Two strawberry miR159 family members display developmental-specific expression patterns in the fruit receptacle and cooperatively regulate *Fa-GAMYB*

Fabiana Csukasi, Livia Donaire, Ana Casañal, Llúcia Martínez-Priego, Miguel A. Botella, Nieves Medina-Escobar, César Llave, Victoriano Valpuesta
First published: 11 April 2012
https://doi.org/10.1111/j.1469-8137.2012.04134.x
Citations: 56
Author for correspondence:
Victoriano Valpuesta
Tel: +34 952 131 932
Email: valpuesta@uma.es

Summary

We have reported previously that the gibberellin (GA) content in strawberry receptacle is high, peaking at specific stages, pointing to a role of this hormone in fruit development. In Arabidopsis, miR159 levels are dependent on GA concentration. This prompted us to investigate the role of two members of the miR159 family and their putative strawberry target gene, *GAMYB*, in relation to changes in GA content during the course of fruit development.

The highest expression level of the two *Fa-MIR159* genes was in the fruit's receptacle tissue, with dramatic changes observed throughout development. The lowest levels of total mature miR159 (a and b) were observed during the white stage of receptacle development, which was concurrent with the highest expression of *Fa-GAMYB*. A functional interaction between miR159 and *Fa-GAMYB* has been demonstrated in receptacle tissue.

The application of bioactive GA (i.e. GA_3) to strawberry plants caused the down-regulated expression of *Fa-MIR159a*, but the expression of *Fa-MIR159b* was not affected significantly. Clear discrepancies between *Fa-MIR159b* and mature Fa-miR159b levels were indicative of post-transcriptional regulation of *Fa-MIR159b* gene expression.

We propose that Fa-miR159a and Fa-miR159b interact with *Fa-GAMYB* during the course of strawberry receptacle development, and that they act in a cooperative fashion to respond, in part, to changes in GA endogenous levels.

Introduction

Strawberries (*Fragaria × ananassa*) are a commercial crop in the Rosaceae family in the genus *Fragaria*. This edible fruit is highly appreciated for its organoleptic properties and health-promoting benefits (Seeram, 2008). The strawberry fruit is, in reality, a false fruit composed of the achenes (true fruits) and the receptacle, which arises from the development of the flower receptacle. During strawberry development, both the achenes and the receptacle follow separate patterns, but their developmental programmes are coordinated. Indeed, a recent study performed parallel profiling of primary and secondary metabolism in both the strawberry receptacle and the achene across several developmental stages. The results displayed a high level of metabolic synchrony and specialization during development (Fait *et al.*, 2008), and it has been known for some time that auxin plays a major role in this developmental coordination. Auxin is synthesized in the achenes and promotes receptacle growth during the early stages of fruit development (Nitsch, 1950). Free auxin reaches its maximum level in both the achene and the receptacle during the green stage and subsequently declines as the fruit ripens (Manning, 1998). The involvement of other hormones during strawberry ripening and the obligated interaction between the achene and the receptacle are unclear. Many endogenous gibberellins (GAs) have been identified in strawberry fruit (Blake *et al.*, 2000), but there have been no conclusive results on the role of this hormone during fruit development and ripening (Bustamante *et al.*, 2009). Recently, we have reported that bioactive GA_4 is notably abundant in the receptacle, with peak levels being observed during

the white stage of development. We also identified the GID1-DELLA pathway as being operative in this tissue (Csukasi *et al.*, 2011).

The importance of microRNA (miRNA)-directed gene regulation during plant development has been widely documented (Jones-Rhoades *et al.*, 2006). Specifically, miRNAs regulate gene expression in animals and plants through the degradation and translational arrest of their target mRNAs. In plants, the 'mature' miRNAs are *c.* 21 nucleotides long and control such processes as stem cell formation, organ identity, leaf polarity, vascular differentiation and cell division patterns (Jones-Rhoades *et al.*, 2006). The mature miRNAs are processed by Dicer (DCL1), a type III RNase, from the longer, noncoding RNA precursors, which are known as primary (pri) miRNAs. The *Arabidopsis* genome contains *c.* 180 miRNA loci, many of which are highly conserved between plant species (Axtell & Bartel, 2005; Rajagopalan *et al.*, 2006). However, information on the role of miRNAs in the most important fruit crops, and their contributions to processes such as fruit development and ripening, is limited. Northern blotting and deep sequencing of short RNAs in tomato fruit identified several conserved and nonconserved miRNAs, and several mRNAs associated with fruit development were validated as tomato miRNA targets (Pilcher *et al.*, 2007; Moxon *et al.*, 2008). Similarly, several conserved miRNAs and grape-specific miRNAs were identified by massive sequencing in grapes and were functionally validated experimentally. Interestingly, the expression levels of the nonconserved miRNAs were slightly higher in ripe berries than in vegetative tissues (Carra *et al.*, 2009). Using microarray technology, differential miRNA expression was observed in both conventional and micropropagated strawberries (Li *et al.*, 2009).

Several studies have demonstrated the interaction between miRNAs and phytohormones in various plant responses (Liu & Chen, 2009). In Arabidopsis, GA and abscisic acid (ABA) modulate miR159 expression during anther development (Achard *et al.*, 2004) and seed germination (Reyes & Chua, 2007), respectively. Interestingly, miR159 cleaves *GAMYB* transcripts in both Arabidopsis and rice (Achard *et al.*, 2004; Tsuji *et al.*, 2006; Allen *et al.*, 2007). The *GAMYB* genes, initially identified in barley aleurone cells (Gubler *et al.*, 1995), regulate the transcription of GA-responsive genes by binding to a GA-response element (GARE) in their promoter regions (Gubler *et al.*, 1999).

In this study, we investigated the involvement of two members of the Fa-miR159 family and one *Fa-GAMYB* member during strawberry fruit development and ripening. These three genes showed tissue- and developmental stage-specific expression patterns, notably in the receptacle of the fruit. A functional interaction between Fa-miR159 and *Fa-GAMYB* was also demonstrated in this tissue. Our results point to a role of two Fa-miR159 family members in receptacle development. The Fa-miR159 signal is channelled through *Fa-GAMYB*, possibly as a response to changes in the endogenous GA levels in this tissue.

Materials and Methods

Plant material, growth conditions and hormone treatment

Strawberry plants (*Fragaria* × *ananassa* Duchesne) of cv Camarosa were grown under field conditions in Huelva in the southwest of Spain. Samples were harvested at five different developmental stages, namely the closed and open flowers (CF, OF) and during the green (G), white (W) and red (R) fruit stages. The vegetative tissues collected for analysis were the leaves (L) and roots (Rt). All tissues were flash frozen immediately in liquid nitrogen and stored at −80°C until use.

For the GA_3 treatment, plants of the same cultivar were grown *in vitro* in Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) supplemented with 15 g l^{-1} sucrose and 6% agar (w/v), and a solution of 100 μM GA_3 in water was added. Control plants were grown in the same medium without GA_3. Growth conditions for all the *in vitro* strawberry plants, as well as the Arabidopsis plants, were identical. Short days (SDs) were made up of16/8 h of light (200 μmol m^{-2} s^{-2})/dark and a 24/17°C regimen, and long days (LDs) were 8/16 h and 23/17°C.

RNA extraction and gene expression analysis

Total RNA was extracted from strawberry tissues as described previously (Manning, 1991). Total RNA from Arabidopsis was extracted from the rosette tissues using TRIzol® Reagent (http://www.invitrogen.com/) according to the manufacturer's instructions. DNAseI (Sigma, AMPD1)

was applied to the samples to eliminate DNA from RNA samples according to the manufacturer's instructions. Reverse transcription-polymerase chain reactions (RT-PCRs) were performed with 1 μg of total RNA using the iScript cDNA Synthesis kit from Bio-Rad (http://www.bio-rad.com) according to the manufacturer's instructions.

