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MicroRNAs and their diverse functions in plants

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Abstract microRNAs (miRNAs) are an extensive class of newly identified small RNAs, which regulate gene expression at the post-transcriptional level by mRNA cleavage or translation inhibition. Currently, there are 3,070 miRNAs deposited in the public available miRNA database; these miRNAs were obtained from 43 plant species using both computational (comparative genomics) and experimental (direct cloning and deep sequencing) approaches. Like other signaling molecules, plant miRNAs can also be moved from one tissue to another through the vascular system. These mobile miRNAs may play an important role in plant nutrient homeostasis and response to environmental biotic and abiotic stresses. In addition, miRNAs also control a wide range of biological and metabolic processes, including developmental timing, tissue-specific development, and stem cell maintenance and differentiation. Currently, a majority of plant miRNA-related researches are purely descriptive, and provide no further detailed mechanistic insight into miRNA-mediated gene regulation and other functions. To better understand the function and regulatory mechanisms of plant miRNAs, more strategies need to be employed to investigate the functions of miRNAs and their associated signaling pathways and gene networks. Elucidating the evolutionary mechanism of miRNAs is also important. It is possible to develop a novel miRNA-based biotechnology for improving plant yield, quality and tolerance to environmental biotic and abiotic stresses besides focusing on basic genetic studies.

Keywords microRNAs · Gene regulation · Identification · Evolution · Plants · Functions · Stresses

Introduction

Temporal and spatial expression of the whole set of genes in cells allows normal plant growth and development. However, it was unclear for some time how cells control the expression of genes in different tissues at different times. For the past several decades, scientists have been trying to elucidate the regulatory mechanisms of gene expression, with one of the most distinguishable discoveries being micorRNAs (miRNAs).

miRNAs are a newly identified and extensive class of endogenous non-coding small RNAs, which negatively regulate gene expression at the post-transcriptional level (Bartel 2007; Filipowicz et al. 2008; He and Hannon 2004). Although the diverse fundamental functions of miRNAs have now been well demonstrated in both plants and animals over the past several years, little attention has been paid to this class of small RNAs for about a decade after the first miRNAs were identified in the soil nematode *Caenorhabditis elegans* in 1993 (Lee et al. 1993). Currently, it is well known that miRNAs are widely present in plants, animals and some viruses (He et al. 2008; Pfeffer et al. 2004; Siomi and Siomi 2010), and miRNAs are involved in regulating almost all biological and metabolic processes, such as stem cell maintenance and differentiation, organ development, signaling pathways, disease, and response to environmental stress (Bushati and Cohen 2007; Jones-Rhoades et al. 2006; Leung and Sharp 2010;

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Willmann and Poethig 2007; Zhang et al. 2007). Investigations suggest that miRNAs control the gene expression of at least 30% of the protein-coding genes in human beings (Lewis et al. 2005). Although they have only been functionally discovered for a decade, miRNAs have become known as one of the most important gene regulators. In this review, we will first present the biogenesis of miRNAs in plants, followed by the current status of miRNA identification and classification in the plant kingdom. We will then focus on the diverse functions of miRNAs in plant growth, development and response to environmental stress. Finally, we will point out the future direction of this field and the potential application of miRNAs in plant genetic studies and crop improvement.

Biogenesis of plant miRNAs

miRNAs are coded by miRNA genes. miRNAs are very small and only have 16–29 nucleotides with an average of 22 nucleotides in length. Although a majority of miRNAs are 21–23 nucleotides in length (Bartel 2007; Zhang et al. 2007), miRNA genes are usually very long and it is not certain how long miRNA genes are. The majority of plant miRNA genesare predominantly located at intergenic regions; however, animal miRNA genes can be located anywhere in the genome, including coding sequences (Millar and Waterhouse 2005; Zhang et al. 2007). Similar to protein coding genes, miRNA genes are also transcribed by RNA polymerase II (RNA Pol II) (Jones-Rhoades et al. 2006; Lee et al. 2004); however, some miRNAs can be transcribed by RNA Pol III (Faller and Guo 2008). The initial miRNA transcripts are called primary miRNAs (pri-miRNAs). RNA Pol II generates capped and polyadenylated pri-miRNAs in both plants and animals (Lee et al. 2004).

The process of generating functionally mature miRNAs from pri-miRNAs is a complicated process because several steps are involved as well as several enzymes being associated with the steps of biogenesis. miRNA biogenesis has been well studied in animals (Kim et al. 2009; Siomi and Siomi 2010). In comparison with animal miRNAs, it is similar for plants to produce mature miRNAs from pri-miRNAs although no evidence shows that animal and plant miRNAs have a common ancestor (Zhang et al. 2007). Currently, a majority of the homologs of genes coding for enzymes involved in animal miRNA biogenesis have been found in plants except for Drosha (a RNase III endonuclease) (Bartel 2007; Jung et al. 2009; Voinnet 2009).

After pri-miRNAs are transcribed, the RNA-binding protein DAWDLE (DDL) binds to the pri-miRNAs and may stabilize them from degradation as well enhance the conversion from pri-miRNAs to miRNA precursors (pre-miRNAs) and further to mature miRNAs. Thedicer-like 1 (DCL1) enzyme cuts off the imperfectly-folded ends of pri-miRNAs to generate pre-miRNAs with stem-loop hairpin secondary structures (Kurihara et al. 2006; Kurihara and Watanabe 2004). This process is ongoing in a nuclear processing center called the D-body or SmD3/SmB-body, which requires the concerted action and physical interactions of several enzymes and/or proteins, including the double-stranded RNA-binding protein HYPONASTIC LEAVES1 (HYL1), the C2H2-zinc finger protein SERRATE (SE), the nuclear cap-binding complex (CBC), and DCL1 (Voinnet 2009; Xie 2010; Zhu 2008). Lost-of-function of DCL1 and SE are usually embryonic-lethal and cause plant death at an early stage because of no mature miRNA accumulation in plant cells (Lobbes et al. 2006; Xie et al. 2003). The *ddl* and *cbc* mutants also cause low miRNA accumulation (Kim et al. 2008). However, some evidence suggests that the DDL protein may have other functions than just miRNA biogenesis because the *ddl* mutant caused stronger abnormalities in plant development than DCL1 caused (Yu et al. 2008).

Although it is not clear how long a pri-miRNA is, almost all pri-miRNAs can fold into a stem-loop hairpin secondary structure (Meyers et al. 2008), and then are processed into pre-miRNAs by DCL1 in plants (Chen 2005). Having a stem-loop structure is one important characteristic for miRNAs, however many other RNAs can also be formed into a similar hairpin structure (Zhang et al. 2006). Pre-miRNAs are further cleaved by DCL1 and HYL1 into a miRNA:miRNA* duplex in nucleus (Kurihara et al. 2006; Song et al. 2007). Newly processed miRNA:miRNA* duplexes have 2 nucleotides overhanging at the 3′ end of each strand and are easily degraded by a class of exonucleases called SMALL RNA DEGRADING NUCLEASE (SDN) (Ramachandran and Chen 2008). To stabilize the miRNA:miRNA* duplexes, a small RNA methyl transferase, called Hua Enhancer 1 (HEN1) immediately methylates the 3′ terminal nucleotides of each strand of the duplexes to prevent their uridylation and subsequent degradation (Yu et al. 2005). In *hen1* mutants, miRNAs fail to accumulate or only accumulate at a lower level, which suggests the role of HEN1 in protection of miRNAs from degradation (Li et al. 2005).

After it is released from the pre-miRNA, the miRNA/miRNA* duplex is transported from nucleus into cytoplasm by HASTY through the nuclear pore in an ATP-dependent manner (Park et al. 2005). HASTY is the plant homolog of exportin-5 that transports pre-miRNAs from nucleus into cytoplasm in animals (Murchison and Hannon 2004). However, the exact functions of HASTY are not fully understood. The *hasty* mutants only show decreased accumulation of partial miRNAs in cytoplasm, which suggests that miRNAs may be also transported by other

mechanisms (Voinnet 2009). It is also unclear what forms of miRNAs are transported, as both forms of miRNA/miRNA* duplexes or miRNA-RISC duplexes are possible subjects of transportation.

