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Original Article

Cloning and Functional Characterization of *CsUGD2* in Cucumber (*Cucumis sativus* L.)

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Abstract

UGD gene encodes UDP-glucose dehydrogenase (UGD) which is a key enzyme in the biosynthesis of cell wall, and it catalyses the irreversible oxidation of UDP-glucose (UDP-Glc) into UDP-glucuronic acid (UDP-GlcA). In cucumber, the expression level of *CsUGD2* genes was higher in phloem tissues of pedicel and fruit than that in stalk. This study investigated the function of *CsUGD2* in cucumber by different methods. Structure analysis indicated that *CsUGD2* gene only has an exon with a length of 1,443 bp. Protein alignment suggested that UGD protein was highly conservative in different species. Phylogenetic analysis showed that *CsUGD2* protein and *CmoUGD2* protein form a same clade which is far away from UGDs in *Arabidopsis*. Real-time fluorescence quantitative analysis of *CsUGD2* in different tissues of cucumber in the same period showed that *CsUGD2* expressed highest in the root of cucumber. When we transformed *CsUGD2* into wild type *Arabidopsis*, the roots of transgenic plants were shorter and the flowering time was delayed. These results suggested that *CsUGD2* may play an important role in the development of cucumber roots and only act on the development of cucumber fruit when compared with other plants fruits. However, the role of *CsUGD2* in regulating the growth and development process of cucumber need to further study.

Keywords: cell wall; *CsUGD2*; cucumber; expression analysis; transgenic plants

Introduction

Cell wall is one of the main structures of plant cells, which plays an important role in plant growth and development. Its main ingredients include cellulose and pectin. The main component of cellulose is linear 1,4-β-D glucan which is accompanied by xyloglucan (Desprez *et al*., 2002). Cellulose leads its organization to possess chemical reducibility, mechanical support and elasticity (Peter, 1991; Sakurai, 1991). The main components of pectin polysaccharide which is a complex network structure are RG-I (rhamnogalactoside I), RG-II (rhamnogalactoside II), HGA (homogalacturonan) and galactan (Hubber *et al*., 1983; Jarvis, 1984). Some specific processes of growth and development of plants are achieved by adjusting the structure of pectin networks, such as root cell differentiation (Wen *et al*., 1999). *UGD* gene encodes UDP-glucose dehydrogenase (UGD), which is a key enzyme in the biosynthesis of cellulose and pectin polysaccharides (Oka and Jigami, 2006). 40-50% of the plant cell wall substance is UDP-Sugars (Oka and Jigami, 2006). UGD catalyzes UDP-Glc (uridine diphosphate glucose) to generate UDP-GlcA (uridine diphosphate glucuronic acid) which is an important precursor of UDP-Sugars. Recent studies have shown that generation of UDP-GlcA may be the rate limit step of the biosynthesis of cell wall precursor thus UGD is important in the production and maintenance of plant cell wall(Amino et al. 1985; Robertson *et al*., 1995). *UGD* genes are highly conservative among different plants (Sieberth *et al*., 1995). There are four genes encoding UGD protein in *Arabidopsis*, UGD1, UGD2, UGD3 and UGD4 (Klinghammer and Tenhaken, 2007).

In the model plant *Arabidopsis thaliana*, *UGD2* gene is mainly expressed in the root tip during the seedling stage, and *UGD2* is highly expressed in the root of the lateral root and the middle column. At the mature stage, *UGD2* gene is expressed predominantly in the roots and begins to express in other parts of the vascular system (Seitz *et al*., 2000) with *UGD2* strongly expressed in the bottom nectary of the anther (Seitz *et al*., 2000). Previous studies indicated that *UGD2* plays an important role in plant growth and development (Xu *et al*., 1995).

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In recent years, some progress of the effects of *UGD* gene on cell wall synthesis has been reported, such as Soybean *UGD* which is a key gene for regulating the supply of soybean hemicellulose precursor (Tenhaken, 1996), Tobacco *UGD* which is highly expressed in tissues that undergoes the synthesis of secondary wall (Bindschedler *et al*., 2005), Sorghum *UGD* which is an important gene involved in cell wall synthesis (Paterson *et al*., 2009) and so on. Cucumber (*Cucumis sativus* L.) is one of the world's important vegetables with good taste and high nutritional value. The vascular system of cucumber is well developed thus it's a model species for the study of vascular bundles (Zhang and Zambryski, 2010). Cucumber phloem plays an important role of the growth and development of cucumber with transporting nutrients produced by photosynthesis of the source organ to the sink organ such as fruit and root (Zhang and Zambryski, 2010). At present, studies on cell wall of phloem of cucumber have rarely been reported. In this study, *CsUGD2* gene of cucumber was cloned, and the function of *CsUGD2* in cucumber growth and development was explored through sequence analysis of protein, phylogenetic characterization, expression analysis and genetic transformation of *Arabidopsis thaliana*.

