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REVIEW

Abiotic stress-associated microRNAs in plants: discovery, expression analysis, and evolution

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Abstract **Abiotic stresses such as drought, cold, and high salinity are among the most adverse factors that affect plant growth and yield in the field. MicroRNAs are small RNA molecules that regulate gene expression in a sequence-specific manner and play an important role in plant stress response. Identifying abiotic stress-associated microRNAs and understanding their function will help develop new strategies for improvement of plant stress tolerance. Here we highlight recent advances in our understanding of abiotic stress-associated miRNAs in various plants, with focus on their discovery, expression analysis, and evolution.**

Keywords microRNA, abiotic stress, epigenetics, gene expression, evolution

Plant response to aboitic stresses

Due to their sessile nature, plants face a myriad of adverse factors in their natural environment, affecting cellular growth, development, and productivity. Suboptimal abiotic conditions, such as drought, flooding, cold, heat, below and above normal illumination, low oxygen availability, heavy metals, radiation, nutrient deficiency, high salinity, and mechanic damage, are among the main factors that cause stress to plants, either alone or in combination (Boyer, 1982). To cope with these stresses, plants have evolved highly complex and well-honed responses that include reprogramming gene expression, readjusting metabolism and water relations, and protecting cellular structures from the damages of stresses (Zhu, 2002). The expression of a large set of genes is usually affected in response to abiotic stresses, indicating that stress responses involve a complex regulatory network (Seki et al., 2002). Within this network, gene expression is temporally and spatially coordinated at the transcriptional, post-transcriptional, and translational levels. MicroRNAs (miRNAs) are an integral component of the stress response network because they regulate the expression of their target protein coding genes, many of which encode transcription factors that regulate the expression of other genes. In addition, the expression of miRNA genes themselves is regulated by upstream transcription factors.

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Biogenesis of miRNAs and other small RNAs

Jacob and Monod (1961) had anticipated that gene products, probably RNA molecules, could act as repressors of genes at the DNA level or directly over mRNAs. Nowadays it has been proven that 20–24 nt small RNA molecules perform active roles as regulators of gene expression. Small RNAs mediate processes such as RNA interference, gene silencing, co-suppression and quelling, via repression of target mRNAs or translation inhibition (Dalmay, 2006; Jones-Rhoades et al., 2006). During these processes, small RNAs recognize target mRNAs via sequence complementarity.

In plants, small RNAs (sRNAs) are classified into two groups based on their biogenesis and function (Vaucheret, 2006; Vazquez et al., 2010). The first group contains various small interfering RNAs (siRNAs), including heterochromatic siRNAs (hc-siRNAs) (Reinhart and Bartel, 2002), trans-acting siRNAs (ta-siRNAs) (Vazquez et al., 2004), natural antisense siRNAs (nat-siRNAs) (Borsani et al., 2005), and long siRNAs (Katiyar-Agarwal et al., 2007). The second group contains miRNAs (Jones-Rhoades et al., 2006) and natural antisense miRNAs (nat-miRNAs) (Lu et al., 2008a).

Whereas hc-siRNAs are involved in DNA methylation and histone modification in a process known as transcriptional gene silencing (TGS) (Chinnusamy and Zhu, 2009), ta-siRNAs and nat-siRNAs function at the postranscriptional level by mediating the cleavage or translational repression of target mRNAs. Ta-siRNAs are sRNAs that are generated in phase from a single-stranded primary transcript that is targeted by a miRNA. The products of this cleavage are then converted to double-stranded RNAs by RDR6 and processed by DCL4 to produce ta-siRNAs (Allen et al., 2005). Nat-siRNAs are generated by overlapping genes that are transcribed in opposite directions. The transcripts of overlapping genes hybridize in the overlapping region and form double-stranded RNAs that are further processed by DCL proteins into sRNAs (Borsani et al., 2005). Nat-siRNAs generated by one member of the pair may target the antisense gene and downregulate its expression (Jin et al., 2008; Ron et al., 2010).

The biogenesis of miRNAs is different from that of siRNAs. MiRNA genes are first transcribed by RNA polymerase II into single-stranded primary miRNAs (pri-miRNAs), which then fold into stable hairpin structures (Bartel, 2004). DCL1 or DCL4 proteins cleave pri-miRNAs into miRNA precursors (pre-miRNAs), and then excise further to produce double stranded RNAs that are typically 21 nt long and methylated at the 3′ end by HEN1 (Chen, 2005). After being exported into cytoplasm by HASTY, one strand (miRNA*) is degraded and the other strand (mature miRNA) is incorporated into Argonaut (AGO) proteins, which are part of the RNA-induced silencing complex (RISC). In RISC, target mRNAs are recognized through their nearly perfect sequence complementarity to miRNAs (Rhoades et al., 2002). The binding of miRNAs to target mRNAs would lead to degradation or translation repression of mRNAs. Recently another class of miRNAs, lmiRNAs, was found in plants. These lmiRNAs are typically 24nt long, and are cleaved by DCL3 and sorted into AGO4 clade proteins. lmiRNAs regulate gene expression by directing DNA methylation of target genes (Wu et al., 2010).

