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REVIEW PAPER

Non-cell-autonomous RNA silencing spread in plants

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ABSTRACT. RNA silencing, including gene quelling in fungi and RNA interference in animals, refers to a process of homologous, sequence-specific, RNA-based, post-transcriptional gene silencing triggered by double-stranded RNA that requires a conserved set of gene products. RNA-induced, homology-dependent gene silencing can also spread locally and systemically between cells to orchestrate developmental programs in plants. The mobile RNAi signal could consist of a complex of small RNAs and proteins. In plants, the mobile RNAi signals may traffic beyond sites of initiation through plasmodesmata channels (cell-to-cell movement) and also over long distances through the phloem (systemic movement). Small interfering RNA processed by DICER-like proteins is considered a hallmark of RNAi mobility, and the phloem represents a unique highway for long-distance spreading of RNAi signals. To date, molecular and genetic studies have identified a few RNA molecules and protein components that function in silencing spread mechanisms. However, the processes involved in cell-to-cell movement of RNAi signals remain poorly understood. To gain further insight into non-cell-autonomous RNAi spreading networks, it is critical to discover and characterize the distinct functions of the various genetic and molecular components involved. In this review, we discuss current advances, as well as gaps, in our understanding of cell-to-cell RNAi spread pathways and their implications for fundamental biology in plants.

Keywords: Cell-to-cell; Plasmodesmata; PTGS; RNAi; Silencing spread.

Abbreviations: AGO, ARGONAUTE; **dsRNA,** double-stranded RNA; **miRNA,** micro RNA; **PTGS,** post-transcriptional gene silencing; **RDR6,** RNA-DEPENDENT RNA-POLYMERASE 6; **RISC,** RNA-induced silencing complex; **RNAi,** RNA interference; **SDE3,** SILENCING DEFECTIVE 3; **SGS3,** SUPPRESSOR OF GENE SILENCING 3; **siRNA,** small interfering RNA; **smRNA,** small RNA; **ta-siRNA,** *trans*-acting siRNA.

CONTENTS

INTRODUCTION

Post-transcriptional gene silencing (PTGS) in plants is a homologous sequence-specific and RNA-based silencing process that utilizes a conserved set of gene products. PTGS is also described as quelling in fungi (Cogoni et al., 1996) and RNA interference (RNAi) in animals (Fire et al., 1998), providing a new paradigm for studying gene regulation. Although the pioneering discoveries on RNA silencing began with plants, this process has now been described in almost all eukaryotic organisms, including protozoa, flies, nematodes, insects, mice and humans. A

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number of genetic and molecular factors were initially reported based on RNA phenotypes, such as the formation of aberrant RNAs and structural changes of transgene repeat sequences. It has since become evident that double-stranded RNA (dsRNA) triggers the silencing of genes with homologous sequences through RNAi. dsRNA is produced by the action of RNA-dependent RNA polymerases or by transcription of endogenous or transgenic sequences. It is then processed into 20- to 25-nt siRNAs or microRNAs (miRNAs) by RNaseIII-like enzymes called Dicers. These siRNAs bind to effector protein complexes, termed RISC (RNA-induced silencing complexes) (Hammond et al., 2000), to mediate degradation of cognate mRNA, translational repression or transcriptional silencing. RISCs contain several proteins, including a member of the ARGONAUTE (AGO) protein family, which has a small RNA (smRNA)-binding PAZ domain as well as a PIWI domain that provides slicing activity to RISCs programmed to guide sequence-specific inactivation of complementary RNA or DNA (Song et al., 2004; Liu et al., 2004). Ten homologues of AGO (1–10) are found in *Arabidopsis thaliana* and mainly function as 'slicers' in this pathway (Chapman and Carrington, 2007).

