Plant miRNomics: Novel Insights in Gene Expression and Regulation

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Abstract

Advances in transcriptomics have led to the classification of small RNAs (sRNAs) into mainly three categories: miRNAs, siRNAs and piRNAs. However, there are many new types of sRNAs under exploration. Though such sRNAs differ from one another, they collaborate in their mode of action. Among the sRNAs, microRNAs (miRNAs) widely captured the attention of molecular biologists. miRNAs are short, endogenously expressed and non-translated RNAs. Mature plant miRNAs are in general smaller in size (~22 bp) and considered as negative gene regulatory molecules. In general plant miRNAs have the following features: (a) They are coded by miRNA genes with unknown length and are sequentially cleaved from pri-miRNA and pre-miRNA into a short mature miRNA by *Dicer-like 1 (dcl1)* and several other enzymes.

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(b) All pre-miRNAs can form a stem-looped hairpin secondary structure with the mature miRNA on one arm and the complementary sequence, termed miRNA*, on the another arm with high negative minimum folding free energy (MFE) and MFE index (MFEI). (c) Typically, miRNAs do negatively regulate target gene expression and the miRNA* sequence is degraded by an unknown mechanism. However, in some cases, the miRNA* sequence also can function to target a specific gene. Over the past few years, microarray technologies, large-scale small RNA and whole genome sequencing projects and data mining have provided a wealth of information about the spectrum of plant miRNAs and their targets. Hitherto identified miRNAs in plant kingdom have shown that they are deeply conserved; nevertheless considerable numbers of species-specific miRNAs also exist. Evidences are gradually mounting to notify that miRNAs have key roles in developmental timing, cell proliferation and cell death, organogenesis, patterning of tissues/organ and more importantly, in response to external environmental stimuli. Thus it is very obvious that plant miRNAs are more numerous and their regulatory impact is more pervasive than was previously suspected.

Keywords
miRNA • Small non-coding RNA • miRNA target • Function of plant miRNA

Introduction

The perfect and complete development of a multicellular organism at normal and abnormal conditions requires specific and coordinated expression of genes. The regulation of gene expression is the most basic level which decides the information encoded in the DNA is decoded into phenotypes. As such it involves complex regulatory networks that direct precise cell division and differentiation patterns. Thus, the key factor that is central to the understanding of the biological systems is how dynamic gene regulatory programmes are generated from the static instructions encoded in the DNA. Though single or several regulators of given gene(s) expression(s) have been described earlier, it is not completely unravelled in several complex trait expressions such as organ formation, behaviour or adaptation to a particular environmental conditions.

However, it is clearly evident that plants and other multicellular organisms need precise spatio-temporal control of gene expression, and this regulatory capacity depends, in part, on small RNAs (sRNAs). Although most genes use RNA in the form of mRNA as a coding intermediate for protein production, there are genes whose final products are RNA and do not code for protein. Such non-coding RNAs range from the transfer and ribosomal RNAs that are involved in protein-synthesising machinery to the more recently discovered regulatory sRNAs. There are several kinds of sRNAs and have specific role in coordinated control of gene expression (see below). This chapter specifically focuses on plant miRNAs, a class of sRNAs and their involvement in negative regulation of plant genes.

miRNAs are small (~20–22 bp in length) RNAs that negatively regulate the expression of genes through specific base pairing with cognate target mRNAs and thereby inducing target mRNA degradation or translational repression or both (Sun 2011). miRNAs were first reported in plants (*Arabidopsis*) during mid-2002 by four different research groups as tiny RNAs with miRNA characteristics (Llave et al. 2002; Mette et al. 2002; Park et al. 2002; Reinhart et al. 2002). Studying the expression profiles of miRNAs, their targets and function in biological system using biotechnological and bioinformatic tools are collectively called as ‘miRNomics’. It is expected that integration of miRNomics data with other ‘omics’ data would help in comprehensive understanding of the precise spatio-temporal control of gene expression and more essentially the specific role of miRNA in gene regulation. Such understanding would also help to develop novel application tools in medicine, agriculture and industries. For example, the miRNA expression profiles provide a powerful

support for the characterisation of disease development in plants. The development of micro array technologies, specific for miRNAs, can help to obtain a miRNomics profile for different organs under disease, and high-throughput proteomics analysis can help to identify the miRNAs that control the differential expression of proteins.

Classification and Comparison of Small RNAs

Until a couple of decades before, it has been considered that the most important components of biological systems are DNA (the foundation of heredity) and proteins (the players of the cellular machinery). During those periods, RNA was recognised as an intermediate molecule that bridges the gap between DNA and protein (especially mRNA) or has functional role in splicing (snRNA) or translation machinery (tRNA and rRNA).

On the other hand, advances in molecular biology have led to classify the RNAs into coding and non-coding RNAs. Coding RNAs comprise of messenger RNAs (mRNAs). Non-coding RNAs are subdivided into ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and small RNAs (sRNAs). Various types of sRNAs have been identified such as microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), small temporal RNAs (stRNAs), tiny non-coding RNAs (tncRNAs) and small modular RNAs (smRNAs). Among them, miRNAs and siRNAs have been well characterised in plant and animal systems (the characteristics of these two sRNAs are described in Table 1) whereas piRNAs have been identified only in animals. Various other types of siRNAs have also been identified, including trans-acting siRNAs (Ta-siRNAs), repeat-associated siRNAs (Ra-siRNAs) and natural-antisense transcript-derived siRNAs (Nat-siRNAs) based on their distinct biogenesis and functions (Kruszka et al. 2012).

Table 1 General features of miRNAs and siRNAs

Property	miRNAs	siRNAs
Description	Regulators of endogenous genes	Defenders of genome integrity when foreign or invasive nucleic acids enter into the cell
Size	20–22 bp	21–24 bp
Biogenesis: precursors	Hairpin-shaped ssRNAs	Long dsRNAs
Biogenesis: nature of precursors	Endogenous precursor such as gene(s) of host's genome	Exogenous precursors such as transposons, transgenes, repeat elements or viruses
Cellular role	mRNA degradation, translational repression	DNA methylation, histone modification and mRNA degradation
Gene regulation mechanism	Post-transcriptionally with partial or full complementarity with target mRNA	Transcriptionally as well as post-transcriptionally with full complementarity with target mRNA
Target genes	miRNAs cannot regulate the genes from which they originate (*cis*) because they are identical, not complementary, to their precursor RNAs	siRNAs can potentially act in both *cis* and *trans* by targeting the elements from which they derive (*cis*) as well as unlinked elements that exhibit substantial complementarity to their sequence (*trans*)
Functions	Cell development, cell differentiation, regulation of developmental process, biotic and abiotic stress response	Defence against transposons and viruses and stress adaptation
First report published in	1993	1999

Thus the latest discovery of an increasing number of small non-coding RNAs with specific regulatory roles has added additional role to the RNA molecules, and it has changed our view on gene expression. It has been shown that small RNAs are known to play essential roles in the multicellular organisms with the surprising exception of the *Saccharomyces cerevisiae* (Sun et al. 2012). sRNAs are involved in a variety of occasions with diverse modes of actions that are essential for genome stability, development and adaptive responses to biotic and abiotic stresses (Jones-Rhoades 2012). For example, they guide DNA elimination during the formation of the macronucleus in protists and heterochromatin assembly in fungi and plants. They target endogenous mRNAs for cleavage and translational repression in plants and animals. sRNAs protect both plant and animal cells against virus infection through an RNA-based immune system. They also control the movement of transposable elements at the transcriptional and post-transcriptional levels in plants and animals.

sRNAs are produced from endogenous genomic loci and repeats and in response to external stimuli such as viruses. sRNAs are invariably bound by argonaute proteins, some of which have endonuclease activity to effect sRNA-guided cleavage of target mRNAs. Argonaute proteins belong to at least two phylogenetic groups: the argonaute subfamily, which binds miRNAs and siRNAs, and the piwi subfamily, which binds piRNAs. Thus the major types of sRNAs are distinguished by their different modes of biogenesis and action (Kruszka et al. 2012). Despite different modes of biogenesis, the sRNAs share similar molecular functions. For example, miRNA, siRNA and piRNAs can direct the cleavage of complementary RNAs. miRNAs and siRNAs can both result in translational inhibition of target mRNAs. siRNAs and piRNAs can both direct chromatin modifications (Chen 2009). Since small RNAs are repressors of gene expression, small RNA-mediated regulation is often referred to as RNA silencing, gene silencing or RNA interference (RNAi). RNA silencing was discovered in plants more than 15 years ago during the course of transgenic experiments that eventually led to silencing of the introduced transgene and, in some cases, of homologous endogenous genes or resident transgenes either by transcription inhibition (transcriptional gene silencing) or RNA degradation (post-transcriptional gene silencing) (Sun et al. 2012).

Among the small RNAs, miRNAs have a number of discrete features as compared to other functional RNA species (Jones-Rhoades 2012). First, most of the known miRNAs are encoded as polycistronic transcripts, proposing that members of the same miRNA family may evolve concurrently and develop in similar ways. Second, it has been known that a significant number of miRNAs are highly conserved in sequences among different related and unrelated organisms. Conserved miRNAs possess a special 'seed' sequence in their 5′ terminus; such conservation suggests that these molecules participate in key cellular processes. Third, miRNAs tend to target and regulate a set of mRNAs instead of a specific mRNA. Fourth, direct experimental evidence supports the notion that the miRNA pathway is an ancient regulatory mechanism evolved before the divergence of multicellular and unicellular organisms. Finally, special cases among viruses are worth mentioning: due to their higher mutation rates and faster evolution processes, most viral miRNAs do not seem to share significant homology with those of their vertebrate counterparts, even within members of the same family, and the lack of homology poses challenges for computational biologists to precisely predict miRNAs based on sequence conservations alone in viruses (Cai et al. 2009).

The past decade has witnessed rapid progress in revealing miRNA diversity, uncovering their mechanisms of action and understanding their biological functions. Here I review our current knowledge of onmiRNAs, with an emphasis on their biogenesis and function in plants. At the same time, I would like to highlight that with the development of the next-generation sequencing technologies and other advances in molecular

biology, miRNAs from various species would shoot up in an inconceivable speed in the near future and such discoveries can greatly enrich our knowledge on miRNAs and their function with new dimensions.

