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RNA silencing movement in plants

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Abstract Multicellular organisms, like higher plants, need to coordinate their growth and development and to cope with environmental cues. To achieve this, various signal molecules are transported between neighboring cells and distant organs to control the fate of the recipient cells and organs. RNA silencing produces cell non-autonomous signal molecules that can move over short or long distances leading to the sequence specific silencing of a target gene in a well defined area of cells or throughout the entire plant, respectively. The nature of these signal molecules, the route of silencing spread, and the genes involved in their production, movement and reception are discussed in this review. Additionally, a short section on features of silencing spread in animal models is presented at the end of this review.

Keywords: Cell non-autonomous; post-transcriptional gene silencing; RNA interference; short range silencing spread; systemic silencing; plant RNA silencing; transcriptional gene silencing
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Invited Expert Review

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INTRODUCTION

Higher eukaryotes present a sequence-specific RNA degradation mechanism termed RNA silencing. RNA silencing encompasses post-transcriptional gene silencing (PTGS) a term often used in plants, RNA interference (RNAi) usually used in metazoa in relation to the small interfering RNA (siRNA)-mediated pathway, but also suppressive regulation by other types of small RNAs (sRNAs) such as microRNAs (miRNAs) and heterochromatic siRNAs (hc-siRNAs). In the siRNA pathway RNase III-type nucleases, Dicer proteins, generate siRNAs from the specific cleavage of double-stranded RNAs (dsRNAs). siRNAs are incorporated into an ARGONAUTE (AGO)-containing complex known as RNA induced silencing complex (RISC). After strand separation, the remaining single-stranded sRNAs guide the sequence-specific cleavage of the target transcripts. The siRNA-mediated silencing pathway targets exogenous sequences, such as viruses and transgenes as well as endogenous sequences leading to post-transcriptional and/or transcriptional silencing of the targeted sequences. For a recent overview of sRNA pathways in plants refer to Pyott and Molnar (Pyott and Molnar 2015). Despite evolutionary conserved features of the siRNA pathway in plants and animals important cross-kingdom differences have been described, and have been previously reviewed (Meister and Tuschl 2004; Mello and Conte 2004; Baulcombe 2006; Kim et al. 2009; Sarkies and Miska 2014).

miRNAs are the best characterized sRNA regulating endogenous RNAs. miRNAs are processed from primary transcripts with extensive fold-back structures which are transcribed from specific endogenous non protein-coding sequences (*MIRNA* genes) either from intergenic sequences or in metazoa also within introns. In plants, *MIRNA* genes are transcribed by RNA polymerase II (Xie et al. 2005a; Megraw et al. 2006) and are expressed in a tissue- and time-specific manner (Lai 2005; Valoczi et al. 2006). Plant miRNAs, function by providing sequence specificity to RISC, which usually slices mRNAs complementary to the miRNAs in a similar way to siRNAs (Llave et al. 2002; Rhoades et al. 2002; Kasschau et al. 2003; Jones-Rhoades et al. 2006), although translational suppression has also been shown (Brodersen et al. 2008). Plant miRNAs have been demonstrated to hold important roles in various processes including growth, stress response and development. miRNA biogenesis and function in both plants and animals have been extensively reviewed elsewhere (Bartel 2004; He and Hannon 2004; Murchison and Hannon 2004; Chen 2005; Siomi and Siomi 2010; Ha and Kim 2014).

In plants, in addition to the well-characterized categories of siRNAs and miRNAs, other types of small RNAs such as trans-acting siRNAs (tasiRNAs) have also been studied. Biosynthetic pathways of tasiRNAs combine features and genetic requirements of siRNAs and miRNAs. tasiRNAs are derived from non protein-coding transcripts containing one or two miRNAs sites. The miRNA cleavage products are converted into a double-strand form by RNA-DIRECTED RNA POLYMERASE 6 (RDR6) (Vazquez et al. 2004; Allen et al. 2005). SUPPRESSOR OF GENE SILENCING 3 (SGS3) is required in this step to protect the miRNA cleavage products from degradation (Yoshikawa et al. 2005). The double stranded

intermediate is then processed by DICER-LIKE 4 (DCL4) and DEDICATED dsRNA-BINDING PROTEIN 4 (DRB4) to phased 21nt tasiRNAs which are incorporated into AGO-containing complexes to target complementary sequences (Vazquez et al. 2004; Allen et al. 2005; Gasciolli et al. 2005; Xie et al. 2005b; Yoshikawa et al. 2005; Adenot et al. 2006). AGO1 (Peragine et al. 2004; Vazquez et al. 2004; Allen et al. 2005; Yoshikawa et al. 2005) and AGO7 (Adenot et al. 2006; Fahlgren et al. 2006; Montgomery et al. 2008) proteins have been found to be involved in tasiRNA function. The miRNA cleavage site determines the phase and is critical for the production of specific tasiRNAs (Allen et al. 2005). In *Arabidopsis thaliana* there are at least eight tasiRNA producing loci that fall into four *TAS* groups, TAS1-4. Unlike other *TAS* loci which are restricted/ limited to the Brassicaceae family, *TAS3* is widely present in land plants.

RNA silencing phenomena include epigenetic modifications directed by DCL3-produced 24nt siRNAs, also known as heterochromatic-siRNAs (hc-siRNAs). hc-siRNAs are the most abundant class of sRNAs in plant cells. They are mostly produced from loci transcribed by RNA Polymerase IV (PolIV) (Mosher et al. 2008; Matzke and Mosher 2014). RNA PolIV products are converted to dsRNAs by RNA dependent RNA Polymerase 2 (RDR2) and sliced into hc-siRNAs by DCL3 (Daxinger et al. 2009). hc-siRNAs are then bound mainly by AGO4, and less often by AGO6 and AGO9 (Zilberman et al. 2003; Qi et al. 2006; Zheng et al. 2007; Havecker et al. 2010; Olmedo-Monfil et al. 2010). hc-siRNA bound to AGOs target homologous sequences directing methylation in a process requiring RNA polymerase V (PolV) (Wierzbicki et al. 2008; Wierzbicki et al. 2009). This RNA-directed DNA methylation (RdDM) is responsible for the transcriptional gene silencing (TGS) of repetitive DNA sequences and transposable elements (TE) in a process that remains unclear.

