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REVIEW

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The molecular characterization and function of miRNAs on mediation of target gene silencing in plants

Abstract MiRNAs belong to one type of noncoding RNAs involved in developmental regulation, genome maintenance, and defense in eukaryotes. In plants, the miRNAs are involved in many molecular interactions, including interfere with expression of mRNAs encoding factors that control developmental processes, stem cell maintenances, auxin responses, and other developmental and physiologic processes. In this paper, the molecular characterization and the functions of miRNAs on mediation of target gene silencing in plants have been overviewed. Further studies on the miRNAs will be helpful for elucidation of the molecular mechanism of post-transcriptional gene silencing in plants.

Keywords miRNAs, molecular characterization, RNA-induced silencing complex (RISC), target gene

1 Introduction

RNA silencing (post-transcriptional gene silencing, PTGS) refers to a family of gene silencing effects that results in the expression of one or more genes to be downregulated or entirely suppressed by the introduction of an antisense RNA molecule. The most common and well-studied example in RNA interference cases is endogenously expressed miRNA or exogenously derived small interfering RNA that induces the degradation of complementary mRNA. There are several types of RNA-silencing mechanisms, including RNA interference (RNAi), the micro RNA (miRNA) pathway, and RNA-directed chromatin silencing (Baulcombe, 2004).

Acting as the post-transcriptional regulators, MiRNAs bind to complementary sequences in the three prime untranslated regions (3′ UTRs) or the open-reading frames of target mRNA transcripts, usually resulting in gene silencing (Bartel, 2004; Baulcombe, 2004). MiRNAs are short RNA molecules, on average of 22 nucleotides in length. The studies in human genome suggest that there are over 1000 miRNAs (Bentwich et al., 2004), generally abundant in many cell types and targeting about 60% of mammalian genes (Lim et al., 2003; Friedman et al., 2005). Each miRNA in human is involved in the repression of hundreds of mRNAs (Brennecke et al., 2005; Lim et al., 2005). In the previous decade, it is well known that miRNAs are well conserved in eukaryotic organisms and are thought to be a vital and evolutionarily ancient component of genetic regulation (Grosshans et al., 2002; Tanzer et al., 2004; Lee et al., 2007; Molnar et al., 2007; Kren et al., 2009).

The miRNAs were early characterized in the early 1990s, but they were not recognized as a distinct class of biologic regulators with conserved functions until the early 2000s. Since then, it is revealed that miRNAs have multiple roles in negative regulation, such as transcript degradation and sequestering, and translational suppression. By affecting gene regulation, miRNAs play crucial roles in regulations of most biologic processes in eukaryotic organisms (Brennecke et al., 2003; Lagos-Quintana et al., 2003; Chen et al., 2004; Poy et al., 2004; Cuellar et al., 2005; Harfe et al., 2005; Lim et al., 2005; Wilfred et al., 2007).

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2 Action mechanisms of miRNAs in induction of gene silencing

As early as the last 90s, it is clearly known that the double-stranded RNA (dsRNA) could induce the posttranscriptional silencing (PTGS) of the corresponding gene via the degradation of homologous RNA (Fire et al., 1998; Waterhouse et al., 1998; Tuschl et al., 1999). This process

of RNA interference (RNAi) is recognized to have an ancestral function in the defense against viruses and transposable elements (TEs), based on the mutant analysis that deficient in PTGS has increased susceptibility to viral infection (Voinnet et al., 1999; Mourrain et al., 2000), and some RNAi-deficient mutants of *Caenorhabditis elegans* also show increased transposon activation (Tijsterman et al., 2002).

