



A survey of microRNA length variants contributing to miRNome complexity in peach (*Prunus persica* L.)

Moreno Colaiacovo[†], Letizia Bernardo[†], Isabella Centomani, Cristina Crosatti, Lorenzo Giusti, Luigi Orrù, Gianni Tacconi, Antonella Lamontanara, Luigi Cattivelli and Primetta Faccioli*

CRA Genomics Research Centre, Fiorenzuola d'Arda, Italy

Edited by:

Takuji Sasaki, National Institute of Agrobiological Sciences, Japan

Reviewed by:

Zhixi Tian, Chinese Academy of Sciences, China

Takeshi Itoh, National Institute of Agrobiological Sciences, Japan

*Correspondence:

Primetta Faccioli, CRA Genomics Research Centre, via S. Protaso 302, I-29017 Fiorenzuola d'Arda (Pc), Italy.
e-mail: primetta.faccioli@entecra.it

[†]Moreno Colaiacovo and Letizia Bernardo have contributed equally to this work.

MicroRNAs (miRNAs) are short non-coding RNA molecules produced from hairpin structures and involved in gene expression regulation with major roles in plant development and stress response. Although each annotated miRNA in miRBase (www.mirbase.org) is a single defined sequence with no further details on possible variable sequence length, isomiRs – namely the population of variants of miRNAs coming from the same precursors – have been identified in several species and could represent a way of broadening the regulatory network of the cell. Next-gen-based sequencing makes it possible to comprehensively and accurately assess the entire miRNA repertoire including isomiRs. The aim of this work was to survey the complexity of the peach miRNome by carrying out Illumina high-throughput sequencing of miRNAs in three replicates of five biological samples arising from a set of different peach organs and/or phenological stages. Three hundred-ninety-two isomiRs (miRNA and miRNA*-related) corresponding to 26 putative miRNA coding loci, have been highlighted by mirDeep-P and analyzed. The presence of the same isomiRs in different biological replicates of a sample and in different tissues demonstrates that the generation of most of the detected isomiRs is not random. The degree of mature sequence heterogeneity is very different for each individual locus. Results obtained in the present work can thus contribute to a deeper view of the miRNome complexity and to better explore the mechanism of action of these tiny regulators.

Keywords: microRNA, isomiRs, next generation sequencing

INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNA molecules produced from hairpin structures and involved in gene expression regulation with major roles in plant development and stress response. MiRNAs are transcribed into a primary transcript which folds into a bulge with stem-loop conformation that is then cleaved by a Dicer-like (DCL) RNase III enzyme named DCL1. The cleavage results in a short duplex: one of the two strands forming the duplex and designated as miRNA* is then typically degraded while the other strand is incorporated into the RNA-induced silencing (RISC) complex where it mediates mRNA recognition and cleavage or translational repression (Jones-Rhoades et al., 2006; Voinnet, 2009; Xie et al., 2010).

Although each annotated miRNA in miRBase¹ is a single defined sequence with no further details on possible variable sequence length, isomiRs – namely the population of variants of miRNAs coming from the same precursors – have been identified in several species and could represent a way of broadening the cell regulatory network (Ebhardt et al., 2009; Guo and Lu, 2010).

Vaucheret (2009) demonstrated the biological significance of mature miRNA length heterogeneity in *Arabidopsis* where the ath-miR168 can be processed as two different miRNA isoforms of 21 nt and 22 nt in length with different activities on AGO1 homeostasis

(AGO1 is the Argonaute1 protein, a component of RISC complex that catalyzes broad miRNA- and siRNA-guided mRNA cleavage and translation repression Voinnet, 2009).

Alteration in miRNA end sequences can have strong effects on miRNA function due to the fact that the identity of the first 5' nucleotide is the major determinant for AGO protein association (Takeda et al., 2008). As an example, Mi et al. (2008) found that AGO1 (which predominates in the miRNAs-mediated pathway) harbors miRNAs that favor a 5' terminal uridine. A change at the 5' terminal nucleotide of a miRNA predictably redirected it into a different AGO complex and altered its biological activity. Additionally, it was reported that the thermodynamic stability at the 5' end of the strand is likely to affect the loading in the AGO complex (Eamens et al., 2009).

An accurate profile of the entire miRNA population of a biological sample provides useful information on miRNA activity and it can be used to compare the distribution of miRNA sequence variants in different samples. In fact, although the distribution of isomiRs across samples has been previously shown to be generally similar, examples in which the dominant isomiR is different from sample to sample have been found in animals (Lee et al., 2012) and could imply a functional role for specific isomiR sequences, besides affecting the accuracy and consistency of miRNA measurement.

This work aims to survey, by carrying out Illumina high-throughput sequencing, the complexity of peach miRNome

¹www.mirbase.org

through the analysis of the miRNA population of a set of samples representative of different tissues and developmental stages.

MATERIALS AND METHODS

PLANT MATERIAL AND RNA EXTRACTION

A 12-year-old tree grafted on wild seedling of the yellow-fleshed cv. Maycrest (*Prunus persica* (L.) Batsch), grown in Palazzolo di Sona, Verona, Italy (45.457°N, 10.822°E), was used as plant source material. Each sample was collected pooling together material from three different branches of the same plant. Four phenological stages (Chapman and Catlin, 1976) were considered: swollen bud, half-inch green, pink, bloom. Leaf and flower swollen buds were collected 41 days before flowering (DBF), half-inch leaves were collected 21 DBF, pink flower buds were collected six DBF. Codes were assigned to each samples: BF, pink; F, bloom; GF, swollen flower bud; O, half-inch green; GL, swollen leaf bud. Tissues were frozen in liquid nitrogen immediately after drawing. Total RNA was extracted from three independent samples with the Plant Total RNA Purification Kit (NORGEN Biotek Corp., Thorold, ON, Canada) following manufacturer instructions. RNA quality and concentration were evaluated with the Agilent 2100 Bioanalyzer RNA 6000 Nano assay (Agilent Technologies, Santa Clara, CA, USA).

SMALL RNA LIBRARIES CONSTRUCTION AND SEQUENCING

Preparation of small RNA libraries was performed with the TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA) following manufacturer instructions. Briefly, 1 µg of total RNA was ligated with adapters at 3' and 5' ends, without any size fractionation. Adapter-ligated RNA was reverse-transcribed with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), then PCR-amplified (15 cycles). Samples were barcoded using 15 variants of the reverse primer provided with the kit. Libraries were pooled together and then purified on a 6% TBE PAGE gel after electrophoresis. Libraries quality and concentration were evaluated with the Agilent 2100 Bioanalyzer DNA 1000 assay. The obtained cDNAs were sequenced using the Illumina Genome Analyzer IIx.

DATA ANALYSIS

Reads were filtered with UEA sRNA plant toolkit² (Moxon et al., 2008) to remove adaptor sequences, reads shorter than 18 nt or longer than 24, low-complexity reads, reads matching rRNAs or tRNAs and reads that did not match the peach genomic sequence available at “The International Peach Genome Initiative – www.rosaceae.org/peach/genome” (only those sequences with a full-length perfect match to the selected genome were retained). Reads from one replicate (randomly chosen) of each biological sample were then analyzed with the software miRDeep-P (Yang and Li, 2011, default parameters) to identify miRNA loci expressed in all the five tested samples (GF, GL, B, F, O). Reads associated to these loci were then also screened in the remaining two replicates.

Read counts for each variant were divided by the total number of reads with a match in the peach genome in each sample and normalized to 1,000,000 reads. Reads that could be related to

more than one locus were assigned by MiRDeep-P to all possible involved loci.

IsomiRs for each putative locus were blasted against miR-Base (release 18) to search for the loci related to previously known miRNAs (Kozomara and Griffith-Jones, 2011). Blast vs. mature sequences was based on the following parameters: outfmt 6, task blastn, dust “no,” *e*-value 10, word_size 7, reward 2, num_alignments 10. Blast vs. precursor sequences was based on the following parameters: outfmt 6, task blastn, dust “no,” *e*-value 10e−3, num_alignments 10.

The correlation between biological replicates was evaluated by calculating the Pearson coefficient for all the possible pairs of replicates belonging to the same biological sample, as well as samples from different tissues for sake of comparison. We decided to remove from the set a sequence (HE860285) whose expression level was abnormally high, because its presence caused the Pearson correlation to be almost one in every comparison, irrespective of the tissue.

To identify miRNAs isomiRs that were differentially expressed among the biological samples, a *t*-test was performed for all the possible comparisons. An isomiR was considered as differentially expressed in a specific comparison if its *p*-value was less than 0.05. The whole set of reads associated with the miRNA loci was then used to perform a hierarchical clustering with R software, by applying the Canberra metric to calculate the distances between the expression vectors of the samples across the reads.

MiRNA target identification was carried out by psRNATarget³ (Dai and Zhao, 2011), with default parameters. To score the complementarity between small RNA and their target transcript, psRNATarget applies the scoring schema of miRU by Zhang (2005). The *maximum expectation* is the threshold of the score. A small RNA/target site pair will be discarded if its score is greater than the threshold. The default cut-off threshold is 3.0.

The accessibility of the mRNA target site to small RNA has been identified as one of the important factors involved in target recognition because the secondary structure (stem, etc.) around the target site will prevent the small RNA and mRNA target from contacting. The psRNATarget server employs RNAup to calculate target accessibility, which is represented by the energy required to open (unpair) secondary structure around the target site (usually the complementary region with small RNA and up/downstream) on target mRNA. The lower the energy the higher the possibility that small RNA is able to contact (and cleave) target mRNA. PsRNATarget uses a software, namely RNAup, described by Mückstein et al. (2006) to calculate this value, denoted as UPE.

All the miRNA-related sequences were submitted to the EMBL database, whereas the sequencing raw reads were submitted to the NCBI SRA (BioProject accession: PRJNA167962).

RESULTS

SEQUENCING PEACH SMALL RNA LIBRARIES

Illumina deep sequencing was used to profile the whole miRNA set of five different samples corresponding to different organs and/or phenological stages. Three replicates were analyzed for

²<http://srna-tools.cmp.uea.ac.uk/plant/cgi-bin/srna-tools.cgi>

³<http://plantgrn.noble.org/psRNATarget>

each sample. A total number of 40,764,330 sequence reads were obtained and filtered as reported in Section “Materials and Methods.” Details on the results of each filtering step are reported in **Table 1**. On average, 2,717,622 raw reads and 664,777 clean reads perfectly matching the genome were obtained in each of the 15 samples.

