Growing Diversity of Plant MicroRNAs and *MIR*-Derived Small RNAs

Mariyana Gozmanova, Vesselin Baev, Elena Apostolova, Gaurav Sablok, and Galina Yahubyan

Abstract Small, noncoding RNAs are essential regulatory molecules of plant genome. Small RNAs (sRNAs) have been classified on the basis of their biogenesis and mode of action in two major types—microRNAs (miRNAs) and small interfering RNAs (siRNAs). Plant miRNAs are typically 21 nucleotides in length and derive from unique genetic loci (*MIR* genes). Next-generation sequencing approaches have increased significantly the number of known plant miRNAs and have revealed that *MIR* genes frequently produce sRNAs, known as miRNA variants, isoforms or isomiRs, which exhibit differences from their corresponding "reference" mature sequences. The main mechanism of action of canonical miRNAs is sequence-specific repression of gene expression on posttranscriptional level. Recent studies have revealed that noncanonical miRNAs and *MIR*-derived siRNAs (a particular subset of isomiRs) can act as well in sequence-specific transcriptional silencing thus influencing genome function through DNA methylation. Moreover, miRNAs can be regulated by epigenetic alteration such as DNA methylation and histone modifications of *MIR* genes. Having profound role in genetic and epigenetic control, plant miRNAs, and *MIR*-derived siRNAs can potentially participate in most developmental processes, plant stress response, and adaptation. In this chapter, we discuss the biogenesis of miRNAs and *MIR*-derived sRNAs and their regulatory impact on plant gene expression.

M. Gozmanova (✉) • V. Baev • G. Yahubyan
Department of Plant Physiology and Molecular Biology, University of Plovdiv, 24 Tsar Assen Str, Plovdiv, Bulgaria

Institute of Molecular Biology and Biotechnologies, 105, Ruski Blvd., 4000 Plovdiv, Bulgaria
e-mail: mgozmanova@gmail.com

E. Apostolova
Department of Plant Physiology and Molecular Biology, University of Plovdiv, 24 Tsar Assen Str, Plovdiv, Bulgaria

G. Sablok
Plant Functional Biology and Climate Change Cluster (C3), University of Technology, Sydney, PO Box 123, Broadway, NSW 2007, Australia

The algorithms for accurate annotation of novel miRNAs and isomiR sequences are still a challenging task, requiring integration of experimental and computational approaches. Here, we shortly present some recent tools which have been developed to facilitate this task by providing friendly user interface, without requiring computing skills for the purpose of the analysis.

Keywords miRNAs • *MIR*-derived siRNAs • Isomirs • Long miRNAs • DNA methylation • Histone modifications

Contents

1 Introduction

RNA-mediated processes together with DNA methylation and histone modifications are considered as the main molecular mechanisms constructing the epigenetic regulatory network which has an essential role in plant developmental programs, stress response and adaptation, transposon silencing, signaling pathways, and various non-Mendelian patterns of inheritance (Grant-Downton and Dickinson 2005; Avramova 2011).

2 Micro RNAs in the Plant Small RNA World

Plant small RNAs (sRNAs), which range in size from approximately 20 to 30 nucleotides (nt), can be distinguished both by their biogenesis and mode of action. They include microRNAs (miRNAs) and small interfering RNAs (siRNAs) such as repeat-associated siRNAs (ra-siRNAs), trans-acting siRNAs (ta-siRNAs), and natural antisense transcript siRNAs (nat-siRNAs) (Vaucheret 2006). Each type of sRNAs is unique with respect to the molecular size, the plant DICER-like (DCL) RNaseIII enzyme which is involved in its biogenesis, and the ARGONAUTE (AGO) protein which is directed by the sRNA for silencing of the target gene expression. Most siRNAs target the same locus they are derived from, except for miRNAs and ta-siRNAs, which target mRNAs produced from different loci. Plant

sRNA molecules function as negative regulators of gene expression on a transcriptional or posttranscriptional level (Ruiz-Ferrer and Voinnet 2009).

In general, ra-siRNAs (or heterochromatic siRNAs, hc-siRNAs) are 21–24 nt in length, and their biogenesis involves DCL2, DCL3, and DCL4 (Kasschau et al. 2007; Xie et al. 2004). These sRNAs are mainly loaded into AGO3 and are known to be involved in DNA and histone methylation (Xie et al. 2004; Zheng et al. 2007; Zilberman et al. 2003). ta-siRNAs are phased 21 nt RNA molecules whose production involves only DCL4 and is triggered by miRNA-directed cleavage of the TAS transcripts. Further ta-siRNAs are loaded into AGO1 and, like miRNAs, promote sequence-specific cleavage of their targeted gene transcripts (Allen et al. 2005, 2006). The biogenesis of 21 and 24 ntnat-siRNAs involves one of DCL1, DCL2, or DCL3, and a subgroup of nat-siRNAs is dependent on RDR2 and PolIV (Borsani et al. 2005; Katiyar-Agarwal et al. 2006; Zhang et al. 2012). A category of sRNAs ranging from 30 to 40 nt in size, referred to as long siRNAs (lsiRNAs), has been subsequently identified (Katiyar-Agarwal et al. 2007). siRNAs act as transcriptional repressors of a subset of transposons and genes through triggering de novo methylation of homologous DNA in the process of RNA-dependent DNA methylation (RdDM) (Wassenegger et al. 1994; Law and Jacobsen 2010).

First plant sRNAs with miRNA characteristics were recognized in *Arabidopsis* in 2002 (Llave et al. 2002; Mette et al. 2002; Park et al. 2002; Reinhart et al. 2002). Canonical miRNAs are typically 21 nt in length, and their precursors (pri-miRNAs) are transcribed by RNA polymerase II (PolII) (occasionally by RNA Polymerase III) from miRNA (*MIR*) genes (Jones-Rhoades et al. 2006). Some precursors were found produced from the spliced introns of the gene transcripts in *Arabidopsis* and rice (Meng and Shao 2012). The single-stranded precursor forms a hairpin structure which is cut out by DCL1 releasing double-stranded miRNA with an approximately 2-nt 3′-end overhang. Since other DCL proteins (DCL2, DCL3, or DCL4) may recognize and process miRNA precursors, miRNAs of diverse sizes ranging from 20- to 24-nt could be generated (Margis et al. 2006). The double-stranded miRNA is subsequently 2′-*O*-methylated by the methyl transferase Hua enhancer1 (HEN1) and protected by RNA degradation (Yu et al. 2005; Molnár et al. 2007; Abe et al. 2010). Only one strand of the duplex is selected to become the functional miRNA (guide strand, mature miRNA) while the other strand is degraded (passenger strand, miRNA*). In some cases, the passenger strand can be differentially expressed in different tissues and developmental stages and can be functionally active (Okamura et al. 2008). In *Arabidopsis*, miR396a-5p is the mature miRNA which is preferentially expressed in root to target growth-regulating factors (GRFs) family (Jones-Rhoades and Bartel 2004), while miR396a-3p is preferentially expressed in flower (Jeong et al. 2013). There are examples of multiple mature miRNA production from a single precursor as in the case of closely related miR161.1, miR161.2, and miR161.3 targeting genes encoding pentatricopeptide repeat proteins (PPRs) in *Arabidopsis* (Allen et al. 2004; Jeong et al. 2013).

