

Article

Fan-miR159 Family Targets Two Types of Genes to Potentially Regulate the Development of Strawberry

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Abstract

MicroRNAs (miRNAs) represent the most abundant class of small RNAs in plants, in which the miR159 family demonstrates a high degree of conservation across various plant species and plays a crucial role in regulating anther, silique, and seed development by targeting *GAMYB-like*. The members of the Fan-miR159 family in cultivated strawberry target not only *GAMYB-like* genes but also *Auxin Response Factor (ARF)* family members, which are annotated as “*ARF23-like*” in the genome. In this study, we firstly analyzed the structural features, evolutionary conservation, and expression patterns of the miR159 family in *Fragaria × ananassa*. Then, we reannotated the “*ARF23-like*” genes in cultivated strawberry and found that they actually belong to the “*ARF2b*” subfamily. Finally, we utilized RT-qPCR and co-transformation experiments to analyze and validate our observation. The results showed the suppression of *FaARF2b-2* expression by fan-miR159n and fan-miR159r in both tobacco and strawberry. Moreover, two transcripts of the *FaARF2b-2* gene were detected in cultivated strawberry, *FaARF2b-2R* and *FaARF2b-2M*. The expression levels of both *FaARF2b-2M* and *FaARF2b-2R* increased following the abscisic acid (ABA) treatment, indicating that they may be positively regulated by ABA. In addition, *FaARF2b-2M* exhibits significantly higher expression levels in the anther compared to *FaARF2b-2R*, which potentially plays an important role in the male reproductive development. Our findings enhance the understanding of the miR159 family in cultivated strawberry and expand the knowledge regarding its novel targets.

Keywords: miR159; *ARF*; target genes; strawberry



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1. Introduction

MicroRNAs (miRNAs) serve as master regulators in plant development. A wide array of miRNAs has been identified across various plant species through direct cloning and high-throughput sequencing techniques. These miRNAs are intricately involved in multiple developmental processes, such as meristem maintenance, establishment of leaf polarity and shape, flowering phase transition, and determination of flower patterning [1]. For instance, the miR165, miR166, and miR394 families are integral to the regulation of the shoot apical meristem (SAM). Specifically, the activities of miR165 and miR166 are quenched by

Argonaute10 (AGO10), which promotes the maintenance of SAM [2]. Meanwhile, miR394 is a mobile signal that impacts stem cell competence to the distal meristem by suppressing the F-box protein, LEAF CURLING RESPONSIVENESS, during the formation of shoot meristem [3]. Since leaves originate from the SAM in flowering plants, miR165 and miR166 are also involved in leaf development, by a miR165/166-HD-ZIP III-AGO1/10 pathway to regulate the adaxial–abaxial polarity of leaves [4,5]. Additionally, miR156 serves as a quantitative modulator to regulate *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* expression in the SAM, thereby controlling leaf plastochron length in *Arabidopsis* [6]. The miR164-*CUC2* and miR396-*GROWTH-REGULATING FACTOR* pathways are involved in the regulation of leaf shape, specifically influencing leaf margin serration [7] and leaf size [8], respectively. Moreover, miR169 and miR172 play crucial roles in regulating floral patterning by, respectively, restricted C class homeotic gene *AGAMOUS* and A class homeotic gene *APETALA2* [9,10]. miRNAs also serve as important regulators involved in reproductive development. For example, the *MIR167a*-overexpressing lines showed female sterility in tomato due to defects in the stigma and stylar trichome, which prevent pollen germination on the stigma surface and hinder pollen tube growth [11]. Reciprocally, miR156 and miR399 affect male fertility in *Arabidopsis* [12] and *Citrus* [13], respectively. Among them, the down-regulation of miR399a.1 caused collapsed pollen and decreased pollen fertility in pummelo [13]. Recent studies have illuminated the valid roles of miRNAs in the maternal or paternal control of embryogenesis and seed development. For instance, *MIR167A* functions as a maternally expressed gene controlling embryogenesis, where *mir167a* (♀) × wild type (♂) crosses resulted in abnormal embryos and endosperm development [14]. Conversely, miR159 acts as a paternal factor in the clearance of maternal barriers. In this context, sperm-transmitted miR159 inhibits the targets of central cell-transmitted miR159, thereby promoting nuclear division in the endosperm [15].

Plant miRNAs are generally classified into three categories: conserved miRNAs, less-conserved miRNAs, and species-specific miRNAs [16]. Among these categories, the miR159 family is notably highly conserved and demonstrates the highest abundance in land plants [17]. The characteristics and functions of the miR159 family have been intensively investigated in *Arabidopsis thaliana*. miR159 targets mRNAs that encode gibberellin (GA)-induced *MYB* (*GAMYB*) or *GAMYB-like* transcription factors [18–20]. These transcription factors play a crucial role in regulating anther development and seed germination within the GA-signaling pathway. For example, *MYB33* and *MYB65*, along with *MYB101*, participate in GA-induced pathways that promote programmed cell death in the aleurone of seeds [19] as well as in the tapetum of anthers [18]. Besides targeting *GAMYB*, miR159 has also been reported to target other transcripts. In *Arabidopsis*, these include *OPT1* and *CSD3* [21,22]; in tomato, *SGN-U567133* [23]; and in rose, *Cytokinin Oxidase/Dehydrogenase6* (*CKX6*) [24]. Notably, *OPT1*, *SGN-U567133*, and *CKX6* are all associated with flower development. *OPT1* functions as a peptide transporter that is potentially involved in the transport of peptides from the maternal tissues to nourish the growth of pollen tubes [25]; *SGN-U567133* raises the post-transcriptional regulation by miR159 in the tomato leaf and flower development [23]; and the miR159-*CKX6* module governs the duration of the cell division by controlling cytokinin catabolism in petals [24]. Additionally, *CSD3* is a copper/zinc superoxide dismutase in a peroxisome isoform [26].

In our previous work, we identified a class of “*Auxin Response Factor23* (*ARF23*)-like” genes as potential targets of miR159 in cultivated strawberry (*Fragaria × ananassa*), which is not related to the *MYB* or *GAMYB* family members [27]. *ARF23* is a pseudogene based on its inability to dimerize and its localization near the entomere, as part of a recently duplicated cluster in *A. thaliana* [28]. Here, we elucidated the true identity of these “*ARF23-like*” genes on the basis of genome-wide identification and characterization of the *ARF* gene

family. We also cloned one of the target genes, named *FaARF2-2b*, and found that it has two transcripts in cultivated strawberry, which may play different roles in the development of strawberry. Through co-transformation experiments conducted in both tobacco and strawberry, we discovered that mature miR159s—fan-miR159n and fan-miR159r—targeted transcripts from *FaARF2-2b* in leaves of tobacco and receptacles of strawberry, respectively. Collectively, this study verifies a novel target gene of miR159 in strawberry, *FaARF2-2b*, which enhances the understanding of miR159 in seed development within this species.

2. Materials and Methods

2.1. Plant Materials and Growth Condition

Two cultivated strawberry (*F. × ananassa*) cultivars, specifically ‘Benihoppe’ and ‘Sweet Charlie’, were selected as experimental materials for sRNA identification. These cultivars were grown at the Baima Teaching and Scientific Research Base of Nanjing Agricultural University, located in Nanjing, China. Additionally, *F. × ananassa* ‘Ningyu’ and *Nicotiana benthamiana* were employed for co-transformation experiments; the former was cultivated in the greenhouse of Jiangsu Academy of Agricultural Sciences, while the latter was maintained under controlled conditions in an illuminating incubator at Nanjing Agricultural University.

2.2. RNA and DNA Isolation

The genomic DNA was extracted from various tissue samples utilizing the Plant Genomic DNA Kit (Tiangen, Beijing, China). Simultaneously, total RNA was isolated from the same tissue samples with the RNA Extraction Kit (Tiangen, Beijing, China). Following this, the extracted RNA samples underwent reverse transcription to generate complementary DNA (cDNA) through the application of the PrimeScript RT reagent kit (TaKaRa, Dalian, China).

2.3. The Expression Level of Fan-miR159 Family Precursor Was Analyzed by RT-qPCR

The internal reference gene for strawberry was 26S rRNA in RT-qPCR using gene-specific primers (Table S1) [29]. The expression level of fan-miR159a—r precursor in strawberry leaves was detected, and RT-qPCR was performed with an SYBR premix EX Taq™ reagent kit (TaKaRa, Kyoto, Japan). The reaction procedure was 95 °C for 10 min; 95 °C 15 s, 58 °C 15 s, 72 °C 10 s, 40 cycles. The relative expression of fan-miR159a—r precursor in strawberry leaves was calculated by the $2^{-\Delta\Delta C_t}$ method [30], with all experiments being repeated three times. A complete list of all primers employed in this study is provided in Table S1.

