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Review

miRNA Biogenesis: A Dynamic Pathway

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MicroRNAs (miRNAs) modulate plant homeostasis through the inactivation of specific mRNAs, especially those encoding transcription factors. A delicate spatial/temporal balance between a miRNA and its targets is central to achieving the appropriate biological outcomes. In this review we discuss our growing understanding of the dynamic regulation of miRNA biogenesis. We put special emphasis on crosstalk between miRNA biogenesis and other cellular processes such as transcription and splicing. We also discuss how the pathway is regulated in specific tissues to achieve harmonious plant development through a subtle balance between gene expression and silencing.

Trends

The assembly of the miRNA biogenesis machinery starts during miRNA gene transcription.

There is crosstalk between components of the miRNA biogenesis and mRNA splicing machineries, which mutually regulate each other.

miRNA production is regulated tissue-specifically and varies depending on each pri-miRNA.

The miRNA pathway is a fluid, dynamic, and interconnected process.

Plant miRNA Biogenesis

MicroRNAs (miRNAs) (see Glossary) are essential components of the gene silencing machinery in most eukaryotic organisms. The plant miRNA pathway, both at the biogenesis and silencing levels, differs substantially from its counterpart in mammals. In plants, miRNAs are produced from a primary miRNA transcript (pri-miRNA), which includes a foldback structure, by the nuclear RNase **DICER-LIKE 1** (DCL1) and its accessory proteins SERRATE (SE) and HYPONASTIC LEAVES1 (HYL1). The central components of the plant miRNA biogenesis pathway, such as DCL1, HYL1, and SE, were identified long ago and served as the foundation of the earliest models of miRNA biogenesis [1]. Only recently has the true complexity of miRNA biogenesis pathway, especially its plasticity, regulation, and crosstalk with other biological processes, become apparent. We now know that the production of miRNAs is a tissue-specific process, is tightly associated with transcription and splicing, and even varies between miRNA precursors. Many aspects of the miRNA pathway, such as biogenesis, turnover, subcellular compartmentalization, and response to the environment, have been reviewed in recent years [1–4]. Instead, in this review we explore the pathway from a different perspective. We center our discussion on the dynamics of miRNA production, its crosstalk with other cellular processes, and how it is regulated in a very subtle and specific manner. We ultimately aim to help the reader construct a fresh view of miRNA biogenesis as a fluid and adaptable process.

Early Transcriptional Events

As for any coding mRNA, the pri-miRNAs are transcribed by the RNA polymerase II (RNAPII) in a process regulated by the MEDIATOR complex and by phosphorylation of the **C-terminal domain of RNA polymerase II** (RNAPII-CTD) largest subunit by CYCLIN-DEPENDENT KINASES (CDKF1 and CDKDs) [5,6]. Similarly to the transcriptional regulation of coding genes, the expression of each individual miRNA gene is controlled by specific transcription factors. However, some transcription factors have been described to affect the miRNA pathway as a whole. NEGATIVE ON TATA LESS 2 (NOT2) interacts with the RNAPII and regulates the transcription of miRNA genes [7]. The MYB-related protein CELL DIVISION CYCLE 5 (CDC5) associates with miRNA gene promoters to increase RNAPII occupancy and transcription [8]. PLEIOTROPIC REGULATORY LOCUS 1 (PRL1) interacts with DCL1 and pri-miRNAs [9]. Such interactions

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enhance pri-miRNA processing potentially by stabilizing the **processing complex**. Because PRL1 also interacts with RNAPII and CDC5, it is possible that this cofactor is also recruited to the nascent pri-miRNA co-transcriptionally [9,10]. In a less well understood process, the cycling DOF transcription factor (CDF2) directly interacts with a subset of miRNA gene promoters where it can act as either a positive or negative regulator [11]. Transcription factors regulating the expression of core components of the biogenesis machinery have a general impact on the production of miRNAs. This is the case of XAP5 CIRCADIAN TIMEKEEPER (XCT), a nuclear protein that modulates **small RNA** production by regulating the transcription of DCL coding genes [12]. HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) is an ubiquitin E3 ligase that promotes RNAPII occupancy at the miRNA168b (miR168b) promoter and enhances its transcription. Even when HOS1 only regulates miR168b, it ultimately affects the accumulation of AGO1 (target of miR168b) and thus the activity of most miRNAs [13]. Similarly, any transcription factor that regulates the expression of miR162 and miR863, which target DCL1 and SE respectively, will affect miRNA production. In another layer of regulation, it was recently discovered that pri-miRNA can encode self-regulating small peptides (miPEP) [14]. At the chromatin level it has been shown that the ATP-dependent SWR1 chromatin-remodeling complex (SWR1-C) regulates the transcription of miRNA genes by altering nucleosome dynamics [15]. Another example of epigenetic regulation is the indirect repression of DCL1, SE, HYL1, and **ARGONAUTE1** (AGO1) by the histone acetyltransferase GENERAL CONTROL NON-REPRESSED PROTEIN 5 (GCN5) [16].

