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Overexpression of UDP-glucose pyrophosphorylase gene could increase cellulose content in Jute (*Corchorus capsularis* L.)

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ABSTRACT

In this study, the full-length cDNA of the UDP-glucose pyrophosphorylase gene was isolated from jute by homologous cloning (primers were designed according to the sequence of *UGPase* gene of other plants) and modified RACE techniques; the cloned gene was designated *CcUGPase*. Using bioinformatic analysis, the gene was identified as a member of the *UGPase* gene family. Real-time PCR analysis revealed differential spatial and temporal expression of the *CcUGPase* gene, with the highest expression levels at 40 and 120 d. PCR and Southern hybridization results indicate that the gene was integrated into the jute genome. Overexpression of *CcUGPase* gene in jute revealed increased height and cellulose content compared with control lines, although the lignin content remained unchanged. The results indicate that the jute *UGPase* gene participates in cellulose biosynthesis. These data provide an important basis for the application of the *CcUGPase* gene in the improvement of jute fiber quality.

1. Introduction

UDP-glucose pyrophosphorylase (UGPase) is an important regulatory enzyme in glucose metabolism in plants, animals and fungi, which was first cloned in yeast cells in 1953 [1,2]. It catalyzes the glucose-1-phosphate (Glc-1-P) reaction with uridine triphosphate (UTP) to form uridine diphosphate glucose (UDPG) and pyrophosphate (PPi), which is the key precursor in the synthesis of cellulose, hemicellulose, pectin, glycolipids and glycoproteins biosynthesis metabolism [3] in plants. UDPG can also be converted to ADPG by the coupled reactions of UGP and cytosolic AGPase in starch synthesis [3]. Uridine diphosphate glucose pyrophosphorylase (UDP-glucose pyrophosphorylase, UGPase) is a precursor of catalytic cellulose-uridine diphosphate glucose (UDP-glucose, UDPG) synthesis. UGPase plays important roles in carbohydrate metabolism and secondary cell wall biosynthesis [4,5]. To date, *UGPase* gene have been cloned in potato [6], banana [7], Barley [3], Rice [8,9], Melon [10], Aspen [11] and Cotton [12].

Current studies show that *UGPase* gene closely associated with cellulose biosynthesis and that the cellulose content increases following transfer of the bacterial *UGPase* gene into tobacco, although the lignin content was unaffected [13]. Transfer of the cotton *UGPase* gene increased cellulose content in *Arabidopsis thaliana* [12]. Furthermore, overexpression of *UGPase* gene in *Streptococcus zooepidemicus* resulted in a slight alteration in virulence and a reduction in the cell envelope hyaluronic acid yield [14].

The jute (*Corchorus capsularis* L.; Family, Tiliaceae) species, also known as Luo Ma, is an annual bast fiber crop. Jute is one of the most important fiber crops in the world, with the planting area and yield almost equal to that of cotton. Jute fibers exhibit a characteristically high luster, good moisture absorption performance, rapid water loss capacity and easy degradation. Consequently, jute has become known as the "gold fiber" in recent years. As a result of the continuous efforts of breeders, jute fiber production has been greatly improved; however, jute fiber cell wall lignification and other limiting factors render the short, coarse fibers suitable only for the production of rope and sacking. These characteristics seriously restrict the field application of jute fiber in the textile industry.

The quality of jute fiber is very poor mainly due to the cellulose content is less (jute: 57–60%, ramie: 65–75%, cotton: 87–90%) [15]. So cloning and functional analysis of the jute *UGPase* gene will provide new insights into potential improvements in the quality of jute fibers and cellulose biosynthesis. However, the complete cDNA of *UGPase* gene and its functional analysis has not been reported at present. So, the purpose of this study is to isolate the full-length cDNA of *UGPase* gene and analyze its function. Studied the variation of *UGPase* gene expression in different tissues and at

Abbreviations: UDP, uridine diphosphate; UGPase, UDP-glucose pyrophosphorylase; UDPG, uridine diphosphate glucose; PPi, pyrophosphate; UTP, uridine triphosphate; AGPase, ADP-glucose pyrophosphorylase; d, days.

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different stages of growth and evaluated its critical role in cellulose synthesis following overexpression in jute.

2. Materials and methods

2.1. Plant material and treatments

Jute seeds (huangma 179) were conserved in the laboratory of genetic improvement of Fujian Agriculture and Forestry University (China). Intact seeds were selected, planted in nutrient soil and cultured in a growth chamber with a light/dark photoperiod of 14 h/10 h and the day/night temperature of 34 °C/26 °C.

