

Focus Review

Intercellular and systemic movement of RNA silencing signals

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In most eukaryotes, double-stranded RNA is processed into small RNAs that are potent regulators of gene expression. This gene silencing process is known as RNA silencing or RNA interference (RNAi) and, in plants and nematodes, it is associated with the production of a mobile signal that can travel from cell-to-cell and over long distances. The sequence-specific nature of systemic RNA silencing indicates that a nucleic acid is a component of the signalling complex. Recent work has shed light on the mobile RNA species, the genes involved in the production and transport of the signal. This review discusses the advances in systemic RNAi and presents the current challenges and questions in this rapidly evolving field.

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Introduction

Development, disease resistance and stress responses in multicellular organisms depend on local and systemic movement of various signal molecules including hormones, transcription factors and other macromolecules. Until the last decade, RNA was ruled out from these signalling pathways because it is susceptible to degradation by cellular nucleases. Exceptional examples of mobile RNA involving non-encapsidated viroids or viral RNAs were known (Harrison and Robinson, 1986; Culver and Dawson, 1989; Hamilton and Baulcombe, 1989), but there was only one suggestion that these pathogen RNAs could indicate how endogenous genes are controlled (Zimmern, 1982).

However, the mobile RNA proposal was revived following the discovery that transgenes triggered a potent gene silencing signal that moved from cell-to-cell and over long distances in plants (Palauqui *et al*, 1997; Voinnet and Baulcombe, 1997). The signal could not be detected directly but, as the effect was dependent on nucleotide (nt) sequence similarity of the transgene and its targets, it seemed likely that an RNA would be

involved (Palauqui *et al*, 1997; Voinnet and Baulcombe, 1997; Fire *et al*, 1998). The possibility of mobile RNA regulators was reinforced by the finding that injection of double-stranded RNA (dsRNA) could initiate both local and systemic RNA silencing in *Caenorhabditis elegans* (Fire *et al*, 1998).

Genetic, molecular and biochemical analyses then revealed RNA silencing pathways in plants, fungi and animals in which dsRNA is processed into double-stranded small (s)RNAs (20–30 nt) by Dicer or Dicer-like (DCL) endonucleases (Bernstein *et al*, 2001). The sRNAs load into Argonaute (AGO) proteins and guide them to complementary nucleic acids by Watson–Crick base-pairing (Tabara *et al*, 1999; Hammond *et al*, 2001; Martinez *et al*, 2002; Liu *et al*, 2004). If the targeted molecule is an RNA, there can be posttranscriptional gene silencing via RNA cleavage, translational repression or mRNA destabilization (Ghildiyal and Zamore, 2009). In addition, in plants and fission yeast, the AGO–sRNA complex can direct epigenetic modifications to the DNA or histones in transcriptional gene silencing (Moazed, 2009; Law and Jacobsen, 2010).

In principle, a mobile RNA could be the long dsRNA precursor of the sRNAs or the double- and single-stranded sRNAs in the primary RNA silencing pathway. In some organisms including plants, fission yeast and worms, there are additional candidate RNAs because sRNA initiate secondary sRNA production in a mechanism that involves RNA-dependent RNA polymerases (RDRs). In plants, a long secondary dsRNA is diced into secondary sRNAs (Mlotshwa *et al*, 2008), whereas in *C. elegans*, the secondary sRNA is produced directly without Dicer (Gent *et al*, 2010). This RDR-mediated phase has the effect of amplifying the effect of RNA silencing (Wassenegger and Krczal, 2006) and any of the secondary RNAs could be the mobile RNA.

In *Arabidopsis* and other plants, there are multiple AGOs, DCLs and RDRs (Baulcombe, 2004) and one scenario was that there are specialized RNA silencing pathways for movement. At present there is good evidence for at least four different types of RNA silencing pathway in plants involving different types of sRNA referred to as micro (mi)RNA and small interfering (si) RNA. These pathways involve miRNA, heterochromatin-associated siRNA (hc-siRNA), *trans*-acting siRNA (tasiRNA) and viral siRNA.

The miRNA pathway uses DCL1 to cleave an imperfectly matched hairpin RNA to generate 21 nt miRNAs, which load primarily into AGO1 to target mRNAs for cleavage (Llave *et al*, 2002; Reinhart *et al*, 2002). The hc-siRNA pathway generates 24 nt siRNAs via the production of transcripts by a plant-specific POL IV, which are made double stranded by RDR2 and are diced by DCL3 (Xie *et al*, 2004; Herr *et al*, 2005; Onodera *et al*, 2005; Pontes *et al*, 2006). These hc-siRNAs are loaded into AGO4, AGO6 and/or AGO9, recruit chromatin modifiers and target transcripts generated by the

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plant-specific POL V to direct DNA methylation at cytosine residues (Zilberman *et al*, 2003; Wierzbicki *et al*, 2008; Havecker *et al*, 2010). The tasiRNA (tasiRNA) pathway uses the miRNA-directed cleavage of a TAS gene to initiate phased 21 siRNA production through the combined actions of RDR6 and DCL4 (Allen *et al*, 2005; Xie *et al*, 2005; Yoshikawa *et al*, 2005). Lastly, plants possess a silencing pathway to suppress invading viral nucleic acids. Double-stranded viral RNA is diced by DCL4 and DCL2 to generate 21–22 nt siRNAs, which target the viral RNA for degradation (Blevins *et al*, 2006; Deleris *et al*, 2006).

However, signal movement is not specific to any of these pathways. Each of the miRNA, siRNA and tasiRNA pathways is associated with mobile RNA silencing, as described below. Here, we discuss recent work in plants that has identified the RNAs associated with the silencing signal, and we compare the progress in plants with developments towards understanding mobile RNA in *C. elegans*.

