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# The initiation of RNA interference (RNAi) in plants

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When an mRNA enters into the RNA degradation pathway called RNA interference (RNAi), it is cleaved into small interfering RNAs (siRNAs) that then target complementary mRNAs for destruction. The consequence of entry into RNAi is mRNA degradation, post-transcriptional silencing and in some cases transcriptional silencing. RNAi functions as a defense against transposable element and virus activity, and in plants, RNAi additionally plays a role in development by regulating some genes. However, it is unknown how specific transcripts are selected for RNAi, and how most genic mRNAs steer clear. This *Current Opinion* article explores the key question of how RNAs are selected for entry into RNAi, and proposes models that enable the cell to distinguish between transcripts to translate versus destroy.

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## Introduction

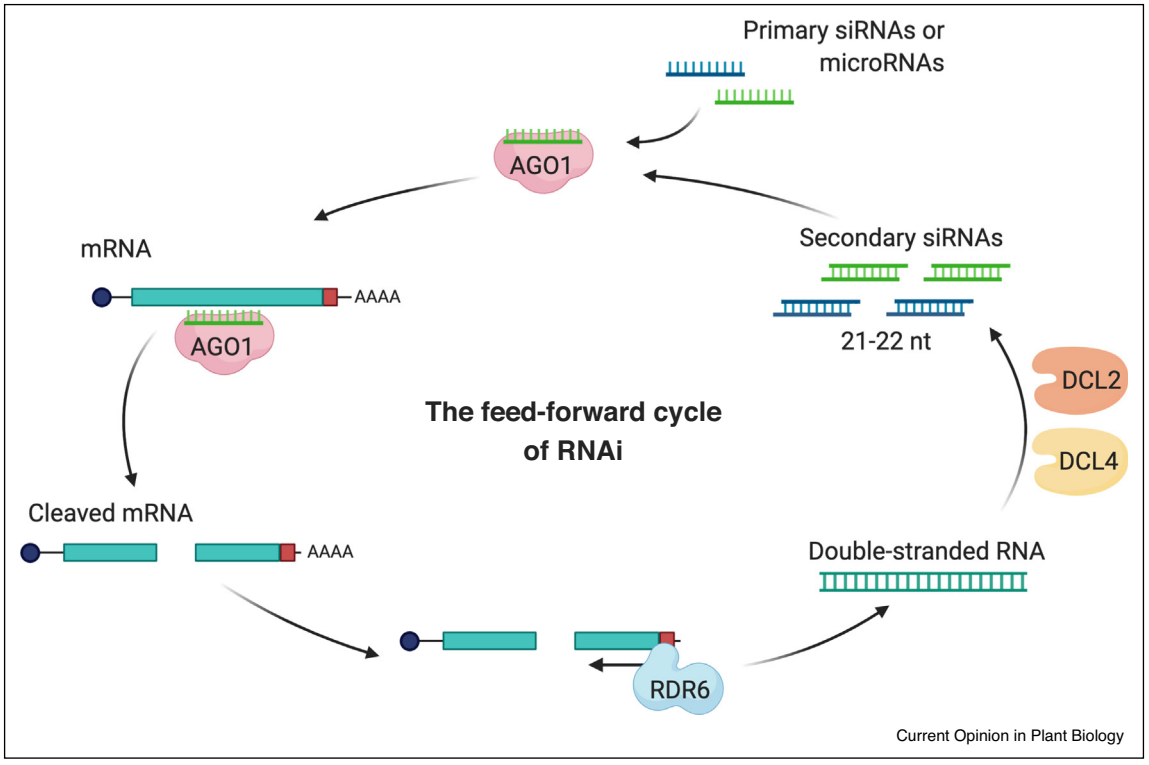
After transcription, there are a number of regulatory steps that guide mRNAs to different fates. The primary fate of genic mRNAs is translation into proteins and eventual decay. mRNA decay is a constant process that removes mRNAs that have either translated a number of times, or become stuck in the first round of translation [1,2]. The recruitment efficiency of the mRNA degradation machinery to the mRNA therefore determines the longevity of an mRNA. mRNA decay starts from the ends of the mRNA, which involves the removal of the polyA tail by deadenylases and 5′ cap by decapping enzymes, then the subsequent 5′ > 3′ and 3′ > 5′ degradation of the mRNA body by exonucleases [3–6].

The key difference between the degradation of mRNA by decay versus the distinct pathway of RNA interference (RNAi) is that the degradation products generated during RNAi have an additional role to target the next round of transcript degradation. In RNAi, rather than exonucleases degrading the mRNA from the ends, the RNA takes on a double-stranded (dsRNA) conformation, which is the substrate for DICER-family proteins to process the dsRNA into small interfering RNAs (siRNAs) 21-22 nucleotides (nt) in length [7,8] (Figure 1). The siRNAs are incorporated into an ARGONAUTE (AGO)-family protein, and they provide AGO with sequence specificity for which mRNA to target through the base-pairing complementarity of the siRNA. In Arabidopsis, the two AGO proteins most often associated with RNAi are AGO1 (which is also used for microRNA function) and AGO2 [9]. After the AGO protein incorporates either a siRNA or miRNA, it associates with other proteins to form the RNA-Induced Silencing Complex (RISC) [9]. The RISC will either translationally repress or cleave the target mRNA. A cleaved mRNA fragment can be the template of another round of dsRNA formation by an RNA-dependent RNA Polymerase (RdRP) family protein, starting the second cycle of RNAi and producing secondary siRNAs (Figure 1). RNAi is a feed-forward self-reinforcing cycle of mRNA degradation and siRNA production (Figure 1). Ultimately, the consequence of RNAi is the amplification of siRNAs which act to further degrade similar mRNAs, lowering the level of their translated protein in a process called post-transcriptional silencing.

In contrast to most genic mRNAs that are decayed, mRNAs produced from transposable elements (TEs), viruses and a few plant endogenous genes are targeted by RNAi and post-transcriptionally silenced. When TEs are transcriptionally activated, their mRNAs can be processed by RNAi, repressing TE activity and re-targeting the TE for chromatin modification through a process of small RNA-directed DNA methylation [10–12]. RNA viruses, which often take a dsRNA form, are processed by RNAi, while mutants of RNAi are highly susceptible to viral infection [13–16]. In addition, RNAi in plants functions on a set of endogenous loci and produces siRNAs with a common periodic or 'phased' pattern. Termed phasiRNA loci, these are often disease resistance genes, and in some cases the secondary siRNAs produced from phasiRNA loci can target a second gene's mRNA. These gene regulatory phasiRNAs are termed trans-acting siRNAs (tasiRNAs), some of which have been selected to play a key role in leaf expansion and other aspects of normal plant development and defense [17–20].

