

RESEARCH ARTICLE

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Unique expression, processing regulation, and regulatory network of peach (*Prunus persica*) miRNAs

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Abstract

Background: MicroRNAs (miRNAs) have recently emerged as important gene regulators in plants. MiRNAs and their targets have been extensively studied in *Arabidopsis* and rice. However, relatively little is known about the characterization of miRNAs and their target genes in peach (*Prunus persica*), which is a complex crop with unique developmental programs.

Results: We performed small RNA deep sequencing and identified 47 peach-specific and 47 known miRNAs or families with distinct expression patterns. Together, the identified miRNAs targeted 80 genes, many of which have not been reported previously. Like the model plant systems, peach has two of the three conserved *trans*-acting siRNA biogenesis pathways with similar mechanistic features and target specificity. Unique to peach, three of the miRNAs collectively target 49 MYBs, 19 of which are known to regulate phenylpropanoid metabolism, a key pathway associated with stone hardening and fruit color development, highlighting a critical role of miRNAs in the regulation of peach fruit development and ripening. We also found that the majority of the miRNAs were differentially regulated in different tissues, in part due to differential processing of miRNA precursors. Up to 16% of the peach-specific miRNAs were differentially processed from their precursors in a tissue specific fashion, which has been rarely observed in plant cells. The miRNA precursor processing activity appeared not to be coupled with its transcriptional activity but rather acted independently in peach.

Conclusions: Collectively, the data characterizes the unique expression pattern and processing regulation of peach miRNAs and demonstrates the presence of a complex, multi-level miRNA regulatory network capable of targeting a wide variety of biological functions, including phenylpropanoid pathways which play a multifaceted spatial-temporal role in peach fruit development.

Keywords: miRNA, Deep sequencing, *Prunus persica*, Pre-miRNA processing, *Trans*-acting siRNA, MYB

Background

There are many mechanisms by which plants regulate gene expression to ensure normal development and appropriate responses to both biotic and abiotic signals. One regulatory mechanism involves endogenous small RNA (sRNA) molecules, 20~24-nt in length [1,2], which act by silencing gene expression. In plants, sRNAs have been classified based on their biogenesis, including

microRNAs (miRNAs), heterochromatic siRNAs (hc-siRNAs), *trans*-acting siRNAs (tasiRNAs) and natural anti-sense siRNAs (nat-siRNAs) [1,3-6]. TasiRNA biogenesis from *TAS* loci depends on miRNA-directed cleavage of their transcripts [4,7,8] and three tasiRNA pathways have been characterized in *Arabidopsis* [7,9]. Although miRNAs only constitute a small fraction in the sRNA population [10,11], the miRNA-guided post-transcriptional gene regulation is one of the most conserved and well-characterized gene regulatory mechanisms [6,10,12]. Increasing evidence shows that miRNAs negatively regulate their target genes, which function in a wide range of biological processes, including organogenesis, signal transduction and stress responses [13,14].

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MiRNAs are derived from a precursor sequence of ~70 bases that usually forms a 21 bp duplex with a conserved stem and variable loops which is excised to produce the mature miRNA. The 21 bp sequence matches one or more target sequences for cleavage [10,13]. MiRNAs were initially identified by direct cloning with bioinformatic prediction or Sanger sequencing of relatively small-sized cDNA libraries [15,16]. The application of deep sequencing has greatly facilitated the pace of miRNA identification in plants. In addition to *Arabidopsis* and rice [9,17], miRNAs have been identified in many other plant species, including poplar [18], tomato [19], maize [20], grape [21], peanut [22] and soybean [23]. Comparative analysis reveals that some of the miRNA families are highly conserved among all plant species while others have diverged and evolved, generating abundant family- and species-specific miRNAs [10,24,25]. These dynamic and evolving miRNAs could serve as a driving force for the selection of improved and novel traits in plants.

Peach (*Prunus persica*) is a model species for genomics studies in the Rosaceae family, which includes a number of economically important fruit tree species such as apple, cherry and plum. It has a relatively small and well annotated genome (~230 Mb), diploid, and there are numerous EST sequences. Peach have a number of unique biological facets not commonly found in model organisms such as a 3–5 year juvenility period before the trees flower and fruit [26]. In addition, as temperate zone plants, the reproductive cycle is one year with flower buds initiating during the previous summer. They enter dormancy triggered by cold weather and/or short photoperiod in the fall, and continue developing when released by the seasonal accumulation of chilling stimulus to bloom in the spring [27,28]. Another distinct feature of peach fruit relative to *Arabidopsis* is the formation of fleshy fruit with hardened endocarp or stone surrounding the seed. Stone formation is closely coordinated with fruit development [29,30]. Lastly, peach can be productive for several decades under changing conditions in the orchard. Conceivably, all these developmental programs require an array of sophisticated regulatory networks, involving numerous players presumably including miRNAs.

Recently, Zhang *et al.* [31] initiated the exploration of miRNAs in peach by computationally identifying 22 miRNAs and experimentally verifying miRNAs for seven conserved miRNA families. We wanted to know if peach has evolved novel miRNAs to correspond with its potentially novel development and growth, and what their targets are. To address this, we performed a comprehensive analysis of peach miRNAs from different tissues by deep sequencing, computational prediction, and molecular approaches. We were able to identify novel and conserved peach miRNAs as well as their targets. A

majority of the miRNAs showed tissue-specific expression and 16% of them were found to be regulated at the post-transcriptional level. In addition, peach conserves two of the three *trans*-acting siRNA pathways and we identified additional protein-coding transcripts as tasiRNA biogenesis loci.

Results

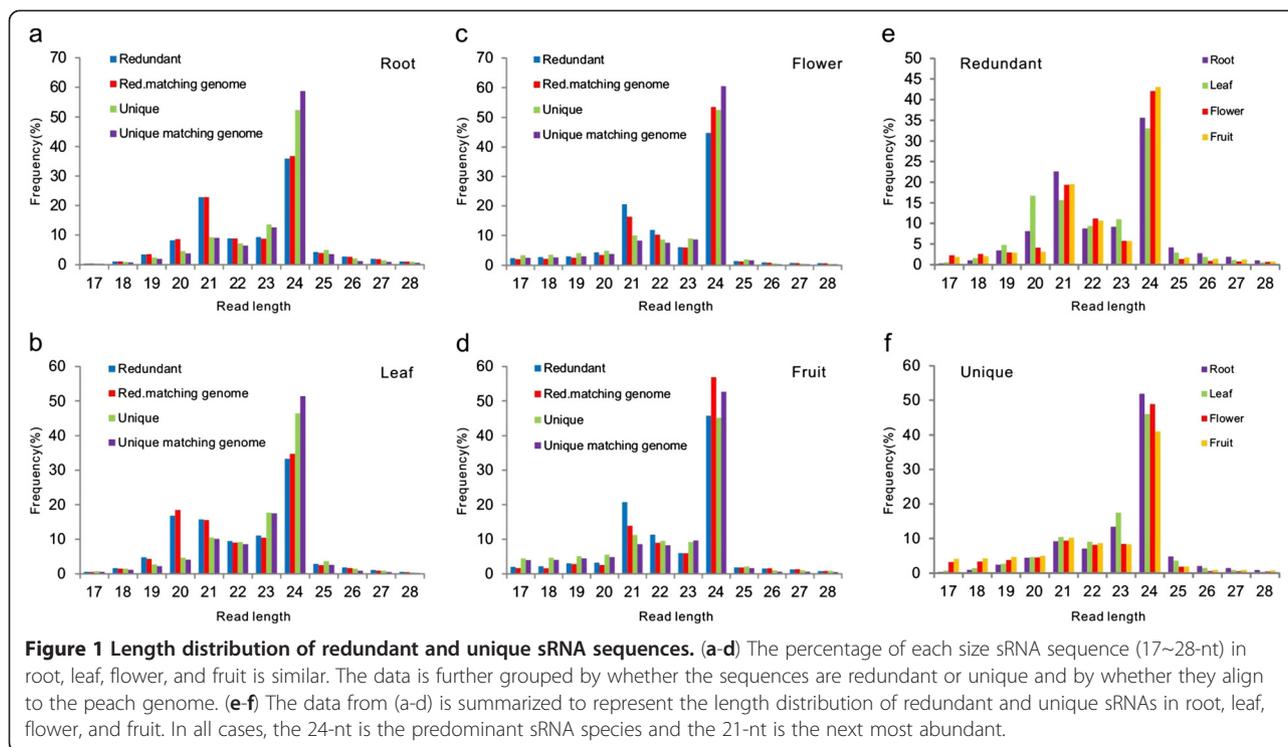
sRNA population in peach

Four cDNA libraries made from peach root, leaf, flower and mixed fruit sRNAs yielded 50 million high quality reads. Among 10 million unique reads, ranging from 15- to 31-nt, 70% were perfectly matched to at least one locus in the peach genome (Peach Genome V1.0 scaffolds, <http://www.rosaceae.org/peach/genome>). These reads were used for further analysis (Additional file 1: Table S1). The 20~24-nt sRNAs constituted over 90% of the identified peach sRNAs, and the 24-nt class of sRNAs was the most abundant class in all tissues (Figure 1a-d). The redundant 24-nt sRNAs were more abundant in flower and mixed fruit than in root and leaf, and the redundant 21-nt sRNAs were more highly expressed in root than other tissues (Figure 1e). Notably, the expression of the unique 24-nt sRNAs was much higher than the 21-nt class in all tissues, especially in root (Figure 1f).

