

Comparative analysis of non-autonomous effects of tasiRNAs and miRNAs in *Arabidopsis thaliana*

Felipe Fenselau de Felippes, Felix Ott and Detlef Weigel*

Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany

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ABSTRACT

In plants, small interfering RNAs (siRNAs) can trigger a silencing signal that may spread within a tissue to adjacent cells or even systemically to other organs. Movement of the signal is initially limited to a few cells, but in some cases the signal can be amplified and travel over larger distances. How far silencing initiated by other classes of plant small RNAs (sRNAs) than siRNAs can extend has been less clear. Using a system based on the silencing of the *CH42* gene, we have tracked the mobility of silencing signals initiated in phloem companion cells by artificial microRNAs (miRNA) and *trans*-acting siRNA (tasiRNA) that have the same primary sequence. In this system, both the ta-siRNA and the miRNA act at a distance. Non-autonomous effects of the miRNA can be triggered by several different miRNA precursors deployed as backbones. While the tasiRNA also acts non-autonomously, it has a much greater range than the miRNA or hairpin-derived siRNAs directed against *CH42*, indicating that biogenesis can determine the non-autonomous effects of sRNAs. In agreement with this hypothesis, the silencing signals initiated by different sRNAs differ in their genetic requirements.

INTRODUCTION

Plants produce a variety of small RNAs (sRNAs), including microRNAs (miRNAs), small interfering RNAs (siRNAs) and *trans*-acting siRNAs (tasiRNAs), to regulate many different processes, such as development, stress and nutritional responses, chromatin structure and pathogen defense (1–5). A common theme in sRNA biogenesis is the processing of a double stranded RNA (dsRNA) by DICER-LIKE (DCL) enzymes into 21–24 nt long molecules. The sRNAs are then loaded onto one of several ARGONAUTE (AGO) proteins that drive transcriptional or post-transcriptional gene silencing (3,6–9).

SiRNAs are produced from perfectly-paired dsRNAs with endogenous (transposons, repetitive sequences) or exogenous (virus, transgenes) origins (3,7,8), while miRNAs originate from endogenous transcripts that include an imperfect foldback. Different from the other classes of sRNAs, a miRNA precursor often spawns just one functional sRNA. MiRNAs can trigger cleavage of target transcripts, or interfere with their translation (9). In the case of *TAS* targets, miRNA-initiated cleavage primes the synthesis of dsRNA by RNA DEPENDENT RNA POLYMERASE 6 (RDR6) and SUPPRESSOR OF GENE SILENCING 3 (SGS3), followed by DCL4-dependent processing of the dsRNA into 21 nt long tasiRNAs (10–15).

An important property of plant siRNAs is their non-cell autonomous activity. Even before the association of gene silencing with sRNAs was recognized, it became clear that co-suppression and post-transcriptional gene silencing (PGTS) could spread from one part of the plant to the other (16–18). Systemic silencing is transmitted via the phloem and it is dependent on RDR6 for amplification and reception of the silencing signal in other tissues (19–23). Silencing triggered by siRNAs likely moves from one cell to the other via plasmodesmata, channels that connect the cytoplasm of adjacent cells (16,20,23,24). In a first step, duplexes of 21 nt long siRNAs produced by DCL4 move 10 to 15 cells from their production site (24–26). In some cases, the primary silencing signal can spread further, relying on an RDR6- and SILENCING DEFECTIVE 3 (SDE3)-dependent amplification mechanism that supports the production of secondary siRNAs (24). Although amplification of the silencing signals is preferentially triggered by foreign RNAs, such as those derived from transgenes or from viruses (22,24), there are endogenous hairpin loci that behave very similarly (27). Furthermore, additional factors required for cell-to-cell movement of siRNA-triggered silencing include RDR2, the NRPD1a subunit of RNA polymerase IVa and CLASSY1, a SNF2 domain-containing protein (25,28,29). Grafting and deep sequencing of small RNA pools have revealed that endogenous 24 nt siRNAs can travel long distances in the plant (27,30).

*To whom correspondence should be addressed. Tel: +49 7071 601 1411; Fax: +49 7071 601 1412; Email: weigel@weigelworld.org

While the mobility of siRNAs and its consequences are well documented, less is known about the mechanisms underlying non-autonomous effects of other classes of sRNAs, such as miRNAs and tasiRNAs. Several experiments with miRNA sensors and tissue-specific expression of natural or artificial miRNAs have indicated that the non-autonomous effects of most miRNAs do not extend very far (31–36). There are, however, several notable exceptions. MiR399 acts as a long distance signal in phosphate homeostasis (37), while miR390 accumulates in different tissues than its precursor (38). In addition, miRNAs have been detected in the phloem sap of several species (39,40). Since the phloem cells are enucleate and cannot produce RNAs, such miRNAs would need to be delivered from other cells such as phloem companion cells. Similarly, several strong lines of evidence indicate that miR165 and miR166 can move radially within the root, and thereby contribute to the patterning of root tissues (36). Finally, the precursor of tasiRNAs that regulate *AUXIN RESPONSE FACTOR3* (*ARF3*) is transcribed in a narrow domain at the adaxial side of the leaf, but the mature tasiRNAs accumulate in a gradient that extends through much of the leaf (41,42).

