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An endogenous, systemic RNAi pathway in plants

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Recent work on metazoans has uncovered the existence of an endogenous RNA-silencing pathway that functionally recapitulates the effects of experimental RNA interference (RNAi) used for gene knockdown in organisms such as *Caenorhabditis elegans* and *Drosophila*. The endogenous short interfering (si)RNA involved in this pathway are processed by Dicer-like nucleases from genomic loci re-arranged to form extended inverted repeats (*IR*s) that produce perfect or near-perfect dsRNA molecules. Although such *IR* loci are commonly detected in plant genomes, their genetics, evolution and potential contribution to plant biology through endogenous silencing have remained largely unexplored. Through an exhaustive analysis performed using *Arabidopsis*, we provide here evidence that at least two such endogenous *IRs* are genetically virtually indistinguishable from the transgene constructs commonly used for RNAi in plants. We show how these loci can be useful probes of the cellular mechanism and fluidity of RNA-silencing pathways in plants, and provide evidence that they may arise and disappear on an ecotype scale, show highly cell-specific expression patterns and respond to various stresses. *IR* loci thus have the potential to act as molecular sensors of the local environments found within distinct ecological plant niches. We further show that the various siRNA size classes produced by at least one of these *IR* loci are functionally loaded into cognate effector proteins and mediate both post-transcriptional gene silencing and RNA-directed DNA methylation (RdDM) of endogenous as well as exogenous targets. Finally, and as previously reported during plant experimental RNAi, we provide evidence that endogenous *IR*-derived siRNAs of all size classes are not cell-autonomous and can be transported through graft junctions over long distances, in target tissues where they are functional, at least in mediating RdDM. Collectively, these results define the existence of a bona fide, endogenous and systemic RNAi pathway in plants that may have implications in adaptation, epiallelism and trans-generational memory.

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Introduction

In the model plant *Arabidopsis*, four paralogues of the RNaseIII enzyme Dicer are at the core of multiple RNA-silencing pathways with specialized functions (Baulcombe, 2004). Dicer-like (DCL)-1 mainly produces 19- to 24-nt-long micro (mi)RNAs from non-coding and mostly intergenic, imperfect stem–loop precursor RNAs (Bartel, 2004). miRNAs incorporate into an RNA-induced silencing complex (RISC) that contains Argonaute-1 (AGO1), one of 10 AGO proteins that effect RNA silencing in *Arabidopsis* (Vaucheret, 2008). The miRNA-loaded AGO1 then guides the post-transcriptional gene silencing of complementary mRNA that includes transcription factor mRNAs and transcripts encoding proteins involved in metabolic or hormonal pathways (Voinnet, 2009). Unlike miRNAs, the 24-nt-long short interfering (si)RNAs produced by DCL3 are believed to act mostly in *cis* upon their incorporation into AGO4 or its surrogate, AGO6, to direct cytosine methylation and chromatin modifications at endogenous loci, including transposons, DNA repeats and other complex gene arrays (Zilberman *et al*, 2003; Zheng *et al*, 2007). The 21-nt siRNA products of DCL4 guide the AGO1-dependent post-transcriptional gene silencing of viral RNA or endogenous transcripts, including those involved in phase transitions, through production of *trans*-acting (tasi) RNAs (Vaucheret, 2005; Ding and Voinnet, 2007). Finally, the 22-nt siRNA products of DCL2 are usually considered as having surrogate roles when DCL4 activity is genetically compromised or suppressed, as in antiviral defence (Bouche *et al*, 2006; Deleris *et al*, 2006). Unlike DCL1, DCL2, DCL3 and DCL4 have higher affinity for perfectly or near-perfect double-stranded (ds)RNA molecules produced by the action of endogenous RNA-dependent RNA polymerases (RDRs), by sense/antisense transcription, converging transcription or by folding of inverted-repeat (*IR*) transcripts.

Genome-wide surveys in *Arabidopsis* have unravelled the existence of many discrete loci that are configured as *IR*s of variable lengths, and often associated with production of siRNAs of all size classes, suggesting the processing of a long dsRNA by the three siRNA-producing DCLs (Kasschau *et al*, 2007; Lindow *et al*, 2007). In *Caenorhabditis elegans* and *Drosophila*, endogenous (endo)siRNAs produced from similar, extended fold-back loci have been shown to target genes in *trans*, unravelling a cellular role for the canonical RNA interference (RNAi) pathway used for experimental, dsRNA-mediated gene knockdown (reviewed by Okamura and Lai, 2008). Comparatively, the evolution, genetics and biological implications of *IR* loci have remained largely unexplored in plants, although previous genetic analyses of

experimental RNAi in *Arabidopsis* have led us to propose the existence of a bona fide endogenous RNAi pathway in this species (Dunoyer *et al*, 2007). These analyses involved the use of a companion cell-specific promoter (AtSUC2, referred thereafter as to SUC) to drive an *IR* transgene designed to produce a long dsRNA targeted against the ubiquitously expressed *SULPHUR* (*SUL*) transcript. We showed that RNAi of *SUL*, diagnosed through development of chlorosis, was manifested several cells away from the vasculature, indicating the existence of a non-cell-autonomous RNAi signal that moves between plant cells (Himber *et al*, 2003; Dunoyer *et al*, 2005). We recently showed that DCL4-dependent 21-nt *SUL* siRNAs act as RNAi signals, and are both necessary and sufficient to recapitulate non-cell-autonomous post-transcriptional gene silencing of *SUL* (Dunoyer *et al*, 2010). Using a bombardment procedure, we further showed that 21-bp siRNA duplexes are also sufficient to ensure mobile RNAi between plant cells, and that they show, in addition, the potential to reach the plant vasculature, and thus, to mediate long-distance RNAi through the phloem (Dunoyer *et al*, 2010). Consistent with this idea, graft transmission of transgene-triggered RNAi has been documented in tobacco and *Arabidopsis* (Palauqui *et al*, 1997; Brosnan *et al*, 2007), although the exact nature of the nucleic acid involved in the long-distance transport process has remained so far ill defined.

The above studies have left a number of important issues unanswered. Firstly, because the experimental set up of the *SUL* or bombardment experiments can only be used to report post-transcriptional gene-silencing events, it remains uncertain if bombarded 24-bp siRNA duplexes, which normally direct chromatin modifications through AGO4, also have the potential to move between cells and reach the vasculature. The same question applies to 22-bp siRNA duplexes in the RNAi pathway. Secondly, it is unclear whether siRNA duplexes of any given size can move systemically to direct transcriptional and/or post-transcriptional gene silencing in distant organs. Thirdly, and perhaps more importantly, it remains unknown if endogenous, as opposed to transgenic, loci also have the potential to trigger cell-to-cell and long-distance silencing, perhaps to orchestrate endogenous gene regulation at a distance.

Here, we provide an in-depth study of two representative endogenous *IR* loci of *Arabidopsis*. Using the many RNA-silencing mutants available in this species, we show that these loci are genetically indiscernible from the fold-back transgene constructs used to trigger experimental RNAi in plants. Furthermore, we provide evidence that the siRNAs produced by at least one of these *IR* loci are functional in mediating the gene silencing of endogenous and exogenous targets, at both the transcriptional and post-transcriptional levels. Using a micro-grafting procedure, we further show that all size classes of siRNAs derived from those endogenous *IR* loci can move through the vasculature, and that at least the 24-nt siRNAs can trigger sequence-specific *de novo* methylation at a distance. Together, these findings support the existence of a bona fide, systemic, endogenous RNAi pathway in *Arabidopsis*. Given the evolutionary features of *IR* loci further uncovered in this study, we propose that this pathway might have important implications in adaptation to stress, epiallelism and epigenetic memory.

Results

Several endogenous Arabidopsis loci seem genetically equivalent to the IR transgene constructs used in experimental RNAi

Genome-wide analyses and inspection of publicly available small RNA deep-sequencing data indicate that gene inversions and duplications are frequent in *Arabidopsis* (Allen *et al*, 2004; Lindow *et al*, 2007). These include presumptive young *MIRNA* precursors that have already acquired bulges and mismatches (Fahlgren *et al*, 2007). Some of these loci, however, also correspond to more recent duplications events, which, pending transcriptional activity, should produce near perfectly base-paired dsRNA of varying size (Lindow and Krogh, 2005; Lindow *et al*, 2007) resembling the products of exogenous *IR* transgenes used in experimental RNAi. We focused our attention on two such endogenous *IR* loci, *IR71* and *IR2039*, both located on chromosome 3 (Henderson *et al*, 2006). Deep-sequencing data analysis indeed unravelled a strikingly symmetrical distribution of highly abundant small (s)RNA species at both loci, consistent with the intra-molecular folding and subsequent dicing of a long dsRNA (Figure 1A and B, and Supplementary Figure S1 and S2). While the sequenced sRNA derived from *IR71* showed a bias towards the 22- and 24-nt sRNA species, those derived from *IR2039* were sequenced as 21-, 22- and 24-nt sRNA species, the cognate products of DCL4, DCL2 and DCL3, respectively (Figure 1A and B).

To experimentally validate the sequencing data, we used available *Arabidopsis* mutants carrying genetic lesions in each of the four *DCL* genes (*DCL1–4*), or combinations thereof. We assayed sRNA accumulation by northern analyses using labelled DNA probes corresponding to large regions of *IR71* or *IR2039* (Figure 1); in both cases the results were similar. In the wild-type (WT) background, the presumed *IR*-derived dsRNA was processed into two major sRNA species. The 22-nt-long sRNA is made by DCL2 whereas the 24-nt-long is made by DCL3 because they were absent in *dcl2* or *dcl3* single mutants, respectively (Figure 1C). While the sRNA accumulation pattern was unchanged in single *dcl4* mutants, accumulation of a 21-nt sRNA species was most evident in *dcl2/dcl3* double mutants. This 21-nt sRNA is the product of DCL4 because it was lost in *dcl2/dcl4* as well as *dcl3/dcl4* double mutants, and also in *dcl2/dcl3/dcl4* triple mutants, in which only the activity of the miRNA-processing enzyme DCL1 remains (Figure 1C). We note that the deep-sequencing data for *IR2039* are only in partial agreement with the molecular analysis, which unravels a bias towards 22- and 24-nt sRNA production for *IR2039*, as seen for *IR71*. We conclude that both *IR71*- and *IR2039*-derived dsRNA are hierarchically processed into siRNA first by DCL2 and DCL3, then secondarily by DCL4, and that the role of DCL1 in siRNA biogenesis is negligible at both loci. Strikingly, hierarchical *DCL* usage and poor DCL1 contribution to siRNA processing were also observed in the genetic analyses of the exogenous, phloem companion cell-specific *IR* SUC:*SUL* (Dunoyer *et al*, 2007), which triggers non-cell-autonomous RNAi of the ubiquitously expressed *SUL* mRNA. A notable difference between the exogenous SUC:*SUL* and the endogenous *IR71* and *IR2039*, however, is that the SUC:*SUL* dsRNA is mostly processed by DCL4 and DCL3, and then secondarily by DCL2 (Dunoyer *et al*, 2007).