As mentioned previously, the role of a miRNA in gene regulation is implemented via complementary to a part of one or more mRNAs. In animals, miRNAs are usually complementary to a site in the 3' UTR. However, the plant miRNAs are usually complementary to coding regions of mRNAs (Wang et al., 2004). It is observed that the perfect or near-perfect base pairing with the target RNA could promote cleavage of the target RNAs (Kawasaki et al., 2004). In addition, the miRNAs in animals are more often only partial to base pair and inhibit protein translation of the target RNAs (Moxon et al., 2008). For partially complementary miRNA to recognize their targets, the nucleotides 2–7 of the miRNA (referred to the seed region) need to be perfectly complementary (Williams et al., 2008). The miRNAs that are partially complementary to the target can also speed up deadenylation, causing mRNAs to be degraded sooner (Eulalio et al., 2009). In eukaryotic organisms, miRNAs occasionally also cause histone modification and DNA methylation of promoter sites and therefore affecting the expression of targeted genes (Mazière et al., 2007; Tan et al., 2009).

In plants, miRNAs are processed from larger noncoding RNA precursors that contain stem-loop structures processed by Dicer-like proteins (DCLs). It is shown that miRNAs in plants are highly conserved in sequence, expression, and function so far. MiRNAs act through several possible mechanisms in plants, including post-transcriptional cleavage of mRNA (Llave et al., 2002; Kasschau et al., 2003; Palatnik et al., 2003), inhibition of translation (Aukerman and Sakai, 2003; Chen, 2004), and RdRP-mediated second-strand synthesis and *trans*-acting siRNA (ta-siRNA) production that are initiated by miRNA action (Volpe et al., 2002; Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005).

The cleavage of target genes via miRNA pathway has been documented to be regardless of the action mode. It has some evidence now that a transcriptional feedback mechanism may also be active (Schwab et al., 2005). However, the prevalence of the putative translational block has not been assessed systematically (Jones-Rhoades and Bartel, 2004).

3 miRNA biogenesis and functional pathways

3.1 DCLs that involved in the pre-miRNAs processing

In eukaryote, miRNA biogenesis and functional pathways have been widely examined and described. First, miRNA primary transcripts (pri-miRNAs) are trimmed in the nucleus into miRNA precursors (pre-miRNAs) by an RNase III-like enzyme called Drosha (Lee et al., 2003). After the initial processing, the pre-miRNAs are exported to the cytoplasm by the function of exportin-5 (Yi et al., 2003; Lund et al., 2004) and are cleaved there to generate mature miRNAs by Dicer, another RNase III-like enzyme (Bernstein et al., 2001). These miRNAs are then incorporated into the RNA-induced silencing complex (RISC) endonuclease, which seems to negatively regulate the genes required for several developmental processes by promoting RISC-mediated degradation of target mRNAs, or the inhibition of target mRNA translation (Carrington et al., 2003).

Up to date, the extensive studies of miRNA biogenesis on plant species have been conducted in *Arabidopsis*. It is determined that in total, four Dicer-like (DCL) enzymes (designated DCL1-DCL4) are involved in the pre-miRNAs processing (Schauer et al., 2002). Of these, DCL1 is shown to be involved in miRNA accumulation (Reinhart et al., 2002). It is also found that DCL1 mRNA is subject to negative feedback regulation by miR162-guided mRNA degradation (Xie, 2003). DCL2 and DCL3 are revealed to be partly involved in viral short interfering RNA (siRNA) biogenesis and endogenous siRNA biogenesis, such as retrotransposon siRNA, respectively (Xie et al., 2004).

The mechanism of plant miRNA biogenesis, including the step where DCL1 acts, has gradually been detected. As one example, it is observed that miR163 biogenesis requires at least three cleavage steps by RNase III-like enzymes. Among them, DCL1 catalyzes at least the first and second cleavage steps in which the domains of DCL1 that bind the double-stranded RNA are involved in positioning of the cleavage sites. Of the three cleavage steps, step one is from pri-miR163 to long miR163 precursor (premiR163), then step two is from long pre-miR163 to short premiR163, and step three is from short pre-miR163 to mature miR163 and the remnant. Through the whole process, four small RNAs including miR163 are released. This result provides the direct evidence that DCL1 is involved in processing of pri- and pre-miRNA (Kurihara et al., 2004).