Known miRNA families and their expression in peach

To identify known miRNA families in peach, we blasted all sRNA sequences against miRBase (release 18). A total of 258 unique sRNA sequences (20~22-nt) were identified belonging to 23 miRNA families that are conserved in both angiosperms and coniferophyta lineages [25] and referred to as conserved miRNAs in this study. These conserved miRNAs varied greatly in expression levels (Additional file 2: Table S2). In addition, most conserved miRNA families showed differential expression among root, leaf, flower and mixed fruit tissues. An additional eighty-three miRNA sequences belonged to 24 miRNA families (Additional file 3: Table S3) that have been identified and reported in at least one plant species or family [10]. These are referred to as less-conserved miRNAs in this work. A canonical predicted stem-loop structure could be identified in seven of the 24 less-conserved miRNA families (Additional file 4: Table S4, Additional file 5: Figure S1). Overall, all the less-conserved miRNAs displayed relatively low expression levels compared to the conserved miRNAs except for miR535, and they, like the conserved miRNAs, displayed differential expression among tissues (Additional file 3: Table S3).

A random subset of the conserved and less-conserved miRNAs was analyzed with RNA blot to validate the expression data from the deep sequencing, utilizing miR172 as a standard (Figure 2a). The tissue-specific



expression patterns were presented for the conserved miRNAs, miR160, miR167, miR169, miR319, miR390 and miR396, and the less-conserved miRNAs, miR828, miR858 and miR2118 (Figure 2b).

Peach-specific miRNAs

After excluding sRNA reads homologous to known miRNAs and other non-coding RNAs, the remaining 20~22-nt sRNA reads were selected for secondary structure prediction. Those with canonical stem-loop structures and sequence specificity (>75% of the reads mapped to the unique locus) were considered as putative new miRNAs (Additional file 5: Figure S1). In total, 47 sRNAs from 134 loci were identified. 29 of these sRNAs qualified as novel peach miRNAs since their star stands were identified and the remaining 18 sRNAs without star strand detected were classified as candidate peach miRNAs (Table 1 and Additional file 6: Table S5). Collectively, we refer to them as peach-specific miRNAs as they at present have only been found in peach. Of the 47 peach-specific miRNAs, 29 were 21-nt, 17 were 22-nt, and one was 20-nt (Table 1). Thirty-eight of the miRNAs were transcribed from single loci and the remaining nine matched 2 to 15 loci. Approximately 67% of the peach-specific miRNAs mapped to the sense strand of genome scaffolds while only 33% of them mapped to the anti-sense strand (Additional file 6: Table S5).

The expression of all peach-specific miRNAs was analyzed and compared by both sRNA-seq and RNA blot

analyses. In general, peach-specific miRNAs had low expression which varied in different tissues (Table 1 and Figure 2c). We detected signals for 24 of the 47 peach-specific miRNAs by RNA blot analyses in one or all seven tissues tested, and the hybridization signal intensity was, in general, correlated with the reads per million (rpm) values as demonstrated for miRC1 and miRC7 with some exceptions (Figure 2c).

Detection of miRNA precursors and tissue- and development-specific differential processing in peach

In plants, miRNA transcription and processing appear to be closely coupled as the transcripts are immediately processed [32,33]. This appeared to hold true for most of the peach miRNAs we analyzed. However, in the case of nine miRNAs, miRC1, miRC9, miRC11, miRC14, miRC16, miRC17, miRC26, miRC31 and miRC34 (Figure 3a,b and Additional file 7: Figure S2) two fragments were detected by RNA blot analyses; one corresponding to the expected 21~22-nt miRNA species and the other corresponding to RNA species ranging from 90 to 130-nt in size, which is the predicted size range of most miRNA precursors. To assess if these large fragment were miRNA precursors, we designed two 21-nt oligo probes (designated as Non-miRC1 and Non-miRC26) complementary only with non-miRNA sequences within stem loops of the selected miRC1 and miRC26 (Figure 3c,d). These probes should detect only the large fragment because they were not complementary with the miRNA sequences. Indeed, only the large fragment

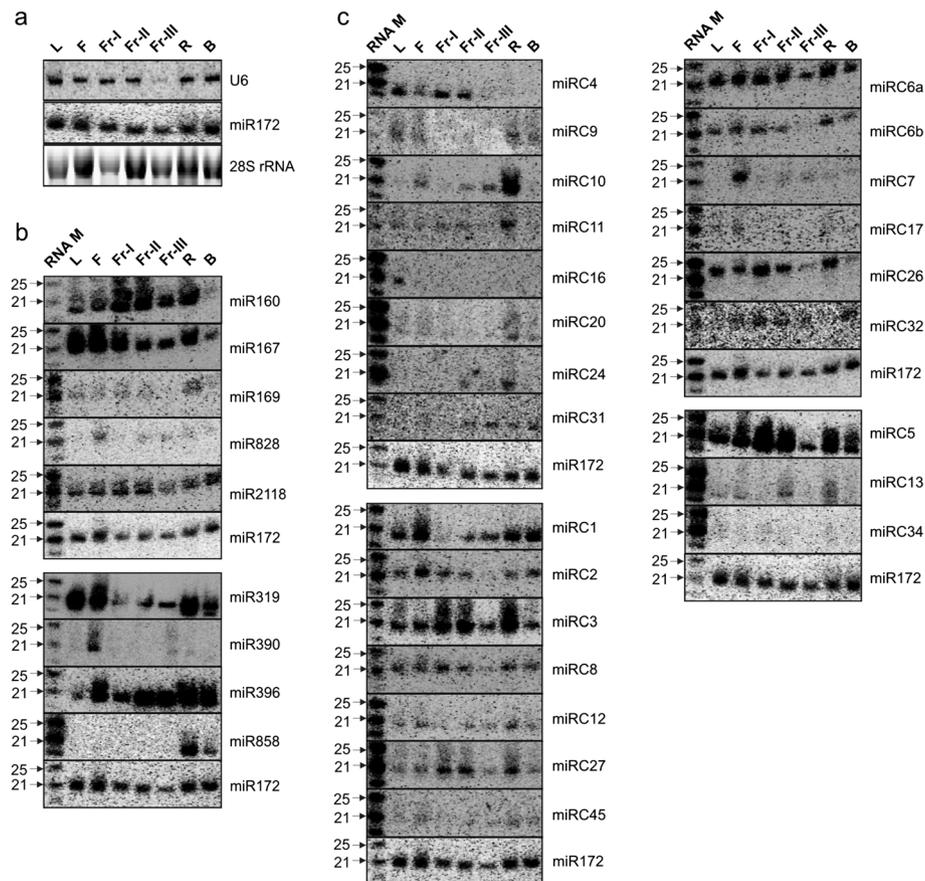


Figure 2 RNA blot analysis of miRNA expression. (a) The expression profiles of three molecules for normalizing gel loading were used to determine the one most uniformly expressed in the seven different RNA samples. MiR172 was chosen as the loading control throughout all RNA blots in this study instead of peach U6 because the U6 proved to be expressed at a substantially lower level in the ripest fruit tissue in these experiments. (b-c) The expression of selected previously-known and peach-specific miRNAs in different peach tissues. All hybridization results from the same membrane are grouped. 25 µg of total RNA isolated from each tissue was separated, transferred to nylon membranes and hybridized using $\gamma^{32}\text{P}$ -labeled oligo probe complementary to RNA marker sequence and along with the probe to the indicated miRNA or gene sequence. For all the blots shown, L, leaf; F, flower; Fr-I, fruit at 19 Day After Bloom (DAB); Fr-II, fruit at 40 DAB; Fr-III, fruit at 82 DAB; R, root; B, bark.

was detected with each of these probes (Figure 3e,f). Re-probing the same blot with another miRNA did not detect the large fragment, indicating that it was not an artifact (Figure 3g,h). Therefore, the detected large fragments likely represented miRNA transcript precursors.

A comparison between large and small fragment intensity among tissues revealed that their relative ratios varied among tissues. For example, miRC26 was more abundant than its precursor in young fruit (Fr-I) while the opposite was true in bark tissue (Figure 3b). These findings suggest that these miRNAs are differentially processed in different tissues. To compare miRNA processing efficiency, we quantified both small and large fragment signal intensity and calculated the percentage of the small fragment intensity relative to the total fragment intensity (APE, arbitrary processing efficiency), as listed in bottom panels of Figure 3a,b. The APE for both miRC1 and miRC26

showed substantial variations among tissues, as over 80% of transcript precursors were processed in flower (F), root (R) and bark (B) but only 11% was processed in young fruit (Fr-I) for miRC1 (Figure 3a). Similarly, 63% of miRC26 precursors was processed in young fruit (Fr-I) while as little as 5% was processed in bark tissue (Figure 3b). In addition to tissue-specific regulation, miRNA processing was also influenced by fruit development stage as the APE for miRC1 increased from 11% to 92% during development from young fruit (Fr-I) to mature fruit (Fr-III) (Figure 3a) while an opposite trend was observed for miRC26 (Figure 3b). Apparently, the expression of these peach miRNAs was subjected to differential processing regulation in a tissue-specific and development-dependent fashion.

