microRNA mediated regulation in fruit quality

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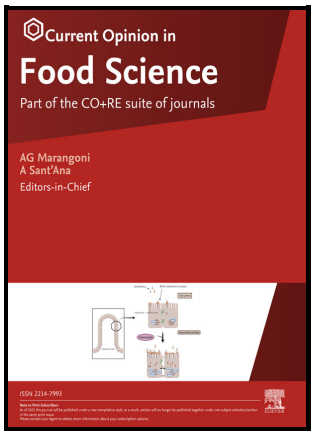

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Abstract

Fruits, the reproductive organs in flowering plants, are an important source of food for humans. Complete fruit development and ripening featuring by a remarkable phenotypic plasticity are orchestrated by versatile genetic factors. microRNAs and phasiRNAs play central roles in regulating diverse biological processes via transcriptionally or post-transcriptionally modulating the expression of target genes. Apart from the their conserved function in model plants, microRNA/phasiRNA-mediated regulation has been widely investigated in fruit crops, with many novel molecular mechanisms. Here, we survey the regulatory mechanisms and biological functions of microRNAs and phasiRNAs in fruit development, with a particular focus on their roles in fruit quality formation. We also discuss their potential application in improving fruit quality.

Introduction

Fruits, the edible part of plants, are rich in carbohydrates, inorganic salts, vitamins, and other nutrients. They are an increasing part of the human diet [1,2]. Botanically, a fruit is a ripened ovary or carpel containing seeds. Besides yield, other horticultural traits, such as nutrient content, texture, flavor, aroma and color, also affect the market value of fruits [1]. The formation of these valuable horticulturaltraits is based on fruit development and the underlying molecular regulatory mechanisms [3]. To harvest a high yield of quality fruit, researchers have investigated fruit development, quality formation, and fruit maintenance for several decades. Studies have thus far involved cultivation improvement, post-harvest techniques, and basic research on biochemistry, genetics, and molecular biology. Among these researches, small RNAs, especially microRNA (miRNA) and phased secondary small interfering RNA (phasiRNA), play a significant role in modulating the biological processes implicated in fruit development and quality formation [4].

Plant miRNAs are a class of short regulatory RNAs with 20-22 nucleotides [5,6]. After the *MIR* genes are transcribed by RNA Polymerase II (Pol II) and processed by DICER-LIKE1 (DCL1), mature miRNAs are incorporated into ARGONAUTE1 (AGO1) to form an RNA-induced silencing complex (RISC), which directs the silencing of target genes [6]. When the RISC was loaded with 22-nucleotides (nt) mature miRNAs, it triggers the biogenesis of phasiRNA from its target transcripts [7]. These phasiRNAs negatively regulate target transcripts during plant development as miRNA does [7]. Over the past two decades, miRNAs and phasiRNAs have been well studied. As negative regulators, they play crucial roles in many aspects of plant development.

In this review, we summarized the latest information regarding miRNA and phasiRNA mediated regulatory networks involved in fruit development in model plants, as well as horticultural crops, with the majority of new information concerning their roles in fruit quality formation.

miRNA and fruit development

Generally, fruit development occurs in three stages: fruit set, fruit growth and fruit ripening and senescence. These developmental stages and the underlying regulatory networks contribute to fruit production and quality formation. Here, we summarized the major fruit development related miRNAs in Table 1.

miRNAs involved in fruit set

Fruit set is the process in which a flower becomes a fruit and potential fruit number and size is determined. As a conserved and well-studied miRNA, miR156/157 are involved in almost all stages of fruit development (Figure 1) [8-13,14**,15*, 16-18]. The miR156-*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) gene pathway maintains the meristematic state of tomato ovary tissues, thereby coordinating the initial steps of fleshy fruit development and determinacy [9].

