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Double-stranded RNA binding proteins DRB2 and DRB4 have an antagonistic impact on polymerase IV-dependent siRNA levels in *Arabidopsis*

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ABSTRACT

Biogenesis of the vast majority of plant siRNAs depends on the activity of the plant-specific RNA polymerase IV (PolIV) enzyme. As part of the RNA-dependent DNA methylation (RdDM) process, PolIV-dependent siRNAs (p4-siRNAs) are loaded onto an ARGONAUTE4-containing complex and guide de novo DNA methyltransferases to target loci. Here we show that the double-stranded RNA binding proteins DRB2 and DRB4 are required for proper accumulation of p4-siRNAs. In flowers, loss of DRB2 results in increased accumulation of p4-siRNAs but not ta-siRNAs, inverted repeat (IR)-derived siRNAs, or miRNA. Loss of DRB2 does not impair uniparental expression of p4-dependent siRNAs in developing endosperm, indicating that p4-siRNA increased accumulation is not the result of the activation of the polIV pathway in the male gametophyte. In contrast to *drb2*, *drb4* mutants exhibit reduced p4-siRNA levels, but the extent of this reduction is variable, according to the nature and size of the p4-siRNAs. Loss of DRB4 also leads to a spectacular increase of p4-independent IR-derived 24-nt siRNAs, suggesting a reallocation of factors from p4-dependent to p4-independent siRNA pathways in *drb4*. Opposite effects of *drb2* and *drb4* mutations on the accumulation of p4-siRNAs were also observed in vegetative tissues. Moreover, transgenic plants overexpressing DRB2 mimicked *drb4* mutants at the morphological and molecular levels, confirming the antagonistic roles of DRB2 and DRB4.

Keywords: RNAi; siRNA; double-stranded RNA binding protein; non-coding RNA; Dicer

INTRODUCTION

Plants contain complex populations of small RNAs involved in different RNA-based silencing pathways that function at the transcriptional and/or post-transcriptional level to control genes, viruses, and transposable elements (Jamalkandi and Masoudi-Nejad 2009; Ruiz-Ferrer and Voinnet 2009; Voinnet 2009; Law and Jacobsen 2010). *Arabidopsis* small RNAs are produced by the activity of four Dicer-like ribonucleases (DCLs) on double-stranded RNA precursors of different origins and structures (Bouche et al. 2006; Henderson et al. 2006; Mlotshwa et al. 2008; Liu et al. 2009). The vast majority of *Arabidopsis* small RNAs (>90%

of the plant global small RNA mass) consists of siRNAs that depend for their biogenesis on the capacity of RNA polymerase IV (PolIV), a homolog of DNA-dependent RNA polymerase II (PolII), to transcribe thousands of intergenic loci (Rajagopalan et al. 2006; Kasschau et al. 2007; Zhang et al. 2007). PolIV-derived single-stranded RNA precursors are converted to long double-stranded molecules by the action of the RNA-dependent RNA polymerase 2 (RDR2) and are mainly cleaved in small 24-nt dimers by the action of DCL3 (Lahmy et al. 2010). The accumulation of some, but not all, PolIV-dependent small RNAs (p4-siRNAs) depends on the activity of RNA polymerase V (PolV), an enzyme related to PolIV but presenting a specific carboxy-terminal domain containing evolutionarily conserved GW/WG repeats (El-Shami et al. 2007). P4-siRNAs have been classified in two categories named type I and type II (Mosher et al. 2009). Type I p4-siRNAs are produced exclusively in flowers and siliques, while type II p4-siRNAs are produced in almost all plant tissues with, again, a maximum of expression in flowers and siliques (Kasschau et al. 2007; Mosher

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et al. 2009). P4-siRNAs are completely absent from the paternal lineage, and all p4-siRNAs accumulating in the developing seeds are inherited maternally (Mosher et al. 2009). P4-siRNAs associate with ARGONAUTE 4 (AGO4), AGO6, or AGO9 (Havecker et al. 2010) and play a critical role in the RNA-directed DNA methylation (RdDM) process, as they can guide a complex set of proteins leading to DNA methylation and chromatin structure modifications at RdDM loci (Chinnusamy and Zhu 2009).

MicroRNAs (miRNAs) represent the second largest population of plant small RNAs. MiRNAs are produced from PolIII (and not polIV) transcripts that can adopt a foldback structure (Rajagopalan et al. 2006; Kasschau et al. 2007). Most miRNAs result from the cleavage of this precursor by a complex composed of DCL1, the double-stranded RNA binding protein (dsRBP) DRB1, and the zinc-finger protein SERRATE (Voinnet 2009). A few miRNA precursors escape this rule, as they are processed by DCL4 instead of DCL1 (Rajagopalan et al. 2006). Although miRNAs represent only ~5% of the plant global small RNA mass, they are important regulators of mRNA translation and degradation, and their contribution is critical for normal plant development.

