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Review

Diverse small RNA-directed silencing pathways in plants

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ABSTRACT

Small silencing RNAs of 21- to 24-nucleotide (nt) in length are essential regulatory components expressed in most eukaryotic organisms. These regulatory small RNAs are produced through pathways that involve several evolutionarily conserved protein families, including DICER (DCR) or DICER-LIKE (DCL), ARGONAUTE (AGO), and RNA-DEPENDENT RNA POLYMERASE (RDR). Plants possess multiple functional DCL, RDR, and AGO proteins. Genetic analyses in the model plant *Arabidopsis thaliana* have revealed multiple small RNA pathways, each utilizing a distinct set of RNA silencing factors. In this short review, mainly based on the work done on *A. thaliana*, we give a brief overview on the multiple small RNA-directed silencing pathways in plants, which includes the biogenesis and function of microRNAs (miRNAs), trans-acting siRNAs (ta-siRNAs), natural *cis*-antisense transcripts-associated siRNAs (nat-siRNAs), and heterochromatic siRNAs.

1. Introduction

Small silencing RNAs are recently identified essential regulatory components that are expressed by most eukaryotic genomes [1]. In plants, small RNAs regulate the expression of target transcripts, direct the epigenetic modification of DNA and histones at specific chromosomal domains, and mediate the defense against invading viruses, all of which operates through a sequence-specific silencing mechanism (reviewed in [2–5]). Recent small RNA deep sequencing efforts in the model plant *Arabidopsis thaliana* have led to the discovery of many thousands of endogenous small RNAs and established a genome-wide small RNA landscape [6–8]. Based on their biogenesis characteristics, these endogenous small RNAs are divided into two broad categories: the microRNAs (miRNAs), which are the focus for this issue of the journal, and the small interfering RNAs (siRNAs), which include multiple classes of siRNAs produced through distinct pathways. Significant progress has been made in our understanding of small RNA-directed regulatory mechanisms, as well as in revealing the genetic complexity of the regulatory small RNA component itself. This is truly remarkable given the short history of small RNA research. In this short review, we will give a brief introduction to the ever-expanding plant small RNA world, with a focus on the diverse small RNA-directed silencing pathways in plants.

2. The RNA silencing machinery

2.1. The conserved core RNA silencing factors

RNA silencing, also known as RNA interference (RNAi), is a conserved regulatory mechanism which typically involves the emergence and the subsequent destruction of a double-stranded RNA (dsRNA) trigger [9,10]. A hallmark of RNA silencing is the generation of small RNAs of 21- to 24-nucleotide (nt) in length from the dsRNA precursor. These small RNAs are then incorporated into an effector complex to guide target recognition and downstream silencing events in a sequence-specific manner [1,3]. Genetic and biochemical studies have revealed a framework for the core RNA silencing machinery, which involves a set of evolutionarily conserved protein families, including DICER (DCR) or DICER-LIKE (DCL), ARGONAUTE (AGO), and RNA-DEPENDENT RNA POLYMERASE (RDR) [3]. Members of these protein families are found in a diverse set of eukaryotes ranging from the unicellular yeast (*Schizosaccharomyces pombe*) and green alga (*Chlamydomonas reinhardtii*) to multicellular plants and animals [3,11,12]. The budding yeast (*Saccharomyces cerevisiae*) is among the few known examples that lack the entire RNA silencing machinery [13], which is consistent with the lack of endogenous small silencing RNAs in this organism.

2.2. The RNA silencing machinery in plants

Plants have several remarkable features in RNA silencing, which have been most clearly demonstrated in the model plant *Arabidopsis*. First, several core RNA silencing factors have been proliferated and functionally diversified during the evolutionary scale of time, likely resulting from genome-wide or segmental gene duplication events followed by specialization [2,3]. For example, the *Arabidopsis thaliana* genome contains four expressed DCL genes, along with six RDR genes and ten AGO genes [2]. This is in contrary to only one (in mammals and worms) or two (in flies and some fungi) DCRs in other systems [2,3]. Second, the RNA silencing machinery in plants includes several factors that appear to be unique to the plant kingdom. One notable example is the nuclear RNA polymerase IV (Pol IV, also known as NRPD), which

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functions in the biogenesis of certain endogenous siRNAs [14,15]. These features have almost certainly contributed to the apparent high complexity of the regulatory small RNA component in plants. Here we provide a brief description of several key RNA silencing factors in plants.

