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Isolation and Characterization of a Gene Encoding Hexokinase from Loquat (*Eriobotrya japonica* Lindl.)

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Abstract. Hexokinase is the first enzyme in the hexose assimilation pathway; it acts as a sensor for plant sugar responses, and it is also important in determining the fruit sugar levels. The full-length cDNA of a hexokinase gene was isolated from loquat through reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends, which was designated as *EjHXK*1. *EjHXK*1 is 1,839 bp long and contains an entire open reading frame encoding 497 amino acids. The predicted protein of *EjHXK*1 shares 72%-81% similarity with other plant hexokinases. Phylogeny analysis indicated that *EjHXK*1 is closely related to maize and rice hexokinases. Transient expression of the 35S: *EjHXK*1-GFP fusion protein was observed on the cell membrane and cytoplasm. Real-time RT-PCR indicated that *EjHXK*1 is expressed in loquat leaves, stems, flowers, and fruits. *EjHXK*1 transcripts were higher during early fruit development, but decreases before maturation, which is consistent with hexokinase enzyme activity during fruit development and conducive for hexose accumulation in mature fruits. These results imply that *EjHXK*1 may play important roles in the regulation of sugar flux during fruit ripening.

***Additional key words*:** glycolysis, hexose metabolism, subcellular locatoin

Introduction

Loquat (*Eriobotrya japonica* Lindl.), which belongs to subfamily Maloideae of the Rosaceae family, is an evergreen fruit tree originally from southeastern China (Qiu and Zhang, 1996). The tree was introduced to Japan, India, and the Mediterranean Basin, among others, and is presently cultivated in Japan, Israel, and Brazil. The loquat fruit is popular not only because of its flavor, which is succulent, flavorful, and acidic, but also because it ripens in spring or early summer, during which no other fresh fruit is usually available.

Their sugar and acid content give the fruits their characteristic flavor. During ripening, fruit acids are degraded, the sugar content increases, and the sugar/acid ratio increases. Sweetness is the basic taste for fruits. Sweeter loquat fruits are preferred (Cañete et al., 2007). Therefore, understanding the mechanism of fruit sugar metabolism is of great importance. Several reports on the mechanism of loquat sugar accumulation have been proposed. Sucrose, glucose, fructose, and sorbitol are the major sugar components of most loquat fruits (Bantog et al., 1999, 2000; Hasegawa et al., 2010; Shaw and Wilson, 1981). Sorbitol-6-phosphate dehydrogenase (S6PDH), sorbitol dehydrogenase (SDH), cell wall-bound acid invertase (bAIV), soluble acid invertase (sAIV), sucrose synthase (SS), and sucrose phosphate synthase (SPS) are evidently related to sugar accumulation (Bantog et al., 1999, 2000). Sugar increase is paralleled by the rise in enzymatic activity during fruit ripening. S6PDH and NAD-SDH are

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found to regulate sugar accumulation mainly at the transcriptional level (Bantog et al., 1999, 2000). However, all these reported enzymes are mainly involved in the metabolism of loquat disaccharide sugars, sucrose, and sorbitol. Only a few studies have been done on the enzymes involved in loquat hexose metabolism.

Hexokinase (HXK, EC 2.7.1.1) catalyzes the ATP-dependent conversion of hexoses to hexose 6-phosphates (Jang et al., 1997). This phosphorylation is the first step in glycolysis, and is also the first metabolizing step for hexoses. More importantly, the reaction catalyzed by hexokinases is irreversible. Therefore, hexokinases have regulatory as well as catalytic roles, serving as a control site in the metabolic pathway. Hexokinases also act as a sugar sensor, perceiving the level and phosphorylation status of sugars and transmitting this information to the nucleus through a signal transduction pathway (Jang et al., 1997; Karve and Moore, 2009; Smeekens, 1998). Glucokinase and fructokinase are also hexose-phosphorylation enzymes, but glucokinase only phosphorylates glucose, and fructokinase has a much higher affinity for fructose, whereas hexokinase is generally capable of phosphorylating a range of hexoses (Renz and Stitt, 1993).