Mature miRNA strand, or occasionally miRNA* strand, is loaded into an AGO protein to form a RNA-induced silencing complex (RISC). Most plant miRNAs possess 5′ uridine, which serves as a sign for association with AGO1 (Mi et al. 2008; Montgomery et al. 2008; Takeda et al. 2008). MiRNA directs RISC to target

mRNAs in a sequence-dependent manner. Plant miRNAs share perfect or nearly perfect complementarity with their targets (Rhoades et al. 2002) and bind to complementary sequences in the 3′UTRs of target mRNAs. While miRNAs with near perfect complementarity repress predominantly translation machinery, those with perfect complementarity induce transcript cleavage (Hutvágner and Zamore 2002; Llave et al. 2002; Bartel 2004).

Though miRNA and miRNA* are the most predominant species from a precursor, high-throughput sequencing (HTS) data show that there are often low-frequency positional and length variations from miRNA hairpin structures (Meyers et al. 2008; Morin et al. 2008). These length and sequence variants of canonical miRNAs have been widely demonstrated as isoforms or isomiRs. Based on the homology assessment methods, they have been primarily classified into templated (miRNAs length variants having homology to parent genes) or non-templated (nucleotide additions and/or posttranscriptional RNA edits resulting in no homology to parent genes) isomiRs (Neilsen et al. 2012; Rogans and Rey 2016). Although the precise mechanism by which these isomiRs originate has not yet been established, it has been laid forth that the isomiR biogenesis is due to the imprecise cleavage activity of DCL1 (Bartel 2004), 3′ uridylation by nucleotidyl transferases such as UTP:RNA uridylyltransferase (URT1) and HEN1 suppressor1 (HESO1) (Tu et al. 2015; Wang et al. 2015), or 3′-5′ exoribinuclease activity of Small RNA Degrading Nucleases (SDNs) (Ramachandran and Chen 2008), producing isomiRs with 5′-, 3′-nt additions or deletions. The regulatory roles of isomiRs have already been established in tissue specificity, development, leaf senescence, and stress response (Colaiacovo et al. 2012; Hackenberg et al. 2013; Xu et al. 2014). In *Arabidopsis*, the comparative assessment of the genome-wide sRNA profiles induced by temperature stress displayed differential expression of a specific subset of mature miRNAs and a variety of isomiRs (Baev et al. 2014). For example, the miR160c precursor gave rise to numbers of isomiRs which were found to be differentially expressed upon high- and low-temperature treatment (Fig. 1). The isomiR with the highest copy number was the most considerably upregulated during stress exposure implying its likely functional significance for plant stress response (Baev et al. 2014).

Many miRNAs come in families, in which *MIR* loci are often closely related and occasionally produce identical miRNAs. Some of annotated miRNA families are conserved across vast phylogenetic scales, while others are family- or species-specific (Cuperus et al. 2011). Like miRNAs, numerous isomiRs have been found conserved across species and are likely to participate in regulation of important biological processes (Ameres and Zamore 2013).

3   MiRNA-Mediated DNA Methylation

The increasing evidences of isomiRs complexity in plants have prompted searching for biologically significant variants amongst the large number of *MIR*-derived sRNAs (Sablok et al. 2015). The well-recognized mechanism of action of miRNAs

Fig. 1 miR160c and its isomiRs in *Arabidopsis*. The profile was generated from NGS datasets derived from temperature conditional responses (*NT* normal temperature, *LT* low temperature, *HT* high temperature). The mature miRNA indexed in miRBase is shown in bold; the isomiR having the highest copy number is underlined

is sequence-specific repression of gene expression on posttranscriptional level. Recent findings have revealed that newly identified miRNAs and *MIR*-derived sRNAs can act as well in sequence-specific transcriptional silencing thus influencing genome function through DNA methylation (Bao et al. 2004; Chellappan et al. 2010; Wu et al. 2010; Khraiwesh et al. 2010).

3.1 First Evidences for an Indirect Link Between MiRNAs and DNA Methylation

The first evidence for involvement of miRNAs in DNA methylation came from the study of Bao et al. (2004) in *Arabidopsis* that intended to answer whether the miRNA complementary site in the target mRNA affected the chromatin state of corresponding gene. For that, they compared DNA methylation between wild-type plants and two mutant lines—*phb-1d* and *phv-1d* in which dominant mutations in the *PHABULOSA* (*PHB*) and PHAVOLUTA (*PHV*) genes disrupted the complementarity site of miRNAs 165 and 166 in *PHB* and *PHV* mRNAs (McConnell et al. 2001; Emery et al. 2003). In the two genes, the miR165/166 complementary site is split by an intron, and the downstream exons were found heavy methylated in the most cells of wild type. Decreased methylation at the *PHB* and *PHV* loci in *phb-1d* and *phv-1d,* respectively, revealed indirect interaction between miR165/166 and the *PHB* and *PHV* templates. *It was hypothesized that the miRNA binds to the complementary site of the processed, nascent mRNA and recruits in trans a chromatin-modifying complex to the closely located template locus* (Bao et al. 2004).

Intriguingly, the *PHB* and *PHV* methylation was not affected in *dcl1* and *ago1* mutants (Bao et al. 2004) that raised the question of whether other players, different but still related to miRNAs, could mediate DNA methylation.

The hypothesis proposed by Bao and collaborators (2004) has been confirmed in moss *Physcomitrella patens* through analyzing the interaction between miR166 and its target mRNAs—*PpC3HDZIP1* and *PpHB10* (Khraiwesh et al. 2010). Similarly to the *PHB* and *PHV* genes in *Arabidopsis*, the miRNA binding site contains an intron and is reconstituted upon splicing of the *PpC3HDZIP1* and *PpHB10* primary transcripts. It was shown in moss mutants without DCL1b that, although the miRNA level was unchanged, the target transcripts were significantly downregulated without being cleaved. Evidences were obtained for accumulation of stable miRNA:target mRNA complexes and for hypermethylation of target loci in these mutants, and was proposed that the ratio between miRNAs and their targets was determining for recruitment *in trans* of the DNA-methylation effector molecules to template loci (Khraiwesh et al. 2010).

3.2 *MIR-Derived sRNAs: The Real Players in MiRNA-Mediated DNA Methylation*

MIR-Derived sRNA Diversity and Biogenesis In *Arabidopsis*, a novel class of 23- to 27-nt sRNAs was identified in *Arabidopsis* that was produced together with the canonical 20- to 22-nt miRNAs from number of *MIR* genes (Vazquez et al. 2008; Chellappan et al. 2010). Unlike canonical miRNAs which originate from ancient, highly conserved *MIR* genes, the long *MIR*-derived sRNAs originate from recently evolved *MIR* genes (Vazquez et al. 2008). These studies demonstrate that canonical miRNAs and *MIR*-derived sRNAs can be generated independently from the same hairpins by DCL1 and DCL3, respectively. Mutational analysis revealed that the accumulation of *MIR*-derived sRNAs was dependent not only on DCL3 but also on RDR2 and PolIV in *Arabidopsis* (Chellappan et al. 2010). The involvement of PolIV, RDR2, and DCL3, which are the main components of the small interfering RNA (siRNA) biogenesis, in the processing of *MIR*-derived sRNAs is the reason to refer to these sRNAs as *MIR*-derived siRNAs (Chellappan et al. 2010). The question what is the activity of PolIV and RDR2 in *MIR*-derived siRNAs biogenesis in *Arabidopsis* remains still open.

