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The Sound of Silence in Plants

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ABSTRACT

RNA silencing appears as one of the most exciting discoveries in the field of molecular biology of the last decade. Double-stranded small RNA species ranging from 20 to 26 nt, named siRNAs, were initially identified as a plant defense mechanism to target viral RNA upon infection. siRNAs can bind to a complementary RNA and induce its degradation. In addition, single stranded miRNAs have been characterized as endogenous small non-coding RNAs involved in mRNA inhibition of translation or cleavage in both plants and animals. A broad range of physiological processes are related to this class of RNAs such as control of development, defense against viral infection and cancer. RNA silencing involves several RNA processing enzymes which are well conserved between plants and animals. However plants have developed specific features of RNA silencing that will be reviewed here. Plants and *C. elegans* are the only organisms capable of producing double stranded RNA from single stranded molecules when these possess specific qualitative and quantitative features. RNA dependent RNA polymerases (RDR) are the critical enzymes for this step. A special focus will be made in this review on the recent progress in characterizing the ability of plants to send short and long distance signals from the initially silenced cell.

Keywords: RNA, silencing, systemic, phloem transport, siRNA, PTGS
Abbreviations: cRNA, complementary RNA; **DCL**, Dicer Like Enzyme; **dsRNA**, double strand RNA; **GFP**, green fluorescent protein; **RDR**, RNA dependent RNA polymerase; **siRNA**, short interfering RNA; **sRNA**, small RNA; **ssRNA**, single strand RNA; **tasiRNAs**, trans-acting siRNAs; **TGS**, transcriptional gene silencing

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INTRODUCTION

The sequence specific inhibition of gene expression, mediated by homologous RNA molecules, is known today as RNA silencing. This term involves a diverse set of mechanisms which affect mRNA either at the level of transcription, stability or translation. In every case this is accomplished by small RNA species (sRNAs) that are bound to the appropriate effector complexes and share a considerable degree of complementarity with the targeted RNA or DNA (reviewed in Brodersen and Voinnet 2006).

Back in the early 1990s, efforts in producing multicolored transgenic petunias eventuated in some cases to the opposite result. Both the transgene and the endogenous gene had become silenced (Napoli *et al.* 1990). This founding observation on RNA silencing became the trigger for many works to follow in this direction. Experiments in *C. elegans* proved that the double stranded RNA (dsRNA) was the causal agent in RNA silencing assays (Fire *et al.* 1998). One year later, the small interfering RNAs (siRNAs) were discovered and therefore the hallmark of RNA silencing was identified (Hamilton and Baulcombe 1999).

In this review, we aim to present the current information on RNA silencing mechanisms in plants and then stress their exquisite ability to produce short and long distance signals for silencing.

RNA SILENCING

It is now well accepted that RNA molecules do not only act as information carriers. RNA is involved in several biological processes such as the defense against viral invaders, regulation of endogenous gene expression, developmental control, cell signaling, transposon silencing and heterochromatin formation.

Several distinct pathways entail the formation of fully or partially complementary dsRNA. These structures are processed into small double stranded RNAs ranging from 20 to 26 nt with the aid of an RNAse-III type enzyme named Dicer (Bernstein *et al.* 2001). Dicer proteins contain dsRNA

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binding, RNA helicase, RNAseIII, DexD, Duf283 and small RNA binding (PAZ) domains. *Arabidopsis thaliana* has 4 *dicer-like* genes (dcl) whereas poplar and rice genome analysis revealed the existence of 5 and 6 respectively (Margis *et al.* 2006).

