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Molecular cloning and characterization of a cotton phosphoenolpyruvate carboxylase gene

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Abstract

Phosphoenolpyruvate carboxylase (PEPC) plays diverse physiological functions during plant development. In this study, a new phosphoenolpyruvate carboxylase gene *GhPEPC2* is isolated from cotton (*Gossypium hirsutum* cv. zhongmian 35) by RACE-PCR. The cloned cDNA of *GhPEPC2* is 3364 bp in length, and has an open reading frame of 2913 bp, encoding for 971 putative amino acids with a calculated molecular mass of 110.6 kD and p*I* of 5.56. The deduced amino acid sequence of GhPEPC2 shares high similarity with other reported plant PEPCs. Southern blot analysis indicates that the cotton PEPC exists as a small gene family and the *GhPEPC2* might have two copies in the cotton genome. The semi-quantitative RT-PCR reveals that *GhPEPC2* constitutively expresses in all the tissues of cotton and accumulated highly in roots, flowers and embryos but relatively low in stems and fibers. In addition, the recombinant GhPEPC2 has been purified by expressing it in *Escherichia coli* and the catalytic properties of it were also investigated. The results showed that GhPEPC2 is a typical C_3 PEPC with a higher K_m (83.6 μM) and lower V_{max} (8.0 μmol min^{-1} mg^{-1}) compared with the C_3 PEPCs previously reported.

Keywords: Phosphoenolpyruvate carboxylase; Recombinant protein; Southern blot; Cotton

1. Introduction

Phosphoenolpyruvate carboxylase (PEPC), one of the CO_2-fixing enzymes, is ubiquitously distributed in plants, algae, and bacteria, but has not been found in fungi, yeasts and animals so far. The native PEPCs usually act as a homotetramer with a total molecular mass about 400 kD and catalyze the formation of oxaloacetate (OAA) by irreversible carboxylation of phosphoenolpyruvate (PEP) in the presence of Mg^{2+}. Usually, the OAA will be rapidly converted to malate by malate dehydrogenase. Because both the OAA and the malate are important intermediates of the tricarboxylic acid cycle, the primary function of PEPC is to replenish the intermediates of this cycle in most organisms [1].

The plant PEPCs function in the cytoplasm and play diverse physiological roles during the plant development. In C_4 and Crassulacean acid metabolism (CAM) plants, a specific isoform of PEPC expresses highly in photosynthetic tissues and initializes the first step of carbon assimilation. Because of the importance of this reaction, this process has been studied intensively [2]. In C_3 plants and the non-photosynthetic tissues of C_4 and CAM plants, the PEPCs are not involved in photosynthesis; but they have been proved to play key roles in various metabolic processes, such as replenishing the intermediates of the tricarboxylic acid cycle, maintaining the cellular pH and osmotic pressure, regulating the movement of guard cell [1], recapturing the CO_2 produced during respiration

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[3,4], producing carbon skeletons for lipid synthesis during seed development [5] and assimilating nitrogen [6]. In addition, the non-photosynthesis related PEPCs also display different catalytic properties, such as higher affinities for substrate PEP, compared with the photosynthesis related PEPCs [7].

In 1984, the first PEPC gene was cloned from *Escherichia coli* [8]. From then on, a large number of genes encoding PEPC have been isolated from a variety of organisms, including bacteria, cyanobacteria, algae and higher plants. In higher plants, the *PEPC*s exist as a small gene family. For example, in *Arabidopsis*, the PEPC gene family is composed of four members, three of which are typical plant PEPCs and the remaining one is a bacterial-type PEPC [9]. And each member of PEPC family might be associated with different physiological functions. In addition, another breakthrough about the PEPC study was the determination of the three-dimensional structures of PEPCs from *E. coli* [10] and *Zea mays* [11]. This structural information, together with other information obtained by site-directed mutagenesis, has made us further understand the molecular catalytic mechanisms of PEPC. Cotton is one of the most important economic crops worldwide. In 1997, the first PEPC gene sequence from cotton was deposited in GenBank (AF008939), but no detailed analysis about this gene was carried out. In this report, we present the isolation and characterization of a new PEPC gene, *GhPEPC2*, from cotton.

