Recovery of *dicer-like 1*-late flowering phenotype by miR172 expressed by the noncanonical DCL4-dependent biogenesis pathway

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ABSTRACT

MicroRNAs (miRNAs) act as down-regulators of gene expression, and play a dominant role in eukaryote development. In *Arabidopsis thaliana*, DICER-LIKE 1 (DCL1) is the main processor in miRNA biogenesis, and *dcl1* mutants show various developmental defects at the early stage of embryogenesis or at gamete formation. However, miRNAs responsible for the respective developmental stages of the *dcl1* defects have not been identified. Here, we developed a DCL1-independent miRNA expression system using the unique DCL4-dependent miRNA, miR839. By replacing the mature sequence in the miR839 precursor sequence with that of miR172, one of the most widely conserved miRNAs in angiosperms, we succeeded in expressing miR172 from a chimeric miR839 precursor in *dcl1-7* plants and observed the repression of miR172 target gene expression. In parallel, the DCL4-dependent miR172 expression rescued the late flowering phenotype of *dcl1-7* by acceleration of flowering. We established the DCL1-independent miRNA expression system, and revealed that the reduction of miR172 expression is responsible for the *dcl1-7* late flowering phenotype.

Keywords: RNA; microRNA; *Arabidopsis*; development; DCL1; DCL4; miR839

INTRODUCTION

MicroRNAs (miRNAs) are endogenous noncoding RNAs 18–24 nt long, and are involved in RNA silencing by repression of the expression of target gene mRNAs having complementary sequences (Bartel 2004). In plants, a miRNA and ARGONAUTE 1 (AGO1), an effector protein of RNA silencing, constitute the major miRNA-induced silencing complex (miRISC) as the functional machinery (Iki et al. 2010). miRISCs bind to target mRNAs by miRNA guidance and suppress the target gene expression by cleavage or translational repression in plants (Llave et al. 2002; Brodersen et al. 2008; Li et al. 2013). The biogenesis of plant miRNAs occurs via two-step processing in nuclei: The first process converts the primary miRNA transcript (pri-miRNA) to the precursor miRNA (pre-miRNA), and the second process converts the pre-miRNA to mature ~21-nt miRNA/miRNA* duplex. DICER-LIKE 1 (DCL1), a homolog of animal Dicer, is an RNase III-like protein and is the core enzyme of the two-step processing in collaboration with HYPONASTIC LEAVES 1 (HYL1) and SERRATE (SE) (Kurihara and Watanabe 2004; Kurihara et al. 2006; Yang et al. 2006).

In *Arabidopsis thaliana*, many miRNAs have important regulatory roles in various developmental events, including the vegetative-to-reproductive phase transition and the development of flowers and roots (Nag and Jack 2010; Huijser and Schmid 2011; Petricka et al. 2012). Although it is common that one animal miRNA targets more than 100 genes, plant miRNAs tend to target one or several particular mRNAs. Notably, many such target mRNAs encode transcription factors (Axtell 2008). These transcription factors, whose expression levels are balanced with cognate miRNAs, control cell differentiation and the cell division pattern during plant development.

Arabidopsis dcl1 mutants show a wide range of developmental defects (Schauer et al. 2002). Mutants of weak *dcl1* alleles, like *dcl1-7* (*sin1-1: short integument1-1*) and *dcl1-9* (*caf-1: carpel factory-1*), show pleiotropic phenotypes including female sterility, late flowering, and small leaves. Null mutants, like *dcl1-5* (*sus1-5: suspensor1-5*), are embryonic lethal. These developmental defects suggest that most if not all miRNAs are indispensable determinants for plant

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development. However, we do not know which species of miRNAs are required for plant development.

The expression levels of almost all miRNAs decrease in *dcl1* mutants; contributions of individual miRNAs remain enigmatic. Double mutants between the *dcl1* mutant and a mutant of a target gene of the particular miRNA might provide a rare clue. Nodine and Bartel (2010) reported that *dcl1-5*, one of the *dcl1* null mutants, could be rescued by crossing with miR156-target *SPL10-RNAi/spl11-1* mutant only with regard to the early embryonic patterning. However, this approach is restricted because the crossed mutant had mutations only in a part of the miRNA target genes, and has difficulty in crossing the mutants of other miRNAs also. Thus, to screen out the essential miRNA(s) for normal plant development directly, we need to recover *dcl1* mutants by decreased expression of miRNAs via an alternative pathway that is independent of DCL1.

