Olga Pontes · Hailing Jin *Editors*

Nuclear Functions in Plant Transcription, Signaling and Development

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Preface for Nuclear Functions (eds. Pontes and Jin)

Plants provide us with food and are the source of several other by-products such as compounds used in pharmaceutics or biofuels. Therefore, it comes as no surprise that plants are crucial in solving major challenges now facing humanity, namely, food productivity/security, increasing energy demands, and environmental changes. There has been a dramatic increase for plant-derived food and feed products as the world population grows exponentially. Plants are also playing a role in filling our ever-increasing energy needs and these bio-energy crops are expected to provide a sustainable, CO_2-neutral emission solution in the near future. Yet such crops will need to be compatible with food and feed agriculture production and must preserve Earth's ecosystems. So the question is: how can we face all of these challenges?

To meet our planet's needs, we need to improve and further develop sustainable methods for plant production by incorporating both biotechnology and sustainable agricultural practices. In this context, we must first establish a baseline understanding of different molecular and cellular mechanisms underlying plant development and response to stress, so we can then apply it to practical advances in plant production across the globe. Surprisingly, the biological networks underpinning plant yield are still poorly understood, particularly regarding the master regulator of cellular function: the nucleus.

The nucleus harbors the large majority of the plant's DNA, the linear sequence of which is the blueprint of every living organism. However, this is only the beginning: how this DNA is expressed and regulated depends on a variety of other interacting factors that play crucial roles in shaping its organization and function. With the sequencing of several plant genomes and recent advances in high-throughput technologies, plant nuclear biologists have been able to unveil many of the mechanisms underlying genome regulation.

For instance, epigenetic modifications, such as histone post-translational modifications and DNA methylation, directly impact gene expression and genome defense by regulating the organization and function of the genome. Importantly, while evolutionary processes take place at a timescale that does not allow plants to respond and adapt to climate-induced stress, we are starting to recognize that epigenetic mechanisms can confer phenotypic plasticity. Epigenetics enables a heritable control of

phenotypes that can change rapidly in response to environmental cues—sometimes over the course of just two to three generations. This epigenetic timescale of change has tremendous implications for how environmentally altered phenotypes are acquired and inherited at the organism and eventually at the population levels.

Another exciting recent discovery that came about through plant biology research is the previously unacknowledged role of noncoding small RNAs in gene expression. These small molecules have been increasingly recognized as players in the establishment of epigenetic modifications, as well as in genome defense and integrity. Noncoding small RNAs impact normal growth, development, and stress responses in diverse plant species, including staple crops such as rice and maize. Small noncoding RNAs are already playing key roles in plant biotechnology applications including directing the specific and enhanced expression of selected genes. These molecules are therefore of great interest in the context of bioengineering, and have enormous potential for enhancing crop productivity in a wide range of ecosystems. Yet, there is still a great deal left to learn about how small noncoding RNAs are integrated into plants' feedback loops, which direct epigenetic modifications throughout development and the stress response process.

Finally, genomes are dynamic structures as their functional properties are strongly determined by their spatial organization over time. Similarly, changes in higher order nuclear organization alter the functional properties of genomic regions. Various types of subcellular physical domains have been identified in the nucleus, the known nuclear bodies or subcompartments, and these structures are associated with transcription factors, RNA-processing proteins, and epigenetic regulators. Interestingly, these nuclear domains display different behaviors in response to the environment, yet it is still a matter of debate how nuclear organization functionally relates to plant biological processes.

The mechanisms and processes described above make it clear that a true understanding of genome function requires integrating the genomic sequence with what we are still discovering about how epigenetics, small noncoding RNAs, and dynamic nuclear organization modify genomes. It is the goal of this book to compile a series of landmark discussions on the recent advances in plant nuclear biology research and offer new perspectives into the functional relevance of the arrangement of genomes and nuclear processes that impact plant physiology and development. The following chapters will provide insights as to how genes are switched on/off and are tuned to specific expression levels, which will allow us to better predict plant phenotypes. Overall, a better understanding of the fundamentals of plant gene expression will aid in the more efficient design of numerous biotechnological applications and plant-breeding programs. This new knowledge will thus provide a foundation for solving both agricultural and environmental problems as well as developing practices that enable global sustainability. Lastly, plant biology is also relevant to human biology as several aspects of underlying mechanisms are conserved between both organisms. Understanding this shared biology will shed light on human diseases and could lead to better therapies for cancer and genetic diseases.

