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Plant microRNAs: new players in functional genomics

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Abstract: MicroRNAs (miRNAs) are small, endogenously expressed, and nonprotein coding RNAs that regulate gene expression via post-transcriptional inhibition and cleavage. To date, several plant miRNAs have been identified via direct cloning, high-throughput sequencing, and bioinformatics analyses. The miRNAs participate in RNA-induced gene silencing complex, and specifically repress the target gene transcripts. Thus, miRNAs regulate the expression of genes playing diverse roles in plants, such as root initiation, leaf morphology, flower development, and response to environmental stimuli. A number of miRNAs have been identified and functionally characterized in eukaryotes. In this review, we discuss the functional roles of miRNAs in plant development as well as stress response to biotic and abiotic environmental factors. Additionally, we present brief information about miRNA detection and discovery techniques.

Key words: High-throughput next generation sequencing, plant development, stress response

1. Introduction

MicroRNAs (miRNAs) are endogenous, small, noncoding RNA molecules playing crucial roles in the regulation of gene expression at the post-transcriptional level in eukaryotes and viruses (Carrington and Ambros 2003; Bartel 2004; Schwab et al. 2005; Unver et al. 2009). These regulatory small RNA molecules achieve their roles through sequence-specific interactions with complementary sites of target mRNA that lead to their degradation (cleavage) or translational repression. Plant miRNAs are approximately 21–24 nucleotides in length, generally having a high degree of complementarity (near-perfect) between miRNAs and their targets, whereas animal miRNAs usually display partial complementarities to their targets; however, that is not the only difference between plant and animal miRNAs (Millar and Waterhouse 2005; Axtell et al. 2011). Although miRNAs share similarities in general, plant pre-miRNAs have larger and more variable stem–loop structures. Mature plant miRNAs often recognize a single target site in the coding region, pair their target sites with near-perfect complementarity, and guide the mRNA to cleavage, suggesting that plant miRNAs may act like siRNAs due to this specificity (Yang et al. 2007). To date, a total of 21,643 mature miRNAs have been identified from 168 species including viruses, a filamentous brown alga (*Ectocarpus siliculosus*), a diatome (*Phaeodactylum tricornutum*), a soil-living amoeba (*Dictyostelium discoideum*), a green alga (*Chlamydomonas reinhardtii*), plants, and animals. Identified miRNAs have been deposited in the publicly available miRNA database (miRBase v18 release November 2011; http://www.mirbase.org) (Kozomara and Griffiths-Jones 2011). Now, totally 4014 miRNAs belonging to 52 plant species have been loaded since the discovery of miRNAs in plants in 2002 (Park et al. 2002; Reinhart et al. 2002).

2. Biogenesis of plant miRNA

The miRNAs are expressed from their own genes located in the intergenic (between protein-coding genes) or intragenic region (within protein-coding genes, in an exonic or intronic manner) on the chromosomes (Lagos-Quintana et al. 2001; Wang and Blelloch 2009). In plants, most miRNA genes are intergenic and transcribed individually from their own region, but a few genes are organized into polycistronic transcription units and co-transcribed from a single promoter at the end of a miRNA gene cluster (Bartel 2004; He and Hannon 2004; Voinnet 2009). It has been also reported that most miRNA genes in plants and animals have TATA box motifs upstream of their transcription start sites (TSSs), which are transcribed, 3′-poly-adenylated, and 5′ capped by RNA Polymerase II (POL II) like most protein-coding genes (Houbaviy et al. 2005; Xie et al. 2005; Megraw et al. 2006). Biogenesis of plant miRNAs requires a multiple biological process

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to generate functional mature miRNAs by recruiting several conserved protein families. Plant miRNA genes are principally transcribed into primary miRNA transcripts (pri-miRNA) by RNA polymerase II (Lee et al. 2004). The pri-miRNA molecules typically contain a region of imperfect self-complementarity and are processed into stem-loop secondary structures (pre-miRNAs) including mature miRNAs in one arm of the secondary structures. A recent study indicated that pri-miRNAs are stabilized by a nuclear-localized FHA domain-containing protein Dawdle (DDL) in *Arabidopsis* (Yu et al. 2008). Following transcription, these long (up to 3 kb) and stabilized primer transcripts are cleaved to hairpin-like miRNA precursors "pre-miRNAs" by RNAse III enzyme Dicer like1 (DCL1) with the aid of double-stranded RNA (dsRNA) binding protein Hyponastic leaves1 (HYL1) and a C2H2 zinc-finger protein Serrate (SE) (Park et al. 2002; Reinhart et al. 2002; Hiraguri et al. 2005). A previous study reported that these dsRNA-binding proteins (HYL1 and SE) are necessary for maintaining plant miRNAs biogenesis properly (Dong et al. 2008). Since SE and HYL1 proteins might play an important role in the recognition and directing of pri-miRNAs, they help DCL1 for loading as well as positioning (Kurihara et al. 2006; Lobbes et al. 2006; Yang et al. 2006). The SE and HYL1 proteins interact with DCL1 in special regions in the nucleus called dicing bodies (D-bodies) or SmD3/SmB-bodies in which stabilized pri-miRNAs are collected and also serve as nuclear processing centers (Fang and Spector 2007; Fujioka et al. 2007). After DCL1-mediated processing of pri-miRNAs in the nucleus, miRNA:miRNA* duplexes, containing a 2-nt overhang at the 3′-end, are formed and methylated by the 3′-methyltransferase Hua enhancer1 (HEN1) that adds methyl groups to 2′-OH of the 3′-terminal nucleotide of each strand (Yang et al. 2006). The methylated 2 nucleotide overhang formation at the 3′-end of miRNA/miRNA* duplex provides the biochemical requirement for the Dicer cleavage activity. Methylation is a crucial step in miRNA biogenesis that prevents miRNA uridylation and degradation from exonuclease activity of the small RNA degrading nuclease (SDN) family (Li et al. 2005; Yu et al. 2005; Ramachandran and Chen 2008). The transportation of the methylated miRNAs/miRNAs* (pre-miRNAs) from the nucleus into the cytoplasm requires a protein known as Hasty (Hst), an *Arabidopsis* homolog of exportin-5 that seems to be cooperating with Ran-GTP (Bollman et al. 2003; Park et al. 2005). Following the detachment of miRNA duplex in the cytoplasm, one strand (mostly miRNA strand) of the miRNA/miRNA* duplex is selectively incorporated with RNA-induced silencing complex (RISC) containing an Argonaute (AGO) family protein, while the other strand (the miRNAs*) is degraded (Baumberger and Baulcombe 2005). Among the 10 AGO family members, AGO1 was found to be one of the most important members for miRNA biogenesis and processing in plants (Vaucheret et al. 2004; Kinder and Martienssen 2005; Ronemus et al. 2006; Mi et al. 2008; Takeda et al. 2008). Argonaute proteins have 2 conserved RNA binding domains: an N-terminal PAZ domain that can bind to the 3-end of single-stranded RNAs, and a PIWI domain that is structurally similar to RNase H in the carboxy-terminus, which is responsible for the endonuclease activity of RISC (Cerutti et al. 2000; Liu et al. 2004; Parker et al. 2004; Miyoshi et al. 2005). Selection of a convenient RNA strand of a miRNA:miRNA* duplex loaded into AGO-containing RISC complex is dependent on several factors: (i) preferential association of AGO1 with miRNA having 5′ terminal uridine residues, (ii) differential thermostability of the miRNA/miRNA* duplex, and (iii) accessory proteins in D-bodies such as HYL1/DRB1. Eventually, functional mature miRNA with AGO1 protein guides the cleavage or the translational repression of target mRNAs through base-pairing interactions (Mi et al. 2008; Takeda et al. 2008; Eamens et al. 2009). In Figure 1, the general steps and important players in plant miRNA biogenesis are depicted. As explained above, only one strand of miRNA/miRNA, miRNA strand (guide strand) successfully incorporated an AGO1-containing RISC, whereas the other strand, considered as a passenger strand or miRNA* (miRNA star) is short-lived and mostly eliminated. Strand selection is largely determined by the thermodynamic features of the miRNA duplex (Khvorova et al. 2003; Schwarz et al. 2003). Due to its rapid degradation, miRNA* sequence is generally low-abundance molecules as compared with its corresponding miRNA partner; however, its expression level can be directly assessed by deep sequencing approach. In addition to their stability differences, plant miRNA shows a high degree of sequence complementarity with miRNA* unlike animal miRNA (Jones-Rhoades et al. 2006). It was also noted that some miRNA* strands could be functional and are also phylogenetically conserved as their miRNAs. As developmental and evolutionary implications, phylogenetically conserved miRNA* might play an important role in providing plentiful miRNA at specific times (Guo and Lu 2010).