Gene expression analysis was performed by real-time quantitative PCR using the fluorescent intercalating dye, SYBR Green, in an iCycler detection system (Bio-Rad). Reactions were performed in triplicate. The absence of primer dimers was confirmed by melting curve examination. *FaRib413* was used to normalize the data because it was identified by northern analysis to be constitutively expressed in a variety of strawberry plant tissues, including during the various fruit stages (Casado-Diaz *et al.*, 2006). Relative expression level quantification was performed using the comparative C_t method (Pfaffl, 2001), where a value of unity was given to the sample with the minimum expression. Primers used for expression analysis are shown in Supporting Information Table S1; the minimal efficiency considered for the primers was 90%. The specificity of the primers was verified before their use for quantitative RT-PCR; no amplification of *FaMIR159a* was observed when *FaMIR159b* primers were used and vice versa; also no amplification of *AtMIR159* genes was observed with strawberry primers.

For the quantitative stem-loop RT-PCR of the mature miR159, RT reactions were performed in the presence of 2 mM stem-loop miR159a RT primer (5′-GCCTCTCATGCTGACGAATTTTGAGAGGCTAGAGCTCC-3′, Roche) or miR59b RT primer (5′-GCCTCTCATGCTGACGAATTTTGAGAGGCTAGATCA-3′) as described previously with several modifications (Bombarely *et al.*, 2010). RT reactions were incubated in a thermocycler for 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. All reactions, including RT minus controls, were performed in duplicate. The RT products were diluted to a final volume of 200 ng μl^{-1} to avoid potential primer interference in the subsequent quantitative PCR. Next, quantitative PCR was performed on a Corbett RG6000 thermocycler in a total volume of 15 μl. The reaction included 3 μl of diluted RT product, 2 × FastStart Universal Probe Master Mix (Roche), 0.2 mM of TaqMan probe complementary to miR159a (5′ FAM-TTGAGAGGCTAGAGCTCCCTTCA-BBQ 3′, Roche) or to miR159b (5′ FAM-TTGAGAGGCTAGAGCTCCCTTCA-BBQ 3′, Roche) and 0.5 mM of each PCR primer (i.e. miR159a-F 5′-GAATTCGACCCTTTGGATTG-3′, miR159b-F 5′-GAATTCGACCCTTTGGATTG-3′ and miR159-R 5′-GCCTCTCATGCTGACGAAT-3′ (Roche)). The reactions were incubated in 0.1-ml tubes at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. All reactions were performed in duplicate in three different PCR runs. The concentration of Fa-miR159 was calculated by converting the C_t value into an absolute copy number using a standard curve generated from a diluted series of synthetic miR159a or miR159b RNA oligos and RotorGene 6000 software (Corbett).

miRNA-guided cleavage validation

A modified RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) protocol was used to map internal miRNA-directed cleavage sites of predicted cDNA targets (Llave *et al.*, 2002). Total RNA was directly ligated to the 5′ RNA adapter without any further enzymatic pretreatment. Ligated RNA was reverse transcribed using gene-specific primers that annealed *c.* 300 nucleotides downstream of the predicted cleavage site of the target mRNA. PCR amplification of the first-strand cDNA was performed using a reverse gene-specific primer and a forward primer derived from the RNA adapter sequence. The 5′ RACE amplification products were gel purified, cloned and sequenced.

Plasmid construction and plant transformation

A partial cDNA clone of the miR159a primary transcript was isolated from a cDNA library prepared from the green fruits of strawberries after extensive sequencing of several thousand clones (Bombarely *et al.*, 2010). A full-length clone was obtained using the Invitrogen 5′ RACE system for the Rapid Amplification of cDNA Ends, version 2.0, according to the manufacturer's instructions. Full-length cDNAs of the *Fa-MIR159b* and *Fa-GAMYB* genes were obtained by PCR from cDNA prepared from the RNA extracted from fruits during different developmental stages. Gene-specific primers were based on the sequences of the *Fragaria vesca* expressed sequence tags (ESTs) DY667997.1 and DY669997.1, which displayed high homology to the Arabidopsis pre-miR159 and *GAMYB*-like genes.

For the *35S::Fa-MIR159a* construct, the full-length sequence of *Fa-MIR159a* was PCR amplified using the primers described in Table S1, subcloned into the pGEM®-T Easy vector (Promega, http://www.promega.com/) and introduced into the *Not*I/*Asc*I sites of the pENTR/D-TOPO® vector (Invitrogen). The clone was finally inserted into the pKGCS-StrepII vector using the LR reaction (Invitrogen). This vector was introduced into *Agrobacterium tumefaciens* (GV3101-pMP90-RK) by electroporation (Sambrook & Maniatis, 1989) and transformed into Arabidopsis using the floral dip method (Clough, 2004).

Northern blot hybridization

RNA blot analysis using 5 μg of total RNA was performed as described by Llave *et al.* (2000). Briefly, a radiolabelled DNA probe from the cloned 5′ RACE *Fa-GAMYB* product was created by a random priming reaction in the presence of ^{32}P-dCTP. The blot was both prehybridized and hybridized at 38°C using the Perfect-Hybrid buffer (Sigma). Ethidium bromide staining of the gel before the blot transfer was used as the loading control. The hybridization signals were measured using densitometry on the RNA blot exposed to autoradiography films.

Locus names and accession numbers

TAIR: At-miR159a, AT1G73687; pre-At-miR159b, AT1G18075; pre-At-miR159c, AT2G46255; AtMYB33, AT5G06100; AtMYB65, AT3G11440; AtMYB101, AT2G32460; AtMYB81, AT2G26960; AtMYB104, AT2G26950; AtMYB97, AT4G26930; AtMYB120, AT5G55020.

GenBank: Pri-Fv-miR159b, DY667997.1; pri-Os-miR159a, CT846637.1; pri-Sl-miR159, EG553913.1; pri-Vv-miR159, EE104779.1; pri-Md-miR159, CO755162.1; FvGAMYB, EX660375.1.

Results

Identification of strawberry miR159 and *GAMYB* gene homologues

The high content of active GA_4 in the receptacle of strawberry fruit (Csukasi *et al.*, 2011) and the fact that the GA-DELLA signalling pathway regulates miR159 in Arabidopsis (Achard *et al.*, 2004) prompted us to investigate a possible role for this miRNA during strawberry fruit development. The complete cDNA sequences corresponding to the homologues of two Arabidopsis *MIR159* genes (*Fa-MIR159a* and *Fa-MIR159b*) were cloned from strawberries. Sequence analyses revealed striking differences in their nucleotide composition. Pairwise sequence alignment of the primary transcript regions corresponding to the Fa-miR159 precursors (pre-Fa-miR159a and pre-Fa-miR159b) revealed a degree of identity of 53% between them (Fig. 1a). As observed in Arabidopsis (Allen *et al.*, 2007), different polyadenylation sites were identified in the strawberry primary transcripts of Fa-miR159a and Fa-miR159b (Supporting Information Fig. S1a). Four putative polyadenylation sites were identified in *Fa-MIR159a*, with a 790-nucleotide segment being the largest possible transcript. Three putative polyadenylation sites were predicted for *Fa-MIR159b*, with the largest transcript being 890 nucleotides in length. The putative Fa-miR159-generating precursors were inferred from secondary structure analysis. The MFOLD program (http://mfold.bioinfo.rpi.edu/) predicted a characteristic stem-loop structure for each pre-Fa-miR159 with free energy values in a range similar to those calculated previously for miRNA precursors (Fig. 1b). As reported for members of the same miRNA family in other species (Jones-Rhoades *et al.*, 2006), the mature miRNA of both precursors was located in the identical stem arm (Fig. 1b). The canonical, mature miR159 sequences derived from each precursor differed in both their terminal 5′ and 3′ end nucleotide positions (Fig. 1d).

A phylogenetic tree was generated using the stem-loop regions of the pre-miR159 sequences of several different species (Fig. 1c). The putative fold-back pre-miR159 sequences from tomatoes (*Solanum lycopersicum*), grapes (*Vitis vinifera*) and apples (*Malus domestica*) were inferred after performing a BLAST search. As shown in Fig. 1(c), pre-Fa-miR159a was grouped with pre-Os-miR159 and pre-Vv-miR159, whereas pre-Fa-miR159b was clustered with pre-At-miR159a and pre-At-miR159b. The mature Fa-miR159a and Fa-miR159b sequences were similar to those of both Arabidopsis and rice (Fig. 1d).