Soon after small RNA (sRNA) duplexes are diced out, they carry 5′ phosphate groups and two 3′ nucleotide overhangs (Nykanen *et al.* 2001; Chapman *et al.* 2004). Then, the duplex is unwound from the most thermodynamically unstable side and the single stranded short RNA (sshRNA) is loaded onto a multi-component protein effector complex originally termed RNA Induced Silencing Complex (RISC) (Khvorova *et al.* 2003; Schwarz *et al.* 2003). It seems possible that more than one effector complex exist depending on the nature of the sRNA they are bound to, and the composition of proteins present there. In all the cases, the core RISC protein is a member of the Argonaute (AGO) family of proteins. Ten *ago* homologues are expressed in *Arabidopsis* but so far only *ago1*, *ago4* and *ago7* have been partially characterized. AGO1 is the core protein of effector complexes that acts in the cytoplasm whereas AGO4 and AGO7 act in the nucleus (Liu *et al.* 2003; Zilberman *et al.* 2003). AGO proteins contain PAZ and Piwi domains (Lingel *et al.* 2003). The PAZ domain recognizes and binds sRNAs whereas Piwi is responsible for the endonucleolytic cleavage of targeted transcripts (Song *et al.* 2004; Qi *et al.* 2005). Other appropriate effector complexes possibly take over the translational repression of transcripts, the induction of transcript de-adenylation or the Transcriptional Gene Silencing (TGS) (Meister and Tuschl 2004; Noma *et al.* 2004; Chu and Rana 2006; Giraldez *et al.* 2006).

Sense and inverted repeat silencing

In many cases the sense overexpression of a gene or gene fragment is adequate for the induction of the silencing machinery against both the transgene and the *cis* homologous sequences. This evidently requires a dsRNA formation step. Sense RNA silencing has been subjected to mutational analysis in order to reveal the relevant endogenous plant silencing players. In such a screen, RDR6 protein was found to be indispensable as it catalyses the RNA dependent polymerization of the complementary RNA strand (cRNA) (Dalmay *et al*. 2000). Several RDR have been identified in plants, but up to now only RDR6 seems to play a role in silencing (Wassenegger and Krczal 2006).

Transcripts that possess atypical features resulting from incomplete RNA maturation are good candidates to become templates for RDR6. In fact 5′ uncapped mRNAs were turned into double stranded by RDR6 in the absence of XRN4, which normally degrades this type of messages (Gazzani *et al.* 2004). Escapers from XRN4 may become captured by RDR6 but still more atypical marks of abnormal RNA templates remain to be elucidated.

Nonetheless, the easiest way for a dsRNA molecule to be produced inside a plant cell is the transgenic introducetion and expression of inverted repeat sequences or hairpin RNA (Waterhouse *et al.* 1998). This is a common strategy used in functional genomics to target a specific RNA for degradation or for the generation of virus resistant plants (Waterhouse *et al.* 1998; Kalantidis *et al.* 2002). However, little is known about which DCL protein cleaves the hairpin RNA produced. It is assumed that all four DCLs participate (Gasciolli *et al.* 2005; Xie *et al.* 2005). The 21 and 24 nt siRNAs produced from such structures are probably generated from the action of DCL4 and DCL3 respectively. The first class guides the cleavage of mRNAs in the cytoplasm and the second mediates chromatin modifications in the nucleus that result in TGS (Hamilton *et al.* 2002; Zilberman *et al.* 2003).

