

RNA silencing movement in plants

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Higher eukaryotes have developed a mechanism of sequence-specific RNA degradation which is known as RNA silencing. In plants and some animals, similar to the nematode *Caenorhabditis elegans*, RNA silencing is a non-cell-autonomous event. Hence, silencing initiation in one or a few cells leads progressively to the sequence-specific suppression of homologous sequences in neighbouring cells in an RNA-mediated fashion. Spreading of silencing in plants occurs through plasmodesmata and results from a cell-to-cell movement of a short-range silencing signal, most probably 21-nt siRNAs (short interfering RNAs) that are produced by one of the plant Dicer enzymes. In addition, silencing spreads systemically through the phloem system of the plants, which also translocates metabolites from source to sink tissues. Unlike the short-range silencing signal, there is little known about the mediators of systemic silencing. Recent studies have revealed various and sometimes surprising genetic elements of the short-range silencing spread pathway, elucidating several aspects of the processes involved. In this review we attempt to clarify commonalities and differences between the individual silencing pathways of RNA silencing spread in plants.

Introduction

Higher eukaryotes have developed a mechanism of sequence-specific RNA degradation called 'RNA silencing', an idiom that combines the terms PTGS (post-transcriptional gene silencing) and RNAi (RNA interference). The central part of the RNA degradation pathway is the generation of siRNAs (short interfering RNAs) from dsRNA (double-stranded RNA) by an RNase III-type nuclease, Dicer. The siRNAs are incorporated into the RISC (RNA-induced silencing complex), and, after strand separation, the remaining single-stranded RNA guides the sequence-specific cleavage of a target RNA. Despite common features of RNA silencing, there are differences between the animal and plant kingdoms and also between species (reviewed in Meister and Tuschl, 2004; Mello and Conte, 2004; Baulcombe, 2006).

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Key words: green fluorescent protein (GFP) transgene, local silencing, plant, RNA silencing, short-range silencing, systemic silencing.

Abbreviations used: AGO, Argonaute; AS-silencing, antisense-induced silencing; CLSY1, CLASSY1; DCL, Dicer-like; dsRNA, double-stranded RNA; DRB, dedicated dsRNA-binding protein; GFP, green fluorescent protein; GUS, β -glucuronidase; HEN1, HUA enhancer 1; miRNA, microRNA; nat-siRNA, natural antisense transcript-derived siRNA; NRPD1A, DNA-dependent RNA polymerase IV large subunit; POLIV, DNA-dependent RNA-polymerase IV; RDR, RNA-directed RNA polymerase; RISC, RNA-induced silencing complex; RNAi, RNA interference; SGS, suppressor of gene silencing; siRNA, short interfering RNA; S-silencing, sense-induced silencing; SDE3, suppressor of defective silencing 3; tasiRNA, *trans*-acting siRNA; VIGS, virus-induced gene silencing.

In addition to the RNAi pathway described above, which characterizes the response of cells to exogenous sequences (viruses, transgenes etc.), multiple silencing pathways that result from the processing of endogenous RNAs operate in plants. miRNAs (microRNAs) are probably the best characterized category of these small RNAs. They are processed from longer primary transcripts, with extensive fold-back structures that are transcribed from specific endogenous non-protein-coding RNA genes. These miRNA genes are under the control of regular polymerase II promoters (Xie et al., 2005a; Megraw et al., 2006) and are expressed in a tissue- and time-specific manner (Valoczi et al., 2006). In plants, miRNAs are considered to function by providing sequence specificity to a protein complex, which slices mRNAs complementary to the miRNAs in a manner similar to siRNAs. Moreover, they have been shown to have important roles in plant growth, stress response and development. miRNA biogenesis and function both in plants and animals have been extensively reviewed elsewhere (Bartel, 2004; He and Hannon, 2004; Murchison and Hannon, 2004; Chen, 2005).

RNase III enzymes: These specifically bind to and cleave dsRNA. RNAi- and miRNA-related enzymes that contain an RNase III domain include the Drosha and all Dicer proteins.

In addition to the well-characterized categories of siRNAs and miRNAs, new kinds of small RNAs have more recently been revealed in plants. These small RNAs, tasiRNAs (*trans*-acting siRNAs) and nat-siRNAs (natural antisense transcript-derived siRNAs), have biosynthetic pathways distinct from siRNAs and miRNAs. tasiRNAs are derived from non-protein-coding transcripts that are targeted by miRNAs, and one of the two cleavage products is converted into a double-strand by RDR6 (RNA-directed RNA polymerase 6) (Vazquez et al., 2004; Allen et al., 2005). SGS3 (suppressor of gene silencing 3) is required in this step, as it seems to protect the miRNA cleavage products from degradation (Yoshikawa et al., 2005). The double-stranded intermediate is then processed by DCL4 (Dicer-like 4), in a complex with DRB4 (dedicated dsRNA-binding protein 4), to phased 21-nt tasiRNAs that are incorporated into AGO (Argonaute)-containing complexes to target complementary sequences (Vazquez et al., 2004; Allen et al., 2005; Gascioli et al., 2005; Xie et al., 2005b; Yoshikawa et al., 2005; Adenot et al., 2006). The miRNA cleavage determines the phase and is critical for the production of specific tasiRNAs (Allen et al., 2005). In *Arabidopsis thaliana* there are at least five tasiRNA-producing loci, *TAS1a–TAS1c*, *TAS2* and *TAS3*. In contrast with *TAS1* and *TAS2* which do not seem to be found in other organisms, *TAS3* is conserved in land plants. nat-siRNAs are derived from overlapping transcripts. In the one example (Borsani et al., 2005) salt-stress-induced *SRO5* (similar to RCD-one 5) overlaps with *P5CDH* (Δ 1-pyrroline-5-carboxylate dehydrogenase), which is implicated in proline catabolism. Under salt-stress conditions the overlapping region gives rise to a 24-nt nat-siRNA, whose production depends on *DCL2*, *RDR6*, *SGS3* and *NRPD1A* (DNA-dependent RNA polymerase IV large subunit). Since a considerable percentage of genes in eukaryotes come in overlapping pairs, the above mechanism could be mediating various cellular responses.

