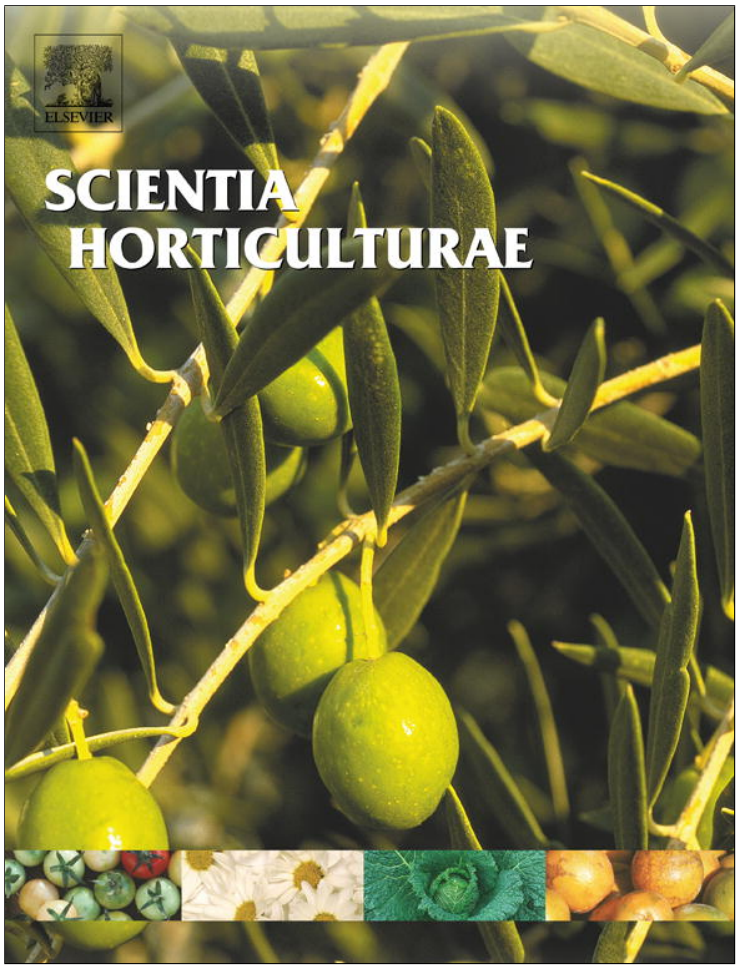

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Characterization of the miR165 family and its target gene *Pp-ATHB8* in *Prunus persica*

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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

Table 1
Primers used for miR-5′ RACE, miR-3′ RACE, and qRT-PCR of ppe-miRNAs.

miRNAs	ppe-miR165a
GSP1 (5′ → 3′)	TTTTTTTTTTGGGGGATGAAGCCTGGT
GSP2 (5′ → 3′)	GGAGTAGAAATCGGACCAGGCTTCATC
GSP3 (5′ → 3′)	TCGGACCAGGCTTCATCCCCC

GSP1 is the specific primer for miR-5′ RACE, and the underlined region base pairs with the 3′ poly(A); GSP2 is the specific primer used for miR-3′ RACE, and the underlined region base pairs with the 5′ adaptor; GSP3 is the specific primer used for qRT-PCR of miRNA.

with 1 μl of $(dT)_{30}$ RT primer and 1 μl dNTP mix (10 mM each) at 65 °C for 5 min to remove any RNA secondary structures. The reactions were chilled on ice for at least 2 min, then the remaining reagents [5 × buffers, dithiothreitol (DTT), RNaseout, SuperScript III] were added as specified in the SuperScript III manual, and the reaction left to proceed for 60 min at 50 °C. Finally, the reverse transcriptase was inactivated by incubation for 15 min at 70 °C. After preparation of miRNA libraries from individual peach organs and tissues, similar quantities of these library samples were pooled for further PCR amplification reactions as done by Song et al. (2010b).