Endogenous sRNAs

MicroRNAs comprise a large class of endogenous small non-coding RNAs with distinctive roles in post-transcriptional gene regulation. They measure about 22 nt and are found in all multicellular organisms and surprisingly in some viruses (Bartel 2004; Cullen 2006). In plants, the microRNA genes (MIR) reside in independent transcriptional units located between protein-coding genes (Du and Zamore 2005). They are transcribed by RNA Pol II where a stem loop structure is produced (Lee *et al.* 2004). DCl1 and HYL1 remove the flanking regions of the primary hairpin transcript and hence release an imperfect miRNA duplex (Han *et al.* 2004). HEN1 methylates the 2′ hydroxyl termini of the duplex providing protection from degradation (Yu *et al.* 2005). Then HASTY, an EXP5 homologue, is said to take over the export of either the mature or duplex miRNA into the cytoplasm (Park *et al.* 2005). Similarly to siRNAs, the mature miRNA will be bound by an AGO1 leading complex (Llave *et al.* 2002; Kasschau *et al.* 2003). This activated machinery will slice all the mRNA targets that share a high level of complementarity with the given miRNA (Ambros 2004; Bartel 2004). So far, *Arabidopsis* is predicted to have approximately 21 conserved families of miRNAs as well as 48 non-conserved in comparison with the rice and poplar genome (Fahlgren *et al.* 2007). Initially, it seemed that plant miRNAs targeted exclusively transcription factors (Palatnik *et al.* 2003; Juarez *et al.* 2004; Kidner and Martienssen 2004; Mallory *et al.* 2004). Recently, this image has been changed, as more and more evidence illustrated the role of miRNAs in hormone signaling as well as in abiotic stress (Jones-Rhoades and Bartel 2004). Auxin sensing is affected by MIR393 which targets the F-box receptors TIR-1, AFB2 and AFB3 whereas MIR160 cleaves the transcript of *auxin transcription factor* ARF16 (Wang *et al.* 2005; Navarro *et al.* 2006). Furthermore two closely related superoxide dismutases CSD1 and CSD2, recruited in plant's defense against oxidative stress, are posttranscriptionally fine-tuned by MIR398 (Sunkar *et al.* 2006).

Trans-acting siRNAs (tasiRNAs) is a newly identified class of endogenous sRNAs that has so far only been found in *Arabidopsis*. TasiRNAs biogenesis takes after a mixed si-miRNA generation model. A primary long transcript named pri-tasiRNA is targeted by a miRNA. Soon after, sequence-specific cleavage occurs, RDR6 and SGS3 polymerize the complementary RNA strand of both 5′ and 3′ cleavage products. Then, DCL4 using the cleaved end as the starting point, cleaves the dsRNA in 21 nt increments (Peragine *et al.* 2004; Vazquez *et al.* 2004; Allen *et al.* 2005). Similarly to microRNAs, tasiRNAs are methylated by HEN1 (Li *et al.* 2005). In this way mature tasiRNAs are produced which will later drive cleavage of fully or partially complementary targets. *Arabidopsis* plants lacking tasiRNAs exhibit an accelerated juvenile to adult phase transition phenotype (Gasciolli *et al.* 2005; Xie *et al.* 2005). More information remains to be revealed for the function of this form of endogenous sRNAs species.

RNA-mediated viral defense

RNA silencing was quickly given a role as a defense mechanism against invading RNA such as viruses and retrotransposons (Voinnet 2001; Lecellier and Voinnet 2004). The replication intermediates of many RNA viruses are postulated to be suitable substrates for DCL. Primarily DCL4 and then DCL2 sense the viral dsRNA and process it into 21 nt and 22 nt siRNAs, which are then bound by AGO proteins. Thus virus antagonizing RISC complexes are formed and target the invading pathogen (Xie *et al.* 2005; Deleris *et al.* 2006). Plant DNA viruses may also be targeted by RNA silencing as it is assumed that dsRNA is formed by overlapping complementary transcripts (Baulcombe 2004).

On the contrary, plant RNA and DNA viruses counteract this innate immune mechanism by expressing specific RNA silencing suppressor proteins. The most well studied suppressors are proteins p19 and p21 encoded from beet western yellow virus and tomato bushy stunt virus. Both

proteins bind to sRNAs and inhibit them from being loaded onto RISC (Chapman *et al.* 2004; Dunoyer *et al.* 2004). More than 30 viral suppressors of silencing are identified and such proteins have become an invaluable tool for the study of RNA silencing in plants (Dunoyer *et al.* 2004; Voinnet 2005). Each one of them seems to act at a different level in order to encumber the silencing process: HcPRO from tobacco etch virus inhibits local silencing by interfering with sRNA production but is inefficient to prevent the spread of systemic silencing (Chapman *et al*. 2004; Dunoyer *et al*. 2004). In contrast, the 2B protein encoded by the cucumber mosaic virus (CMV), the P25 protein from potato virus X (PVX) and the P50 protein from apple chlorotic leaf spot virus block the spread of silencing by inhibiting the movement and/or production of the silencing signal (Voinnet *et al.* 2000; Guo and Ding 2002; Yaegashi *et al.* 2007).