In plants and some animals silencing is a cell non-autonomous event (Hunter et al. 2006; Hinas et al. 2012). In these systems, silencing initiated in one cell eventually leads to silencing of homologous sequences in a group of cells or even throughout the whole organism. Although the mechanisms of silencing spread have commonalities between plants and animals, the details of silencing movement seem to be largely divergent (Voinnet 2005; Sarkies and Miska 2014). The present review will focus mainly on silencing spread in plants. A more extensive review of silencing spread can be found in Mermigka et al. (Mermigka et al. 2015). The mobile nature of RNA silencing was first demonstrated by grafting experiments and agroinfiltration assays in transgenic plants (Palauqui et al. 1997; Voinnet and Baulcombe 1997). In both cases the spread of silencing was observed from the lower silenced leaves to the upper non-silenced leaves leading to the suppression of the targeted gene expression. Progression of silencing spread was manifested initially in the vein area of the systemic leaves and gradually spread throughout the leaf area. Based on the sequence specificity of the mobile signal in the recipient cells, the authors suggested that the signal is a nucleic acid. Depending on the pattern of silencing spread, it is distinguished in short range and systemic silencing spread (Figure 1). The characteristics of short range and systemic silencing spread are presented in Table 1.

SHORT RANGE SPREAD OF RNA SILENCING

Short range spread of RNA silencing (SRS) as opposed to systemic spread (SS) (see below) manifests as silencing of a specific gene in a limited and defined area of cells (Figure 1). This area has been estimated to extend approximately 10–15 cells further from the cells where silencing initiation took place (Himber et al. 2003). It has been observed upon induction of silencing of both transgenes and endogenes (Schwach et al. 2005; Dunoyer et al. 2007; Smith et al. 2007), and spontaneously during transgene expression (Kalantidis et al. 2006). Short range silencing of transgenes and endogenes can be induced but only transgenes seem to be spontaneously targeted by this type of silencing.

THE SHORT RANGE SILENCING SIGNAL

Initial work using viral suppressors of silencing suggested that DCL3-produced 24nt siRNAs mediate short range silencing spread (Hamilton et al. 2002). Later work using an *Arabidopsis thaliana dcl4* mutant line, however, clearly demonstrated a primary role for DCL4-produced 21nt siRNA in SRS (Dunoyer et al. 2005). Additionally, it was proposed that the silencing signal moves cell-to-cell as dsRNA (Dunoyer et al. 2010b). The role of 21nt siRNAs as a short range signal is now supported by multiple findings: (i) there is a genetic requirement for DCL4 but not for any of the other DCLs for silencing to spread from companion cells (CC) to adjacent cells (Dunoyer et al. 2007; Smith et al. 2007). Complementation of *dcl4* mutants with DCL4 specifically expressed in CCs was able to re-establish short range signaling indicating that DCL4 was needed to generate siRNAs which served as the short range signal (Dunoyer et al. 2010b), (ii) biolistically delivered fluorescent 21nt and 24nt siRNAs could spread locally to the anticipated range of 10–15 cells from the spot of insertion but only 21nt siRNAs lead to silencing outside the bombarded area (Dunoyer et al. 2010b) and (iii) CC specific expression of silencing suppressor p19 of *Carnation italian ringspot virus* (CIRV) which shows higher affinity for 21nt over 24nt siRNAs prevented SR RNA silencing (Dunoyer et al. 2010b).

Besides 21nt siRNAs, whose role in short range silencing has been to a certain extent characterized, hc-siRNAs, tasiRNAs and miRNAs can also spread cell-to-cell. Hc-siRNAs do not seem to trigger PTGS in the recipient cell but may induce TGS that somehow fails to manifest phenotypically (Brosnan and Voinnet 2011). Recent studies in *Arabidopsis* pollen have led to the hypothesis that heritable epigenetic silencing of transposable elements may require the movement of hc-siRNA from the vegetative nucleus to the sperm cells (Slotkin et al. 2009). Similarly, AGO9-dependant transport of TE-derived 24 nt siRNAs out of the somatic companion cells has been shown to play a crucial role in the specification of gametic cells by repressing TE expression in the female ovule (Olmedo-Monfil et al. 2010). Mobile hc-siRNAs have been also found to participate in the imprinting of paternal or maternal alleles during embryo development (Calarco et al. 2012).

The degree of miRNA mobility and their function as cell non-autonomous regulators of gene expression has been