Cotranscriptional Assembly of the Processing Complex

In plants, pri-miRNA transcription and processing were long believed to be separated processes taking place one after the other. Recent evidence suggests that both processes are tightly associated, with at least the assembly of the processing complex starting during transcription. One of the strongest lines of evidence for coupling between miRNA gene transcription and processing was the discovery of the Elongator complex as a key component in the miRNA biogenesis [17]. This complex is required for RNAPII occupancy at miRNA loci and interacts with DCL1 and SE. More strikingly, DCL1 was found to associate, in an Elongator-dependent manner, with the chromatin of miRNA loci [17]. This suggests that DCL1, and probably SE, are recruited to the nascent pri-miRNAs co-transcriptionally, likely during transcript elongation. Supporting this idea, C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 and 2 (CPL1 and CPL2) are able to interact both with the RNAPII-CTD and the miRNA processing machinery, specifically with SE. CPL1 and CPL2 modulate RNAPII activity by dephosphorylating its CTD and regulating miRNA biogenesis by modulating HYL1 activity [18,19]. Owing to its dual interaction with the RNAPII-CTD and SE, we speculate that CPL proteins are also recruited into the processing complex during miRNA gene transcription. NOT2, CDC5, PRL1, and CDF2, that associate with RNAPII and miRNA gene promoters as described above, also interact with components of the miRNA processing machinery [7,8,11]. CDC5, PRL1 and NOT2 interact with DCL1 and SE to promote pri-miRNA processing. It would not be surprising if these proteins acted by assisting the recruitment of processing factors to the nascent pri-miRNAs [7,8]. Similarly, TOUGH (TGH), which was originally described to interact with TATA binding proteins, also interacts with pri-miRNAs, DCL1, HYL1, and SE [20,21]. Interestingly, TGH may modulate both the interaction of pri-miRNAs with the DCL1-containing complex as well as the activity of DCL proteins [21].

The **nuclear cap-binding complex** (CBC) binds to the 5′ cap of nascent pri-miRNAs leading to the proper production of miRNAs [22–24]. CBP20 and CBP80 were shown to interact with SE, CBP20 also with NOT2b, linking the CBC and the processing machinery [7,25]. However, it is still unclear if the CBC acts as a scaffold to recruit SE and NOT2b to the nascent pri-miRNAs or if such interactions take place after complex formation to stabilize the pri-miRNAs.

Glossary

ARGONAUTE (AGO): the AGO proteins, loaded with small RNAs (sRNAs), catalyze the transcriptional or post-transcriptional silencing of target genes. There are 10 members of the AGO family of proteins in *Arabidopsis*.
C-terminal domain of RNA polymerase II (RNAPII-CTD): highly conserved tandem heptapeptide repeats (Y–S–P–T–S–P–S) located within the C-terminal end of the largest subunit of RNAPII. Post-translational modifications of the RNAPII-CTD regulate RNAPII activity, the recruitment of transcription factors, and RNA processing.
DICER-LIKE (DCL): homologs of the human DICER protein. DCLs are RNase III endonucleases that process long double-stranded RNA (dsRNA) into sRNA.
Dicing body (D-body): subnuclear speckles that contain pri-miRNAs and several components of the miRNA biogenesis machinery.
HUA ENHANCER1 (HEN1): a methyltransferase that methylates the 3′ nucleotides of sRNA duplexes, protecting them from uridylation-triggered degradation.
MicroRNAs (miRNAs): DCL1-dependent ~21 nt sRNAs that guide the RISC complex to target genes, inducing their cleavage or translational inhibition.
Nuclear cap-binding complex (CBC): a protein complex formed by two subunits, CBP20 and CBP80, that binds to the mRNA 5′-cap. The CBC acts in many aspects of mRNA metabolism including mRNA splicing, export, and decapping protection.
Processing complex: defined as the set of proteins required for the processing of a pri-miRNA into a mature miRNA duplex.
RNA-induced silencing complex (RISC): protein complex responsible for triggering sRNA-mediated gene silencing. It contains an Argonaute protein, a loaded sRNA, and accessory proteins.
Small RNA: group of small (20–25 nt) single-stranded RNAs derived from dsRNA precursors that induce transcriptional (TGS) and post-transcriptional (PTGS) gene silencing.

Even though we can only speculate about the dynamics and sequence of the processing complex formation (Figure 1), it is clear now that it takes place, at least partially, during the transcription of pri-miRNAs.