It is widely accepted that the main biological functions of RNA silencing are to assist in the resistance to viruses and other exogenous RNAs (for example, viroids or RNA from agrobacterial T-DNA-encoded genes), secure the stability of the genome through the suppression of transposon activity and provide an additional mechanism for the regulation of endogenes (Baulcombe, 2004).

In some organisms, including plants and the nematode *Caenorhabditis elegans*, silencing is a non-cell-autonomous event. In these systems, silencing initiation in one cell eventually leads to the silencing of the same sequences in a whole group of cells or even in the whole organism. Although the mechanisms of silencing spread have commonalities between the two systems, such as the involvement of RDRs in silencing spread, the details of silencing movement seem to be largely divergent (Voinnet, 2005). In a recent review, Hunter et al. (2006) summarize the silencing spread in *C. elegans*, whereas the present review will concentrate on silencing spread only in plants. We will attempt to clarify the distinctions between the three different types of silencing spread, short-range silencing spread, extensive local silencing spread and systemic silencing. The genetic and molecular factors involved in systemic silencing are largely unknown. However, three recent studies have advanced our understanding of short and extensive local spread, using *Arabidopsis* as a model system (Dunoyer et al., 2005, 2007; Smith et al., 2007).

Exogenous silencing activation

Manifestations of silencing spread

Systemic spread of silencing was first convincingly shown in *Nicotiana tabacum* and *Nicotiana benthamiana* (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Palauqui and Vaucheret, 1998; and reviewed in Fagard and Vaucheret, 2000). It was shown to manifest as the sequence-specific suppression of an ectopically overexpressed gene whether the sequence was of endogenous origin (Palauqui et al., 1997; Fagard and Vaucheret, 2000) or of exogenous origin

Argonaute (AGO) proteins: These are the catalytic components of the RISC, which is responsible for the post-transcriptional gene silencing phenomena. AGO proteins bind siRNAs and have endonuclease activity, which is known as 'slicer' activity, directed against mRNA strands that are complementary to their bound siRNA fragment.

T-DNA: This is a DNA fragment of the *Agrobacterium tumefaciens* Ti plasmid which can be transferred by non-homologous recombination into the genomic DNA of the host cell.

(Voinnet et al., 1998). Grafting experiments have also demonstrated silencing spread in tomato (Shaharuddin et al., 2006), cucumber (Yoo et al., 2004) and *Helianthus* (Hewezi et al., 2005). Grafting experiments, in this respect, are essential, because they allow for a clear separation between the silencing-signal-producing (source) tissues from the signal-receiving (sink) tissues (Kalantidis, 2004). Furthermore, in such experiments a wild-type segment can be interspaced between the rootstock and scion to ensure that cell-to-cell amplification of the signal is not taking place. In these original systemic silencing experiments, the silencing inducer and the targeted sequence were regulated by a very strong promoter (e.g. 35S promoter). Although grafting experiments were not reported, non-cell-autonomous movement of silencing has also been described in *Arabidopsis* (Guo et al., 2003; Himber et al., 2003; Dunoyer et al., 2007; Smith et al., 2007), *Petunia* (Que et al., 1998), *Medicago* (Limpens et al., 2004) and even ferns (Rutherford et al., 2004).

In all the above experiments sequences homologous to the transgenic inducers of silencing, whether endogenous or transgenes, are suppressed post-transcriptionally in an RNA-mediated fashion. Once initiated the process is stably maintained in the silenced tissues and is not inherited.

Initiation of silencing spread

Silencing spread has been shown to follow the initiation of silencing by overexpression of sense or antisense RNA, but most efficiently by the expression of dsRNA (Brodersen and Voinnet, 2006). In transgenic plants efficient suppression of the transgene was achieved by infiltration of RNA from silenced tissues (Hewezi et al., 2005) or even direct bombardment with dsRNA and siRNAs (Klahre et al., 2002). There have not been any reports of the spontaneous initiation of endogene silencing. Therefore, spontaneous initiation of silencing is characteristic of transgenic sequences, transposons, viruses and viroids. The downstream steps of silencing following dsRNA formation have to a great extent been elucidated (reviewed in Brodersen and Voinnet, 2006). However, the mechanisms of S-silencing (sense-induced silencing) and AS-silencing (antisense-induced silen-

