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REVIEW

Recent advances in the regulation of plant miRNA biogenesis

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ABSTRACT

MicroRNAs (miRNAs) are essential non-coding riboregulators of gene expression in plants and animals. In plants, miRNAs guide their effector protein named ARGONAUTE (AGO) to find target RNAs for gene silencing through target RNA cleavage or translational inhibition. miRNAs are derived from primary miRNA transcripts (pri-miRNAs), most of which are transcribed by the DNA-dependent RNA polymerase II. In plants, an RNase III enzyme DICER-LIKE1-containing complex processes pri-miRNAs in the nucleus into miRNAs. To ensure proper function of miRNAs, plants use multiple mechanisms to control miRNA accumulation. On one hand, pri-miRNA levels are controlled through transcription and stability. On the other hand, the activities of the DCL1 complex are regulated by many protein factors at transcriptional, post-transcriptional and post-translational levels. Notably, recent studies reveal that pri-miRNA structure/sequence features and modifications also play important roles in miRNA biogenesis. In this review, we summarize recent progresses on the mechanisms regulating miRNA biogenesis.

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1. Introduction

Small non-coding RNAs (sRNAs) are riboregulators of gene expression and play crucial roles in various biological processes [1–5]. In eukaryotes, small RNAs can be classified into four groups based on their origins: microRNA (miRNA), small interfering RNA(siRNA), PIWI-interacting RNA (piRNA) and transfer RNA-derived small RNA (tsRNA) [1,2,6–8]. These small RNAs can repress gene expression at the transcriptional (TGS) and/or post-transcriptional (PTGS) levels [3,9]. miRNAs, ~20-24 nucleotides (nt) in size, which are encoded by plants, animals and some viruses, are distinguished from other small RNAs by their highly precise excision from imperfect stem-loop residing in the primary miRNA transcripts (pri-miRNAs) [10]. In plants, miRNAs mainly repress gene expression through mediating target RNA cleavage or translational inhibition [1,2].

Most plant miRNAs are encoded by genes (*MIRs*) residing in the intergenic regions, while some miRNAs are derived from introns of protein-coding genes and other non-coding RNAs [1,11,12]. The majority of plant *MIRs* are transcribed by the DNA-dependent RNA polymerase II (Pol II) to generate pri-miRNAs [1,2]. The imperfect stem-loop structure of pri-miRNAs is recognized and processed by the Dicer-like RNase III endonuclease 1 (DCL1) into short precursors (pre-miRNAs) with the help of the dsRNA-binding protein HYPONASTIC LEAVES 1 (HYL1) and the zinc finger protein SERRATE (SE). Pre-miRNAs are further processed by DCL1 to generate imperfect miRNA/miRNA* duplexes [2,13], which consist of a duplex region and 2-nt 3′ overhangs at each end, in the nucleus [13]. After processing, each strand of the duplex is 2′-*O*-methalated at the 3′ end by the small RNA methyltransferase HUA ENHANCER 1 (HEN1), which protects miRNAs from degradation [14]. Then, one strand (miRNA) of the miRNA/miRNA* duplex is selected as the guide strand and loaded into Argonaute-1 (AGO1) to assemble miRNA-induced silencing complex (miRISC), while the passenger strand (miRNA*) is eliminated [13]. It should be noted that some miRNAs are loaded into AGO1 homologs instead of AGO1 in plants [15–17]. Recent studies suggest that miRISC assembles in the nucleus and is exported to the cytosol in a CRM1(EXPO1)/NES-dependent manner [18,19]. However, another study shows that some unloaded miRNAs are present in the cytoplasm, indicating the miRISC assembly may also occur in the cytoplasm [20].

To date, hundreds of miRNAs have been identified in plants. These miRNAs modulate development and physiology of plants such as the development of seed, root, shoot and flower, phase transition, and responses to biotic and abiotic stresses [1,3,21,22]. Misregulation of miRNA accumulation has been shown to cause developmental defects or diseases, suggesting that miRNA biogenesis is a precisely controlled process. Indeed, studies have found that miRNA biogenesis is modulated at both transcriptional and post-transcriptional levels. In this review, we seek to summarize recent progresses in the regulation of miRNA biogenesis.