In recent years, a major breakthrough in the study of RNA silencing in plants came with the discovery that RNA silencing is generally non-cell autonomous (Hyun et al., 2011). Plants can generate extensive RNA silencing through non-metabolic, gene-specific diffusible signals with sequence-specific information that travel from cell to cell through plasmodesmata (channels that connect most plant cells) (Lucas et al., 2009) and over long distances through the vascular phloem (tissue that distributes nutrients throughout the plant) to different organs (Voinnet et al., 1998; Mlotshwa et al., 2002; Himber et al., 2003; Voinnet, 2005). Non-coding small RNAs (miRNAs, siRNAs) play diverse and inherently integrative roles in the cellular system. Recent analyses of RNA silencing suggest that direct transport of regulatory RNAs is necessary for non-local gene silencing (Hamilton and Baulcombe, 1999; Lucas et al., 2001; Tournier et al., 2006). The degree of movement of silencing signals depends on the physiological conditions and surrounding environment of the tissue. In this review, we summarize our current understanding of the molecular and biological roles of non-coding RNA-mediated transport of silencing information between cells, highlighting exciting recent discoveries.

SMALL RNA—A HALLMARK OF RNAi SIGNALING

A wealth of research has demonstrated that a set of non-coding cellular smRNAs, including miRNAs, siRNAs and *trans*-acting siRNAs (ta-siRNAs), ranging from 20 to 25 nucleotides in length, are involved in RNAi pathways in plants. These small RNAs can be distinguished based on their origin, biogenesis and mode of action in cellular systems. Recent studies indicate that smRNAs can be considered to be signal-transmitting molecules that can move between cells and even enter the phloem for long distance transport from source to sink organs (Yoo et al., 2004; Chen and Kim, 2006; Lough and Lucas, 2006; Kehr and Buhtz, 2008).

siRNAs are excised from long, linear dsRNAs of foreign genes, viruses, transposons and natural sense/antisense RNA, and are then incorporated into RISCs to guide target mRNA through Watson-Crick base pairing. In plants, siRNA (silencing inducer) can be transmitted from cell to cell (Himber et al., 2003; Dunoyer et al., 2010a) and over long distances through the vascular system (Yoo et al., 2004; Kalantidis et al., 2008).

In contrast, microRNAs (miRNAs) are endogenously and deliberately expressed products of the genome of an organism. miRNAs are usually 19-22 nt long, are derived from the stem loop of folded precursor dsRNAs by the DCL1 endonuclease, and post-transcriptionally regulate eukaryotic gene expression, as shown by strong developmental defects in several miRNA overexpression and loss-of-function mutants (Bartel, 2004; Kurihara et al., 2006; Meyers et al., 2008; Schwab and Voinnet, 2009). Most plant miRNAs are relatively immobile and cell autonomous (Parizotto et al., 2004; Alvarez et al., 2006), but some miRNAs have been recently reported to function in non-cell-autonomous RNAi. Carlsbecker et al. (2010) suggested that the non-cell autonomous silencing activity of miRNA165/166 over a short distance is crucial for regulation of the HD-ZIP III transcription factor in a dosage-dependent manner in the root, and is also involved in xylem patterning.

Additionally, a novel form of endogenous small RNA, termed trans-acting siRNA (ta-siRNA), was recently discovered (Peragine et al., 2004; Allen et al., 2005; Williams et al., 2005). ta-siRNA generation involves components from both the siRNA and miRNA pathways, via a two-step process. First, miRNAs cleave non-coding *TAS* precursor transcripts (*pri-tasiRNA*), resulting in cleavage products that serve as substrates for endogenous RNA-DEPENDENT RNA POLYMERASE 6 (RDR6). A putative RNA binding protein, SUPPRESSOR OF GENE SILENCING 3 (SGS3) is also involved in this step, by stabilizing the cleavage fragments. The resulting dsRNAs are cut, likely by DCL4, into 21-nt long ta-siRNAs. These products have been shown to be involved in the degradation of homologous cellular mRNA by AGO family protein to control leaf polarity and developmental phase changes (reviewed by Vaucheret, 2005; Adenot et al., 2006; Curaba and Chen, 2008). Two recent studies in *Arabidopsis* strongly suggest that ta-siRNAs are potential candidates responsible for non-cell-autonomous gene regulation, moving beyond cellular initiation sites (Chitwood et al., 2009; Schwab et al., 2009). Chitwood et al. (2009) have demonstrated incongruities between expression patterns of precursor and mature small RNAs during development and provided evidence for the movement of ta-siRNAs from adaxial to abaxial leaf domains. Based on *in situ* hybridization and