In addition to p4-siRNAs and miRNAs, three minor populations of small RNAs are present in plants. Transacting siRNAs (ta-siRNAs) originate from the targeting of long PolIII precursors by specific miRNAs (Allen and Howell 2010). The biogenesis of ta-siRNAs requires the RNA-dependent RNA polymerase 6 (RDR6), DCL4, and the dsRBP DRB4. Like miRNAs, ta-siRNAs target mRNA for degradation and are critical regulators of plant development (Allen and Howell 2010). Inverted repeat (IR)-derived siRNAs are transcribed from loci that generate long RNA foldback structures that are mainly cleaved by DCL2 and DCL3 to generate 22-nt and 24-nt siRNAs, respectively (Dunoyer et al. 2010). The biogenesis of these endogenous IR-derived siRNAs is completely independent of PolIV, yet these small RNAs can participate in RdDM as well as post-transcriptional gene silencing (PTGS) (Dunoyer et al. 2010). Finally, overlapping natural *cis*-antisense transcripts can generate double-stranded RNA molecules that can be cleaved by DCL1 or DCL2 in a two-step process to generate small natural antisense RNAs (nat-siRNAs) (Borsani et al. 2005; Jen et al. 2005; Jin et al. 2008). Curiously, despite the production of double-stranded molecules by the pairing of the two antisense transcripts, the biogenesis of nat-siRNAs requires PolIV and RDR6 (Borsani et al. 2005). In most cases, the overlapping natural *cis*-antisense transcripts are only simultaneously produced under specific environmental conditions (Jin et al. 2008), so that nat-siRNAs are not found in plants grown in standard conditions. Nat-siRNAs usually target one of the two generating transcripts for degradation (Borsani et al. 2005; Jen et al. 2005; Jin et al. 2008).

Arabidopsis possesses a small family of five closely related dsRBP (DRB1 to DRB5) (Hiraguri et al. 2005). DRB1, also known as HYL1, is required for DCL1-mediated processing

of most miRNA precursors (Kurihara et al. 2006). DRB1 probably binds as a dimer to the miRNA/miRNA* duplex and recruits DCL1, in cooperation with SERRATE (Yang et al. 2010). It is likely that DRB1 guides the selection of the miRNA strand (Eamens et al. 2009). DRB4 is a partner of DCL4 and is involved in the biogenesis of ta-siRNAs and DCL4-dependent miRNAs (Adenot et al. 2006; Nakazawa et al. 2007; Pouch-Pélessier et al. 2008; Eamens et al. 2009). The DRB4/DCL4 complex is also required to produce siRNAs from exogenous viral dsRNAs. Recently, DRB4 was found to be essential *in vitro* for DCL4 activity (Qu et al. 2008; Fukudome et al. 2011). DRB2, DRB3, and DRB5 were reported not to be involved in small RNA biogenesis (Curtin et al. 2008), and their functions remained elusive. Here, we show that DRB2 and DRB4 are both involved in the production of p4-siRNAs and have antagonistic effects on this pathway.

RESULTS

DRB2 and DRB4 are required for proper accumulation of p4-siRNAs

Accumulation of p4-siRNAs in immature flowers was analyzed in a large number of *Arabidopsis thaliana* mutants to identify new actors involved in their biogenesis. Immature flowers were chosen because both type I and type II p4-siRNAs accumulate in floral tissues (Mosher et al. 2009). In addition to PolIV, PolV, RDR2, DCL3, and AGO4, this screen identified DRB2 and DRB4 as important players in this pathway (Fig. 1). *drb2* mutants accumulated two- to 2.5-fold more p4-siRNAs (type I and II) than wild-type plants (Fig. 1A), whereas *drb4* mutants exhibited reduced accumulation of p4-siRNAs. This reduction was more pronounced for type I p4-siRNAs (0.3- to 0.5-fold) than type II p4-siRNAs (0.6- to 0.7-fold). *drb2drb4* double mutants exhibited a pattern similar to *drb2* single mutants, indicating that *drb2* is epistatic to *drb4* for p4-siRNAs accumulation. miRNA accumulation was largely unaffected by the loss of DRB2 and/or DRB4 as reported previously (Curtin et al. 2008). Also, as previously found (Adenot et al. 2006; Nakazawa et al. 2007; Curtin et al. 2008), accumulation of ta-siRNAs was unaffected by the loss of DRB2 but was reduced in *drb4* and in the *drb2drb4* double mutant.

Western blot hybridization using specific antibodies raised against DCL3 and AGO4 revealed that these two major actors of the polIV pathway accumulated in similar amounts in *drb2* and *drb4* compared to wild type (data not shown), indicating that the changes in p4-siRNA accumulation observed in *drb2* and *drb4* did not result from changes in DCL3 or AGO4 levels. Consistently, 24-nt but not 21-, 22-, or 23-nt p4-siRNAs were lost in *dcl3* (Fig. 1B), whereas siRNAs of the four sizes were simultaneously affected in *drb2* and *drb4*. Interestingly, the accumulation of both type I and type II p4-siRNAs was reduced to the same extent in *dcl4*

