Published in final edited form as:
*New Phytol*. 2010 April ; 186(1): 207–215. doi:10.1111/j.1469-8137.2010.03187.x.

# RNAi-mediated down-regulation of *DCL1* and *AGO1* induces developmental changes in resynthesized *Arabidopsis* allotetraploids

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## Summary

- Both natural and newly synthesized allopolyploids display nonadditive gene expression changes through genetic and epigenetic mechanisms. The nonadditively expressed genes include many microRNA (miRNA) targets, suggesting a role for miRNAs and their targets in morphological variation in the allopolyploids and their progenitors.
- We produced dominant-negative transgenic allotetraploid plants in *Arabidopsis* using RNA interference (RNAi) that downregulates the expression of miRNA biogenesis genes, including *DCL1* and *AGO1*.
- RNAi of *DCL1* and *AGO1* led to dominant negative phenotypes and decreased accumulation of several miRNAs and a tasiRNA tested in the transgenic resynthesized allotetraploids.
- The results demonstrated that miRNA biogenesis genes are effectively downregulated in the resynthesized allotetraploids containing redundant homoeologous genes that are difficult to be manipulated by conventional mutation screens. These lines will be useful for studying the effects of miRNA biogenesis genes on growth and developmental variation in the allopolyploids.

### Keywords

*AGO1*; *DCL1*; polyploidy; hybrid vigor; microRNA; RNAi; small RNA

## Introduction

Polyploids can be formed by whole-genome duplication within a species (autopolyploids) or by hybridization between related species followed by chromosomes doubling (allopolyploids) (Chen, 2007; Doyle *et al.*, 2008). Estimates indicate that over 75% of



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flowering plants are polyploids (Masterson, 1994), and many important crops such as wheat, cotton, and canola are allopolyploids. *Arabidopsis* allotetraploids are readily formed by hybridizing two autotetraploids, *Arabidopsis thaliana* and *Arabidopsis arenosa* (Comai *et al.*, 2000; Wang *et al.*, 2004). Genome-wide analysis of gene expression indicates *c*. 15% (up to *c*. 35%) of the transcriptome divergence between *A. thaliana* and *A. arenosa* (Wang *et al.*, 2006b). The majority of differentially expressed genes (*c*. 68%) between the parents are nonadditively expressed in two independently derived allotetraploids. Nonadditive expression refers to that the expression level of a gene (two homoeologous loci) in the allotetraploid is not equal to the sum of the two parental loci. It is considered to be a major source of nonadditive or novel phenotypic variation in the allotetraploids. For example, nonadditive regulation of *FRI* and *FLC* loci mediates flowering-time variation in *Arabidopsis* resynthesized and natural allopolyploids (Wang *et al.*, 2006a). A recent study showed that nonadditive expression of the genes involved in the circadian clock regulation leads to vigorous growth through altered expression of downstream metabolic genes in the allotetraploids (Ni *et al.*, 2009). Many microRNA (miRNA) target genes encode transcription factors and proteins that are important to plant growth and development (Carrington & Ambros, 2003; Kidner & Martienssen, 2005; Chuck *et al.*, 2009). Interestingly, many miRNAs and their targets are nonadditively expressed in the allotetraploids (Wang *et al.*, 2006b; Ha *et al.*, 2009), suggesting their role in morphological and phenotypic variation in the allopolyploids.

One of the difficulties in studying gene function and epigenetic regulation in allotetraploids is genetic redundancy, because two or more homoeologous loci of the progenitors' genes are present in an allopolyploid. Although Transfer DNA (T-DNA) insertion and Ethane Methyl Sulfonate (EMS) mutagenesis have been widely used to create gene knockout or knockdown mutants for genetic studies in plants, including *Arabidopsis* (Alonso *et al.*, 2003; Maple & Moller, 2007), these conventional genetic screening strategies do not work well in polyploids. The dominant negative strategy using RNA interference (RNAi) or hairpin RNA (hp-RNA) is an effective approach to overcome genetic redundancy and has been used to downregulate the expression of *MET1*, *DDM1* and *HDT1* in natural allotetraploid, *Arabidopsis suecica* (Lawrence & Pikaard, 2003; Lawrence *et al.*, 2004; Wang *et al.*, 2004). Resynthesized *Arabidopsis* allotetraploids have not been transformed, and miRNA biogenesis genes have not been subjected to RNAi in allotetraploids. It is likely that RNAi for miRNA biogenesis genes may not work well in diploids because the small RNA biogenesis genes such as *DCL1* are essential for growth and development and can only be maintained in heterozygous states (Gasciolli *et al.*, 2005). In *Arabidopsis*, DICER-LIKE 1 (DCL1) is an RNaseIII-type enzyme and functions in the sequential processing of primary miRNAs (pri-miRNAs) to pre-mature miRNAs (pre-miRNAs) and finally mature miRNAs. The guide strand of a miRNA is then incorporated into the RNA-induced silencing complex (RISC) containing ARGONAUTE 1 (AGO1) for target gene downregulation (Chapman & Carrington, 2007b). In a previous study we showed that *DCL1* and *AGO1* are nonadditively expressed in allotetraploids, suggesting that miRNA biogenesis genes have a role in nonadditive accumulation of miRNAs in allopolyploids (Ha *et al.*, 2009b). In this study, we used gene-specific hp-RNAs to downregulate *DCL1* or *AGO1* expression in *Arabidopsis* resynthesized allotetraploids, namely, Allo733 (Wang *et al.*, 2006b). The RNAi of *DCL1* and *AGO1* allotetraploids displayed dominant negative phenotypes that are characteristic of these miRNA biogenesis genes in diploids. These lines are valuable materials for testing the effects of miRNAs and their targets on growth and developmental variation in resynthesized and natural allotetraploids.