2.4. Verification of the precise sequence of ppe-miR165 by miR-RACE

For validation of ppe-miR165 using rapid amplification of cDNA ends for miRNA (miR-RACE, Song et al., 2010a; Wang et al., 2011), a cDNA library of the sRNA pool from the RNA samples isolated from various organs and tissues (Fu et al., 2005) was amplified with mir-Racer 5′ primer (5′- GGACACTGACATGGACTGAAGGAGTA-3′) and the mirRacer 3′ primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATG-3′) to generate a pool of non gene specific products. These amplification reactions were carried out for 35 cycles at a final annealing temperature of 61 °C. 5′end reactions were performed with the mirRacer 5′ primer and miRNA-gene-specific forward primers (GSP1), while 3′ end reactions were carried out with the mirRacer 3′ primer and miRNA gene specific reverse primers (GSP2). GSP1 and GSP2 were complementary to seventeen nucleotide length sequences of the potential ppe-miRNAs and a part of Poly (T) and 5′ adaptor (Table 1). In each case, a unique gene-specific DNA fragment was amplified. After amplification, the 5′ and 3′ end PCR products were separated in 2.5% agarose gels stained with ethidium bromide (EtBr). The gel slices containing DNA with a size of about 56 bp (5′ end product) and 87 bp (3′ end product) were excised and the DNA fragments were purified using an agarose gel DNA purification kit (Takara, Japan), according to the manufacturer's instructions. The DNA fragment was directly subcloned with the TOPO TA cloning Kit (Invitrogen, USA) and Colony PCR was performed using the PCR-specific primer pairs just as done above 5′ end and 3′ end clones having PCR products of about 56 bp and 87 bp long were sequenced respectively.

2.5. Prediction of potential targets for members from the ppe-miR165 family

Previous studies have shown that miRNAs bind to the protein coding region of their mRNA targets with perfect or near-perfect sequence complementarity, and degrade the target mRNAs in a way akin to RNA interference (Wang et al., 2004). Schwab et al. (2005) designed a set of rules for predicting miRNA targets based on transcriptome analysis in transgenic Arabidopsis plants that over-expressed miRNAs. These criteria include allowance for one mismatch in the region complementary to nucleotides 2–12 of the miRNA, but not at the cleavage site (nucleotides 10 and 11), and three additional mismatches permitted between nucleotides 12 and 21, but ensuring that no more than two continuous mismatches occur within this region. By adopting and slightly modifying these rules in predicting targets for newly identified miRNA in peach, we allowed one mismatch between nucleotide positions 1–9 from the 5′ end of the miRNA, no mismatches for positions 10 and 11, and another two mismatches were allowed between positions 12 and 21/24. The number of allowed mismatches at complementary sites between miRNA sequences and potential mRNA targets was no more than four, and no gaps were allowed at the complementary sites.

2.6. Expression analysis of ppe-miR165 with qRT-PCR

The template for qRT-PCR was reverse transcribed cDNAs of the miRNA-enriched libraries used as above, and the precise sequences of ppe-miR165 was used as the forward primers and the mir-Racer 3′ primer as the reverse ones (Table 1) (Song et al., 2010a). qRT-PCR was conducted in a Rotor-Gene 3000 cycler (Corbett Robotics, Australia) employing the Rotor-Gene software version 6.1 (Wang et al., 2004). For each reaction, 1 μl of diluted cDNA (equivalent to about 100 pg of total RNA) was mixed with 10 μl of SYBR® Green qRT-PCR Master Mix (Toyobo, Osaka, Japan), and 5 pmol each of the forward and the reverse primers were added in a final volume of 20 μl. The conditions for the PCR amplification were as follows: polymerase activation at 95 °C for 1 min, then 95 °C for 1 min, followed by 50 cycles of 95 °C for 15 s, 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 20 s. A comparative quantification procedure was used to determine relative expression levels whereby 5.8S rRNA was taken as a reference gene as previously done in *A. thaliana* (Shi and Chiang, 2005) and the relevant data analyzed with an $R^2 > 0.998$ using the LinRegPCR program (Ramakers et al., 2003).

2.7. Expression analysis of Pp-ATHB8 by qRT-PCR

The expression of *Pp-ATHB8*, a ppe-miR165 target gene was assayed by qRT-PCR as previously described by Wilson et al. (2005). The reverse transcription products were amplified using gene-specific primers that overlapped the known or predicted cleavage sites (Table 2). Reactions were performed in triplicate on a Rotor-Gene 3000 cycler (Corbett Robotics, Australia) and *UBI* was used as a reference gene in the qPCR detection of mRNAs as previously done in *A. thaliana.*