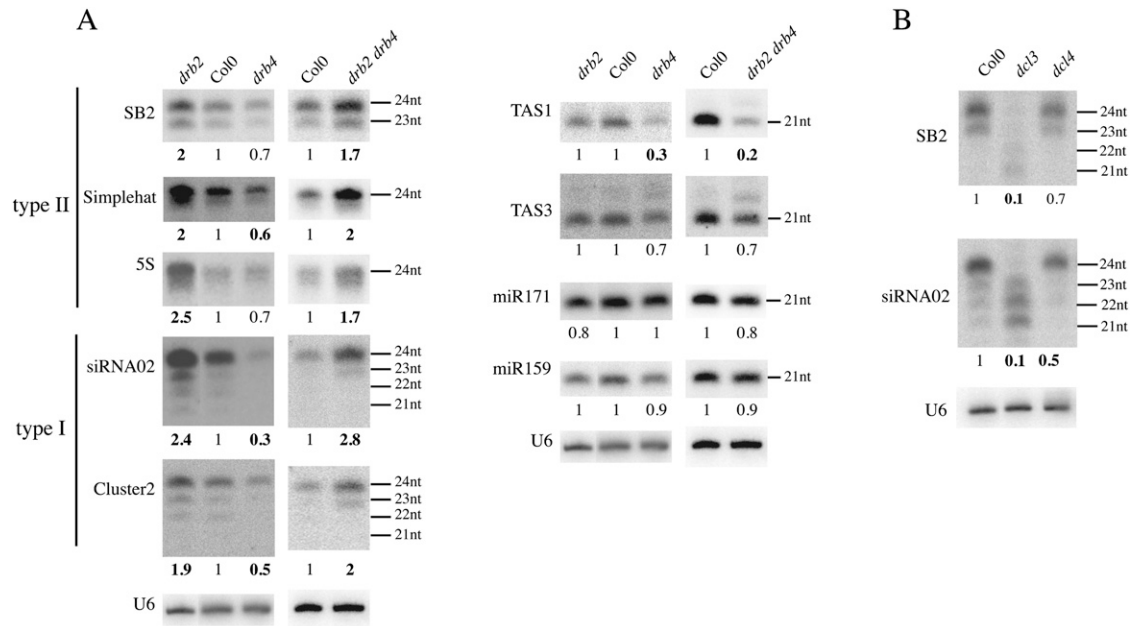


FIGURE 1. DRB2, DRB4, DCL3, and DCL4 are required for proper accumulation of polymerase IV-dependent (p4)-siRNAs. (A) Impact of the absence of DRB2, DRB4 or DRB2, and DRB4 on accumulation of different small RNA species. RNA extracts were obtained from immature flowers, and three independent biological replicates were performed for each sample. Values are normalized to U6 RNA and are expressed as a ratio relative to the wild-type Col0. For p4-siRNAs, only the 24-nt species was used for normalization. Probe sequences are given in Supplemental Table S1. The SB2 probe targets one of the six *Arabidopsis thaliana* SINE dispersed repeat families (Deragon and Zhang 2006). (B) Impact of DCL3 and DCL4 on p4-siRNA accumulation.

and *drb4* (Fig. 1B), suggesting a coordinated action of DRB4 and DCL4 in the p4-siRNA pathway.

Sequencing of small RNA populations from the *drb2* and *drb4* mutants

To extend our analysis of the role of DRB2 and DRB4 in the p4-siRNA pathway, low molecular weight RNAs from wild type, *drb2*, and *drb4* immature flower extracts were reverse-transcribed and sequenced using the Illumina HiSeq 2000 system. After elimination of background RNA and normalization (see Materials and Methods), the global distribution of 21- to 24-nt RNAs was analyzed (Fig. 2A). As expected, the 24-nt population (mainly composed of p4-siRNAs (Rajagopalan et al. 2006; Kasschau et al. 2007; Zhang et al. 2007)) was by far the major component. Compared to wild type, the 24-nt population clearly was increased (2.3-fold) in *drb2* and reduced (0.75-fold) in *drb4*. The 21-nt population (mainly composed of miRNAs) (Rajagopalan et al. 2006; Kasschau et al. 2007) represented the second largest population of small RNAs. In our global analysis, the 21-nt population showed a modest increase in *drb2* (1.2-fold) and a small decrease in *drb4* (0.87-fold). To test if these small variations were the result of a change in the miRNA population, we analyzed miRNAs independently in the two mutants (Fig. 2B). We observed that 21-nt miRNA populations were unchanged in *drb2* and *drb4* (0.98- and 0.99-fold, respectively) compared to wild type. This observation is

in agreement with our Northern hybridization results (Fig. 1) and suggests that the small variations of the 21-nt population observed in the global analysis were not the result of changes in miRNA populations. Finally, the 22- and 23-nt small RNA populations behaved similarly to the 24-nt population in the two mutants, although the 22-nt population shows a stronger reduction in *drb4* (0.48-fold) compared to wild type.

To test if global variations of 21- to 24-nt RNAs in *drb2* and *drb4* result mainly from changes in type I and II p4-siRNAs, we analyzed the distribution of small RNAs originating from various genomic loci. First, populations of type II p4-siRNAs from eight transposable element (TE) families were studied (Fig. 2C). All TE-related type II siRNAs (21- to 24-nt) were increased two- to threefold in the *drb2* mutant. In contrast, the TE-related type II siRNAs of different sizes were affected differently by the loss of DRB4. Indeed, the 23- and 24-nt populations were less affected by the loss of DRB4 (0.7-fold reduction) compared to the 21-nt (0.4-fold) and the 22-nt (0.2-fold). Next, populations of type I p4-siRNAs from five genomic loci (Mosher et al. 2009) were analyzed (Fig. 2D). Here again, all type I siRNAs (21- to 24-nt) were increased two- to threefold in the *drb2* mutant. Type I siRNAs were more affected by the loss of DRB4 than type II, with, again, strong differences depending on RNA sizes. Indeed, 23- and 24-nt type I siRNAs showed a 0.4-fold reduction, while 21-nt exhibited a 0.2-fold reduction and 22-nt accumulated to less than 0.1-fold compared to the wild type.

