DRB2 is Required for MicroRNA Biogenesis in Arabidopsis thaliana

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Abstract

Background: The Arabidopsis thaliana (Arabidopsis) DOUBLE-STRANDED RNA BINDING (DRB) protein family consists of five members, DRB1 to DRB5. The biogenesis of two developmentally important small RNA (sRNA) species, the microRNAs (miRNAs) and trans-acting small interfering RNAs (tasiRNAs) by DICER-LIKE (DCL) endonucleases requires the assistance of DRB1 and DRB4 respectively. The importance of miRNA-directed target gene expression in plant development is exemplified by the phenotypic consequence of loss of DRB1 activity (drb1 plants).

Principal Findings: Here we report that the developmental phenotype of the drb235 triple mutant plant is the result of deregulated miRNA biogenesis in the shoot apical meristem (SAM) region. The expression of DRB2, DRB3 and DRB5 in wild-type seedlings is restricted to the SAM region. Small RNA sequencing of the corresponding tissue of drb235 plants revealed altered miRNA accumulation. Approximately half of the miRNAs detected remained at levels equivalent to those of wild-type plants. However, the accumulation of the remaining miRNAs was either elevated or reduced in the triple mutant. Examination of different single and multiple drb mutants revealed a clear association between the loss of DRB2 activity and altered accumulation for both the elevated and reduced miRNA classes. Furthermore, we show that the constitutive over-expression of DRB2 outside of its wild-type expression domain can compensate for the loss of DRB1 activity in drb1 plants.

Conclusions/Significance: Our results suggest that in the SAM region, DRB2 is both antagonistic and synergistic to the role of DRB1 in miRNA biogenesis, adding an additional layer of gene regulatory complexity in this developmentally important tissue.

Introduction

In Arabidopsis thaliana (Arabidopsis), the various classes of endogenous 21–24 nucleotide (nt) small RNA (sRNA) are processed from structurally distinct double-stranded RNA (dsRNA) precursors by four DICER-LIKE (DCL) proteins, an RNAse III-like family of endonucleases [1,2]. Two functionally well-characterized sRNA classes, the microRNAs (miRNA) and trans-acting small-interfering RNAs (tasiRNAs), both of which predominantly direct cleavage of highly complementary mRNAs into small interfering RNA (siRNA) species, are processed by DRB1-assisted DCL1 in specialized nuclear dicing bodies (D-bodies) [14,15,16]. The precice and efficient processing of these dsRNA substrates by DCL1 and DCL4 is mediated by HYPONASTIC LEAVES1 (HYL1; referred to as DRB1 from here on) and DRB4 activity respectively, two of the five members of the Arabidopsis drsRNA BINDING (DRB) protein family [7,9,10,11]. The functional importance of these two DCL/DRB protein partnerships on plant development is exemplified by the phenotypes displayed by dcl1, dcl4, drb1 and drb4 plants [7,9,12,13].

In miRNA biogenesis, the primary-miRNA (pri-miRNA) transcript, a long non-protein-coding RNA containing a region of self-complementarity allowing for stem-loop formation, is processed by DRB1-assisted DCL1 in specialized nuclear dicing bodies (D-bodies) [14,15,16]. The initial cleavage step of the miRNA biogenesis pathway produces a smaller stem-loop intermediate, the precursor-miRNA (pre-miRNA). The DCL1/DRB1 partnership also directs the second cleavage step of the miRNA biogenesis pathway to liberate the miRNA/miRNA* duplex from the pre-miRNA stem-loop sequence [7,12,17]. The 2’-nt overhang of each duplex strand is methylated by the sRNA-specific methyltransferase HUA ENHANCER1 (HEN1) and exported to the cytoplasm where the miRNA guide strand is separated from the miRNA* passenger strand [3,18]. The liberated miRNA sRNA is loaded onto the ARGONAUTE1 (AGO1)-catalyzed RNA-induced silencing complex (RISC) to predominantly direct cleavage of highly complementary miRNAs loci [13,19].

In the closely related tasiRNA biogenesis pathway, three miRNAs, miR173, miR390 and miR828, guide AGO1 (miR173 and miR828) or AGO7 (miR390)-catalyzed cleavage of TAS transcripts, long non-protein-coding RNAs transcribed from TAS loci [20,21,22]. This identifies the cleaved TAS transcript as a...
MicroRNA Biogenesis Requires DRB2

Results

DRB family member expression and mutant phenotypes

In *Arabidopsis*, miRNAs typically require the DCL1/DRB1 protein complex for their biogenesis [4,22]. In accordance with reduced mature miRNA accumulation, *drb1* plants express a pleiotropic phenotype, characterized by upwardly curled (hyponasty) rosette leaves, and reduced organ size, growth and fertility [7,12,25,28]. The loss of DRB2 also results in changes to rosette leaf morphology (Figure 1A). Compared to wild-type plants, *dbh* rosette leaves are ovoid, flatter and darker in color due to increased anthocyanin production. As *dbh* plants mature, the margins of their rosette leaves become highly serrated. Furthermore, the *dbh* phenotype is epistatic to those expressed by *dbh3* and *dbh5* plants. Plants defective for the activity of these two DRB family members are essentially wild-type in appearance, as is the double mutant *dbh3dbh5* (Figure 1A).

Our previous analyses demonstrated that DRB2, DRB3 and DRB5 are highly similar at both the genomic and amino acid level [25] suggesting that these three family members may be functionally redundant. As described for the *dbh1* plant line with reduced miRNA accumulation, the *dbh235* triple mutant also expresses a pleiotropic phenotype. Although the margins of *dbh235* rosette leaves remain serrated, they do not display the dark-green coloration and ovular shape of *dbh2* rosette leaves. Instead, they are pale green in color with dark green venation of the vasculature, have a lanceolate shape and are downturned at their tips (epinasty). Furthermore, the petioles of *dbh235* rosette leaves are markedly shorter than those of wild-type plants, and this combined with their downward curvature leads to the formation of a compact rosette (Figure 1A). In contrast to all other single, double or triple *dbh* mutant plants, which essentially display the architecture of a wild-type inflorescence stem, the stems of *dbh235* plants are fused (Figure 1B).