One technical replicate (randomly chosen and numbered as “1”) of each sample was subsequently analyzed with miRDeep-P which highlighted the putative miRNA coding loci of the peach genome expressed in the five tested samples (reported in Files S1–S5 in Supplementary Material and summarized in File S6 in Supplementary Material). Twenty-six putative miRNA coding loci were expressed in all samples according to miRDeep-P results. The length of the putative precursors was between 41 nt and 227 nt (average length of 104 nt), while average mature miRNAs size was 22 nt.

These 26 miRNAs were selected and, for each of them, the corresponding associated reads were searched in all the replicates of each sample. The results (miRNAs and miRNAs* associated reads) are reported in **Table 2** for each locus, the link between locus name and locus position can be found in File S7 in Supplementary Material and retrieved at www.rosaceae.org/peach/genome.

ISOMIRs IDENTIFICATION AND ANALYSIS

IsomiRs at each locus were blasted against miRBase. In some cases no mismatches were reported with the conserved sequences present in miRBase (e.g., miR403, miR394, miR166, miR156) while in some others mismatches were present and related to differences in the sequence and/or in its length. Detailed blast results are reported in File S8 in Supplementary Material which reports blast results based both on mature sequences (sheet “mature”) and precursor sequences (sheet “precursors”) deposited in miRBase. The file reports the matching sequence with the lowest *e*-value. When more than one matching sequence, belonging to different

miRNA families, were found to have the same *e*-value all of them were reported.

Some miRNA families have more than one putative locus, therefore miRDeepP assigned common reads to all the possible loci. Both miRNA and miRNA*-related reads were identified at each locus. In some cases putative miRNAs* were identified on the basis of the alignment orientation (\pm with miRNA mature sequence deposited in miRBase) in some others the miRNA* sequences were already deposited in miRBase. The results of **Table 2** highlight that some loci are characterized by a larger set of variants than others.

In the majority of the loci the most frequent read for a specific locus was the same in all the tested samples and across all the replicates of a sample (**Table 2**). Only in a few cases were some differences detected among samples or among replicates belonging to the same sample. Locus named 3_16 is particularly interesting because all the replicates of sample O have as the most frequent read the one corresponding to miRNA* (**Table 2**).

In some loci also the second most frequent read referred to the mature miRNA was the same in all the replicates of a sample and in all the samples. The second most frequent read was often obtained by a different cutting site at 5' or 3' ends. As reported above, miRNA*-related reads have also been identified by miRDeep-P for most of the 26 loci and length variability was detected for both 5' and 3' ends.

Target analysis was carried out by psRNATarget. The whole set of targets identified is reported in File S9 in Supplementary Material.

INTRA- AND INTER-SAMPLES ANALYSIS

The average Pearson correlation between all the possible pairs of replicates belonging to the same biological sample was calculated, in order to evaluate whether it was in agreement with the “Standards, guidelines, and best practices for RNA-seq” adopted

Table 1 | Reports the number of filtered reads perfectly matching the peach genome in each of the tested samples.

Sample	Raw reads	Matching adaptors	Matching adaptors (18–24 nt)	Low-complexity filtered (non-redundant)	rRNA/tRNA removed (non-redundant)	Matching peach genome (non-redundant)
BF1	2842653	2332661	1592671	1582689 (356161)	1335567 (333478)	797297 (207233)
BF2	2553116	2124140	1570583	1560824 (370103)	1401892 (351620)	817438 (220223)
BF3	2641037	2184480	1646360	1635958 (387915)	1466005 (368373)	853043 (235873)
F1	2523898	1819981	1180424	1173273 (215571)	858130 (194546)	368958 (78332)
F2	2898014	2361457	1389585	1381108 (233414)	1040918 (214222)	630870 (115644)
F3	3613383	2923242	1768783	1757830 (320484)	1362239 (297258)	700729 (143030)
GF1	2696289	2170904	1354915	1346476 (307349)	1200810 (289976)	774146 (190043)
GF2	3722325	2848155	1962881	1950819 (429497)	1570664 (405303)	928251 (248399)
GF3	2952035	2254276	1327544	1319295 (308967)	1145330 (291401)	609728 (168939)
GL1	2357377	1707677	1072569	1065997 (241873)	870779 (220909)	481523 (122824)
GL2	2304406	1729282	1080322	1073580 (253835)	929602 (234995)	460632 (125267)
GL3	1822754	1345521	745352	740785 (207198)	616318 (190449)	338168 (111111)
O1	3704334	2907197	1811057	1799817 (327514)	1384994 (302970)	890186 (182703)
O2	1896816	1509747	1068609	1061991 (226499)	911190 (208620)	624084 (129672)
O3	2235893	1753142	1286833	1278775 (282053)	1093002 (263673)	696601 (162637)

BF, pink; F, bloom; GF, swollen flower bud; O, half-inch green; GL, swollen leaf bud.

Table 2 | Reports the read count (divided by the total number of reads with a perfect match to the peach genome and normalized to 1,000,000 reads) of 26 putative miRNA coding loci that were expressed in all the 15 samples according to miRDeep-P results.

miRNA	Reads	EMBL accession number	BF1	BF2	BF3	F1	F2	F3	GF1	GF2	GF3	GL1	GL2	GL3	O1	O2	O3	
1_10	AGTTTGTGCGTGAATCGAACC	HE862997	2.5	1.2	1.2	35.2	11.1	45.7	2.6	4.3	4.9	0	10.9	5.9	1.1	0	0	
	CAGTTTGTGCGTGAATCGAAC	HE860429	6.3	9.8	19.9	24.4	7.9	20	3.9	10.8	14.8	8.3	17.4	5.9	3.4	0	8.6	
	TTAGATTCACGGCACAAC	HE862999	0	0	0	0	0	0	1.3	2.2	1.6	0	0	0	0	0	0	
	TTAGATTCACGGCACAAC	HE860429	3.8	6.1	4.7	5.4	0	4.3	3.9	4.3	3.3	2.1	6.5	0	1.1	1.6	0	
	TTAGATTCACGGCACAAC	HE863001	2.5	6.1	4.7	8.1	0	5.7	1.3	4.3	1.6	0	4.3	0	4.5	3.2	4.3	
	TTAGATTCACGGCACAAC	HE860293	66.5	93	83.2	208.7	71.3	125.6	67.2	106.7	132.8	105.9	125.9	230.7	27	40.1	58.9	
	AACCAAAATCTCTGGACTCCTG	HE860430	1.3	0	1.2	0	0	0	0	1.3	1.1	0	0	0	0	0	1.6	0
	AAGAGATTTGGTACTCAC	HE863003	0	0	1.2	0	0	0	0	1.3	2.2	1.6	0	4.3	3	0	1.6	1.4
	AAGAGATTTGGTACTCAC	HE860430	2.5	2.4	0	0	0	1.4	0	1.1	1.1	0	0	0	0	0	0	0
	AAGAGATTTGGTACTCAC	HE863005	0	7.3	1.2	0	0	0	0	2.6	1.1	1.6	0	0	1.1	0	0	2.9
	AAGAGATTTGGTACTCAC	HE860431	12.5	18.4	12.9	5.4	3.2	2.9	14.2	9.7	6.6	4.2	2.2	0	6.7	6.4	7.2	
	AAGATTTGGTACTCAC	HE863007	6.3	2.4	3.5	2.7	0	0	0	1.1	0	0	2.1	0	1.1	1.6	1.4	
	AGAGATTTGGTACTCAC	HE860431	1.3	4.9	4.7	0	1.6	0	0	2.6	0	0	2.1	0	3	1.1	4.8	
	AGAGATTTGGTACTCAC	HE863009	0	1.2	2.3	0	1.6	0	0	1.1	1.6	0	0	0	2.2	0	2.9	
	AGAGATTTGGTACTCAC	HE860297	117.9	162.7	126.6	271	36.5	25.7	71	65.7	49.2	45.7	43.4	20.7	80.9	52.9	34.5	
ATTAATCCAAACGGTGAATCAC	HE860432	0	0	0	0	0	0	0	0	0	0	2.1	2.2	0	0	0		
CAAGAGATTTGGTACTCAC	HE863011	0	0	0	0	1.6	0	0	0	0	0	0	0	3	1.1	0	0	
CAAGAGATTTGGTACTCAC	HE860432	1.3	0	1.2	0	0	0	0	0	2.2	0	0	0	0	1.1	0	0	
CAAGAGATTTGGTACTCAC	HE863013	0	2.4	2.3	2.7	3.2	4.3	0	2.6	2.2	0	0	0	0	0	0	0	
CAAAGATTTGGTACTCAC	HE860433	0	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0	
TCCAAGAGATTTGGTACTCAC	HE863015	1.3	0	0	0	0	1.4	0	0	0	0	2.1	0	0	0	0	1.4	
CGAAACCTCCCATCCAA	HE860433	1.3	0	0	0	0	0	0	1.3	2.2	0	2.1	2.2	3	0	0	0	
GAGAGTTGCGGAAAGA	HE863017	0	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0	
GGTGAGAGTTGCGGAAAGA	HE860434	0	0	0	0	0	0	0	0	0	0	2.1	0	0	0	1.6	4.3	
GGTGAGAGTTGCGGAAAGA	HE863019	2.5	9.8	16.4	0	0	0	0	10.3	14	8.2	12.5	15.2	11.8	15.7	30.4	38.8	
GGTGAGAGTTGCGGAAAGA	HE860434	32.6	24.5	52.8	0	3.2	0	96.9	96.9	206.8	157.4	105.9	121.6	106.5	75.3	91.3	208.2	
GGTGAGAGTTGCGGAAAGA	HE863021	0	0	0	0	0	0	0	1.3	0	0	0	0	3	0	0	0	
GGTGAGAGTTGCGGAAAGA	HE860435	0	0	0	0	0	0	0	0	0	0	4.2	0	0	0	0	0	
TCCGAAACCTCCCATCCAA	HE863023	1.3	0	1.2	0	0	1.4	0	3.9	0	0	2.1	0	0	2.2	0	1.4	
TCCGAAACCTCCCATCCAA	HE860435	3.8	1.2	1.2	0	0	0	0	1.3	1.1	0	0	0	0	0	0	1.4	
TCCGAAACCTCCCATCCAA	HE863025	0	0	0	0	0	0	0	3.9	0	0	0	0	0	0	0	0	
TCCGAAACCTCCCATCCAA	HE860436	17.6	30.6	17.6	5.4	6.3	15.7	29.7	29.7	49.6	27.9	33.2	39.1	26.6	13.5	11.2	8.6	
TCCGAAACCTCCCATCCAA	HE863027	1.3	2.4	1.2	2.7	0	1.4	0	6.5	1.1	3.3	0	2.2	3	0	3.2	0	
TGGGTGAGAGTTGCGGAAAGA	HE860436	0	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0	
TGGGTGAGAGTTGCGGAAAGA	HE863029	2.5	0	0	0	0	2.9	0	1.3	0	0	0	0	0	0	0	0	
TTCCGAAACCTCCCATCCAA	HE860437	2.5	2.4	1.2	0	0	1.4	0	1.4	0	1.6	0	4.3	3	1.1	0	0	
TTCCGAAACCTCCCATCCAA	HE863031	3.8	1.2	4.7	0	0	8.6	7.8	5.4	3.3	2.1	4.3	3	1.1	0	0	0	
TTCCGAAACCTCCCATCCAA	HE860437	8.8	8.6	23.4	5.4	9.5	17.1	34.9	34.9	23.7	23	10.4	21.7	3	2.2	4.8	4.3	
TTCCGAAACCTCCCATCCAA	HE863033	15.1	11	12.9	0	4.8	5.7	29.7	22.6	13.1	16.6	16.6	21.7	3	1.1	0	5.7	
TTCCGAAACCTCCCATCCAA	HE860304	1135.1	1196.4	1134.8	401.1	391.5	687.9	1802	1802	2047.9	1895.2	1277.2	1417.6	777.7	433.6	387.8	446.5	
TTCCGAAACCTCCCATCCAA	HE860438	18.8	15.9	16.4	8.1	6.3	12.8	14.2	14.2	21.5	14.8	20.8	28.2	3	3.4	4.8	5.7	

