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Characterization of the miR165 family and its target gene *Pp-ATHB8* in *Prunus persica*

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ABSTRACT

A study to identify and validate *Prunus persica* microRNAs belonging to the ppe-miR165 family was conducted using miR-RACE where the expression of these miRNAs could be detected in leaves, flower buds, flowers and fruits of *P. persica* by quantitative real-time PCR (qRT-PCR) with some members of this miRNA family exhibiting tissue-specific expression. In addition, *Pp-ATHB8* which is one of the potential target genes for ppe-miR165 family was experimentally verified by PPM-RACE and RLM-RACE, from which the cleavage sites of the target mRNA were mapped and the expression patterns of cleaved fragments also detected, thus demonstrating the mode by which ppe-miR165 regulates the target gene *Pp-ATHB8*. Furthermore, spatiotemporal expression levels of the target gene and its cleaved fragments were analyzed by qRT-PCR, where they were found to exhibit expression trends at variance from ppe-miR165, thus indicating the cleavage mode of this miRNA on its target gene. The characterization of this important miRNA and its interaction with an equally important target gene in *P. persica*, further deepens our understanding of the role of ppe-miR165 in *P. persica* and expands the knowledge of small RNA-mediated regulation in this fruit crop.

1. Introduction

With the rapid development in molecular biology, research on microRNAs (miRNAs) has received growing attention owing to the apparent influence of these small RNAs on plant growth and development processes. miRNAs are a group of small endogenous 20–22 nt long non-coding RNAs which play very important roles both in plants and animals. In plants, miRNAs are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) (Chen et al., 2005; Zhang et al., 2006a), which are cut into miRNA precursors (pre-miRNAs) with typical hairpin structures. Mature miRNAs are generated from the stem portion of the single stranded stem-loop precursor by a complex containing the nuclear RNase III enzyme and the ribonuclease III-like enzyme Dicer (DCL1) (Kurihara and Watanabe, 2004), then the mature miRNA is incorporated into the RNA-induced silencing complex (RISC) and guides RISC to complementary mRNA targets. miRNAs regulate activities of their target genes through degradation of target mRNAs or repression of translation in targeted genes by base pairing with their target genes (Bartel, 2004; Mallory and Vaucheret, 2004; Carrington and Ambros, 2003; Navarro et al., 2006; Chekulaeva and Filipowicz, 2009; Krol et al., 2010). In plants, miRNAs generally interact with their targets through perfect or near-perfect complementarities with increasing evidence demonstrating that plant miRNAs can post-transcriptionally regulate target genes having critical roles in plant pathways and processes which include developmental patterning, stem-cell identity, hormone signaling, miRNA biogenesis and stress responses (Jones-Rhoades et al., 2006), leaf organ morphogenesis and polarity (Juarez et al., 2004; Xu et al., 2007), floral differentiation and development (Nag et al., 2009; Nag and Jack, 2010), auxin signaling and boundary formation/organ separation (Sunkar and Zhu, 2004).

Many known miRNAs are evolutionarily conserved in the plant species ranging from mosses and ferns to higher flowering plants. This has made it possible to perform computational searches for the homologs or orthologs of miRNAs based on the highly conserved sequences in mature miRNAs as well as long hairpin structures in

Abbreviations: ATHB, *Arabidopsis thaliana* homeobox; DTT, Dithiothreitol; EtBr, Ethidium bromide; GSP, Gene-specific forward primers; HB, Homeobox; HD, Homeodomain; HD-Zip protein, Homeodomain-leucine zipper protein; HMW RNA, High molecular weight RNA; LMW RNA, Low molecular weight RNA; miRNA, microRNA; miR-RACE, Rapid amplification of cDNA ends for microRNA; ppe-miRNAs, *Prunus persica* microRNAs; PPM-RACE, Poly (A) polymerase-mediated 3′ rapid amplification of cDNA ends; qRT-PCR, Quantitative real-time PCR; RACE, Rapid amplification of cDNA ends; RISC, RNA-induced silencing complex; RLM-RACE, RNA ligase-mediated 5′ rapid amplification of cDNA ends; RT-PCR, Reverse transcriptase polymerase chain reaction; sRNA, Small RNA.

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precursors (Wang et al., 2004). The successful bioinformatics prediction of miRNAs in tomato, maize, cotton, soybean, citrus, grape, and other plants (Yin et al., 2008; Zhang et al., 2006b, 2008; Zhang et al., 2007; Song et al., 2009; Carra et al., 2009; Zeng et al., 2010; Xie et al., 2007; Qiu et al., 2007; Gleave et al., 2008; Yu et al., 2010; Zhang et al., 2012) confirms the efficiency of computational or bioinformatics-based approach in prediction of miRNAs in various plants. In addition, the targeting of mRNAs by miRNAs is achieved through base pairing between the seed sequences of miRNAs to the complementary sites on their targets. This aspect can also make computational identification of miRNA targets a valuable method.