In the cytoplasm, the miRNA/miRNA* duplex is separated; the miRNA strand is incorporated into the RNA-induced silencing complex (RISC) and forms a miR-RISC complex (Chen 2005), in which ARGONAUTE 1 (AGO1) cleaves the target mRNA in the middle of the mRNA-miRNA duplex. On the contrary, the miRNA* sequence is degraded by an unknown mechanism. However, some miRNA* may also function as regular miRNA sequences to target the expression of specific genes (Guo and Lu 2010). Unlike miRNAs in animals, the majority of plant miRNAs cleave their target mRNAs instead of inhibiting protein translation (Zhang et al. 2007).

miRNAs in plants

The discovery of extensive miRNAs in animals prompted scientists to search for miRNAs in plants. In 2002, several research groups independently identified more than 100 miRNAs in *Arabidopsis* by using direct cloning technology (Llave et al. 2002; Reinhart et al. 2002); some of those miRNAs are highly conserved in plants and also can be found in rice, corn and tobacco (Jones-Rhoades and Bartel 2004; Sunkar et al. 2005; Zhang et al. 2005). This suggests that miRNAs also exist widely in plant systems. Since the first plant miRNAs were discovered in *Arabidopsis*, identification and functional analysis of plant miRNAs has become one of the hottest research fields in plant biology. As a result, in the past several years, great progress has been made in this field. According to the publicly available miRNA database, miRBase, currently 3,070 plant miRNAs have been identified and deposited in the database (Release 16 September 2010) (Griffiths-Jones et al. 2008). The majority of these miRNAs have been validated using different experimental approaches, including deep sequencing, northern blotting, and/or quantitative real time PCR (qRT-PCR). These 3,070 miRNAs were obtained from 43 plant species, which include 10 moncots (monocotyledons), 28 dicots (dicotyledons), 2 species from conifers (coniferopsida), 2 species from moss (bryophyte) and one species from green algae (chlorophyta) (Table 1). Of them, 1,769 miRNAs were obtained from dicots, 887 from monocots and 414 from lower plant species. Currently, there are ten plant species with more than 100 identified miRNAs, which are rice (*Oryza sativa*, 462), barrel (*Medicago truncatula*, 375), black cottonwood (*Populus trichocarpa*, 234), moss (*Physcomitrella patens*, 229), *Arabidopsis thaliana* (213), soybean (*Glycine max*, 203), *Arabidopsis lyrata* (201), maize (*Zea mays*, 170), wine grape (*Vitis vinifera*, 163), and sorghum (*Sorghum bicolor*, 148). A total of 2,398 (78%) plant miRNAs were obtained from these 10 plant species, suggesting that plant miRNA research is still limited to a few plant species with a majority of them being model species. Among these species, the largest number of miRNAs, 462, was identified in rice; however, there are only 213 were identified from *Arabidopsis* with the reason for the difference in number being unclear.

Methods for identifying plant miRNAs

Both experimental methods and computational approaches have been adopted to identify miRNAs in plants. First, scientists adopted a direct cloning approach, which is widely used to discover animal miRNAs, and is also used to discover miRNAs in different plant species (Llave et al. 2002; Reinhart et al. 2002). Then, as more was understood about plant miRNAs, particularly on their conservation and secondary structure, computational approaches were developed to identify more miRNAs from a wider range of plant species (Jones-Rhoades and Bartel 2004; Zhang et al. 2005). Recently, as deep sequencing technologies have become more available and affordable (Lu et al. 2006), more and more studies have been employing deep sequencing to discover and functionally analyze miRNAs in plants (Creighton et al. 2009; Fahlgren et al. 2007; Sunkar et al. 2005). All of these three approaches have been widely used to identify miRNAs in plants. Table 2 summarizes the major advantages and disadvantages of these three methods.

Evidences show that many miRNAs are highly evolutionarily conserved in plants from moss to land plants (Arazi et al. 2005; Floyd and Bowman 2004). This provides a powerful approach to identify miRNAs in a new plant species through identifying homologues through a Blastn search using known miRNAs in other plant species. However, sequence similarity can not guarantee that the obtained sequences are real miRNAs due to the fact that miRNAs are only about 20–22 nt in length and the high identity percentage in pairwise alignment may be caused by a stochastic match rather than by sequence homology. To better identify miRNAs and reduce the false positives, homolog searches always need to be combined with the major characteristics of miRNAs, particularly the stem-loop hairpin structure. Based on the conservation and features of miRNAs, Zhang et al. (2005) developed an EST analysis to identify miRNAs in a wide range of plant species (Zhang et al. 2005). By using this approach, they identified a total of 481 miRNAs, belonging to 37 miRNA families in 71 different plant species from more than 6 million EST sequences in plants. They also extended the EST analysis to other genomic sequences, including the

Table 1 Currently available miRNAs and their distribution in the publicly available miRNA database, miRBase

Species	Phylum	Class	Order	Family	Number of miRNAs
Arabidopsis lyrata	Streptophyta	Dicotyledons	Brassicales	Brassicaceae	128
Arabidopsis thaliana	Streptophyta	Dicotyledons	Brassicales	Brassicaceae	144
Brassica napus	Streptophyta	Dicotyledons	Brassicales	Brassicaceae	15
Brassica oleracea	Streptophyta	Dicotyledons	Brassicales	Brassicaceae	4
Brassica rapa	Streptophyta	Dicotyledons	Brassicales	Brassicaceae	9
Carica papaya	Streptophyta	Dicotyledons	Brassicales	Caricaceae	1
Glycine max	Streptophyta	Dicotyledons	Fabales	Fabaceae	151
Glycine soja	Streptophyta	Dicotyledons	Fabales	Fabaceae	9
Arachis hypogaea	Streptophyta	Dicotyledons	Fabales	Fabaceae	29
Lotus japonicus	Streptophyta	Dicotyledons	Fabales	Fabaceae	3
Medicago truncatula	Streptophyta	Dicotyledons	Fabales	Fabaceae	19
Phaseolus vulgaris	Streptophyta	Dicotyledons	Fabales	Fabaceae	5
Vigna unguiculata	Streptophyta	Dicotyledons	Fabales	Fabaceae	1
Populus euphratica	Streptophyta	Dicotyledons	Malpighiales	Salicaceae	4
Populus trichocarpa	Streptophyta	Dicotyledons	Malpighiales	Salicaceae	38
Ricinus communis	Streptophyta	Dicotyledons	Malpighiales	Euphorbiaceae	19
Gossypium arboreum	Streptophyta	Dicotyledons	Malvales	Malvaceae	1
Gossypium herbecium	Streptophyta	Dicotyledons	Malvales	Malvaceae	2
Gossypium hirsutum	Streptophyta	Dicotyledons	Malvales	Malvaceae	18
Gossypium raimondii	Streptophyta	Dicotyledons	Malvales	Malvaceae	2
Aquilegia coerulea	Streptophyta	Dicotyledons	Ranunculales	Ranunculaceae	17
Malus domestica	Streptophyta	Dicotyledons	Rosales	Rosaceae	1
Citrus clementina	Streptophyta	Dicotyledons	Sapindales	Rutaceae	4
Citrus reticulata	Streptophyta	Dicotyledons	Sapindales	Rutaceae	3
Citrus sinensis	Streptophyta	Dicotyledons	Sapindales	Rutaceae	37
Citrus trifoliata	Streptophyta	Dicotyledons	Sapindales	Rutaceae	5
Solanum lycopersicum	Streptophyta	Dicotyledons	Solanales	Solanaceae	16
Vitis vinifera	Streptophyta	Dicotyledons	Vitales	Vitaceae	47
Aegilops tauschii	Streptophyta	Monocotyledons	Poales	Poaceae	3
Brachypodium distachyon	Streptophyta	Monocotyledons	Poales	Poaceae	16
Festuca arundinacea	Streptophyta	Monocotyledons	Poales	Poaceae	14
Hordeum vulgare	Streptophyta	Monocotyledons	Poales	Poaceae	13
Oryza sativa	Streptophyta	Monocotyledons	Poales	Poaceae	188
Saccharum officinarum	Streptophyta	Monocotyledons	Poales	Poaceae	6
Sorghum bicolor	Streptophyta	Monocotyledons	Poales	Poaceae	26
Triticum aestivum	Streptophyta	Monocotyledons	Poales	Poaceae	33
Triticum turgidum	Streptophyta	Monocotyledons	Poales	Poaceae	1
Zea mays	Streptophyta	Monocotyledons	Poales	Poaceae	25
Picea abies	Streptophyta	Coniferopsida	Coniferales	Pinaceae	34
Pinus taeda	Streptophyta	Coniferopsida	Coniferales	Pinaceae	25
Physcomitrella patens	Streptophyta	Bryopsida	Funariales	Funariaceae	142
Selaginella moellendorffii	Streptophyta	Isoetopsida	Selaginellales	Selaginellaceae	50
Chlamydomonas reinhardtii	Chlorophyta	Chlorophyceae	Chlamydomonadales	Chlamydomonadaceae	71

genome survey sequence database (GSS) (Pan et al. 2007). Currently, this approach has been widely used by different laboratories, and many miRNAs have been identified from more plant species, including some important crops, such as cotton, corn, wheat, oilseed, soybean, tobacco, switchgrass and apple (Dezulian et al. 2006; Frazier et al. 2010; Matts et al. 2010; Xie et al. 2007; Yu et al. 2011; Zhang et al. 2007, 2008).