Table 2 | Continued

miRNA Reads	EMBL accession number	BF1	BF2	BF3	F1	F2	F3	GF1	GF2	GF3	GL1	GL2	GL3	O1	O2	O3
TTTTGAAGCAGATGATGGAAC	HE860448	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0
TTTGCAACCCCGCCCAT	HE863077	0	2.4	0	0	0	0	1.3	0	3.3	0	2.2	0	0	0	1.4
TTTGCAACCCCGCCCAT	HE860449	2.5	0	0	2.7	0	0	6.5	0	8.2	2.1	2.2	0	0	6.4	0
TTTGCAACCCCGCCCAT	HE863079	5	1.2	1.2	2.7	0	1.4	3.9	2.2	8.2	4.2	0	3	2.2	4.8	1.4
TTTGCAACCCCGCCCAT	HE860449	20.1	19.6	14.1	10.8	7.9	12.8	67.2	26.9	73.8	33.2	41.2	50.3	15.7	19.2	7.2
TTTGCAACCCCGCCCAT	HE863081	5	8.6	3.5	2.7	1.6	1.4	22	9.7	13.1	10.4	13	5.9	5.6	3.2	5.7
TTTGCAACCCCGCCCAT	HE860450	115.4	71	70.3	59.6	36.5	75.6	148.6	136.8	239.5	240.9	223.6	171.5	57.3	134.6	83.3
TTTGCAACCCCGCCCAT	HE863083	0	1.2	1.2	0	0	0	3.9	2.2	11.5	0	4.3	11.8	0	0	0
AGTTGGCATACTGCCAACTG	HE860450	3.8	2.4	2.3	13.6	4.8	10	1.3	0	0	0	0	0	3.4	1.6	1.4
ATGGCAATCTGTCCACCTCC	HE863085	0	1.2	0	0	1.6	0	0	0	0	0	0	0	1.1	0	0
TGGCATTGTCCACCTCC	HE860451	1.3	0	0	0	0	1.4	0	0	0	0	2.2	0	0	0	0
TTGGCATTGTCCACCT	HE863087	6.3	6.1	7	13.6	11.1	7.1	2.6	1.1	1.6	2.1	0	5.9	0	0	0
TTGGCATTGTCCACCT	HE860451	18.8	24.5	15.2	13.6	28.5	27.1	1.3	5.4	8.2	2.1	4.3	0	0	1.6	8.6
TTGGCATTGTCCACCT	HE860307	89.1	121.1	89.1	127.4	141.1	225.5	80.1	53.9	59	27	26.1	20.7	20.2	35.3	25.8
TTGGCATTGTCCACCT	HE863089	36.4	29.4	36.3	10.8	26.9	62.8	23.3	15.1	13.1	2.1	4.3	3	4.5	4.8	1.4
TTGGCATTGTCCACCT	HE860452	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AACATGATCATCCGAATGAT	HE863091	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0
AATGCTGTGGTTCGAGA	HE860452	1.3	2.4	1.2	0	1.6	2.9	0	1.1	3.3	2.1	2.2	0	0	1.6	1.4
ACCAGGCTTCATCCCC	HE863093	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ATCCGAATGATTCGGACAGGCT	HE860453	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0
ATCGGACCCAGGCTTCATCCCC	HE863095	6.3	14.7	17.6	0	9.5	11.4	2.6	1.1	0	6.2	6.5	0	1.1	4.8	5.7
ATGCTGTGGTTCGAGA	HE860453	0	0	0	2.7	0	0	0	0	0	0	0	0	0	0	0
CGGACCCAGGCTTCATCC	HE863097	0	0	0	0	0	0	0	1.1	0	0	2.2	0	2.2	0	0
CGGACCCAGGCTTCATCC	HE860454	1.3	2.4	0	2.7	1.6	0	1.3	5.4	1.6	0	0	3	1.1	0	0
CGGACCCAGGCTTCATCC	HE863099	282.2	208	289.6	336.1	187	182.7	235.1	213.3	203.4	265.8	251.8	174.5	449.3	299.6	328.7
CGGACCCAGGCTTCATCC	HE860454	1.3	1.2	0	2.7	3.2	1.4	1.3	1.1	0	2.1	4.3	0	0	1.6	0
CTGGACCCAGGCTTCATCC	HE863101	0	2.4	1.2	2.7	1.6	1.4	1.3	0	1.6	0	0	0	1.1	0	0
CTGGACCCAGGCTTCATCC	HE860455	66.5	83.2	65.6	132.8	42.8	129.9	9	9.7	9.8	10.4	17.4	17.7	16.9	46.5	43.1
CTGGACCCAGGCTTCATCC	HE863103	151.8	165.2	175.8	192.4	130	189.8	165.3	159.4	146	126.7	147.6	136	75.3	187.5	183.7
CTGGACCCAGGCTTCATCC	HE860455	0	0	0	0	0	1.4	1.3	0	0	2.1	0	0	0	0	0
GAATGCTGTGGTTCGAGAC	HE863105	3.8	1.2	0	5.4	0	1.4	0	0	0	0	0	0	1.1	1.6	0
GACCAGGCTTCATCC	HE860456	1.3	0	0	0	0	0	0	0	1.6	0	0	0	0	0	0
GAAATGCTGTGGTTCGA	HE863107	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0
GGAATGCTGTGGTTCGAGA	HE860456	12.5	17.1	12.9	24.4	4.8	30	3.9	4.3	4.9	8.3	17.4	29.6	4.5	0	10
GGAATGCTGTGGTTCGAGAC	HE863109	5	6.1	8.2	2.7	4.8	17.1	0	1.1	3.3	4.2	4.3	0	0	0	2.9
GGACCCAGGCTTCATCC	HE860457	1.3	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0
GGACCCAGGCTTCATCC	HE863111	146.7	154.1	143	184.3	136.3	148.4	155	161.6	134.5	189	191	147.9	171.9	174.7	193.8
TGGACCCAGGCTTCATCC	HE860457	58.9	64.8	65.6	43.4	28.5	31.4	42.6	54.9	39.4	24.9	28.2	29.6	47.2	54.5	40.2
TGGACCCAGGCTTCATCC	HE863113	440.2	408.6	385.7	417.4	261.5	412.4	384.9	339.3	332.9	180.7	256.2	283.9	159.5	280.4	254.1
TGGACCCAGGCTTCATCC	HE860458	706.1	675.3	720.9	441.8	321.8	449.5	586.5	627	546.1	388.4	525.4	387.4	410	512.8	541.2
TGGACCCAGGCTTCATCC	HE860285	282744.1	279717.9	296598.2	273500	243934.6	239560.7	306036.6	275122.8	248117.2	282794.4	317557.2	199637.5	217491.6	348033.3	350352.6
TGGACCCAGGCTTCATCC	HE863115	209.5	212.9	233.3	384.9	160.1	276.9	170.5	137.9	165.6	272.1	191	168.6	174.1	282	236.9