Central to this *Current Opinion* article is the molecular branchpoint that differentiates RNA decay from RNAi.

**Figure 1**




The feed-forward cycle of RNAi.
ARGONAUTE1 (AGO1) targets mRNAs for cleavage (slicing) guided by the sequence complementary of an incorporated primary siRNA or microRNA. The RNA-dependent RNA Polymerase RDR6 then recognizes the sliced mRNAs and produces double-stranded RNA (dsRNA). The dsRNAs are cleaved into secondary siRNAs by the DICER-LIKE family proteins DCL2 and DCL4. The secondary siRNAs can feed into AGO1 and target additional mRNAs for cleavage, resulting in a feed-forward cycle of RNAi that results in post-transcriptional silencing. Navy circle – 5′-cap; aqua box – open reading frame; red box – terminator sequence. All figures created with BioRender.com.

Both processes start by degrading an RNA, but their functional outcomes are very different: RNA decay is not amplified and therefore allows for future mRNAs of this gene to be translated into proteins, while RNAi leads to the continued and amplified targeted destruction of mRNAs that possess this sequence, and in some cases transcriptional silencing of the locus that generates these RNAs. A key example of how important this branchpoint is can be found when investigating the processing of transgene-derived mRNAs. These transcripts routinely enter RNAi rather than or in addition to the pathway of translation and decay [21,22]. The functional consequence of transgene RNAi is the undesired post-transcriptional silencing of the desired transgene product and trait. The goal of this *Current Opinion* article is to review how RNAs are selected to enter into RNAi, placing them on a path to repression and silencing rather than translation and decay.

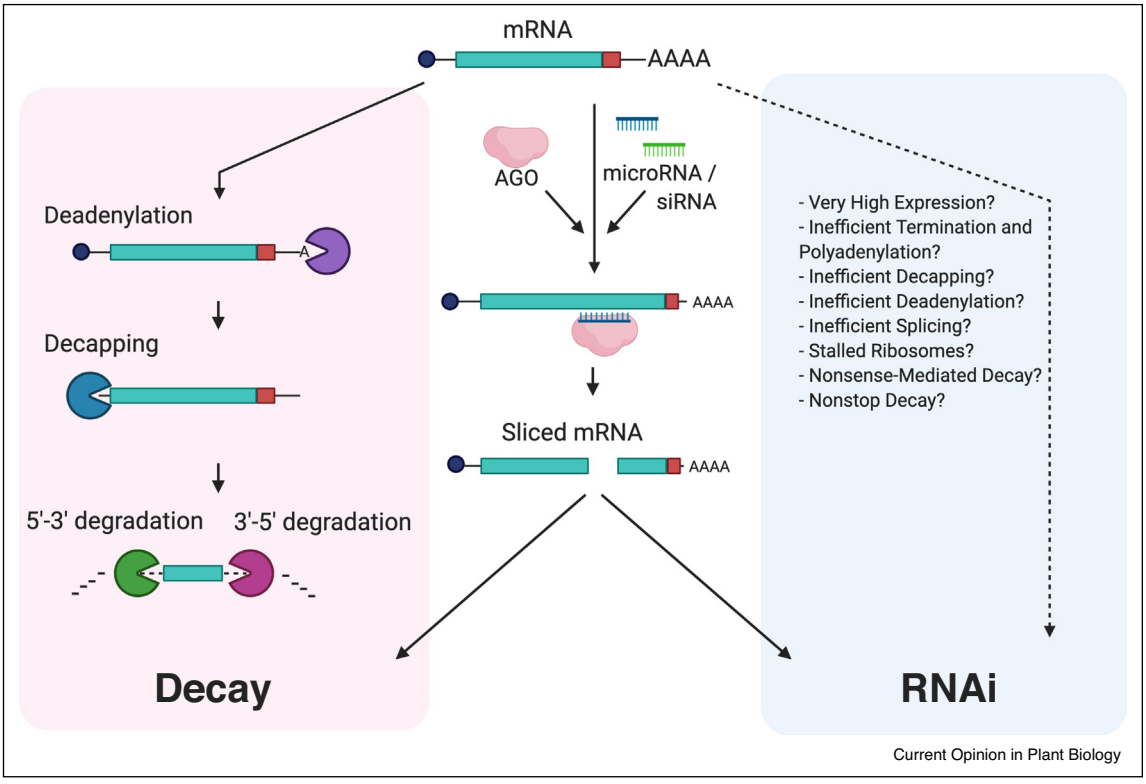
## Entry of cleaved mRNAs into RNAi

The investigation into how particular mRNAs are selected to undergo RNAi is best understood for mRNAs that have already been sensitized for RNAi by internal cleavage by an endonuclease. One such endonuclease is the RISC complex itself, which can be guided to internally cleave (slice) an mRNA guided by the incorporated microRNA or siRNA. The resulting sliced mRNA can then either be degraded by exonucleases (decayed) or converted into dsRNA by RdRP proteins. This processing pathway branchpoint therefore determines whether this mRNA is decayed (while other copies of this mRNA are left to translate) or enters into the feed-forward cycle of RNAi where copies of this mRNA are targeted by secondary siRNAs (Figure 2).

### Small RNA triggers of RNAi

Whether the sliced mRNA enters into RNAi is programmed by the microRNA or primary siRNA itself. Three non-mutually exclusive mechanisms exist. First, Axtell *et al.* [23] proposed that mRNA transcripts that are targeted by two distinct microRNAs will enter into RNAi. Generally, both of these microRNAs will cleave the target before entry into RNAi, but in this 'Dual-Hit' model, only one of the two microRNAs needs to cleave the target,

**Figure 2**




Decision point for entry of RNAs into RNA decay or RNAi.
Two potential outcomes for mRNAs: decay versus RNAi. AGO-sliced mRNAs fit between these two categories, and can potentially enter either pathway. The decay pathway is well-studied and the molecular mechanisms have been defined. In contrast, why and how particular RNA transcripts enter into RNAi without first being sliced by an AGO protein remains enigmatic (dashed line) due to the large number of triggers described in the literature (bullet points).

while the other can be cleavage-deficient and still cause the mRNA to enter into RNAi. This mechanism would allow for nearly all genic mRNA targets of microRNAs to escape RNAi because they are only targeted at a single site, while mRNAs that are targeted multiple times such as TE mRNAs will enter into RNAi [23]. The second mechanism is based on the amount of asymmetry in the microRNA duplex structure. Manavella *et al.* [24] engineered the length, number of non-base paired bulges and bulge position in microRNA duplexes and observed that as long as the microRNA duplex has an asymmetrical structure, it is sufficient to trigger RNAi. These findings prompted the authors to propose that the microRNA duplex structural asymmetry reprograms the RISC to target RNAi even before the passenger microRNA strand is released.

