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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-

tial heat-denaturing step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 56 °C for 20 s, and 72 °C for 20 s. Fluorescence was measured at the end of each cycle. Melting-curve analysis was performed by heating the PCR product from 55 °C to 95 °C. The expression data for these genes were presented as relative units after normalization to the reference genes and used as the internal control by employing the $2^{-\Delta\Delta CT}$ method (Liu et al., 2015; Su et al., 2013). Mean expression and standard deviation (SD) values were calculated from the results of three independent replicates.

2.7. Enzyme activity assays

The frozen berries were ground in a tenfold volume of cold Tris–HCl buffer (pH 7.5) containing 10 mM NH_4Cl, 10 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100 and 4% polyvinylpolypyrrolidone (PVPP). The homogenates were centrifuged (10000*g*, 20 min at 4 °C), and the supernatant was collected and was used for determining the enzyme activities.

The VcMIPS enzyme was assayed colorimetrically by the periodate oxidation method of Barnett, Brice, and Corina (1970) and further corroborated by the inositol 1-phosphatase assay as described by Adhikari, Majumder, Bhaduri, Dasgupta, and Majumder (1987). For VcIMP activity, the colorimetric estimation of released inorganic phosphate (Pi) was performed following the methods by Baykov, Evtushenko, and Avaeva (1988). The reactions were carried out in 50 mM Tris–HCl buffer (pH 7.5) containing 3 mM $MgCl_2$, 30 mM substrate and 20 μg partially purified proteins at 37 °C for 1 h. Afterward, 700 μL of deionized water and 200 μL of malachite green solution were added to develop the color and the released inorganic phosphate was subsequently determined spectrophotometrically at *A*630. MIOX activity was determined by referring to the method of Reddy, Swan, and Hamilton (1981). Partially purified proteins were incubated for 30 min at 30 °C in a buffer containing 50 mM sodium acetate, 1 mM ferrous ammonium sulfate, 2 mM L-cysteine, and 60 mM *myo*-inositol. The reaction was stopped by boiling for 10 min and the denatured proteins were removed by centrifugation. The D-GlcUA in the supernatant was measured as described by Van den Hoogen et al. (1998). Protein concentration was determined according to Bradford (1976) using bovine serum albumin as standard. In each case, values are mean ± SD of three independent replicates.

2.8. Statistical analysis

Statistical analyses were performed using SigmaPlot11.0 and SPSS13.0 software. Mean values ± SD of at least three replicates were reported, and significant differences were defined as $P < 0.05$ in Duncan's analysis.

3. Results and discussion

3.1. Changes in MI levels during blueberry fruit development

Fruits from two commercial cultivars, cv 'Berkeley' and 'Bluecrop' were selected again for the present study. As shown in our previous study (Liu et al., 2015), fruits were harvested in batches during the harvest season and sorted into six developmental stages according to fruit size and color. From stages 1–3, young fruits were hard and dark green and differed primarily in size, whereas from stages 4–6, enlarged light green fruits began to soften and accumulate red and then blue pigments.

MI concentrations in fruit samples at the above six stages were measured by HPLC. As shown in Fig. 1, the MI content was the highest at stage 1 in both 'Berkeley' and 'Bluecrop'. The high

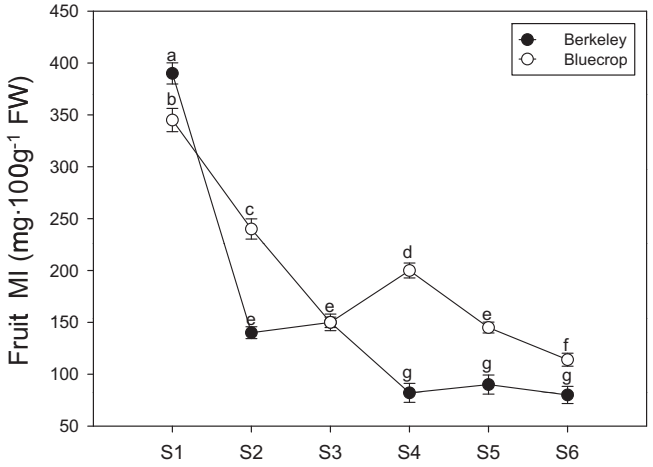

Fig. 1. Changes in MI accumulation during blueberry fruit development. Values are means of three replicates and different letters indicate significant differences (Duncan's test, $P < 0.05$).

