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Identification and Validation of Potential Conserved microRNAs and Their Targets in Peach (*Prunus persica*)

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MicroRNAs are a class of small, endogenous, non-coding RNA molecules that negatively regulate gene expression at the transcriptional or the post-transcriptional level. Although a large number of miRNAs have been identified in many plant species, especially from model plants and crops, they remain largely unknown in peach. In this study, 110 potential miRNAs belonging to 37 families were identified using computational methods. A total of 43 potential targets were found for 21 families based on near-perfect or perfect complementarity between the plant miRNA and the target sequences. A majority of the targets were transcription factors which play important roles in peach development. qRT-PCR analysis of RNA samples prepared from different peach tissues for 25 miRNA families revealed that miRNAs were differentially expressed in different tissues. Furthermore, two target genes were experimentally verified by detection of the miRNA-mediated mRNA cleavage sites in peach using RNA ligase-mediated 5' rapid amplification of cDNA ends (RLM-RACE). Finally, we studied the expression pattern of the two target genes in three different tissues of peach to further understand the mechanism of the interaction between miRNAs and their target genes.

INTRODUCTION

MicroRNAs (miRNAs) are a large group of endogenous ~21 nt small RNAs that play essential regulatory roles in various biological and metabolic processes, including development, signal transduction, cell fate identity, organ differentiation and stress responses by targeting messenger RNAs (mRNAs) for degradation or translational repression (Bartel, 2004; Carrington and Ambros, 2003; Jones-Rhoades et al., 2006). miRNA genes are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) (Bartel, 2004; Carrington and Ambros, 2003). Following transcription and several post-transcriptional modifications using a set of Dicer-like enzymes, miRNA precursors (pre-miRNAs) and eventually mature miRNAs are generated (Schauer et al., 2002). Subsequently, mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) to modulate the expression of target genes (Carrington and Ambros, 2003).

Recently, miRNAs have been identified by three methods: genetic screens, cloning and computational approaches. Although miRNAs are initially discovered using genetic screening technology (Lee et al., 1993; Wightman et al., 1993), this method is limited as it is expensive, time consuming and dominated by chance (Zhang et al., 2006b). Direct cloning of miRNAs, followed by construction and sequencing of a small RNA library, has been successfully employed to identify miRNAs in plants (Feng et al., 2009; Jian et al., 2010; Sunkar et al., 2005; Yao et al., 2007). However, only abundant miRNA genes can be easily detected by cloning or Northern blot. To find low-expression miRNA genes, computational prediction provides a convenient, valid strategy. A large number of miRNAs have been identified using computational approaches based on the high degree of conservation in plants (Dhandapani et al., 2011; Jones-Rhoades and Bartel, 2004; Lindow and Krogh, 2005; Zhang et al., 2006a).

miRNA regulation mechanisms include repression of translation (Aukerman and Sakai, 2003; Chen, 2004), cleavage of targeted mRNAs (Schwab et al., 2005; Sunkar et al., 2005) and chromatin modification (Jeong et al., 2011). The known miRNA targets have a high degree of complementarity to miRNAs and cleavage of the target mRNA typically occurs at the centre of the paired region, especially for plant miRNAs (German et al., 2008). This allows for the prediction of miRNA targets by computational approaches. Several studies have utilised this fact and identified many target genes in different plant species while allowing 1-3 nucleotide mismatches between the target mRNAs and miRNAs (Song et al., 2009; Wang et al., 2012; Zhang et al., 2008).

A growing number of miRNAs and their targets have been predicted and/or experimentally discovered in many plants; however, the miRNAs in peach are largely unknown despite a previous study reporting a few miRNAs in this species (Zhang et al., 2011). The systematic identification and characterisation of miRNAs in peach using the peach genome would help in

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identifying more miRNAs. We could better understand the genetic and morphological diversity of this species though analysing the functions of miRNAs. In addition, miRNA gene expression analyses have provided a good method to discern complex biological processes in plants. Peach is an economically important group of cultivated fruits and is considered one of the genetically most well-characterised species in the Rosaceae. The doubled haploid cultivar 'Lovell' Genome Sequencing Project was completed by employing the accurate Sanger methodology in 2010, which generated a 219 Mbp genome sequence and a 37.2 Mbp mRNAs sequence. There are a total of 202 scaffolds in this assembly of peach and are 26,938 CDS sequences (http://www.rosaceae.org/node/355). In this study, we predicted peach miRNAs and their targets by computational approaches using peach genome sequences. These potential miRNAs and their targets needed to be further validated and characterised by detecting and quantifying their expression in different tissues. A simple, accurate, special and sensitive method for miRNA detection and target expression profiling is in high demand. Here, we used a qRT-PCR-based method.

MATERIALS AND METHODS

Plant materials

Samples of young leaves, stems and flowers were collected from one-year old grafted 'Lovell' (*Prunus persica*) trees that were grown in the garden of Nanjing Agricultural University, China. After collection, all the samples were immediately frozen in liquid nitrogen and stored at -70°C for the following study.

Reference set of miRNA and peach genome sequences

A total of 2675 previously known mature plant miRNAs and their precursor sequences from 43 plant species were downloaded from the miRBase (Griffiths-Jones, 2006; Griffiths-Jones et al., 2008). Peach genome sequences and mRNA sequences were obtained from GDR (http://www.rosaceae.org/node/355).

Analysis software

A computer program, microHARVESTER (Dezulian et al., 2006), was used to identify potential miRNAs. The prediction of secondary structures and the stability of miRNA precursors were assessed by RNAfold (Hofacker et al., 1994) and Mfold-3.1.2 (Zuker, 2003).

Prediction of potential *Prunus persica* miRNAs (ppe-miRNAs) and targets

We used the microHARVESTER program to predict peach miRNAs. This approach, with excellent sensitivity and specificity, was based on a homology search followed by a set of structural filters. First, BLASTn (Altschul et al., 1997) and the Smith-Waterman pairwise alignment algorithm (Smith and Waterman, 1981) were employed to precisely determine the precursor and mature sequence candidates with a sensitive BLAST parameter setting (word-length 7 and E-value cutoff 10). We discarded those candidates whose aligned segments did not span most of the mature segment of the known precursor sequences and whose mature segments differed by more than four mistakes with a previously known mature miRNAs. Second, we used RNAfold to predict the minimal free energy structure of the candidate sequence. We discarded a candidate if more than six nucleotides of its miRNA* did not form bonds with its mature miRNA. Finally, Mfold was used to predict whether the remaining precursors had high negative minimal folding free energy (MFE), adjusted minimum folding free energy (AMFE) and a high minimal folding free energy index (MFEI) or not (Zhang et al., 2006c).

Previous studies have shown that all plant miRNAs mediate gene expression by targeting mRNA sequences at a perfect or near-perfect complementary site. This allowed the prediction of miRNA targets by computational approaches. To identify potential regulatory targets, we tested the 110 identified miRNAs against the peach mRNA sequence using a BLASTn search. Gaps were not allowed and G:U and other non-canonical pairs were treated as mismatches. The number of allowed mismatches at complementary sites between miRNA sequences and potential mRNA targets was no more than three. BLASTx was performed with the selected target transcripts to identify the functions of miRNAs.

Low molecular weight RNA extraction

Low molecular weight (LMW) RNA was independently isolated from different tissues by using CTAB reagent according to the procedure previously described by Wang et al. (2010). The concentration of RNA was measured by a UV-1800 spectrophotometer (Eppendorf, Germany) at 260 and 280 nm and visually ascertained on a 1.5% agarose gel.