2. Regulation of pri-miRNA accumulation

2.1 *Pri-miRNA transcription*

Like mRNAs, the Pol II-dependent pri-miRNAs undergo capping, splicing and polyadenylation [23–25]. Their transcription and processing are regulated by general and specific

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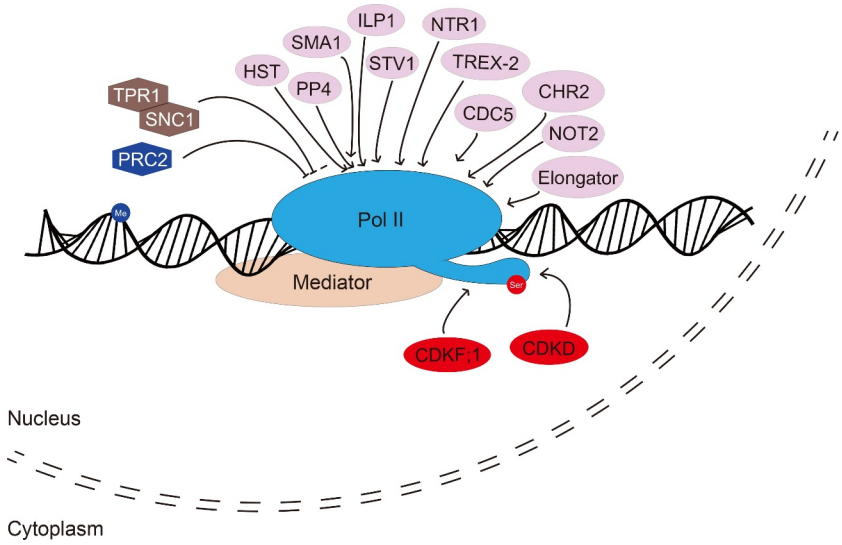

Figure 1. Regulation of *MIR* transcription.

Multiple transcription factors are required for the regulation of RNA polymerase II (Pol II)-mediated *MIR* transcription. The activity of Pol II is also regulated by phosphorylation at the C-terminal domain (CTD) of its largest subunit. Positive and negative regulators are marked with ellipse and hexagon, respectively. Regulators involved in phosphorylation of CTD and histone methylation of *MIR* promoters are marked with red colour and blue colour, respectively. Me: methylated DNA; ser: serine amino acid within the CTD.

transcription factors (Fig. 1) [25,26]. In addition, the transcription of some pri-miRNAs is spatiotemporally modulated to ensure proper function of corresponding miRNAs.

The Mediator complex is a general transcriptional coactivator facilitating the recruitment of RNA Pol II to *MIR* promoters (Fig. 1) [27]. Many additional proteins have been shown to stimulate *MIR* transcription. These factors include ELP2 and ELP5 of the Elongator complex [28], the transcription factor NEGATIVE ON TATA LESS 2 (NOT2) [29], the DNA-binding protein CELL DIVISION CYCLE 5 (CDC5) [30], the ribosomal protein SHORT VALVE 1 (STV1) [31], PROTEIN PHOSPHATASE 4 (PP4) [32], the splicing factor SMALL1(SMA1) [33], the ATPase CHROMATIN REMODELLING FACTOR 2 (CHR2) [34], the exportin 5 homolog HASTY (HST) [35], the pore-associated proteins THO/HRP1 PHENOTYPE 1 (THP1) and SUPPRESSOR OF ACTIN 3A (SAC3A) of the TREX-2 complex (Fig. 1) [19]. In the loss-of-function mutants of these proteins, the levels of pri-miRNAs, *MIR* promoter activities and/or the Pol II occupancy at *MIR* promoters are reduced, suggesting that these proteins modulate *MIR* transcription by directly or indirectly promoting the recruitment of Pol II to *MIR* promoters [19,-28–35]. In addition, some proteins negatively regulate miRNA transcription (Fig. 1). Disruption of the F-Box protein CONSTITUTIVE EXPRESSER OF PR GENE 1 (CPR1) increases the transcript levels of the disease resistance protein *SUPPRESSOR OF npr1-1, CONSTITUTIVE 1* (*SNC1*), resulting in reduced pri-miRNA transcription. Moreover, overexpression of *SNC1* and the transcriptional corepressor *TOPLESS RELATED 1* (*TPR1*) represses the transcription of *MIRs* (Fig. 1) [36]. These results suggest that SNC1 is a repressor of *MIR* transcription. However, some protein factors can act as both a transcriptional activator and repressor. For instance, mutations in the *CYCLING DOF TRANSCRIPTION FACTORS 2* (*CDF2*) increases the transcription of some *MIRs*, while represses that of others [37].

Besides affecting *MIR* promoter activity, some protein factors, such as INCREASED LEVEL OF POLYPLOIDY1-1D (ILP1) and NTC-RELATED PROTEIN 1 (NTR1), facilitate transcriptional elongation of *MIR* genes (Fig. 1) [38]. Notably, the RNA silencing effector protein AGO1 can negatively affect the transcription of *MIR161* and *MIR173* via disassembling Pol II under salt stress conditions [39]. Moreover, phosphorylation of the C-terminal domain (CTD) of the largest subunit of Pol II is also required for efficient *MIR* transcription (Fig. 1). Disruption of CYCLIN-DEPENDENT KINASE F;1 (CDKF;1) and CDKD kinases, which catalyse Ser phosphorylation of CTD, impairs *MIR* transcription, 5′-capping, 3′-processing and splicing [40]. Chromatin features such as histone acetylation and methylation can also influence *MIR* transcription. For example, Polycomb Repressive Complex 2 (PRC2) represses *MIR156A/C* transcription through increasing H3K27me3 deposition at their promoters [41].

