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RESEARCH PAPER

Phylogenetic tree-informed microRNAome analysis uncovers conserved and lineage-specific miRNAs in *Camellia* during floral organ development

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Abstract

In plants, miRNAs are endogenous small RNAs derived from single-stranded precursors with hairpin structures. The evolution of miRNAs and their targets represents one of the most dynamic circuits directing gene expression, which may play fundamental roles in shaping the development of distinct plant organs. Here we performed high-throughput small RNA sequencing in five organ types of *Camellia azalea* to capture the spatial profile of small non-coding RNA. In total we obtained >227 million high-quality reads and identified 175 miRNAs with mature and precursor sequences. We aligned the miRNAs to known miRNA databases and revealed some conserved as well as 'newly evolved' miRNA genes. Twelve miRNAs were identified to be specific in the genus *Camellia*, supporting the lineage-specific manner of expansion of 'young' miRNAs. Through differential expression analysis, we showed that many miRNAs were preferentially abundant in certain organ types. Moreover, hierarchical clustering analysis revealed distinctive expression patterns of tissue-specific miRNAs. Gene Ontology enrichment analysis of targets of stamen- and carpel-specific miRNA subclusters showed that miRNA–target regulatory circuits were involved in many important biological processes, enabling their proper specification and organogenesis, such as 'DNA integration' and 'fruit development'. Further, quantitative PCR of key miRNAs and their target genes revealed anti-correlated patterns, and uncovered the functions of key miRNA–target pairs in different floral organs. Taken together, this work yielded valuable information on miRNA–target regulation in the control of floral organ development and sheds light on the evolution of lineage-specific miRNAs in *Camellia*.

Key words: *Camellia*, differential expression, floral development, lineage specific, microRNA identification, miRNA–target regulation.

Introduction

The plant miRNAs are small non-coding RNAs with diverse regulatory functions through silencing of their targeted transcripts (Axtell and Bowman, 2008). In contrast to other double-stranded silencing RNAs such as siRNA, miRNAs are primarily transcribed from plant genomes (Axtell and Bartel, 2005; Axtell, 2013), and mature miRNAs are processed from single-stranded precursors with hairpin structures (Axtell and Bartel, 2005; Axtell, 2013).

Genome-wide analyses of many plants species have proved that some miRNA gene families were highly conserved in

Abbreviations: FB, floral bud; FDR, false discovery rate; GO, Gene Ontology; qPCR, quantitative PCR;YL, young leaf.

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terms of sequences, secondary structures of precursor RNA, and target genes (Meyers *et al.*, 2008; Cuperus *et al.*, 2011), while more miRNA gene families appeared to be lineage specific and diverse (Cuperus *et al.*, 2011; Nozawa *et al.*, 2012). Several miRNA families, including miRNA156, miRNA160, and miRNA166, were found from fern to flowering plants and shared conserved miRNA sequences and target genes (Axtell and Bartel, 2005; Axtell *et al.*, 2007; Cuperus *et al.*, 2011). These deeply conserved miRNAs often had critical functions in plant development and were probably under stringent selection pressure (Gu *et al.*, 2012). It was recognized that newly evolved or lineage-specific miRNAs were less selected during evolution (Voinnet, 2009; Gu *et al.*, 2012). Evolutionary analysis in *Arabidopsis thaliana* and *Arabidopsis lyrata* identified some newly evolved miRNAs, which were more divergent compared with deeply conserved miRNAs (Fahlgren *et al.*, 2010).

It has been shown that miRNA160 regulates the auxin signaling pathway by targeting *Auxin Response Factor* (*ARF*) genes to control plant development; and miRNA165/166 directed the organ polarity pattern by suppressing *Homeodomain Leucine Zipper* (*HD-ZIP*) transcription factor genes (Willmann and Poethig, 2007). Some of the conserved miRNA–target regulations, in both monocots and dicots, were also found to be critical in floral development (Luo *et al.*, 2013). For example, the miRNA172–APETALA2 (AP2) regulatory circuit in Arabidopsis expanded our knowledge of the classic ABC model in the regulation of floral organ identity determination (Chen, 2004), and similar combinations were also evident in other plant species including maize, rice, wheat, and potato, which were involved in different developmental processes (Nair *et al.*, 2010; Lee and An, 2012; Karlova *et al.*, 2013). The floral homeotic genes *FISTULATA* and *BLIND* from snapdragon and petunia, respectively, were revealed to encode miRNA169 family members (Cartolano *et al.*, 2007). Both miRNAs targeted the nuclear transcription factor Y alpha (NF-YA) transcription factors which were required for C class gene activation, and therefore negatively modulated C function (Cartolano *et al.*, 2007). The miRNA169–NF-YA regulation was found in other higher plants including Arabidopsis, but its role in determining C function gene expression was not observed (Wollmann *et al.*, 2010), suggesting that the conservation might be relatively lineage specific. Hence, the rapid expansion of newly evolved miRNAs could contribute to the introduction of novel functions, given the great diversity of floral forms. In addition to adding post-transcriptional regulation to ABC model genes, some miRNAs were found to specify tissue-specific cell types during the later stages of floral organ morphogenesis (Nag and Jack, 2010). It has been found that miRNA156 and its targeted *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes acted in parallel with *SPL8* (not targeted by miRNA156) to control sporogenic tissue formation during anther development in Arabidopsis (Xing *et al.*, 2010). The accurate, harmonic regulation of *SPL* genes by miRNA156 was required for proper formation of the septum during gynoecium development (Xing *et al.*, 2013). Moreover, the regulation of miRNA156-targeted *SPL9* was also found to be involved in modulating the biosynthesis of anthocyanin and potentially fine-tuning the secondary metabolism of floral organ development (Gou *et al.*, 2011).

With the rapid development of next-generation sequencing technologies and bioinformatics tools, more and more miRNA genes were identified in a much wider range of plant species, presenting an extensive platform for comparative analysis. Moreover, the recent progress in plant genome sequencing enabled a deep understanding of miRNA biogenesis, function, and evolution. For instance, a comprehensive comparison was conducted in the closely related species Arabidopsis and *Capsella*, both belonging to the Camelineae, which revealed surprising variations of characteristics in miRNA–target pairs (Smith *et al.*, 2015). Although substantial advances in understanding the evolutionary history of ancient miRNA genes in plants have been made, little is known regarding the mechanism of extensive expansion of lineage-specific miRNAs and how they could contribute to the complexity of plant development.

The *Camellia* genus contains many economically important woody species, of which *C. japonica* is a famous ornamental species. There are five major types of double flowers classified based on petal number, arrangement, and shape, and degree of petaloid stamens (Sealy, 1958; Sun *et al.*, 2014*b*). The generation of extra petals in different double flower types was the driving force in ornamental flowers, greatly promoting their aesthetic value. Molecular genetics in several ornamental plants underscored the significance of repression of C class functions in double flower formation (Dubois *et al.*, 2010; Galimba *et al.*, 2012). However, the formation of distinctive double flower types in *Camellia* appeared to involve certain novel alterations in C class gene expression (Sun *et al.*, 2014*a*). Therefore, understanding the molecular mechanism of floral organ development in *Camellia* may provide insights in genetic engineering of new varieties. However, genomics information on *Camellia* is still limited, and the lack of a genome-wide investigation of miRNA and its targets is a major problem for further understanding of the underlying mechanism.