## Materials and Methods

### Plant materials and growth conditions

The resynthesized allotetraploids were derived from a cross of *A. thaliana* (L.) Heynh. tetraploid pollinated with pollen from *A. arenosa* (L.) Lawalrée tetraploid and used for gene expression analysis in the $F_6$ generation (Comai *et al.*, 2000; Wang *et al.*, 2004). One of the stable allotetraploids was self-pollinated to the $F_8$ generation and used to generate transgenic plants. *dcl4-2* T-DNA mutant (CS6954) was obtained from the Arabidopsis Biological Resource Center (ABRC). All plants were grown at 22°C under a 16 h light and 8 h dark cycle. All samples were collected between 10 o'clock in the morning and noon. for gene expression analyses.

### RNAi constructs and plant transformation

Partial cDNA fragment of *DCL1* (436-bp; *At1g01040*) and *AGO1* (470-bp; *At1g48410*) were amplified from *A. thaliana* cDNA using the primer pairs with flanking 5′ attB1 and 3′ attB2 sites (Supporting Information Table S1). Individually amplified PCR product was cloned into pDONR221 using Gateway technology (Invitrogen) to generate an entry construct for subsequent recombination into the destination vector, pAGRIKOLA (Hilson *et al.*, 2004). The resulting hp-RNA construct contained the partial cDNA fragment that was cloned into sense and antisense orientation flanking the *Pdk* and *Cat* introns. Individual RNAi construct was co-transformed with the helper plasmid, pSoup, into *Agrobacterium tumefaciens* GV3101 by electroporation. For plant transformation, Allo733 plants with many flower buds (*c*. 10 wk old) were dipped twice (with 1 wk apart) in *Agrobacterium* cultures containing the *DCL1* or *AGO1* RNAi construct using floral dip method (Clough & Bent, 1998). The plants were grown four more weeks after transformation, and the seeds were harvested for screening transgenics using herbicides. Primary transformants were selected on Gamborg's B-5 Basal Medium (G5893; Sigma) containing 20 mg $l^{-1}$ glufosinate-ammonium (Sigma). Genomic DNA was extracted from 4-wk-old leaves of selected transformants for PCR genotyping using a 5′ gene-specific primer (with the attB2 site) and a 3′-primer target the OCS terminator (Table S1). Here T1 seeds were referred to a mixture of transformed and untransformed seeds from floral dipped plants (T0). The transformant plants were selected from T1 seeds, and T2 plants were obtained from progenies (T2 seeds) of a T1 plant.

### Total RNA isolation and qRT-PCR

Total RNA was extracted from leaves of 7–8 wk old plants (*c*. 100 mg tissues) using Trizol Reagent (Invitrogen) and treated with Turbo DNase (Ambion) according to the manufacturers' instructions. An aliquot (1 μg) of DNase-treated RNAs was prepared for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) in the presence of 25 ng $\mu l^{-1}$ oligo dT (12–18) primer. The synthesized cDNAs were diluted to 100 μl, and 1 μl of the diluted cDNA was used for quantitative (q)PCR using power SYBR green PCR mix (Applied Biosystems, Foster City, CA, USA) in the presence of 0.5 $\mu_M$ gene specific primers (Table S1). The qPCR was performed using 7500 Real Time PCR thermal cycler (Applied Biosystems). Expression levels of the endogenous target genes in RNAi lines were normalized against the *Ubiquitin10* transcript levels. Relative expression levels of the target transcripts were derived by comparisons between an RNAi line and control transgenic lines that contain a plasmid vector only. A Student *t*-test was used to determine the significance of target gene downregulation in the RNAi lines relative to the control transgenic plants in three biological replications.

### Small RNA detection

Total RNA (10 μg) was separated in a 15% polyacrylamide gel and transferred to a Hybond N+ membrane (GE Healthcare, Piscataway, NJ, USA) using a Mini-Protean II wet-transfer cell (BioRad). After ultraviolet (UV) crosslinking, the membrane was prehybridized in Church buffer (Church & Gilbert, 1984) at 37°C for 1 h. An oligonucleotide with the sequence complementary to the target mature miRNA was end-labeled with γ-P32 ATP (6000 Ci $mmol^{-1}$) and purified using a G-25 Sephadex column (Pharmacia Fine Chemicals Inc., Piscataway, NJ, USA). The antisense U6 oligonucleotide was also end-labeled using 2 mCi $ml^{-1}$ γ-P32. For hybridization, the small RNA blot was incubated overnight at 40°C in the presence of radioactive labeled probes. After hybridization, the blot was rinsed with 2× standard saline citrate (SSC) for 5 min, then washed twice with 2× SSC and 0.1% sodium dodecyl sulfate (SDS) for 15 min at 40 °C. Small RNA hybridization signals were detected by exposing the blot to a Phosphor imaging plate from 4 h to overnight and quantified using IMAGEQUANT (Bio-Rad). Each hybridization experiment was replicated once. For detection of other miRNAs, the same blot was stripped in 0.1× SSC and 0.1% SDS solution at 95°C for 1 h and reprobed with an antisense oligonucleotide probe of another miRNA, as previously described, following a 1-h prehybridization in Church buffer at 37°C.