Some specific transcription factors regulate the transcription of individual *MIR* genes in a spatiotemporal manner. For instance, flower development can be coordinately regulated by several protein factors that affect the transcription of *MIR172*. The SANT-domain-containing protein *POWERDRESS (PWR)* can facilitate Pol II occupancy at the promoters of *MIR172a, b* and *c* but not *MIR172d* or *e* [42]. The transcription factor APETALA 2 (AP2)-dependent recruitment of the transcription co-repressors LEUNIG (LUG) and SEUSS (SEU) represses *MIR172* expression [43]. During shade avoidance, PHYTOCHROMOE INTERACTING FACTORS (PIFs) bind five *MIR156* promoters and repress their expression [44]. *MIR156* can also be positively regulated by the B3 domain transcription factor FUSCA3 (FUS3) during the transition from embryo to seedling development [45]. Interestingly,

another B3 transcription factor ABSCISIC ACID INSENSETIVE3 (ABI3) promotes the expression of *MIR156* during the early stages of seed development while repressing their expression during late development [46]. In addition, ABI3 negatively regulates the transcription of *MIR160B* [46]. During cell wall remodelling, the transcription factor for photomorphogenesis HYPOCOTYL5 (HY5) negatively regulates *MIR775A* in aerial organs whereas positively in roots of *Arabidopsis* [47,48].

2.2 Pri-miRNA stability

Eukaryotes possess nuclear RNA surveillance machineries that cooperate with RNA processing to control maturation and degradation of RNAs [49,50]. Previous studies demonstrated that the 5′-to-3′ exoribonucleases (XRNs) and the 3′-to-5′ exoribonuclease complex exosome are responsible for the elimination of pri-miRNA processing intermediates [51–53]. Notably, most pri-miRNAs are non-coding RNAs that contain premature stop codon, and are potential targets of the nuclear RNA machineries. Thus, protection of pri-miRNAs from degradation during co-transcriptional processing is likely crucial for maintaining the amounts of pri-miRNAs required for miRNA biogenesis. Indeed, accelerated degradation of pri-miRNAs has been shown to reduce miRNA accumulation [54–59].

DAWDLE (a forkhead-associated domain-containing protein, DDL) has been shown to stabilize pri-miRNAs in plants (Fig. 2) [54]. DDL is an RNA-binding protein and interacts with both DCL1 and pri-miRNAs. Lack of DDL reduces the accumulation of pr-miRNAs and miRNAs without affecting *MIR* promoter activity and transcription, suggesting that DDL may protect pri-miRNAs from degradation. Besides DDL, several components of the MOS4-associated complex (MAC), including PRL1 (PLEIOTROPIC REGULATORY LOCUS1, a conserved WD-40 protein), MAC3 (a U-box type E3 ubiquitin ligase), also play a role in stabilizing pri-miRNAs (Fig. 2). MAC is a conserved complex. Its homolog complexes include the CDC5-SNEV^{PRP} [19]-^{PSO} [4] complex of human and the Nineteen complex (NTC) of yeast are associated with the spliceosome and participate in splicing [60,61]. Like DDL, these MAC proteins associate with the DCL1 complex and are required for the accumulation of pri-miRNAs but not for *MIR* transcription [55,56]. However, how these proteins stabilize pri-miRNAs are still poorly understood.

Recently, several enzymes that degrades pri-miRNAs in the nucleus have been identified. Over accumulation of 3′-phosphadenosine 5′-phosphate (PAP), an inhibitor of the XRNs, increases pri-miRNA levels and stability [57]. Moreover, the introduction of loss-of-function *xrn2* or *xrn3* mutation into *mac5a*, in which lack of MAC5A, another component of MAC, reduces pri-miRNA stability, increases pri-miRNA half-lives, and partially recovers the accumulation of pri-miRNAs [58]. These results show that XRN2/XRN3 catalyzes the degradation of pri-miRNAs in the nucleus (Fig. 2). In addition, the levels of some pri-miRNAs are increased in the *hen2* mutant, which lacks HUA1 EHANCHER2 (HEN2), a component of the nuclear exosome targeting complex (NEXT) that promotes RNA degradation by nuclear exosome, suggesting that exosome is also responsible for the degradation of pri-miRNAs (Fig. 2) [59,62].