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Chapter 1
RNA-Directed DNA Methylation and Transcriptional Silencing in *Arabidopsis*

Xian-Yang Deng and Xin-Jian He

Introduction

DNA methylation is an important chromatin marker that is involved in transcriptional regulation in plants, fungi, and mammals [1, 2]. In plants, most DNA methylation occurs at transposable elements and other repetitive DNA sequences and is required for the transcriptional silencing of these regions [3, 4]. DNA methylation occurs in all the three cytosine contexts: the symmetric CG and CHG contexts (in which H = A, T, or C) and the asymmetric CHH context. CG methylation is maintained by methyltransferase 1 (MET1), a homolog of mammalian DNA methyltransferase 1 (DNMT1) [5, 6]. The plant-specific chromomethylase 3 (CMT3) specifically catalyzes CHG methylation [7, 8]. Domains rearranged methyltransferase 2 (DRM2) and its homologs are responsible for establishing CHH methylation and to a lesser extent CG and CHG methylation [9, 10]. CG methylation is present in transposable elements and DNA repeats, as well as in genic regions, but CHG and CHH methylation is almost exclusively present in transposable elements and DNA repeats [3, 4].

Small interfering RNAs (siRNAs) and long noncoding RNAs (ncRNAs) are responsible for establishing DNA methylation and/or repressive histone H3K9 methylation at transposable elements and DNA repeats in plants, fungi, and mammals [1, 11, 12]. In *Arabidopsis,* DNA methylation can be established through a well-described RNA-directed DNA methylation (RdDM) pathway [2]. RdDM plays important roles in development, stress response, and genome evolution [2, 12, 13]. RdDM requires canonical components in the conserved RNA interference (RNAi) machinery; these components are members of the Dicer and Argonaute families and RNA-dependent RNA polymerase 2 (RDR2) [14, 15]. Moreover, plant-specific DNA-dependent RNA polymerases IV and V (Pol IV and Pol V), DNA methyltransferase DRM2, and several other proteins are required for RdDM [13, 14, 16–20]. In the past few years, our knowledge of RdDM has been

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greatly improved by genetic, biochemical, and structural studies. In this chapter, we describe the recent insights into the mechanisms underlying RdDM.

Pol IV-Dependent siRNAs

Two atypical, plant-specific polymerases, Pol IV and Pol V, are required for the biogenesis of siRNAs and long ncRNAs, respectively; both Pol IV and Pol V consist of multiple subunits [20, 21]. Nuclear RNA polymerase D1 (NRPD1; formerly named NRPD1a) is the largest subunit of Pol IV, and nuclear RNA polymerase E1 (NRPE1; formerly named NRPD1b) is the largest subunit of Pol V [20]. Nuclear RNA polymerase D/E2 (NRPD/E2) is the second largest subunit of both Pol IV and Pol V. Some subunits are only present in one RNA polymerase, but others are shared by Pol IV, Pol V, and/or Pol II [16, 17, 19, 20, 22].

RdDM is thought to be initiated by 24-nt siRNAs. As indicated in Fig. 1.1, the biogenesis of the 24-nt siRNAs is dependent on Pol IV [14, 16, 18], RDR2, and Dicer-like 3 (DCL3) [14, 16, 18, 23]. Pol IV is responsible for producing single-stranded RNAs (ssRNAs), which are converted into double-stranded RNAs