## Results

### RNAi-mediated downregulation of *DCL1* and *AGO1* in allotetraploids

Nonadditive expression of miRNAs in the allotetraploids may be caused by transcriptional regulation of homoeologous miRNA precursor loci and/or posttranscriptional regulation of miRNA biogenesis genes (Ha *et al.*, 2009). In *A. thaliana*, DCL1 and AGO1 are two key components of the miRNA biogenesis pathway that play a critical role in growth and development (Meins *et al.*, 2005; Ramachandran & Chen, 2008). Interestingly, several miRNA biogenesis genes, including *DCL1* and *AGO1*, are nonadditively expressed in the allotetraploids, which may lead to differential accumulation of miRNAs and growth and developmental variation in resynthesized and natural allotetraploids (Ha *et al.*, 2009). To test the role of miRNA biogenesis genes in miRNA production in allotetraploids, we produced dominant negative transgenic plants using RNAi of *DCL1* and *AGO1*. The transgenic allotetraploid plants were generated using two hp-RNA constructs targeting *A. thaliana DCL1* and *AGO1*, respectively, with pAGRIKOLA (Hilson *et al.*, 2004) as the destination vector (Fig. 1a). To target *DCL1*, a 436-bp *AtDCL1* cDNA region corresponding to amino acid residues 1–146 of AtDCL1 (*At1g01040*) (Fig. 1b) was used for the hp-RNA construct. A 470-bp cDNA encoding amino acid residues 675–831 of AtAGO1 (*At1g48410*) (Fig. 1c) was used as the hp-RNA construct to target *AGO1*. The nucleotide sequence alignment between *AtDCL1* and *AtAGO1* cDNA fragments and the corresponding *A. arenosa* homoeologous BAC sequences (Fig. 1b,c) showed 97.3% and 97% identities, respectively. With the hairpin construct, a 21- to 24-nt RNA predicted by computational analysis showed a perfect match with both *AtDCL1* and *AaDCL1*. By contrast, the *AtDCL1* fragment in the hp-RNA construct had no homology with other members (*AtDCL2*, *AtDCL3* and *AtDCL4*) of the gene family in *A. thaliana* (data not shown). Among the 10 *AtAGO* genes, *AGO1*, *AGO5* and *AGO10* are in the same clade. The *AtAGO1* sequence used in the hairpin construct is 64.9% identical to *AGO10* and 64.5% identical to *AGO5* (Fig. S1). Computational analysis did not find a region with continuous 21- to 24-bp that could target a sequence region other than *AGO1*. Outside the clade, the *AtAGO1* fragment has a low sequence identity with other AGO family members (e.g. *AGO4* and *AGO6*).

Two hairpin constructs were transformed into the stable resynthesized allotetraploid line (Allo733). We produced 10 *AGO1* (*ago1*(Allo):RNAi) and 11 *DCL1* (*dcl1*(Allo):RNAi) transgenic plants. The transgenic plants were examined for the presence of transgenes using

PCR and genomic DNA (data not shown). The T1 transgenic plants were self-pollinated, and one-third of them died or did not produce seeds. The expression levels of endogenous *DCL1* and *AGO1* were examined using quantitative reverse-transcription (qRT-)PCR analysis (Fig. 1d,e). Among the *dcl1*(Allo):RNAi lines tested, endogenous *DCL1* expression levels were reduced, ranging from *c*. 8% in the *dcl1-1*(Allo):RNAi line to *c*. 40% in the *dcl1-3*(Allo):RNAi line relative to the control transgenic plants that contained a plasmid vector construct (Fig. 1d). On average, the *DCL1* expression levels were reduced by 23% in the *DCL1* RNAi lines (0.77 ± 0.05; $n = 5$; $P = 0.035$) compared with the *DCL1* expression levels in the control transgenic plants. Downregulation of *DCL1* expression by RNAi increased the number of leaves in some *dcl1*(Allo):RNAi lines (Fig. 2b), suggesting a role for *DCL1* in cell proliferation and development, as observed in the *caf-1* (CARPEL FACTORY, a DCL1-like homolog) mutants (Jacobsen *et al.*, 1999; Park *et al.*, 2002). Similar to homozygous *dcl* (*sin1*) mutants in *Arabidopsis* diploids (Ray *et al.*, 1996), flowering time was delayed by 1–3 wk in the *dcl1*(Allo):RNAi lines compared with the control plants (flowered in *c*. 8 wk) in the T1 generation (Fig. 2f). However, flowering time of several *dcl1*(Allo):RNAi lines in the T2 generation was variable, suggesting that the RNAi effects on *DCL1* repression are relatively unstable. Further investigation is required to fully characterize the effects of *DCL1* downregulation on phenotypic variation, including flowering time.

