Global effects of the small RNA biogenesis machinery on the *Arabidopsis thaliana* transcriptome

Sascha Laubinger[a,b,1], Georg Zeller[a,c,1], Stefan R. Henz[a], Sabine Buechel[a,b], Timo Sachsenberg[a], Jia-Wei Wang[a], Gunnar Rätsch[c], and Detlef Weigel[a,2]

[a]Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany; [b]Center for Plant Molecular Biology (ZMBP), University of Tübingen, 72076 Tübingen, Germany; and [c]Friedrich Miescher Laboratory of the Max Planck Society, 72076 Tübingen, Germany

This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2009.

Contributed by Detlef Weigel, August 31, 2010 (sent for review August 8, 2009)

In *Arabidopsis thaliana*, four different dicer-like (DCL) proteins have distinct but partially overlapping functions in the biogenesis of microRNAs (miRNAs) and siRNAs from longer, noncoding precursor RNAs. To analyze the impact of different components of the small RNA biogenesis machinery on the transcriptome, we subjected *dcl* and other mutants impaired in small RNA biogenesis to whole-genome tiling array analysis. We compared both protein-coding genes and noncoding transcripts, including most pri-miRNAs, in two tissues and several stress conditions. Our analysis revealed a surprising number of common targets in *dcl1* and *dcl2 dcl3 dcl4* triple mutants. Furthermore, our results suggest that the DCL1 is not only involved in miRNA action but also contributes to silencing of a subset of transposons, apparently through an effect on DNA methylation.

dicer-like | DNA methylation | miRNA | transposon

Like other plants, the widely used model species *Arabidopsis thaliana* produces a complex population of small RNAs (sRNAs). These sRNAs come in two major flavors: microRNAs (miRNAs), most of which are 20–22 nt long, and siRNAs, with a typical length of 23–24 nt. Most sRNAs are derived from longer precursor RNAs, which are either dsRNA molecules or ssRNA molecules that form a self-complementary fold-back structure. These RNAs are processed to sRNAs by four different dicer-like (DCL) proteins, DCL1, DCL2, DCL3, and DCL4 (reviewed in ref. 1).

DCL1 is mainly involved in the generation of miRNAs, which are derived from longer primary miRNA (pri-miRNA) transcripts that are transcribed by polymerase II (polII) (2). Pri-miRNAs are first trimmed by DCL1 to precursor miRNAs (pre-miRNAs), from which DCL1 further excises the miRNA/miRNA* duplexes (3). DCL1 interacts with the dsRNA binding protein hyponastic leaves 1 (HYL1) and the zinc-finger protein serrate (SE) (4–10). Formation of this complex occurs in nuclear dicing bodies and is required for accurate processing activity of DCL1 (10, 11). The core miRNA biogenesis machinery probably acts in concert with associated factors that ensure proper processing of pri-miRNAs. These include the forkhead-associated domain containing protein dawdle (DDL) and the components of the nuclear cap binding complex abscisic acid ABA hypersensitive 1 (ABH1)/Cap-Binding Protein (CBP) 80 and CBP20 (12–15). Processed miRNAs subsequently associate with one of the ten *Arabidopsis* argonaute (AGO) proteins to regulate their target mRNAs by transcript cleavage and/or inhibition of translation (16–22) until the miRNA is degraded by specific sRNA degradation nuclease (SDN) proteins (23).

Other classes of sRNAs are mainly produced by DCL2, DCL3, and DCL4. SiRNAs derived from natural antisense transcripts (nat-siRNAs) are generated by DCL1 and DCL2 (24). DCL4 mainly acts in the biogenesis of transacting siRNAs (tasiRNAs) and in the generation of mobile siRNAs that communicate silencing effects between cells, but DCL4 also generates miRNAs from almost perfectly complementary miRNA fold backs (25–29). DCL3 acts in concert with RNA-dependent RNA polymerase 2 (RDR2) to generate heterochromatic siRNAs (30, 31). These 24-nt-long sRNAs guide DNA methylation, and mutations in any of the biogenesis factors cause decreased levels of DNA methylation, with subsequent loss of histone methylation (31–33). The main targets of RNA-directed DNA methylation (RdDM) in plants are pseudogenes, transposable elements, and other repetitive sequences (34, 35). Methylation of cytosines depends on the sequence context. For instance, maintenance and de novo methylation of CHG and CHH sites often require a persistent sRNA trigger, whereas symmetric CG methylation, after it is induced by sRNAs, can be maintained by RNA-independent mechanisms (33, 36–38).

Although the four DCL proteins have distinctive functions in many different sRNA-generating pathways, there is functional overlap (29, 39–43). In addition, there is an interwoven network of miRNA and siRNA pathways that requires the function of different DCL proteins. The most prominent example is the tasiRNA pathway, which relies on the coordinated action of DCL1 and DCL4 together with several other specific components (5, 27, 28, 44–46). Regulation of AGO1 brings miRNA and siRNA pathways together as well, because *AGO1* mRNA, cleaved by miR168, is a source of secondary siRNA (47).

A comprehensive side-by-side comparison that investigates to what extent miRNA and siRNA pathways regulate common sets of transcripts has been missing, although subsets of mutants have been analyzed by conventional protein-coding, gene-focused expression arrays or tiling arrays (2, 48, 49). Here, we present a comparative whole-genome tiling array analysis of RNA populations from wild-type plants, different *dcl* mutants, and two other miRNA biogenesis mutants, *hyl1* and *se*. Our study, which included two tissues and several stress treatments, led to the discovery of previously unknown targets of *Arabidopsis* DCL proteins and provided insights into overlapping activities among DCL proteins.

Results

Expression Analysis of miRNA Precursors. We analyzed RNA populations in three biological replicates from two different tissues of wild-type plants and *dcl1*-100, *hyl1*-2, and *se*-3 mutants with

Author contributions: S.L., G.Z., G.R., and D.W. designed research; S.L., S.B., and J.-W.W. performed research; S.L., G.Z., S.R.H., and T.S. analyzed data; and S.L., G.Z., and D.W. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/geo (accession no. GSE21685).

[1]S.L. and G.Z. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: weigel@weigelworld.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1012891107/-/DCSupplemental.