2.8. Mapping of mRNA cleavage sites using RLM-RACE and PPM-RACE

To map miRNA-mediated cleavage products, the methods of RNA ligase-mediated rapid amplification of 5′cDNA ends (RLM-RACE) and Poly (A) polymerase-mediated 3′ rapid amplification of cDNA ends (PPM-RACE) were employed. The methods comprise of the following main steps: HMW RNAs polyadenylation at 37 °C for 60 min in a 50 μl reaction mixture with 5 μg of HMW RNAs, 1 mM ATP, 2.5 mM $MgCl_2$, 5 mM 5 × buffer and 8 U poly (A) polymerase (Ambion, Austin, TX); Ligation of HMW RNAs to a 5′ adapter (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) using T4 RNA ligase (Invitrogen, Carlsbad, CA). Poly (A)-tailed HMW RNA and adapter-ligated HMW RNA were then recovered by phenol/chloroform extraction followed by ethanol precipitation. Reverse transcription was performed using 5 μg each of Poly (A)-tailed HMW RNA and adapter-ligated HMW RNA, 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). Poly (A)-tailed HMW RNA and adapter-ligated HMW RNA (10 μl total volume) were incubated

Table 2
Primers used for experiments.

Primer number	Target gene	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Amplified size (bp)
1	*Pp-ATHB8*	ATGATGGCGGTGACGTCAGCCT	GACAAATGACCAGTTGATGAACATGAAGC	2520
3	*Pp-ATHB8*	CAACACCATTTGACCCCTCAGCATC	TAAGGATTTCAGCGACTCTTGTAGGC	253
5	*Pp-ATHB8*	TCTATGAGAACAGCTATTTCCGCCAG	ATTCTAGAGGCCGAGGCGGCCGACATG	221
7	*Pp-ATHB8*	AGGACACTGACATGGACTGAAGGAGTAG	CATACTTGGACCATTCTGAGTGTTGTT	343

1,2 is the primer pair used to amplify *Pp-ATHB8* ORFs; 3,4 is the primer pair for qRT-PCR of *Pp-ATHB8*; 5,6 is the primer sequences of 5′ products of miRNA cleaved *Pp-ATHB8* for PPM-RACE; 7,8 is the primers sequences of 3′ products of miRNA cleaved *Pp-ATHB8* for RLM-RACE.

Table 3
List of predicted and verified ppe-miR165 from the *Prunus persica* genome database.

miRNA name	Predicted miRNA sequence ppe-miR165	Verified miRNA sequence	Chr.	Gene ID	Location	Target predicted from peach genome v1.0 predicted CDS	Targeted protein class
ppe-miR165a	UCGGACCAGGCUUCAUUCCC	UCGGACCAGGCUUCAUCCCCC	1	2213246	3′	ppa001386m(ATHB8)	Class III
ppe-miR165b	UCGGACCAGGCUUCAUUCCC	UCGGACCAGGCUUCAUCCCCC	2	19693035	3′	ppa001405m(ATHB15)	
ppe-miR165c	UCGGACCAGGCUUCAUUCCC	UCGGACCAGGCUUCAUCCCCC	2	26094764	3′	ppa001378m(ATHB1)	HD-Zip
ppe-miR165d	UCGGACCAGGCUUCAUUCCC	UCGGACCAGGCUUCAUCCCCC	5	12581757	3′	ppa001343m(ATHB14)	
ppe-miR165e	UCGGACCAGGCUUCAUUCCC	UCGGACCAGGCUUCAUCCCCC	8	19800602	3′	ppa020309m(unknown protein) ppa019833m(unknown protein)	Protein

with 1 μl of $(dT)_{30}$ RT primer and 1 μl dNTP mix (10 mM each) at 65 °C for 5 min to remove any RNA secondary structures. The reactions were chilled on ice for at least 2 min, then the remaining reagents [5 × buffers, dithiothreitol (DTT), RNaseout, SuperScript III] were added as specified in the SuperScript III manual, and the reaction proceeded for 60 min at 50 °C. Finally, the reverse transcriptase was inactivated by 15 min incubation at 70 °C. After the preparation of miRNA cleaved target mRNA libraries from various organs and tissues, similar quantities of these library samples were pooled for further PCR and qRT-PCR amplification reactions. The PPM-RACE and RLM-RACE amplifications were performed using the GeneRacer 5′, 3′primer and the gene-specific primers (Table 2). The amplification products were gel purified, cloned, and at least eight independent clones were sequenced.