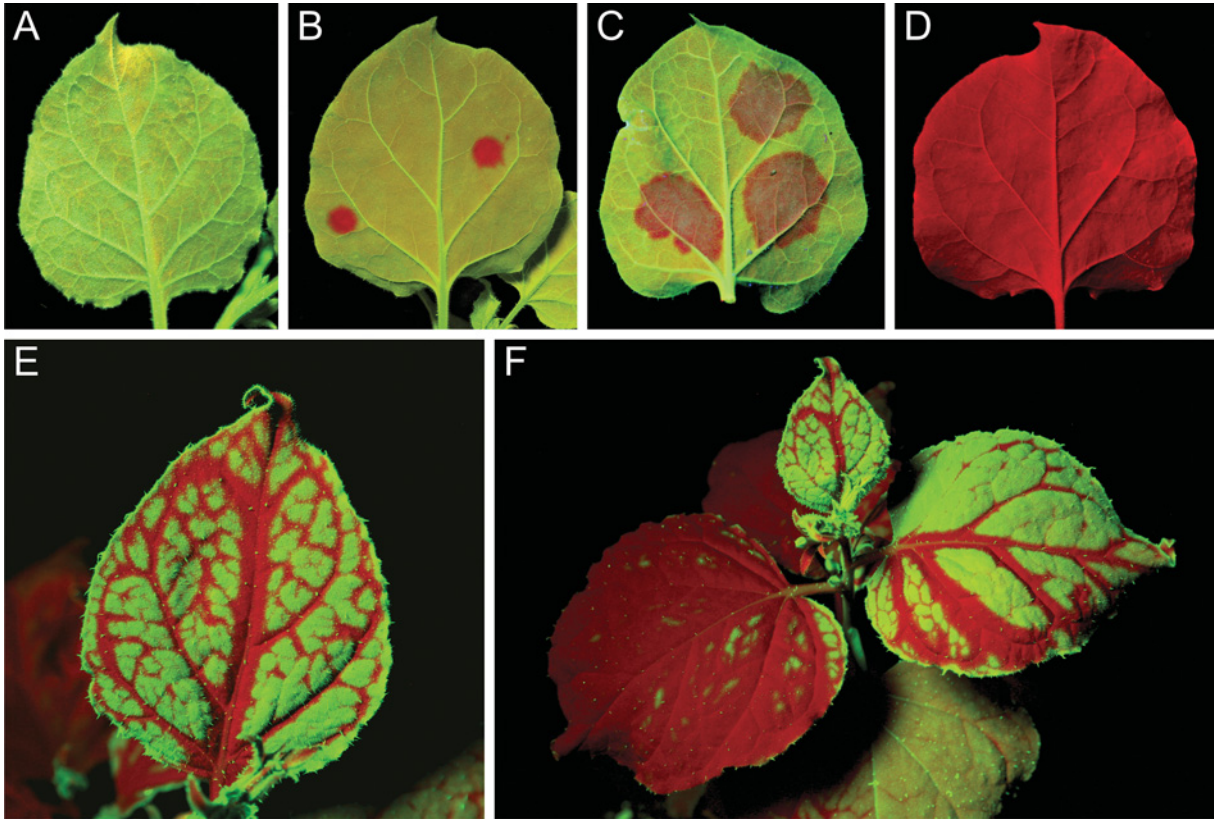
cing) remain obscure. It is believed that in these cases silencing is initiated through the activation of *RDRs* (Wassenegger and Krczal, 2006) that transform a single-stranded RNA to dsRNA. *Arabidopsis* has six *RDR* genes, but their involvement in RNA silencing pathways has only been partially elucidated for *RDR6* and *RDR2*. A term favoured for non-characterized RNA inducers of silencing is that of 'aberrant' RNA, where aberrancy may be a qualitative feature (problematic 3' or 5' ends, secondary structure etc.) (Herr et al., 2006; Luo and Chen, 2007) or even quantitative features, such as excess amounts of transcript (Palauqui and Vaucheret, 1998; Schubert et al., 2004), typical of transgenes expressed from the 35S promoter. It is not clear how either silencing-inducing qualitative or quantitative features of RNA molecules are sensed inside the plant cell. It is interesting to note that unlike silencing induced by dsRNA, silencing induction in plants overexpressing sense or antisense transgenic sequences is stochastic (Palauqui et al., 1996; Kim and Palukaitis, 1997; Holtorf et al., 1999; Qin et al., 2003; Kalantidis et al., 2006). In other words, uncharacterized factors affect the efficiency of silencing initiation in these plants. One such factor is potentially temperature, which has been shown to affect the efficiency of silencing induction (Szittyta et al., 2003,) possibly through enhanced *RDR6* transcript levels (Qu et al., 2005); although the efficiency of the silencing mechanism was shown to be affected by temperature also downstream of dsRNA formation (Kalantidis et al., 2002). It should be noted that even though all RNA-mediated silencing phenomena seem to share a common dsRNA step (Beclin et al., 2002), S-silencing, AS-silencing and IR-silencing (inverted repeat silencing) have been repeatedly reported not to produce identical silencing phenotypes (Que et al., 1998; Shaharuddin et al., 2006). It is generally accepted that the cell 'senses' as inducers of silencing any RNA sequences that have features characteristic of what are considered to be natural targets of the silencing mechanism in plants: T-DNA-containing genes (Dunoyer et al., 2006), viruses and transposons (Baulcombe, 2004; Ding et al., 2004). What these features are, however, is not clear.

In respect to the issues addressed in this review, it is particularly interesting that, whatever the inducer

Grafting: This is a method of artificial transfer of part of one plant to a new position on another plant of the same or even a different species. It is a method widely used in plant propagation in which the tissues of one plant are encouraged to fuse with those of another.

Figure 1 | UV phenotypes of RNA silencing of a GFP transgene in *N. benthamiana*

(A) Not silenced; (B) spontaneous short-range local silencing; (C) induced local silencing; (D) fully silenced; (E) systemic silencing; (F) systemic (centre and right-hand leaves) and extensive local silencing (left-hand leaf).



of silencing is, in plants there is always some spreading of silencing taking place, from a few cells around the silencing source cell to the whole plant; RNA silencing in plants manifests as a non-cell-autonomous event. Nevertheless, there are important dissimilarities between different types of silencing spreading, and it is convenient to differentiate between short-range local silencing spread (short-range spread), extensive local silencing spread (extensive local spread) and systemic silencing. The phenotypes of the three different categories of silencing for a GFP (green fluorescent protein) transgene in *N. benthamiana* are shown in Figure 1.