Approaches for plant miRNA discovery

The approaches for miRNA discovery in plants can be roughly classified into two categories: direct cloning and bioinformatics. The method of choice for miRNA discovery has been cloning and sequencing the expressed mature miRNAs. The basic procedure includes cloning and sequencing of small RNAs, mapping small RNAs to reference genomic sequences, examining the secondary structure of candidate precursors, and checking for precise cleavage of precursors (Barrera-Figueroa et al., 2011). Early application of cloning and capillary sequencing led to the discovery of dozens of miRNAs in *Arabidopsis* (Llave et al., 2002; Sunkar and Zhu, 2004) and rice (Sunkar et al., 2005). To overcome the laborious cloning procedure and the expense of capillary DNA sequencing, and to increase throughput, next-generation sequencing techniques such as massively parallel signature sequencing (MPSS) and 454 pyrosequencing have been used for small RNA profiling. The deep coverage from these techniques makes it possible to identify miRNAs that are expressed in rare conditions or at low abundance, leading to the identification of many novel miRNAs (Lu et al., 2005a; Lu et al., 2006; Rajagopalan et al., 2006; Fahlgren et al., 2007; Johnson et al., 2007; Nobuta et al., 2007; Lu et al., 2008a; Sunkar and Jagadeeswaran, 2008; Sunkar et al., 2008). Recently, researchers began to apply the Illumina sequencing platform for miRNA discovery (Wei et al., 2009; Zhang et al., 2009a; Barrera-Figueroa et al., 2011). The Illumina sequencing platform produces millions of reads from a single run and thus provides two orders of magnitude greater depth than prior high-throughput sequencing techniques and allows better miRNA identification and profiling.

Cloning of small RNAs is straightforward. Small RNAs (sRNAs) are first separated from total RNA or pre-purified low-weight RNA fractions, by resolution in a gel. Small RNAs of 18–30 nt long are excised and eluted from the gel and sequentially ligated to 5′ and 3′ adaptors. The ligation products are then reverse-transcribed, amplified, and sequenced (Lu et al., 2007). In addition to miRNAs, cloned sRNAs include rRNAs, mRNAs, tRNAs, siRNAs, and chloroplastic sRNAs. Discrimination of miRNAs from other kinds of sRNAs is a key step in the discovery of genuine miRNAs.

To enhance the efficiency of miRNA cloning, the cloning procedure has been subject to several modifications and improvements. During the process of sRNA cloning, it is critical to reduce the amount of undesirable ligation products, in which sRNAs or adaptors are ligated in tandem. Specifications were included in the first protocols for sRNA cloning to prevent self-ligations of adaptors (Llave et al., 2002). The 3′ adapter is phosphorylated at the 5′ end so that it can be ligated to the 3′ end of sRNAs. The 3′ end of the 3′ adaptor is modified to an inverted deoxythymidine (idT) to avoid self-ligation of 3′ adaptors. The 5′ adaptors are hydroxylated at both 5′ and 3′ ends to prevent their self-ligation. The formation of empty products by ligation of 3′and 5′adaptors is prevented by a purification procedure after the ligation of each adaptor to eliminate residual adaptor molecules (Lu et al., 2007). To prevent sRNA-sRNA self-ligation, the 3′ adaptor may be preadenylated at the 5′ end (Lau et al., 2001; Pak and Fire, 2007). Preadenylation permits the ligation of adaptor to sRNAs in absence of ATP. Without ATP, 5′ phosphate of sRNAs cannot be adenylated and sRNA-sRNA self-ligation cannot occur (Vigneault et al., 2008).

Once small RNA sequences are obtained, computational tools such as SOAP (Li et al., 2009) and BOWTIE (Langmead et al., 2009) are used to map small RNA reads to reference genomic sequences. For each mapped small

RNA, the segments around the mapping location are extracted and fold into secondary structure. A series of established criteria can be applied to determine whether the extracted segment is likely a miRNA precursor (Ambros et al., 2003; Meyers et al., 2008). These criteria discriminate miRNAs from other small RNAs or mRNA degradation products. A distinct feature of miRNAs is that mature miRNAs are expressed predominantly from one strand and precisely excised from a hairpin region.

The second approach for miRNA discovery is the bioinformatics approach (Mendes et al., 2009). Because it is not a prerequisite to obtain mature miRNA sequences, the bioinformatics approach remains a useful complement to the cloning-based approach, especially for identifying miRNAs that are rarely or lowly expressed and miRNAs in plants with limited genomic resources. Homology search can be used to identify miRNAs that are conserved between two plant species. Mature miRNAs from one species are used to query genomic sequences, expressed sequence tags (ESTs), or small RNA sequences from another species to find miRNAs that match the known miRNAs, usually allowing up to two mismatches. Homology search has been used to identify miRNAs that exist in ESTs, genomic survey sequences, and genome assemblies in diverse plant species (Dezulian et al., 2006; Zhang et al., 2006; Sunkar and Jagadeeswaran, 2008; Zhang et al., 2009b). An obvious limitation of this approach is that it cannot identify novel miRNAs that have not been found in any species yet.

Attempts have also been made to make *de novo* identification of miRNAs. They can be classified into two types. The first type takes advantage of the structural features of miRNA genes. It first identifies all potential hairpin structures that exist in the genome, and then uses a set of filters such as loop length, number of mismatches between putative miRNA and the corresponding miRNA* sequence, and GC content to find hairpins that are most likely real miRNA genes. The conservation of hairpin sequences between two plants may be required. Using this approach, some of known miRNAs were confirmed and new candidates were suggested (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004; Wang et al., 2004). Features of pre-miRNA structures can also be used to build support vector machine (SVM), which is trained with a set of known plant miRNAs and then used to select real miRNAs from candidate hairpin sequences with high accuracy (Xuan et al., 2011). The second approach for *de novo* miRNA identification takes advantage of the fact that plant miRNAs and their target regions have nearly perfect match (up to 4 mismatches) (Rhoades et al., 2002). It first detects a match between an intergenic region and an annotated protein coding gene transcript, and then expands the matched intergenic region and evaluates it for characteristics of miRNA genes (Adai et al., 2005; Lindow and Krogh, 2005).