In *Arabidopsis*, a few miRNAs are expressed independently of DCL1, unlike other general miRNAs. The expression of miR822, miR839, and miR859 are dependent on DCL4, one of the DCL1 homologs in plants (Rajagopalan et al. 2006; Ben Amor et al. 2009). *Arabidopsis* has four DCL proteins and they have distinct characters, respectively. Whereas DCL1 cognates a hairpin RNA structure of miRNA precursors, DCL2 and DCL4 produce endogenous and virus-derived small interfering RNAs (siRNAs) from long double-stranded RNAs (dsRNAs), which down-regulate the target RNA accumulation level. DCL3 also cognates dsRNAs and produces siRNAs working in RNA-directed DNA methylation. The exceptional substrate preference of DCL4 for the noncanonical DCL4-dependent miRNAs is considered to be due to their relatively long and high-complementary precursors (Chapman and Carrington 2007).

In this study, we established an artificial precursor construct based on a DCL4-dependent miRNA, miR839, to express a miRNA in *dcl1* mutants. To take advantage of the system, we tested miRNAs responsible for the *dcl1* mutant phenotypes in terms of phenotypic recovery. By replacing the mature sequence in the precursor of miR839 with the miR172 mature sequence, miR172 was expressed in a DCL4-dependent manner. miR172 processed with the aid of DCL4 could suppress the expression of the known miR172 target genes and rescue some, but not all, of the *dcl1-7* late flowering phenotype. Thus, our study provided technical progress for future research, and the role of one miRNA in *Arabidopsis* development was clarified through miRNA complementation in the *dcl1-7* mutant.

RESULTS

dcl1-7 and *dcl1-9* mutants show distinct patterns in their miRNA expression profiles

Some miRNA families are conserved widely among angiosperm species. It is accepted that DCL1 is involved in the pro-

cessing of almost all miRNA precursors. Accordingly, many *dcl1* mutants of different alleles show developmental phenotypes in flowering time and reproduction. It is suspected that such defects are caused by decreases in the levels of miRNAs with regulatory roles in plant development, and that the imbalance of target mRNAs accumulation causes uncoordinated cell differentiation and morphogenesis.

We suspected that weak allele mutants of *dcl1* have some biased inability to express some miRNAs, resulting in allele-specific phenotypic changes. Thus, we checked the expression levels of miRNAs of 12 conserved families (miR156, miR159/319, miR160, miR162, miR164, miR165/166, miR167, miR168, miR169, miR170/171, miR172, and miR390) in the inflorescences of mutants *dcl1-7* and *dcl1-9* (Fig. 1A) and in wild-type (WT) Col-0 plants. As a result of ANOVA, it was indicated that the levels of most miRNAs have a difference between WT, *dcl1-7*, and *dcl1-9*, except for miR168 ($P < 0.05$).

FIGURE 1. Expression levels of miRNAs conserved widely among angiosperms were decreased in *dcl1* mutants. (A) Northern blotting was used to detect the expressions of 12 miRNA families in *dcl1-7* and *dcl1-9* inflorescences. The gel patterns are representatives of three experimental replicates, and the numbers below the gel pattern indicate relative fold levels averaged over three replicates and standard errors. U6 snRNA was used as the internal control. (B) The table summarizes the results of statistic analysis of A by Tukey's test ($n = 3$). (*) $P < 0.05$; (-) no significant difference.

Then, analysis using Tukey's test indicated that the levels of most miRNAs were reduced significantly in both *dcl1-7* and *dcl1-9*, except for miR165/166 and miR319 in *dcl1-9* ($n = 3$, $P < 0.05$) (Fig. 1B). In addition, the two mutants showed distinct patterns of miRNA expression levels. The levels of some miRNAs (miR156, miR159, miR162, and miR172) were reduced more significantly in *dcl1-9* than in *dcl1-7*. In contrast, the levels of miR165/166 and miR319 were significantly higher in *dcl1-9* than in *dcl1-7* ($n = 3$, $P < 0.05$) (Fig. 1B).