Sending the silencing signal

Plants have developed a special capacity to send silencing systemically outside the area where it was initiated. During a viral infection, silencing will enable the yet non-affected area of the plant to be prepared and protected from the imminent viral intrusion (Voinnet *et al.* 2000; Scholthof 2005; Voinnet 2005a; Xie and Guo 2006). A systemic signal was first identified 13 years ago (Boerjan *et al.* 1994). Tobacco plants that overexpress *S*-adenosyl-L-methionine synthetase (SAM-S) were used and it was observed, without explanation, that once silencing is initiated in lower leaves, it spreads to upper ones as well. Later, the use of graft methodology revealed that rootstocks silenced for nitrate reductase (Palauqui *et al.* 1997) or GFP (Voinnet and Baulcombe 1997) could induce silencing in a top grafted scion containing the homologous sequence. The silencing signal could even pass through a piece of stem which did not contain the homologous sequence, suggesting that during the transport the signal is not re-amplified in each cell. It was then identified as a non-cell autonomous phenomenon (Palauqui *et al.* 1997). Since those early works, intense research has been conducted in order to identify the molecule responsible for the long distance silencing spread. However, to this date, its exact nature remains a puzzling question. Two major reviews (Mlotshwa *et al.* 2002; Voinnet 2005b) have marked out the progress in understanding the plant systemic silencing signal. Here, our aim is to present the growth of information on silencing signal and comment on how recent observations have helped into better understanding the mechanism.

Short distance spread of silencing

Initiation of silencing occurs when an "aberrant" RNA is detected. It could be a double stranded RNA or a single strand one with abnormal features but in this last case, the detection mechanism is still poorly understood (**Fig. 1**) (Baulcombe 2004). Transgenic plants overexpressing a sense transgene could be divided in three classes: those with stable low expression (class I), those with unstable high transgene expression leading to spontaneous silencing (class II) and those with permanent silencing (class III) (Palauqui *et al*. 1996; Di Serio *et al*. 2001; Kalantidis *et al*. 2006). In a variable percentage of class II plants, silencing occurs without specific trigger. From initial foci corresponding to short distance spread (10 to 15 cells) silencing can then move long distances throughout the plant.

The mechanism of short distance movement is dependent on 21 nt siRNAs that most probably diffuse through

