Next-generation Sequencing Sheds New Light on Small RNAs in Plant Reproductive Development

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Abstract

Reproductive development is a key step of the plant life cycles and indicates the start of a new life cycle. The reproductive organs including flower, fruit and seed, have diverse and complex structures, which is a syndrome in the evolution of angiosperms. The development of plant reproductive organs depends on the correct spatial and temporal expression of numerous genes acting in concert to form regulatory networks. Small non-coding RNAs (sRNAs) play a key role in the reproductive development through different modes of sequence-specific interaction with their targets. The sRNAs guide transcriptional and post-transcriptional regulation to intensively integrate into the complex process. Next-generation sequencing (NGS) techniques have greatly extended the scientist's capabilities to identify and quantify sRNAs by supplying a massive number of sequences. In turn this has led to a greater understanding of the many complex roles and interactions that sRNAs are involved with during reproductive development. In this review, we provide an overview of the biogenesis and classification of plant sRNAs, and summarize the recent progress in the understanding of plant sRNA in the flower, and fruit/seed development. Also, we discuss NGS approaches to profile global sRNA expression, as well as the application of sRNA-seq/degradome-seq approaches to identify novel sRNAs and verify their targets related to the above development processes.

Introduction

In plants, small, non-coding RNAs have been characterized as an important factor in post-transcriptional regulation. Regulatory small RNAs (sRNAs) generally range in size from 20 to 24 nucleotides (nt). They are products of their larger RNA precursors that have double-stranded duplexes and are sliced by endonuclease Dicer-like (DCL) proteins (Axtell, 2013). One strand of the duplex, the mature sRNA, is bounded to the Argonaute (AGO) protein to assemble a RNA-induced Silencing Complex (RISC), which allows hybridizing with the target RNAs. The target RNAs are silenced by repressing chromatin modifications, decreasing RNA stability, and lowering translational efficiency (Vaucheret, 2006). The AGO-bounded small-RNA assembly in plant works as a reservoir of sequence-specific negative regulators deployed for a wide variety of functions in many aspects of plant development (Axtell, 2013). The mutation or overexpression of sRNAs always leads to pleiotropic phenotypes including the developmental defects in leaves, flowers, and fruits (Buxdorf *et al.*, 2010; Wang *et al.*, 2011b). The underlying mechanisms of sRNA pleiotropism could be contributed to the spatial and temporal of action of sRNA and their downstream targets. The variability of sRNA expression patterns and targets may endow them with diverse functions in different organs or developmental stages. In this review, we overview the formation and action of plant sRNAs, and discuss

next-generation sequencing (NGS) approaches (e.g. sRNA-seq/degradome-seq) to identify the sRNA and verify their targets. Mostly, we summarize the recent progress in understanding the roles of plant sRNA in reproductive regulation. We focus on the regulation of flowering time, male and female organ development, and fruit/seed development by sRNAs and their targets. Understanding how sRNAs function in plant reproductive development could provide new insights into improving the important agronomic traits during plant breeding

Plant sRNA biogenesis and classification

Generally, plant sRNA can be categorized into two different groups, based on their biogenesis and function. One group, hairpin RNAs (hpRNAs), is derived from the single-stranded precursors that process an intramolecular and self-complementary 'hairpin structure'. The other group, small interfering RNAs (siRNAs), is derived from double-stranded precursors. Plants hpRNAs include microRNAs (miRNA) and some non-microRNAs (Fig. 9.1). In contrast to most animal miRNAs which bind their targets with imprecise complementarity to repress translation (Ambros *et al*., 2003; Seggerson *et al*., 2002), plant miRNAs commonly interact with their targets through perfect or near-perfect complementarity to directly degrade mRNA target rather than repress translation (Llave *et al*., 2002; Rhoades *et al*., 2002). Although chemically similar to miRNAs, siRNAs arise from double-stranded RNAs instead of specific fold-back structures (hairpin). During RNA interference (RNAi), siRNAs guide sequence-specific cleavage of RNAs (Denli and Hannon, 2003), as well nuclear events including histone and DNA methylation, resulting in transcriptional silencing (Reinhart and Bartel, 2002; Volpe *et al*., 2002; Zilberman *et al*., 2003). Despite the differences in origin, miRNAs and siRNAs are functionally interchangeable. Like miRNAs, most known plant siRNAs function in guiding target RNA degradation (Llave *et al*., 2002; Reinhart and Bartel, 2002; Tang *et al*., 2003).

Currently, most of known plant siRNAs fall into one of the three secondary groups: heterochromatic siRNAs (hc-siRNAs), secondary siRNAs (typically, trans-acting siRNA or ta-siRNA), or natural antisense transcript-derived siRNAs (natural-antisense siRNAs, or nat-siRNAs) (Fig. 9.1) (Axtell, 2013). Hc-siRNAs, the most abundant type of sRNAs, are derived from transposons or other repetitive sequences (Wei *et al*., 2012). Their single-stranded RNA transcripts are transcribed by the DNA-dependent RNA polymerase IV (Pol IV) (Herr *et al*., 2005; Onodera *et al*., 2005), and converted into dsRNAs by RNA-dependent RNA polymerase 2 (RDR2) (Xie *et al*., 2004). These dsRNAs are then processed by DCL3 to generate 23- to 24-nt-long hc-siRNAs (Wroblewski *et al*., 2014). Secondary siRNAs production tends to be triggered by one or more upstream sRNAs, which could be either miRNAs or other siRNAs. The upstream sRNAs can also help recruit an RDR to synthesize complementary double-stranded RNA, which are then processed into secondary siRNA by DCL (Allen *et al*., 2005; Axtell, 2013; Yoshikawa *et al*., 2005). Ta-siRNAs are a representative class of the secondary siRNAs. Ta-siRNAs are transcribed from TAS transcripts by RNA polymerase II (Pol II). The TAS transcripts either go through AGO-mediated and miRNA-guided cleavage or are converted to double-stranded RNA by RDR6 and Suppressor of Gene Silencing 3 (SGS3) (Talmor-Neiman *et al*., 2006). The resulting dsRNAs are further processed by DCL4 to produce 21-nt length siRNAs (Allen *et al*., 2005). Nat-siRNAs precursors are formed by the hybridization of independently transcribed and complementary RNAs, which relies on the plant-specific RNA polymerase IV (PolIV) (Zhang *et al*., 2012). An initial nat-siRNA from the complementary region of two NAT genes is produced through the action of DCL and RDR, and then the initial nat-siRNAs guide the cleavage of one NAT gene thus forms the sequential production of new nat-siRNAs. However, these new nat-siRNAs are not required for further cleavage, unlike tas-siRNA playing a role in targeting unlinked transcripts (Vaucheret, 2006). Nat-siRNAs are derived either from the opposite strands of the same locus (*cis*-Nat-siRNAs), or from genes with no overlap (*trans*-Nat-siRNAs). However, *trans*-NAT-siRNAs have not been found in plants until recently (Axtell, 2013). In previous *Arabidopsis* studies, the biogenesis of biotic- and abiotic-induced nat-siRNAs is dependent on DCL1 and/or DCL2, and RDR6 (Borsani *et al*., 2005; Katiyar-Agarwal *et al*., 2006). The different result in *Arabidopsis* and rice shows that the production of 23- to 28-nt nat-siRNAs are