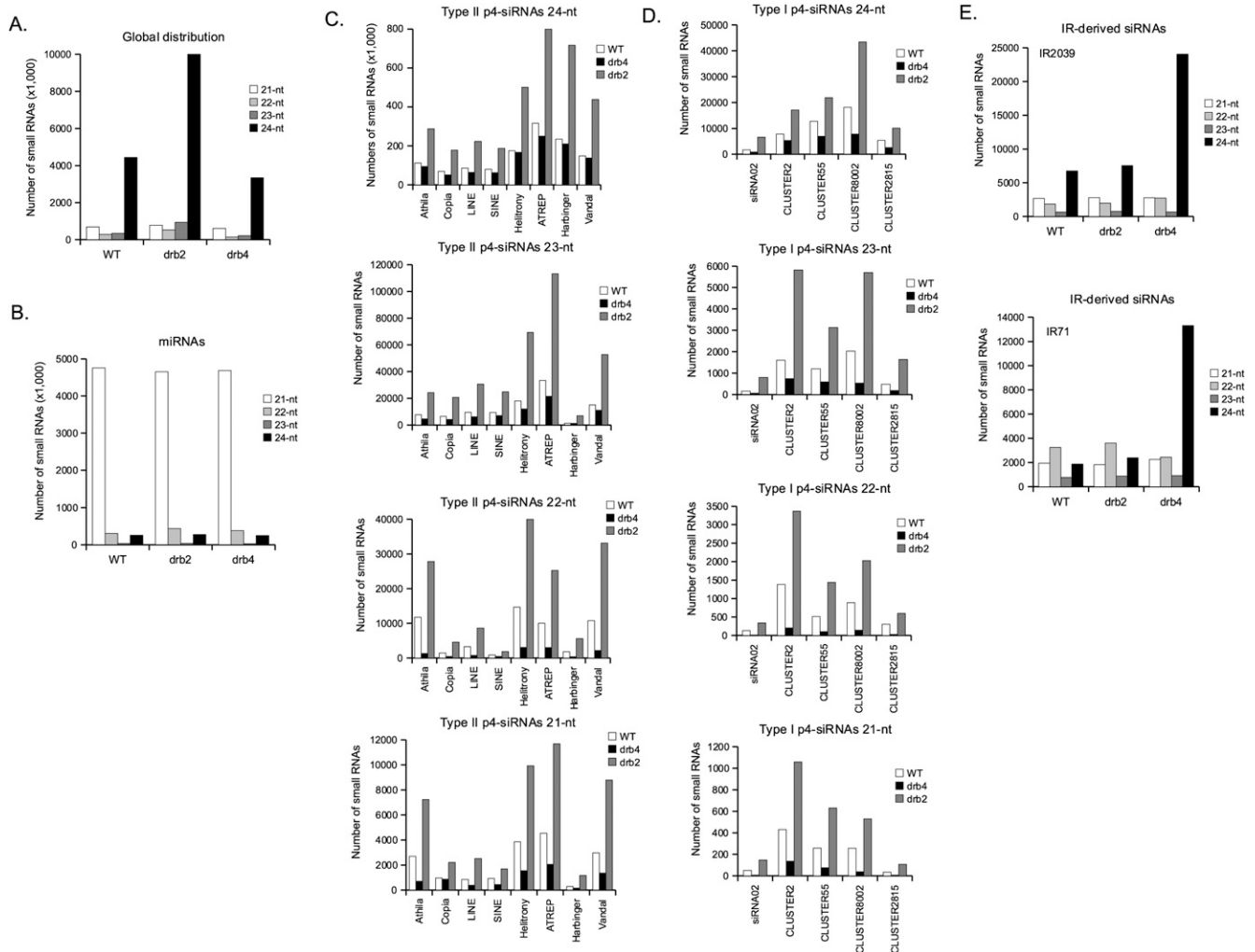


FIGURE 2. Genome-wide profiling of the different populations of small RNAs in the *drb2* and *drb4* mutants compared to wild-type (Col0). (A) Global distribution of 21–24-nt small RNAs. (B) General distribution of miRNAs. (C) Distribution of 21–24-nt type II p4-siRNAs issued from different dispersed repeat families. (D) Distribution of 21–24-nt type I p4-siRNAs issued from five different genomic loci. (E) Distribution of 21–24-nt small RNAs from two representative endogenous inverted repeats (IR) loci.

To evaluate the impact of DRB2 and DRB4 on the accumulation of p4-independent IR-derived siRNAs, populations of siRNAs from two IR loci were analyzed in wild type and in the *drb2* and *drb4* mutants (Fig. 2E). In sharp contrast to p4-dependant siRNAs, loss of DRB2 did not affect significantly IR-derived siRNAs, suggesting that DRB2 is not involved in the accumulation of p4-independent siRNAs. In contrast, loss of DRB4 resulted in increased levels (five- to sixfold) of 24-nt, but not 21- to 23-nt IR-derived siRNA (Fig. 2E), similar to a previous report (Dunoyer et al. 2010).

Both DRB2 and DRB4 are involved in the accumulation of DCL4-dependent miRNAs

Young miRNAs such as miR822, miR839, or miR840 are generated by DCL4 instead of DCL1 (Rajagopalan et al.