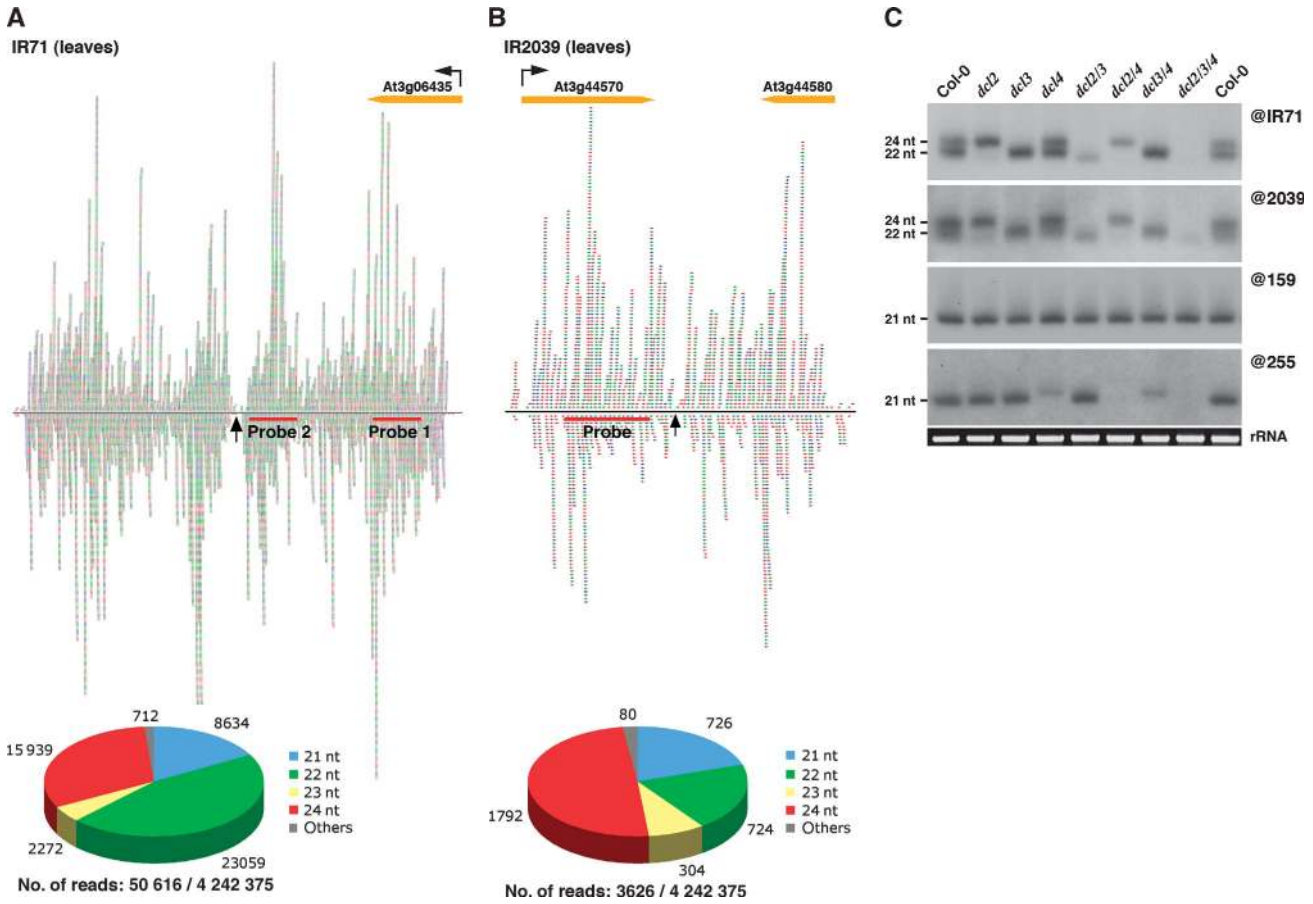

Figure 1 Small-RNA populations and DICER usage at endogenous *IR* loci. A representation of sequenced sRNAs derived from either the *IR71* (**A**) or *IR2039* (**B**) endogenous hairpins. Indicated are the number of each size class of sRNA and the number of reads compared with the total number of sequenced sRNAs derived from *IR71* (**A**) or *IR2039* (**B**). Shown are the location of the probes used in panel **C** and the location and orientation of the genes adjacent to the respective hairpins. The arrow indicates the predicted terminal loop of the folded RNA. (**C**) RNA gel blot analysis of *DCL* usage at *IR71* (@IR71) and *IR2039* (@2039). miR159 (@159) serves as a control for miRNA levels and *trans*-acting siRNA255 (@255) as a control for other siRNAs in this and subsequent blots.

Having established that both *IR71*- and *IR2039*-associated siRNAs are processed by DCL2, DCL3 and DCL4, we reasoned that plants carrying simultaneous lesions in each of these factors should accumulate the putative long dsRNA substrate of DCLs to higher levels than in WT individuals. As shown in Figure 2A, an RNA species covering the near full-length of *IR71* or *IR2039* (6800 and 2100 bp, respectively) could be detected by northern analysis, and was clearly more abundant in *dcl2/dcl3/dcl4* triple mutants than it was in WT *Arabidopsis*. This result strongly suggests that both *IR71*- and *IR2039*-derived siRNAs are diced from extensively base-paired, long single-stranded (ss)RNA precursors resembling the fold-back transcripts produced from the SUC:*SUL* experimental construct (Dunoyer *et al*, 2005). Further supporting this idea, mutations in genes encoding RDRs that convert long ssRNA with no intrinsic fold-back potential into dsRNA, had no effect on siRNA accumulation from either endogenous loci or from SUC:*SUL* (Figure 2B; Dunoyer *et al*, 2007). Furthermore, the same was true of additional mutations that alter siRNA production from sense but not *IR* transgenes, including *xrn4*, *sgs3*, *sde3* and *sde5* (Figure 2B; Supplementary Figure S3A; Beclin *et al*, 2002; Himber *et al*, 2003; Gazzani *et al*, 2004; Hernandez-Pinzon *et al*, 2007). We conclude that *IR71* and *IR2039* are likely endogenous equivalents to the *IR* transgenes used in experimental RNAi, both in terms of fold-back RNA production and subsequent siRNA processing. On the basis of high occurrence of loci producing symmetrically organized and heterogeneous siRNA populations in the *Arabidopsis* genome (Lindow *et al*, 2007), we infer that many transcribed endogenous hairpins, in addition to *IR71* and *IR2039*, share the above features.

Further similarities in the processing and stability of siRNA derived from endogenous and exogenous IR loci

Optimal activity of DCLs in plants requires cofactors that include a family of dsRNA-binding proteins of which there are five paralogues (DRB1–5) in *Arabidopsis* (Hiraguri *et al*, 2005). Inspection of siRNA processing from *IR71* and *IR2039* in *drb2*, *drb3* and *drb5* mutant backgrounds showed no differences as compared with that in WT plants (Figure 2B, left panel). However, a loss-of-function mutation in the DCL4-interacting protein, DRB4, caused a dramatic gain in the accumulation of the DCL3-dependent, 24-nt siRNAs derived from both endogenous *IR*s (Figure 2B, left panel), as previously reported for SUC:*SUL*-derived siRNAs (Figure 2B, right panel; Dunoyer *et al*, 2007). To explain this effect, we proposed that DCL3 normally competes with the DRB4–DCL4 complex for access to the *SUL* dsRNA (Dunoyer *et al*, 2007). Accordingly, loss of DRB4 would not only reduce