Coupling Between miRNA Processing and Splicing

Increasing evidence indicates that the production of miRNA and the RNA splicing are linked processes. One of the first insights into such crosstalk was the identification of the dual roles of the CBC and SE in mRNA splicing and pri-miRNA processing [23,25,26]. The dual roles are probably due to in transcript stabilization, assistance with spliceosome assembly, and potential recruitment of pri-miRNA processing components. This observation suggests that both splicing and pri-miRNA processing may take place in a coordinated manner. We now know that both processes share common factors and that miRNA biogenesis may be affected by RNA splicing and vice versa. Proteins such as SE, CBP20, CBP80, SICKLE (SIC), STABILIZED1 (STA1), the THO/TREX complex, GLYCINE-RICH RNA-BINDING PROTEIN 7 (GRP7), and REGULATOR OF CBF GENE EXPRESSION 3 (RCF3, also known as HOS5 and SHI1) have been shown to participate both in mRNA splicing and miRNA biogenesis [23,27–35]. STA1, a pre-mRNA processing factor 6 homolog, is required for miRNA production, probably by regulating DCL1 levels, and for proper pri-miRNA splicing [27]. Similarly, plants containing mutations in the *SIC* gene exhibit reduced levels of miRNAs and abnormal mRNA splicing. Interestingly, *SIC* mutants accumulate non-degraded spliced introns rather than the unspliced pre-mRNAs. The authors proposed that SIC binds to the spliced intron lariats and pri-miRNAs loops to regulate their degradation and processing [35]. RCF3 represents another example of shared function in pre-mRNA splicing and pri-miRNA processing. It interacts with two serine/arginine-rich splicing factors, RS40 and RS41, and modulates intron splicing, preferentially during stress [28]. RCF3 is recruited into subnuclear speckles (Box 1) probably by the interaction with CPL1 [28]. In these nuclear bodies RCF3 binds to pri-miRNAs and colocalizes with HYL1 and SE, affecting the biogenesis of a subset of miRNAs [29,34]. The protein GRP7, known to alter mRNA alternative splicing, was found to interact with a subset of pri-miRNAs and repress their processing [32,33]. Both THO2 and EMU, members THO/TREX complex, regulate miRNA production and mRNA splicing. This dual role is probably the consequence of their complex functions in RNA transport and stability [30,31].

In agreement with the idea of coupling between these processes, it has been shown that pri-miRNA introns located at the 3′, but not at the 5′, of the stem-loop positively affect the abundance of the derived miRNAs [36,37]. This phenomenon depends on the 5′ splice site rather than on a genuine splicing event, suggesting a communication between components of both machineries. Interestingly, Schwab and colleagues [37] showed that this regulatory mechanism is bidirectional. In this sense, not only does the presence of a 3′ intron in the pri-miRNA regulate miRNA biogenesis but also deficient miRNA processing affects adjacent intron splicing. miR400 provides another example of splicing-dependent miRNA production, where heat-induced alternative splicing provokes the retention of the miR400 precursor in the host gene transcript, thereby reducing its processing efficiency [38].

The evident bidirectional regulation between the miRNA processing and splicing machineries implies that mutation of any miRNA-processing factor will probably affect the splicing of adjacent introns, and vice versa for splicing factors. However, it remains unclear how the miRNA processing machinery affects the splicing in *trans* of introns not located in miRNA genes.

Hierarchical Actions in the Pathway

Several cases of hierarchical action and even redundancy have been described for components of the miRNA pathway. CPLs, KH-containing proteins, AGOs, DCLs, and double-stranded (ds) RNA-binding proteins (DRBs) are some of the most prominent cases.