Historical Perspectives of miRNA

Although miRNAs were first discovered in the early 1990s (Lee et al. 1993; Wightman et al. 1993), they were not recognised as miRNAs until 2001 (Lee and Ambros 2001; Lau et al. 2001; Lagos-Quintana et al. 2001). Since then, miRNAs have attracted a huge interest from scientists, and large numbers of miRNAs were identified in almost all the biological systems (Bartel 2004). In the year of 1993, the Ambros and Ruvkun labs investigated the first endogenous, non-protein-coding 22 nt RNAs in nematode *Caenorhabditis elegans* as *lin-4* and *let-7*, both of which are key regulatory molecules in the pathway controlling the timing of larval development. *lin-4* is recognised as the founding member of a new class of sRNAs called miRNAs (Lee and Ambros 2001; Lau et al. 2001; Lagos-Quintana et al. 2001). Compared with animal miRNAs, plant miRNAs were identified several years later. In 2002, several researchers identified plant miRNAs by cloning sRNAs and reported the molecular mechanisms of miRNA biogenesis and function in plants (Llave et al. 2002; Mette et al. 2002; Park et al. 2002; Reinhart et al. 2002). In the past couple of years, the total number of miRNAs in economically important crops has dramatically increased as the approaches for efficient identification species-specific miRNAs were developed. The first miRNAs detected in a viral genome were reported in 2004 by Pfeffer and colleagues in Epstein–Barr virus. To date, Release 19 of the miRBase sequence database contains 21264 entries representing hairpin precursor miRNAs, expressing 25141 mature miRNA products, in 193 species. The data are freely available to all through the web interface at http://www.mirbase.org/872 (verified on 08th May, 2013).

Biogenesis of miRNA

miRNA biogenesis is a multi-step enzymatic process which includes transcription, processing, modification and RISC loading (Fig. 1; Jones-Rhoades 2012). First, a miRNA gene is transcribed to a primary miRNA (pri-miRNA), which is usually a long sequence of more than several hundred nucleotides (Fig. 1). This step is controlled by Pol II enzymes (Bartel 2004). After analysing several miRNA-related sequences in *Arabidopsis*, Allen et al. (2004) presented a model for miRNA origin. In their model, plant pre-miRNAs originated from their target genes by formation of inverted duplications which have been transcribed but not modified further. miRNAs may originate anywhere within the plant genome; many genomic regions were found to be sites of miRNAs although these regions were previously considered featureless. These findings suggest that miRNA origin may be more complicated than previously thought by involving many mechanisms such as inversion and duplication (see below).

In the second step, the pri-miRNA is cleaved to a stem-loop intermediate called miRNA precursor or pre-miRNA. This step is controlled by the Drosha RNase III endonuclease in animals (Bartel 2004) or by Dicer-like 1 enzyme (DCL1) in plants (Tang et al. 2003). In animals, pre-miRNAs are then transported by exportin 5 from the nucleus into the cytoplasm (Sun et al. 2010), followed by formation of miRNA–miRNA* duplex and mature miRNAs by another RNase III-like enzyme called Dicer (Bartel 2004). However plant miRNAs differ from animals in this step. Plant miRNAs are cleaved into miRNA–miRNA* duplex possibly by Dicer-like enzyme 1 (DCL1) in the nucleus rather than in the cytoplasm (Bartel 2004); then the duplex is translocated into the cytoplasm by HASTY, the plant orthologue of export in 5 (Jones-Rhoades 2012). DCL1 acts with two partner proteins: HYL1 (a double-stranded RNA binding protein) and SE (a zinc-finger protein). These three proteins co-localise in nuclear Dicing bodies in vivo. SE also localises in numerous nuclear speckles and acts in the splic-

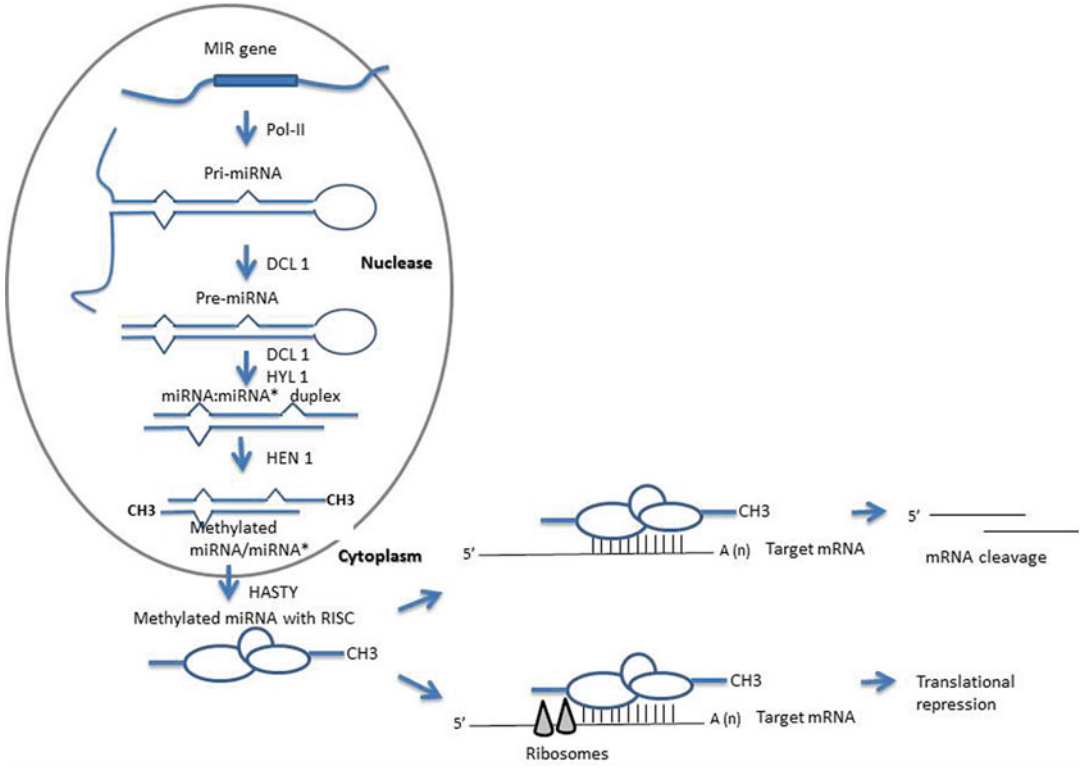

Fig. 1 Successive steps involved in biogenesis of miRNA to target recognition and function

ing of pre-mRNAs. In plants, it is also noticed that the miRNAs are methylated on the ribose of the last nucleotide by the methyltransferase (Chen 2009). The miRNA–miRNA* duplex is methylated on the 2′ OH of the 3′ terminal nucleotides by HEN1. The miRNA is exported from the nucleus to the cytoplasm through export factors including HASTY. In the cytoplasm, both plant and animal miRNAs are unwound into single-strand mature miRNAs by helicase (Bartel 2004).

Finally, the mature miRNAs enter a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC) (Bartel 2004; Chen 2009) where they regulate targeted gene expression. The sRNA degrading nuclease (SDN1) family of exonucleases degrades single-stranded miRNAs to limit their steady-state levels (Chen 2009). Recently it was reported that a large number of 5′ or 3′ truncated miRNAs from various tissues of *Populus trichocarpa* can be isolated. Thus it is suggesting the occurrence of exonucleolytic degradation of miRNAs (Lu et al. 2005a, b). They also showed that a significant portion of the isolated miRNAs contains one or a few post-transcriptionally added adenylic acid residues at the 3′-end. The short adenylate tail of miRNAs is distinct from the longer polyadenylate tail added to other plant RNAs for the exosome-mediated degradation. Results of in vitro miRNA degradation assays revealed that the addition of adenylic acid residues on the 3′-end reduced miRNA degradation rate (Lu et al. 2005a, b).

Current Molecular Understanding of Plant Gene Regulation by miRNAs

In the RISC complex, miRNAs bind to target mRNA and inhibit gene expression through perfect or near-perfect complementarity between the miRNA and the mRNA (Bartel 2004). This

causes gene silencing (Almeida and Allshire 2005) and it termed differently in diverse organisms. For example, this process is referred to as RNA interference (RNAi) in animals (Hannon 2002), quelling in fungi (Cogoni et al. 1996) and post-transcriptional gene silencing in plants (Baulcombe 2004). In plants, most target mRNAs contain one single miRNA complementary site, and most corresponding miRNAs typically perfectly complement to these sites and cleave the target mRNAs (Fig. 1; Bartel 2004). Unlike animal miRNA targets, the complementary sites in plants can exist anywhere along the target mRNA rather than at the 3′ UTR. Yet another mechanism was also identified in plant miRNA regulation. Although some miRNAs can perfectly complement to target mRNAs, they regulate gene expression by repressing gene translation possibly through inhibition of ribosome movement (Fig. 1; Chen 2009). This proposes a hypothesis that miRNAs may be involved in more complicated mechanisms to control gene expression in plants than in animals, i.e. plant miRNAs regulate gene expression at the post-transcriptional level not only by repression of mRNA translation but also by direct cleavage of mRNAs.

In general, miRNA*, the complementary strand of mature functional miRNA, is thought to degrade rapidly or accumulate at only very low levels, suggesting that it may not be functional. However, several recent studies have shown that plant miRNA* tends to accumulate at high levels under certain conditions (reviewed in Sunkar et al. 2012) and regulates a different target, i.e. other targets that are regulated by its cognate mature miRNA (Zhang et al. 2011a; Sunkar et al. 2012).

Molecular Structural Features of miRNA Genes and Its Expression

Like that of protein-coding genes, the core structure of a miRNA gene is also composed of a Pol II promoter, a transcribed region and a terminator. These basic structural elements of miRNA genes have been well studied in recent years except for the terminator region. MiRNA promoters contain the basic TATA, CAAT boxes as well as binding sites for specific transcription factors (TFs) (Xie et al. 2005). The promoter activity of an miRNA gene, such as miR390 and miR165/166, can be monitored by fusing it with a reporter gene (e.g. *GUS* or *GFP*) using plant transgenic technology (Montgomery et al. 2008). Like the promoters of protein-coding genes, miRNA promoters also behave in a tissue-specific or a temporally specific manner. Interestingly, the majority of plant miRNAs regulate the expression of TFs (Jones-Rhoades 2012). As a general way of auto-regulation, the promoters of some miRNAs, such as miR156 and miR172, contain the binding sites of their target TFs, further demonstrating a fine-tuning role of miRNAs in their regulatory networks (Wu et al. 2010). The transcribed region of the miRNA, the mature miRNA and the immediate miRNA precursor (pre-miRNA) are small; however, the primary miRNA (pri-miRNA) transcripts tend to be large in size (please see Fig. 1). Thus, the transcribed region of a miRNA gene is usually longer than necessary for miRNA biogenesis. Compared to the promoter and terminator regions, at least part of the miRNA gene transcribed region plays a key role in miRNA biogenesis and is thus an essential part in miRNA gene evolution (Tang et al. 2007). As stated above, unlike miRNA promoters and transcribed regions, miRNA terminators have not been well studied. Their existence is inferred from the fact that pri-miRNAs are polyadenylated. However, it is not known how the terminators or the polyA tail affects miRNA biogenesis or the stability of pri-miRNAs, and thus it necessitates further research (Tang et al. 2007).