While mobility of a variety of small RNAs is now accepted, their non-autonomous effects appear to differ. For example, movement of miRNAs appears to be much more limited than that of siRNAs (31–36). Because the investigated sRNAs differed in sequence in previous work, it has been difficult to disentangle the effects of sRNA sequence from the consequences of different sRNA histories due to divergent biogenesis mechanisms. We have compared sRNAs of identical sequence, but generated by either the miRNA or tasiRNA pathway. We show that similar to siRNAs, the silencing effects of miRNA can spread 10 to 15 cells from phloem companion cells to mesophyll cells, while a tasiRNA of the same sequence has much more far-reaching non-autonomous effects. Importantly, the genetic requirements for the mobile silencing signals triggered by miRNAs, tasiRNAs and siRNAs differ.

MATERIALS AND METHODS

Plant material

Arabidopsis thaliana Columbia (Col-0) is referred to as wild type. The *dcl2 dcl3 dcl4* (*dcl234*'), *dcl1-100*, *rdr6-15*, *rdr2-1* and *npr1a-3* mutants and the *SUC2:3xYFP* transgenic lines have been previously described (43–47). Mutants expressing atasiR-SUL, amiR-SUL and siR-SUL were selected from F₂ plants by PCR-based genotyping for the transgene and the mutations. F₁ hybrids containing both *SUC2:amiR-SUL* and *SUC2:3xYFP* were isolated by double antibiotic selection.

Transgenic lines

The sRNA targeting the *SUL* homolog *CH42* (At4g18480), UUAAGUGUCACGGAAAUCCCU, was designed with the WMD tool (33,48). Overlapping PCR was used to replace the mature miRNA and miRNA* in the MIR319a (AT4G23713), MIR156c (AT4G31877),

MIR164b (AT2G47585) and MIR167a (AT3G22886) backbones. The same approach was used to generate the atasiR-SUL constructs, by replacing siR255 in the three members of the *TASI* family, *TASIa* (AT2G27400), *TASIb* (AT1G50055) and *TASIc* (AT2G39675), respectively (48). For the siR-SUL construct, we cloned the same *CH42* fragment (TAIR9 coordinates chromosome 4, 10, 202, 162–10, 202, 350) in both sense and antisense orientation into the pHANNIBAL vector (49). All constructs were shuttled into a modified version of the pGreen vector (50) containing the *CaMV35S* and *SUC2* promoters (51–53). Binary constructs (Supplementary Table S3) were introduced into *Agrobacterium tumefaciens* strain ASE (54), which was used for floral dip transformation (55) (see Supplementary Data for additional details).

RNA analysis

Total RNA was isolated from two-week old plants using TRIZOL (Invitrogen, Carlsbad, CA, USA). sRNA blots were prepared by resolving 10–20 µg of total RNA on a 17% PAGE gel under denaturing conditions (7 M urea) and subsequent transfer to a positively charged nylon membrane. Membranes were hybridized with DNA oligonucleotide probes that had been radioactively labeled with γ -³²P-ATP and OptiKinase™ (USB, Cleveland, OH, USA). For detection of sRNAs derived from the siR-SUL construct, we employed a DNA probe consisting of the *CH42* fragment in the RNAi triggering vector, which was labeled with α -³²P-dCTP using the Prime-a-genes kit (Promega, Madison, WI, USA). cDNA for RT-PCR was synthesized with the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Canada). See Supplementary Table S4 for probes.

Small RNA sequencing

Small RNA libraries were constructed following a protocol described elsewhere (56) with modifications (Supplementary Data) and sequenced on the Illumina GAI platform (San Diego, CA, USA). Two independent libraries (biological replicates) were analyzed for the amiR-SUL and atasiR-SUL lines. The sRNA sequence tags were filtered and mapped back to the *A. thaliana* reference genome using SHORE (57), yielding 5.7–6.5 million aligned sRNA tags. We then calculated coverage graphs allowing or disallowing up to two mismatches to the *CH42* locus. The effect of excluding repetitive matches was investigated, but found to be negligible (data not shown). We tested the significance of the secondary sRNA population observed in the *SUC2:atasi-SUL* line as follows. First, we defined a 500-bp region for the *CH42* locus where secondary sRNAs were highly increased (Chr4:10201701.10202200, excluding the amiR/atasi-SUL region). We then determined the total number of reads for this region in both samples, which were 99 in *SUC2:atasiR-SUL* and 22 in *SUC2:amiR-SUL*. Next, starting from this region, we divided the genome in both directions, in 500-bp bins, counted the total sRNA reads in the two lines and calculated the fold change for each bin with more than 60 reads across both lines (50% of that in

the *CH42* bin). To avoid division by zero, we added a pseudo count of one to each bin.