In rice, the function of DCL proteins (OsDCLs) in the miRNAs biogenesis has been also explored. Knocked down of *OsDCL1* and *OsDCL4*, respectively, via an RNA interference approach resulted in developmental variations largely at the seedling stage. Transgenic analysis suggested that *OsDCL1* plays a critical role in miRNA processing in rice. Based on the direct-cloning method, the roles of *OsDCL1* and *OsDCL4* in miRNA/siRNA biogenesis and rice development have been evaluated. Analysis of *OsDCL1IR* and *OsDCL4IR* transformants generated by the RNAi approach with loss of functions of these genes suggested that *OsDCL1*, but not *OsDCL4*, is important for miRNA accumulation. In the meantime, it is observed that

the production of siRNAs from transgenic inverted repeats and endogenous CentO regions is not affected in either *OsDCL1IR* or *OsDCL4IR* transformants, suggesting that the production of miRNAs and siRNAs is via distinct OsDCLs in rice (Liu et al., 2005).

Till now, the structure features of DCLs have also been gradually understood. As the typical signatures, an N-terminal helicase domain, two tandemly repeated RNase III domains, and one or more C-terminal dsRNA binding domains are highly conserved among DCL proteins. However, the number of DCL proteins varies among different organisms. In mouse (*Mus musculus*) and human (*Homo sapiens*), a single DCL is responsible for the generation of both miRNAs and siRNAs, whereas in plants, multiple DCL proteins exist (Liu et al., 2005).

3.2 Proteins of AGO and HYL acted as the key components of RNA-induced silencing complex (RISC)

After being processed into miRNAs from pre-miRNAs by Dicer-like (DCL)1 (Kurihara and Watanabe, 2004), the miRNAs are assembled into a protein complex, referred to RNA-induced silencing complex (RISC). It has been shown that ARGONAUTE1 (AGO1), a key component of RISC, is indispensable for the function of miRNAs (Vaucheret et al., 2004). In *Arabidopsis*, the AGO family comprises 10 members (Fagard et al., 2000; Carmell et al., 2002). Among them, the most studied *Arabidopsis* AGO protein is AGO1. It is shown that AGO1 is associated with the miRNA pathway and transgene-silencing pathway (Fagard et al., 2000; Vaucheret et al., 2004). Mutants of loss-of-function with strong *ago1* alleles verified that AGO1 significantly affects miRNA accumulation and miRNAs target regulation (Vaucheret et al., 2004). The *ago1* mutants are also impaired in spontaneous silencing of a foreign transgene (cosuppression) (Fagard et al., 2000) and exhibit hypersusceptibility to Cucumber Mosaic Virus (CMV) (Morel et al., 2002). The studies on other AGO members suggested that *AGO4* with endogenous siRNAs playing roles in affection of epigenetic silencing (Zilberman et al., 2003; Zilberman et al., 2004) and AGO7 and AGO10 have a function in the transition from juvenile to adult phases of plant growth (Hunter et al., 2003) and meristem maintenance (Moussian et al., 1998; Lynn et al., 1999), respectively.

Acting as a member of a family of miRNA biogenesis proteins, HYL1 also participates in miRNA accumulation in coordination with DCL1 and HEN1. It is detected that the HYL1 protein has two dsRBD domains, dsRBD1 and dsRBD2, respectively, in its N-terminal half. *In vitro* studies suggested that a preferential binding to the dsRNA could occur via the mediation of these domains (Lu and Fedoroff, 2000). HYL1 also has a putative nuclear localization site (NLS) and a putative protein–protein interaction (PPI) domain. Although HYL1 has been postulated to be located in the nucleus via the guidance of NLS, the function of the NLS in the localization of HYL1 for miRNA biogenesis has not been identified.