In the *ago1*(Allo):RNAi lines tested, the expression levels of endogenous *AGO1* were reduced 33–52% (Fig. 1e). The *ago1*(Allo):RNAi lines showed an average of 44% reduction in *AGO1* expression levels (0.56 ± 0.04; $n = 5$; $P = 0.006$) compared with the control transgenic plants. Phenotypically, some *ago1*(Allo):RNAi lines flowered late (Fig. 2f), and others displayed dramatic changes in leaf morphology with reduced levels of leaf serration and curled leaf phenotypes, which curled toward the abaxial side of the leaves (Fig. 2c,d,e). Interestingly, the curled leaf phenotype in *ago1*(Allo):RNAi lines resembles that in *dcl4-2* mutant in *A. thaliana* diploids (Fig. 2d,e) (Xie *et al.*, 2005;Yoshikawa *et al.*, 2005). In addition, like *dcl1*(Allo):RNAi lines, down-regulation of *AGO1* in Allo733 led to late flowering in several transgenic lines.

### *DCL1* and *AGO1* RNAi lines showed decreased accumulation of small RNAs in allotetraploids

DCL1 and AGO1 are the key components of miRNA biogenesis pathway (Ramachandran & Chen, 2008). Consistent with the reduced expression levels of *DCL1* and *AGO1*, accumulation of selected miRNAs was affected in both *dcl1*(Allo):RNAi and *ago1*(Allo):RNAi lines (Fig. 3a). In general, miRNA and tasiRNA levels were downregulated 20–50% in the *dcl1*(Allo):RNAi and *ago1*(Allo):RNAi lines. The amounts of miR159a, miR159b and miR403 were reduced more in *dcl1*(Allo):RNAi lines than in *ago1*(Allo):RNAi lines (Fig. 3b). The data suggest that RNAi of *dcl1* and *ago1* correlates with reduced accumulation of RNA and tasiRNA , consistent with the hypomorphic phenotypes observed.

## Discussion

Many miRNAs and their targets control cell patterning, proliferation, growth and development in plants and animals (Pasquinelli & Ruvkun, 2002; Carrington & Ambros, 2003; Kidner & Martienssen, 2003; Chuck *et al.*, 2009). In resynthesized allotetraploids derived from *A. thaliana* and its related species *A. arenosa*, many miRNAs and their targets that are differentially expressed between *A. thaliana* and *A. arenosa* are nonadditively expressed, suggesting an important role of miRNAs in morphological and developmental variation between closely related species and allopolyploids (Ha *et al.*, 2009). Both EMS and T-DNA insertional mutants of *DCL1* or *AGO1* have been identified and characterized in

several *A. thaliana* ecotypes (Col, L*er*, Ws and C24) (Bohmert *et al.*, 1998; Jacobsen *et al.*, 1999; Lynn *et al.*, 1999; Golden *et al.*, 2002; Schauer *et al.*, 2002; Baumberger & Baulcombe, 2005; Sorin *et al.*, 2005; Yang *et al.*, 2006; Brodersen *et al.*, 2008). Unlike in *A. thaliana* diploids, T-DNA insertion and EMS mutagenesis approaches do not work effectively on reducing the expression of target genes in allotetraploids because of genetic redundancy. Therefore, dominant negative strategy has been employed to downregulate the expression of homoeologous genes including several chromatin genes in the allotetraploids. Stable RNAi lines of natural *A. suecica* have been generated for *MET1, DDM1* and *HDT1*. RNAi of *met1* and *ddm1* in a natural allotetraploid *A. suecica* leads to activation of many transposons and heterochromatic genes (Wang *et al.*, 2004; Chen *et al.*, 2008), whereas silenced rDNA was reactivated in RNAi of *hdt1* lines (Lawrence & Pikaard, 2003; Lawrence *et al.*, 2004). The phenotypic abnormality of RNAi of *met1* and *ddm1* was severe in the selfing progeny, consistent with the *met1* and *ddm1* mutant lines in diploids (Vongs *et al.*, 1993; Kankel *et al.*, 2003). In this study, RNAi of *dcl1* and *ago1* in the resynthesized allotetraploids (Allo733) results in dramatic changes in leaf morphology and flowering time in the T1 generation. However, almost all *dcl1*(Allo):RNAi and *ago1*(Allo):RNAi lines with severe developmental phenotypes were not viable in T2 generation. It is possible that RNAi of *dcl1* and *ago1* may lead to embryonic lethality as observed in several *dcl1* mutants in *A. thaliana* diploids (Jacobsen *et al.*, 1999; McElver *et al.*, 2001; Schauer *et al.*, 2002). Alternatively, additional mutations may accumulate during selfing in the *dcl1*(Allo):RNAi lines as observed in the *ddm1* mutants (Stokes & Richards, 2002). Only transgenic lines with low RNAi penetrance might have survived through meiotic transmission during selfing. Compared with *ddm1* and *met1 A. suecica* RNAi lines, RNAi of *dcl1* and *ago1* in resynthesized allotetraploids gives rise to severe effects on growth and development in early generations of polyploidization, suggesting tight regulation of RNAi pathway on repression of *DCL1* and *AGO1*. In addition, RNAi of chromatin genes and small RNA-biogenesis genes might have different effects in resynthesized and natural allotetraploids, and the resynthesized allotetraploids may be more sensitive to epigenetic perturbations than natural allotetraploids.

