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Myo-Inositol content determined by *myo*-inositol biosynthesis and oxidation in blueberry fruit

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ABSTRACT

Myo-inositol metabolism in plant edible organs has become the focus of many recent studies because of its benefits to human health and unique functions in plant development. In this study, *myo*-inositol contents were analyzed during the development of two blueberry cultivars, cv 'Berkeley' and cv 'Bluecrop'. Furthermore, two *VcMIPS 1/2* (*Vaccinium corymbosum MIPS*) genes, one *VcIMP* (*Vaccinium corymbosum IMP*) gene and one *VcMIOX* (*Vaccinium corymbosum MIOX*) gene were isolated for the first time from blueberry. The expression patterns of *VcMIPS2*, *VcIMP* and *VcMIOX* genes showed a relationship with the change profiles of *myo*-inositol content during fruit ripening. The results were further confirmed by the analyses of the enzyme activity. Results indicated that both *myo*-inositol biosynthesis and oxidation played important roles in determining of *myo*-inositol levels during the development of blueberry. To our knowledge, this report is the first to discuss *myo*-inositol levels in fruits in terms of biosynthesis and catabolism.

1. Introduction

As a metabolite, the importance of *myo*-inositol (MI) is paramount for almost all biological systems. For plants, MI is the precursor of many critical molecules, such as phosphatidylinositol, members of the raffinose family, MI hexaphosphate (also known as phytic acid, PA), and ascorbic acid (AsA). MI also plays important roles in signal transduction, cell wall formation, phosphate storage, osmotic regulation, and antioxidation (Chatterjee et al., 2006; Donahue et al., 2010; Loewus & Murthy, 2000). Furthermore, recent data showed that both MI and PA, as dietary supplements, were effective antioxidant, hypolipidemic, anticarcinogenic, and antidiabetic agents, although PA has been traditionally considered as an antinutrient (Croze et al., 2012; Kumar, Sinha, Makkar, & Becker, 2010; Okazakia & Katayamab, 2014). MI synthesis and catabolism in plant edible organs have become the focus of many recent works because of the benefits of MI to human health, as well as its unique functions in normal plant development.

De novo synthesis of MI consists of two steps. D-glucose-6-phosphate is first catalyzed by L-*myo*-inositol-1-phosphate synthase (MIPS; EC 5.5.1.4) (Loewus, Bedgar, & Loewus, 1984). This reaction is followed by dephosphorylation of L-*myo*-inositol 1-phosphate to free MI, which is catalyzed by the MI monophosphatase (IMP; EC 3.1.3.25) (Loewus & Loewus, 1983; Torabinejad, Donahue, Gunesekera, Allen-Daniels, & Gillaspy, 2009). These two reactions comprise the Loewus pathway, which was first studied in plants and is the only documented biosynthetic route to produce MI (Donahue et al., 2010). In this process, MIPS is the rate-limiting enzyme. Numerous studies have focused on the relationship between the *MIPS* expression level and abiotic stresses in plants (Cui, Liang, Wu, Ma, & Lei, 2013; Wang et al., 2011). However, the regulation mechanisms of *MIPS* expression during fruit ripening have not been thoroughly studied.

Aside from synthesis, another important regulatory point of MI homeostasis is the MI oxidation catalyzed by the *myo*-inositol oxygenase (MIOX; EC 1.13.99.1). This enzyme utilizes molecular oxygen and irreversibly catalyzes the oxygenative cleavage of MI to D-glucuronic acid (D-GlcUA), which is an important precursor for both plant cell wall and an alternative AsA pathway in plants. Thus, enzyme MIOX is a good candidate to control the flux of carbohydrates through this pathway (Lorence, Chevone, Mendes, & Nessler, 2004; Torabinejad & Gillaspy, 2006). However, compared

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with MIPS, few data are available about enzyme MIOX. Until now, only few plant *MIOX* genes have been characterized. Thus, identification of the *MIOX* genes from various plants will help us understand the catabolism of inositol in organisms and reveal the underlying mechanisms (Alford, Rangarajan, Williams, & Gillaspy, 2012; Duan et al., 2012; Torabinejad & Gillaspy, 2006).

Highbush blueberry (*Vaccinium corymbosum* L.) is one of the most economically important fruit crops worldwide, particularly in North America (Zifkin et al., 2012). In recent years, the planting area of highbush blueberry has annually increased in North China. Blueberries are among the richest sources of health-promoting compounds and antioxidants in fruits (Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001). Therefore, the metabolism of the bioactive compounds in blueberry fruits is of great interest. However, to date, several studies have focused on the metabolism of the flavonoid and phenolic acid compounds in blueberries (Rasmussen, Frederiksen, Struntze Krogholm, & Poulsen, 2005; Tan et al., 2014; Taverniti et al., 2014).

