Short Silencing RNA: The Dark Matter of Genetics?

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Plants and animals have single-stranded silencing RNAs (sRNAs) of 21–25 nucleotides in length that are derived from a double-stranded (ds)RNA precursor by Dicer (DCL) processing. These RNAs are the guide RNA for nucleases of the AGO class that cleave targeted RNA in a nucleotide sequence-specific manner. The cleaved RNAs are then degraded further or they are the template for an RNA-dependent RNA polymerase (RDR) that generates a dsRNA. In this paper, I discuss the possibility that this RDR-generated dsRNA initiates a cascade in which there are multiple rounds of secondary sRNA production. I propose that these secondary sRNAs feature in mechanisms that can either buffer mRNA populations against change or, in certain circumstances, mediate extensive changes in mRNA populations. The RNA cascades may also have RNA-mediated epigenetic characteristics in addition to the DNA and chromatin transcriptional silencing potential that has been previously linked with RNA silencing.

In 1993, the Ruvkun and Ambros laboratories described two different regulatory short RNAs affecting the timing of developmental transitions in *Caenorhabditis elegans* (Lee et al. 1993; Wightman et al. 1993). They referred to these RNAs as short temporal (st) RNAs, and they discovered that they mediate gene silencing by base-pairing to a complementary nucleotide sequence in the 3'UTR of target mRNAs. For many years, they were the only known example of regulatory RNA acting in this manner. However, in 2001 and 2002, when short RNA was sequenced from animals and plants, it became apparent that the stRNAs are typical of a large class of short regulatory RNAs that are now referred to as micro (mi)RNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart et al. 2002). Ruvkun described this new family of RNAs using a dark matter metaphor because, like the hypothetical dispersed mass in the physical universe, they were recently discovered, abundant, and functionally significant (Ruvkun 2001). This dark matter metaphor remains appropriate because, as discussed below, there are many previously unidentified families of these RNAs, in addition to miRNAs, that mediate gene silencing. I refer generically to these RNAs as silencing RNAs (sRNAs). Correspondingly, I refer to silencing mechanisms based on these RNAs as examples of “RNA silencing.”

The sRNAs are processed from a longer dsRNA precursor by an RNase III homolog known as dicer or dicer-like (DCL) (Bernstein et al. 2001; Schauer et al. 2002). DCL cleaves these precursor RNAs into a 20- to 24-nucleotide dsRNA with 5' phosphate and 3' hydroxyl groups that is recruited by a second nuclease known as slicer (Fig. 1) (Liu et al. 2004). Slicer, a member of the Argonaute (AGO) family of proteins, cleaves one of the two strands, and the cleavage products are released (Matranga et al. 2005). The remaining single-stranded sRNA is then available for a targeting mechanism in which slicer is guided by Watson-Crick base-pairing to a complementary target RNA or DNA.

Figure 1. The core mechanism of RNA silencing in plants. Single-stranded RNA (ssRNA) is converted into a double-stranded (ds) form by an RDR protein. The dsRNA is cleaved by DCL and the single-stranded sRNA is retained in an AGO ribonucleoprotein complex where it has the role of a guide RNA.

The consequence of the RNA targeting mechanism is either RNA cleavage by AGO, translational arrest, or both (Schwab et al. 2005). The DNA-targeted mechanism leads to heterochromatin formation and, in some instances, DNA methylation at the target locus (Lippman and Martienssen 2004). The DNA-targeted mechanism is less well understood than RNA-targeted silencing, although it does require an AGO protein (Volpe et al. 2002; Zilberman et al. 2003; Li et al. 2006). In principle, the DNA-targeted mechanism could involve base-pairing of an sRNA with one of the DNA strands at the target locus. However, in cells in which nuclear DNA is not replicating, this mechanism would only succeed if there was unwinding of the DNA double helix. A more plausible mechanism involves indirect targeting of DNA: The direct target of the sRNA interaction could be a nascent RNA that is still attached to the transcribed chromatin, rather than DNA. In fission yeast, it is likely that sRNA is targeted at a nascent RNA (Bayne and Allshire 2005), but in plant systems, the evidence is not conclusive.