3. Functional roles of the plant microRNAs

Studies showed that many predicted and experimentally confirmed miRNA targets are genes encoding regulatory proteins, showing that miRNAs play a role at the core of gene regulatory networks. Functional genomics studies have shown the involvement of plant miRNAs in many developmental processes (Jones-Rhoades et al. 2006; Jung et al. 2009) and their diverse roles in stress responses (Sunkar et al. 2012). Molecular mechanisms regulating developmental transitions, such as seed germination,

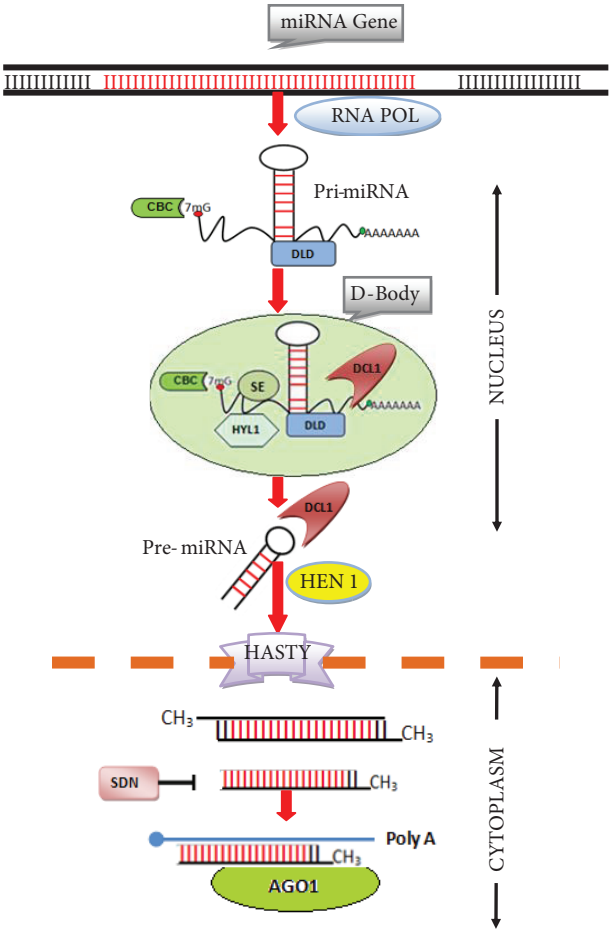


Figure 1. Biogenesis of plant miRNA. The general steps in plant microRNA (miRNA) biogenesis pathway: the primary transcripts (pri-miRNA) of plant-miRNA are transcribed by RNA polymerase II (POL II) from their own chromosomal loci located between protein-coding genes (mostly). Pri-miRNA genes have 5' caps and 3' poly-A tails that are the hallmark of RNA polymerase II transcription. Pri-miRNA generates one (or more) stem-loop (hairpin) secondary structures and is likely stabilized by a nuclear RNA binding protein Dawdle (DLD) until their processing has been completed in the D-body. Following transcription, pri-miRNA is converted to pre-miRNA by RNAse III enzyme Dicer like1 (DCL1) with the help of Hyponasty leaves1 (HYL1), Serrate (SE), and nuclear cap-binding complex (CBC). DCL1 is responsible for processing both the pri-miRNA and pre-miRNA sequences within the nucleus due to lack of Drosha homologs in plants. Then the miRNA/miRNA* duplex is methylated at the 3'-ends by the methyl transferase HEN1, which protects the duplex from small RNA degrading nuclease (SDN) and exported to the cytoplasm via exportin-5-like protein Hasty. Eventually, the methylated miRNA/miRNA* duplex is selectively incorporated with RISC containing Argonaute (AGO) to repress or cleavage target mRNA.

vegetative and reproductive phase changes, flowering initiation, and seed production have been extensively studied (Wu et al. 2006; Jung et al. 2009; Nodine and Bartel 2010; Wu et al. 2011; Yang et al. 2011). One of the regulatory players for developmental processes is miRNAs (Table 1). In addition to their important functions in organ development, such as leaf morphogenesis, floral organ identity, and root development, plant miRNAs play a crucial role in feedback regulation of small RNA pathways and in the biogenesis of some siRNAs. Additionally, plant miRNAs are involved in various stress responses, such as salinity, drought, mineral-nutrient, mechanical, and pathogen stresses (Liu et al. 2008; Zhou et al. 2008; Trindade et al. 2010; Sunkar et al. 2012; Thiebaut et al. 2012) as well as metabolism, signal transduction, and protein degradation processes (Unver et al. 2010a) (Table 2).

4. Regulation of plant development by miRNAs

Plant miRNAs are involved in regulatory networks to coordinate gene expression, playing important roles in developmental processes. Several developmental programs were detected that involved miRNAs, such as root initiation and development (Montgomery et al. 2008; Marin et al. 2010), vascular development (Yu et al. 2005; Donner et al. 2009), leaf morphogenesis and polarity (Palatnik et al. 2003; Mallory et al. 2004), floral differentiation (Chuck et al. 2008), and phase transition from vegetative growth to reproductive growth (Yang et al. 2007; Yant et al. 2010). Loss-of-function in some miRNA genes and miRNA complementary sites of target genes as well as in the genes related to miRNA biogenesis lead to abnormalities in plant development and growth, such as abnormal leaf development (Aukerman et al. 2003; Palatnik et al. 2003), vascular development (Kim et al. 2005), and flower morphology (Chen 2004); male sterility (Xing et al. 2010); and early flowering (Aukerman et al. 2003; Chen 2004). The *dcl1* and *hasty* are important genes for plant miRNA biogenesis, and their loss-of-function results in abnormalities in plant growth and development, such as altered leaf morphology, delayed floral transition, female sterility, and early stage embryo arrest (Dugas and Bartel 2004; Rohades et al. 2006; Zhang et al. 2006b). Additionally, several defects, such as leaf shape disruption and altered flower morphology, reduced fertility, and differentiated vegetative phase transition (Bollman et al. 2003) were detected in plants having loss-of-function in the *hasty* gene because of reduced levels of most miRNAs (Park et al. 2005) (Table 1).

4.1. Root initiation and development

The plant's root system is crucial for stability and interaction with the environment as well as nutrient and water uptake. Lateral roots initiate from a small number of pericycle cells and, depending on the environmental conditions, they differentiate into a primordia and grow out of the primary root. Transcription induced by auxin

Table 1. MicroRNAs in plant developmental processes.

Plant	Plant organ	miRNA	Target gene	Function	References
Arabidopsis	Root	miR390	*tas3*, *arf*	Root development	Montgomery et al. (2008), Marin et al. (2010), Yoon et al. (2010)
Arabidopsis	Root, leaf, flower, seed	miR164	*nac1, cuc2*	Lateral root emergence, leaf morphology, petal development regulation, floral meristem activity, seed development	Mallory et al. (2004), Laufs et al. (2004), Guo et al. (2005), Nikovics et al. (2006), Jung et al. (2009)
Arabidopsis, rice, barley	Root, seed	miR160	*arf10*, *arf16*	Lateral root initiation, seed development	Mallory et al. (2005), Sorin et al. (2005), Xue et al. (2009), Kantar et al. (2010), Nodine and Bartel (2010)
Arabidopsis, rice, barley, opium poppy	Root, flower	miR171	*scl6*	Root and flower development	Xue et al. (2009), Kantar et al. (2010), Unver et al. (2010b)
Arabidopsis	Leaf, vascular system, flower, seed	miR165/166	*hd-zip III* gene family, *phb*, *phv*	Leaf development and morphology, meristem, seed development	Mallory et al. (2004), Kim et al. (2005), Williams et al. (2005), Zhang et al. (2006b)
Antirrhinum majus, Arabidopsis	Leaf, seed, flower	miR159	*cin*, *tcp, gamyb*	Leaf morphogenesis, seed development and germination, flowering time regulation, reproductive development	Palatnik et al. (2003), Chen (2005), Allen et al. (2007), Jung et al. (2009)
Arabidopsis, rice, poplar	Leaf, flower, seed	miR156 and miR157	*sbp box*	Leaf morphogenesis, phase transition, flowering time regulation, seed development	Rhoades et al. (2002), Wu and Poethig (2006), Gandikota et al. (2007), Wang et al. (2008), Nodine and Bartel (2010), Zhao et al. (2010), Wang et al. (2011a), Kim et al. (2012)
Arabidopsis	Leaf	miR396	*grf*	Leaf development	Liu et al. (2009), Rodriguez et al. (2010)
Peanut	Root and leaf	miR3508	*ppo*	Root and leaf development	Chi et al. (2011)
Arabidopsis, rice, maize	Flower	miR172	*toe1*, *toe2*, *toe3, ids1*, *gloy15*	Flowering time, vegetative phase change	Aukerman and Sakai (2003), Lauter et al. (2005), Chuck et al. (2007), Yang et al. (2007)
Opium poppy	Flower	miR169	*cbf-b/nf-ya*	Flowering time	Unver et al. (2010b)
Arabidopsis, rice	Flower, root, seed	miR167	*arf6*, *arf8*	Root elongation, gynoecium and stamen development, seed development	Gutierrez et al. (2009), Jung et al. (2009), Xue et al. (2009), Vidal et al. (2010)
Arabidopsis, rice	Flower	miR444	*mads-box*	Flower development	Li et al. (2010)
Rice	Seed	miR397 and miR528	*ao*	Seed development	Xue et al. (2009)
Arabidopsis, tomato	Seed, leaf	miR319	*tcp4, lanceolate*	Seed development, leaf development and morphology	Palatnik et al. (2003), Jones-Rhoades et al. (2006), Efroni et al. (2008), Nodine and Bartel (2010), Shleizer-Burko et al. (2011)
Soybean	Seed	miR1530	*tkt*	Seed development	Song et al. (2011)