The *MIR839A* gene is possibly transcribed like other miRNA genes, and the transcript is processed independently of DCL1

Unlike other miRNAs, miR839 is expressed independently of DCL1, but is dependent on DCL4 (Rajagopalan et al. 2006). To confirm this, we performed Northern blotting. miR839 expression was detected at the same level in the three different mutants (*dcl1-7*, *dcl1-9*, and *dcl1-100*), but not in *dcl4-2* (Fig. 2A), as reported in previous studies (Rajagopalan et al. 2006).

The *MIR839A* locus in the *Arabidopsis* genome is shown in Figure 2B. We performed RNA ligase-mediated rapid amplification of cDNA ends (RACE) to identify the transcription start site (+1 site), which was 735-nt upstream of the 5'-end of the mature miR839 sequence, and the termination site, which was 364-nt downstream from the 3' end of the mature sequence. It is possible that the primary transcript of the *MIR839A* gene is transcribed as other *MIRNA* genes.

Ectopically expressed miR839 has gene-silencing ability like other miRNAs

We then tested whether the miR839 precursor functioned like conventional miRNAs to suppress target mRNAs. For this purpose we chose a transient expression system using *Nicotiana benthamiana* leaves to observe whether simultaneous introduction of the miR839 gene could suppress ectopic expression of an mRNA with the miR839 target site at its 3' UTR.

First, we cloned the genomic sequence of the miR839 precursor into plasmid pAT006 between the CaMV35S promot-

FIGURE 2. Characterization of DCL4-dependent miR839 expression revealed that miR839 has silencing ability. (A) Northern blotting to detect the expression of miR839 in florescences of *dcl4* and *dcl1* mutants. (B) Genome mapping of *Arabidopsis* *MIR839A* gene. Rapid amplification of cDNA ends (RACE) was used to determine the transcription start site and termination site, which are represented as +1 and +1121, respectively. (C) Illustration of the cloning of pre-miR839 into vector pAT006 and *Agrobacterium*-mediated transient expression in *N. benthamiana*. (D) Ectopic expression of miR839 in *N. benthamiana* mediated by *Agrobacterium*. (E) Illustration of the luciferase constructs used in Dual-luciferase reporter assay in *N. benthamiana*. (F) Repression of firefly luciferase expression by miR839 ectopic expression in *N. benthamiana*. The mean fold value of F-luc/R-luc is represented ($n = 4$). Error bar, SE. (*) $P < 0.05$. (G) 5'-RACE assay to determine the 5' ends of the 3' cleavage product of firefly luciferase mRNA. Arrowheads represent the 5'-end position and the number of RACE-clones.

er and terminator to obtain pAT006-pre-miR839a (Fig. 2C). pAT006-pre-miR839a was then transfected into *N. benthamiana* leaves. Two days later, we detected ectopic expression of miR839 in the *N. benthamiana* leaves (Fig. 2D). This result confirmed that the miR839 precursor was transcribed and processed correctly into the precise mature miR839.

We then confirmed whether or not miR839 was able to reduce the target gene expression. We checked the ability of miR839 to suppress its target mRNAs using the Dual-luciferase reporter assay in *N. benthamiana* leaves. The complete complementary nucleotide sequences of miR839 were inserted downstream from the firefly luciferase gene (Fig. 2E). The

result showed that the expression of firefly luciferase was significantly decreased in the presence of mature miR839 ($n = 4$, $P < 0.05$, Student's t -test), but not in its absence (Fig. 2F).

We also used 5' RACE to check whether or not the cleavage of firefly luciferase mRNA occurred precisely at the miR839 target site (Fig. 2G). The result showed that nine of 10 RACE-clones were cleaved at the AGO1 slicing position between nucleotides 10 and 11 of the miRNA sequence (Fig. 2G). Based on these results, we judged that the miR839 precursor could be used for ectopic expression, as reported using other miRNAs dependent on DCL1 (Parizotto et al. 2004).

Precursor construct of miR839 could express a noncognate miRNA in a *dcl1* mutant and suppress its target gene expression

The miR839 precursor was not processed by DCL1, but possibly by DCL4, so we hypothesized that the miR839 precursor structure could be used to express otherwise DCL1-dependent miRNAs.