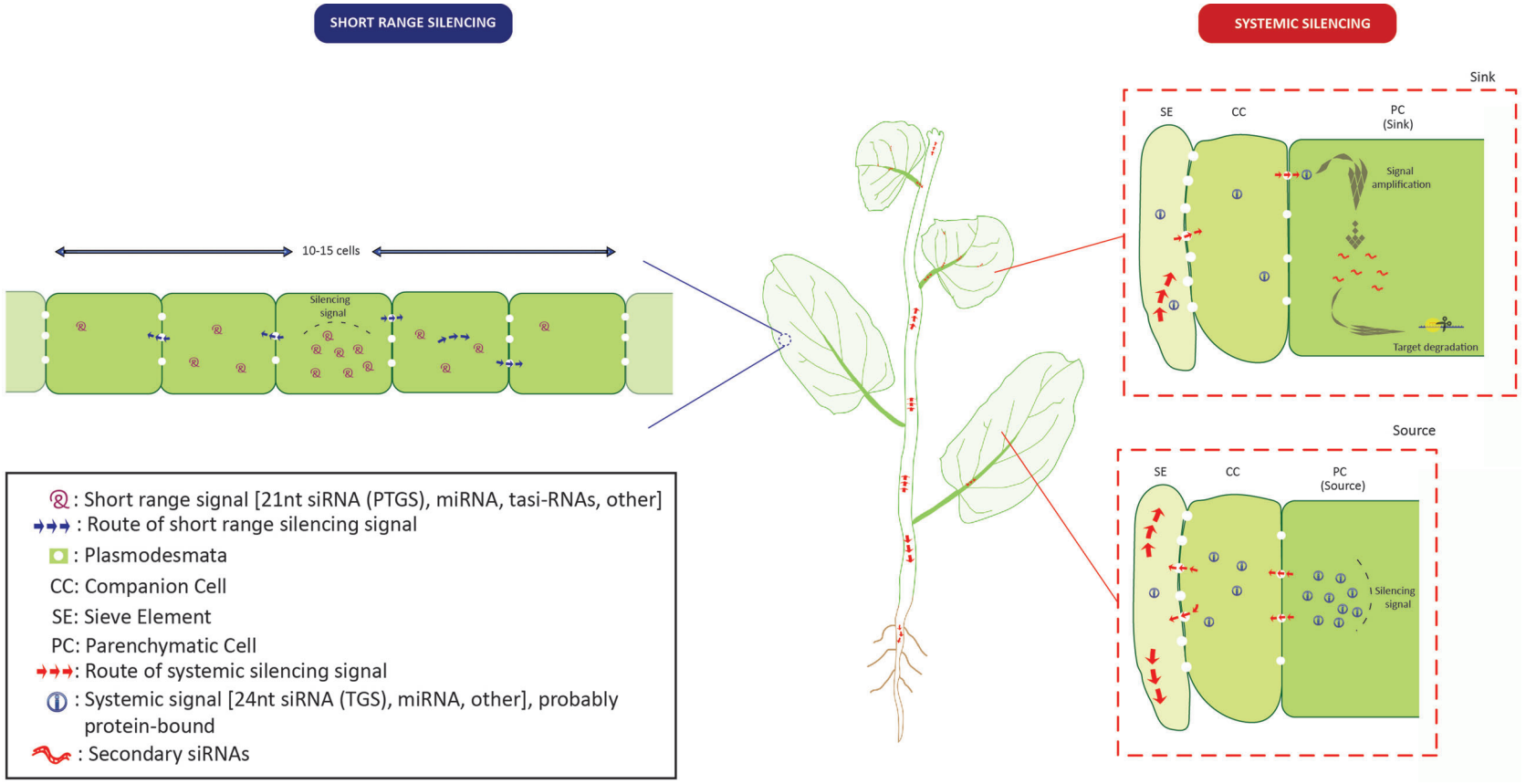

Figure 1. Route of short range and systemic silencing signal

In short range silencing spread the silencing molecules (21nt siRNAs, miRNAs, tasiRNAs or other type) most probably follow the symplastic route. The silencing spread is limited to 10–15 cells beyond the production site and does not require an amplification mechanism. In systemic silencing spread the silencing molecules (24nt siRNA, miRNA or other type) follow to symplastic route until reaching the Sieve elements (SE), which are specialized cells of the phloem. Silencing molecules are then transported with high velocity through the phloem pathway from the source (mature leaf) to the sink tissues (newly emerging leaves, meristem, flowers or root). Once in the sink tissues, the perception of the silencing molecule requires an amplification mechanism.

Table 1. Short range vs long range silencing spread

Features	Short range	Long range
Extent of silencing	10–15 cells (diffusion)	Systemically
Identity of mobile signal	21nt siRNA miRNA tasiRNA other (?)	24nt siRNA miRNA other (?)
Route of mobile signal	Symplastic (plasmodesmata) Apoplastic (?)	Symplastic (plasmodesmata and phloem)
Genes participating	*DCL4* *AGO1* *NRPD1a* *CLSY1* *RDR2* *HEN1* *DCL1* *JMJ14*	*DCL3* *RDR6* *NRPD1a* *PSRP1* *DCL1* (?) *CLSY1* (?) *AGO4* (?) *RDR2* (?)
Requirement of amplification process in recipient cells/ tissues	NO	YES
Requirement of amplification process throughout the translocation stream	No data	NO

controversial. Evidence for and against a role for miRNA as mobile regulatory molecules are summarized in Brosnan and Voinnet (Brosnan and Voinnet 2011). In *A. thaliana* roots miR166 has been shown to move at least one cell layer from the endodermis to the central vascular cylinder. There, it triggers the degradation of the HD-ZIPIII transcription factor PHABULOSA, to prevent the differentiation of protoxylem to xylem cells and ensure the correct development of the root cylinder (Carlsbecker et al. 2010). Similarly, there is evidence for a cell non-autonomous function of miR394. This miRNA is expressed in the epidermal cell layer of the shoot apical meristem but moves to the internal meristem cell layers to downregulate the expression of the F-Box coding gene *LEAF CURLING RESPONSIVENESS*. This downregulation is essential to ensure the responsiveness of these cell layers to the homeodomain transcription factor *WUSCHEL*, a stem cell promoting factor (Knauer et al. 2013) (reviewed by Benkovics and Timmermans 2014).

A role for mobile regulatory sRNAs has also been attributed to tasiRNAs produced by the plant conserved *TAS3* locus. *TAS3* produced tasiRNAs are important for the regulation of the auxin response factors ARF2, ARF3 and ARF4. It has been shown that these tasiRNAs are mobile over short distances playing an important role in the development of leaf polarity and lateral root growth (Chitwood et al. 2009; Schwab et al. 2009; Marin et al. 2010; de Felippes et al. 2011; Benkovics and Timmermans 2014). It has been suggested that tasiRNAs may be the cell non-autonomous equivalent to miRNAs (Chen 2009; Dunoyer and Voinnet 2009). Recent findings, regarding the cell non-autonomous function of miRNAs, however challenge this hypothesis (see section below). In any case the genetic requirements for the short-range movement of endogenous sRNAs, such as miRNAs and tasiRNAs differ substantially from those of siRNAs of exogenous origin (reviewed by Brosnan and Voinnet 2011).

SHORT-RANGE SILENCING CAN SPREAD THROUGH PLASMODESMATA

Short range spread of the silencing signal involves cell-to-cell movement of the signal through the symplast or/and the apoplast. Symplastic movement involves trafficking of the silencing signal through plasmodesmata (PD) (Figure 1) which are plasma membrane-lined channels observed only in higher plant cells (reviewed in Lucas et al. 2009; Maule et al. 2011). Evidence supporting the movement of silencing signals through PD has accumulated over the last years. The first evidence came through the observation that the guard cells of stomata which, at that stage of development are symplastically isolated due to blockage of PD, are the only cell type escaping short-range spread (Voinnet et al. 1998; Himber et al. 2003; Kalantidis et al. 2006). The simplest explanation for this observation is that the silencing signal cannot spread to these cells due to the lack of plasmodesmatal connections, which would otherwise allow the symplastic transport of the silencing signal. Apoplastic movement on the other hand involves export from the cell, movement through cell walls and intracellular space, and then import into the cell. Although it cannot be excluded, direct evidence for an apoplastic movement of the silencing signal is still lacking.

Analyses of RNA silencing spread in *Arabidopsis* embryos revealed that the ability of the short-range signal to spread is affected by PD aperture. Using size-specific tracers, it was shown that the short-range signal moves to a similar extent as soluble proteins with a size range between 27–54 kDa (Kobayashi and Zambryski 2007). In the same work, transgene-triggered PTGS movement correlated positively with PD aperture and was comparable to that of soluble protein tracers moving by diffusion rather than active gating. Interestingly, the authors also showed that in embryonic tissues with larger PD aperture, SRS could spread through a 35-cell distance (Kobayashi and Zambryski 2007). These results are in agreement with the general assumption that the extent of silencing signals in SRS is governed by diffusion and regulated by PD aperture.

GENES INVOLVED IN SHORT RANGE SPREAD

Most of the information available on the genes required for short range spread comes from genetic screens. Two similar,

but independent experimental settings in *Arabidopsis* have shed light on SRS. In both settings the CC specific promoter SUC2 was used to differentiate the cell(s) producing from the one receiving the signal. In both cases the targeted endogenous RNAs, *i.e., PHYTOENE DESATURASE* (*PDS*) and *SULFUR* (*SUL*) caused a visible chlorotic phenotype (Dunoyer et al. 2007; Smith et al. 2007). The inducer was a SUC2 driven (and therefore CC specific) fragment of the target RNA expressed as inverted repeat. These screens identified a number of factors necessary for short range spread.