Camellia azalea is a species closely related to *C. japonica* with a unique summer-flowering habit (Ye, 1985). It was widely used as a valuable resource for flowering time breeding in cultivated *C. japonica*. We previously performed an in-depth transcriptome analysis to capture the gene expression profiles during floral bud development (Fan *et al.*, 2015). Here, in order to identify the miRNAs and their potential targets in the regulation of floral organ development, we employed small RNA sequencing in five tissue types (young leaf, stamen, petal, carpel, and floral bud) with three biological replicates. In total, we generated >273 million high-quality reads from *C. azalea* and identified 175 miRNA genes through bioinformatics analysis. We focused on comparisons between floral organs and vegetative tissues, and identified subsets of miRNAs which were differentially expressed. Further, we characterized the global expression profiles of miRNAs by hierarchical clustering and examined functional properties of stamen- and carpel-specific miRNA-targeted genes. We revealed that 'DNA integration' and 'fruit development' were

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the most enriched Gene Ontology (GO) categories for stamen and carpel development, respectively, related to miRNA regulation. Through mapping of short reads to the miRNA database (miRBase 21), we found a group of 12 high confidence miRNAs that have not been described in other plant species. A similarity search for miRNA precursors against RNA sequencing (RNA-seq) data sets in related species supported that those were potentially novel and lineage-specific miRNAs in *Camellia*. Finally, quantitative PCR (qPCR) experiments showed that some key targets of miRNAs showed anti-correlated expression patterns with their cognate miRNA during flower development. This work provided a comprehensive analysis of miRNA genes in *Camellia* species, and could be informative for understanding the lineage-specific manner of expansion and evolution of miRNAs in eudicots.

Materials and methods

Plant materials and growth conditions

Camellia plants used in this study were grown in the greenhouse of the Research Institute of Subtropical Forestry in Fuyang (Hangzhou city, Zhejiang, China) under natural light conditions. The floral buds of *C. azalea* between 12 mm and 15 mm in length were collected and dissected under a microscope (Leica DFC295, Leica Microsystems, Germany). At this stage, floral organs were still maturing and could be distinguished by manual separation. Floral buds were collected from three independent plants and each sample was made up of >6 buds. After sample collection, the tissues were frozen immediately in liquid nitrogen and stored in −80 °C freezers before use. Three biological replicates were collected, and each biological replicate contained samples from at least three plants.

RNA extraction

Total RNA was extracted from floral buds by using the EASYspin Plant RNA Extration kit (RN09, Aidlab, Beijing, China) and treated with Column DNA Erasol to avoid DNA contamination. RNA quality and quantity were determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and Bioanalyzer RNA nano chip (Agilent Technologies, Singapore). Only the RNA samples with a 260/280 ratio between 1.8 and 2.0, a 260/230 ratio between 2.0 to 2.5, and a RIN (RNA integrity number) >8.0 were used for sequencing.

Small RNA sequencing and data processing

Approximately, 15 μg of total RNA for each tissue sample was used for the construction of libraries using a Small RNA Library Preparation kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. Each library was barcoded with different indices and stored in a −80 °C freezer before sequencing. Sequencing was performed in a v3 flowcell on an Illumina HiSeq 2500 sequencer, generating 1×36 bp reads. All sequencing data were deposited in the NCBI Short Read Archive under BioProject ID PRJNA257896 (accession no. SRP045386). We quantified transcript levels by TPM (transcripts per million) methods (Fahlgren *et al.*, 2007).

miRNA identification

The sequencing reads were processed to remove adaptors, and cleaned by Q30 value. Reads with >20% bases <Q30, and N base >10% were filtered. The remaining reads were further filtered if read length was >30 bp or <18 bp. The derived reads were mapped to various RNA database including Silva, GtRNAdb, Rfam, and Repbase by Bowtie (Langmead *et al.*, 2009) for annotation. The unannotated reads containing small regulatory RNAs were processed for miRNA identification by miRDeep2 (Friedlander *et al.*, 2012). To satisfy the parameters of miRNAs in plant species as described (Meyers *et al.*, 2008), the remaining reads were mapped onto a previous transcriptome assembly (Fan *et al.*, 2015) and guided the extraction of potential precursors at a size of 200 bp (Yang *et al.*, 2011). The secondary structure and mapping information was processed as described in the miRDP package (Yang and Li, 2011). The precursors were aligned to short reads, and statistical analyses of read distributions were generated for each miRNA gene for evaluation of miRNA. The miRDeep2 output figures were manually checked by the existence of miRNA* sequences, and the precision of editing of mature sequences was as described previously (Taylor *et al.*, 2014). To quantify the abundance of miRNA, the TPM value was defined as 'counts of reads mapped to miRNA×1 000 000'/'reads mapped to the reference genome' (Fahlgren *et al.*, 2007).

Phylogenetic analysis

To construct a phylogenetic tree of miR160 genes from *Camellia*, the precursor sequence of conservative_c38436.graph_c0_79977 was searched through BLAST to the PNDP database (Yi *et al.*, 2015). The high-similarity genes were retrived by the cut-off e-value 1e-10. The miR160 phylogenetic trees were made by MEGA5 using the Maximum Likelihood (ML) method according to the manual (Tamura *et al.*, 2011). To construct a phylogeny tree of the miRNA family of *Camellia*, the validated sequences of plant miRNAs were downloaded (http://palaeo.gly.bris.ac.uk/donoghue/) and searched with sequences from *Camellia*. The miRNA family tree with various plant species was adapated as reported (Taylor *et al.*, 2014).

Target prediction qPCR

The miRNA targets were annotated by standard settings of psRNATarget (Dai and Zhao, 2011) with maximum expectation value 3.0. The primers of candidate genes of miRNA targets were designed by PrimerExpress2.0 with default settings (Supplementary Table S1 at *JXB* online). RNA samples were transcribed and amplified using a PrimeScript reagent Kit (PR037Q, Takara, China) on a QuantStudio 7 Flex Real-Time machine (Applied Biosystems, USA).

Expression analysis and GO enrichment analysis

To identify differentially expressed miRNAs, DESeq2 software was used, and expression fold change >2 and a [Benjamini–Hochberg false discovery rate (FDR) corrected] *P*-value <0.01 were selected (Love *et al.*, 2014). The miRNA expression data were normalized by the row *z*-score method. Hierarchical clustering of gene expression was performed by the clustergram function in the Matlab Bioinformatics toolbox with minor changes. GO enrichment analysis was performed as previously described, and visualized using the ReviGO tool (Supek *et al.*, 2011).

Results

High-throughput small RNA sequencing of five organ types in *Camellia azalea*

In order to identify small regulatory RNAs and their distribution patterns in *C. azalea*, 15 small RNA libraries containing five different organs (young leaf, stamen, carpel, petal, and floral bud) with three biological replicates were generated and sequenced by next-generation sequencing (see the Materials and methods for detailed sample information). For each

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individual sample, the retrieved raw reads ranged between 18.9 million and 28.2 million (Table 1), which presented a deep resource for extensive discovery of small regulatory RNAs. In total, >348 million raw reads were obtained (Table 1) and processed for miRNA identification. In each sample, we focused on read lengths from 18 bp to 30 bp, and mapped them to Silva, GtRNAdb, Rfam, and Repbase databases (Table 2) to annotate the composition of the small RNA population. rRNAs were the most abundantly annotated RNAs, accounting for about half of total reads, and the unannotated RNAs were potentially novel regulatory small RNAs (Table 2). We further predicted miRNAs with their pre-miRNA sequences. Initially, the clean reads were mapped to the miRBase database to identify known miRNAs. The average percentage of total cleans reads mapped to miRBase was 9.37%, and 21 bp and 22 bp reads had a higher mapping rate than others (Table 3).