Hexokinases have been identified in many plants, such as Arabidopsis, rice, tomato, among others (Cho et al., 2006; Dai et al., 1999; Jiang et al., 2003; Roessner-Tunali et al., 2003). Both Arabidopsis and rice have more than two hexokinase genes (Jang, 1997). *AtHXK*1 overexpression in tomato causes a reduction of rapid senescence (Dai et al., 1999). However, the influence of hexokinase on tomato metabolism is highly dependent on the developmental and/or environmental situation, as the influence on primary metabolism diminishes markedly over developmental time (Roessner-Tunali et al., 2003). The antisense repression of hexokinase 1 leads to overaccumulation of transitory starches in potato (Veramendi et al., 1999, 2002). Hexokinases are shown to associate with various subcellular compartments, such as chloroplasts, mitochondria, Golgi complexes, endoplasmic reticula, plasma membranes, and cytosols, which indicate numerous distinct intracellular functions (Cho et al., 2006a; Damari-Weissler et al., 2007; Frommer et al., 2003; Giese et al., 2005; Kandel-Kfir et al., 2006; Olsson et al., 2003; Rezende et al., 2006; Schleucher et al., 1998; Wiese et al., 1999).

Given the importance of hexokinases for the phosphorylation of hexoses for the glycolytic pathway, a putative hexokinase was isolated from loquat in the present study. The subcellular location of the protein, as well as its relationship with hexose during fruit development was characterized and investigated. The results may provide important insight into hexose metabolism in loquat fruit.

Material and Methods

Plant Material

All the experimental materials were obtained from a ten-year-old *Eriobotrya japonica* Lindl. (loquat) tree, growing in the loquat garden at Tangxi, Hangzhou, China. Three trees with similar conditions were chosen for obtaining the materials. Leaves, flowers, stems, and fruits at different developmental stages were collected and immediately frozen in liquid nitrogen (N_2) and stored at -80℃ until analysis. Loquat fruits were selectively collected at 30, 75, 124, 136 days after flowering (DAF), corresponding to the slow development stage, fast development stage, color breaking stage, and the ripening stage, respectively.

cDNA Cloning and Sequence Analysis

Total RNA was extracted from loquat tissues using a modified CTAB method (Gasic et al., 2004). RNA quality was checked by spectrophotometer and gel electrophoresis. First strand cDNA was synthesized using a RevertAid first strand cDNA synthesis kit (Fermentas). To isolate the hexokinase gene from loquat, degenerate primers were designed according to the conserved region by alignment of putative hexokinase genes from GenBank. The forward degenerate primer was 5′-TATGC(G/A)(T/C)TGGA(T/C)CT(A/T)GG(T/G/A)GG(A/G/C)ACAAA(C/T)T-3′, and the reverse primer was 5′-AG(TCA)GC(T/A)GC(T/A)CCAATGCC(T/A)GA(C/T/A)CCATCATT-3′. About 1,160 bp fragment was amplified using this primer pair. For amplification of unknown 5′ end and 3′ end of the putative loquat hexokinase gene, specific primers were designed according to the sequenced fragment, the 3′-Full Rapid Amplification of cDNA Ends (RACE) Core Set, and the 5′-Full RACE kit (TaKaRa). The primer for 3′-RACE was 5′-GCGATGCATCACGATGCATCCTCTG-3′ and 5′-GGGTGTACTGAAAAAGCTGGGAA-3′; the primers for 5′-RACE were 5′-TCGTCCACTTAATAAGGGTCCCCG-3′ and 5′-CAAAGAGTGCATCTGAAGTCCCGACC-3′. The polymerase chain reaction (PCR) products were extracted and cloned into pUC18-T vector (Sangon, Shanghai), transformed into *E. coli* DH5α competent cells, and sequenced by Sangon, Shanghai. To construct a phylogenetic tree, putative plant hexokinase sequences were retrieved from NCBI or Swiss-Prot. The sequences were analyzed using NCBI BLASTX and ClustalW. The phylogenetic tree was drawn using the Maximum Likelihood method of MEGA5 (http://megasoftware.net/).