Materials and Methods

Material and sampling

The high generation inbred line '9930' of cucumber was used as the material. After soaking in 55 °C warm soup, it was germinated in a 28 °C incubator and sown in a nutrient bowl two days later. When the seedlings grew two true leaves, they were planted in the Science Park of China Agricultural University. The roots, stems, leaves, male flower buds, female flower buds and fruits of the same growth stage were harvested and the RNA was extracted from the cucumber at the same growth stage and stored at -80 °C.

The wild type *Arabidopsis thaliana Col* was used as the material, the seeds were sterilized with 0.03% Triton-X and then sowed in MS solid medium. After treated at 4 °C for 3 days, the seeds were placed in a light culture room and transplanted into the sterilized nutrition soil 10 days later. *Arabidopsis* culture conditions were 16 h light / 8 h dark, 22 °C.

Cloning and structure analysis of CsUGD2 gene in cucumber

Total RNA was extracted from each part of cucumber using RNA extraction kit (provided by HuaYueYang Co., Ltd.), and single-stranded cDNA was synthesized using TUREscript H-RTase reverse transcriptase (supplied by Advai).

The CDS sequences and protein sequences of the *Csa4G094000* gene were obtained in the cucumber database (http://cucurbitgenomics.org/). The alignment of the CDS and protein sequences in the *Arabidopsis* database (Lamesch *et al*., 2012) revealed that the gene is a homologue of the *Arabidopsis AtUGD2* (*AT3G29360.1*) and named it *CsUGD2*. CDS sequences were designed using Primer 5 software. The sequences of the full-length primers were 5'-ATGGTGAAGATCTGTTGCATTGG-3' and 5'-CTAAGCCACAGCCGGCATG-3' respectively. Then, *CsUGD2* gene was cloned from cucumber high generation inbred line '9930'.

AtUGD2 and *CsUGD2* were genotyped using online software GSDS2.0 (http://gsds.cbi.pku.edu.cn/).

Protein sequence analysis and phylogenetic tree analysis

There were six UGD homologous protein sequences, including *CsUGD2*, aligned using the Boxshade website (http://www.ch.embnet.org/software/BOX_form.html) with the following parameters: Font Size: 8; Input sequence format: other. Then, the phylogenetic tree of UGD homologues from 17 species was constructed by using the MEGA 5 software using the neighbor-joining method. The parameters were: Statistical Method: Neighbor-joining; No. of Bootstrap Replications: 1,000.

Real-time fluorescence quantitative PCR

Real-time quantitative PCR (qRT-PCR) was performed using the ABI PRISM 7500 real-time PCR system (Applied Biosystems China). Each gene in qRT-PCR was subjected to 3 biological and 3 technical replicates. The cucumber *Ubiquitin* (*Csa000874*) gene was used as an internal control (primers 5'-CACCAAGCCCAAGAAGATC-3' and 5'-TAAACCTAATCACCACCAGC-3') and fluorescent quantitative *CsUGD2* primers (5'-TTGCATCCTGACCGAGTGG-3' and 5'-CGAGAACACGATGAAGCCG-3'). The cucumber root, stem, leaf, female flower bud, male flower bud and fruit on the day of flowering were used as template.

Construction and genetic transformation of cucumber CsUGD2

The plasmid of *CsUGD2*-pMD19-T was screened and sequenced. The plasmid was digested by *BamH* Ⅰ and *Xba* Ⅰ restriction enzymes simultaneously with PBI121 vector. The gel was recovered and ligated with T4 ligase. After cooling for 30 min, heat shock was performed at 42 °C for 90 s in *E. coli* DH5*α*, and then the positive bacterial liquid was sent for sequencing. Recombinant plasmids were transformed into Agrobacterium C58 by electroporation after plasmid extraction, and Agrobacterium carrying *CsUGD2*-PBI121 plasmid was selected using PCR screening. Thereafter, it was transformed into *Arabidopsis* by floral dip method (Clough *et al*., 1998).