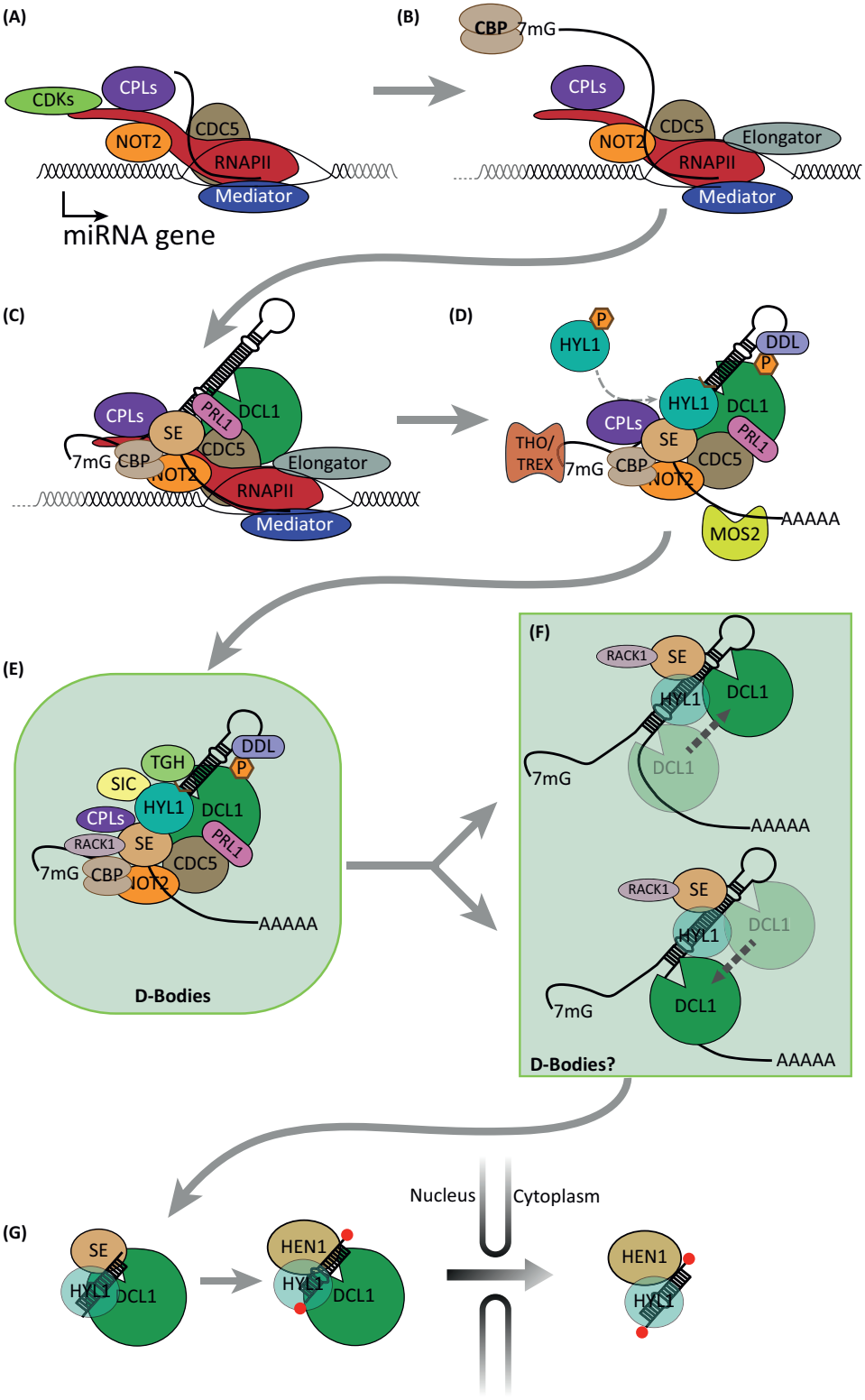

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Figure 1. Cotranscriptional Assembly of the miRNA Processing Complex. The figure represents the tentative sequence of recruitment and assembly of the miRNA processing machinery. (A) At the early stage of miRNA gene

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Box 1. Where Are Pri-miRNAs Processed?

Even if the miRNA biogenesis machinery is recruited early during transcription, it does not imply that pri-miRNA processing takes place co-transcriptionally. The pri-miRNAs maturation was suggested to occur in **dicing bodies** (D-bodies) [53,75]. This hypothesis is consistent with the localization of most miRNA processing components, such as DCL1, CBC, SE, HYL1, PRL1, SIC, RECEPTOR OF ACTIVATED C KINASE 1 (RACK1), TGH, CDC5, CDF2, and CPL, in nuclear speckles [8,9,11,19,21,25,35,53,64,76,77]. At least two proteins, MODIFIER OF SNC1 2 (MOS2) and the THO/TREX complex, seem to be involved in the transport/assembly of the components of the D-bodies. Both MOS2 and the THO/TREX core protein THO2 are involved in miRNA production [30,78]. In contrast to most miRNA biogenesis cofactors, none of these proteins interacts with the miRNA processing machinery nor colocalizes within D-bodies. Instead, MOS2 and THO2 bind to pri-miRNAs, facilitating their recruitment by HYL1. Notably, the formation of D-bodies depends on MOS2-mediated HYL1 recruitment of pri-miRNA, a phenomenon not tested for THO2. NOT2, a potential scaffold for DCL1 recruitment during transcription, regulates the number of DCL1-containing bodies [7]. This observation suggests that the assembly of the bodies could also take place co-transcriptionally. This idea is supported by the fact that plants lacking components of the Elongator complex show a drastic reduction in the number of DCL1-containing D-bodies [17].

However, it is also possible that nascent pri-miRNA transcripts are processed before they are released in the nucleoplasm, leaving the D-bodies as a potential pri-miRNA–HYL1 reservoir. However, there is insufficient evidence that miRNA processing takes place exclusively/predominantly in these structures. All studies on pri-miRNA processing have been conducted in nuclear extracts and thus do not address the location of processing. *In vitro* assays suggest that D-bodies are not essential for pri-miRNA processing [46,79].

The interaction between AGO1 and HYL1 in D-bodies [53] suggests that these speckles could act in some other aspect of the miRNA pathway, such as miRNA sorting, AGO1 reprogramming, or as a miRNA reservoir. In any case further investigation will be necessary to precisely understand the dynamics of the miRNA biogenesis, the sequence of assembly of the complex, and the precise moment and place where the pri-miRNAs are processed.

Dicer-Like Proteins

Arabidopsis thaliana encodes four Dicer-like proteins. DCL2, DCL3, and DCL4 process long dsRNAs into 22, 24, and 21 nt small RNAs, respectively, whereas DCL1 participates in the production of 21 nt miRNAs. It is well documented that DCL2, DCL3, and DCL4 can hierarchically process the same dsRNAs with different outcomes (reviewed in [39]). Even though the importance of DCL1 in the production of miRNAs is clear, reflected in the lethality of the *dcl1* null mutants and the strong reduction of miRNA production in hypomorphic alleles, other DCL proteins are able to process some pri-miRNAs circumstantially. The best example is the partial competition between DCL1 and DCL3 to produce 21 or 24 nt miRNAs from the same precursors in tissues where DCL3 is particularly abundant [40]. DCL3-dependent long miRNAs associate with AGO4 and trigger DNA methylation, effects that are distinct from those of DCL1-dependent 21 nt miRNAs produced from the same precursor [41]. The long and high-complementarity precursors of miR822, miR839, and miR869 are mainly processed by DCL4 via a DCL1-independent pathway [42–44]. It remains to be elucidated if such pri-miRNAs can be also processed by DCL1, DCL2, or DCL3 in some circumstances. It has been shown that the activity of the DCL proteins is regulated by ion concentrations, which at the cellular level are controlled by