Transcript Detection by Quantitative Real-time RT-PCR

Real-time reverse transcription (RT)-PCR primers were designed using Primer3 software (Rozen et al., 2000). The forward primer used for loquat hexokinase was 5′-TAG CTACGTCGACAATCTCC-3′; the reverse primer was 5′ -CCGCAATGTAGTCAAAGAGT-3′. The loquat actin gene (GenBank ID: FJ481118) was used as the internal standard. The forward primer for actin was 5′-TGGTCGTACAAC AGGTAT-3′; the reverse primer was 5′-GGGCAACATA TGCAAGCT-3′. QuantiTect SYBR Green PCR Kits (Qiagen) and Applied Biosystems 7300 Real Time PCR System (ABI) were used for the gene expression analysis. Three replicates were performed for each sample.

Subcellular Localization of GFP-tagged Proteins in Onion Epidermal Cells

A 35S: *EjHXK*1-GFP fusion gene was constructed by inserting the open reading frame (ORF) of the full length cDNA, except the stop codon, into the pCAMBIA-1302 binary vector downstream of the constitutive CaMV 35S promoter and into the upstream of GFP. The forward primer used for the PCR amplification of the ORF was 5′ -CGGGGGACTCTTGACATGGGGAAGAAGGCGGTGA TAATC-3′; the reverse primer was 5′-ACTAGTCAGATC TACCATGGAGGATTCATCGATTCCTAGGTATTGTG AG-3′. The underlined sequences were appended for the expression vector construction. The binary vector pCAMBIA-1302 was digested with *Nco*I. The PCR product was extracted from the gel and the purified cDNA was cloned in the *Nco*I-digested pCAMBIA-1302 using the seamless cloning method (GeneArt Seamless Cloning and Assembly Kit, Invitrogen). Onion epidermal segments were peeled and placed on a Murashige and Skoog medium (MS) plate. The recombinant pCAMBIA-35S: *EjHXK*1-GFP plasmids were transiently expressed in the onion epidermal cells using PDS-1000/He Biolistic Particle Delivery System (Bio-Rad). After 16 h to 24 h, the transformed cells were observed under a Zeiss LSM 510 Meta Confocal Microscope.

Determination of Sugar Content

Loquat fruits were ground and extracted according to Qin et al. (2004). The extraction was repeated three times. The crude extract was filtered on a Sep-Pak C18 column to eliminate pigments and other non-sugar compounds, and finally analyzed using high-performance liquid chromatography (HPLC, Waters 1500, USA) equipped with a Sugar-Pak column and detected by a differential refractive index detector (Waters 2414, USA). The mobile phase was distilled water, and the flow rate was 0.6 mL·min^{-1}.

Hexokinase Activity Assay

The enzymatic assay for loquat HXK was performed as described by Fox et al. (1998). The assay for individual samples was repeated three times.

Results

Isolation of Loquat Hexokinase Gene and Sequence Analysis

A partial cDNA fragment was isolated from loquat using degenerate primers designed according to the alignment of plant hexokinase genes from GenBank. Furthermore, the full-length cDNA was obtained through 5′-RACE and 3′ -RACE. The isolated full-length cDNA was designated as *EjHXK*1 (GenBank ID: JF414121). *EjHXK*1 is 1,839 bp, with a 57 bp 5′-UTR and a 291 bp 3′-UTR, and a 1,491 bp ORF that encodes 497 amino acids. The predicted protein