amount of MI in young fruits may be associated with the retention of turgor during the cell expansion phase (Cui et al., 2013). Moreover, some differences exist in the change profiles of the MI levels between the two cultivars. In 'Berkeley', the MI level declined in both stages 2 and 4 but more significantly in stage 2. However, in 'Bluecrop' the MI content decreased gradually until stage 3, which was followed by a slight peak in stage 4. To our knowledge, few works on other plants, except for kiwifruits, have focused on the change patterns of MI accumulation during fruit ripening. Cui et al. (2013) compared the MI accumulation among four *Actinidia* species, namely *Actinidia arguta*, *Actinidia eriantha*, *Actinidia rufa*, and *A. deliciosa*, and found that MI concentrations remained relatively stable in *A. deliciosa*, *A. eriantha*, *and A. rufa* throughout the fruit developmental stages. However, in *Actinidia argute*, the concentration significantly increased until day 45 after flowering. In the present study, differences were observed in the MI accumulation during fruit development of the two blueberry cultivars. These results indicated that MI accumulation might vary between species and even between genotypes of particular species.

Furthermore, we found that a close correlation exists between the levels of MI and AsA in the two blueberry cultivars. In our previous study, 'Bluecrop' exhibited a larger AsA content than 'Berkeley' at stage 2 and from stage 4 until the fruit ripened (Liu et al., 2015). Similarly, compared with 'Berkeley', 'Bluecrop' presented obvious advantages in terms of MI content at the aforementioned stages. Thus, we wondered whether the higher MI accumulation may, to some extent, contribute to the higher AsA content in 'Bluecrop' than in 'Berkeley'. To date, the L-galactose pathway has been confirmed to be the predominant biosynthetic route to AsA in plants. However, whether MI acts as a precursor to AsA in an "animal-like" pathway in plants has not been established with certainty (Duan et al., 2012; Endres & Tenhaken, 2009; Lorence et al., 2004; Zhang, Gruszewski, Chevone, & Nessler, 2008). In this pathway, MI was supposed to be oxidized to D-GlcUA by MIOX and further converted into AsA. To reveal the mechanisms of MI accumulation and the relationship between the levels of MI and AsA during blueberry fruit development, we further compared the expression patterns of the key genes involved in MI metabolism between the two blueberry cultivars.

3.2. Identification of the MI biosynthetic genes from blueberry

Given that the rate-limiting step of MI synthesis is catalyzed by MIPS, we intended to identify the *MIPS* genes from the two blueberry cultivars to gain insights into the molecular mechanisms

underlying the MI content changes during fruit ripening. To date, no sequences of blueberry *MIPS* are available. Thus, the objective genes were obtained using homologous cloning techniques. Two corresponding full-length cDNAs were obtained and named *VcMIPS1* and *VcMIPS2*. Both *VcMIPS1* and *VcMIPS2* contained only ORF of 1533 bp encoding 510 amino acids (aa), which shared 93% identity with each other. As shown in Supplementary Fig. S1, four conserved domains, namely, GWGGNNG, LWTANTER, NGSPQNTFVPGL, and SYNHLGNNDG, existed in the two VcMIPS proteins, which specifically belong to the MIPS family (Chatterjee et al., 2006; Cui et al., 2013; Majumder, Chatterjee, Dastidar, & Majee, 2003). A phylogenetic analysis of selected members from the plant MIPS family showed that VcMIPS1 and VcMIPS2 were closely related to AdMIPS from kiwifruit (Fig. 2A).