Table 2 Comparison of three major approaches for identifying plant miRNAs

	Direct cloning	Deep sequencing	EST/GSS analysis
Cost	Moderate	High	Low
Efficiency	Low	High	Moderate
Time required	More	More	Less
Technology required	Moderate	High	Low
False positive possibility	Low	Low	High
Need experimental confirmation	No	No	Somewhat
Possibility for new miRNAs	Yes	Yes	No
Comprehensive	No	Yes	Yes
miRNA quantitative information	No	Yes	Somewhat
Easy adoption	Moderate	No	Yes
Need genome sequence	Yes	Yes	No

Although EST/GSS analysis can be used to identify miRNAs from different species, this approach only can be used to identify conserved miRNAs and it is impossible to identify new miRNAs, particularly species-specific miRNAs. Direct cloning was first used to clone and identify miRNAs in animals. In this method, scientists first isolated small RNAs from a total RNA population and then made a small RNA cDNA library. After sequencing the small RNAs, the small RNA sequences would be matched to genome sequences for their potential precursors. If the potential precursor could be folded into a stem-loop hairpin secondary structure and also met other criteria, the small RNAs were annotated as miRNAs (Billoud et al. 2005; Llave et al. 2002; Reinhart et al. 2002). At first, all plant miRNAs were identified by direct cloning. However, it is time consuming, lab extensive, and cost inefficient to discover miRNAs using this method. Currently, direct cloning has been quickly replaced by deep sequencing technology for identifying miRNAs.

Deep sequencing technology is a powerful technology for identifying and analyzing the expression of miRNAs. Since this technology was developed, it was employed to the field of miRNAs (Lu et al. 2006). Deep sequencing of miRNAs has more advantages than sequencing protein-coding RNAs because miRNAs are short sequences. Both 454 and Illumina platforms have been employed to identify miRNAs and it seems that the Illumina technology is better in sequencing small RNAs. During deep sequencing, several million reads will be produced for each sample and it is possible to identify all potential conserved and species-specific miRNAs, which are usually unsaturated by direct cloning. Currently, by using deep sequencing technology, thousands of miRNAs have been identified from many plant species, including wheat, cotton, *Arabidopsis*, and rice (Fahlgren et al. 2007; Pang et al. 2009; Sunkar et al. 2008; Wei et al. 2009).

Origin and evaluation of miRNAs in plants

Several investigations indicate that many miRNA families are evolutionarily conserved across all major lineages of plants, including mosses, gymnosperms, monocots and eudicots (Arazi et al. 2005; Floyd and Bowman 2004). This suggests that regulation of gene expression by miRNAs appears to have existed at the earliest stages of plant evolution and has been tightly constrained (functionally) for more than 425 million years.

The origin of miRNAs in plants includes two aspects, the origin of the first miRNA(s) in plants and the origin of newly evolved miRNAs. Answers in regards to the origin of the first miRNA(s) in plants were always associated with the phylogenetic distribution of miRNAs in other eukaryotic lineages. Currently, miRNAs are only validated in plants and animals. Although some protists, including the social amoeba (*Dictyostelium discoideum*), trichomonads (*Trichomonas vaginalis*), and diplomonads (*Giardia intestinalis*) were reported to possess miRNAs (Hinas et al. 2007; Lin et al. 2009; Zhang et al. 2009), the evidence for this opinion only comes from computational analyses and the expression of predicted genes. These methods can only document that they are small RNAs and cannot provide convincing enough evidence to distinguish whether they are indeed bona fide miRNAs or siRNAs. In addition, the origin of miRNAs does not only focus on miRNA genes but it is also about how the whole miRNA processing system and gene regulation network originate. The multiple step processing during miRNA biogenesis, the complexity of miRNA-directed gene regulation networks, and the conservation of important components including Dicer, DCL, and AGO (Carthew and Sontheimer 2009; Liu et al. 2009; Mallory and Vaucheret 2010) make it hard to believe that they all originated more than once in the evolutionary history (Axtell and Bowman 2008). One of the key points

in the origin of miRNAs is whether animals and plants once shared a common set of miRNA genes. The current limited phylogenetic distribution of miRNAs and no observed sequence similarity between plant and animal miRNAs supports the independent origin hypothesis, which postulates that the miRNA(s) in plants and animals have independently originated from an ancient sRNA processing pathway (Axtell and Bowman 2008). However, low conservation of miRNAs fosters the common origin hypothesis, which postulates that plant miRNAs have a common origin with animal miRNAs and the extensive diversification of miRNAs lead to no significant similarity between plants and animals, which also cause the high divergence between green algae and land plants. Despite the fact that the common origin hypothesis requires gene loss in fungi and makes it less parsimonious, the highly dynamic nature of newly evolved miRNA genes in plants (see below) make it more plausible than the independent origin hypothesis. The independent origin hypothesis also implies that the miRNAs in protists (if present) would show great divergence with current known miRNAs and as a result, computational approaches may not be suited for identifying miRNAs in these early divergent species.

Despite no convincing data to illustrate the origin of the first miRNAs in plants, lines of evidence have been discovered for the origin of newly evolved miRNAs in plants. Currently, three different models have been proposed for the origin of new miRNAs: inverted gene duplication of target genes, spontaneous evolution, and derivation from transposable elements (Voinnet 2009). These three models differ in the sources of stem-loop structures of pre-miRNA precursors, which can reflected in their names, and are consistent with the occurrence of gene drift or fragment loss outside the mature miRNA regions, selection in the mature miRNA regions, and co-evolution of miRNAs with their original or new targets in the later time (Tang 2010; Voinnet 2009). Among these three models, except for the spontaneous evolution model (De Felippes et al. 2008), the other two models have solid evidence to support their hypotheses (Allen et al. 2004; Piriyapongsa and Jordan 2008). This indicates that different miRNAs have their own mode of origin. Investigations on two *Arabidopsis* genomes revealed that 32 of a total of 107 miRNA families show similarities with their target genes in the extension foldback regions, which indicates most miRNAs stem from the model of inverted gene duplication of target genes (Fahlgren et al. 2010). In addition, studies on *Arabidopsis* also revealed that non-conserved miRNAs have a relative higher divergent rate than the conserved miRNAs and most of them probably target other proteins rather than their original ones (Ma et al. 2010). The highly dynamic nature of miRNAs may explain the great diversity of miRNAs in plants.

Although the green alga *C. reinhardtii* also has multiple miRNAs and its miRNA silencing systems may have evolved from the common ancestor with land plants, no direct sequence similarity is detected among the miRNA genes of green algae and land plants (Molnar et al. 2007; Zhao et al. 2007). Two categories of miRNAs, conserved and non-conserved or less conserved miRNAs, are present in land plants. Conserved miRNAs are relatively distributed widely throughout different land plant lineages, are highly expressed, and often have multiple loci acquired by extensive genome duplication. Otherwise, non-conserved miRNAs are usually either species-specific or are distributed within lower taxonomic groups, are lowly expressed, and only single copies are present (Voinnet 2009). Currently, only a few miRNA families were found to belong to the conserved miRNA category in miRBase. These families consisted of 12 deeply conserved through moss to angiosperms, 21 moderately conserved in seed plants, 38 conserved in angiosperms (Tables 3, 4; Fig. 1). Whole genome analysis of miRNAs in two closely related species, *A. thaliana* and *A. lyrata*, revealed that about 80% of miRNA families are conserved between them and 13% of miRNA genes are species-specific (Fahlgren et al. 2010). Given the divergence time of *A. thaliana* and *A. lyrata*, it is estimated that 1.2–3.3 miRNAs arose every million years (Fahlgren et al. 2010).

