A MicroRNA as a Translational Repressor of *APETALA2* in *Arabidopsis* Flower Development

Xuemei Chen

Waksman Institute, Rutgers University, Piscataway, NJ 08854, USA

Xuemei Chen: xuemei@waksman.rutgers.edu

Abstract

Plant microRNAs (miRNAs) show a high degree of sequence complementarity to, and are believed to guide the cleavage of, their target messenger RNAs. Here, I show that miRNA172, which can base-pair with the messenger RNA of a floral homeotic gene, *APETALA2*, regulates *APETALA2* expression primarily through translational inhibition. Elevated miRNA172 accumulation results in floral organ identity defects similar to those in loss-of-function *apetala2* mutants. Elevated levels of mutant *APETALA2* RNA with disrupted miRNA172 base pairing, but not wild-type *APETALA2* RNA, result in elevated levels of APETALA2 protein and severe floral patterning defects. Therefore, miRNA172 likely acts in cell-fate specification as a translational repressor of *APETALA2* in *Arabidopsis* flower development.

MicroRNAs (miRNAs), ~22-nucleotide non-coding RNAs that regulate protein-coding RNAs, are processed from longer hairpin transcripts by the enzyme Dicer [reviewed in (1, 2)]. In *Arabidopsis*, the accumulation of miRNAs requires a Dicer homolog, DCL1, and a novel protein, HEN1 [reviewed in (3)]. The two founding members of *Caenorhabditis elegans* miRNAs, *lin-4* and *let-7*, inhibit the translation of their target mRNAs through imperfect base-pairing interactions with their targets (4, 5). *Arabidopsis* miRNAs show a higher degree of sequence complementarity to their potential targets, and, like short interfering RNAs, several plant miRNAs direct the cleavage of their target RNAs (6–9). Here, I report the role of an *Arabidopsis* miRNA, miRNA172, in the control of floral organ identity and floral stem cell proliferation as a potential translational repressor of a floral homeotic gene.

Four organ types are specified in the floral meristem by the combinatorial actions of three classes of transcription factors, the floral A, B, and C genes [reviewed in (10)]. *APETALA2* (*AP2*, a class A gene) and *AGAMOUS* (*AG*, a class C gene) specify the identities of the perianth and reproductive organs, respectively, and act antagonistically to restrict each other's activities to their proper domains of action within the floral meristem. We have identified *HEN1* as a gene required for class C activity in the flower, because recessive *hen1*

mutations result in reproductive-to-perianth organ transformation in the *hua1-1 hua2-1* background, which is partially compromised in class C activity (11, 12). The requirement of *HEN1* for the accumulation of miRNAs (13) suggests that the absence of a miRNA(s) is responsible for the floral homeotic transformation in *hua1-1 hua2-1 hen1* flowers.

MiRNA172, a miRNA highly complementary to a region in the mRNAs of *AP2* (14) (Fig. 1A), At2g28550, At5g60120, and At5g67180, which encode proteins with AP2 domains, is a candidate miRNA in flower development. The putative miRNA172 binding sites are located within the coding regions of the genes but are outside of the conserved AP2 domains. To test whether *AP2* is regulated by miRNA172, I first studied *AP2* expression in genotypes in which miRNA172 accumulates to low levels, such as *hen1* and *dcl1* mutants (Fig. 1B). *AP2* RNA abundance in these genotypes was similar to that in wild type (Fig. 1C) (9). AP2 protein amounts in *hen1* and *dcl1* inflorescences, however, were about 2 to 3 times that in wild type (Fig. 1D), suggesting that *AP2* is regulated at the translational or posttranslational levels by a miRNA(s) absent in *hen1* and *dcl1* plants. Elevated AP2 accumulation is likely the cause of the homeotic transformation in *hua1-1 hua2-1 hen1-1* flowers, because a severe loss-of-function *ap2* allele, *ap2-2* (15), rescues the *hua1-1 hua2-1 hen1-1* floral homeotic phenotypes (12).