The homeobox (HB), a 183 bp DNA sequence element, was originally identified as a region of sequence similarity shared by several genes involved in the control of Drosophila development (Gehring, 1987). The HB sequence encodes a 61amino acid sequence, known as the homeodomain (HD). HB genes have now been identified in many animal and plant species, including *Arabidopsis thaliana*, maize (Ruberti et al., 1991; Hake, 1992). Especially, HB genes in *A. thaliana* also contain a second element that potentially codes for a leucine zipper motif (Zip), located immediately 3′ to the homeobox. Therefore these gene products have been designated as homeodomain-leucine zipper proteins (HD-Zip proteins, Ruberti et al., 1991). All members of the miR165 family in *Arabidopsis* have been shown to target Class III homeodomain-leucine zipper proteins, which is a class of transcription factors specific to plants (Ruberti et al., 1991; Schena and Davis, 1992; Sessa et al., 1998). These proteins regulate critical aspects of plant development, including lateral organ polarity, apical and lateral meristem formation, and vascular development. For example, the mutations in the maize HB gene *Knotted-1* cause alterations in maize leaf morphology (Hake, 1992). The mutations in the HB gene *GLABRA2* result in abnormal trichome expansion in *A. thaliana* (Rerie et al., 1994). *A. thaliana* homeobox *8* (*ATHB8*), a member of HD-Zip transcription factor family, is one of the earliest known molecular markers of procambium and it is induced by auxin. This gene promotes procambial and cambial cell differentiation into xylem tissues (Baima et al., 2001) and participates in a positive feedback loop in which auxin signaling induces the expression of *ATHB8*, which in turn positively modulates the activity of procambial and cambial cells leading to differentiation.

Peach (*Prunus persica*) is one of the most economically important fruit crops worldwide and it has a long history of cultivation in China. The public release of the entire genome sequence of peach in April 1, 2010 (http://www.rosaceae.org/peach/genome) has made prediction and characterization of *P. persica* microRNAs (ppe-miRNAs) both a necessary and practicable research. Although several ppe-miRNAs have been predicted and validated based on the peach EST database (Zhang et al., 2012), they are however, very few for conclusive research on peach miRNA and thus more ppe-miRNAs still need to be identified and characterized. According to miRNA conservation among different plant species and considering the critical aspects in plant development of HD-Zip protein, it is necessary to predict and characterize the miRNA members of ppe-miR165 family. There are no previous reports on isolation and characterization of members of HD-Zip protein family in *P. persica.* Due to these aspects, members of the ppe-miR165 family together with one of their target genes (*Pp-ATHB8*) were focused on in this study, and they were computationally predicted according to the conservation characteristics of these miRNAs and their targets in different plant species. The precise sequences of the predicted ppe-miR165 members were validated by miR-RACE and the sequences of the target gene *Pp-ATHB8* was cloned and sequenced. The expression of the verified ppe-miR165 members and the target gene *Pp-ATHB8* were also studied in different tissues of peach by quantitative real-time PCR (qRT-PCR). Considering the mode by which most plant miRNAs direct the cleavage of their mRNA targets when these mRNA have extensive complementarity to the miRNAs (Palatnik et al., 2003; Floyd and Bowman, 2004; Mallory et al., 2005; Li et al., 2010), the regulatory mode of the verified ppe-miR165 members on the target was also studied whereby ppe-miR165-mediated cleavage products were mapped through PPM-RACE and RLM-RACE. PPM-RACE and RLM-RACE which is a strategy comprising the preparation of an enriched library of 5′ and 3′ products of miRNA-cleaved target mRNAs, 5′ RACE and 3′ RACE for accurate amplification and qRT-PCR of miRNA-cleaved target mRNA. Our results point to a need for extensive studies on more peach miRNAs so as to facilitate further understanding on the functions of their target genes.

2. Materials and methods

2.1. Plant materials

Young (one month) and old (six months) leaves, flower buds, small (semi-open) and big size (fully open) flowers as well as fruits at different stages of development (1 cm, 3 cm and 4.5 cm diameter) were collected from peach cv. 'Yoshihime' grown at the National Peach Germplasm Repository in Nanjing, China. After collection, all the samples were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Prediction of potential ppe-miR165 family members

The prediction procedure used followed that outlined by Song et al. (2009) and the core principles and parameters used in finding sequences conserved in different plant species was based on similar work carried out in other plant species (Song et al., 2010b; Bonnet et al., 2004; Zhang et al., 2006a; Sunkar and Jagadeeswaran, 2008; Sunkar et al., 2008). Sequences of members of the miR165 family in other plants were downloaded from the miRBase (Release 18.0, November 2011) while the peach genome sequence was downloaded from the Peach Genome (v1.0, April 2011; http://www.rosaceae.org/peach/genome). Target mRNAs were predicted from the peach genome v1.0 predicted CDS (http://www.rosaceae.org/node/355) based on precise sequences of the ppe-miR165 members.

2.3. RNA extraction and construction of small RNA cDNA libraries

Total RNA was isolated from 100 mg of each sampled tissue using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The RNA was then treated with DNase I according to the manufacturer's instructions (Takara) so as to remove contamination by genomic DNA. Low and high molecular weight (LMW and HMW) RNA were separated with 4 M LiCl as described in other reports (Adai et al., 2005; Song et al., 2010a).

The construction of cDNA libraries of small RNAs (sRNAs) to generate the miRNA-enriched libraries was done as previously reported by Song et al. (2009). LMW RNAs were polyadenylated at 37 °C for 60 min in a 50 μl reaction mixture with 1.5 μg of total RNA, 1 mM ATP, 2.5 mM $MgCl_2$, and 4 U poly(A) polymerase (Ambion, Austin, TX). Poly (A)-tailed sRNAs were recovered by phenol/chloroform extraction and ethanol precipitation. 5′adapter (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) was ligated to the poly (A)-tailed RNA using T4 RNA ligase (Invitrogen, Carlsbad, CA), and the ligation products recovered by phenol/chloroform extraction followed by ethanol precipitation. Reverse transcription was performed using 1.5 μg of sRNA and 1 μg of $(dT)_{30}$ RT primer (ATTCTAGAGGCCGAGGCGGCCGACATG-d$(T)_{30}$ (A, G or C) (A, G, C, or T)) with 200 U of SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). 10 μl of sRNA was incubated