Local movement of an RNA silencing signal

In plants and *C. elegans*, the silencing signal moves initially into adjacent cells. In some instances, the signal spreads throughout the organism so that the target gene is silenced in most cell types. In plants, the systemic movement involves the vascular system comprising the phloem and xylem but, once the signal reaches the destination tissue, it again can spread by movement between adjacent cells. First, we consider the local movement mechanisms involving translocation between adjacent cells and in the following sections we describe systemic movement.

Molecular transport in plants can occur either symplastically through channels that connect adjacent cells called

plasmodesmata (symplastic movement; Figure 1) or apoplastically through a process that involves transfer across the cell membrane, the cell walls and intercellular spaces (apoplastic movement). The precedent with proteins and viruses indicated that local movement of a silencing signal is likely to be symplastic (Lough and Lucas, 2006). Consistent with this hypothesis, the signal is excluded from stomatal guard cells that are symplastically isolated from mesophyll cells in leaves (Voinnet *et al*, 1998).

The size limit for molecules passing through the plasmodesmata is around 27 kDa but plasmodesmata can dynamically change their size and selectivity (Imlau *et al*, 1999) to allow passage of larger molecules including the KNOTTED1 transcription factor and viral ribonucleoproteins (Lucas *et al*, 1995; Carrington *et al*, 1996). Viruses encode proteins that can change the exclusion limit of plasmodesmata and presumably there are cellular proteins acting similarly to allow movement of larger molecules.

Genetic screens in *Arabidopsis* were designed to identify the proteins involved in the movement of silencing signals including those that might have a role in opening the plasmodesmata (Himber *et al*, 2003; Smith *et al*, 2007). A silencer transgene used in these screens was expressed specifically in phloem companion cells (Figure 1) using the phloem-specific promoter of the *SUC2* gene. The targeted genes gave chlorotic or photobleached phenotypes when silenced so that the spread of the signal was apparent from the appearance of cells adjacent to the phloem (Figure 1) and, in both instances, there was silencing in the cells surrounding the veins. Mutant derivatives of these lines exhibited either reduced or enhanced silencing phenotypes and genes corresponding to these mutants have been mapped and identified (Table 1).

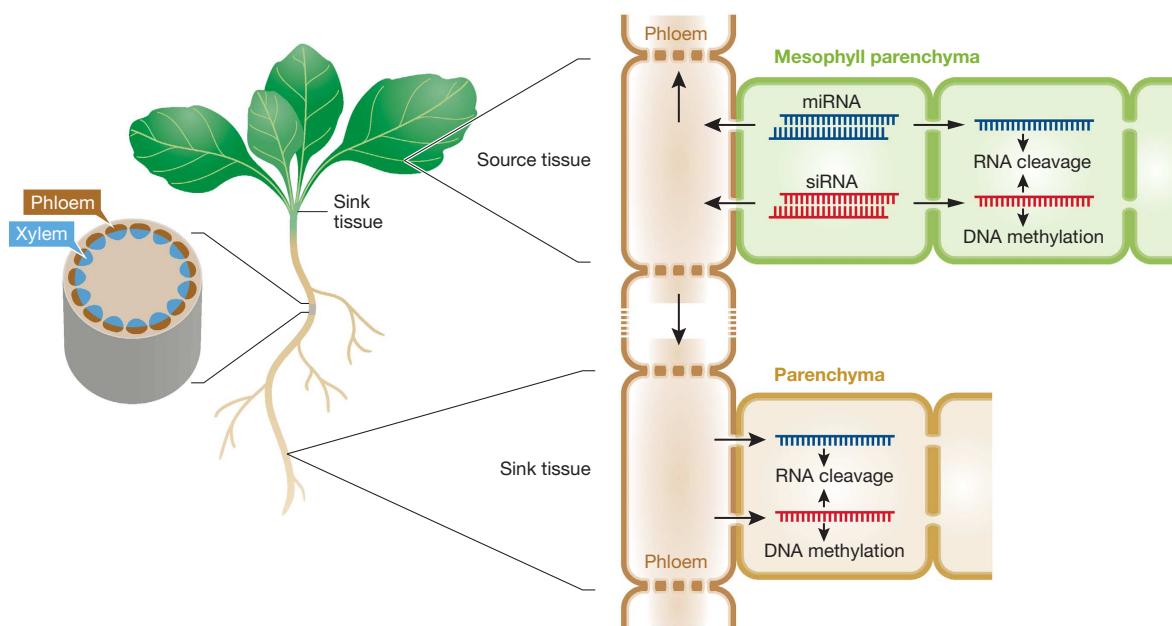


Figure 1 sRNA movement in *Arabidopsis*. sRNAs produced in photosynthetic source tissues such as the leaves move to growing points and photosynthetic sinks through the phloem, the tissue that carries proteins, hormones and nucleic acids (Lough and Lucas, 2006). The phloem contains two cell types, the enucleated sieve elements, which are porous and allow movement of solutes, and the phloem companion cells, which support the sieve elements. The xylem transports water and ions. sRNAs unload from the phloem and move from cell-to-cell through plasmodesmata. Some 21 nt miRNAs are likely mobile and cleave complementary mRNAs (Pant *et al*, 2008; Carlsbecker *et al*, 2010) whereas mobile 24 nt siRNAs direct DNA methylation (Molnar *et al*, 2010) and 21 nt siRNAs target RNAs for degradation (Chitwood *et al*, 2009). The parenchyma represents the bulk of the plant tissue. The mesophyll parenchyma is present in leaves and is the site of photosynthesis.

