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Identification and comparative analysis of the *MCU* gene family in pear and its functions during fruit ripening

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ABSTRACT

Mitochondrial calcium uniporter (MCU) plays an important role in cell senescence and aging in mammals. However, the function of MCU homologs during the ripening and senescence of postharvest fruit had not been characterized until recently. In this study, a comprehensive study was conducted on the characteristics of the *MCU* family genes in the pear genome. In total, seven PbrMCU genes were identified and classified into two subgroups. Whole-genome duplication (WGD)/segmental duplication is the main driving force behind their expansion. The genes contained various conserved motifs and *cis*-acting elements, and the correspondent proteins possessed a serial of conserved motifs. A total of six *PbrMCUs* with diverse expression patterns were detected as the pear fruit ripened. Following the results of the impact of postharvest treatments (ethrel and 1-methylcyclopropene) and transient overexpression of 1-aminocyclopropane-1-carboxylate oxidase 1 gene (*PbrACO1*), the candidate genes *PbrMCU3*, *PbrMCU4*, and *PbrMCU7* were determined to be involved in pear ripening. Moreover, the response of mitochondrial calcium uptake 1 (*PbrMICU1*) transcription to the postharvest treatments was similar to that of *PbrMCU4*.

1. Introduction

Mitochondrial Ca^{2+} ($[Ca^{2+}]m$), a positive effector of ATP synthesis at appropriate concentrations, plays critical roles in diverse cellular processes, ranging from energy metabolism to cell death (Giacomello et al., 2007; McCormack et al., 1990; Rizzuto and Pozzan, 2006; Szabadkai and Duchen, 2008).

The molecular mechanisms of Ca^{2+} uptake into mitochondria have been widely studied in mammals. To date, several protein families making up the mitochondrial Ca^{2+} uniporter complex have been identified, including the pore-forming mitochondrial calcium uniporter (MCU; Baughman et al., 2011; De et al., 2011; Raffaello et al., 2013). Furthermore, several regulatory elements, such as MICUs (mitochondrial calcium uptake) (MICU1, 2, and 3; Perocchi et al., 2010; Plovanich et al., 2013), EMRE (essential MCU regulator) (Sancak et al., 2013), and MICUR (mitochondrial calcium uniporter regulator) (Madesh, 2015; Paupe et al., 2015), have been well characterized.

It has been shown that $[Ca^{2+}]m$ elevation in mitochondria can promote ATP synthesis; however, excessive and persistent $[Ca^{2+}]m$ elevation results in $[Ca^{2+}]m$ overload and aberrant electron leakage, promoting mitochondrial ROS (mROS) production and cellular oxidative damage (Brookes et al., 2004; Görlach et al., 2015). Recently, the Madesh group reported that MCU activity was also positively modulated by the luminal mROS; oxidative stress results in further $[Ca^{2+}]m$ elevation, which leads to a further increase in mROS (Alevriadou et al., 2017). Taken together, mitochondrial Ca^{2+} uptake and mROS generation are interdependent phenomena that contribute to cell function in a 'mutual crosstalk' (Alevriadou et al., 2017).

The mitochondrial uptake of Ca^{2+} in plants has, as in mammals, been investigated extensively by biochemical means for a half-century, and the roles of Ca^{2+} in plant mitochondria appear to be consistent with those reported for mammals (Arpagaus et al., 2002; Virolainen et al., 2002). Ca^{2+} inside the mitochondria positively affects ATP production by upregulating the major limiting enzymes of the citric acid cycle, but Ca^{2+} overload can lead to mitochondrial dysfunction and cell death. Thus, the MCU homologs presented in plants are good candidates for involvement in mitochondrial Ca^{2+} homeostasis, regulation, and signaling.

Fruit ripening and senescence is regarded as an oxidative process, which involves the accumulation of ROS (Wang et al., 2009). Tomato

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cultivar 'Selection-7', exhibiting higher oxidative stress, ripened faster than did cultivar 'ARTH-3', which has a lower redox state (Mondal et al., 2004). Furthermore, it has been reported that ethylene, which advances the ripening process, is correlated with the metabolism of ROS by regulating the activities of antioxidant enzymes (Ke et al., 1998; Masia, 2010; Zhang et al., 2016). Thus, considering the relationship among ethylene, ROS, and Ca^{2+} uptake (Brookes et al., 2004; Görlach et al., 2015), a hypothesis was proposed that the MCU homologs in plants, together with ethylene and ROS, might be involved in the regulation of fruit ripening and senescence.

Pear is cultivated on six continents, with China being the leading producer, and the initiation of the ripening of pear fruit (a climacteric fruit) is characterized by an increase in respiration rate, in association with the accumulation of ethylene (Trinchero et al., 2004). In the present study, the isolation and characterization of the *MCU* gene family in the pear genome was carried out to analyze genomic organization, gene structure, and motif composition, as well as *cis*-acting element composition. Meanwhile, the expression profiles of the *PbrMCUs* and their regulator, *PbrMICUs*, during fruit ripening were assayed in control fruits and in fruits treated with ethrel or 1-methylcyclopropene (1-MCP), which would help us to identify the candidate *PbrMCU* genes involved in the pear ripening process. Ethrel accelerated the ripening and senescence process of postharvest pear fruit, while the reverse effect was observed after 1-MCP treatment.