Known miR159 targets include the Arabidopsis *GAMYB*-like genes *MYB33* and *MYB65* (Millar & Gubler, 2005), which belong to a gene family encoding transcription factors that are commonly involved in diverse developmental processes. This finding led us to search for members of the *GAMYB*-like gene family in EST sequences from *F. vesca*. The high degree of sequence similarity of the *F. vesca* and *Fragaria* × *ananassa* ESTs (Bombarely *et al.*, 2010) allowed for the design of sequence-specific primers to clone the corresponding full-length cDNA from *F. ananassa*. Nucleotide sequence analyses revealed the presence of a unique miR159 cleavage site that exhibited extended complementarity to both Fa-miR159a and Fa-miR159b. Because the deduced amino acid sequence of the Fa-GAMYB-encoded protein possessed an R2R3 domain (Achard *et al.*, 2004; Tsuji *et al.*, 2006; Gong & Bewley, 2008) and box 1, 2 and 3 conserved motifs (Achard *et al.*, 2004; Tsuji *et al.*, 2006; Gong & Bewley, 2008), both of which are characteristic of transcription factors encoded by *GAMYB*-like genes, this gene was referred to as *Fa-GAMYB* (Fig. S1b).

The amino acid sequence of the Fa-GAMYB protein was aligned to the Arabidopsis MYB proteins encoded by genes containing a predicted miR159 binding site (Millar & Gubler, 2005). Interestingly, the Fa-GAMYB protein exhibited the highest degree of similarity to the Arabidopsis *GAMYB*-like gene-encoded proteins MYB33 and MYB65 (Fig. 2a), and base paired with near-perfect complementarity with Fa-miR159a and Fa-miR159b (Fig. 2b). Both MYB33 and MYB65 were sensitive to miR159-directed regulation via endonucleolytic cleavage of their mRNAs (Achard *et al.*, 2004). In conclusion, we revealed the existence of at least two miR159 loci in the strawberry genome and a putative target gene encoding Fa-GAMYB.

Ectopic expression of Fa-miR159a restores a *mir159*-defective phenotype in Arabidopsis

We tested the hypothesis that the predicted *Fa-MIR159a* gene was competent to yield functional, mature miR159 sequences. Genetic transformation often constitutes an overwhelming proof-of-function for a gene product; however, the inherent difficulty associated with strawberry transformation and the lack of appropriate tissue-specific promoters in this species prompted us to use a heterologous system. It has been reported that the Arabidopsis *mir159a* and *mir159b* single mutants do not show any severe developmental defects, suggesting that they are functionally redundant (Allen *et al.*, 2007). However, *mir159a*, *mir159b* double mutants present reduced apical dominance, curled leaves, shorter siliques and smaller seeds with an irregular shape as a result of the inactivation of the miR159-guided regulatory pathway (Allen *et al.*, 2007). The Arabidopsis *mir159a*, *mir159b* double mutant, as well as the wild-type (WT) Columbia ecotype, were transformed with the full-length *Fa-MIR159a* sequence under the control of the *35S* promoter (*35S::Fa-MIR159a*). The defective *mir159a*, *mir159b* phenotype was rescued by expression of *Fa-MIR159a* in Arabidopsis plants grown using an SD regimen (Fig. 3a). The same complementation pattern was observed in plants cultivated using LD conditions (Fig. S2). These results provided experimental evidence that *Fa-MIR159a* was effective at complementing both *miR159a* and *mir159b* loss-of-function genes in *Arabidopsis*.

We investigated the phenotypic rescue in miR159 double mutants to determine whether it was the consequence of Fa-miR159-guided negative regulation of Arabidopsis *GAMYB* target genes. We first confirmed the presence of the *Fa-MIR159a* transgene in the Arabidopsis *mir159a*, *mir159b* double mutants (Fig. 3b). The relative expression of *AtMYB65*, a known target of miR159 in Arabidopsis (Achard *et al.*, 2004), was determined in both the *miR159a*, *miR159b* double mutant plants and in the *Fa-MIR159a*-complemented transgenic line. *AtMYB65* mRNA levels were elevated in the *mir159a*, *mir159b* double mutants compared with the WT plants. This result was consistent with the lack of miR159 activity in the mutant (Fig. 3c). By contrast, ectopic expression of the orthologous *Fa-MIR159a* in the mutant background reduced the expression of *AtMYB65* to WT levels (Fig. 3c). Furthermore, ectopic expression of *Fa-MIR159a* in a WT Arabidopsis background resulted in a down-regulation of the *AtMYB65* transcripts to levels below those in the WT (Fig. 3c). Collectively, these results demonstrated that transcripts derived from the predicted strawberry *MIR159a* locus compensated for the lack of endogenous miR159 activity in Arabidopsis, thereby suggesting that *Fa-MIR159a* was effectively processed into a functional mature miR159a. Furthermore, transgenic restoration of a miR159-driven regulatory pathway targeting the *AtMYB65* mRNA was sufficient to alleviate the developmental defects observed in the *miR159a*, *mir159b* double mutant Arabidopsis plants (Fig. 3a).

Fa-GAMYB is a validated target of Fa-miR159 in strawberry fruit

To experimentally validate a functional interaction between Fa-miR159 and its predicted target, *Fa-GAMYB*, we used a modified 5′ RLM-RACE assay (Llave *et al.*, 2002). To determine whether the miR159-directed cleavage of *Fa-GAMYB* transcripts was tissue- and/or developmental stage-specific, we used poly-A(+) RNA purified from the G, W and R receptacles. The 5′ end sequencing of the amplified products revealed a predominant cleavage site in the middle of the Fa-miR159 complementarity region (Fig. 4a). Cleavage occurred at an identical position in all tested samples, except in the R receptacle (RR), where we identified a secondary cleavage site one nucleotide upstream of the preferential cleavage site. These results confirmed that *Fa-GAMYB* is an authentic target of Fa-miR159, and *Fa-GAMYB* was subjected to miR159-mediated down-regulation in all tissues tested.

Next, we performed an RNA blot analysis using a 3′ proximal *Fa-GAMYB* radiolabelled probe to assess the extent of miR159-guided negative regulation in all three samples interrogated. Our assay detected hybridization bands consistent with both the full-length *Fa-GAMYB* mRNA and a shorter 3′ RNA cleavage product, the result of the miR159-guided processing of full-length mRNA in the strawberry fruit receptacle at the three developmental stages sampled (Fig. 4b). Furthermore, the ratio of full-length mRNA to shorter cleavage products was comparable at the G, W and R stages, suggesting an active role for miR159 in the control of *Fa-GAMYB* mRNA levels during fruit development.

Fa-miR159a and Fa-miR159b have different expression patterns during strawberry fruit development, and their transcript levels are opposite that of *Fa-GAMYB*

To gain an insight into the outcome of the miR159-*Fa-GAMYB* mRNA interaction during strawberry fruit development, relative primary transcript levels of both Fa-miR159a and Fa-miR159b were measured by quantitative RT-PCR at several developmental stages (CF and OF, and in G, W and R fruits) and in two vegetative tissues (i.e. L and Rt). *Fa-MIR159a* exhibited a significant maximum expression in G fruits (Fig. 5a), whereas *Fa-MIR159b* reached its peak in R fruits (Fig. 5b).

The common ‘strawberry fruit’ is actually a false fruit composed of the achenes and the receptacle, which greatly differ in their origin, cell identities and physiological roles. To gain precision in our analysis, we investigated the expression of the two *Fa-MIR159* genes separately, in the achenes or the receptacle, during the G, W and R stages. The expression patterns of both *Fa-MIR159a* and *Fa-MIR159b* were similar in the achenes during the three developmental stages (Fig. 5c,d). By contrast, *Fa-MIR159a* and *Fa-MIR159b* transcripts were more abundant in the receptacle than in the achenes and exhibited substantial differences during development. The *Fa-MIR159a* transcript reached its highest expression level during the G stage and subsequently decreased significantly during the W and R stages (Fig. 5c). By contrast, the expression of *Fa-MIR159b* in the receptacle increased gradually from the G through the W and R stages (Fig. 5d).