2.2. Explant preparation for genetic transformation

The jute seeds were rinsed under running water for 10 min and subsequently surface-sterilized with 70% (v/v) ethanol for 5 min and 5% sodium hypochlorite for 10 min. The seeds were rinsed three times with sterile water. All of the sterilized seeds were germinated in 30 mL of MS medium [1] without growth regulators. The culture conditions used were 25–28 °C with a 16-h photoperiod for 5–7 d. In this procedure, the entire cotyledonary node was excised from the sterile seedlings using sterile surgical blades, the primary leaves were kept, and approximately 0.5–0.8-cm-long epicotyls were removed.

2.3. The cDNA clone of the CcUGPase gene

Total RNA of jute plants was extracted using the OMEGA isolation kit (R6827-01, USA). First strand cDNA was synthesized using the Reverse Transcription Kit (Pimerscript™RT DRR037S TAKARA CHINA). Degenerate primers were designed according to the UGPase gene sequence of other plants (Supplementary Materials) as follows: (sense primer, CF: 5′-TGGWSNAARATHCARACNCCNACNG-3′; antisense primers, CR: 5′-GTYTCNARYTGNARNACYTT-3′). The key fragments of the cDNA were amplified based on degenerate primer. The 5′RACE primers (Cc-R1:5′-CAGTGTGTCATCATGGGTGTTG-3′, Cc-R2: 5′-CTTCAATGACGGACTTCGGT-3′) were designed using the Primer 5.0 design software. In the first PCR reaction, cDNA (1 μL) was used as templates in a reaction system containing the upstream and downstream primers (1 μL, 10 μmol L^{-1}), 10 × Buffer (2.5 μL), dNTPs (2 μL, 10 mM each), Taq polymerase (0.2 μL, 10 U/μL), and made up to 25 μL with ddH_2O. The PCR (Eppendorf 5334 Mastercycler® Gradient)conditions were as follows: 94 °C for 3 min, 94 °C for 30 s, 60 °C for 1 min, 72 °C for 2 min (35 cycles) with a final extension incubation at 72 °C for 10 min. Terminated reactions were maintained at 4 °C. In the second PCR, 1 μL of the first PCR product was used as the template under identical conditions. Subsequently, the PCR products (10 μL) were analyzed by 1% agarose gel electrophoresis, The sequence were sub-cloned into the pUC19 vector following standard cloning method [16]. The sequence were by sequenced, resulting sequences used in a homology search in NCBI webset (http://www.ncbi.nlm.nih.gov/).

The 3′RACE primers: (Cc3-F1:5′-CAACACCCATGATGACACACTG-3′, Cc3-F2: 5′-AATCAGAGCCAGTATCCCCG-3′), PCR reaction system and conditions are same to 5′RACE. The DNAMAN software was used to assemble the key sequence, 5′RACE and 3′RACE results into full-length cDNA of CcUGPase gene. The sequence was used to design specific primers (CcQF: 5′-CGTCTCCAGTCCACACCAAATCC-3′; CcQR: 5′-GAAACTCTCCCGCAACATACACAA-3′) for amplification of the full-length cDNA. The PCR reaction system containing 1 μL cDNA, the upstream and downstream primers (1 μL, 10 μmol L^{-1}), 10 × Buffer (2.5 μL), dNTP (2 μL, 2.5 μL, 10 mM each), Taq polymerase (0.2 μL, 10 U/μL), and made up to 25 μL with ddH_2O. The PCR conditions were as follows: 94 °C for 2 min, 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min (35 cycles) with a final extension incubation at 72 °C for 10 min. Terminated reactions were maintained at 4 °C. Subsequently, the PCR products (20 μL) were analyzed by 1% agarose gel electrophoresis, purified and ligated into the pMD19-T vector. The resulting construct vector was transformed into *Escherichia coli DH5α*. The sequence were characterized by sequencing followed by homology search in NCBI (http://www.ncbi.nlm.nih.gov/).

2.4. Sequence and phylogenetic analysis

DNA sequencing was performed by BGI (Shanghai, China) and analyzed using BioEdit software [17] and DNAman software (http://www.lynnon.com/). The alignment of cDNA and amino acids was carried out by BLAST in the NCBI. Phylogenetic analysis was performed with the mature protein sequence using the MEGA 4 program [18].