Although a large number of miRNA genes disperse over the genome, clustered ones are found co-expressed as polycistronic units that may have functional relationships. In addition, more than half of miRNAs reside in introns of their host genes and co-express with their neighbouring protein-coding sequences, and some may derive from common primary transcripts and even share the same promoters (Jones-Rhoades 2012). Nevertheless, a sizable number of miRNA genes come from regions that are distal from previously annotated protein-coding

sequences, and such locations imply that they probably derive from independent transcription units with their own promoters (Cai et al. 2009).

In all the plant species examined, miRNA genes are scattered throughout the genome. A majority (84 %) of miRNA genes are located in intergenic regions. This situation is contrary to that of *Drosophila* species, in which nearly half of the miRNA genes are located in introns (Nozawa et al. 2010). It should be noted that the proportion of intergenic miRNA genes is not correlated with the genome size of the plant species examined. In fact, *Arabidopsis* shows a high proportion of intergenic miRNA genes (86 %) even though its genome size is as small as that of *D. melanogaster* (Nozawa et al. 2010).

A range of techniques are available for miRNA gene expression analysis or quantification, including Northern blotting, dot blotting, RNase protection assay, primer extension analysis, invader assay and quantitative PCR (reviewed in Kim and Nam 2006). Large-scale cDNA cloning can also provide information on the relative expression level of miRNAs in diverse samples. However, most of these techniques involve laborious procedures, making it difficult to determine the level of all known miRNAs. Currently, the most widely used method is based on microarrays. However, the problem of potential cross-hybridisation of related miRNAs still remains unresolved. In addition, systematic bias could be introduced during reverse transcription, PCR amplification, enzymatic labelling or fluorescence tag ligation. These problems were successfully avoided by developing a new procedure called the RNA-primed array-based Klenow enzyme (RAKE) assay (Kim and Nam 2006). The most recent innovation in miRNA detection involves the bead-based flow cytometric method (see below).

Combining all the experimental results, the existence of miRNAs in plant cell is now being verified by the following criteria: (1) a band of about 22-nucleotide mature miRNA could be detected by Northern blotting, usually with precursor miRNA of about 70 nucleotides; (2) the precursor forms a hairpin structure and the mature miRNA is present in one arm of the hairpin; (3) both the mature and the precursor miRNAs are usually phylogenetically conserved; and (4) the precursor miRNAs should be observed when Dicer function is disturbed (Li et al. 2010a, b). Interesting additional mechanisms (such as single nucleotide polymorphisms, RNA editing, methylation and circadian clock) that play important roles in controlling the expression and function of miRNAs were also described by Cai et al. (2009).

In summary, the loci that encode plant miRNAs, the MIR genes and their expression are clearly distinct from previously annotated genes, but their promoters, primary transcripts and responsible RNA polymerase remain to be identified.

Evolution of miRNA

Most plant species contain more than 100 miRNA genes in their genome, and estimation of the numbers of miRNA genes and gene families in ancestral species indicated that the numbers of miRNA genes and gene families increased considerably in the lineage to flowering plants after the divergence from green algae (Jones-Rhoades 2012). With respect to the evolutionary origin of miRNA genes, there are several different mechanisms have been proposed (Jones-Rhoades 2012): (1) miRNA genes may be generated from duplicates of protein-coding genes. This is an attractive hypothesis because amiRNA gene generated from a protein-coding gene would bind to the transcript of the protein-coding gene. In fact, there seem to be a number of miRNA genes generated in this way (Allen et al. 2004; Rajagopalan et al. 2006; Fahlgren et al. 2007). In plants more than 90 % of miRNA gene families which are conserved in nine or more species are multigene families, whereas only 23 % of species-specific miRNA genes from multigene families were reported. These observations suggest that gene duplication has played important roles in increasing the number of miRNA genes in plants (Nozawa et al. 2011). (2) Transposable elements (TEs) may become miRNA genes. In particular, miniature inverted-repeat transposable elements

(MITEs) have a potential to become miRNA genes because they have inverted repeats with a short internal sequence, which can potentially turn into the hairpin structure of amiRNA gene. It has been proposed that dozens of miRNA genes were originated from MITEs or other TEs in *Arabidopsis* and rice (Piriyapongsa and Jordan 2008). (3) New miRNA genes may be generated by duplication of pre-existing miRNA genes with subsequent mutations. This mechanism also seems to be important in plants because each miRNA gene family on average consists of several miRNA genes and there is experimental evidence to support this mechanism (Jones-Rhoades 2012). (4) miRNA genes naturally arise by spontaneous mutations from hairpin structures in the genome. Some miRNA genes have been generated in this way in *Arabidopsis* (De Felippes et al. 2008).

Conservation and Divergence of Plant microRNA Genes

According to published data in miRBase (Griffiths-Jones et al. 2008), in general, miRNAs can be placed into two different categories: the highly abundant and conserved miRNAs and the rare and species-specific miRNAs. In one earlier study, it was found that miRNAs with identification numbers from miR156 to miR408 are exceptionally conserved and their expression levels are generally high. The remaining miRNAs after miR408 in the series are expressed at a low level, species specific or induced by specific conditions (Ma et al. 2010). While the former class contains mostly the ancient miRNAs that are important in plant development and responses to stress, the latter class might be composed of newly evolved miRNAs with their functions being non-essential due to their low activities. For example, in plants, ~20 miRNA families which are well conserved between dicots and monocots are known. Of these, 7 miRNA families, i.e., miR156/157, miR160, miR159, miR319, miR165/166, miR390 and miR408, have been also found in primitive land plants such as *Physcomitrella* and *Selaginella* which suggest that these are deeply conserved (Nozawa et al. 2011). In addition, *Arabidopsis*, rice, *Populus* and *Physcomitrella* possess many non-conserved lineage- or plant species-specific miRNA families (Nozawa et al. 2011).

As stated earlier, experimental and computational analysis has indicated that many plant miRNAs and their targets are conserved between monocots and dicots. Monocots and dicots diverged about 125 million years ago; thus, miRNAs should have existed at that time when they diverged from the same ancestor (Jones-Rhoades 2012). Further, employment of microarray technology and EST database to analyse the expression of several miRNAs in different plant species found that some miRNAs existed not only in dicots and monocots but also in ferns, lycopods and mosses (Zhang et al. 2006a). Both miRNA conservation and miRNA target conservation indicate that plant miRNAs have a very deep origin in plant phylogeny, at least since the last common ancestor of bryophytes and seed plants. Gene regulation by miRNA is an ancient evolutionary mechanism to control gene expression. It is one part of the global gene regulation mechanisms. This suggests that miRNA-mediated gene regulation existed more than 425 million years ago in the plant kingdom (Jones-Rhoades 2012). The age of plant miRNA is comparable to the age of miRNA regulation in metazoans; however, no evidence yet shows that plant miRNAs and animal miRNAs have a common ancestor. However, it is also expected that some non-conserved or lowly conserved miRNAs may be documented in the near future as more miRNAs are discovered in different plants at different developmental stages or under biotic and abiotic stresses. Non-conserved miRNAs may play more specific roles in specific plant species, such as the differentiation and elongation of cotton fibres. Several numbers of novel and differentially expressed miRNAs in contrasting cotton cultivars exposed to water stress were noticed in our laboratory (Boopathi et al. in preparation).

The entire miRNA transcribed region can be divided into two subregions in general: the evolutionarily stable subregion and the variable subregion (Jones-Rhoades 2012). The

stable subregion is small in size and includes mainly the mature miRNA and the miRNA, whereas the loop and other sequences beyond the stable subregions, which represent most of the miRNA gene, belong to the variable subregions. The stable and variable subregions are the outcome of evolution over a long period. Except for the stable subregion, the overall sequences of most ancient miRNA genes tend to be variable due to genetic drift, natural selection and fixation in evolution. Sequence variations along the variable subregions of most miRNA genes are helpful in keeping the miRNA genes active (Nozawa et al. 2011). All miRNAs have similar secondary hairpin structures; many of these are evolutionarily conserved. This suggests a powerful approach to predict the existence of new miRNA orthologues or homologues in other species.

Identification of miRNA Genes

There are at least four different methods that are available for identifying miRNAs, and they are based on the major characteristics of miRNAs such as: (a) all miRNAs are small non-coding RNAs, usually consisting of ~20–22 nucleotides for animals and ~ 20–24 nt for plants; (b) all miRNA precursors have a well-predicted stem-loop hairpin structure, and this fold-back hairpin structure has a low free energy; and (c) many miRNAs are evolutionarily conserved, some from worm to human or from ferns to core eudicots or monocots in plants (Zhang et al. 2006a, b). Plant miRNAs are less conserved than animal miRNAs. Usually, only the mature miRNAs are conserved in plants instead of miRNA precursors that are usually conserved in animals. Therefore, when an sRNA (see above) is considered as an miRNA, all of these major characteristics should be included. Further, to evade labelling other sRNAs or fragments of other RNAs as miRNAs, Ambros et al. (2003) developed combined criteria to identify new miRNAs. These combined criteria include both biogenesis and expression criteria, neither of which on its own is sufficient for identifying a candidate gene as a new miRNA (Ambros et al. 2003). So far, all newly predicted or identified miRNAs have conformed to these rules.

The four approaches that are employed for identifying miRNAs are genetic screening or forward genetics (Lee et al. 1993; Wightman et al. 1993), direct cloning and sequencing after isolation of small RNAs (Lu et al. 2005a, b), computational strategy (Brown and Sanseau 2005) and expressed sequence tags (ESTs) analysis (Zhang et al. 2005). Characteristics, advantages and limitations of these methods are given in Table 2, and more details on individual methods are provided below. Earlier studies of miRNA profiling depended on Northern blotting, RT-PCR and cloning, which are labour intensive and time consuming, and they cannot obtain about global miRNA expression patterns. Compared with other methods of protein-coding gene prediction, predicting and annotating miRNA genes still need more work, and hence it is generally suggested that combined use of the above methods will improve the efficiency of miRNA profiling and characterisation.

Genetic Screening or Forward Genetics

This is the first approach by which the first two miRNAs (i.e. the founding members of the miRNAs: *lin4* and *let7*) were identified in *C. elegans* (Lee et al. 1993; Wightman et al. 1993). Since genetic studies are based on clear phenotypes, the in vivo functions of genetically identified miRNAs are well established. This method was similar to methods for identifying other traditional genes. Although this method was useful for identifying some miRNAs, application of this strategy was limited because it is expensive, time consuming and dominated by chance. To overcome some of the shortcomings of genetic screening, another experimental approach was recently described for isolating and identifying new miRNAs. This approach involves direct cloning after isolation of sRNAs.