2006; for miR840, see the ASRP database <http://asrp.cgrb.oregonstate.edu/>). Two of these RNAs (miR822 and miR839) accumulate at lower levels in *drb4* (Pouch-Pélessier et al. 2008; Eamens et al. 2009), suggesting that their biogenesis requires the coordinate action of DCL4 and DRB4. Our sequencing data confirmed that the accumulation of these three DCL4-dependent miRNAs was strongly reduced in the absence of DRB4 (Fig. 3A). A two- to threefold reduced accumulation of these miRNAs was also observed in *drb2*, suggesting that DRB2 is also required for the proper biogenesis of DCL4-dependent miRNAs (Fig. 3A). To confirm the specific action of DRB2 and DRB4 on DCL4-dependent miRNAs, Northern blot hybridization was performed using *drb2*, *drb3*, *drb4*, and *drb5* mutants. Accumulation of miR839 was unchanged in *drb3* and *drb5* but was reduced in *drb2* and *drb4*, consistent with sequencing data. In contrast, the accumulation of miR173,

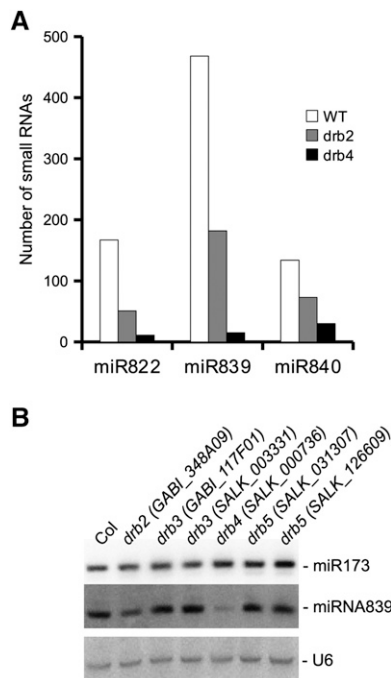


FIGURE 3. Impact of DRB2 and DRB4 on DCL4-dependent miRNAs. (A) The three miRNAs are strongly reduced in the small RNA population of *drb4* and are also significantly affected in the *drb2* background. (B) Northern hybridization using RNA extracts from immature flowers, confirming a reduction of miRNA839 accumulation in *drb2* and *drb4* but not in two independent *drb3* and *drb5* mutant lines. References for the different mutant lines are indicated in brackets. The accumulation of DCL1-dependent miRNA173 was unaffected in these different conditions.

a DCL1-dependent miRNA, was unchanged in *drb2*, *drb3*, *drb4*, and *drb5* (Fig. 3B).

DRB2 does not repress p4-siRNA expression in the paternal lineage

The paternal lineage does not contribute to p4-siRNA populations in developing seeds of *Arabidopsis* (Mosher et al. 2009). This uniparental expression of p4-siRNAs could be explained if an activating factor is produced in the maternal lineage and/or if a repressive factor is expressed in the paternal lineage (Mosher et al. 2009). Because it is highly abundant in anthers and pollen (Curtin et al. 2008), DRB2 is a good candidate for the shutdown of the p4-siRNA pathway in the paternal lineage, potentially explaining the twofold increased accumulation of p4-siRNAs observed in *drb2*. To test this hypothesis, we performed reciprocal crosses between *drb2* and *polIV* mutants and analyzed the accumulation of type I and type II p4-siRNAs five days post-anthesis (Fig. 4). If DRB2 represses p4-siRNAs in the male lineage, crossing *drb2* as male with *polIV* as female should result in the accumulation of paternally inherited p4-siRNAs in the resulting siliques. However, siliques

resulting from crosses between *polIV* as female and *drb2* as male did not accumulate p4-siRNAs, suggesting that DRB2 is not acting as a repressor in the paternal lineage. The reciprocal cross (*polIV* as male and *drb2* as female) resulted in a higher level of p4-siRNAs in siliques compared to wild type but in a level similar to the one found in *drb2* siliques, suggesting that the antagonistic effect of DRB2 on p4-siRNAs levels occurs in the maternal lineage.

DRB2 and DRB4 are required for proper accumulation of p4-siRNAs in vegetative tissues

Whereas type I p4-siRNAs accumulate only in flowers and siliques, type II p4-siRNAs are additionally produced in vegetative tissues, allowing analysis of the impact of the loss of DRB2 and DRB4 in these later tissues. Changes in type II p4-siRNA, ta-siRNA, and miRNA accumulation in *drb2*, *drb4*, and *drb2drb4* 17-day-old seedlings were similar to those observed in flowers (cf. Figs. 1 and 5). Although reproducible, the small increase in type II p4-siRNAs in *drb2* was much less pronounced in seedlings than in flowers. Therefore, the impact of the loss of DRB2 is lower in vegetative tissues compared to flowers. Apart from this difference, our results suggest that DRB2 and DRB4 modulate similarly the biogenesis of p4-siRNAs in flowers and vegetative tissues.