There are two variations of the sRNA biogenesis mechanism. In the first of these mechanisms, the sRNA pre-

cursor is an RNA with partially complementary internal repeats (Fig. 2). The inverted repeat regions anneal to form an incompletely double-stranded RNA in which mismatched regions in the RNA precursor are likely to provide structural cues that guide Dicer to a single site in the precursor RNA. It is also possible that these mismatch sites exclude DCL from the flanking regions so that each precursor normally gives rise to only a single sRNA (Han et al. 2006). The sRNAs generated by this process are the miRNAs referred to above.

In the second variation, the DCL substrate is completely double-stranded, and several sRNAs are generated from each dsRNA precursor (Fig. 2). Presumably, the absence of unpaired bases means that there are no structural cues to guide DCL to any one region of its substrate RNA. Consequently, the cleavage of dsRNA is less targeted than with the miRNA precursors. The dsRNA may be produced either by annealing of complementary single-stranded RNAs, by intramolecular base-pairing of self-complementary inverted repeat regions, or by the action of an RNA-dependent RNA polymerase (RDR) on a single-stranded RNA template. The sRNAs produced by the cleavage of the completely double-stranded RNA are referred to as short interfering (si) RNAs (Carmell and Hannon 2004).

In this paper, I speculate about the possible biological roles and mechanisms of sRNA-mediated silencing. My discussion is based primarily on the sRNAs of plants, although some of the mechanisms proposed may also be relevant to animal and fungal systems. First, I summarize the genetic and molecular approaches to characterize the sRNAs in *Arabidopsis*. I then describe how these sRNAs may form interaction cascades and networks affecting genetic and epigenetic systems in growth and development.

GENETICS AND THE HIGH-THROUGHPUT SEQUENCING REVOLUTION

In *Arabidopsis*, the analysis of biogenesis and targeting of sRNAs has benefited from a classic mutation analysis combined with a more recent approach—the Hubble tele-

Figure 2. The difference between miRNA and siRNA. miRNAs (*upper diagram*) are generated from precursor dsRNAs in which sequence mismatches provide structural cues so that DCL releases only a single short RNA after cleavage. The precursors of siRNAs, in contrast (*lower diagram*), are completely dsRNA, and the absence of structural cues means that DCL releases siRNAs from many positions.

scope of the RNA universe—that uses novel high-throughput cDNA sequencing technologies (Lu et al. 2005; Henderson et al. 2006). The picture emerging from these analyses, as described below, is of multiple variations on the core RNA silencing mechanisms.

The mutation analysis involving both forward and reverse genetics reveals the existence of multigene families encoding the three key proteins—RDR, DCL, and AGO—in RNA silencing (Baulcombe 2004). Many, if not all, of these isoforms are involved in variations of RNA silencing. DCL1, for example, generates miRNAs (Baulcombe 2004), whereas DCL2, 3, and 4 feature in the siRNA biogenesis pathways (Dunoyer et al. 2005; Gascioli et al. 2005). DCL3 has the most distinct function because it generates longer siRNAs—24 nucleotides rather than 21 or 22 nucleotides—that are associated with heterochromatin silencing and DNA modification (Xie et al. 2004). RDR2 and AGO4 are similarly distinct from their homologs in that they also are primarily involved in heterochromatin rather than mRNA targeting (Xie et al. 2004; Zilberman et al. 2004). Mutants at four of the six RDR loci and eight of the ten AGO loci have yet to be characterized in terms of their RNA silencing phenotypes.