The structural gene coding for MIPS was first identified in yeast. The *MIPS* genes were subsequently cloned and characterized from a wide range of animals and plants. Yeast and animal genomes contain a single gene-encoding MIPS, whereas plants contain multiple *MIPS* genes. For example, the *MIPS* gene family includes three members in *Arabidopsis* and at least three and four in sesame (*Sesamum indicum*) and soybean (*Glycine max*), respectively (Donahue et al., 2010; Torabinejad & Gillaspy, 2006). In this study, we reported for the first time the isolation of two *MIPS* genes, *VcMIPS1* and *VcMIPS2*, from the cDNA of blueberries. The multiplicity of *MIPS* genes may indicate the presence of a complex temporal and/or spatial regulation of the first step of MI synthesis in plants. Although a complete characterization of the MIPS gene family in a single plant species has been rarely performed to date, expression studies have revealed the possibility of specialized roles for individual enzyme isoforms (Donahue et al., 2010; Torabinejad & Gillaspy, 2006).

We also used homologous cloning strategies to isolate the *IMP* gene from blueberry. A full-length target cDNA, designated as *VcIMP*, was obtained from blueberry fruits. The predicated VcIMP protein consisted of 270 aa and possessed three conserved motifs (Supplementary Fig. S2): motif A (DPLDGT), motif B (WDXAAG), and motif C (GEET), which were characteristic of a lithium-sensitive phosphatase super family. This super family mainly consists of three kinds of members, namely, IMP, inositol polyphosphate 1-phosphatase, and fructose 1,6-bisphosphatase (Atack, Broughton, & Pollack, 1995). To further clarify whether the predicated protein belonged to IMP family or not, a rooted phylogenetic tree was constructed. As shown in Fig. 2B, the VcIMP clustered together with the IMP proteins of other plant species.

3.3. Identification of VcMIOX in MI catabolism from blueberry

To maintain the MI content, the most important index is another enzyme named MIOX, which plays a vital role in MI catabolism. In this study, we for the first time isolated a *MIOX* gene, *VcMIOX* from blueberry fruits. The ORF of the obtained *VcMIOX* encoded a protein of 304 aa. Sequence alignment showed that the predicted amino acid sequence of the VcMIOX protein is highly identical to other plant MIOX proteins, such as AtMIOX1 (74.2%) of *Arabidopsis*, OsMIOX (72.1%) of *Oryza sativa* and TcMIOX1 (75.56%) of *Theobroma cacao*. Furthermore, similar to other MIOX proteins, VcMIOX contained a conserved domain referred to as the DUF706 domain in the Pfam database (Supplementary Fig. S3), which was the putative equivalent of a MIOX activity domain (Torabinejad & Gillaspy, 2006). Further phylogenetic analysis showed that VcMIOX were closely related to PpMIOX from *Prunus persica* (Fig. 3).

The MIOX enzyme was first measured in rat kidney and later shown to be present also in plants (Alford et al., 2012; Torabinejad & Gillaspy, 2006). This enzyme directly and irreversibly oxidizes MI in the presence of molecular oxygen, without