Screening and identification of transgenic Arabidopsis plants

The seeds of T_0 generation *Arabidopsis thaliana* were harvested and sterilized with 0.03% Triton-X. The seeds were disseminated on MS solid medium containing 0.1% kanamycin and stored in a light incubator at 4 °C for 3 days. After 10 days, the positive seedlings were transplanted to sterilize nutrition soil. PCR identification was performed on DNA extracted from plant leaves of T_1 generation, and ramets of T_1 generation seeds were harvested and then sowed on MS solid medium containing 0.1% kanamycin. After 4 °C for 3 days, they were placed in light incubator for 10 d, and T_2 generation of transgenic plants were gained. Taking statistical analysis and phenotype analysis on the root length and bolting time of T_2 generation transgenic plants.

Results

Structure analysis, protein sequence analysis and phylogenetic analysis of CsUGD2 gene

The CDS sequence of cucumber *CsUGD2* was cloned, which was similar to *Arabidopsis AtUGD2* gene and contained only one exon. The length of the two exons were highly conserved, both of which were 1,443 bp.

The UGD homologous protein sequences of melon, soybean and rice were found by NCBI. The protein sequence alignment was carried out using Boxshade website. The result showed that *CsUGD2* has the same length as other UGD proteins and has 480 amino acids (Fig. 1). *CsUGD2* was highly conserved on the protein sequence. Both of cucumber and melon belonged to Cucurbitaceae, and *CsUGD2* and *CmoUGD2* had 18 conserved amino acids which was, however, different from other species (Fig. 1). Phylogenetic tree analysis of UGD protein sequences of 17 species, including cucumber, was performed using MEGA 5.0 software. *CsUGD2* was on the same clade as *CmoUGD2* of melon, while relatively far away from *AtUGDs* (Fig. 2).

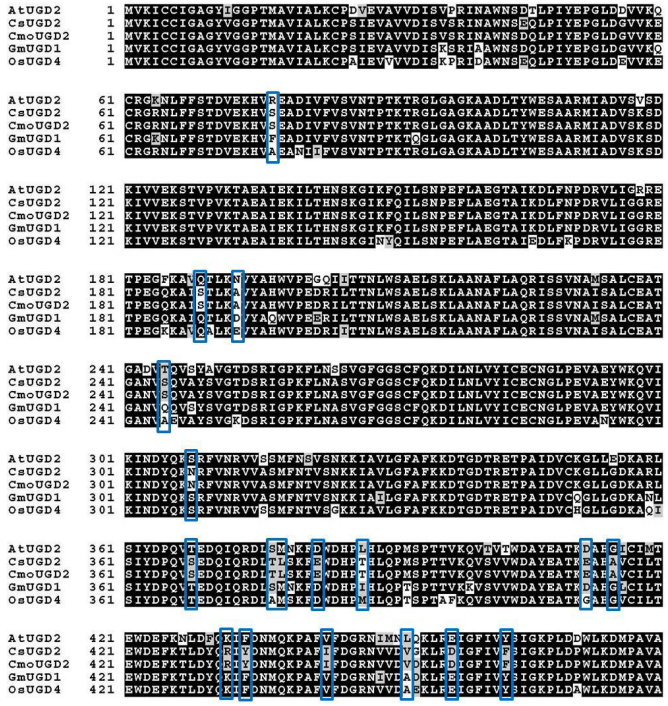

Fig. 1. Alignment of *CsUGD2* and *UGD* homologs in other species *AtUGD2*: *Arabidopsis thaliana UGD2* (AT3G29360.1); *CsUGD2*: *Cucumis sativus UGD2* (Csa4G094000); *CmUGD2*: *Cucumis melo* UGD2 (XP008451675.1); *GmUGD1*: *Glycine max UGD1* (NP001238410.1); *OsUGD4*: *Oryza sativa* UGD4 (XP_015620155.1); Blue rectangles represent conservative amino acids in cucurbits

