

# Identification and characterization of microRNAs at different flowering developmental stages in moso bamboo (*Phyllostachys edulis*) by high-throughput sequencing

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**Abstract** Researching moso bamboo flowering has been difficult because of its unknown flowering interval and the rarity of florescent samples. To identify microRNAs (miRNAs) and study their expression patterns during the flower developmental process of moso bamboo, small RNAs from non-flowering leaves and four flower developmental periods were sequenced using Illumina technology. In total, 409 known miRNAs and 492 differentially expressed novel miRNAs were identified in moso bamboo. Of the known miRNAs that were differentially expressed between non-flowering and flowering samples, 64 were predicted to have a total of 308 targets. Among the miRNAs, seven known and five novel miRNAs were selected, as were four of their target genes, and their expression profiles were validated using qRT-PCR. The results indicated

that the miRNA expression levels were negatively correlated with those of their targets. The research comprehensively revealed that the differentially expressed miRNAs and their targets participated in diverse biological pathways and played significant regulatory roles in moso bamboo flowering. The data provide a significant resource for understanding the molecular mechanisms in moso bamboo flowering and senescence, and serve as the primary foundation for further studies on metabolic regulatory networks that involve miRNAs.

**Keywords** Moso bamboo · Flower development · Inflorescence morphogenesis · microRNA · Illumina sequencing

## Abbreviations

ARF	Auxin response factor
GAMYB	Gibberellin MYB gene
GO	Gene Ontology
GH3	Gretchen Hagen3
HD-ZIPIII	Homeodomain-leucine zipperIII
MADS-box	The initials of MCMI, AGAMOUS, DEFICIENS and SRF 4
MFE	Minimum free energy
mRNA	Messenger RNA
miRNAs	microRNAs
NAC	NAM, ATAF1/2 and CUC2 domain-containing transcription factors
qRT-PCR	Quantitative real-time RT-PCR
siRNA	Small interfering RNA
SPL	Squamosa promoter binding protein-like
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
sRNA	Small RNA
TIP41	Tonoplast intrinsic protein 41

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## Introduction

Moso bamboo (*Phyllostachys edulis*) is a large woody species that is the most valued, ecologically, economically, and culturally, bamboo in Asia. It occupies an area of ~3,000,000 hm<sup>2</sup>, accounting for 20 % of total forest area worldwide (Li et al. 2012). Its high economic value results from its culm and shoots. The most interesting characteristic of moso bamboo is its peculiar flowering habit, which often leads to severe economic and ecological problems. The flowering of moso bamboo is scarce, random and uncertain. These special characteristics are the major obstacles to studying and using moso bamboo. Moso bamboo is monocarpic and flowering rarely occurs under normal conditions. However, they do flower at the end of very long vegetative growth phases, although the flowered clumps in a large area then usually die (Lin and Mao 2007). It was proposed that in monocarpic plants, such as moso bamboo, the sink strength of the flowers and fruits led to death by exhausting the plant (Herold 1980). Therefore, inhibiting flowering and fruiting could postpone death and senescence by preventing some of the metabolic declines normally associated with monocarpic senescence. Moso bamboo flowering has been recorded and studied extensively for a long time, and many researchers thought that changing the environmental signals, nutrient substances, mineral elements and endogenous hormones could cause it to flower (Zhan and Li 2007; Zheng et al. 2003). However, little is known about the mechanism of bamboo senescence and cell death after flowering at the molecular regulatory level because of the uncertainty of moso bamboo flowering and the difficulty in collecting fluorescent samples. Research on the molecular mechanisms of moso bamboo flowering is of great importance because of the huge economic loss and ecological crisis caused by its flowering. In recent years, the regulatory role of miRNAs in plant development has attracted attention. In this research, we have collected full-scale moso bamboo flowering samples after several years and conducted comprehensive miRNAs analyses to provide a foundation for evaluating the crucial regulatory roles of miRNAs in moso bamboo flowering.

miRNAs are small regulatory RNAs that play important roles in plant development (Chitwood et al. 2009; Nogueira et al. 2007; Rubio-Somoza et al. 2009), signal transduction and protein degradation (Guo et al. 2005; Zhang et al. 2006), as well as biotic and abiotic stress responses (Ruiz-Ferrer and Voinnet 2009; Shukla et al. 2008). Most predicted targets of miRNAs in plants known are putative transcription factors with functions in development, indicating a role for miRNAs in the center of gene regulatory networks (Jones-Rhoades et al. 2006). A large number of miRNAs have been identified in different

plant tissues at various developmental stages (Lu et al. 2008; Sunkar et al. 2005; Zhu et al. 2008). High-throughput sequencing technologies provide useful tools for identifying and quantifying miRNAs and allowed the exploration of small RNA (sRNA) populations in some model and economically important species, such as *Oryza sativa* (Nobuta et al. 2007; Zhou et al. 2010), *Glycine max* (Song et al. 2011) and *Arabidopsis thaliana* (Fahlgren et al. 2007; Rajagopalan et al. 2006). Pelaez et al. (2012) constructed sRNA libraries using roots, flowers, leaves and seedlings of *Phaseolus vulgaris* and studied organ-specific miRNA family expression levels in different organs through sequencing analyses. Wang et al. (2009) defined an endogenous flowering pathway in *A. thaliana* which contained miR156-regulated Squamosa promoter binding protein-like (SPL) transcription factors. Yu et al. (2012) found that the transcriptional repressor DELLA directly bound to the SPL transcription factor that was the target gene of miR156. The interaction between DELLA and SPL inhibited SPL's transcriptional activity. The expression levels of miR172 and MADS box genes were inhibited to delay the plant blossoming process. Thus, miRNAs play important regulatory roles in plant developmental and flowering processes. However, until now, there has been no systematic investigation of the roles of miRNAs in moso bamboo flowering.

In the present research, we used a paraffin sectioning technique to investigate the morphogenesis of moso bamboo inflorescences for the first time. Illumina sequencing technology was used to identify known and novel miRNAs based on libraries generated from non-flowering samples and from diverse developmental stages of flowering samples. The differential expression of known and novel miRNAs between non-flowering and flowering samples was analyzed in detail. The targets of differentially expressed miRNAs were predicted and subjected to a GO functional enrichment analysis, to further reveal potential regulatory roles in the moso bamboo flowering process. The research extends our molecular knowledge of moso bamboo populations and provides a comprehensive miRNA profile. The data provide a foundation for evaluating the crucial regulatory roles of miRNAs in moso bamboo flowering.

## Materials and methods

### Sample preparation and anatomical observations of plant tissues

Flowering moso bamboo samples at different stages and non-flowering moso bamboo leaves (ck) were collected in Guilin (E 110°17'–110°47'; N 25°04'–25°48') in the Guangxi Zhuang Autonomous Region from April to

August, 2013. The samples were stored in stationary liquid formaldehyde:glacial acetic acid:70 % alcohol (1:1:18). The morphogenesis of inflorescences in moso bamboo was observed using a paraffin sectioning technique. The four developmental stages were based on the anatomical structures of the floral organs: F-1, floral bud formation, during which a plant transits from the vegetative to reproductive stage; F-2, inflorescence axis continues to stretch, and lateral buds start to differentiate; F-3, bloom stage, flowers with both pistils and stamens emerge from glumes; and F-4, embryo formation. In addition, a large number of flowering moso bamboo samples at different stages and non-flowering moso bamboo samples were frozen in liquid nitrogen immediately and then stored at  $-80^{\circ}\text{C}$  for further research.

### RNA extraction, small RNA library construction, sequencing and data analyses

Total RNA from each group of frozen samples (ck, F-1, F-2, F-3 and F-4) was separately isolated using Trizol reagent (Invitrogen, USA), according to the manufacturer's instructions. Five sRNA libraries constructed from moso bamboo were sequenced using Illumina high-seq 2000 by the Beijing Genomics Institute (BGI) (Shenzhen, Guangdong Province, China). Briefly, 18–30 nt sRNAs were isolated from the total RNA using 15 % TBE–urea denaturing polyacrylamide gels. Then, 5' and 3' RNA adaptors were ligated to these sRNAs and reverse transcribed into cDNAs. These cDNAs were amplified by PCR and subjected to Illumina sequencing. Following the removal of low-quality reads, modified sequences from 18 to 30 nt were used for further analyses. At the beginning, rRNA, tRNA, snRNA and snoRNA were discarded from the sRNA sequences. The remaining sequences were used as query with the BLAST algorithm against NCBI GenBank and Rfam databases (Burge et al. 2013). Then, the unique sRNA sequences were used as query with the BLASTN algorithm to search the miRNA database. The standard analysis annotated the clean tags into different categories and labeled those that could not be annotated as predicted novel miRNAs. Then, target predictions for miRNAs and a GO enrichment analysis of target genes were performed.

### Identification of known miRNAs in moso bamboo

Unique sRNAs were aligned to the miRNA precursors of corresponding species in miRBase to obtain a miRNA count. The detailed criteria are as follows: (1) align the tags to the miRNA precursors in miRBase with no mismatches; and (2) based on the first criterion, align the tags to the mature miRNAs in miRBase with at least 16 snt overlap-allowing offsets. Those miRNAs satisfying both the above criteria will be counted as expressing identified known

miRNAs. To compare miRNA expression data under the five libraries, initially, each identified miRNA read count was normalized to the total number of reads in each given sample. After that, Bayesian method was used to appraise the statistical significance ( $P$  value). A specific miRNA was considered to be differentially expressed if its  $P$  value  $\leq 0.01$  and its normalized sequence counts changed more than twofold.

### Differential expression analysis of miRNAs in moso bamboo

The read count of each identified miRNA in moso bamboo was normalized to obtain the expression of transcript per million (TPM), which was used to evaluate the miRNAs expression patterns in the five different libraries. The normalization formula used is as follows: Normalized expression = Actual miRNA count/total count of clean reads  $\times 1,000,000$ . The fold changes (the log<sub>2</sub> scale values) and  $P$  values were calculated from the normalized expression data. Then, the log<sub>2</sub> ratio was generated. miRNAs with similar expression patterns were clustered using Gene Cluster 3.0.