transcription NOT2, CDC5, and Mediator are required for RNA polymerase II (RNAPII) recruitment to these loci. Following recruitment, changes in phosphorylation of the RNAPII C-terminal domain (CTD), mediated by CDKs and CPLs, regulate transcription initiation. (B) The Elongator complex interacts with RNAPII while the 5′-CAP of nascent pri-miRNA is bound by the nuclear cap-binding complex (CBC). (C) Elongator acts as a scaffold that recruits DCL1 to the nascent pri-miRNA with the assistance of PRL1. The interactions of SE with the CBC, CPLs, NOT2, DCL1, and CDC5 make it hard to predict when and through which interaction SE is recruited. (D) The transcribed pri-miRNA is stabilized by DDL upon dual interaction with a putative phosphorylated domain of DCL1 and the transcript. The dephosphorylation of HYL1, by CPLs, induces its activity and recruitment to the complex. MOS2 and the THO/TREX complex then probably transport the pri-miRNA to the D-bodies. (E) Several cofactors, such as RACK1, SIC, and TGH, are incorporated into the processing complex. (F) DCL1 starts processing the pri-miRNA from the base or loop depending on each miRNA structure and length. Panel (F) shows a simplified version of the processing complex (E) focusing on how DCL1 processes the pri-miRNAs. (G) Once the mature miRNA duplex is produced, HEN1 interacts with DCL1 and HYL1 to displace SE from the complex. The 3′ termini of both miRNA strands in the duplex are then 2′-*O*-methylated by HEN1 and exported to the cytoplasm. Both Figures 1 and 2 depict interactions that have been experimentally tested and some that are only presumed to exist. A detailed list of confirmed interactions can be found in recent reviews [4,39].

environmental conditions [45,46]. This observation suggests that in specific conditions/tissues the balance and hierarchy between DCL proteins may change.

HYL1 and DRB Proteins

There are five members of the DRB family of proteins in *A. thaliana* and at least eight in rice [47,48]. While HYL1, also known as DRB1, is a well-known miRNA biogenesis cofactor, the other DRB proteins were believed not to be involved in miRNA biogenesis [49]. However, an analysis of the miRNA accumulation in DRB mutants, focusing only in tissues where these proteins are expressed, revealed that DRB2 acts, in such tissues, antagonistically and synergistically to HYL1 for a specific subset of miRNAs [50]. DRB2 was also shown to repress HYL1 expression and to induce the miRNA-mediated translation inhibition of targeted genes [51]. Despite not affecting the production of miRNAs, DRB3 and DRB5 cooperate with DRB2 to silence genes [52]. DRB3 and DRB5 may also modulate DRB2-mediated inhibition of translation, noting that both proteins localize in the cytoplasm where DRB2-mediated translation inhibition is expected to take place. In addition to its nuclear functions, HYL1 is also located in the cytoplasm where no functions have yet been attributed [19,53]. It is not excluded that cytoplasmic HYL1 might be a further target for DRB3 and DRB5 regulation.

DRB4 was shown to assist DCL4 in the production of tasiRNAs, miRNAs, and RNA polymerase IV-dependent siRNAs [54,55]. Interestingly, DRB2 is antagonistic to DRB4 in the production of siRNAs but is required for the production of DRB4/DCL4-dependent miRNAs [55].

Specificity of the Pathway

It has been shown that the miRNA precursors can be processed in at least five different ways depending on their structure. Short precursors are processed by DCL1 via a single cleavage, either from the base to the loop or from the loop to the base. Long precursors are processed from the base to the loop, from the loop to the base, or bidirectionally by subsequent DCL1-mediated cleavages [56–59]. DCL1 exhibits a remarkable flexibility in the recognition and processing of plant miRNA precursors. This flexibility may be a characteristic feature of DCL1 given that it possesses a unique, intrinsically disordered, dsRNA-binding domain that folds upon binding, conferring on the protein adaptability to different substrates [60]. Alternatively, it might be the result of a distinctive combination of cofactors acting on each type of miRNA precursor. DCL1 is known to rely on the assistance of cofactors, such as SE and HYL1, to precisely process pri-miRNAs [19,46,61]. Deep sequencing of small RNAs in plants lacking miRNA biogenesis proteins have revealed that most, if not all, only regulate the production of specific miRNA subsets [16,19,21,30,35,50,62–64]. It is likely that a combination of features in the pri-miRNA, such as different structures, sizes, and sequences, result in the specific requirement for a given group of cofactors. It is also possible that the apparent specificity of some cofactors for a set of miRNAs is the consequence of overlapping expression of a given protein and a subset of miRNA genes in particular tissues. This hypothesis is supported by the existence of several tissue-specific factors. RCF3/HOS5 has been shown to affect the biogenesis of miRNAs only in particular tissues [29,34]. Considering that both RCF3 and DRB2 regulate HYL1 in overlapping niches, it will be interesting to investigate if crosstalk between these factors takes place. Another example of tissue specificity is the competition between AGO10 and AGO1 for loading of miR165/166. Sequestration of these miRNAs impairs AGO1-mediated regulation of HD-ZIP III transcription factors that promote shoot apical meristem maintenance [65,66]. In addition to revealing a new layer of regulation by AGO-mediated miRNA sequestration, these reports point to the importance of narrow, and tissue-specific, regulation of the pathway in plant development. The rice AGO18 protein works in a similar way. Upon virus infection, AGO18 sequesters miR168, leading to a derepression of AGO1, a natural target of this miRNA, and a burst in antiviral small RNA-mediated defense [67]. This is an outstanding example of a regulatory mechanism that has evolved to respond to a very specific stimulus. It remains to