Unlike *Arabidopsis*, numbers of *MIR* genes were found to produce both canonical miRNAs and 24 ntsRNAs, or only 24 ntsRNAs in rice (Zhu et al. 2008; Wu et al. 2009). The two types of sRNA can be processed from the same hairpins by the cooperative action of DCL1 and DCL3, or, as in some cases, a hairpin precursors can be processed by DCL3 giving rise to only 24-nt long sRNA species called long miRNAs or lmiRNAs (Wu et al. 2010). 31 of the 54 lmiRNAs identified by Wu and coworkers in rice (2010) were observed later to be located in the intronic regions of protein-coding genes (Tong et al. 2013). Contrasting to the *Arabidopsis MIR*-

derived siRNAs, there is no evidence to suggest that the formation of lmiRNAs needs RDR2 and PolIV. In tomato, 10 loci encoding putative 24 ntlmiRNAs were predicted, and the expression of four of them was proved to be DCL3-dependent confirming their identity as lmiRNAs (Kravchik et al. 2014). In addition, the expression profiles of two of the tomato lmiRNAs in different organs showed their involvement in tomato reproductive development.

MIR-Derived sRNA Effector Complexes and DNA Methylation Canonical miRNAs and MIR-derived sRNA are loaded on functionally different argonaute complexes—canonical miRNAs associate specifically with AGO1 while MIR-derived siRNAs are sorted in AGO4 clade proteins. The reduced levels of *MIR*-derived siRNAs in *ago4-1* mutant and AGO4-coimmunoprecipitation assay have revealed that these sRNAs could associate with AGO4 in *Arabidopsis* (Chellappan et al. 2010). The observations of Wu et al. (2010) in rice suggest that lmiRNAs initiated with adenine sort into AGO4a, AGO4b, and AGO16, and those beginning with uracil are loaded on AGO4b.The association of *MIR*-derived siRNAs with AGO4 clade proteins suggested their involvement in DNA methylation.

There are increasing evidences that some *MIR*-derived siRNAs are indispensable for DNA methylation of their target loci *in trans* and/or of their own *MIR* loci *in cis*. In the *Arabidopsis* mutant *nrpd1-3* lacking the Pol IV largest subunit, where 23- to 26-nt sRNAs were absent, reduced DNA methylation of the putative target mRNAs At4g16580 and At5g08490 for the recently evolved miR2328 and miR2831-5P, respectively, correlated with upregulated mRNA expression (Chellappan et al. 2010). In the same mutant, the DNA methylation of *SPL2* (squamosa-promoter binding protein-like), a target of the canonical miR156, was found reduced up- and downstream of the miR target site compared to wild type. To examine the DNA methylation of lmiRNA-producing and target loci in rice, two mutant lines *dcl3a-17* and *rdr2-2* were subjected to bisulfite sequencing (Wu et al. 2010). Since the lmiRNAs biosynthesis requires DCL3, but not RDR2, the comparison of methylation profiles of these mutants would allow for differentiation between siRNA- and lmiRNA-dependent DNA methylation. In that way, it was demonstrated that miR1873 directed the methylation at its own locus as well as that miR1863, miR820.2, miR1873.1, and miR1876 mediated the methylation of their target genes (Wu et al. 2010). Unlike siRNA-dependent DNA methylation spreading in the 3′-direction only, lmiRNAs induce DNA methylation bidirectionally from the miRNA-binding site in target genesas exemplified by bisulfite sequencing of the target genes of miR1862c, miR1863b, miR1867, miR2121b, miR5150, and miR5831 in rice (Hu et al. 2014) and of miR160 and miR166 in moss (Kravchik et al. 2014).

The described variety of plant canonical and non-canonical miRNAs and *MIR*-derived siRNAs, with the particularities of their biogenesis, AGO incorporation, and mode of action, is schematically presented in Fig. 2.
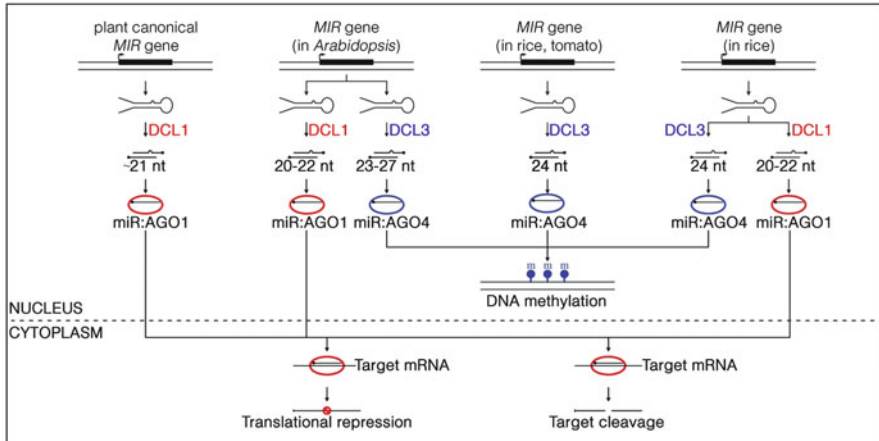
Fig. 2 Diversity of plant *MIR*-derived sRNAs. The hairpin precursors, transcribed from most plant *MIR* genes, are cut out by DCL1 to produce canonical mature ~21 nt miRNAs which associate with AGO1 and mediate target mRNA cleavage or translational repression. In addition to this classical pathway, some plant *MIR* genes can generate sRNA species that differ from the canonical miRNAs. In *Arabidopsis*, two sRNA species—canonical miRNAs and *MIR*-derived siRNAs (23–27 nt)—can be generated independently from different molecules of the same hairpin population by DCL1 and DCL3, respectively (Chellappan et al. 2010). In rice and tomato, some *MIR* genes produce only 24 ntlmiRNAs using DCL3, while other *MIR* genes can produce canonical miRNA and lmiRNA species simultaneously by coordinate activities of DCL1 and DCL3 on the same molecule (Wu et al. 2010; Kravchik et al. 2014). *MIR*-derived siRNAs and lmiRNAs associate predominantly with AGO4 and mediate DNA methylation of target genes or their own *MIR* genes. The DCL1/AGO1 pathway is depicted in *red* color, while the DCL3/AGO4 pathway is depicted in *blue* color

4 Epigenetic Control of *MIR* Genes

Plant miRNA expression might be regulated on transcriptional level by chromatin remodeling due to histone modification or DNA methylation of the corresponding *MIR* loci.