3. Results

3.1. Computational prediction and precise sequence validation of members in the ppe-miR165 family

Five potential ppe-miRNAs with sequences belonging to ppe-miR165 family were detected from the peach genome by computational screening and the true-to-type members from this group were successfully verified (Table 3). The precursors of these ppe-miRNAs could be folded into the typical secondary structures which have been accepted as an important validation parameter for predicted miRNA genes (Fig. 1). For sequence uniformity in the miR-RACE results, **e**ight clones of each PCR product were sequenced with the PCR reaction template being a DNA library of sRNAs (≤200 nt) constructed from different types and stages of *P. persica* organs following the method reported by Song et al. (2009) and Fu et al. (2005). The difference between the method employed here and traditional RACE lies in the gene-specific primers used (Table 1). The PCR products were cloned and sequenced when they yielded reliable bands (Fig. 2) and the sequencing results were used to confirm the actuality of the predicted ppe-miR165 members and to identify their precise end sequences (Table 3). The sequence identity between the miRNAs in *Arabidopsis* and validated ppe-miRNAs (Table 4) and the regions in the corresponding precursors were also used to confirm the success of the 5′ miR- and 3′ miR-RACE technique for precise determination of the miRNA sequences.

3.2. Expression analysis of ppe-miR165 family members by qRT-PCR

Preferential expression of miRNAs in some organs and tissues not only supports the existence of the miRNAs in the organism, but also provides leads to their probable physiological functions. The detection and measurement of miRNA expression levels is one of the most important works carried out in miRNA studies in various plants, for which qRT-PCR as a method has been found to be preferred due to its inherent reliability. In this study, miRNA libraries isolated from different organs and tissues of peach were used in detection of members from the ppe-miR165 family. The primers for the qRT-PCR were synthesized based on the precise sequence of the identified ppe-miR165 (Table 3). From the results of qRT-PCR, it is discernible that ppe-miR165 was expressed ubiquitously in all tissues with distinct tissue, and/or growth stage-specific expression patterns (Fig. 3). This expression of ppe-miR165 can be characterized as high in young leaves, and lower in other tissues hence confirming existence of stage- and organ-specificity in expression. These qRT-PCR data provide strong evidence confirming the existence of members of the ppe-miR165 family in peach.

3.3. Prediction and cloning of targets for ppe-miR165

The potential targets of ppe-miR165 were first predicted using a homology search following the procedure for prediction of potential miRNA targets in a protein-coding gene database (see Section 2). The ppe-miR165 sequence was tested against the peach Genome v1.0 for predicted CDS (http://www.rosaceae.org/node/355) using BLASTn search (Altschul et al., 1997). The predicted results show that the 6 targets of ppe-miR165 were highly conserved to those in *Arabidopsis*, with all targets being class III HD-Zip proteins (Table 3). Among these 6 targets ppa001386 (*Pp-ATHB8*) was cloned, and its sequence deposited and assigned accession No. JQ712870. The sequence of *Pp-ATHB8* has sites complementary to the sequence of ppe-miR165 (Fig. 4).

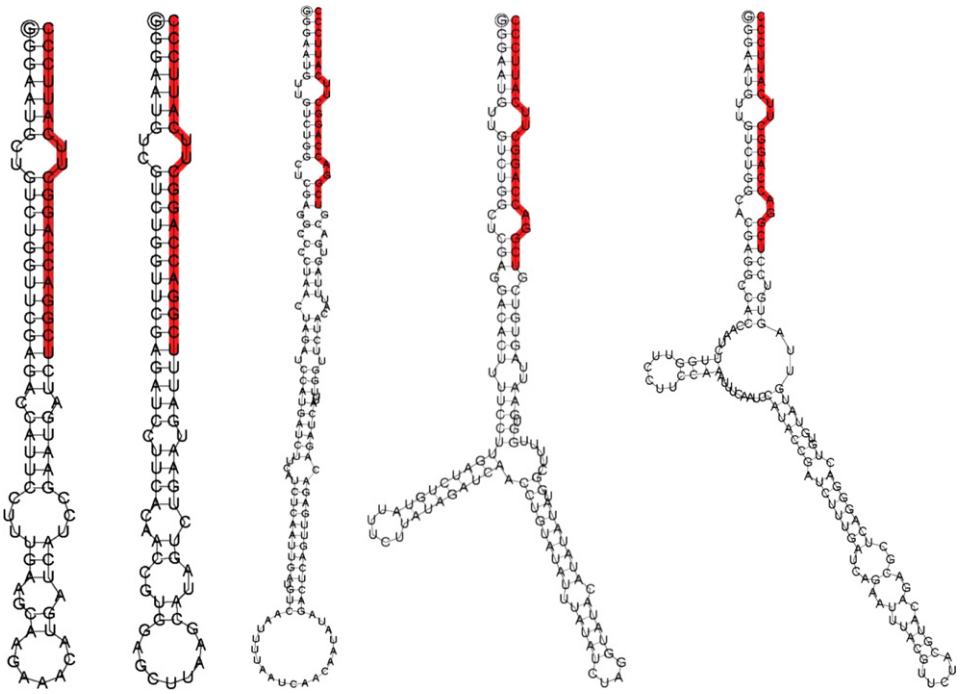

Fig. 1. Predicted fold-back structures of members of the ppe-miR165 family. The mature miRNA sequences are shaded. From left to right is "ppe-miR165 a, b, c, d and e".