Identification of miRNA targets in plants

miRNAs function indirectly by targeting protein-coding genes. Many investigations have demonstrated that miRNAs inhibit gene expression by binding to mRNAs. When a miRNA perfectly or near-perfectly binds to mRNAs, miRNAs mediate the cleavage of mRNAs and therefore repress gene expression, while a miRNA that imperfectly binds to a mRNA inhibits translation. In plants, a majority of miRNAs perfectly or near-perfectly bind to their targeted mRNAs, thus, mRNA cleavage is regarded as the major mechanism of miRNA-mediated gene regulation in plants (Rhoades et al. 2002; Schwab et al. 2005).

Identification of miRNA targets is the first critical step in investigating miRNA functions. Because of the unique interactions between miRNAs and their targets, Blastn searching has been widely used to predict miRNA targets, followed by RACE-PCR which is employed to find the cleavage site for validating the predicted targets. However, Blastn searching needs to be modified in order to predict miRNA targets because the miRNA sequences are so small and traditional Blastn searching is developed for use in long sequence alignment. Based on this principle, computational tools may need to be designed to efficiently predict miRNA targets.

Table 3 Distribution of 38 conserved miRNAs in plants

miRNA family	Number of members[a]	Number of species[b]	Dicots[c]	Monocots[c]	Conifers[c]	Mosses[c]
miR156	162	29	17	8	2	2
miR170	122	26	16	7	1	2
miR165	143	25	15	6	2	2
miR159	110	25	15	7	1	2
miR396	60	21	13	5	2	1
miR160	70	22	12	7	1	2
miR390	29	15	10	3	1	1
miR395	121	15	9	4	1	1
miR408	23	17	8	6	1	2
miR167	86	25	18	6		1
miR530	16	5	3	1		1
miR414	3	3	1	1		1
miR419	3	3	1	1		1
miR447	25	5	4			1
miR1028	3	3	2			1
miR1061	2	2	1			1
miR472	56	17	13	2	2	
miR398	33	18	13	4	1	
miR397	21	14	8	5	1	
miR845	13	5	3	1	1	
miR783	2	2	1		1	
miR169	245	22	14	8		
miR172	61	18	13	5		
miR164	52	17	12	5		
miR169	189	19	12	7		
miR162	19	14	11	3		
miR168	22	16	11	5		
miR393	24	13	10	3		
miR394	18	10	7	3		
miR827	11	7	5	2		
miR413	3	3	2	1		
miR415	2	2	1	1		
miR416	2	2	1	1		
miR417	2	2	1	1		
miR418	2	2	1	1		
miR420	2	2	1	1		
miR426	2	2	1	1		
miR529	4	4	1	3		

[a] This number includes all identified microRNAs in the 43 species

[b] This number includes all species that contain the special miRNA family

[c] This number includes the species number in each group

Although many computational tools have been developed for predicting animal miRNA targets, few tools are available for identifying plant miRNA targets (Zhang et al. 2006). There are currently four tools, miRU (Zhang 2005), Helper tools (Moxon et al. 2008), TAPIR (Bonnet et al. 2010), and Target-align (Xie and Zhang 2010), available for predicting miRNA targets in plants. All four tools predict plant miRNA targets based on very strictly limited criteria, which include the allowed number and location of mismatches between miRNAs and their predicted mRNAs. Compared with the other three tools, Target-align is user-friendly and allows users to set a variety of parameters within their search including the alignment score setting, maximum score, number of consecutive mismatches, base site restrictions, number of G:U wobbles, and number of gaps (Xie and Zhang 2010).

Computational prediction of miRNA targets has provided some clues on miRNA targets and also plays a role in elucidating miRNA function. However, insights into the intrinsic function of miRNAs are taken based on the

Table 4 Group- or species-specific microRNAs in plants

Groups	Number of group-specific microRNAs	Number of species-specific microRNAs	Total
Dicots/ dicotyledons	56	334	390
Monocots/ monocotyledons	18	156	174
Conifers/ coniferopsida	4	33	37
Mosses/ bryophyta	1	168	169
Green algae/ chlorophyta	0	71	71

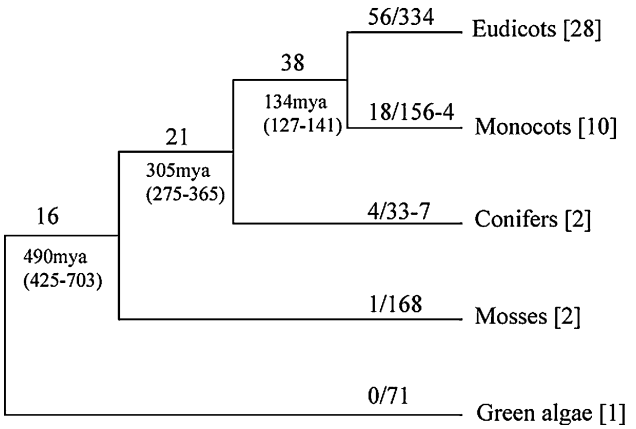

Fig. 1 Conserved and group or species-specific microRNA families in plants. The divergent order of major plant groups is shown by a schematic tree. The involved species number in each group is displayed within the *brackets* after the group names. The *numbers below the branches* indicate the most reliable estimate of the divergent time of each group in the geological time scale (million years ago, mya), and the *following numbers within the parentheses* indicate the estimate range published for each node. The *numbers above each terminal branch* indicate the putative group-specific (*before the slash*), species-specific (*after the slash*), and lost (if it has, *after dash*) microRNA family numbers. The *numbers above the internal branches* indicate the conserved microRNA family number

experimentally validated targets. All computational prediction needs experimental validation and is confined by limited available genome sequences. In plants, the majority of miRNA targets were validated using the cleavage character of miRNAs to their targets. Four methods were used to detect such cleaved products: modified 5′-rapid amplification of cDNA Ends (5′-RACE), miRNA-resistant targets, Agro infiltration, and in vitro cleavage. To date, the most widely used method for validation of miRNA targets is to determine the miRNA cleavage site by 5′RACE. This method shows the 5′-position of the target mRNAs, which generally occurs opposite and between the 10th and 11th position of the corresponding miRNA (Elbashir et al. 2001; German et al. 2009). However, all of these methods, including RACE, have shortages in that the potential targets must be validated one by one. To overcome this pitfall, currently the 5′RACE method has been expended to high-throughput sequencing technologies for both miRNA target identification and validation (Addo-Quaye et al. 2008; German et al. 2008; Gregory et al. 2008). Of them, degradome sequencing, or Parallel Analysis of RNA Ends (PARE) sequencing technology, is a direct approach for combining miRNA target identification and confirmation (Addo-Quaye et al. 2008; German et al. 2008). Using PARE sequencing technology, many undiscovered targets have been identified in some model plant species over the past several years, which include *A. thaliana* (Addo-Quaye et al. 2008; German et al. 2008), *Glycine max* (Song et al. 2011), *Vitis vinifera* (Pantaleo et al. 2010), *Oryza sativa* (Zhou et al. 2010), *Physcomitrella patens* (Addo-Quaye et al. 2009). Table 5 and Supplemental Table 1 summarize the major validated targets with known funcitons in plants using this method and others. Based on the summarzied targets, we found that more than 80% of the targets in these five species could be detected by the PARE method.The remaining targets may be expressed in a special developmental stage or stressed environment. Investigation of these miRNA-target pairs would help to elucidate what kind of functions miRNAs have in plants.

