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·Invited Review·

Micro RNAs and Short-interfering RNAs in Plants

Ramanjulu Sunkar[1*] and Jian-Kang Zhu[2]

([1]*Department of Biochemistry and Molecular Biology*, *Oklahoma State University*, Stillwater, OK 74078, USA;
[2]*Department of Botany and Plant Sciences*, *University of California*, Riverside, CA 95251, USA)

Abstract

Gene silencing can occur either at the transcriptional level or post-transcriptional level or both. Many instances of sequence-specific silencing requires small RNAs that can be divided into two major classes: microRNAs (miRNAs) and short-interfering RNAs (siRNAs). miRNAs function in post-transcriptional gene silencing by guiding mRNA degradation or translational repression. Endogenous siRNAs are more diverse in plants than in animals and can direct post-transcriptional gene silencing through mRNA degradation or transcriptional gene silencing by triggering DNA methylation and histone modifications. This review discusses recent advances in the field of small RNA-guided gene silencing in plants including rice.

Key words: gene silencing; microRNAs; short-interfering RNAs; plants.

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Small RNAs and Gene Silencing

Endogenous small RNAs potentially involved in gene silencing can be divided into two major classes: microRNAs (miRNAs) and short-interfering RNAs (siRNAs). miRNAs were first identified in *Caenorhabditis elegans* through genetic screens for aberrant development (Lee et al. 1993; Wightman et al. 1993) and were later found in a number of multicellular eukaryotes (Bartel 2004). MicroRNAs are genome encoded ~20–22-nt, small non-coding RNAs. A duplex structure containing miRNA and miRNA* with 2-nt 3' overhangs is generated from a longer single stranded hairpin precursor by the Ribonuclease III-like enzyme, Dicer-like 1 (DCL1) in association with other proteins such as HYL1 and Serrate (SE) in plants (Figure 1; Jones-Rhoades et al. 2006; Yang et al. 2006a; Lobbes et al. 2006). HEN1, a plant-specific methyltransferase provides stability to the miRNA duplex by adding methyl groups to the 3' ends (Yu et al. 2005). The miRNA* duplex produced in the nucleus may be exported to the cytosol by HASTY (HST). Only the miRNA but not the miRNA is loaded onto an Argonaute-containing RNA-induced silencing complex (RISC). The RISC complex will be guided by miRNAs to complementary or partially complementary mRNAs for degradation or translational repression (Figure 1). Thus far, ~20 deeply conserved microRNA families are known in plants (Jones-Rhoades and Bartel 2006; Mallory and Vaucheret 2006). In addition to conserved miRNAs, high throughput sequencing of small RNA libraries revealed many non-conserved miRNAs in *Arabidopsis* (Lu et al. 2005, 2006; Rajagopalan et al. 2006).

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*Author for correspondence.

Tel: +1 405 744 7896;

Fax: +1 405 744 7799;

E-mail: <rsunkar@biochem.okstate.edu>.

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Roles of miRNAs in Plant Development and Stress Responses

Target predictions of plant miRNAs revealed the possible involvement of miRNAs in various aspects of plant development, including auxin signaling, meristem boundary formation and organ separation, leaf development and polarity, seedling development, embryo development, phyllotaxy, lateral root formation, transition from the juvenile to adult vegetative phase and from the vegetative to flowering phase, formation of floral organ identity, petal number and reproduction (Mallory and

Vaucheret 2006; Jones-Rhoades et al. 2006). Also, plant miRNAs are key regulators in biotic and abiotic stress responses and in nutrient homeostasis (Jones-Rhoades and Bartel 2004; Fujii et al. 2005; Chiou et al. 2006; Navarro et al. 2006; Sunkar et al. 2006, 2007).

