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Chapter 6

MicroRNAs Sequencing for Understanding the Genetic Regulation of Plant Genomes

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Abstract

MicroRNAs (miRNAs) are endogenous non-coding RNAs that play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. Small RNAs are classified into different types by their biogenesis and mode of action, such as miRNAs, siRNAs, piRNAs, and snoRNAs. In the case of miRNAs, this specific type regulates gene expression in plants and animals by targeting mRNAs for cleavage and translational repression, respectively. Diverse miRNAs regulate plant development, metabolism, and responses to biotic and abiotic stresses. The identification of miRNAs has been accomplished in diverse species, organs and developmental or diverse biotic and abiotic stress conditions. Novel massive sequencing techniques and further bioinformatics analysis have allowed the identification of hundreds of miRNAs in *Arabidopsis thaliana*, *Oryza sativa*, *Malus domestica*, *Zea mays*, *Solanum lycopersicum*, and other plants. Functional characterization of a given miRNA in a specific biological context has shown their role in the fine-tuning mechanisms of posttranscriptional gene regulation. In this chapter, besides making a summary of genome-wide miRNA profiling in plants, we describe how gain and loss of function approaches influence plant phenotypes that affect development, physiology or stress responses, pointing to miRNAs as effective tools for the generation of new plant phenotypes that improve plant productivity and conservation.

Keywords: Gene expression, Plant development, mRNA targeting, miRNA, sRNAs

1. Introduction

MicroRNAs (miRNAs) are a class of non-coding endogenous small RNAs (sRNAs) that have attracted a huge interest from scientists. Experimental and computational approaches have demostrated that miRNAs play crucial roles during plant growth and development. Expression of miRNAs is highly regulated at both the transcriptional and postranscriptional level.

The development of new sequencing technologies has been crucial for the identify of novel miRNAs and to understand their function in specific process including the adaptation of plants to extreme environments. Plant development and of course, in the improvement of plants for human consumption. This book chapter highlights research progress on plant miRNAs and their various functions on plant growth, development, and stress responses.

2. Plant sRNAs´classification

Regulatory sRNAs are ubiquitous components of endogenous plant transcriptomes, as well as common responses to exogenous viral infections and introduced double-stranded RNA molecules (dsRNA). They range from 20 to 24 nucleotides in length. Endogenous sRNAs are processed from dsRNA precursors sRNAs can be classified based on their origin and function. In plants, those derived from single stranded precursors capable of adquiring an imperfect extensive nearly perfect dsRNA precursors hairpin are called hairpin small RNAs (hpRNAS) and those derived from dsRNA are referred to as small interfering RNAs (siRNAs). Frequently, siRNAs fall into one of three additional groups: heterochromatic siRNAs, secondary siRNAs, and natural antisense transcript siRNAS (NAT-siRNAs; Figure 1) [1].

2.1. Micro-RNAs (miRNAs)

miRNAs are a well-studied subset of hpRNAs defined by the highly precise excision of one or sometimes a few functional products, which correspond to the mature miRNAs (Figure 1) [1, 2]. miRNAs have a defined set of miRNA targets [1, 3], and individual miRNA families can be evolutionary conserved [1, 4]. Most plant miRNAs require a member of the DCL1-clade for their biogenesis, and a member of the AGO1-clade to exert their function, although some exceptions have been described [1, 5].

Several miRNA families are conserved in plant species, and some are conserved from mosses to flowering plants [1, 6]. Conserved miRNAs have homologous target mRNAs in several species, showing that miRNA/target relationships are very stable during plant evolution [1, 3]. However, some relationships between plant miRNAs and their targets can be novel; for example, miR159 is a highly conserved miRNA that targets MYB transcription factors in several plant species. Buxdorf *et al*. [7]; found that miR159 in tomato, also targets SGN-U567133, a non-MYB mRNA. Expression of SGN-U567133 causes developmental defects, suggesting that regulation of gene expression trough miR159 of this non-canonical target has a functional consequence [1].

However, not all plant miRNAs are conserved; some of the miRNAs present in any given plant species seem to be unique to that species, and some other miRNAs are conserved only be-

tween closely related species [1]. The lineage-specific miRNAs are different in some ways from the more conserved miRNAs. These miRNA have more heterogeneous processing from their hairpin precursors, and have low abundance, and they are generally encoded by single genes instead of multiple paralogs [1, 8]. These differences suggest that some lineage-specific miRNAs could be transient, nonfunctional entities, and categorize them as a distinct subset of miRNAs (Figure 1) [1].