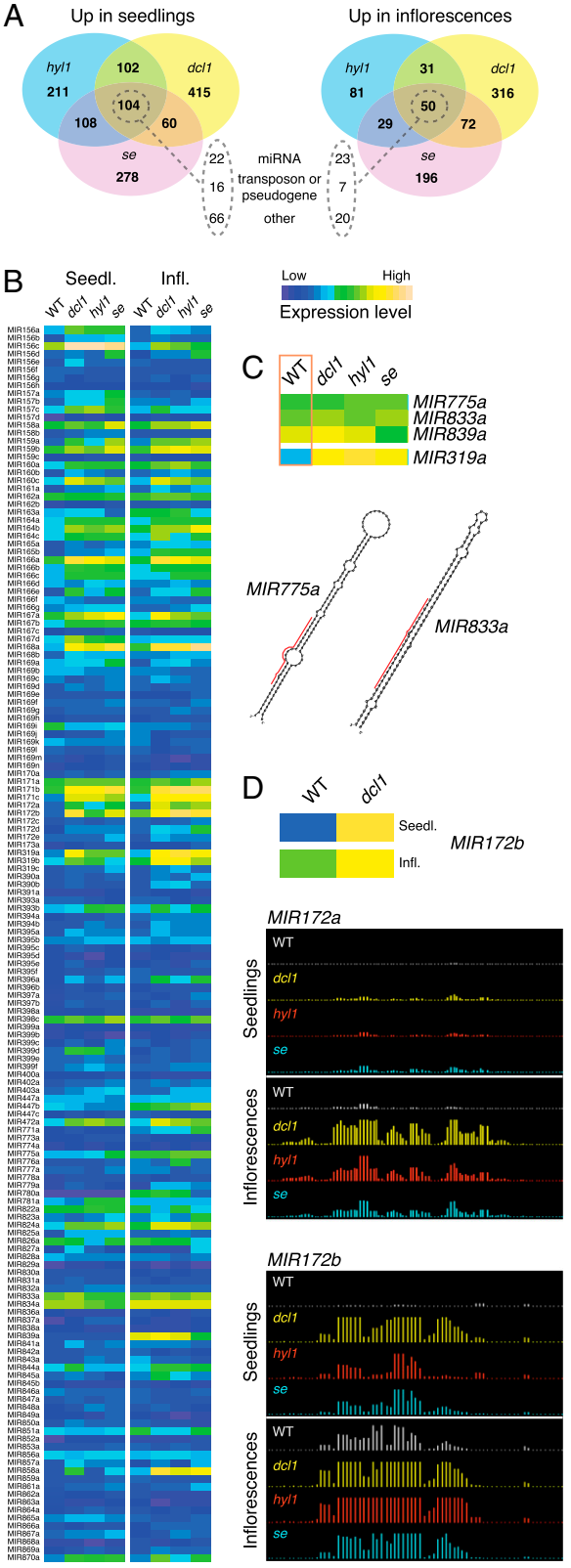

Fig. 1. Global gene expression profiles in miRNA biogenesis mutants determined with tiling arrays. (*A*) Comparison of annotated genes up-regulated in *dcl1*, *hyl1*, and *se* mutants. (*B*) Heat map of pri-miRNA expression. (*C*) Comparison of three pri-miRNAs that are highly expressed in both wild-type plants and mutants with a canonical pri-miRNA, *MIR319a*, which accumulates only in miRNA-processing mutants. Secondary structure predictions below, indicating extensive doublestrandedness of the precursors, are from the Arabidopsis Small RNA Project (ASRP) (53). (*D*) Tiling array hybridization intensities for two miR172 precursors are averaged across three biological replicates.

Affymetrix Arabidopsis Tiling1.0R whole-genome arrays, focusing first on annotated coding and noncoding genes (*dcl1*-100 described in Fig. S1). Because we expected RNAs that are turned over by the miRNA biogenesis machinery in wild type to be more abundant in the mutants, we first looked at genes with increased expression in the mutants. Consistent with miRNA precursors (pri-miRNAs) being stabilized in miRNA biogenesis mutants, these comprised the largest group of induced genes. In addition, several transposons and pseudogenes were expressed at higher levels in the mutants (Fig. 1*A*). In agreement with previous work with microarrays (48), very few miRNA target genes were significantly up-regulated in miRNA biogenesis mutants. Only a single miRNA target, *AGO2*, was significantly increased across all mutants investigated.

For most miRNA precursors, only the pre-miRNA fold backs are annotated. Where known, we, therefore, made use of published information about transcript start and end positions of pri-miRNAs (2). In cases where such information was not available, we included the signal intensities of the three probes up- and downstream of the annotated fold back. Using this extended approach, we detected 30–54 putative pri-miRNAs in seedlings and 38–44 pri-miRNAs in inflorescences that were significantly up-regulated in *dcl1*, *hyl1*, or *se* mutants (Fig. 1*B*).

There were some cases where the pri-miRNAs were detectable at similarly high levels in wild-type and mutant plants (Fig. 1*C*), suggesting that they were not efficiently processed by DCL1. Among these were *pri-miR839*, a substrate of DCL4 (25), and *pri-miR833*, which, like *pri-miR839*, has an almost perfect fold-back structure. *Pri-miR869*, another known substrate of DCL4 (50, 51), was not detectable in any of the mutants or conditions that we investigated. The fold back of another largely unaffected pri-miRNA, *pri-miR775*, exhibits an unusual four-base bulge in the miRNA/miRNA* complementary site (Fig. 1*C*). We also observed differences in the processing efficiency in the tissues analyzed. *Pri-miR172b* accumulated to similarly high levels in both seedlings and inflorescences of *dcl1* mutants. In contrast, its abundance in the corresponding wild-type tissues differs remarkably: it was detectable in wild-type inflorescences but completely turned over in young wild-type seedlings (Fig. 1*D*). These results imply that processing efficiency can be modulated in a tissue- and precursor-specific manner.

miRNA Precursor Expression in Response to Abiotic Stresses. Because the abundance of several mature miRNAs has been reported to be affected by abiotic stresses, we used the tiling array platform to investigate *dcl1* mutants exposed to salt, osmotic, cold, and heat stress as well as the stress hormone ABA for 1 and 12 h. Only a minority of pri-miRNAs responded to different stresses, including miR395 (52) (Fig. 2 *A* and *B*). Most changes were detected in response to heat stress, for example, for miR775, miR838, or miR844 (Fig. 2*B*). We also observed that potential miRNA exonucleases of the *SDN1* family were, on average, more stress-responsive than other factors involved in miRNA processing, methylation, or transport (Fig. 2*C*).

Differential Effects of DCL1, HYL1, and SE on Unannotated Transcriptionally Active Regions. We also identified unannotated transcriptionally regions (TARs) using previously described computational tools (54, 55). Between 181 and 1,306 kb, or 0.2–1.1% of the genome, were transcribed at significantly higher levels in *dcl1*, *hyl1*, or *se* mutants than in wild type. Conversely, 295–3,279 kb, or 0.2–2.7% of the genome, were transcribed at lower levels in at least one of the three mutants.