High-throughput sequencing technologies are still being refined, but at present, the most useful method involves a mechanical cloning procedure and an automated pyro-sequencing of DNA (Margulies et al. 2005). Other suitable techniques use single-molecule DNA sequencing. The power of these methods derives from the absence of a requirement for the labor-intensive manipulations associated with cloning of genomic and complementary DNA in plasmid and phage vectors. It is therefore relatively straightforward to generate sequence data from hundreds of thousands of sRNAs. The current versions of these technologies have a limitation for analysis of genomic DNA in that the sequence data are only short: 20–100 nucleotides. However, this limitation is not relevant to the analysis of short sRNAs.

These sequence data reveal that the *Arabidopsis* sRNAs are predominantly in the size range of 20–26 nucleotides with prominent size groups of 21 and 24 nucleotides (Lu et al. 2005; Henderson et al. 2006). Alignment of the sRNA sequences shows that they are not randomly distributed copies of genomic DNA, and I refer to the regions of the genome with a match to many individual sRNA sequences as sRNA loci. Some of these loci correspond to single-copy genome sequences, and they are the genes from which the sRNAs are produced. Other sRNAs correspond to repeated DNA sequences from which the sRNA genes cannot be identified with certainty. Any of these repeated sRNA loci could be either a source gene of sRNAs or a target of sRNA-mediated silencing. It could also be that they are completely inactive DNA with the same sequence as transcribed regions.

At present, the classification of these loci is an ongoing task in several laboratories using data from various tissues in wild type and from RNA silencing mutants at RDR, DCL, AGO, and other genes (Lu et al. 2005; Henderson et al. 2006). The absence or hyper-accumulation of sRNA in these mutant plants may be informative about the function of sRNA encoded at the locus. My laboratory and

others have also used the association of sRNAs with an AGO protein as a parameter in the classification process (Baumberger and Baulcombe 2005; Qi et al. 2005, 2006). Epitope-tagged versions of the AGO proteins are immunoprecipitated, and the associated sRNAs are characterized using the high-throughput sequencing technology. The information produced by the high-throughput sequencing approaches is a major advance because it is informative about sRNAs and sRNA loci that would not be accessible through simple genetics and phenotype analysis or by using smaller-scale sequencing technology.

The extensive sRNA profile from the high-throughput sequence analysis includes all of the recognized classes of sRNA, including miRNAs and various types of siRNA. However, a new point to emerge is that these sRNA classes are represented by more loci than had been recognized previously (Lu et al. 2005; Henderson et al. 2006). At present, we do not have precise information about the numbers of each type of sRNA because the analysis of the high-throughput sequence data is at an early stage: The numbers change as new data are produced or when the parameters of the computational analysis are modified. However, for the purposes of this paper, it is sufficient to know that there are many thousands of sRNA loci in the *Arabidopsis* and presumably other genomes. Nor is it necessary, for the discussion, to have complete knowledge of all types of sRNA. My interpretation requires only the knowledge that there are many different types of sRNA and that the targets include both mRNAs and heterochromatic loci.

CASCADES AND NETWORKS OF RNA SILENCING

sRNA interactions may lead to AGO-mediated silencing of mRNA or DNA as described above. However, these interactions can also lead to secondary sRNA production through a process involving RDR proteins (Vaistij et al. 2002; Xie et al. 2005; Yoshikawa et al. 2005). In this section, I first discuss secondary siRNAs. I then assess the implications of these two outcomes of sRNA interactions—silencing or secondary sRNA production—for the potential to form cascades and networks of RNA silencing.

Secondary siRNA production in plants and *C. elegans* involves RDR proteins (Sijen et al. 2001; Vaistij et al. 2002). A single-stranded RNA is targeted by an sRNA-slicer complex and is then converted into a dsRNA form by an RDR (Fig. 3). In plants, one of the best-understood secondary siRNA systems leads to the production of trans-acting (ta)siRNAs (Xie et al. 2005; Yoshikawa et al. 2005). The primary sRNAs are miRNAs that direct the cleavage of a single-stranded noncoding RNA. The cleavage process involves the same AGO-mediated mechanism that leads to mRNA degradation, but the cleaved RNA becomes a substrate for RDR-mediated dsRNA production, DCL cleavage, and production of secondary sRNAs—the tasiRNAs.