Somewhat surprisingly, genes not related to the 21nt siRNA pathways were also identified. In addition to DCL4 and AGO1, which are directly related to the 21nt siRNA pathway, NRPD1a and RDR2 known to be components of the hc-siRNA pathway were also found to affect short range silencing. RDR2 and NRPD1a encode a nuclear RDR and the largest subunit of the plant-specific heterochromatic RNA polymerase-IV, respectively (Dunoyer et al. 2007; Smith et al. 2007). One of the screens identified a SNF2-domain-containing protein, named CLASSY1 (CLSY1), which, based on subcellular localization studies, was proposed to have a role at a step between NRPD1a and RDR2. CLSY1 contains a DNA-binding region and mutations identified to affect silencing spread are likely to affect its nucleic-acid-binding abilities (Smith et al. 2007).

The nuclear dsRNA binding protein HUA ENHANCER 1 (HEN1) is responsible for methylation of siRNAs and miRNAs, and *Arabidopsis hen1* mutants are defective in SRS, presumably resulting from the absence of 21nt siRNAs stabilization (Dunoyer et al. 2007). Involvement of DCL1 in short range silencing was reported although the specificities of this involvement are still not clear. Finally, JMJ14, a histone-H3K4 demethylase promoting non-CG methylation, was identified in a genetic screen to participate in SRS of RNA silencing. The authors propose that JMJ14 acts downstream of the Argonaute effector complex to demethylate histone H3K4 at the targeted DNA thus, presumably, affecting SRS (Searle et al. 2010). In a more recent paper Le Masson and coworkers elaborated on the role of JMJ14 in S-PTGS and suggested that this protein likely acts on transgenes by reducing transcription levels thus preventing S-PTGS triggering (Le Masson et al. 2012). Nevertheless, there are still important open questions regarding the function of genes involved in hc-siRNA pathway in silencing spread (see last section).

SHORT RANGE SILENCING DOES NOT REQUIRE SIGNAL AMPLIFICATION

SRS does not require amplification of the silencing signal. As stated above, in most tissues SRS expands to up to 15 cells away from the cell(s) in which it initiated (Himber et al. 2003; Kalantidis et al. 2006; Dunoyer et al. 2010b). It appears as if the silencing signal produced in the initial cell(s), diffuses to the adjacent cells through PD and fades out after 15 cells. Why silencing is not amplified in the adjacent cells in these cases remains unclear. It has been suggested that the lack of amplification may be related to the low amount of initial signal produced. This suggestion, however, is unlikely since spreading of transgene silencing induced by the strong expression of a homologous dsRNA via agroinfiltration is restricted to a narrow layer of cells adjacent to the agroinfiltrated area (Himber et al. 2003; Kalantidis et al. 2006). It should be noted, however, that apart from SRS there is also extensive local spread (see also (Kalantidis et al. 2008), where cell-to-cell spread further than the 15 cell limit is observed. Such spreading is, for example, seen at the receiving tissues of systemically silenced leaves and in tasiRNA spread (Himber et al. 2003; Schwach et al. 2005; de Felippes et al. 2011). It likely requires a relay mechanism of secondary siRNA generation whose prerequisites have not been fully understood.

SYSTEMIC SILENCING

While in SRS the mobile signal is limited to a group of cells surrounding the site of its production, in systemic silencing the signal is transmitted to distant organs adopting a phloem based movement. The systemic nature of this mechanism was demonstrated in grafting experiments and agroinfiltration assays in transgenic plants (Palauqui et al. 1997; Voinnet and Baulcombe 1997). In both cases the spread of silencing was observed from the lower silenced leaves to the upper non-silenced leaves leading to the suppression of the targeted gene. Progression of silencing spread was manifested initially in the vein area of the systemic leaves and gradually spread throughout the leaf area. Based on the sequence specificity of the mobile signal in the recipient cells, the authors suggested that the signal is a nucleic acid.

IDENTITY AND MOVEMENT OF THE MOBILE SIGNAL

Analysis of phloem sap content (Yoo et al. 2004; Buhtz et al. 2008; Buhtz et al. 2010; Varkonyi-Gasic et al. 2010; Rodriguez-Medina et al. 2011) and grafting experiments involving mutant lines for components of RNA silencing (Himber et al. 2003; Brosnan et al. 2007; Dunoyer et al. 2010a; Molnar et al. 2010; Melnyk et al. 2011a) have provided pivotal information on the nature of the systemic silencing signal. From the above studies it has become clear that siRNAs and some miRNAs move systemically.

Although the seminal identification of small RNAs in the phloem sap of cucurbits by W. Lucas' group (Yoo et al. 2004) suggested their mobility, it was D. Baulcombe's (Molnar et al. 2010; Melnyk et al. 2011a) and O. Voinnet's groups (Himber et al. 2003; Dunoyer et al. 2010a) that proved that all classes of siRNAs (21–24nt) move systemically and that 24nt siRNAs are most probably the systemic signal. Indeed, grafting experiment revealed that source tissues deficient in the biosynthetic pathway of 24nt siRNAs could not transmit anymore the silencing state to sink tissues. Additionally, it was shown that the 24nt siRNAs was the most abundant class of siRNAs in sink tissues. Whether 24nt siRNAs are the only mobile signal or if other types of RNAs are involved in SS such as aberrant RNAs awaits verification.

Systemic movement of miRNAs and their activity in sink tissues are supported by several studies (reviewed by Marin-Gonzalez and Suarez-Lopez 2012). Phloem sap analysis conducted initially in cucurbits (Yoo et al. 2004) and later in the phloem sap of other plant species (Buhtz et al. 2008; Buhtz et al. 2010; Varkonyi-Gasic et al. 2010; Rodriguez-Medina

et al. 2011) detected miRNAs. Since phloem sap sampling techniques are prone to contamination during the wounding process, a more robust demonstration of miRNA mobility was obtained from grafting experiments. By this approach miR399, miR395, miR172 and miR156 were shown to move long distance through the graft union (Lin et al. 2008; Pant et al. 2008; Buhtz et al. 2010; Kasai et al. 2010; Bhogale et al. 2014). The systemic mobility of other miRNAs (e.g. miR168, miR169 and others) has not yet been confirmed.

Little is known concerning the form by which siRNAs and miRNAs move systemically (double or single stranded, naked or in complex with proteins). Experiments conducted by Lucas' group in cucurbits have identified a phloem protein, PHLOEM SMALL RNA BINDING PROTEIN 1 (*Cm*PSRP1), presenting a high affinity to sRNAs and a preference for ssRNAs (Yoo et al. 2004). The same group and others also found a bias towards one miRNAs strand in the phloem sap (Yoo et al. 2004; Buhtz et al. 2008). These observations imply that mobile systemic sRNAs are single stranded and possibly in complex with phloem protein(s) as opposed to the proposed double stranded form by which sRNAs move in cell-to-cell silencing (Dunoyer et al. 2010b). Genetic evidence supporting a role for *Cm*PSRP1 in the transport of mobile systemic sRNAs in the phloem flow, however, is still lacking.