We next implemented miRDeep2 core algorithms with modifications for plant miRNAs to analyze the unknown reads for discovery of new miRNAs (Yang and Li, 2011; Friedlander *et al.*, 2012). In total we identified 175 potential miRNAs in *C. azalea* (Supplementary Table S2). The length distribution of mature miRNAs along with the abundance of reads was revealed (Fig. 1A) of which 21 bp and 22 bp RNAs were most abundant. We also determined the frequency of the first base of mature miRNAs, and showed that the 21 bp and 22 bp miRNAs preferentially started with 'U' (62.2% and 64.7%, respectively) (Fig. 1B), while 23 bp and 24 bp miRNAs preferred 'A' at the first base (85.7% and 66.7%, respectively) (Fig. 1B).

Structural and phylogenetic analysis of MIR160 family members in Camellia azalea

To access the characteristics of deeply conserved miRNAs in *C. azalea*, we aligned the mature miRNAs to the Plant ncRNA Database (PNRD) (Yi *et al.*, 2015) to isolate the highly conserved miRNAs. We found that 19 miRNAs were highly homologous to miRNAs in other plant species, but not all of them were among the deeply conserved miRNA families (Supplementary Table S2). For example, the miRNA 'unconservative_c73833.graph_c1_694101' was very closely related to 'vvi-MIR3633a' (only one mismatch, Supplementary Table S2), while this family was not expanded in other eudicot species. It has been reported that several miRNA families are deeply conserved among the plant kingdom, such as MIR160, MIR159, MIR172, etc., and most of those conserved miRNAs tend to be abundant (Cuperus *et al.*, 2011). In the 19 highly conserved miRNAs, the most abundant miRNA was 'conservative_c66064.graph_c4_364506' (1,942,415 counts) corresponding to 'ath-MIR472' in Arabidopsis targeting CC-NBS-LRR class genes (Gonzalez *et al.*, 2015). The members of MIR172 and MIR156 were also relatively abundant (Supplementary Table S3).

Table 2. *Summary of statistics of small RNA annotation*

Total clean reads were mapped to RNA public databases.

RNA types	All samples	Percentage
rRNA	112 839 497	49.702%
snRNA	41 805	0.018%
snoRNA	10 737	0.005%
tRNA	957 825	0.422%
Repbase	235 683	0.104%
Unannotated	112 946 288	49.749%
Total	227 031 835	

snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; Repbase, repetitive elements database; unannotated, reads without match.

Table 1. *Summary statistics of small RNA sequencing in different tissue samples*

Five tissue types with three biological replicates were sequenced.

Samples	Raw reads	Containing 'N' reads	<18 nt reads	>30 nt reads	Clean reads	Q30 (%)
Sta1	27 231 911	4422	5 706 748	2 262 944	19 257 797	91.23
Sta2	25 087 127	4108	4 047 332	2 698 047	18 337 640	91.33
Sta3	27 730 283	4530	6 738 174	2 185 731	18 801 848	91.39
YL1	24 932 396	2327	3 893 974	3 352 934	17 683 161	91.28
YL2	24 595 622	2233	4 136 824	3 698 979	16 757 586	91.57
YL3	28 169 163	2526	7 620 002	2 169 134	18 377 501	91.43
Car1	19 526 996	923	4 674 920	1 463 632	13 387 521	93.65
Car2	20 912 484	915	7 941 796	1 109 149	11 860 624	93.67
Car3	22 586 242	3060	8 595 396	2 038 668	11 949 118	91.97
FB1	22 570 580	1019	4 113 887	2 393 223	16 062 451	93.1
FB2	18 857 803	2901	5 539 079	2 280 349	11 035 474	90.64
FB3	23 181 785	3403	4 663 062	4 201 519	14 313 801	92.04
Pet1	20 986 358	879	3 685 447	2 487 776	14 812 256	92.99
Pet2	21 675 882	952	9 262 100	1 294 813	11 118 017	94.02
Pet3	20 094 282	937	4 838 116	1 978 189	13 277 040	93.19
Total	348 138 914	35135	85 456 857	35 615 087	227 031 835	92

Sta, stamen; YL, young leaf; Car, carpel; FB, floral bud; Pet, petal.

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To gain further volutionary insights into conserved miRNAs in *Camellia*, we investigated the MIR160 family in detail (Fig. 2). The mature miRNA sequence (conservative_c38436.graph_c0_79977) was identical to MIR160 members in maize (zma-MIR160g), and there were 6465 read counts that matched the pre-miRNA precursor (Fig. 2B). The analysis of the secondary structure of the precursor showed that it had a canonical stem–loop structure of mature miRNA processing (Fig. 2A). The distribution pattern also revealed that the mature miRNA region had the most abundant reads coverage, and the corresponding complementary region (* region) also had enriched reads matching (Fig. 2B) its precursor. As the sequence similarities of conserved miRNAs also lay in their precursors, we retrieved pre-miRNA sequences of the plant MIR160 family and constructed a phylogenetic tree (Fig. 2C). It showed that conservative_c38436.graph_c0_79977 formed a subclade with members from *Vitis*, *Populus*, *Manihot*, and *Lotus*, which correlated well with its phylogenetic placement (Fig. 2C).

Table 3. *Statistics of clean short reads mapped to miRBase*

The cleaned reads ranging from 18 bp to 30 bp were mapped to the miRBase database. The number and percentage of mapped reads of different lengths are shown.

Reads length (bp)	Clean reads	Mapped reads	Percentage
18	17 189 587	1 296 764	7.54%
19	16 672 261	1 459 746	8.76%
20	17 572 797	1 520 413	8.65%
21	30 736 304	4 897 255	15.93%
22	24 377 606	4 250 732	17.44%
23	17 510 820	1 280 248	7.31%
24	50 694 233	3 030 527	5.98%
25	14 063 149	749 276	5.33%
26	13 074 839	855 849	6.55%
27	8 138 012	607 917	7.47%
28	6 882 139	465 344	6.76%
29	5 688 455	448144	7.88%
30	4 431 633	417 502	9.42%
Total	227 031 835	21 279 717	9.37%

Differentially expressed miRNAs among five tissue types in Camellia azalea

Plant miRNAs play pivotal roles in directing plant growth, development, and responses to environmental conditions by post-transcriptionally regulating their target genes. Compares with animal miRNAs, the complementarity between mature miRNAs and target transcripts was usually high and stringent (Parizotto *et al.*, 2004). Hence, spatiotemporal expression patterns of miRNAs are of great importance to the functions of miRNA genes, as well as to their targets. We showed that the global expression of miRNAs in replicates had high correlations between each other, suggesting that the data sets were ideal for statistical analysis for identifying significant variations (Supplementary Fig. S1). To identify differentially expressed miRNAs between tissues, we performed quantification of miRNAs in each sample and obtained significant differential miRNAs between organs (*P*-value <0.05; Benjamini–Hochberg FDR corrected, Supplementary Table S4). We focused on distinctions between some vegetative and reproductive organs and identified differentially expressed miRNAs in the following comparisons: young leaf–stamen, young leaf–petal, stamen–carpel, stamen–petal, and carpel–petal (Fig. 3). A Venn diagram of differentially expressed miRNAs in those comparisons showed the common and specific miRNAs (Fig. 3). Some known miRNAs with regulatory roles in floral organ development were identified, such as miRNA167, miRNA156, and miRNA159 (Supplementary Table S4). It is worth noting that the number of differential miRNAs did not change much but only a few specific differential miRNAs in a certain combination were found (Fig. 3).