MiRNAs in plants are primarily regulated at the transcriptional level. We quantified the changes in

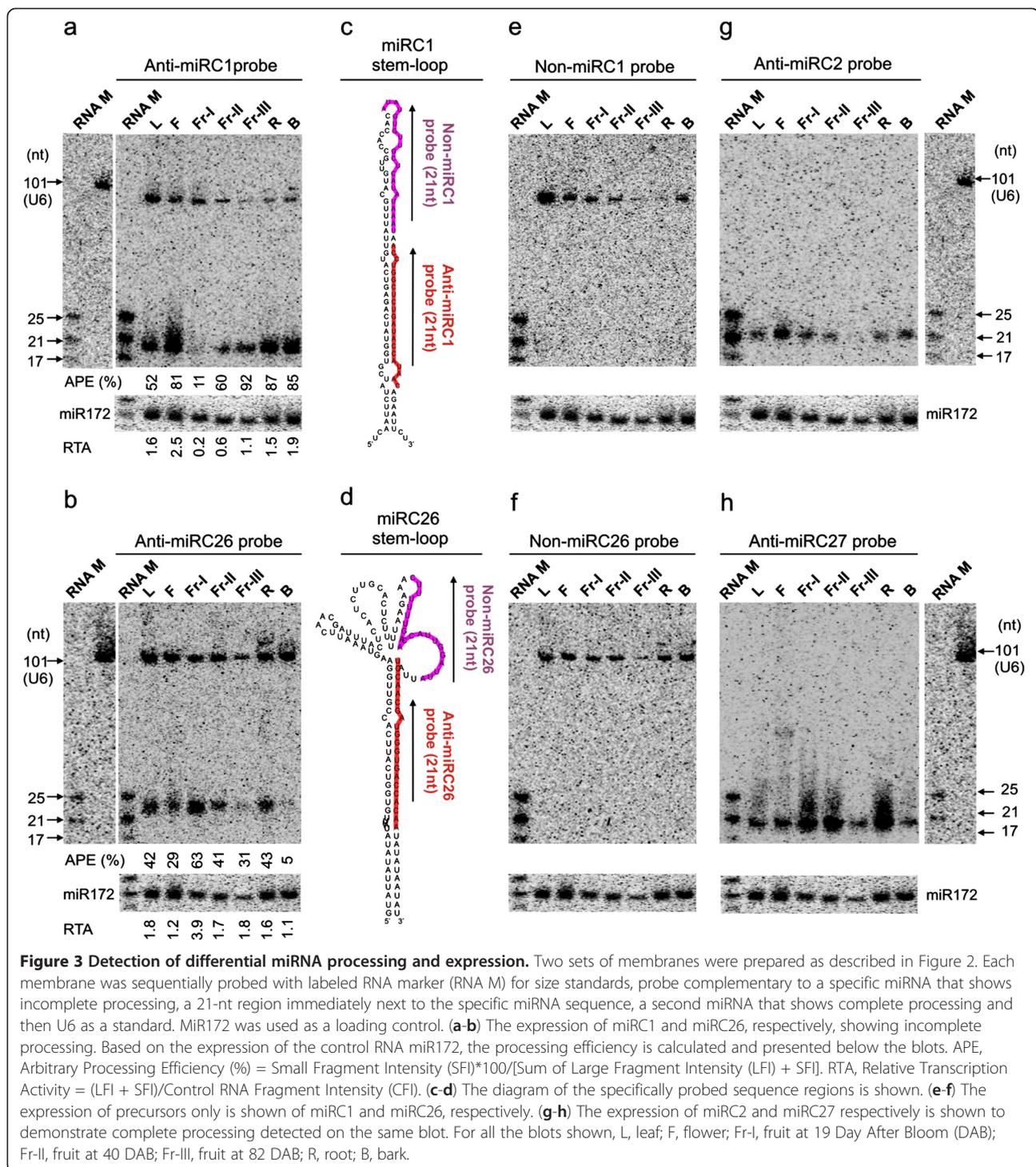
Table 1 Novel or candidate miRNAs identified from *P. persica* sRNA libraries

miRNA	miRNA sequence (5'-3')	Length	Scaffold ^a	Match site	Strand	Normalized abundance (rpm ^b)					miRNA* sequence (5'-3')
						Root	Leaf	Flower	Fruit	Total	
miRC1	ACCUGGCUCUGAUACCAU AAC	21	Scaffold_3	8575480	+	1571	908	13139	7818	23436	CGUGGUUAUCAGAGUCAUGUUA
miRC2	UGAAGGAAGAUUUGUGAAAG	21	Scaffold_7	18921374	-	1573	3962	117	53	5705	UUCACAGAUUUUCCUCAUU
miRC3	CUUCCCAAACCUCCCAUUCUA	22	Scaffold_1	29648613	+	40	33	1288	2804	4165	GGAUUGGGAGAUUGGGAAAA
miRC4	UGAGCAAUGGCACACAGCCCU	21	Scaffold_3	2185580	+	0	0	1273	1837	3110	UUGUGCCAUUGCUC AAGC
miRC5	UUUCCGAAACCUCCCAUUC CAA	22	Scaffold_1	29646139	+	60	3	515	2080	2658	GGGUGAGAGGUUGCCGAAAGA
miRC6a	UUUAUACAAUGAAAUACG GCGCCG	22	Scaffold_1	2254120	+	1286	309	249	328	2172	GCCGUGUUUUUGUAUAAAAG
miRC6b	UUUAUACAAUGAAAUACG GUGCG	22	Scaffold_1	2244523	+	97	15	58	10	180	CCGUGUUUCCUUGUAUAAAAG
miRC7	UGGCACCAUGAUACCAAG UUU	22	Scaffold_7	18801272	-	0	0	986	404	1390	ACUUGGUUAUCUUGGUGCCGGU
miRC8	CAGGAAAGAAUGUGAUGAGUA	21	Scaffold_2	2899303	+	24	469	11	0	504	UUUGCUCGUCACAUUCUUUCC
miRC9	UCGCAGGAGAGAUUGGCACUGUC	22	Scaffold_3	19986823	-	109	16	0	0	125	UGGUGUCAUCCCUCCUGUGACC
miRC10	CGAACUUAUUGCAACUAGCUU	21	Scaffold_4	6342241	+	3	19	0	56	78	GCUAGGUGCAACAAGUUC AAU
miRC11	GGAGCGACCUUGGGAUCACAUG	21	Scaffold_4	23714205	+	12	14	1	22	49	UGUGUUCUCAGGUCGCCCCUG
miRC12	UCUGAGUCAGAUUACUGAAUA	21	Scaffold_6	8360495	+	10	32	1	0	43	UUCAGAUUUUUGACUCAGAA
miRC13	ACUCUCCCUCAAAGGCUUCUAG	22	Scaffold_5	11892663	+	7	14	4	12	37	CGAAGCCUUGGGGAGAGUAA
miRC14	UAGAGAGAUGGUCAGCAAUGU	21	Scaffold_4	14225624	+	3	3	1	26	33	AUUGCUGAUCACCUCUCUAAU
miRC15	CCACAUUUUAGAUUACCUUG	21	Scaffold_7	10402743	-	0	0	9	20	29	CAAGGUAGUUUAAAUGUGG
miRC16	UUCAAAGGGUACAUCCACAGU	21	Scaffold_2	18505840	+	0	5	8	9	22	CAACUGUGGACAUACCCUUUG
miRC17	UCUGUCGUAGGAGAGAUGGCGC	22	Scaffold_3	19984391	-	9	0	0	12	21	UCAUCUCUCCUCGACUGAA
miRC18	UCGUGGGGAGAGAUCAAUCG	21	Scaffold_7	18333173	-	0	0	6	12	18	AUUAGACCUCUCCCGACGAAA
miRC19	CCUCCAUGCCACGCAUUUCUA	22	Scaffold_8	10608407	-	0	0	11	6	17	GAGAUGGGUGGCGGGAAGGA
miRC20	AUUUCGACUAAUAACACAAUG	21	Scaffold_7	1039113	+	3	0	2	11	16	UUGUGUUUUGGCCGAAAAUAG
miRC21	AUAAUAAUGUCCGGAGUCAA	21	Scaffold_6	19901315	+	0	11	0	0	11	GAUAUCCGCACAUUAAUUG
miRC22	CCCUUCCAGUAAGGCACCCCC	21	Scaffold_5	13061652	+	0	0	1	10	11	GGGUUCCUUGUUGGAAGGACU
miRC23	AUUUCAGCUAAGUUGAGUUGU	21	Scaffold_3	13433851	+	0	1	8	1	10	AAUCAACUCAGCUUAGCUGAACUG
miRC24	UCCCUCAAGGGCUCCCAUUAU	22	Scaffold_3	9747805	-	2	0	0	8	10	UGUUGGGGCGUCUUUUG
miRC25	UCAAUUAGAAAUGAUAAAGUG	21	Scaffold_6	7122989	+	0	0	5	2	7	CUUGUUUUUUUUAAUUGAUU
miRC26	UCCAACGAUGGGUGACCACAA	21	Scaffold_7	16562805	-	0	0	2	5	7	UUUGUGGUCAUUCACCGUUGGA
miRC27	UCCUGUGCGAACGUCCAGAAG	21	Scaffold_1	498129	+	3	4	0	0	7	UAACUCCGAACGUCCGCAUA
miRC28	CUUGUUUUUUUUAAUUGAUU	21	Scaffold_6	9969911	-	0	0	4	1	5	AACCAAUUAGAAAUAACAAGUGG
miRC29	AAAGACUAAAUAACCUUGA	20	Scaffold_5	6250039	-	8	6	45	1	60	None detected
miRC30	UACUUGACCCACAACUGGUU	21	Scaffold_1	27613921	+	1	6	13	24	44	None detected
miRC31	UGGGCACGCCAGAAUAAAGCAA	22	Scaffold_7	13727378	+	12	27	0	0	39	None detected