Table 2 Characteristics, advantages and limitations of different approaches used for miRNA identification

Particulars	Genetic screening/ forward genetics	Direct cloning and sequencing after small RNA isolation	Computational approaches	EST analysis
Requirement of genome sequence	Not required	Not required	Required	Required
Use of specific software	Not required	Not required	Required	Required
Cost of the experiment	High	High but less than genetic screening	Moderate	Low
Efficiency in identification of miRNA	Low	High	Low	High
Experimental confirmation of identified miRNA	Not required	Not required	Required	Required
Efficiency in identification of new miRNA	High	High	High	Low
Can be applied across the species?	Yes	Yes	Yes	No
Can quantitative information on miRNA be obtained?	No	Yes	No	Somewhat
Reference	Lee et al. (1993) and Wightman et al. (1993)	Lu et al. (2005a, b)	Brown and Sanseau (2005)	Zhang et al. (2005)

Direct Cloning and Sequencing

In this approach, sRNA molecules are first isolated by size fractionation. Then these small RNAs are ligated to RNA adapters at their 5′ and 3′ ends (Lu et al. 2005a, b). In the successive steps, they are reverse transcribed into cDNA, which is then amplified and sequenced. Because only sRNAs are isolated and screened by this method, it is a more efficient way to obtain miRNAs than general genetic screening. This method can be further refined by combining it with massively parallel signature sequencing. This method is not only suitable for identifying plant miRNAs but also can quantify miRNA abundance at the same time.

Deep Sequencing

The emergence of next-generation technologies, capable of generating 10^5–10^7 sequences in a single experiment, has revolutionised our ability to meaningfully describe the populations of miRNAs (and other sRNAs) expressed in plant cells (Jones-Rhoades 2012). Deep sequencing has dramatically expanded the number of miRNA families known to exist in plants and has led to revised guidelines of evidence required to justify annotation of plant miRNAs (Meyers et al. 2008).

A simple and apt reason that describes the utility of deep sequencing in miRNA identification is that analysing large numbers of reads increases the chance of recovering rare transcripts. On the other hand, analysing a few hundred reads is often sufficient to obtain clones of highly expressed miRNAs. Since miRNA abundance varies by at least several orders of magnitude, much larger data sets are needed to recover a substantial fraction of lower abundance miRNAs. A second powerful reason for using deep sequencing is that categorising the cloned RNAs is more straightforward when patterns of RNA expression are apparent (Jones-Rhoades 2012). Specifically, the observation of patterns of sRNA accumulation consistent with Dicer processing of a hairpin intermediate (i.e. detection of an miRNA–miRNA* pair, as defined by the predicted secondary structure of a single-stranded precursor) is strong evidence for miRNA-like biogenesis (Meyers et al. 2008). Examples of miRNA–miRNA* pairs are sometimes observed

at low depth of sequencing for abundant miRNAs (Reinhart et al. 2002), but deep sequencing makes it feasible for comparatively rare miRNAs (Rajagopalan et al. 2006). At the same time it should be noted that there is considerable evidence that miRNA discovery by deep sequencing has not reached saturation even in well-sampled species like *Arabidopsis* (Fahlgren et al. 2007; Jones-Rhoades 2012).

Computational Methods

The third approach that is used to identify miRNA is the computational approach. This approach is based on a genome sequence and bioinformatic tools (Jones-Rhoades and Bartel 2004). The first miRNA search algorithm was MiRscan, which successfully predicted miRNA genes that display close homology in two nematode worms: *C. elegans* and *C. briggsae*. MiRscan was further improved by defining conserved sequence motifs found in the vicinity of nematode miRNA genes. Since then several tools were developed, and the following are some of the commonly used public resources of machine learning-based approaches for finding miRNA genes: MiRAlign, Mir-abela, Tirplet SVM, MiPred, RNAmicro, Microprocessor SVM, Bayes miRNA find, ProMiR and MiRFinder (Please see Table 3). They have successfully predicted miRNA genes in plants and animals. However, it is generally believed that computational approach is slightly inefficient and certainly not comprehensive. The predicted miRNAs need to be confirmed by experiments such as cloning or Northern blotting.

EST Analysis

The fourth approach is an expressed sequence tag (EST) analysis approach. It is well recognised that several miRNAs are evolutionarily conserved from species to species (Llave et al. 2002; Reinhart et al. 2002; Zhang et al. 2005). This suggests a powerful approach to predict homologies or orthologues of previously known miRNAs (Jones-Rhoades and Bartel 2004). More importantly, this approach is very useful for predicting miRNAs in multiple species, especially in species whose genomes are unknown. It advocates that EST analysis is a good alternative method for identifying miRNAs, especially for species whose genome is poorly understood. However, this method can only identify conserved miRNAs. miRNAs that are more likely non-conserved cannot be identified based on the EST approach.

A novel innovation in miRNA detection involves the bead-based flow cytometric method (Lu et al. 2005a). Each individual bead is marked with fluorescence tags (which can yield up to 100 colours, each representing a single miRNA) and coupled to probes that are complementary to miRNAs of interest. miRNAs are ligated to the 5′ and 3′ adaptors, reverse transcribed, amplified by PCR using a common biotinylated primer, hybridised to the capture beads and stained with streptavidin–phycoerythrin. The beads are then analysed using a flow cytometer capable of measuring bead colour (denoting miRNA identity) and phycoerythrin intensity (denoting miRNA abundance). Since hybridisation takes place in solution, this method offers more specific detection of closely related miRNAs compared with conventional glass-slide microarrays (Lu et al. 2005a).

Documented miRNAs in Plants

Despite notable recent progress in identifying miRNAs, including detailed data on miRNA expression pattern and target genes of miRNAs, they remain widely distributed in published literature. To this end, a cohesive database system is profoundly needed for data deposit and further application. Thus, the availability of a large amount of miRNA information, including the sequence information, structural features and annotated functional roles in crop plants, necessitates in developing databases with specific goals and tools. There are several miRNA and their target databases available in the World Wide Web. Some notable databases are miRBase (the home of microRNA data; the miRBase sequence

Table 3 Publically available resources for miRNA identification using computational methods

Name of the programme/algorithm	Features	Website/reference
Mir-abela	Computed from the entire hairpin structure	http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi
Triplet-SVM	Each hairpin is encoded as a set of 32 triplet elements	http://bioinfo.au.tsinghua.edu.cn/miRNAsvm/
MiPred	32 global and intrinsic hairpin folding attributes based on sequence, structural, statistical thermodynamics and topology	http://web.bii.a-star.edu.sg/_stanley/Publications
RNAmicro	12 features based on structure, sequence composition, conservation, thermodynamic stability and structural conservation	http://www.bioinf.unileipzig.de/~jana/software/
Microprocessor SVM	686 features from structure and sequence; 7 additional Drosha processing sites features	https://demo1.interagon.com/miRNA/
Bayes miRNA find	Secondary structure and sequence features	https://bioinfo.wistar.upenn.edu/miRNA/miRNA/login.php
ProMiR	A hairpin structure is represented as a pairwise sequence; each position of the pairwise sequence has two states: structural and hidden	http://cbit.snu.ac.kr/~ProMiR2/
MiPred	Contiguous structure-sequence composition, minimum of free energy of the secondary structure	http://www.bioinf.seu.edu.cn/miRNA
MiRFinder	18 parameters, including the local secondary structure differences of the stem region of miRNA and the secondary structures of pre-miRNAs	http://www.bioinformatics.org/mirfinder
srnaloop	Use of sequential and structural properties	http://arep.med.harvard.edu/miRNA/pgmlicense.html
miRseeker	Comparative analysis, stem-loop conservation	http://www.ncrna.org/login_form
ERPIN (Easy RNA Profile IdentificatioN)	Sequence or structural alignment	http://tagc.univ-mrs.fr/erpin/
findMiRNA	Seed match, comparative analysis	http://www.molquest.com/help/2.3/programs/Find-miRNA/description.html
MiRAlign	Sequence or structural alignment	http://bioinfo.au.tsinghua.edu.cn/MiRAlign/
PalGrade	Sequential and structural properties	Barad et al. (2004)
miRTour	Automated homology-based discovery of plant miRNA and their targets from sequencing data sets (EST, GSS, SRA, etc.)	http://bio2server.bioinfo.uniplovdiv.bg/miRTour/
miRDeep	Identifies miRNAs using deep-sequencing technique (for worm)	http://www.mdc-berlin.de/en/research/research_teams/systems biology of generegulatory_elements/projects/miRDeep/index.html
miRanalyzer	Identifies miRNAs using deep-sequencing technique (for worm, fly and animals)	http://web.bioinformatics.cicbiogune.es/microRNA/

database is a searchable database of published miRNA sequences and annotation), PMRD (plant microRNA database; this database includes all publicly known plant miRNA sequences – including those in miRBase), MicroCosm Targets (formerly miRBase Targets; a web resource containing computationally predicted targets for microRNAs across many species), miR2DiseaseBase (manually curated database documenting known relationships of miRNA dysregulation and human disease), miRecords (manually curated database of experimentally validated miRNA–target interactions), miRvar (database for genomic variations in microRNAs), PASmiR (a literature-curated and web-accessible database, which provides detailed, searchable descriptions of miRNA molecular regulation in different plant abiotic stresses), psRNATarget (a Plant Small RNA Target Analysis Server) and TargetScan (which predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA), ASRP (collection of known miRNAs in plants), miRNA map (collection of computationally identified miRNA in metazoan genomes), miRCen (collection and identification of animal miRNA–target interactions using multiple target-prediction programmes), CoGemiR (comparative genomics of miRNAs) and TarBase (collection of experimental miRNA targets).

miRNA Nomenclature

Since hundreds of miRNAs specific to particular crop and thousands across other species are being identified, a system of nomenclature has been adopted and names are designated to specific miRNAs before publication of their discovery (Ambros et al. 2003). Experimentally confirmed microRNAs are given a number that is attached to the prefix mir followed by a dash, e.g. mir-123. The uncapitalised mir- refers to the pre-miRNA and the capitalised miR- refers to the mature form. MiRNAs with similar structures with differences at 1 or 2 nucleotides are noted with added lowercase letter, e.g. miR-1a and miR-1b. It is possible for miRNAs at different loci to produce the same miRNA, and these are shown with additional number, e.g. miR-1-1 and miR-1-2. Strictly speaking, microRNA nomenclature should also be preceded by first letter of the genus and first two letters of the species in which they are observed, e.g. *hsa*-miR-156, i.e. miR156 which is identified in *Homo sapiens*. On the other hand, common miRNA species are also having distinct nomenclature, e.g. viral v-miRNA and drosophila d-miRNA. microRNAs originating from the 3′ end or 5′ end are often denoted with a -3p or 5p suffix, e.g. miR-142-5p and miR-142-3p.

miRNA Targets

As stated earlier, the very first miRNA target was noticed through genetic screens performed by the Ambros laboratory to characterise the heterochronic gene pathway (i.e. the temporal progression of developmental events in *C. elegans*). They uncovered a 22 nt non-coding RNA as the product of the *lin-4* gene (Lee et al. 1993). *lin-4* RNA repressed the protein levels of *lin-14*, a gene that functions in the same developmental pathway. The *lin-4* RNA had the potential to bind, with partial antisense complementarity, to sequences found in the 3′-untranslated region (3′-UTR) of *lin-14* mRNA and repress its translation (Lee et al. 1993). A similar kind of regulation was later noticed when the discovery by the Ruvkun laboratory, of *let-7*, a second 22nt RNA that also functioned in the heterochronic gene pathway, was published (Wightman et al. 1993). As that of *lin-4, let-7* RNA recognised sequences present in the 3′-UTR of its *lin-41* mRNA target and repressed LIN-41 protein levels. Thus it is obvious that each miRNA has its own target gene(s), whose expression(s) is/are negatively regulated by the specific miRNA.