Construction of small RNA cDNA libraries

Small RNAs were isolated from three peach tissues including young leaves, young stems and flowers. The small RNAs were polyadenylated using a poly(A) polymerase (NEB, USA) and then the poly(A)-tailed RNAs were recovered by phenol/chloroform extraction followed by ethanol precipitation. Reverse transcription was performed using MLV reverse transcriptase (Promega, USA), 1 μg of RT primers (Table 1) and 1 μg of poly(A)-tailed RNA to synthesise the small RNA cDNAs following the manufacturer's instructions (Ro et al., 2006).

qRT-PCR analysis of peach miRNAs

The templates used for qRT-PCR were the miRNA-enriched cDNA libraries generated from young leaves, stems and flowers. A miRNA-specific primer and a universal reverse primer, URP, were used for real-time quantitative PCR (Table 1). For real-time PCR, cDNA was mixed with 2× SYBR Green Mix (Takara, Japan) and each of the miRNA specific primers and a universal reverse primer in a final volume of 20 μl. PCR runs were 40 cycles each at 95°C for 10 s, 60°C for 20 s and 72°C for 45 s. Each reaction was repeated three times. The relative miRNA expression was quantified using the comparative ΔΔCT method (Livak and Schmittgen, 2001). All expression profiles were normalised to expression levels in the stem. 5.8S rRNAs (Design, 2005), was used as an internal control. The primer sequences are shown in Table 1.

Validation of miRNA target genes using RLM-RACE

To find the internal cleavage site in ppa005013m (targeted by miR156) and ppa005230m (targeted by miR172), RLM-RACE was performed using the 5′-Full Race Kit (Takara, Japan). A modified procedure for RLM-RACE was carried out following the instruction of the kit without calf intestinal phosphatase and tobacco acid pyrophosphatase treatment. Total RNA was extracted from young leaves, young stems and flowers using the CTAB method. Poly $(A)^+$ mRNA was purified from pooled tissue RNA using the Oligotex™ -dT30<Super> mRNA Purification Kit (Takara) according to the manufacturer's instructions. The adapter was directly ligated to the mRNA, then first strand cDNA was synthesised in a reverse transcription reaction. An amplification step, the same as the one used for gene-specific

Table 1. Primers used for qRT-PCR

Name	Sequences (5′ → 3′)	Name	Sequences (5′ → 3′)
ppe-miR1511	AACCTGGCTCTGATACCATA	ppe-miR172a	AGAATCTTGATGATGCTGCATAA
ppe-miR172b	GGAATCTTGATGATGCTGCAG	ppe-miR171a	TGATTGAGCCGCGTCAAT
ppe-miR171b	TGATTGAGCCACGCCAAC	ppe-miR171c	AGATTGAGCCGCGCC
ppe-miR171d	TGATTGAGCCGTGCCAA	ppe-miR167b	TGAAGCTGCCAGCATGATCT
ppe-miR2275	TTTAGTTTCCTCCAATATCTCA	ppe-miR398a	TGTGTTCTCAGGCATCACAC
ppe-miR398b	TGTGATCTCAGGTCACCCC	ppe-miR398c	TGTGTTCTCAGGTCGCC
ppe-miR164c	TGGAGAAGGGGAGCACG	ppe-miR169d	CAGCCAAGGATGACTTGC
ppe-miR169g	TAGCCAAGGATGACTTGCC	ppe-miR169i	TGAGCCAAGAATGACTTGCT
ppe-miR403	TTAGATTCACGCACAAACTC	ppe-miR396b	TTTCACAGCTTTCTTGAACTG
ppe-miR396c	TTCCACAGCTTTCTTGAACT	ppe-miR166e	GCGAACCAGACAGCATTC
ppe-miR408	ATGCACTGCCTCTTCCCT	ppe-miR482a	TCTTTCCGAAACCTCCC
ppe-miR319f	TTGGACTGAAGGGAGCTC	ppe-miR395d	ATGAAGTGTTCAAGGGAACTC
ppe-miR395g	CTGAAGTGTTTGGGGGAAC	ppe-miR160a	TGCCTGGTTCCCTGTATG
ppe-miR390	AAGCTCAGGAGGGATAGC	ppe-miR156e	TTGACAGAAGATAGAGAGCACA
ppe-miR156i	TGACAGAAGATAGAGAGCACAA	ppe-miR827	GTAGATGACCATAAACAAACAA
ppe-miR447	ACTCTCCCTCAAGGGCT	ppe-miR2118	CTACCGATTCCACCCATTC
ppe-miR164a	TGGAGAAGCAGGGCACG	ppe-miR393a	TCCCAAGGGATCGCATCG
ppe-miR535a	TGACGACGAGAGAGAGCAC	ppe-miR397	TCATTGAGTGCAGCGTTGAT
ppe-miR477	CTCTCCCTCAAAGGCTTC	URP	CCAGTAGCGTATGATGAGCA
5.8s rRNA forward primer	CTCGGCAACGGATATCTCG		
5.8s rRNA reverse primer	CTAATGGCTTGGGGCG		
ppa005013m forward primer	TTGCTGATGGAGTGGAAT		
ppa005013m reverse primer	TCTGCTGGTTGTAACTTCT		
ppa005230m forward primer	TTCAACTCCTCCTCCAAC		
ppa005230m reverse prime	ATGACGACGAAGAAGAAGA		
PRII forward primer	TGAAGCATACACCTATGATGATGAAG		
PRII reverse primer	CTTTGACAGCACCAGTAGATTCC		
RT-primer	CCAGTAGCGTATGATGAGCACAGAGTCTGAGATCACTCGTAGCGAGG-d$(T)_{33}$-V(A/C/G)N(A/C/G/T)		

RACE, was recommended by the manufacturer and included a 5′ nest primer and a 3′ gene specific primer. The primer sequences were as follows: 5′ RACE outer primer (CATGGCT ACATGCTGACAGCCTA), 5′ RACE inner primer (CGCGGA TCCACAGCCTACTGATGATCAGTCGATG), *ppa005013m* 3′ outer primer (GAATTGGTTGGTTTGAGAACCAAAC), *ppa005013m* 3′ inner primer (GGTTGCTGCCATTACTGTGTGAGTG), *ppa 005230m* 3′ outer primer (AGCTCCTGCAATAGAAACCGGG TAT) and *ppa005230m* 3′ inner primer (CGATGGTGAAAA ATGGTGGTGGAGA). After amplification, the 5′ RACE products were gel-purified and cloned, and at least 10 independent clones were randomly chosen and sequenced.

The expression analysis of miRNA targets

Reverse transcription of the total RNA that was extracted from young leaves, young stems and flowers was performed using the PrimeScript® RT reagent Kit. cDNA was mixed with 2× SYBR Green Mix (Takara, Japan) and each of the miRNA target-specific primers in a final volume of 20 μl for qRT-PCR. *PRII* (Tong et al., 2009) was used as an internal control. The primer sequences are shown in Table 1.

RESULTS

Computational prediction of potential ppe-miRNAs

Since the beginning of abundant miRNA identification and annotation, it has been well-recognised that miRNAs are evolutionarily conserved in plants and animals. Based on the conserved sequences and secondary structures, 290 potential candidates were selected through a computer program, microHARVESTER. Of these, 207 candidates had fewer than four mismatches with known mature miRNA sequences. After carefully evaluating the secondary structures using the criteria described in the Methods section, there remained 110 potential miRNAs (Table 2).