Other conserved miRNA pathways, such as miR159-*GAMYB1/2* and miR166-*HB15A*, also are involved in ovary development [19, 20]. miR160, which targets the *AUXIN RESPONSE FACTORS* (*ARFs*), regulates longan embryo development, which is critical for fruit set [21]. miR397 regulates the number of seeds and lignin biosynthesis of Arabidopsis via targeting *LACCASES* (*LACs*) [22]. miR393 regulates two *TOLL/INTERLEUKIN-1 RECEPTOR-LIKE* (*TIR1*-like) genes *CsTIR1* and *CsAFB2*, which are essential for seed germination and the parthenocarpic fruit of cucumber during fruit set [23] (Figure 1 and Table 1).

miRNAs involved in fruit growth

Fruit growth is the process of fruit enlargement, which affects fruit morphology and size. During fruit growth, the miR156-*SPL2* regulatory module adjusts the elongation of siliques in Arabidopsis [18]. miR160 targets *ARFs* and affects fruit morphology and placental thinning in tomatoes [24], while miR166-*REV* were involved in tomato fruit shape formation [25]. miR397-targeted *LACs* regulate the fruit cell lignification in pears during fruit growth [26**]. Regulatory modules mediated by other conserved miRNAs, such as miR159-*MYBs* [27], miR172-*AP2* [28,29**,30], miR164-*NACs* [12,27,31**], miR396-*GRFs* [32], are related to fruit growth (Figure 1 and Table 1).

miRNAs involved in fruit ripening and senescence

Diverse biological processes occurs during fruit ripening and senescence, such as flavor formation, coloration, browning and so on. At the ripening stage of a tomato, miR157 targets *COLOURLESS NON-RIPENING* (*CNR*), an *SPL* gene critical for ripening, impacting fruit softening after the red ripe stage [11]. Conserved miRNAs, such as miR159-*MYBs* [33], miR172-*AP2* [28,29**,30], and miR164-*NACs* [12,27,31**] are also related to fruit ripening or senescence (Figure 1). In the banana, decreasing miR528 results in upregulated expression of *POLYPHENOL OXIDASE*

(*PPO*) genes encoding polyphenol oxidase, leading to ROS surge and subsequent browning of the banana fruit in cold conditions [34**] (Table 1). Specifically, miR396g may regulate the target genes associated with glycosylation and in solubilization of tannin precursors during the persimmon ripening [16] (Table 1). Non-conserved miRNA pathways, such as miR1917-*CARBON DIOXIDE TRANSFER RATE 4* (*CTR4*) [35], miR2991-*ANTIDIURETIC HORMONES* (*ADHs*) [16], and miR7125-*COMPLEX CHROMOSOME REARRANGEMENTS* (*CCRs*) [36**], play specific roles in different biological processes during fruit ripening (Table 1).

Interestingly, some miRNA pathways work in the same biological process during fruit ripening and senescence. For instance, both the miR156-*SPLs* [13-16] and miR828/858-*MYBs* [16,17,37-41] module are involved in fruit coloration. miR164, miR156, miR319, miR6478 [12], miR528 [34**] and miR159 [42] are associated with fruit browning in apples, bananas, and strawberry (Table 1).

miRNA and fruit quality formation

As mentioned above, miRNAs are involved in many aspects of fruit development, contributing to fruit quality formation. Here, we particularly focused on miRNAs that affect fruit (1) size and shape, (2) flavor, (3) coloration, and (4) texture.

miRNAs involved in fruit size and shape

Arabidopsis fruit constitutes an ovary containing three primary tissue types: the valve, the replum and the valve margin [28]. *AtAPETALA2* (*AP2*), a transcription repressor, suppresses the expression of downstream genes that identify the valve margin and replum and inhibits the growth of the valve margin [28]. miR172c targets *AtAP2* and affects the fruit size of Arabidopsis. The valve growth is blocked in Arabidopsis plants with decreased miR172 activity via overexpressing target mimicry or expressing a miR172-resistant *AP2*, resulting in smaller fruit [28]. Although the accumulation of miR172 enhances fruit size in Arabidopsis [28], the miR172-*AP2*