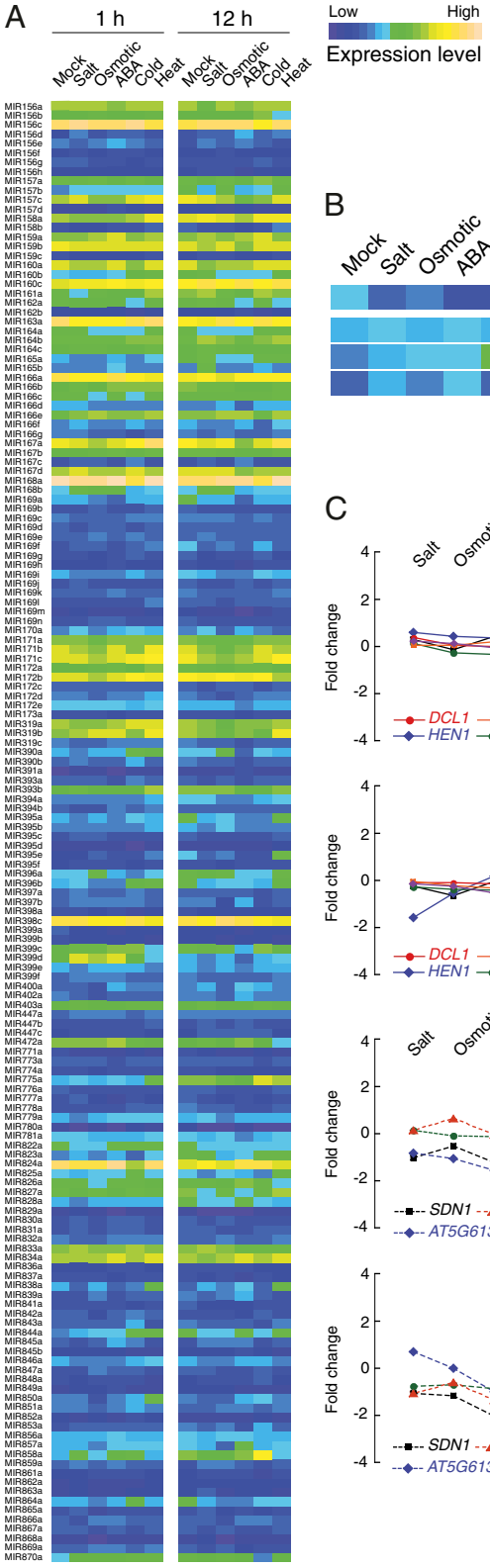

Fig. 2. Expression of *pri-miRNAs* and genes encoding miRNA biogenesis factors in stress-treated *dcl1* mutants. (*A*) Heat map of pri-miRNA expression in *dcl1* mutant seedlings. (*B*) Examples of pri-miRNAs specifically up-regulated in response to heat stress. (*C*) Expression changes of genes involved in miRNA processing and action (*DCL1*, *HYL1*, *SE*, *HEN1*, *HASTY*, and *AGO1*) and genes encoding a set of related RNA exonucleases (*SDN1*, At2g48100, At5g61390, and At1g74390).

Underexpressed TARs tended to be close to annotated genes, suggesting that these corresponded to unannotated parts of known genes, whereas TARs that were more abundant in the mutants were often far from annotated genes (Fig. 3*A*), suggesting that the miRNA biogenesis machinery has a role in managing the results of inappropriate transcription. Many of the newly identified TARs were up-regulated in all three mutants, but some were specifically more abundant only in a single mutant, indicating functional specialization (Fig. 3*B*). Whereas SE is known to have DCL1-independent roles (8, 9, 12, 13, 56), the only function described for HYL1 has been as a DCL1 cofactor (4–7, 11). It was, therefore, surprising that there were TARs that were up-regulated in *hyl1* but not *dcl1* mutants (up to 50% of all differentially expressed TARs in *hyl1* mutants) (Fig. 3 *B* and *C*). Because *hyl1*-2 mutants are phenotypically less severe than the *dcl1*-100 mutants, this cannot be explained by quantitative differences. Rather, it indicates qualitative differences, implying distinct functions for DCL1, HYL1, and SE in addition to their shared action in RNA processing.

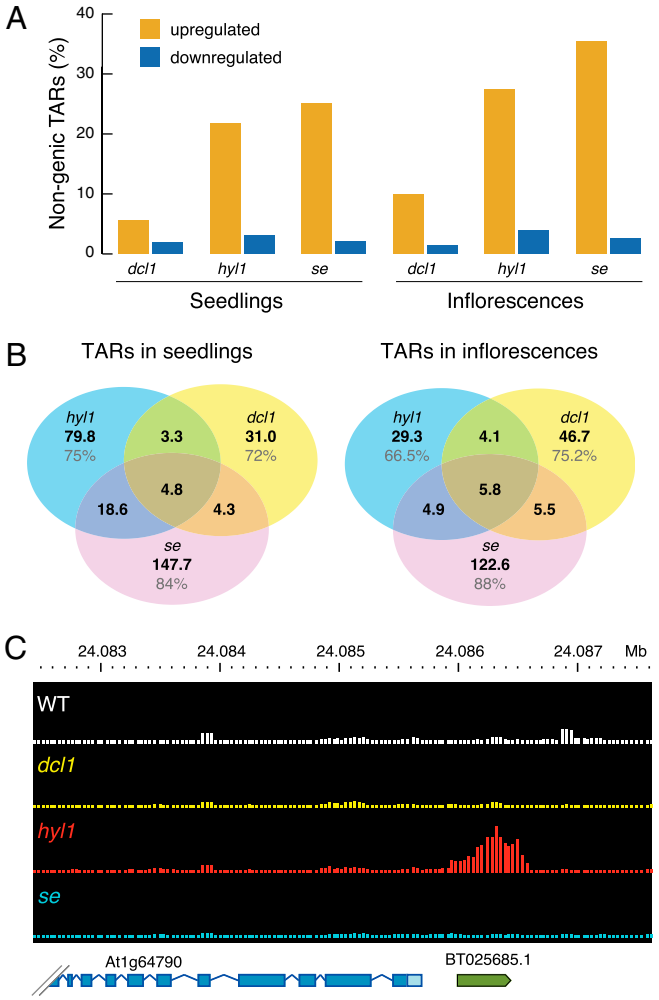

Fig. 3. Transcriptionally active regions (TARs) that specifically appear in miRNA-processing mutants. (*A*) Fractions of intergenic TARs among those that were significantly induced or repressed relative to wild type (Mann–Whitney U test, $\alpha \leq 5\%$). (*B*) Overlap of total length (in kilobases) among unannotated TARs with significantly higher expression in *dcl1*, *hyl1*, and *se* compared to wild type. Fractions of unique TARs are indicated in gray. (*C*) Hybridization intensities on tiling arrays for an unannotated TAR detected exclusively in *hyl1* mutants. An unannotated expressed sequence tag clone is shown in green.

Length of pri-miRNA Transcripts. pri-miRNAs are variable in size and often contain introns. We, therefore, asked whether some of the TARs identified in *dcl1*, *hyl1*, and *se* mutants might constitute unannotated exons of annotated miRNAs genes. Between 10% and 30% of unannotated TARs that were uregulated in inflorescences were found in a 0- to 5-kb window around annotated miRNA genes (Fig. 4*A*), suggesting that miRNA transcripts are often much longer than previously thought.