Consistent with its role as a cofactor for DCL1 in miRNA biogenesis, *DRB1pro:GUS* plants showed constitutive and ubiquitous reporter gene expression. GUS expression was restricted to the SAM region of plants transformed with the *DRB3pro:GUS* and *DRB5pro:GUS* vectors (Figure 1C). Analysis of plants transformed with our original *DRB2pro:GUS* vector, containing a 1.7 kb putative promoter fragment, suggested that; i) DRB2 expression was restricted to developing anthers, pollen and germinating seed, and; ii) DRB2 expression did not overlap with DRB3 and DRB5 [25]. However, extending the putative promoter region to include 4 kb upstream of the transcription start site of *DRB2* showed that in vegetative tissue *DRB2* expression is concentrated in the SAM region, an expression domain that overlaps with DRB3 and DRB5 (Figure 1C).

miRNA accumulation in the SAM region of *dbh235* plants

The developmental phenotypes expressed by *dbh1* and *dbh235* plants, in addition to the overlapping expression of *DRB2*, *DRB3* and *DRB5* led us to investigate miRNA accumulation in whole plant samples for comparison to the specific tissues where these three DRB family members are expressed. Alterations to leaf shape, curvature and margin serration have been associated with expression changes to members of several well-characterized miRNA gene (*MIR* gene) families or their target genes [29,30,31,32]. Northern blotting and RT-PCR were used to determine if one, or all three DRB proteins are involved in miRNA biogenesis or action in whole plants. However, these analyses performed on the aerial tissue of 4 week old *dbh* mutant plants failed to identify any significant changes to either mature miRNA accumulation (Figure S1A), or expression of their cognate targets (Figure S1B).

We next used sRNA sequencing to quantify mature miRNA levels in the SAM region of *dbh235* plants. Selection of this tissue was determined by reporter gene expression analysis of plants transformed with the *DRB2pro:GUS*, *DRB3pro:GUS* and *DRB5pro:GUS* vectors (Figure 1C). Following normalization (normalized to the total number of 20–24 nt sRNA reads mapping to the *Arabidopsis* genome for each sample), sequencing identified 440895 and 307153 sRNA reads in the Col-0 and *dbh235* samples respectively that perfectly matched 140 of the 189 mature miRNA sequences entered into the miRBase database (http://www.mirbase.org/) at the time of analysis (Table S1). The 140 mature miRNA sequences detected in both the Col-0 and *dbh235* samples by sRNA sequencing represented 57 *MIR* gene families (Table S2). When individual family member reads were combined to give an overall *MIR* gene family score, miRNA accumulation for 8 (14.0%), 26 (45.6%) and 23 (40.4%) *MIR* gene families was elevated, at approximate wild-type levels and reduced respectively in the SAM region of *dbh235* plants (Table S3). Table 1 shows the five *MIR* gene families in *dbh235* plants with the most significantly elevated or reduced accumulation in the specific tissues assessed by sRNA sequencing. Five miRNAs with unchanged accumulation in *dbh235* plants are also listed in Table 1 and were included as wild-type controls. Northern blotting was used to confirm the accumulation profile of each of the 15 miRNAs listed in Table 1...
Three miRNAs, including miR164, miR168 and miR169, all of which form highly conserved, well-characterized MIR gene families were selected as representatives for further analysis of the elevated, unchanged and reduced miRNA accumulation classes in *drb235* plants.

In the SAM region miRNA accumulation requires DRB2 activity

To determine whether the deregulated miRNA accumulation observed in the SAM region of the *dbb233* triple mutant was a result of the loss of activity of one or multiple DRB proteins, mature miRNA accumulation as well as pri-miRNA and target gene expression was assessed in the same tissues used for sRNA sequencing sampled from all possible *dbb2, dbb3* and *dbb5* mutant combinations. Northern blotting revealed a clear association between the loss of DRB2 activity (Figure S3A) and enhanced mature sRNA accumulation for the elevated miRNA class representative, miR164 (Figure 2A). RT-PCR analysis of pri-miRNA expression suggested that the enhanced accumulation of miR164 observed in the *dbb2, dbb3* and *dbb5* mutant backgrounds resulted from more efficient processing of the precursor transcripts, *PRI-MIR164A* and *PRI-MIR164B* in the absence of DRB2 activity (Figure 2A). Furthermore, RT-PCR analysis revealed that in accordance with enhanced precursor transcript processing and mature miRNA accumulation, the expression levels of two of the targets of miR164, namely *CUC1* and *CUC2* were reduced. *CUC1* was undetectable in all *dbb2*-containing plant lines. Intriguingly, *CUC2* was only undetectable in *dbb233* plants. In *dbb2, dbb23* and *dbb25* plants *CUC2* was detectable, but at significantly reduced levels. Taken together, these results suggest that these two closely related targets have different DRB requirements for regulation of their wild-type expression by miR164. The expression of *CUC3*, a closely related member of the same transcription factor family as *CUC1* and *CUC2* was also assessed by RT-PCR. Unlike *CUC1* and *CUC2*, *CUC3* does not contain a miR164 target sequence, however, *CUC3* expression has been shown to be regulated by these two closely related family members [33,34]. The observed reduction in *CUC3* levels in all four of the analyzed *dbb2*-containing backgrounds suggested that miR164 target gene expression was indeed reduced in the same tissues where miR164 levels were shown to be elevated by northern blotting.

MiR168 was selected as the unchanged miRNA class representative, as we have previously demonstrated that the accumulation of this miRNA is largely unaffected by the loss of activity of any of the five members of the *Arabidopsis* DRB protein family in whole plant samples [25,35]. Northern blotting and RT-PCR analyses confirmed our previous findings, demonstrating that precursor transcript (*PRI-MIR168A*) processing efficiency, mature miRNA accumulation and target gene (*AGO1*) expression all remained at approximate wild-type levels in the absence of DRB2, DRB3 and DRB5 activity (Figures 2B and S3A). However, contrary to the reported independence of miR168 accumulation in the absence of DRB activity in whole plants, these analyses also...
revealed that in the specific tissue analyzed by sRNA sequencing, the SAM region, DRB1 is required for wild-type miR168 accumulation and target gene expression regulation (Figure 2B).

As demonstrated for the elevated class of miRNAs, accumulation of the *drb235* reduced miRNA class representative, miR169 was associated with the loss of DRB2 activity (Figure S3A). MiR169 levels were reduced in *drb2*, *drb23*, *drb25* and *drb235* plants (Figure 2C). The *Arabidopsis* miR169 family consists of 14 members and our sRNA sequencing revealed miR169a to be the most prevalent of the five family members detected (Table S1). The precursor transcript and target gene of miR169a, PRI-MIR169A and NFTYA5 respectively [36] were therefore included in our analyses. RT-PCR analysis suggested that the observed reductions in miR169 accumulation in *drb2*, *drb23*, *drb25* and *drb235* plants was a result of inefficient primary transcript processing with higher levels of PRI-MIR169A detected in all four of these DRB2 deficient backgrounds (Figure 2C). The reductions in mature miR169 accumulation due to inefficient PRI-MIR169A processing observed in *drb2*, *drb23*, *drb25* and *drb235* plants was in turn demonstrated to result in deregulated target gene expression with NFTYA5 levels elevated in all four of these plant lines lacking DRB2 activity (Figures 2C and S3A).