Table 1 Novel or candidate miRNAs identified from *P. persica* sRNA libraries (Continued)

miRC32	UAAGGUUGAGCCGGAAUCGGA	22	Scaffold_6	8366918	+	2	3	2	19	26	None detected
miRC33	CUCUUAUCGUUGGAUCAAUU	22	Scaffold_5	10113319	-	0	0	0	22	22	None detected
miRC34	UGCUGUUGAGAUGUGCGGUU	21	Scaffold_8	6836158	+	17	1	0	1	19	None detected
miRC35	UGUGUUAUCGUAGAAAAUUAU	21	Scaffold_1	27061839	+	6	2	8	1	17	None detected
miRC36	AAUGUCACCUCCACACUCCU	21	Scaffold_4	23445268	+	0	0	0	16	16	None detected
miRC37	UGGACGUCUAGAAAAUACGG	21	Scaffold_4	23035470	+	7	8	0	0	15	None detected
miRC38	UUAAGCCCAAGAAAGCCCGAC	21	Scaffold_4	23839384	+	0	0	0	14	14	None detected
miRC39	ACCUCUUAUAGAUAGUCCCA	21	Scaffold_3	193497	+	0	0	0	12	12	None detected
miRC40	AGACAGGUUCUUUAUCUCAUG	22	Scaffold_1	22425519	-	0	0	3	9	12	None detected
miRC41	UCGAUUUAUGUUUAAGUAUC	22	Scaffold_4	22126310	+	0	0	6	5	11	None detected
miRC42	UCUGACUUUACCAGAAUCUGA	22	Scaffold_5	11020912	+	3	0	0	5	8	None detected
miRC43	CAUUAGAGCGGUGGUACACAA	21	Scaffold_1	30892686	+	1	4	2	0	7	None detected
miRC44	UGCCAAGAAAGAGUUGCCCUA	21	Scaffold_3	1327042	-	0	3	2	0	5	None detected
miRC45	ACCUCUCAUUCUAAACCCUCA	22	Scaffold_1	29656462	-	0	0	0	4	4	None detected
miRC46	UGCAUGCACCUUGAUAGAUGU	21	Scaffold_5	17169215	-	0	0	0	4	4	None detected

^aOnly a single mapped scaffold is shown here. All matched scaffolds are included in Additional file 6: Table S5. ^brpm, reads per million.



transcription of the specific miRNAs among tissues by relative transcription activity (RTA) of miRC1 and miRC26, in regard to miR172 as the loading control. Specifically, miRC1 had the highest RTA in flower (F) and the lowest RTA in young fruit (Fr-I), while miRC26 had the highest RTA in young fruit (Fr-I) and the lowest RTA in bark (B) (Figure 3a,b). MiRNA transcription and

processing shared similar activity in some tissues but different in other tissues, indicating that the two regulatory mechanisms could be uncoupled.

Identification of miRNA targets in peach

We identified 64 target genes for known miRNAs through sequencing of a peach degradome library and

Table 2 Targets for known miRNA in peach identified by degradome sequencing

miRNA	Target gene ^a	Align score ^b	Normalized reads at cleavage site (tpb) ^c	Category ^d	Target gene annotation
Conserved targets for conserved miRNAs					
miR156	ppa006611m	3	150.2	3	Squamosa promoter-binding-like protein
miR156	ppa007056m	2	500.7	2	Squamosa promoter-binding-like protein
miR156	ppa021582m	2	1151.5	2	Squamosa promoter-binding-like protein
miR156	ppa024285m	3	3704.8	0	Squamosa promoter-binding-like protein
miR159	ppa003628m	3.5	2252.9	0	MYB transcription factor
miR160	ppa002082m	1	1301.7	2	Auxin response factor
miR160	ppa002710m	1	1251.6	2	Auxin response factor
miR164	ppa007653m	2.5	11214.7	0	NAC domain-containing protein
miR165	ppa001343m	2.5	2353.1	2	Homeobox-leucine zipper protein
miR165	ppa001378m	2.5	4856.3	0	Homeobox-leucine zipper protein
miR166	ppa001343m	3	2353.1	2	Homeobox-leucine zipper protein
miR166	ppa001378m	3	4856.3	0	Homeobox-leucine zipper protein
miR167	ppa001179m	4.5	1201.6	2	Auxin response factor
miR168	ppa000619m	4	275.4	2	Argonaute protein
miR168	ppa000900m	4	275.4	2	Argonaute protein
miR169	ppa006634m	3.5	1226.6	2	Nuclear transcription factor Y subunit A
miR390	AJ875750 (EST)				PpTAS3
miR393	ppa003344m	1	1401.8	2	Auxin signaling F-box protein
miR393	ppa003465m	2	550.7	3	Auxin signaling F-box protein
miR395	ppa002425m	2	600.8	2	Sulfate transmembrane transporter
miR396	ppa003017m	3.5	4005.2	0	Growth-regulating factor
miR396	ppa006912m	3	4956.5	2	Growth-regulating factor
miR396	ppa011917m	3.5	7960.4	0	Growth-regulating factor
miR396	ppa019623m	3	9312.2	0	Growth-regulating factor
miR396	ppa021277m	4	22179.0	0	Growth-regulating factor
miR396	ppa022199m	3	28086.7	0	Growth-regulating factor
miR396	ppa024293m	3	700.9	2	Growth-regulating factor
miR397	ppa003308m	4	550.7	2	Laccase
miR397	ppa003408m	3	300.4	3	Laccase
miR397	ppa003646m	1.5	9011.8	0	Laccase
miR397	ppa003714m	3	400.5	3	Laccase
miR397	ppa022440m	1.5	250.3	3	Laccase
miR408	ppa018507m	1	1051.4	2	Copper ion binding protein
miR408	ppa021383m	3	3104.1	2	Copper ion binding protein
Tas3-siRNA	ppa001557m	2	650.9	2	Auxin response factor
Tas3-siRNA	ppa001392m	1.5	22529.4	2	Auxin response factor
Novel targets for conserved miRNAs					
miR396	ppa003643m	4	400.5	3	Rho guanyl-nucleotide exchange factor
miR408	ppa004802m	3.5	500.7	3	Selenium-binding protein
miR408	ppa007350m	4	3404.4	2	Cyclin D3

Table 2 Targets for known miRNA in peach identified by degradome sequencing (Continued)

Targets for other known miRNAs					
miR505	ppa012208m	4.5	275.4	2	ATP synthase
miR505	ppa012241m	4.5	275.4	2	ATP synthase
miR530	ppa004922m	4	50.1	4	ATP binding
miR828	ppa010908m	1	55247.2	0	MYB transcription factor
miR828	ppa016135m	3	2152.8	2	MYB transcription factor
miR828	ppa024533m	2	1852.4	2	MYB transcription factor
miR858	ppa005421m	4.5	851.1	2	3-ketoacyl-CoA thiolase
miR858	ppa006057m	4.5	851.1	2	3-ketoacyl-CoA thiolase
miR858	ppa006769m	4	150.2	3	MYB transcription factor
miR858	ppa009143m	4	150.2	3	MYB transcription factor
miR858	ppa010252m	4	3754.9	2	MYB transcription factor
miR858	ppa015883m	3	650.9	0	MYB transcription factor
miR858	ppa016135m	3.5	16481.5	2	MYB transcription factor
miR858	ppa016385m	4	1201.6	0	MYB transcription factor
miR858	ppa016708m	4.5	650.9	2	MYB transcription factor
miR858	ppa017136m	3.5	35.0	3	MYB transcription factor
miR858	ppa018561m	4	1201.6	2	MYB transcription factor
miR858	ppa019380m	4	35.0	1	MYB transcription factor
miR858	ppa022205m	4	4070.3	0	MYB transcription factor
miR858	ppa022431m	4.5	6959.1	2	MYB transcription factor
miR858	ppa022465m	3.5	16481.5	0	MYB transcription factor
miR858	ppa023768m	2.5	3704.8	2	MYB transcription factor
miR858	ppa023812m	3.5	4070.3	0	MYB transcription factor
miR858	ppa024074m	4.5	4070.3	0	MYB transcription factor
miR894	ppa005211m	4	300.4	3	Ankyrin repeat family protein
miR2478	ppa024560m	4.5	300.4	3	Disease resistance-responsive protein
miR2911	ppa004713m	4.5	2052.7	2	Vacuolar processing enzyme
miR2916	ppa008099m	4.5	200.3	2	Galacturonosyltransferase-like protein
miR4171-5	ppa010474m	4.5	650.9	2	C3HC4-type RING finger family protein
miR4171-5	ppa012554m	4	150.2	3	Universal stress protein (USP)

^a Target gene is the identified transcript from the peach transcriptome (<http://www.rosaceae.org/node/35>) or the EST sequence in the case of miR390.

^b Align score is calculated according to [7].

^c tpb, transcripts per billion, according to [21].