### Quantitative real-time PCR (qRT-PCR) analysis of miRNAs and target genes in moso bamboo

Total RNA was extracted from each groups' frozen sample (ck, F-1, F-2, F-3 and F-4). cDNAs were synthesized from total RNA using miRNA-specific stem-loop RT primers. The reaction was incubated at  $16^{\circ}\text{C}$  for 30 min,  $42^{\circ}\text{C}$  for 30 min,  $85^{\circ}\text{C}$  for 5 min and  $4^{\circ}\text{C}$  for 5 min. qRT-PCR was carried out in a Light Cycler 480 machine (Roche, Switzerland) using a SYBR Green I Master Kit (Roche, Switzerland). The final volume was 20  $\mu\text{l}$ , containing 10  $\mu\text{l}$   $2\times$  SYBR Premix Ex Taq, 7.2  $\mu\text{l}$  of nuclease-free water, 0.4  $\mu\text{l}$  of each primer (10  $\mu\text{M}$ ) and 2  $\mu\text{l}$  of cDNA. The amplification was carried out as follows: initial denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 43 cycles at  $95^{\circ}\text{C}$  for 10 s,  $58^{\circ}\text{C}$  for 20 s and  $72^{\circ}\text{C}$  for 10 s. The melting curves were obtained at  $95^{\circ}\text{C}$  for 5 s and  $58^{\circ}\text{C}$  for 1 min and then cooled to  $40^{\circ}\text{C}$  for 30 s (Unver and Budak 2009). All reactions were performed in triplicate. U6 snRNAs were chosen as internal controls for the miRNAs (Ding et al. 2011). The stem-loop reverse transcription primers were designed following the method described by Chen et al. (2005). PCR primers, including a miRNA-specific forward primer and a reverse primer, were then added to amplify the PCR products.

The expression analyses of several target genes were also examined using qRT-PCR. Reverse transcription reactions were performed using 2 mg of RNA by M-MLVRT (Promega, USA) according to the manufacturer's instructions.

**Table 1** Primers used in miRNAs and targets qRT-PCR

Name	Primer	Sequence
phe-miR167a	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGTCAGATCA
	Forward primer	ACGGGCTGAAGCTGCCAGCAT
phe-miR164a	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGTGCACGTG
	Forward primer	AGCCCTGGAGAAGCAGGGCA
phe-miR166a	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGGGGGAATG
	Forward primer	GCGGTCCGACCAGGCTTCA
phe-miR529-5p	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGAGGCTGTA
	Forward primer	GCGGGCAGAAGAGAGAGAGTA
phe-miR535a	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGGCGTGCTC
	Forward primer	AGCCCGTGACAACGAGAGAG
phe-miR156 k	RT Primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGGTGCTCAC
	Forward primer	ACGGGCTTGACAGAAGAGAGT
phe-miR172a	RT Primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGATGCAGCA
	Forward primer	AGCCCGAGAATCTTGATGATG
novel_miR_14	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGAACCGTTG
	Forward primer	ACGGGCAGTGTCCCTGTAGC
novel_miR_70	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGTCTCCTTC
	Forward primer	TGCCCGCTGGCCTGATAGTA
novel_miR_17	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGGTGAATCT
	Forward primer	AGCCCGCAGCACCATCAAG
novel_miR_198	RT Primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGTACAATGA
	Forward Primer	GCGGGCTTGGAGTGTATCATC
novel_miR_381	RT primer	CTCAACTGGTGTCTGCGTGGAGTCGGCAATTCAGTTGAGCAACCCCC
	Forward primer	ACTCACAGGTAGGCGACGCG
U6	Universal reverse primer	CTCAACTGGTGTCTGCGTGGAGTC
	Forward primer	GGACATCCGATAAAAATTGGAACGATACAG
PH01000041G2170	Reverse primer	AATTTGGACCATTCTCGATTTATGCGTGT
	Forward primer	ACGTAGCCATCTTCCCTC
PH01000722G0020	Reverse primer	GAGGAGGCTAGGCTGTGATG
	Forward primer	GAAAGAGAGGAGCCGGGATT
PH01003375G0030	Reverse primer	TTACTCCGGGCCGTCAGATT
	Forward primer	GGGCTCACAAGAAGTATGCG
PH01001056G0640	Reverse primer	AGGATCCATGGCTCGTACTG
	Forward primer	CACGAGATCTTGAAGCTGC
TIP 41	Reverse primer	CAAAGGCTACTCTTGCGACC
	Forward primer	AAAATCATTGTAGGCCATTGTTCG
	Reverse primer	ACTAAATTAAGCCAGCGGGAGTG

The sequences of four selected target genes were acquired from the moso bamboo genome database (<http://www.ncgr.ac.cn/bamboo>). *TIP41* was chosen as the internal house-keeping control gene (Fan et al. 2013). qRT-PCR was again carried out in a Light Cycler 480 machine (Roche, Switzerland) using a SYBR Green I Master Kit (Roche, Switzerland). The 20  $\mu$ l reaction mixture contained 10  $\mu$ l 2 $\times$  SYBR Premix Ex Taq, 7.2  $\mu$ l of nuclease-free water, 0.4  $\mu$ l of each primer (10  $\mu$ M) and 2  $\mu$ l of cDNA. Amplification reactions were performed as follows: 95  $^{\circ}$ C for 10 s, 60  $^{\circ}$ C

for 10 s and 72  $^{\circ}$ C for 20 s. All reactions were performed in triplicate. The primers used in all of the qRT-PCR experiments are listed in Table 1.

### Prediction of novel miRNAs in moso bamboo

The miRNA hairpins were predominantly located in intergenic regions, introns or reverse repeat sequence of the coding sequence. The characteristic miRNA precursor's hairpin structure can be used to predict novel miRNAs.

**Table 2** Primers for 5'RACE

Target gene	Primer	Sequence
PH01000041G2170	Outer primer	GCCACCGTACCCGTTGCTCT
	Inner primer	GTCATGCCGTGGTCTTGGT
PH01000722G0020	Outer primer	ACTCCGGGCCGTCAGATTGG
	Inner primer	TTAAGATCGCCTGCCGTGGG
PH01003375G0030	Outer primer	TAGAAGGAGTACGCCAGCCTGTG
	Inner primer	CTTCGCTAACTTGAGAAATGC
PH01001056G0640	Outer primer	CTGCCTGATTGCCACTGTCTG
	Inner primer	AGAACAGTAGCGGTCTGGTATG

The prediction software Mireap (<http://sourceforge.net/projects/mireap/>) was used to predict novel miRNAs by exploring secondary structures, minimum free energies and the Dicer cleavage sites of the unannotated sRNA tags that could be mapped to genome. The key conditions were as follows: (1) the miRNA and miRNA\* were derived from opposite stem arms such that they formed a duplex with two nucleotide long 3' overhangs; (2) base pairing between the miRNA and the other arm of the hairpin, which includes the miRNA\*, is extensive such that there are typically four or fewer mismatched miRNA bases; (3) asymmetric bulges were minimal in size (one or two bases) and frequency (typically one or less), especially within the miRNA/miRNA\* duplex (Meyers et al. 2008); (4) the secondary structures of the hairpins were steady, with a free energy of hybridization lower than or equal to  $-18$  kcal/mol; and (5) the number of mature miRNAs with predicted hairpins must be no fewer than five in the alignment results. The novel miRNA expression level was calculated by summing the counts of miRNAs with no more than three mismatches on the 5' and 3' ends and with no mismatches in the middle based on the alignment results. Finally, the prediction of novel miRNA candidates was summarized, including the base bias at each position among all sRNA candidates.

### Prediction of potential miRNA targets in moso bamboo

The rules used for target prediction in plants are based on those suggested by Allen et al. (2005) and Schwab et al. (2005). Some key conditions are as follows: (1) no more than four mismatches between the sRNA and its target (G-U bases count as 0.5 mismatches); (2) no more than two adjacent mismatches in the miRNA/target duplex; (3) no adjacent mismatches in positions 2–12 of the miRNA/target duplex (5' of miRNA); (4) no mismatches in positions 10–11 of miRNA/target duplex; (5) no more than 2.5 mismatches in positions 1–12 of the miRNA/target duplex (5' of miRNA); and (6) the minimum free energy (MFE) of the miRNA/target duplex should be  $\geq 75$  % of the MFE of the miRNA bound to its perfect complement.

### Analysis of 5'RACE

The 5'RNA ligase-mediated rapid amplification of cDNA ends (RACE) reactions were essentially performed according to the manufacturer's protocol (Invitrogen, USA). Briefly, 200 ng enriched mRNA samples were extracted from moso bamboo tissues without the calf intestine alkaline phosphatase plus tobacco acid pyrophosphatase treatment and used to perform RT-PCR with oligo (T) primers. The PCR reactions used gene-specific and nested primers, followed by RNA adaptor ligation. All amplified PCR products were gel purified, inserted into the pGEM-T easy vector (Promega, USA) and confirmed by sequencing. The primers used in RACE are listed in Table 2.

### Functional analyses of miRNA targets

Gene ontology (GO) is now widely accepted for use in large-scale gene annotation projects. The potential targets of known miRNAs and novel miRNAs in moso bamboo that showed obvious differential expression patterns in the five samples (ck, F-1, F-2, F-3 and F-4) were subjected to a GO functional enrichment analysis. GO categorizes the potential functions of the predicted target genes. There are three ontologies in GO: biological processes, cellular components and molecular functions. This method first maps all target gene candidates to GO terms in the database (<http://www.geneontology.org/>), calculating gene numbers for each term, then uses a hypergeometric test to find significantly enriched GO terms among the target gene candidates comparing with the reference gene background. The numbers of genes from the three ontologies are then used to infer the functions of the miRNAs.

## Results

### Characterization of inflorescence morphogenesis in moso bamboo

The moso bamboo flowers are "fake" inflorescences and spike inflorescences, with average lengths of  $\sim 7$ – $9$  cm.