(1). RDRs. Genes encoding RDR family members [RDR2 (At4g11130) and RDR6 (also known as SDE1/SGS2, At3g49500)] were recovered in several independent genetic screens for mutants that are impaired in transgene-induced RNA silencing in *Arabidopsis* [14,16–18]. As mentioned earlier, the *Arabidopsis* genome contains six identifiable *RDRs*. Three of them, namely *RDR3* (At2g19910), *RDR4* (At2g19920), and *RDR5* (At2g19950) are organized as a tandemly-linked cluster on chromosome II and evidence for their function is still lacking. RDR1 (At1g14790), whose expression is inducible upon virus infection or salicylic acid treatment, has been shown to play a role in antiviral defense [19], although it remains unclear if RDR1 is directly involved in siRNA generation. On the other hand, recent genetic data suggest that RDR2 and RDR6 play a direct role in distinct endogenous small RNA pathways [20–22], presumably in converting an RNA template into dsRNAs that serve as substrates for DCLs. Biochemical analysis of RDR6 has confirmed its RNA polymerase activity which converts single-stranded RNA template into dsRNA in a primer-independent manner [23].

(2). DCLs. DCRs in animals and DCLs in plants are multi-domain RNase III-like ribonucleases that process dsRNA substrates into small RNAs [24]. The N-terminal RNA helicase domain, the dual RNase III domain and dsRNA-binding motif (s) at the C-terminus, as well as the central Piwi/Argonaute/Zwill (PAZ) domain are among the conserved structural features that are characteristic to the DCR/DCL family members [24,25]. The central role of DCR/DCL family proteins in RNA silencing was first demonstrated biochemically in an in vitro system of *Drosophila* cell-free extracts [24]. *Arabidopsis* DCL1 (At1g01040), which is one of the four DCLs (DCL1-4) encoded by the genome, was recovered in several independent forward genetic screens for mutants that are defective in flower or embryo development [25]. The genetic requirement of DCL1 for miRNA biogenesis has been firmly demonstrated using the loss-of-function *dcl1* alleles [25–27]. The role for each of the remaining three DCLs in biogenesis of endogenous small RNAs have also been shown genetically [22,28–31]. Biochemical characterization of DCL1 and DCL3 (At3g43920) using immunoprecipitated proteins supported their activities inferred from the genetic data [32].

(3). HYPONASTIC LEAVES1 (HYL1)/dsRNA-binding (DRB) family proteins. Members of this family include five dsRNA-binding domain-containing proteins (HYL1 [At1g09700], and DRB2-5) [5]. They appear to function as DCL-interacting proteins and assist DCLs in processing dsRNA substrates into small RNAs. HYL1, the founding member of this family has been shown to specifically interact and colocalize with DCL1 in the nucleus [33–36], while DRB4(At3g62800) specifically interacts with DCL4(At5g20320) [37,38]. It seems reasonable to speculate that other members in this family may also function through interaction with specific DCLs.

(4). HUA ENHANCER1 (HEN1). HEN1 (At4g20910) is a small RNA methylase which is responsible for methylation of the 2′-hydroxyl group on the ribose of 3′ terminal nucleotide [39,40]. Methylation at the terminal nucleotide is a remarkable feature of functional plant small RNAs, although similar modification in certain DCR-independent small RNAs by an HEN1 homolog has been reported in other systems [41,42]. In vitro analysis revealed small RNA duplexes with the characteristic 2-nt 3′ overhang as the preferred HEN1substrates [40], indicating that methylation likely takes place immediately after DCL processing. The HEN1-dependent methylation appears to be critical for protecting plant small RNAs from nucleolytic activity or other types of modifications including oligouridination [43].

(5). AGOs. AGO proteins have two identifiable conserved domains, a PAZ domain proximal to the N-terminus and a PIWI domain at the C-terminus [44,45]. Members of the AGO family proteins have been identified in the multi-protein effector complexes known as RNA-induced silencing complexes (RISCs) [44,45]. Structural analysis of animal AGO family members revealed PAZ as a small RNA-binding domain [46–48] and PIWI as a ribonucleolytic "slicer" which resembles "RNase H" in its tertiary structure [49,50]. The PIWI domain of certain AGO family members is therefore assumed responsible for the "slicer" activity of a RISC. Among the ten predicted *Arabidopsis* AGO family members (AGO1-10), the role of AGO1 (At1g48410), AGO4 (At2g27040), AGO6 (At2g32940), and AGO7 (At1g69440) in small RNA-directed silencing has been genetically established [51–54]. The "slicer" activity for both AGO1 [32,55] and AGO4 [56] has also been biochemically demonstrated.