2.4. The Cloning of Pre-Fan-miR159b/n/r and Their Targets

Strawberry leaves were used as the experimental material for DNA extraction. The full-length DNA sequences encoding the precursor regions of miR159b/n/r were amplified using gene-specific primers via PCR. According to the target gene sequences in the genome of *F. × ananassa*, specific primers were designed within the coding regions (CDS) using Primer Premier 5 software [31] (Table S1). Using cDNA derived from strawberry leaf tissue as a template, PCR amplification was carried out to clone these genes. The PCR amplification protocol consisted of an initial denaturation at 98 °C for 3 min, followed by 35 cycles of 98 °C for 10 s, 54 °C for 20 s, and 72 °C for 1 min, a final extension at 72 °C for 5 min, and storage at 4 °C. PCR products were analyzed by agarose gel electrophoresis, and the correctly sized bands were purified and recovered according to the manufacturer’s instructions for the Gel Extraction Kit (TaKaRa, Kyoto, Japan).

2.5. Plasmid Construction

Nine recombinant plasmids were constructed in this study. For the construction of 35SCaMV::*fan-MIR159b*, 35SCaMV::*fan-MIR159n*, and 35SCaMV::*fan-MIR159r*, the full-length sequence of *fan-MIR159b*, *fan-MIR159n*, and *fan-MIR159r* was introduced into the *Hind* III/*Eco* R I sites of the pBI121 vector, respectively. And for 35SCaMV::*FaMYB101-GUS*, 35SCaMV::*FaARF2b-2R-GUS*, and 35SCaMV::*FaARF2b-2M-GUS*, the full-length sequence of *FaMYB101*, *FaARF2b-2R*, and 35SCaMV::*FaARF2b-2M* was introduced into the *Xba* I/*Bam* HI sites of the pBI121 vector, respectively.

For 35SCaMV::*mFaARF2b-2R-GUS* and 35SCaMV::*mFaARF2b-2M-GUS*, we used 35SCaMV::*FaARF2b-2R-GUS* and 35SCaMV::*FaARF2b-2R-GUS* as a template, and we mutated it with 5 bases in the complementary pairing sequence of the mature miR159 sequence without changing the amino acid type corresponding to the mutated codon. For 35SCaMV::*mFaMYB101-GUS*, we used 35SCaMV::*FaMYB101-GUS* as a template, and mutated it with 8 bases in the complementary pairing sequence of the mature miR159b sequence without changing the amino acid type corresponding to the mutated codon.

2.6. Plasmids Transformed Instantaneously into Tobacco and Strawberry

These plasmids were introduced into *Agrobacterium tumefaciens* (GV3101) by the freeze-thaw method, and they were transformed into *N. benthamiana* and *F. × ananassa* ‘Ningyu’, according to the methods previously described by Sparkes et al. [32] and Jia et al. [33].

2.7. GUS Staining and Activity Assays

GUS staining was performed on plant tissues according to the established protocol [34]. After 48 h, the staining solution was removed through a series of washes with 70% ethanol. The 4-methylumbelliferyl- β -D-glucuronide (MUG) assay was used for the quantitative measurement of GUS activity. One unit of GUS activity is defined as the amount of enzyme that catalyzes the production of 1 nmol of 4-MUG from 1 mg of soluble protein per minute. *Agrobacterium* suspensions containing each recombinant vector were infiltrated into five tobacco leaves or three strawberry receptacles, and the samples were subsequently analyzed for GUS activity. The experiment was independently repeated three times.

The reaction was carried out in a 1 mL reaction mixture containing 1 mM 4-MUG and protein extract, with a total volume of 1 mL. The reaction solution was incubated at 37 °C, and 100 μ L aliquots were removed at 10, 20, and 30 min, respectively. Each aliquot was immediately mixed with 900 μ L of 0.2 M Na₂CO₃ to terminate the enzymatic reaction. The fluorescence of the samples was measured at an excitation wavelength of 365 nm using a UV-visible spectrophotometer, and a standard curve was generated. The fluorescence value of the test sample was determined based on the standard curve, and GUS activity was subsequently calculated.

2.8. Identification and Characterization of ARFs in *Fragaria*

The genome information files of diploid strawberry *F. vesca* (v4.0.a2) [35] and octoploid strawberry *F. × ananassa* (v1.0.a1) [36] were obtained from the Genome Database for Rosaceae [37]. The protein sequences of the *AtARF* gene family were downloaded from TAIR (<https://www.arabidopsis.org/>) (<https://www.arabidopsis.org/>) (accessed on 18 September 2022) and used to perform a BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 25 November 2025) (E-value 1×10^{-10}) search against strawberry genome sequences to obtain *Fragaria* orthologous genes. Then, through the Pfam database (<http://smart.embl-heidelberg.de/>) (accessed on 19 September 2022) [38] and Conserved Domain Database [39] in NCBI (<http://www.ncbi.nlm.nih.gov/cdd>) (accessed on 19 September 2022), we performed an analysis of candidate genes of conservative structure

domain Auxin-resp and B3. We removed sequences with a conserved domain integrity < 70%. The physical and chemical parameters of the proteins were calculated using the ProtParam tool (<https://web.expasy.org/protparam/>) (accessed on 24 February 2023). And motif analysis was performed using MEME Suite [40].

2.9. Phylogenetic Analysis

The miR159 precursor sequences from various plants were obtained from miRbase [41]. These precursor sequences were aligned using MEGA 11 [42], and a phylogenetic tree was constructed based on the Maximum Likelihood method. The ARF protein sequences were also aligned using MEGA 11 [42], and a phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replication.

2.10. Analysis of Tissue Expression Patterns of *FaARF2b-2*

Total RNA was extracted from various strawberry tissues, including the root, stem, leaf, calyx, petal, anther, receptacle, ovary, style, 12 days after pollination (DAP) embryo, endosperm, and seed coat, and it was subsequently reverse transcribed into cDNA. RT-qPCR primers were designed according to the CDS of *FaARF2b-2R* and *FaARF2b-2M*, as well as the region encompassing nucleotide sequence differences (Table S1). The *EF1-α* gene was used as the internal reference gene for strawberry. The expression level of *FaARF2b-2* in various strawberry tissues was analyzed using RT-qPCR with the SYBR premix EX Taq™ reagent kit (TaKaRa, Japan). The reaction system and procedure were identical to those described previously [27]. The relative expression of *FaARF2b-2* in different tissues was calculated using the $2^{-\Delta\Delta C_t}$ method [30], and all experiments were performed in triplicate.

2.11. Expression Patterns of *FaARF2b-2R* and *FaARF2b-2M* Under Abiotic Stress

The ‘Benihoppe’ with uniform growth conditions were subjected to a low temperature (4 °C), 2 mM ABA, drought (300 mM mannitol), and salt (100 mM NaCl), and leaves were collected at specific time points (0 h, 12 h, 24 h, 48 h, 72 h, and 96 h) after treatment initiation. Immediately after collection, samples were flash-frozen in liquid nitrogen and stored at −80 °C for subsequent experimental analysis. The expression patterns of *FaARF2b-2R* and *FaARF2b-2M* under various stress treatments were analyzed by RT-qPCR using cDNA from strawberry leaves.

2.12. Statistical Analyses

Each experiment was repeated independently three times. The Statistics Analysis System (SAS) (<https://welcome.oda.sas.com/>) (accessed on 15 March 2023) was used for the statistical analysis of the data. The mean and standard deviation (SD) of samples were calculated. Significance difference analysis of different groups was performed by two-tailed Student’s *t* tests. The lowercase letters indicate significant differences at $p < 0.05$.

3. Results

3.1. Two Classes of miR159 Members in the Embryos and Endosperm of Strawberry

Our previous study identified a total of 18 fan-miR159 members in the embryos and endosperm of strawberry [27]. Among these, 12 members (fan-miR159a/b/c/d/e/f/g/h/i/j/k/l) exhibited high expression levels in embryo tissue and were predicted to target *GAMYB* family members, whereas the remaining 6 members (fan-miR159m/n/o/p/q/r) displayed endosperm-specific expression and were predicted to target a class of genes annotated as “ARF23-like” in the genome (Figure 1A; Table S2). The mature sequences of fan-miR159c/d/e/f were identical to that of ath-miR159a. However, the six mature miRNAs—fan-miR159m/n/o/p/q/r—which share a common sequence among them—

selves, differ from other miR159 family members. These differences are particularly evident at nucleotide positions 5–7, 13, and 21 (Figure 1B). The pre-fan-miR159 precursors corresponding to the mature miR159 m/n/o/p/q/r are located on chromosomes 7-1, 7-2, 7-3, and 7-4, respectively, while the other pre-fan-miR159s are located on chromosomes 3-2 and 3-3 (miR159a/b), 5-1, 5-2, 5-3, and 5-4 (miR159c/d/e/f), 6-1, 6-2, 6-3, and 6-4 (miR159g/h/i/j/k/l) (Figure 1C). The secondary stem-loop structures of these pre-fan-miR159 sequences were further predicted using RNAfold (Figure S1). To investigate the evolutionary conservation of the miR159 family, a phylogenetic tree was constructed based on the stem-loop regions using pre-miR159 sequences from various dicotyledonous and monocotyledonous plant species. The resulting tree classified these pre-miR159s into five distinct clades (I–V), with pre-fan-miR159s distributed across clades I, II, IV, and V (Figure 2). In plants, ARF transcription factors are typically targeted by the miR160 (ARF10/16/17/18) and miR167 (ARF6/8/12) families [43–45]. Our analysis revealed that, unlike fan-miR159m/n/o/p/q/r, most fan-miR160 and fan-miR167 members exhibited higher expression in the embryo than in the endosperm, with the exception of fan-miR167j/k/l [27]. In summary, these findings are considered intriguing, prompting further investigation.