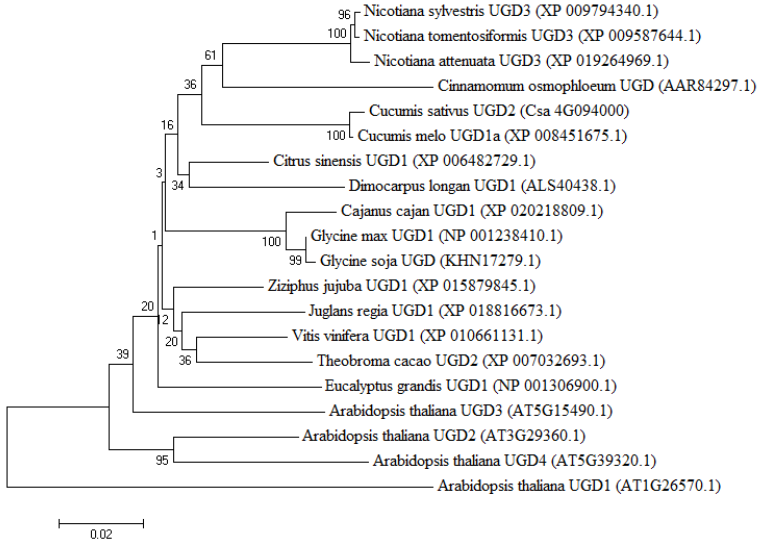

Fig. 2. Phylogenetic analysis of *CsUGD2* and *UGD* homologs in other species

Expression analysis of CsUGD2

The expression of *CsUGD2* in different parts of cucumber was analyzed by real-time quantitative PCR (Quantitative Real-time PCR). *CsUGD2* had the highest expression in roots, and gradually decreased in fruits, leaves and stems, and was significantly lower in female flower buds and male flower buds than in other parts (Fig. 3).

Transformation and expression analysis of CsUGD2

35S promoter was used to construct the *CsUGD2* overexpression vector. After sequencing by PCR, *CsUGD2*-PBI121 was successfully constructed. Agrobacterium containing *CsUGD2*-PBI121 plasmid was used to genetically transform wild-type *Arabidopsis Col*. After T_1 generation was obtained, four transgenic plants were obtained by RT-PCR and DNA identification (Fig. 4A, B). However, there was no significant difference in the expression of *CsUGD2* between strain #4 and wild type *Col* (Fig. 4A). Therefore, we performed phenotypic analysis of strain # 1-3.

The root length (8 strains per strain) was measured and transplanted into sterile nutrient soil when the true leaves of T_2 plants of strain #1-3 were present. The bolting time of every strain was recorded. the root length of the transgenic lines averaged 8.37 mm, which was significantly shorter than that of the wild type ($P < 0.01$) (Table 1, Fig. 5A). The average bolting time was 32.47 days, which was significantly later than that of the wild-type control plants ($P < 0.01$), and the growth was weaker than the wild type (Table 1, Fig. 5B).

Discussion

The *CsUGD2* gene contains one exon and two untranslated regions and its structure is highly similar to that of *Arabidopsis thaliana* and several other species. Based on the protein sequence alignment analysis (Fig. 1), it was found that the UGD amino acid sequences of different species are highly conserved, while *CsUGD2* and *CmoUGD2* still have 18 amino acids conserved in Cucurbitaceae but not consistent with other species (Fig. 1). And UGDs of Cucurbitaceae may have special biological functions.

AtUGD2 of *Arabidopsis* is mainly expressed in the roots during *Arabidopsis* seedling stage and mainly in the vascular system during maturation (Siddique *et al*., 2012). In this study, real-time PCR analysis of different parts of cucumber showed that *CsUGD2* was highly expressed in the roots of cucumber, followed by fruits, leaves and stems (Fig. 3), which was similar to that of *Arabidopsis*. The function of *AtUGD2* in *Arabidopsis* has some reference significance for studying the regulation of *CsUGD2* in the development of the phloem of cucumber. It can be seen that *CsUGD2* heterologous overexpression resulted in very short roots and more bolting time than the wild type in *CsUGD2* transgenic plants (Fig. 5, Table 1), indicating that *CsUGD2* had a major effect on the growth of *Arabidopsis* root development and indirectly led to vegetative growth retardation and weak growth.

Table 1. Length of the seedling roots and bolting time from *Col* and *CsUGD2*-pBI121 *Arabidopsis* plants

Genotype	Line	Number of plants	Length of the seedling roots	Bolting time (d)
35SCsUGD2/Col	#1	8	10.4 ± 0.4**	32.1 ± 0.7**
	#2	8	6.5 ± 0.3**	32.9 ± 0.7**
	#3	8	8.2 ± 0.4**	32.4 ± 0.4**
Col	#1	8	30.8 ± 1.0	26.0 ± 1.2

Note: ** means significantly difference at 1%.