Diverse functions of miRNAs in plants

Although plant miRNAs have only been discovered in the last decade, miRNA-related research has become one of the hottest research fields in plant sciences and miRNA technology has become the most quickly developed technology in plant biology history. Currently, more and more investigations have demonstrated that miRNAs play critical functions in almost all biological and metabolic processes in plants.

miRNAs control cell fate and plant morphology

The determination of a cell to a particular fate is critical for plant development. Totipotency of cells provides the power for any single cell the potential to differentiate into any type of cell. However, a cell always follows the prescribed order to differentiate into a specific cell and tissue. This process is controlled by a complicated mechanism that is quite unclear. Recent studies show that miRNAs may play an important role in cell fate and further control plant development and morphology. Carlsbecker et al. (2010) demonstrated that miR165 could serve as a cell signaling molecule and it guided cells in the exchange of positional information for proper patterning during root development,

Table 5 Validated miRNA targets with known functions in plants

miRNA family	Target family[a]	Functions	Methods[b]	Species[c]
miR156/ miR157	Squamosa-promoter binding protein-like*	Plant development	1, 5	Ath, Gma, Osa, Rco, Mes, Pvu, Ptr, Sly, Pta, Ppa, Vvi
miR159	MYB transcription factor*	Signaling pathway and development	1, 2, 3, 5	Ath, Osa, Gma,Vvi, Pta, Ppa
	1-aminocyclopropane-1-carboxylate synthase	Propanoate metabolism	1	Ath
	Melanocyte-specific gene related gene*	Regulation of gene expression	1	Ath
	Oligopeptide transport gene	Oligopeptide transporter	1	Ath
	Saur family protein	Auxin response	5	Vvi
miR319	TCP transcription factor	Leaf morphogenesis, cell differentiation, embryonic development	1, 2, 5	Ath, Osa, Vvi, Gma, Pvu
	Cyclin	Cell division	1	Ppa
	Transposon protein	Transposon	5	Osa
miR160	Auxin response factor*	Signaling pathway, flower development	1, 4, 3, 2, 5	Ath, Osa, Gma, Rco, Mes, Sly, Pta, Mtr
	Aux/IAA	Biotic stress	1	Pta
miR161	Pentatricopeptide repeat protein	RNA editing	1, 5	Ath
	UDP-D-glucuronate 4-epimerase	Pectin synthesis	1	Ath
miR162	Dicer-like	Flower development, virus induced gene silencing, production of lsiRNA/ta-siRNAs	1, 5	Ath, Osa, Mtr
miR163	S-adenosylmethionine-dependent methyltransferase		1, 5	Ath
miR164	NAC domain transcription factor*	Signaling pathway, root development, response to oxidative stress	1, 4, 3, 2, 5	Ath, Osa, Gma, Rco, Sly, Mes, Pvu, Mtr, Vvi
	Phytosulfokines	Signaling pathway	5	Osa
miR165/ miR166	HD-ZIPIII transcription factor*	Leaf and vascular development	1, 4, 2, 5	Ath, Osa, Gma, Sly, Cri, Tgl, Ppa, Skr, Vvi
	Transporter	Transport	5	Osa
miR167	Auxin response factor*	Signaling pathway, flower development	1, 5	Ath, Osa, Gma, Sly, Mtr, Vvi
miR168	Argonaute	Signal transduction, development, stress response	1, 2, 5	Ath, Osa, Gma, Mtr
miR169	CCAAT-binding transcription factor*	Flowering timing	1, 5	Ath, Osa, Gma
	Chloroplast photosystem ii subunit x	Photosynthesis	5	Vvi
miR170/ miR171	Scarecrow-like transcription factor*	Shoot branching	1, 5	Ath, Osa, Pvu, Sly, Ppa, Mtr, Vvi
	Nodulation signaling pathway 2 protein	Signaling pathway	1, 3, 5	Osa
miR172	Apetala2-like transcription factor*	Signaling pathway, development, stress response	1, 2, 5	Ath, Osa, Gma, Pvu, Sly, Mtr, Vvi
	Auxin response factor*	Signaling pathway, flower development	5	Osa
	Helix-loop-helix DNA-binding domain containing protein*	BR regulation of plant development	5	Osa
miR173	Cylicin		5	Ath
	tasiRNA-generating locus	Plant development	1, 5	Ath
miR390/ miR391	tasiRNA-generating locus	Plant development	1, 5	Ath, Osa, Pta, Ppa, Mtr
miR393	bHLH transcription factor*	BR regulation of plant development	1	Ath
	Transport inhibitor response 1/auxin F-box	Defense response, response to phosphate starvation, signaling pathway, flower/root development	1, 5	Ath, Osa, Gma, Rco, Mes, Pvu, Mtr

Table 5 continued

miRNA family	Target family[a]	Functions	Methods[b]	Species[c]
miR394	F-box*	Signal transduction and regulation of the cell cycle	1, 5	Ath, Osa
	RNA polymerase sigma factor	Transcription	5	Osa
miR395	ATP-sulfurylase	Sulphur assimilation, response to cadmium ion	1	Ath, Mtr
	Cytochrome b5-like heme/steroid binding domain containing protein	Fatty acid metabolism	5	Osa
	Sulfate transporter	Sulfate transport	1, 5	Ath, Mtr
miR396	50S ribosomal protein L20	Translation	5	Osa
	Growth regulating factor	Leaf development, response to red light	1, 5	Ath, Osa, Gma
	Transcription factor X1*		5	Osa
miR397	ice1 (inducer of cbf expression 1) transcription factor*	Cold stress	5	Vvi
	Laccase	Copper homeostasis, response to water deprivation, cell wall biogenesis	1	Ath, Mtr
miR398	Copper chaperone for superoxide dismutase	Copper homeostasis	1	Ath
	Copper superoxide dismutase	Biotic and abiotic stresses	1, 5	Ath, Osa, Gma
	Cytochrome-c oxidase	Energy metabolism	1	Ath
miR399	PHOSPHATE/E2 ubiquitin-conjugating protein	Phosphate homeostasis	1, 5	Ath,Osa, Mtr
	Protein induced by phosphate starvation	Phosphate homeostasis	2	Ath
miR400	Pentatricopeptide repeat protein	RNA editing	5	Ath
miR402	Demeter-like/repressor of silencing 1-like	Base-excision repair, maintenance of DNA methylation	1	Ath
miR403	Argonaute	Gene silencing	1, 5	Ath, Sly
miR408	Copper ion binding protein	Copper homeostasis	1, 5	Ath, Osa, Mtr, Ppa
	Laccase	Copper homeostasis, response to water deprivation, cell wall biogenesis	1	Ath
	Plantacyanin	Plant reproduction	1	Osa, Ppa
miR414	14-3-3	Signal transduction, stress response, transcriptional regulation	5	Osa
	Pentatricopeptide repeat proteins	RNA-editing	5	Osa
	Tubulin/FtsZ domain containing protein	Assembly and disassembly of plant microtubules	5	Osa
miR415	40S ribosomal protein S10	Translation	5	Osa
miR419	Atkinesin-13A/kinesin-13A	Formation of Golgi vesicles	5	Osa
miR444	C3HC4 type family protein	Growth, development, and stress response	5	Osa
	MADS-box transcription factor*	Regulation of flowering time	1, 5	Osa
miR447	2-phosphoglycerate kinase	Carbohydrate metabolism	1	Ath
miR472	CC-NBS-LRR	Detection of diverse pathogens	1	Ath
miR473	GRAS domain—containing protein*	Signal transduction and development	1	Ptr
miR474	Kinesin	Mitosis, meiosis and transport	1	Ptr
	Pentatricopeptide repeat protein	RNA editing	1	Ptr
miR475	Pentatricopeptide repeat protein	RNA editing	1	Ptr
miR477	CONSTANS-like transcription factor*	Regulators of gene expression	1	Ptr
	GRAS domain—containing protein*	Signal transduction and development	1	Ptr
	Heat-shock protein	Stress response	5	Ppa
	Helix-loop-helix (HLH) transcription factor*	BR regulation of plant development	1	Ppa
	Ribosomal protein L29	Translation	5	Ppa
	Zinc-finger CCT-domain proteins	Light signal transduction	5	Ppa