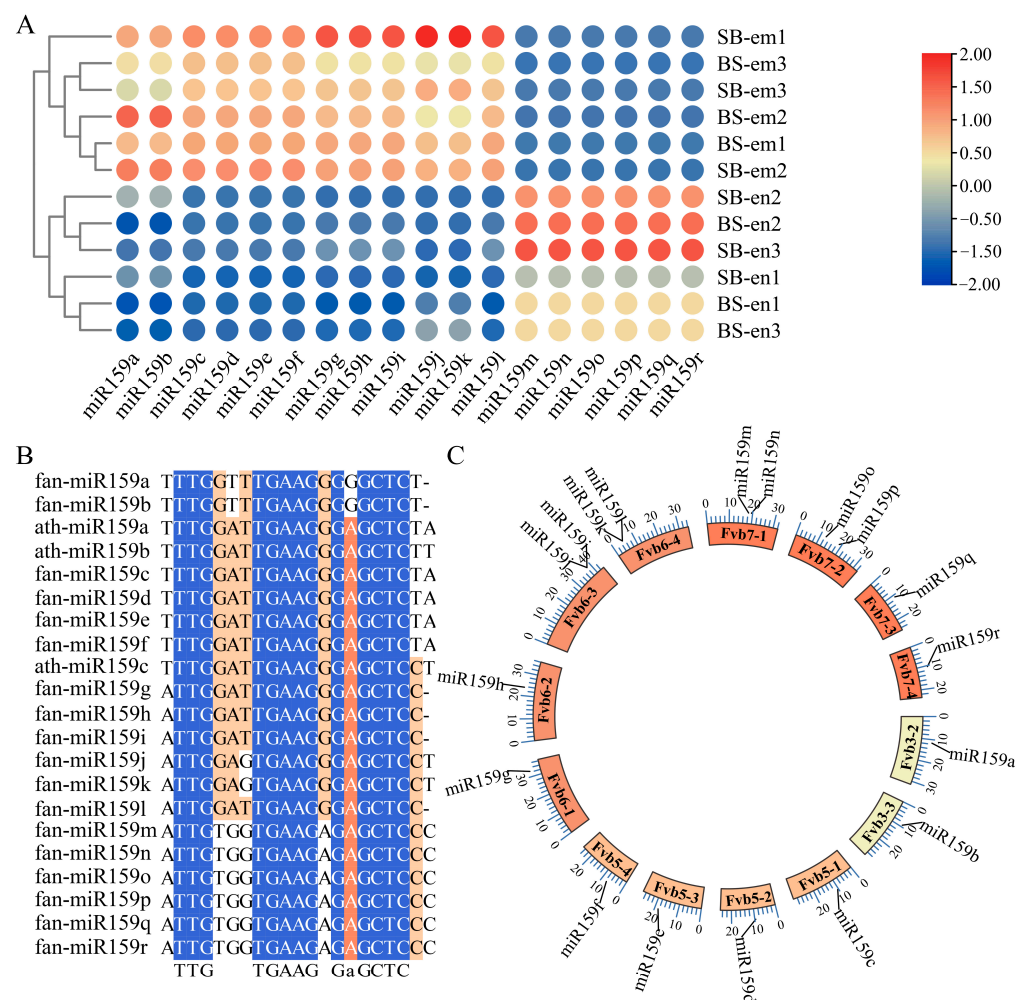


Figure 1. A total of 18 fan-miR159 members in *F. x ananassa*. (A) The expression pattern of fan-miR159 family in the embryos and endosperm of strawberry. BS: 'Benihoppe' (♀) × 'Sweet Charlie' (♂); SB: 'Sweet Charlie' (♀) × 'Benihoppe' (♂); em: embryo tissue; en: endosperm tissue. For each tissue, three biological replicates were performed. (B) Mature sequences of miR159 members from *F. x ananassa* and *A. thaliana*. (C) The genomic location of pre-fan-miR159s of mature fan-miR159 family.

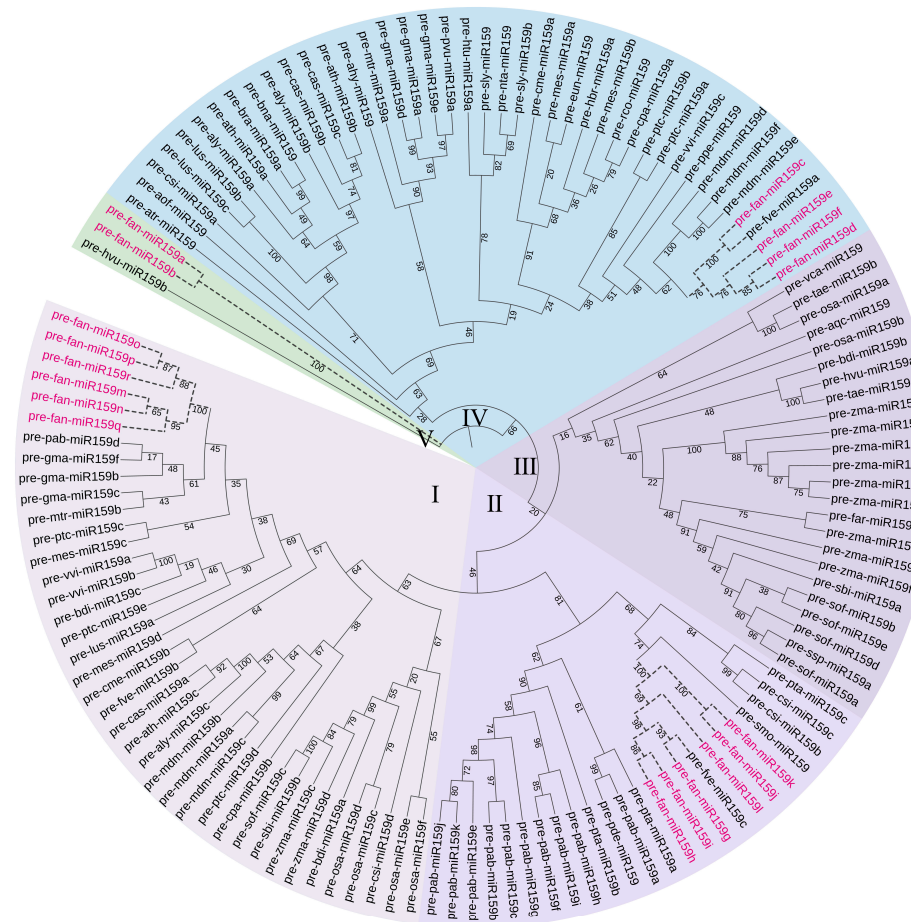


Figure 2. The miR159 precursors from various plants were divided into five groups in the Maximum Likelihood tree, including I (Alice blue), II (lavender), III (medium purple), IV (sky blue), and V (dark sea green).

3.2. The “FaARF23-like” Genes Are Members of FaARF2 Subfamily

ARF23 is a pseudogene in *A. thaliana*, and its biological function is still unknown [28]. In order to determine the true identity of “ARF23-like” genes in the strawberry, we conducted a comprehensive genome-wide identification of the ARF gene family in both *F. × ananassa* and the diploid model species *F. vesca*. The results showed that a total of 17 *FvARFs* (*FvARF1*~*FvARF18*) and 62 *FaARFs* (*FaARF2a-1*~*FaARF19*) were identified in *F. vesca* and *F. × ananassa*, respectively (Table S3). These genes were named based on their sequence homology with *Arabidopsis* ARF genes. Notably, the “ARF23-like” genes from *F. × ananassa* exhibited high sequence conservation with *AtARF2* in *Arabidopsis* and *FvARF2b* in *F. vesca*. Consequently, they were designated as *FaARF2b-1*, *FaARF2b-2*, *FaARF2b-3*, and *FaARF2b-4*. Like most ARF genes, *FaARF2b-1/2/3/4* contain three representative domains: a B3-like DNA-binding domain, an Auxin response domain, and an Aux/IAA dimerization domain (Figure S2). To investigate potential structural differences between *FaARF2b* members and other ARF2 subfamily members, we conducted a motif analysis and found that, compared with the *ARF2a* members in *F. vesca* and *F. × ananassa*, *ARF2b* genes lack motif 12 but possess an additional motif 19, with the exception of *FvARF2b* and *FaARF2b-4* (Figure S3). Furthermore, physicochemical property analysis showed that the theoretical pI and grand average of hydropathicity of *FaARF2b* proteins are higher than those of *FaARF2a* proteins (Table S4).

