsis thaliana* (84%). Phylogenetic analyses of UGPase consisted of five main branches (Fig. 3), in which cotton

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    ATGGAAAAGCTGGAACACCTCAAATCTGCCGTCGCTGCTCTTTCTGAAATCAGTGAAAAT  60
1     M  E  K  L  E  H  L  K  S  A  V  A  A  L  S  E  I  S  E  N
    GAGAAAAACGGATTCATCAACCTTGTCTCCCGCTATCTCAGTGGAGAAGCTCAGCACATA 120
21    E  K  N  G  F  I  N  L  V  S  R  Y  L  S  G  E  A  Q  H  I
    GAGTGGAGTAAGATCCAGACTCCAACTGATGAAGTGGTTGTTCCTTATGACACCTTGTCT 180
41    E  W  S  K  I  Q  T  P  T  D  E  V  V  V  P  Y  D  T  L  S
    CCCTCTCCTGATGATCCTGCTGAAACCAAGAAGCTCTTGGACAAACTTGTTGTCTTAAAG 240
61    P  S  P  D  D  P  A  E  T  K  K  L  L  D  K  L  V  V  L  K
    CTTAATGGAGGTCTCGGAACCACTATGGGATGTACTGGTCCCAAATCCGTCATTGAAGTT 300
81    L  N  G  G  L  G  T  T  M  G  C  T  G  P  K  S  V  I  E  V
    CGCAATGGCTTGACTTTTCTTGACCTAATTGTTATTCAGATCGAGAATCTTAATTCTAAA 360
101   R  N  G  L  T  F  L  D  L  I  V  I  Q  I  E  N  L  N  S  K
    TACGGATGTAATGTTCCGTTGGTTCTGATGAACTCATTCAACACCCATGATGACACATTG 420
121   Y  G  C  N  V  P  L  V  L  M  N  S  F  N  T  H  D  D  T  L
    AAGATTGTCGACAAGTACTCAAATTCAAACATTGAGATTCATACTTTTAATCAGAGCCAA 480
141   K  I  V  D  K  Y  S  N  S  N  I  E  I  H  T  F  N  Q  S  Q
    TATCCTCGTCTGGTTGTTGAAGATTTTGCTCCATTACCAAGCAAAGGCCAGCATGGCAAG 540
161   Y  P  R  L  V  V  E  D  F  A  P  L  P  S  K  G  Q  H  G  K
    GATGGATGGTACCCTCCTGGTCATGGTGATGTGTTCCCATCTCTAATGAACAGTGGCAAG 600
181   D  G  W  Y  P  P  G  H  G  D  V  F  P  S  L  M  N  S  G  K
    CTTGATGCTTTCTTATCACAGGGCAAGGAGTATGTCTTCGTTGCAAATTCAGACAATTTG 660
201   L  D  A  F  L  S  Q  G  K  E  Y  V  F  V  A  N  S  D  N  L
    GGTGCTATTGTTGACTTGAAAATCTTAAACCATTTGGTCCAAAACAAGAATGAATATTGT 720
221   G  A  I  V  D  L  K  I  L  N  H  L  V  Q  N  K  N  E  Y  C
    ATGGAGGTTACACCCAAAACCCTAGCTGATGTCAAGGGTGGTACTCTTATTTCTTATGAA 780
241   M  E  V  T  P  K  T  L  A  D  V  K  G  G  T  L  I  S  Y  E
    GGAAAAGTTCAGCTCCTTGAAATTGCTCAAGTCCCTGATGAACATGTCAATGAGTTCAAG 840
261   G  K  V  Q  L  L  E  I  A  Q  V  P  D  E  H  V  N  E  F  K
    TCTATAGAAAAGTTTAAAATTTTCAATACGAACAATTTGTGGGTCAACCTGAATGCTATC 900
281   S  I  E  K  F  K  I  F  N  T  N  N  L  W  V  N  L  N  A  I
    AAGAGGCTTGTGGAAGCTGATGAACTCAAGATGGAGATCATTCCAAACCCAAAGGAGGTC 960
301   K  R  L  V  E  A  D  E  L  K  M  E  I  I  P  N  P  K  E  V
    AATGGAATTAAGGTTCTTCAACTGGAAACTGCAGCTGGTGCAGCAATTAGGTTCTTTGAT 1020
321   N  G  I  K  V  L  Q  L  E  T  A  A  G  A  A  I  R  F  F  D
    CATGCTATTGGTATCAACGTACCTCGATCGCGATTCCTTCCAGTGAAGGCAACTTCAGAT 1080
341   H  A  I  G  I  N  V  P  R  S  R  F  L  P  V  K  A  T  S  D
    TTGCTTCTTGTTCAGTCTGACCTTTACACCTTAGTTGATGGATTTGTTATCCGGAATAAA 1140
361   L  L  L  V  Q  S  D  L  Y  T  L  V  D  G  F  V  I  R  N  K
    GATAGAGCGAATCCTACAAACCCATCCATAGAATTGGGGCCTGAATTCAAGAAGGTTGGT 1200
381   D  R  A  N  P  T  N  P  S  I  E  L  G  P  E  F  K  K  V  G
    AACTTCTTAAGTCGATTCAAGTCAATCCCGAGCATCATTGGGCTTGATAGCCTTAAGGTG 1260
401   N  F  L  S  R  F  K  S  I  P  S  I  I  G  L  D  S  L  K  V
    ACTGGTGATGTTTGGTTTGGTGCTGGCATTGTGCTCAAGGGGAAAGTGAGTATCGCTGCA 1320
421   T  G  D  V  W  F  G  A  G  I  V  L  K  G  K  V  S  I  A  A
    AAACCCGGGGTGAAGTTGGAGATTCCCGATGGAGCTGTAATTGAGAACAAGGAAATTAAT 1380
441   K  P  G  V  K  L  E  I  P  D  G  A  V  I  E  N  K  E  I  N
    GGCCCCGAAGACATCTGAGagaggcccgtctgccagccgaagtttccctgattttggtgt 1440
461   G  P  E  D  I  *
    gtcgcagtagataacgaacgcatcttttatatagataggaagtaaaataaaataaaaata 1500
    cttggaacagaagtagtatttgcgtttcatatcacatatatatgttgtatgtcttgcggg 1560
    agtttcccttgaattactatttttcgaggtatgatgaaaaacagtgttctgaatgttgta 1620
    tctacttttgcaccaaacaacttagccccttttaccaaaaaaaaaaa              1668
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Fig. 2 Full-length GhUGP cDNA and putative amino acid sequence. *Capital letters* and *small letters* are denoted as coding region and non-coding region, respectively. Nucleotides are numbered on the *right* and amino acids are *left*. *Asterisks* mean the termination codon. Motif of putative *N*-glycosylation is *boxed*. A polyadenylation signal is *underlined*. Several key amino acids of nucleotide-binding (NB) loop are displayed with *double underline*. Main residues are shown with *shadow* and *bold*