2. Materials and methods

2.1. Plant materials and nucleic acid extraction

Cotton (*Gossypium hirsutum* cv. zhongmian 35) plants were grown with a 16 h light and 8 h dark cycle at 25 °C in a controlled environment. Leaves, cotyledons, stems and roots were collected from 2-week-old seedlings. Embryo buds and flowers were prepared from 90-day-old mature cotton plants. Fibers or seeds were harvested at 5 or 40 days post-anthesis, respectively. All the samples for nucleic acid extraction were frozen immediately in liquid nitrogen and stored at −70 °C. Total genomic DNA was prepared as described by Paterson et al. [12]. Total RNA was extracted from various tissues by PURESCRIPT® RNA purification kit (Gentra, Minneapolis, USA) according to the manufacturer's manual.

2.2. Molecular cloning and sequence analysis of GhPEPC2

The RACE system (3′-Full RACE Core Set, TaKaRa, Dalian, China) was applied to isolate the *GhPEPC2* according to the manufacturer's instructions. We first searched the cotton EST database (National Center for Biotechnology Information BLAST search program) and found an EST (AI725699) which might derive from a new PEPC gene. Based on this EST, we designed a pair of gene specific primers (PEP2-F1: 5-ATGGA TCTTT GCCTG GACAC AG-3′ and PEP2-F2: 5′-ATGCT GCAGG AGATG TACAA TG-3′) and applied nested-PCR to get the 761 bp fragment of the *GhPEPC2*s 3′ region. Then we designed a *GhPEPC2* specific primer (PEP2-R1: 5′-TTTCT TCAAA GTTGG TTCTC AACC-3′) based on this fragment and a universal primer (PEP2-F3: 5′-CGATA TTCTT CAGGA TTTGC ATGG-3′) according to the most conserved region in the 5′ part of plant *PEPC*s. Using the two primers, we cloned the 2435 bp fragment of the *GhPEPC2*s central region. Finally, we cloned the remaining 5′ region of *GhPEPC2* by chromosome walking (Genome Walker Kit, Clontech) using two primers (PEP2-WK1: 5′-CCAAG TTAAG CATGT GGGAG AAAGC C-3′ and PEP2-WK2: 5′-TCCTC AAGTT TCTTG GGGGT ACTCT TC-3′). The PCR-amplified products were cloned into pMD18-T vector (TaKaRa, Dalian, China) and then sequenced. The entire *GhPEPC2* was obtained by ligating these fragments together using proper restriction enzyme sites.

The deduced protein sequences were analyzed by Clustal X [13] and the phylogenetic tree was drawn by TreeView [14]. The accession numbers of the sequences used in this paper were *E. coli* (P00864), *Arabidopsis thaliana-1* (AJ532901), *A. thaliana-2* (AJ532902), *A. thaliana-3* (AF071788), *A. thaliana-4* (AJ532903), *Gossypium hirsutum-1* (AF008939), *G. hirsutum-2* (EU032328, the present study), *Zea mays* C4 (P04711), *Z. mays* C3 (X61489), *Glycine max* (Q02909), *Solanum tuberosum* (CAA62469), *Nicotiana tabacum* (CAA41758), *Sorghum bicolor* (CAA42549), *Oraza sativa-1* (AF271995), *O. sativa-b* (AP002882), *Flaveria pringlei* C3 (Z48966), *F. trinervia* C4 (P30694), *Alternanthera pungens* C4 (AY950665), *A. ficoidea* C3/C4 (AY950666), *A. sessilis* C3 (AY950667), *Brassica napus* (BAA03094), *Lasianthus japonicus* (BAC20365), *Medicago truncatula* (ABE82904), *Thermosynechococcus elongatus* (BAC09464), *Caulobacter crescentus* (NP_420304), *Deinococcus radiodurans* (NP_295007), *Haemophilus influenzae* (NP_439778) and *Rhodopseudomonas palustris* (D89668).

