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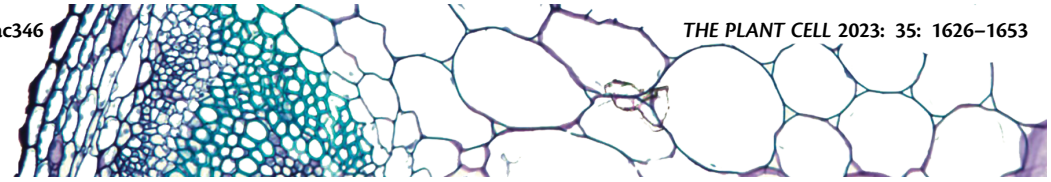

Review

Beyond transcription: compelling open questions in plant RNA biology

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Abstract

The study of RNAs has become one of the most influential research fields in contemporary biology and biomedicine. In the last few years, new sequencing technologies have produced an explosion of new and exciting discoveries in the field but have also given rise to many open questions. Defining these questions, together with old, long-standing gaps in our knowledge, is the spirit of this article. The breadth of topics within RNA biology research is vast, and every aspect of the biology of these molecules contains countless exciting open questions. Here, we asked 12 groups to discuss their most compelling question among some plant RNA biology topics. The following vignettes cover RNA alternative splicing; RNA dynamics; RNA translation; RNA structures; R-loops; epitranscriptomics; long non-coding RNAs; small RNA production and their functions in crops; small RNAs during gametogenesis and in cross-kingdom RNA interference; and RNA-directed DNA methylation. In each section, we will present the current state-of-the-art in plant RNA biology research before asking the questions that will surely motivate future discoveries in the field. We hope this article will spark a debate about the future perspective on RNA biology and provoke novel reflections in the reader.

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Introduction

(Written by Pablo A. Manavella)

In all living organisms, DNA is the molecule storing all genetic information, while RNA carries this data to the ribosomes to be translated into proteins. While DNA is omnipresent in our imagination, making star appearances in movies, TV shows, and books, the contribution of RNA to life is less recognized by society. However, as a consequence of the recent COVID pandemic, the word "RNA" has reached most people on the planet as they learned about the RNA-based genome of the virus and the therapeutic use of RNA vaccines. Thus, the concept of "information flow", that is the decoding of DNA to protein using an RNA intermediate, has suddenly become the center of attention and conversations. What remains largely unknown to the general audience is that the advent of sequencing technologies has made it clear that RNA is not only a coding molecule but also has various other functions, mostly in the form of cellular non-coding RNA transcripts.

The study of RNAs has emerged as a particularly important research field in contemporary biology, especially in plant biology, where these molecules execute many actions during development and response to the environment. Advances in sequencing technologies have allowed the global analysis of RNA modifications, the resolution of RNA secondary structures, the mapping of epigenetic modifications, the identification of RNA-edited sequences, and the discovery of novel classes of RNAs resulting in a revolution in molecular biology that is just starting.

In this article, we gathered 12 experts in different aspects of plant RNA biology to discuss some of the most compelling open questions in the field. Each section discusses long-standing open questions of the field as well as questions that have only begun to emerge from break-through discoveries. We hope this article helps stimulate the community and sparks new ideas and research projects that will expand the frontiers of RNA biology knowledge in plants.

How does light control RNA alternative splicing in plants?

(Written by Micaela Godoy Herz and Alberto Kornblihtt)

Plants rely on light as their main source of energy, but light also regulates many developmental and physiological responses during the plant life cycle (Arsovski et al., 2012). Furthermore, light signals induce a massive reprogramming of gene expression in plants (Tognacca et al., 2020). Alternative splicing produces multiple mRNA variants from a single locus. Splicing and alternative splicing are coupled with transcription, and factors that regulate transcription also affect alternative splicing (Kornblihtt et al., 2013).

Our lab showed how light regulates plant alternative splicing through the chloroplast (Petrillo et al., 2014). Light and dark conditions affect alternative splicing of a subset of Arabidopsis (*Arabidopsis thaliana*) genes preferentially encoding proteins involved in RNA processing. This effect requires functional chloroplasts: treatment of Arabidopsis seedlings with drugs that impair the chloroplast photosynthetic transport chain inhibit the effect of light on alternative splicing. Moreover, the effect of light is also observed in roots when communication with leaves—the photosynthetic tissue—is not interrupted (Petrillo et al., 2014). Light, sensed by the chloroplast, indeed triggers a retrograde signal that regulates alternative splicing not only in leaves, but also in roots.

How does light cause splicing responses in roots? In a recent work, Riegler and collaborators investigated this shoot-to-root signaling: they showed that alternative splicing responses in roots are not directly caused by light, but are instead most likely triggered by sugars. The kinase TARGET OF RAPAMYCIN (TOR) plays a key role in this signaling pathway. Sugars activate the TOR pathway and act as mobile signals to coordinate alternative splicing responses throughout the plant (Riegler et al., 2021).

These results afforded us a better understanding of how mobile signals regulate alternative splicing throughout the entire plant in response to light. One remaining outstanding open question is what happens in the nucleus: that is, what are the mechanisms involved in this regulation of alternative splicing in plants?

We performed different experiments to address the role of transcription elongation and determined that the light control of alternative splicing responds to a kinetic coupling mechanism (Godoy Herz et al., 2019). Briefly, the kinetic coupling model explains how changes in RNA Polymerase II (Pol II) elongation rate influence alternative splicing. Each splice site consists of a consensus sequence that is recognized by spliceosomal components, although "strong" splice sites (those that are close to the consensus sequence) are more efficiently recognized than "weak" splice sites, which are suboptimal. In the example illustrated in Figure 1, there is an alternative splicing event with two 3′ splice sites: a weak upstream 3′ splice site, and a strong downstream splice site. If Pol II elongation rate is fast, both sites are presented to the splicing machinery at the same time, and the strong 3′ splice site is recognized by the splicing machinery more efficiently, resulting in exon skipping. However, if Pol II transcription rate is slow, the splicing machinery will recognize the upstream, weaker, 3′ splice site first, and afterwards the strong 3′ splice site, which leads to exon inclusion (Godoy Herz et al., 2019). We showed by different experimental approaches that light promoted transcription elongation in Arabidopsis, while Pol II elongation was slower in darkness. Furthermore, the light control of alternative splicing and elongation was abolished in plants lacking function for TRANSCRIPTION FACTOR II S (TFIIS) in a previous report (Dolata et al., 2015): These TFIIS mutant plants did not respond to light signaling on a group of alternative splicing events. This result demonstrated that coupling between transcription and splicing is important for a whole organism to respond to environmental cues (Figure 1).

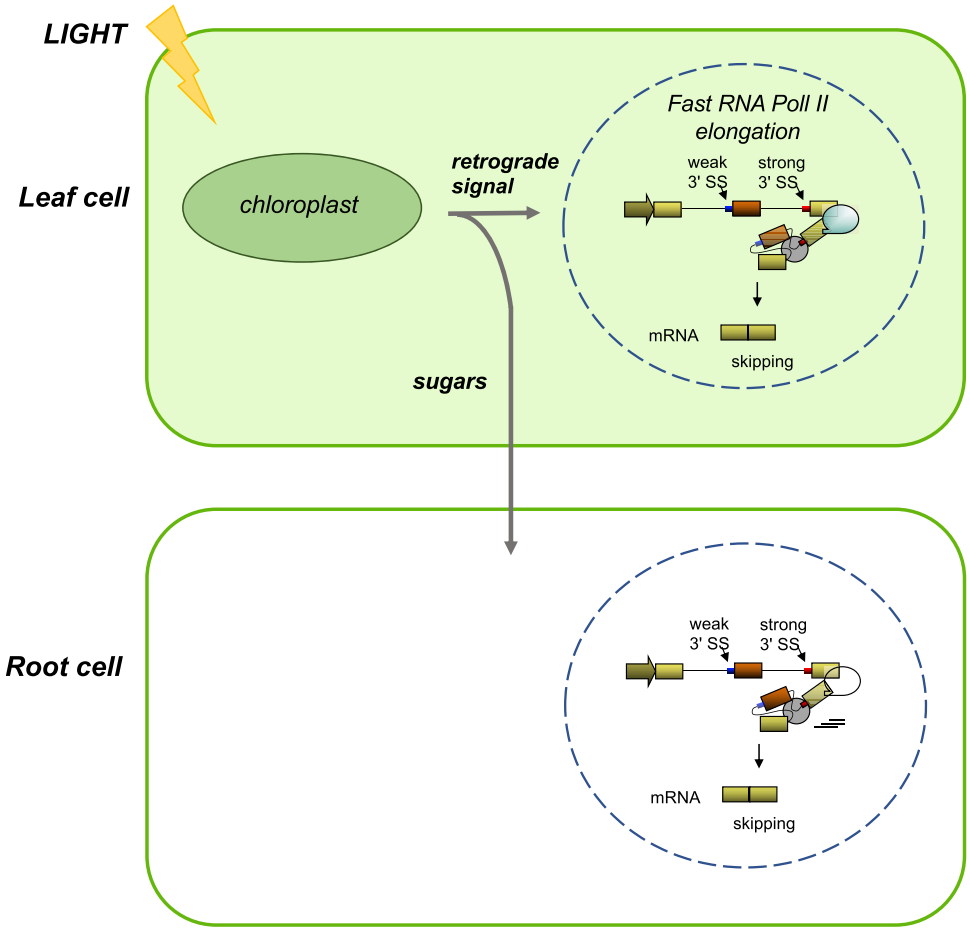

Figure 1 Light, sensed by the chloroplast, triggers a retrograde signal that regulates alternative splicing in the nucleus. In the light, RNA polymerase II (Poll II) elongation rate is fast, resulting in exon skipping. Leaf cells produce sugars that act as mobile signals to coordinate alternative splicing responses throughout the whole plant, thus reaching root cells.

Plant lines with higher Pol II transcription activity were recently generated by introducing point mutations in NRPB2, the second largest subunit of Pol II. As a result, an accelerated Pol II elongation rate increased the polymerase signal in gene bodies, which appeared to modulate alternative splicing choices (Leng et al., 2020).

Even though our knowledge of alternative splicing in plants has grown significantly in the last decade, many important open questions remain. It has been shown that, in response to light, sugars activate the TOR pathway, which in turn regulates alternative splicing. But how does TOR regulate alternative splicing in the nucleus? In the chloroplast, the exact nature of the chloroplast retrograde signal that regulates alternative splicing remain unknown, although it may be triggered by the oxidation state of the plastoquinone pool connecting both photosystems (Petrillo et al., 2014).

Moving forward, inside the nucleus, how light promotes Pol II elongation is unknown: what makes Pol II elongation faster in the light, and slower in darkness? There are many possible mechanisms that might explain how the chloroplast regulates transcription elongation. Furthermore, the role of chromatin modifications on the regulation of alternative splicing in plants remains an interesting field to investigate. Previous studies in mammalian systems have shown that histone post-translational modifications play a key role in the regulation of alternative splicing decisions. Treating cell cultures with drugs that open chromatin structure promoted changes in alternative splicing by facilitating Pol II elongation and exon skipping (Schor et al., 2009). By contrast, cell differentiation results in an increase in intragenic silencing chromatin marks that raised the rate of higher exon inclusion (Schor et al., 2013). In our work, histone acetylation mimics the effect of light on alternative splicing, but light does not affect the levels of this histone modification (Godoy Herz et al., 2019). Future experiments will be needed to address the role of chromatin structure in splicing regulation in plants.

Moreover, coupling between transcription elongation and alternative splicing may also act in response to other environmental stimuli, like temperature. A recent work shows that the TFIIS elongation factor is required for thermal adaptation in Arabidopsis (Szadeczky-Kardoss et al., 2022). Furthermore,

analyses of plant native elongation transcript sequencing (plaNET-seq) experiments in response to cold showed changes in Pol II promoter-proximal stalling and at the 3′ end of genes (Kindgren et al., 2020).

Finally, it would be interesting to study if these mechanisms of gene expression regulation are also conserved in other plants and other photosynthetic organisms like algae. Future work from different groups will be needed to address these questions.

The invisible world of RNA dynamics

(Written by Reed Sorenson and Leslie E. Sieburth)

Transcriptomics has transformed our understanding of molecular responses to signals. The abundance of many mRNAs can be robustly upregulated or downregulated, and many regulated genes bring about changes in development or physiology. Indeed, measurements of RNA abundance are so ingrained in our thinking that changes in RNA levels are frequently referred to as *gene expression* or *transcriptional responses*. However, alongside regulatory events that lead to changes in mRNA levels, there lurks the largely unseen layer of mRNA decay rate regulation. In addition to RNAs with modified rates of decay and changes in their abundance, rates of decay can also be modified for mRNAs whose abundances are held steady. This largely invisible dynamic regulation is just beginning to be investigated, and so there are numerous unanswered questions, including why decay rates are modified independently of changes in abundance, how this modulation occurs, and whether this regulation has implications for mRNAs that do show changes in their abundance.

RNA abundances are influenced by both synthesis (transcription) and decay, and the rate of RNA turnover is called flux (Figure 2A). Wide variations in flux have been observed in all deep RNA decay analyses, but whether flux rates affect RNA abundances and/or regulation is still an open question. A special case of mRNA flux regulation occurs when both the transcription and decay rates of an mRNA are modulated,

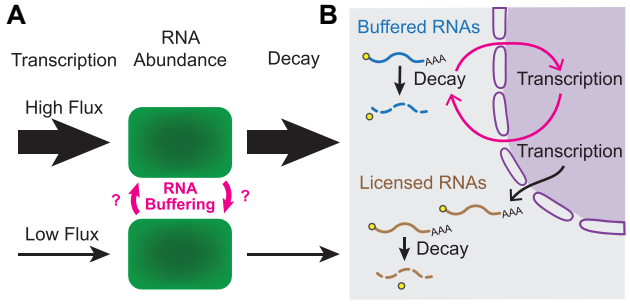

Figure 2 RNA buffering is a flux-mediated regulatory mechanism that maintains some mRNAs at a stable abundance. A, RNA abundance is influenced by the balance between RNA synthesis and degradation. B, RNA flux describes the turnover rate of an mRNA, RNA buffering occurs when the flux of an RNA shifts, but not its abundance.

and yet the mRNA abundance does not change. This phenomenon is called "RNA buffering" because transcription and decay rates are balanced to maintain steady abundances (Figure 2B). RNA buffering has been documented in Arabidopsis, but we are at the very beginning of understanding all aspects of this process, including both how and why some mRNAs become buffered.