Northern blotting was further applied to confirm the association between the loss of DRB2 activity and alterations to miRNA accumulation for both the *drb235* elevated and reduced miRNA classes. As demonstrated for miR164 and miR169, Figure S3B shows that the levels of two additional miRNAs, specifically miR841 and miR170 (Table 1), are elevated and reduced respectively in the absence of DRB2 expression (Figure S3A). Furthermore, and as illustrated for miR168, miR162 accumulation remained at wild-type levels in the absence of DRB2, DRB3 and DRB5 expression (Figure S3). Concurrent examination of these analyses strongly indicated that DRB2 activity is associated with the observed changes to miRNA accumulation for both the *drb235* elevated and reduced classes of miRNA, and that in these specific tissues DRB family members DRB2, DRB3 and DRB5 are not involved in the biogenesis of miRNAs exhibiting wild-type levels of accumulation.

The *drb235* phenotype results from the tissue-specific elevation of miR164 accumulation

The *drb235* developmental phenotype is primarily characterized by rosette leaf margin serration and inflorescence stem fusion (Figures 1A and 1B). Changes to miR164 accumulation and/or the expression of two of its target genes, *CUC1* and *CUC2* are associated with alterations to rosette leaf margin serration as well as defects in SAM formation and cotyledon, sepal and stamen separation [31,37,38]. MiR164 accumulation, along with DRB2, *CUC1* and *CUC2* expression was therefore assessed in specific tissues of Col-0 and *drb235* plants to correlate these expression changes with the *drb235* phenotype. Northern blotting showed that in wild-type plants, miR164 levels are spatiotemporally regulated. The miR164 sRNA accumulated to detectable levels in all Col-0 tissues evaluated, with the highest levels of accumulation detected in the inflorescence stem and floral tissue (Figure 3A). RT-PCR analysis revealed that DRB2, together with the miR164 targets *CUC1* and *CUC2* showed high levels of overlapping expression in the Col-0 SAM region sample (Figure 3A). Northern blotting showed that miR164 accumulation was highly elevated in the corresponding tissue of *drb235* plants and in accordance RT-PCR revealed a corresponding loss of target gene expression. A similar trend was also observed for the *drb235* inflorescence stem sample where the loss of DRB2 activity was demonstrated to result in enhanced miR164 accumulation and a corresponding loss of *CUC1* and *CUC2* expression (Figure 3A).

These analyses also revealed that enhanced mature miR164 accumulation in the absence of DRB2 expression is tissue-specific. Although DRB2 was expressed at low levels in Col-0 seedlings and floral tissues, no change in miR164 accumulation or target gene expression was observed in the corresponding tissue of *drb235* plants. Detection of DRB2 expression in Col-0 seedlings was not unexpected as the analyzed tissue would also contain the SAM region where promoter-GUS (Figure 1C) and RT-PCR analyses (Figure 3A) showed DRB2 expression to be localized. This suggests that other tissues collected as part of the seedling sample, including cotyledons, young leaves and roots are masking the observed changes in miR164, *CUC1* and *CUC2* levels in the SAM region of *drb235* seedlings. Plants lacking miR164 accumulation or ectopically expressing either the MIR164A or MIR164B precursor transcript produce abnormal floral organs as a consequence of altered CUC1 and CUC2 activities [30,31,39]. No floral defects are observed in *drb235* plants and furthermore no change in miR164c level was detected by sRNA sequencing (Table S1). This suggests that in wild-type plants, DRB2 does not interact with the PRE-MIR164C transcript and that the tissue-specific elevation of miR164 accumulation observed in plants lacking the activity of DRB2 results from a loss of the repressive effects of DRB2 on DCL1/DRB1-mediated, PRE-MIR164A and PRE-MIR164B processing (Figure 2A).

To further test the association between tissue-specific elevation of miR164 accumulation with the *drb235* developmental pheno-
type, miR164, CUC1 and CUC2 levels were assessed in additional drb mutants that also have altered miR164 accumulation but do not express the rosette leaf margin serration or inflorescence stem fusion defects of drb235 plants. In addition to Col-0, the drb35 double mutant was also included in these analyses as a wild-type control for miR164 accumulation, target gene expression (Figure 2A), leaf margin serration and inflorescence stem architecture (Figures 1A and 1B). We and others have previously shown that DRB1 is required for the biogenesis and wild-type accumulation of the miR164 sRNA [7,40]. The analyses presented in Figure 3B show that in the SAM region of drb1 plants, miR164 accumulation is significantly reduced and that the expression of its
target genes, CUC1 and CUC2 is proportionately elevated. MiR164 accumulation and target gene expression are also reduced and up-regulated respectively in drb12 plants, however these changes are not as severe as those detected in drb1 plants. These four plant lines with either wild-type miR164 and target gene levels (Figure S4A; Col-0 and drb35), or reduced miR164 accumulation and up-regulated target gene expression (Figure S4B; drb1 and drb12), all develop rosette leaves with smooth margins and inflorescence stems that are not fused.

Unlike drb1 and drb12 plants, altered miR164 accumulation correlates with the observed changes to rosette leaf margin serration and/or inflorescence stem architecture in drb2 and drb235 plants. Elevated miR164 accumulation in the SAM region of drb2 plants leads to the loss of CUC1 expression and significantly reduced levels of CUC2 (Figure 3B). In the same tissues in the drb235 triple mutant however, enhanced miR164 accumulation results in the complete loss of both CUC1 and CUC2 expression. The additional loss of CUC2 expression in drb235 plants, compared to the loss of CUC1 only in drb2, appears to direct the differences in inflorescence stem architecture displayed by these two DRB2-defective plant lines (Figures 1B and S4C). These analyses also suggest that the observed reductions to CUC2 expression and the complete loss of CUC1 are responsible for the development of rosette leaf margin serration in all drb2-containing backgrounds.

DRB1 and DRB2 are required for miRNA biogenesis in the SAM region

To determine the contribution of DRB1 and DRB2 activity to miRNA biogenesis in the SAM region northern blotting and RT-PCR were used to assess miRNA accumulation, precursor transcript processing and target gene expression for the drb235 elevated, unchanged and reduced miRNA class representatives in drb1, drb2 and drb12 plants. Figure 4A shows that compared to drb1 and drb2 plants, miR164 accumulation is elevated and reduced, respectively, in the drb12 double mutant. RT-PCR revealed a direct correlation between precursor transcript processing efficiency, PRI-MIR164A and PRI-MIR164B expression, and miRNA accumulation in these three drb mutant lines. Target gene expression was also reflective of precursor transcript processing efficiency and mature miRNA accumulation. The data presented in Figure 4A suggests that in the absence of DRB2 activity in drb12 plants, miR164 precursor transcripts are more freely available to enter the canonical miRNA biogenesis pathway mediated by the DCL1/DRB1 partnership, but in this double mutant plant DCL1 cannot efficiently process the increased levels of available substrate as it is also defective in DRB1 activity.