Table I Mutations that affect the cell-to-cell RNA silencing spreading phenotype in *Arabidopsis*

Reduced RNA silencing spread	Enhanced RNA silencing spread	Do not affect <i>PDS</i> and/or <i>SUL</i> silencing spread
<i>ago1</i> ^a	<i>ago4</i> ^{b,c,d}	<i>ago4</i> ^{a,d}
<i>clsy1</i> ^b	<i>dcl3</i> ^{b,d}	<i>dcl2</i> ^a
<i>dcl1</i> ^a		<i>dcl3</i> ^{a,d}
<i>dcl4</i> ^{b,e}		<i>drb4</i> ^a
<i>fca</i> ^f		<i>hst1</i> ^g
<i>fpa</i> ^f		<i>hyl1</i> ^a
<i>fj</i> ^h		<i>rdr1</i> ^a
<i>jmj14</i> ^c		<i>rdr6</i> ⁱ
<i>hen1</i> ^a		<i>sde3</i> ^j
<i>hpr1</i> ⁱ		<i>sde5</i> ^{j,k}
<i>nRPD1</i> ^{a,b}		
<i>nRPD2a</i> ^b		
<i>rdr2</i> ^{a,b}		
<i>tex1</i> ^l		

Mutations are classified as reducing, enhancing or not affecting PHYTOENE DESATURASE (*PDS*) and/or SULPHUR (*SUL*) RNA silencing.

^aDunoyer *et al* (2007).

^bSmith *et al* (2007).

^cSearle *et al* (2010).

^dAffects *PDS* silencing spread but not *SUL* silencing spread.

^eDunoyer *et al* (2005).

^fBaurle *et al* (2007).

^gDunoyer *et al* (2010a).

^hManzano *et al* (2009).

ⁱHimber *et al* (2003).

^jJauvion *et al* (2010).

^kHernandez-Pinzon *et al* (2007).

^lYelina *et al* (2010).

A conclusion from these genetic screens is that the production and the movement of a silencing signal involves more than one of the previously identified silencing pathways. Mutations in *NRPD1* and *RDR2*, in the hc-siRNA pathway, affected mobile silencing, whereas other components in the same pathway, *AGO4* and *DCL3*, did not (Dunoyer *et al*, 2007). Similarly, *DCL4* and *AGO1*, but not *RDR6*, in the tasiRNA pathway were also required (Table I).

However, a limitation of these genetic screens is the absence of spatial information. The mutant genes could act either in the phloem companion cells in which the signal was generated or in the recipient cells, or both. To address this issue, Dunoyer *et al* (2010b) expressed *DCL4* specifically in the phloem companion cells in the *dcl4* mutant background. Mobile silencing was restored in these plants, demonstrating that *DCL4* was required in cells that generate the silencing signal (Dunoyer *et al*, 2010b) and by extrapolation the 21-nt siRNAs that are the product of *DCL4* were implicated as the signal. Expression of the P19 viral suppressor of silencing in these cells prevented the mobile silencing phenotype and reinforced this conclusion (Dunoyer *et al*, 2010b). P19 binds 21 nt siRNAs (Silhavy *et al*, 2002) and would prevent their recruitment into the RNA silencing pathway (Vargason *et al*, 2003). Companion cell-specific expression of *AGO1* did not, however, complement the loss of mobile silencing in *ago1* mutants of the transgenic silencer lines, indicating that *AGO1* acts in cells that receive the silencing signal (Dunoyer *et al*, 2010b).

An interpretation of these various genetic data is that 21 nt duplex sRNAs are produced in the cells that generate the

signal and that they move into the recipient cells where they are recruited into an *AGO1* complex. To test this hypothesis, Dunoyer *et al* (2010a,b) bombarded fluorescently labelled sRNAs and found that 21 and 24 nt sRNAs move between cells in a double-stranded but not single-stranded form. In contrast, the delivery of sRNA by microinjection techniques failed to demonstrate 21 or 25 nt dsRNA movement from cell-to-cell (Yoo *et al*, 2004). Microinjection has been used extensively to analyse the effect of viral movement proteins on plasmodesmata size exclusion (Carrington *et al*, 1996) but it is subject to artefacts caused by high pressure damage of subcellular structures (Storms *et al*, 1998). Our cautious interpretation of the conflicting data is that double-stranded 21 nt siRNA is a signal of mobile silencing but that further analysis is needed. We also note that none of the experiments rule out the possibility that the mobile RNA signal exists in several forms of which the 21-nt RNA duplex is just one.

In addition to the hc-siRNA and tasiRNA pathway components, the genetic analyses also implicated *TEX1* and *HPR1* in mobile silencing. These proteins are associated with an intracellular RNA transport complex known as THO/TREX in mammals (Strasser *et al*, 2002; Rehwinkel *et al*, 2004; Jauvion *et al*, 2010; Yelina *et al*, 2010) (Table I). *TEX1* and *HPR1* may export or process a signal from the nucleus into a compartment for cell-to-cell movement. Alternatively, these proteins could traffic imported RNA in the cells that receive a silencing signal.

It is striking that, until now, the genetic screens have not revealed proteins associated either with plasmodesmata or with cytoskeletal features that would influence symplastic movement of silencing RNA. This failure is probably because symplastic movement of various endogenous molecules including RNA are essential for normal growth and development and mutants would be difficult to detect because they would exhibit limited or no viability. In leaf development, for example, there is evidence that tasiRNAs produced on the adaxial side of the leaf diffuse to the abaxial side to regulate the leaf development gene *AUXIN RESPONSE FACTOR 3* (Chitwood *et al*, 2009; Schwab *et al*, 2009). Similarly, there are miRNAs with an effect between cells on transcription factor expression in the root stele (Carlsbecker *et al*, 2010) although it is not known whether the mobile factor is the miRNA or its precursor.