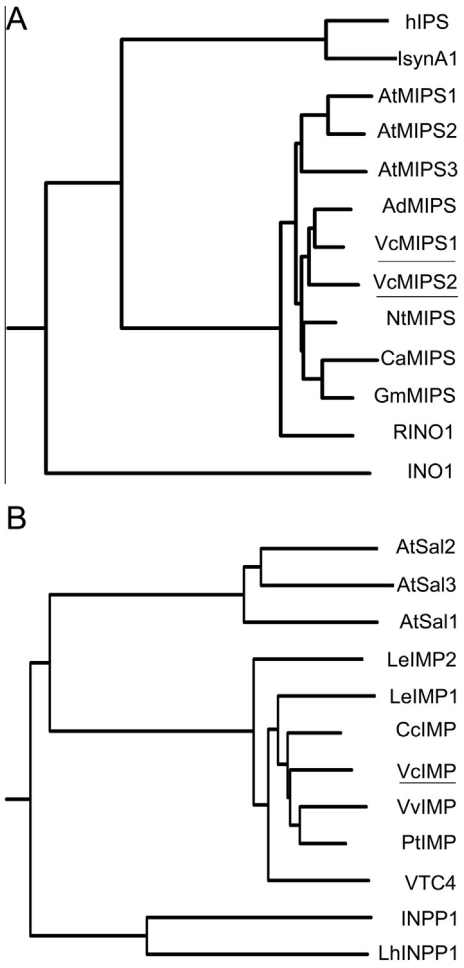

Fig. 2. Phylogenetic trees of VcMIPS1/2 and VcIMP with the related protein, respectively. The rooted tree is generated with the distance matrix using the Neighbor-Joining method of the DNAMAN MASED program. The length of the branch line indicates the extent of difference calculated from 1000 replicates. (A) Phylogenetic tree of VcMIPS1 and VcMIPS2 with the related protein. The proteins are as follows: AtMIPS1 (NM_120143); AtMIPS2 (Q38862); AtMIPS3 (NM_121055); AdMIPS (JX122766); NtMIPS (AB009881); CaMIPS (NP_001265952); GmMIPS (ABC55421); RINO1(AB012107). Besides, two animal MIPS proteins, IsynA1 (AF288525) from *Mus musculus* and hIPS (AF207640) from *Homo sapiens*, and one yeast MIPS protein, INO1 (L23520) from *Saccharomyces cerevisiae* were included as reference. (B) Phylogenetic tree of VcIMP with the related protein. The proteins are as follows: LeIMP2 (AAP15454); LeIMP1 (AAP15455); CcIMP (CDP04491); VvIMP (XP_010655423); VTC4 (Q9M8S8). Besides, three members of fructose 1, 6-bisphosphatase: AtSal1 (NP_201203); AtSal2 (O49623); AtSal3 (Q8GY63) from *Arabidopsis* and two members of inositol polyphosphate 1-phosphatase: INPP1 (XP_010655423) from *Homo sapiens*; LhINPP1 (JAG11081) from *Lygus hesperus* were included as references.

other cofactors. According to previous research, plants may contain more than one *MIOX* gene, which is similar to *MIPS*. For instance, four *MIOX* genes have been reported in *Arabidopsis*. However, little is known about *MIOX* gene family in other plants (Duan et al., 2012; Lorence et al., 2004). The *VcMIOX* gene sequence obtained from blueberries in the current study will provide molecular tools to analyze the roles of MIOX in carbohydrate metabolism.

3.4. Expression analyses of genes in MI biosynthesis and oxidation during fruit ripening

To study the transcriptional regulation of MI metabolism in highbush blueberry, the expression patterns of the two *VcMIPS* genes, one *VcIMP* gene and one *VcMIOX* gene were profiled over the six stages of development by qRT-PCR. Considering the broad

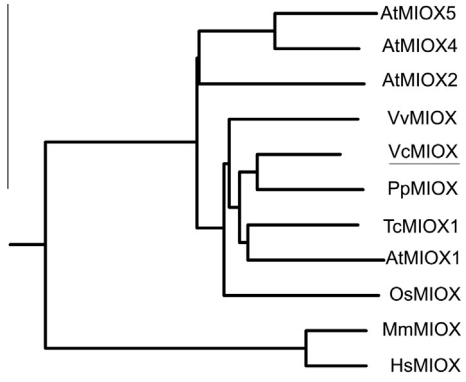

Fig. 3. Phylogenetic tree of VcMIOX and the related proteins. The rooted tree is generated with the distance matrix using the Neighbor-Joining method of the DNAMAN MASED program. The length of the branch line indicates the extent of difference calculated from 1000 replicates. The proteins are as follows: AtMIOX1 (NP_172904); AtMIOX2 (NP_565459); AtMIOX4 (NP_1943556); AtMIOX5 (NP_200475); TcMIOX1 (XM_007012893) from *Theobroma cacao*; OsMIOX (NM_001064406); VvMIOX (XM_002283083); PpMIOX (XM_007215664) from *Prunus persica*. Besides, two animal MIOX proteins, HsMIOX (NP_060054) from *Homo sapiens* and MmMIOX (NP_064361) from *Mus musculus* were included as reference.