To further determine the identity of these “ARF23” genes, a neighbor-joining phylogenetic tree was constructed using MEGA11 [42] based on the protein sequences of the ARF

gene family from multiple species, including *A. thaliana* [46], tomato [47], castor bean [48], and rosaceous plants—such as apple [49], pear [50], Siberian apricot [51], and strawberry. The phylogenetic analysis revealed that approximately 200 ARF genes were divided into four major classes: Class I, Class II, Class III, and Class IV. Each class included genes from all species analyzed, with Class I containing the highest number of genes (68) and Class II the lowest (25) (Figure 3). Class I was further divided into four subclasses: class Ia, class Ib, class Ic, and class Id. Class II and Class IV were each split into two subgroups, and Class III was divided into three subgroups. The tree indicated that the four “FaARF23-like” genes (*FaARF2b-1/2/3/4*) belonged to class Ia, which may have similar evolutionary relationships to *MdARF1*, *MdARF4*, *MdARF5*, and *RcARF2a*.

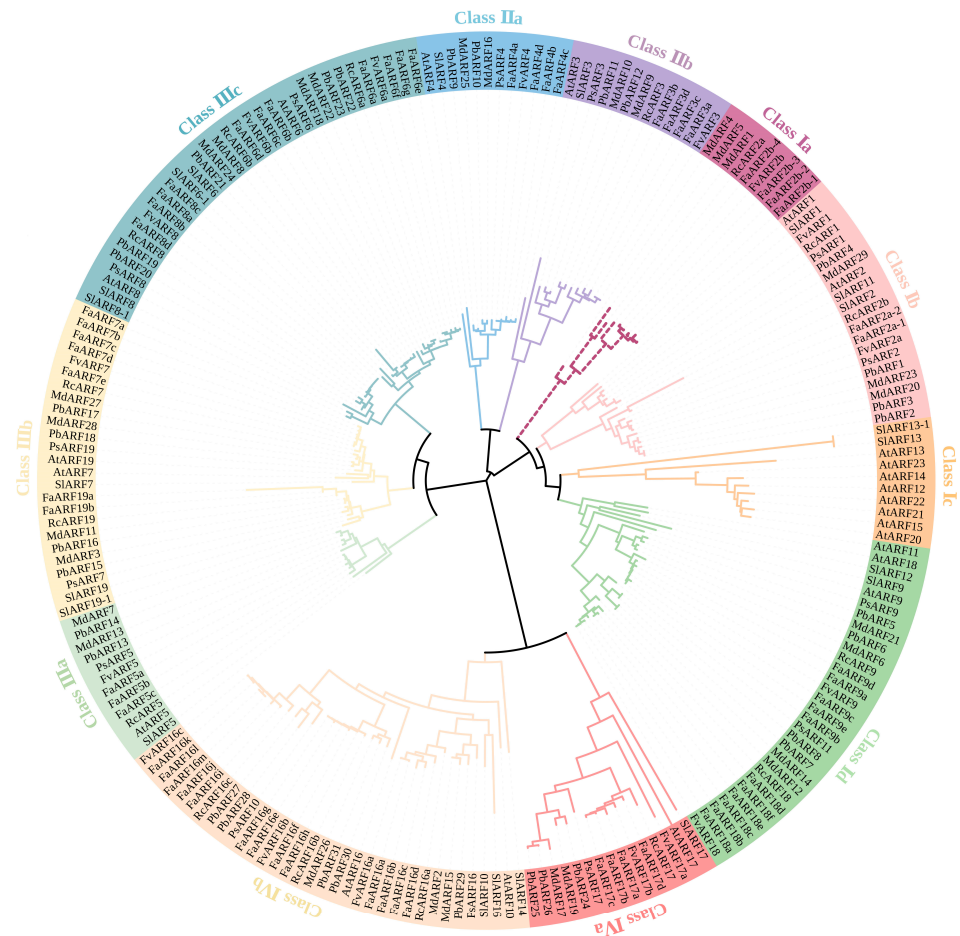


Figure 3. NJ-tree of ARF gene family in seven plants, and these ARFs were divided into four clades. At: *A. thaliana*; Fv: *F. vesca*; Fa: *F. x ananassa*; Pb: *Pyrus bretschneideri*; Ps: *Prunus sibirica*; Rc: *Ricinus communis*; Sl: *Solanum lycopersicum*.

3.3. Expression Levels of Pre-Fan-miR159a–r in Strawberry

In order to compare the regulation of precursors of all fan-miR159 members based on the expression level of mature fan-miR159 at the transcription level, we employed RT-qPCR to detect the expression levels of miR159a–r precursors in the leaves of two strawberry cultivars, ‘Benihoppe’ and ‘Sweet Charlie’. The results demonstrated that the relative expression levels of fan-miR159b and fan-miR159n were higher in leaves of both ‘Benihoppe’ (Figure 4A) and ‘Sweet Charlie’ strawberries (Figure 4B). This suggests that the precursors of fan-miR159b and fan-miR159n exert stronger regulatory effects on the expression level of mature fan-miR159 in the leaves of these two cultivars compared to other precursors. Therefore, fan-miR159b and fan-miR159n were selected for further research.

Additionally, fan-miR159r, which shared the same target genes as fan-miR159n, was also chosen for subsequent studies.

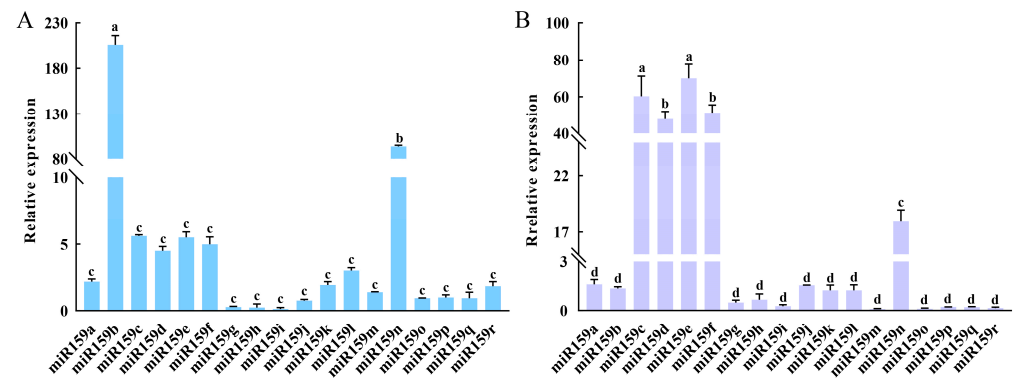


Figure 4. The expression levels of precursors of all fan-miR159 members in the leaves of strawberries 'Benihoppe' (A) and 'Sweet Charlie' (B). The lowercase letters indicate significant differences at $p < 0.05$.

3.4. Cloning of Pre-Fan-miR159 Members and Their Target Genes

Following PCR amplification and 1.5% gel electrophoresis detection, the precursor fragments of fan-miR159a–r were successfully amplified (Figure 5), including a 302 bp of pre-fan-miR159b and 198 bp fragments for pre-fan-miR159n and pre-fan-miR159r. The PCR products were then ligated into a cloning vector, and Sanger sequencing confirmed that these sequences were consistent with the results of sRNA-seq analysis.

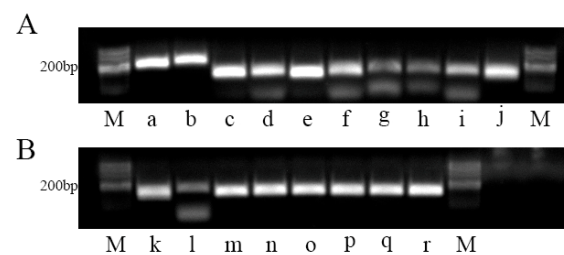


Figure 5. The precursor fragments of fan-miR159a–j (A) and fan-miR159k–r (B) were obtained after PCR amplification and 1.5% gel electrophoresis detection. M, GeneRuler 500 bp DNA ladder.

We further compared the expression levels of genes targeted by fan-miR159 members, including fan-miR159b, fan-miR159n, and fan-miR159r. Based on the RNA-seq data from strawberry embryo and endosperm tissues, the result showed that maker-Fvb6-3-augustus-gene-58.39 (*FaMYB101*) exhibited the highest expression level among the target genes of fan-miR159b (Figure S4A). Meanwhile, maker-Fvb2-4-augustus-gene-182.52 (*FaARF2b-2*) displayed the highest expression level among the target genes of fan-miR159n and fan-miR159r (Figure S4B). Therefore, *FaMYB101* and *FaARF2b-2* were cloned for subsequent verification experiments (Figure S4C,D). PCR amplification and Sanger sequencing results confirmed that the CDS sequence of *FaMYB101* was consistent with the reference genome. However, during the cloning and sequencing of *FaARF2b-2*, in addition to obtaining a gene with a 2028 bp sequence identical to the reference genome, we also identified an alternative variant containing three additional bases (TAG). The gene with a sequence length matching the reference genome was designated as *FaARF2b-2R*, while the variant with the three-base insertion was named *FaARF2b-2M*.