Because the expression of miRNA precursors does not always correlate with the steady-state levels of the mature miRNAs (Nogueira *et al.*, 2009), we quantified the mature Fa-miR159a and Fa-miR159b transcript levels in the strawberry receptacles during the G, W and R stages using stem-loop quantitative RT-PCR. Absolute accumulation levels of the two Fa-miRNAs were calculated by converting C_t into an absolute copy number using a standard curve derived from a diluted series of synthetic Fa-miR159a or Fa-miR159b RNA oligonucleotides. The results of the quantitative RT-PCRs suggested a predominant accumulation of Fa-miR159b compared with Fa-miR159a at all stages analysed (Fig. 6a). Quantitative RT-PCR also identified an accumulation pattern of Fa-miR159a that virtually mimicked that of the *Fa-MIR159a* transcript, displaying maximum levels during the G stage and gradually decreasing towards the R stage. Interestingly, the Fa-miR159b accumulation pattern deviated from that observed for *Fa-MIR159b*. The level of mature Fa-miR159b was significantly lower in the W receptacle compared with the G and R stages, in contrast with the *Fa-MIR159b* transcript, which was significantly lower during the G stage. This discrepancy between *Fa-MIR159b* and Fa-miR159b patterns may reflect differential processing efficiency of the miR159b primary transcript dependent on the developmental stage. In summary, our results indicated that *Fa-MIR159a* was modestly expressed and converted into mature miR159a during strawberry receptacle development. By contrast, *Fa-MIR159b* was actively and preferentially transcribed and processed into mature Fa-miR159b at every stage of receptacle

development, and its absolute content was 8–25-fold higher than that of Fa-miR159a, depending on the developmental stage analysed (Fig. 6a).

Next, we investigated whether the expression profile of Fa-miR159 members delineated the expression of the predicted target gene, *Fa-GAMYB*, in the aforementioned tissues and developmental stages. Our results from quantitative RT-PCR revealed that *Fa-GAMYB* mRNA was expressed in the receptacle at levels significantly higher than in the achenes (Fig. 6b). Furthermore, the highest transcript level in the receptacle was repeatedly observed during the W stage, when less miR159 transcript was detected. This observation regarding *Fa-GAMYB* mRNA was not so clear in the northern blotting studies of Fig. 4(b), probably because of the different sensitivity and/or specificity of these two techniques for the quantification of transcript levels. Conversely, *Fa-GAMYB* mRNA levels in the receptacle were substantially diminished during the G and R stages; in these stages, the total miR159 levels were higher (Fig. 6a). On the basis of our data, we can conclude that the relative accumulation of *GAMYB* mRNA is strongly and negatively dependent on the abundance of miR159 in the strawberry receptacle. These receptacle expression patterns are suggestive of Fa-miR159a and Fa-miR159b controlling *Fa-GAMYB* expression in strawberry fruit.

Because miR159 was reported to be up-regulated by GA in Arabidopsis (Achard *et al.*, 2004), we hypothesized that this hormone might influence, at least partially, the expression of strawberry *Fa-MIR159a* and *Fa-MIR159b*. To test this possibility, *in vitro* strawberry plantlets were treated with bioactive GA (GA_3). The direct GA_3 treatment of strawberry fruits was not feasible because, similar to other fruits, they are recalcitrant to standardized external hormone application, mostly because of the thickness of the fruit cuticle. After 6 h of GA_3 treatment, the relative accumulation of *Fa-MIR159a* was drastically reduced by nearly 25-fold relative to the level in untreated control plants (Fig. 7a). Conversely, *Fa-MIR159b* transcripts did not respond at such a high level to GA_3 application (Fig. 7b). At this time, no significant effect on *Fa-GAMYB* expression was observed (Fig. 7c), but this was somehow expected, as a 24–36-h delay has been reported for a change in *GAMYB* expression in response to GA treatment (Tsuji *et al.*, 2006; Gong & Bewley, 2008). Treatment effectiveness was confirmed by monitoring the effect of GA_3 application on the transcript accumulation of two GA-responsive genes, *FaGA3ox* and *FaGA2ox*. After 2 h of GA_3 treatment, the two genes were down- and up-regulated, respectively (Fig. 7d,e), as predicted on the basis of previous reports (Yamaguchi, 2008). In conclusion, in the strawberry plant, each miR159-generating locus responded differently to GA treatment.

Discussion

miRNAs have a great impact on the biological processes of plants, mainly during development and the stress response (Jones-Rhoades *et al.*, 2006). However, information about their function during fruit growth and ripening is limited. Although there have been reports on their function in several species (Pilcher *et al.*, 2007; Carra *et al.*, 2009), this is not the case in strawberries. We report that two strawberry genes, *Fa-MIR159a* and *Fa-MIR159b*, display tissue- and developmental stage-specific expression patterns during fruit ripening, with their highest expression being identified in the receptacle vs the achenes. However, analysis of the mature Fa-miR159 in the receptacle revealed that the levels of Fa-miR159b were, at every stage, much higher than those of Fa-miR159a. In addition, the lowest levels of Fa-miR159b were observed during the W stage, differing from the lowest expression of *Fa-MIR159b*, which was observed in the G receptacle. Similar discrepancies between pri-/pre-miRNA and mature miRNA levels have been well documented in both plants and animals (Kim *et al.*, 2009; Voinnet, 2009; Correa *et al.*, 2010). Three members of the miR159 family have been identified in Arabidopsis (Allen *et al.*, 2007), and At-miR159a and At-miR159b are likely to act in a redundant manner (Allen *et al.*, 2007). In the strawberry, there are no data to assess the possible redundancy of these two miR159 family members. Our expression studies indicate that the down-regulation of mature Fa-miR159 may contribute to the regulation of receptacle development. The receptacle is not the true fruit but, rather, is the result of the engrossment of the flower receptacle. The major phenotypic events in the transition from the G to W stage are marked by a dramatic increase in cell size and a loss of photosynthesizing capacity (Perkins-Veazie, 1995). These critical changes are the result of a programme that is accompanied by both dramatic gene expression and metabolic changes (Aharoni *et al.*, 2002). This transition coincides with a significant decrease in mature Fa-miR159.

Clearly, miRNAs play important roles during plant development through their regulation of the expression levels of their target genes. Indeed, miR159 targets have been functionally analysed in both

Arabidopsis and rice. In Arabidopsis, seven *GAMYB*-like genes contain potential miR159 binding sites (Rhoades *et al.*, 2002), but only *MYB33* and *MYB65* were deregulated in the *mir159a miR159b* double mutant plants (Millar & Gubler, 2005; Allen *et al.*, 2007). In rice, three genes encoding GAMYB-like transcription factors contained a miR159 complementary sequence (Jones-Rhoades & Bartel, 2004; Tsuji *et al.*, 2006). Interestingly, a new miR159 target gene, which is unrelated to MYB, was identified in the tomato (Buxdorf *et al.*, 2010). In strawberry, we provide evidence that a *GAMYB*-like gene, *Fa-GAMYB*, is the target of the mature Fa-miR159. It is important to note that the search for *Fa-GAMYB* homologues in *F. vesca* (Shulaev *et al.*, 2011), the wild diploid relative of the cultivated strawberry (*F.* × *ananassa*), which has been proposed as a model for the genus (Shulaev *et al.*, 2011), identified only one putative homologue.

The *GAMYB* and *GAMYB*-like genes encode a highly conserved family of R2R3 MYB domain transcription factors (Gubler *et al.*, 1999). *GAMYB* was initially identified in the cereal aleurone (Gubler *et al.*, 1995) and is strongly expressed in the anthers, especially in the tapetum (Murray *et al.*, 2003; Aya *et al.*, 2009). In this tissue, miR159 is co-expressed with *GAMYB* and finely regulates the levels of this transcription factor (Tsuji *et al.*, 2006). It has been demonstrated that the interplay of miR159 and its target, *GAMYB*, is involved in the regulation of vegetative growth, flowering time, anther development, seed shape and germination (Millar & Gubler, 2005; Allen *et al.*, 2007; Reyes & Chua, 2007). In the strawberry receptacle, *Fa-MIR159a* and *Fa-MIR159b* are co-expressed with *Fa-GAMYB*, and their opposite expression patterns indicate a possible role in the regulation of the levels of the transcription factor *Fa-GAMYB*. However, only the levels of the mature miR159b clearly correlate with the *Fa-GAMYB* expression pattern in the receptacle, suggesting that miR159a might have a more prominent role in other strawberry tissues. Despite many studies of strawberry fruit development, only a small number of regulatory genes relevant for this process have been identified (Aharoni *et al.*, 2001).