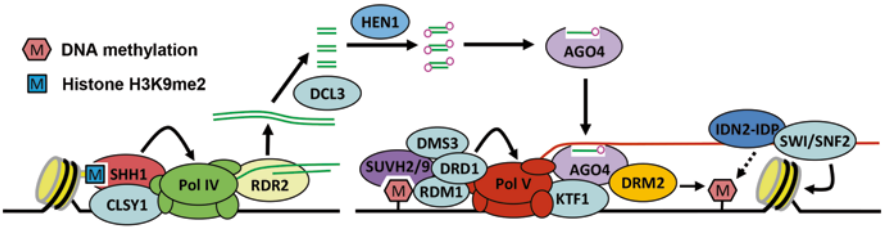

Fig. 1.1 Model for RNA-directed DNA methylation (RdDM) in *Arabidopsis*. In the RdDM pathway, RNA polymerase IV (Pol IV) transcribes single-stranded RNAs that are immediately converted into double-stranded RNAs by RDR2. DCL3 cleaves the double-stranded RNAs into 24-nt siRNAs that are methylated at their 3′-ends by HEN1. A single strand of the siRNA duplex associates with AGO4 and forms the AGO4–siRNA complex. SHH1 recognizes histone H3K9 methylation and then recruits Pol IV to RdDM target loci. CLSY1 interacts with SHH1 and may assist Pol IV recruitment. RNA polymerase V (Pol V) transcribes long noncoding RNAs that act as scaffold RNAs. The recruitment of Pol V to target loci is dependent on the DDR complex, which is composed of DMS3, DRD1, and RDM1. The SRA domain- and SET domain-containing proteins SUVH2 and SUVH9 bind methylated DNA and interact with the DDR complex, thereby facilitating the recruitment of Pol V. Pol V-produced noncoding scaffold RNAs base-pair with siRNAs in the AGO4–siRNA complex, whereas NRPE1 and KTF1 interact with AGO4 through their C-terminal WG/GW domains. The AGO4–siRNA complex guides the de novo DNA methyltransferase DRM2, which catalyzes DNA methylation at the loci. IDN2 interacts with its paralogs IDP1 and IDP2, and forms tetramers that bind Pol V-produced scaffold RNAs. IDN2 associates with the SWI/SNF complex and mediates nucleosome positioning for Pol V-stabilized nucleosomes. DCL3 - Dicer-like 3, RDR2 - RNA-dependent RNA polymerase 2, siRNA - small interfering RNAs, HEN1 - hua enhancer 1, AGO4 - Argonaute protein 4, SHH1 Sawadee homeodomain homolog, NRPE1 - nuclear RNA polymerase E1, IDN2 - involved in de novo 2, SWI/SNF switch/sucrose nonfermentable, CLSY1 - chromatin-remodeling protein CLASSY1, DMS3 defective in meristem silencing 3, DRD1 - defective in RdDM,SRA SET- and RING-associated, SUVH SU(VAR) homologs, KTF1 - KOW-containing transcription factor 1

(dsRNAs) by RDR2. The dsRNAs are cleaved into 24-nt siRNAs mainly by DCL3, which is partially redundant with two other DCL enzymes, DCL2 and DCL4 [14, 23, 24]. The 3′-OH groups of 24-nt siRNAs are methylated by HUA ENHANCER 1 (HEN1), which stabilizes 24-nt siRNAs in vivo [25]. The 24-nt siRNAs are loaded onto ARGONAUTE proteins AGO4, AGO6, or AGO9 [15, 26–28] and are assembled into RNA-induced transcriptional silencing (RITS) complexes that signal de novo DNA methylation and transcriptional silencing at target regions.

Polymerase activity in vitro has been documented for Pol IV [29], and mutations in the conserved catalytic site of NRPD1 abolish the abundance of 24-nt siRNAs; together, these results suggest that Pol IV is an active RNA polymerase in vivo [29]. Unlike Pol II, Pol IV activity in vitro requires an RNA primer and is insensitive to alpha-amanitin [29]. Pol IV and RDR2 associate in vivo, but Pol IV does not require RDR2 for activity, whereas RDR2 is nonfunctional in the absence of associated Pol IV. The coupling of Pol IV and RDR2 results in the channeled synthesis of double-stranded precursors for 24-nt siRNA biogenesis [29].