To determine whether *AP2* is regulated by miRNA172, I tested the effect of elevated miRNA172 level on *AP2* expression in planta. Five genes in the *Arabidopsis* genome (table S1) can give rise to three miRNA172 species with one nucleotide difference. All three miRNA172 species can base-pair with *AP2* RNA with no or one mismatch (Fig. 1A). Each of four *MIR172* genes, *MIR172a-1*, *a-2*, *b-2*, and *c* (13), was fused to two copies of the cauliflower mosaic virus 35S enhancer, which drives high-level and near ubiquitous expression, and was introduced into wild-type plants. Most T1 transgenic lines containing each *MIR172* gene but not *MIR173* (an unrelated miRNA gene) or the vector alone displayed accelerated floral transition (Fig. 2A). In addition, plants harboring *35S::MIR172a-1*, *a-2*, or *b-2*, but not *35S::MIR172c*, the vector DNA, or *35S::MIR173*, showed floral homeotic phenotypes similar to those of *ap2* loss-of-function mutants, such as *ap2-2* or *ap2-9* (15) (Figs. 2B and 3, B and C). Similar phenotypes were also observed in an activation-tagging line that over-expressed miRNA172a-2 (16). Some cauline leaves of *35S::MIR172a-1*, *35S::MIR172a-2*, and *35S::MIR172b-2* lines had stigmatic tissues (Fig. 3D), suggesting elevated *AG* expression. Indeed, *AG* RNA accumulation was elevated in the cauline leaves of *35S::MIR172a-1* plants relative to control transgenic plants (fig. S1). Some *35S::MIR172a-1* and *a-2* plants also had rosette leaves that curled upward (Fig. 3E), a phenotype found in plants expressing *AG* in leaves (17, 18). Because severe loss-of-function *ap2* mutants do not have curly leaves or carpelloid leaves, the leaf phenotypes of *35S::MIR172* lines indicate that miRNA172 regulates, in addition to *AP2*, other genes that repress *AG* in leaves.

RNA filter hybridization showed that these transgenic lines had higher levels of miRNA172 (Fig. 2, C to E). Because the abundance of miRNA172 in wild-type plants is low in young seedlings and high in inflorescences (13), the increase in miRNA172 abundance in *35S::MIR172* lines was more evident in young seedlings than in inflorescences (Fig. 2, C and E). Despite the presence of *ap2*-like phenotypes in these lines, *AP2* RNA accumulation

was similar to that in control transgenic plants (Fig. 2F). AP2 protein, although readily detectable in wild type, was undetectable in *35S::MIR172a-1* inflorescences (Fig. 1D). This indicates that miRNA172 regulates *AP2* expression at the translational level.

Expression of *MIR172* with the 35S enhancer demonstrated the ability of *MIR172* to regulate *AP2* expression in planta when it is expressed from an exogenous promoter. Is miRNA172 normally present at the expected time during flower development? A modified in situ hybridization procedure showed that the antisense miRNA172 probe gave strong signals in stage 1 floral primordia (Fig. 3F). The signal persisted in all four floral whorls until stage 7, when miRNA172 appeared to be concentrated in the inner two floral whorls (Fig. 3H). The signal was unlikely to be a result of the miRNA precursor (fig. S2). The sense probe did not give any hybridization signals (Fig. 3, G and I). The presence of miRNA172 in developing flowers indicates that miRNA172 can regulate *AP2* in floral patterning. In fact, the later accumulation of miRNA172 in the inner two whorls may cause AP2 protein to be concentrated in the outer two floral whorls, where AP2 acts to specify perianth identities.