be addressed if AGO18-mediated regulation of AGO1 also takes place during plant development, perhaps in a specific cell niche, as is the case for AGO10 in *A. thaliana*.

Other cases of tissue specificity have been reported. The biogenesis of miR168 was thought to be largely unaffected by the absence of HYL1. However, it has been recently shown that HYL1 is necessary to produce this miRNA, but only in the SAM, where the genetic interaction with DRB2 is probably crucial [49,50]. miR171 strand selection and gene silencing were shown to be regulated tissue-specifically by unknown determinants [68]. SIC represents another case of a miRNA biogenesis cofactor that regulates, tissue-specifically, the production of a subset of miRNAs [35]. Analysis of small RNA sequencing and miRNA gene expression profiles allowed the identification of dozens of organ-specific miRNAs [69]. It was suggested that tissue-specific or stress-induced alternative splicing, and variations of polyA-site usage in pri-miRNAs, could influence miRNA accumulation [37,38]. In the future it will be necessary to study the miRNA biogenesis pathway using cell-specific approaches to achieve a finer understanding of the pathway. In addition, there are emerging overlaps between miRNA processing and the **RNA-induced silencing complex** (RISC) that warrant further investigation.

Post-Translational Regulation

Considering the important role of miRNAs in gene regulation, it is not surprising that the biogenesis and function of these small RNAs are highly regulated. Post-translational modification of proteins is a common regulatory mechanism in most cellular processes. Protein phosphorylation is one of the central reversible post-translational modifications that regulate cellular metabolism, protein–protein interactions, enzyme activities, and protein degradation [70]. In animals, post-translational modifications of miRNA biogenesis cofactors are essential for proper functioning of the pathway [71]. The plant miRNA pathway is also subject to this type of regulation. The phosphatases CPL1 and CPL2 dephosphorylate HYL1 to enhance its activity, and thus modulate miRNA production and strand selection [19]. CPL1 also dephosphorylates RCF3, which is in turn involved in miRNA biogenesis and splicing [29]. In addition to affecting their activity, CPL1-mediated dephosphorylation of HYL1 and RCF3 alters their nuclear localization, suggesting that **D-body** formation may also be subject to post-translational regulation [19,29,34]. Interestingly, HYL1 dephosphorylation by CPL1 seems to be RCF3-dependent only in a subset of tissues [34]. The evidence suggests that, at least in some cell types, HYL1, DCL1, SE, CPL1, CPL2, and RCF3 are part of the same protein complex [19,29,34]. Thus, a role for CPL1 and RCF3 in the phosphorylation of SE and DCL1 is plausible but has not yet been explored. Counteracting CPL1 activity, the MITOGEN ACTIVATED PROTEIN KINASE 3 (MPK3) was reported to mediate the phosphorylation of HYL1 and to impair miRNA biogenesis [48]. However, MPK3-mediated HYL1 phosphorylation was only demonstrated *in vitro*, and it remains to be investigated whether this kinase is indeed sufficient and necessary to fully catalyze HYL1 phosphorylation *in vivo*. In addition to phospho-regulation, HYL1 is also post-translationally regulated at the protein level. During daytime, the E3 ubiquitin ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) translocates from the nucleus to cytoplasm to suppress an unknown protease and thus prevents HYL1 degradation [72].

The RNA-binding protein DAWDLE (DDL) recognizes pri-miRNAs and facilitates, upon direct protein–protein interaction, DCL1 association with pri-miRNAs [63]. DDL contains a forkhead-associated (FHA) domain, which is known to mediate protein–protein interactions by targeting motifs that contain phospho-threonine [73]. Machida and Yuan demonstrated that the DDL FHA domain interacts, through its phospho-threonine binding cleft, with a region of the DCL1 helicase domain that contains potential phospho-threonine motifs [74]. This finding suggests that DCL1 may need to be phosphorylated to interact with DDL and thus efficiently process pri-miRNAs. Post-translational regulation of the miRNA biogenesis machinery is depicted in Figure 2.