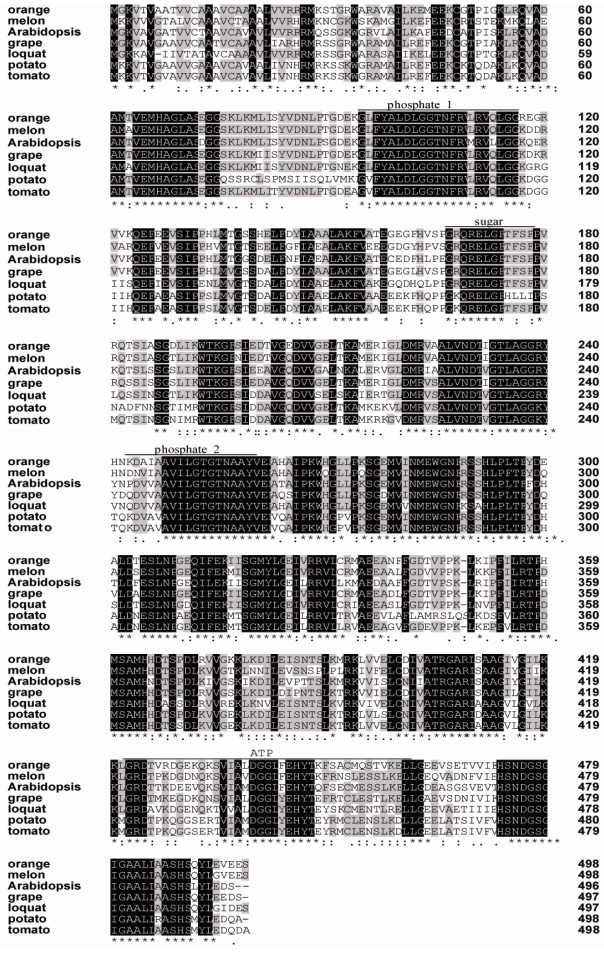

Fig. 1. Comparison of the predicted amino acid sequences of loquat hexokinase with other plant hexokinases. Same amino acids are highlighted in black. Loquat hexokinase was from this work. ATP, two phosphate binding sites, and sugar recognition site are marked (Menu et al., 2001). GenBank accession numbers are orange HXK, AAG28503; grape HXK1, XP_002283608; Arabidopsis HXK2, NP_179576; Arabidopsis HXK1, NP_194642; potato HK1, O64390; tomato HXK, NP_001233957; melon HXK2, ACJ04705.

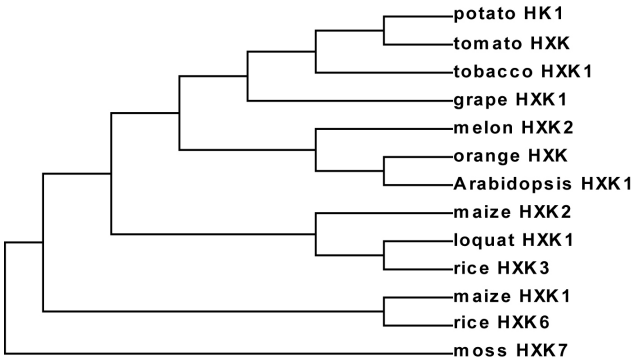

Fig. 2. Phylogenetic tree of putative hexokinase proteins from plants. The tree was generated by using Maximum Likelihood method.

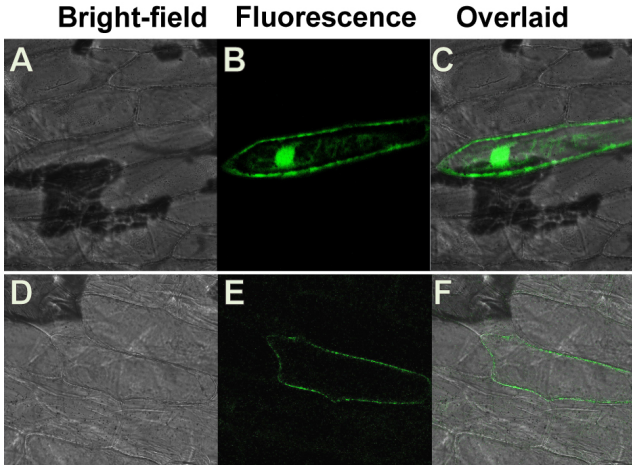

Fig. 3. Transient expression of *EjHXK*1-GFP fusion proteins in onion epidermal cells. 35S: GFP (A, B, and C) and 35S: *EjHXK*1-GFP (D, E, and F) was transiently expressed in onion epidermal cells bombarded by gene gun. Images were obtained under laser-scanning confocal microscope. A, D, bright-field images; B, E, images under the confocal microscope; C, F, overlaid images of A and B, D and E, respectively.

of *EjHXK*1 shared 72%-81% similarity with other plant hexokinases, such as orange and grape (Fig. 1). The ATP binding site, two phosphate binding sites, and sugar recognition site were located in the same highly conserved section in all plant hexokinases, including *EjHXK*1 (Fig. 1). A phylogenetic tree of putative plant hexokinases was drawn using the Maximum Likelihood method (Fig. 2). *EjHXK*1 was clustered into the subgroup with rice HXK3 and maize HXK2; that subgroup was different from the subgroup of grape, tomato, tobacco, melon, and orange hexokinases.