reporter fusions, Schwab et al. (2009) reported that *AUXIN RESPONSE FACTOR* (*ARF*) controlling ta-siRNAs behave non-cell autonomously to control ARF activities. Slotkin et al. (2009) proposed that siRNAs processed from transposable elements could also move from pollen vegetative nuclei to sperm cells to mediate transposon silencing in the germ line. Another intriguing result was recently published by Olmedo-Monfil et al. (2010) on the movement of 24 nt-long ta-siRNAs during female gametogenesis in *Arabidopsis.* Thus, various small RNAs have emerged as potential mediators of non-cell autonomous RNA silencing.

SILENCING SIGNAL SPREAD BETWEEN CELLS

The expansion of RNA silencing into neighboring plant cells can be divided into two distinct types of mechanisms, namely cell-to-cell and long-distance spread. Cell-to-cell spread consists of two sequential events, local (limited) and extensive silencing (Figure 1).

Local silencing spread

Local cell-to-cell transport of RNA silencing can be distinguished from extensive silencing spread by the activity of cellular RNA-dependent RNA polymerases (RDRs). In the absence of amplification triggered by RDR activity, the extent of silencing through plasmodesmata is limited to as few as 10-15 cells beyond the site of initiation, which is known as 'local' or 'limited' cell-to-cell silencing spread. Plasmodesmata are unique intercellular channels found in most plant cells, with the exception of mature guard cells and cells at the maternal/filial boundary (Lucas and Lee, 2004; Oparka, 2004; Kim and Zambryski, 2005; Maule, 2008). Interestingly, the local movement mechanism relies on the DCL4-dependent production of 21-nucleotide siRNA when endogenous genes are targeted in an AGO1-dependent manner (Himber et al., 2003; Parizotto et al., 2004). Cell-to-cell spread of RNA silencing in *Arabidopsis* occurs during embryogenesis through distinct plasmodesmata apertures (Kobayashi and Zambryski, 2007). Furthermore, using size-specific GFP tracers, the extent of silencing signal was found to be similar to that of soluble proteins between 27 to 54 kDa (Kobayashi and Zambryski, 2007).

When a double stranded *SULPHUR* gene fragment was expressed using a phloem companion cell-specific promoter, the non-cell autonomous silencing of the endogenous *SULPHUR* gene was manifested by a yellow chlorosis (reduction of chlorophyll concentrations) phenotype observed in companion cells and in the 10-15 adjacent cells