To test this, we designed a chimeric miR839 precursor sequence whose mature sequence and its complementary sequence (miRNA*) were easily replaced by specific miRNAs and their complementary sequences by introduction of BsaI restriction enzyme site (Fig. 3A,B). In addition, the 35S promoter sequence was placed upstream of the artificial miR839 precursor sequence to drive its transcription. If this strategy worked, DCL1-dependent miRNAs could be expressed by DCL4 activity from the chimeric miR839 precursor in *dcl1* mutants.

miR172, which is one of the conserved miRNA families in angiosperms, has a role in flower development and the promotion of flowering time by repressing expression of the *AP2* gene family (Aukerman and Sakai 2003; Chen 2004; Wu et al. 2009). Moreover, the level of miR172 was strongly decreased in the two *dcl1* mutants that showed the late flowering phenotype (Fig. 1). Based on such observations and earlier results, we determined that miR172 was a candidate miRNA for the *dcl1* phenotype. We constructed a chimeric miR839 precursor construct in which the mature miR839 sequence was replaced with miR172a to obtain pWAT206-pre-miR839-172. To examine the efficiency of the pre-miR839-172 construct, we transformed *dcl1-7* heterozygous plants with this vector using *Agrobacterium*. After selection of T1 plants heterozygous for

FIGURE 3. miR172 was expressed from the pre-miR839 backbone and repressed the target gene expression. (A) Schematic procedure of the chimeric vector construction using the type IIS restriction enzyme BsaI. (B) Schematic diagram of the chimeric pre-miR839 constructs. (C) Northern blotting to detect DCL4-dependent expression of miR172 in *dcl1-7*. miR167 was detected to confirm the specificity of miR172 expression. Numbers below the band pattern indicate the relative fold level averaged over three replicates. U6 snRNA was used as the internal control; #1 and #2 indicate two independent lines of transgenic plants. (D) Relative accumulation of mRNA of the miR172-target *TOE2* by qRT-PCR; #1 and #2 indicate the same lines as in C. Error bar, SE. (**) $P < 0.01$.

both the *dcl1-7* mutation and the 35S-pre-miR839-172 transgene, T2 seeds were harvested. Such seeds were planted and their flowering times observed. We selected two transgenic plant lines, designated #1 and #2. In general, the late flowering phenotype of *dcl1-7* was rescued in the 35S: pre-miR839-172/*dcl1-7* plants (Fig. 4A), while the sterile phenotype was not complemented (data not shown). Then we performed a statistical analysis comparing the flowering time of transgenic plants #1 and #2 with that of *dcl1-7*. As a result, both transgenic lines #1 and #2 flowered significantly earlier than *dcl1-7* plants ($P < 0.001$, Mann-Whitney test with Bonferroni correction; F-test showed the inequality between variances) (Fig. 4B, C). This result, therefore, suggests that the transgene rescued the *dcl1-7* late flowering phenotype.

We then checked the expression of miR172 in the 35S-pre-miR839-172 transformants #1 and #2. Untransformed *dcl1-7*

FIGURE 4. DCL1-independent expression of miR172 recovered the late flowering phenotype of *dcl1-7*. (A) Image of a *dcl1-7* homozygous plant at 47 d showing rescued phenotype of late flowering by 35S-pre-miR839-172 transgene. White characters indicate each genotype. Wild-type (WT) and *dcl1-7* plants without transgenes were used as controls. (B) Overlap dot plots with box plots showing the distribution of flowering time in each genotype. The top and bottom of the box show the first and third quartile each, and the band inside the box shows the second quartile. The ends of whiskers show the highest datum within the 1.5 interquartile range (IQR) of the upper quartile and the lowest datum within the 1.5 IQR of the lower quartile; #1 and #2 indicate the same lines as in Figure 3C. (C) The table summarizes the flowering time shown in B. Numbers show the averages and standard errors.

accumulated a low level of miR172 and miR167. In contrast, 35S: pre-miR839-172/*dcl1-7* #1 and #2 plants expressed a significantly higher level of miR172 than *dcl1-7*, but not miR167 ($n = 3$, $P < 0.01$, Tukey's test) (Fig. 3C). This result showed that the 35S: pre-miR839-172 transgene could express miR172, even in the absence of fully functional DCL1. The earlier flowering phenotype correlated with the expression of mature miR172.