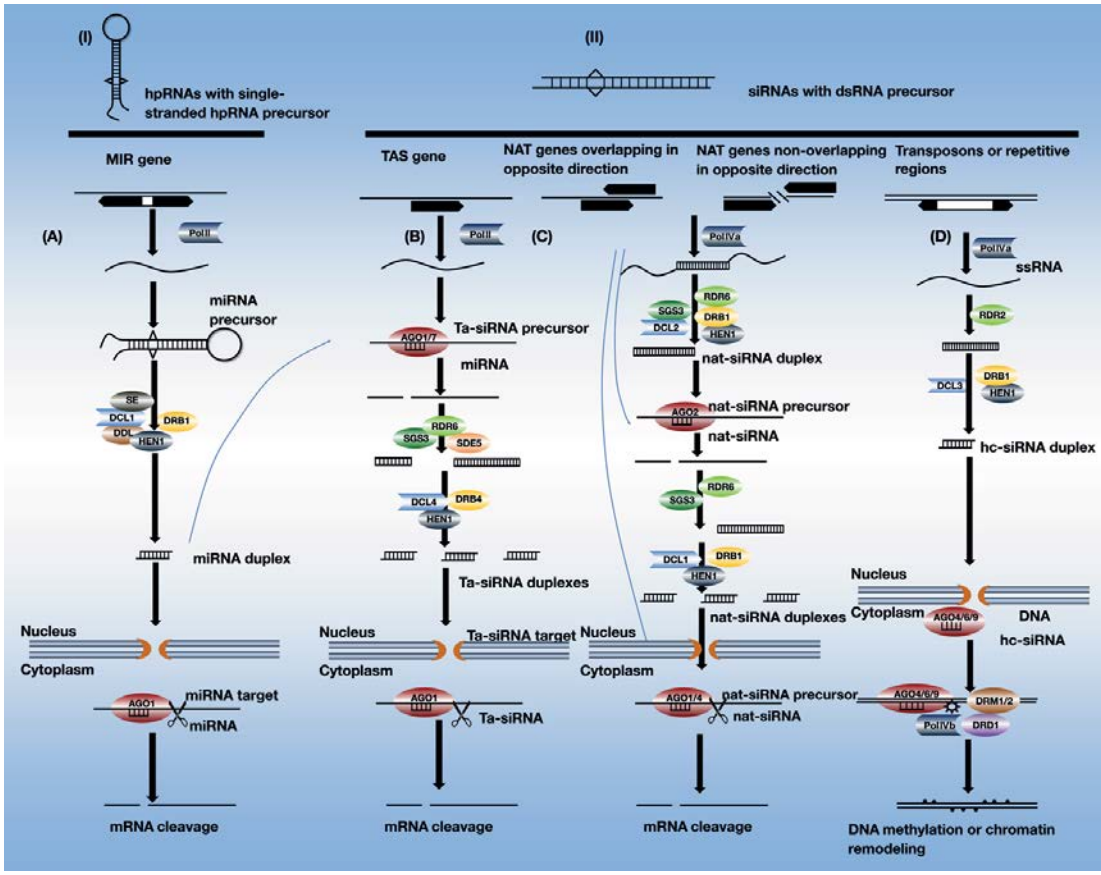

Figure 9.1 miRNA, ta-siRNA, nat-siRNA, and hc-siRNA pathways in plants. The same colour is used to indicate members of the same gene family. Plant small RNAs have two groups. (I) One is derived from single-stranded precursors that process an intramolecular and self-complementary 'hairpin structure', called hairpin RNAs (hpRNAs), typically, miRNA, and (II) the other is derived from double-stranded precursors, and called small interfering RNAs (siRNAs), including ta-siRNA, nat-siRNA, and hc-siRNA. (A) The miRNA pathway: MIRNA genes are transcribed by Pol II into precursor RNAs that form hairpin stem–loops due to their self-complementary structure, a classical trait of miRNA precursors. Processing of miRNA precursors into a 21-to 22-nucleotide miRNA duplex with 2-nucleotide 39 overhangs requires Cap binding proteins CBP20 and CBP80, DAWDLE (DDL), SERRATE (SE)SE (a zinc finger protein), DRB1 (a dsRNA binding protein), and DCL1 (an RNaseIII). miRNA duplexes are methylated by HEN1 to protect against SDN exonuclease degradation. One strand of the miRNA duplex binds to AGO1. The miRNA-AGO1 are transported to the cytoplasm to complement mRNA targets, and AGO1 cleaves the target mRNAs. (B) The ta-siRNA pathway: ta-siRNAs are also transcribed by Pol II. miRNAs binds to AGO1 or AGO7 to guide cleavage of ta-siRNA precursors. After cleavage, fragments of ta-siRNA precursor are synthesized into dsRNA by RDR6, SGS3, and SILENCING DEFECTIVE5 (SDE5), and the dsRNA is cleaved into 21- to 22-nucleotide ta-siRNAs by the DRB4/DCL4/HEN1 complex. (C) nat-siRNA pathway: a pair of natural cis-antisense transcripts (overlapping or non-overlapping) are transcribed by Pol IV. Two transcripts generate a dsRNA molecular with their complementary end sequences. The dsRNA are cleaved by DCL2, assisted by NRPD1a, RDR6, and SGS3, and turned into a stable 24-nt nat-siRNA. This single 24-nucleotide nat-siRNA subsequently targets one of the cis-antisense gene pair transcripts for cleavage, and the cleaved RNA molecule is converted to dsRNA by RDR6 and SGS3. The RDR6/SGS3-synthesized dsRNA molecule is then processed into phased 21- nucleotide nat-siRNAs by the action of DCL1, assisted by DRB1 and HEN1. The phased 21-nucleotide nat-siRNAs, like the ta-siRNA class of endogenous sRNAs, are in turn used as guides to direct sequence-specific silencing of homologous mRNAs. However, the production of these 21-nt nat-siRNAs does not appear to be required for down-regulating the constitutive transcript. (D) Heterochromatin pathway: transposons and repeat DNA are transcribed by PolIV, which is then converted into dsRNA by RDR2-mediated synthesis of dsRNA. The dsRNA is processed by DCL3 into 24-nucleotide siRNAs. These siRNAs are methylated by HEN1 and loaded onto AGO4, AGO6, and AGO9, which induce DNA methylation through the DNA methyltransferase DRM1/2. DNA methylation of some loci also requires PolIV and the SWI2/SNF2-like chromatin remodelling factor DRD1.

dependent on DCL3 and RDR2, while 20- to 22-nt nat-siRNAs are processed by DCL1 and RDR6, (Borsani *et al.*, 2005; Ron *et al.*, 2010; Zhang *et al.*, 2012).

NGS accelerating plant sRNAomics

Many efforts have been made to discover and identify sRNA from diverse plant species in recent years. To date, three main approaches, including forward genetics, bioinformatics prediction and direct cloning and sequencing, are used to identify sRNA and their targets, and define sRNA function in plants, (Liu *et al.*, 2014a). The methods of forward genetics primarily rely on mapping, isolating, cloning, and sequencing small cellular RNAs. Although these endeavours have drastically increased the amount of information available for sRNA and their target mRNAs, it is still impractical to count on these traditional genetic methods to empirically explore a large scale of complex and diverse sRNAs and their targets mRNAs (Liu *et al.*, 2014a). Thanks to the pairing between sRNAs and their targets, bioinformatic methods have become feasible and effective in identifying sRNAs. The precise or near-precise complementarity between miRNAs and mRNAs in plants, as well as their high conservation, makes systematic target identification easier in plants than in animals (Axtell and Bartel, 2005; Rhoades *et al.*, 2002). However, a number of non-conserved miRNAs are specifically expressed in certain species, tissue or developmental stages but at low levels, which makes it difficult to identify them (Mao *et al.*, 2012; Xu *et al.*, 2012).

In recent years, the next-generation sequencing (NGS) technologies have replaced many of the traditional methods for identifying sRNA and their targets. NGS has dramatically accelerated genomic studies by supplying a massive amount of sequences data, which allows comprehensive analysis of genomes, transcriptomes, and sRNAomics. The current NGS platforms include 454 Genome Sequencers (Roche Applied Science), Solexa Genome Analyser (Illumina), the SOLiD platform (Applied Biosystems), and the more recent Polonator (Dover/Harvard) and the Helicos sequencer (Helicos) and the Ion Torrent Personal Genome Machine (PGM) (Life Technologies). These NGS platforms have reduced the cost and time of required for traditional Sanger sequencing (Liu *et al.*, 2012; Loman *et al.*, 2012; Metzker, 2010; Shendure and Ji, 2008). The application of NGS has considerably advanced miRNA discovery in several non-model plants as it has provided a large amount of low-cost genomic and transcriptomic data for these plant species (Fang *et al.*, 2014). Furthermore, NGS also provides a new method for the validation of the splicing targets throughout the whole genome, i.e. degradome sequencing. Degradome sequencing can precisely identify sRNA target genes and explore their interactions which aids in clarifying the functions of these sRNAs in the diverse biological processes. NGS has revolutionized the traditional computational target prediction, and been successfully applied to screen for miRNA targets in many plants, for example *Arabidopsis* (Addo-Quaye *et al.*, 2008; German *et al.*, 2008), grapevine (Pantaleo *et al.*, 2010) maize (Zhao *et al.*, 2012), cucumber (Mao *et al.*, 2012), oilseed rape (Xu *et al.*, 2012), rice (Sun *et al.*, 2015) and soybean (Chen *et al.*, 2016).

Among the NGS platforms, Illumina-Solexa has been a popular platform, due to its relatively low cost, relatively short running time and relatively long reads. ABI SOLID yields the highest accuracy but requires a long time for sequencing runs. Compared with the other methods, Roche 454 generates the longest reads, which allows for more accurate read assembly and can span larger repetitive regions within the genome. However, Roche 454 is costly and low throughput and has a high error rate. Ion torrent is notable for its three different priced sequencing-chip reagents based on the throughout requirement, so that scientists have different choices according to their experiment design. However, like Roche 454, Ion Torrent PGM is apt to produce homopolymer-associated indel errors (Loman *et al.*, 2012). HeliScope overcomes the bias representation of templates and mutations in clonally amplified templates, but expensive and introduces higher error rates compared with other technologies (Metzker, 2010). Polonator is an open source platform with free software and protocols so that users are required to maintain and control reagents qualities. It may be the least expensive platform, but yields the shortest reads so far (Metzker, 2010). Interested readers can refer to several excellent reviews which introduce these NGS platforms in more detail (Liu *et al.*, 2012;

Loman *et al*., 2012; Metzker, 2010; Shendure and Ji, 2008).

Since NGS' steps into the model species *Arabidopsis* (Fahlgren *et al*., 2007; Lu *et al*., 2005; Rajagopalan *et al*., 2006; Sunkar and Zhu, 2004) and rice (Jones-Rhoades and Bartel, 2004; Sunkar *et al*., 2005) for studying sRNAs, it has been also successfully applied in many other non-model plant species (Bi *et al*., 2015; Khaldun *et al*., 2015; Wu *et al*., 2014; Xin *et al*., 2015; Xu *et al*., 2013; Yao *et al*., 2015a; Zeng *et al*., 2015). Plant miRNAs are one of the most widely studied groups of sRNAs. To date, a total of 8496 miRNAs from 73 Viridiplantae species have been registered in the latest miRBase (version 21, June 2014) (www.mirbase.org/) (Kozomara and Griffiths-Jones, 2014). For comparison, a total of 8,433 miRNAs from 121 plant species as well as the possible target genes and predicted interaction site have been deposited in the Plant Microrna Database (PMRD) (http://bioinformatics.cau.edu.cn/PMRD/) (Zhang *et al*., 2010). Another resource for predicting and comparing plant microRNAs is MicroPC (μPC) (www3a.biotec.or.th/micropc/index.html), which provides the comprehensive information of plant miRNAs from two sources including the previously reported miRNAs from miRBase and the predicted miRNAs. It also collects the targets of these miRNA based on a large-scale expressed sequence tags (ESTs) analysis (Mhuantong and Wichadakul, 2009). In addition, a plant sRNA target analysis server (psRNATarget) (http://plantgrn.noble.org/psRNATarget/) has incorporated a series of recently discovered miRNA target recognition sites in plants (Dai and Zhao, 2011). To relieve the growing pressure of handling massive quantities of short-read sequences, numerous NGS-based sRNA transcriptome bioinformatics analysis tools have been developed (Li *et al*., 2012; Zhou *et al*., 2011). These tools include the standalone software used for deep sequencing miRNA data, such as MicroRazerS (Emde *et al*., 2010), CASHX pipeline (Fahlgren *et al*., 2009), mirDeep/mirDeep2 (Friedländer *et al*., 2008, 2012), miRTRAP (Hendrix *et al*., 2010), MIREAP (Song *et al*., 2010), miRNAkey (Ronen *et al*., 2010) MIReNA (Mathelier and Carbone, 2010), miRExpress (Wang *et al*., 2009b), miREvo (Wen *et al*., 2012), and miRVine (Belli Kullan *et al*., 2015), as well as some web servers, such a mirTools (Zhu *et al*., 2010), UEA sRNA toolkit (Moxon *et al*., 2008b), and SeqBuster (Pantano *et al*., 2010). Moreover, several databases offer the serves for miRNAs search and sRNAs annotation, such as deepBase (Yang *et al*., 2010), miRBase (Griffiths-Jones *et al*., 2008), and FANTOM4 EdgeExpressDB (Severin *et al*., 2009). Among these tools, mirTools, UEA sRNA Toolkit, miRNAkey, miRExpress, miRExpress, FANTOM4 EdgeExpressDB, and miRVine can also be used to compare the expression of sRNAs based on NGS' data. Performance comparison and evaluation of some software tools are available in some previous reviews (Li *et al*., 2012; Zhou *et al*., 2011). Notably, several particular tools, such as psRNATarget, UEA sRNA tools (Stocks *et al*., 2012), Target-Align (Xie and Zhang, 2010), mirplant (An *et al*., 2014) and PsRobot (Wu *et al*., 2012) are tailored to deal with plant sRNAs based on species specificity.