Several miRNAs (miR156a, miR159a, miR159b, miR171a and miR403) and a tasiRNA (tasiR255) accumulate in low amounts in both *dcl1*(Allo):RNAi and *ago1*(Allo):RNA lines, confirming the functional consequences of *DCL1*/*AGO1* repression by RNAi. In *Arabidopsis*, small RNAs biogenesis is mediated by four distinct members of the DCL protein family (Henderson *et al.*, 2006). Although four DCL proteins have partially redundant functions (Gasciolli *et al.*, 2005), each DCL protein is associated with the production of a predominant class of small RNAs, including miRNAs by DCL1, siRNAs by DCL2 and DCL3, and tasiRNAs by DCL4 (Vazquez, 2006; Chapman & Carrington, 2007). No recovery of severe *dcl1*(Allo):RNAi lines is probably the result of specific silencing of *DCL1* instead of other *DCL* genes because *DCL1* has very low sequence identity with other *DCLs* in the RNAi target regions, and *AtDCL1* and *AaDCL1* are highly conserved in the RNAi target regions. The perfect match of predicted 21- to 24-nt RNA within the hairpin RNA with both *AtDCL1* and *AaDCL1* suggests that the homoeologous *DCL1* loci are downregulated in the allotetraploids.

It is notable that the level of miRNA reduction does not necessarily correspond to the level of *DCL1* repression. Several factors may affect the abundance of mature miRNA in allotetraploids. In addition to miRNA biogenesis factors such as DCL1 and AGO1, miRNA loci may be regulated at the transcriptional level (Ha *et al.*, 2009). Homoeologous miRNA precursors in the allotetraploids may also be processed differently, which alters the abundance of mature miRNA.

Specific small RNAs are associated with different AGOs (effector proteins) within the RISC for direct mRNA cleavage, translation repression or transcriptional gene silencing (Chapman & Carrington, 2007; Hutvagner & Simard, 2008). *Arabidopsis* contains 10 AGO proteins that are classified into three lineages with AGO1, AGO5 and AGO10 within the same clade (Zheng *et al.*, 2007; Hock & Meister, 2008). Both *AtAGO5* and *AtAGO10* share 65% nucleotide identity with the *AtAGO1* hairpin construct. This may cause some complications with RNAi of *ago1* in allotetraploids. *AGO5* and *AGO10* may not be severely repressed by RNAi of *ago1*. Mild developmental defects observed in *ago1*(Allo):RNAi lines are probably associated with *AGO1*, *AGO5* and *AGO10* redundancy (Lynn *et al.*, 1999). Many genes, including *DCL1* and *AGO1*, are nonadditively expressed in the allotetraploids (Ha *et al.*, 2009), which may alter the effectiveness of RNAi on target (*ago1* or *dcl1*) genes. The high levels of survival RNAi lines in the T2 generation may be related to partial expression of *A. arenosa DCL1* and/or *AGO1*, leading to different levels of phenotypic abnormalities in these RNAi lines. In addition, downregulating *AGO1* expression might have disrupted the homoeostasis of this protein and feedback regulation by miR168 (Vaucheret *et al.*, 2006). The effects of *AGO1* downregulation on target gene expression in the RNAi lines should be characterized in future studies.

The accumulation levels of miRNAs are more severely affected in *dcl1*(Allo):RNAi lines than in *ago1*(Allo):RNAi lines, which is consistent with targeted repression of *DCL1* and redundant *AGO1*, *AGO5* and *AGO10* that have moderate levels of sequence identities. Moreover, DCL1 plays a major role in miRNA biogenesis, whereas AGO1 is an effector for both miRNA and siRNA pathways (Chapman & Carrington, 2007b). AGO1 functions in mediating miRNA-directed target mRNA cleavage, and incorporation of miRNA into AGO1 may prevent rapid turnover of miRNA. The decrease in miRNA accumulation in *ago1*(Allo):RNAi lines probably results from indirect effects of *AGO1* repression or feedback regulation by miR168 (Vaucheret *et al.*, 2006). The similar curled-leaf phenotypes between diploid *dcl4-2* mutant and *ago1*(Allo):RNAi lines may suggest interactive roles of DCLs and AGOs in miRNA biogenesis and developmental regulation. For example, miRNA-directed cleavage of *TAS* transcripts (involving DCL1 and AGO1) may lead to DCL4-dependent tasiRNA production (Peragine *et al.*, 2004; Vazquez *et al.*, 2004; Xie *et al.*, 2005). Flowering time variation in the *dcl1* and *ago1*(Allo):RNAi lines can be caused by multiple factors, including environmental conditions and downregulation of miRNAs. For example, the late-flowering phenotype might be related to downregulation of miR172 in the RNAi lines because upregulation of miR172 induces early flowering in diploids (Aukerman & Sakai, 2003). Further investigation is required to fully characterize the effects of *DCL1* and *AGO1* downregulation on flowering time and other phenotypic changes.

In summary, we have produced a set of dominant negative RNAi lines of miRNA biogenesis genes (*DCL1* and *AGO1*) in resynthesized allotetraploids. Although these lines need further characterization and investigation, the *dcl1*(Allo):RNAi and *ago1*(Allo):RNAi lines provide useful materials for studying different and specific roles of DCL1 and AGO1 in growth and developmental variation in the allopolyploids and their progenitors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Changqing Zhang and other members of the Chen laboratory for helpful discussion, and Mahek Mehta and Ngan Nguyen for plant care and technical assistance. The work was supported by the National Institutes of Health (GM067015) (Z.J.C.).