There may also be movement of sRNA or sRNA precursors between vegetative and sperm cells in *Arabidopsis* pollen (Slotkin *et al*, 2009). However, there are unlikely to be plasmodesmata in the sperm cell wall (Russell and Cass, 1981) and this process would have to be apoplastic rather than symplastic. Apoplastic movement has also been proposed to explain how maternally expressed siRNAs could be transferred from the endosperm of developing seeds into the symplastically isolated embryo (Martienssen, 2010; Mosher and Melnyk, 2010) but direct evidence for this is lacking.

Evidence for movement of RNA into sperm cells is based on experiments using artificial miRNAs (amiRNA) expressed specifically in the microspore precursor and pollen vegetative cell but not the sperm (Slotkin *et al*, 2009). The interpretation of these data depends crucially on the amiRNA promoter being completely inactive in the pollen sperm cell. It also requires that amiRNAs produced in the microspore precursors are degraded before sperm cell differentiation (Le Trionnaire *et al*, 2010). To remove any ambiguity, it

would be helpful to have confirmatory experiments based on different methods.

Systemic movement of RNA silencing

Movement of a silencing signal between different organs of the plant requires loading into the vascular system, transport and then unloading in the recipient tissue or cells (Figure 1). This long distance or systemic movement of the silencing signal takes place over days (Voinnet *et al*, 1998) and it is generally from photosynthetic sources (i.e. leaves) to sucrose sinks (i.e. roots and growing points) through a bulk flow process that is characteristic of the phloem (Voinnet *et al*, 1998; Van Bel, 2003). For this reason, the phloem rather than the xylem is generally considered as the conduit for movement of the silencing signal. Notably, the xylem sap, which transports water and ions, is free of RNA (Figure 1; Buhtz *et al*, 2008).

The systemic signal, as with a local signal, could include various forms of nucleic acid including sRNAs and sRNA precursors. Approaches to identification of the systemic RNA silencing signal include direct sampling of phloem sap (Yoo *et al*, 2004; Buhtz *et al*, 2008) and detection of RNAs in stocks and scions of grafted plants (Palauqui *et al*, 1997; Schwach *et al*, 2005; Brosnan *et al*, 2007; Molnar *et al*, 2010; Dunoyer *et al*, 2010a). Both approaches, as described below, support the involvement of sRNA as part of the RNA silencing signal.

The direct sampling approach in cucurbits and *Brassica* allowed the detection of 18–25 nt siRNAs and miRNAs (Yoo *et al*, 2004) including those associated with nutrient homeostasis (Buhtz *et al*, 2008). Assays on sRNAs found in the phloem suggest that they are present as single-stranded RNAs (Yoo *et al*, 2004; Buhtz *et al*, 2008) but both miRNA and the complementary miRNA* sequences were present. In non-vascular cells, one of the two strands is destroyed as the miRNAs are loaded into AGO and the presence of both strands is, therefore, an indication that sRNAs may not be associated with AGO in the phloem. The phloem sap is a benign RNase-free environment so the sRNAs could be stable in an unbound form (Sasaki *et al*, 1998; Doering-Saad *et al*, 2002) or they could be associated with proteins other than AGO.

A protein—CmPSRP1—in pumpkin phloem binds specifically to 25 nt single-stranded RNAs (Yoo *et al*, 2004). It promoted the intercellular movement of this RNA in a single but not double-stranded form in a microinjection assay. However, there is no genetic evidence to support the function of this protein and the microinjection assay, as discussed above (Storms *et al*, 1998), is prone to artefacts and addresses cell-to-cell rather than long distance movement. Thus, further analysis of this protein is required.

The use of plant grafting is a useful supplement to direct sampling because it allows the source of the silencing signal to be genetically distinct from the recipient tissue and nucleotide sequence data can be used to identify RNA that has moved across the graft union. For example, Pant *et al* (2008) overexpressed miR399 in shoots and, following grafting to wild-type roots, they observed an increase in miR399 abundance in the roots. Dunoyer *et al* (2010a) similarly used an *Arabidopsis* line that lacked endogenous siRNAs from part of an inverted repeat transcript (IR71). Roots of this line contained very few IR71 siRNAs from this region unless

they were grafted to shoots of wild-type plants in which those siRNA were abundant.

Both of these analyses were an advance over the simple direct sampling approach because they showed genetically that RNA has moved from shoot to root and they are consistent with the idea that sRNAs are mobile. However, none of the analyses described above has been conclusive about molecular form of the mobile RNA. The sampling of phloem sap was not linked to a functional analysis of the mobile RNA and the grafting assays were equally consistent with movement of sRNAs or of their precursors. The picture was further complicated by the analysis of plants in which roots undergoing RNA silencing of *GFP* were grafted to non-silenced *GFP* shoots. The shoot *GFP* was silenced in these experiments irrespective of whether the root contained mutations in the *dcl1* mutant or the *dcl2 dcl3 dcl4* triple mutant (*dcl2,3,4*) in the initiating tissue and the authors of that study concluded that DCLs are not required. They inferred that long RNAs are the mobile RNA species (Brosnan *et al*, 2007). However, the interpretation of these experiments was complicated by functional redundancy of DCLs in *Arabidopsis* (Deleris *et al*, 2006) and it remained possible that sRNAs are the mobile species.

A more conclusive grafting analysis exploited the absence of 22–24 nt sRNAs in the *Arabidopsis dcl2,3,4* triple mutant (Molnar *et al*, 2010) and used next generation sequencing to monitor movement of a silencing signal from shoots to root. This approach was clearly informative about 24 nt sRNAs from transgenes and endogenous loci: they were absent in recipient *dcl2,3,4* roots if they were grafted to *dcl2,3,4* shoots due to the lack of DCL3 but they were present if the mutant roots were grafted to wild-type shoots. Similarly, the 24-nt sRNAs were more abundant in wild-type roots grafted to wild-type shoots than in wild-type roots grafted to *dcl2,3,4* shoots. The clear conclusion is that 24 nt sRNAs move systemically and that a substantial proportion of the 24-nt sRNA in roots has moved from the shoots. A further conclusion from this analysis was that, at least from a *GFP* transgene and from selected endogenous loci, the 24 nt rather than 21 nt sRNAs were selectively mobile through the systemic pathway.