Subcellular Location of *EjHXK*1

The ORF of *EjHXK*1 was ligated into the upstream of the GFP coding region of pCAMBIA-1302 binary vector.

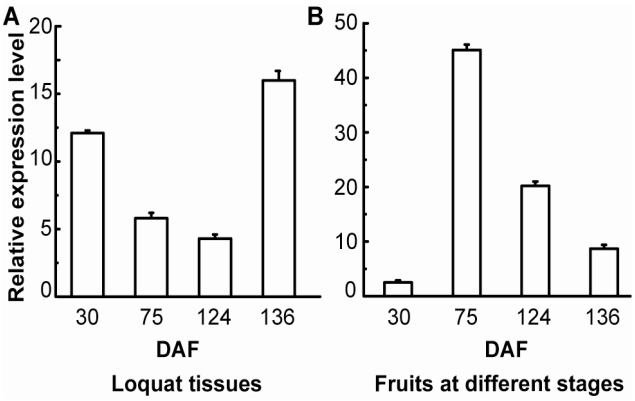

Fig. 4. Transcript analysis of *EjHXK* in loquat tissues by real-time RT-PCR. A, different organs; B, fruits at different developmental stages. Loquat actin was used as an internal reference. Three replicates were carried out for each sample.

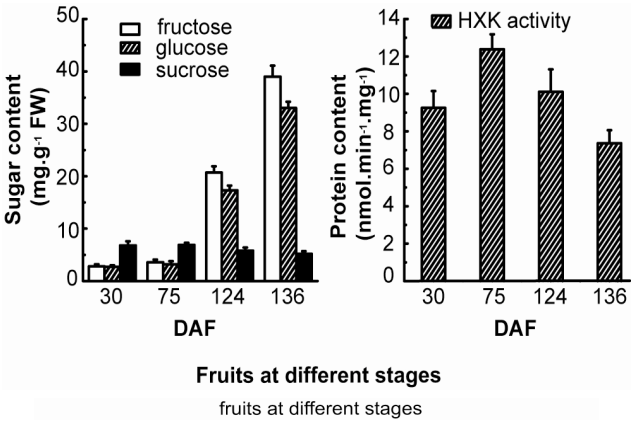

Fig. 5. Changes of hexokinase activity and sugar contents during loquat fruit development.

CaMV 35S was the promoter and the construction was expressed as a GFP-fused protein. The recombinant plasmids were transformed into onion epidermal cells using a gene gun and then GFP signal observed under a confocal microscope. Confocal imaging of the GFP fluorescence revealed that the 35S: *EjHXK*1-GFP fusion protein mainly on the cell membrane (Fig. 3). However, the putative *EjHXK*1 encoded protein was predicted to be located in the chloroplast, mitochondrial, and also in the endoplasmic reticulum by WoLF PSORT (http://woldpsort.org), which predicts the subcellular localization sites of proteins based on their amino acid sequences.

Expression of *EjHXK*1 in Loquat Tissues

Real-time RT-PCR was carried out to examine the expression *EjHXK*1 levels in various organs, young leaves, mature leaves, stems, and flowers of the loquat tree. *EjHXK*1 transcripts were detected in all the experimental materials, among which the flowers possessed the highest levels (Fig. 4A).

Expression of *EjHXK*1 was lower at 30 DAF, but increased at 75 DAF, and then decreased till fruit maturation (Fig. 4B).

HXK Enzymatic Changes and Sugar Accumulation during Fruit Development

Dynamic changes of hexokinase enzyme activity and fructose, glucose, and sucrose contents during loquat fruit development were measured. The hexokinase enzyme activity was at a higher level during early fruit developmental stages, and then decreased during fruit maturation. Hexoses, including glucose and fructose, were kept at a very low level during the early developmental stages, and then increased as the fruit matured (Fig. 5).