We then checked the silencing ability of miR172 expressed in a DCL4-dependent manner. Both *TOE1* and *TOE2* are target genes of miR172, and have an effect on repression of flowering time (Aukerman and Sakai 2003). Using qRT-PCR, we quantified the level of *TOE1* and *TOE2* mRNAs in 35S: pre-miR839-172/*dcl1-7* #1 and #2 plants and compared them with those of WT and *dcl1-7* plants. *TOE2* mRNA in *dcl1-7* significantly increased by ~ 3.6 -fold compared with WT plants, whereas in 35S: pre-miR839-172/*dcl1-7* #1 and #2 plants, the *TOE2* mRNA level was the same as that in the WT ($n = 3$, $P < 0.05$, Tukey's test) (Fig. 3D). In contrast, the level of *TOE1* mRNA was not statistically different between WT, *dcl1-7*, and 35S-pre-miR839-172/*dcl1-7* plants ($n = 3$, $P > 0.05$, ANOVA) (Supplemental Fig. S1).

Taken together, the results suggested that miR172 expressed from the pre-miR839-172 construct could suppress the target gene in the absence of full DCL1 function and resulted in an earlier flowering time. In addition, our data indicated that the decrease of miR172 expression level is the main cause of the late flowering phenotype of *dcl1-7*; however, it is not the cause of other *dcl1* pleiotropic defects.

DISCUSSION

The study of specific miRNAs using alternative miRNA biogenesis pathways has been awaited in the analysis of miRNA biogenesis mutants (Yang and Lai 2011). In such cases, where the levels of most miRNAs decreased, it is difficult to analyze the contribution of respective miRNAs in detail. miRNA expression by a noncanonical pathway could overcome this problem and make it possible to test the roles of each miRNA. Until now, the ectopic expression of miRNAs by a noncanonical pathway has not been successful in plant cells, in contrast to animals (Maurin et al. 2012; Yang et al. 2012). In this study, we expressed miR172 in *dcl1-7* via the noncanonical DCL4-dependent biogenesis pathway using

miR839 precursor backbone. This is the first report of the use of a noncanonical miRNA expression system in plants. Additionally, we also established transformants expressing one miRNA specifically via the noncanonical pathway and succeeded in detecting its direct role in vivo.

Knockout (KO) mutants of the main factors in the miRNA biogenesis pathway, such as *dicerKO* and *droshaKO*, often show severe developmental defect phenotypes in plants and animals (Schauer et al. 2002; Bernstein et al. 2003). In *Arabidopsis*, *dcl1* null mutants are embryonic lethal, whereas weak *dcl1* mutant alleles have a wide range of developmental defect levels. Although both *dcl1-7* and *dcl1-9*, two such weak alleles, are sterile because female reproductive organs are developmentally defective, there is a slight difference between them; *dcl1-7* shows abnormal ovule development, while *dcl1-9* shows excessive cell proliferation in the third and fourth whorls of the floral organs (Schauer et al. 2002). Between these two alleles, we observed differences in the expression levels of widely conserved miRNA families (Fig. 1). These distinct expression patterns explained some, if not all, of the phenotypic differences between them.

As the first step for comprehensive understanding, we applied the system to express one miRNA in a *dcl1* mutant