module shows the opposite function in apples. The fruit growth of an apple is negatively regulated by the overexpression of miR172, leading to a dramatic reduction in fruit size (Figure 2) [29**]. This is mainly because the fruits of different plants develop from different tissues. Unlike Arabidopsis and tomato fruits, both of which are derived from ovaries [43], apple fruits are mostly derived from the hypanthium that is hypothesized to consist of the fused bases of the sepals, petals, and stamens, while the inferior ovary becomes the core of the fruit. Interestingly, in tomatoes, the carpel-only flowers developed into parthenocarpic fruits owing to the overexpression of the *MIR172* gene [43]. These studies illustrate that the effect of specific miRNA modules on fruit growth might be specific to fruit type and plant species. In the tomato, research has revealed novel roles of miR396 in regulating sepals and fruit size by targeting *SlGRFs* and provided a novel, potential way to improve tomato fruit yield [32].

miRNAs also help to form fruit shape. In the tomato, overexpression of a miR166-resistant *SlREVOLUTA* (*SlREV*) gives rise to ectopic fruit formation on receptacles and most of the secondary fruits are irregularly spaced without placenta and ovules (Figure 2) [25]. A transgenic tomato with ectopic expression of miR160-insensitive *ARF10* results in cone-shaped fruit, with a sharper angle at the distal fruit edge and a higher ratio of proximal/distal diameters in transgenic fruits (Figure 2) [44]. Simultaneously, reduction of miR160 substantially increases expression of miR160-targeted *ARF10A*/*10B*/*17*, which causes elongated, pear-shaped fruit [24]. Modifying the miRNA expression pattern of scion can alter the fruit shape of *Cucurbita pepo* cultivars via intra-species grafting [27]. miR159, miR164 and miR171 play a negative role in the regulation of fruit shape, resulting in smaller *C. pepo* heterograft fruit [27].

miRNAs involved in fruit flavor

Besides fruit size and shape, other traits, such as flavor and color, contribute to a fruit's market value. Fruit flavor is a combination of taste and aroma, which is

increased by the accumulation of primary metabolites (such as sugars and acids) and secondary metabolites (such as flavonoids and phenolics) [45,46].

In the woodland strawberry, overexpression of miR399 increases phosphate uptake [47,48], which facilitates the fructose, glucose, and soluble solid content in ripening fruit [48]. Three novel miRNAs (Novel_miR65, Novel_miR75 and Novel_miR92) target the gene encoding galacturonosyltransferase involved in 'sugar metabolism' in the hot pepper [49]. In persimmon, miR395p-3p and miR858b regulate *bHLH* and *MYB* respectively, which synergistically regulate the structural genes responsible for tannin biosynthesis [16]. In persimmon fruit ripening, miR156j-5p-*SPL* regulates the stabilization of the MYB-bHLH-WD40 complex, decreasing *PROANTHOCYANIDINS* (*PAs*, or called tannin) production [16].

miRNAs involved in fruit coloration

Fruit coloration during ripening can be achieved by chlorophyll breakdown and the accumulation of pigments such as anthocyanins [14**]. miR828/miR858-*MYBs* [37-41] and miR156/157-*SPLs* [13,14**,15*,50**] are conserved pathway to regulate coloration in various fruit crops.

miR828, with a length of 22-nt, targets *MYB* or *TAS4* and triggers the biogenesis of phasiRNAs that *in trans* or *in cis* regulate multiple *MYBs* while miR858 directly regulates the expression of *MYBs*. These *MYBs* belong to the R2R3 class, which is integrated with multiple biological processes, particularly in plant anthocyanin biosynthesis [51]. This sophisticated regulatory network might provide the accurate regulation in anthocyanin biosynthesis in different plants. This pathway has been reported to regulate the coloration of various fruit including litchi [15*], sea buckthorn [17], grapes [38], tomatoes [39], and kiwifruit [40].