The system where RNA buffering is best understood is budding yeast (*Saccharomyces cerevisiae*). A mysterious observation led to its discovery: mutants with defects in either RNA decay or transcription were found to maintain normal mRNA abundances. It turned out that the initial defect, in e.g. RNA decay, was accompanied by a compensatory change (e.g. in transcription). That is, normal abundances of mRNAs in many transcription and RNA decay mutants were maintained by precisely balanced changes through RNA buffering (Haimovich et al., 2013; Sun et al., 2013a; Timmers and Tora, 2018; Hartenian and Glaunsinger, 2019). Because most mRNA decay occurs in the cytoplasm, while transcription takes place in the nucleus, RNA buffering requires not just precise regulation, but also communication between the nucleus and the cytoplasm. Mechanisms underlying this regulation are still emerging and somewhat controversial, but studies in yeast have revealed RNA decay proteins relocating to the nucleus and displaying novel functions. For example, Sun and colleagues showed that the yeast 5′→3′ EXORIBONUCLEASE 1 (XRN1) moves from the cytoplasm to the nucleus, where it binds DNA and influences transcription of buffered RNAs (Sun et al., 2013a). Similarly, upon nuclear RNA exosome dysfunction, RNA buffering was activated by global attenuation of transcription via stabilization of the mRNA encoding HISTONE SIRTUIN DEACETYLASE (HST3) (Bryll and Peterson, 2022). RNA buffering has also been observed in Drosophila (*Drosophila melanogaster*), where it was used for gene dosage compensation (Faucillion et al., 2022).

It was a similarly mysterious observation that led us to discover RNA buffering in Arabidopsis (Sorenson et al., 2018). Cytoplasmic mRNA decay initiates through deadenylation, and decay in the 3′→5′ direction can be catalyzed by either the RNA exosome or SUPPRESSOR OF VARICOSE (SOV)/DIS3-LIKE EXONUCLEASE 2 (DIS3L2), while decay in the 5′→3′ direction is initiated by decapping followed by exoribonucleolytic digestion by XRN4 (Labno et al., 2016). Because the popular wild-type accession Columbia-0 (Col-0) harbors a *sov* loss-of-function mutation (Zhang et al., 2010) possible functions of this decay pathway were mysterious. To understand why *sov* mutants did not show an abnormal phenotype, and also identify mRNA substrates of decapping and SOV, we compared genome-wide RNA decay rates for wild type and RNA decay mutants. In *varicose* (*vcs*) mutants (which lack mRNA decapping), the expectation that mRNA decapping substrates would decay more slowly was largely observed. The most common decay pattern (seen in >7,000 RNAs) was half-lives that were longer in *vcs*, and longer still in *vcs sov* double mutants, indicating that these RNAs

were typically degraded by VCS, but upon loss of VCS, SOV provided back-up. However, many mRNAs in *sov* mutants showed a surprising shift to shorter half-lives. Moreover, mRNA decapping (via VCS) was required to sustain these shorter half-lives. This unusual decay rate shift had no significant effect on RNA abundances, indicative of RNA buffering and explaining the lack of phenotypic consequences in *sov* mutants in Col-0. Data suggestive of RNA buffering was also identified in an Arabidopsis study of cold response (Arae et al., 2017). We do not know whether plants use a mechanism similar to that of yeast for RNA buffering; however, there are no reports of XRN4 being found in the nucleus (suggesting that RNA buffering in Arabidopsis might differ mechanistically from yeast), and the shifting of SOV substrates to decapping via VCS has not been described previously.

Conventional views of gene expression place all the action on those mRNAs whose abundances are altered. However, RNA buffering turns this conventional view on its head by demonstrating that many mRNAs with stable unchanging abundances also undergo complex regulation. And this observation leads to an even bigger open question: how are some mRNAs buffered so that their abundances do not vary, while others appear to be able to freely increase or decrease in abundance? If only specific mRNAs are buffered, perhaps they share a common sequence motif, e.g. for a regulatory RNA-binding protein. Alternatively, buffering of RNA abundances may be a default state, and some sort of licensing might be required to allow mRNAs to undergo changes in abundance (Figure 2B). What distinguishes RNAs to be buffered from those licensed to undergo alterations in their abundance? Attractive candidates can be found in the expanding world of RNA modifications, from differing caps to covalent modifications such as N^6-methyladenosine (m^6A) or structure (Kwok et al., 2013; Mauer et al., 2017; Anderson et al., 2018; Reichel et al., 2019; Wang et al., 2019; Zhang et al., 2019a). Addressing these many open questions will require much deeper understanding of RNA kinetics.

Stabilization of mRNA and translational regulation by stress granules in response to environmental conditions

(Written by Kentaro Nakaminami and Motoaki Seki)

Current technologies used to analyze gene expression have enabled a high-level of resolution on the expression of thousands of genes. Advancements in proteomic technologies have also greatly improved the comprehensive analysis of proteins. Thus, it has become possible to analyze plant physiology and metabolism in great detail using various analytical methods. Collectively, these studies have empirically indicated that gene and protein abundance patterns are not always identical based on the results of multiomics analyses. Major factors contributing to the observed differences between mRNA and protein patterns are post-transcriptional regulation of mRNA and translational regulation of proteins. Both mechanisms fine-tune which mRNAs are translated into proteins to regulate the physiology and metabolism of living organisms.

Various events occur between mRNA transcription and translation via the activity of RNA-binding proteins (RBPs) that determine whether proteins are synthesized (Burjoski and Reddy, 2021). These events begin with quality control of transcribed mRNAs, followed by degradation of unnecessary or aberrant mRNAs, or protein translation. Additionally, mRNAs can be temporarily stored via a stabilization system for subsequent activation in response to environmental changes and other stimuli. Although mRNA abundance is affected by the balance between transcription and degradation, the amount of protein is not always proportional to mRNA abundance, and is affected by post-transcriptional regulatory mechanisms such as the speed of translation and translational inhibition. mRNA degradation is regulated by mRNA-protein (mRNP) complexes called processing bodies (PBs); translation is carried out by ribosome complexes (poly-ribosomes or polysomes), while mRNA stabilization or storage occurs in stress granules (SGs) (Chantarachot and Bailey-Serres, 2018; Maruri-Lopez et al., 2021). These granules are not organelles but rather membraneless RNA granules formed via liquid–liquid phase separation (LLPS) (Emenecker et al., 2020). They have been reported to be present in both animals and plants and are becoming a growing research focus. This section of the present review discusses the nature of SGs, which are responsible for the mechanisms of translational regulation, and how mRNAs are stabilized and stored in these bodies.

Plants suppress mRNA translation when they are subjected to severe stress (Merchante et al., 2017). This strategy reduces energy expenditure under stress conditions, as only essential proteins are synthesized. Since translation requires considerable energy, reducing energy requirements during stress contributes to increased survival rates. Importantly, active but selective translation must operate during stress response in plants since essential proteins are still translated. The temporary storage of mRNA in SGs during stress conditions can be rapidly reversed, with mRNAs being released in a translationally active form (polysomes) as plants recover from stress conditions (Kosmacz et al., 2019). The mechanisms responsible for determining target selectivity and translation timing by mRNP complexes, however, have not been clearly elucidated.

SG complexes that form in the cytoplasm during stress are conserved in eukaryotes (Maruri-Lopez et al., 2021). SG formation in plants is triggered by a variety of stresses, including high temperature, hypoxia, high salinity, and darkness (Chantarachot and Bailey-Serres, 2018; Hamada et al., 2018). An SG is composed of translationally arrested mRNAs and proteins related to the initiation of translation, such as translation initiation factors, small subunits of ribosomal RNA (rRNA), poly(A)-binding proteins, as well as regulatory RBPs that inhibit translation. Recently, hundreds of

proteins have been characterized as SG components by combining immunoprecipitation (IP) and genome-wide mRNA-binding interactome capture methods with proteomic analyses (Chantarachot and Bailey-Serres, 2018; Kosmacz et al., 2019; Marondedze et al., 2019; Gutierrez-Beltran et al., 2021; Maruri-Lopez et al., 2021). These results have suggested that SGs are formed not only upon heat and hypoxia stresses, but also by drought stress, resulting in translational repression. The components discovered in these studies were not revealed based on their homology to SG components in animals and yeasts as in previous studies, but rather were directly identified by the indicated methodologies as components of SGs. Although many SG components have been isolated with this approach, proteins within SGs also include translation-promoting proteins such as translation initiation factors, and not all are related to translation inhibition. It is necessary to consider the components and functions of SGs, including spatiotemporal factors such as the dynamics of SG formation/dissociation, timing and localization. SGs suppress translation and protect transcribed mRNAs from degradation by temporarily storing selected mRNAs. SGs can be disassembled during stress recovery and the stored mRNAs then become accessible for immediate translation. This rapid reactivation is believed to be a response to environmental changes. Previous studies have identified SG-regulated target mRNAs by analyzing RBPs present in SGs. The identification of untranslated target mRNAs stored in SG has provided information on the translational control or selective translation mechanism that occurs in response to stress. In previous studies using hypoxic and heat-stress samples, various direct target mRNA identifications have been performed with multiomics analyses such as RNA immunoprecipitation followed by sequencing (RIP-seq) analysis, transcriptome analysis, translatome, and mRNA degradation rate analysis (Sorenson and Bailey-Serres, 2014; Nguyen et al., 2016; Tian et al., 2022; Zhu et al., 2022). Although many mRNA targets that are thought to be regulated by SGs have been revealed, their subsequent fates, such as when translation-inhibited mRNAs are finally translated into proteins, still remains unclear at this time.

While multiomics analyses, such as an RIP analysis combined with translatome and polysomal analyses, have enabled the identification of SG components and direct target mRNAs that are SG-regulated, there are still many open questions that are await clarification. For example, OLIGOURIDYLATE BINDING PROTEIN 1b (UBP1b), an SG component, localizes to the nucleus under non-stress conditions. UBP1b-stress granules (UBP1b-SG) are induced to form in the cytoplasm in response to heat stress, and candidate target mRNAs for UBP1b have been identified. UBP1b is present in both the nucleus and cytoplasm, but its precise location where it exerts its mRNA stabilization role remains unclear (Figure 3). In addition, PBs and SGs co-localize, suggesting that their constituent components might interact (Hamada et al., 2018). Although many mRNAs have been described as SG targets, not all will be translationally inhibited. It is plausible that some targets might be degraded by PBs rather than stabilized by SGs; the underlying mechanism of recruitment of target mRNAs remains to be elucidated. Future studies should elucidate how targeted mRNAs are exactly regulated by SGs. Clarifying the mechanism(s) of selective translation will be a major step forward in understanding stress responses in plants.

The pervasive function of RNA structure in plant growth and development

(Written by Yiliang Ding)

Plant growth and development is a continuous process starting with embryogenesis, and the formation of the embryonic root and shoot, followed by organogenesis of diverse organs such as roots, leaves, branches, and flowers. Plants rely on gene expression regulation to achieve specific cell differentiation and elongation to form different organs. This extremely high coordination of gene expression at both temporal and spatial levels requires diverse regulatory mechanisms to achieve evolutionary fitness. Furthermore, plants have evolved to adapt to wide-ranging environmental conditions, acquiring highly dynamic regulation of gene expression in response to different environmental factors. In addition to gene sequence content, RNA structure is another important property of genes that can dynamically regulate gene expression at the post-transcriptional level (Zhang and Ding, 2021).

Recent advances in RNA structure studies have enabled unprecedented opportunities to determine the functional importance of RNA structure across varied aspects of plant growth and development. For instance, the antisense long non-coding RNA (lncRNA), *COOLAIR*, folds into a complex RNA structure (Hawkes et al., 2016) that was suggested to suppress transcription of the key flowering gene, *FLOWERING LOCUS C* (*FLC*) and promote flowering following vernalization. Another key regulator of plant vascular development, *JULGI* (*JUL*), was shown to limit phloem differentiation through its direct interaction with an RNA tertiary structure motif, RNA G-quadruplex, on the 5′ untranslated regions (5′ UTRs) of *SUPPRESSOR OF MAX2 1-LIKE4/5* (*SMXL4/5*) mRNA to suppress their translation (Cho et al., 2018). Other studies have shown that RNA G-quadruplex affects plant root growth and development (Foley et al., 2017; Zhang et al., 2019b; Yang et al., 2020a). Extensive studies have indicated that RNA structural conformations change in response to temperature (Su et al., 2018; Chung et al., 2020), light (Gawronski et al., 2021), salinity stress (Kramer et al., 2020; Tack et al., 2020), and phosphate starvation (Reis et al., 2021). These changes subsequently alter gene expression at the post-transcriptional level such as translation and RNA degradation (Su et al., 2018; Chung et al., 2020; Kramer et al., 2020; Tack et al., 2020; Gawronski et al., 2021; Reis et al., 2021). These recent studies have focused on either identifying a specific RNA structural element on a specific transcript, or determining global associations between RNA

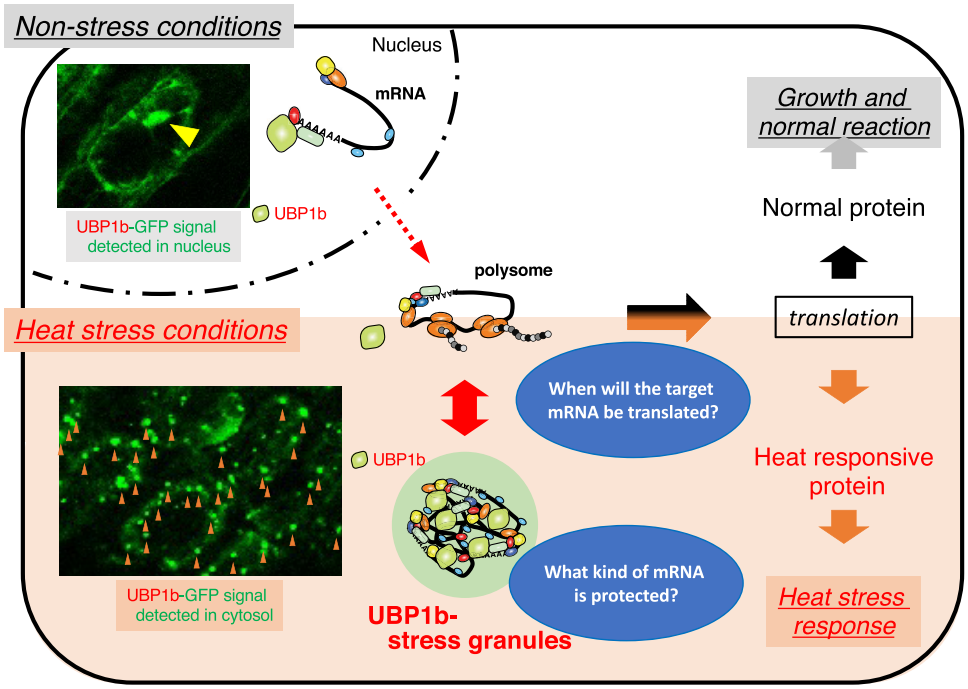

Figure 3 Hypothetical model of stress granule function. UBP1b localizes to the nucleus under non-stress conditions. UBP1b-stress granules (SGs) are induced to form in the cytoplasm in response to heat stress. UBP1b-SGs protect target mRNA from degradation during stress. Elucidation of the mechanism of target mRNA recruitment and the timing of the translation of the protected mRNA will provide critical information on the selective translation mechanisms utilized in plants in response to stress.

structure features and corresponding molecular functions, and further support the growing evidence that highlights the importance of RNA structure across diverse aspects of plant growth and development.