The third mechanism is the size of the microRNA/siRNA itself. Most microRNAs are 21 nt and do not target RNAi. Some microRNAs are 22 nt, and the targets of these 22 nt microRNAs correlate with the production of secondary siRNAs [25,26]. In 2010, two papers demonstrated that this one nucleotide size difference was sufficient to mediate RNA decay of the sliced RNA (21 nt) versus RNAi (22 nt) [25,26]. This mechanism provides several interesting potential points of regulation. First, selection of microRNA length will differentiate between the functional outcome of the sliced mRNA. Second, since 22 nt siRNAs are typically generated by the Dicer family protein DICER-LIKE 2 (DCL2), and the 21 nt siRNAs by DCL4, these two proteins can act as an accelerator (DCL2) and brake (DCL4) on the strength of the RNAi response. DCL2 and DCL4 function on the same dsRNA substrates, and they are known to have an overlapping yet hierarchical relationship [27,28]. Therefore, the amount of 22 nt siRNAs observed by RNA gel blot or small RNA sequencing is often used as a measurement of the strength of ongoing RNAi.

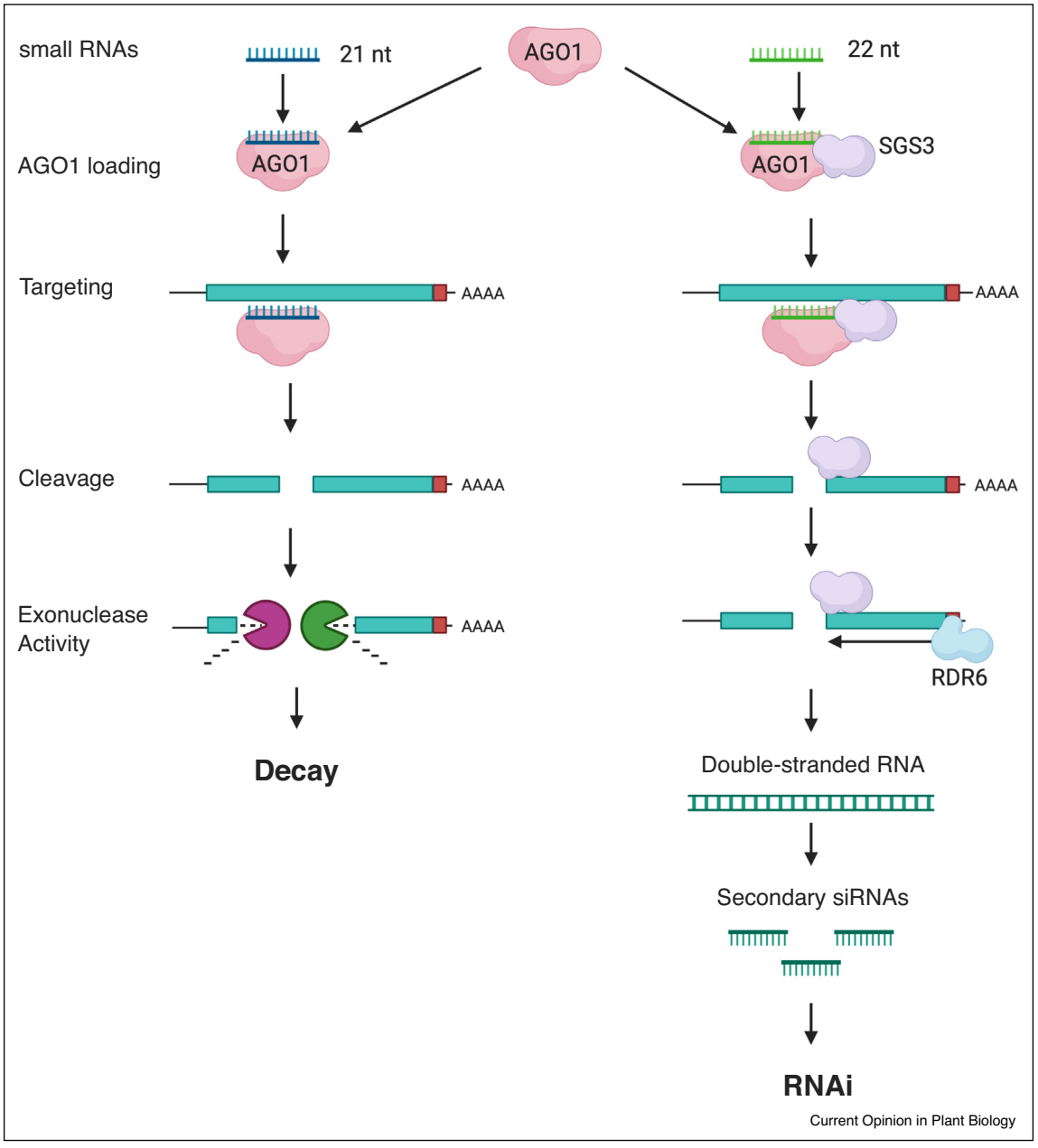
## Protein determinants of RNAi

Two key proteins are known to mediate the distinction between RNA decay of the RISC-sliced transcript and conversion into dsRNA. First, the RNA-DEPENDENT RNA POLYMERASE 6 (RDR6) protein (or potentially any member of the RdRP protein family) may act on the sliced transcript and convert single-stranded RNA into

dsRNA. Since dsRNA will be efficiently cleaved by Dicer family proteins (see above), the synthesis of dsRNA by RdRP proteins is the key control point for the entry into RNAi (Figure 3). Additionally, many RNA viruses encode their own RdRP proteins and require a dsRNA form as part of their lifecycle [29], making them obvious targets of RNAi. Repression of viruses is considered the evolutionary origin of RNAi [30,31], and many (if not all) viruses encode suppressors of RNAi in order to complete their dsRNA lifecycle [32].