The 110 predicted peach miRNAs belong to 37 miRNA families. The largest miRNA family size identified was miR399 that consisted of 11 members. miR156, miR169 and miR395 possessed nine, nine and eight members, respectively, whereas, 17 miRNA families had only one member identified in this study (Fig. 1A). The length of the mature miRNAs ranged from 17 to 23 nt (Table 2 and Fig. 1B). The majority of miRNAs were 21 nt long (60.0%) followed by 20 (21.8%), 22 (15.5%), 23 (1.8%) and 17 (0.9%) (Fig. 1B).

Following peach miRNA identification, diversity could be

Table 2. Details of identified ppe-miRNAs and structural information

miRNA	Location	Mature miRNA	MAS	MN (nt)	ML (nt)	PL (nt)	(A+U)%	MFEs	AMFEs	MFEIs
ppe-miR156a	scaffold_7:22103897-22104005	UGACAGAAGAGAGUGAGCAC	5′	0	20	109	50.46	47.70	43.76	0.810
ppe-miR156b	scaffold_1:33986381-33986484	UGACAGAAGAGAGUGAGCAC	5′	0	20	104	53.85	53.70	51.63	1.076
ppe-miR156c	scaffold_7:19399614-19399720	UGACAGAUAGAGAGAGAGCAC	5′	2	21	107	57.94	52.20	48.79	1.084
ppe-miR156d	scaffold_1:34819687-34819794	UGACAGAUAGAGAGUAAGCAC	5′	1	21	108	52.78	47.40	43.89	0.861
ppe-miR156e	scaffold_3:10599623-10599738	UUGACAGAAGAUAGAGAGCAC	5′	0	21	116	52.59	48.30	41.64	0.757
ppe-miR156f	scaffold_5:17453185-17453288	UUGACAGAAGAUAGAGAGCAC	5′	0	21	104	57.69	47.70	45.87	1.040
ppe-miR156g	scaffold_3:21012691-21012815	UUGGCAGAAGAAAAGAGAGCAC	5′	3	22	125	60.80	49.00	39.2	0.800
ppe-miR156h	scaffold_6:3280057-3280163	UUGACAGAAGAAAGAGAGCAC	5′	1	21	107	52.34	53.30	49.81	0.977
ppe-miR156i	scaffold_3:10599079-10599182	UGACAGAAGAUAGAGAGCACA	5′	1	21	104	55.77	47.40	45.58	0.991
ppe-miR159a	scaffold_2:18908762-18908950	AUUGGAGUGAAGGGAGCUCC	3′	2	20	189	52.91	92.10	48.73	0.548
ppe-miR159b	scaffold_5:17562391-17562575	UUUGGAUUGAAGGGAGCUCUA	3′	1	21	185	51.89	78.70	42.54	0.478
ppe-miR159c	scaffold_4:13455964-13456050	CUUGGCUUGAAGGGAGCUCCG	3′	2	21	87	48.28	24.40	28.05	0.623
ppe-miR160a	scaffold_6:21902556-21902657	UGCCUGGUUCCCUGUAUGCCA	5′	1	21	102	50.00	45.30	44.41	0.871
ppe-miR160b	scaffold_4:5325323-5325427	UGCCUGGCUCCCUGUAUGCCA	5′	0	21	105	46.67	63.80	60.76	1.085
ppe-miR162	scaffold_5:16885784-16885886	UCGAUGAACCGCUGCCUCCAG	3′	3	21	103	52.43	43.30	42.04	0.858
ppe-miR164a	scaffold_6:26465850-26465969	UGGAGAAGCAGGGCACGUGCA	5′	0	21	120	53.33	60.00	50.00	0.893
ppe-miR164b	scaffold_6:24710611-24710792	UGGAGAAGCAGGGCACGUGCA	5′	0	21	182	59.89	56.60	31.10	0.426
ppe-miR164c	scaffold_8:21367800-21367915	UGGAGAAGGGGAGCACGUGCA	5′	3	21	116	50.86	53.80	46.38	0.814
ppe-miR164d	scaffold_3:1784022-1784116	UGGAGAGCUAGAGCACAUGCA	5′	4	21	95	54.74	41.30	43.47	1.011
ppe-miR166a	scaffold_8:19800532-19800631	UCGGACCAGGCUUCAUUCCC	3′	0	20	100	51.00	55.40	55.40	1.131
ppe-miR166b	scaffold_5:12581627-12581786	UCGGACCAGGCUUCAUUCCC	3′	0	20	160	59.38	59.60	37.25	0.573
ppe-miR166c	scaffold_2:26094634-26094793	UCGGACCAGGCUUCAUUCCC	3′	0	20	160	53.13	63.40	39.63	0.528
ppe-miR166d	scaffold_2:19692915-19693064	UCGGACCAGGCUUCAUUCCC	3′	0	20	150	61.33	64.20	42.80	0.738
ppe-miR166e	scaffold_1:2213219-2213318	UCGAACCAGACAGCAUUCCC	3′	4	20	100	53.00	49.50	49.50	1.053
ppe-miR167a	scaffold_4:5610366-5610741	UGAAGCUGCAAGAUGACCUG	5′	4	20	376	69.95	102.20	27.18	0.241
ppe-miR167b	scaffold_6:27656223-27656309	UGAAGCUGCCAGCAUGAUCUG	5′	0	21	87	58.62	43.00	49.43	1.373
ppe-miR167c	scaffold_1:1563822-1563911	UGAAGCUGCCAGCAUGAUCUU	5′	1	21	90	56.67	45.60	50.67	1.299
ppe-miR167d	scaffold_8:20014988-20015080	UGAAGCUACCACAUGAUCUG	5′	3	20	93	51.61	42.80	46.02	1.023
ppe-miR169a	scaffold_3:19573376-19573494	UAGCCAGAGACGACUUGCCGA	5′	4	21	119	50.42	46.80	39.33	0.667
ppe-miR169b	scaffold_4:16645761-16645853	GAGCCAAGGAUGACUUGCCA	5′	2	20	93	53.76	47.30	50.86	1.183
ppe-miR169c	scaffold_4:16664507-16664599	GAGCCAAGGAUGACUUGCCA	5′	2	20	93	53.76	45.10	48.49	1.128
ppe-miR169d	scaffold_4:16676120-16676220	CAGCCAAGGAUGACUUGCCGG	5′	3	21	101	53.47	47.00	46.53	0.990
ppe-miR169e	scaffold_4:16648995-16649095	CAGCCAAGGAUGACUUGCCGG	5′	3	21	101	52.48	48.60	48.12	1.002
ppe-miR169f	scaffold_3:21677982-21678091	UAGCCAAGGAUGACUUGCCUG	5′	0	21	110	50.91	43.60	39.64	0.734
ppe-miR169g	scaffold_4:10137181-10137297	UAGCCAAGGAUGACUUGCCUGC	5′	0	22	117	53.85	45.20	38.63	0.715
ppe-miR169h	scaffold_1:22425535-22425679	UAGCCAAGGAGACUGCCUGU	5′	3	20	145	51.03	55.80	38.48	0.542
ppe-miR169i	scaffold_4:16691539-16691653	UGAGCCAAGAAUGACUUGCUG	5′	2	21	115	58.26	46.90	40.78	0.850
ppe-miR171a	scaffold_3:16525609-16525709	UGAUUGAGCCGCGUCAAUAUC	3′	1	21	101	57.43	41.90	41.49	0.965
ppe-miR171b	scaffold_3:21505900-21505996	UGAUUGAGCCACGCCAACAUC	3′	2	21	97	61.86	37.50	38.66	1.045
ppe-miR171c	scaffold_7:21451283-21451383	AGAUUGAGCCGCGCCAAUAUC	3′	1	21	101	53.47	48.60	48.12	1.024
ppe-miR171d	scaffold_3:16557260-16557354	UGAUUGAGCCGUGCCAAUAUC	3′	1	21	95	54.74	49.60	52.21	1.214
ppe-miR171e	scaffold_1:32200679-32200763	UGAUUGAGCCGUGCCAAUAUC	3′	1	21	85	52.94	38.30	45.06	1.126
ppe-miR172a	scaffold_2:20686356-20686484	AGAAUCUUGAUGAUGCUGCAU	3′	0	21	129	59.69	53.10	41.16	0.792
ppe-miR172b	scaffold_2:22285766-22285872	GGAAUCUUGAUGAUGCUGCAG	3′	2	21	107	55.14	49.50	46.26	0.964
ppe-miR172c	scaffold_6:4912768-4912951	UGAAUCUUGAUGAUGCCGCAC	3′	3	21	184	59.24	58.50	31.79	0.424
ppe-miR319a	scaffold_1:29856933-29857147	UUGGACUGAAGGGAGCUCCU	3′	1	20	215	51.63	102.90	47.86	0.460
ppe-miR319b	scaffold_2:23738870-23739096	UUGGACUGAAGGGAGCUCCUC	3′	1	20	227	57.71	88.40	38.94	0.406
ppe-miR319c	scaffold_2:18914576-18914767	UUGGAUUGAAGGGAGCUCCA	3′	2	20	192	48.96	100.40	52.29	0.534
ppe-miR319d	scaffold_5:17220685-17220922	UUGGACUGAAGGGAGCUCCC	3′	0	20	238	49.16	111.20	46.72	0.386
ppe-miR319e	scaffold_6:2304460-2304649	UUGGACUGAAGGGAGCUCCC	3′	0	20	190	56.84	78.30	41.21	0.503
ppe-miR319f	scaffold_8:17944925-17945032	UUGGACUGAAGGGAGCUCUCA	3′	2	21	108	50.93	40.40	37.41	0.706
ppe-miR390a	scaffold_6:24551362-24551453	AAGCUCAGGAGGGAUAGCGCC	5′	0	21	92	54.35	44.30	48.15	1.146
ppe-miR390b	scaffold_6:1728631-1728735	AAGCUCAGGAGGGAUAGCGCC	5′	0	21	105	61.90	48.50	46.19	1.155
ppe-miR393a	scaffold_2:25056675-25056781	UCCCAAGGGAUCGCAUCGAUCC	5′	2	22	107	57.94	39.50	36.92	0.820
ppe-miR393b	scaffold_2:22650086-22650180	UCCAAAGGGAUCGCAUUGAUCC	5′	0	22	95	56.84	40.80	42.95	1.047
ppe-miR394a	scaffold_1:43600159-43600272	UUGGCAUUCUGUCCACCUCCAU	5′	0	22	114	58.77	49.60	43.51	0.926
ppe-miR394b	scaffold_1:32136128-32136229	UUGGCAGUAUGCCCACCUCCAC	5′	3	22	102	53.92	37.50	36.76	0.782
ppe-miR395a	scaffold_1:26767775-26767862	CUGAAGUGUUUGGGGGGACC	3′	1	20	88	52.27	37.80	42.95	1.023
ppe-miR395b	scaffold_1:26799377-26799487	CUGAAGUGUUUGGGGGGACC	3′	1	20	111	55.86	43.80	39.46	0.805
ppe-miR395c	scaffold_1:26765063-26765158	AUGAAGUGAGUGAGGGAACUC	3′	4	21	96	57.29	42.60	44.38	1.082
ppe-miR395d	scaffold_1:26805384-26805488	AUGAAGUGUUCAAGGGAACUC	3′	4	21	105	59.05	39.40	37.52	0.873
ppe-miR395e	scaffold_1:26780279-26780383	AUGAAGUGUUCAAGGGAACUC	3′	4	21	105	59.05	35.20	33.52	0.780