Since every mRNA is capable of folding into a particular RNA structure, this question has stimulated interest to explore the pervasive role of RNA structure in individual genes to gain a more comprehensive understanding of RNA structure-mediated regulation in plant growth and development. To achieve this in-depth understanding, the strategies employed for studying RNA structure functionality need to reach a new level. A promising approach may be the capability of achieving specific cell-type resolution. In plants, although stem cells are pluripotent, their cellular trajectories are limited in scope because the identity of any given cell depends on its position relative to its neighbors. For instance, root growth starts from sets of stem cell initials in the quiescent center (QC), which generate continuous parallel files of epidermal cells that divide in a transverse, anticlinal orientation. Cells then divide in the meristematic zone before starting to elongate into the differentiation zone of the mature root. After division, cells remain in the same position and belong to the same lineage (Costa, 2016). Interestingly, all these cell types share the same genetic information encoded in their DNA, but with diverse cellular conditions. The folding status of RNA structure is highly dependent on cellular conditions such as ion concentrations and interacting proteins (Zhang and Ding, 2021). Thus, it is likely that RNAs may fold differently to specify gene function in different cell types, resulting in unique cell identities (Figure 4). Future research could focus on dissecting the extent of RNA structure diversities across individual cell types. Indeed, the development of single-cell RNA structure profiling will advance our understanding of RNA structural dynamics in plant cells.

Another future perspective could be to elucidate how RNA structures serve as environmental sensors. During growth, plants are constantly challenged by fluctuating environmental conditions such as biotic and abiotic stresses. Other abiotic stresses such as flooding and drought are likely to affect the folding status of RNA structures due to changing molecular concentrations in the cells (Zhang and Ding, 2021). During pathogen infection, many metabolites are significantly altered that may also influence RNA folding (Zhang and Ding, 2021). Additional research could focus on dissecting the detailed mechanisms of RNA structure-mediated stress responses including comprehensive assessments of different stresses, or different degrees and duration of stress. Finally, it may be possible to assess the evolution of RNA structures across the plant kingdom. Previous studies have illustrated the evidence of evolutionary selection of certain RNA structure motifs (Yang et al., 2020a) and distinguished RNA structure features in specific species (Deng et al., 2018; Yang et al., 2021). Studies of evolutionary RNA structures may shed novel insight into understanding nucleotide diversities in non-coding regions and at synonymous codon

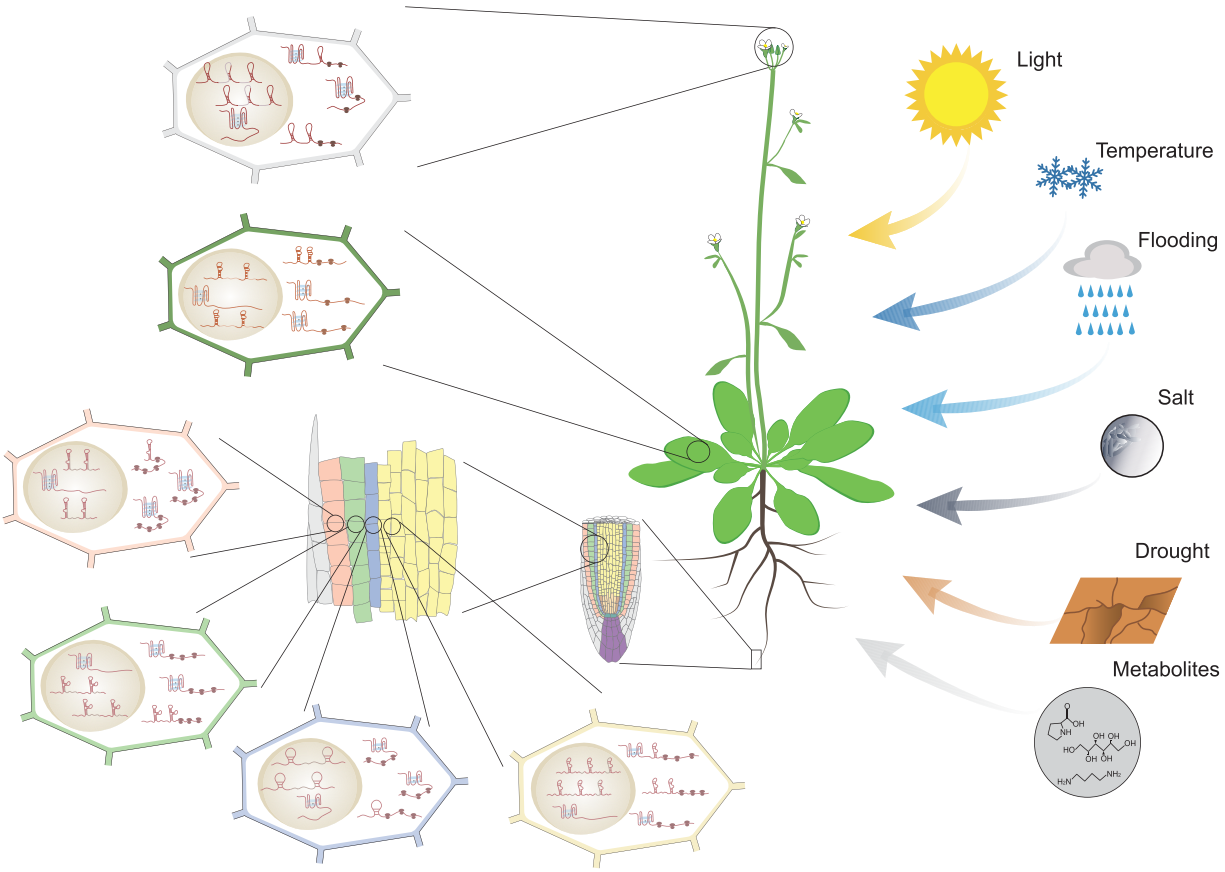

Figure 4 RNA structure may pervasively function in plant growth and development. RNAs may fold into diverse RNA structures in different cell types and under different environmental conditions. These dynamic and diverse RNA structures facilitate the regulatory specificities of gene expression at post-transcriptional levels.

positions. Extension of RNA structure studies with a phylogenomic perspective may provide an evolutionary perspective on RNA structure functionality.

With the rise of transcriptome-wide RNA structuromes, large volumes of RNA structure data now provide the necessary scope for deep learning applications with the potential for translating fundamental knowledge into RNA structure-based molecular design. For instance, from transcriptome-wide RNA structure and RNA stability data, we can now learn and predict what kind of RNA structure features are responsible for RNA stability. It may be possible to customize these RNA structure features into more or less stable RNAs of interest. Where RNA structure acts as a post-transcriptional regulator, directly affecting protein production, RNA structure-guided molecular design may offer the potential for new avenues in synthetic biology.

Recent technological advances have significantly pushed the discovery of RNA structure functionalities forward. Further innovations in PacBio and Nanopore technologies to study RNA structures may offer more accurate RNA structure information at single-base resolution. These upcoming developments will invigorate RNA structure views to individual RNA structure conformations rather than the familiar bulk conformation. RNA structures may be a type of hidden "codon" embedded in every gene that facilitates the complexity of gene expression regulation. The rapid growth of RNA structure research may ultimately reveal the regulatory power of RNA structures in every aspect of plant growth and development.

Sensing, regulation, and functions of r-loops in plants

(Written by Qianwen Sun)

The R-loop, a three-stranded chromatin structure comprising one single-stranded DNA molecule and one RNA:DNA hybrid duplex, is widely distributed in the genome, with essential roles in multiple cellular and disease processes (Garcia-Muse and Aguilera, 2019; Brickner et al., 2022; Petermann et al., 2022). Recent advances in genome-wide detection methods have broadened our understanding of the distribution and dynamic patterns of R-loops (Xu et al., 2022b). R-loops are involved in many biological processes related to genome regulation, including transcription, replication, DNA damage and repair, and chromatin organization

(Zhou et al., 2022a). The biological study of R-loops in plants began in 2013 when we discovered that an R-loop formed on the promoter of the antisense lncRNA *COOLAIR* and affected the expression of *FLC* (Sun et al., 2013b). In 2017, following the development of ssDRIP-seq (single-strand DNA ligation-based library construction after DNA:RNA hybrid IP, followed by sequencing), the localization of R-loops in the Arabidopsis genome was revealed (Xu et al., 2017). The R-loop profiles of other plants have since been disclosed (Figure 5). Through analysis of genome-wide data (Xu et al., 2017, 2020b), some unique features of R-loop distribution in the nuclear genomes of plants have emerged, prompting intriguing research directions in plant R-loop biology.

While analyzing the genome-wide distribution of R-loops, we identified a unique group of R-loops formed by antisense lncRNAs near transcription start sites (TSS) named asTSS_R-loops (Xu et al., 2017). Similar patterns of R-loop distribution have also been observed in other plant species, such as rice (*Oryza sativa*) and maize (*Zea mays*) (Figure 5, and summarized in Zhou et al., (2022a)). These conserved patterns raise several questions: what are the functions of these asTSS_R-loops; how are they transcribed, and is the transcriptional initiation of the antisense lncRNAs specific to particular physiological or pathological responses? Another notable finding was the prevalence of transfer RNA (tRNA)-promoted sense R-loops throughout the genome (Xu et al., 2017). We discovered that these intragenic R-loops orchestrated transcriptional interference between Pol II and Pol III, thus regulating the expression of oxidative-responsive genes (Liu and Sun, 2021). Surprisingly, a large proportion of R-loops is located in constitutive pericentromeric heterochromatin and overlaps with H3K9me2 and H3K27me1 heterochromatic marks in Arabidopsis (Xu et al., 2017). This observation raises intriguing questions about the functions of R-loops in heterochromatin formation and organization in plants.

R-loops play important roles in cellular reprogramming in mammals (Yan et al., 2020; Li et al., 2020b). During the life-cycle of Arabidopsis, R-loops showed a range of dynamic changes during generational switches (such as flowering and germination) and during recovery from long-term heat-stress treatment (Xu et al., 2020b). During the transition from vegetative growth to flowering, R-loop formation decreased dramatically, whereas from flower development to germination, R-loop formation gradually increased, suggesting that the global reprogramming of R-loops also occurs in Arabidopsis. These dramatic changes in R-loop formation likely co-occur with other events of genome regulation, such as DNA replication and transcriptional reprogramming. It will be important to explore the biological functions and regulatory mechanisms of R-loop reprogramming during key developmental transitions in plants. Conversely, R-loops likely function in transcriptional reprogramming during physiological and pathological processes. Interestingly, Moore *et al.* proposed a model of R-loop-mediated transcriptional reprogramming during plant defense responses (Moore et al., 2011), although experimental evidence is still lacking.

Most R-loops form and function in *cis*. However, *trans*-formed R-loops may also play important roles in plants. For example, the lncRNA *APOLO* promoted *trans*-R-loop formation and altered chromatin loop conformation (Ariel et al., 2020). Current detection methods cannot provide information about whether R-loops form in *cis* or in *trans*, underscoring the need to develop a high-throughput technique for distinguishing *cis*- or *trans*-R-loops globally. Moreover, it would be useful to alter the levels of R-loops at specific genomic loci (Liu and Sun, 2021), but there is currently no efficient way to modulate an entire group of R-loops (such as asTSS_R-loops) jointly. asTSS_R-loops were recently proposed to promote co-transcriptional micro RNA (miRNA) processing (Gonzalo et al., 2022). However, the lack of tools for modulating R-loops makes it challenging to study the functions of particular groups of R-loops with similar distribution patterns in the genome. Alternatively, identifying the specific regulators of a particular group of R-loops could help solve this problem.

To date, several R-loop modulators have been identified in plants (Zhou et al., 2022a). Among these, the evolutionarily conserved RNase H1 proteins specifically remove the RNA moiety in RNA:DNA hybrids, thus resolving R-loops efficiently. The Arabidopsis genome encodes three RNase H1 proteins: AtRNH1A, AtRNH1B, and AtRNH1C (Yang et al., 2017). While AtRNH1B and AtRNH1C are involved in stabilizing the genome integrity of semi-autonomous organelles (mitochondria and chloroplasts) (Yang et al., 2017; Cheng et al., 2021; Wang et al., 2021b), the biological function of nucleus-localized AtRNH1A is still unclear. The biological functions of RNase H1 proteins and other R-loop regulators in different plant species also need to be further explored.