An inflorescence has several spikelets, and each spikelet has one to six florets. A floret has two glumes, one palea, one lemma, three lodicules, three stamens and one pistil. The morphogenesis of inflorescences can be divided into four periods: floral bud formation (F-1), inflorescence growth (F-2), bloom (F-3) and embryo formation (F-4).

**The floral bud formation stage (F-1)** The external environment induces the inhibition of the vegetative leaf growth of physiologically mature bamboo plants. The vegetative growth cone was converted to a reproductive growth cone, and bud formation marked the beginning of flower morphogenesis (Jiang 2002). Moso bamboo inflorescences stemmed from the dormant buds at the leaf base. During germination, growth cones gradually dilated and flower buds formed. Afterward, the tip of the growth cone formed a conical projection and bract primordium (Fig. 1a1, a2). At this time, the formation of the main inflorescence axes indicated that the moso bamboo switched from vegetative to reproductive growth.

**Inflorescence growing stage (F-2)** Inflorescence axes started to elongate after apical flower buds formed. New projections appeared on the other side of the spindle tips and then changed into the new buds that would become lateral spikelet primordia (Fig. 1b1, b2).

**Bloom stage (F-3)** Stamen and pistil primordia differentiated into stamens and pistils, respectively. Afterward, they transformed into the young flower organs until the spikelet differentiation was completed (Fig. 1c1, c2). As the first floret differentiated, the spikelet primordium increased rapidly and continued to differentiate into the new lateral spikelet.

**Embryo formation stage (F-4)** The anthers became mature and spread pollen, which fell on the stigmas. Ovules developed into seeds following the double fertilization. The mature embryos of moso bamboo had a piece of developed scutellum and degraded scales (Fig. 1d1, d2).

### sRNA sequencing analysis

sRNA libraries from non-flowering and flowering moso bamboo samples at different stages were sequenced using Illumina technology to identify known and novel miRNAs involved in the development of moso bamboo flowers. sRNAs are classified into two categories: small interfering RNAs (siRNAs) and miRNAs (Carthew and Sontheimer 2009). miRNAs are non-coding RNAs, 21–24 nt long, which regulate gene expression at the post-transcriptional level. miRNAs direct the cleavage or translational inhibition of mRNA based on their base pair complementation with target mRNAs (Bushati and Cohen 2007). After filtering out the reads lacking sRNA sequences, 18,761,783, 23,055,864, 15,304,493, 17,497,326 and 16,874,894 reads, ranging from 18 to 30 nt in length, were obtained from

ck, F-1, F-2, F-3 and F-4 libraries, respectively (Online Resource 1). The sRNA length distribution (16–30 nt) in each library revealed that the most diverse and abundant species were 21–24 nt long, which is a representative size range for Dicer-derived products (Online Resource 2). The 24 nt sRNA sequences were the most abundant in all five libraries, followed by the 21 nt sRNA sequences, which were the second most abundant in four libraries except the F-1 library. After further eliminating the unannotated sRNAs and non-coding RNAs, including rRNAs, snRNAs, snoRNAs and tRNAs, 3529,502, 1567,476, 1951,752, 1831,760 and 1,549,099 miRNA sequences, accounting for 18.81, 6.8, 12.75, 10.47 and 9.18 %, respectively, of the total sRNAs in ck, F-1, F-2, F-3 and F-4 libraries, respectively, were identified. The miRNA sequences in the ck library were significantly more abundant than those in the other libraries, indicating that more miRNAs may be involved in regulating the early developmental stage of moso bamboo and the initiation of flowering.

### Identification of known miRNAs in moso bamboo

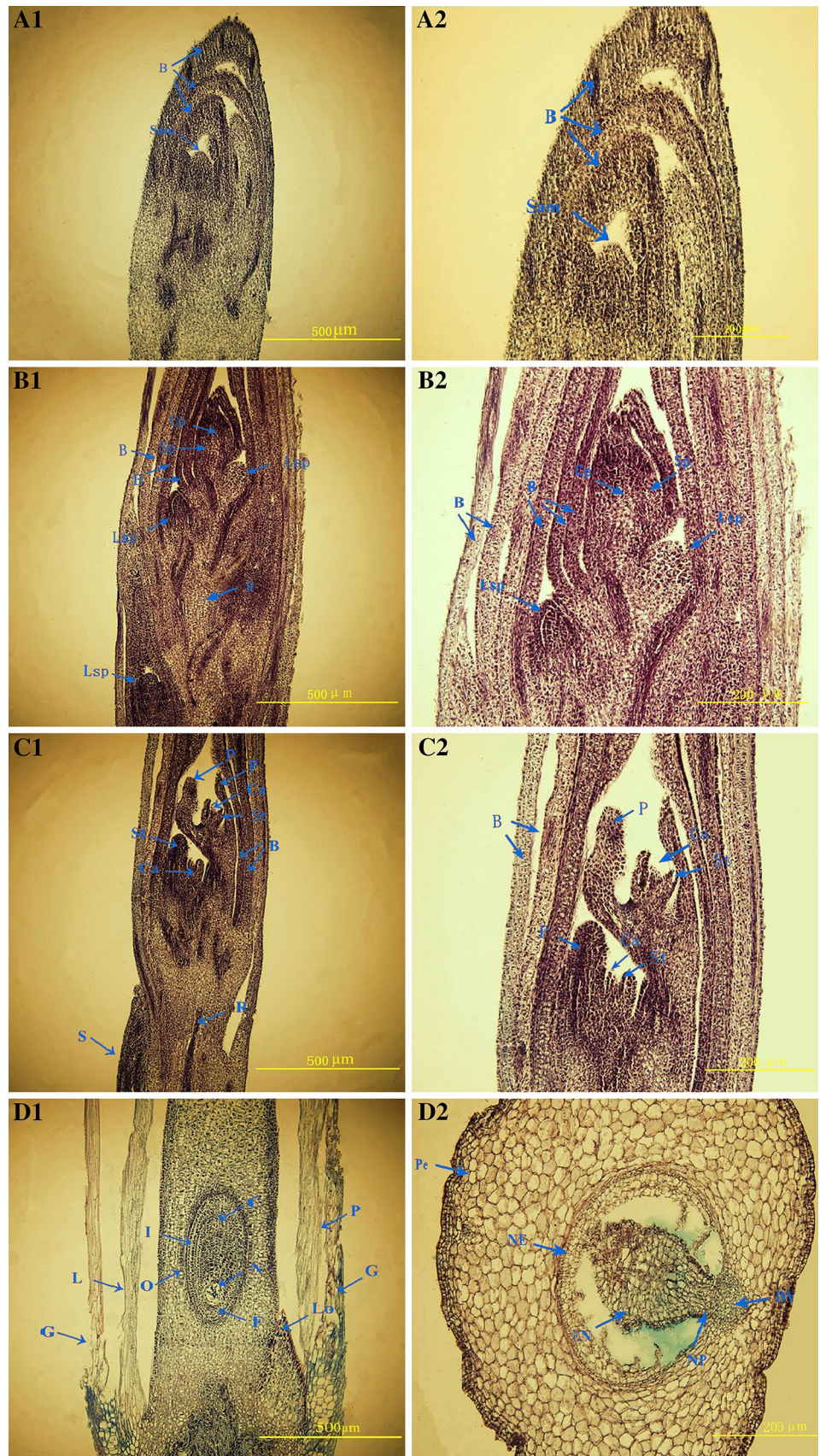
The sRNA libraries were used as query to search miRBase Version 21.0 using the BLASTN algorithm for unique mature plant miRNA sequences, thus identifying the known miRNAs in moso bamboo. Using a series of strict filtering criteria and further analysis, we identified 409 known miRNAs from moso bamboo (Online Resource 3). Interestingly, a differential expression analysis indicated that the 409 known miRNAs were differentially expressed in the five samples. Several conserved miRNAs, such as miR166a, miR167a and miR535a, had quite high expression levels. However, the levels of miR165a-3p, miR319b, miR393b-3p and some other miRNAs were rather low.

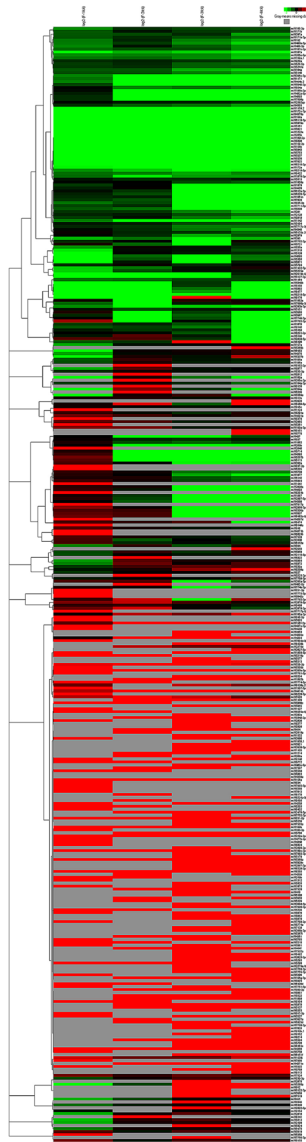
The relative expression patterns of mature miRNAs, and the sequence expression of a number of known miRNAs at different developmental stages, were essential for proper organ developmental regulation (Megraw et al. 2006; Parizotto et al. 2004; Valoczi et al. 2006). We could study the miRNA-mediated flowering regulatory pathways by understanding the miRNA expression patterns at the different developmental stages of moso bamboo flowers. The 409 known miRNAs in moso bamboo were clustered based on their expression profiles at different developmental flowering stages (Fig. 2). The results indicated that the expression levels of a number of miRNAs, such as miR164a, miR166a, miR167a and miR535a, in the ck library were significantly higher than those of the F-1, F-2, F-3 and F-4 libraries, while some miRNAs exhibited the opposite expression pattern. For example, miR169b, miR395 h-5p, miR529-3p and several other miRNAs were up-regulated in flowering compared with non-flowering samples.