4.1 Impact of Histone Modifications of MIR Loci on MiRNA Expression

The studies of Kim et al. (2009) performed with the *Arabidopsis* mutants, *gcn5-1* and *gcn 5-2*, revealed that acetylation of histone H3 lysine 14 at numbers of miRNA loci interfered with miRNA production. The analysis of pri-miRNA/mature miRNA accumulation levels suggested a role of GCN5 (histone acetyltransferase) at both pri-miRNAs maturation and miRNA expression.

Briefly, the reduced expression of miRNA processing genes—DCL1, SE, HYL, and AGO1, correlated with low accumulation of pri-miRNAs and high levels of mature miRNAs in the wild type. In *gcn5* mutants, the expression of these components of miRNA processing pathway was upregulated and correlated with increased levels of miRNAs and decreased levels of corresponding pre-miRNAs. No direct interaction between GCN5 and DCL1, SE, HYL, and AGO1 genes was identified. These data inspired the hypothesis of the presence of a common repressor of these genes, the activity of which was influenced by GCN5.

Furthermore, a direct interaction was described between GCN5 and four *MIR* genes (miR165a, miR172a, miR395e, and miR399d) in the wild-type *Arabidopsis*. Based on studies of *gcn 5-2* mutant, the specific miRNA-protein interaction was mapped to the bromodomain of GCN5. Moreover, some histone deacetylases (HDA9 and HDA19) were also seen as factors regulating miRNA accumulation.

Nosaka et al. (2012) have found that miR820 cleaves *OsDRM2* mRNA and induce DNA methylation at the miR820 target site in *OsDRM2* gene. On the other hand, it became clear that miR820 is encoded by CACTA TEs (five copies, located on different chromosomes) (Nosaka et al. 2013). The heterochromatic mark (H3K9 dimethylation) is detected in CACTA copies carrying miR820 genes suggesting repression of miR820. However, in one of the CACTA copies, that resides in chromosome 7 and produces transcripts, low levels of active histone marks (H3K4 di/tri methylation and H3K9 acetylation) correlate with high level of asymmetric cytosine methylation (CHH) in the same region, but still the transcription of miR820 from the this locus is allowed.

In *Brachypodium distachyon*, a representative of Pooideae plants, a newly evolved, species-specific miRNA-miR5200 was identified to target the mRNAs of two florigen genes, FTL1 and FTL2 (Wu et al. 2013a, b). The miR5200 expression was found changed under different day lengths being upregulated under short day (SD) and downregulated under long day (LD). The authors claimed that differential expression of miR5200 might due to changes in chromatin modifications at the *MIR* genes, MIR5200a and MIR5200b. A repressive histone mark H3K27tri-methylated was enriched in the MIR5200 genes under LD conditions. Thus, epigenetics control of miR5200 expression was found to participate in the photoperiodic regulation of the transition from vegetative toward reproductive stage in *B. distachyon* (Wu et al. 2013a, b).

4.2 DNA Methylation of MIR Genes Affects MiRNA Expression

DNA methylation status at CG, CHG, and CHH contexts has been explored in both promoter region and gene body of *MIR* genes in rice. These parameters were compared between conserved and species-specific miRNA (Hu et al. 2014). The highest rate of methylation was determined in CG context of both promoters and

gene bodies of non-conserved *MIR* genes. It was found that the genes of majority of highly expressed, conserved miRNAs were constitutively hypomethylated, while the genes of recently evolved miRNAs were hypermethylated at both promoters and gene bodies. The authors suggested that a strong control orchestrated by DNA methylation had been established in plant evolution as a means for repression of newly evolved species-specific miRNAs.

DNA methylation landscape was monitored in five different rice tissues – embryo, endosperm, root, shoot, and mature leaves (Hu et al. 2014). The promoters and gene bodies of non-conserved *MIR* genes showed the same trend in their methylation profile in CG context among the studied tissues. Hypomethylation was detected in all contexts in endosperm. A higher rate of methylation was detected only in CHH context in embryo and mature leaves. DNA methylation of promoters and gene bodies of conserved miRNAs didn't show significant differences in all tissues except for the endosperm where reduced methylation in CHG and CHH context was observed.

In poplar, a relationship between DNA methylation and gene expression was described for both long noncoding RNAs (lncRNA) >200 bp and miRNAs (Song et al. 2016).The forth exon of lncRNA00268512 gene was observed on the complementary strand of the first exon of the protein-coding gene Potri.018G127000, in which miR396e was located. Due to this substantial overlap, it was proposed that the lncRNA00268512 and miR396e may interact (Song et al. 2016). Moreover, a stress-specific differentially methylated region (SDMR 162) was identified in the first exon of Potri.018G127000. The decreased DNA methylation of SDMR 162 correlated with increased levels of lncRNA upon cold and osmotic stress. It was suggested that the excess lncRNA molecules could captured miRNA396e-3p and caused its lower abundance in response to stress.

4.3   Link Between MIR Gene DNA Methylation and Plant Stress Response

Abiotic stress-responsive DNA methylation was observed in five conserved *MIR* genes (miR167-3p, miR6445a, miRNA319c, miR156f, and miR472a) and eleven non-conserved *MIR* genes in *Populus simonii* (Song et al. 2016). A long-term impact of DNA methylation on gene expression of miRNAs was seen in response to short-term abiotic stress in ~15% of de novo methylated sites. Stress-induced DNA demethylation was reported at two *MIR* genes encoding miR156f and miR472b transcripts. Approximately, 11% of demethylated sites that responded to abiotic stress were found preserved 6 months later (Song et al. 2016).

Ci et al. (2015) identified 1066 stress-specific DNA methylated sites in poplarin response to heat and cold stress. Analysis of DNA methylation levels showed 150 stress-specific DNA methylated sites per each type of stress and 100 DNA methylated sites common to both stress types. Seven *MIR* genes (miR156i,

miR156j, miR167h, miR390c, miR393a, miR396e, and miR396g) were found to be differentially methylated in response to temperature stress. Moreover, their expression was influenced by the cytosine methylation pattern. Most temperature responsive *MIR* genes that carry CNG methylation pattern displayed higher expression than those having CG methylation.

5 Computational Tools for Plant MiRNA Analysis from NGS Datasets

The identification of the entire repertoire of sRNAs and miRNAs has been made possible by the next-generation sequencing (NGS) techniques in an efficient and cost-effective fashion. Due to the big data output of these methods, the computational approaches are a necessary step to depict miRNAs in outgrowing datasets.

A typical bioinformatics miRNA identification algorithm based on NGS datasets involves several steps including, but not limited to (1) quality filtering and adapter trimming; (2) mapping of sRNA reads to identify corresponding genomic loci; (3) estimating the miRNA expression based on copy number; (4) exploring the 2D RNA structure of the loci for identification of pre-miRNA; etc. sRNA sequencing libraries usually also contain other non-miRNA RNA molecules, such as other sRNAs, degradation reads from protein-coding genes, rRNA reads, etc. The computational miRNA identification also compels filtering these non-miRNAs as much as possible from the reads in the library to make analysis more accurate and the "background" to be discarded. Each step in the process may result in a change of the output of the analysis, but perhaps the most crucial part is how the mapping stage is done. The software pipelines available so far vary in terms of user interface, user control, parameters, the input format data, reference databases, and how they adopt each of the above analysis stages.