3.5. Co-Expression of *FaMYB101* and Fan-miR159b in Tobacco Leaves

Four recombinant vectors for tobacco co-transformation were constructed: 35SCaMV::G-US, 35SCaMV::fan-MIR159b, 35SCaMV::FaMYB101-GUS, and 35SCaMV::mFaMYB101-GUS

(Figure 6A). Among these, synonymous mutations were introduced into the target site of *FaMYB101* in the *35SCaMV::mFaMYB101-GUS* vector to damage its sensitivity for *fan-miR159b* (Figure 6B). The constructed recombinant vectors were transformed into *Agrobacterium* for activation, infiltrated into tobacco leaves, and the *GUS* gene activity was quantitatively detected by histochemical staining and *GUS* activity. The results showed that the expression level of the *GUS* gene was relatively high in the leaves transformed with *35SCaMV::GUS*, *35SCaMV::FaMYB101-GUS*, and *35SCaMV::mFaMYB101-GUS*, but no expression was observed in the leaves transformed with *35SCaMV::fan-MIR159b*. The expression of *GUS* was significantly reduced in the leaves co-transformed with *35SCaMV::fan-MIR159b* and *35SCaMV::FaMYB101-GUS*, while it was not affected in the leaves co-expressed with *35SCaMV::fan-MIR159b* and *35SCaMV::mFaMYB101-GUS* or in those co-expressed with *35SCaMV::GUS* and *35SCaMV::FaMYB101-GUS* (Figure 6C,D). The negative regulatory effect of *fan-miR159b* on the target gene *FaMYB101* was verified through histochemical staining and quantitative *GUS* activity assays, which was consistent with previous research results [52].

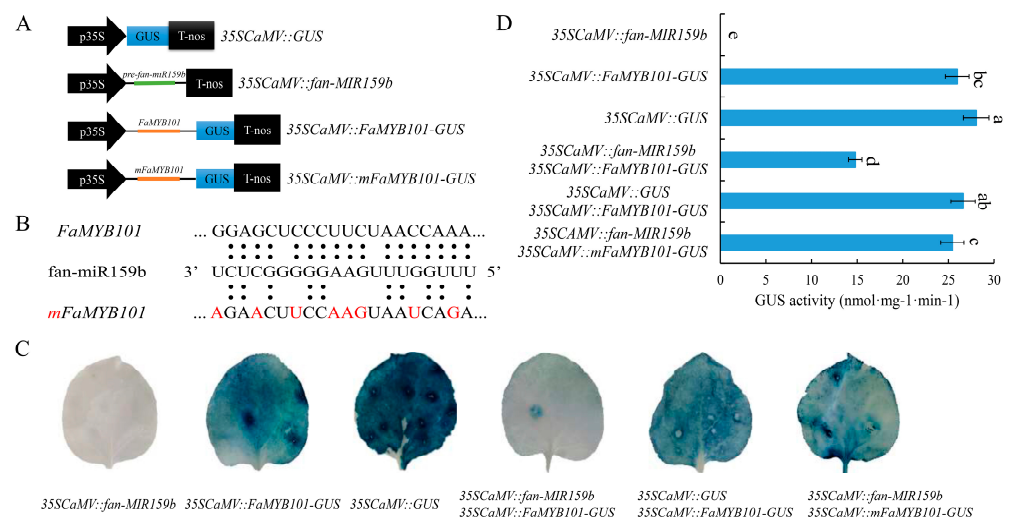


Figure 6. Fan-miR159b targets *FaMYB101*. (A) Four recombinant vectors were constructed for the co-transformation in leaves of tobacco. (B) Synonymous mutations in the targeted sequence region of *FaMYB101*. The red fonts represent synonymous mutations. (C) *GUS* staining of leaves in the tobacco. (D) *GUS* activity in the leaves of tobacco. The lowercase letters indicate significant differences at $p < 0.05$.

3.6. Co-Expression of Two Transcripts of *FaARF2b-2* and *Fan-miR159n/r* in Tobacco Leaves

Seven recombinant vectors were constructed to validate the target relationship of two transcript variants of *FaARF2b-2* (*FaARF2b-2R* and *FaARF2b-2M*) and two miR159 family members (*fan-miR159n* and *fan-miR159r*), including *35SCaMV::GUS*, *35SCaMV::fan-MIR159n*, *35SCaMV::fan-MIR159r*, *35SCaMV::FaARF2b-2R-GUS*, *35SCaMV::FaARF2b-2M-GUS*, *35SCaMV::mFaARF2b-2R-GUS*, and *35SCaMV::mFaARF2b-2M-GUS* (Figure 7A). Synonymous mutations were introduced into the target sites of *FaARF2b-2R* and *FaARF2b-2M* in *35SCaMV::mFaARF2b-2R-GUS* and *35SCaMV::mFaARF2b-2M-GUS* (Figure 7A). Subsequently, the recombinant vectors were transiently transformed into tobacco leaves, and *GUS* gene activity was quantitatively assessed by histochemical staining and *GUS* activity. The results showed that the *GUS* gene was highly expressed in the leaves transformed with *35SCaMV::GUS*, *35SCaMV::FaARF2b-2R-GUS*, *35SCaMV::FaARF2b-2M-GUS*, *35SCaMV::mFaARF2b-2R-GUS*, and *35SCaMV::mFaARF2b-2M-GUS*, but was not expressed in leaves transformed with *35SCaMV::fan-MIR159*. *GUS* expression was significantly reduced in leaves co-transformed with *35SCaMV::fan-MIR159n* and ei-

ther 35SCaMV::FaARF2b-2R-GUS or 35SCaMV::FaARF2b-2M-GUS. However, GUS expression remained unaffected when 35SCaMV::fan-MIR159n was co-expressed with 35SCaMV::mFaARF2b-2R-GUS or 35SCaMV::mFaARF2b-2M-GUS, or when 35SCaMV::GUS was co-expressed with 35SCaMV::FaARF2b-2R-GUS or 35SCaMV::FaARF2b-2M-GUS (Figure 7B). GUS activity assays revealed that GUS activity was significantly reduced in the leaves of tobacco that was co-transformed with 35SCaMV::fan-MIR159n and 35SCaMV::FaARF2b-2M-GUS (Figure 7C). Consistent with this, the leaves co-transformed with 35SCaMV::fan-MIR159n and 35SCaMV::FaARF2b-2R-GUS also showed lower GUS activity (Figure 7D). These findings were confirmed by histochemical staining and quantitative GUS activity assays, and similar results were observed for fan-MIR159r (Figure S5). The results clearly demonstrate the negative regulatory effect of fan-miR159n and fan-miR159r on the target genes FaARF2b-2R and FaARF2b-2M.

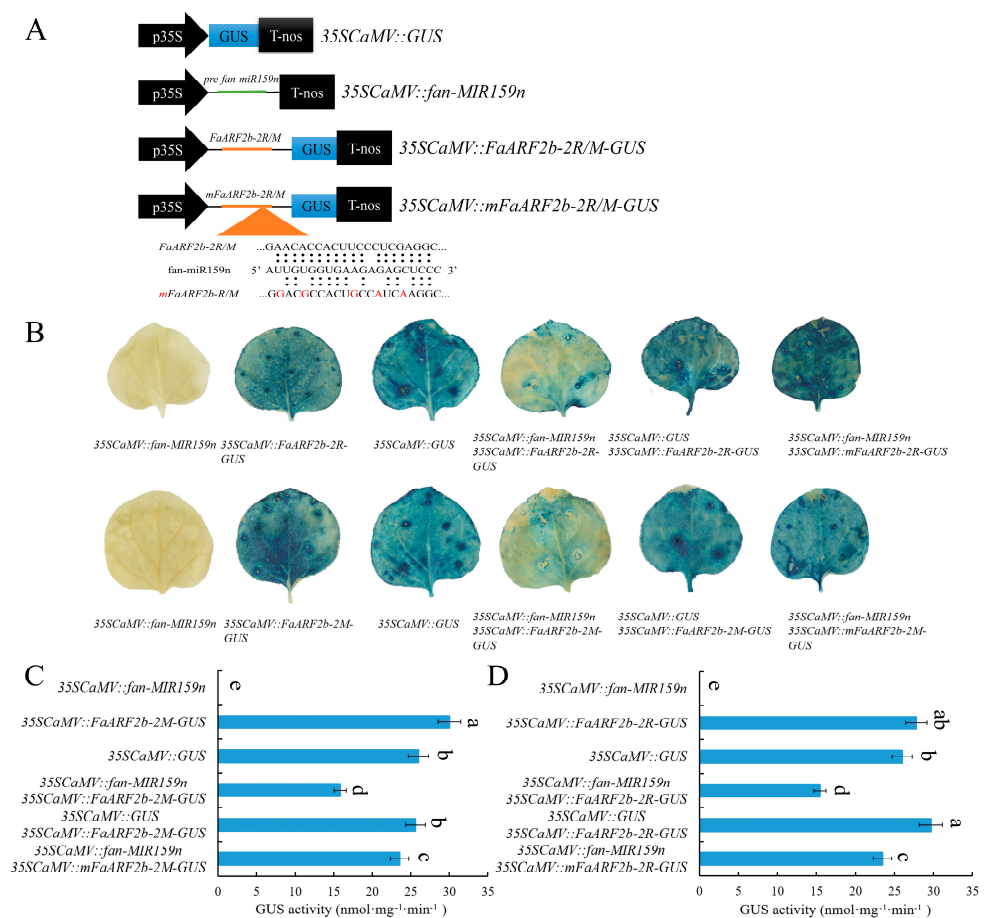


Figure 7. Fan-miR159n targets two transcripts of FaARF2b-2. (A) Six recombinant vectors were constructed for the co-transformation in leaves of tobacco, and synonym mutations in the targeted sequence region of FaARF2b-2M and FaARF2b-2R. Synonymous mutations are in red fonts. (B) GUS staining of leaves in the tobacco. (C) GUS activity in the leaves of tobacco was used for the target relationship of fan-miR159n and FaARF2b-2M. (D) GUS activity in the leaves of tobacco was used for the target relationship of fan-miR159n and FaARF2b-2R. The lowercase letters indicate significant differences at $p < 0.05$.