(continued)

miRNA	Location	Mature miRNA	MAS	MN (nt)	ML (nt)	PL (nt)	(A+U)%	MFEs	AMFEs	MFEIs
ppe-miR395f	scaffold_1:26764880-26764975	AUGAAGUGUUCAAGGGAACUC	3′	4	21	96	55.21	43.40	45.21	1.051
ppe-miR395g	scaffold_1:26806562-26806681	CUGAAGUGUUUGGGGGAACUC	3′	1	21	120	60.00	50.60	42.17	0.878
ppe-miR395h	scaffold_1:26748442-26748561	CUGAAGUGUUUGGGGGAACUC	3′	1	21	120	60.00	38.30	31.92	0.665
ppe-miR396a	scaffold_7:21479083-21479229	UUCCCACAGCUUUAUUGAACCG	5′	4	22	147	54.42	51.60	35.10	0.524
ppe-miR396b	scaffold_1:39280926-39281045	UUUCACAGCUUUCUUGAACUGU	5′	2	22	120	58.33	55.90	46.58	0.932
ppe-miR396c	scaffold_7:21474174-21474288	UUCCACAGCUUUCUUGAACUU	5′	0	21	115	56.52	51.30	44.61	0.892
ppe-miR397	scaffold_4:1619240-1619325	UCAUUGAGUGCAGCGUUGAUG	5′	1	21	86	62.79	42.60	49.53	1.548
ppe-miR398a	scaffold_1:27542748-27542857	UGUGUUCUCAGGCAUCACACCUU	3′	4	23	110	57.27	55.40	50.36	1.072
ppe-miR398b	scaffold_4:22864986-22865110	UGUGAUCUCAGGUCACCCCUGU	3′	3	22	125	42.40	76.50	61.20	0.85
ppe-miR398c	scaffold_4:23714196-23714310	UGUGUUCUCAGGUCGCCCCUG	3′	2	21	115	46.09	51.20	44.52	0.718
ppe-miR399a	scaffold_4:3161730-3161872	UGCCAAAGGAGUAAUUGCCCAG	3′	2	22	143	55.24	50.50	35.31	0.552
ppe-miR399b	scaffold_4:3186380-3186502	UGCCAAAGGAGAAUUGCCCUG	3′	0	21	123	60.98	60.50	49.19	1.025
ppe-miR399c	scaffold_4:3187835-3187944	UGCCACUAGAGAAUUGCCCUG	3′	3	21	110	51.82	49.80	45.27	0.854
ppe-miR399d	scaffold_4:3192972-3193111	UGCCAGAGGAGACUUUGCCCUG	3′	3	22	140	56.43	51.40	36.71	0.602
ppe-miR399e	scaffold_3:4392605-4392744	UGCCAGAGGAGACUUUGCCCUG	3′	3	22	140	55.00	55.20	39.43	0.626
ppe-miR399f	scaffold_3:541261-541369	UGCCAAAGAAGAGUUGCCCUA	3′	2	21	109	54.13	51.30	47.06	0.941
ppe-miR399g	scaffold_3:584777-584885	UGCCAAAGAAGAGUUGCCCUA	3′	2	21	109	53.21	48.50	44.50	0.872
ppe-miR399h	scaffold_3:574913-575021	UGCCAAAGAAGAGUUGCCCUA	3′	2	21	109	54.13	50.00	45.87	0.917
ppe-miR399i	scaffold_1:46720492-46720600	UGCCAAAGAAGAGUUGCCCUA	3′	2	21	109	54.13	54.20	49.72	0.994
ppe-miR399j	scaffold_5:9980708-9980803	UGCCAAUGGAGAGACGCCCUA	3′	4	21	96	53.13	35.90	37.40	0.831
ppe-miR399k	scaffold_4:3179684-3179797	UGCCAAAGGAGAAUUGCCGUG	3′	1	21	114	58.77	34.30	30.09	0.640
ppe-miR403	scaffold_1:8677734-8677847	UUAGAUUCACGCACAAACUCG	3′	0	21	114	54.39	44.80	39.30	0.756
ppe-miR408	scaffold_10:245026-245130	AUGCACUGCCUCUUCCCUGGC	3′	2	21	105	48.57	46.60	44.38	0.822
ppe-miR414	scaffold_7:21519316-21519594	UCAUCAUCAUCAUCAUCGUCU	5′	2	21	279	47.67	121.50	43.55	0.298
ppe-miR447	scaffold_5:11892465-11892595	ACUCUCCCUCAAGGGCUUCUCAG	5′	3	23	131	54.96	55.90	42.67	0.723
ppe-miR477	scaffold_5:11892655-11892736	CUCUCCCUCAAAGGCUUCUA	5′	1	20	82	54.88	43.30	52.80	1.427
ppe-miR482a	scaffold_1:29646074-29646169	UCUUUCCGAAACCUCCCAUUCC	3′	3	22	96	65.63	32.20	33.54	1.016
ppe-miR482b	scaffold_1:29651603-29651699	CCUACUCCACCCAUUCC	3′	1	17	97	55.67	43.70	45.05	1.048
ppe-miR482c	scaffold_3:10579299-10579395	UCUUCCCAAGCCCGCCCAUUCC	3′	1	22	97	62.89	42.50	43.81	1.217
ppe-miR535a	scaffold_8:17685868-17685968	UGACGACGAGAGAGAGCACGC	5′	1	21	101	48.51	61.90	61.29	1.179
ppe-miR535b	scaffold_8:17689597-17689697	UGACAACGAGAGAGAGCACGC	5′	0	21	101	48.51	62.30	61.68	1.186
ppe-miR538	scaffold_4:4636437-4636570	UUGCAUGCAGUCUAUGUCUGGG	5′	2	22	134	61.19	36.70	27.39	0.527