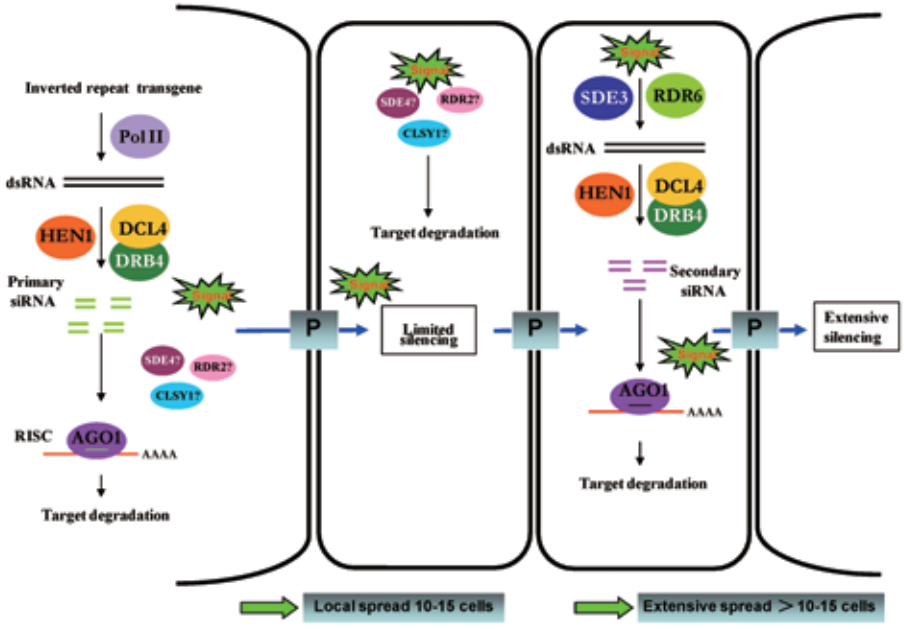

Figure 1. A simple model for inverted repeat transgene mediated non-cell-autonomous RNA silencing in plants. When an inverted repeat transgene is introduced into plants, dsRNAs are synthesized by polymerase II (Pol II), then cleaved by DCL4 with the cooperation of DRB4 to generate 21-nt long primary siRNAs. The siRNAs are stabilized by HEN1-mediated methylation and uploaded into effector protein complexes (RISCs), which degrade target mRNA in an AGO1-dependent manner. The 21nt siRNA, dsRNA or aberrant mRNA may able to move into neighboring (10-15) cells to enhance silencing machinery through plasmodesmata (P) by an unknown mechanism. SDE4/NRPD1a, RDR2, and CLSY1 have been reported to be involved in modifying the signal in donor cells or recipient cells via this local silencing pathway (details in text). Alternatively, an unknown signal could be amplified through the combined action of cellular RDR6 and SDE3 to synthesize new dsRNA, which is processed by the silencing machinery. This process was reiterated to induce extensive silencing spreads (more than 10-15 cells) in *Arabidopsis* through plasmodesmata. This model is based on Himber et al. (2003) and Dunoyer et al. (2007).

in the leaf (Himber et al., 2003). This phenotype was not altered in the *rdr6/sde1* mutant background, suggesting that signal amplification through RDR6 is not required (Himber et al., 2003). Instead, silencing was lost in the *dcl4* mutant, which is impaired in the production of 21-nt siRNA, but not 24-nt siRNA (Hamilton et al., 2002; Dunoyer et al., 2005). The extent of cell-to-cell local silencing correlates with the level of 21-nt long primary siRNA accumulation at the site of silencing initiation, but the detailed mechanism of such signal transmission is still unclear. A very recent study suggested that DCL4-dependent 21-nt siRNA duplexes are necessary and sufficient for non-cell autonomous RNAi (Dunoyer et al., 2010a). Their bombardment experiment confirmed that transgene-derived siRNAs not only mediate cell-to-cell RNA silencing through plasmodesmata, but also induce long distance RNAi signaling through the phloem (Dunoyer et al., 2010a). Molnar et al. (2010) proposed that DCL3 dependent 24-nt smRNAs also spread across the graft union from shoot to root, where they normally direct chromatin modifications in recipient cells through AGO4. However, there is no clear evidence regarding the 'non-cell-autonomous' function of smRNAs derived from endogenous loci. Interestingly, another study by Dunoyer et al. (2010b) addressing the issue of endogenous inverted repeats (IR) using different RNAi mutants in *Arabidopsis* suggests that two endogenous, as opposed to transgenic, loci (*IR-71*, *IR-2039*) have the potential to trigger local and systemic RNA silencing (Dunoyer et al., 2010b). Using micro-grafting experiments, they also revealed that all size classes of smRNAs produced from these *IR* loci could traffic through the vascular system, and that 24-nt siRNAs could trigger cognate-specific *de novo* methylation at a distance (Dunoyer et al., 2010b). This study clearly establishes a genetic overlap for both endogenous and exogenous *IR* loci-mediated siRNA processing, stability and function.