SRAs profiling by NGS in plant reproductive development

Plant development goes through two distinct phases: vegetative and reproductive growth. Reproduction in angiosperms is referred to as a continuous process characterized by three distinct phases, i.e. flowering and pollination, fruits and seed dispersal, seed and seedling establishment. Flowers have their unique appearances, as a result of angiosperms' evolutionary success (Li *et al*., 2016b). Their specialized and complex perianth morphologies have evolved in response to the selective pressure from pollination. The outmost whorl of flower (sepals) plays a protective role, while the inner whorl, the corolla, attracts insects with showy petals. The fruit develops from the flower, which is also the result of angiosperms' evolutionary success. The fruit primarily grows to accommodate the seeds, protects and nourishes them, and assists in their dispersal at maturity.

Recently, intensive studies on sRNA transcriptome have shed light on understanding the molecular mechanisms that control flower and fruit/seed development in both model (Chen *et al*., 2011a; Lee *et al*., 2010; Mohorianu *et al*., 2011; Moxon *et al*., 2008a; Zuo *et al*., 2012, 2013) and non-model plants (Bi *et al*., 2015; Khaldun *et al*., 2015; Li *et al*., 2015a; Liu *et al*., 2014d; Xin *et al*., 2015; Xu *et al*., 2013; Yao *et al*., 2015a; Zeng *et al*., 2015). High-throughput parallel sequencing

technologies have greatly improved the efficiency of finding novel sRNA functions under various conditions and from plant species. sRNA families have also been identified across various plant species, many of which are universal. These sRNAs are often involved in numerous crucial roles at each major stage of development and target the regulatory genes, such as transcription factors.

sRNAs related to flower development have been profiled in many crops, vegetable, fruit trees and ornamental flowers. In wild rice (*O. rufipogon*), 512 conserved rice miRNAs in 214 miRNA families and 290 new predicted miRNAs have been identified from three libraries generated at the vegetative and flowering stages (Chen *et al.*, 2013). Among these miRNAs, 187 miRNAs regulate the expression of flowering-related genes, including flowering-related miRNAs, such as oru-miR97, oru-miR117, oru-miR135, and orumiR137, etc., and early-flowering-related miRNAs, such as osa-miR160f, osa-miR164d, osa-miR167d, osa-miR169a, osa-miR172b, and oru-miR4, etc. In hickory (*Carya cathayensis Sarg.*), 39 known miRNAs in 23 families, as well as two novel and ten potential novel miRNAs in nine families, have been identified from two floral differentiation stages (Wang *et al.*, 2012b). A diverse set of miRNAs and their target genes may be related to hickory flower development. In radish, 94 known miRNAs in 21 conserved and 13 non-conserved miRNA families, as well as 44 potential novel miRNAs, have been identified from the two libraries at vegetative and reproductive stages (Nie *et al.*, 2015). Among them, 42 known and 17 novel miRNAs with different expression have been identified as bolting and flowering-related miRNAs, and 154 target transcripts for these miRNAs have been shown to be involved in plant development, signal transduction, and transcriptional regulation. In four varieties of Roses (*Rosa* sp.), 25 novel and 242 conserved miRNAs have been identified from floral tissues (Kim *et al.*, 2012a). Combinative analysis with transcriptomic data identified a serial of shared and species-specific miRNAs. They are involved in flower development. Degradome and sRNA analysis in the leaves, stalks and flower buds of *Phalaenopsis* exposed to low ambient temperature have identified 46 sRNA groups and their targets that are involved in flowering time and developmental processes under (An and Chan, 2012). In cymbidium, 48 conserved mature conserved miRNA in 20 families, and 45 novel miRNAs, as well as two ta-siRNAs, have been identified from flower tissue at different stages (Li *et al.*, 2015a). Being assisted with transcriptomic data, flower-development-related unigenes have been identified, including the MADS-box transcription factors targeted by miR156, miR172 and miR5179, as well as various hormone responding factors targeted by miR159.

Many sRNA have been discovered to be involved in a model plant-tomato fruit development. A large amount of the targets of the sRNAs are involved in ethylene (ET) or auxin (AUX) pathway, such as ethylene response factors (*AP2/ERF* and *ERF4*) or a key enzyme in ET biosynthesis (*1-aminocyclopropane-1-carboxylate oxidase 1*, *ACO1*), as well as two splice variants of constitutive *triple response 4* (*LeCTR4* and *LeCTR4*) regulated by miR1917 (Moxon *et al.*, 2008a). In another study, seven novel miRNA families, as well as 24 families including 103 conserved miRNAs and ten non-conserved miRNAs have been identified at three different fruit ripening stages and fruits by exogenous ET treatment (Zuo *et al.*, 2012). The targets of many these miRNAs are predicted to be transcription factors, such as *CNR* or *AP2a*, regulated by miR156/157 or miR172, respectively. Some targets are putatively involved in fruit ripening and softening, such as *pectate lyase* and *beta-galactosidase*, while a few are putatively involved in ET biosynthesis and signalling pathways, such as *ACS*, *EIN2* and *CTR1*. Two members of the TAS3 family (miRZ8 and miRZ9), 590 putative phased sRNAs and 125 cis-natural antisenses (nat-siRNAs) have also been identified in tomato fruit ripening process (Zuo *et al.*, 2013). Furthermore, the degradome has identified a total of 119 pairs of miRNA-mRNA e.g. AGO1/miR168, 106 of which are new, e.g. TAS3-mRNA/miR390 (Karlova *et al.*, 2013). AGO1/miR168 is involved in miRNA biogenesis, indicating a feedback loop regulation of fruit development. Moreover, the miRNAs/targets responding to ET also results in tomato pedicel abscission (Xu *et al.*, 2015). The correlation can vary between a miRNA and its target during tomato fruit ripening. Since miRNAs always negatively regulate their targets, the high number of positively correlated miRNA/target pairs suggests that mutual exclusion could be as widespread as temporal regulation. For example, miR164 and NAMCUC2 are positively related in flower opening, whereas, as the fruit develops,

miR164 becomes negatively related to *NAM-CUC2* (Lopez-Gomollon *et al.*, 2012).

Next-generation sequencing has also been applied to identify sRNA and their expression pattern in the seed of many important crops, as well as miRNA target cleavage. In rice, 434 known miRNAs have been obtained from different rice grain filling stages. The predicted targets of the differentially expressed miRNAs may participate in signal transduction, carbohydrate, and nitrogen metabolism, the response to stimuli and epigenetic regulation (Yi *et al.*, 2013). Similarly, 457 known miRNAs and 13 novel miRNAs have been identified from superior and inferior spikelets during grain development stages. Genes targeted by those differentially expressed miRNAs, i.e. miR156, miR164, miR167, miR397, miR1861, and miR1867, have been considered to play roles in multiple developmental and signalling pathways related to plant hormone homeostasis and starch accumulation (Peng *et al.*, 2014). In maize, 40 known and 162 novel miRNA families have been identified from developing grain. Further analysis shows miR159, miR164, miR166, miR171, miR390, miR399, and miR529 families have putative roles in the embryogenesis of maize grain development by participating in transcriptional regulation and morphogenesis, while miR167 and miR528 families participate in metabolism process and stress response during nutrient storage (Li *et al.*, 2016a). In the developing fibres of cotton, sRNA sequencing has identified 47 conserved miRNA families and seven new miRNAs (Liu *et al.*, 2014d). Together with degradome sequencing, 140 targets of 30 conserved miRNAs and 38 targets of five new miRNAs have been confirmed to affect fibre development, including *SBP* and *MYB*, *LEUCINE-RICH RECEPTOR-LIKE PROTEIN KINASE* (*LRR-RLK*), *PECTATE LYASE* (*PLL*), *TUBULIN*, *UDP-GLUCURONIC ACID DECARBOXYLASE* (*UXS1*) and *CYTOCHROME C OXIDASE SUBUNIT 1* (*CO1*). miRNA156/157 in ovule and fibre development regulate mature fibre length.

Next-generation sequencing has considerably advanced fruit miRNA discovery in fruit species. In strawberry, 88 known and 1224 new miRNAs have been obtained, as well as 103 targets cleaved by 19 known and 55 new miRNAs families. Among them, 14 targets, including *NAC* transcription factor, *ARF*, and *MYB* transcription factors have been found to be involved in regulating fruit senescence (Xu *et al.*, 2013). In apple, 23 conserved, 10 less-conserved and 42 apple-specific miRNAs or families have been discovered from fruit and other vegetative organs (Xia *et al.*, 2012b). The miRNAs target 118 genes with a wide range of enzymatic and regulatory activities. In addition, over 70 diverse genes targeted by 100 miRNAs have been confirmed by degradome analysis. The confirmed genes are potentially involved in diverse aspects of plant growth and development. In pear, 2216 target genes of 188 known miRNAs and 1127 target genes of 184 novel miRNAs have been identified. The miRNAs are widely involved in the regulation of fruit development, including 11 miRNAs in the pathway of lignin biosynthesis, nine miRNAs in sugar and acid metabolism, and one regulating AUX response factor (Wu *et al.*, 2014). In date palm, 238 conserved miRNAs and defined 78 fruit-development-associated miRNAs have been identified. These miRNAs function mainly in regulating genes involved in starch/sucrose metabolisms and other carbon metabolic pathways (Xin *et al.*, 2015). In litchi, 296 miRNAs in 49 known miRNA families have been identified from pericarp under ambient storage and post-cold storage, as well as their 197 targets Of these, 14 miRNA–target pairs are actively involved in litchi fruit senescence-related processes, including energy regulation, anthocyanin metabolism, hormone signalling, and pathogen infection defence (Yao *et al.*, 2015a). In *Lycium chinense*, 60 conserved miRNAs in 31 families and 30 novel miRNAs have been identified from shoot tip and fruit. Five miRNAs are related to fruit maturation, lycopene biosynthesis and signalling pathways (Khaldun *et al.*, 2015). In another *Lycium* species, *L. barbarum*, 50 novel miRNAs, and 38 known miRNAs are from fruit at different developmental stages. The miRNA–target interactions for *L. barbarum* ripening regulators include miR156/157-*LbCNR* and -*LbWRKY8*, and miR171-*LbGRAS*. Regulatory interactions potentially control fruit quality and nutritional value via sugar and secondary metabolite accumulation, including miR156 targeting of *FRUCTOKINASE* and *1-deoxy-D-xylulose-5-phosphate synthase* (*DXS*) and miR164 targeting of *BETA-FRUCTOFURANOSIDASE* (Zeng *et al.*, 2015). In banana, a total of 125 known miRNAs and 26 novel miRNAs have been identified from fruit under ET or *1-methylcyclopropene* (1-MCP) treatment. The differentially

expressed 82 miRNAs have been predicted to target 815 genes. Some of the target genes encode transcription factors and other functional proteins, including *SPL*, *AP2*, *EIN3*, *E3 ubiquitin ligase*, *β-galactosidase*, and *β-glucosidase* (Bi *et al*., 2015).