Seven known miRNAs, miR164a, miR166a, miR167a, miR529-3p, miR535a, miR156 k and miR172a, were

**Fig. 1** Morphogenesis of inflorescence in moso bamboo by paraffin sectioning technique.

**a1, a2** Floral bud formation, **b1, b2** inflorescence growing, **c1, c2** spikelets differentiation, **d1, d2** embryo formation. *B* bract, *Sam* shoot apical meristem, *Cp* calyx primordia, *Sp* stamen primordia, *Lsp* lateral spikelet primordia, *R* rachis, *St* stamen, *Ca* carpel, *S* spikelet, *P* palea, *G* glume, *L* lemma, *P* Palea, *Lo* lodicule, *O* ovule, *I* integument, *N* nucellus, *C* chalaza, *F* funicle, *Pe* peel, *EN* endosperm, *NE* nucellar epidermis, *DV* dorsum vascular, *NP* nucellar projection





**Fig. 2** Differential expression analysis of known miRNAs. Heatmap for clustering analysis of moso bamboo miRNAs. Bar the scale of the expression levels of the miRNAs (log2)

selected for further validation experiments (Fig. 3). Their expression levels were analyzed by qRT-PCR. The qRT-PCR results indicated that miR164a, miR166a and miR167a exhibited trends of significant down-regulation, while miR529-3p showed a trend of increasing expression that progressed from the non-flowering samples through the flowering stages, which was consistent with the sequencing data. The four miRNAs could play vital regulatory roles in the moso bamboo blooming process. The expression of miR535a in the ck library was much higher than in the F-1 library. However, it was rapidly up-regulated in the F-2 sample, presenting no apparent difference from the ck sample, and then was significantly down-regulated in

**Fig. 3** The expression profiles of seven selected known miRNAs from flowering tissues at different flower developmental stages and leaves of non-flowering of moso bamboo. U6 snRNA was used as a reference in qRT-PCR. The level of every miRNA in the control was set at 1.0. Error bars the standard deviation of three replicates. **a** miR164a, **b** miR166a, **c** miR167a, **d** miR529-3p, **e** miR535a, **f** miR156 k, **g** miR172a

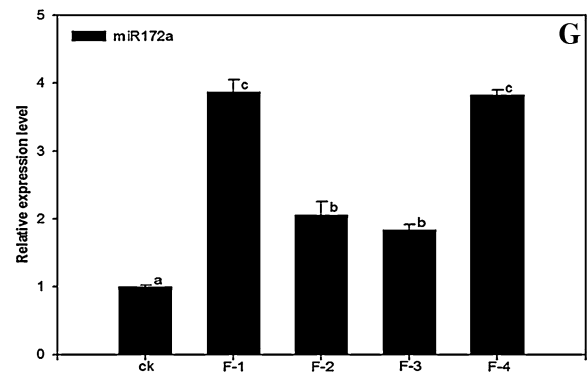
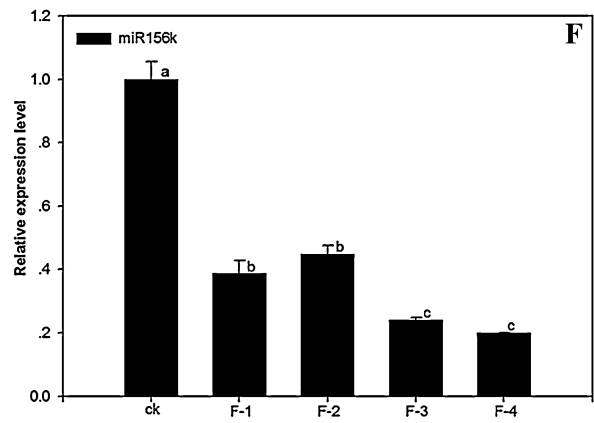
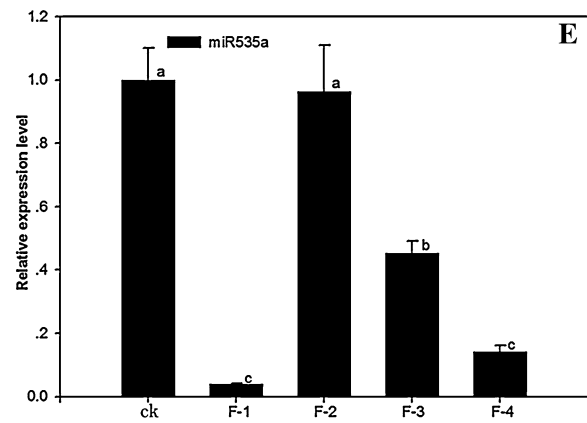
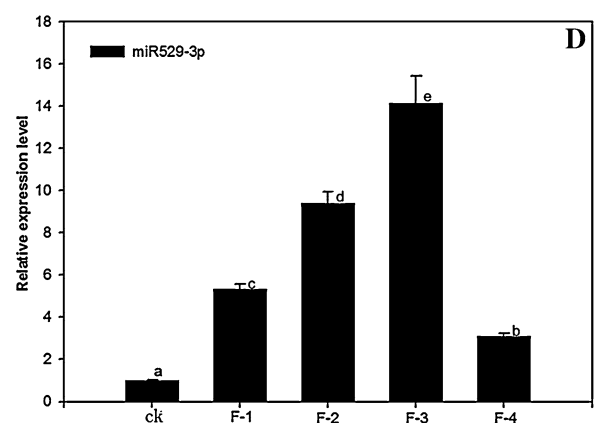
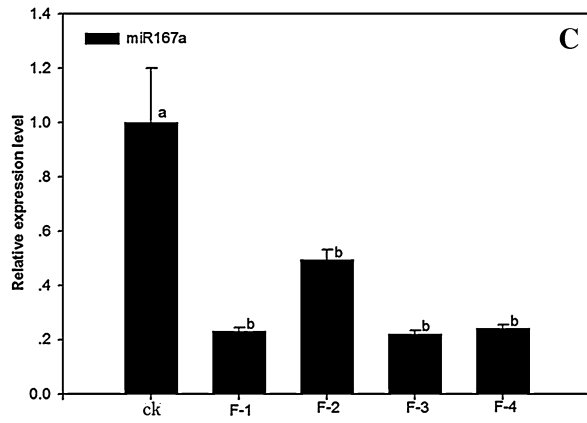
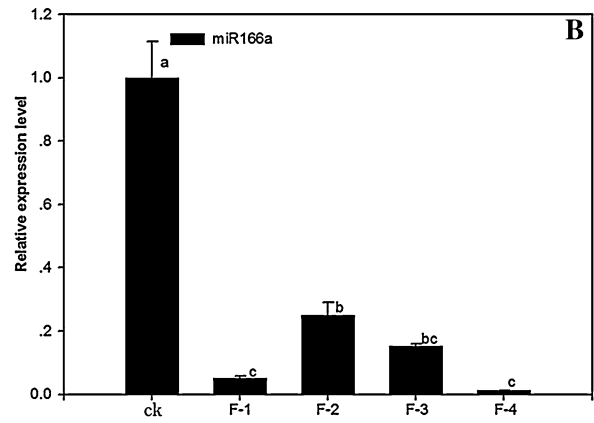
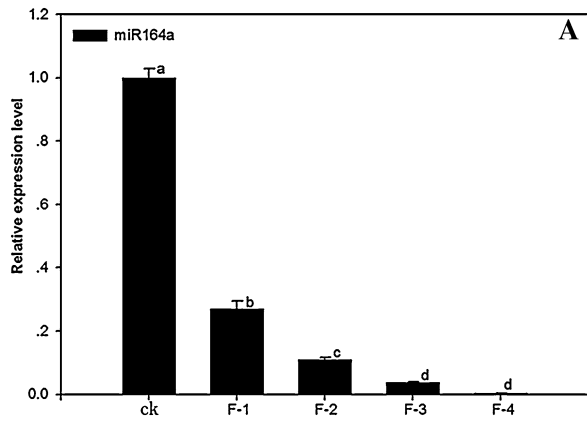
the F-3 and F-4 samples. The results clearly indicated that miR535a potentially played an important regulatory role at the inflorescence growth stage of moso bamboo. We found, using qRT-PCR, that the expression of miR156 k showed a trend of significant down-regulation, while miR172a exhibited an increasing expression level progressing from non-flowering leaves through the flowering stages. It may be that miR156 k played a regulatory role in the vegetative growth of moso bamboo, and the miR172a expression gradually increased with flower development.

### Identification of novel miRNAs in moso bamboo

The genome sequences of moso bamboo were used to predict its potential novel miRNAs. After eliminating the known miRNAs, a sequence alignment indicated there were 492 differentially expressed novel miRNAs in moso bamboo. All of the predicted novel miRNAs corresponded to their precursor sequences. If the novel miRNA was located in one arm of the precursor and its complementary sequence was located in the other arm, then the complementary sequence was regarded as a miRNA\* sequence. In total, 29 star miRNAs, corresponding to 27 novel miRNAs, were also predicted in moso bamboo (Table 3). The nucleotide bias at each position of novel miRNA candidates in moso bamboo was predicted. The majority of these novel miRNA candidates had 21 and 22 nt lengths and started with a 5'U (Online Resource 4). The uridine (U) abundance was the highest, especially at the 5' end of the novel miRNAs, and adenosine (A) was the second most predominant nucleotide (Online Resource 5).