Short-range spread

Cell-to-cell movement of silencing without signal amplification

When an endogenous gene is targeted for post-transcriptional gene silencing, the effect of suppression

is only observed in the cells where the inducer of suppression, usually a dsRNA or an appropriately modified virus, is active and in a thin layer of cells surrounding them. This thin layer is estimated to be 10–15 cells wide and is considered to be the result of spreading of silencing without amplification of the silencing signal (Himber et al., 2003). It has been shown that endogenes are ‘protected’ from the amplification ability of RDR enzymes and, as a result, spreading of silencing cannot use a relay mechanism for the silencing signal. Therefore, spreading of silencing of endogenes has to rely on the spreading of the original silencing signal produced in the cells containing the silencing inducer. Evidence of the inability of RDR to function with endogenous sequences comes from two sources, from transitivity studies (reviewed in Bleys et al., 2005) and from *rdr* mutants (Himber et al., 2003; Schwach et al., 2005). The phenomenon of silencing spreading along the

mRNA sequence is termed transitive RNA silencing and is characteristic of plants and the nematode *C. elegans* (Sijen et al., 2001; Vaistij et al., 2002; Van Houdt et al., 2003). It is believed to be the result of RDR6 activity and the subsequent generation of secondary siRNAs (Himber et al., 2003; Bleys et al., 2005). In contrast with *C. elegans*, in plants it has been shown repeatedly that unlike transgenes, endogenes resist extension of silencing to sequences outside the one originally targeted by RNAi (Vaistij et al., 2002; Koscianska et al., 2005; Miki et al., 2005; Petersen and Albrechtsen, 2005). This insulation of endogenes from RDR activity is not due to gene-sequence specificities, since the same sequence is prone to transitivity when expressed as a transgene (Koscianska et al., 2005). One important difference between transgenes and endogenes in this respect are the RNA steady-state levels of the targeted sequences, since transgenic transcription is usually regulated by promoters much stronger than those regulating endogene expression. Indeed, transitivity has been reported for an endogene with high levels of expression (Bleys et al., 2006). On the other hand, in *Arabidopsis rdr6*-null mutants, silencing spreads only to the limited 10–15 cells around the silencing source cells (Himber et al., 2003). Similar results were reported by Schwach et al. (2005) following knock-down of the endogenous *RDR6* gene in *N. benthamiana* by RNAi. In this manner, suppression of *GFP*-transgene by silencing spread was reminiscent of the endogene silencing phenotype, with silencing in systemic tissues not spreading further out of the veins than the usual short-range spread of 15 cells (Schwach et al., 2005). However, this short-range spreading of silencing that does not require an amplification step has not been exclusively observed in endogene silencing or transgene silencing of *rdr* mutants. Short-range silencing of transgenic sequences with no further spread outside the 15-cell zone has been described for transgenes after bombardment with DNA (Palauqui and Balzergue, 1999) or siRNA (Klahre et al., 2002), or even after induction of silencing by a viral vector (Ryabov et al., 2004). Even more surprisingly, silencing without systemic spread has been described as a spontaneous event in some *GFP* transgenic lines (Kalantidis et al., 2006). We suggested that, in the above case, the amount of silencing signal produced was not sufficient to initiate the relay mechanism necessary for further spread of silencing (Kalantidis

et al., 2006). This is in accordance with the general notion of RNA silencing as a molecular immune system (Bagasra and Prilliman, 2004); a threshold for systemic silencing would prevent an over-reaction to a minor threat.

Short-range silencing signal

On the basis of work with isolated viral suppressors of silencing, a role in silencing spread was attributed to the longer siRNAs (24-nt long, mostly products of *DCL3*) (Hamilton et al., 2002). However, more recent genetic evidence shows clearly that the 24-nt siRNAs are not involved in cell-to-cell silencing. Instead, cell-to-cell spread of silencing is lost in *Arabidopsis* plants with lesions in the *DCL4* gene, the *Arabidopsis* Dicer paralogue responsible for the generation of 21-nt siRNAs (Dunoyer et al., 2005). In accordance with this finding, the spontaneous short-range silencing of the *GFP* transgene could be reverted by viral suppressors of silencing that bind and presumably sequester siRNAs (Kalantidis et al., 2006). Nevertheless, for obvious technical reasons it has not been possible yet to isolate siRNAs spreading to this small zone of less than 15 cells surrounding the primarily silenced tissue.

There is indirect evidence that short-range spreading of silencing takes place through the plasmodesmata, which are fine cytoplasmic tubes connecting adjacent plant cells. This evidence comes from the fact that during short-range spread, the only cells that escape silencing are the guard cells of stomata which at that stage of development are symplastically isolated due to the blockage of these specific channels (Voinnet et al., 1998; Himber et al., 2003; Kalantidis et al., 2006). Although in spontaneous short-range silencing some silenced guard cells could be observed in the centre of larger silenced foci, it could be argued that the silencing signal had entered these cells before the plasmodesmata blockage took place (Kalantidis et al., 2006). More recent analyses of RNA silencing spread in *Arabidopsis* embryos revealed that the ability of the short-range signal to spread is influenced by the aperture of plasmodesmata present in the specific tissue where silencing is spreading. By using size-specific tracers, it was shown that the short-range signal moves to a similar extent as soluble proteins between 27–54 kDa (Kobayashi and Zambryski, 2007).

Plant genes involved in short-range silencing spread

As indicated above, *RDR6* was not found to be necessary for short-range spread of silencing, unlike *DCL4* whose absence leads to loss of silencing spread. Two large genetic screens conducted in parallel by Dunoyer et al. (2005, 2007) and Smith et al. (2007) revealed additional important and/or essential players in cell-to-cell silencing spread. In both screens an endogenous gene was targeted by RNAi using a hairpin construct driven by the phloem-specific *SUC2* (sucrose transporter 2) promoter. Both screens relied on the chlorotic phenotype produced in the tissues where RNAi was effective. Using this phloem-specific promoter, it was possible to differentiate between the primary silencing events taking place in the phloem only and the secondary silencing events as a result of silencing spread. In addition to *DCL4*, which was identified in a previous report from the same genetic screen (Dunoyer et al., 2005), both groups identified two rather surprising genes necessary for the cell-to-cell spread of silencing (Dunoyer et al., 2007; Smith et al., 2007): *NRPD1a*, the gene encoding the large subunit for a putative specific POLIV (DNA-dependent RNA-polymerase IV) (Herr et al., 2005; Kanno et al., 2005; Onodera et al., 2005; Pontier et al., 2005) and *RDR2* (Xie et al., 2004; Herr et al., 2005; Pikaard, 2006), the gene encoding an RDR associated with the POLIV pathway (Herr et al., 2005; Pikaard, 2006). It is still unclear in what way these two genes involved in methylation and transcriptional silencing pathways can affect short-range spread of silencing. It was suggested that POLIV and RDR2 may assist in the generation of dsRNA which is then processed by *DCL4* to generate 21-nt siRNAs, which act as the short-range signal (Smith et al., 2007). On the other hand, Dunoyer et al. (2007) suggest that *NRPD1a* and *RDR2* affect silencing downstream of *DCL4*, either by promoting physical silencing spread between cells or by affecting the reception of the signal in the cells where the silencing signal enters. Their system allowed them to differentiate between primary siRNAs produced by the transgene sequences and secondary siRNAs produced by endogenous sequences. Since secondary siRNAs were not detected, they presumed that, in this respect, *NRPD1a* and *RDR2* are not involved in secondary siRNA synthesis from endogenous transcripts (Dunoyer et al., 2007). Additional genes af-