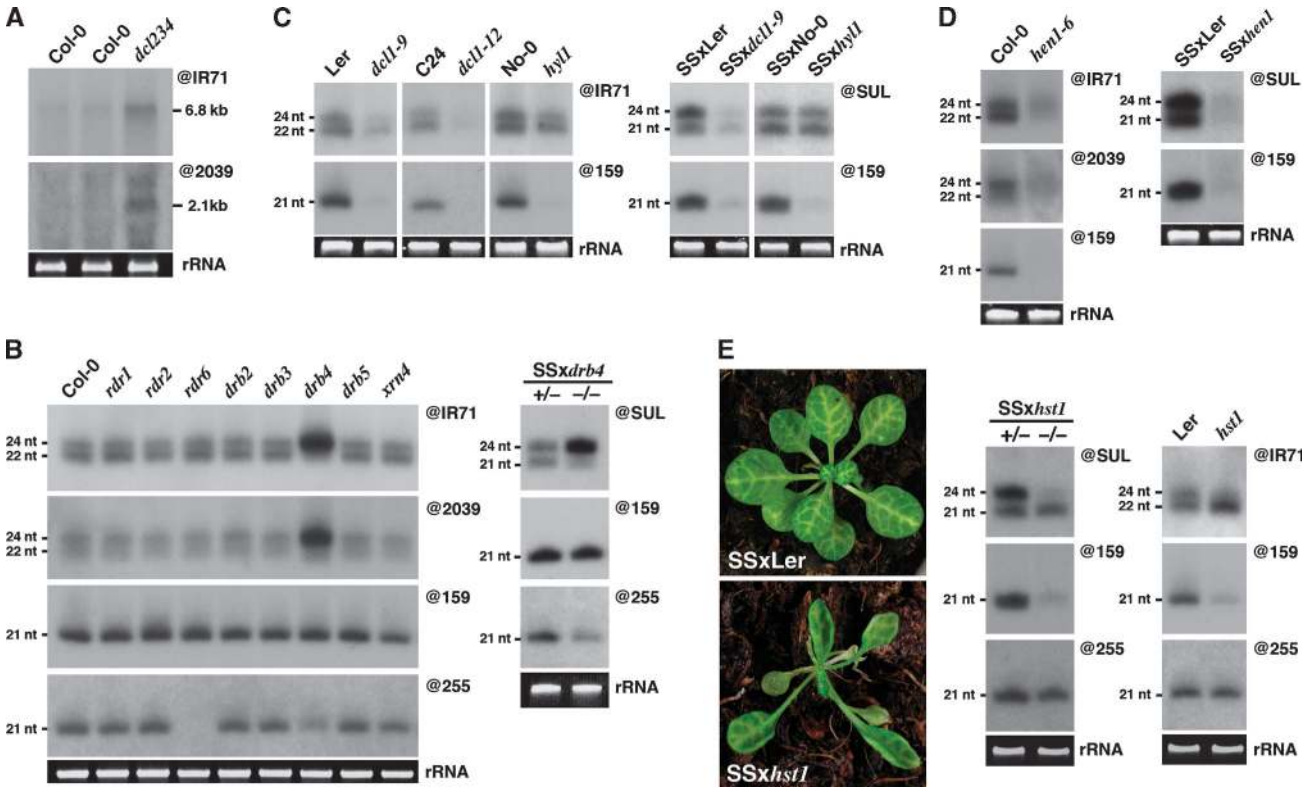

Figure 2 Genetic requirements for siRNA biogenesis from exogenous and endogenous *IR*. (**A**) RNA gel blot analysis of the predicted full-length fold-back hairpin RNA corresponding to *IR71* and *IR2039* in Col-0 and triple *dcl2/dcl3/dcl4* mutants, respectively. (**B**) Accumulation of siRNAs from endogenous *IR*s is largely unaffected in *rdr*, *drb* and *xrn* mutant backgrounds unlike in *drb4* mutant background where 24-nt siRNAs strongly over-accumulate. The right panel shows similar affects of *drb4* on *SUL* siRNA accumulation. (**C**) Northern analysis (left) showing the effect of *dcl1* and *hyl1* mutations on *IR71* siRNA accumulation. The same analyses in a SUC:*SUL* background are shown in the right panel. (**D**) RNA blot analysis in *hen1* mutants in Col-0 (*IR71* and *IR2039*, left) and Ler (*SUL*, right). (**E**) Phenotype and northern analysis of *SUL* siRNA (@SUL) in plants heterozygous or homozygous for the *hst1* mutation. The same analysis is shown on the right for the *IR71* loci in Ler and *hst1*.

DCL4 activity but also concurrently stimulate the DCL3-mediated processing of 24 nt siRNAs, a scheme that, evidently, also likely applies to the dsRNA produced from the endogenous *IR71* and *IR2039* loci (Figure 2B, left panel).

Despite its negligible role in the processing of siRNAs, we previously reported that DCL1 somehow facilitates the accumulation of siRNAs derived from the SUC:*SUL IR* locus (Dunoyer *et al*, 2007). This was diagnosed by a strong reduction in the levels of all *SUL* siRNA size classes in hypomorphic *dcl1* mutant backgrounds (Figure 2C, right panel; Dunoyer *et al*, 2007). We experimentally ascribed this effect to a known activity of DCL1, which liberates miRNA imperfect fold-backs (called pre-miRNA) from their longer primary transcripts (pri-miRNA), a reaction orchestrated in metazoans by the RNaseIII enzyme Drosha (Lee *et al*, 2003). In plants, this 'Drosha-like' activity of DCL1 is prerequisite to the consequent DCL1-mediated processing of mature miRNA duplexes in the nucleus (Kurihara and Watanabe, 2004). Similarly, we proposed that DCL1 liberates the perfect or near-perfect stem–loops from *IR*-derived primary transcripts, and thereby facilitates their subsequent access and hierarchical processing by the three siRNA-generating DCLs (Dunoyer *et al*, 2007). We could not test this idea for *IR2039*, because it is absent in the genome of *Arabidopsis* accessions carrying available hypomorphic *dcl1* mutations (the same caveat applied to studies of mutations in *HYL1* and *HASTY*, see later in this study). However, northern analyses of *IR71* in the *dcl1-9* (ecotype Ler; Jacobsen *et al*, 1999) or *dcl1-12* (ecotype C24; Brodersen *et al*, 2008) hypomorphic mutants clearly indicated a facilitating role for DCL1 in *IR71*-derived siRNA biogenesis (Figure 2C, left panel). Previous analysis of *SUL* dsRNA processing did not show any significant effect of mutations in *DRB1*, also known as *HYL1*, which assists pri-to pre-miRNA processing by DCL1 in the nucleus (Figure 2C, right panel; Han *et al*, 2004). Accordingly, the *hyl1* mutation only caused a slight reduction in *IR71*-derived siRNA accumulation (Figure 2C, left panel). Collectively, the results obtained with *drb4*, *dcl1* and *hyl1* mutations emphasize the striking similarities found in the processing of exogenous and endogenous *IR*-derived dsRNA.

Upon their processing by DCLs, all classes of *Arabidopsis* sRNAs are methylated at their 3′ ends by the SAM-methyl transferase HEN1, which protects them from urydilation and subsequent degradation (Li *et al*, 2005; Yu *et al*, 2005). As shown in Figure 2D (left panel), and as previously reported for the *SUL* siRNAs (right panel, Dunoyer *et al*, 2007), both *IR71*- and *IR2039*-derived siRNAs were sensitive to the *hen1* mutation. Coincident or subsequent to their stabilization by HEN1, some *Arabidopsis* sRNAs exit the nucleus by mechanisms that might include recruitment of the EXPORTIN-5 homolog HASTY (HST). On the basis of metazoan studies, HST was previously proposed to be necessary for the nucleocytoplasmic transport of some, albeit not all, mature miRNA duplexes of *Arabidopsis* (Park *et al*, 2005). We thus used *hst1* mutants in the Ler ecotype to measure the accumulation of both SUC:*SUL*- and *IR71*-derived siRNAs in transgenic and

non-transgenic plants, respectively. While we confirmed the previously reported decrease of some endogenous miRNAs in *hst1* mutants (Figure 2E; Park *et al*, 2005), accumulation of both SUC:*SUL*- (left panel) and IR71-derived siRNA (right panel) of the 24-nt size class (DCL3 products) was dramatically and specifically reduced. Strikingly, this effect was also observed with endogenous DCL3 products involved in the heterochromatic silencing pathway (including sRNA1003), and with 24-nt siRNA derived from an RNA virus (Supplementary Figure S4). Previous analyses of the ASRP02 and AtSIN1 heterochromatic siRNAs (not tested here) had not shown such an effect of *hst1*; however, a clear reduction in accumulation of the 24-nt siRNA ASRP1003 was observed (Park *et al*, 2005). Thus, unexpectedly, the *hst1* mutation affects at least some classes of DCL3 products, clearly implicating HST in processes beyond mere miRNA transport, which will deserve further investigation. These results nonetheless show that nearly identical mechanisms underlie not only the processing but also the intracellular transport or stability of siRNAs that derive from both exogenous and endogenous *IR* loci.

Mutations in the RNA-directed DNA methylation pathway do no affect the processing or stability of endogenous IR-derived siRNAs

Previous studies of two independent transgenic *IR* loci, including SUC:*SUL*, unexpectedly uncovered the contribution of several components of the RNA-directed DNA methylation (RdDM) pathway to non-cell-autonomous RNAi (Dunoyer *et al*, 2007; Smith *et al*, 2007). Notably, mutations in *NRPD1*, but not in *NRPE1*, encoding the largest subunit of plant-specific, heterochromatic RNA polymerase-IV and V, respectively, caused a loss of vein-centred *SUL* silencing (Dunoyer *et al*, 2007). Similar observations were made with mutations in *RDR2* and *CLSY1*, an *SNF2* domain-containing gene, which, together with *RDR2* and *NRPD1*, but unlike *NRPE1*, is required for biogenesis of 24-nt siRNA at many endogenous, heterochromatic loci (Supplementary Figure S3B; Xie *et al*, 2004; Herr *et al*, 2005; Kanno *et al*, 2005; Pontier *et al*, 2005; Smith *et al*, 2007). None of the above mutations, however, altered *SUL* siRNA processing by DCL4 and DCL3, or prevented *SUL* silencing within the companion cells, suggesting that NRPD1, RDR2 and CLSY act downstream of DCL and AGO, being presumably required for movement or sensing of RNAi in recipient cells (Supplementary Figure S3B; Dunoyer *et al*, 2007). In agreement with these previous findings and further emphasizing the genetic equivalence of endogenous and exogenous *IRs*, none of the above mutations affected the production of 21-, 22- or 24-nt siRNAs from either the *IR71* or *IR2039* loci (Supplementary Figure S3C).

Endogenous IR-derived siRNAs can effect RdDM and post-transcriptional gene silencing

In the SUC:*SUL* system, the 21-nt *SUL* siRNAs are necessary and sufficient to mediate both intracellular and non-cell-autonomous post-transcriptional gene silencing upon their specific incorporation into AGO1, one of 10 *Arabidopsis* AGO effector proteins (Dunoyer *et al*, 2007). The 24-nt *SUL* siRNAs, by contrast, recruit AGO4 (Figure 3A), presumably to mediate RdDM at homologous loci, and are completely dispensable for both intracellular and non-cell-autonomous RNAi (Dunoyer *et al*, 2007). We thus investigated what AGO loading rules applied to the 22-nt (as opposed to 21-nt in SUC:*SUL*) and 24-nt siRNAs that accumulate preferentially at the *IR71* and *IR2039* loci. We found, indeed, that the 22-nt siRNA, but not the 24-nt siRNA, produced at both endogenous loci are loaded into AGO1, as assessed by immunoprecipitation (Figure 3A). Conversely, the 24-nt siRNA from *IR71* and *IR2039*, but not their 22-nt counterparts, were found in AGO4 immunoprecipitates (IPs) (Figure 3A, and data not shown). We then asked whether these AGO1-loaded 22-nt siRNAs and AGO4-loaded 24-nt siRNAs could function in the RNAi and RdDM pathways, respectively. Our analysis focused on *IR71* because we could retrieve a T-DNA insertion that disrupts the basal part of the fold-back, causing loss of siRNA accumulation from this region as assessed by northern analysis and siRNA deep-sequencing (*IR71*-T-DNA; Figure 3B, probe-1). The T-DNA does not, however, eliminate siRNA production from the distal part of the *IR71* fold-back where siRNA accumulation is nonetheless reduced as compared with that in WT plants (Figure 3B, probe-2), presumably as a consequence of suboptimal dsRNA folding in this region.