However, there is not a simple relationship of 24 nt siRNA and systemic movement. Only 35% of sRNA loci in *Arabidopsis* (7179 loci) produced sRNAs that are associated with mobility and other locus-specific factors must be required (Molnar *et al*, 2010). In addition, the selectivity against 21 nt RNA does not always apply, as indicated by systemic silencing associated with a mobile 21 nt miRNA (Pant *et al*, 2008) and siRNA (Dunoyer *et al*, 2010a). A type of 21 nt tasiRNA also acts non-autonomously over a much greater range than some miRNAs or transgene-derived siRNAs (de Felippes *et al*, 2011). It is therefore clear that sRNA size is not the only determinant of local or systemic movement. There must be other factors that could be associated, for example, with the genetic locus from which the sRNAs are transcribed or with the long precursor RNA.

Separate mechanisms for local and systemic movement?

In principle, there could be separate mechanisms for local and systemic movement such that RNA from any single locus

could move only between cells or only within the phloem. Supporting this idea there is the preferential association of 21 nt sRNA and 24 nt sRNA with local and systemic movement, respectively (Dunoyer *et al*, 2007; Smith *et al*, 2007; Molnar *et al*, 2010). An analysis of viral suppressor proteins is also consistent with separate mechanisms: suppressors that blocked both 21 and 24 nt sRNA pathways prevented both local and systemic silencing (p19; Hamilton *et al*, 2002) whereas those affecting specifically the 24-nt sRNA pathways blocked only systemic silencing (Hc and P1; Hamilton *et al*, 2002).

Why would systemic movement be distinct from local movement? One trivial explanation could be quantitative: an sRNA signal from a weak source would be diluted over a few cells and would not spread further than a few cells. In such a situation, a viral suppressor leaving a low level of residual sRNA would block systemic but not local silencing, whereas a suppressor causing a complete reduction would block both systemic and local spread. The influence of quantitative effects in local movement is illustrated clearly by the spread of silencing from the *SUC2* promoter construct. Most of the transgenic lines with this construct exhibited silencing that spread over tens of cells beyond the veins but, in mutant lines in which the transgene promoter is derepressed, the targeted gene was suppressed in all or most cells of the leaf (Smith *et al*, 2007).

Qualitative differences of local and systemic silencing are also possible and they could be influenced, for example, by mechanisms for entry in, translocation through, or exit from the phloem. Virus movement is a useful model because it too has distinct mechanisms for local and systemic spread that can be differentiated through the use of low levels of cadmium. Cadmium inhibits systemic but not local spread of both gene silencing and viruses (Ueki and Citovsky, 2001) and the differential effect is probably related to enhancing callose accumulation in the cells surrounding the vascular tissue (Ueki and Citovsky, 2002).

Surprisingly, the low levels of cadmium did not affect plant development. It is unlikely therefore that systemic movement of an RNA silencing signal is required for coordination of growth and development, at least in *Arabidopsis* and *Nicotiana* species. However, there is good evidence for roles of the systemic silencing in phosphate homeostasis and virus resistance. Phosphate homeostasis is influenced by a mobile miRNA or miRNA precursor (miR399) that targets the *PHO2* mRNA (Pant *et al*, 2008) encoding a suppressor of phosphate uptake. There is a complex feedback system in which the phosphate-induced miR399 or its precursor is translocated to the root where it blocks further production of phosphate uptake proteins. Further fine tuning of this system involves a non-coding RNA that sequesters the miRNA in a non-productive interaction (Doerner, 2008).

In virus resistance, a systemic silencing RNA signal is involved in exclusion of the virus from the shoot apex. The signal is produced in the initially infected cells and moves into the meristem and surrounding cells of the shoot apex either together with or ahead of the systemically mobile virus (Ratcliff *et al*, 1997; Schwach *et al*, 2005). The signal would then be available to suppress the virus as it enters the meristematic cells so that infection is never established. The signal is presumed to include viral sRNA but, until now, there is no definitive evidence to rule out longer sRNA

precursors. For RNA viruses, it is likely that 21 and 22 nt sRNAs or their precursors would be involved because they normally mediate posttranscriptional mechanisms (Ruiz-Ferrer and Voinnet, 2009). For DNA viruses, the 24-nt size class or their precursors could mediate this meristem exclusion by acting either at the posttranscriptional or at the transcriptional level (Ruiz-Ferrer and Voinnet, 2009).

Meristems and persistent effects of a silencing signal

RNA silencing in meristems is complex. It clearly takes place as evidenced by the analysis of meristem exclusion of viruses and by the use of a transgene that silences a promoter active in meristems (Kanno *et al*, 2008). However, there is also evidence of transgenes that escape silencing in meristems or that are only weakly silent in dividing cells (Mittelsten Scheid *et al*, 1991; Vaucheret, 1994). Unravelling this complexity is important because the effect of systemic silencing in shoot apices could be important not only in terms of virus resistance. The systemic silencing signals corresponding to endogenous sequences could, at least in principle, move into shoot meristems and initiate effects that persist in the cells as they differentiate into the main organs of the plant. The persistence of effects due to systemic RNA silencing signals could be mediated by mechanisms acting either on DNA or at the RNA level.