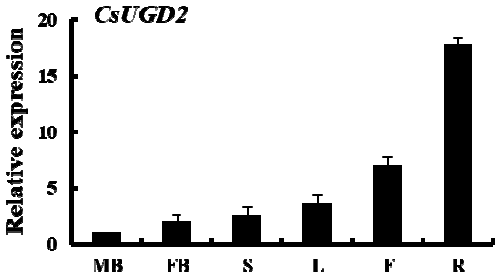

Fig. 3. Expression of *CsUGD2* in different organs of cucumber; MB: male buds; FB: female buds; S: stem; L: leaf; F: fruit; R: root

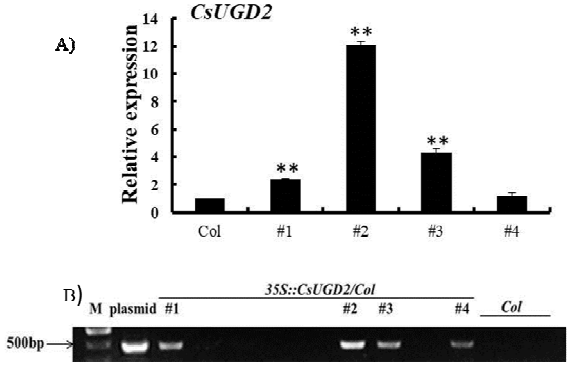

Fig. 4. Real time PCR analysis of *CsUGD2* expression in transgenic *Arabidopsis* and DNA verification of transgenic *Arabidopsis* (#1-#4); A: Real time PCR analysis of *CsUGD2* expression in transgenic *Arabidopsis*; Col: wild type; #1: Line 1; #2: Line 2; #3: Line 3; #4: Line 4; B: DNA verification of *CsUGD2* in transgenic *Arabidopsis* (#1-#4); M: 2000 bp DNA marker; plasmid: Positive control; *Col*: Wild type *Arabidopsis*

Fig. 5. Phenotypes of *CsUGD2*-pBI121 of transgenic Arabidopsis; A: Comparison of 10-day-old transgenic plants (bottom) and wild type (top) plants; B: Comparison of 32-day-old transgenic plants (right) and wild type (left) plants

In addition, heterologous overexpression of *CsUGD2* may affect the synthesis of cell wall in the phloem of *Arabidopsis thaliana*, which may affect the development of phloem as well as the growth and development of *Arabidopsis thaliana*.

It has been reported that the expression of *CsUGD2* (*Csa4G094000*) in the phloem of fruit stalks was low in the differentially expressed genes of phloem in different parts of cucumber fruit and higher in phloem parts of fruit (Zhao *et al*., 2015). Therefore, *CsUGD2* might play a fine regulatory role in the development of cucumber phloem and have an important effect on the development of cucumber fruit. However, the transgenic *Arabidopsis* plants of *CsUGD2* did not show significant differences in fruit while root length was significantly shorter than the wild type (Fig. 5A, Table 1). Quantitative real-time PCR revealed that *CsUGD2* had the highest expression in the roots. In addition, *CsUGD2* may be differentially expressed in different regions of root phloem. Therefore, *CsUGD2* played a very important role in root development of cucumber. Although *CsUGD2* was differentially expressed in phloem of different parts of cucumber fruit, there are significant differences between cucumber and *Arabidopsis* fruits, for cucumber fruits including stalks, stems and fruit, while *Arabidopsis* fruit without stalks. Consequently, *CsUGD2* may only act on development of cucumber fruit. The specific function of *CsUGD2* in cucumber growth and development needs further study.

Conclusions

CsUGD2 was highly expressed in the roots of cucumber, followed by fruits, leaves and stems detected by real-time PCR analysis of different parts of cucumber. Heterologous overexpression of *CsUGD2* resulted in very short roots and more bolting time than the wild type in *CsUGD2* transgenic plants while Quantitative Real-time PCR revealed that *CsUGD2* had the highest expression in the roots. However, the transgenic Arabidopsis plants of *CsUGD2* did not show significant differences in fruit while root length was significantly shorter than the wild type. These results enable to show that *CsUGD2* has a major effect on the growth of Arabidopsis root development, plays a very important role in root development of cucumber and indirectly led to vegetative growth retardation and weak growth. And this study could function to facilitate further studies of specific function of *CsUGD2* in cucumber growth and development.

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