(6). Pol IV. Like Pol II, a Pol IV holoenzyme presumably consists of multiple subunits. As mentioned earlier, Pol IV has only been found in plants. *Arabidopsis* expresses two forms of Pol IV termed Pol IVa and Pol IVb. These two forms differ at least by their largest subunit [NRPD1a (At1g63020) and NRPD1b (At2g40030 and At2g20040; a miss annotation for the actual single locus)], but share the same second largest subunit [NRPD2a (At3g23780)] [14,15,57,58]. The Role of Pol IV in small RNA-directed silencing was shown in several independent studies which used either forward or reverse genetics approach [14,15,57,58]. *NRPD1a* (also known as *SDE4*) was recovered from a genetic screen for mutants defective in transgene-induced silencing [14]. An independent screen for mutants defective in RNA-directed DNA methylation recovered both *NRPD1b* and *NRPD2a* [57].

3. Multiple small RNA-directed silencing pathways in plants

The existence of multiple RDRs, DCLs, and AGOs in plants could reflect the operation of more than one functional small RNA-directed silencing pathway in this kingdom, because a subset of these key silencing factors could in principle assemble into different functional "modules". Indeed, mounting evidence supported the idea that evolution has shaped several distinct small RNA pathways in plants through proliferation and subsequent functional specialization of several key RNA silencing factors [2,3].

3.1. miRNAs and miRNA-directed gene regulation

Mature miRNAs in plants are predominately 21-nt small RNA species [4]. They arise from defined genetic loci — the *MIRNA* genes, which are found predominantly within genomic segments previously annotated as intergenic regions (IGRs) [4]. The expression of a *MIRNA* gene begins with Pol II transcription to yield a primary miRNA transcript (pri-miRNA) that is capable of forming the characteristic imperfect "foldback" hairpin structure [59,60]. A pri-miRNA is believed to be first processed into a "stem-loop" precursor (pre-miRNA), followed by the excision of a small RNA duplex that contains the mature miRNA and the miRNA* (the small RNA that resides on the opposite side of the duplex) [4]. A miRNA may reside on either the 5′ or the 3′ arm of the stem-loop precursor, depending on the thermodynamic feature of a miRNA:miRNA* duplex [61,62]. The ribonucleolytic activity of DCL1 is involved in this multi-step processing [59]. HYL1, and perhaps also SERRATE (SE), a zinc finger protein, are also required in

this process [35,63,64]. HEN1, which recognizes the miRNA:miRNA* duplex as its substrate, methylates the 2′-hydroxyl group on the ribose of 3′ terminal nucleotide in each strand. The fact that DCL1, HYL1, and SE as well as HEN1 are all localized in the nucleus suggests that plant miRNAs are born in the nucleus [22,35,36,63,64].

A miRNA:miRNA* duplex dissociates upon the selective incorporation of the mature miRNA into an AGO1-containing RISC, a process known as RISC assembly [61,62]. The miRNA* is typically short-lived and often not detectible in vivo. It is generally believed that most, if not all, mature miRNAs function in the cytoplasm, where they direct the cleavage of their target transcripts [4], although it remains unclear at precisely what stage the miRNAs are transported from the nucleus to the cytoplasm. HASTY (HST, At3g05040), an *Arabidopsis* ortholog of the mammalian Exportin 5 likely plays a role in transporting miRNAs to the cytoplasm [65,66].

Molecular cloning and computational prediction have identified over 180 *MIRNA* loci in *Arabidopsis*, representing over 80 miRNA families [4,8,67,68]. For about twenty families of the *Arabidopsis* miRNAs so far identified, orthologs are also present in other plant species including both dicotyledonous species, such as poplar, and monocotyledous species, such as rice [67]. A small subset which includes members of the miR156, miR319, and miR390 families appears to be deeply conserved across all the land plants examined, suggesting their ancient origin during evolution [69]. There are also numerous miRNAs that appear to be specific to *Arabidopsis* [8,68]. Analyses on some of the non-conserved miRNAs have revealed evolutionary footprints that suggest a mechanism for miRNA origin through inverted duplication of founder gene sequences [70].