Irrespective of the exact nature of the silencing signal, it must move from the production site to the receptive tissue where it will exert its action. Multiple lines of evidence support a symplastic movement of the signal following the photoassimilate translocation route from source to sink tissues (Figure 1). This involves cell-to-cell movement though PD until reaching specialized cells of the phloem tissue, the companion cells (CC). From there signal molecules are uploaded to the other phloem-specific types of cells, called sieve elements (SE), which are elongated, end-to-end connected cells, creating a conduit through which fast communication of distant organs is achieved. Analyses of exudates from xylem (i.e. the other tissue of the vascular system) identified only minerals and macromolecules such as peptides and proteins (reviewed by Turnbull and Lopez-Cobollo 2013) but not sRNAs (Buhtz et al. 2008) while the only ribonucleic acids detected are viral RNAs (Opalka et al. 1998; Verchot et al. 2001) in the form of ribonucleic-protein complexes but not as naked RNAs. In the phloem sap, on the other hand, different types of RNAs such as sRNAs, endogenous and viral RNAs have been detected (reviewed by Kehr and Buhtz 2008) favoring the theory of a phloem based movement.

A phloem based source to sink movement of the systemic signal is also supported in agroinfiltration experiments in which a silencing inducer expressed in mature leaves (source tissues) resulted in silencing of the targeted gene in young emerging leaves (sink tissues) but not in already fully expanded leaves (source tissues) (Voinnet et al. 1998). This progression of silencing was paralleled by the authors to the route observed by a previous study (Roberts et al. 1997) for a phloem-transported dye in *N. benthamiana* plants. Similar results were obtained in grafting experiments using leaf removal/shading approach and phloem tracker (Tournier et al. 2006). In each case, the direction of silencing movement followed the source to sink direction and correlated with the phloem tracker route. At this point it should be clarified that as long as source to sink relations exist systemic silencing moves either from the upper to the lower part of the plant or *vice versa*. (Voinnet et al. 1998; Tournier et al. 2006; Molnar et al. 2010; Bai et al. 2011). While unidirectionality (Palauqui et al. 1997; Sohn et al. 2014) or directionality-depended efficiency of silencing spread (Molnar et al. 2010) have been reported, other factors might have an impact on the silencing spread efficiency, such as the age or the developmental stage of the plants (Liang et al. 2012), or the grafting method (Crete et al. 2001).

A recent study conducted in *Arabidopsis* has suggested that signal movement from roots to shoots might occur through PD and not through the phloem (Liang et al. 2012), in a manner analogous to short range silencing spread. This result might explain both the lower efficiencies for root to shoot silencing spread commonly observed in *Arabidopsis* (Molnar et al. 2010) and its distinctive spreading pattern. Indeed, root to shoot silencing spread expanded from the base to the tip of the leaf and not through the veins (Brosnan et al. 2007; Liang et al. 2012).

GENES INVOLVED IN SYSTEMIC RNA SILENCING

DCL proteins

Although all classes of siRNAs move systemically DCL3 seems to be the only DCL protein essential for systemic silencing spread. Micro grafting experiments in *Arabidopsis* have shown that silencing-inducing scions (source) with a *dcl3* mutant background were unable to induce transgene silencing in the roots (sink) (Melnyk et al. 2011a). Conversely, WT silencing-inducing scions can efficiently silence rootstocks with a *dcl2*, *dcl3*, *dcl4* background (Molnar et al. 2010; Melnyk et al. 2011a). Taken together, this suggests that DCL3 is necessary for the production but not the perception of the silencing signal. This finding, however, is in disagreement with a previous study showing that neither DCL3 nor any other DCL are exclusively responsible for root to shoot systemic silencing (Brosnan et al. 2007). This apparent discrepancy likely results from the opposite directions of the systemic silencing analyzed in each study (discussed in detail below).

RDR6

Initial experiments using *rdr6* mutants suggested a role for RNA Dependent RNA polymerase 6 in the spreading and maintenance of the silencing state (Vaistij et al. 2002) (Himber et al. 2003). Grafting experiments using transgenic plants knocked down for RDR6 RNAi (RDR6i plants) demonstrated that RDR6 is mainly involved in the perception of the silencing signal (Schwach et al. 2005) (further discussed in the next section).

Genes of the heterochromatic silencing pathway

nrpd1a mutants used as source tissues in grafting experiments lead to reduced amounts of 24nt siRNAs and DNA methylation of endogenous loci in recipient roots, supporting its role in the production of the mobile silencing production (Molnar et al. 2010). In another report, *CLSY1*, *AGO4*, *RDR2*, and *NRPD1a* have been identified as genes involved in silencing spread (Smith et al. 2007) but due to the nature of the experiments it cannot

be concluded whether they are indeed involved in systemic spread and if so in which exact step they participate. Grafting experiments using different mutants will again clarify these aspects.

PSRP1

As discussed above PSRP1, is a phloem specific protein present in cucurbits, exhibiting high affinity for sRNAs and facilitating their movement through PD (Yoo et al. 2004). PSRP1 orthologs are absent in tobacco and *Arabidopsis*.

OTHER ASPECTS OF SYSTEMIC RNA SILENCING

Signal amplification through the translocation stream is not mandatory for the long distance movement of the systemic silencing signal

As shown in grafting and agroinfiltration experiments, the produced silencing signal can move systemically even in the absence of homologous sequences in the recipient tissues. The first evidence came from a triple grafting approach in which a WT stem separated two transgenic lines, one serving as the inducer and the other as the recipient of the silencing signal (Palauqui et al. 1997). Suppression of the transgene in the recipient tissue could be observed even when the WT stem spacer was up to 30 cm long. However, the transgenic lines used in this study, overexpressed an endogene that was also expressed in the intermediate WT segment of the graft. Thus, in this case, a definitive physical distance between sites acting as inducers and recipients might be questioned. To clarify this, triple graft experiments in which silencing was directed towards a transgene were implemented by Voinnet et al. (Voinnet et al. 1998). Long distance silencing through the WT segment was detected, confirming the results of Palauqui et al. (Palauqui et al. 1997).