Comparisons of the expression levels of the novel miRNAs between the F libraries and the ck library were performed (Fig. 4). Interestingly, the analysis indicated that all 492 novel miRNAs in moso bamboo were differentially expressed between the F libraries and the ck library. A large number of novel miRNAs were more highly expressed in the ck library than in the F libraries. A cluster analysis of the novel miRNAs in moso bamboo showed that the novel miRNAs were mainly classified into two categories (Fig. 5). The expression patterns of the novel miRNAs were different from those of known miRNAs. The expression levels of some novel miRNAs, such as novel\_miR\_14, novel\_miR\_70 and novel\_miR\_137, in the ck library were apparently lower than in the F-1, F-2, F-3 and F-4 libraries, while a large number of novel miRNAs showed the opposite expression pattern. For example, novel\_miR\_17, novel\_miR\_198, novel\_miR\_381





**Table 3** Novel miRNAs and miRNA\*s sequences from moso bamboo

miRNA name	miRNA sequence	miRNA* sequence	miRNA* counts				
			ck	F-1	F-2	F-3	F-4
novel_miR_2	TACCGACTTGTTCGATTAATCGTC	GATTAGTCGGCAAGTCGGGC	37	0	23	23	0
		CGATTAGTCGGCAAGTCGGGC	140	0	101	55	46
		TGATTAGTTGGCAAGTCGGGCGA	30	0	0	0	0
novel_miR_18	TTGTACAGCCACGGATCTCGG	AAGATCCGTGGCGTGACAACA	0	0	0	5	0
novel_miR_67	GATTAGTCGGCAAGTCGGGC	ACCGACTTGTTCGATTAATCGT	31	0	14	0	0
novel_miR_144	TGAGCGAGTTGGACACTTGTC	CTAGTCCAAGTCGCTCAAC	12	0	10	17	10
novel_miR_150	TCAGAATTTAGATGCTATGAA	GATAGCATATAAAATTCTGAAA	0	0	0	7	0
novel_miR_152	TTCTCTGCAAGGTATCAGGTG	ACCTGATATGGTGTAGGGAACC	0	12	0	0	0
novel_miR_236	TGGGCAAGTCTCCTCGGCTACC	TAGCCAAGGATGATTTGCCTGTA	0	20	0	11	0
novel_miR_241	TGATTAGTTGGCAAGTCGGGCGA	TACCGACTTGTTCGATTAATCGTC	31	0	14	0	0
novel_miR_294	GCCGGCGGTGGCGTCTCTTC	AGATGACGCCACGCCGGCCA	0	0	0	9	0
novel_miR_356	TGATAGCATATAAAATTCTGA	AGAATTTAGATGCTATGAAA	0	6	0	0	0
novel_miR_497	AGGAGTCGTCACGCCTGGCCATG	TGGTCAATGGGCGAGATCGTGG	0	0	21	0	0
novel_miR_556	TGAAGTGTGGGGGAACTC	GTTCCCTCAAGCACTTCACG	10	0	0	0	0
novel_miR_574	TTTTGCAAGTTCGGGGTGTCAA	TCGATACTGAGGGGGTGAATG	0	5	0	0	0
novel_miR_575	TCGATACTGAGGGGGTGAATG	TTTTGCAAGTTCGGGGTGTCAA	0	5	0	0	0
novel_miR_652	AGAATTTAGATGCTATGAAA	TGATAGCATATAAAATTCTGA	8	5	0	0	0
novel_miR_706	TAGCCAAGGATGATTTGCCTGTA	TGGGCAAGTCTCCTCGGCTACC	18	0	0	0	0
novel_miR_989	TCCCGGACGGCAGCAACCGAG	AGGTGCTGCCAACCGGGACG	0	0	25	0	0
novel_miR_1183	CCCTACAATTTGAACCTCCAT	GGAGTTCAAATGTAGGGTA	0	0	167	21	34
novel_miR_1221	GGAGGTTCAAATGTAGGGTA	CCCTACAATTTGAACCTCCAT	0	0	5	0	0
novel_miR_1252	GGGCTTCTACTTTGGCAAG	TGCCAAAGGAGAGCTGCCCTA	0	0	0	0	18
novel_miR_1255	TGGTCAATGGGCGAGATCGTGG	AGGAGTCGTCACGCCTGGCCATG	29	48	0	0	0
novel_miR_1273	AGGTGCTGCCAACCGGGACG	TCCCGGACGGCAGCAACCGAG	0	0	9	0	0
novel_miR_1362	GATAGCATATAAAATTCTGAAA	TCAGAATTTAGATGCTATGAA	12	0	0	6	0
novel_miR_1368	AAGATCCGTGGCGTGACAACA	TTGTCACGCCACGGATCTCGG	164	61	70	65	0
novel_miR_1399	TACCGACTTGTTCGATTAATCGT	TGATTAGTCGGCAAGTCGGGC	0	0	0	0	15
novel_miR_1425	AGATGACGCCACGCCGGCCA	GCCGGCGGTGGCGTCTCTTC	6	0	0	0	0
novel_miR_1714	TGCCAAAGGAGAGCTGCCCTA	GGGCTTCTACTTTGGCAAG	0	0	8	0	0

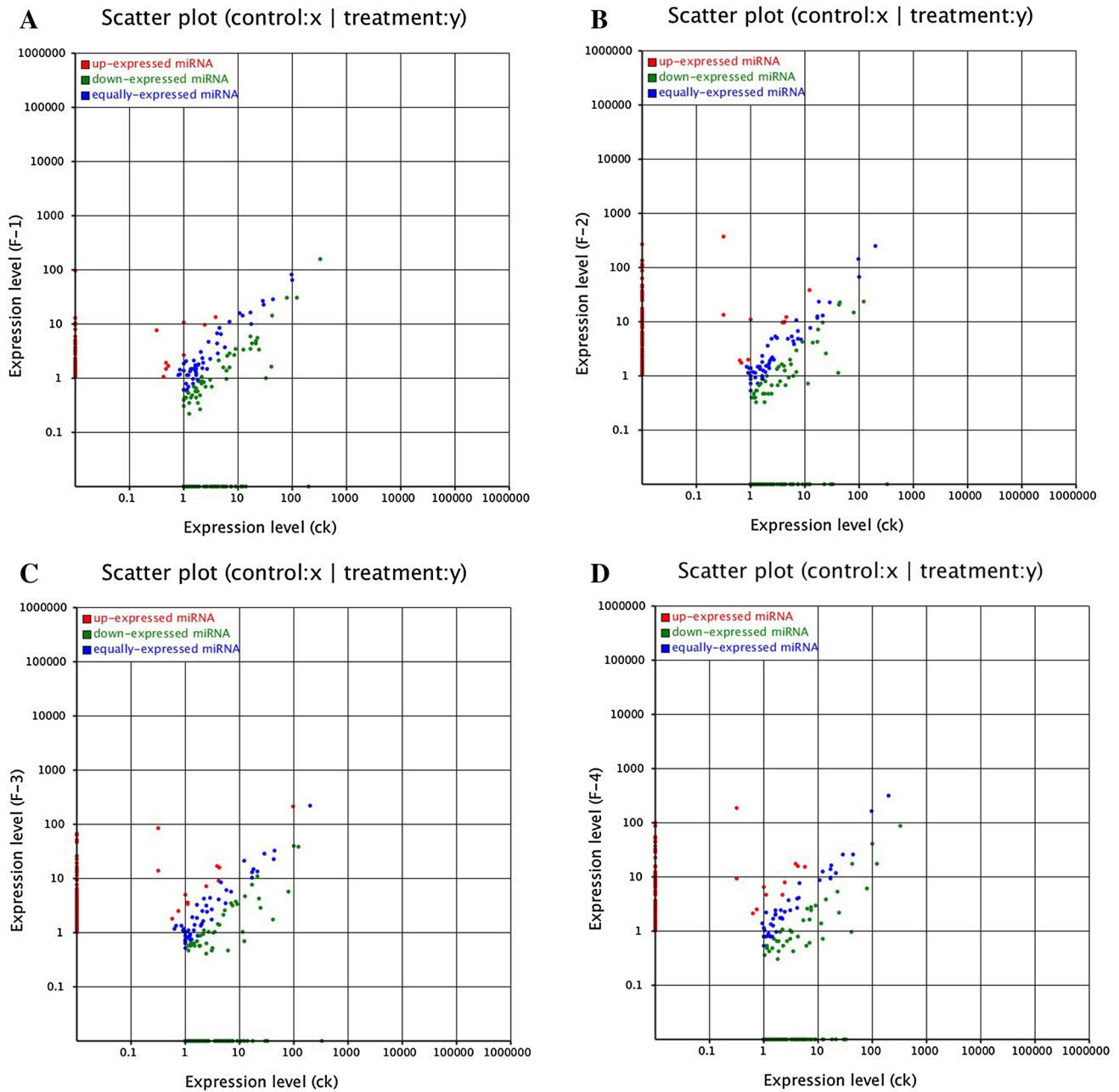
and many other novel miRNAs exhibited a trend of significant down-regulation from non-flowering samples through the flowering stages. We validated the expression profiles of novel\_miR\_14, novel\_miR\_17, novel\_miR\_70, novel\_miR\_198 and novel\_miR\_381 at different developmental stages of moso bamboo flowers using qRT-PCR (Fig. 6). These differentially expressed novel miRNAs may play important and specific potential regulatory roles in the moso bamboo blooming process.

### Prediction of differentially expressed miRNA targets and target functional analyses in moso bamboo

We predicted 308 targets of the 64 known miRNAs differentially expressed between the ck library and the F libraries (Online Resource 6). Additionally, we also predicted

specifically expressed known miRNAs and novel miRNAs and their targets at different developmental stages (Supplementary Tables 4, 5). Interestingly, the number of specifically expressed novel miRNAs and target genes was much larger than those of known miRNAs in moso bamboo.

There are three GO categories, biological processes, cellular components and molecular functions, to which all of the target genes of differentially expressed miRNAs in moso bamboo were assigned (Online Resource 7; Fig. 7). miRNAs participated in diverse regulatory events, so the frequencies of ‘cellular progress’, ‘metabolic progress’ and ‘response to stimulus’ terms were quite high (Sunkar and Zhu 2004). The target genes of differentially expressed miRNAs existed in ‘cell, organelle and membrane’, and chiefly functioned in ‘binding, catalytic activity and transcription factor activity’.