In summary, comprehensive analyses of the transcriptome, sRNAs and degradome using NGS provides a useful platform for investigating miRNA-directed and non-miRNA-directed endonucleolytic cleavage in plants, and is a viable tool for exploring sRNA–target interactions involved in reproductive development.

Roles of sRNAs in flowering time

Flowering is a crucial step of the reproduction in angiosperms. It starts from the floral transition and the floral patterning and go to the development of floral organs (Luo *et al*., 2013). Floral transition is the switch from the vegetative stage to the reproductive growth. The process is influenced by diverse endogenous and exogenous cues such as age, hormones, photoperiod, and temperature (Andrés and Coupland, 2012; Bäurle and Dean, 2006; Srikanth and Schmid, 2011). Molecular and genetic studies of the model plant, *Arabidopsis*, have suggested there are five flowering time pathways, including age, autonomous, gibberellins (GAs), photoperiod and vernalization pathways (Amasino and Michaels, 2010). Further evidence indicates the extensive crosstalks, feedback or feed-forward loops between the components of these pathways. Many of these components are transcription factors, and a typical example is the MADS-box genes determining the identity of floral organ and patterned to the classic ABCDE model (Krizek and Fletcher, 2005; Li *et al*., 2016b; Li *et al*., 2015a; Li *et al*., 2013b). The class A genes (e.g. *APETALA1*, *AP1*), B genes (e.g. *PISTILLATA*, *PI*, and *APETALA3*, *AP3*), C genes (e.g. *AGAMOUS*, *AG*), D genes (e.g. *SEEDSTICK*, *STK* and *SHATTERPROOF*, *SHP*), and E genes (e.g. *SEPALLATA*, *SEP*) are interacted and orchestrated to control the formation of the four whorls, i.e. sepal, petals, stamen, and ovule (Krizek and Fletcher, 2005; Theissen, 2001). In addition, other MADS-box genes, e.g. *FLOWERING LOCUS T (FT)* (Lee *et al*., 2013) and *FLOWERING LOCUS C (FLC)* (Deng *et al*., 2011), *SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1)* (Lee and Lee, 2010), *FRUITFULL (FUL)* and *LEAFY (LFY)* (Yamaguchi *et al*., 2009) have been reported to regulate flowering time, while *AGAMOUS-LIKE 15 (AGL15)*, *AGL18* (Adamczyk *et al*., 2007) and *FOREVER YOUNG FLOWER (FYF)* (Chen *et al*., 2011b) play some roles in flower senescence and abscission.

sRNAs work as the mediators of transcription factors for crosstalk to regulate the floral transition, and they are finally integrated into flowering pathways by feedback or feed-forward loops. In vascular plants, current studies have focused on miRNAs, such as the well-studied miR156 and miR172 (Fig. 9.2). The two miRNAs are a part of highly conserved regulatory module across all angiosperms (Huijser and Schmid, 2011). They arise independently but are negatively regulated by each other (Li and Zhang, 2016). miR156 targets a group of transcription factors, called *SQUAMOSA PROMOTER BINDING likes* (*SPB* or *SPLs*) family genes, such as *SPL3* and *SPL9* (Rhoades *et al*., 2002; Xing *et al*., 2010) (Table 9.1). The expression of miR156 enriches at the seedling stage and then drops progressively, which is observed in *Arabidopsis*, rice, maize, and poplar (Chuck *et al*., 2007a; Wang *et al*., 2011a; Wu and Poethig, 2006; Xie *et al*., 2012). The decrease in miR156 expression results in accumulation of *SPLs*, which in turn promotes flowering (Kim *et al*., 2012b). For example, *SPL9* promotes the transcription of *FUL*, *SOC1*, and *AGL42* (Wang *et al*., 2009a), *SPL3* promotes the transcription of *FUL*, *LFY*, and *AP1* (Wang *et al*., 2009a; Yamaguchi *et al*., 2009), and *SPL4* and *SPL5* promote the transcription of *FUL*, *SOC1*, *LFY*, *AP1*, and *FT* (Yamaguchi *et al*., 2009). Interestingly, these studies also indicate that *SPL3* and *SPL9* act in parallel to the floral inducer, *FT* (Fig. 9.2). In contrast to miR156, miR172 is scarcely expressed in plant juvenile phases of plants and accumulates over the developmental time. MiR172 acts downstream of miR156 and promotes flowering (Aukerman and Sakai, 2003; Chen, 2004; Jung *et al*., 2007; Wu *et al*., 2009). miR172 is regulated by miR156 through a bridge of *SPLs*. *SPLs* directly regulates the transcription of miR172 by binding its promoter region (Wu *et al*., 2009). miR172 specifically targets *AP2*, *TOE1*, *TOE2*, *TOE3*, *SMZ*, and *SNZ* in *Arabidopsis* (Aukerman and Sakai, 2003; Mathieu *et al*., 2009; Schmid *et al*., 2003), and six *AP2-like* genes in maize, including *GL15* (Chuck *et al*., 2007b) (Table 9.1). These genes act the

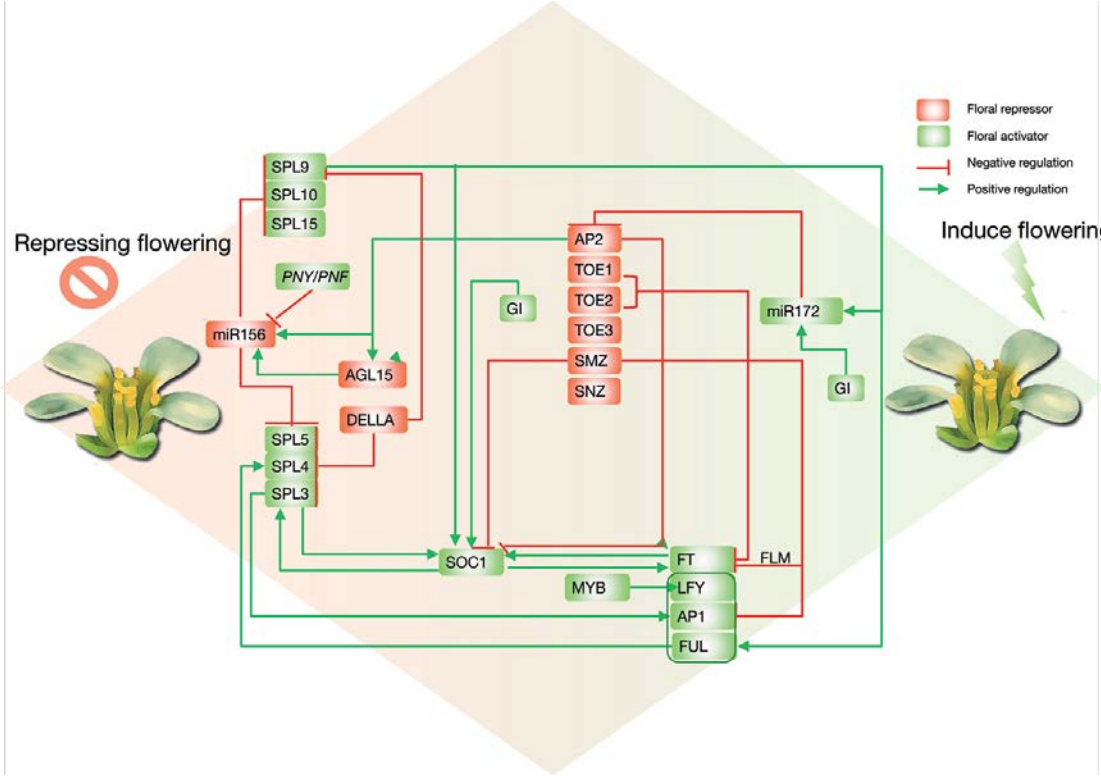

Figure 9.2 The *miR156–SPL* and *miR172–AP2* modules participating in the regulatory networks of flowering. The miR156 pathway is shown on the left and the miR172 pathway on the right. miR156 down-regulates its target genes, SPL family TFs, while miR172 down-regulates the target genes of the *AP2*-like family. Flowering is induced by up-regulating expression of *SPL3*, *SPL4*, and *SPL5* genes. The *spl4* expression is activated by FUL. SPL3 regulates the expression of LFY, *AP1*, *FT*, and *FUL*, and *SPL9* directly regulates the expression of *AP1*, *SOC1*, and *FUL*. *The spl4* expression is activated by FUL. Flowering can be suppressed by expression of AP2-like genes, *AP2*, *SMZ*, *SNZ*, and *TOE1–3*. miR156 is positively regulated by *AP2* and *AGL15*. *TOE1* and *TOE3* repress flowering by down-regulating *FT*. *SMZ* negatively regulates the expression of *AP1*, *SOC1*, and *FT*, the latter through *FLM*. The *DELLA* protein represses *SPL9* and the expression of SPL3/4/5. SPL3/4/5, are highly induced by *PNY* and *PNF* through negative regulation of miR156. Green arrows and green boxes represent activation and floral activators, respectively. Red arrows and red boxes represent repression and flower repressors, respectively. *AGL15*, *AGAMOUS-LIKE15; AP2*, *APETALA2; PNY*, *PENNYWISE; PNF*, *POUND-FOOLISH; FT*, *FLOWERING LOCUS T; FLM*, *FLOWERING LOCUS M; FUL*, *FRUTIFUL; LFY*, *LEAFY; SOC1*, *SUPPRESSOR OF CONSTANS1; SMZ*, *SCHLAFMUTZE; SNZ*, *SCHNARCHZAPFEN; SPL*, *SQUAMOSA PROMOTER-BINDING PROTEIN-LIKE; TOE1–3*, *TARGET OF EAT1–3.*

downstream targets of miR156 (Wu *et al.*, 2009) and negatively regulate flowering time (Aukerman and Sakai, 2003; Jung *et al.*, 2007; Lauter *et al.*, 2005). Thus, miR156 and miR172 form a feedback loop for fine tuning the flowering response (Yant *et al.*, 2010).