fecting the process of cell-to-cell silencing spreading in *Arabidopsis* were identified during these genetic screens, such as an SNF2-domain-containing protein named *CLASSY1* (*CLSY1*), which on the basis of sub-cellular localization studies, was proposed to have a possible role at a step between *NRPD1a* and *RDR2*. In agreement with this hypothesis, *RDR2* localization was severely disrupted in *clsy1* mutants, whereas *NRPD1a* localization was only mildly affected (Smith et al., 2007). *CLSY1* contains a DNA-binding region and the mutations identified to affect silencing spread are likely to affect its nucleic-acid-binding abilities. However, the roles of *CLSY1*, *NRPD1a* and *RDR2*, three proteins primarily localized in the nucleolus, in cell-to-cell spread is far from clear. In contrast with *NRPD1a* and *RDR2*, which positively affect cell-to-cell movement of the local silencing signal, two other genes of the POLIV pathway, *DCL3* and *AGO4*, were suggested either to negatively affect this process or not to affect it at all (Dunoyer et al., 2007). Other genetic factors with a positive effect on short-range signal movement are genes related to *DCL4* silencing (Dunoyer et al., 2007), *HEN1* (Hiraguri et al., 2005; Yang et al., 2006) required for the stabilization of siRNAs, *DRB4*, associated with optimizing processing of *DCL4* substrate (Hiraguri et al., 2005; Adenot et al., 2006; Nakazawa et al., 2007), and *AGO1* which is responsible for the execution of the sequence-specific slicing of silencing target mRNAs (Baumberger and Baulcombe, 2005; Qi et al., 2005). A previous work in *N. benthamiana* presented evidence for a role that *AGO1* may have in cell-to-cell silencing spread (Jones et al., 2006). Unexpectedly, however, one of the screens identified *DCL1*, the Dicer paralogue associated with the generation of miRNAs to enhance the spreading of silencing, possibly by processing the original hairpin transcript to a substrate more suitable for *DCL4*-directed siRNA-generation activity (Dunoyer et al., 2007). Therefore, it is now becoming evident that the individual RNA silencing pathways in plants are not as independent as originally anticipated. *DCL4* has been characterized as the Dicer paralogue with a primary role in antiviral defence and tasiRNA production (Xie et al., 2005b; Deleris et al., 2006), *DCL1* has been implicated in the miRNA, as well as tasiRNA pathways (Vazquez et al., 2004; Xie et al., 2004; Allen et al., 2005; Yoshikawa et al., 2005), *HEN1* (HUA enhancer 1) was implicated in the stabilization of most, if not all, small RNA species

by methylation, and *AGO1* has been shown to be involved in various silencing pathways (Blevins et al., 2006). Now these four genetic elements have been found to be involved in the cell-to-cell spreading of silencing, and essential roles in this process are attributed to *NRPD1a* and *RDR2* which had been previously only connected to siRNA-directed heterochromatin formation. On this basis silencing pathways are envisaged to function through downstream and upstream modules that can combine to form different pathways (Smith et al., 2007). The model shown in Figure 2 summarizes these recent findings.