Several functional studies in *Arabidopsis* demonstrated the role of miRNAs in developmental processes. One of the first reports provided the genetic basis of a microRNA involvement in leaf development (Palatnik et al. 2003). An activation tagging approach in *Arabidopsis* led to the overexpression of miR319 (also known as miRJAW) (Palatnik et al. 2003). miRJAW is complementary to a highly conserved sequence motif found in several *TCP* genes (a large family of proteins sharing a common "TCP domain" (Teosinte branched 1, Cycloidea and PCF (proliferating cellular nuclear antigen factors)). The miRJAW gain-of-function phenotype is caused by down-regulation of the *TCP* genes. This was confirmed in microarray studies in the mutant background and the cleavage sites were mapped to the JAW-complementary site in multiple *TCP* genes (Palatnik et al. 2003). In a second report, two independent studies provided *in vivo* significance of *Arabidopsis* miR172-guided regulation of its target gene *APETALA2*, in floral homeotic patterning (Aukerman and Sakai 2003; Chen 2004). There are three classes of transcription factors whose combinatorial activities specify the four organ types in the floral meristem. One of these, APETALA2 (AP2), regulates the identity of perianth organs. AP2 protein levels are elevated in mutant backgrounds that affect miR172 accumulation, and the *AP2* mRNA contains a target site for miR172. Overexpression of miR172 in transgenic plants accelerated floral transition and induced homeotic

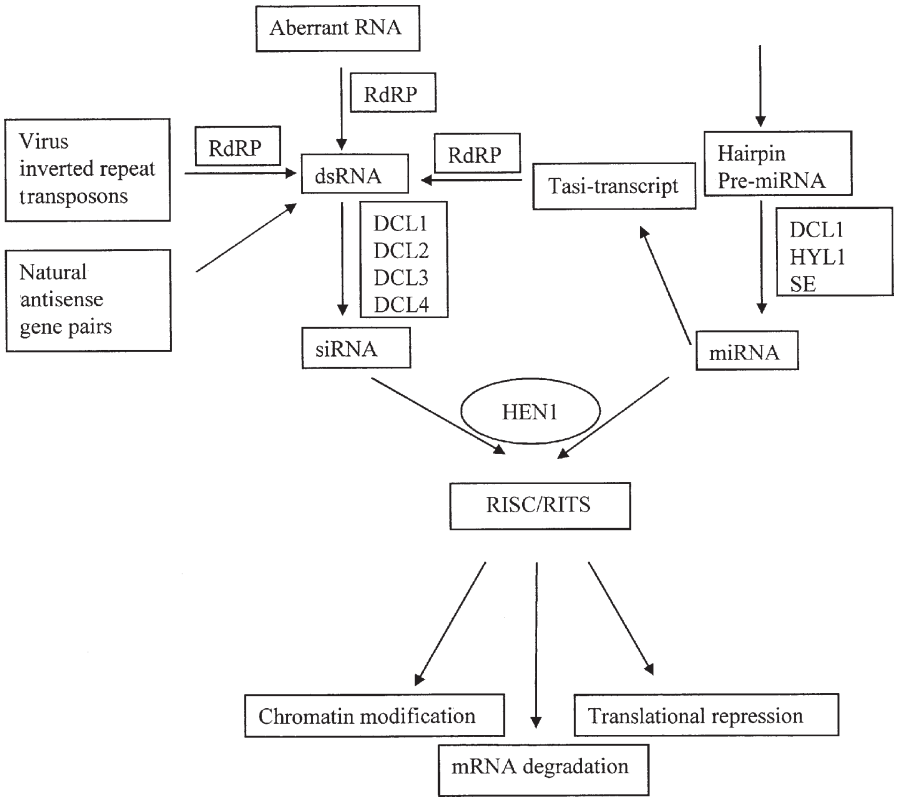

Figure 1. Biogenesis and mode of action of small RNAs in plants.

Mature microRNAs (miRNAs) are derived from a coordinated action of Dicer-like 1 (DCL1), HYL1 and Serrate SE. The processed 21-nt duplex is methylated at its 3' end by HEN1. Methylated miRNAs are loaded into RISC (RNA-induced silencing complex) and serve as a guide molecule for recognizing the target mRNAs. RISC can cause post-transcriptional gene silencing either by mRNA degradation or by inhibiting translation of target mRNAs. Biogenesis of endogenous siRNAs (*Trans*-acting siRNAs (tasiRNAs), natural-antisense transcripts-derived siRNAs (natisRNAs) and heterochromatic short-interfering (si) RNAs) or viral siRNAs requires the activity of one of the RNA-dependent RNA polymerases (RdRPs) for converting single-stranded RNA (ssRNA) into double-stranded RNA (dsRNA). The dsRNA is recognized and diced by one of the DCLs (DCL2, DCL3, DCL4 or even DCL1). The processed siRNAs are loaded into a RISC or RITS (RNA-induced transcriptional silencing complex) and can cause mRNA degradation (post-transcriptional gene silencing), or DNA/histone modifications causing transcriptional gene silencing of the target gene.