Direct cloning and bioinformatics approaches start with different data sets and use different rules. However, they are by no means separate or exclusive from each other. These two approaches work synergistically in the miRNA discovery process. On the one hand, when small RNAs are sequenced in the direct cloning approach, bioinformatics tools and methods are needed to analyze high throughput data sets. On the other hand, after miRNA candidates are identified through bioinformatics approach, small RNA data can be used to validate these candidates (Zhang et al., 2009b). In addition, experimental procedures such as northern blots remain to be the most reliable way for validating the expression of candidate miRNAs.

Finding abiotic stress-associated miRNAs

A few years after the discovery of miRNAs in plants, a pioneer study reported the involvement of miRNAs in the molecular response to abiotic stresses. Sunkar and Zhu (2004) found that miRNA393, miR402, and miR319c were induced by cold, dehydration, salt, and plant stress hormone abscisic acid (ABA), whereas miR398 was downregulated. It was later revealed that miR398 was also downregulated by oxidative stress (Sunkar et al., 2006). MiR398 regulates the expression of two homologous Cu/Zn superoxide dismutase genes that are involved in protecting cells from damage of toxic superoxide radicals. Further studies showed that miR399 was upregulated by inorganic phosphate starvation (Chiou et al., 2006), miR169 was downregulated by drought stress (Li et al., 2008) and nitrogen starvation (Zhao et al., 2011) in *Arabidopsis*, and several miRNAs were regulated by mechanical stresses in *Populus* (Lu et al., 2005b). These early studies established that miRNAs play active roles in plant response to abiotic stresses. Because traditional sequencing technology yielded very limited depth, read counts did not provide reliable measure for miRNA expression. In these early studies, northern blot assays were the only feasible way to determine whether a miRNA was regulated by a stress. Due to the low resolution of northern blot analysis, it was hard to identify stress-associated miRNAs that were expressed at relatively low abundance.

The general principle for identifying stress-regulated miRNAs is simple: the expression levels of miRNAs are measured and compared in stress-treated and control samples, and miRNAs that show significantly higher or lower expression in the stress-treated samples are up- or down-regulated miRNAs, respectively. Illumina and other next-generation sequencing platforms provide deep coverage of mature miRNAs and thus make it feasible to detect differentially expressed miRNAs based on comparison of normalized read counts in stress and control libraries. This is also known as digital expression analysis and was used before next-generation sequencing to analyze expression patterns in cDNA libraries (Audic and Claverie, 1997). Because sequence depths in sRNA libraries are much higher than those in cDNA libraries, the statistical test tends to identify

significant expression among too many miRNAs. In practice, more criteria such as minimum fold change and minimum expression level are used to narrow down number of stress-regulated miRNAs (Barrera-Figueroa et al., 2011). Due to high sequencing cost, deep sequencing of sRNA libraries are usually performed without biologic replicates. As the sequencing capacity keeps improving with the use of more powerful sequencing machines such as Illumina HiSeq2000, and sequencing cost going down, it will be feasible to sequence sRNA libraries with biologic replicates (McCormick et al., 2011). With biologic replicates, we can more reliably identify differentially expressed miRNAs with software packages such as EdgeR (Robinson et al., 2010).

Genome-wide analysis of miRNA expression can also be achieved with microarray technology (Liu et al., 2008; Lu et al., 2008b; Ge et al., 2010; Ding et al., 2011; Kantar et al., 2011). Mature sequences of known miRNAs are placed on a microarray chip and stress and control samples are labeled and hybridized to the microarray. MiRNAs that differ significant in hybridization signal intensities are detected as stress-regulated miRNAs. Comparing to digital expression analysis through deep sequencing, miRNA microarray can be more cost-effective when number of samples is large. However, expression analysis is limited to known miRNAs that have been placed on the chip. Therefore, microarray approach is only feasible for plants species that have been subject to extensive miRNA discovery (Liu et al., 2008). In some studies, especially those using plants that have not been investigated before, known miRNAs belonging to different species are included in the microarray to expand the analysis (Jia et al., 2009; Valdés-López et al., 2010). However, this approach can only used for detecting miRNAs that are well conserved between species. A recent comparison study showed that digital expression analysis is usually consistent with miRNA microarrays (Pradervand et al., 2010), hence we expect that both approaches will continue to be widely used.

After putative stress-regulated miRNAs are detected with sequencing data or microarrays, the next step is detailed expression analysis of individual miRNAs. Northern blotting is often the preferred method. However, it may not be an option if the miRNA is lowly expressed or the amount of RNA sample is limited. Real-time qPCR may also be used for confirmation of differential miRNA expression through quantification of miRNA precursors or mature sequences (Chen et al., 2011).

Identification of miRNA targets

MiRNAs perform cellular functions through regulation of their target protein-coding genes. To illustrate molecular mechanisms of plant stress response and delimit exact functional roles of stress-regulated miRNAs, it is necessary to identify miRNA targets. Because of nearly perfect complementarity between miRNAs and their targets in plants, computational prediction of miRNA targets can achieve decent accuracy (Rhoades et al., 2002; Dai et al., 2011). Nonetheless, subsequent validations through experiments are necessary to eliminate false positives and to find targets that are missed by computational prediction. The standard method for target validation is a modified procedure of Rapid Amplification of cDNA Ends (RACE) (Llave et al., 2011). However, this low throughput, laborious procedure does not scale well if many targets need to be validated.