allele: miR172 in *dcl1-7*. miR172 is a well-known plant miRNA involved in flowering promotion and flower development (Aukerman and Sakai 2003; Chen 2004). The expression level of miR172 was low in *dcl1-7* (Fig. 1). In this study, miR172 was expressed in a DCL1-independent manner and could rescue the *dcl1-7* late flowering defect (Fig. 4A–C). Our data are consistent with miR172's role in the promotion of flowering. Thus, the DCL4-dependent miRNA biogenesis pathway could be used to complement canonical DCL1-dependent miRNAs. In addition, miR172 is functionally related to miR156, which has a reciprocal role in inhibiting maturation. *SPL* genes targeted by miR156 promote the expression of miR172 and this sequential relation induces maturation and flowering (Wu et al. 2009). While both miR156 and miR172 decreased in *dcl1-7* plants (Fig. 1), *dcl1-7* shows the late flowering phenotype and the sole expression of miR172 rescued the late flowering phenotype (Fig. 4A–C). This observation suggested that expression of miR172 is epistatic to the effect of miR156 in the determination of flowering time. Comparative genome analysis shows that miR156 is conserved among almost all land plants, whereas miR172 exists only in angiosperms, and not in mosses and lycopods (Axtell and Bartel 2005). Therefore, the regulatory system involving miR172 must have evolved during the environmental adaptation of angiosperms on land. In light of the physiological and morphological differences among them, it is thought that miR172 emerged in an ancestor of angiosperms, and then obtained a dominant role over miR156 in developmental timing for promotion of reproduction with seeds.

Finally, we obtained partial recovery of the *dcl1-7* phenotype by expressing miR172; however, we could not attain complete complementation of *dcl1-7*'s sterile phenotype. To realize complete rescue of *dcl1* plant fertility, more miRNAs are required in addition to miR172. Taking advantage of our DCL1-independent miRNA expression system, stepwise rescue of these multiple *dcl1* mutant defects by specific miRNA will make it possible to dissect each miRNA's role in *Arabidopsis* developmental transition in detail. The identification of miRNAs indispensable for plant reproduction is important in both basic and applied plant research. Further studies are required to fulfill the promise of our developed DCL1-independent miRNA expression system.

MATERIALS AND METHODS

Plant materials and growth conditions

The *dcl1-7* allele was kindly provided by James C. Carrington and is previously described (Xie et al. 2005). The *dcl1-9* allele was backcrossed six times with Col-0 plants. *dcl1-100* (GABI_098F10), *dcl4-2* (GABI_160G05), and *mir839a* (SAIL_21_B06) seeds were obtained from the ABRC and NASC stock centers. WT plants were Columbia-0 (Col-0) ecotype.

Arabidopsis thaliana plants were grown on soil under 22°C long-day conditions (16 h light/8 h dark) to analyze progression of devel-

opment or continuous light for other analysis. All *N. benthamiana* plants were grown on soil at 24°C under continuous light.

Plasmids construction

Vectors pAT and pWAT were used for plant transformation in this study. Vector pAT has a pMDC background and has the same multiple cloning site as pGREEN II 0000. Vector pWAT is a modified version of pGREEN II 0000 and is previously described (Kumakura et al. 2013). Both pAT006 and pWAT206 have a CaMV 35S promoter and terminator in the multiple cloning site, while pAT010 has a NOS promoter and terminator. pWAT206 has a BASTA resistant gene.

The precursor sequence of miR839 was amplified from Col-0 genomic DNA and cloned into pAT006 via two restriction enzymes, SalI and SpeI, to obtain pAT006-pre-miR839.

The chimeric miR839 precursor was synthesized with the type IIS restriction enzyme BsaI (Fig. 3A). First, the PCR fragment containing the base of miR839 precursor sequence and two inverted BsaI sites was amplified by overlapping PCR and inserted into SalI–SpeI sites in pWAT206 to obtain pWAT206-pre-miR839Bsa. Second, the PCR fragment of chimeric pre-miR839-172 with the BsaI sites in both ends was amplified from pAT006-pre-miR839 and ligated into pWAT206-pre-miR839Bsa after BsaI cutting to obtain pWAT206-pre-miR839-172.

It was reported that miR156 acts on the target sequence of *SPL3* mRNA at its 3' UTR (Gandikota et al. 2007); it is a rare case in plant miRNA, because most miRNAs have target sequences in the coding regions of their target mRNAs. In consideration of the future analysis of this line, we tried to mimic the natural context for miRNA silencing to suppress the target sequence in the 3' UTR of the mRNA. For this purpose, the 3' UTR and following terminator sequence of *SPL3* was cloned downstream from the stop codon of the firefly luciferase gene. The native miR156-target sequence was replaced with the miR839-complementary sequence, without any additional sequence insertion, using BsaI.

All primers used for construction are listed in Supplemental Table S1.