1_32	HE860458	110.4	1798	155.9	273.7	190.2	332.5	111.6	8.6	14.8	24.9	99.9	11.8	28.1	57.7	45.9
	HE863117	0	1.2	1.2	5.4	0	5.7	0	0	0	0	2.2	0	0	1.6	1.4
	HE860459	2.5	11	9.4	8.1	4.8	12.8	9	4.3	8.2	6.2	4.3	5.9	4.5	9.6	10
	HE863119	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HE860459	1.3	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0
	HE863121	1.3	1.2	0	0	0	0	0	0	0	0	0	0	0	0	0
	HE860460	5	2.4	7	2.7	4.8	4.3	0	1.1	0	2.1	0	0	1.1	1.6	1.4
	HE863123	2.5	0	0	0	0	1.4	0	0	0	0	0	0	0	0	0
	HE860460	1.3	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0
	HE860311	71.5	80.7	97.3	181.6	38	44.2	29.7	53.9	31.2	49.8	17.4	11.8	11.2	11.2	20.1
	HE863125	2.5	3.7	0	0	1.6	0	0	0	1.6	2.1	0	0	0	0	0
	HE860461	0	2.4	2.3	8.1	1.6	0	1.3	0	0	2.1	0	0	0	1.6	0
	HE863127	8.8	14.7	15.2	21.7	3.2	7.1	2.6	0	1.6	10.4	0	0	0	0	0
	HE860461	3.8	2.4	3.5	2.7	0	0	5.2	9.7	0	0	0	3	2.2	3.2	5.7
	HE860451	1.3	0	0	0	0	1.4	0	0	0	0	2.2	0	0	0	0
	HE863087	6.3	6.1	7	13.6	11.1	7.1	2.6	1.1	1.6	2.1	0	5.9	0	0	0
	HE860451	18.8	24.5	15.2	13.6	28.5	27.1	1.3	5.4	8.2	2.1	4.3	0	0	1.6	8.6
	HE860307	89.1	12.11	89.1	127.4	141.1	225.5	80.1	53.9	59	27	26.1	20.7	20.2	35.3	25.8
	HE863129	1.3	0	1.2	0	0	0	1.3	1.1	1.6	2.1	0	0	1.1	0	0
	HE860462	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0
	HE863131	3.8	2.4	2.3	2.7	0	0	2.6	2.2	0	2.1	4.3	0	2.2	1.6	1.4
	HE860462	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HE860288	8.8	23.2	27	5.4	79	7.1	14.2	15.1	3.3	16.6	23.9	20.7	20.2	17.6	18.7
	HE860287	125.4	174.9	219.2	70.5	130	81.3	107.2	131.4	147.6	280.4	230.1	378.5	179.7	126.6	277.1
	HE860286	129.2	116.2	148.9	29.8	71.3	37.1	63.3	75.4	62.3	170.3	141.1	195.2	85.4	83.3	208.2
	HE863133	2.5	1.2	2.3	0	0	0	0	1.1	0	0	0	0	1.1	1.6	0
	HE860463	2.5	3.7	8.2	21.7	0	2.9	1.3	2.2	0	0	0	0	0	4.8	0
	HE863135	2.5	3.7	4.7	2.7	1.6	5.7	1.3	0	0	0	0	0	2.2	8	1.4
	HE860463	11.3	3.7	7	5.4	0	8.6	3.9	3.2	0	0	0	3	9	4.8	5.7
	HE863137	26.3	22	36.3	16.3	1.6	8.6	7.8	10.8	3.3	2.1	0	3	6.7	24	11.5
	HE860464	47.7	35.5	32.8	146.4	14.3	65.6	11.6	12.9	8.2	2.1	8.7	8.9	15.7	16	15.8
	HE860428	154.3	137	137.2	168	53.9	119.9	95.6	72.2	90.2	16.6	19.5	14.8	83.1	81.7	64.6
	HE863139	2.5	6.1	3.5	10.8	1.6	5.7	1.3	2.2	1.6	0	2.2	0	2.2	1.6	4.3
	HE860464	0	0	0	2.7	1.6	0	0	0	0	0	0	0	0	0	0
	HE863141	0	1.2	1.2	0	0	0	0	0	0	2.1	0	0	0	0	0
	HE860465	0	1.2	0	0	0	1.4	1.3	1.1	0	0	0	0	0	0	0
	HE860334	12.5	22	18.8	46.1	44.4	44.2	37.5	43.1	16.4	22.8	23.9	41.4	1.1	3.2	7.2
	HE863143	3.8	12.2	15.2	19	20.6	11.4	11.6	19.4	8.2	8.3	4.3	3	1.1	0	2.9
	HE860465	0	0	0	5.4	1.6	4.3	0	0	0	0	0	0	0	0	0
	HE863145	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	HE860337	1.3	4.9	3.5	67.8	30.1	55.7	1.3	4.3	1.6	12.5	6.5	3	2.2	1.6	5.7
	HE860466	0	0	0	2.7	1.6	0	0	0	0	0	0	0	0	0	0

(Continued)

Table 2 | Continued

miRNA	Reads	EMBL accession number	BF1	BF2	BF3	F1	F2	F3	GF1	GF2	GF3	GL1	GL2	GL3	O1	O2	O3	
3_16	3_16	TGATATTGGATCGATGCGATC	HE863147	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	
		ATGTAGGAATGGGCTGTTTG	HE860466	2.5	0	0	0	0	1.3	0	0	0	0	0	0	0	0	
		CCCAAGCCCGCCATTCC	HE863149	0	0	0	0	0	2.6	0	0	0	0	0	0	0	0	
		CCCAAGCCCGCCATTCCA	HE860467	0	0	0	0	0	1.3	1.1	0	0	0	0	0	0	0	
		CTTCCCAAGCCCGCCATTCCA	HE863151	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	
		GGAATGGGCTGTTGGGA	HE860467	13.8	7.3	17.6	16.3	28.5	12.8	3.9	7.5	11.5	10.4	17.4	20.7	11.2	14.4	10
		GGAATGGGCTGTTGGGAT	HE863153	5	1.2	4.7	0	1.6	2.9	1.3	2.2	0	6.2	4.3	0	2.2	1.6	5.7
		GGAATGGGCTGTTGGGATG	HE860468	70.2	56.3	92.6	168	68.2	92.8	10.3	24.8	23	22.8	17.4	20.7	69.6	56.1	61.7
		GGAATGGGCTGTTGGGATGA	HE860347	100.3	84.4	150.1	311.7	111	177	22	49.6	50.8	47.8	34.7	53.2	197.7	86.5	104.8
		GGAATGGGCTGTTGGGATGAA	HE863155	0	0	0	2.7	0	1.4	0	0	0	0	0	0	1.1	0	0
		GGAATGGGCTGTTGGGATGAAAG	HE860468	0	0	0	2.7	0	0	0	0	0	0	0	0	2.2	0	1.4
		TAGGAATGGGCTGTTGGGA	HE863157	2.5	1.2	3.5	0	1.6	0	0	0	0	0	0	0	0	0	1.4
		TTCCCAAGCCCGCCATT	HE860469	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0
		TTCCCAAGCCCGCCATTCC	HE863159	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		TTCCCAAGCCCGCCATTCC	HE860469	0	3.7	0	10.8	0	7.1	6.5	4.3	4.9	6.2	8.7	3	0	0	1.4
		TTCCCAAGCCCGCCATTCCA	HE863161	0	2.3	0	0	1.6	1.4	0	0	1.6	2.1	0	0	0	0	0
3_28	3_28	TTCCCAAGCCCGCCATTCCA	HE860348	112.9	130.9	99.6	216.8	123.6	182.7	217	225.2	278.3	212.8	115.3	47.2	38.5	63.2	
		TTGTAGGAATGGGCTGTTGGGA	HE860470	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0	
		TTTCTTCATCCCAACACGC	HE863163	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	
		ATGGTGTATCCCTCTGTGACC	HE860470	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0	
		CCAAATTTAGAGAGAGAGAGAG	HE863165	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	
		CCATCTTCCTGTGACATGAC	HE860471	0	0	0	2.7	0	1.4	0	0	0	2.1	0	0	0	0	
		CGCAGAGAGATGGCAGTG	HE863167	0	0	0	0	0	0	0	0	0	2.1	2.2	0	0	0	
		GGTGTATCCCTCTGTGACC	HE860471	0	0	0	0	1.6	0	0	0	0	2.1	2.2	0	0	0	
		TCCATCTTCCTGTGACATGA	HE863169	0	0	0	2.7	0	0	0	0	1.6	2.1	2.2	3	0	0	
		TCGCAGGAGATGGCAC	HE860472	2.5	0	0	0	0	0	0	0	0	0	4.3	3	0	0	
		TCGCAGGAGATGGCACTG	HE863171	7.5	2.4	2.3	5.4	0	0	5.2	14	9.8	35.3	39.1	47.3	0	0	5.7
		TCGCAGGAGATGGCACTGT	HE860472	1.3	0	2.3	8.1	0	10	1.3	5.4	3.3	8.3	15.2	17.7	1.1	0	1.4
		TCGCAGGAGATGGCACTGTC	HE860356	15.1	9.8	15.2	51.5	19	38.5	40	74.3	44.3	110.1	165	139	3.4	4.8	7.2
		TCGCAGGAGATGGCACTGTCT	HE863173	0	0	0	2.7	0	0	0	0	1.6	0	0	0	0	0	0
		TGGTGTATCCCTCTGTGACC	HE860473	0	0	0	0	0	4.3	0	2.2	4.9	10.4	8.7	29.6	1.1	0	1.4
		TTCCATCTTCCTGTGACATGA	HE863175	0	0	0	2.7	3.2	1.4	2.6	1.1	1.6	2.1	4.3	23.7	0	0	0
4_21	4_21	TTCCCAAGCCCGCCATT	HE860473	0	0	0	0	0	2.6	0	0	2.1	2.2	3	0	0	0	
		TTCCCAAGCCCGCCATT	HE860473	0	0	0	0	0	2.6	1.1	1.6	2.1	2.2	3	0	0	1.4	
		TTCCCAAGCCCGCCATT	HE863177	0	0	1.2	0	1.4	0	0	0	0	0	0	0	0	0	
		CCCTGCAGTACCTCTTTIACCC	HE860474	0	0	1.2	2.7	0	0	0	0	0	0	0	0	0	0	
		GGAGCGACTGGGATCACATG	HE863179	0	1.2	1.2	21.7	1.6	0	0	0	0	0	0	0	1.1	0	0
		GTGTCTCAGGTCGCCCTG	HE860474	0	0	2.3	0	0	1.4	0	1.1	3.3	0	0	0	2.2	0	0
		TGTGTCTCAGGTCGCCCTG	HE863181	3.8	2.4	3.5	8.1	4.8	4.3	0	0	0	2.1	0	3	0	14.4	7.2
		TGTGTCTCAGGTCGCCCTG	HE860475	0	1.2	0	2.7	0	0	0	2.2	0	0	0	0	3.4	1.6	8.6
		TGTGTCTCAGGTCGCCCTG	HE860366	356.2	210.4	280.2	311.7	313.9	216.9	31	71.1	54.1	60.2	36.9	35.5	540.3	700.2	723.5
		AATGTGTCTGGCTCGAG	HE863183	0	1.2	3.5	0	0	1.4	0	0	0	0	0	0	1.1	1.6	1.4
	AATGTGTCTGGCTCGAGG	HE860475	6.3	13.5	7	8.1	0	1.4	6.5	8.6	0	6.2	10.9	0	6.7	28.8	11.5	