Table 5 continued

miRNA family	Target family[a]	Functions	Methods[b]	Species[c]
miR478	Organic anion transporter-like protein	Organic anion transporter	1	Ptr
miR480	Proton-dependent oligopeptide transport family protein	Proton transporter	1	Ptr
miR482	Cytochrome p450	Cellular metabolism	5	Vvi
	Disease resistance proteins	Biotic stress	1	Ptr
miR528	F-box domain and LRR containing protein	Signal transduction and regulation of the cell cycle	5	Osa
	F-box/LRR-repeat MAX2	Pant development	5	Osa
	L-ascorbate oxidase	Ascorbate metabolism	5	Osa
miR529	Apetala2-like transcription factor*	Signaling pathway	1	Ppa
	Squamosa-promoter binding protein-like	Plant development	5	Osa
miR530	Zinc finger, C3HC4 type family protein	Growth, development, and stress response	5	Osa
miR534	Blade on petiole 2-like BTB and ankyrin-domain proteins	Gametophyte transition	5	Vvi
miR538	MADS box transcription factor*	Regulation of flowering time	1, 5	Ppa
miR773	DNA (cytosine-5-)-methyltransferase	DNA methylation	1	Ath
miR774	F-box*	Signal transduction and regulation of the cell cycle	1	Ath
miR775	Galactosyltransferase	Protein amino acid glycosylation	1	Ath
miR778	SET-domain factor*	Histone methylation	1	Ath
miR780	Cation/hydrogen exchanger	Cation transport	1	Ath
miR806	Transposon protein	Transposon	5	Osa
miR823	Chromomethylase	DNA methylation	1, 5	Ath
miR824	MADS-box transcripUion factor*	Regulation of flowering time	1, 5	Ath
miR827	SPX (SYG1/Pho81/XPR1) domain/Zinc finger (C3HC4-Uype)	Defense response to bacterium, response to salicylic acid stimulus	1, 5	Ath
miR828	MYB transcription factor*	Signaling pathway and development	1, 5	Ath, Vvi
	tasiRNA-generating locus	Plant development	1	Ath
miR837	Lipoic acid synthase family protein	Lipoic acid synthesis	5	Ath
miR842	Jacalin lectin	Vegetative storage	1	Ath
miR844	Protein kinase	Protein ubiquitination	1	Ath
miR846	Jacalin lectin	Vegetative storage	1	Ath
miR856	Cation/hydrogen exchanger	Cation transport	1	Ath
miR857	Laccase	Copper homeostasis, response to water deprivation, cell wall biogenesis	1	Ath
miR858	MYB transcription factor*	Signaling pathway and development	1, 5	Ath, Sly
miR859	F-box*	signal transduction and regulation of the cell cycle	1	Ath
miR894	Glycolate oxidase	Photosynthesis	5	Vvi
	Thiazole biosynthetic enzyme	Thiamine biosynthesis, stress response	5	Vvi
miR902	Helix-loop-helix (HLH) transcription factor*	BR regulation of plant development	1, 5	Ppa
miR946	Disease resistance protein	Biotic stress	1	Pta
miR1023	STOP1 transcription factors*	Protect from proton and aluminum toxicities	5	Ppa
miR1028	Protein arginine N-methyltransferase	Flowering time	5	Ppa
miR1029	Apetala2-like transcription factor*	Signaling pathway	1	Ppa
	DRE-binding transcription factor*	Abiotic stress	5	Ppa
miR1038	Histone deacetylase 2	Transcriptional regulation, cell cycle progression and developmental events	5	Ppa
miR1039	Vesicle-associated membrane family proteins	Vesicle fusion	5	Ppa

Table 5 continued

miRNA family	Target family[a]	Functions	Methods[b]	Species[c]
miR1043	Alcohol dehydrogenase	Cellular metabolism	5	Ppa
	WLIM1 transcription factor*	Regulation of gene expression	5	Ppa
miR1049	PPR-repeat protein	RNA-editing	5	Ppa
miR1065	WIP4 transcription factor*	Regulation of gene expression	5	Ppa
miR1073	Cu/Zn superoxide dismutase	Stress response	5	Ppa
miR1211	Molybdate transporter	Molybdate transporter	5	Ppa
miR1215	CRE1/WOL/AHK4 histidine kinase	Sulfate transport	1	Ppa
miR1216	Gravitropic in the light	Plant growth	5	Ppa
miR1218	NAM/ATAF/CUC3 (NAC3) protein	Signaling pathway, root development, response to oxidative stress	1	Ppa
miR1219	Auxin response factor*	Signaling pathway, flower development	1	Ppa
miR1223	NAC domain transcription factor*	Signaling pathway, root development, response to oxidative stress	1	Ppa
miR1425	Release factor 1	Fertility restorer	5	Osa
miR1428	Carbon catabolite derepressing protein kinase	Carbohydrate metabolism	5	Osa
miR1436	F-box domain containing protein	Signal transduction and regulation of the cell cycle	5	Osa
	Serine carboxypeptidase homologue	F-box domain containing protein	5	Osa
	Transposable element protein	Transposon	5	Osa
	WRKY transcription factors*	Biotic and abiotic stresses	5	Osa
miR1439	Disease resistance RPP13-like protein 1	Biotic stress	5	Osa
miR1442	bZIP transcription factor*	Pathogen defence, signal transduction, seed maturation and flower development	5	Osa
miR1444	Polyphenol oxidase	Synthesis of phenolic compounds	1	Ptr
miR1446	Gibberellin (GA) response modulator-like protein	Signal transduction	1	Ptr
miR1448	Disease resistance protein	Biotic stress	1	Ptr
miR1511	60S ribosomal protein	Translation	5	Gma
miR1514	NAC domain transcription factor*	Signaling pathway, root development, response to oxidative stress	5	Gma
miR1515	Autophagy protein	Autophagy	5	Gma
miR1516	Disulfide isomerase	Protein folding	5	Gma
miR1530	Auxin inducible transcription factor*	Signaling pathway, flower development	5	Gma
	Transketolase	Carbohydrate metabolism	5	Gma
miR1536	Ribulose-1,5-bisphosphate carboxylase	Calvin cycle	5	Gma
miR1857	Splicing factor U2AF	RNA splicing	5	Osa
miR1884	CP12-2	Calvin cycle regulation	5	Osa
	Transposon protein, CACTA, En/Spm sub-class	Nransposon	5	Osa
	Uricase	Nitrogen fixation	5	Osa
miR1917	Triple response 4	Ethylene responses	1	Sly
miR2101	CPUORF7 basic helix-loop-helix (bHLH) protein	BR regulation of plant development	5	Osa

Table 5 continued

miRNA family	Target family[a]	Functions	Methods[b]	Species[c]
miR2868	Protein kinase Kelch	Signal transduction	5	Osa

The most validated miRNA-target pairs were collected from the following sources: (1) the collection of Dr. Jim Carrington (as of Oct 2007, Carrington_MIRNA_TAS_data.xls) downloaded from ftp://ftp.arabidopsis.org/home/tair/Genes/SmallRNAs; (2) miRNA-target pairs collected by Bonnet et al. (2010), which includes the data from degradome analysis or parallel analysis of RNA ends (PARE) in *Arabidopsis thaliana* (Bonnet et al. 2010); (3) degradome analysis of rice *Oryza sativa*, moss *Physcomitrella patens*, grapevine *Vitis vinifera*, and soybean *Glycine max*. The * indicates that the target family belongs to transcription factors. For the data from degradome sequencing, targets with low abundant tags (category III) have low confidence of real targets and are removed from the table

[a] Only the targets with known function are retained in the table

[b] Methods used to valid the targets include: (1) 5′-RACE; (2) miRNA-resistant target; (3) agro infiltration; (4) in vitro cleavage; (5) degradome sequencing

[c] The species names are abbreviated as following: Ath, *Arabidopsis thaliana*; Cri, *Ceratopteris richardii*; Gma, *Glycine max*; Mes, *Manihot esculenta*; Mtr, *Medicago truncatula;* Osa, *Oryza sativa*; Ppa, *Physcomitrella patens*; Pta, *Pinus taeda*; Ptr, *Populus trichocarpa*; Pvu, *Phaseolus vulgaris*; Rco, *Ricinus communis*; Sly, *Solanum* lycopersicum; Skr, *Selaginella kraussiana*; Tgl, *Taxus globosa*; Vvi, *Vitis vinifera*

in which two water conducting cell types, protoxylem and metaxylem are patterned centripetally (Carlsbecker et al. 2010). This pattern occurrs through the crosstalk between the vascular cylinder and the surrounding endodermis; the crosstalk is mediated by cell-to-cell movement that is controlled by both a transcription factor and miR165/166 (Carlsbecker et al. 2010). Over-expression of miR165 increased the number of stele cells and all the xylem precursors acquired peripheral fate differentiating exclusively as protoxylem in *Arabidopsis* wild type roots as well in the *shr-2* mutant (Carlsbecker et al. 2010).

miRNAs control plant development and morphology through controling plant cell differentiation and proliferation. DCL1 is one important enzyme controlling miRNA biogenesis; the aberrant expression of *dcl1* gene affects miRNA biogenesis and causes a downstream influence on miRNA function (Zhang et al. 2006). Several investigations show that loss-of-function of the *dcl1* gene caused plant developmental abnormalities, including female sterility, aberrant embryo development arrested at an early stage, altered leaf shape, and delayed phase change (Liu et al. 2005; Park et al. 2002). This suggests that miRNAs are involved in controlling plant development and morphology.