Requirement of an amplification process in the recipient tissues

Requirement of an amplification process in the recipient tissues was initially suggested by studies using *Arabidopsis* mutants deficient in the production of secondary siRNAs (Vaistij et al. 2002; Himber et al. 2003). Vaistij et al. (Vaistij et al. 2002) showed in *rdr6 Arabidopsis* mutant, which present primary but not secondary siRNAs, that silencing could be initiated but not maintained. Involvement of RDR6 in the systemic silencing was also demonstrated using the phloem specific SUC2 promoter expressing part of the GFP gene as an inverted repeat (SUC2: GF-FG) (Himber et al. 2003). When Arabidopsis plants stably expressing GFP were supertransformed with the SUC2: GF-FG construct the GFP transgene was silenced throughout the entire plants. In a *rdr6* mutant, however, silencing of the GFP transgene triggered by the SUC2:GF FP construct was restricted near the vein area in a pattern reminiscent of short-range silencing spread. Using a similar approach, the same group identified the putative RNA helicase SDE3 as another protein involved in the systemic spread, albeit with a lower importance than RDR6.

Although the above experiments pointed to a role of RDR6 in systemic silencing spread, the exact step in which RDR6 participated remained unclear. To disentangle whether RDR6 is involved in the production or perception of the signal, grafting experiments were conducted in *N. benthamiana RDR6i* mutants stably expressing GFP (GFP/RDR6i line) (Schwach et al. 2005). When these plants were used as scions on a GFP silencing-inducing rootstock silencing spread was either completely abolished or in a few cases vein restricted indicating that RDR6 is necessary for the perception of the signal. In the same study an effect on the production/transmission of the systemic signal due to the lack of RDR6 was not observed since rootstocks of GFP/RDR6i plants in which silencing of the transgene was induced could transmit the systemic signal to the stably expressing GFP scions.

Additional components participating in the reception of the signal in the root to shoot silencing spread were later documented by Brosnan *et al*. (Brosnan et al. 2007). In their experiments, mutants in the heterochromatic siRNA pathway (*nrpd1*, *dcl3*, *ago4* and *rdr2*) were used in grafting experiments either as inducers or receivers of the silencing signal. Since silencing spread was compromised only when *nrpd1a*, *dcl3*, *ago4* or *rdr2* single mutants were used as scions the authors concluded that these proteins were required in the perception but not the production of the signal. It should be noted that subsequent grafting experiments from another group using the *dcl3* mutant background as inducers of the signal, reported its requirement for the production of the signal (Melnyk et al. 2011a). These contradictory results regarding the role of DCL3 in both production and reception of the mobile signal could be explained by the difference in the silencing spread direction used by the two groups. Brosnan *et al*. (Brosnan et al. 2007) studied root to shoot silencing spread while Melnyk et al. (Melnyk et al. 2011a) studied silencing spread to the opposite direction. Since Liang et al. (Liang et al. 2012) showed that root to shoot silencing spread in *Arabidopsis* adopts a cell-to-cell movement rather than a phloem based pathway, it seems reasonable to speculate that DCL3 is indeed involved in the production of the systemic signal.

Systemic silencing is mediated by both PTGS and TGS

Another aspect of systemic spread refers to the involvement of post-transcriptional and transcriptional gene silencing as pathways for mediating systemic spread. At least two studies (Brosnan et al. 2007; Liang et al. 2012) favor a PTGS-mediated establishment of silencing in the recipient tissues, since they could detect background transcript levels of the silenced targets but no epigenetic modifications in the coding region. Other studies (Molnar et al. 2010; Melnyk et al. 2011b), on the other hand, detected changes in the methylation status of promoter or downstream regions in the recipient tissues. These changes were observed in endogenous and exogenous sequences. Similarly, Bai et al. (Bai et al. 2011) and Dunoyer et al. (Dunoyer et al. 2010a) observed increase in the methylation of the target region.

Evidence supporting the involvement of both pathways in the establishment of systemic silencing in recipient tissues comes also from studies using virus induced gene silencing (VIGS) (Baulcombe 1999). VIGS shares many similarities with systemic silencing spread and acts at both posttranscriptional and transcriptional level (Jones et al. 1998; Kanazawa et al. 2011; Bond and Baulcombe 2015).

Silencing spread efficiency of endogenous and exogenous sequences

For the study of cell non-autonomous silencing a wide variety of sequences have been used as targets of silencing including exogenous genes like *GFP* or *GUS* but also endogenes like *NITRATE REDUCTASE* (*NR*), *PDS* or *SUL*. It appears that the efficiency of silencing spread is higher for exogenous than for endogenous genes (Palauqui et al. 1997; Palauqui and Balzergue 1999; Dunoyer et al. 2007; Smith et al. 2007). To our knowledge, the reasons for this apparent difference remain poorly understood.

One model for explaining this phenomenon involves the so called "threshold" model, which refers to the levels of aberrant transcripts below which silencing signal amplification does not take place (Voinnet 2005). Within this model, endogenes would be protected from systemic silencing spread (Kalantidis et al. 2008). Accordingly, localized events triggering silencing of an endogene will not lead to its systemic silencing, protecting the plant from an over-reaction to a minor threat. Stochastic local silencing of a transgene not followed by systemic spread has been reported (Kalantidis et al. 2006) and might reflect this "minor threat" response. Since exogenous genes presenting comparable expression level than endogenes can be still systemically silenced (Himber et al. 2003) the level of target transcripts might not be the main determinant.

In addition to the transcript level, internal attribute(s) of endogenes (e.g., introns, UTRs, etc.) might prevent the initiation of the amplification mechanism. Silencing of Rubisco and PDS has been shown to involve primary but not secondary siRNA pointing out the non-transitive nature of the silencing mechanism (Vaistij et al. 2002). The notion that endogene features might impinge on the silencing spread efficiency was recently shown by (Dadami et al. 2013). The authors hypothesized that if endogenous features such as promoter, intron and UTR protect endogenes from silencing amplification then a transgene harboring the same features might be more resilient to silencing. Indeed, the addition of endogenous features onto a GFP transgene delayed the onset and spread of systemic silencing in agroinfiltration assay. It should be noted that the above result does not preclude that specific features of exogenous sequences may be related to the generation of aberrant RNAs. A better understanding on the effect of endogenous features on silencing efficiency will be of great value. In addition to the features of the targeted gene environmental conditions (i.e., temperature and light intensity) seem to influence the silencing spread efficiency (Szittya et al. 2003; Kotakis et al. 2010; Kotakis et al. 2011; Patil and Fauquet 2015).

ROLES OF MOBILE SRNAS IN PLANTS

Studies have suggested a role for cell non-autonomous RNA silencing during plant development and viral defense. As stated above, miR165/166, miR390 and its cleavage product tasiARF are involved in root and leaf development. In addition TE-derived long siRNAs produced in the vegetative nucleus during gametogenesis seem to participate in suppressing transcription of TEs in sperm cells (Schwab et al. 2009; Slotkin et al. 2009; Carlsbecker et al. 2010; Marin et al. 2010).