^d Category 0: >1 raw read at the position, abundance at position is equal to the maximum on the transcript, and there is only one maximum on the transcript; Category 1: >1 raw read at the position, abundance at position is equal to the maximum on the transcript, and there is more than one maximum position on the transcript; Category 2: >1 raw read at the position, abundance at position is less than the maximum but higher than the median for the transcript; Category 3: >1 raw read at the position, abundance at position is equal to or less than the median for the transcript; Category 4: Only 1 raw read at the position.

found that most of them were abundantly represented as conserved miRNA targets (Table 2). Thirty-five target genes were identified for 15 of the conserved miRNA families, and 29 target genes for the nine less-conserved miRNA families. The target transcripts were then classified into five categories (0–4) based on their abundance (Table 2 and Table 3) [34]. Ten targets for the conserved miRNAs fell into the category 0, the most abundant, and half of the targets fell into category 2. We could not identify any targets for some conserved miRNA families regardless of whether the expression level of the

corresponding miRNAs was low or high, indicating there is no clear correlation between miRNA level and the efficiency of cleavage of its target transcript.

A large number of the identified targets were members of transcription factor gene families, including SPL, MYB, ARF, NAC and GRF, while others were related to sRNA binding (AGO), auxin signaling (TIR/AFB), sulfate transport (AST) and redox reactions (LAC and ARPN) (Table 2). For a given miRNA with multiple conserved target transcripts, the frequency of each target in the degradome varied. More importantly, novel targets were

Table 3 Targets for peach-specific miRNAs identified by degradome sequencing

miRNA	Target gene ^a	Align score ^b	Normalized reads at cleavage site (tpb) ^c	Category ^d	Target gene annotation
miRC2	ppa010261m	5	751.0	2	Zinc finger protein
miRC3	ppb024266m	3.5	350.5	2	NBS-LRR class disease resistance protein
miRC4	ppa012465m	4.5	10263.4	2	Unknown protein
miRC5	ppb024266m	5	350.5	2	NBS-LRR class disease resistance protein
miRC6a	ppa019098m	4	40.1	2	Pentatricopeptide (PPR) repeat-containing protein
miRC6a	ppa020475m	1	5997.8	0	Pentatricopeptide (PPR) repeat-containing protein
miRC6a	ppa023796m	5	15.0	4	Pentatricopeptide (PPR) repeat-containing protein
miRC6b	ppa019098m	4.5	40.1	2	Pentatricopeptide (PPR) repeat-containing protein
miRC6b	ppa020475m	1.5	5997.8	0	Pentatricopeptide (PPR) repeat-containing protein
miRC12	ppa000294m	5	600.8	2	Protein kinase family protein
miRC12	ppa000823m	5	851.1	2	Translation initiation factor
miRC13	ppa008890m	5	2353.1	2	Esterase/lipase/thioesterase family protein
miRC13	ppa010397m	4.5	100.1	3	Allene-oxide cyclase
miRC16	ppa018545m	5	100.1	3	FAR1-related sequence 3; zinc ion binding
miRC16	ppa022612m	5	100.1	3	FAR1-related sequence 3; zinc ion binding
miRC29	ppa002618m	5	50.1	3	RNA binding / translation initiation factor
miRC29	ppa002620m	5	50.1	3	RNA binding / translation initiation factor
miRC30	ppa004763m	4.5	25.0	4	Catalase
miRC45	ppa002666m	5	1552.0	2	Vernalization independence; DNA binding

^a Target gene is the identified transcript from the peach transcriptome (<http://www.rosaceae.org/node/35>).

^b Align score is calculated according to [7].

^c tpb, transcripts per billion, according to [21].

^d Category 0: >1 raw read at the position, abundance at position is equal to the maximum on the transcript, and there is only one maximum on the transcript; Category 1: >1 raw read at the position, abundance at position is equal to the maximum on the transcript, and there is more than one maximum position on the transcript; Category 2: >1 raw read at the position, abundance at position is less than the maximum but higher than the median for the transcript; Category 3: >1 raw read at the position, abundance at position is equal to or less than the median for the transcript; Category 4: Only 1 raw read at the position.

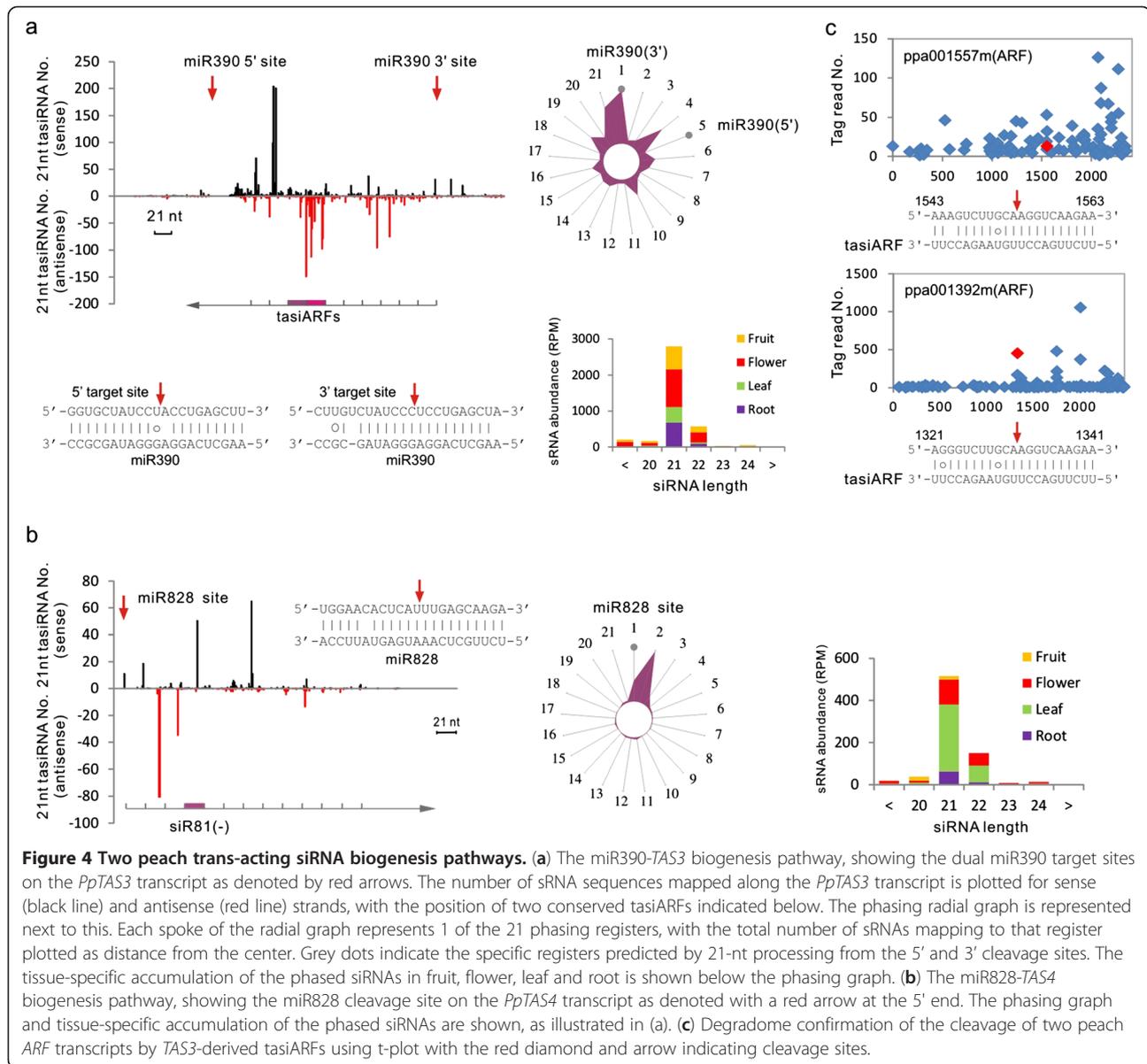
also identified for at least two conserved miRNAs, i.e. miR396 and miR408. The miR396 target encodes a rho guanyl-nucleotide exchange factor and the two miR408 targets encode a selenium-binding protein and a D-type cyclin (Table 2). We also identified either single or multiple targets for the nine less-conserved miRNAs in peach (Table 2). Both miR828 and miR858 targeted MYB family genes. MiR828 could cleave three MYB genes while miR858 targeted 18, among which they shared one common target. In addition, miR858 was found to target two other genes both encoding peroxisomal 3-ketoacyl-CoA thiolases that have critical roles in fatty acid metabolism [35] (Table 2). Notably, miR858 had the most gene targets identified in this study.

A total of 16 targets were identified for 12 of the peach-specific miRNAs (Table 3). Among the identified targets, only one fell into category 0; eight into category 2 and seven into category 3 or 4 (Table 3). MiRC6a and miRC6b shared two of three gene targets identified while miRC3 and miRC5 targeted the same gene transcript. MiRC12 and miRC13 target different genes while miRC16 and miRC29 target two genes from the same gene family. The other four peach-specific miRNAs were found to target

single genes. The identified 16 gene targets encode diverse proteins including zinc finger, NBS-LRR class disease resistance, PPR containing, protein kinase, FAR1-related, RNA binding, catalase and vernalization-related proteins (Table 3), suggesting that these peach-specific miRNAs are likely involved in regulation of a wide range of biological processes or metabolic pathways.