2.3. Southern blot and semi-quantitative RT-PCR analysis

Genomic DNA (30 μg per sample) was digested with DraI, EcoRI, EcoRV, HindIII and XbaI at 37 °C overnight, separated on 0.8% agarose gels and then transferred onto Hybond-N^+ nylon membranes (GE Healthcare, Buckinghamshire, UK). Two probes were used in Southern blot analysis: one was amplified from the conserved coding region of *GhPEPC2* about 1000 bp in length with two primers (5′-CTCAA GAGAC TTGTG GTTGA TCTCA AG-3′ and 5′-TTTGT TCTTC AGACC ACTCT CGGC-3′); the other was from the 3′ untranslated region of *GhPEPC2* about 310 bp in length using the other two primers (5′-CACCG ACCTA CTACA CGAGG TGTG-3′ and 5′-AGAAG CCTCA AAAGG CATTC CTTG-3′). The two probes were labeled with [α-^{32}P]dCTP (Random Primer DNA Labeling Kit Ver. 2, Takara, China). After

hybridization, the membrane was washed at 65 °C in the following buffers twice: 2× SSC with 0.1% SDS and 0.5× SSC with 0.1% SDS, and exposed to X-ray films at −70 °C for 24 h.

Semi-quantitative RT-PCR analysis was carried out according to the manufacturer's instructions (Reverse Transcription System, Promega, USA). A pair of primers (RT-1: 5′-CACCG ACCTA CTACA CGAGG TGTG-3′ and RT-2: 5′-AGAAG CCTCA AAAGG CATTC CTTG-3′) was designed for semi-quantitative RT-PCR. In order to normalize the template amount of different samples, two primers (UBQ7-1: 5′-AGGCA TTCCA CCTGA CCAAC-3′ and UBQ7-2: 5′-GCTTG ACCTT CTTCT TCTTG TGC-3′) were used to amplify the *Ubiquitin7*, a house-keeping gene, as the internal control. The PCR products were separated by electrophoresis on 1.2% agarose gels.

2.4. Expression, purification and enzyme activity assay of recombinant GhPEPC2

The open reading frame (ORF) of *GhPEPC2* was amplified by a pair of primers (Exp-F1: 5′-GGTAC CGAAT TCATG GCGAG TTTTA ATAAT-3′ and Exp-R1: 5′-GTCGA CTCGA GTTAA CCGGT GTTTT GCAT-3′). The amplified PCR fragment was digested by EcoRI and SalI, and then inserted into the corresponding restriction sites of expression vector pGEX-6P-1 (GE Healthcare, UK). The constructed plasmid was confirmed by sequencing and then transformed into *E. coli* strain BL21. The recombinant GhPEPC2 protein was purified according to the manufacturer's instructions, except some modifications: the cells were grown at 25 °C and induced by 50 μM isopropyl thio-β-D-galactoside (IPTG). The enzymatic activity of recombinant GhPEPC2 was measured as described previously [15]. The kinetic parameters, Michaelis constants (K_m) and limiting velocities (V_{max}) of recombinant GhPEPC2 were calculated based on the Michaelis–Menton equation using the software SigmaPlot 8.0.

3. Results and discussion

3.1. Isolation and sequence analysis of GhPEPC2

The sequence analysis indicated that the cloned fragment was 3364 bp in length, which contained an ORF of 2913 bp encoding 971 amino acids with a calculated molecular mass of 110.6 kD and p*I* of 5.56 (Fig. 1). The deduced amino acid sequence was highly homologous to other plant PEPCs reported previously (Table 1), which suggested that it might be a new member of the PEPC gene family. So we designated this gene as *GhPEPC2* (*G. hirsutum* phosphoenolpyruvate carboxylase 2) and deposited it in GenBank database with the assigned Accession No. EU032328. The DNA sequence analysis showed that there was an in-frame stop codon (position 36–38 nt) upstream of the putative ATG (position 103–105 nt) start codon, which indicated that we had cloned the entire ORF of *GhPEPC2*. Interestingly, a cytoplasmic polyadenylation element (TTTATAT) was found near the 3′ end of the *GhPEPC2* (Fig. 1), which suggested that the gene might be subtly regulated at the post-transcriptional level.