Microscopy

YFP expression and natural fluorescence of chlorophyll were analyzed with a Leica MZ FLIII microscope (Leica Microsystems, Wetzlar, Germany) fitted with wide- and band-pass YFP filters and an AxioCam HRc (Zeiss, Jena, Germany) digital camera with Zeiss AxioVision software version 3.1.

RESULTS

Non-autonomous effects of miRNAs

To investigate movement of a silencing signal, we employed sRNAs targeting *CHLORINA42* (*CH42*), the *A. thaliana* homolog of tobacco *SULPHUR* (*SUL*). Inactivation of *CH42* causes bleaching of green plant tissues (58), resulting in an easily-scorable phenotype. We targeted *CH42* with an artificial miRNA, amiR-SUL (33). We compared the effects of amiR-SUL with those of siRNAs spawned from a transcribed inverted repeat of *CH42* sequences (siR-SUL) (49). Both constructs were introduced into *A. thaliana* plants under the control of the *SUCROSE-PROTON SYMPORTER 2* (*SUC2*) promoter, which confers strong expression in phloem companion cells (51,52).

Himber and colleagues (24) have shown that the effects of siRNAs can extend 10 to 15 cells from their production site. Consistent with this report, there was prominent bleaching of green mesophyll cells along the leaf veins in *SUC2:siR-SUL* plants (Figure 1A). A very similar phenotype was seen in *SUC2:amiR-SUL* plants, suggesting that silencing initiated by miRNAs spreads over a range comparable of that of siRNAs. Closer analysis of chlorophyll autofluorescence in *SUC2:amiR-SUL* plants confirmed that the bleached regions extended beyond the veins (Figure 1B). To determine directly how far the silencing had spread beyond the cells in which the *SUC2* promoter is active, we crossed *SUC2:amiR-SUL* to a plant expressing three tandem copies of yellow fluorescent protein in the *SUC2* domain (*SUC2:3xYFP*) (43). The large size of 3xYFP traps it inside cells, allowing precise localization of *SUC2* promoter activity. The bleached area around the veins in *SUC2:amiR-SUL* was indeed much larger than the *SUC2* expression domain (Figure 1C). Most plants carrying the *SUC2:amiR-SUL* construct presented different degrees of bleaching around the vascular tissue (Figure 1D, Supplementary Figure S1). The levels of amiR-SUL were positively correlated with the severity of bleaching (Figure 1E), but the extent of the bleached area around the vasculature was similar in all lines.

While miRNAs are produced mainly through the action of DCL1, several factors, such as secondary structure of the pre-miRNA and the tissue where the miRNA is expressed, can lead to miRNA precursors being processed by different DCLs, resulting in the production of siRNAs instead (59–61). Therefore, the non-autonomous effects in *SUC2:amiR-SUL* plants might be not caused by true miRNA-mediated silencing, but through

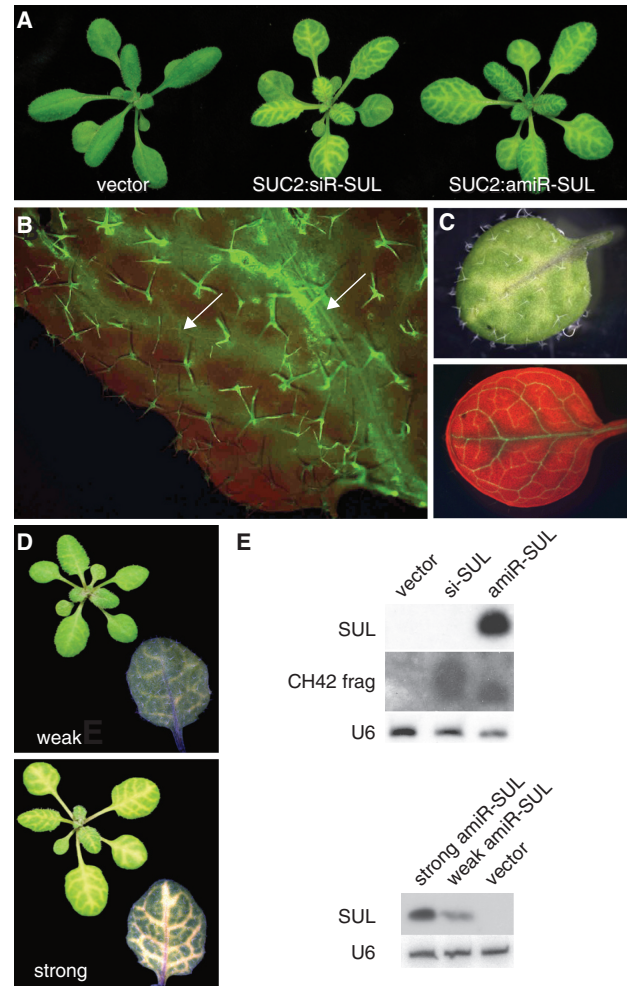


Figure 1. Spreading of miRNA-triggered silencing from phloem companion cells. (A) *SUC2:amiR-SUL* and *SUC2:siR-SUL* plants present similar bleaching patterns. (B) UV-induced red chlorophyll autofluorescence is suppressed in bleached areas, which appear light green in a *SUC2:amiR-SUL* leaf. Arrows point to leaf veins. (C) *SUC2:amiR-SUL SUC2:3xYFP* leaf. Top, visible light; bottom, UV fluorescence. Bright green YFP signal is more restricted than the bleached areas that are dark. (D) Comparison of mild and severely bleached plants. A single leaf is shown in detail. (E) sRNA blots probed with an oligonucleotide specific for amiR-SUL (SUL) or a *CH42* fragment (CH42 frag). U6 was used as loading control.