AAATGTTGCTGGCTCGAGGCC	HE863185	0	0	2.7	0	0	0	1.3	1.1	1.6	0	4.3	0	1.1	1.6	0	0
AAATGTTGCTGGCTCGAGGCC	HE860476	0	0	0	0	0	0	1.3	1.1	0	0	0	0	0	0	0	0
AAATGTTGCTGGCTCGAGGCCCT	HE863187	0	0	0	0	0	0	0	2.2	3.3	2.1	0	3	0	0	0	0
ACGAGCCTCATCCGCC	HE863093	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
ACGTCGACACAGGCTTCATTC	HE860476	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0	0
ACGTCGACACAGGCTTCATCC	HE863189	1.3	0	0	0	0	0	1.3	0	1.6	0	0	0	0	1.6	1.4	0
ATGTTGCTGGCTCGAGG	HE860477	1.3	2.4	3.5	0	0	0	0	0	0	0	2.2	0	1.1	1.6	2.9	0
ATTTGGTTACATTTAGTGAC	HE863191	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CGACACAGGCTTCATCC	HE863097	0	0	0	0	0	0	0	1.1	0	0	2.2	0	2.2	0	0	0
CGACACAGGCTTCATCC	HE860454	1.3	2.4	0	2.7	1.6	0	1.3	5.4	1.6	0	0	3	1.1	0	0	0
CGACACAGGCTTCATCC	HE863099	282.2	208	289.6	336.1	187	182.7	235.1	213.3	203.4	265.8	251.8	174.5	449.3	299.6	328.7	0
CGACACAGGCTTCATCC	HE860454	1.3	1.2	2.7	0	1.6	1.4	1.3	1.1	0	2.1	4.3	0	0	1.6	0	0
CGTCGACACAGGCTTCATCC	HE860477	1.3	0	0	0	0	0	0	2.2	0	0	0	0	0	0	0	0
CGTCGACACAGGCTTCATCC	HE863193	0	0	0	0	0	1.4	0	0	0	0	0	0	3.4	0	0	0
CGTCGACACAGGCTTCATCC	HE860478	3.8	2.4	1.2	0	0	0	1.3	0	3.3	0	2.2	0	2.2	1.6	4.3	0
GAATGTTGCTGGCTCGA	HE863195	1.3	1.2	0	0	0	0	1.3	0	1.6	0	0	0	1.1	0	0	0
GAATGTTGCTGGCTCGAGG	HE860478	5	13.5	7	10.8	1.6	2.9	1.3	2.2	0	0	2.2	3	3.4	14.4	5.7	0
GAATGTTGCTGGCTCGAGG	HE863197	0	1.2	2.3	2.7	0	0	0	2.2	0	0	0	3	0	0	1.4	0
GAATGTTGCTGGCTCGAGG	HE860479	0	2.4	0	0	0	1.4	0	0	0	0	0	0	1.1	1.6	0	0
GAATGTTGCTGGCTCGAGG	HE863199	0	0	0	0	0	0	0	0	3.3	2.1	0	0	0	0	0	0
GACACAGGCTTCATCC	HE860456	1.3	0	0	0	0	0	0	0	1.6	0	0	0	0	0	0	0
GAATGTTGCTGGCTCG	HE860479	38.9	24.5	44.5	16.3	1.6	5.7	9	26.9	18	14.5	21.7	20.7	22.5	49.7	63.2	0
GAATGTTGCTGGCTCGA	HE863201	10	11	24.6	5.4	4.8	1.4	11.6	16.2	14.8	22.8	8.7	32.5	5.6	8	24.4	0
GAATGTTGCTGGCTCGAG	HE860480	6.3	3.7	10.6	16.3	4.8	0	6.5	8.6	3.3	8.3	4.3	11.8	4.5	6.4	14.4	0
GAATGTTGCTGGCTCGAGG	HE863203	165.6	156.6	174.7	73.2	26.9	49.9	74.9	106.7	80.4	58.1	117.2	263.2	175.2	211.5	353.1	0
GAATGTTGCTGGCTCGAGG	HE860480	1.3	1.2	1.2	0	0	0	2.6	1.1	1.6	2.1	0	3	0	4.8	0	0
GGACACAGGCTTCATCC	HE860457	1.3	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0	0
GGACACAGGCTTCATCC	HE863111	146.7	154.1	143	184.3	136.3	148.4	155	161.6	134.5	189	191	147.9	171.9	174.7	193.8	0
GTCGACACAGGCTTCATC	HE863205	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	0
GTCGACACAGGCTTCATCC	HE860481	0	0	0	0	0	0	0	1.1	0	0	0	0	1.1	0	0	0
GTCGACACAGGCTTCATCC	HE863207	64	117.4	86.7	86.7	49.1	109.9	49.1	56	42.6	29.1	28.2	3	97.7	94.5	68.9	0
GTCGACACAGGCTTCATCC	HE860481	21.3	26.9	29.3	29.8	9.5	14.3	31	29.1	23	33.2	30.4	17.7	51.7	62.5	37.3	0
GTCGACACAGGCTTCATCC	HE863209	0	2.4	7	0	0	1.4	78	8.6	0	2.1	4.3	0	4.5	4.8	7.2	0
GTTGCTGGCTCGAGGCC	HE860482	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	0
TAAATGTAGAACCAATGATCT	HE863211	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TCACATAATGTAGAACCAATG	HE860482	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0	0
TCGACACAGGCTTCATC	HE860457	58.9	64.8	65.6	43.4	28.5	31.4	42.6	54.9	39.4	24.9	28.2	29.6	47.2	54.5	40.2	0
TCGACACAGGCTTCATCC	HE863113	440.2	408.6	385.7	417.4	261.5	412.4	384.9	339.3	332.9	180.7	256.2	283.9	159.5	280.4	254.1	0
TCGACACAGGCTTCATCC	HE860458	706.1	675.3	720.9	441.8	321.8	449.5	586.5	627	546.1	388.4	525.4	387.4	410	512.8	541.2	0
TCGACACAGGCTTCATCC	HE860285	282744.1	279717.9	296598.2	273500	243934.6	239360.7	306036.6	275122.8	248117.2	282794.4	317557.2	199637.5	217491.6	348033.3	350352.6	0
TCGACACAGGCTTCATCC	HE863115	209.5	212.9	233.3	384.9	160.1	276.9	170.5	137.9	165.6	272.1	191	168.6	174.1	282	236.9	0
TGCTGGCTCGAGGCCCTA	HE863213	0	0	2.7	0	0	0	0	0	0	0	0	0	0	0	0	0

(Continued)

Table 2 | Continued

miRNA Reads	EMBL accession number	BF1	BF2	BF3	F1	F2	F3	GF1	GF2	GF3	GL1	GL2	GL3	O1	O2	O3	
5_3	CCCGCCTTGATCAACTG	HE860483	0	2.3	0	0	0	0	1.1	0	6.2	0	3	0	0	0	
	CCCGCCTTGATCAACTGAA	HE863215	0	1.2	0	0	2.9	1.3	1.1	3.3	6.2	8.7	5.9	0	1.6	2.9	
	CCCGCCTTGATCAACTGAAT	HE860483	35.1	44	38.7	75.9	30.1	95.6	263.9	242.7	255.4	455.9	2573	20.2	139.4	208.2	
	CCGCTTGCATCAACTGAAT	HE863217	2.5	0	1.2	0	0	0	2.6	0	2.1	4.3	0	0	0	0	0
	CGTTGGTGCAGGTCGGGA	HE860484	0	0	0	0	0	0	0	1.1	1.6	2.1	0	3	0	0	0
	CGTTGGTGCAGGTCGGGAA	HE863219	0	0	0	0	0	0	0	1.1	3.3	6.2	2.2	3	1.1	1.4	0
	CGTTGGTGCAGGTCGGGAA	HE860484	0	0	0	2.7	0	0	0	0	0	0	2.2	0	0	0	0
	CGTTGGTGCAGGTCGGGAA	HE863221	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0
	GGTCCCGCCTTGATCAACTGAA	HE860485	0	0	0	0	0	0	0	0	0	4.2	2.2	0	0	0	0
	GGTCCCGCCTTGATCAACTGAA	HE863223	0	0	2.3	0	0	0	0	0	0	2.1	2.2	0	0	0	0
	TCGCTTGGTGCAGGTCGGGA	HE860485	11.3	20.8	15.2	48.8	6.3	14.3	27.1	26.9	68.9	78.9	56.4	65.1	5.6	19.2	18.7
	TCGCTTGGTGCAGGTCGGGAA	HE860370	259.6	254.5	184	219.5	136.3	186.9	193.8	339.3	501.9	494.3	579.6	520.5	104.5	208.3	249.8
	TCGCTTGGTGCAGGTCGGGAA	HE863225	0	0	0	1.6	0	0	3.9	1.1	8.2	10.4	4.3	3	0	1.6	4.3
	TGGTCCCGCCTTGATCAACT	HE860486	6.3	9.8	12.9	24.4	14.3	28.5	28.4	24.8	39.4	45.7	52.1	82.8	4.5	12.8	10
	TGGTCCCGCCTTGATCAACT	HE863227	0	0	0	2.7	0	0	0	0	1.6	0	0	0	0	0	0
	TGGTCCCGCCTTGATCAACT	HE860486	1.3	1.2	1.2	0	0	0	0	0	0	4.2	0	0	0	0	0
	TTGGTCGGTGCAGGTCGGGAA	HE863229	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0
	AAGTCAGGAGGATAGC	HE860487	1.3	0	0	2.7	0	1.4	0	1.1	1.6	0	0	0	0	0	0
	AAGTCAGGAGGATAGCGC	HE863231	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	AAGTCAGGAGGATAGCGC	HE860388	70.2	64.8	86.7	178.9	218.7	118.4	153.7	149.7	173.8	211.8	254	224.7	141.5	158.6	113.4
AGCTCAGGAGGATAGCGC	HE860487	0	1.2	0	5.4	1.6	0	0	0	0	2.1	0	0	1.1	0	0	
CGCTATCCATCCTGAGTTTC	HE863233	0	1.2	1.2	8.1	19	2.9	0	0	1.6	0	0	0	0	0	0	
CGCTATCCATCCTGAGTTTCA	HE860488	1.3	1.2	4.7	13.6	42.8	27.1	2.6	5.4	1.6	0	2.2	0	1.1	0	0	
TAITGGCTATCCATCCTGAGTT	HE863235	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	
TCCATCCTGAGTTTTCATGGCT	HE860488	1.3	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0	
TTGGCTATCCATCCTGAG	HE863237	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0	
AAGTCGAGGATAGCTGAGC	HE860489	0	0	0	0	0	0	0	0	0	0	0	0	1.1	4.8	0	
AGATCAITGGTGGTTCATC	HE863239	5	0	0	0	0	0	0	2.2	1.6	0	0	0	1.1	6.4	7.2	
CTAGATCATGTGGTAGTTTCATC	HE860489	1.3	0	0	0	0	0	1.3	0	1.6	0	0	0	1.1	1.6	0	
GAAGCTGCCAGCATGATCTG	HE863241	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	2.9	
GAAGCTGCCAGCATGATCTGA	HE860490	1.3	0	1.2	0	0	0	0	0	0	0	0	0	1.1	0	2.9	
GATCATGTGGTAGTTTCATC	HE863243	15.1	11	14.1	0	0	0	10.3	8.6	8.2	0	0	0	37.1	51.3	41.6	
GCTFAGATCATGTGGTAGTTTCATC	HE860490	0	0	0	0	1.6	0	0	0	0	0	0	0	1.1	0	4.3	
TAGATCATGTGGTAGTTTCATC	HE863245	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0	
TGAAGCTGCCAGCATGAT	HE860491	0	0	0	0	0	0	0	0	0	0	0	0	1.1	0	0	
TGAAGCTGCCAGCATGATC	HE863247	76.5	48.9	62.1	2.7	4.8	2.9	25.8	45.2	41	2.1	8.7	0	21.3	25.6	31.6	
TGAAGCTGCCAGCATGATCT	HE860284	170.6	172.5	161.8	19	17.4	30	153.7	153	88.6	10.4	15.2	14.8	35.9	36.9	48.8	
TGAAGCTGCCAGCATGATCTG	HE860399	1434.8	1105.9	1518.1	43.4	174.4	99.9	496	627	321.5	10.4	13	11.8	775.1	796.4	785.2	
TGAAGCTGCCAGCATGATCTGA	HE860400	1056.1	677.7	907.3	59.6	57.1	34.3	511.5	667.9	408.4	16.6	6.5	3	302.2	427.8	447.9	
TGAAGCTGCCAGCATGATCTGAGC	HE860491	2.5	0	0	0	0	0	1.3	2.2	1.6	0	0	0	2.2	1.6	2.9	
TGTTGAAGCTGCCAGCATGATC	HE863249	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
AAGTCAGGAGGATAGC	HE860487	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