In addition, the miR156-*SPL* module negatively regulates anthocyanin biosynthesis in Arabidopsis by destabilizing the MYB-bHLH-WD40 transcriptional activation complex [13]. This regulatory mechanism has also been observed in fruits.

For instance, the miR156a-*SPL12* module manipulates the accumulation of chlorophylls and anthocyanins during fruit ripening in the blueberry, in which *VcSPL12* interacted with *VcMYBPA1* (Figure2) [14**]. Similarly, in pear, miR156-targeted *SPLs* interfere with the MYB-bHLH-WD40 complex in anthocyanin biosynthesis [52]. In litchi, miR156a-targeted *LcSPL1*, interacting with *LcMYB1*, are vital in anthocyanin biosynthesis [15*].

In addition to the abovementioned two conserved pathways, other miRNAs are also involved in fruit coloration. In the blueberry, miR396 and a novel miRNA (miR_n10) target chloroplast *FILAMENTATION TEMPERATURE-SENSITIVE PROTEIN Z* (*FtsZs*) and chloroplastic *BCL-2-ASSOCIATED ATHANOGENE 1* (*BAG1*), respectively, therefore modulating coloration [53] (Figure2). In the litchi, a novel microRNA (NEW41) involved in fruit coloration is differentially expressed, and may function in anthocyanin biosynthesis by targeting *CHALCONE ISOMERASE* (*LcCHI*) [15*]. Recently, a novel mechanism suggests that the *MdMYB16/MdMYB1*-miR7125-*MdCCR* module regulates the homeostasis between lignin and anthocyanin biosynthesis in the coloration of apple fruit during light induction (Figure 2) [36**]. Some research has shown that long non-coding RNA (lncRNA) could regulate miRNAs as endogenous target mimics (eTMs) and participate in anthocyanin accumulation. In the apple, *MLNC3.2* and *MLNC4.6* (two lncRNAs), function as eTMs by blocking the miR156 mediated cleavage of *SPL* (Figure 2) [50**]. In the sea buckthorn, two lncRNAs (*LNC1* and *LNC2*) act as the eTMs of miR156a and miR828a respectively. Silencing *LNC1* and *LNC2* leads to increased and decreased anthocyanin content in the berries [17].

miRNAs involved in fruit texture

Texture is another chief fruit quality that consumers consider [54]. An interesting study shows that Pbr-miR397a, inhibits the expression of the laccase gene (*PbrLAC1*/*2*/*18*), regulates fruit cell lignification in pear fruits (Figure2) [26**].

Based on whole genome resequencing of 60 pear varieties, researchers found that the promoter region of *MIR397a* contains the TCA-element (salicylic acid response element), which possesses a single base (A-G) mutation. This SNP is associated with low levels of fruit lignin [26**].

Fruit softening is also believed to affect the fruit texture, where cell wall degradation is critical [55]. In grape, miRNA-mediated regulations that repress the target genes involved in cell wall degradation are vital for fruit softening [56]; miR479, miR399g, miR397a, miR3627-5p, miR2950, and novel_miR22 are involved in grape berry softening, via regulation of their target genes associated with fruit softening and membrane lipid peroxidation, including *β-GALACTOSIDASE* (*BGA*), *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE 3* (*ACO3*), *LIPOXYGENASE* (*LOX*), *RIPENING-INDUCED PROTEIN GRIP22* (*Grip22*)/*PHENYLALANINE MMONIA-LYASE* (*PAL*), *CHALCONE SYNTHASE* (*CHS*), and *PECTINESTERASE* (*PE*) [56]. SlymiR157 and SlymiR156 are involved in the regulation of the *LeSPL-CNR*, which belongs to the *SPL* TF family, contributing greatly to tomato fruit softening [11].