The ability of miRNA172 to repress *AP2* expression in vivo and the presence of miRNA172 in the flower suggest that miRNA172 normally keeps *AP2* expression level low during flower development. To assess the importance of this regulation in flower development and to determine whether this regulation is direct, I introduced wild-type *AP2* cDNA or *AP2m1*, a mutant *AP2* cDNA with six mismatches to miRNA172 (Fig. 1A), into wild-type plants and expressed them under the control of a 35S promoter. As a result, 100 and 82 independent transgenic plants for *35S::AP2* and *35S::AP2m1*, respectively, were obtained. Dramatic differences were found in the severity and frequency of floral defects between the two transgenic T1 populations (Fig. 4A). About 60% of *35S::AP2m1* plants had flowers with an enlarged floral meristem surrounded by many whorls of staminoid organs or petals (Fig. 3, J and K). Some floral defects, such as the loss of floral determinacy and the transformation of stamens to petals, resemble those in *ag* severe loss-of-function mutants, which is consistent with the ability of *AP2* to negatively regulate *AG*. Other floral defects, such as the enlargement of the floral meristem and the extreme excess of stamens, suggest that *AP2* regulates other floral patterning genes. Alternatively, these phenotypes may have resulted from elevated *AP2* expression and do not represent the natural functions of *AP2*. In contrast to *35S::AP2m1* plants, the majority of *35S::AP2* plants had normal flowers (Fig. 4A). Only a small number of *35S::AP2* plants showed mild floral defects (Fig. 3L). The *35S::AP2* plants with normal flowers had elevated *AP2* RNA accumulation comparable to those in *35S::AP2m1* plants with severe floral defects (Fig. 4C and fig. S3). However, AP2 protein abundance was elevated in *35S::AP2m1* but not *35S::AP2* plants (Fig. 1D). The similarity in *AP2* RNA abundance but difference in AP2 protein levels indicates that miRNA172 regulates *AP2* expression at the translational level. Note that AP2 protein abundance in *35S::AP2m1* lines is similar to that in *hen1* or *dcl1* plants (Fig. 1D), although the *hen1* or *dcl1* floral defects are much weaker than those of *35S::AP2m1* plants. This may be a result of reduced accumulation of many miR-NAs, some of which may regulate targets that act antagonistically to *AP2*, in *hen1* or *dcl1* plants. Alternatively, some *35S::AP2m1* phenotypes may be a result of the exogenous promoter used in the study.

Many *35S::AP2* and *35S::AP2m1* plants also exhibited delayed floral transition (Fig. 4B). Interestingly, about a quarter of *35S::AP2* plants, although not exhibiting any floral phenotypes, were later flowering than *35S::AP2m1* plants, including those with severe floral defects (Fig. 4B). Although *AP2* does not appear to function in floral transition, two closely related genes, At2g28550 (*TOE1*) and At5g60120 (*TOE2*), which also have a miRNA172 binding site, act as repressors of flowering (16). Given that *35S::AP2* and *35S::AP2m1* lines with moderately or severely delayed floral transition showed high levels of *AP2* RNA (Fig. 4C and fig. S3) but only *35S::AP2m1* lines had high levels of AP2 protein (Fig. 1D), it is likely that the late-flowering phenotypes in *35S::AP2* and *35S::AP2m1* plants were caused by two distinct mechanisms. The increased abundance of AP2 protein in *35S::AP2m1* may allow AP2 to act through the TOE pathway to delay floral transition. On the other hand, the increased abundance of *AP2* RNA in *35S::AP2* lines may compete with the *TOE* RNAs for miRNA172 regulation such that more TOE proteins accumulate and delay floral transition. Consistent with this hypothesis, *35S::MIR172a-1* rescues the late-flowering phenotype of *35S::AP2* plants (19).

In addition to disrupting the miRNA172 binding site, the *AP2m1* mutation also introduced an amino acid substitution (Phe to Leu, Fig. 1). To evaluate whether the phenotypes of *35S::AP2m1* plants were caused by the amino acid change, I generated *35S::AP2m3* plants, which harbor a truly silent mutation in the miRNA172 binding site (Fig. 1). Analysis of 88 independent transgenic plants showed that *35S::AP2m3* had nearly identical phenotypes to those of *35S::AP2m1* (fig. S4).