First, miRNAs regulate plant leaf development and leaf morphology. To date, at least 5 miRNAs (miR156, miR159, miR165, miR166, and miR319) have been demonstrated to control the pattern and development of leaves in *Arabidopsis*, maize, and other plant species (Jung et al. 2009; Kanehira et al. 2010; Kim et al. 2009; Millar and Waterhouse 2005; Pant et al. 2008). These miRNAs regulate leaf development by targeting the homeodomain leucine zipper (HD-ZIP) and the TCP transcription factor genes. miR319, originally reported as miR159, is the first miRNA experimentally shown function during leaf development (Palatnik et al. 2003, 2007). Overexpression of miR319 resulted in jaw-D phenotypes, including uneven leaf shape and curvature (Palatnik et al. 2003). The reason is that miR319 targets the TCP transcription factor, which regulates leaf development. miR165/166 regulates the developmental polarity of the leaf by targeting the HD-ZIP genes *PHAVOLUTA* (*PHV*), *PHABULOSA* (*PHB*) and *REVOLUTA* (*REV*), whose accumulation alters in adaxial and abaxial regions (Kidner 2010; Rubio-Somoza and Weigel 2011; Williams et al. 2005). In addition to the conserved miRNAs, non-conserved miRNAs may also play roles in leaf development. One example is miR824 that has been reported to play a role in stomatal development (Kutter et al. 2007). Overexpression of one single miRNA, miR156, significantly increases leaf initiation and plant biomass in *Arabidopsis* (Schwab et al. 2005). This suggests a novel miRNA-based biotechnology for improvement of plant biomass for agriculture purposes and also for biofuel production.

Secondly, miRNAs regulate shot and root development. Currently, at least five members of the NAM/ATAF/CUC (NAC)-domain transcription factor family were shown to be targeted by miR164. Of these NAC transcription factors, NAC1 is involved in lateral root development (Xie et al. 2002), whereas CUC1 and CUC2 regulate meristem development and separation of aerial organs (Aida et al. 1997). Overexpression of miR164 resulted in reduced root development and aberrant aboveground development (Guo et al. 2005; Mallory et al. 2004). In contrast, inhibition of miR164 expression induced *Arabidopsis* to produce more lateral roots (Guo et al. 2005). Studies have also demonstrated that several auxin response transcription factors (ARF) are targeted by miRNAs; ARFs are a class of plant-specific DNA-binding proteins, which control auxin-regulated transcription and function in many plant development, particularly on root and shoot development. There are 23 AFR genes in *Arabidopsis* and it has demonstrated that at least 5 of them are targeted by miRNAs. ARF 10, ARF 16 and ARF 17 were

targeted by miR160. Disruption of miRNA:ARF relationship resulted root growth defects (Guo et al. 2005; Mallory et al. 2004; Wang et al. 2005).

miRNAs regulate phage change from vegetative growth to reproductive growth and floral development. One well studied miRNA is miR172 (Aukerman and Sakai 2003; Chen 2004). During plant vegetative growth, the expression level of miR172 is very low; as flowering approaches, miR172 expression levels increase significantly and reach the highest at flowering time. Over-expression of miR172 inhibits translation of *ap2* and *ap2*-like mRNAs, resulting in early flowering and disruption of the specification of floral organ identity (Aukerman and Sakai 2003; Chen 2004). miR159 was implicated in floral and anther development by targeting the expression of MYB33 and MYB55 (Achard et al. 2004).

The control of miRNAs in cell fate and morphology is implemented through a complex network. On one hand, the same miRNAs may function in different tissues, such as miR165/166 that may involve in the developmental polarity of leaves and roots (Carlsbecker et al. 2010; Rubio-Somoza and Weigel 2011), and different pathways, like miR160 which has been reported to be involved in signaling pathways, flower development, and seed germination (Liu et al. 2007; Mallory et al. 2005). On the other hand, certain miRNA targets may be regulated by other miRNAs' targets, such as expression of miR172 target AP2-like proteins being promoted by AFR3 which is regulated by the target of miR390, TAS3 (Rubio-Somoza and Weigel 2011).

miRNAs control plant response to environmental stress

Environmental stresses, including biotic and abiotic stress, are important factors limiting plant growth and development and also further limit crop yield and quality. Investigations on model plant species and crops have shown that drought and salinity stress induce differential expression of thousands of protein-coding genes (Aprile et al. 2009; Kreps et al. 2002; Kurihara et al. 2009; Matsui et al. 2008; Zeller et al. 2009; Zhu 2002). Some of the identified stress-induced genes have been used to improve crop tolerance to stress using transgenic technology (Hasegawa et al. 2000; Umezawa et al. 2006). However, the regulatory mechanism of these protein-coding genes is quite unclear.

Recently identified miRNAs may play an important role in response to environmental stress as well as the regulation of the expression of protein-coding genes during plants exposed to biotic and abiotic stress. During a genome-wide EST analysis, Zhang et al. (2005) found that 25.8% of ESTs containing miRNAs were obtained from stress-induced plant tissues; the stresses being drought, salinity, cold and high temperature, and pathogen infection (Zhang et al. 2005). Although this is not direct evidence for miRNA-involved plant response to environmental stress, it at least gives clues to the role of miRNAs in plant responses to stress. Several recent experimental studies have shown that abiotic stresses (drought, salinity, high and low temperature, and osmotic stress) induce differential expression of a set of miRNAs. miR395 was over-expressed under sulfate starvation condition (Jones-Rhoades and Bartel 2004). miR395 targets APT sulfurylase (APS) which catalyzes the first step of inorganic sulfate assimilation (Jones-Rhoades and Bartel 2004). Abiotic stresses (drought, cold, and salinity) strongly induced miR402 over-expression (Sunkar and Zhu 2004). Other miRNAs, such as miR319, are induced by either cold or other stresses (Sunkar and Zhu 2004). Several other studies also demonstrated that drought and salinity stresses induce significantly differential expression of miRNAs in a variety of plant species, including *Arabidopsis* (Jagadeeswaran et al. 2009; Jia et al. 2009; Liu et al. 2008), rice (Zhao et al. 2007), maize (Ding et al. 2009), Populus (Jia et al. 2009; Lu et al. 2005), and tobacco (Frazier et al. 2011). Currently, a number of miRNAs have been found to be induced by drought and salinity stress in several different plant species. These miRNAs include miR156, miR159, miR165, miR167, miR168, miR169, miR319, miR393, miR395, miR396, miR398, miR399, and miR402. Over-expression of the single miRNA miR169 confers enhanced drought tolerance in tomato (Zhang et al. 2011). At same dehydrated condition, the non-transgenic plants show obvious dehydration symptoms but the transgenic plants with over-expression of miR169 grow very well. Physiological tests show that transgenic tomatoes have reduced stomatal openings and a decreased transpiration rate which aids in preventing leaf water loss.

Almost all of these stress-induced miRNAs are evolutionarily conserved, which suggests that miRNA-mediated regulatory mechanisms in responding to environmental stresses in plants may be evolutionarily conserved.

miRNAs involve in signal transduction

Plant development involves extensive communication between cells, tissues and organs. This communication is usually mediated by mobile molecules. At the early stage, hormones, small peptides, and even some proteins can serve as mobile molecules for mediating cell-to-cell communication. Although it is well-known that mRNA molecules can be transported a long distance in animals (Du et al. 2007), the evidence for mRNA translocation over long distance initially emerged in 1999 by identifying a set of mRNAs in phloem sap (Ruiz-Medrano et al. 1999), in which scientists showed that 8 different transcripts can be transported through the phloem in mature pumpkin leaves

and stems. Following this study, more direct evidences on plant mRNA long distance transportation have appeared. Grafting experiments, in situ RT-PCR analysis, and laser capture microdissection provide direct evidence for mRNA movement from the plant rootstock into a grafted section, such as an apical meristem (Kanehira et al. 2010; Kim et al. 2001; Notaguchi et al. 2008; Ruiz-Medrano et al. 1999; Xoconostle-Cazares et al. 1999).