Evidences also reveal that the interplay between protein involved in miRNA biogenesis and ribonucleases leads to pri-miRNA degradation and stabilization. SE, a key factor in the DCL1 complex, appears to have a role in recruiting ribonucleases to pri-miRNAs (Fig. 2), given the fact that SE interacts with both NEXT and XRN2 [58,59,62]. Supporting this notion, in *mac5*, the degradation of pri-miRNAs by XRN2 or XRN3 requires a functional SE [58]. This result also suggests that the function of MAC5 is to protect pri-miRNAs from SE-dependent 5′-to-3′ ribonucleases (Fig. 2). Interestingly, *hen2* and *sop1* (mutation in another component of NEXT) increase stability and accumulation of some pri-miRNAs in *hyl1*, resulting in elevated levels of some miRNAs

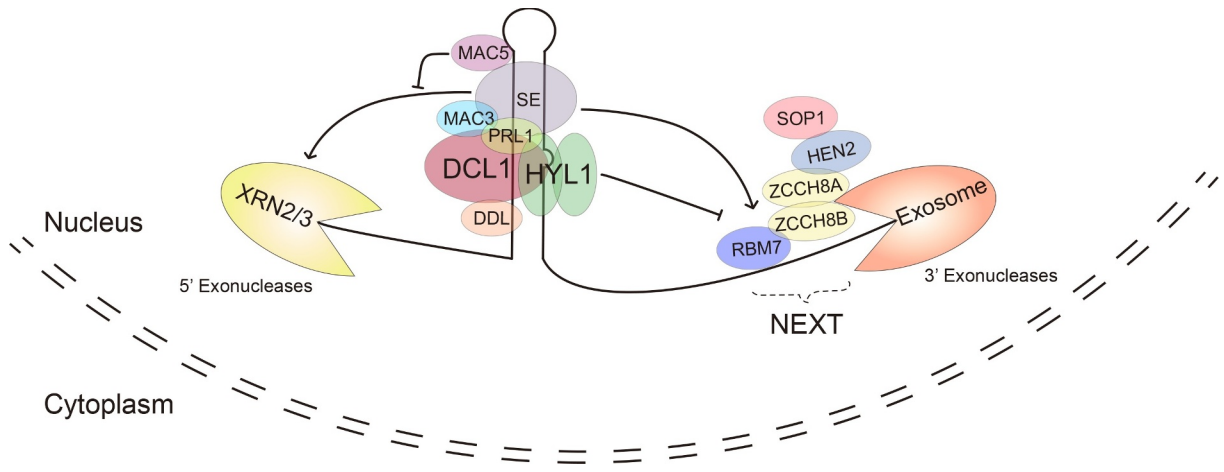

Figure 2. Regulation of pri-miRNA stability.

Multiple protein factors are required for the regulation of pri-miRNA stability. Unprocessed pri-miRNA can be degraded by 5′-3′ exoribonucleases (XRNs) and 3′-5′ exosomes in an SE-dependent manner. MAC5 and HYL1 protect pri-miRNAs from degradation by XRNs and exosomes, respectively. The functional mechanisms of DDL, PRL1 and MAC3 in the regulation of pri-miRNA stability are still unknown.

and partial recovery of developmental defects of *hyl1* [62]. In contrast, these two mutations do not have a similar impact on *dcl1* [62]. These results suggest that HYL1 is able to protect some pri-miRNAs from SE-dependent exosome activities (Fig. 2), in addition to its role in pri-miRNA processing [62]. Taken together, these recent findings suggest that plants may use SE to recruit enzymes to degrade pri-miRNA processing intermediates. However, at the same time, plants also employ other components of the DCL1 complex such as DDL, HYL1 and MAC to coordinately protect unprocessed pri-miRNAs from degradation by these ribonucleases.

3 Regulation of miRNA biogenesis by pri-miRNA structure features, splicing and modification

As the substrates of the DCL1 complex, pri-miRNAs themselves also affect the processing efficiency in various ways. It has been shown that pri-miRNA structure is an import factor for the cleavage site selection and processing efficiency of DCL1. Moreover, splicing and alternative splicing of pri-miRNAs also contribute to the processing efficiency of some pri-miRNAs. Interestingly, nucleotide modifications within the pri-miRNAs can also modulate the production of some miRNAs.

3.1 Sequence and structure features of pri-miRNAs

The stem-loop of pri-miRNAs varies in length, structure and positioning of the miRNA/miRNA* duplex. These features play crucial roles in determining processing efficiency and manner. Most pri-miRNAs have an imperfect lower stem of ~15 bp, which is often followed by a large bulge, below the miRNA/miRNA* duplex [63–65]. This structure is required for the accurate initial cleavage distal to loop (base-to-loop cleavage). In contrast, some pri-miRNAs such as pri-miR159a and pri-miR319a possess long upper stem, resulting in loop-to-base cleavage [66–68].