Table 2. Common miRNA related with stress response in plants.

Plant	Stress	miRNA	Target genes and proteins	References
Arabidopsis, barley, rice, *Populus*	Drought, salt, cold, fungal pathogen	miR156	SBP family TFs	Liu et al. (2008), Lu et al. (2008), Kantar et al. (2010), Zhou et al. (2010), Li et al. (2011), Xin et al. (2011),
Arabidopsis, bean	Drought, ABA, salt, bacterial pathogen	miR159	MYB33 and MYB101, MYB33 and MYB65 TFs	Reyes and Chua (2007); Frazier et al. (2011), Alonso-Peral et al. (2012)
Populus, maize, rice	Drought, salt	miR162	*dcl1*	Zhou et al. (2010), Li et al. (2011a)
Populus	Mechanical stress	miR164	NAC TFs	Lu et al. (2005)
Arabidopsis, barley, soybean, wheat, maize	Salt, cold, drought heat, fungal and bacterial pathogen	miR165/ miR166	HD-ZIP III TFs, PHABULOSA, *homeobox* genes	Liu et al. (2008), Zhou et al. (2008), Lv et al. (2010), Ding et al. (2009), Kantar et al. (2010), Xin et al. (2011), Li et al. (2011b), Zhang et al. (2011a)
Arabidopsis, maize, rice	Drought, salt, cold, nutrient deprivation, bacterial pathogen	miR167	ARF6 and ARF8 TFs	Liu et al. (2008), Li et al. (2010), Lv et al. (2010), Zhou et al. (2010), Frazier et al. (2011), Zhang et al. (2011a), Sunkar et al. (2012),
Arabidopsis, rice, *Populus,* wheat	Drought, salt, cold, heat	miR168	*ago1*	Liu et al. (2008), Lu et al. (2008), Zhou et al. (2010), Xin et al. (2011)
Arabidopsis, rice	Drought, salt, cold	miR169	NF subunit Y, CCAAT-BOX Binding Factors	Zhao et al. (2007), Zhang et al. (2009), Li et al. (2010), Frazier et al. (2011)
Arabidopsis, Populus, barley, rice	Drought, mechanical, salt, cold	miR171	SCL TFs	Reyes et al. (2007), Liu et al. (2008), Kantar et al. (2010), Lv et al. (2010), Frazier et al. (2011)
Arabidopsis, Populus, barley, rice	Cold, drought	miR172	AP2 TF	Liu et al. (2008), Zhang et al. (2009), Frazier et al. (2011), Xin et al. (2011)
Arabidopsis, rice	Salt, drought, cold, bacterial pathogen	miR319	TCP family TFs	Liu et al. (2008), Lv et al. (2010), Zhang et al. (2011a)
Arabidopsis, Populus	Cold, bacterial pathogen, drought	miR390, miR476	GLUTAMATE RECEPTOR proteins	Liu et al. (2008), Lu et al. (2008), Lv et al. (2010), Zhang et al. (2011a)
Arabidopsis, rice, wheat	Salt, heat, cold, ABA, drought, nutrient deprivation, bacterial and fungal pathogen	miR393	F-BOX protein, TRANSPORT INHIBITOR RESPONSE1 protein, *tir1*, *afb2*, and *afb3* genes	Navarro et al. (2006), Reyes et al. (2007), Zhao et al. (2007), Liu et al. (2008), Arenas-Huertero et al. (2009), Li et al. (2010), Frazier et al. (2011), Xin et al. (2011), Sunkar et al. (2012)
Arabidopsis, rice	Nutrient deprivation, drought	miR395	*aps*	Bartel (2004), Rhoades and Bartel (2004), Zhou et al. (2010)
Arabidopsis, barley	Drought, salt, cold	miR396	GRF family TFs	Liu et al. (2008), Kantar et al. (2010), Chi et al. (2011), Frazier et al. (2011)
Arabidopsis, rice, soybean, *Brachypodium*	Cold, ABA, salt	miR397b	LACCASEs	Sunkar and Zhu (2004), Zhao et al. (2007), Liu et al. (2008), Zhang et al. (2009), Zhang et al. (2009)
Arabidopsis	Nutrient deprivation, oxidative stress, bacterial pathogen	miR398	*csd1, csd2, ccs1, cox5b.1*	Sunkar et al. (2006, 2012), Abdel-Ghany et al. (2008), Gifford et al. (2008), Trindade et al. (2010), (Li et al. 2010a), Li et al. (2011c), Zhang et al. (2011a), Zhu et al. (2011)
Arabidopsis	Nutrient deprivation	miR399	*ubc*	Lu and Huang (2008)
Arabidopsis	Cold, ABA, salt	miR402	*DML3,* ARGONAUTE 2	Sunkar and Zhu (2004), Zhou et al. (2008)
Populus, barley	Mechanical stress, drought	miR408	PLANTACYANIN	Lu et al. (2005), Kantar et al. (2010), Trindade et al. (2010)
Populus	Cold	miR474	PPRs	Lu et al. (2008)
Rice	Cadmium stress	miR166	Homeodomain-leucine Zipper (HD-Zip) TFs	Ding et al. (2011)
Rice	Aluminum stress	miR528	F-BOX/LRR-MAX2	Lima et al. (2011)
Arabidopsis	Bacterial	miR825	REMORINA, ZF, homeobox	Li et al. (2010a).
Arabidopsis	Drought, ABA	miR2118	*Unknown gene*	Arenas-Huertero et al. (2009)

hormone forms the lateral root development through transcription factors of the auxin response factor (ARF) family (Moreno-Risueno et al. 2010). Several miRNAs connect with auxin signaling (Rubio-Somoza et al. 2009): for lateral root initiation, miR164 targeting *nac1* (Mallory et al. 2004; Guo et al. 2005) and miR160 targeting *arf10/arf16/arf17* play regulatory roles (Xue et al. 2009), while miR390 targeting *tas3-arf* genes (Marin et al. 2010; Yoon et al. 2010), miR167 targeting *arf8*, and miR393 targeting *afb3* (Vidal et al. 2010) provide proper root elongation. *Arabidopsis* miR164 targets *nac1* gene transcript, which transduces auxin signals for lateral root emergence. In loss-of-function miR164 mutants, *nac1* mRNA level was found to be accumulated and resulted in more lateral roots. Conversely, miR164 overexpressor *Arabidopsis* showed decreased lateral root emergence with suppressed *nac1* mRNA level (Guo et al. 2005). Another miRNA is miR160, functioning in root development and growth. The *arf10, arf16*, and *arf17* mRNAs are targets of miR160 and their expression levels increase in loss-of-function miR160 *Arabidopsis*. Through miR160 suppression, several developmental defects including rooting formation were observed (Mallory et al. 2005). Additionally, miR160-resistant plants cause root tip defects (Wang et al. 2005). The miR167 targets *arf6* and *arf8*. The *arf6* induces miR160 to repress *arf17*. In contrast, *arf17* may activate miR167 to coordinate a*rf6* and *arf8* expression and root development (Table 1). Therefore, miR160 and miR167 targets have opposite roles in auxin regulation (Gutierrez et al. 2009).