6_7

7_23

7_24

(Continued)

Table 2 | Continued

miRNA Reads	EMBL accession number	BF1	BF2	BF3	F1	F2	F3	GF1	GF2	GF3	GL1	GL2	GL3	O1	O2	O3
ACGTTATGTTGCAAAATGTC	HE863287	0	0	0	0	0	0	1.3	1.1	0	0	0	0	0	0	0
ATGTTGCAAAATGTC	HE860501	0	0	0	0	1.6	1.4	1.3	0	1.6	0	0	5.9	0	0	0
CAACGTGACAACAACGAGC	HE863289	1.3	0	1.2	0	0	2.9	0	0	1.6	0	0	0	0	0	0
CAACGTGACAACAACGAGCC	HE860502	10	15.9	12.9	8.1	7.9	12.8	2.6	5.4	6.6	8.3	0	0	0	1.6	2.9
CACGTTATGTTGCAAAATGTC	HE863291	0	0	0	2.7	0	1.4	0	0	0	0	0	0	0	0	0
TAATGTTGCAAAATGTC	HE860502	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0
TATGTTGCAAAATGTC	HE860414	16.3	9.8	24.6	24.4	12.7	20	24.5	21.5	29.5	10.4	17.4	14.8	12.4	4.8	5.7
TGAACAACAATAATGCCCCG	HE863293	0	0	0	0	0	0	1.3	0	0	0	0	0	0	0	0
TTGACAACGTGACAACAAC	HE860503	0	1.2	1.2	0	0	0	5.2	2.2	1.6	0	4.3	3	0	0	0
GACAGAAGAGAGTGAGCAC	HE863121	1.3	1.2	0	0	0	0	0	0	0	0	0	0	0	0	0
GCTCACCTCTCTGTGTCAGC	HE863295	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0
TGACAGAAGAGAGTGAGCA	HE860460	1.3	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0
TGACAGAAGAGAGTGAGCA	HE860311	71.5	80.7	97.3	181.6	38	44.2	29.7	53.9	31.2	49.8	17.4	11.8	11.2	11.2	20.1
TGACAGAAGAGAGTGAGCA	HE863125	2.5	3.7	0	0	1.6	0	0	0	1.6	2.1	0	0	0	0	0
CCGACAAGCGTCTCTCTCGTT	HE860503	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GTGCTCTCTGTTGTCATG	HE863297	1.3	3.7	4.7	13.6	4.8	0	1.3	0	3.3	2.1	6.5	0	2.2	6.4	5.7
TGACAACGAGAGAGAGCAC	HE860504	2.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TGACAACGAGAGAGAGCAC	HE863299	0	0	0	2.7	0	1.4	0	0	3.3	0	0	0	0	0	0
TGACAACGAGAGAGAGCAC	HE860423	75.3	35.5	21.1	29.8	23.8	41.4	20.7	45.2	32.8	39.5	69.5	62.1	5.6	14.4	8.6
TTGACAACGAGAGAGAGCAC	HE860504	2.5	1.2	1.2	0	0	1.4	0	0	1.6	0	2.2	0	0	0	1.4
TTGACAACGAGAGAGAGCAC	HE863301	6.3	0	0	8.1	4.8	2.9	3.9	6.5	3.3	2.1	10.9	0	1.1	1.6	0
TTGACAACGAGAGAGAGCAC	HE860505	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TTGCGCACCCATGAAAGGGCCA	HE863303	0	0	0	2.7	0	0	0	0	0	0	0	0	0	0	0
TTTGACAACGAGAGAGAGCAC	HE860505	2.5	0	1.2	2.7	0	4.3	1.3	3.2	1.6	4.2	6.5	3	1.1	1.6	0
AATGCTGCTGGTTCGAGA	HE863305	0	0	1.2	2.7	3.2	1.4	0	0	0	0	0	0	0	1.6	0
AATGCTGCTGGTTCGAGATC	HE860506	1.3	0	0	0	0	1.4	0	0	0	0	0	0	0	0	0
ATTCGGACCAGGCTCATTCC	HE863307	3.8	3.7	3.5	2.7	1.6	4.3	1.3	0	0	0	0	3	0	0	0
ATTCGGACCAGGCTCATTCC	HE860506	0	1.2	0	2.7	0	0	0	0	0	0	2.2	0	0	0	0
CGGACCAGGCTCATTCC	HE863097	0	0	0	0	0	0	0	1.1	0	0	2.2	0	2.2	0	0
CGGACCAGGCTCATTCC	HE860454	1.3	2.4	0	2.7	1.6	0	1.3	5.4	1.6	0	0	3	1.1	0	0
CGGACCAGGCTCATTCC	HE863099	282.2	208	289.6	336.1	187	182.7	235.1	213.3	203.4	265.8	251.8	174.5	449.3	299.6	328.7
CGGACCAGGCTCATTCC	HE863309	2.5	6.1	1.2	8.1	15.9	4.3	0	1.1	4.9	2.1	2.2	0	1.1	3.2	0
GAATGCTGCTGGTTCGAGA	HE860507	1.3	7.3	2.3	2.7	0	8.6	0	0	0	0	0	0	0	0	2.9
GACCAGGCTCATTCC	HE860456	1.3	0	0	0	0	0	0	0	1.6	0	0	0	0	0	0
GACCAGGCTCATTCC	HE863311	0	0	0	2.7	0	0	0	0	0	0	0	0	0	0	0
GATTCGGACCAGGCTCATTCC	HE860507	1.3	0	0	2.7	0	0	0	0	0	0	0	0	0	0	0
GGAATGCTGCTGGTTCGA	HE863313	1.3	1.2	0	0	1.6	2.9	1.3	1.1	1.6	2.1	0	3	1.1	0	1.4
GGAATGCTGCTGGTTCGAGA	HE860508	20.1	15.9	19.9	21.7	28.5	35.7	2.6	5.4	8.2	4.2	0	3	3.4	4.8	5.7
GGAATGCTGCTGGTTCGAGAT	HE863315	0	0	0	2.7	0	0	0	0	0	0	0	0	0	0	0
GGACCAGGCTCATTCC	HE860457	1.3	0	1.2	0	0	0	0	0	0	0	0	0	0	0	0
GGACCAGGCTCATTCC	HE863111	146.7	154.1	143	184.3	136.3	148.4	155	161.6	134.5	189	191	147.9	171.9	174.7	193.8
GGGAATGCTGCTGGTTCGAG	HE860508	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0