Organisms must integrate and coordinate the activities of different tissues and cell types. Precisely analyzing the genome-wide patterns of R-loops from ultralow input samples is difficult using current methods (Zhou et al., 2022a). It is imperative to establish ultralow-input (or even single-cell) R-loop profiling techniques to systematically explore the functions of R-loops in critical genomic events in complex tissues. Such tools could be powerful for dissecting R-loop distribution and dynamics in specific cell types and during specific differentiation programs, such as double fertilization in plants. As the topological state of the genome could affect R-loop formation, it would be useful to develop tools to quantitatively measure topological conformation and explore how the 3D genome organization influences R-loop formation. Advanced computational predictions of R-loops genome-wide could complement experimental approaches for species with available genome sequence information.

Chloroplasts and mitochondria are semi-autonomous organelles of endosymbiotic origin with their own genetic materials. In the face of complex external environmental conditions and internal growth and developmental factors,

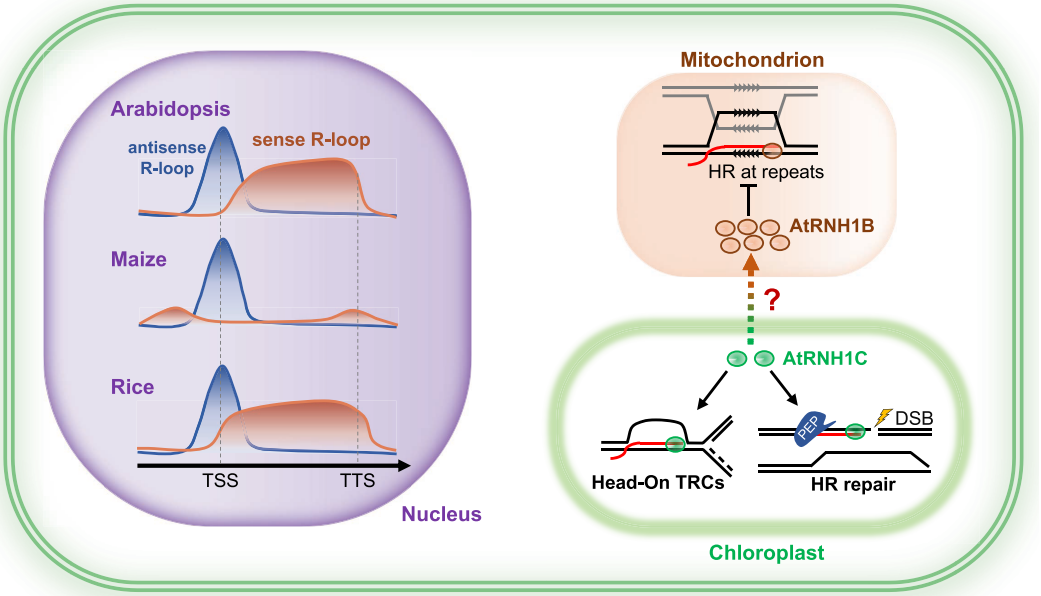

Figure 5 R-loops in plant cells. Left, different distribution patterns of nuclear R-loops along the gene body in the genomes of Arabidopsis, maize, and rice. Right, chloroplast-localized AtRNH1C restricts RNA:DNA hybrid formation to release head-on transcription-replication conflicts (TRCs) and to promote homologous recombination (HR) repair in chloroplasts. Mitochondrion-localized AtRNH1B inhibits HR at repeats in the mitochondrial genome by suppressing RNA:DNA hybrid formation. In the absence of AtRNH1B, high levels of mitochondrial R-loops stimulate the relocation of AtRNH1C to mitochondria.

how these organelles maintain their genome stability has long been unclear. We recently discovered that R-loops act as regulatory centers in determining the stability of organelle genomes (Figure 5). R-loops play both positive and negative roles in maintaining the stability of the organellar genome, which not only causes genome instability by modulating head-on transcription-replication conflicts (Yang et al., 2017, 2020b) but also promotes DNA damage repair (Cheng et al., 2021; Wang et al., 2021b). However, our knowledge about how R-loop levels are sensed and adjusted to maintain normal organellar function is still in its infancy. For example, in cells lacking mitochondrion-localized AtRNH1B, chloroplast-localized AtRNH1C sensed high R-loop levels and relocalized to mitochondria via an unknown mechanism (Figure 5). This observation raises the following intriguing question: Do plants sense R-loops during chloroplast-mitochondria communication? Furthermore, how do plants coordinate and adjust R-loop levels inside and between cells, and how is this process managed in response to physiological and pathological processes?

Epitranscriptomic mRNA modification: a potent regulatory mechanism in plant development and stress responses

(Written by Hunseung Kang)

Epitranscriptomic RNA modifications, which are analogous to epigenetic regulation that involves DNA methylation and histone modifications, are emerging as a new layer of gene regulation. These modifications play a pivotal role in fine-tuning plant development and fitness to changing environmental cues. At least 160 mRNA modifications have been identified to date, among which N^6-methyladenosine (m^6A), N^1-methyladenosine (m^1A), and 5-methylcytidine (m^5C) are common and abundant internal modifications observed in coding RNAs; m^6A is the most prevalent internal modification in eukaryotic mRNAs (Boccaletto et al., 2018). Methyltransferases (referred to as "writers"), demethylases (referred to as "erasers"), and RNA-binding proteins (referred to as "readers") are cellular components responsible for the installation, removal, and interpretation of m^6A marks, respectively (Figure 6). Recent transcriptome-wide m^6A mapping, as well as the identification and characterization of m^6A writers, readers, and erasers in Arabidopsis and model crops, have enhanced our understanding of the dynamics, distribution, regulatory mechanisms, and biological functions of m^6A methylation in plant development and stress responses.

Transcriptome-wide analyses of m^6A methylation patterns in plants have led to the identification of an RR(m^6A)CH (R = A/G; H = A/C/U) motif found in all eukaryotes (Luo et al., 2014; Duan et al., 2017; Hu et al., 2021) and a URU(m^6A)Y (Y = C/U) motif unique to plants (Arribas-Hernandez et al., 2018; Hu et al., 2021; Arribas-Hernandez et al., 2021a; Arribas-Hernandez et al., 2021b; Hu et al., 2022). The presence of the plant-specific m^6A motif, as well as the common m^6A motif conserved across all eukaryotes, suggests that m^6A modifications exert multifaceted functions in plants.

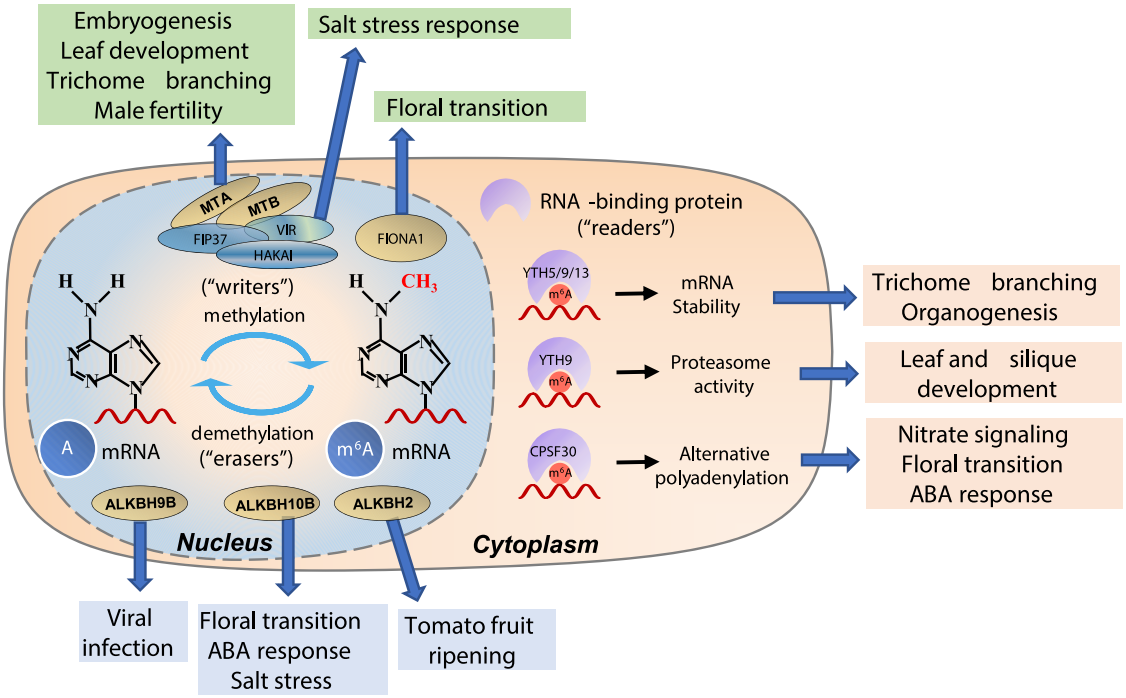

Figure 6 Regulatory roles of N^6-methyladenosine (m^6A) writers, erasers, and readers in RNA metabolism, plant development, and stress responses. The cellular components responsible for installation, removal, and interpretation of m^6A marks are methyltransferases (writers), demethylases (erasers), and RNA-binding proteins (readers), respectively. The m^6A reader proteins YTH5, YTH9, and YTH13 are also known as ECT4, ECT2, and ECT3, respectively. RNA methylation affects all aspects of RNA metabolism, including stability, export, intron splicing, and translational control, which are crucial for plant development and stress responses. Several potential m^6A erasers and readers are yet to be identified.

The m^6A writers responsible for these modifications include METHYLTRANSFERASE A (MTA), MTB, FKBP12-interacting protein 37 (FIP37), VIRILIZER (VIR), the E3 ubiquitin ligase HAKAI, and FIONA1 (FIO1) (Ruzicka et al., 2017); reviewed in (Hu et al., 2019; Xu et al., 2022a). The three m^6A erasers, AlkB homolog 2 (ALKBH2), ALKBH9B, and ALKBH10B, have been confirmed as m^6A demethylases (Duan et al., 2017; Martinez-Perez et al., 2017; Zhou et al., 2019) (Figure 6). YT521-B homology (YTH)-domain proteins have been characterized as m^6A readers that recognize m^6A marks and affect the stability, translation, nucleus-to-cytoplasm movement, and alternative polyadenylation of m^6A-modified transcripts (Arribas-Hernandez et al., 2018; Scutenaire et al., 2018; Wei et al., 2018; Hu et al., 2019; Song et al., 2021; Arribas-Hernandez et al., 2021a; Arribas-Hernandez et al., 2021b; Hou et al., 2022) (Figure 6). Dynamic and reversible m^6A methylation play vital roles in embryogenesis, morphogenesis, trichome morphology, root development, and fruit ripening (Ruzicka et al., 2017; Arribas-Hernandez et al., 2018; Hu et al., 2019; Zhou et al., 2019; Hu et al., 2022) (Figure 6). Accumulating evidence has highlighted the pivotal roles of m^6A modifications in plant growth and development. However, several questions, including the mechanism by which m^6A is added to, or removed from, mRNA transcripts in a growth stage-dependent manner and differentially regulates the abundance of transcripts crucial for plant development, remain unanswered.

Mapping and characterization of mRNA modifications in plant stress responses are currently at the nascent stage. Bioinformatics analyses revealed that the expression levels of m^6A writers, erasers, and readers change differentially in response to diverse stresses (Hu et al., 2019), suggesting a vital role for m^6A methylation in plant stress responses. Recent molecular evidence has established a link between mRNA modifications and transcript levels involved in plant stress responses (Hou et al., 2021, 2022; Hu et al., 2021). Notably, m^6A modifications play crucial roles in plant responses to diverse stresses, including salt, drought, and nutrient (nitrate) starvation, by affecting mRNA stability, alternative polyadenylation, and translation efficiency of stress-responsive genes (Hou et al., 2021, 2022; Hu et al., 2021). However, the precise mechanism underlying RNA modification-mediated gene regulation during stress adaptation requires further investigation. Therefore, the crucial aspects that remain unexplored are the mechanisms by which RNA modification patterns vary under specific stress conditions and the association of these modifications with stress-induced alterations in transcript and protein levels.

Most studies conducted thus far have focused on the cellular components responsible for RNA methylation and their roles in the nucleus and cytoplasm. Chloroplast and mitochondrial RNAs are highly m^6A-methylated, accounting for 98%–100% and 86%–90% of the transcripts in chloroplasts and mitochondria, respectively (Luo et al., 2014; Wang

et al., 2017b). Therefore, RNA methylation might likely exert crucial roles in plant organelles. However, the nature and identity of writers, erasers, and readers in chloroplasts and mitochondria, except m^4C and m^6_2A rRNA writers in chloroplasts, are largely unknown (reviewed in Manduzio and Kang (2021)). Analysis of chloroplast proteomes by liquid chromatography-tandem mass spectrometry and prediction of organelle-localized proteins have revealed that the m^6A writer components MTA, MTB, and FIP37 found in plant nuclei were also possibly localized in chloroplasts and mitochondria and several putative S-adenosyl methionine (SAM)-dependent methyltransferase proteins are present in the chloroplasts of Arabidopsis (reviewed in Manduzio and Kang (2021)). Further verification of the methyltransferase activity of these putative writer proteins, as well as the previously unknown erasers or readers in chloroplasts and mitochondria, will help elucidate the significance of RNA modifications in plant organelles.

Rapid progress in transcriptome-wide mapping and the identification of writers, readers, and erasers have unraveled the regulatory roles of m^6A modification in plant development and stress responses. Nonetheless, many challenges remain in mapping m^6A modifications at single-base resolution using recently advanced sequencing methods, including Nanopore direct transcriptome deep sequencing (RNA-seq), MAZTER-seq, m^6A-REF-seq (m^6A-sensitive RNA-Endoribonuclease-Facilitated sequencing), and miCLIP-seq (m^6A individual-nucleotide-resolution cross-linking and IP combined with high-throughput sequencing). Furthermore, characterizing novel cellular components of writers, readers, and erasers in crops will help firmly establish the molecular link between m^6A, crop productivity, and stress adaptation. Recent findings have suggested that m^6A is associated with LLPS, which expands the repertoire of regulatory mechanisms crucial for cellular responses to developmental and environmental cues (Scutenaire et al., 2018; Ries et al., 2019; Song et al., 2021). Integrating these molecular insights to the regulatory roles of m^6A modification with novel genome-editing technologies, including A-to-G base editing to modify potential m^6A sites and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease 13 (Cas13)-based targeted RNA methylation (Liu et al., 2019; Li et al., 2020a), will greatly facilitate epitranscriptomics research and lead to the development of a potential strategy for breeding stress-tolerant crops via precisely engineered RNA modifications. Further exploration of this field is warranted, and we anticipate exciting discoveries in the near future.