In plants miRNAs were described first in *Arabidopsis* and later in other species. To date, there are 5399 plant miRNAs from 61 species available at the miRBase (www.http://www.mirbase.org/cgi-bin/browse.pl; verified on 09th May, 2013). Most of these miRNAs target transcrip-

tion factors and thus are implicated in diverse aspects of plant growth and development (Jones-Rhoades 2012). The major challenge in determining miRNA functions is to identify their regulatory targets. By analogy to *lin-4* and *let-7* RNAs, it is reasonable to suppose that miRNAs generally recognise their regulatory targets through base pairing. An indication that target prediction for certain plant miRNAs might be more straightforward came with the identification of miR171, a plant miRNA with perfect antisense complementarity to the mRNAs of three scarecrow-like transcription factors (Llave et al. 2002; Reinhart et al. 2002).

Since thousands of miRNAs are being reported regularly, identification of miRNA targets becomes a crucial phase in appreciating their regulatory function. The methods to do this can be generally classified into two different groups according to their generations (some selective list of computational methods for target prediction are given in Table 4). The first-generation methods such as miRanda, DIANA-microT, RNAhybrid, MicroInspector and TargetScans are based mainly on three characteristic properties: (1) The 5′ seed of the miRNA (nucleotide positions 2–8 of the miRNA) is complementary to the 3′ UTR of the target mRNA. (2) The RNA–RNA duplex has a higher negative folding free energy. (3) Mature miRNAs, binding sites of miRNA to mRNA and miRNA–mRNA duplex all are highly conserved from species to species, particularly within the same kingdom (Li et al. 2010a, b).

The new generation of methods often utilises machine learning-based approaches. PicTar is a typical example. This algorithm scans the alignments of 3′ UTRs for those displaying seed matches to miRNA and then filters the alignments according to their thermodynamic stability. Each predicted target is scored by using a HMM maximum-likelihood fit approach. In the PicTar model, synergistic effects of multiple binding sites of one miRNA or several miRNAs acting together are accounted for along with appropriate scoring of overlapping site and background for binding (Li et al. 2010a, b). The probabilities are assigned according to experimental and computational results. Some experimental studies also suggested that site accessibility was a critical factor for efficient repression. Excellent comparisons of computational methods used to predict miRNAs and their targets are provided in Li et al. (2010a, b).

In plants, the successful targeting reaction requires complementarity of the miRNA at most of the residues (Mallory and Bouché 2008). The consequence of the targeting reaction depends on the nature of the targeted RNA and the extent of complementarity with the miRNA. The target RNA is cleaved and the level of the protein product is reduced if there is near complete complementarity, including positions 9 and 10 of the miRNA. Translational suppression without turnover of the target RNA is mediated by miRNAs with incomplete complementarity to their target (Lanet et al. 2009). In addition, there may be miRNA-mediated targeting of chromatin-associated RNAs that leads directly or indirectly to targeted epigenetic modification (Wu et al. 2010). In some instances, miRNA-mediated gene silencing is a simple negative switch: whenever the miRNA gene is active, the target mRNA is silent. However, these versatile RNA regulators may also participate in feedback loops and carry out more subtle roles in genetic regulation (MacLean et al. 2010). They might dampen fluctuations in target gene expression, for example, influence temporal changes. In some instances, the miRNAs or their precursors may move through plasmodesmata and different stages in the feedback system occur in adjacent cells or in separate roots and shoots. MiRNAs may also initiate regulatory cascades with multiple mRNA targets (MacLean et al. 2010). These cascades involve secondary siRNAs (see above) that associate with argonaute (AGO) proteins, similarly to miRNAs.

It has been shown that an individual miRNA is able to control the expression of more than one target mRNAs and that each mRNA may be regulated by multiple miRNAs (Jones-Rhoades and Bartel 2004). The 5′ region of miRNA usually contributes more to the specificity and activity in binding targets. The interactions between miRNA and mRNA are usually restricted to the ‘seed’

Table 4 Resources used to find miRNA targets using computational strategies

Name of the tool	Characteristic features	Website
PicTar	HMM maximum-likelihood fit approach	http://pictar.mdc-berlin.de/
TargetBoost	Boosted genetic programming algorithm to create weighted sequence motifs	https://demo1.interagon.com/demo
RNA22	Exhaustive pattern discovery based on locally conserved signatures	http://cbcsrv.watson.ibm.com/rna22.html
PITA	Incorporates the role of target-site accessibility within traditional seed finding procedures	http://genie.weizmann.ac.il/pubs/mir07
miTarget	SVM based on structural, thermodynamic and positional features	http://cbit.snu.ac.kr/~miTarget
MicroTar	Complementarity of miRNAs to their target and thermodynamic data	http://tiger.dbs.nus.edu.sg/microtar/
NBmiRTar	Naive Bayes classifier based on sequence and duplex structures feature	http://wotan.wistar.upenn.edu/NBmiRTar
mirWIP	Structural accessibility of target sequences, total free energy of miRNA–target hybridisation and topology of base pairing to the 5′ seed region of the miRNA	http://146.189.76.171/query.php
Sylamer	Nucleotide pattern analysis based on expression profiling data sets	www.ebi.ac.uk/enright/sylamer/
GenMiR++	Bayesian algorithm based on expression data sets	http://www.psi.toronto.edu/genmir
TargetScanS	Seed match (SM), sequence complementarity (SC) and minimal free energy (MFE) of miRNA/target duplex; sequence preferences of target sites	http://www.targetscan.org/
miRanda	SM, SC and MFE	http://www.microrna.org/
RNAhybrid	Measures SC, MFE and statistical significance of miRNA–target interactions	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/
MirTarget2	Based on machine learning technique; a computational model was trained by a variety of features concerning miRNA–target interactions	http://mirdb.org/miRDB/
DIANA-micro	SM, SC, MFE and sequence preferences of target sites	http://www.diana.pcbi.upenn.edu/cgibin/microt.cgi
miRcheck	Sequence complementarity, allowing gap in miRNA/target duplex (for plants)	http://web.wi.mit.edu/bartel/pub/software.Html
miRU	Sequence complementarity, allowing gap in miRNA/target duplex (for plants)	http://bioinfo3.noble.org/miRNA/miRU.htm
findMiRNA	Sequence complementarity; gap not allowed (for plants)	http://sundarlab.ucdavis.edu/miRNA/

sequence near the 5′ terminus in animals despite the fact that most plant miRNAs regulate their targets based on complete complementarity. The ~6 to 8 nt 'seed' sequence is highly conserved among species and even a slight change in sequence may alter its target spectra. It is also suggested that the location of central loop in the miRNA–mRNA duplexes may play a key role in affecting the efficiency of gene regulation mediated by miRNAs (Jones-Rhoades 2012).

miRNAs control the target expression by base pairing to sequence motifs in the 3′UTR of mRNAs with perfect or near-perfect complementarities (Jones-Rhoades and Bartel 2004). Certain AU-rich elements in 3′UTR were uncovered to interact with miRNAs and act both directly and indirectly as potent post-transcriptional regulatory signals. Analysis of the miRNA target sites indicated that genes with longer 3′UTRs usually have higher density of miRNA-binding sites and are mainly involved in developmental modulations, whereas genes with shorter 3′UTRs usually have lower density of miRNA-binding sites and tend to be involved in basic cellular processes (Jones-Rhoades and Bartel 2004). These facts emphasise the importance of 3′UTR in interacting with miRNAs. It is also claimed that a small subset of miRNAs from plants and animals exert repression regulation by specifically targeting the 3′ UTR of some mRNAs. In addition, candidate target sites of miRNAs falling in the protein-coding regions are also identifiable based on computational and experimental approaches. Hence, it is obvious that miRNAs identify their targets by multiple pathways or modes (Cai et al. 2009). Thus the impact of the interaction between miRNAs and their targets is further complicated than it was thought.

Current target-prediction programmes depend on the information from sequence, structure-associated free energy and evolutionary conservation to predict candidate mRNAs. Those bioinformatic methods usually result in the prediction of tens or hundreds of targets for each miRNA with high false positive rates. Therefore, further experiments of gain-of-function and loss-of-function are still needed and will determine how many of these predicted targets are genuinely targeted by miRNAs.

A number of experimental techniques have been used to validate miRNA–target relationships that are predicted by bioinformatics. These include the detection of cleavage activity in vivo or in vitro, the detection of target abundance (at the RNA and/or protein level) following perturbation of miRNA function and the effects of reducing the complementarity between the miRNA and target (Jones-Rhoades et al. 2006). Another approach is to use microarrays or RNA sequencing to globally monitor abundance of RNAs following perturbation of miRNA expression. Another powerful method to empirically identify targets of miRNAs (and other small RNAs) is to sequence libraries of 5′ fragments from uncapped, polyadenylated transcripts (which is also referred to as degredome sequencing). A large number of miRNA–target interactions initially identified via bioinformatics now have confirmed roles in specific aspects of plant biology (Chen 2009). Until now, miRNA target identification has relied mainly on computational approaches since genome-wide experimental approaches have not been developed due to the limitation of technology. This problem has recently been overcome by approaches that combine HITS-CLIP (high-throughput sequencing of RNAs isolated by cross-linking immunoprecipitation) with bioinformatic analysis (Chi et al. 2009).