We analyzed the expression pattern of a particularly long miRNA transcript, pri-miR865, in more detail. The *miR865* fold back is located downstream of the constans (*CO*) transcription unit, for which enhanced expression of an antisense RNA in *abh1*/*cbp80* mutants has been reported (57). RT-PCR analysis confirmed that pri-miR865 accumulated to increased levels in *dcl1* seedlings and inflorescences. Additionally, we found that the pri-miR865 transcript extended all of the way into the *CO* promoter region (Fig. 4*B*). Mapping with RACE revealed that a major transcript with two introns terminated 1,677 nt upstream of the *CO* start codon. The *CO* promoter region antisense RNA is much more abundant in *dcl1* mutants than in wild type, paralleling the behavior of *pri-miR865* (Fig. 4*B*). The long *pri-miR865* transcript accumulated not only in *dcl1* mutants but also in *se*, *abh1*, and to a lesser extent, *hyl1* mutants (Fig. 4*C*). As a control, we analyzed plants with a mutation in *AGO1*, the major downstream effector of the miRNA pathway; no change was seen, further supporting the notion that the miRNA biogenesis machinery directly affects stability of the long *pri-mi865* isoform. Taken together, our results indicate that miRNA genes can produce very long transcripts that can overlap with adjacent protein coding genes.

Effects of DCL1 on Transposon Transcripts. Some of the nongenic TARs that accumulate in miRNA biogenesis mutants overlapped with annotated transposons. We analyzed two helitron-type transposons, AT1TE36060 and AT1TE93270, in more detail.

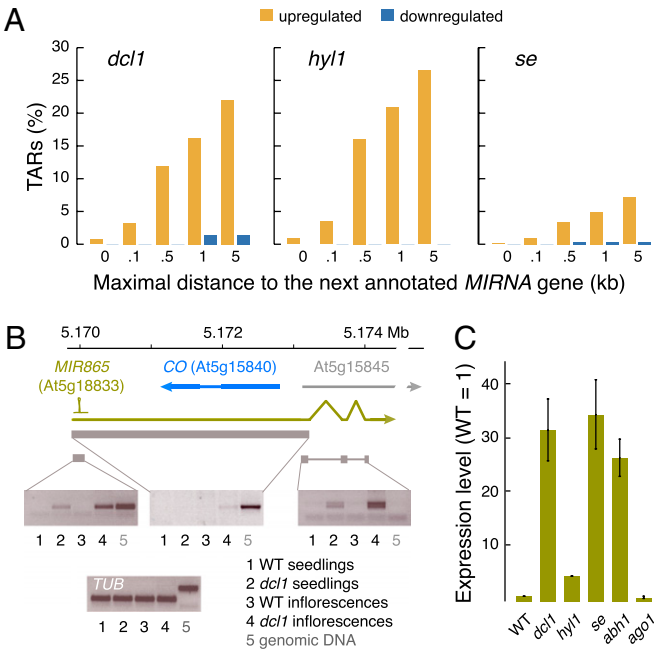

Fig. 4. Many TARs likely identify unannotated portions of pri-miRNAs. (*A*) Distances of unannotated TARs with induced expression in mutant inflorescences from annotated *miRNA* genes. (*B*) RT-PCR analysis of *pri-miRNA865*. Primers for the reaction on the right spanned splice junctions. (*C*) Quantitative RT-PCR analysis of *pri-miRNA865*. Error bars indicate the range of two independent biological experiments.

In *dcl1* mutants, we detected transcripts that partially covered the two transposons (Fig. 5*A*). Both were also detected in *hyl1* mutants, whereas only AT1TE93270 was induced in *se* mutants (Fig. 5 *A* and *B*).

The differential effects suggest distinct silencing mechanisms that silence these transposons in wild type. To investigate this further, we analyzed their expression in other sRNA-related mutants. AT1TE36060 but not AT1TE93275 expression was detected in plants lacking the three other DCL proteins, DCL2, DCL3, and DCL4 (Fig. 5 *B* and *C*). This implies that DCL1 acts in concert with other DCL proteins to repress AT1TE36060 but that AT1TE93275 is an exclusive client of DCL1. In agreement, AT1TE93275 is derepressed in *abh1* and *ago1* mutants that are affected in miRNA biogenesis or function (Fig. 5*C*).

Because transposon silencing often relies on DNA methylation (58, 59), we analyzed mutants that are impaired in siRNA-mediated de novo methylation or maintenance methylation. Whereas there were only modest changes in AT1TE36060 expression, large amounts of AT1TE93275 transcripts accumulated in all of the DNA methylation mutants investigated (Fig. 5*D*). To investigate DNA methylation directly, we first used methylation-sensitive restriction enzymes HpaII (for CG and CHG methylation) and PstI (for CHG methylation) to analyze AT1TE93275 (Fig. 5*E*). DNA methylation at AT1TE93275 was indeed strongly reduced in *dcl1* mutants, which was confirmed by bisulfite sequencing of genomic DNA (Fig. 5 *E* and *F*). These results suggest that *DCL1* can affect DNA methylation like other members of the *DCL* family do.

Comparison of DCL1 and DCL2/DCL3/DCL4 Effects. Because of overlapping effects on at least one transposon, we directly compared the transcriptomes of *dcl1* and *dcl2 dcl3 dcl4* mutants. Expression analysis of annotated genes revealed that 45 and 31 genes, respectively, were up-regulated in seedlings and inflorescences of both *dcl1* and *dcl2 dcl3 dcl4* mutants. This group included *TAS1c* as well as several targets of tasi-RNAs; all of these are known be under the direct or indirect control of *DCL1* and *DCL4*. Also, the expression of some miRNAs is increased in both *dcl1* and *dcl2 dcl3 dcl4* mutants (Fig. 6*A* and Fig. S2). About one-quarter of common targets were pseudogenes or transposable elements (Fig. 6*A*), further supporting the idea that *DCL1* acts in concert with other *DCLs* to regulate the expression of some transposons. However, *DCL1* is apparently required only for the silencing of a small subset of transposons, because many more were induced in *dcl2 dcl3 dcl4* than in *dcl1* mutants.

We also performed de novo TAR identification in *dcl2 dcl3 dcl4* plants. In total, up to 507 kb of the genome were expressed at higher levels in the triple mutants, whereas up to 3,403 kb were underexpressed. However, a much larger fraction of the induced TARs are located in intergenic regions than is the case for the underexpressed TARs (Fig. 6*B*). We observed very little overlap between unannotated TARs detected in *dcl2 dcl3 dcl4* and in *dcl1* mutants (Fig. 6*C*). We then compared genome-wide effects of loss of either *DCL1* or *DCL2* and *DCL3* and *DCL4* on transposon transcription. TARs that were more abundant in *dcl2 dcl3 dcl4* mutants compared with wild type more often overlapped with transposable elements than in the case of *dcl1* (Fig. 6*D* and Fig. S3). Taken together, these results suggest that all four DCL proteins act cooperatively on some transposons but that, otherwise, DCL1 functions largely independent of DCL2, DCL3, and DCL4.