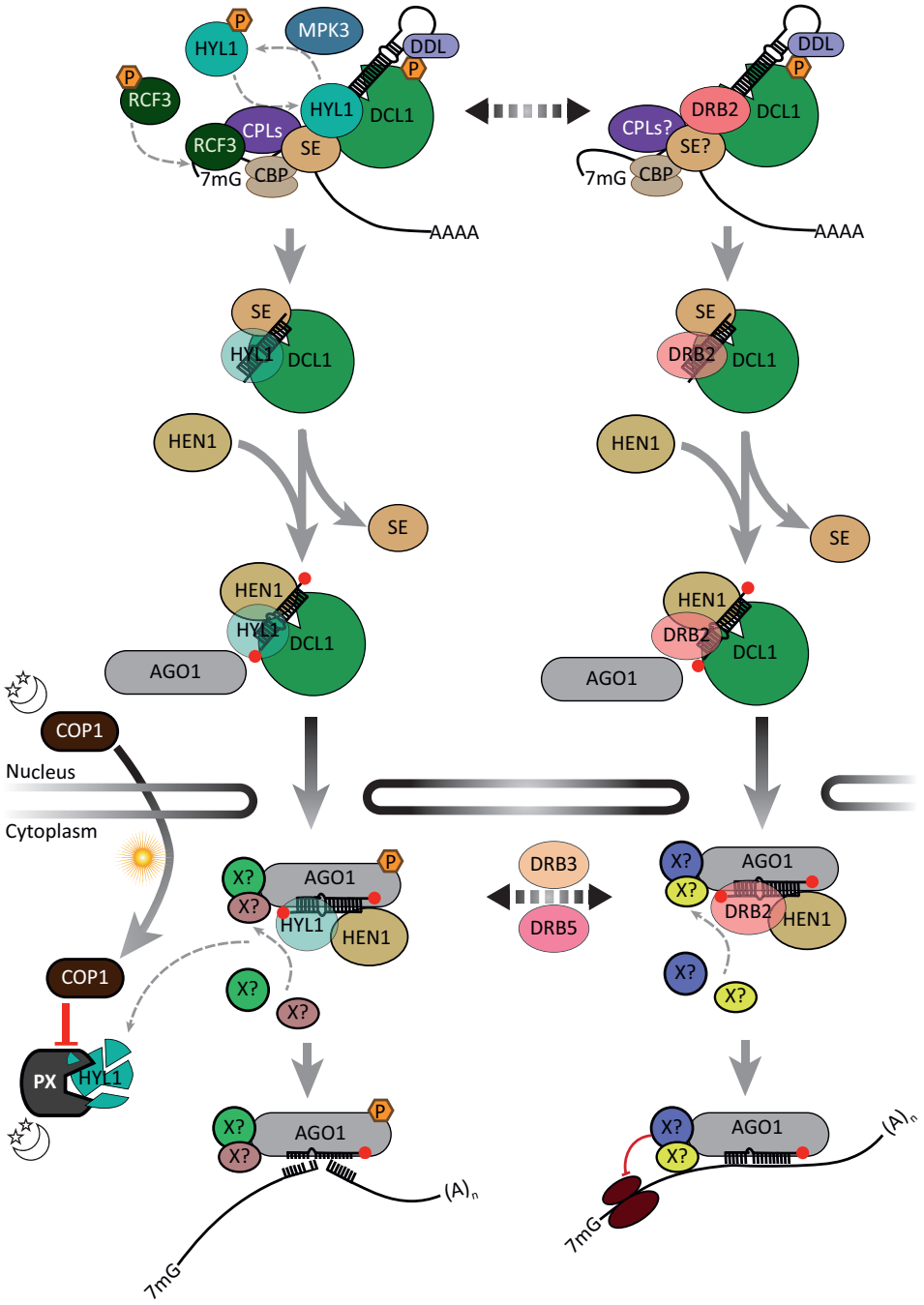

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Figure 2. Post-Translational Regulation of the miRNA Biogenesis Machinery. Phosphorylation plays an important role in the production of mature miRNAs. The putative phosphorylation of DCL1 helicase domain allows its interaction with DDL and the stabilization of the pri-miRNA. CPLs dephosphorylate HYL1 and RCF3, allowing precise processing by DCL1 and ultimately correct strand selection by AGO1. Competition between HYL1 and DRB2 seems to take place in tissues where both proteins are expressed. The figure shows a simplified version of the processing complex (Figure 1E) focusing on the post-translational regulation of the machinery. After the miRNA duplex is produced, HEN1 displaces SE from the complex and methylates both miRNA strands. Crosstalk between HYL1/DRB2 and AGO1 takes place either in the cytoplasm or the nucleus. Such interactions reprogram AGO1 to a specific silencing pathway. Such an effect is probably a consequence of HYL1/DRB2 triggered post-translational modifications or switching between AGO1-interacting cofactors (X). HYL1 dictates AGO1 strand selection and triggers AGO1-mediated target cleavage. DRB2-dependant miRNAs block

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Links Between miRNA Processing and the RISC