Conclusion and prospects

Great progress has been made in miRNA research over the last two decades due to next-generation sequencing and increasingly powerful bioinformatics tools. Many miRNA/phasiRNAs engaged in fruit regulation have been unveiled. Besides the conserved miRNA and phasiRNA pathways, lineage- or species-specific miRNAs/phasiRNAs also regulate traits that might be vital in a certain species, such as stone cell development in the pear and tannin metabolism in persimmon, which is beneficial to practical breeding programs. Other horticultural plants are less understood so far. The biological function of miRNAs in fruit traits are obscure, owing to the lack of an effective transformation system in woody fruit trees. Hence, function validations through overexpression, RNAi, and CRISPR out are hardly used in woody fruit trees. Instead, VIGS with a TRSV-based expression vector could

express and suppress (via STTM) the target miRNAs, and has been becoming a powerful tool to study the function of miRNA and its target genes [57].

Small RNAs, especially phasiRNAs, are capable of cell-to-cell movement and long-distance migration through the phloem [58]. Grafting is widely used in fruit crops. In a grafting plant, the vascular tissues of the stock and scion are placed in contact with each other. With the long-distance mobility of phasiRNAs, we might deliver a silencing signal from a scion that overexpresses a *PHAS* locus harboring serval phasiRNAs to the rootstock to remotely regulate the expression level of horticultural trait related genes or stress-resistant genes. This might be a useful tool to manipulate value traits of scion or rootstock during orcharding.

Conflict of interest statement

The authors declare no conflict of interests.

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Figure legends

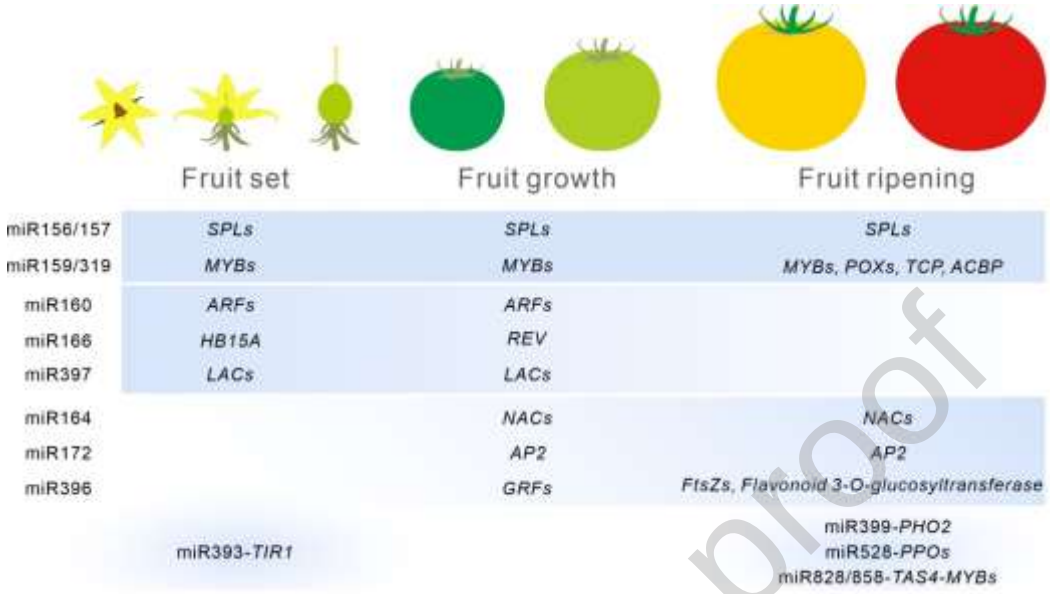

Figure 1. Conserved miRNA pathways involved in the fruit development. miRNAs and their different target genes were sorted by the fruit development stages that they were involved. miR156/157 and miR159/319 mediated regulatory pathways were involved in all stages of fruit development. miR160, miR166 and miR397 affect fruit set and growth while miR172, miR164, and miR396 affect fruit growth and fruit ripening. miR393-*TIR1* were involved only in fruit set while miR399, miR528, and miR828/858 mediated pathways were involved in fruit ripening.