Discussion

We have analyzed a core set of mutants impaired in proteins required for miRNA (*DCL1*, *HYL1*, and *SE*) and siRNA biogenesis (*DCL2*/*DCL3*/*DCL4*). With genome-wide tiling arrays, we have been able to detect pri-miRNAs and other unstable transcripts that are processed by these factors. Our results are

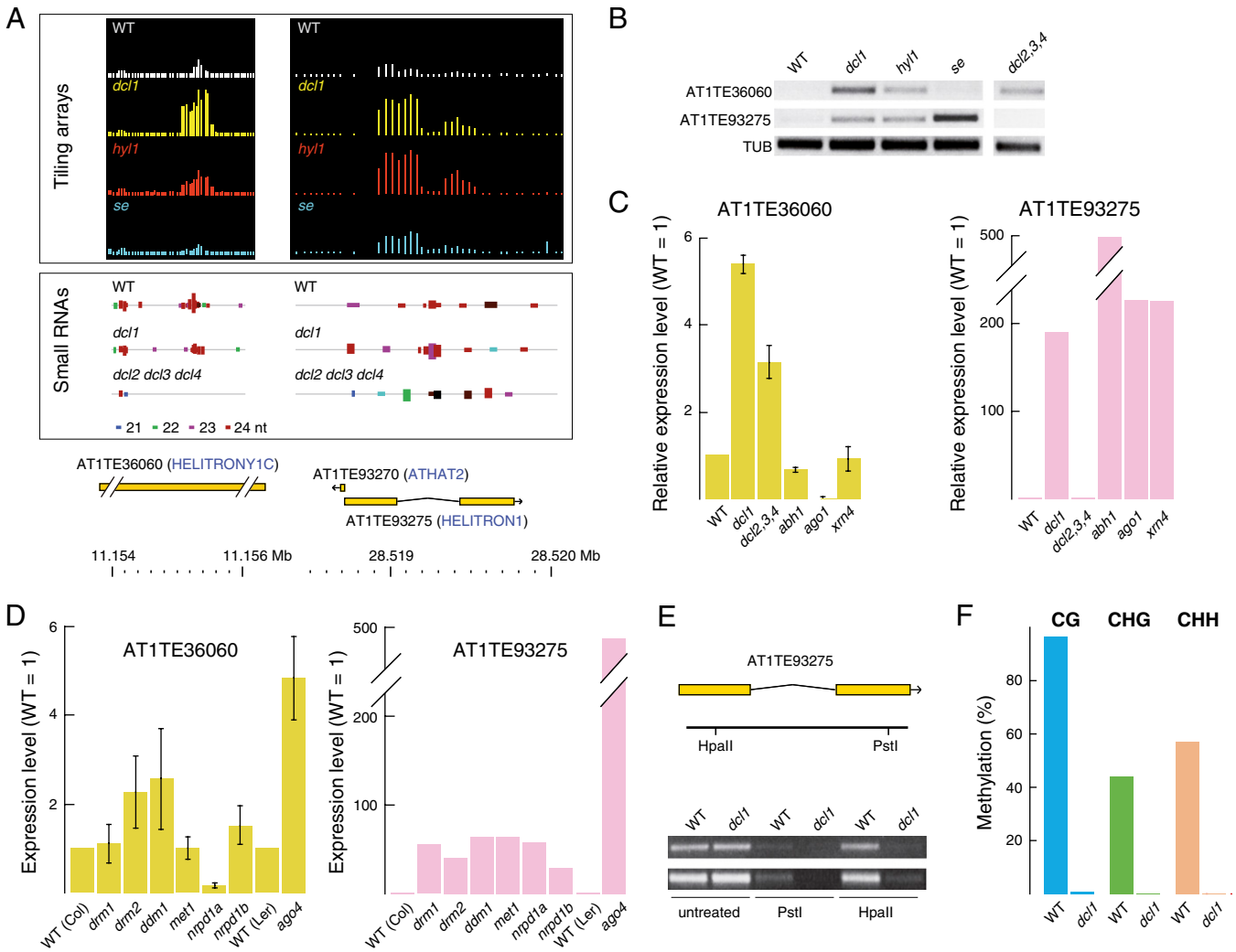

Fig. 5. Analysis of two helitron-type transposons. (*A*) Comparison of tiling array expression analysis and small RNA profiles from ref. 53. (*B*) RT-PCR analysis. (*C* and *D*) Quantitative RT-PCR analysis. (*E* and *F*) Analysis of DNA methylation using digest of genomic DNA with methylation-sensitive restriction enzymes followed by PCR (*E*) or sequencing of bisulfate-treated genomic DNA (*F*). For the latter, at least 30 clones were sequenced for each genotype. Sequence contexts are shown above.

reminiscent of studies of other RNA-processing mutants, which also revealed many transcripts that are not detectable in wild-type plants and that would have escaped detection with conventional expression arrays (12, 58, 60–62).

Expression of miRNA Precursors. Many pri-miRNAs were not obviously affected by mutations in *DCL1*, *HYL1*, or *SE*. Our observations are in agreement with a recent report that many pri-miRNAs are relatively insensitive to loss of *HYL1* (63). A trivial explanation could be that expression of pri-miRNAs is simply too low for detection on microarrays. However, there was no clear correlation between background levels in wild type and increased expression in one of the three mutants. It is possible that expression changes are obscured by feedback regulation or that other factors contribute to the stability of pri-miRNA transcripts. In addition, both *dcl1* and *se* alleles are not complete null alleles, and residual functions of the mutant proteins might be sufficient for processing of some miRNAs.

Several miRNAs are regulated by biotic and abiotic stresses (19, 52, 64–70), but we detected only a small number of pri-miRNAs that responded robustly to different stresses. This finding is consistent with what has been reported for miR159a, the levels of which are induced by ABA without an effect on the corresponding pri-miRNAs (66). These observations suggest that mature miRNAs might be differentially turned over in a given tissue or under certain stress conditions. SDN1, which belongs to a family of 15 proteins with an exonuclease domain, degrades mature miRNAs (23). Four of the genes in this family, including SDN1, were affected by at least one of the stresses that we examined. Therefore, we hypothesize that changes in miRNA turnover and stability contribute to the overall changes of the sRNA inventory under stress.