Interestingly, a few of pri-miRNAs contain multibranched terminal loops [69]. This structure heterogeneity results in bi-directional processing of pri-miRNAs, although the base-to-loop processing more efficiently produces miRNAs [69]. However, the efficient processing pri-miR157c depends on its branched terminal loop, suggesting that the DCL1 complex can recognize alternative structures of pri-miRNAs under some circumstances [69,70]. In addition, the presence of specific GC-rich sequence in the miRNA:miRNA* region has been observed and may be required for efficient and accurate processing of pri-miRNAs [71]. Moreover, certain nucleotide pairs at pri-miRNAs' cleavage site are preferred for efficient processing pri-miRNAs [72]. Interestingly, the identity of the nucleotides at mismatched positions of the stem is also important for efficient pri-miRNA processing [72]. Furthermore, some conserved sequence and structures of pri-miRNAs proximal to the miRNA/miRNA* duplex have been identified from various plant species and likely are important for miRNA biogenesis [73].

3.2 Splicing of pri-miRNAs

Like protein-coding genes, many *MIRs* contain introns [74]. The splicing of introns has important roles in the processing of some pri-miRNAs. Splicing of introns can change the structure of some pri-miRNAs, which in turn can affect processing. In rice, the presence of intron disrupts the formation of stem-loop structure of pri-miR842 and pri-miR846, and therefore, splicing of these introns is required for their processing [75]. In contrast, the stem-loop of pri-miR162a is composed of unspliced intron and exon [76]. As a result, splicing of intron inhibits the production of miR162 [76]. In addition, the introns downstream of stem loop from pri-miR161, pri-miR163 and pri-miR172b are required for efficient production of corresponding miRNAs [77,78], although the introns can be replaced by unrelated ones. Further study shows that the 5′ splicing site, but not the 3′ splicing site, of these introns, stimulates processing efficiency by affecting the selection of polyadenylation site [77–79].

Alternative splicing also plays a role in regulating miRNA biogenesis. In rice, *MIR528* contains two alternative splicing sites, resulting in two forms of pri-miR528 [80]. One has a long 3′ end and less efficiently processed, while the other one has a short 3′ end and efficiently processed [80]. The levels of two forms change during development, resulting in differential accumulation of miR528 [80]. In Arabidopsis, pri-miR400 localizes in the intron of a host gene and needs to be spliced out for efficient processing [81]. However, heat stress can cause retention of pri-miR400 containing intron in the host gene transcript, resulting in less accumulation of miR400 [81].

3.3 Pri miRNA modifications

RNA modifications have recently emerged as important post-transcriptional regulators [82]. Particularly, the *N* [6]-methyadenosine (m^6A) modification is crucial to regulate gene expression in mammals and plants [83,84]. A recent study links m^6A to miRNA biogenesis [85,86]. mRNA adenosine methylase (MTA) is responsible for the deposition of m^6A into RNA molecules [87]. MTA is able to interact with Pol II and Tough (TGH), a known miRNA processing regulator [85,86]. In the *mta* mutant, the abundances of m^6A and miRNAs are significantly reduced. Further evidences show that a subset of pri-miRNAs is bound and methylated by MTA, which in turn ultimately promotes pri-miRNA processing [85]. Low level of m^6A may affect stem-loop region of pri-miRNAs, and thus reduce the association of pri-miRNAs with HYL1 [85]. Intriguingly, untemplated cytidine or uridine addition on the trimmed pre-miRNAs can restore the intact length pre-miRNAs, and therefore, promote their processing [88].

4. Regulation of the DCL1 complex

The levels and activities of the DCL1 complex are modulated at multiple levels to control miRNA biogenesis [1,4,5]. On one hand, the recognition and processing of pri-miRNAs by the DCL1 complex require the assistance of many protein factors. On the other hand, DCL1, HYL1 and SE themselves are regulated at transcriptional, post-transcriptional and post-translational levels.