siRNAs. To examine this possibility, we transformed *dcl234* triple mutants with the *SUC2:amiR-SUL* construct. Inactivation of *DCL2*, *DCL3* and *DCL4* did not affect the bleaching phenotype (Figure 2A). As a control, we introduced the *SUC2:amiR-SUL* construct into *dcl1* plants by crossing. In these plants, no bleaching occurred (Supplementary Figure S2).

In some cases, miRNA-triggered cleavage of targets can initiate transitive action of the miRNA, in which the cleaved target transcript is converted to dsRNA by RDR6 and subsequently processed into secondary siRNAs by DCLs (31,62). To test whether the cell-autonomous effect of amiR-SUL was due to transitivity, we crossed *SUC2:amiR-SUL* to *rdr6-15* mutants, which do not generate secondary siRNAs. The *SUC2:amiR-*

SUL-induced bleaching phenotype was unaffected by the *rdr6* mutation (Figure 2B). The presence of the *rdr6* mutation was confirmed both by the gross phenotype, and by the absence of tasiR255 production (Figure 2B and C). Taken together, these results suggest that the mobile silencing triggered by the *SUC2:amiR-SUL* is due to bona fide miRNA action.

A potential concern when using transgenes to characterize an endogenous mechanism is that expression levels much higher than those seen for endogenous miRNAs contribute to the observed effects. While commonly employed for assays of non-autonomous action of proteins or sRNAs (25,27,28,35,43,63), the *SUC2* promoter is known to be strong (52,64,65). Abnormally high expression of a miRNA under control of the *SUC2* promoter might saturate the processing machinery. This could in turn result in miRNA processing through pathways that are not DCL1 dependent. We therefore compared the expression levels of amiR-SUL to endogenous miRNAs by deep sequencing of the sRNA population. As reported in Supplementary Table S1, many miRNAs were expressed more strongly than amiR-SUL, with steady-state levels of some being more than two orders of magnitude higher. We conclude that our system reflects the natural action of the sRNA machinery.

Non-autonomous miRNA effects are not precursor specific

In phloem sap of the *A. thaliana* relative *Brassica napus*, a distinct subset of plant miRNAs has been identified (39,40), raising the possibility that only certain miRNA precursors can produce miRNAs that leave the cell of origin. To test the effects of the precursor, if any, on

non-autonomous effects of the mature miRNA, we produced amiR-SUL from different miRNA precursors. We engineered the MIR156c, MIR164b and MIR167a precursors to produce the same mature miRNA sequence as our original amiR-SUL construct, which was in the MIR319a backbone; the corresponding constructs were named amiR-SUL_156, amiR-SUL_164 and amiR-SUL_167 (Supplementary Figure S3). We chose miR156, because it represents one of the families found in phloem sap (39,40). We chose MIR164 and MIR167, because it has been suggested that amiRNAs produced from these backbones in phloem companion cells do not have non-autonomous effects (35).

To determine the efficiency of amiRNA production from the different precursors, we first expressed these from the CaMV 35S promoter (53). Like plants that expressed amiR-SUL ubiquitously from the MIR319a precursor, *35S:amiR-SUL_156* and *35S:amiR-SUL_167* plants were very small and strongly bleached, like the original *35S:SUC2:amiR-SUL* lines (Figure 3A). *35S:amiR-SUL_164* plants were larger, with variable bleaching, flowered normally and were fertile (Figure 3A). RNA blots indicated that amiR-SUL was only very inefficiently processed from the MIR164 precursor, even though it was expressed at a similar level as the other precursors (Figure 3B and C).