Thus it can be concluded that the scope of miRNA-mediated regulation in plants, as currently understood, is much more limited in terms of the numbers of genes targeted. Well-characterised plant miRNA–target interactions (as identified by bioinformatic and experimental approaches outlined above) involve extensive complementarity and are relatively few in number. Most plant miRNA families have 0–10 known targets (usually from the same gene family) in a single genome, implying that less than 1 % of plant genes are miRNA targets. It has also been suggested that plant miRNAs, including miRNAs for which verified targets exist, might also have large numbers of yet unidentified targets.

Regulation of the Regulators: Genetic Control of miRNA Biogenesis

Since the discovery of miRNA, bioinformatic and experimental analysis has focused mainly on the identification and predicted target of miRNAs, whereas regulation of miRNA gene expression remained largely unexplored. As more experimental evidence indicated that miRNAs played an important role in the development stage and mechanism of biotic and abiotic responses, it was urgent to understand the regulation process of this petite regulator itself. The bioinformatic studies on the regulation of miRNA could roughly be divided into two parts. The first part focused on prediction of transcription factors (TFs) that governed the expression of miRNAs or location of the promoter region upstream of miRNA sequences. The second part focused on the crosstalk between miRNAs and TFs based on the experimentally proved or computationally predicted TFs and miRNA regulation relationship data sets. miRNA is coordinatively modulated by multifarious effectors such as SNP, miRNA editing, methylation and circadian clock. Cai et al. (2009) and Li et al. (2010a, b) provided a detailed description of such regulations in miRNA biogenesis and function.

Role of miRNA in Plant Growth and Development

miRNAs are playing highly significant roles in regulatory mechanisms operating in plants, including developmental timing, cell differentiation, proliferation and apoptosis. As stated above, miRNAs have several distinct features that impart specific molecular role to them as compared to other regulatory RNA species. Most of the known miRNAs are encoded as polycistronic transcripts and tend to target and regulate a set of mRNAs instead of a specific mRNA substrate. The cross species conserved miRNAs possess a special 'seed' sequence in their 5′ terminus; such conservation suggests that these molecules participate in critical cellular processes (Jones-Rhoades and Bartel 2004; Cai et al. 2009; Jones-Rhoades 2012).

Table 5 illustrates some selected examples of miRNAs and their specific role in plant growth and development. Altogether, these results suggest that miRNAs are implicated in various regulatory processes and signalling pathways that are required for organogenesis in crop plants. Additional investigation of miRNA diversity in tissues or organs of various species may serve to discover new miRNAs, to reveal new targets of conserved miRNAs and to suggest additional roles of miRNAs in these crop species. It has been proposed elsewhere that these new miRNA-modulated patterns may be influential to cellular developmental pattern and that miRNAs may be highly interesting targets to understand the control of cell growth and development in plants (Achard et al. 2004). For example, it was predicted that one of the miR396 targets was the *callose synthase* catalytic subunit (*CFL1, AF085717*), a gene that is differentially expressed during cotton fibre development (Cui et al. 2001). It was also observed that miR396 was expressed in different organs, including fibre and ovules. This suggests that miR396 plays a role in cotton fibre differentiation and development. Further study of the regulatory mechanisms of miRNAs on *CFL1* and other fibre-related genes will allow better understanding of the molecular mechanism of fibre development in cotton. This can lead to efficiently improve cotton fibre yield and quality more precisely and quickly than ever.

Role of miRNA in Biotic and Abiotic Stress Resistance in Plants

Besides the findings of miRNAs acting as master regulators of plant growth and development, other evidences also suggest that miRNAs play a key role in plant stress responses (Kedde et al. 2012). In addition, the expression profiles of most miRNAs that are implicated in plant growth and development are significantly altered during stress. This clearly implies that attenuated plant growth and development under stress may be

Table 5 Representative examples of miRNAs involved in plant growth and development

Name of the miRNA	Crop	Predicted target	Experimentally validated target	Function of miRNA	Reference
miR163	*Arabidopsis*	Members of the plant SABATH methyltransferase family	–	Species divergence and genome hybridisation	Ha et al. (2009)
miR164	*Arabidopsis*	Cup-shaped cotyledon (CUC) genes, namely, CUC1, CUC2 and CUC3	–	Organ initiation from meristematic tissues	Laufs et al. (2004)
miR171 miR396a	Cotton	Regulatory genes such as a predicted histone deacetylase and probable WRKY transcription factor 20 as well as structural genes such as beta-tubulin 7	–	Early boll development	Pang et al. (2011)
miR396	*Arabidopsis*	Growth-regulating factor (GRF) family of transcription factors	The transcription factor bHLH74	Cell proliferation in leaves	Debernardi et al. (2012)
miR165/166	*Arabidopsis*	–	PHABULOSA (PHB) and PHAVOLUTA (PHV) proteins (which are class III homeodomain leucine zipper (HD-ZIP) TFs)	Leaf polarity, shoot apical meristem formation and vascular tissue differentiation	Khan et al. (2009)
miR160	*Arabidopsis*	–	Auxin-responsive factors (ARF10, 16 and 17)	Root development	Khan et al. (2009)
miR396	*Medicagotruncatula*	Growth regulation factors (GRF), which are involved in the promotion of cell proliferation	–	Root tips	Lelandais-Brière et al. (2009)
miR167	Rice	–	Genes of auxin-related pathway	Positive regulator of adventitious root development	Meng et al. (2009)

(continued)

Table 5 (continued)

Name of the miRNA	Crop	Predicted target	Experimentally validated target	Function of miRNA	Reference
miR482	Soybean	–	Resistance (R) gene receptor kinases	Nodule initiation	Li et al. (2010a, b)
miR408	*Populustrichocarpa*	–	Plastocyanin-like, early-responsive to dehydration-related protein	Development of leaves, phloem, xylem, tension xylem and opposite xylem	Lu et al. (2005b)
miR172	*Arabidopsis* and maize	–	APETALA 2 (AP2) and AP2-like mRNAs	Early flowering and disrupting the specification of floral organ identity	Zhang et al. (2007)
miR393	*Citrus trifoliate*	–	Transport inhibitor response-like protein (TIR)	Roots, stems and leaves development	Song et al. (2009)
miR165 and miR166	*Arabidopsis*	–	Class-III homeodomain leucine zipper (HD-ZIP) transcription factor genes: PHABULOSA (PHB), PHAVOLUTA (PHV) and REVOLUTA (REV)	Radialization and adaxialization of leaf and vascular bundles in the stem	Zhang et al. (2007)

under the control of stress-responsive miRNAs. Even subtle and transient changes in miRNA expression during stress can have profound physiological effects (Mendoza-Soto et al. 2012).

Abiotic and biotic stresses are a big issue for plant growth and development and are major constraints to agricultural productivity worldwide. Examples of abiotic stress or adverse environmental factors include drought, submergence, salinity, solar radiation (excess light or high light intensities, UV light), extreme temperatures (heat and low temperature/freezing stress) and pollutants (heavy metals, herbicides). In addition, low concentrations of essential macro- and micronutrients or conditions that result in poor uptake of these nutrients are also perceived as stress by plants. Several field studies showed that environmental stress caused about 20–30 % yield loss and some may completely destroy crop yield (Gepstein and Glick 2013). Similarly, the biotic stresses such pests and diseases also cause huge loss to the farm productivity.

During the evolution, crops have developed different mechanisms to resist different environmental stresses, including salinity, cold, drought, pests and diseases. The molecular basis of plant tolerance to these stresses has been profoundly explored over several decades. These research efforts have identified numerous genes that are induced under such abiotic stress, with the hope that over expression of stress-responsive genes would improve plant stress tolerance. However, these transgenic plants exhibited very small improvements in stress tolerance or no improvement at all (Bartels and Sunkar 2005), largely because the complicated genetic interactions underlying plant stress tolerance are not completely understood, i.e. although several genes have been identified and isolated from plants (http://www.plantstress.com/biotech/index.asp?Flag=1), the principle mechanism of plant resistance still remains unknown. In addition to protein-coding genes, the expression of miRNAs in plants is altered during conditions of stress. Increasing evidences suggest that miRNAs may play an important role in plant response to biotic and abiotic stresses (Tables 6, 7 and 8). The miRNAs that have been identified as stress responsive

Table 6 Representative examples of miRNAs involved in biotic stress response

Name of the miRNA	Crop	Predicted target	Experimentally validated target	Function of miRNA	Reference
miR393	*Arabidopsis*	Genes involved in auxin perception and signalling	–	Protecting plants against pathogenic bacteria	Navarro et al. (2006)
miR482 and miR2118	Tomato	–	Disease resistance proteins with nucleotide binding site (NBS) and leucine-rich repeat (LRR) motifs	Contributes to a novel layer of defence against pathogen attack	Shivaprasad et al. (2012)
miR1885	*Brassica rapa*	–	TIR–NBS–LRR class disease-resistant transcripts	Induced specifically by turnip mosaic virus (TuMV) infection	He et al. (2008)
miR160, miR393 and miR1510	Soybean	–	12 resistance-related genes	In response to soybean mosaic virus infection	Yin et al. (2013)
miR156 variants	*Arabidopsis*	–	At3G15270 (SPL family members)	In response to *Brevicoryne brassicae* attack and *Pseudomonas syringae* infestation	Barah et al. (2013)

Table 7 Representative examples of miRNAs involved in abiotic stress response

Name of the miRNA	Crop	Predicted target	Experimentally validated target	Function of miRNA	Reference
miR169	*Arabidopsis*	–	*NFYA5*	Down-regulation of this miRNA is crucial for adaptation to drought stress	Li et al. (2008)
miR169	*Arabidopsis*	At1g48500 which encodes a ZIM (zinc-finger protein expressed in inflorescence meristem) TF	–	Cold-responsive miRNA	Zhou et al. (2008)
miR827 and miR2111	*Arabidopsis*	–	E3 ligases (At1g02860 and At1g63010 by miR827 and At3g27150 bymiR2111)	Induced during phosphate deprivation	Fujii et al. (2005)
miR399	*Arabidopsis*	–	*PHO2*/*UBC24* (an E2 ubiquitin-conjugating enzyme)	Mobilisation of internal phosphate from older to younger leaves	Fujii et al. (2005)
miR395	*Arabidopsis*	Low-affinity sulphate transporter (*SULTR2*;*1*/*AST68*) and three members of the ATP sulfurylase family (*APS1, APS3, APS4*)	–	Sulphate deprivation	Jones-Rhoades and Bartel (2004)
miR397, miR398, miR408 and miR857	*Arabidopsis*	–	Copper containing proteins such as Cu/Zn SODs (CSDs), plantacyanin and several laccases (laccase-2, laccase-3, laccase-4, laccase-7, laccase-12, laccase-13 and laccase-17)	Save copper for more essential proteins, such as plastocyanin and cytochrome c oxidase, by silencing CSDs, plantacyanin and laccases	Yamasaki et al. (2007)
miR1446	*Populustrichocarpa*	–	GCN5-related N-acetyltransferase (GNAT) family protein, gibberellin response modulator-like protein	In response to cold, drought, hydration, salinity stresses	Lu et al. (2008)
miR319, miR390, miR393, miR398	*Arabidopsis*	TCP (teosinte branched/cycloidea/PCF) TF, TAS3, F-box auxin receptors, TIR1/AFBs and bHLH transcription factors, Cu/Zn superoxide dismutases (CSD) enzymes: cytosolic CSD1 and plastidic CSD2 such as the COX5b.1, the 5b subunit of mitochondrial cytochrome oxidase	–	In response to metal toxicity	Reviewed by Mendoza-Soto et al. (2012)