4.1 The recognition and processing of pri-miRNAs by DCL1

Besides DCL1, HYL1 and SE are two core components for miRNA biogenesis. HYL1 is a dsRNA-bind protein that recognizes the stem region of pri-miRNA. Studies show that HYL1 plays important roles in positioning of pri-miRNAs into DCL1. Lack of HYL1 causes miscleavage of pri-miRNAs by DCL1 and reduces the accumulation of miRNAs at global levels. SE is a C2H2 zinc finger domain and binds pri-miRNAs. Disruption of SE also causes reduced miRNA levels at global levels. It has been proposed that DCL1, HYL1 and SE form a subnuclear loci called the D-body [2,89–91]. A recent study shows that SE-mediated phase separation is crucial for D-body assembly [92]. SE contains three intrinsically disordered regions (IDRs), which are required for phase separation and efficient miRNA processing [92]. D-body co-transcriptionally processes pri-miRNAs [2,89–91]. The co-transcriptional recruitment of DCL1 to pri-miRNAs requires the Elongator complex [28], NOT2 [29], MAC3 [55] and MAC7 [93]. Mutations in these proteins reduce the amount of the DCL1 complex without affecting the protein levels of DCL1, HYL1 and SE, suggesting that they facilitate the assembly of the DCL1 complex [28,29,55,93]. It should be noted that the ubiquitin ligase activity of MAC3 is required for miRNA biogenesis [55], suggesting that protein ubiquitination may play a role in the assembly of the DCL1 complex. The DEAH-box helicase PHYTOPHTHORA SUPPRESSOR OF RNA SILENCING 1 (PINP1) [94] and the RNA binding protein MODIFIER OF SNC1, 2 (MOS2) [95] also affect the formation of the DCL1 complex. MOS2 [95], STV1 [31] and TGH [86] bind pri-miRNAs and assist the recruitment of the DCL1 complex to pri-miRNAs.

In the past decades, many protein factors modulating DCL1 activities have been identified. NOT2 and Elongator, in addition to their role in pri-miRNA transcription, interact with the DCL1 complex to facilitate pri-miRNA processing (Fig. 3) [28,29]. In addition, CDC5 [30], PRL1 [56], MAC3 [55], MAC5 [58] and MAC7 [93], also interact with the DCL1 complex and promotes its activity (Fig. 3). These five proteins are components of MAC, suggesting that MAC plays a multiple role in pri-miRNA processing. Additional factors include TGH [86], DDL [54] and MOS2 [95], which also interact with DCL1 to enhance its activity (Fig. 3). Moreover, several pre-mRNA processing factors including the CAP-BINDING PROTEIN 20 (CBP20) and CBP80 [96] SMA1 [33], the pre-mRNA processing factor 6 homolog STABILIZED1 (STA1) [97], HIGH OSMOTIC STRESS GENE EXPRESSION 5 (HOS5) [98], ARGININE/SERINE-RICH SPLICING FACTOR 40 (RS40) and RS41 [98], the U1 snRNP Subunit LETHAL UNLESS CBC 7 RL (LUC7rl) [11], the THO2 in the THO/TREX complex [99], GLYCINE-RICH RBP 7 (GPR7) [100], SICKLE (SIC, a proline-rich protein) [101], the PRE-MRNA-PROCESSING PROTEIN (PRP)39b, PRP40a, PRP40b [11], also interact with the DCL1 complex and positively regulate pri-miRNA processing (Fig. 3). In addition, the RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1) interacts with SE to promotes pri-miRNA processing (Fig. 3) [102]. Notably, these protein factors also modulate the splicing. These discoveries suggest that miRNA biogenesis and general RNA processing are interconnected. Some specific factors modulate the activity of DCL in a spatiotemporal manner. For example, the DEAD-BOX RNA HELICASE 27 (RH27) interacts with DDL, HYL1 and SE, and promotes miRNA biogenesis in embryos, shoot apical meristem and root apical meristem [103]. Besides positive factors, negative factors of the DCL1 complex are also identified. Despite of its positive role in promoting *MIR* transcription, CHR2, an DNA/RNA helicase, is able to unwinds pri-miRNAs, which in turn represses pri-miRNA processing (Fig. 3) [34]. CDF2 decoys the DCL and HYL1 to inhibit the activity of the DCL1 complex [37].

4.2 Regulation of DCL1, HYL1 and SE

Studies also reveal that the expression and activities of DCL1, HYL1 and SE is controlled at transcriptional, post-transcriptional and post-translational levels. These multifaceted regulations appear to be crucial for proper accumulation of miRNAs.