Hierarchical analysis of miRNAs in different organs to identify tissue-specific miRNAs

To investigate further the expression patterns of miRNAs in different organs in *C. azalea*, we performed hierarchical

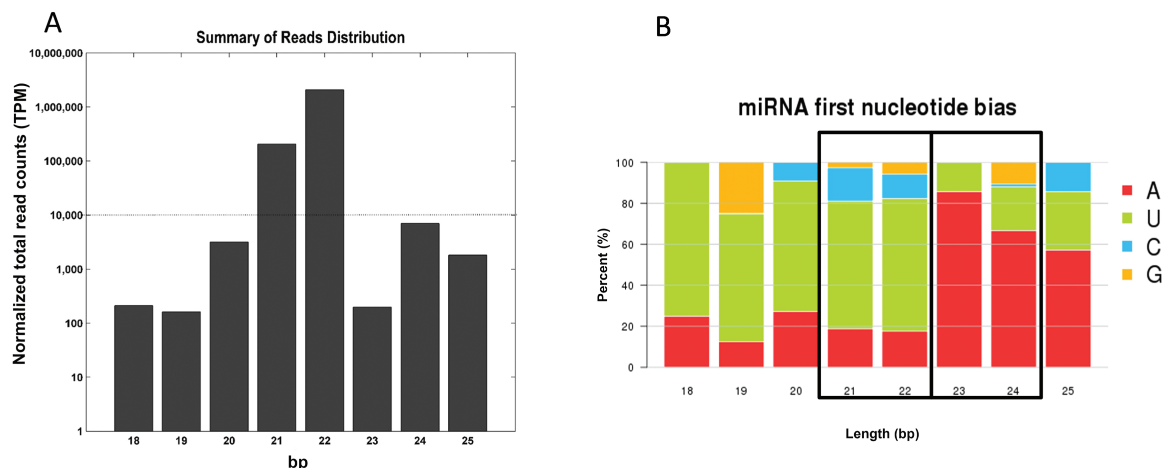

Fig. 1. Characterization of identified miRNAs of *Camellia azalea.* (A) The distribution of reads along with mature miRNA length. (B) The presence of the first nucleotide of miRNAs along with the mature miRNA length.

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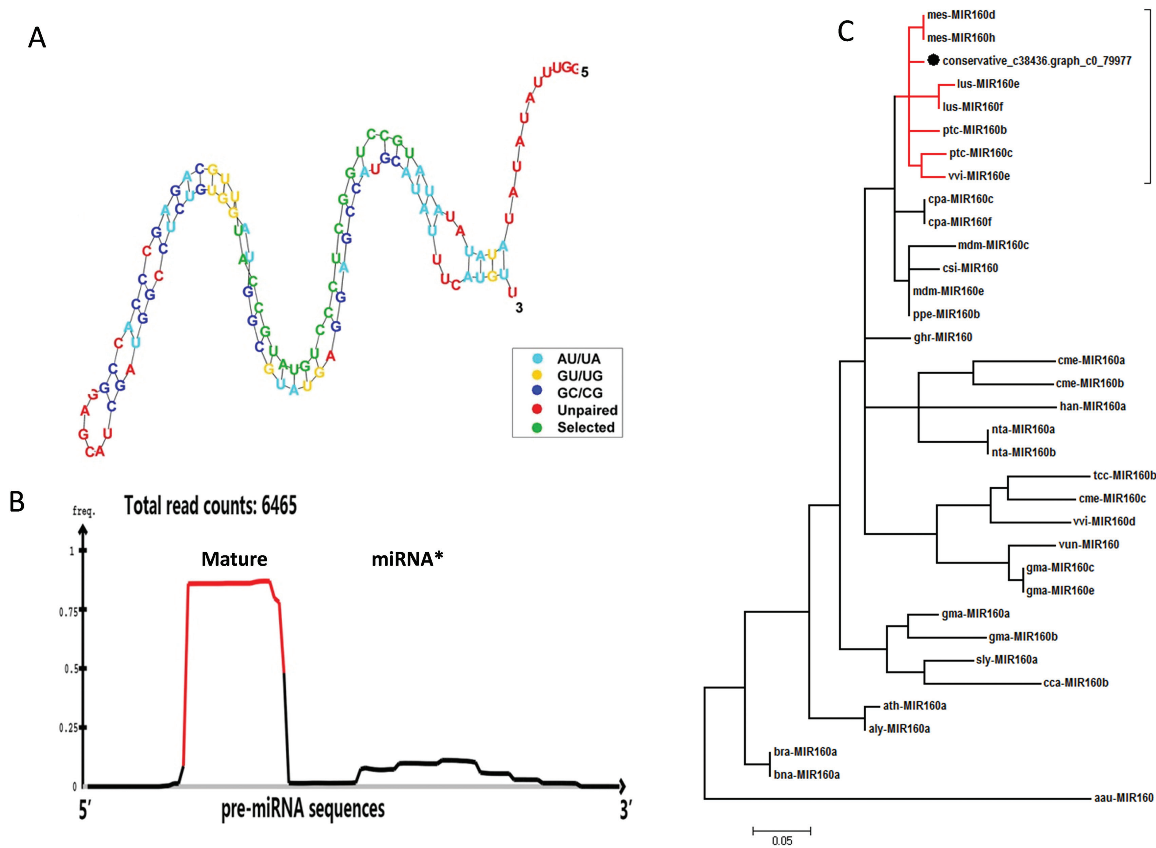

Fig. 2. Characterization of MIR160 family member (conservative_c38436.graph_c0_79977) of *Camellia azalea*. (A) The secondary structure of the precursor of miRNA160 displaying the canonical stem–loop feature of miRNAs. Green color indicates the mature miRNA sequences. (B) Analysis of sequencing reads to miRNA precursors. The *y*-axis denotes the percentage of reads to locations; the red line indicates the mature miRNA, and the complementary area is denoted as miRNA*. (C) A phylogenetic tree was constructed by precursors of miRNA160 from diverse plant species. The red lines indicate the subclade containing miRNA160 members of *C. azalea*. miRNAs from other species were retrieved from the PNRD.

clustering analysis of expression of all identified miRNAs (Fig. 4). We showed that most miRNAs were preferentially expressed in cetain tissues. Despite the radical changes of expression abundance between different miRNAs, the expression levels among tissue types varied significantly (Fig. 4A), suggesting diverse functions of miRNA–target regulation in the control of organ development. The clustering of miRNA expression resulted in several major subclusters with distinct expression patterns (Fig. 4A). Among these, we concentrated on two subclusters, which were carpel and stamen specific (Fig. 4B, C). The development of the stamen and carpel was recognized as one of the most important stages of organogenesis regarding sexual reproduction processes in higher plants (Pinyopich *et al.*, 2003). To capture the functional properties of carpel- and stamen-specific miRNAs, we first predicted the targets of all miRNAs through bioinformatics analysis, and 2379 transcripts were revealed to be targeted by miRNAs (Supplementary Table S5). We also performed GO enrichment analysis to study the specific miRNA–target regulation in stamen and carpel (Fig. 5) by functional annotation of target genes. We identified 109 and 57 GO terms which were significantly enriched (P-value <0.05, FDR <0.25) in stamen- and carpel-specific genes, respectively (Supplementary Table S6). GO terms were also grouped and visualized (Supek *et al.*, 2011) to reveal the representative biological processes that were potentially involved in miRNA functions (Fig. 5).