The *GAMYB*-like genes have been implicated in GA signal transduction (Gubler *et al.*, 1995; Murray *et al.*, 2003; Aya *et al.*, 2009). In Arabidopsis, both *MYB33* and *MYB65* have been implicated in the GA signalling pathway which regulates flowering during SD conditions (Gocal *et al.*, 2001; Achard *et al.*, 2004). We recently determined a critical role for GA in the development of the strawberry receptacle by demonstrating that, during the transition from the G to W stage, there is a dramatic peak of GA_4 (Csukasi *et al.*, 2011). We also reported that, during the change from the G to W receptacle, the expression of both *FaGID1* and *FaRGA* is correlated with the activation of GA signalling (Csukasi *et al.*, 2011). Recent studies on *GAMYB*'s regulation of anther development in rice have proposed that GA control occurs via GID1/DELLA (Aya *et al.*, 2009). In this study, we report that the GA increase is coincident with the maximal expression of *Fa-GAMYB*; however, a functional relationship between GA and Fa-GAMYB production needs to be formally demonstrated. Our results depict a model in which mature Fa-miR159 may arise from two precursors, specifically pre-Fa-miR159a and pre-Fa-miR159b, which display different regulatory mechanisms. Although the expression of *Fa-MIR159a* is GA dependent, *Fa-MIR159b* expression is post-transcriptionally regulated. Different post-transcriptional regulations for the miR159 family members have been reported previously (Li *et al.*, 2011). During receptacle development, this post-transcriptional regulation constitutes an alternative, GA-independent mechanism to regulate *Fa-GAMYB* expression mediated by Fa-miR159b. In Arabidopsis, the expression of miR159 is regulated by GA (Achard *et al.*, 2004), whereas, in rice, miR159 expression has been shown to be GA independent (Tsuji *et al.*, 2006). However, analyses of the effect of GA in specific members of the miR159 family have not been performed in these species. The occurrence of both GA-dependent and GA-independent control of mature Fa-miR159 is an example of specialization within the miR159 gene family in the strawberry. Their sequential operation during receptacle development secures a minimum for mature Fa-miR159 during the W stage.

Acknowledgements

This work was supported by BIO2010-15630 (V.V.) and BIO2009-12004 (C.L.) (Ministerio de Ciencia e Innovacion (MICINN), Spain). F.C. was granted a FPI fellowship from MICINN (Spain). The authors thank Dr Anthony A. Millar for providing *Arabidopsis thaliana mir159a*, *b* double-mutant seeds.

References

Achard P, Herr A, Baulcombe DC, Harberd NP. 2004. Modulation of floral development by a gibberellin-regulated microRNA. Development 131: 3357–3365.

Aharoni A, Keizer LCP, Van Den Broeck HC, Blanco-Portales R, Munoz-Blanco J, Bois G, Smit P, De Vos RCH, O'Connell AP. 2002. Novel insight into vascular, stress, and auxin-dependent and -independent gene expression programs in strawberry, a non-climacteric fruit. Plant Physiology 129: 1019–1031.

Aharoni A, Vos CHRD, Wein M, Sun Z, Greco R, Kroon A, Mol JNM, O'Connell AP. 2001. The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant Journal 28: 319–332.

Allen RS, Li J, Stahle MI, Dubroue´ A, Gubler F, Millar AA. 2007. Genetic analysis reveals functional redundancy and the major target genes of the Arabidopsis miR159 family. PNAS 104: 16371–16376.

Axtell MJ, Bartel DP. 2005. Antiquity of microRNAs and their targets in land plants. Plant Cell 17: 1658–1673.

Aya K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, Nishimura M, Matsuoka M. 2009. Gibberellin modulates anther development in rice via the transcriptional regulation of gamyb. Plant Cell 21: 1453–1472.

Blake PS, Taylor DR, Crisp CM, Mander LN, Owen DJ. 2000. Identification of endogenous gibberellins in strawberry, including the novel gibberellins GA123, GA124 and GA125. Phytochemistry 55: 887–890.

Bombarely A, Merchante C, Csukasi F, Cruz-Rus E, Caballero J, Medina-Escobar N, Blanco-Portales R, Botella M, Munoz-Blanco J, Sanchez-Sevilla J et al. 2010. Generation and analysis of ESTs from strawberry (Fragaria · ananassa) fruits and evaluation of their utility in genetic and molecular studies. BMC Genomics 11: 503.

Bustamante CA, Civello PM, Martínez GA. 2009. Cloning of the promoter region of b-xylosidase (FaXyl1) gene and effect of plant growth regulators on the expression of FaXyl1 in strawberry fruit. Plant Science 177: 49–56.

Buxdorf K, Hendelman A, Stav R, Lapidot M, Ori N, Arazi T. 2010. Identification and characterization of a novel miR159 target not related to MYB in tomato. Planta 232: 1009–1022.

Carra A, Mica E, Gambino G, Pindo M, Moser C, Pe` ME, Schubert A. 2009. Cloning and characterization of small non-coding RNAs from grape. Plant Journal 59: 750–763.

Casado-Diaz A, Encinas-Villarejo S, Santos B, Schiliro E, Yubero-Serrano E, Amil-Ruiz F, Pocovi M, Pliego-Alfaro F, Dorado G, Rey M et al. 2006. Analysis of strawberry genes differentially expressed in response to Colletotrichum infection. Physiologia Plantarum 128: 633–650.

Clough SJ. 2004. Floral Dip: Agrobacterium-Mediated Germ Line Transformation. In: Leandro P, ed. Methods in Molecular Biology, Vol. 286. Transgenic Plants: Methods and Protocol. Totowa, NJ, USA: Humana Press Inc., 91–101.

Correa RL, Steiner FA, Berezikov E, Ketting RF. 2010. MicroRNA directed siRNA biogenesis in *Caenorhabditis elegans*. PLoS Genetics 6: e1000903.

Csukasi F, Osorio S, Gutierrez JR, Kitamura J, Giavalisco P, Nakajima M, Fernie AR, Rathjen JP, Botella MA, Valpuesta V et al. 2011. Gibberellin biosynthesis and signalling during development of the strawberry receptacle. New Phytologist 191: 376–390.

Fait A, Hanhineva K, Beleggia R, Dai N, Rogachev I, Nikiforova VJ, Fernie AR, Aharoni A. 2008. Reconfiguration of the achene and receptacle metabolic networks during strawberry fruit development. Plant Physiology 148: 730–750.

Gocal GFW, Sheldon CC, Gubler F, Moritz T, Bagnall DJ, MacMillan CP, Li SF, Parish RW, Dennis ES, Weigel D et al. 2001. GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis. Plant Physiology 127: 1682–1693.

Gong X, Bewley JD. 2008. A GAMYB like gene in tomato and its expression during seed germination. Planta 228: 563–572.

Gubler F, Kalla R, Roberts JK, Jacobsen JV. 1995. Gibberellin-regulated expression of a myb gene in barley aleurone cells: evidence for MYB transactivation of a high-pl a-amylase gene promoter. Plant Cell 7: 1879–1891.

Gubler F, Raventos D, Keys M, Watts R, Mundy J, Jacobsen JV. 1999. Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone. Plant Journal 17: 1–9.

Jones-Rhoades MW, Bartel DP. 2004. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Molecular Cell 14: 787–799.

Jones-Rhoades MW, Bartel DP, Bartel B. 2006. MicroRNAs and their regulatory roles in plants. Annual Review of Plant Biology 57: 19–53.