Similarly, *SUC2:amiR-SUL_156* and *SUC2:amiR-SUL_167* plants were strongly bleached, like the original *SUC2:amiR-SUL* lines (Figure 3A), but *SUC2:amiR-SUL_164* plants were largely normal. While our results suggest that there are no specific miRNA precursor requirements for non-autonomous miRNA effects, the absence of extensive bleaching in *SUC2:amiR-SUL_164* plants, apparently due to inefficient miRNA processing,

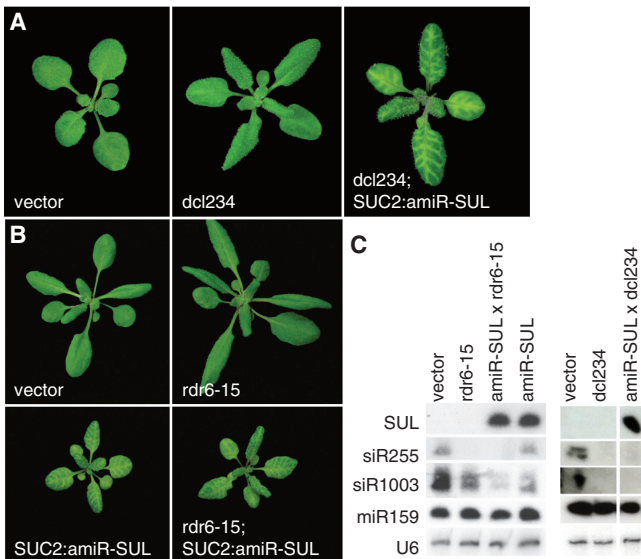


Figure 2. Confirmation of amiR-SUL-triggered silencing. (A) amiR-SUL production in *dcl234* triple mutant background. (B) RDR6-independent spreading of amiR-SUL-triggered silencing. (C) sRNA blots. Probes are indicated on the right. siR255 production is RDR6 and DCL4-dependent, siR1003 is DCL3-dependent but RDR6-independent. MiR159 was used as an additional control. Note characteristic leaf shape of *rdr6* and *dcl234* mutants in (A) and (B).

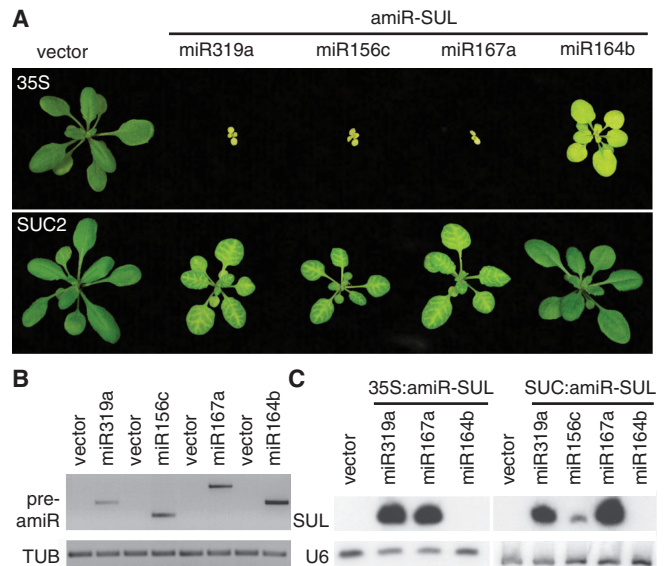


Figure 3. Effect of *MIRNA* backbone on spreading of the silencing signal. (A) Whole-rosette phenotypes of plants expressing amiR-SUL from different precursors, with promoters indicated on the left. (B) Precursor expression monitored by RT-PCR with β -*TUBULIN*-2 (*TUB*) as control. (C) sRNA blots.

indicates that expression levels are important in determining non-autonomous effects, consistent with non-selective movement of the silencing signal, similar to what appears to be the default for many proteins (66,67).

Non-autonomous effects of tasiRNAs

TasiRNAs, which like miRNAs are normally 21 nt long, are generated from *TAS* precursor transcripts. The phase of tasiRNA formation is determined by the miRNA cleavage event that triggers tasiRNA formation (12). This feature allows the design of artificial tasiRNAs (ataiRNAs) with specific sequences (68–70). We have previously developed a *TAS1a* derivative that produces an atasiRNA, atasiR-SUL_1a, with the same sequence as our amiR-SUL (48), therefore allowing a direct comparison of both sRNAs. The *TAS1b* and *TAS1c* derivatives atasiR-SUL_1b and atasiR-SUL_1c also produce the same sRNA.

SUC2:ataiR-SUL transgenic plants were much more severely affected than *SUC2:amiR-SUL* plants. In the most extreme cases, the phenotype of *SUC2:ataiR-SUL* plants began to approach that of *35S:ataiR-SUL* plants, with pervasive bleaching throughout the entire leaf and much reduced stature (Figure 4A and Supplementary Figure S4). In weaker lines, which were more intensely bleached around the veins than in the remainder of the leaf, the boundary between affected and unaffected tissue was nevertheless much more diffuse than in *SUC2:amiR-SUL* plants (Figure 4A). The phenotypic differences between *SUC2:ataiR-SUL* and *SUC2:amiR-SUL* plants suggest that the biogenesis pathway of an sRNA, rather than its expression levels, has a major effect on its non-autonomous activity. This hypothesis was corroborated by a direct comparison of mature sRNA accumulation in the *SUC2:ataiR-SUL* and *SUC2:amiR-SUL* lines, with the first having much lower levels (Figure 6B).

siRNA-triggered silencing can spread across long distances, by means of an RDR6-dependent amplification mechanism termed transitivity (24). Unfortunately, one cannot test directly RDR6-dependence of non-cell autonomous tasiRNA effects, because tasiRNA generation itself requires RDR6 (10,11). We therefore designed an atasiR-SUL in which the two or three last nucleotides, respectively, do not pair with the target transcript (ataiR-SUL_2mm and atasiR-SUL_3mm) (Supplementary Data), based on a proposal (62) that this reduces 3' 5' transitivity, which depends on priming by the sRNA.