The key step in NGS data analysis is the mapping the huge amount of short reads to a given genome. Several algorithms and software modules have been specifically designed for dealing with the alignment of millions of reads. Some of the most used tools for the alignment to the reference genome are Bowtie (http://bowtie.cbcb.umd.edu/) (Langmead et al. 2009), BWA (http://maq.sourceforge.net/) (Li and Durbin 2009), MAQ (http://maq.sourceforge.net/) (Li et al. 2008), and SOAP (http://soap.genomics.org.cn/) (Li et al. 2008).

The standard approach for identification of plant miRNAs involves cross-species discovery of the conserved miRNAs in the sample due to the fact that large population of miRNAs has orthologues in the plant kingdom. However, this is limited to organisms where known reference genome and miRNAs genes are available.

In such comparative analysis, the NGS short reads are aligned to a known reference database. These miRNA databases are the source for known miRNA sequence (mature and hairpin sequences) and annotation information. They also are

essential for expression profiling of miRNAs. The most popular miRNA databases include, but not limited to: miRBase (Griffiths-Jones 2004), deep Base (Yang and Qu 2012), microRNA.org (Betel et al. 2008), miRGen (Megraw et al. 2007), miRNAMap (Hsu et al. 2006), miRNEST (Szcześniak et al. 2012), and PMRD (Zhang et al. 2010); among them the most comprehensive ones for plant miRNAs are miRBase and PMRD.

The more challenging analysis is to try to identify novel mature and precursor miRNA sequences directly from NGS read data without any dependence upon homologous references of known conserved plant miRNAs. Such methods usually include deep investigation of the mapping results and the clustering of the sRNAs produced from miRNA/miRNA* regions. To discover the mature miRNA among such clusters of sRNAs expressed from the precursor region, only those reads which fit a specific criteria (e.g., could form duplex, maximum with four mismatches, observed 3′ overhangs, duplex length stayed within the range of 18–24 bp, and high copy number) are considered as miRNA candidates (Meyers et al. 2008). However, such rules have limitations and necessitate experimental validation over large amount of datasets. Based on PCR experiments which aimed to validate de novo predicted miRNA candidates, some authors reported that 40% of them were false positive (Wei et al. 2009). All these studies have urged the researchers in the recent years to try to optimize the existing and develop new methods to decrease the false positive rate of the output.

A variety of web-based and stand-alone software have been developed for analyses of plant miRNA data. The list of some of the available software that are designed specifically for plant miRNAs or can be used with plant miRNAs datasets can be found in Table 1.

The recent discovery that miRNAs can both regulate and be regulated by target interactions has a key role for understanding their roles in gene regulation (Salmena et al. 2011). A unique and remaining task in the field is the capacity to identify miRNA targets with high confidence. Moreover, finding the real functional miRNA targets is still puzzling even though the biological rules of miRNA targeting have been shown experimentally and computationally.

The classical way of identifying a plant miRNA target lays on the complementarity between itself and its mRNA site defined by the stability of the duplex which has been utilized widely as a main feature in the analysis step by computational tools. Some of the tools that utilize these algorithms are psRNATarget (Dai and Zhao 2011) and imiRTP (Ding et al. 2012), which predict the functional type of miRNA based on the complementary at the central region of the miRNA:target pair.

Recently in the era of NGS, degradome datasets have been used to find evidence of cleaved miRNA targets without relying on computational RNA folding predictions. Experimental methods have shown that miRNA AGO-mediated cleavage of mRNA happens exactly between the 10th and 11th nucleotide of miRNA–mRNA duplex. The subsequent upstream molecule of the cleaved target is degraded, but the downstream fragment is shown to be stable (Llave et al. 2002). And therefore, NGS techniques involving the capturing of these downstream fragments (Addo-Quaye et al. 2008; German et al. 2008; Gregory et al. 2008) and

Table 1 Bioinformatics tools for plant miRNA analysis from NGS datasets

Tool name	Reference	Web page
PsRobot	Wu et al. (2012)	http://omicslab.genetics.ac.cn/psRobot/
Semirna	Muñoz-Mérida et al. (2012)	http://www.bioinfocabd.upo.es/semirna/
miRDeepFinder	Xie et al. (2012)	http://www.leonxie.com/DeepFinder.php
psRNATarget	Dai and Zhao (2011)	http://plantgrn.noble.org/psRNATarget/
miRDeep-P	Yang and Li. (2011)	http://faculty.virginia.edu/lilab/miRDP/
miRA	Evers et al. (2015)	https://github.com/mhuttner/miRA
plantDARIO	Patra et al. (2014)	https://github.com/mhuttner/miRA
CAP-miRSeq	Sun et al. (2014)	http://bioinformaticstools.mayo.edu/research/cap-mirseq/
miREvo	Wen et al. (2012)	http://evolution.sysu.edu.cn/software/mirevo.htm
miRanalyser	Hackenberg et al. (2011)	http://bioinfo2.ugr.es/miRanalyzer/miRanalyzer.php
miR-PREFeR	Lei and Sun. (2014)	https://github.com/hangelwen/miR-PREFeR
miRPlant	An et al. (2014)	http://sourceforge.net/projects/mirplant/
mirTools 2.0	Wu et al. (2013a, b)	http://centre.bioinformatics.zj.cn/mr2_dev/index.php
sRNAtoolbox	Rueda et al. (2015)	http://bioinfo5.ugr.es/srnatoolbox/index
UEA sRNA Workbench	Stocks et al. (2012)	http://srna-workbench.cmp.uea.ac.uk/
ShortStack	Axtell (2013)	http://sites.psu.edu/axtell/software/shortstack/
CleaveLand	Addo-Quaye et al. (2009)	http://www.bio.psu.edu/people/faculty/Axtell/AxtellLab/Software.html
PAREsnip	Folkes et al. (2012)	http://srna-workbench.cmp.uea.ac.uk/tools/analysis-tools/paresnip/
PatMaN	Prüfer et al. (2008)	http://bioinf.eva.mpg.de/patman
SoMART	Li et al. (2012)	http://bakerlab.berkeley.edu/somart-webserver-mirna-sirna-analysis
StarScan	Liu et al. (2015)	http://mirlab.sysu.edu.cn/starscan/

the following bioinformatics analysis of such datasets can be used to identify the miRNA targets. Recently, several tools have been developing that use degradome data in order to predict miRNA targets. Among the most popular software are CleaveLand (Addo-Quaye et al. 2009), SeqTar (Zheng et al. 2012), PAREsnip (Folkes et al. 2012), PatMaN (Prüfer et al. 2008), SoMART (Li et al. 2012), and StarScan (Liu et al. 2015).