2. Materials and methods

2.1. Sequence retrieval and annotation of MCU genes

The sequences of the published MCU and MICU proteins from *Arabidopsis* were downloaded from the Arabidopsis Information Resource (TAIRrelease10, http://www.arabidopsis.org). They were used as query sequences in BLASTP searches with a stringent E-value cut-off ($\leq 1E^{-10}$) against the pear genome database (http://peargenome.njau.edu.cn) (Wu et al., 2013). All significant hits were then subjected to the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/), Pfam (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) databases to confirm the presence of the diagnostic domain. A local BLASTN against pear EST libraries (http://peargenome.njau.edu.cn) was conducted to find the corresponding record for each putative *PbrMCU* and *PbrMICU* with maximum identity > 95%, length > 200 bp, and E-value < $1E^{-10}$ (Qiao et al., 2015). The physicochemical parameters of the full-length proteins were calculated using the Protparam tool (http://web.expasy.org/protparam/) (Bhalla et al., 2015), and the subcellular localization of PbrMCUs was predicted by the SLP-Local server (http://sunflower.kuicr.kyoto-u.ac.jp/smatsuda/slplocal.html) (Dong et al., 2016).

2.2. Phylogenetic analysis, gene structure, and protein motif analysis

The phylogenetic tree was constructed using the Minimum Evolution (ME) method with a bootstrap analysis of 1000 replicates and the Jones-Taylor-Thornton model with Gamma-Distributed (G) substitution rates using MEGA7.0 software (Kumar et al., 2016).

Gene structures of *PbrMCUs* were obtained by alignment of the open reading frames (ORF) with corresponding genomic sequences with the gene structure display server (GSDS) program (http://gsds.cbi.pku.edu.cn/) (Hu et al., 2015).

Conserved protein motifs were predicted with MEME Suite (http://meme-suite.org/tools/meme). The settings were: maximum numbers of different motifs, 9; minimum motif width, 20; and maximum motif width, 50. The identified motifs were annotated using SMART protein analyzing software (Schultz et al., 2000).

2.3. Location of PbrMCUs on chromosomes, synteny, and Ka/Ks analysis

The chromosomal locations of *PbrMCUs* were determined based on genome annotation data and then plotted using Circos (Krzywinski et al., 2009). A method similar to that used for the Plant Genome Duplication Database (PGDD) (http://chibba.agtec.uga.edu/duplication/) was applied to analyze the syntenic relationships in pear (Lee et al., 2013), and the duplicated *PbrMCUs* were categorized into the following types: whole genome duplication (WGD)/segmental, tandem, singleton, proximal, and dispersed.

MCScanX downstream analysis tools were used to annotate the Ka and Ks substitution rates of syntenic gene pairs (Qiao et al., 2015). KaKs Calculator 2.0 was used to determine Ka and Ks by the Nei-Gojobori (NG) method (Wang et al., 2010). The date of WGD/segmental duplication event was calculated according to the method of Qiao et al. (2015).

2.4. Prediction of PbrMCU regulatory elements

Cis-acting regulatory elements presented in the promoter (1.5 kbp upstream of the translational start sites) were identified using the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002). Divergence between upstream sequences of each paralogous gene pair was measured by the GATA program (Nix and Eisen, 2005), with window size set at 7 and lower cutoff score at 12 bits.

2.5. Plant material and treatment

Pear (*Pyrus seratina* Reheder cv. Housui) fruits at the commercially mature stage were harvested from homogeneous trees in the experimental orchard in Nanjing, and immediately transported to the laboratory. Uniform and defect-free fruits were then randomly divided into three treatments with 100 fruits per replicate each. The treatments were as follows: (1) 0.5 mL/L H_2O immersion for 5 min, (2) 1.5 ppm 1-MCP incubation for 24 h, and (3) 0.5 mL/L ethrel immersion for 5 min. Subsequently, fruits were preserved in polythene bags at 25 °C. Samples were taken every six days until the decay rate exceeded 20%.

2.6. ROS content determination

ROS content was analyzed according to the method of Sunkar (2010), and the result was expressed as fluorescence units Kg^{-1} protein. Protein determination was carried out based on the BCA method (Brown et al., 1989) with a protein assay kit (A045-4, Nanjing Jiancheng Bioengineering Institute, China).

2.7. RNA isolation, cDNA synthesis, and quantitative RT-PCR

Total RNA was isolated using TRIzol Reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA quality was confirmed by agarose gel electrophoresis and spectrophotometry (Nanodrop 1000, Thermo Fisher Scientific, Wilmington, DE, USA). Followed by RNase-free DNase treatment (Qiagen, Valencia, CA, USA), approximately 2 μg total RNA was used for first-strand cDNA synthesis using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TRANSGEN, Beijing, China). q-PCR was performed in 20 μL reaction mixture containing 80–100 ng cDNA, 200 nM of each primer, and 10 μL LightCycler 480 SYBR GREEN I Master Mix (Roche). All the reactions were carried out in a CFX96 Real-Time System (Roche), following a three-step standard protocol (45 cycles of 10 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C) with a melting curve analysis. *PbrTubulin* gene was used as the reference gene. With the exception of PbrMCU2, which had not been cloned from pear, the specific primers of the other six *PbrMCUs* and *PbrACO1* were designed by the primer designing tool of NCBI as listed in Table S1. The expression levels of *PbrMCU* genes were