Decoding the grammar of plant long non-coding RNAs

(Written by Federico D. Ariel and Martin Crespi)

The inspection of the presence and combination of domains within a protein is generally a good starting point to infer its potential molecular action. This information is then complemented with subcellular localization studies, biochemical characterization, analysis of expression patterns of the encoding gene across multicellular organisms and genetic approaches to propose a biological role of the given gene in plants. By contrast, the comprehensive functional characterization of lncRNAs (Wierzbicki et al., 2021) is a challenging task that should take into account (i) their promiscuous or specific interaction with other molecules based on their sequence and/or structure; (ii) their redundancy with other unrelated transcripts; (iii) their subcellular localization; (iv) their role within molecular regulatory networks; and (v) an eventual RNA biological activity (Figure 7). In the last 15 years, thousands of lncRNAs have been annotated from a growing number of plant species, although their functional characterization lags behind, thus severely hindering the differentiation between transcriptional noise and biologically relevant non-coding transcripts. Identifying general molecular features linking specific lncRNAs with their targets have uncovered certain mechanisms. For instance, target mimicry of miRNAs (RNA molecules acting as decoy of miRNAs blocking their activity) was demonstrated for *INDUCED BY PHOSPHATE STARVATION 1* (*IPS1*) and could be later predicted *in silico* for other lncRNAs across species (Franco-Zorrilla et al., 2007). However, for the large majority of lncRNAs acting through other molecular mechanisms, there is no evident features to define their targets in order to dissect the molecular basis governing their action in plants.

Upon extensive annotation of lncRNAs across species, future screenings for biological functions, likely based on systematic CRISPR-derived approaches, may empower the selection of novel relevant lncRNAs for in-depth molecular characterization. In addition, integration of lncRNA expression patterns from transcriptomic data of multiple wild-type plants, mutants, and natural accessions in response to environmental and developmental cues will position the lncRNA of interest within particular regulatory networks driving plant development and/or adaptation to the environment.

Specific lncRNAs have been shown to interact with protein partners in ribonucleoprotein (RNP) complexes (modulating their stability, subcellular localization, or their activity), DNA (forming RNA–DNA duplexes known as R-loops), or other transcripts (such as antisense RNAs, forming paired RNA regions triggering mRNA degradation or promoting translation) (Lucero et al., 2021). Future research to generalize these interactions may include global identification of RNAs forming R-loops or interacting with specific RNP complexes involved in splicing modulation (Rigo et al., 2020) or the translational machinery (Bazin et al., 2017). Another emerging mechanism is the interaction of lncRNAs with chromatin-related proteins linked to epigenetic regulations such as LIKE HETEROCHROMATIN PROTEIN 1 (LHP1), CURLY LEAF (CLF), MEIOTIC F-BOX (MOF), ARABIDOPSIS TRITHORAX-LIKE PROTEIN1 (ATX1), or WD40 REPEAT 5A (WDR5a) (Fonouni-Farde et al., 2021) although their binding

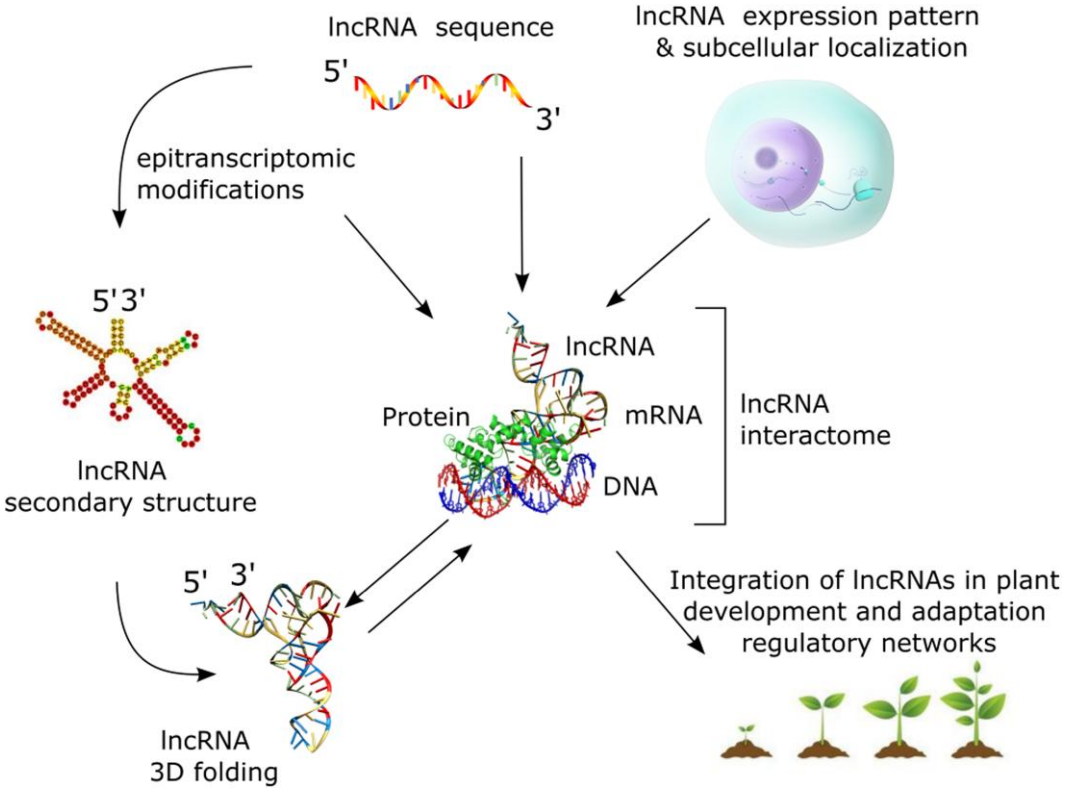

Figure 7 Plant lncRNA grammar is determined by the transcript interactome. Multiple features contribute to the interaction of lncRNAs with DNA, protein partners, or other RNA molecules. First, their expression pattern and their subcellular localization will restrict the range of potential partners. Second, the lncRNA-interacting capacity depends on its sequence, post-transcriptional modifications, and secondary and tertiary structure adopted, which is, in turn, modulated by the interaction with partner molecules. Third, the resulting lncRNA interactome participates in the regulatory networks behind plant development and adaptation to the environment as all these factors can be responsive to environmental cues.

specificity remains uncertain, or even with transcription factors (e.g. WRKY42, (Moison et al., 2021)). In addition, the identification of nascent RNAs (Kindgren et al., 2020) as well as chromatin-associated lncRNAs based on chromatin isolation and high-throughput sequencing techniques will further contribute to creating a matrix of lncRNA features underlying their function. Altogether, mapping lncRNA interactions with DNA, chromatin, and proteins involved in a wide range of mechanisms in model and crop plants should set the stage for a comprehensive classification of lncRNAs enabling the search of singularities and commonalities behind the functions of non-coding transcripts.

The identification of protein partners and lncRNA-interacting nucleic acids using biotinylated probes for the purification of lncRNA-containing complexes followed by mass spectrometry or DNA sequencing is an initial key goal to define the lncRNA interactome, despite the potential artifacts linked to these approaches (Machyna and Simon, 2018). Alongside the genome-wide identification of lncRNAs participating in alternative RNP complexes, the detailed characterization of selected lncRNA actions on these complexes remains essential to better understand the diversity of regulatory mechanisms involving non-coding transcription.

Another major question in lncRNA biology and biochemistry concerns transcript structure (Zhu et al., 2021). Secondary and tertiary structures of RNAs are very likely determinant features for their dynamic interaction with proteins and other partners. Considering that plants cannot modulate their body temperature, the structure of lncRNAs may serve as potential versatile molecules acting as thermosensors in order to rapidly adjust epigenomic features and alternative splicing, two major processes affected by ambient temperature (John et al., 2021; Perrella et al., 2022). A growing number of prediction tools based on classical and machine learning approaches have shed light on this field (Bugnon et al., 2022), although the biochemical characterization of individual or groups of plant lncRNAs is just starting. In general, genome-wide approaches for the mapping of double-stranded RNAs (dsRNAs) or chemical degradation profiles to reconstruct transcript structures fail to deliver enough data about low-abundance lncRNAs. However, in vitro transcription of selected lncRNAs followed by biochemical approaches ignores the enormous collection of epitranscriptomic modifications as well as their in vivo interaction with partner molecules, which are likely to affect RNA structure (Miller et al., 2022).

Similar to the study of metazoan lncRNAs, cell biology techniques, notably single molecule RNA (smRNA) fluorescence in situ hybridization (FISH) (Duncan et al., 2017), can contribute to our understanding of the mechanisms involving specific lncRNAs. As a complement to subcellular fractionation studies followed by high-throughput sequencing, smRNA FISH may not only indicate whether a given lncRNA accumulates in the nucleus or the cytoplasm, but also reveal its distribution in "speckles", or localization in specific loci, in subcellular compartments, in non-membranous organelles or particles. However, the technical difficulties related to the presence of cell wall barriers in plant tissues prevents the accessibility of fluorescent oligonucleotide probes, thus delaying the massive use of this approach by most plant RNA biology groups, in comparison to labs working on mammalian cell culture models.

The fields of plant RNA biology and biochemistry will need to integrate cell biology, RNP proteomics, genomic and genetic approaches to unveil the function and evolution of the non-coding transcriptome, in particular during differentiation and environmental stress responses. Evolutionary analysis at a global level (e.g. involving synteny) of lncRNAs exhibiting common features (e.g. integration into specific RNPs), together with the in-depth characterization of specific leading cases, will achieve a better understanding of the structures and sequences (likely very short) setting the specificity rules of their interaction with partner molecules. As the RNA interactome ultimately determines their function, these integrated approaches will hopefully help us uncover the grammar of plant lncRNAs.

Emerging and long-standing questions about miRNA biogenesis in plants

(Written by Axel Giudicatti and Pablo A. Manavella)

From the point of view of RNA biology, miRNAs are exciting molecules. Not only do mature miRNAs target other RNA molecules to block their translation or trigger their degradation, but their precursors undergo nearly all the regulatory features described in this article. For instance, many *MIRNA* genes contain introns that affect the processing of the primary transcripts (pri-miRNAs) (Stepien et al., 2017); the ribonucleotides of miRNA precursors can be modified or edited to change their regulatory outcome (Mingardi et al., 2018; Bhat et al., 2020); pri-miRNA secondary structure fluctuations define miRNA biogenesis (Wang et al., 2018b; Re et al., 2019); even asTSS_R-loops were recently shown to promote co-transcriptional processing of miRNAs (Gonzalo et al., 2022). These features make miRNAs a unique entity where many aspects of RNA biology converge. Even after more than 20 years of research, all these aspects of miRNA biology present unresolved questions and intriguing gaps in our knowledge. For instance, although we know that there is a crosstalk between splicing and pri-miRNA processing (Stepien et al., 2017), it is unclear how these two processes interact. The transcription and processing of pri-miRNAs is coupled (Fang et al., 2015; Gonzalo et al., 2022). This observation opens the possibility that the crosstalk between pri-miRNA processing and splicing only exists for miRNAs processed co-transcriptionally where both machineries, the spliceosome and microprocessor, meet. In this scenario, it is unclear whether the miRNA processing factors and splicing factors act cooperatively or simply interfere entropically with each other over the nascent pri-miRNAs during maturation. Advances in RNA sequencing technologies, especially of nascent RNAs, will help our understanding of how these two processes are connected (Figure 8C).

On its own, the discovery of coupling between transcription and miRNA processing, initially suggested by the ground-breaking work of Fang et al. (2015) and further confirmed in 2022 (Gonzalo et al., 2022), opened many exciting new avenues of inquiry. The recruitment of the microprocessor to *MIRNA* loci is a well-reported phenomenon (Fang et al., 2015; Cambiagno et al., 2021). However, how the microprocessor specifically recognizes these loci over any other Pol II-transcribed region remains an enigma. Still, the association of the microprocessor to *MIRNA*s requires the presence of the pri-miRNA transcript (Fang et al., 2015). Thus, it is possible that the Dicing complex recognizes the stem-loop structure within pri-miRNA transcripts, thereby giving specificity to the system. Co-transcriptional miRNA processing appeared favored in those loci containing asTSS_R-loops (Gonzalo et al., 2022). These three-stranded chromatin structures may also provide an initial signal promoting the recruitment of the microprocessor to these loci, although their functions in this process are still merely hypothetical. Nevertheless, this result raises the possibility that the three-stranded hybrid is the platform upon which the microprocessor is built. It will be interesting to study whether any of the proteins proposed to link the microprocessor to chromatin have affinity for R-loops, either for the single-stranded DNA or the RNA/DNA hybrid (Figure 8B). The assembly of the processing complex also presents a challenging, but very relevant, problem to solve; which is the hierarchical order of recruitment of the microprocessor components to *MIRNA* loci? Another compelling question raised from the discovery of the processing of nascent pri-miRNAs is whether co-transcriptionally processed miRNAs have distinct functions. In this sense, it was recently shown that the protein HASTY (HST) is required for both the assembly of the microprocessor at *MIRNA* loci and to promote the non-cell-autonomous function of miRNAs (Brioudes et al., 2021; Cambiagno et al., 2021). It is therefore possible that miRNAs processed during transcription take a particular road that makes them mobile molecules (Figure 8E). Perhaps this pool of miRNAs somehow avoids loading into ARGONAUTE 1 (AGO1), an event proposed to lock miRNAs inside the cell, preventing their movement (Devers et al., 2020; Fan et al., 2022; Voinnet, 2022). It is curious that the precise mechanisms of miRNA movement between cells and whether such movement is chaperoned, still