Kim VN, Han J, Siomi MC. 2009. Biogenesis of small RNAs in animals. Nature Reviews Molecular Cell Biology 10: 126–139.

Li Y, Li C, Ding G, Jin Y. 2011. Evolution of MIR159/319 microRNA genes and their post-transcriptional regulatory link to siRNA pathways. BMC Evolutionary Biology 11: 122.

Liu Q, Chen Y-Q. 2009. Insights into the mechanism of plant development: interactions of miRNAs pathway with phytohormone response. Biochemical and Biophysical Research Communications 384: 1–5.

Llave C, Kasschau KD, Carrington JC. 2000. Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proceedings of the National Academy of Sciences, USA 97: 13401–13406.

Llave C, Kasschau KD, Rector MA, Carrington JC. 2002. Endogenous and silencing-associated small RNAs in plants. Plant Cell 14: 1605–1619.

Manning K. 1991. Isolation of nucleic acids from plants by differential solvent precipitation. Analytical Biochemistry 195: 45–50.

Manning K. 1998. Isolation of a set of ripening-related genes from strawberry: their identification and possible relationship to fruit quality traits. Planta 205: 622–631.

Millar AA, Gubler F. 2005. The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell 17: 705–721.

Moxon S, Jing R, Szittya G, Schwach F, Rusholme Pilcher RL, Moulton V, Dalmay T. 2008. Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. Genome Research 18: 1602–1609.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497.

Murray F, Kalla R, Jacobsen J, Gubler F. 2003. A role for HvGAMYB in anther development. Plant Journal 33: 481–491.

Nitsch JP. 1950. Growth and morphogenesis of the strawberry as related to auxin. American Journal of Botany 37: 211–215.

Nogueira FTS, Chitwood DH, Madi S, Ohtsu K, Schnable PS, Scanlon MJ, Timmermans MCP. 2009. Regulation of small RNA accumulation in the maize shoot apex. PLoS Genetics 5: e1000320.

Perkins-Veazie P. 1995. Growth and ripening of strawberry fruit. In: Janick J, ed. Vol 17. Horticultural reviews. Hoboken, NJ, USA: John Wiley & Sons, Inc. 267–297.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29: e45.

Pilcher RL, Moxon S, Pakseresht N, Moulton V, Manning K, Seymour G, Dalmay T. 2007. Identification of novel small RNAs in tomato (*Solanum lycopersicum*). Planta 226: 709–717.

Rajagopalan R, Vaucheret H, Trejo J, Bartel DP. 2006. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes & Development 20: 3407–3425.

Reyes JL, Chua N-H. 2007. ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. Plant Journal 49: 592–606.

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. 2002. Prediction of plant microRNA targets. Cell 110: 513–520.

Sambrook JFE, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd edn. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.

Seeram NP. 2008. Berry fruits: compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. Journal of Agricultural and Food Chemistry 56: 627–629.

Shulaev V, Sargent DJ, Crowhurst RN, Mockler TC, Folkerts O, Delcher AL, Jaiswal P, Mockaitis K, Liston A, Mane SP et al. 2011. The genome of woodland strawberry (*Fragaria vesca*). Nature Genetics 43: 109–116.

Tsuji H, Aya K, Ueguchi-Tanaka M, Shimada Y, Nakazono M, Watanabe R, Nishizawa NK, Gomi K, Shimada A, Kitano H et al. 2006. GAMYB controls different sets of genes and is differentially regulated by microRNA in aleurone cells and anthers. Plant Journal 47: 427–444.

Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. Cell 136: 669–687.

Yamaguchi S. 2008. Gibberellin metabolism and its regulation. Annual Review of Plant Biology 59: 225–251.

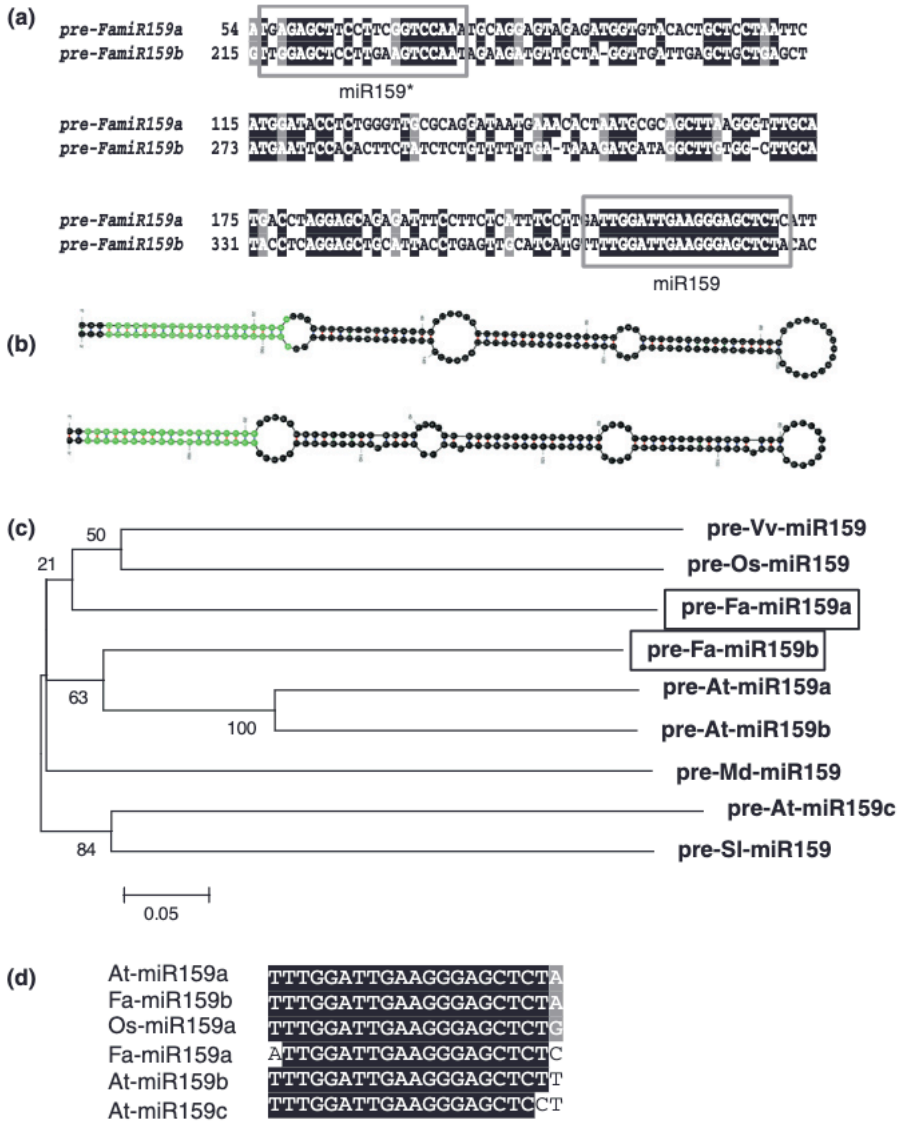

Fig. 1. Analysis of miR159 sequences.

(a) *Sequence alignment* of pre-Fa-miR159a and pre-Fa-miR159b. Grey boxes highlight the mature sequences of miR159a and miR159b in both arms of the precursors (miR159 and miR159*).

(b) *Secondary structure* of pre-Fa-miR159a (top) and pre-Fa-miR159b (bottom) as predicted by MFOLD (http://mfold.bioinfo.rpi.edu/), along with their corresponding ΔG values for loop free energy decomposition: −94.6 kcal/mol and −77.9 kcal/mol, respectively. The grey shading represents the mature miR159a and miR159b sequences in both arms of the precursors.

(c) *Phylogenetic tree* of miR159 precursors from rice (Os), strawberry (Fa), Arabidopsis (At), tomato (Sl), apple (Md), and grape (Vv). A neighbour-joining tree was constructed, and the bootstrap values from 1000 trials are indicated. Black boxes highlight pre-Fa-miR159a and pre-Fa-miR159b.

(d) *Sequence alignment* of mature miR159 from strawberry with the corresponding sequences of other species in which functionality on mRNA targets has been reported.