As stated above a systemic movement of some miRNAs has been documented (Lin et al. 2008; Pant et al. 2008; Buhtz et al. 2010; Kasai et al. 2010; Bhogale et al. 2014). Shoot to root translocation of miR395 in WT/hen1 grated plants has been shown to downregulate one of its targets, ATP sulfurylase 4 (ASP4) (Buhtz et al. 2010). In grafted plants using a miR399 overexpressing line as a scion and WT line as a stock, shoot to root translocation of miR399 has been shown to downregulate *PHO2*, a critical component for Pi homeostasis, suggesting a role for miRNA399 in the long distance regulation of phosphate homeostasis (Lin et al. 2008; Pant et al. 2008). Similarly, stocks of potato plants grafted on transgenic plants overexpressing miR156 showed morphological alterations similar to those observed in miR156 overexpressing plants together with increased levels of mature miR156 implying a role of miR156 as a systemic signal (Bhogale et al. 2014). Despite the above and other studies (Martin et al. 2009), a *bona fide* role of miRNAs as a systemic cue can only be attributed to miR395 since it is the only miRNA in which WT plants were used as a source of this type of sRNA.

Systemic movement of miRNAs may also play a role during the plant responses to biotic stresses. Phloem sap collected from *Brasicca napus* and/or *Cucurbita maxima* plants deprived of inorganic phosphate (Pi) had increased amounts of miR399 (Pant et al. 2008) and miR2111 (Pant et al. 2009) compared to plants grown under normal conditions. Similarly, increases in miR395, miR398 and miR399 in both non-vascular tissues and the phloem sap have been measured during sulfate, copper and Pi starvation, respectively (Buhtz et al. 2008; Buhtz et al. 2010). Further experiments are needed to assess if the increase in miRNA level in the phloem sap could regulate plant development.

Mobile sRNAs also seem to be implicated in transferring epigenetic changes in recipient tissues. To investigate this aspect Molnar et al. (2010) have conducted grafting experiments between *Arabidopsis* ecotypes Columbia and C24, which were shown in the same study to exhibit different methylation patterns at certain genomic loci (hypermethylation at some loci were observed in C24 ecotype compared to Columbia ecotype). They observed that hypermethylation at some loci in roots of C24/Col-dcl2,3,4 (deficient in the production of 24nt siRNA) root compared to Col-dcl2,3,4/Col-dcl2,3,4 coincided with the shoot to root translocation of 24nt siRNAs. When *sde4* mutants (impaired in the heterochromatic pathway) were used as scions the authors observed a decrease in the production of 24nt siRNAs and no methylation of the specific loci in the rootstocks. A functional role of mobile siRNAs was also suggested by Zhang et al. (Zhang et al. 2014). With the use of heterografts the authors observed that translocation of a mobile signal from transgenic *Nicotiana tabacum* plants, expressing an inverted repeat of DISRUPTED MEIOTIC *cDNA 1* (*DMC1*), a meiosis-specific cell-cycle factor, to WT scions caused suppression of *DCM1* in the anthers of the first flowers of the scions. The authors suggested that siRNAs derived from the inverted repeat could be the mobile signal since they were detected in the flowers of the WT scions (Zhang et al. 2014).

The role of silencing spread in plant antiviral response is now well supported, and may explain the mechanism of "cross protection" against viruses first reported almost a century ago (Wingard 1928). It has been shown that modified

viruses unable to move systemically and containing part of a host gene can induce silencing in systemic leaves (Voinnet et al. 2000) or to neighboring cells (Himber et al. 2003) suggesting that a mobile signal produced during viral infection moves systemically or to short distances and activate the antiviral mechanism in the recipient cells. Additionally, Schwach et al. (Schwach et al. 2005) suggested a requirement for RDR6 in the resistance against Potato virus X (PVX) and the perception of the mobile signaling in systemic leaves.

RNA SILENCING SPREAD IN ANIMALS

Here we summarize important findings of RNA silencing spread in animal models, mainly *Caenorhabditis elegans*, insects and mammals. This short section is indented to provide readers with the basis to elaborate comparison between silencing spread in plants and animals. For further reading in the field of RNA silencing in animals, readers are invited to recent reviews covering this topics in more details (Turchinovitch 2013; Feng and Guang 2013; Zhuang and Hunter 2011; Grishok 2013.

While silencing of endogenes and transgenes is pervasive in animals, systemic spread of silencing has been found in few animal species. To date, the nematode *C. elegans* is the animal model in which spread of RNA silencing has been best studied. Cell structures and genes involved in RNA silencing spread in animals are different to those characterized in plants. Indeed, membrane channels, gap junction and intercellular bridges replace plasmodesmata in controlling intercellular siRNA transport while a RDR-based signal amplification relay is apparently absent in animals.

RNA SILENCING SPREAD IN *C. elegans*

Components involved in intercellular movement of RNA silencing in *C. elegans*

Injection, endogenous expression and ingestion of dsRNA trigger systemic RNA silencing in *C. elegans* (for review see Jose and Hunter 2011). Forward genetic screens for mutants defective in systemic RNA silencing have identified five genes (*sid1-5*). SID1-3 and SID-5 have been relatively well characterized while the molecular identity and physiological role of SID-4 still remain elusive. SID-1 is a transmembrane specific dsRNA-gating channel involved in the cellular import but not export of the RNA silencing signal (Jose et al. 2009). SID-2 is localized at the apical membrane of the cells facing the intestinal lumen (Hinas et al. 2012). Contrarily to SID-1, SID-2 activity is ATP dependent favoring substrates of up to 500 bp long (McEwan et al. 2012). SID-3 is a widely expressed Cdc-42-associated tyrosine kinase (Ack) required for the cellular import of dsRNAs (Jose et al. 2012). SID-5 is widely expressed and colocalizes with endosomal markers, suggesting that vesicle-mediated transport of RNA may play a role in systemic RNA silencing (Hinas et al. 2012).

The current model for the route of systemic RNA silencing in *C. elegans* indicates that dsRNAs located in the intestinal lumen are bound by SID-2, enter the intestinal cells by endocytosis, and are then unloaded into the cytosol through the SID-1 channel (Winston et al. 2007; McEwan et al. 2012). SID-1, however, is dispensable for the transport of the silencing signal across the intestine barrier (Jose et al. 2009). In this alternative pathway, dsRNA-SID-2 complexes are transported between cells via SID-5 dependent endocytic vesicles without being unloaded into the cytosol of the intestinal cells.

