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Molecular Cloning and Expression Analysis of a Hexokinase Gene, *MdHXK1* in Apple

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Abstract

A hexokinase gene named *MdHXK1* (MDP0000309677) was cloned from 'Gala' apple (*Malus* × *domestica* Borkh.). Sequence analysis showed that the *MdHXK1* gene was 1 497 bp long and encoded 499 amino acids. The predicted molecular mass of this protein was 54.05 kD, and the pI was 5.76. A phylogenetic tree indicated apple MdHXK1 exhibited the highest sequence similarity to *Pyrus bretschneideri* PbHXK1. Analysis of the functional domain showed that the MdHXK1 protein included two conserved kinase domains. The prediction of subcellular localization suggested that the MdHXK1 protein was mainly localized in the cytoplasm. There was an indication that *MdHXK1* existed as one copy in the apple genome by Southern blotting. Silico analysis suggested that the promoter sequence contained several typical *cis*-acting elements, including defense, sugar signaling and phytohormone responsive elements. Quantitative real-time PCR analysis demonstrated that the *MdHXK1* gene was mainly expressed in stem and flower tissues. During the development of apple fruits, the expression of the *MdHXK1* gene initially increased and then decreased. The changes on Glc phosphorylation relative activity and glucose concentration showed the same trend. In addition, the expression of this gene was induced by salt stress, low temperature, and abscisic acid (ABA). Finally, we obtained and purified the fused MdHXK1 protein by recombinant prokaryotic expression. Studies have demonstrated that *MdHXK1* may participate in sugar metabolism in apple fruits. Enzyme encoded by *MdHXK1* is a key factor in the mediation of sugar accumulation. Recently, researchers on hexokinase at home and abroad mainly focused on model plants, such as *Arabidopsis*, tobacco and rice, but orchard fruit like apple were underresearched. Our research established the foundation for the further study of the functions of *MdHXK1*.

Keywords: apple; hexokinase; *MdHXK1*; molecular clone; expression analysis; prokaryotic expression

1. Introduction

The glucose sensor, hexokinase (HXK), phosphorylates hexose in plant metabolism. Hexose must be phosphorylated by HXKs to produce hexose-6-P that can then be used for metabolic processes in the glycolytic pathway, which provides energy and intermediate metabolites for the physiological activities of plants. Therefore, the phosphorylation of hexose is essential to sustain carbon flow for starch synthesis and respiration (Claeyssen and Rivoal, 2007). Studies have found that sugars prominently repress the expression of genes involved in photosynthesis and glyoxylate metabolism in cucumber culture cells and maize protoplast and this type of repression can be reverted by a specific HXK competitive inhibitor (Karve et al., 2008), which reveals that HXKs play a part in sugar sensing. In *Arabidopsis*, the HXK family consists of six identified members (AtHXK1, AtHXK2, AtHXK3, AtHKL1, AtHKL2 and AtHKL3), and three of these with catalytic function that can phosphorylate glucose (Jang and Sheen, 1994). AtHXK1 has been proven to be a glucose sensor in *Arabidopsis*. Analyses of transgenic *Arabidopsis* plants with gain or loss of AtHXK function show that the wild type *Arabidopsis* shows abnormal greening of the cotyledons, developmental delay and stunted hypocotyls and roots on half-strength Murashige and Skoog (MS) plates containing 6% glucose. Transgenic plants overexpressing *AtHXK1* show sugar hypersensitivity and antisense plants show sugar hyposensitivity (Jang et al., 1997). In a previous study, the AtHXK1 protein without catalytic function was obtained using a site-specific mutagenic approach. Then, the mutant gene was transferred into an *Arabidopsis* HXK1 mutant *gin2-1* (glucose insensitive 2-1), and the results show that the transgenetic

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line without catalytic function still can sense sugar signal, which demonstrates that the sugar sensing function and catalytic function of AtHXK1 are uncoupled (Moore et al., 2003).

In addition to the regulation function in signal transduction, HXK1 has been involved in the regulation of plant growth and development as well as in hormone signal conduction (Moore et al., 2003; Qin et al., 2003; Rolland et al., 2006; Kunz et al., 2014). The use of a *gin2-1* mutant reveals the importance of glucose sensing and signal conduction in plant growth. Under diverse environmental conditions, AtHXK1 has different functions, e.g. it may repress plant growth through restraining seed germination and seedling growth or promote the development and growth of adult plants; the growth promoting function will accelerate senescence and shorten the plant life (Smeekens et al., 2010). Wild type *Arabidopsis* grows rapidly and shows premature aging under strong light conditions. However, the mutant *gin2-1* line is small in size, dark green and exhibits less cell expansion, which reflects the fact that HXK1 promotes cell expansion and the growth of root, leaf and inflorescence under strong light conditions (Loreti et al., 2001). In transgenetic tomato plants, overexpression of *AtHXK1* caused a stunted phenotype and premature senility. These results show that sugar signaling mediated by HXK1 can promote or repress plant development and growth; in addition, the different roles of HXK1 are decided by the inherent glucose level and the sensitivity of plants to glucose (Granot, 2007). The sugar signal mediated by HXK1 represses the expression of *OsCIPK15* [calcineurin B-like (CBL) interacting protein kinase 15] by oxidative phosphorylation (Yim et al., 2012).