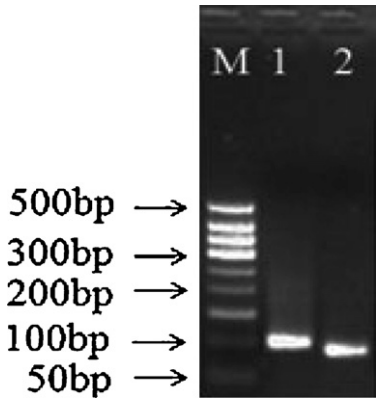

Fig. 2. 3′ RACE and 5′ RACE products of ppe-miRNAs amplified by PCR as shown in an ethidium bromide-stained agarose gel. Lane 1 is 3′ RACE product of ppe-miR165a, and lane 2 is the 5′RACE product.

3.4. Expression analysis of Pp-ATHB8

Expression analysis of miRNA targets in specific tissues not only provides clues on physiological functions of these target genes, but also helps in understanding the role of the miRNAs. To elucidate the functions of *Pp-ATHB8*, which is one of the genes targeted by members of the ppe-miR165 family, its spatiotemporal expression was studied using qRT-PCR. As shown in Fig. 3, *Pp-ATHB8* was expressed in all tissues studied with some tissue- and/or growth-stage-specific aspects as reflected by the different expression levels. *Pp-ATHB8* was found to exhibit inverse relationship in expression with ppe-miR165 (Fig. 3) as expected for most miRNA target genes, suggesting that this gene might be actively cleaved by ppe-miR165 at some levels. Specifically, *Pp-ATHB8* was highly expressed in old leaves and big fruits, moderately in young leaves and big flowers and lowly in flower buds, small and medium sized fruits, and much lower in other tissues (Fig. 3). This situation could be explained by the probability that ppe-miR165 regulated *Pp-ATHB8* by degrading its mRNA through direct cleavage.

3.5. Experimental verification of ppe-miR165-guided cleavage of Pp-ATHB8 mRNA

Based on the fact that most plant miRNAs negatively regulate their target genes through miRNA directed cleavage within the region of complementarity (Palatnik et al., 2007; Mallory and Bouché, 2008; Llave et al., 2002; Jones-Rhoades and Bartel, 2004), we employed the PPM-RACE and RLM-RACE procedure to test whether *Pp-ATHB8* can also be cleaved and to map the cleavage sites in this predicted target gene. All the primers used for PPM-RACE and RLM-RACE are as listed in Table 2. After PPM-RACE and RLM-RACE analysis, PCR products showing anticipated bands on agarose gels were sliced and sequenced. Results indicate that ppe-miR165 can guide cleavage of *pp-ATHB8* mRNA, yielding 3′ and 5′ cleavage fragments. The cleavage on *Pp-ATHB8* was found to occur at the tenth nucleotide from the 5′ end in the binding region (Fig. 4). Similar behavior has been reported in the cleavage of miRNA targets in other plants (Palatnik et al., 2003, 2007; Llave et al., 2002; Jones-Rhoades and Bartel, 2004).

In order to further characterize the mode by which ppe-miR165 regulates *Pp-ATHB8* and the level of degradation of the *Pp-ATHB8* mRNA, we analyzed the 5′ end and 3′ end products of the cleaved

Table 4
Sequence alignment between ppe-miR165 and its ortholog in *Arabidopsis*.

miRNA(5′ → 3′)	Nucleotide order																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
ppe-miR165	U	C	G	G	A	C	C	A	G	G	C	U	U	C	A	U	C	C	C	C	C	
ath-miR394a	U	C	G	G	A	C	C	A	G	G	C	U	U	C	A	U	C	C	C	C	C	