Trans-acting siRNAs in peach

In this study, we found that both miR390-TAS3 and miR828-TAS4 tasiRNA pathways are conserved in peach as evidenced by the identification of miR390 and miR828, TAS3 and TAS4 transcripts and the generation of phased 21-nt siRNAs along both TAS3 and TAS4 transcripts (Figure 4a,b). RNA blot analysis showed that both miR390 and miR828 had detectable expression in various peach tissues (Figure 2b). MiR390's cleavage target, a TAS3 ortholog (EST: AJ875750, defined as *PpTAS3*) was identified in peach, and shared similar dual miR390 target sites with its *Arabidopsis* counterpart. Mapping of sRNA reads against *PpTAS3* defined a similar tasiRNA generation region and pattern between these dual target sites. Mostly 21-nt tasiRNAs were generated



in the flower tissue (Figure 4a), which correlated with flower-specific expression of miR390 (Figure 2b). Of these siRNA populations, two siRNAs shared >95% sequence identity with the characterized *Arabidopsis* tasiARFs, which were shown to target *AtARF2*, *AtARF3*, and *AtARF4* that negatively regulate auxin signaling [36]. Our degradome analysis also showed that these two peach tasiARFs targeted two ARF transcription factors (ppa001557m and ppa001392m) (Figure 4c), indicating functional conservation in peach.

A peach *TAS4* ortholog, defined as *PpTAS4*, was also identified. Its transcript bore a miR828 signature binding site at the 5' end with a similar siRNA biogenesis pattern (Figure 4b) and *PpTAS4* siRNA was preferentially produced in the leaf and flower (Figure 4b). One of *TAS4*-

derived siRNAs, *TAS4*-siRNA(-81), shared extensive sequence identity with its *Arabidopsis* counterpart which has been shown to target at least three *MYBs* that up-regulate anthocyanin production [37]. Our degradome analysis did not identify any target for the peach *TAS4*-siRNA(-81), but *in silico* analysis predicted at least two *MYBs* (ppa024617m and ppa022808m) as targets for peach *TAS4*-siRNA(-81). These predicted *MYB* targets are closely related to *AtMYB113* which is targeted by *Arabidopsis TAS4*-siRNA(-81) [38].

Multiple miRNA targeting of MYB transcription factors with diverse functions in peach

In *Arabidopsis*, miR159, miR828 and miR858 target at least 13 *MYB* genes [39]. Our degradome data found

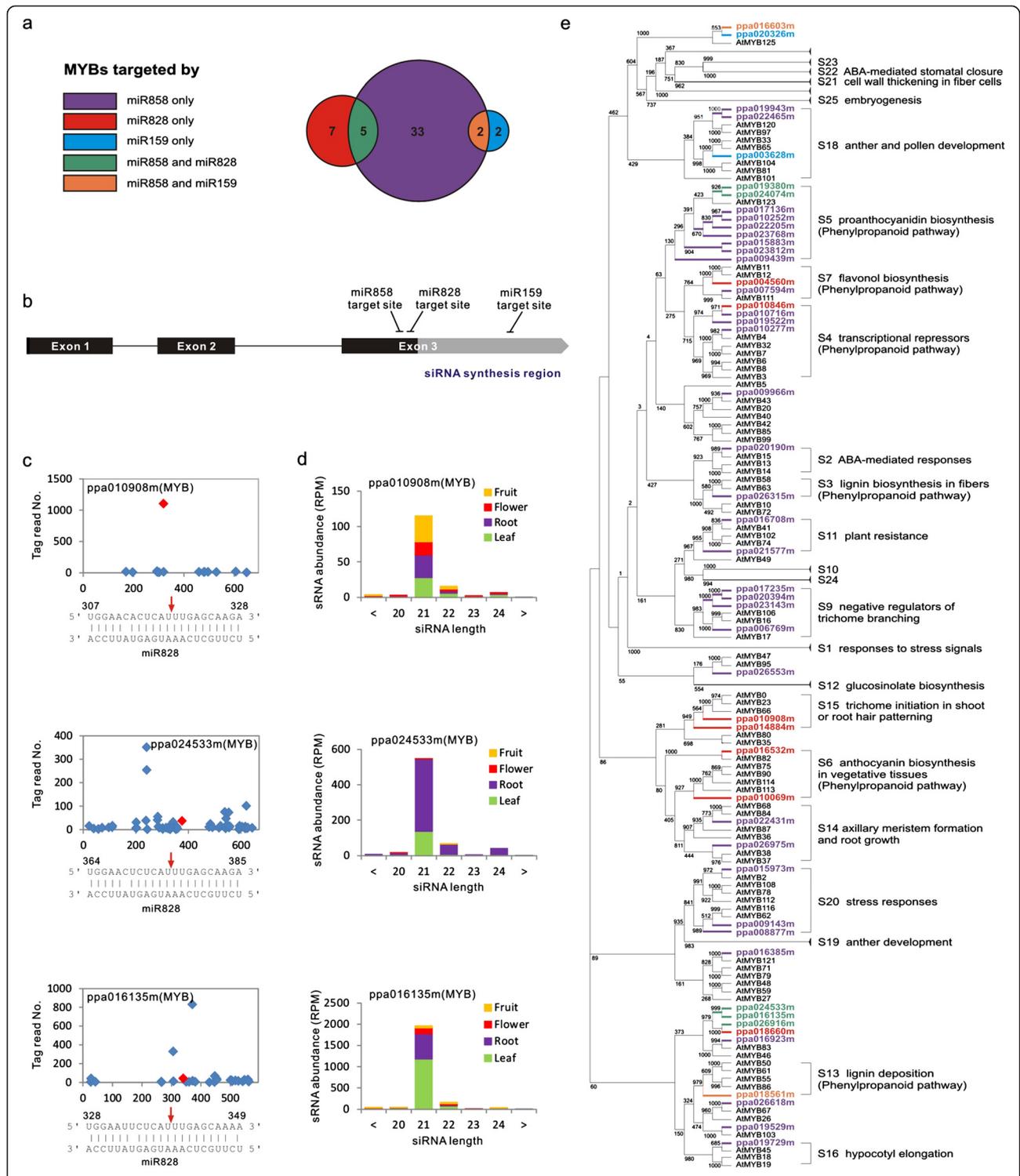


Figure 5 Three miRNAs target 49 peach MYBs. (a) It was found that 49 MYBs were targeted by peach miR159, miR828 and miR858, some of which were targeted by more than one of the miRNAs. MiR858 targeted the majority of these MYBs. (b) Genomic organization of R2R3 MYB genes, location of target sites of miR159, miR828 and miR858, as well as a potential siRNA generation region. The highly conserved sequences are denoted by black area while the diverged sequence by gray box along the MYB coding regions. (c) Degradome confirmation of miR828 cleavage in three MYB transcripts. The red diamond and arrow indicate the cleavage site. (d) The tissue-specific accumulation of the phased siRNAs produced from the miR828-cleaved MYB transcripts is shown, as illustrated in Figure 4. (e) Phylogenetic analysis of functional relationship between miRNA-targeted peach R2R3 MYBs and the characterized Arabidopsis R2R3 MYBs according to previously work [39,40]. MYB genes targeted by specific miRNA are differentiated by the same colors, as illustrated in (a).

that these three miRNAs collectively targeted 19 *MYBs* in peach (Table 2). However, considering that the miRNA target number was underestimated, due to no or low expression of target genes in the specific tissues, it is likely that more *MYB* gene targets exist. Therefore, we performed *in silico* target prediction and identified an additional three, nine and 24 *MYB* genes for miR159, miR828 and miR858, respectively, with an align score of less than 5. Thus, a total of 49 *MYB* target genes were found, four for miR159, 12 for miR828 and 40 for miR858. MiR858 shared five targeted *MYBs* with miR828 and two with miR159 (Figure 5a). Most *MYB* genes that we confirmed or predicted as miRNA targets belonged to the R2R3-*MYB* class, sharing a similar genomic organization with a conserved 5' region and a divergent region at the 3' end (Figure 5b). Further analysis revealed that miR828 and miR858 target sites were separated by 12 nucleotides and co-located in the conserved region of the third exon while the miR159 target site was located in the divergent region of the co-targeted *MYBs* (Figure 5b). Furthermore, we found that the miR828-cleaved transcripts of three *MYBs* underwent phased 21-nt siRNA biogenesis production (Figure 5c). These three *MYB* transcripts shared similar miR828 cleavage positions, tasiRNA generation regions and patterns, intron-exon structure and sequence conservation (Figure 5b). The produced tasiRNAs displayed quite different tissue specificity; the majority of ppa024533m-derived tasiRNAs were found in root, the majority of ppa016135m-derived tasiRNAs were primarily in leaf and ppa010908m-derived tasiRNAs were distributed mostly in fruit (Figure 5d).

A large number of R2R3 *MYB* genes comprising 25 subgroups have been functionally characterized in *Arabidopsis* [39,40]. Accordingly, we did a phylogenetic analysis for all the miRNA-targeted peach *MYB* genes and found that of the four miR159-targeted *MYBs*, one was in *MYB* subgroup 18 - anther and pollen development; another co-targeted by miR858 in subgroup 13 - lignin deposition, mucilage production and stomatal aperture [39], and the remaining two were ungrouped. Twelve miR828-targeted *MYBs* grouped into five subgroups, i.e. S4, S5, S6, S7 - anthocyanin biosynthesis and S15 - trichome initiation (Figure 5e). The 40 *MYBs* targeted by miR858 fell into 11 subgroups, which were shown to regulate diverse biological processes, including organ morphogenesis, lignification, anthocyanin biosynthesis and plant response to stresses in *Arabidopsis* (Figure 5e). Therefore, miR858 could play a more fundamental and diverse role in peach, compared to miR159 or miR828. Interestingly, of the 49 *MYBs*, 19 were related to phenylpropanoid metabolism, which is a key pathway associated with stone hardening and fruit color development, suggesting an important role

of these miRNAs in regulation of peach fruit development and ripening.