Extensive silencing spread

The mechanism underlying cell-to-cell spreading of silencing beyond 10-15 cells, termed 'extensive silencing,' has only been determined for transgenic target genes. Extensive RNA silencing in plants is dependent on the combined activities of cellular RDR6 and a putative RNA helicase-like protein (SDE3). This process can amplify RNAi signals through the bi-directional transitivity phenomenon (Voinnet et al., 1998; Vaistij et al., 2002). 5′ to 3′ transitivity is primer-dependent, whereas 3′ to 5′ is primer-independent. An aberrant RNA that accumulates as a result of erroneous transcription of sense transgene-derived transcripts (Dalmay et al., 2000), transposons, or viruses (Mourrain et al., 2000) is used to synthesize the 3′ complementary strand via RDR6 activity. In 3′ to 5′ transitivity, dsRNAs are generated via antisense transcription (Luo et al., 2009). Both processes require the action of RDR6, SDE3 and the coiled-coil protein SGS3 (Mourrain et al., 2000; Vaistij et al., 2002; Himber et al., 2003). The resulting dsRNAs are chopped by DCL4 to synthesize secondary siRNA (Vaistij et al., 2002; Himber et al., 2003; Schwach et al. 2005). It has been reported that secondary siRNAs are not overlapping in sequence with the primary siRNAs. Silencing over greater distances may be achieved by a relay amplification of original local cell-to-cell signaling event in waves of 10–15 cells at a time (Himber et al., 2003).

Silencing at a distance of greater than 10-15 cells can be clearly observed when the trigger and target genes are both exogenous. When an inverted repeat construct corresponding to the 5′ part (GF) of *GFP* driven by phloem-specific *AtSUC2* promoter was introduced into a *GFP* transgenic line (GFP142), silencing of *GFP* occurred from the vascular tissue to the whole leaf (Himber et al., 2003). *GFP* silencing was observed only around the vascular area (~10-15 cells) in *rdr6* mutants, indicating that extensive, but not limited, silencing spread was critically dependent upon RDR6. Similar experiments also implicate SDE3 in the extensive spread of silencing (Himber et al., 2003). smRNA analysis indicated that the accumulation of 21-nt and 24-nt siRNA corresponding to 'GF' was similar to the wild type in *rdr6* and *sde3*. On the contrary, 'P' specific 21-nt siRNAs were higher in wild type than in mutant plants. Based on these results, Himber et al. (2003) proposed a strong correlation between the synthesis of transitive siRNA and the extent of silencing spread.

GENETIC COMPONENTS REQUIRED FOR CELL-TO-CELL RNA SILENCING SIGNAL

To date, genetic and molecular studies have identified several genes that affect cell-to-cell RNA silencing. As mentioned above, extensive local spreading of silencing was totally abolished in *rdr6* mutants (Himber et al., 2003; Schwach et al., 2005), suggesting that RDR6 plays an important role in this kind of spreading through an RNA amplification mechanism. In addition, SDE2/SGS3 binds and stabilizes RNA templates to initiate RDR6-mediated dsRNA synthesis. Recently, Kumakura et al. (2009) observed co-localization of SGS3 and RDR6 and their interaction in specific cytoplasmic granules, termed SGS3/RDR6-bodies. Biochemical studies suggest that RDR6 has terminal nucleotidyltransferase activity along with primer-independent RNA polymerase activity on single-stranded RNAs (Curaba and Chen, 2008). Furthermore, 21-nt primary siRNAs have not been detected in *dcl4*, suggesting that DCL4 is the key enzyme in this pathway (Dunoyer et al., 2005).