4.2. Leaf development

Leaf morphogenesis and development are also regulated by miRNAs (Palatnik et al. 2003). Studies have identified some miRNAs that are related to leaf development such as miR396 (Liu et al. 2009; Rodriguez et al. 2010), miR319 (Schommer et al. 2008), miR164a (Nikovics et al. 2006), and miR156 (Wang et al. 2008). The *Arabidopsis* miRNA, miR396, has been found to be a regulator of plant-specific transcription factor (growth regulating factor) genes (Jones-Rhoades and Bartel 2004). Smaller leaf size occurred upon overexpression of miR396 in *Arabidopsis* (Liu et al. 2009), and the miR396-resistant *grf2* provided bigger leaf size as compared to the wild type (Rodriguez et al. 2010). The miR396 antagonizes the expression of *grf2* in the distal part of the leaf, attenuating cell proliferation in developing leaves by down-regulation of GRF activity. Its expression is also found at low level in meristem to regulate cell proliferation and size (Rodriguez et al. 2010). The miR319, also called miRJAW in *Arabidopsis*, regulates *tcp* genes, which are plant-specific transcription factors involved in leaf development (Palatnik et al. 2003; Schommer et al. 2008). The *tcp4* mRNA is targeted to be cleaved by miR319, and expression of miR319-resistant *tcp4* in *Arabidopsis* resulted in abnormal leaf morphology (Palatnik et al. 2003; Efroni et al. 2008). Moreover, overexpression of miR319 in tomato produced super-compound leaves (Ori et al. 2007). The tomato *tcp* homolog *lanceolate* gene expression correlated with diverse type of leaf shapes (Shleizer-Burko et al. 2011). Interestingly, the *lanceolate* escaped from miR319 regulation via differential expression level. Therefore, stage-specific expression of miR319 or a repressor version of *lanceolate* resulted in different leaf shapes (Shleizer-Burko et al. 2011). The *cincinnata* (*cin*) gene from snapdragon (*Antirrhinum majus)* encodes a member of the TCP family of transcription factors, required for the differential regulation of cell division during leaf morphogenesis to form a flat leaf (Nath et al. 2003). Mutation in the *cin* gene resulted in morphological changes (Crawford et al. 2004). The *Arabidopsis* genome contains at least 2 regions with the potential to produce miRJAW, as well as several potential precursors for a family of 3 almost identical miRNAs (miR159a–c) that are related to miRJAW (Palatnik et al. 2003). miR165/166 and miR159/JAW are essential for controlling the pattern and development of leaves by direct regulation of 2 classes of transcription factor genes (*hd-zip* and *tcp*) (Zhang et al. 2006a). In addition, miR156a and miR157a, located on chromosome 2 and chromosome 1, respectively, target mRNA coding for the squamosa promoter-binding protein (Sbp) box, which is involved in leaf morphogenesis (Table 1) (Zhao et al. 2010). The miR396 targets GRF transcription factors required for coordination of cell division and differentiation during leaf development in *Arabidopsis* (Chi et al. 2011). In peanut, a legume-specific miRNA, miR3508, targets polyphenol oxidase (*ppo*) genes in the roots and leaves. PPO is localized on the thylakoids of chloroplasts and in vesicles or other bodies in non-green plastid types, and catalyzes the oxygen-dependent oxidation of phenols to quinones (Chi et al. 2011).

4.3. Vascular development

Vascular development is initiated by the formation of provascular cells that subsequently develop into procambium, from which both conducting tissues are eventually differentiated (Jung et al. 2008; Donner et al. 2009). A subset of class III homeodomain-leucine zipper (HD-ZIP III) transcription factors play a role in vascular development. In *Arabidopsis*, the *hd-zip III* gene family includes 5 members: *athb15*, *athb8*, *phavoluta* (*phv*), *phabulosa* (*phb*), and *revoluta* (*rev*), which are regulated by miR165 and miR166. The *athb15* and *athb8* are predominantly expressed in vascular tissue, suggesting that they may have some role in vascular development. The *athb15* mRNA is cleaved in standard wheat germ extracts and in *Arabidopsis*, and its cleavage is mediated by miR166 in *Nicotiana benthamiana* cells. Overexpression of miR166a resulted in decreasing *athb15* mRNA levels and caused accelerated vascular cell differentiation from cambial/procambial cells, and

consequently produced an altered vascular system with expanded xylem tissue and an interfascicular region. The miR166/165 complementary sequence is highly conserved in mRNAs of *athb15* and its gene homologs from various plant species. It is, therefore, likely that miR166-mediated *hd-zip III* gene repression is conserved in all vascular plants (Kim et al. 2005; Zhang et al. 2006a).

4.4. Flower development

The genes required for proper flower development and timing are regulated by miRNAs. Gain-of-function and loss-of-function analyses indicated that 2 of the *ap2*-like target genes normally act as floral repressors, which are regulated by miR172. Early flowering and disrupted floral organ identity phenotypes were obtained in miR172 overexpressing plants (Zhu et al. 2010). Several *ap2*-like genes such as *target of eat1* (*toe1*), *toe2*, and *toe3* genes in *Arabidopsis*, and *indeterminate spikelet1* (*ids1*) and *gloy15* in *Zea mays* are also regulated by miR172 (Lauter et al. 2005). The *ap2* gene mutants result in the same morphology and improper flowering time (Aukerman and Sakai 2003; Chen 2004). The loss-of-function *toe1-1* mutation results in slightly early flowering and the *toe1-1 toe2-1* double mutant flowers much earlier than the wild type. On the other hand, overexpression of *toe1* (*toe1-1D*) leads to delayed flowering, while overexpression of miR172 from the 35S promoter causes early flowering, and overcomes the late flowering phenotype of *toe1-1D* (Yang et al. 2007). The role of miR164 in flowering was determined by loss-of-function allele (Guo et al. 2005). The early extra petals1 (Eep1) phenotype, caused by a transposon insertion of miR164c, resulted in flowers with extra petals in *Arabidopsis*. The transcription factor CUC1 and CUC2 accumulation upon miR164c mutation led to flower development defects (Baker et al., 2005). The roles of miR165/166 in floral structure architecture were also discovered. The miR165/166-overproducing *Arabidopsis* plants have floral development defects the same as *men1* and *jba-1D* mutants (Williams et al. 2005; Jung et al. 2007). Auxin signals are found to be closely related to floral organogenesis. The miR167 targets *arf6* and *arf8*, which play a role in gynoecium and stamen development and fertility. Overexpression of miR167 causes defects in floral organ development (Wu et al. 2006). In opium poppy, miR169b and miR169n target CCAAT-binding transcription factor (Cbf-b/Nf-ya) regulating flowering time, and miR171 targets a plant-specific scarecrow-like transcription factor 6 (SCL6) regulating floral development (Unver et al. 2010b).

4.5. Phase transition

In plants, the timing of phase transition between the vegetative phase and the reproductive phase is tightly controlled. Regulation and control of the phase change are both dependent on endogene expression and environmental signals. Genetic studies have discovered positive and negative regulators of phase transition control. A group of genes encoding squamosa promoter-binding protein-like (SPL) transcription factors, such as SPL3, SPL4, and SPL5 responsible for initiation of flowering, are negatively regulated by miR156 (Jones-Rhoades et al. 2002; Wu and Poethig 2006; Gandikota et al. 2007; Wang et al. 2008). Mutations of the genes related to miRNA biogenesis and transport result in altered juvenile-to-adult vegetative phase change and vegetative-to-reproductive phase change. Overexpression of the *miR156a* gene causes late flowering and delays in vegetative phase change in *Arabidopsis* (Wu and Poethig 2006; Shikata et al. 2009), rice (Xie et al. 2005), and poplar (Wang et al. 2011a). On the other hand, overexpression of miR156-resistant *spl3/4/5* genes results in early flowering (Kim et al. 2012). The endogenous level of miR156 is temporally regulated, which is very high in the early juvenile vegetative phase but decreases rapidly before the onset of the adult juvenile phase (Wu and Poethig 2006). miR156 also targets *spl9* and *spl15* genes responsible for shoot maturation (Kantar et al. 2010). Additionally, it has been detected that SPL13 represses the phase transition in the miR156-resistant version of *spl13* (r*spl13*) in *Arabidopsis* (Martin et al. 2010). The transition from juvenile to adult leaf identity is also controlled by miR172 targeting the apetala2 class of transcription factors (Chen 2004). The maize mutant *glossy15*, miR172-target *ap2* gene mutant (Lauter et al. 2005), shows the characteristics of adult leaf cells in the juvenile phase. The *glossy15* mRNA is accumulated in juvenile leaves and decreased in mature leaves. On the other hand, the level of miR172 became detectable only when leaves started showing adult characteristics and gradually increased subsequently (Lauter et al. 2005; Chuck et al. 2007).