We used available deep-sequencing data from whole-plant AGO1 immunoprecipitates (AGO1-IP; Mi *et al*, 2008) together with target prediction algorithms to identify abundantly loaded ($>6000/1\,683\,581$ reads), 22-nt-long siRNA derived from *IR71*, that showed near-perfect complementarity to endogenous transcripts. Microarray and quantitative RT–PCR analyses in WT versus *IR71*-T-DNA plants identified two such endogenous mRNA as potential targets of *IR71* (Supplementary Figure S5). However, given the uncertainty of their expression patterns and the lack of antibodies against the corresponding proteins, we resorted to using a ubiquitously expressed sensor transgene to ascertain this issue. To that aim, the region complementary to the abundant, 22-nt-long siRNAs found in AGO1-IP was fused to the 3′-UTR of a GFP transgene (Figure 3C). The resulting *IR71* sensor was then placed under the control of the ubiquitous 35S promoter and transformed into WT or *dcl2/dcl3/dcl4* triple mutant *Arabidopsis*. We found that GFP expression and sensor mRNA accumulation were low or below detection limit in all independent transgenic lines in a WT background, but they where high in all independent lines generated in the *dcl2/dcl3/dcl4* background (Figure 3C), which prevents accumulation of all siRNA classes derived from *IR71* (Figure 1C). These results strongly suggest that 22-nt-long, *IR71*-derived siRNAs can effect post-transcriptional gene silencing upon their loading into AGO1.

To investigate whether the 24-nt-long siRNA can trigger RdDM through AGO4, we exploited available genome-wide DNA methylation data and the known capacity of endogenous DCL3 products to guide cytosine methylation in *cis*, in all sequence contexts (Cokus *et al*, 2008; Lister *et al*, 2008). We identified a region present in *IR71*, but disrupted in *IR71* T-DNA, that was densely populated by siRNA of the 24-nt size class. This region, furthermore, contained several cytosine methylation peaks that matched at least two methylation-sensitive restriction sites, which we therefore used in semi-quantitative (sqPCR) and quantitative (qPCR)-based, DNA methylation assays (Cokus *et al*, 2008; Lister *et al*, 2008; Figure 3D). These analyses showed a clear disparity in the methylated status of both sites in WT versus *IR71*-T-DNA plants (Figure 3D), which differ only in their

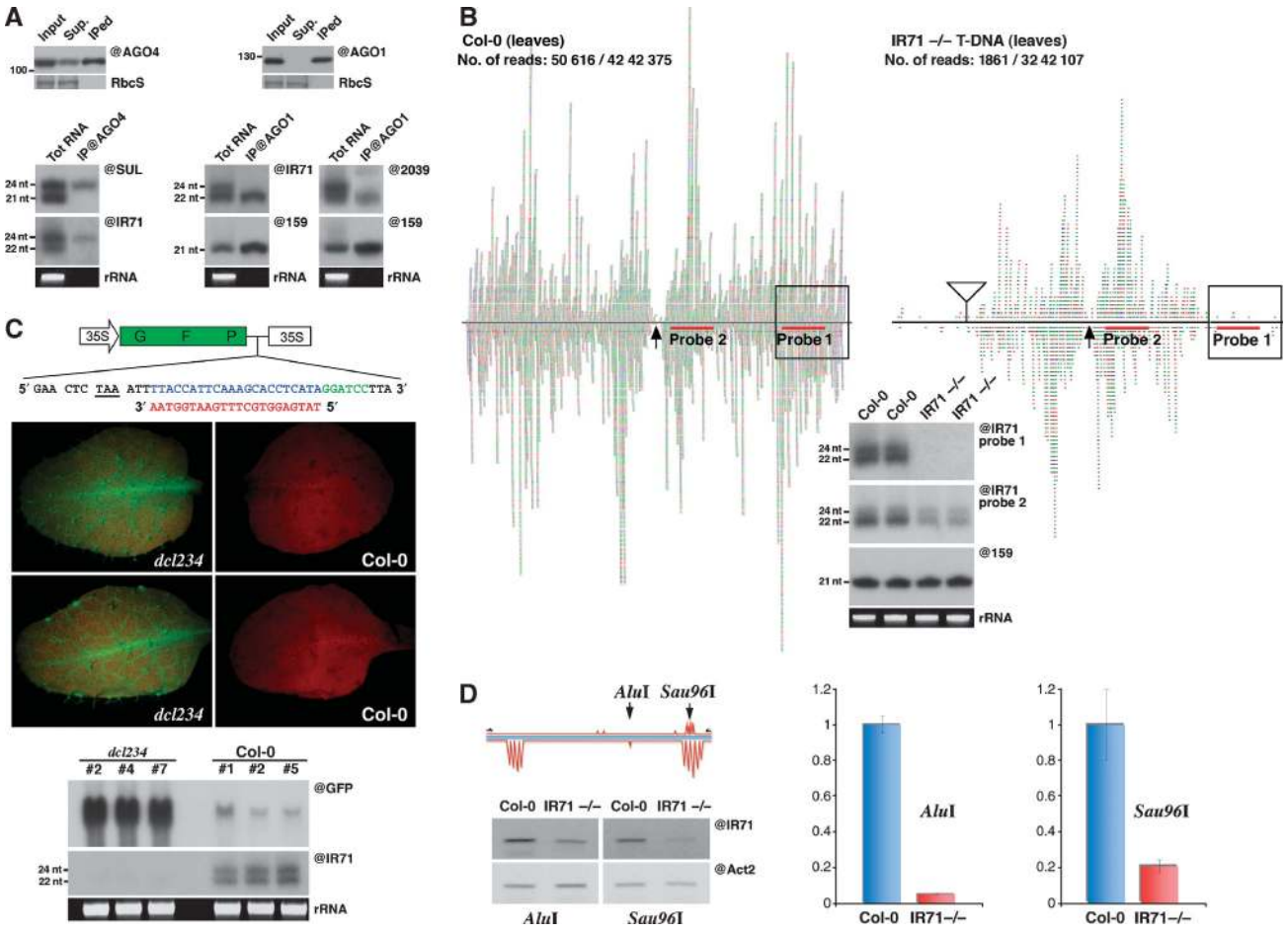

Figure 3 *IR*-derived siRNAs are loaded into cognate AGO proteins and can function at both post-transcriptional gene silencing and RNA-directed DNA methylation levels. (**A**) Immunoprecipitation experiments were conducted using either an AGO4- or AGO1-specific antibody. The presence of either AGO1 or AGO4 in each IP was confirmed by protein blot analysis (upper panels). Total RNA extracted from the respective IPs was subjected to northern analysis using the indicated probes. (**B**) Sequencing and molecular confirmation of siRNAs from Col-0 and a T-DNA insertion line at the *IR71* locus. siRNAs sequenced from the aforementioned genotypes, including the predicted terminal loop (arrow), the number of *IR71* reads compared with the total number of reads and the location of probes used in the gel blot analysis. Also shown is the location of the T-DNA insertion (triangle), with the boxed region representing the predicted region of the *IR* fold-back structure that would be disrupted by the insertion. (**C**) A schematic representation of the 35S:*GFP* sensor used to assay the post-transcriptional silencing ability of AGO1-loaded *IR71*-derived siRNAs. A recognition sequence (blue) for the highly AGO1-loaded siRNA (red) was inserted three bases after the stop codon of *GFP* at the start of the 3′UTR. The middle panels show the GFP sensor fluorescence after transformation into either a *dcl2/dcl3/dcl4* triple mutant (left) or Col-0 plant (right). Northern blot analysis (bottom panel) confirms the strong *GFP* mRNA decrease and the presence of *IR71*-derived siRNAs in silenced Col-0 plants, and the converse for non-silenced *dcl2/dcl3/dcl4* plants. (**D**) Analysis of DNA methylation induced by *IR71*-derived siRNAs. A schematic representation of the predicted regions of methylation within a 300-nt portion of the *IR71* fold-back disrupted by the T-DNA insertion, including the location of the primer and restriction sites used. sqPCR analysis (@IR71) of DNA extracted from the indicated genotypes after digestion with the methylation-sensitive enzyme *Alu*I or *Sau*96I. Equal input of DNA was confirmed by amplification of a region of actin-2 (@Act2) lacking either restriction site. Quantitative real-time PCR analysis (right) confirmed the results of the semi-quantitative approach.

capacity to produce siRNA at those sites. The results thus suggest that *IR71*-derived siRNAs can effect RdDM, at least in *cis*, upon their incorporation into AGO4.

Evolutionary fluidity and regulated expression patterns of endogenous IR loci

The near-perfect double-strandedness (Supplementary Figure S1 and S2) of the predicted fold-backs of *IR71* and *IR2039* shows that those structures have not yet acquired many mutations, suggesting that they have evolved very recently. To investigate this issue, we studied the accumulation of siRNAs from *IR71* and *IR2039* in a variety of *Arabidopsis* accessions that have evolved in distinct ecological niches and are thought to represent the genetic diversity found within the *Arabidopsis thaliana* species (Nordborg *et al*, 2005). Remarkably, among the accessions tested, Col-0 was the only ecotype in which siRNA production from *IR2039* could be detected by northern analysis, even after long exposures (Figure 4A). This result suggests that the gene duplication and/or inversion at the *IR2039* locus is Col-0-specific and represents, therefore, an extremely recent event. By contrast, siRNA could be detected from the *IR71* locus in all ecotypes tested, with the notable exception of Kondura and Se-0, respectively isolated from central Asia and Spain, and belonging to two closely related phylogenetic clades (Figure 4A and Nordborg *et al*, 2005). Neither *IR71*-derived nor *IR2039*-derived siRNAs could be detected in closely related species *Capsella rubella* or *Ophiorrhiza pumila* (Figure 4A). Thus,