In our previous study, the AsA content in fruits was compared with the expression profiles of AsA biosynthetic and recycling genes between 'Berkeley' and 'Bluecrop' cultivars, which were found to contain different levels of AsA in ripe fruits. Our results indicated that the L-galactose pathway was the predominant route of ascorbic acid biosynthesis in blueberry fruits (Liu et al., 2015). Considering that MI may be also a precursor for an alternate AsA biosynthetic route in plants, which is known as MI pathway in animals, we analyzed the MI accumulation patterns during the fruit development of the two blueberry cultivars, namely, 'Berkeley' and 'Bluecrop'. Furthermore, the expression profiles of the key genes, including the *VcMIPS* (*Vaccinium corymbosum MIPS*), *VcIMP* (*Vaccinium corymbosum IMP*) and *VcMIOX* (*Vaccinium corymbosum MIOX*) were systematically compared between the two cultivars. In addition, the change patterns of the corresponding enzyme activity were analyzed. To our knowledge, the present research is the first to report the MI metabolism of blueberries. The current results will provide new information for us to understand the mechanisms regulating MI accumulation and the roles of MI in AsA biosynthesis during the development of blueberries.

2. Materials and methods

2.1. Plant materials

Two highbush blueberry cultivars, 'Berkeley' and 'Bluecrop' planted in an organic blueberry farm in Yantai, Shandong Province, China (37°31′N, 121°21′E) were used in this study. Six 6-year-old trees of each cultivar were selected and randomly divided into three groups, with two trees in each group. Fruit set and ripening initiation of blueberries were asynchronous; therefore, the fruits were randomly harvested in batches during the harvest season in 2013 and 2014 and sorted into six stages according to size and fruit color, following the validated methods used for sorting blueberries (Zifkin et al., 2012). Fruits at the same stage, which were collected from two trees belonging to the same group, were mixed together as one sample. The number of fruits from each tree was equal. Thus, each cultivar consisted of three sample replicates at each stage. Each sample was placed in a centrifugal tube, immediately frozen in liquid nitrogen, and then stored at −80 °C until use.

2.2. Measurements of MI concentrations

MI contents were determined using high-performance liquid chromatography (HPLC), as described by Liang et al. (2011) and Li, Wang, Li, Yao, and Hao (2013). Berries measuring 2 g were homogenized in cold 80% (w/v) ethanol, extracted at 60 °C for 30 min, and then centrifuged at 4 °C. Pellets were washed with 80% (w/v) ethanol and centrifuged twice. Supernatants of the three groups were combined and evaporated under vacuum until ethanol was removed. Sugars were resuspended in double-distilled water and filtered through a SEP-C18 cartridge and then a 0.45-μm nylon filter. The injection volume was 10 μL. Sugars were detected using a SHIMADZU RID-10A refractive index detector with reference cell maintained at 40 °C. MI concentration was calculated as milligrams per gram of fresh weight.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the fruits at different stages through a modified CTAB method and treated with RNase-free DNase I. Two micrograms of the total RNA were used to synthesize the first-strand cDNA by using a PrimeScript First Strand cDNA Synthesis Kit (Takara, China).

2.4. Isolation of the target genes by reverse-transcription PCR (RT-PCR)

To date, the sequences of blueberry *MIPS*, *IMP* and *MIOX* remain unavailable. To clone *VcMIPS*, degenerate primers (Pmips) were designed using homologous gene sequences from other plants, such as *Arabidopsis*, kiwifruit (*Actinidia deliciosa*), and chickpea (*Cicer arietinum*), which were downloaded from the National Center for Biotechnology Information. RT-PCR was performed as follows: 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min. Two fragments of approximately 1.7 kb were amplified from cDNA by the degenerate primers. Comparison with sequences in the databases revealed that each sequence had a complete open reading frame (ORF), and was highly homologous to *MIPS* genes from other plant species. The two genes were named *VcMIPS1* and *VcMIPS2*, respectively. For isolating *VcIMP* and *VcMIOX*, we firstly designed two degenerate primers (Pimp1 and Pmiox1) and performed 3′ RACE PCR, respectively. Subsequently, 5′ RACE PCRs were performed to obtain the 5′ regions of *VcIMP* and *VcMIOX* using the gene-specific primers, Pimp2 and Pmiox2, respectively. 3′ and 5′ RACE PCRs were carried out in accordance with the manufacturer's instructions (SMART RACE cDNA Amplification Kit, Clontech). To obtain the full-length cDNA sequence of the two genes, RT-PCR was performed using their own 5′ and 3′ specific primers. All primers used in this study are listed in Supplementary Table S1.

2.5. Sequence comparison and phylogenetic analysis

Multiple protein sequences were aligned using the Clustal W method in DNAMAN software package. A phylogenetic tree was constructed using the neighbor-joining method of MEGA 3.1 with 1000 bootstrap replicates (Su, Zhang, Yin, Zhu, & Han, 2015; Su et al., 2013).

2.6. qRT-PCR analysis

Real-time RT-PCR (qRT-PCR) was performed to detect the expression profiles of the target genes. Gene-specific primers were designed (Supplementary Table S2) using the non-conservative regions at the 3′ end. Primer specificity was confirmed by corresponding melting curves with a single sharp peak or a single amplified fragment with the correct predicted length. To further verify the PCR results, PCR fragments were inserted into the pGEM-T vector and then sequenced. Blueberry *GAPDH* and *SAND* were selected as reference genes in accordance with our previous study (Liu et al., 2015). The qRT-PCR reaction was performed in 25 μL volumes containing 10 μM of each primer, 50 ng of cDNA, and 12.5 μL of SYBR Premix Ex Taq II. The PCR amplification conditions included an ini-