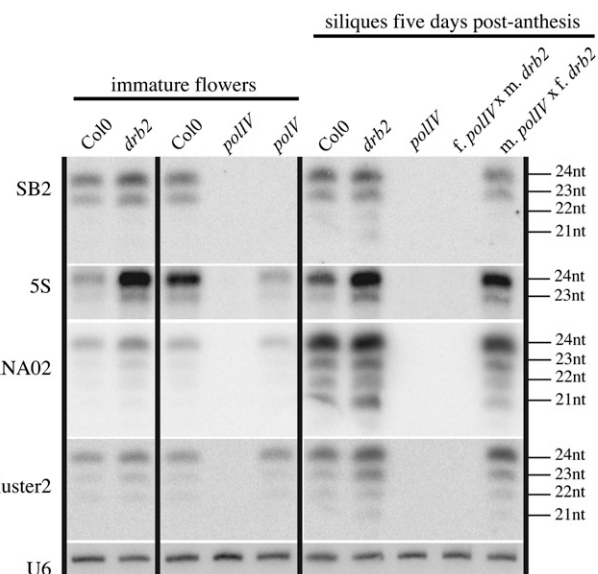


FIGURE 4. DRB2 does not repress p4-siRNA expression in the paternal lineage. For reciprocal crosses, emasculated flowers were pollinated manually, and RNA was extracted from the developing cross at five days after fertilization. Loss of DRB2 function does not induce paternal-specific expression of p4-siRNAs in the developing seeds. As reported previously, type II p4-siRNAs (SB2, 5S) are sensitive to PolV action, while type I p4-siRNAs (siRNA02 and Cluster2) accumulation is affected weakly, or not at all, in the *polIV* mutant (Pontier et al. 2005; Zheng et al. 2009).

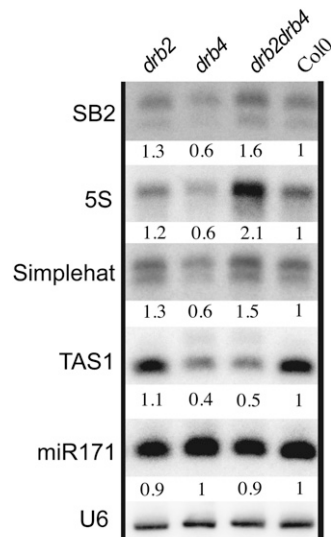


FIGURE 5. DRB2 and DRB4 also impact p4-siRNA expression in vegetative tissues. Seventeen-day-old plantlets showed accumulation defects of type II p4-siRNAs similar to that observed with flowers but with a lower impact in the *drb2* background. Hybridization signals are normalized to U6 RNA and expressed as a ratio relative to the wild-type Col0.

DRB2 overexpression in seedlings mimics a *drb4* mutation at the morphological and molecular levels

To determine how DRB2 limits the production/accumulation of p4-siRNAs in flowers and vegetative tissues, we constitutively expressed a tagged-DRB2 protein under the control of the 35S promoter and measured levels of p4-siRNAs. Seventeen-day-old seedlings accumulating low levels of tagged-DRB2 (cPTPDRB2-17) looked similar to wild-type plants (Fig. 6A). In contrast, plants accumulating high levels of tagged-DRB2 (cPTPDRB2-4) and unchanged levels of DRB4 (Fig. 6C) exhibited a zippy phenotype (Fig. 6A), similar to *drb4* and *dcl4* mutants that have a zippy phenotype due to reduced accumulation of TAS3 ta-siRNAs (Adenot et al. 2006). Consistently, the level of TAS3 ta-siRNAs was reduced in plants accumulating tagged-DRB2 (Fig. 6B). The accumulation of tagged-DRB2 also correlated with a slight reduction of p4-siRNA accumulation (Fig. 6B), similar to that observed in *drb4* (Fig. 5), indicating that DRB2 over-accumulation functionally antagonizes DRB4.

DISCUSSION

Our study reveals that a correct balance between DRB2 and DRB4 is necessary for proper accumulation of p4-siRNAs. Loss of DRB2 results in a general two- to threefold increase in p4-siRNA accumulation (Figs. 1, 2), whereas over-accumulation of DRB2 results in a reduction of p4-siRNA accumulation (Fig. 6B), similar to that observed in *drb4* (Figs. 1, 2). Uniparental expression of p4-dependent

siRNAs in developing endosperm is not affected by the *drb2* mutation (Fig. 4), indicating that p4-siRNA increased accumulation does not result from the reactivation of the polIV pathway in the male gametophyte. Interestingly, all sizes of p4-siRNAs over-accumulate in *drb2*. By far, DCL3-dependent 24-nt siRNAs represent the most abundant form of p4-siRNAs (Fig. 1B). However, 21-, 22-, and 23-nt p4-siRNAs are also found. The enzymatic machinery responsible for their production in wild-type plants remains unclear. Analysis of double, triple, and quadruple *dcl* mutants revealed that, in the absence of DCL3, DCL2 produces 22- and 23-nt p4-siRNAs, while DCL4 produces 21-nt p4-siRNAs. Moreover, DCL1 appears able to produce 21-nt p4-siRNAs in the absence of DCL2, DCL3, and DCL4