Compared to wild-type plants, no change in the levels of miR168, the precursor transcript PRI-MIR168A or the target gene AGO1 were observed in drb2 plants (Figure 4B). Changes in precursor transcript processing efficiency, mature miRNA accumulation and target gene expression were observed in drb1 and
red yet detectable levels in *dbh1* and *dbh2* plants, but below detection sensitivities in the *dbh12* double mutant (Figure 4C). RT-PCR assessment of PRI-MIR169A expression showed that the observed reduction to miR169 accumulation in *dbh1* and *dbh2* plants was a result of inefficient precursor transcript processing. The detection of even higher levels of precursor transcript, in combination with the failure to detect miR169 by northern blotting, in *dbh12* plants strongly indicated that the activity of both DRB family members is a requirement for miRNA biogenesis in the SAM region of *Arabidopsis* plants.

An artificial miRNA directing PHYTOENE DESATURASE silencing is differently processed from individual miRNA precursor transcripts

The results presented in Figure 4 indicated that the involvement of DRB1 and DRB2 in miRNA biogenesis in the SAM region of *Arabidopsis* plants is determined at the miRNA precursor level. To further assess the influence of the miRNA precursor transcript on DRB1- and DRB2-mediated miRNA-directed silencing, the endogenous miRNA sequences of PRI-MIR164B and PRI-MIR169A were replaced with an identical artificial miRNA (amiRNA) sequence targeting PHYTOENE DESATURASE (PDS; amiR-PDS) for amiRNA-directed RNA silencing. These two plant expression vectors, amiR164B-PDS and amiR169A-PDS, were generated via overlapping PCR-based cloning [41,42] and used to transform wild-type Col-0 plants and *dbh* mutants, *dbh1*, *dbh2* and *dbh235*. Transformation of Col-0 with either PDS-targeting amiRNA vector generated plants with rosette leaves that were completely photo-bleached (Figures 5A and 5C). Both amiR-PDS vectors were also introduced into *dbh1* plants that lack the activity of DRB1, the preferred partner protein of DCL1 in miRNA biogenesis. Unlike Col-0, introduction of either amiRNA vector into *dbh1* plants resulted in the generation of transformant lines that were essentially *dbh1* in appearance, lacking any observable photo-bleaching or further arrest of *dbh1* development. Northern blot and RT-PCR analyses of amiR-PDS accumulation, precursor transcript processing and target transcript expression demonstrated that the differences in silencing efficiencies directed by either amiRNA vector in Col-0 and *dbh1* plants was a result of inefficient precursor transcript processing in the absence of DRB1 activity, leading to reduced mature amiR-PDS accumulation and defective silencing of the PDS target gene in *dbh1* transformant lines (Figures 5B and 5D).

The photo-bleached phenotype of Col-0/amiR169A-PDS plants was uniformly expressed by *dbh2* and *dbh235* plants following transformation with the amiR169A-PDS vector (Figure 5A). However, molecular analyses, as demonstrated by northern blotting and RT-PCR, showed that the over-accumulation of the PRI-MIR164B-delivered sRNA in the absence of DRB2 activity, resulted in additional severe reductions to overall plant growth and development in *dbh2* amiR169A-PDS and *dbh235* amiR169B-PDS plants. All recovered *dbh2* amiR169A/PDS and *dbh235* amiR169B-PDS transformants were further reduced in size compared to Col-0/amiR169A-PDS transformants (Figure 5A). Taken together, these results again associated the loss of DRB2 activity with enhanced PRI-MIR164B processing, mature miR164 (amiR-PDS) accumulation and sRNA-directed target gene (PDS) silencing (Figure 5B).

As reported for Col-0/amiR169A-PDS plants, the cotyledons and first few leaf pairs of amiR169A-PDS-transformed *dbh2* plants were completely photo-bleached. However, as these transformants matured, rosette leaves with green tissue emerged from the SAM region (Figure 5C). Interestingly, this is the same tissue where reporter gene expression was observed in DRB2 promoter-driven

Figure 4. DRB1 and DRB2 are required for miRNA biogenesis in the SAM region. (A) miR164 accumulation, MIR164 precursor transcript processing and target gene expression in the SAM region of *dbh1*, *dbh2* and *dbh12* plants. (B) miR168 accumulation and PRI-MIR168A and AGO1 expression in *dbh1*, *dbh2* and *dbh12*. (C) The accumulation of miR169 and the expression of PRI-MIR169A and NFYA5 in *dbh1*, *dbh2* and *dbh12* plants.

dbh12. However, the molecular profile of the *dbh12* double mutant exactly matched that of *dbh1* plants demonstrating that DRB2 is not involved in the biogenesis of miRNAs with unchanged accumulation in the SAM region of *dbh235* plants (Table S1).

The accumulation of the *dbh235* reduced miRNA class representative, miR169 was reduced in all three *dbh* mutant backgrounds analyzed. Compared to Col-0, miR169 was at
In accordance with the emergence of green tissue, amiR-PDS accumulation was reduced in \textit{drb2}/amiR169A-PDS plants, and furthermore, precursor transcript processing and target gene expression were determined to be reduced and elevated respectively in the \textit{drb2} background (Figure 5D).

Figure 5. Artificial miRNA-directed silencing of \textit{PDS}. (A) Photo-bleached phenotypes expressed by Col-0 and \textit{drb} mutants transformed with the amiR\textsuperscript{164B}-PDS plant expression vector. Scale bars = 7.5 mm. (B) amiR-PDS accumulation and \textit{PRI-MIR164B-PDS} and \textit{PDS} expression in amiR\textsuperscript{164B}-PDS expressing plants. (C) amiR\textsuperscript{169A}-PDS-directed PDS silencing in wild-type plants and \textit{drb} mutants. Red arrows indicate the small sectors of photo-bleaching displayed by \textit{drb235}/amiR\textsuperscript{169A}-PDS plants. Scale bars = 7.5 mm. (D) amiR-PDS accumulation and target transcript expression in plants expressing the modified \textit{PRI-MIR169A} transcript targeting \textit{PDS} for amiRNA-directed silencing.