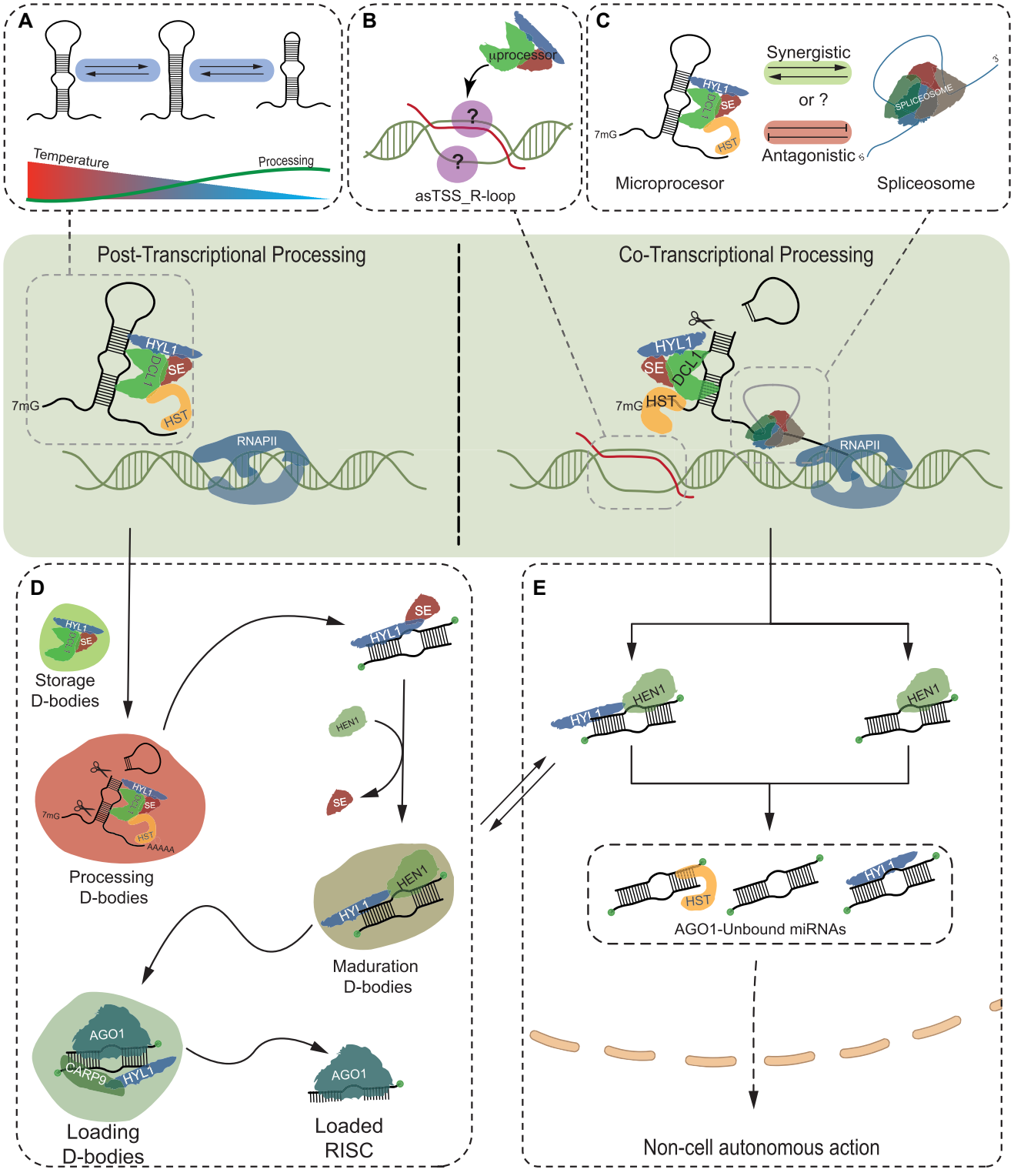

Figure 8 Unanswered questions of miRNA biogenesis. A, Can the alterations of processing efficiency caused by pri-miRNA refolding upon temperature change act as thermosensors during the plant response to heat? B, Can proteins specifically binding to the ssDNA or RNA/DNA strands of R-loops act as scaffold to recruit the microprocessor to *MIRNA* loci? C, How does the microprocessor and spliceosome interact? D, Can we define different D-bodies? And if so, can we establish the precise biochemistry within D-bodies during their maturation? E, Are co-transcriptionally processed miRNAs functionally different from their siblings produced post-transcriptionally, perhaps defining mobile miRNAs?.

remains unknown. In fact, this question is probably one of the longest-standing questions in the field.

Among the four DICER-Like (DCL) enzymes in *Arabidopsis*, DCL1 is the main actor in miRNA processing, due to its nuclear localization and preference to process imperfect stem-loop folded RNAs. Within the pri-miRNA stem-loop, DCL1 recognizes structural features that guide processing to release a unique miRNA duplex (Bologna et al., 2013; Manavella et al., 2019). It was recently shown that the folding of pri-miRNAs can be altered, consequently modifying processing efficiency (Wang et al., 2018b). In addition, nucleotides at pri-miRNAs can be modified and even edited, although the influence that these events have over the miRNA processing were not demonstrated in plants (Mingardi et al., 2018; Bhat et al., 2020). The role of RNA editing, modification, and refolding in miRNA processing are just emerging as important regulatory mechanisms and deserve our attention. It is expected, as plants are non-thermogenic organisms, that the secondary structure of plant pri-miRNAs will fluctuate with ambient temperature, likely affecting their processing. Thus, it can be envisioned that some miRNAs may even act as thermosensors (Figure 8A). Although we do have some evidence that temperature changes how miRNAs are processed (Re et al., 2019), much more needs to be done on this subject.

D-bodies are one of the most intriguing elements in the miRNA pathway. These discrete membraneless nuclear speckles are the typical localization of many fluorescently tagged miRNA biogenesis proteins (Fang and Spector, 2007). The localization of these proteins led to the proposal that D-bodies are the center of miRNA processing in plants. A recent study showed that D-bodies arise through SERRATE (SE)-mediated phase separation (Xie et al., 2021). Disruption of SE phase separation, and thus D-body formation, by deleting the N-terminal intrinsically disordered region (IDR) of SE reduces miRNA accumulation, supporting the idea that D-bodies are sites of pri-miRNA processing. The role of D-bodies in miRNA processing is also supported by several studies showing a correlation between D-body formation and miRNA production. Intriguingly, other studies have shown that the disappearance of D-bodies does not affect the ability of the cell to produce miRNAs (discussed by Mencia et al. (2022)). This observation suggests that D-bodies are not the sole place of miRNA processing and raises the possibility that compensatory mechanisms act to offset the reduction of miRNAs caused by the loss of D-bodies. While we now know that miRNA can be processed co-transcriptionally (Fang et al., 2015; Cambiagno et al., 2021; Gonzalo et al., 2022), many pri-miRNAs are partially or entirely processed in the nucleoplasm, likely in D-bodies (Gonzalo et al., 2022). Thus, a balance between these two processing sites may buffer any fluctuation in processing and maintain stable levels of miRNAs. Given the current data, it is hard to simply categorize D-bodies as the only place where miRNA biogenesis occurs. It is also possible that D-bodies are not unique entities but rather a collection of small micro-reactors of different compositions and functions (Figure 8D). This idea goes along with the finding that despite localizing to D-bodies, some miRNA factors do not co-localize with each other (Tomassi et al., 2020). We previously discussed several possible scenarios for D-body functions (Mencia et al., 2022). Defining the nature and role of D-bodies and their crosstalk with co-transcriptional processing is at the frontier of miRNA research, although it is a technically challenging goal. Future studies applying state-of-the-art in vivo immunostaining and biochemical approaches will certainly surprise us with new discoveries about these nuclear speckles.

These are only a few of the many open questions regarding how miRNAs are produced and do not even consider the equally large number of questions we have regarding how miRNAs act once loaded into the RNA-induced silencing complex (RISC) and how these molecules are stabilized or degraded when necessary.

Cross-kingdom RNAi

(Written by Qiang Cai and Hailing Jin)

Over the years, studies on extracellular RNAs, including small RNAs (sRNAs), have focused mostly on their movement between cells and tissues within an organism (Liu and Chen, 2018; Huang et al., 2019). Naturally occurring sRNA trafficking across organismal boundaries between interacting organisms that induces gene silencing in the counter party, a biological phenomenon named cross-kingdom/species RNA interference (RNAi), was first described in plant–fungal interactions (Weiberg et al., 2013). During infection, the gray mold fungal pathogen *Botrytis cinerea* delivers its sRNAs, called sRNA effectors, into plant cells and hijacks the plant RNAi machinery to silence those host genes that are involved in plant immunity (Weiberg et al., 2013). Similar phenomena were later observed in many plant pathogens and parasites. For example, sRNAs from the fungal pathogens *Verticillium dahlia* (causing verticillium wilt of cotton [*Gossypium hirsutum*]) and *Puccinia striiformis* (causing stripe rust of wheat [*Triticum aestivum*]) can move into their plant host and silence plant defense genes (Wang et al., 2016, 2017a). Similarly, oomycete pathogens, such as *Hyaloperonospora arabidopsidis* (causing downy mildew of Arabidopsis) (Dunker et al., 2020), also utilize cross-kingdom RNAi to achieve aggressive infection. Furthermore, miRNAs from parasitic plant dodders (*Cuscuta campestris*) act as cross-species regulators of gene expression in their plant hosts, suggesting that mobile sRNAs act as virulence factors during parasitism (Shahid et al., 2018). Cross-kingdom RNAi is not limited to pathogenic interactions but also exists in symbiotic interacting systems. A recently discovered fungal miRNA from the beneficial ectomycorrhizal fungus *Pisolithus microcarpus* enters *Eucalyptus grandis* root cells and stabilizes the symbiotic interaction by silencing several nucleotide-binding (NB)-ARC domain-containing proteins from the host (Wong-Bajracharya et al., 2022). Even for prokaryotic

pathogens that do not have a canonical RNAi pathway, rhizobial tRNA-derived short RNAs act as functional sRNAs moving into plant cells to silence nodulation-related target genes (Ren et al., 2019). Most strikingly, the molecular mechanism underlying cross-kingdom RNAi is also conserved. The sRNAs from the fungal pathogens *B. cinerea* and *V. dahlia*, the oomycete pathogen *H. arabidopsidis*, and the rhizobium were all found to be loaded into the plant host AGO1 to silence host target genes (Weiberg et al., 2013; Wang et al., 2016; Ren et al., 2019; Dunker et al., 2020).

Recent studies have shown that cross-kingdom RNAi is bidirectional, and many plant species can also transport endogenous sRNAs into their interacting pathogens (Cai et al., 2021; Liu et al., 2021). For example, Arabidopsis plants send miRNAs, phased secondary small interfering RNAs (phasiRNAs), and other endogenous short interfering RNAs (siRNAs) into interacting *B. cinerea* cells (Cai et al., 2018). These transported host sRNAs can silence *B. cinerea* virulence-related genes, many of which are involved in fungal vesicle-trafficking pathways (Cai et al., 2018). Cross-kingdom sRNA trafficking from host plants into pathogens was also observed in other plant–fungal systems, such as cotton-*V. dahliae* and wheat-*F. graminearum* interaction systems (Cai et al., 2021).

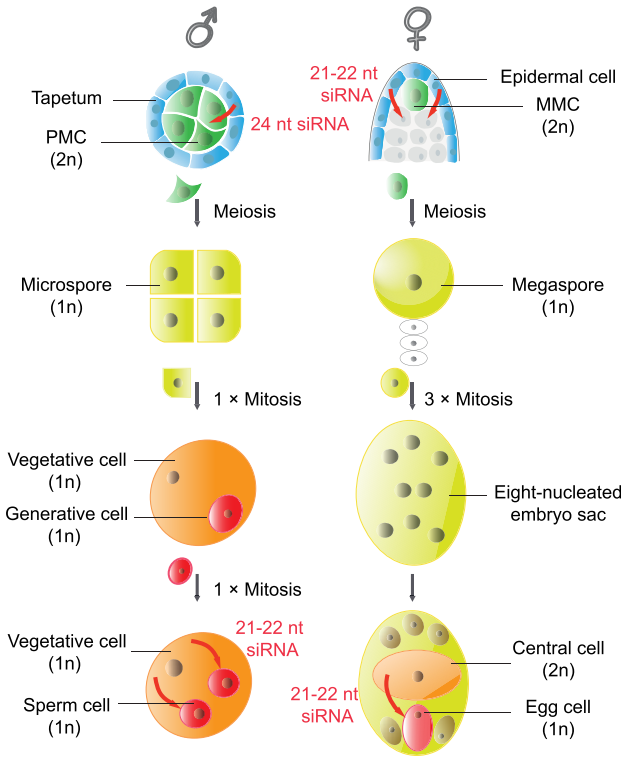

Figure 9 siRNA movement during Male and female germline development in Arabidopsis. Here we use a more relaxed definition of the germline to indicate the cell lineage that undergoes meiosis and produces the gamete(s). Arrows mark the direction of the proposed siRNA movement. PMC, pollen mother cells; MMC, megaspore mother cells.

It has been demonstrated that plant sRNAs are transported into fungal cells mainly by extracellular vesicles (EVs) (Cai et al., 2018). EVs are heterogeneous membrane-encapsulated structures that transport different RNA and protein cargoes between cells (Mathieu et al., 2019). EVs play an important role in sRNA trafficking between cells and tissues in both animal and plant systems (Cai et al., 2019; Mathieu et al., 2019). Like animal cells, a heterogeneous population of EVs exists in plants (Cai et al., 2019; Huang et al., 2021b). In Arabidopsis, a distinct class of EVs, called tetraspanin (TET)-positive exosomes, are responsible for secretion and transport of functional sRNAs and play a significant role in cross-kingdom RNAi and plant–microbial interactions (Cai et al., 2018; He et al., 2021).