Other mechanisms of secondary siRNA production have also been proposed. It could be, for example, that the

Figure 3. Secondary siRNA production. An AGO slicer with primary sRNA cleaves a target RNA. The cleaved RNA is then an RDR substrate so that dsRNA and sRNA are produced from the whole length of the targeted RNA (*upper diagram*). sRNA-primed synthesis of dsRNA would generate sRNAs only on the 5' side of the primary sRNA target (*lower panel*) and is not consistent with observations from plant systems.

primary siRNA anneals to its target RNA and is used as a primer by the RDR rather than as a guide for a slicer nuclease (Fig. 3) (Sijen et al. 2001; Makeyev and Bamford 2002). This priming mechanism has been invoked as an explanation for secondary sRNA production in *C. elegans*. However, it is not likely that this priming method is involved in plants because the secondary sRNAs are produced on both the 3' and 5' sides of the primary sRNA target (Voinnet et al. 1998; Vaistij et al. 2002).

Another possible mechanism of secondary siRNA production involves the heterochromatin-targeted siRNAs and a recently discovered RNA polymerase: pol IV. pol IV is a structural homolog of the well-known DNA-dependent RNA polymerases I–III, and genetic analysis indicates that there are two isoforms—pol IVa and pol IVb (Herr et al. 2005; Kanno et al. 2005; Onodera et al. 2005). These isoforms share a common second-largest subunit, but they have largest subunits with different carboxy-terminal domains. From the siRNA phenotype of RNA silencing mutants, it seems that pol IV provides a template RNA that is converted into dsRNA by an RDR and then into siRNA by a DCL (Herr et al. 2005; Pontier et al. 2005).

These mutant phenotypes also indicate that sRNA production by pol IVb is dependent on pol IVa but not vice versa (Pontier et al. 2005). A plausible mechanism that explains this interdependence is similar to the amplification of tasiRNAs: A primary siRNA produced in a pol IVa pathway would cleave a pol IVb transcript so that it becomes a template for dsRNA production by an RDR. siRNA would then be produced in the normal way by a DCL. A second possible mechanism for amplification of

pol-IV-dependent siRNAs involves the RNA-directed heterochromatinization process and requires that pol IVb preferentially transcribe heterochromatin. According to this model, the pol-IVa-dependent sRNAs would direct heterochromatinization of a locus so that it then has the potential to be transcribed by pol IVb. In this scenario, the pol-IVb-generated precursor of secondary siRNAs would be dependent on the primary siRNAs.

The above descriptions involve mechanisms in which primary sRNAs initiate a single round of secondary sRNA production. However, there is no reason, in principle, that there should be just a single round of secondary sRNA production: There could be many layers in a cascade of secondary sRNA production. As a framework for analysis of RNA silencing cascades, I describe a hypothetical situation in which there are component RNA species referred to as initiators, nodes, and end points (Fig. 4). In the simplest situation, the initiator would be an RNA, such as a miRNA precursor with an inverted repeat region. Primary 21- to 24-nucleotide sRNA would be produced by DCL-mediated cleavage of the self-complementary region in this RNA. End point RNAs, exemplified by most miRNA targets in plants, would be cleaved and degraded by interaction with the sRNA (Fig. 4). These end point RNAs would not be substrates for RDR, and they would not direct secondary sRNA production.

The node RNAs would be recruited into the silencing network if they are targeted and cleaved by AGO slicer enzymes associated with siRNAs derived from the initiator (Fig. 4). These node RNAs would be complementary to the primary sRNAs according to miRNA targeting rules (Schwab et al. 2005, 2006), and after sRNA-directed cleavage, they could be substrates for RDR and further rounds of sRNA production. As each primary sRNA would have generated multiple secondary sRNAs, this involvement of node RNAs results in amplification of

silencing pathways. The production of secondary siRNA is an amplification process in silencing pathways.