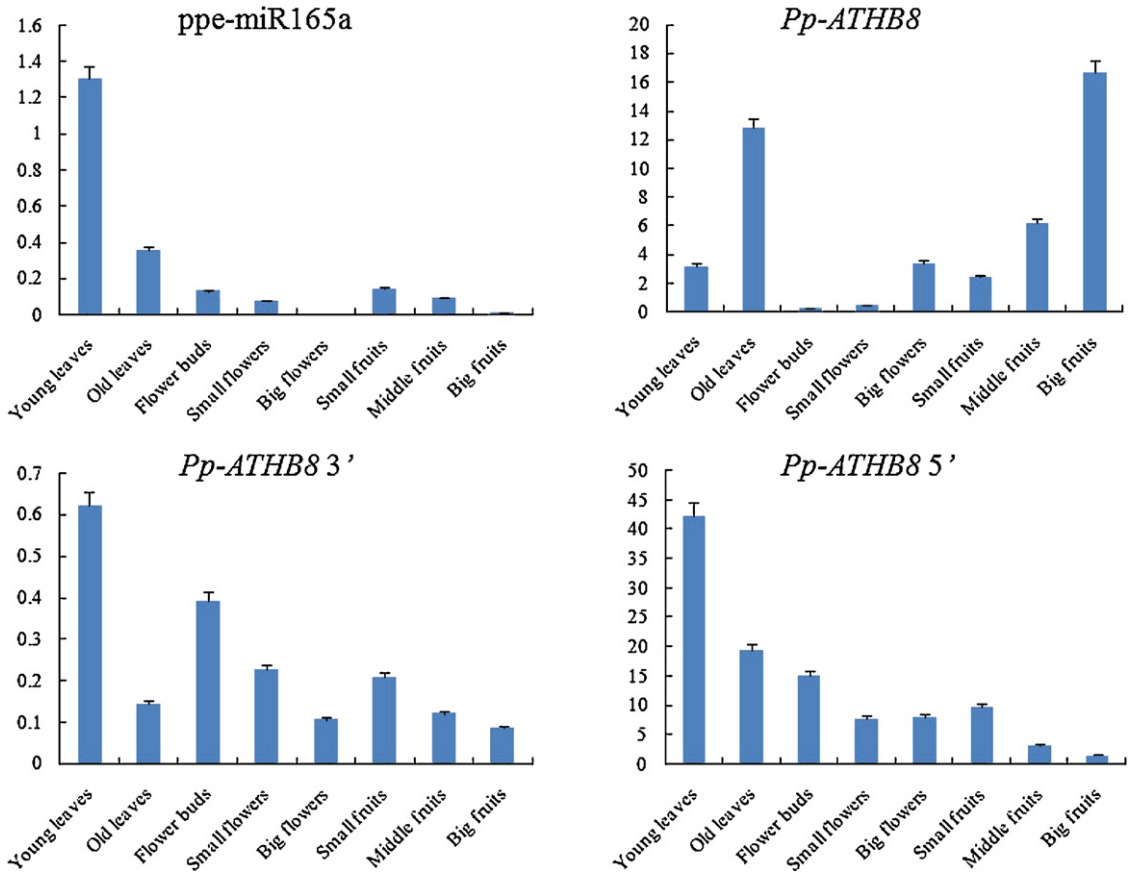

Fig. 3. Relative expression levels of ppe-miR165a and its target *Pp-ATHB8* and miRNA cleaved target gene fragments. Each reaction was repeated three times.