In the past few years, cohorts of tools have been developed to identify isomiRs, which are either stand-alone or web-based (Table 2). Among the tools used to profile isomiRs in plants are SeqCluster (Pantano et al. 2011), miRSeqNovel (Qian et al. 2012), isomiRID (de Oliveira et al. 2013), sRNAtoolbox (Rueda et al. 2015), isomiRex (Sablok et al. 2013), and isomiRage (Muller et al. 2014). Although these tools allow the identification of the isomiRs, they suffer some omissions such as isomiR profiling that would take into account the sequencing artifacts, expression-

Table 2 Recently developed classification tools for identifying isomiRs

Tool name	Reference	Web page
SeqCluster	Pantano et al. (2011)	https://github.com/lpantano/seqbuster
miRSeqNovel	Qian et al. (2012)	http://sourceforge.net/projects/mirseq/files
isomiRID	de Oliveira et al. (2013)	http://www.ufrgs.br/RNAi/isomiRID/
isomiRex	Sablok et al. (2013)	bioinfo1.uni-plovdiv.bg/isomiRex/
miRspring	Humphreys and Suter (2013)	http://mirspring.victorchang.edu.au
IsomiRage	Muller et al. (2014)	http://cru.genomics.iit.it/Isomirage/
sRNAtoolbox	Rueda et al. (2015)	http://bioinfo5.ugr.es/srnatoolbox
isomiR-SEA	Urgese et al. (2016)	http://eda.polito.it/isomir-sea
miR-isomiRExp	Guo et al. (2016)	http://mirisomirexp.aliapp.com
DeAnnoIso	Zhang et al. (2016)	http://mcg.ustc.edu.cn/bsc/deanniso/
IsomiRbank	Zhang et al. (2016)	http://mcg.ustc.edu.cn/bsc/isomir/

based read support, visualization of the isomiRs with respect to read depth and mapping, target predictions, and functional enrichment. Some tools such as isomiRex (Sablok et al. 2013) are web-based, and without allowing the identification of novel miRNAs, provide support for isomiRs visualization based on read depth, whereas stand-alone tools lack for isomiRs visualization, but support the PARE-Seq-based targets predictions. Although the recently published isomiR Detection tool DeAnnoIso (Zhang et al. 2016) allows for the detection of isomiRs, it can be only accessible through the web interface and lacks to detect the isomiRs across a wide range of plant species (only four plant species are supported). IsomiRage can group the functionally relevant isomiRs according to the adenylation, uridylation, and other respective events in response to biological context (Muller et al. 2014). Other tools such as miR-isomiRExp (Guo et al. 2016) and isomiR-SEA (Urgese et al. 2016) are yet to be assessed for their efficiency in plant isomiR profiling. In terms of the pre-analyzed isomiRs, only one such database tool isomiRBank (Zhang et al. 2016) exists, providing the pre-compiled set of isomiRs across four plant species.

Conclusions The use of genome-wide technologies has enabled the identification of novel miRNAs and a large number of isomiRs that transform the plant miRNAome in an increasingly complex world. Though some of the emerging *MIR*-derived siRNAs resemble canonical miRNAs, they show deviation from the conventional view of miRNA biogenesis, AGO incorporation, mode of action, and regulatory effect on gene expression. LmiRNAs are an excellent example of the biological significance of such *MIR*-derived siRNAs, that being mediators in a noncanonical RdDM pathway, lead to the fine-tuning of epigenetic control in plants. Furthermore, the variability in DNA methylation patterns of genes encoding novel, species-specific miRNAs, induced by different types of stress, upgrades our view about the regulatory networks associated with plant response, adaptability, and tolerance.

References

Abe M, Yoshikawa T, Nosaka M et al (2010) WAVY LEAF1, an ortholog of arabidopsis HEN1, regulates shoot development by maintaining microRNA and trans-acting small interfering RNA accumulation in rice. Plant Physiol 154:1335–1346

Addo-Quaye C, Eshoo TW, Bartel DP et al (2008) Endogenous siRNA and microRNA targets identified by sequencing of the Arabidopsis degradome. Curr Biol 18:758–762

Addo-Quaye C, Miller W, Axtell MJ (2009) CleaveLand: a pipeline for using degradome data to find cleaved small RNA targets. Bioinformatics 25:130–131

Allen E, Xie Z, Gustafson AM et al (2004) Evolution of microRNA genes by inverted duplication of target gene sequences in Arabidopsis thaliana. Nat Genet 36:1282–1290

Allen E, Xie Z, Gustafson AM et al (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell 121:207–221

Ameres SL, Zamore PD (2013) Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol 14:475–488

An J, Lai J, Sajjanhar A et al (2014) miRPlant: an integrated tool for identification of plant miRNA from RNA sequencing data. BMC Bioinformatics 15:275

Avramova Z (2011) Handbook of epigenetics. In: Tollefsbol T (ed) The new molecular and medical genetics. Elsevier, Amsterdam, pp 251–278

Axtell MJ (2013) ShortStack: comprehensive annotation and quantification of small RNA genes. RNA 19:740–751

Axtell MJ, Jan C, Rajagopalan R et al (2006) A two-hit trigger for siRNA biogenesis in plants. Cell 127:565–577

Baev V, Milev I, Naydenov M et al (2014) Insight into small RNA abundance and expression in high- and low-temperature stress response using deep sequencing in Arabidopsis. Plant Physiol Biochem 84:105–114

Bao N, Lye K-W, Barton MK (2004) MicroRNA binding sites in Arabidopsis class III HD-ZIP mRNAs are required for methylation of the template chromosome. Dev Cell 7:653–662

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116:281–297

Betel D, Wilson M, Gabow A et al (2008) The microRNA.org resource: targets and expression. Nucleic Acids Res 36:D149–D153

Borsani O, Zhu J, Verslues PE et al (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. Cell 123:1279–1291

Chellappan P, Xia J, Zhou X et al (2010) siRNAs from miRNA sites mediate DNA methylation of target genes. Nucleic Acids Res 38:6883–6894

Ci D, Song Y, Tian M et al (2015) Methylation of miRNA genes in the response to temperature stress in Populussimonii. Front Plant Sci 6:921

Colaiacovo M, Bernardo L, Centomani I et al (2012) A survey of MicroRNA length variants contributing to miRNome complexity in peach (PrunusPersica L.) front. Plant Sci 3:165

Cuperus JT, Fahlgren N, Carrington JC (2011) Evolution and functional diversification of MIRNA genes. Plant Cell 23:431–442

Dai X, Zhao PX (2011) psRNATarget: a plant small RNA target analysis server. Nucleic Acids Res 39:W155–W159

de Oliveira LF, Christoff AP, Margis R (2013) isomiRID: a framework to identify microRNA isoforms. Bioinformatics 29:2521–2523

Ding J, Li D, Ohler U et al (2012) Genome-wide search for miRNA-target interactions in Arabidopsis thaliana with an integrated approach. BMC Genomics 13:S3–S3

Emery JF, Floyd SK, Alvarez J et al (2003) Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr Biol 13:1768–1774

Evers M, Huttner M, Dueck A et al (2015) miRA: adaptable novel miRNA identification in plants using small RNA sequencing data. BMC Bioinformatics 16:370

Folkes L, Moxon S, Woolfenden HC et al (2012) PAREsnip: a tool for rapid genome-wide discovery of small RNA/target interactions evidenced through degradome sequencing. Nucleic Acids Res 40:e103–e103