phenotypes that resemble *ap2* loss-of-function mutants. Conversely, expression of *AP2* with a disrupted miR172 target site but not the wild type *AP2*, caused strong defects in floral patterning. Many studies support the notion that the degree of complementarity between an miRNA and its target mRNA determines the type of regulatory mode: a high degree of complementarity leads to mRNA cleavage, whereas a low degree of complementarity may lead to translational repression (Doench et al. 2003). However, *AP2* regulation guided by miR172 in *Arabidopsis* suggests that miRNA and target mRNA interactions are more complicated. *Arabidopsis* miR172 directs both cleavage (Kassachau et al. 2003; Schwab et al. 2005) and translational repression (Aukerman and Sakai 2003; Chen 2004) of *AP2*, and possibly a feed-back loop (Schwab et al. 2005). Whether such a complexity is widespread or is just restricted to the miR172-*AP2* pair is unknown. Nevertheless, these findings suggest that the rules governing the mode of miRNA-guided target gene regulation is not adequately understood.

The disruption of plant miRNA regulation has been shown to cause striking developmental abnormalities in several cases. Dominant gain-of-function mutations in homeodomain-leucine zipper (HD-ZIP) transcription factor genes, *PHABULOSA*, *PHAVULOTA*, and *REVOLUTA* that destabilize pairing to miR165/miR166 cause loss of adaxial/abaxial polarity in developing leaves (Emery et al. 2003). Similar mutations in the HD-ZIP gene, *ROLLED LEAF1* in maize, also cause the adaxialization of the abaxial surface of leaves, indicating that the miR165/miR166 family has a conserved role in determining leaf polarity despite the morphological differences between *Arabidopsis* and maize leaves (Juarez et al. 2004).

Plants impaired in miR164-mediated regulation of *CUP-SHAPED COTYLEDON1* (*CUC1*) have altered patterns of embryonic, vegetative and floral development and boundary formation during organ initiation from meristems (Mallory et al. 2004; Laufs et al. 2004). miR164b-overexpressing plants display organ fusion phenotypes (Mallory et al. 2004). Analysis of an miR164a loss-of-function mutant defined a specific role in leaf margin formation. The miR164a loss-of-function mutant shows deep serrate margins. In contrast, miR164a gain-of-function results in smooth margins (Nikovics et al. 2006). miR164c seems to regulate *CUC1* and *CUC2* in a tissue-specific manner in early developing flowers and the interaction determines petal number (Baker et al. 2005). miR164 also negatively regulates auxin-mediated lateral root development by targeting *NAC1* transcripts. A close inverse correlation was found between changes in the miR164 level and changes in both *NAC1* transcript level and the number of lateral roots (Guo et al. 2005). Both miR164a and miR164b loss-of-function mutants produce more lateral roots compared to wild-type seedlings. The miR164 level is modulated by auxin, suggesting a homeostatic mechanism by which miRNA mediates the clearance of NAC1 mRNA to terminate auxin signaling (Guo et al. 2005).

Auxin is a phytohormone implicated in many aspects of plant growth and development. Many auxin effects are mediated by Auxin Response Factors (ARFs) in plants. miR160 targets *ARF10*, *ARF16* and *ARF17* in plants. *Arabidopsis* transgenic plants expressing an miR160-resistant version of *ARF17* displayed several developmental defects including distorted embryo symmetry, altered phyllotaxy, abnormal stamens and sterility (Mallory et al. 2005). Another miRNA, miR167 negatively regulates several other members of the *ARF* gene family (*ARF6* and *ARF8*) that are involved in plant development (Jones-Rhoades and Bartel 2004). Additionally, miRNA-dependent *trans*-acting siRNAs also target *ARF* genes in plants (Allen et al. 2005; Hunter et al. 2006; Fahlgren et al. 2006).