Extensive local spread

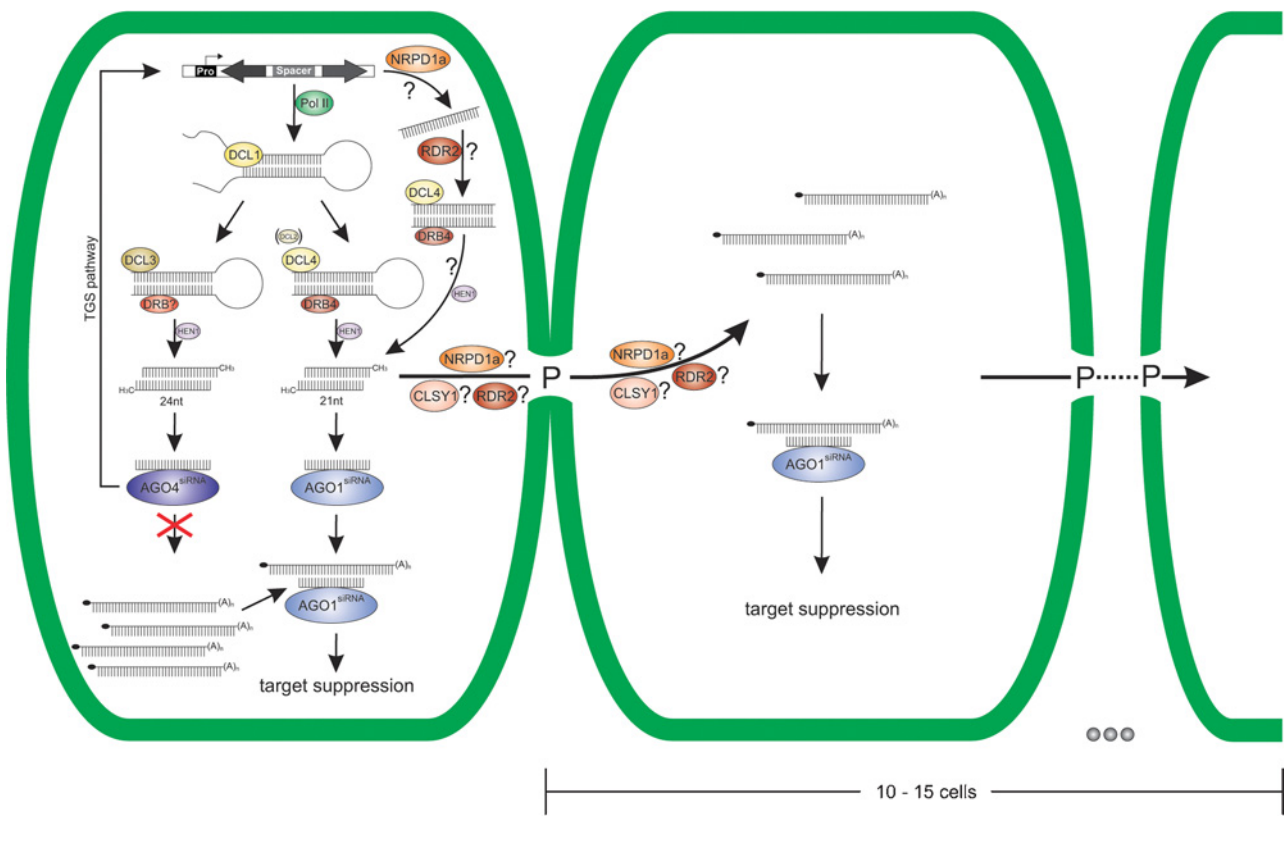
This type of silencing is the one most difficult to differentiate. It refers to cell-to-cell silencing spread that exceeds the maximum of 15-cell-long spreading of the short-range silencing signal. It is characteristic for sink leaves of transgenic plants receiving the systemic silencing signal against the transgene: silencing arrives through the veins (see below) and expands throughout the whole leaf lamina (Figure 1F). It has only been described to operate against transcripts of transgenes (Dunoyer et al., 2007; Smith et al., 2007), and there is evidence indicating that it is mediated by the activity of *RDR6*. In *rdr6* mutants, it does not occur even for transgenes (Himber et al., 2003; Schwach et al., 2005), indicating that the extensive local spread operates through an RNA amplification mechanism. The putative RNA helicase *SDE3* (suppressor of defective silencing 3), identified as a necessary factor for transgene silencing (Dalmay et al., 2001), was also shown to contribute to extensive local spread, although to a smaller extent than *RDR6* (Himber et al., 2003). Himber et al. (2003) were able to differentiate between primary and secondary siRNAs produced in a GFP-expressing *Arabidopsis* line (line GFP142) by an elegant experimental setup. They induced silencing of the *GFP* transgene using a hairpin construct containing only a part of the *GFP* target sequence designated as 'GF', whereas the non-targeted *GFP* sequence was designated as 'P'. Therefore 'P' siRNAs could only be secondary siRNAs produced through transitivity. They showed that the amounts of primary 21- and 24-nt siRNAs that originated from the RNAi-targeted 'GF' region were the same in *GFP142* and *GFP142/rdr6* or *GFP142/sde3* mutant lines, despite the lack of extensive local silencing

spread in the mutant plants. In contrast, secondary 'P' 21-nt-long siRNAs, but not 24-nt-long siRNAs, were only found in *GFP142* plants. They concluded that secondary 21-nt siRNA levels were directly proportional to the degree of silencing movement (Himber et al., 2003). Hence, the extensive short-range spread seems to operate through a relay mechanism of the short-range local spread on the basis of amplification of the 21-nt-long siRNAs produced through the activities of *RDR6*, *SDE3* and presumably *DCL4*. Since *RDR6* has been shown to function primarily on transgenes, extensive local spreading of silencing is not observed when targeting endogene-produced mRNAs. As mentioned above it was shown recently in transitivity studies that *RDR6* may exceptionally also act on endogenes expressed at high levels (Bleys et al., 2006). It would therefore be interesting to look for extensive local silencing against these specific endogenes.

An intriguing fact about silencing spread driven against a highly expressed transgene is that in the leaf where induction of silencing occurs extensive local spread has not been reported. Extensive local spread of silencing has always been connected with the spreading of transgene silencing in the receiving tissues. This is surprising as: (i) there is a substantial amount of primary siRNA produced, (ii) there is abundance of template mRNA for *RDR6* to act on and (iii) therefore enough substrate for the activity of *DCL4* in order to generate the secondary 21-nt-long siRNAs implicated to be involved in extensive local spread. Why then is extensive cell-to-cell spread not observed in the silencing signal source leaf? A plausible explanation is that this inability for extensive local spread is due to sink source relationships. The cells where silencing is induced generate primary siRNAs, which by simple diffusion may spread 15 cells around them, but the actual spreading signal is exclusively exported to the phloem for systemic spread (see below). At the other end, the sink tissues receiving the systemic signal initiate cell-to-cell spread, but now all the cells in the sink leaf are recipients of metabolites and the silencing signal that moves with them (Tournier et al., 2006) (see below). Nevertheless, sink source relationships alone are unlikely to be enough to explain this disparity. If mere diffusion of the amplified signal through the plasmodesmata was able to mediate extensive local silencing then in different experimental setups, a