In addition, multiple sequence alignment revealed that GhPEPC2 protein had all the conserved functional residues that had been reported previously in plant PEPCs [16] (Fig. 1). For example, an alanine residue was found at position 780 of the GhPEPC2. Previous results indicated that all non-photosynthetic and CAM PEPCs contained an alanine residue at this position while this site was occupied by a serine in all C_4 PEPCs. These suggested that this position is of central importance for the evolution of C_4 PEPC [17]. In addition, a reversible phosphorylation site was identified at the serine residue near the N terminus of GhPEPC2 (Fig. 1). This site was found in all the plant-type PEPCs but absent in bacterial-type PEPCs reported so far. So it was regarded as a hallmark used to distinguish the two forms of PEPCs [18]. The analysis also revealed that the deduced amino acid sequence of GhPEPC2 displayed very similar sizes and high identity with other plant PEPCs, but showed relatively low similarity with the bacterial-type PEPCs (Table 1).

Furthermore, we performed the phylogenetic analysis using the PEPCs from plants, bacteria and algae found in GenBank (Fig. 2). The results showed two distinct branches: the plant-type PEPCs were exclusively grouped in one branch; the bacterial-type PEPCs, including those isolated from *Arabidopsis* and rice, were grouped in another branch. These demonstrated that all the PEPC evolved from a common ancestor. In the plant-type PEPC branch, GhPEPC2 is closely related to one PEPC isolated from potato (CAA62469, 91.3% identical amino acids), but not to the PEPC previously obtained from cotton (AF008939, Table 1). Similarly, the three plant-type PEPCs of *Arabidopsis* were also grouped into two separate branches (Fig. 2), suggesting that the plant-type PEPCs in the same plant species might diverge with different evolutionary patterns. It was interesting that all the C_4 PEPCs of the dicot species were closely related to their own non-C_4-type PEPCs. In contrast, the monocot C_3 and C_4 PEPC (maize, in this study) were grouped into very remote two branches, implying that the C_4 dicot plants might have evolved more recently than the C_4 monocot plants.

3.2. Southern blot and expression pattern of GhPEPC2

In *Arabidopsis*, the PEPC family is composed of four members. Because *G. hirsutum* is an allotetraploid with two distinct subgenomes (A and D) from two ancestors [19], we supposed that there would be several genes encoding PEPC in the *G. hirsutum* genome. In order to determine the approximate gene number of *GhPEPC2* in the cotton genome, we applied the Southern blot analysis with the probes generated from the coding region (Fig. 3(a)) or