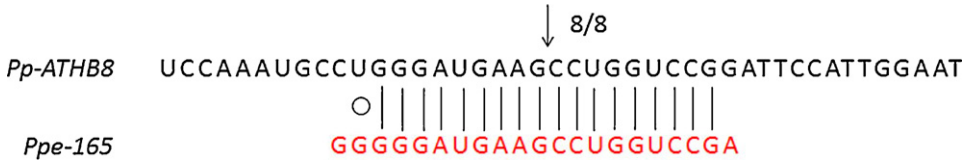

Fig. 4. Mapping of the mRNA cleavage sites by PPM-RACE and RLM-RACE. The top strand (black) depicts a miRNA complementary site, while the bottom strand depicts the miRNA (red). Watson-Crick pairing (vertical dashes) and G: U wobble pairing (circles) are indicated. The arrows indicate the 5′ termini of mRNA fragments isolated from *P. persica*, as identified by cloned PPM-RACE and RLM-RACE products, with the frequency of clones shown. Only the cloned sequences that matched the correct gene and had 5′ ends within a 100 nt window centered on the miRNA validation are included. The partial mRNA sequences from the target genes were aligned with the miRNAs. The numbers indicate the fraction of cloned PCR products terminating at different positions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Pp-ATHB8 mRNAs from PPM-RACE and RLM-RACE and determined their expression levels by qRT-PCRs amplification (Fig. 3). The 3′ end of *Pp-ATHB8* was found to be highly expressed in young leaves, flower buds, small flowers and small fruits, and had moderate or relatively low expression in the other tissues while the 5′ end product of this gene was expressed highly in both young and old leaves, but at moderate or relatively low levels in other organs/tissues (Fig. 3). This indicates a contrast in the expression trends between 5′ and 3′ ends of *Pp-ATHB8* and *Pp-ATHB8* itself, which in turn points to the possible degradation of *Pp-ATHB8* by ppe-miR165. This information related to the 5′ and 3′ ends from the cleaved mRNA of *Pp-ATHB8* is necessary for comprehensive understanding of the regulatory relationship between ppe-miR165and *Pp-ATHB8*. The information is also useful in the wider understanding of interactions between ppe-miRNAs and their target genes. The expression of the cleaved *Pp-ATHB8* mRNA products followed that of ppe-miR165 (Fig. 3) where the products existed in all tissues and expressed tissue-, and/or growth-stage-specific expression characteristics.

4. Discussion

Although plant miRNAs have been intensively studied in the recent past, few studies have been conducted on miRNAs in *P. persica*, despite its importance as a fruit crop in many parts of the world. Both extensive and intensive studies on ppe-miRNAs are necessary and should be expedited. The availability of *P. persica* genome sequence released on April, 2010 (http://www.rosaceae.org/peach/genome) can facilitate the identification and characterization of ppe-miRNAs and their target genes as has been the situation for miRNAs in *A. thaliana* and other plants. The findings of this research are a useful addition to functional genomics and biology in peach. Computational prediction of miRNAs has been found to be efficient and even though a number of methods following this strategy have been developed, they have some disadvantages which include their inability to determine the precise sequences of miRNAs. This limitation can however be overcome using miR-RACE as used in this study

to validate ppe-miR165 through small RNA library construction, cloning and sequencing. From the sequencing results we can see that miR-RACE can be used to confirm and identify the precise end sequences of the predicted miRNA. The sequence identity between the miR165 family in *A. thaliana* (downloaded from miRBase 18.0, http://www.mirbase.org/) and validated members of the ppe-miR165 family (Table 4) show that their sequences were identical. This further shows the effectiveness of mi-RACE as a method for verification of predicted miRNAs. The ubiquitous nature of members from the ppe-miR165 family is evidenced by qRT-PCR detection in different tissues of peach ranging from developing leaves, flowers and fruits with some tissue-, and/or growth-stage-specificity in expression being recorded (Fig. 3). Our qRT-PCR analysis results confirm the existence of ppe-miR165 in *P. persica* and the observed preferential expression of this important miRNA can provide important clues on its probable functions.

Target identification is an important step necessary for assessment and assignment of putative functions to miRNAs in plants. Currently, the most efficient tool available for this is the bioinformatics approach facilitated by the high degree of homology between miRNAs and their targets sequences in plants (Rhoades et al., 2002). Based on this, we predicted and verified the targets of ppe-miR165. We also described the isolation and characterization of a target gene of ppe-miR165, *Pp-ATHB8*, encoding a homeodomain-leucine zipper protein of 840 amino acids which belongs to the HD-ZIP III family previously described. To our knowledge, this is the first report on prediction of target genes and their targeted proteins for members of the miR165 family. Furthermore, *Pp-ATHB8* is the first gene encoding HD-Zip protein isolated from *P. persica* to be reported. Multiple sequence alignment against the NCBI database indicates that *Pp-ATHB8* shares a high degree of nucleotide identity with their homologs in the Rosaceae family or other species. *Pp-ATHB8* shares 91% homology with putative HB8 HD-Zip III mRNA of *Malus x domestica* (Accession no. FJ177427.1) and BZIP18 of *Malus x domestica* (Accession no. HM122470.1), 84% homology with putative HD-Zip protein 8 (ATHB8) of *Poncirus trifoliata* (Accession no. FJ502239.1) and HD-Zip protein 8 (ATHB8) of *Populus trichocarpa* (Accession no. AY919623.1). All these observations confirm that *Pp-ATHB8* does indeed exist in peach. The expression results revealed that its predicted targets in peach might have a conserved function with their orthologs in *A. thaliana* (Rhoades et al., 2002). This is the first report on prediction of members of the miR165 family and their distribution on chromosomes in peach which showed high conservation with those in *A. thaliana*.