Figure 2 | A speculative model for short-range spreading of RNA silencing

This figure is based on Dunoyer et al. (2007) and attempts to integrate suggested models from three recent papers (Dunoyer et al., 2005, 2007; Smith et al., 2007). [Adapted with permission from Macmillan Publishers Ltd: Nature Genetics, Dunoyer et al. (2007) © 2007]. The primary transcript produced by the transgene is processed by DCL1 in a Drosha-like fashion, facilitating its subsequent processing by DCL4 and/or DCL3. The DCL proteins act in concert with respective DRBs, and may be competing with each other for the same dsRNA substrate. Processing of the hairpin by DCL3 gives rise to 24-nt-long RNAs, which are then stabilized by HEN1 through 2'-O-methylation of their 3' ends, and can be used in the AGO4 pathway negatively affecting the transgene activity. Processing of the hairpin by DCL4 will give rise to 21-nt-long small RNAs which are also stabilized by HEN1. DCL4-produced siRNAs are then loaded into the AGO1-containing RISC, mediating cleavage of complementary mRNAs. The 21-nt-long siRNAs have been proposed to mediate the cell-to-cell movement either alone or in a ribonucleoprotein complex moving through the plasmodesmata (P) connecting neighbouring cells. Smith et al. (2007) proposed that the 21-nt siRNAs may also be produced from dsRNA generated by *RDR2*, once the NRDP1A-related TGS (transcriptional gene silencing) pathway has been initiated through the activity of the AGO4-containing RISC. Cell-to-cell silencing spread was shown to require the activity of NRDP1a, CLSY1 and RDR2 either at the donor or/and at the recipient cell.



variability in the length of the silenced zone surrounding the infiltrated tissues should be expected. However, the length of the silenced zone surrounding the tissue where silencing is induced, is more or less a constant approx. 10–15 cells (K. Kalantidis and M. Tabler, unpublished data). This aspect of silencing spread therefore awaits additional experimentation, possibly involving chimaeric tissues.

Systemic silencing

RNA silencing in plants has the ability to spread systemically. This is not a gradual cell-to-cell spread. Instead, after silencing of a transgene is initiated in a few cells of a source leaf, a silencing signal travels along the vascular system and induces silencing in sink leaves. There, by extensive local movement, silencing spreads throughout the sink leaf tissues.

This was first shown convincingly by grafting experiments in *Nicotiana* plants. In addition, it was shown that movement of the signal could pass through tissues not expressing homologous sequences (Palauqui et al., 1997; Voinnet et al., 1998). Although not tested, it is believed that the systemic silencing signal can be generated against both transgenes and endogenes, but that since in endogenes amplification of the signal will not follow, silencing of endogenes fails to manifest outside the vascular tissues of source leaves.

Further evidence that systemic silencing spread is a separate process from local spread came from experiments using cadmium as an inhibitor of silencing spread. At non-toxic concentrations cadmium inhibited systemic, but not cell-to-cell, spread (Ueki and Citovsky, 2001).

It is widely accepted that systemic silencing serves an antiviral function, initiating the plant's silencing response in tissues the virus has not reached yet (Schwach et al., 2005). In accordance with this notion, the 2b protein of CMV (cucumber mosaic virus) suppresses silencing by blocking the systemic spread. Using grafting experiments, Guo and Ding (2002) showed that a 2b-expressing transgenic plant segment could interfere with systemic spread. In a triple grafting experiment either a wild-type or a 2b-expressing scion were grafted on top of a GUS (β -glucuronidase)-silenced rootstock. On top of this, a second-tier GUS-expressing upper scion was placed. The 2b-expressing lower scion, unlike the wild-type scion, prevented silencing appearing in the GUS-expressing upper scion. It had been shown previously that for 2b to exert its suppressor properties there is a strict requirement for nuclear localization (Lucy et al., 2000), which for years remained puzzling (Baulcombe, 2002). In contrast, it was demonstrated that 2b does not interfere with local silencing spread. In recent work, it was convincingly shown that 2b exerts its silencing suppression activity through the specific interference with the AGO1 slicing function (Zhang et al., 2006). Since AGO1 is likely the slicing component of the hypothetical RISC, this activity of the 2b protein is probably related with the antiviral role such a complex may have in the plant. There is now strong evidence for the existence and function of a RISC programmed by virus-specific siRNAs (Lakatos et al., 2006). However, it is difficult to reconcile this activity of 2b with its ability to speci-

fically suppress systemic silencing. It was proposed that suppression of systemic silencing by 2b could be explained, if this viral protein interfered with other components of the silencing machinery necessary for spread (Ruiz-Ferrer and Voinnet, 2007). This suggestion, however, awaits experimental evidence. A comprehensive list of viral silencing suppressors found to interfere with silencing movement is given in Table 1. Suppressors shown to affect silencing spread in VIGS (virus-induced gene silencing) assays were not included in this list. Since VIGS involves the systemic movement of the viral vectors to the sites of silencing suppression these assays do not distinguish between the suppression of local silencing and of systemic silencing movement.

It should be noted that systemic silencing has not been unequivocally shown in *Arabidopsis* (Dunoyer et al., 2005). This is possibly due to the life cycle of the *Arabidopsis* plant, which is a rather difficult model to study systemic processes. As a result, genes necessary for systemic spread have not been recovered from genetic screens and most of the data on systemic silencing comes from works on *Nicotiana* sp., with very little genetic evidence. The generation of *Nicotiana* plants deficient in some key genes of the silencing pathways will be useful to identify factors of the systemic signalling process. What also remains elusive is the identity of the systemic signalling molecule. It is accepted that it is an RNA molecule (Jorgensen et al., 1998), although its exact nature still remains a mystery (Mlotshwa et al., 2002; Dunoyer et al., 2005). It was shown that siRNAs were unlikely to be the systemic signal, since experiments with the HC-Pro suppressor of silencing, which sequesters all three sizes of siRNAs (Lakatos et al., 2006), were unable to block the systemic movement of the silencing signal (Mallory et al., 2003). It had been suggested that there may not be a single signal of systemic silencing, but more than one type of RNA molecules may serve as the mobile signal (Fagard and Vaucheret, 2000; Voinnet, 2005). Whatever the identity of the systemic signals, it was early on assumed to move through the phloem (Voinnet and Baulcombe, 1997; Fagard and Vaucheret, 2000; Mlotshwa et al., 2002). This was later confirmed by studies using a phloem flow tracer, which also allowed for a more detailed analysis of the specific properties of RNA spread (Tournier et al., 2006). In this study (Tournier et al., 2006), it was also conclusively shown that

Table 1 | Known suppressors of RNA silencing and their effects on silencing movement in plants

ND: not determined; PA: patch assay, as described by Lu et al. (2004).