Second, the RNA-binding protein SUPPRESSOR OF GENE SILENCING 3 (SGS3) is required for secondary siRNA production, RNAi and natural virus resistance

**Figure 3**




Model of SGS3 protection of sliced RNA and entry into RNAi.
The incorporation of a 22 nucleotide (nt) siRNA or microRNA into AGO1 results in the binding of SGS3 [37]. After mRNA cleavage, SGS3 remains associated with the target mRNA and protects the mRNA ends from degradation by exonucleases via the RNA decay pathway. The protected mRNA is the substrate for RDR6 production of a double-stranded RNA molecule, which is the trigger for secondary siRNA production and RNAi. Model adapted from Ref. [38].

[33,34]. SGS3 acts after RISC slicing but before RDR6 to stabilize the cleaved mRNA and protect the RNA end from exonucleases of the RNA decay pathway (Figure 3) [35,36]. In 2013, Yoshikawa *et al.* [37] performed a series of *in vitro* experiments demonstrating that SGS3 interacts with AGO1 before the RISC slices its target mRNA. Once cleaved, SGS3 remains associated with the cleaved transcript, potentially protecting it from the decay exonucleases. In the current model (Figure 3), the SGS3/AGO1 interaction only occurs when AGO1 is loaded with specifically 22 nt microRNAs/siRNAs, and therefore only mRNA transcripts cleaved by the 22 nt form are protected from exonuclease degradation and are available templates for RDR6 activity [38]. This protection and stabilization of the sliced end correlates with RDR6′s preference for deadenylated mRNAs or non-polyadenylated RNAs as substrates *in vitro* [39]. A recent paper suggests that the RISC-SGS3 complex stalls ribosomes and triggers siRNA production [77]. The current model postulates a conformation change generated by AGO1 caused by carrying a 22 nt microRNA/siRNA versus 21 nt version, allowing for SGS3 binding to AGO1. A similar model of RISC conformation change and binding to SGS3 is suggested not due to small RNA length, but rather the asymmetry of the microRNA duplex (see above) [24]. There are many aspects of this model that remain unknown, and several of the key experiments supporting these models were performed *in vitro* and either were not attempted or not successful *in vivo*.

## Entry of uncleaved transcripts into RNAi

It is clear from the literature that RNAi can be triggered on RNAs without the requirement that they are first sliced by a microRNA or *trans*-acting siRNA. There are at least two mechanisms that can trigger RNAi without a RISC-induced slicing event, one of which is well understood, and the second one is currently enigmatic. In the first mechanism, the trigger for RNAi is again dsRNA, this time generated by the secondary structure of the RNA itself [40]. Inverted repeats or overlapping antisense transcripts can generate their own dsRNA without requiring the activity of an RdRP. These transcripts are cleaved by Dicer proteins into siRNAs (including 24 nt siRNAs), which can trigger RNAi and RNA-directed DNA methylation both in *cis* and *trans* [8].

Second, a genic (or transgenic) RNA that fails RNA quality control (RQC) can enter into RNAi. Many studies have suggested that imperfect '*aberrant*' RNAs can be acted upon by RdRP and/or Dicer family proteins to trigger siRNA production and entry into RNAi [22,41–43]. Although often blamed for triggering RNAi, the molecular identity and mechanism of these aberrant RNAs remains elusive. This lack of clarity is not due to a shortage of research, positive data or publications. Rather, many features of the RNA have been identified and suggested to be *the* trigger of RNAi, but it is unclear how these features are interrelated and which are causes (triggers) versus downstream consequences of RNAi.

### Weak mRNA termination triggers RNAi

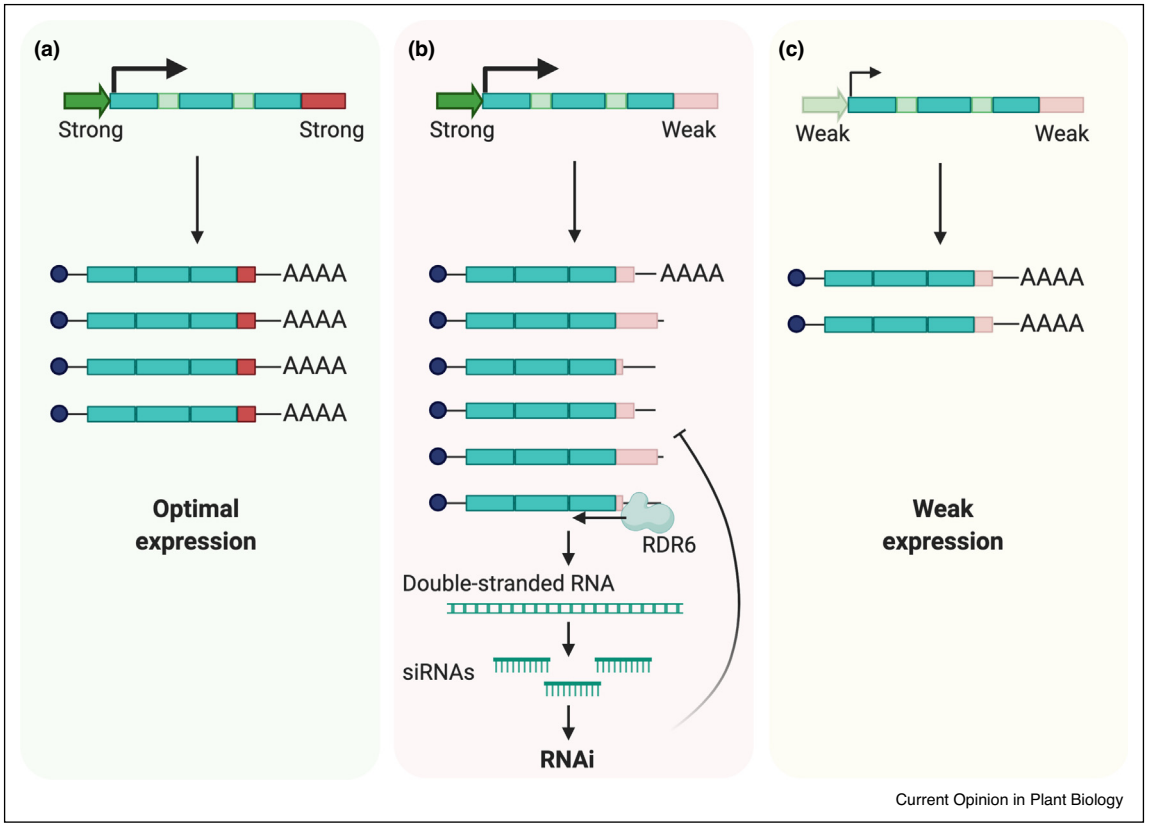
Many characteristics of non-sliced mRNA transcripts themselves have been suggested to mediate the direct entry of that transcript into RNAi (Figure 2). For example, since overexpressed transgene-derived mRNAs tend to be targeted by RNAi, the level of mRNA expression above a threshold is thought to be a trigger [44–46]. In addition, mRNA splicing [43,47–49], stalled ribosomes [50•,77], decapping [51], nonsense-mediated decay [42] and nonstop decay [52] all have evidence of affecting whether a transgenic mRNA enters into RNAi. In this case, the problem is not a lack of data demonstrating which RNA processing pathway leads to RNAi, but many conflicting reports that have each focused on different transgene RNAs or endogenous transcripts.