A plant miRNA typically functions as a negative regulator of gene expression by guiding the cleavage of its target transcripts [4]. The near-perfect sequence complementarity between a plant miRNA and its target has facilitated the target identification by computational prediction [71]. In *Arabidopsis*, hundreds of miRNA targets have been identified [8,68,71,72]. A significant subset has been subjected to rigorous experimental validation. Firmly established plant miRNA targets include mRNAs encoding a variety of transcription factors, small RNA metabolic factors, ubiquitin-mediated protein degradation pathway components, putative disease resistance genes, as well as several non-protein-coding transcripts (see the section 3.2 below) [8,68,71,72]. It is interesting to notice that DCL1 and AGO1, two known factors in the miRNA pathway, as well as AGO2 (At1g31280), an AGO family member with unknown function, are under negative feedback regulation by distinct miRNAs [70,73,74]. The essential role of miRNAs in plant development is evidenced by the severe developmental defects seen in several miRNA-deficient mutants such as *hen1*, *dcl1*, and *ago1* [25,26,51,75]. In fact, null alleles of *dcl1* and *ago1* mutation are embryonic lethal [25,51].

3.2. Trans-acting siRNAs (ta-siRNAs) and their regulatory roles

ta-siRNAs also arise from defined genetic loci (*TAS* loci) through a remarkable, miRNA-dependent biogenesis pathway [5,76]. The expression of ta-siRNAs is initiated by Pol II transcription to yield *TAS* transcripts that contain miRNA target site(s). The miRNA-directed cleavage of a *TAS* transcript is thought to generate critical features that are recognized by RDR6 [72]. The cleaved *TAS* transcript is converted into dsRNA by RDR6, a process that also requires the SUPRESSOR OF GENE SILENING 3 (SGS3, At5g23570) [20,21]. Successive cleavage of the resulting dsRNA by DCL4 produces a phased array of 21-nt siRNAs [29–31]. Some of these siRNAs (the ta-siRNAs) are incorporated into RISCs to direct the cleavage of mRNA targets [20,21]. The term ta-siRNA was so coined mainly because of the fact that the mRNAs targeted by a ta-siRNA differ from the *TAS* transcript in both genomic origin and overall sequence. As one would expect, factors that are required for miRNA biogenesis and function, including DCL1, HYL1, HEN1, and AGO1 are also required for ta-siRNA biogenesis, although a requirement for HEN1 should be both direct and indirect since ta-siRNAs are also methylated in vivo [5]. While both RDR6 and DCL4 have been shown to possess nuclear localization signal [77,78], it is currently unclear if the ta-siRNA biogenesis pathway operates exclusively in the nucleus. The *Arabidopsis* SILENCING DEFFECTIVE5 (SDE5, At3g15390), a putative homolog of human mRNA export factor has been shown to be required for ta-siRNA accumulation, raising the possibility that the ta-siRNA biogenesis may involve more than one subcellular compartment [79].

Eight *TAS* loci belonging to four families (*TAS1*, *TAS2*, *TAS3*, and *TAS4*) have been identified in *Arabidopsis* [8,20,21,29–31,72]. As mentioned earlier, just like a miRNA, a ta-siRNA negatively regulates gene expression by guiding the cleavage of a target mRNA. The known targets of ta-siRNAs include genes encoding auxin responsive transcription factors (ARF3, ARF4), pentatricopeptide repeat (PPR) family proteins, putative MYB transcription factors and proteins of unknown function [8,37,80,81]. Interestingly, accumulation of TAS3 ta-siRNAs requires AGO7, suggesting that these siRNAs likely function in AGO7-containing RISCs to guide target cleavage [37,80]. The ta-siRNA-mediated regulatory mechanism appears to be conserved in a wide range of plant species [82].