Currently, biochemical deletion analysis indicates that HYL1 interacts strongly with DCL1 in which the dsRBD1 domain is essential for dsRNA binding *in vitro*, whereas dsRBD2 contributes to its PPI activity (Hiraguri et al., 2005). The interaction between DCL1 and HYL1 is important for the efficient and precise processing of pri-miRNA during plant miRNA biogenesis (Kurihara et al., 2006). However, the question still remains whether HYL1 without NLS and PPI domains would be able to function in the production of miRNAs and in the establishment of various developmental processes (Wu et al., 2007).

4 Genes encoding miRNAs in eukaryotic organisms

MiRNA genes are found in intergenic regions or in antisense orientation to genes (Lau et al., 2001), containing their own miRNA gene promoter and regulatory units (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee et al., 2001; Lee et al., 2004). Studies suggested that as much as 40% of miRNA genes may lie in the introns of protein and non-protein coding genes or even in exons, and frequently arrange in a sense orientation (Cai et al., 2004; Rodriguez al., 2004; Weber 2005). There are some miRNA genes showing a common promoter, including the 42%–48% of all miRNAs originating from polycistronic units containing 2–7 discrete loops from which mature miRNAs are processed (Lee et al., 2004; Altuvia et al., 2005). But, this does not mean that the mature miRNAs within the same family will be homologous in structure and function. The promoters of miRNAs have been shown to have some similarities in their motifs to promoters of other genes transcribed by RNA polymerase II such as protein coding genes (Lee et al., 2004; Zhou et al., 2007).

With the progress of miRNAs studies and the increased knowledge in the related scope, it is demonstrated that many genomic regions previously considered featureless were found to be sites of numerous small RNAs (Lu et al., 2005). Currently, it is known that the number of miRNAs per eukaryotic genome varies by species. For example, miRBase presently lists 114, 117, and 326 miRNA genes in *Arabidopsis*, *Caenorhabditis elegans*, and humans, respectively (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006). In contrast to animals who have many unique mature miRNA sequences and small miRNA families, the situation is reversed in plants, which typically have a small number of unique miRNA sequences and large miRNA families (Li and Mao, 2007). In rice, one of the most important crop species in the world, totally 20 miRNA families have already been identified through computational approaches based on conservation with known miRNAs from *Arabidopsis* (Llave et al., 2002; Park et al., 2002; Reinhart et al., 2002; Jones-Rhoades and Bartel,

2004; Sunkar and Zhu, 2004; Wang et al., 2004; Adai et al., 2005; Sunkar et al., 2005).

The total number of genes targeted by miRNAs is possibly highly variable and genome specific. In *Arabidopsis*, it is reported that only 1% of all protein-coding genes appear to be miRNA targets (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004), in contrast to at least 20% of all protein-coding genes that are likely miRNA targets in animals (Lewis et al., 2003, 2005 ; Grun et al., 2005; Krek et al., 2005; Lall et al., 2006). Furthermore, differences also exist between plants and animals in the number of genes targeted by each miRNA. For example, each *Drosophila* miRNA has an average of over 50 predicted targets (Grun et al., 2005), but most *Arabidopsis* miRNAs have six targets or fewer (Jones-Rhoades et al., 2006).

5 Microevolution of miRNA-target site interactions and roles in phenotype evolution

Though existed as only about 22 nt in length, there are also sequence variations at miRNAs sequence in a plant species. Based on the analysis of the characterized binding sites for these miRNAs in *Arabidopsis*, it is found that both miRNAs and their target binding sites have very low nucleotide variation and divergence compared to their flanking sequences, indicating strong purifying selection on these sites in this species. However, sequence data flanking the mature miRNAs exhibit normal levels of polymorphism for the accessions. In addition, nonneutral evolution or subtle effects on predicted pre-miRNA secondary structure have been detected in some cases, suggesting that there is a raw material for the differential function of miRNA alleles (Ehrenreich et al., 2008). Overall, these results show that despite differences in the architecture of miRNA-based regulation, miRNAs and their targets are similarly constrained in both plants and animals.