An important recent study by de Felippes *et al.* [53••] demonstrated that intron splicing can affect entry into RNAi, but this was dependent on which specific terminator element was used in this transgene. This work built upon previous findings [54,55,56••] and demonstrated that the terminator is the most important characteristic of a transgene that determines whether the mRNA will be targeted by RNAi. At the same time, they did a forward mutant screen for proteins that affect the entry of an mRNA into RNAi and identified four genes that all encode termination factors. Although the terminator has a clear role in triggering RNAi, the molecular mechanism by which it acts is not known. The current model suggests that a weak terminator, or a moderate terminator that has been paired with a promoter that overpowers the ability of the terminator to function efficiently, results in dispersed locations of polyadenylation and potentially the production of transcripts that are not polyadenylated. These terminated, but not polyadenylated, transcripts may be the direct substrates for RdRP proteins, similar to the cleaved mRNAs generated by microRNA/siRNA cleavage (Figure 4). This model connects with work from Baeg *et al.* that showed *in vitro* that RDR6 prefers non-polyadenylated RNAs [39].

### Base modifications, non-canonical 5′ caps and RNAi

A wide range of nucleotide base modifications occur on RNA transcripts. These modifications have been appreciated on tRNA and rRNA bases for decades, while more recently they have become the subject of interest on transcripts derived from RNA Polymerase II. Unfortunately termed '*epitranscriptomics*' (as most often there is no evidence for the essential heritability of epigenetics), the field of RNA base modifications has expanded both the number of modifications identified and their function (reviewed in Ref. [57]). While studies in both animals and plants have linked RNA modifications to a variety of functions, including RNA decay [58–60], there is

**Figure 4**




Model of improperly terminated transcripts directly entering into RNAi.
**(a)** Strong terminators lead to efficient transcript termination, polyadenylation and translation. **(b)** A weak terminator can be overpowered by a strong promoter and result in inefficient termination and non-uniform polyadenylation. Some of these improperly terminated transcripts are the substrates of RDR6, which converts them into double-stranded RNA, which is the trigger for siRNA production and RNAi. **(c)** A weak terminator does not always produce RNAi. When the weak terminator is paired with a weak promoter, the terminator functions correctly and efficient termination is restored. Dark/light green arrow – promoter; aqua boxes – open reading frame; dark/light red box – terminator sequence.

currently no strong evidence of a particular RNA base modification leading to RNAi [61]. However, in addition to acquiring internal base modifications, RNA transcripts can obtain non-canonical 5′ caps such as nicotinamide adenine dinucleotide (NAD+) [62]. Recent studies demonstrated that the loss of the enzyme responsible for removing the NAD+ cap results in increased siRNA levels [63], and that NAD+ capped transcripts are more likely to be processed by RDR6 into siRNAs [64]. This data suggests that this post-transcriptional addition of a non-canonical 5′ cap to an RNA precedes RNAi.

## The role of GC content in protecting transcripts against RNAi

The common dogma for codon optimization of transgene coding regions is to match the GC content and codon usage (common versus rare codons) of the species in which the transgene will be used [65,66]. However, in 2017 Sidorenko *et al.* [67••] found that encoding the same protein with the highest possible GC content (by selectively using a GC at the codon 3rd or 'wobble' position without changing the amino acid encoded) reduced the chance that this transgene transcript would enter into RNAi. The high GC content was successful at reducing RNAi in high GC plant genomes, as well as in genomes with standard GC content, where the GC level of the transgene was a clear outlier. Although convincing in its effectiveness, the molecular mechanism responsible for the protection against RNAi is unknown. Theories have ranged from an effect on both transcriptional and translational rates and more efficient termination of high GC transcripts. A recent study demonstrated that rice TEs, which are common targets of RNAi, have a lower GC content at the third codon than genes. The authors then suggested that this codon usage results in ribosome stalling, RNA cleavage and subsequent siRNA production of TE transcripts [50•]. Together, these studies imply a relationship between codon usage, translation efficiency and entry into RNAi.

## Antagonism between RNAi and RNA decay

Although the function of RNA decay and RNAi are distinct, they are not completely independent. In fact, RNA decay and RNAi can compete for substrate mRNAs and work in parallel to remove all of a given mRNA from the cell. For example, when RNA decay function is reduced due to mutation, some transcripts that are normally not targeted by RNAi enter into the RNAi pathway [46,51,68,69]. A screen for transgene silencing identified three RNA deadenylation and 3′ degradation proteins that act to decay the transgene transcript and inhibit RNAi [70]. Without this normal decay pathway, RNAi was activated and siRNA produced from the transgene mRNA. Similarly, a separate study found that the proteasome subunit RPT2a inhibits the RQC pathway, leading to transgene transcripts feeding into RNAi [71••]. Oppositely, when RNAi is mutated, mRNAs that were degraded by RNAi now are translated and/or enter into the RNA decay pathway (for example Ref. [72]). In an interesting example, Conti *et al.* found that the tobacco mosaic virus specifically targets an increase in the cellular RNA decay pathway, leading the authors to suggest that the purpose of this increase in RNA decay is to avoid the plant's viral RNAi defense [73].

## Conclusion

We have summarized several known triggers for funneling transcripts into the cycle of RNAi, and we proposed models to explain how particular transcripts are selected. It remains to be determined if multiple entry mechanisms exist and function mutually exclusively, or if each factor indirectly influences one key trigger. In the future, additional studies like that of de Felippes *et al.* [53••] should be performed that combinatorially test multiple factors (promoter, splicing, termination, GC levels).