3.7. Co-Expression of Two Transcripts of FaARF2b-2 and Fan-miR159n/r in the Fruits of Strawberry

To further validate the regulatory relationships between fan-miR159n/r and their target genes FaARF2b-2R and FaARF2b-2M in strawberry, this study continued to perform co-transformation experiments in the cultivated strawberry 'Ningyu'. The constructed recombinant vectors were transiently infiltrated into a receptacle, and the GUS gene activity was subsequently detected by histochemical staining and quantitative

GUS activity assays. The results (Figure 8A) showed that GUS expression was high in receptacles transformed with *35SCaMV::GUS*, *35SCaMV::FaARF2b-2R/M-GUS*, and *35SCaMV::mFaARF2b-2R/M-GUS*, but was not detected in receptacles transformed with *35SCaMV::fan-MIR159n*. And GUS expression was significantly reduced in receptacles co-transformed with *35SCaMV::fan-MIR159n* and either *35SCaMV::FaARF2b-2R-GUS* or *35SCaMV::FaARF2b-2M-GUS*. However, GUS expression remained unaffected in receptacles co-transformed with *35SCaMV::fan-MIR159n* and either *35SCaMV::mFaARF2b-2R-GUS* or *35SCaMV::mFaARF2b-2M-GUS*, or with *35SCaMV::GUS* and either *35SCaMV::FaARF2b-2R-GUS* or *35SCaMV::FaARF2b-2M-GUS*, which was consistent with the results of tobacco co-expression experiments. The results of receptacle co-expression were further confirmed by detecting the expression levels of *FaARF2b-2* in receptacles. Total RNA was extracted from infiltrated receptacles of strawberry, and the expression levels of target genes *FaARF2b-2R* and *FaARF2b-2M* were detected by using RT-qPCR. The results (Figure 8B,C) showed that *FaARF2b-2R* and *FaARF2b-2M* had the weak expression levels in receptacles co-transformed with *35SCaMV::fan-MIR159n* and *35SCaMV::FaARF2b-2R-GUS* or *35SCaMV::FaARF2b-2M-GUS*, as indicated by GUS staining. In contrast, their expression was significantly enhanced in receptacles co-transformed with *35SCaMV::mFaARF2b-2R-GUS* or *35SCaMV::mFaARF2b-2M-GUS* and *35SCaMV::fan-MIR159n*. This was similar to the results observed in receptacles co-transformed with *35SCaMV::fan-MIR159r* and either *35SCaMV::FaARF2b-2R-GUS* or *35SCaMV::FaARF2b-2M-GUS* in strawberry (Figure S6). These finding further confirm the negative regulatory effects of *fan-miR159n* and *fan-miR159r* on two target genes, *FaARF2b-2R* and *FaARF2b-2M*.

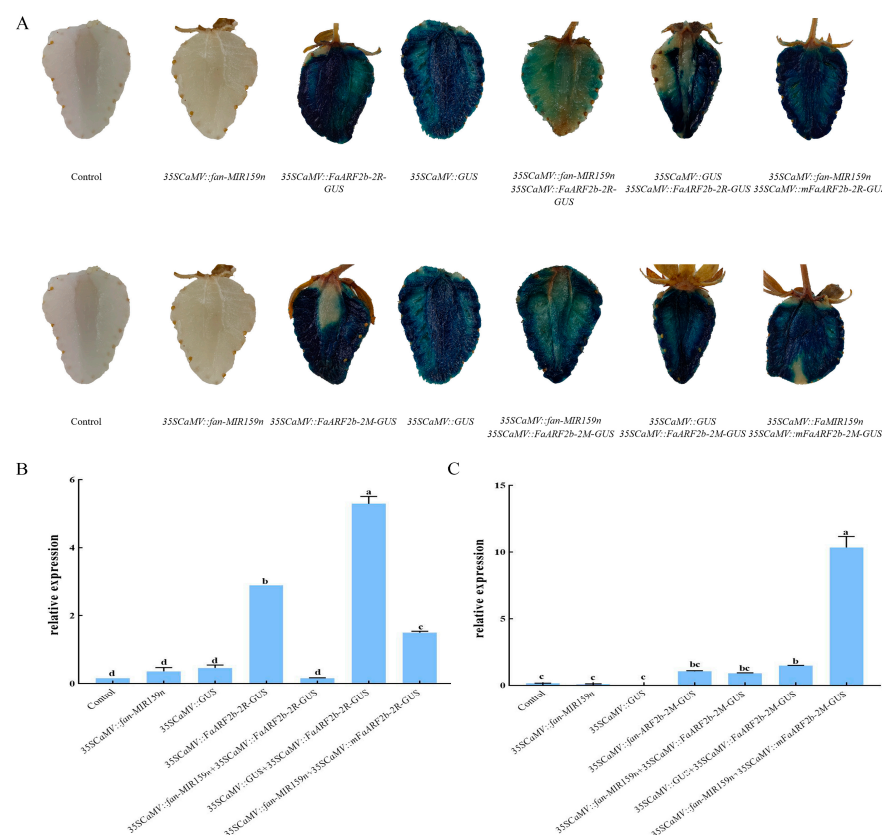


Figure 8. The co-expression of two transcripts of *FaARF2b-2* and *fan-miR159n* in strawberry fruits. (A) Co-transformation in the receptacles of strawberry. (B) The expression level of *FaARF2b-2R* in receptacles. (C) Expression pattern of *FaARF2b-2M* in strawberry receptacles. The receptacles that were injected with the infection buffer without *Agrobacterium* cells served as the control. Lowercase letters indicate significant differences at $p < 0.05$.

3.8. The Tissue-Specific Expression Patterns of *FaARF2b-2R* and *FaARF2b-2M*

In order to clarify the expression characteristics of *FaARF2b-2R* and *FaARF2b-2M* in cultivated strawberry, the relative expression levels of these two transcripts were analyzed by RT-qPCR in various tissues, including the root, short stem, leaves, calyx, petal, anther, receptor, ovary, style, embryo, endosperm, and seed coat from 12 DAP of cultivated strawberry (Figure 9). The results showed that both *FaARF2b-2R* and *FaARF2b-2M* were most highly expressed in the endosperm, with *FaARF2b-2M* exhibiting a higher expression level than *FaARF2b-2R*. *FaARF2b-2R* was moderately expressed in the seed coat, leaf, embryo, and calyx, and lowest in petal. *FaARF2b-2M* showed relatively high expression in the anther, leaf, embryo, and seed coat and the lowest expression in petal. This case showed that two transcripts exhibit distinct tissue-specific expression patterns, and *FaARF2b-2M* displays higher expression than *FaARF2b-2R* in several tissues. This differential expression may be attributed to the three-base insertion.

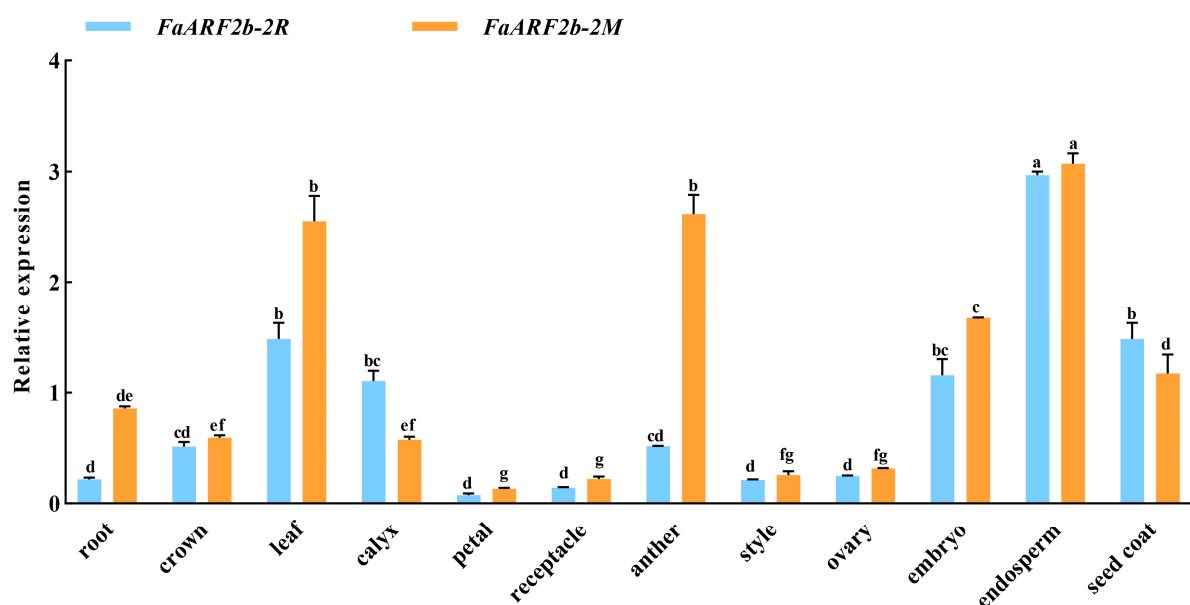


Figure 9. The expression patterns of two transcripts of *FaARF2b-2* in the tissues of strawberry. Lowercase letters in sky blue and orange indicate a significant difference at $p < 0.05$ within gene expression levels in *FaARF2b-2R* and *FaARF2b-2M*, respectively.