In recent years a new approach based on next-generation sequencing has been developed for genome-wide identification or validation of miRNA targets. Because target mRNAs are recognized by corresponding miRNAs in specific complementary region, they are cleaved at specific locations. The 5′ end portion of the cleavage or degradation products can be cloned and sequenced with next-generation sequencing technology. When sequencing reads are mapped back to mRNA targets, they are predominantly accumulated at the cleavage location. This procedure, termed PARE (Parallel Analysis of RNA Ends) or degradome analysis, has been used successfully to validate hundreds of miRNA targets in several plants, including *Arabidopsis* (Addo-Quaye et al., 2008; German et al., 2008), rice (Wu et al., 2009; Li et al., 2010), soybean (Song et al., 2011), *Populus euphratica* (Li et al., 2011a), *Medicago truncatula* (Devers et al., 2011), and *Vitis vinifera* (Pantaleo et al., 2010). However, because most of these degradome analyses were performed using plants that grew under control conditions, we would not be able to validate a miRNA target if either the miRNA or the target gene was expressed only or predominantly in plants that grew under stress conditions. To validate targets of tissue- or stress-specific miRNAs, we need to perform degradome analyses with specific tissues from plants that grow under specific conditions.

Evolution and functional diversification of stress-associated miRNAs

Like protein coding genes, miRNA genes are subject to the same evolutionary processes such as substitution, insertion, deletion, recombination, and natural selection. Extensive miRNA discovery in a diverse set of plant lineages makes it possible to investigate their evolutionary patterns. Comparing to protein coding genes, miRNAs display much faster evolutionary rate. Among a large number of miRNA families, only around 20 families are conserved in angiosperms (Axtell and Bowman, 2008). The majority of miRNA families show very limited taxonomic distributions (Cuperus et al., 2011). For example, a detailed study of miRNA gene catalogs in *Arabidopsis thaliana* and *Arabidopsis lyrata* showed that at least 13% of miRNA genes in each species are unique, despite that the two species diverged only around 10 million years ago (Fahlgren et al., 2010). Indeed, it has been a common theme to find a high proportion of species-specific miRNAs in

almost all miRNA discovery projects in plants (Axtell et al., 2007; Sunkar et al., 2008; Szittya et al., 2008; Zhu et al., 2008; Barrera-Figueroa et al., 2011). These data suggest that miRNA genes are frequently created and lost. In the *Arabidopsis* lineage, it has been estimated that the rate of flux is 1.2 to 3.3 miRNA genes per millions years (Fahlgren et al., 2010).

How did miRNA genes originate in plants? A couple of mechanisms have been proposed. MiRNA genes may be created from duplicated protein coding genes that were arranged in tandem and in inverted directions (Allen et al., 2004). A strong support for this hypothesis is that the homology between some young miRNA genes and their targets extended beyond mature miRNA region (Allen et al., 2004; Fahlgren et al., 2010). Another rich sources for miRNA origination are inverted repeats that are found abundantly in plant genomes (Jones-Rhoades and Bartel, 2004; de Felippes et al., 2008). Some of these repeats belong to non-autonomous DNA transposons or miniature inverted-repeat transposable elements (MITEs) (Bureau and Wessler, 1992; Jiang et al., 2004). These inverted repeats, if transcribed, can form hairpin structures that may be cleaved by DCL proteins into short double strand RNAs. Some small RNAs that were generated from inverted-repeat loci have been shown to possess typical miRNA characteristics (Piriyapongsa and Jordan, 2008; Li et al., 2011b).

Once miRNAs were created, their fate depended on how they would function in the cellular system. The majority of young miRNAs did not find a target gene or did not regulate targets in such a way that plant fitness was enhanced, and they were quickly lost during evolution. This was supported by recent evolutionary analysis of miRNAs in *Arabidopsis thaliana* and *Arabidopsis lyrata* where most young miRNAs were weakly expressed, lacked targets, and appeared to be selectively neutral (Fahlgren et al., 2010). Only a small number of young miRNAs were favored by natural selection and became conserved miRNAs.

Not surprisingly, among abiotic stress-associated miRNAs discovered in various plants (Supplemental Materials Table S1), most are conserved miRNA families. These miRNAs were conserved through natural selection because they helped plants to cope better with abiotic stresses. However, this does not mean conserved miRNAs always perform their function in exactly the same way. Although most conserved miRNAs target the same set of homologs (Axtell and Bowman, 2008), functional diversifications are evident at several levels.

First, orthologous miRNAs may function quite differently under the same stress in different species. For example, we compared the list of miRNAs that were identified as drought-associated miRNAs in six plants that have been subject to genome-wide miRNA expression profiling under drought stress conditions (Fig. 1). It is apparent that conserved miRNAs function quite differently under the same stress: conserved miRNAs were often not only regulated in some species but not in others, but also regulated in different directions (upregulated in one species, but downregulated in another species). Although part of the difference can be attributed to different expression profiling approaches or different criteria for selecting stress-regulated miRNAs, functional diversification of conserved miRNAs most likely plays a major role.

Second, orthologous miRNAs may function differently under the same stress even in different genotypes of the same species. For example, we compared the expression profiles of drought-associated miRNAs in a drought-tolerant genotype and a drought-sensitive genotype in cowpea and found that nine miRNAs were predominantly or exclusively expressed in only one genotype and that 11 miRNAs were drought-regulated in only one genotype, but not the other (Barrera-Figueroa et al., 2011).

Lastly, different members of the same miRNA family may function differently after duplication. For example, Lelandais-Brière et al. (2009) have shown that several miRNA isoforms had tissue-specific expression patterns, suggesting functional diversification of different isoforms.