Systems with initiator, node, and end point RNAs would expand with time because additional nodes would be recruited into the cascade. In addition, they could form a closed network if downstream secondary sRNAs are targeted at initiator or node RNAs involved at upper levels of the cascade. An RNA silencing network would influence the cell in which it was initiated because the initiator, node, and end point RNA may be protein-coding mRNAs. In addition, through the DNA targeting mechanism, the RNA silencing systems would directly affect heterochromatinization of certain regions and have an indirect effect on gene expression and chromosome behavior. The cascade could also operate between cells because sRNAs or sRNA precursors have the potential to spread through plasmodesmata (Voinnet et al. 1998; Palauqui and Balzergue 1999).

Analysis of high-throughput sRNA sequence data indicates that there may be many thousands of node RNAs including hundreds of mRNAs (Lu et al. 2005; Henderson et al. 2006). The hallmark of these node RNAs is that they would match multiple secondary sRNAs and be RDR dependent (Lu et al. 2006). However, although there may be many of these RNAs, the analysis of sRNA profiles indicates that there are fewer mRNA nodes than would be expected on the basis of a simple statistical analysis in which it is assumed that secondary sRNA production requires targeting by a single sRNA with a sequence match in 15/21 positions or more. This value corresponds approximately to the match between bona fide miRNAs and their targets (Schwab et al. 2005, 2006). To explain lower than expected frequency of mRNA nodes, I propose that a single sRNA targeting event is not sufficient to initiate secondary sRNA production: There must be additional factors. This requirement for additional factors would explain the absence of transitivity and secondary sRNA production on endogenous mRNAs targeted by transgenic sRNAs or miRNAs (Vaistij et al. 2002; Parizotto et al. 2004).

In some situations, an additional factor that predisposes an RNA to behave as a node could be structural “aberrancy.” “Aberrant” RNA was invoked originally as an explanation for transgene RNA silencing: It was proposed that transgene RNAs would be more aberrant than endogenous RNAs, although it was difficult originally to define aberrancy. Recently, based on analysis of *Arabidopsis* mutants, there is evidence that aberrancy could be perturbation of 5' cap or 3' end mRNA features that would influence the ribonucleoprotein (RNP) status of an mRNA (Gazzani et al. 2004; Herr et al. 2006). An interpretation of these findings is that the normal messenger ribonucleoprotein (RNP) structure would inhibit RDR. With the disrupted RNP of an aberrant RNA, the enzyme would have fuller access to its template.

By extrapolation from these findings, I propose that an RNA with aberrancy at either the 5' or 3' end would behave as a node if it is targeted by a single sRNA. It could also be, as indicated in a very recent report, that a “normal” RNA with two sRNA target sites could also become a source of secondary sRNAs and, consequently, a node (Axtell et al. 2006). RNAs with aberrancy at both ends might also generate sRNAs in the absence of target-

Figure 4. The components of RNA silencing cascades. The three parts of the figure illustrate the processes associated with initiator, node, and end point RNAs. The siRNAs produced by DCL from the initiator RNA are the primary sRNAs. The primary siRNA then directs AGO-mediated cleavage of a node RNA, and the RDR-mediated synthesis of dsRNA and secondary sRNA then follows. The nucleoprotein complex at the bottom of the node RNA column is the AGO slicer protein associated with a secondary sRNA. The AGO slicer at the top of the end point RNA column could be associated either with primary or secondary sRNAs.

ing by a primary sRNA: The presence of the second aberrancy would substitute for sRNA targeting. In this scenario, the doubly aberrant RNA would be an initiator RNA rather than a node because the sRNA production would be independent of an sRNA. Correspondingly, the sRNAs from this doubly aberrant RNA would be primary rather than secondary. One of the major current challenges in sRNA studies is to find out how much of the RDR-dependent sRNA in *Arabidopsis* is primary or secondary. This information will be informative about the extent of sRNA networks and cascades.