ppe-miR827	scaffold_7:22580971-22581076	GUAGAUGACCAUAAACAAACA	3′	2	21	106	70.75	35.20	33.21	1.071
ppe-miR858	scaffold_5:17626942-17627247	UCUCGUUGUCUGUUCGACCUU	5′	1	21	306	66.67	67.30	21.99	0.216
ppe-miR1030	scaffold_6:7773855-7774079	UCUGCAUUUGCACCUGCACUU	5′	3	21	225	59.11	76.30	33.91	0.369
ppe-miR1446	scaffold_3:21872535-21872639	UUCUUAACUCUCUCCCUCAUA	5′	2	21	105	59.05	44.00	41.90	0.975
ppe-miR1511	scaffold_3:8575412-8575509	AACCUGGCUCUGAUACCAUA	3′	2	20	98	63.27	41.30	42.14	1.17
ppe-miR2111	scaffold_4:5129206-5129292	UAAUCUGCAUCCUGAGGUUUA	5′	0	21	87	51.72	52.80	60.69	1.445
ppe-miR2118	scaffold_1:29644637-29644733	CUACCGAUUCCACCCAUUCCGA	3′	2	22	97	58.76	39.90	41.13	1.028
ppe-miR2275	scaffold_3:19748539-19748641	UUUAGUUUCCUCCAAUAUCUCA	3′	1	22	103	60.19	47.00	45.63	1.113
ppe-miR3627	scaffold_3:19985286-19985440	UGGUCGCAUAGCGACGGCACU	5′	4	21	155	43.23	55.20	35.61	0.405
ppe-miR3629a	scaffold_4:14155746-14155846	GGCUGCCGAGAAAGUGUGGGA	5′	3	21	101	53.47	27.00	26.73	0.659
ppe-miR3629a	scaffold_6:6832326-6832628	GGUUGCUGAGAAAAUGCAGGA	5′	2	21	303	61.72	84.30	27.82	0.240
ppe-miR3629a	scaffold_6:6555645-6555802	GGUUGAUGAGAAAAUGAAGGA	5′	3	21	158	70.25	39.10	24.75	0.527
ppe-miR3629a	scaffold_2:19091880-19092047	GGCUGCUGAGAAAUCUGGGA	5′	3	20	168	64.29	48.80	29.05	0.484
ppe-miR3629a	scaffold_5:12208693-12208905	GGUUACUGAGAAAAUGAAGGA	5′	3	21	213	63.38	71.40	33.52	0.430
ppe-miR3629a	scaffold_8:14952639-14952764	GGUUGCUGAGAAAAUGGAGGA	3′	2	21	126	57.94	27.20	21.59	0.407
ppe-miR3629a	scaffold_7:14342466-14342736	GGUUGCUGAGAAAGUGUGGGA	3′	3	21	271	53.14	88.00	30.66	0.241

MAS, mature miRNA arm sided in hairpin secondary structure; MN, mismatch number; ML, mature miRNA length; PL, precursor miRNA length; MFE, minimum folding free energy; AMFE, adjusted minimum folding free energy; MFEI, minimum folding free energy index.

found not only in the numbers within each miRNA family, but also in other aspects, such as the location of the mature miRNAs and the length of miRNA precursors. The 49.1% of the mature miRNA sequences were located at the 5′-end of the miRNA precursor sequence, with the others at the 3′-end. The length of the miRNA precursors varied from 82 to 376 nucleotides with an average of 128.6. However, the majority of the identified miRNAs (73.6%) had 82-129 nucleotides, with more than half of the miRNAs (59.1%) at 90-119 nucleotides (Fig. 1C). Compared with animal miRNAs, which have a consistent nucleotide length (~70-80 nt) (Ambros, 2004; Bartel, 2004), the length of plant (including peach) miRNA precursors varies. Although all identified ppe-miRNAs had similar predicted stem-loop hairpin structures, their hairpin shapes varied due to differences in length (Fig. 2). The percentage AU content ranged from 42.4% to 70.75% with an average of 55.68% (Table 2). Several studies have found that miRNA precursors have low folding free energy, and have suggested that low free energy is an important characteristic of miRNAs (Dhandapani et al., 2011; Zhang et al., 2006b). The MFE of the 110 identified ppe-

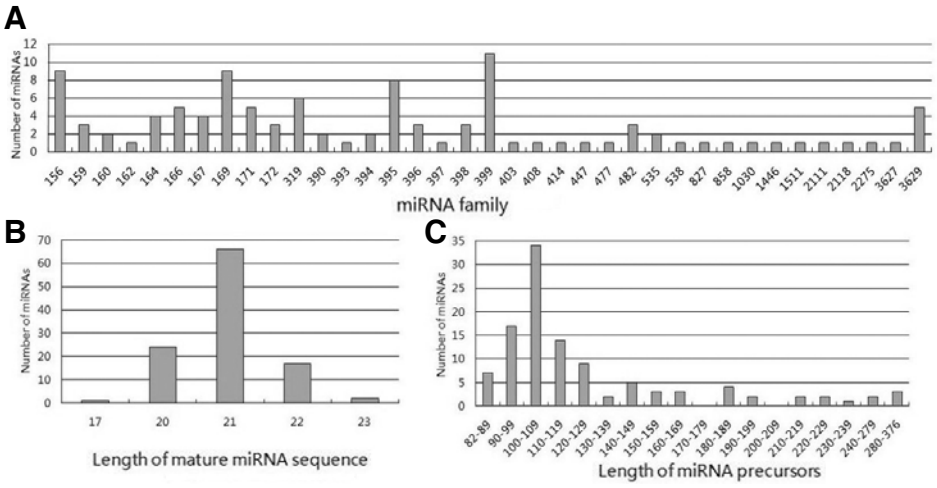

Fig. 1. Analysis of potential miRNAs in peach (A) miRNA family in peach (B) Size distribution of miRNAs in peach (C) Number of peach miRNAs against different lengths of mature miRNAs.