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stem of *drb235/amiR<sup>169A</sup>-PDS* transformants. Accumulation of the amiR-PDS sRNA was reduced in *drb235* transformant lines and RT-PCR suggested that this reduction resulted from inefficient PRI-MIR169A-PDS processing in the triple mutant, leading to deregulated target gene expression (Figure 5D). When compared with amiR<sup>169A</sup>-PDS transformed *dbb*2 plants, the almost complete lack of photo-bleaching in *dbb235/amiR<sup>169A</sup>-PDS* transformants strongly indicates that the action of all three of these closely-related DRB family members is required for wild-type sRNA-mediated target gene expression regulation for miRNAs with reduced accumulation in *dbb235* plants.

The constitutive over-expression of DRB2 can compensate for the loss of DRB1

To confirm the requirement of DRB2 activity in miRNA biogenesis, *dbh1* plants were transformed with the DRB2 coding sequence under the control of the 35S promoter (Figure S5A). We have previously shown that the *dbh1* developmental phenotype can be fully complemented via the introduction of a plant expression vector that constitutively over-expresses *DRB1* [25]. This transformant line was therefore included in our analyses as a positive control for reversion to wild-type miRNA accumulation and target gene expression. Similarly to the positive control line *dbh1/DRB1*, the constitutive over-expression of DRB2 (Figure 5B); in the absence of DRB1 activity, fully complemented the *dbh1* phenotype (Figure 6A). Northern blotting (Figure 6B) and RT-PCR (Figure 6C) analyses further demonstrated that the molecular profile of *dbh1/DRB2* plants closely matched those of Col-0 and *dbh1/DRB1* plants. The coding sequences of *DRB3* and *DRB5* were also constitutively expressed in the *dbh1* background to determine if these two DRB family members could also compensate for the loss of DRB1 activity (Figures S5A and S5B). However, no transformant line expressing a wild-type phenotype was recovered following transformation of *dbh1* plants with either vector (Figure 6A). In addition to displaying the *dbh1* phenotype, mature miRNA accumulation and target gene expression remained at *dbh1* levels in *dbh1/DRB3* and *dbh1/DRB5* plants (Figures 6B and 6C).

To determine why the constitutive over-expression of DRB2, and not DRB3 or DRB5 allowed for *dbh1* complementation, the coding sequences of *DRB2, DRB3* and *DRB5* were fused in frame with the *YELLOW FLOURESCENT PROTEIN* (YFP) reporter gene and transiently expressed in *Nicotiana benthamiana* (*N. benthamiana*) leaves via *Agrobacterium*-infiltration. Previous studies have shown DRB1, DCL1 and their miRNA precursor substrates to be localized in nuclear D-bodies [14,15,16]. The *DRB1* coding sequence was therefore fused to the *CIAN FLOURESCENT PROTEIN* (CFP) reporter gene for; i) co-expression with the DRB2-YFP, DRB3-YFP and DRB5-YFP vectors, and; ii) confirmation of nuclear localization. As an additional positive control for nuclear localization, we used the Histone 2B (H2B) protein fused in frame to YFP (H2B-YFP) which has been demonstrated previously to exclusively localize YFP florescence to the nucleus [43]. As expected, co-infiltration of *N. benthamiana* leaves with the DRB1-CFP and H2B-YFP constructs showed overlapping expression in the nucleus of infiltrated cells (Figure 6D). Reporter gene expression also overlapped in the nucleus when the DRB1-CFP and DRB2-YFP vectors were co-infiltrated with YFP fluorescence concentrated in small nuclear compartments and adjacent to the nuclear membrane. However, YFP and CFP fluorescence did not overlap when either the DRB3-YFP or DRB5-YFP vector was co-expressed with DRB1-CFP. YFP was observed throughout the cytoplasm of cells expressing DRB3-YFP. Fluorescence was also observed in the cytoplasm of DRB3-YFP expressing cells, concentrating in chloroplasts (Figure 6D). Taken together, the results presented in Figure 6 suggest that DRB3 and DRB5 are not able to substitute for DRB1 activity in the DCL1-catalyzed dsRNA processing stages of the *Arabidopsis* miRNA biogenesis pathway as these two family members are excluded from the appropriate cellular compartment, namely nuclear D-bodies. Furthermore, these analyses also demonstrated that when expressed constitutively outside of its wild-type functional domain, nuclear-localized DRB2 can compensate for the loss of DRB1 activity in *dbh1* plants.

Discussion

In this study, we have demonstrated that the pleiotropic phenotype displayed by *dbh235* plants is a result of altered miRNA accumulation and target gene expression in specific tissues where DRB2 is expressed in wild-type plants. Extension of our original DRB2 putative promoter region from 1.7 kb [25] to 4 kb upstream of the DRB2 transcription start site showed that these additional regulatory elements directed DRB2 expression to overlap with DRB3 and DRB5 in the SAM region (Figure 1C). The *dbh235* developmental phenotype when compared to those displayed by *dbcl* hypomorphic and *dbh1* null mutants suggested that DRB2, DRB3 and DRB5 could be functioning redundantly in the *Arabidopsis* miRNA biogenesis pathway. Small RNA sequencing of the specific tissue where these three genes are expressed in wild-type plants identified three distinct miRNA accumulation classes in *dbh235* plants, those that were elevated, unchanged or reduced. Northern blotting (Figures 2 and S3) revealed a clear association between the loss of DRB2 activity and altered sRNA levels for both the elevated and reduced *dbh235* miRNA classes to suggest that DRB2 under some circumstances is antagonistic and under other circumstances is synergistic to the function of DRB1 in miRNA biogenesis in this developmentally important tissue.

The accumulation of the elevated miRNA class representative, miR164, was enhanced in all plant lines lacking DRB2 activity (Figures 2A and S3A). However, the phenotype of the *dbh235* triple mutant is distinct to *dbh2, db23* and *dbh25* and all other *dbh* mutant combinations, developing fused inflorescence stems (Figure 1B). In this tissue (Figure 3A), and in the SAM region of *dbh235* plants (Figures 2A and S3A), the elevated levels of miR164 completely represses *CUC1* and *CUC2* expression. Previous genetic analyses have shown that *CUC1* and *CUC2* are functionally redundant and that plants defective for *CUC1* and *CUC2* activity, including the *cuc1 cuc2* double mutant, or plants engineered to constitutively and ubiquitously express either the *MIR164A* or *MIR164B* precursor transcript, display vegetative and floral organ fusion defects [30,33,37,38,44]. This indicates that the inflorescence stem fusions observed in *dbh235* plants result from tissue-specific elevation of miR164 accumulation and a corresponding loss of *CUC1* and *CUC2* expression.