Some meaningful biological processes were revealed in stamen-specific miRNA–target analysis including DNA integration and the oxidation–reduction process (GO:0055114) (Fig. 5A) in which DNA integration was most significant. In stamen, the development of male gamete cells was an essential course throughout sequential steps of cell proliferation, including germ cell initiation, meiosis, and tapetum formation. This result suggested that miRNA–target regulation might be involved in the process of DNA replication and cell division. The enriched biological processes in carpel were distinct from those of stamen, but the ‘oxidation–reduction’ process was also significantly enriched (Fig. 5B). Interestingly, another group of GO terms was revealed as ‘fruit development’ which was highly enriched for carpel (Fig. 5B). The development of the carpel is a complex process not only giving rise to distinctive tissues such as integument, ovules, and ovary walls, but also establishing floral meristem determinacy. We further traced the annotation of fruit development by investigating the functional properties of carpel-specific miRNA–target pairs, and found that the pair miRNA160–ARF (conservative_c38436.graph_c0_79977–c69414.graph_c0) was potentially associated

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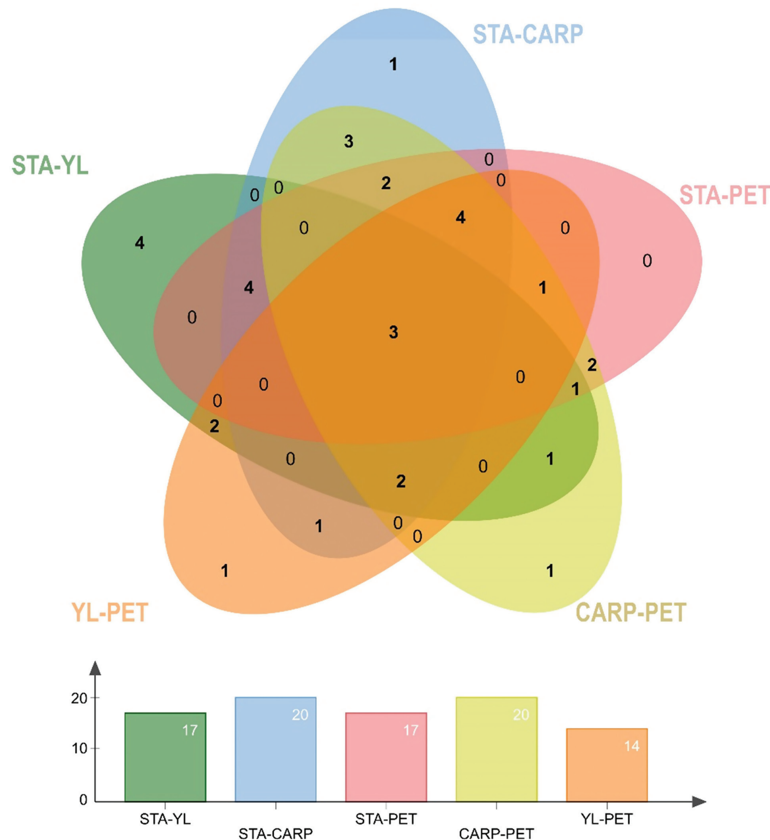

Fig. 3. A Venn digram of differentially expressed miRNAs between organs. Sta, stamen; YL, young leaf; Car, carpel; FB, floral bud; Pet, petal. Five combinations of differential expressed miRNAs were analyzed. The total number of differential miRNAs is listed at the bottom. (This figure is available in colour at *JXB* online.)

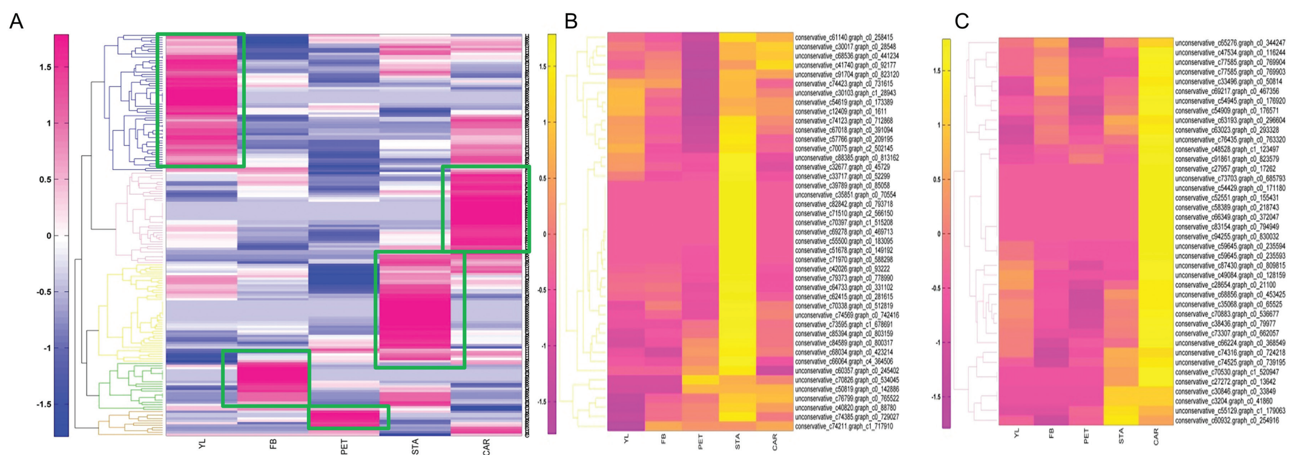

Fig. 4. Heat map of hierarchical clustering analysis of miRNA expression in *Camellia azalea*. (A) A global view of all miRNAs expression levels by hierarchical clustering. Mean values of three biological replicates were used and normalized by row *z*-score for clustering. Five major clusters with distinct expression patterns are denoted by different colors and green rectangles. (B) Close-up view of the stamen-specific miRNA cluster. (C) Close-up view of the carpel-specific miRNA cluster.

with carpel development (Supplementary Table S5). The miRNA160 gene had its highest expression levels in carpels and targeted an ARF (Fig. 4B; Supplementary Table S3). In Arabidopsis, it has been shown that three ARFs were targeted by miRNA160 members during Arabidopsis reproductive organ development (Luo *et al.*, 2013), suggesting conserved miRNA–target regulation and shared regulatory functions in *Camellia*.