Both *SUC2:ataiR-SUL_2mm* and *SUC2:ataiR-SUL_3mm* plants suffered from the same widespread bleaching as the original *SUC2:ataiR-SUL* plants (Supplementary Figure S5). This experiment, however, does not address the possibility of 5' 3' or priming-independent transitivity. We therefore sequenced the 19 to 25 nt sRNA population around the *CH42* locus in these transgenic lines (Figure 5A). We found very few novel sRNAs matching the *CH42* locus in *SUC2:siR-SUL* and *SUC2:amiR-SUL* plants that did not correspond to the sRNAs generated from the triggering transgene (Figure 5B and Supplementary Figure S6). Although the most abundant species was still the original atasiR-SUL trigger, the level of novel sRNAs was more than four times higher in *SUC2:ataiR-SUL* compared to *SUC2:amiR-SUL* plants (Supplementary Table S2). To confirm that this variation was not fortuitous, we compared the ratios of sRNAs reads between these two lines for different regions of the genome. We identified 1971 bins of length 500 bp in which the sum of the read counts for both samples was at least 50% of the read count for the *CH42* bin. Only 60 bins (3%) had a read ratio between the two lines equal or higher than half of the ratio at the *CH42* locus, and only 14 (0.7%) had a similar or higher ratio (Supplementary Figure S7). This comparison suggested that the increase in secondary sRNAs in the *SUC2:ataiR-SUL* line is indeed significant and that transitivity has potentially a role in tasiRNA spreading.

Genetic requirements for non-autonomous effects of different sRNAs

Mutations in several genes, including *RDR2*, *NRPD1a* and *CLASSY* compromise the non-autonomous effects of sRNAs (28,29). To determine whether the different classes of sRNAs rely on the same genetic system for spreading of the silencing signal, we crossed siR-SUL, amiR-SUL and atasiR-SUL producing lines to *rdr2-1* and *nrdp1a-3* mutants. As expected from previous work (28,29), the bleaching in *SUC2:siR-SUL* plants was completely suppressed in both mutant backgrounds. In contrast, bleaching triggered by *SUC2:amiR-SUL* and *SUC2:ataiR-SUL* was not affected in these mutants (Figure 6A), indicating that the non-autonomous effects of the different sRNAs have differential genetic requirements.



Figure 4. Non-autonomous effects of atasiR-SUL. (A) Whole-rosette phenotype of *SUC2:ataiR-SUL_1c*. (B) sRNA blots. MiR173, which triggers *TASI* processing, was used as an additional control.