Independent of the trigger, RNAi has important consequences for gene, TE, and virus regulation. If RNAi is not initiated correctly, active TEs will not be silenced and will mutagenize the genome as they spread, proper regulation of phasiRNA production and development will not occur, and the plants may succumb to virus infection. Alternatively, if triggered at too high of a rate, the RNAi becomes *transitive*, creating new secondary siRNAs and targeting new off-target mRNAs. RNAi can also affect transcript regulation transgenerationally, by either guiding heritable chromatin modifications in RNA-directed DNA methylation [74,75] or by sustaining the cycle of RNAi over multiple generations [76•].

The known entry points into RNAi suggest mechanisms that researchers should use to avoid the post-transcriptional silencing of the gene products that they aim to engineer. The expressed mRNAs should not carry sequences complementary to known primary microRNAs/siRNAs, and they should have a high GC content. The DNA used to make this mRNA should contain several introns so the mRNA is spliced. Researchers should avoid convergent promoters and inverted repeats that will form dsRNA, and they should use a promoter and terminator that have matched strengths so the transcript is terminated and polyadenylated efficiently. These design features, and perhaps others yet to be discovered, should favor the mRNA into the desired translation and decay pathway rather than RNAi.

## Conflict of interest statement

Nothing declared.

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## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Ghosh S, Jacobson A: **RNA decay modulates gene expression and controls its fidelity**. *Wiley Interdiscip Rev RNA* 2010, **1**:351-361.
2. Radhakrishnan A, Green R: **Connections underlying translation and mRNA stability**. *J Mol Biol* 2016, **428**:3558-3564.
3. Moore MJ: **From birth to death: the complex lives of eukaryotic mRNAs**. *Science* 2005, **309**:1514-1518.
4. Houseley J, Tollervey D: **The many pathways of RNA degradation**. *Cell* 2009, **136**:763-776.
5. Chiba Y, Green PJ: **mRNA degradation machinery in plants**. *J Plant Biol* 2009, **52**:114-124.
6. Li B, Wu H, Guo H: **Plant mRNA decay: extended roles and potential determinants**. *Curr Opin Plant Biol* 2018, **45**:178-184.
7. Hutvágner G, Zamore PD: **RNAi: nature abhors a double-strand**. *Curr Opin Genet Dev* 2002, **12**:225-232.
8. Bologna NG, Voinnet O: **The diversity, biogenesis, and activities of endogenous silencing small RNAs in Arabidopsis**. *Annu Rev Plant Biol* 2014, **65**:473-503.
9. Fang X, Qi Y: **RNAi in plants: an Argonaute-centered view**. *Plant Cell* 2016, **28**:272-285.
10. McCue AD, Nuthikattu S, Reeder SH, Slotkin RK: **Gene expression and stress response mediated by the epigenetic regulation of a transposable element small RNA**. *PLoS Genet* 2012, **8**:e1002474.
11. McCue AD, Panda K, Nuthikattu S, Choudury SG, Thomas EN, Slotkin RK: **ARGONAUTE 6 bridges transposable element mRNA-derived siRNAs to the establishment of DNA methylation**. *EMBO J* 2015, **34**:20-35.
12. Cuerda-Gil D, Slotkin RK: **Non-canonical RNA-directed DNA methylation**. *Nat Plants* 2016, **2**:16163.
13. Morel J-B, Godon C, Mourrain P, Béclin C, Boutet S, Feuerbach F, Proux F, Vaucheret H: **Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance**. *Plant Cell* 2002, **14**:629-639.
14. Ding S-W, Voinnet O: **Antiviral immunity directed by small RNAs**. *Cell* 2007, **130**:413-426.
15. Garcia-Ruiz H, Takeda A, Chapman EJ, Sullivan CM, Fahlgren N, Brempelis KJ, Carrington JC: **Arabidopsis RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and**

**small interfering RNA biogenesis during turnip mosaic virus infection**. *Plant Cell* 2010, **22**:481-496.

16. Pumplin N, Voinnet O: **RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence**. *Nat Rev Microbiol* 2013, **11**:745-760.

17. Adenot X, Elmayan T, Lauressergues D, Boutet S, Bouché N, Gasciolli V, Vaucheret H: **DRB4-dependent TAS3 trans-acting siRNAs control leaf morphology through AGO7**. *Curr Biol* 2006, **16**:927-932.

18. Garcia D, Collier SA, Byrne ME, Martienssen RA: **Specification of leaf polarity in Arabidopsis via the trans-acting siRNA pathway**. *Curr Biol* 2006, **16**:933-938.

19. Fahlgren N, Montgomery TA, Howell MD, Allen E, Dvorak SK, Alexander AL, Carrington JC: **Regulation of AUXIN RESPONSE FACTOR3 by TAS3 ta-siRNA affects developmental timing and patterning in Arabidopsis**. *Curr Biol* 2006, **16**:939-944.

20. Hunter C, Willmann MR, Wu G, Yoshikawa M, Gutiérrez-Nava M de la L, Poethig SR: **Trans-acting siRNA-mediated repression of ETTIN and ARF4 regulates heteroblasty in Arabidopsis**. *Development* 2006, **133**:2973-2981.

21. Béclin C, Boutet S, Waterhouse P, Vaucheret H: **A branched pathway for transgene-induced RNA silencing in plants**. *Curr Biol* 2002, **12**:684-688.

22. Luo Z, Chen Z: **Improperly terminated, unpolyadenylated mRNA of sense transgenes is targeted by RDR6-mediated RNA silencing in Arabidopsis**. *Plant Cell* 2007, **19**:943-958.

23. Axtell MJ, Jan C, Rajagopalan R, Bartel DP: **A two-hit trigger for siRNA biogenesis in plants**. *Cell* 2006, **127**:565-577.

24. Manavella PA, Koenig D, Weigel D: **Plant secondary siRNA production determined by microRNA-duplex structure**. *Proc Natl Acad Sci U S A* 2012, **109**:2461-2466.

25. Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, Takeda A, Sullivan CM, Gilbert SD, Montgomery TA, Carrington JC: **Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in Arabidopsis**. *Nat Struct Mol Biol* 2010, **17**:997-1003.

26. Chen H-M, Chen L-T, Patel K, Li Y-H, Baulcombe DC, Wu S-H: **22-nucleotide RNAs trigger secondary siRNA biogenesis in plants**. *Proc Natl Acad Sci U S A* 2010, **107**:15269-15274.

27. Bouché N, Lauressergues D, Gasciolli V, Vaucheret H: **An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs**. *EMBO J* 2006, **25**:3347-3356.

28. Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O: **Hierarchical action and inhibition of plant dicer-like proteins in antiviral defense**. *Science* 2006, **313**:68-71.