Discussion

Peach miRNAs and their targets with diverse biological significance

In plants, miRNAs are both highly conserved and rapidly evolving, and these features have been characterized in a variety of lineages [25]. While extensive research has been carried out on model plants, less is known about the characterization and functional analysis of miRNAs from plant species with agricultural and horticultural significance such as peach and apple [31,41,42]. An earlier study reported computational and experimental identification of eight miRNAs from seven conserved miRNA families in peach [31], not extensive compared to those identified in other plant species [18-21]. Here we provided a comprehensive analysis of peach miRNAs produced in different tissues and characterized their expression patterns by both sRNA-seq and RNA blot analyses. Most of the identified peach miRNAs were found to be tissue-specific, as previously observed for other plant species [9,19-21]. Many known miRNAs targets, mostly transcription factors, control diverse physiological processes and genetic programs associated with leaf polarity, lateral root formation, flowering, metabolism and stress responses [36,39,43,44]. In this study, a majority of the targets for peach miRNAs have counterparts previously identified in other species [18-23]. For example, two gene targets identified for miR160 in peach are homologous to those in *Arabidopsis* encoding two auxin response factors (ARFs) that act as repressors in auxin signaling, while a target for miR167 in peach is homologous to *AtARF6* which is an activator in the same pathway [36]. Peach tasiARFs target genes that encode auxin signal repressors as their *Arabidopsis* counterpart does. Further, miR393 that targets TIR1/AFB2 Auxin Receptor (TAAR) Clade, another class of key players for auxin signaling [45] was also found to target three TAAR homologs in peach. Evidently, miRNA- and tasiARF-mediated regulation of the auxin signaling pathway previously characterized in *Arabidopsis* is also conserved in peach. However, some of the identified known miRNAs were found to have additional or novel gene targets in peach (Table 2). For example, miR396 that is known to target a conserved family of growth-regulating factor genes also targeted a rho guanyl-nucleotide exchange factor that is involved with phytochrome signaling. Likewise, miR408, besides targeting the conserved copper ion binding protein, could also target two genes encoding a selenium-binding protein and a D-type cyclin, which regulate plant defense and growth, respectively [46,47]. These results suggest that some known

miRNAs have either acquired new targets or expanded their regulatory functions in peach.

Our work found 47 peach-specific miRNAs, which were differentially regulated in various tissues and/or developmental stages (Figure 2c) and targeted a variety of genes with a wide range of biological functions. For example, miRC6a/b targeted two genes encoding pentatricopeptide (PPR) repeat-containing proteins involved in RNA editing, splicing and translation within mitochondria and chloroplasts [48,49]. MiRC3 and miRC5 both targeted the same NBS-LRR disease resistance protein [50]. Other peach-specific miRNAs were found to target genes associated with transcription/translation initiation, protein kinases, and esterase/lipase/thioesterase family proteins. MiRC45 was found to target a transcript encoding *VIP* (*VERNALIZATION INDEPENDENCE*), a gene that modulates vernalization in *Arabidopsis* [51,52]. We identified targets for only 12 of the 47 peach-specific miRNAs, which raises a question of whether these remaining miRNAs are non-functional, or alternatively regulate gene expression by translational repression, not direct cleavage of target mRNAs [53,54]. Conceivably, further improvement of degradome analysis sensitivity and detailed examination of possible translational repression functions of these miRNAs in peach or transgenic plants would provide information of whether or not these miRNAs are functional in peach.

Differential processing of miRNA precursors in peach

In animals, many miRNAs with distinct spatio-temporal expression patterns are modulated by transcriptional and/or post-transcription regulation [55]. In plants though, miRNA transcripts are thought to be immediately processed into mature miRNAs through the processing machinery (DCL1, HYL1, or SE) inside the nucleus, hence the transcript precursor processing is coupled with the transcription [6]. However, a recent study in maize showed that transcription was not closely associated with transcript precursor processing for two miRNAs in specific tissues. The accumulation of abundant miR166a transcript precursors but not mature miR166a was detected in the tip of the maize shoot apical meristem [56]. Transcript precursors were observed for miR390 by RT-PCR in the L1 but not the L2 layer of the shoot apical meristem while similar levels of mature miR390 was revealed in the same L1 and L2 layers by *in situ* hybridization [56]. These results suggest that both miR166a and miR390 precursors could be differentially processed in various cell types or tissues, although other possibilities, including miRNA stability and mobility, could not be ruled out [56,57]. In this study, we detected differential accumulation of mature miRNAs and their transcript precursors for nine peach miRNAs (Additional file 7: Figure S2). By comparing the miRNA

precursor processing efficiency among tissues for two chosen miRNAs (miRC1 and miRC26), we found that the processing efficiency of their transcript precursors varied in root, bark, leaf, flower, as well as during fruit development (Figure 3), demonstrating that differential processing modulates miRC1 and miRC26 expression. We also found that there was no correlation between miRNA transcription and precursor processing activities thereby demonstrating the two processes can likely be uncoupled. Our detection of these larger transcripts provided substantial evidence for differential processing, though still not conclusive, suggesting this might be part of the basis for the miRNA expression in specific tissues or developmental stages in peach. Similar observations were found during apple miRNA identification indicating such a regulatory mechanism may be common in fruit crops.

Although the mechanism underlying differential processing of miRNA precursors in plants remains unknown, it has been characterized in animal cells where miRNA biogenesis is initiated by processing the pri-miRNA transcript into miRNA precursors (pre-miRNA) by the microprocessor complex containing the dsRNA-binding protein DGCR8 and the RNase III enzyme Drosha as well as multiple accessory proteins [55,58]. The processed pre-miRNAs are exported to the cytoplasm and cleaved into mature 22-nt miRNAs by Dicer proteins. Two groups of protein factors that utilize distinct strategies to modulate differential processing of miRNA precursors have been identified and characterized. Group one factors (SMADs and p53) interact with the microprocessor complex and accessory factors (EWSR1, p68 and p72) to activate or repress the processing of pri-miRNAs [59-62]. Group two factors (Lin28, hnRNPs, KSRP and TRBP) recognize and bind to the terminal loop of specific miRNA precursors to facilitate the processing or degradation of the bound miRNA precursors in specific tissues or cell types [63-65]. Conceivably, various factors expressed in specific tissues or developmental stages could directly or indirectly interact in the same manner to modulate the processing of specific miRNA precursors in peach. Our findings here implied a prevailing differential stabilization of miRNA precursors, most likely to be dependent on the tissue-specific action of DCLs, HYLs, HENs and other RNA-binding proteins.

The conserved *trans*-acting siRNA pathway in peach

To date, four *TAS* gene families have been characterized in *Arabidopsis*, of which the miR390-*TAS3* and miR828-*TAS4* pathways are conserved in plants [3,4]. Here we identified both *TAS3* and *TAS4* peach orthologs, together with their corresponding trigger miRNAs (Figure 4a,b). We also found similar siRNA biogenesis

patterns in the cleaved *TAS3* and *TAS4* transcripts. The tasiRNA species are conserved as evidenced by the identification of homologous tasiARFs and *TAS4*-siRNA(-81) and their targets either confirmed by degradome analysis (Figure 4c) and/or *in silico* prediction. Together, these data indicate that both miR390-*TAS3* and miR828-*TAS4* biogenesis pathways and functions are at least partially conserved in peach. Since auxin signaling and modulation is essential for diverse biological processes in peach, especially for fruit development and ripening [66,67], miR390-*TAS3* biogenesis-derived tasiARFs in specific tissues could orchestrate auxin signaling that could be directly relevant to fruit growth and development. In *Arabidopsis*, *TAS4*-siRNA(-81) has been shown to target genes (*AtMYB75*, *AtMYB90* and *AtMYB113*) that positively regulate anthocyanin production [9,38]. The induction of *AtMYB75* along with anthocyanin accumulation activates miR828, *TAS4* and *TAS4*-siRNA(-81) [37]. This feedback regulatory loop is proposed to maintain proper anthocyanin levels in plant tissues under nutrient stress condition [38]. This role of *TAS4*-siRNA(-81) could also be important in peach, where anthocyanin production is directly related to fruit color, which can be highly variable and is considered as an important fruit quality trait [29,68]. While direct gene targets for *TAS4*-siRNA(-81) have not been confirmed by the degradome analysis, *in silico* prediction showed that *TAS4*-siRNA(-81) can target at least two peach *MYBs* highly homologous to *AtMYB113*. Thus, miR828, *TAS4*, *TAS4*-siRNA(-81) and the targeted *MYBs* could form a similar feedback regulatory circle that control anthocyanin accumulation and possibly fruit coloration during peach fruit ripening, which is further supported by the observation of detectable miR828 expression in the mature but not the young fruit (Figure 2b).

miRNA-mediated MYB regulatory networks in peach and their biological relevance

While *MYBs* are known to be targeted by miRNAs in other species [9,21,38], the target number is limited [39]. In peach, at least 49 *MYBs* can be potentially targeted by miR159, miR828 and miR858 (Figure 5a). This expansion may have to do with the specialized developmental programs that potentially are regulated through specific *MYBs*, including but not limited to the lignin synthesis and deposition that orchestrates the stone hardening process and the flavonoid synthesis that is important to fruit color, nutritive properties and disease resistance [29,69-71]. The lignin and flavonoid biosynthesis pathways are biochemically competitive, drawing from the same phenylpropanoid precursors. Thus, they need to be tightly coordinated during peach fruit

development to enable efficient phenylpropanoid metabolism in a tissue specific fashion [29]. While we were only able to detect miR828 expression during fruit development (Figure 2b), we cannot rule out the potential roles of miR858 and miR159 since they could be highly cell- or tissue-, or stage-specific during fruit development, and their expression window period might be missed in this study. Still, the potential regulatory roles of miR858, miR159 and miR828 in lignin, cell wall and flavonoid metabolism and synthesis pathways provides evidence for a significant role of sRNA in coordinating fruit development.