2.5. Real-time PCR analysis

Total RNA was isolated from different jute tissues (root, stem, leaf) at different developmental stages (20, 40, 60, 120 and 150 d) and cDNA was generated using a PrimeScript™ reverse transcription kit (TaKaRa) according to the manufacturer's instructions. The resultant cDNA was diluted ten times for use as the template in expression analyses. Primers for expression analysis were designed according to the full-length cDNA sequence of the *CcUGPase* gene (CPF:5′-GAGGTCTGGGGACTACAATG-3′, CPR: 5′-TCAACAACCAAACGGGGATA-3′) and the jute 18sRNA gene, which served as internal reference (18s-F: 5′-GTGGAGCGATTTGTCTGGTT-3′, 18s-R: 5′-TGTACAAAGGGCAGGGACGT-3′). Expression analysis was conducted using the ABI 7300 real-time PCR system with a reaction system containing upstream and downstream primers (0.5 μL, 10 μmol L^{-1}), 2 × SYBR Green Master Mix (1 μL), cDNA (1 μL) and made up to a total volume of 20 μL with ddH_2O. Quantitative real-time PCR analysis was performed using the following reaction conditions: 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s, 60 °C for 50 s (40 cycles) using an ABI 7500 fluorescence quantitative PCR instrument. All experiments were repeated three times and relative gene expression was calculated by the $2^{-\Delta\Delta CT}$method.

2.6. Plasmid construction

The open reading frame (ORF) of *CcUGPase* gene was amplificated using the following primers: Sense primer, 5′-GAAGATCTCGTCTCCAGTCCACACCAAATCC-3′ and antisense, 5′-GGGTAACCGAAACTCTCCCGCAACATACACAA-3′ (*BglII* and *BstEII* restriction endonuclease sites are shown in underlined red letters). The amplified PCR product was *digested with BglII and BstEII a*nd inserted into a binary vector pCAMBIA 1301 containing a hygromycin phosphotransferase (*hph*) gene, for expression under the control of the CaMV35S promoter. Sequence analysis confirmed the correct insertion of the *UGPase* gene into the vector.

2.7. Transformation of jute and screening

The constructed vector containing the *CcUGPase* gene was introduced into *Agrobacterium tumefaciens* strain EHA105 by the freeze–thaw method [16]. Using agrobacterium-mediated transformation of cotyledonary node of jute [19,20]. Transgenic lines were obtained by selection on MS culture medium containing hygromycin (30 mg/L) and confirmed by PCR amplification using a gene-specific primer 1304-F (5′-TGTTCTCTCCAAATGAAATGAACT-3′) and a vector-specific primer:1304-R (5′-AATCAGAGCCAGTATCCCCG-3′)

under the following conditions: denaturation at 94 °C for 5 min, followed by 94 °C for 50 s, 55 °C for 50 s, 72 °C for 1.5 min (35 cycles) with a final extension incubation at 72 °C for 10 min. Terminated reactions were maintained at 4 °C. The PCR product was detected in 1% agarose gel electrophoresis. Chose positive plants confirmed by PCR for southern bolt detection, according to the transformation vector plasmid pCAMBIA1301, choose three single restriction sites, respectively is: *BSTEII*, *BglII* and *NcoI*, 5–10 μg genomic DNA was digested overnight, after electrophoresis, transfer film, print, pre hybridization, hybridization. hygromycin probe labeled with alkaline phosphatase Labeling Kit (ROCHE company). The hygromycin probe primer (R: 5′-CATACTTGAGACCAGTGTTT-3′, F: 5′-CCGACCTTAACTAGCTCAAT-3′), the results was collected use the gel imaging system FluorChem SP 50 mm f11.4 lens.

2.8. Phenotype analysis of transgenic jute

The morphology and growth rate of transgenic and control plants was analyzed. Each group comprised five strains and the analysis was repeated three times. The results were analyzed by SPSS LSD variance analysis.

2.9. Determination of lignin content

Lignin content was determined using the method described by Huntley et al. [21]. Powder of 80-day-old transgenic and wild jute stems (100 mg) were passed through a μm mesh screen and extracted with acetone using Soxhlet apparatus for 6 h. The acetone extract (100 mg) was treated with 5 mL 72% H_2SO_4 for 2 h at the room temperature, and then mixed in 112 mL distilled water and steamed for 1 h. The mixture was filtered through a 40 μm mesh screen, rinsed with 100 °C water, dried and weighed. The lignin content was calculated as a percentage of the total weight.