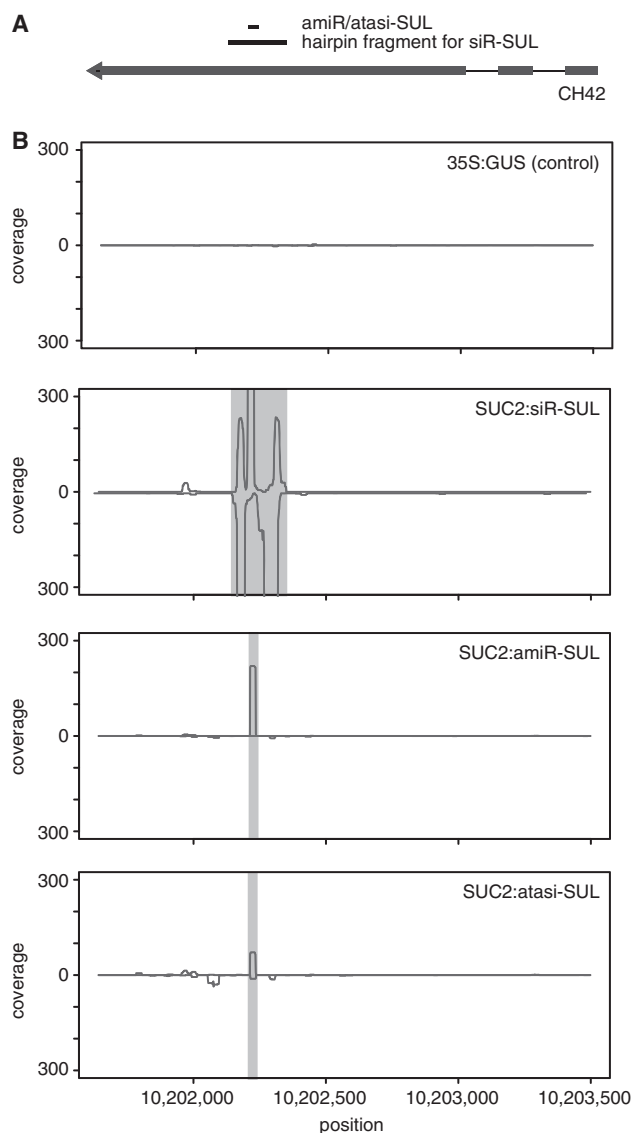


Figure 5. Secondary sRNAs at the *CH42* locus. (A) Diagram of *CH42* locus. Exons are indicated as thick lines. Regions targeted by primary sRNAs from siR-SUL and amiR/ataSiR-SUL transgenes are shown. (B) Small RNA populations at the *CH42* locus. About 19–26 nt sRNAs, with a maximum of two mismatches (as in amiR/ataSiR-SUL), are shown. See Supplementary Figure S6 for perfect-match sRNAs only. Grey regions indicate origin of primary siR-SUL and amiR/ataSiR-SUL, respectively.

DISCUSSION

Here, we have documented that the silencing effects of an amiRNA, amiR-SUL, can extend 10 to 15 cells from the site of its production in phloem companion cells, which is in the same range as observed for transgene-derived siRNAs. In contrast to siRNAs (25,28,29), the non-autonomous effects of amiR-SUL do not depend on RDR2 and NRPD1a. While the specific precursor from which the amiRNA is processed does not seem to be essential for non-autonomous silencing, the biogenesis pathway through which a 21 nt sRNA is generated plays a crucial role, since the silencing effects of an

ataSiR-SUL of identical sequence as amiR-SUL extend much further.

Recently, Tretter *et al.* (35) examined non-autonomous effects of sRNAs, using sRNAs targeting *PHYTOENE DESATURASE (PDS)*, downregulation of which produces a similar phenotype as *CH42/SUL* knockdown. They reported that expression of siRNAs, but not amiRNAs, under indirect control of the *SUC2* promoter via the LhG4 transactivator (71), resulted in bleaching beyond the veins. One reason for apparent failure to detect amiRNA non-autonomy could be relatively low sRNA expression levels due to the LhG4 system, which is known to suffer from variable efficacy (71,72). Such a scenario is in line with our finding that efficiency of amiRNA processing affects the detection of non-autonomous effects, similar to what has been reported before for siRNAs (25,29). In support of this explanation, Tretter *et al.* (35) did observe non-autonomous effects after simultaneous expression of two amiRNAs under direct control of the *SUC2* promoter, which caused a very similar phenotype as seen in the majority of our *SUC2:amiR-SUL* lines.

Perhaps our most intriguing finding is that amiR-SUL and atasiR-SUL, despite having identical sequences, caused distinct silencing phenotypes in our system. Which factors could be responsible for defining how far sRNA-triggered silencing spreads? The most obvious difference is the pathway that generates the sRNA. Mallory *et al.* (73) have reported a case in which siRNAs produced from inverted repeats could spread systemically, while siRNAs for the same target, but derived from viral amplicons, were not able to move. It is likely that different DCLs and co-factors, which load AGO-containing RNA induced silencing complexes (RISCs), or AGOs and their co-factors, play a major role in defining the range of the silencing signal (9). This is at least indirectly supported by the different genetic requirements for miRNA- and tasiRNA-triggered silencing signals in our system.

Given several recent reports in which production and effect of sRNAs were directly examined (27,30), it seems likely that the mobile signal is the triggering sRNA itself. Differences in biogenesis could impact the production of secondary sRNAs, which in turn can mediate mobility of the silencing signal (24). We have detected more secondary sRNAs in tasiR-SUL than in amiR-SUL or siR-SUL expressing plants, showing that limited transitivity might contribute to the spreading of tasiRNA silencing.

In contrast to siR-SUL, transmission of the silencing signal triggered by an amiRNA or an atasiRNA does not rely on RDR2 and NRPD1a. Genetic screens using two different trigger loci have previously identified these two factors as being required for movement of siRNA-silencing signals (25,28,29). Some observations (28) indicate that RDR2 and NRPD1a act downstream of siRNA production, either by supporting the translocation of the silencing signal or its reception in other cells. Smith and colleagues (29), on the other hand, suggested that both proteins are involved in the amplification and/or generation of the signal. In any case, that the non-autonomous effects of amiR-SUL and atasiR-SUL are insensitive to loss of RDR2 or NRPD1a shows that not