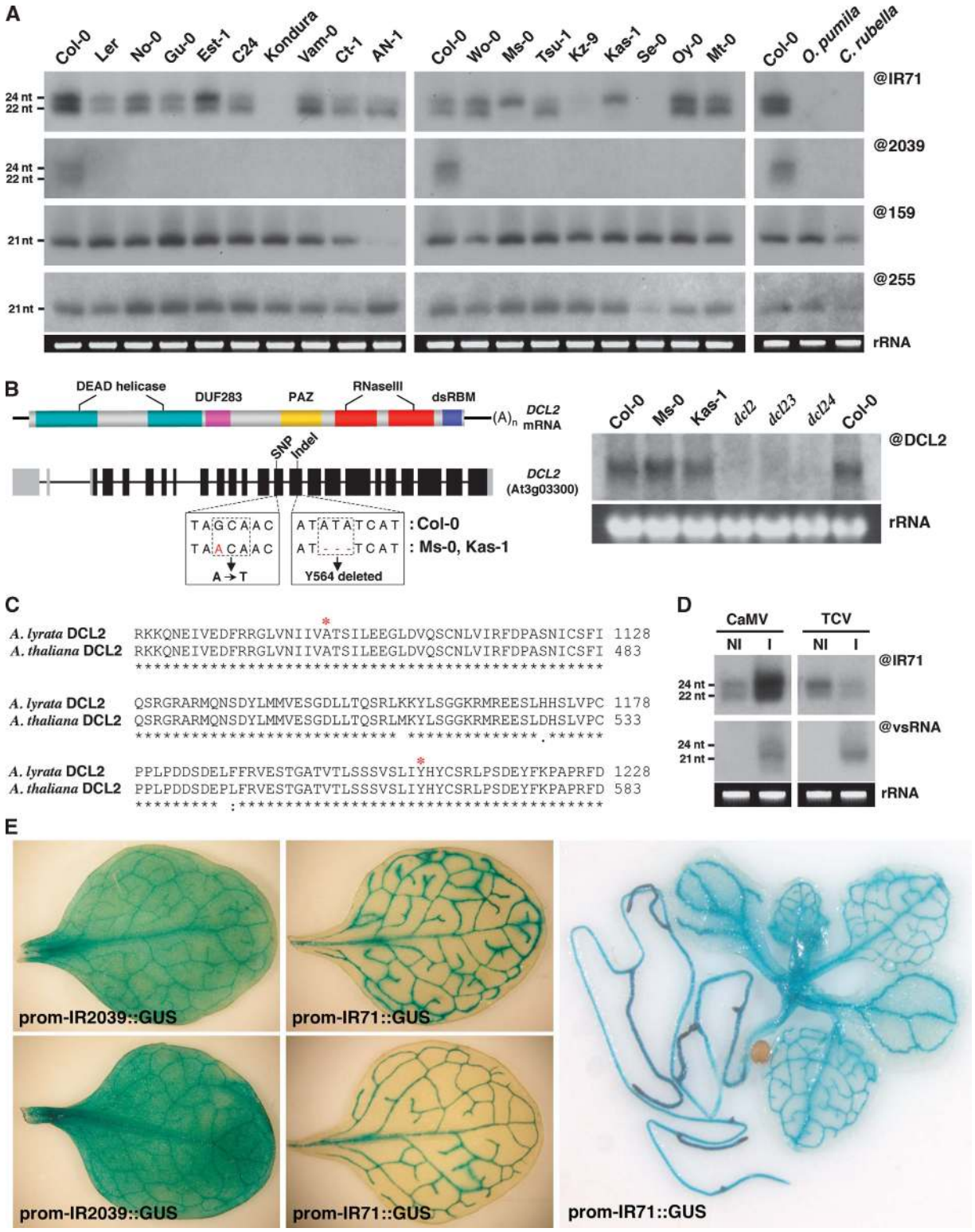

Figure 4 Rapid evolution and regulated expression of endogenous *IR* loci. (**A**) Northern analysis of *IR71*- and *IR2039*-derived siRNAs in various *Arabidopsis* accessions representing the genetic diversity within the *A. thaliana* species. Also shown are miRNA (@159) and *trans*-acting siRNA (@255) in each accession. (**B**) A schematic representation of the *DCL2* mRNA showing the domain structure and genomic region with the indicated SNP and indel in ecotypes Ms-0 and Kas-1 (boxed). The right panel shows that the mutations in the *DCL2* genomic sequence do not affect mRNA accumulation (@DCL2) in the indicated accessions as assessed by RNA gel blot analysis. (**C**) Alignment of DCL2 amino acid sequence covering both the predicted amino acid substitution and deletion (marked *) between a representative sequence present in most *Arabidopsis* accessions (except Ms-0 and Kas-1) and the distinct species *A. lyrata*. (**D**) Northern analysis of virus-infected Col-0 plants probed with either *IR71*- (@IR71) or virus siRNA (vsRNA)-specific probes. NI, non-infected and I, infected with either CaMV (Cauliflower mosaic virus) or TCV (Turnip crinkle virus). (**E**) GUS staining of leaves from promoter::GUS fusions of two independent transgenic lines containing ~1.5-kb upstream promoter regions from either *IR2039* (left) or *IR71* (middle). The right panel shows GUS staining of a whole plant representative of the *IR71* promoter::GUS fusion lines.

compared with *IR2039*, the gene duplication event at the *IR71* loci must have occurred earlier in evolution.

Interestingly, *IR71*-derived 22-nt siRNAs were below the detection limit of northern analysis in two phylogenetically related *Arabidopsis* accessions, Ms-0 and Kas-1 (Figure 4A and Nordborg *et al*, 2005). DNA sequencing showed single-nucleotide polymorphisms (SNPs) in the introns and in the open-reading frame of *DCL2* in both accessions (ORF; Figure 4B). Intronic SNPs were not found at splicing sites and, accordingly, accumulation and electrophoretic mobility of the *DCL2* mRNA were unchanged, as compared with Col-0 (Figure 4B). However, an SNP and an indel within the *DCL2* ORF were predicted to substitute an alanine for a threonine ($A_{454}T$) in the DCL2 helicase domain, and to cause the deletion of a tyrosine (Y_{564}) in the DUF283 domain. Both amino acids were highly conserved in all other *Arabidopsis* accessions inspected, and even in the distinct species *Arabidopsis lyrata* (Figure 4C; data not shown). This observation suggests that accessions Ms-0 and Kas-1 produce loss-of-function variants of the DCL2 protein. This further suggests that post-transcriptional gene silencing from *IR71*, and perhaps other *IR* loci, is not operational in those accessions, while the RdDM potential of *IR71* remains, in principle, unaltered. Therefore, endogenous *IR*s can arise and abort on an ecotype scale and can even be processed alternatively, giving rise to possible contrasted outputs on gene regulation depending on specific ecotypes. Consistent with a potential role for endogenous *IR* in evolving with, and therefore sensing, the direct environment of plants, we found that sRNA production from both *IR71* and *IR2039* was strongly altered by a variety of stresses, including viral infection and treatments with a flagellin-derived peptide that elicits basal defence reactions in plants (Figure 4D and data not shown).

Next, we assessed the expression patterns of *IR71* and *IR2039*. A 1.5-kb stretch of sequence located upstream from the predicted transcription start of the dsRNA produced at each locus was fused transcriptionally to the GUS reporter gene. The resulting transgenes were then transformed into WT *Arabidopsis* plants. In several independent lines, the expression pattern of *IR2039* was found to be ubiquitous; leaves, in particular showed faint, uniform blue staining (Figure 4E, left panel). By contrast, independent transgenic lines expressing the *IR71* reporter construct showed a highly vascular-specific blue staining in leaves. The staining was more uniform in the stems and roots, where it was particularly pronounced at the tips (Figure 4E, right panel). Therefore, rapidly evolving *IR*s may respond to the plant environment and may have specific expression patterns.

IR71-derived siRNAs are mobile and functional over long distances

The vascular-specific transcriptional pattern of *IR71* in leaves was in sharp contrast with the uniformity of the silencing of the GFP-based *IR71* sensor construct in those organs (Figure 3C). This disparity suggested that silencing from the endogenous *IR71* is not cell-autonomous, and moves from the vasculature to adjacent cells, as seen with the exogenous *IR* SUC:*SUL*. In plants, transgene silencing can not only move from cell to cell, but also over long distances through the phloem, the latter process being mostly evident through grafting experiments (Palauqui *et al*, 1997; Voinnet *et al*, 1998; Brosnan *et al*, 2007). To investigate whether *IR71* does indeed have the potential to trigger non-cell-autonomous gene silencing, we exploited a previously described micro-grafting procedure that was successfully used to monitor long-distance transgene silencing in *Arabidopsis* (Brosnan *et al*, 2007).

We reasoned that long-distance silencing movement from the endogenous *IR71* locus would be diagnosed as a gain of siRNA species in tissues of *IR71*-T-DNA plants upon their grafting to WT plants. This gain should be particularly evident over the region disrupted by the T-DNA, which accumulates background levels of siRNAs in *IR71*-T-DNA plants, as shown by deep-sequencing and northern analyses (Figure 3B). The aerial parts or roots of WT plants were used as 'silencing donor' tissues in grafting experiments involving 'silencing recipient' tissues (roots, Figure 5A; or shoots, data not shown) from *IR71*-T-DNA plants. Twenty-eight days after grafting, the recipient tissues were then sampled and total sRNAs were subjected to deep-sequencing analyses. Sequencing of sRNAs isolated from corresponding, non-grafted tissues of WT or *IR71*-T-DNA plants was performed in parallel, as reference. Remarkably, siRNA species of all size classes corresponding to the T-DNA-disrupted region of *IR71* were sequenced well above background frequencies in the tissues of recipient *IR71*-T-DNA plants used as root material (Figure 5B). Although statistically significant, the phenomenon was not as pronounced if *IR71*-T-DNA tissues were used as aerial material in the grafting experiments (data not shown), suggesting preferential shoot-to-root transmission of RNA silencing from WT to *IR71*-T-DNA plants. Northern analyses using *IR71*-specific probes corresponding to the T-DNA-disrupted region confirmed detection of new siRNAs in recipient tissues of *IR71*-T-DNA plants (Figure 5D). The prevalent siRNA species detected by northern analysis were of the 24-nt size class, but longer exposures confirmed detection of 21- to 22-nt siRNAs as well, in agreement with the deep-sequencing data (Figure 5D).