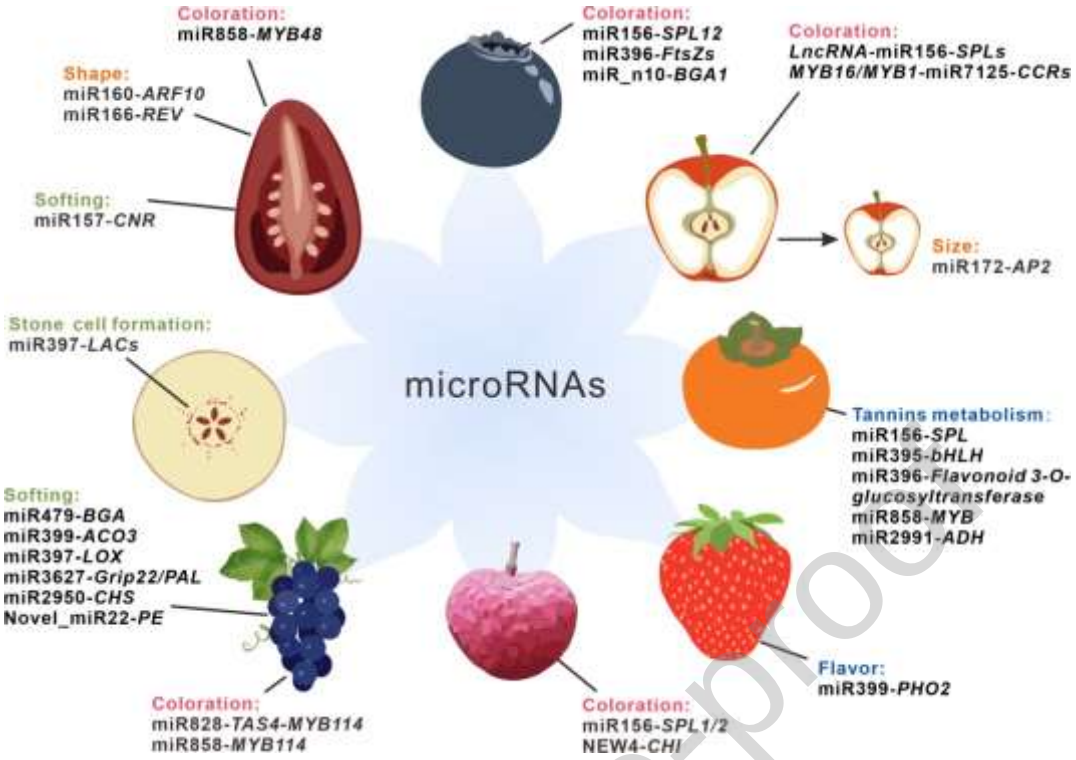

Figure 2. Main miRNA pathways involved in fruit quality. miRNA pathways involved in fruit size and shape were highlighted in orange, miRNA pathways involved in fruit flavor were highlighted in blue, miRNA pathways involved in fruit coloration were highlighted in red, miRNA pathways involved in fruit texture were highlighted in green.

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miRNA family	Target	Species	Quality Traits	Evidence Grade*	References
miR156	*SPL*	Arabidopsis	Gynoecium patterning	A	8
miR156	*SPL*	Tomato	Ovary development	A	9
miR160	*ARF10/16/17*	Longan	Embryo development	B	21
miR160	*ARF10A 10B and ARF17*	Tomato	Ovary / Placenta development	A	24
miR159	*GAMYB1/2*	Tomato	Ovule development	A	19
miR166	*HB15A*	Tomato	Ovule development	A	20
miR397	*LAC*	Arabidopsis	Seeds number	A	22
miR156	*SPL2*	Arabidopsis	Silique development	A	18
miR393	*CsTIR1 and*	Cucu	Seed ger	A	23