4.6. Seed development

Seed development in plants initiates with tissue differentiation at early stages, and maturation programs provide seed desiccation, dispersal, and germination in later phases. A number of transcription factors and genes are involved in regular seed development and maturation programs requiring transcriptional, post-transcriptional, and post-translational regulations. Therefore, miRNA-based transcriptional regulation is also critical for normal seed development. Plants with loss-of-function in miRNA biogenesis and function generate acute embryonic defects and lethality. Several studies indicated the roles of miRNA in embryogenesis and seed development (Wu et al. 2006; Yang et al. 2006; Allen et al. 2007; Bowman and Floyd 2008; Nodine and Bartel 2010; Willmann et al. 2011). The genes related to miRNA biogenesis are found to be responsible for regular seed development, e.g. *dcl1-15* mutants mature earlier (Willmann et al. 2011). The 2 genes, *lec2* (Leafy-cotyledon2) and *fus3* (Fusca3), responsible for seed

maturation were detected as increased in *dcl1-15* mutants. It has been observed that Dcl1 represses *fus3* and *lec2* in the embryo during early embryogenesis. On the other hand, double mutant phenotypic analyses showed that *lec2-1* and *dcl1-15* as well as *fus3-3* and *dcl1-15* double mutants reduced the *dcl1-15* phenotypes. Therefore, LEC2 and FUS3 are essential for the early maturation phenotype of *dcl1-15*. The SE and HYL1 proteins are required for miRNA processing (Laubinger et al. 2008). It has been detected that either *se* or *hyl1* mutations in *Arabidopsis* have elevated levels of pri-miRNA, reduced levels of miRNA, and abnormal embryo development (Yang et al. 2006). The miR168 targets *ago1* mRNA (Jones-Rhoades et al. 2002), which is essential for miRNA biogenesis and appropriate embryo development. The miR168 resistant *ago1* mutants, accumulating the *ago1* mRNA, present a phenotype similar to that of *dcl1, hen1*, and *hyl1* mutants (Vaucheret et al. 2004). Another study related to miRNA regulation of embryo development in plants showed that the *dcl1* mutant *Arabidopsis* overexpresses ~50 miRNA targets. Of those, 9 genes encoding transcription factors were up-regulated around the 8-cell-embryo stage in this mutant. These genes are predicted to be the targets of miR156, miR159, miR160, miR166, miR319, or miR824. An additional 6 targets of miR168, miR393, miR400, or miR778 were up-regulated at the globular stage. Therefore, the miRNA regulation is essential for proper embryo patterning (Nodine and Bartel 2010). Auxin plays crucial roles during seed development. Its level and regulation of auxin-responsive genes are important for pattern formation during embryogenesis (Bowman and Floyd 2008). The mutation of *arf2* causes increased seed size and weight in *Arabidopsis* (Xue et al. 2009). The miR164 targets 5 members of Nac (No apical meristem; Ataf1/2; and Cup-shaped cotyledon2)-domain transcription factors important for suitable patterning in early embryogenesis. The miR164-resistant *cuc1* results in alterations in embryonic development (Mallory et al. 2004). The seed size and seed development are affected by the miRNA159/miR319 family. The mir159ab double mutant seeds have reduced size and irregular shape because of *gamyb* gene deregulation (Allen et al. 2007). The *myb33* and *myb101* mRNAs regulating seed germination are suppressed by miR159 activity. Overexpression of miR159 results in hyposensitivity to abscisic acid (ABA) during seed germination, which is also observed in the *myb33* and *myb101* mutants (Jung et al. 2009). Overexpression of miR166g targeting HD-ZIP transcription factors results in seedling arrest (Williams et al. 2005) in *Arabidopsis*.

5. Stress responsive microRNAs in plants

Plants are exposed to serious biotic and abiotic stresses such as drought, salinity, alkalinity, cold, pathogen infections, and diseases, which are the predominant cause of decreased crop yields. Plants use adaptive responses operating at the transcriptional, post-transcriptional, translational, and post-translational levels to cope with these environmental challenges (Sunkar 2012). As a post-transcriptional gene regulator, a number of miRNAs (Table 2) play roles in multiple stress responses in plants. There is much evidence showing the direct link between miRNA regulation and stress response in plants. Expression of plant miRNAs has been detected as up- and down-regulated levels upon treatment of diverse stress conditions. Since some of the genes related to stress response are post-transcriptionally regulated by miRNAs, the NFYA5 transcription factor, which promotes drought resistance in *Arabidopsis* (Li et al. 2008), is found to be regulated by miR169 (Li et al. 2008; Trindade et al. 2010). Another differentially expressed miRNA upon stress is miR528, which targets copper ion binding protein genes, and its expression has been found to be increased in glyphosate (an herbicide) inoculated tolerant festuca plant leaves (Unver et al. 2010a). Although many studies have been conducted on the functions of miRNAs on stress regulation in plants, we discuss some of the stress conditions and regulation of miRNAs here.

5.1. Drought stress

Drought is a major limitation for plant development and productivity. Several genomics studies including expression level measurements and transgenic approaches have been applied to better investigate drought tolerance mechanism in plants. To date, many genes related to water deficiency tolerance were identified (Ni et al. 2009). Additionally, a number of studies examining miRNA regulation on drought tolerance in plants have obtained valuable information. For example, miR167, miR168, miR171, and miR396 were found to be drought stress responsive miRNAs in *Arabidopsis* (Liu et al. 2008). Moreover, miR159-regulated *myb33* and *myb101* were identified as important genes playing roles in response to ABA accumulation under drought stress (Reyes and Chua 2007). The miR169 targets the Nfya5 transcription factor, and is down-regulated upon water deficiency (Li et al. 2008). Overexpression of a miR169-resistant *nfya5* transgene significantly improves drought tolerance by promoting stomatal closure under drought stress (Li et al. 2008). The miR169 also targets 8 NF transcription factor Y subunit mRNAs in rice (Li et al. 2010b). Trindade et al. (2010) reported that the levels of miR398 and miR408 are increased in *Medicago truncatula* plants upon water deficiency and then cause down-regulation of their respective targets, *cox5b* and plantacyanin, suggesting that these miRNAs play a role in regulating *M. truncatula* responses to drought stress. Some of the barley miRNAs, such as miR156, miR166, miR171, and miR408, were observed as differentially expressed upon dehydration (Kantar et al. 2010). The expression of miR159.2, miR1514,

miR2118, and miR2119 was induced in *Phaseolus vulgaris* by abiotic stress, especially drought and ABA treatment (Arenas-Huertero et al. 2009). Moreover, expression of miR102, miR156, miR162, miR167, and miR473 was up-regulated under drought-influenced conditions in poplar (Li et al. 2011a) (Table 2).

5.2. Salt stress

Salt stress is another important constraint for plants. It negatively affects plant growth and productivity all over the world. To date, several studies have been performed to measure and detect the differential expression level of plant miRNA upon salt treatment. In an expression-based study, Liu et al. (2008) reported that several miRNA, such as miR396, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158, and miR169, responded to salt treatment (Liu et al. 2008). miR396 is a salt responsive plant miRNA targeting growth regulating factor (GRF) transcription factors. Overexpression of miR396 in rice and *Arabidopsis* results in reduced salt and alkali stress tolerance compared to that of wild-type plants (Chi et al. 2011). In a recent study, expression level of miR398, targeting 2 Cu/Zn superoxide dismutases (CSD1 and CSD2), was differentiated in response to salt stress (Jagadeeswaran et al. 2009). In addition, miR395 regulates the *asp1* gene for sulfur accumulation and allocation (Liang et al. 2010). Furthermore, miR530, miR1445, miR1446, miR1447, and miR171 were down-regulated, whereas miR482.2 and miR1450 were up-regulated during salt stress in poplar (Lu et al. 2008).