Table 8 Selective examples of miRNAs that are expressed in response to multiple stresses

Name of the miRNA	Stress under which it was identified	Reference
miR156	Drought, salt, cold, heat, ABA, oxidative, hypoxia, UV B	Reviewed by Sunkar et al. (2012) and Kruszka et al. (2012)
miR159	Biotic, drought, salt, cold, heat, ABA, hypoxia, UV B	
miR160	Biotic, drought, salt, cold, heat, ABA, hypoxia, UV B	
miR162	Drought, salt, cold, hypoxia	
miR165/166	Biotic, drought, cold, heat, hypoxia, UV B	
miR167	Biotic, drought, salt, cold, ABA, hypoxia, UV B	
miR393	Biotic, drought, salt, cold, heat, ABA, UV B	
miR398	Oxidative, Cu and phosphate deficiency, UV, salt, ABA, water deficit, addition of sucrose, paraquat, ozone or plant pathogens	Abdel-Ghany and Pilon (2008), Jagadeeswaran et al. (2009), Jia et al. (2009), Sunkar et al. (2005), and Yamasaki et al. (2007)

in diverse plant species are too numerous to be covered comprehensively here, and hence only selective examples are listed.

On several occasions, miRNAs are shown to be key regulators in plant disease development (Tables 6 and 8). Some of the miRNAs may get involved in virus-induced gene silencing. Helper component-proteinases *(HC-Pro), p19, p21* and *p69*, are unrelated viral suppressors of gene silencing, and they play important roles in the virus response to plant antiviral silencing response (Zhang et al. 2007). Several investigations demonstrated that quite a lot of miRNAs are related to the activity of these viral suppressors. *HC-Pro* inhibited the expression level and activity of miR171 and caused miR171-related developmental deficiency. *P69* enhanced the expression and activity of miRNAs and caused rapid degradation of miRNA-targeted mRNAs and consequently enhance plant resistance to pathogens (Zhang et al. 2007). miRNAs responsive to plant infection by viruses were also identified in many plant species, such as *Brassica rapa* (He et al. 2008), rice (Du et al. 2011), *Arabidopsis* (Hu et al. 2011) and tomato (Lang et al. 2011). The role of individual miRNAs in plant resistance to viruses has not yet been demonstrated. Rather, plants may use the general RNA silencing machinery to degrade viral RNAs or target viral DNAs for methylation (Hohn and Vazquez 2011).

While the role of RNA silencing in defence against viruses was unravelled several years ago, the involvement of miRNA-guided regulations has emerged only recently as one of the many strategies developed by plants to protect against bacterial pathogens (Navarro et al. 2006). Perception of flagellin is crucial for plant resistance to *Pseudomonas syringae* bacterium (Gómez-Gómez and Boller 2002). The miRNA miR393 seems to play an important role in this process (Navarro et al. 2006). Indeed, flagellin-derived peptide induces the expression of miR393a and down-regulates the expression of the auxin receptors AFB1 at the transcriptional level and TIR1, AFB2 and AFB3 at the post-transcriptional level. Although the direct role of miR393 in bacterial resistance has not yet been demonstrated, auxin signalling is important for resistance, and the model predicts that repression of auxin signalling by increased miR393 function would restrict *P. syringae* growth (Navarro et al. 2006). Recently, a group of bacteria-regulated miRNAs that targets genes encoding proteins of the auxin, abscisic acid and jasmonic acid biosynthetic and/or signalling pathways was identified (Zhang et al. 2011b). The expression of 12 target genes, including *ARF8, ARF10, ARF16, ARF17, TIR1, AFB2, AFB3, MYB33* and *MYB65* which are the targets of miR160, miR167, miR393 and miR159, was negatively correlated with the accumulation of their miRNAs upon *Pseudomonas* infection (Zhang et al. 2011b). The results suggested an important role of these miRNAs in plant defence signalling by regulating and fine-tuning multiple plant hormone pathways.

Similarly, high-throughput sequencing has facilitated identification of miRNAs affected in genotype sensitive to Asian soybean rust (ASR), a foliar disease caused by *Phakopsora pachyrhizi* in soybean and powdery mildew strain *Erysiphe graminis* f. sp. *tritici* in wheat (Kulcheski et al. 2011). MIR-Seq11, MIR-Seq13 and MIR-Seq15, which are predicted to target *peroxidases, oxidoreductases* and translational initiation factor transcripts, are down-regulated upon ASR infection. In wheat, 24 miRNAs responsive to powdery mildew infection have also been identified (e.g. miR156, miR164, miR167 and miR393; Kruszka et al. 2012).

Similarly, various miRNAs were identified in *Arabidopsis*, *Oryza*, *Nicotiana*, *Z. mays*, Sorghum, *Populus, Gossypium, Brassica, Vitis, Physcomitrella* and *Chrysanthemum*, and their target genes were found to encode various transcriptional factors or important functional enzymes that play critical roles in plant response to various abiotic stresses (Tables 7 and 8). Specific miRNAs have also been discovered with key roles in protecting the plants against particular abiotic stress.

For example, genome profiling of drought-stressed rice has been carried out at various developmental stages to reveal drought-responsive miRNAs (Zhou et al. 2010). It has led to the identification of 30 miRNA families, which was significantly either up-regulated (such as miR395, miR474, miR845, miR851, miR854, miR901, miR903 and miR1125) or down-regulated (such as miR170, miR172, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088 and miR1126) during drought. Prediction and validation of target genes corresponding to these miRNAs and study of their regulation at the level of transcription factors have evidenced the role of these miRNAs in drought tolerance (Zhou et al. 2010).

In another maize study, 39 miRNAs have been identified with altered expression under submergence stress (Zhang et al. 2008). Among them, expression of 19 miRNAs was up-regulated during the early stages (0–12 h) of submergence, which recovered to normal levels during later stages. However, the expression of 12 miRNAs was down-regulated during the initial stages and up-regulated after 24 h of submergence. Interestingly, seven of these 39 miRNAs were dramatically induced between 24 and 36 h of post-submergence (Zhang, et al. 2008). These miRNAs targeted genes that actively participate in eliminating reactive oxygen species (ROS) and aldehyde groups. Also, target genes possess a *cis-acting* element that is essential to cope with anaerobic conditions. The predicted targets of these miRNAs were classified into three categories (Zhang, et al. 2008). The first category includes various transcription factors involved in plant development and organ formation. For example, *ZAG1*, an agamous-like gene, was detected as a target of miR159. In addition, *HD-ZIP* is a target for miR166 and scarecrow-like family (*SCL*) is a target for miR171. The second category includes several targets of miRNAs that are involved in phytohormone cascade such as GA, Myb and auxin-responsive factors (*ARF12, ARF17* and *ARF25*). The third category includes targets encoding the proteins involved in physiological processes. The predicted targets of submergence stress-responsive miRNAs are involved in carbohydrate and energy metabolism, including *starch synthase, invertase*, malic enzyme and *ATPase*, as well as in elimination of ROS and acetaldehyde (*ALDH*) (Zhang et al. 2008). These findings have highlighted the complexity of adaptive plant responses. These adaptation strategies are helpful for survival of maize seedlings under submergence conditions.

From *Arabidopsis*, sRNA library was constructed to identify sRNAs involved in cold, dehydration and salt stress (Sunkar and Zhu 2004). This study has come out with the identification of two previously known miRNAs (miR171b and miR319c), 24 novel miRNAs constituting 15 new families and 102 novel endogenous siRNAs. From the identified miRNAs, miR393, miR397b and miR402 were up-regulated during cold, dehydration and salt stress whereas miR389a.1 was down-regulated. miR319c was found to be specifically up-regulated during cold stress (Sunkar and Zhu 2004). Microarray-based profiling of cold-responsive miRNAs has also been carried out

from rice (Lv et al. 2010). Most of the identified miRNAs were down-regulated during cold stress. miRNAs from miR167 and miR319 families were down-regulated, while miR171 families were reported for variable expression profiles (Lv et al. 2010).

Similarly, salt-responsive miRNAs have also been identified from maize roots (Ding et al. 2009). miRNA microarray hybridisation has led to the identification of 98 salt-responsive miRNAs from 27 plant miRNA families. These miRNAs showed differential expression during salt stress. While 18 miRNAs were expressed in maize salt-tolerant species, 25 miRNAs showed delayed expression in maize salt-sensitive species (Ding et al. 2009). Most of the miRNAs responsive to salt stress directly regulate transcription factors. From *Z. mays*, miR159a/b, miR164a/b/c/d and miR1661m have been cloned that target transcription factors *Myb, NAC1* and homeodomain leucine zipper protein (HD-ZIP) (Ding et al. 2009). Other salt-responsive transcription factors targeted by miRNAs included MADS-box proteins and zinc-finger proteins. Further experimentation has led to the cloning of miRNAs belonging to miR474, miR395 and miR396 families from *Z. mays*. miR474 and miR395 were reported to target negative regulators of salt tolerance. They were up-regulated during salt stress, causing suppression of the respective factors. On the contrary, miR396 was reported to down-regulate in the presence of salt stress (Ding et al. 2009).

In a yet another study, deep-sequencing technology was chosen to determine the small RNA transcriptome of *Saccharum* sp. cultivars grown on saline conditions (Bottino et al. 2013). They constructed four small RNAs libraries prepared from plants grown on hydroponic culture exposed to 170 mMNaCl and harvested after 1 h, 6 h and 24 h. Each library was sequenced individually and together generated more than 50 million short reads, and there were 98 conserved miRNAs and 33 miRNAs*. Several of the microRNA showed considerable differences of expression in the four libraries. The results showed that miRNAs had higher expression in samples treated with severe salt treatment compared to the mild one. Furthermore, the majority of the predicted target genes had an inverse regulation with their correspondent miRNAs. The targets encode a wide range of proteins, including transcription factors, metabolic enzymes and genes involved in hormone signalling, probably assisting the plants to develop tolerance to salinity (Bottino et al. 2013).