	CsAFB2	mber	mination and parthenocarpic fruit		
miR156	*SPL2\9\10\16*	Grape		B	10
miR157	*LeSPL-CNR(SPL)*	Tomato		B	11
miR164	*NAC6\7*	Kiwifruit	Fruit ripening	B	31**
miR172	*AP2*	Tomato		A	30
miR9177	*SlCTR4*	Tomato		A	35
miR156	*SPL*				
miR164	*NAC*				
miR319	*TCP4/GAMYB/ACBP4*	Apple	Fruit browning	C	12
miR6478	*PLP2*				
miR528	*PPO*	Banana	Fruit browning	B	34**
miR159	*POX1\2*	Strawberry	Fruit senescence	B	42
miR159/319/8	*MYB*	Litchi	Fruit senesc enc	B	33

28/858			e		
miR2188	*PGK/Ca^{2+}-ATPase*	Litchi			
miR172	*AP2*	Arabidopsis	Fruit size	A	28
miR172	*AP2*	Apple	Fruit size	A	29**
miR396	*SlGRFs*	Tomato	Fruit size	A	32
miR166	*REV*	Tomato	Fruit shape	A	25
miR160	*ARF10*	Tomato	Fruit shape	A	44
miR164 miR159	*NAC* *GAMYBs*	Wintersquash and Summersquash	Fruit shape	B	27
miR171	*GRASs*				
miR399	*PHO2*	Strawberry	Flavor	A	48
Novel-miR65,75,92	*Galactu ronosyl transferase*	Hot pepper	Sugar metabolism	B	49
miR2991	*ADH*	Persimmon	Tannins metabo	B	16
mi	*SPL*				

miR156			lism		
miR396	*Flavonoid 3-O-glucosyltransferase*				
miR858	*MYB*				
miR395	*bHLH*				
miR156	*SPL9*	Arabidopsis	Fruit coloration	A	13
miR156	*SPL12*	Blueberry	Fruit coloration	A	14**
miR156	*SPL1/2*	Litchi	Fruit coloration	B	15*
lncRNA-miR156	*SPL2-like/SPL3*	Apple	Fruit coloration	A	50**
miR156	*SPLs*	Pear	Anthocyanin biosynthesis	B	52
miR156a miR828a	*SPL9* *MYB114*	Seabuckthorn	Anthocyanin biosynthesis	B	17
miR828	*MYB82*	Arabidopsis	Fruit coloration	A	37
miR858a	*HY5-MYBL2*	Arabidopsis	Fruit coloration	A	41

miR828 miR858	*TAS4-MYB114* *MYB114*	Grape	Fruit coloration	B	38
miR858	*MYB48*	Tomato	Fruit coloration	A	39
miR858	*MYBC1*	Kiwifruit	Fruit coloration	B	40
miR7125	*CCRs*	Apple	Fruit coloration	B	36**
miR396miR_n10	*FtsZs* *BAG1*	Blueberry	Fruit coloration	B	53
NEW41	*CHI*	Litchi	Fruit coloration	B	15*
miR156/157	*SPL-CNR*	Tomato	Fruit softing	B	112
miR397	*LAC1\2\18*	Pear	Stone cell	A	26**
miR479	*BGA*				
miR399	*ACO3*				
miR397 miR3627	*LOX* *Grip22/PAL*	Grape	Fruit texture	B	56
miR2950	*CHS*				
Novel_m	*PE*				

iR
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miRNA pathways involved in fruit development were highlighted in purple, miRNA pathways involved in fruit size and shape were highlighted in orange, miRNA pathways involved in fruit flavor were highlighted in blue, miRNA pathways involved in fruit coloration were highlighted in red, miRNA pathways involved in fruit texture were highlighted in green.

*Grade A indicates the miRNA regulatory pathway is validated by multiple solid experiments including transgenic assay. Grade B indicates the miRNA regulatory pathway is validated by multiple experiments and/or illumina sequencing, but without transgenic assay. Grade C indicates the miRNA regulatory pathway were identified by illumina sequencing only.