A simple interpretation of those results is that endogenous, *IR71*-derived siRNAs had moved from shoots to roots, although movement of long RNA, including the long dsRNA precursor of those molecules (Figure 2A), could not be formally excluded. Nonetheless, of the two possibilities, we favour the former, because we have recently shown that mechanically delivered siRNAs do recapitulate mobile silencing between *Arabidopsis* cells and can reach the vascular system (Dunoyer *et al*, 2010). This previous study more specifically identified the DCL4-dependent, 21-nt siRNA as being both necessary and sufficient to mediate non-cell-autonomous RNAi, leaving open the possibility that 24-nt siRNAs also can move and mediate RdDM at a distance (Dunoyer *et al*, 2010). The identification of 24-nt-long siRNAs in the recipient *IR71*-T-DNA grafted material thus prompted us (i) to assay for their possible mobility upon mechanical delivery and (ii) to test their functionality in grafted, recipient tissues. To investigate the first point, we used chemically synthesized 24-bp siRNA duplexes covalently labelled at their 3′-end with the fluorescent dye ALEXA555 to enable their observation *in planta* upon biolistic delivery, as described in our parallel study (Dunoyer *et al*, 2010). Twenty hours post bombardment (hpb) of the leaves of WT seedlings, the delivered siRNA duplexes had formed foci that were indistinguishable from those observed upon bombardment of 21-bp siRNA duplexes (Figure 5C; Dunoyer *et al*,

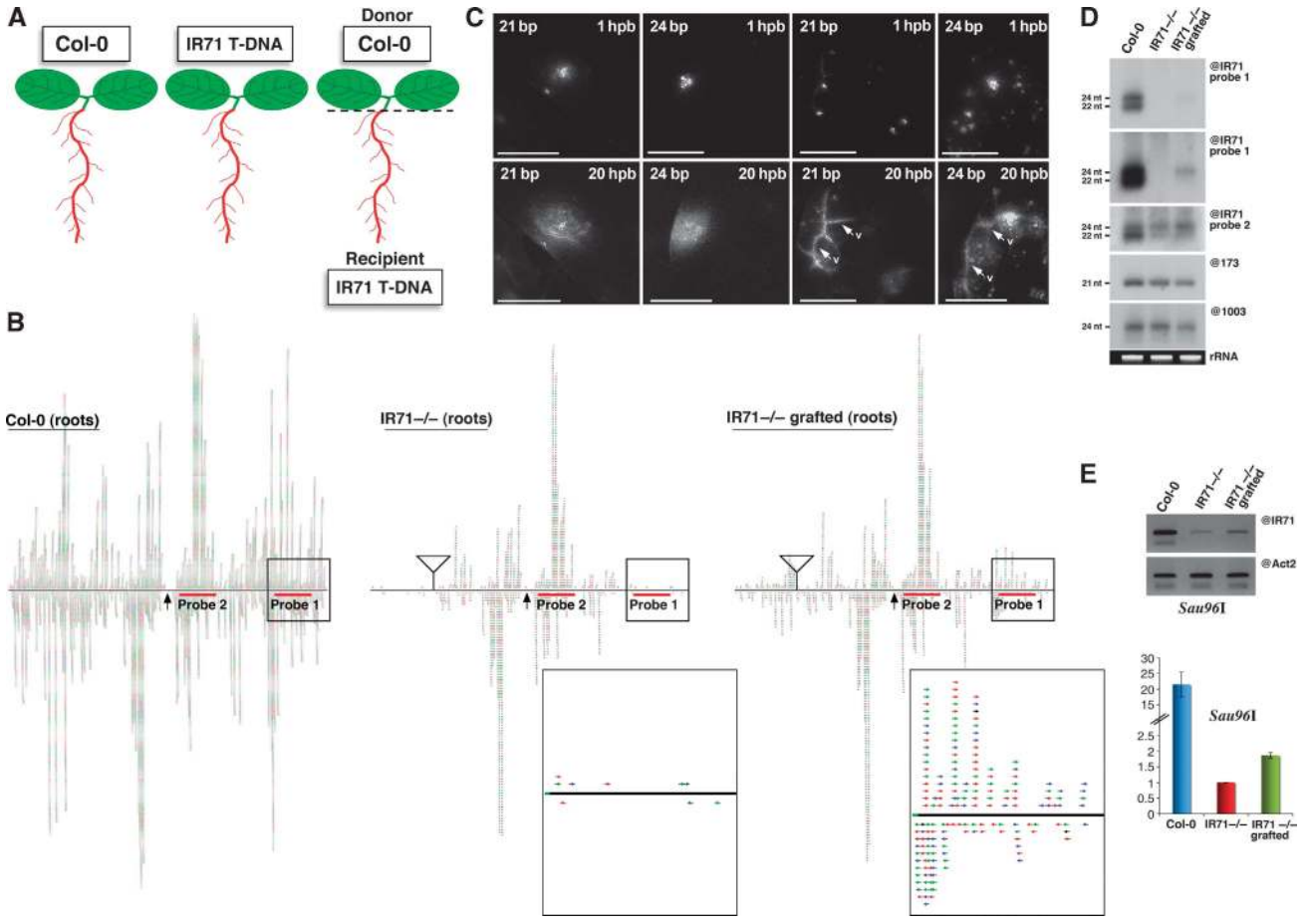

Figure 5 *IR71*-derived siRNAs are mobile and functional over long distances. (**A**) A schematic representation of the genotypes Col-0 and *IR71* T-DNA (referred to subsequently as *IR71*−/−), and grafting used to detect the long-distance movement of *IR71*-derived siRNAs. (**B**) Sequencing of siRNA populations in the roots of Col-0, IR71−/− and grafted roots as represented in panel A. The inset boxes show a close-up comparison of the siRNA populations in the IR71−/− line and the siRNAs received in the IR71−/− line once grafted onto the donor Col-0 line. Blue, green and red correspond to 21-, 22- and 24-nt size classes respectively. (**C**) *In planta* biolistic delivery of ALEXA555-labelled 21- and 24-bp siRNA duplexes. The top four panels show fluorescence 1 hpb for the indicated siRNA size at locations distal (left two panels) or proximal (right two panels) to the leaf veins. The same is shown 20 hpb in the lower four panels. Note that both 21- and 24-bp siRNAs, when delivered proximal to veins (V), have the ability to enter the vasculature and potentially move over longer distances. (**D**) Northern analysis of the genotypes shown in panel A confirms the sequencing results shown in panel B, with two IR71-specific probes (@IR71 probe 1 and @IR71 probe 2) as well as controls for miRNA accumulation (@173) and heterochromatic siRNA accumulation (@1003). (**E**) Semi-quantitative (top panel) and quantitative (bottom panel) methylation-sensitive PCR of Col-0, IR71−/− and grafted root tissue. Genomic DNA extracted from the indicated genotypes was digested with the methylation-sensitive enzyme *Sau*96I and amplified using the primers as shown in Figure 3D. Actin-2 serves as a loading control.

2010). Furthermore, several expanding foci could clearly reach the vasculature (Figure 5C), showing that exogenously delivered 24-bp siRNA, just like their 21-bp counterparts, have the potential to move from cell to cell and over long distances, in agreement with the grafting data.

To investigate the functionality of the endogenous, graft-transmitted 24-nt siRNA, we used the approach described in Figure 3D, and investigated the methylation status of the DNA comprised within the disrupted region of the *IR71* locus in *IR71*-T-DNA tissues, either before or after grafting to WT plants. This analysis (Figure 5D) showed that DNA methylation in silencing recipient tissues was intermediate to that seen in WT plants (fully methylated state) and in *IR71*-T-DNA plants (unmethylated state). This result was expected from the moderate, albeit clearly significant gain in the abundance of *IR71*-derived siRNA after grafting (Figure 5B). We conclude that endogenous, 24-nt siRNAs are mobile and can mediate RdDM over long distances.

Discussion

This study has uncovered a near-perfect genetic overlap between the processing, stability, loading and activity of siRNAs generated from exogenous and endogenous *IR* loci. This finding therefore establishes RNAi as an authentic, naturally occurring silencing pathway in plants, as has been recently shown in metazoans, including fly and worm reviewed in Okamura and Lai, 2008. Although our analysis focused on two specific endogenous *IR*, it is likely that the genetics and long-distance signalling capacities described here will apply to many additional genomic loci of *Arabidopsis*. These notably include abundant loci that form perfect or near-perfect stem–loops of various sizes and map to remnant or active protein-coding genes and to transposons (Lindow *et al*, 2007). In the following sections, we discuss how the genetic and evolutionary features of endogenous *IR*s might have implications in the broad contexts of phenotypic

plasticity, adaptation and epiallelism in plants, and how, more pragmatically, they might also help clarify issues pertaining to the mechanism of RNA silencing in plants.

Endogenous IR loci as molecular probes of RNA-silencing mechanisms

Previous studies of transgenic *Arabidopsis* have raised some issues regarding the role of specific RNA-silencing pathway components in non-cell-autonomous RNAi (Dunoyer *et al*, 2007; Smith *et al*, 2007). These analyses involved two distinct SUC-driven *IR* transgenes, including SUC:*SUL* used here, or a related construct targeting the phytoene desaturase (PDS) transcript (SUC:*PDS*; Smith *et al*, 2007). Both studies converged in establishing that mutations in *NRPD1*, *RDR2* and *CLSY1* abolish the cell-to-cell signalling of RNA silencing, an unexpected result given the known endogenous role of these factors in *cis*-acting, heterochromatic silencing mediated by 24-nt siRNAs. The two analyses diverged, however, in the identification, in the SUC:*PDS* study, of *dcl3* and *ago4* as mutations that enhanced cell-to-cell silencing movement from the veins to adjacent cells (Smith *et al*, 2007). Accordingly, in WT plants, the SUC:*PDS* hairpin was found to generate vastly disproportionate amounts of 24-nt siRNAs, unlike in the SUC:*SUL* system in which 21- and 24-nt siRNAs accumulate to similar levels, as seen here with the 22- and 24-nt siRNA species derived from *IR71* and *IR2039*. On the basis of this and other results, the authors of the SUC:*PDS* study proposed that 24-nt siRNAs have roles in RNAi signalling, and positioned *NRPD1*, *RDR2* and *CLSY1* upstream of *DCLs* in the movement pathway, because mutations in those genes caused a strong decrease in *IR*-derived, 24-nt siRNA accumulation (Smith *et al*, 2007). By contrast, none of those mutations affected the accumulation of siRNAs derived from the SUC:*SUL* construct, nor did they impinge AGO1-dependent intracellular silencing of *SUL* (Dunoyer *et al*, 2007). Likewise, mutations in *NRPD1*, *RDR2* or *CLSY1* did not affect siRNA production from *IR71* and *IR2039* (Supplementary Figure S3C). On the basis of these observations and the finding that 24-nt siRNAs can induce dose-dependent, *cis*-methylation of endogenous *IR* loci, we propose an alternative explanation to the results obtained with the SUC:*PDS* system. Most likely, the SUC:*PDS* transgene, unlike the SUC:*SUL* transgene, is integrated into a region of the genome that was already prone to heterochromatic silencing and methylation. This feature probably attracts NRPD1 onto the transgenic *IR* DNA to produce, through RDR2 and CLSY1, large amounts of dsRNA templates of DCL3. This, in turn, leads to in-*cis* production of excessive levels of 24-nt siRNAs, subsequently targeted back to the *IR* DNA. This would increase methylation and thereby strengthen the heterochromatic state of the locus. Accordingly, inactivating DCL3 or AGO4 (the effector of 24-nt siRNAs) would release heterochromatin at the SUC:*PDS* locus, and thus enhance the production of DCL4-dependent, 21-nt siRNAs to perform exacerbated, non-cell-autonomous RNAi. Based on the converging results from the analyses of endogenous IRs and of the SUC:SUL IR, we propose, therefore, that NRPD1, RDR2 and CLSY1 are required downstream from DCL4 and AGO1 in cell-to-cell and possibly long-distance post-transcriptional gene silencing (Dunoyer *et al*, 2007). Consistent with this idea, our unpublished data suggest an effect for those factors at the level of signal sensing, in target cells, as established from tissue-specific rescue experiments akin to those published by our laboratory recently (Dunoyer *et al*, 2010). This example therefore illustrates how, beyond their potential effect on various aspects of plant biology (see below), endogenous *IR* loci constitute useful molecular probes of the mechanisms of RNA silencing.