physiological and cellular changes occurring during fruit development, *GAPDH* and *SAND* were selected as simultaneous reference genes, as described in our previous study (Liu et al., 2015). According to Zifkin et al. (2012), these genes were the most constant reference genes across the blueberry developmental stages.

As shown in Fig. 4, *VcMIPS1* slightly fluctuated throughout fruit development, and no significant differences were observed in *VcMIPS1* expression levels at different stages between 'Bluecrop' and 'Berkeley'. By contrast, *VcMIPS2* expression was regulated strictly in the process of blueberry fruit development (Fig. 4). 'Bluecrop' and 'Berkeley' shared a similar expression pattern of *VcMIPS2*, which was a declining trend in stages 1–6. However, differences between the two cultivars were still observed. In stages 1–5, the expression levels of *VcMIPS2* continued a rapid decline in 'Berkeley', whereas those of *VcMIPS2* in 'Bluecrop' exhibited a mild decrease in stages 3–4. Therefore, although 'Berkeley' showed more *VcMIPS2* transcripts than 'Bluecrop' from stages 1–3, the situation was the opposite in stages 4–6.

As for the *VcIMP* gene, the expression levels had no significant changes from stage 1–2, and then decreased obviously until stage 4, and finally lowered slightly from stage 4–6 in the two cultivars (Fig. 4). Besides, it was observed that 'Bluecrop' had higher *VcIMP* expression level than 'Berkeley' from stage 4 until fruit ripening.

The expression patterns of the *VcMIOX* gene, encoding the enzyme responsible for MI oxidation, were further compared between the two blueberry cultivars. In 'Berkeley', *VcMIOX* expression level first increased and peaked at stage 2 and then fell until stage 5. In stages 5 and 6, *VcMIOX* transcripts maintained a steady state. By contrast, in 'Bluecrop' the *VcMIOX* expression level