Factors that control the transcript levels of *DCL1, HYL1* and/or *SE* have been identified. XAP5 CIRCADIAN TIMEKEEPER (XCT), a nuclear-localized protein and STA1 have been shown to promote the expression of *DCL* genes including *DCL1* [97,104], while the histone acetyltransferase GENERAL CONTROL NON-REPRESSED PROTEIN 5 (GCN5) appears to repress the transcription of *DCL1, HYL1* and *SE* (Fig. 3) [105]. Moreover, the DELAY OF GERMINATION1 (DOG1) promotes the transcription of *DCL1, HYL1, SE, TGH* and *CDC5* [106]. In addition, proper splicing of the ninth intron of *DCL1* requires the splicing factor SMA1 [33], which may affect *DCL1* expression. The expression of *DCL1* and *SE* is also subjected to feedback regulation. miR162 and miR863, which are generated by the DCL1 complex, directly target *DCL1* and *SE* for cleavage, respectively, which in turn ensures the correct *DCL1* and *SE* transcript levels [107,108]. Interestingly, the *DCL1* transcript levels are also negatively controlled by the DCL1-mediated processing of *MIR838*, which is derived from the 14th intron of *DCL1* pre-mRNAs [109]. Interestingly, the DCL1 protein can be stabilized by light during de-etiolation [110]. However, elevated DCL1 protein levels do not alter miRNA levels in the de-etiolated plants relative to etiolated plants, suggesting the presence of a light-induced suppressor of DCL1 [110]. Indeed, a light-stabilized FORHEAD-ASSOCIATED DOMAIN 2 (FHA2) protein was recently found to inhibit DCL1 activity by limiting its access to pri-miRNAs [111].

Notably, plants also use RNA-based mechanisms to decoy the components of the DCL1 complex. Intron lariat RNAs derived splicing by product, when failing to be disassociated from spliceosome, bind the DCL1 complex and prevent pri-miRNA processing (Fig. 3) [112]. Interestingly, ILP1 and NTR1, two splicing factors, interact with DCL1 and SE, remove intron lariat RNAs from spliceosome, and promotes the accumulation of miRNAs (Fig. 3) [38]. Another example is the transcripts generated from the short-interspersed elements (SINEs). SINE transcripts form a structure similar to