During flower development, miRNA172 represses the expression of *AP2*. This regulation is likely crucial for the proper development of the reproductive organs and for the timely termination of floral stem cells. Furthermore, this regulation appears to be mediated by direct sequence complementarity between *AP2* mRNA and miRNA172 and occurs primarily at the level of translation. MiRNA172-guided cleavage of *AP2* mRNA may also occur at a low level, because potential cleavage products, presumably mediated by miRNA172, have been previously detected by rapid amplification of cDNA ends–polymerase chain reaction (9, 15). This and other studies show that plant miRNAs can regulate their target mRNAs through one or both mechanisms, translational inhibition and transcript cleavage.

Acknowledgments

References and Notes

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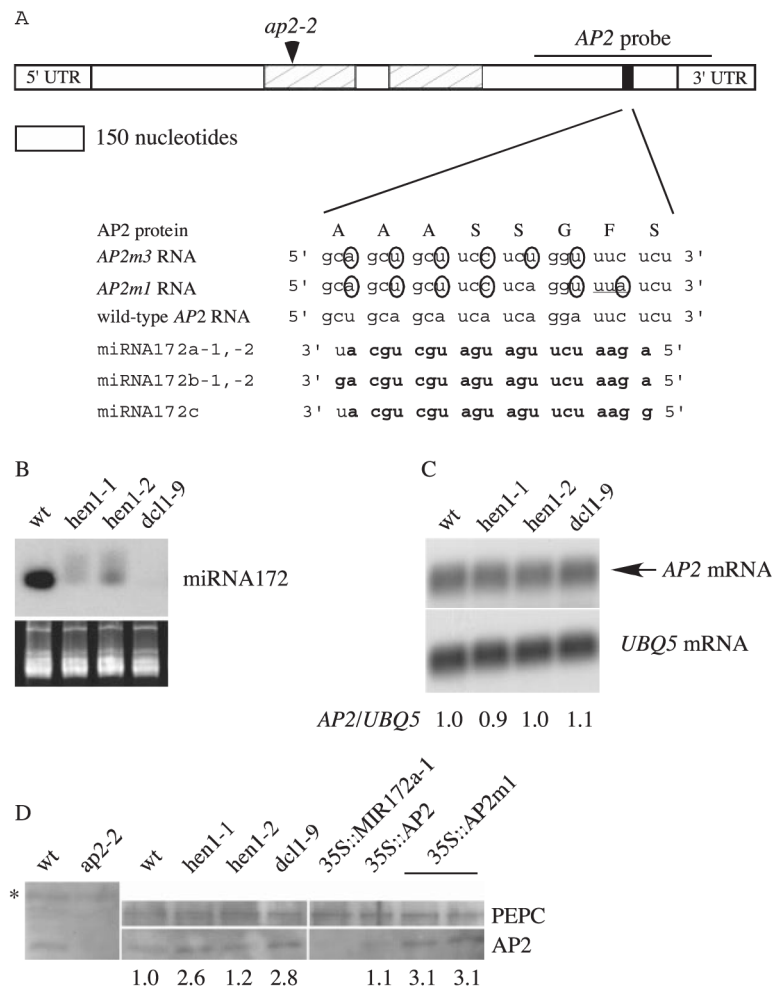
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Fig. 1.
Sequence complementarity between *AP2* RNA and miRNA172 and *AP2* RNA and protein accumulation in various genotypes. (**A**) A diagram of the *AP2* mRNA showing the AP2 domains (hatched rectangles) and the miRNA172 binding site (black rectangle). The mutant nucleotides in *AP2m1* and *AP2m3* are circled. Although *AP2m1* causes a Phe-to-Leu amino acid substitution (underlined codon), *AP2m3* does not change the amino acid sequence. Nucleotides in miRNA172 that can base-pair with *AP2* RNA (with G:U pairing allowed) are in bold. The *ap2-2* allele is a splice acceptor site mutation that would generate a stop codon at the indicated position (arrowhead). 