In addition, miR156 and miR172 appear to mediate the interactions within other flowering pathways (Bergonzi *et al.*, 2013; Zhou *et al.*, 2013). In the GA pathway of *Arabidopsis*, GA promotes flowering through the degradation of several *DELLA* proteins, GA repressors, which can interact with *SPL9* (Harberd *et al.*, 2009; Yu *et al.*, 2012) (Fig. 9.2). The *SPL*-mediated activation of miR172 and floral identity genes eventually leads to floral transition (Yu *et al.*, 2012). The stability of *DELLA*, which directly affects *SPLs* and miR172 activity, is not only regulated by the GA signal but also by several other phytohormones, including AUX, ET, and abscisic acid (ABA) (Vanstraelen and Benkova, 2012). In the photoperiod pathway, miR172 is regulated by photoperiod through *GIGANTEA* (*GI*), a key regulator that mediates photoperiodic flowering (Jung *et al.*, 2007) (Fig. 9.2). *GI* can

Table 9.1 SRNAs and their target genes involved in reproductive development, including flower and fruit/seed development

miRNA	Target genes	Species	Function(s) of target genes in reproductive development	References
miR156	*SPL2*, *SPL3*, *SPL4*, *SPL5*, *SPL9*, *SPL10*, *SPL15*	*Arabidopsis thaliana*	Floral organ identity and flowering time	Gandikota *et al*., 2007; Schwab *et al*., 2005; Schwarz *et al*., 2008; Wang *et al*., 2008; Wu *et al*., 2009; Wu and Poethig, 2006; Yamaguchi *et al*., 2009
	SPL9–2	*Brassica rapa* ssp. *pekinensis*	Controls the heading time by shortening the seedling and rosette stages	Wang *et al*., 2014b
	SBP1	*Antirrhinum majus*	Controls flowering time	Preston and Hileman, 2010
	SBP3	*Physcomitrella patens*	Negatively regulates the vegetative developmental transition from protonemata to leafy gametophores	Cho *et al*., 2012
	Teosinte glume architecture1 (tga1)	*Zea mays*	Promotes flowering	Chuck *et al*., 2007a
	SPL	*Panicum virgatum* L. (switchgrass)	Promotes flowering	Chuck *et al*., 2011; Fu *et al*., 2012
	SBP	*Solanum lycopersicum*	Promotes normal phase change	Salinas *et al*., 2012; Zhang *et al*., 2011
	SPL	*Oryza sativa*	Promotes flowering	Xie *et al*., 2006, 2012
	SPL	*Acacia confusa*, *Acacia colei*, *Eucalyptus globulus*, *Hedera helix*, *Quercus acutissima*, *Populus* × *canadensis*	Promotes juvenile-to-adult vegetative phase change	Wang *et al*., 2011a
	AaSPL	*Arabis alpina*	Promotes flowering	Bergonzi *et al*., 2013
	NtSPL	*Nicotiana tabacum*	Promotes flowering	Zhang *et al*., 2015
miR156/157	*SlySBP2*, *SlySBP6b*, *SlySBP10*, *SlySBP13*, *SlySBP15*	*Solanum lycopersicum L.*	Flower and fruit morphology, fruit ripening	Ferreira e Silva *et al*., 2014; Karlova *et al*., 2013; Moxon *et al*., 2008a
	CNR		Fruit development and ripening	Karlova *et al*., 2013; Moxon *et al*., 2008a
miR157	*SPL*	*Torenia fournieri*	Promotes normal phase change and branching	Shikata *et al*., 2012
miR159	*AtMYB: AtMYB33*, *AtMYB65*, *AtMYB97*, *AtMYB101*, *AtMYB104*, *AtMYB120*	*Arabidopsis thaliana*	Promote flowering in response to GA and length of photoperiod	Achard *et al*., 2004; Alonso-Peral *et al*., 2010
	OsGAMYB OsGAMYBL1	*Oryza sativa*	Promotes heading	Tsuji *et al*., 2006
	LtGAMYB	*Lolium temulentum*	Promotes flowering	Achard *et al*., 2004; Woodger *et al*., 2003
	SsGAMYB	*Sinningia speciosa*	Promotes flowering	Li *et al*., 2013a
miR160	*ARF16*, *ARF17*, *ARF10*	*Solanum lycopersicum L.*	Fruit development and ripening	Karlova *et al*., 2013

Table 9.1 Continued

miRNA	Target genes	Species	Function(s) of target genes in reproductive development	References
miR164	*CUC2*	*Solanum lycopersicum L.*	Fruit development and ripening	Moxon *et al*., 2008a
	CUC1, *CUC2*	*Arabidopsis thaliana*	Flower development	Baker *et al*., 2005; Laufs *et al*., 2004
	GOBLET (GOB)	*Arabidopsis thaliana*	Flower development	Baker *et al*., 2005; Sieber *et al*., 2007
miR165/166	*HD-ZIP III genes: ATHB15*, *ATHB8*, *REVOLUTA(REV)*, *PHABULOSA(PHB)*, *PHAVOLUTA(PHV)*	*Arabidopsis thaliana*	Flower development, embryo patterning, postembryonic and axial meristem initiation	Floyd and Bowman, 2004; Kim *et al*., 2005; Reinhart *et al*., 2002; Zhou *et al*., 2007
miR167	*ARF6*, *ARF8*	*Arabidopsis thaliana*	Flower development	Ru *et al*., 2006; Wu *et al*., 2006
	GAMyb-like1, *GAMyb-like2*	*Solanum lycopersicum* L.	Fruit development and ripening	Karlova *et al*., 2013
miR168	*ARGONAUTE 1*	*Solanum lycopersicum* L.	Fruit development and ripening	Karlova *et al*., 2013
miR169	*AtNF-Y family of TFs*	*Arabidopsis thaliana*	Repress and promote flowering in response to abiotic stress	Xu *et al*., 2014
	NF-YA family of TFs	Petunia and *Antirrhinum*	Flower development	Jones-Rhoades and Bartel, 2004; Zhao *et al*., 2009
miR171	*LOST MERISTEM 1*	*Arabidopsis thaliana*	Delays flowering	Xue *et al*., 2014
	SCARECROW-LIKE	*Hordeum vulgare*	Promotes flowering	Curaba *et al*., 2013
miR170/ miR171	*GRAS/SCR (Scarecrow-related)*	*Solanum lycopersicum* L.	Fruit development and ripening	Karlova *et al*., 2013; Moxon *et al*., 2008a
miR172	*AP2-like: TOE1*, *TOE2*, *TOE3*, *SMZ*, *SNZ*	*Arabidopsis thaliana*	Negatively regulates induction of flowering	Aukerman and Sakai, 2003; Jung *et al*., 2007; Mathieu *et al*., 2009; Schmid *et al*., 2003; Schwab *et al*., 2005; Yant *et al*., 2010
	CfTOE1	*Cardamine flexuosa*	Repressor of flowering	Zhou *et al*., 2013
	GLOSSY15	*Zea mays*	Repressor of flowering	Lauter *et al*., 2005
	ZmTOE1	*Zea mays*	Repressor of flowering	Salvi *et al*., 2007
	InAP2-like	*Ipomoea nil*	Involved in photoperiodic flower induction	Glazińska *et al*., 2009
	RELATED TO APETALA2 1(RAP1)	*Solanum tuberosum*	Possible repressor of flowering and tuberization	Martin *et al*., 2009
	SlAP2a, *SlAP2b*, *SlAP2c*, *SlAP2e*, *SlAP2d*	*Solanum lycopersicum* L.	Fruit development and ripening	Itaya *et al*., 2008; Karlova *et al*., 2013; Moxon *et al*., 2008a
	Sly-TASI3a, *Sly-TASI3b*	*Solanum lycopersicum* L.	Fruit development and ripening	Karlova *et al*., 2013
	Cleistogamy1(Cly1)	*Hordeum vulgare*	Promotes cleistogamy	Nair *et al*., 2010
	AP2	*Malus X domestica*	Fruit development and ripening	
miR319	*TCP family of TFs*	*Arabidopsis thaliana*	Promotes flowering	Palatnik *et al*., 2003