Pol IV-dependent 24-nt siRNAs are the most abundant class of small RNAs in *Arabidopsis*. These siRNAs are mainly produced at thousands of discrete transposable elements and repetitive DNA elements located at pericentromeric heterochromatin [18, 30, 31]. It is important to determine how Pol IV-dependent siRNAs are produced at specific chromatin regions rather than at others. Sawadee homeodomain homolog 1 (SHH1)/DNA-binding transcription factor 1 (DTF1) was independently identified by a forward genetic screen and a Pol IV affinity purification [32, 33]. SHH1/DTF1 specifically associates with Pol IV but not with Pol V, and the accumulation of most Pol IV-dependent siRNAs is markedly decreased in the *shh1/dtf1* mutants [32, 34, 35], suggesting a role of SHH1 in Pol IV transcription. SHH1 contains a SAWADEE domain that preferentially binds to unmethylated K4 and methylated K9 modifications on the histone H3 [34]. Pol IV ChIP accompanied by DNA deep sequencing indicated that SHH1 is required for the association of Pol IV with chromatin [34]. When critical residues in the SAWADEE domain are mutated, both 24-nt siRNA and DNA methylation levels are decreased [34]. These results suggest that SHH1 is responsible for targeting Pol IV to chromatin by associating with RdDM target loci that have unmethylated K4 and methylated K9 on histone H3. The chromatin-remodeling protein CLASSY1 (CLSY1) was primarily identified as an RdDM component by a forward genetic screen, and Pol IV-dependent siRNA accumulation is drastically decreased in the *clsy1* mutant [36]. CLSY1 is purified by Pol IV affinity purification [32], suggesting that CLSY1 associates with Pol IV. The functional and physical association of CLSY1 with Pol IV suggests that chromatin remodeling is involved in Pol IV transcription.

Pol V-Dependent ncRNAs

Like Pol IV, Pol V is also required for siRNA accumulation. However, the effect of Pol V on siRNA accumulation is limited to a subset of Pol IV-dependent siRNAs and is likely a result of its effect on DNA methylation [30, 34]. Researchers have demonstrated that Pol V-produced ncRNAs function as scaffolds for the recruitment

of the silencing machinery and help siRNAs recognize their target loci; the latter function is possibly facilitated by base-pairing between AGO4-bound siRNAs and nascent Pol V-produced transcripts [37, 38]. Polymerase activity in vitro and in vivo has been shown for Pol V [29, 37]. That Pol V carries out transcription using the bipartite oligonucleotide template but not the tripartite template suggests an inability to disrupt downstream dsDNA during transcription [29].

Pol V-dependent ncRNAs help recruit the RdDM silencing machinery and are required for siRNA-mediated DNA methylation and transcriptional silencing. A genome-wide ChIP-seq analysis indicated that the largest subunit of Pol V, NRPE1, is enriched at promoters of protein-coding genes and at recently evolved transposons [39]. This localization pattern is highly correlated with Pol V-dependent DNA methylation and 24-nt siRNA accumulation [39–41]. The vast majority of Pol V-enriched regions are usually shorter than 250 bp [39]. The association of Pol V with promoters of protein-coding genes indicates that Pol V is likely originated from ancient RNA polymerase II (Pol II) [39], which is consistent with the finding that Pol II shares several conserved subunits with Pol IV and Pol V [20, 21]. A small proportion of ncRNAs are produced by Pol II and these ncRNAs are involved in DNA methylation and transcriptional gene silencing through the RdDM pathway [6, 42], supporting the notion that Pol IV and Pol V are originally evolved from Pol II.

A number of transcription factors are required for Pol II transcription. It is interesting to determine whether Pol V transcription requires transcription factors. RDM4 (RNA-directed DNA methylation 4) / DMS4 (defective in meristem silencing 4), a homolog of the yeast Pol II-dependent transcription factor IWR1, has been identified as a canonical RdDM component by two independent genetic screens [43, 44]. Pol V-produced ncRNAs are decreased in the *rdm4* mutant, suggesting that RDM4 is required for Pol V transcription [43]. Unlike other canonical RdDM mutants, the *rdm4* mutant has pleiotropic developmental defects. RDM4 is a transcription factor that is shared by Pol II, Pol IV, and Pol V [32, 43].