2.10. Determination of cellulose content

The cellulose content was determined using the sulfuric acid digestion method by [22].The powder of 80-day-old transgenic and control lines stems (100 mg) was added to a flask containing 100 mL 60% sulfuric acid and shaken for 30 min. The mixture was filtered and 5 mL of the filtrate was added to 0.2 mL 2% (v/v) anthrone before being hydrolyzed with 5 mL 67% sulfuric acid (v/v) at 100 °C for 10 min. The resulting solution was immediately cooled on ice, and the cellulose content of a sample was measured using a TU-1810 spectrophotometer at A_{650}.

3. Results and discussion

3.1. The cDNA cloning and analysis of CcUGPase gene

Jute is a plant rich in polysaccharides, polyphenols and other secondary materials. RNA extraction is difficult [23], we extracted high quality RNA use OMEGA isolate Kit, precipitation with isopropyl alcohol at last. The integrity of the total RNA was evaluated by 0.8% agarose gel electrophoresis, the electrophoresis pattern included three bands (28S, 18S, 5S) (Fig. 1A). The brightness of the 28S bands was 2X that of the 18S, indicating almost no RNA degradation, or protein contamination, occurred, resulting in high quality RNA suitable for next experiment.

Approximately 1901 bp (Fig. 1B) cDNA of *UGPase* gene was amplified using homologous clone and modified RACE techniques,

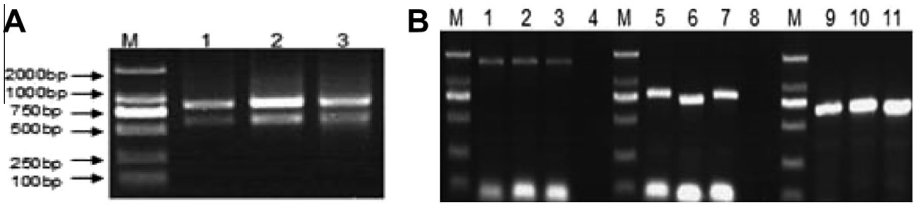

Fig. 1. (A) Agarose gel electropherogram of total RNA of jute. M: Marker DL2000. 1, 2, 3: The RNA of Stem bark from jute. (B) The result of PCR amplification M: Marker DL2000. 1, 2, 3: Product of full length of *CcUGP gene.* 4, 8: Positive control. 5, 6, 7: 5′RACE PCR product. 9, 10, 11: 3′RACE PCR product.