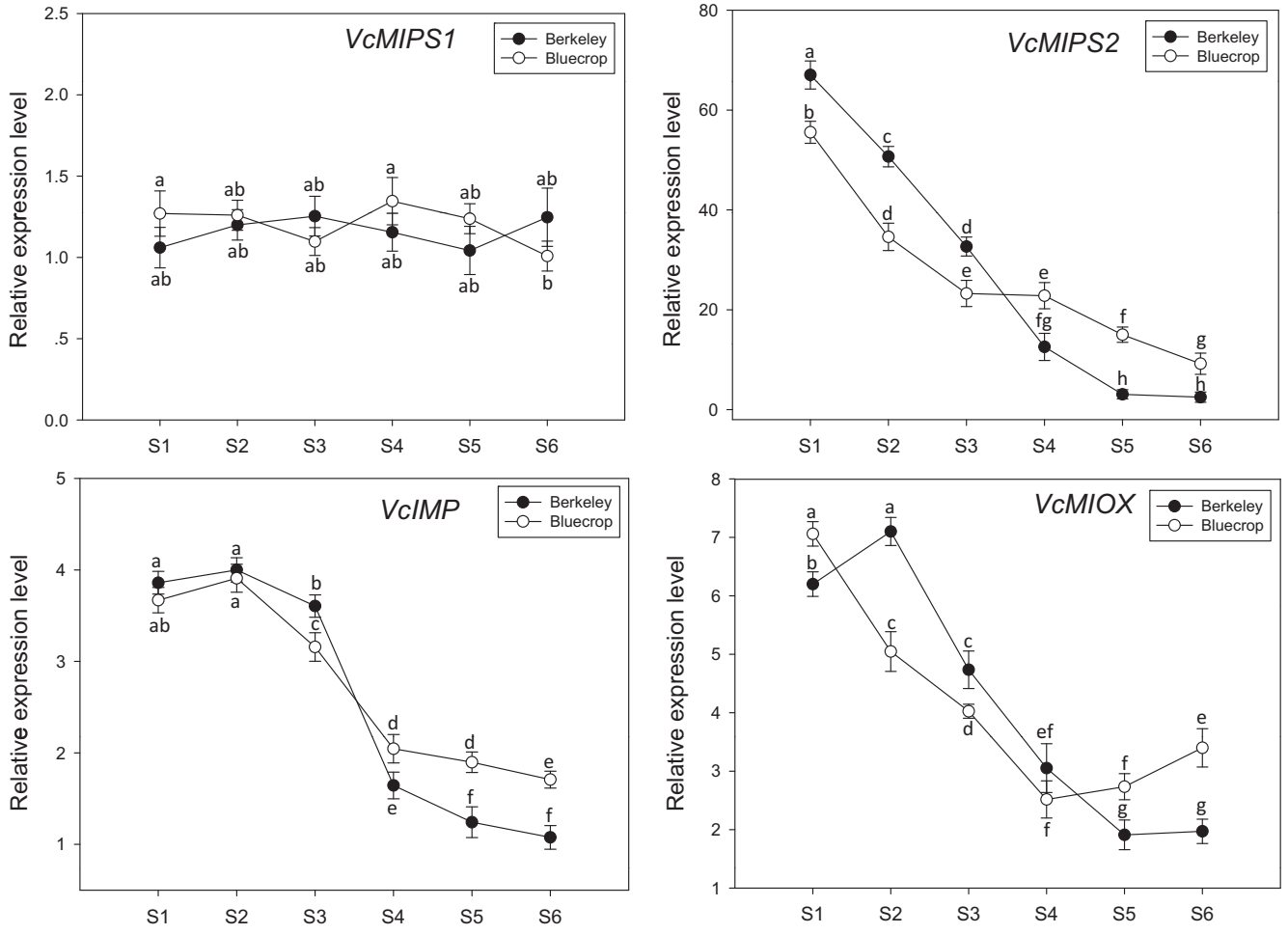

Fig. 4. Expression analyses of genes in MI biosynthesis and oxidation during fruit development using qRT-PCR. Values are means of three replicates ± SD and different letters indicate significant differences (Duncan's test, $P < 0.05$).

decreased continuously and reached the minimum at stage 4. Afterward, an increasing trend followed (Fig. 4).

3.5. Change patterns of enzyme activities in MI biosynthesis and oxidation during fruit ripening

To further confirm the relationships between the expression of the three key genes and MI accumulation, the change patterns of VcMIPS, VcIMP and VcMIOX enzyme activities were analyzed during fruit ripening. As shown in Fig. 5, VcMIPS activity shared similar patterns with *VcMIPS2* expression in the two cultivars. Chang patterns of VcIMP and VcMIOX activities were also basically consistent with those of *VcIMP* and *VcMIOX* expression, respectively.

The MIPS enzyme, which converted D-glucose-6-phosphate to L-*myo*-inositol 1-phosphate, was suggested as a key rate-limiting enzyme in MI biosynthesis. According to the literature, MI content could be controlled by increasing or decreasing *MIPS* expression in plants. For example, overexpression of *TUR1*, a *MIPS* gene from *Spirodela polyrrhiza*, in *Arabidopsis* led to the increase in free MI pool, whereas antisense suppression of *MIPS* in transgenic potato resulted in a sevenfold reduction in MI (Donahue et al., 2010; Torabinejad & Gillaspy, 2006; Wang et al., 2011). IMP is the second important enzyme in de novo MI synthesis, which is also required for the MI recycling from MI phosphate signaling molecules. In plants, *IMP* gene expression is developmentally regulated, with maximal levels being present in plant tissues undergoing rapid cell divisions. It has been shown that a loss of function in the *VTC4*, one *IMP* gene in *Arabidopsis* gene leaded to a decrease in MI contents (Torabinejad et al., 2009).