5.3. Nutrient deficiency

Nutrient availability is an important abiotic stress factor for plant yield and productivity. To cope with nutrient deprivation, plants use several metabolic, physiological, and molecular mechanisms. Transcriptional regulation upon nutrient limitation in plants via miRNA-mediated gene expression adjustment has been reported for several element deficiencies, such as phosphate (Pant et al. 2008; Hsieh et al. 2009), sulfate (Rausch et al. 2005), and copper (Yamasaki et al. 2007; Abdel-Ghany and Pilon 2008). Several miRNAs, found to be differentially expressed upon phosphate deprivation, are reported as miR156, miR169, miR395, miR398, miR778, miR827 miR828, and miR2111 (Sunkar et al. 2012). In addition, miR395 plays a role in sulfate metabolism, being complementary to mRNA of ATP sulfurylase (APS) proteins. Expression of miR395 was induced by low external sulfate concentrations (Jones-Rhoades and Bartel 2004). Moreover, in response to low Cu^{2+} availability, miR398 is induced, leading to decreased levels of *csd* transcripts to save the Cu^{2+} ions for plastocyanin (Shukla et al. 2008), and other miRNAs (miR397, miR408, and miR857) were detected as copper responsive (Yamasaki et al. 2007). To adopt nitrogen availability, the miR393/Afb3 and miR167/Arf8 modules are implicated in regulating root system architecture in plants (Vidal et al. 2010). In *M. truncatula*, miR169a targets *hap2-1* encoding a subunit of hetero-trimeric CCAATBOX-BINDING transcription factor complex to the nodule meristematic zone. Nodule development is arrested by the overexpression of miR169a or suppression of *hap2-1*, resulting in a deficient nitrogen-fixation phenotype (Combier et al. 2006).

5.4. Cold stress

Cold stress or low temperature is another challenge for plant growth and productivity. It has been shown that expression levels of miRNA are also affected by low temperature in several plants. Lv et al. (2010) used a rice-specific miRNA microarray chip and found that 15 miRNA families are differentially regulated by cold stress. In their study, response of miR171 family members to cold stress was observed, and they found that the miR171 family members are differently regulated. While the expression of pri-miR171a/e/f/i is down-regulated, that of pri-miR171c/d/h is up-regulated. In other studies, expression levels of miR165/166, miR169, miR172, miR393, miR396, miR397, miR402, and miR408 were detected as increased, and that of miR398 was measured as decreased in cold stress applied *Arabidopsis* plants (Sunkar and Zhu 2004; Zhou et al. 2008). In *Brachypodium*, a total of 25 miRNAs, including a few novel ones, were measured as differential expressed upon cold treatment (Zhang et al. 2009). The miR319 was detected as decreased in both roots and shoots, although strong induction was observed in plantlets in sugarcane (Thiebaut et al. 2012). Additionally, miR2118 was observed as induced under cold stress in beans (Arenas-Huertero et al. 2009).

5.5. Oxidative stress

Elevated reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2) and free radicals are found in plant cells under biotic and abiotic stress conditions. The enzymatic scavenging mechanism of oxidative stress response is composed of superoxide dismutases (SODs), peroxidases, and catalases to detoxify the ROS (Shukla et al. 2008). The expression level of miR398 was found to be decreased by oxidative stress since it targets copper/zinc superoxide dismutases (Cu/Zn-SODs), cytosolic *csd1*, and chloroplast localized *csd2*, thereby causing an increase in the accumulation of CSD1 and CSD2 in *Arabidopsis* (Sunkar et al. 2006) and rice (Li et al. 2010b). miR169, miR397, miR528, miR1425, miR827, miR319a.2, and miR408-5p were observed as H_2O_2 responsive and differently expressed in rice seedlings (Li et al. 2010b). Deep sequencing showed that the expression levels of miR169, miR397, miR827, and miR1425 were increased while that of miR528 was decreased by H_2O_2 treatments (Li et al. 2011c).

5.6. Response to biotic stresses

Biotic factors such as bacteria, viruses, fungi, and insects affect plant growth and productivity. Being sessile organisms plants respond to diseases via their physiologic and molecular mechanisms. In several studies, the roles of small RNA in disease resistance responses were revealed (Navarro et al. 2006; Fahlgren et al. 2007; Jin 2008; Katiyar-Agarwal and Jin 2010; Li et al. 2010a; Zhang et al. 2011a, 2011b; Zhou et al. 2012). miR393 is the first reported responsive miRNA upon bacterial inoculation in plants (Navarro et al. 2006). Bacterial elicitor flg22 results in the induction of miR393 expression level to suppress auxin signaling. This miRNA has been also measured as 10-fold increased when *Arabidopsis* leaves were inoculated with nonpathogenic *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 hrcC mutant, defective in TTSS (Fahlgren et al. 2007; Zhang et al. 2011b). Additionally, expression levels of miR160 and miR167 were discovered to be increased in the same nonpathogenic inoculation (Fahlgren et al. 2007). Zhang et al. (2011a) performed a high throughput small RNA deep sequencing experiment using 3 different inoculations including *Pst* DC3000 hrcC (nonpathogenic), *Pst* DC3000 EV (virulent), and *Pst* DC3000 avrRpt2 (avirulent), and they identified 15, 27, and 20 differentially expressed miRNA families respectively in *Arabidopsis* (Zhang et al. 2011a). miR398 was found to be suppressed upon oxidative stress treatment via bacterial pathogen infection when *Arabidopsis* plants were inoculated with *Pst* avirulent strains (*Pst* DC3000 carrying avrRpm1 and avrRpt2), but not with the virulent strain (Jagadeeswaran et al. 2009). miR393* also plays a critical role in plant antibacterial response by targeting and suppressing expression of a Golgi-localized Snare protein, MEMB12. Inhibition of the expression of *memb12* leads to increased exocytosis of pathogenesis-related protein PR1; consequently, it prevents pathogen growth (Bednarek et al. 2010). Expression level measurement of miRNAs was also studied in fungal pathogen inoculation, e.g., nonprotein coding (npc)-RNA molecules were measured in powdery mildew infected wheat (Xin et al. 2010), and a total of 125 putative wheat stress responsive long npc-RNAs were identified. Some of the predicted npc-RNAs such as miR675, miR167, and miR2004 corresponding to miRNAs were found to be fungal-stress responsive.

6. Methods for miRNA detection and measurement

Understanding the miRNA-mediated gene regulation is largely dependent on the availability of innovative strategies and methodological approaches for accurate detection of miRNA expression levels in specific tissues. Identification of differentially expressed miRNA genes in cell transcriptome directly reflects the dynamic cell behavior under changing conditions. The miRNA and target mRNA expression level measurement presents valuable information about the miRNA's functions. So far, numerous methodologies have been developed for rapid, sensitive, specific, and genome-wide detection of miRNAs. These methods fall under 4 categories: direct cloning, real-time quantitative PCR, high-throughput deep-sequencing, and hybridization-based methods including northern blotting, in situ hybridization, and miRNA-microarray methods. Each method has its own advantages and drawbacks, such as direct cloning and next-generation sequencing technologies with high sensitivity, which are used in both the discovery and confirmation of miRNAs, whereas qRT-PCR and microarray are used only for expression patterning. Differentially expressed miRNAs obtained from genome-wide miRNA expression profiling methods such as microarray or deep-sequencing generally require more validation done with real-time quantitative PCR studies. Despite the progress made in developing methods for the quantification of miRNAs, rapid and highly sensitive methods are still needed for overcoming known obstacles of miRNAs, including short-length, low abundance, and the presence of miRNA families whose members have roughly the same sequence compositions and represent similar expression patterns. Below, we will briefly summarize some of these methods routinely used for measuring miRNA levels.