UGPase was grouped with *Solanum tuberosum* (D00667 and Z18924). It suggested that they may have similar features and functions.

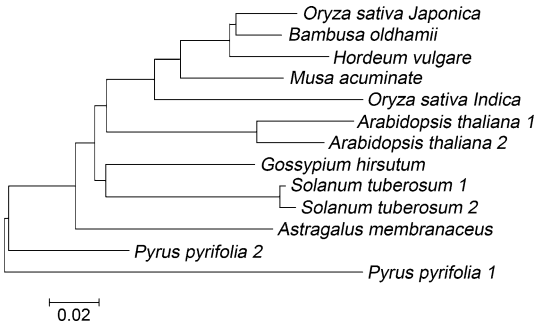

Fig. 3 Phylogenetic tree of UGPase from cotton based on the full amino acid sequences. The tree was constructed by the neighbor-joining method. Amino acid sequence of UGPase come from *Gossypium hirsutum* (GU067484), *Oryza sativa* Japonica (AB062606), *Oryza sativa* Indica (AAF62555), *Musa acuminata* (AAF19422), *Solanum tuberosum* 1(D00677) and *Solanum tuberosum* 2 (Z18924), *Bambusa oldhamii* (AY178448), *Astragalus membranaceus* (AF281081), *Hordeum vulgare* (X91347), *Pyrus pyrifolia* 1 (AB027617), *Pyrus pyrifolia* 2 (AB013353), *Arabidopsis thaliana* 1 (At3g03250) and *Arabidopsis thaliana* 2 (At5g17360)