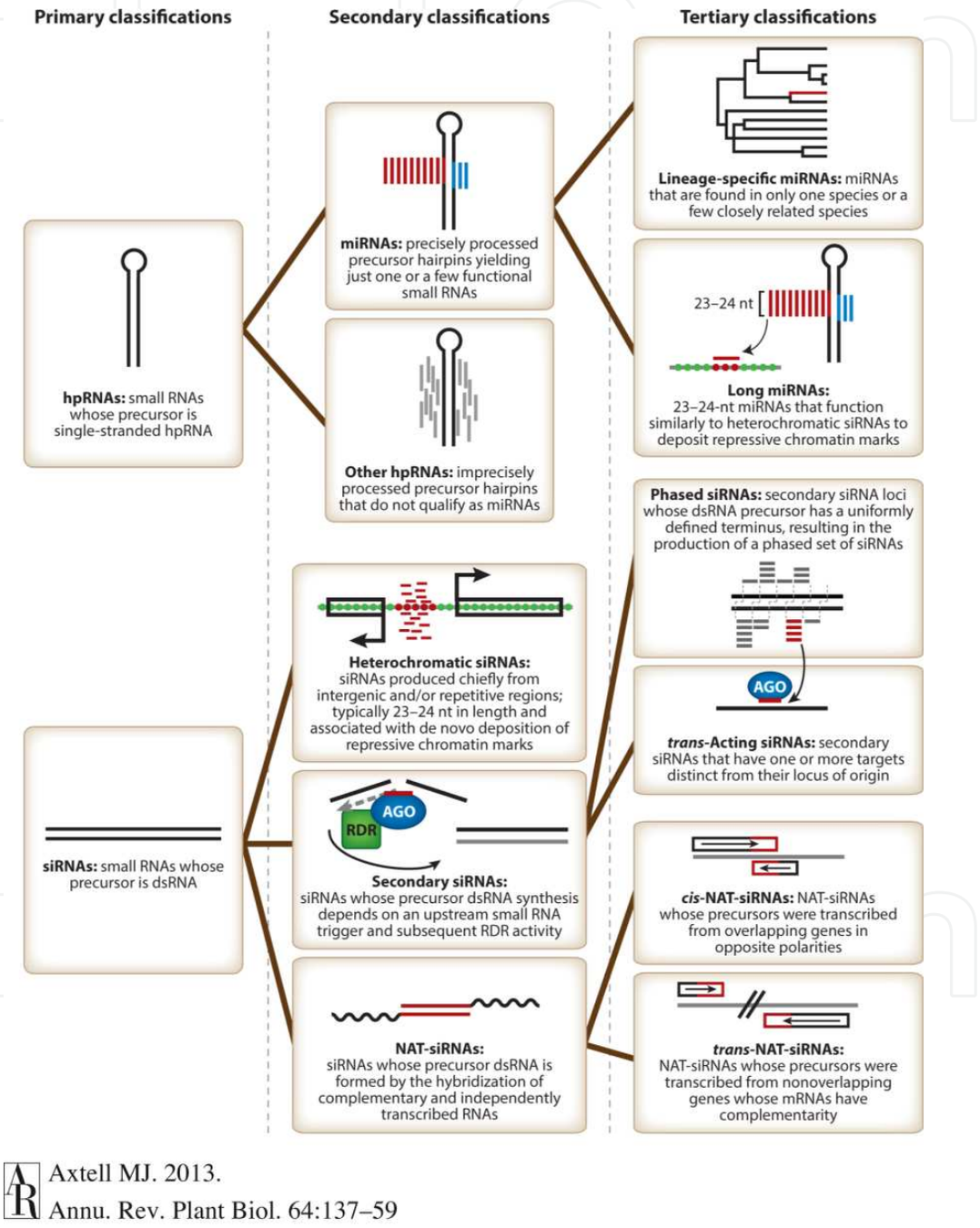

Figure 1. Hierarchical classification system for endogenous plant small RNAs. Thick black lines indicate hierarchical relationships. Abbreviations: double-stranded RNA (dsRNA); hairpin RNA (hpRNA); microRNA (miRNA); natural antisense transcript small interfering RNA (NAT-siRNA); small interfering RNA (siRNA) [1].