The DNA/chromatin mechanism is associated predominantly with the 24-nt hc-siRNAs that direct DNA methylation in the recipient cells (Molnar *et al*, 2010) including those in the meristem (Molnar *et al*, 2010) (Melnyk *et al*, unpublished data). Presumably, the 24-nt siRNAs are taken into the nucleus of the recipient cells where they are stabilized by the activity of an exportin-5 homologue, *HASTY* (*HST*) (Park *et al*, 2005; Dunoyer *et al*, 2010a). Once initiated in an sRNA-dependent manner, these effects would persist because they would be maintained by maintenance DNA methylases (Jones *et al*, 2001).

Persistent effects of a systemic RNA signal at the RNA level would involve RDR proteins in a mechanism that is like that of the tasiRNA pathway. A primary sRNA in this mechanism would be the signal or derived from the signal and it would be functionally equivalent to the initiator miRNA in the tasiRNA pathway. A long RNA in the meristem would be targeted by the primary sRNA in the same way that the tasiRNA precursor is targeted by a miRNA and it would then be converted into dsRNA and secondary sRNAs by RDR and DCL proteins. Such a mechanism would be persistent if the secondary sRNAs could substitute for the initiator sRNA in subsequent rounds and it is likely to explain the properties of a graft-transmissible transgene silencing signal in *Arabidopsis* (Himber *et al*, 2003; Brosnan *et al*, 2007) and *Nicotiana benthamiana* that is amplified by RDR6 (Schwach *et al*, 2005). This RNA-based mechanism might also be implicated in RNA silencing mediated resistance against viruses in plants that have 'recovered' from virus disease. Recovered plants are disease free in the upper regions and exhibit RNA-mediated resistance against secondary infections (Ratcliff *et al*, 1997). It is likely that the resistance is mediated by viral RNAs that moved into the meristem from the lower diseased regions of the plant. There might also be

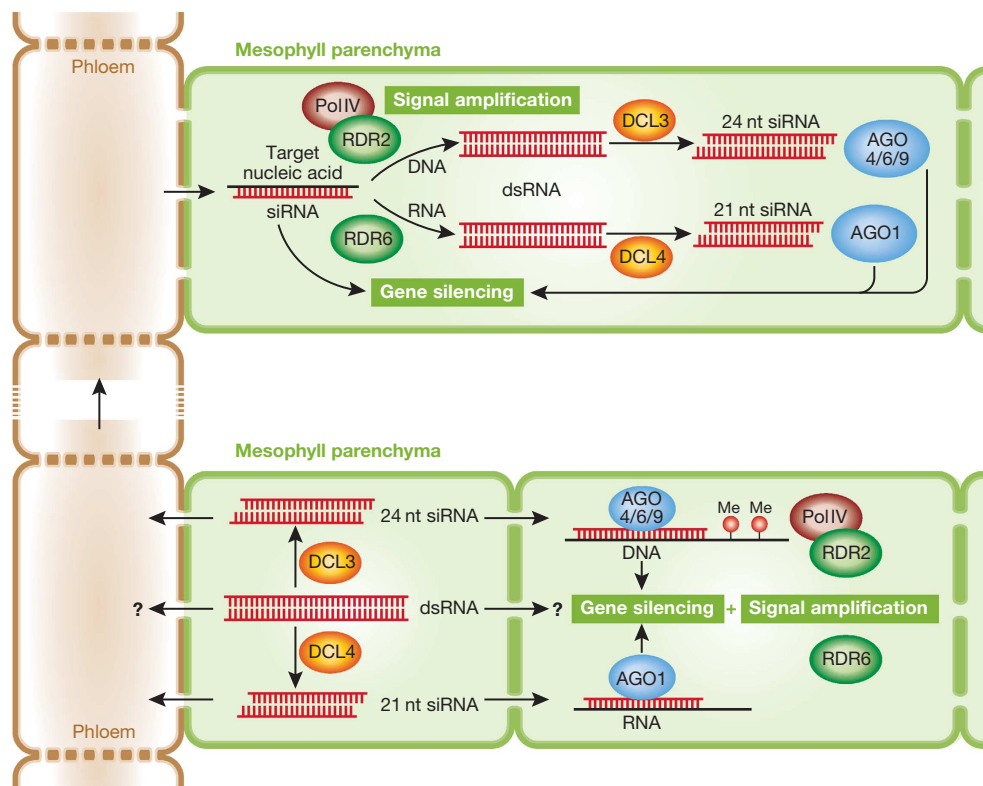


Figure 2 The genetic requirements of siRNA movement in *Arabidopsis*. siRNAs generated by DCL3 or DCL4 likely move from cell-to-cell through plasmodesmata and long distances through the phloem. Mobile 21 nt siRNAs associate with AGO1 to direct posttranscriptional gene silencing (Dunoyer *et al*, 2005, 2007, 2010b), whereas mobile 24 nt siRNAs may associate with AGO4, 6 or 9 to direct DNA methylation (Me) or other chromatin marks associated with transcriptional gene silencing (Zilberman *et al*, 2003; Havecker *et al*, 2010). Either sRNA can likely initiate signal amplification in recipient tissues via the action of RNA Polymerase IV and/or RDRs (Schwach *et al*, 2005; Brosnan *et al*, 2007). The contribution of long RNAs to systemic RNA silencing in plants is unknown.

RNA-mediated amplification as a component of systemic RNA-mediated silencing through the hc-siRNA pathway (Figure 2) as evidenced by the involvement of RDR2 (Brosnan *et al*, 2007).