Another level of regulation of miRNA accumulation is the processing efficiency of pri- and pre-miRNAs. The RNA binding protein Lin-28 selectively blocks processing of *let-7* pre-miRNA in stem cells by directly binding to the loop region of the fold back and by uridylation of the pre-miRNA (71–75). We observed high levels of *pri-miR172b* in both *dcl1* seedlings and inflorescences, indicating that the precursor is transcribed in both tissues. As with other pri-miRNAs, *pri-miR172b* was not detectable in wild-type seedlings but accumulated to high levels in wild-type inflorescences. This suggests that pri-miRNA172b processing is at least partially suppressed in inflorescences or that specific factors promote processing in seedlings. Further

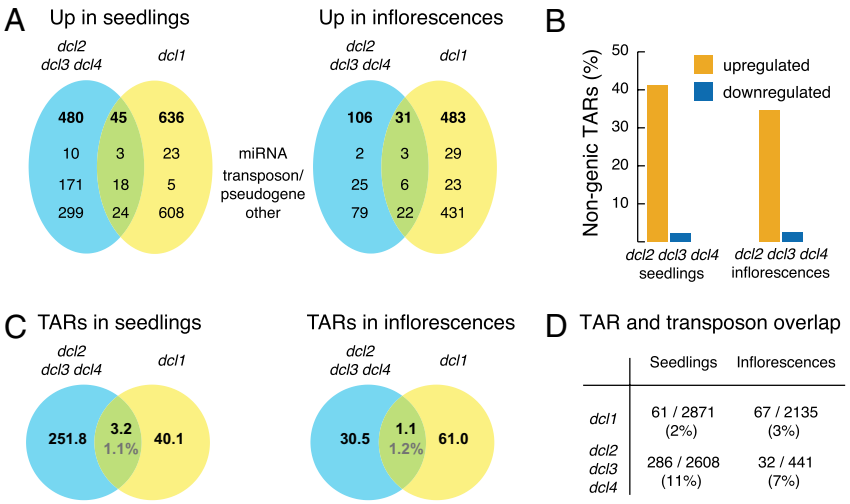

Fig. 6. Comparison of *dcl1* and *dcl2 dcl3 dcl4* mutants. (*A*) Overlap in annotated genes that were up-regulated relative to wild type at a false-discovery rate (FDR) of 0.1. (*B*) Fraction of intergenic TARs among all TARs that were significantly induced or repressed relative to wild type (Mann–Whitney U test, $\alpha \leq 5\%$). (*C*) Overlap of total length (in kilobases) among TARs that are unannotated and induced in *dcl1* and *dcl2 dcl3 dcl4* mutants. (*D*) Fractions of TARs that overlap with annotated transposable elements among TARs that were induced relative to wild type.

analysis of this locus will help to identify *Arabidopsis* proteins involved in miRNA processing efficiency. More generally, careful expression analysis of miRNA genes under different conditions might reveal other pri-miRNAs that are likely subject to posttranscriptional regulation.

In this regard, it is also of interest that some pri-miRNA transcripts are quite long, with many opportunities for recruitment of regulatory proteins. Very long pri-miRNA transcripts themselves might play additional roles not related to miRNA function, especially if they overlap with adjacent genes such as *pri-miR865*, which is part of a long antisense transcript at the *CO* locus. This locus might also be another example of a pri-miRNAs with several isoforms, because the transcript that we identified differed in length from the *CO* antisense transcripts reported before (57), with the potential caveat that these authors investigated a different strain of *A. thaliana*. One can speculate that, compared with protein coding genes, there is less selection on the length and presence of specific introns in pri-miRNAs.

Overlapping Effects of the Four DCLs on Transposons. Comparative transcriptome analysis of *dcl1* and *dcl2 dcl3 dcl4* mutants supports the conclusion that DCL1 acts mainly in miRNA processing and that it fulfills this role largely independently of other DCL proteins. However, the four DCLs also have overlapping functions; for instance, they are all involved in RNA silencing of viral transcripts, and DCL1 and DCL3 act redundantly in the control of *FLOWERING LOCUS C* expression (29, 41, 76). To this, we can now add coordinated action of DCLs in silencing a subset of transposons, exemplified by AT1TE36060. What then makes some transcripts a substrate for multiple DCL proteins? It is conceivable that DCL1 creates an initial cut in some transposon-derived RNAs, which it does in the generation of pre-miRNAs and they are processed from much longer pri-miRNAs. The cleaved, aberrant transcripts might then enter the RNAi pathway executed by other DCL proteins.

Surprisingly, transcripts derived from the AT1TE93275 transposon only require DCL1 function for a complete turnover. There is no evidence that the effect of DCL1 on transposon expression and methylation is indirect. Interestingly, several sRNAs larger than 21 nt, the typical DCL1 product, are derived from this region of the genome, even in the absence of DCL2, DCL3, and DCL4 (Fig. 5*A* and a detailed view in Fig. S4) (53, 77), consistent with DCL1 being able to generate sRNAs larger than 21 nt from certain substrates. The fact that methylation of the AT1TE93275 locus is DCL1-dependent may imply that DCL1-derived small RNAs can guide RdDM.

miRNAs can evolve from transposons (78–81). A common route for miRNA origin is from perfectly complementary fold backs that undergo a shift from DCL4- to DCL1-mediated processing. If evolution of miRNAs from transposable elements is a general phenomenon, one might expect to identify more such miRNAs in plant genomes with more transposons than in the relatively streamlined *A. thaliana* genome. Transcripts derived from transposons can also interfere with the activity of the miRNA biogenesis machinery. Intriguingly, a short interspersed transposable element RNA introduced through transgenesis can reduce HYL1 activity (82). Whether endogenous, transcribed transposons play a role in modulating DCL1, HYL1, and SE activity remains to be elucidated.

Materials and Methods

Plant Material. All mutants used in this study were in the Columbia (Col-0) background. The *dcl1*-100 (Fig. S1), *hyl1*-2, *se*-1, *se*-3, *abh1*-285, and *ago1*-27 mutants and the *dcl2*-1 *dcl3*-1 *dcl4*-2 triple mutants have been described (5, 13, 43, 56, 83, 84). *nrpd1a*-3 (SALK_128428), *nrpd2b*-1 (SALK_008535), *drm1*-2 (SALK_031705), *drm2*-2 (SALK_150863), *ddm1* (SALK_024844), *met1*-7 (SALK_076522), and *ago4*-1 (N3854) were ordered from NASC, and homozygous mutants were isolated.

Tiling Array Analyses. RNA was extracted from whole seedlings or inflorescences using the RNeasy Plant Mini Kit (Qiagen). RNA integrity was determined on a Bioanalyzer using the RNA 6000 Series II Nano Kit (Agilent). *SI Materials and Methods* has hybridization to Affymetrix Arabidopsis Tiling1.0R arrays and data analyses. Raw array data files are located in the Gene Expression Omnibus (accession number GSE21685).

RT-PCR and DNA Methylation Analyses. Please see *SI Materials and Methods* for information on RT-PCR and DNA methylation analyses.