Various mechanical stresses involving wind, water or any other entity imposing physical forces upon the plant body have also been found to down- or up-regulate certain miRNAs. For example, a comparative analysis of miRNA expression was performed in *Populus trichocarpa* subjected to mechanical stress via bending the plant stem in an arch for 4 d (Lu et al. 2005b). The expression of miR156, miR162, miR164, miR475, miR480 and miR481 was found to be down-regulated whereas miR408 was up-regulated in the xylem tissue of mechanically stressed plants as compared to the unstressed control (Lu et al. 2005b).

The above findings suggest that miRNA profiles are unique in closely related genotypes with contrasting stress sensitivities. Thus, it is likely that a more comprehensive analysis, including the impact of such regulation on miRNA targets, would provide better insights into miRNA-guided gene regulation that differs in stress-tolerant genotypes. Such molecular mechanisms could then be incorporated into strategies for improving the stress tolerance of crop plants.

miRNAs Responsive to Multiple Stresses

There are some miRNAs that are expressed in response to multiple biotic and abiotic stresses (Table 8). A specific well-studied example is miR398 which is involved in responses to diverse stresses such as oxidative stress, Cu and phosphate deficiency, ultraviolet (UV) stress, salt stress, ABA stress and water deficit and addition of sucrose, paraquat, ozone or plant pathogens (Abdel-Ghany and Pilon 2008; Jagadeeswaran et al. 2009; Jia et al. 2009; Sunkar et al. 2005; Yamasaki et al. 2007). Thus understanding the

miR398-mediated stress regulatory network would provide new potential tools for genetic improvement of combined stress tolerance in plants.

Forthcoming Perceptions

The biological functions of the majority of plant miRNAs, including non-conserved and conserved miRNAs in plants, have yet to be uncovered. Up to now, a large body of evidence supports the idea that miRNAs are involved in a broad spectrum of biological progresses involving negative post-transcriptional gene regulation. Based on increasing numbers of specific miRNA functional study, it is indispensable for us to construct a global view about miRNA regulation mechanisms and understand miRNA in different angles (Cai et al. 2009). Thus the regulation of miRNA biogenesis or activity will be a major area of interest. As described by Chen (2009), there are several issues to be resolved including the following: 'Is the processing of specific miRNAs regulated? Are the activities of specific miRNAs regulated? What determines when amiRNA inhibits the translation of its target mRNA rather than cleaving it? How does amiRNA inhibit the translation of its target mRNA?' Similarly, we are yet to know the fates of these miRNAs, i.e. after repressing their targets, what are the molecular mechanisms to get rid of these miRNAs?

In view of the important roles of miRNA in the regulation of gene expression and hence tissue functions and phenotypes, investigations of miRNA offer many opportunities. One application is the use of concepts and techniques for gene targeting to achieve the inhibition of miRNAs in vitro and in vivo (Sun et al. 2010). In the complementary approach, the development of tools for the delivery of miRNAs to suppress the expression of target genes involved in pathogenesis is equally important. These concepts have been adopted for the development of drugs using miRNAs (Sun et al. 2010).

It is generally conceived that miRNAs downregulate gene expression by cleaving mRNA or by repressing mRNA translation. Such understanding has several applications in agriculture. As such, it may be possible to design artificial miRNAs to suppress target gene expression in order to study gene function, similar to the use of antisense mRNA and RNAi which are widely used as tools for studying gene function (see below). Another possibility is the use of miRNA knowledge to improve plant yields, quality or resistance to various environmental stresses including insect and pathogen infection. For example, crop resistance to drought could be improved by down-regulating miR169 (Table 7). Further study of miRNAs could provide us with new tools for increasing crop yield and/or quality.

Antagomirs, a group of modified anti-miRNA oligonucleotides, are currently the most readily available tools for miRNA inhibition. They have been applied successfully to inhibit specific endogenous miRNAs in cell cultures and mice (Krutzfeldt et al. 2005). To enhance the delivery efficiency of antagomirs to target tissues, several techniques were used to conjugate or package the antagomirs. These include methods based on the uses of lipids (e.g. cholesterol or liposomes), peptides (e.g. TAT leading sequences), proteins (e.g. binding proteins or antibodies), viruses (e.g. retroviral and adenoviral vectors), hydrogel and nanoparticles (Sun et al. 2010). Further developments in antagomir oligonucleotide design, packaging and local delivery through novel principles and technologies will serve to enhance the effectiveness of these antagomirs.

In another strategy, synthetic RNAs containing miRNA-targeted sites can serve as a 'decoy' or 'sponge' to compete with miRNA in binding to its target mRNA and thus inhibit miRNA functions. The concept of amiRNA sponge was reported first by Ebert et al. (2007), who engineered the tandem repeats of the putative miRNA-binding sites into the 3′ UTR of green fluorescent protein or luciferase reporter genes. Their results demonstrated that the miRNA sponge effectively suppresses the expression of the reporter gene. Notably, Ebert et al. (2007) have shown that the miRNA sponge outperforms antagomir in most miRNAs tested and that the combination of sponges and antagomir exhibits a synergistic

effect. These studies indicate that miRNA sponges can effectively modulate the endogenous miRNA and their target functions. Future directions for bioengineers interested in this area include designing and manufacturing miRNA-sponge expression systems, engineering miRNA sponges to be inducible and tissue specific and developing and improving various delivery vehicles and tools (Sun et al. 2010).

It is possible that the engineering of miRNA constructs may use some of the strategies established for other small RNAs. An example is the tunable RNA interference (RNAi) construct (Deans et al. 2007) with the use of two coupled repressor proteins: one controlling the small hairpin RNA (shRNA) gene expression and another controlling the target gene expression. The shRNA and target gene expressions can thus be controlled by adding inducers specific to their coupled repressors. With such multi-repressor modules, the target gene can be temporally tuned in the presence or absence of shRNA. The various components in the construct are modular in nature, thus allowing regulation of a desired gene in tissue-specific and inducible manners. Although originally designed for shRNA targeting, such a strategy may be applicable for engineering an miRNA-based gene switch (Sun et al. 2010).

As stated in the beginning, artificial miRNAs (amiRNA) that act on specific targets can also be created and they have potential applications in plant functional genomics (Parizotto et al. 2004). Such amiRNAs can reduce the abundance of gene transcripts containing a complementary sequence. Web-based resources have been developed to aid in plant amiRNA design, such as WMD3 (Schwab et al. 2006; http://wmd3.weigelworld.org/). The amiRNA sequence is then integrated into a modified miRNA precursor within a functional miRNA transcript. This amiRNA precursor can be inserted into a transformation vector and introduced into plants for expression. Similar to the native miRNAs, amiRNAs can be processed to their mature forms and direct the RISC to down-regulate the target genes. For plant species where whole genomes have been sequenced, it is possible to avoid off-target suppression by selecting an amiRNA sequence that can distinguish between closely related genes. The amiRNA technique is of great value in functional genomics for crop species because amiRNAs act as dominant suppressors in the first generation of plant transformation. Sun et al. (2012) stated that amiRNA transgenics are more stable and efficient than those created by RNA interference (RNAi).

The greatest gap in our knowledge remains in the unknown functions of the majority of genes in plants. Future miRNA studies will likely be directed to finding functions for genes and dissecting functional redundancy. Thus, miRNAs and amiRNAs are powerful tools for basic research and for genetic modifications. miRNA-based technology will allow the specific down-regulation of a great many genes of unknown function. Using gene-specific suppression with miRNAs or amiRNAs, it is possible to distinguish functions of redundant genes. Genetic manipulation or engineering of new miRNAs could allow the specific regulation of candidate genes for modification of metabolism, growth, development and adaptation of plants. Such modifications would advance breeding programmes in agriculture, horticulture and forestry and improve productivity or response to climate change.

Closing Comments

A tiny spark can burst into mighty flame. Similarly, miRNAs, an evolutionary conserved class of small (~22 nt) non-coding RNAs, have huge roles in cellular processes, and it has recently created much attention among the molecular researchers. During earlier days, several computational approaches have estimated that every biological system may contain ~ 1 % miRNA genes of the total protein-coding genes. However, later bioinformatic studies indicated that the proportion of genes in the genome under miRNA regulation may be much larger than previously thought. For example, it is now considered that about 30 % of all human genes may be regulated by miRNAs (Jones-Rhoades 2012).

However, only few hundred miRNAs have been deposited to date in the miRNA database. This is far from the predicted miRNA number, and it requires more comprehensive strategies to capture all the miRNAs.

Further, there are many questions that remain to be addressed (see above). Although much work is focused on sRNAs, similar mechanisms may also account for the inheritance of other non-coding RNAs, including long non-coding RNAs (lnRNAs). We now have data on miRNAs and miRNA targets in representative species from green algae, non-seed plants and gymnosperms. But we do not have much information about the extent of diversity or conservation of miRNA expression within any of these lineages. Similarly, the analysis of more examples of closely related species and accessions will provide a clearer picture of how miRNAs evolve over shorter time frames. Besides it is unclear whether plants under stress use both modes of target gene regulation, i.e. degradation of transcripts and translational repression, or whether one mode is preferred over the other.

Identification of stress-responsive miRNAs is largely dependent on sequence-based profiling, which is known to have some bias, and thus requires independent validation. Small RNA blot analysis, although lacking sensitivity, is a gold standard for validation. Most published studies have not systematically confirmed profiling data using small RNA blot analysis. Implementation of highly reliable and rigorous assays is essential for firm characterisation of stress-responsive miRNAs in plants. Examining the effect of stress-regulated miRNA on its mRNA target using degradome analysis can provide robust confirmation of the stress responsiveness of miRNA (Sun et al. 2012). In addition to identifying miRNA targets, by analysing degradome libraries from control and stressed samples, it should be possible to quantify the impact of a stress-responsive miRNA on its mRNA target.

Further, studying stress-responsive miRNAs and their target gene expression in individual cell types will provide greater insights into miRNA target networks that operate in a cell- or tissue-specific manner during stress. As our understanding of the roles of miRNAs during stress deepens, the possibilities for using miRNA-mediated gene regulation to enhance plant stress tolerance will become enormous.

Thus, investigations on miRNA offer new and exciting opportunities for scientists. Manipulation of miRNA activities can lead to the integrative understanding of the molecular basis of regulation to systems levels and also help in developing new ways to design the miRNA sequences for enhancement of their agricultural applications and to improve the innovative algorithms and analysis methods for the further advancement of this novel research field. For example, a better understanding of small RNA regulation in hybrids and allopolyploids will help us effectively select the best combinations of parents for producing hybrids and allopolyploid plants and manipulate small RNA expression to overcome species barriers and produce superior hybrids to meet the growing demand in food, feed, cloths and biofuels.

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