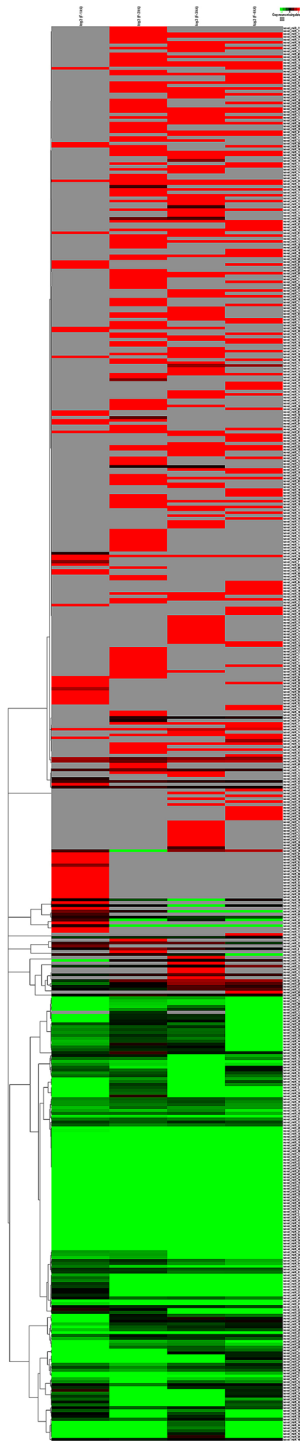
**Fig. 4** Comparisons of the different expression of novel miRNAs between F libraries and ck library. Each point in the *scatter plots* represents one miRNA. The *x*-axis and *y*-axis individually show the expression levels of miRNAs in ck library and F libraries. *Red points* fold change  $>1$ , *P* value  $<0.05$ , and more abundant in F libraries; *blue points*  $-1 < \text{fold change} < 1$ , *P* value  $>0.05$ , and equally expressed in ck library and F libraries; *green points* fold change less than  $-1$ ,

*P* value  $<0.05$ , and more abundant in ck library. **a** Comparison of the different expression of novel miRNAs between F-1 library and ck library. **b** Comparison of the different expression of novel miRNAs between F-2 library and ck library. **c** Comparison of the different expression of novel miRNAs between F-3 library and ck library. **d** Comparison of the different expression of novel miRNAs between F-4 library and ck library (color figure online)

### Expression analyses of target genes

The expression levels of four targets [PH01000041G2170 (no apical meristem protein, the target of miR164a), PH01000722G0020 (OsGrx\_A2—glutaredoxin subgroup III, the target of miR529-3p), PH01003375G0030 (GIL1,

the target of novel\_miR\_17) and PH01001056G0640 (1,3-beta-glucan synthase component domain-containing protein, the target of novel\_miR\_381)] were measured during the development of moso bamboo flowers to study whether the target genes were actually regulated by corresponding miRNAs. The relative expression



**Fig. 5** Differential expression analysis of novel miRNAs Heatmap for clustering analysis of *P. edulis* novel miRNAs. Bar the scale of the expression levels of the novel miRNAs ( $\log_2$ )

levels of PH01000041G2170, PH01003375G0030 and PH01001056G0640 increased from non-flowering samples to flowering samples, while those of miR164a, novel\_miR\_17 and novel\_miR\_381 were inhibited (Fig. 8). In

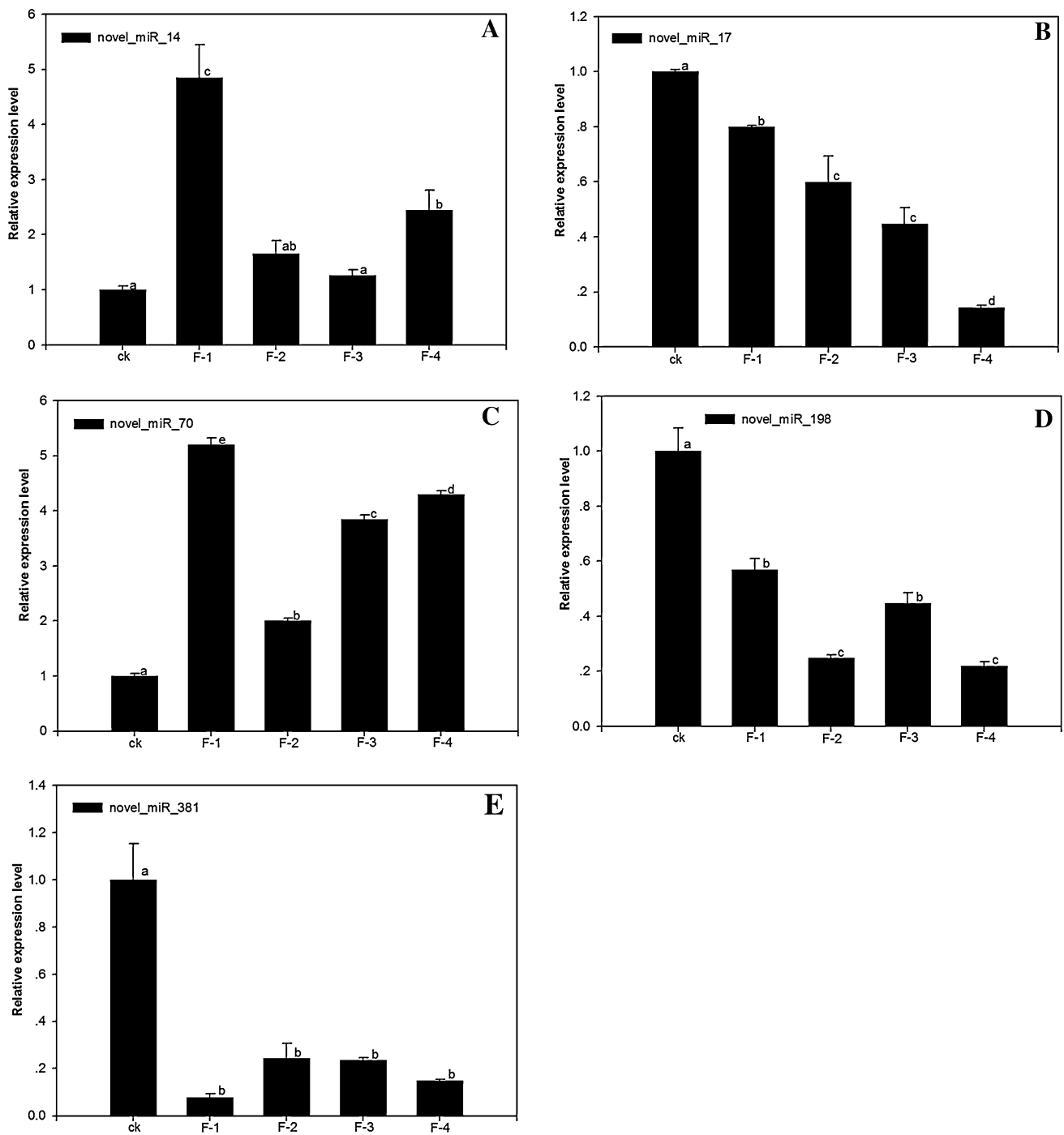
addition, the variation in miR529-3p abundance had a negative effect on the abundance of PH01000722G0020. The expression profiles of miRNAs and their target genes were complementary. Additionally, we performed 5'RACE reactions. These results suggested that we had successfully amplified the four miRNA-target genes, and they were consistent with the target prediction results (Fig. 9).

## Discussion

Research on model plants has indicated that the timing of floral induction is controlled by complex regulatory networks, which contain photoperiod and circadian clock, gibberellin, autonomous, age and ambient temperature pathways (Fabio et al. 2010; Simpson and Dean 2002). The irregular and extraordinary characteristics of moso bamboo flowering have drawn more and more attention and research interest (Janzen 1976). Nonetheless, few studies have addressed the molecular functions and regulatory mechanisms of moso bamboo flowering because it is very difficult to collect florescent samples. We successfully collected florescent samples and divided them into four developmental periods to be studied using a paraffin sectioning technique. The draft genome of the fast-growing non-timber forest species moso bamboo and the floral transcriptome of moso bamboo at different developmental stages of flowering are available (Gao et al. 2014; Peng et al. 2013).

The moso bamboo inflorescence is a 'fake' inflorescence, thus its morphogenesis is more complex than other Gramineous plants, such as *Oryza sativa* and *Triticum aestivum*. Previous studies on the morphogenesis of *Phyllostachys violascens* inflorescence have been performed (Lin et al. 2012), but there is no research report on moso bamboo inflorescence morphogenesis. We used a paraffin sectioning technique to study moso bamboo inflorescence morphogenesis for the first time, and it has provided a good theoretical foundation for research on moso bamboo flower development. Based on references and the moso bamboo flower's anatomical structure, we divided the inflorescences' morphogenesis into four periods: F-1, F-2, F-3 and embryo F-4. In F-1, moso bamboo transforms from vegetative to reproductive growth. After the top bud primordium forms the spikelet primordium, the lateral shoot primordia continue to differentiate until the principal inflorescence axis stops elongating. The lateral shoot primordia then form new false spikelets that continue to differentiate into new false spikelets.