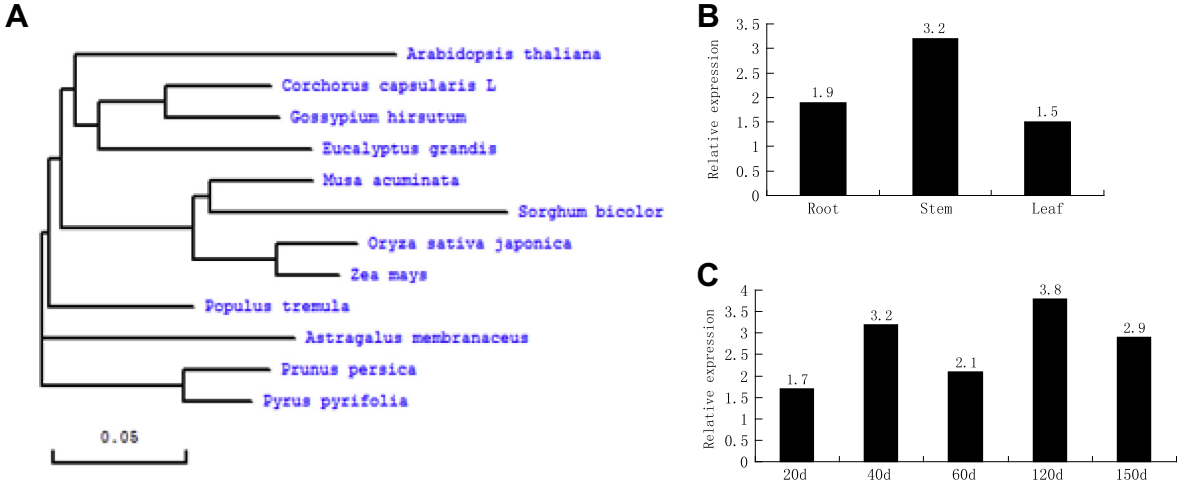

Fig. 2. (A) The phylogenetic tree of *UGPase* gene based on the full amino acid sequences from different plants. The tree was constructed by the neighbor-joining method. Amino acid sequence of UGPase come from *Astragalus membranaceus* (AAF86501.1), *Arabidopsis thaliana* (AT5G17310), *Musa acuminate* (AAF19422.1), *Oryza sativa japonica group* (BAB69069.1), *Sorghum bicolor* (XP_002453185.1), *Zea mays* (ACL52500.1), *Pyrus pyrifolia* (BAA25917.1), *Eucalyptus grandis* (ACF04278.1), *Prunus persica* (AGH25528.1), *Populus tremula* (ABB88893.1), *Gossypium hirsutum* (ACJ11711.1). (B) Real-time PCR was used to measure the relative expression of *CcUGPase* gene. Expression pattern of *CcUGPase* gene in jute at different tissues. (C) Expression pattern of *CcUGPase* gene in jute at different stages.

sequencing confirmed that this gene belonged to *UGPase* gene family. incorporating a 1706 bp ORF, and encoding 465 amino acids. After analyzing deduced amino acid sequence of jute UGPase by BLAST in NCBI, we found the UGPase had high identity with *Gossypium hirsutum* (FJ415165.1) (90%), *Populus tremula* (DQ302093.1) (84%), *Prunus persica* (KC339525.1) (83%), *Eucalyptus grandis* (EU737107.1) (82%), *Pyrus pyrifolia* (AB013353.1) (82%) and *Vitis vinifera* (FQ387683.1) (80%). A phylogenetic tree (Fig. 2A) was constructed based on the amino acid sequences of the UGPase proteins of *C. capsularis* L., *E. grandis*, *G. hirsutum*, *P. tremula*, *P. persica*, *A. thaliana*, *Zea mays*, *Sorghum bicolor*, *P. pyrifolia*, *Oryza sativa japonica*, *Astragalus membranaceus* and *Musa acuminata*. The results showed that the *CcUGPase* gene exhibited the closest relationship with *G. hirsutum*, and the most distant relationship with *P. pyrifolia*, *P. persica* and *A. membranaceus*.

3.2. Differential spatial and temporal expression of CcUGPase gene

Fluorescent quantitative PCR analysis showed that the *CcUGPase* gene was expressed in root, stem and leaf (Fig. 2B). The highest expression of the *CcUGPase* gene was detected in the stem. At present, we mainly harvest cellulose in the stem of jute, the stem contains a lot of fiber, which is associate with *CcUGPase* gene with highest expression levels in stem. The development of jute can be divided into four growth cycles (stable stage, the rapid growth stage, stable stage and the mature stage) [24]. Analysis of *CcUGPase* gene in the stem at different stages of growth showed *CcUGPase* gene with highest expression during the rapid growth period (40 and 120 d) (Fig. 2C), at this stages through increase the *CcUGPase* gene expression to improve the rate of cellulose synthesis. While at stable stages (40 and 60 d), the *CcUGPase* gene expression is decreased. Previous research has shown expression of the *UGPase* gene can activate cell wall synthesis, wall loosening, or cytoskeleton arrangement [25,26].

In the (Fig. 3), we exhibit the explant regeneration and transformation process. In the screening of transgenic jute, To ascertain the expression of *CcUGPase* genes in transgenic seedlings, total RNA was isolated and RT-PCR was performed using primers specific for *CcUGPase* gene transcripts. The PCR-positive (Fig. 4A) plants were selected as putative transgenic jute. Genomic DNA was isolated from jute leaves of randomly selected three PCR-positive plants and used for Southern blot analysis with hygromycin

Fig. 3. Plant regeneration of jute via direct adventitious shoot formation. (A) Cotyledonary node were culture after 1 week. (B) Adventitious shoots formation after 3 weeks of culture. (C) Adventitious shoots were 2-cm long after 4 weeks. (D) Roots were induced of adventitious shoots after 7 weeks. (E) Plantlet was transferred into soil. (F) Plantlets grow normal in soil.