Recent investigations have also demonstrated that small RNAs including miRNAs are mobile and can be transported through the phloem system from one organ to another (Chitwood and Timmermans 2010). Several investigations show that miRNAs could be involved in long-distance signal transduction (Buhtz et al. 2008; Kanehira et al. 2010; Pant et al. 2008). Buhtz et al. (2008) identified 32 annotated miRNAs, belong to 18 families from oilseed rape vascular exudates; of them, three well known miRNAs, miR395, miR398 and miR399, were found to be highly abundant in phloem sap under nutrient starvation conditions (Buhtz et al. 2008). Pant et al. (2008) also demonstrated that miR399 is highly present in the phloem sap of two diverse plant species, rapeseed and pumpkin. Micro-grafting experiments, using model plant species *Arabidopsis*, further indicated that miR399 accumulated in a high expression level by over-expression of miR399 in the shoot; however, only high levels of mature miR399 species were detected in the wild type roots. The authors did not determine the pre-miR399 in the roots (Pant et al. 2008), therefore this suggests that miR399 can be transported from the shoots to the roots through the vascular system. The observation that high abundance of non-vascular tissue produced miRNAs exist in the vascular tissue suggests that these miRNAs are generated in non-vascular tissue and then exuded into the vascular tissue and transported to other tissues for function.

Many major identified phloem-mobile miRNAs, such as miR395, miR398 and miR399, have been demonstrated to be induced by stresses, including nutrient deprivation, oxidative stress, drought and salinity stress. This suggests that mobile miRNAs may play an important role in plant response to different environmental abiotic and biotic stresses as well as plant nutrient homeostasis. miR395 has already been shown to be strongly induced by drought and sulphate deprivation; two confirmed targets of miR395 are ATP sulfurylase (APS) and the sulfate transporter SULTR2;1, which both play some role in sulphate homeostasis in plants (Chiou 2007; Jones-Rhoades and Bartel 2004). APS knockdown mutants accumulate twice as much sulfate than that in wild type plants. Both SULTR2;1 loss mutants and miR395-over-expression plants show impaired sulfate allocation from old to young leaves (Liang et al. 2010). miR398 has three targets, cytochrome-c oxidase, copper superoxide dismutase, and acopper chaperone for superoxide dismutase, which confers the role of miR398 in biotic and abiotic stress response (Zhu et al. 2011). Pant et al. (2008) demonstrated that shoot-generated miR399 negatively regulates PHO2, a critical component for maintenance of Pi homeostasis in roots.

Plant hormones play an important role in plant growth and development, including shoot, root, and flower differentiation. Many of these processes are involved in auxin signaling. Current studies have demonstrated that several miRNAs are associated with and target auxin signaling pathways. It is demonstrated that two conformed miR393 targets, TIR and AFB, are receptors for the plant hormone auxin (Dharmasiri et al. 2005; Sunkar and Zhu 2004). A 22-amino acid peptide (flg22) from the N terminus of eubacterial flagellin induced a two fold increase in miR393 expression in *Arabidopsis* after 20 and 60 min of infection (Navarro et al. 2006). Overexpressing miR393 showed a five fold lower bacterial titer in *Arabidopsis*, suggesting that miR393 inhibits the growth of virulent Pto DC3000 (Navarro et al. 2006). The reason causing the bacterial resistance of plants with over expression of miR393 is that miR393 negatively regulates TIR1 which represses auxin signaling and further enhances bacterial disease resistance (Navarro et al. 2006). TIR1 is one part of the ubiquitin ligase complex SCF^{TIR1} that interacts with Aux/IAA proteins to promote Aux/IAA protein degradation (Gray et al. 2001). Aux/IAA proteins repress auxin signaling through heterodimerization with Auxin Response Factors (ARFs) that are down regulated by flg22 triggers events (Navarro et al. 2006). Thus, overexpression of a single miRNA may enhance plant immune response and disease resistance.

Concluding remarks

Although the first plant miRNAs were discovered less than one decade ago, miRNA-related research has become one of the hottest research fields in plant science and miRNAs have become known as one of the most important regulators in plant gene regulation. In the past several years, hundreds of miRNAs have been identified from different plant species using various experimental and computational approaches. Many investigations have demonstrated that miRNAs are involved in almost all biological and metabolic processes in plants, including plant development and response to different environmental abiotic and biotic stresses. Although much progress has been made in this field, miRNA-related research is still in its infantry within plant kingdom. To better understand the functional and regulatory mechanism of miRNAs in plants, several research directions should be considered.

miRNA-related research needs to switch from descriptive research to mechanistic research

Although much research is currently being performed on plant miRNAs, a majority of the research is purely descriptive, and provides no mechanistic insight into miRNA function and miRNA-mediated gene regulation. The majority of this research includes the identification of miRNAs by simply using deep sequencing technology as well as in silico computational approaches. Currently a list of miRNAs has been identified in many plant species, however only very few miRNAs have clear functions in plant development. In the future, scientists should more focus on the functional analysis of miRNAs instead of simply identifying miRNAs. These research should include, but should not limited to (1) miRNA functional analyses, with possibly more transgenic technology beingused to overexpress or knockdown a specific miRNA in order to investigate their function and their regulatory mechanisms on plant growth and development; (2) the regulatory mechanisms of miRNA-mediated plant development and gene regulation. Although it is known that miRNAs control plant development through miRNA-mediated gene regulation, it is unclear how this process is regulated and how miRNAs interact with specific targets; (3) using miRNAs and/or artificial miRNAs as a tool for improving plant yield, quality and tolerance to environmental stress. Many studies have shown that miRNAs control plant development, affect biomass, as well aid in response to abiotic and biotic stresses, however, no single study has been performed to establish a miRNA-based biotechnology for improving crop yields.

Enhancement of the study of miRNA target identification

miRNAs work through targeting one or more protein-coding genes. Thus, identifying miRNA targets is the first as well as the critical step in studying miRNA function. However, one of the major issues is that miRNA research in plants is more behind than research in animals because there is a lack of knowledge about miRNA targets. Currently we do not know exactly what mechanisms cause the miRNA:mRNA interactions and how many mRNAs are targeted by a single miRNA. In the past years, several computational tools have been developed for identifying miRNA targets in plants. These tools include miRU (Zhang 2005), Helper tools (Moxon et al. 2008), TAPIR (Bonnet et al. 2010), and Target-align (Xie and Zhang 2010). All of these tools are based on currently available knowledge on miRNA:mRNA interaction, such as complementarity between mRNAs and corresponding miRNAs, maximal folding free energy, functional positions and conservation. However, because of our limited knowledge of this interaction, it is hard to design a perfect tool for identifying miRNA targets. In the future, the interaction mechanisms of miRNA and its targets need to be better understood in order to design a better tool for identifying plant miRNA targets.

Deep sequencing technology provides a new powerful tool for enhancing miRNA target identification. Based on the fact that a majority of miRNAs are either perfectly or near-perfectly complementary to their targets and cleave their targets, RACE has been employed to identify the cleavage sites since the very beginning. Currently, RACE has been expended to directly identify miRNA targets by deep sequencing combined with extensive bioinformatics work (Addo-Quaye et al. 2008; German et al. 2008; Gregory et al. 2008). Of them, degradome sequencing technology is a newly developed approach for miRNA target identification and confirmation (Addo-Quaye et al. 2008; German et al. 2008). However, this approach only can be used to identify the targets of already known miRNAs and only for the miRNAs that cleave their targets. However, recent studies show that some miRNAs may inhibit translation by non-perfectly binding to target mRNAs with more potential target sites (Brodersen et al. 2008; Montgomery et al. 2008; Yu and Wang 2010). Thus, a new approach needs to be developed for identifying plant miRNAs with more flexible criteria.

miRNA evolution

There are several evolutionary problems faced by scientists. The first issue is finding out what the relationship is between plant and animal miRNAs. Many miRNAs are conserved from species to species. However, the origin and evolution of miRNAs is still unknown. Although miRNAs are produced in both plant and animal systems and the biogenesis of miRNAs is similar to each other, there are currently no clues to whether the miRNAs have same ancestor or not for both plants and animals.

The second issue is how new miRNAs originate quickly. With the exception of conserved miRNAs, there are many species-specific miRNAs. It is unclear how these miRNAs originated and when they diverged from each other. Unlike miRNAs in animals, the genes encoding plant miRNAs are varied except for the mature sequences and some miRNA* sequences. It is unlikely, at least for many miRNAs, that miRNAs are evaluated following the traditional roles.

In plants, there are two gene silencing pathways; one is miRNA-mediated gene regulation and another one is small interfering RNA (siRNA)-mediated gene regulation. These two pathways are so similar in plants, what is their relationship during the long evolutionary history? How are

they separated from each other? Solving these evolution questions will provide a better understanding of miRNA-mediated gene regulation and allow a better application of this knowledge for improving crop yields.

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