Overexpression of miR159 results in decreased *MYB33* and *LEAFY* transcript levels leading to defective anthers, decreased fertility and delayed flowering under short day conditions (Achard et al. 2004). Abolishing interaction between miR159 and *MYB33* resulted in pleiotropic developmental defects such as inability to expand cotyledons, arrest of seedling development, reduced size and shorter internodes (Millar and Gubler 2005). miR156 overexpression causes a moderate delay in flowering (Schwab et al. 2005; Gandikota et al. 2007), whereas the overexpression of *SPL3* and an miR156-resistant *SPL3*, one of the target genes of miR156, results in accelerated flowering (Cardon et al. 1997; Wu and Poethig 2006; Gandikota et al. 2007).

In addition to their roles in development, functional studies indicated that miRNAs play important roles in plant responses to biotic and abiotic stresses (Jones-Rhoades and Bartel 2004; Fujii et al. 2005; Navarro et al. 2006; Sunkar et al. 2006; Sunkar et al. 2007). For instance, miR395 is not detectable in plants grown under standard conditions, but is induced by low-sulfate stress. miR395 targets *ATP* sulfurylases (*APS*) that catalyze the first step of inorganic sulfate assimilation, and the accumulation of *APS1* mRNA is decreased under low-sulfate stress (Jones-Rhoades and Bartel 2004). Similarly, miR399 is not detectable in plants grown under standard nutrient rich conditions, but is induced under low-phosphate stress. miR399 targets a transcript encoding ubiquitin-conjugating enzyme (UBC), and *UBC* mRNA accumulation is decreased in response to low-phosphate stress. Down-regulation of *UBC* is important to induce the phosphate transporter gene *AtPT1* and attenuate primary-root elongation (Fujii et al. 2005; Chiou et al. 2006). In addition, miR395 and miR399 also target transporter genes for sulfate and phosphate, respectively (Jones-Rhoades et al. 2004; Allen et al. 2005).

In contrast to the induction of miR395 and miR399, miR398 that targets two Cu/Zn-superoxide dismutase (*CSD2* and *CSD1*) transcripts is decreased in response to diverse abiotic stresses. Nuclear run-on assays in *Arabidopsis* indicated that *CSD2* as well as *CSD1* transcripts are not induced at the transcriptional level during stress (Sunkar et al. 2006), but the up-regulation

of these two *CSD* genes is dependent on changes in miR398 levels. In response to oxidative stress, miR398 is down-regulated to release its suppression of *CSD1* and *CSD2* genes (Sunkar et al. 2006). These examples have clearly shown that the regulation of miRNA expression alters target gene expression in response to environmental stress, and this is important for plant acclimation to stress conditions. Thus, miRNAs induced in response to stress are likely to target negative regulators of stress tolerance, whereas miRNAs decreased under stress conditions may target positive regulators.

MicroRNAs that can target *DCL1*, required for miRNA processing or *Argonaute-1* (*AGO1*), a key component in RISC (RNA-induced silencing complex), have been found in plants. miR162 and miR168 direct the cleavage of *DCL1* and *AGO1* transcripts, respectively (Xie et al. 2003; Vaucheret et al. 2004). The targeting of *AGO1* mRNA by miR168 is required for proper plant development, illustrating the importance of feedback control by miR168 (Vaucheret et al. 2004). *DCL1* transcripts are found to be targeted by another recently found miRNA, miR838 in *Arabidopsis* (Rajagopalan et al. 2006). miR838 is derived from the intron 14 of *DCL1* pre-mRNA. This finding revealed an additional regulatory mechanism in maintaining DCL1 homeostasis (Rajagopalan et al. 2006). Another miRNA, miR403, which targets *AGO2* (Argonaute-2) mRNA, was found in *Arabidopsis* and *Populus* (Sunkar and Zhu 2004; Allen et al. 2005). It is not known yet, whether AGO2 functions in miRNA or siRNA pathways in plants. The implication is that these miRNAs might provide a negative feedback regulation of genes that control miRNA/siRNA processing and action.