To date, few data related to the expression analyses of genes involved in MI biosynthesis with fruit ripening are available. Cui et al. (2013) only reported the expression levels of *MIPS* in the development of four *Actinidia* species. During kiwifruit development, *MIPS* transcripts were generally the most at the early stages, and then decreased gradually until the fruits ripened. However, the *MIPS* gene expression levels showed no clear association with MI concentrations in fruits. In our study, either *VcMIPS2* or *VcIMP* expression patterns did not exert a completely positive correlation with the changing profiles of MI content during the development of two blueberry cultivars. For instance, 'Berkeley' had higher *VcMIPS2* expression levels, while lower MI contents than 'Bluecrop' at stage 2. Nevertheless, the expression levels of both *VcMIPS2* and *VcIMP* were still related to the MI content in the two cultivars. Specially, in the late stage of fruit development (from stage 4 to 6), higher expression levels of both *VcMIPS2* and *VcIMP* were associated with higher MI content in 'Bluecrop' compared with 'Berkeley' (Fig. 4). Furthermore, the results of enzyme activity analysis also confirmed that 'Bluecrop' have higher VcMIPS and VcIMP activities than 'Berkeley' during the same period (Fig. 5). These results indicated that the efficiency of MI biosynthesis was partially responsible for the MI accumulation during the development of blueberries. Therefore, MI accumulation in fruits may be affected by many other aspects, including recycling from inositol-containing compounds, as well as oxidization, with the exception of de novo synthesis (Cui et al., 2013).

MIOX, a unique monooxygenase, catalyzes the oxidation of MI to D-GlcUA. Overexpression of the *MIOX4* gene in *Arabidopsis* was shown to increase AsA levels by twofold to threefold. Thus, Lorence et al. (2004) proposed that *MIOX* offered a possible entry point into plant AsA biosynthesis, and MI acts as a precursor to AsA in an "animal-like" pathway in plants. However, Endres and Tenhaken (2009) showed that overexpression of *MIOX* did not increase the AsA content, and D-GlcUA played a negligible role in AsA biosynthesis. To date, whether MIOX participates in AsA biosynthesis remains unclear (Duan et al., 2012). In our previous study, the AsA contents of both cultivars have been shown to peak