Currently, more and more microevolution of miRNA-target site interactions has been reported. It is shown that a single nucleotide polymorphism (SNP) has underlain a quantitative trait locus for muscularity in erkoyartic organisms (Clop et al., 2006). Similarly, an SNP in an existing miRNA binding site causing Tourette's syndrome has also been reported in humans (Abelson et al., 2005). In addition, an SNP has been identified in *Human herpesvirus* that affects Drosha processing of a miRNA precursor (Gottwein et al., 2006).

Genome-wide surveys of miRNA and miRNA binding site polymorphism have only been largely conducted in humans. The studies have shown levels of polymorphism at miRNAs, and their targets are lower than at coding or neutral regions. The mutations at these sites are shown to exhibit a general signature of purifying selection (Chen and Rajewsky, 2006; Saunders et al., 2007). One of these studies has also shown evidence for positive selection on some miRNA binding sites based on long-range haplotype signatures (Saunders et al., 2007), implying that beneficial miRNA-target site polymorphisms may exist.

Although the extent to which miRNAs contribute to phenotypic evolution is largely unclear thus far, evidence suggests that miRNAs could play an important role during the process. It is suggested that there were essential miRNA-target site interactions and have been conserved for more than 400 million years in plants and animals, e.g. miR165/166 and Class III HD-ZIP (homeodomain Leu zipper) genes in land plants (Floyd and Bowman, 2004) and the *let-7* miRNA and the *lin-41* mRNA in metazoans (Pasquinelli et al., 2000). There are also others that appear to be species- or clade-specific and may function in evolutionarily derived traits (Bonnet et al., 2004; Axtell and Bartel, 2005).

Currently, predictions can be made about the expected level of miRNA and miRNA binding site sequence variation in plants relative to humans, based on analysis of the functional differences between plant and animal miRNAs. Owing to plant miRNAs typically having fewer mRNA targets and more miRNA family members than animal miRNAs, it may lead to reduced constraint on and higher sequence diversity in miRNA sequences in plants. However, as most plant miRNAs perform important functions in development and physiology, and spatiotemporal functional differences may exist among plant miRNA family members making each independently essential, the constraint on plant miRNAs may parallel that observed in humans (Ehrenreich et al., 2008). As for miRNA binding sites, the constraint is likely to be strong across the entire miRNA binding site in plants due to the importance of the entire binding site in miRNA-mRNA pairing. On the other hand, the miRNA binding sites may experience an additional constraint due to the presence of these sites largely in coding exons in plants (Ehrenreich et al., 2008).

By resequencing more than half of the characterized miRNAs and binding targets in this species from 24 diverse accessions, Ehrenreich et al. (2008) have assessed the levels and patterns of nucleotide polymorphism in miRNAs and their binding sites in *Arabidopsis*. It is observed that there were significantly reduced genetic variations at these sites relative to flanking sequence, with only four SNPs and an insertion/deletion (indel) present in the sample. Four miRNAs that exhibit nonneutral patterns of molecular variation and numerous SNPs are predicted to have subtle effects on the pre-miRNA secondary structure. These results suggest that mutations within mature miRNAs and their binding sites do not contribute substantially to gene expression and phenotypic variation in plant species. The ample variation flanks mature miRNAs that could contribute to the evolutionary diversification of these key regulatory genes.

6 Roles of miRNAs in regulation of plant development

6.1 Part of transcription factor genes functional on development are regulated via miRNAs action pathway

Acting as indispensable components in the control of gene expression during development, transcription factors involve perception and integration of cellular and environmental signals. Till now, many plant miRNA targets encode transcription factors involved in cell fate determination and development have been identified (Rhoades et al., 2002; Jones-Rhoades et al., 2006).