BIOLOGICAL IMPLICATIONS OF sRNA CASCADES

The existence of RNA silencing cascades and networks would have profound implications for cellular regulation for several reasons. These systems would have inherent feedback characteristics, and they would provide a mechanism through which a change in the expression level of a node RNA would influence secondary sRNAs targeted at other nodes or end points in the cascade or network. In addition, the RNA silencing networks could have epigenetic characteristics. In this section, I consider the biological implications that are related and consequent to these characteristics of RNA silencing cascades and networks.

The simplest manifestation of negative feedback in RNA silencing cascades and networks would operate if the transcription of a node RNA was reduced as a consequence of an external stimulus or due to mutation of a promoter or transcription factor. The amount of RNA available for secondary sRNA production would also be reduced as a result of this change and, correspondingly, there would be less RNA silencing targeted by this sRNA. Since the targets of this sRNA would include the original node RNA, the reduced silencing activity would compensate, at least partially, for the reduced transcription. The converse would apply if the transcription of the node RNA was increased: The increased RNA silencing would offset the effects of the increased transcription (Baulcombe 2004). In addition, there could be more complex negative feedback systems, as discussed previously, whenever sRNAs target mRNAs for proteins that are required for silencing or for transcription factors that are required for production of sRNAs (Achard et al. 2004; Vaucheret et al. 2004, 2006).

These examples of negative feedback illustrate how RNA silencing loci and cascades would provide a buffer against the influence of the environment or mutation on the expression of node RNAs. In some respects, this system could provide an RNA-level equivalent of the heat shock protein (HSP)-mediated buffering mechanism that protects against the effect of misfolded proteins caused by mutation or environmental stress (Queitsch et al. 2002).

The RNA silencing could also mediate large-scale changes to the regulatory state of a cell if perturbations result in the introduction of a novel source of sRNAs. The new sRNAs would feed into a new cascade and, consequently, there could be silencing of multiple node and end point RNAs. Situations in which cascades could be activated by novel sources of sRNA could involve inducible genes or transposons. Transposons are abundant sources

of sRNA (Lu et al. 2005; Henderson et al. 2006) and they may be the major components of RNA silencing cascades either as initiators or nodes. Phenotypes of transposon-induced silencing could be manifested, for example, in hybrids between related plants with different transposon populations. Such transposon-induced silencing could explain, in part, the unexpected phenotypes that frequently arise in hybrid plants (Tanksley and McCouch 1997).

Viruses might also initiate RNA silencing cascades or networks. If a virus-induced cascade targets mRNAs, there could be viral symptoms that are caused by silencing. There are several examples of viral or viroid RNAs that have properties predicted of silencing RNAs in that they induce symptoms but do not encode proteins (Devic et al. 1989; Jaegle et al. 1990; Wang et al. 2004). However, there is no reason, in principle, that coding sequence viral RNAs could not also induce symptoms by silencing.

If viral RNAs have inverted repeats, they could serve as initiators of silencing cascades. However, even without inverted repeat regions, they could participate in cascades as nodes if they have endogenous sRNA target sites or a combination of sRNA target sites and aberrancy, as discussed above. In such a situation, a cascade that includes viral sRNAs would have been initiated by an endogenous sRNA. Since the viral sRNAs would have an antiviral role, this type of process illustrates how the endogenous sRNAs would, in effect, be the initiators of an immune response. A precedent for the antiviral role of endogenous sRNAs exists in animal cells producing miRNA targeted at a retrovirus, although there does not appear to be secondary sRNA produced in this system (Lecellier et al. 2005).