Curiously, the phenotypic consequences of altered miR164, *CUC1* and *CUC2* levels on rosette leaf margin development reported here contrast with those described previously. The leaf margins of *cuc1-13* plants are indistinguishable from those of Col-0 plants and rosette leaves with smooth margins are displayed by the *cuc2-3* mutant or by plants ectopically over-expressing miR164 precursor transcripts [30,31,33]. In addition, T-DNA insertion knockouts of the *MIR164A* locus or plants engineered to express a miR164-resistant version of *CUC2* (*CUC2*<sup>miR164</sup>-plants), develop highly serrated rosette leaves [31,34,45]. Taken together, these studies demonstrate that miR164a-mediated regulation of *CUC2* expression is required for rosette leaf margin development. In our series of *dbh* mutants, including *dbh2, db23, db25* and *dbh235*
Figure 6. The constitutive over-expression of DRB2 can compensate for the loss of DRB1 activity. (A) Phenotypes expressed by homozygous 2 week old plants following the transformation of drb1 with the DRB1, DRB2, DRB3 and DRB5 over-expression vectors. Scale bars = 7.5 mm. (B) miR164, miR168 and miR169 accumulation in DRB over-expression vector transformed drb1 whole plants. (C) CUC2, AGO1 and NFYA5 expression, the target genes of miR164, miR168 and miR169 respectively in DRB over-expression vector transformed drb1 whole plants. (D) Cellular localisation of Arabidopsis DRB1, DRB2, DRB3 and DRB5 fluorescent reporter gene fusion vectors transiently expressed in N. benthamiana leaves. Scale bars = 20 μm.

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miRNA accumulation and target gene expression profile of complemented the drb1 expression was reduced in drb2 processing efficiency (Figure 4). The observed changes to miR164 and miR169 levels in these three mutant lines resulted in further arrests to plant development due to an overexpressing the second amiR-PDS vector. As shown for PRI-MIR169A members, failure to detect a miR169 signal by northern blotting correlated the efficiency of PDS-directed transcript processing stage of miRNA biogenesis is required to correlate with elevated miR164 accumulation in the double DRB2 activity in addition to DRB1 function is required for the pri-miRNA level. Compared to Col-0 and drb1 plants, all of which were shown to have elevated miR164 levels and corresponding reductions or complete loss of CUC1 and CUC2 expression, developed rosette leaves with serrated margins (Figures 1A, 3B and 5A). In contrast, miR164-mediated CUC1 and CUC2 expression is deregulated in dbh1 and dbh2 plants where miR164 levels are reduced and both of these mutant lines display rosette leaves with smooth margins (Figures 3B and 5B). Our analyses suggest that loss of CUC1 expression and reduced CUC2 levels in the specific tissues where miR164 accumulation is elevated in the absence of DRB2 activity, namely the SAM region, directs the rosette leaf margin serration phenotype expressed by dbh2, dbh25, dbh25 and dbh25 plants. These tissue-specific alterations to miR164 accumulation and target transcript expression could account for the phenotypic differences displayed by our dbh mutant lines and those previously characterized for plants lines where miR164, CUC1 and CUC2 levels are altered in all tissues and throughout all stages of development.

RT-PCR analysis of dbh1, dbh2 and dbh12 plants revealed that the observed changes to miR164 and miR169 levels in these three mutant lines was a result of alterations to precursor transcript processing efficiency (Figure 4). PRI-MIR164A and PRI-MIR164B expression was reduced in dbh2, but the level of each precursor transcript was increased in dbh1 and dbh12 plants. The moderate increase in PRI-MIR164A and PRI-MIR164B levels in dbh1 plants compared to their higher levels of expression in dbh1 plants correlated with elevated miR164 accumulation in the double mutant (Figure 4A). These analyses indicate that DRB2 antagonism of the DCL1/DRB1 partnership during the precursor transcript processing stage of miRNA biogenesis is required to regulate the accumulation of a subset of miRNAs in the SAM region of wild-type Arabidopsis plants. Compared to wild-type plants, miR169 accumulation was reduced to a similar level in dbh1 and dbh2 (Figure 4C). In accordance with miR169 levels, PRI-MIR169A expression was elevated in both of these dbh mutant plants to suggest that DRB1 and DRB2 are required for DCL1-catalyzed processing of the precursor transcripts of MIR169 family members. Failure to detect a miR169 signal by northern blotting and detection of further elevated PRI-MIR169A and NFTA5 expression in the dbh12 double mutant by RT-PCR confirmed that DRB2 activity in addition to DRB1 function is required for the biogenesis of a subset of miRNAs in the SAM region of Arabidopsis plants.

Modification of PRI-MIR164B and PRI-MIR169A to replace their endogenous sRNA silencing signals with the same PDS-targeting amiRNA confirmed that the antagonistic and synergistic action of DRB2 on DRB1 function in miRNA biogenesis occurs at the pri-miRNA level. Compared to Col-0 and dbh1 plants, transformation of dbh2 and dbh255 with the amiR164-PDS vector resulted in further arrests to plant development due to an overaccumulation of the amiR-PDS (Figures 5A and 3B). A different PDS silencing profile was displayed by the same dbh mutants when expressing the anti-miR-PDS vector. As shown for dbh1/amiR164-PDS plants, PDS silencing was severely deregulated in dbh235 plants expressing the amiR164-PDS vector. AmiR164-PDS-directed PDS silencing was also disrupted in dbh2 plants, but only in the specific tissue where DRB2 is expressed in wild-type plants (Figures 1C and 5C). Northern blotting and RT-PCR analyses directly correlated the efficiency of PDS silencing directed by either amiR-PDS vector with pri-miRNA processing efficiency and amiRNA accumulation in dbh2 and dbh235 plants.