The production of Pol V-dependent ncRNAs is also dependent on DRD1 (defective in RNA-directed DNA methylation 1), DMS3 (defective in meristem silencing 3), and RDM1 (RNA-directed DNA methylation 1) [37, 45–47], which form a DDR (DRD1, DMS3, and RDM1) complex in vivo [11]. The DDR complex is required for the association of Pol V with chromatin, and the association facilitates the transcription of Pol V-dependent noncoding RNAs [39]. The SU(VAR)3–9 homologs SUVH2 and SUVH9 act redundantly in RdDM and transcriptional silencing [48, 49]. By associating with the DDR complex, SUVH2 and SUVH9 are involved in the association of Pol V with chromatin [50, 51]. SUVH2 and SUVH9 contain an SET- and RING-associated (SRA) domain that directly binds to methylated DNA [48]. SUVH2 and SUVH9 act in RdDM by directing the DDR complex and Pol V to RdDM target loci (Fig. 1.1). The binding of SUVH2 and SUVH9 to methylated DNA facilitates the formation of a self-reinforcing loop of DNA methylation and Pol V transcription.

Microrchidia 6 (MORC6)/DMS11 (defective in meristem silencing 11) and MORC1 are the members of the conserved microrchidia adenosine triphosphatase (ATPase) family with a GHKL (gyrase, Hsp90, histidine kinase, MutL) ATPase domain. The *morc1* and *morc6* mutants show decondensation of pericentromeric

heterochromatin and increased interaction between the pericentromeric regions and the rest of the genome [52], whereas the *morc6*/*dms11* mutant shows slight decreases in siRNA accumulation and DNA methylation [53, 54]. A recent study indicate that MORC1 and MORC6 interact with the DDR complex and with SUVH2 and/or SUVH9 [50], which is consistent with the effect of the *morc6*/*dms11* mutation on Pol V-dependent transcripts [54].

Recruitment of RdDM Effector to Chromatin

Pol IV-dependent siRNAs associate with AGO4, thereby facilitating the formation of an RdDM effecter complex that is required for DNA methylation. The assembly of the RdDM effector complex in *Arabidopsis* is similar to the RITS complex in fission yeast [1, 55]. In *Arabidopsis*, 24-nt siRNAs produced by DCL3 are subjected to a sorting process, and the specificity of RNA sorting may be associated with the terminal nucleotide of the siRNA and duplex properties, such as thermodynamic asymmetry or degree of base-pairing [56, 57]. Twenty-four-nucleotide siRNAs are loaded onto AGO4 [58, 59]. The loading process occurs in the cytoplasm with assistance from the ATP-bound HSP90, and ATP hydrolysis induces the dissociation of the siRNA passenger strand and results in a conformational change in AGO4, leading to the importation of the AGO4–siRNA complex into the nucleus [60]. Pol V-dependent noncoding RNAs recruit the AGO4–siRNA complex to chromatin by base-pairing with siRNAs [38]. DRM2 is a key de novo DNA methyltransferase that is responsible for DNA methylation at RdDM target loci, but how DRM2 is recruited to chromatin is poorly understood. A recent study demonstrated that DRM2 exists in a complex with AGO4 and preferentially methylates one DNA strand, which acts as the template for Pol V transcription [61]. The results support a model, in which DRM2 is guided to target loci in a strand-specific manner. The AGO4–siRNA complex and Pol V-dependent ncRNAs may be required for the recruitment of DRM2 to chromatin (Fig. 1.1).

Immunolocalization experiments with isolated nuclei have shown that RDR2, DCL3, and AGO4 localize in the Cajal body [62, 63]. The Cajal body is involved in a variety of functions including pre-mRNA splicing, rRNA processing, and telomere maintenance [64]. It is possible that the biogenesis of siRNAs and long ncRNAs, and the assembly of protein–RNA complexes may involve the function of the Cajal body. Further study is required to understand the detailed role of the Cajal body in RdDM. A portion of the NRPE1 signals colocalize at target loci, together with DRD1, a protein required for Pol V transcription [59, 62], which is consistent with the function association of Pol V and DRD1 at the downstream step of RdDM.