The potential for RNA silencing cascades to have an epigenetic characteristic involves the well-characterized mechanisms involving RNA-directed DNA methylation and heterochromatin formation (Lippman and Martienssen 2004). There is also the potential for a second epigenetic mechanism at the RNA level. To illustrate this second mechanism, I consider a silencing system in which the initiator RNA is expressed transiently and in which the node RNAs would be expressed continuously (Fig. 5). The epigenetic silencing would be initiated when these primary sRNAs interact with and direct cleavage of node RNAs. Secondary sRNAs would be produced in these initiator rounds of silencing, and they would have the potential to target other node RNAs. However, they would also have the potential to target further “maintenance” rounds of sRNA production on the original node RNAs (Fig. 5). The property of plant silencing systems, that secondary sRNAs are produced on both the 5′ and 3′ sides of the sRNA target, means that these maintenance rounds of silencing and the downstream cascade would persist, even in the absence of the primary sRNA. If secondary sRNAs were produced only on the 5′ side of an sRNA target, the sRNAs would be progressively more restricted in each maintenance round and the silencing would not persist (Bergstrom et al. 2003) unless there are direct sequence repeats in the node RNA, as discussed by Martienssen (2003).

An experimental example of an RNA-mediated epigenetic mechanism involves viruses inoculated to plants carrying a viral transgene. Some of these lines are not ini-

Figure 5. RNA-mediated epigenetic silencing. An RNA-mediated epigenetic mechanism is shown as a series of rounds involving primary and secondary sRNAs. In the initiator round, the primary sRNA triggers the production of secondary sRNAs on a node RNA. These secondary sRNAs then target other RNAs in the cascade in processes not shown in the diagram. However, they can also target the node RNA again and supplant the role of the primary sRNA in the subsequent rounds of secondary sRNA production. These subsequent rounds are referred to as maintenance rounds because they perpetuate the silencing mechanism even if the primary sRNA is no longer being produced. In effect, these maintenance rounds of silencing provide a molecular memory of the primary sRNA.

tially resistant to the virus, despite the presence of complete sequence identity between the virus and the transgene. The plants manifested viral symptoms on the inoculated leaves and the first leaves to become systemically infected. However, on the upper leaves of the plant, the virus was absent and the transgene was silent (Lindbo et al. 1993). It is likely in this situation that the viral RNA was the initiator producing sRNAs that targeted a transgene node RNA. This node RNA remained silent and, presumably, was a source of secondary siRNAs even after elimination of the initiator viral RNA. In a second example, the initiator of GFP RNA silencing was transiently expressed in one leaf of a plant. Once a signal of silencing had spread out of this leaf, the silencing persisted for the lifetime of the plant, even after the leaf had been removed (Voinnet and Baulcombe 1997; Voinnet et al. 1998). This effect was dependent on an RDR (Schwach et al. 2005). These examples illustrate the principle that there can be an RNA-mediated epigenetic mechanism although they do not involve cascades involving multiple nodes.

CONCLUSION: FUTURE ANALYSES OF CASCADES

In this paper, I speculated on the existence of sRNA cascades. I have justified this speculation by describing data to support the existence of the component molecules and processes in these cascades, including primary and secondary sRNA, transitivity, and end point RNAs. To support my speculation, I describe the limited evidence that primary sRNAs initiate secondary sRNA production. There is also evidence for enormous numbers of sRNAs in plants, and their dependency on RDR activity is consistent with, but not proof that, the idea that many of them are secondary. These sRNA cascades would have the buffering, integrative, and epigenetic characteristics described above. However, their extent is unknown. Do they encompass all

or most of the RNA in a cell either as initiator, node, or end point molecules—or just a minority of the cellular RNAs? Are there a few cascades or many, and do the cascades interact with each other? Fortunately, we are now in a position to address these questions using the full genome sequence of *Arabidopsis* and the power of the high-throughput sequencing technologies. For the first time, using these resources, we have the capability to determine the sequence profile of sRNA populations associated with AGO proteins, in RNA silencing mutants, in *Arabidopsis* genotypes, and after biotic and abiotic stimuli. We also have genetic tools—mutants in many RNA silencing genes and the potential to isolate insertion mutants in sRNA loci so that the small RNA profiles can be dissected in detail.

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