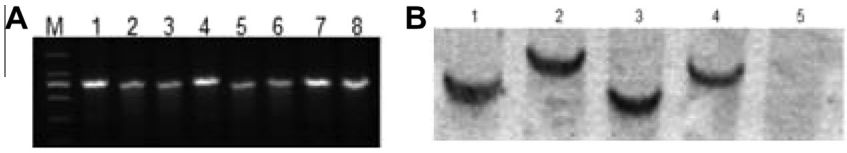

Fig. 4. (A) The result of PCR amplification of hygromycin gene in jute M: DL2000 Marker. 1, 2, 3, 4, 5, 6, 7, 8: The different samples. (B) The result of Southern hybridization 1: Positive control of pCAMBIA1301 vector. 2: Digested by *BSTEII*. 3: Digested by *BglII*. 4: Digested by *NcoI*. 5: The negative control.

Table 1
Morphology, cellulose and lignin analysis of wild-type and transgenic jute.

Lines	Height (m)	Cellulose (%)	Lignin (%)
Wild-type	2.29 ± 0.08[b]	20.8 ± 0.9[b]	17.2 ± 0.5[a]
Transgenic line 1	2.85 ± 0.07[a]	24.7 ± 1.1[a]	17.7 ± 1.0[a]
Transgenic line 2	2.84 ± 0.084[a]	25.5 ± 1.9[a]	16.8 ± 1.4[a]
Transgenic line 3	2.89 ± 0.052[a]	25.3 ± 1.0[a]	17.2 ± 0.4[a]

Each value represents mean of five replicates ± SD. Means were compared using ANOVA. The different letter after data within a column represents significant difference at 5% probability level.
[a,b] $P<0.05$.

specific probe further confirmed the success of the transgenic jute. The results (Fig. 4B) indicated that all the selected plants have T-DNA integrated in Arabidopsis genome and cotain one insertion sites of the transgene, this will remain stable in genetic process of transgenic offsprings. No hybridization bands were detected in the non-transgene plants.

3.3. Phenotype observation, lignin and cellulose contents of transgenic lines

Significant phenotypic differences were observed between with *CcUGPase* gene transgenic lines and the wild-type jute at different stages, transgenic jute exhibiting a faster growth rate and increased height (Table 1). More detailed analysis of the differences between the transgenic and non-transgenic lines was carried out by determining the plant height at 120 d (Table 1). The results indicated a significant increase in plant height of the transgenic lines compared with the wild-type. The data indicate that *CcUGPase* gene involved in plant growth and development [12]. This is consistent with previous studies on the insertion of an exogenous *UGPase* gene into tobacco, which resulted in increased growth rate and plant height [27,28]. These findings suggest that UGPase plays a key role in the strength of sink tissues and the increased growth rate resulted in greater production of fibers compared with non transgenic plants [27]. Some research report that UDP-glucose pyrophosphorylase is not rate limiting for sucrose/starch and cell wall synthesis, but is essential in Arabidopsis [29,30].

Cellulose is a polysaccharide composed of glucose and widely used in paper making and textile industry. Measurement of the cellulose and lignin contents (Table 1) of transgenic and control plants at 100 d revealed no significant differences in the lignin contents (Wild-type: 17.2%; Transgenic line 1: 17.7%; Transgenic line 2: 16.8; Transgenic line 3: 17.2), while the cellulose content was markedly higher in the transgenic lines than that in control (Table 1). These results are consistent with those of previous studies [12]. UGPase catalyzes the conversion of uridine triphosphate into uridine diphosphate glucose, which serves as the glucosyl donor involved in cellulose synthesis.

The cellulose content in jute is much lower than cotton, ramie [15,31], if we can greatly improve the cellulose content, jute is likely to be like cotton applicated in textile industry. Therefore, it can be speculated that *CcUGPase* gene affects plant cellulose synthesis based on the results obtained in this study, we will improve the fiber quality of jute use this gene in the future.

4. Conclusions

In summary, the *UGPase* gene from jute was identified and analyzed. Bioinformatics analysis showed the gene is one of members of UGPase gene family. Real-time PCR showed *CcUGPase* gene with the highest expression level in the stem at 40 and 120 d. PCR and southern hybridization results indicate exogenous gene was integrated into the jute genome. Over-expression of the *CcUGPase* gene in jute resulted transgenic plants exhibited a faster growth rate and increased cellulose content compared with the non-transgenic control plants. These results suggest that the *CcUGPase* gene plays a key role in cellulose synthesis. This information will provide the basis of further investigations aimed at improving jute crop fiber quality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.053.

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