Discussion

Hexokinases not only catalyze the irreversible conversion of hexoses into hexose 6-phosphates, which is the first step in glycolysis, but also serve as a glucose sensor, regulating plant metabolism (Jang, 1997). In the present study, a full-length cDNA of hexokinase gene, *EjHXK*1, was isolated from loquat by RT-PCR and RACE technology. *EjHXK*1 contained an intact ORF, encoding 497 amino acids. The predicted protein of *EjHXK*1 shared 72%-81% similarity with hexokinases from grape, rice, and so on (Fig. 1). However, *EjHXK*1 was clustered with rice HXK3 and maize HXK2, a subgroup separate from that of grape, tomato, tobacco, melon, and orange (Fig. 2). Karve et al. (2010) indicated that the plant hexokinase family contains 5-11 genes, including metabolic catalysts, glucose signaling proteins, and non-catalytic apparent regulatory enzyme homologs. *EjHXK*1 possibly functions as any of these roles, which needs to be further elucidated through overexpression or antisense repression analysis.

Hexokinases are suggested to have numerous distinct intracellular functions and associated with various subcellular compartments, like chloroplasts, mitochondria, the plasma membrane, and the cytosol, among others (Cho et al., 2006; Damari-Weissler et al., 2007; Kandel-Kfir et al., 2006; Rezende et al., 2006). However, in the present study, transient expression of the 35S: *EjHXK*1-GFP fusion protein was observed mainly on the cell membrane. There was no plastid fluorescence signal observed (Fig. 3) based on observing at least twenty individual transformed cells, but the *EjHXK*1 encoded protein was predicted to be located in the chloroplast, mitochondria, and also in the endoplasmic reticulum by WoLF PSORT. The differences between the experimental results and WoLF PSORT prediction probably are due to WoLF PSORT performs based on protein amino acid sequences, which result in about 80% prediction accuracy.

In plants, hexokinase catalyzes the ATP-dependent conversion of hexoses into hexose 6-phosphates. This catalysis is the first step of glycolysis, and is also the first metabolic step for hexoses. Hexokinase genes from one plant show different characteristics. Rice hexokinase genes show special expression patterns. *OsHXK*2 through *OsHXK*9 are expressed in various organs, whereas *OsHXK*10 is pollen specific. Sugars can induce the expression of some but not all rice hexokinase genes (Cho et al., 2006). *LeHXK*2 mRNA was detected in tomato roots, leaves, flowers, and developing fruit, most of which were highly expressed in flowers (Menu et al., 2001). *LeHXK*3 is membrane-associated. *LeHXK*4 is plastidic (Kandel-Kfir et al., 2006). *LeHXK*1 and *LeHXK*4 are the dominant hexokinases in all tomato tissues. *LeHXK*1 expression is very low in leaves and very high in young tomato fruits (Dai et al., 2002). In the present study, *EjHXK*1 was expressed in all experimental loquat materials, including leaves, stems, flowers, and fruits. The number of *EjHXK*1 transcripts was higher at 75 DAF, which was the early fruit developmental stages, but decreased before fruit maturation, which was consistent with the hexokinase enzyme activity during fruit development (Figs. 4 and 5). The high expression of hexokinase mRNA and high enzymatic activity could not make up for the hexose accumulation in young fruits. Only a reduction in mRNA level and enzymatic activity would be conducive to the hexose accumulation in a mature fruit. These results implied that *EjHXK*1 may play important roles on the regulation of sugar flux during fruit ripening.

Hexokinase genes are proposed to be sugar sensors based on studies with overexpressed *AtHXK*1 transgenic Arabidopsis plants (Jang et al., 1997). *AtHXK*1 overexpression in tomato plants exhibits dramatic changes in phenotypes, growth inhibition, and accelerated senescence. These results indicate that hexokinase also mediates the tomato sugar signaling pathways (Dai et al., 1999). However, *StHXK*1 overexpression or repression in potato plants induces significant changes in hexokinase activity, but does not affect the overall physiology of the transgenic plants. In the present study, conserved hexokinase-binding domains for sugar, phosphate, and ATP were identified in *EjHXK*1, which had higher homology with *Arabidopsis* and tomato hexokinases. Over-expression and antisense repression of *EjHXK*1 are under-taken to explore whether *EjHXK*1 is involved in loquat sugar sensing and catalyzing.

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