Table 9.1 Continued

miRNA	Target genes	Species	Function(s) of target genes in reproductive development	References
miR390	*TAS3*	*Arabidopsis thaliana*	Promotes juvenility by negative regulation of*ARF3*	Fahlgren *et al*., 2006
miR393	*AtTIR1*, *AFB 1–3*	*Arabidopsis thaliana*	Repressor of flowering	Chen *et al*., 2011c
	OsTIR1, *OsAFB2*	*Oryza sativa*	Repressor of flowering	Xia *et al*., 2012a
	SlTIR1	*Solanum lycopersicum* L.	Flower-to-fruit transition	Karlova *et al*., 2013; Ren *et al*., 2011
miR396	*GRF1*, *GRF2*, *GRF3*, *GRF4*, *GRF7*, *GRF8*, *GRF9*	*Arabidopsis thaliana*	Pistil development	Liang *et al*., 2014
	GRF6, *GRF10*	*Oryza sativa*	Inflorescence and spikelet development	Liu *et al*., 2014b
miR399	*PHOSPHATE 2*	*Arabidopsis thaliana*	Ambient temperature-responsive repressor of flowering	Kim *et al*., 2011
miR824	*AGL16*	*Arabidopsis thaliana*	Represses flowering in certain genetic backgrounds and environmental conditions	Hu *et al*., 2014
miR858/ miR828	*MYB2*	*Gossypium*	Fibre development	Guan *et al*., 2014
miR5200	*FTL1*, *FTL2*	*Brachypodium distachyon*	Promotes flowering in response to photoperiod	Wu *et al*., 2013
miR4376	*ACA10*	*Solanum lycopersicum* L.	Flower morphology and fruit yield	Wang *et al*., 2011b
TAS3	*ARF3*, *ARF4*	*Solanum lycopersicum* L.	Fruit development and ripening	Karlova *et al*., 2013
	ARF3/ETTIN, *ARF4*	*Arabidopsis thaliana*	Fruit development and ripening	Fahlgren *et al*., 2006
cis-nat-siRNA	*ARI14*	*Arabidopsis thaliana*	Facilitates sperm formation and double fertilization	Ron *et al*., 2010
Nat-siRNA	*DGD2*	*Phalaenopsis aphrodite*	Spike initiation and flowering	An and Chan, 2012

also positively regulate *CO* transcription, the core component responsible for measuring the distinction of day length (Fowler *et al.*, 1999; Park *et al.*, 1999). Notably, the miR172-dependent pathway in photoperiodic induction is independent to *CO* but requires the functional *FT*, the target of *CO* (Jung *et al.*, 2007). Moreover, many miR156-targeted *SPLs* are able to respond to the photoperiodic changes (Wang *et al.*, 2014a). For example, *SPL3*, *SPL4*, and *SPL5* are highly induced by photoperiod in a *PENNYWISE (PNY)*- and *POUND-FOOLISH (PNF)*- dependent manner (Lal *et al.*, 2011; Schmid *et al.*, 2003), and this process is carried out through negative regulation of miR156 (Lal *et al.*, 2011) (Fig. 9.2). Taken together, miR156/miR172 may integrate the signals from multiple phytohormones to coordinate floral development in response to environmental changes.

miR159 is another important component mediating the flowering pathway, which targets at least three *MYB* transcription factors in *Arabidopsis*, including *MYB33*, *MYB65* and *MYB101* (Achard *et al.*, 2004). *MYB33* responding to photoperiods or GA treatment promotes the floral transition through activation of *LFY* (Gocal *et al.*, 2001). Similar to miR172, miR159 can be promoted by GA

through overcoming *DELLA*-mediated repression. In contrary to miR172, increased level of miR159 delays flowering only in short day plants (Achard *et al.*, 2004). However, another report is contradictory and states that overexpression of miR159 does not alter flowering time (Alonso-Simon *et al.*, 2010; Schwab *et al.*, 2005). Thus, *MYB33* and the related *MYB* genes' roles in flowering await further investigation.

miR393 is another regulator of flowering and targets two AUX receptors, *OsTIR1* and *OsAFB2* in rice. It promotes flowering by suppressing the sensitivity of *OsTIR1* and *OsAFB2* to AUX (Xia *et al.*, 2012a), suggesting a potential role of AUX signalling in flowering. Similarly to miR393, miR399 can induce early flowering by degrading its target transcription factors, *PHOSPHATE 2* (*PHO2*) in *Arabidopsis*. *PHO2* is known to maintain phosphate homeostasis and regulate flowering time (Kim *et al.*, 2011).

One particular gene, *DIGALACTOSYLDIACYLGLYCEROL SYNTHASE 2* (*DGD2*), has been identified to be targeted by the NATs unique to *Phalaenopsis* (An and Chan, 2012). *DGD2* affect the lipid content of chloroplast membranes under phosphate-starvation conditions (Ge *et al.*, 2011). In *Phalaenopsis*, it may play a unique role in regulating the lipid composition of the chloroplast membrane under low ambient temperature treatment. Low ambient temperature is required for spikelet initiation in *Phalaenopsis* (Blanchard and Runkle, 2006; Chen *et al.*, 2008), suggesting that the NATs play a key role in flowering time and/or developmental processes during the flowering phase transition.

Roles of sRNAs in androecium development

The androecium is a male organ with an anther on the top. The anther contains haploid microspores derived from the meiosis of diploid sporogenous cells. The cytoplasm and cytoskeletons of microspores are reorganized and eventually develop into pollen grains. Pollen development is essential for male fertility, which relies heavily on somatic anther tissues, such as the tapetum. The formation of the tapetum ensures early pollen development, after which tapetum degeneration nourishes pollen into maturation (Twell, 2011). Genome-wide studies have revealed that many sRNA are involved in male organ development in plant species (Wei *et al.*, 2011; Yin and Shen, 2010; Zhang *et al.*, 2009). Some of these sRNAs participate in tapetum formation and degeneration, microspore formation, and pollen release (Table 9.1).

miR156-targeted *SPLs* (miR156 non-targeted SPL8) are necessary for maintaining anther fertility in *Arabidopsis*. Loss of miR156-targeted *SPLs* leads to a semi-sterile anther due to an abnormality of primary tapetum cells and primary sporogenous cells (Xing *et al.*, 2010).

miR159 post-transcriptionally regulates the conserved *GAMYB-like* genes which respond to GA signal (Gubler *et al.*, 2002; Murray *et al.*, 2003). Overexpression of miR159 or disruption of the GA biosynthesis pathway both delays flowering and reduces fertility (Achard *et al.*, 2004; Cheng *et al.*, 2004). In *Arabidopsis* and rice, the proper development of the pollen cells relies on the spatial restriction of *GAMYB-like* gene expression in the anthers (Millar and Gubler, 2005; Tsuji *et al.*, 2006). miR159 overexpression down-regulates anther *MYB33/MYB65* in *Arabidopsis* and anther *TaGAMYB1* in wheat, and suppresses tapetum degradation other than forms hypertrophic tapetum, which therefore affects microspore development and eventually leads to male sterility (Millar and Gubler, 2005; Tsuji *et al.*, 2006; Wang *et al.*, 2012a).

miR165 and miR166 differ by only one nucleotide. In *Arabidopsis*, it is involved in microspore development by regulating their target gene *REVOLUTA (REV)*, an *HD-ZIP III* family gene (Table 9.1). The expression of *REV* is negatively associated with that of *FILAMENTOUS FLOWER* (*FIL*). *REV* negatively regulates the establishment of anther polarity, while *FIL* regulates the development of microsporangia and microspore mother cells (Lian *et al.*, 2013). The biogenesis of plant miR165/166 requires HYPONASTIC LEAVES1 (HYL1), a double-stranded RNA-binding protein. HYL1 mediates the accurate processing of pre-miRNAs into mature miRNAs via DCL1. HYL1 deficiency-suppressing miR165/166 reduces the microsporangia by half (four in wild type) and decreases male fertility in *Arabidopsis* (Lian *et al.*, 2013). The balance between *REV* and *FIL* expression is regulated by HY1-dependent miR165/166

and required for the formation of inner microsporangia and anther connectives during stamen development.

MiR167 controls pollen development by directly targeting two *auxin response factor* genes (*ARFs*) *ARF6* and *ARF8* (Tabata *et al.*, 2010). In *Arabidopsis*, the dysfunction of miR167 indulges *ARF6* and *ARF8* ectopic expression in the connective cells of anther. The connective cells become too large to break for pollen release (Ru *et al.*, 2006; Wu *et al.*, 2006). Deep sequencing shows a high expression of tae-miR166/167 in the spikelet tissue of thermo-sensitive male-sterile wheat line suffered from cold-induced male sterility, where members of *ARF* families are the targets of tae-miR167 (Tang *et al.*, 2012). Similarly, nineteen members including the diverse isomiRs, have been identified for miR167 and most of them have shown a significant up-regulation in the anther tissue of male sterile mutants in citrus (Fang *et al.*, 2014).

MiR169 family members target the gene family *NF-YA* transcription factor. In Petunia and *Antirrhinum*, *NF-YA* transcription factors belong to the homeotic C-class genes during flower development (Jones-Rhoades and Bartel, 2004; Zhao *et al.*, 2009). These miR169-encoding genes are referred as *BLIND* (*BL*) in Petunia and *FISTULATA* (*FIS*) in *Antirrhinum*, and both of them repress the expression of *NF-YA* genes in inner two floral whorls during flower development. Loss of *FIS* and *BL*, or the lack of miR169, result in the conversion of petals to stamens in the second whorl (Cartolano *et al.*, 2007). Notably, such a role of miR169 in regulating of C-gene activity in Petunia and *Antirrhinum* does not appear in *Arabidopsis* (Cartolano *et al.*, 2007).

Cis-nat-siRNAs are also involved in pollen development (Ron *et al.*, 2010). This kind of sRNAs has only been reported in the previous studies on plants exposed to environmental stress (Borsani *et al.*, 2005; Katiyar-Agarwal *et al.*, 2006). In a recent study on *Arabidopsis*, cis-nat-siRNA-based regulation plays some key roles in reproductive function, as it facilitates sperm formation and double fertilization (Ron *et al.*, 2010). Two inversely transcribed genes, *KOKOPELLI* (*KPL*) and *ARIADNE14* (*ARI14*), generate a pair of sperm-specific nat-siRNAs. In the absence of *KPL*, the *ARI14* RNA level in sperm is increased and fertilization is impaired. Finally, the accumulation of *ARI14* transcripts leads to reduced seed set.

In addition, miR166 is highly accumulated in developing rice pollen, and eight other miRNAs (OsmiR528, OsmiR5793, OsmiR1432, OsmiR159, OsmiR812d, OsmiR2118c, OsmiR172d, and miR5498) are differentially expressed in rice anthers between a cytoplasmic male sterility (CMS) line and a maintainer line, suggesting these miRNAs play some roles in rice pollen development (Wei *et al.*, 2011). Eight miRNAs, Zma-miR601, Zma-miR602, Zma-miR603, Zma-miR604, Zma-miR605, Zma-miR606, Zma-miR607, and Zma-miR397 families have also been identified in a maize cytoplasmic male sterile line. Their 18 potential targets of these miRNAs are considered to be involved in a number of biological processes during pollen development (Shen *et al.*, 2011). Four other families, miR397, miR399, miR408, and miR535 have exhibited a significantly different expression between male sterile mutant and wild type in citrus (Fang *et al.*, 2014). Three members of miR397 (crt-miR397.1, crtmiR397.2, crt-miR397.3) have been shown to be up-regulated in the anther tissue of male sterile mutant in citrus and they targeted *BRANCHED-CHAIN-AMINOACID AMINOTRANSFERASE 2* (*BCAT2*) (Fang *et al.*, 2014). miR397 also targets some oxidative genes like *LACCASES*, *PEROXIDASE*, and *TUBULIN*, which play an important role in pollen viability and cell wall formation during pollen development in *Arabidopsis* (Khraiwesh *et al.*, 2012).