3. Plant miRNAs´ biogenesis

The mode of action of miRNAs between plant and animal kingdoms is different [9]. The miRNA biogenesis in plants has been documented using *Arabidopsis thaliana* as a model plant [10]. Primary miRNAs (pri-miRNAs) are transcribed by the RNA polymerase II (Figure 2), from regions in between coding genes [11]. The pri-miRNA adquires a hairpin secondary structure, and its length ranges from approximately 70 to many hundreds of bases [10]. The protein Dawdle (DDL) is an RNA-binding protein that stabilizes a subset of pri-miRNAs for the subsequent export to D-bodies [12]. D-bodies are compartments where miRNAs are processed by the joint action of Serrate (SE) and Hyponastic leaves 1 (HYL1) that together with Dicer-like 1 (process the pri-miRNA into a precursor-miRNA (pre-miRNA). Susequently, DCL1 releases the miRNA duplex containing the mature miRNA and the passenger miRNA* (Figure 2) [13].

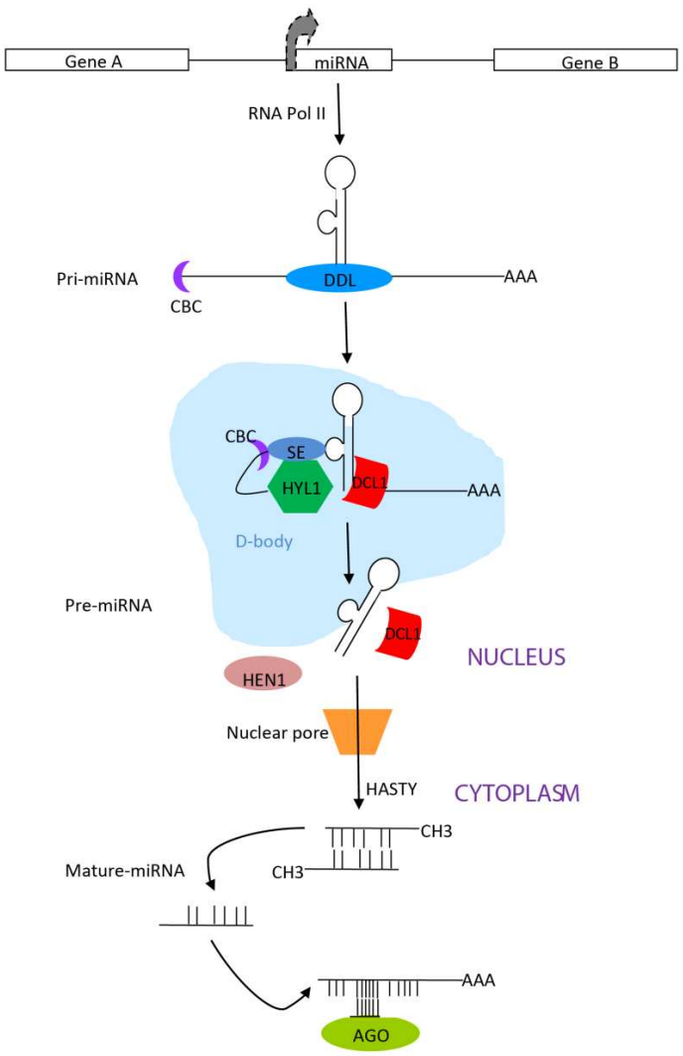

Figure 2. miRNA biosynthesis in plants. miRNA biosynthesis complex in nucleus and cytoplasm Dawdle protein (DDL), binding complex (CDC), Serrate protein (SE), Hyponastic leaves 1 (HYL1), Dicer-like 1 (DCL1), exportin-5 ortholog (HASTY), methylation protein (HEN1), and Argonaute (AGO)

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The miRNA released by DCL1 is exported out of the nucleus through the exportin - 5′ortholog (HASTY). The pre-miRNA is converted into a mature miRNA, but the double strand persist, so one of the two strands is the guide strand and the other is the passenger (that gets degraded), the mature guide miRNA is methylated by HEN1 to prevent the degradation by sRNA degrading nuclease [14].