TCGACCAGGCTTCATTC	HE860457	58.9	64.8	65.6	43.4	28.5	31.4	42.6	54.9	39.4	24.9	28.2	29.6	47.2	54.5	40.2
TCGGACAGGCTTCATTC	HE863113	440.2	408.6	385.7	417.4	261.5	412.4	384.9	339.3	332.9	180.7	256.2	283.9	159.5	280.4	254.1
TCGGACAGGCTTCATTC	HE860458	706.1	675.3	720.9	441.8	321.8	449.5	586.5	627	546.1	388.4	525.4	387.4	410	512.8	541.2
TCGGACAGGCTTCATTC	HE860285	282744.1	2797179	296598.2	273500	243934.6	239360.7	306036.6	275122.8	248117.2	282794.4	317557.2	199637.5	217491.6	348033.3	350352.6
TCGGACAGGCTTCATTC	HE863317	376	416	39.9	27.1	12.7	21.4	59.4	47.4	41	31.2	28.2	35.5	33.7	32	40.2
TCGGACAGGCTTCATTC	HE860509	0	0	1.2	0	0	0	0	0	0	2.1	0	0	0	0	0
TCGGACAGGCTTCATTC	HE863319	1.3	0	2.3	0	4.8	1.4	0	1.1	0	0	0	0	0	0	0
TCGGACAGGCTTCATTC	HE860509	184.4	223.9	195.8	273.7	416.9	299.7	49.1	62.5	47.6	24.9	17.4	23.7	35.9	49.7	43.1
TCGGACAGGCTTCATTC	HE863321	153	168.8	158.3	219.5	280.6	191.2	117.5	101.3	95.1	27	32.6	20.7	35.9	57.7	44.5
TCGGACAGGCTTCATTC	HE860510	1.3	0	0	0	1.6	0	0	0	0	0	0	0	0	0	0
TTGAGGGAATGCTGTGG	HE863323	1.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TTTGGACCAGGCTTCATTC	HE860510	67.7	104	85.6	103	187	114.2	41.3	25.9	24.6	4.2	6.5	20.7	14.6	11.2	15.8
CACGTGCTCCCTTCTCC	HE863325	0	0	0	0	0	0	0	0	0	2.1	0	0	0	0	0
CACGTGCTCCCTTCTCCAAC	HE860511	2.5	1.2	2.3	0	4.8	10	10.3	4.3	11.5	12.5	17.4	20.7	3.4	1.6	4.3
TGGAGAAGCAGGCGAGTGCA	HE860424	55.2	30.6	24.6	62.3	7.9	45.7	14.2	28	21.3	20.8	17.4	29.6	6.7	8	4.3

BF, pink; F, bloom; GF, swollen flower bud; O, half-inch green; GL, swollen leaf bud.

by ENCODE Consortium.⁴ Average correlation coefficients were equal to 0.98 for BF, 0.95 for F, 0.98 for GF, 0.95 for GL, and 0.97 for O. For the sake of completeness and in order to allow a comparison between related and unrelated samples, we also calculated the average Pearson correlation between samples of different tissues, which was equal to 0.66 on the basis of the reads reported in **Table 2**. All the Pearson coefficients are reported in File S10 in Supplementary Material. **Figure 1A** reports the results obtained from clustering the five samples on the basis of all the reads frequencies (average frequencies of three replicates, reads included miRNA*-related reads; reads assigned by miRDeep-P to more than one locus were counted once) at the 26 loci analyzed. Additionally, a clustering analysis was performed by considering only the count of the most frequent read in each locus. The analysis included those loci where the most frequent read was the same in all the samples (16 different reads, **Figure 1B**). **Figure A1** in Appendix reports clustering results obtained without averaging the three replicates of each sample. As it can be seen, replicates are always grouped correctly.

A *t*-test was also performed for all the possible comparisons of biological samples (File S11 in Supplementary Material). The most frequent isomiR (highlighted in yellow in File S11 in Supplementary Material) is frequently the one able to distinguish the higher number of samples (e.g., locus 4_21, locus 6_4). Some miRNA-related reads are able to differentiate most of the analyzed samples: e.g., miR398 and miR167 got 8 significant comparisons out of 10.

DISCUSSION

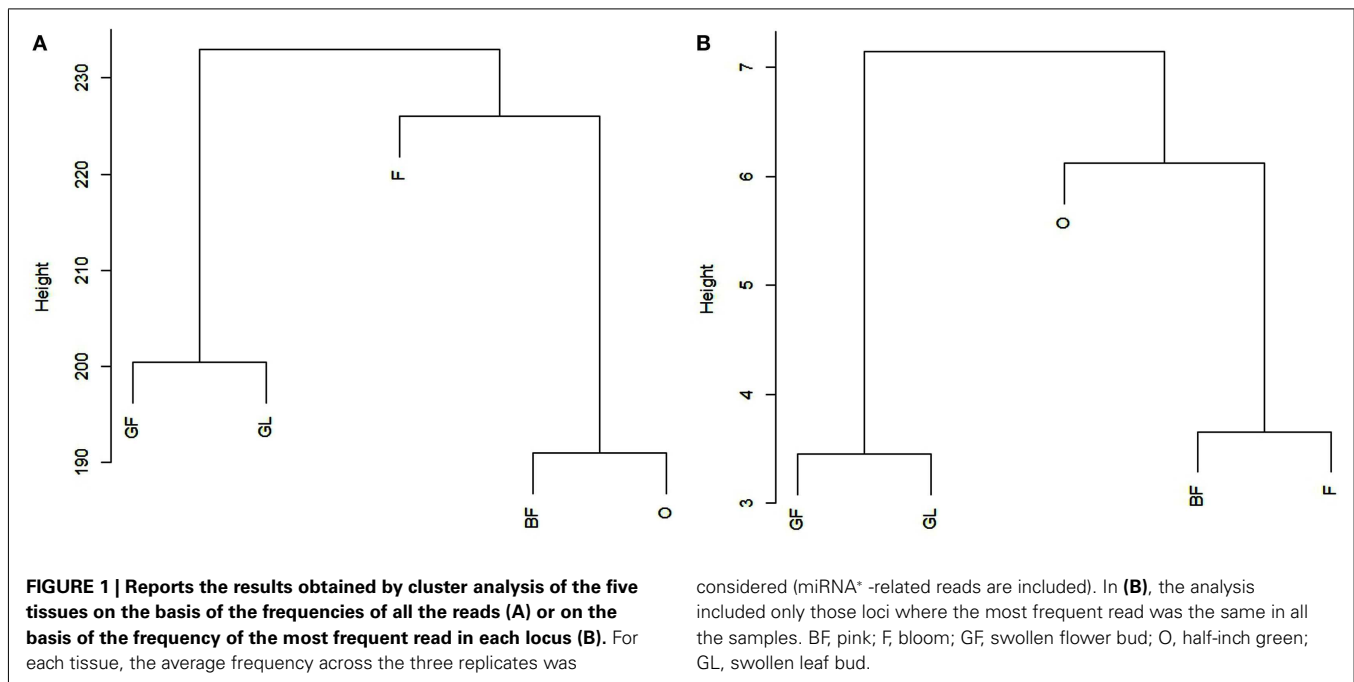
To assess the putative biological significance of isomiRs in peach, in the present study we carried out miRNAs profiling by sequencing three replicates of five biological samples arising from a set of different organs and/or phenological stages. Actually, variants of miRNAs are commonly found in deep sequencing experiments but their functional meaning and stability is still under investigation in plants.

Twenty-six miRNA putative loci expressed in all samples analyzed have been identified by miRdeep-P and analyzed for miRNA population heterogeneity. The average length of miRNA associated reads was included between 18 nt and 24 nt. Several previous works reported a miRNA length in plants included between 22 nt and 24 nt. The identification of miRNA* associated reads provides more evidence about reliability of the loci identified by miRDeep-P.

All the analyzed loci show miRNA length variants but tend to maintain the uridine at the 5' end, in those cases where uridine is the first base of the most abundant isomiR. As reported above, uridine is the most frequent nucleotide in AGO1 association, perhaps explaining the drive to maintain it at the 5' end. Ebhardt et al. (2009) reported examples of miRNA with 5' deletions and 3' uridine additions that create a different distribution in AGO complexes. As an example, ath-miR822 was determined to reside almost exclusively in the AGO1 complex while its modified variant with a U deletion at 5' end and a UU addition at 3' end was found equally in AGO1 and AGO4 complexes.

The difference in read count between the first most frequent read and the second most frequent read varies among loci being

⁴http://encodeproject.org/ENCODE/protocols/dataStandards/RNA_standards_v1_2011_May.pdf



in some cases minimal (e.g., locus 1_5) while in some others it is quite consistent (e.g., loci 4_21, 6_4). In some loci the second most frequent read was the same in all the replicates of a sample and in all the different samples. The presence of the same isomiRs in different biological replicates of a sample and in different tissues demonstrate that the generation of most of the detected isomiRs is not random. The importance of evaluating the correlation between biological replicates from RNA-seq experiments has been discussed previously in several papers (Oshlack et al., 2010; Hansen et al., 2011). As above reported, the correlation among biological replicates has been calculated to check the reliability of the experiment on the basis of the “Standards, guidelines, and best practices for RNA-seq” adopted by ENCODE Consortium which requires that the Pearson correlation of gene expression between two biological replicates for RNAs that are detected in both samples using RPKM or read counts should be between 0.92 and 0.98. Regarding the present work, the average Pearson correlation between all the possible pairs of replicates belonging to the same biological sample was greater than or equal to 0.95 for all the tested samples, in agreement with the required standards. Clustering results and *t*-test reported in **Figure 1** and File S11 in Supplementary Material, respectively, show that it is possible to clearly distinguish among samples and to group them in a functional way. However, when considering **Figure A1** in Appendix obtained without averaging replicates of each sample, it should be noted that clustering results seem to be more confident when only the most frequent read is taken into account: BF (pink) and F (bloom) are more strictly related being two subsequent phenological stages so it is expected to find a closer relationship between them.

The co-existence of different variants with a similar level of expression could imply a biological role for all of them. Locus 1_26 shows such an example: in this case there are two prevalent isomiRs (HE860305 and HE860450) that differ for one T at the

5' end. For both the isomiRs there are then variants at the 3' end with different lengths.

Target analysis carried out by psRNATarget (File S9 in Supplementary Material) revealed that in many cases isomiRs share the same target. However, because AGO invariably catalyzes the cleavage of targets opposite the bond between nucleotides 10 and 11 from the 5' end of the miRNA, the cleavage products are different when there is a shift toward the 5' end or nucleotide addition at the 5' end of the miRNA mature sequence. Differences in cleavage sites among members of the same miRNA family have been recently studied in rice by Jeong et al. (2011) highlighting a different abundance of specific cleavage sites among plant organs.