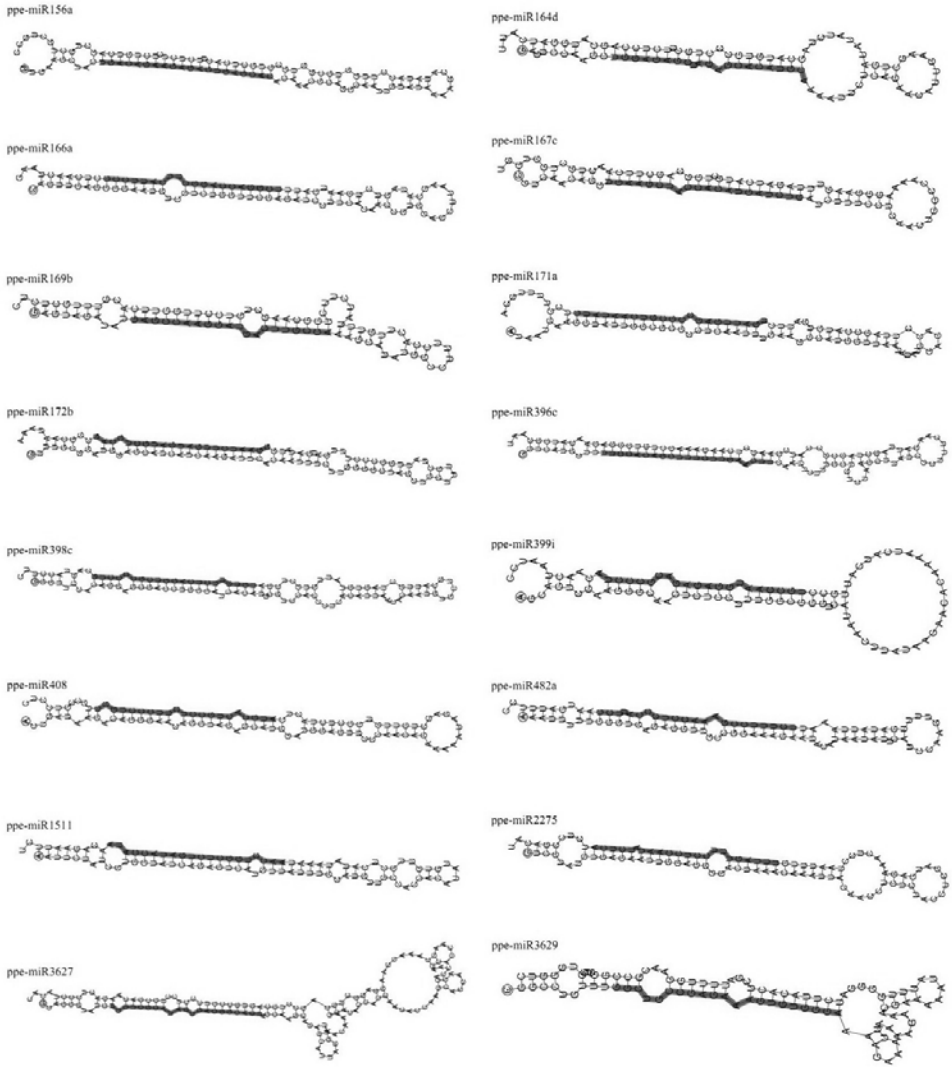

Fig. 2. Stem-loop hairpin structures of representative miRNA families. Red indicates mature miRNA sequences.

miRNAs ranged from -121.5 to -24.4 kcal/mol with an average of -52.82 kcal/mol. However, minimal folding free energy depends on the length of the RNA sequence (Seffens and Digby, 1999). Thus, to avoid the effect of using minimal folding free

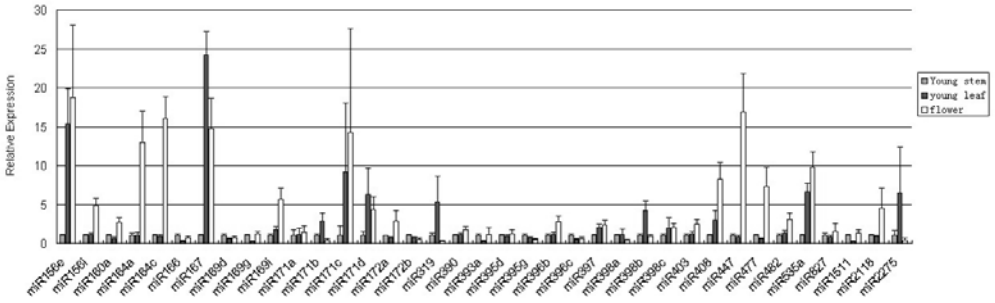

Fig. 3. Relative expression levels of peach miRNAs in different tissues.

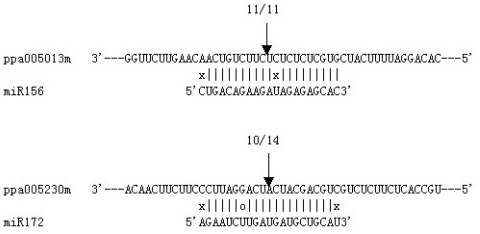

Fig. 4. Mapping of mRNA cleavage sites by RNA ligase-mediated 5′ RACE. Each top strand depicts a miRNA-complementary site in the target mRNA, and each bottom strand depicts the miRNA. Watson-Crick pairing (vertical dashes), G:U wobble pairing (circles) and mismatched base pairing (X) are indicated. Arrows indicate the 5′ termini of mRNA fragments isolated from peach. Numbers indicate the fraction of terminating cloned PCR products.

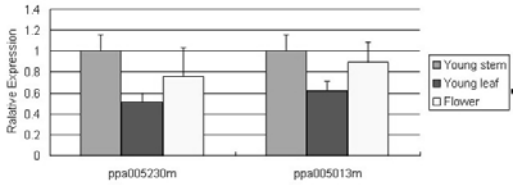

Fig. 5. Relative expression levels of peach miRNA target genes in different tissues.

energy as the one and only criterion to identify new miRNAs (Adai et al., 2005), MFEI was used to distinguish miRNAs from other non-coding and coding RNAs. The MFEI values in our study for precursor miRNA sequences ranged from 0.216 to 1.548 with an average of 0.835.