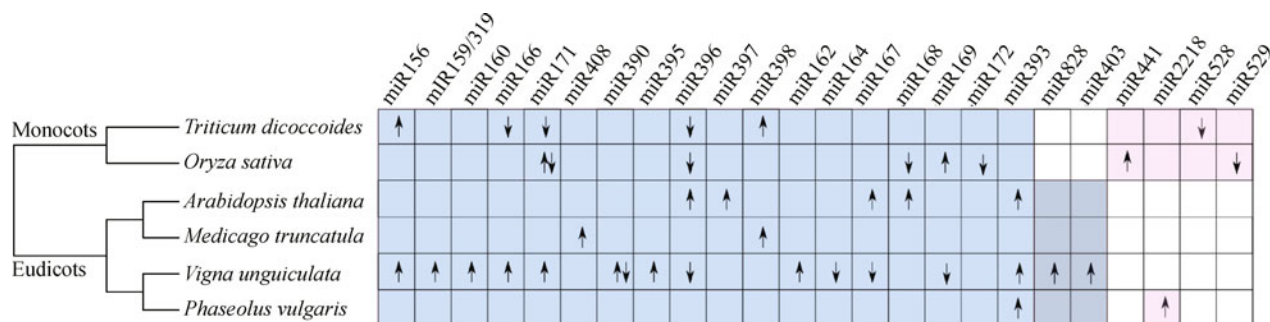

Figure 1 Functional diversification of drought-associated miRNAs. Six plants have been subject to genome-wide surveys for drought-associated miRNAs and only conserved miRNAs that were regulated by drought in at least one plant were shown. Only conserved miRNAs shown in Figure 1 of Cuperus et al. (2011) were considered. Boxes were colored according to range of conservation. The absence of a miRNA in a species was denoted with white color and presence was denoted with other colors. Up- and down-arrows in the boxes indicate whether miRNAs were up- or down-regulated by drought stress. Two arrows in a single box means the expression of the same miRNA family changed in different directions under drought stress in the same species from two or more independent studies.

Future directions

The studies of abiotic stress-associated plant miRNAs in the last few years have focused on miRNA discovery, especially at the whole genome scale. We now have a long list of abiotic stress-associated miRNAs under different stresses and in a variety of plants. These efforts clearly demonstrated the important roles that miRNAs play in stress response and built a solid foundation for future research. Whereas genome-wide expression profiling will likely to continue in the near future, there is an urgent need for detailed functional characterizations of individual miRNAs. Mutants can be created with altered expression of a particular miRNA and effects of the mutation are observed at the molecular and phenotypic levels. So far, only a few miRNAs have been investigated in details in the model plant *Arabidopsis* (Sunkar et al., 2006; Li et al., 2008; Gou et al., 2011). Additional functional studies of miRNAs are needed, especially in economically important crops, with the aim to find ways to modify crops genetically for better stress tolerance.

Like other molecules in the cellular system, miRNAs do not act alone, but as an integral part of complex gene regulation network. Like protein coding genes, the transcription of miRNA genes are regulated by upstream transcription factors. However, very little is known about upstream regulators and regulatory motifs of miRNA genes, as well as miRNA-mediated regulatory circuits. Recently the upstream boundaries of a few dozen miRNAs genes were determined in *Arabidopsis* (Xie et al., 2005) and maize (Zhang et al., 2009b). Several attempts have been made to find regulatory motifs in the upstream regions of plant miRNAs (Megraw et al., 2006; Joung and Fei, 2009; Liu et al., 2010). Further investigations on upstream regulatory motifs and transcription factors and continuous validation of targets of stress-associated miRNAs will allow us to construct a detailed miRNA-mediated gene regulatory network (Meng et al., 2011), which is critical for full understanding of plant stress response.

Closely related plant species or different genotypes of the same species often differ appreciably in their capacities of stress tolerance. Comparative analysis of the expression of miRNAs in these plants may allow us to detect differential expression of some stress-associated miRNAs (Barrera-Figueroa et al., 2011). Functional analysis of these miRNAs may provide clues to the mechanisms of natural variation in stress tolerance and may allow us to find new means for crop improvement.

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Compliance with ethics guidelines

Blanca E. Barrera-Figueroa, Zhigang Wu, and Renyi Liu declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Adai A, Johnson C, Mlotshwa S, Archer-Evans S, Manocha V, Vance V, Sundaresan V (2005). Computational prediction of miRNAs in *Arabidopsis thaliana*. Genome Res, 15(1): 78–91

Addo-Quaye C, Eshoo T W, Bartel D P, Axtell M J (2008). Endogenous siRNA and miRNA targets identified by sequencing of the *Arabidopsis* degradome. Curr Biol, 18(10): 758–762

Allen E, Xie Z, Gustafson A M, Carrington J C (2005). microRNA-directed phasing during trans-acting siRNA biogenesis in plants. Cell, 121(2): 207–221

Allen E, Xie Z, Gustafson A M, Sung G H, Spatafora J W, Carrington J C (2004). Evolution of microRNA genes by inverted duplication of target gene sequences in *Arabidopsis thaliana.* Nat Genet, 36(12): 1282–1290

Ambros V, Bartel B, Bartel D P, Burge C B, Carrington J C, Chen X, Dreyfuss G, Eddy S R, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T (2003). A uniform system for microRNA annotation. RNA, 9(3): 277–279

Audic S, Claverie J M (1997). The significance of digital gene expression profiles. Genome Res, 7(10): 986–995

Axtell M J, Bowman J L (2008). Evolution of plant microRNAs and their targets. Trends Plant Sci, 13(7): 343–349

Axtell M J, Snyder J A, Bartel D P (2007). Common functions for diverse small RNAs of land plants. Plant Cell, 19(6): 1750–1769

Barrera-Figueroa B E, Gao L, Diop N N, Wu Z, Ehlers J D, Roberts P A, Close T J, Zhu J K, Liu R (2011). Identification and comparative analysis of drought-associated microRNAs in two cowpea genotypes. BMC Plant Biol, 11(1): 127

Bartel D P (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116(2): 281–297

Bonnet E, Wuyts J, Rouzé P, Van de Peer Y (2004). Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. Proc Natl Acad Sci USA, 101(31): 11511–11516

Borsani O, Zhu J, Verslues P E, Sunkar R, Zhu J K (2005). Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. Cell, 123(7): 1279–1291

Boyer J S (1982). Plant productivity and environment. Science, 218 (4571): 443–448