The next step is the recruiting of the miRNA by an Argonaute (AGO) protein. In *Arabidopsis thaliana* 10 different AGO proteins were are present [15]. the AGO/mature miRNA complex its finds target mRNA based on the complementarity between the guide sequence and the mRNA, then it cleaves its target the target thanks to AGO's slicer activity (Figure 2).

4. Plant miRNAs′ function and plant development

A miRNA is a 21–24 nucleotide RNA product of a non-protein-coding gene. Plants, like animals, have a large number of miRNA-encoding genes in their genomes. Plant miRNAs have been predicted or confirmed to regulate a variety of processes, such as development, metabolism, and stress responses (Table 1). Plant miRNAs have been predicted or confirmed to regulate genes encoding various types of proteins. A major category of miRNA target genes consists of transcription factors or other regulatory proteins that function in plant development or signal transduction [16]. The first evidence that sRNAs play roles in plant development, came from mutants with impaired sRNA biogenesis or function [17].

4.1. Auxin signaling

Auxins are critical for plant development and their interaction with the environment. Local concentration of auxins, as established by polar auxin transport, provides the growth pattern on the axis of the embryo from root stem cells, and controls primordia outgrowth from meristems, as well as initiate, lateral root formation, and gravitropic responses.

A number of genes in auxin signaling are confirmed or predicted targets of miRNAs. The TIR1 auxin receptor is a predicted target of miR393 [17] (Table 1). Several auxin response factors, such as ARF10, ARF16, and ARF17, contain potential binding sites for miR160 [18] and ARF6; ARF8 have sites for miR167 [19]. In *in vivo* assays, miR160 can guide the cleavage of ARF10 and ARF16, and miR167 guide the cleavage of ARF8 mRNA [20]. The expression of a miR160-resistant version of ARF17 (5mARF17) leads to developmental defects with abnormalities, such as leaf serration, leaf curling, early flowering, altered floral morphology, and reduced fertility [20]. This indicates that regulation of ARF17 mediated by miR160 is crucial for different aspects of plant development (Table 1).

4.2. Plant organ boundary formation

Three members of the NAC gene family, *CUP-SHAPED COTYLEDON* (*CUC*) *1*, *2*, and *3*, have overlapping functions on organ boundary formation and in the initiation of shoot apical meristem (SAM). These three genes are expressed first in the boundaries of embryo cotyledons and later in the boundaries of floral organs [21].

miRNA	TARGET FAMILY	TARGETS	FUNCTION IN PLANTS
mir156	SBP	*SPL2, SPL3, SPL4, SPL10*	Apical dominance
mir159	MYB TCP	*MYB33, MYB65,* *TCP2, TCP3, TCP4,* *TCP10, TCP24*	Male sterility
mir160	ARF	*ARF10,ARF16, ARF17*	Root development
mir164	NAC	*CUC1, CUC2, NAC1,* *At5g07680, At5g61430*	Aging induced cell death. Senescence
mir166	HD-ZIPIII	*PHB, PHV, REV, ATHB-8,* *ATHB-15*	Female sterility Organ polarity
mir167	ARF	*ARF6, ARF8*	Auxin signaling
mir169	HAP2	*At1g17590, At1g72830,* *At1g54160, At3g05690*	Root architecture Stress response
mir171	SCL	*SCL6-III, SCL6-IV*	Developmental patterning
mir172	AP2	*AP2, TOE1, TOE2, TOE3*	Flower development
mir393	bZIP	*At1g27340* *arf10, arf16 and arf17*	Auxin signaling Root development
mir396	GRF	*GRL1, GRL2, GRL3, GRL7,* *GRL8, GRL9*	Cell proliferation Leaf development
mir444	MADS	*Os02g49840*	Defense response

Table 1. miRNA targets and their associated function

miR164 targets CUC1 and CUC2 but not CUC3 [17]. Assays of miR164 overexpression in wild-type plants (under the control of CaMV35S promoter) lead to floral organ fusion [20] and cotyledon fusion, in a lower frequency [22]. Expressing a CUC2 resistan version to miR164 can restore sepal separation in miR164 overexpressing lines [22]. A wild-type plant expression of CUC1 resistant to miR164 results in changes in sepal (reduced) and petal number (increased) and broadened leaves [20].