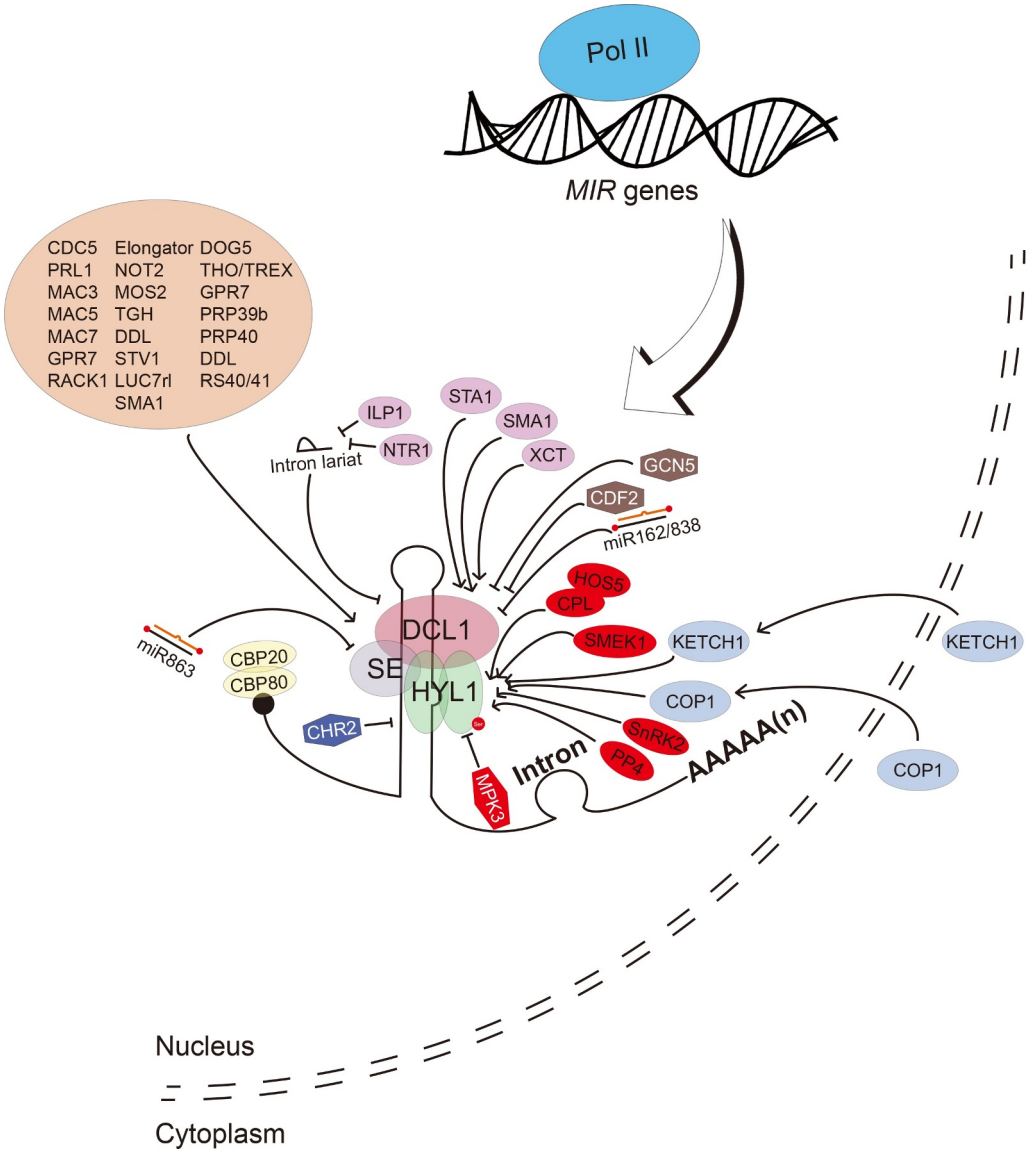

Figure 3. Regulation of pri-miRNA processing.

Processing of pri-miRNAs requires the core dicing body (DCL1, SE and HYL1) as well as many other accessory factors (brown ellipse). The abundance and activities of DCL1, SE and HYL1 are regulated by multiple protein factors and miRNAs. Positive and negative regulators are marked with ellipse and hexagon, respectively. Regulators involved in phosphorylation of HYL1 are marked with red colour.

pri-miRNAs, which in turn sequesters HYL1 from pri-miRNA processing [113].

Post-translational modifications also play critical roles in modulating the activities of the DCL1 complex. HYL1 is subjected to phosphorylation modification. The MITOGEN-ACTIVATED PROTEIN KINASE 3 (MPK3) and the SNF1-RELATED PROTEIN KINASE 2 (SnRK2) interact with and phosphorylate HYL1 (Fig. 3) [114,115]. Phosphorylation has been shown to inhibit HYL1 function, because dephosphorylation of HYL1 by the C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 (CPL1) and its homolog CPL2, is required for HYL1 localization to D-bodies and pri-miRNA processing (Fig. 3) [116]. CPL1 and CPL2 require the assistance of HOS5 to dephosphorylate HYL1 in young vegetative and reproductive tissues [117]. Moreover, SUPPRESSOR OF MEK 1 (SMEK1), together with the Protein Phosphatase 4 (PP4), also dephosphorylates HYL1, which in turn prevents HYL1 degradation (Fig. 3) [118]. Interestingly, phosphorylation also stabilizes HYL1 in the dark by keeping it in the nucleus so that during dark-to-light transition, light-mediated dephosphorylation is able to active HYL1 for miRNA biogenesis [119]. In addition, Constitutive Photomorphogenic 1 (COP1), a RING-finger E3 ligase, moves from the nucleus to the cytoplasm and stabilizes HYL1 in the light by repressing unknown protease activity [120]. SE is also phosphorylated by *in vitro* [29,115], but the functional significance of this modification remains to be identified.

The activity of SE and HYL1 is modulated at additional layers. An importin-beta protein KARYOPHERIN ENABLING THE TRANSPORT OF THE CYTOPLASMIC HYL1 (KETCH1) transports HYL1 from the cytoplasm into the nucleus to promote miRNA production (Fig. 3) [121]. Moreover, a recent study shows that SE interacts with the 20S core proteasome, which is able to degrade dysfunctional SE in a ubiquitin-independent

manner [122]. The elimination of disordered SE is required for the protection of the functional DCL1 complex.

Conclusion and perspectives

In the past decades, many protein factors modulating miRNA biogenesis have been identified. Analyses on these proteins and corresponding mutants have led to a better understanding of the process generating miRNAs. However, huge challenges remain to fully understand miRNA biogenesis. The biochemical functions for most of these protein factors are still unknown. Many of these factors have multiple roles in miRNA biogenesis. It is unclear whether a complex or multiple subcomplexes are required for a specific component to perform multiple tasks. Moreover, how the protein factors act coordinately to control the biogenesis of individual miRNAs based on pri-miRNA sequence and structure is still an unexplored field. In addition, it is unknown if miRNA biogenesis is spatiotemporally controlled at single cell levels, if so, how this is achieved. Clearly solving these questions requires novel techniques including single-cell biology and *in vitro* and *in vivo* systems to reconstitute miRNA biogenesis pathway. Studies also reveal that pri-miRNA stability is determined by the interplay between proteins involved in miRNA processing and ribonucleases, which provide a new regulatory layer of miRNA biogenesis. Further investigation on these observations will lead to a better understanding of how miRNA processing machinery and RNA decay machinery coordinate during miRNA biogenesis. The contribution of m^6A methylation on miRNA biogenesis and the fact that many factors involved in miRNA biogenesis function in transcription and splicing of mRNAs suggest that miRNA biogenesis is interconnected with other cellular processes. It will be interesting to further investigate how miRNA biogenesis is integrated into these processes.

Disclosure statement

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