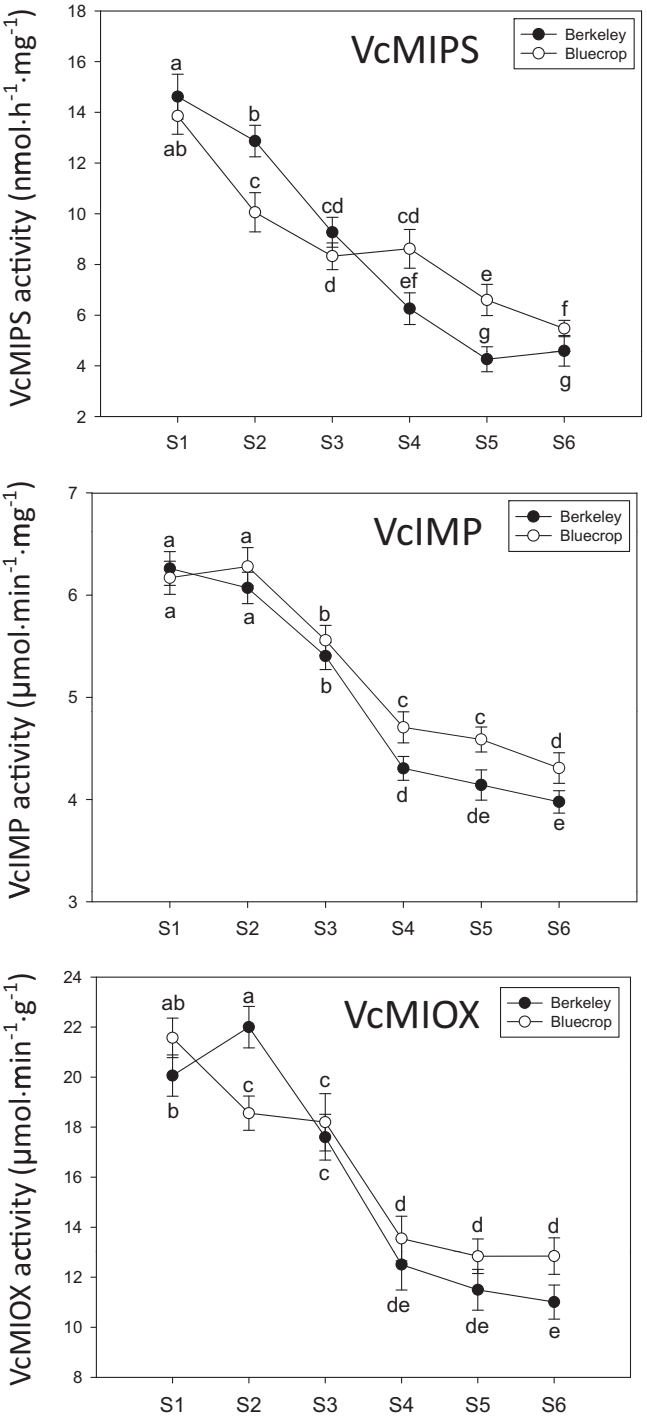

Fig. 5. Change patterns of VcMIPS, VcIMP and VcMIOX activities during fruit development. In each case values are mean ± SD of three independent replicates, and different letters indicate significant differences (Duncan's test, $P < 0.05$).

at stage 3, and then decline along with fruit ripening (Liu et al., 2015). We did not find a clear correlation between *VcMIOX* expression level and AsA content in the present study (Figs. 4 and 5). However, compared with 'Berkeley', the higher expression level of *MIOX* might, to some extent, contribute to the higher AsA content in 'Bluecrop' at stages 4–6.

Although increased MIOX activity plays an ambiguous role in AsA biosynthesis, the enzyme clearly controls the MI level in plants (Endres & Tenhaken, 2009). In the present study, a sharp decline in MI content was observed from stages 1–2 in 'Berkeley'. This

phenomenon might be partially caused by the improvement of MI oxidation catalyzed by VcMIOX during the period. Besides, although both the expression levels of *VcMIPS* and *VcMIOX* decreased between stages 3 and 4, the decline rate of *VcMIOX* expression was higher than that of *VcMIPS*, which may explain the emergence of a small peak in MI contents at stage 4 in 'Bluecrop' (Fig. 5). Therefore, the current results indicated that VcMIOX played important roles in determining the MI level during fruit development in either 'Berkeley' or 'Bluecrop'.

In conclusion, MI contents in fruits were analyzed in cv 'Bluecrop' and cv 'Berkeley', along with the expression profiles of the key genes involved in MI biosynthesis and catabolism. Besides, the change patterns of the corresponding enzyme activities were analyzed. The results showed that some differences exist in the change profiles of the MI levels between the two cultivars. Furthermore, two *VcMIPS* genes, one *VcIMP* gene and one *VcMIOX* gene were isolated for the first time from blueberry fruits. Specifically, *VcMIPS 1/2* and *VcMIOX* encoded the rate-limiting enzymes in MI biosynthesis and oxidation, respectively. The expression patterns of *VcMIPS2*, *VcIMP* and *VcMIOX* genes showed a relationship with the change profiles of MI content during fruit ripening. The results were further confirmed by the analyses of the enzyme activities. Overall, the results indicated that both MI biosynthesis and oxidation played important roles in determining MI levels in the development of blueberries.

Conflict of interest

The authors declare no competing financial interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.04.099.

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