Expression of the CUC2 mir164-resistan version also increased the width of the boundary domain between sepals [22]. A similar effect was observed in miRNA mutants such as *dcl1*, *hen1,* and *hyl1* [22]. The sepal boundary expansion could explain the phenotypes related to narrow sepals in *dcl*, *hen1,* and *hyl1* mutants and reduced sepal number in CUC genes. miR164 is potentially encoded by a gene family of three members (MIR164 family), where MIR164c was identified as a regulator of petal number in flowers [23].

4.3. Polarity at the leaves and floral organs

Leaves and floral organs, are initiated as primordia on the SAM or floral meristems. These lateral organs have polar structures that develop in the adaxial side in the primordium, and

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they differ from the side that faces away from the meristem (called the abaxial side) [16]. The polarity of lateral organs is established through antagonistic interactions between two groups of genes: the class III homeodomain leucine zipper (HD-zip) including *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*); the other group includes the KANADI family (*KAN1*, *2*, and *3*) [16, 24]. The HD-zip genes are expressed in the adaxial domain, and the *KAN* genes are expressed in the abaxial domain in a differential way. Mutations in *PHB*, *PHV,* and *REV* genes result in adaxialized leaves and floral organs, and also contribute to the polarity of the vasculature [16].

Analysis of a particular set of mutants in PHB, PHV and REV revealed their regulation by miR165/miR166 the wild types, and shown that mutations in the binding sites of miRNA165/166 affecting the regulation of these genes at the miRNA level rather than the activity of the proteins [16, 18]. In a gain-of-function assays with the *phb-d* allele, the expression domain of the gene expands into the abaxial region [16, 25]. It shows that a regulation mediated by miRNA inhibited the PHB expression in the adaxial domain. The mechanism would be that degradation of the HD-zip mRNAs by miR165/166 in the abaxial domain clears the mRNAs from this domain. Also, it was found that miR165/166 causes DNA methylation of the PHB and PHV genes [26]. The miR165/166-mediated regulation of HD-zip genes is highly conserved, and the miR165/166-binding site is very similar among angiosperms, gymnosperms, ferns, and mosses [27].

4.4. Organ identity in flowers

Floral organs are organized in whorls at the floral meristem. The identities of the floral primordia are directed by the activities of floral homeotic genes known as the A, B, and C genes, and the regulation is known as the ABC model [16]. The A and C genes specify the identities of the perianth and reproductive organs, respectively. Mutations in AGAMOUS (class C gene) replace the reproductive organs by perianth organs, and mutations in APETALA2 (class A gene) lead to the loss of perianth. It suggests that A and C genes are antagonists to their activity within the floral meristem [16].

AP2 contains a binding site for miR172 and is regulated by miR172 *in vivo* (Table 1). Overexpression of miR172 under the control of CaMV35S promoter causes a reduction in the levels of AP2 protein, and the phenotypes at the floral structure are similar to those in ap2 mutants [28]. The overexpression of a miR172 resistant form of AP2 cDNA replaces the reproductive organs by perianth organs [28]. The expression assays using the AP2 promoter result in severe floral patterning defects [16, 28]. These results indicate the importance of miR172 in repressing AP2 in the inner two whorls in floral initiation. As in the HD-zip/KAN/miR165/166 system, miRNA serves as a negative regulator of one of the two antagonist functions.

Another miRNA, miR159 plays a role in reproductive development (Table 1) by regulating two MYB-domain transcription factor genes, MYB33 and MYB65. These two genes act redundantly to prevent the hypertrophy of the tapetum during anther development [29]. miR159 reduces the MYB33 and MYB65 expression to anthers. Transformed plants with MYB33 (a miR159-resistant version) under its own promoter arrested plant growth at various stages, suggesting that MYB33 expression by miR159 is critical for plant development [29].