3.9. The Expression Patterns of *FaARF2b-2R* and *FaARF2b-2M* After Stress Treatments

To further investigate the functional characteristics of *FaARF2b-2R* and *FaARF2b-2M*, and to explore how the insertion of three bases alters the function of *FaARF2b-2R* relative to the reference genome, we analyzed the relative expression levels of *FaARF2b-2R* and *FaARF2b-2M* in strawberry leaves following stress treatments (Figure 10). The results showed that under low-temperature treatment, both *FaARF2b-2R* and *FaARF2b-2M* exhibited a trend of initial downregulation followed by upregulation, and the expression of *FaARF2b-2R* was significantly affected by low temperature. Oppositely, the expression of *FaARF2b-2R* showed an initial upregulation followed by downregulation after the ABA treatment. Its expression level reached its peak at 72 h and then declined, but it remained higher than that of the control. This indicates that ABA treatment significantly upregulated the expression level of *FaARF2b-2R* (Figure 10A). This pattern is also similar to that of *FaARF2b-2R* after the ABA treatment (Figure 10B). *FaARF2b-2R* and *FaARF2b-2M* exhibited the same expression trend after the drought treatment. At 12 h of treatment with 300 mM mannitol, their expression increased and reached the peak. Compared with *FaARF2b-2M*, *FaARF2b-2R* was more susceptible to the effects of drought. We also analyzed the effects

of salt stress on these two transcripts. RT-qPCR analysis revealed that the effect of salt stress on the expression levels of *FaARF2b-2R* and *FaARF2b-2M* was not as significant as that of low-temperature stress, drought stress, and ABA treatment. Based on the above analysis, we speculated that *FaARF2b-2* may be involved in regulating processes such as low temperature and drought stress, as well as ABA response.

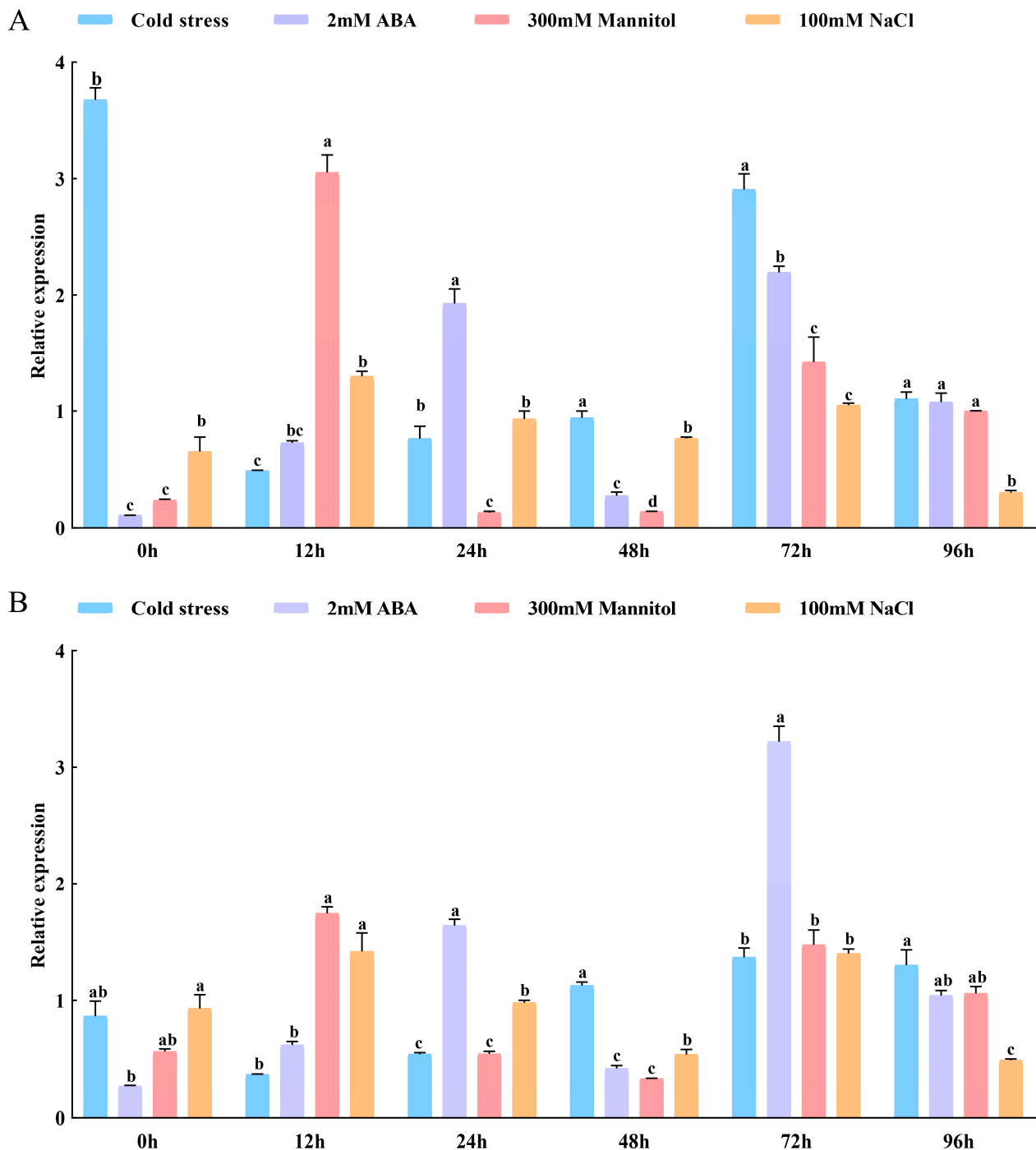


Figure 10. Relative expression profiles of *FaARF2b-2R* (A) and *FaARF2b-2M* (B) from the leaves of *F. × ananassa* under stress treatments. Significance difference analysis of different groups was performed by two-tailed Student's *t* tests. The lowercase letters indicate significant differences at $p < 0.05$.

4. Discussion

4.1. miR159 Family in Plants

MiRNAs are a class of small non-coding RNAs, typically 20–24 nucleotides in length, that are classified into families based on the nucleotide sequences of their mature forms [53,54]. Among these, the miR159 family exhibits a significant size preference for 21-nucleotide sequences and is widely conserved across tracheophyte species [55]. The *Arabidopsis* miR159 family comprises three members: miR159a, miR159b, and miR159c [56]. These miRNAs are 21 nucleotides long and differ only at their 3' termini, with a single-nucleotide difference between miR159a and miR159b and two-nucleotide differences between miR159a and miR159c [57]. Previous studies have shown that the size of the miR159 family is influenced by the evolutionary dynamics. For instance, four members of the miR159 family have been identified in *Populus* 'Nanlin895' [58], six in rice [59], and ten and eleven members in *Dendrobium officinale* [60] and maize [61], respectively. Among these, some members exhibit greater sequence variation, with 3–5-nucleotide differences, a pattern also observed in this study. Of the 18 mature fan-miR159s, fan-miR159a/b, fan-miR159c/d/e/f, fan-miR159g/h/i, fan-miR159j/k, and fan-miR159m/n/o/p/q/r share identical sequences within each group (Figure 1B). We found that fan-miR159c/d/e/f are completely consistent with *Arabidopsis* miR159a, differing from fan-miR159m/n/o/p/q/r by seven nucleotide variations located at the 5' and 3' termini, as well as at the fifth, seventh, and thirteenth positions of the sequence. These sequence variations result in differences in their target gene specificity. To explore the evolutionary relationships of fan-miR159m/n/o/p/q/r, a phylogenetic tree was constructed based on the stem-loop regions of pre-miR159 from a broad range of land plants (Figure 2). The tree showed that the strawberry miR159m/n/o/p/q/r precursors are highly similar, suggesting functional redundancy among them. In addition, these precursors show up closer to pre-pab-miR159d, pre-gma-miR159b/c/f, and pre-mtr-miR159b in the ML tree. We have conducted a comprehensive review of the relevant literature pertaining to these miRNAs, including pab-miR159d [62], gma-miR159b/c/f [63], and mtr-miR159b [64]. However, no predicted target genes were identified within Norway spruce, soybean, and *Medicago truncatula*. Furthermore, pre-fan-miR159c and pre-fan-miR159g have a closer distance with pre-fve-miR159a and pre-fve-miR159c in the tree, respectively. We found that these miRNAs share identical target genes in *F. × ananassa* and *F. vesca* [65], specifically GAMYB-related genes, indicating a high degree of evolutionary conservation among these miRNAs.