German MA, Pillay M, Jeong D-H et al (2008) Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. Nat Biotech 26:941–946

Grant-Downton RT, Dickinson HG (2005) Epigenetics and its implications for plant biology. 1. The epigenetic network in plants. Ann Bot 96:143–1164

Gregory BD, O'Malley RC, Lister R, Urich MA, Tonti-Filippini J, Chen H, Millar AH, Ecker JR (2008) A link between RNA metabolism and silencing affecting Arabidopsis development. Develop Cell 14:854–866

Griffiths-Jones S (2004) The microRNA registry. Nucleic Acids Res 32:D109–D111

Guo L, Yu J, Liang T et al (2016) miR-isomiRExp: a web-server for the analysis of expression of miRNA at the miRNA/isomiR levels. Scientific Rep 6:23700

Hackenberg M, Rodríguez-Ezpeleta N, Aransay AM (2011) miRanalyzer: an update on the detection and analysis of microRNAs in high-throughput sequencing experiments. Nucleic Acids Res 39:W132–W138

Hackenberg M, Shi B-J, Gustafson P et al (2013) Characterization of phosphorus-regulated miR399 and miR827 and their isomirs in barley under phosphorus-sufficient and phosphorus-deficient conditions. BMC Plant Biol 13:214–214

Hsu PWC, Huang H-D, Hsu S-D et al (2006) miRNAMap: genomic maps of microRNA genes and their target genes in mammalian genomes. Nucleic Acids Res 34:D135–D139

Hu W, Wang T, Xu J et al (2014) MicroRNA mediates DNA methylation of target genes. Biochem Biophys Res Commun 444:676–681

Humphreys DT, Suter CM (2013) miRspring: a compact standalone research tool for analyzing miRNA-seq data. Nucleic Acids Res 41:e147

Hutvágner G, Zamore PD (2002) A microRNA in a multiple-turnover RNAi enzyme complex. Science 297:2056

Jeong DH, Thatcher SR, Brown RS et al (2013) Comprehensive investigation of microRNAs enhanced by analysis of sequence variants, expression patterns, ARGONAUTE loading, and target cleavage. Plant Physiol 162:1225–1245

Jones-Rhoades Matthew W, Bartel David P (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14:787–799

Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57:19–53

Kasschau KD, Fahlgren N, Chapman EJ et al (2007) Genome-wide profiling and analysis of Arabidopsis siRNAs. PLoS Biol 5:e57

Katiyar-Agarwal S, Morgan R, Dahlbeck D et al (2006) A pathogen-inducible endogenous siRNA in plant immunity. Proc Nat Acad Sci U S A 103:18002–18007

Katiyar-Agarwal S, Gao S, Vivian-Smith A et al (2007) A novel class of bacteria-induced small RNAs in Arabidopsis. Genes Dev 21:3123–3134

Khraiwesh B, Arif MA, Seumel GI et al (2010) Transcriptional control of gene expression by microRNAs. Cell 140:111–122

Kim W, Benhamed M, Servet C et al (2009) Histone acetyltransferase GCN5 interferes with the miRNA pathway in Arabidopsis. Cell Res 19:899–909

Kravchik M, Damodharan S, Stav R et al (2014) Generation and characterization of a tomato DCL3-silencing mutant. Plant Sci 221–222:81–89

Langmead B, Trapnell C, Pop M et al (2009) Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 10:R25–R25

Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11:204–220

Lei J, Sun Y (2014) miR-PREFeR: an accurate, fast and easy-to-use plant miRNA prediction tool using small RNA-Seq data. Bioinformatics 30:2837–2839

Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows–Wheeler transform. Bioinformatics 25:1754–1760

Li R, Li Y, Kristiansen K et al (2008) SOAP: short oligonucleotide alignment program. Bioinformatics 24:713–714

Li F, Orban R, Baker B (2012) SoMART: a web server for plant miRNA, tasiRNA and target gene analysis. Plant J 70:891–901

Liu X, Hao L, Li D et al (2015) Long non-coding RNAs and their biological roles in plants. Genomics Proteomics Bioinformatics 13:137–147

Llave C, Xie Z, Kasschau KD et al (2002) Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science 297:2053–2056

Margis R, Fusaro AF, Smith NA et al (2006) The evolution and diversification of Dicers in plants. FEBS Lett 580:2442–2450

McConnell JR, Emery J, Eshed Y et al (2001) Role of PHABULOSA and PHAVOLUTA in determining radial patterning in shoots. Nature 411:709–713

Megraw M, Sethupathy P, Corda B et al (2007) miRGen: a database for the study of animal microRNA genomic organization and function. Nucleic Acids Res 35:D149–D155

Meng Y, Shao C (2012) Large-scale identification of mirtrons in Arabidopsis and rice. PLoS ONE 7:e31163

Mette MF, van der Winden J, Matzke M et al (2002) Short RNAs can identify new candidate transposable element families in Arabidopsis. Plant Physiol 130:6–9

Meyers BC, Axtell MJ, Bartel B et al (2008) Criteria for annotation of plant microRNAs. Plant Cell 20:3186–3190

Mi S, Cai T, Hu Y et al (2008) Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5′ terminal nucleotide. Cell 133:116–127

Molnár A, Schwach F, Studholme DJ et al (2007) miRNAs control gene expression in the single-cell alga Chlamydomonasreinhardtii. Nature 447:1126–1129

Montgomery TA, Howell MD, Cuperus JT et al (2008) Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell 133:128–141

Morin RD, O'Connor MD, Griffith M et al (2008) Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. Genome Res 18:610–621

Muller H, Marzi MJ, Nicassio F (2014) IsomiRage: from functional classification to differential expression of miRNA isoforms. Front Bioeng Biotechnol 2:38

Muñoz-Mérida A, Perkins JR, Viguera E et al (2012) Semirna: searching for plant miRNAs using target sequences. OMICS 16:168–177

Neilsen CT, Goodall GJ, Bracken CP (2012) IsomiRs—the overlooked repertoire in the dynamic microRNAome. Trends Genet 28:544–549

Nosaka M, Itoh J-I, Nagato Y et al (2012) Role of transposon-derived small RNAs in the interplay between genomes and parasitic DNA in rice. PLoS Genet 8:e1002953

Nosaka M, Ono A, Ishiwata A et al (2013) Expression of the rice microRNA miR820 is associated with epigenetic modifications at its own locus. Genes Genet Syst 88:105–112

Okamura K, Phillips MD, Tyler DM et al (2008) The regulatory activity of microRNA* species has substantial influence on microRNA and 3′ UTR evolution. Nat Struct Mol Biol 15:354–363

Pantano L, Estivill X, Martí E (2011) A non-biased framework for the annotation and classification of the non-miRNA small RNA transcriptome. Bioinformatics 27:3202–3203

Park W, Li J, Song R et al (2002) CARPEL FACTORY, a dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol 12:1484–1495

Patra D, Fasold M, Langenberger D et al (2014) plantDARIO: web based quantitative and qualitative analysis of small RNA-seq data in plants. Front Plant Sci 5:708