29. Ahlquist P: **RNA-dependent RNA polymerases, viruses, and RNA silencing**. *Science* 2002, **296**:1270-1273.

30. Obbard DJ, Gordon KHJ, Buck AH, Jiggins FM: **The evolution of RNAi as a defence against viruses and transposable elements**. *Philos Trans R Soc B Biol Sci* 2009, **364**:99-115.

31. Zhou R, Rana TM: **RNA-based mechanisms regulating host–virus interactions**. *Immunol Rev* 2013, **253**:97-111.

32. Burgyán J, Havelda Z: **Viral suppressors of RNA silencing**. *Trends Plant Sci* 2011, **16**:265-272.

33. Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, Jouette D, Lacombe A-M, Nikic S, Picault N *et al.*: **Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance**. *Cell* 2000, **101**:533-542.

34. Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS: **SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis**. *Gene Dev* 2004, **18**:2368-2379.

35. Souret FF, Kastenmayer JP, Green PJ: **AtXRN4 degrades mRNA in Arabidopsis and its substrates include selected miRNA targets**. *Mol Cell* 2004, **15**:173-183.

36. Yoshikawa M, Peragine A, Park MY, Poethig RS: **A pathway for the biogenesis of trans-acting siRNAs in Arabidopsis**. *Gene Dev* 2005, **19**:2164-2175.

37. Yoshikawa M, Iki T, Tsutsui Y, Miyashita K, Poethig RS, Habu Y, Ishikawa M: **3′ fragment of miR173-programmed RISC-cleaved RNA is protected from degradation in a complex with RISC and SGS3**. *Proc Natl Acad Sci U S A* 2013, **110**:4117-4122.

38. Yoshikawa M, Iki T, Numa H, Miyashita K, Meshi T, Ishikawa M: **A short open reading frame encompassing the microRNA173 target site plays a role in trans-acting small interfering RNA biogenesis**. *Plant Physiol* 2016, **171**:359-368.

39. Baeg K, Iwakawa H, Tomari Y: **The poly(A) tail blocks RDR6 from converting self mRNAs into substrates for gene silencing**. *Nat Plants* 2017, **3**:17036.

40. Li F, Zheng Q, Vandivier LE, Willmann MR, Chen Y, Gregory BD: **Regulatory impact of RNA secondary structure across the Arabidopsis transcriptome**. *Plant Cell* 2012, **24**:4346-4359.

41. Herr AJ, Molnàr A, Jones A, Baulcombe DC: **Defective RNA processing enhances RNA silencing and influences flowering of Arabidopsis**. *Proc Natl Acad Sci U S A* 2006, **103**:14994-15001.

42. Moreno AB, de Alba AEM, Bardou F, Crespi MD, Vaucheret H, Maizel A, Mallory AC: **Cytoplasmic and nuclear quality control and turnover of single-stranded RNA modulate post-transcriptional gene silencing in plants**. *Nucleic Acids Res* 2013, **41**:4699-4708.

43. Dalakouras A, Lauter A, Bassler A, Krczal G, Wassenegger M: **Transient expression of intron-containing transgenes generates non-spliced aberrant pre-mRNAs that are processed into siRNAs**. *Planta* 2018, **249**:457-468.

44. Que Q, Wang HY, English JJ, Jorgensen RA: **The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence**. *Plant Cell* 1997, **9**:1357-1368.

45. Bleys A, Houdt HV, Depicker A: **Down-regulation of endogenes mediated by a transitive silencing signal**. *RNA* 2006, **12**:1633-1639.

46. Zhang X, Zhu Y, Liu X, Hong X, Xu Y, Zhu P, Shen Y, Wu H, Ji Y, Wen X *et al.*: **Suppression of endogenous gene silencing by bidirectional cytoplasmic RNA decay in Arabidopsis**. *Science* 2015, **348**:120-123.

47. Christie M, Croft LJ, Carroll BJ: **Intron splicing suppresses RNA silencing in Arabidopsis**. *Plant J Cell Mol Biol* 2011, **68**:159-167.

48. Dadami E, Moser M, Zwiebel M, Krczal G, Wassenegger M, Dalakouras A: **An endogene-resembling transgene delays the onset of silencing and limits siRNA accumulation**. *FEBS Lett* 2013, **587**:706-710.

49. Elvira-Matelot E, Bardou F, Ariel F, Jauvion V, Bouteiller N, Masson IL, Cao J, Crespi MD, Vaucheret H: **The nuclear ribonucleoprotein SmD1 interplays with splicing, RNA quality control, and posttranscriptional gene silencing in Arabidopsis**. *Plant Cell* 2016, **28**:426-438.

50. • Kim E, Wang L, Lei Z, Li H, Fan W, Cho J: **Translational inhibition and phase separation primes the epigenetic silencing of transposons**. *bioRxiv* 2020 http://dx.doi.org/10.1101/2020.04.08.032953

This study demonstrated that plant transposable elements have less favorable codon usage which results in ribosome stalling, RNA truncation, and localization to the siRNA body. This study suggests a molecular mechanism of how the plants discriminate TE transcripts from genic transcripts for silencing.

51. Martínez de Alba AE, Moreno AB, Gabriel M, Mallory AC, Christ A, Bounon R, Balzergue S, Aubourg S, Gautheret D, Crespi MD *et al.*: **In plants, decapping prevents RDR6-dependent production of small interfering RNAs from endogenous mRNAs**. *Nucleic Acids Res* 2015, **43**:2902-2913.

52. Szádeczky-Kardoss I, Csorba T, Auber A, Schamberger A, Nyikó T, Taller J, Orbán TI, Burgyán J, Silhavy D: **The nonstop decay and the RNA silencing systems operate cooperatively in plants**. *Nucleic Acids Res* 2018, **46**:4632-4648.

53. •• Felippes F, de, McHale M, Doran RL, Ro den S, Eamens AL, Finnegan EJ, Waterhouse PM: **The key role of terminators on the expression and post-transcriptional gene silencing of transgenes**. *Plant J* 2020, **104**:96-112 http://dx.doi.org/10.1111/tpj.14907
This study combinatorially tested several factors including splicing and demonstrated that the efficiency of transcript termination is the key for entry into RNAi.

54. Nagaya S, Kawamura K, Shinmyo A, Kato K: **The HSP terminator of *Arabidopsis thaliana* increases gene expression in plant cells**. *Plant Cell Physiol* 2010, **51**:328-332.