Expression level of *GhUGP* by real-time PCR

The transcription levels of *GhUGP* were detected in root, stem, leaf and flower (Fig. 4a). Among several parts of cotton, GhUGP mRNA was most abundant in flower (6.50-fold than in leaf), lower level in root (4.23-fold), more lower in stem (2.58-fold) and lowest in leaf. It indicated that the GhUGP is mainly distributed in storage organs of plants and, probably, involved in polysaccharide biosynthesis.

The mRNA level of *GhUGP* was measured to determine the effect of different treatments, such as sucrose, light, low temperature, flooding and ethylene. After cotton leaves were fed in different concentrations of sucrose solutions for 12 h, the expression of *GhUGP* was up-regulated in all treatments and reached the highest level at 300 mM sucrose. The strong induction of *GhUGP* could be observed at 150 mM sucrose feeding (Fig. 4b). Light exposure for 8 h and 150 mM sucrose feeding increased the transcription level of *GhUGP* to higher level than individual treatment with sucrose or light (Fig. 4c). When the cotton seedlings were exposed at 4°C, the transcription level of *GhUGP* increased markedly after 7 days (Fig. 4d). Under flooding or O_2-deficiency conditions, the expression of *GhUGP* decreased gradually (Fig. 4e). The transcription of *GhUGP* was increased correlated to ethylene induction after 1 h and continued till 12 h. The responses of *GhUGP* to ethylene in cotton ovules were shown in Fig. 4f.

Phenotype analysis of transgenic lines

GhUGP transgenic *Arabidopsis* line displayed different phenotypes as compared to wild type: higher height and faster rate of growth at different development stage