3′UTR, 3′ untranslated region; 5′UTR, 5′ untranslated region. (**B**) Accumulation of miRNA172 in inflorescences. The stained gel below indicates the amount of RNA in each lane. wt, wild type. (**C**) *AP2* RNA abundance in inflorescences. The numbers indicate the relative abundance of *AP2* RNA in various genotypes. (**D**) AP2 protein abundance in inflorescences. Although the AP2 antisera reacted with a number of proteins (one indicated by the asterisk), the identity of the AP2 signal was revealed by its absence in the *ap2-2* mutant. The numbers indicate the abundance of AP2, as calculated by using phosphoenolpyruvate carboxylase (PEPC) as the loading control.
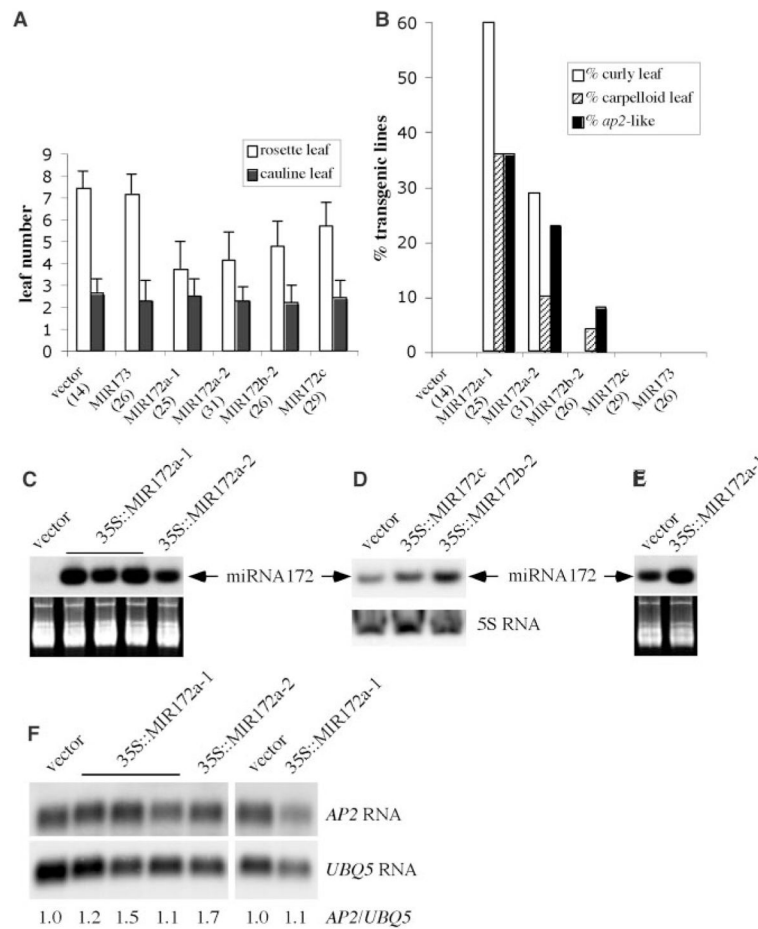
Fig. 2.
Analyses of *35S::MIR172* transgenic lines. (**A**) Leaf numbers of T1 transgenic lines. (**B**)
Percentage of T1 transgenic lines with phenotypes that resemble plants with ectopic *AG*
expression, such as curly leaves, carpelloid leaves, and *ap2*-like flowers. The total number of
independent lines analyzed in (A) and (B) is in parentheses. The miRNA172 abundance in
(**C**) 20-day seedlings of T2 lines, (**D**) 30-day T1 lines, and (**E**) inflorescences of T2 lines.
The stained gels in (C) and (E) and the 5S rRNA hybridization in (D) indicate the amount of
total RNA used. (**F**) *AP2* RNA accumulation in 20-day seedlings (the five lanes on the left)
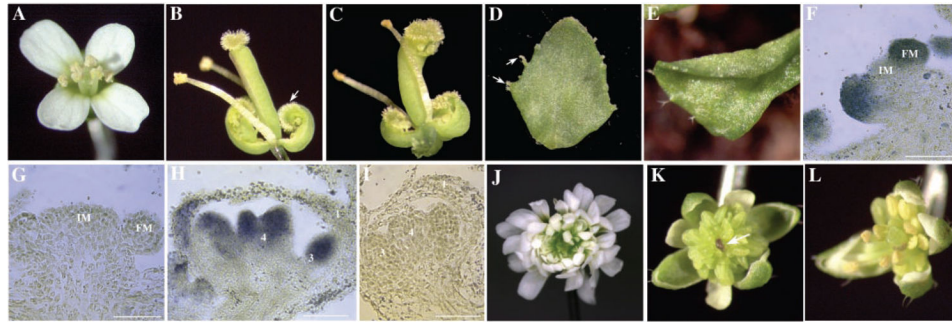and in inflorescences (the two lanes on the right) of T2 lines.