Roles of sRNAs in gynoecium development

The gynoecium is the female reproductive organ and is composed of one or more fused carpels. Carpels have three parts: a stigma at the top, a style, and an ovary. The ovary accommodates ovules, which are differentiated for fertilization and subsequent embryogenesis. The gametocyte development and morphological maturity for pollination determine female fertility.

Many of the miRNAs in androecium also have roles in the gynoecium. The miR156-targeted *SPL*s not only negatively regulate anther fertility but also redundantly control gynoecium patterning (Table 9.1). In *Arabidopsis*, down-regulation of

miR156-targeted *SPLs* clearly results in a shortened style and an apically swollen ovary narrowing into an elongated gynophore (Xing *et al.*, 2013). The up-regulation of miR156 and down-regulation of *SPL8* (miR156 non-targeting *SPL8*) both enhance the impairment of pollen tube in *ETTIN* mutant. In addition, *ETTIN* is an *ARF3*, possibly facilitates AUX production or accumulation, and determines apical-basal patterning during ovary formation (Xing *et al.*, 2013). Thus, miR156-targeted *SPLS* and *SPL8* redundantly control gynoecium patterning through interfering with AUX homeostasis and signalling. Additionally, miR167-targeted *ARF6* and *ARF8* both regulate gynoecium and stamen development in immature flowers (Ru *et al.*, 2006). In tomato, the down-regulation of miR167-targeted *ARF6* and *ARF8* leads to the absence of trichomes on the styles, the failure of pollen tube formation and sterility (Liu *et al.*, 2014c). The roles of miR167 and *ARFs* are conserved across different species (Li *et al.*, 2015b). In *Arabidopsis*, miR165 and miR166 also both regulate gynoecium development. Both of them target the same *HD-ZIP III* genes *ATHB15*, *ATHB8*, *REV*, *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) (Table 9.1) (Floyd and Bowman, 2004; Reinhart *et al.*, 2002; Zhou *et al.*, 2007), and regulate these target genes in different ways (Jung *et al.*, 2007). Overexpression of miR165 in *Arabidopsis MERISTEM ENLARGEMENT 1* (*MEN1*) mutant results in a decrease in the expression of all of these *HD-ZIP III* target genes and causes developmental defects in the SAM such as an enlarged apical meristem and short and sterile carpels (Kim *et al.*, 2005). MiR166 has also been shown to control embryonic SAM development in rice and maize (Nagasaki *et al.*, 2007; Nogueira *et al.*, 2007).

A set of miRNAs in vegetative growth also function in gynoecium formation and female fertility. In *Arabidopsis*, miR396 has an essential roles in leaf growth by regulating *GROWTH REGULATING FACTOR* (*GRF*), which interacts with *GRF-interacting factor 1* (*GIF1*) to participate in cell proliferation (Liu *et al.*, 2009). Besides, miR396 controls carpel number by down-regulating its targets *GRF6/10* (Liang *et al.*, 2014). MiR164 is another sRNA regulating vegetative and gynoecium development. miR164 targets *Arabidopsis CUP-SHAPED COTYLEDON* (*CUC*) genes *CUC1* and *CUC2* to control the boundary size of meristems (Laufs *et al.*, 2004), shoot apical meristem formation, and the initiation of leaf margin development (Hibara *et al.*, 2003; Kamiuchi *et al.*, 2014; Nikovics *et al.*, 2006). Moreover, miR164 are involved in tomato boundary formation in different organs by regulating two *NAM* genes, *GOBLET* (*GOB*) and *SlNAM2*. In leaf, *GOB* down-regulation causes the leaflets to have a smoother margin. Similarly, in *Arabidopsis* flowers, *SINAM2* down-regulation leads to an abnormal fusion of the sepal and whorl (Berger *et al.*, 2009; Hendelman *et al.*, 2013) and a defect in carpel closure (Baker *et al.*, 2005; Sieber *et al.*, 2007). In maize, the *TASSELSEED4* (*TS4*) gene, encodes *zma*-miR172, controls tassel formation and induces carpel abortion by degrading *INDETERMINATE SPIKELET1* (*IDS1*), an *APETALA2* (*AP2*)-*LIKE* transcription factor. However, in ears, *IDS1* expression is abundant at the base of the initiating primordial carpel. Thus, the regulation of *IDS1* by zma-miR172 results in the differences in primordial initiation, and sex determination (Chuck *et al.*, 2007b). In addition, the down-regulation of *SBPs* by *zma*-miR156 results in feminized male tassels (Hultquist and Dorweiler, 2008). The regulatory networks of sRNAs are pervasive and appear to cross regulate among many vegetative growth and reproductive growth pathways.

Roles of sRNAs in fruit/seed development

Fruits/seeds are derived from the maturation of one or more flower(s), and the gynoecium of the flower(s) forms the whole or part of the fruit. The gynoecium expands to appropriately accommodate the seeds, while developing into a fruit, and the walls of ovary become pericarps. The fruit set and growth is determined in part by the seeds. In such a way, seed and fruit development occur as a coordinated processes.

To date, the gene regulation networks underlying fruit development have been largely unravelled for model plants, such as *Arabidopsis* and tomato. *Arabidopsis* has dehiscent fruits (standard silique), composed of the three main elements along the fruit mediolateral axis: the valve, the valve margin, and the replum. The fruit elements are determined by the genes during the early stages of gynoecium development and the genes in subsequent specific domains. Many of these genes encode transcription factors. For example, an MADS-Box

transcription factor, *RIPENING-INHIBITOR* (*RIN*), is involved in regulating most of the tomato ripening phenomena, e.g. pro-vitamin A and carotenoid accumulation, softening, and the production of flavour compounds, etc. The *RIN* also acts upstream of both ET- and non-ethylene-mediated ripening control (Vrebalov *et al.*, 2002). An *SBP*-box transcription factor, *COLORLESS NONRIPENING* (*CNR*), is a positive regulator of tomato fruit ripening, and plays roles in tomato fruit colour formation and pericarp texture (Manning *et al.*, 2006). Another transcription factor, *AP2*, is a negative regulator of ripening in tomato in response to ET (Karlova *et al.*, 2011). In addition to these fruit-ripening genes, some others control tomato fruit size and shape, for instance, *Fw2.2* encodes a plant-specific protein and participates in cell division in fruit specifically to regulate fruit size (Cong and Tanksley, 2006). An orthologue *Arabidopsis YABBY2*, *FASCIATED* (*FAS*) is associated with a flat tomato fruit shape and the number of locules (Cong *et al.*, 2008; Rodríguez *et al.*, 2011). *LOCULE NUMBER* (*LC*), is near to the tomato orthologue of *Arabidopsis WUS*, is a homeodomain transcription factor gene also regulating the number of locules (Mayer *et al.*, 1998). Another two genes, *SUN* and *OVATE*, control fruit elongation shape (Liu *et al.*, 2002; Xiao *et al.*, 2008). More information on tomato fruit development can be obtained from a previous review (Karlova *et al.*, 2014).

Many transcription factor mRNAs are the targets of sRNA. Through bridging these transcription factors, sRNAs are extensively integrated into genetic networks to regulate fruit development (Table 9.1). In *Arabidopsis*, miR172 plays a critical role in fruit morphogenesis, allowing proper fruit growth after fertilization by targeting the transcriptional repressor *AP2* (and *TOE3*) (José Ripoll *et al.*, 2015). *AP2* (and *TOE3*) represses ovary and silique growth by modulating cell division and expansion through inhibiting the expression of *AG* and *FUL* (Fig. 9.3) (José Ripoll *et al.*, 2015; Yant *et al.*, 2010). In the absence of AP2, FUL acts together with ARF proteins (such as ARF8) to directly activate the

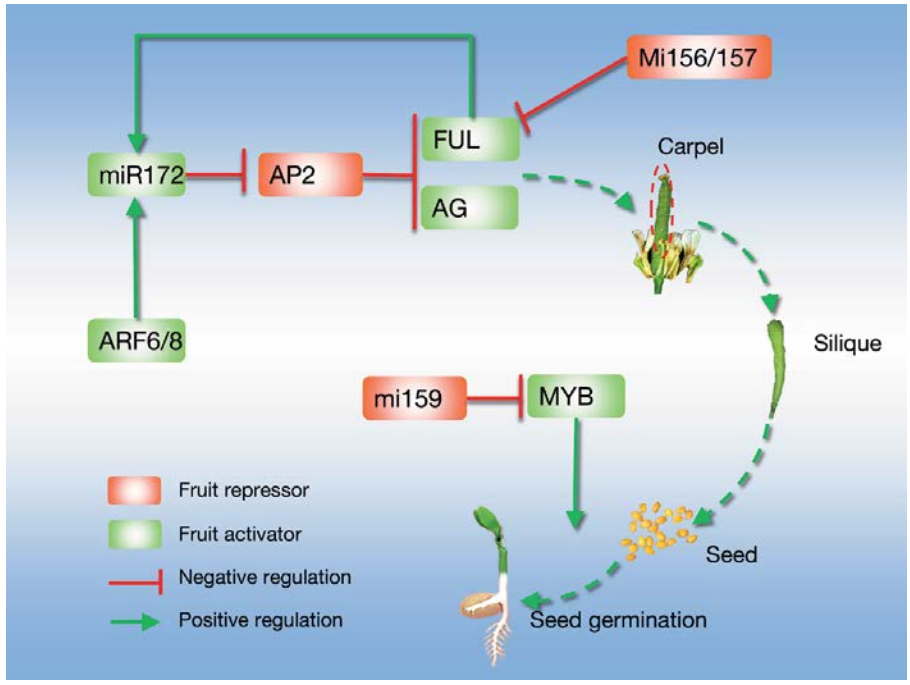

Figure 9.3 Model for the regulation of fruit growth in *Arabidopsis* by small RNA. MiRNA172 negatively regulates AP2 by inhibiting its translation. AP2 directly binds to the promoter and represses the expression of both FUL and AG which are important for carpel and *Arabidopsis* silique development. An elevated level of miRNA172 inhibits *Arabidopsis* silique growth. In seeds, the regulation of MYB by miR159 and GA for the aleurone cells development and death is required for germination. FUL is a well-characterized regulator of cell differentiation by miR156- and miR157- during early stages of *Arabidopsis* fruit development.

miR172 valve-specific miR172-encoding gene so as to promote valve growth (three main domains outside of silique: the valves, the valve margin, and the replum). Interestingly, the opposite impact of miRNA172 occurs in apple, and is that miRNA172 inhibits apple fruit growth through negative regulation of AP2, which is required for sepal and fruit flesh cortex development (Yao *et al.*, 2015b). In apple, overexpression of miRNA172 results in a decreased number of hypanthium cells before pollination and small size of it after pollination. In conclusion, the impact of miRNA172 on fruit growth may be achieved initially by influencing the size of floral organs, which contribute tissues to fruit development and then modulating cell division and expansion in these tissues in response to fertilization-induced signals and signal transduction. However, its roles are species specific.