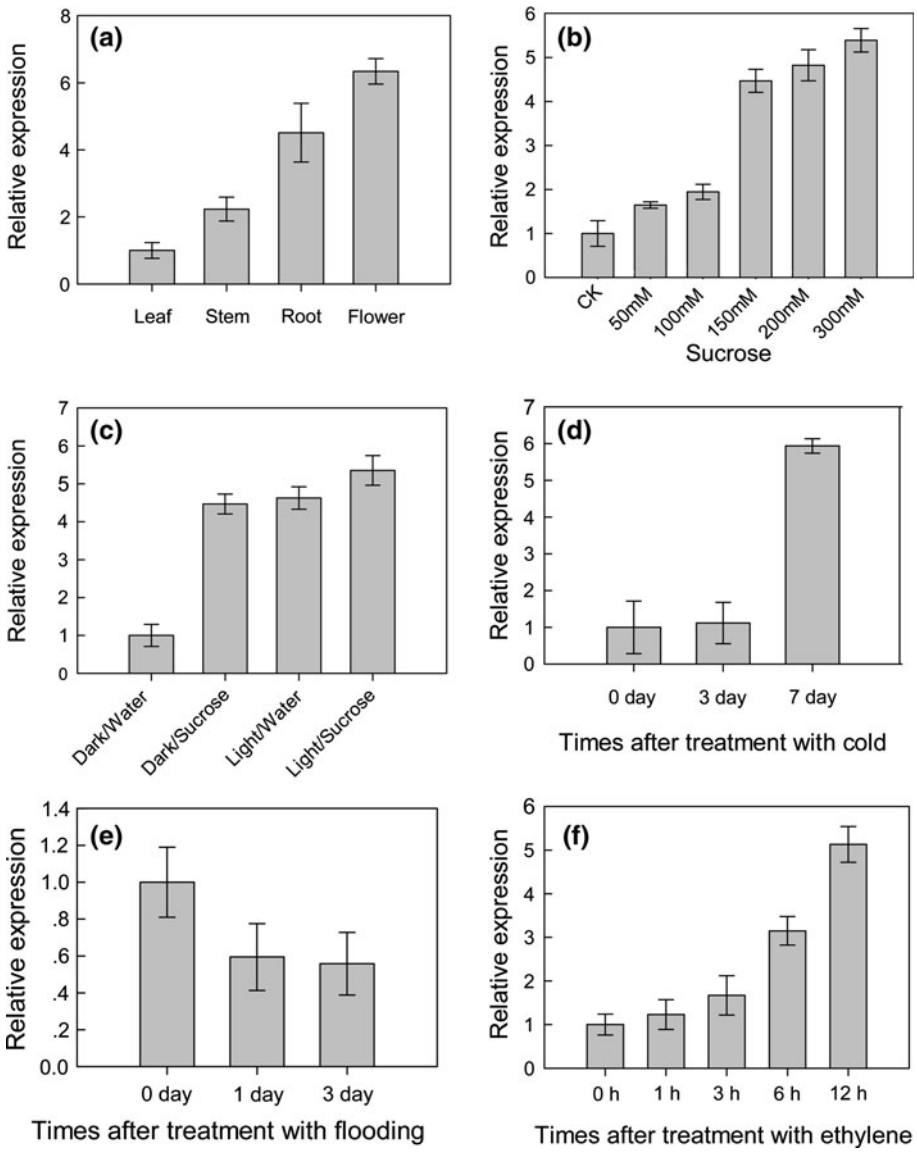

Fig. 4 Real-time PCR was used to measure the relative expression of GhUGP. **a** Tissue-specific expression pattern of GhUGP in cotton. **b** Effect of sucrose on GhUGP expression in cotton leaves. **c** Effect of light on GhUGP expression in cotton leaves. **d** Effect of low-temperature stress on GhUGP expression in cotton. **e** Effect of flooding stress on GhUGP expression in cotton. **f** Effect of ethylene on GhUGP expression in cotton ovules

(Fig. 5). To precisely analysis, the discrepancy of phenotypes between transgenic and wild-type Arabidopsis, we determined the leaf area, cauline branch number, internode distance and plant height at 40-day-old Arabidopsis (Table 1). The heights of all transgenic lines were also increased evidently in the comparison between *GhUGP* transgenic and wild-type *Arabidopsis* of 40 days . There were no significant difference in leaf area and cauline branch number between transgenic Arabidopsis lines and control. But the increase in internode distance in the transgenic line (D) was significant.

Carbohydrate analysis

Three soluble sugars (sucrose, fructose and glucose) and starch were measured in the rosette in wild and transgenic Arabidopsis grown for 40 days (Table 2). Sucrose had significantly increased in five *GhUGP* transgenic lines as compared to the wild type, while glucose and fructose were significantly increased in three of the five transgenic lines (C, D and E). As for starch, three of the five transgenic lines (A, C and E) had significantly increased in the rosette as compared to control. Three of the five GhUGP transgenic lines (C, D and E) had significantly elevated the total soluble surge content relative to control lines.

Determination of lignin and cellulose contents in transgenic Arabidopsis

The lignin and cellulose content in 40-day-old wild and transgenic plants were measured (Table 3). Compared with wild type (18.5%), the content of lignin determined by