Potential targets of peach miRNA

In this study, we identified a total of 43 potential target genes, involved in different biological functions, for the 21 identified miRNA families in peach based on the fact that miRNAs perfectly or near-perfectly complement their target sequences in peach. We could not identify the target genes for the following 16 miRNA families: ppe-miR 2275, 398, 169, 403, 3627, 396, 482, 390, 827, 1446, 414, 447, 535, 162, 538, 477 and 858. The majority of these miRNA targets were various transcription factor genes including *SBP*, *MYB*, *ARF*, *NAC*, *AP2*, *PHB*, *F-box*, *GRAS* and *PPR* that are known to regulate plant development. Some miRNA targets included the inorganic phosphate transporter (miR399), the sulphate transporter (miR395) and laccase (miR397). Other targets were uncharacterised (miR408) and hypothetical proteins (miR1511, miR3629, miR1030).

The identified target genes were conserved in several plants, including the squamosa promoter-binding-like (SPL) genes of miR156, the MYB domain containing gene of miR159, the NAC-domain containing gene of miR164, HD-ZipIII transcription factors of miR166, auxin responsive factors (ARFs) of miR160, scarecrow-like transcription factor of miR171, *AP2* domain-containing transcription factor of miR172 and laccase of miR397 (Table 3). This analysis revealed that the majority of target transcripts were highly correlated with plant development and metabolic processes. Several of the well-annotated target transcripts such as *MYB*, *NAC1*, *PHB* and *ARF*s have putative functions involved in floral organ formation. miR164 and miR166 are root-associated miRNAs that regulate the *NAC* and *HD-ZIP* transcription factor genes, respectively. *HD-ZIP* proteins also regulate vascular development as well as lateral organ polarity and meristem formation. miR156, which targets eight squamosa promoter binding protein-like (SPL) transcription factor genes, is involved in flowering time modulation and leaf morphogenesis. miR159 and miR319 both target *MYB* which is involved in flower development. Auxin responsive factors (ARFs) were also a class of targets of miRNA160. ARFs are important components of auxin signal transduction. Unfortunately, the function of the targets of miR1511, miR3629, miR408, miR2118 and miR1030 are currently unknown.

Expression analysis of peach miRNAs by qRT-PCR

To validate the existence and spatiotemporal expression of miRNAs in organisms and to assess their potential roles in regulating the expression of the genes, we analysed the expression of a sample of 37 miRNA sequences belonging to 25 families using qRT-PCR in young leaves, stems and flowers of 'Lovell'. The qRT-PCR analyses demonstrated that all miRNAs were expressed in all the three tissues tested. However, while analysing the results from qRT-PCR, we observed that the expression level of miRNAs differed from each other in the three peach tissues tested (Fig. 3). The qRT-PCR results showed that miR160a, miR166e, miR169d/g, miR171a/b, miR172a/b, miR390, miR393a, miR395d/g, miR396b/c, miR397, miR398a//b/c, miR403, miR482a, miR827 and miR1511 expression levels were not significantly different in all tested tissues. Several miRNAs had different expression patterns in leaf, stem and flower tissues. miR319f and miR2275 accumulated in young leaves, while miR156a, miR164, miR408, miR447, miR477 and miR2118 were expressed predominantly in flowers. miR156i, miR167b, miR169i, miR171c/d and miR535 were all expressed more abundantly in young leaves and flowers in peach com-

Table 3. Potential targets of identified peach miRNAs

miRNA family	Targeted genes	Targeted protein	Target function
miR156	ppa024285m	Probable receptor-like protein kinase	Adjust protein kinase activity
	ppa005013m	Squamosa promoter-binding-like protein 12-like	Transcription factor (TF)
	ppa023657m	Squamosa promoter-binding-like protein 16	TF
	ppa007056m	SPL domain class transcription factor	TF
	ppa006611m	SPL domain class transcription factor	TF
	ppa021582m	SPL domain class transcription factor	TF
	ppa017695m	Squamosa promoter-binding-like protein 6-like	TF
	ppa003644m	Squamosa promoter-binding-like protein 6-like	TF
	ppa007202m	Squamosa promoter-binding-like protein 16-like	TF
miR159	ppa003628m	Transcription factor GAMYB	TF
miR160	ppa002710m	Auxin response factor 18	TF
	ppa002082m	Auxin response factor 18-like	TF
	ppa002195m	Auxin response factor 16	TF
	ppa003136m	Auxin response factor	TF
miR164	ppa007653m	NAC domain-containing protein	TF
miR166	ppa001405m	HB15 HD-ZipIII transcription factors	TF
miR167	ppa017885m	Pentatricopeptide repeat-containing protein (PPR)	TF
miR171	ppa001781m	GRAS family transcription factor (SCARECROW-like)	TF
	ppa001561m	GRAS family transcription factor (SCARECROW-like)	TF
miR172	ppa021782m	Ethylene-responsive transcription factor RAP2-7-like	TF
	ppa005230m	AP2 domain-containing transcription factor	TF
	ppa003783m	AP2 domain class transcription factor	TF
	ppa018704m	AP2 domain class transcription factor	TF
miR319	ppa003628m	Transcription factor GAMYB	TF
miR393	ppa003465m	Protein auxin signalling F-box 3	TF
	ppa003344m	Transport inhibitor response 1 protein	TF
miR394	ppa004699m	F-box family protein	TF
miR395	ppa002425m	Sulphate transporter 2.1-like	Adjusts nutrient balance
miR397	ppa015544m	Laccase-11-like	Oxidoreductase
	ppa017222m	Laccase-11-like	Oxidoreductase
	ppa003590m	Laccase-11-like	Oxidoreductase
	ppa003714m	Laccase-17	Oxidoreductase
	ppa003296m	Laccase-2-like	Oxidoreductase
miR399	ppa025234m	Probable inorganic phosphate transporter 1-3	Adjusts nutrient balance
miR408	ppa018507m	Uncharacterised protein LOC100305588 precursor	Unknown
miR477	ppa016418m	GRAS family transcription factor	TF
	ppa026722m	GRAS family transcription factor	TF
	ppa025123m	Hypothetical protein	Unknown
miR1030	ppa000744m	Hypothetical protein	Unknown
miR1511	ppb023395m	Hypothetical protein	Unknown
miR2111	ppa023821m	F-box family protein	TF
miR2118	ppa013258m	Unknown	Unknown
miR3629	ppa018289m	Hypothetical protein	Unknown

pared with the expression in stems, whereas the expression of miR166e, miR169g, miR393a and miR1511 in young leaves was lower than in young stems and flowers.

The results show that different family members, even different members of the same family, display drastically different expression levels. Abundance comparisons of different members in one miRNA family in various peach tissues may provide valuable information on the role played by miRNAs in plant growth.

Identification of miRNA-guided cleavage and expression analysis of miRNA targets in peach

To verify the potential miRNA targets and better comprehend how ppe-miRNAs regulate their target genes, the cleavage sites of the miRNA target and its expression in three tissues were identified and analysed. The RLM-RACE procedure was successfully used to map the cleavage sites in two predicted ppe-miRNA target genes. *Ppa005013m* and *ppa005230m* were confirmed as the real targets of ppe-miR156 and ppe-miR172, respectively, since all the 5′-ends in the mRNA fragments mapped to the nucleotide that pairs to the tenth nucleotide of each miRNA with higher frequencies than depicted for each

pairing oligo (Fig. 4). The two predicted targets were found to have specific cleavage sites corresponding to the miRNA complementary sequences (Fig. 4) and might be regulated by the two ppe-miRNAs. *Ppa005013m* and *ppa005230m* are similar to Arabidopsis proteins coded by the SPL domain class and AP2 domain-containing transcription factors, respectively (Table 3). To further assess the regulatory action between the miRNA and its target, we analysed the expression of two miRNA target genes, *ppa005013m* and *ppa005230m*, using qRT-PCR. The qRT-PCR analysis demonstrated that all miRNA target genes were expressed in all the three tissues tested. However, the expression levels of miRNA target genes were different from each other in the three peach tissues tested (Fig. 5). The two genes were both expressed more abundantly in young stems and were lower in young leaves. Their expression levels were not significantly different in the three tested tissues.