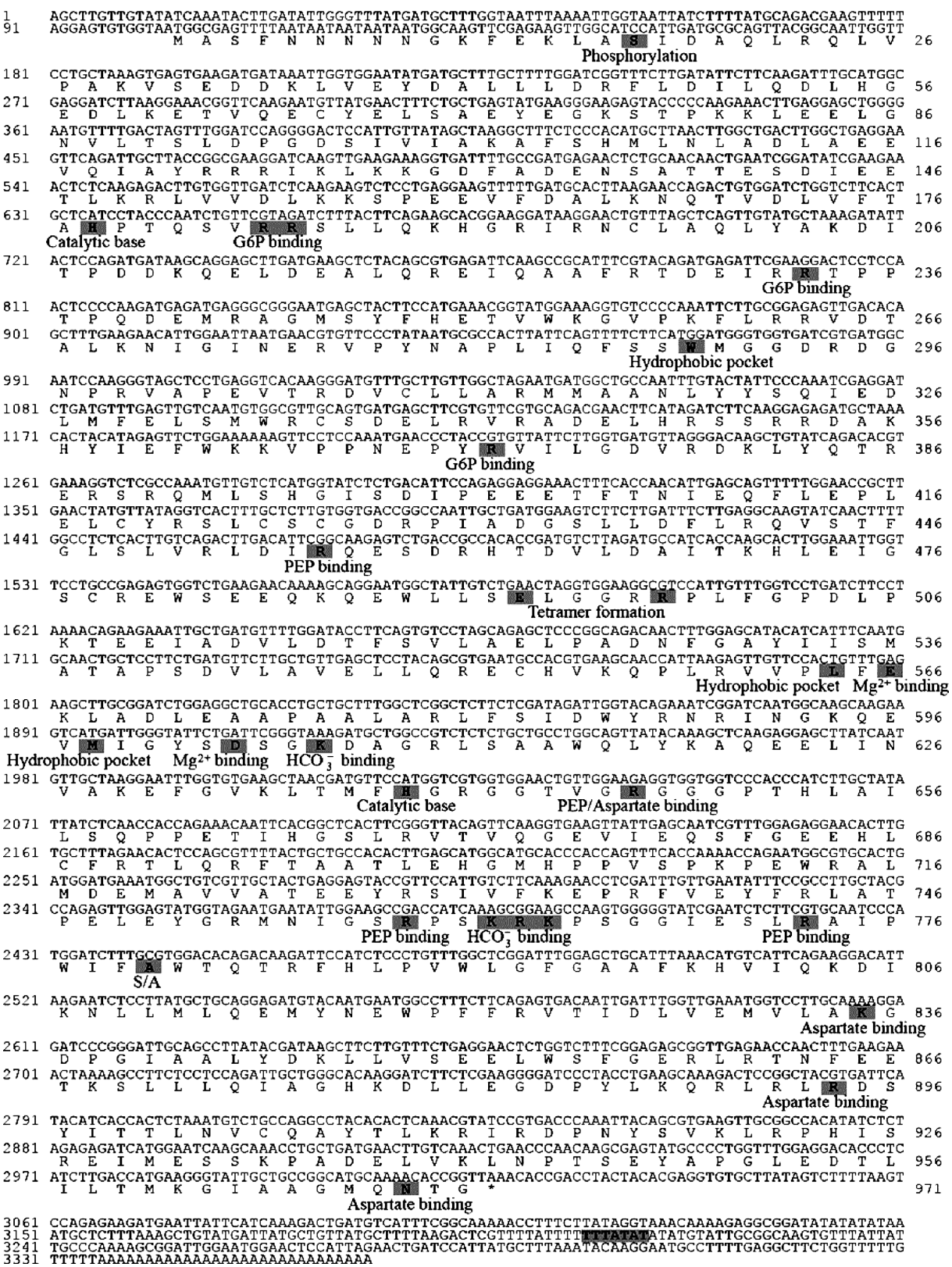

Fig. 1. Sequences analysis of the *GhPEPC2*. Nucleotides and amino acids sequences are numbered on the left and right, respectively. The functional amino acid residues are displayed in gray boxes with annotations under them. The putative cytoplasmic polyadenylation element in the 3′ end is also labeled by gray boxes.

the 3′ untranslated region (Fig. 3(b)) of *GhPEPC2*. As shown in Fig. 3(a), we found complex hybridization bands in each lane while using the probe amplified from the coding region of *GhPEPC2*, which indicated that there were

Table 1
The homologies (% identical amino acids) with other PEPCs

	G. hirsutum-1[a]	*A. thaliana-1*	*A. thaliana-4*	*Z. mays C3*	*Z. mays C4*	*E. coli*
G. hirsutum-2	89.7	88.3	36.6	87.9	78.3	38.1
G. hirsutum-1		89.3	37.2	88.0	78.6	38.7
A. thaliana-1			36.9	85.4	76.6	38.4
A. thaliana-4				36.7	35.9	36.5
Z. mays C3					77.0	38.6
Z. mays C4						37.9

[a] The accession numbers are described in Materials and methods.