Molecular sensors of the environment?

So far, *IR*s with an extended fold-back structure have been mostly considered as relatively ill-defined, primary steps in the evolution of young *MIRNA* loci (Allen *et al*, 2004; Fahlgren *et al*, 2007). *IR* loci have also been regarded as having little regulatory potential of their own, notably because they are thought to be expressed at low or very low levels to avoid the off-targeting effects of their associated siRNA populations (Allen *et al*, 2004; Voinnet, 2009). On the basis of their near-perfect genetic and functional analogy to exogenous RNAi transgenes, we propose, on the contrary, that *IR* loci represent bona fide regulators of gene expression, which might allow plants to sense, respond to, and perhaps memorize, changes in their direct environment. Thus, we have shown that *IR* loci may arise and collapse at an ecotype-based scale, and represent, therefore, some of the fastest evolving genes of plants. We have also shown that siRNAs derived from such loci are functional in mediating gene silencing in *cis* and *trans*, at both transcriptional and post-transcriptional levels. Notably, we could identify several endogenous transcripts that probably undergo RNAi through *IR71*-derived 22-nt siRNAs, which are abundantly loaded into AGO1 ($>6000/1\,683\,581$ reads in IPs), the cognate effector of post-transcriptional gene silencing. We could also show that *IR*-derived 24-nt siRNAs can direct DNA methylation over long distances, as evidenced by transmission of their epigenetic effects through grafting. Finally, we have shown that *IR* loci may display highly specific expression patterns and may be transcribed at high levels, particularly in response to external stimuli. These evolutionary and functional features have at least two foreseeable effects on plant biology. Firstly, it is conceivable that induction/repression of siRNA populations at *IR* loci has roles in adapting sequence-specific plant responses to stress, not only at the sites of its induction, but also at the level of the entire organism owing to the mobility of the siRNAs involved. The high level evolutionary fluidity of the *IR* loci is ideally suited to their possible function as 'molecular sensors', because it allows novel regulatory siRNA populations to evolve or collapse within discrete ecological niches. siRNAs from such populations may thus be probed for functionality and some might be potentially fixed by positive selection if they have adaptive value. The system might be given further flexibility through concerted or random evolution of DCL usage, which might modify the regulatory output of *IR*-derived siRNAs as shown here with *IR71* in the Ms-0 and Kas-1 accessions of *Arabidopsis*. It will be interesting to determine the extent to which evolution of endogenous *IR* loci is driven by the environment and results from natural selection rather than genetic drift.

Endogenous IRs and their potential role in epiallelism

The second foreseeable biological implication of *IR* loci is as potential sources of epialleles. Although only a few natural cases of epilallelism have been documented so far, the potential reversibility and flexibility of the process has

prompted speculation that it might constitute a widespread, and perhaps preferential, form of gene regulation in sessile organisms such as land plants (Finnegan, 2002; Vaillant and Paszkowski, 2007). A role for a transposon-derived *IR*, in essence similar to that described here with *IR71* and *IR2039*, has already been uncovered with the characterization of the *Mu killer (Muk) IR* locus (Slotkin *et al*, 2005; Lisch, 2009), which was shown to trigger heritable, *trans*-silencing of the entire *MuDR* transposon family in maize. Transposons have been linked to the establishment, maintenance and erasure of epigenetic states, with potentially profound phenotypic consequences (Banks *et al*, 1988) notably through control of gene expression, consistent with the visionary model of Barbara McClintock (McClintock, 1956). Thus, the findings made using *Muk* raise the interesting concept where epigenetic states at a genome-wide scale might be controlled through a single *IR* locus. This concept is even more attractive given the fact that, in the few cases investigated, extra-genic sources of epialleles are not readily identifiable, so that chromatin states seem to be maintained throughout generations independently of an elusive primary triggering event (Cubas *et al*, 1999; Finnegan, 2002; Vaillant and Paszkowski, 2007). Owing to (i) their *cis* and long-distance DNA methylation properties, (ii) the nucleotide-sequence specificity of their effects and—perhaps more critically—(iii) their rapid pace of birth and death, we propose that endogenous *IR* loci might constitute discrete, and possibly evolutionarily transient, sources of heritable epigenetic modifications.

De novo formation of an *IR* locus through genomic duplication–inversion events occurring, say, in one particular *Arabidopsis* accession, could trigger the accumulation of ecotype-specific siRNAs. These could have a *cis*- and possibly *trans*-methylation potential not only in somatic, but also in reproductive tissues, pending on *IR* expression patterns and/or IR-derived siRNA movement. Gametophytic *trans*-methylation patterns could then be propagated in progenies through the action of maintenance methylases such as MET1 (Ronemus *et al*, 1996; Saze *et al*, 2003; Mathieu *et al*, 2007) and could potentially become independent of the primary source of siRNA, that is, the *IR* locus itself. Independence could be achieved through out-breeding, or simply through lack of positive selection necessary to maintain the integrity of the initial *IR* locus, which could then become eventually invisible in nonetheless epigenetically modified progenies. In essence, the existence of such a process under natural conditions has been confirmed by the finding that the effects of the *MuK* locus on the epigenetic state of *MuDR* could be maintained upon segregation of the *MuK IR* (Slotkin *et al*, 2005). This sort of a scenario could also explain why DNA methylation of most repeat elements can be maintained in the apparent absence of siRNAs in *Arabidopsis* (Teixeira *et al*, 2009). The possibility of such a process is also evidenced in artificial settings utilizing tobacco plants expressing a reporter transgene under control of the 35S promoter (Jones *et al*, 2001). When these plants were infected with a meristem-infecting recombinant virus carrying sequences of the 35S promoter, transgene promoter methylation and ensuing transcriptional gene silencing (TGS) were not only manifested in the apexes, but also in the progenies, over several generations. Moreover, the rate of TGS transmission exceeded by far the rate of viral seed transmission and was dependent upon the integrity of the tobacco homologue of MET1, required for maintenance of CG methylation at the 35S promoter (Jones *et al*, 2001). In the light of the above examples, the widespread occurrence of endogenous hairpins akin to *IR71* or *IR2039* makes it possible that evolutionarily transient, long dsRNA contributes significantly to stable epiallelism.

Mobility of endogenous RNA silencing

We conclude from this and another study (Dunoyer *et al*, 2010; Molnar *et al*, 2010) that all size classes of endogenous siRNA have the potential to move between cells and over long distances in *Arabidopsis* and probably other plant species. In principle, there is no reason to exclude the movement of other types of endogenous small RNAs, including those that are generated from loci, which, unlike *IR*s, are not genetically designed to form extensive dsRNA. Hence, experiments performed using transgenes support the possibility of phloem-transmission of some stress-modulated miRNAs (Pant *et al*, 2008), while recent studies suggest that physical movement of heterochromatic siRNAs from vegetative to reproductive nuclei accounts for epigenetic reprogramming in the male gametophyte (Slotkin *et al*, 2009). siRNA mobility between mature plant tissues can be rationalized under various biological circumstances that include long-distance gene regulation, for instance as part of stress adaptation (evoked in the previous section) or developmental patterning, as well as antiviral defence, where cell-to-cell and long-distance movement of virus-derived siRNAs probably immunizes naive cells ahead of the infection front (Havelda *et al*, 2003; Chitwood *et al*, 2009). The finding that all siRNA size classes might be potentially mobile is interesting in the prospect of antiviral defence, as it suggests that the 24-nt siRNA products of DCL3, which typically accumulate to high levels during DNA virus infection, might account for systemic, transcriptional gene silencing of viral episomes or mini-chromosomes through cytosine methylation (Raja *et al*, 2008).

While it can be anticipated that systemic antiviral defence will be relatively unrestricted by the host to keep pace with the high replication rates of viruses, the prospect of endogenous gene regulation by mobile siRNAs raises the important issue as to how the process might be regulated at the cell-to-cell and long-distance levels. At present, little information is available about the fate of mobile siRNA within and outside incipient cells, yet specific sub-cellular compartmentalization might constitute a principal, preliminary step in the channelling of endogenous siRNAs towards mobility as opposed to cellular retention. In this respect, the biolistic procedure described here and elsewhere (Dunoyer *et al*, 2010) now provides a handle to the cell biology of siRNAs, present not only in delivered, but also in adjacent cells, including the vascular cells of bombarded leaves.