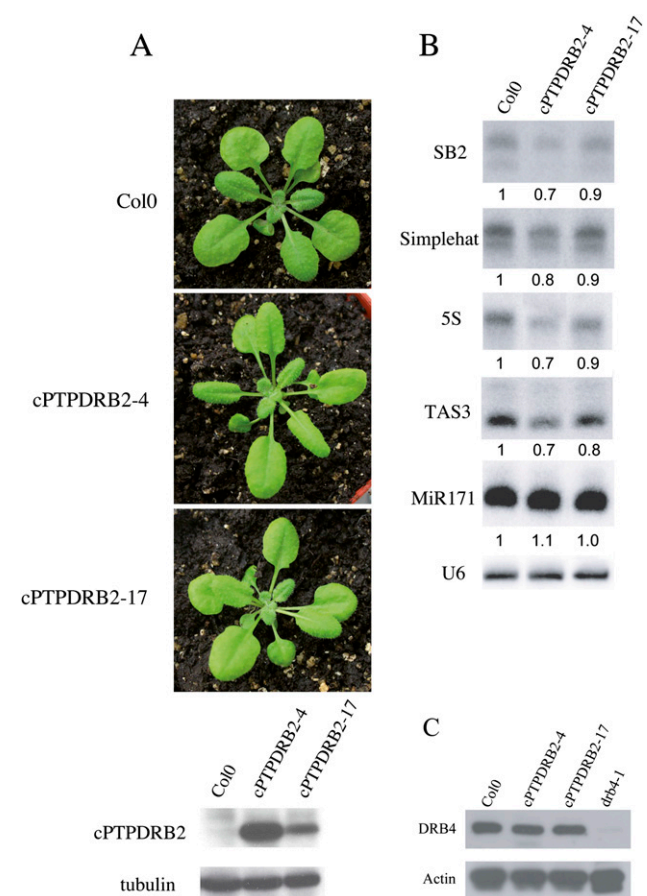


FIGURE 6. Constitutive expression of DRB2 is associated with a zippy-like phenotype and a reduction of p4-siRNAs. (A) A downward-curved leaf margins phenotype was clearly associated with plant lines that express elevated levels of PTP-tagged DRB2 protein under the control of the 35S promoter. (B) Plants accumulating tagged-DRB2 present a slight reduction of p4-siRNAs, a situation similar to that observed in *drb4* (Fig. 5). The level of TAS3 ta-siRNAs is also reduced in these plants. (C) Western blot probed with an antibody raised against DRB4. DRB4 accumulates to a similar level in Col0 and in the two DRB2 overexpressing lines, ruling out the possibility that the effects observed result from a reduction of DRB4 levels when DRB2 levels are increased. As expected, no specific signal was detected when a protein extract from the *drb4* mutant line was used.

(Bouche et al. 2006; Henderson et al. 2006; Liu et al. 2009). Although these results suggest that 21-, 22-, and 23-nt p4-siRNAs are produced by DCL2 and DCL4, it remains possible that DCL3 produces all sizes of p4-siRNAs in wild-type plants. Over-accumulation of all sizes of p4-siRNAs in *drb2* mutants could be explained by an antagonistic effect of DRB2 on DCL3 and other DCL(s) involved in p4-siRNA biogenesis. However, *drb2* mutants exhibit unchanged accumulation of polymerase IV-independent miRNAs, ta-siRNAs, and endogenous IR-derived siRNAs, which are processed by DCL1, DCL2, DCL3, and DCL4, indicating that the antagonistic effect of DRB2 is strictly limited to the p4-siRNA pathway. Therefore, the antagonistic effect of DRB2 on the p4-siRNA pathway likely occurs upstream of the DCL(s). One possibility could be that DRB2 interacts with RDR2 and binds to RDR2-derived dsRNA, thus limiting their processing by DCL(s). Alternatively, PolIV primary transcripts or RDR2-derived dsRNA bound to DRB2 could enter a degradation pathway involving RNases that remain to be identified. Under any of these two scenarios, loss of DRB2 would allow more RDR2-derived dsRNA to be processed into all sizes of p4-siRNAs by DCL(s).

Loss of DRB4 results in reduced accumulation of DCL4-dependent miRNAs and ta-siRNAs because DCL4 functions with DRB4 for their processing (Adenot et al. 2006; Pouch-Pélissier et al. 2008). Loss of DRB4 also results in reduced accumulation of all sizes of p4-siRNAs. Type I p4-siRNAs are more affected than type II p4-siRNAs, and 22-nt are more affected than 21-nt, which themselves are more affected than 23- and 24-nt (Figs. 1, 2). One possibility to explain these results is that, in addition to DCL4, DCL2 and DCL3 could also require DRB4 for efficient dsRNA processing. However, we failed to immunoprecipitate DCL3 from a transgenic line expressing a tagged-DRB4 fusion protein in conditions where we can readily immunoprecipitate DCL4 (see Supplemental Fig. S1) suggesting that DRB4 is not associated with DCL3 in vivo. Because type I and type II p4-siRNAs accumulate at reduced levels in both *dcl4* and *drb4* mutants, it is more likely DRB4 controls p4-siRNA levels through DCL4. How the DRB4/DCL4 complex regulates the accumulation of all sizes of p4-siRNAs remains to be determined. A spectacular increase in the accumulation of 24-nt p4-independent IR-derived siRNAs is also observed in *drb4* (Fig. 2E), similar to a previous report (Dunoyer et al. 2010). In this case, DRB4 acts independently of DCL4 because IR-derived siRNA levels and patterns are unchanged in the *dcl4* mutant (Dunoyer et al. 2010). How DRB4 limits the production of IR-derived siRNAs is unknown. One possibility is that lowering the global efficiency of the p4-dependent siRNA pathway in the *drb4* mutant could lead to the reallocation of factors from the p4-dependent to the p4-independent siRNA pathway. Alternatively, DRB4 could directly bind IR precursor RNAs and limit their cleavage by DCL3.