55. Hirai T, Kurokawa N, Duhita N, Hiwasa-Tanase K, Kato K, Kato K, Ezura H: **The HSP terminator of *Arabidopsis thaliana* induces a high level of miraculin accumulation in transgenic tomatoes**. *J Agric Food Chem* 2011, **59**:9942-9949.

56. •• Pérez-González A, Caro E: **Effect of transcription terminator usage on the establishment of transgene transcriptional gene silencing**. *BMC Res Notes* 2018, **11**:511
The authors analyzed the effect of different usage of terminators on transgene expression and DNA methylation. This study shows that terminator efficiency determines the RNA expression and abnormal RNA production, which can trigger RNAi and accumulation of DNA methylation in transgene promoters.

57. Roundtree IA, Evans ME, Pan T, He C: **Dynamic RNA modifications in gene expression regulation**. *Cell* 2017, **169**:1187-1200.

58. Wang X, Lu Z, Gomez A, Hon GC, Yue Y, Han D, Fu Y, Parisien M, Dai Q, Jia G *et al.*: **N6-methyladenosine-dependent regulation of messenger RNA stability**. *Nature* 2014, **505**:117-120.

59. Shen L, Liang Z, Gu X, Chen Y, Teo ZWN, Hou X, Cai WM, Dedon PC, Liu L, Yu H: **N6-methyladenosine RNA modification regulates shoot stem cell fate in Arabidopsis**. *Dev Cell* 2016, **38**:186-200.

60. Kramer MC, Anderson SJ, Gregory BD: **The nucleotides they are a-changin': function of RNA binding proteins in post-transcriptional messenger RNA editing and modification in Arabidopsis**. *Curr Opin Plant Biol* 2018, **45**:88-95.

61. Yu X, Sharma B, Gregory BD: **The impact of epitranscriptomic marks on post-transcriptional regulation in plants**. *Brief Funct Genomics* 2020 http://dx.doi.org/10.1093/bfgp/elaa021. Online ahead of print.

62. Wang Y, Li S, Zhao Y, You C, Le B, Gong Z, Mo B, Xia Y, Chen X: **NAD+-capped RNAs are widespread in the Arabidopsis transcriptome and can probably be translated**. *Proc Natl Acad Sci U S A* 2019, **116** 201903682.

63. Pan S, Li K, Huang W, Zhong H, Wu H, Wang Y, Zhang H, Cai Z, Guo H, Chen X *et al.*: **Arabidopsis DXO1 possesses deNADding and exonuclease activities and its mutation affects defense-related and photosynthetic gene expression**. *J Integr Plant Biol* 2020, **62**:967-983.

64. Yu X, Willmann MR, Vandivier LE, Trefely S, Kramer MC, Shapiro J, Guo R, Lyons E, Snyder NW, Gregory BD: **Messenger RNA 5′ NAD+ capping is a dynamic regulatory epitranscriptome mark that is required for proper response to abscisic acid in Arabidopsis**. *Dev Cell* 2021, **56**:125-140.e6.

65. Parret AH, Besir H, Meijers R: **Critical reflections on synthetic gene design for recombinant protein expression**. *Curr Opin Struct Biol* 2016, **38**:155-162.

66. Brule CE, Grayhack EJ: **Synonymous codons: choose wisely for expression**. *Trends Genet* 2017, **33**:283-297.

67. •• Sidorenko LV, Lee T, Woosley A, Moskal WA, Bevan SA, Merlo PAO, Walsh TA, Wang X, Weaver S, Glancy TP *et al.*: **GC-rich coding sequences reduce transposon-like, small RNA-mediated transgene silencing**. *Nat Plants* 2017, **3**:875-884
This study makes the observation across several plant species that transgenes designed with the highest GC levels are most resistant to RNAi.

68. Irvine DV, Zaratiegui M, Tolia NH, Goto DB, Chitwood DH, Vaughn MW, Joshua-Tor L, Martienssen RA: **Argonaute slicing is required for heterochromatic silencing and spreading**. *Science* 2006, **313**:1134-1137.

69. Liu L, Chen X: **RNA quality control as a key to suppressing RNA silencing of endogenous genes in plants**. *Mol Plant* 2016, **9**:826-836.

70. Li T, Natran A, Chen Y, Vercruysse J, Wang K, Gonzalez N, Dubois M, Inzé D: **A genetics screen highlights emerging roles for CPL3, RST1 and URT1 in RNA metabolism and silencing**. *Nat Plants* 2019, **5**:539-550.

71. •• Kim M-H, Jeon J, Lee S, Lee JH, Gao L, Lee B-H, Park JM, Kim YJ, Kwak JM: **Proteasome subunit RPT2a promotes PTGS through repressing RNA quality control in Arabidopsis**. *Nat Plants* 2019, **5**:1273-1282
This work reinforces the concept that RNA decay and RNAi compete for RNA substrates, and demonstrates that a proteasome subunit can repress RNA decay and therefore indirectly promote RNAi.

72. Li F, Wang Y, Zhou X: **SGS3 cooperates with RDR6 in triggering geminivirus-induced gene silencing and in suppressing geminivirus infection in *Nicotiana benthamiana***. *Viruses* 2017, **9**:247.

73. Conti G, Zavallo D, Venturuzzi AL, Rodriguez MC, Crespi M, Asurmendi S: **TMV induces RNA decay pathways to modulate gene silencing and disease symptoms**. *Plant J* 2017, **89**:73-84.

74. Slotkin RK, Martienssen R: **Transposable elements and the epigenetic regulation of the genome**. *Nat Rev Genet* 2007, **8**:272-285.

75. Law JA, Jacobsen SE: **Establishing, maintaining and modifying DNA methylation patterns in plants and animals**. *Nat Rev Genet* 2010, **11**:204-220.

76. • Hu Q, Ailhas J, Blevins T, Klahre U, Vazquez F, Mette MF, Meins F: **Epigenetic transgenerational effects on RNAi in Arabidopsis**. *bioRxiv* 2020 http://dx.doi.org/10.1101/2020.04.05.026641
This manuscript demonstrates the transgenerational nature of RNAi in plants is similar to *Caenorhabditis elegans* as it decays over generations if the signal is not transferred to chromatin modification and transcriptional silencing.

77. Iwakawa H *et al.*: **Ribosome stalling caused by the Argonaute-miRNA-SGS3 complex regulates production of secondary siRNA biogenesis in plants**. *bioRxiv* 2020 http://dx.doi.org/10.1101/2020.09.10.288902.11:14. 2020.09.10.288902.