ACKNOWLEDGMENTS. We thank Jim Carrington, Eunyoung Chae, Noah Fahlgren, Joffrey Fitz, Josef Kuhn, Yasushi Kobayashi, Julian Schroeder, Chris Sullivan, and Team miRNA in the Weigel laboratory for helpful discussion. This work was supported by Deutsche Forschungsgemeinschaft Grant LA2633/1 (to S.L.), European Community FP6 IP SIROCCO Contract LSHG-CT-2006-037900 (to D.W.), a Gottfried Wilhelm Leibniz award from the Deutsche Forschungsgemeinschaft (to D.W.), and the Max Planck Society (G.R. and D.W.).

1. Chapman EJ, Carrington JC (2007) Specialization and evolution of endogenous small RNA pathways. *Nat Rev Genet* 8:884–896.
2. Xie Z, et al. (2005) Expression of *Arabidopsis MIRNA* genes. *Plant Physiol* 138: 2145–2154.
3. Kurihara Y, Watanabe Y (2004) *Arabidopsis* micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc Natl Acad Sci USA* 101:12753–12758.
4. Han MH, Goud S, Song L, Fedoroff N (2004) The *Arabidopsis* double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation. *Proc Natl Acad Sci USA* 101:1093–1098.
5. Vazquez F, Gasciolli V, Crété P, Vaucheret H (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr Biol* 14:346–351.
6. Hiraguri A, et al. (2005) Specific interactions between Dicer-like proteins and HYL1/DRB-family dsRNA-binding proteins in *Arabidopsis thaliana*. *Plant Mol Biol* 57:173–188.
7. Kurihara Y, Takashi Y, Watanabe Y (2006) The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. *RNA* 12:206–212.
8. Lobbes D, Rallapalli G, Schmidt DD, Martin C, Clarke J (2006) SERRATE: A new player on the plant microRNA scene. *EMBO Rep* 7:1052–1058.
9. Yang L, Liu Z, Lu F, Dong A, Huang H (2006) SERRATE is a novel nuclear regulator in primary microRNA processing in *Arabidopsis*. *Plant J* 47:841–850.
10. Fang Y, Spector DL (2007) Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living *Arabidopsis* plants. *Curr Biol* 17:818–823.
11. Dong Z, Han MH, Fedoroff N (2008) The RNA-binding proteins HYL1 and SE promote accurate in vitro processing of pri-miRNA by DCL1. *Proc Natl Acad Sci USA* 105: 9970–9975.
12. Gregory BD, et al. (2008) A link between RNA metabolism and silencing affecting *Arabidopsis* development. *Dev Cell* 14:854–866.
13. Laubinger S, et al. (2008) Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 105:8795–8800.
14. Kim S, et al. (2008) Two cap-binding proteins CBP20 and CBP80 are involved in processing primary microRNAs. *Plant Cell Physiol* 49:1634–1644.
15. Yu B, et al. (2008) The FHA domain proteins DAWDLE in *Arabidopsis* and SNIP1 in humans act in small RNA biogenesis. *Proc Natl Acad Sci USA* 105:10073–10078.
16. Llave C, Xie Z, Kasschau KD, Carrington JC (2002) Cleavage of *Scarecrow*-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* 297:2053–2056.
17. Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a MicroRNA and its *APETALA2*-like target genes. *Plant Cell* 15:2730–2741.
18. Chen X (2004) A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. *Science* 303:2022–2025.
19. Dugas DV, Bartel B (2008) Sucrose induction of *Arabidopsis* miR398 represses two Cu/Zn superoxide dismutases. *Plant Mol Biol* 67:403–417.
20. Brodersen P, et al. (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science* 320:1185–1190.
21. Gandikota M, et al. (2007) The miRNA156/157 recognition element in the 3′ UTR of the Arabidopsis SBP box gene *SPL3* prevents early flowering by translational inhibition in seedlings. *Plant J* 49:683–693.
22. Lanet E, et al. (2009) Biochemical evidence for translational repression by *Arabidopsis* microRNAs. *Plant Cell* 21:1762–1768.
23. Ramachandran V, Chen X (2008) Degradation of microRNAs by a family of exoribonucleases in *Arabidopsis*. *Science* 321:1490–1492.
24. Borsani O, Zhu J, Verslues PE, Sunkar R, Zhu JK (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell* 123:1279–1291.
25. Rajagopalan R, Vaucheret H, Trejo J, Bartel DP (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes Dev* 20:3407–3425.
26. Dunoyer P, Himber C, Voinnet O (2005) DICER-LIKE 4 is required for RNA interference and produces the 21-nucleotide small interfering RNA component of the plant cell-to-cell silencing signal. *Nat Genet* 37:1356–1360.
27. Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. *Genes Dev* 19:2164–2175.
28. Xie Z, Allen E, Wilken A, Carrington JC (2005) DICER-LIKE 4 functions in trans-acting small interfering RNA biogenesis and vegetative phase change in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 102:12984–12989.
29. Gasciolli V, Mallory AC, Bartel DP, Vaucheret H (2005) Partially redundant functions of *Arabidopsis* DICER-like enzymes and a role for DCL4 in producing trans-acting siRNAs. *Curr Biol* 15:1494–1500.
30. Kasschau KD, et al. (2007) Genome-wide profiling and analysis of *Arabidopsis* siRNAs. *PLoS Biol* 5:e57.
31. Xie Z, et al. (2004) Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol* 2:e104.
32. Chan SW, et al. (2004) RNA silencing genes control de novo DNA methylation. *Science* 303:1336.
33. Lister R, et al. (2008) Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. *Cell* 133:523–536.
34. Rabinowicz PD, et al. (2003) Genes and transposons are differentially methylated in plants, but not in mammals. *Genome Res* 13:2658–2664.
35. Matzke M, Kanno T, Huettel B, Daxinger L, Matzke AJ (2007) Targets of RNA-directed DNA methylation. *Curr Opin Plant Biol* 10:512–519.
36. Henderson IR, Jacobsen SE (2007) Epigenetic inheritance in plants. *Nature* 447: 418–424.
37. Cokus SJ, et al. (2008) Shotgun bisulphite sequencing of the *Arabidopsis* genome reveals DNA methylation patterning. *Nature* 452:215–219.
38. Teixeira FK, et al. (2009) A role for RNAi in the selective correction of DNA methylation defects. *Science* 323:1600–1604.
39. Blevins T, et al. (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res* 34:6233–6246.
40. Fusaro AF, et al. (2006) RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. *EMBO Rep* 7:1168–1175.
41. Moissiard G, Voinnet O (2006) RNA silencing of host transcripts by cauliflower mosaic virus requires coordinated action of the four *Arabidopsis* Dicer-like proteins. *Proc Natl Acad Sci USA* 103:19593–19598.
42. Bouché N, Lauressergues D, Gasciolli V, Vaucheret H (2006) An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J* 25:3347–3356.
43. Deleris A, et al. (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* 313:68–71.
44. Allen E, Xie Z, Gustafson AM, Carrington JC (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell* 121:207–221.
45. Peragine A, Yoshikawa M, Wu G, Albrecht HL, Poethig RS (2004) *SGS3* and *SGS2/SDE1/RDR6* are required for juvenile development and the production of trans-acting siRNAs in *Arabidopsis*. *Genes Dev* 18:2368–2379.
46. Adenot X, et al. (2006) DRB4-dependent *TAS3* trans-acting siRNAs control leaf morphology through AGO7. *Curr Biol* 16:927–932.
47. Mallory AC, Vaucheret H (2009) *ARGONAUTE 1* homeostasis invokes the coordinate action of the microRNA and siRNA pathways. *EMBO Rep* 10:521–526.
48. Ronemus M, Vaughn MW, Martienssen RA (2006) MicroRNA-targeted and small interfering RNA-mediated mRNA degradation is regulated by argonaute, dicer, and RNA-dependent RNA polymerase in *Arabidopsis*. *Plant Cell* 18:1559–1574.
49. Kurihara Y, et al. (2009) Transcriptome analyses revealed diverse expression changes in *ago1* and *hyl1 Arabidopsis* mutants. *Plant Cell Physiol* 50:1715–1720.
50. Fahlgren N, et al. (2007) High-throughput sequencing of *Arabidopsis* microRNAs: Evidence for frequent birth and death of *MIRNA* genes. *PLoS ONE* 2:e219.
51. Ben Amor B, et al. (2009) Novel long non-protein coding RNAs involved in *Arabidopsis* differentiation and stress responses. *Genome Res* 19:57–69.
52. Jones-Rhoades MW, Bartel DP (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol Cell* 14:787–799.
53. Backman TW, et al. (2008) Update of ASRP: The Arabidopsis Small RNA Project database. *Nucleic Acids Res* 36:D982–D985.
54. Laubinger S, et al. (2008) At-TAX: A whole genome tiling array resource for developmental expression analysis and transcript identification in *Arabidopsis thaliana*. *Genome Biol* 9, 10.1186/gb-2008-9-7-r112.
55. Zeller G, Henz SR, Laubinger S, Weigel D, Rätsch G (2008) Transcript normalization and segmentation of tiling array data. *Pac Symp Biocomput* 13:527–538.
56. Grigg SP, Canales C, Hay A, Tsiantis M (2005) *SERRATE* coordinates shoot meristem function and leaf axial patterning in *Arabidopsis*. *Nature* 437:1022–1026.
57. Kuhn JM, Breton G, Schroeder JI (2007) mRNA metabolism of flowering-time regulators in wild-type *Arabidopsis* revealed by a nuclear cap binding protein mutant, *abh1*. *Plant J* 50:1049–1062.
58. Zhang X, et al. (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in *arabidopsis*. *Cell* 126:1189–1201.
59. Slotkin RK, Martienssen R (2007) Transposable elements and the epigenetic regulation of the genome. *Nat Rev Genet* 8:272–285.
60. Kurihara Y, et al. (2009) Genome-wide suppression of aberrant mRNA-like noncoding RNAs by NMD in *Arabidopsis*. *Proc Natl Acad Sci USA* 106:2453–2458.
61. Kurihara Y, et al. (2008) Identification of the candidate genes regulated by RNA-directed DNA methylation in *Arabidopsis*. *Biochem Biophys Res Commun* 376:553–557.
62. Chekanova JA, et al. (2007) Genome-wide high-resolution mapping of exosome substrates reveals hidden features in the *Arabidopsis* transcriptome. *Cell* 131:1340–1353.
63. Szarzynska B, et al. (2009) Gene structures and processing of *Arabidopsis thaliana* HYL1-dependent pri-miRNAs. *Nucleic Acids Res* 37:3083–3093.
64. Jagadeeswaran G, Saini A, Sunkar R (2009) Biotic and abiotic stress down-regulate miR398 expression in *Arabidopsis*. *Planta* 229:1009–1014.
65. Liu HH, Tian X, Li YJ, Wu CA, Zheng CC (2008) Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA* 14:836–843.
66. Reyes JL, Chua NH (2007) ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. *Plant J* 49:592–606.
67. Sunkar R, Kapoor A, Zhu JK (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18:2051–2065.
68. Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* 16:2001–2019.
69. Zhou X, Wang G, Sutoh K, Zhu JK, Zhang W (2008) Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochim Biophys Acta* 1779:780–788.
70. Zhou X, Wang G, Zhang W (2007) UV-B responsive microRNA genes in *Arabidopsis thaliana*. *Mol Syst Biol* 3:103.
71. Heo I, et al. (2008) Lin28 mediates the terminal uridylation of *let-7* precursor MicroRNA. *Mol Cell* 32:276–284.
72. Newman MA, Thomson JM, Hammond SM (2008) Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA* 14:1539–1549.
73. Piskounova E, et al. (2008) Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. *J Biol Chem* 283:21310–21314.
74. Rybak A, et al. (2008) A feedback loop comprising *lin-28* and *let-7* controls pre-*let-7* maturation during neural stem-cell commitment. *Nat Cell Biol* 10:987–993.