Expression and correlation analyses of target genes

To evaluate the potential regulation of miRNA targets, we performed real-time qPCR experiments to check the expression patterns of target genes. We found the ARF10-like gene (c69414.graph_c0) displaying an anticorrelated pattern (cor −0.35) with its cognate miRNA160 (conservative_c38436.graph_c0_79977) (Fig. 6). Another miRNA160 member

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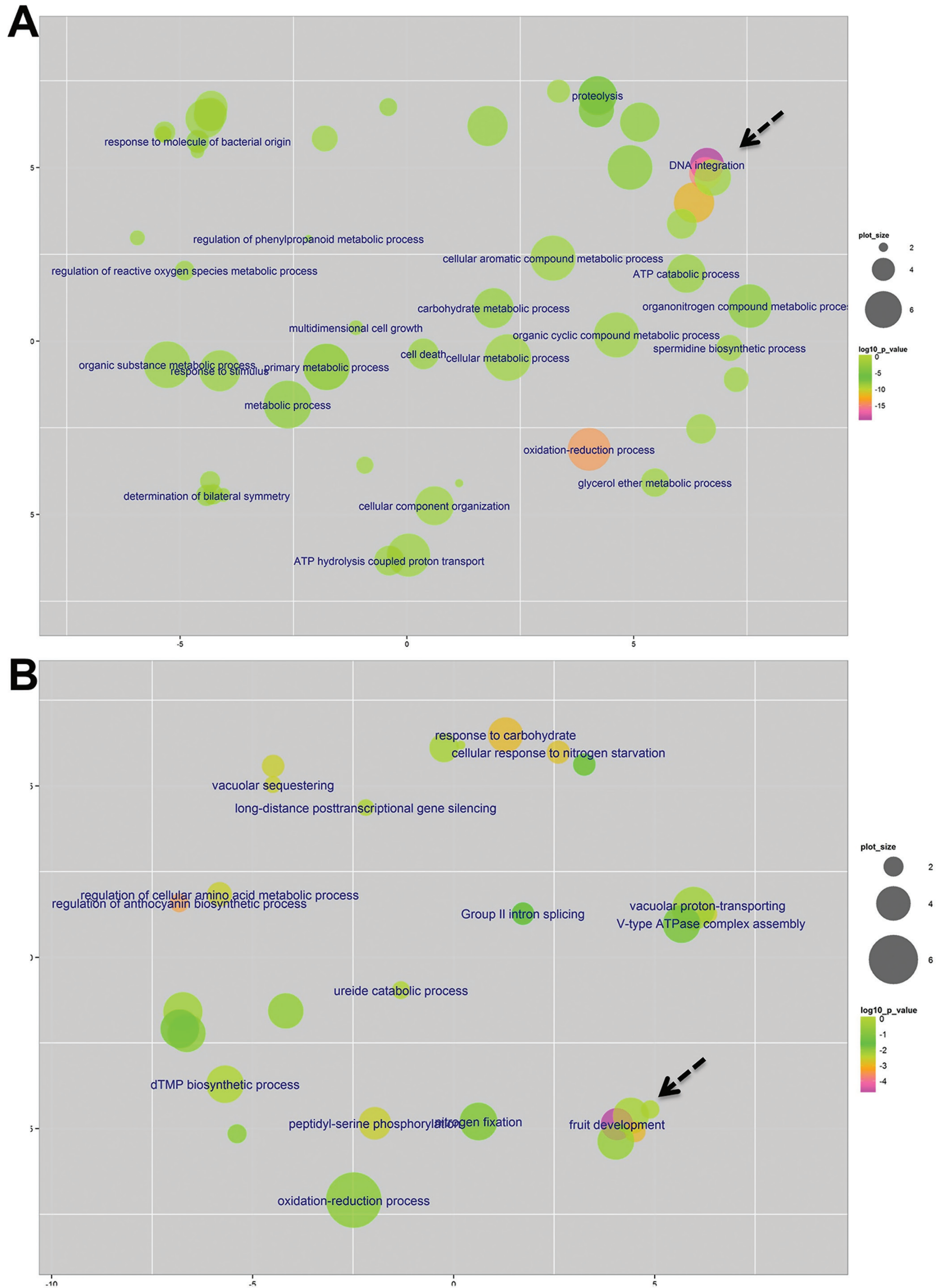

Fig. 5. GO enrichment analysis of stamen- and carpel-specific miRNA targets. (A) The enriched GO terms of stamen-specific miRNA-targeted genes. The arrow indicates the most significant GO groups including 'DNA integration'. (B) The enriched GO terms of carpel-specific miRNA-targeted genes. The arrow indicates the most significant GO groups including 'fruit development'.

(conservative_c67358.graph_c0_401366) did not show a negative correlation with the potential target gene (c68145.graph_c0). We also checked two novel miRNAs (unconservative_c68536.graph_c0_441234 and unconservative_c62043.graph_c0_274234) and their targets (c66994.graph_c0 and c73273.graph_c0). We found that the latter pair, but not the first pair, had a negative correlation (Fig. 6). The miRNA167 potentially targeted two SPL genes in *C. azalea*; we tested

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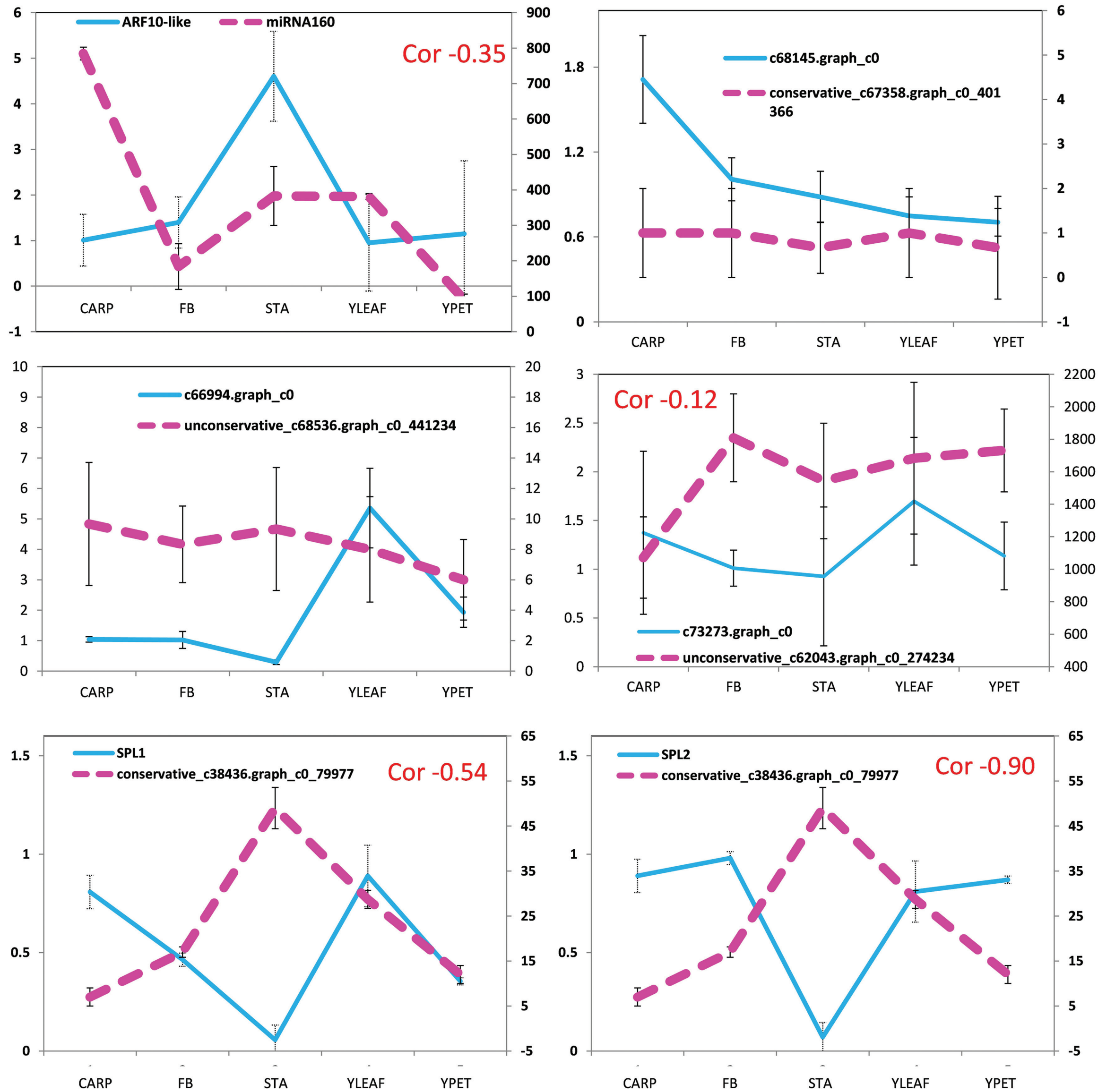