By comparing the expression of *Pp-ATHB8* in the vegetative leaves of the plant with that in the flower and in the fruit, we can draw conclusion that the *Pp-ATHB8* expression has higher level in mature tissues or organs than younger ones. As mentioned in *A. thaliana* (Baima et al., 1995), this gene may be a positive regulator of proliferation and differentiation, and participates in a positive feedback loop in which auxin signaling induces the expression of *Pp-ATHB8*, which in turn positively modulates differentiation of procambial and cambial cells. However, the detailed function of this gene in *P. persica* is still not clear and need to be studied further. Our computational identification result of *Pp-ATHB8* can also verify ppe-miR165, whereby the identity levels and the annotated functions of *Pp-ATHB8* with its orthologs in *A. thaliana* or other plants can provide powerful reference to support its prediction as a ppe-miR165 target gene.

Previous studies have shown that miRNAs bind to the protein coding region of their mRNA targets with perfect or near-perfect sequence complementarity, and degrade the target mRNAs in a way akin to RNA interference (Wang et al., 2004). Though miRNAs generally function as negative regulators of gene expression by mediating the cleavage of target mRNAs or by repressing their translation, the cleavage of target mRNAs appears to be the predominant mode of gene regulation in plants (Llave et al., 2002; Sunkar et al., 2005). Finding the cleavage site of a target gene supposedly located in its sequence that is complementary to that of the miRNA is essential in verification of the cleavage of target genes, and the outcome also provides powerful data supporting the authenticity of the identified miRNA target. Detection of the cleaved products of the target genes of miRNAs is also necessary in the study of the regulatory mechanisms of miRNAs on their target genes. The original and popular technique for this detection is Northern Blotting, whereby discovery of fragments having the anticipated size may indicate that the miRNA regulates its target through degradation of the target mRNA. Using Northern Blotting, 3′ end products of the miRNA cleaved target mRNAs may be detected, while the 5′ end products are usually not detected (Chen, 2008). In addition, the detection of presumed cleavage loci using Northern Blotting is not easy. Generally, use of Northern Blotting may also be affected by contamination, low sensitivity and is time-consuming. To address these shortcomings of Northern Blotting, 5′ RLM-RACE is needed and therefore for this study, we used PPM-RACE and RLM-RACE to detect and clone fragments precisely corresponding to the predicted ppe-miR165 guided cleavage products of *Pp-ATHB8* mRNA. PPM-RACE and RLM-RACE, takes advantage of modified 5′ RACE and 3′ RACE, to co-analyze the 5′ and 3′ end cleaved products of the target gene of a miRNA. PPM-RACE and RLM-RACE analysis on *Pp-ATHB8* targeted by ppe-miR165 indicates that this predicted target gene had specific cleavage sites corresponding to complementary sites in the ppe-miR165 sequence (Fig. 4), with the 5′ end of the *Pp-ATHB8* mRNA fragments mapping to the nucleotide that pairs with the 10th nucleotide from the 5′ end of the ppe-miR165. Similar behavior has been reported in the cleavage of miRNA targets in other plants (Palatnik et al., 2003, 2007; Llave et al., 2002; Jones-Rhoades and Bartel, 2004). The qRT-PCR results of cleaved *Pp-ATHB8* mRNA show that both the 3′ and 5′ fragments existed in different tissues, with specific expression patterns in different growth stages or organs. The 5′ and 3′ ends of the target genes showed relatively consistent trends. The fact that expression of the two products from *Pp-ATHB8* cleavage as regulated by ppe-miR165 followed that of ppe-miR165 and contrasted with the expression of *Pp-ATHB8* (Fig. 3), indicates that ppe-miR165 indeed directed cleavage of its target mRNA. In conclusion, we show that ppe-miR165 family members and their target gene *Pp-ATHB8* exist in peach tissues and we further demonstrate that PPM-RACE and RLM-RACE can be successfully used to validate the mechanisms by which ppe-miRNAs cleave their target mRNAs based on the expression of the 3′ and 5′ end cleaved fragments. The findings on spatiotemporal expression analysis of ppe-miR165 and *Pp-ATHB8* as well as the cleaved fragments of *Pp-ATHB8* further confirms the importance of ppe-miRNAs in regulating peach development. It is recommended that more intensive research on this aspect of peach genomics be done so as to discover more ppe-miRNAs and check the role of miRNAs on the control of peach development as well as to assess a putative function for miRNA in plants. This will in turn broaden the knowledge of sRNA-mediated regulation in peach. This study also offered the foundation for isolation of other target genes of members in the ppe-miR165 family and for studying the functions and interaction of these target genes in regulation of critical aspects of plant development.

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