Endogenous siRNAs

Large scale sequencing of small RNA libraries has revealed an unexpected diversity of endogenous siRNAs in *Arabidopsis* and rice (Llave et al. 2002; Lu et al. 2005; Sunkar and Zhu 2004; Sunkar et al. 2005b; Rajagopalan et al. 2006; Johnson et al. 2007). siRNAs are structurally related to miRNAs but differ in their biogenesis (Doench et al. 2003; Tang et al. 2003). siRNAs are 21–24-nt small RNAs derived from the processing of typically long dsRNAs (Waterhouse et al. 2001; Plasterk 2002), which are themselves products of specific RNA-dependent RNA polymerase (RDR) activities. The dsRNA is processed by the DCL family of enzymes (DCL2, DCL3 and DCL4) to produce the predominant 21 and 24-nt siRNAs. These endogenous siRNAs can fall into different classes such as tasiRNAs, natsiRNAs and heterochromatic siRNAs, based on their biogenesis and function.

Trans-acting siRNAs

In general, the term “siRNA” refers to small RNAs that silence transcriptionally or post-transcriptionally the same locus from which they derive. *Trans*-acting siRNAs (tasiRNAs) down-regulate protein coding genes from a different locus at the post-transcriptional level, which is similar to miRNAs (Vazquez et al. 2004; Peragine et al. 2004). These are a class of endogenous 21-nt regulatory siRNAs. An initial DCL1-dependent, miRNA-guided cleavage of tasiRNA primary transcript defines the 5’ and 3' ends of the transcript which can be converted into dsRNA by RDR6 and SGS3 and sets the 21-nt phase for accurate tasiRNA formation by DCL4 in *Arabidopsis* (Allen et al. 2005; Gasciolli et al. 2005; Yoshikawa et al. 2005; Xie et al. 2005; Axtell et al. 2006). Thus, two DCLs (i.e. DCL1 and DCL4) and RDR6 are required for tasiRNA biogenesis. Additionally, biogenesis of tasiRNAs requires AGO1, HEN1, HYL1 and SGS3 (Vazquez et al. 2004; Peragine et al. 2004). This pathway differs from the heterochromatic siRNA pathway, which requires DCL3 and RDR2 but not SGS3 and RDR6, but resembles the miRNA pathway, which requires AGO1, DCL1, HEN1, and HYL1, because tasiRNA primary transcripts are targeted by miRNAs, miR173 and miR390 in plants (Allen et al. 2005; Xie et al. 2005; Gasciolli et al. 2005; Yoshikawa et al. 2005; Axtell et al. 2006). Thus far, tasiRNAs are found only in plants.

Natural-antisense Transcripts-derived siRNAs

Genome-wide analyses revealed a widespread existence of natural *cis*-antisense gene pairs (NATs) in the eukaryotic genomes (Werner and Berdel 2005; Makalowska et al. 2005). Despite large intergenic spaces in the genome, more than 1 000 pairs of NATs exist in *Arabidopsis* (Wang et al. 2005) and about 600 NATs were reported in the rice genome (Osato et al. 2003). The widespread occurrence of NAT gene pairs raised the possibility of siRNAs being generated from such gene pairs. Recently, it was shown that a pair of NAT genes, *Arabidopsis P5CDH* and *SRO5*, an overlapping gene pair can give rise to siRNAs in response to salt stress (Borsani et al. 2005). These natsiRNAs regulate salt stress resistance in *Arabidopsis* (Borsani et al. 2005). Another recently reported endogenous natsiRNA-ATGB2, is specifically induced by the bacterial pathogen *Pseudomonas syringae* carrying effector avrRpt2. This nat-siRNA plays an important role in resistance (R) gene *RPS2*-mediated plant immunity (Katiyar-Agarwal et al. 2006). Accumulation of the 22-nt *Pst (avrRpt2)*-induced natsiRNA depends on DCL1 and RDR6, whereas the accumulation of the 24-nt *SRO5-P5CDH* nat-siRNA depends on DCL2 and RDR6 (Katiyar-Agarwal et al. 2006). Several potential natsiRNAs can be found in *Arabidopsis* small RNA databases such as the MPSS and ASRP (unpubl. data; Gustafson et al. 2005; http://mpss.udel.edu/) databases.