How specific plant sRNAs are selectively loaded into EVs has long remained poorly understood. A recent study identified a list of EV-localized RNA-binding proteins, including AGO1, DEAD-box RNA helicases (RH11, RH37, and RH52), and ANNEXIN 1 and 2 (ANN1 and ANN2) (He et al., 2021). AGO1, RH11, and RH37 were shown to selectively bind to a set of sRNAs that are found in EVs, and contribute to selective sRNA loading into EVs, mostly TET-positive exosomes, whereas ANN1/2 bind to RNAs non-specifically. The level of sRNAs is reduced in EVs isolated from *ann1 ann2* mutants, which indicates that ANN1/2 are involved in sRNA stabilization in EVs, although they do not contribute to selective sRNA loading (He et al., 2021).

Research on cross-kingdom RNAi and sRNA trafficking is still in its infancy, and increasing studies demonstrate that mobile RNA molecules are important regulatory elements of the interaction between hosts and interacting organisms (Huang et al., 2019). Bidirectional cross-kingdom RNAi has been developed during the co-evolutionary arms race between hosts and pathogens, which has become a widespread molecular regulatory mechanism in plant–microbial interaction and plays a significant role in host immunity and pathogen virulence (Huang et al., 2019). Current studies show that EVs are essential in transporting sRNAs from the plant hosts to pathogens (Cai et al., 2021). EV-mediated sRNA transport has evolved in both plant and animal systems, suggesting that it is likely a conserved mechanism for cell-to-cell communication. The current understanding of cross-kingdom RNA transport is just the tip of the iceberg. Many questions remaining to be answered in this field are: i) Can pathogen sRNAs act as effector molecules, and can plants sense them as pathogen-associated molecular patterns (PAMPs)? ii) Do plant EVs also transport other classes of RNAs, i.e. mRNAs and lncRNAs, into pathogen cells to inhibit virulence? iii) Are there other mechanisms by which plant RNAs are selectively loaded into EVs? iv) Do fungal pathogens also utilize EVs to deliver RNAs into host plants? v) Besides EVs, do other pathways contribute to cross-kingdom RNA transport? A better understanding of RNA communications between interacting organisms will contribute to the development of new strategies for disease control and crop protection, such as EV-based sRNA fungicides.

Why does germline development require specialized small RNAs?

(Written by Xiaoqi Feng)

Small interfering RNAs (siRNAs) move between cells and exert regulatory functions during plant and animal development (Chen and Rechavi, 2022). Specialized, somatically produced siRNAs play essential roles during plant germline development. Similarly, a special army of siRNAs operates in the animal germline, called Piwi-interacting sRNAs (piRNAs) (Ozata et al., 2019). A central question arising from these reproductive-cell-specific siRNAs is why such specificity? What is intrinsic about sexual reproduction that requires specialized siRNAs? This is arguably one of the most exciting questions in RNA and reproductive biology. As these siRNAs have diverse, pleiotropic roles during reproductive development, investigation of multiple eukaryotic lineages is necessary to resolve this question.

Pioneering evidence of soma-germ siRNA movement in plants came from Arabidopsis pollen where 21-nt siRNAs associate with derepressed transposable elements (TEs) in the sperm companion cell, the vegetative cell (Slotkin et al., 2009) (VC; Figure 9). These siRNAs, but not the TE transcripts, accumulate in the sperm cell, suggesting that VC siRNAs can move into the sperm to reinforce TE silencing (Figure 9). Such TE reactivation (and hence associated siRNAs) is likely confined to gamete companion cells, as it is largely driven by a DNA demethylase, DEMETER (DME) (He et al., 2019), whose encoding gene is specifically expressed in companion cells (Feng et al., 2013). Indeed, TEs that are demethylated by DME in the VC are hypermethylated in sperm (where *DME* is not expressed) in a DME-dependent manner (Ibarra et al., 2012). *DME* is also expressed in the egg companion cell, the central cell, and likely activates siRNAs moving into the egg (Ibarra et al., 2012; Feng et al., 2013) (Figure 9).

Since the above-mentioned study by Slotkin *et al.*, it has become clear that somatic cells surrounding the germline produce distinct populations of siRNAs. An example is a variant form of the small RNA-directed DNA methylation pathway (RdDM) in meiocyte nurse cells (the tapetum; Figure 9). RdDM methylates TEs using 24-nt siRNAs transcribed by RNA Polymerase IV (Pol IV), which is recruited by putative chromatin remodelers, CLASSY1-4 (CLSY1-4). Somatic tissues mainly express *CLSY1* and *CLSY2*, and their proteins recruit RdDM to thousands of repeats. In tapetal cells and ovules, *CLSY3* is expressed at much higher levels than *CLSY1/2*, leading to a distinct 24-nt siRNA profile with the vast majority of siRNAs coming from a few hundred loci (Long et al., 2021; Zhou et al., 2022b).

Although these germline siRNAs were discovered due to their roles in TE silencing, increasing evidence links them to gene regulation, for example, during pollen development in *Capsella* (Wang et al., 2020). 24-nt siRNAs produced by tapetal cells methylate genes with similar but not identical sequences in male meiocytes (Walker et al., 2018; Long et al., 2021) (Figure 9), thereby regulating the splicing of a meiotic gene and facilitating meiosis (Walker et al., 2018; Long et al., 2021). As the TE-silencing and gene regulatory functions of germline siRNAs go hand in hand, it is tantalizing but difficult to tease apart which is the primary function, if such a distinction is possible.

Compounding the complexity, germline siRNA biogenesis varies among plant species. Although Arabidopsis meiotic 24-nt siRNAs are produced by Pol IV and RdDM, similarly abundant 24-nt phased secondary siRNAs (phasiRNAs) in maize and rice tapetal cells are produced by cleavage of non-coding Pol II transcripts by a miRNA (Liu et al., 2020). Monocot anther wall cells also accumulate an earlier wave of 21-nt phasiRNAs. Both 21-nt and 24-nt phasiRNAs have been proposed to move into meiotic cells and are important for male fertility, especially under certain environmental conditions, although it is still unclear why (Liu et al., 2020; Zhou et al., 2022c).

Another challenge is to elucidate the link between siRNA-mediated gene regulation and germ cell differentiation. The most well-understood example is the differentiation of female meiocytes, called megaspore mother cells (MMCs). Normally, only one subepidermal (L2) cell adopts MMC fate and undergoes meiosis in each ovule (Figure 9). Multiple MMCs differentiate in mutants of RdDM or 21-22 nt *trans*-acting siRNA (tasiRNA) pathways (Olmedo-Monfil et al., 2010; Su et al., 2020). Key components of both pathways are specifically expressed in apical epidermal (L1) cells, suggesting that these L1-produced siRNAs are essential for suppressing MMC fate in L2 cells (Figure 9). Importantly, causal links were made between L1-produced tasiRNAs, the repression of *AUXIN RESPONSE FACTOR 3* (*ARF3*) in L2 cells, and the suppression of MMC fate (Su et al., 2017, 2020). However, this is unlikely the sole regulatory mechanism for MMC differentiation, as mutations of other epigenetic pathways, such as METHYLTRANSFERASE 1 (MET1)-mediated DNA methylation maintenance (Li et al., 2017), also cause a similar supernumerary MMC phenotype. An indirect mechanism is also plausible, e.g. failure of epigenetic silencing interferes with MMC meiosis or function, which activates neighboring cells to adopt MMC fate as a compensating mechanism.

A converging feature of germline siRNAs is their non-cell-autonomy, which raises the question of why germ cells do not produce the siRNAs themselves, but instead rely on neighboring companion/nurse cells. Many ideas have arisen: perhaps siRNA biosynthesis exposes certain risks as it generally involves transcription of TEs, or nurse cells might afford to sensitize their chromatin environment to unfurl their genome and reveal potentially hazardous TEs (Feng et al., 2013), or maybe it is a question of why not, as nurse cells are already geared to provide a wide range of nutrients and other molecules to germ cells. These are exciting concepts ripe for exploration.

For Arabidopsis tapetal siRNAs, non-cell-autonomy may allow more precise control of germline transcriptional

regulation. Canonical RdDM is self-reinforcing, as DNA methylation promotes the generation of methylation-inducing siRNAs by recruiting Pol IV. The methylation arm of RdDM is tuned more aggressively in meiocytes to target broader sequences, which allows the targeting of genes and fast-evolving TEs (Long et al., 2021). However, given the self-reinforcing nature of RdDM, this broad-targeting ability needs to be tightly controlled to prevent the long-term establishment of RdDM at inappropriate genomic regions. Such control is achievable by cellular compartmentalization: 24-nt siRNA biogenesis is confined to the tapetum, whereas broad-targeting competence is restricted to male meiocytes (Long et al., 2021).

Understanding how germline siRNAs move between cells remains technically challenging. Plasmodesmata provide symplastic connections between daughter cells and are known to prevail in several scenarios of germline siRNA movement (Liu et al., 2020; Long et al., 2021). However, in which form(s) and how does the silencing signal move (Chen and Rechavi, 2022)? Furthermore, one cannot exclude the possibility of an apoplastic transport mechanism (reviewed before in the context of cross-kingdom RNAi), warranting further investigation.

Finally, germline siRNAs undoubtedly have functions beyond those in germ cells. siRNAs in sperm can act as quantitative measures of paternal genome dosage, whose imbalance with maternal dosage causes seed abortion (Wang et al., 2018a). Similarly, encountering gamete siRNAs in the zygote could, in theory, assess the compatibility of parental genomes, leading to hybridization barriers (Bourc'his and Voinnet, 2010). Although debated, endosperm siRNAs have also been proposed to move into the embryo, where they may exert a transgenerational effect (Bourc'his and Voinnet, 2010). siRNA pathways are known to be environmentally sensitive and malleable. Thus, germline siRNAs might be inherited by the next generation to facilitate memory of the environment and regulate the development of the offspring accordingly. The transgenerational effect of siRNAs (if any) remains an exciting area for future investigation.

The roles of small RNAs in the regulation of agronomic traits of crops

(Written by Yijun Qi)

Our knowledge of the biogenesis, action mode and biological roles of small RNAs has mostly been obtained from studies in Arabidopsis. However, findings in Arabidopsis cannot always be reasonably extrapolated to crops. Studies in crops, despite still being limited, have revealed that small RNAs play unexpected roles, particularly in the regulation of traits of agronomic significance.

Dozens of miRNAs have been shown to regulate crop development, metabolism, and stress responses. For instance, miR156, one of the most conserved miRNAs among plant species, regulates juvenile to adult transition in Arabidopsis (Wang et al., 2009; Wu et al., 2009a). However, in rice, miR156 not only helps shape plant architecture but also regulates grain development and filling (Jiao et al., 2010). The conserved miR396, which in Arabidopsis regulates plant development, targets and regulates the transcription factor gene *HaWRKY6* in sunflower (*Helianthus annuus*) during heat response (Giacomelli et al., 2012). There are many species-specific miRNAs in crops. For example, miR528, a monocot-specific miRNA, targets a number of genes involved in a variety of developmental processes or biotic and abiotic stress responses (Chen et al., 2019). How conserved miRNAs gain more regulatory functions and how species-specific miRNAs have been acquired by certain crops remain to be fully elucidated. Dissection of diversified roles of miRNAs in crops will greatly improve our understanding of the range of miRNA-mediated regulation.

In addition to canonical 21-nt miRNAs, there is a distinct class of 24-nt long miRNAs, referred to as lmiRNAs, in rice (Wu et al., 2009b). lmiRNAs regulate transcription via directing DNA methylation at target sites (Wu et al., 2010). It remains unclear how prevalent lmiRNAs are among crops. lmiRNAs that have been functionally characterized were all found to regulate rice biotic stress responses (Zhou et al., 2020; Jiang et al., 2020a; Campo et al., 2021). This result raises the question as to whether lmiRNAs evolved for plant stress responses and adaptation to environmental changes. Systematic identification of lmiRNAs and their target genes in different crops will be necessary for a better understanding of lmiRNA evolution and function.

Twenty-four-nt siRNAs are produced mainly from TEs and direct DNA methylation at target loci through RdDM. While Arabidopsis mutants lacking RdDM do not show obvious phenotypes, rice RdDM mutants have pleiotropic alterations, including dwarfism, an increase in rice tillering and a reduction in rice panicle size (Wei et al., 2014; Xu et al., 2020a). In maize, loss of 24-nt siRNAs leads to dwarfism, altered leaf polarity, and development of feminized tassels (Alleman et al., 2006). These findings indicate that 24-nt siRNAs are important regulators of agronomic traits in crops. The more prevailing regulatory role of 24-nt siRNAs in rice and maize could be explained by the fact that TEs are very abundant and dispersed in euchromatic regions in these plants, which greatly increases the likelihood that RdDM at TEs regulates nearby genes. Indeed, increased tillering in rice RdDM mutants is attributed to loss of RdDM at miniature inverted-repeat transposable elements (MITEs) near *MIR156d/j* and *D14*, which control rice tillering (Xu et al., 2020a). Interestingly, it has recently been shown that 24-nt siRNA can direct DNA methylation at imperfectly matched targets in Arabidopsis and cabbage (*Brassica rapa*) (Fei et al., 2021; Long et al., 2021; Burgess et al., 2022), which may greatly increase the range and complexity of RdDM-mediated gene regulation. For most 24-nt siRNAs, their tissue-specific expression, their targets, and the effects of their loss remain unknown.

PhasiRNAs, secondary siRNAs that are produced following miRNA-directed target mRNA cleavage, can be 21 or 24 nt in

length, depending on the miRNA trigger. PhasiRNAs are the predominant type of small RNAs in anthers in monocots, suggesting that they play a pivotal role in crop reproduction. Supporting this notion, loss of 21-nt phasiRNAs, or their activity, in rice leads to pollen sterility (Jiang et al., 2020b), and overproduction of 21-nt phasiRNAs at the *Pms1* locus results in photoperiod-sensitive male sterility, which allows the establishment of a two-line system for hybrid rice breeding (Fan et al., 2016). 21-nt phasiRNAs were found to facilitate the progression of meiosis by directing target mRNA cleavage (Zhang et al., 2020; Jiang et al., 2020b). As these targets are regulated for successful meiosis, investigation of their functions could be a shortcut to discovering genes and mechanisms important for crop reproduction. Loss of 24-nt phasiRNAs causes reduced pollen fertility and seed-setting rate in rice and temperature-sensitive male sterility in maize. There is some evidence supporting the idea that 24-nt phasiRNAs direct DNA methylation in *cis* (Zhang et al., 2021). Whether they can direct DNA methylation in *trans* and whether DNA methylation, if established, can be passed to next generation and regulates grain development remain to be explored.

tRNA-derived small RNAs (tsRNAs) and rRNA-derived small RNAs (rsRNAs) are two classes of small RNAs that have recently been identified. Whereas we still have limited information about the expression profile, modes of action, and biological roles of rsRNAs in plants, tsRNAs have been profiled in Arabidopsis (Ma et al., 2021). tsRNA levels appear to undergo dynamic changes in response to abiotic and biotic stresses. A 19-nt 5′ tsRNA produced from tRNA-Ala regulates anti-fungal defense in Arabidopsis (Gu et al., 2022). tsRNAs have not been well characterized in crops and their functions remain to be revealed. It will be also interesting to investigate whether they are widely involved in stress responses in crops.