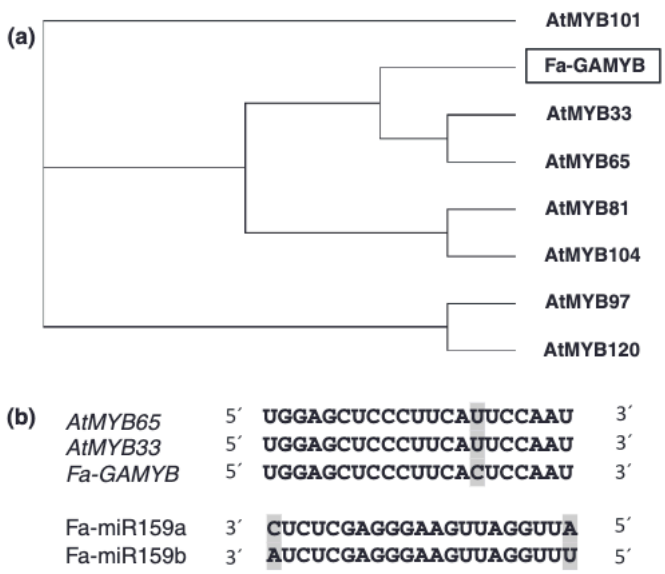

Fig. 2. Analysis of GAMYB-like sequences from Arabidopsis and strawberry.

(a) *Dendrogram* resulting from the alignment of MYB proteins in Arabidopsis (At) encoded by genes with a putative target sequence of miR159, and Fa-GAMYB.

(b) *Base pairing interaction* between Fa-miR159a and Fa-miR159b and AtMYB33, AtMYB65, and Fa-GAMYB. The grey background indicates differences in nucleotides among the aligned sequences.

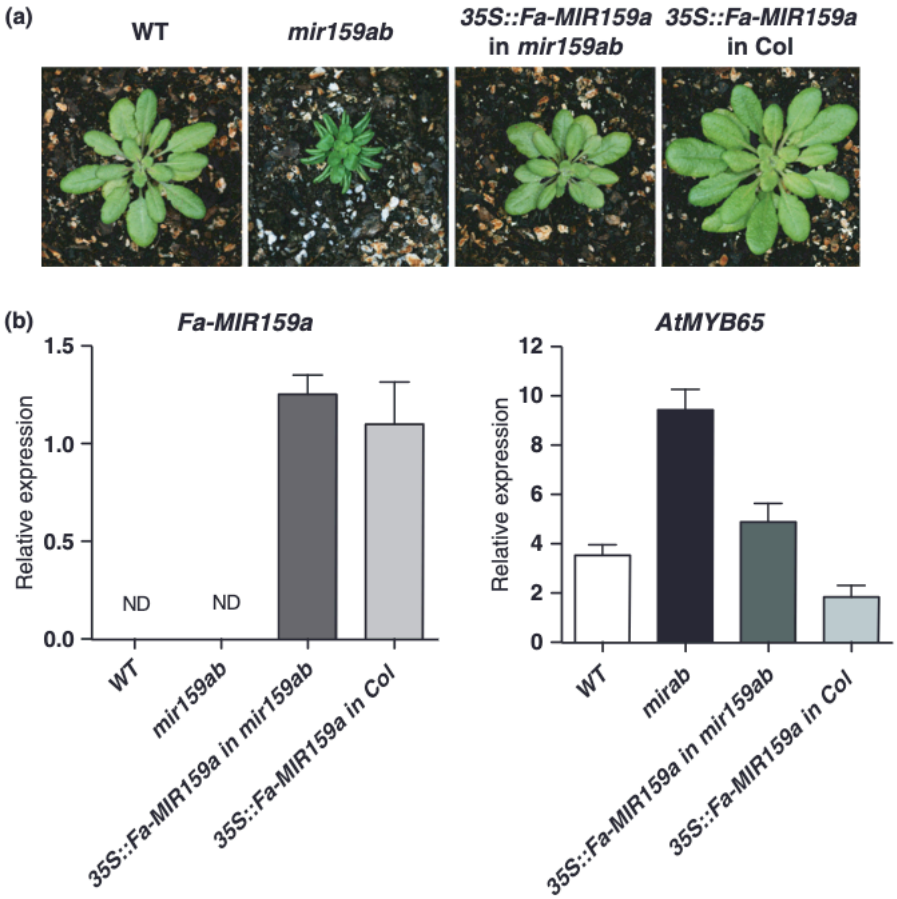

Fig. 3. Functional analysis of Fa-MIR159a.

(a) *Phenotype* in short-day (SD) growth conditions of Arabidopsis wild-type (WT), double mutant (mir159ab), double mutant complemented with Fa-MIR159a under the control of the 35S promoter (35S::Fa-MIR159a in mir159ab), and Columbia ecotype of Arabidopsis expressing Fa-MIR159a (35S::Fa-MIR159a in Col).

(b, c) *Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)* analysis of strawberry miR159a precursor and AtMYB65. Total RNA was extracted from the rosettes of the same lines as in (a) and the relative transcript levels of Fa-MIR159a (b) and AtMYB65 (c) were determined by qRT-PCR. Bars represent the means of two independent biological replicates ± SE. ND, not detected.

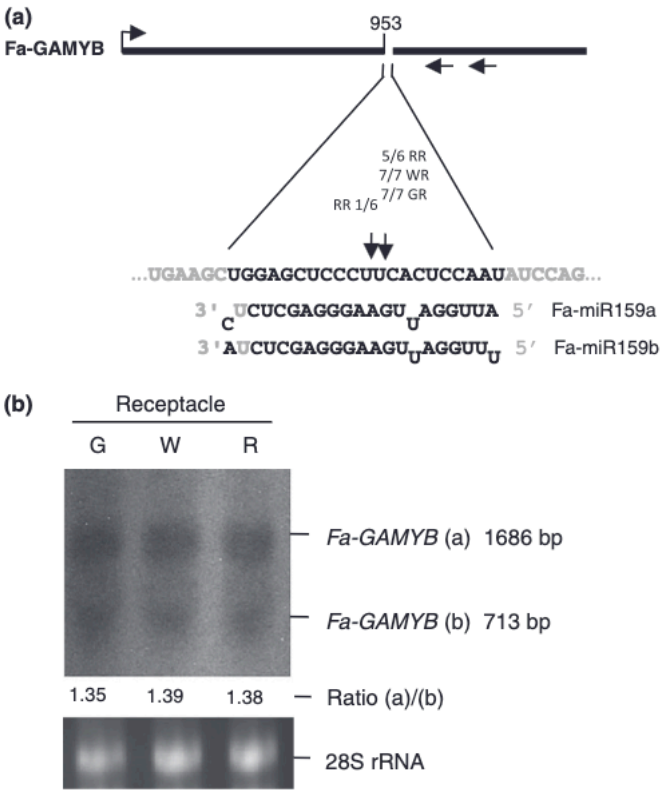

Fig. 4. Targeting of Fa-GAMYB by Fa-miR159.

(a) *Mapping of Fa-miR159-guided cleavage sites in Fa-GAMYB mRNA by RNA ligase-mediated amplification of 5¢ cDNA ends (RLM-RACE).* Sequences of Fa-miR159a and Fa-miR159b are shown. Vertical arrows indicate the 5¢ positions of the cleaved mRNA fragments and the numbers indicate the number of independent clones analyzed in the different tissues.

(b) *Fa-GAMYB mRNA cleavage during strawberry receptacle development at the green (G), white (W), and red (R) stages.* RNA blot assay with a probe corresponding to the 3¢ end of the Fa-GAMYB gene. Both Fa-GAMYB full-length mRNA (a) and the Fa-miR159-guided cleavage product (b) are detected. Ethidium bromide-stained RNA before transfer was used as a loading control.