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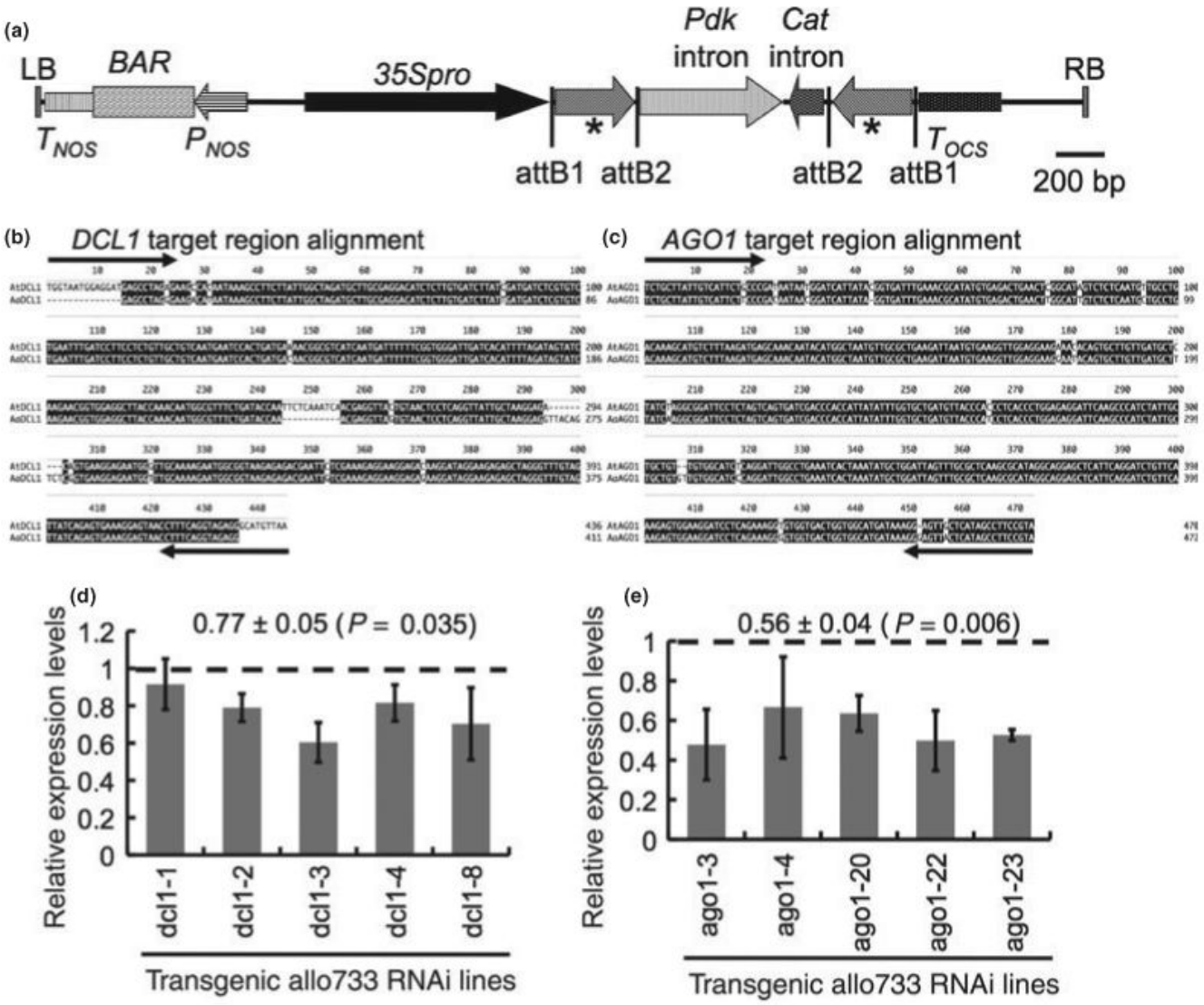


**Fig. 1.**

Hairpin RNA constructs for *DCL1* and *AGO1* and downregulation of *DCL1* and *AGO1* in transgenic plants. (a) Schematic diagram of T-DNA region of the hairpin RNA construct in pAGRIKOLA (Hilson *et al.*, 2004). The hairpin RNA cassette was produced using partial cDNA fragments of *DCL1* (436 bp) or *AGO1* (470 bp) that was cloned into the T-DNA region flanking the *Pdk* and *Cat* introns using Gateway cloning vector. *35Spro*, cauliflower mosaic virus 35S promoter; attB1 and attB2, recombination attachment sites from Gateway cloning vector; *BAR*, bialaphos resistance gene; *Cat*, intron of the castor bean *Cat* gene; LB, left border; *Pdk*, 2nd intron of the *Flaveria Pdk* gene; $P_{NOS}$, nopaline synthase promoter; RB, right border; $T_{NOS}$, nopaline synthase terminator; $T_{OCS}$, octopine synthase terminator. Asterisks indicate the *DCL1* or *AGO1* partial cDNA fragments in the sense and antisense orientation. (b,c) CLUSTAL W alignment of target *AtDCL1* (b) or *AtAGO1* (c) fragment used in the RNAi construct with the corresponding bacterial artificial chromosome (BAC) sequence from *Arabidopsis arenosa*. Arrows show the primer binding sites in generating the hairpin constructs. (d,e) quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) showing downregulation of *DCL1* and *AGO1* transcripts in *dcl1*(Allo):RNAi lines (d) and *ago1*(Allo):RNAi lines (e) compared with the average expression level of the corresponding

transcripts in two control transgenic lines containing a plasmid vector only (dashed line). Relative expression levels were estimated using *Ubiquitin10* gene as an internal control. Values at top indicate the mean ± SE and *P*-value of target transcript reduction in the RNAi lines compared with the control transgenic lines. Error bars indicate standard deviation from replicated experiments.