4.5. Developmental transitions

The SAM generates leaves during the vegetative phase and flowers during the reproductive phase. The types of leaves produced at vegetative stage that are put out earlier (juvenile leaves) differ from the ones produced later (adult leaves). These changes between the developmental states are regulated by sRNAs[16].

miR172 regulates some other AP2-like genes, such as *TOE1*, *TOE2*, *TOE3*, *SMZ*, and *SNZ*, in addition to AP2. The *toe1-1* mutation results in an early flowering phenotype. The *toe2-1* mutant does not show a phenotype in flowering time, although the *toe1-1* and *toe2-1* double mutant flowers much earlier than the wild type, this indicates that TOE1 and TOE2 are redundant repressors of the transition from vegetative to reproductive stages [28]. The overexpression of *TOE1* (toe1-1D) provokes a delayed flowering time phenotype. A similar late flowering phenotype is caused by SMZ and SNZ overexpression [30]. When miR172 is overexpressed in TOE overexpressed lines, it results in early flowering phenotype, changing the late flowering phenotype of *toe1-1D* [28]. Hence, miR172 regulates the transition from vegetative to reproductive stage through the *TOE* genes regulation, via translational inhibition. However, overexpression of miR172 does not lead to a decrease of *TOE1* mRNA level, suggesting that this miRNA regulates different targets with different mechanisms [30].

miR156 overexpression also affects flowering time. 35S::MIR156 plants show a late flowering phenotype [31]. miR156 targets *Squamosa* promoter binding protein-like (SPL) transcription factor genes [18]; the role of *spl* genes in floral transition needs to be studied. Overexpression of miR319/Jaw results also in delayed flowering. miR319/Jaw targets to TCP transcription factors, and the participation of these factors in flowering is unknown [32]. miR159 overexpression leads to delayed flowering under short day conditions [33].

A pronounced vegetative phase change is observed in maize; an *AP2-like* gene *glossy*15 promotes juvenile leaf identity and its RNA is only found in juvenile leaves [34]. *glossy*15 contains a miR172-binding site suggesting that *glossy*15 is a target of miR172 as demonstrate *in vivo*. miR172 expression is correlated with the specification of adult leaf characteristics. It seems that miR172 clears glossy15 mRNA in adult leaves to promote the vegetative phase change [35].

4.6. Leaf development

The *CINCINNATA* (*CIN*) gene from snapdragon is required for differential regulation of cell division in leaf morphogenesis, where the resultant phenotype is a flat leaf [36]. *CIN* is a member of the TCP family of transcription factors. The overexpression of miR319/Jaw in Arabidopsis results in the reduction of the mRNA levels in five *TCP* genes containing miR319/Jaw-binding sites [32]. Overexpression of a resistant version of *TCP2 to* miR319/Jaw restores phenotype of miR319/Jaw overexpression [32].

4.7. sRNA metabolism

DCL1 contains a binding site for miR162 and miR162-guided cleavage products of DCL1 mRNA are detected *in vivo* [37]. *DCL1* mRNA abundance is augmented in mutants defective

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in miRNA biogenesis (such as *dcl1* or *hen1*) [37]. The *AGO1* gene is targeted by miR168. Overexpression of miR168-resistant version of *AGO1* affects miRNA function, the phenotype is a miRNA that targets overaccumulation, and the plants show phenotypes similar to miRNA biogenesis mutants, such as *dcl1*, *hen1*, and *hyl1* [38]. AGO2, an argonaute gene, contains a binding site for miR403 in its 3′-UTR [39]. The regulation of genes involved in sRNA metabolism or function by miRNAs involves a feedback mechanism to ensure an adequate level of activity for the different the sRNA pathways.

5. miRNAs sequencing and prediction

Since the early beginning of noncoding RNA findings in developmental patterning, researchers have emphasized the bioinformatic challenges [40-42], not only in the miRNA discovery but also in the target prediction in order to better understand the expression, processing, and mechanism of regulation through base pairing recognition [12, 43, 44].

Genetic screening and direct cloning approaches work for simple miRNA candidate per event; therefore, these technologies have been replaced with Northern blotting, qRT-PCR and miRNA array assays. However, since the availability of next generation sequencing, this kind of technologies has been more frequently used as an efficient strategy for detailed research on plant miRNA of a wide variety of species because of the generation of millions of sequences per run. It can not only identify miRNA but also generate expression profiles. Moreover, big data analyses require a support of computational tools in order to extract relevant and refined information [45, 46].