Fig. 3.
Phenotypes of *35S::MIR172*, *35S::AP2*, and *35S::AP2m1* lines and in situ localization of miRNA172. (**A**) A wild-type flower. (**B**) An *ap2-9* flower with first-whorl organs transformed into carpels (arrow). (**C**) A *35S::MIR172a-1* flower that closely resembles *ap2* flowers. A cauline leaf with stigmatic tissues (**D**, arrows) and a curly rosette leaf (**E**) from *35S::MIR172a-1* plants. In situ hybridization using an anti-sense [(**F**) and (**H**)] or sense [(**G**) and (**I**)] miRNA172 probe on longitudinal sections of inflorescence meristems (IM) with two young floral meristems (FM) [(**F**) and (**G**)] and stage 7 flowers [(**H**) and (**I**)]. Hybridization signals are represented by the blue color. Size bar, 50 μm. The numbers in (H) and (I) represent the positions of the organs, with 1 being the outermost whorl. (**J**) A *35S::AP2m1* flower with numerous petals and loss of floral determinacy. (**K**) A *35S::AP2m1* flower with many staminoid organs and an enlarged floral meristem (arrow). (**L**) A *35S::AP2* flower with more stamens than the wild type.
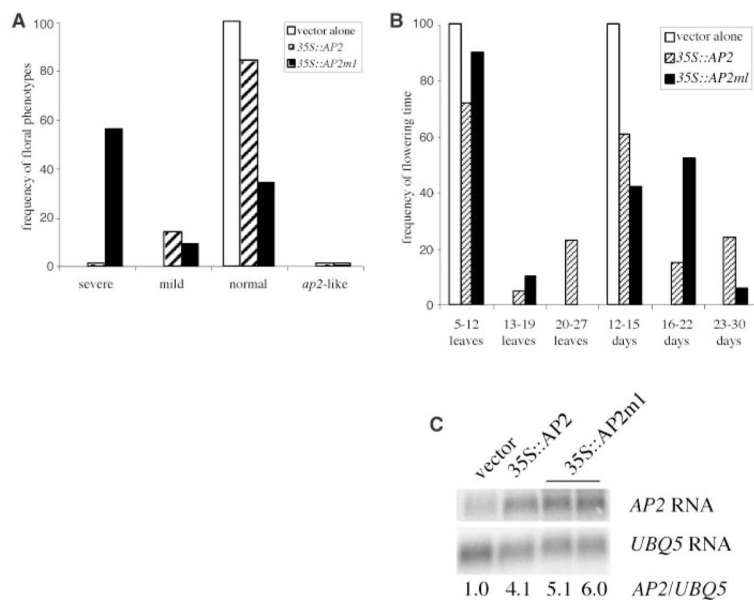
Fig. 4.
Analyses of *35S::AP2* and *35S::AP2m1* transgenic T1 lines. (**A**) The frequency of floral defects in *35S::AP2* and *35S::AP2m1* lines. (**B**) Flowering time as represented by the numbers of total leaves produced before flowering or the numbers of days it takes for the stem to reach 0.5 cm in length. (**C**) *AP2* RNA accumulation in inflorescences from T1 transgenic plants containing the vector alone, *35S::AP2* (with normal flowers but flowers extremely late), or *35S::AP2m1* (with severe floral defects and flowers moderately late). Note that the tissues used were the same as the ones used in AP2 protein analysis shown in Fig. 1D. Numbers at the bottom indicate the relative amount of *AP2* RNA in the various genotypes.