A very interesting finding is related to the biological role of miRNA*. Despite the general consensus that miRNAs* have no regulatory activity, several recent publications have provided evidence about their biological function (Mah et al., 2010). In our results, isomiRs have been found also for miRNAs*. As an example, at locus 3_16 the conserved miRNA* has a high number of length variants, most due to a variable 3' end. Locus 3_16 codes for miR482: the miRNA* sequence deposited in miRBase was actually the most frequent read (HE860347) in all the three replicates of sample O (half-inch green) with an average ratio miRNA/miRNA* equal to 0.4. GF and GL showed an average ratio of miRNA/miRNA* equal to 5.7, while in BF and F the ratio was close to one in two out of three replicates. Similar results have been previously found in mammals by Kuchenbauer et al. (2012) that classified miRNA/miRNA* ratios into groups showing that about 50% of all miRNA duplexes revealed high ratios (>100) consistent with a strong preferential processing of one dominant miRNA strand. About 24% had intermediate ratios (between 100 and 10), about 13% showed low ratios (between 10 and 1), while another 13% showed inverted ratios (<1). The finding that miRNAs can

display tissue-dependent miRNA arm selection opposes the general consensus that only one strand is highly dominant for any given miRNA duplex and opens insights into the possible biological function of selective accumulation of miRNA*. A recent review of Sunkar et al. (2012), discusses several studies showing that miRNA* tend to accumulate at a high level under particular conditions. As an example, miR393* accumulates at a high level during infection of *P. syringae* in *Arabidopsis* leaves and promotes plant resistance to bacterial infection. Mir399* is accumulated at high levels during phosphate deprivation in *Arabidopsis* and miR395* accumulates at high levels in *Sorghum* grown in optimal nutrient conditions.

PsRNATarget has been used to investigate possible target genes for miR482 and miR482* at locus 3_16. MiRNA482 target a peach sequence coding for a “probable receptor-like protein kinase” (expectation = 2, target accessibility = 17.288), while miRNA482* targets a NADH dehydrogenase gene (expectation = 3, target accessibility = 8.463). Examples of different targets for a pair of miRNA/miRNA* are reported in previous studies (Sunkar et al., 2012). Mir393 and miR393* target two entirely different gene families (TIR1 and SNARE) both involved in pathogen resistance of host plant. The possibility that a target-dependent strand selection based on the presence in the cell of miRNA or miRNA* targets might influence the selection of the active miRNA arm has been discussed by other authors. For instance Chatterjee and Grosshans (2009) reported that mRNAs can stabilize their cognate miRNAs thus suggesting coordinated RISC assembly which depends on a miRNA and its target levels.

Results obtained in the present work contribute to a deeper view of the miRNome complexity and to a better exploitation of the mechanism of action of these tiny regulators. The exact definition of the entire repertoire of peach miRNAs is in fact a prerequisite for a correct description of miRNAs whose expression is altered in response to specific developmental conditions or environmental stimuli. Future experiments based on small RNA-seq coupled with RNA-seq on the same samples will be carried out to highlight more clearly the possible biological role of miRNA isomiRs in plants.

REFERENCES

- Chapman, P. J., and Catlin, G. A. (1976). Growth stages in fruit trees from dormant to fruit set. *N. Y. Food Life Sci. Bull.* 58.
- Chatterjee, S., and Grosshans, H. (2009). Active turnover modulates mature miRNA activity in *Caenorhabditis elegans*. *Nature* 461, 546–549.
- Dai, X., and Zhao, P. X. (2011). PsRNATarget: a plant small RNA target analysis server. *Nucleic Acids Res.* 39, W155–W159.
- Eamens, A. L., Smith, N. A., Curtin, S. J., Wang, M. B., and Waterhouse, P. M. (2009). The *Arabidopsis thaliana* double-stranded RNA binding protein DRB1 directs guide strand selection from microRNA duplexes. *RNA* 15, 2219–2235.
- Ebhardt, H. A., Tsang, H. H., Dai, D. C., Liu, Y., Bostan, B., and Fahlman, P. (2009). Meta-analysis of small RNA-sequencing errors reveals ubiquitous post-transcriptional RNA modifications. *Nucleic Acids Res.* 37, 2461–2470.
- Guo, L., and Lu, Z. (2010). Global expression analysis of miRNA gene cluster and family based on isomiRs from deep sequencing data. *Comput. Biol. Chem.* 34, 165–171.
- Hansen, K. D., Wu, Z., Irizarry, R. A., and Leek, J. T. (2011). Sequencing technology does not eliminate biological variability. *Nature Biotechnol.* 29, 572–573.
- Jeong, D.-H., Park, S., Zhai, J., Gurazada, S. G. R., de Paoli, E., Meyers, B. C., and Green, P. J. (2011). Massive analysis of rice small RNAs: mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage. *Plant Cell* 23, 4185–4207.
- Jones-Rhoades, M. W., Bartel, D. P., and Bartel, B. (2006). MicroRNAs and their regulatory roles in plants. *Annu. Rev. Plant Biol.* 57, 19–53.
- Kozomara, A., and Griffith-Jones, S. (2011). miRBase: Integrating microRNA annotation and deep-sequencing data. *Nucleic Acids Res.* 39, D152–D157.
- Kuchenbauer, F., Mah, S. M., Heuser, M., McPherson, A., Rüschemann, J., Rouhi, A., Berg, T., Bullinger, L., Argiropoulos, B., Morin, R. D., Lai, D., Starczynowski, D. T., Karsan, A., Eaves, C. J., Watahiki, A., Wang, Y., Aparicio, S. A., Ganser, A., Krauter, J., Döhner, H., Döhner, K., Marra, M. A., Camargo, F. D., Palmqvist, L., Buske, C., and Humphries, R. K. (2012). Comprehensive analysis of mammalian miRNA* species and their role in myeloid cells. *Blood* 118, 3350–3358.
- Lee, L. W., Zhang, S., Etheridge, A., Ma, L., Martin, D., and Galas, D. (2012). Complexity of the microRNA repertoire revealed by next-generation sequencing. *RNA* 16, 2170–2180.
- Mah, S. M., Buske, C., Humphries, R. K., and Kuchenbauer, F. (2010). miRNA*: a passenger stranded in RNA-induced silencing complex? *Crit. Rev. Eukaryot. Gene Expr.* 20, 141–148.
- Mi, S., Cai, T., Hu, Y., Chen, Y., Hodges, E., Ni, F., Wu, L., Li, S., Zhou, H., Long, C., Chen, S., Hannon, G. J., and Qi, Y. (2008). Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* 133, 116–127.

ACKNOWLEDGMENTS

We thank Keith Anthony Grimaldi for helping with the preparation of the manuscript. The present work has been supported by Drupomics Project (Italian Ministry for Agriculture). We acknowledge the International Peach Genome Initiative for pre-publication access to the peach genome sequence.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Genetics_and_Genomics/10.3389/fpls.2012.00165/abstract

File S1 | Reports the miRNA coding loci identified by miRDeep-P in pink sample.

File S2 | Reports the miRNA coding loci identified by miRDeep-P in bloom sample.

File S3 | Reports the miRNA coding loci identified by miRDeep-P in swollen flower bud sample.

File S4 | Reports the miRNA coding loci identified by miRDeep-P in half-green sample.

File S5 | Reports the miRNA coding loci identified by miRDeep-P in swollen leaf bud sample.

File S6 | Reports a summary of the miRNA coding loci identified by miRDeep-P.

File S7 | Reports the link between locus name and locus position.

File S8 | Reports the results of the blast analysis against known plant miRNAs.

File S9 | Reports target analysis for all the identified isomiRs.

File S10 | Reports Pearson correlation coefficients between all the possible pairs of replicates belonging to the same biological sample, as well as samples from different tissues.

File S11 | Reports the results of the t-test which was performed for all the possible comparisons of biological samples. The most frequent isomiR in each locus is highlighted in yellow.

- Moxon, S., Schwach, E., Dalmay, T., MacLean, D., Studholme, D. J., and Moulton, V. (2008). A toolkit for analyzing large-scale plant small RNA datasets. *Bioinformatics* 24, 2252–2253.
- Mückstein, U., Tafer, H., Hackermüller, J., Bernhart, S. H., Stadler, P. F., and Hofacker, I. L. (2006). Thermodynamics of RNA–RNA binding. *Bioinformatics* 22, 1177–1182.
- Oshlack, A., Robinson, M. D., and Young, M. D. (2010). From RNA-seq reads to differential expression results. *Genome Biol.* 11, 220.
- Sunkar, R., Li, Y.-F., and Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. *Trends Plant Sci.* 17, 196–203.
- Takeda, A., Iwasaki, S., Watanabe, T., Utsumi, M., and Watanabe, Y. (2008). The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. *Plant Cell Physiol.* 49, 493–500.
- Vaucheret, H. (2009). AGO1 homeostasis involves differential production of 21-nt and 22-nt miR168 species by miR168a and miR168b. *PLoS ONE* 4, e6442. doi:10.1371/journal.pone.0006442
- Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell* 136, 669–687.
- Xie, Z., Khanna, K., and Ruan, S. (2010). Expression of microRNAs and its regulation in plants. *Semin. Cell Dev. Biol.* 21, 790–797.
- Yang, X., and Li, L. (2011). miRDeep-P: A computational tool for analyzing the microRNA transcriptome in plants. *Bioinformatics* 27, 2614–2615.
- Zhang, Y. (2005). miRU: An automated plant miRNA target prediction server. *Nucleic Acids Res.* 33(Suppl. 2), W701–W704.
- Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
- Received: 03 April 2012; accepted: 04 July 2012; published online: 26 July 2012.
Citation: Colaiacovo M, Bernardo L, Centomani I, Crosatti C, Giusti L, Orrù L, Tacconi G, Lamontanara A, Cattivelli L and Faccioli P (2012) A survey of microRNA length variants contributing to miRNome complexity in peach (*Prunus persica* L.). *Front. Plant Sci.* 3:165. doi: 10.3389/fpls.2012.00165
This article was submitted to *Frontiers in Plant Genetics and Genomics, a specialty of Frontiers in Plant Science.*
Copyright © 2012 Colaiacovo, Bernardo, Centomani, Crosatti, Giusti, Orrù, Tacconi, Lamontanara, Cattivelli and Faccioli. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.

APPENDIX