The precise control of cell differentiation is very important to guarantee the successive production of lateral organs for normal plant development. Therefore, the balance between meristem maintenance and organ formation needs to be rigorously regulated so as to maintain the potential for

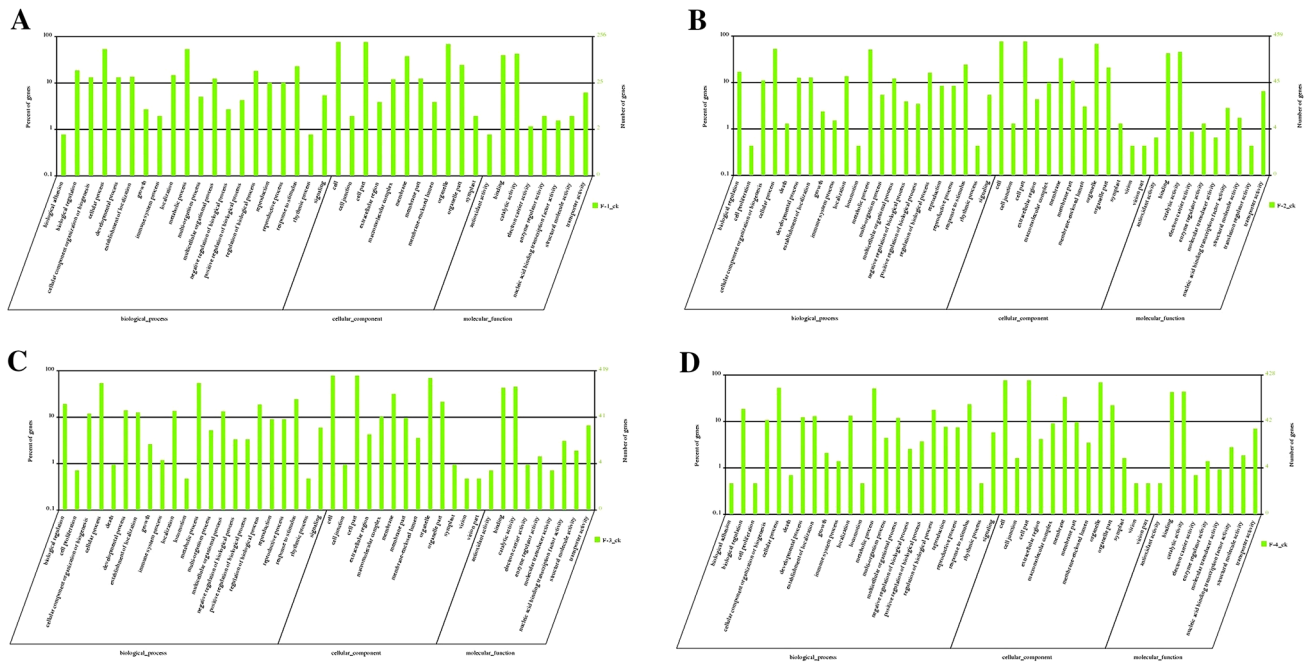


**Fig. 6** The expression profiles of five selected novel miRNAs from flowering tissues at different flower developmental stages and leaves of non-flowering of moso bamboo. U6 snRNA was used as a reference in qRT-PCR. The level of every miRNA in the control was set

at 1.0. *Error bars* the standard deviation of three replicates. **a** novel\_miR\_14, **b** novel\_miR\_17, **c** novel\_miR\_70, **d** novel\_miR\_198, **e** novel\_miR\_381

indeterminate growth. miRNAs have vital functions in regulating nearly all of plant development. During the past few years, researchers have witnessed the quick discovery of miRNAs that regulate gene expression in various organisms, and the miRNA regulatory mechanisms in different plant

developmental processes have been examined. For example, Xue et al. (2009) identified 241 known and 26 novel miRNAs via parallel signature sequencing of short RNAs from rice grains at 3–12 days after flowering. Moreover, both deep sequencing and microarray analyses were used to analyze



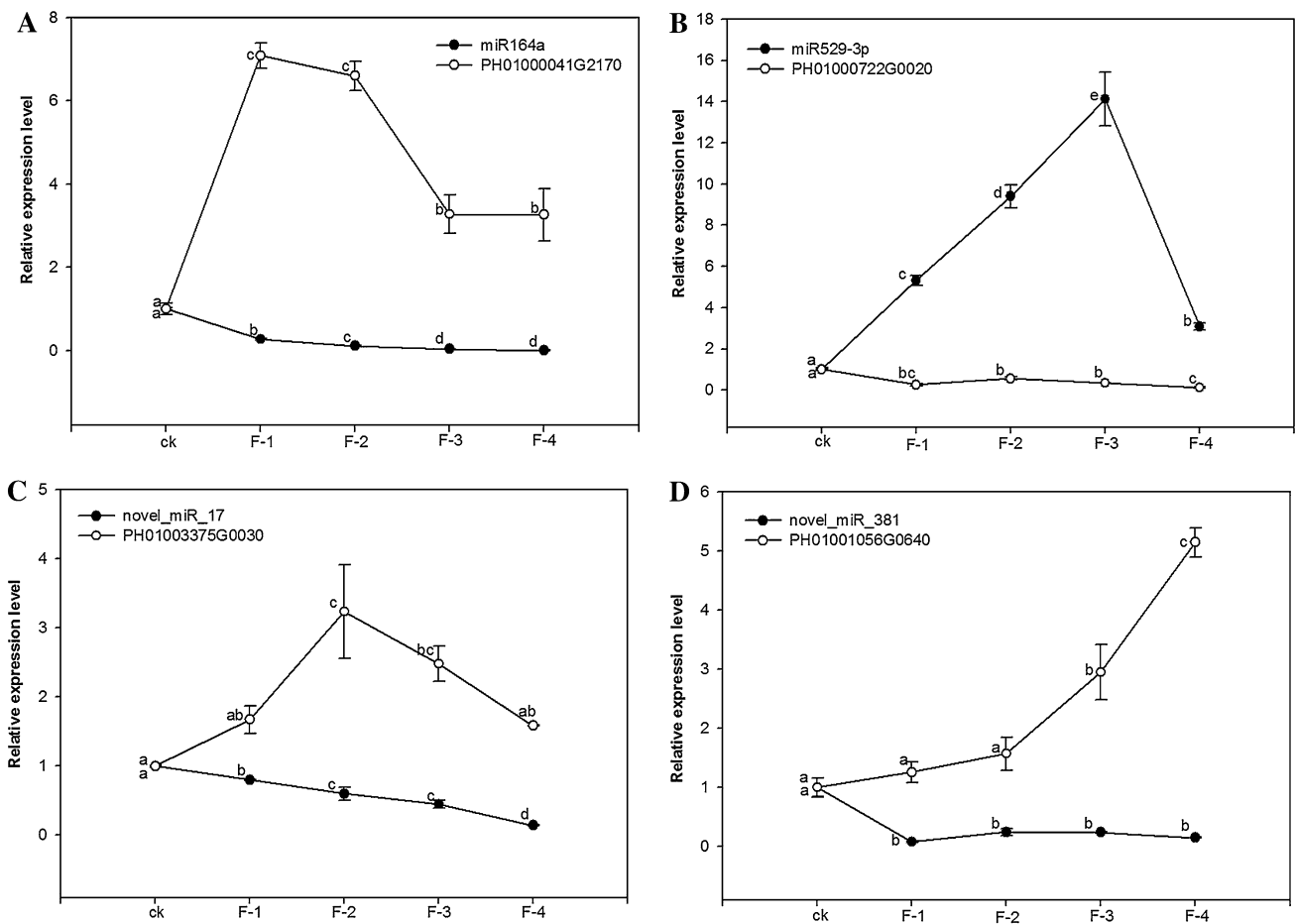
**Fig. 7** Gene ontology of the predicted targets for differentially expressed novel miRNAs. Categorization of miRNA-target genes was performed according to the biological process, cellular component and molecular function. **a** Gene ontology of the predicted targets for differentially expressed novel miRNAs between F-1 library and ck library. **b** Gene ontology of the predicted targets for differentially

expressed novel miRNAs between F-2 library and ck library. **c** Gene ontology of the predicted targets for differentially expressed novel miRNAs between F-3 library and ck library. **d** Gene ontology of the predicted targets for differentially expressed novel miRNAs between F-4 library and ck library

a miRNA library generated from rice grain, and 102 known miRNAs and 11 novel miRNAs were identified (Lan et al. 2012). An endogenous flowering pathway in *A. thaliana* was defined by miR156-regulated SPL transcription factors (Wang et al. 2009). High-throughput sequencing has quickened miRNA expression analyses and guided the discovery of many miRNAs, including the differentiation-specific miRNAs (Morin et al. 2008). In the present study, using Illumina sequencing technology and bioinformatics analyses, we obtained 18,761,783, 23,055,864, 15,304,493, 17,497,326 and 16,874,894 sequence reads from ck, F-1, F-2, F-3 and F-4 libraries, respectively, and identified 409 known miRNAs, and 492 novel miRNAs in moso bamboo. The Illumina sequencing of sRNAs in moso bamboo revealed that 24 nt sRNAs were the dominant form in the five libraries of moso bamboo, with the second highest being 21 nt sequences. This has been shown in many other plant species, such as *Medicago truncatula* (Szittyta et al. 2008; Wang et al. 2011a, 2011b), *A. thaliana* (Fahlgren et al. 2007) and *Citrus sinensis* (Xu et al. 2010). The sequencing results indicated that the most abundant sRNAs are miRNAs and siRNAs in moso bamboo.

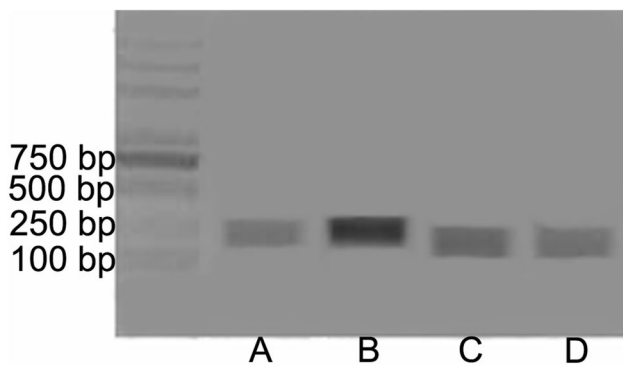
Most of the known miRNAs were found to be expressed in all five libraries of moso bamboo. However, a number of known miRNAs were expressed in a stage-specific pattern, indicating that the expression of known miRNAs

varied at different developmental stages of moso bamboo flowering. Most of the known miRNAs presented differential expression patterns between the ck library and the other four libraries. The cluster analysis results showed that the known miRNAs were divided into down-regulated and up-regulated categories with the expression trend progressing from non-flowering samples through the flowering stages. The number in the down-regulated category was significantly larger than that of the up-regulated category. Thus, the differential expression patterns, rather than the composition of miRNAs, may play the primary regulatory role in the moso bamboo flowering process. The expression patterns of miRNAs in tissues and organs could provide vital keys to their regulatory and biological functions. Consequently, the relative expression levels of seven selected miRNAs, which were identified in our research, were assessed using qRT-PCR. The relative expression levels of miR164a, miR166a and miR167a in the ck library were significantly higher than those in the F-1, F-2, F-3 F-4 libraries, while miR529-3p exhibited the opposite expression pattern. Interestingly, the relative expression level of miR535a in the ck library was apparently higher than that in the flowering samples, except in the F-2 stage. The relative expression level of miR535a was rapidly up-regulated in the F-2 sample and presented no apparent difference