Heterochromatic siRNAs

DNA methylation is a conserved epigenetic modification of the genome that serves the dual roles of gene regulation and control of repetitive elements, such as transposons. Plant genomes contain relatively high levels of 5-methylcytosine (5mC), ranging from 5% to 25% of total cytosine, depending on the species (Rangwala and Richards 2004). In contrast, 5mC content in human genomes is approximately 4%. Whereas the bulk of DNA methylation in mammals is confined to CGs, DNA methylation in plant genomes is found in three different sequence contexts, CG, CNG (where N is any base), and asymmetric CHH (where H = A, T, or C) (Bender 2004). Different pathways control the maintenance and establishment of cytosine methylation. The maintenance of CG methylation requires the DNA methyltransferase MET1. Maintenance of non-CG methylation (CNG and CHH) requires the methyltransferases CHROMOMETHYLASE 3 (CMT3) and domains rearranged methyltransferase 1 and 2 (DRM1/DRM2), which function with varying degrees of redundancy. Non-CG DNA methylation is directed partly by RNAi factors such as DRD1 and histone H3 lysine 9 methyltransferases (Chan et al. 2006). The putatively siRNA-directed DRM and histone methylation-directed CMT3 activities are dynamic and play important roles in regulating the expression of endogenous genes (Zhang et al. 2006).

Plants are known to undergo RNA-dependent DNA methylation of transgenes, and double-stranded RNA and siRNAs are thought to be important in mediating this process (Aufsatz et al. 2002; Matzke et al. 2004). Genomic mapping of cloned small RNAs, specifically 24-nt siRNAs in *Arabidopsis* and rice revealed that these are derived from many types of retro elements, transposons, repeat-rich regions and heterochromatic regions (Hamilton et al. 2002; Llave et al. 2002; Mette et al. 2002; Sunkar and Zhu 2004; Xie et al. 2004; Sunkar et al. 2005b; Gustafson et al. 2005; Rajagopalan et al. 2006).

Recent studies indicated that two isoforms of DNA-dependent RNA polymerases IV (NRPD1a and NRPD1b) are essential for heterochromatic silencing (Herr et al. 2005; Onodera et al. 2005; Pontier et al. 2005). The 5S rRNA genes and the AtSN1 SINE retrotransposons are heavily methylated and have siRNAs in wild-type plants. Selective disruption of *nrpd1a* or *nrpd1b* indicated that efficient silencing at transposons and highly repeated sequences requires the concerted action of both PolIV forms, while a basal level of silencing at low repetitive DNA is only dependent on NRPD1a (Herr et al. 2005; Onodera et al. 2005; Pontier et al. 2005).

In Drosophila, a large number of endogenous siRNAs referred to as repeat-associated small interfering RNAs (rasiRNAs) can be mapped to transposons. At least 25% of small RNAs cloned from *Drosophila* were homologous to one of the family of repetitive DNA, mostly retro elements (Aravin et al. 2003). However, the biogenesis of these siRNAs is independent of RDR activity. A possible source for this class of siRNAs is double-stranded RNA generated by the annealing of sense and antisense transcripts of transposable elements (Aravin and Tuschl 2005). In plants, 24-nt size siRNAs that resemble rasiRNAs have been identified (Xie et al. 2004; Sunkar et al. 2005b; MPSS (Massive parallel signature sequencing) data). The biogenesis of rasiRNAs requires two RNA polymerases PolIV complexes (NRPD1a and NRPD1b) and RDR2 (Herr et al. 2005; Pontes et al. 2006). In *Arabidopsis*, the Argonaute protein AGO4, along with the 24-nt long heterochromatic siRNAs, are required for the silencing of transposons and other repetitive elements through DNA methylation and heterochromatic histone modifications (Zilbermann et al. 2003; Chan et al. 2004).

In *Schizosaccharomyces pombe*, siRNAs produced by Dicer are able to direct heterochromatin formation *via* a protein complex called RITS (RNA-induced initiation of transcriptional silencing) (Verdel et al. 2004). RITS directly links the siRNA produced by Dicer to heterochromatin because it contains both a previously known chromodomain protein Chp1 that binds centromeres and the *Saccharomyces pombe* Argonaute homolog, Ago1. In post-transcriptional RNA silencing, Argonaute has an important role in the RISC complex as the slicer of target mRNAs and is directed by siRNAs or miRNAs. It is proposed that RITS could play an analogous role by targeting specific regions of chromatin for heterochromatin formation. In fission yeast, silencing of centromeric heterochromatic repeats is accomplished by methylation of lysine 9 of histone H3 (H3K9) and subsequent binding of Swi6 (heterochromatin protein 1), which results in transcriptional repression (Volpe et al. 2002).