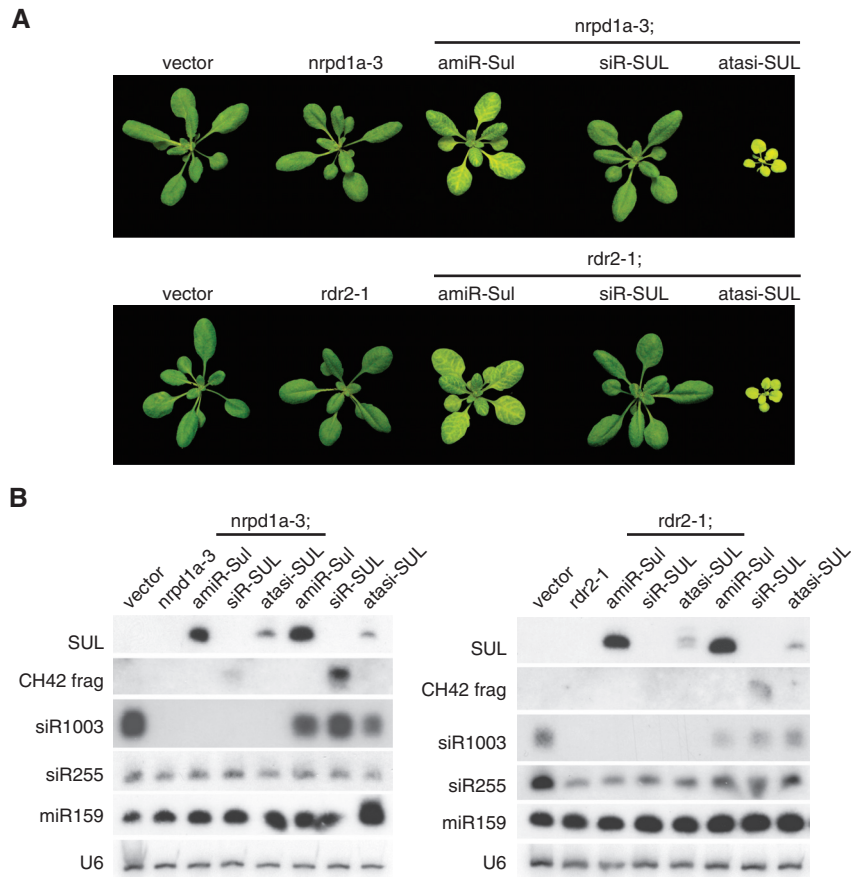


Figure 6. Differential genetic requirements for non-autonomous effects of amiR-SUL and atasiR-SUL initiated silencing. (A) Whole-rosette phenotypes. (B) sRNA blots.

all sRNAs require these two factors for transmission of their effects to neighboring cells.

Together with previous studies (31–38), our work highlights that non-autonomous action of miRNAs is likely to be context-dependent. One of the responsible factors might be the expression level of an miRNA. Among mutants that alter cell-to-cell spreading of siRNA-mediated gene silencing, those with more extensive movement of the silencing signal also have higher siRNA levels, while one of the classes lacking non-autonomous siRNA effects no longer accumulates 21 nt siRNAs (25). The same correlation has been observed for systemic movement of siRNAs, where higher copy number of the triggering transgene may lead to more efficient systemic acquired silencing (20). As discussed above, this could be one of the reasons why different levels of non-autonomy have been detected for the same amiRNAs (35).

Plants expressing higher levels of amiR-SUL present stronger bleaching, but silencing does not appear to spread further than in more weakly bleached lines. Nonetheless, expression levels still seem to be an important feature, since it affects the extent to which the neighboring cells are affected. Corroborating this idea, there are various types of published evidence for non-cell autonomy of 13 of the 19 miRNA families that are expressed more highly than amiR-SUL in *SUC2:amiR-SUL* plants (Supplementary Table S1) (36–40).

A second factor affecting miRNA non-autonomy could relate to time and place of expression. Both selective and non-selective intercellular mobility of molecules are affected by the tissue and developmental stage of the plant (66,74–76). In addition, trafficking of the silencing signal may depend on the cell type. RNAi initiated in epidermal cells has been shown to spread only locally, while expression of the same RNAi trigger in an entire leaf engenders systemic silencing (77). It is possible that, compared to other cell types, miRNAs expressed in phloem companion cells, as in this study, can more easily initiate non-autonomous silencing, or move themselves to adjacent cells, e.g. because phloem companion cells contain factors that promote non-autonomous behavior.

A third, less often considered possibility could be tissue- or cell type-specific processing of the precursor. Some miRNA precursors that are mainly processed by DCL1 in leaves can be processed by DCL3 in inflorescences, where they spawn a distinct class of miRNAs that are 23 to 25 nt long (61). Spreading of silencing triggered by amiR-SUL and atasiR-SUL is not due to the sRNA sequence, but more likely caused by biogenesis factors or effectors engaged in the miRNA and tasiRNA pathways. In analogy, the production of miRNAs through tissue-specific pathways could result in differential non-autonomous effects.

In summary, we propose that the question of cell-autonomy versus non-autonomy of sRNAs does not have a simple answer, but rather that it is contingent on several circumstances that include time, place and level of expression, which may interact with biogenesis and translocation pathways in a complex manner. Depending on the setting, miRNA behavior might therefore range from strictly cell-autonomous action, to local spreading that generates morphogenetic gradients, and even long-distance systemic silencing (36–38,42). The apparent behavior of tasiRNAs might be even more complex, as the non-autonomous effect of tasiRNA might depend both on tasiRNA-specific factors and on the action of the upstream triggering miRNAs.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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