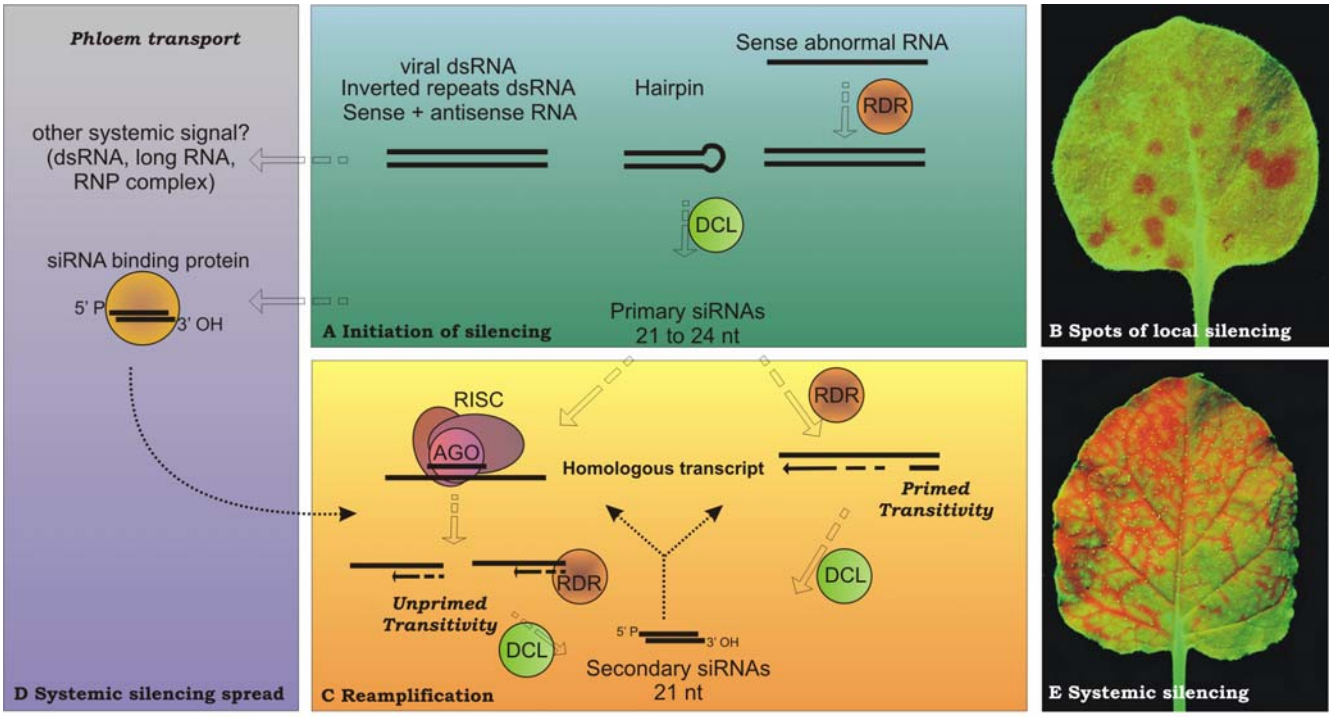

Fig. 1 RNA silencing detection and spread in plants. (**A**) Silencing is initiated once a double stranded RNA (dsRNA) of various origins is detected. Single stranded sense RNA with abnormal features could be processed by an RNA dependent RNA polymerase (RDR) that synthesizes the complementary strand. Every dsRNA is cleaved by Dicer Like Enzymes (DCL) into small primary siRNAs of 21 to 24nt length. (**B**) Initiation of silencing results in a small area of silencing as observed in this leaf of *Nicotiana benthamiana* expressing Green Fluorescent Protein (line 5.4) with stable spots of spontaneous short range silencing (SRS) (Kalantidis *et al.* 2006). (**C**) Once silencing has bypassed a certain threshold it could be re-amplified through transitivity. SiRNAs could serve as primers for RDR that will synthesize a complementary RNA (cRNA) to any homologous targets. Alternatively siRNAs are loaded into the RISC complex bound to Argonaute protein (AGO) in order to target and slice homologous transcripts. Cleavage products could serve as abnormal RNA for RDR to synthesize dsRNA. Re-amplification results in dsRNA processed by DCL enzyme into secondary siRNAs of 21nt length. (**D**) A systemic silencing signal of unclear nature is produced from initial dsRNA and travels at long distance through the phloem. It could be siRNAs associated to a siRNA binding protein, or another unidentified ribonucleic particle (RNP). In the recipient cells the translocated signal is reamplified by an RDR dependent mechanism to result in systemic silencing. (**E**) A typical phenotype of systemic silencing observed in a leaf of *N. benthamiana* expressing GFP (line 12.1) upon induction of silencing by agroinfiltration of a GFP hairpin producing construct in the lower leaves (Kalantidis *et al.* 2006).

plasmodesmata (Himber *et al.* 2003). If silencing is below a certain threshold it is diluted from cell-to-cell until it stops rendering a spotty phenotype of silencing (**Fig. 1B**) (Kalantidis *et al.* 2006) or a border of a few silenced cells ahead of the initially silenced agroinfiltrated area (Himber *et al.* 2003). Recently, Kalantidis *et al.* (2006) have reported transgenic *N. benthamiana* expressing GFP that display a stable spot phenotype without systemic spread. This illustrates indubitably that short and long distance silencing rely on different mechanisms. Interestingly, the spots required a low level of siRNAs to be maintained.