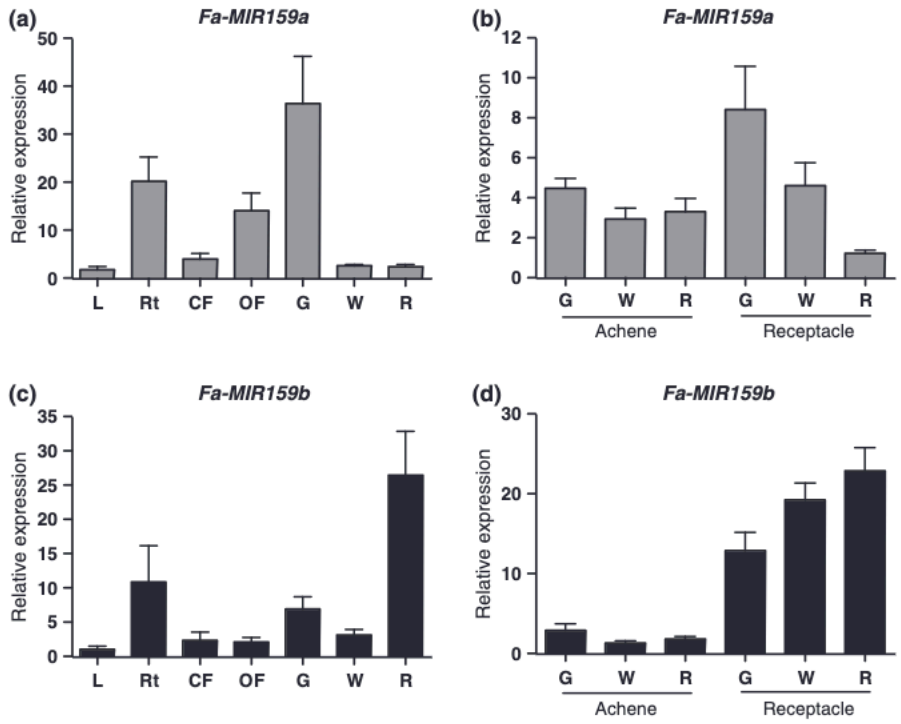

Fig. 5. Relative expression levels of Fa-MIR159a and Fa-MIR159b in different strawberry plant tissues.

Total RNA was extracted from different strawberry tissues, and the relative transcript levels of Fa-MIR159a and Fa-MIR159b were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Fa-MIR159a (a) and Fa-MIR159b (c) in different plant tissues: CF, closed flower; OF, open flower; G, green fruit; W, white fruit; R, red fruit; L, leaves; Rt, roots.

Fa-MIR159a (b) and Fa-MIR159b (d) in achene and receptacle separately at the green (G), white (W), and red (R) stages.

Bars represent the means of two independent biological samples ± SE.

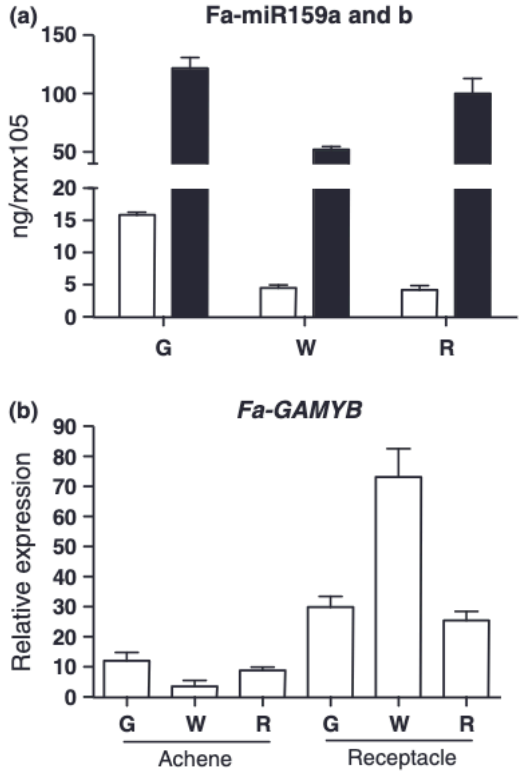

Fig. 6. Absolute levels of mature Fa-miR159a and Fa-miR159b and relative transcript levels of Fa-GAMYB during strawberry fruit development.

(a) Stem-loop quantitative reverse transcription-polymerase chain reaction (qRT-PCR) quantification of mature Fa-miR159a and Fa-miR159b in receptacle tissues (G, green; W, white; R, red). Histograms show the calculated concentration of Fa-miR159a (open bars) and Fa-miR159b (closed bars) by absolute quantification based on synthetic miR159a and miR159b standard curves. Data are the average of two biological replicates ± SE.

(b) Relative transcript levels of Fa-GAMYB as determined by qRT-PCR in achene and receptacle separately in G, W, and R stages.

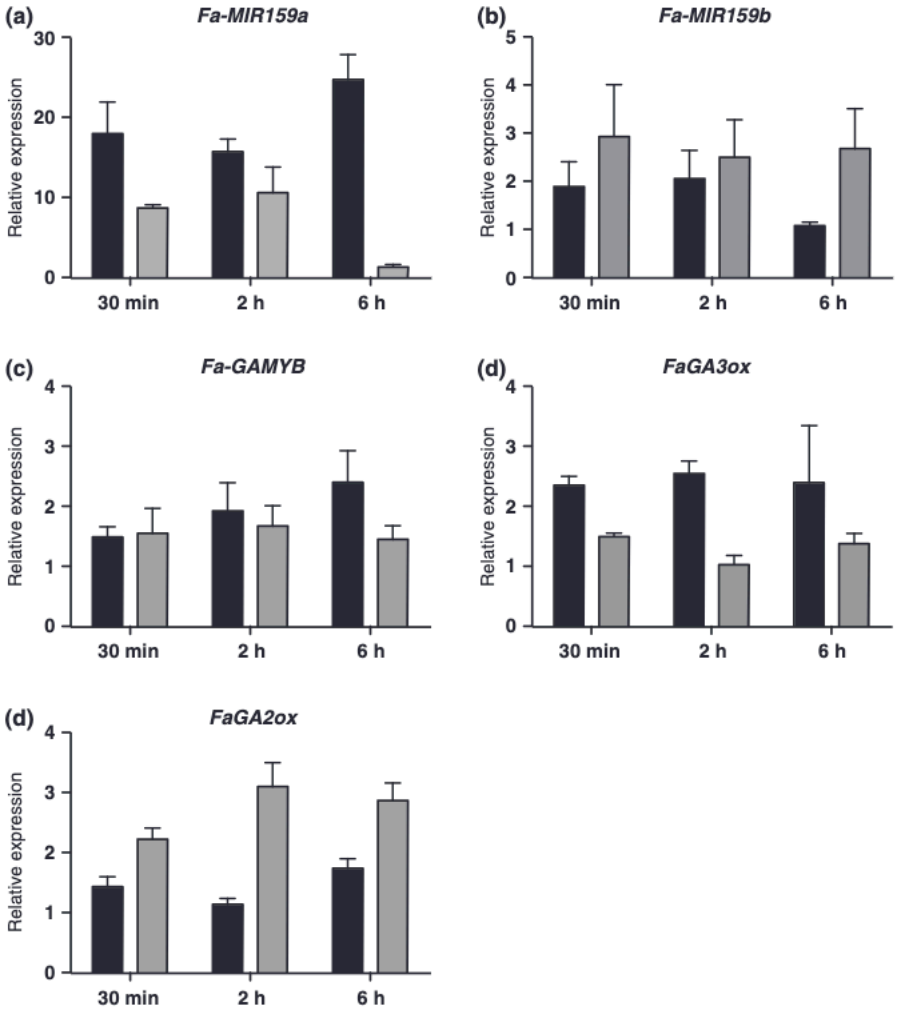

Fig. 7. Effect of bioactive gibberellin (GA3) treatment on the expression of Fa-MIR159a and Fa-MIR159b.

Total RNA was extracted from strawberry plants cultured in vitro and treated with or without 100 μM GA3, and the relative transcript levels of Fa-MIR159a (a), Fa-MIR159b (b), and Fa-GAMYB (c) were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The relative expression of FaGA3ox (d) and FaGA2ox (e) was used as a control of the treatment. Expression studies were performed at different time points: 30 min, 2, and 6 h. Black bars, -GA3; grey bars, +GA3.