Several families of transcription factors that depend on these conserved miRNAs to exert their roles on regulation of developmental functions are included as follows: Transcription factor TCP on regulation of leaf shape via miR159 (Palatnik et al., 2003), NAC determination of organ boundaries by miR164 (Laufs et al., 2004), APETALA2-like regulation of flowering time and specification of floral whorl identity by miR172 (Aukerman and Sakai, 2003; Chen, 2003), HD-ZIPIII regulation of organ polarity and the arrangement of vascular tissues by miR166 (Williams et al., 2005), MIKC-type MADS box protein AGAMOUS-LIKE16 (AGL16) regulation of proper flower formation via miRNA and miR824 (Rajagopalan et al., 2006; Fahlgren et al., 2007), HD-Zip transcription factors PHV and PHB determination of the establishment of adaxial (upper surface)–abaxial (lower surface) polarity via miR165 and miR166 (Zhou et al., 2007), and R2R3 MYB domain proteins *MYB33* and *MYB65* regulation of anther development via miR159 (Millar et al., 2005). These findings demonstrate that miRNAs pathway plays crucial roles on regulation of various types of transcription factors which further implicate the plant development.

6.2 Plant growth regulated via RISC components

As indispensable components of the RNA-induced gene silencing complex, proteins of AGO and HYL have functions involved in many development aspects in plants. In *Arabidopsis*, loss-of-function *dcl1* mutants were resulted in markedly variations of development status, including the embryo and vegetative and reproductive organs (Schauer et al., 2002). Defects associated with *dcl1* mutants include overproliferation of meristems (which contain pluripotent stem cells), conversion of normally determinate floral meristems into indeterminate meristems, delayed flower timing, and overproliferation of embryonic suspensor cells (Schauer et al., 2002). *dcl1* mutants have reduced levels of miRNAs and ectopically express miRNA target genes (Park et al., 2002; Reinhart et al., 2002; Kasschau et al., 2003). Similarly, mutations in other genes that are involved in miRNA formation (*HEN1*), or that are suspected to function in miRNA pathways (*AGO1*), also cause *dcl1*-like phenotypes and leaf polarity defects (Park et al., 2002; Kidner et al., 2003). Further, the RNA-silencing suppressor encoded by Turnip Mosaic Virus interferes with miRNA activity, causes ectopic expression of miRNA target genes, and induces a spectrum of defects that overlap with those of *dcl1, ago1*, and *hen1* mutants (Kasschau et al., 2003). These data suggest that miRNAs functions in acting as negative regulators to control meristem cell identity, organ polarity, and other developmental processes via the coordination with other RISC components.

7 Various important roles of miRNAs currently identified that involved in regulation of plant development and stress responses

7.1 Roles in regulation of vascular cambium and structure of apical meristem

Lateral organ primordia are initiated at the periphery of the shoot apical meristem (SAM) as cell clusters making a fundamental transition to determinacy, a process associated with the downregulation of *KNOX* genes (Jackson et al., 1994). It is known that the leaf initiation in the peripheral zone of the shoot apical meristem involves a transition to determinate cell fate, but indeterminacy is maintained in the vascular cambium, a tissue critical to the continuous growth of vascular tissue in leaves and stems. In the past several years, it is shown that the orientation of cambial growth is regulated by miRNA-directed cleavage of mRNA from the *Nicotiana sylvestris* ortholog of *PHAVOLUTA* (*NsPHAV*). Under the condition of loss of miRNA regulation in semidominant *phv1* mutants, the lateral growth of leaf midveins and stem vasculature were misdirected away from the shoot, disrupting vascular connections in stem nodes (McHale et al., 2004). In the meantime, the *phv1* mutation also expands the central zone in vegetative and inflorescence meristems, implicating that miRNA and *NsPHAV* play vital roles in regulation of the meristem structure. In flowers, *phv1* causes reiteration of carpel initiation, a phenocopy for loss of *CARPEL FACTORY*/*DICER LIKE1*, indicating that miRNA is also critical to the termination of indeterminacy in floral meristems (McHale et al., 2004). These results point to a common role for miRNA in spatial and temporal restriction of *HD-ZIPIII* mediated indeterminacy in apical and vascular meristems.