6.1. High-throughput sequencing of miRNAs

The emergence of next-generation high-throughput cDNA and direct-RNA sequencing techniques has revolutionized whole-transcriptome analysis at an unprecedented depth, accuracy, and resolution. New-generation sequencing (NGS) technologies have been successfully applied in a variety of areas, covering de novo genome sequencing (Velasco et al. 2010; Vogel et al. 2010), genome re-sequencing (Ashelford et al. 2011) (DNA-Seq), transcriptomics (Wei et al. 2011; Hansey et al. 2012; Mutasa-Gottgens et al. 2012) (RNA-Seq), and epigenetic modification, such as DNA methylation or DNA–protein interactions (Chip-Seq) (Kaufmann et al. 2010), and recently they have been extensively used in genome-wide identification and quantification of known and novel miRNAs and other noncoding small RNAs in a single instrument run. Currently, there are several commercially available NGS platforms including Illumina Solexa sequencing, Roche 454 GS FLX, Applied Biosystems SOLiD, Helicos HeliScope, Ion Torrent PGM–Applied Biosystems, and single molecule real-time sequencing (SMRT)–Pacific Bioscience (the last 3 sequencers are called third-generation DNA sequencing instruments). When compared with previous sequencing technologies, third-generation sequencing technologies offer significant advantages in terms of simplified library construction,

small amounts of starting material, and longer read lengths. However, more importantly, these technologies do not require the conversion of RNA into cDNA or ligation/pre-amplification steps. Therefore, they can be effectively used for direct sequencing of RNA without the need for cDNA conversion process causing the cDNA synthesis-based artifacts and pre-amplification experiments leading to biases and errors (Schadt et al. 2010). Each NGS platform was comparatively differentiated from each other in terms of template preparation, read length, run time, sequencing chemistry, depth of coverage, raw accuracy, and cost per run (Metzker 2010; Egan et al. 2012). Nowadays, 3 major deep sequencing platforms, ABI/Solid, Roche 454 Pyrosequencer, and especially Illumina's miRNA-Seq, account for the vast majority of small RNA sequencing all over the world. The deep sequencing approach can easily eliminate some technical challenges and obstacles sourced from intrinsic properties of miRNAs, such as small read size, low-abundance, instability, and contamination with other RNA fragments. In addition to providing comprehensive information about the miRNA transcriptome on a genome-wide scale (miRNAome), the NGS technologies also allow for the large-scale identification of cells (Judson et al. 2009; Fehniger et al. 2010; Wei et al. 2011), tissue (Mica et al. 2010; An et al. 2011; Chi et al. 2011; Gonzalez-Ibeas et al. 2011), stage (Song et al. 2010; Zhang et al. 2012), condition (Zhang et al. 2009; Du et al. 2011; Li et al. 2011), and species-specific miRNAs (Jones-Rhoades et al. 2006; Zhang et al. 2006a; Voinnet et al. 2009). In recent years, NGS has been applied to determine the populations of variants of known miRNAs (isomiRs) that are mainly generated from alternative cleavage of Dicer Like-1 (DCL1) during biogenesis, and they can have 5′ and/or 3′ cleavage variations, insertions, and deletions (Lelandais-Brière et al. 2009). One intriguing application area of NGS technologies in miRNA research is "degradome sequencing", a next-generation sequencing method integrated with modified 5′ RACE (rapid amplification of cDNA ends) in order to identify miRNA-directed mRNA cleavage sites (Addo-Quaye et al. 2008; German et al. 2008; Meng et al. 2010). This method has been widely used to identify the genome-wide analysis of miRNA-mediated cleavages in plants; hence, detailed functional information could be obtained about patterns of RNA degradation (Pantaleo et al. 2010; Zhou et al. 2010; Mao et al. 2012). Moreover, deep sequencing allows us to determine the miRNAs whose expression profiles could be differentiated under a variety of stress conditions including drought (Barrera-Figueroa et al. 2011; Wang et al. 2011b), cold (Zhang et al. 2009), phosphate deficiency (Hsieh et al. 2009), sulfate deficiency (Huang et al. 2010), and fungal infection (Xin et al. 2011).

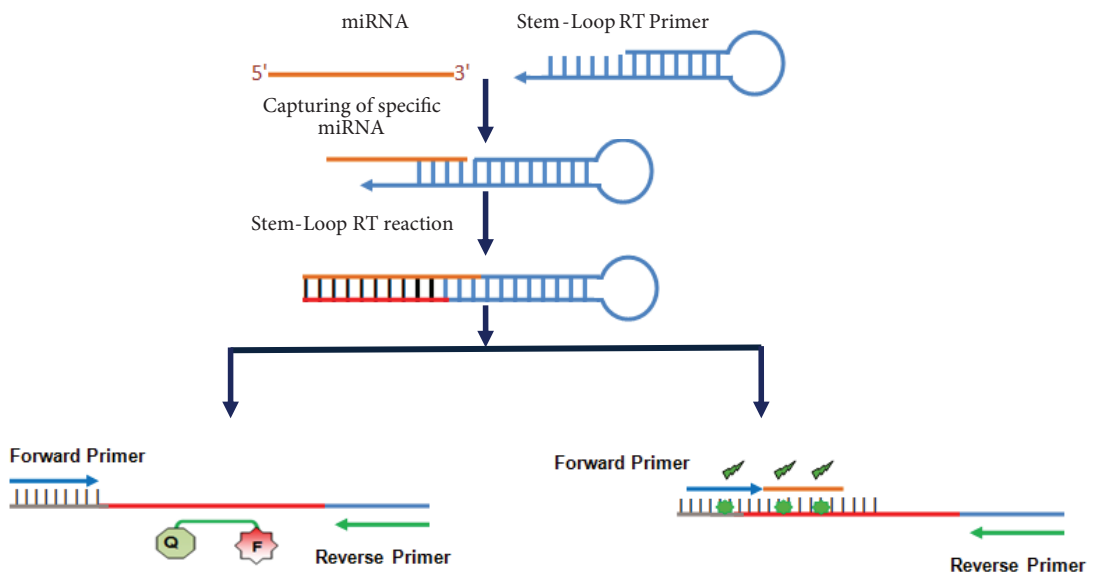
6.2. MicroRNA profiling using microarrays

Microarray technology is one of the most powerful and versatile high-throughput tools. This technique has been used successfully to measure genome-wide coding and/or small noncoding gene expression changes in a single experiment. Unlike other nucleic acid hybridization methods, it is a genome-wide hybridization method that is currently used in various areas of molecular biology including (i) characterization and determination of differentially expressed genes on a genomic scale, (ii) global interrogation of genomic structural variations by using array-comparative genomic hybridization (array based-CGH), (iii) revealing and measuring both DNA polymorphism and dosage changes among individuals by using single-nucleotide polymorphism (SNP) array, (iv) detecting epigenetic modifications, such as DNA methylation status, genome-wide protein–DNA interactions, and chromatin modifications using ChIP-chip array, and (v) profiling the genome-wide miRNA expression pattern by using miRNA-microarray (Davison et al. 2006; Liu et al. 2008; Yin et al. 2008). A number of different commercially available miRNA array platforms (Agilent Technologies miRNA profiling system, Illumina MicroRNA Expression Profiling BeadChips arrays, Affymetrix's GeneChip miRNA Array, µParaflo Microfluidic Array Technology, etc.) (Chen et al. 2012; D'Andrade and Fulmer-Smentek 2012; Dee and Getts 2012; Zhou et al. 2012) have been developed to date, but there are some technical differences in these available platforms including probe design, probe immobilization chemistry, slide formats, sample labeling, signal detection chemistries, and cost, although the general procedures of a miRNA-microarray experiment (cDNA preparation, sample labeling, array construction, hybridization, fluorescent detection, and data analysis) are somewhat similar. The probe design has been an integral component of the microarray experiment and an appropriate probe should match some criteria, such as high specificity, affinity, and consistent melting temperature (Tm) with other probes. However, miRNA-microarray probe design is quietly challenging because of the short length, low abundance, and a wide range of melting temperatures (between 45 and 74 °C) of mature miRNAs as well as different miRNAs belonging to same family sharing similar nucleotide sequences (Yin et al. 2008). In order to overcome this obstacle, as with other hybridization-based methods, recently developed locked nucleic acid (LNA)-modified capture probes are widely used to normalize effectively melting temperatures on slides, and it also enables sequence discrimination with single nucleotide resolution (Castoldi et al. 2006, 2007). Although miRNA-microarray technology allows researchers to provide pivotal information about genome-wide noncoding gene

expression profiling in parallel as well as being a relatively cheap technique on a per gene basis, it has some time-consuming processes to solve, namely quality assessment, data analysis, background correction and normalization, and appropriate statistical methods as well as mature miRNA innate characteristic as mentioned above. In plants, miRNA-microarray technology has been extensively used in numerous studies for understanding the functional roles of miRNAs in different organs (Dongdong et al. 2009; Amiteye et al. 2011; Peng 2012), and under variable growth conditions like biotic (Guo et al. 2011) and abiotic stresses, such as drought, cold, and heavy metals (Liu et al. 2008; Ding et al. 2009; De-Kang et al. 2010; Ding et al. 2011; Unver et al. 2010a; Li et al. 2011).