Assembly of the RdDM effector complexes is mediated by multiple protein–protein and protein–RNA interactions that recruit proteins to specific genomic regions. NRPE1 contains WG/GW repeats in its C-terminal domain, which is thought to act as an AGO4 hook motif [65]. KOW-containing transcription factor 1 (KTF1)/SPT5-like protein (SPT5L) also contains conserved WG/GW repeats in its C-terminal domain [66, 67]. Moreover, KTF1/SPT5L is capable of binding to

noncoding scaffold RNAs produced by Pol V [67]. Chromatin binding of AGO4 and KTF1 occurs downstream to Pol V [38, 68]. KTF1 and AGO4 are recruited to chromatin in parallel and partially independently of each other, whereas KTF1 enhances AGO4 chromatin binding at a subset of RdDM sites [68]. The chromatin binding of KTF1/SPT5L and AGO4 may create a platform for the recruitment of DRM2 to chromatin at RdDM targets [68].

IDN2 (involved in de novo 2)/RDM12 (RNA-directed DNA methylation 12), another factor that is thought to act as a downstream RdDM effector, is homologous to suppressor of gene silencing 3 (SGS3), a protein involved in the accumulation of viral siRNAs, ta-siRNAs, and nat-siRNAs for posttranscriptional gene silencing. IDN2 contains an N-terminal C2H2-type zinc finger domain, an XS domain, a coiled-coil domain, and a specific XH domain [69–71]. The XS domain is required for the binding of IDN2 to dsRNAs [70]. A possible RNA substrate for IDN2 is the duplex that is formed between AGO4-bound siRNAs and Pol V-dependent noncoding transcripts. The duplex could be a signal that aids in the recruitment of DRM2 to establish DNA methylation.

IDN2 can form a homodimer and associate with two IDN2 paralogs, IDP1 (IDN2 paralog 1)/IDNL1 (IDN2-LIKE 1) and IDP2/IDNL2 [69, 71]. IDP1 is required for siRNA accumulation, de novo DNA methylation, and transcriptional gene silencing, whereas the roles of IDP2 partially overlap with those of IDP1 [71, 72]. The coiled-coil domain of IDN2 is essential for the homodimerization of IDN2 with itself but is not required for IDN2 association with IDP1 and IDP2, whereas the uncharacterized XH domain of IDN2 is required for association with IDP1 and IDP2 but not for IDN2 homodimerization [71]. Unlike IDN2, IDP1 and IDP2 are incapable of binding to double-stranded RNA, suggesting that IDP1 and IDP2 have distinct roles in the IDN2–IDP1/IDP2 complex [71]. The IDN2–IDP1/IDP2 complex may facilitate the recruitment of the double-stranded RNA-containing RdDM effector complex to specific chromatin regions at a downstream step of the RdDM pathway.

IDN2 was shown to interact with SWI3B, a component of the SWI/SNF (switch/sucrose nonfermentable) ATP-dependent nucleosome-remodeling complex [73]. The SWI/SNF complex affects Pol V-stabilized nucleosome positioning and contributes to transcriptional silencing. The study suggests that IDN2 acts as an adaptor protein connecting Pol V-produced ncRNAs and the SWI/SNF complex, thereby guiding the SWI/SNF complex and mediating nucleosome positioning (Fig. 1.1). Pol V-produced ncRNAs may be not only required for DNA methylation but also required for repressive nucleosome positioning at RdDM target loci.

Conclusion and Perspective

RdDM is involved in the transcriptional silencing of noncoding genomic regions as well as of protein-coding genes flanked by noncoding transposable elements and other repetitive DNA sequences. Disruption of RdDM components not only affects

transposable element silencing and genome stability but also affects protein-coding gene expression and imprinting, stress response, and various developmental stages. An important objective for future research is to determine how RdDM acts in these processes. Although most RdDM components have been cloned and preliminarily characterized, some critical questions remain. How are Pol IV- and Pol V-dependent noncoding RNAs differentiated for distinct functions? Which chromatin superstructure features are preferentially targeted by RdDM? How is DNA methylation established for introduced unmethylated transgenes? How are DNA methylation, histone modification, heterochromatin condensation, and nucleosome positioning concomitantly regulated at RdDM target loci? Recently, several pre-mRNA splicing factors were demonstrated to be involved in RdDM and transcriptional silencing [74–77]. Similarly, RNA splicing and processing is required for RITS in fission yeast [78, 79]. It will be interesting to determine how these splicing factors coordinate with the RdDM machinery and act in transcriptional silencing. Further studies on RdDM will undoubtedly clarify the roles of noncoding genomic regions in plants and will of course benefit the breeding of agricultural crops.

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