Because many agronomic traits are controlled by small RNAs, manipulation of small RNA-mediated gene regulation has emerged as an important strategy to achieving desired agronomic traits. Unlike overexpressing or knocking out a gene, manipulation of small RNA activity allows us to fine-tune or precisely control the expression of a gene. Such changes in gene expression can be more physiologically relevant and may overcome side effects induced by all-or-nothing approaches. Thus, this offers a great new strategy to improve agronomic traits in crops.

Open questions in the study of RNA-directed DNA methylation

(Written by Craig S. Pikaard)

Eukaryotic cells protect themselves against TEs, viruses and other selfish genetic elements using RNAi pathways dependent on siRNAs. In plants, siRNAs range in size from 21 to 24 nt and mediate both post-transcriptional gene silencing (PTGS) and transcriptional gene silencing. RdDM is an important aspect of transcriptional gene silencing, involving siRNAs to bring about cytosine methylation of complementary DNA sequences (Erdmann and Picard, 2020). Chemical modifications of histone proteins also occur, in crosstalk with DNA methylation (Law and Jacobsen, 2010). Collectively, DNA and histone modifications result in chromatin environments that suppress promoter-dependent gene activation, but exactly how is not clear.

Most of what we know about RNA-dependent silencing in plants comes from studies of Arabidopsis. At least two pathways contribute to RdDM: an initiation pathway that acts on transcriptionally active transposons or invading viruses and a maintenance pathway that perpetuates cytosine methylation at thousands of transposon loci throughout the genome (Figure 10). The establishment pathway overlaps with the pathway for PTGS (Nuthikattu et al., 2013) and begins with transposon, virus, or transgene transcripts that are somehow recognized as being different from other cellular RNAs (Hung and Slotkin, 2021), triggering their conversion into double-stranded RNA (dsRNA) by RNA-DEPENDENT RNA POLYMERASE 6 (RDR6). The dsRNAs are then cut (diced) into 21- or 22-nt siRNAs by the Dicer-like endonucleases DCL4 or DCL2 and loaded into an Argonaute family protein, primarily AGO1 or AGO6 (Ariel and Manavella, 2021). siRNA-AGO1 complexes can bind complementary target mRNAs to cause their destruction or interfere with their translation, thus achieving PTGS. In parallel, 21-22-nt siRNAs bound to AGO6 guide low-level cytosine methylation at complementary DNA sequences in partnership with multisubunit RNA Polymerase V (Pol V) and the DNA methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2). Low-level methylation is not sufficient for transcriptional gene silencing but serves as a signal to recruit the machinery of the maintenance pathway, which accounts for the vast majority of RdDM activity (Figure 10). This pathway involves RNA Polymerase IV (Pol IV), RNA-DEPENDENT RNA POLYMERASE 2 (RDR2), DCL3, 24-nt siRNAs, AGO4, Pol V, DRM2, and numerous helper activities implicated in Pol IV or Pol V recruitment or chromatin modification and is dependent on 24-nt siRNAs (Figure 10).

The biogenesis of 24-nt siRNAs is understood in some detail, having been recapitulated in vitro (Singh et al., 2019) using purified enzymes whose structures have recently been resolved (Fukudome et al., 2021; Huang et al., 2021a; Wang et al., 2021a), yet questions still remain. Pol IV acts first in the pathway, presumably initiating RNA biosynthesis within the context of a melted DNA transcription bubble, as is the case for other DNA-dependent RNA polymerases. However, Pol IV is unable to sustain transcriptional elongation over more than ~12–16 nt into the double-stranded DNA beyond the initiation bubble (Singh et al., 2019), for reasons that are not yet clear. This behavior causes the polymerase to stall and then retreat, sliding backward along the DNA template as the template and non-template strands reanneal (Fukudome et al., 2021; Huang et al., 2021a), a phenomenon known as polymerase backtracking. As Pol IV backtracks, the 3′ end of its short (~30 nt) transcript becomes unpaired

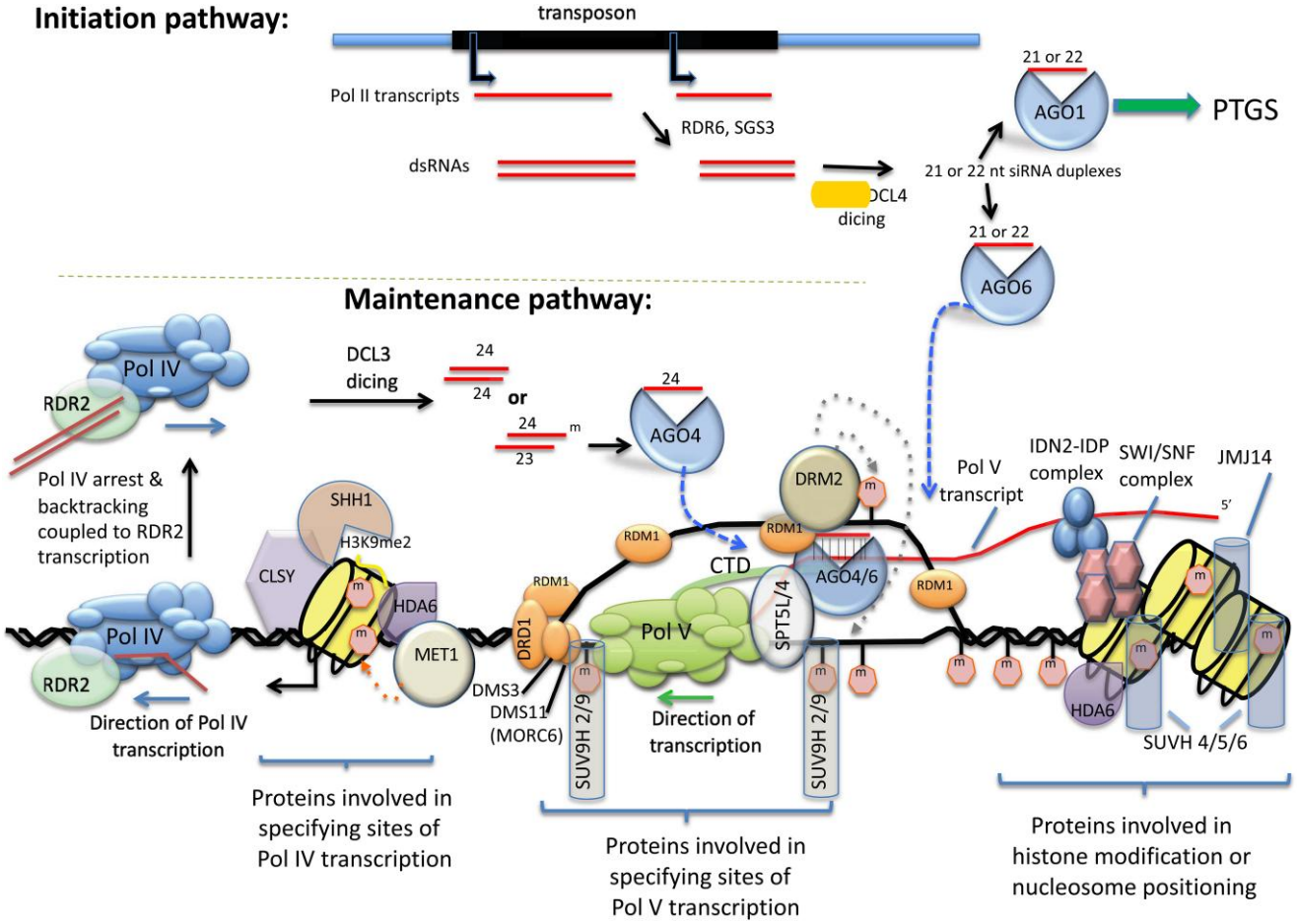

Figure 10 Establishment and maintenance of DNA methylation by RdDM. 21- and 22-nt siRNAs that are generated by DCL4 and DCL2 can bind to AGO1 to target mRNAs for post-transcriptional silencing (PTGS) or bind to AGO6 to initiate RdDM in partnership with Pol V and DRM2. The latter enzymes are also key to the major RdDM pathway that maintains silencing of thousands of loci and requires 24 -t siRNAs that are generated by the Pol IV-RDR2 complex and DCL3 and loaded primarily into AGO4. CG maintenance methylation, requiring MET1 and HDA6, is important for both Pol IV and Pol V recruitment, and correlates with histone H3 lysine 9 dimethylation (H3K9me2) among associated nucleosomes. Proteins that interact with these marks and are implicated in Pol IV or Pol V transcriptional activity are indicated, as are histone modifying enzymes involved in establishing repressive chromatin environments. The figure is an update of the transcription fork model originally published in 2013 (Pikaard et al., 2012), revised in 2017 (Wendte and Pikaard, 2017) and also adapted by other authors (Matzke and Mosher, 2014).

from the template DNA strand and is extruded and becomes engaged by RDR2 (Huang et al., 2021a), which uses the RNA as a template and initiates transcription 1–2 nt internal to its 3′ end (Fukudome et al., 2021). Whether the physical interactions of RDR2 with specific Pol IV subunits stimulate Pol IV backtracking and disfavors Pol IV elongation remains unclear, but is testable. Upon completing transcription of the Pol IV strand to generate a dsRNA, RDR2 has an intrinsic terminal transferase activity that adds an extra untemplated nucleotide to the 3′ end of its transcript, and then RDR2 releases the resulting dsRNA (Singh et al., 2019). Due to initiation by RDR2 internal to the 3′ end of the Pol IV transcript and its addition of an untemplated nucleotide to the 3′ end of its transcript, the resulting dsRNA has 3′ overhangs of 1–2 nt at each end. These overhangs, together with 5′ nucleotide preferences, program alternative DCL3 dicing reactions from either end of the dsRNAs, yielding siRNA duplexes that consist of a 24-nt strand paired with a 23-nt strand or a pair of 24-nt strands (Loffer et al., 2022) (Figure 10). In the case of 24/23 duplexes, the 23-nt RNAs serve as so-called passenger strands that help specify that the paired 24-nt strands are loaded into AGO4 to serve as guide strands (Wang et al., 2022). The passenger strand is then sliced by AGO and partially released. It is not clear how, or why, 24-nt siRNAs are specifically loaded as guide strands given that 21-, 22-, 23-, or 24-nt RNAs can be loaded into recombinant AGO4 and guide slicing of target RNAs with similar efficiency (Wang et al., 2022). One speculation is that a dsRNA-binding chaperone activity that can discriminate between 3′ overhangs of 1 or 2 nt orients the siRNA duplex such that the strand with the 2-nt overhang is loaded into AGO4 as the guide strand. In the case of asymmetric 24/23 duplexes, the 24-nt strand would be oriented to become the guide whereas for symmetrical 24/24 duplexes, with 2-nt overhangs at each end, guide

strand choice would presumably be random. Experiments are needed to test this hypothesis.

What happens following AGO4-siRNA loading is not clear. Early studies showed that AGO4 localization at RdDM loci is dependent on Pol V transcription, that AGO4 can be chemically crosslinked to Pol V transcripts (Wierzbicki et al., 2009) and that cytosine methylation occurs where siRNAs overlap sites of Pol V occupancy (Wierzbicki et al., 2012). Other studies have revealed that AGO4 can bind the C-terminal domain (CTD) of the Pol V largest subunit and/or the Pol V-associated protein, SPT5L (Suppressor of Ty insertion 5-like) (El-Shami et al., 2007; Bies-Etheve et al., 2009). Thus, AGO4-RNA and AGO4-protein interactions are both likely to be important, but whether they occur simultaneously or sequentially is unknown. And how does DNA methylation, and/or the histone modifications that correlate with DNA methylation, ensue from these siRNA-AGO4-Pol V interactions? There is co-IP evidence that DRM2 and AGO4 can directly interact (Zhong et al., 2014), but RdDM has not yet been achieved in vitro. Biochemical and structural studies that could reveal the spatial positions of the proteins, RNAs, and DNA strands when RdDM occurs would be break-through studies for the field.

Other major unanswered questions pertain to how Pol IV and Pol V transcription is initiated. Bacterial and archaeal multisubunit RNA polymerases, as well as eukaryotic RNA polymerases I, II, and III require DNA-binding transcription factors that recruit the polymerase to promoters, melt the DNA in the vicinity of the start site and position the polymerase to initiate transcription of one of the two DNA strands. However, conventional transcription factors and promoters have not been implicated in Pol IV or Pol V transcription. Instead, the evidence suggests that pre-existing chromatin modifications serve as recruitment signals, with cytosine methylation in the CG context, requiring MET1 and HISTONE DEACETYLASE 6 (HDA6) (Blevins et al., 2014), methyl cytosine binding by SUPPRESSOR OF VARIEGATION 3-9 HOMOLOG PROTEIN 2/9 (SUVH2/9), or binding of methylated histone H3 lysine 9 (H3K9) by SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) implicated in Pol IV and/or Pol V recruitment (Figure 10) (Erdmann and Picard, 2020). ATP-dependent DNA translocases are also implicated, including the CLSY protein family in the case of Pol IV and DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) in the case of Pol V. However, there is currently no biochemical evidence to suggest how promoter-independent DNA melting, polymerase positioning or transcription initiation occurs for Pol IV or Pol V. Once again, in vitro experiments with purified components will be needed to move from knowing the list of proteins involved to knowing what they do and how they work.

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