However, apart from the virus resistance and recovery, there is very little information about the biological role of systemic silencing effects in meristems that lead to persistent changes in daughter cells and differentiated organs. In *Arabidopsis*, key functions in growth and development can be ruled out because, as mentioned above, most RNA silencing mutants grow normally. However, we could envision that induced stress tolerance or resistance to disease is due to a silencing signal that moves from parts of the plant that perceive the inducing stimulus to the meristem. A variation of this idea is based on the potential of RNA silencing to induce heritable epigenetic changes and the emerging evidence for *trans*-generational inheritance of phenotypes induced in response to environmental changes (Molinier *et al*, 2006; Boyko *et al*, 2007; Whittle *et al*, 2009; Lang-Mladek *et al*, 2010; Ito *et al*, 2011). Perhaps, the silencing signal induces heritable epigenetic effects in the meristem that then persist through the pollen and egg cells into the next generation (see Box 1)? These possibilities have implications for understanding of adaption of plants and plant populations to extreme environments and they can be easily explored using mutants in various RNA silencing pathways.

Box 1 Outstanding questions

- Do mobile sRNAs contribute to heritable epigenetic phenomena in plants?
- In what form does RNA move long distances in plants?
- Why are some endogenous siRNAs mobile in plants, while others are not?
- What proteins are required for long distance transport in plants?
- What proteins are required for RNA export in *C. elegans* and other animals?
- Does endogenous systemic RNA silencing exist outside of plants?
- Can the mobile RNA in *C. elegans* be physically detected?
- Is there widespread apoplasmic movement of RNA signals in plants?

Movement of RNA between organisms

The effects of RNA silencing can spread out of a plant. In a biotechnological context, this movement is illustrated by the ability of RNA silencing transgenes to target genes in invertebrate and fungal pathogens when they feed on the plant (Huang *et al*, 2006; Baum *et al*, 2007; Mao *et al*, 2007; Nowara, 2010; Tinoco *et al*, 2010). The assumption in these examples is that sRNAs are transferred between organisms but, for the reasons described above, we cannot rule out that sRNA precursors are the mobile form and that they are processed in the pest. In the case of transfer to sucking

insects and nematodes, a stylet extracts the contents of plant cells or the phloem sap, presumably including the RNA silencing signal. With *Coleoptera*, for which this gene targeting strategy is also successful (Baum *et al*, 2007), the cells are broken open when the insect feeds. In this instance, the RNA silencing signal remains functional in an extracellular environment, indicating that it is protected by association with proteins. The subsequent transfer into the insect is likely to involve transfer of the signal across a membrane and is discussed below in the section about silencing in animals.

Hemi-parasitic plants such as *Triphysaria* can also take up silencing RNA from their host plant. Wild-type *Triphysaria*, for example, can transfer an RNA silencing signal from a *GUS* silenced lettuce to a *GUS* expressing line (Tomilov *et al*, 2008) and presumably the mobile RNA could also have silenced *Triphysaria* genes *in transit*. Unlike the examples with insects, this transfer is likely to be completely symplastic because there are plasmodesmata formed across the cell wall between the two species (Vaughn, 2003).

These examples with invertebrate pests and parasitic plants are both artificial, but there is no reason in principle why they could not have natural significance if sRNAs in the plant have chance similarity to essential genes in the pest or parasite. In that scenario, the RNA silencing would be part of a natural defense system, as it is with viruses.

Systemic RNAi in animals

Although first identified in plants, systemic RNA silencing also occurs in diverse animals including worms (*C. elegans*; Fire *et al*, 1998), insects (*Tribolium*; Bucher *et al*, 2002) and *Planaria* (Newmark *et al*, 2003). Unlike plants, *C. elegans* and *Planaria* can uptake dsRNA from the environment by feeding or soaking in a dsRNA solution. The exogenously supplied or microinjected dsRNA can trigger sequence-specific gene silencing in the treated and adjacent tissues and silence the progeny of injected animals. Thus, the long dsRNA or its derivatives must move across the cell membrane from cell-to-cell, unlike the situation in plants where the movement of the silencing signal occurs through a shared cytoplasm. This phenomenon implies the need for membrane-bound transporters and, indeed, a transmembrane protein called SID-1 has been identified in a forward genetic screen that is required for systemic RNA interference (RNAi) in *C. elegans* (Winston *et al*, 2002).

SID-1 homologues exist in most animals but not plants, suggesting that systemic RNAi may also occur in other animals including mammals (Winston *et al*, 2002; Wolfrum *et al*, 2007). Since SID-1 has higher affinity for longer dsRNA than sRNA duplexes (Feinberg and Hunter, 2003), it is highly likely that long dsRNA is the main component of the RNAi-associated silencing signal in worms. Consistent with this idea, a mutation in RDE-4, which prevents the processing of dsRNA into siRNA (Parrish and Fire, 2001), is not required for movement of a heritable silencing signal (Grishok *et al*, 2000).

Although the movement of exogenous and transgenic dsRNA is well documented in *C. elegans*, it remains unknown whether endogenous sRNAs are transported in animals (see Box 1). Sources of dsRNA and siRNAs are present in the *C. elegans* genome (Ambros *et al*, 2003; Zhao *et al*, 2007) so it is likely that they are mobile. Consistent with this idea,

SID-1 imports synthetic pre-miRNAs and long hairpin molecules, suggesting that it may also transport endogenous dsRNAs (Shih and Hunter, 2011).

Notably, SID-1 mutants have no obvious developmental phenotypes (Winston *et al*, 2002), suggesting that SID-1-mediated dsRNA transport does not have a role in essential developmental processes. SID-1 is not required for RNA export (Winston *et al*, 2002; Jose *et al*, 2009) so uncharacterized RNA export or import pathways in *C. elegans* may contribute to developmentally important processes. The recent discovery of a virus in *C. elegans* (Felix *et al*, 2011) may shed light on the endogenous function of SID-1 and systemic RNA movement in antiviral defence.