6.3. Quantitative real-time PCR (qRT-PCR)

With its high level of sensitivity, specificity, accuracy, and practical use, quantitative real-time PCR (qRT-PCR) has been considered the gold standard for gene expression measurements; hence, it has been extensively used in simultaneous measurement of gene expression patterns of miRNAs (Varkonyi-Gasic et al. 2007; Schmittgen et al. 2008; Unver et al. 2009, 2010a, 2010b; Bin et al. 2011). This method allows us to perform accurate and efficient quantification of miRNA by using as little as 25 pg of total RNA. The method also enables direct and rapid quantification of miRNA from a single cell without RNA isolation or any further downstream processing (Chen et al. 2005). Nevertheless, there are significant limitations that hamper the broader use and applicability of qRT-PCR. These limitations are mostly sourced from the nature of miRNA: (i) mature miRNAs are short (average miRNA is 19-24 nucleotides in length), (ii) GC-content of mature miRNA sequences can be heterogeneous in quality, creating a gap between melting points (Tm) of each miRNA, (iii) reverse transcription of mature miRNAs is generally difficult due to the lack of distinctive characters like poly-A tail, and (iv) to distinguish between miRNAs in the same family that differ in only one base could be difficult (Benes and Castoldi 2010; Balcells et al. 2011). In general, there are 2 common qRT-PCR methods: SYBR-Green-based miRNA RT-qPCR assays and Stem-loop RT-based TaqMan, which are differentiated from each other in terms of chemical reaction. The SYBR-based assays use fluorescent double-stranded DNA binding dye, which can intercalate into strands of amplification products, and measuring the increase in fluorescence during PCR cycles monitors detection of amplified miRNAs. However, the detection of expression level of any miRNA by using SYBR Green assay presents several disadvantages because dye can bind to any double-stranded DNA regardless of amplicon or nonspecific cDNAs; thus, it may generate false positive signals. However, the SYBR-based assay is widely used for detection of the expression profile of well-known miRNAs because of its cost per sample, sensitivity, and no requirement for probes (Raymond et al. 2005; Varkonyi-Gasic et al. 2007; Sharbati-Tehrani et al. 2008). Unlike SYBR-based qRT-PCR methods, the stem-loop RT-based TaqMan method uses a target-specific fluorogenic probe that enables the rapid detection and quantification of desired miRNAs (Figure 2) (Chen et al. 2005; Mestdagh



A) TaqMan based Real-Time PCR

B) SYBR Green Real-Time PCR

Figure 2. A schematic illustration of TaqMan probe and SYBR Green real-time PCR assay for quantification of mature miRNA (Varkonyi-Gasic et al. 2007; Wang et al. 2010).

et al. 2008). Chen et al. (2005) developed an unusual and mature miRNA-specific stem-loop RT primer that can easily hybridize in a sequence-specific manner with its target mature miRNA. It was also reported that the stem-loop RT primers used in the reverse-transcription step are considerably better when compared with conventional ones. The use of miRNA gene-specific TaqMan probes with mature miRNA-specific stem-loop RT primer increases the sensitivity and specificity of the methods; therefore, stem-loop RT based TaqMan is now considered the gold standard among the miRNA detection methods (Mestdagh et al. 2008; Schmittgen et al. 2008; Becker et al. 2010).

6.4. In situ hybridization

In situ hybridization (ISH) has emerged as a powerful technique that combines molecular biological techniques with histological and cytological approaches for detection of specific nucleic acid sequences in samples at the cellular, tissue, and even whole organism levels (Valoczi et al. 2004; Kloosterman et al. 2006). Among the other hybridization-based methods, only in situ hybridization has the potential to accurately measure the "spatial" and "temporal" expression patterns of miRNAs and their targets within fixed tissues and cells or even cellular compartments. Contrary to the conventional hybridization methods that require multiple steps including: (i) the isolation of total or small RNAs, (ii) gel electrophoresis for sorting of RNAs based on size and charge, (iii) transferring small RNA samples to a sheet of special membrane, nitrocellulose in general (blotting step), and (iv) detecting the small RNAs with a hybridization probe complementary to part of or the entire target RNA sequence, in this method, we can directly hybridize mature miRNAs with synthetic RNA probes (riboprobes) in order to obtain more information about the localization and relative expression levels of miRNAs of interest without any further processing steps. One of the most important considerations in the miRNA ISH process is developing and designing special and high-affinity binding of the probes that can easily hybridize with mature miRNAs because of the short sequences of mature miRNAs and the longer precursor of miRNAs (pre-miRNAs) having the same sequences as mature ones. Recently, the development of locked nucleic acid (LNA) oligonucleotide probes, which have a high degree of complementary with the miRNA, has paved the way for efficient detection of animal and plant miRNAs when compared with conventional RNA probes (Valoczi et al. 2004; Vester and Wengel 2004). It has been emphasized that the LNA-modified probe shows a strong ability to bind to mature miRNAs rather than pri- or pre-miRNAs, subsequently resulting in detection of high-level mature miRNAs. Another advantage of it is that LNA-modified probes and conventional antisense riboprobes (for target) can be used collaboratively to detect both a mature miRNA and its target or desired mRNA simultaneously (Sweetman et al. 2008; Sweetman 2011). Moreover, as mentioned above, this technique has been widely and successfully applied to cultured cells (Deo et al. 2006; Thompson et al. 2007; Pearson et al. 2009), tissue sections (Nelson et al. 2006; Wulczyn et al. 2007), and formalin-fixed and paraffin-embedded tissues (Nuovo et al., 2009) as well as whole mounts (Wienholds et al. 2005; Ason et al. 2006; Kloosterman et al. 2006). Kinder and Timmermans (2006) utilized miRNA in situ hybridization in order to elucidate the role of miRNAs in the developmental process of *Arabidopsis* and maize; they also gave a comprehensive protocol about the in situ hybridization technique for plants (Juarez et al. 2004; Kidner and Martienssen 2004; Kidner and Timmermans 2006). Additionally, Várallyay and Havelda (2011) described a step-by-step plant miRNA-ISH protocol for *A. thaliana* and *N. benthamiana* by using highly sensitive LNA-modified oligonucleotide probes; in particular, they touch upon almost every aspect of protocols (Várallyay and Havelda 2011). In conclusion, miRNA-ISH supplied by LNA-oligonucleotide probes enables the spatial and temporal expression patterns of miRNA with high sensitivity cells, tissues, or organs belonging to both animals and plants.

6.5. Northern blotting

Northern blotting is a hybridization-based technique that can be used to detect expression levels of miRNAs of interest, indicating the relative amounts of target miRNAs in the total RNA pool. The technique consists of 3 steps: (i) the separation of RNA samples using denaturing polyacrylamide gel electrophoresis (PAGE) according to their size, (ii) transferring the RNAs to solid nylon membranes, and (iii) hybridization of RNAs with a radioactive probe that is complementary to all or part of the RNA sequence, and samples are then exposed to X-rays to obtain information. By using this method, we can easily determine the size of miRNAs and validate computationally predicted miRNAs; the technique also allows us to compare the multiple samples in one run. However, this technique has some drawbacks, such as requirement of large amounts (5–25 mg for each sample) and high-quality RNA samples, and having a considerably time-consuming protocol (Pall and Hamilton 2008; López-Gomollón 2011). It was previously reported not to be suitable for clinical studies to detect and measure the expression level of hundreds of miRNAs. Valoczi et al. (2004) made some alteration in northern blotting by using modified oligonucleotides using locked nucleic acid (LNA), which increases the method's sensitivity for the detection of mature miRNAs by at least 10 times (Valoczi et al. 2004; Varallyay et al. 2008). Koscianska et al. (2011) used a novel high-resolution northern-blotting technique for detecting endogenous and exogenous RNAs

and compared the result with data obtained from miRNA deep sequencing. In conclusion, using the high resolution northern blotting instead of conventional methods enables analysis of endogenous and exogenous RNAs in the range of 20-70 nt and it also helps in detecting the length heterogeneity analysis of miRNAs and their precursors (Koscianska et al. 2011).

7. Conclusion and future perspectives

To date, numerous miRNAs from diverse plant species have been identified and characterized with the help of genome scale studies and developing technologies. miRNA expression level differentiation analyses upon environmental stimuli have been performed and developmental changes have been measured in distinct species. Moreover, functional roles of several plant miRNAs in development and stress response were discovered. Since the functionality studies of miRNAs concentrated on conserved miRNAs, still there is a lack of information about the roles of many miRNAs. Targets of identified plant miRNAs were found to involve diverse biological processes such as flowering timing, vegetative phase change, root initiation, and stress response to biotic and abiotic factors. It will be possible to determine the regulation of other biosynthetic processes and possible involvement of miRNA in diverse physiological mechanisms. By the aid of next-generation sequencing and microarray technologies, many more miRNAs from various plants will be characterized and measured. Rapidly evolving in silico methods will help to enable us to identify more miRNAs and their targets for better understanding of the nature of miRNAs in the near future.

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