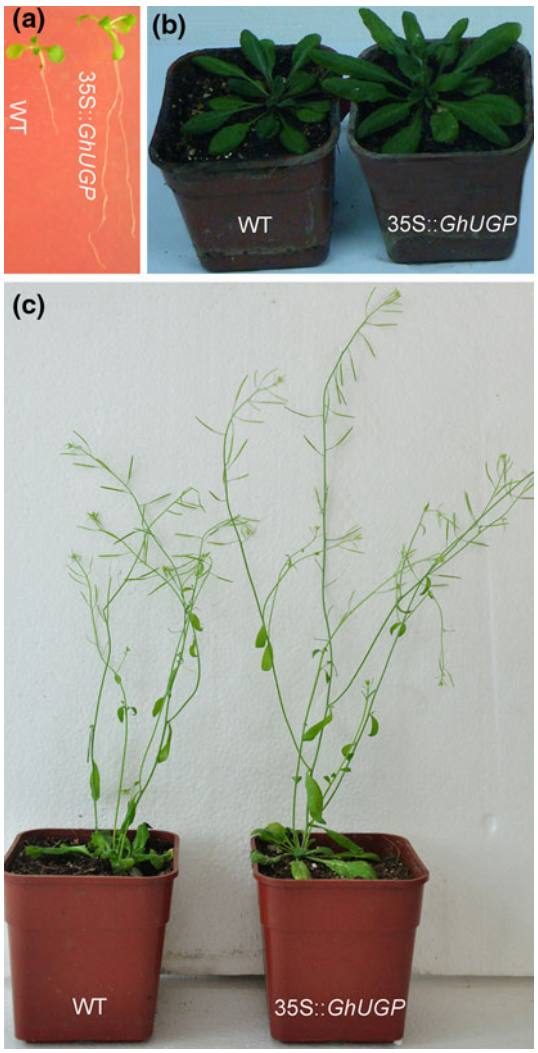

Fig. 5 Morphology of wild type and transgenic Arabidopsis. **a** Phenotype of 10-day-old plants. The three transgenic seedlings in the right side had longer roots than wild type (*left*). **b** Transgenic plant (*right*) displayed faster rate than wild type (*left*) after 3 weeks. **c** The transgenic plants (*right*) were much higher than wild type (*left*) after 40 days

modified micro-Klason method had not significantly changed in transgenic lines (16.9–19.3%). The cellulose content in stems of the transgenic lines was found to be significant higher ranged from 2.1 to 6.3% than in the control lines.

Discussion

In this paper, *GhUGP* (GU067484) from cotton was characterized. Based on the alignment with *Solanum tuberosum* and other plants, several functional consensus motifs identified in various eukaryotic organisms were found in the deduced GhUGP full-length sequence. These conserved motifs have been demonstrated to be essential for substrate binding and catalytic activity. The NB region was essential for UGPase activity. Recent research on barley UGPase proved the functions of the loop through domain deletion approaches, resulting in completely inactive enzyme (Meng et al. 2009a) and Cys-99 was replaced (C99S mutant) to affect PPi binding (Martz et al. 2002). These important amino acids in NB loop of cotton UGPase corresponded to 88-92 aa, and Cys-91 may affect substrate binding. Another plant UGPase motif N-X-S/T (X represents any amino acid other than proline) involved in membrane-bound activity. The potato UGPase had putative glycosylation sites at amino acid positions 168 (N-Q-S) and 307 (N-L-S). The similar two motifs were observed in rice UGPase, one at 162 (N-Q-S) and the other at 201 (N-N-S) (Kleczkowski 1994). The UGPase from cotton had only one potential glycosylation site at 157 (N-Q-S). In addition, several key Lys residues of UGPase from potato had been demonstrated to be important according to the site-directed mutagenesis, particularly Lys-367 may be essential for catalytic activity, Lys-263 and Lys-329 for binding with MgPPi or glucose-1-P substrate (Katsube et al. 1991). Abe et al. (2002) reported that UGPase from rice had Lys-257, Lys-323 and Lys-361 relatively positioned with the three

Table 1 Morphology analysis of wide-type and transgenic Arabidopsis

Lines	Leaf area (cm^2)	Cauline branch number	Intermode distance (cm)	Height (cm)
Control	1.55 ± 0.32	5.1 ± 0.8	5.3 ± 0.4	25.7 ± 1.1
35S::UGPase A	1.57 ± 0.34	5.0 ± 0.4	5.7 ± 0.3	28.3 ± 1.2[a]
35S::UGPase B	1.53 ± 0.64	4.8 ± 0.5	5.8 ± 0.7	29.6 ± 2.1[a]
35S::UGPase C	1.54 ± 0.47	5.2 ± 0.9	5.7 ± 0.6	28.9 ± 0.7[a]
35S::UGPase D	1.57 ± 0.68	5.3 ± 0.6	6.3 ± 0.4[a]	31.5 ± 0.9[b]
35S::UGPase E	1.58 ± 0.79	5.0 ± 0.8	6.0 ± 0.8	30.4 ± 1.0[a]