Two previously characterized genes, *NRPD1a* (an DNA-dependent RNA-polymerase IV) (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005) and *RDR2* (an RNA dependent RNA polymerase 2) (Xie et al., 2004; Herr et al., 2005; Pikaard, 2006), are well-known to be involved in transcriptional gene silencing (TGS) and RNA directed DNA Methylation (RdDM) pathways. Interestingly, the participation of these two genes has been characterized in non-cell-autonomous pathways by two groups (Dunoyer et al., 2007; Smith et al., 2007). Dunoyer et al. (2007) proposed that RDR2 and NRPD1a affect silencing downstream of DCL4 and

AGO1, either by boosting physical trafficking of silencing signals between cells or by stimulating detection or modification in recipient cells. Moreover, in addition to *NRPD1a* and *RDR2,* Smith et al. (2007) identified a new component, CLASSY1 (an SNF2 domain–containing protein), required for cell-to-cell spreading of RNAi. Cytochemical analysis indicates that CLASSY1 most likely functions in the nucleus with RDR2 and NRPD1a in the upstream portion of the RNAi signaling pathway (Smith et al., 2007). Another interesting finding of these two genetic screens is that the other downstream factors of the heterochromatin silencing pathway, DCL3 and AGO4, are not required for the spreading of silencing signals (Dunoyer et al., 2007; Smith et al., 2007). Other molecular genetic components that affect cell-to-cell RNAi spread include *HEN1* (Hiraguri et al., 2005; Yang et al., 2006), which is essential for siRNA stability by methylation, *DRB4*, which is involved in the refining of *DCL4* substrate (Hiraguri et al., 2005; Adenot et al., 2006; Nakazawa et al., 2007), and *AGO1*, which is required for cognate specific slicing (Baumberger and Baulcombe, 2005; Qi et al., 2005). Olmedo-Monfil et al. (2010) reported that regulation of female gametogenesis initiation is controlled by ARGONAUTE 9 (AGO9), which is preferentially expressed in reproductive companion cells, but not in the associated male or female gametes or their precursors, in a dosage-dependent, non-cell-autonomous manner in *Arabidopsis*. They proposed that AGO9 has bias to interact with transposable element-derived 24-nt siRNAs, and that consequently these smRNAs are silenced in ovules and other accessory cells of female gametes.

As yet, however, uncovering the detailed mechanism of silencing with an RNA specificity determinant remains a challenge. More advanced approaches, including genetic, biochemical and molecular biological studies, are needed to identify potential components involved in non-cell-autonomous RNA silencing spread.

PHLOEM—A UNIQUE HIGHWAY FOR SYSTEMIC TRANSPORT OF RNAi SIGNALS

Land plants co-ordinate their growth and development, responses to environmental conditions, and defense against pathogens through the vascular system tissues, xylem and phloem. The phloem provides the essential conduit for nutrients (photoassimilates), defensive compounds and informational signal transport, while the xylem mediates mineral and water transport. Recent evidence indicates that in addition to gradual cell-to-cell spreading of RNA silencing signal, plants have the ability to transport RNAi signals to distant tissues, called systemic or long-distance spread of silencing. A signal initiated in a few cells of a source leaf can be actively spread over long distances through specialized phloem sieve tubes to induce silencing in sink leaves. This process was first intensively investigated using grafting experiments in tobacco plants (Palauqui et al., 1997). Later, systemic silencing spread was also found in other plants, such as cucumber (Yoo et al., 2004), *Helianthus* (Hewezi et al., 2005), tomato (Shaharuddin et al., 2006), and *Arabidopsis* (Brosnan et al., 2007) using grafting approaches.