Over-expressing DRB2 in the absence of DRB1 activity fully complemented the dbh1 phenotype (Figure 6A). The wild-type miRNA accumulation and target gene expression profile of dbh1/ DRB2 whole plant samples (Figures 6B and 6C) suggests that the involvement of DRB2 in miRNA biogenesis is restricted by its tissue-specific expression (Figures 1C and 3A). The expression of individual MIR gene family members is also regulated both spatially and temporally [31,32,46], and although many of these pri-miRNA transcripts could potentially express the same structural features or sequence motifs as those demonstrated to require DRB2 for their biogenesis here, their wild-type accumulation in other tissues is only dependent on the ubiquitously expressed DRB1. Fusion of the DRB2 coding sequence to the YFP reporter gene showed that DRB2 is a nuclear protein (Figure 6D). DCL1 and DRB1 are also nuclear-localized and function in concert in D-bodies to direct cleavage of miRNA/miRNA* duplexes in a sequential two-step process from the dsRNA stem-loop regions of pri-miRNA and pre-miRNA transcripts [14,16]. Furthermore, like DRB1, DRB2 has been demonstrated to interact with DCL1 and dsRNA in vitro [8] providing further support for the requirement of DRB2 activity in the biogenesis of a subset of miRNAs in specific tissues. It has recently been reported that DRB2 is antagonistic to DRB4 in the production of all sRNA size classes processed from PolIV generated transcripts and that DRB2 is synergistic to DRB4 in the biogenesis of DCL4-dependent miRNAs [26]. These findings parallel those reported here on DRB2 antagonism and synergism in DCL1/DRB1-directed miRNA biogenesis and suggest that the dsRNA intermediates derived from PolIV generated transcripts, or the stem-loop structures of DCL4/DRB4-dependent miRNA precursor transcripts also express structural features or sequence motifs that direct their interaction with DRB2 as well as with DRB4. Alternatively, DRB2 could be competing with DRB1 and DRB4 for interaction with their partnering proteins DCL1 and DCL4. Taken together, the results presented here and those of [26] suggest that DCL/DRB partners and/or DRB dsRNA interactions in the endogenous sRNA biogenesis pathways of Arabidopsis are more complex than previously thought.

As suggested by our northern blotting data (Figures 2C and 5D), DRB3 are DRB5 play no role in the processing steps of miRNA biogenesis and the constitutive and ubiquitous expression of these two DRB proteins failed to compensate for the loss of DRB1 activity in dbh1 plants (Figures 6A and 6B). YFP fusion to the DRB3 and DRB5 coding sequences revealed that they are not able to compensate for the loss of nuclear-localized DRB1 activity as they are both expressed in the cytoplasm (Figure 6D). However, these two cytoplasmic DRBs do appear to be involved in regulating the expression of specific target genes of DRB2-associated miRNAs. For example, the combined loss of DRB3 and DRB5 activity was demonstrated to result in the complete repression of CUC2 expression in the SAM region of dbh235 plants (Figure 2A). Furthermore, the almost complete absence of photo-bleaching in dbh253/amiR164-PDS transformants compared to the tissue-specific loss of amiR164-PDS-directed silencing only in dbh2/amiR164-PDS plants suggests that the activity of all three of these closely-related DRB family members is required for wild-type expression regulation of DRB2-associated miRNA target genes. The exact role that these two cytoplasmically-localized DRBs play in miRNA biogenesis or action remains to be functionally characterized.

We propose that, in Arabidopsis, DRB2 is performing a dual regulatory role in miRNA biogenesis in specific tissues (Figure 7). MiRNAs with wild-type accumulation in dbh235 plants are produced by the canonical miRNA biogenesis pathway mediated by the DCL1/DRB1 partnership (Figure 7; upper middle dark grey panel). Following their export to the cytoplasm the DCL1/DRB1-generated miRNA is loaded onto AGO1-catalyzed RISC to guide silencing of cognate mRNAs. For miRNAs with elevated
accumulation in *drb235* plants our results suggest that their precursor transcripts harbor structural features or sequence motifs that direct their interaction with DRB2. For DRB2 to direct its antagonistic effect on the DCL1/DRB1 biogenesis pathway, the DRB2-associated signal is predicted to be positioned within the pri-miRNA sequence that would prevent pre-miRNA stem-loop formation. In the event that the DRB2-associated signal was positioned outside of this region, the pri-miRNA would still have the capacity to fold and be recognized for entry into the canonical miRNA biogenesis pathway (Figure 7; upper left light grey panel). DRB2 is also synergistic to DRB1 for the biogenesis of another distinct class of miRNAs. The precursor transcripts of miRNAs with reduced accumulation in *drb235* plants express associating signals that could direct their interaction with either DRB1 or DRB2 (Figure 7; upper right light grey panel). Directing different DRB/pri-miRNA transcript associations in tissues where DRB2 is expressed would not only add an additional layer of regulatory complexity to miRNA-mediated target gene expression, but would also ensure the viability of plants in which either DRB1 or DRB2 activity is temporarily suppressed, for example by some environmental or pathogen-mediated stress.

**Materials and Methods**

**Vector construction**

The DRB1, DRB3 and DRB5 promoter driven GUS reporter gene plant expression vectors are previously described [25]. The new DRB2 promoter-driven GUS reporter gene plant expression vector, pDRB2pro:GUS was generated by PCR amplification of a 4 kb genomic fragment upstream of the transcription start site of DRB2 with primers pDRB2-PRO-F and pDRB2-PRO-R (Table S5). This PCR product was flanked by 5′ BamHI and 3′ NoI restriction sites and was cloned into the pGEM-T Easy cloning vector (Promega) to produce pGEM-T-DRB2pro. This vector was digested with BamHI and NoI to release the DRB2 promoter fragment that was cloned into the similarly digested vector pRITA [47] to produce pRITA:DRB2pro-GUS. The pRITA:DRB2pro-GUS vector was digested with NoI to release the DRB2pro-GUS fragment which was cloned into the similarly digested plant expression vector pBART [47] to produce pDRB2pro:GUS.

Genomic fragments containing the PRI-MIR164B (AT3G01747) and PRI-MIR169A (AT3G13405) sequences and flanking regulatory domains were amplified by PCR with primer pairs pMIR164B-F1/R1 and pMIR169A-F1/R1 and cloned into the pGEM-T Easy cloning vector to produce pGEM-T:MIR164B and pGEM-T:MIR169A respectively. These two vectors were used as templates for the construction of the PDS-targeting amiRNA plant expression vectors pamiR164B-3′/5′-PDS and pamiR169A-3′/5′-PDS by overlapping PCR-based cloning, essentially as described previously [41,42]. The modified PRI-MIR164B and PRI-MIR169A sequences were cloned into pART7 using the introduced XhoI/ EcoRI and XhoI/HindIII restriction sites. These two amiR-PDS shuttle vectors were digested with NoI and the resulting restriction fragments cloned into the similarly digested plant expression vector pBART to produce pamiR164B-3′/5′-PDS and pamiR169A-3′/5′-PDS. All primers used in the construction of the amiR-PDS vectors are listed in Table S5.

To produce the DRB over-expression vectors used to transform *db1* plants, the DRB1, DRB2, DRB3 and DRB5 coding sequences were generated using the Qiagen OneStep RT-PCR kit according to the manufacturer’s instructions (primer sequences listed in Table S5). Each product was flanked by a 5′ KpnI and 3′ XhoI restriction site and these products cloned into the pGEM-T Easy cloning vector to produce vectors pGEM-T:DRB1/2/3/5.