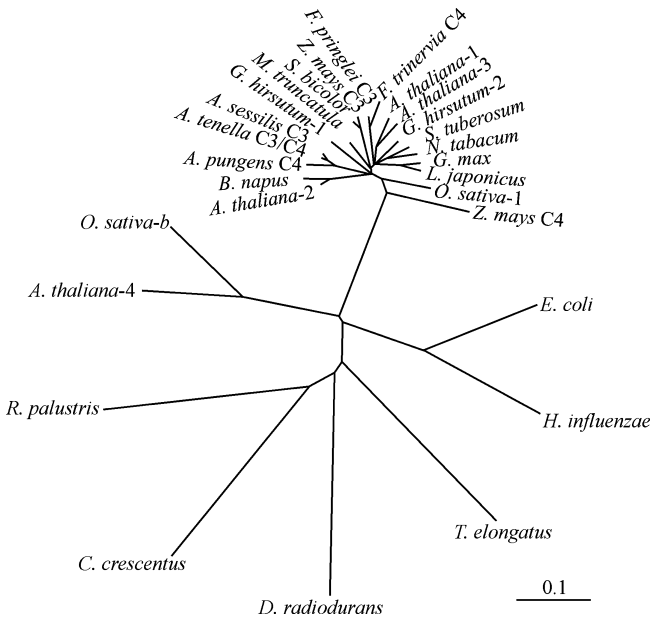

Fig. 2. Phylogenetic analysis of PEPCs. The accession numbers are described in Materials and methods.

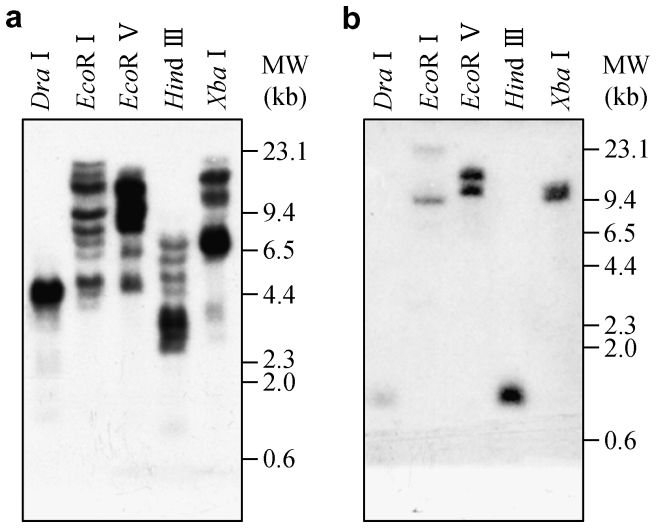

Fig. 3. Southern blot analysis of *GhPEPC2*. The genomic DNA, which was digested with different restriction enzymes, was hybridized with conserved coding region (a) or 3′ untranslated region (b) of *GhPEPC2*.

several genes encoding PEPC in the cotton genome. In contrast, when using the 3′ untranslated region of *GhPEPC2* as the probe, there were only one or two bands in the lanes digested with EcoRI, EcoRV, HindIII or XbaI (Fig. 3(b)). It should also be noted that no clear band was detected in the lane digested with DraI. The reason may be that there was one DraI restriction site within the 3′ untranslated region of *GhPEPC2*, and the minimum probe length required by the Random Primer DNA Labeling Kit (Ver. 2, Takara, China) was 300 bp. So the restriction site of DraI within this region might destroy the effect of the probe. Furthermore, there was no intron existed within the 3′ untranslated region of *GhPEPC2* (data not shown). So we concluded that *GhPEPC2* might exist as a two-copy gene in the cotton genome.