The biological roles of siRNA-mediated RNA silencing include protection of the genome against mobile DNA elements (Tabara et al. 1999; Ketting et al. 1999; Wu-Scharf et al. 2000) and resistance against viruses (Voinnet 2001; Waterhouse et al. 2001) and bacterial pathogens (Katiyar-Agarwal et al. 2006), and plant acclimation to abiotic stress (Borsani et al. 2005).

Arabidopsis DCLs Function in a Partially Redundant Manner

The biogenesis of endogenous small RNAs in plants is especially complex, as reflected by the large numbers of enzymes potentially involved. *Arabidopsis* has four Dicer-like (DCL) genes, poplar has five DCL-like genes and rice has six DCL-like genes (Margis et al. 2006). Additional DCLs in poplar and rice appear to be due to divergence found in the DCL2 and DCL3 genes (Margis et al. 2006). In *Arabidopsis*, the generation of tasiRNAs, natsiRNAs and heterochromatic siRNAs is dependent on distinct DCLs (DCL2, DCL3 and DCL4) and RDRs (RDR2 and RDR6) (Vaucheret 2006). *Arabidopsis* DCL1 and DCL4 function in tasiRNA biogenesis (Allen et al. 2005; Gasciolli et al.

2005; Yoshikawa et al. 2005; Xie et al. 2005; Axtell et al. 2006). DCL2 is involved in the production of some viral siRNAs (Xie et al. 2004; Bouche et al. 2006; Blevins et al.2006) and 24-nt natsiRNAs (Borsani et al. 2005). DCL3 and RDR2, as well as the Pol IV (DNA-dependent RNA polymerase IV) complexes involving both NRPD1a and NRPD1b, cooperate in generating heterochromatin-associated 24-nt siRNAs (Herr et al. 2005; Onodera et al. 2005; Pontier et al. 2005). Nonetheless, in the absence of one of these DCLs (i.e. DCL2, DCL3 or DCL4), different classes of siRNAs still accumulate although to a lesser extent indicating that these three DCLs have partially redundant functions (Gasciolli et al. 2005; Bouche et al. 2006). Recent studies have shown that DCL1, which was previously thought to be involved exclusively in miRNA biogenesis, has also been found to play a role in the biogenesis of natsiRNAs (Borsani et al. 2005; Katiyar-Agarwal 2006) and 21-nt siRNAs from CaMV-derived dsRNA (Blevins et al. 2006; Moissiard and Vionnet 2006). On the other hand, in addition to processing tasiRNAs, DCL4 was found to be involved in biogenesis of two miRNAs, miR882 and miR839 in *Arabidopsis* (Rajagopalan et al. 2006). These observations suggest that DCLs have specific as well as partially overlapping functions.

Small RNAs in Rice

Large scale sequencing of rice small RNA libraries has identified miRNA homologs (miR396d,e, miR437 and miR444), which are conserved in barley, maize, wheat, sorghum or sugarcane, but not in *Arabidopsis* or *Populus*, suggesting that these miRNAs are specific to monocotyledonous plants (Sunkar et al. 2005a). Since the sequencing of small RNA libraries has not been saturated and deep sequencing of rice small RNA libraries is ongoing, it is likely that many more new miRNAs conserved only in monocots or specific to rice will be found. Similar to *Arabidopsis* DCL1, a rice DCL1 ortholog has been shown to be involved in miRNA production and the *dcl1* knock-down mutants showed abnormal developmental and reproductive defects (Liu et al. 2005).

The sequencing of small RNA libraries also led to the discovery of several hundreds of endogenous siRNAs in rice (Sunkar et al. 2005b; Liu et al. 2005; Luo et al. 2006; Johnson et al. 2007) and more will certainly be discovered soon. Some of these endogenous siRNAs are abundantly expressed in diverse tissues, as they could be detected using small RNA blot analysis (Sunkar et al. 2005b). These endogenous siRNAs are of ~21- to 24-nt in size, and their possible involvement in transcriptional and post-transcriptional gene silencing in rice is highly likely.