Silencing re-amplification

The short distance silencing signal can be re-amplified, if it bypasses a certain intensity threshold, in a pathway requiring RDR6 in *Arabidopsis* and *N. benthamiana* (Himber *et al.* 2003; Schwach *et al.* 2005). Once an internal transgene fragment is targeted by homologous 'primary' siRNAs, RDR6 synthesizes the cRNA and secondary siRNAs will be produced from the 5′ and 3′ *cis* flanking regions of the full transcript (Voinnet *et al.* 2000; Vastij *et al.* 2002). The secondary siRNAs are exclusively 21nt and are probably produced by the DCL4 (Deleris *et al.* 2006). Secondary siRNAs are produced from a broader region than the primary ones, a mechanism that is named transitivity (Vaistij *et al.* 2002). A first model was raised in which RDR6 uses siRNAs as primers to initiate a 3′ to 5′ transitivity by homology to *C. elegans* model (Vaistij *et al.* 2002). However, results are often contradictory from one team to another, as several works identified transitivity in both directions while others observed only 5′ to 3′ transitivity (Vaistij *et al.* 2002; van Houdt *et al.* 2003; Garcia-Perez *et al.* 2004; Shimamura *et al.* 2007). This 5′ to 3′ spread of silencing along the RNA target is dependent on a second model for which RDR6 action relies on the unprimed synthesis of cRNA (Shimamura *et al.* 2007). To our knowledge, there is no hypothesis to explain how, in this case, RDR6 selects a specific target to synthesize its complementary RNA. RDR6 is a critical enzyme for sense mediated silencing but is dispensable for hairpin or dsRNA mediated silencing (Dalmay *et al.* 2000; Mourrain *et al.* 2000). If local silencing bypasses a certain threshold, then systemic spread to distant parts of the plant is initiated (Himber *et al.* 2003; Garcia-Perez *et al.* 2004; Kalantidis *et al.* 2006). In cells receiving the long distance systemic signal, RDR6 is required to re-amplify the signal. This was shown by the rdr6 mutants whose capacity to perceive a systemic trigger was lost (Himber *et al.* 2003; Schwach *et al.* 2005).

Long distance spread of silencing

The pattern of long distance spread of silencing is reminiscent of viral spread through the phloem (Voinnet and Baulcombe 1997; Voinnet *et al.* 1998). It was hypothesized that the systemic signal is phloem transported. However, there was some inconsistent data: in grafted plants virus can spread from scion to stock via phloem flow moving from upper source leaves to lower sink roots (Hull 2002). Nevertheless, in grafts between silenced and non-silenced plants, the spreading of silencing signal from scion to rootstock was reported to be impossible (Palauqui *et al.* 1997; Voinnet and Baulcombe 1997) or very inefficient (Sonoda and Nishiguchi 2000). All together these works showed that systemic silencing spread to scion appeared in stochastic manners in only few scion leaves and only in a given percentage of the grafts carried out. We recently resolved those inconsistencies by marking the phloem flow with radiolabeled phosphate $^{32}P_i$ in GFP plants undergoing systemic silencing (Tournier *et al.* 2006). We have shown that silencing spread into a systemic leaf is correlated to phloem input from the area where silencing is initiated. The grafts, in any direction, that do not transmit silencing were those that could not establish a phloem connection from the silenced area toward the GFP expressing ones. We showed that it is possible to stimulate the establishment of phloem and silencing flow in any direction by pruning the older leaves in the sink area. Under those conditions, silencing could be efficiently transmitted to rootstock as well. Collectively, those data showed that the long distance silencing signal is univocally transported through the phloem (Tournier *et al.* 2006).

Another surprising result is the competence of different transgenes to send out the silencing signal. The inverted repeat silenced rootstock or the sense expressing class II line (autonomously silenced) showed the strongest potential to send silencing (Crete *et al.* 2001; Mallory *et al.* 2003). Inversely, antisense silenced rootstock or amplicon silenced ones were partially or fully inefficient in sending silencing under normal conditions though they are also silenced and produce similar quantities of siRNAs (Crete *et al.* 2001; Mallory *et al.* 2003; Kalantidis 2004). However, recent reports have begin to shed light on this issue when they observed that like sense transgenes, antisense ones are able to send systemic signal though they require longer time (Shaharuddin *et al.* 2006). They hypothesized that the molecular mechanisms in sense or antisense silencing are the same, but with lower intensity of systemic trigger production in antisense loci. The more difficult detection of antisense systemic silencing was overcome in this last paper by using tomato plant overexpressing ACO1 as a signal sensor scion. The specificities of silencing transmission may be dependent on plant architecture and development. Tomato plants have a longer life and a different capacity to cicatrize graft junction than *N. benthamiana*, which could result in better detection of low signal transmission.