Fig. 6. Expression profiles of miRNA and targets. Each panel contains the mean expression profiles of miRNA by RNA sequencing (dashed line). The predicted target of miRNA was analyzed by qPCR analysis, shown by the solid line. The negative correlation between miRNA and targets is shown in red.

the expression and showed that both displayed strong negative correlations with miRNA167 (Fig. 6). The negative correlations between miRNA and targets provided evidence of target prediction programs, and the levels of the miRNA and target pair might also be important to define regulatory functions. For example, miRNA167 was highly expressed in stamens, while its potential targets (*SPL1* and *SPL2*) were almost not detectable (Fig. 6). This suggested that the post-transcriptional silencing of *SPL* genes was critical for stamen specification.

Lineage-specific miRNAs in Camellia

The expansion of some miRNA family members in eudicots was uncovered in a lineage-specific manner (Nozawa *et al.*, 2012; Taylor *et al.*, 2014). To access the known miRNA families in *Camellia*, a phylogeny tree of miRNA families of various plant species was used for evaluation. Through sequence analysis, we found most of the conserved miRNA families (except miR2950 in spermatophyta) in angiospermae (Fig. 7). Three miRNA families (miR3627, miR4376, and miR4414) at node eudicotyledons were missed, probably due to incorrect reference sequences (Fig. 7). We also found two *Vitis*-specific families (miR3633 and miR3629) in *Camellia*, and miR3633 was highly abundant with high identification confidence, suggesting that it may occur before the separation of *Vitis* and *Camellia* (Supplementary Table S2; Fig. 7). The miRNAs in *Camellia* were aligned to the plant miRNA database, and some of them were not conserved, showing <16 bp matched bases to other miRNAs. They were considered as lineage-specific miRNAs. Given the fact that bioinformatics prediction might yield false discoveries of miRNAs, we re-evaluated the miRNAs by stringent criteria (Taylor *et al.*, 2014) (Supplementary Fig. S2) and categorized 39 high confident miRNAs (Supplementary Table S2). Among these, 12 miRNAs were identified as *Camellia* lineage-specific

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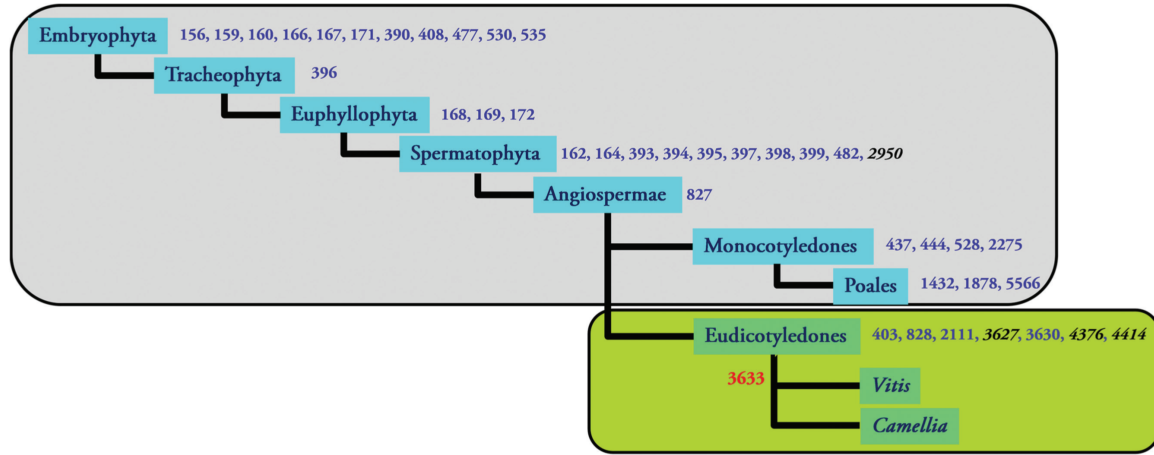

Fig. 7. A phylogeny tree of various plant species and miRNA families containing *Camellia*. The validated miRNA sequences were downloaded (http://palaeo.gly.bris.ac.uk/donoghue/) and searched with *Camellia* miRNAs and reference sequences. Black and italic numbers indicate missing miRNA families. MiR3633 is highlighted in red, suggesting that it may have occurred before the separation of *Vitis* and *Camellia*.

Table 4. *Summary of lineage-specific miRNAs in Camellia.*

The precursors of miRNAs were searched in public RNA-seq databases from the genera *Camellia* and *Vitis*. 'Complete' indicates the full-length sequence was covered with high similarity. '*C. azalea* only' indicates it was only found in this study not in other *Camellia* species. 'Partial' indicates part of the precursor sequences were covered in other *Camellia* species.

Read count	Length (bp)	Precursor (bp)	Original ID	*Camellia*	*Vitis*
57	19	90	unconservative_c59645.graph_c0_235594	Complete	Not found
258	20	101	conservative_c46619.graph_c0_112462	Partial	Not found
399	20	91	conservative_c64733.graph_c0_331102	Partial	Not found
383	21	108	conservative_c33717.graph_c0_52299	Complete	Not found
18	21	111	conservative_c62980.graph_c0_292527	Complete	Not found
169	21	111	conservative_c69217.graph_c0_467356	Complete	Not found
1138	21	91	unconservative_c54945.graph_c0_176920	*C. azalea* only	Not found
89	21	111	unconservative_c68751.graph_c1_448843	*C. azalea* only	Not found
991	21	92	unconservative_c91704.graph_c0_823120	*C. azalea* only	Not found
38	22	112	conservative_c48528.graph_c1_123497	Complete	Not found
15	22	112	conservative_c73595.graph_c1_678691	*C. azalea* only	Not found
24167	22	112	unconservative_c62043.graph_c0_274234	Complete	Not found

miRNAs (Supplementary Table S2). To validate further the lineage-specific miRNAs, we aligned the precursor sequences to the public transcriptomics database from *Camellia* and *Vitis* (NCBI SRA 08/2015), and we found that no miRNA gene was supported by *Vitis* data sets, but most of them were discovered in other *Camellia* species (Table 4). This result supported the notion that the lineage-specific miRNAs could evolve within the genus *Camellia*.