Bureau T E, Wessler S R (1992). Tourist: a large family of small inverted repeat elements frequently associated with maize genes. Plant Cell, 4 (10): 1283–1294

Chen C, Tan R, Wong L, Fekete R, Halsey J (2011). Quantitation of microRNAs by real-time RT-qPCR. Methods Mol Biol, 687: 113–134

Chen X (2005). MicroRNA biogenesis and function in plants. FEBS Lett, 579(26): 5923–5931

Chinnusamy V, Zhu J K (2009). RNA-directed DNA methylation and demethylation in plants. Sci China C Life Sci, 52(4): 331–343

Chiou T J, Aung K, Lin S I, Wu C C, Chiang S F, Su C L (2006). Regulation of phosphate homeostasis by MicroRNA in *Arabidopsis*. Plant Cell, 18(2): 412–421

Cuperus J T, Fahlgren N, Carrington J C (2011). Evolution and functional diversification of MIRNA genes. Plant Cell, 23(2): 431–442

Dai X, Zhuang Z, Zhao P X (2011). Computational analysis of miRNA targets in plants: current status and challenges. Brief Bioinform, 12 (2): 115–121

Dalmay T (2006). Short RNAs in environmental adaptation. Proc Biol Sci, 273(1594): 1579–1585

Devers E A, Branscheid A, May P, Krajinski F (2011). Stars and symbiosis: microRNA- and microRNA*-mediated transcript cleavage involved in *Arbuscular mycorrhizal* symbiosis. Plant Physiol, 156(4): 1990–2010

Dezulian T, Remmert M, Palatnik J F, Weigel D, Huson D H (2006). Identification of plant microRNA homologs. Bioinformatics, 22(3): 359–360

Ding Y, Chen Z, Zhu C (2011). Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*). J Exp Bot, 62(10): 3563–3573

Fahlgren N, Howell M D, Kasschau K D, Chapman E J, Sullivan C M, Cumbie J S, Givan S A, Law T F, Grant S R, Dangl J L, Carrington J C (2007). High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes. PLoS ONE, 2 (2): e219

Fahlgren N, Jogdeo S, Kasschau K D, Sullivan C M, Chapman E J, Laubinger S, Smith L M, Dasenko M, Givan S A, Weigel D, Carrington J C (2010). MicroRNA gene evolution in *Arabidopsis lyrata* and *Arabidopsis thaliana.* Plant Cell, 22(4): 1074–1089

Felippes F F, Schneeberger K, Dezulian T, Huson D H, Weigel D (2008). Evolution of *Arabidopsis thaliana* microRNAs from random sequences. RNA, 14(12): 2455–2459

Ge Y, Li Y, Zhu Y M, Bai X, Lv D K, Guo D, Ji W, Cai H (2010). Global transcriptome profiling of wild soybean (*Glycine soja*) roots under NaHCO3 treatment. BMC Plant Biol, 10(1): 153

German M A, Pillay M, Jeong D H, Hetawal A, Luo S, Janardhanan P, Kannan V, Rymarquis L A, Nobuta K, German R, De Paoli E, Lu C, Schroth G, Meyers B C, Green P J (2008). Global identification of microRNA-target RNA pairs by parallel analysis of RNA ends. Nat Biotechnol, 26(8): 941–946

Gou J Y, Felippes F F, Liu C J, Weigel D, Wang J W (2011). Negative regulation of anthocyanin biosynthesis in *Arabidopsis* by a miR156-targeted SPL transcription factor. Plant Cell, 23(4): 1512–1522

Jacob F, Monod J (1961). Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol, 3(3): 318–356

Jia X, Wang W X, Ren L, Chen Q J, Mendu V, Willcut B, Dinkins R, Tang X, Tang G (2009). Differential and dynamic regulation of miR398 in response to ABA and salt stress in *Populus tremula* and *Arabidopsis thaliana.* Plant Mol Biol, 71(1–2): 51–59

Jiang N, Feschotte C, Zhang X, Wessler S R (2004). Using rice to understand the origin and amplification of miniature inverted repeat transposable elements (MITEs). Curr Opin Plant Biol, 7(2): 115–119

Jin H, Vacic V, Girke T, Lonardi S, Zhu J K (2008). Small RNAs and the regulation of cis-natural antisense transcripts in *Arabidopsis*. BMC Mol Biol, 9(1): 6

Johnson C, Bowman L, Adai A T, Vance V, Sundaresan V (2007). CSRDB: a small RNA integrated database and browser resource for cereals. Nucleic Acids Res, 35 (Database issue): D829–D833

Jones-Rhoades M W, Bartel D P (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell, 14(6): 787–799

Jones-Rhoades M W, Bartel D P, Bartel B (2006). MicroRNAS and their regulatory roles in plants. Annu Rev Plant Biol, 57(1): 19–53

Joung J G, Fei Z (2009). Identification of microRNA regulatory modules in *Arabidopsis* via a probabilistic graphical model. Bioinformatics, 25(3): 387–393

Kantar M, Lucas S J, Budak H (2011). miRNA expression patterns of *Triticum dicoccoides* in response to shock drought stress. Planta, 233 (3): 471–484

Katiyar-Agarwal S, Gao S, Vivian-Smith A, Jin H (2007). A novel class of bacteria-induced small RNAs in *Arabidopsis*. Genes Dev, 21(23): 3123–3134

Langmead B, Trapnell C, Pop M, Salzberg S L (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol, 10(3): R25

Lau N C, Lim L P, Weinstein E G, Bartel D P (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans.* Science, 294(5543): 858–862

Lelandais-Brière C, Naya L, Sallet E, Calenge F, Frugier F, Hartmann C, Gouzy J, Crespi M (2009). Genome-wide *Medicago truncatula* small RNA analysis revealed novel microRNAs and isoforms differentially regulated in roots and nodules. Plant Cell, 21(9): 2780–2796