DISCUSSION

miRNAs have been extensively studied in recent years, and thousands of miRNA genes in the plant kingdom, from mosses and ferns to higher flowering plants, have been computationally predicted and/or experimentally cloned either by traditional genetic approaches or by the recently developed next-generation sequencing (NGS) strategy (Meng et al., 2011). However, only 22 miRNAs belonging to seven miRNA families were computationally predicted in peach using peach EST sequences (Zhang et al., 2011). A systematic study of miRNAs has not been completely performed in peach using peach genome sequence. The identification of entire sets of peach miRNA genes and, subsequently, their targets will lay the foundation for unravelling the complex miRNA-mediated regulatory networks controlling development and other physiological processes. Computational approach and high-throughput sequencing approach are the two main methods used to identify miRNAs (Song et al., 2009; 2010b; Yu et al., 2011; Zhao et al., 2010). In our study, we used the peach genome sequence to predict miRNAs and their targets and found 110 potential miRNAs and 43 presumed miRNA targets. Of these predicted miRNAs, 23 miRNA families were conserved, often over broad evolutionary distances, while 19 miRNAs belonging to 14 miRNA families were not conserved, as they exist in only a small number of species. Furthermore, miR3629 and miR3627 have been found only in *Vitis vinifera*. miR1511, miR2275, mi1446, miR1030, miR538, miR414, miR447 and miR858 were also very rare miRNAs, only found in one or two species. Our results indicated that many miRNAs were specific to small groups of related species and we speculated that they could play a part in speciation. The high-throughput sequencing approach was also employed to identify peach miRNAs. The 631 known miRNA families and 341 potential novel miRNAs were identified (unpublished data). In the known miRNAs, 34 (31%) miRNAs predicted by computational method were the same as the miRNAs identified using high-throughput sequencing approach. In the remaining different miRNAs, 21 miRNAs (miR160a, miR164c, miR169d/i, miR171a/b/c, miR393a, miR395d/g, miR396b/c, miR398a/b/c, miR408, miR447, miR482, miR827, miR1511, miR2275) were verified by qRT-PCR methods. That is to say 55 (50%) miRNA sequences were valid at least. In addition, four miRNA families, miR538/1030/1446/3629, were not found in miRNAs that produces by high-throughput sequencing, while these miRNA families were predicted by computational methods. It is confirmed that our computational method is efficient and reliable and can help identify miRNAs irrespective of expression conditions.

The identification of target genes for miRNAs is an important step in understanding the regulation of miRNA *via* structural genes. Although thousands of miRNAs have been identified in plants, the targets for these miRNAs have not been tested and verified due to the fact that there has been no large-scale experimental method available (Zhang et al., 2006b). We first predicted miRNA targets, then verified two miRNA target genes by RLM-RACE. We searched candidate targets of peach miRNAs using a BLASTn search with 110 identified miRNAs against peach mRNA sequences. Our analysis revealed that most of the predicted targets in peach have a conserved function with miRNA targets in Arabidopsis (Rhoades et al., 2002) and a wide variety of plant species (Dhandapani et al., 2011; Jones-Rhoades and Bartel, 2004). Consistent with previous reports, most of these targets in peach were plant-specific transcription factors, such as *AP2*, *NAC*, *SBP*, *MYB* and the *ARF* family. Nonetheless, the discovery that miRNAs regulate genes such as the sulphate transporter, the inorganic phosphate transporter and laccases showed that miRNAs also have a crucial role in regulating other aspects of plant biology. Upregulation of miR395 could suppress the corresponding target genes during sulphate starvation and miR399 may control Pi homeostasis by regulating the expression of a ubiquitin-conjugating E2 enzyme in *Arabidopsis* (Chiou, 2006). These miRNAs may play important roles in plant nutrient homeostasis and responses to environmental biotic and abiotic stresses. Finding a cleavage site supposedly located in the sequence of the target gene complementary to the miRNA is necessary to verify the cleavage of target mRNAs. Among the methods used to observe miRNA-dependent cleavage of targets, RLM-RACE is the most useful (Llave et al., 2002; Song et al., 2009). Our results show that two potential target genes for the two ppe-miRNAs had specific cleavage sites corresponding to their miRNA complementary sequences. Furthermore, it was also observed that, consistent with previous reports (Debernardi et al., 2012; Song et al., 2010b; Wang et al., 2012), ppe-miRNA targets have an miRNA-complementary site located in their coding regions.

Currently, the major outlines of the functional interactions of plant miRNAs are gradually becoming known and the functions of miRNAs are being generally investigated by altering miRNA expression or by analysing mutant target genes lacking miRNA binding sites (Sun et al., 2011). The expression of miRNAs and their target gene pattern might provide clues about miRNA functions. Previous reports have demonstrated that several *Arabidopsis*, *Oryza* and *Populus* miRNAs are expressed ubiquitously while the expression of others is regulated by development and show preferential accumulation in certain tissues, while some others are regulated in response to stress (Yao et al., 2007). In this study, we used qRT-PCR to validate the existence and spatiotemporal expression of miRNAs and their target genes. In our work, 37 miRNAs in 25 families were identified. miR156, miR171 and miR408 have been tested and verified in peach (Zhang et al., 2011), and the expression of these miRNAs in different tissues corresponded with our study with the exception of miR156. Zhang et al. (2011) reported that the expression of miR156 is higher in young leaves than in flowers; however, our results were exactly the opposite. It is apparent that the expression of miRNAs may differ in diverse varieties. In trifoliate orange, miR156 was accumulated more in flowers than leaves and stems while miR156 was expressed more abundantly in leaves compared with flowers and young shoots in citrus (Song et al., 2009; 2010a). In apple, miR156 were high

expression in stems and low in flowers and leaves (Yu et al., 2011). It is revealed that the same miRNA family have variations expression patterns to facilitate functional specialization in different plant. To further understand the mechanism of the interaction between miRNAs and their target genes, we studied the expression of miRNA target genes (*ppa005013* and *ppa 005230*) in three tissues. The expression levels of the target genes of miR156 and miR172, *ppa005013m* and *ppa005230m*, were not significantly different in the three tested tissues. However, it is interesting that the expression level tendency of ppemiRNAs (miR156 and miR172) and their target genes (*ppa 005013m* and *ppa005230m*) were not opposite in three tissues. miR156 accumulated more in flowers and less in young stems, while the expression of the miR156 target gene, *ppa005013m*, was higher in young stems and lower in young leaves. The expression of miR172a and miR172b in the same family was different in the three peach tissues. miR172a was expressed more abundantly in flowers than young stems and leaves, while miR172b was expressed more abundantly in young stems than young leaves and flowers. Their common target gene, *ppa 005230m*, was expressed more abundantly in young stems than in young leaves and flowers. It seems that *ppa005013m* and *ppa005230m* are not just regulated by miR156 and miR172, respectively. They may regulated by other miRNAs or genes. The emerging picture of miRNA regulation is a complex and comprehensive gene regulatory network. Comprehensive characterisation of all the identified peach miRNAs and their target genes in different tissues would be helpful to understand the tissue-specific expression of all the miRNAs as well as their regulatory roles with respect to different tissues, organs, and conditions.

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