Overall, our analyses reveal specialized, redundant, and antagonistic functions for DRB2 and DRB4. Analysis of *drb2* and *drb4* mutants points to an antagonistic effect in the DCL3-dependent p4-siRNA pathway. In addition, plants over-expressing DRB2 mimic the zippy phenotype of *drb4* mutants and display reduced levels of p4-siRNAs (Fig. 6), supporting an antagonistic effect of DRB2 on DRB4 and pointing to a potential interference of ectopically expressed DRB2 in the DRB4/DCL4-dependent ta-siRNA pathway. In contrast, both *drb2* and *drb4* mutations cause a reduction in the accumulation of young DCL4-dependent miRNAs (Fig. 3), suggesting partial redundancy in this pathway. Lastly, loss of DRB2 does not affect IR-derived siRNA accumulation, whereas loss of DRB4 results in increased levels of DCL3-dependent 24-nt but not DCL2/4-dependent 21- to 23-nt IR-derived siRNA, indicating specialized functions for DRB4. Together, these results suggest that DRB proteins are part of multiple complexes, allowing independent actions in different pathways. Elucidation of the DRB interaction network will shed light on the functioning of this complex family of proteins.

MATERIALS AND METHODS

Genetic stocks and plant growth conditions

The stocks of *drb2-1*, *drb4-1*, *drb2-1drb4-1*, *dcl4-2*, *dcl3-1*, *nprpd1a-4* (*polIV*), and *nripe1-11* (*polV*) used in this study are in a Columbia (Col0) genetic background and were described previously (Xie et al. 2004; Pontier et al. 2005; Xie et al. 2005; Curtin et al. 2008). For ectopic DRB2 expression, the *DRB2* coding sequence was associated to a C-terminal PTP (protC-TEV-protA) tag (Schimanski et al. 2005) and placed under the 35S promoter expression. Wild-type *Arabidopsis* Col0 plants were transformed as previously described (Clough and Bent 1998), and transformed lines displaying different levels of DRB2 expression were identified using PAP antibody. Plant seeds were stratified for two days at 4°C before growth in chambers on soil at 23°C under a 16-h-light/8-h-dark cycle. For in vitro analyses, seeds were sterilized and sowed on solid Murashige and Skoog (MS) medium containing 1% sucrose (w/v) and grown under continuous light at 20°C.

RNA isolation and hybridization

Total RNA was extracted as described elsewhere (Pélissier et al. 2004), using immature inflorescences (stages 1–12), siliques five days post-anthesis, or rosette from in vitro growing 17-day-old plantlets. For the detection of small RNAs, 12–18 µg of total RNA samples were heat-treated in 1.5 volume of standard formamide buffer and loaded on 15% polyacrylamide (19:1 acrylamide:bis-acrylamide)–8.3 M urea–0.5X TBE gel and separated by electrophoresis. The samples were electroblotted to Hybond-NX membranes (GE Healthcare) and fixed following a carbodiimide-mediated cross-linking procedure (Pall et al. 2007). Pre-hybridization and hybridization were carried out in 5X SSC, 20 mM Na₂HPO₄ pH7.2, 7% SDS, 2X Denhardt solution, 50 mg/mL herring DNA at 50°C. Sequences of the different probes used are given in Supplemental Table S1. Filters were washed twice with 3X SSC,

25 mM NaH₂PO₄ pH7.5, 5% SDS at 50°C for 10 min, followed by one to two washes with 1X SSC, 1% SDS at 50°C for 10 min. Signals were visualized using a phosphorimager (Molecular Imager FX ; Bio-Rad) for quantification.

Sequencing of small RNAs and data analysis

Total RNAs were separated on polyacrylamide gel electrophoresis as above and small RNAs (18–36 nucleotides) were cut out of the gels, used to construct libraries according to the manufacturer's protocol (Illumina Small RNA Sample Prep Kits), and sequenced on a HiSeq 2000 system (Illumina) on the MGX platform (<http://www.mgx.cnrs.fr/>). Fourteen and 0.6 million small RNA reads were obtained for Col0, 16.1 million for *drb2*, and 10.4 million for *drb4*. Small read counts were normalized by taking highly expressed conserved miRNAs that were shown by northern hybridization to be invariant in Col0 (Fig. 1) and calculating normalization factors for each condition. The normalized data was cross-confirmed by calculating the normalization factor for all significantly expressed 36-nt (that represent general breakdown RNA products), which yielded similar values. Only small RNAs having 10 or more read counts in at least one condition were considered in Figures 2 and 3.

Immunoblot analysis

Protein extracts were obtained by grinding frozen tissues in liquid nitrogen; After resuspension in 4X Laemli Buffer, the extracts were treated for 5 min at 95°C and centrifuged before loading on SDS/PAGE gels. Proteins were transferred onto PVDF membrane (Immobilon-P; Millipore), and proTA fusion proteins were visualized using the PAP (Peroxidase-Anti-Peroxidase) soluble complex (Sigma) diluted at 1/10,000. The level of DRB4 was evaluated using a custom made specific antibody (Eurogentec) diluted at 1/500. Equal protein loading was assessed using anti-tubulin or anti-actin specific antibody.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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