Agrobacterium-mediated transient expression assay

Agrobacterium tumefaciens (GV3101) was infiltrated into *N. benthamiana* leaves as described previously (Takeda et al. 2008). The concentration of *Agrobacterium* was normalized to 0.8 OD₆₀₀. Leaves were sampled 48 h after infiltration.

To estimate the silencing efficiency of miRNAs in plants, vectors containing firefly luciferase or *Renilla* luciferase gene as the internal control were coinfiltrated with the precursor of miRNA into *N. benthamiana* leaves by an *Agrobacterium*-mediated transient expression assay. The ratio of the suspension of *Agrobacterium* transformed by each vector (pAT006-pre-miR839 [or empty vector] : pAT006-F-luc-839target : pAT010-R-luc) was 8:1:1.

In planta transformation of *Arabidopsis* by *Agrobacterium*

Agrobacterium in planta transformation was used to establish *Arabidopsis* transformants. *Arabidopsis* inflorescences were dipped in *Agrobacterium* transformed by a transgene-containing vector

suspended in 5% sucrose and 0.04% Silwet L77 solution. *dcl1-7* heterozygous plants were selected by genotyping for transformation.

RNA extraction and small RNA Northern blot analysis

The RNAiso Plus reagent (TaKaRa) was used to isolate total RNA from *Arabidopsis* inflorescence or *N. benthamiana* infiltrated leaves. A total of 10 µg of total RNA was separated on a denaturing 17.5% polyacrylamide gel (7 M urea) in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA at pH 8.0). Separated RNA was electroblotted onto Hybond-N⁺ membranes (GE Healthcare). Radiolabeled DNA oligonucleotide probes were constructed by end-labeling with [γ -³²P]ATP by using T4 polynucleotide kinase. Hybridization was performed at 42°C with PerfectHyb Plus (SIGMA Aldrich). U6 snRNA was used as the internal control. Primers used for the radio-labeled probe are listed in Supplemental Table S1.

Dual-luciferase assay

The activity of each luciferase was detected using the Dual-Luciferase reporter assay system and Glomax 20/20 Luminometer (Promega).

Forty-eight hours after infiltration, leaf discs were excised and placed in 1.5-mL tubes as samples. The leaf discs were ground in liquid nitrogen, 300 µL of 1 × Passive Lysis Buffer was added and the samples were mixed thoroughly. Following centrifugation at 22,600g at 4°C for 3 min, 4 µL of supernatant was mixed into 40 µL of Luciferase Assay Reagent II and the firefly luciferase activity was measured. Forty microliters of Stop & Glo Reagent was added and *Renilla* luciferase activity was measured. Passive Lysis Buffer, Luciferase Assay Reagent II, and Stop & Glo Reagent are all parts of the Dual-Luciferase Reporter Assay System's kit. Four biological replicates were performed for each sample.

RNA ligase-mediated RACE

5' and 3' RACE were performed using the GeneRacer Kit (Invitrogen; Life Technologies), according to the manufacturer's protocol with GeneRacer oligo dT primer. To determine the full-length of the *MIR839A* transcript, total RNA from Col-0 inflorescences was used. To detect the cleavage sites in the miRNA target sequence of the firefly luciferase mRNA, treatment of total RNA from infiltrated *N. benthamiana* leaves was started from the RNA ligase reaction, except for the CIP and TAP reactions to select only cleaved RNA fragments. Primers used for PCR amplification are listed in Supplemental Table S1.

Quantitative RT-PCR (qRT-PCR)

qRT-PCR was used to quantify miRNA-target gene mRNAs. Total RNA was extracted from *Arabidopsis* inflorescences and the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa) was used to synthesize the cDNA according to the manufacturer's protocol. qPCR was performed with the StepOnePlus Real Time PCR System (Applied Biosystems; Life Technologies) using a KAPA SYBR Fast qPCR kit (KAPA BIOSYSTEMS) and reaction conditions as previously described (Motomura et al. 2012). *ACTIN2* was used as the internal control gene. Expression levels were averaged by two

technical replicates and three biological replicates. Error bars in the relevant figure represented the standard error of the mean. Primer sets for each PCR amplification are listed in Supplemental Table S1. Primers to detect *TOE1* and *TOE2* mRNA were previously described (Wu et al. 2009).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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