The initial long dsRNAs, which triggers RNA silencing and the primary but not the secondary siRNA are conveying the silencing signal (Tabara et al. 1999; Parrish and Fire 2001; Jose et al. 2011; Jose et al. 2012). Once in the recipient cell, primary siRNAs are bound by the Argonaute RDE-1 and recruit RNA-dependent RNA polymerases (RDR) to the targeted gene engaging *de novo* synthesis of secondary siRNAs (Sijen et al. 2001; Pak and Fire 2007; Sijen et al. 2007; Gu et al. 2012). Secondary siRNAs bound by secondary Argonautes (SAGO) are responsible for the bulk of silencing activity, which can take place in both the cytosol and the nucleus (Yigit et al. 2006; Guang et al. 2008; Guang et al. 2010; Vasale et al. 2010; Buckley et al. 2012). Systemic RNA silencing can reach the germline and establish a silencing state lasting for at least five generations (Alcazar et al. 2008; Ashe et al. 2012; Buckley et al. 2012). This inherited silencing is thought to be established and maintained through the action of small RNA-directed epigenetic changes and chromatin modifications.

RNA SILENCING SPREAD IN INSECTS

dsRNA uptake from the culture media into cultured *Drosophila melanogaster* S2 cells involves genes related to endocytosis, scavenger receptors and lipid-modifying enzymes (Saleh et al. 2006; Ulvila et al. 2006). Their direct involvement in the transport of RNA silencing across the plasma membrane, however, remains to be established. At the organism level, *D. melanogaster* exhibits short range spread but not systemic RNA silencing or transitivity (Roignant et al. 2003). Short range spread of RNA silencing can be triggered by intra-abdominal injection of dsRNAs but not by transgene expression (Dzitoyeva et al. 2001; Roignant et al. 2003). The lack of SID-1 and RDRs in *D. melanogaster* may explain the apparent absence of systemic silencing in this organism (Roignant et al. 2003).

In the beetle *Tribolium castaneum*, injection of dsRNAs into the body cavity of larvae has been shown to trigger a robust systemic RNA silencing response lasting into the adult stage (Tomoyasu and Denell 2004). Although *T. castaneum* and *D. melanogaster* genomes present both a similar number of *Dicer* and *Argonaute* encoded genes and lack RDRs, *T. castaneum* genome includes three *sid-1*-like genes (*SilA-C*). *T. castaneum SilA-C* genes, however, exhibit some homology with another *C. elegans* gene, *Tag130*, which does not seem to be involved in systemic RNA silencing.

RNA SILENCING SPREAD IN MAMMALS

Introduction of long dsRNAs (above 30 bp in length) in mammalian cells generally does not trigger RNA silencing but instead induces a non-sequence-specific immune response known as interferon response which is related to virus infection (Samuel 2001). Mammals lack RDR but *sid-1*

homologs are present in human and mice. Overexpression of the human homolog SID-1 in cultured cell lines increases both siRNA uptake and RNA silencing efficiency (Duxbury et al. 2005; Overhoff and Sczakiel 2005). Furthermore, SID-1 overexpression has been recently shown to promote contact-dependent bidirectional transfer of both siRNAs and functional miRNAs between human cells in culture (Elhassan et al. 2012).

Documentation of small RNA mobility in mammals mostly concerns miRNAs (reviewed in Chen et al. 2012; Turchinovich and Burwinkel 2012; Turchinovich et al. 2013). Short distance transfer of intracellular miRNAs via gap junctions and intercellular bridges has been reported in various cell types *in vitro* (reviewed in Mittelbrunn and Sanchez-Madrid 2012). Microvesicle encapsulated and AGO2 bound circulating miRNAs protected from nuclease attack have been found in various body fluids including urine, serum, plasma and milk (Arroyo et al. 2011; Turchinovich et al. 2011; Turchinovich and Burwinkel 2012).

BIOLOGICAL ROLES OF RNA SILENCING SPREAD IN ANIMALS

The biological roles of RNA silencing spread and intercellular transfer of small RNAs in animals remain elusive. In *C. elegans*, RNA silencing spread from endogenous loci has not yet been reported and *sid* mutants exhibit standard growth and development. A possible role for RNA silencing spread in conveying parental experience into the progeny, providing a rapid adaptive response, is tempting but awaits demonstration. Although it has been shown that RNA silencing plays a role in antiviral immunity in *C. elegans*, the direct involvement of RNA silencing spread *per se* in limiting virus infection has not yet been proven (Lu et al. 2005; Schott et al. 2005; Wilkins et al. 2005; Felix et al. 2011; Ashe et al. 2013).

In mammals, studies on the intercellular transfer of small RNAs have been mostly conducted in cell lines. miRNA-based intercellular communication has been shown to play a role during cancer progression, viral infection, immune response and neurological processes *in vitro* (Skog et al. 2008; Ohshima et al. 2010; Pegtel et al. 2010; Mittelbrunn et al. 2011; Xin et al. 2012). *In vivo*, intercellular transfer of miRNA may support paracrine and autocrine but less likely endocrine signaling because the concentration of miRNAs measured in blood is too low to transmit biological activity into recipient cells. Surprisingly, recent studies have shown that miRNAs can also function in intercellular signaling via their binding onto plasma membrane Toll-like receptors of recipient cells promoting the progression of central nervous system damage (Fabbri et al. 2012; Lehmann et al. 2012).

SUMMARY AND OUTSTANDING QUESTIONS

It is now established that RNA silencing in plants can spread short range and systemically with the two processes exhibiting similarities but also important differences. Short range silencing spreads from cell-to-cell through the symplastic movement of 21nt siRNAs (PTGS). Besides siRNA, tasi- and miRNAs have also been reported to move over short distances. Systemic silencing on the other hand follows a phloem-based translocation pathway from (metabolic) source to sink tissues. Systemic spread has been documented for both TGS and PTGS phenomena involving the movement of 24nt hc-siRNA and 21nt siRNA, respectively. Some of the genes involved in short range and systemic silencing have been identified; surprisingly genes of the TGS pathway also participate in short range silencing spread. Systemic silencing requires a distinct set of genes in the tissues producing and tissues receiving the signal.

In the last few years we have witnessed significant progress in deciphering the mechanisms of RNA silencing spread. Nevertheless, important questions still remain unanswered including: (i) in which form the silencing signal molecules move (*i.e.* naked or protein-bound)?; (ii) in addition to sRNAs, can longer dsRNAs act as signaling molecules during PTGS?; (iii) are all miRNAs capable of non cell-autonomous silencing? If not, what are the features enabling some miRNAs to act in a non cell-autonomous manner and others not? In addition, are non cell-autonomous tasiRNAs, present in model plants other than *Arabidopsis*; (iv) it is clear that the extent of silencing spread, whether SRS (or even extensive SRS) or SS is affected by various parameters including the nature of the trigger, possibly it's quantity but also potential endogenous regulators (yet to be identified), as well as environmental factors. None of these parameters have been yet comprehensively deciphered. As a result, the extent of silencing spread still holds a level of stochasticity; (v) is silencing spread involving both TGS and PTGS genes and pathways? Work on the above open questions holds promise for exciting findings in the near future.

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