**Fig. 8** The expression profiles of four target genes and their corresponding miRNAs from flowering tissues at different flower developmental stages and leaves of non-flowering of moso bamboo. *TIP41* was chosen as the internal housekeeping gene control for target genes. The level of every gene in the control was set at 1.0. Error

bars the standard deviation of three replicates. **a** PH01000041G2170 and miR164a, **b** PH01000722G0020 and miR529-3p, **c** PH01003375G0030 and novel\_miR\_17, **d** PH01001056G0640 and novel\_miR\_381



**Fig. 9** Agarose gel electrophoresis of 5'RACE product. *a* 5'RACE product of PH01003375G0030, *b* 5'RACE product of PH01000041G2170, *c* 5'RACE product of PH01001056G0640, *d* 5'RACE product of PH01000722G0020

from its expression in the ck. The results indicated that miR535a plays a vital and potentially regulatory role in the inflorescence growth stage of moso bamboo. miR156 k and miR172a were known to be involved in the vegetative-reproductive shift. miR156 k was the main regulatory gene that altered the plant growth cycle. It not only controlled the transformation from vegetative to reproductive growth, but also controlled the transformation from the juvenile to adult phase. miR156 k directly repressed the expression of the SPL transcription factor (Schwab et al. 2005). Overexpression of miR156 k caused a flowering delay and decreased the expression of the SPL family (Klein et al. 1996). The qRT-PCR results indicated that miR156 k plays a regulatory role in the vegetative growth of moso bamboo, while miR172a mainly plays a regulatory role in the reproductive growth of moso bamboo. miR172a controlled the flowering

time and the formation of floral organs through the regulation of the *AP2*-like transcription factor. miR172 acted downstream of miR156, which controlled the expression of miR172. The target gene (*SPL9*) of miR156 could directly promote transcription of miR172 to regulate plant flowering (Wu et al. 2009). The targets of miR164a are *CUC1* and *CUC2*, belonging to the nuclear NAC transcription factor family. *CUC2* prompts the development of floral organs (Takada et al. 2001). miR164a negatively regulates *CUC1* and *CUC2* to inhibit meristem formation and floral development, influencing the establishment of organ primordial boundaries (Mallory et al. 2004). miR166a could also regulate the morphogenesis of inflorescence. miR166a regulated the START domain-containing protein and the transcription factor HD-ZIPIII, affecting the development of vascular tissues in inflorescences and the polarity of the floral organs (Kim et al. 2005). The flower structures of mutants were seriously damaged as a result of the overexpression of miR166 (Jung and Park 2007; Williams et al. 2005). The targets of miR167a are auxin response factor (ARF) genes *ARF6* and *ARF8*, belonging to the ARF family. ARF completes the response to auxin signaling by combining with the auxin response gene *GH3* (Wu et al. 2006). The overexpression of miR167a inhibited flower development, including the stamen filament shortening, anther inability to spread pollen and female sterility (Nagpal et al. 2005; Wu et al. 2006). Therefore, the overexpression of miR164a, miR166a, miR167a, miR535a and miR156 k could inhibit moso bamboo inflorescence morphogenesis. Their relative expression levels were gradually down-regulated during the moso bamboo flowering process. miR529-3p targets zinc finger (C3HC4-type RING finger) family protein and OsGrx\_A2—glutaredoxin subgroup III genes (Barakat et al. 2007). The relative expression levels of miR529-3p and miR172a were gradually up-regulated during the moso bamboo flowering process, indicating that they played positive regulatory roles. A number of conserved miRNAs of moso bamboo were similar to those of other monocotyledons. Moso bamboo species have a close relationship with *O. sativa*, and many of their miRNAs were homologous to those in *O. sativa*. However, the known miRNAs of moso bamboo are more diverse and many of them presented one or two substitutions compared with those of *O. sativa*.

In our research, 492 differentially expressed novel miRNAs were predicted from five libraries of moso bamboo, using the moso bamboo genome data as a reference. Most of the novel miRNAs were 21 and 22 nt in length and began with a 5' U. The abundance of U was the highest, especially at the 5' end of the novel miRNAs. U and A were specifically generated by different Dicer proteins and recognized by Argonaute 1 and 2 proteins, respectively (Czech and Hannon 2011; Mi et al. 2008). The sequencing reads may reflect the expression levels of the

sequences. The sequencing results and validated expression profiles indicated that the expression levels of many novel miRNAs, such as novel\_miR\_17, novel\_miR\_198 and novel\_miR\_381, in the ck library were significantly higher than those in the F libraries, and showed a trend of down-regulation from the non-flowering samples through the flowering stages. Gene expression research indicated that the metabolic pathways of plants related to cell enlargement and histological differentiation in the early developmental phase (Okawa et al. 2003; Zhu et al. 2003). The expression levels decreased at the flowering stages when moso bamboo started to lose nutritional components and the metabolism switched to senescence, which might be closely related to the down-regulation of a large number of novel miRNAs. The differentially expressed novel miRNAs exhibited apparent changes between the ck library and the F libraries, indicating specific potential regulatory roles in moso bamboo flowering. Additionally, we found the specifically expressed miRNAs and their targets at different stages. The number of specifically expressed miRNAs in the ck sample was greater than in the F samples. An analysis of this expression revealed that the specific miRNAs only played regulatory roles at particular flower developmental stages. Also, 29 miRNAs\* sequences corresponding to 27 novel miRNAs were sequenced in moso bamboo. Both novel miRNAs and miRNAs\*s were derived from stem-loop hairpin structures. miRNAs were stable while miRNAs\*s were often destroyed when released from the pre-miRNA arm. Therefore, the abundance of miRNAs\* was very low (Khvorova et al. 2003). Illumina high-seq sequencing is a useful tool to detect novel miRNAs and miRNAs\* (Git et al. 2010). The low abundance of miRNA\* in moso bamboo could be the result of the rapid miRNA\* degradation rate. The relationship between miRNA\* and its flexible expression may reveal a special regulatory role for miRNA\* in moso bamboo flowering.

Identifying the regulatory targets of miRNAs could reveal their functions. We identified the targets of the differentially expressed known and novel miRNAs. The target annotation corroborated other research that had shown that many of the predicted targets were associated with transcription factors (Song et al. 2011; Yin and Shen 2010). In addition, targets participating in cellular progress, metabolic progress, response to stimulus, development process, transportation and other biological processes were also represented. This may be related to the ongoing cell proliferation in the meristems of flower buds necessary to build floral organ primordia, causing the strong metabolic activity found in the flower buds (Sun et al. 2014). Moreover, among the GO terms, 'binding' was dominant in the major category of 'molecular function', and 'nucleic acid binding transcription factor activity' was also found to be statistically significant. Moso bamboo flowering is a complicated



process, containing a great many cellular events related to unique transcriptomic profiles. Previous research indicated that plant transcription factors play vital roles in the complex network of transcriptional regulation during development (Duan et al. 2005). Many predicted targets of the differentially expressed miRNAs encode transcription factors, such as SPL (miR156), GAMYB (miR159), NAC (miR164a), AP2 domain-containing protein (miR172a) and auxin response factor (ARF, miR160a), indicating that the differentially expressed miRNAs function as primary regulators in the moso bamboo flowering process by regulating the expression of transcription factors. miR166 regulates the temporal program of floral stem cells by targeting *HD-ZipIII* and *APETALA2* genes (Ji et al. 2011). miR159, miR164, miR167 and miR319 specify unique cell types at the later stages of flower development (Nag and Jack 2010). It is thought that miR167 and miR160 target the ARF DNA-binding protein that regulates transcription in response to the phytohormone auxin (Rajagopalan et al. 2006; Reinhart et al. 2002), which regulates the development of leaves and flowers (Lim et al. 2010; Liu et al. 2010; Wang et al. 2011a, b). Transcriptional regulation is vital for a lot of the diverse developmental responses to auxin signals, including cell elongation, division and differentiation in both roots and shoots (Licausi et al. 2011). Interestingly, a large number of predicted targets of the differentially expressed known and novel miRNAs were observed to respond to stimuli. Previous research showed that moso bamboo flowering might be associated with an environment stress response. The phenomenon of moso bamboo flowering may itself represent the plant's stress response to an external stress. Our research could provide reference data for this hypothesis. The GO analysis revealed that these miRNAs and their targets may be involved in diverse biological processes in moso bamboo flowering. Additionally, we demonstrated a modulation in selected known and novel miRNA-target expression levels during the development of moso bamboo flowers. The results showed that the expression profiles of miRNAs were negatively correlated with those of their targets, which further corroborated the regulatory role of miRNAs on their targets and indicated the roles of miRNAs in moso bamboo flowering and senescence.

The identification and characterization of known and novel miRNAs involved in the regulation of moso bamboo flower development will facilitate our understanding of the molecular mechanisms regulating the process. Because moso bamboo is ecologically and economically valuable, it is of great importance to explore and study the rare moso bamboo flowering process. We identified 409 known miRNAs and 492 differentially expressed novel miRNAs. A large number of known and novel miRNAs were either specifically or differentially expressed in non-flowering leaves and flowering samples, respectively, indicating that they play primary

regulatory roles in the initiation of flowering and the developmental processes of moso bamboo flowers. Most importantly, our investigation provides a vital foundation from which to explore sRNA-based regulatory mechanisms in moso bamboo, and related species, flowering. Further work will include analyzing and validating the particular regulatory effects of several key miRNAs and the construction of metabolic regulatory networks involving miRNAs.

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**Conflict of interest** Jian Gao declares that she has no conflict of interest. Wei Ge declares that he has no conflict of interest. Ying Zhang declares that she has no conflict of interest. Zhanchao Cheng declares that he has no conflict of interest. Long Li declares that he has no conflict of interest. Dan Hou declares that she has no conflict of interest. Chenglin Hou declares that he has no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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