75. Viswanathan SR, Daley GQ, Gregory RI (2008) Selective blockade of microRNA processing by Lin28. *Science* 320:97–100.
76. Schmitz RJ, Hong L, Fitzpatrick KE, Amasino RM (2007) *DICER-LIKE 1* and *DICER-LIKE 3* redundantly act to promote flowering via repression of *FLOWERING LOCUS C* in *Arabidopsis thaliana*. *Genetics* 176:1359–1362.
77. Fahlgren N, et al. (2009) Computational and analytical framework for small RNA profiling by high-throughput sequencing. *RNA* 15:992–1002.
78. Moxon S, et al. (2008) Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening. *Genome Res* 18:1602–1609.
79. Piriyapongsa J, Mariño-Ramírez L, Jordan IK (2007) Origin and evolution of human microRNAs from transposable elements. *Genetics* 176:1323–1337.
80. Piriyapongsa J, Jordan IK (2008) Dual coding of siRNAs and miRNAs by plant transposable elements. *RNA* 14:814–821.
81. Piriyapongsa J, Jordan IK (2007) A family of human microRNA genes from miniature inverted-repeat transposable elements. *PLoS ONE* 2:e203.
82. Pouch-Pélissier MN, et al. (2008) SINE RNA induces severe developmental defects in *Arabidopsis thaliana* and interacts with HYL1 (DRB1), a key member of the DCL1 complex. *PLoS Genet* 4:e1000096.
83. Prigge MJ, Wagner DR (2001) The arabidopsis *serrate* gene encodes a zinc-finger protein required for normal shoot development. *Plant Cell* 13:1263–1279.
84. Morel JB, et al. (2002) Fertile hypomorphic *ARGONAUTE* (*ago1*) mutants impaired in post-transcriptional gene silencing and virus resistance. *Plant Cell* 14: 629–639.