3.3. Natural cis-antisense transcripts-associated siRNAs (nat-siRNAs) and their role in plant stress responses

Nat-siRNAs originate from dsRNA precursors formed by convergent bidirectional transcription of two partially overlapping genes [28,83]. The *Arabidopsis* genome contains nearly a thousand gene pairs that could potentially give rise to transcripts in such a *cis*-antisense configuration [28]. Based on the two published cases, the biogenesis of nat-siRNAs appears to involve two phases: an initiation phase and a reinforcement phase. In the initiation phase, the dsRNA formed by the *cis*-antisense transcripts is processed into a primary nat-siRNA species by either DCL1 or DCL2 (At3g03300) activity. In both reported cases, one of the overlapping genes is constitutively expressed. The induced expression of the other genes therefore governs the formation of the dsRNA, which triggers the initiation phase. The induction was responsive to high salinity conditions in one case [28], and to the infection by a bacterial pathogen in the other [83]. The primary nat-siRNA directs the cleavage of the complementary transcript which is constitutively expressed, setting the stage for the next phase. The reinforcement phase is mechanistically similar to the biogenesis of ta-siRNAs. The cleaved transcript is converted into dsRNA in a RDR6- and SGS3-dependent manner. DCL1 processing of this dsRNA produces a phased array of secondary nat-siRNA species, which further promote the silencing of the constitutive transcripts. Intriguingly, the production of the secondary nat-siRNA species also involved Pol IVa, a factor that is not required for ta-siRNA biogenesis.

The nat-siRNA pathway appears to function as a plant adaptive protection mechanism in response to either abiotic or biotic stress. In the case of high salinity responsive nat-siRNAs, cleavage of the constitutively expressed transcript for Δ^1-pyrroline-5-carboxylate dehydrogenase (P5CDH, At5g62530) leads to proline accumulation, which activates a pathway that would alleviate the stress [28]. Similarly, the bacterial pathogen-induced nat-siRNAs enhance the host defense response by repressing a putative negative regulator of the disease resistance pathway [83].

3.4. Heterochromatic siRNAs and their regulatory roles

Heterochromatic siRNAs in plants are typically 24-nt small RNAs that are associated with genomic repetitive sequences such as transposons, retroelements, rDNAs, and centromeric repeats [84–86]. Biogenesis of heterochromatic siRNAs requires the activity of Pol IVa, RDR2, DCL3, and HEN1 [7,22,14,15,22,84]. Current model for heterochromatic siRNA biogenesis states that Pol IVa produces single-stranded RNA transcripts from certain genomic loci including

transposable elements and other repetitive regions. The Pol IVa transcripts move to the nucleolus where they are converted into dsRNAs by RDR2, and subsequently processed by DCL3 to yield heterochromatic siRNAs in the nucleolar RNA processing center that colocalizes with Cajal bodies [87,88]. Small RNA expression profiling in both wild type *Arabidopsis* and small RNA-deficient mutant lines by deep sequencing strategy strongly supported the current model for heterochromatic siRNA biogenesis [6,7,86,89].

The heterochromatic siRNAs are known to direct DNA and histone methylation in an AGO4-dependent pathway [54,90]. The heterochromatic siRNA-directed DNA methylation also requires the Pol IVb [14,15,57,58], the SNF2-like ATPase DRD1(At2g16390) [91,92], and the de novo cytosine methyltransferase DRM2 (At5g14620)[90,93]. Since AGO4 has been shown to physically interact with the largest subunit of Pol IVb (NRPD1b), and DCL3, AGO4, and Pol IVb colocalize in the nucleolar RNA processing center, Pol IVb may be part of the AGO4-containing RISC in the chromatin RNA silencing pathway [87]. It has been proposed that the AGO4/NRPD1b/siRNA complex directs target recognition for DRD1- and DRM2-dependent DNA methylation at specific loci [87,88]. AGO6, another member of the *Arabidopsis* AGO family, has been recently shown to have partially redundant functions with AGO4 in heterochromatic siRNA-directed silencing [94]. It is currently unclear as to what extent the expression or function of AGO4 and AGO6 overlap.

4. Conclusions and perspectives

The complexity of the regulatory small RNA component in plants is apparently much higher than we have thought before and may be higher than those found in other systems as well. This could be partly attributable to the sessile nature of land plants which are constantly exposed to the changing environment and forced to evolve novel mechanisms to cope with biotic and abiotic stress. In fact, several well-characterized small RNAs including miRNAs and siRNAs are known to function in alleviating stress conditions such as pathogen attack, nutrients deprivation, and high salinity [95]. A recently reported novel class of bacterial pathogen-inducible, longer (30–40-nt) siRNA species also appears to play a role in disease resistance [96]. It is reasonable to anticipate discovery of novel regulatory small RNAs by applying the deep sequencing strategy to plants challenged with biotic or abiotic stress conditions.

Remarkable advances have been made in our understanding of the small RNA-directed silencing pathways in plants within the last few years. The four major pathways which we summarized above, however, are no more than an overly simplified picture of what nature has invented. Further characterization on the key RNA silencing factors for their biochemical activity, tissues-specific expression domain, as well as high-resolution subcellular localization will be informative and shall lead to a clearer picture.

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