In addition to reports demonstrating crosstalk between the transcriptional, splicing, and miRNA biogenesis machineries, it is also becoming evident that there is communication between miRNA biogenesis and the **RNA-induced silencing complex**. The nuclear interaction between HYL1 and AGO1 was probably the first clue of such a connection [53]. Among its functions HYL1 influences the AGO1 miRNA/miRNA* strand selection, a process that takes place once the miRNA duplex is loaded into the AGO complex [19,80]. This implies crosstalk between HYL1 and AGO1, probably in the D-bodies before miRNA loading. However, HYL1 is also diffusely located in the nucleoplasm and cytoplasm [19,53]. No functions have been attributed to HYL1 in such locations where it could interact directly or indirectly with AGO1 and affect its activity.

Similar to HYL1, its homolog DRB2, which also localizes in the nucleus and cytoplasm, seems to influence RISC fate by inducing miRNA-target translation inhibition [19,53]. Another case of early RISC reprogramming, possibly triggered by HYL1, was suggested to take place before AGO1 miRNA/miRNA* strand selection [81]. The size and structure of the miRNA/miRNA* duplex affect the fate of the AGO1-cleaved mRNAs even when the miRNA* strand is no longer present in the RISC [81–83]. RACK1 was found to interact with SE to direct the processing of some pri-miRNAs, but it is also part of the AGO1 complex both in the cytoplasm and the nucleus [64].

A dynamic flux of the pathway, rather than the traditional stepwise view, is also evident for miRNA methylation by **HUA ENHANCER1** (HEN1). This protein, which localizes in nucleus and cytoplasm [53], interacts with DCL1 and HYL1 [84]. This suggests that there is a mechanistic link between progression from miRNA production to HEN1-mediated miRNA methylation. It was shown that HEN1 and SE interact with the same region of DCL1, implying that SE must dissociate from the complex to allow HEN1 binding. Evidence suggesting that HYL1, as a dimer, binds to the miRNA/miRNA* region of the pri-miRNA suggests that this protein could be a potential platform to anchor and stabilize the mature miRNAs once produced [61,85].

Current understanding points to a very dynamic and fluid pathway. However, the sequence of recruitment, assembly, action, and disassembly of the components of the processing and RISC complexes is still unknown and may only be conjectured (Figure 2).

Concluding Remarks

For years the miRNA biogenesis pathway has been viewed as linear process. This view was influenced by the animal miRNA pathway in which the different processing stages require dedicated enzymes and cellular compartmentalization. Separation between the different stages in the plant miRNA pathway is less evident, resulting in tight interconnection and co-regulation. Accumulating evidence indicates that each pri-miRNA is differently processed in a progressive and bidirectional manner [58,59]. This implies that the progression from pri-miRNA to mature miRNA should be considered as a continuous multistep process without defined stages.

Despite significant progress in recent years, many aspects of the pathway remain to be addressed (see Outstanding Questions). Further effort will be necessary to study the dynamics of recruitment, assembly, action, and disassembly of the components of the miRNA machinery. An avalanche of new miRNA biogenesis cofactors have been described in recent years. However, it is not always clear, based on the data presented, whether these cofactors are directly involved in miRNA biogenesis. In some cases a relatively weak reduction in the steady-state levels of miRNAs or indirect effects observed upon mutation of a particular gene are the

Outstanding Questions

What is the precise order of recruitment of cofactors during miRNA processing complex assembly?

When and where are the miRNAs processed? Are D-bodies processing centers or do they have a different function?

How does the miRNA biogenesis machinery affect the splicing of non-miRNA genes?

How are miRNA genes differentiated from other RNAPII-transcribed genes during the recruitment of DCL1?

What is the role of HYL1 outside the D-bodies and in the cytoplasm? In addition, what is the role of AGO1 in the nucleus?

Are DCL1, SE, and AGO1 post-translationally regulated?

Is there any role for the phosphorylated version of HYL1?

Even when we know the substrate preference for all DCL proteins it is not clear how each DCL is specifically recruited to their respective substrates. In the case of DCL1 we wonder what makes pri-miRNA special. Although imperfect RNA foldback structures are not uncommon secondary features of mRNA molecules, DCL1 is only recruited to miRNA transcripts.

How do the miRNA cofactors act mechanistically in the miRNA pathway? For most miRNA cofactors we know their protein partners, cellular localization, RNA-binding capacity, and effects on the steady-state levels of miRNA, but the precise molecular mechanisms underlying their roles in the miRNA pathway remain unknown.

the translation of AGO1-targeted mRNAs. Both paths are potential targets of DRB3 and DRB5 cytoplasmic regulation. HYL1 is actively degraded in the cytoplasm by an unknown protease (PX) during the night. This regulatory process is blocked by COP1 upon nucleus–cytoplasm translocation during the day.

only evidence supporting its role in miRNA biogenesis. The possibility that such cofactors act indirectly in miRNA biogenesis or even at a different stage of the pathway cannot be excluded. Future research will need to elucidate the precise mechanism of action of the cofactors already identified to better understand the regulation of the pathway.

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