[a] Significant difference from control values at $\alpha = 0.10$

[b] Significant difference at $\alpha = 0.05$; mean (±SE) were calculated from fifteen plants per line

Table 2 Carbohydrate content of the whole rosette in wild and transgenic Arabidopsis

Lines	Sucrose (mg g^{-1} FW)	Glucose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)	Total soluble sugars (mg g^{-1} FW)	Starch (mg g^{-1} FW)
Control	0.110 ± 0.028	0.380 ± 0.012	0.305 ± 0.040	0.798 ± 0.037	0.175 ± 0.014
35S::UGPase A	0.260 ± 0.034[a]	0.476 ± 0.023	0.428 ± 0.016	1.164 ± 0.025	0.324 ± 0.028[a]
35S::UGPase B	0.284 ± 0.041[a]	0.485 ± 0.018	0.425 ± 0.021	1.201 ± 0.032	0.294 ± 0.015
35S::UGPase C	0.260 ± 0.019[a]	0.645 ± 0.037[a]	0.658 ± 0.035[a]	1.546 ± 0.025[a]	0.335 ± 0.036[a]
35S::UGPase D	0.254 ± 0.023[a]	0.679 ± 0.023[a]	0.627 ± 0.026[a]	1.563 ± 0.031[a]	0.276 ± 0.039
35S::UGPase E	0.285 ± 0.034[a]	0.644 ± 0.027[a]	0.566 ± 0.019[a]	1.508 ± 0.022[a]	0.380 ± 0.024[a]

[a] Significant difference from control values at $\alpha = 0.10$. Mean (±SE) were calculated from fifteen plants per line

Table 3 Lignin and cellulose content in wild and transgenic Arabidopsis

Lines	Lignin (%)	Cellulose (%)
Control	18.5 ± 0.1	23.3 ± 0.2
35S::UGPase A	17.8 ± 0.5	25.6 ± 0.5[a]
35S::UGPase B	19.3 ± 0.1	27.5 ± 0.7[a]
35S::UGPase C	16.9 ± 0.2	26.6 ± 0.2[a]
35S::UGPase D	18.4 ± 0.1	29.6 ± 0.9[a]
35S::UGPase E	18.9 ± 0.7	25.4 ± 0.2[a]

[a] Significant difference from control values at $\alpha = 0.10$. Mean (± SE) were calculated from fifteen plants per line

Lys residues in potato and exercising similar functions. Meng et al. (2009a) demonstrated that Lys-260 mutant had low activity and high K_m for PPi and Trp-191 and Trp-302 involved in UDPG binding. Analogously, cotton UGPase had the relative position of Lys-252, Lys-318, Lys-356, Try-183 and Try-294 with those two plants. It suggested that Lys-356 may be responsible for the catalytic activity of UGPase, and Lys-252, Lys-318, Try-183 and Try-294 for substrate binding in cotton UGPase.

The expression of *GhUGP* of cotton was markedly up-regulated on feeding sucrose and light exposure after dark-adapted cotton leaves (Fig. 3b, c). The sucrose effect was mimicked by exposing leaves to light, because of the sucrose formation during photosynthesis (Ciereszko et al. 2001b). Sucrose stimulation was also observed for *UGP* expression from Arabidopsis and potato tubers (Ciereszko et al. 2001a; Spychalla et al. 1994). Studies have found that sucrose specific signaling may have different components from HXK-mediated glucose signaling or osmoticum signal pathways (Rook et al. 1998; Loreti et al. 2001). In Arabidopsis, the sucrose up-regulation on *UGP* was independent of HXK status, and was completely blocked by okadaic acid (OKA) (Ciereszko et al. 2001b). The expression of *UGP* gene under sucrose and light treatment suggested the UGPase participated in the catabolism of sucrose into glucose-1-phosphate. Cool stress was another important factor that affected *UGP* gene expression in plants. Low temperature resulted in up-regulation on *UGP* and protein content as well as UGPase activity in Arabidopsis by an ABA-independent pathway (Ciereszko et al. 2001a). In potato tubers, cold treatment generally led to a rapid sugar accumulation and this phenomenon was known as "cold sweetening" (Sowokinos et al.1997). The *UGP* gene from cotton was up-regulated in cold stress (Fig. 3d), which suggested that low temperature may have direct effect on "cold sweetening" phenomenon. The transcription of *GhUGP* was down-regulated by flooding (Fig. 3e), which is consistent with Arabidopsis UGPase (Ciereszko et al. 2001b). It was worth to mention that the expression of *GhUGP* was manifestly up-regulated by ethylene in cotton ovules. Although it was a little reported *UGP* gene response to ethylene in plants, ethylene may promote cotton fiber elongation by activating genes that mediate cell wall synthesis, wall loosening, or cytoskeleton arrangement (Shi et al. 2006). It suggested that increasing the expression of *UGP* from cotton may promote the development of fibers cell.