In order to study the expression pattern of *GhPEPC2* in different organs under the normal growth conditions, we performed the semi-quantitative RT-PCR using the total RNA extracted from the roots, stems, true leaves, cotyledons, flowers, seeds, embryo buds and fibers with primers specific for *GhPEPC2*. All of the RT-PCR experiments were repeated at least twice. As shown in Fig. 4, the *GhPEPC2* transcripts were widely distributed in all of the cotton organs, which indicated that the *GhPEPC2* might be a house-keeping gene. In addition, the *GhPEPC2* transcripts were abundant especially in roots, flowers and embryos, but relatively low in other organs. In *Arabidopsis*, the four members of PEPCs showed different spatial

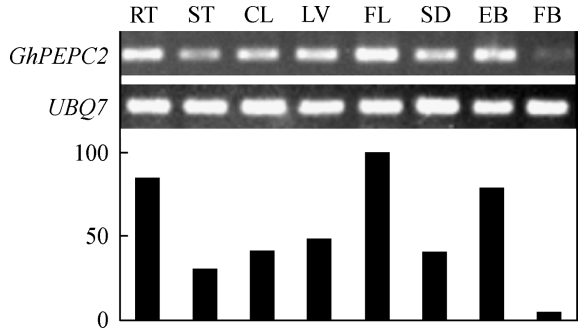

Fig. 4. Expression profiles of *GhPEPC2* in various organs of cotton. Total RNA was isolated from roots (RT), stems (ST), true leaves (LV), cotyledons (CL), flowers (FL), seeds (SD), embryo buds (EB) and fibers (FB) of cotton. The *UBQ7*, the abbreviation of *Ubiquitin7*, was used as the internal control. The results are shown graphically relative to the transcript level of the flowers (value arbitrarily set to 100).

Table 2
Kinetic parameters of GhPEPC2 and previously reported PEPCs

	G. hirsutum-2[a]	*A. sessilis* C3	*A. tenella* C3/C4	*A. pungens* C4	*F. pringlei* C3	*F. trinervia* C4
K_m (μM)	83.6	36.0	42.0	157.0	61.0	652.0
V_{max} (μmol min^{-1} mg^{-1})	8.0	12.0	18.0	21.0	27.0	29.0

[a] The accession numbers are described in "Materials and methods".

expression patterns and each member might play particular physiological functions [20]. Besides, the C_3 PEPC exerted important roles in roots, such as enhancing the aluminum tolerance by exudating organic acids from roots [21], nitrogen assimilation [6] and participating in the phosphorus deficiency reaction [22]. Our results showed that the *GhPEPC2* expressed highly in roots and flowers, therefore we supposed that it might play important functions in these organs. But further studies should be carried out to prove this hypothesis.

3.3. Expression and kinetic properties of recombinant GhPEPC2

In order to analyze the catalytic properties of GhPEPC2, we subcloned the coding region of *GhPEPC2* into the prokaryotic expression vector pGEX-6P-1 and the recombinant GhPEPC2 was produced in the *E. coli* strain BL21. The catalytic properties of purified recombinant GhPEPC2 were investigated and the results are listed in Table 2 (the catalytic kinetic properties of previously reported C_3, C_3/C_4 and C_4 PEPCs are included). The phylogenetic results suggested that the C_4 PEPCs evolved from the C_3 PEPCs, but they showed different expression levels and tissue specificities. In addition, they also differed in their catalytic properties concerning substrate affinity. As a general tendency, the C_4 isoforms displayed lower affinities for PEP compared with the C_3 PEPCs. For example, in the genus *Flaveria*, the K_m (for PEP) of C_4 PEPC was about 10 times higher than the C_3 isoform [7]. The enzymatic assay showed that the GhPEPC2 had a K_m of 83.6 μM and V_{max} of 8.0 μmol min^{-1} mg^{-1}, which were similar with the C_3 PEPCs described previously (Table 2). But it should also be noted that the GhPEPC2 showed a higher K_m value (about two times) than other C_3 PEPCs in Table 2. The reason should be that GhPEPC2 might participate in some special physiological processes during cotton development, but further investigations were needed to support this suggestion.

Acknowledgments

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