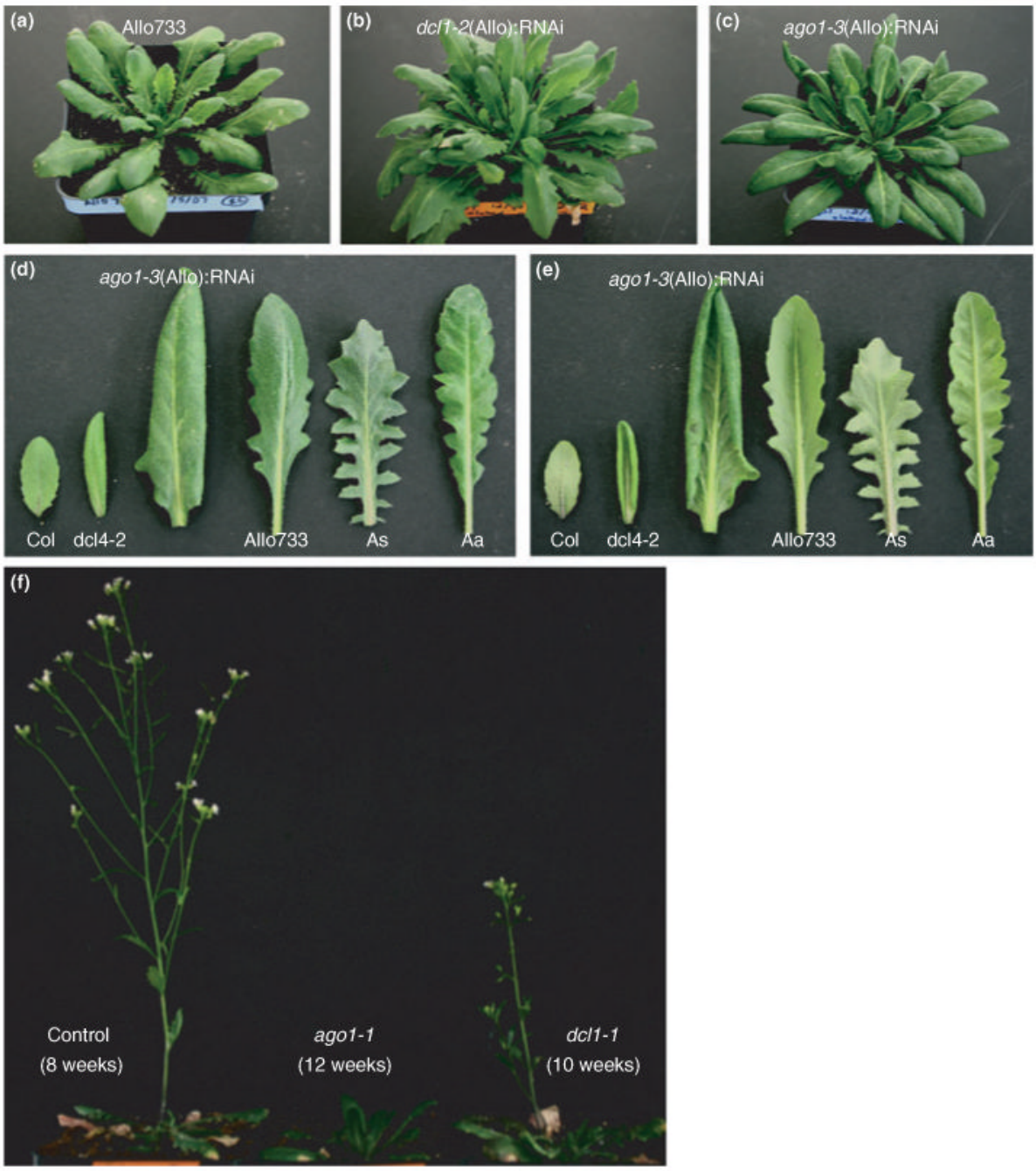


**Fig. 2.**

RNAi-induced phenotypes in *dcl1*(Allo):RNAi and *ago1*(Allo):RNAi transgenic allotetraploids (Allo733, 8 wk old). (a) Wild-type control: resynthesized allotetraploids (Allo733) derived from *Arabidopsis thaliana* and *Arabidopsis arenosa* tetraploids. (b) *dcl1-2*(Allo):RNAi transgenic plants showing increased number of rosette leaves. (c) *ago1-3*(Allo):RNAi transgenic plant displaying an increased number of rosette leaves and an altered pattern of leaf serration. (d) Adaxial and (e) abaxial leaf morphology comparison between *ago1*(Allo):RNAi transgenic plants and other lines including *A. thaliana* diploid Col-0 and *dcl4-2* mutant, tetraploid *A. arenosa* (Aa), natural allotetraploid *Arabidopsis*

*suecica* (As), and resynthesized allotetraploid (Allo733). (f) Late-flowering phenotypes of *dcl1-1*(Allo) and *ago1-1*(Allo) RNAi lines compared with the control plant.