Li B, Qin Y, Duan H, Yin W, Xia X (2011a). Genome-wide characterization of new and drought stress responsive microRNAs in *Populus euphratica.* J Exp Bot, 62(11): 3765–3779

Li R, Yu C, Li Y, Lam T W, Yiu S M, Kristiansen K, Wang J (2009). SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics, 25(15): 1966–1967

Li W X, Oono Y, Zhu J, He X J, Wu J M, Iida K, Lu X Y, Cui X, Jin H, Zhu J K (2008). The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. Plant Cell, 20(8): 2238–2251

Li Y, Li C, Xia J, Jin Y (2011b). Domestication of transposable elements into MicroRNA genes in plants. PLoS ONE, 6(5): e19212

Li Y F, Zheng Y, Addo-Quaye C, Zhang L, Saini A, Jagadeeswaran G, Axtell M J, Zhang W, Sunkar R (2010). Transcriptome-wide identification of microRNA targets in rice. Plant J, 62(5): 742–759

Lindow M, Krogh A (2005). Computational evidence for hundreds of non-conserved plant microRNAs. BMC Genomics, 6(1): 119

Liu B, Liu L, Tsykin A, Goodall G J, Green J E, Zhu M, Kim C H, Li J (2010). Identifying functional miRNA-mRNA regulatory modules with correspondence latent dirichlet allocation. Bioinformatics, 26 (24): 3105–3111

Liu H H, Tian X, Li Y J, Wu C A, Zheng C C (2008). Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana.* RNA, 14(5): 836–843

Llave C, Franco-Zorrilla J M, Solano R, Barajas D (2011). Target validation of plant microRNAs. Methods Mol Biol, 732: 187–208

Llave C, Kasschau K D, Rector M A, Carrington J C (2002). Endogenous and silencing-associated small RNAs in plants. Plant Cell, 14(7): 1605–1619

Lu C, Jeong D H, Kulkarni K, Pillay M, Nobuta K, German R, Thatcher S R, Maher C, Zhang L, Ware D, Liu B, Cao X, Meyers B C, Green P J (2008a). Genome-wide analysis for discovery of rice microRNAs

reveals natural antisense microRNAs (nat-miRNAs). Proc Natl Acad Sci USA, 105(12): 4951–4956

Lu C, Kulkarni K, Souret F F, MuthuValliappan R, Tej S S, Poethig R S, Henderson I R, Jacobsen S E, Wang W, Green P J, Meyers B C (2006). MicroRNAs and other small RNAs enriched in the *Arabidopsis* RNA-dependent RNA polymerase-2 mutant. Genome Res, 16(10): 1276–1288

Lu C, Meyers B C, Green P J (2007). Construction of small RNA cDNA libraries for deep sequencing. Methods, 43(2): 110–117

Lu C, Tej S S, Luo S J, Haudenschild C D, Meyers B C, Green P J (2005a). Elucidation of the small RNA component of the transcriptome. Science, 309(5740): 1567–1569

Lu S, Sun Y H, Chiang V L (2008b). Stress-responsive microRNAs in Populus. Plant J, 55(1): 131–151

Lu S, Sun Y H, Shi R, Clark C, Li L, Chiang V L (2005b). Novel and mechanical stress-responsive MicroRNAs in *Populus trichocarpa* that are absent from Arabidopsis. Plant Cell, 17(8): 2186–2203

McCormick K P, Willmann M R, Meyers B C (2011). Experimental design, preprocessing, normalization and differential expression analysis of small RNA sequencing experiments. Silence, 2(1): 2

Megraw M, Baev V, Rusinov V, Jensen S T, Kalantidis K, Hatzigeorgiou A G (2006). MicroRNA promoter element discovery in *Arabidopsis*. RNA, 12(9): 1612–1619

Mendes N D, Freitas A T, Sagot M F (2009). Current tools for the identification of miRNA genes and their targets. Nucleic Acids Res, 37(8): 2419–2433

Meng Y, Shao C, Chen M (2011). Toward microRNA-mediated gene regulatory networks in plants. Brief Bioinform, 12(6): 645–659

Meyers B C, Axtell M J, Bartel B, Bartel D P, Baulcombe D, Bowman J L, Cao X, Carrington J C, Chen X, Green P J, Griffiths-Jones S, Jacobsen S E, Mallory A C, Martienssen R A, Poethig R S, Qi Y, Vaucheret H, Voinnet O, Watanabe Y, Weigel D, Zhu J K (2008). Criteria for annotation of plant MicroRNAs. Plant Cell, 20(12): 3186–3190

Nobuta K, Venu R C, Lu C, Beló A, Vemaraju K, Kulkarni K, Wang W Z, Pillay M, Green P J, Wang G L, Meyers B C (2007). An expression atlas of rice mRNAs and small RNAs. Nat Biotechnol, 25(4): 473–477

Pak J, Fire A (2007). Distinct populations of primary and secondary effectors during RNAi in *C. elegans.* Science, 315(5809): 241–244

Pantaleo V, Szittya G, Moxon S, Miozzi L, Moulton V, Dalmay T, Burgyan J (2010). Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. Plant J, 62(6): 960–976

Piriyapongsa J, Jordan I K (2008). Dual coding of siRNAs and miRNAs by plant transposable elements. RNA, 14(5): 814–821

Pradervand S, Weber J, Lemoine F, Consales F, Paillusson A, Dupasquier M, Thomas J, Richter H, Kaessmann H, Beaudoing E, Hagenbüchle O, Harshman K (2010). Concordance among digital gene expression, microarrays, and qPCR when measuring differential expression of microRNAs. Biotechniques, 48(3): 219–222