Prüfer K, Stenzel U, Dannemann M et al (2008) PatMaN: rapid alignment of short sequences to large databases. Bioinformatics 24:1530–1531

Qian K, Auvinen E, Greco D et al (2012) miRSeqNovel: An R based workflow for analyzing miRNA sequencing data. Mol Cell Probes 26:208–2011

Ramachandran V, Chen X (2008) Degradation of microRNAs by a family of exoribonucleases in Arabidopsis. Science 321:490–1492

Reinhart BJ, Weinstein EG, Rhoades MW et al (2002) MicroRNAs in plants. Genes Develop 16:1616–1626

Rhoades MW, Reinhart BJ, Lim LP et al (2002) Prediction of plant microRNA targets. Cell 110:513–520

Rogans SJ, Rey C (2016) Unveiling the micronome of cassava (Manihot esculenta Crantz). PLoS One 11:e0147251

Rueda A, Barturen G, Lebrón R et al (2015) sRNAtoolbox: an integrated collection of small RNA research tools. Nucleic Acids Res 43:W467–W473

Ruiz-Ferrer V, Voinnet O (2009) Roles of plant small RNAs in biotic stress responses. Annu Rev Plant Biol 60:485–510

Sablok G, Milev I, Minkov G et al (2013) isomiRex: web-based identification of microRNAs, isomiR variations and differential expression using next-generation sequencing datasets. FEBS Lett 587:2629–2634

Sablok G, Srivastva AK, Suprasanna P et al (2015) isomiRs: increasing evidences of isomiRs complexity in plant stress functional biology. Front Plant Sci 6:949–949

Salmena L, Poliseno L, Tay Y et al (2011) AceRNA hypothesis: the Rosetta stone of a hidden RNA language? Cell 146:353–358

Song Y, Ci D, Tian M et al (2016) Stable methylation of a non-coding RNA gene regulates gene expression in response to abiotic stress in Populussimonii. J Exp Bot 67:1477–1492

Stocks MB, Moxon S, Mapleson D et al (2012) The UEA sRNA workbench: a suite of tools for analysing and visualizing next generation sequencing microRNA and small RNA datasets. Bioinformatics 28:2059–2061

Sun Z, Evans J, Bhagwate A et al (2014) CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. BMC Genomics 15:423

Szcześniak MW, Deorowicz S, Gapski J et al (2012) miRNEST database: an integrative approach in microRNA search and annotation. Nucleic Acids Res 40:D198–D204

Takeda A, Iwasaki S, Watanabe T et al (2008) The mechanism selecting the guide strand from small RNA duplexes is different among argonaute proteins. Plant Cell Physiol 49:493–500

Tong Y-A, Peng H, Zhan C et al (2013) Genome-wide analysis reveals diversity of rice intronic miRNAs in sequence structure, biogenesis and function. PLoS One 8:63938

Tu B, Liu L, Xu C et al (2015) Distinct and cooperative activities of HESO1 and URT1 nucleotidyl transferases in microRNA turnover in Arabidopsis. PLoS Genet 11:e1005119

Urgese G, Paciello G, Acquaviva A et al (2016) isomiR-SEA: an RNA-Seq analysis tool for miRNAs/isomiRs expression level profiling and miRNA-mRNA interaction sites evaluation. BMC Bioinformatics 17:148

Vaucheret H (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Develop 20:759–771

Vazquez F, Blevins T, Ailhas J et al (2008) Evolution of Arabidopsis MIR genes generates novel microRNA classes. Nucleic Acids Res 36:6429–6438

Wang X, Zhang S, Dou Y et al (2015) Synergistic and independent actions of multiple terminal nucleotidyl transferases in the 3′ tailing of small RNAs in Arabidopsis. PLoS Genet 11: e1005091

Wassenegger M, Heimes S, Riedel L et al (1994) RNA-directed de novo methylation of genomic sequences in plants. Cell 76:567–576

Wei L, Zhang D, Xiang F et al (2009) Differentially expressed miRNAs potentially involved in the regulation of defense mechanism to drought stress in maize seedlings. Int J Plant Sci 170:979–989

Wen M, Shen Y, Shi S et al (2012) miREvo: an integrative microRNA evolutionary analysis platform for next-generation sequencing experiments. BMC Bioinformatics 13:140

Wu L, Zhang Q, Zhou H et al (2009) Rice microRNA effector complexes and targets. Plant Cell 21:3421–3435

Wu L, Zhou H, Zhang Q et al (2010) DNA methylation mediated by a microRNA pathway. Mol Cell 38:465–475

Wu H-J, Ma Y-K, Chen T et al (2012) PsRobot: a web-based plant small RNA meta-analysis toolbox. Nucleic Acids Res 40:W22–W28

Wu J, Liu Q, Wang X et al (2013a) mirTools 2.0 for non-coding RNA discovery, profiling, and functional annotation based on high-throughput sequencing. RNA Biol 10:1087–1092

Wu L, Liu D, Wu J et al (2013b) Regulation of FLOWERING LOCUS T by a microRNA in Brachypodium distachyon. Plant Cell 25:4363–4377

Xie Z, Johansen LK, Gustafson AM et al (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol 2:e104

Xie F, Xiao P, Chen D et al (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80:75–84

Xu X, Bai H, Liu C, Chen E, Chen Q, Zhuang J et al (2014) Genome-wide analysis of microRNAs and their target genes related to leaf senescence of rice. PLoS One 9(12):e114313

Yang X, Li L (2011) miRDeep-P: a computational tool for analyzing the microRNA transcriptome in plants. Bioinformatics 27:2614–2615

Yang J-H, Qu L-H (2012) Deepbase: annotation and discovery of microRNAs and other noncoding RNAs from deep-sequencing data. In: Fan J-B (ed) Next-generation microRNA expression profiling technology: methods and protocols. Humana Press, Totowa, NJ, pp 233–248

Yu B, Yang Z, Li J et al (2005) Methylation as a crucial step in plant microRNA biogenesis. Science 307:932

Zhang Z, Yu J, Li D et al (2010) PMRD: plant microRNA database. Nucleic Acids Res 38:D806–D813

Zhang X, Xia J, Lii YE et al (2012) Genome-wide analysis of plant nat-siRNAs reveals insights into their distribution, biogenesis and function. Genome Biol 13:R20

Zhang Y, Zang Q, Zhang H et al (2016) DeAnnIso: a tool for online detection and annotation of isomiRs from small RNA sequencing data. Nucleic Acids Res 44:W166–W175

Zheng X, Zhu J, Kapoor A et al (2007) Role of Arabidopsis AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. EMBO J 26:1691–1701

Zheng Y, Li Y-F, Sunkar R et al (2012) SeqTar: an effective method for identifying microRNA guided cleavage sites from degradome of polyadenylated transcripts in plants. Nucleic Acids Res 40:e28

Zhu Q-H, Spriggs A, Matthew L et al (2008) A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. Genome Res 18:1456–1465

Zilberman D, Cao X, Jacobsen SE (2003) Control of locus-specific siRNA accumulation and DNA and histone methylation. Science 299:716