With more in-depth research, blast searches of the *Malus* Genome Database using *Arabidopsis thaliana HK* sequences as a query was performed and MDP0000309677 was denominated *MdHXK1*. Li et al. (2012) noted that *MdHXK1* may take part in processes related to sugar metabolism and accumulation. However, it remains unclear whether *MdHXK1* responds to abiotic stresses or not. Feng et al. (2015) investigated the biochemical characterization and the protein structure of AtHXK1, and laid a foundation for further research on HXK1. To date, little research has been conducted on *HXKs* in woody fruit crops, e.g. apple tree, and future research on the function of *HXKs* in sugar metabolism will contribute to breed improvement and improve apple quality. In this study, we used 'Gala' apple (*Malus* × *domestica* Borkh.) as study materials, and isolated the *MdHXK1* gene by gene cloning. Its bioinformatics and Southern blotting were analyzed. Then, we detected its expression patterns in different tissues, different fruit growth stages and in response to various stresses using qRT-PCR. In addition, Glc phosphorylation relative activity of MdHXK1 and glucose concentration were detected during apple fruit development. Lastly, recombinant protein was obtained by prokaryotic inducement. The goal of the present study was to establish the foundation for the further study of the functions of MdHXK1 protein.

2. Materials and methods

2.1. Plant materials and treatments

The 10-year-old 'Gala' apple trees were planted in the Experimental Orchard of the Shandong Institute of Pomology (Tai'an, China). Samples were taken at different fruit growth stages (30, 60, 90, 120 d after blossom) starting in April 2014. Growing root, caulicle, new leaves, flowers at the initial bloom stage and young fruit of 30 d after blossom to be used for gene expression were quickly frozen in liquid nitrogen and stored at −80 °C after sampling. Tissue cultured seedlings were used for analyzing the effects of *MdHXK1* expression in response to abiotic stresses. Tissue cultured 'Gala' seedlings were submerged in medium with the inclusion of 100 mmol · L^{-1} NaCl, at 10 °C, 100 μmol · L^{-1} abscisic acid (ABA) and samples were collected at 0, 1, 3, 6, 9, 12, 24, and 48 h after treatment for the expression analysis.

2.2. Quantitative RT-PCR analysis of gene expression

Total RNA of each sample was extracted using an RNA plant plus reagent (Tiangen Biotech Co., Ltd., Beijing, China). First-strand cDNA was synthesized using a PrimeScript® RT reagent Kit (TaKaRa Biotechnology, Tokyo, Japan). Quantitative RT-PCR was conducted using cDNA templates to detect the expression level of *MdHXK1* in apple. Apple *18S rRNA* (the sense primer: 5′-ACACGGGGAGGTAGTGACAA-3′ and the reverse primer: 5′-CCTCCAATGGATCCTCGTTA-3′) was used as loading controls. The specific primer sequences (the sense primer: 5′-CTGAAAGTGGTCGGGAGCAAA-3′ and the reverse primer: 5′-TGCACGAGTGGCAACTATGTCG-3′) were used for PCR analysis. Fluorescence quantitative PCR reactions were conducted using an Ultra SYBR Mixture (with ROX) Kit (CWBIO Co. Ltd., Beijing, China). The 20 μL reaction system consisted of 2 × Ultra SYBR Mixture 10.0 μL, the sense primer (10 μmol · L^{-1}) 1.0 μL, the reverse primer (10 μmol · L^{-1}) 1.0 μL, cDNA 1.0 μL, and ddH_2O 7.0 μL. All of the samples were tested in three technical replicates. The programs were pre-denaturated at 95 °C for 10 min; and then 40 cycles containing denaturation 15 s at 95 °C, annealing 15 s at 56 °C, and elongation 10 s at 65 °C; fluorescence was detected and collected at the third step per cycle. Specific mRNA level was quantified using the cycle threshold (Ct) $2^{-\Delta\Delta Ct}$ method (Hu et al., 2012).

2.3. Molecular cloning and bioinformatics analysis of the MdHXK1

One pair of primers was designed and synthesized based on the published *MdHXK1* sequences, and PCR amplification was performed using the cDNA of 'Gala' cultured seedlings as the template. The PCR amplification procedure was as follows: predenature at 94 °C for 5 min, then 94 °C 30 s, 60 °C 30 s, 72 °C 90 s, for 32 cycles, finally extended at 72 °C for 10 min. The PCR product was separated by 1.25% agarose gel electrophoresis, isolated by a TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.4.0 (TaKaRa Biotechnology, Tokyo, Japan), then cloned into the pMD18-T vector and sequenced.

Chemicophysical properties, protein structure and function domain, cellular localization and genomic structure were predicted and analyzed on-line using the following websites: http://web.expasy.org/protparam/; http://smart.embl-heidelberg.de/; and http://gsds.cbi.pku.edu.cn/index.php/, respectively.

2.4. Southern blotting

The 'Gala' cultured seedlings genomic DNA was extracted using the cetyl-trimethylammonium bromide method. One pair