Functional studies of miRNAs and their targets in rice were initiated only recently. Thus far, the functional characterization of three miRNAs (miR156, miR159 and miR167) has been reported (Tsuji et al. 2006; Xie et al. 2006; Yang et al. 2006). miR156 is one of the more highly conserved miRNAs and it targets members of the *SPL* (squamosa promoter binding protein-like family) gene family in plants (Jones-Rhoades et al. 2006). In general, the expression of miRNA and its target genes are negatively correlated (Sunkar et al. 2006). Temporal and spatial expression analysis of miR156 and the *SPL* genes revealed a negative correlation in rice (Xie et al. 2006). Overexpression of one of either two miR156 precursors (miR156b and miR156h) resulted in suppression of several *SPL* genes (*SPL2*, *SPL12*, *SPL13*, *SPL14* and *SPL16*). These transgenic rice plants showed severe developmental abnormalities including dwarfism, increased number of tillers and delayed flowering (Xie et al. 2006). To some extent, these phenotypes resembled the transgenic plants overexpressing miR156 in *Arabidopsis* (Schwab et al. 2005).

Arabidopsis miR159 was found to be regulated by gibberellin and abscisic acid (ABA) treatment (Achard et al. 2004; Reyes and Chua 2007). In contrast, miR159 expression in rice seedlings was unaltered in response to gibberellin application (Tsuji et al. 2006), suggesting that the regulation of conserved miRNAs may not be similar in diverse plant species. *OsGAMYB* and *OsGAMYBL-1* are regulated by miR156 in rice. Transgenic rice plants overproducing the miR159a precursor are severely affected in their internode elongation, and have malformed flowers and shrunken and brownish pistils and glumes (Tsuji et al. 2006). miRNA overexpression mimics the effect of target gene loss-of-function. The miR156 overexpression rice phenotypes are similar to but more severe than those of *gamyb* knockout mutants. This could be attributed to the possibility that in addition to *GAMYB* and *GAMYBL-1,* miR159 probably also targets other genes in rice.

Proper miRNA-mediated auxin signaling is essential for guiding normal auxin-mediated plant responses. miR167 is another highly conserved miRNA and its target genes, ARFs, are involved in auxin signaling in plants. ARFs are transcription factors that bind to the auxin responsive *cis*-acting elements in early auxin responsive genes such as *Aux/IAA*, *SAUR* and *GH3*. In rice cultured cells, miR167 levels were found to be depleted in response to auxin but not cytokinin, ABA or gibberellin application, suggesting that auxin specifically suppresses miR167 expression (Yang et al. 2006b). The decreased miR167 in auxin-treated rice cells is also accompanied by reduced degradation of *ARF8* mRNA and increased expression of GH3-2 transcripts (Yang et al. 2006b). These findings indicated that rice miR167 regulates the expression of *OsGH3* levels by targeting *OsARF8* in an auxin-dependent manner (Yang et al. 2006b).

Conclusions and Outlook

A large set of *Arabidopsis* miRNAs have been conserved over

a great length of evolutionary distance, but at the same time an emerging picture from small RNA cloning studies clearly indicated that many more miRNAs are not conserved (Sunkar et al. 2005; Lu et al. 2005; Rajagopalan et al. 2006). The roles of miRNAs in plants are quite diverse, and encompass functions in development, nutrient homeostasis and stress responses. An understanding of miRNA-mediated gene regulation could lead to novel strategies for improving plant traits such as stress tolerance. For example, we have shown recently that plant abiotic stress tolerance can be improved using the knowledge gained from miR398-mediated regulation of its target genes in *Arabidopsis* (Sunkar et al. 2006). Similarly, the unexpected enormous diversity of endogenous siRNAs in plants is an indication that siRNAs may have important functions in diverse plant processes. With the exception of a few repetitive element-associated 24-nt siRNAs in heterochromatin formation and silencing of transposons and repetitive elements (Zilberman et al. 2003; Chan et al. 2004; Xie et al. 2004), 21-nt tasiRNAs in plant development (Fahlgren et al. 2006; Hunter et al. 2006) and two natsiRNAs implicated in stress responses (Borsani et al. 2005; Katiyar-Agarwal et al. 2006), the function of most endogenous siRNAs is currently unknown. New challenges lie ahead for plant biologists to discover new small RNAs and define small RNA function in development and physiology.

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