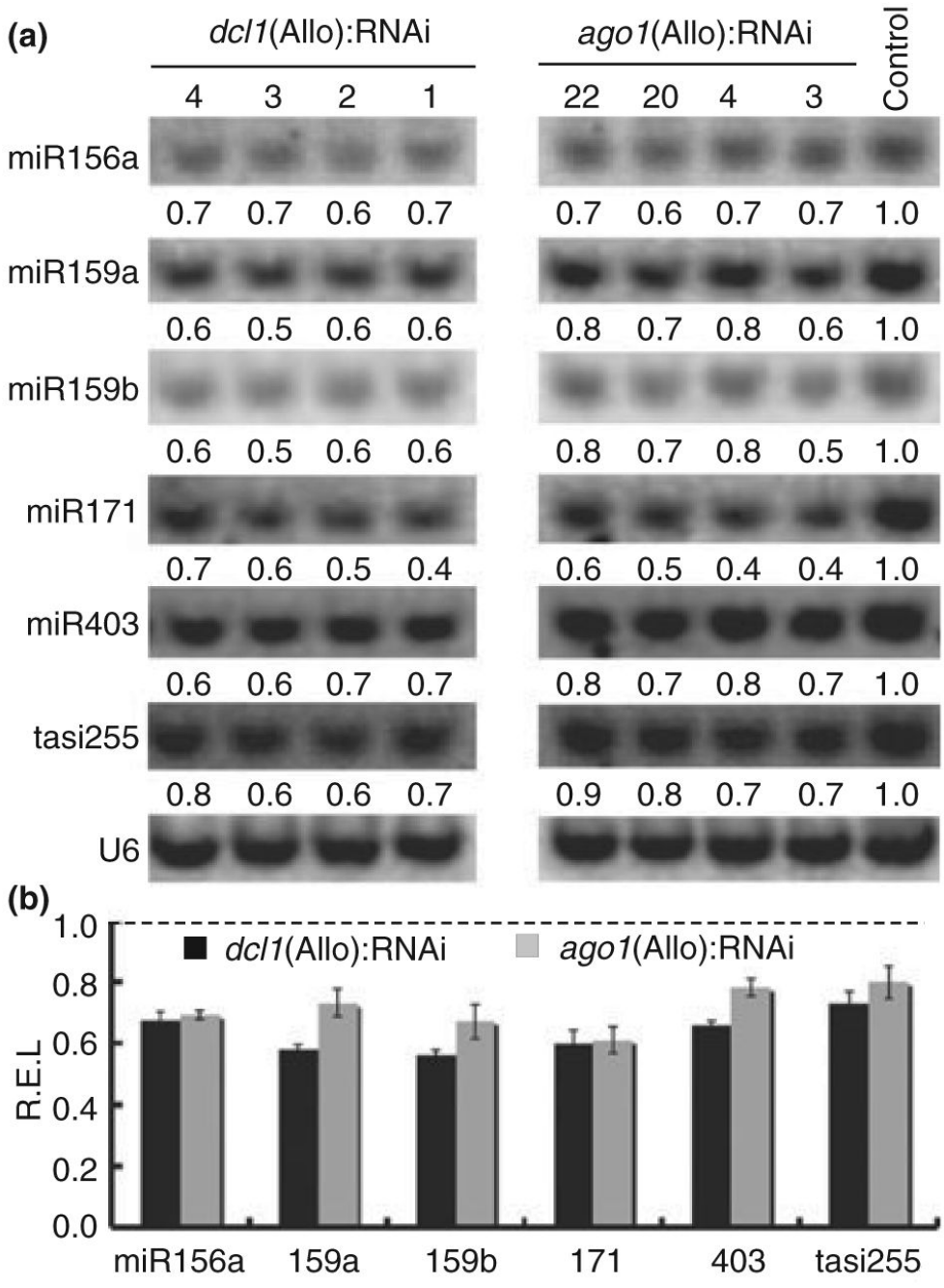


**Fig. 3.**

Decreased levels of miRNA accumulation in *dcl1*(Allo) and *ago1*(Allo) RNAi lines. (a) Representative small RNA blots of selected miRNAs (miR156a, miR159a, miR159b, miR171a, and miR403) and tasiRNA (tasiR255) in *dcl1*(Allo):RNAi and *ago1*(Allo):RNAi lines. Averaged numbers (from two independent blots) indicate relative densitometric intensities of miRNA levels between the RNAi lines and a control transgenic plant. The hybridization signals were normalized using the U6 as an endogenous control. Densitometric intensities were obtained using IMAGEQUANT software (Bio-Rad). (b) Averaged relative expression levels (REL) (mean ± SE) of miRNAs and tasiRNA shown in (a). The REL of each miRNA or tasiRNA was averaged from densitometric intensities in four

*dcl1*(Allo):RNAi lines or *ago1*(Allo):RNAi lines, which was normalized to the transgenic line containing empty vector construct (dashed line).