Rajagopalan R, Vaucheret H, Trejo J, Bartel D P (2006). A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana.* Genes Dev, 20(24): 3407–3425

Reinhart B J, Bartel D P (2002). Small RNAs correspond to centromere heterochromatic repeats. Science, 297(5588): 1831

Rhoades M W, Reinhart B J, Lim L P, Burge C B, Bartel B, Bartel D P (2002). Prediction of plant microRNA targets. Cell, 110(4): 513–520

Robinson M D, McCarthy D J, Smyth G K (2010). EdgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics, 26(1): 139–140

Ron M, Alandete Saez M, Eshed Williams L, Fletcher J C, McCormick S (2010). Proper regulation of a sperm-specific cis-nat-siRNA is essential for double fertilization in *Arabidopsis*. Genes Dev, 24(10): 1010–1021

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002). Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J, 31(3): 279–292

Song Q X, Liu Y F, Hu X Y, Zhang W K, Ma B, Chen S Y, Zhang J S (2011). Identification of miRNAs and their target genes in developing soybean seeds by deep sequencing. BMC Plant Biol, 11(1): 5

Sunkar R, Girke T, Jain P K, Zhu J K (2005). Cloning and characterization of microRNAs from rice. Plant Cell, 17(5): 1397–1411

Sunkar R, Jagadeeswaran G (2008). *In silico* identification of conserved microRNAs in large number of diverse plant species. BMC Plant Biol, 8(1): 37

Sunkar R, Kapoor A, Zhu J K (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell, 18(8): 2051–2065

Sunkar R, Zhou X F, Zheng Y, Zhang W X, Zhu J K (2008). Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol, 8(1): 25

Sunkar R, Zhu J K (2004). Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. Plant Cell, 16(8): 2001–2019

Szittya G, Moxon S, Santos D M, Jing R, Fevereiro M P, Moulton V, Dalmay T (2008). High-throughput sequencing of *Medicago truncatula* short RNAs identifies eight new miRNA families. BMC Genomics, 9(1): 593

Valdés-López O, Yang S S, Aparicio-Fabre R, Graham P H, Reyes J L, Vance C P, Hernández G (2010). MicroRNA expression profile in common bean (*Phaseolus vulgaris*) under nutrient deficiency stresses and manganese toxicity. New Phytol, 187(3): 805–818

Vaucheret H (2006). Post-transcriptional small RNA pathways in plants: mechanisms and regulations. Genes Dev, 20(7): 759–771

Vazquez F, Legrand S, Windels D (2010). The biosynthetic pathways and biological scopes of plant small RNAs. Trends Plant Sci, 15(6): 337–345

Vazquez F, Vaucheret H, Rajagopalan R, Lepers C, Gasciolli V, Mallory A C, Hilbert J L, Bartel D P, Crété P (2004). Endogenous *trans*-acting siRNAs regulate the accumulation of *Arabidopsis* mRNAs. Mol Cell, 16(1): 69–79

Vigneault F, Sismour A M, Church G M (2008). Efficient microRNA capture and bar-coding via enzymatic oligonucleotide adenylation. Nat Methods, 5(9): 777–779

Wang X J, Reyes J L, Chua N H, Gaasterland T (2004). Prediction and identification of *Arabidopsis thaliana* microRNAs and their mRNA targets. Genome Biol, 5(9): R65

Wei B, Cai T, Zhang R, Li A, Huo N, Li S, Gu Y Q, Vogel J, Jia J, Qi Y, Mao L (2009). Novel microRNAs uncovered by deep sequencing of

small RNA transcriptomes in bread wheat (*Triticum aestivum* L.) and *Brachypodium distachyon* (L.) Beauv. Funct Integr Genomics, 9(4): 499–511

Wu L, Zhang Q, Zhou H, Ni F, Wu X, Qi Y (2009). Rice MicroRNA effector complexes and targets. Plant Cell, 21(11): 3421–3435

Wu L, Zhou H, Zhang Q, Zhang J, Ni F, Liu C, Qi Y (2010). DNA methylation mediated by a microRNA pathway. Mol Cell, 38(3): 465–475

Xie Z, Allen E, Fahlgren N, Calamar A, Givan S A, Carrington J C (2005). Expression of *Arabidopsis* MIRNA genes. Plant Physiol, 138 (4): 2145–2154

Xuan P, Guo M, Liu X, Huang Y, Li W, Huang Y (2011). PlantMiRNAPred: efficient classification of real and pseudo plant pre-miRNAs. Bioinformatics, 27(10): 1368–1376

Zhang B H, Pan X P, Cannon C H, Cobb G P, Anderson T A (2006). Conservation and divergence of plant microRNA genes. Plant J, 46 (2): 243–259

Zhang J Y, Xu Y Y, Huan Q, Chong K (2009a). Deep sequencing of *Brachypodium* small RNAs at the global genome level identifies microRNAs involved in cold stress response. BMC Genomics, 10(1): 449

Zhang L F, Chia J M, Kumari S, Stein J C, Liu Z J, Narechania A, Maher C A, Guill K, McMullen M D, Ware D (2009b). A genome-wide characterization of microRNA genes in maize. PLoS Genet, 5(11): e1000716

Zhao M, Ding H, Zhu J K, Zhang F, Li W X (2011). Involvement of miR169 in the nitrogen-starvation responses in *Arabidopsis*. New Phytol, 190(4): 906–915

Zhu J K (2002). Salt and drought stress signal transduction in plants. Annu Rev Plant Biol, 53(1): 247–273

Zhu Q H, Spriggs A, Matthew L, Fan L, Kennedy G, Gubler F, Helliwell C (2008). A diverse set of microRNAs and microRNA-like small RNAs in developing rice grains. Genome Res, 18(9): 1456–1465