One puzzling aspect that might indicate specific regulation of long-distance transport is the observation, made by us and by others, that graft-transmission of endogenous small RNAs in *Arabidopsis* appears to be much more efficient from shoots to roots, unlike for transgene-derived or virus-derived siRNAs, which seem to move both ways (Palauqui *et al*, 1997; Voinnet *et al*, 1998, 2000; Brosnan *et al*, 2007). The phenomenon could merely reflect the physiological state of the sink-to-source allocation of photo-assimilates in *Arabidopsis*. It might, however, equally indicate the existence

of tighter control devices for the entry of endogenous small RNA in aerial tissue, as opposed to root apexes, particularly in the meristems, the plant stem cell niches that give rise to all tissues, including gametophytes. The existence of such devices might notably ensure selectivity in the type of siRNAs that reach the reproductive organs. This might be particularly crucial if some of these molecules, such as the *IR*-derived 24-nt siRNAs, have the potential to mediate DNA methylation, and therefore, to trigger potentially heritable epigenetic changes according to the schemes described in the previous section of this discussion. In *C. elegans*, feeding or 'environmental' RNAi can disseminate to virtually all of the worm's organs, including the germ line, such that sequence-specific gene silencing can be transmitted to, and maintained in progenies over several generations through mechanisms that likely involve chromatin modifications (Alcazar *et al*, 2008). On the basis of these findings in the worm, it is thus conceivable that the endogenous, systemic and sequence-specific silencing process uncovered in this study is part of an elaborated epigenetic mechanism that allows plants to memorize, as progeny populations, information perceived in the soma of individual progenitors.

Materials and methods

Plant material

A. thaliana mutants *dcl1-9*, *dcl2-1*, *dcl3-1*, *dcl4-2*, *rdr1-1*, *rdr2-1*, *rdr6-15*, *drb4-1*, *xrn4-1*, *hen1-1*, *hen1-6*, *hyl1-1*, *hst1*, *ago4-3*, *nrpd1a-1*, *nrpd1b-1*, *sgs3-14*, *sde5-2* and the SUC:*SUL* reference line have been described previously (Lu and Fedoroff, 2000; Zilberman *et al*, 2003; Peragine *et al*, 2004; Park *et al*, 2005; Pontier *et al*, 2005; Adenot *et al*, 2006; Bouche *et al*, 2006; Deleris *et al*, 2006; Hernandez-Pinzon *et al*, 2007; Curtin *et al*, 2008). *drb2-1* (GABI_348A09), *drb3-2* (Salk_022644), *drb5-2* (Salk_126609), *clsy1-7* (Salk_018319), *sde3-5* (Salk_003347) were obtained from the Salk Institute Genome Analysis Laboratory (La Jolla, CA) or from the GABI-Kat collection. The *A. thaliana* reference ecotypes used were Columbia (Col-0), Landsberg erecta (Ler) and Nossen (No-0). After the mutants were crossed with the SUC:*SUL* reference line, the progeny were selfed and homozygous mutant genotypes were selected by allele-specific PCR using the F2 population. The *dcl2-dcl3*, *dcl2-dcl4*, *dcl3-dcl4* and *dcl2-dcl3-dcl4* mutant lines were as described previously (Deleris *et al*, 2006). Other *Arabidopsis* accessions were obtained from the Nottingham Arabidopsis Stock Centre.

The GFP sensor for *IR71* was constructed following the procedure previously described by Parizotto *et al*, 2004. The transgene was introduced into the appropriate background by the floral dip method, according to Bechtold and Pelletier (1998). For GFP imaging, pictures were taken using a Nikon SMZ1500 dissecting microscope coupled to a 100-W epifluorescence module (Nikon).

RNA analysis

Total RNA was extracted from *Arabidopsis* tissues using the Tri-Reagent (Sigma, St Louis, MO) according to the manufacturer's instructions. RNA gel blot analysis of high- and low-molecular-weight RNA was performed using 10 and 30 μg of total RNA, respectively, and was performed as described previously (Dunoyer *et al*, 2004). Ethidium bromide staining of total RNA before transfer was used to confirm equal loading. Radiolabelled probes for detection of the *SUL*, *IR71* or *IR2039* siRNAs were made by random priming reactions in the presence of α-32P-dCTP (Amersham). The template used was a 400-bp-long (for *SUL*), 650-bp-long (for IR71 probe 1), 372-bp-long (for *IR71* probe 2) and 670-bp-long (for *IR2039*) PCR product amplified from the *Arabidopsis* cDNA. DNA oligonucleotides complementary to miRNAs, *trans*-acting siRNAs or heterochromatic siRNAs were end-labelled with γ-32P-ATP using T4 PNK (New England Biolabs, Beverly, MA).

Protein analysis

Total proteins were extracted from 4-week-old seedlings or from the flower buds of *Arabidopsis* and resolved by SDS–PAGE. After electroblotting on an Immobilon-P membrane (Millipore), protein gel blot analysis was performed using the appropriate antiserum. Rabbit antisera were raised against a peptide designed in AGO4 (CELKKRNPNENGEFET) and affinity-purified by Eugentec (Eurogentec SA, Belgium).

Immunoprecipitation

The peptide used to raise rabbit polyclonal antibodies against AGO1 was described previously (Qi *et al*, 2005). Antibodies were affinity-purified before use. For immunoprecipitation, 1 g of 3- to 4-week-old seedlings or 0.3 g of flower buds were ground in liquid nitrogen and homogenized in 3 ml/g of extraction buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP-40) containing 1 tablet/10 ml of protease inhibitor cocktail (Roche) for 1 h at 4°C. Cell debris were removed by centrifugation at 12 000 *g* at 4°C for 30 min. Extracts were precleared by incubation with protein-A–agarose (Roche) at 4°C for 1 h. The precleared extracts were then incubated with affinity-purified AGO1 or AG04-specific antibodies and protein-A–agarose overnight at 4°C. The IPs were washed three times (20 min each) in extraction buffer. Aliquots of the IPs and of the supernatant were collected before the first wash to assess the efficiency of immunoprecipitation by Western blot analysis. For RNA analysis, immune complexes were subjected to Tri-Reagent extraction (Sigma).

sqPCR and qPCR

Semi-quantitative methylation-sensitive PCR was performed using approximately 50 ng of genomic DNA from the indicated tissue (either leaf or root) and genotype, and digested with either the methylation-sensitive enzymes *Alu*I or *Sau*96I. After digestion samples were extracted with phenol:chloroform, precipitated and amplified using the primers IR71meth-F and IR71meth-R (primers sequences available upon request) to assess *IR71* methylation. As a loading control the same DNA was amplified using the primers act2-F and act2-R, with the resulting amplicon lacking both restriction sites. As control for digestion, PCR products containing either *Alu*I or *Sau*96I from actin-2 were amplified (data not shown) using the primers act2-AluI-F and act2-AluI-R, and act2-Sau96I-F and act2-Sau96I-R, respectively. Each PCR was repeated on duplicate DNA extractions with similar results obtained.

Real-time qPCR was performed using the same samples described above using a 2 × LightCycler 480 SYBER Green master mix (Roche) in a LightCycler 480II machine. Methylation was assessed using the same primers described for sqPCR, including those used for digestion confirmation. Duplicate digested DNA extractions, each in triplicate, were subjected to the following cycling 45 times as follows: 95°C for 10 s, 60°C for 15 s and 72°C for 30 s. The number of cycles after which fluorescence reached a set threshold (C_t value) was averaged for each triplicate and expressed as a ratio to the actin-2 loading control.

Biolistic delivery

siRNAs corresponding to regions of mGFP5 were purchased from Invitrogen and were obtained as annealed double-stranded molecules. The positions of 5′ and 3′ ends relative to the ATG start codon are indicated in parentheses: sense siRNA 21 nt, 5′(476)-GCCACAA GUUGGAAUACAA-3′(494); antisense siRNA 21 nt, 5′(494)-UUGUAU UCCAACUUGUGGC-3′(476); sense siRNA 24 nt, 5′(473)-UCGGCCA CAAGUUGGAAUACAA-3′(494); antisense siRNA 24 nt, 5′(494)-UUG UAUUCCAACUUGUGGCCGA-3′(473); 3′ terminal TT were added systemically according to Elbashir *et al* (2001).

In vitro-grown *Arabidopsis* seedlings 12–15 days after germination were bombarded using a PDS-1000/He particle delivery system (Bio-Rad). siRNAs were loaded on gold particles and were delivered at 1300 psi following the manufacturer's recommendations. Movement of ALEXA555-labelled siRNAs was imaged using a Leica Z16APO Macrofluo equipped with a × 5 Plan Apo main objective and × 20– × 30 additional zoom factor, using a Leica DFC360FX camera and LAS 3.4.1 imaging software. For ALEXA555, a 546/12, 560, 605/75 nm (excitation, dichromatic, emission) filterset was used.

Solexa deep-sequencing

Three weeks after grafting, leaves or roots were havested and total RNA was extracted using Tri-Reagent. A 10 μg weight of total RNA

was used for preparation of small-RNA libraries (18–26 nt in length). After 5′ and 3′ linker ligation followed by RT–PCR, the libraries were sequenced using a Illumina Genome Analyser and the resulting reads were analysed for their ability to perfectly match the Col-0 genome sequences (Fasteris, Switzerland).

GUS staining

The prom-IR71:GUS and prom-IR2039:GUS constructs were created by PCR amplifying the 1500-bp upstream region of At3g06435 (for *IR71*) and At3g44570 (for the *IR2039* locus), and cloning into the pENTR1A vector between the *Eco*RI and *Xho*I sites. The promoter fragment was then remobilized by LR clonase-II recombination (Invitrogen) into pMDC162 (Curtis and Grossniklaus, 2003). Histochemical staining was performed as described previously by Jefferson (1987).

Grafting of Arabidopsis

Grafting was performed by the butt grafting method described by Turnbull *et al* (2002), with some modifications. Seedlings were germinated on Murashige and Skoog medium, with the plates orientated vertically. The grafting procedure was performed on a single 0.45 μm nitrocellulose filter (Millipore, Bedford, MA) on top of two pieces of moist Whatman (Maidstone, UK) no. 1 filter paper in a 90 mm Petri dish. Scions were produced by using a no. 15 scalpel blade, slicing within about a millimeter of the apex of the seedling. When necessary, one cotyledon was removed to orientate the scion as close to the membrane as possible. Rootstocks were generated by the same cutting procedure that was used to produce scions. Grafts were aligned by using a dissecting microscope and plates were sealed with parafilm and incubated vertically at 21°C for 7 days. The grafted plants were then transferred to soil and grown under long-day length at 21°C for 3 weeks.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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