7.2 Roles in regulation of root cap formation via mediation of auxin responses

The phytohormone auxin plays critical roles during plant growth, many of which are mediated by the auxin response factor (ARF) family. So far, it is observed that miRNAs could target several mRNAs implicated in auxin responses.

MiR160 targets *ARF10*, *ARF16*, and *ARF17*, three of the 23 *Arabidopsis thaliana ARF* genes. Studies suggested that the overexpression of miRNAs and the expression of miRNA-resistant targets *in vivo* have allowed assignment of developmental roles to miR159/miR319/JAW, miR164, miR165/166, and miR172 miRNA families (Mallory et al., 2005). It is found that disruption of miR160 could result in the increases of *ARF17* mRNA levels, further leading to severe developmental abnormalities, such as the defects in embryonic, root, vegetative and floral development, and alters *GH3*-like gene expression (Mallory et al., 2005). Together, these results clearly indicate that miR160-directed regulation is critical for the developmental functions of ARF17 and expose ARF17 as a possible transcriptional regulator of *GH3*-like early auxin-response genes.

The plant root cap mediates the direction of root tip growth and protects internal cells (Dolan et al., 1993). Root cap cells are continuously produced from distal stem cells, and the phytohormone auxin provides position information for root distal organization (Sabatini et al., 1999). In *Arabidopsis*, it is also observed that the auxin response factors ARF10 and ARF16 were targeted by miR160, acting as the controller of root cap cell formation. Transgenic plants in which the expression of *ARF10* and *ARF16* is repressed and the *arf10-2 arf16-2* double mutants display the same root tip defect, with uncontrolled cell division and blocked cell differentiation in the root distal region, show a tumor-like root apex and loss of gravity-sensing (Wang et al., 2005). In the meantime, the expressions of *ARF10* and *ARF16* genes in roots were independently regulated by auxin and miR160, generating a pattern that is consistent with root cap development. It is also found that miR160-uncoupled production of ARF16 exerts pleiotropic effects on plant phenotypes, and miR160 plays an essential role in regulating *Arabidopsis* development and growth (Wang et al., 2005).

7.3 Roles in regulation of Cu/Zn superoxide dismutases involved in stress responses

It is well known that the environmental stresses induce the expression of many genes. As one of the important abiotic stresses, reactive oxygen species (ROS) is becoming a major cause of loss of crop productivity (Allen et al., 1997; Mittler, 2002; Apel and Hirt, 2004; Bartels and Sunkar, 2005; Foyer and Noctor, 2005). ROS affects many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation (Foyer et al., 1994). Stress-induced ROS accumulation is counteracted by intrinsic antioxidant systems in plants that include a variety of enzymatic scavengers, such as superoxide dismutase, ascorbate peroxidase, glutathione peroxidase, glutathione *S*-transferase, and catalase (Sunkar et al., 2006).

Currently, it is found that the induction of some of the genes important for stress adaptation might be mediated by a downregulation of miRNAs. Increasing evidence points to a potential role of miRNAs in diverse physiologic processes. Under stress condition, two closely related Cu/Zn superoxide dismutases, cytosolic CSD1 and chloroplastic CSD2, respectively, were induced by the miR398 regulated pathway in *Arabidopsis* (Sunkar et al., 2006). Studies of the miR398 expression pattern detected that this miRNA was downregulated transcriptionally by oxidative stresses, and this downregulation is important for post-transcriptional *CSD1* and *CSD2* mRNA accumulation and oxidative stress tolerance. The miR398 also played an important role on specifying the spatial and temporal expression patterns of *CSD1* and *CSD2* mRNAs (Sunkar et al., 2006). These results suggest that *CSD1* and *CSD2* expression is fine-tuned by miR398-directed mRNA cleavage pathway. It could be an effective new approach to improving plant productivity via relieving miR398-guided suppression of *CSD2* in transgenic plants under oxidative stress conditions.