Plants and animals share the core silencing machinery, including the Dicer and AGO nucleases and in some instances the RNA-dependent RDRs that are involved in the amplification of the silencing signal and in the production of secondary siRNAs. In worms, RDRs can generate dsRNA that includes sequences upstream of the trigger dsRNA sequence, and these secondary dsRNAs can also act as or generate a transported silencing signal (Sijen *et al*, 2001; Alder *et al*, 2003). Intriguingly, injection of short antisense RNA can trigger gene silencing in non-injected tissues (Tijsterman *et al*, 2002). These experiments revealed that both long and short RNAs can act as mobile signals and might indicate the involvement of multiple as yet uncharacterized transport pathways in systemic RNAi. Ultimately, physical detection of mobile species in *C. elegans* will be necessary to understand the contribution of short and long RNAs to gene silencing.

The movement of a signal associated with RNA silencing also occurs at the intracellular level in both plant and animals. sRNAs are enriched at distinct foci in the cytoplasm called P bodies and GW bodies (Jakymiw *et al*, 2005; Liu *et al*, 2005; Pillai *et al*, 2005; Sen and Blau, 2005), which suggests that localization is actively regulated. In *C. elegans*, the AGO protein NRDE-3 binds to siRNAs in the cytoplasm and transports them to the nucleus (Guang *et al*, 2008). A loss of NRDE-3 prevents nuclear gene silencing, suggesting that this protein is absolutely required (Guang *et al*, 2008). No plant AGOs have been identified that can transport specific classes of RNAs to discrete cellular locations, but it is conceivable that such mechanisms exist. One proposal is that the endolysosomal system contributes to the transport of sRNAs (Gibbins and Voinnet, 2010). Conceivably, sRNAs could be compartmentalized in vesicles for transport either intra or intercellularly. Supporting this hypothesis in *Drosophila* cells, the uptake of dsRNAs from the environment requires receptor-mediated endocytosis (Saleh *et al*, 2006) while gene silencing by miRNAs and siRNAs is linked to endosomal trafficking (Lee *et al*, 2009).

Systemic RNA silencing may also exist in other animals including vertebrates. Modified siRNAs injected in the tail vein of mice silenced endogenous targets in various tissues (Soutschek *et al*, 2004) and a subset of miRNAs are present in vesicles of human blood, suggesting that they have a non-cell autonomous function (Hunter *et al*, 2008). A recent study found that the majority of miRNAs in human blood associated in protein complexes with AGO2 (Arroyo *et al*, 2011). The identification of extracellular AGO2-miRNA effectors suggests that these complexes may be functional and provides a plausible explanation for how sRNAs could move

systemically in mammals and other organisms. Determining whether endogenous sRNA movement occurs in mammals and understanding what types of RNAs move long distances will aid in the development of systemic RNA drug therapies (Whitehead *et al*, 2009).

Concluding remarks

RNA is now generally recognized as an important signal in plants in connection with gene silencing. The discovery that siRNA is a component of the signal was not surprising but the early evidence did not anticipate that it would be associated with epigenetic modifications as well as with posttranscriptional events: the first indications of a mobile signal in silencing were only associated with posttranscriptional mechanisms.

Most evidence indicates symplastic movement of the signal in plants but not animals, although there may be instances, in pollen for example, of apoplastic movement involving transport across membranes. Other aspects of the mechanism are poorly defined. We know, for example, that 21 and 24 nt siRNAs can be mobile in a duplex form in artificial systems but we do not yet have the definitive data to show that endogenous RNAs move in that form. To rule out that siRNA or miRNA precursors are mobile, we need additional data using, perhaps, genetic mosaics. Cell type specific promoters to express sRNA precursors or to complement *DCL* mutations might also be effective. The approaches used to analyse of mobile RNA also need to account for observation that the translocation mechanism is selective for subtypes of mobile RNA. Only some 24 nt siRNA loci or miRNA loci produce mobile sRNAs or sRNA precursors and there must be subtle RNA- or genetic locus-specific factors that influence the potential for RNA movement.

There is also much to be done about the identification of proteins required for the movement process and in the comparative analysis of local and systemic movement. That many of these proteins may be necessary for other transport functions, in addition to that of RNA, will complicate this analysis.

However, the major challenge concerns the function of mobile RNA silencing. A review on this topic referred to

an 'information superhighway' in the expectation that the flowering hormone florigen, morphogenetic gradients and defense signals would all have an RNA component (Jorgensen *et al*, 1998). The defense signalling concept has survived, at least in virus disease, and there may be some instances of morphogenetic gradients with an RNA component in embryos, leaves and root. The RNA 'superhighway' concept remains valid but it probably now needs revision to take into account the recent data.

A particularly puzzling aspect of the question about function relates to the 24-nt siRNAs. They are by far the most abundant component of the systemically mobile sRNA but, in *Arabidopsis*, their absence in mutants does not have a conspicuous phenotype. One reaction to this result would be that they are an artefact or do not have an important function. Alternatively they could have a role that has not yet been revealed. We favour the second view given that they are so abundant, that the movement mechanism is selective for 24 nt sRNAs from some but not all loci and because the mobile RNAs have the potential to initiate DNA methylation at targeted loci. Taking these factors into account, a reasonable hypothesis is that these mobile RNAs initiate epigenetic effects in meristematic tissue. Such effects could be associated with changes in gene expression. They could also be related to genome defense if the mobile RNA is a sentinel of active transposons in non-meristematic cells. There is also the intriguing possibility that the mobile sRNA could initiate transgenerational effects and affect the adaptation of a plant lineage or population to changed conditions. Clearly, these issues are fundamental to plant biology. They will also have practical application because the mobile sRNAs could be harnessed in crop improvement.

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Conflict of interest

The authors declare that they have no conflict of interest.

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