In grafting, silencing signals produced from the transgenic source stock could be transmitted into the non-silenced scion by crossing the graft junction. In a three-way grafting experiment, a wild type plant stem was grafted between silenced transgenic root stock and non-silenced transgenic scion, and a silencing signal was transmitted through the non-transgenic wild type plant (Palauqui et al., 1997). Therefore, amplification steps need not be involved in phloem transport (Schwach et al., 2005). However, high amounts of target transcripts were required for reception of the silencing signal over long distances (Garcia-Perez et al., 2004; Schwach et al., 2005). There is growing evidence that small RNAs synthesized from a virus infection or a transgene may act as signaling molecules, as they could be observed in silenced phloem but not in non-silenced plants (Yoo et al., 2004), and can propagate beyond their site of origin. siRNAs larger than 21 nt (24-26 nt), are proposed to be essential for long-distance spreading of silencing signals (Himber et al., 2003).

Interestingly, some miRNAs have been predicted to be potential mediators for signaling directly involved in information transfer over long distances through the phloem. Gradual spreading of miRNA166 expression during leaf development, and its accumulation in phloem tissue, indicate a possible role in signal movement from a signaling source below the incipient maize leaf (Juarez et al., 2004). The combination of inorganic phosphate (Pi) alteration and coexpression of PHO2 and miR399s in the phloem indicates the potential involvement for these gene products in systemic silencing (Lin et al., 2008). The translocation of systemic miR399 silencing signals from shoots to roots was reported to be critical for increasing the uptake of Pi and transformation in the initial stages of Pi depletion (Lin et al., 2008).

Proteins involved in systemic silencing spread remain largely unknown. Yoo et al., (2004) first discovered a PHLOEM SMALL RNA BINDING PROTEIN 1 (PSRP1) from pumpkin phloem sap using biochemical analyses in which small RNAs were bound with high affinity. To date, though, no clear homologues have been identified in *Arabidopsis* or *N. benthamiana*. The discovery of smRNA carrier proteins in the phloem will shed light on the mechanism of long-distance trafficking of RNAi signals through the plant vascular system.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Increased knowledge of non-cell-autonomous RNA silencing will help to explain a number of aspects of plant development and pathogen defense. Recent rapid advances in the understanding of non-cell-autonomous RNA silencing would have been impossible without the genetic

resources of the model plant *Arabidopsis*. Although a range of mechanistic functions for this phenomenon have been described in the last decade, the molecular and biochemical nature of silencing spread, and the mechanisms that regulate these functions, have only recently begun to emerge. It is now clear that plants employ two distinct processes to transmit silencing signals between cells, namely, cell-to-cell and systemic spreading. In the first mechanism, 21-nt smRNAs are well-documented as potential silencing signal molecules. Therefore, a future challenge is to dissect the molecular and cellular regulatory components that are required for cell-to-cell spread of RNA trafficking in plants. Genetic mutant screening for non-cell-autonomous RNA silencing has uncovered a number of heterochromatin gene-silencing components but so far has failed to isolate components that directly regulate cell-to-cell smRNA movement. Furthermore, an as-yet unknown systemic silencing signal can be transported over long distances via the phloem tissue of the plant vascular system. The molecular mechanisms underlying phloem-dependent RNA translocation and function represent a significant challenge to our understanding of this process. Our knowledge of the components deciding the entry or exit of RNA is also still limited. Phloem-derived non-coding RNA molecules also have yet to be characterized. Investigating the genetic basis and molecular mechanisms of RNA phloem loading and transport, as well as identifying the internal and external triggers that induce phloem mobile trafficking, offers new possibilities for exploring systemic gene silencing in higher plants.

The existence of endogenous *IR* loci demonstrates that post-transcriptional gene silencing is a genuine, commonly utilized process in plants, as has already been shown in other organisms, such as flies and worms. These *IR* loci might have biological significance for phenotypic variation and adjustment of environmental stress responses in plants. Ongoing and future investigations will certainly reveal more exciting insights as to the extent of mechanisms and functions shared by *IR* loci-mediated non-cell autonomous RNA silencing pathways across the eukaryotic kingdom. Our picture of smRNA-mediated cell-to-cell and systemic RNAi has been dramatically reshaped by the flood of new findings, but we still have a lot left to discover.

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