7.4 Roles in regulation of phosphate homeostasis

Direct sequencing of small RNAs from a stress-induced library has revealed several new miRNA families (Sunkar and Zhu, 2004). This suggests that these small RNAs play roles in responses and adaptations of abiotic stress. Among the miRNAs responding to abiotic stresses in *Arabidopsis*, the expression of miR393 and miR159 has been shown to be regulated by abscisic acid (Sunkar and Zhu, 2004) and gibberellin (Achard et al., 2004), respectively. This suggests that they are possibly involved in the regulation of genes responding to distinct abiotic stress via the mRNA degradation pathway.

Low P availability in crop production has been becoming one of the problems to overcome worldwide. Plants have evolved a series of adaptive responses to maintain P homeostasis (Raghothama, 1999). These responses include conservation and remobilize the internal P and enhanced acquisition of external P (Raghothama, 1999; Poirier and Bucher, 2002), which involves rapid and distinct changes in gene expression. So far, although many P-responsive genes have been reported in large-scale expression profiling (Hammond et al., 2003, Wasaki et al., 2003; Wu et al., 2003; Misson et al., 2005), the molecular mechanisms regulating these P starvation-responsive genes remain to be determined.

Currently, evidence shows that a mechanism that involves the suppression of an ubiquitin-conjugating E2 enzyme by a specific microRNA, miR399, exists in plants, by which plants regulate inorganic phosphate (Pi) homeostasis to adapt to environmental changes in Pi availability. Upon Pi starvation, the miR399 is upregulated, and its target gene, an ubiquitin-conjugating E2 enzyme, is simultaneously downregulated in *Arabidopsis*. Further, accumulation of the E2 transcript is suppressed in transgenic *Arabidopsis* overexpressing miR399. Transgenic

plants accumulated five to six times the normal Pi level in shoots and displayed Pi toxicity symptoms that were phenocopied by a loss-of-function E2 mutant. Moreover, unlike wild-type plants, in which Pi in old leaves was readily retranslocated to other developing young tissues, remobilization of Pi in miR399-overexpressing plants was impaired (Chiou et al., 2005). These results provide evidence that miRNA controls Pi homeostasis by regulating the expression of a component of the proteolysis machinery in plants.

8 Conclusions

Plant miRNAs often have extensive, evolutionarily conserved complementarity to plant mRNAs. This observation has enabled numerous regulatory targets to be confidently predicted and experimentally validated. Progress on miRNAs suggests that besides controlling transcription factor mRNA abundance, miRNAs may also regulate a large number of other cellular processes. To date, the functions of plant miRNAs have been deduced from overexpression of precursor sequences encoding miRNAs and/or expression of target mRNAs that have been rendered cleavage-resistant by mutations. Overexpression of miRNA precursors in transgenic plants leads to increased miRNA levels and decreased target mRNA levels. This provides a powerful approach for systematically identifying the distinct miRNAs roles in the future. With more and more mutants to be molecularly identified, further clarification of the biologic roles of miRNAs in plants will be aided by analysis of the loss-of-function way that are directly affected in miRNA genes.

Currently, direct sequencing of small RNAs from a stress-induced library has revealed several new miRNA families, suggesting a role of these small RNAs in abiotic stress responses. Experimental analysis demonstrated that distinct miRNAs play important roles on regulation of the phosphorus homeostasis in plants. But little results of miRNAs mediated abiotic/abiotic responses in plants have been reported, in contrast to those on development regulation. More and more studies are necessary to be conducted for the identification of the miRNAs that impose roles in regulation of biotic and abiotic stress responses in plants.

While target identification reveals the breadth of developmental processes that are under miRNA regulation in plants, challenges still exist for us to cope with. Do all plant miRNAs act as repressors, and do they all affect translation by similar biochemical mechanisms? What developmental signals control miRNA gene transcription and miRNA processing? Do plant miRNAs also act by nondegradative modes? Based on further and widely studies on above questions, we anticipate that the study of these small RNAs will continue to deliver many interesting findings in the future.

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