The over-expression *GhUGP* in Arabidopsis under the control of a single CaMV 35S promoter showed visible phenotypic differences compared to the wild type (Fig. 5; Table 1). Transgenic lines showed significant increase in height and internode distance. It is consistent with the previous work, which shown that over-expressing the *UGP* from *Acetobacter xylinum* under the control of the 2× CaMV 35S promoter increase plant height growth in tobacco (Coleman et al. 2006). These findings suggested UGPase played an important role in the strength of sink tissues. Plant displaying increased growth resulted in the formation of fibers at a higher rate than control plants (Coleman et al. 2006). The current research further supported previous fact by the increased cellulose content in transgenic Arabidopsis (Table 3).

Significant increases in all soluble carbohydrates were observed in the leaves rosette of *GhUGP* transgenic Arabidopsis (Table 2), which indicated that UGPase from cotton could modify the carbon partitioning towards

soluble sugars. Similarly, in tobacco, the over-expressing *UGP* from *Acetobacter xylinum* increased glucose in stems and fructose in leaves (Coleman et al. 2006). In transgenic poplar, the over-expression of the *UGP* significantly increased in sucrose, glucose and fructose in the leaf tissues (Coleman et al. 2007). In addition, the GhUGP-transformed lines showed the higher level of starch than in wild-type Arabidopsis, which suggested that carbohydrates accumulation in the source leaves resulted from the pooling of available soluble sugars. The better explanation was the exogenous UGPase in leaf tissues resulted in the increase in carbohydrates, and was probably working coordinately with SPS in the synthesis of sucrose to accumulate the storage carbohydrates (Kleczkowski 1994). Other studies also supported that UGPase could provide the substrate-Glc-1-P for starch biosynthesis (Johansson 2003).

Cellulose and lignin are main components in cell wall. Compared with the control lines, the cellulose content in five transgenic Arabidopsis lines had been significantly higher (Table 3). It indicated that cotton UGPase activity may be linked with the activity of the cellulose synthase complex in cell wall. Previous studies had also predicted the possible role of UGPase in cellulose biosynthesis (Coleman et al. 2006, 2007). The feature of UGPase was reminiscent of sucrose synthase (SuSy), which could bind to plasmalemma and directly provide UDPG to cellulose synthase (Amor et al. 1995). Furthermore, the SuSy has been shown to be strongly associated with cellulose formation in cotton and play important roles in cotton fiber cell initiation and elongation (Ruan et al. 2003). Although it was not reported the role of UGPase in cotton fiber cell biosynthesis, these findings of cotton UGPase indicated it may affect the developing of cotton fibers cell.

In summary, the new cotton gene *GhUGP* has high identify with UGPase from other plants. Real-time PCR revealed that expression of *GhUGP* was affected by sucrose, low temperature and O_2-deficiency, which suggested that cotton UGPase was involved in sucrose/polysaccharides metabolism. The overexpression of *GhUGP* in Arabidopsis influences the growth of plants but also altered soluble sugar, starch and cellulose contents. Findings suggested cotton *GhUGP* was a functional UDP-glucose pyrophosphorylase gene and might play an important role in the allocation of carbon especially in cellulose biosynthesis. Interestingly, transcription of *GhUGP* mRNA was manifestly up-regulated by ethylene in cotton ovules, which indicated *GhUGP* from cotton may affected the developing of cotton fibers cell.

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