Parallel to the development of deep sequencing strategies, sophisticated computational tools, and refined databases have played a major role in the effort to obtain a genome-wide profiling of miRNAs. Because of such effort, MiRBase, TargetScan, Plant Non-coding RNA Database (PNRD), miRNEST 2.0, and miRDeepFinder have been developed as free-access tools available for the study of miRNAs [46, 47].

miRBase Database [48] is a public repository first established in 2002. Nowadays, it is managed by Griffiths-Jones lab at the University of Manchester. In one of its latest version (released v. 21, June 2014) there are a total of 28,645 hairpin precursors and 35,828 mature products all over across 223 species. Besides, the miRBase includes a functional miRNA information connected with Wikipedia resource [48].

Tha Plant miRNA Database (PMRD, http://bioinformatics.cau.edu.cn/PMRD/) was created in 2009 by Prof. Zhen Su´s lab to integrate only plant miRNA data from public databases to keep together sequence information, secondary structure, target genes, and expression profiles [49]. New findings for ncRNA were identified, such as epigenetic regulators. The central focus of miRNA on the PMRD became limited for regulatory repository of data. PNRD (http://structuralbiology.cau.edu.cn/PNRD/index.php) is the updated version on PMRD released with improvements in functional analysis and service [50].

miRNESt was developed in 2012 by the Laboratory of Functional Genomics as a comprehensive repository for plant, animal and virus miRNAs. In consulted version (miRNEST2.0, http://

rhesus.amu.edu.pl/mirnest/copy/home.php) are included 522 miRNA from animal and plant with prediction data cross to 15 external databases, predicted targets for plant candidates supported by experimental validation, miRNA gene structure, and degradome data [51].

miRDeepFinder is a software developed in order to identify miRNA cross their target from deep sequencing. This package also provides to analyze miRNA functionality and it is a specific tool for plant species. From biogenesis, gene regulation as well as target recognition, abundance miRNA/miRNA* analysis, miRDeepFinder is capable to analyze RNA deep sequencing.

There are some computational approaches available for miRNA expression as well as miRNA target determination. Moreover, the false positive data are estimated for around 24-70%,therefore the experimental validation is required to characterize miRNA function [52].

There are methodologies for miRNA function validation at different levels: protein level by Western blot and mRNA level by qRT-PCR [52]. qRT-PCR is one of the most used techniques for detection of miRNA expression due for its high sensitivity to miRNA detection, the capability to identify single nucleotide changes [53]. miR-RACE (PCR-based) is an effective method to determine the precise sequence of miRNA at their 5′ and 3′ ends, which can distinguish between members of a miRNA family, and they can determine expression patterns at different family member levels [53, 54].

Parallel analysis of RNA ends (PARE) is a modification of miR-RACE, deep sequencing, and bioinformatic analysis for high-throughput sequencing. PARE is also known as degradome or genome-wide mapping of uncapped transcripts. PARE is used to plant systems manly to identify large subsets of miRNA targets to direct cleavage [55].

6. Conclusions and perspectives

Genetic, biochemical, and genomic studies have revealed a diverse array of endogenous sRNAs in plants, and resulted in the identification of several distinct classes of sRNAs. The study of these sRNAs has increased our knowledge regarding the function of these gene regulatory molecules.

Traditional computational approaches have made great progress in predicting new miRNAs in combination with molecular analysis. Most of the miRNAs are likely to be non conserved and/or species specific; this makes it hard to adapt the current approaches to predict non-conserved miRNA genes, and how to identify non-conserved miRNAs in non-model species where a reference genome is not available is an area of intense research.

Studies on miRNA target identification represents a big challenge beyond the identification of miRNA genes; total number of miRNA targets per miRNA family is still unknown and a large number of predicted miRNAs have not been validated experimentally. The identification of miRNA targets will improve our understanding of miRNA-mediated regulation of plant growth and development. miRNAs regulate gene expression by cleaving mRNA or by repressing mRNA translation; thus, now it is possible to design artificial miRNAs to suppress

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the expression of a target gene in order to study gene function, similar to the use of alternative molecular tools (i.e. antisense mRNA) used for studying gene functions.

The use of miRNA to improve plant yields, quality, or resistance to various environmental stresses including insect and pathogen infection will come with the understanding of miRNA regulation over specific processes. Future study of miRNAs will provide us with tools for improving crop growth and quality.

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