Discussion

Camellia species are of great importance to horticulture and their miRNAs could serve as targets of genetic engineering-based breeding. In this work, we used next-generation sequencing technology to identify conserved and novel lineage-specific miRNAs that may possess critical roles in floral development. The potential application of miRNA-based genetic engineering could facilitate the molecular breeding of new ornamental varieties with desired floral forms in camellias. Without a high-quality reference genome in the genus *Camellia*, it is challenging to distinguish authentic miRNAs and other small RNAs despite very abundant overall sequencing depth. It is recognized that the 'young' or newly evolved miRNA genes were often less abundant (Fahlgren *et al.*, 2007, 2010). From our data sets combining 15 independent sequencing libraries, many potential miRNAs were still expressed at a low level (Supplementary Table S3). Therefore, further deep sequencing or enrichment approaches might be required to ensure the discrimination of authentic miRNA genes in *Camellia* species before the complete genome becomes available. The miRDeep algorithm was developed for animal miRNA discovery, and modifications for plant species were developed, allowing the discovery of true miRNA genes even under poor sequencing depth as tested in Arabidopsis (Yang *et al.*, 2011; Breakfield *et al.*, 2012; Li *et al.*, 2012; Jain *et al.*, 2014). However, it remained challenging to distinguish miRNAs and other types of interfering RNAs by using the small RNA sequencing data sets (Coruh *et al.*, 2014). It appeared critical to employ several key

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characteristic features for accurate identification of canonical miRNA genes, such as a high degree of complementary area, and the presence and precision editing of miRNA* sequences (Taylor *et al.*, 2014). Due to the high rate of misannotation of miRNA families present in miRBase, a phylogeny tree of 'close' and 'far' lineages provided systematic outlines of miRNA evolution (Taylor *et al.*, 2014), which may reconcile the unreliable alignments of similarity searches. Indeed, in this study, searching the miRNA family in *Camellia* fitted well with its phylogenetic placement (Fig. 7). We also identified a group of lineage-specific miRNAs (Table 4), which were potentially newly evolved in Ericales. Some of these miRNAs were not covered in depth (<100 counts); hence, further analyses such as genomic loci identification or cloning of mature and precursor sequences might be necessary.

miRNA–target regulation plays key roles in plant development, and some conserved pairs of both miRNA and targets were found to be involved in diverse processes of plant morphogenesis (Rubio-Somoza and Weigel, 2011). Several key miRNA families and targets were identified in the regulation of floral development, such as miRNA172–AP2, miRNA164–CUP-SHAPED COTYLEDON (CUC), and miRNA156–SPL which displayed conserved functions in various plant species (Nag and Jack, 2010; Luo *et al.*, 2013). Stamen and carpel are two distinct reproductive organs in which development of male and female gametes occurs. Several conserved miRNAs and their targets were revealed to participate in differentiation of cell types in male and female floral organs. For example, in Arabidopsis, miRNA167 targeted ARF genes, miRNA159 targeted MYB genes, and miRNA319 targeted *TCP*- (*TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL NUCLEAR ANTIGEN FACTOR*) like transcription factors, all of which were involved in anther development and maturation (Goetz *et al.*, 2006; Cecchetti *et al.*, 2008; Rubio-Somoza and Weigel, 2013). miRNA156-targeted *SPL* genes were found to regulate gynoecium differentiation (Xing *et al.*, 2013). More and more miRNAs were discovered by deep sequencing in non-model species that potentially regulated species-specific aspects of plant growth by targeting novel genes (Chae *et al.*, 2015). In this work, the analysis of differentially expressed miRNAs among different organ types yielded valuable information concerning tissue-specific miRNAs in *Camellia* and was of value for finding important regulatory miRNA–target pairs for floral organ development (Fig. 3). For instance, a miRNA159 member (conservative_c60714.graph_c0_251578) was abundantly detected in carpel tissues, at >10-fold higher levels, when compared with stamen, suggesting its role in the differentiation of gynoecium tissues despite the fact that miRNA159 was highly expressed in anthers to regulate cell differentiation in Arabidopsis (Millar and Gubler, 2005). Many other important regulatory pairs, such as miRNA319–TCP, miRNA156–SPL, and miRNA167–ARF, were discovered by the differential profiling analysis (Supplementary Table S5), suggesting conserved functions in *Camellia* species. It might also be interesting to investigate some new roles of miRNAs due to their interesting expression patterns. For example, miRNA319 (conservative_c34554.graph_c0_60739), miRNA160 (conservative_c38436.graph_c0_79977), and miRNA167 (conservative_c42026.graph_c0_93222) were found to be highly expressed in both young leaf and carpel, but expressed at low levels in other tissues (Supplementary Table S3). It was recognized that the carpel was a highly derived organ, and molecular mechanisms controlling its development shared many common characters with leaf development (Mathews and Kramer, 2012); therefore, those miRNA mediated-pathways could participate in common regulatory pathways underlying the basic organization of the leaf and carpel, such as polarity determination and cell type differentiation. It is recognized that miRNA319 belongs to the larger miRNA159 family, while another three copies of miRNA159 (conservative_c28654.graph_c0_21100, conservative_c61500.graph_c0_264442, and conservative_c31373.graph_c0_37688) did not display strong tissue-specific patterns (Supplementary Table S3). These findings imply that the functional diversification of the miRNA319 subfamily could be related to its evolutionary relationship. The GO enrichment analysis in stamen and carpel found many biological processes that were potentially regulated through miRNA actions (Fig. 5). One common GO process was identified as 'oxidation–reduction processes'. One explanation was that the development of the stamen and carpel might require suppression of the photosynthesis pathway to reach a hypoxic state (Chen *et al.*, 2015).

We identified a group of lineage-specific miRNAs in *Camellia* species which were supported by RNA-seq data sets from other species of *Camellia* but not *Vitis* (Table 3). These miRNA genes were potentially 'young' or 'newly evolved' and absent in the plant miRNA database. The miRDeep2 analysis of these miRNA genes revealed that the precision of miRNA and miRNA* sequencing was low; –the 2 nt overhang editing was not significant. It indicated that the biosynthesis of lineage-specific miRNAs might have some non-canonical features when they are compared with highly conserved miRNAs. Whether the lineage-specific miRNAs play special roles in development and growth and how they evolved are key questions to be addressed.

Understanding floral development in ornamental *Camellia* species could provide useful information to facilitate the genetic breeding process. The miRNA-mediated regulation was found to be critical in floral organ identity determination, elaboration of organ shape, and cell type differentiation (Luo *et al.*, 2013). Further, they are also an ideal biotechnology target of artificial manipulation of gene function (Zhang and Wang, 2015). Previous studies showed that the formation of different types of double flowers in *Camellia* was involved in alterations of expression patterns of key floral homeotic genes (Sun *et al.*, 2014*a*), but the underlying mechanism remained elusive. It is possible that miRNA-regulated gene expression may contribute to the double flower development. The present study has provided a genome-wide collection of miRNAs in *Camellia* with expression profiles in multiple organs, which can help us to uncover the roles of miRNA genes in domestication of ornamental camellias.

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Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Clustering of sample correlations

Figure S2. The summary of the distribution of miRNAs as described in Supplementary Table S2.

Table S1. Primers used in this study.

Table S2. Summary of miRNAs in *Camellia azalea*.

Table S3. The expression levels of miRNA in *Camellia azalea*.

Table S4. Differentially expressed miRNAs between organ types.

Table S5. The prediction of targeted genes of miRNAs.

Table S6. The enriched GO terms of stamen- and carpel-specific miRNA targets.

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