Vectors were digested with *KpnI* and *XhoI* and the four resulting restriction fragments cloned into the similarly digested vector pART7 to produce pART7:DRB1/2/3/5. The pART7:DRB vector series was digested with *NoI* and cloned into the *NoI* site of pBART to produce the plant expression vectors pDRB1, pDRB2, pDRB3 and pDRB5.

For construction of the DRB fluorescent reporter gene fusion vector series, the DRB over-expression vectors were used as templates for PCR amplification of the respective DRB coding sequences, minus their stop codons. The antisense primer used in these PCR reactions introduced a *XhoI* restriction site at the 3′ terminus of each PCR product and these 3′ no stop codon (NSC) DRB restriction fragments (DRB-NSC) were cloned into the pGEM-T Easy cloning vector to produce the pGEM-T:DRB1/2/3/5-NSC vectors. The *CFP* (DRB1) and *YFP* (DRB2, DRB3 and DRB5) reporter genes were also amplified by PCR with forward and reverse primers that contained *XhoI* restriction sites (Table S5). These two PCR products were cloned into the pGEM-T Easy cloning vector to produce pGEM-T-CFP and pGEM-T-YFP respectively and all vectors were subsequently digested with *XhoI*. The CFP/*XhoI* and YFP/*XhoI* restriction fragments were gel purified and cloned into the respective *XhoI* linearized pGEM-T:DRB1/2/3/5-NSC vectors. pGEM-T-DRB-NSC vectors containing reporter gene inserts in the desired 5′ to 3′ orientation were digested with *EcoRI* and cloned into the similarly digested pART7 to produce pART7:DRB1-CFP and pART7:DRB2/3/5-YFP respectively. These four vectors were digested with *NoI* and the resulting restriction fragments cloned into the *NoI* digested plant expression vector pBART to produce pDRB1-CFP, pDRB2-YFP, pDRB3-YFP and pDRB5-YFP.

**Plant material, growth conditions and transformations**

The *db6* T-DNA insertion lines used in this study have been described previously [25]. *Arabidopsis* and *N. benthamiana* plants were grown under standard glasshouse conditions of 16 h of light/8 h of dark at 24°C. All plant expression vectors used in this study were transformed into *Agrobacterium tumefaciens* (strain GV3101) via electroporation. *Agrobacterium* cultures were used to transform *db1* plants with the DRB series of over-expression vectors, wild-type plants (Col-0) with the DRB2 promoter-driven GUS reporter gene vector and Col-0, *drb1*, *drb2* and *drb235* plants with the amiR-PDS vectors by floral dipping as described previously [48]. Transformants were selected by germinating the dippled seed on plant growth media supplemented with 10 mg/mL of phosphinothricin. For stable transformations of *Arabidopsis*, the number of primary transformants expressing the phenotypes reported here are listed in Table S6. Leaf infiltrations of *N. benthamiana* plants for the transient expression of the DRB fluorescent reporter gene fusion series of plant expression vectors via *Agrobacterium*-mediated transformation was performed as previously described [35].

**Reporter gene expression analyses**

Screening for GUS expression in 4 week old plants expressing the *DRB1*, *DRB2*, *DRB3* or *DRB5* promoter-driven GUS reporter gene plant expression vectors using 3-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) was conducted according to [49]. The *CFP* and *YFP* reporter gene vectors were transiently expressed in *Agrobacterium*-infiltrated *N. benthamiana* leaves for 72 hr. Following this incubation period, *Agrobacterium*-infiltrated leaves were removed from the plant and CFP and YFP fluorescence examined by confocal microscopy under constant illumination.
Small RNA sequencing

Total RNA was isolated from the SAM region of 4 week old Col-0 and drb235 plants using TRIzol Reagent according to the manufacturer’s instructions (Invitrogen). Twenty micrograms (20 µg) of total RNA was shipped to the Victor Chang Cardiac Research Institute for processing. The sequences of sRNA (20–24 nt in length) that exactly matched those deposited into the miRBase database (http://www.mirbase.org/) for Arabidopsis thaliana were determined using SOLiD color space technology.

Northern blot analysis of miRNA accumulation

Northern blot analysis to assess miRNA accumulation in Arabidopsis was essentially performed as described previously [40]. In brief, total RNA was isolated from pooled plant tissues using TRIzol Reagent according to the manufacturer’s instructions. Twenty micrograms (20 µg) of total RNA was separated on 15% denaturing (10 M urea) polyacrylamide gels by electrophoresis and transferred to HyBond-N* membrane (Amersham) by electroblotting. DNA oligonucleotide probes specific for each miRNA assessed by northern blotting were end-labeled using
Terminal deoxynucleotidyl transferase (Fermentas) and $\gamma^{32}\text{P}$ CTP. All DNA oligonucleotide probes used in this study are listed in Table S4.

RT-PCR and qRT-PCR analysis of pri-miRNA and miRNA target gene expression

A 5 µg aliquot of the same total RNA isolation used for northern blotting was digested with 5 units of RQ1 RNase-free DNase (Promega) at 37°C for 30 min and purified using an RNeasy Mini kit (Qiagen). Purified RNA (1 µg) was used to synthesize cDNA with SuperScript III reverse transcriptase (Invitrogen) and Oligo (dT)23 according to the manufacturer’s instructions. Each cDNA was diluted to 50 ng/µL and 3 µL of this dilution was used as template in a 25 µL PCR reaction, and each experiment was repeated three times. TUBULIN (TUBULIN BETA6; AT5G23860) was used as the housekeeping control for pri-miRNA expression analysis and ACTIN (ACTIN2; AT3G18780) was used as the housekeeping control for miRNA target gene expression. All primers used in this study for RT-PCR assessment of pri-miRNA and miRNA target gene expression are listed in Table S5. To confirm RT-PCR assessment of precursor transcript and miRNA target gene expression, the same cDNA samples were analyzed by quantitative RT-PCR (qRT-PCR) according to [10]. All qRT-PCR expression data is provided in Table S7.

Supporting Information

Figure S1 Accumulation of miRNAs involved in leaf shape development and expression of their target genes in 4 week old whole plant samples. (A) miR159, miR164 and miR319 accumulation in 

Figure S2 miRNA accumulation in the SAM region of 

Figure S3 Comparison of miR841, miR162 and miR170 accumulation in the SAM region 

Figure S4 Leaf margin phenotypes displayed by 

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Author Contributions

Conceived and designed the experiments: ALE PMW. Performed the experiments: ALE KWK. Analyzed the data: ALE SJC PMW. Contributed reagents/materials/analysis tools: SJC. Wrote the paper: ALE PMW.
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