MiR159's regulations on *GAMYB-like* genes act not only in leaf and flower (including anthers), but also in seeds through GA signalling pathway (Gubler *et al.*, 2002; Murray *et al.*, 2003). In *Arabidopsis* seeds, the regulation of three *GAMYB-LIKE* genes (*AtMYB33/65/101*) by miR159 and GA controlling the aleurone cells development and death, is required for germination (Fig. 9.3) (Alonso-Simon *et al.*, 2010). The seed dormancy/germination process is well known to be antagonistically regulated by both GA and ABA. Accordingly, miR159 acts as a negative regulator, while *AtMYB33* and *AtMYB101* serve as positive regulators of germination responding to ABA (Reyes and Chua, 2007). Some *MYBs* are synchronously targeted by other miRNAs, such as miR858, and miR828 (Guan *et al.*, 2014; Pang *et al.*, 2009). miR828 and miR858 have been proven to coordinate *Arabidopsis* trichome and cotton fibre development via *MYBs* (Guan *et al.*, 2014). Cotton fibre is a kind of special single-celled trichomes initiated from the epidermal layer of cotton ovule and its development shares some similar regulatory mechanisms with leaf trichome development (Wan *et al.*, 2014). *MYB* family transcription factors are the critical regulator in fibre development, such as the *Arabidopsis R2R3 MYB* domain transcription factor *GLABROUS1* (*GL1*) (Larkin *et al.*, 1993), and a cotton *GL1-LIKE MYB* transcription factor (referred as *GaMYB2*) (Wang *et al.*, 2004). Interestingly, in cotton, the reduced expression of *GhMYB25-like* gene leads to a fibreless phenotype but no effect on the formation of other trichomes (Walford *et al.*, 2011). In apple, miR159-*MYBs* may regulate both male organ and embryo development, while MiR828- and miR858-*MYBs* are linked with diverse biological processes and metabolism pathways underlying cell wall formation, lignification, anthocyanin biosynthesis, cell fate and identity, plant development and the response to biotic and abiotic stresses (Xia *et al.*, 2012b).

Many miRNA-target regulations are active from flowering to fruiting. For example, miR156-SPL/SBP box node, works not only in flower development, but also in diverse aspects of fruit development, including phase transition, plant architecture, trichome distribution, embryonic patterning and anthocyanin biosynthesis (Chuck *et al.*, 2007a; Gou *et al.*, 2011; Mohorianu *et al.*, 2011; Nodine and Bartel, 2010; Schwab *et al.*, 2005; Wang *et al.*, 2009a; Yu *et al.*, 2010). In tomato, *S. lycopersicum SBP* box genes (*SlySBP*) have been identified to be the target of miR156 and miR157 (Moxon *et al.*, 2008b; Salinas *et al.*, 2012). As discussed in flower development, miR156-targeted *SPL3* positively and directly regulates the MADS box genes *APETALA1* (*AP1*), *FUL* and *LFY* (the central regulator of flowering) (Fig. 9.3) (Yamaguchi *et al.*, 2009). Interestingly, *FUL* is a well-characterized regulator of cell differentiation by miR156- and miR157- during the early stages of *Arabidopsis* fruit development (Fig. 9.3) (Gu *et al.*, 1998). Besides, *FUL*-like genes play important roles in the development of the fruit wall in two basal eudicot Papaveraceae species during fruit maturation (Pabón-Mora *et al.*, 2012). Tomato MADS box gene *FUL1/TDR4*, an orthologue of *AtFUL*, is induced during ripening and probably the target of *CNR*, a *SlySBP* family member (Bemer *et al.*, 2012; Seymour *et al.*, 2013). Another target of tomato *SBPs* target, the MADS-box gene *MACROCALYX* (*MC*), not only controls sepal and inflorescence, but also regulates the tomato fruit abscission zone by working with another MADS box gene, *JOINTLESS* (Nakano *et al.*, 2012; Vrebalov *et al.*, 2002). Moreover, overexpressing the *AtMIR156b* precursor indirectly induces the expression of *LeT6/TKN2* (a *KNOX*-like class I gene) and *GOB* (a *NAM/CUC*-like gene) in developing the ovaries of transgenic plants and the ovaries of the natural mutant *Mouse-ear* (*Me*), which exhibits abnormal flower and fruit morphologies (Ferreira e Silva *et al.*, 2014). The abnormal morphologies include

extra carpels and ectopic structures in fruits. In another example, miR396 targets *GRF6/10*, which combines with *GIF1* to form a transcription co-activator. In rice, overexpressing OsmiR396 and knocking down *GRF6* result in phenotypes with open husks and sterile lemma in seed (Liu *et al*., 2014b). *GRF6* and *GRF10* transactivate the rice *JMJD2* family *JMJC* gene *706* (*OsJMJ706*) and *CRINKLY4 RECEPTOR-LIKE KINASE* (*OsCR4*) responding to GA, which is required for floral development into normal seed (Liu *et al*., 2014b). miR397 regulates rice *LACCASE* (*LAC*), a regulator involved in brassinosteroids signalling, and also plays a crucial role in the productivity of other crop plants (Zhang *et al*., 2013). Through genome-wide identification and screening, OsmiR397 is highly expressed in rice seeds, and implicated in the regulation of several key yield-related factors, such as vascular bundle formation, panicle branches numbers, effective grains and tiller numbers, grain hull, and endosperm size (Chen *et al*., 2011a; Zhang *et al*., 2013). The yield-related factors, in turn, increased grain yield in miR397 up-regulating plants. Additionally, the regulatory interaction between miR397 and *LAC* has been predicted to be conserved in many species, including tobacco, populus, and *Arabidopsis* (Jones-Rhoades and Bartel, 2004). miR4376 regulates the expression of an auto-inhibited Ca^{2+}-ATPase in tomato, *ACA10* which plays a critical role in tomato flower morphology and fruit yield (Wang *et al*., 2011b). Ca^{2+} signatures, and oscillations, in the cytoplasm or organelles are critical for signal transduction and are regulated by Ca^{2+} influx through the activities of Ca^{2+} channels and Ca^{2+} efflux through the activities of high affinity Ca^{2+}-ATPases (pumps) or low-affinity Ca^{2+} exchangers (Dodd *et al*., 2010; Kudla *et al*., 2010; McAinsh and Pittman, 2009). There are 14 Ca^{2+}-ATPases in *Arabidopsis* and rice. Four of them are endoplasmic reticulum-type Ca^{2+}-ATPases and ten are ACAs (Baxter *et al*., 2003). Based on sequence alignments and intron positions, *Arabidopsis ACA10*, *ACA8*, and *ACA9* are clustered together and their encoded proteins are located in the plasma membrane (Baxter *et al*., 2003). In *Arabidopsis*, ACA9 is necessary for normal pollen tube growth and fertilization (Schiøtt *et al*., 2004), whereas ACA10 is critical for inflorescence growth and juvenile-to-adult phase transition under long-day growth conditions (George *et al*., 2008). miR4376 is highly accumulated in leaves and young flower buds but later diminished in young green fruits and almost absent in mature fruits. The most significant impact of miR4376 or *SlACA10* overexpression is the drastically yield-loss of mature fruits (Wang *et al*., 2011b).

Conclusions and future perspectives

The discoveries of sRNAs in plants and the growing evidence of their involvement in a variety of functional roles have offered a new insight into plant reproductive biology. Notably, different sRNA families and their different members can make very specific contributions to the spatial and temporal control of their targets. The confirmed sRNAs targets are highly interconnected. The spatial and temporal expression patterns of sRNA and the cross-talks between various sRNA-regulated modules may enables sRNAs to function in different organs or developmental stages. For example, miR156 and miR172 have essential functions in the whole reproductive processes, including androecium, gynoecium, and fruit development. Thus, altering the expression levels of specific plant sRNAs makes it possible to regulate the key transcription factors and entire downstream gene regulatory networks. In practice, some important processes such as plant growth and stature, flowering, seed set and yield are obvious targets for genetic engineering. sRNA networks play a central role in these important processes, which makes them appeal biotechnologies for developing the varieties with improved key agronomic traits. For example, male fertility-related miRNAs have the potential to be utilized in hybrid breeding. In numerous studies, both sRNA targets have been quantitatively and spatially manipulated, e.g. introducing mutations into target genes or specific sRNA loci that fine-tune sRNAs control. In contrast, the mutants or transgenic varieties provide the evidence that sRNA regulate nodulation in regulating many plant reproductive developmental processes.

The sRNA-mediated regulation networks underlying plant reproductive development are much more complicated than initially expected. The high-throughput data, such as transcriptome and degradome, have been widely used to identify miRNA sequences and to validate their target genes

in plants. Being assisted by the appropriate bioinformatics tools, people can predict the sRNAs of interests and their targets. However, many sRNA sequences remain uncharacterized in sequencing projects, indicating far more sRNA loci than previously expected. These uncharacterized sRNAs are likely to have a broader range of functions in plant reproductive development than currently known. The large gap between uncharacterized sRNA and the confirmation of their functions awaits more experimental and computational studies to fill in future.

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