4.2. miR159s and Their Targets in Plants

In *Arabidopsis*, computational predictions suggest that miR159 mainly targets eight MYB genes: MYB33, MYB65, MYB81, MYB97, MYB101, MYB104, MYB120, and MYB125 (*DUO1*) [66]. These genes contain highly conserved miR159 binding sites and encode conserved GAMYB-like genes of transcription factors, with the exception of *DUO1* [67]. Silencing efficacy assays have shown that MYB81, MYB97, MYB101, MYB104, and *DUO1* are poorly silenced, whereas MYB33 and MYB65 are potently silenced [68]. The miR159:GAMYB regulatory module has been experimentally validated in various plant species, including *Arabidopsis* [52,69,70], soybean [71], cotton [72], tobacco [73], polar [58], as well as horticultural plants such as orchids [74], gloxinia [75], peach [76], and tomato [77]. The functional role of this module has been extensively studied and is known to be involved in the male reproductive ability, seed development, vegetative growth, and flowering-time control [20]. In *Arabidopsis*, functional redundancy exists between MYB33 and MYB65, based not only on their high sequence similarity and identical expression patterns in flowers but also on the absence of phenotypic alterations in either single mutant [18]. A similar pattern is observed for miR159a and miR159b, where *MIR159a* and *MIR159b* have simi-

lar expression patterns consistent with *MYB33* repression, and only the *mir159ab* double mutant exhibits pleiotropic development defects, including stunted growth, curled leaves, and reduced apical dominance [70]. The miR159:*MYB33* module can also promote drought tolerance and is correlated with the accumulation of the osmoprotective compounds proline and putrescine in tomato [78]. Moreover, recent studies have shown that this module, in conjunction with ABA signaling pathway, regulates adventitious rooting in poplar [58] and bud dormancy in apple [79].

In addition to the canonical *GAMYB* targets, miR159 also recognizes additional non-canonical targets. For instance, *SGN-U567133*, which is unrelated to *MYB* genes and contains a NOZZLE-like domain, has been shown to be post-transcriptionally regulated by miR159 in the tomato leaf and flower development [23]. A non-canonical target of miR159, *CKX6*, was uncovered in rose, where the miR159-*RhCKX6* module regulates the duration of the cell division phase by modulating cytokinin catabolism in petals [24]. In this study, we also identified additional targets of miR159 that belongs to the *AFR* gene family. To confirm these candidate targets, we further investigated the case that the suppression of *FaARF2b-2* expression by fan-miR159n and fan-miR159r in both tobacco and strawberry (Figures 7 and 8). However, the transient overexpression experiments do not provide direct evidence supporting the targeted relationship between fan-miR159 and *FaARF2b-2*. The 5' RNA ligase-mediated rapid amplification of cDNA ends (5' RLM-RACE) assay is a key method for identifying miRNA-directed target cleavage sites and has been widely regarded as the standard approach for validating authentic miRNA-mRNA interactions [80]. We made multiple attempts to perform this assay; however, it was unsuccessful, likely due to the unique characteristics of endosperm tissue. Therefore, additional experiments are required to further confirm this targeting relationship, such as degradome sequencing and silencing *FaARF2b-2* via a virus-induced gene silencing (VIGS) approach. Furthermore, what regulatory role does the miR159n/r-*FaARF2b-2* module play in strawberry development, and which downstream genes are affected during this regulatory process? These are intriguing scientific questions that warrant further investigation. In subsequent research, it is imperative to establish VIGS and overexpression lines of miR159 and *FaARF2b-2* in strawberries to elucidate these mechanisms.

4.3. Two Transcripts of *FaARF2b-2* May Play Different Roles in the Development of Strawberry

In this study, two mRNA isoforms of the *FaARF2b-2* gene were detected in cultivated strawberry. Polyploidy and alternative splicing greatly contribute to producing multiple transcript isoforms in eukaryotes [81]. *F. × ananassa* is an allo-octoploid derived from spontaneous hybrids [82]. Previous studies have shown that 7204 genes have five or more splice isoforms in *F. × ananassa*, with the *gene06434-FvARF2* protein-coding locus giving rise to 34 transcripts [83]. Due to gene expression redundancy associated with polyploidy, we examined the expression patterns of *FaARF2b-2R* and *FaARF2b-2M* across various strawberry tissues (Figure 9). The results showed that *FaARF2b-2R* and *FaARF2b-2M* exhibit a similar expression pattern, with higher expression levels observed in leaf and seed-related tissues, such as the embryo, endosperm, and seed coat. In *Arabidopsis*, three *arf2* insertion mutants exhibit similar pleiotropic developmental phenotypes, including an enlarged leaf size, elongated and thickened inflorescence stems, abnormal flower morphology, sterility, delayed senescence, delayed abscission, etc. [84]. Therefore, we speculate that the strawberry *ARF2* may also possess similar functions. In addition, *FaARF2b-2M* exhibits significantly higher expression levels in the anther compared to *FaARF2b-2R*, suggesting a potentially important role of *FaARF2b-2M* in male reproductive development. Moreover, the expression levels of both *FaARF2b-2M* and *FaARF2b-2R* increased following ABA treatment, indicating that ABA may promote the expression of *FaARF2b-2* (Figure 10). However,

previous studies have shown that *ARF2* acts as a negative regulator in the ABA response pathway, controlling seed germination and primary root growth [85]. Consequently, the function of *FaARF2b-2* will be a focus of our further research.

In general, this study validates previous findings that the miR159 family likely targets two classes of genes, *GAMYBs* and *ARFs*, in strawberry. We have initially demonstrated the targeting relationship between fan-miR159 and *FaARF2b-2* and have preliminarily characterized the functional role of *FaARF2b-2*.

5. Conclusions

This study reveals that certain members of the miR159 family in cultivated strawberry target genes belonging to the *ARF2b* subfamily, rather than *ARF23-like* genes as previously annotated. The target gene *FaARF2b-2* generates two distinct transcripts, which potentially have functions in the development of the anther and endosperm, as well as mediating positive responses to stress treatments in strawberry. These findings not only enhance our molecular understanding of the miR159-*ARF2b-2* module in strawberry but also expand the known functional scope of this conserved miRNA family. Moreover, they provide initial insights into the biological roles of *FaARF2b-2*.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/horticulturae11121443/s1>, Figure S1: The stem-loop structures of pre-fan-miR159 were predicted by the RNA fold; Figure S2: Conserved domain of *ARF* gene family in strawberries, *F. vesca* (A) and *F. × ananassa* (B); Figure S3: Motif analysis of *ARF* proteins in strawberries; Figure S4: Cloning and expression patterns of fan-miR159 target genes in strawberry. (A) The expression pattern of target genes of fan-miR159b. (B) The expression level of target genes of fan-miR159n and fan-miR159r. (C) The CDS sequence of *FaMYB101* was cloned by PCR amplification. M, GeneRuler 5000 bp DNA ladder. (D) *FaARF2b-2* was cloned by PCR amplification, M, GeneRuler 5000 bp DNA ladder; Figure S5: Fan-miR159r targets two transcripts of *FaARF2b-2*. (A) Recombinant vectors were constructed for the co-transformation in leaves of tobacco. (B) *GUS* staining of tobacco leaves. (C) *GUS* activity in the leaves of tobacco was used to the target relationship of fan-miR159r and *FaARF2b-2M*. (D) *GUS* activity in the leaves of tobacco was used to the target relationship of fan-miR159r and *FaARF2b-2R*; Figure S6: The co-expression of two transcripts of *FaARF2b-2* and fan-miR159r in strawberry fruits. (A) Co-transformation the *FaARF2b-2R* and fan-miR159r in the receptacles of strawberry. (B) Co-transformation the *FaARF2b-2M* and fan-miR159r in the receptacles. (C) Expression pattern of *FaARF2-2R* and *FaARF2-2M* in strawberry receptacles; Table S1: The primers in this study; Table S2: The fan-miR159 members and their candidate target genes; Table S3: Basic information on *ARF* family genes in *Fragaria vesca* and *Fragaria × ananassa*; Table S4: Physical and chemical parameters of *ARF* proteins in *Fragaria*.

Author Contributions: Conceptualization, X.J., X.H. and Y.Q.; methodology, X.J. and X.H.; software, X.J. and X.H.; validation, X.J., X.H. and Q.A.; formal analysis, X.J. and X.H.; investigation, C.N., E.W. and Y.Q.; resources, X.J., Q.Z. and Y.Q.; data curation, X.J. and X.H.; writing—original draft preparation, X.J.; writing—review and editing, X.J., Q.Z. and J.L.; visualization, X.J. and X.H.; supervision, Y.Q.; project administration, H.Y. and Y.Q.; funding acquisition, Y.Q. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

5' RLM-RACE	5' RNA ligase-mediated rapid amplification of cDNA ends
AGO	Argonaute
ARF	Auxin Response Factor
cDNA	complementary DNA
CDS	coding regions
CKX6	Cytokinin Oxidase/Dehydrogenase6
DAP	days after pollination
GAMYB	encode gibberellin-induced MYB
miRNA	microRNA
MUG	methylumbelliferyl- β -D-glucuronide
SAM	shoot apical meristem

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