Virus group	Virus species	Abbreviation	Suppressor	Interferes with . . .		Reference
				Short-range movement	Systemic movement	
Bromoviridae	Cucumber mosaic virus	CMV	2b	No (PA)	Yes (PA)	Guo et al. (2004); Hamilton et al. (2002); Li et al. (2002)
Bunyaviridae	Tomato spotted wilt virus	TSWV	NS _s	ND	Yes (PA)	Takeda et al. (2002)
Closteroviridae	Citrus tristeza virus	CTV	CP	No (PA)	Yes (grafts)	Lu et al. (2004)
	Citrus tristeza virus		P20	No (PA)	Yes (grafts)	Lu et al. (2004)
	Sweet potato chlorotic stunt virus	SPCSV	p22	Yes (PA)	Yes (PA)	Kreuze et al. (2005)
Comoviridae	Cowpea mosaic virus	CPMV	S CP	Yes (PA)	VIGS assay	Cañizares et al. (2004); Liu et al. (2004)
Flexiviridae	Potato virus X	PVX	p25	ND	Yes (PA)	Hamilton et al. (2002)
Geminiviridae	African cassava mosaic virus	ACMV	AC2	Yes (PA)	No (PA), yes (VIGS assay)	Voinnet et al. (1999); Hamilton et al. (2002); Himber et al. (2003); Vanitharani et al. (2004)
Hypoviridae	Cryphonectria hypovirus 1	CHV1	P29	No (PA)	Yes (PA)	Segers et al. (2006)
Orthomyxoviridae	Influenza A virus	FLUAVA	NS1*	ND	Yes, to some extent	Bucher et al. (2004); Delgadillo et al. (2004)
Potyviridae	Potato virus Y; Tobacco etch virus	PVY; TEV	HC-Pro	Yes (PA)	No (PA, grafts)	Mallory et al. (2001); Hamilton et al. (2002); Liu et al. (2004)
Reoviridae	Rice dwarf phyto-reovirus	RDV	Pns10	Yes, only sense-induced (PA)	Yes, sense-induced (PA)	Cao et al. (2005)
Sobemovirus	Rice yellow mottle virus	RYMV	P1	ND	Yes (PA)	Voinnet et al. (1999); Hamilton et al. (2002)
Tombusviridae	Pothos latent virus	PoLV	P14	Yes (data not shown)	Yes (data not shown)	Mérai et al. (2005)
	Tomato bushy stunt virus	TBSV	P19	Yes (PA)	Yes (PA)	Hamilton et al. (2002); Silhavy et al. (2002); Himber et al. (2003)
	Turnip crinkle virus	TCV	CP (P38)	Yes (<i>SUC-SUL</i> assay)	Yes (PA)	Qu et al. (2003); Thomas et al. (2003); Deleris et al. (2006)

*Recombinantly expressed in *N. benthamiana*.

the silencing signal may move from scion to root-stock, if the sink–source relationship in the plant is altered appropriately. Although the systemic silencing signal moves via the phloem, most probably as fast as the phloem flow, silencing in the sink tissues manifests only a few days later and after a certain time is independent of the signal source. This indicates that induction of silencing in the systemic tissues may require overcoming a hypothetical silencing threshold.

A comprehensive analysis of RNA molecules present in the phloem sap was carried out by Yoo et al. (2004). For their analysis they used cucurbits from which analytical quantities of phloem sap can be collected. A large population of small RNA molecules with the characteristic 3' and 5' ends of Dicer proteins are present in the sap. These include known miRNAs, but also various other endogenous siRNA species (Yoo et al., 2004). Yoo et al. (2004) further identified PSRP1 (phloem small-RNA-binding

protein 1) in the phloem sap, which was subsequently shown to bind and facilitate movement of single-stranded small RNA molecules between cells. However, an unequivocal orthologue of this gene has not been found in *Arabidopsis* or *Nicotiana* sp. and therefore its role in the systems where silencing has been studied in greater detail might not be determined.

Conclusions

In plants, it was very soon realized that silencing is a non-cell-autonomous event (Palauqui et al., 1997; Voinnet and Baulcombe, 1997; Jorgensen et al., 1998). It took longer to recognize that at least two different and likely separate mechanisms may operate, one for short-range spread and one for systemic spread (Himber et al., 2003; Kalantidis et al., 2006). As we have seen local spread can be either amplified, mainly when transgenic transcripts are targeted, or not amplified, usually, but not exclusively, when endogenous sequence transcripts are targeted. In the latter case, silencing eventually also spreads to a mere 10–15 cells, most probably through plasmodesmata (Voinnet et al., 1998). For a more extensive spread of silencing the activity of RDR6 is required, possibly for the amplification of the signal. Extensive local spread is characteristic of sink tissues, although the reasons behind this restriction have not been fully addressed. Both the short-range and extensive local spread are most probably mediated by mainly the 21-nt-long siRNA products of *DCL4* (Dunoyer et al., 2005). Under specific circumstances, potentially a threshold level that is exceeded (Schubert et al., 2004; Kalantidis et al., 2006), silencing may spread systemically through the phloem from metabolic source tissues to metabolic sink tissues (Tournier et al., 2006). The systemic silencing signal is believed to be an RNA molecule whose specificities, however, remain elusive. Silencing signals are unlikely to be the only or even the main RNA signals moving through the plant. In fact, it has been shown that various RNAs of potentially multiple functions may travel systemically (Lough and Lucas, 2006), and also RNA viruses and viroids travel systemically through the plants. Of particular interest may be the understanding of viroid systemic movement, since these subviral particles do not encode for any proteins and therefore for their biological cycle, including systemic spread, rely on host factors (Tabler and Tsagris, 2004; Ding et al.,

2005; Flores et al., 2005; Daros et al., 2006; Ding and Itaya, 2007).

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