Over the past years, the mechanism of long silencing transport has been intensely studied, but how much progress has been made in understanding the molecular nature of the silencing signal? On the one hand, siRNAs immediately appeared to be good candidates as the systemic agent of silencing since they are small enough to be easily transported via plasmodesmata and long enough to be sequence specific. SiRNAs were cloned from phloem sap confirming that they could be uploaded in sieves elements (Yoo *et al.* 2004). On the other hand, strong evidence was raised against this initial hypothesis. Indeed, when HC-pro from tobacco etch virus was expressed in plants it suppressed silencing. SiRNAs generated from sense co-suppression or hairpin disappear but not the systemic silencing (Mallory *et al.* 2001, 2002). Similar results were again obtained when the silencing inhibition potential of protein P1, a partner of HC-pro, was tested (Valli *et al.* 2006). Amplicon silenced lines are normally unable to trigger systemic silencing. However, upon HC-pro action local silencing is suppressed and systemic silencing is induced (Mallory *et al.* 2003). Other recent work noticed a poor correlation between siRNA level and the capacity for a systemic silencing signal to be sent: when agroinfiltrating a Virp1 (viroid binding protein 1) hairpin in a *N. benthamiana* VIRP1 overexpressing line, Koscianska *et al.* (2005) observed high siRNA levels in the treated leaves but no systemic silencing. In another report, ACO1 siRNAs were detected in tomato lines that stably overexpressed ACO1 mRNA (Shaharuddin *et al.* 2006). These non-silenced plants were, however, receptive to systemic silencing spread since they were easily silenced upon grafting onto a silenced rootstock. The authors hypothesized that siRNAs may not be the systemic signal because otherwise silencing would start in those plants independently of grafting (Shaharuddin *et al.* 2006).

A single report, but of critical importance, identified a molecule, other than siRNAs, with high silencing potential (Boutla *et al.* 2002). It was shown that an 80nt RNA species extracted from GFP silenced plants was more efficient in inducing silencing than siRNAs themselves when injected in GFP expressing *C. elegans.* The exact nature of the signal was not identified, probably because it is extremely rare.

It has to be pointed out that almost any foreign nucleic acid introduced ectopically induces silencing of homol-

ogous targets. It can be single stranded or double strand RNA or promoterless DNA, and certainly siRNAs (Voinnet *et al.* 1998; Palauqui and Balzergue 1999; Hewezi *et al.* 2005). This high sensitivity of tissue to react to foreign nucleic acids has led to confusing results: siRNA may not be the bona-fide systemic trigger or at least not the single one. It is conceivable that several RNA species work together.

CONCLUSION

Over the past years and despite intensive research, the identity of the plant systemic silencing trigger has remained elusive. Even if an unquestionable role is played by siRNAs, they might not be the only or main molecular signal. Lessons from characterization of macromolecules transport in the phloem raised the existence of traveling ribonuclear particles (RNP) containing endogenous, viral or viroid RNAs (Tabler and Tsagris 2004; Scholthof 2005; Lough and Lucas 2006). Recently, several general RNA binding proteins have been cloned from phloem sap (Ruiz-Medrano *et al.* 2004; Gomez *et al.* 2005; Giavalisco *et al.* 2006) and noteworthy siRNAs were found in *Cucurbita* species phloem as well as a small RNA binding protein (CmPSRP1) (Yoo *et al.* 2004). Rarely are RNAs found naked in the cell and any future attempt to identify the systemic silencing signal should probably seek for an RNP rather than for a single RNA molecule.

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