The finding that miR858 shares five *MYB* targets with miR828 and two with miR159, respectively, and three miR828-targeted *MYBs* undergo siRNA biogenesis supports the notion of the evolution of a miRNA- and siRNA-mediated silencing reinforcement regulatory mechanism in peach. The co-targeting of the same *MYBs* by two miRNAs is expected to strengthen their silencing function while miRNA cleavage followed by siRNA biogenesis reinforces the same silencing effect. Although these are two unrelated biological events, they achieve the same goal, executing a strong responsive regulatory function; hence the miRNA-mediated co-targeting could also enable the targeted *MYBs* to be under refined spatio-temporal regulation. Thus the observed distinct expression patterns of miR828 and miR858 among various tissues and fruit developmental stages would modulate the co-targeted *MYB* expression in an exquisite spatio-temporal manner, to precisely regulate the co-ordination of lignification in stone and mesocarp- and ectocarp-specific fruit coloring during peach fruit development and ripening.

Conclusions

We characterized miRNAs and their potential targets in peach to provide a comprehensive list of peach miRNAs and reveal the potential scope of their regulatory functions. We show that peach has both conserved and species-specific miRNAs with distinct expression patterns, and that these miRNAs potentially target dozens of genes with a wide range of biological functions. Quite a few of the peach-specific miRNA precursors are subject to differential processing in various tissues and during fruit development, indicating possible mechanisms that define the extent of miRNA accumulation in a spatio-temporal manner. Further, both miR390-*TAS3* and miR828-*TAS4* siRNA biogenesis pathways and their functions appear to be conserved in peach; miR828 cleavage is capable of activating siRNA biogenesis in *PpTAS4* and three *MYB* protein-coding transcripts, indicating a silencing reinforcement in peach. In addition, we found that miR159, miR828 and miR858 collectively target 49 *MYBs*, 19 of which are known to regulate

phenylpropanoid metabolism, a key pathway involved in stone hardening and fruit color development. In summary, we extensively characterize the unique expression pattern and processing regulation of peach miRNAs, and demonstrate the presence of a complex miRNA regulatory network capable of targeting a wide variety of biological functions in peach. Our results provide new and valuable information for deciphering the intricate roles of peach miRNAs and tasiRNAs in gene regulation, which will be useful for the further investigation of miRNA and tasiRNA functions in other crop species.

Methods

Plant materials

Root, bark, leaf, flower and fruits of different developmental stages (19, 40 and 82 days after bloom) were collected from *Prunus persica* cv. Lovell peach trees, planted at Appalachian Fruit Research Station, Kearneysville, WV. All the samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

RNA preparation and deep sequencing

Total RNA was extracted, using Plant RNA Purification Reagent (Invitrogen, CA, USA), from four different peach tissues, i.e. root, leaf, flower and mixed fruits of various developmental stages. The small RNA and degradome library construction were performed by BGI (Beijing Genomics Institute, China). In brief, the small RNAs of 18~30-nt were isolated from total RNA and ligated to a 5' RNA adapter and a 3' RNA adapter, as described previously [72]. A reverse transcription reaction followed by PCR was performed and the amplified library then underwent gel purification prior to sequencing on SOLID system or Illumina Hiseq 2000 platform.

The peach degradome library was constructed as previously described [73], with pooled total RNA from peach root, leaf, flower and mixed fruits. In brief, poly (A) RNA was extracted and ligated to a 5' RNA adapter and the products were digested after RT-PCR and ligated to a 3' dsDNA adapter. The amplified library was then gel-purified for sequencing on Illumina Hiseq 2000 platform.

The small RNA library and degradome library sequencing data are available under NCBI-GEO accession no: GSE38535.

RNA blot analysis

For RNA blot analysis, 25 μg of total RNA from leaf, flower, fruit at 19 day after bloom (DAB), 40 DAB, 82 DAB, root and bark was separated on a denaturing 15% polyacrylamide gel. The RNA was blotted onto Amersham HybondTM-NX membranes (GE Healthcare, Waukesha, WI, USA) and crosslinked using EDC (Sigma, St.

Louis, MO, USA). DNA oligonucleotides probes (Additional file 8: Table S6) that are reverse complementary to miRNAs were labeled with $\gamma^{32}\text{P}$ -ATP using T4 polynucleotide kinase (NEB, Beverly, MA, USA). MicroRNA Marker Probe (NEB, Beverly, MA, USA) was used for size determination. MiR172 was selected as a loading control for all the RNA blots because it gave the most consistent signal among different tissues compared to other genes we tested, including U6 (Figure 2a). The membranes were hybridized at 42°C for overnight and washed twice at 55°C with washing buffer containing $2 \times \text{SSC}$ and 2% SDS. The membranes were then exposed to the phosphorscreens and scanned with Typhoon TRIO Variable Mode Imager (GE Healthcare, Waukesha, WI, USA). We stripped the membranes with probe stripping solution and then exposed the stripped membranes overnight to the phosphorscreens to ensure that there was no trace of radiation signal detected before re-hybridizing with a new probe. Many of the hybridizations were repeated with a second blot to verify the results.

Bioinformatics analysis

All the sequencing data was processed by removing the 3' adaptor using CLC Genomic Workbench 4.9 (CLC Bio., Aarhus, Denmark). Any sequences without adaptor sequence were excluded from analyses. Reads homologous to non-coding RNAs and conserved miRNAs were removed by BLATN alignment against Rfam 10 (<http://www.sanger.ac.uk/resources/databases/rfam.html>) and mature miRNAs in miRBase (<http://www.mirbase.org>, release 18), allowing up to two mismatches. The remaining sRNAs were subjected to peach-specific miRNA identification. Read mapping was conducted using Bowtie [74], and Vienna RNA package was used for miRNA secondary structure prediction [75]. Those sRNAs (20~22-nt) with a canonical stem-loop structure (no more than four mismatches, and no more than one central bulge) and a miRNA/miRNA* pair accounting for over 75% of the reads matching to the respective precursor locus were considered as potential peach-specific miRNAs. A detailed screening criterion was applied according to Meyers *et al.* [76]. The total number of the reads perfectly matching the peach genome in a given library was used for the normalization of read abundance, denoted as rpm (reads per million). The degradome analysis and target categorization were performed using CLEAVE-LAND pipeline 2.0 [34,77] and Targetfinder 1.6 (<http://carringtonlab.org/resources/targetfinder>). The alignment score threshold was set to 4.5 for conserved and less-conserved miRNAs, and to 5 for novel and candidate miRNAs. The peach genome scaffold, CDS sequence and gene annotation information were retrieved from GDR (www.rosaceae.org).

Multiple alignment and phylogenetic analysis

Multiple alignments were conducted using CLUSTAL X2 [78]. All the peach *MYB* targets for miR828, miR858, and miR159 were predicted by Targetfinder 1.6 with an align-score of no more than 5. Amino acid sequences of *MYB* factors in *Arabidopsis* were retrieved from TAIR (<http://www.arabidopsis.org>) and a phylogenetic tree was generated using the neighbor-joining method and 1000 bootstraps with putative full-length sequences using CLUSTAL X2 [78]. The subgroup and function annotation were designated according to Dubos et al. [39].

Additional files

- Additional file 1: Table S1.** Statistics of sRNA sequences from peach root, leaf, flower and fruit.
- Additional file 2: Table S2.** Read length distribution for each conserved miRNA family recovered from peach sRNA libraries.
- Additional file 3: Table S3.** Peach homologs of known miRNAs.
- Additional file 4: Table S4.** Known miRNAs with canonical stem-loop structure predicted from peach sRNA libraries.
- Additional file 5: Figure S1.** Stem-loop structures for peach miRNAs.
- Additional file 6: Table S5.** A detailed list of peach-specific miRNAs.
- Additional file 7: Figure S2.** Detection of pre-miRNAs in peach.
- Additional file 8: Table S6.** Peach miRNA probes for RNA blot analysis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HZ and ZL initiated the research. HZ, ZL, BZ and YA designed the experiments. RX performed the computational analyses. HZ and ZL carried out the biological experiments, interpreted the results and prepared the manuscript. CDD and AMC provided extensive intellectual suggestion for the manuscript organization and writing. All authors critically read and approved the final version of the manuscript.

Acknowledgements

We would like to thank Mr. Dennis Bennett for his excellent technical assistance, and Ms. Linda Dunn for providing peach root and leaf tissues.

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Received: 16 March 2012 Accepted: 13 August 2012

Published: 21 August 2012

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doi:10.1186/1471-2229-12-149

Cite this article as: Zhu et al.: Unique expression, processing regulation, and regulatory network of peach (*Prunus persica*) miRNAs. *BMC Plant Biology* 2012 **12**:149.